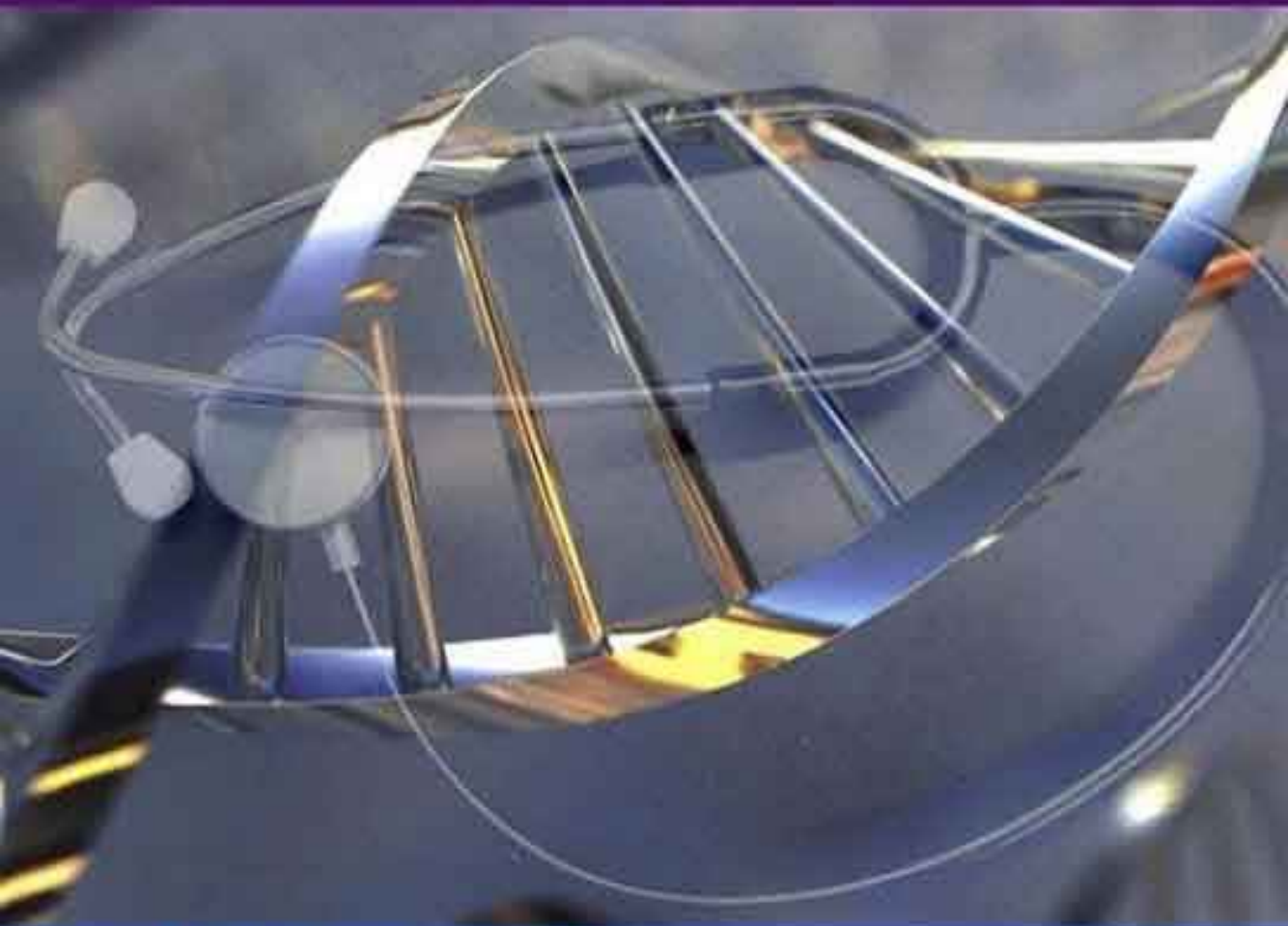


# EMERY AND RIMOIN'S PRINCIPLES AND PRACTICE OF MEDICAL GENETICS SIXTH EDITION

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# PREFACE

In the three decades that have elapsed since the first edition of this book was published, the field of medical genetics has experienced explosive growth. The rate of change in the science and application of medical genetics that occurred in the period spanning the first through the fifth editions of this book has markedly accelerated. In the 1980s, genetics was viewed by most practitioners as an important but obscure corner of medicine. Now it is widely recognized that virtually all human disorders have a genetic component, and genetics is viewed as the key basic science in uncovering the mysteries of disease pathogenesis. The tools of molecular genetics have matured remarkably over these past three decades, to the point where the mutations that underlie most single-gene disorders are being reported week by week. This has resulted in insights into disease mechanisms that have solved medical puzzles that have existed for centuries. These advances are also providing new clinical approaches that vastly improve the ability to accurately diagnose these disorders, and in many instances offer new hope for treatment and prevention. Of even greater importance for the medical and public health communities, and the public, is the increasing ability to dissect the genetic contributions to common disorders. The relationships between nuclear and mitochondrial genotypes, epigenetics, environment and chance remain dauntingly complex, but powerful molecular and computational tools are now being applied to these problems. The promise of new approaches to prevention and treatment represents a new paradigm, which includes what some call “personalized medicine,” that will transform health care.

The sequencing of the human genome has captured public attention and raised both hopes and concerns. Clinical applications of genomics have expanded dramatically since the last edition of this book, most notably in cytogenomics and whole-exome or whole-genome sequencing. Nevertheless, it remains to be seen how quickly and to what extent the genetic approach will be incorporated into the day-to-day practice of medicine. The complexity of translating scientific developments

in genetics to clinical application is being increasingly recognized, particularly with respect to legal, ethical, psychological, economic, and social implications. The need to inform colleagues throughout medicine about advances in genetics and the principles of their clinical application has never been greater.

The continued excitement and progress in our field are reflected in further expansion of this book with several new chapters. The number of genetic disorders understood at the molecular level continues to increase and common disorders not traditionally viewed as “genetic” are included as genetic contributions are coming to light. The sheer mass of new information precludes continued publication of the complete chapters in hard copy. The print volume contains short summaries of each chapter along with a few illustrations and recent references of general relevance to the topic. The complete chapters are available on the PPMG6e website. Authors are expected to update the online version at least semiannually.

We continue to be grateful for the comments about previous editions provided by our colleagues. This edition is better for their input, and we accept full responsibility for the deficiencies that remain.

We warmly acknowledge, besides our contributing authors, the assistance of staff at Elsevier Health Sciences and our personal assistants, especially Sue Lief in Los Angeles. We appreciate the continued moral, scholarly, and spiritual support of Professor Alan EH Emery, one of the originators of this book.

Finally, just as this edition was being finalized, the other originator and senior editor of this book, David Rimoin, died after a brief illness. There have been many memorials and tributes to Dr Rimoin, and others will emerge in the future. This sixth edition of *Principles and Practice of Medical Genetics* is one such memorial, to which he made tremendous intellectual and organizational contributions.

Reed E Pyeritz  
Bruce R Korf  
August 2012

# FOREWORD

In the previous five editions of *Principles and Practice of Medical Genetics*, our late colleague, Victor McKusick, masterfully reviewed the advances that had occurred during the years between the various editions. This sixth edition, which will be published 30 years after the first volumes in 1983, finds the practice of medical genetics in a new environment.

The search for and identification of disease genes have continued to be important in our understanding of genetic disorders. Such discoveries, first reported with the groundbreaking discovery in 1986 of the genes underlying chronic granulomatous disease and Duchenne muscular dystrophy, are now so frequently reported that they will not usually merit publication in a major journal.

Undoubtedly, sequencing of an individual's whole exome or genome is currently forcing dramatic changes in the way genetics is practiced on the clinical and research level. There will be not a single subfield of genetics that will not be profoundly reshaped in the coming years as a consequence of these new technologies. We already observe and welcome fundamental discoveries that revolutionize our understanding of human disease, evolution, and ancestry. Similarly, diagnosis, prognosis and therapeutics have been markedly improved. Even the relationship to our endobiome has to be rethought as a consequence of discoveries in the new field of metagenomics.

In practice, we now increasingly order sophisticated genetic tests to confirm a diagnosis with great accuracy that is already suspected clinically. For example, based on the careful study of the dystrophin gene, we are able not only to confirm a diagnosis but also to predict with considerable accuracy whether a young boy presenting with muscle disease is likely to have the more severe Duchenne muscular dystrophy, or the milder Becker phenotype. This ability to use widely available genetic testing in many clinical situations to dissect out clinical phenotypes with specific gene mutations is invaluable.

The most common genetic disease that we recognize, inherited or acquired, is cancer. Now genetic tests are ordered in families known or thought to be at risk for breast (and other) cancers. Treatment decisions are made for these patients who are found to carry mutant genes that confer increased risk for malignancy, and who might have already presented with cancer. Although we have a vastly greater understanding of tumor biology, as well as predictive laboratory tools, we still lack the ideal cancer treatment, as the tumor in each patient is unique. However, some genetic discoveries related to cancer treatment are among the first to define what many call "personalized medicine".

We are now increasingly using genetic technologies in a more preventive mode across a population, not just in a person in whom we are trying to confirm a diagnosis. The most frequent genetic testing done in the United States and the rest of the developed world in this fashion is that involved with newborn screening. We screen in the public health sector all 4.2 million babies born in the United States for more than 30 disorders, nearly of which are genetically determined. These tests are largely done with metabolite analysis using mass spectroscopy, but there are increasingly direct genetic analyses done as secondary tests. This genetic testing differs in that it is aimed at healthy babies, in an effort to identify and treat serious, often fatal genetic disorders before symptoms appear. The US Center for Disease Control and Prevention has recognized the expansion of newborn screening as one of the most important public health developments of the past 10 years.

The application of next generation sequencing to newborn screening is now in pilot studies and will ultimately be applied at all ages. The actual sequencing, which has been the most daunting issue, is rapidly becoming practical. The costs to sequence the whole exome or genome have dropped markedly, making it feasible to use these resources in an attempt to better understand the outcome of a known condition. For example, by looking across the whole genome of an infant who screens positive for sickle cell disease, it might be possible to identify phenotype-modifying loci and much better predict the course of this severe and variable condition.

Challenges to the broad use of next generation sequencing remain. These include the management of the flood of information that comes from such sequencing and filling gaps in phenotypic annotations of variations in a whole genome. It is likely that we will soon enter the time when information about the person's entire genome will be a part of the medical record and as such a part of medical practice. Such information will require the widespread adoption of the electronic medical record. With the genome sequence in one's medical record one can inquire about the genome over a lifetime, and annotations made to greatly enhance our understanding.

We currently lack the scientific, logistical, ethical and legal framework required for the appropriate and effective use of such information. We will most surely identify modifiers that affect disease states (some of which can possibly be clues to therapies), but we will have a wide array of findings, identifying disorders, which are late in onset and many that lack therapies or any other actionable outcomes. And, while the profession wrestles with issues about what to return of research results and incidental findings from next generation sequencing,

several companies have begun offering these genetic tests directly to the consumer.

Health professionals and the general public must have and will benefit from much expanded genetic education. Eventually, everyone will want to understand better their genetic makeup, their risks for disease, how to prevent serious consequences of disease predispositions, and the most appropriate treatments for overt disease. This sixth

edition of *Emery and Rimoin's Principles and Practice of Medical Genetics* is one effective mechanism for providing this education.

**R. Rodney Howell**

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# PERSONAL MEMORIES OF DAVID RIMOIN

David and I first met in 1963 when we were both research fellows in Victor McKusick's Unit at Johns Hopkins Hospital. He studied dwarfism and I studied muscular dystrophies. He had an office opposite mine and we shared many experiences and events together until I graduated PhD in 1964 and returned to the UK. David was awarded his PhD a year or two later. We remained on close personal terms thereafter—including a wonderful stay at his home in Beverly Hills when I was Boeckmann Visiting Professor at UCLA. On another occasion we attended a meeting in Moscow (before perestroika and glasnost), which was very challenging for both of us. It was at this meeting when we conceived the notion of editing a new textbook on medical genetics.

Over the years, we worked closely together on *PPMG* and on several occasions he visited Edinburgh where I

was then working. In 2006, he was invited to give the annual *Emery Lecture* at Green College, Oxford University. He gave an excellent talk entitled "The skeletal dysplasias: clinical–molecular correlations" which was well-received by everyone.

I shall miss David greatly—his warmth, generosity and friendship. And of course his collaboration with the *Emery & Rimoin's Principles and Practice of Medical Genetics*.

Alan E H Emery  
University of Edinburgh;  
Honorary Fellow, Green Templeton College,  
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June 2012

## History of Medical Genetics

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## 1.1 PREFACE (PETER HARPER)

Victor McKusick's chapter "History of Medical Genetics," which first appeared in the third edition of this textbook in 1996, has provided readers with a masterly account of the origins and development of the field, containing a remarkable amount of information in the limited space available. This has been a historical account as seen by somebody who has lived through most of it, and who has perhaps shaped the development of medical genetics more than any other individual. While this may at times have given a personal slant to the areas emphasized, it provides an unrivaled perspective on the field—indeed for many years it has been the only substantive account, no detailed history of medical genetics having appeared until recently (Harper, 2008).

McKusick's death in 2008 has sadly removed the possibility of him providing a revised edition of his chapter, but his continued active attention to, and involvement in, the detailed evolution of medical genetics, up to the very end of his life, seen particularly in his interest in the Human Genome Project (HGP) and in the updating of his catalog OMIM, not to mention his many unique photographs, means that the chapter is still of the greatest value, and that it would be wrong, as well as regrettable, were it to be discarded. At the same time though, the highly condensed nature of the text makes it difficult, if not impossible to edit without making radical changes.

Thus, for the present edition of this book, a compromise has been reached, whereby the chapter remains essentially intact, but is framed by this introduction and by a postscript outlining some of the relevant topics that the original chapter does not cover. The web-based format of this new edition will allow the chapter to

evolve progressively over the coming years, in keeping with changes in the field, which have not only seen continuing new advances, but have also shown the urgent need for the history of its recent past to be recorded and preserved.

## 1.2 INTRODUCTION

Medical genetics is the science of human biologic variation as it relates to health and disease. Clinical genetics is that part of medical genetics concerned with the health of individual humans and their families. Alternatively, clinical genetics can be defined as the science and practice of diagnosis, prevention, and management of genetic disorders.

Within recent years, medical genetics has become established as a clinical specialty, as the culmination of developments that began in 1956 with the description of the correct chromosome number of the human. With the discovery of specific microscopically visible chromosomal changes associated with clinical disorders, beginning with Down syndrome in January 1959, medical genetics acquired an anatomic base. Medical geneticists now had their specific organ—the genome—just as cardiologists had the heart and neurologists had the nervous system.

The anatomic base of medical genetics was greatly extended with the mapping of genes to chromosomes and specific chromosomal regions, at an ever-accelerating pace, during the past 30 years. Gene mapping has not only enlarged the base for medical genetics but, indeed, as pointed out to me by Charles Scriver (personal communication, 1980), has also provided a neo-Vesalian basis for all of medicine (1). Medical historians tell us that the anatomy of Vesalius published in 1543 was of pivotal importance in the development of modern medicine. It was the basis of the physiology of William Harvey (1628) and the morbid anatomy of Morgagni (1761). Similarly,

\*Deceased.

human gene mapping constitutes an approach to the study of abnormal gene function in all diseases; the gene mapping approach has been adopted by researchers in almost all branches of medicine in the study of their most puzzling disorders. Through mapping, they have sought the basic defect in these disorders, and their clinical colleagues have used mapping information for diagnosis and carrier detection. The ultimate anatomic basis for medical genetics, the DNA sequence, is provided by the HGP.

In this brief history of medical genetics, I trace the foundations of the field that were laid between 1865, when Mendel published his work, and 1956, when the correct chromosome number was reported. I then discuss the events of the past 50 years that have seen the main evolution of the discipline. Finally, I attempt some projections for the future.

### 1.3 FOUNDATIONS OF MEDICAL GENETICS BEFORE 1956

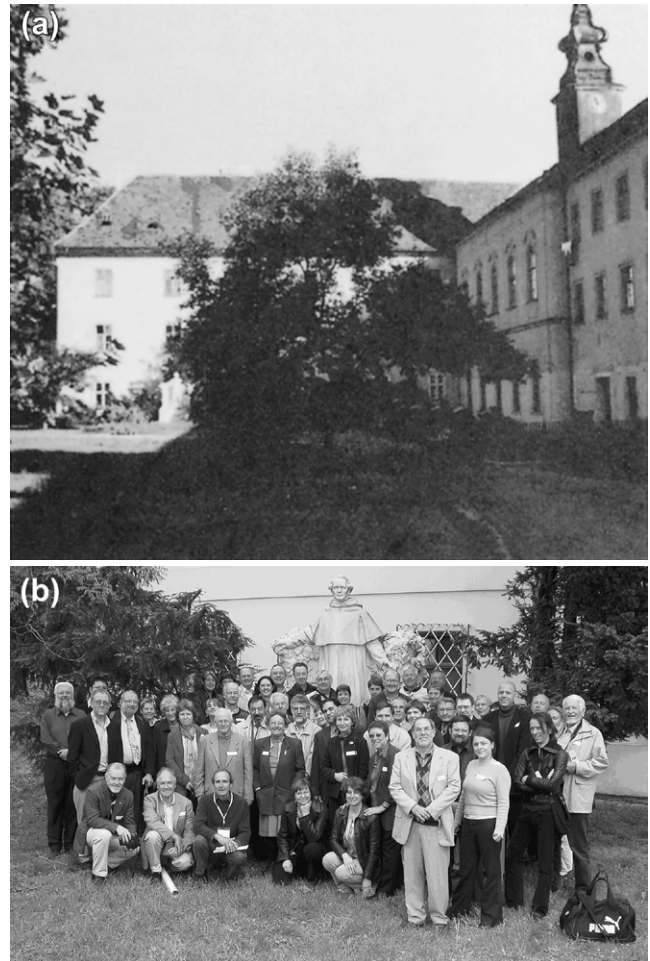
Medical genetics in many developed countries is now a recognized specialty. In the United States, for example, the American Board of Medical Genetics certifies practitioners in the field, including PhD medical geneticists; in 1991, the American Board of Medical Genetics became the 24th organization to join the family of certifying American specialty boards. Medical genetics is a rather unusual branch of clinical medicine; indeed, it may be nearly unique in that it originated out of a basic science. Most specialties started as crafts (or out of a technological advance such as radiography) and only subsequently acquired basic science foundations.

The basic science that developed before 1956 and served as the foundation for the developments of the past 50 years included Mendelism, cytogenetics, biochemical genetics, immunogenetics, and statistical, formal, and population genetics.

#### 1.3.1 Mendelism

The demonstration of the particulate nature of inheritance was the contribution of Gregor Mendel (1822–1884), a monk and later Abbot in an Augustinian monastery (Figure 1-1) in Brunn (now Brno), Moravia (now the Czech Republic). The terms dominant and recessive were his. The delay in recognition of his work has been attributed to various factors, but the most likely is poor timing; in 1865, when Mendel reported his findings and conclusions, the chromosomes had not yet been discovered. Because its physical basis, meiosis, had not yet been described, Mendelism had no plausible basis to qualify it over other possible mechanisms of inheritance such as blending inheritance, which was favored by Francis Galton (1822–1911) another of Mendel's contemporaries (151).

R. A. Fisher (1890–1962) (2) raised a question whether Mendel's results were “too good”; that is, the data agreed too closely with the conclusions (see the

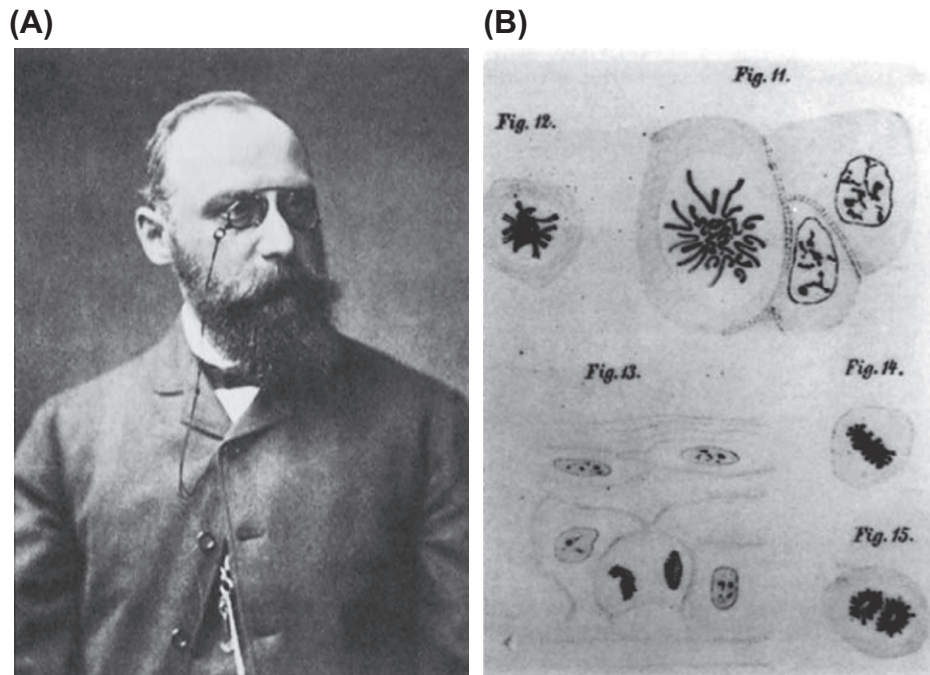


**FIGURE 1-1** (a) Mendel's monastery as it appeared in 1971. (b) Participants in the 2005 International Workshop on Genetics, Medicine and History, held at Mendel's Abbey, Brno.

discussion of this matter by Novitski (3)). (Mendel's “round” [R] versus “wrinkled” [r] trait in the garden pea has been shown to be due to a transposon insertion in the R gene for a starch-branching enzyme. The wrinkled state is due to lack of an osmotic effect present when the normally functioning enzyme is present. The first demonstration of mutation in the human due to insertion of a transposon was provided by the Kazazian group (4): a movable element from chromosome 22 was inserted into the factor VIII gene causing hemophilia A.)

Human chromosomes, visualized in tumor cells in mitosis, were pictured in a paper by Walther Flemming (Figure 1-2A), professor of anatomy in Kiel, in 1882 (Figure 1-2B). The term chromosome was introduced by Waldeyer in 1888. Mitosis and meiosis were described in the last quarter of the nineteenth century. (The term meiosis was introduced in 1905 by Farmer and Moore; the process had been previously referred to as the reduction divisions. The word meiosis, usually taken to mean “reduction in size of the pupil,” is from the same root. It is fortunate that the words are spelled differently in the two usages. In fact, Farmer and Moore spelled it “maiosis.” They wrote as follows: “We propose to apply





**FIGURE 1-2** A, Walther Flemming (1843–1905), discoverer of chromosomes (152). B, First illustration of human chromosomes, by Flemming (1882) (courtesy of the Genetics and Medicine Historical Network).

the terms Meiosis or Meiotic phase to cover the whole series of nuclear changes included in the two divisions that were designated as Heterotype and Homotype by Flemming.”)

During the 1880s, Roux, deVries, and Weismann developed the theory that the chromosomes carry determinants of heredity and development. The state of cytogenetics before the discovery of Mendel’s work was reviewed by E. B. Wilson (Figure 1-3) in his classic text (5). In a discussion of the Roux–deVries–Weismann theory (pp. 182–185), Wilson (5) wrote that “the chromatin is a congeries or colony of invisible self-propagating vital units ..., each of which has the power of determining the development of a particular quality. Weismann conceives these units ... [to be] associated in linear groups to form the ... chromosomes.”

In 1900, Mendelism was rediscovered independently by Hugo deVries (1848–1935) in Amsterdam, The Netherlands, Carl F. J. E. Correns (1864–1933) in Tübingen, Germany, and Erich von Tschermak (1871–1962) in Vienna, Austria. The chromosome theory of Mendelism was put forward about 1903 by Walter S. Sutton (1877–1916) (Figure 1-4), then a graduate student at Columbia under E. B. Wilson (6,7), and by Theodor Boveri (1862–1915) of Würzburg, Germany, a leading cytologist to whom Wilson dedicated his landmark book (1896). Sutton, a Kansas farm boy, had studied meiosis in the Kansas grasshopper at Kansas University under C. E. McClung. He was in New York in 1902 when William Bateson, the leading English champion for Mendelism, lectured there on the subject. Sutton promptly recognized the behavior of Mendel’s so-called factors in transmission from one generation to the next as exactly what



**FIGURE 1-3** E. B. Wilson (1856–1939), noted cytologist and author of *The Cell in Development and Inheritance* (1896).

one would expect if they were located on the chromosomes. The segregation of alleles and assortment of non-alleles were precisely what one would anticipate given the observed behavior of the chromosomes in meiosis.

William Bateson (1861–1926) had a difficult time converting biometricians to the Mendelian view of heredity.



**FIGURE 1-4** Walter S. Sutton (1877–1916), codeveloper of the chromosome theory of Mendelism.

These were the disciples of Francis Galton, including Karl Pearson (1856–1936), who favored blending inheritance. They were led to this view through experiences with the study of quantitative traits that we would call multifactorial. Their recalcitrance is evident in the following statement (8):

*As we have seen in the course of this work, albinism is a graded character, and we have every reason to believe that both in man and dogs separate grades are hereditary ... Mendelism is at present the mode—no other conception of heredity can even obtain a hearing. Yet one of the present writers at least believes that a reaction will shortly set in, and that the views of Galton will again come by their own.*

The views of the biometricians and Mendelists were reconciled by R. A. Fisher in a classic paper published in 1918 (9). Fisher showed that the Mendelian behavior of multiple genes functioning together can explain the findings of the biometricians in regard to quantitative traits. (In his 1918 paper, Fisher introduced the term “variance.”)

Bateson made many contributions to genetics, including the introduction of the term. In a letter written in 1905, related to a projected new professorship at Oxford

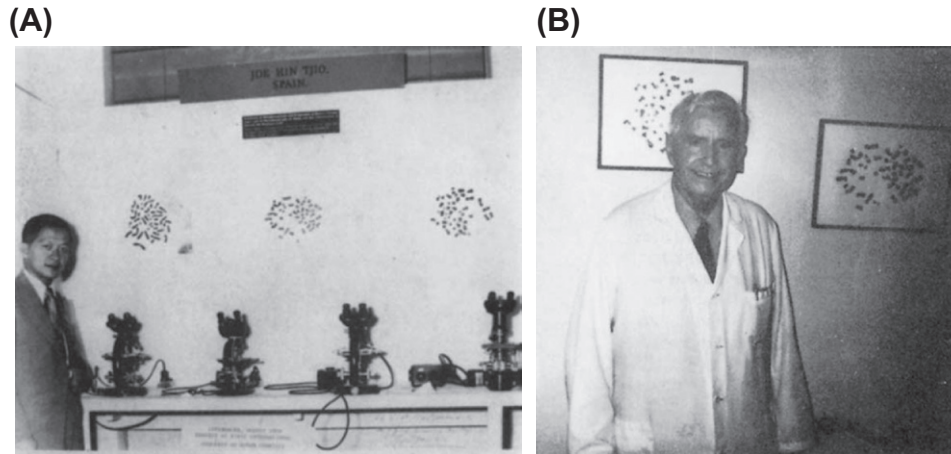


**FIGURE 1-5** Thomas Hunt Morgan (1866–1945), student of linkage and the first American-born Nobel laureate in medicine or physiology. Photo ca. 1910.

University (which he did not receive), Bateson wrote as follows:

*If the Quick Fund were used for the foundation of a Professorship relating to Heredity and Variation the best title would, I think, be “The Quick Professorship of the Study of Heredity.” No single word in common use quite gives this meaning. Such a word is badly wanted and if it were desirable to coin one “Genetics” might do. Either expression clearly includes Variation and the cognate phenomena.*

Another of Bateson’s accomplishments was the discovery (with Punnett) of linkage in the domestic fowl. They misinterpreted the phenomenon and assigned terms that have been perpetuated, however, coupling and repulsion signify whether the two mutant genes of particular interest are on the same chromosome or on opposite chromosomes, respectively. The true significance of linkage and the application of linkage and crossing over to chromosome mapping were contributions of Thomas Hunt Morgan et al. (1866–1945) working in the famous “fly room” at Columbia (Figure 1-5). Alfred Sturtevant was then a college undergraduate; Calvin Bridges and Hermann Muller were junior associates of Morgan. The unit of genetic map distance, the centimorgan (cM), was named for Morgan. He obtained his PhD in 1892 from Johns Hopkins University, he was the first native-born American to be awarded the Nobel prize in physiology or



**FIGURE 1-6** A, Exhibit by J. H. Tjio at the First World Congress of Human Genetics in Copenhagen (1956). Demonstration of  $2N=46$  in mitotic cells. B, Albert Levan in Lund, Sweden, in 1989. Collaborator of Tjio in work published in 1956.

medicine(1933),givenforhiscontributionstotheconceptof the gene.

In 1927, Hermann J. Muller (1890–1967) demonstrated that X-irradiation produces an increase in the rate of mutation in *Drosophila*. For this work, he received the Nobel prize in 1946.

### 1.3.2 Cytogenetics

During the 50 years after Flemming’s first picturing of human chromosomes in 1882, several attempts were made to determine the chromosome number in the human and to determine the human sex chromosome constitution. A particularly definitive paper appeared to be that of Painter (10), in which the diploid number of 48 was arrived at from study of meiotic chromosomes in testicular material from a hanged criminal in Texas. (There is probably no basis for the favorite suggestion of graduate students that the man may have had the XYY syndrome and therefore appeared to have 24, rather than 23, bivalents.) Painter commented that in some of his best preparations, the diploid number appeared to be 46. As an important contribution of the paper, Painter established the human XY sex chromosome mechanism.

In 1949, Murray Barr, working with Bertram in London, Ontario, discovered the sex chromatin, otherwise known as the Barr body, or X chromatin. The distinctive body was visualized in the nucleus of neural cells of the cat. A neuroanatomist, Barr, was studying changes in neural cells with repetitive stimulation of the nerves. Fortunately, he had sufficiently good records that he could establish that the unusual body in the interphase nuclei occurred only in female cats. Ten years later, when the XXY Klinefelter syndrome and the XO Turner syndrome, as well as other sex chromosome aneuploidies, were described, the correlation of the number of Barr bodies with the number of X chromosomes in excess of one was established. The correlation with X-inactivation and the Lyon hypothesis were likewise elaborated.

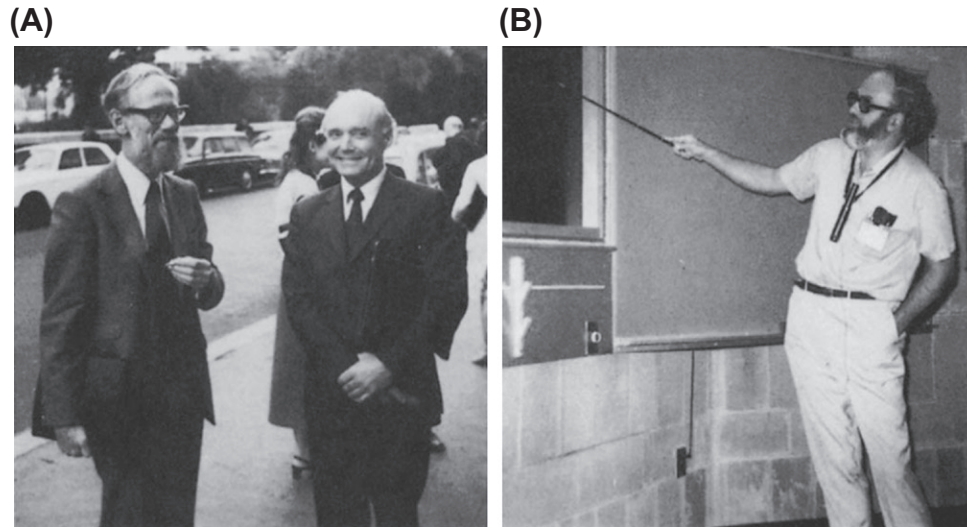
Twenty years after the paper of Barr and Bertram (1949, (11)), the fluorescent (F) body, or Y chromatin, was discovered in interphase nuclei by Caspersson et al. (12–14).

Three new techniques developed in the 1950s and early 1960s facilitated the burgeoning of human cytogenetics, including clinical cytogenetics. During the early 1950s, T. C. Hsu (sometimes known to his colleagues and friends as “tissue culture Hsu”) was working in Galveston when he accidentally discovered, through an error in the preparation of solutions, that hypotonicity causes nuclei to swell with dispersion of the mitotic chromosomes, improving visibility. In his paper, Hsu acknowledged that Hughes at Cambridge University had independently made the same discovery. This technical “trick” was used by J. H. Tjio and Albert Levan (15) (Figure 1.6 A and B) in studies of the chromosomes of fetal lung cells in mitosis and by Charles Ford (Figure 1.7A) and John Hamerton (Figure 1.7B) in studies of meiotic testicular cells (1956) to determine that the correct chromosome number of the human is  $2N=46$ .

In the early days of clinical cytogenetics, it was the practice to do bone marrow aspiration in order to get an adequate number of dividing cells for analysis. This requirement was avoided by the introduction of phytohemagglutinin by Peter Nowell (16–18). As its name implies, phytohemagglutinin is of plant origin and causes the agglutination of red cells. Its use was introduced by Edwin E. Osgood (1899–1970) of Portland, Oregon, for the purpose of separating white blood cells from red blood cells. It was Nowell’s observation that circulating lymphocytes exposed to phytohemagglutinin were stimulated to divide. Thus, it was possible to obtain from a sample of peripheral blood adequate numbers of dividing cells for chromosome studies.

The third technique of particular value to cytogenetics was the use of colchicine to arrest cell division in mitosis. Combined with phytohemagglutinin, it further helped ensure adequate numbers of cells at a stage of division optimal for chromosome identification and enumeration.





**FIGURE 1-7** Charles Ford (A) (at right) and John Hamerton (B) confirmed the  $2N=46$  in meiotic cells in 1956. Photographs taken in 1971 and 1974, respectively.

### 1.3.3 Biochemical Genetics

The acknowledged father of biochemical genetics is Archibald Garrod (1858–1936), who introduced the concept of inborn errors of metabolism, as well as the term (19). Alkaptonuria was the first of the disorders he investigated. In his paper on this condition in 1902, after coaching by Bateson, he recognized that its inheritance was probably Mendelian recessive because of the occurrence in both males and females with normal parents who were often consanguineous. In his famous Croonian lectures (delivered in 1908 and published in 1909), he formally unveiled the concept of inborn errors of metabolism and discussed three other conditions: pentosuria, albinism, and cystinuria. The nature of the enzymatic defect in all four of these disorders is now known. La Du et al. (20) confirmed Garrod's prediction of an enzyme deficiency in alkaptonuria by demonstrating deficiency of homogentisic acid oxidase in a liver sample. The gene mutant in alkaptonuria has been mapped to chromosome 3 by linkage studies; the gene encoding homogentisic acid oxidase has been cloned and a number of disease-causing mutations identified therein (OMIM 203500). The enzyme deficiency of tyrosinase in classic oculocutaneous albinism has been characterized all the way to the DNA level, and the enzymatic and genetic defects in other forms of albinism have been defined. On the basis of classic biochemistry, the enzyme defect in pentosuria is known to involve xylitol dehydrogenase (L-xylulose reductase); the gene has not been mapped. Cystinuria is not, strictly speaking, a Garrodian inborn error of metabolism but a defect in renal (and intestinal) transport of dibasic amino acids. It is caused by mutation in an amino acid transporter gene located on chromosome 2.

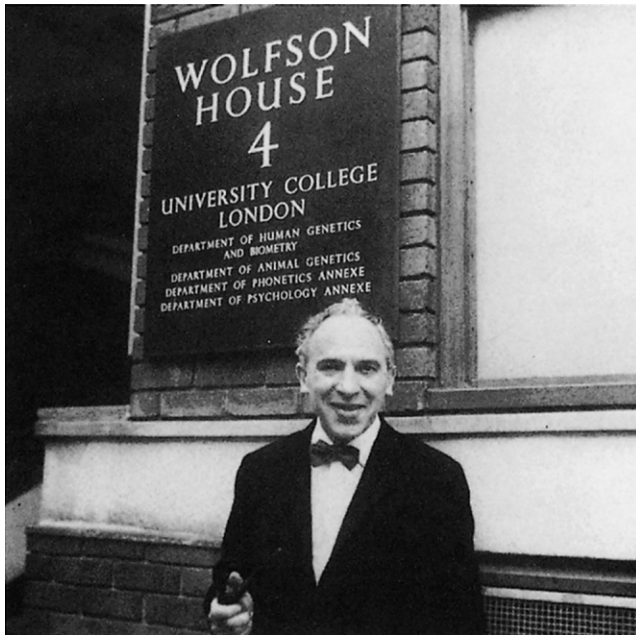
Bateson, Garrod's contemporary and advisor in matters genetical, is credited with the useful piece of advice: "Treasure your exceptions!" He was referring to findings

in experimental genetics and perhaps was emphasizing also the value of investigating rare mutants. The same message was conveyed by Archibald Garrod (21). He quoted a letter written by William Harvey a few months before his death in 1657:

*Nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows traces of her workings apart from the beaten path; nor is there any better way to advance the proper practice of medicine than to give our minds to the discovery of the usual law of nature by careful investigation of cases of rarer forms of disease. For it has been found, in almost all things, that what they contain of useful or applicable nature is hardly perceived unless we are deprived of them, or they become deranged in some way.*

Garrod was William Osler's immediate successor as Regius Professor of Medicine at Oxford University. He seems to have been sensitive to the prevailing view that the disorders he studied were unimportant because they were rare, especially in contrast to the infections and nutritional diseases rampant at that time. It was after his retirement from the Regius chair in 1927 that Garrod (22) wrote "The Inborn Factors in Disease, expanding his earlier Inborn Errors of Metabolism (23) to the full-blown theory of biochemical individuality." Indeed, as pointed out by Barton Childs (24), there was prophetic symbolism in the succession from Osler, who emphasized the disease that affected the patient, to Garrod, who focused on the patient affected by the disease.

Alkaptonuria was not the first inborn error of metabolism in which a specific enzyme deficiency was demonstrated. The first was hepatic glucose-6-phosphatase (G6P) deficiency demonstrated in glycogen storage



**FIGURE 1-8** Harry Harris (ca. 1974).

disease type 1 by Carl and Gerty Cori (25). (Diaphorase deficiency in methemoglobinemia was demonstrated by Gibson in 1948, but perhaps this cannot be considered a Garrodian inborn error of intermediary metabolism.) Deficiency of phenylalanine hydroxylase in phenylketonuria (PKU) was demonstrated soon after by Jervis (26). Asbjørn Følling in Norway had described PKU in 1934. The molecular nature of a mutation of the gene encoding phenylalanine hydroxylase (PAH; MIM 261600) in PKU was first described by Savio Woo et al. in 1986 (27).

Linus Pauling (1901–1994) introduced his concept of molecular disease during the late 1940s. It was an outgrowth of his focus on protein structure and specifically his work on sickle-cell anemia (with Itano) (28) demonstrating by electrophoresis that the hemoglobin molecule had an abnormal structure. By 1956, Vernon Ingram had narrowed the abnormality of the sickle hemoglobin molecule to a single peptide and by 1957 to a single amino acid difference.

By 1956 also, Oliver Smithies was writing about starch gel electrophoresis, opening up the study of human variation at the protein level. With this and similar methods, Harry Harris (1919–1994) at the Galton Laboratory extended the Garrodian concept of biochemical individuality (Figure 1-8).

As studies of the heredity of biochemical differences were going on, investigations of the chemistry of heredity were underway that would be the basis of what we now call molecular genetics. DNA had been discovered in fish sperm by Miescher in the nineteenth century. At the Rockefeller Institute for Medical Research in 1944, Avery et al. (29) demonstrated that the transforming factor, which changed the pneumococcus from a rough

form to a smooth form, is DNA. In 1953, Watson and Crick suggested a model for the structure of DNA consistent with its X-ray crystallography and with its biologic properties, including the capacity for replication.

### 1.3.4 Immunogenetics

Immunogenetics can be said to have had its start with the discovery of the ABO blood groups. These were demonstrated through the existence of “natural” antibodies (isoantibodies) by K. Landsteiner in 1901. From the distribution of ABO blood types in populations, Felix Bernstein in the early 1920s derived support for the multiple allele, one-locus explanation rather than the alternative two-locus (A, non-A and B, non-B) hypothesis. Yamamoto et al. (30) defined the molecular differences between the genes for blood groups O and A and between those for A and B.

The next-to-be-discovered blood group was MN, found by Landsteiner and Levine (31). These workers injected rabbits with different samples of human red cells and absorbed the resulting rabbit immune serum with other red cell samples until they found antibodies that distinguished human blood of the same ABO type.

Yet other blood group systems were discovered on the basis of antibodies (antisera) from mothers immunized to red cell antigens the fetus inherited from the father and antibodies from recipients of mismatched blood transfusions. The Rh blood group system is an example. As recounted by Race and Sanger (32), Levine and Stetson (33), in a brief but historic paper, described how the mother of a stillborn fetus suffered a severe hemolytic reaction to the transfusion of blood from her husband. The mother’s serum agglutinated the cells of her husband and those of 80 of 104 other ABO compatible donors. The antigen responsible was shown to be independent of the ABO, MN, and P groups. In 1940, Landsteiner and Weiner (34), having immunized rabbits and guinea pigs with the blood of the monkey *Macacus rhesus*, made the surprising discovery that the antibodies agglutinated not only the monkey red cells but also the red cells of about 85% of New York City white people, who were said to be Rh positive. Because the anti-Rh antibody was found in the blood of persons who had suffered reaction to the transfusion of ABO-matched blood, and the antibody of Levine and Stetson (33) was apparently identical to that raised in rabbits by injection of rhesus blood, the system was called rhesus (Rh). Levine et al. (35) showed that erythroblastosis fetalis is the result of Rh incompatibility between mother and child. Years later it became known that rabbit anti-rhesus and human anti-Rh antibodies are in fact not the same, but it was too late to change the name. Instead, the rabbit anti-rhesus antibody was called anti-LW, in honor of Landsteiner and Weiner. The LW antigen was later shown to be encoded by a gene on chromosome 19 that is quite distinct from the RH gene on chromosome 1.

Before 1956, blood groups provided some of the clearest examples of the role of Mendelism in the human, as well as some of the most important examples of the application of genetic principles in human health and disease, particularly blood transfusion and maternofetal Rh incompatibility.

### 1.3.5 Statistical, Formal, and Population Genetics

A cornerstone of population genetics is the Hardy-Weinberg principle, named for Godfrey Harold Hardy (1877–1947), distinguished mathematician of Cambridge University, and Wilhelm Weinberg (1862–1937), physician of Stuttgart, Germany, each publishing it independently in 1908. Hardy (36) was stimulated to write a short paper to explain why a dominant gene would not, with the passage of generations, become inevitably and progressively more frequent. He published the paper in the *American Journal of Science*, perhaps because he considered it a trivial contribution and would be embarrassed to publish it in a British journal.

R. A. Fisher, J. B. S. Haldane (1892–1964), and Sewall Wright (1889–1988) were the great triumvirate of population genetics. Sewall Wright is noted for the concept and term random genetic drift. J. B. S. Haldane (37) (Figure 1-9) made many contributions, including, with Julia Bell (38), the first attempt at quantitation of linkage of two human traits: color blindness and hemophilia. Fisher proposed a multilocus, closely linked hypothesis for Rh blood groups and worked on methods for correcting for the bias of ascertainment affecting segregation analysis of autosomal recessive traits.



**FIGURE 1-9** J. B. S. Haldane with Helen Spurway and Marcello Siniscalco at the Second World Congress of Human Genetics, Rome, 1961.

To test the recessive hypothesis for mode of inheritance in a given disorder in humans, the results of different types of matings must be observed as they are found, rather than being set up by design. In those families in which both parents are heterozygous carriers of a rare recessive trait, the presence of the recessive gene is often not recognizable unless a homozygote is included among the offspring. Thus, the ascertained families are a truncated sample of the whole. Furthermore, under the usual social circumstances, families with both parents heterozygous may be more likely to be ascertained if two, three, or four children are affected than they are if only one child is affected. Corrections for these so-called biases of ascertainment were devised by Weinberg (of the Hardy-Weinberg law), Bernstein (of ABO fame), and Fritz Lenz and Lancelot Hogben (whose names are combined in the Lenz-Hogben correction), as well as by Fisher, Norman Bailey, and Newton E. Morton. With the development of methods for identifying the presence of the recessive gene biochemically and ultimately by analysis of the DNA itself, such corrections became less often necessary.

Pre-1956 studies of genetic linkage in the human for the purpose of chromosome mapping are discussed later as part of a review of the history of that aspect of human genetics.

## 1.4 GROWTH AND DEVELOPMENT OF MEDICAL GENETICS: 1956 TO THE PRESENT

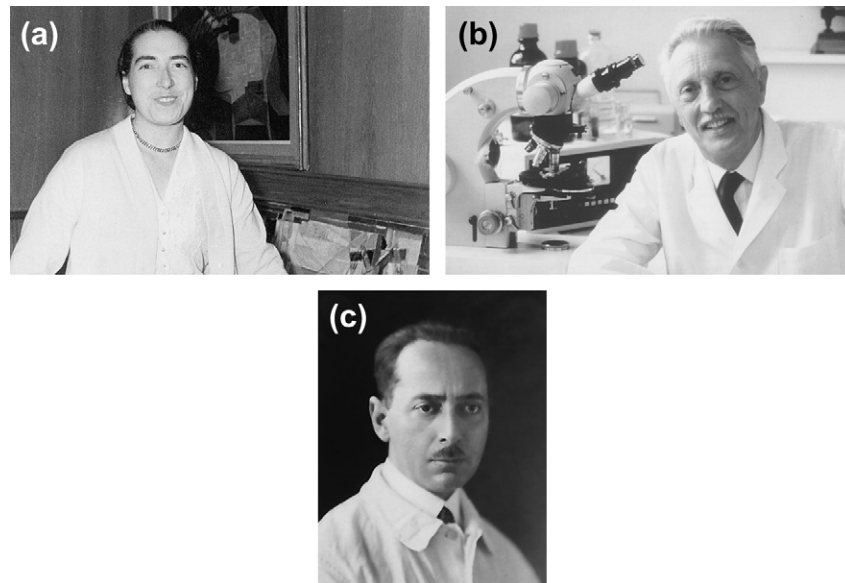
During the past 50 years, medical genetics has developed through a convergence of Mendelism, cytogenetics, biochemical genetics, immunogenetics, and statistical, formal, and population genetics. The development in each of these areas is traced in the preceding part of this chapter. Since 1956, medical genetics, in building on these foundations, has been blessed with three methodologies more or less specific to the field. These are “chromosomology” (beginning about 1956), somatic cell genetics (beginning about 1966), and molecular genetics (beginning about 1976). As will be indicated later, transgenic mice and all methods for transfer of genes into cultured cells or whole organisms, beginning about 1986, constitute a fourth methodological approach. The gene transfer methods, in combination with directed mutation and gene “knock-out,” have already proved particularly useful in the analysis of the function of genes, normal and abnormal.

Two further major methodological advances were database searching (research in silico or cybergenomics) as a primary method of genetic and genomic research and microarray technology for profiling of gene function. These began in the mid or late 1990s.

### 1.4.1 Chromosomology

Following the lead of Margery Shaw (personal communication, 1971), I divide the history of human cytogenetics into five ages (1) 1882–1956 (gestation), the period





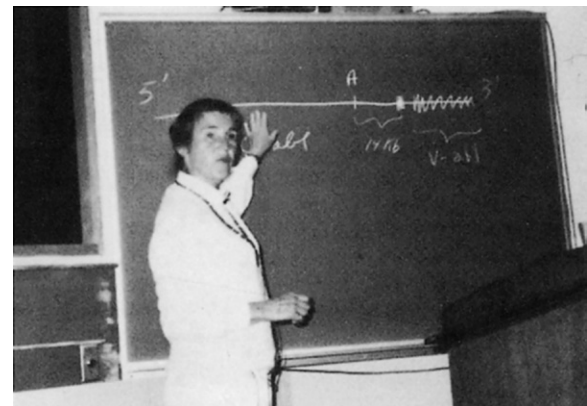
**FIGURE 1-10** Discoverers of trisomy (a) Marthe Gautier (b) Jerome Lejeune (c) Raymond Turpin. 1984 (from Harper (2008), courtesy of Oxford University Press).

from the first publication on human chromosomes to the reports of the correct chromosome number; (2) 1956–1966 (a golden age of human clinical cytogenetics); (3) 1966–1969 (resting phase), a period when the field seemed to be “in the doldrums,” with little progress; (4) 1969–1977 (the banding era); and (5) 1977 to the present (the era of molecular cytogenetics).

Jerome Lejeune (1926–1994) and colleagues (Figure 1-10) opened up the field of clinical cytogenetics with their report in January 1959 of the extra small chromosome in mongoloid idiocy, as Down syndrome was then called. (A letter in *The Lancet* in 1961, containing a list of 19 signatories resembling a short Who’s Who in Human Genetics (39), established the eponymic designation as the preferred one. This is a prime example of the triumph of an eponym.)

The quinacrine fluorescence method was the first of the banding methods developed by Torbjørn Caspersson et al. (12–14) and exploited by Peter Pearson and others. This was followed by the various methods of Giemsa staining following alkali and other treatments for the so-called G-banding and by the method called reverse banding, or R-banding, because the Giemsa-light bands were stained.

The banding techniques permitted the unique identification of each human chromosome. This was immensely useful in experimental situations such as the study of rodent/human somatic cell hybrids (see Chapters 102, 103, and 139) and in the precise characterization of chromosomal aberrations. An early result was the demonstration that the smallest autosome is not number 22, but rather number 21, the autosome trisomic in Down syndrome. Jerome Lejeune had thought that Down syndrome is trisomy 21 of the next to the smallest autosome, not the smallest. Furthermore, it was demonstrated by Janet Rowley (40) (Figure 1-11) that the Philadelphia (Ph) chromosome involves the non-Down syndrome



**FIGURE 1-11** Janet Rowley at Bar Harbor Course, 1983.

chromosome, number 22, and that it represents a reciprocal translocation (with chromosome 9), not a deletion. The precise delineation of deletions and other aberrations permitted deletion mapping and mapping by dosage effects. An early, perhaps the first, example was the assignment of the locus for red cell acid phosphatase (MIM 171500) to 2p25 by Ferguson-Smith et al. (41). The refined cytogenetic delineation of aberrations in the chromosomes also permitted the recognition of “new” chromosomal syndromes. Because one could be reasonably certain of having a “pure culture” series of cases with the same anomaly, it was possible to establish karyotype–phenotype correlations. The trisomy 8 syndrome, the 5q– syndrome, the Pallister–Killian syndrome, and the Jacobson syndrome were examples.

By 1977, high-resolution cytogenetics involving the banding of extended chromosome in cells arrested in prophase or prometaphase had been introduced independently by Jorge Yunis (42), Uta Francke (43), and the Manilovs. This improved further the identification

of microdeletions in solid tumors such as Wilms' tumor and retinoblastoma, in 11p13 and 13q14, respectively. It revealed specific chromosomal abnormalities in congenital disorders of previously obscure etiology, including Langer–Giedion syndrome, Prader–Willi syndrome, DiGeorge syndrome, and Beckwith–Wiedemann syndrome. It provided the basis for the concept of contiguous gene syndromes put forward by Roy Schmickel (44).

The era of molecular cytogenetics, which persists to this day, began about 1977. With improved methods for studying DNA, the biochemical basis of banding was elucidated. The GC-rich nature of the Giemsa-light bands was demonstrated, and this information was correlated with evidence that these bands are also gene rich. Chromosomal *in situ* hybridization with radiolabeled DNA probes was first made to work reliably for single-copy genes in 1981, through the work of Mary Harper and Grady Saunders, Cynthia Morton, Malcolm A. Ferguson-Smith (Figure 1-12), and others. Because of high background noise and perhaps other factors, erroneous results had been previously obtained in experiments that attempted to map single-copy genes. Harper used dextran to create a “wad” of the isotopically labeled probe, thereby achieving a signal at the site of the particular gene that was well above the background level. In the initial paper by Harper et al. (45), the method was used to map the insulin gene to the tip of the short arm of chromosome 11. Fluorescent *in situ* hybridization, a non-isotopic method, was developed by Ward et al., Landegent et al., and others in about 1985.

The combination of molecular techniques with cytogenetic techniques permitted chromosome mapping of oncogenes and identification of their role in hematologic

malignancies associated with translocations. The MYC oncogene on chromosome 8 in Burkitt lymphoma was an early example elucidated by Carlo Croce, Philip Leder (Figure 1-13), and others; the ABL oncogene on 9q, involved in chronic myeloid leukemia, was worked out by Rowley, Heisterkamp, Grosveld (46), and others. Chromosome sorting with fluorescence-activated devices, followed by analysis of gene content by molecular genetic methods, was developed; for example, Lebo et al. (47) used this method specifically for gene mapping.

Deletions were also found in association with neoplasms, usually solid tumors. The classic example is retinoblastoma. Lionel Penrose et al. (48) were the first to find a deletion in any neoplasm, a deletion in chromosome 13 in retinoblastoma. (Deletions in retinoblastoma played a role in proof of the Knudson hypothesis (49) and in positional cloning of the RB1 gene.)

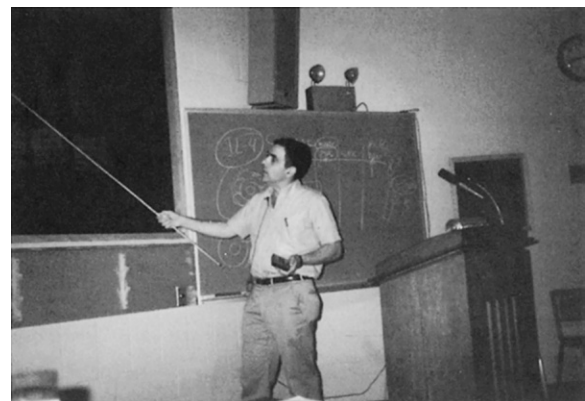
Chromosome microdissection, another method of physical mapping, was developed for collection of DNA from specific regions that could then be subjected to molecular genetic studies. For example, the approach was used by the Horsthemke group (50) to study the gene content of the region of 8q involved in Langer–Giedion syndrome, a contiguous gene syndrome (44).

## 1.4.2 Somatic Cell Genetics

A second large methodological advance in the era of human genetics since 1956 was somatic cell genetics. This has been contributory in several ways. In formal genetic analysis, it permitted the mapping of genes to specific human chromosomes or chromosome regions by the study of interspecies hybrids (e.g. between the human and the mouse). It permitted the differentiation of allelism and nonallelism disorders on the basis of noncomplementation or complementation, respectively, when cells from different patients with a given disorder (e.g. xeroderma pigmentosum) were mixed. In a third place, it permitted the study of the biochemical essence of many inborn errors of metabolism in cultured cells,



**FIGURE 1-12** Malcolm A. Ferguson-Smith with Marie G. Ferguson-Smith in Glasgow, 1981.



**FIGURE 1-13** Philip Leder at Bar Harbor Course, 1988.

usually skin fibroblasts. In a fourth and perhaps its most important application, somatic cell genetics provided an effective approach to the investigation of that vast category of somatic cell genetic disease—neoplasia.

Somatic cell genetics can be said to have gotten its start in the mid-1960s. The techniques that had been developed for culturing cells during the previous decades and the findings of studies of cultured cells were a useful background. No cell line has been subjected to more extensive study than has the HeLa cell. This cell line was isolated from the cervical carcinoma of a patient named Henrietta Lacks ([Figure 1-14](#)), who presented to the Johns Hopkins Hospital in early 1951 at the age of 31. Hers was one of some two dozen cervical carcinomas in which George O. Gey (1899–1970) attempted to establish a cell line and the only one yielding a successful result. The fact that it was an unusual cancer, indeed an adenosquamous carcinoma rather than the usual squamous cervical carcinoma, was found on review of the histology by Jones et al. ([51](#)). It had an unusual fungating appearance suggesting a venereal lesion and prompting a dark-field analysis for spirochetes (which were not found). Although there was no evidence of invasion or metastasis at the time she was first seen and despite radium therapy, Mrs Lacks was dead in 8 months. The genetic characteristics of the HeLa cell line, including HLA types, were determined by Susan Hsu et al. ([52](#)) and compared with the findings in surviving members of her family. That Mrs Lacks was a heterozygote for glucose-6-phosphate dehydrogenase (G6PD) deficiency (G6PD A/B) was established by the fact that she had both G6PD-deficient and G6PD-normal sons. The HeLa cell line is G6PD deficient (G6PD-A), indicating its monoclonal origin; this fact was established by Philip Fialkow ([53](#)) in studies of the monoclonality of cancers. The

vigor of the HeLa cell line is attested to by the extent to which it has contaminated other cell lines in laboratories around the world ([51](#)).

Based on the information acquired from studies of cultured cells, the HeLa cell being the prototypic human cell line, cell culture achieved wide use in studies of inborn errors of metabolism in the 1960s. Among the first of such studies, based on the wide enzymatic repertoire of the fibroblast, was that of galactosemia by Bias and Kalckar and by Robert Krooth in the late 1950s and early 1960s. Later in the 1960s, when Seegmiller with Rosenbloom and Kelley was defining the deficiency of hypoxanthine phosphoribosyl transferase (HPRT) in the Lesch–Nyhan syndrome ([54](#)), he would refer to making morning rounds on his tissue cultures. He alluded to the fact that cultured skin fibroblasts captured the essence of the patients' inborn errors of metabolism for study. Another notable example of the use of cultured cells in genetic studies was the Goldstein and Brown characterization, in the 1970s, of the low-density lipoprotein receptor and its role in normal cholesterol metabolism and familial hypercholesterolemia.

The development of prenatal diagnosis by amniocentesis was dependent on the fortunate circumstance that the amniocyte for the most part demonstrates the same enzymatic activities (or deficiencies) in cell culture as do other cells and tissues of the patient. Exceptions, however, include conditions such as type 1 glycogen storage disease (von Gierke disease) in which deficiency of G6P had been demonstrated by the Coris. Phenylketonuria proved to be another exception.

An important application of somatic cell genetics to formal genetics was the clonal proof of the Lyon hypothesis. Ronald Davidson, Harold Nitowsky, and Barton Childs ([55](#)) provided the most compelling evidence in the human. These investigators cloned two classes of cells from cultures of females heterozygous for the A/B electrophoretic variant of G6PD, one class of cells being type A and the other type B.

All cancer is genetic disease—somatic cell genetic disease. The chromosome theory of cancer was first clearly enunciated by Theodor Boveri in his monograph of 1914 (see [Reference 56](#) for biographic details).

Somatic cell genetics has played a big role in the proof and clarification of the genetic basis of cancer. It provided the methods by which the clonal nature of cancers was proved. Much of the molecular genetics of cancer such as the demonstration of oncogenes has been discovered through somatic cell genetics. The triad of methodologies that have been successively employed beginning in 1956—chromosomology, somatic cell genetics, and molecular genetics—have all played a role.

A particularly substantial contribution of somatic cell genetics has been to gene mapping (see later), specifically physical mapping, that is, the assignment of genes



**FIGURE 1-14** Henrietta Lacks, whose cervical carcinoma was the source of the clonal HeLa cell line.





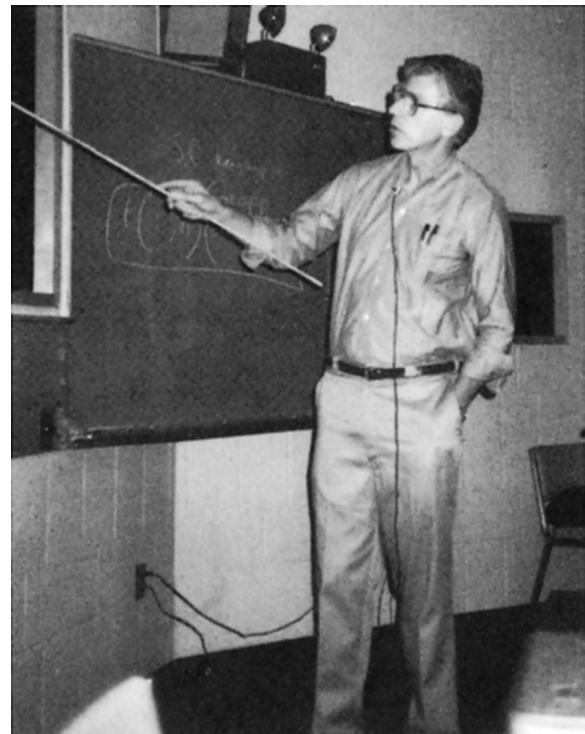
**FIGURE 1-15** Guido Pontecorvo (right) with Dirk Bootsma at Third Human Gene Mapping Workshop, Baltimore, 1975.

to specific chromosomes and chromosomal regions, as opposed to genetic mapping, the determination of the interval between gene loci on the basis of amount of crossing over during meiosis. The method substituted interspecies (e.g. mouse and human) differences for interallelic differences used in meiosis-based linkage mapping. Somatic cell hybridization was what Haldane (Figure 1-9) called a substitute for sex and Pontecorvo (Figure 1-15) termed a parasexual method.

Somatic cell hybridization was made practical by the discovery of fusogens: first a virus, Sendai, by Y. Okada (57) and later a chemical, propylene glycol, by Guido Pontecorvo (58) (Figure 1-15). Both damage the plasma membrane of the cocultivated rodent and human cells, encouraging fusion. In the second place, the development of selection media allowed the isolation of hybrid cells from among the numerically overwhelming parental cells. The first of the selection media was hypoxanthine-aminopterin-thymidine (HAT), adapted to this use by John Littlefield (59) (Figure 1-16). The first human gene to be mapped by the method of somatic cell hybridization was that which encodes the cytosolic isozyme of thymidine kinase (TK1; MIM 188300). Mary Weiss and Howard Green (60), who mapped TK1 to a specific chromosome, recognized the power of somatic cell hybridization for chromosome mapping. Barbara Migeon and C. S. Miller (61) identified the TK1-bearing chromosome as belonging to group E, and after the advent of chromosome banding, Miller et al. (62) identified the chromosome as number 17. Through the work of Frank Ruddle (Figure 1-17), Walter Bodmer (Figure 1-18), and many



**FIGURE 1-16** John Littlefield at Bar Harbor Course, 1964.



**FIGURE 1-17** Frank Ruddle at Bar Harbor Course, 1987.

others, the application of somatic cell genetics to chromosome mapping was exploited to the fullest. During the 1970s, the somatic hybrid cell method accounted for the largest part of the progress in gene mapping that was collated in the regular Human Gene Mapping Workshops begun in 1973 (63).



**FIGURE 1-18** Walter Bodmer at Bar Harbor Course, 1979.

### 1.4.3 Molecular Genetics

The foundations of molecular genetics were laid in the pre-1956 era by the discovery of DNA, called nuclein, by Miescher in 1867; the demonstration that the pneumococcal transforming factor is DNA by Oswald Avery, Colin MacLeod, and Maclyn McCarty (1944); and the solution of the structure of DNA by Watson and Crick (64) (Figure 1-19). Working out the three-letter code of DNA was begun by Marshall Nirenberg and Heinrich Matthaei, who in 1961 observed the synthesis of a polypeptide composed solely of phenylalanine residues, when they used artificially synthesized RNA consisting only of uracil. Thereafter, the code was “cracked,” one trinucleotide at a time, with the complete Rosetta stone of molecular genetics becoming available by 1966.

Restriction enzymes that cut DNA at the site of specific sequences were discovered by Werner Arber and Hamilton O. Smith in about 1970, and Daniel Nathans showed that one can use the enzymes for mapping DNA, the so-called restriction map, first developed for SV40 viral DNA. The Southern blot method for displaying fragments of DNA created by dissection with restriction enzymes was developed by Edwin M. Southern in 1975 (65).

The discovery of specific restriction endonucleases made possible the isolation of discrete molecular fragments of naturally occurring DNA for the first time. This capability was fundamental to the development of molecular cloning (66), which opened up the era of recombinant DNA. The combination of molecular cloning and endonuclease restriction allowed the synthesis and isolation of any naturally occurring DNA that could be cloned into a useful vector and on the basis of flanking restriction sites, excised from it. The availability of a large variety



**FIGURE 1-19** Francis Crick (left) and James Watson at Cavendish Laboratory, University of Cambridge, 1953. (From *Photo Researchers*, with permission.)

of restriction enzymes significantly expanded the value of these methods.

The polymerase chain reaction (PCR) was a major addition to the molecular genetics armamentarium. PCR was formally unveiled in full technical detail by Mullis et al. (67) at the same historic *Cold Spring Harbor Symposium on Quantitative Biology*, Molecular Biology of *Homo sapiens* at which the HGP was discussed in a historic rump session. Combined with specific restriction enzyme cleavage at the mutation site, the method had been used in the diagnosis of sickle-cell anemia by Saiki et al. (68).

The first human gene to be cloned was chorionic somatomammotropin, by Shine et al. (69). About the same time, Tom Maniatis cloned two of the smallest human genes, those for the  $\alpha$ - and  $\beta$ -chains of hemoglobin. Most genes are at least 20 times bigger, and a few are as much as 1000 times bigger, containing much non-coding DNA (i.e. introns). After the discovery of reverse transcriptase, it was possible to take a shortcut and clone only the coding part of the gene complementary to the processed messenger RNA (mRNA), the so-called complementary DNA (cDNA). The cloning of large segments of DNA (up to a megabase or more) came in 1987 with the invention of YAC (yeast artificial chromosome) cloning by David Burke, Maynard Olson, and others (1987).

Two advanced methods for DNA sequencing were reported simultaneously in 1977, by Fred Sanger (70) and Maxam and Gilbert (71). Sanger and Gilbert shared the Nobel prize in chemistry in 1980. The Sanger dideoxy method for DNA sequencing remained the basic technology upon which the genetics revolution of the last vintage of the twentieth century was based, albeit major advances in automation and other modifications were made.



### 1.4.4 Chromosome Mapping

The first assignment of a specific gene to a specific human chromosome can be credited to E. B. Wilson (Figure 1-3), Columbia University professor and colleague of Thomas Hunt Morgan (Figure 1-5). Wilson (72) wrote as follows:

*In the case of color blindness, for example, all the facts seem to follow under this assumption if the male be digametic (as Guyer's observations show to be the case in man). For in fertilization this character will pass with the affected X chromosome from the male into the female, and from the female into half her offspring of both sexes. Color blindness, being a recessive character, should therefore appear in neither daughters nor granddaughters, but in half the grandsons, as seems to be actually the case.*

John Dalton (1766–1844), whose name is memorialized in the unit of molecular mass, had described his own color blindness in the 1790s; Swiss ophthalmologist Horner had described the pedigree patterns we now recognize as typical of X-linked recessive inheritance. (Even earlier, as indicated by Rushton in 1994 (73), Pliny Earle, a pioneer Philadelphia psychiatrist, in 1845 delineated the inheritance pattern of color blindness on the basis of his own family.) The sex chromosome constitution of the human had been assumed to be either XY or XO in the male and XX in the female, as a result of studies by Wilson's colleague N. M. Stevens.

In a famous paper in 1915, J. B. S. Haldane (1892–1964), with his sister Naomi and fellow student A. D. Sprunt, reported the first example of autosomal genetic linkage in a mammal. This was the linkage of “pink eye” (p) with albinism ©. The authors did not use the term linkage; the title of their paper was “Reduplication in mice.” It was published in the early stages of the “War of 1914” (World War I), and in the first paragraph the authors stated: “Owing to the war it has been necessary to publish prematurely, as unfortunately one of us (ADS) has already been killed in France.”

The first human genetic map interval to be estimated was that for color blindness and hemophilia. The transmission of hemophilia in the characteristic X-linked pedigree pattern was described in New England families early in the nineteenth century (74). J. B. S. Haldane with Julia Bell (38) in 1937 attempted an estimate of the interval on the basis of published pedigrees, and Haldane with C. A. B. Smith revised the estimate in 1947 (75). They placed the interval in the vicinity of 10cM; the fact that there are two forms of X-linked hemophilia (A, or classic hemophilia, and B, or Christmas disease) was not found until 1952, when in the Christmas issue of the *British Medical Journal*, Biggs et al. in Oxford, England, reported the case of a 5-year-old boy named Christmas with hemophilia of a distinctive type (76). It was distinguishable from classic hemophilia by the fact that serum from Master Christmas corrected the clotting defect in the blood of patients with classic hemophilia.

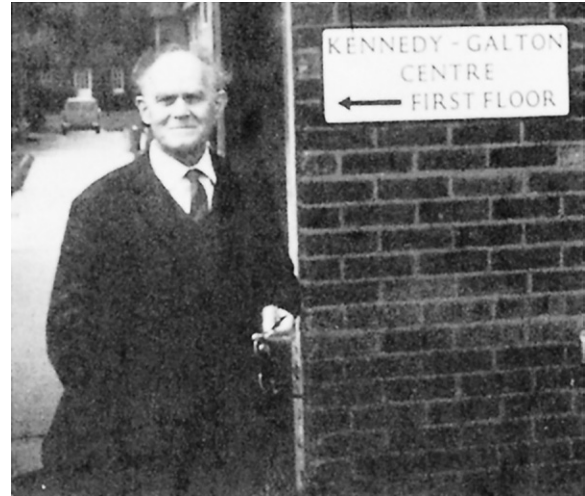


FIGURE 1-20 Lionel Penrose, 1973.

Subsequent studies of the linkage between hemophilia A and color blindness showed very tight linkage, and a reanalysis of the published hemophilia/cb data used originally by Haldane et al. led C. A. B. Smith to conclude that the series was a mixture of two forms of hemophilia, one very tightly linked to color blindness and one loosely linked or not linked at all. We now know that hemophilia B maps to the long arm of the X chromosome, but at a distance proximal to hemophilia A and color blindness, which are at the very tip of the long arm.

During the 1930s and 1940s, considerable thought was given to what approaches might be used to identify genetic linkage, recognizing that it is necessary in the human to use matings as they are found; one cannot, of course, create the matings that are most informative as one can in mouse and fruit fly. Lionel Penrose (1898–1972) (Figure 1-20) elaborated a sib-pair method in the 1930s (77). The first autosomal linkage in the human was not identified until the early 1950s, when Jan Mohr (Figure 1-21) demonstrated linkage of the Lutheran blood group and secretor factor loci as part of his doctoral thesis in the institute headed up by Tage Kemp (Figure 1-21) in Copenhagen. Mohr was subsequently the director of that institute, and it was in his department that the first-to-be-discovered autosomal linkage group (Lutheran and secretor) was determined to be located on chromosome 19 (78).

The method logarithm of the odds (lods) was developed by C. A. B. Smith in the 1950s and elaborated by Newton Morton. Using these methods, Morton (79) demonstrated linkage of the elliptocytosis and Rh blood group loci (including the demonstration of genetic heterogeneity, as some families did not show linkage). Renwick and others showed linkage of the ABO blood group to the nail–patella syndrome. All three of the early examples of autosomal linkage were established with blood groups (or, in the case of secretor factor, an “honorary” blood group), illustrating the pathetically limited repertoire of markers, a major handicap to gene mapping in that era.

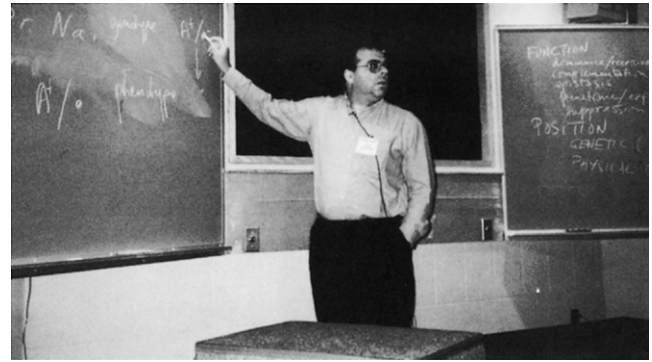


**FIGURE 1-21** Jan Mohr (left) and Victor McKusick with plaque honoring Tage Kemp (1896–1964), Copenhagen, 1979. Mohr, Kemp's successor, was the director of the Institute of Human Genetics in Copenhagen. Kemp was the convener of the First World Congress of Human Genetics (1956).

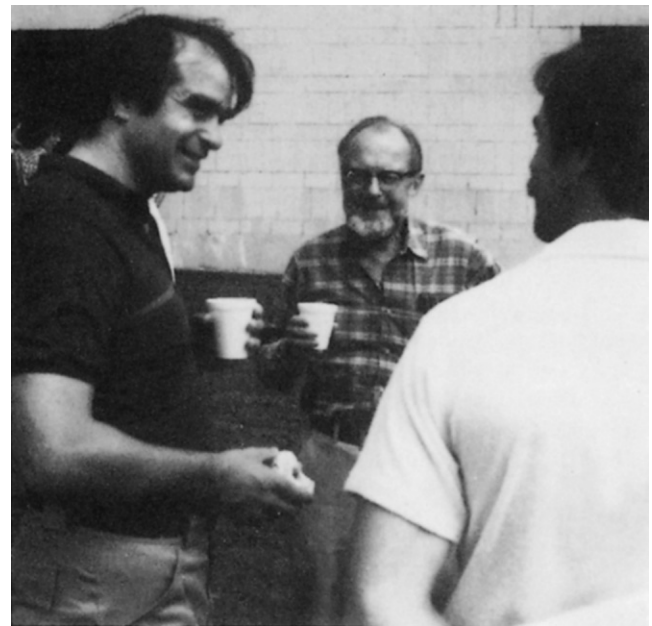
By 1951, when the first autosomal linkage was discovered in the human, at least eight autosomal linkage groups had been identified in the mouse. On the other hand, it was about the same time in the early 1950s that the first X-linked locus was identified in the mouse, whereas three dozen or more loci were known to be on the human X chromosome because of X-linked pedigree patterns.

The first gene to be assigned to a specific autosome was the Duffy blood group locus, which was localized to chromosome 1 by Donahue et al. (80). At that time, R. P. Donahue was a candidate for the doctoral degree in human genetics at Johns Hopkins. As every graduate student in human genetics should probably do, he determined his own karyotype, although his thesis research was not on a cytogenetic problem. He identified a heteromorphism of the chromosome 1 pair. One chromosome 1 showed what looked like an uncoiled region (this was in the prebanding era) in the proximal portion of the long arm. Donahue had both the wit and the gumption to do a linkage study: the wit to sense that this might be a mendelizing character in his family, and the gumption to collect blood samples from widely scattered relatives, to determine marker traits, and to analyze the data. The analysis suggested linkage of the Duffy blood group locus to the “uncoiler” trait. Although the lod score was far below the value of 3.0 usually accepted as evidence for linkage, confirmation of the assignment came quickly from other workers.

After 1971, abundant information was collected on chromosome mapping by the method of somatic cell hybridization. Radiation by hybrid mapping made use of hybridization with rodent cells to “rescue” human cells in which random fragmentation of chromosomes had been produced by radiation. In situ hybridization of DNA probes to chromosomes, first with radioactively



**FIGURE 1-22** David Botstein at Bar Harbor Course, 1987.



**FIGURE 1-23** Ray White (left), James Neel (middle), and Joseph Nadeau at Bar Harbor, 1983.

labeled probes and later with fluorescent probes, was a mapping method first made to work reliably for single-copy genes in 1981.

About 1980, molecular genetics entered the chromosome mapping field. It contributed to the field in three ways: (1) it permitted direct identification of the human gene in somatic cell hybrids, so that it was possible to go directly for the gene, rather than requiring expression of the human gene in the hybrid cell; (2) it provided probes for chromosomal in situ hybridization, which was made to work for the first time in a reliable way for single-copy genes in 1981; and (3) it provided DNA polymorphisms as markers for family linkage study, thereby providing a virtually limitless repertoire.

In a seminal paper (81), David Botstein (Figure 1-22), Ray White (Figure 1-23), Michael Skolnick, and Ron Davis formally outlined the value of what they called restriction fragment length polymorphisms (RFLPs or “riflips”) as linkage markers in family studies. In fact, the first RFLP had been discovered by Kan and Dozy (82),

an HpaI variant 3' to the  $\beta$ -globin locus, and Ellen Solomon and Walter Bodmer (83) had suggested the value of such DNA variants as linkage markers:

*Given the range of available restriction enzymes, one can envisage finding enough markers to cover systematically the whole human genome. Thus, only 200 to 300 suitably selected probes might be needed to provide a genetic marker for, say, every 10% recombination. Such a set of genetic markers could revolutionise our ability to study the genetic determination of complex attributes and to follow the inheritance of traits.*

The late Allan C. Wilson (1934–1991) decried the use of the term restriction fragment length polymorphism, arguing that length mutations are one large class of mutations, the other being point mutations, and that it would be better to refer to these simply as restriction fragment polymorphisms. In mice they are referred to as restriction fragment length variants (RFLVs). Indeed this is what they are, differences between inbred strains and not polymorphisms in the strict sense as defined by E. B. Ford of Oxford University as the occurrence together in the same habitat of two or more distinct forms of a species in such proportions that the rarest of them cannot be maintained by recurrent mutation. Indeed, RFLPs in the human, when first discovered in a small number of samples, could not be known to be polymorphisms in the terms of this definition; 1% is usually taken as the minimal frequency of the rarer allele. However, it turns out that most DNA variants are indeed highly variable and qualify as polymorphisms within the Ford definition.

A second form of DNA variation to be discovered was variable number of tandem repeats (VNTRs), introduced as hypervariable DNA markers for linkage studies, as well as for use in forensic science by Jeffreys et al. (84) and by Nakamura et al. (85). The third major, and still more variable, type of DNA polymorphism is represented by the dinucleotide repeats, for example (CA)<sub>n</sub>, the so-called microsatellites. These were discovered and developed by James Weber at the Marshfield Clinic in Marshfield, Wisconsin, and by Michael Litt in Portland, Oregon; they are extensively exploited by many, particularly Jean Weissenbach in Paris, for the creation of genetic maps and the mapping of disease genes.

The first major successful application of RFLPs in linkage study was the mapping of the Huntington disease (HD) locus to the tip of chromosome 4 through linkage to the so-called GS8 RFLP; this marker at a locus designated D4S10 in the human gene mapping nomenclature was, in turn, mapped to that region of chromosome 4 by in situ hybridization. The initial demonstration of linkage was achieved by James Gusella et al. (86) on samples assembled by Nancy Wexler (Figure 1-24) from an extraordinarily large and extensively affected kindred with HD in the Lake Maracaibo region of Venezuela.



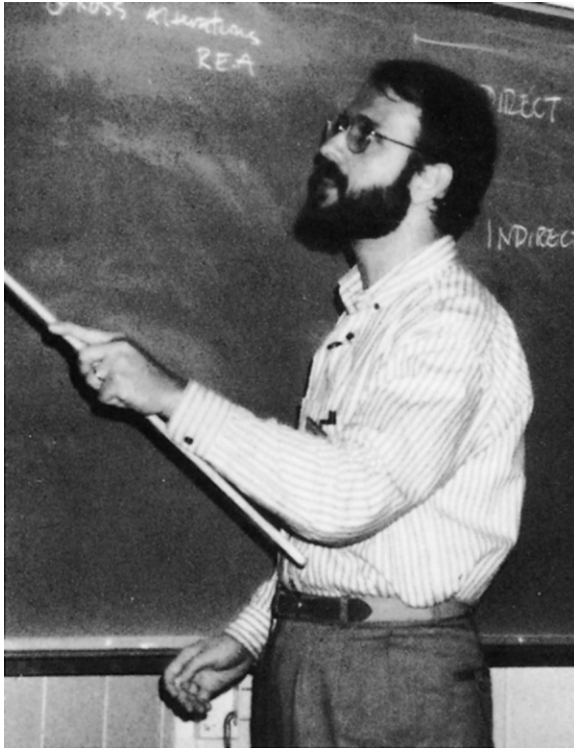
**FIGURE 1-24** Frank Ruddle (left) and Nancy Wexler at Bar Harbor, 1983.



**FIGURE 1-25** Haig Kazazian at Bar Harbor, 1976.

Haplotyping is the determination of markers close to, and on the same chromosome as, the mutation of interest. The term haplotype was first used in connection with the major histocompatibility types, HLA types. Haplotyping was developed particularly extensively by Haig Kazazian (Figure 1-25) and Stylianos Antonarakis (Figure 1-26) at Johns Hopkins University, in collaboration with Stuart Orkin in Boston and others, for identifying the presence of the gene for thalassemia in particular individuals. Because haplotypes represent closely linked markers, it is not unexpected that in families and even in population groups, common forms of thalassemia are associated with the same haplotype. This important application of the linkage principle in diagnosis and in population genetics (for tracing the multiple origins, e.g. of the sickle hemoglobin gene) was extended from the



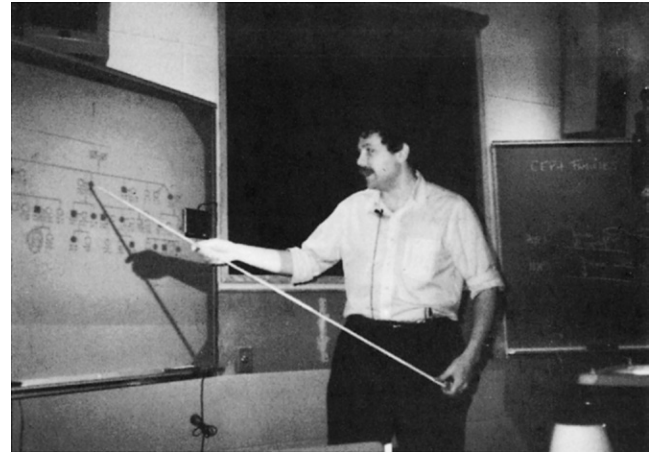


**FIGURE 1-26** Stylianos Antonarakis at Bar Harbor, 1988.

hemoglobinopathies to many inborn errors of metabolism, particularly cystic fibrosis. Because of the many different mutations capable of causing cystic fibrosis and because of the relatively high frequency of the “cystic fibrosis” gene, tracing it in families through the haplotype has been useful.

In some populations, such as the Finns and the Old Order Amish, many usually rare disorders occur in relatively high frequency because of a founder effect. In such populations, most individuals affected with a recessive disorder that is rare in the general population are homozygous for a particular haplotype. Indeed, as was theorized by C. A. B. Smith in 1953 and elaborated more recently by Lander and Botstein (87) (Figure 1-27), patients with any rare autosomal recessive disorder, if the parents are consanguineous, will be expected to be homozygous for closely linked markers, that is, homozygous for a particular haplotype. This has indeed been shown to be the case in several disorders, including Bloom syndrome, diastrophic dysplasia, and alkaptonuria (88). Of course, in founder populations, a specific haplotype is likely to be shared in common by persons affected with an autosomal dominant or X-linked recessive as well.

Linkage analysis by the study of PCR-amplified DNA from individual sperm was introduced by Norman Arnheim in 1988 (89). Essentially, one could fill in the sort of information contained in stick diagrams used to explain linkage in textbooks of genetics. With this approach, it was not necessary to have observations on diploid offspring to identify recombinants among the gametes produced by the parent.



**FIGURE 1-27** Eric Lander at Bar Harbor, 1989.

Comparative mapping, most extensively pursued between mouse and humans, has been exploited as a clue to the likely location of genes in the human. A great deal of syntenic homology has been found between these two extensively studied species. The X chromosome in the human and in all other placental mammals has essentially the same genetic content, as was pointed out during the 1960s by Ohno (90), who also pointed out that the X chromosome is about the same size in all these organisms. The extensive syntenic homology, in the case of the autosomes, between the human and the mouse came as a surprise to many. What I have chosen to call the Oxford grid was developed by John H. Edwards, professor of genetics at Oxford University, and collaborators, particularly A. G. Searle, at nearby Harwell.

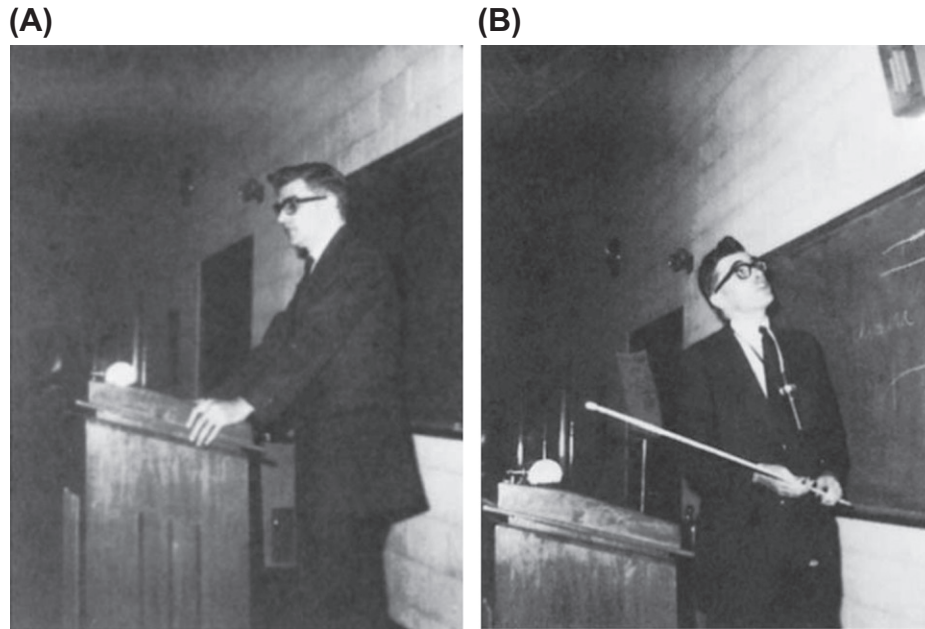
The term synteny, meaning “on the same chromosome,” was introduced by James H. Renwick (1926–1994) (Figure 1-28) in about 1970. It proved to be a useful term of distinction from linkage; two loci may be syntenic but not linked in the strict genetic sense because they are so far apart on the same chromosome that they assort independently through crossing over.

Renwick was also responsible for developing an early program for computer-assisted linkage analysis. Jurg Ott (91) also developed programs, as have others. These facilitated widespread analysis of linkage data.

A development of great importance for advancement of genetic mapping was the creation of the Centre d’Études du Polymorphisme Humain (CEPH) in Paris by Jean Dausset in 1983. CEPH created and maintains a reference panel of family DNAs for mapping by linkage. The three-generation family units consist of the four grandparents, two parents, and a minimum of eight children. The data on markers, as they are determined in these reference families in many laboratories, are collated, and new markers and genetic disorders can be mapped against the reference panel.

Radiation hybrid mapping was first devised by Goss and Harris (92) as a modification of mapping with





**FIGURE 1-28** A and B, James Renwick at Bar Harbor, 1972.

somatic cell hybrids. It involved fragmentation of the human chromosomes by irradiation of cultured cells and the “rescue” of these cells by fusion with mouse cells. The method was revived and revamped about 1990 by Peter Goodfellow of Cambridge University (93) and David Cox of Stanford University (94). Both created DNA panels from cells with various human chromosomal content.

The creation of clone libraries, for example, YACs, whose map location was known from radiation hybrid mapping or other methods made it possible to map a newly found sequence by hybridization or by linkage to a marker such as a sequence-tagged site (STS) or gene known to be on the same clone fragment. STSs were proposed by Maynard Olson et al. (95) as useful markers in mapping (96).

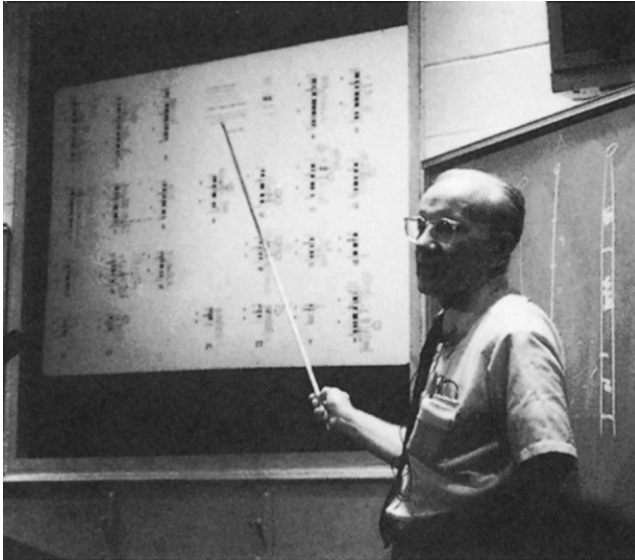
### 1.4.5 The HGP and Genomics

The goal of the HGP is to locate all the genes and sequence the entire haploid. It is primarily a mapping project; even sequencing was determined the ultimate map. In its initial form, however, it was not formally conceived as a mapping project. When proposed by Robert Sinsheimer, Walter Gilbert, Renato Dulbecco, and others in the period 1985–1986, the HGP was viewed as a project for complete sequencing of the genome. Previous progress in gene mapping and the value of the results were apparently unfamiliar to the leading promulgators, who were molecular geneticists. In part, objections to the project raised between 1985, when it was first proposed, and 1990, its official start date, resulted from the impression that it was an enterprise of mindless sequencing without much biology. (A factor also contributing to the unhappiness of many scientists with the project was

the price tag. The National Research Council/National Academy of Sciences (NRC/NAS) Committee on Mapping and Sequencing the Human Genome, commissioned in late 1986, and reporting out in February 1988, estimated that the “job” could be done in 15 years for \$200 million a year. Journalists in particular advertised the HGP as a \$3.0 billion project, which biologists coping with funding problems found disturbing. Of course, the annual budget of the National Institutes of Health (NIH) is approximately 50 times that figure (in 1988 dollars)!)

At a birth defects congress in The Hague in 1969, complete mapping of the genes on the human chromosomes had been proposed as an effective approach to solution of problems of congenital malformations and genetic disorders in general (97) (Figure 1-29). The proposal came close on the heels of the first manned Moon landing in July 1969; complete genome mapping was seen as the next Moon shot. The potential benefit of complete mapping was frequently emphasized thereafter. For example, it was advanced as an achievable goal for the last “vintade” of the twentieth century (98). Human Gene Mapping Workshops, initiated by Frank Ruddle in New Haven, Connecticut, in 1973, continued in the same form until 1991, for collation of the world’s experience in gene mapping. The function of these workshops was taken over by HUGO (see later).

It is true that the 1969 proposal had little apparent impact even though it was repeatedly restated over the next 15 years (1,98). In 1969, the methodology that would make the HGP possible was not yet at hand. As outlined elsewhere, recombinant DNA technology and methods of efficient DNA sequencing came in the 1970s; improved methods for both genetic and physical mapping



**FIGURE 1-29** Victor McKusick and the human gene map, Bar Harbor Course, 1981.



**FIGURE 1-30** At Howard Hughes Medical Institute-sponsored NIH conference on the proposed HGP, July 1986. Left to right: Victor McKusick, L. H. (Holly) Smith, James Watson, Walter Gilbert, Robert Sinsheimer.

came in the 1980s. As pointed out by R. Cook-Deegan ((99), p. 10) in his history of the HGP, whatever revisionist history might be written, the facts are that the HGP was conceived and promoted, not by medical geneticists, but by molecular biologists such as Robert Sinsheimer, Walter Gilbert (Figure 1-30), and Renato Dulbecco, who worked predominantly on organisms other than the human. And it was conceived and promoted apparently in ignorance, for the most part, of what had been accomplished and proposed in the area of gene mapping. It is true that in the 1986 Cold Spring Harbor Symposium devoted to the human genome, a long review was given of the status of the human gene map (100). This and the accompanying chromosome-by-chromosome lists of genes then mapped were an eye-opener to the molecular geneticists present. Also at that meeting, a rump session



**FIGURE 1-31** Francis Collins (left) with Leroy Hood, Cold Spring Harbor, 1993.

led by Gilbert and Paul Berg discussed the pros and cons of the proposed genome project (see Reference (99) for photographs of Gilbert, Berg, and Botstein at that crucial meeting and for a summary of the discussion).

If the period 1985–1990 was a gestational period for the HGP, the NRC/NAS committee (1988) was the midwife. The official birth date for the project in the United States was October 1, 1990, with federal funding jointly through the Department of Energy (DOE) (about one-third) and the NIH (about two-thirds). (The DOE through its national laboratories has long had a mandate to study the biologic effects of radiation. With the leadership of Charles DeLisi, then with the DOE, and the legislative initiative of Senator Pete Domenici of New Mexico, DOE became involved both intramurally (in its national laboratories) and extramurally in the HGP.)

James G. Watson (Figure 1-30) of Watson–Crick fame led the NIH program for its first 3 years, bringing to it both his prestige and his wisdom. At the same time he directed the newly created National Center for Human Genome Research (NCHGR), which functioned in much the same way as the individual institutes, he continued to direct the Cold Spring Harbor Laboratory on Long Island, New York. In 1993, Francis Collins (Figure 1-31) became the director of the NCHGR and proceeded to develop an intramural program in medical genetics. This reflects his conviction that the HGP is primarily a gene-finding enterprise and is linked to clinical medicine as both an efficient way to find genes and an effective way to realize potential benefits in diagnosis, prevention, and management of genetic disorders. The “center” became an institute, the National Human Genome Research Institute (NHGRI), on January 1, 1995.

In parallel with the HGP in the United States, genome programs were developing in many other countries, particularly the United Kingdom, France, and Japan. In September 1988, a group of 32 scientists from 14 countries met in Montreux, Switzerland, to found the Human Genome



**FIGURE 1-32** Founding council for the Human Genome Organization (HUGO) at Montreux, Switzerland, September 1988 (11 members were absent). First row (left to right): Matsubara, Shows, Tocchini-Valentini, Honjo, Shimizu, McKusick (with Swiss cowbell), Lyon, Gilbert, Cantor, Robson, Karpov (observer). Second row: Hirt, Ruddle, Collins, Zinder, Sutherland, Cavanee, Hinton (staff), Strayer (staff), Tooze, Hood, Frézal, Cahill, Ferguson-Smith. Third row: Pearson, Dulbecco, Philipson, Jacob, Mirzabekov, Goodfellow (observer), Dausset, Watson, Worton, Southern, Grzeschik.

Organization (HUGO) (Figure 1-32). As a member of the organizing group, Norton Zinder, put it, “HUGO is a UN for the human genome.” It is a coordinating organization. Its founder president Victor McKusick was succeeded by Walter Bodmer in 1990, by Thomas Caskey in 1993, by Grant Sutherland in 1996, and thereafter by Gert-Jan van Ommen followed by Lap-Chee Tsui.

The HGP progressed even faster than predicted, mainly because of new methods: PCR (101), YACs (102), STSs (95), microsatellite linkage markers (103), and others. The official start date for the HGP in the United States was October 1, 1990. The completion date predicted by the 15-year estimate made by the NRC/NAS Committee on Mapping and Sequencing the Human Genome (104) was September 30, 2005. At the 5-year mark, Francis Collins (105) could report that the HGP was “ahead of schedule and under budget.”

A phenomenal speeding up occurred in the last part of the 1990s, with announcement of a “first draft” of the complete sequence in June 2000. The acceleration can be credited to the development of improved high-throughput sequencers that used capillaries for the dideoxy method developed by Sanger in 1977; and particularly to the application to the human of “shotgun” sequencing, which had been successful in the first complete sequencing of a free-living organism, *Haemophilus influenzae*, by the group of Hamilton Smith (106). Previously, the approach of the publicly funded HGP had been the creation of successive maps: a genetic linkage map of many genetic markers (103), a physical map of overlapping DNA segments (e.g. YACs; (102)), and finally the ultimate map, the DNA sequence. As opposed to this top-down approach, a bottom-up strategy, sequencing random fragments with assembly thereafter, was used by Venter et al. in the private genome project of Celera to get the complete genome sequence of microorganisms and subsequently of *Drosophila* (107) and the human.

The Human Mitochondrial Genome Project (HMGP), although never called that, had been completed long before. The HMGP had been done in an order opposite to the way the HGP was conducted. The complete sequencing was done first, by the group of Fred Sanger at Cambridge University (108). Then all the genes were identified, and finally disorders related to mutations in those genes were characterized.

The term genome appears to have been first used by Winkler in 1920 and to have been created by the elision of GENes and chromosOMEs to signify the set of chromosomes and the genes they contain (63). Thus, it is an irregular hybrid from two Greek roots. The term genomics is of more recent minting, having been proposed in July 1986 by Thomas H. Roderick of The Jackson Laboratory to designate the field of gene mapping and sequencing and specifically a new journal. The journal’s inaugural editorial (63) was entitled “A new discipline, a new name, a new journal.” The next 10 years showed a remarkable growth of the field and widespread use of the term genomics, so that in 1996 an editorial was entitled “An established discipline, a commonly used name, a mature journal” (109).

Goodfellow (1997) stated that he “would define genetics as the study of inheritance and genomics as the study of genomes.” Adjectives added to the word genomics have indicated specific aspects or applications of genomics. Mapping and sequencing constitute structural genomics. “Functional genomics,” continuing with Goodfellow’s definitions, “is the attachment of information about function to knowledge of DNA sequence.” Pharmacogenomics (110), toxicogenomics, comparative genomics, and physiologic genomics are some of the derivative terms for subfields of genomics (111).

Leading approaches for functional genomics are transgenic methods, which involve manipulation of the particular sequence in transgenic animals. Another



is database searching for similar sequences in other organisms where function is already known or can be determined more readily than is possible in the human. The latter is comparative genomics. Database searching has been referred to as research *in silico*, or cybergenomics.

Already before the HGP was completed, intense attention was directed to the function of the genome in a global sense as reflected by the protein gene products. Proteomics was a term invented about 1995 by Mark Russell who used two-dimensional display methods for peptides (112). Physiomics, a further derivation, is also called systems analysis or systems biology and refers further to function.

Functional genomics has also involved the study of gene expression as reflected by mRNA. Description of coordinated gene expression in various tissues, at various stages of development and in various physiological states, became possible with the development of micro-assay methods (chip technology) and other methods such as SAGE (serial analysis of gene expression) in the latter part of the 1990s. These methods for profiling gene expression represent the sixth of the major methodologies that have advanced medical genetics since 1956.

#### 1.4.6 Clinical Applications of Gene Mapping

Gene mapping, defined as the location of a gene to a specific chromosome site and/or the identification of markers that are close neighbors, has clinical value in diagnosis by the linkage principle and usefulness in identifying the nature of the basic defect either through positional cloning or through the candidate gene approach. J. B. S. Haldane (Figure 1-9) suggested in the 1920s that diagnosis by the linkage principle would be both possible and useful. In 1956, after Fuchs had suggested amniocentesis as a method of diagnosis of fetal sex based on the presence or absence of a Barr body in amniocytes, John H. Edwards (113) suggested that with amniocentesis one could do prenatal diagnosis by the linkage principle. The idea was further developed by McKusick (114), and McCurdy (115) demonstrated its practicability in connection with the hemophilia A carrier status, using the very closely linked G6PD marker. Schrott et al. applied the approach in connection with the closely linked myotonic dystrophy and secretor loci, the secretor status of the fetus being determinable in amniotic fluid (116,117). But the full strength of the linkage approach was not realized until the 1980s, when abundant DNA markers became available. As indicated earlier, haplotypes (i.e. clustered markers around the disease locus) were useful in the thalassemias and in some inborn errors of metabolism. Usually, however, linkage is an arduous, and not always practicable, approach because it requires the availability

of multiple family members and heterozygosity of the marker traits in key individuals. Furthermore, even under ideal circumstances, the available markers may be a distance from the disease locus, creating the possibility of a small, but finite chance of recombination, yielding false results. Thus, it is preferable to go for DNA diagnosis, that is, diagnosis based on the precise gene lesion.

In those disorders in which an enzyme deficiency or other protein abnormality has been identified, the wild-type gene can be cloned and defects in the gene identified. In the case of many Mendelian disorders, however, the nature of the biochemical defect was a mystery until the introduction of the mapping approach to identify the basic derangement. The first of the mystery diseases to be mapped by linkage to DNA markers was HD (86). It was, however, almost exactly 10 years to the week before the nature of the gene defect was reported (118).

Map-based gene discovery involves positional cloning and the candidate gene approach. Positional cloning was referred to initially as reverse genetics (119). Starting with the phenotype, mapping it and then going to the gene is, however, the approach of classic genetics; true reverse genetics will be increasingly practiced as fragments of DNA with transcriptional and other characteristics of a gene are investigated to determine their role in the phenotype, by methods such as transgenic, or “knockout,” mice. For this reason, Francis Collins (120) (Figure 1-31) suggested that the approach be called positional cloning, not reverse genetics (119,121). Table 1-1 lists, in chronological order of discovery, the disease-producing genes identified by positional cloning (that is, “walking in” on the gene from flanking markers) up to January 1994.

Whereas positional cloning and the candidate gene approach are map-based cloning, functional cloning starts with the functional gene product and uses reverse transcriptase to create a cDNA corresponding to the mRNA encoded by the particular gene. The candidate gene approach involves mapping the disease phenotype to a particular chromosomal location and then scrutinizing that chromosomal region for genes encoding enzymes or other proteins that might plausibly be implicated in the disease in question. Support for the involvement of the candidate gene in the disease is provided by the demonstration of absolute linkage of a RFLP or microsatellite marker within the gene with the disease in question, and the proof is clinched by the demonstration of a specific point mutation or other intragenic lesion. Elucidation of the defect in one form of hypertrophic cardiomyopathy by Seidman et al. (122,123) is an early example.

Many methods for identification of intragenic lesions have been identified over the past 10 years. By late 1994, disease-producing point mutations had been identified in almost 350 genes, and in many of these (e.g. CFTR, HBB) they numbered in the hundreds.



**TABLE 1-1 Map-Based Gene Discovery: Positional Cloning (Selected Examples)<sup>a</sup>**

Disorder	Gene	Location	MIM No.	Year
Chronic granulomatous disease, X-linked	CYBB	Xp21.1	306400	1986
Duchenne muscular dystrophy	DMD	Xp21.2	310200	1986
Retinoblastoma	RB1	13q14.1–q14.2	180200	1986
Cystic fibrosis	CFTR	7q31.25	219700	1989
Wilms' tumor	WT1	11p13	194070	1989
Neurofibromatosis type 1	NF1	17q11.2	162200	1990
Gonadal dysgenesis, XY female type	TDF, SRY	Yp11.3	480000	1990
Choroideremia	CHM	Xq21.2	303100	1990
Fragile X syndrome	FMR1	Xq27.3	309550	1991
Myotonic dystrophy	DM	19q13.2–q13.3	160900	1992
X-linked agammaglobulinemia	AGMX2	Xp22	300310	1993
Neurofibromatosis type 2	NF2	22q12.2	101000	1993
HD	HD	4p16.3	143100	1993
Multiple endocrine neoplastic type 2	RET	10q11.2	171400	1993
Breast cancer, familial, type 1	BRCA1	17q21	113705	1994
Polycystic kidney disease-1	PKD1	16p13.3	173900	1994
Tuberous sclerosis-2	TSC2	16p13	191092	1994
Breast cancer, familial, type 2	BRCA2	13q	600185	1995
Werner syndrome	WRN	8p	277700	1996
Multiple endocrine neoplasia, type 1	MEN1	11q	131100	1997
Peutz–Jeghers syndrome	STK11	19p	175200	1998
Rett syndrome	RTT	Xq	312750	1999
Ellis–van Creveld syndrome	EVC	4p	225500	2000
Cartilage–hair hypoplasia	RMRP	9p	250250	2001
Alstrom syndrome	ALMS1	2p13	203800	2002
Hemochromatosis, juvenile	HJV	1q21	602390	2003
Pernicious anemia, congenital	GIF	11q13	261000	2004
Roberts syndrome	ESCO2	8p21.1	268300	2005
Methylmalonic aciduria, cblC type, with homocystinuria	MMACHC	1p34.1	277400	2006

<sup>a</sup>In addition to these Mendelian disorders, many genes with somatic mutations involved in neoplasia have also been identified by positional cloning, starting, for example, from translocation breakpoints.

MIM, *Mendelian Inheritance in Man* (144).

## 1.4.7 Evolution of Clinical Genetics

Knut Faber (124) attributed to Mendel a major role in shaping our thinking about nosology, particularly the classification of disease and the delineation of distinct disease entities. The advent of the bacteriologic era in the decades immediately after Mendel also had a powerful effect on nosology. Both developments sharpened the focus on etiology: the role of specific microorganisms or the role of specific mutant genes. Until little more than a century ago, jaundice, dropsy, anemia, and other disorders were treated like disease entities in medical textbooks and in medical thinking and practice. Although Mendelian thinking contributed importantly to the general concepts in medicine, genetics did not become involved significantly in clinical medicine until after the acquisition of an anatomic base, beginning in the late 1950s.

One can point to several examples of pre-Mendelian “pedigree genetics.” Patterns characteristic of autosomal dominant and autosomal recessive inheritance were commented on by Maupertuis in the 1750s, by Adams in

1814 (125), and by Sedgwick in the 1860s. The X-linked recessive pattern of hemophilia was noted in a newspaper account in the 1790s and in medical reports by Otto in 1803 and Hay in 1813 of early New England families (74, 126). Similarly, the X-linked recessive pedigree pattern of color blindness was described clearly by Swiss ophthalmologist Horner in Zurich in 1876 and even earlier by Earle (127) in Philadelphia.

The relationship of consanguinity to an increased frequency of genetic defects was demonstrated by Bemiss in 1857 in studies of congenital deafness.

Early post-Mendelian examples of pedigree genetics include a report of albinism as a recessive by William E. Castle at Harvard in 1902. Farabee, a graduate student with Castle, described brachydactyly as a Mendelian dominant trait, basing his thesis research on a family in his home town of Old Concord, Pennsylvania. The family was subsequently updated by Haws and McKusick (128). Harvey Cushing, the neurosurgeon and biographer of William Osler, published a large kindred with symphalangism (his term for ankylosis of the phalanges)

in a paper with accompanying foldout pedigree in the first issue of *Genetics* in 1916, which also contained the famous paper by Calvin Bridges on nondisjunction.

The three main principles of clinical genetics (perhaps they should be called the three main phenomena of significance to clinical genetics) are pleiotropism, genetic heterogeneity, and variability. The history of our understanding of each can be traced.

Pleiotropism refers to multiple phenotypic (i.e. clinical) effects of a single mutant gene. This phenomenon is important to clinical medicine because often an external feature that is part of the pleiotropism and that may be in itself benign and insignificant may point to the presence of serious internal disease and/or to the fact that the person is a carrier of the mutant gene. The term was introduced by Plate in 1910. Hadorn developed the concept in considerable detail on the basis of studies in *Drosophila*. In his *Animal Genetics and Medicine* (129), Hans Grüneberg gave numerous examples, particularly from the mouse, and presented what he called “pedigrees of causes” relating all features of the syndrome back to a unitary defect. Analysis of pleiotropism, with the demonstration of plausible pedigrees of causes, was an important aspect of heritable disorders of connective tissue (130). The evidence for a unitary basic defect was fundamental to research in Marfan syndrome and other disorders. It took more than 35 years for the prediction of a unitary connective tissue defect in Marfan syndrome to be substantiated and particularized (131,132).

Genetic heterogeneity means that any one of several genetic mechanisms can lead to the same or similar phenotype. The idea was implicit in the work of Johannsen of Copenhagen, who in the first decade of this century distinguished phenotype and genotype. It was he who introduced the two terms (as well as the word “gene”) and put forward the concept that the phenotype is no necessary indication of the genotype. Genetic heterogeneity is obviously of practical importance in clinical medicine, as the prognosis, appropriate genetic counseling, and effective treatment may vary among the several genetic forms of a given disorder. Many examples of genetic heterogeneity have been uncovered during the past 50 years, especially through the application of biochemical and molecular methods. A striking example was homocystinuria, which simulates Marfan syndrome closely because of dislocated lenses and skeletal features such as increased height, scoliosis, and deformity of the anterior chest, but homocystinuria has recessive, not dominant, inheritance; has thrombotic, not aortic, complications in the cardiovascular system; and, of course, has a biochemical marker in the form of homocysteine in the urine.

Baur, Lenz, and Fischer, in their textbook in the 1930s, recognized genetic heterogeneity. William Allen, who is honored by the William Allen Award of the American Society of Human Genetics, wrote about genetic heterogeneity when he pointed out that some disorders, such as Charcot-Marie-Tooth disease, occur in autosomal

dominant, autosomal recessive, and X-linked forms. Allen pointed out that as a generalization the autosomal recessive form is most severe, the autosomal dominant form mildest (and most variable), and the X-linked recessive form intermediate in severity. This generalization is sometimes called Lenz’s law, after Fritz Lenz of the Baur, Lenz, and Fischer textbook (and father of Widukind Lenz, medical geneticist of Münster, Germany).

Harry Harris (133) (Figure 1-8) of London, emphasized the heterogeneity of apparently simple “characters.” One of the problems central to all studies in human genetics arises from the difficulty of knowing whether a particular difference has been characterized in, as it were, a “chemically pure” form. What appears at first sight to be a homogeneous entity readily identifiable by a particular technique, and presumably having a unitary genetical causation, turns out, with the application of newer techniques to the problem, to consist of more than one quite distinct phenomenon ... The condition known as “cystinuria” provides a simple illustration of this point.

As one aspect of the “darker side” of heredity counseling, F. Clarke Fraser (134) pointed to genetic heterogeneity of clinical entities. He wrote “A lot of difficulty comes from the fact that for many diseases two clinically similar cases may be genetically different, and thus have different genetic prognoses.”

Newton E. Morton (79) demonstrated genetic heterogeneity in elliptocytosis when he found that the disorder is linked to the Rh blood group locus in some families and not in others.

Variation in the clinical picture is a characteristic of disease of all etiologies, both genetic and nongenetic. If the clinical picture resulting from a particular etiologic factor were invariant, clinical medicine would be child’s play. Learning clinical medicine is, to a large extent, learning how to cope with variation, as well as pointing out the significance of pleiotropism, Hans Grüneberg (129) emphasized that variability can depend on the genetic background of the particular mutation. A frequently cited example from the mouse was provided by the work of L. C. Dunn (1893–1974), who found that the brachyury mutation, usually manifested by a short tail, was accompanied by an almost normal tail in some genetic stocks.

Penetrance and expressivity are aspects of variation. The terms and the concepts they signify were introduced by Vogt in 1926 (135) and were used by Timofeeff-Ressovsky soon thereafter while he was working in Berlin with Vogt. (Timofeeff-Ressovsky, still in Berlin at the time the Russians captured the city in 1944, was sent to a gulag, where he was a fellow prisoner of Solzhenitsyn; see Solzhenitsyn’s *Gulag Archipelago* for references to the evening intellectual interactions by which they helped maintain each other’s sanity. Timofeeff-Ressovsky was partially “rehabilitated” toward the end of his life and attended the World Congress of Genetics in Moscow in 1978.)

Penetrance is an all-or-none phenomenon. Expressivity is variation in severity of a genetic disorder or trait. When expressivity is so low that the disorder cannot be recognized, the gene is said to be nonpenetrant. Obviously, nonpenetrance is to some extent related to the power of the methods for studying the phenotype. The more penetrating the method, the lower the frequency of nonpenetrance.

### 1.4.8 Clinical Armamentarium of Medical Genetics

Part of the reason for the creation of the American Board of Medical Genetics and comparable agencies in some other countries is the fact that since 1956 there is so much more that clinical geneticists can do. Thus, a mechanism is needed for oversight of the training and certification of practitioners.

Bradford Hill, the British biostatistician, suggested that the practice of medicine consists of seeking answers to three questions: What is wrong? The answer is diagnosis. What is going to happen? The answer is prognosis. What can be done about it? The answer is treatment. (David Danks of Melbourne suggested to me that the health professional should always keep a fourth question in mind: Why did it happen? The answer is etiology and pathogenesis, on which prevention and treatment can be based.)

Advances in diagnosis have come from both the clinic and the laboratory. Because the individual genetic disorders, of which there are many, are rare, clinical geneticists play a key role in diagnosis. For many of these disorders, there are not yet specific biochemical or other tests. Syndromology and dysmorphology are important aspects of the clinical geneticist's work. Cytogenetic diagnosis is his specific responsibility, at least for non-malignant disorders. The oncologist makes heavy use of cytogenetic diagnosis, particularly in relation to hematologic malignancies.

Prognosis in clinical genetics has somewhat different implications than prognosis in other parts of clinical medicine. The question—What is going to happen?—relates not only to the person at hand but also to other members of the family, particularly an unborn child. Testing for the carrier status in a condition such as Duchenne muscular dystrophy or hemophilia or for a disease in its presymptomatic stages as in HD is obviously of great importance to prognostication.

Neonatal screening and population-based screening for specific genetic diseases, and prenatal screening for Down syndrome in mothers over 35 years of age, do not fit neatly into the Bradford Hill paradigm of clinical medicine. Although they do address the question—What is going to happen?—the question—What is wrong?—has not been asked. The question is not even—Is something wrong?—as all genetic disorders are not tested for.

These procedures are at the interface between clinical medicine and public health, or at least between clinical

medicine and preventive medicine. I would not wish to suggest that I recognize a distinction between preventive medicine and clinical medicine; preventive medicine is an integral and exceedingly important aspect of clinical medicine.

Treatment is generally viewed as the “short suit” of clinical genetics. However, more can be accomplished than is realized, and I would emphasize that management is a better designation for the medical geneticist's role than is treatment, which implies a repertoire of measures almost exclusively pharmaceutical or surgical.

With this overview as a preamble, let me outline the development of the medical geneticist's clinical armamentarium during the past 50 years.

An important item in the armamentarium of the clinical geneticist is command of syndromology and dysmorphology. Genetic diseases include a large number of individually rare disorders many of which have little basis of diagnosis other than their particular clinical features. Syndromology is the art and science of recognizing distinct genetic entities by characteristic combinations of clinical manifestations. Robert Gorlin of Minneapolis and John Opitz of Madison, Wisconsin (later of Helena, Montana, and Salt Lake City), are two American syndromologists from among the many capable ones. Dysmorphology is the term introduced by David W. Smith (1926–1981) of Seattle, Washington, as an improvement on clinical teratology. It implies syndromology and as well encompasses considerations of etiology and pathogenesis; for example, the mechanisms by which the several features of malformation syndrome occur together were part of David Smith's focus, and his delineation of the fetal alcohol syndrome illustrated his attention not only to distinctive clinical features but also to causation. *Smith's Recognizable Patterns of Malformation Syndromes* (136) is a classic.

Many malformation syndromes and genetic disorders carry the name of a person, not always the first, who described the condition as a distinct entity. These eponyms have advantages when the basic nature of the disorder is unknown and thus a specific label based thereon is not possible. Usefully, eponyms remind medical geneticists of the roots of their field (137). Place names (toponyms) likewise link the disorder to the geographic or ethnic setting of the first (or early) description of the disorder or protein (e.g. hemoglobin) variant. Tangier disease and familial Mediterranean fever are examples.

Neither syndromology nor dysmorphology is limited to genetic disorders, but congenital anomalies of all etiologies are the responsibility of clinical geneticists. They must keep in mind all causation, both genetic and non-genetic and an interaction of the two. It was no accident that the teratogenic action of thalidomide was detected by a medical geneticist, Widukind Lenz, in the early 1960s. It was easy for a nongeneticist to consider phocomelia a genetic disorder; a geneticist would be more likely to recognize that the distribution of cases in time, place, and families was not consistent with a genetic basis.

Annual conferences entitled the Clinical Delineation of Birth Defects were initiated in 1968 at Johns Hopkins University with financial support of the March of Dimes. During their heyday, from 1968 to about 1978, these gatherings of the aficionados illustrated the attention given by clinical geneticists to the development of the field of syndromology/dysmorphology. The annual David W. Smith conferences continue in the same tradition.

Several computerized databases have been developed during the past 10–15 years as an aid to the syndromologist: the London Dysmorphology Database of Robin Winter; GenDiag of Ségolène Amye, Paris; and POSSUM of Agnes Bankier and David Danks of Melbourne.

### 1.4.9 A Synthesis: 1956–2001

Interestingly, 1956 was not only the year that human chromosomology got off to a firm start but also the date of the First World Congress of Human Genetics, which took place in Copenhagen under the presidency of Tage Kemp. The 10th and the most recent of these quinquennial congresses was held in 2001. The first congress was a splendid survey of the status of human genetics at the time. The subsequent congresses were milestones measuring progress since 1956 (138).

At the first congress in 1956, Tjio and Levan (Figure 1-6A and B) and Ford and Hamerton (139) (Figure 1-7A and B) were getting the chromosome number right. Newton Morton was writing on linkage analysis. Oliver Smithies was beginning to write on starch gel electrophoresis. Vernon Ingram was narrowing down the molecular defect in sickle hemoglobin to a single peptide, and the first edition of my *Heritable Disorders of Connective Tissue* (130) was published.

Behold what happened between the first congress and the eighth held in Washington in 1991. From the anatomy of the chromosomes at the most elementary level of enumeration, we had gone to their dissection by both mechanical and molecular methods. Genetic linkage had enjoyed a phenomenal renaissance, and linkage was by then analyzed on populations of sperm studied individually by direct molecular methods. Rather than studying variation in proteins by electrophoresis, we were examining variation in the DNA itself. From the one example of sickle hemoglobin, the known mutational repertoire of the  $\beta$ -globin gene had expanded to more than 400, for example. At least one disease-related point mutation had been defined in more than 170 different genes, a count that had passed the 1000 mark by the end of 2000 (140).

Among the heritable disorders of connective tissue, clinical delineation had been refined by molecular definition. For example, in the mucopolysaccharidoses, lumped under the Hurler syndrome in 1956, at least 10 enzymatically distinct entities had been defined and, in the disorders of the fibrous elements of connective tissue, precise intragenic lesions had been described in

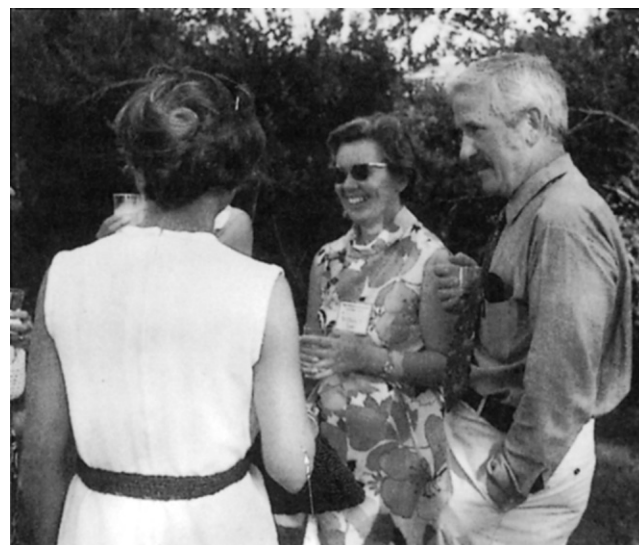
osteogenesis imperfecta, in some of the Ehlers–Danlos syndromes and skeletal dysplasias, and in the Marfan syndrome.

By the 1961 congress in Rome, chaired by Luigi Gedda, clinical chromosomology had arrived. From the findings in Turner syndrome and Klinefelter syndrome, the role of the Y chromosome in sex determination was realized; the existence of testis-determining factor (TDF) was deduced. The hypothesis of the single-active X chromosome, advanced by Mary Lyon (141,142) (Figure 1-33), was the intellectually provocative new concept. Electrophoretic polymorphisms of serum proteins and red cell enzymes were being described. The “Philadelphia chromosome” was found in chronic myelogenous leukemia, one of the first pieces of evidence in humans supporting the chromosome theory of cancer (18).

By 1991, imprinting had taken the place of lyonization. TDF had been cloned and characterized under the label “sex-determining region of Y.” The fundamental basis of specific forms of cancer had been traced to specific genes—sometimes multiple, sequentially collaborating genes—and to specific mutations within genes.

The Chicago congress in 1966 was under the presidency of Lionel S. Penrose of London (Figure 1-20). The genetic code had been completely deciphered that year. Somatic cell genetics had entered the scene for the study of inborn errors of metabolism. For example, it provided the strongest proof of the Lyon hypothesis and, through the study of cultured amniotic cells, opened the way for prenatal diagnosis by amniocentesis. The concept of lysosomal diseases had emerged, and the first edition of *Mendelian Inheritance in Man* (MIM) (143), already computer based, was published earlier that year.

Review of the growth of successive editions of MIM provides an opportunity to engage in some scientometrics. As indicated by the original subtitle “Catalogs



**FIGURE 1-33** Mary Lyon (center) with Wesley Whitten at Bar Harbor, 1971.



of Autosomal Dominant, Autosomal Recessive, and X-linked Phenotypes,” MIM was an encyclopedia of phenotypes—genetic traits and disorders. But early on it was the intent that there should be one entry per gene locus, based largely on the philosophy that, if two genetic diseases or traits result from mutation at different loci, they are fundamentally distinct, however similar in phenotype they may be. Genetics is finding genes—gene hunting. The number of entries in successive editions of MIM can serve as one basis of quantifying progress in our field in the past 40 years. There were about 1500 entries in the first edition. In the 1960s, the only way we had to identify separate entries (read “gene loci”) was by mendelizing phenotypes, sometimes aided by biochemical or immunologic characteristics or by genetic features such as linkage. In the 1970s, the rate of accession was accelerated by a parasexual method for gene identification and mapping, namely, somatic cell hybridization. By the 1980s, cloning of human genes was practiced. Accordingly, entries in MIM were created for genes when they were cloned, sequenced, and mapped, even though no Mendelian phenotype had been associated. By the 12th edition (144), entries in MIM numbered 8587, an impressive figure but still a long way from the 60,000 to 70,000 expressed genes the human was then thought to have (145). (By fall 2000, the number in the continuously updated online version, OMIM, was approaching 12,000.) Furthermore, by the 1990s, the evolution of the content of MIM as outlined above had led to a self-redefinition that prompted change of the subtitle to “*A Catalog of Human Genes and Genetic Disorders*” in the 11th edition (1994) and thereafter.

By the Paris congress in 1971, the study of inborn errors of metabolism in cultured cells had paid off. For example, elucidation of the defect in Lesch–Nyhan syndrome and the differentiation of various mucopolysaccharidoses had been accomplished through the study of cultured cells. Chromosome banding had been introduced, and the first genes were being assigned to specific autosomes by linkage to chromosomal heteromorphisms and rearrangements and by interspecies somatic cell hybridization. There were four autosomal gene assignments by 1971.

Plotting the growth of the human gene map since 1971 is another exercise in scientometrics. By June 1976, just before the fifth congress in Mexico City, at least one gene had been assigned to every chromosome. This was largely through the application of somatic cell hybridization. By the 1981 congress in Jerusalem, molecular genetic methods for gene mapping had entered the scene and were responsible for further acceleration of mapping in the 1980s. Molecular genetics provided probes for identification of human genes in rodent/human somatic cell hybrids. It provided probes for in situ hybridization to chromosomes and, importantly, it provided DNA markers (e.g. RFLPs, VNTRs) for family linkage studies, for mapping Mendelian disorders of unknown biochemical basis. By the time of the 1991 congress, a total of at

least 2300 genes had been mapped to specific chromosomes and, for most, to specific chromosomal regions.

By the fifth congress in Mexico City in 1976, other advances of note included the concept of receptor diseases—disorders such as familial hypercholesterolemia (146) and androgen insensitivity—and the Knudson hypothesis of hereditary/sporadic tumors (49,147). The Philadelphia chromosome had been reinterpreted as a reciprocal translocation rather than a deletion.

By the 1981 congress in Jerusalem, under the presidency of James V. Neel (Figure 1-34), in addition to the advances in the methods and results of gene mapping, human genes were being cloned, the genetic basis of antibody diversity was well on the way to elucidation, human variation was being studied with monoclonal antibodies, and one human chromosome had been completely sequenced—the mitochondrial chromosome.

By 1986 and the congress in Berlin, presided over by Arno Motulsky (Figure 1-34), HD had been mapped using RFLPs—the first disorder of unknown biochemical basis mapped by this approach. VNTRs were a new class of markers. The previous spring, PCR had been unveiled at the Cold Spring Harbor Symposium, which had been devoted to the human genome. PFGE, DGGE, and CVS were introduced as acronyms for other new techniques or diagnostic methods. Contiguous gene syndromes were conceptualized and the deletions underlying them used for gene mapping and gene isolation. The approach, then called “reverse genetics,” had succeeded in isolation of the gene for chronic granulomatous disease and at the very time of the congress was well on the way to characterization of the large gene that is mutant in Duchenne muscular dystrophy. Transgenic mice expressing human genes had been created. By molecular methods, the Knudson hypothesis had been proved for retinoblastoma; and the specific oncogenic molecular changes in the Philadelphia chromosome of chronic myeloid leukemia had been worked out, as well as those in the translocations underlying Burkitt lymphoma.



**FIGURE 1-34** Three presidents of the World Congress of Human Genetics (left to right): Arno Motulsky (Berlin, 1986), James V. Neel (Jerusalem, 1981), Victor McKusick (Washington, 1991). Photo from 1991. Neel died in 2000 at the age of 85.

By the time of the eighth congress in 1991 (chaired by Victor McKusick; Figure 1-34), the HGP had been launched. The HGP had been in a stage of debate and planning in 1986. The fruitfulness of positional cloning (alias reverse genetics) had been established for Duchenne muscular dystrophy, cystic fibrosis, neurofibromatosis, polyposis coli, and others. The candidate gene approach had also paid off for retinitis pigmentosa, hypertrophic cardiomyopathy, Marfan syndrome, and malignant hyperthermia. The genetics of common cancers such as colon cancer had been greatly clarified. TDF had been cloned. Specific mutations in the mitochondrial chromosome had been related to specific diseases. Imprinting and uniparental disomy were challenging concepts.

Between the eighth congress and the ninth (1996), in Rio de Janeiro, Brazil, with Newton Morton as president, ESTs (expressed sequence tags) were introduced (148) as a shortcut to the expressed part of the genome, the first free-living organism (*H. influenzae*) was completely sequenced (1995), a detailed linkage reference map using DNA markers was completed, a physical map of YAC contigs was published, radiation hybrid mapping came into wide use, the role of folic acid in birth defects was accepted as established and folic acid supplementation of food products introduced into practice. Preimplantation diagnosis at a four- or eight-cell stage was added to in vitro fertilization (IVF), as a method for selection of non-mutant concepti.

Between the ninth congress in Rio de Janeiro and the 10th in Vienna in 2001 (G. Utermann, president), database “mining” (research in silico or cybergenomics) came in as a primary method of genomic research. As a result of positional cloning and related approaches to disease gene discovery, well over 1000 genes had been found to have one or more disease-related mutations (140). Microassay methods (chip technology) had been introduced for profiling gene expression and for gene diagnosis (149). Yeast became the first eukaryote to be completely sequenced (in spring 1996 shortly before the Rio congress). *Caenorhabditis elegans*, a nematode, was the first metazoan completely sequenced (in 1999), followed by *Drosophila* (in 2000). On June 26, 2000, completion of a first draft of the complete human sequence was announced jointly by Venter and Collins, for the private and public projects, respectively, in a historic ceremony in the East Room of the White House, with connection by satellite to 10 Downing Street in London where Prime Minister Blair echoed President Clinton in emphasizing the significance of the event.

During the 1996–2001 interval, rapid progress toward the goal of the HGP was accompanied by the following paradigm shifts:

- from structural genomics to functional genomics;
- from map-based gene discovery to sequence-based gene discovery;
- from genetic disease diagnosis to detection of genetic predisposition of common disorders;
- from etiology (specific causation) to pathogenesis (mechanism);
- from a one-gene-at-a-time approach to study of systems, pathways, and families of genes;
- from genomics to proteomics.

Another paradigm shift was from medical genetics to genetic medicine. This was not merely a change of name of institutions, although that did occur, as in the program at Johns Hopkins, which began in 1957 as the Division of Medical Genetics in the Department of Internal Medicine, became in 1989 a multidisciplinary Center for Medical Genetics, and in 1999 was transformed into an institute of genetic medicine. Beyond a change in name, this shift is a broadening of perspective. Medical genetics tends to imply an exclusive focus on rare Mendelian disorders and chromosomal aberrations; genetic medicine reflects the fact that genetics pervades all parts of clinical medicine, that genetic factors are involved in all disease, and that genetic predisposition in the etiology of common disorders (complex traits) is an important part of the science and practice of medical genetics.

Since 1956, human genetics has become medicalized, to use Motulsky’s term, to an enormous extent. It has become subspecialized. Medical genetics has become professionalized through the development of clinical colleges and certifying organizations. During the past decade, human genetics has also become intensely molecularized. Molecular genetics pervades all aspects of human and medical genetics. Human genetics has become commercialized to an extent we might not have predicted. The field has also become democratized and universalized; its implications are felt in all aspects of society. It has become consumerized; consumerism is evident in the role of genetic support groups and foundations for funding of research on single genetic diseases.

#### 1.4.10 Forty Years in the History of Medical Genetics

*Mendelian Inheritance in Man* (MIM) has recorded in detail the advances in medical genetics in the period since it was first initiated in 1960 as a catalog of X-linked traits (126). The catalog of recessives was undertaken in late 1962 in connection with studies of an inbred group, the Old Order Amish. The dominant catalog was created in 1963. The catalogs were placed on the computer in 1964. The first print edition of all three catalogs (a pioneer in computer-based publication) appeared in 1966, the 12th in 1998. The 12 editions represent serial cross-sections of the field of medical genetics over four decades.

MIM was made publicly available online (under the designation OMIM) in 1987. OMIM (<http://www.ncbi.nlm.nih.gov/omim>) has the virtue of timeliness (it is updated

almost daily) and easy searchability. The print version has usefulness in a nonelectronic setting and as an archive.

The evolution of medical genetics since the early 1960s is illustrated in many ways by MIM (and OMIM). The subtitle at the beginning was “*Catalogs of Autosomal Dominant, Autosomal Recessive, and X-linked Phenotypes*.” Although the gene behind the phenotype was always in mind (e.g. the X-linked catalog was created as an indication of the genetic constitution of the X chromosome), almost the only way to identify a gene was through a mendelizing phenotype, at the beginning. With molecular genetic advances, the subtitle in later print editions became “*A Catalog of Human Genes and Genetic Disorders*.”

## 1.5 THE FUTURE

Since the late 1980s, thought and discussion have been devoted to the future of human genetics to an unprecedented extent—largely growing out of the debate over the HGP and the planning for it. Rarely, if ever, has the future of a scientific field and its implications for society been given such wide and intense attention. An unusual and important feature of the federally funded HGP in the United States was support of research projects in the ethical, legal, and societal implications (ELSI) of genome mapping and sequencing.

ELSI was a proactive effort to avoid misuse and abuse of newfound genomic information. The history of eugenics and its misguided, unethical, and immoral practices based on primitive knowledge of human genetics in the first half of the twentieth century was a warning signal. The atrocities in Nazi Germany (150) were only the most flagrant, inhuman, and abominable examples. Furthermore, it was anticipated that the power of information on the full genome of individuals and population groups would raise novel issues for society, medicine, and the law.

Completion of the HGP provides a source book of the human that will be the basis for study in human biology and medicine for a long time to come. When all the genes have been found, we certainly still will not know, for most of them, their function even in solo, let alone in concert with the rest of the genome. True reverse genetics, in the New Genetics according to Walter Gilbert, David Botstein, and others, will involve working from specific DNA sequences of unknown function to the phenotype. Having in the past 35 years worked progressively from the phenotype to DNA, we will in the next 35 years be returning from DNA to the phenotype by determining the function of specific DNA sequences. Just as the function of much of the DNA remains to be determined even though the full sequence is known, the worldwide variation in that DNA is largely unknown, and long study will be required of the relationship between DNA variation and variation in function, critical to the understanding of evolution and the genetics of disease susceptibility and performance.

Study of the source book provided by the HGP should be particularly useful in the two great frontiers of human biology: how the mind works and how development is programmed. In the area of health and medicine, great benefit can be expected from the better understanding of genetic factors in multifactorial disorders, which tend to be the common conditions such as hypertension and major mental illness. The source book will surely be very useful to the understanding of somatic cell genetic disease. Gene mapping and related studies have been primarily responsible for appreciation that in addition to the three classical categories of genetic disease—single gene disorders, multifactorial disorders, and chromosomal aberrations—somatic cell genetic disease is a large fourth category. This has been most extensively and definitively established through the definition of mutation as the basis of cancer. (Facetiously, it is suggested that medical genetics is taking over oncology.) Somatic cell mutations are likely to occupy us increasingly as the basis of congenital malformations, autoimmune processes, and even aging. (McFarlane Burnett suggested about 1960 that somatic cell mutation is the basis of autoimmune processes, and a somatic mutation theory of aging has been entertained for a long time.) The connection between oncogenesis and teratogenesis—between oncogenes and teratogenes, if you will—is already adumbrated by the examples of Wilms’ tumor and Greig cephalosyndactyly syndrome, to mention two. It is to be assumed that somatic cell gene therapy not only for inherited diseases but perhaps also for some of these acquired somatic cell genetic diseases, especially neoplasia, will become available during the next decade.

Two scientific and technologic revolutions, the biologic revolution and the information revolution, converged in the human genome initiative. Information is power. Risks can accompany both the political and the scientific changes. Appropriately, ethical, legal, and societal implications of the human genome initiative are being examined in many parts of the world.

The methods developed by the HGP will allow the rapid and economical generation of information on the genomes of individuals. In the medical area, this information will widen the gap between what we know how to diagnose and what we know how to treat, between what we can diagnose in the presymptomatic state and what we can prevent. We have had that problem, for example, in the case of HD. The complete map and sequence are also likely to increase the gap between what we think we know and what we really know. But by this second gap, I refer in part to the likelihood that weak associations will be found between particular genomic constitutions and certain presumed characteristics such as criminality or alcoholism or elements of intelligence or performance. Some of these associations are likely to be spurious. Other weak associations may be found to be statistically valid but will be blown out of all proper proportion to the detriment of individuals



and of groups. As geneticists, we have a responsibility to avoid unfounded conclusions and overblown interpretations and to inculcate the profound respect for the genetic variability that is the strength of the species and indeed of the individual—referring to the differences in the two genomes each of us has, one from the father and one from the mother.

The mere existence of the complete reference gene map and DNA sequence down to the last nucleotide may lead to the absurdity of reductionism, the misconception that we then know everything it means to be human, or to the absurdity of genetic determinism, that what we are is a direct and inevitable consequence of what our genome is. Our phenotypes are not “hard-wired” to our genotypes. Risk figures that state the chance of given common disorder in an individual based on a genome screen are probabilities, not certainties. They are more analogous to a weather report than to a road map.

Thus, information on the reference gene map and sequence of the human may represent per se a hazard, if it distorts the way we think about ourselves and our fellow human beings. The ability to analyze the genomes of individuals is accompanied by risks of information misuse and abuse. We must be alert to the need to protect the privacy and confidentiality of the information that the HGP will allow to be collected on the genetic constitution of individuals. We must make every effort to avoid the misuse or abuse of such information by third parties, and our governments may need to take measures to assure these protections under law.

Near the end of his terms of office, President Eisenhower warned against the dangers of the military industrial complex. It is appropriate to warn of a potential hazard of the genetic–commercial complex. The increasing availability of tests for presumed genetic quality or poor quality could lead the commercial sector and the Madison Avenue publicist to bring subtle or not so subtle pressure on couples to make value judgments in the choice of their gametes for reproduction. Autonomy in reproductive choice is a cornerstone of the ethics of genetic counseling. That reproductive choice would not be autonomous if subjected to the Madison Avenue type of pressure. Especially, trivialization of reproductive choices should be avoided.

As human geneticists, we are privileged to work in a scientifically important field and a field of intellectual challenge. Human genetics is a field that holds particular fascination because it involves the most fundamental and pervasive aspects of our own species, an added fascination that the physical sciences or pure mathematics, for example, cannot share. To have combined with this intellectual and anthropocentric fascination the opportunity to contribute to human welfare and to be of service to families and individuals through medical genetics and clinical genetics is a privilege. The privilege carries with it responsibilities to which I have already referred.

## 1.6 ADDENDUM (PSH)

### 1.6.1 The Founders of Clinical Genetics

The section headed “the evolution of Clinical Genetics” in McKusick’s original chapter tells us much about the advances in the field over the past half century, but almost nothing about the people responsible or the pattern of evolution across different countries. Perhaps this is not too surprising, for he would have unavoidably had to give much prominence to himself. Now there need not be any such inhibitions and an account, necessarily brief, of the founders of the field forms an important part of its history.

While there were medically trained researchers in human genetics from the beginning of the twentieth century, such as Julia Bell in Britain (105) and Madge Macklin and William Allan in America (Soltan, 1992), Clinical Genetics as a medical discipline did not take shape in any significant way until the 1950s, when medical workers in several countries began to recognize that the scientific foundations of human genetics were now substantial enough for the systematic development of both clinically oriented research and genetic services.

In North America the initial step can be seen as the founding in 1950 of a medical genetics unit at Montreal Children’s Hospital by F. Clarke Fraser, followed in 1957 by academic departments (technically Divisions of Departments of Medicine) in Baltimore and Seattle, by Victor McKusick and Arno Motulsky, respectively. In Europe, France took an early lead, with Pediatrician Maurice Lamy appointed as Professor of Medical Genetics at Hôpital Necker, Paris in 1953, succeeded by Jean Frézal. Britain, in the form of Lionel Penrose’s Galton Laboratory at University College, London, had provided a focal point from 1945 for training and inspiring many of the founders from elsewhere, but did not itself develop as a clinical genetics unit, concentrating on basic human genetics research after Penrose’s retirement, while John Fraser Roberts, active from the 1930s and founding the first UK genetic counseling clinic in 1948, also restricted his clinical role to genetic counseling alone, as did his successor Cedric Carter. The two major British founders in the field of clinical genetics were Pediatrician Paul Polani, based at Guy’s Hospital, London, and Cyril Clarke, internist and head of Medicine at the Liverpool Medical School. It is of interest to note that the medical background of these founders in both Britain and North America was at least as much from adult medicine as from pediatrics.

In many countries, development of clinical genetic services was considerably slower; for Germany this is not unexpected, given the legacy of abuses from eugenics under the third Reich, but it is more surprising in the case of the Scandinavian countries, which had from an early stage been leaders in human genetics research. From the very beginning, though, close international links have

been a strong feature of medical genetics, encouraged and reinforced by the numerous visiting workers at Penrose's Galton Laboratory and by the trainees from many different countries with Victor McKusick in Baltimore, most of whom became leaders in their own countries on their return and were strongly influential in determining the pattern and ethos of genetic services which they and their successors established.

### 1.6.2 Victor McKusick and the History of Medical Genetics

The role of Victor McKusick in the history of Medical Genetics is a threefold one. As a key founder of the field, he and his own work, over a period of more than 50 years (114), form a major strand in its history and development; secondly, his periodic reviews on the current status of medical genetics chronicle successive advances ((126); Mckusick, 1989) and many of his other writings have a strongly historical approach. Finally from the beginning of his career he took a keen interest in the history of medicine and published a series of studies on specific physicians whose early reports formed important landmarks in the documentation of inherited disorders (143). A fuller assessment of McKusick's role as a historian of Medical Genetics can be found in the chapter by Harper in the book of Dronamraju and Francomano (2012).

Another, more unusual contribution has come from McKusick's habit of taking numerous photographs at conferences, courses and all other possible occasions, both formal and informal. Many of the then young and unknown scientists and clinicians who appear in these informal "snaps" have since risen to fame. This collection must amount to several thousand images over the years and it is greatly to be hoped that they will be carefully preserved and cataloged along with his other personal scientific records at the Johns Hopkins Archive.

Only a few have been published, some in this chapter, others in relation to the Bar Harbor "short course" on medical genetics (1).

Altogether medical genetics is fortunate to have had such an assiduous and objective chronicler of its first half century as Victor McKusick has been.

### 1.6.3 Preserving the History of Medical Genetics

The inevitable loss of the founders of Medical Genetics reminds us how much of its history we lose when they die. Not all of the key workers have such a keen historical sense as did Victor McKusick; many confine their writing to current scientific or clinical activities. The records of past discoveries, like old personal scientific records and correspondence, are often destroyed or lost, while increasingly, older workers continue with their research up to an advanced age and give less thought to documenting or reflecting on their past achievements.

For these and other reasons a coordinated effort is required if the history of medical genetics is to be fully recorded for posterity, rather than future historians being left with a few, perhaps unrepresentative, fragments on which to base their future analyzes. Fortunately, it is not too late for much to be salvaged, and several significant initiatives have already been started, both in Europe and America. Table 1-2 lists some of these; increasingly they are web-based, making the end results accessible to all. Oral history, as well as written records, forms a key element, especially recorded interviews with early workers in the field.

At the same time though, the pace at which "current advances" turn into "history" is quickening, so it is essential that documenting the history of a particular area begins as early as possible; a prime example is the HGP, but the beginnings of human molecular genetics in medicine are likely to be of equal significance.

**TABLE 1-2 History of Human and Medical Genetics Sources and Resources**

#### Records of individual workers

American Philosophical Society Genetics Collection (APS) (<[www.amphilsoc.org](http://www.amphilsoc.org)>).

UK Genetics Archive Project (<[www.genmedhist.org/Records](http://www.genmedhist.org/Records)>).

Cold Spring Harbor Archive (<<http://library.cshl.edu/>>).

#### Oral history

Oral history of human genetics project (<[www.socgen.ucla.edu/hgp/](http://www.socgen.ucla.edu/hgp/)>).

Interviews with human and medical geneticists (<<http://www.genmedhist.org/Interviews>>).

*Talking of Genetics* (Gitschier, 2010)

Witness Seminars. See <[www.ucl.ac.uk/histmed/publications/wellcome\\_witnesses\\_c20th\\_med](http://www.ucl.ac.uk/histmed/publications/wellcome_witnesses_c20th_med)> for the transcripts of several seminars relevant to medical genetics.

#### Books

See "Further reading"; also Harper (2008), Chapter 18 and Appendix 1 for details.

#### Book collections

Human Genetics Historical Library (see <<http://www.genmedhist.org/HumanHistLib/>>). A collection of over 3000 books.

Electronic Scholarly Publishing (<[www.esp.org](http://www.esp.org)>) has digitized a series of books and papers on classical genetics.

Cold Spring Harbor Laboratory Press has published a number of important historical books on genetics and eugenics.

## 1.7 A TIMELINE FOR MEDICAL GENETICS

Modern medical genetics as a well-defined field of medicine has developed so rapidly since its beginnings half a century ago, that it is often forgotten how far back in time its roots and origins go (Table 1-3). It can be reasonably argued that genetics overall was based in considerable measure on problems of human inheritance and inherited disease, and studies of this extend back long before the twentieth century acceptance of Mendelism. Thus medical genetics, when thought of in the

widest sense, is perhaps the oldest area of genetics, and certainly not the recent addition that it is sometimes portrayed as.

This “timeline” gives some of what I consider to be the main landmarks along this lengthy course. Not all of these can be considered to be directly part of “medical genetics,” even on the broadest definition, but they are all relevant to it in one way or another. I have also included some more general “world events” that have particularly impacted on the development of the field. I shall welcome suggestions for other items that might be included in future updates.

**TABLE 1-3 A Timeline for Human and Medical Genetics. Based on the Timeline of the Genetics and Medicine Historical Network (<[www.genmedhist.org](http://www.genmedhist.org)>). The Original Version First Appeared in Harper PS (2008): A Short History of Medical Genetics, OUP**

1651	William Harvey's book <i>De Generatione Animalium</i> studies the egg and early embryo in different species and states: “Ex ovo omnium” (all things from the egg).
1677	Microscopic observations of human sperm (Leeuwenhoek).
1699	Albinism noted in “Moskito Indians” of Central America (Wafer)
1735	Linnaeus, <i>Systema Naturae</i> . First “natural” classification of plants and animals.
1751	Maupertuis proposes equal contributions of both sexes to inheritance and a “Particulate” concept of heredity.
1753	Maupertuis describes polydactyly in Ruhe family; first estimate of likelihood for it being hereditary.
1794	John Dalton. Color blindness described in himself and others. Limited to males. Erasmus Darwin publishes <i>Zoonomia</i> . Progressive evolution from primeval organisms recognized.
1803	Hemophilia in males and its inheritance through females described (Otto).
1809	Inherited blindness described in multiple generations (Martin). Lamarck supports evolution, including man, based on inheritance of acquired characteristics.
1814	Joseph Adams, Concepts of “predisposition” and “disposition”; “congenital” and “hereditary.”
1852	First clear description of Duchenne muscular dystrophy (Meryon).
1853	Hemophilic son, Leopold, born to Queen Victoria in England.
1858	Charles Darwin and Alfred Russel Wallace. Papers to Linnean Society on Natural Selection.
1859	Charles Darwin publishes <i>On the Origin of Species</i> .
1865	Gregor Mendel's experiments on plant hybridization presented to Brunn Natural History Society.
1866	Mendel's report formally published.
1868	Charles Darwin's “provisional hypothesis of pangenesis.” Charles Darwin collects details of inherited disorders in <i>Animals and Plants under Domestication</i> .
1871	Friedrich Miescher isolates and characterizes “nucleic acid.”
1872	George Huntington describes “Huntington's disease.”
1882	First illustration of human chromosomes (Flemming).
1885	“Continuity of the germ plasm” (August Weismann).
1887	Boveri shows constancy of chromosomes through successive generations.
1888	Waldeyer coins term “chromosome.” Weismann presents evidence against inheritance of acquired characteristics.
1889	Francis Galton's <i>Law of Ancestral Inheritance</i> .
1891	Henking identifies and names “X chromosome.”
1894	Bateson's book <i>Material for the Study of Variation</i> .
1896	EB Wilson's book <i>The Cell in Development and Inheritance</i> .
1899	Archibald Garrod's first paper on alkaptonuria.
1900	Mendel's work rediscovered (deVries, Correns and Tschermak).
1901	Karl Landsteiner discovers ABO blood group system. Archibald Garrod notes occurrence in sibs and consanguinity in alkaptonuria.
1902	Bateson and Saunders' note on alkaptonuria as an autosomal recessive disorder. Bateson and Garrod correspond. Garrod's definitive paper on alkaptonuria an example of “chemical individuality.” Bateson's <i>Mendel's Principles of Heredity</i> . A <i>Defence</i> supports Mendelism against attacks of biometricians. Chromosome theory of heredity (Boveri, Sutton).
1903	American Breeders Association formed. Includes section of eugenics from 1909. Cuénot in France shows Mendelian basis and multiple alleles, for albinism in mice. Castle and Farabee show autosomal recessive inheritance in human albinism. Farabee shows autosomal dominant inheritance in brachydactyly.

*Continued*



**TABLE 1-3 A Timeline for Human and Medical Genetics. Based on the Timeline of the Genetics and Medicine Historical Network (<[www.genmedhist.org](http://www.genmedhist.org)>). The Original Version First Appeared in Harper PS (2008): A Short History of Medical Genetics, OUP—cont'd**

1905	Stevens and Wilson separately show inequality of sex chromosomes and involvement in sex determination in insects. Bateson coins term "genetics."
1906	First International Genetics Congress held in London.
1908	Garrod's Croonian lectures on "inborn errors of metabolism." Royal Society of Medicine, London, "Debate on Heredity and Disease." Hardy and Weinberg independently show relationship and stability of gene and genotype frequencies (Hardy–Weinberg equilibrium).
1909	Bateson's book <i>Mendel's Principles of Heredity</i> documents a series of human diseases following Mendelian inheritance. Karl Pearson initiates <i>The Treasury of Human Inheritance</i> . Wilhelm Johannsen introduces term "gene."
1910	Thomas Hunt Morgan discovers X-linked "white eye" <i>Drosophila</i> mutant. Eugenics Record Office established at Cold Spring Harbor under Charles Davenport.
1911	Wilson's definitive paper on sex determination shows X-linked inheritance for hemophilia and color blindness.
1912	Winiwarter proposes diploid human chromosome number as approximately 47. First satisfactory human chromosome analysis. First International Eugenics congress (London).
1913	Alfred Sturtevant constructs first genetic map of <i>Drosophila</i> X-chromosome loci. American Genetics Society formed as successor to American Breeders Association.
1914	Boveri proposes chromosomal basis for cancer. (Outbreak of World War I).
1915	Haldane et al. show first mammalian genetic linkage in mouse.
1916	Relationship between frequency of a recessive disease and of consanguinity (F. Lenz). Calvin Bridges shows non-disjunction in <i>Drosophila</i> .
1918	Anticipation first recognized in myotonic dystrophy (Fleischer). R.A. Fisher shows compatibility of Mendelism and quantitative inheritance.
1919	Hirszfeld and Hirszfeld show ABO blood group differences between populations. Genetical Society founded in UK by William Bateson.
1922	Inherited eye disease volumes of <i>Treasury of Human Inheritance</i> (Julia Bell).
1923	Painter recognizes human Y chromosome; proposes human diploid chromosome number of 48.
1927	Hermann Muller shows production of mutations by X-irradiation in <i>Drosophila</i> . Compulsory sterilization on eugenic grounds upheld by courts in America (Buck v. Bell).
1928	Stadler shows radiation-induced mutation in maize and barley. Griffiths discovers "transformation" in <i>Pneumococcus</i> .
1929	Blakeslee shows effect of chromosomal trisomy in <i>Datura</i> , the thorn apple.
1930	R.A. Fisher's <i>Genetical Theory of Natural Selection</i> . Beginning of major Russian contributions to human cytogenetics. Haldane's book <i>Enzymes</i> attempts to keep biochemistry and genetics linked.
1931	Archibald Garrod's second book <i>Inborn Factors in Disease</i> . UK Medical Research Council establishes Research Committee on Human Genetics (Chairman J.B.S. Haldane).
1933	Nazi eugenics law enacted in Germany.
1934	Fölling in Norway discovers phenylketonuria (PKU). <i>Treasury of Human Inheritance</i> volume on Huntington's disease (Julia Bell). O.L. Mohr's book <i>Genetics and Disease</i> . Mitochondrial inheritance proposed for Leber's optic atrophy (Imai and Moriwaki, Japan).
1935	First estimate of mutation rate for a human gene (hemophilia; J.B.S. Haldane). A preliminary estimate had been made by Haldane in 1932. R.A. Fisher (amongst others) suggests use of linked genetic markers in disease prediction.
1937	First human genetic linkage—hemophilia and color blindness (Bell and Haldane). Moscow Medical Genetics Institute closed; director Levit and others arrested and later executed. Destruction of Russian genetics begins. Seventh International Genetics Congress, Moscow canceled. Max Perutz begins crystallographic studies of hemoglobin in Cambridge.
1938	Lionel Penrose publishes "Colchester Survey" of genetic basis of mental handicap.
1939	Seventh International Genetics Congress held in Edinburgh. "Geneticists' Manifesto" issued. (Outbreak of World War II) Cold Spring Harbor Eugenics Record Office closed. Rh blood group system discovered (Landsteiner and Wiener).
1941	Beadle and Tatum produce first nutritional mutants in <i>Neurospora</i> and confirm "one gene—one enzyme" principle. Charlotte Auerbach discovers chemical mutagens in Edinburgh (not published until the end of the war).
1943	Nikolai Vavilov, leader of Russian genetics, dies in Soviet prison camp. First American genetic counseling clinic. Mutation first demonstrated in bacteria (Luria).

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1944	Schrödinger's book <i>What Is Life?</i> provides inspiration for the first molecular biologists. Avery shows bacterial transformation is due to DNA, not protein.
1945	Lionel Penrose appointed as head of Galton Laboratory, London, founds modern human genetics as a specific discipline. (Hiroshima and Nagasaki atomic explosions.) Genetic study of effects of radiation initiated on survivors of the atomic explosions (J.V. Neel director).
1946	Penrose's inaugural lecture at University College, London, uses PKU as paradigm for human genetics. John Fraser Roberts begins first UK genetic counseling clinic in London. Sexual processes first shown in bacteria (Lederberg).
1948	Total ban on all genetics (including human genetics) teaching and research in Russia. American Society of Human Genetics founded. H.J. Muller, President. J.B.S. Haldane suggests selective advantage in thalassemia due to malaria.
1949	<i>American Journal of Human Genetics</i> begun. Charles Cotterman, first editor. Linus Pauling et al. show sickle-cell disease to have a molecular basis. J.V. Neel shows it to be recessively inherited. Barr and Bertram (London, Ontario) discover the sex chromatin body.
1950	Curt Stern's Book <i>Human Genetics</i> . Frank Clark Fraser initiates Medical Genetics at McGill University, Montreal.
1951	Linus Pauling shows triple helical structure of collagen. HELA cell line established from cervical cancer tissue of Baltimore patient Henrietta Lacks.
1952	First human inborn error shown to result from enzyme deficiency (glycogen storage disease type 1, Cori and Cori). Rosalind Franklin's X-ray crystallography shows helical structure of B form of DNA.
1953	Model for structure of DNA as a double helix (Watson and Crick). Bickel et al. initiate dietary treatment for PKU. Enzymatic basis of PKU established (Jervis). Specific chair in Medical Genetics founded (first holder Maurice Lamy, Paris).
1954	Allison proves selective advantage for sickle-cell disease in relation to malaria.
1955	Sheldon Reed's book <i>Counselling in Medical Genetics</i> . Oliver Smithies develops starch gel electrophoresis for separation of human proteins. Fine structure analysis of bacteriophage genome (Benzer).
1956	Tjio and Levan show normal human chromosome number to be 46, not 48. First International Congress of Human Genetics (Copenhagen). Amniocentesis first validated for fetal sexing in hemophilia (Fuchs and Riis).
1957	Ingram shows specific molecular defect in sickle-cell disease. Specific Medical Genetics departments opened in Baltimore (Victor McKusick) and Seattle (Arno Motulsky).
1958	First HLA antigen detected (Dausset).
1959	Harry Harris' book <i>Human Biochemical Genetics</i> Perutz completes structure of hemoglobin. First human chromosome abnormalities identified in: Down's syndrome (Lejeune et al.). Turner syndrome (Ford et al). Klinefelter syndrome (Jacobs and Strong).
1960	Trisomies 13 and 18 identified (Patau et al. and Edwards et al.). First edition of <i>Metabolic Basis of Inherited Disease</i> . Role of mRNA recognized. First specific cytogenetic abnormality in human malignancy (Nowell and Hungerford, "Philadelphia chromosome"). Chromosome analysis on peripheral blood allows rapid development of diagnostic clinical cytogenetics (Moorhead et al.). Denver conference on human cytogenetic nomenclature. First full UK Medical Genetics Institute opened (under Paul Polani, Guy's Hospital, London). First Bar Harbor course in Medical Genetics, under Victor McKusick.
1961	Prevention of rhesus hemolytic disease by isoimmunization (Clarke et al., Liverpool). Mary Lyon proposes X-chromosome inactivation in females. Cultured fibroblasts used to establish biochemical basis of galactosemia (Krooth and Weinberg), establishing value of somatic cell genetics. "Genetic Code" linking DNA and protein established (Nirenberg and Matthaei).
1963	Population screening for PKU in newborns (Guthrie and Susi).
1964	Ultrasound used in early pregnancy monitoring (Donald). First journal specifically for medical genetics ( <i>Journal of Medical Genetics</i> ). Genetics restored as a science in USSR after Nikita Khrushchev dismissed. First HLA workshop (Durham, North Carolina).
1965	High frequency of chromosome abnormalities found in spontaneous abortions (Carr, London, Ontario). Human-rodent hybrid cell lines developed (Harris and Watkins).

Continued

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1966	First chromosomal prenatal diagnosis (Steele and Breg). First edition of McKusick's <i>Mendelian Inheritance in Man</i> . Recognition of dominantly inherited cancer families (Lynch).
1967	Application of hybrid cell lines to human gene mapping (Weiss and Green).
1968	First autosomal human gene assignment to a specific chromosome (Duffy blood group on chromosome 1) by Donahue et al.
1969	First use of "Bayesian" risk estimation in genetic counseling (Murphy and Matalik). First Masters degree course in genetic counseling (Sarah Lawrence College, New York).
1970	Fluorescent chromosome banding allows unique identification of all human chromosomes (Zech, Caspersson et al.).
1971	"Two-hit" hypothesis for familial tumors, based on retinoblastoma (Knudson). Giemsa chromosome banding suitable for clinical cytogenetic use (Seabright). First use of restriction enzymes in molecular genetics (Danna and Nathans).
1972	Population screening for Tay-Sachs disease (Kaback and Zeiger).
1973	Prenatal diagnosis of neural tube defects by raised alpha fetoprotein (Brock). First Human Gene Mapping Workshop (Yale University).
1975	DNA hybridization (Southern) "Southern blot."
1977	Human beta-globin gene cloned.
1978	Prenatal diagnosis of sickle-cell disease through specific RFLP (Kan and Dozy). First mutation causing a human inherited disease characterized (beta-thalassemia). First birth following IVF (Steptoe and Edwards).
1979	Vogel and Motulsky's textbook <i>Human Genetics, Problems and Approaches</i> .
1980	Primary prevention of neural tube defects by preconceptional multivitamins (Smithells et al.). Detailed proposal for mapping the human genome (Botstein et al.).
1981	Human mitochondrial genome sequenced (Anderson et al.).
1982	Linkage of DNA markers on X chromosome to Duchenne muscular dystrophy (Murray et al.).
1983	First autosomal linkage using DNA markers for Huntington's disease (Gusella et al.).
1983	First general use of chorionic villus sampling in early prenatal diagnosis.
1984	DNA fingerprinting discovered (Jeffreys).
1985	Application of DNA markers in genetic prediction of Huntington's disease. First initiatives toward total sequencing of human genome (US Dept of Energy and Cold Spring Harbor meetings).
1986	PCR for amplifying short DNA sequences (Mullis).
1988	International Human Genome Organisation (HUGO) established. US congress funds HGP.
1989	Cystic fibrosis gene isolated. First use of preimplantation genetic diagnosis.
1990	First attempts at gene therapy in immunodeficiencies. Fluorescent in situ hybridization introduced to cytogenetic analysis.
1991	Discovery of unstable DNA and trinucleotide repeat expansion (fragile X).
1992	Isolation of <i>PKU</i> (phenylalanine hydroxylase) gene (Woo et al.). First complete map of human genome produced by French <i>Généthon</i> initiative (Weissenbach et al.).
1993	Huntington's disease gene and mutation identified. <i>BRCA 1</i> gene for hereditary breast-ovarian cancer identified.
1996	"Bermuda Agreement" giving immediate public access to all HGP data.
1997	First cloned animal (Dolly the sheep), Roslin Institute, Edinburgh.
1998	Total sequence of model organism <i>Caenorhabditis elegans</i> . Isolation of embryonic stem cells.
1999	Sequence of first human chromosome (22).
2000	"Draft sequence" of human genome announced jointly by International Human Genome Consortium and by Celera. Correction of defect in inherited immune deficiency (SCID) by gene therapy, (but subsequent development of leukemia).
2002	Discovery of microRNAs.
2003	Complete sequence of human genome achieved and published.
2005	Sequencing of chimpanzee genome.
2006	Prenatal detection of free fetal DNA in maternal blood clinically feasible.
2007	First genome-wide association studies giving robust findings for common multifactorial disorders.
2008	First specific individual human genomes sequenced.
2010	Sequencing of Neanderthal genome. Diagnostic use of human exome sequencing.
2011	Modern human genome shown to contain sequence from other ancient hominins (Neanderthal and Denisovan).



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### Biography

**Peter Harper** was born in Barnstaple, UK; he trained in Medicine at Oxford University and in London, qualifying in 1964. After working at the Liverpool Institute of Medical Genetics with Cyril Clarke he spent 2 years as a research fellow with Victor McKusick at Johns Hopkins Hospital, Baltimore, before returning to Britain to found the Wales Institute of Medical Genetics at Cardiff where he became the Professor of Medical Genetics at the same institute. On retirement from this post in 2004, he was appointed as University Research Professor of Human Genetics at Cardiff University to develop his work on the history of human and medical genetics.

Dr Harper's main research interests have been in the clinical and molecular genetics of inherited neurological disorders, notably myotonic dystrophy and Huntington's disease. He has also been extensively involved with service delivery, policy issues and social and ethical aspects of medical genetics at a national and international level.

During the past decade he has led an international initiative to preserve and record the history of human and medical genetics; his historical books include *A Short History of Medical Genetics* (2008, revised edition 2013), *First Years of Human Chromosomes* (2006) and *Landmarks in Medical Genetics* (2004).

# CHAPTER

# 2

## Medicine in a Genetic Context

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### 2.1 INTRODUCTION

The history of science is characterized by an exponential rate of expansion (1). No aspect has escaped, but biology, which is relatively new, has by all accounts exploded. Naturally, these changes are reflected in new principles, new thinking, and new ways of handling new information. Among the problems created is that of making these novelties available to practitioners of science of all kinds.

Among the ways suitable to medicine are massive volumes that contain detailed summaries of diseases, usually of one class, such as endocrine, gastroenterological, or, as in the case of *Principles and Practice of Medical Genetics* (PPMG), inherited. And of course, the pace of change requires revisions, always characterized by increases that reflect the rate of accumulation. Fission adds volumes whose pages, chapters, contributors, and diseases all do their best to obey the exponential imperative. Each chapter represents one topic more or less, an expansible topic capable of embracing new disorders with each new edition, so the number of chapters is no guide to the number of diseases. In addition to new diseases, new paths of basic science are added, a characteristic of books that mirror progress in reductionist investigation. But where is reductionist biology taking us? Clearly, one direction is toward fragmentation; more and more is learned about increasingly restricted fields so that even specialties bifurcate and medicine becomes ever more splintered. But despite such assaults, whatever it is we call medicine has at the bottom some integrity, some consistency, and common grounds that are clearly revealed in PPMG as well as in its sister enterprise, *The Metabolic and Molecular Basis of Inherited Disease* (MMBID) (2). One such common ground is genetics. And as there is included in both these books disorders of such a striking variety of cellular structures and metabolic mechanisms engaging every organ and organ system, it is easy to imagine that genetic variation is at the

basis of all diseases. This idea is far from new, having been suggested even in the eighteenth and nineteenth centuries, when it took the form of diathesis and idiosyncrasy (3). Then, in this century, it appears in the shape of a continuity between clear-cut segregating monogenic diseases and varying degrees of familial aggregation of cases that suggest the outcomes of the actions of more than one gene acting in environments favorable for the onset of a disease. But now, with the advent of genomics, which makes possible the study of the genetics of diseases of complex origin in families of patients who have affected relatives, as well as in those who do not, we are learning that genetic variation underlies the latter no less than the former. So the continuity of segregating to nonsegregating familial aggregation is extended to include cases where there is neither segregation nor aggregation (4). Perhaps we should require a disease to be shown not to be associated with any genetic variation, before saying it has no genetic basis.

All professions undergoing rapid change and increasing specialism face the same dilemma. The generalists who, as generalists, must keep up, find the density of new information daunting, even impossible to sort out and retain. So, books such as the PPMG are intended to present this information in an orderly way and in relation to specific diseases. But the job is no sooner done than even newer information arrives to change how the various disorders are perceived, and, of course, treated. Furthermore, new diseases have been described and must be included. Hence another edition must appear. And that's not all. The various sciences that contribute to our understanding are all changing, too, providing new insights that challenge conventional thinking. Editors respond to this intense pressure by including articles that present not only new information but also new insights, new ways of thinking about groups of diseases or perhaps all, and these usually appear at the beginning, hinting strongly that the reader of any later chapter would do well to read these preliminary ones.

It would seem that the editors of PPMG suppose that, for example, the principles of chromosomal organization, genomics or the investigations of diseases of complex

<sup>†</sup>Deceased.



origin would help the reader better to understand the chapters on developmental anomalies, the origins of high blood pressure, or inborn errors. And this may happen, given the effort. But each reader who makes this synthesis for himself or herself is likely to do it in the context of some specific disorder rather than to generalize the principles to all diseases. Indeed, we lack a clearly articulated set of principles of disease as opposed to diseases. That is not to say that medicine lacks principles; the idea of the body as a machine that breaks and needs fixing is one, and the medical history, diagnosis, pathogenesis, treatment, prognosis, and prevention all have a conceptual basis, as do the basic sciences related to medicine. But disease as a concept seems to be taken for granted. Textbooks of medicine, pediatrics, and pathology often begin with generalities, but not about disease. Medical texts sometimes begin by defining the practice of medicine, the meaning of signs and symptoms, doctor–patient relationships and medical ethics, whereas pediatric texts include growth and development, relations with parents, and the special quality of the medicine of infants and children. Perhaps it is an expression of medicine’s pragmatism. Why try to define something you know cannot be deciphered at a satisfactory level, even though you know that the level must be molecular? This is the view of the editors of the twenty-first edition of the *Cecil Textbook of Medicine*, published in 2000 (5). The editors say “true understanding of disease processes depends upon levels of scientific knowledge that are just being discovered.” Pathology probably comes nearest to touching on the principles of disease in generalizing on cell injury, inflammation, cell death, and tissue repair. But no book suggests that a student of medicine (and we are all students throughout the length of our careers) might take profit in an account of disease as opposed to diseases, including why we have it, who is likely to be affected and how, and when in the lifetime and what forms can it take, as well as what are its constraints? That is, what are the explanatory generalizations that compose a context within which to fit all diseases?

Similarly, definitions of disease have fallen by the wayside. It is true that many such definitions have been offered; there is a sizable literature on the subject (6–9). Perhaps today’s reluctance stems from physicians’ perception that we have not had the wherewithal for any but descriptions based on signs and symptoms rather than anything at its core. But today we are satisfied with a definition of a disease when pathogenesis is explained by reference to abnormality of some metabolic or homeostatic system, and we can describe the qualities of the proteins that compose the system. Now, if that is so, why may we not define disease as a consequence of incongruence of a metabolic or homeostatic system with conditions of life? And as all such systems are composed of proteins capable of reflecting the variations of their genetic origins, is it not appropriate to agree with Vogel and Motulsky who, in the third edition of their book, *Human Genetics*, proclaimed genetics as the principal “basic science for medicine” (10)?

If genetics is the basic science for medicine, it should be possible to construct a set of principles that characterize

disease in a genetic context. That is, a set of generalizations shared by all diseases and framed in genetic terms. And there should be hierarchies of principles, inclusive and of increasing generality and forming a matrix embracing them all. What follows is one such matrix.

## 2.2 THE PRINCIPLES OF DISEASE

A foundation for developing principles of disease exists in the ideas of Ernst Mayr (11). Mayr perceives biology as divided into two areas differing in concept and method. One, functional biology, is concerned with the operation and interaction of molecules, systems, and organisms. Causes are proximate, the viewpoint is inward, and questions are commonly preceded by how; how does the organism function? The other area, Mayr calls evolutionary. It is concerned with the history of functional biology, its causes are called ultimate, and its questions are prefaced by why; why in the sense of, what is the history of organisms, what are the conditions of the past that have made it possible to ask for answers to the how questions? The two areas of biology meet, or overlap, at the level of the DNA, so that the functional deals with everything after transcription, whereas the evolutionary centers on the history of the DNA as well as, presumably, with the evolution of the conditions of the environment within which organisms have attained their current state.

Mayr did not include disease in his description of the two biologies, but disease is no less biological than the ideal state, so there should be no difficulty in applying his principles to biological abnormality. So in relation to disease, the proximate causes are (1) the products of the variant genes and (2) the experiences of the environment with which they are incongruent. Ultimate or remote causes are (1) the mechanisms of mutation and the causes of fluctuations through time of the elements of the gene pool, including selection, mating systems, founder effects, and drift and (2) the means whereby cultures and social organization evolve. In disease, the variant gene products and the experiences of the environment with which they are incongruent account for characteristic signs and symptoms, but in making available the particular proximate causes assembled by chance in particular patients, it is the remote causes that impart the stamp of individuality to the case.

So the model relates disease to causes, to the gene pool and ultimately to biological evolution as well as to the evolution of cultures, and to individuality, the latter a consequence of the specificities of both causes. Here there are also elements for constructing a context of principles of disease, always remembering that the word context derives from the Latin word *contexere*, meaning to weave. That is, the principles must be seen to be related and interdependent so as to form a network of ideas within which to compose one’s thoughts about each specific example of each disease.

There is a further feature of Mayr’s views on biology, also crucial in its application to disease (12). It is the state of mind in which to observe patients. In medicine, we tend to think of patients in relation to their disease,

that is, as a class of people characterized by the name of the disease. This is what Mayr calls typological thinking. Although patients do differ somewhat from one another, they all share an essence: the disease. In contrast, Mayr proposes population thinking, in recognition that populations consist not of types but of unique individuals. So, in this context, disease has no essence; its variety is imparted by that of the unique individuals who experience it, each in their own private version, and the name of the disease is a convenience, an acknowledgment of the necessity to group patients for logistical purposes. The fruits of the Human Genome Project (HGP) can be accommodated only with such a populational perspective.

Why do we need such principles? Physicians are pragmatic; their way is determined by what they see before them, and students and especially residents are intolerant of anything they can label “philosophical.” But the principles are there, explaining the qualities and behaviors of diseases, and they await exposition.

But have we not already discovered them? Medicine is at the peak of success in diagnosis and treatment and moving rapidly to ever new heights of achievement. But all changes may not be equally evident. For example, the analysis of pathogenesis, traditionally a top-down process, is beginning to give way to a bottom-up approach in which discovery of variant genes leads to variant protein products and thence to the same molecular analysis of pathogenesis (see Chapter 16). Also, the genetic heterogeneity and individuality of disease are not easily accommodated in traditional thinking. So we are changing how we look at disease, how we define and classify it, and the language we use in describing it. For example, genomics and proteomics are new words that embody ways of thinking new in the past several decades (4,13,14). These developments are changing our relationships to biology and society. Biologists are expressing interest in the fates of the molecules they discover, and the public is becoming aware of what molecular biology and genetics mean to them, as risk factors, for example (15,16). So, because this same molecular genetics gives us new insights into the principles that govern—and have always governed—disease, should we not articulate those principles and weave them into our thinking?

Reasons for doing so lie in the need for coherence in medicine, coherence in the face of reductionist dispersion, coherence in bringing new developments to the whole of the medical enterprise and to the public, and coherence in medical education and the thinking that goes into it. No one can possibly know all the information there is, but we all need a context that can supply both a substrate on which to apply the new and a receptacle within which to encompass our own field. The principles of disease bear a relationship to diseases that resemble the relationship of military strategy to tactics, of historiography to the practice of history, or of grammar to precision in learning and speaking a language. Once such principles have sunk into the unconscious, they remain there as a context

and a basis from which the conscious thinking about the subject takes off. They are no longer “philosophy” but the basis for daily thought.

## 2.3 DEFINING DISEASE

If we are to define disease, it must be as loss of adaptation; the open system has had difficulty in maintaining homeostasis. So our question is how is this failure of adaptation attained? The straightforward answer is to say that a variation in a homeostatic system was incongruent with its environment, whether within the cell or outside, and the mechanisms for compensation were inadequate, momentarily or permanently, to restore congruence (see Chapter 16). As a result, other systems were affected, and then still others. But this only tells us that the machine broke down. If we would define disease, we must know what variations can lead to what levels of incongruence. We must know the weaknesses in the evolution of organisms, or if not weaknesses, the degrees of flexibility. That is, the origins of human disease lie in both human evolution and the environment with which the human species has evolved to be congruent. And because both biological and cultural evolution are continuous, although at markedly different rates, congruence must be relative and changing.

Such questions have always been germane to the definition of disease, however infrequently posed, but they assume a new relevance now because of the frequent assertion that one needs to know only the molecular form of the incongruence to devise an appropriate treatment. That is, all our problems could be solved at the molecular level. If this were true, we do not need to define disease except molecularly, and that is the vision we pursue. But before committing wholly to such a concept, it is as well to probe further into the question, what is disease?

It may seem odd to ask such a question; surely it has been answered again and again. And so it has, and many times, but always within the descriptive limits of the period. Descriptions and definitions of disease have in history proceeded from top down and from outside in. That is, from a history of the illness only, to history plus inspection, then on to physical examination in life and at autopsy, to increasingly intimate inspections by radiological and newer visual means, as well as biochemical examination, and now molecular analysis. Now that we can proceed from the bottom up, beginning with genes identified by genomics to their protein products and to the homeostatic systems into which they are integrated, the definition of disease should be reconsidered.

History reveals two opposing definitions. One, called essentialist, proposes that diseases exist somehow and in some way as entities that attack their victims. The other, called nominalist, is represented as a change within, an altered state, or a deviation in response to some stimulus. In the essentialist view, the patient is healthy and is brought low by the disease, whereas in the nominalist, the disease is an expression of the particularity of the

individual response to a stimulus. This modern-sounding construction was popular in the nineteenth century in the form of “diathesis,” in which there was suggested some element of heredity as well as individual vulnerability (3,17). It was swept aside by the essentialist version, which emerged, in the later part of the nineteenth and early twentieth centuries, when microorganisms were discovered. Then the nominalist began to regain favor as, first, biochemistry, then genetics, and then molecular biology flourished. Today, although we still experience microbial scourges, the nominalist view prevails, perhaps because we can so easily see that the responses to diseases, even to microbial assaults, are a product of the individuality of the systems of homeostasis and because we are more perceptive of the interrelationships of proximate and remote causes. But there is a lingering residuum of essentialism in molecular diagnosis, so often proclaimed to be a preliminary to some “designer” treatment, usually to be concocted by pharmaceutical companies who have made the word pharmacogenetics their own. Such a diagnosis is unobjectionable as far as it goes, but it has implications. That is, it is an essentialist view insofar as (1) it emphasizes the disease without differentiating the patient; (2) it includes only proximate causes, the gene and its product are perceived as no less essentialist than the microbe that attacks; and (3) it is typological. Even though allelic heterogeneity may be acknowledged, the variation is around the expressions of the “classical case.” There is no recognition that each patient will respond to the effects of the products of each gene individually, to say nothing of each designer drug. For many years, the monogenic diseases were regarded in an essentialist typological vein, but we have become more nominalist, more prone to population thinking, and more ready to recognize the significant effects of variability of both the genetic and the environmental settings in which the principal gene effect is measured (18). So, in defining disease, we must not only take into account the gene that seems most relevant to the phenotype—after which the phenotype may be neglected in the interest of molecular treatment—but also keep alive the relationships of genes (or better, of their products) and phenotypes, the better to grasp the individuality of each, so as to tailor the particularity of the molecular treatment to the biological individuality of a very particular patient. Then, having that principle in mind, the necessity to group patients for treatment can be managed rationally.

So, in the end, how shall we define disease? The elements of Mayr’s model must be satisfied. That is, the definition must include remote as well as proximate causes and the relationship of both to DNA. Also, it must be populational in concept rather than typological, which is to say, it must be nominalist. So, one way of expressing it is as follows: disease is a consequence of incongruence between genetically variable homeostatic systems and the kinds, intensities, and durations of exposures to elements of the environments to which they are called upon to adapt.

No doubt objections will be raised. Is cyanide poisoning a disease? No human variation is needed. Nor is there variation in susceptibility to scurvy, although it is unquestionably a disease. But although cyanide will extinguish all life, scurvy is a disease of species; only we and the guinea pig among mammals are vulnerable. Still, it is fair to say that human homeostasis is uniformly incongruent in the presence of cyanide and the absence of ascorbic acid, and so both qualify for this definition. Others see poisoning and trauma as something other than disease and call them by other names—accidents for one. But again the human constitution is incongruent with bullets and car crashes and so is vulnerable. How about infections? We have genetically determined mechanisms, both well developed and efficient, in coping with microbial invaders. Here, the variability includes individual vulnerability in the many immunodeficiencies, as well as individual invulnerability, both relative and absolute, in individuals who are immune to infections caused by many organisms including one malarial parasite, the tubercle bacillus, and the polio virus (19).

And if there are those that are immune to, who can doubt that there are individualities susceptible to particular organisms? In general, microorganisms attack at cellular sites we define as strengths but which they have defined for their purposes as our weaknesses; for example, cell surface molecules designed for high efficiency as elements of metabolism, but which the organisms have adapted themselves to use as means of access. The point is that it is usually variation in the microbial, rather than the human, cell that brings particularity to the encounter, although there may be both. So, because variation in either human victim or microbial attacker or both determine the nature of the encounter, infections fulfill the nominalist definition, even while as entities they are compatible with, and are even the prototype of, the essentialist definition. This suggests that both definitions are of historical interest only, suitable for the levels of description of disease that we have left behind. But they are still of value in showing how the more intimate we become with the human body, organ, and molecule, the more our concepts change and the more we need to shed old ideas and their locutions and adapt to what the new is telling us. But still we should observe that much of what is new was foreshadowed in the old. A leisurely reading of the chapter “The inborn factors in infective diseases” in Archibald Garrod’s book, *Inborn Factors in Disease*, published in 1931, underlines the validity of that observation (20).

## 2.4 THE HOW QUESTIONS

In the Mayr model, it is by way of DNA that remote causes leave their imprint on the proximate and it is the protein gene products that are the effectors of both. Once we spoke of gene–environment interactions, but now we know that the actual contact between these sources of variation is by way of those proteins. Indeed, one way



of perceiving the DNA is as a molecule that is helpless without proteins that carry out all its ends, including transcription and translation too (21). So the proteins carry on the life of the cell as elements in integrated systems, responding to influences from adjacent cells, distant organs, and the outside, to maintain the open system in its uncertain relationships in life. They are, therefore, unit steps of homeostasis, and as such are pivotal in concepts of life, development, aging, health, and disease.

Such a list of attainments is banal without explanation and illustration. In the following section, there are several ways in which the unit steps of homeostasis fulfill the purposes of the cell. They are called unit steps to convey their elemental state as units of pathways, and cascades, structural elements, protein machines, transducers in signaling systems, and transporters or receptors of molecules that are going somewhere. The phrase further implies units of integration into systems intended to maintain the organism's steady state; they are the node between nature and nurture, and the phrase has the virtue of being indifferent to whether or not the specific protein fulfills a useful purpose or is disruptive. And finally, the unit steps have an important historical meaning, representing the central idea of Garrod's inborn error, Beadle and Tatum's one gene-one peptide, and Pauling's molecular disease (22–24).

## 2.5.1 Some Qualities of the Unit Step of Homeostasis

**2.5.1.1 As a Unit of History.** Clearly, DNA is an instrument of memory, a memory that in preserving the past, gives guidance for the future. That is, the future must always reflect the past, and the means whereby this Janus vision is attained is the protein gene product that repeats its phylogenetic history in its current composition and function and predicts its future in its reincarnation through subsequent generations as itself or in the form of variants. Some of the variants have no future and their incongruence is noted by natural selection. Others are contingent, favorable for some conditions and inappropriate for others. And then there are those proteins that have hardly changed from microbial ancestry and that represent core functions. In human society, political and religious systems have similar capacities for endurance, revealing fundamental unchanged dogma associated with adaptation in ways that promote the cause with little change in the fundamentals. So the proteins that constitute our proteomes descend to us not only from our parents and other human antecedents but also, with variable conservation, from both the ancient and recent past.

**2.5.1.2 As Effectors of Gene Intention.** We often speak of a gene or genes as being “for” something, by which we indicate some sort of direct relationship to a phenotype. That is, we seem to be saying that the gene's influence is determining. And so it is, if by determining

we mean the sequences of bases in mRNA and of amino acids in a protein product. In this sense, the gene is indeed “for” something. But each gene product has in addition an emergent career of its own, not predicted at all, or only indirectly, by its gene. It assumes a position in the homeostatic device to which it belongs and can now be said to be “for” that system, as the factor VIII gene is “for” both the factor VIII protein and “for” clotting. But it is far from determining of clotting; all the other elements are needed, too, or, as we all well-know, life-threatening bleeding occurs. We also know that in physiology system is integrated with system in hierarchical relationships, so that the farther away from the steps of translation and first integration, the more dilute the gene's determining power becomes. No doubt the genes are involved wherever their products are to be found, but indirectly, and any one may have little power to shape the ultimate phenotype. In another sense, the genes appear to be hardly involved at all beyond transcription because it is the quality of the protein product that determines its role in the economy of the cell, a role that is determined by how the protein folds and takes shape, a shape that must accommodate to the shapes of the products of other genes and they with still others. No doubt the protein's folding and shape reflect the information residing in its parent gene, but its gene has no control over the shapes of those other proteins with which it fits, to say nothing of how multiprotein machines work (25). Here is a question not of genes but of how proteins interact. It is a matter of physiology. Indeed, it is possible that as the fruits of the HGP and the proteomists filter into medicine, we will hear a good deal less about genes and more about proteins (26). This could be less than ideal were the proteins not perceived to be as closely identified with the concept of variation as the genes. Let us see to it that they are.

**2.5.1.3 As a Unit of Development.** T. H. Morgan adopted *Drosophila* as an organism suitable for the study of development (27). But it did not work out that way, and his students led the way to the operational definition of the gene. So it is ironic that modern technology has made the fruit fly ideal for the very study that defied Morgan's efforts (28,29).

In development, the genes fulfill their intentions in the ways just described. Their products are the units of developmental change, assuming positions in systems appropriate for their conformation so as to give each organism a matrix, embodying a trajectory of change that is a product of how the embryo, fetus, and baby meet and respond to experiences of intrauterine and external environments. That is, development is a historical process; what the organism is today is built upon what it was yesterday and leads to what it will be tomorrow (30). And because the genes see to the continuity of their products throughout the changes of a lifetime, it is hardly likely that the influences of the past, however distant, would fail to influence the present. So if we would understand the origins and

expressions of disease, it must be in the context of three timescales, all at once: that of phylogeny, that of development maturation and aging, and that of the present (31). To know what we begin with is to know potential incongruence; to know where development is taking, or has taken, us is to clothe the potential with the probable, one way or the other; and to know where we are at the moment is to know the strengths and weaknesses that we will face tomorrow. There is an increasing interest in the idea that some diseases of middle life have precursors, manifestations dating to early, even intrauterine, life. These expressions may not appear to relate to the disease they are said to characterize. Rather, they may represent subtle changes in trajectories that, if pursued, emerge finally as disease (32). How else could birth weight be related to type II diabetes or heart attack?

**2.5.1.4 As a Unit of Individuality.** In medicine, patients are seen one at a time. Each one is biologically unique, has different experiences, and tells a different story. These expressions, together with the help of the laboratory and observations over time, are compared with those of the classical case to reach a diagnosis, and, allowing something for variation, treatment or management is devised. This thinking is typological, individuality is usually ignored, and the doctor is in thrall to nosology. The method works well enough, but heterogeneity of proximate cause may be overlooked and patients are likely to be aware when they are being perceived as representative of a class rather than as their unique selves. Now, molecular biology has given us the wherewithal to observe molecular individuality, that is, the capacity to make comparisons between individuals of variations in base pairs in the DNA and differences in amino acid sequences in proteins. The unit of individuality is the unit step of homeostasis, and the expression of uniqueness lies in how the variant proteins affect each its own system and the integrations of the latter with others, as well as how the systems respond to nongenetic proximate causes. Genomic analysis of single nucleotide polymorphisms (SNPs) suggests that the number of polymorphic loci expressed in amino acid substitution in proteins will turn out to be somewhat greater than the 30% we are accustomed to (33,34). This is the substrate of variability within which additional “private” variants as well as clearly bad mutants express their effects, and all this variation is manifested in how the integrated homeostatic devices are fulfilling their duties. So, if each human being is unique by virtue of the variant proteins in his or her whole physiological apparatus, why should not each such human being express an experience with disease as variously as a career of health.

Variation contributed by variant proteins is far from all. Such variability is compounded by the individuality of the developmental and maturational trajectory characteristic of each person, a path determined no less strongly by the kinds, intensities, and durations

of experiences than by the protein gene products with which they interact. But the final arbiter of individuality is the remote causes, which determine the specificity of both genes and experiences. The variation in the parental gene pool is a sample of what is available to the species, but it is necessarily limited, characterized by ethnicity and made local by founder effect, migration, and mating customs. These are all influences that determine the particularity of an individual’s genetic endowment. But if genetic individuality is both determined and constrained by the genetic raw material inherited at conception, so is the variety of experiences made possible and limited by the mores of the social and cultural milieu, itself often inherited, which shape our likes and dislikes, our indulgences and restraints, in short, the qualities and quantities of the experiences we encounter. So, in the end, it is the remote causes that confer the specificity of individuality, but the unit steps of homeostasis that supply the substrate. Of course, the idea of variant proteins as units of individuality is not a new one, having been proposed by Archibald Garrod as “chemical individuality” as early as 1902 (23).

**2.5.1.5 The Unit Steps as Effectors of Disease.** If the gene product is the implement of homeostasis, it follows that it is the effector of disease; certain of its variants are in some degree incongruent with the environment, inside the cell or out. That is, wherever the origins and mechanisms of pathogenesis have been laid bare, there are proteins at the root of it. How could it be otherwise, given that both structures and motivators of the functions of cells are proteins, and disease is a consequence of homeostatic incongruence. A critic might suggest infections as exceptions, but it is the congruence of the microorganism’s structures with our unit steps of homeostasis that allows them to attach themselves to cell surfaces and then to release toxin or to gain access to the cell’s interior and to reproduce there. It is they who define our strengths as weaknesses and our congruence as incongruence. And they do so by using the human gene products, the human unit steps of homeostasis.

The history of the realization of this role of the unit step in disease is of interest; it paralleled the successive descriptions and definitions of both genes and proteins (6). We all know that Archibald Garrod was the first to call attention to alkaptonuria as a hereditary alternative form of metabolism because of failure of an enzymatically catalyzed step (23). He called this, and other such metabolic aberrations, inborn errors to distinguish them from diseases. This was an insight of extraordinary penetration in which he recognized that the differences in protein composition that distinguished species must also differentiate individuals within species (35). But even by 1909 when his first book, *Inborn Errors of Metabolism*, was published, he could go no farther (17,35,36). Little was known about protein structure and nothing of sequence of amino acids, and it was not even established yet, to everyone’s satisfaction, that enzymes were proteins (37).

As for the gene in 1909, it was still defined statistically, and although phenotype and genotype were differentiated in that year, the gene was an unknown entity, perceived by Johannsen as “an accounting or calculating unit.” But by 1915 the gene had been defined operationally, so by then Garrod could have proposed the inborn errors as products of mutants of single genes. But he never did. Even in his 1931 book, *Inborn Factors in Disease*, he did not use the word gene despite a general recognition that genes were involved somehow in some diseases (20,35). For example, in 1927 Barker reported that, “No less than 223 heritable anomalies have been described in man already” (38). And others, not in medicine, recognized a biochemical relationship between genes and phenotypes: Wright in coat colors of guinea pigs and Wheldale in flower pigments (39,40). Then in the late 1930s and early 1940s, the studies of Ephrussi, Beadle, and Tatum, first in *Drosophila*, then in *Neurospora*, provided a functional definition of the gene that brought gene and protein unequivocally together to clarify Garrod’s observations, and capitalizing on rapid advances in biochemistry, to begin in the 1950s an era of biochemical genetics (22). Biochemical genetics was an ecumenical enterprise. If Garrod was its icon and Harry Harris its chief expositor, there were also contributions of nongeneticists, including Pauling’s concept of molecular disease and the elaboration of the enzyme deficiencies in (type I) glycogen storage disease, galactosemia and other disorders, all classical inborn errors, described by biochemists with no primary interest in genetics and who made no reference to Garrod or to Beadle–Tatum (24,41,42). But whatever the influence, the list of inborn errors expanded rapidly, soon attaining an exponential rate of increase that has never slackened.

It is worth noting that biochemical genetics flourished before the impact of the discovery of the double helix could be felt. But the later developments led first to Yanofsky’s definition of the structural gene with its correspondence to sequences in amino acids in proteins (43) and later to the definition of the gene that includes both transcribed and nontranscribed DNA. And this led, in turn, to the development of genomics as an analytical method. Thus biochemical genetics, whose analysis proceeds from the phenotype to the protein and its gene, met genomics, whose analysis proceeds from the gene toward the phenotype by way of its protein product (44). And in time, the glamor passed from biochemical genetics to genomics, perhaps principally because the former had no way to tackle the genetics of complex disorders. Actually both are needed because phenotypes are not necessarily explained on discovery of the gene or genes whose products are acting as proximate causes.

As the focal point in pathogenesis, the protein gene product provides an economical answer to the question of the origins of monogenic diseases. But the question of the moment is how to explain those called complex. The approach includes genomics, by which salient genes can

be found and characterized (14). Further steps involve discovery of their proteins and the homeostatic devices to which they belong, after which the pathophysiology may be elucidated. Additional participation by genetically inclined thinkers lies in sorting out the heterogeneity by means of appropriate family studies, work that must be done before, or together with, efforts to tie treatments to the consequences of particular protein variants.

Today we scoff at such diagnostic “entities” as dropsy and consumption, having begun long since to resolve their heterogeneity. But the HGP will provide the means to show how much more we have to go to characterize distinctive versions of, say, heart attack and stroke. So numerous are the genetic contributors likely to be that a case might be made for everyone having his or her own version of heart attack, stroke, or other multigenic multifactorial disorders. So family studies are vital for deciding which genes play important roles in which versions of the disease. The results will resemble those in the study of monogenic disorders; the heterogeneity will be of both loci and alleles, and the sets thereof will vary from family to family and individual to individual (18). This kind of genetic thinking, not yet routine in medicine, is crucial to our understanding and represents an important principle of disease.

**2.5.1.6 The Protein Product as a Unit of Selection.** Neodarwinism is the outcome of a debate in which geneticists agreed that the object of selection must be phenotype, not genes, whereas evolutionary biologists, to whom the phenotype had been that object all along, agreed that both phenotypes, and their variation, originated in the genes (45–47). If so, although the phenotype remains the unit of selection, it is the variable unit step or steps that cause it to qualify for that fate. In medicine, we are not much concerned with the selection by which species attain their characteristics but with what evolutionary biologists call “purifying” selection, that which removes “undesirable” genes prior to reproduction. So here again the protein product of the gene occupies a central position between two aspects of human biology. And here is yet another example of the cleavage between biology and medicine. The irony in the word purifying is not lost on the physician to whom the protection of life is uppermost, while to biology, with no stake in the individual, the question is purely one of understanding the rise and decline of species. But, in fact, variations in unit steps of homeostasis are no less the stuff of positive selection than negative.

**2.5.1.7 As a Hedge against Genetic Determinism.** Institutions change and renew themselves but they always retain residual signs of their origins. No one would deny that all the genetics of today stems from the concepts elaborated in the fly room at Columbia, or that we continue to use both concepts and language appropriate to the drosophilists’ definition of the gene (21). Theirs was an operational definition in which authority for both heredity and cellular function was accorded to



the gene. In his book, *What is Life*, Schroedinger spoke of the gene as “law code and executive power” as well as “architect’s plan and builder’s craft in one” (48). So the language of *Drosophila* genetics included such locutions as genes “for” gene–environment interaction, modifiers, penetrance, and pleiotropy, all of which are perceived as properties of the gene, although we know now that they refer to events mediated by the protein unit steps of homeostasis. There is no question of the latter’s specification by the genes, but in folding and assuming an appropriate position in a relevant homeostatic device, they become a part of mechanisms that regulate both themselves and the DNA (see Chapter 16). Thus it is not the genes that are penetrant, pleiotropic, or that interact with the environment, it is the proteins that do these actions that are removed from the genes’ control.

It might be correct to speak of a “gene for” say an enzyme or even its pathway; for example, there is a “gene for” phenylalanine hydroxylase and “for” phenylalanine degradation. But in their further integration, proteins lose their identity in those of integrated functions, for which any single gene can no longer be perceived to have any authority.

There is another way in which the locution “gene for” is used. When we observe that a disease segregates, we say there is a “gene for” that disease, that one or more mutants act as proximate cause. That is exactly what the drosophilists did for their mutants, unconcerned with their ignorance of how a gene could shorten bristles or deform wings. We continue to use their discourse, even though we know that the protein product is the actual agent of function (21). But “genes for” is a tricky phrase. When we use it unthinkingly, as in genes for high blood pressure, say, we obscure our own inner view of the reality, whereas when we speak of variant proteins, there springs immediately to mind pathways, cascades, receptors, transducers, and feedback loops (see Chapters 16, BP). Incidentally, it is amusing to imagine that had Archibald Garrod come to alkaptonuria thinking like a geneticist of the time, he would probably have perceived it only as a recessive character, not an inborn error. But he came to it as a biochemist and saw it for what it was: a metabolic alternative due to the absence of an enzyme. He used the genetic evidence expressed in consanguinity to support the idea of heredity, not as evidence of a gene. So, rather than perceiving his lack of interest in genetics as a shortcoming, we should be glad of it because the idea of a “gene for” alkaptonuria could have stood in the way of his biochemical insights. But equally, had he pondered the work of the drosophilists emerging in print from 1905 to 1920, and which included their operational definition of the gene, the second edition of his *Inborn Errors of Metabolism* published in 1926 must surely have anticipated the Beadle–Tatum one gene–one enzyme principle (49).

So, if we human geneticists of today revert occasionally to the drosophiline mentality, how likely are patients, their families, and the public to escape? How are they to

know that the words “gene for,” say IQ, artistic ability, or criminal behavior obscure the unfathomable complexity of the identity and actions of gene products integrated in hierarchies to compose cells, organs, and whole organisms, all in touch with one another and with the outside? The extremes to which “genes for” can go are summarized in a book called *The DNA Mystique: The Gene as a Cultural Icon* by Nelkin and Lindee (50). But fortunately we have our mental image of the products of the genes, the unit steps of homeostasis, with their multifarious behaviors as a bulwark against loose thinking.

**2.5.1.8 As the Goal of HGP.** One road to the discovery of new principles of disease is the HGP (51). Lander has suggested that this bears the same relationship to biology that the periodic table bears to chemistry (14). So it compels our attention. Furthermore, it is the ultimate identifier of those homeostatic units that lie at the basis of pathogenesis.

About 20,000 or so genes and their products have been identified, and sooner or later, the products’ roles in homeostasis will follow, with obvious benefits for investigation of pathogenesis, treatment, and prevention. In addition, definitive samples of gene products, useful in defining disease, will be available for characterizing human biological properties hitherto unknown. A few examples of questions that are being asked are

1. How variable is the human genome? Is it more, or less, than the estimates of Harris and Lewontin? Studies of SNPs suggest more as do the results of the Thousand Genomes Project (33,34). Nothing could be more useful than this answer because it is the common genes that so often act as modifiers and furnish the wherewithal for complex diseases.
2. Is there an inborn error for every locus? And are all classes of proteins equally involved in diseases? In a comparison of 348 mutant proteins associated with inborn errors listed in MMBID, seventh edition, and a list of 3000 “core” proteins shared by yeast and *Caenorhabditis elegans*, the distribution of protein types in the two samples was remarkably similar (52). Although indirect, the suggestion is there that all protein types are involved in inborn errors, but we cannot yet say that there is an inborn error for every locus, however plausible the idea may be.
3. Are diseases characterized by the qualities of the proteins that are their proximate causes? For example, do enzyme deficiencies differ in some systematic way from disorders associated with receptors, transcription factors, or structural proteins?
4. Are conserved genes over- or underrepresented in disease? One might expect them to be overrepresented on the assumption that they fulfill critical functions or underrepresented because their mutants might be so often lethal.
5. What is the role, if any, of developmental constraints in fostering or suppressing disease? These are limitations on the evolution of phenotypic variation

expressed in developmental blind alleys. Kirschner and Gerhart have examined ways by which such constraints are loosened to allow new mutation and evolutionary progress. But would some of the latter be disease (53)? And Rutherford showed how, in *Drosophila*, such constraint was exerted by a heat shock protein (54). When altered by mutation, the constraining force was lifted and the effects of mutants suppressed by the wild-type protein were observed. Some of these effects were developmental anomalies.

6. Are diseases characterized by the evolutionary age of the proteins that lie at their root? That is, we might suppose that inborn errors of housekeeping genes shared by remotely related species were the oldest. Do they differ in any particular from diseases of the most recent mammalian or human genes?
7. What are the implications for aging? Are some proteins more frequently the object of aging processes, or is it random? Errors in the mitotic machinery that led to multiple abnormalities of regulation of dozens of enzymes increase with age (55). So, will aging, which has been perceived by some as dishomeostasis, turn out to have the same molecular basis as disease?

Many other questions are being asked, many no doubt not now askable because the contexts in which they are relevant are unknown. As more and more diseases are given molecular definition, we will surely classify them differently, departing from the current anatomical, organ system, age-related rubrics, moving to more molecular designations. As heterogeneity is laid bare, old classes will go and new ones will come, reflecting a sharp revision in how we will see disease itself. In addition, our language will change. It is likely that we will refer less to genes and more to proteins, so our residual *drosophiline* language is likely to go, too. Of what use are words such as modifier, epistasis, penetrance, pleiotropy, and the like when visualizing the reality as actions and interactions of proteins, in say, multiunit machines, or even in whole systems (14)? This also suggests that we in medicine will be thinking less in units and more in multiunit devices (proteomes, metabolomes, etc.). Linear thinking may be out as complexity moves in. But maybe the most significant change in our thinking will be compelled by the definitive evidence of human variation and individuality. Typological thinking will give way to population thinking. No doubt there will always be use for the former at one level; that of the value of means and the classical case, but only as a preliminary to the population thinking that perceives the extent and impact of variation on human individuality.

**2.5.1.9 Social Impact.** The unit step of homeostasis is attaining increasing prominence as a risk factor and signal for preventive action, and medicine has been adapting not only to their potential use but also to their impact on their possessors' lives. These concerns are well-known to readers of PPMG; they have been the subject of many

papers, books, and committee reports and they touch on counseling, ethics, legal matters, and psychological impact (15,16,56–58). They are mentioned here because of the potential uses of such risk factors in prevention. If the HGP fulfills its promise, there is the possibility to know the protein products of all genes known to participate in pathogenesis. Many scenarios as to the use of these markers have been offered. Only time will tell which, if any, is practical, but we would be wise to continue our study and preparation. How to use information, available at birth, about many variant genes, perhaps dozens in single individuals, and known to be associated with diseases all across the life span is something entirely new in medicine. Is it consonant with good medicine? Is it acceptable to the public? How do we prepare the public to make rational decisions about it? How do we prepare individuals to accept and use constructively such emotionally loaded information? These questions can be answered only in colloquy with the public.

**2.5.1.10 As a Source of Coherence.** In concentrating on the specificities of the pathogenesis of each disease, reductionist investigation emphasizes the separateness of diseases. It exerts a centrifugal effect that adds to that of our conventional nosology, which divides medicine into specialties across which we interact collaboratively, when at all. But the concept of the unit step of homeostasis as the central focus of all pathogenesis provides a principle of disease that exerts a contrary centripetal force that unifies the thinking about both disease and diseases. It is the difference between analysis and synthesis. Medical thinking, until recently, was mainly synthetic. It dealt with the body as a whole, no doubt because of ignorance of its parts. In contrast, in the thinking of today, the emphasis is on analysis; our attention is directed more to microunitary parts with less attention to the whole. But in acting as units of the mechanisms whereby an open system maintains its adaptation to an indifferent environment, the protein gene product is the effective link between those proximate and remote causes portrayed in the Mayr model. And that link is no less evident when unit step and environment are incongruent than when congruent. In the Mayr model, the proximate causes are consequent on the DNA and pose questions prefaced by how, whereas the remote causes lead up to the DNA and pose questions prefaced by why. In this summary of the role of the protein gene product, we have dealt with how questions. In the next section, we examine the why questions and the principles they illustrate.

## 2.6 THE WHY QUESTIONS

Just as the questions preceded by “how” are answered by reference to proximate causes, so are the “why” questions answered by reference to remote causes. And the answers to the why questions contribute no less to the specificity of identity than the proximate. Indeed, they are the enablers, the ultimate arbiters of that specificity. So we are what we are by virtue of endowment, experience, development,

maturation, and aging, but the bounds of what we can be and the precise description of what we are, are determined by how the remote causes came to be what they are and how they were sampled in the making of the individual. So the answers to the why questions are likely to probe more widely and deeply than those preceded by “how.” In fact, they begin with the latter to give them specificity. And yet we spend most of our energies and money on seeking proximate causes. The imperatives of treatment and prevention require it, yet it should be observed that it is the “why” questions that are most often asked by the public, particularly by those affected by disease. Medical education would do well to include them.

### 2.6.1 Why do we have Disease?

Why is it that after all these eons of evolution, species have not evolved to perfect attunement with the environment? First, it must be said that we are remarkably well adapted. Increasing longevity suggests that in the developed world we are moving toward the ideal rectangular survival curve (59). That is, we are moving in the direction, at least, of some probably unattainable minimal amount of disease compatible with some necessary degree of genetic diversity. In addition, the environment changes, requiring new adaptation, and then observation suggests that nature never reaches for perfection but for some compromise that ensures perpetuation of species usually at the expense of individuals. For example, the fecundity of our own species is presumed to be of the order of only 25%, and not all of that makes it to maturity (60). But that a principal hazard to the global ecology is a surplus of human beings is testimony to nature’s way of doing business.

Because evolution proceeds by the intervention of natural selection, there must be something to select, and again, although there are mechanisms of astonishing precision to ensure the accuracy of replication of DNA, they are not perfect either, and so, after the removal of individuals unlikely ever to survive or reproduce, we are still left with sufficient variation among individuals to adapt to the randomness of change in the environment. This includes genes that are either neutral under all conditions or contingent, that is, adaptive under some conditions but nonadaptive and conducive to incongruence and disease under others. So disease must be presumed to be a by-product of the necessity to have enough variability for all conditions.

### 2.6.2 Why this Disease?

Patients often ask this question. “Why,” they ask, “should I have diabetes or cancer?” or “Why should my baby have this bizarre disease I’ve never even heard of?” The media have spread the news of genetics, so their question may be, “Is it in my genes, and if so where in the world did such genes come from?” So the questions are directed to both proximate and remote causes. First

to the proximate, which include the genetic endowment received by the patient at conception and which, together with the kinds, duration, and intensities of experiences of the environment, have created a developmental and maturational matrix that is an expression of individual potential from day to day. Then, the parental contribution to this matrix reflects the specificity of their genes, themselves representative of one or more gene pools, each with its own variable composition and history. The contributions of experiences are representative of qualities of the society the patient inhabits, qualities that vary with cultural history. And finally, the contribution of development and maturation is that of a trajectory whose specificity is derived from both endowment and experiences as they create and characterize the evolving matrix within which the incongruence and disease are engendered. So the answer to the question of “why this disease” requires us to acknowledge that while the disease is a consequence of incongruence between proximate causes, it is the remote causes that account for the existence, availability, and particularity of those proximate factors.

### 2.6.3 Why this Person?

If patients are baffled by the disease they experience, they are angered by its apparent choice of themselves. “Why me?” is their injured cry. Of course, the reasons reside in the origins and specificities of genes, experiences, and development given in answer to the previous question, but “Why me?” is a profoundly different question because, no matter how specific the genes, the experiences, and the development, so long as it is only the disease itself that commands our attention, there is the possibility that we miss the full impact of the individuality of the patient, its multifariousness, its history, and its uniqueness. There is always a healthy side to a sick patient with its own proximate and remote causes and its diversity. And it is in noting those qualities that some clinicians are distinguished from others to whom the particularity of the patient lies only in his or her variant molecules. In fact, it is in appealing to the particularity of the whole patient, molecules included, that the physician is able to help the patient to discover and to mobilize resources that may make the difference between a timely and a delayed recovery, or even between life and death.

### 2.6.4 Why at this Time?

Perhaps the least understood by its victims is disease’s apparent caprice in choosing when it strikes: the infant, blooming and full of promise, who wilts and dies; the robust, active college student who dies in one or two days of meningococcal meningitis; the busy, tireless 50-year-old who is felled by cancer. No doubt in old age disease and death are less anomalous, more expected, and yet we must ask why one person is privileged to die at 90 years, whereas others have died untimely. But we know that



there are reasons for ages at onset of diseases and that they are accommodated within the nominalist definition of disease. These reasons are embodied in human mortality curves that, in the developed world, are U-shaped, declining sharply after birth, reaching a nadir at adolescence, and beginning to rise again in young adult life. If we were to include life before birth, the postnatal decline would be seen to be the end of a steep drop through intrauterine life.

Table 2-1 lists and contrasts qualities of the diseases experienced on the two sides of the U. How do we explain the differences? Again, appeal to remote causes gives the answers. All differences are those expected if the heritability of disease were to fall throughout life. The table tells us that indeed it does decline; the incidence of monogenic disease drops sharply before the nadir of the U, which suggests strong selection against disorders that imperil reproduction, leaving postpubertal disease to be

associated mainly with the kind of genetic variation that is contingent, implicated in disease only in the presence of nongenetic proximate cause, and representative of the kind of variation successful species exhibit. But, although the distribution of mortalities is U-shaped, the principle of continuity is not defied; monogenic disease continues to occur even in late life, and diseases of complex origin are known in childhood, even in utero (Figure 2-1).

Another way of perceiving in more detail the decline in prominence of the genetic impulse in disease is to express it as a decline in a gradient of selective effect. The gradient is not of genes but of phenotypes, and the weight of selection is heaviest in utero and least in old age. Burden is measured in risk to life, curtailment of reproduction, and permanent disability. These are biological burdens such that loss of life in utero, even before implantation, is more burdensome than the death of a bread-winning parent at age of 40 years with all its social upheaval. The apparent discrepancy in these burdens is a consequence of a natural ambiguity in human life in which we have two selves: one biological and the other social. It is as if we live two lives, one in obedience to the biological imperative to survive to reproduce and the other to exploit endowment and opportunity to have a fulfilling social life. Obviously these lives are intertwined, but when in opposition, there is a schism that may lead to disease.

The gradient is at its peak in utero where for reasons we can only guess, the majority of conceptuses are found wanting and die. Because intrauterine life is protected from the outside, most of this mayhem is likely to be genetic; for example, known early losses are most frequently associated with chromosomal anomalies (60). But there must be inborn errors as well, involving among others, proteins specific to development. Then there are many known inborn errors with onset early in postnatal life and constituting a significant proportion of disease in newborns. In keeping with the biological desirability of a population of reproducers

TABLE 2-1	Differences in Pre- and Postpubertal Diseases	
	Prepubertal	Postpubertal
Mode of inheritance	Monogenic	Multifactorial
Age at onset	Early	Late
Frequency	Rare	Frequent
Latency	Short	Long
Affected relatives	Numerous	Few
Diagnostic specificity	High	Low
Number of diseases	Very many	Fewer
Burden	Great	Less
Sex differences	Occasional	Frequent
Influence of migration	No	Yes
Secular change	No	Yes
Effects of SES	Some	More
Success in treatment	Some	More
Heritability	High	Low
Predictability	High	Low

SES, socioeconomic status.

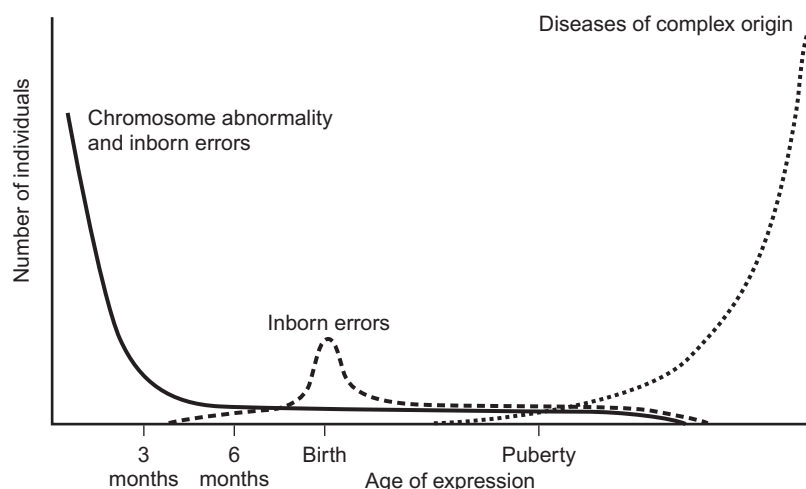


FIGURE 2-1 Continuity of disease across the lifetime.

unencumbered by such mutants, 90% of single-gene diseases will have been disclosed by puberty and 99% by the end of reproduction (61). As teenage passes into early adult life and that into midlife, the residual monogenic disease consists mainly of the cases of earliest onset of complex diseases, the most severe, life-threatening and resistant to treatment, whereas those with later onset, milder, more responsive to treatment are of complex origin, often irregularly familial or sporadic, a continuity commensurate with a high degree of heterogeneity of proximate cause. At the lowest end of the gradient are degrees of health expressed in resistance, completing the continuity that began with the mayhem in utero. Resistance to disease is little noticed in medicine where appeals to the doctor are only made when the patient is sick, but it is well-known in infections.

In keeping with the concept of declining heritability, we expect an increasing contribution of nongenetic variation. Most cancer takes its origin from mutation, but it is mainly somatic and so may be counted as of environmental origin, perhaps as an aspect of aging. Aging is perceived as dishomeostasis, a product of cell loss and dysfunction due to denaturation of proteins. So while gerontologists, no doubt correctly, decline to see aging as disease, its expressions are associated with failure of the same gene products we associate with disease.

The decline in heritability is not monotonic but rises and falls according to developmental phase (see Figure 2-1) (61). For example, the genetically determined intra-uterine disease has an early onset, whereas fetal disease late in pregnancy is more likely to be of maternal origin; prematurity, placenta previa, hypertension, premature placental detachment, and the like. Then there is a fresh spate of genetic disease in infancy, with deaths in childhood being more likely to be due to poisons, infections, accidents, and in some places, homicide. In adult life, early-onset cases of complex disorders are strongly conditioned by genetic variation, whereas late-onset cases are likely to be nonfamilial (62).

Reasons for this phenomenon are conjectural, but it makes sense if the effects of new sets of genes are exposed to selection at the beginning of each developmental phase. It is an observation that needs study. Another kind of continuity exhibited by the gradient of selective effect is the overlap of disorders characteristic of different developmental phases (see Figure 2-1). For example, most chromosomal anomalies are lethal in utero, but for some there is a backwash of live-born babies. But not all malformations are due to chromosomal aberration; some are of complex origin and some are harmless and may be recognized only at autopsy or emerge only under conditions of unusual stress, such as urinary tract infection in a patient with a single kidney, perhaps in adult life. Prepubertal life is characterized by monogenic disease, but type I diabetes, asthma, inflammatory bowel disease, and other disorders of complex origin sometimes have onset then too. When a disease has onset over a broad range of ages, we must always wonder whether all victims to

whose disorder we give the same name actually have the same disease. So in complex disorders, the problem of naming will be with us until we are able to sort out the heterogeneity. In the end, it seems likely that the number of diseases of adult life will be no less than those of the prepubertal years. No doubt genomics will identify the genes, and the issue of names will be resolved.

One reason why this description of the decline in heritability, with its stops and starts and overlaps, has been given prominence here is to illustrate the principle of the continuity of life, a continuity often ignored in both concept and organization. Hospitals, medical schools, and medical education are arranged around age and organ system for perfectly good reasons, and the system has proved its worth. But molecular genetics provides a continuity that brings the specialties together in taking a longitudinal view of human life.

## 2.7 PREVENTION AND TREATMENT

One might suppose a priori that the Mayr model could not accommodate prevention and treatment of human disease. Biology is concerned with what is and does not accommodate intervention; the minute one intervenes, what is no longer is. And what could be more unnatural than government agencies that regulate how we deal with nature, or surgical transplantation of organs as a treatment, to say nothing of the idea of designer drugs for molecular defects. But we cannot escape our biological heritage, nor do we wish to, so perhaps the model is apposite after all.

All organisms are capable of adapting to, or otherwise defending themselves against, uncongenial environments. Some call upon homeostatic flexibility when under stress, whereas others move to evade it; evolution has seen to these self-protective capabilities. For example, many organisms have molecular mechanisms to withstand stress; heat shock proteins are one (63). Others are enzymes that detoxify foreign substances, and a third is up- and down-regulation of metabolic systems. Some organisms remove themselves from threats. In addition, animals that can, choose surroundings appropriate for their physiology and improve them too (64). So, in the sense that they know what experiences to avoid, animals practice prevention, and when attacked by disease or hurt in the course of the day's work, they fall back on natural mechanisms by way of treatment. The difference between them and us is that we consciously intervene in both. But in the degree to which we seek out proximate causes intending to alter them by prevention or treatment, and in the degree to which we try consciously to influence social and cultural conditions with an eye to changing remote causes, we do fulfill the expectation of the model.

### 2.7.1 Prevention

Constituted separately from medicine and described as improvement in diet, housing, and other living

conditions, prevention has saved more lives than treatment (65). And when microorganisms were identified as proximate causes, they became the target of prevention by quarantine and immunization; the latter remains a staple of medical care. These preventive measures were and are in the hands mainly of local government, whereas progress in fostering the ideas and promoting education in preventive medicine were and are the work of university-based schools of public health and hygiene. After the 1950s, when antibiotics reduced the mortality of infections, other diseases such as diabetes, cancer, and kidney disorders came into focus. Then the ideas of preventive medicine and epidemiology, which had been restricted to the control of infectious diseases in populations, began to include prevention of noninfectious disease in individuals, leading to the establishment of organizations that included patients, their families, and the public, and that were devoted to education and counseling of patients and relatives as well as the general public with the intention to prevent and to learn enough to treat these common disorders. The American Cancer Society, the American Diabetes Society, and The March of Dimes come readily to mind. In the 1960s and 1970s as more and more inborn errors were described, this principle was also applied to the generation of a multitude of disease-related societies, each dedicated to education, treatment, and prevention of one disease, the latter in the form of reproductive counseling, antenatal diagnosis, and sometimes abortion. Then, as the molecular basis of these disorders was discovered, newborn screening for inborn errors was offered by many state health departments and intensive studies were undertaken of every aspect of this form of preventive medicine including screening, counseling, and issues both ethical and legal (56,66–70). The question then arose of testing relatives of patients with inborn errors with an eye to reproductive advice, and the triumph of Tay–Sachs testing is one result (15,56). And now that rapid progress is being made in unraveling the genomics and proteomics of complex disease, time will give us more risk factors in the way of variant genes and proteins. These developments are reviewed here in this detail to call attention to the movement of the focus of prevention away from populations to individuals, and now to the molecular emphasis in both prevention and treatment. Just as the discovery of genes associated with disease suggests the possibility of cure, so does it suggest prevention by testing of relatives and populations. Indeed, the logic of prevention is even more powerful than that of cure. That is, unlike treatment, which is always after the fact and is occasionally as threatening as the disease it is designed to combat, prevention spares the organism such rigors even while far less disruptive of social and economic life. On the other hand, in keeping with the principle of continuity, the two are sometimes indistinguishable.

So, may we expect miracles of prevention now that we can identify proximate causes? Readers of PPMG

know that we may not (15,16,57,58). It is a matter of the continuity of the gradient of selective effect. At one end, the virtual elimination of Tay–Sachs disease among Jews and the prevention of a few other inborn errors by the same means represent successes of the high technology promoted by Lewis Thomas (71). At the other end are healthy centenarians who attribute their robust health to some idiosyncratic behavior. But in between are those genes and their variant products whose virtues are a sometime thing, depending on, on the one hand, the specificities of experiences over the lifetime, and on the other, their support, or reinforcement in failure, by the variant products of other genes. So the same variant gene product may be adequate in one person and fail in another even in the same family. Or it may be within the same person adequate under one circumstance and insufficient under another. Thus, as a predictor, a gene may be of only limited use to an individual even while accepted as a significant risk in a population. This is a frequent problem of epidemiologically designated risk factors; it is not always clear to whom among their possessors the trait is actually risky—to say nothing of gradations in risk. It is the problem also of evidence-based medicine, which, however valuable in increasing the rigor of diagnosis and treatment provides recommendations suitable for populations, not individuals (72–74). It is a matter of typological, as opposed to population, thinking. Of course, the HGP has added greatly to the list of our genes and their proteins so that the exact identity of all of the units in pathways and other homeostatic devices will be known, improving thereby the predictive value of various combinations of variants (75). And, assuming increasing identification of exterior proximate causes, the accuracy and usefulness of preventive predictions may improve remarkably. The necessity for the advancement of knowledge of nongenetic proximate causes cannot be exaggerated. We need a project of similar scope and ambition to that of the HGP. In the meantime, we should do what we can where we can, and for the rest, fall back on an aspect of medicine that may have become unfashionable in modern times but which is perhaps more than ever needed: helping patients to live with uncertainty.

A far less likely, but more effective, means of health promotion is the control of remote causes. The virtue of such an approach is clearly indicated in the Mayr model wherein the relationship of the two kinds of causes and the two kinds of biology is so lucidly stated. To influence by law the distributions of genes is both unconstitutional and in strong opposition to “liberty and the pursuit of happiness,” but to influence the organization of society and culture for the betterment of health is not only possible but also already the aim of numerous government agencies, the even more numerous private disease-related societies, as well as physicians who have advised their patients to practice healthy ways. But what has not been emphasized, at least in the United States, is the power of corporate action, of putting the weight of the whole



medical profession socially and politically on the side of health promotion. Today in the United States this is impossible, and some will say it is undesirable, but the point to be made here is the logic of the position. On one side is the power of remote causes in the origin of disease (let us think here of the evolution, growth, organization, penetration, and political powers of the tobacco industry) and on the other is the power of societies to organize themselves to influence those remote causes. In the United States, it was public opinion followed by legal action that began the descent of the authority of tobacco. There are other social conditions likely to retard the advance of prevention. One of these is the combination in the public mind of a superficial grasp of progress in biology and genetics and an unreasoning belief in the limitless potential of that progress. But for a recipient to respond realistically to the offer of prevention requires the ability (1) to differentiate between personal and populational probabilities, (2) to grasp its potential for success or failure, and (3) to participate constructively with a knowledgeable and sympathetic physician in greeting either success or failure. We do not often think of the evolution of education, or of the public grasp and acceptance of advances in medicine as remote causes of success or failure, but we should.

### 2.7.2 Treatment

The essence of Lewis Thomas' concept of high technology in treatment lay in the discovery of the exact point or points in the machine that were broken and that could be repaired by a single, simple, straightforward maneuver (71). One of his examples of such a treatment was the use of steroid in the adrenogenital syndrome. And after the 1950s, amid the rapid accumulation of newly described inborn errors, there was optimism that such diseases would be brought under control (76). But results so far have been something less. In the 1980s, Hayes tabulated successes and failures in 65 inborn errors, a part of a larger randomized sample of monogenic diseases taken from MIM 5 (77). Table 2-2 shows that for 12%, treatments were successful in rendering the patient normal or essentially so, whereas in 40% there was some improvement, often not very impressive, and about 48% showed no success at all. A further examination of success or failure in treatment of the same 65 diseases 10 years later was reported by Treacy with results shown in Table 2-2 (78). There was no increase in the number of very successful treatments, but some of the previously resistant disorders now yielded in some degree, often to such rigorous therapies as tissue and organ transplantation. These, Thomas saw as middle technologies, sometimes effective, but expensive and perhaps hard on the patient. A third look in 1999, this time including 517 inborn errors listed in the seventh edition of MMBID, gave much the same results (see Table 2-2) (79).

**TABLE 2-2 Effectiveness of Treatment of Inborn Errors**

	1983 Hayes	1993 Treacy	2000 Treacy
Fully beneficial	12 <sup>a</sup>	12	12
Partially beneficial	40	57	54
No benefit	48	31	34

<sup>a</sup>Percentage of total.

Given the qualities of the inborn errors that were the object of treatments, the record is perhaps not surprising. Most are at or near the top of the gradient of selective effect, some are lethal, and some are permanently crippling. And all are heterogeneous, some as to loci, all as to alleles. And we now know that we must expect equal heterogeneity among those shadowy modifiers we presume to exist (18). So these disorders are simply the most intractable. But farther down the gradient, the diseases are more amenable, not necessarily to cure, but certainly to management. This seems to be telling us that there is a relationship between heritability and success in therapy. When the heritability is high in a population, we expect less of treatment than when it is low (i.e. fewer patients are likely to respond satisfactorily). The history of treatment of rickets with vitamin D is exemplary. When, after the mid-1940s rickets almost disappeared, nearly all that was left were several different kinds of monogenic vitamin D-resistant rickets (20). This experience seems to furnish medicine, especially preventive medicine, an aim, even a motto. We work to drive the heritability of disease toward 1.0. And we fervently hope that the gene therapists will confound the motto by inventing high-technology treatments that subdue even the most "genetic" and even the most refractory of those disorders that continue to resist every effort to contain them. As for the complex disorders, they are resistant, too, but in a different way. If every individual has his or her own set of proximate causes, the complexity is of a high degree. The problem is one of discovery of the nongenetic causes and trying to eliminate them, as well as discovering which sets of genetic causes pose vulnerability to the threats of those nongenetic influences, both in general and in each affected individual. No easy job, but we are up against a wily opponent. Many years ago, Max Delbrück observed that "any living cell carries with it the experiences of a billion years of experimentation by its ancestors. You cannot expect to explain so wise an old bird in a few simple words" (80). We are definitely embarked on an effort to expose that wisdom. The next two or three editions of PPMG should show how wise the old bird is.

## 2.8 CONCLUSION

The explanatory principles of disease begin with the capacity of the species for genetic variability, a capacity

that is required for survival of species and that is experienced randomly resulting in genes whose products have, through time, conferred upon their recipients a status of congruence with equally variable environments. But sometimes the result is incongruence, which can lead to disease. It is in the gene products, the protein unit steps of homeostasis, that this species variability is expressed in congruence or incongruence in health or disease. This expression occurs within a biochemical and molecular cellular matrix conditioned by interaction between such protein products and experiences of the environment through development, maturation, and aging. Accordingly, analysis of pathogenesis must be pursued in three timescales all at once: that of phylogeny whence the genes and their products were derived; that of ontogeny, maturation and aging, which condition the ever-changing matrix; and that of the moment representing the impact of today's events. This principle, embracing the three timescales, also incorporates two kinds of causes, proximate and remote, which are expressed in the uniqueness of individuals. The incongruent proximate causes, variable protein unit steps of homeostasis and varying kinds, amounts and durations of experiences of the environment, account for the expressions of disease phenotypes that are subjected to selection and incur the social stigmas that complicate the lives of their victims. Remote causes are composed of the evolution and dynamics of both biological and social milieux that account for the nature and local availability of proximate causes, their unique assembly as genotypes and availability as experiences, to form combinations favorable for disease. And it is this particularity that determines who gets which disease at what time in life. The qualities of diseases are expressions of unique and variable human genomes arranged in a gradient of selective effect, a representation of the removal in early life of those unlikely to reproduce, and in postreproductive life, a less-intense test of survival in a variable environment. It is in the latter part of the range of the gradient that both prevention and treatment are likely to be most effective; prevention because changes in environment can be effective in avoiding disease, and treatment because the homeostasis can be characterized as inefficient and in need of a boost, rather than broken. The logic of prevention is more powerful than that of treatment, but we need both a more comprehensive knowledge of nongenetic proximate causes and, in time, to learn, understand, and adjust to the social dislocations any sudden spate of preventions could bring. But it is in part in the grasp of the possibility and plausibilities of both prevention and treatment, and in part in understanding the meaning in medicine of individuality and the virtues of population thinking in relation to it, that we may be able at once to pursue the reductionist path we have so successfully traversed and return to embrace the integration, the humanity, of patients who appeal to us for relief of both the consequences of their molecular incongruities and the injury of the disease to that integrated humanity.

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### Biographies



**Barton Childs, MD** (1916–2010) was emeritus professor of pediatrics at the Johns Hopkins University School of Medicine. He attended medical school there and, with brief sojourns studying at Boston Children’s Hospital and University College London, spent nearly 70 years as a devoted clinician, teacher, mentor and investigator in Baltimore. He was the first to prove that X-inactivation occurred during early human embryogenesis, thus confirming the Lyon hypothesis. He was one of the first to develop and endorse genetic counseling as a clinical specialty before the advent of dedicated training programs and formal certification. Perhaps his greatest contribution was his commitment to introducing genetics into educational curricula at all levels of instruction, especially throughout medical school. The current curriculum at Johns Hopkins, “From Genes to Society,” is based largely on his perceptions. His numerous awards included the William Allan Award of the American Society of Human Genetics, the Johns Howland Award in pediatrics, and the Research Career Award of the National Institutes of Health. He worked avidly and incessantly for nearly 30 years after attaining emeritus status. During this time he published *Genetic Medicine: A Logic of Disease*, which should be required reading for any student of medicine or human biology.



**Reed Pyeritz** completed his PhD and MD at Harvard and trained in internal medicine at the Peter Bent Brigham Hospital in Boston and medical genetics at the Johns Hopkins Hospital in Baltimore. He is a professor of Medicine and Genetics at the Raymond and Ruth Perelman School of Medicine of the University of Pennsylvania in Philadelphia, where he directs the Center for the Integration of Genetic Healthcare Technologies and serves as vice-chair for academic affairs of the Department of Medicine. He is a senior fellow in both the Center for Bioethics and the Leonard Davis Institute for Health Economics.

His research has focused on several areas, including clinical investigations of heritable disorders of connective tissue and cardiovascular diseases, especially those affecting the thoracic aorta. Currently he is studying how more refined methods of investigating genetic variation, such as cytogenomic arrays and whole genome sequencing, actually increase the level of uncertainty in applying the results clinically.

# CHAPTER

# 3

## Nature and Frequency of Genetic Disease

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### 3.1 INTRODUCTION

Genes are major determinants of human variation. Genome-sequencing studies have shown that an individual genome includes 10,000–11,000 differences from the reference genome that change an amino acid in a protein (non-synonymous change) and an approximately equal number of synonymous variants. Any individual is heterozygous for 50–100 variants that have been associated with genetic disorders (1). The impact of genetically determined characteristics spans a continuum and may be nil at one extreme or lethal at the other. In determining a trait, one or two alleles may be all important, but more commonly genes interact with one another and with one or more environmental factors. This interaction between genetic and environmental factors is now apparent for numerous conditions and includes many previously believed to have a purely environmental etiology. For example, genetically determined susceptibility has now been identified for several infections, for many drug-induced idiosyncrasies, and for several carcinogens (e.g. bladder cancer in aniline dye workers who are slow acetylators). We suspect that such interactions are commonplace and that relatively few conditions are solely environmental in causation. In addition to the environment, interactions with the microbiome, epigenetic modifications resulting in altered gene expression,

and chance all influence how the genome translates into a phenotype.

### 3.2 FREQUENCY OF GENETIC DISEASE

For definitions of the types and frequency of genetic disorders, we use the currently available information, with the proviso that these are all the minimum frequencies and are based on imperfect categorization. Interpretation of what constitutes a genetic disorder will depend on the situation (e.g. red–green color blindness may be a serious disability to the hunter-gatherer) or on the public perception (e.g. persons with albinism are considered blessed in some populations). Hence, the distinction between normal variation and disease is blurred and, as variable criteria have been used in different surveys of genetic disease, this must be considered in their interpretation. The situation is further complicated by the continued delineation of new phenotypic subtypes and by population variations in the frequencies of different genetic disorders. Thus, most of the available data pertain to specific conditions either in a sample of the general population or in specific populations (e.g. the learning disabled); extrapolation to the overall population is difficult.

#### 3.2.1 Chromosomal Disorders

By definition, a chromosomal disorder is present if there is a visible alteration in the number or structure of the chromosomes. Using routine light microscopy and a moderate level of chromosome banding, the frequency

<sup>†</sup>Deceased.

of balanced and unbalanced structural rearrangements in newborns has been estimated at about 9.2/1000 (2). Some of those with unbalanced rearrangements will have congenital anomalies and/or intellectual disabilities. A proportion of those with balanced changes will, in adult life, be at increased risk of either miscarriage or having a disabled child. The incidence of aneuploidy in newborns is about 3/1000, but the frequency increases dramatically among stillbirths or in spontaneous abortions (3). Different types of chromosomal abnormalities predominate in spontaneous abortions as compared with live-born infants. For example, trisomy 16 is the commonest autosomal trisomy in abortions, whereas trisomies for chromosomes 21, 18, and 13 are the only autosomal trisomies occurring at appreciable frequencies in live-born infants. Monosomy for the X chromosome (45,X) occurs in about 1% of all conceptions, but 98% of those affected do not reach term. Triploidy is also frequent in abortions, but is exceptional in newborns. The high frequency of chromosomally abnormal conceptions is mirrored by results of chromosome analysis in gametes, which reveal an approximate abnormality rate of 4–5% in sperm (4) and 12–15% in oocytes (5).

Routine light microscopy cannot resolve small amounts of missing or additional material (less than 4Mb of DNA). The advent of genomic microarray analysis has revealed a high frequency of submicroscopic deletions and duplications and other copy-number variations, including both apparently benign and pathological changes (6). Multiple microdeletion and microduplication disorders have been defined in recent years and undoubtedly more await discovery (see Chapter 29). Such microdeletions, which epistemologically link “chromosomal disorders” with single-gene disorders, account for a proportion of currently unexplained learning disability and multiple malformation syndromes (7).

### 3.2.2 Single-Gene Disorders

By definition, single-gene disorders arise as a result of mutations in one or both alleles of a gene on an autosome or sex chromosome or in a mitochondrial gene. There have been many investigations into the overall frequency of single-gene disorders. Many early estimates were misleadingly low due to under-ascertainment, especially of late-onset disorders (e.g. familial hypercholesterolemia, adult polycystic kidney disease, and Huntington disease). Carter (8) reviewed the earlier literature and estimated an overall incidence of autosomal dominant traits of 7.0 in 1000 live births, of autosomal recessive traits of 2.5 in 1000 live births, and of X-linked disorders of 0.5 in 1000 live births. This gave a combined frequency of 10 in 1000 live births (1%). At that time, approximately 2500 single-gene disorders had been delineated. The number of recognized Mendelian phenotypes has since almost doubled, and these new entities include several particularly common conditions (e.g. familial breast cancer syndromes, with a combined

estimated frequency of 5 in 1000; hereditary nonpolyposis colon cancer syndromes, with a combined frequency of 5 in 1000). In addition, new technologies for DNA analysis have revealed a higher-than-expected frequency of generally asymptomatic people with one or two mutant alleles at a locus (1). For example, up to 1% of the population has a mutant allele for von Willebrand factor, but many of these people have a few or no symptoms, so again there is the problem of the imprecise and variable boundary between a harmless variant and a clinically important one. Furthermore, DNA analysis has shown that for several important disorders, including myotonic dystrophy and fragile X syndrome, relatives of an affected individual may harbor a premutation that, although not detrimental to the carrier, has the potential for expansion to a full deleterious mutation in an offspring. The prevalence of such premutation carriers may be as high as 1 in 178 females for fragile X syndrome (9).

The frequencies of many single-gene disorders show population variation. Geographic variation may be explained by selection or by founder effects or may be attributed to random genetic drift. Selection has resulted in a carrier frequency of 1 in 3 for sickle cell anemia in parts of equatorial Africa, and the Afrikaners of South Africa have a high frequency of variegate porphyria and familial hypercholesterolaemia due to a founder effect. The carrier frequency for mutations in *HFE*, one of the genes responsible for hemochromatosis, is 1 in 10 in individuals of Celtic ancestry. Undoubtedly, more single-gene disorders are going to be delineated. In theory, at least one per locus will eventually be recognized (about 20,000) minus those with no or a mild phenotype and minus those incompatible with establishment/continuance of a pregnancy. Increasing the total are those loci for which different mutations cause entirely different phenotypes. For example, mutations in *LMNA* can result in at least 13 distinct disorders (10). There is also an overlap with the multifactorial category. For example, many patients with acute intermittent porphyria are asymptomatic in the absence of an environmental trigger, and epistatic involvement of other genes is believed to contribute to intrafamilial phenotypic variation for patients with the same mutation. As more gene–environment and gene–gene interactions are identified, the boundary between single-gene disorders and multifactorial disorders will become further blurred.

### 3.2.3 Multifactorial Disorders

Multifactorial disorders result from an interaction of one or more genes with one or more environmental factors. Thus, in effect, the genetic contribution predisposes the individual to the actions of environmental agents. Such an interaction is suspected when conditions show an increased recurrence risk within families, which does not reach the level of risk or pattern seen for single-gene disorders and



when identical twin concordance exceeds that for non-identical twins, but is less than 100% (see Chapter 14). For most multifactorial disorders, however, the nature of the environmental agent(s) and the genetic predisposition are currently unclear and are the subject of intensive research efforts. The ability to conduct genome-wide association studies has accelerated progress in this area.

Multifactorial disorders are believed to account for approximately one-half of all congenital malformations and to be relevant to many common chronic disorders of adulthood, including hypertension, rheumatoid arthritis, psychoses, and atherosclerosis (complex common disorders). The former group had an estimated frequency of 46.4 per 1000 in the British Columbia Health Surveillance Registry (11). In addition, a multifactorial etiology is suspected for many common psychological disorders of childhood, including dyslexia (5–10% of the population), specific language impairment (5% of children), and attention deficit-hyperactivity disorder (4–10% of children). Hence, the multifactorial disorder category represents the commonest type of genetic disorder in both children and adults. Multifactorial disorders also show considerable ethnic and geographic variation. For example, talipes equinovarus is some six times more common among Maoris than among Europeans, and neural tube defects were once 10 times more frequent in Ireland than in North America.

Often ignored, both intellectually and in research, are genotypes that reduce susceptibility to potentially harmful environmental factors. An understanding of alleles that provide protection from disease or increase longevity will yield insight into pathogenesis as well as novel approaches to therapy and prevention.

### 3.2.4 Somatic Cell Genetic Disorders

Somatic cell mutation is a natural developmental process in the immune system, but is also responsible for a significant burden of genetic disease. This includes somatic or germline mosaicism for single-gene disorders (12), as well as mutations that give rise to cancer (see Chapter 21). Cancer cells tend to have accumulated multiple mutations; the first step in the cascade of mutations may be inherited (i.e. involving germ cells and all somatic cells). Carcinogens are important causes of noninherited mutations, and genetic susceptibility is suspected to account for individual variation in risk on exposure. Somatic-cell genetic disorders might also be involved in other clinical conditions such as autoimmune disorders and the aging process.

## 3.3 MORBIDITY AND MORTALITY DUE TO GENETIC DISEASE

The same general difficulties that pertain to the frequency estimates for genetic disorders also apply to estimates of

the contribution of the various types of genetic disorders to morbidity and mortality during pregnancy, in childhood, and in adulthood. Hence, these figures should be taken as minimum estimates.

### 3.3.1 Conception and Pregnancy

One in 15 recognized pregnancies spontaneously leads to miscarriage, and a higher percentage (up to 50%) of conceptions is lost before recognition of the pregnancy. The majority of these losses are caused by numerical chromosomal abnormalities.

### 3.3.2 Childhood

Since the turn of the century in many Western countries, advances in medicine and public health have resulted in a gradual decline in the contribution of environmental factors to childhood morbidity and mortality. The result of these changes has been to throw genetic disorders into greater prominence. By contrast, in developing countries, non-genetic causes of childhood mortality continue to predominate. An idea of the contribution of genetic disease to morbidity can be judged from the prevalence of such diseases among pediatric inpatients. In reviewing 4115 inpatients, Hall and colleagues (13) found multifactorial disease in 22.1%, a single-gene disorder in 3.9%, and a chromosomal disorder in 0.6%. Thus, more than 1 in 4 pediatric inpatients have a genetic disorder in one of these categories, as compared with the general population frequency estimate of 1 in 20 by 25 years of age. This does not include the morbidity that does not lead to inpatient admission. An updated survey by McCandless et al. revealed 71% of children admitted to the hospital to have a disorder with a significant genetic component (14). Stevenson and Carey (15) have found that 34.4% of deaths among children hospitalized in a tertiary care center could be attributed to congenital anomalies, of which 16.7% were due to chromosomal abnormality and 11.7% due to a recognized malformation syndrome.

### 3.3.3 Adulthood

In Western countries, the commonest causes of death are cancer and cardiovascular disease. All cancers are now known to have a cumulative somatic-cell genetic basis, and there is evidence for a major genetic contribution to cardiovascular disease. Single-gene disorders causing diabetes or high blood pressure are relatively uncommon, but multifactorial inheritance accounts for a large proportion of patients with premature vascular disease and systemic hypertension. Similarly, there is a growing recognition of the importance of multifactorial inheritance for many other common disorders of adulthood responsible for both morbidity and mortality.

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## Biographies



**Bruce R Korf, MD, PhD.** Dr Korf received his MD degree from Cornell University Medical College and his Ph.D. degree in genetics and cell biology from Rockefeller University. He then completed training in pediatrics, pediatric neurology, and genetics at Children's Hospital, Boston. He served as clinical director in the Division of Genetics at Children's Hospital from 1986 to 1999 and as the medical director of the Harvard-Partners Center for Genetics and Genomics from 1999 to 2002. Currently he is the Wayne H and Sara Crews Finley Chair in Medical Genetics, Professor and Chair, Department of Genetics and Director, Heflin Center for Genomic Sciences at University of Alabama at Birmingham. Dr Korf has completed terms as president of the Association of Professors of Human and Medical Genetics, President of the American College of Medical Genetics, member of the boards of directors of the American College of Medical Genetics and the American Society of Human Genetics, member of the Liaison Committee on Medical Education, and the National Cancer Institute Board of Scientific Counselors. He currently is President of the ACMG Foundation for Genetic and Genomic Medicine and serves on the Board of Scientific Counselors of the National Human Genome Research Institute.



**Reed Pyeritz** completed his PhD and MD at Harvard and trained in internal medicine at the Peter Bent Brigham Hospital in Boston and medical genetics at the Johns Hopkins Hospital in Baltimore. He is a professor of Medicine and Genetics at the Raymond and Ruth Perelman School of Medicine of the University of Pennsylvania in Philadelphia, where he directs the Center for the Integration of Genetic Healthcare Technologies and serves as vice-chair for academic affairs of the Department of Medicine. He is a senior fellow in both the Center for Bioethics and the Leonard Davis Institute for Health Economics.

His research has focused on several areas, including clinical investigations of heritable disorders of connective tissue and cardiovascular diseases, especially those affecting the thoracic aorta. Currently he is studying how more refined methods of investigating genetic variation, such as cytogenomic arrays and whole genome sequencing, actually increase the level of uncertainty in applying the results clinically.

# CHAPTER

# 4

## Genomics and Proteomics

*Raju Kucherlapati*

### 4.1 GENES AND HUMAN DISEASE

The last century has witnessed the increasing realization that genetics plays an important role in human health and disease. The principles underlying genetics were discovered by Mendel in 1867, but remained obscure until the beginning of the twentieth century. Archibald Garrod first enumerated the significance of these genetic principles to human disease in 1902 (1).

The initial focus of human genetics was the class of diseases and disorders that were the result of mutations in single genes. Because the inheritance of these disorders follows the rules established by Mendel's discoveries, these disorders are referred to as Mendelian disorders. Because the disorders are the result of mutations in single genes, they are also referred to as single-gene disorders. Much of the twentieth century was devoted to the description of a large number of such disorders. A compilation of these disorders that is available online (Online Mendelian Inheritance in Man (OMIM) (2)) lists more than 10,000 such disorders. Many of these disorders were discovered in newborn children. Although all of these single-gene disorders collectively constitute a significant health burden on the population, the relative rarity of each of the disorders made human genetics an esoteric specialty of medicine.

The realization that genetics plays an important role in common as well as rare disorders is changing the importance of this science in research and clinical medicine. Assessment of risk factors for many common disorders, such as cancer, cardiovascular disease, obesity, diabetes, and neurologic/neurodegenerative diseases, to name only a few, has revealed that one of the greatest risk factors for developing a disease is having a close relative who is affected by the disorder. Since close relatives share many genes in common, it is reasonable to conclude that genes play an important role in the etiology of these disorders. Dramatic illustration of the role of genetics in common disorders came from the studies of twins. Identical twins share all of their genetic information and fraternal twins are like siblings, who, on the average, have a 50% probability of being identical at any genetic locus. Therefore, if one member of the twin has a disorder that has a genetic

basis, the probability that the second member also has the disease (concordance) would be very high in identical twins and no more than 50% in fraternal twins (3). For example, the concordance rate of diabetes and obesity in identical twins is as high as 90%, while in fraternal twins it is less than 50% (4). These types of studies have shown that many of the common disorders have a genetic basis.

In addition to the changes of genes in the germ line, somatic genetic changes also play a very critical role in many diseases, especially those that involve clonal expansion of cells, such as cancer. For example, somatic mutations in the gene *TP53* are found in more than 50% of all human cancers.

Germ line or somatic changes that involve whole chromosomes or parts of chromosomes are also important for human disease. Complete or partial trisomy (three copies of a chromosome) of chromosome 21 leads to Down syndrome, and chromosome number changes (aneuploidy) and segmental amplifications or loss of individual genes or areas of the genome are common features in many solid tumors.

Detection of chromosomal, sub-chromosomal, or individual gene changes can help understand the etiology of many human diseases. In addition, the ability to accurately detect these changes also has implications for diagnosis, prognosis, and treatment of human disease.

### 4.2 GENOMICS

The term genomics was first coined by Thomas Roderick of the Jackson Laboratory and was used to create a new scientific journal in 1987. This term is now used to define an area of science. While genetics is the study of patterns of inheritance and the study of single genes, genomics is the study of the organization of the entire genome as well as the study of the functions and interactions of large sets of genes or the whole genome.

One of the early contributions of genomics is the facilitation of disease gene identification. The identification and study of disease genes required novel technologies. The advent of recombinant DNA technologies and the polymerase chain reaction (PCR) in the last third of

the twentieth century allowed identification of several disease genes. Protein sequencing methods identified the basis for certain diseases such as sickle cell anemia, and the cloning of genes corresponding to abundant messenger RNAs allowed confirmation of the genetic basis for some blood-borne diseases. This approach, however, required that we have the knowledge about the protein whose lack or alteration in function causes the disorder. Since the molecular basis for many of the human diseases is not known, and in many cases even the particular organs where the gene product is critical are not known, a directed approach for gene cloning is not possible.

Positional cloning, an alternative approach to cloning genes based on their position in the genome, has been developed (5). In this approach, genetic methods are used to localize the disease locus to a relatively small segment of the genome, all of the genes in that interval are identified, and the sequence of each of the genes is compared between affected and unaffected individuals, preferably among closely related individuals. The gene that shows a mutation among the affected individuals would be the disease-causing gene.

To facilitate disease gene discovery and to form the foundation for understanding the complete set of genetic instructions in the human genome, an international effort was initiated in the early 1990s to map and sequence the human genome. These efforts culminated in the development of a draft sequence of the human genome in 2001 (6) and the completed sequence in 2003 (7), which coincided with the 50th anniversary of the discovery of the structure of DNA.

### 4.3 MAPPING THE HUMAN GENOME

The maps of the human genome that were constructed can be generally classified into two categories: genetic maps and physical maps.

#### 4.3.1 Genetic Maps

Construction of genetic maps requires three components:

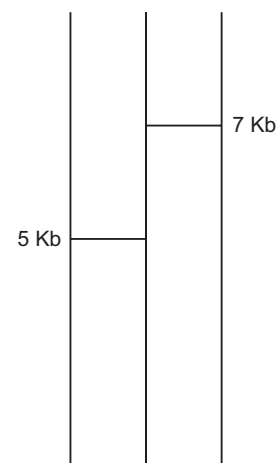
1. Genetic markers that are polymorphic in the population.
2. Robust methods for analyzing the markers in an appropriate set of pedigrees.
3. Analytical methods to construct the maps.

Early markers for construction of genetic maps were immunologic markers such as the ABO, MN, and Rh blood groups. In the 1970s, the discovery that enzymes could exhibit polymorphisms that resulted from changes in charge (isoenzymes) led to widespread use of such markers. A revolution in genetic mapping of humans was facilitated by the discovery of DNA-based polymorphisms. The focus on such markers was based on natural variation in the presence or absence of restriction enzyme

recognition sites. Let us consider a fragment of DNA that can be recognized by the restriction enzyme EcoRI to be 5 kbp in length. If, in a certain subset of the population, there were a single nucleotide change at one of the two recognition sites that define the particular 5 kbp fragment, EcoRI digestion would result in a fragment that is larger than the 5 kbp fragment (Figure 4-1).

Such polymorphisms are termed restriction fragment length polymorphisms (8). Because this is a DNA-based assay, use of a single restriction enzyme and an array of unique probes from different portions of the genome enable the detection of many different regions of variation. Such polymorphisms can only be bimorphic (presence or absence of the recognition site). For genetic analysis, the degree of heterozygosity of the markers determines their utility. If there are two alleles of a locus and they are in Hardy–Weinberg equilibrium, the degree of the heterozygosity can be ascertained by the simple formula  $2pq$ , where  $p$  = the frequency of one allele and  $q$  = the frequency of the second allele. If the two alleles are present with equal frequency ( $p$  and  $q = 0.5$ ), the degree of heterozygosity would be  $2(0.5)(0.5) = 0.5$ . If one allele is present at low frequency (e.g.  $p = 0.9$  and  $q = 0.1$ ), the heterozygosity would be much lower:  $2(0.9)(0.1) = 0.18$ . Therefore, for a biallelic marker the maximum heterozygosity would be 0.5.

The discovery and the use of microsatellite markers (9,10) made genetic mapping easier. This was based upon the observations that there are a variety of repetitive sequences that are located within the genome and that the number of individual elements within the repeats is highly polymorphic in the population. For example, there are regions in the genome that contain one (mono; e.g. AAAAAA), two (di; e.g. CACACACA), three (tri), and four (tetra) nucleotide repeats. Very often the number of the individual repetitive elements at these regions is highly variable. Since such variations have occurred



**FIGURE 4-1** As a result of a single nucleotide change at one of the two recognition sites that define a particular 5 kbp fragment, EcoRI digestion results in a fragment that is larger than the 5 kbp fragment.



many, many generations ago, the repeat sizes are stably transmitted from one generation to the next. The variation in repeats can be analyzed by using PCR primers for unique sequences that flank the repeats and the separation of the products in high-resolution gels that can distinguish small differences in the length of the products (11). The high degree of polymorphism of these markers results in a high degree of heterozygosity in the population (sometimes as much as 0.8 or more).

The microsatellite markers are now being replaced by single nucleotide polymorphisms (SNPs). Sequencing of individual genes or portions of other regions revealed that any two unrelated individuals differ at approximately 1 in every 1000 nucleotides (9). Although such differences are usually bimorphic and heterozygosity at any one marker might be low, such polymorphisms can be easily and inexpensively assayed. Strategies for using such markers in genetic analysis were proposed (12–14). Methods to detect a very large number of such polymorphisms are now becoming available.

The use of all of these different types of markers has enabled the construction of several different genetic maps of the human genome. The strategy for construction of genetic maps involved the use of DNA samples from a set of multigenerational families and using the information to assess frequencies of recombination between pairs of markers. The genetic map is a reflection of the frequency of genetic recombination. Any Mendelian disease that is segregating in a family or a set of families can be considered as a genetic marker and can be mapped and placed within the genetic map. The unit of genetic recombination is percent recombination, and 1% recombination is equal to a genetic distance of 1 centimorgan (cM). The length of the human genome in recombination units is 3000 cM. Since the physical size of the human genome (see later) is about  $3 \times 10^9$  bp, 1% recombination frequency corresponds to approximately  $10^6$  bp of DNA.

### 4.3.2 Physical Maps

Physical maps of the genome proved to be valuable as a prelude to clone-by-clone-based sequencing and now serve as useful tools for identifying and examining small, well-defined cloned portions of the genome.

The first physical map of a eukaryote that was constructed was that of the yeast *Saccharomyces cerevisiae*. To construct a physical map, a genomic phage library (fragments of DNA that were cloned into a bacteriophage vector) was made. DNA from individual clones was digested with a restriction endonuclease and the products were separated by gel electrophoresis. The resulting pattern from each clone is defined as a “fingerprint.” If two clones have sequence overlaps, they would share a part of the fingerprint profile (15). Therefore, overlaps between clones can be established. As overlaps are established, the clones begin to coalesce and form

contiguous segments referred to as “contigs.” At early stages of assembly, each individual clone will constitute a contig, and therefore the size of individual contigs will be equal to the size of the clone. As the contigs merge, their size increases; ultimately, each chromosome will be represented by a single contig.

Although clone-based fingerprinting played an important role in human genome mapping (16,17), the much larger size of the genome required the development of novel strategies and approaches to accomplish the goal. Two methods became particularly useful: radiation hybrid (RH) mapping and sequence-tagged site (STS) mapping.

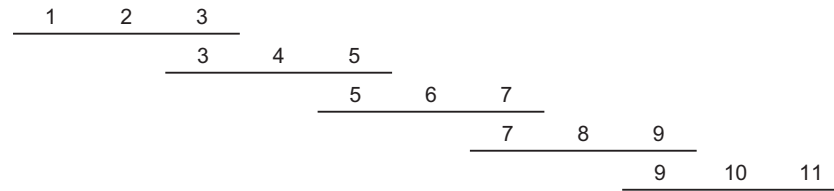
**4.3.2.1 RH Mapping.** When human cells are irradiated with ionizing radiation, they suffer chromosomal breaks. The number of such double-stranded breaks per cell is proportional to the level of irradiation. Levels of irradiation that cause one or more breaks per chromosome are lethal to the cells; however, the irradiated human cells can be rescued by fusion to a normal cell. Goss and Harris showed that this feature could be used to map genes (18). A normal human cell type is irradiated and fused with a rodent cell. The somatic cell hybrids retain a random subset of the chromosomal fragments. A panel of independent RHs would retain different and random segments of the human genome. The closer two markers are along the length of a chromosome, the lower the possibility of breakage between them. Therefore, frequency of co-retention of any two markers would reflect their relative distance apart.

A panel of RH cell lines was generated and screened with PCR-based markers. Like genetic maps, co-segregation of markers was reflected as units that are called centirays for a given level of irradiation (19).

**4.3.2.2 STS Maps.** Construction of RH maps and other physical maps required easily detectable markers. STS markers are one such set of markers. A pair of PCR primers that amplify a unique region of the genome defines each of the STS markers. Such markers are very robust because they define a unique region of the genome, and they can correspond to genes, polymorphic markers, or anonymous regions of nonrepetitive DNA (17). A global physical map of the human genome was constructed using this approach.

STS markers that corresponded to polymorphic regions were used for genetic mapping, and all types of STS markers were used for RH mapping and for identifying individual-cloned DNA fragments from libraries and for establishing clone overlaps and building contigs.

A number of different types of genomic DNA libraries proved useful in making physical maps. Unlike yeast maps, in which short-insert libraries played an important role, human physical map construction required large-insert clones. Initial efforts were focused on the use of yeast artificial chromosome (YAC) libraries (20). Although YAC vectors could take DNA fragments from as low as a few hundred kilobases to as large a segment



**FIGURE 4-2** If two YACs share a set of STS markers, they are considered to have an overlapping DNA sequence. The order of the markers and the relative orientation of the YACs can be deduced by the patterns of overlap. The strategy used to accomplish this goal is shown here.

as a megabase ( $10^6$ bp) or more, they proved to be unstable and had a tendency to be chimeric. Nevertheless, they proved to be valuable in constructing physical maps. Genomic libraries of human DNA in YACs were screened with large numbers of STS markers. Each YAC was defined by the set of STS markers it contained. If two YACs share a set of STS markers, they would be considered to have an overlapping DNA sequence. The order of the markers and the relative orientation of the YACs can be deduced by the patterns of overlap. The strategy that was used to accomplish this goal is shown in Figure 4-2. An actual physical map for a part of human chromosome 12 is shown in Figure 4-3.

The RH-based and YAC-based maps, in combination with genetic maps, provided an excellent framework for more detailed physical maps of the human genome that were based on the use of inserts from a set of bacterial cloning vectors such as cosmids and bacterial artificial chromosomes (21,22).

#### 4.4 SEQUENCING THE HUMAN GENOME

Two different approaches were used to obtain the sequence of the human genome. In one approach, the basic unit of sequencing was a cloned segment of DNA and the second approach involved a genomic shotgun approach. In the clone-based approach, DNA from a defined segment of the DNA that corresponded to a particular human chromosome was used to construct a random fragment (shotgun), small-insert library. Members of this library were sequenced, and automated methods were used to assess overlaps and to assemble the sequence into a contig that corresponded to the original large-insert clone. Sequencing overlapping clones allowed assembly of the sequence for each chromosome (23). In the random clone-sequence approach, total human DNA was randomly fragmented, cloned into sequencing vectors, and sequenced. Automated methods were used to assess overlaps of sequence information from different clones and to assemble the sequence (6).

Draft sequences of the human genome were published in 2001, and a completed version of the sequence was described in 2004 (6,7,23). The human genome is composed of approximately  $2.7 \times 10^9$ bp of DNA, and current estimates suggest that it encodes approximately 23,000 genes.

#### 4.5 CURRENT APPROACHES TO SEQUENCE PARTS OR THE WHOLE HUMAN GENOME

There has been a revolution in the methodologies and the cost of DNA sequencing that in turn is greatly facilitating the identification of human disease genes. Much of the first human genome sequencing was achieved by the use of dideoxynucleotide sequencing, also referred to as Sanger sequencing named after the Nobel Prize-winning biochemist Frederick Sanger. In this method, a sequencing reaction mixture contained normal nucleotides and small portions of a dideoxynucleotides. Whenever a dideoxynucleotide is incorporated into the DNA sequence, it prevents the addition of additional nucleotide, thus generating DNA molecules of varying size that end at the site of the incorporation of the dideoxynucleotide. Separation of these molecules on the basis of their size on automated DNA sequencers enables rapid sequencing of the molecule. In the later stages of the human genome project, DNA was fractionated in 96 capillary gel matrices and the sequence lengths of several hundreds of nucleotides per capillary were achieved.

The rapidity with which DNA sequences can be generated and the cost of obtaining them has dramatically changed since 2001. Although there are several different platforms for rapidly sequencing DNA, the basic principle involves generating millions of microscopic spots in a small region, each corresponding to a single DNA molecule, deposited in a matrix and the positions of all of the molecules recorded optically. In one method, a single-tagged nucleotide is added to the reaction mixture and the polymerase is allowed to add that single nucleotide to the appropriate DNA molecule. Imaging after this reaction would record all the spots that have incorporated that nucleotide. A second nucleotide is added to the reaction mixture and the spots that incorporate the nucleotide are recorded. This process is completed with all four nucleotides and the process repeated again. Deconvolution of the images allows the deduction of the sequence of DNA at each spot. Alignment of the resulting sequences to a standard genomic sequence would allow assembly of the DNA sequence. As millions of DNA molecules can be simultaneously sequenced, large amounts of DNA sequence can be generated in a relatively small amount of time at a significantly reduced cost (24). For example, it was estimated that the completion of the human genome



sequence cost US\$2–3 billion while current technologies can accomplish the same goal for US\$10,000 or less.

During the past few years, technologies to capture specific subsets of the human genome have also been developed. Using these methods, it is possible to sequence tens to hundreds of genes or the exomes of all genes for a fraction of the cost of the whole genome sequencing. These technologies are revolutionizing identification of human disease genes.

## 4.6 AN APPROACH FOR CLONING HUMAN DISEASE GENES

For Mendelian disorders, a well-established approach to clone the disease gene is to identify one or a set of multi-generational families in which this disorder is segregating and map the disease locus onto the genetic map. Once a disease gene locus is mapped to a large interval, it is possible to narrow the location by using additional markers in that region. Once the location is narrowed down to a 1–2cM interval, it is possible to identify all of the genes encoded in that region and sequence them in the affected individuals. A gene that consistently has a mutation in the affected individuals but not in the unaffected individuals is most likely the disease gene (5). This approach has been successfully used to clone a large number of genes. Among the early genes that were cloned are those for cystic fibrosis (13), neurofibromatosis (25,26), and Huntington disease (27). In the early stages of disease gene cloning based upon the location of the gene, it was necessary to narrow down the region in which the disease gene was likely to be present to a relatively small region and therefore a small number of genes. The advent of massively parallel sequencing approaches described above are changing the landscape, and examples of whole-exome sequencing or whole genome sequencing of a single affected individual are becoming more commonplace (28).

### 4.6.1 Association Studies

The availability of the human DNA sequence has also allowed us to define the genetic variation among individuals. That definition, in turn, is permitting us to identify the genes that may be involved in complex disorders.

Sequencing genes or parts of the genomes from different individuals revealed that there is natural variation in the DNA sequence. On average, any two individuals differ at approximately 1 of every 1000 nucleotides (9,11,12,14). Most such variation is at the single nucleotide level and is referred to as a SNP. The frequency of any single polymorphism within a given population is variable and depends on the time of its origin in the human history: the older the origin, the more frequent it is in the population. Each of the DNA sequence variants can be used as a polymorphism and can serve as a genetic marker. SNPs can be used like any other genetic marker

to map a genetic locus. SNPs can also be used to conduct association studies. If a common disease has a genetic basis, the disease-causing allele and genetic markers that are closely linked to it will be present at much higher frequencies in the affected cohort of individuals. Examination of the DNA from a set of cases and controls for a large set of SNPs would reveal any association between the disease and genetic markers. A large number of such associations have been described and confirmed (29).

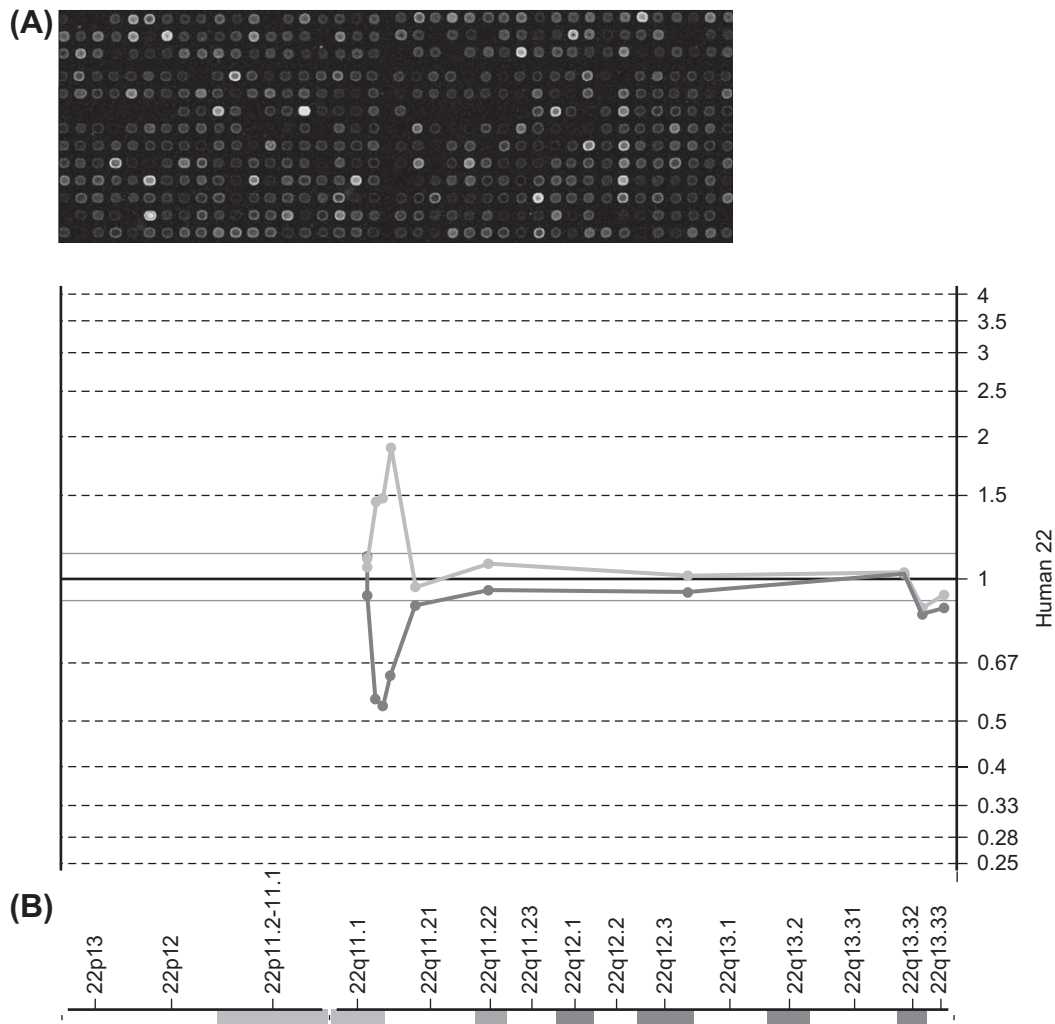
### 4.6.2 Genomic Changes

Alterations in the genome in the germ line or soma may have profound implications for disease. For example, certain types of chromosome number abnormalities result in specific disorders. Increase in the number of copies of chromosome 21 from two to three results in Down syndrome. Reduction in the copy number of a portion of chromosome 22 from two to one causes DiGeorge/velocardiofacial syndrome (30). Increase in the copy number of the same region from two to three results in der(22) syndrome (31). Whole chromosome number changes are very common in many solid tumors and increases of copy numbers of specific regions of the genome thorough amplification or loss of heterozygosity of specific regions or particular chromosomes are also common features in solid tumors (32). Detection of these chromosomal changes has implications for diagnosis and prognosis of disease. Different methods are available to detect specific chromosomal or sub-chromosomal changes.

**4.6.2.1 Cytogenetics Methods.** Examination of metaphase chromosomes is a common method for determining chromosomal and sub-chromosomal changes. Because chromosomes are highly condensed during metaphase, the resolution of this method for detecting chromosomal changes is several megabases of DNA. Preparation of metaphase chromosomes for examination requires that the cells be undergoing mitosis. Nonmitotic or interphase cells can also be used for detection of copy number changes by hybridization of the cells' DNA with targeted probes that correspond to known regions of the genome. This method is referred to as fluorescence in situ hybridization. For example, if a chromosome 21-specific probe is used for hybridization, cells from a normal individual reveal two spots corresponding to the two chromosomes. If the cells are from a patient or fetus with Down syndrome, the probe may reveal three spots.

**4.6.2.2 Array-Based Comparative Genomic Hybridization.** A high-resolution method for detecting copy number changes is available. In this method, sequences corresponding to specific known portions of human DNA can be made into an array. DNA from a normal individual labeled with a fluorophore (e.g. green) and DNA from the test sample labeled with a second fluorophore (e.g. red) can be combined and hybridized to the array. The two DNA samples compete with each other to hybridize to the target immobilized DNA. If a particular





**FIGURE 4-4** The relative intensities of two fluorophores can be measured with a high degree of accuracy, providing a sensitive method for detection of copy number changes. An example of how this method was used to detect copy number changes in a part of human chromosome 22 is shown here. (A) Image of an array (B) Relative intensities of the two colors in the hybridization reaction. The region of the spike reveals loss of one copy of that segment.

segment of the DNA is present in equal parts in the reference and sample DNA, the two fluorophores will be equally represented and the spots will be yellow in color (a combination of green and red). If the sequence is overrepresented in the reference, the spot(s) corresponding to it will be green; if it is overrepresented in the test sample, the spot(s) will be red. The relative intensities of the fluorophores can be measured with a high degree of accuracy, thus providing a sensitive method for detection of copy number changes (33). An example of how this method was used to detect copy number changes in a part of human chromosome 22 is shown in Figure 4-4.

#### 4.7 SEQUENCE-BASED METHODS FOR DETECTING CHROMOSOMAL ABNORMALITIES

The new technologies of DNA sequencing described above are also facilitating the rapid discovery of

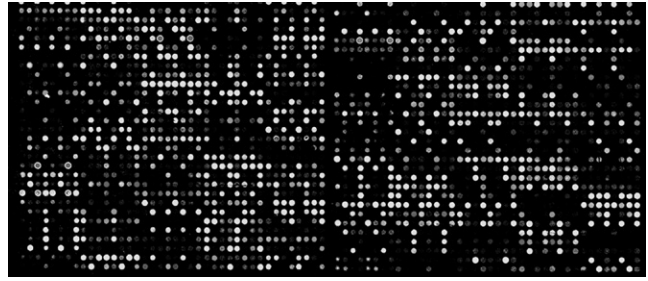
structural aberrations in DNA. In one approach to DNA sequencing, genomic libraries with insert sizes of 200–300 nucleotides are generated and each molecule is sequenced from both ends and a sequence of 35, 50, or 100 nucleotides is generated. Once the sequence is obtained with a sufficient amount of redundancy and the short reads aligned to the reference genome, it is possible to segment the genome into segments of a desired size (e.g. segments of 1,000 bp) and count the number of reads in each segment. The number of such reads can be compared to a single or pooled control samples. If there is no copy number difference in one or a series of adjacent segments in the control and test samples, the ratio of test/control would be 1.0. If there are copy number variations or amplification of the region of the genome, the ratio would exceed 1.0 and the actual number would depend on the extent of the amplification (a ratio of 1.5 would indicate that the test sample has three copies versus the normal two copies, a ratio of 2 would indicate that

the test sample has four copies versus the normal two copies, etc.). A ratio of less than 1.0 would indicate reduction in copy number. Because the whole genome can be sampled by these sequencing methods, the accuracy and resolution afforded by these methods can be significantly greater than FISH-based or array-based methods.

If the average size of the inserts in the library is 250 bp and 50 nucleotides of sequence was obtained from each end, the pair of reads (mate pairs) would map back to the same chromosome and the distance between the ends of the sequence reads would, on the average, be 150 nucleotides. If there is a 10kb deletion in the genome being sequenced, the mate pairs would map to the same chromosome but would map to regions that are nearly 10kb apart. Since the deletion breakpoints can be mapped to a very small region of the genome, developing appropriate primers that flank the deletion breakpoint, amplifying the region from the test sample, and sequencing that short region would reveal the precise breakpoint at the resolution of a single nucleotide. If the mate pairs map to different chromosomes, it would suggest a translocation. Other chromosomal abnormalities such as inversions and duplications can also be detected by these sequencing methods. The same general approach can be used to sequence RNA. Counting the number of times a transcript is represented in the sequence reads indicates its abundance. Mate pairs would allow detection of alternative splicing products.

### 4.7.1 Gene Expression Profiling

Different genes are expressed in different tissues and cell types. Pathologic changes in particular cells or tissues are often accompanied by changes in patterns of gene expression. Methods to detect changes in expression levels of individual genes are now available. They include Northern blotting, wherein RNA from a target cell type is fractionated by gel electrophoresis, transferred to a nylon membrane, and hybridized with a labeled gene-specific probe. Band intensity is used as a measure of abundance of the corresponding transcript. Quantitative reverse transcriptase-PCR is also a very useful method. The development of array-based methods allows examination of virtually all transcripts in any cell or tissue type. In this method, RNA from a reference sample is converted to its corresponding complementary DNA or RNA, labeled with a fluorophore, mixed with a corresponding complementary DNA or RNA from the test sample labeled with a different fluorophore, and hybridized to an array of DNA fragments corresponding to individual genes (Figure 4-5). Measurement of fluorescence intensities can be correlated to levels of gene expression (34). Sequence-based methodologies are beginning to be used for detecting transcript abundance. When RNA is converted into complementary DNA and subjected to sequencing, the relative number of times a



**FIGURE 4-5** RNA from a reference sample is converted to its corresponding complementary DNA or RNA, labeled with a fluorophore, mixed with a corresponding complementary DNA or RNA from the test sample labeled with a different fluorophore, and hybridized to an array of DNA fragments corresponding to individual genes.

particular RNA is sequenced provides a measure of its abundance.

## 4.8 PROTEOMICS

Proteins are the functional units of cells. Rapid and accurate identification of proteins in cells or body fluids can provide significant information about the functional status of the cell, tissue, or organism. A number of newer methods are becoming available for such analysis.

A proteome is defined as the complete protein complement of a cell, tissue, or organism. The study of the proteome is referred to as proteomics. Although the study of proteins in detail preceded studies of DNA and RNA of organisms, high-throughput methods for detecting and quantitating proteins on a large scale have recently emerged and are continuing to develop. Detection of proteins relied on fractionating methods and gel electrophoresis. Large-scale protein analysis relied on two-dimensional electrophoresis (2DE) and imaging of the spots that result from such a method. Although 2DE is a powerful method, it does not provide for unambiguous assignment of each of the bands to a specific protein. A number of protein analytic methods are now available that are useful in identification of large numbers of proteins. Several of these methods use some type of fractionation of a complex mixture of proteins or peptide fragments followed by mass spectrometry methods to identify individual proteins. In some methods, the mass of a large number of proteins is assessed, thus providing for a profile of the mixture. Differences in the profiles can be used for diagnostic or discovery purposes. In another method, a protein mixture is fractionated and individual fractions are digested with a protease; mass spectrometry is used to deduce the mass of the peptide as well as to sequence that peptide. The availability of genomic sequence allows us to predict the proteins that the genome encodes, and computational comparison of the database containing all of the predicted proteins and peptides allows for unambiguous identification of the origin of the peptide that was sequenced by the mass spectrometer.

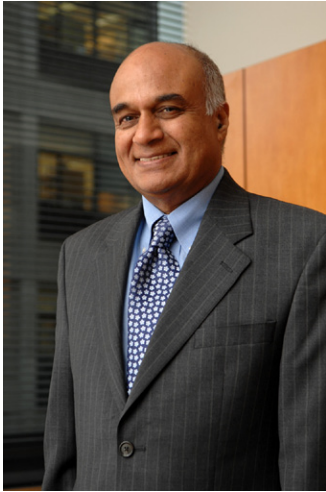
## GLOSSARY

- Association Studies** – assessing relationships based on specific genetic or physical features with particular traits.
- Comparative Genomic Hybridization** – a method to detect changes in copy number in different individuals or on the development of tumors.
- Genetic Maps** – maps based on genetic recombination frequencies.
- Genomics** – study of the organization of the entire genome as well as the study of the functions and interactions of large sets of genes or the whole genome.
- Physical Maps** – maps based on physically defined entities such as fragments of DNA.
- Proteomics** – study of protein composition on a large scale.

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### Biography



**Dr Raju Kucherlapati, PhD**, is the Paul C Cabot professor in the Harvard Medical School Department of Genetics. He is also a professor in the Department of Medicine at Brigham and Women's Hospital. Dr Kucherlapati was the first scientific director of the Harvard Medical School–Partners Healthcare Center for Genetics and Genomics. His research focuses on gene mapping, gene modification, and cloning disease genes. During 1989–2001, Dr Kucherlapati was the Lola and Saul Kramer professor of Molecular Genetics and the Chairman of the Department of Molecular Genetics at the Albert Einstein College of Medicine in New York. He was previously a professor in the Department of Genetics at the University of Illinois, College of Medicine. He began his research as an assistant professor in the Department of Biochemical Sciences at Princeton University. He has chaired numerous NIH committees and served on the National Advisory Council for Human Genome Research and the NCI Mouse Models for Human Cancer Consortium. He is also a member of the Cancer Genome Atlas project of the National Institutes of Health. He is a member of the Institute of Medicine of the National Academy of Sciences and a fellow of the American Association for the Advancement of Science. He is a member of Presidential Commission for the Study of Bioethical Issues. Dr Kucherlapati received his BS and MS in Biology from universities in India, and he received his PhD from the University of Illinois at Urbana, as well as conducting postdoctoral work at Yale University.



# CHAPTER

# 5

## Genome and Gene Structure

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### 5.1 INTRODUCTION

The chemical structure of genetic material as well as the storage, processing, and transfer of genetic information from one generation to the next are similar in all living organisms. Thus, it was expected that the complexity of the human phenotype would be explained by a significantly higher number of genes in humans when compared with simpler organisms. Surprisingly, instead of the predicted 50,000–150,000 human genes, the sequence of the human genome revealed about 20–25,000 genes, similar to the number of genes in many other organisms. However, analysis of the genome and its products has revealed complexity in the form of exquisite temporal and spatial regulation of gene expression, alternative transcripts derived from a single locus, multiple splice variants (including tissue-specific variants) for most genes, and complex posttranslational modifications of proteins, all of which create endless diversity in gene products and their functions.

Since the initial sequence of the human genome was determined, we have gained tremendous new insights into genome structure such as how the sequence and structure define the complex functions of human cells and how genome architecture can be altered to produce disease. Additionally, how our genome is compared with the sequences of the genomes of closely and distantly related organisms has provided unique insights into evolutionary conservation of gene and protein functions as well as our origins as a species. Technological innovations have facilitated defining the genomic sequences of many individual humans, particularly the polymorphic differences that distinguish individuals, allowing a fuller description of the history of our species and the traits we manifest. We are also at the point where we can conceive of understanding at molecular level the genetic contributions to disease across the human population, facilitating targeted medical intervention based on these findings.

### 5.2 DOUBLE HELIX STRUCTURE, DNA REPLICATION, TRANSCRIPTION, AND MEIOTIC RECOMBINATION

#### 5.2.1 Double Helix

The function of the human genome is to transfer information reliably from parent cells to daughter cells and from one generation to the next. This is carried out in a semiconservative manner. One of the two parental DNA strands of a double helix remains intact in every cell division, serving as a template for copying the sequence. The two DNA strands form the double helix by hydrogen bonding between the nitrogenous bases: guanine (G) pairs with cytosine (C) and adenine (A) pairs with thymine (T) (Figure 5-1). The hydrogen bonds formed between these pyrimidine–purine pairs (guanine and adenine are purines; cytosine and thymine are pyrimidines) stabilize the double helix and ensure that the two complementary strands remain together and in register. The strands are oriented antiparallel to each other, meaning that they run in opposite directions: One strand is oriented in a 5′–3′ direction, whereas the other in a 3′–5′ direction.

#### 5.2.2 Replication

Genetic information is preserved and transmitted by DNA replication, a process that produces two identical copies of the DNA. During this process, the two parental strands separate, and each serves as a template for synthesis of a new complementary strand by an enzyme called DNA polymerase (Figure 5-2). As a consequence, each daughter cell inherits one strand of the parental duplex. Every DNA molecule thus contains a “young” strand that was synthesized in the parental cell during DNA replication and an “old” strand that was inherited from the parental cell and synthesized in the grandparental cell. This semiconservative manner of replication guarantees transmission of intact information from one generation to the next. Remarkably, the genome copies itself through

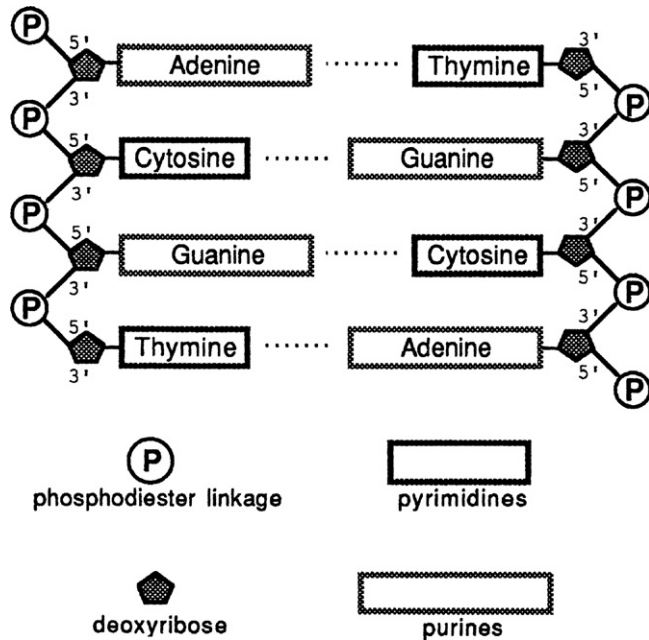


FIGURE 5-1 Complementary structure of double-stranded DNA.

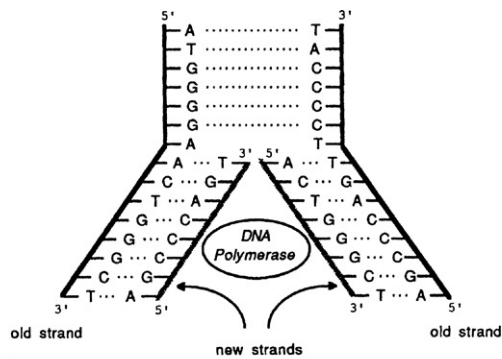
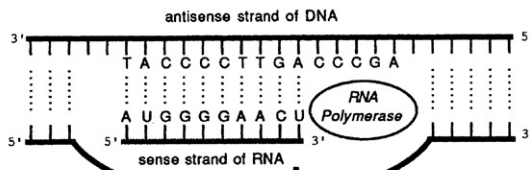
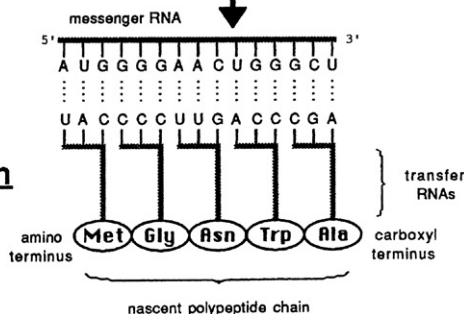
**Replication****Transcription****Translation**

FIGURE 5-2 Flow of genetic information.

millions of cell divisions during an individual's life with amazing precision. The error rate of about  $1 \times 10^{-8}$  per base pair (bp) per generation means that replication of  $3 \times 10^9$  bps comprising the human genome leads to about 60 new single base mutations per individual.

**5.2.3 Transcription**

Only about 1–1.5% of the genome is reflected in the population of mature protein-coding transcripts. Protein-coding genes are transcribed from DNA into messenger RNA (mRNA) (see Figure 5-2). A single gene can give rise to multiple transcripts by means of alternative splicing and alternative sites of transcription initiation and termination, generating functional diversity. During transcription, the DNA duplex unwinds, and one of the strands serves as the template for synthesis of a complementary RNA strand. RNA is distinguished from DNA by the presence of uracil instead of thymine, ribose instead of deoxyribose, and a different three-dimensional folding pattern. The mRNA molecules are single stranded and function as the vehicle for translating genomic information into a protein.

In eukaryotes, genes are transcribed by one of the three different RNA polymerases (I, II and III, respectively). RNA polymerase I transcribes ribosomal RNAs (rRNAs) (except for 5S rRNA), RNA polymerase II transcribes mRNAs and micro RNAs (miRNAs), and RNA polymerase III transcribes 5S rRNA, transfer RNA (tRNA) and other small RNAs. In addition to RNA polymerase, initiation of gene transcription requires other proteins, so that multiple factors form the complex responsible for transcriptional initiation. This complex gets attached to the initiation site of transcription at the 5' end of the gene (the promoter) and determines which genes are transcribed in different cell types or during different developmental stages. The transcription factors (TFs), along with the activities of *cis*-acting enhancer and inhibitor sequences, also determine the level of gene expression. The enhancers and inhibitors can be located near the promoter of a gene, at the 5' or 3' side of the promoter or at significant distances away from the transcription start site. Such sequences are commonly found within first introns of many mammalian genes.

Most typically, only one of the two DNA strands gets transcribed, and the DNA strand that is similar to the transcribed mRNA sequence is referred to as the sense strand. The DNA sequence that serves as the transcriptional template is referred to as the antisense strand.

**5.2.4 Meiotic Recombination**

Meiotic recombination (1) refers to the reciprocal physical exchange of chromosomal DNA between the parental chromosomes and occurs at meiosis during spermatogenesis and oogenesis, serving to ensure proper chromosome segregation. During the four-strand stage of meiosis, two duplex DNA molecules (one from each parent) form a

hybrid, and a single strand of one duplex is paired with its complement from the other duplex. Single-stranded DNA is exchanged between the homologous chromosomes, and the process involves DNA strand breakage and resealing, resulting in the precise recombination and exchange of DNA sequences between the two homologous chromosomes. This process is highly efficient and does not usually result in mutations at the sites of recombination. Recombination thus shuffles genetic material between homologous chromosomes, generating much of the genetic diversity that characterizes differences between individuals, even within the same family.

The frequency of recombination between two loci along a chromosome is proportional to the physical distance between them, and historically, this provided the basis for defining the genetic distance between loci, allowing genetic maps to be constructed. The genetic proximity of two loci is measured by the percentage of recombination between them; a map distance of 1centimorgan (cM) indicates 1% recombination frequency between the two loci. The human genome sequence has made it possible to compare genetic and physical distances and to analyze variations in recombination frequency in different chromosomal regions. On average, 1 million base pairs (1Mb) correspond to 1cM (1% recombination frequency). However, there is a tremendous local variation between individual chromosomes and among particular chromosomal regions. For example, the average recombination rate is higher in the short arms of chromosomes and at the distal segments of the arms, but overall is suppressed near the centromeres. There is also a significant variation in the recombination rates between the sexes, with 1.6-fold more recombination on average in females relative to males. On average, female recombination is higher at the centromeres and male recombination at the telomeres (2).

### 5.2.5 Direction of DNA and RNA Synthesis

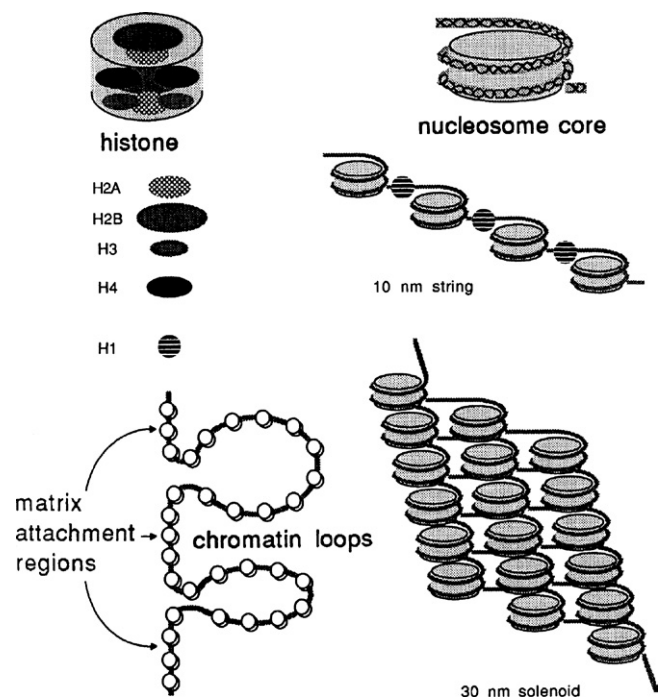
Each chromosome in the human cell consists of a continuous double-helical DNA strand; an average chromosome contains some 4–5 cm of DNA. The polarity of a single-stranded nucleic acid is defined by the position of the phosphodiester bonds, which connect the 3' hydroxyl group of one nucleoside to the 5' hydroxyl group of the next (see Figure 5-1). A nucleoside is composed of a purine or pyrimidine base and a deoxyribose (in DNA) or ribose (in RNA), and a nucleotide is composed of a nucleoside and one or more phosphate groups. The phosphate groups in a single nucleoside triphosphate are attached to the ribose or deoxyribose moiety via the 5' hydroxyl residue, and two of the three phosphates are removed during incorporation of each nucleoside triphosphate into DNA or RNA. When the new DNA strand is synthesized during replication or copied during transcription, the polymerase enzymes add new nucleotides to the 3' hydroxyl group of a growing polynucleotide chain. The new strand of DNA or RNA is thus

synthesized in the 5'–3' direction, and so the parental or template DNA strand is read in the 3'–5' direction. If two proteins are encoded by adjacent genes that lie on different strands along the chromosome, those genes are said to have opposite transcriptional orientations.

## 5.3 ORGANIZATION OF GENOMIC DNA

The 3 billion bps that constitute the human genome are packaged into 22 pairs of autosomes and the X and Y sex chromosomes. The chromosomal DNA can be divided into regions of heterochromatin and euchromatin: heterochromatin represents the “tightly” packed regions of chromosomal DNA and euchromatin represents the “loose” regions, which are generally the actively transcribed DNA regions. Using cytogenetic staining methods, differently packed chromosomal regions can be viewed as G (Giemsa staining)-bands, with the banding pattern characteristic of each individual chromosome providing the basis for the cytogenetic identification of each human chromosome. From early on, the dark G-bands were considered to reflect “gene-poor and GC-poor” regions, and the sequence of the human genome has proven this concept to be accurate. However, human genome sequence information has revealed that there can be a tremendous variation in the GC content across chromosomal regions.

Each of the 23 pairs of human chromosomes contains a single DNA duplex extending between the two telomeres. When the DNA in the human genome is stretched from one end to the other, its length would be more than 3 ft long! Remarkably, compacting the DNA by over 100,000-fold, which is required to fit the chromosomes



**FIGURE 5-3** Packaging of DNA into chromatin.

into the nucleus, is achieved by coiling and folding the double helix into a series of progressively shorter and thicker structures (Figure 5-3). Proteins that bind to DNA help direct and organize this folding, and the folded complex of DNA and protein is referred to as chromatin.

### 5.3.1 Nucleosomes and Higher Order Chromatin Structure

In addition to compacting the genetic material to fit into the nucleus, chromatin condensation can also regulate accessibility of the DNA for transcription and other processes. The simplest level of chromatin structure is the organization of DNA and histones into nucleosomes (3). Each nucleosome is a 147-bp long segment of DNA tightly wrapped almost two times around an octamer histone core. This octamer core contains two molecules each of the histones H2A, H2B, H3, and H4. Nucleosomes are the fundamental feature of all eukaryotic DNA and the sequences of the core histones are well conserved among even, distantly related species. A fifth histone, H1, binds to the DNA just outside each nucleosome and its sequence is less well conserved. A region of linker DNA, about 60bp in length in humans, usually separates adjacent nucleosomes, so that nucleosomes are for the most part regularly spaced.

Nucleosomes represent the first level in the packaging of naked DNA into chromatin and appear in the electron microscope as strings of 10-nm “beads.” The next level in packaging (see Figure 5-4) is the coiling of the nucleosomes, which, in some models, forms a solenoid that contains six nucleosomes per turn, measures

30nm in diameter, and is stabilized by interactions between H1 molecules bound to adjacent nucleosomes. Other models propose alternative patterns of nucleosomal stacking, with different internucleosomal interactions stabilizing the structure. Epigenetic modifications, primarily DNA methylation, and histone modifications also regulate the structure and dynamics of chromatin folding. Additional levels of folding can compress DNA into 300-nm fibers and a nearly 1000-nm metaphase chromatid.

### 5.3.2 Euchromatin and Heterochromatin

During metaphase, the entire chromosome is highly condensed but at other times, most chromatin is organized into fibers of intermediate diameter (30–300 nm), termed euchromatin. However, some portions of the genome remain highly condensed throughout the entire cell cycle, replicate late during S phase, and are termed heterochromatin. Many areas of heterochromatin are located close to chromosome centromeres and at the telomeres of acrocentric chromosomes, contain highly repetitive or simple-sequence DNA instead of genes, and may play a structural role in chromosome organization. A special form of heterochromatin is found in the inactive X chromosome in female cells, which contains genes but generally does not express them. This is partially due to a high degree of chromatin condensation and histone modification that does not allow access to the DNA by the transcriptional machinery. Inactive X heterochromatin remains highly condensed during the lifetime

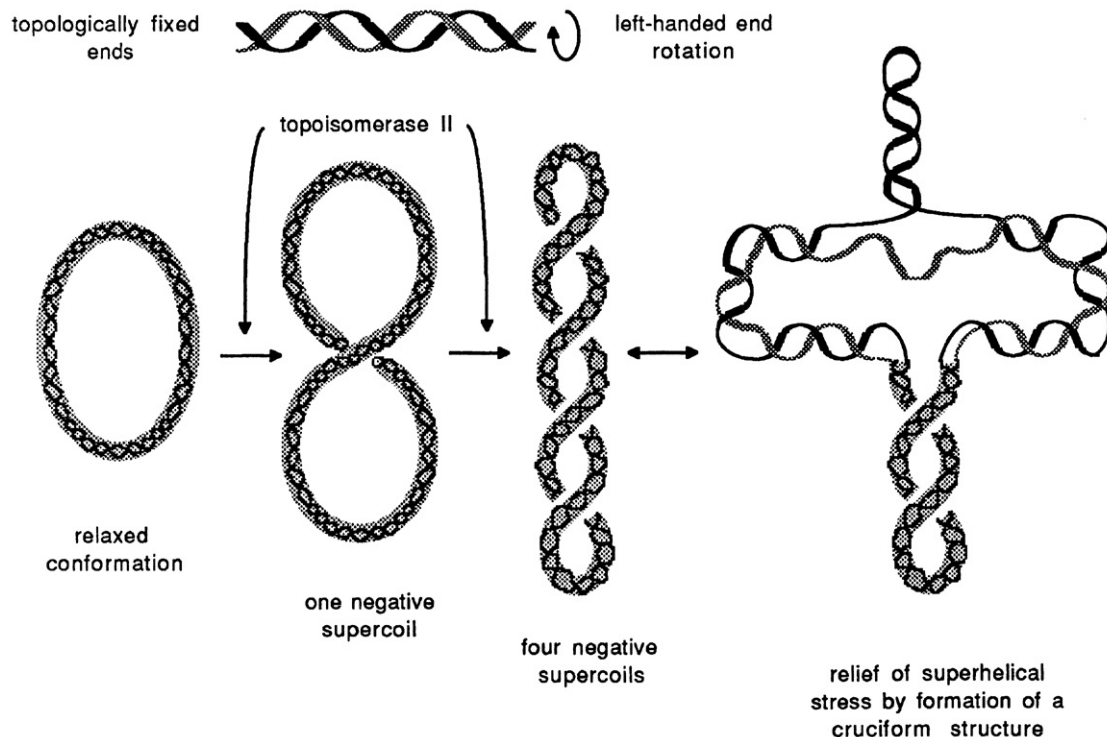


FIGURE 5-4 Superhelical turns in DNA.



of somatic cells, but in germ cells, during oogenesis, it becomes active and euchromatic by the time of entrance into meiosis.

Formation of heterochromatin involves proteins that help direct condensation and packaging to achieve assembly of these proteins and DNA into heterochromatin. The molecular mechanisms behind heterochromatin formation have been revealed by studies of inactivation of the second X chromosome in female cells (4). Heterochromatin spreading is also thought to be involved in the initiation of X-chromosome inactivation that occurs in all female cells because condensation begins from a specific site on the X chromosome, the X-inactivation center, which in humans is located on Xq13. At this center, the X-inactivation-specific transcript (XIST) gene encodes a 17- to 19-kb long noncoding RNA, which is transcribed only from the inactive X chromosome and acts in *cis* to initiate X-inactivation. During early embryonic development, X-inactivation is random, and so females are mosaics with respect to whether the maternal or paternal X is active in each cell. Once established in somatic cells, the inactive X is stable through replication and cell division; i.e. the same inactive X will be inactive in all daughter cells.

XIST RNA inactivates genes at a significant distance from the gene that encodes it. Although XIST is essential for X-inactivation, regulatory genes including TSIX and a variety of TFs control XIST. While XIST is essential for the initiation of X-chromosome inactivation, it is not required for the maintenance of X-inactivation. The detailed analyses of the process of X-inactivation have informed more general aspects of heterochromatin formation and gene inactivation.

### 5.3.3 Centromeres and Telomeres

Special features of the DNA molecule that comprises each human chromosome are required at the centromeres and the telomeres. Located close to most centromeres are many copies of a 171-bp  $\alpha$ -satellite repeat that forms the core of the centromere. These sequences bind structural proteins that serve as a site for kinetochore formation and spindle attachment during metaphase. Certain alphoid repeats are found close to the centromeres of all chromosomes, while others are specific for one or a small number of chromosomes. The proteins and DNA sequences that make up the centromeres must also ensure that the two daughter chromatids are partitioned to different cells during cytokinesis (5).

As template-directed replication of DNA can only be performed in a 5'–3' direction, one strand of each duplex cannot be fully replicated at its 3' terminus by the DNA polymerase. Therefore, the telomeres of each human chromosome contain many copies of a short repeat 5'-TTAGGG-3', which can be replicated using the enzyme telomerase (6). This enzyme has an RNA component, which itself serves as a template and can elongate

the 5'-TTAGGG-3' repeat in a manner that does not depend on the DNA strand.

**5.3.3.1 Repeat Content of the Human Genome.** Less than 5% of the human genome sequence encodes proteins, whereas repeat sequences account for at least 50% of the sequence. The repeats fall into five categories: (i) transposon-derived repeats, often referred to as interspersed repeats; (ii) inactive retroposed copies of cellular genes (referred to as processed pseudogenes); (iii) segmental duplications consisting of blocks of around 10–300 kb that have been copied from one region of the genome into another; (iv) blocks of tandemly repeated sequences such as centromeres and ribosomal gene clusters; and (v) simple-sequence repeats consisting of direct repeats of short sequences such as (CA)<sub>n</sub> or (CGG)<sub>n</sub>, which have been extremely important for human genetic studies as they have been used as genetic markers (see “Interindividual Variations in the Human Genome”).

**Transposable elements** in humans, as in all mammals, fall into four types: long interspersed elements (LINEs), short interspersed elements (SINEs, including Alu sequences), long terminal repeat (LTR) retrotransposons, and DNA transposons. Both the number and age of transposable elements in the human genome are strikingly different from those in other species. The density of transposable elements is much higher in humans than in other species, and the human genome contains more ancient transposons than do other species. It thus appears that these repeats have survived because of a significant evolutionary advantage although their selective advantage and precise function are not well understood. Some chromosomes are extremely crowded with repeat elements (e.g. a 500-kb region on the short arm of the X chromosome has an overall transposable element density of 89%), whereas other chromosomal regions are nearly devoid of repeats (e.g. the homeobox gene clusters).

**Pseudogenes** are regions of DNA with many sequence elements of a potential transcriptional unit (e.g. promoter, protein-coding region, splice junctions, etc.), yet do not code for a functional product. They can originate after gene duplication when the duplicated sequence acquires a mutation that prevents its expression. For example, a member of the  $\alpha$ -globin gene family,  $\psi\zeta$ , has all the sequence characteristics of a functional globin gene, but the protein-coding region contains a point mutation that prevents the expression of a full-length globin (7). A second way in which pseudogenes originate is by the pathway of reverse transcription and integration. If the mRNA of a cellular gene is converted into complementary DNA by reverse transcriptase, a duplex DNA molecule can be formed that lacks introns and contains a poly(A) tract. Pseudogenes with this pattern are commonly found in genomic DNA, showing that cellular mRNAs are occasional substrates for reverse transcriptase and that the DNA products can integrate back into the genome.

**Large segmental duplications** (8) are especially enriched in pericentromeric and subtelomeric regions of chromosomes. These interchromosomal duplications are sequences from elsewhere in the genome, sized at 1–200 kb, and are much more common in humans than in yeast, flies or worms, suggesting a relatively recent origin for these genomic elements.

**5.3.3.2 Interindividual Variations in the Human Genome.** Sequencing of the human genome has exposed multiple interindividual variations. These variants include both simple-sequence repeat polymorphisms and single nucleotide polymorphisms (SNPs). Repeat polymorphisms can represent di- (mostly  $CA_n$ ), tri-, or tetranucleotide repeats, and they form the basis of the genetic map of the human genome. These repeat markers are multiallelic; the alleles differ in the number of repeat units and thus can be used to identify the maternal and paternal alleles of individuals as well as to define recombinations between marker loci. The high degree of length polymorphism among simple-sequence repeats within the human population is due to frequent slippage by DNA polymerase during replication. These repeats comprise about 3% of the human genome, and there is approximately one such repeat per 2 kb of genomic sequence. The large number and wide distribution of these repeats has facilitated mapping and identification of many genes associated with inherited human disorders solely based on their chromosomal position.

Certain specific triplet repeats can be unstable, expanding and contracting during meiosis and/or mitosis. If the repeat becomes excessively long, it can cause diseases such as Huntington disease or spinocerebellar ataxia, which both are caused by the expanded repeat CAG in the coding region of a gene and result in a long polyglutamine tract within the gene product. Some expanded repeats occur in 5'- and 3'-untranslated regions (UTRs) and also result in a disease due to an inhibitory effect on gene expression.

SNPs are also nonrandomly distributed in the human genome. Millions of SNPs have been identified in the genome sequence but only a small fraction are predicted to affect the protein sequence. This limits the extent to which such genetic variations contribute to the structural diversity of human polypeptides, but regulatory effects on gene expression may cause or result in susceptibility to a variety of human phenotypes.

Copy number variation (CNV) describes the variation identified within the population or associated with human diseases in genomic segments larger than the SNP and simple-sequence repeat polymorphisms but smaller than cytogenetically visible chromosomal abnormalities (9). An appreciation of the number and diversity of such variants has primarily been a product of comparative genomic hybridization studies in normal individuals (copy number polymorphisms) and in patients with a wide variety of genetic disorders. Although the number of sites that vary is small when

compared with the SNPs and simple-sequence repeat polymorphisms, the number of bps involved may be as much as two orders of magnitude greater (10). Similar to the SNP variations, CNVs may be associated with susceptibility to particular disorders or may be causative, especially when they arise *de novo* in an individual.

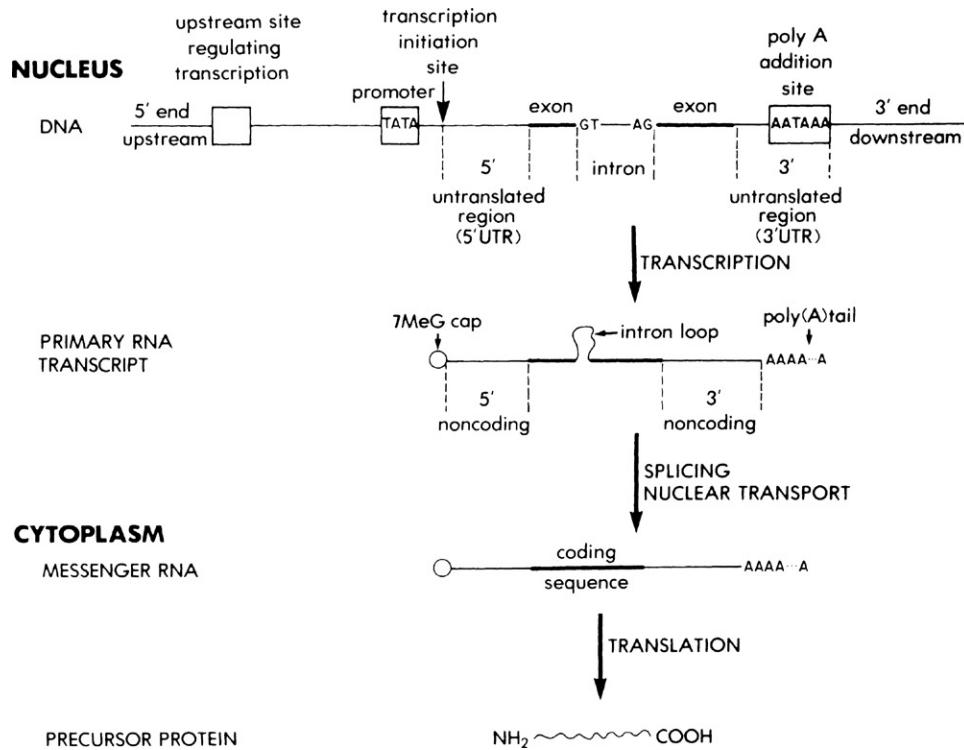
## 5.4 GENE STRUCTURE AND THE MOLECULAR PATHWAY OF GENE EXPRESSION

### 5.4.1 Structure of Transcriptional Units: Exons and mRNA

Sequences coding for a single eukaryotic mRNA molecule are typically separated by noncoding sequences into noncontiguous segments along the chromosomal DNA strand (Figure 5-5). The segments that are retained in the mature mRNA are referred to as exons. During transcription, the exons are spliced together from a larger precursor RNA that contains, in addition to the exons, interspersed noncoding segments referred to as introns. The number of exons coding for a single mRNA molecule depends on the gene and the organism, but ranges from 1 to more than 100. Human genes tend to have small exons, with a median value of only 123 bps. The exons are separated by introns, which can be less than 100 bp but can also exceed 10 kb. The size distribution of exons and introns of human genes based on the analyzed sequence information and comparison to worm and fly sequences are provided in Table 5-1.

Individual exons may correspond to structural and/or functional domains of the proteins for which they code, such as the signal peptide of secreted polypeptides or the heme-binding domain of globin. For some complex proteins, domains encoded by single exons often appear in apparently unrelated proteins, suggesting that the evolution of these proteins may have been facilitated by the ability to bring together different protein subdomains by exon shuffling. The origin of intron/exon structure is thought to be extremely ancient and to predate the divergence of eukaryotes and prokaryotes. However, prokaryotes and small eukaryotes (e.g. yeast) have lost their introns during evolution, perhaps because of the strong selective pressure on these organisms to retain a small genome size.

**5.4.1.1 Gene Expression.** The expression of individual genes can be regulated at multiple levels. Before a gene sequence gets translated into a polypeptide sequence, multiple events take place: activation of the local DNA structure, initiation and completion of transcription, processing of the primary transcript, transport of the mature transcript to the cytoplasm, and translation of the mRNA. All these steps can be the target of regulation and thus are potential control points for altering gene expression. Some genes are needed in all cell and tissue



**FIGURE 5-5** Eukaryotic gene structure and the pathway of gene expression.

<b>TABLE 5-1 Characteristics of Human Genes</b>	
	<b>Average</b>
Internal exon	123bp
Exon number	9
Introns	3300bp
3'-UTR	770bp
5'-UTR	300bp
Coding sequence	1340bp
Polypeptide	447aa
Overall size	27kb

aa, amino acids; bp, base pairs; kb, kilobase pairs; UTR, untranslated region.

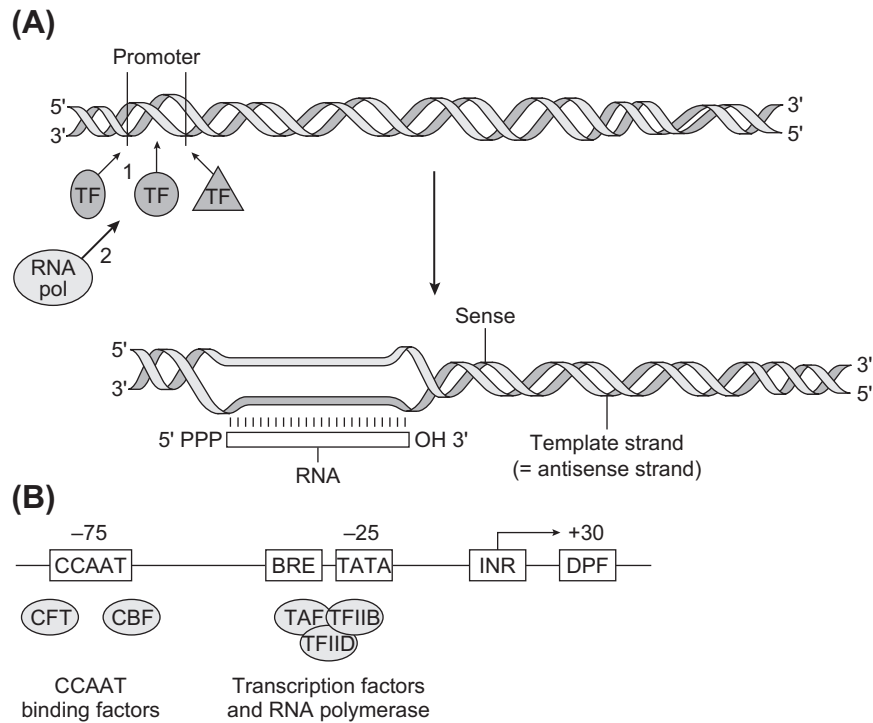
types as they encode a crucial gene product. Such genes are often referred to as “housekeeping genes.” However, numerous human and mammalian genes show highly restricted cell- or tissue-specific expression patterns, and this spatial and/or temporal restriction of gene expression can also be regulated at multiple levels.

**5.4.1.2 Transcription.** Initiation of transcription happens when the compact DNA structure is loosened and short sequence elements in the 5' end of the gene guide and activate RNA polymerase (Figure 5-6). A group of such sequences is often clustered upstream of the transcription initiation site to form the promoter. The promoter is a region of DNA at the 5' end of the genes that bind RNA polymerase.

There are different types of promoters for RNA polymerases I, II, and III. RNA polymerases I and III are dedicated to transcribing genes encoding RNA molecules (rRNA and tRNA), which assist in the translation of the

polypeptide-coding genes. All RNA polymerases are large proteins and appear as aggregates consisting of 8–14 subunits. Significant amounts of information exist on promoter sequences specific for these polymerases. The basal apparatus, the generic minimal promoter sequence that is sufficient to initiate transcription of any protein-coding gene, contains an RNA polymerase II recognition signal as well as signals for general TFs needed for the binding of the polymerase by most genes. This minimal promoter contains a consensus sequence (5'-TATA-3', referred to as the TATA box), some 25 bp upstream of the site at which transcription begins, surrounded by GC-rich sequences, as well as the B recognition elements (BRE) sequence (TF recognition element), the Inr (initiator) sequence at the start site of transcription, and the DPE (downstream promoter element) at some 30 bp 3' from the transcription initiation site. Furthermore, some 50–200 bp upstream is the CAAT box, to which several TFs bind. The usual nomenclature of the numerous transcription factors is TF followed by a roman numeral to indicate the associated RNA polymerase. The general TFs, such as TFIIB, TFIID, TFIIIE, TFIIF, and TFIIF, facilitate the binding and activation of RNA polymerase II into an activated transcriptional complex.

Genes are constitutively expressed at some basal minimum rate determined by the core promoter. However, transcription can be increased or totally switched off by additional positive or negative elements (enhancers or silencers), which regulate the efficiency and specificity with which a promoter is recognized by the transcriptional apparatus. These *cis*-acting regulatory elements are typically short sequences located at some 200 bp

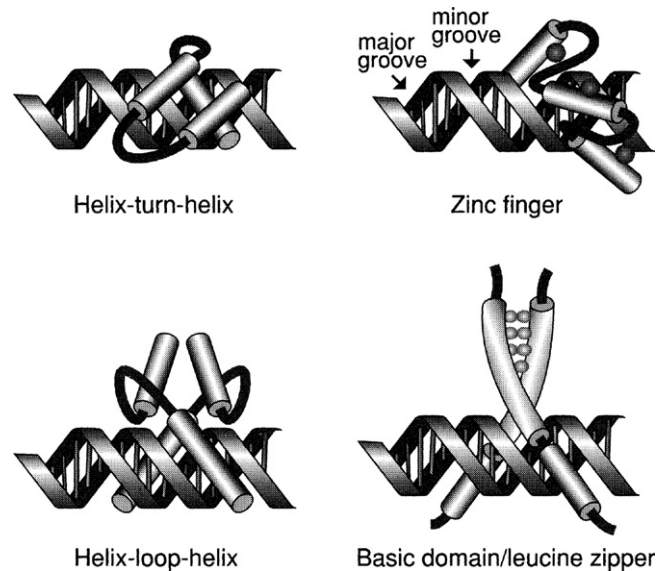


**FIGURE 5-6** A. Transcription of eukaryotic genes. B. Basic promoter elements in eukaryotes.

upstream from the promoter sequence but may also be placed at more distant locations. Finally, gene expression can be regulated by elements that respond to external stimuli. These response elements are often within 1000 bp upstream from the transcription start site. Genes under common control share similar response elements, recognized by regulatory TFs. Some response elements are extremely well characterized, such as heat shock response elements or glucocorticoid response elements.

Promoters do not necessarily have to lie upstream of the transcription initiation site. For example, most promoters for RNA polymerase III, including the 5S RNA promoter, lie downstream of the transcription start site within the coding sequence (11). This promoter binds the general transcription factor TFIID, a large protein with several zinc fingers, which then, along with the factors TFIIB and TFIIC, binds RNA polymerase III in a manner such that the polymerase is positioned at the exact spot where transcription begins. Although the mechanism of TFIID binding appears to be a common one, promoters that lie in exon sequences may be limited to the special situations in which multicopy genes such as 5S RNA or tRNA are subject to coordinate regulation.

Comparisons among many TFs have exposed some structural domains characteristic of the DNA-binding character of these proteins (Figure 5-7). These include zinc finger motifs, helix-turn-helix motifs, helix-loop-helix motifs, and leucine zipper motifs. The zinc finger motif binds a zinc ion with four highly conserved amino acids, two cysteines and two histidines ( $C_2H_2$ ) to form a finger-like loop (12). The typical loop is 25 amino acids long,



**FIGURE 5-7** Three-dimensional structure of DNA helix bound to TFs.

and the finger structure is often tandemly repeated. The helix-turn-helix motif is a common element of homeobox proteins. It consists of two short  $\alpha$ -helices separated by a short linker region and confers sequence specificity to DNA binding (13). The helix-loop-helix motif consists of two  $\alpha$ -helices separated by a loop that is flexible enough to allow two helices to pack against each other. The contact of the helix-loop-helix motif with DNA is considered to be looser than other TFs. The leucine zipper motif is a helical stretch of amino acids, with leucine at every



seventh amino acid position and occurring once in every two turns of the helix. Characteristic of most TFs is that they recognize and bind a short nucleotide sequence and their binding surfaces have extensive complementarity to the surface of the DNA double helix. Typically, eukaryotic TFs have two functional domains: a DNA-binding domain that binds to the DNA of the target gene and an activation domain that interacts with other proteins, which regulate transcription.

#### 5.4.1.3 Enhancers and *cis*-Acting Regulatory Elements.

Enhancers are defined as *cis*-acting sequences that increase transcriptional initiation but, unlike promoters, are not dependent on their orientation or their distance from the transcriptional start site (14). They may be found within the introns of the genes they regulate, within adjacent genes or, in extreme cases, an Mb or more away. Enhancer sequences are generally short, on the order of 20–30 bp, and bind specific TFs. When there is a mechanistic diversity in enhancer function, many enhancers facilitate the assembly of an activated transcriptional complex at the promoter via a chromatin looping mechanism. Other mechanisms involve recruitment of RNA polymerase II by enhancers or transcription of enhancer sequences to generate long noncoding RNAs that can facilitate transcription. Enhancers have roles in differentiation, tissue specification and tissue-selective gene expression, playing important roles during development and in specific cell types.

Silencers are another class of *cis*-acting regulatory elements that reduce transcription levels (15). They are less well characterized than enhancers, and some of them are position dependent while others seem to be position independent. They can bind TFs that act in transcriptional initiation, and many genes contain a combination of both positive and negative upstream regulatory elements that act in concert on a single promoter. This diversity of regulatory elements has the potential to precisely modulate gene expression with regard to cell type, developmental stage, and environmental conditions. Boundary elements are insulators, most of which block or isolate the effects of enhancers or silencers, limiting their action to the target genes.

Mutations of gene promoters or enhancers can alter the pattern of gene expression but not the structure of a particular gene product. While such mutations are much less frequent than structural mutations in genes, they provide insight into the elements of transcriptional regulation. For example, point mutations and partial deletions of the  $\beta$ -globin gene cluster that affect upstream regulatory sequences lead to reduced expression of adult  $\beta$  chains in  $\beta$ -thalassemia and/or increased expression of fetal  $\gamma$  chains in hereditary persistence of fetal hemoglobin (16).

### 5.4.2 Parts of a Gene that are Transcribed but not Translated

**5.4.2.1 5'-Untranslated Sequences.** Shortly after initiation of mRNA transcription, a 7-methylguanosine

residue is added to the 5' end of the primary transcript (see Figure 5-5). This 5' cap is a characteristic of nearly every mRNA molecule (17). Many functions have been ascribed to the cap, the most notable of which is protection of the mRNA from degradation by exonucleases. The cap may also promote splicing and nuclear export of the RNA and is recognized by the translational machinery. The 5'-UTR extends from the capping site to the beginning of the protein-coding sequence and can be several hundred base pairs in length. The 5'-UTR regions of most mRNAs contain a consensus sequence, 5'-CCA/GCCAUGG-3', known as a Kozak consensus sequence, involved in the initiation of protein synthesis. In addition, some 5'-UTRs contain upstream AUG codons that can affect the initiation of protein synthesis and thus could serve to control expression of selected genes at the translational level.

**5.4.2.2 Introns and Splice Junctions.** The number of introns in a simple transcriptional unit will be one less than the number of exons. More complicated arrangements exist in which an upstream exon can be spliced to any of several different downstream exons, or in which a complete transcriptional unit is nested inside an intron of a second transcriptional unit. In these situations, the same DNA sequence can be used as both exon and intron, depending on the transcriptional unit. Regardless of how the transcriptional unit is organized, the boundaries between potential exons and introns share common features that are important in the splicing process. Beginning from the upstream or 5' exon, these splice junctions have the sequence

```
...ag[GTaagt.....(>80 bp).....  
pyNpyPyPuApy...(~8–20 bp)..PyAG]...
```

where the brackets define the exon–intron junctions, the underlined nucleotides represent the splice donor, branch point and splice acceptor sequences, respectively, the upper case sequences are virtually invariant characteristics of every splice junction, and the lower case sequences are other conserved bases within the consensus splice sites. These conserved intron sequences serve a critical role in the splicing process, and many inherited diseases are caused by mutations of the consensus splice junctions. Most splicing mutations alter one of the invariant GT or AG nucleotides of the splice donor or splice acceptor (18) and result in abnormal splicing and either loss of the gene product or synthesis of an abnormal polypeptide chain.

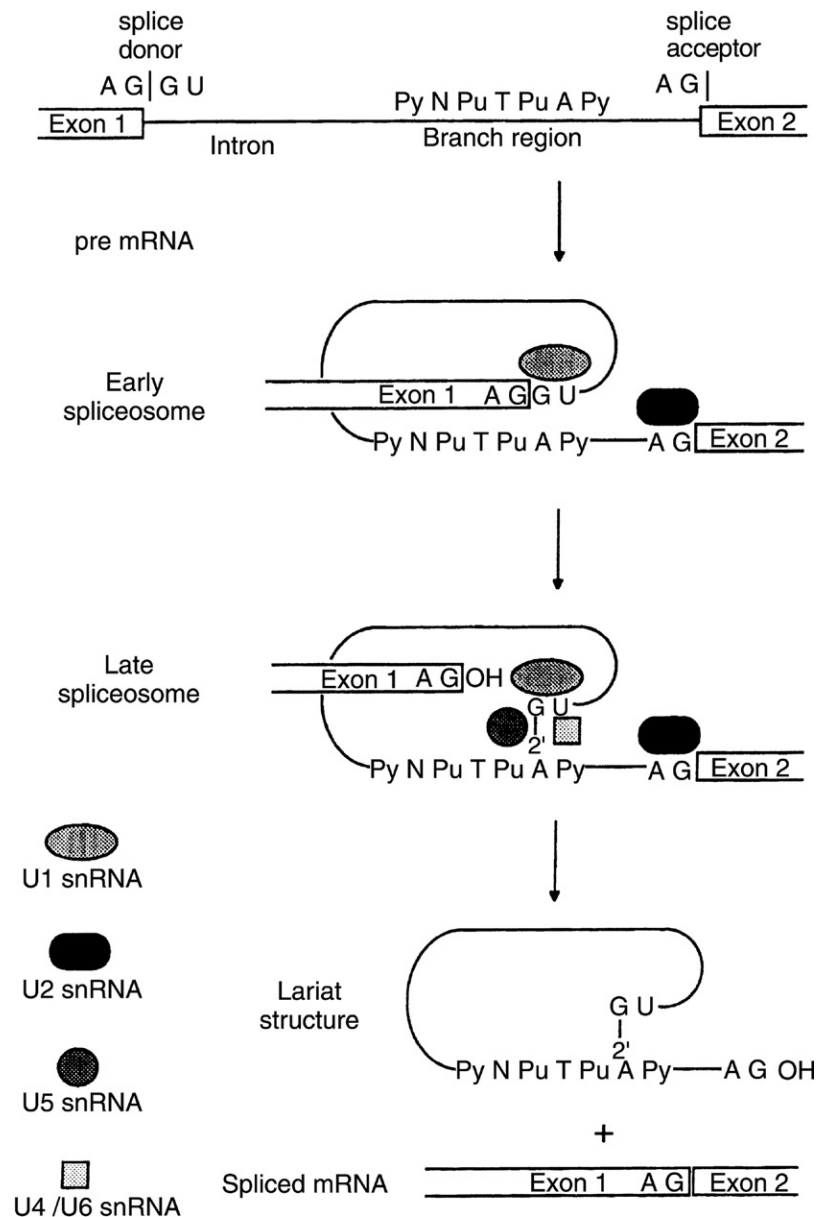
A transcribed precursor RNA molecule must have its introns spliced out and its ends modified before export to the cytoplasm as mature mRNA. The spliceosome, which is composed of small nuclear ribonucleoproteins (snRNPs), mediates the splicing of the large number of pre-mRNA transcripts, collectively referred to as heterogeneous nuclear RNA (hnRNA). Spliceosomes are multienzyme complexes that both catalyze the splicing reaction and stabilize the intermediates in the splicing process. The snRNPs comprising the spliceosome consist of a set of five integral snRNA molecules (U1, U2, U4, U5, and U6) tightly associated with a large number of

proteins (19). RNA molecules in the snRNPs are among the most highly evolutionarily conserved sequences among eukaryotes. An initial intermediate of the splicing reaction is formed when the 5' guanylate end of an intron (the splice donor) is joined to an adenylate residue near the 3' end of the intron (the branch point) through a 2'–5' phosphodiester linkage (Figure 5-8). After the completion of exon–exon fusion, the excised intron is released as a “lariat structure” by cleavage at the splice acceptor.

The genes encoding rRNA and tRNA also contain exons and introns, but are spliced by different mechanisms than those required for mRNA splicing. Self-splicing of RNA without any protein factors is known to happen in prokaryotes, which suggests that introns have an extremely ancient evolutionary origin, predating not only the eukaryote/prokaryote divergence, but also perhaps the origin of proteins as well.

**5.4.2.3 3'-Untranslated Sequences and Transcriptional Termination.** The 3' ends of primary transcripts are determined by transcriptional termination signals located downstream of the ends of each coding region. However, the 3' ends of mature mRNA molecules are created by cleavage of each primary precursor RNA and the addition of a several hundred nucleotide polyadenylate (poly(A)) tails (see Figure 5-5). The cleavage site is marked by the sequence 5'-AAUAAA-3' located 15–20 nucleotides upstream of the poly(A) site and by additional GU-rich sequences 10–30 nucleotides downstream. Histone mRNAs, which do not have poly(A) tails, have stem-loop structures instead with cleavage of the primary transcript mediated by a distinct protein complex that includes the U7 snRNP (20).

Some complex transcriptional units contain several potential polyadenylation and/or transcription termination



**FIGURE 5-8** Splicing of mRNA.

sites. It is often difficult to distinguish the latter from the former as the product available for analysis (mRNA) has lost the portion of the 3' terminus originally transcribed by RNA polymerase. Alternative polyadenylation (or termination) sites can determine final protein structure if the longer precursor RNA contains an exon not found in the shorter precursor RNA. In a simple case, two proteins with different carboxyl termini are formed. But if alternative exon splice sites are made available in the longer precursor RNA, proteins with entirely different sequences can be produced.

The region from the translation termination codon to the poly(A) addition site may contain up to several hundred nucleotides of a 3'-UTR, which includes signals that affect mRNA processing and stability. Many mRNAs that are known to have a very short half-life contain AU-rich elements, 50–150bp sequences containing AUUUA motifs that regulate mRNA stability (21). Other, less well-characterized sequences can have similar effects. Removal or alteration of these sequences can prolong the half-life of mRNA, indicating that such elements represent a general regulatory feature of mRNAs whose level of expression can be rapidly altered.

### 5.4.3 Translation of RNA into Protein

**5.4.3.1 Genetic Code.** After intron sequences are spliced out of the primary RNA transcript and the 3' terminus is generated (in most cases, by the addition of a poly(A) tail), the mature mRNA is transported from the nucleus to the cytoplasm, where it is translated into a polypeptide chain. In the cytoplasm, tRNA molecules provide a bridge between mRNA and free amino acids (see Figure 5-2). Adjacent groups of three nucleotide sequences in the mRNA (codons) each bind to complementary three nucleotide sequences in tRNA (anticodons). Unlike most other nucleic acids, tRNA molecules have rigid tertiary structures. All tRNAs are L-shaped, with the anticodon located at one end and the amino acid binding site at the other end. Modified nucleotides, such as methylguanosine (mG) and pseudouridine ( $\psi$ ), are common in tRNA and help determine the specific three-dimensional characteristics of tRNA molecules. Aminoacyl tRNA synthetases specifically recognize different tRNAs and attach each tRNA to the correct amino acid. The last base in each codon is followed by the first base in the next, and thus the first codon in an mRNA molecule determines the reading frame for all subsequent codons.

The relationship between codon and amino acid sequence is referred to as the genetic code (Figure 5-9). Different tertiary structures of each tRNA are specifically recognized by the proper tRNA synthetase, ensuring the accuracy of the code. As the anticodon sequence itself does not determine tRNA tertiary structure, each amino acid may have several possible codons recognized by tRNAs with different anticodons but similar tertiary structures; that is, they are recognized by the same tRNA

UUU } Phe UUC UUA } Leu UUG	UCU } Ser UCC UCA UCG	UAU } Tyr UAC UAA - Stop UAG - Stop	UGU } Cys UGC UGA - Stop UGG - Trp
CUU } Leu CUC CUA CUG	CCU } Pro CCC CCA CCG	CAU } His CAC CAA CAG	CGU } Arg CGC CGA CGG
AUU } Ile AUC AUA } Met AUG	ACU } Thr ACC ACA ACG	AAU } Asn AAC AAA } Lys AAG	AGU } Ser AGC AGA } Arg AGG
GUU } Val GUC GUA GUG	GCU } Ala GCC GCA GCG	GAU } Asp GAC GAA GAG	GGU } Gly GGC GGA GGG

FIGURE 5-9 The genetic code.

synthetase. For example, 5'-AAA-3' tRNA<sup>Phe</sup> (the tRNA coding for phenylalanine with the anticodon 5'-AAA-3') has the same tertiary structure and is charged by the same tRNA synthetase as 5'-GAA-3' tRNA<sup>Phe</sup>. Thus, both codons 5'-UUU-3' and 5'-UUC-3' code for phenylalanine using different tRNAs but the same tRNA synthetase. Additional redundancy in the genetic code arises because the third base in each codon–anticodon duplex (which is the first base from the 5' end of the anticodon) can be flexible according to the rules of Watson–Crick base pairing. In particular, G:U or U:G base pairs are often found in the third position of a codon–anticodon duplex, and the guanine analog inosine, found only in tRNA, can pair or wobble with A, C, or U in the codon. Despite the redundancy of the genetic code, synonymous codons are not used with equal frequency, and the pattern of codon usage (codon bias) may vary tremendously among different species and between nuclear and mitochondrial mRNAs.

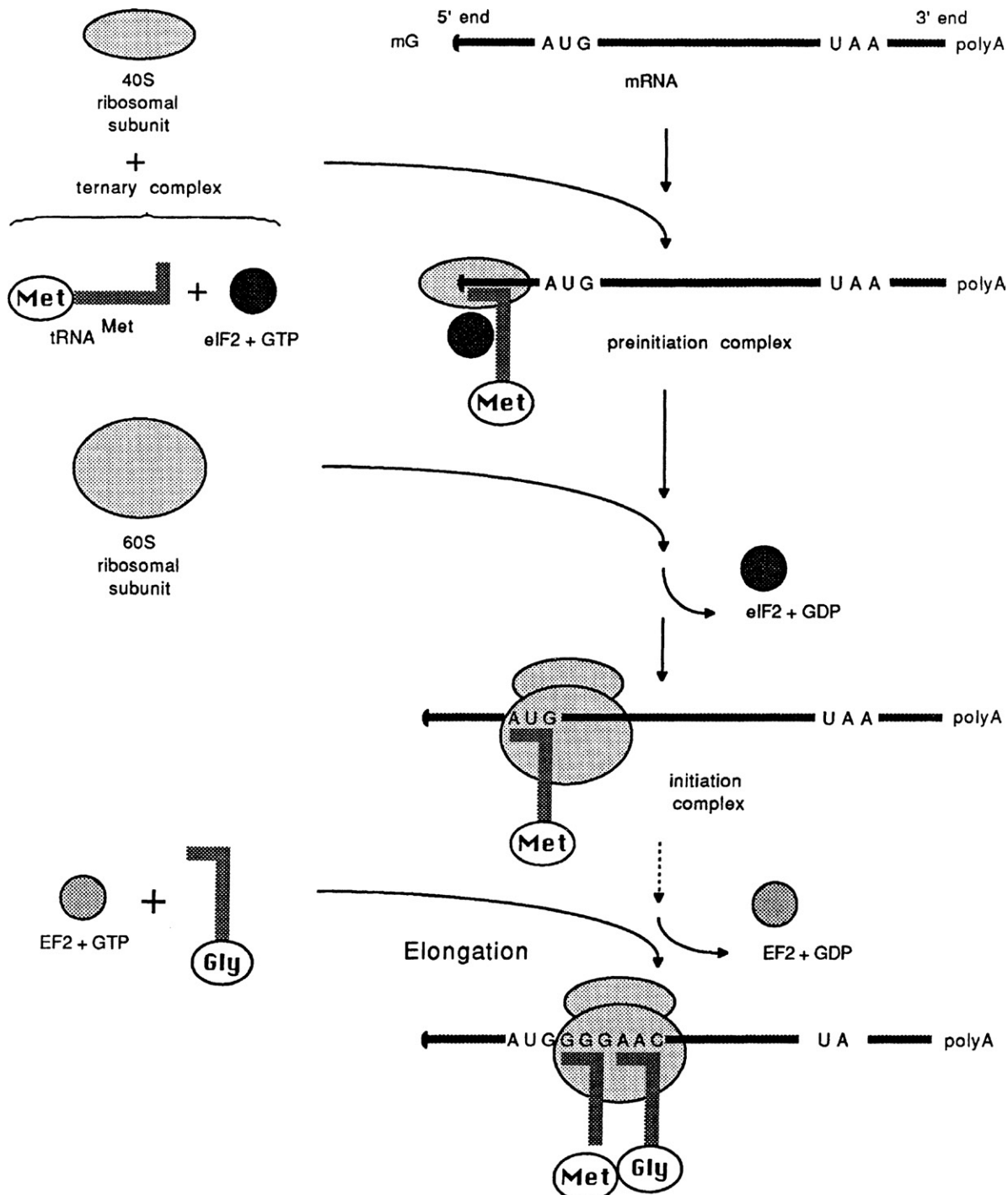
The AUG codon, which codes for methionine, nearly always begins the protein-coding portion of each mRNA molecule. Therefore, the vast majority of newly synthesized peptides begin with methionine. The tRNA<sup>Met</sup> for the initiator AUG codon has a different tertiary structure from all other tRNAs, including the tRNA<sup>Met</sup> that functions in elongation. Translation of most mRNAs generally begins with the first AUG from the 5' end, which is typically embedded within a Kozak consensus sequence (5'-CCA/GCCAUGG-3') and establishes the reading frame (22). The UAA, UAG, and UGA codons are stop codons and have no cognate tRNAs. Thus recognition of any one of these codons by the protein synthesis machinery terminates the protein-coding portion of every mRNA molecule.

Mutations that change a codon into a different codon and would therefore encode a different amino acid result in a protein with an amino acid substitution, and these are described as missense mutations. However, the UAA, UAG, and UGA codons do not code for an amino acid but instead serve as a signal to terminate protein synthesis.

Mutations that produce one of these codons in the middle of a normal reading frame cause truncation of the newly synthesized protein during protein synthesis and are referred to as nonsense mutations. Frequently transcripts with a premature termination codon are degraded by a process called nonsense-mediated decay, so that no protein product is synthesized from the mutant allele, resulting in haploinsufficiency for the gene product.

**5.4.3.2 Protein Synthesis.** The biochemistry of protein synthesis (Figure 5-10) can be divided into the stages of

initiation, elongation, and termination. All three processes occur on ribosomes, cytoplasmic particles of protein and rRNA that align the different substrates of each reaction. When inactive, ribosomes exist as separate pools of the two ribosome subunits, described by their size or sedimentation coefficient (S value). The small 40S ribosomal subunit contains 18S rRNA and ~33 different proteins, and the large 60S subunit contains 28S rRNA, 5.8S rRNA, 5S rRNA, and ~50 different proteins. Beyond these structural components, there is a wealth of additional factors,



**FIGURE 5-10** Translation of mRNA into protein.



including both proteins and functional RNA molecules, that are required for ribosome biogenesis (23). Translation begins with the formation of a preinitiation complex that contains the 40S ribosomal subunit, initiator tRNA<sup>Met</sup>, GTP, and several protein initiation factors. An mRNA molecule initially binds to the preinitiation complex in conjunction with several initiation factors that interact with the 5' cap structure. The canonical model for identification of the AUG start codon involves scanning the mRNA in a 5'–3' direction until the consensus sequence 5'-CCA/GCCAUGG-3' is reached. However, internal ribosome entry sites (IRES) mediate ribosome recruitment and translational initiation for uncapped mRNA molecules and for translation when cap-dependent processes are inhibited (24). Binding of the 60S ribosomal subunit and dissociation of several initiation factors generate a complex of proteins and subcellular particles poised to begin synthesis of the first peptide bond.

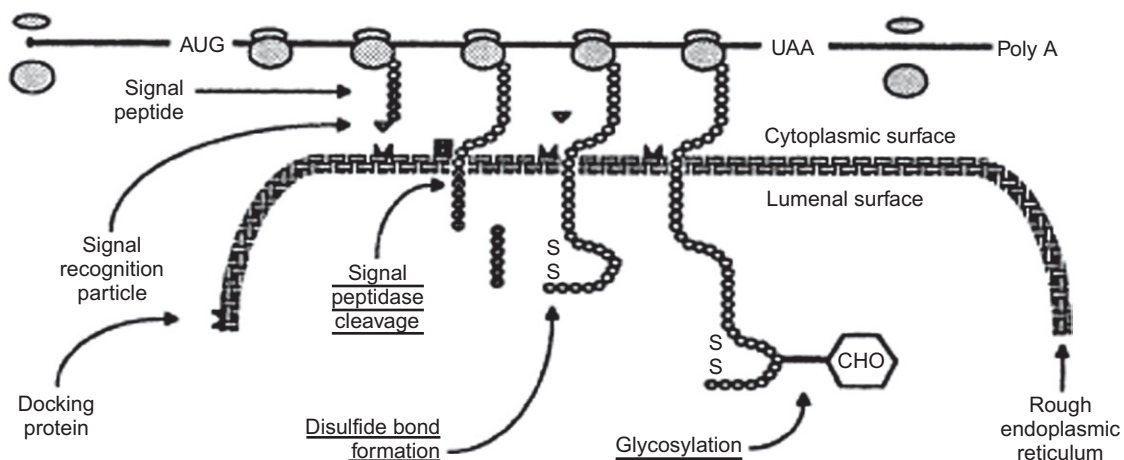
A ribosome contains room for two tRNAs and their respective amino acids (see Figure 5-10). One tRNA at the peptidyl or P site is attached to the amino acid that has just been incorporated into a nascent peptide chain, and another tRNA at the aminoacyl or A site is attached to its cognate amino acid and is ready to participate in protein synthesis. During elongation, a peptide bond is formed between the two adjacent amino acids, the ribosome moves to the next codon in the mRNA, the tRNA at the P site dissociates from the nascent peptide chain, and the tRNA at the A site is translocated to the P site. This series of reactions is dependent on elongation factor 1 (EF1), which binds to free charged tRNAs, and EF2, which facilitates translocation from the A site to the P site. A single mRNA can be simultaneously translated by several active ribosomes, forming a polysome that can contain as many as 50 ribosomes.

When a codon signifying termination of protein synthesis (UAA, UAG, or UGA) is reached, the completed polypeptide separates from the tRNA at the P site, and the ribosome dissociates. In bacteria and yeast, a unique group of suppressor tRNA mutations is caused by

changes in an anticodon that then permit binding of a charged tRNA to a termination codon. Point mutations in an mRNA that would normally lead to premature termination codon (e.g. by changing a UUA codon into a UAA codon) are then partially suppressed by the mutant tRNA, allowing synthesis of a full-length protein with a missense change at the position of the mutant codon. The principle of suppression of nonsense codons, which represent about 30% of disease-causing mutations in humans, can be mimicked by treatment with aminoglycoside antibiotics and other pharmaceutical compounds (25). Used as a therapeutic approach, such treatment has the potential to affect therapy in a wide variety of genetic disorders.

**5.4.3.3 Protein Localization.** Gene products function in particular cellular compartments. For example, histones, tubulin, glycosyltransferases, peptide hormone receptors, and collagen are specifically localized to the nucleus, cytosol, Golgi apparatus, cell membrane, and extracellular space, respectively. Although many membranes contain pores large enough to accommodate a linear polypeptide chain, completely folded proteins are generally too large to fit through these pores. In addition to the problem of translocating soluble proteins across membranes, proteins that remain attached to the membrane must be placed and oriented in specific ways. These problems—protein sorting, translocation, and membrane orientation—have been solved by complex biochemical mechanisms that depend in part on short peptide sequences in each protein. One of the most well-understood pathways is the initial sorting of gene products into those that will remain inside the cytosol or nucleus and those that pass across the endoplasmic reticulum (ER) membrane, and which are then available for secretion into the extracellular space. This initial sorting is determined early in the translation of proteins destined to cross the ER by the presence of a specialized hydrophobic signal sequence of 20–30 amino acids (26) usually located at the N-terminus (Figure 5-11).

The signal sequence is first recognized when about 25 amino acids of the growing polypeptide have emerged



**FIGURE 5-11** Translocation of newly synthesized proteins across the endoplasmic reticulum.

from the ribosome and bind to a protein–RNA complex called the signal recognition particle (SRP). The SRP stops further translation until bound to a docking protein complex, the translocon, which is located on the surface of the ER and forms a hydrophilic membrane pore (27). The signal peptide passes through the pore, translation recommences, and the growing polypeptide crosses the membrane co-translationally. After the protein has passed into the lumen of the ER, signal peptidase, a protein on the luminal surface of the ER, cleaves the signal peptide to complete the initial phase of protein sorting.

Proteins extruded from the RER pass through the Golgi apparatus into secretory vesicles for transport to the cell surface. Proteins destined for the extracellular matrix are secreted from the cell when the vesicles fuse with the plasma membrane. Insertion and orientation of proteins destined for the cell membrane, however, require additional sequences that function either to stop transfer across the membrane (stop transfer sequences) or to initiate transfer of an internal loop of the nascent polypeptide chain (start transfer sequences). Start transfer sequences are recognized by SRP-like N-terminal signal sequences but are not cleaved from the protein after translocation. The number, order, and orientation of start transfer and stop transfer sequences determines the conformation of complex integral membrane proteins that span the membrane multiple times. Some soluble proteins that contain the short peptide sequence Lys–Asp–Glu–Leu (KDEL) remain in the lumen of the RER, such as binding protein (BiP) and protein disulfide isomerase (PDI). Both BiP and PDI facilitate the folding of newly synthesized proteins in the RER. PDI catalyzes the rearrangement of Cys–Cys disulfide bonds; BiP is a so-called chaperone that binds temporarily to portions of other proteins normally not exposed to the surface and, in doing so, prevents partially folded proteins from misfolding and/or aggregating. Soluble proteins destined for specialized compartments inside the cell, such as lysosomes or peroxisomes, use a signal sequence to gain access to the ER lumen but require additional mechanisms for proper subcellular localization. Lysosomal sorting depends on amino acid sequences that specify posttranslational addition of a mannose 6-phosphate residue. Proteins containing this modification are selectively transferred from the Golgi apparatus to the lysosomal interior. Failure to modify proteins destined for the lysosome in this way is responsible for the inherited disease mucopolipidosis II (I-cell disease).

There are over 1000 mitochondrial proteins, the great majority of which are encoded in the nucleus and synthesized in the cytosol. Similar to the proteins destined for the ER, transport of proteins across the mitochondrial membrane also depends on a signal sequence and utilizes several translocator complexes (28,29). The targeting sequence is usually located at the amino terminus and is cleaved upon import, but sometimes is internal to the protein and is therefore not cleaved. Unlike RER proteins, for which translation and translocation are

codependent, mitochondrial proteins are first translated completely, released into the cytosol, and then translocated into the mitochondrial membranes, intermembrane space or matrix. The potential problem of translocating a completely folded protein across a membrane pore is solved for mitochondria by complexes that include chaperone proteins of the Hsp70 family, which bind to proteins destined for the mitochondria and stabilize them in the unfolded state until after they have passed across the mitochondrial membrane, after which they assume their folded conformation.

**5.4.3.4 Posttranslational Modification.** Alterations to protein structure that occur after translation include the formation of disulfide bonds, hydroxylation, glycosylation, proteolytic cleavage, and phosphorylation. Phosphorylation of serine, tyrosine, and threonine residues is a common reversible modification that alters protein–protein interactions or controls enzymatic activity, mostly of intracellular proteins. The formation of disulfide bonds, hydroxylation, glycosylation, and proteolytic cleavage are generally not reversible and mostly involve extracellular proteins.

Intramolecular disulfide bond formation can begin co-translationally as the growing polypeptide chain enters the lumen of the ER. Some proteins, such as immunoglobulin light chains, have a sequential pattern of intra-chain disulfide bonds (e.g. between the first and second cysteines or third and fourth cysteines). Other proteins, such as proinsulin, have a more complicated pattern. Protein folding and establishment of the correct arrangement of disulfide bonds are critical steps in synthesizing a three-dimensional protein structure. Glycosylation of newly synthesized proteins may be O-linked via serine, threonine, or hydroxylysine residues, or N-linked via asparagine residues. O-linked glycosylation is catalyzed by glycosyltransferases located on the luminal surface of the Golgi apparatus. N-linked glycosylation begins with transfer of a 14-residue oligosaccharide from a lipid molecule (dolichol) embedded in the RER membrane to the asparagine residue of a growing polypeptide chain. At some sites, the oligosaccharide is highly modified by removal of some carbohydrates and addition of other carbohydrates to form a complex glycoprotein modification. Other sites are less modified and contain the original high mannose composition of the dolichol intermediate. Many glycoprotein modifications help determine the specificity of extracellular protein–protein interactions, such as antigen–antibody binding or attachment of cells to the extracellular matrix.

Proteoglycans are a specialized class of extensively glycosylated proteins that contain a protein core with long disaccharide chains branching off at regular intervals and can contain as much as 95% carbohydrate by weight. Proteoglycans are extremely hydrophilic and form hydrated gels that provide structural integrity to the extracellular space. During growth and development, extracellular remodeling is accompanied by endocytosis

and degradation of proteoglycans by lysosomal enzymes specific for different disaccharide chains; absence of these lysosomal enzymes produces mucopolysaccharidoses, such as Hunter syndrome or Hurler syndrome.

#### 5.4.4 Expression of Housekeeping and Tissue-Specific Genes

Many proteins that operate in basic metabolic functions such as energy generation or nutrient transport are found in all cells, and the genes that encode these proteins are described as housekeeping genes. They are characteristically expressed at a relatively constant level in all cells. More specialized genes that are not housekeeping are used only at specific times and places during development or in one or a limited set of tissues. The sequence of the human genome has revealed that the most common genes in our genome are those encoding TFs and nucleic acid binding proteins, which together encode 26.8% of the proteins with known or putative function. Other highly represented genes encode receptors, transferases, signaling molecules and transporters (30). These and other housekeeping genes usually account for 90% or more of the transcripts expressed in any particular cell type.

Analysis of genome sequence data has revealed that segments of DNA with a relatively large proportion of 5'-CpG-3' dinucleotide pairs and a very high GC content are frequently located near the 5' ends of all housekeeping and a proportion of tissue-selective genes (31). These CpG islands thus mark promoters and are present adjacent to about 70% of annotated genes, accounting for about half of the known CpG islands. Some of the remaining CpG islands have been associated with transcriptional activity, suggesting that these sequences mark the promoters of unknown transcripts and genes. On average, CpG islands are about 1000-bp long and most are less than 2000 bp in length. The CpG sequences within each island are generally unmethylated and are thought to facilitate transcription either by preferential binding of TFs or by altering chromatin structure to a nucleosome-deficient and transcriptionally permissive state. Methylation of CpG islands is associated with reduced transcriptional activity, such as on the inactive X chromosome, although the methylation event appears to follow rather than initiate transcriptional silencing.

**5.4.4.1 Gene Families.** Gene families consist of structurally (and usually functionally) related genes with a common evolutionary origin. Multiple levels of hierarchical subfamily structure are common. Examples include the immunoglobulin variable region ( $\kappa$  and  $\lambda$ ), collagen (interstitial and basement membrane), and globin ( $\alpha$  and  $\beta$ ) gene families. Some gene family members, such as collagens, are dispersed among different chromosomal locations, but many others, such as the  $\kappa$  or  $\lambda$  variable region genes, are physically linked. Among gene families that exhibit linkage, members are usually oriented in the same direction. In some cases, linkage is thought to be an evolutionary

footprint without functional significance, suggesting that evolutionary divergence of the gene family occurred through successive rounds of duplication in tandem arrays. However, in other gene families, such as the immunoglobulins, linkage has been conserved during evolution because it provides a mechanism for coordinated or regulated control of gene expression. Even when dispersed, coordinated expression of members of gene families can be regulated by similar control mechanisms by carrying similar response elements in their 5' regulatory regions.

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### Biography



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# CHAPTER

# 6

## Epigenetics

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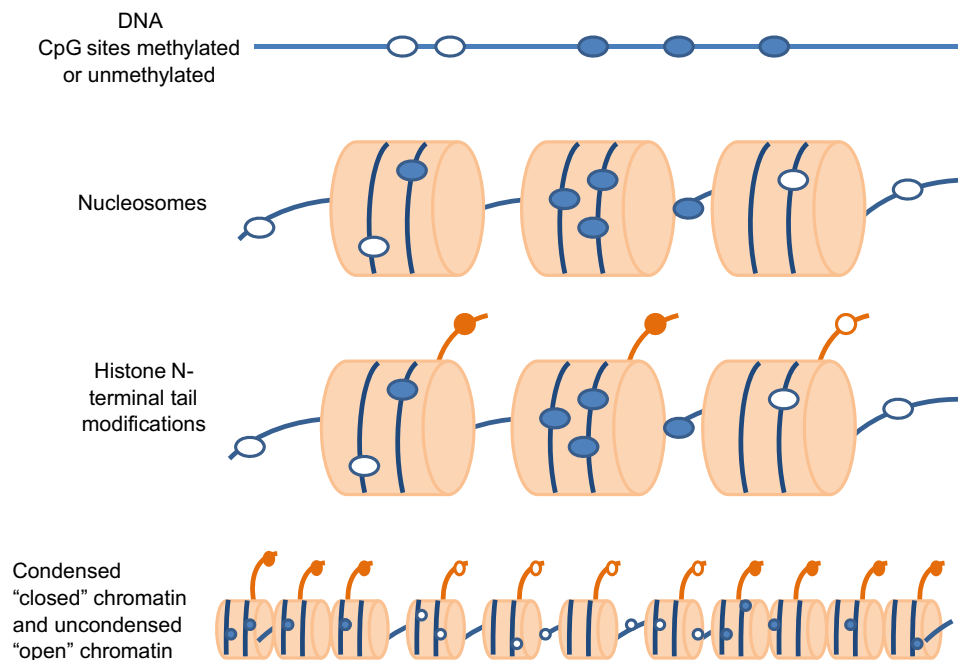
### 6.1 INTRODUCTION

Despite the tremendous advances in human genetics enabled by the original public and private human genome projects and brought to fruition with high-throughput genotyping and “Nextgen” DNA sequencing, many aspects of human biology still cannot be adequately explained by nucleotide sequences alone. Normal human development requires the specification of a multitude of cell types/organs that depend on transcriptional regulation programmed by epigenetic mechanisms. Epigenetics refers to modifications to DNA and its associated proteins that define the distinct gene-expression profiles for individual cell types at specific developmental stages. Disruption of such control mechanisms is associated with a variety of diseases with behavioral, endocrine, or neurologic manifestations, and quite strikingly with disorders of tissue growth, including cancer. While the involvement of epigenetic alterations in many of these diseases has been known to specialists for some time, the importance of epigenetics in clinical medicine has only just begun to emerge. Current research is focused on characterizing *cis*- and *trans*-acting influences of the genetic background on epigenetic marks, delineating cell type or tissue-specific epigenetic marks in human health and disease, studying the interaction between epigenetic marks and the environment especially with respect to fetal programming and risks for common adult onset disorders, and modulating adverse epigenetic states by drug-based and nutritional therapies.

### 6.2 EPIGENETIC MECHANISMS: CHROMATIN, DNA METHYLATION AND LONG NONCODING RNAs

An epigenetic trait is defined as a “stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence” (1). Epigenetic patterns, essential for controlling gene expression in normal growth and development, are established by a number of mechanisms including DNA methylation at cytosine residues in CpG dinucleotides and covalent modifications of histone proteins, as well as by less well-understood mechanisms controlling long-range chromatin architecture within the cell nucleus.

DNA in most eukaryotic cells is packaged with histone proteins to form nucleosomes—the beads in the well-known “beads-on-a-string” structure of chromatin. Most double-helical DNA is wrapped around an octamer core of four histone homodimers, H2A, H2B, H3, and H4, which through multiple levels of packaging establish chromatin conformations that can be relaxed or tightened to either facilitate or repress transcription in specific cells at critical times in development. Condensed states of chromatin (heterochromatin) inhibit transcription, while relaxed states (euchromatin) are conducive to transcription. For instance, non-transcribed telomeric and centromeric repeat regions are often silenced due to their compact heterochromatin environment, while highly active genes, usually located within euchromatin, are often expressed due to a more open chromatin environment, often with a short nucleosome-free segment of DNA



**FIGURE 6-1** Epigenetic organization of chromatin: Layering of DNA methylation, histone modification to control gene expression. DNA of a gene promoter can be unmethylated (white circles) and in most cases the gene is expressed or the promoter can be methylated (blue circles) and in most cases the gene is not expressed. DNA is not independent of its associated histone proteins. Histone modifications are established and maintained independently or dependently on the DNA methylation state of a region. These protein modifications can activate (open orange circles) or repress (filled orange circles) gene transcription. Although not shown in this figure, but mentioned in the text, additional epigenetic processes, including microRNA and long noncoding RNAs, also contribute to gene regulation. The DNA/histone protein nucleosome core is further compacted to form higher-order chromatin structures that also contribute to gene regulation.

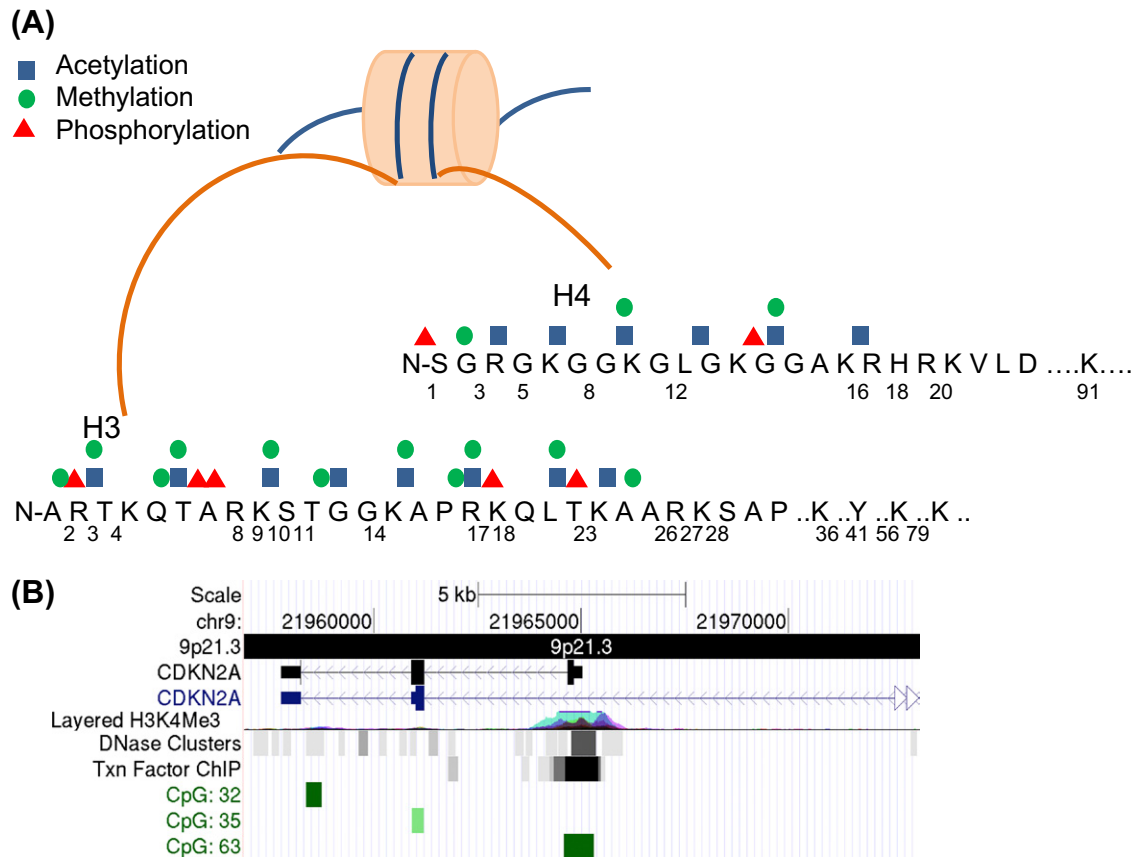
near the transcriptional start site. Further regulation is accomplished by assembling promoter–enhancer complexes via long-range chromatin looping (2), a process that is regulated by specific DNA sequences called insulators, which are often located at the points of intra-chromosomal contacts from which the loops emanate.

The core histones are subject to diverse posttranslational modifications, including methylation and acetylation, often at lysine and arginine amino acid residues in the loosely structured N-terminal histone tails that project from the tightly structured nucleosome cores (Figure 6-1). Depending on their pattern of modifications, these N-terminal histone tails are recognized by other chromatin proteins that activate or repress transcription, and therefore certain histone modifications can establish, and potentially maintain, active or silent epigenetic states (Figure 6-2). The cellular enzymes that catalyze histone modifications are therefore important modifiers of gene expression. Mutations in some of these genes can lead to human disease, which will be discussed in detail in later sections of this chapter. Examples include histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases, and histone kinases. The modifications catalyzed by this large array of enzymes can be sequential and interdependent, mutually exclusive, or independent. Despite this complexity, data from individual labs and

large-scale mapping projects such as ENCODE are starting to reveal some general rules for the histone code, with a finite number of specific combinations of histone modifications correlating with genes that are expressed, repressed, or poised for expression but not yet expressed (3,4).

DNA methylation involves the transfer of a methyl group to cytosine in a CpG dinucleotide, catalyzed by DNA methyltransferase enzymes that establish and maintain these patterns through cell division. Importantly, DNMT1, the major maintenance methylase, has a high affinity for hemimethylated DNA (5) and it therefore acts to propagate the original methylation patterns. One stage-specific isoform of DNMT1, DNMT1O, is an oocyte-derived protein that enters cell nuclei only at the eight-cell stage of the early embryo, and has an essential role in the maintenance of epigenetic marks (6).

Independent of maintenance methylation, methyl groups must also be added *de novo* at various times during development; for example, to establish parental imprints on the DNA (7), to methylate centromeric DNA and other constitutive heterochromatin, and to defend the host against foreign DNA integration and expression. The DNMT3 family includes two *de novo* DNA methyltransferases: DNMT3A and DNMT3B. These enzymes can efficiently methylate CpGs that are not in hemimethylated DNA. Another member of the DNMT3



**FIGURE 6-2** (A) Histone modifications of histone H3 and H4 N-terminal tails. Posttranslational modifications of N-terminal tails (these can also occur in the C-terminal domain, but are not shown here) can occur in combination and are read by the appropriate protein to establish local and global decondensed or open and condensed or closed chromatin states: Ac, acetylation (blue squares); Me, methylation (green circles); and P, phosphorylation (red triangles). (B) Snapshot from UCSC genome browser representing H3K4 methylation in the promoter of the tumor-suppressor gene CDKN2A. This diagram is an example of epigenetic data available in UCSC genome browser. The description of each genomic feature is shown on the left. Two isoforms of CDKN2A are shown in black and blue, here we focus on a shorter (black) isoform. Enrichment for active histone H3K4me3 is shown by multiple colors in 9 cell lines. The peak of H3K4me3 coincides with transcription start site of CDKN2A, CpG island (green), as well transcription-factor-binding sites (TxN factor ChIP) and DNase clusters, which are indicators of open chromatin. All of these marks—H3K4me3, transcription-factor binding as well DNase clusters—indicate that CDKN2A is active transcribed in these cell lines. Information about other histone marks and DNA methylation levels is available from the UCSC genome browser under multiple tracks from the Regulation section. (This image was downloaded from UCSC genome browser <<http://genome.ucsc.edu/>> (Ref. (313)). The ENCODE Regulation data is from Ref. (314).

family—DNMT3L—has no catalytic activity, but can bind to and activate DNMT3A and is required to maintain allele-specific methylation in imprinted regions of the genome (8). The function of DNMT2 appears to be primarily in RNA methylation (9).

In mammalian cells, DNA methylation occurs predominantly on cytosines located in CpG dinucleotides within repetitive elements and in some non-repetitive sequences in intergenic and intragenic regions, while CpG methylation is usually excluded from gene promoter regions that are especially CpG-rich, referred to as CpG islands (10). In 98% of the genome, CpG dinucleotides appear at a low frequency of 1/80 nucleotides, but in the remaining 2% of the genome they are found in CpG islands of from 200 bp to several kb in length, with a much higher CpG density. Approximately 50–60% of genes contain CpG islands, typically though not always in their proximal promoters. Such CpG islands are almost always

unmethylated in normal tissues, with the exception of imprinted genes, X-inactivated genes, retrotransposons, and a few genes with tissue-specific silencing (11,12). DNA methylation at CpG island-associated promoters usually causes gene silencing, either by directly interacting with transcription factors or by recruiting methyl-binding proteins that then recruit histone-modifying enzymes to transform chromatin to a repressive state (13). Importantly, the general protection of CpG islands from methylation can break down in pathological conditions, particularly in cancer cells, leading to aberrant gene silencing (14).

Although DNA methylation and histone modifications are regulated by different sets of enzymes, crosstalk among these modifications occurs through interactions of enzymes and other proteins that create and recognize these patterns (15). The relationship between these two central types of epigenetic modifications is known to

be bi-directional, with histone marks being more labile and DNA methylation more stable (15–20). Thus DNA methylation can act to “lock in” epigenetic states. However, regulating metastable states of gene expression is so crucial in development and tissue homeostasis that other mechanisms, in addition to histone modifications and DNA methylation, come into play to establish and maintain epigenetic states. Regulatory noncoding RNAs, including small interfering RNA (siRNA), microRNAs (miRNAs), and long noncoding RNAs (lncRNAs) play important roles in the regulation of gene expression at several levels of transcription, mRNA degradation, splicing, transport and translation (21). The main function of siRNAs and miRNAs is posttranscriptional regulation. They pair with homologous mRNAs and may cause translational repression or more generally degradation (21). In addition, both types of small RNA molecules are implicated in transcriptional gene regulation through modification of epigenetic marks. Though more research is required, preliminary data suggest that this miRNA-mediated transcriptional regulation is coupled with other epigenetic regulatory mechanisms as well. The *POLR3D* gene has been shown to be silenced in *cis* by the miRNA mir-320, encoded within the *POLR3D* locus, through recruitment of the H3K27 methyltransferase EZH2 (22).

lncRNAs are found at various locations in relation to protein-coding genes. They may be antisense, intronic, intergenic, promoter or enhancer associated and can regulate transcription both in *cis* and *trans* by a number of different mechanisms (23). Specifically, lncRNAs have been shown to establish specialized nuclear compartments devoid of RNA-polymerase II. Chromatin-associated polycomb repressive complexes mediating epigenetic changes are found in these nuclear compartments (21,23), as exemplified by the *XIST* lncRNA that is essential for initiating X-chromosome silencing (24). lncRNAs are also found in imprinted gene clusters where the expression of monoallelically expressed lncRNAs results in spreading of repressive chromatin marks such as DNA methylation, H3K27, and H3K9 methylation in *cis* for distances up to 1kb (25). The functions of lncRNAs seem to be quite diverse; in contrast to the in *cis* silencing mediated by *XIST* and imprinted lncRNAs, non-imprinted lncRNA *HOTAIR* expressed from the *HOXC* gene was shown to recruit polycomb repressive complex2 and histone demethylase LSD1 resulting in acquisition in *trans* of silencing histone marks at genes within the *HOXD* gene cluster located on another chromosome (26). In spite of different details in their functions, it has been estimated that 20% of lncRNA expressed in human cells is bound by polycomb group proteins, suggesting a shared biochemical mechanism for their role in epigenetic silencing (27).

In addition to 5-methylcytosine (5mC), mammalian DNA contains a related modified base—5-hydroxymethylcytosine (5hmC). Both of these modifications have been known for decades, but have received very different

levels of attention in the scientific literature. 5mC has been studied extensively, and its role as an epigenetic modification involved in gene regulation, X-chromosome inactivation (XCI), genomic imprinting, silencing of transposons, and cancer development is well described. On the other hand, 5hmC has only recently entered center stage when it was shown that proteins in the ten-eleven translocation (TET) family of oxygenases catalyze the conversion of 5mC to 5hmC, and that the gene for one of these enzymes, *TET2*, is sometimes mutated in human cancers, notably in cases of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). The formation of 5hmC can in principle lead to demethylation of DNA, either by a passive process (failure of remethylation of these sites in S-phase) or by an active mechanism (direct demethylation of 5hmC or base excision of a further modified product such as carboxy-mC), so it has been suggested that TET family oxidases probably contribute to the dynamics of DNA methylation. 5hmC has been found in many cell types and tissues, with particularly high levels in the brain and in embryonic stem (ES) cells. As discussed in the next section on epigenetic reprogramming, TET1 has been shown to be important for self-renewal and maintenance of ES cells.

### 6.3 EPIGENETIC REPROGRAMMING

Patterns of DNA methylation differ extensively between the sperm and the egg, and cytosine methylation in DNA is dynamically reprogrammed both during germ cell and early embryonic development. This process has been well studied at the whole genome level and for specific imprinted genes, leading to the conclusion that most areas of the diploid genome become equalized in their epigenetic marks on the paternal and maternal alleles by the time early preimplantation development is completed, with strong allelic asymmetries persisting mainly at imprinted loci (28) and at certain other loci with haplotype-dependent allele-specific methylation. This equalization of the two alleles at most autosomal loci, which is essential for classical Mendelian transmission of human genetic disorders, involves early post-zygotic reductions in DNA methylation, which seem to result both from active demethylation, via a mechanism that is still being researched, and passive demethylation, in which CpG methylation is diluted through early rounds of DNA replication in the presence of relatively low-maintenance methylase activity.

Genomic levels of the “sixth base” 5hmC also change during development, probably as a function of the activity of TET family enzymes, which are particularly highly expressed in ES cells (29–31). In the mouse genome, 5hmC was shown to be widely distributed throughout non-repetitive regions, whereas satellite repeats (which are located in heterochromatin) are highly enriched for 5mC but substantially less for 5hmC (32). Distinct 5hmC and 5mC patterns are observed at CpG islands overlapping



with gene promoters, whereas 5 mC is depleted from these regions, and 5 hmC is well represented. Interestingly, the presence of 5 hmC and depletion of 5 mC at CGI promoters is associated with increased transcription in ES cells (32). Consistent with these observations, active histone marks H3K4me3 are enriched in promoters with high 5 hmC, suggesting that enriched 5 hmC at CGI promoters is positively correlated with active transcription. Decline of TET oxidase levels during differentiation of ES cells is accompanied by reduced promoter 5 hmC and increased 5 mC levels correlating with silencing of certain key developmental regulatory genes (32,33). Thus, one current hypothesis is that hydroxymethylation and the TET proteins could play a role in erasing methylation marks from promoters of pluripotency-related genes during differentiation (32,34). Hydroxymethylation may also play a role in epigenetic reprogramming of primordial germ cells (paternal genome) and early embryos (35,36).

## 6.4 EPIGENETIC REGULATION OF X INACTIVATION

Inactivation of one of the two X chromosomes in female cells was first described by Mary Lyon in 1961 (37) and since then remains the outstanding example of epigenetic silencing of nearly an entire chromosome, which is regulated in *cis* by a small control region in the DNA of this chromosome known as the X inactivation center (XIC). XCI has evolved in placental mammals to achieve dosage compensation of X-linked genes between female cells with two X chromosomes and male cells carrying only one X and one (small and rudimentary) Y chromosome. It is hypothesized that the X and Y chromosomes have evolved from a pair of autosomes, coinciding in time with the evolution of the placenta and driven by the acquisition of the sex determining region Y (*SRY*) gene. In the course of mammalian evolution, the *SRY*-carrying Y chromosome has been significantly reduced in size and lost most of its active genes, retaining in addition to *SRY* a few other genes playing a role in male reproduction, whereas the X chromosome has acquired additional genetic material through translocations from autosomes. This scenario resulted in homology between the X and Y chromosomes being reduced to two small pseudo-autosomal regions (PAR1 and PAR2) (reviewed in (38,39)). It is estimated that the human Y chromosome contains ~45 expressed genes, whereas the X chromosome has ~1300 known genes (38,40). The resulting major bias in copy number for X-linked genes between males and females is mostly transcriptionally compensated via XCI.

X-chromosome deletion and translocation mapping in mouse and humans defined a critical region for the XIC covering ~1Mb and mapping to chromosome band Xq13 in humans (41,42). Further discovery within this region of a lncRNA, the X-inactive specific transcript *XIST*, which is expressed from the XIC at high levels only on the X chromosome destined for inactivation, and which

quickly spreads to coat the entire inactive X in female cells, was a major breakthrough in understanding the mechanism of XCI (43–45). The majority of the work on mechanisms of X inactivation was done using mouse preimplantation embryos and ES cells. Before turning to the details of this mechanism, as useful as mouse models have been in unraveling XCI, there are a number of substantial differences in the XCI process between mice and humans. In mice, there are two waves of XCI; the first is imprinted inactivation of the paternal X, which is initiated shortly after fertilization. This pattern of paternal X-chromosome silencing is maintained in extra-embryonic tissues, but is erased and reestablished in a random manner in the inner cell mass (ICM) of blastocysts, that gives rise to the embryo proper (46). In contrast, in human embryos XCI is initiated at the blastocyst stage and is random in both ICM and trophoblast (47).

Random XCI is a multi-step process that can be divided into three steps: initiation, spreading, and maintenance. Initiation involves counting the number of X chromosomes per cell so that one X remains active per diploid number of autosomes. In other words, XY males and XO females keep their single X chromosome active, whereas XX, XXX, and XXXX females and XXY males inactivate all but one X, up-regulating *XIST* RNA on all of the X chromosomes destined to become inactive. The spreading of inactivation is achieved through sequential acquisition of epigenetic marks starting with *XIST* RNA, followed by polycomb repressor complex2 recruitment, shift to late replication timing, enrichment of histone macro H2A, silencing of chromatin marks such as histone H3 and H4 hypoacetylation, H3 Lysine27 methylation, and finally DNA methylation of CpG-rich promoters (48,49). Once established early in embryonic development, the inactive state of an X chromosome is maintained through somatic cell divisions utilizing epigenetic modifications of DNA and histones (50).

Regulation of *Xist/XIST* expression itself is a complex process involving multiple *cis*- and *trans*-acting factors. In mice, pluripotency transcription factors Oct4 and Nanog are negative regulators of *Xist* expression, such that a decrease in their expression early in post-zygotic development coincides with cell differentiation, upregulation of *Xist*, and the onset of XCI (51–53). Several sub-regions within the mouse Xic are involved in *Xist* regulation: *Tsix*—an antisense transcript of *Xist*—is a negative regulator of its expression and it protects the active X from inactivation (54,55); noncoding RNA *Jpx* (56), X-pairing region (Xpr) (57,58), and protein-coding *Rnf12* (59,60) are positive regulators of *Xist* expression. There are a number of differences in the organization of the mouse and human XIC/Xic, and there is little sequence conservation between mouse and humans (42,61). Based on comparative analysis of Xic in several mammalian species, it seems that Xic is an evolutionary labile locus and the orthologues across different mammalian species act via multiple diverse strategies (47).

For example, in mouse *Jpx* is located 9kb upstream of *Xist*, whereas in humans it is separated by 90kb (42,61). Furthermore, human *TSIX* shows little conservation with mouse, and is not transcribed through the entire *Xist* as in mouse (62). Also, in human fetal cells *XIST* and *TSIX* are co-expressed from the inactive X (63), suggesting that *TSIX*-mediated downregulation of *XIST* might be nonfunctional in humans.

More research is required to understand the regulation of XCI in human embryonic development. Despite the elegant picture that has emerged, some steps of the XCI process are still not completely understood. A lot of intriguing questions remain, such as how the cell counts the number of Xs and decides how many to inactivate; how X chromosomes communicate between each other in order to retain one active X and to avoid a lethal state with two active or inactive X chromosomes; how *XIST* RNA recruits repressive chromatin markings; and how spreading of the inactivated state occurs along the inactive X chromosome. These are active areas of current research (46,64).

#### 6.4.1 Special Aspects of X Inactivation Relevant to Human Genetic Diseases

Not all X-linked genes are subject to X inactivation; some genes are robustly expressed from both Xa and Xi. It is estimated that 3% and 15% of genes escape XCI in mouse and humans, respectively (65). Again, there are fundamental differences between human and mouse genes that escape X inactivation. Mouse genes escaping X inactivation are randomly distributed along X, whereas in humans they tend to cluster together. Only six of such genes overlap between mouse and humans (42). Expression analysis of ~600 X-linked genes in human fibroblasts with nonrandom X inactivation and in rodent-human hybrid cells carrying an inactive human X have shown that ~15% of the X-linked gene escape X inactivation; ~10% of genes show heterogeneous escape status among different individuals. Genes that escape XCI are frequently expressed at lower levels from Xi than from Xa (66). Further, based on DNA methylation analysis of X-linked promoters, it is estimated that 12% of the X-linked genes show variable inactivation status among different somatic tissues (67). Most but not all genes that escape XCI have Y-linked homologs either within or outside of PAR regions, some are functional whereas others are pseudogenes. On the human X chromosome, the location of genes that escape XCI is seemingly not random, as the majority of the genes are clustered within regions of X which have the highest degree of homology to the Y chromosome (66).

Genes that escape XCI are important potential contributors to phenotypes of X-chromosome aneuploidy, both in the relatively common situation of X-chromosome deficiency (females with Turner syndrome, karyotype 45,X; often abbreviated XO) and in

supernumery X-chromosome syndromes. Less than 1% of XO conceptuses survive to birth (68), with surviving individuals having a female phenotype and manifesting Turner syndrome—namely short stature, ovarian dysfunction, and a variety of somatic abnormalities such as webbed neck, high arched palate, increased carrying angle of elbows, aortic coarctation, renal malformations, and cognitive problems with visual-spatial perception, and social interactions (69). These problems are presumed to be due to haploinsufficiency of the few genes that normally escape X inactivation (i.e. are present in two active copies in normal XX females). Most of these genes are in the PARs: one of the genes within PAR1, *SHOX*, has been implicated in the short-stature phenotype of females with Turner syndrome, and short stature is observed in individuals with sub-chromosomal deletions encompassing PAR1 on either the X or Y chromosomes (70). Individuals carrying supernumerary X chromosomes—XXX and XXY—have increased mortality rates, possibly resulting from overexpression of X-linked genes that escape XCI (42).

Interestingly, the human X chromosome is enriched for genes expressed in brain, with more than 80 such genes identified to date (71,72). Thus, many X-linked conditions present clinically as syndromic or non-syndromic ID. Sex-chromosome dimorphism makes the inheritance of X-chromosome conditions more complex than patterns observed for autosomal dominant or recessive inheritance. The majority of X-linked disorders affect males, with carrier females either being unaffected or mildly affected, depending on whether there is skewed or random XCI in critical tissues and its impact on the phenotype. Normally, XCI is random, resulting in cellular mosaicism, with two approximately equal populations of cells that express either paternal or maternal X. However, there are cases of significant skewing of X inactivation. Rare mutations within XIC may result in failure to inactivate the X that carries these mutations, with preferential inactivation of the other X chromosome. This type of skewing occurs at the onset of XCI and is termed “primary skewing” (73). Less rare is a mechanism that confers a selective advantage for cells with one of the active Xs, resulting in “secondary skewing.” This situation frequently occurs when part of an X chromosome is deleted (42). Usually skewed XCI selection favors cells with a normal active X, selectively silencing the mutation-bearing X chromosome (74). However, there are rare examples of preferential activation of a mutant X chromosome in female carriers, resulting in more severe disease phenotype, such as rare cases of Duchenne muscular dystrophy in females (75), adrenoleukodystrophy caused by *ABCD1* mutation (76), or Xp11.22–23 duplication in females with ID, speech delay, and autism (77,78). These situations can result from stochastic factors, presence of a genetic mutation or autosomal translocation on another X, or faster proliferation of cells carrying the mutation on the active

X (74). There are two known exceptional situations in which females carriers of X-linked mutations are more severely affected than males: mutations in the *ephrin-B1* gene (79) and *EFMR* (80).

Another example of females affected by an X-linked disorder is the severe neurodevelopmental disorder Rett syndrome, caused by heterozygous mutations of the X-linked *MECP2* gene. *MECP2* mutations are extremely rare in XY males, either because of early embryonic lethality or due to the fact that sporadic *MECP2* mutations almost exclusively occur on the X-chromosome transmitted from fathers to daughters, possibly secondary to deleterious effects of *MECP2* mutation on the oocyte (81). In classical Rett syndrome patients, X inactivation is generally not skewed in brain tissue of affected girls (82), but skewed XCI has been reported in females with very mild symptoms of Rett syndrome (83,84).

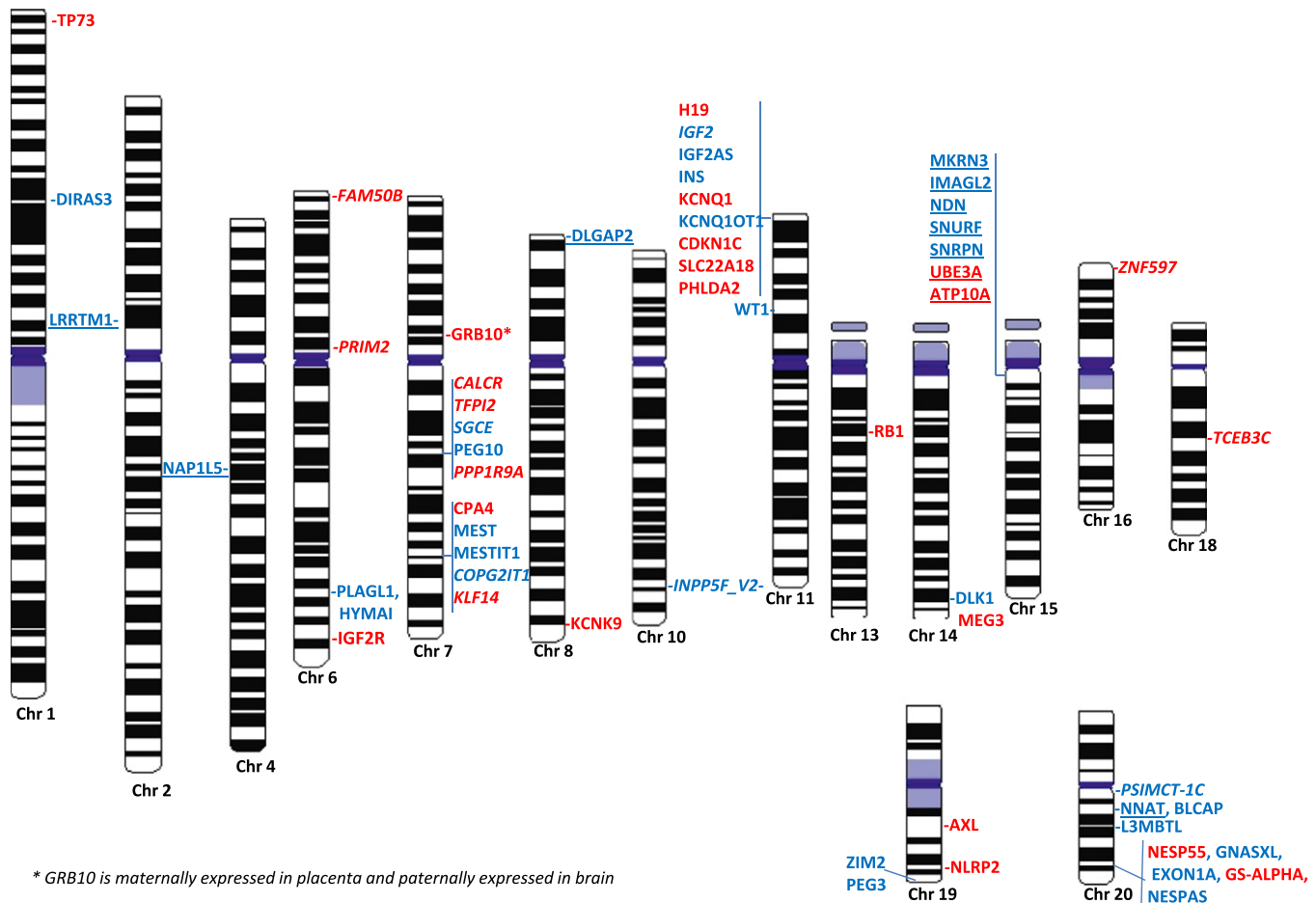
For all these reasons, when counseling families with suspected or known X-linked conditions, knowledge of the inactivation status of the gene, as well as the presence of XCI skewing are important factors. Testing for skewing of X inactivation based on DNA methylation analysis of a polymorphic CAG repeat within exon1 of the *AR* gene (85) is routinely used in molecular diagnostic laboratories to address this question. However, it should be kept in mind that usually this test is performed on clinically accessible tissues, such as blood or buccal swabs, and the degree of skewing might vary among different tissues (74).

## 6.5 GENOMIC IMPRINTING

Genomic or parental imprinting was discovered some 30 years ago, but the phenomenon of parent of origin-specific inheritance was observed 3 millennia ago when mule breeders found that mating a female horse to a male donkey gave rise to a “mule” whereas the reciprocal cross yields a phenotypically distinct equine, called a “hinny” (86). Thus, in violation of classical Mendelian principles, two animals with exactly the same diploid genome can be phenotypically distinct depending only on the parental origins of each haploid chromosome complement. This phenomenon can now be explained by epigenetics. The first embryological evidence of genomic imprinting came from experiments done in the mid-1980s in which attempts to reconstitute a viable mouse embryo entirely from either the maternal germline (gynogenetic conceptus derived from the fusion of two female pronuclei) or the paternal germline (androgenetic conceptus from two male pronuclei) were uniformly unsuccessful (87,88). From these results, the investigators immediately postulated that the maternally transmitted and paternally transmitted genomes (really epigenomes) were not functionally equivalent. Subsequent results analyzing mouse transgenes suggested that different DNA methylation on maternal vs. paternal alleles might be an important mechanism accounting for this nonequivalence.

Imprinting has turned out to affect a relatively small, but important subset of mammalian genes. The first endogenous imprinted genes, including the maternally expressed mannose-6-phosphate/insulin-like growth factor 2 receptor (*Igf2r*) gene, the paternally expressed insulin-like growth factor 2 (*Igf2*) gene, and the maternally expressed non-translated *H19* RNA, were discovered and studied in the early 1990s, first in mice and quickly thereafter in humans. Imprinting tends to be well conserved between the two species, but there are some exceptions in which specific genes, for example, *Igf2r*, are functionally imprinted in mice but not in humans. Recently updated catalogs of imprinted genes in human and mouse (<http://www.geneimprint.com>, <http://www.otago.ac.nz/IGC> and <http://www.mgu.har.mrc.ac.uk/imprinting/imprinting.html>) show >100 imprinted transcripts with ~63 strongly imprinted genes documented in humans (Figure 6-3). Many of these imprinted genes play vital roles in embryonic growth, neonatal behavior, or both, and these genes sometimes exhibit tissue or developmental stage-specific monoallelic expression patterns (89). This parent-specific expression of imprinted genes imposes a functionally haploid state at imprinted loci in normal tissues, and hence deleterious effects from heterozygous mutations or hemizygous DNA deletions at these loci. This situation underlies a variety of human disorders that are inherited in a non-Mendelian fashion; that is, with phenotypic effects seen only after transmission of the mutant allele from one type of parent (90,91).

Thus, while most autosomal genes are expressed roughly equally from the two parental alleles, imprinted genes are expressed preferentially or completely from only one allele—paternal or maternal—depending on the specific imprinted gene under consideration. The complex molecular mechanisms involved ultimately produce differential epigenetic marks on the two parental chromosomes: typically allele-specific DNA methylation (ASM) and/or allele-specific histone modifications. As noted previously, these epigenetic marks are often different in male vs. female gametes, and for imprinted genes this allelic asymmetry is preserved, albeit with some post-zygotic changes, in one or more tissues of the offspring (92). In other words, imprinted genes are an important exception to the general rule that epigenetic marks become equalized on the two alleles during mammalian post-zygotic development. Interestingly, most imprinted genes have been found to reside in 100 kb to several Mb-sized gene clusters, which define sub-chromosomal imprinted domains. All of the best-studied imprinted domains have their own kilobase-scale *cis*-acting imprinting control region, often referred to as an imprinting center (IC) or a germline differentially methylated region (DMR). These ICs have acquired a crucial DNA methylation mark in the paternal or maternal germline, and they preserve this mono-allelic mark in one or more tissues of the offspring, leading by several different



**FIGURE 6-3** Ideograms of human imprinted genes. Ideograms were generated using <http://www.dna-rainbow.org/ideograms/>. Ideogram of each human chromosome known to have an imprinted gene based on the imprinted gene catalog last updated January 2011 (<http://igc.otago.ac.nz>) and recent literature. The G-bands—areas with proportional more A–T base pairs—are normally colored black in schematic representations. To compare the schematic ideograms with our rendered chromosomes, we colored the A–T bases black and the G–C bases white. Blue areas in the rendered chromosomes identify bases not known yet. Blue genes are paternally expressed and red genes are maternally expressed. Bold genes are implicated in growth and the underlined genes play roles in neurodevelopment. Genes in italics have no reported function in growth or neurodevelopment.

downstream mechanisms to mono-allelic expression in *cis* of several clustered imprinted genes.

Geneticists have long debated the *raison d'être* for this surprising non-Mendelian phenomenon. The paternal–maternal intergenomic conflict hypothesis was proposed early on by evolutionary biologists to explain the observed patterns of imprinting for growth-related imprinted genes, and it seems to be standing the test of time as the most likely biological rationale for imprinting (89). Key to this hypothesis is the well-substantiated observation that imprinted genes that are expressed from paternal alleles tend to drive increased growth of the fetus and placenta and/or promote neonatal activity including suckling, thereby placing a greater demand on maternal resources while, in contrast, imprinted genes expressed from maternal alleles tend to inhibit growth and down-regulate demands on the mother (93). Less well understood are the origins of imprinted gene expressions in the brain, and their effects on cognition and behavior. Emerging studies point to an as yet underinvestigated

role for a subset of brain-specific imprinted genes in normal neurodevelopment, cognition, and behavior, including certain major psychiatric disorders (94).

### 6.5.1 Androgenetic and Gynogenetic Tumors: Hydatidiform Moles and Ovarian Teratomas

Complete hydatidiform moles (CHMs) are trophoblast tumors arising from a defective oocyte that has been fertilized by one or rarely two sperms, followed by the retention of a diploid set of paternal chromosomes during early cell divisions and loss of the maternal chromosomes (95). This scenario typically leads to paternal uniparental disomy (UPD) for all chromosomes: thus almost all CHMs are androgenetic tumors, and the dys-regulated growth of these neoplasms is consistent with the frequent physiological roles of paternally expressed imprinted genes in promoting trophoblast proliferation (93). Women with CHM need to be followed after



evacuation of the mole, since these lesions can progress to malignant choriocarcinoma. Diagnosis is often straightforward but occasionally difficult; immunostaining of histological sections from suspected placental neoplasms for the proteins encoded by maternally expressed imprinted genes, including *CDKN1C* ( $p57^{KIP2}$ ) and *PHLDA2*, can be useful for diagnosis as CHMs will be negative for their expression while normal placenta and so-called partial moles will retain expression of these markers (96,97). In nearly all hydatidiform moles, the maternal chromosomes have been physically lost, but rare examples of biparental moles that retain the maternal genome have been described, which occur in families and/or repeatedly in successive pregnancies, suggesting a genetic predisposition. In these tumors, nonetheless there is markedly reduced expression of paternally imprinted/maternally expressed genes, indicating that the androgenetic gene expression state in these variant cases has resulted from failure of maternal imprinting, and that this state is biologically essential for tumor formation (98). Germline mutations in the *NLRP7* and *C6orf221* oocyte-expressed genes have now been identified in some women with this syndrome of familial biparental hydatidiform moles (99,100).

Mature cystic teratomas originate from a parthenogenetically activated oocyte after first meiosis, and are one of the most common types of benign ovarian tumors. The result is the formation of a cyst containing mature tissues from each of the three germ-cell layers. They usually contain disorganized, tridermal, mature components, in which ectodermal tissue predominates (101–103). Muter describes genetic imprinting as a major factor in the development of some of these tumors (103). We have recently identified a genome-wide disruption of normal methylation profiles at ICs in mature ovarian cystic teratomas validating early reports (104).

## 6.5.2 Genomic Imprinting and Human Disease

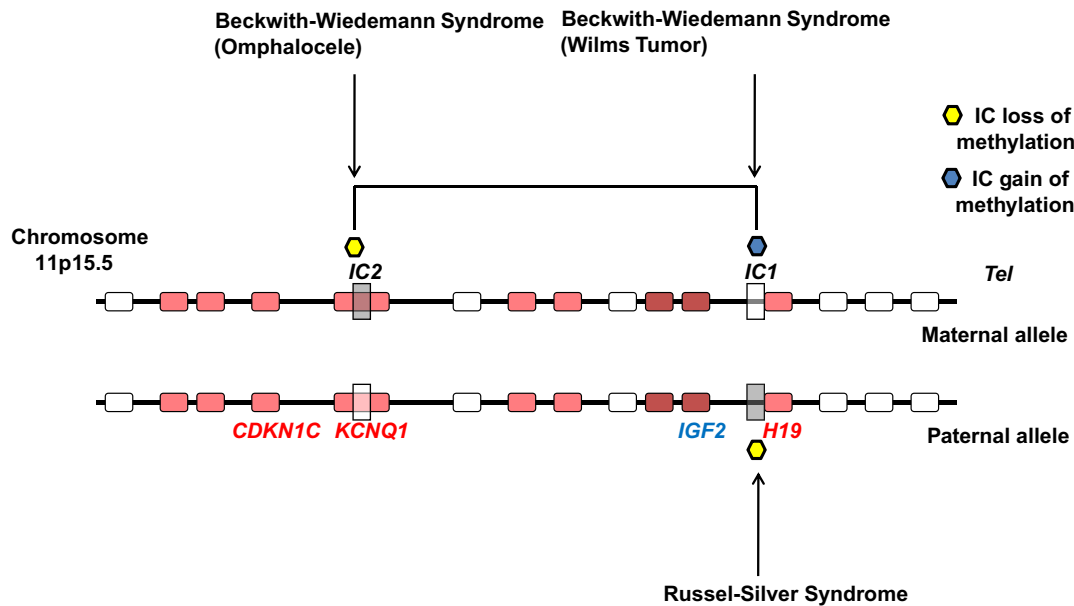
Imprinted genes typically function in growth regulation and neurodevelopment, and the corresponding disease phenotypes due to genetic or epigenetic aberrations in these genes indeed entail major abnormalities of intra-uterine growth or postnatal cognition and behavior (Figure 6-3). We will first discuss imprinting disorders that feature overgrowth or growth restriction as one of their major clinical characteristics.

**6.5.2.1 Beckwith–Wiedemann Syndrome.** This disorder is a rare, often sporadic, heterogeneous congenital overgrowth disorder with an incidence of ~1/13,700 live births (90,105,106). However, this is likely an underestimate as milder and overlapping phenotypes may not be ascertained. Clinically, diagnostic criteria include macrosomia (somatic overgrowth), macroglossia (large tongue), abdominal wall defects (omphalocele, often requiring surgical repair), ear creases and pits, kidney

malformations, neonatal hypoglycemia, visceromegaly, and somatic hemihyperplasia. Certain tissues and organs can also become disproportionately large (kidneys, liver). There is also an increased incidence of embryonal tumors (7.5%). Most common are Wilms tumor and hepatoblastoma, but a variety of other tumor types are seen, including neuroblastoma, rhabdomyosarcoma, and adrenocortical carcinoma.

Beckwith–Wiedemann Syndrome (BWS) is caused by epigenomic and/or genomic alterations in the imprinted gene clusters on chromosome band 11p15.5 (107). The 11p15.5 region can be subdivided into two distinct imprinted domains separated by a non-imprinted region (Figure 6-4), and lesions in each of these imprinted domains can cause BWS, albeit with some phenotypic differences between the two variants of this syndrome. Notably, Wilms tumors are more common in children with BWS due to epigenetic lesions in the *IGF2/H19*-imprinted domain (gain of CpG methylation in the *H19* DMR), while omphalocele is usually more severe in the larger group of cases of BWS that are caused by epigenetic lesions in the *KCNQ1/CDKN1C*-imprinted domain (108). *IGF2*, pathologically activated on the maternal allele in some cases of BWS, encodes a growth factor, while *CDKN1C*, repressed in many cases of BWS, encodes a cyclin-cdk inhibitor. Both of these genes are normally highly expressed in fetal development and have been shown to control growth in mouse models. Very importantly, most cases of BWS are due to purely epigenetic lesions (“epimutations”), either gain of CpG methylation on the maternal allele of the *H19* upstream DMR, which silences *H19* and activates the expression of *IGF2*, or the loss of methylation on the *KCNQ1* intronic DMR, which silences *CDKN1C* plus several nearby maternally expressed genes. These epimutations occur as epigenetic programming errors early in post-zygotic development, often resulting in tissue mosaicism for the cells carrying the epimutation. Cases of BWS with paternal UPD or trisomy encompassing band 11p15.5 are also well documented. Importantly, rare but informative BWS cases have also been described with structural DNA lesions (micro-deletions) in the *H19* DMR, or point mutations in the coding region of *CDKN1C*, providing genetic proof of the major causal roles of *CDKN1C* deficiency and *IGF2* biallelic expression, respectively, in the two main classes of this syndrome.

**6.5.2.2 Russell–Silver Syndrome.** This disorder is characteristically diagnosed in individuals with severe prenatal and postnatal growth compromise (109). Most cases of Russell–Silver Syndrome (RSS) are sporadic; occasionally there are familial cases. Prenatal growth restriction, with or without postnatal short stature, has been the most consistent feature described in patients categorized as RSS or RSS-like. Significant variation occurs in the remaining phenotypic features such as asymmetric growth of the extremities. Molecular results should therefore be examined in the context



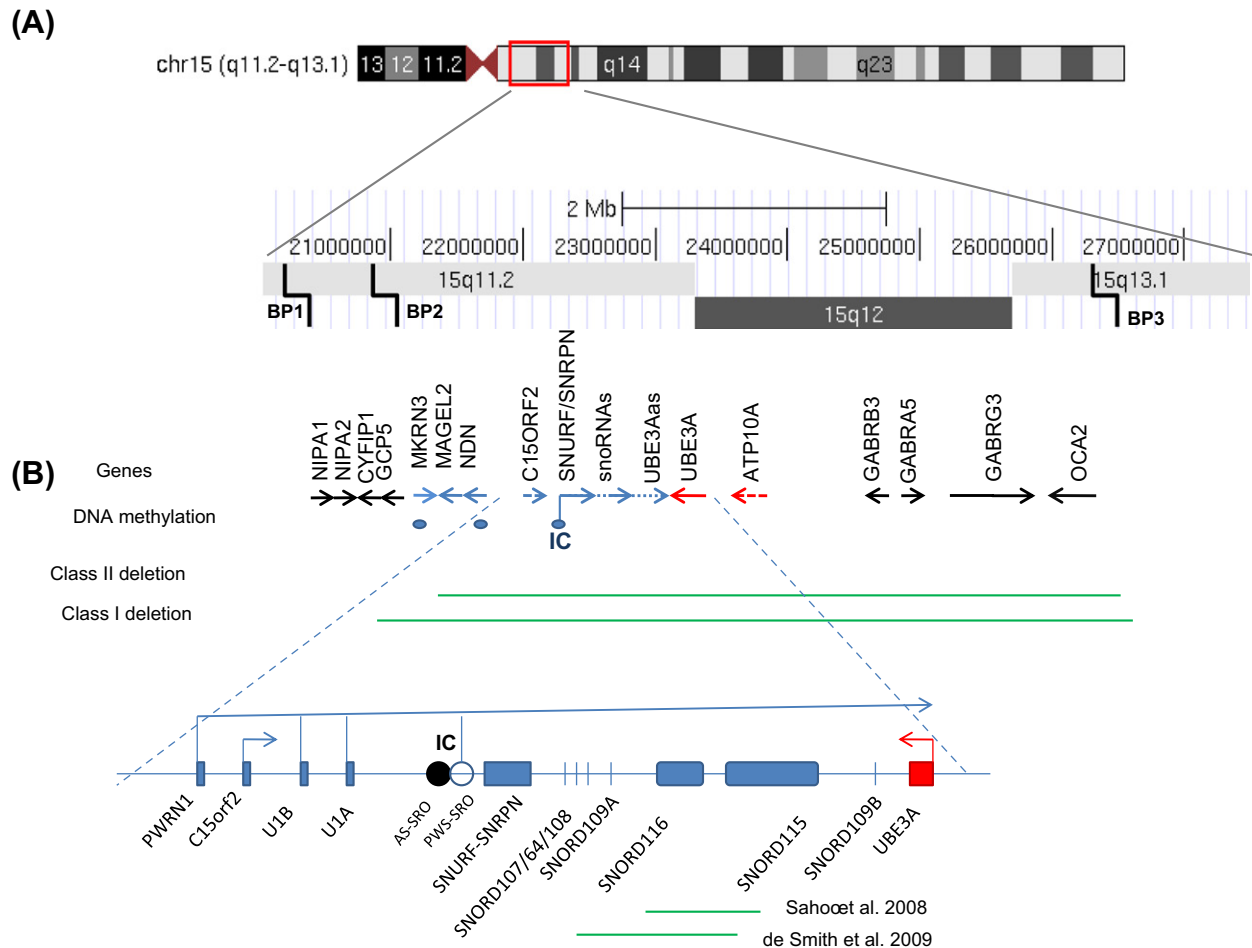
**FIGURE 6-4** Schematic representation of imprinted gene clusters on human chromosome 11p15.5. Imprinted genes are indicated as filled boxes and non-imprinted genes as empty boxes. Paternally expressed genes are indicated in blue and maternally expressed genes in red color. Hollow rectangles show the location on normally unmethylated IC and filled rectangles indicate that the IC is normally methylated. Methylation alterations—such as loss of methylation (yellow hexagon) and gain of methylation (blue hexagon)—show the locations of these changes in each of the two syndromes: BWS and RSS spectrum. In the telomeric domain are two imprinted genes: *H19* and insulin-like growth factor 2 (*IGF2*). *IGF2* is a paternally expressed fetal growth factor and *H19* is a noncoding RNA. IC1 is usually methylated on the paternal chromosome and unmethylated on the maternal chromosome. Normally, the *H19* gene is expressed from the maternal allele and *IGF2* from the paternal allele. Loss of methylation (LOM) at IC1 leads to biallelic expression of *H19* and no expression of *IGF2*, resulting in RSS. Conversely, gain of methylation (GOM) at IC1 leads to biallelic expression of *IGF2* and no expression of *H19* resulting in BWS. The centromeric domain contains several imprinted genes, including *KCNQ1*, *KCNQ1OT1* (long noncoding RNA within the *KCNQ1* gene, not shown in this figure), and *CDKN1C*. IC2 at the promoter for *KCNQ1OT1* regulates the expression of *KCNQ1OT1*, which is a paternally expressed noncoding transcript that further regulates *in cis* the expression of the maternally expressed imprinted genes in the centromeric domain. LOM at IC2 leading to biallelic expression of *KCNQ1OT1* is found in 50% of BWS patients. This epigenetic alteration leads to reduced expression of the growth-regulating gene *CDKN1C*.

of the clinical criteria used for ascertainment of the subjects. Maternal UPD for chromosome 7, a chromosome with known imprinted loci, has been found in 7–10% of RSS cases ascertained using stringent diagnostic criteria. Imprint dysregulation on human chromosome 7 can also cause the RSS phenotype. Epigenetic abnormalities at the *IGF2/H19* locus on chromosome 11 have been found in 20–40% of RSS cases depending on the selection criteria. The main phenotypic feature consistently seen among patients with *H19* DMR hypomethylation is the severe prenatal growth restriction with the postnatal maintenance of short stature (more than 2 SD below the mean). Hence, this form of RSS is essentially opposite, both in phenotype and epigenotype, to cases of BWS with *H19* DMR hypermethylation and somatic overgrowth. Duplication of maternal chromosome band 11p15 has also been reported in individuals with reasonably stringent RSS diagnostic criteria; again representing an opposite situation to a subgroup of BWS cases with duplication of the paternal copy of this same chromosomal region. Thus, a well-defined subgroup of RSS cases seems to result from *IGF2* deficiency. Whether loss of the noncoding *H19* RNA or a microRNA (miR-675) encoded within this gene, which occurs coordinately with the *IGF2* gene activation in both UPD and epimutation cases, also plays

a physiological role in some aspects of RSS and BWS is still under investigation.

### 6.5.2.3 Prader-Willi and Angelman Syndromes.

These two disorders are discussed together because they both map to the imprinted gene cluster on chromosome band 15q11–q13 (Figure 6-5). These are two distinct neurogenetic disorders, both occurring at a frequency of 1 in 15,000–25,000 live births (110). Prader-Willi Syndrome (PWS) is characterized by hypotonia and feeding difficulties early in life, with failure to thrive in early infancy, followed by a shift to excessive eating that can lead to morbid obesity at 1–6 years of age. Individuals with PWS also exhibit developmental delay, mild to moderate ID, a distinctive behavioral phenotype including temper tantrums and obsessive-compulsive features, short stature, hypogonadism, characteristic facial features, scoliosis, and non-insulin-dependent diabetes mellitus. Consensus diagnostic criteria for PWS were first developed by Holm et al. (111), and further revised based on molecular diagnostics (112). In contrast, Angelman Syndrome (AS) is characterized by microcephaly, severe ID, severe speech impairment, gait ataxia, seizures, and a unique behavioral profile including frequent laughter, smiling and excitability. Consensus diagnostic criteria were developed by Williams et al. (113).



**FIGURE 6-5** Schematic maps of the imprinted domains on chromosome bands 15q11–q13. (A) Arrows represent the genes (note that sizes of genes are not to scale). Colors of the arrows represent pattern of expression, blue: paternal, red: maternal, black: biallelic, and dashed arrows show unconfirmed monoallelic patterns of expression. The names of the genes are shown above the respective arrows. IC is an IC located upstream of *SNURF-SNRPN*. Blue circles show regions of differential DNA methylation on the maternal chromosome. BP1, BP2 and BP3 are recurrent breakpoints. Green lines indicate regions of typical deletions (class I and II) associated with Angelman syndrome (maternal deletions) and Prader-Willi syndrome (paternal deletions), and maternal duplications associated with ASD. (B) Zoom into the *SNURF-SNRPN-UBE3A* region. Circles are IC critical elements, black circle is the AS smallest overlapping region (AS-SRO), white circle is PWS-SRO. Boxes are genes, colors of boxes represent pattern of expression, blue: paternal and red: maternal. Arrows denote the direction of expression. *SNURF-SNRPN* is a multi-exonic gene, expressed in multiple isoforms, with the first 3 coding exons encoding SNURF, a protein of unknown function, and *SNRPN* encoding SmN, a spliceosomal protein involved in mRNA splicing. PWRN1, u1A, and u1B are alternative transcription start sites of *SNURF-SNRPN*. SnoRNAs are encoded within introns of *SNURF-SNRPN*, with individual genes for SNORD 107, 64, 108, 109A and 109B, while SNORD 116 and 115 are multi-copy gene clusters. The function of the snoRNAs is not completely understood, they are possibly involved in modulating alternative splicing/regulation nucleolar size. Some of the splice variants of *SNURF-SNRPN* span *UBE3A* (*UBE3A-as*), which possibly regulates imprinted expression of *UBE3A*. Green lines are small atypical deletions associated with PWS.

That these two distinct disorders both map to the imprinted domain on chromosome band 15q11–q13 is explained by the fact that this large (~2.5 Mb) imprinted region contains several paternally expressed (i.e. expressed in an imprinted fashion only from the paternal allele) genes, including *MKRN3*, *MAGEL2*, *NDN*, *C15ORF2*, *SNURF-SNRPN*, and a cluster of C/D small nucleolar (sno-) RNAs, plus a maternally expressed imprinted gene—*UBE3A*—and the *ATP10C* gene that exhibits polymorphic maternal allele expression (110,114). The expression of genes within the 15q11–q13 imprinted domain is regulated by an IC, containing two critical control elements located at the 5' end of *SNURF-SNRPN* (Figure 6-5). The differentially

methyated IC and the promoter regions of *MKRN3* and *NDN* are methylated only on the maternal allele.

Accumulated molecular data have shown that AS results from functional loss, via mutation, paternal UPD, or maternal deletion, or in rare but informative cases IC microdeletions or epimutations, of the maternally expressed *UBE3A* gene. Conversely, PWS arises from the functional loss of paternally expressed genes, no single candidate gene was identified to date; however, atypical microdeletions suggest the important role of sno-RNA *SNORD116*. The most frequent cause of both syndromes (~70%) is a de novo ~5–7 Mb deletion, typically visible by standard cytogenetics. These interstitial deletions involve the entire imprinting domain and several

nonimprinted genes (class I and II deletions) (110,115). In PWS, deletions occur on the paternal chromosome, whereas in AS deletions occur on the maternal chromosome. In 25–30% of PWS cases, loss of the active paternal 15q11–q13 genes result from maternal UPD for this chromosomal region, and conversely 2–5% of AS results from paternal UPD (110). No mutations in single genes have been demonstrated to date in PWS, but in 10% of AS cases mutations of the maternal copy of *UBE3A* have been documented (116,117). This gene encodes an E3 ubiquitin ligase a protein that functions in protein degradation. About 80% of the mutations occur de novo, and about 20% are inherited from unaffected mothers (118). By a mechanism that is not yet fully understood, maternal expression of *UBE3A* is only observed in the brain but not in other tissues, accounting for the neurobehavioral AS phenotype of deficient expression (119,120).

An IC defect is reported in 1–3% of PWS and 2–4% AS patients. This type of defect results from an acquisition of maternal-type imprint (gain of DNA methylation) on the paternal chromosome in PWS and conversely the loss of the maternal imprint (loss of DNA methylation) on the maternal chromosome in AS. In some cases the IC defects are pure epimutations, while in a small but informative group the IC defects are secondary to DNA microdeletions within the bipartite IC. The location of microdeletions is distinct in AS vs. PWS cases; the smallest region of overlap (SRO) of the microdeletions in PWS is 4.3 kb in size and is located within the *SNURF-SNRPN* exon1/promoter (121), while the AS SRO is 880 bp and is located more centromeric, about 35 kb upstream of exon1 of *SNURF-SNRPN* (122). For both syndromes, a microdeletion can be de novo, or inherited from an unaffected carrier parent, through the male germline in PWS and through the female germline in AS (110). However, as noted above, the majority of IC defects in both disorders are due to primary epigenetic alterations (epimutations), which can result from failure of imprint erasure/acquisition or maintenance. Failure of imprint maintenance occurs post-fertilization and results in somatic mosaicism, such that not all cells are affected by the IC defect. In PWS, somatic mosaicism is very rare, whereas in AS it occurs in about 40% of cases with IC defects (110). The percentage of normally methylated cells can vary from less than 1–40% and patients having more cells with normal methylation tend to have milder clinical symptoms (123). Of interest, the proportion of AS patients with an IC defect is increased to 25% in children born to sub-fertile couples or undergoing fertility treatments (124).

The relative contributions of specific paternally expressed genes to PWS are still not clear. Atypical small deletions (125,126) and balanced translocations within the *SNURF-SNRPN* locus (127–132) point to the importance of sno-RNA *SNORD116*, as they usually involve either deletion or transcriptional dysregulation of this gene, whereas DNA methylation and expression of the

genes centromeric to *SNURF-SNRPN* remain intact (110). However, as there are some atypical features seen in patients with microdeletions, it is likely that other genes within the imprinted cluster contribute to the PWS phenotype, as well as having potentially important roles in other clinical phenotypes. For example, an unbalanced translocation associated with the deletion of the paternal copies of *MKRN3*, *MAGEL2*, and *NDN* result in obesity and ID, without other features of PWS (133). Lastly, in contrast to PWS where most cases can be explained by genetic and/or epigenetic alterations at the 15q11–q13 imprinted cluster, 10–15% of suspected AS cases have no identifiable molecular alteration (110).

### 6.5.3 Differential Diagnosis, Epigenotype–Phenotype Correlations, and Genetic Counseling in Imprinting Disorders

Several disorders clinically resembling AS and PWS and could be considered if the molecular testing for AS and PWS is negative (110). For AS, often the most clinically relevant disorder in the differential is Rett syndrome, and for PWS it is maternal UPD of chromosome 14 (110). As discussed previously for BWS, there are certain epigenotype–phenotype correlations for PWS and AS. Haploinsufficiency for non-imprinted genes located within the deleted regions can contribute to more severe phenotypes compared to other genetic/epigenetic lesions cases for both AS and PWS (110). For example, hypopigmentation is a frequent finding in PWS patients with deletions due to loss of one copy of the *OCA2* gene (134). Haploinsufficiency of the *GABRB3* gene is suggested to play a role in susceptibility to severe seizures in AS deletion patients (135). The frequency of autism and psychosis is increased in UPD cases of PWS, but the molecular basis is unknown (136,137). Notably, the AS- and PWS-associated 15q11–q13 imprinted cluster plays an important role in autism spectrum disorder (ASD) susceptibility. Maternal duplications of 15q11–q13 with the same breakpoints observed in AS and PWS are currently the most frequent known genetic causes of autism, found in 1–2% of ASD case (138).

Identifying specific molecular defects in imprinting disorders provides important information for patient management and for estimating recurrence risk. Molecular diagnosis, which often consists of testing for abnormal DNA methylation in the relevant imprinted domains, can be done in an increasing number of imprinting disorders and is already widely applied in PWS/AS (domain in chromosome band 15q11–q13) and BWS/SRS (domains in chromosome band 11p15.5). The majority of molecular alterations within imprinting domains (UPDs, IC epimutations, and microdeletions) can be diagnosed in a simple fashion by assaying DNA methylation in the respective IC. Techniques such as Southern blot or PCR-based assays for DNA methylation (139,140) are useful in addition to the more recently



developed method— multiplex ligation-dependent probe amplification (141). The advantage of using MPLA is that it detects both methylation levels and copy number across several sites within the imprinted cluster. Thus, it can identify microdeletions within the IC in PWS and AS, which are important as they could be inherited from unaffected parents and are associated with 50% recurrence risk. MPLA cannot distinguish UPD from primary IC defects; however, in BWS, simultaneous gain of DNA methylation at H19 DMR and the loss of DNA methylation at *KCNQ1* intronic DMR strongly suggest the presence of chromosome 11p15 UPD. Additional testing for chromosome 11p15 UPD using either a PCR-based dosage assay or microsatellite genotyping should be undertaken if this molecular change is suspected, especially given the high frequency of somatic mosaicism. A normal DNA methylation pattern at chromosome 15q11–q13 makes the diagnosis of PWS highly unlikely as ~99% of PWS cases have de novo deletions, UPD, or IC defects. For AS and BWS, if no loss of DNA methylation at respective ICs is detected, sequencing of *UBE3A* or *CDKN1C*, respectively, should be performed. For SRS, DNA methylation is usually performed for the distal (H19) IC on chromosome 11p15.5 and the imprinted genomic regions at 7p13 and 7q32 (142).

If no molecular defects are identified by methylation and mutation screening, comparative genome hybridization arrays to identify small atypical deletions or duplications could be pursued. It should be kept in mind that identification of small deletions or duplications is dependent on the resolution of microarrays, which can vary significantly among different diagnostic laboratories. Although chromosome translocations, inversions, and duplication infrequently cause imprinting disorders, their presence is associated with a real risk of recurrence. A chromosome abnormality associated with an imprinting disorder may or may not have an associated methylation defect. Therefore, whether or not a methylation defect is present, high-resolution banding of the critical chromosomal region(s) should be considered for all individuals who have imprinting disorders.

Individuals with imprinting disorders and UPD have not to date been reported to transmit the molecular alteration or imprinting disorder to the next generation. In fact, theoretically this is very unlikely. Recurrence risk is usually low (<1%) in individuals with IC epimutations and imprinting disorders. This low risk implies that, in most cases, the IC defect can be rectified by normal germline reprogramming mechanisms. However, there are a small percentage of cases with IC defects that are heritable. These can be recognized from a positive family history or an associated genomic alteration. When inherited genetic alterations are present such as IC microdeletions in AS and PWS, *UBE3A* mutation in AS and *CDKN1C* mutation in BWS, the associated recurrence risk rises to 50%. Such genetic alterations segregate in a Mendelian fashion, but the penetrance of the imprinting disorder depends on which parent transmits the mutation. For example, a parent carrying a mutation in *CDKN1C* (BWS) or *UBE3A* (AS) has a 50% chance of transmitting the mutation, but the imprinting disorder is expressed only if the mother transmits the mutation, since the paternally transmitted gene, whether it carries a mutation or not, is normally silenced in the male germline.

## 6.6 GENETIC DISORDERS DUE TO GENES AFFECTING CHROMATIN STRUCTURE

A number of disorders have been described with mutations or deletions in genes that are important for maintaining normal epigenetic regulation. Loss of function of these genes can disrupt normal establishment, maintenance, or reading of epigenetic marks, thereby resulting in altered chromatin structure and gene expression. In most conditions of this type, we still do not understand precisely how the mutation is related to the phenotype of the human disease. Many of these disorders are associated with ID; other features include facial dysmorphism and various congenital anomalies (143–145). Examples of disorders caused by loss of function of such genes are shown in Table 6-1 and are described in the following.

**TABLE 6-1 X: Genes Causing Chromatin Disorders**

Gene	Function	Locus	Disorder	OMIM	References
<i>ATRX</i>	ATPase/Helicase	Xq21.1	Thalassemia/mental retardation syndrome, X-linked	#301040	Gibbons et al. (315)
<i>CHD7</i>	ATPase/Helicase	8q12.2	CHARGE syndrome	#214800	Vissers et al. (153)
<i>KDM5C</i>	H3K4 demethylase	Xp11.22	X-linked ID	#300534	Rujirabanjerd et al. (162)
<i>EHMT1</i>	H3K9 methyltransferase	9q34.3	Kleefstra syndrome	#610253	Kleefstra et al. (170)
<i>NSD1</i>	H3K36 methyltransferase	5q35.3	Sotos syndrome Weaver syndrome	#117550, #277590	Baujat and Cormier-Daire (175) Douglas et al. (176)
<i>MLL2</i>	H3K4 methyltransferase	12q13.12	Kabuki syndrome	#147920	Ng et al. (181)
<i>CREBBP</i>	Histone acetyltransferase	16p13.3	Rubinstein–Taybi syndrome	#180849	Petrij et al. (316)
<i>EP300</i>	Histone acetyltransferase	22q13.2	Rubinstein–Taybi syndrome	#180849	Roelfsema et al. (317)
<i>DNMT3B</i>	DNA methyltransferase	20q11.21	ICF syndrome	#242860	Xu et al. (203)
<i>MECP2</i>	Methyl binding protein	Xq28	Rett syndrome	#312750	Amir et al. (204)

## 6.6.1 Diseases Due to Aberrant Chromatin

**6.6.1.1 ATR-X Syndrome (*ATRX*).** Mutations in an X-linked gene—*ATRX*—cause  $\alpha$ -thalassemia/mental retardation syndrome, X-linked (ATR-X). This syndrome is characterized by severe ID, facial dysmorphism, urogenital anomalies, and  $\alpha$ -thalassemia. As the *ATRX* gene normally undergoes X inactivation, affected individuals are almost exclusively males, while females usually are unaffected due to preferential X inactivation of the chromosome with the *ATRX* mutation (146). The *ATRX* protein is involved in epigenetic regulation through two functional domains: an ATP/helicase domain and an ADD domain that shares homology with de novo methyltransferases. The ATP/helicase domain is proposed to be involved in nucleosome repositioning and making DNA more accessible for protein binding (147) while the ADD domain has been shown to bind histone H3 tails with the silencing mark H3K9me3, but not the active mark H3K4me3/2 (148). In terms of genomic targets, *ATRX* has been shown to localize to the nucleus in heterochromatin, telomeric/subtelomeric chromosomal regions, rDNA, and promyelocytic leukemia bodies (149–151). Furthermore, peripheral blood cells of ATR-X patients exhibit changes in DNA methylation of rDNA, sub-telomeric repeats, and Y chromosome-specific satellites (149). By ChIP-sequencing, it was established that in erythroid cells *ATRX* binds to CpG-rich tandem repeat sequences clustered at sub-telomeric regions, thereby affecting the expression of associated genes including  $\alpha$ -globin, which accounts for the  $\alpha$ -thalassemia phenotype of ATR-X syndrome (152).

**6.6.1.2 CHARGE Association (*CHD7*).** Nonsense or missense mutations and deletions resulting in haploinsufficiency of the chromodomain helicase DNA-binding protein *CHD7* cause the majority of cases of CHARGE association (*CHD7*) (153,154). Clinical diagnosis of *CHD7* is based on nonrandom associations of the following congenital abnormalities: Coloboma of the eye, Hear defects, Atresia of the nasal choanae, Retarded growth and development, Genital abnormalities, and Ear abnormalities/deafness/vestibular disorder (153,155). Studies in model organisms—*Drosophila* and mouse—have found phenotypes that overlap those found in humans (156,157). In *Drosophila*, reduced expression of *kismet/CHD7* results in deficits in axonal pruning, guidance and extension, as well as defects in memory and motor function. *Kismet* has also been shown to regulate the repressive histone H3 methylation mark of lysine 27 (158) and the loss of *kismet/CHD7* expression results in increased repressive chromatin marks, thereby repressing the expression of other genes than it would normally regulate. Similarly, in human cell lines, *CHD7* has been shown to bind to chromatin regions that are active as demonstrated by histone H3 lysine 4 (H3K4) methylation and DNase1 hypersensitivity of these binding sites (158).

### 6.6.1.3 X-linked Mental Retardation (*KDM5C*).

Mutations in the X-linked gene *KDM5C*, encoding a histone demethylase, cause a spectrum of phenotypes, ranging from syndromic to non-syndromic ID. The clinical features in males with *KDM5C* mutations include mild to severe ID, epilepsy, short stature, hyperreflexia, aggressive behaviors, and microcephaly (159–164). *KDM5C* escapes X inactivation, and has a functional Y-linked homolog—*KDM5D*—so female heterozygous mutation carriers are usually unaffected but sometimes demonstrate mild ID or learning difficulties (72). *KDM5C* have several conserved functional domains, including the Bright/ARID domain responsible for DNA binding; the catalytic JmjC domain; and two PHD domains responsible for histone binding (165,166). *KDM5C* can bind to the repressive histone mark H3K9me3 and can remove the active epigenetic mark H3K4me3/2, thus establishing a repressive chromatin state (167,168). *KDM5C* point mutations found in patients can suppress demethylase activity and/or H3K9me3 binding in vitro, depending on the location of the mutation (168). Chromatin immunoprecipitation (ChIP) in cell lines showed that *JARID1C* co-localizes with REST, a transcriptional repressor in the neuron-restrictive silencing elements, in the promoters of a subset of REST target genes, including *BDNF* and *SCN2A*, suggesting that the loss of *JARID1C* activity impairs REST-mediated neuronal gene regulation (169).

**6.6.1.4 Kleefstra Syndrome (*EHMT1*).** Haploinsufficiency of the *EHMT1* gene due to heterozygous deletions or mutations causes the 9q subtelomeric deletion syndrome, also known as Kleefstra syndrome (*EHMT1*). These individuals demonstrate moderate to severe ID, childhood hypotonia, and facial dysmorphism (170). *EHMT1* encodes a histone methyltransferase catalyzing mono- and dimethylation of H3K9 through its catalytic SET and PreSET domains (171,172). H3K9me2 is a euchromatic silencing mark (173). It has been shown in mice that *Ehmt1* forms a heteromeric complex with another H3K9 methyltransferase, *G9a*, and knockouts of either of these genes lead to very similar phenotypes in mice, including embryonic lethality and loss of H3K9 methylation (171,172). In conditional knockouts in the forebrain of *Ehmt1*, *G9a*, or both, behavioral abnormalities, including defects in learning, motivation, and environmental adaptation were observed (174). Furthermore, *Ehmt1/G9a* deficiency in the forebrain led to derepression of non-neuronal genes, suggesting that the role of the *Ehmt1/G9a* complex is to protect neurons from transcriptional noise. Distortion of this transcriptional homeostasis has been proposed to lead to the ID phenotype (174).

**6.6.1.5 Sotos Syndrome (*NSD1*).** Haploinsufficiency due to mutations or deletions of the *NSD1* gene, encoding a histone methyltransferase, causes Sotos syndrome (*NSD1*), an overgrowth condition associated with macrocephaly, facial dysmorphism, advanced bone age, and learning difficulties or mild ID (175). Some

mutations of this gene are associated with another overgrowth condition, Weaver syndrome (176). *NSD1* has a catalytic lysine methyltransferase SET domain and four zinc-binding PHD domains and functions primarily to mono- and dimethylate H3K36 (177). The role of H3K36 methylation is not completely understood; in model organisms it has been found within gene bodies of expressed genes and is associated with the suppression of intragenic transcriptional initiation (178). ChIP-CHIP experiments using promoter microarrays have shown that NSD1 binds to promoters of genes playing a role in various processes, such as cell growth/cancer, keratin biology, and bone morphogenesis (179). In addition, it was found that four of the NSD1 PHD domains bind histone H3 methylated at K4 and K9, and that the large majority of point mutations found in Sotos syndrome disrupt this binding (180).

**6.6.1.6 Kabuki Syndrome (*MLL2*).** Recently, the whole exome sequencing has uncovered heterozygous mutations in the *MLL2* gene as the cause of Kabuki syndrome, characterized by mild to moderate ID, multiple congenital anomalies, short stature, and typical facial features (181). *MLL2* belongs to the SET1 family of histone H3K4 methyltransferases. It has a catalytic SET domain, five PHD domains, and an HMG-I binding motif (182). *MLL2* is a part of a multi-protein complex that catalyzes mono-, di-, and trimethylation of H3K4 (183). H3K4 trimethylation is associated with active transcription (178) and the reduction of *MLL2* in human HeLa cells results in downregulation of a number of genes involved in cell adhesion, cytoskeleton organization, transcriptional regulation, and development (183). Interestingly in mice, *Mll2* has been shown to be crucial for the epigenetic re-programming that takes place before fertilization in oocytes by trimethylation H3K4, with deficiency of *Mll2* resulting in anovulation (184).

**6.6.1.7 Rubinstein–Taybi Syndrome (*CREBBP/EP300*).** Haploinsufficiency of chromosome 16p13.3 due to microdeletion or mutation in either the *CREB-binding protein* (*CREBBP*) or *E1A-binding protein* (*p300*) results in Rubinstein–Taybi syndrome (RSTS), characterized by multiple congenital anomalies, including postnatal growth deficiency, microcephaly, specific facial characteristics, broad thumbs, and big toes, and ID (185). Both of these homologous proteins contain a HAT domain and have been demonstrated to have overlapping functions, but there are some differences in expression patterns and necessity for specific processes and signaling molecule responsiveness (186–190). Using mouse models, the HAT activity has been demonstrated to be important for long-term potentiation, learning, and memory (191,192), which are in part regulated by histone acetylation-dependent transcription (193,194).

**6.6.1.8 ICF Syndrome (*DNMT3B* and *ZBTB24*).** Immunodeficiency, centromere instability, and facial anomalies (ICF) syndrome is a very rare disorder caused by mutations in *DNMT3B* in the majority of cases, and of

the epigenetic regulator gene *ZBTB24* in a minority of cases (195,196). Patients with ICF have low levels of immunoglobulins and reduced B- and T-lymphocyte counts (197). Most patients have DNA hypomethylation and chromatin under-condensation localized to juxtacentromeric (adjacent to the centromere) regions of chromosomes 1, 9, and 16, probably accounting for the diagnostic secondary chromosomal fusions observed in metaphase analyzes from affected lymphocytes (198–201). Aberrant hypomethylation also occurs in alpha satellite DNA, constitutive heterochromatin, Alu sequences, and some imprinted genes (200–203).

## 6.6.2 Disease Due to Abnormal Reading of Epigenetic Marks

**6.6.2.1 Rett Syndrome (*MECP2*).** Heterozygous mutations of the X-linked gene *MECP2* cause Rett syndrome in girls. Rett syndrome is characterized by developmental arrest between 5 and 18 months of age, followed by regression of acquired skills, loss of speech, stereotypical movements, microcephaly, seizures, and severe ID (204). The function of *MECP2* has been very extensively studied, but the mechanism by which its deficiency results in the phenotypes of Rett syndrome remains incompletely understood. Initially, *MECP2* was identified as a protein capable of binding methylated DNA (205). It was found to have abundant binding sites distributed throughout the genome and was demonstrated to function in the repression of transcription (206–208). The best-established mechanism by which *MECP2* downregulates gene expression is through recruitment of HDACs, which transform chromatin into a repressive state by removing acetyl groups from histones H3 and H4 (206,208,209). However, there is growing evidence that the role of *MECP2* in transcription regulation is more complex; for example, in mice it was shown to bind to the transcriptional activator CREB to activate transcription of a large number of genes in the hypothalamus (210). *MECP2* deficiency can lead to Rett syndrome through dysregulation of specific genes—such as *BDNF*—which has been shown to have an *MECP2*-binding site. Furthermore, reduction of *Bdnf* in mice mimics some features of the *Mecp2*-null mice phenotype (211) and *Bdnf* overexpression in *Mecp2* knockout mice can partially rescue the phenotype by improving their locomotor function, extending lifespan (212), and rescuing synaptic dysfunction (213). These data suggest that *BDNF* is indeed an important and clinically relevant *Mecp2* transcriptional target. Recent findings suggest that *MECP2* is almost as abundant as histone H1 in mouse neurons but not in glia (214), so *MECP2* function in neurons might affect genome-wide chromatin remodeling rather than only the regulation of the expression of specific genes. In addition, targeted deletion of *Mecp2* in mice results in increased expression of repetitive elements in neurons (214), prompting investigators to suggest



that this affects overall transcriptional noise in neurons, and the ability of neurons to respond adequately to environmental signals (215).

Lastly, it is relevant to consider that known functional interactions between the proteins involved in chromatin disorders suggest that their targets can in part overlap leading to shared or overlapping phenotypes such as ID. For example MECP2 and ATRX are components of the same chromatin remodeling complex (216,217). EHMT2, a partner of EHMT1, has been shown to be a component of the same protein complex as KDM5C (169). Identification of dysregulated genes and epigenetic marks in these chromatin disorders is the subject of ongoing research.

## 6.7 METHODS FOR STUDYING EPIGENETIC MARKS

### 6.7.1 Mapping DNA Methylation

Many techniques have been developed to study DNA methylation. One of the first methods to score DNA methylation at a specific locus was Southern blotting of genomic DNA digested with methylation-sensitive restriction enzymes (218). Certain restriction enzymes (e.g. *HpaII*, *SmaI*, *NotI*) that contain CpG as part of their recognition sequences do not cut that site when the C is methylated. Therefore, failure to cleave by a methyl-sensitive restriction enzyme is an evidence of DNA methylation at that site. Restriction enzymes can also be used in combination with microarray platforms to evaluate genome-wide DNA methylation patterns, including promoter methylation and allele-specific methylation (219,220).

The gold standard that allows for the comprehensive analysis of CpG sites is sodium bisulfite chemical conversion of DNA. Sodium bisulfite deaminates non-methylated dCs to dU residues; during subsequent PCR amplification, the latter are converted to A/T base pairs. However, if the C is methylated, the DNA sequence does not change (221). A number of methods have been developed to determine the levels of DNA methylation across multiple CpG sites. PCR primers can be designed to amplify specific genomic regions. Methylation-specific PCR provides a semi-quantitative measurement of DNA methylation levels. An alternative approach involves amplification of bisulfite PCR products followed by cloning and sequencing. This more-thorough approach permits DNA methylation levels of a larger number of individual CpG sites to be quantified, and the precise patterns of methylation to be displayed. One of the newer technologies—pyrosequencing—determines an absolute value for DNA methylation at individual CpG sites across a region. By combining sodium bisulfite conversion and microarrays or massively parallel Nextgen sequencing, genome-wide DNA methylation patterns can be determined for a large number of CpG sites (104,222).

### 6.7.2 Mapping Histone Modifications and Chromatin Structure

To determine the interaction of histone proteins with DNA, specifically histones that carry various modifications, chromatin immunoprecipitation (ChIP) is employed. This methodology detects the covalent modifications of histones bound to either active or inactive genes. Generally, cells or tissues are briefly fixed with formaldehyde to crosslink the proteins to the DNA. The fixed chromatin is fragmented, usually by sonication, to enable targeted analysis. The chromatin is next subjected to immunoprecipitation (IP) with an antibody specific for a given protein or, more often, a unique covalent modification of a certain residue (e.g. acetylation or methylation of lysine 9 of histone H3). After IP and the removal of the crosslinks, one can then amplify specific regions of interest by quantitative or semi-quantitative PCR. A PCR product indicates that the protein with that particular modification was associated with the DNA of the targeted genome region. Alternatively, ChIP can be combined with microarray technology or Nextgen sequencing to define genome-wide histone modification locations in various tissues and disease states. A large-scale project—the NIH Roadmap Epigenetics Mapping Consortium—has begun to map DNA methylation and a large number of histone modifications in a number of tissues, including normal, cancerous, and pluripotent cells, and the data from this project are being made publically available (223). Also important in the study of chromatin is the three-dimensional long-range interaction of regions of DNA and their associated proteins. To determine the three-dimensional interaction of chromatin segments at long distances from each other, a method called chromatin conformation capture (3C) has been developed (224). Newer modifications of the 3C method have been derived to facilitate genome-wide interaction mapping using next-generation sequencing (225).

## 6.8 CANCER EPIGENETICS

As amply demonstrated by the disorders discussed above, epigenetic aberrations can result in a diverse array of non-neoplastic human diseases. The contribution of epigenetic alterations to a number of different cancers has been studied even longer and equally intensively and is too broad to cover comprehensively; instead we restrict our discussion here to general principles, with selected illustrations. Changes in DNA methylation were the first epigenetic alteration identified in cancer (226), and subsequent work over three decades has shown that both hyper- and hypomethylation are important and pervasive pathogenic mechanisms both in early and late stages of human neoplasia (227). Not surprisingly, histone modifications and miRNA expression are also altered in cancer (228). It has become increasingly apparent recently that proteins regulating epigenetic marks, including histone



methyltransferases and demethylases, DNA methyltransferases, and chromatin remodeling SWI–SNF complexes, are also dysregulated, not just by over- or under-expression but also by recurrent cancer-associated somatic mutations. DNA methylation and histone methylation profiles, as well as miRNA signatures, are being used as epigenetic biomarkers to diagnose and predict recurrence risk for a number of tumor types, and it is hoped that such profiles will also become useful for individualizing anticancer therapies (229).

### 6.8.1 DNA Hypermethylation in Cancer

CpG hypermethylation in gene promoters is the best characterized epigenetic abnormality in human malignancies. A common paradigm in cancer epigenetics is hypermethylation of the CpG-rich promoter regions of tumor-suppressor genes, resulting in epigenetic silencing of these genes (14). Indeed, for some of the most important tumor suppressors—such as the *CDKN2A* gene encoding the p16 cell cycle inhibitor—promoter hypermethylation can be the most common mechanism underlying their functional loss during tumor formation, with the corresponding genetic pathways for loss of function (deletion/mutation) being utilized less commonly (230). Hypermethylated promoter DNA is associated with virtually every type of human tumor, with each type of tumor having its own signature of methylated genes, such as the methylation of *GSTP1* in prostate cancer, the von Hippel–Landau syndrome gene *VHL* in renal cancer, the mismatch repair gene *MLH1* in colon and endometrial cancers, and sometimes *BRCA1* in breast cancer (231–236). In some of these examples, the same tumor-suppressor gene is mutated or methylated as alternative pathways in the same tumor type: loss-of-function mutations in *MLH1* and *VHL* are found in the germlines of patients with hereditary colon and renal cancer, respectively, and these same genes are hypermethylated and silenced in sporadic tumors of the same histologic type (231,237).

While gain of DNA methylation is often discussed as a late event in tumor progression, CpG hypermethylation in specific sequences often occurs early in cancer formation, sometimes preceding tumorigenesis. Examples of early epigenetic aberrations can also be cited in other adult malignancies: in cigarette smokers, *CDKN2A* promoter methylation occurs in dysplastic bronchial epithelial cells prior to the formation of overt lung cancers (238), promoter hypermethylation of tumor suppressor genes is already detectable in the pre-malignant lesion Barrett esophagus (239,240). One of the best substantiated examples of a very early epigenetic lesion predisposing to subsequent tumor formation is the gain of methylation of the *H19* DMR on the maternal allele, which leads to the loss of imprinting of *IGF2* expression and can often be detected in non-neoplastic kidney cells both in BWS-associated and sporadic cases of the pediatric kidney cancer Wilms tumor (227).

DNA hypermethylation has attracted much attention as a biomarker for cancer detection and classification. To be clinically applicable, an ideal tumor biomarker must be specific for cancer, and readily detectable in clinical specimens obtained through minimally invasive procedures. DNA hypermethylation seems to fulfill these requirements and has been considered to be a promising biomarker. Examining the methylation of a subset of genes (*GSTP1*, *APC*, *RASSF1*, and *MDR1*) distinguished primary prostate cancer from benign prostate tissues with sensitivities and specificities of greater than 90% (241,242). DNA methylation alterations can be detected and used as biomarkers in feces for colorectal cancer, urine for bladder cancer screening, and sputum to predict the occurrence of lung cancer (243–245). However, for reasons that are complex but partly financial, these types of tests largely remain at the research stage and have not yet been widely adopted in clinical practice.

### 6.8.2 DNA Hypomethylation in Cancer

Global DNA hypomethylation in cancer cells was in fact identified prior to promoter hypermethylation (246), with studies indicating that genome-wide 5-methyl-C is reduced on an average of 10% in a number of different tumor types (226,247). Thus, the net decrease in the genomic methyl-C content in cancer cells often exceeds the localized increases in DNA methylation (248). There is some evidence that hypomethylation of DNA can result in genomic instability leading to mutations, deletions, amplifications, inversions, and translocations (249). Poor prognosis in colon cancer is associated with hypomethylation of repetitive elements (250). Hypomethylation can also lead to the reactivation of silenced genes and miRNAs leading to a cascade of aberrant expression (251,252). While most abnormalities in DNA methylation in human cancers cannot yet be explained by a clear genetic mechanism, there are at least several recurrent cancer-associated somatic mutations, in the *IDH1/2*, *TET2*, and *DNMT3A* genes, that have been proposed as candidates for explaining altered DNA methylation in certain tumor types such as AMLs and MDS (253).

### 6.8.3 Abnormalities of Histone Modifications in Cancer

Epigenetic alterations in cancer are not restricted to DNA methylation. Genome-wide mapping of histone marks has demonstrated global reductions in acetylated H4 lysine 16 (H4K16ac) and H4 lysine 20 trimethylation (H4K20me3), both resulting in or correlating with repression of gene expression (254). HDACs have been found to be overexpressed in a number of cancer types, and in some cancers that can be the dysregulation of HATs due to translocations resulting in deleterious gene-fusion products (255–257). Aberrant histone methylation of H3K9 and H3K27 also results in gene silencing

in many cancers (258,259). EZH2, a histone methyltransferase of H3K27, is frequently overexpressed in breast and prostate tumors, in addition to other tumors (260–262). As is true for aberrant DNA methylation, most abnormalities in histone modifications in cancer are not yet explained by a single genetic lesion. However, with high-coverage sequencing technologies an increasing number of chromatin-modifying enzymes, such as CREB, JARID1C, EZH2 and SWI/SNF family proteins hSNF5/INI1 and PBRM1, are now being found mutated in specific types of human cancers (263–267).

#### 6.8.4 Aberrant miRNA Expression in Cancer

Comparisons of tumor tissues and corresponding normal tissues have revealed global changes in miRNA expression during tumorigenesis (268). In chronic lymphocytic leukemia, *miR-15* and *-16*, which target the anti-apoptotic gene *BCL2*, are downregulated (269). Similarly, *let-7* that targets the oncogene *RAS* is downregulated in lung cancer (269). Upregulation of *miR-21* that targets *PTEN* has been shown to occur in glioblastoma (270). These alterations in miRNA expression may occur through a number of mechanisms including chromosomal abnormalities, transcription-factor binding, and epigenetic alterations (271). Silencing of miRNA expression has been shown to occur by aberrant hypermethylation in a number of cancers (272,273). As is true for the other epigenetic factors discussed above, the role of miRNA dysregulation in cancer has been validated genetically by findings of DNA deletions encompassing miRNA genes, for example, on chromosome 13 in chronic lymphocytic leukemias (274).

#### 6.8.5 Therapies Targeting Epigenetic Modifications in Cancer

Two classes of medications—DNA methylation inhibitors and HDAC inhibitors—have been approved by the US Food and Drug Administration as treatment for cancer (275). The DNA methylation inhibitors—5-azacytidine (azacytidine) and 5-aza-2'-deoxycytidine (decitabine)—are nucleoside analogs that get incorporated into the genomes of growing tumor cells and act as suicide inhibitors of DNA methyltransferase enzymes, leading to progressive loss of DNA methylation with each S-phase of the cell cycle. These medications have been approved for use in the treatment of MDS and have shown some promise for treating AML and other hematological malignancies (276). The HDAC inhibitor vorinostat (suberoylanilide hydroxamic acid) is being used with good results in treating patients with cutaneous T-cell lymphoma in the United States (277). Beyond treatment for cancers, such medications are being used and developed to treat a wider spectrum of diseases. Resveratrol, a natural compound in red wine, which inhibits sirtuins (a family of HDACs) is being evaluated as treatment for

type II diabetes and metabolic syndrome (278). Valproic acid (VPA), also an HDAC inhibitor, is currently used to treat seizures and mood disorders (279,280), and VPA in combination with other medications has been shown to inhibit cancers in vitro (281). The wide array of epigenetic alterations identified in human disease could present valuable targets for approved medications as well as for novel ones that have yet to be developed.

### 6.9 ENVIRONMENTAL INFLUENCES ON EPIGENETIC TRAITS

Transgenerational effects of the environment on our epigenome were recently documented for two tragic historical events: the Dutch hunger winter (1944) and the great Chinese famine (1958–1961). Studies of children born following these periods are reported to have an increase risk for schizophrenia (282,283). Children who were conceived during the Dutch famine had six decade later changes in DNA methylation in the *IGF2* gene which encodes a growth hormone critical for normal embryonic development (284). These data constitute the first concrete evidence that in humans the mother's diet early in pregnancy can directly affect the programming of the epigenome early in utero that shape our development later in life.

The role of diet and environment in the expression of imprinted genes has not been extensively explored. We are only beginning to understand which environmental signals can alter methylation of specific genes or the genome as a whole. There is evidence that environmental exposure to compounds such as cadmium (285) and arsenic (286) may be a predisposing factor that leads to epigenetic instability, aging, and cancer.

Both folate and the enzyme methyltetrahydrofolate reductase (MTHFR) are important for DNA synthesis and methylation. In fact, mice lacking the enzyme MTHFR have been shown to have decreased global DNA methylation. In humans, MTHFR enzyme activity depends on an individual's genotype for the functional polymorphism MTHFR 1298A>C and correlates positively with the level of global DNA methylation (287). Further, humans on a folate-depleted diet demonstrate decreased global DNA methylation (288). Conversely, in a study of adult males on hemodialysis, adding an exogenous source of folate led to an increase in both global and locus-specific DNA methylation, including *H19*, *IGF2*, and *SYDL1* (289). Lastly, dietary supplementation with folic acid and B vitamins has been clearly shown to modify tumor incidence in mouse models (290,291).

### 6.10 ABNORMALITIES IN EPIGENETIC PROGRAMMING LINKED TO INFERTILITY AND ASSISTED REPRODUCTION

In mice and humans, oocytes retrieved following hormonal induction or embryos studied after in vitro culture have shown methylation and/or expression anomalies in

several imprinted genes (292–295). Studies of human oocytes harvested after medical hormonal induction showed loss of methylation at the maternal *MEST/PEG1* DMR on chromosome band 7q33 (296) and gain of methylation at the maternal *H19* DMR (IC1) on chromosome band 11p15.5 (295). Increasing attention has recently been focused on reports of increased rates of epigenetic errors in human following infertility/ART. In particular, two rare epigenetic disorders—BWS and AS—exhibited an increased incidence in retrospective studies (odds-ratios 6–17 and 6–12, respectively) in children born following infertility/ART (297–302). The data are especially compelling in that the increased incidence is attributable to an increase in specific epigenetic errors at two different chromosomal locations, with both locations being affected by abnormal imprinting on the maternal (oocyte-derived) alleles. Furthermore, in ART-conceived AS and BWS patients, loss of maternal methylation at their respective DMRs (chromosomes 15q11 and 11p15) occurs 8 and 1.9 times more often, respectively, than in individuals born from spontaneous conceptions (124,303,304). Such evidence supports the hypothesis that ART-conceived children have an increased rate of epigenetic errors over those in the general population.

In humans, it is still unclear whether maternal loss of methylation observed in children post ART is the result of the procedure itself or of an underlying infertility with oocyte abnormalities in the couple seeking ART interventions, or both. However, idiopathic male infertility is also associated with aberrant methylation at both maternal and paternal alleles, suggesting that male germ cells represent another potential source for methylation defects in children conceived via ART (305,306). Recently, ovarian stimulation (part of subfertility/infertility treatment) was linked to perturbed genomic imprinting at both maternally and paternally expressed genes. These data demonstrate that superovulation has dual effects during oogenesis: disruption of imprint acquisition in growing oocytes, and the disruption of a maternal-effect gene product subsequently required for imprint maintenance during preimplantation development (307).

### 6.11 IN UTERO EPIGENETIC PROGRAMMING OF ADULT TRAITS AND DISEASE

The Developmental Origins of Health and Disease (DOHAD) hypothesis, pioneered by David Barker (308), has predicted among other things that maternal stress during pregnancy (dietary inadequacy, toxic exposures, and perhaps psychological stress) might lead to persistent epigenetic changes in the fetus, which could play a role in modulating the subsequent onset of adult cardiovascular, metabolic, and psychiatric diseases. In mice, maternal behavior in the neonatal period may correlate with epigenetic programming of adult behavior. Recent studies have indicated that mothers showing strong

nurturing behavior toward their pups, by frequently licking and grooming their offspring, produce alterations in the patterns of DNA methylation; for example, in the promoter of the glucocorticoid receptor gene, in the hippocampus of their pups (309). This area of research has now become quite active and it will be important to follow progress in this area over the next several years.

### 6.12 GENETIC–EPIGENETIC INTERACTIONS

With the exponentially increasing volume of human genetic data from SNP and DNA copy number analyses on microarrays, genome-wide association studies (GWAS), and “post-GWAS” studies such as whole exome and whole genome sequencing, it becomes crucial to consider interactions between the genome and the epigenome. In principle, these interactions can be of two types: interactions in *cis*, in which the local DNA sequence and haplotype can affect the pattern of epigenetic marks on a given allele, and interactions in *trans*, in which the overall genome, including mutations, DNA gains and losses, and whole chromosomal aneuploidies, can affect epigenetic patterns at various sites distributed across all the chromosomes. A small but increasing number of studies are starting to address these central questions about genome–epigenome interactions. By analyzing multiple tissue samples from multiple human individuals using high-throughput genetic and epigenetic profiling methods, these studies are starting to uncover recurrent and highly predictable genome–epigenome interactions.

In 2008, Kerkel et al. (220) used the MSNP method; the pre-digestion of genomic DNA by methylation-sensitive restriction enzyme(s) followed by probe synthesis and hybridization to SNP arrays, to examine ASM in several human tissues. Their study was designed to detect new examples of imprinted genes, but instead they found numerous examples of previously unsuspected ASM at loci outside of imprinted regions. Most of these examples of non-imprinted ASM showed a strong correlation of CpG methylation patterns with local SNP genotypes, indicating *cis*-regulation of this epigenetic phenomenon. That paper was quickly followed by other reports examining various types of human cells and tissues for ASM or similar phenomena of methylation quantitative trait loci (mQTLs) and allele-specific transcription factor binding (ASTF). All of these papers confirmed that for the majority of genes and intergenic regions showing strong ASM, mQTLs, or ASTF, the allelic asymmetry is dictated not by parent of origin but rather by local SNPs; that is, by the haplotype in which the epigenetic pattern is embedded (310). Thus, while ASM due to parental imprinting is a potent mechanism for regulating functional gene dosage, it affects fewer genes than this more newly recognized phenomenon of haplotype-dependent ASM. In parallel with this work, many laboratories have used



microarrays and Nextgen sequencing to map the related phenomena of haplotype-dependent allele-specific RNA expression (ASE) and eQTLs, which turn out to affect up to 10% of human genes.

Importantly for clinical genetics, these widespread phenomena of haplotype-dependent ASM, ASTF, and ASE, in contrast to imprinting, do not violate any Mendelian principles: while the allele-specific epigenetic patterns are not actually passed through the germline, in each generation these patterns are reestablished and maintained in the fetal and adult tissues under the strong *cis*-acting influence of the local DNA sequence. So for counseling purposes, each locus with haplotype-dependent epigenetic asymmetry can be thought of as inherited with the DNA sequence as a Mendelian trait.

Recent data have indicated that there can also be *trans*-acting effects of chromosomal aneuploidies on epigenetic patterns in human tissues. In particular, the chromosomal aneuploidy that causes Down syndrome (trisomy 21) has been shown to produce gene-specific and highly recurrent changes in DNA methylation in blood leukocytes including T-lymphocytes (311). Additional studies are in progress to test for this phenomenon in brain cells with trisomy 21, and in other situations such as cancer cells with recurrent simple chromosomal aneuploidies.

### 6.13 THE FUTURE: EPIGENOMICS

As we have highlighted throughout this chapter, the role of epigenetic marks in translating the primary genomic sequence has now moved to the forefront of human genetics, with clear implications for our understanding of human development and disease. A number of initiatives have now been implemented to define human epigenetic patterns at high resolution with complete genomic coverage. The technology is now available to investigate multiple tissue-specific epigenomes in humans, and the NIH Roadmap Epigenomics Mapping Consortium ([www.roadmapepigenomics.org](http://www.roadmapepigenomics.org)) was launched to produce a public resource of human epigenomic data to catalyze basic biology and disease-oriented research (223). Another parallel initiative is the NIH Epigenomics of Health and Disease Roadmap Program, which funds investigator-initiated research. While a good part of what we know so far about epigenetics in disease has come from cancer research, it is telling that most of the initial research grants from this program have been targeted to other complex diseases ranging from Alzheimer disease to adult heart disease and diabetes to autism. These initiatives interface with the International Human Epigenomics Consortium, which was established to accelerate and coordinate epigenomics research worldwide (312). These data should provide keys to unravel genetic and environmental factors that impinge on epigenomes to affect normal processes such as development and aging and lead to human diseases when these processes go awry.

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Otago Imprinted Genes: <http://igc.otago.ac.nz>.  
 UCSC Genome Browser: <http://genome.ucsc.edu/>.

### Biographies



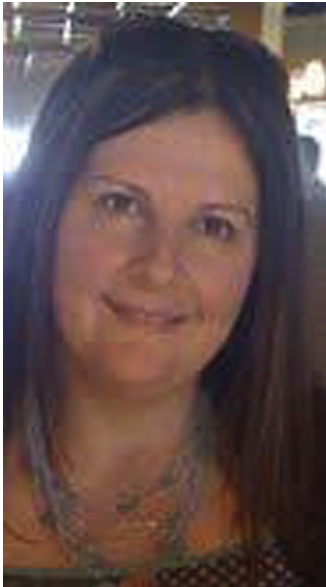
**Rosanna Weksberg, MD, PhD**, is a Professor of Pediatrics and Medical Genetics at the Hospital for Sick Children and the University of Toronto. She has worked on human imprinting disorders and growth-related conditions since 1995. Her research has, more recently, included elucidation of the epigenetic basis and environmental contributors to neurodevelopmental disorders such as early onset psychosis and autism, as well as the identification of new imprinted genes. An important complementary research focus of the laboratory involves the characterization of the effects of both genetic variation and environmental exposures on epigenotype.



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**Dr. Benjamin Tycko, MD, PhD**, is a Professor of Pathology and Cell Biology in the Institute for Cancer Genetics and Taub Institute on Alzheimer's disease and the Aging Brain at Columbia University. Dr. Tycko's lab has had a continuous research program on the role of epigenetics and DNA methylation in normal mammalian development and in diverse human disease processes and developmental disorders, including cancer, Down syndrome, Alzheimer's disease, and most recently autoimmune diseases. Key areas of current emphasis include (i) mechanistic studies on genetic-epigenetic interactions that occur in cis and in trans in human tissues and (ii) mouse models to elucidate the function of differentially methylated genes.

# CHAPTER 7

## Human Gene Mutation in Inherited Disease: Molecular Mechanisms and Clinical Consequences

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### 7.1 INTRODUCTION

A wide variety of different types of pathogenic mutations have been found to cause human inherited disease, with many diverse mechanisms being responsible for their generation. These types of mutations include single base-pair substitutions in coding, regulatory, and splicing-relevant regions of human genes, as well as micro-deletions, micro-insertions, duplications, repeat expansions, combined micro-insertions/-deletions (“indels”), inversions, gross deletions and insertions, and complex rearrangements. A major goal of molecular genetic medicine is to be able to predict the nature of the clinical phenotype through ascertainment of the genotype. However, the extent to which this is feasible in medical genetics is very much disease-, gene-, and mutation-dependent. The study of mutations in human genes is nevertheless of paramount importance for understanding the pathophysiology of inherited disorders, for optimizing diagnostic testing as well as in guiding the design of new therapeutic approaches.

The first description of the precise molecular defect in a human disease (the sickle cell mutation, a Glu to Val substitution at the 6th codon of the  $\beta$ -globin (*HBB*) gene) was identified by Ingram in 1956 (1) who found that the difference between hemoglobin A and hemoglobin S lays in a single tryptic peptide. His analysis was made possible by the methods developed by Sanger for determining the structure of insulin and Edman to effect the stepwise degradation of peptides. This was followed, just over 30 years ago, by the characterization of the first heritable human gene mutations at the DNA level: gross deletions

of the human  $\alpha$ -globin (*HBA*) and  $\beta$ -globin (*HBB*) gene clusters giving rise to  $\alpha$ - and  $\beta$ -thalassaemia (2) and a single base-pair substitution (Lys17Term) in the human  $\beta$ -globin (*HBB*) gene causing  $\beta$ -thalassaemia (3). Since then, continuous technical advances have potentiated the identification of numerous disease-related genes and the discovery of thousands of underlying pathological lesions (4). Single base-pair substitutions (67%) and micro-deletions (15.6%) are the most frequently encountered mutations in the human genome, the remainder comprising an assortment of micro-insertions (6.5%), indels (1.5%), gross deletions (6.6%), gross insertions and duplications (1.4%), inversions, repeat expansions (0.3%), and complex rearrangements (1.0%).

The vast majority of mutations listed in Human Gene Mutation Database (HGMD) reside within the coding region (86%), the remainder being located in either intronic (11%) or regulatory (3%, promoter, untranslated, or flanking regions) sequences. Mutations may interfere with any stage in the pathway of expression from gene activation to synthesis and secretion of the mature protein product. The question of the proportion of possible mutations within human disease genes that are likely to be of pathological significance, is one that is difficult to address because it is dependent not only on the type and location of the mutation but also on the functionality of the nucleotides involved (itself dependent in part upon the amino acid residues that they encode), which is often hard to assess (5–10). In addition, some types of mutations are likely to be much more comprehensively ascertained than others, making observational



comparisons between mutation types an inherently hazardous undertaking.

Different types of human gene mutations may vary in size, from structural variants (SVs) to single base-pair substitutions, but what they all have in common is that their nature, size, and location are often determined either by specific characteristics of the local DNA sequence environment or by higher order features of the genomic architecture (11). This chapter attempts to provide an overview of the nature of mutations causing human genetic disease and then considers their consequences for the clinical phenotype. Two online databases, which interested readers may consult, contain information on known disease-related (pathogenic) mutations: the HGMD (<http://www.hgmd.org>) and *Mendelian Inheritance in Man* (<http://www.ncbi.nlm.nih.gov/Omim/> and <http://omim.org/>).

## 7.2 MOLECULAR MECHANISMS OF MUTATION CAUSING HUMAN INHERITED DISEASE

### 7.2.1 “Neutral Variation”/DNA Polymorphisms

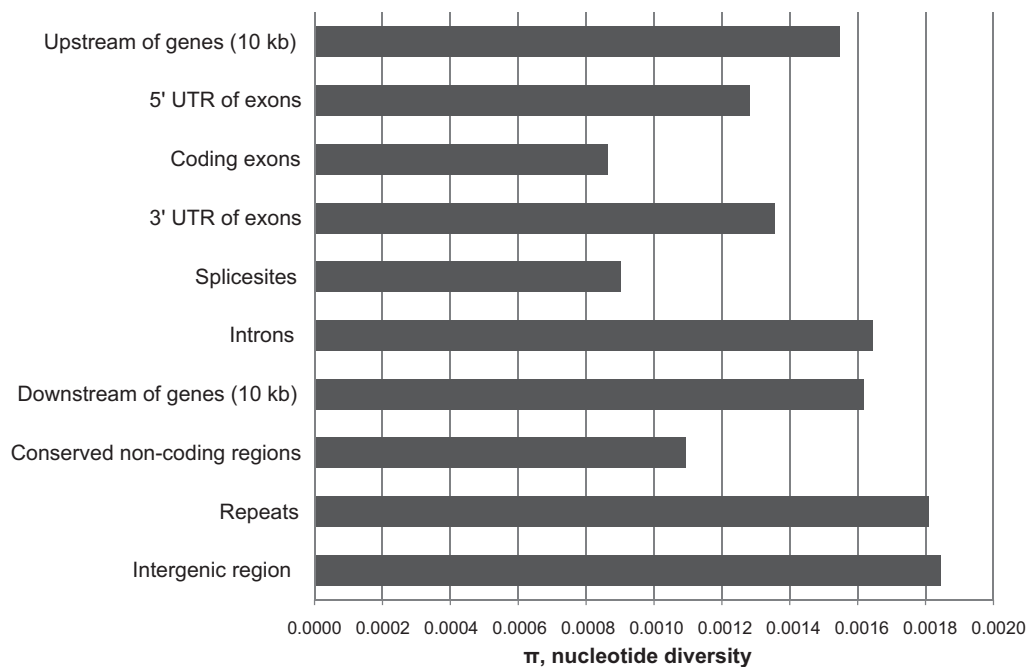
The term *polymorphism* has been defined (12) as a “Mendelian trait that exists in the population in at least two phenotypes, neither of which occurs at a frequency of less than 1%” Polymorphisms are not therefore rare. Indeed, there is enormous variation in the DNA sequences of any two randomly chosen human haploid genomes. Clearly, not all variations within a gene result in the abnormal expression of protein products. Indeed, single nucleotide substitutions/polymorphisms (SNPs) occur in 1/~600–1200 nucleotides in intervening sequences and flanking DNA (13–18). These substitutions represent the most common form of DNA polymorphism that can be used as markers for specific regions of the human genome. Similarly, some single nucleotide substitutions in the coding regions of genes may also be normal (nonpathogenic) polymorphic variants even if they result in nonsynonymous substitutions of the polypeptide product (19). For example, there are three common forms of the  $\beta$ -globin (*HBB*) gene on chromosome 11p; these forms differ at five nucleotides, one of which lies within the first exon of the gene and results in a synonymous codon. The average human gene contains >120 biallelic polymorphisms, 46 of which occur with a frequency >5%, with five occurring within the coding region (20).

Some polymorphisms entail the alteration of an encoded amino acid, for example, the Lewis *Le* alleles of the *FUT3* gene (21), whereas others may introduce a stop codon that serves to inactivate the gene in question, for example the secretor *se* allele of the *FUT2* gene present in 20% of the population (22). However, not all polymorphisms are SNPs. Examples of other types of gene-associated polymorphisms in the human genome include triplet

repeat copy number (e.g. in the *FMR1* gene; see 9.2.1.3), gross gene deletion (e.g. *GSTM1* and *GSTT1*; (23)), gene duplication (e.g. *HBG2*; (24)), intragenic duplication (e.g. *IVL*; (25)), micro-insertion/deletion (e.g. *PAIL1*; (26)), indel (e.g. *APOE*; (27)), gross insertion (e.g. the inserted *Alu* sequence in intron 16 of the *ACE* gene; (28)), inversion (e.g., the 48 kb Xq28 inversion involving the *EMD* and *FLN1* genes; (29)), and gene fusion (e.g. between the *RCP* and *GCP* visual pigment genes; (30)). Functional polymorphisms may occur within the coding region (31) or regulatory regions (32) of a gene or may impact on pre-messenger RNA (mRNA) splicing (33) and can therefore have consequences for protein structure/function, gene expression, or mRNA splicing. It can be seen that the mutational spectrum of polymorphisms in the human genome is qualitatively different to that underlying human disease; they may vary in terms of location and frequency but otherwise they display remarkable similarities indicative of the same underlying mutational mechanisms.

It is likely that some SNPs, whether frequent or rare, alter the risk of common complex human phenotypes (“functional SNPs”). A public SNP database now contains more than 10 million entries (dbSNP; <http://www.ncbi.nlm.nih.gov/SNP/index.html>). An international project has recently been completed, termed the “HapMap project” (34–36), the goal of which was to define the patterns of common SNP genetic variation in a sample of 270 DNAs from individuals of European, African, Chinese, and Japanese origin (<http://www.hapmap.org/>). The data obtained from this project constitute ~2.8 million SNPs and are publicly available. The results of this project are likely to contribute significantly to our understanding of both common and rare human genetic disorders and traits. Furthermore, recent advances in high-throughput sequencing have led to the discovery of a large number of individually rare polymorphic variants in samples from the 1000 genomes (37) and other projects. The recent version of the dbSNP database (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) contains more than 12.5 million variants. The protein-coding regions of a typical human genome (also known as the exome) contain 9000–11,000 nonsynonymous variants, ~100 nonsense codons, and 35 splice variants (38). Analysis of 54 human genomes, recently sequenced by Complete Genomics (<http://www.completegenomics.com>), revealed 3,700,000–4,700,000 single nucleotide variants per genome; the frequency of these variants was not identical in various fractions of the genome and is related to regional evolutionary constraints. Figure 7-1 illustrates that the protein-coding fraction of the genome, which is under evolutionary pressure, contains the smallest number of variants per kilobase (kb); by contrast, the repeat fraction of the genome and the intergenic regions, which presumably evolve neutrally, contain almost double the number of variants per kilobase.

### Nucleotide diversity in different genomic regions



- Genomic variants from CompleteGenomics, 54 unrelated individuals from different ethnic groups ([www.completegenomics.com](http://www.completegenomics.com))
- Genomic regions drawn from UCSC genome browser (<http://genome.ucsc.edu>)

**FIGURE 7-1** Nucleotide diversity (equivalent to frequency of polymorphic variants) in different genomic regions. The genomic variants analyzed are from the whole genome sequences of 54 unrelated human genomes (see text).

Another form of polymorphic variation in our genome is the presence of variable numbers of tandem repeats. The repeat unit can be 10–60 nucleotides in length and many different alleles may exist at a given locus (39,40). The combination of a VNTR and single nucleotide substitutions within the repeat unit results in an extremely high level of polymorphic variability, which can be used as a unique bar code to distinguish different individuals (41). The introduction of the polymerase chain reaction (PCR) (42) permitted the rapid detection and analysis of variation in short sequence repeats (SSRs), for example (GT)<sub>n</sub> repeats (43,44). These are common polymorphisms that occur on average once for every 50kb of genomic DNA. The SSRs also display many alleles and the repeat unit can be two, three, four, five, or more nucleotides. Poly(A) tracts may also be polymorphic, exhibiting variation in the number of A residues (45); many of these polymorphisms are localized at the ends of *Alu* repetitive elements. Another kind of polymorphism in the human genome involves the presence or absence of retrotransposons (i.e. *Alu* or LINE repetitive elements or pseudogenes) at specific locations (46,47). Duplicational polymorphisms in some human genes, for example *HBA1*, *PRB1-4*, *HBZ*, *CYP21/C4A/C4B* have been known for some time (47,48). More recently, however, the use of comparative genomic hybridization against BAC or oligonucleotide arrays has revealed extensive copy number polymorphism/variation (CNP

or CNV) of sizeable genomic regions (49–51). Details of many thousands of such genomic variants may be found in the following databases: *CNV Project*, <http://www.sanger.ac.uk/humgen/cnv>, and *Database of Genomic Variants*, <http://projects.tcag.ca/variation>. A first CNV map of the human genome of the 270 “HapMap” individuals revealed a total of 1440 CNV regions covering ~360 megabases (Mbs) (12% of the genome) (52). More recently, high-resolution tiling oligonucleotide microarrays have been used to generate comprehensive genomic maps of >10,000 CNVs (53,54). The functional significance, if any, of most of these polymorphic variants is, however, unknown. What is clear, however, is that no single individual genome contains the full complement of functional genes (55), a paradigm shift that strikes at the heart of the concept of a “reference genome” (56).

Deletional polymorphisms are also remarkably frequent in the human genome: a typical individual has been estimated to be hemizygous for some 30–50 deletions >5 kb, spanning >550 kb in total, and encompassing >250 known or predicted genes (57,58). Since such deletions appear to be in linkage disequilibrium with neighboring SNPs, we may surmise that they share a common evolutionary history (59).

Human DNA polymorphisms have proven extremely useful in developing linkage maps, for mapping monogenic and polygenic complex disorders, for determining the origin of aneuploidies and chromosomal

abnormalities, for distinguishing normal from mutant chromosomes in genetic diagnoses, for performing forensic, paternity, and transplantation studies, for studying the evolution of the genome, the loss of heterozygosity in certain malignancies, the detection of uniparental disomy, the instability of the genome in certain tumors, recombination at the level of the genome, the study of allelic expression imbalance, and the development of haplotype maps of the genome. However, in studying the role of a candidate gene in a given disorder, it is imperative to distinguish between pathogenic mutations that cause a clinical phenotype and the polymorphic variability of the normal genome.

### 7.2.2 Nonsense SNPs

The loss of a particular gene/allele is not invariably associated with a readily discernible clinical phenotype (60,61). This assertion is supported by the identification of more than 1000 putative nonsense SNPs (i.e. nonsense mutations that have attained polymorphic frequencies) in human populations (62,63). About half of these nonsense SNPs have been validated by dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP>), a process that involves the exclusion of mutations in pseudogenes and of artifacts caused by sequencing errors. *Bona fide* nonsense SNPs are expected either to lead to the synthesis of a truncated protein product or alternatively to the greatly reduced synthesis of the truncated protein product (if the mRNA bearing them is subject to nonsense-mediated mRNA decay (NMD)). Based on the relative locations of the nonsense SNPs and the exon–intron structures of the affected genes, Yamaguchi-Kabata et al. (2008) (64) concluded that 49% of nonsense SNPs would be predicted to elicit NMD, whereas 51% would be predicted to yield truncated proteins. Some of these nonsense SNPs have been found to occur in the homozygous state in normal populations (62), attesting to the likely functional redundancy of the corresponding genes. At the very least, genes harboring nonsense SNPs may be assumed to be only under weak selection (63).

It should be appreciated that nonsense SNPs may even occur in “essential” genes, yet still fail to come to clinical attention (or give rise to a detectable phenotype) if these genes are subject to CNV (see CNVs and copy number mutations below) that masks any deleterious consequences by ensuring an adequate level of gene expression from additional wild-type copies either in *cis* or in *trans*. Thus, CNV might serve to “rescue” the full or partial loss of gene function brought about by the nonsense mutations, thereby accounting for the occurrence of the latter at polymorphic frequencies. Consistent with this postulate, Ng et al. (2008) (63) reported that ~30% of nonsense SNPs occur in genes residing within segmental duplications, a proportion some three-fold larger than that noted for synonymous SNPs. Genes harboring nonsense SNPs were also found to belong to

gene families of higher than average size (63), suggesting that some functional redundancy may exist between paralogous human genes. In support of this idea, Hsiao and Vitkup (2008) (65) reported that those human genes that have a homolog with  $\geq 90\%$  sequence similarity are approximately three times less likely (66) to harbor disease-causing mutations than genes with less closely related homologs. They interpreted their findings in terms of “genetic robustness” against null mutations, with the duplicated sequences providing “back-up” by potentiating the functional compensation/ complementation of homologous genes in the event that they acquire deleterious mutations.

## 7.3 DISEASE-CAUSING MUTATIONS

### 7.3.1 The Nature of Mutation

Figure 7-2 depicts the frequencies of the various mutation types responsible for molecularly characterized human genetic disorders, as recorded in HGMD (<http://www.hgmd.org>) and Refs. (67,68). HGMD records each mutation *once* regardless of the number of independent occurrences of that lesion. Figure 7-2 shows the frequency of the first mutation per disease recorded in MIM (<http://www.ncbi.nlm.nih.gov/Omim> and <http://omim.org/>) and Ref. (69). As of December 2011, HGMD contained some 120,000 different disease-causing mutations and disease-associated/functional polymorphisms in 4411 human genes (Figure 7-2), whereas MIM contained selected examples of allelic variants in 3329 human genes associated with a specific phenotype.

### 7.3.2 Nucleotide Substitutions

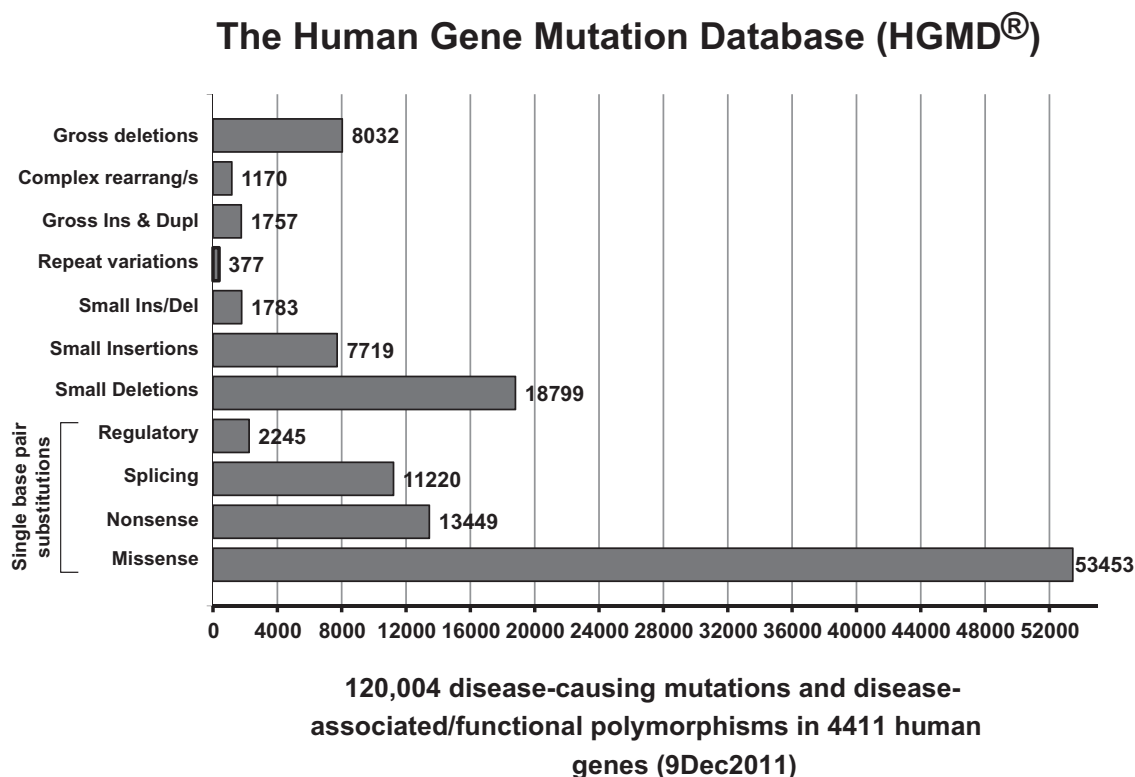
Single nucleotide substitutions are the most frequent pathological mutations in the human genome (Figure 7-2). Most of these alterations occur during DNA replication, which is an accurate yet error-prone multistep process. The accuracy of DNA replication depends on the fidelity of the replicative step and the efficiency of the subsequent error correction mechanisms (70). Analysis of more than 7000 missense and nonsense mutations associated with human disease has indicated that the most common nucleotide substitution for T (thymine) is to C (cytosine), for C it is to T, for A (adenine) it is to G (guanine), and for G it is to A (71). Transitions are, therefore, much more common than transversions. Some 61% of the missense and nonsense mutations currently logged in HGMD are transitions (T to C, C to T, A to G, G to A) while 39% are transversions (T to A or G, A to T or C, G to C or T, C to G or A).

Among single nucleotide substitutions, there is one that clearly predominates and represents the most common type of mutational lesion in the human genome: CpG dinucleotides mutate to TpG at a frequency that is about five times higher than mutations in all other

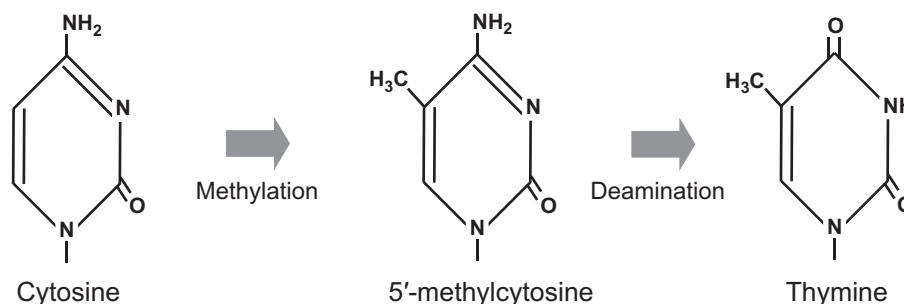
dinucleotides (71–74). This substitution, which when it occurs on one DNA strand generates TG, and on the other, CA (the “CG to TG or CA rule”) represents a major cause of human genetic disease. This phenomenon was first observed in the factor VIII (*F8*) gene in cases of hemophilia A (73), but it was soon noted in the studies of many other genes (75). In hemophilia A, CG to TG or CA mutations account for 46% of point mutations in unrelated patients (76). In the HGMD (68) (<http://www.hgmd.org>), such mutations currently account for ~18% of the total number of missense and nonsense mutations (66). Among CpG dinucleotide mutations, transitions to TG or CA account for ~90% of substitutions. The mechanism of this common type of mutation appears to be methylation-mediated deamination of 5-methylcytosine

(5mC). In eukaryotic genomes, 5mC occurs predominantly in CpG dinucleotides, most of which appear to be methylated (see (77) for review). 5mC then undergoes spontaneous nonenzymatic deamination to form thymine (Figure 7-3). There is a bias in terms of the origin of CpG to TpG mutations: most occur in male germ cells (the male:female ratio is 7:1). One reason for this may be that sperm DNA is heavily methylated, whereas oocyte DNA is comparatively undermethylated (78). Another reason may be the considerably higher number of germline cell divisions in males as compared to females (79).

Cytosine methylation also occurs in the context of CpNpG sites (80). If we assume not only that CpNpG methylation occurs in the germline but also that 5mC deamination can occur within a CpNpG context, then it



**FIGURE 7-2** Spectrum of different types of human disease-causing mutations and disease-associated/functional polymorphisms logged in the HGMD as of December 2011.



**FIGURE 7-3** Schematic representation of cytosine, 5'-methylcytosine and thymine, and the chemical events involved in the mutational transformation of cytosine to thymine.



follows that methylated CpHpG sites are also very likely to constitute mutation hotspots causing human inherited disease. Initial evidence that this might indeed be the case came from the observation that disproportionately high numbers of C>T and G>A transitions occur at CpNpG sites in studies of the human genes, *NF1* (81) and *BRCA1* (82). Further, ~9.9% of 54,625 missense and nonsense mutations from 2113 genes causing inherited disease (HGMD) are C>T and G>A transitions located within CpNpG trinucleotides, approximately twofold higher proportion than would have been expected by chance alone (66). Some 5% of missense or nonsense mutations causing human inherited disease may, therefore, be attributable to methylation-mediated deamination of 5 mC within a CpNpG context.

In a recent analysis, the average direct estimate of the combined rate of all mutations was  $1.8 \times 10^{-8}$  per nucleotide per generation (83). Single nucleotide substitutions were found to be ~25 times more common than all other mutations, while deletions were ~3 times more common than insertions; complex mutations were very rare and the CpG context was found to increase substitution rates by an order of magnitude (84). Rates of different kinds of mutations were also found to be strongly correlated across different loci (84).

It has been estimated that ~20% of new missense mutations in humans result in a loss of function, whereas 53% have mildly deleterious effects, and 27% are effectively neutral with respect to phenotype (85). These estimates have received independent support, at least qualitatively, from a study of human coding SNPs by Boyko et al. (86), who predicted that 27–29% of missense mutations would be neutral or near neutral, 30–42% would be moderately deleterious, with most of the rest (i.e. 29–43%) being highly deleterious or lethal, and by Eyre-Walker et al. (87) who estimated that >50% of mutations would be likely to exert only a mild effect on the phenotype.

A recent study using human osteosarcoma cell lines has shown that noncanonical (non-B) DNA conformations are capable of increasing the overall spectrum of single base-pair substitutions in a reporter gene in *cis* by exposing those DNA sequences to oxidative damage (88). In this study, the spectrum of single base-pair substitutions was shown to be indistinguishable from that induced by other conditions known to lead to an hyperoxidative state (such as WRN deficiency and lung tumorigenesis), an observation which lends support to a model whereby DNA bases become oxidized, followed by the transfer of their oxidized state (“hole migration”) to target neighboring bases. If these observations are eventually found to be relevant in the context of “natural” chromatin during meiosis, then the impact of non-B DNA conformations on human inherited disease, both with respect to single base-pair substitutions and gross rearrangements (see Section 7.3.6), could be quite significant. Further, since non-B DNA structures can

interfere with DNA replication and repair, and may serve to increase mutation frequencies in generalized fashion, they have the potential to serve as a unifying concept in studies of mutational mechanisms underlying human inherited disease.

### 7.3.3 Synonymous Nucleotide Substitutions

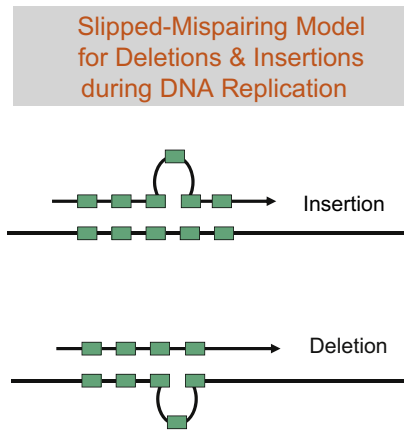
Synonymous (“silent”) mutations, although not altering the amino acid sequence of the encoded protein directly, can still influence splicing accuracy or efficiency (89–94). It has become increasingly clear that apparently silent SNPs may also become distinctly “audible” in the context of mRNA stability or even protein structure and function. Thus, three common haplotypes of the human *COMT* gene, which differ in terms of two synonymous and one nonsynonymous substitution, confer differences in *COMT* enzymatic activity and pain sensitivity (95,96). The major *COMT* haplotypes differed with respect to the stability of the *COMT* mRNA local stem-loop structures, the most stable being associated with the lowest levels of *COMT* protein and enzymatic activity (95). In similar vein, synonymous SNPs in the *ABCB1* gene have been shown to alter *ABCB1* protein structure and activity (97), possibly by changing the timing of protein folding following extended ribosomal pause times at rare codons (98). Finally, it should be understood that although the deleteriousness of the average synonymous mutation is always likely to be less than that of a nonsynonymous (missense) mutation (86), the higher prevalence of synonymous mutations means that they may actually make a significantly greater contribution to the phenotype than nonsynonymous mutations (99).

### 7.3.4 Micro-Deletions and Micro-Insertions

Deletions or insertions of a few nucleotides are also fairly common as a cause of human inherited disease. Most of these are less than 20 base pairs (bp) in length. Indeed, the majority of micro-deletions involve <5 nucleotides. In HGMD, the deletion of 1 bp accounts for 48% of small deletions while an additional 30% involve two or three nucleotides. The majority of micro-deletions recorded (78%) result in an alteration of the reading frame. Most micro-deletions occur in regions that contain direct repeats of 2 bp or more. The most common length of direct repeat is 3 bp (48% of direct repeats associated with short deletions (74)). The most plausible mechanism for small deletions mediated by the presence of direct repeats is the slipped mispairing model (100) (Figure 7-4). In addition, deletions of one or a few nucleotides frequently occur in runs of the same nucleotide, for example, a poly(T) region (101). Finally, inverted repeats and “symmetric elements” are also frequently found in the immediate vicinity of micro-deletions (102,103). Krawczak and Cooper (104) identified a

consensus sequence—TG(A/G)(A/G)(G/T)(A/C)—which they claimed to represent a deletion hotspot.

Micro-insertions (again up to 20 nucleotides) are rarer than micro-deletions; thus, in HGMD there are three times as many micro-deletions as micro-insertions

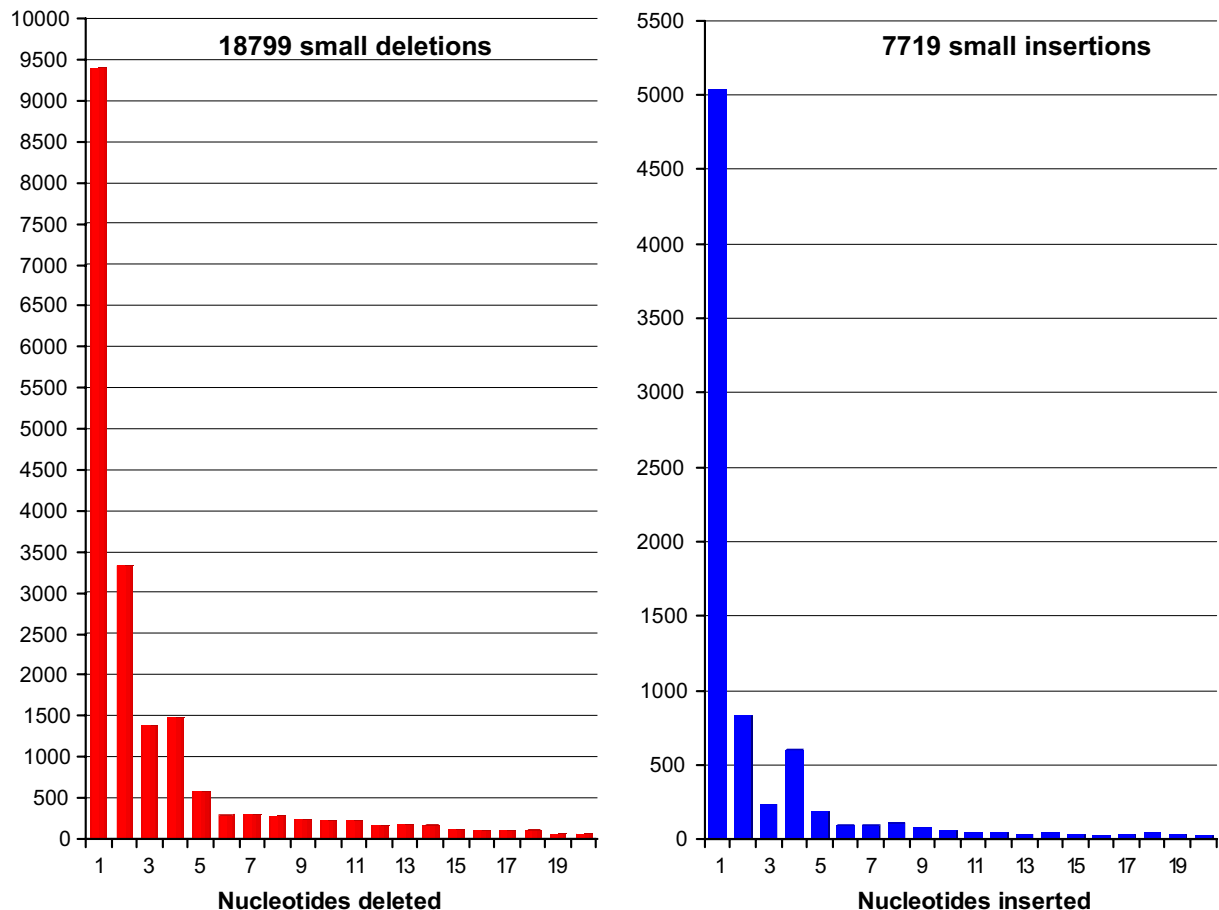


**FIGURE 7-4** Schematic representation of the slipped mispairing model for deletions and insertions during DNA replication.

(Figure 7-2). Nearly half of these involve the insertion of only one nucleotide (Figure 7-5). As is the case with micro-deletions, most micro-insertions lead to alterations of the reading frame and are located in regions containing direct or inverted repeats or runs of the same nucleotide. Details of possible mechanisms of generation during replication can be found in (105). However, there are as-yet insufficient data available to estimate the frequency ratio of micro-insertions or micro-deletions in male or female germ cells. In the case of such lesions in factor VIII (*F8*) gene, 56% of micro-deletions/-insertions have been reported to occur in DNA regions harboring direct repeats or runs of the same nucleotide (76).

HGMD data (3767 micro-deletions and 1960 micro-insertions) were used to perform a meta-analysis of micro-deletions and micro-insertions causing inherited disease, both defined as involving  $\leq 20$ bp DNA (106). A positive correlation was noted between the micro-deletion and micro-insertion frequencies for 564 genes in which both micro-deletions and micro-insertions have been reported. This is consistent with the view

### HGMD Small Deletions and Insertions (1-Dec-11)



**FIGURE 7-5** Size distribution of short (<20 bp) pathogenic human gene deletions and insertions (HGMD; <http://www.hgmd.org>; 5 January 2007).

that the propensity of a given gene/sequence to undergo micro-deletion is related to its propensity to undergo micro-insertion. While micro-deletions and micro-insertions of 1 bp constitute, respectively, 48% and 66% of the corresponding totals, the relative frequency of the remaining lesions correlates negatively with the length of the DNA sequence deleted or inserted. Many micro-deletions and micro-insertions of >1 bp are potentially explicable in terms of slippage mutagenesis, involving the addition or removal of one copy of a mono-, di-, or trinucleotide tandem repeat. The frequency of in-frame 3 bp and 6 bp micro-insertions and micro-deletions was, however, found to be significantly lower than that of mutations of other lengths, suggesting that some of these in-frame lesions may not have come to clinical attention. Various sequence motifs were found to be overrepresented in the vicinity of both micro-insertions and micro-deletions, including the heptanucleotide CCCCCTG that shares homology with the complement of the 8-bp human minisatellite conserved sequence/chi-like element (GCWGGWGG). The “indel hotspot” GTAAGT (and its complement ACTTAC) were also found to be overrepresented in the vicinity of both micro-insertions and micro-deletions, thereby providing a first example of a mutational hotspot that is common to different types of gene lesions. Other motifs overrepresented in the vicinity of micro-deletions and micro-insertions included DNA polymerase pause sites and topoisomerase cleavage sites. Several novel micro-deletion/micro-insertion hotspots were noted and some of these exhibited sufficient similarity to one another to justify terming them “super-hotspot” motifs. Analysis of DNA sequence complexity also demonstrated that a combination of slipped mispairing mediated by direct repeats, and secondary structure formation promoted by symmetric elements, can account for the majority of micro-deletions and micro-insertions. Thus, micro-insertions and micro-deletions exhibit strong similarities in terms of the characteristics of their flanking DNA sequences, implying that they are generated by very similar underlying mechanisms.

A similar analysis on micro-deletions and micro-insertions in 19 human genes presented evidence for an elevated micro-deletion rate at YYYYTG and an elevated micro-insertion rate at TACCRC and ATMMGCC (107). These authors also found that ~45% of micro-deletions led to the removal of a repeated sequence, an event they termed “deduplication” in order to highlight the identity of the deleted sequence and the sequence abutting the site of deletion.

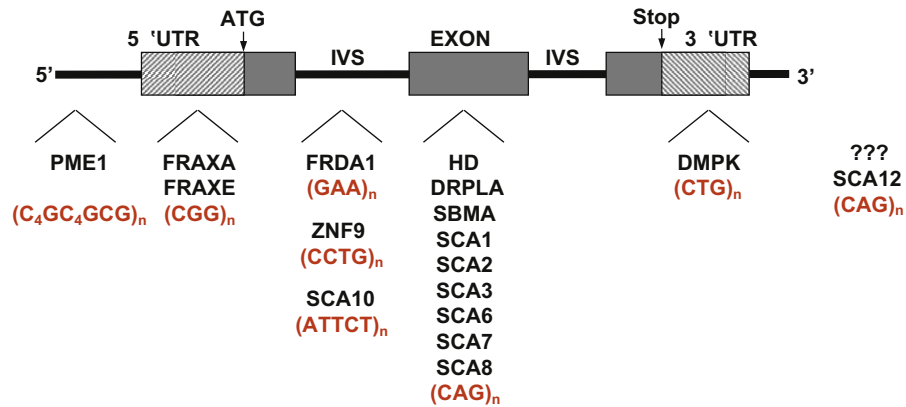
### 7.3.5 Expansion/Copy Number Variation of Trinucleotide (and Other) Repeat Sequences

Another mechanism of human gene mutation causing hereditary disease is the instability of repeat (mainly trinucleotide) sequences and their expansion in affected

genes (108–110). A growing number of repeat expansion disorders (in excess of 66 are now recorded in HGMD), the majority of which involve neuromuscular tissue, have been found to be due to, or associated with, the expansion of repeat sequences; of these, 53 are expansions of triplet repeats. The first such disease was fragile X, a common cause of male mental retardation, which mapped to chromosome Xq27.3. Some examples of these disorders include Huntington disease, myotonic dystrophy, spinobulbar muscular atrophy, spinocerebellar ataxia 1, spinocerebellar ataxia 3 or Machado–Joseph disease, the fragile E site, and dentatorubral pallidolysian atrophy. Genetic “anticipation” (the earlier onset and increasingly severe phenotype in successive generations) is a common phenomenon in these disorders (111). The trinucleotide involved is usually either CAG or CGG but occasionally CTG, GCG, or GAA. It can be located in the 5′ untranslated region (UTR) as in the case of the *FMR1* gene underlying fragile X, within the coding region (as in Huntington disease, spinocerebellar ataxia 1 (SCA1), SCA3, and Kennedy disease) where it encodes poly(Gln), in an intron as in Friedreich ataxia (*FXN*) and myotonic dystrophy type 2 (*ZNF9*), or in the 3′ UTR as in myotonic dystrophy type 1 (*DMPK*) (Figure 7-6). The expansion of the triplet repeat either prevents the expression of the associated gene (112), results in a dominant gain-of-function mutation mediated by the longer poly(Gln) peptide (113), or alters the RNA processing of other genes (114,115).

Trinucleotide repeats are usually polymorphic in human populations. Rarely, however, the number of trinucleotide repeats lies within a high risk category that is termed a “premutation.” In such a case, the premutation exhibits a high probability of further expansion (instability) to yield disease-related alleles (“full mutation”). In fragile X, for example, the normal polymorphic alleles of the CGG repeat contain between 10 and 50 triplets, the premutation between 50 and 200 triplets, and the full mutation more than 200 triplets (116). Expansion of premutations to full mutations only occurs during female meiotic transmission. The probability of repeat expansion correlates with repeat copy number in the premutated allele. Since the premutation must precede the appearance of a full mutation, all mothers of affected children carry either a full mutation or a premutation (116). Premutation alleles may also be associated with late-onset movement disorders and premature ovarian failure (117,118).

The precise mechanism of repeat expansion is unclear although it is known that DNA polymerase progression is blocked by CTG and CGG repeats and the resultant idling of the polymerase could serve to catalyse slippage leading to repeat expansion (119). In the case of SCA1, interruption of the CAG repeat with a CAT unit is associated with more stable trinucleotide repeat (120). More details of these “dynamic mutations” can be found in the appropriate sections covering individual disorders, and in Ref. (121). Short expansions of GCG trinucleotide codons encoding Ala have been observed in



### Selected Repeat Expansions in Human Disorders

**FIGURE 7-6** Location of the repeat expansion in selected human disorders.

the *HOXD13* gene causing dominant polydactyly, and in the *PABP2* gene causing oculopharyngeal muscular dystrophy (122,123). These mutations may be due to unequal crossing-over rather than polymerase slippage. Generally speaking, it is likely that repeat instability is a consequence of the resolution of unusual secondary structure intermediates during DNA replication, repair, and recombination (124).

A repeat expansion of 12 nucleotides (CCCCGCCCGCG) in the 5' flanking region of the *CSTB* gene causes one form of the recessive progressive myoclonus epilepsy type 1 (EPM1) (125). This indicates that repeat sequences other than trinucleotides can expand and cause human disorders. This particular expansion silences the *CSTB* gene, probably because it alters the spacing of transcription factor binding sites from each other and/or the transcriptional initiation site (126).

A tetranucleotide repeat expansion (CCTG)<sub>n</sub> in intron 1 of the *ZNF9* gene causes myotonic dystrophy type 2 (115). This expansion can be between 75 and 11,000 repeats in length. The expansion of the pentanucleotide repeat (ATTCT)<sub>n</sub> is responsible for the phenotype of spinocerebellar ataxia 10 (SCA10). The expansion occurs in intron 9 of the *SCA10* gene and can be up to 22.5 kb in length (127). Expansions of even longer repeats have been reported. In Usher syndrome type 1C, for example, there is an expansion of a 45 bp VNTR in intron 5 of the *USH1C* gene (nine tandem repeats instead of the usual less than six such repeats); this expansion has been predicted to inhibit the transcription of the gene (128). There are also cases in which a large repeat expansion is not associated with a particular phenotype, for example, the expansion of an AT-rich 33-mer repeat in the dictamycin-sensitive fragile site 16B (129).

### 7.3.6 Mechanisms of Gross Genomic Rearrangement

Structural variation in the human genome is characterized by a number of different types of gross rearrangements

including deletions, duplications, insertions (termed CNVs) as well as inversions, and translocations. Four major mutational mechanisms account for these SVs: nonallelic homologous recombination (NAHR), nonhomologous end joining (NHEJ), replication-based mechanisms, and L1-retrotransposition (53,54,130).

**NAHR:** Sequence analysis of the breakpoints of 1054 SVs identified in the genomes of 17 healthy human individuals revealed that NAHR accounts for 22% of insertions and deletions as well as 69% of inversions (130). The majority of these SVs are likely to represent neutral polymorphisms but ~1% may be disease associated. However, some apparently neutral SVs appear to predispose to further structural rearrangements, such as deletions and duplications, which then in turn give rise to disease (131–135). Thus, for example, heterozygosity for the ~970 kb inversion polymorphism of the *MAPT* locus at 17q21.3 predisposes to the NAHR events that underlie the 17q21.31 micro-deletion syndrome (136,137). It may be that inversion heterozygosity perturbs the pairing of homologous chromosomes during meiosis, which then promotes interchromosomal NAHR between the inversion-flanking low copy repeats (LCRs), thereby giving rise to the 17q21.3 micro-deletion.

During meiosis, NAHR between sequences that are nonallelic (i.e. paralogous) can result in recurrent deletions and duplications that cause specific genomic disorders. Liu et al. (138) studied two patient cohorts with reciprocal genomic disorders localized to chromosome 17p11.2: the deletion-associated Smith–Magenis syndrome and the duplication-associated Potocki–Lupski syndrome. They reported that complex rearrangements (those with more than one breakpoint) were more prevalent in copy number gains (17.7%) than in copy number losses (2.3%), an observation which supports a role for replicative mechanisms in the formation of complex rearrangements. With respect to the NAHR-mediated recurrent rearrangements, the crossover frequency was found to be positively associated with the flanking LCR length and inversely influenced by the inter-LCR distance. It



would, therefore, appear that the probability of ectopic chromosome synapsis increases with increasing LCR length, with ectopic synapsis being a prerequisite for ectopic crossing-over.

Recent findings also indicate that NAHR represents a major mechanism underlying unbalanced recurrent translocations, which are mediated by either interchromosomal LCRs or segmental duplications located on nonhomologous chromosomes (139).

**NHEJ:** The defining characteristic of NHEJ is the ligation of double-strand break (DSB) ends without the requirement for extensive homology, in stark contrast to the situation pertaining with homologous recombination. The presence of terminal microhomologies (typically 1–3 bp) facilitates NHEJ but this appears not to be an absolute requirement; only 30–50% of all SVs in the human genome have originated through microhomology-mediated NHEJ events (53,140).

Although some NHEJ events would have resulted from the repair of DSBs that originated quasi-randomly, there are also many well-documented cases in which the locations of the NHEJ-initiating DSBs appear to be highly dependent on the local DNA sequence environment. The role of the local DNA sequence context in generating NHEJ-mediated germline mutations is exemplified by the constitutional t (11;22)(q23;q11), the most common type of recurrent non-Robertsonian translocation in humans (141,142). The breakpoint sequences of both chromosomes are characterized by several hundred base pairs of inverted AT-rich repeats; similar sequences have also been identified at the breakpoints of other nonrecurrent translocations (143). It would appear that the NHEJ of two ends from different DSBs requires those ends to be physically located in the immediate vicinity. Indeed, DSBs tend to undergo translocations with those chromosomes with which they share nuclear space (144). This provides strong support for the “contact-first” hypothesis, which proposes that interactions between different DSBs can only take place if they are colocalized at the time of DNA damage (145). Consistent with this hypothesis, close spatial proximity has been observed between several frequent translocation partners (146,147).

A number of recombination-predisposing motifs and non-B DNA-forming sequences have been found to be overrepresented at NHEJ breakpoints, indicative of the sequence-directed nature of many NHEJ-mediated rearrangements (148,149). It has also been observed that at least one of the breakpoints of NHEJ-mediated rearrangements is often located within repetitive elements (such as LTRs, LINE or *Alu* elements) and sequence motifs capable of causing DSBs have been frequently identified in the vicinity of the breakpoints of these NHEJ-mediated rearrangements (150). Importantly, the breakpoints of many nonrecurrent CNVs mediated by NHEJ map to LCRs, suggesting that LCRs can promote genomic instability by inducing certain chromatin secondary structures.

**Replication-based mechanisms:** Replication slippage or template switching during replication account for both small and large deletions and duplications with terminal microhomologies. Recently, relevant replication-based models including serial replication slippage (SRS) (151–153), fork stalling and template switching (FoSTes) (154), and microhomology-mediated break-induced replication (MMBIR) (155), which were collectively termed microhomology-mediated replication-dependent recombination by Chen et al. (156), have been used to explain the generation of a diverse range of complex genomic rearrangements (154,157).

DNA replication stalling-induced chromosome breakage has also been found to be an important mechanism causing deletions at chromosomal ends. Different types of telomeric deletions have been described (158): type A terminal deletions are formed by chromosomal ends that are stabilized by the capture of a telomere from another source, whereas type B deletions are actually interstitial deletions toward the chromosomal ends. By contrast, type C deletions describe the process by which chromosomal ends are stabilized by telomere healing, namely the telomerase-dependent de novo addition of telomeres at non-telomeric sites. Terminal deletions associated with inverted duplications (159) can be classified as either type A or type C. Recently, Hannes et al. (160) succeeded in cloning the breakpoints of nine chromosome 4p terminal deletions. All nine cases were shown to be type C terminal deletions. Bioinformatics analysis of the breakpoint-flanking regions involved in these 9 cases, together with 12 previously fully characterized type C terminal deletions, led to the realization that there is an enrichment in secondary structure-forming sequences and replication stalling site motifs in these regions as compared with a randomly selected sequence dataset (160).

Certain sequence features, such as microsatellites and transposon-rich regions, can serve to induce replication stalling, thereby acting as potential sources of genome instability (161,162). On this basis, Koszul et al. (163) proposed a two-step mechanism to account for the generation of large segmental duplications: “First, a replication fork pauses and collapses generating a chromosome breakage. Second, the double-strand break can be processed into a new replication fork either intra- or inter-molecularly by a break-induced replication-like mechanism that does not necessarily need a long sequence homology.” It was this “microhomology-dependent BIR” model that was subsequently deployed to explain disease-causing copy number mutations. In MMBIR, replication ends with the engagement of a misaligned template instead of reannealing to its original template; the synthesis of the second strand then follows the synthesis of the first (reviewed in (156)). In practice, mutations due to SRS/FoSTes are often indistinguishable from those due to MMBIR. Indeed, the two terms have sometimes been used interchangeably (164,165).

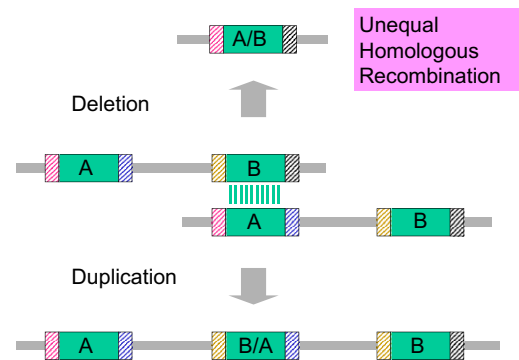
Ankala et al. (166) have recently proposed another mechanism, the aberrant firing of replication origins, to explain a number of complex lesions in a series of 62 intragenic nonrecurrent rearrangements within various genes (mainly in the *DMD* gene). While repetitive sequence elements were noted in only four individual cases, microhomologies (2–10 bp) were observed at breakpoint junctions in 56% of the cases studied; further, insertions ranging from 1 to 48 bp were noted in 16 of the 62 cases. The sequence proximal to the breakpoints in six individual Duchenne muscular dystrophy (DMD) cases was characterized by tandem repetitions of short segments (5–20 bp). The repeated replication of template sequences proximal to the mapped deletion breakpoints was taken as evidence of attempts by the replication machinery to bypass a stalled replication fork. This mutational mechanism, based on the replication rescue model originally suggested by Doksanı et al. (167), constitutes a novel type of template slippage event. Indeed, it can be seen that microhomologies at CNV breakpoints may be attributed to microhomology-mediated end joining (MMEJ), a replication repair mechanism, rather than to a recombination-based mechanism.

### 7.3.7 Gross Deletions

Gross deletions are common causes of certain disorders and rare in others. In most of the X-linked disorders, for example, large deletions account for about 5% of molecular defects. In other disorders, however, such as steroid sulfatase deficiency, large deletions of the *STS* gene account for 84% of patients (168). The same is true for disorders such as DMD, growth hormone deficiency, and  $\alpha$ -thalassemia (169–171).

A considerable number of large deletions are probably generated by mispairing of homologous sequences and unequal recombination (Figure 7-7). One of the best examples of homologous unequal recombination is the case of  $\alpha$ -globin genes on chromosome 16p. As a result of a recent evolutionary duplication of the  $\alpha$ -globin genes, extensive regions of sequence homology exist between the two closely linked  $\alpha$ -genes. Unequal crossover results in either the deletion of one  $\alpha$ -gene or the creation of a fusion hybrid gene (172). The reciprocal product chromosomes carry three  $\alpha$ -genes and are not associated with a clinical phenotype (173). Another example of a fusion gene resulting from an unequal crossover is the case of Hemoglobin Lepore characterized by a hybrid gene between the  $\delta$ - and  $\beta$ -globin genes on chromosome 11p (174). In the case of steroid sulfatase deficiency, the deletion can be as large as 1 Mb (175). In Kallmann syndrome, translocation can occur as a result of unequal mispairing of X and Y homologous sequences (176).

A number of common genetic disorders are due to large deletions (or duplications) caused by unequal crossing-over of homologous sequences. Figure 7-8 depicts various examples that include a 1.5 Mb deletion of



**FIGURE 7-7** Homologous unequal recombination between similar regions of sequences A and B. The recombination events cause either deletions or duplications. In the case of a deletion, a hybrid sequence is generated with the first part from sequence A and the second from sequence B. The middle sequence in the duplication product is also a hybrid sequence; the first part is from sequence B and the second from sequence A.

17p12 in hereditary neuropathy with liability to pressure palsies (HNPP) (177), deletion of 1.5 Mb of 17q11.2 in neurofibromatosis type 1 (178), deletion of 1.6 Mb of 7q11.23 in Williams syndrome (179), deletion of 5 Mb of 17p11.2 in Smith–Magenis syndrome (180), deletion of either 3 Mb or more rarely 1.5 Mb of 22q11 in DiGeorge and velo-cardio-facial syndromes (181,182), and 4 Mb deletions of 15q in Prader–Willi and Angelman syndromes (183). A recurrent deletion of ~0.5 Mb of 17q21.3, which may be mediated by a common inversion polymorphism, has also been described (83,184–186). For a review of chromosomal “duplicons,” the LCRs that mediate deletions and duplications, see Ref. (187). It has been estimated that ~5% of the human genome is duplicated either intra- or interchromosomally (188). The large deletions or duplications (see below) due to duplicon crossover are also termed “genomic disorders.” A recent review of such genomic disorders may be found in Ref. (189).

In many cases of large deletion, homologous unequal crossover occurs between repetitive elements such as *Alu* sequences (190). The *Alu* repeat is the most abundant repetitive element with about  $1.5 \times 10^6$  copies in the human genome (191,192). The element is about 300 bp in length and consists of two similar regions separated by a short A-rich region. Unequal crossover can occur between *Alu* sequences oriented in either the opposite or the same direction. In addition, unequal crossings over have been noted between *Alu* elements and nonrepetitive DNA sequences without homology to *Alus*. The best examples of *Alu*-*Alu* recombination occur in the genes encoding the low-density lipoprotein receptor (*LDLR*) which underlies familial hypercholesterolemia, and complement component 1 inhibitor (*C1I*; (193,194)). All but one of the breakpoints associated with *LDLR* gene deletions occur within *Alu* repeats. By contrast, deletions in other *Alu*-rich genes (e.g. *GLA1*) do not necessarily involve *Alu* repetitive elements (195). This

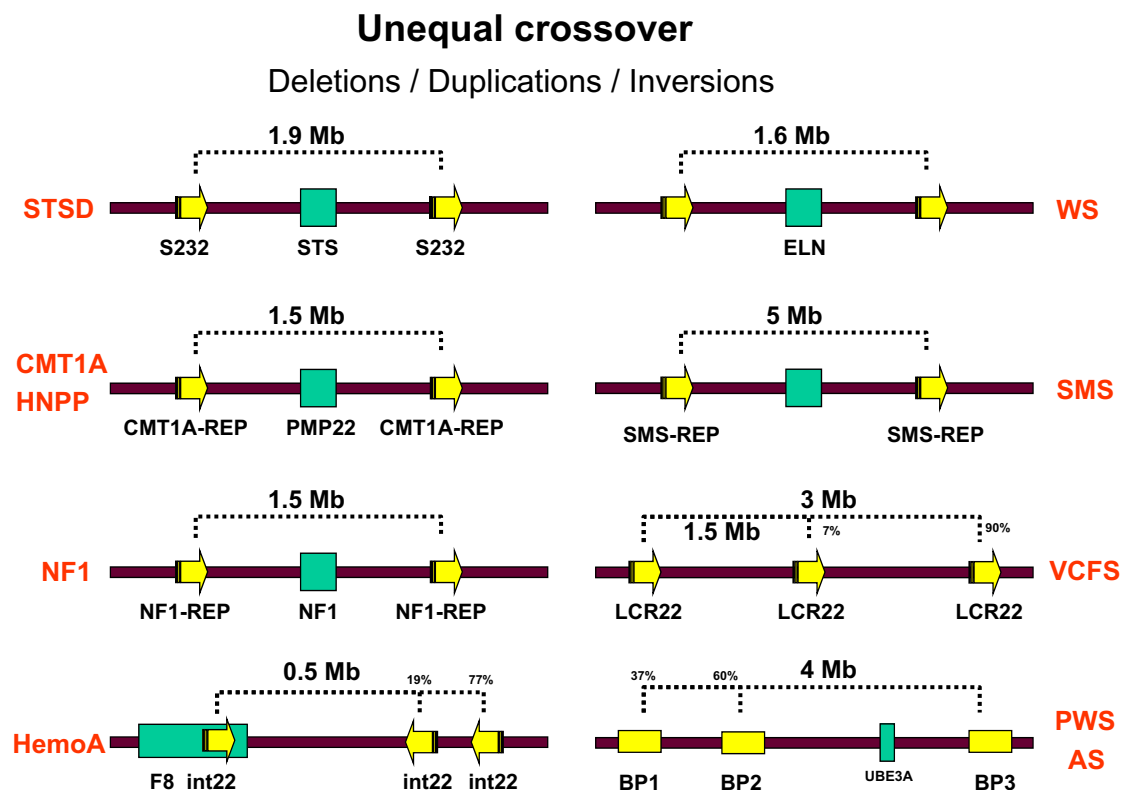
notwithstanding, *Alu*-mediated recombination between nonallelic *Alu* sequences is a fairly frequent cause of gene deletion causing human genetic disease (196–205). It should be appreciated that the importance of *Alu* sequences in the context of mediating genomic deletions does not lie simply with their sheer abundance; *Alu* elements also possess inherent recombination–predisposing properties (206).

Nonhomologous (illegitimate) recombination occurs between two DNA sites that share minimal sequence homology of a few base pairs. This type of recombination during meiosis or alternatively, slipped mispairing during DNA replication mediated by short (2–8) nucleotide direct repeats flanking the deletions is a common finding in many instances of large gene deletions (207). Such deletions have been studied, for example, in hemophilia A; a compilation of 46 junctions from large deletions revealed that about 50% shared 2- to 6-bp homology at the breakpoint junction, as compared with only 17% in which the deletion was due to *Alu*–*Alu* recombination (208). Similar results have been reported from the intron 7 deletion hotspot in the *DMD* gene; 8/9 deletion breakpoints examined were found to be flanked by DNA sequences with minimal homology (209).

It has also been proposed that alternative DNA conformations may trigger genomic rearrangements through

recombination–repair activities. Distance measurements have indicated the significant proximity of alternating purine–pyrimidine and oligo(purine–pyrimidine) tracts to breakpoint junctions in 222 gross deletions and translocations, respectively, involved in human diseases. In 11 deletions analyzed, breakpoints were explicable by non-B DNA structure formation (210).

The Gross Rearrangement Breakpoint Database (GRaBD; <http://www.uwcm.ac.uk/uwcm/mg/grabd/>) was established primarily for the analysis of the sequence context of translocation and deletion breakpoints in a search for characteristics that might have rendered these sequences prone to rearrangement (211). GRaBD, which contains 397 germline and somatic DNA breakpoint junction sequences derived from 219 different rearrangements underlying human inherited disease and cancer, represents a large but not comprehensive collection of sequenced gross gene rearrangement breakpoint junctions. Analysis of these breakpoints has extended our understanding of illegitimate recombination by highlighting the importance of secondary structure formation between single-stranded DNA ends at breakpoint junctions. For example, potential secondary structure was noted between the 5′ flanking sequence of the first breakpoint and the 3′ flanking sequence of the second breakpoint in 49% of rearrangements, and between the



**FIGURE 7-8** Genes, duplons, and diseases. Unequal crossover between homologous sequences (duplons) produce either deletions or duplications of the DNA between the duplons. The duplons are shown by arrows or by clear boxes. Genes included in the duplications/deletions are shown as dark boxes. STSD, steroid sulfatase deficiency; CMT1A, Charcot-Marie-Tooth type A1; HNPP, hereditary neuropathy with liability to pressure palsies; NF1, neurofibromatosis 1; HemoA, hemophilia A; WS, Williams syndrome; SMS, Smith–Magenis syndrome; VCFS, Velo–cardio–facial syndrome; PWS, Prader–Willi syndrome; AS, Angelman syndrome.

5' flanking sequence of the second breakpoint and the 3' flanking sequence of the first breakpoint in 36% of rearrangements (149). In addition, deletion breakpoints were found to be AT-rich whereas translocation breakpoints were GC-rich. Alternating purine-pyrimidine sequences were found to be significantly overrepresented in the vicinity of deletion breakpoints while polypyrimidine tracts were overrepresented at translocation breakpoints (148).

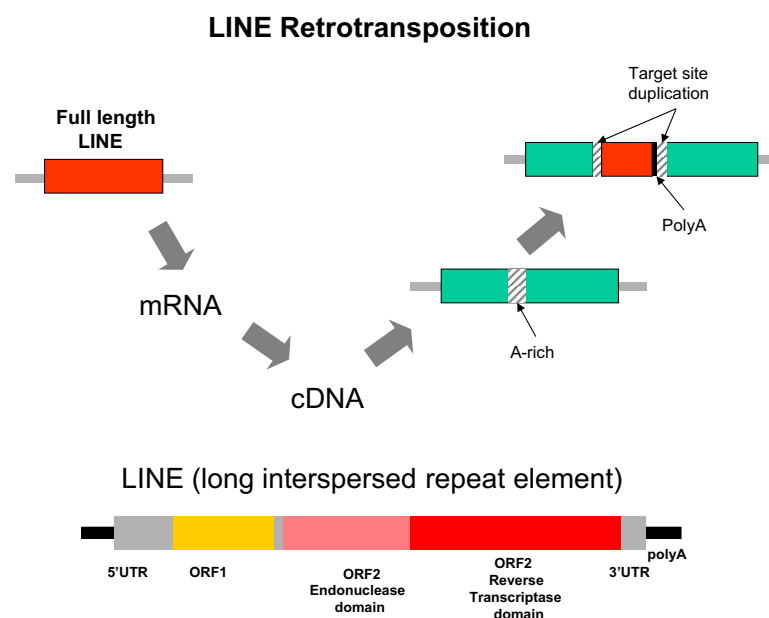
Finally, several examples of pathogenic large genomic deletions caused by the prior L1-mediated insertion of L1 (212,213), *Alu* (214,215), or SVA (216) insertions (see below) have been reported, as well as the first cases of L1-driven pseudogene insertion causing human genetic disease (217,218).

### 7.3.8 Large Retrotranspositional Insertions

A less common but nevertheless still fascinating mechanism of human gene mutation is the de novo insertion of repetitive elements via retrotransposition. The phenomenon was first observed in humans in the factor VIII (*F8*) gene in two unrelated de novo cases of severe hemophilia A (219). Truncated LINE (long interspersed) repetitive elements were introduced into exon 14 of the factor VIII (*F8*) gene where they caused disruption of the reading frame. The inserted elements contained a poly(A) tract and caused a target site duplication of more than 12 nucleotides. Further analysis of these insertions revealed that, in one case, the inserted element was an exact but truncated copy of a full-length LINE element with open reading frames (ORFs) found at chromosome 22q11 (220). The master source gene produces an mRNA that is probably

reverse transcribed (possibly via a reverse transcriptase encoded by itself) and the double-stranded nucleic acid is then reinserted into an A-rich region of the genome (Figure 7-9). LINEs probably integrate into genomic DNA by a process called target-primed reverse transcription (221). The proposed mechanism of LINE retrotransposition is as follows: An active LINE is transcribed in the nucleus and is subsequently transported to, and translated in, the cytoplasm. The two LINE-encoded proteins, ORF1 and ORF2, complex with LINE transcripts in ribonucleoprotein particles. The complexes are then transported to recipient DNA sequences where target-primed reverse transcription occurs. The new, integrated LINE copy is usually truncated at its 5' end. Over evolutionary time, L1s have shaped mammalian genomes through a number of different mechanisms. First, they have greatly expanded the genome both by their own retrotransposition and by providing the machinery necessary for the retrotransposition of other mobile elements, such as *Alu* sequences or SVA elements (153). Second, they have shuffled non-L1 sequence throughout the genome by a process termed transduction. Accidents of retrotransposition can cause disease and a number of such insertions have been reported to date (221,222). It is noteworthy that insertions of these elements within introns of genes or flanking regions are probably not associated with disease, but instead represent rare, private polymorphisms (223).

Similar retrotranspositions that involve members of the *Alu* sequence family have also been reported in several genes (examples include *Alu* insertions into the *NF1* gene causing type 1 neurofibromatosis, into the factor IX (*F9*) gene causing hemophilia B, and into the cholinesterase (*BCHE*) gene in a case of acholinesterasemia;



**FIGURE 7-9** Schematic representation of LINE retrotransposition. A master retrotransposon (full length LINE) from one chromosomal location is transcribed to mRNA; then reverse transcribed to double-stranded DNA and inserted into an adenine-rich region of another chromosomal location. The transposon has a poly(A) tail and produces a target site duplication.



(224–226)). It is likely that LINEs provide the molecular machinery necessary for the retrotransposition of *Alus*. One study using mutation analysis of the *F9* gene has estimated the frequency of retrotransposition to be such that it occurs somewhere in the genome of about 1 in every 17 children born (227).

Some 17% of a collection of gross insertions, all  $\geq 276$  bp in length, were due to LINE-1 (L1) retrotransposition involving different types of elements (L1 *trans*-driven *Alu*, L1 direct, and L1 *trans*-driven SVA) (153). A meta-analysis of 48 recent L1-mediated retrotranspositional events known to have caused human genetic disease revealed that 26 were L1 *trans*-driven *Alu* insertions, 15 were direct L1 insertions, 4 were L1 *trans*-driven SVA insertions, and 3 were associated with simple poly(A) insertions (228). The systematic study of these lesions, when combined with previous in vitro and genome-wide analyses, allowed several conclusions regarding L1-mediated retrotransposition to be drawn: (a) ~25% of L1 insertions are associated with the 3' transduction of adjacent genomic sequences, (b) ~25% of the new L1 inserts are full-length, (c) poly(A) tail length correlates inversely with the age of the element, and (d) the length of target site duplication in vivo is rarely longer than 20 bp. This analysis also suggested that some 10% of L1-mediated retrotranspositional events are associated with significant genomic deletions in humans.

Interestingly, Chen et al. (229) reported an indel in the *CFTR* gene that involved the insertion of a short 41 bp sequence with partial homology to a retrotranspositionally competent LINE-1 element. These authors dubbed such insertions of ultra-short LINE-1 elements “hyphen elements.”

Several instances of the clustering of pathogenic L1-mediated insertion events have also been observed. Thus, three independent *Alu* insertions have been found to be integrated into a 104 bp region of the *FGFR2* gene (230,231), two independent L1 insertions have been reported to have inserted into an 89 bp region of exon 44 of the dystrophin (*DMD*) gene (232,233), while six different insertions were found in a 1.5 kb region of the *NF1* gene (234). It should also be noted that independent L1-retrotransposition elements can integrate at precisely the same chromosomal sites. Thus, two markedly different *Alu* Ya5a2 elements became integrated at precisely the same site in the *F9* gene causing severe hemophilia B (226,235), whereas an SVA element and an *Alu* sequence were inserted at the same site within the coding region of the *BTK* gene (236). These observations are consistent with some genomic locations being exquisitely prone to L1-retrotransposition (228).

### 7.3.9 Large Insertion of Repetitive and Other Elements

The insertion of non-retrotransposons, namely beta-satellite repeats, has been observed in the human genome.

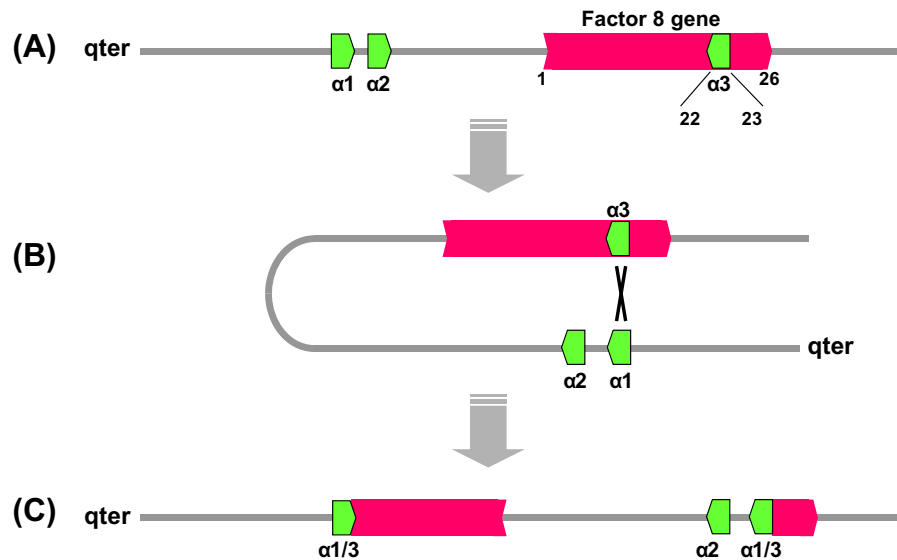
The insertion of 18 copies of the 68 bp monomer of the beta-satellite repeat in exon 11 of the *TMPRSS3* gene on chromosome 21 caused one form of recessive non-syndromic deafness DFNB10 (237). This may have been mediated by invasion of the genomic DNA by a small polydispersed circular DNA.

A patient with a sporadic case of Pallister–Hall syndrome has been shown to have experienced a de novo nucleic acid transfer from the mitochondrial to the nuclear genome. This mutation, a 72 bp insertion into exon 14 of the *GLI3* gene, creates a premature stop codon and predicts a truncated protein product. Both the mechanism and the cause of the mitochondrial-nuclear transfer are however unknown (238). Further examples of pathological mitochondrial-nuclear sequence transfers have been subsequently identified in the *USH1C* gene (153) and the *PAFAH1B1* gene (Millar et al. 2010).

Gross insertions ( $>20$  bp) comprise  $<1\%$  of disease-causing mutations. In an attempt to study these insertions in a systematic way, 158 gross insertions ranging in size between 21 bp and ~10 kb were identified from the HGMD; their study has revealed extensive diversity in terms of the nature of the inserted DNA sequence and has provided new insights into the underlying mutational mechanisms (153). Some 70% of gross insertions were found to represent sequence duplications of different types (tandem, partial tandem, or complex). In the context of a 26 bp insertion into the *ERCC6* gene, the authors also speculated as to whether they had found evidence for another mechanism of human genetic disease, involving the possible capture of DNA oligonucleotides (153).

### 7.3.10 Inversions

The most common inversion found to date is that associated with the factor VIII (*F8*) gene, which occurs via intrachromosomal recombination mediated by a 9.5 kb sequence that is repeated three times in the last megabase of Xqter; once in intron 22 of the *F8* gene and twice about 400 kb telomeric to the first (239,240) (Figure 7-10). Most inversions, which are high frequency independent recurring events, involve the distal sequence. The vast majority of inversions occur in male germ cells (241), perhaps because intrachromosomal recombination is inhibited by the presence of homologous X chromosomes (the male:female ratio was estimated to be about 300:1). Almost all mothers of inversion hemophilia A cases are carriers of the abnormality. DNA diagnosis of the molecular lesion in severe hemophilia A has been greatly facilitated by the frequent occurrence of this common inversion of the *F8* gene (45% of individuals with severe hemophilia A). The frequency of de novo *F8* gene inversion has been estimated to be  $7.2 \times 10^{-6}$  per gamete per generation. Another example of inversion has been described in the *IDS* gene (also on Xq) in about 13% of cases of



**FIGURE 7-10** Common inversion of the factor VIII (*F8*) gene in severe hemophilia A. (A) Schematic representation of the most distal 1 Mb of Xq. Regions  $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$  represent 9.5 kb highly homologous DNA elements. The orientations of these sequences are shown by arrows. (B) Intrachromosomal recombination between elements  $\alpha_1$  and  $\alpha_3$ . (C) The crossover results in the inversion of exons 1–22 of the *F8* gene.

Hunter syndrome (242). Inversions of DNA sequences have also been reported in the  $\beta$ -globin gene cluster on 11p and in the *APOA1-APOC3-APOA4* gene cluster on 11q (243,244).

A meta-analysis of inversions of  $\geq 5$  bp but  $< 1$  kb has been performed by Chen et al. (152). Of the 21 mutations studied, 19 were found to be compatible with a model of intrachromosomal serial replication slippage in *trans* (SRStrans) mediated by short inverted repeats. Eighteen (one simple inversion, six inversions involving sequence replacement by upstream or downstream sequence (DSS), five inversions involving the partial reinsertion of removed sequence, and six inversions that occurred in a more complicated context) of these were found to be consistent with either two steps of intrachromosomal SRStrans or a combination of replication slippage in *cis* plus intrachromosomal SRStrans. The remaining lesion, a 31 kb segmental duplication associated with a small inversion in the *SLC3A1* gene, was explicable in terms of a modified SRS model incorporating the concept of “break-induced replication.” This study has, therefore, lent broad support to the idea that intrachromosomal SRStrans can account for a variety of complex gene rearrangements involving inversions.

### 7.3.11 Duplications

Duplications of whole genes or exons have contributed very significantly to the evolution of the human genome (47). Indeed, most gene clusters (e.g.  $\beta$ -globin, growth hormone, Hox) owe their origin to gene duplications that have occurred during vertebrate evolution. Furthermore, the presence of similar domains in proteins (e.g. immunoglobulin-like domains in many

transmembrane proteins) are due to duplications of certain exons.

Occasionally, however, duplications may also be the cause of genetic disorders. The most frequent mechanism of duplication is homologous unequal crossover as described for large deletions. In fact, most large duplications generated as the reciprocal product of a deletion resulting from homologous unequal crossover. Duplications are less common, however, than their theoretically reciprocal deletions (e.g. see Ref. (245), for the *DMD* gene). This may be due to the nonpathogenicity of a duplication (e.g.  $\alpha$ -globin genes; (173)), elimination of duplications as is the case for the *HPRT1* gene, or to the fact that not all mechanisms that lead to deletions also produce duplications. A large and common duplication has been identified in cases of Charcot-Marie-Tooth disease type 1A (246). This duplication involves 1.5 Mb of DNA on chromosome 17p containing the peripheral myelin protein 22 (*PMP22*) gene. It results from homologous unequal crossover events between 24 kb repeats that flank the duplicated region. The reciprocal deletion product of this recombination event is responsible for a completely different clinical phenotype: HNPP (Figure 7-8). Another notable duplication of at least 500 kb that includes the *PLP1* gene is a frequent cause of Pelizaeus-Merzbacher disease (247). The pathogenetic mechanism of these duplications involves unequal crossing-over in meiosis mediated by “duplicons” in the genome (187).

The molecular defect in the majority of cases with ectrodactyly type SHFM3 on chromosome 10q24, is an approximately 0.5 Mb tandem duplication. The precise pathogenetic mechanism of this duplication is unknown (248). Additional gene duplications causing recognizable syndromes include the *APP* duplication

causing early-onset Alzheimer disease (249), the *SNCA* duplication and Parkinson disease (250) and the triplication of ~605 kb segment containing the *PRSS1* gene in families with hereditary pancreatitis (251).

### 7.3.12 Copy Number Variation in Association with Disease

CNVs are a form of genomic diversity that involves DNA sequences  $\geq 1$  kb in length that are present in the human genome in a variable number of copies. CNVs may be recurrent (which arise by NAHR) or nonrecurrent (which arise by nonhomologous end joining and recombination-based mechanisms) (252, 252a). Such gross duplications/deletions are not only rather abundant but also often occur at polymorphic frequencies. Conrad et al. (53) generated a comprehensive map of >8500 validated CNVs >500 bp (detected in 41 Europeans/West Africans) that together cover a total of 112.7 Mb (3.7% of the genome). These authors estimated that 39% of the validated CNVs overlapped 13% of RefSeq genes (NCBI mRNA reference sequence collection). Further, they concluded that the CNVs detected resulted in the “unambiguous loss of function” of alleles for 267 different genes.

It has been estimated that on average, 73–87 genes vary in terms of their copy number between any two individuals (253). This high degree of interindividual variability with regard to gene copy number has challenged traditional definitions of wild type and “normality” and even the very concept of a “reference genome” itself. High-resolution breakpoint mapping is a prerequisite for the accurate assessment of CNV size, the identification of the genes and regulatory elements affected, and hence, for the determination of the consequences of CNV for gene expression (254) and the phenotypic sequelae (190,255–257). This notwithstanding, it is already becoming clear that these consequences may go far beyond the physical bounds of a given CNV. For example, a CNV involving the human *HBA* gene has a dramatic influence on the expression of the *NME4* gene some 300 kb distant (258). In addition, a 5.5 kb microduplication of a conserved noncoding sequence with demonstrated enhancer function, about 110 kb downstream of the bone morphogenic protein 2 (*BMP2*) gene, has been found to cause brachydactyly type 2A in two families (259).

It may well be that the precise extent and/or location of many CNVs will vary between individuals, thereby further increasing both the mutational and phenotypic heterogeneity. The extent to which CNVs are likely to contribute to the diversity of human phenotypes, including “single gene defects,” genomic disorders, and complex disease, is increasingly being recognized. Thus, CNV of the *FCGR3B* genes is a determinant of susceptibility to immunologically mediated glomerulonephritis (260). CNVs in the *CCL3L1* and *DEFB4* genes have also been found to be associated with increased

susceptibility to HIV infection and Crohn’s disease, respectively (261,262), whereas rare CNVs associated with various complex phenotypes have been identified in studies of schizophrenia (263), epilepsy (264), and severe early-onset obesity (265). CNVs are now being widely recruited to genome-wide association studies (GWAS) with the aim of assessing their influence on human disease causation/susceptibility (256,266,267).

To date, several dozen human disease conditions have been identified, which are either caused by CNVs or whose relative risk is increased by CNVs (256,268). Remarkably, an excess of both rare and de novo CNVs has been identified in patients with psychiatric disorders and obesity (263,265,269–273). These findings point to genetic heterogeneity in these conditions thereby illustrating the likely complexity inherent in identifying all disease-causing CNVs. Intriguingly, Shlien et al. (274) have reported a highly significant increase in CNV number among patients with Li–Fraumeni syndrome, carriers of inherited *TP53* mutations. Hence, it would appear that heritable genetic variants have the potential to modulate the rate of germline CNV formation.

It is already clear that the disease relevance of CNVs represents a continuum, stretching from “neutral” polymorphisms on the one hand to directly pathogenic copy number changes on the other (256). Between these two extremes may lie those CNVs that are capable of acting as predisposing (or protective) factors in relation to complex disease (275,276). Thus, for example, a 117 kb deletion encompassing the *UGT2B17* gene has been found to be associated with an increased risk of osteoporosis (277). Intriguingly, some germline CNVs appear to predispose to disease even although no known genes reside within their boundaries (278,279). Importantly, a 520 kb micro-deletion has been identified at 16p12.1, which predisposes to various neuropsychiatric phenotypes as a single copy number mutation and aggravates neurodevelopmental disorders if it co-occurs together with other large deletions and duplications (276). It remains to be seen whether “CNV equivalents,” <1 kb in size, that actually occur rather more frequently than true CNVs (>1 kb) (53), will also be relevant to disease. What is already clear is that, over the coming years, an increasing number of important CNV-disease associations are going to come to light (257).

### 7.3.13 Gene Conversion

Gene conversion is the modification of one of two alleles by the other. It involves the nonreciprocal correction of an “acceptor” gene or DNA sequence by a “donor” sequence, which itself remains physically unchanged. In most known instances of gene conversion as a cause of human genetic disease, the functional gene has been wholly or partially converted to the sequence of a highly homologous and closely linked pseudogene, which therefore acts as the donor sequence (280). Probable examples include

the genes for steroid 21-hydroxylase (*CYP21*; (281)), polycystic kidney disease (*PKD1*; (282)), neutrophil cytosolic factor p47-*phox* (*NCF1*; (283)), immunoglobulin  $\lambda$ -like polypeptide 1 (*IGLL1*; (284)), glucocerebrosidase (*GBA*; (285)), von Willebrand factor (*VWF*; (286)), and phosphomannomutase (*PMM2*; (287)). These gene/pseudogene pairs are all closely linked with the exception of the *VWF* gene (12p13) and its pseudogene (22q11-q13), and the *PMM2* gene (16p13) and its pseudogene (18p). Together, these two exceptions would seem to establish a precedent for the occasional occurrence of gene conversion between unlinked loci in the human genome.

An *in silico* analysis of the DNA sequence tracts involved in 27 well-characterized nonoverlapping gene conversion events in 19 different genes reported in the context of inherited disease was recently performed (288). It was noted that gene conversion events tended to occur within (C+G)- and CpG-rich regions and that sequences with the potential to form non-B DNA structures (and which might be involved in the generation of double-strand breaks that could, in turn, serve to promote gene conversion) occurred disproportionately within maximal converted tracts and/or short flanking regions. Maximal converted tracts were also found to be enriched in a truncated version of the chi-element (a TGGTGG motif), immunoglobulin heavy chain class switch repeats, translin target sites, and several novel motifs including (or overlapping) the classical meiotic recombination hotspot, CCTCCCCCT (288). Finally, it was found that gene conversions tended to occur in genomic regions that had the potential to fold into stable hairpin conformations. Taken together, these findings support the concept that recombination-inducing motifs, in association with alternative (non-B DNA) conformations, can promote recombination in the human genome.

The large number of duplicated gene sequences in the human genome implies that a considerable number of disease-associated mutations could originate via interlocus gene conversion. A genome-wide computational approach to identify disease-associated mutations derived from interlocus gene conversion events recently revealed hundreds of known pathological mutations that could have been caused by interlocus gene conversion (289). In addition, several dozen high-confidence cases of inherited disease mutations resulting from interlocus gene conversion were identified in about 1% of all genes analyzed. About half of the donor sequences associated with such mutations were functional paralogous genes, suggesting that epistatic interactions or differential expression patterns would determine the impact upon fitness of a single amino acid substitution between duplicated genes. In addition, Casola et al. (289) identified thousands of hitherto undescribed deleterious mutations that could potentially arise via interlocus gene conversion. It would therefore appear as if the impact of interlocus gene conversion upon the spectrum of human inherited disease may be considerably greater than has hitherto been appreciated.

Although mutations that are detrimental to the fitness of individuals are expected to be rapidly purged from the population by natural selection, some pathological mutations are nevertheless retained at high frequencies in human populations. Several hypotheses have been proposed to account for this apparent paradox (high new mutation rate, genetic drift, overdominance, or recent changes in selective pressure). However, there is an additional process that appears to contribute to the spreading of deleterious mutations: GC-biased gene conversion (gBGC), a process associated with recombination that tends to favor the transmission of GC-alleles over AT-alleles. Necsulea et al. (290) have shown that the spectrum of amino acid-altering polymorphisms in human populations exhibits the footprints of gBGC. This pattern is not explicable in terms of selection and is evident with all nonsynonymous mutations, including those predicted to be detrimental to protein structure and function as well as those that have been implicated in the causation of human genetic disease. These results indicate that gBGC meiotic drive contributes to the spreading of deleterious mutations in human populations.

### 7.3.14 Insertion–Deletions (Indels)

A relatively rare type of mutation causing human genetic disease is the *indel*, a complex lesion that appears to represent a combination of micro-deletion and micro-insertion. One example is provided by the 9 deleted base pairs encoding codons 39–41 of the  $\alpha$ 2-globin (*HBA2*) gene that were replaced by 8 inserted bases that served to duplicate the adjacent downstream sequence (DSS) (291). Indels constitute a fairly infrequent type of lesion causing human genetic disease; some 1.5% of lesions in HGMD fall into this category.

Several indel hotspots have been noted in a meta-analysis of HGMD data on 211 different indels underlying genetic disease (292). A GTAAGT motif was found to be significantly overrepresented in the vicinity of the indels studied. The change in complexity consequent to a mutation was also found to be indicative of the type of repeat sequence involved in mediating the event, thereby providing clues as to the underlying mutational mechanism. The majority of indels (>90%) were explicable in terms of a two-step process involving established mutational mechanisms. Indels equivalent to double base-pair substitutions (22% of the total) were found to be mechanistically indistinguishable from the remainder and may therefore be regarded as a special type of indel.

### 7.3.15 Other Types of Complex Rearrangement

Complex mutational events that involve combined gross duplications, deletions, and/or insertions of DNA sequence have been not infrequently observed and



together constitute ~1% of entries in HGMD. One example of this type of gene defect is a 10.9kb deletion coupled with a 95 bp inversion in the factor IX (*F9*) gene causing hemophilia B (293). The molecular characterization of this type of lesion is often extremely complicated, and in most cases, the underlying mutational mechanisms could not be readily inferred.

Recently, however, a meta-analysis of 21 complex gene rearrangements derived from the HGMD revealed that all but one could be accounted for by a model of SRS, involving twin or multiple rounds of replication slippage (151). Thus, of the 20 complex gene rearrangements, 19 (7 simple double deletions, 1 triple deletion, 2 double mutational events comprising a simple deletion and a simple insertion, 6 simple indels that may constitute a novel and noncanonical class of gene conversion, and 3 complex indels) were compatible with the model of SRS in *cis*; by contrast, the remaining indel in the *MECP2* gene appears to have arisen via interchromosomal replication slippage in *trans*.

A novel type of complex genomic rearrangement, comprising intermixed duplications and triplications of genomic segments, has recently been described at both the *MECP2* and *PLP1* loci (294). These complex rearrangements share a common genomic organization viz., a duplication-inverted triplication-duplication (DUP-TRP/INV-DUP), in which the triplicated segment is inverted and located between directly oriented duplicated genomic segments. The DUP-TRP/INV-DUP structures appear to be mediated by inverted repeats, up to >300 kb apart.

### 7.3.16 Multiple Simultaneous Mutations

Transient hypermutability is a general mutational mechanism with the potential to generate multiple synchronous mutations, a phenomenon probably best exemplified by “closely spaced multiple mutations” (CSMMs). From a collection of human inherited disease-causing multiple mutations, Chen et al. (295) retrospectively identified numerous potential examples of pathogenic CSMMs that exhibited marked similarities to the CSMMs reported in other systems. These examples included (i) eight multiple mutations, each comprising three or more components within a sequence tract of <100 bp (*CBS*, *MPZ*, *OPN1LW*, and *STK1* genes), (ii) three possible instances of “mutation showers” in the *PTCH1*, *FANCA*, and *KNG1* genes, respectively, and (iii) numerous highly informative “homocoordinate” mutations (multiple mutations involving the same mutation type).

Recently, a remarkable phenomenon has been reported in multiple cancer samples: The presence of tens to hundreds of genomic rearrangements involving spatially localized genomic regions (296). These complex rearrangements primarily affected a single chromosome, although in some cases, multiple apparently concomitant alterations affected several different chromosomes. Stephens et al. convincingly argued that

these massive, yet spatially localized, genomic rearrangements must have resulted from a single catastrophic event (which they termed “chromothripsis”) rather than from a series of progressive and hence independent alterations. Chromothripsis also appears to be capable of explaining the generation of some complex de novo structural rearrangements in the germline (297). An illustrative example pertains to a highly complex chromosomal rearrangement, identified in a child with severe congenital abnormalities, which comprised at least 12 de novo breakpoint junctions and involved chromosomes 1, 4, and 10. These breakpoints were clustered in small genomic regions of up to 3.5 Mb in size on each chromosome. Reconstruction of the derivative chromosomes indicated that the breakpoints formed concordantly oriented pairs on the reference genome. Both intra- and interchromosomal junction sequence features were compatible with those commonly associated with NHEJ (297). The insights generated from the seminal work of Stephens and his colleagues may well help us to understand the mutational mechanisms underlying some previously reported germline complex rearrangements (298). For example, with respect to the de novo mutational event on chromosome 2 in a patient with Waardenburg syndrome and other congenital defects, the original authors suggested that all five breaks might have occurred simultaneously but were unable to explain why the breakpoints had occurred within a single chromosome (299). In the light of our emerging knowledge of chromothripsis, the idea that this complex rearrangement could have been generated in such a way as to be compatible with the NHEJ repair of simultaneously generated DSBs becomes quite attractive. Liu and colleagues subsequently investigated 17 subjects with various development abnormalities by means of high-resolution genome analysis (300). Constitutional multiple copy number changes, including deletions, duplications, and/or triplications, as well as inversions were observed in all cases. Strikingly, in each case, all rearrangements occurred within a single chromosome; in 15 of the 17 cases, the rearrangements were localized to the distal half of the affected chromosomal arms. FISH and breakpoint junction data indicated that all additional copies of the duplicated and triplicated segments appear to be randomly joined, forming a large “breakpoint junction cluster” on 9q21. By analogy with the phenomenon of chromothripsis, the observation of these extremely complex rearrangements in a single chromosome was also described as a chromosome catastrophe event (300). However, this kind of chromosomal change cannot be easily explained by the previously described NHEJ repair of simultaneously generated DSBs. Instead, Liu and colleagues envisaged the involvement of a replicative mechanism in the generation of this complex chromosome catastrophe event comprising multiple duplications and/or triplications; they regarded MMBIR as the most likely underlying mechanism. They further suggested that a

potential replication fork collapse at 9q21 could account for the breakpoint clustering therein (300).

### 7.3.17 Molecular Misreading

Long runs of adenines (and perhaps other mononucleotides or dinucleotides) promote a phenomenon termed “molecular misreading” by which DNA replication/RNA transcription and/or translation result in erroneous products with different numbers of (A)s derived from the original DNA sequence (301). In a family with hypobetalipoproteinemia, a deletion of one C in the A<sub>5</sub>CA<sub>3</sub> coding sequence of the *APOB* gene results in a run of (A)<sub>8</sub>. The patient, however, did not have severe disease, because some ApoB protein was made. This was the result of molecular misreading in which ~10% of the resulting mRNAs contained (A)<sub>9</sub> instead of the expected (A)<sub>8</sub>; this partially restored the reading frame thereby templating the synthesis of low amounts of normal ApoB (302). Similarly, a family with mild to moderately severe hemophilia A with a deletion of one T within the coding A<sub>8</sub>TA<sub>2</sub> sequence of the *F8* gene has been reported. The partial “correction” of the phenotype was due to restoration of the reading frame because of molecular misreading in which ~5% of the resulting RNAs contained (A)<sub>11</sub> instead of the expected (A)<sub>10</sub>. In this family, there was also evidence for ribosomal frameshifting during translation of the mutant RNA (303).

Another example of this phenomenon was observed in the *APC* gene. A T-to-A transversion is present in the coding A<sub>3</sub>TA<sub>4</sub> sequence of the *APC* gene in 6% of Ashkenazi Jews, and in about 28% of Ashkenazim with a family history of colorectal cancer. This mutation creates a small hypermutable region, indirectly causing cancer predisposition because there are many somatic cells in which stretches of (A)<sub>9</sub> occur instead of the expected (A)<sub>8</sub>; the (A)<sub>9</sub> results in frameshifting and a truncated dysfunctional APC (304). Interestingly, in the neurofibrillary tangles, neuritic plaques, and neuropil threads in the cerebral cortex of Alzheimer disease and Down syndrome, abnormal forms of  $\beta$ -amyloid precursor protein and ubiquitin B have been observed. These aberrant proteins were produced because of +1 frameshifting that resulted from a deletion of AG in a sequence GAGAG that occurred in the coding regions of both genes (*APP* and *UBB*, respectively). This dinucleotide deletion was again the result of molecular misreading during transcription or posttranscriptional editing of RNA (305). This mechanism is likely to yield a considerable quantity of abnormal RNA molecules and protein products in somatic cells (306).

### 7.3.18 Germline Epimutations

*Epimutations* are modifications of DNA that constitute clonally heritable (yet potentially reversible) alterations in the transcriptional status of a gene that lead to the

abnormal silencing of that gene. Epimutations are not mutations *sensu stricto* since they do not alter the gene’s nucleotide sequence. However, germline epimutations of the *MLH1* gene have been reported in individuals with multiple cancers (307) and in the *MLH1* and *MSH2* genes in hereditary nonpolyposis colorectal cancer (308). These heritable inactivating epimutations are characterized by mono-allelic hypermethylation of the *MLH1* or *MSH2* genes and, to all intents and purposes, are functionally equivalent to conventional mutations. A maternal epimutation in the *GNAS* gene has also been reported as a cause of Albright osteodystrophy and parathyroid hormone resistance (309). With the determination of the human methylome (80) and the recent recognition that DNA sequence polymorphisms can exert an effect on gene function via allele-specific methylation in *cis* (310), the number of recognized epimutations should rise quite significantly in the coming years. If eventually shown to be both of pathological significance and heritable, some examples of histone modification (311,312) or RNA editing (313,314) could also turn out to represent “honorary mutations.”

### 7.3.19 Frequency of Disease-Producing Mutations

*Mutation frequency within genes:* The frequency of different molecular defects is not the same for every gene and every disorder. Indeed, human disease genes exhibit very considerable allelic heterogeneity in terms of their mutational spectra; for some genes, a few predominant disease alleles predominate whereas for others, there is a wide range of disease alleles, each relatively rare (315). The mutational spectrum depends very largely on the DNA sequence characteristics of the gene in question (e.g. the presence of repeat units or homologous sequences), and the function of, and evolutionary constraints experienced by, its encoded protein (316). For some genes, deletions predominate; for others, one particular type of lesion such as an inversion may be especially common. Some genes exhibit mainly frameshifts and stop codons associated with a specific disorder, whereas others manifest mainly missense mutations for a given phenotype, or expansions of trinucleotide repeats.

Disease mutations are nonuniformly distributed within genes (317). Such mutations were found to be statistically overrepresented in conserved domains, and underrepresented in variable regions, even after allowing for the amino acid site variability of domains over long-term evolutionary history. This finding suggests that there is a nonadditive influence of amino acid site conservation on the observed intragenic distribution of disease mutations.

*Mutation frequency within human populations:* Population genetic considerations are also likely to be very important in determining why some mutations occur frequently, either within a patient cohort or in

the population at large (see Frequency of Inherited Disorders Database, <http://archive.uwcm.ac.uk/uwcm/mg/fidd/>; FINDbase, <http://www.findbase.org/>). Selection, migration, and genetic drift are all likely to play a role as well as the mutation rate (318–320). Thus, the mutational spectrum of the *PAH* gene underlying phenylketonuria appears to result from a range of different factors including founder effect, range expansion and migration, genetic drift, and possibly also heterozygote advantage (321). Selection can also serve to maintain deleterious mutations at high frequencies in particular populations by overdominant selection (heterozygote advantage). Good examples of this phenomenon are provided by a reduction in risk of severe malaria associated with female heterozygotes and male hemizygotes for mutations in the X-linked *G6PD* gene (322,323), for individuals heterozygous for the  $\beta$ -globin (*HBB*) sickle cell mutation, Glu6Val (324), and for individuals heterozygous and homozygous for  $\alpha^+$ -thalassemia (325). Intriguingly, however, the protection against malaria afforded by sickle cell disease and  $\alpha^+$ -thalassemia when inherited individually is lost when the two conditions are co-inherited (326). Other possible examples of heterozygote advantage include an elevated cortisol response in heterozygous carriers of *CYP21A* mutations (327), higher values for hemoglobin, serum iron and transferrin saturation in women heterozygous for *HFE* gene mutations (328), resistance to prion infection conferred by a common prion protein (*PRNP*) polymorphism (329), resistance to severe sepsis in heterozygous carriers of the factor V Leiden polymorphism, Arg506Gln (330), and increased keratinocyte cell survival in individuals heterozygous for *GJB2* gene mutations (331). Resistance to cholera toxin (332), protection against bronchial asthma (333), and resistance to *Pseudomonas aeruginosa* infection (334) have all been mooted as possible bases for overdominant selection in heterozygous carriers of *CFTR* gene mutations. However, cystic fibrosis heterozygotes have been shown to secrete chloride at the same rate as individuals lacking *CFTR* gene mutations (335).

A number of genetic diseases are known to be particularly prevalent in Jewish populations (336,337). The presence of four distinct lysosomal storage diseases at significant frequencies among Ashkenazi Jews has often been considered as providing evidence for a selective advantage accruing to heterozygotes in this population. However, evidence in support of the idea of genetic drift appears to be more compelling (338,339).

Selection may also act at an extremely early stage to boost the frequency of some mutations that are deleterious at a later stage in development. For example, gain-of-function missense mutations in the fibroblast growth factor receptor 2 (*FGFR2*) gene responsible for Apert syndrome have been shown to confer a selective advantage on spermatogonial cells by promoting the clonal expansion of mutant cells (340,341).

### 7.3.20 Functional Characteristics of Human Disease Genes

Human disease genes appear to be distinguishable from “nondisease genes” (in reality, the latter can only be defined as genes that are not yet known to cause inherited disease) in terms of a range of features including gene structure, gene expression, physicochemical properties, protein structure, and evolutionary conservation (31,316,342–347). Thus, human disease genes are characterized by the greater length of their encoded amino acid sequences, a larger numbers of longer introns, a broader range of tissue expression, and a wider phylogenetic distribution (343,348). Human disease genes are also known to be unevenly distributed between the human chromosomes (349,350). Further, synonymous nucleotide substitutions appear to occur at a higher rate in human disease genes, a finding that may reflect increased mutation rates in the chromosomal regions in which disease genes are found (349). It may be that disease genes are more prevalent in genomic regions that experience elevated rates of mutation (351). Another possible explanation is that the disease gene set may contain a disproportionately lower number of genes expressed in the germline (349). This is because mutations in such genes might be expected to be more effectively repaired by transcription-coupled repair (transcription-coupled repair in the germline appears to account for the strand asymmetry that the human genome exhibits in terms of inherited mutations; (352,353)). Strand asymmetries with respect to the mutation rate may, however, also arise through the influence of DNA replication origins (354), recombination (355,356), and strand-biased repair (357).

### 7.3.21 Mutation Nomenclature

Some consistency in the way in which mutations are described is essential for the accurate and unambiguous reporting and curation of mutation data. Most guidelines on how to describe mutational changes in human genes are to be found in (358) and on the Human Genome Variation Society (HGVS) website (<http://www.hgvs.org/mutnomen/>). A program, *Mutalyzer*, is available to check sequence variation nomenclature (<http://www.lovd.nl/mutalyzer>) using a human genome reference sequence and following the current recommendations of the HGVS; *Mutalyzer* is capable of handling most types of mutations including nucleotide substitutions, deletions, duplications, insertions, indels, and splice-site changes (359).

### 7.3.22 Mutations in Gene Evolution

Mutations in human gene pathology and evolution represent two sides of the same coin in that those same mutational mechanisms that have frequently been implicated in

human pathology have also been involved in potentiating evolutionary change (47). Regardless of whether they are advantageous, disadvantageous, or neutral, these mutational changes and their putative underlying causal mechanisms are very similar. It is now clear that the gene has often been a dynamic entity over evolutionary time, not a static one. Indeed, during vertebrate evolution, many genes have undergone gross rearrangement as a result of the action of a variety of mutational processes including insertion, inversion, duplication, repeat expansion, translocation, or deletion. What links pathology and evolution is the underlying genomic architecture with its hitherto largely unexplored vocabulary of structural elements, and different types and patterns of repetitive DNA sequences (189). It can thus be seen that the mutational spectra of germline mutations responsible for inherited disease, somatic mutations underlying tumorigenesis, polymorphisms (either neutral or functionally significant), and differences between orthologous gene sequences, exhibit remarkable similarities implying that they are very likely to have causal mechanisms in common.

## 7.4 CONSEQUENCES OF MUTATIONS

### 7.4.1 Mutations Affecting the Amino Acid Sequence of the Predicted Protein, but Not Gene Expression

Many missense mutations (i.e. nucleotide substitutions that result in an amino acid substitution) cause hereditary disease in humans. Missense mutations are of importance in understanding the structure or function of a protein since they usually occur in amino acid residues of structural or functional significance (10). Occasionally, however, not only is the mutated residue not conserved in mouse, but also the substituting residue in humans is identical to its wild-type counterpart in the orthologous (e.g. murine) gene (360). It is thought that the most likely explanation for the majority of these cases of fixation of disease mutations in mice is *compensatory mutation*. The chimpanzee genome has been found to harbor a number of examples of potentially compensated mutations (PCMs), defined as human disease-causing or disease-associated missense mutations for which the substituting amino acid is identical to the wild-type amino acid residue at the orthologous position in chimpanzee (361). The absence of strongly deleterious consequences of a specific PCM in chimpanzee would be explicable either by virtue of the very different (simian) environment or by dint of hitherto unidentified variants (“compensatory mutations”) in the chimpanzee genome that have served to epistatically buffer the PCM (361). It should be noted, however, that the PCM may only have become seriously disadvantageous in the human lineage, either as a consequence of other lineage-specific genetic changes or due to changes in the human environment and/or lifestyle (362,363). In this case, it would not have been

necessary for the chimpanzee PCM to be compensated for at any time, which is why the qualifier “potentially” is important when PCMs are defined on the basis of current genetic and clinical data.

It is sometimes difficult to establish a causative link between a missense mutation and a disease phenotype (364). The absence of the mutation in a large sample (usually 200 individuals) from the same ethnic group as the patient serves to exclude the possibility of a common polymorphism. Amino acid substitutions in evolutionarily conserved residues can also be good candidates for true pathogenicity (10). If the function of the protein is known, assessment of the effect of the missense mutation can be performed by *in vitro* mutagenesis and functional assay. Finally, the introduction of the mutation into an entire organism (e.g. transgenic mice) and the study of its systemic effects provide one of the best means to assess its contribution to a particular clinical phenotype. Amino acid substitutions can be shown to reduce or abolish the physiological function of a protein; for example, missense mutations have been identified in factor VIII that abolish thrombin cleavage, which is necessary for its activation (365), interfere with binding to other proteins such as vWF (366), or create or abolish N-glycosylation sites (367). In other proteins, mutations have been identified, for example in DNA binding domains, catalytic domains, transmembrane domains, ATP-binding regions, receptor-ligand contact sites, phosphorylation, or other chemical modification sites. Missense mutations may also affect protein folding causing a dramatic change in secondary and tertiary structures such that the protein can no longer fulfill its physiological function.

A classical example of a missense mutation in the active site of an enzyme is provided by  $\alpha$ 1-antitrypsin, Pittsburgh, found in an individual with a fatal bleeding disorder (368). The underlying mutation in the  $\alpha$ 1-antitrypsin (*SERPINA1*) gene substituted Arg for Met358 within the active site of the molecule. Substitution by Arg served to alter the substrate specificity of  $\alpha$ 1-antitrypsin by converting its “bait loop” (which is specific for elastase) to one that was specific for thrombin. In effect, the molecule lost its anti-elastase activity and became a serine protease inhibitor capable of inhibiting thrombin and factor Xa.

Mutations involving gains of glycosylation have generally been considered rare, and the pathogenic role of the new carbohydrate chains has never been formally established (369); however, the three children identified with Mendelian susceptibility to mycobacterial disease were homozygous with respect to a missense mutation in the *IFNGR2* gene that created a new N-glycosylation site in the IFN $\gamma$ R2 chain. The resulting additional carbohydrate moiety was found to be both necessary and sufficient to abolish the cellular response to IFN $\gamma$ . From 10,047 HGMD mutations in 577 genes encoding proteins trafficked through the secretory pathway, 142 candidate missense



mutations (~1.4%) in 77 genes (~13.3%) for potential gain of *N*-glycosylation were identified. Six mutant proteins were shown to bear new *N*-linked carbohydrate moieties. Thus, it may be that an unexpectedly high proportion of mutations causing human genetic disease do so via the creation of new *N*-glycosylation sites. Indeed, the pathogenic effects of these mutations may be a direct consequence of the addition of *N*-linked carbohydrate.

Missense mutations can result in disease by (1) elimination or reduction of the physiological activity/role of the protein; (2) gain-of function by which the amino acid substitution creates new functional capabilities of the protein in biochemical and developmental processes in which the protein either does not participate or has a different role; (3) change of the target function of another protein as in the case of the mutation in the protein C cleavage site at Arg 506 of coagulation factor V, which is associated with thrombophilia (370), or in the case of a mutation in the thrombin cleavage site of factor VIII that eliminates normal activation of factor VIII (365), or in the case of severe obesity from childhood and R236G in the human pro-opiomelanocortin (*POMC*) gene that disrupts the dibasic cleavage site between beta-melanocyte-stimulating hormone (beta-MSH) and beta-endorphin (371); and (4) participation of the mutant polypeptide in protein complexes, which renders the entire complex abnormal or nonfunctional, as in the case of the triple helical structure of certain collagens in which incorporation of one abnormal collagen chain results in “protein suicide” or an abnormal structure that degrades rapidly (372).

Missense mutations have a multitude of different effects on protein structure and function including (i) introduction of larger residues within the hydrophobic protein core leading to adverse interactions between residues (373,374), (ii) introduction of buried charged residues (373–375), (iii) disruption of protein–protein interactions (376), (iv) disruption of hydrogen bonding (373,375), (v) interference with DNA binding (373), (vi) breakage of disulfide covalent linkages (373), (vii) mutation of catalytic residues (375,377), (viii) perturbation of metal binding (373), (ix) loss of post-translational modification sites (378), (x) gain of intrinsic disorder (374,379), (xi) loss of stability (380,381), and (xii) disruption of quaternary structure (373,382).

Without in-depth analytical studies, missense mutations are often difficult to distinguish from polymorphisms with little or no clinical significance, either in the context of candidate gene sequencing studies (383) or in the context of exome sequencing studies (384). In the “post-genome era,” a substantial amount of human genetic variation will become amenable to high-throughput analysis in the form of SNPs, and many of these SNPs will influence directly the structure, function, or expression of genes and the RNAs/proteins they encode. Prior knowledge as to which SNPs are most likely to be clinically relevant would greatly enhance the power of studies

that aim to identify disease genes through the genotypic screening of patients in both families and populations. Inclusion of structural/functional information could be especially important in the elucidation of multifactorial disease, where genetic heterogeneity and complex interactions between genes and environment have so far limited the success of genetic epidemiological studies (385). Recently, several predictive models have been developed which employ a number of different biophysical parameters to estimate the likely functional impact of an amino acid substitution on the structure and function of a protein (7,380,386,387). These models have been used to distinguish reasonably and successfully between pathological substitutions, functional polymorphisms, and neutral polymorphisms. Vitkup et al. (388) have claimed that mutations at arginine and glycine residues are together responsible for about 30% of cases of genetic disease, whereas random mutations at tryptophan and cysteine have the highest probability of causing disease.

## 7.4.2 Mutations Affecting Gene Expression

Mutations that do not result in amino acid substitution invariably affect gene expression, that is, transcription, RNA processing, and maturation, translation, or protein stability. Total or partial gene deletions, insertions, inversions, and other gross rearrangements obviously result in the loss of gene expression. These types of mutations are usually less frequent unless the genomic sequence environment of specific genes (e.g. presence of repeats) predisposes to such lesions. Disorders with high frequencies of gross rearrangements include  $\alpha$ -thalassemia, DMD, steroid sulfatase deficiency, and hemophilia A. Some partial gene deletions that eliminate one or a few exons in-frame result in milder clinical phenotypes because gene expression is not totally eliminated; the resulting protein may lack an amino acid domain that is not critical for its function (389).

## 7.4.3 Promoter (Transcription Regulatory) Mutations

Microlesions within proximal gene regulatory regions currently comprise only ~1.7% of known mutations causing or associated with human inherited disease (see HGMD). Their relative rarity may be in part because not all regulatory elements occur immediately 5′ to the genes that they regulate. Indeed, many such elements are located within the first exon, within introns (390) or within 5′ or 3′ UTRs. In the same vein, upstream ORFs (uORFs), present in 50% of human genes, often impact on the expression of the primary ORFs; indeed, both mutations and polymorphisms have been reported within uORFs that can modulate or even abolish the expression of the downstream gene (391,392).

Mutations in known promoter motifs usually lead to reduced (or occasionally increased) mRNA levels. Such

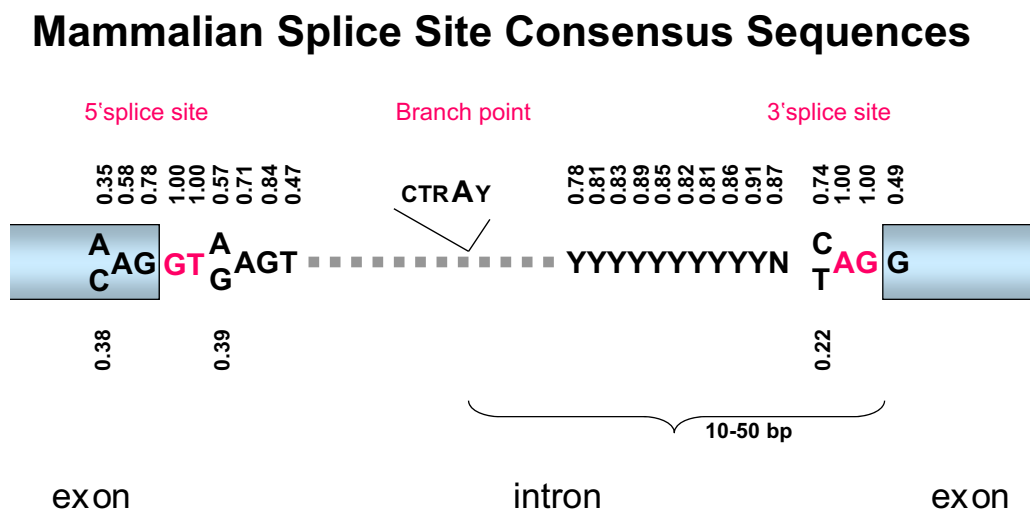
mutations have been studied in the TATA box of the  $\beta$ -globin (*HBB*) gene (393). Other disease-associated nucleotide substitutions occurring within DNA motifs that bind transcription factors include those located in the CACCC motif of the  $\beta$ -globin (*HBB*) gene influencing transcription factor EKLf binding (394,395), several motifs in the  $\gamma$ -globin (*HBB*) genes (396), the CCAAT motif of the *F9* gene influencing C/EBP binding (397), the SP1 motif of the *LDLR* gene promoter (398), the HNF-1 binding site in the *PROC* gene (399), and the binding site for the transcription factor Oct-1 in the lipoprotein lipase (*LPL*) gene (400). These few examples are only representatives of a total of over 2200 known promoter mutations listed in HGMD and causing human genetic disease. The importance of these mutants lies in the specific DNA sequences thereby implicated in binding to transcription factors. Although most of the known mutations reduce the levels of mRNA production, some substitutions actually increase it. Examples include various lesions in the promoters of the  $\gamma$  and  $\text{A}\gamma$  globin (*HBB1* and *HBB2*) genes that cause hereditary persistence of fetal hemoglobin (HPFH) due to the inappropriate continuation of  $\delta$ -globin (*HBB*) gene expression into adult life (401) and a gain-of-function (creates a GATA1 binding site) regulatory SNP, which is located in a non-genic region between the  $\alpha$ -globin genes and their upstream regulatory elements (402). An increase in the distance of promoter elements from the transcriptional start site may also result in gene silencing. Such an example has been found in the promoter elements of the *CSTB* gene in EPM1 (126). Mutations that alter the transcriptional regulation of gene expression have been reviewed in (403).

The concomitant change in local DNA sequence complexity surrounding a substituted nucleotide is directly related to the likelihood of a regulatory mutation coming

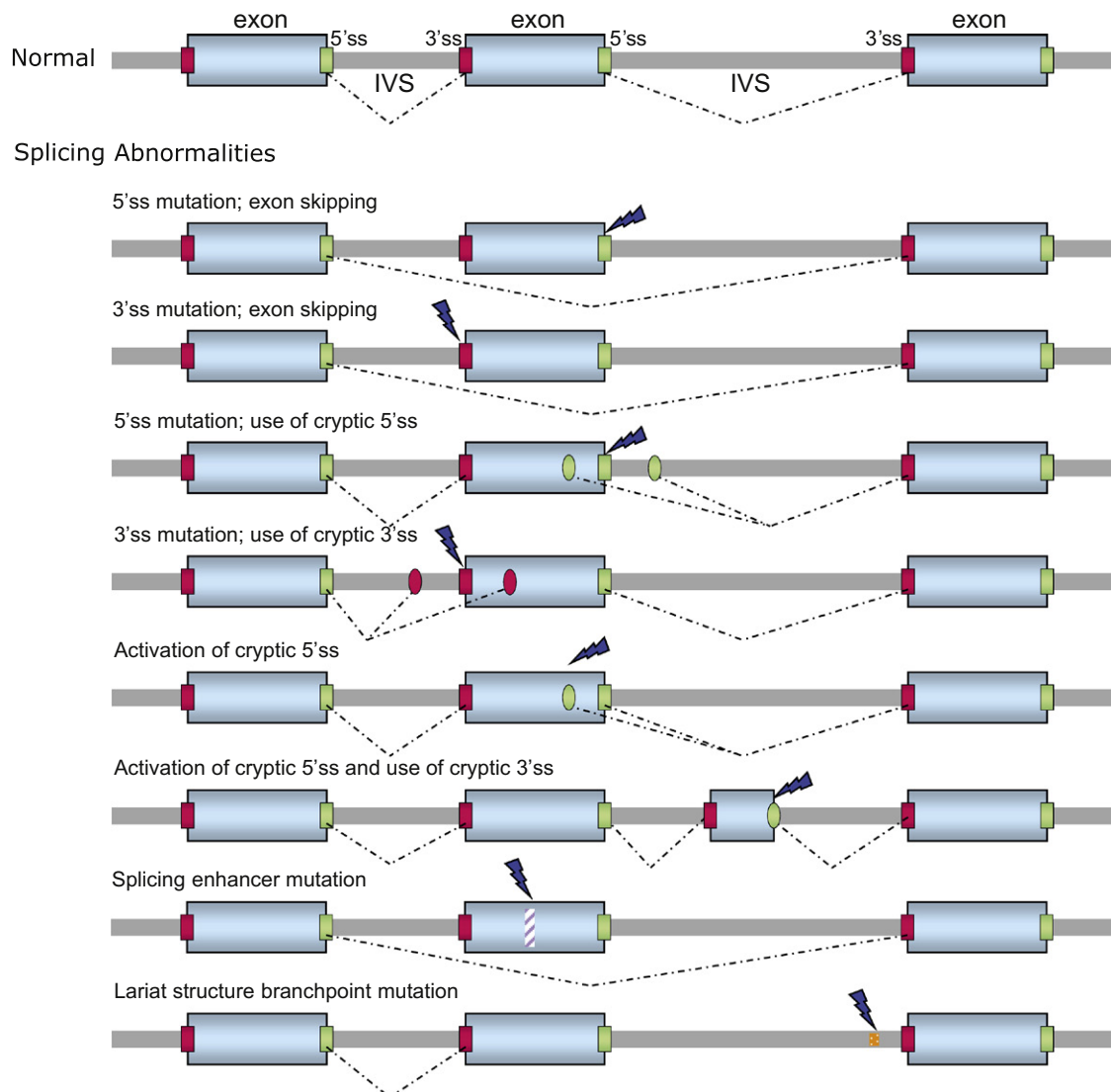
to clinical attention (67). This finding is consistent with the view that DNA sequence complexity is a critical determinant of gene regulatory function and may reflect the internal axial symmetry that frequently characterizes transcription factor binding sites. Polymorphisms in the promoter region that are associated with differential levels of gene expression may predispose to common disorders. For example, a G>A SNP, at nucleotide -6 relative to the transcriptional initiation site of the angiotensin (*AGT*) gene, influences the basal level of transcription and may predispose to essential hypertension (404). In excess of 400 disease-associated promoter polymorphisms are listed in HGMD plus >700 functional promoter polymorphisms that significantly increase or decrease promoter activity but which have not yet been associated with a clinical phenotype.

#### 7.4.4 mRNA Splicing Mutants

Single base-pair substitutions in splice junctions constitute at least 10% of all mutations causing human inherited disease. There are, however, a wide variety of mutations within both introns and exons that can affect normal RNA splicing (see Ref. (405) for review). The different mechanisms by which disruption of pre-mRNA splicing play a role in human disease have been reviewed in Ref. (406). The most commonly found mutations occur in the invariant dinucleotides GT and AG found at the beginning and end of the donor (5') and acceptor (3') consensus splice sequences (see Figure 7-11 for the consensus splice elements and Figure 7-12 for the different kinds of RNA splicing abnormalities). Almost all of these mutations cause either exon skipping or cryptic splice-site utilization resulting in the severe reduction or absence of normally spliced mRNA. In addition, mutations in nucleotides +3, +4, +5, +6, -1, and -2 of



**FIGURE 7-11** Consensus sequences for the donor (5' splice) and acceptor (3' splice) sites and the branchpoint. Numbers above or below the nucleotides correspond to frequencies of a given nucleotide in a large number of mammalian splice-site sequences. Note that the dinucleotides GT and AG (in red) at the beginning and end of the intron are invariant.



**FIGURE 7-12** Examples of splicing abnormalities in introns of human genes. Exons are shown as blue boxes; introns as lines between exons. Green squares denote the normal 5' (donor) splice sites; red squares represent the normal 3' (acceptor) splice sites. Green and red circles denote cryptic 5' and 3' splice sites, respectively. The broken blue wedge represents the site of mutation.

the consensus donor splice site have been frequently observed (Figure 7-13), with variable severity of the RNA splicing defect. Similarly, mutations in positions -3 and the polypyrimidine tract of the consensus acceptor splice site have been noted (Figure 7-13). In the majority of these cases, some normal splicing occurs and the defect is not severe. Utilization of cryptic splice sites leads to the production of abnormal mature mRNA with premature stop codons or to the inclusion of additional amino acids after translation (see Ref. (74) for examples and references cited therein).

Employing a neural network for splice-site recognition, Krawczak et al. (407) performed a meta-analysis of 478 disease-associated splicing mutations, in 38 different genes, for which detailed laboratory-based mRNA phenotype assessment had been performed. Inspection of the  $\pm 50$  bp DNA sequence context of the mutations revealed that exon skipping was the preferred phenotype when

the immediate vicinity of the affected exon-intron junctions was devoid of alternative splice sites. By contrast, in the presence of at least one such motif, cryptic splice-site utilization became more prevalent. This association was, however, confined to donor splice sites. Outside the obligate dinucleotide, the spatial distribution of pathological mutations was found to differ significantly from that of SNPs. Although disease-associated lesions clustered at positions -1 and +3 to +6 for donor sites and -3 for acceptor sites, SNPs were found to be almost evenly distributed over all sequence positions considered. When all putative missense mutations in the vicinity of splice sites were extracted from the HGMD for the 38 studied genes, a significantly higher proportion of changes at donor sites (37/152; 24.3%) than at acceptor splice sites (1/142; 0.7%) was found to reduce the neural network signal emitted by the respective splice site. It was estimated that some 1.6% of disease-causing missense

## HGMD Mutations in Intron splice sites (1-Dec-11)

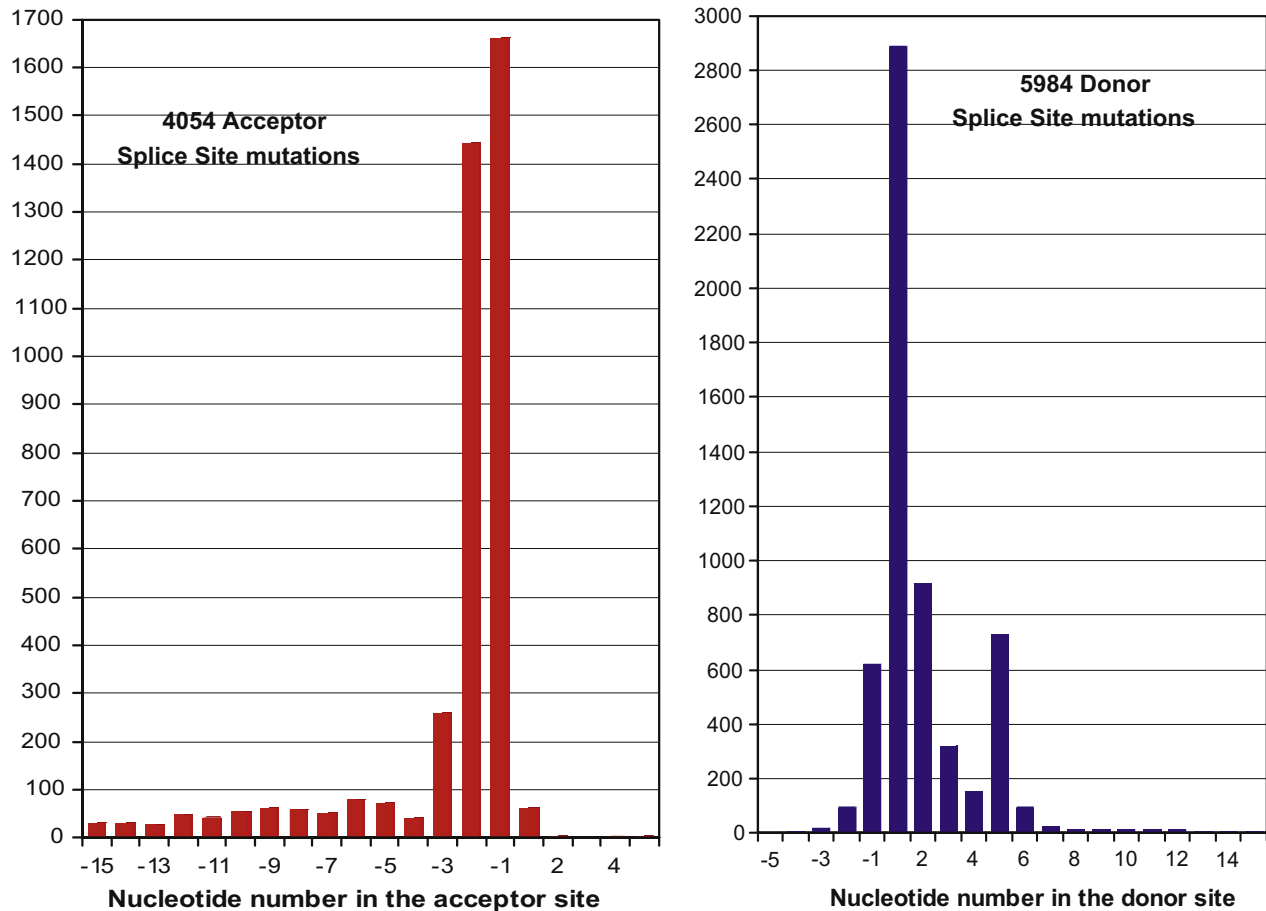


FIGURE 7-13 Mutations in the consensus sequences of splice junctions recorded in the HGMD.

substitutions in human genes are likely to affect the mRNA splicing phenotype (196).

Other kinds of mutations in introns include those that cause the activation of cryptic splice sites (by altering a sequence so as to make it more similar to an authentic consensus splice site) or by creation of new splice sites (408). In both instances, new intron splice patterns occur with consequent introduction of stop codons or abnormal peptides after translation. These mutations do not completely abolish normal splicing and are therefore not associated with the absence of normal mature mRNA. A mutation in a lariat structure branchpoint (409) has been found in the *L1CAM* gene in a patient with X-linked hydrocephalus (410). By contrast, another mutation in intron 5 of the type 2 neurofibromatosis (*NF2*) gene created a consensus branchpoint sequence and led to the activation of a cryptic exon (411).

Some 98.7% of all splice sites in human genes conform to consensus sequences that include the invariant dinucleotides GT and AG at the 5' and 3' ends of the introns, respectively (412). Noncanonical sequences (e.g. GA-AG, GC-AG, and AT-AC) do however occur at human splice junctions, albeit much less frequently (<0.02%, 0.69%, and 0.05%, respectively). Some of

these noncanonical splice sites are nevertheless known to be utilized with high efficiency and may be conserved over quite long stretches of evolutionary time. Such sites have occasionally come to clinical attention when they have harbored mutations causing human inherited disease (413). Moreover, the utilization of a cryptic non-canonical donor splice site within exon 1 of the *HRPT2* gene in a case of familial isolated primary hyperparathyroidism as a consequence of a causative lesion in intron 1 of the gene has been reported (414). RNA isolated from EBV-transformed lymphoblastoid cell lines derived from the patients was utilized to demonstrate the consequences at the level of the mRNA phenotype (the loss of 30 bases from the mRNA transcript).

Single base-pair substitutions within “splicing enhancer” sequences may also perturb splicing by promoting exon skipping; examples include a mutation in intron 3 of the growth hormone (*GH1*) gene causing short stature (415) and a mutation in exon 5 of the adenosine deaminase (*ADA*) gene causing ADA deficiency (416). In patients with frontotemporal dementia with parkinsonism, three heterozygous mutations in a cluster of four nucleotides +13 to +16 of exon 10 of the microtubule-associated protein tau (*MAPT*) gene destabilized a

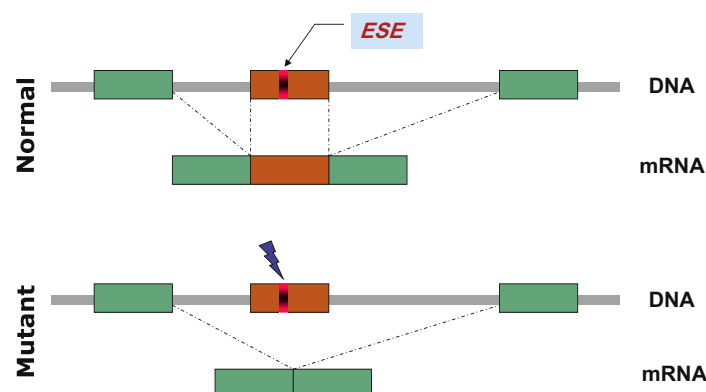


potential stem-loop structure that is probably involved in regulating the alternative splicing of exon 10. This caused more frequent use of the 5' splice site and an increased proportion of tau transcripts that include exon 10. The increase in exon 10+ mRNA increased the proportion of tau protein containing four microtubule-binding repeats, which is consistent with the neuropathology described in families with this type of frontotemporal dementia (417). One mutation found in the *ATM* gene causing ataxia-telangiectasia was a deletion of four nucleotides (GTAA) in intron 20 within an intron-splicing processing element (ISPE) that is complementary to U1 snRNA. This element mediates accurate intron processing and interacts specifically with U1 snRNP particles (418). Finally, the intronic prothrombin (*F2*) gene 19911A>G polymorphism influences splicing efficiency by altering a known functional pentamer CAGGG motif (419). Some nonsense mutations cause skipping of one or more exons, presumably during pre-mRNA splicing in the nucleus. This phenomenon has been termed “nonsense-mediated altered splicing” but its underlying mechanism is unclear. The first such mutation was described in the *FBN1* gene in Marfan syndrome (420). It is now recognized that any nucleotide substitution within exons (nonsense, missense, or translationally silent synonymous point mutation) that disrupts a splicing enhancer or silencer (ESE, enhancer splicing element; composite exonic regulatory element of splicing) or creates an exon splicing silencer (ESS) may affect either the pattern or the efficiency of mRNA splicing (89,421–423) (Figure 7-14). In exon 12 of the *CFTR* gene, about one quarter of synonymous variations result in exon skipping and, hence lead to the synthesis of an inactive CFTR protein (424). For reviews on the effects of exonic variants on splicing, and additional examples of such pathogenic mutations, see (425). It has been estimated that pathogenic effects of ~20% of mutations in the *MSH2* gene result from missense mutations that perturb splicing through the disruption of ESE sites. Similarly, the pathogenic effects of ~16% of missense mutations in the *MLH1* gene are thought to be

ESE related (426). Further, recent studies of exon splicing enhancer mutations have suggested that as many as 25% of known missense and nonsense mutations causing human inherited disease may alter functional splicing signals within exons (427,428). If this estimate is accurate, it suggests a much more widespread role for aberrant mRNA processing in causing human inherited disease than has hitherto been appreciated.

Splice-mediated insertional inactivation involving an *Alu* repeat was first reported by Mitchell et al. (429). Analysis of the ornithine  $\delta$ -aminotransferase (*OAT*) mRNA of a patient with gyrate atrophy revealed a 142 nucleotide insertion at the junction of exons 3 and 4. An *Alu* sequence is normally present in intron 3 of the *OAT* gene, 150 bp downstream of exon 3. The *Alu* sequence found in the cDNA was identical to this one, except that the patient was homozygous for a C→G transversion in the right arm of the *Alu* repeat which served to create a new 5' splice site. This activated an upstream cryptic 3' splice site [the poly(T) complement of the *Alu* poly(A) tail followed by an AG dinucleotide] and a new “exon,” containing the majority of the right arm of the *Alu* sequence, was recognized by the splicing apparatus and incorporated into the mRNA. The splice-mediated insertion of an *Alu* sequence in reverse orientation has also been reported in the *COL4A3* gene causing Alport syndrome (430). Deep intronic mutations, located at some considerable distance from splice sites and known splicing-related sequence elements, generally appear to comprise <1% of known splicing mutations (431–433). Such lesions often create novel splice sites thereby activating cryptic exons (“pseudoexons”). It should be appreciated that the <1% figure is very likely to be an underestimate owing to the inherent difficulty in detecting splicing mutations located outside of (and distant from) exon-intron splice junctions. Thus, for example, when the *NF1* gene was methodically screened for mutations that altered splicing, 5% of the identified lesions that altered splicing were deep intronic mutations (434). Among disease-causing lesions, inclusion of a pseudoexon as

**Exon Skipping due to mutations in enhancer splicing elements**



**FIGURE 7-14** Exon skipping due to nonsense, missense, and silent mutations in enhancer splicing elements (ESE). This element is shown as a darkened segment of the middle exon.

a consequence of cryptic splice-site activation appears to be the most common consequence of deep intronic mutation (435). If we also consider the deep intronic polymorphic variants that have the potential to confer susceptibility to disease (436–438), it is very likely that splicing-relevant intronic variation will have been seriously underascertained thus far.

### 7.4.5 RNA Cleavage-Polyadenylation Mutants

A number of examples of RNA cleavage-polyadenylation mutations have now been described (439). Those reported occur in the sequence AAUAAA, which is 10–30 nucleotides upstream of the polyadenylation site and is important for the endonucleolytic cleavage and polyadenylation of the mRNA. Mutation in this sequence of the  $\beta$ -globin (*HBB*) gene results in mild thalassemia (440). In these cases, normal polyadenylation and cleavage occurs at a level about 10% of normal. Alternative AAUAAA sites downstream of the mutated ones are used, resulting in larger mRNAs that are highly unstable. Other mutations near the poly(A) cleavage sequence may result in mRNA destabilization; one such mutation has been described 12 bp upstream of the AAUAAA sequence of the *HBB* gene in a patient with  $\beta$ -thalassemia (441).

The G>A mutation at the 3'-terminal nucleotide of the 3' UTR of the *F2* (prothrombin) gene mRNA gives rise to an elevated prothrombin plasma level and represents a common genetic risk factor for the occurrence of thromboembolic events. This mutation creates an inefficient 3' end cleavage signal and represents a gain-of-function mutation, causing increased cleavage site recognition, increased 3' end processing and increased mRNA accumulation and protein synthesis (442,443).

### 7.4.6 Mutations in MicroRNA-Binding Sites

MicroRNAs (miRNAs) posttranscriptionally down-regulate gene expression by binding to complementary sequences on the 3' UTRs of their cognate mRNAs, thereby inducing either mRNA degradation or translational repression. Over 700 human miRNAs have so far been identified but many more probably still remain to be discovered. These miRNAs are each likely to down-regulate a large number of different target mRNAs.

The first reported pathological mutation in an miRNA-binding site was a G→A transition in a binding site for miR-189 within the 3' UTR of the *SLITRK1* gene of two apparently unrelated Tourette syndrome patients (444). Experimental confirmation of the functional effect of this mutation came from the demonstration that, in the presence of miRNA-189, in vitro constructs bearing the 3' UTR mutation served to increase repression of a reporter gene by comparison with the wild type. A further example of a functional miRNA-target site variation involves an SNP in the 3' UTR of the human *AGTR1*

gene; although the variant allele is not downregulated by miR155, it has been associated with hypertension in numerous studies (445). An increasing number of genetic variants located in microRNA target sites are being reported, either causing or associated with an increased risk of inherited disease (446–450).

### 7.4.7 Mutations in Nonprotein-Coding Genes

In contrast to the plethora of mutations identified in protein-coding genes, the identification of mutations in nonprotein-coding genes is still very much in its infancy (451). A number of disease-causing or disease-associated mutations have already been reported in various small nucleolar RNA genes (452,453) and miRNA genes (454–457). In addition, mutations have also been documented in the longer noncoding RNA genes *XIST*, *TERC*, *H19*, and *RMRP* (see HGMD for details).

A putative pathological mutation has been described in a “gene” encoding a paternally expressed antisense transcript of the *GNAS* complex locus (*GNASAS*) (458), whereas a functional polymorphism has been reported within an enhancer at the 30 end of the *CDKN2BAS* “gene,” which encodes an antisense RNA transcript (459). A *CRYGE1* pseudogene-reactivating mutation associated with hereditary cataract formation (460) probably also falls into this category. The above examples are likely to comprise only the tip of a fairly large iceberg that still remains essentially unexplored. Thus, for example, both SNP and CNV are likely to impact significantly on miRNA gene expression with myriad potential pathological consequences (446).

### 7.4.8 Mutations in Noncoding Regions of Functional Significance

By adopting a gene-centric view, we have until now largely ignored the extensive nonprotein-coding portion of the human genome in our quest for mutations of pathological significance. As a consequence, we have not only seriously underestimated the extent of the functional component of the genome, but may also have overlooked many mutations within this genomic “dark matter” (461). In both the human and the mouse genomes, many noncoding regions exhibit a similar level of evolutionary conservation to that evident in protein-coding regions (462,463). As yet, however, little is known of the effect that mutations in these regions might have on either the phenotype or on overall fitness. Studies of the most evolutionarily conserved noncoding regions have yielded results that are consistent with the view that most mutations in noncoding regions are only slightly deleterious (463,464).

To obtain a first, necessarily rather crude, estimate of the contribution of variation in human noncoding sequences to phenotypic and/or disease traits, Visel

et al. (465) performed a meta-analysis of ~1200 SNPs that have been identified as the most significantly associated variants in published GWAS. They found that, in 40% of cases, neither the SNP in question nor its associated haplotype block overlapped with any known exons. These authors, therefore, concluded that in at least one-third of detected disease associations, variation in noncoding sequence rather than coding sequence could have causally contributed to the trait in question. We suspect that this could be because the common disease-common variant's hypothesis (466) may be much more likely to apply to noncoding sequence than to coding sequence, owing to the selectional constraints impacting on sufficiently frequent functional variation in the latter. In similar vein, others have also estimated that 39–43% of trait/disease-associated SNPs in GWAS are located within intergenic regions (467,468). This notwithstanding, it should be appreciated that any given variant apparently detected within a noncoding region may actually reside within a hitherto undiscovered exon (469). We should, however, also be aware that rare variants, in *cis* to those found to be associated with a given disease or trait in GWAS studies, may simply by chance give rise to “synthetic associations” that are then attributed to much more common variants (470).

In the context of identifying genetic variants responsible for human inherited disease, Cooper et al. (4) have argued that it will become increasingly important to consider functional elements in the genome (the “functionome”) rather than simply genes *per se*. We employ the term “functionome” here to describe the totality of the biologically functional nucleotide sequences in the human genome, irrespective of whether they are associated with genes or not. Because conserved noncoding sequences in the human genome appear to be ~10-fold more abundant than known genes (471), it is likely that (1) currently known mutations within coding regions are unlikely to be fully representative of the universe of pathological mutations and (2) a whole new grouping of disease-causing mutations may await identification and characterization. Once again, a paradigm shift in our thinking may well be required if we are to maximize the potential of the emerging high-throughput technology to detect new (hitherto latent) types of human gene mutation.

The above notwithstanding, it is rather unlikely that the functional nonprotein-coding portion of the human genome will prove to be quite as mutation-dense as the protein-coding portion. For most inherited disorders, the mutation detection rate is already fairly high (490%), although this success rate is often achieved by combining different mutation detection methodologies, for example, to screen for exon deletions and CNVs as well as more subtle lesions (472). At least some of the “missing lesions” may nevertheless be found by screening extragenic functional elements.

### 7.4.9 Cap Site Mutations

Transcription of the mRNA is initiated at the so-called cap site, which is protected from exonucleolytic degradation by the addition of  $\alpha$ -methylguanine. An A to C transversion at the cap site of the  $\beta$ -globin (*HBB*) gene was found in a patient with  $\beta$ -thalassemia (473). It is not, however, clear if this mutation causes reduced transcription or abnormal initiation of transcription since C is found in 6% of transcriptional initiation sites (474) (the most common nucleotide [76%] at position +1 is A). A functional (C/A) polymorphism of the transcriptional initiation site has been noted in the *APOH* gene; the rarer A allele displayed a carrier frequency of 0.12 and was associated with markedly reduced plasma  $\beta$ 2 glycoprotein I (475).

### 7.4.10 Mutations in 5' UTRs

Sequence motifs in the 5' UTRs of genes are thought to play a role in controlling the translation of the encoding mRNA. The phenotypic effects of lesions in 5' UTRs and their clinical consequences have been reviewed (476). Mutations in the iron response element (IRE) in the 5' UTR of the ferritin (*FTH1*) gene interfere with the post-transcriptional regulation of ferritin synthesis by decreasing the affinity of IRE for IRE-binding protein (477). By contrast, decreases in the steady-state level of  $\beta$ -globin (*HBB*) mRNA have been noted in association with a single base deletion at position +10, a G-to-A substitution at position +22, a C-to-G transversion at position +33, and a 4 bp deletion (AAAC) at position + (40–43) in the *HBB* 5' UTR (478–480).

### 7.4.11 Mutations in 3' Regulatory Regions

Sequences in the 3' regulatory regions (3' RRs) of genes are known to be involved in controlling mRNA cleavage/polyadenylation and determining mRNA stability, nuclear export, intracellular localization, and translational efficiency. Although such regions are rich in regulatory elements, relatively few pathological mutations have been reported (439,481). Although only ~0.2% of mutations currently logged in HGMD are located within 3' RRs, this is likely to represent a rather conservative estimate of their actual prevalence. A typical example is the G→A transition 69 nucleotides downstream of the polyadenylation site of the  $\delta$ -globin (*HBD*) gene causing  $\delta$ -thalassemia (482); the mutation occurs within a GATA motif and serves to increase the binding affinity of the sequence for erythroid-specific DNA binding protein.

In an attempt to study 3' RR mutations systematically, Chen et al. (439,483) performed a systematic analysis of disease-associated variants in the 3' RRs of human protein-coding genes. A total of 121 3' RR variants in 94 human genes were collated including 17 mutations

in the upstream core polyadenylation signal sequence (UCPAS), 79 in the upstream sequence (USS) between the translational termination codon and the UCPAS, 6 in the left arm of the “spacer” sequence between the UCPAS and the pre-mRNA cleavage site, 3 in the right arm of the “spacer” sequence or downstream core polyadenylation signal sequence, and 7 in the DSS of the 3'-flanking region. All the UCPAS mutations and the rather unusual cases of *DMPK*, *SCA8*, *FCMD*, and *GLA* mutations were found to exert a significant effect on the mRNA phenotype and the majority cause monogenic disease. By contrast, most of the remaining variants were polymorphisms, were found to exert a comparatively minor influence on mRNA expression, but may predispose to, protect from, or modify complex clinical phenotypes. The systematic study of these lesions permitted the identification of consistent patterns of secondary structural change that promise to allow the discrimination of nonfunctional USS variants from their functional counterparts.

#### 7.4.12 Translational Initiation Codon Mutations

Mutations in the ATG translational initiation codon have been reported in quite a wide variety of disorders (e.g. Ref. (484)). Instances of substitutions in all three nucleotides have been observed in  $\beta$ -thalassemia, Norrie disease, albinism, phenylketonuria, McArdle disease, and Albright osteodystrophy among others. Indeed, a total of 405 mutations within ATG translational initiation codons are recorded in HGMD representing ~0.7% of the total number of reported coding sequence mutations causing human inherited disease. Almost invariably, the mutation leads to severe reduction of steady-state mRNA levels similar to that associated with nonsense mutations. The mutant mRNA is presumably not translated. The first AUG codon occurs in the context of the so-called Kozak consensus sequence GCCA/GCCAUGG, which is thought to be recognized by the 40S ribosomal subunit (485). Mutations at the initiator methionine ATG may completely abolish translation; however, there are alternative possibilities, namely utilization of the mutant ATG with much reduced efficiency or translational initiation at the next available ATG codon. A C/T polymorphism immediately 5' to the ATG codon within the Kozak sequence of the *CD40* gene is thought to influence translation efficiency (486).

Some diseases are caused by mutations that perturb the initiation step of translation by changing the context around the start AUG codon or introducing upstream AUG codons (see Ref. (487) for review). The scanning mechanism provides a framework for understanding the effects of these changes in mRNAs. The scanning mechanism refers to the entry of the small ribosomal subunit at the (usually capped) 5' end of the mRNA and linear migration until an AUG codon is

encountered. Mutational mechanisms such as (i) reinitiation at an internal start codon (e.g. thrombopoietin, *TPO*) and (ii) leaky scanning (as in the case of the *Rx/rax* gene underlying the mouse eyeless mutation) probably account for such cases.

Naturally occurring mutations in the GCCA/GCCAUGG motif include (for the numbering of the mutant nucleotide, the A of the AUG codon is +1; see references in Ref. (487)): (i) +4 G-to-A in the androgen receptor (*AR*) gene in a family with partial androgen insufficiency; (ii) -1 C-to-T transition in the  $\alpha$ -tocopherol transfer protein (*TTPA*) gene in a family with vitamin E deficiency; (iii) a 2 nt deletion causes an A-to-C change at position -3 of the  $\alpha$ -globin gene (*HBA*) in a patient with  $\alpha$ -thalassemia; (iv) -3 A-to-T transversion in the mouse *Pax6* gene causes defects in eye development; and (v) -3 G-to-C somatic mutation in the *BRCA1* gene in one case of highly aggressive sporadic breast cancer. It is not surprising that most of the naturally occurring mutations involve positions -3 and +4, the positions wherein experimentally induced mutations have the strongest effect.

A meta-analysis of 405 unique (HGMD-derived) single base-pair substitutions, located within the ATG translation initiation codons of 255 different genes, reported to cause human genetic disease has been performed (488). Although these lesions comprised only 0.7% of coding sequence mutations in HGMD, they nevertheless were 3.4-fold overrepresented as compared to other missense mutations. The distance between a translation initiation codon and the next downstream in-frame ATG codon was significantly greater for genes harboring ATG codon mutations than for the remainder of genes in HGMD (control genes). This suggests that the absence of an alternative ATG codon in the vicinity of an ATG translation initiation codon increases the likelihood that a given ATG mutation will come to clinical attention. An additional 42 single base-pair substitutions in 37 different genes were identified in the vicinity of ATG translation initiation codons (positions -6 to +4, comprising the Kozak consensus sequence). These substitutions were however not evenly distributed, being significantly more abundant at position +4. Finally, contrary to the authors' initial expectation, the match between the original translation initiation codon and the Kozak consensus sequence was significantly better (rather than worse) for genes harboring ATG codon mutations than for the HGMD control genes (488).

#### 7.4.13 Termination Codon (“Nonstop”) Mutations

“Nonstop” mutations are single base-pair substitutions that occur within translational termination (stop) codons, which can lead to the continued and inappropriate translation of the mRNA into the 3'-UTR. The classic example of a termination codon mutant is the case of the  $\alpha_2$ -globin Constant Spring, with a mutation in the normal



stop codon; this substitution leads to incorporation of an additional 31 amino acid residues in the  $\alpha_2$ -globin polypeptide chain (489). The resulting protein is unstable and does not interact properly with the  $\beta$ -globin chains of hemoglobin. Some 119 mutations within Term codons (in 87 different genes) have been recorded in HGMD, representing ~0.2% of all missense/nonsense mutations.

A meta-analysis of these 119 nonstop mutations noted a paucity of alternative in-frame stop codons in the immediate vicinity (0–49 nucleotides downstream) of the mutated stop codons as compared with their control counterparts (490). This implies that at least some nonstop mutations with alternative stop codons in close proximity will not have come to clinical attention, possibly because they will have given rise to stable mRNAs (not subject to nonstop mRNA decay) that are translatable into proteins of near-normal length and biological function. A significant excess of downstream in-frame stop codons was, however, noted in the range 150–199 nucleotides from the mutated stop codon (490). The authors speculated that recruitment of an alternative stop codon at greater distance from the mutated stop codon might trigger nonstop mRNA decay, thereby decreasing the amount of protein product and yielding a readily discernible clinical phenotype.

#### 7.4.14 Frameshift Mutations

A large number of frameshift mutations have been described in numerous disease-related genes. All lead to altered translational termination with abnormal polypeptide chains after the frameshifts; severe phenotypes are usually seen (491). Frameshifts occur with micro-deletions or micro-insertions and exon skipping. The mechanisms underlying these mutations were discussed earlier in this chapter.

#### 7.4.15 Nonsense Mutations

Nonsense mutations give rise to premature termination of translation and truncated polypeptides. They account for ~11% of all described gene lesions causing human inherited disease and ~20% of disease-associated single base-pair substitutions affecting gene coding regions (492). Pathological nonsense mutations resulting in TGA (38.5%), TAG (40.4%), and TAA (21.1%) occur in different proportions to naturally occurring stop codons (492). Of the 23 different nucleotide substitutions giving rise to nonsense mutations, the most frequent are CGA → TGA (21%; resulting from methylation-mediated deamination) and CAG → TAG (19%) (492). The differing nonsense mutation frequencies are largely explicable in terms of variable nucleotide substitution rates such that it is unnecessary to invoke differential translational termination efficiency or differential codon usage. Nonsense mutations are usually associated with a reduction in the steady-state level of cytoplasmic mRNA (493).

This mechanism of “NMD” is responsible for the degradation of mRNAs that contain a premature termination codon at a position at least 50 nt upstream of an exon–exon boundary (494) but it is not universal (495). One or more parameters could be affected: the transcription rate, the efficiency of mRNA processing or transport to the cytoplasm, or mRNA stability.

In the majority of described instances of nonsense mutations, the resulting disorders are recessive in nature as a consequence of the haploinsufficiency resulting from the NMD-induced absence of the truncated proteins (which ensures that such polypeptides do not interfere with the function of the wild-type protein). Nonsense mutations that do not elicit NMD can, however, give rise to a dominant negative condition (e.g. mutations in the *SOX10* gene causing Waardenburg Shah syndrome; (496)). Since for NMD to be activated, the nonsense mutation must reside at least 50–55 nt upstream of an exon–exon boundary, it follows that the precise location of the nonsense mutation could be an important factor in predicting the pathogenicity of that lesion. By way of example, nonsense mutations within the last exon of the human  $\beta$ -globin (*HBB*) gene do not elicit NMD. As a consequence, the truncated  $\beta$ -globin product has near-normal abundance, fails to associate properly with  $\alpha$ -globin, and hence gives rise to a dominantly inherited form of  $\alpha$ -thalassemia (24). Different nonsense mutations within the same gene may thus be associated with different clinical phenotypes depending on whether or not NMD is activated. Another example of this is provided by a nonsense mutation (Q37X) in the *DAX1* gene of an adrenal hypoplasia congenita patient; this lesion is associated with a milder-than-expected clinical phenotype on account of the expression of a partially functional, amino terminal-truncated DAX1 protein synthesized from an alternative in-frame translational start site at Met83 (497). In a recent meta-analysis, the proportion of known disease-causing nonsense mutations predicted to elicit NMD was found to be significantly higher than among nonobserved (potential) nonsense mutations, implying that nonsense mutations that elicit NMD are more likely to come to clinical attention (492). In practical terms, the observation of greatly reduced or absent cytoplasmic mRNA associated with nonsense mutations has important implications for mutation screening. Thus, attempts to obtain mRNA for RT-PCR and mutation detection may result in amplification of nucleic acid from only the non-nonsense mutation-bearing allele. Nonsense mutations in the factor VIII (*F8*) gene (hemophilia A) and fibrillin (*FBN1*) gene (Marfan syndrome) have been associated with the skipping of exons containing these mutations (240,420) and this observation has now been extended to other genes; exon skipping is either complete or partial. The mechanism underlying this phenomenon is unknown although a number of intriguing models have been proposed (498).

Some genes are characterized by numerous nonsense mutations but relatively few if any missense mutations (e.g. *CHM*), whereas other genes exhibit many missense mutations but few if any nonsense mutations (e.g. *PSEN1*). Genes in the latter category have a tendency to encode proteins characterized by multimer formation (492). Consistent with the operation of a clinical selection bias, genes exhibiting an excess of nonsense mutations are also likely to display an excess of frameshift mutations (492). Recently, an example of the spontaneous read-through of a premature termination codon was reported in a patient who was a compound heterozygote for two nonsense mutations in the *LAMA3* gene (R943X/R1159X) (499). The patient, who presented with junctional epidermolysis bullosa, was expected to die as a consequence of harboring these nonsense mutations but was “rescued” by spontaneous read-through of the R943X-bearing allele. This patient’s full-length R943X-bearing *LAMA3* mRNA escaped nonsense-mediated decay, thereby ensuring near-normal *LAMA3* mRNA and laminin- $\alpha 3$  protein levels. The genetic context of the *LAMA3* mutation R943X was found to be close to a hypothetical consensus sequence for optimal premature termination codon read-through.

#### 7.4.16 Unstable Protein Mutants

Missense mutations can cause abnormal protein folding and are, therefore, associated with reduced expression owing to instability of the protein. Reviews of mutations that affect protein stability can be found in Refs. (500,501). For proteins that circulate in body fluids, most mutations are associated with “CRM negative” status in which the amount of protein correlates with the amount of activity or “CRM reduced” status in which the amount of activity is still lower than the amount of protein produced. Many such mutations have been seen in factor VIII causing mild/moderate hemophilia A (76).

The nature of the biophysical properties of amino acid substitutions in p53 that increase their likelihood of coming to clinical attention has been explored (502); these include solvent inaccessibility, the number of adverse steric interactions introduced, and a reduction in H-bond number. This study was extended by modeling *in silico* all amino acid replacements that could potentially have arisen from an inherited single base-pair substitution in five human genes encoding arylsulfatase A (*ARSA*), antithrombin III (*SERPINC1*), protein C (*PROC*), phenylalanine hydroxylase (*PAH*), and transthyretin (*TTR*) (386). A total of 9795 possible mutant structures were modeled and 20 different biophysical parameters assessed. Comparison with the HGMD-derived spectra of 469 clinically detected mutations indicated that several types of mutation-associated change affected protein function, including the energy difference between wild-type and mutant structures, solvent accessibility of the mutated residue, and distance from the binding/

active site. These parameters are considered to be important in protein folding which adds support to the view that many missense mutations come to clinical attention by virtue of their consequences for protein folding and stability (503,504).

#### 7.4.17 Mutations in Remote Gene Regulatory Elements

In the  $\beta$ -globin gene cluster, a regulatory region about 10kb upstream of the  $\epsilon$ -globin (*HBE*) gene has been identified that is capable of directing a high level of position-independent  $\beta$ -globin gene expression (505). This region, termed the locus control region (LCR), is thought to organize the entire 60kb  $\beta$ -globin gene cluster into an active chromatin domain and to enhance the transcription of individual globin genes (506). A similar LCR is also present in the  $\alpha$ -globin gene cluster and other gene clusters (507). Deletions of the LCR in the  $\beta$ -globin gene cluster result in silencing of the  $\beta$ -globin (*HBB*) and other genes of the cluster, even although the coding regions of these genes are still intact (401). A particular 25kb deletion, known as Hispanic  $\gamma\delta\beta$ -thalassemia, which deletes sequences 9.5–39kb upstream of the  $\epsilon$ -globin (*HBE*) gene including the LCR renders the *HBB* gene 60kb downstream of the deletion nonfunctional (508). This extraordinary effect of the deletion of the LCR is thought to be due to an altered (DNase I-resistant) state of chromatin associated with nonfunctional genes. Several other examples of similar deletions in the LCR of the  $\alpha$ -globin gene cluster have been reported (509).

Several other examples of remote regulatory elements have come to attention as a consequence of their ablation by gross deletions located at some considerable distance (from 10kb to several megabases) from the genes whose expression they disrupt (150). For instance, a 960-kb deletion of noncoding sequence, lying between 1.477Mb and 517kb upstream of the *SOX9* gene gives rise to the acampomelic form of campomelic dysplasia (510). Such pathological deletions, however, are not necessarily always so large. Indeed, a 7.4kb deletion, located 283kb upstream of the *FOXL2* gene, has been identified as a cause of blepharophimosis syndrome; it disrupts a long noncoding RNA (*PISRT1*) as well as eight conserved noncoding sequences (511). For some conditions, such lesions may actually occur quite frequently, as in the case of the *SHOX* gene where ~22% of Leri–Weill syndrome patients and ~1% of individuals with idiopathic short stature harbor a micro-deletion spanning the upstream enhancer region that leaves the coding region of the *SHOX* gene intact (512).

Over the last few years, a number of other examples of mutations in remote promoter elements have been reported. These include a total of nine mutations within a 1-kb region (termed the long-range or limb-specific enhancer) ~979kb 5' to the transcriptional initiation site of the sonic hedgehog (*SHH*) gene (513)

and a T>C transition 1.44Mb upstream of the *SOX9* gene associated with cleft palate/Pierre Robin sequence (514). Far upstream polymorphic variants that influence gene expression and that are relevant to disease are also beginning to be documented. Thus, for example, the C>T functional SNP 14.5 kb upstream of the *IRF6* gene, associated with cleft palate, alters the binding of transcription factor AP-2 $\alpha$  (515). Similarly, a functional SNP ~6kb upstream of the  $\alpha$ -globin-like *HBM* gene serves to create a binding site for the erythroid-specific transcription factor GATA1 and interferes with the activation of the downstream  $\alpha$ -globin genes (402). A functional SNP ~335 kb upstream of the *MYC* gene increases the risk of colorectal and prostate cancer by increasing the expression of the *MYC* gene by altering the binding strength of transcription factors TCF4 and/or TCF7L2 to a transcriptional enhancer (516–519). Finally, in the context of pointing out the shortcomings of the gene-centric approach to mutation detection, we should be aware that functional SNP rs4988235, located 13.9kb upstream of the lactase (*LCT*) gene and associated with adult-type hypolactasia, actually resides deep within intron 13 (c.19171+326C>T) of the *MCM6* gene (520–522). Given that up to 5% of quantitative trait loci for gene expression lie >20kb upstream of transcriptional initiation sites (523), many more far upstream polymorphic variants that influence gene expression are likely to be identified in the coming years.

Rather fewer pathological mutations are known to be located at a considerable distance downstream of human genes. One example is the C>G transversion 2528 nt 3' to the term codon of the *CDK5R1* gene, which has been postulated to play a role in nonspecific mental retardation (524). Perhaps more dramatic is the A>G SNP (rs2943641), 565981bp 3' to the Term codon of the *IRS1* gene, which is associated with type 2 diabetes, insulin resistance, and hyperinsulinemia; the G allele was found to be associated with a reduced basal level of *IRS1* protein (525).

In the light of the above, it can be seen that the underascertainment of disease-associated mutations within regulatory regions is likely to be quite substantial but can potentially be rectified by emerging high-throughput entire genome sequencing protocols.

#### 7.4.18 Cellular Consequences of Trinucleotide Repeat Expansions

Trinucleotide repeat expansion has been discussed earlier. In the case of fragile X, the (CGG) $_n$  repeat is located in the 5' UTR of the *FMR1* gene, and its expansion to full mutation results in hypermethylation of the promoter region, loss of transcription, and hence silencing of the gene (526). Loss of the encoded protein, fragile X mental retardation protein (FMRP), which is thought to play a role in dendritic mRNA transport and translation, is responsible for the classical fragile X syndrome

phenotype. Gene inactivation can also be caused by altering the spacing of promoter elements from the transcriptional start site as in the case of the 12mer repeat expansion in the *CSTB* gene (126).

When the trinucleotide repeat lies within the gene-coding region as in Huntington disease, its expansion results in an abnormal protein with a gain of function due to the enlargement of the polyglutamine tract. Mutant huntingtin exerts its pathological effects via abnormal protein aggregation, transcriptional dysregulation, mitochondrial dysfunction, excitotoxicity, and abnormal cellular trafficking, leading to neuronal loss particularly in the dorsal substratum (527).

Another example of a gain-of-function mutation is provided by the expansion of the CTG repeat in the 3' UTR of the *DMPK* gene causing type 1 myotonic dystrophy (DM1). This does not abolish transcription but rather causes nuclear retention of RNA transcripts leading to the transcriptional dysregulation of other genes (528). CTG expansion appears to lead to the sequestration of cellular RNA-binding proteins, which in turn gives rise to the abnormal splicing of multiple transcripts (529). DM1 thus exemplifies a disease whose mechanistic basis lies at the RNA level.

#### 7.4.19 Mutations That Give Rise to Inappropriate Gene Expression

HPFH and hereditary persistence of  $\alpha$ -fetoprotein (HPAFP) are two clinical conditions that are prototypes for the inappropriate expression of  $\gamma$ -globin (*HBG1* and *HBG2*) and  $\alpha$ -fetoprotein (*AFP*) genes, respectively. Normally, the levels of fetal hemoglobin (HbF;  $\alpha_2\gamma_2$ ) in adult life are very low, as there is a switch from fetal to adult hemoglobin during the perinatal period. Similarly, AFP is produced at high level in fetal liver but declines rapidly after birth. In HPFH and HPAFP, however, the levels of HbF and AFP, respectively, are inappropriately high in adult life. This is often due to single nucleotide substitutions in the promoter regions of the *HBG2*, *HBG1*, or *AFP* genes. A considerable number of mutations that occur in the region -114 to -202 of the  $\gamma$ -globin genes have been characterized and presumably cause persistent expression of their corresponding genes (401). A similar situation has been observed with a -119 mutation in the *AFP* gene (530). These mutations occur within DNA binding motifs for transcriptional regulators. A very interesting mutational mechanism has been proposed for facioscapulohumeral muscular dystrophy (FSHD), a common autosomal dominant myopathy associated with a typical pattern of muscle weakness. Most FSHD patients carry a large deletion of a 4q35-located polymorphic D4Z4 macrosatellite repeat array and present with fewer than 11 repeats whereas normal individuals possess between 11 and 150 repeats (531). An almost identical D4Z4 repeat array is present at 10q26 (532) and the high sequence homology between these two

arrays can cause difficulties in molecular diagnosis. Each 3.3 kb D4Z4 repeat contains a *DUX4* (double homeobox 4) gene that, among others, is activated on contraction of the 4q35 D4Z4 array due to the induction of chromatin remodeling of the 4qter region. An increasing number of 4q subtelomeric sequence variants are now recognized, although FSHD only occurs in association with three “permissive” haplotypes, each of which are associated with a polyadenylation signal located immediately distal of the last D4Z4 repeat (533). This poly-A signal stabilizes any *DUX4* mRNAs transcribed from this most distal D4Z4 repeat in FSHD muscle cells. Synthesis of both the *DUX4* transcripts and the protein in FSHD muscle cells induces significant cell toxicity. *DUX4* is a transcription factor that targets several genes which results in a deregulation cascade that inhibits myogenesis, sensitizes cells to oxidative stress and induces muscle atrophy, thereby recapitulating many of the key molecular features of FSHD (534).

#### 7.4.20 Position Effect in Human Disorders

In several instances, a DNA alteration is found well outside the putative gene that is primarily involved with a disease. Mutations acting by “positional effect” are those in which the transcription unit and minimal promoter of the gene remain intact, but there is a nearby alteration that influences gene expression (535). These positional effect DNA lesions may involve distal promoter regions, enhancer/silencer elements, or changes in the local chromatin environment. The positional effect could be up to several megabases away from the gene of interest. The examples of the LCR in the  $\beta$ -globin gene cluster and the transcriptional repressor D4Z4 in FSHD are provided elsewhere in this chapter. Most of the position effects are due to chromosomal rearrangements that frequently lead to alteration of the chromatin environment of the gene. Possible mechanisms which may lead to a positional effect include the following: (i) The rearrangement separates the transcription unit from distant *cis*-regulatory elements (enhancer removal results in gene silencing, whereas silencer removal results in inappropriate gene activation); (ii) juxtaposition of the gene with an enhancer element from another part of the genome; (iii) removal of an insulator or boundary element may also lead to inappropriate gene silencing; (iv) enhancer competition of DNA sequences that were juxtaposed to the gene; (v) positional effect variegation in which the chromosomal rearrangement causes the juxtaposition of an euchromatic gene with a region of heterochromatin.

Some examples of positional effect mutations due to translocation breakpoints include genes *PAX6* in aniridia (536), *SOX9* in campomelic dysplasia (537,538), *POU3F4* in X-linked deafness (539), *HOXD* complex in mesomelic dysplasia (540), *FOXL2* in blepharophimosis/ptosis/epicanthus inversus syndrome (BPES) (541,542),

and the *SHH* gene in preaxial polydactyly (543). In these cases, the translocation breakpoints may be in excess of a megabase away from the inappropriately expressed/silenced gene. Indeed, in one example of campomelic dysplasia, the breakpoint maps ~1.3 Mb downstream of the *SOX9* gene, making this the longest-range position effect so far found (538). For a recent review of position effect mutations, see (544).

It is likely that in the majority of cases, the position effect involves a highly conserved *cis*-acting regulatory element. These *conserved noncoding elements* (CNCs; also termed multiple-species conserved sequences; conserved non-genic sequences; the most highly conserved are also called ultra-conserved elements) comprise approximately 1–2% of the human genome and represent potential targets for pathogenic mutations (545–549). An example of such a lesion is provided by the 52 kb deletion of a large noncoding region downstream of the sclerostin (*SOST*) gene in patients with van Buchem disease, leading to altered expression of the *SOST* gene (550). The deletion disrupts a bone-specific enhancer element that drives *SOST* gene expression.

Pathogenic mutation may also occur in nonconserved elements that could become functional after the introduction of the mutant sequence. This pathogenetic mechanism has been described underlying a variant form of  $\alpha$ -thalassemia. Affected individuals from Melanesia have a gain-of-function regulatory single nucleotide polymorphism (rSNP) in a non-genic region between the  $\alpha$ -globin genes and their upstream regulatory elements. The rSNP creates a new promoter-like element that interferes with the normal activation of all downstream  $\alpha$ -like globin genes (402).

#### 7.4.21 Position Effect by an Antisense RNA

An individual with an inherited  $\alpha$ -thalassemia has been described who has a deletion that results in a truncated, widely expressed gene (*LUC7L*) becoming juxtaposed to a structurally normal  $\alpha$ -globin (*HBA2*) gene. Although it retained all of its local and remote *cis*-regulatory elements, expression of the *HBA2* gene was nevertheless silenced and its CpG island became completely methylated at an early stage during development. The antisense RNA of the *LUC7L* gene appears to have been responsible for the silencing of the *HBA2* gene (551).

#### 7.4.22 Abnormal Proteins Due to Fusion of Two Different Genes

The translation of fusion genes results in novel proteins with different or abnormal properties from their parent polypeptides. Fusion genes are either the result of homologous unequal crossing-over or the junction sequences at breakpoints of chromosomal translocations. Hemoglobin Lepore, a fusion of  $\delta$ - and  $\beta$ -globin genes, is the prime example of the first mechanism. Other examples



of abnormal fusion genes due to unequal crossover include the case of glucocorticoid-suppressible hyperaldosteronism (GSH), an autosomal dominant form of hypertension, caused by oversecretion of aldosterone (552); some GSH patients have hybrid genes between *CYP11B1* and *CYP11B2*, two highly homologous cytochrome P450 genes on 8q22. The hybrid gene contains the regulatory elements of *CYP11B1*, expressed in the adrenal gland, and the 3' coding region of *CYP11B2*, which is essential for aldosterone synthesis. Another example is the case of abnormalities of color vision resulting from fusion of the green and red color pigment (*RCP*, *GCP*) genes (553). Recombination between the Kallmann gene on Xp22.3 (*KALX*) and its homolog (*KALY*) at Yp11.21 results in a fusion gene that is transcriptionally inactive and is associated with Kallmann syndrome secondary to an X;Y translocation. Finally, Francis et al. (554) identified a large atypical hemolytic uremic syndrome family in whom a deletion occurred through MMEJ rather than by NAHR. The deletion resulted in the formation of a *CFH/CFHR3* hybrid gene. The protein product of this gene, a 24 short consensus repeat protein, was found to be secreted at slightly lower levels than wild-type factor H, but the decay accelerating and cofactor activities of this protein were significantly impaired. A growing number of hematologic malignancies are associated with abnormal fusion proteins, the genes of which are found at the breakpoints of chromosomal translocations. One of the first reported examples was the case of fusion of the *BCR* and *ABL* genes in the *t* (9;22) known as Philadelphia (Ph) chromosome in chronic myelogenous leukemia. The *BCR* gene is on chromosome 22 and the *ABL* gene is on chromosome 9; after the translocation junction, a fusion gene is created with the promoter elements of the *ABL* gene and the 3' half of the *BCR* gene (555). A new abnormal protein is detected in the leukemia cells, the abnormal function of which probably contributes to the malignant phenotype. Another example is the case of Ewing sarcoma (a solid tumor of bone) in which an 11;22 translocation results in a fusion of the *FLI1* gene on 11q24 with the *EWS* gene on 22q12 (556) and for a classical review see (557). Fusion genes can be readily identified by PCR and can serve either as diagnostic indicators for relapse in the disorders concerned or as indicators of the need for an alternative therapeutic regimen.

#### 7.4.23 Mutations in Genes Involved in Mismatch Repair Associated with Genomic Instability in the Soma

The study of somatic mutation is extremely important both for the study of cancer (558) and for other diseases such as paroxysmal nocturnal hemoglobinuria (559). Mutations that lead to abnormal or abolished function of genes encoding for proteins involved in DNA mismatch repair are of particular importance because they lead to accumulation of mutations throughout the

genome. For example, some forms of hereditary nonpolyposis colon cancer (HNPCC), which may account for up to 10% of colon carcinoma, are due to mutations in genes such as *MSH2* or *MLH1* that encode mismatch repair proteins (560–562). In families with mutations in these genes, the DNA of tumor tissue shows considerable instability as detected by the generation of new alleles for numerous DNA polymorphic markers (563). One of the genes affected by the genomic instability is that encoding the type II transforming growth factor- $\beta$  (TGF- $\beta$ ) receptor, (*TGFB2R*) which has a run of 10 adenines in its coding region. This run of As is altered, resulting in a frameshift and absence of the receptor, which in turn releases the cell from TGF- $\beta$  inhibitory effects and contributes to malignancy (564). The discovery and further study of genes of the mutation repair system will enhance our understanding of both germline and somatic mutations.

#### 7.4.24 Comparison of Germline and Somatic Mutational Spectra

To date, relatively few studies have attempted to compare the germline and somatic mutational spectra for the same genes. This notwithstanding, the mutational mechanisms underlying single base-pair substitutions (565,566), micro-deletions, and micro-insertions (566–568), and even gross gene rearrangements (569,570) often appear to exhibit similarities between the germline and the soma. Ivanov et al. (571) performed a comparison of somatic, germline, shared (found in both soma and germline), and somatic recurrent mutational spectra for 17 human tumor suppressor genes, which focused upon missense single base-pair substitutions and micro-deletions/micro-insertions. The somatic and germline mutational spectra for these genes were similar in relation to C.G>T.A transitions but differed with respect to the frequency of A.T>G.C, A.T>T.A, and C.G>A.T substitutions. Shared missense mutations were found to be characterized by higher mutability rates, greater physicochemical differences between the wild-type and mutant residues, and a tendency to occur in evolutionarily conserved residues and within CpG/CpHpG oligonucleotides. Mononucleotide runs of  $\geq 4$  bp were identified as hotspots for shared micro-deletions/micro-insertions.

#### 7.4.25 Mosaicism

*Germline mosaicism* is a relatively frequent mechanism of inherited disease and provides an explanation for the inheritance pattern in cases where multiple affected offspring are born to clinically and phenotypically normal parents (572). It arises through the occurrence of a mutation de novo in a germline cell or one of its precursors during the early embryonic development of the parent. Since mitotic divisions predominate in both spermatogenesis and oogenesis, most germline mutations are

likely to be mitotic rather than meiotic in origin. *Somatic mosaicism* results from mutations occurring during mitotic cell divisions in the embryo with subsequent clonal expansion of the affected cells (573). The clinical effect of somatic mosaicism depends critically upon the developmental stage at which the mutation occurs. Thus, a mutation that occurs very early on in embryonic development is likely to affect many somatic tissues. By contrast, mutations occurring rather later may give rise to a phenotype that is confined to a single body region or even to a single organ. Somatic mosaicism arising at a very early embryonic stage can involve both somatic cells and germ cells. Such individuals (*gonosomal mosaics*) are at risk of having affected children. Recent data support the postulate that the frequency of mosaicism is increased in cancer predisposition syndromes characterized by high new mutation rates, suggestive of a direct relationship between the mutation rate in the soma and that in the germline (574).

#### 7.4.26 Human Mutation Rates

Recent studies have estimated the human mutation rate per nucleotide per generation to be between  $7.6 \times 10^{-9}$  and  $2.2 \times 10^{-8}$  (575,576). This equates to an average of 50–100 de novo mutations in a newborn genome, which corresponds to  $\sim 0.86$  de novo amino acid altering mutations. The genome-wide CNV mutation rate has also been estimated using SNP microarrays; at a resolution of  $\sim 30$  kb, Itsara et al. observed 9 de novo CNVs from 772 transmissions, corresponding to a mutation rate of  $1.2 \times 10^{-2}$  CNVs per genome per transmission (577).

Sex differences in mutation rates may have a variety of different underlying causes. For *premeiotic mutations*, the single most important factors are likely to be the much higher number of cell divisions during spermatogenesis as compared to oogenesis and the fact that the number of male germ cell divisions experienced is age dependent (578). However, the likelihood of a given mutation having originated in a particular parent is often dependent on the nature of the mutation in question. In general, point mutations tend to display a paternal bias, arising during spermatogenesis, while gross deletions tend to occur predominantly in females having originated during oogenesis (579,580). The ratio of the male to female nucleotide substitution mutation rates has been estimated to be around 6 (79) but may be as high as 20 (581), rather higher than expectation based on the ratio of the relative numbers of male versus female germline cell divisions, and consistent with most mutations being replication driven. Recently, the complete genomes from two parent-offspring trios have been sequenced to  $>22$ -fold mapped depth; a total of 49 and 35 germline de novo mutations were identified in two parent-offspring trios, respectively. In one family, 92% of the de novo mutations originated from the paternal germline, whereas the

equivalent figure from the other family was 36% of de novo mutations (582).

#### 7.4.27 Concepts of Dominance and Recessiveness in Relation to the Underlying Mutations

A genetic character is held to be *dominant*, if it is manifest in the heterozygous state and *recessive* if it is not. Thus, for a truly dominant condition, homozygotes should be clinically and phenotypically indistinguishable from heterozygotes (583). If this is not so, and the homozygote is more seriously affected, then the respective alleles may be regarded as *semidominant* (584).

In general, most recessive alleles are loss-of-function alleles and include gross gene deletions and rearrangements, frameshift mutations, nonsense mutations, and so on. By contrast, dominant alleles are often associated with gain of function, either due to dominant negative mutations (which interfere with and hence abrogate the function of the wild-type allele) or dominant positive mutations (which confer increased, constitutive, novel, or toxic activity on the mutant protein). Examples of dominant negative mutations are to be found in the *GH1* (585) and *KIT* (586) genes, while dominant positive mutations have been reported in the *PMP22* (587), *GNAS1* (588), *DMPK* (589), and *SERPINA1* (368) genes. It should be noted that loss-of-function mutations [e.g. *TERT* (590) and *RUNX2* (591)] can also be associated with dominantly inherited conditions in cases where a 50% reduction in the level of the protein product is sufficient to impede function.

For X-linked diseases, it is probably inappropriate to use the terms dominant and recessive since males are hemizygous and females often display variable expressivity of their heterozygous mutations due to skewed X-inactivation or clonal expansion (592).

#### 7.4.28 Genetic Architecture of Complex Diseases

The study of the genetic architecture of complex disease has revolved around the discussion of two apparently opposing models: the common disease–common variant (CD/CV) hypothesis and the multiple rare variant or common disease–rare variant (CD/RV) hypothesis (466). Since the CD/CV model conceptually underpinned the HapMap Project, GWAS that have employed HapMap data have tended to interrogate the association of common SNPs (MAF  $>5\%$ ) with complex diseases and traits. Initial GWAS data, therefore, strongly supported the involvement of common variants, especially common SNPs in complex phenotypes (468). However, such studies have succeeded in explaining only a small fraction of the heritability of complex phenotypes (593) and this “missing heritability” has tended to challenge the validity of the CD/CV hypothesis. Perhaps not surprisingly,

more recent data are revealing contributions from both common and rare variants to complex phenotypes. Thus, although common SNPs can explain a greater proportion of the heritability than was initially appreciated (594), support for a role for rare variants has also been accumulating from studies of rare SNPs (595,596) and rare CNVs (264,265). This suggests that the genetic architecture of complex phenotypes is likely to comprise both common and rare variants.

## 7.5 GENERAL PRINCIPLES OF GENOTYPE-PHENOTYPE CORRELATIONS

Given knowledge of a specific clinical phenotype, to what extent can the underlying causal genotype be inferred? Conversely, given knowledge of a specific genotype, to what extent is it possible to infer the likely clinical phenotypic consequences (in terms, for example, of the penetrance, age of onset, and severity of the disease)? The study of the genotype-phenotype relationship is essentially an exploration of the actual correspondence between the genotype and the phenotype where any particular genotype usually corresponds to multiple phenotypes whereas many different genotypes can often correspond to a given phenotype. Several general principles have emerged as a result of the intensive study of causative mutations in genetic disorders. The following discussion highlights some of these principles. The reader is encouraged to use the On-line Mendelian Inheritance in Man (OMIM) at <http://www3.ncbi.nlm.nih.gov/Omim> for further information or for specific genes and clinical phenotypes. It is likely that the phenotypic consequences of a given mutation will depend on other genetic variants present in the same gene or in the same genome (597). The review of Wolf (598) provides an excellent guide to the complex issues inherent in the study of the relationship between the mutant genotype and the clinical phenotype.

### 7.5.1 Mutations in the Same Gene May Be Responsible for More Than One Disorder

There are many examples to illustrate the principle that mutations in a single gene can cause different and distinct clinical phenotypes (“allelic heterogeneity”). Historically, the first example is that of the  $\beta$ -globin (*HBB*) gene on 11pter. Mutations of this gene cause  $\beta$ -thalassemia, sickle cell disease, and methemoglobinemia. The *L1CAM* gene on Xq28 has been shown to be mutated in hydrocephalus and stenosis of aqueduct of Sylvius, MASA syndrome (mental retardation, aphasia, shuffling gait, and adducted thumbs), and spastic paraplegia 1. The *COL1A2* gene on 7q21-q22 is involved in four different clinical forms of osteogenesis imperfecta (types II, III, IV, and atypical) as well as Ehlers-Danlos syndrome type VII B. The fibroblast growth factor receptor 2 (*FGFR2*) gene is mutated in three different craniosynostosis syndromes,

namely Pfeiffer, Crouzon, and Jackson-Weiss. The *COL2A1* gene is implicated in Stickler syndrome type 1, SED congenita, Kniest dysplasia, achondrogenesis-hypochondrogenesis type 2, precocious osteoarthritis, Wagner syndrome type 2, and SMED Strudwick type. In a survey of 1014 genes causing disorders in OMIM, 165 genes were associated with two disorders, 52 genes with three disorders, 24 genes with four disorders, and 19 genes with five or more disorders (69).

### 7.5.2 One Disorder May Be Caused by Mutations in More Than One Gene

There are a plethora of similar clinical phenotypes due to mutations in different genes. This observation, also known as “nonallelic” or “locus” heterogeneity, is well understood thanks to linkage analyses for genetic disorders and the search for mutations in different genes. Thus, tuberous sclerosis, a relatively common autosomal dominant disorder, is caused by lesions in at least two different loci: *TSC1* on 9q34 and *TSC2* on 16p13.3. Approximately 60% of TSC families show linkage to the *TSC2* locus and 40% to the *TSC1* locus. HNPCC has been associated with mutations in five different genes: *MLH1* on 3p, *MSH2* on 2p16, *PMS1* on 2q31-q33, *PMS2* on 7p22, and *MSH6* on 2p16. Retinitis pigmentosa has so far been associated with a total of 23 different genes and the list is still growing. We expect that disorders of complex or polygenic phenotypes, such as hypertension, atherosclerosis, diabetes, schizophrenia, and manic-depressive illness, will be associated with a considerable number of genes scattered throughout the genome.

### 7.5.3 One and the Same Mutation May Give Rise to Different Clinical Phenotypes (“Polypheny”)

The clinical phenotype does not only depend on the one mutation in the responsible gene; it can be modified by the action of any of the other ~25,000–30,000 genes in the genome (Wolf, 1997). The environment can also play an important role in the full development of the clinical phenotype. The classic sickle cell disease mutation in the  $\beta$ -globin (*HBB*) gene (Glu6Val) may be associated with severe or mild sickle cell disease. The amelioration of the severe clinical phenotype in this case can be attributed to the increased expression of  $\gamma$ -globin genes and the presence of high levels of HbF. The genomic environment of the  $\beta$ -globin gene cluster may, therefore, modify the severity of sickle cell disease as may genetic variation originating from other loci, for example, the  $\alpha$ -globin genes (103). Another example of this phenomenon has recently been provided by studies of certain craniosynostoses. Both Pfeiffer and Crouzon syndromes can be associated with the same C342Y or C342R mutations in the *FGFR2* gene.

The clinical phenotype associated with the D178N missense mutation in the prion protein (*PRNP*) gene is critically dependent on the presence of the Met or Val129 polymorphic allele to which it is coupled. When D178N lies in *cis* to the Met129 allele, fatal familial insomnia (FFI) results, whereas D178N coupled to the Val129 allele is associated with Creutzfeldt–Jakob disease (599). The Met/Val129 polymorphism also exerts an effect in *trans* through the normal allele since FFI is more severe and of longer duration in patients homozygous for either the Met or the Val allele.

One of the best examples of the contribution of the environment to the clinical phenotype of single gene disorders is that of phenylketonuria due to PAH deficiency. Individual homozygous or compound heterozygous for mutations in the *PAH* gene develop severe mental handicap if fed a normal diet. However, the cognitive status remains normal if these individuals are fed with a special, “phenylalanine-free” diet.

#### 7.5.4 Mutations in More Than One Gene May Be Required to Express a Given Clinical Phenotype (Digenic Inheritance; Triallelic Inheritance)

Digenic inheritance refers to clinical phenotypes caused by the co-inheritance of mutations in two unlinked genes. Thus, one form of retinitis pigmentosa is due to the co-inheritance of mutations in the *RDS* gene on 6p and the *ROM* gene on 11q (600). Individuals with either one or the other mutation do not suffer from the disease. In similar vein, digenic inheritance of mutations in the *MITF* and *TYR* genes has been reported as a cause of Waardenburg syndrome type 2 in conjunction with ocular albinism (601). This phenomenon may be common in polygenic disorders and in disorders with “low penetrance.”

Triallelic inheritance refers to clinical phenotypes, with apparent recessive mode of inheritance, caused by the co-inheritance of three mutant alleles, two in one gene and one in another gene. An example of triallelic inheritance is provided by the Bardet–Biedl syndrome. There are pedigrees in which affected individuals have two mutant alleles in the *BBS6* gene and one mutant allele in the *BBS2* gene. Other pedigrees have two mutant alleles in the *BBS2* gene and one mutant allele in *BBS6* (602). This type of inheritance indicates that some forms of BBS have a complex pattern of inheritance. As above, this phenomenon may be relevant in polygenic disorders and in disorders with “low penetrance.”

#### 7.5.5 Different Mutations in the Same Gene May Give Rise to Distinct Dominant and Recessive Forms of the Same Disease

vWF deficiency is a relatively common monogenic disease of blood coagulation. Many mutations have been studied

in the *VWF* gene on chromosome 12p. A proportion of mutations (usually deletions, nonsense codons, or frame-shift mutations) cause vWF deficiency with a recessive mode of inheritance; other mutations (mostly missense substitutions) are, however, associated with a dominant mode of inheritance of the vWF deficiency (603).

Although the majority of hitherto characterized growth hormone (*GH1*) gene lesions (including gross deletions and missense/nonsense mutations) that underlie familial short stature are inherited in autosomal recessive fashion, there is a group of intron 3 splicing mutations that are characterized by a dominant mode of inheritance (415). These lesions result in the in-frame skipping of exon 3 encoding 40 amino acids including a Cys residue. The dominant negative nature of this type of mutation is thought to be explicable in terms of the participation of the resulting free unpaired cysteine residue in an illegitimate intermolecular disulfide linkage leading to dimerization of the mutant molecule with a normal GH molecule and inhibition of GH secretion.

### 7.6 WHY STUDY MUTATION?

Although the sequencing of the human genome was finished some time ago, its annotation is still far from complete (604–606). Full exploitation of the emerging data, specifically in relation to understanding the etiology of inherited disease and disease predisposition, is however likely to be hampered by our ignorance of the basic processes underlying interindividual, interpopulation, and interspecies genetic diversity. At the population level, such an understanding is seen as essential for any meaningful interpretation of the prevalence/incidence patterns observed for diseases with a genetic basis. Within families, it is a prerequisite for being able to explain how interindividual variation arises and how variable phenotypic expression can be associated with identical gene lesions. Thus, for human genome sequence data to be useful in the context of molecular medicine, they must eventually be related to the genetic variation underlying human inherited disease. To this end, the meta-analysis of pathological germline mutations in human genes should facilitate

1. the assessment of the spectrum of known genetic variation underlying human inherited disease (4),
2. the identification of factors determining the propensity of DNA sequences to undergo germline mutation (150),
3. the optimization of mutational screening strategies (607),
4. improvements in our ability to predict the clinical phenotype from knowledge of the mutant genotype (8,374),
5. the identification of disease states that exhibit incomplete mutational spectra, prompting the search for, and detection of, novel gene lesions associated with different clinical phenotypes (608),



6. extrapolation toward the genetic basis of other, more complex traits and diseases (609),
7. improvements in our understanding of the biological function(s) of a given protein (373),
8. meaningful comparison between the mechanisms of mutagenesis underlying both inherited and somatic diseases (571),
9. studies of human genetic diseases in their evolutionary context (189).

Our genes have evolved slowly, probably via a myriad of meandering and circuitous pathways, escorted through the millennia of erratic environmental influences by the molding force of natural selection. Perhaps this hesitant evolutionary past accounts for present day genes containing, encoded within their nucleotide sequences, the potential seeds of their own destruction. How apt in this context is the poet's description of nature: "so careful of the type she seems, so careless of the single life" (Alfred Lord Tennyson, "In memoriam A.H.H.", 1850).

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### Biographies



**Stylianos E Antonarakis** is currently professor and Chairman of Genetic Medicine at the University of Geneva Medical School. He is a medical, molecular, human geneticist, physician–scientist, who studied extensively the relationship between genomic variation and phenotypic variation. He received his MD (1975) and DSc (1982) from the University of Athens Medical School, and after a specialization in pediatrics in the University Hospital, Athens Greece, he moved to Baltimore, Maryland to become a medical geneticist at the Johns Hopkins University School of Medicine with Haig H Kazazian and Victor McKusick (1980–1983). He joined the faculty of the Johns Hopkins University in 1983 and rose to full professor of pediatric genetics, biology, and medicine in 1990. In 1992, he moved to Geneva, Switzerland to chair Genetic Medicine in the University of Geneva. His research work and accomplishments include the understanding of the molecular bases of monogenic disorders and complex genetic disorders including the  $\beta$ -thalassemias, hemophilias, and trisomy 21. His laboratory participated in the human genome sequence and functional analysis, particularly on chromosome 21. He is an international expert on disorders of chromosome 21, cloning of genes for genetic disorders, development of diagnostic tests, genome structure and function, studies of the genome variability, and conserved noncoding sequences in human DNA. He has published extensively (more than 600 well-cited papers) in the scientific literature and is coeditor of the current edition of the classic textbook *Genetics in Medicine*; he is listed as one of the highly cited scientists by the ISI institute (more than 35,000 citations; h-index 94). He was the President of the European Society of Human Genetics (2001–2002), and member of the HUGO Council, foreign member of the Academy of Athens (2003), and member of EMBO (2006). He was the co-organizer of the European School of Genetic Medicine, and in the last 28 years taught in the Bar Harbor Genetics Course, Maine. He was awarded the Society of Pediatric Research Young Investigator Award (1984), International Jerome Lejeune Prize (2004), the European Society of Human Genetics Award (2005), and was elected to the Society of Scholars of the Johns Hopkins University (2006), and the American Academy of Physicians (2010). He was awarded the Commander of the Order of Phoenix medal from the Hellenic Democracy (2007). More than 70 talented young scientists were trained in his laboratory (graduate students and postdoctoral fellows); in addition, more than 25 young physicians were trained in the Medical Genetics Clinic of his department. With Haig Kazazian he has established one of the first molecular diagnostic laboratories in the United States as early as 1982. He is a member of the Swiss National Science Foundation Research Council, and the Chair of the Genetics Review Panel of the EU ERC. His research laboratory was/is supported by grants from the National Institutes of Health, the European Union (including the European Research Council), and the Swiss National Science Foundation and numerous other Foundations including the Gebert and Lejeune Foundations. He is the originator of the World Down Syndrome Day ([http://en.wikipedia.org/wiki/World\\_Down\\_Syndrome\\_Day](http://en.wikipedia.org/wiki/World_Down_Syndrome_Day)). His current interests and research projects are the functional analysis of the genome, effect of human genetic variation to phenotypic variation, the molecular pathogenesis of trisomy 21 and polygenic phenotypes, the functional characterization of the conserved fraction of the genome, diagnostics and prevention of genetic disorders, and the societal implications of genetics and genome research.



**David N Cooper** is professor of human molecular genetics at Cardiff University. He obtained his PhD in molecular biology from Edinburgh University in 1983. Having worked on the molecular genetics of inherited disorders of thrombosis and hemostasis at the University of London, he took up his present position in 1995. His research interests are largely focused on elucidating the mechanisms of mutagenesis underlying human genetic disease, but include the exploration of genotype–phenotype relationships in various inherited conditions, as well as human evolutionary and population genetics. He has published more than 340 papers in the field of human molecular genetics and has coauthored or coedited a number of books including *Human Gene Mutation* (1993), *Nature Encyclopedia of the Human Genome* (2003), *Molecular Genetics of Lung Cancer* (2005), *Handbook of Human Molecular Evolution* (2008), and *Copy Number Variation and Disease* (2009). He curates the *Human Gene Mutation Database* (<http://www.hgmd.org>), a comprehensive database of mutations causing human inherited disease, which is marketed internationally by BIOBASE GmbH. Professor Cooper is European editor of *Human Genetics* and editor of the Genetics and Disease section of Wiley's *Encyclopedia of Life Sciences*. He is also a member of several editorial boards including the *Journal of Medical Genetics* and *Human Mutation*.

# CHAPTER

# 8

## Genes in Families

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### 8.1 INTRODUCTION

Genetic counseling is the process by which patients and relatives at risk of a disorder that may be hereditary are advised of the consequences of the disorder, the probability of developing and transmitting it, and of the ways in which this may be prevented, avoided, or ameliorated. To achieve these aims, an accurate diagnosis and detailed information regarding the family history are essential. The basis for establishing a diagnosis depends on medical history, examination, and investigation. The diagnostic information is combined with the information obtained from the family pedigree to determine the mode of inheritance of the disorder and to calculate the risk of recurrence so that family members can be appropriately counseled.

A family history of a genetic condition may be due to the following:

1. A mutation within a single nuclear gene
2. A mutation within a mitochondrial gene
3. A contiguous gene deletion or duplication involving anything from a few to a large number of genes
4. A chromosomal rearrangement resulting in unbalanced products at meiosis
5. Multifactorial inheritance

### 8.2 PEDIGREE CONSTRUCTION

Accurate documentation of the family history is an essential part of genetic assessment, and the best method of recording this information is by constructing a family pedigree. Pedigrees are universally used in patients' genetic records, journal articles, and textbooks as the means of relaying information in an easily interpreted visual format. Pedigrees also provide the basis for calculations required for both recurrence risk estimation in individual families and linkage analysis in gene-mapping studies.

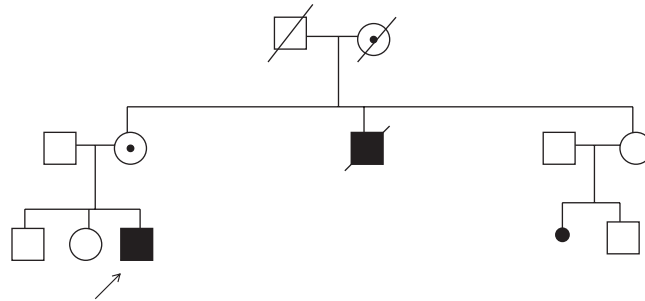
In a pedigree, squares are used to represent males and circles to represent females. Generations are indicated

by Roman numerals and individuals within each generation by Arabic numbers. Despite the universal use of the pedigree as a method of recording information and as an analytical tool, there is still considerable variation in the use of symbols relating to both routine medical information (pregnancy, spontaneous abortion, and termination of pregnancy) and new reproductive technologies (artificial insemination by donor semen, donor ovum, and surrogate motherhood).

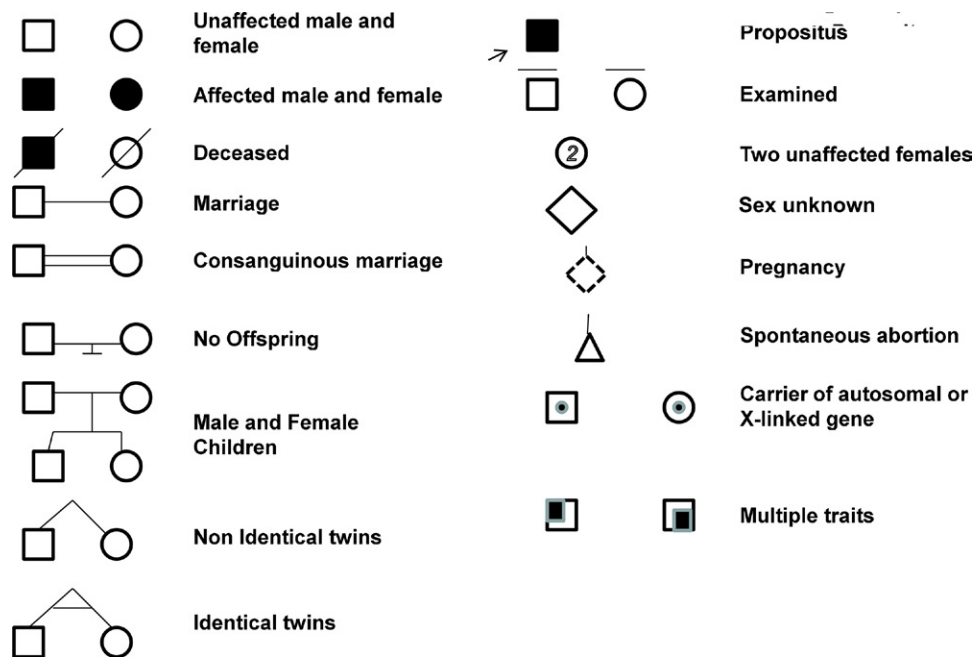
An example of a family pedigree is shown in [Figure 8-1](#), using the symbols illustrated in [Figure 8-2](#). While drawing a pedigree, it is usually simplest to start with the person seeking advice (the consultand). In some cases, the consultand will be an apparently healthy relative seeking information about how the condition may affect him/her or his/her offspring. Pedigree details are completed for both sides of the family, including previous and subsequent generations.

Conventionally, the paternal lineage is placed on the left and the maternal lineage on the right. Within sibships, individuals are listed from left to right in birth order. The affected person (proband), through whom the family has been ascertained, or the relative seeking advice (the consultand), are normally indicated in the pedigree by an arrow. Name, date of birth, and a summary of relevant medical details should be recorded for all family members. Further information, including medical records, may be required for affected individuals to confirm actual diagnosis. Not all relevant information may be volunteered by the consultand, indeed certain sensitive information—such as a previous termination of pregnancy or illegitimacy—may be deliberately withheld if the accompanying partner is not aware of it. Important details that should be asked about include assisted conception, previous miscarriages, stillbirths, terminations, children adopted into or out of the family, and consanguinity. Ethnic origin should be recorded, as





**FIGURE 8-1** Example of a family pedigree for Duchenne muscular dystrophy.



**FIGURE 8-2** Examples of symbols commonly used in drawing a pedigree.

some genetic conditions are more prevalent in particular ethnic groups, and this information may provide a clue to the likely diagnosis. The family history should be completed on both sides of the family for a consultant couple, even if the presenting condition is clearly traced to one side, as unrelated pregnancy losses or handicapping disorders may have occurred on the other side of the family that could have greater reproductive impact for the couple than the disorder for which they are seeking information.

### 8.3 UNIFACTORIAL INHERITANCE/ SINGLE-GENE DISORDERS

Unifactorial inheritance refers to those disorders that are due to the inheritance of a single mutant gene. The first descriptions of unifactorial inheritance were made by Mendel in 1865, when he published the results of his experiments on the garden pea in his paper “Versuche über Pflanzen Hybriden” (“Experiments on Plant

Hybrids”). His work was largely ignored until it was republished by Bateson in 1901, from which time the term Mendelian inheritance became synonymous with unifactorial inheritance.

From the ratios that Mendel described in his experiments on the garden pea, and the work of subsequent researchers, including Bateson, four main conclusions were drawn (1):

1. Genes come in pairs (Mendel termed them factors), one inherited from each parent.
2. Individual genes can have different alleles, some of which (dominant traits) exert their effects over others (recessive traits)—the principle of dominance. In Mendel’s own words, “those characters which are transmitted entire, or almost unchanged in the hybridization, and therefore in themselves constitute the characters of the hybrid, are termed the dominant, and those which become latent in the process, recessive.”

3. At meiosis, alleles segregate from each other with each gamete receiving only one allele—the principle of segregation, or Mendel's first law.
4. The segregation of different pairs of alleles is independent—the principle of independent assortment, or Mendel's second law.

With time, these principles have had to be modified. For example, although most genes come in pairs, for genes on the sex chromosomes males have only one allele; that is, they are termed hemizygous. Also, although alleles of a gene on different chromosomes show independent assortment, genes that are physically close together on the same chromosome do not—a phenomenon that has allowed mapping of genes in the human genome through linkage studies. These principles, however, still form a useful set of rules designed to explain the inheritance of many inherited characteristics and disorders.

Diseases inherited in a Mendelian fashion are categorized according to whether the gene is on an autosome or a sex chromosome and whether the trait is dominant or recessive.

## 8.4 DOMINANCE AND RECESSIVENESS

### 8.4.1 Definition of Dominance

Fundamental to the understanding of Mendelian inheritance are the concepts of dominance and recessiveness. Dominance is not a property intrinsic to a particular allele, but describes the relationship between it and the corresponding allele on the homologous chromosome. If the phenotypes associated with the genotypes AA and AB are the same but differ from the phenotype of BB, allele A is dominant to allele B and, conversely, allele B is recessive to allele A. Therefore, allele A manifests in the heterozygous state. An example of a dominant disease allele is that of Huntington disease, with most individuals affected with the disease being heterozygous for a mutant allele. However, individuals have been identified who have been shown by molecular techniques to be homozygous for the mutant allele by virtue of both parents being affected with Huntington disease. Such individuals do not appear different phenotypically from heterozygotes for the disorder.

**8.4.1.1 Incomplete Dominance.** If the phenotype of the heterozygous state, AB, is intermediate between the phenotypes of AA and BB, allele A is said to be incompletely dominant or semidominant to allele B. The skeletal dysplasia achondroplasia causes rhizomelic shortening of the limbs, a characteristic facies with mid-face hypoplasia, exaggerated lumbar lordosis, limitation of hip and elbow extension, genu varum, and trident hand. It was conventionally thought to be due to a dominant allele, but homozygotes for the mutant gene have a much more severe skeletal dysplasia, resulting in early

death from respiratory obstruction due to a small thoracic cage and neurologic deficit due to hydrocephalus. Therefore, achondroplasia is an example of incomplete or semidominance. Homozygotes for most dominant mutant alleles causing human genetic diseases occur so rarely that it is not known whether they exhibit complete or incomplete dominance.

**8.4.1.2 Codominance.** If the phenotype of AB displays the phenotypic features of both the homozygotic states, then alleles A and B are said to be codominant. The human ABO blood group system exhibits codominance. The system consists of three alleles: A, B, and O. Both A and B are dominant in relation to O, and therefore blood group A can have the genotype AA or AO. Blood group B can have the genotype BB or BO. However, neither A nor B shows dominance over the other, and therefore individuals with the genotype AB have the phenotypic characteristics of both blood group A and blood group B.

### 8.4.2 Mechanisms of Dominance

Most mutations result in an allele that is recessive to the wild-type allele; the phenotype is therefore only expressed in the homozygous state. This is because most mutations result in an inactive gene product, but the reduced level of activity due to the remaining wild-type allele is sufficient to achieve the effects of that gene product. An example is a gene for an enzyme that is only required in small amounts as a catalyst for a metabolic pathway. Although in some recessive inborn errors of metabolism it is possible to identify heterozygotes, more often than not, the only way to identify carriers is by direct mutation analysis, using molecular genetic techniques.

There are several mechanisms by which a mutation can lead to a dominant mutant allele whose phenotype is expressed in the heterozygous state (2).

**8.4.2.1 Loss-of-Function Mutations.** For most mutant alleles, loss of function will usually exhibit recessive behavior. Where a reduced amount or reduced activity of the gene product results in the phenotypic features, this is termed haploinsufficiency (e.g. in a critical rate-limiting step of a metabolic pathway). Any reduction in the amount of gene product will result in that pathway not being able to function at full activity. The same appears to apply to regulatory genes that could have a threshold level of activity. PAX3 is a gene coding for a DNA-binding protein, and point mutations in the gene result in Waardenburg syndrome type 1, characterized by deafness and pigmentary disturbances. Certain mutations in PAX3 have been shown to abolish all protein functions of that allele, so the phenotype must be due to a dosage effect as it manifests in the heterozygous state.

Another example in which the quantitative amount of a gene product is important is the genes that produce proteins in large quantities. An example is the gene for C1 esterase inhibitor, mutations of which cause the

disorder hereditary angioneurotic edema. C1 esterase inhibitor is removed rapidly from the circulation at a rate independent of its concentration. Therefore, although heterozygotes produce 50% of the normal amount, they have only 15–20% of the normal amount in the circulation, leading to the clinical manifestations of the disorder.

#### 8.4.2.2 Gain-of-Function Mutations.

**8.4.2.2.1 Increased Gene Dosage.** This mechanism involves an excess of gene product leading to a disease phenotype. Although gene dosage of critical regions or genes has been invoked as the cause for the phenotypic features associated with autosomal trisomies, there are a few examples involving single-gene disorders. One example involves the *PMP22* gene, which codes for the peripheral myelin protein 22. Duplication of the DNA sequence of one allele is associated with hereditary motor and sensory neuropathy type 1A.

**8.4.2.2.2 Ectopic or Temporally Altered Messenger RNA Expression.** Ectopic or temporally altered messenger RNA is expressed when a mutation occurs that affects the time or place of gene expression and usually involves a regulatory part of the gene. For example, during development in erythroid precursor cells, there is a switch from the production of  $\gamma$ -globin to the production of the  $\delta$ -globin and  $\beta$ -globin. This switch is controlled, at least in part, by the binding of transcription factors to the  $\gamma$ -globin promoter. Point mutations in the globin-promoter region prevent the normal switch, resulting in the disorder of hereditary persistence of fetal hemoglobin.

**8.4.2.2.3 Increased Protein Activity.** Mutations can lead to proteins with a prolonged half-life or proteins that have lost their normal constitutive inhibitory regulatory activity. If a mutation occurs in a part of a gene that codes for the protein sequence acting as the recognition site for proteolytic degradation, this will not take place, with the protein remaining active. Many proteins possess domains that allow their activity to be reversibly inhibited. For example, skeletal muscle sodium channels undergo voltage-sensitive regulation, and mutations in the gene *SCN4A* that codes for the  $\alpha$  subunit of the sodium channels result in the disorder hyperkalemic periodic paralysis, characterized by muscle myotonia and paralysis due to loss of regulatory inactivation of the sodium channel.

**8.4.2.2.4 Dominant-Negative Mutations.** If a mutant allele interferes with the wild-type allele, this is termed a dominant-negative mutation. This could occur in a multimeric protein in which a mutant subunit has an intact binding domain but altered catalytic activity, affecting the function of the entire multimer. If a protein is a dimer, one mutant and one wild-type allele would result in only 25% normal dimers, with up to a 75% reduction in activity.

Many structural proteins are multimers (e.g. the various types of collagen proteins). Each of the collagen

subunit genes has a central portion coding for repeating tripeptide units that are essential for the assembly of the collagen molecule. The disease *osteogenesis imperfecta* is caused by point mutations in the central portion of one of the collagen subunit genes *COL1A1* or *COL1A2* leading to a structural deformation that causes disruption of the whole collagen protein.

**8.4.2.2.5 Toxic Protein Alterations.** Toxic protein alterations are mutations that cause structural alterations in proteins, thus disrupting normal function and leading to toxic products that poison the cell. An example is hereditary amyloidosis, in which mutations in the transthyretin gene lead to resistance to proteolysis, and hence to increased stability of the protein. The protein then undergoes multimerization and accumulates in the cell as fibrils, causing disruption of the cell.

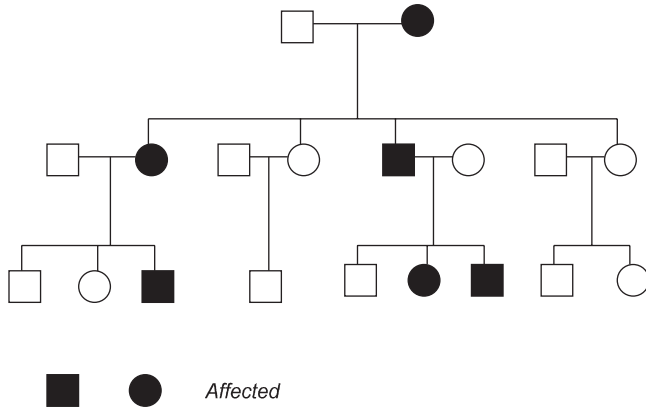
**8.4.2.2.6 New Protein Functions.** Some mutations have been found to confer a new function on a gene product. For example, a fatal bleeding disorder was found to be caused by a missense mutation in the  $\alpha_1$ -antitrypsin gene, in which methionine was replaced by arginine at position 358, the effect of which was to convert  $\alpha_1$ -antitrypsin, normally an inhibitor of elastase, into an inhibitor of thrombin. This thrombin inhibitory activity was not compensated for by an increase in endogenous coagulant production, resulting in a severe bleeding disorder (3).

**8.4.2.3 Recessive Mutations with Dominant Effects.** The mechanisms described so far show how mutations can cause dominant effects at a cellular level by the effects on the proteins produced. It is possible to have mutations that show a dominant pattern of inheritance in families, yet are recessive at the cellular or molecular level; that is, the gene is inactivated but has no other effect. The classic example of this is the retinoblastoma gene *RB1*, inactivation of which can lead to the formation of the developmental eye tumor retinoblastoma. Families can show a dominant mode of inheritance for this disorder, yet cells heterozygous for the mutation are completely normal, the mutation itself being recessive.

The dominant pattern of inheritance of familial retinoblastoma is the result of transmission of a first mutation with a second somatic mutation occurring in the normal allele of at least one retinal cell during a critical period of development, leading to the formation of a retinoblastoma—the “two-hit” hypothesis (4). There are several ways in which the normal allele in somatic cells can be inactivated. These include point mutations, deletions, translocations, and mitotic nondisjunction, resulting in the loss of a whole chromosome. It is now known that the two-hit hypothesis applies to most of the dominantly inherited familial cancer syndromes in which the germ line mutation in a tumor suppressor gene is recessive and a mutation in a somatic cell in the corresponding allele leads to the development of a tumor.

**TABLE 8-1** Characteristics of an Autosomal Dominant Inherited Disorder

Successive or multiple generations in a family are affected.
Males and females are both affected in approximately equal proportions.
Males and females can both be responsible for transmission.
There is at least one instance of male-to-male transmission.

**FIGURE 8-3** Pedigree consistent with autosomal-dominant inheritance.

## 8.5 AUTOSOMAL-DOMINANT INHERITANCE

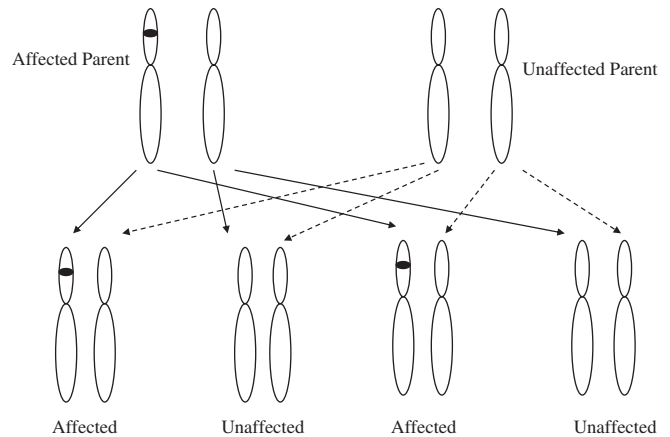
Autosomal-dominant inheritance refers to disorders caused by genes located on the autosomes, thereby affecting both males and females. The disease or mutant alleles are dominant to the wild-type alleles, so the disorder is manifest in the heterozygote (i.e. an individual who possesses both the wild-type and the mutant allele). The necessary characteristics to be certain that a disorder is inherited in an autosomal-dominant manner are listed in Table 8-1 and depicted in Figure 8-3.

### 8.5.1 Recurrence Risks

Since individuals with autosomal-dominant disorders are heterozygous for a mutant and a normal allele, there is a 1-in-2 (50%) chance a gamete will carry the normal allele and a 1-in-2 (50%) chance a gamete will carry the mutant allele. Assuming that the individual's partner will contribute a normal allele, there is a 1-in-2 (50%) chance that the offspring, regardless of sex, will inherit the disorder with each pregnancy (Figure 8-4).

### 8.5.2 Penetrance

There can be marked variability in the clinical manifestations of autosomal-dominant disorders, and they can demonstrate reduced penetrance (i.e. not every person with the mutant allele shows features of the disorder). The penetrance of a disorder is an index of the proportion of individuals with a mutant allele who manifest the

**FIGURE 8-4** Recurrence risks in autosomal-dominant inheritance.

disorder. An allele is said to be nonpenetrant if an individual known to be heterozygous for the allele, either by pedigree analysis or by molecular investigation, shows no signs of the disorder when subjected to appropriate clinical investigation. The penetrance of some genes is dependent on the age of the individual, as in Huntington disease, in which the penetrance is age dependent or is said to show delayed penetrance.

The penetrance (P) of a disorder is usually expressed as a proportion or percentage of the individuals carrying the gene who develop the disorder. If the P value is known for a particular condition, the risk to the offspring of an apparently unaffected individual can be calculated. In practice, the risk is usually less than 10%, as an unaffected relative is not likely to carry the gene if the penetrance is high, and a gene carrier is not likely to develop the disorder if the penetrance is low.

### 8.5.3 Expressivity

The expressivity of a gene is the degree to which a particular phenotype is expressed in an individual. Many autosomal-dominant diseases show variable expressivity such that individuals in the same family who carry an identical mutation can vary considerably in the severity of their disorder. For example, in the autosomal-dominant disorder neurofibromatosis type 1, the number of neurofibromas that an individual develops can vary dramatically from a few to many hundreds even within the same family. The variability seen in autosomal-dominant disorders may present as both inter- and intrafamilial differences. Intrafamilial variability may reflect the action of modifying genes, but interfamilial variability is more likely to be due to allelic heterogeneity at a single locus. A problem encountered in genetic counseling is that a mildly affected individual, such as a parent with only skin manifestations of tuberous sclerosis, may have a severely affected child. This situation is seen in many autosomal-dominant disorders, in that the mildly affected individuals are more likely to reproduce than the severely affected individuals.



### 8.5.4 Anticipation

A disorder is said to demonstrate anticipation if the phenotype of the mutant allele increases in severity as it is passed down the generations. An example of a disorder that demonstrates anticipation is myotonic dystrophy. A typical three-generation family with myotonic dystrophy showing anticipation is shown in [Figure 8-5](#). Another example of anticipation is seen in Huntington disease, in which the onset of symptoms is often seen to occur earlier with each succeeding generation.

### 8.5.5 Sex Influence

Sex influence involves the expression of an autosomal allele that occurs more frequently in one sex than the other. An example in humans is gout, with males affected more frequently than females until after the menopause, an effect probably mediated by hormonal differences.

### 8.5.6 Sex Limitation

Some traits are manifested only in individuals of one sex, an extreme situation known as sex limitation. This can occur when a gene affects an organ only possessed by one of the sexes (e.g. unicornuate uterus or ovarian cancer).

### 8.5.7 Pleiotropy

Pleiotropy refers to the phenomenon in which a single gene is responsible for a number of distinct and seemingly unrelated phenotypic effects. For example, the allele causing neurofibromatosis type 1 can produce abnormalities of skin pigmentation, neurofibromas of the peripheral nerves, short stature, macrocephaly, skeletal abnormalities, and fits. Each of the pleiotropic effects

of an allele can show reduced or nonpenetrance and variable expressivity.

### 8.5.8 Mechanisms of Reduced Penetrance and Variable Expressivity

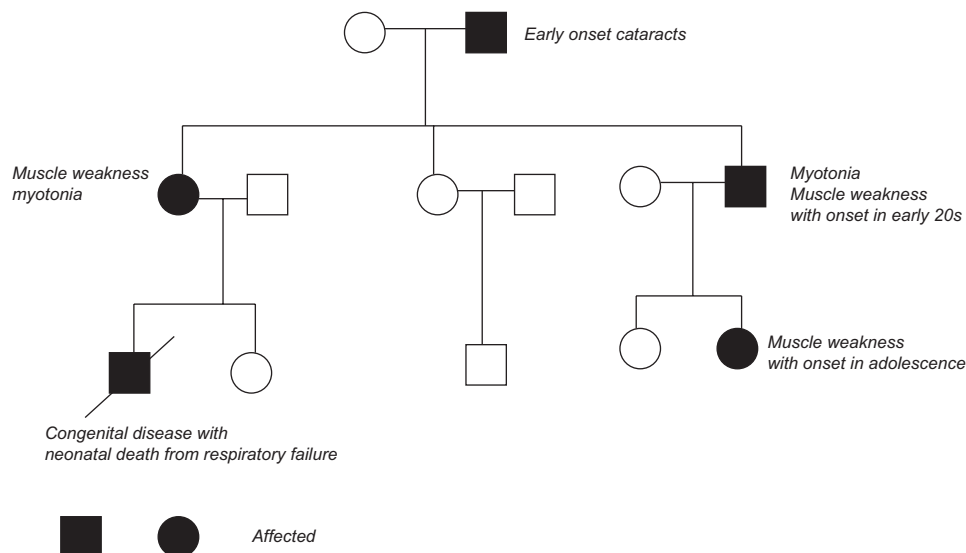
Some of the underlying mechanisms accounting for reduced penetrance and variable expressivity have been known for some time, while others are only now becoming apparent with the advent of modern molecular genetic techniques.

**8.5.8.1 Environmental Factors.** Environmental influences can affect the expression of genes. This can involve factors in the internal environment, such as hormones, or in the external environment, such as the effect of certain drugs and as barbiturates in acute intermittent porphyria. This disorder is characterized by attacks of abdominal pain, constipation, and psychiatric disturbances and is due to a mutation in a gene coding for an enzyme involved in heme biosynthesis. Attacks can be precipitated by certain drugs, including phenobarbital and the sulfonamides. Avoidance of these precipitating factors will result in nonpenetrance of the disorder.

**8.5.8.2 Somatic Mutations.** Retinoblastoma has already been mentioned as an example of one of the familial cancer genes in which a “second-hit” somatic mutation needs to occur in order for the disorder to manifest.

**8.5.8.3 Unstable DNA Triplet Repeat Sequences.** There are a group of dominant genetic disorders in which the mutation is an unstable DNA triplet repeat sequence that expands in successive meioses and whose size correlates with the severity of the disorder. This accounts for the anticipation seen in such disorders as myotonic dystrophy and Huntington disease.

**8.5.8.4 Genetic Background.** Information from studies of inbred animals suggests that it is likely that the genetic background of an individual (i.e. the nature of



**FIGURE 8-5** Pedigree for a family with myotonic dystrophy demonstrating anticipation.

particular alleles at other loci that interact with a mutant allele) will influence the penetrance and expression of a disorder. Analysis of the effect of this type of interaction is complex and is poorly understood at present, although examples are beginning to be delineated. For example, a study suggests that the alleles of the SMAD3 gene, which encodes a key regulatory protein in the transforming growth factor beta signaling pathway and is known to interact indirectly with the breast/ovarian cancer gene BRCA2 may contribute to an increased risk of breast cancer in BRCA2 mutation carriers (5).

### 8.5.9 New Dominant Mutations

While nonpenetrance can be a possible cause of a dominant disorder arising in the offspring of completely normal parents, an alternative explanation is that a mutation has arisen during the transmission of the gene; that is, it represents a new or *de novo* mutation. This appears to be more common for certain disorders than others. For example, in achondroplasia both parents are of normal stature in 80% of families. This also reflects the reduced reproductive fitness of adults with achondroplasia. The observation that achondroplasia occurred more frequently in last-born children of a sibship was suggested by Penrose as attributable to increased paternal age associated with new mutations, on the basis that “older germ-cells, possessed by older parents, might be more likely to show deterioration in the form of genetical changes” (6).

Some dominant mutations are universally lethal before the affected individual reaches reproductive age, and therefore are always seen as new dominant mutations. Several lethal disorders that were previously considered to be recessive are now known in many instances to be due to new dominant mutations, such as the perinatal lethal form (type II) of *osteogenesis imperfecta*.

New dominant mutations that occur during gametogenesis are associated with a negligible recurrence risk for future siblings. However, if the disorder is compatible with survival to reproductive age (and the possibility of reproduction), the recurrence risk for the offspring of the affected individual is 50%.

### 8.5.10 Gonadal or Somatic Mosaicism

In the case of certain new dominant mutations, there is a small but significant risk that a second child will be affected despite both parents being clinically normal. The ability to determine the molecular basis of many of these disorders has shown that this finding can be explained by the phenomenon of somatic mosaicism. This is possible when there are two genetically different types of cell in an individual, one carrying the mutant allele and the other not. If two genetically different types of cells occur within the gonads, this is referred to as gonadal mosaicism. However, there may be no mosaicism present in the gonads, where all cells may carry the mutant gene,

and the mosaicism may be present in other tissues. This situation would more correctly be referred to as somatic mosaicism.

The mutational event occurs after fertilization, with the degree and tissue specificity of the mosaicism being dependent on the time when it occurred. There is evidence that this usually occurs early in development, as commitment of primordial cells to the germ line occurs before tissue allocation, and in studies of individuals with gonadal mosaicism, up to 50% also have the mutation in a somatic cell line.

The frequency of gonadal mosaicism differs between disorders. At present, it is unclear why it is a frequent finding; however, it is now known that genetic heterogeneity with several newly discovered auto-recessive forms of IO can account for a good deal of the increased risks to unaffected parents. In some disorders such as fascioscapulohumeral dystrophy and osteogenesis imperfecta, mosaicism is found in approximately 19% and 15% of all cases, respectively (7), while in others such as achondroplasia, there have only been a few rare case reports. This is an important point to remember while providing recurrence risk advice in genetic counseling.

Somatic mosaicism may also be present in an individual who manifests the phenotype of an autosomal-dominant disorder. A study looking at individuals who were the first members of their families to develop the signs of neurofibromatosis type 2, a disorder characterized by bilateral vestibular schwannomas, estimated that 24.8% of their study cohort were mosaic for the NF2 mutation (8). Therefore, a new dominant mutation may arise during meiosis as a germ line mutation or postconceptually as a somatic mutation that is then transmitted through the germ line.

## 8.6 AUTOSOMAL RECESSIVE INHERITANCE

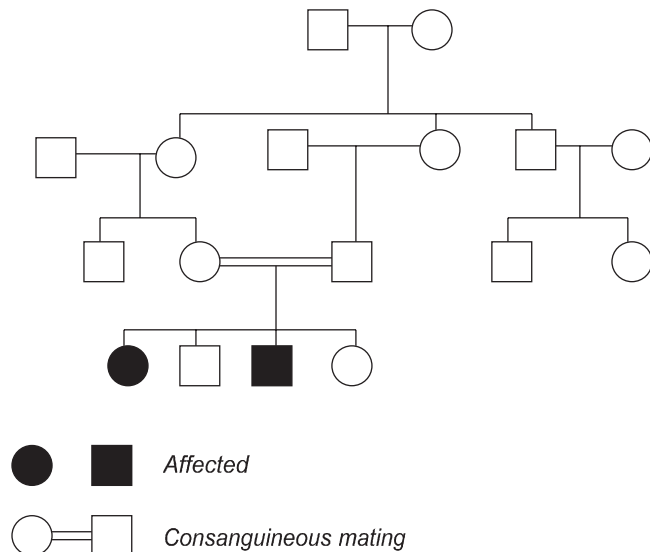
Autosomal recessive inheritance refers to disorders due to genes located on the autosomes, but in which the disease alleles are recessive to the wild-type alleles and are therefore not evident in the heterozygous state, only being manifest in the homozygous state. The necessary characteristics to be certain that a disorder is inherited in an autosomal recessive manner are listed in Table 8-2 and depicted in Figure 8-6. The parents of an individual with an autosomal recessive disorder are heterozygous for the disease allele and are usually referred to as being carriers for the disorder.

### 8.6.1 Consanguinity

If a couple are consanguineous, they have at least one ancestor in common in the preceding few generations. This means that they are more likely to carry identical alleles inherited from this common ancestor and could both transmit an identical allele to their offspring, who

**TABLE 8-2** Characteristics of an Autosomal Recessive Inherited Disorder

Both males and females are affected.
The disorder normally occurs in only one generation, usually within a single sibship.
The parents can be consanguineous.

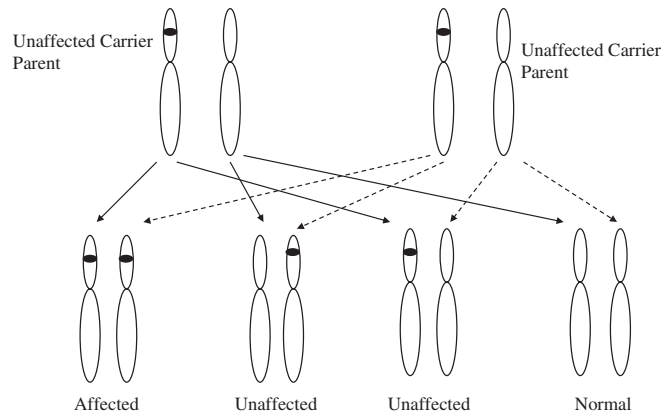
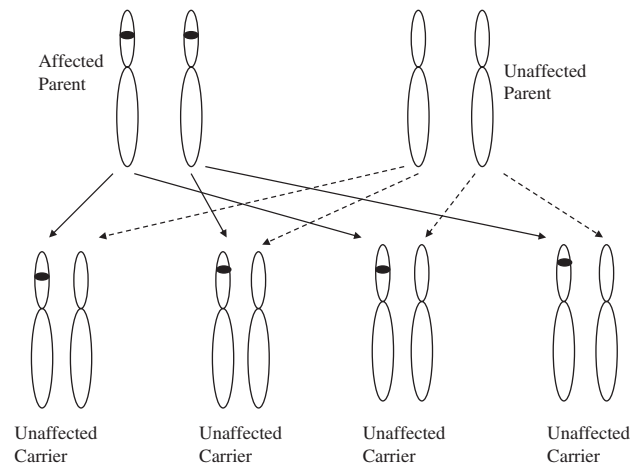
**FIGURE 8-6** Pedigree consistent with autosomal recessive inheritance.

would then be homozygous for that allele. A consanguineous couple have an increased risk that their offspring will be affected with a recessive disorder. The rarer a particular disease is in a population, the more likely the parents are to be consanguineous. For example, cystic fibrosis is a common autosomal recessive disorder in whites in Western Europe, with an incidence of ~1 in 2000. The incidence of consanguinity in the parents of children with cystic fibrosis is not appreciably greater than that in the general population. By contrast, with very rare autosomal recessive disorders such as alkaptonuria, eight of the first 19 families originally described by Garrod were consanguineous (9).

### 8.6.2 Recurrence Risks

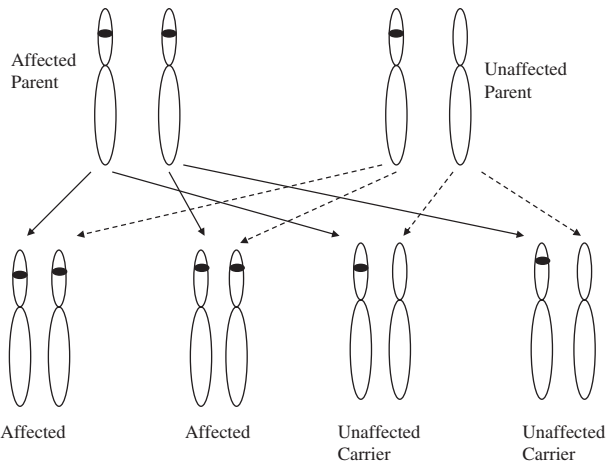
When two parents carrying the same disease allele reproduce, there is an equal chance that gametes will contain the disease or the wild-type allele. There are four possible combinations of these gametes, resulting in a 1-in-4 (25%) chance of having a homozygous affected offspring, a 1-in-2 (50%) chance of having a heterozygous unaffected carrier offspring, and a 1-in-4 (25%) chance of having a homozygous unaffected offspring (Figure 8-7).

When an individual with an autosomal recessive disorder has children, they will only produce gametes

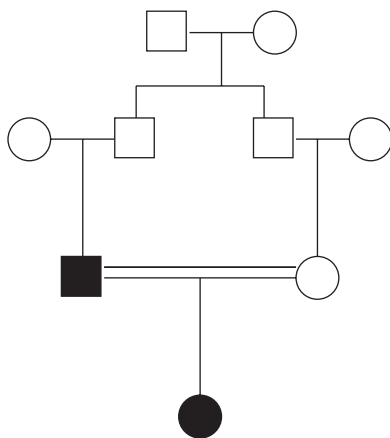
**FIGURE 8-7** Recurrence risks in autosomal recessive inheritance.**FIGURE 8-8** Recurrence risks for an individual with an autosomal recessive disorder and a normal partner.

containing the disease allele. Since it is most likely that their partners will be homozygous for the wild-type allele, the partners will always contribute a normal allele and therefore all the children will be heterozygous carriers and unaffected (Figure 8-8). If, however, an affected individual has children with a partner who happens to be heterozygous for the disease allele, there will be a 50% chance of transmitting the disorder, depending on whether the partner contributes a disease or a wild-type allele (Figure 8-9). Such a pedigree is said to exhibit pseudodominance (Figure 8-10).

In autosomal recessive disorders, the difficulty lies not with risk estimation, but in determining the underlying mode of inheritance, as these disorders usually present as isolated cases with little contributory information to be gleaned from the pedigree. Carrier risks to other relatives can be calculated from the pedigree, and carrier testing may be appropriate for disorders with a high gene frequency or when consanguineous marriages are planned. The risk to members of the extended family for having an affected child will depend on their own risk calculated from pedigree data and the population-based carrier risk



**FIGURE 8-9** Recurrence risks for an individual with an autosomal recessive disorder and a carrier partner.



**FIGURE 8-10** Pedigree of an autosomal recessive disorder showing pseudodominance.

appropriate to their spouse. Higher risks in consanguineous marriages are dependent on the degree of relationship between the partners. In disorders of unknown genetic etiology, consanguinity suggests, but does not prove, an autosomal recessive mode of inheritance.

### 8.6.3 Genetic Heterogeneity

It is not unusual in some recessive disorders, such as sensorineural deafness, for two affected individuals to have children. Assortative mating occurs in such instances because of social circumstances in which individuals with the same disability, such as deafness or visual impairment, are often educated together or share the same social facilities. If their disorder was due to a mutation in the same autosomal recessive gene, all their offspring would be affected. In a number of studies involving the offspring of parents with inherited sensorineural deafness, however, a significant proportion of such matings led to offspring with normal hearing. Although in some instances this could be due to other causes (e.g. acquired

causes being mistaken for inherited deafness), in most instances, the gene causing the deafness in the two parents is different, a phenomenon known as genetic heterogeneity. Each parent will transmit the mutant allele for their own deafness, but a wild-type allele of the gene involved in their partner's deafness. Therefore, the child is heterozygous for the two mutant alleles, what is known as a double heterozygote. This type of genetic heterogeneity involving different genes is known as locus heterogeneity.

Different modes of inheritance have also been documented for a number of clinically defined disorders with similar phenotypes. For example, autosomal dominant, autosomal recessive, and X-linked recessive inheritance have all been documented in hereditary spastic paraplegia, hereditary motor and sensory neuropathy, and retinitis pigmentosa depending on the causative gene. Locus heterogeneity makes it difficult to determine risks of recurrence for phenotypes that follow both dominant and recessive inheritance if the mode of inheritance is not clearly defined by the family pedigree. Laboratory analysis of families is also complicated by locus heterogeneity, as direct mutation analysis may not be practical for conditions in which one of the several/many genes is implicated and the suggestion of linkage between a polymorphic marker and the disease in a small family does not necessarily prove that a mutation at that particular locus is responsible.

Heterogeneity can also exist at the same locus; thus, an individual affected with a recessively inherited disorder can have two different mutations in the two alleles of the gene and is often called a compound heterozygote. Most individuals with recessive disorders are compound heterozygotes unless a specific mutation is especially prevalent in a particular population or the affected individual is the offspring of a consanguineous relationship, in which case the allele is likely to be identical by descent. This is known as allelic or mutational heterogeneity. The specific mutations that an affected individual possesses can, in fact, determine the severity of the disorder, as in cystic fibrosis, in which individuals who are homozygous for the most common mutation in the cystic fibrosis gene,  $\Delta F508$ , have a higher incidence of pancreatic insufficiency. As the underlying mutations causing disease are identified, it is becoming increasingly apparent that in many cases the exact nature of the mutation will determine the phenotype—a phenomenon known as genotype–phenotype correlation.

In some cases, different classes of mutations in a particular gene may act in a dominant or in a recessive manner. For example, a wide variety of phenotypes have been associated with mutations in the LaminA/C gene (10). These include mutations acting in an autosomal-dominant fashion, causing Emery–Dreifuss muscular dystrophy, limb girdle muscular dystrophy type 1B, dilated cardiomyopathy, familial partial lipodystrophy, and Hutchinson–Gilford progeria syndrome, as well as



mutations acting in an autosomal recessive fashion, such as Emery–Dreifuss muscular dystrophy, type 2B1 axonal neuropathy, and mandibuloacral dysplasia.

### 8.6.4 Uniparental Disomy

Uniparental disomy (UPD) refers to the presence of both homologs of a chromosome pair or chromosomal region in a diploid offspring being derived from a single parent. If the two homologs are identical due to an error in meiosis II, this is known as uniparental isodisomy, while if the two homologs are different but still from the same parent due to a meiosis I error, this is known as uniparental heterodisomy. UPD has been reported as a rare cause of the autosomal recessive disorder cystic fibrosis in the offspring of a couple in whom only one parent was a heterozygote carrier of the mutant allele. The affected offspring received both chromosome 7 homologs with the mutant allele from that parent. The recurrence risk in this situation would be negligible.

### 8.6.5 New Mutations

An autosomal recessive disorder may potentially be due to the inheritance of a mutation from one parent, with a *de novo* mutation occurring at the same locus on the chromosome inherited from the other parent. This is likely to be a very rare phenomenon, but accounts for some cases of spinal muscular atrophy due to a predisposition to generate deletions in the 5q13 region involving the SMN1 gene (11). The recurrence risk in this situation would relate to the risk of repeated *de novo* mutation and therefore negligible.

## 8.7 SEX-LINKED INHERITANCE

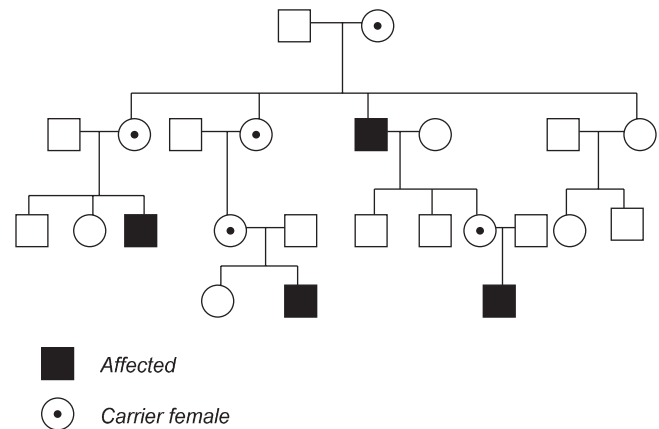
Strictly speaking, sex-linked inheritance refers to the inheritance patterns shown by genes on the sex chromosomes. If the gene is on the X chromosome, it is said to show X-linked inheritance and, if on the Y chromosome, Y-linked or holandric inheritance.

### 8.8 X-LINKED RECESSIVE INHERITANCE

This form of inheritance is conventionally referred to as sex-linked inheritance. It refers to disorders due to recessive genes on the X chromosome. Males have a single X chromosome and are therefore hemizygous for most of the alleles on the X chromosome so that, if they have a mutant allele, they will manifest the disorder. Females, on the other hand, will usually only manifest the disorder if they are homozygous for the disease allele, and if heterozygous will usually be unaffected. Since it is rare for females to be homozygous for a mutant allele, X-linked recessive disorders usually affect males only.

**TABLE 8-3** Characteristics of an X-linked Recessive Inherited Disorder

Males are affected almost exclusively.
Transmission occurs through unaffected or carrier females to their sons.
Male-to-male transmission is not observed.
Affected males are at risk of transmitting the disorder to their grandsons through their obligate carrier daughters.



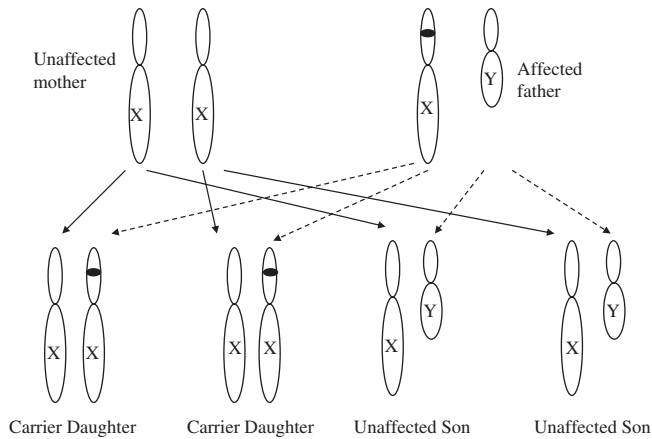
**FIGURE 8-11** Pedigree consistent with X-linked recessive inheritance.

The necessary characteristics to be certain that a disorder is inherited in an X-linked recessive manner are listed in Table 8-3 and portrayed in Figure 8-11.

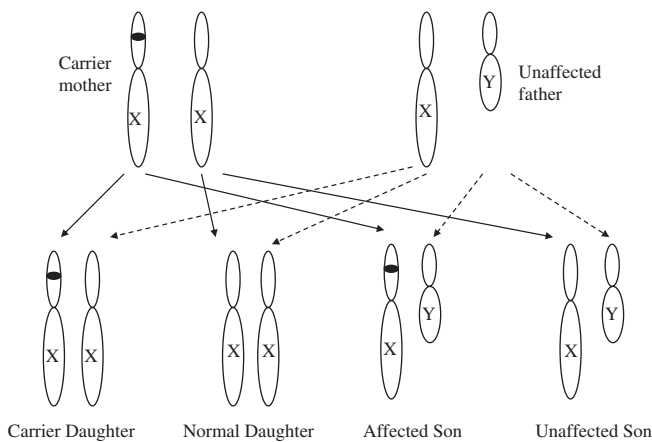
### 8.8.1 Recurrence Risks

If a male affected with an X-linked recessive disorder survives to reproduce, he will always transmit his X chromosome with the mutant allele to his daughters, who will be obligate carriers. An affected male will always transmit his Y chromosome to a son, and therefore none of his sons will be affected (Figure 8-12). A carrier female has one X chromosome with the wild-type allele and one X chromosome with the disease allele; therefore, her sons have a 1-in-2 (50%) chance of being affected, while her daughters have a 1-in-2 (50%) chance of being carriers (Figure 8-13).

For a female to be affected with an X-linked recessive disorder, her mother would have to be a carrier and her father affected with the disorder. Obviously, this situation is encountered only very rarely. Another possibility is that her mother is a carrier and the X chromosome transmitted by her father undergoes a new mutation. A female can also be affected by an X-linked recessive disorder if she has a single X chromosome (i.e. Turner syndrome), in which case she will be hemizygous for alleles on the X chromosome, like a male.



**FIGURE 8-12** Recurrence risks for a male with an X-linked recessive disorder.



**FIGURE 8-13** Recurrence risks for a female carrier of an X-linked recessive disorder.

## 8.8.2 X-Inactivation

Early in embryonic development, one of the X chromosomes in females is inactivated in each cell with the result that the female, like the male, has a single functional X chromosome. In individuals with X chromosome aneuploidies, all but one of the X chromosomes are inactivated in each cell. The process of X-inactivation is controlled by a region of the X chromosome, the X-inactivation center, situated on the proximal portion of the long arm. X-inactivation usually occurs as a random process such that, in approximately 50% of cells in a female, the maternally derived X chromosome is active, and in the other 50%, the paternally derived X chromosome is active. In females who are carriers of an X-linked recessive mutation, one half of the cells will actively express the disease allele. Occasionally, this can be demonstrated clinically. In X-linked *retinitis pigmentosa*, for example, careful fundoscopic examination of a female carrier can show a mosaic pattern of pigmentation.

## 8.8.3 Manifesting Female Carriers of X-Linked Recessive Disorders

Although female carriers of X-linked recessive disorders are usually asymptomatic, they can manifest signs of the disorder. They are usually much less severely affected than males, however. There are a number of different mechanisms by which a female heterozygote can manifest signs of an X-linked recessive disorder, but the underlying cause for each one is nonrandom or skewed X-inactivation. In this situation, there is a departure from the normal random process of X-inactivation, with a greater proportion of one X chromosome being inactivated than the other. If in most cells the active X chromosome is the one with the mutant allele, a female may manifest the disorder.

### 8.8.3.1 Mechanisms of Nonrandom X-Inactivation.

A number of mechanisms can lead to nonrandom X-inactivation:

1. *Chance*: Skewed X-inactivation can occur by chance.
2. *Monozygotic twinning*: There have been several reports of monozygotic female twins, both heterozygous for a dystrophin gene deletion, one of whom was a manifesting carrier for Duchenne-type muscular dystrophy and the other an unaffected carrier. It was demonstrated that, in lymphocytes and fibroblasts of the affected twin, the majority of active X chromosomes had the deletion, while in the unaffected twin, most active X chromosomes possessed the intact gene. It has been postulated that the twinning process could have been a consequence of cell surface differences as a result of X chromosome inactivation, leading to the separation of the two cell masses (12).
3. *Cytogenetic abnormalities*: In females with an X-autosome translocation, the normal X chromosome will be preferentially inactivated, maintaining the diploid state for the autosome involved in the translocation. If the translocation disrupts or interferes with the expression of a gene on the X chromosome, or if the X chromosome involved in the translocation carries a disease allele, that female will manifest the disorder. The finding of X-autosome translocations in manifesting female carriers of Duchenne-type muscular dystrophy was instrumental in the mapping and cloning of the dystrophin gene.
4. *Elimination of cells expressing the mutant allele*: If a gene on the X chromosome is required for cell survival, the normal gene will always be found on the active X chromosome and the defective gene on the inactive X chromosome in the mature cell population, even though X-inactivation occurred as a random process. This has been demonstrated in a number of disorders, including X-linked severe combined immunodeficiency, and can be used in the determination of carrier status for females at risk.

### 8.8.4 Gonadal Mosaicism

As with autosomal-dominant disorders, gonadal mosaicism is an important phenomenon in X-linked recessive disorders, occurring particularly frequently in Duchenne-type muscular dystrophy, in which it has been shown to occur in both male and female gametogenesis. It is important to take this into account when advising mothers of apparently sporadically affected males with Duchenne-type muscular dystrophy of recurrence risks.

Mutations arising during meiosis may occur in male or female germ-cells. If a particular mutation arises largely in male germ-cells, as in Lesch–Nyhan syndrome (13) and hemophilia A (14), the majority of mothers of affected boys will be carriers, and risks to sisters will be 50% regardless of how many unaffected brothers they have. In Duchenne muscular dystrophy, the overall mutation rate appears to be equal in males and females, but it has been suggested that most point mutations occur in spermatogenesis, while most deletions arise in oogenesis (15). In isolated cases, the recurrence risk might therefore be higher in nondeletion cases, as these mothers would be more likely to be carriers.

## 8.9 X-LINKED DOMINANT INHERITANCE

X-linked dominant inheritance is an uncommon form of inheritance and is caused by dominant disease alleles on the X chromosome. The disorder will manifest in both hemizygous males and heterozygous females. Random X-inactivation usually means that the females are less likely to be severely affected than hemizygous males, unless they are homozygous for the disease allele. The characteristics of an X-linked dominant inherited disorder are listed in Table 8-4 and depicted in Figure 8-14.

### 8.9.1 Recurrence Risks

Offspring of either sex have a 1-in-2 (50%) chance of inheriting the disorder from affected females (Figure 8-15). The situation is different for males affected by X-linked dominant disorders, whose daughters will always inherit the gene and whose sons cannot inherit the gene (Figure 8-16). An example of an X-linked dominant disorder is vitamin D-resistant rickets. X-inactivation

**TABLE 8-4** Characteristics of an X-linked Dominant Inherited Disorder

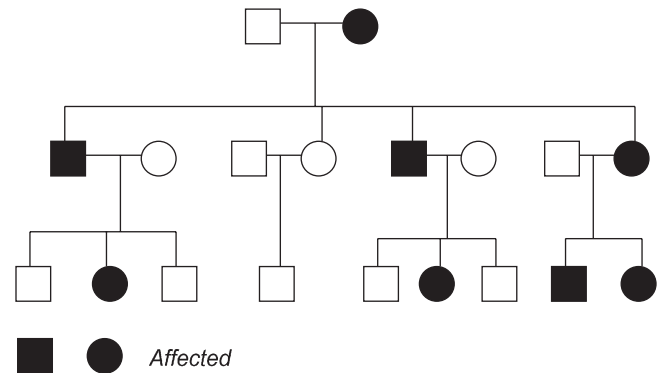
Daughters of affected males always inherit the disorder.
Sons of affected males never inherit the disorder.
Affected females can transmit the disorder to offspring of both sexes.
An excess of affected females exists in pedigrees for the disorder.

results in females with this disorder having less severe skeletal changes than those that occur in affected males.

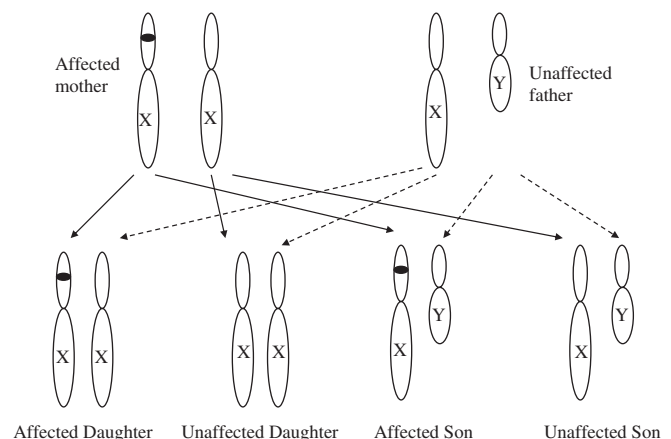
An exception to the rule that females are less severely affected than males is craniofrontonasal dysplasia, in which heterozygous females have a coronal craniosynostosis and affected hemizygous males do not have a craniosynostosis. The condition is caused by mutations in the ephrin B1 gene, and it has been proposed that, in heterozygous females, patchwork loss of ephrin B1 disturbs tissue boundary formation at the developing coronal suture, whereas in males deficient in ephrin B1, an alternative mechanism maintains the normal boundary (16).

### 8.9.2 X-Linked Dominant Lethal Alleles

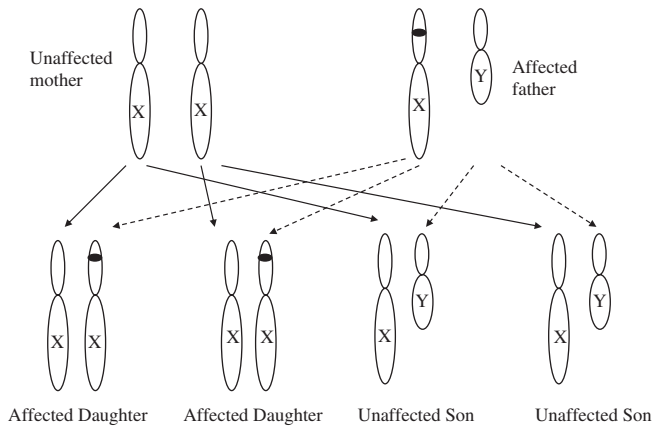
In some disorders due to mutant alleles of genes on the X chromosome, affected males are never or very rarely seen (e.g. incontinentia pigmenti and Goltz syndrome). This is thought to be due to a lethal effect of the mutant disease allele in the hemizygous male, resulting in nonviability of the conceptus during early embryonic development. As a consequence, if an affected female were to have children, one would expect a sex ratio of 2:1, female to male,



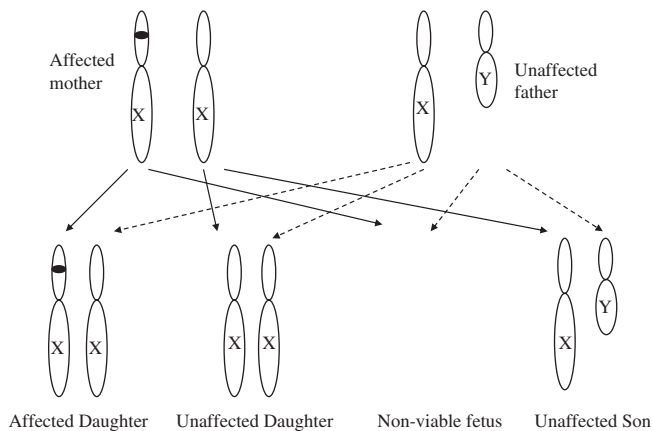
**FIGURE 8-14** Pedigree consistent with X-linked dominant inheritance.



**FIGURE 8-15** Recurrence risks for a female affected with an X-linked dominant disorder.



**FIGURE 8-16** Recurrence risks for a male affected with an X-linked dominant disorder.



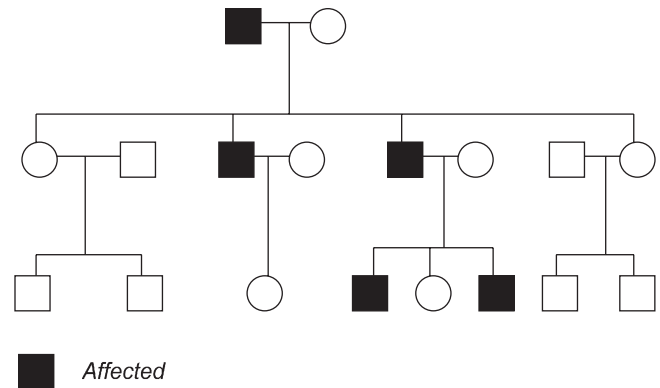
**FIGURE 8-17** Recurrence risks for a female affected with an X-linked dominant disorder lethal in males.

in the offspring and that one half of the females would be affected, while none of the male offspring would be affected (Figure 8-17). The majority of the mothers of females with these X-linked dominant lethal disorders are generally unaffected, and the disease alleles are therefore thought to arise as new mutations.

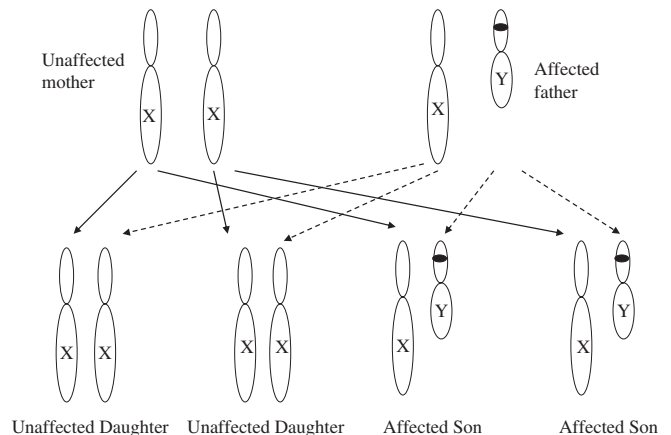
Rett syndrome is an X-linked dominant condition caused by mutations in the MECP2 gene. Girls with classical Rett syndrome have severe mental retardation developing after a period of relatively normal development. The mutations that cause classical Rett syndrome are lethal in males; however, other mutations in the gene can give a pattern of mental retardation in males that behaves as an X-linked recessive condition—another example of genotype–phenotype correlation.

## 8.10 Y-LINKED (HOLANDRIC) INHERITANCE

Y-linked, or holandric, inheritance refers to genes carried on the Y chromosome. They therefore will be present only in males, and the disorder would be passed on to all



**FIGURE 8-18** Pedigree consistent with Y-linked inheritance.



**FIGURE 8-19** Recurrence risks for a male affected with a Y-linked disorder.

their sons but never their daughters (Figure 8-18). Genes involved in spermatogenesis have been mapped to the Y chromosome, but a male with a mutation in a Y-linked gene involved in spermatogenesis would probably be infertile or hypofertile, making it difficult to demonstrate (Figure 8-19) Y-linked inheritance. This situation may well change with the use of techniques such as intracytoplasmic sperm injection (ICSI) to treat male infertility, which will result in the transmission of the infertility to male offspring.

## 8.11 PARTIAL SEX LINKAGE

A small region of sequence identity exists between the X and Y chromosomes located at the tips of the long and short arms, known as the pseudoautosomal regions of the sex chromosomes. A high rate of recombination at the telomeres of the short arms is thought to be obligatory for normal meiosis of these chromosomes. The genes within these regions, known as pseudoautosomal genes, escape X-inactivation in the female; therefore, both sexes have two active alleles at these loci. The pseudoautosomal gene SHOX has been postulated to account for some of the features seen in the numerical sex chromosome disorder, Turner syndrome. As a



result of the high recombination frequency, mutated genes located within the pseudoautosomal region can be transferred from the Y chromosome to the X chromosome and vice versa. Haploinsufficiency of the SHOX gene is the cause of Leri–Weill dyschondrosteosis, and in one study about half of the segregations investigated showed a transfer of the SHOX abnormality to the alternate sex chromosome. Therefore, the condition can be inherited as either an X-linked dominant condition or, more rarely, a Y-linked condition. Affected men can transmit the mutation or deletion to a son as well as to a daughter (17).

### 8.11.1 Genomic Imprinting and Epigenetic Mechanisms

Genomic imprinting is a phenomenon in which gene expression depends on parental origin. Imprinting is due to an epigenetic mechanism, in which the primary DNA sequence of the gene is not changed, but transcriptional regulation is affected by mechanisms such as DNA methylation, histone acetylation, and histone methylation. In contrast to the biallelic expression of most genes, imprinted genes demonstrate monoallelic expression. This process modifies the transmission and expression of certain genetic diseases and should be borne in mind as a possible mechanism underlying disorders that do not follow typical Mendelian inheritance. Many disorders due to defects affecting imprinted genes arise *de novo*, but they can also be familial. For example, the familial paraganglioma syndrome is due to mutations in the succinate dehydrogenase subunits B, C, and D. SDHD is an imprinted gene with the paternal allele active in each cell and the maternal allele inactive. The mutation is dominant and an affected parent (male or female) has a 1-in-2 chance of passing it on to each child, but the child is at increased risk to develop paragangliomas if only the mutation is inherited from the father (Figure 8-20). Conversely, the Angelman's gene

UBE3A is imprinted in the opposite way so that only the maternal allele is active.

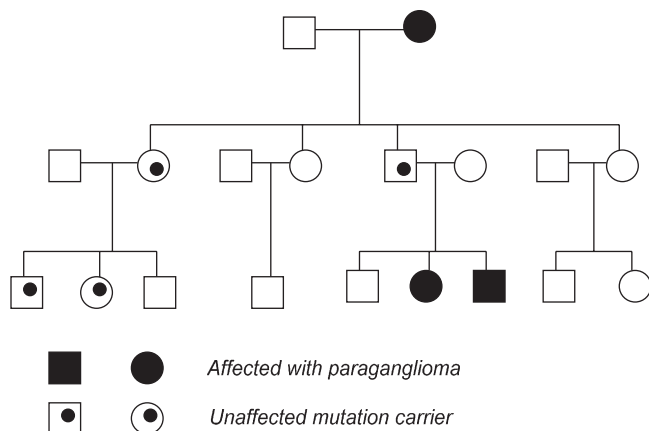
Prader–Willi syndrome (PWS) and Angelman syndrome are probably the best known examples of disorders due to imprinted genes. These disorders affect different genes in the imprinted region at 15q11-13. Loss of expression of paternal candidate genes in this region leads to PWS, and the loss of expression of the maternal UBE3A (ubiquitin protein ligase E3A) gene leads to Angelman syndrome. The underlying mechanism is often a *de novo* deletion or point mutation within the imprinted gene itself, but some cases are due to epigenetic mechanisms such as UPD or the consequence of an imprinting center defect. Maternal UPD results in PWS and paternal UPD in Angelman syndrome. Some familial cases of Angelman syndrome are due to mutations in the gene UBE3A. A mutation inherited from a mother will cause Angelman syndrome, but when inherited from the father the condition will not manifest. The imprint is reset during male and female gametogenesis. Therefore, in familial Angelman's, if an unaffected female carries a mutation in UBE3A on her paternal allele it is reset at gametogenesis so that it is now on the active allele and the children who inherit it will have Angelman syndrome.

Some genes show tissue-specific imprinting; for example, the GNAS gene, which causes Albright hereditary osteodystrophy (AHO). AHO is due to G(s)α inactivating mutations, imprinted in a tissue-specific manner, with expression in the proximal renal tubules, thyroid, pituitary, and ovaries being from the maternal allele. Maternally inherited mutations lead to AHO with endocrine involvement (pseudohypoparathyroidism type 1A), whereas paternally inherited mutations lead to AHO alone. Pseudohypoparathyroidism type 1B (parathormone resistance without AHO) can be caused by a deletion of the imprinted promoter regions of the gene that control gene expression (18).

An increased incidence of imprinted disorders, notably Beckwith–Wiedemann Syndrome, has been reported in children conceived by in vitro fertilization or ICSI techniques, suggesting that the process of resetting the parental imprint may be perturbed by specific elements of assisted reproductive methodology (19).

**8.11.1.1 Digenic Inheritance.** Genetic or locus heterogeneity by which different genes can cause clinically identical disorders has been discussed previously in this chapter. However, these cases are considered to be monogenic in that, in any one family, only one locus is thought to be defective. Reports of families with *retinitis pigmentosa* in which the affected individuals are heterozygous for mutations in two different recessive genes (i.e. double heterozygotes) suggested the possibility of a previously undescribed mode of inheritance, known as digenic inheritance (20).

Although the families described were initially thought to be compatible with autosomal dominant *retinitis*



**FIGURE 8-20** Pedigree showing familial paragangliomas and the effects of imprinting of the SDHD gene.

*pigmentosa* with reduced penetrance, the families showed a number of unusual features:

1. In each family, the disease originated in the offspring of unaffected individuals.
2. Affected individuals transmitted the disorder statistically significantly to fewer than 50% of their offspring.

On molecular testing, it was found that both the affected and the unaffected individuals carried a mutation in the *peripherin/RDS* gene. Affected individuals were also heterozygous for a mutation in the *ROM1* gene. These genes encode two of the polypeptide subunits of an oligomeric transmembrane protein complex present at the photoreceptor outer segment disc rims. Mutant *peripherin/RDS* protein can assemble with wild-type *ROM1* to form structurally normal complexes, but cannot assemble with mutant *ROM1* protein. Therefore, only the combination of the two heterozygous mutations is pathogenic (21).

Digenic inheritance has also been suggested in inherited sensorineural deafness and in arrhythmogenic right ventricular cardiomyopathy (22). Whether this is a true phenomenon and how common it is still to be fully elucidated.

**8.11.1.2 Triallelic Inheritance.** Bardet–Biedl syndrome (BBS) is a multisystem disorder characterized by obesity, retinal degeneration, polydactyly, gonadal, and renal malformations, and behavioral and developmental problems, with a population incidence of 1 in 14,000 to 1 in 16,000. Initially, segregation studies suggested that the inheritance pattern was autosomal recessive. BBS is a genetically heterogeneous condition and so far 14 genes have been identified accounting for about 70% of cases. BBS families demonstrate great intra- and interfamilial variation in the phenotype. A more complex inheritance pattern has emerged with the report that about 5% of cases have three mutations, two in one BBS gene, and a third in another BBS gene. There are a number of possible explanations for this finding. One is triallelic inheritance with the third allele affecting the variability in the phenotype that is seen in BBS. Alternatively, the third allele may represent a heterozygous recessive mutation which has no effect on the phenotype or a rare polymorphism (23).

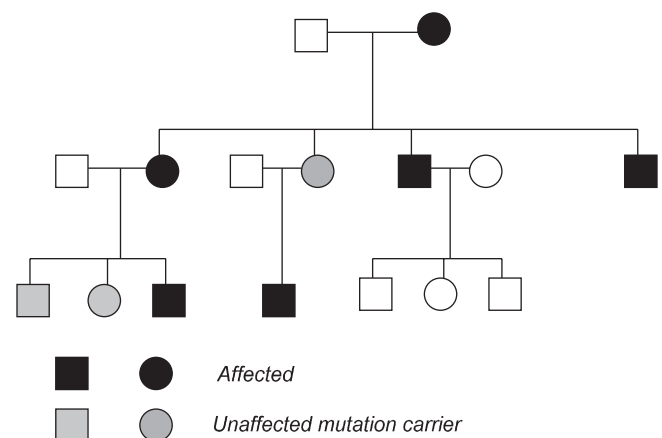
### 8.11.2 Mitochondrial Inheritance

The nuclear chromosomes are not the only source of coding DNA sequences within the cell. Mitochondria possess their own DNA, which, as well as coding for mitochondrial transfer RNA and ribosomal RNA, also carries the genes for 12 structural proteins that are all mitochondrial enzyme subunits. Mutations within these genes have been shown to cause disease (e.g. Leber's hereditary optic neuropathy, LHON). The inheritance pattern of mitochondrial DNA is, however,

very different from that of nuclear DNA. Mitochondria are exclusively maternally inherited. Therefore, mitochondrial mutations can only be transmitted through females, although they can affect both sexes equally. Genetic assessment of mitochondrial diseases is complicated by the great variability of these disorders and a pedigree that is seldom conclusive of maternal transmission, as many cases are due to sporadic mitochondrial DNA mutations or are determined by nuclear genes.

In Leber hereditary optic neuropathy, the pattern of maternal inheritance is well documented. The commonest mitochondrial DNA mutation is a point mutation in base pair 1178 of the ND4 gene of complex I. Two other common mutations have also been described (G3460A and T14484C), which also involve genes encoding complex I subunits of the respiratory chain. More than 95% of the cases are the result of one of these three mutations. Women with the mutation will transmit it to all offspring, but only around 1 in 2 males and 1 in 10 females with the mutation develop loss of vision. Affected and carrier males do not transmit the mutation to their offspring. An example of a pedigree for a family with Leber's is shown in Figure 8-21.

A single cell contains many copies of the mitochondrial genome, and heteroplasmy for a mitochondrial mutation is usual. Heteroplasmy is the presence in the cell of both the wild-type and mutated copies of the gene. The proportion of mutant mitochondrial DNA in leukocytes is not a reliable indicator of which individuals will develop symptoms. As with other mitochondrial disorders, this presents problems in counseling asymptomatic individuals known to carry the mutation. The recurrence risks for mitochondrial disorders are difficult to determine. Large-scale mitochondrial DNA deletions, as in Kearns–Sayre syndrome, are usually sporadic, while point mutations, as in Leber hereditary optic neuropathy, are more likely to be maternally transmitted.



**FIGURE 8-21** Pedigree showing Leber's Hereditary Neuropathy due to a mitochondrial mutation.

### 8.11.3 Chromosomal Disorders

Factors that indicate the possibility of a familial chromosomal disorder in a pedigree include a family history of infertility, multiple spontaneous abortions, and malformed stillbirths or live-born infants with multiple congenital malformations occurring in a pattern that does not conform to that of Mendelian inheritance. **Figure 8-22** illustrates a kindred in which there is the segregation of a reciprocal translocation in both balanced and unbalanced forms. The possibility of a subtle rearrangement not detected by routine cytogenetic analysis but leading to recurrent chromosome imbalance in offspring should be borne in mind and a number of techniques can be used to identify this situation, such as fluorescence in situ hybridization (FISH), telomere FISH, comparative genomic hybridization, and microarray analysis.

It is difficult to give precise reproductive risks to a person known to carry a balanced reciprocal translocation. The risk of viable chromosome imbalance will depend on the size and origin of the unbalanced segment generated, and there is an association between the mode of ascertainment and the risk of recurrence although this is not absolute. Ascertainment through the live birth of an abnormal baby is associated with a higher risk than ascertainment through miscarriage or infertility. Information from an individual pedigree is seldom sufficient to derive a family-specific risk based on reproductive outcomes in known carriers, and the estimation of risk is mainly based on the likely segregation pattern at meiosis and the chance of an imbalance being viable.

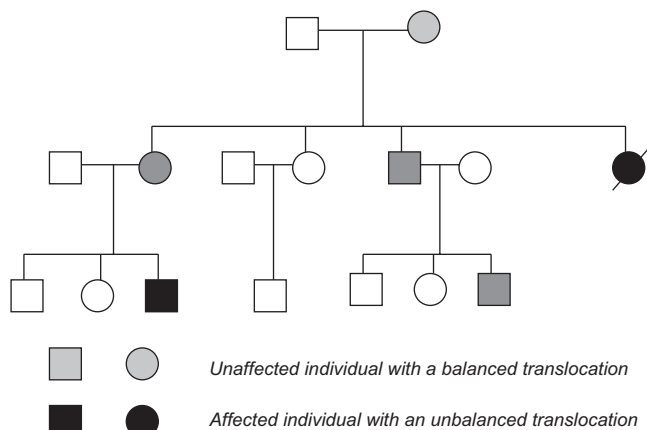
The majority of chromosomal disorders present as sporadic cases. Although very low, the recurrence risk may be increased above the normal age-related risk. After the birth of a child with Down syndrome due to trisomy 21 to a mother under the age of 35 years, the recurrence risk is 0.5% for trisomic Down syndrome and 1% for all chromosomal abnormalities. For mothers over the age of 35 years, the birth of a child with Down syndrome does

not appear to increase recurrence risk significantly compared to the population-related risks. The risk for other family members is not increased.

### 8.11.4 Multifactorial Inheritance

A large group of relatively common disorders have a considerable genetic predisposition, but do not follow clear-cut patterns of inheritance within families. These include many birth defects and chronic diseases of later life. Liability to these disorders appears to be due to the interaction of several genetic and environmental influences. Clusters of cases within a family may simulate a Mendelian pattern of inheritance. It is often difficult to be precise about recurrence risks in multifactorial disorders. The risk is greatest among first-degree relatives, is usually small for second-degree relatives, and often does not exceed the general population risk for third-degree relatives. The risk increases when multiple family members are affected. Risks are also affected by the incidence of the disorder in the general population and the sex of the patient and relatives in disorders that have unequal sex incidence, such as pyloric stenosis and Hirschsprung disease. In these disorders, recurrence is higher in the siblings of the less affected sex probably reflecting a greater genetic load to cause the disease in that sex. The severity of the disorder also influences risk; for example, the recurrence risk is greater for bilateral cleft lip and palate than for unilateral cleft lip alone.

In many disorders, empirical risks have been derived from family studies that provide data on observed recurrences. Risks derived in this way will be less accurate in disorders that are genetically heterogeneous and may not be applicable to populations different from those in which the family studies were performed. Nevertheless, empirical risks provide a useful basis for discussing levels of risk during genetic counseling. Risk tables for some of the common congenital malformations, such as cleft lip, with or without cleft palate, pyloric stenosis, and neural tube defects have been published. Considerable heterogeneity occurs within groups of disorders traditionally considered to be multifactorial or polygenic, such as diabetes, epilepsy, and congenital heart disease, with a proportion of cases attributable to single-gene defects. Identification of a specific genetic etiology, such as the presence of a submicroscopic deletion at chromosome 22q11 in some cases of nonsyndromic congenital heart disease, is important in providing genetic advice appropriate to a particular case. Hirschsprung disease provides a good example of a polygenic disorder in which involvement of several single loci have now been identified in a proportion of families. Data from initial family studies suggested sex-modified polygenic inheritance, and empirical recurrence risks have been produced for genetic counseling based on the sex of the index case and relative



**FIGURE 8-22** Pedigree illustrating the segregation of a balanced and unbalanced reciprocal chromosome translocation.

and the length of the aganglionic segment involved. A mode of inheritance compatible with an incompletely penetrant autosomal-dominant gene was suggested in some families. Linkage to a gene on chromosome 10 was subsequently demonstrated and mutations in the RET oncogene demonstrated. Mutations in other genes, notably the endothelin receptor type B gene, have also been implicated. Thus in a subset of families with Hirschsprung disease, there is a major unifactorial predisposition, a situation likely to be reflected in other multifactorial disorders.

### 8.11.5 Isolated Cases

Many patients presenting to the genetics clinic represent isolated cases within the family, and pedigree information does not contribute to defining the mode of inheritance. Genetic advice in such cases depends on reaching a diagnosis in the affected individual and identifying the underlying etiology. Any of the following situations may apply:

1. The disorder may be due to an autosomal-dominant gene defect, arising by new mutation, transmitted through a nonpenetrant or very mildly affected parent, or by a clinically unaffected parent who carries a mosaic germ line mutation. The situation may also represent nonpaternity. Risk to siblings varies between 0% and 50%, depending on the origin of the mutation, while risk to the offspring of the affected individual would be 50%.
2. The disorder may be caused by an autosomal recessive gene defect with a 25% recurrence risk for siblings unless due to UPD or new mutation. If the carrier state can be confirmed by molecular or biochemical analysis, cascade screening of other family members may be appropriate when the population carrier frequency is high or consanguineous marriages are planned.
3. The disorder may be due to an X-linked gene, usually presenting in a hemizygous male but affecting females if the X-inactivation pattern is skewed or the gene acts dominantly. Isolated cases may represent new mutations, which are frequent in lethal X-linked recessive disorders, or may be transmitted by asymptomatic mothers who are carriers or who have germ line mutations.
4. The disorder may be due to a mitochondrial DNA mutation representing a sporadic case or maternal transmission.
5. The disorder may be due to a chromosomal abnormality. Many of these, including the common trisomies due to nondisjunction, have a low risk of recurrence, but unbalanced karyotypes due to familial chromosomal rearrangements may carry high risks of recurrence, and investigation of the relatives is required.
6. The disorder may be polygenic and recurrence risks depend on the disorder. These are based on empirical data derived from family studies.
7. The disorder may have a nongenetic etiology with no increase in the risk of recurrence, unless due to a teratogenic agent that further pregnancies will also be exposed to.

## CROSS REFERENCES

Epigenetics, Mutations in Human Genetic Disease, Chromosomal Basis of Inheritance, Mitochondrial Genes in Degenerative diseases, Cancer, and Aging, Multifactorial Inheritance and Complex Diseases, Risk Estimation in Genetic Counseling, Genetic Counseling.

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# CHAPTER

# 9

## Analysis of Genetic Linkage

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### 9.1 INTRODUCTION TO LINKAGE ANALYSIS

Linkage analysis is a well-established genetic method used to map the genes for heritable traits to their chromosome locations. It is part of a larger process that has been referred to as “reverse genetics,” because the approach works in the reverse order from our model of how genes operate, biologically. That is, while genes act in a forward fashion to produce a trait, reverse genetics starts with the trait and uses linkage analysis along with other analytic methods to identify the predisposing genes. Reverse genetics became feasible in the 1990s, when a very extensive panel of multi-allelic markers that spanned the human genome was established. During the last 10 years, the genome-wide markers in use have evolved from multi-allelic to bi-allelic single nucleotide polymorphisms (SNPs) where their spacing is much denser. The whole genome is analyzed and the approach is referred to as a full genome linkage scan.

The genome-wide markers are genotyped and tested in a study sample of pedigrees and those showing the strongest statistical evidence of linkage exceeding a predetermined threshold localize the trait gene to the chromosome segment where the markers reside. The resolution at the locus is usually quite poor, as many genes will reside within a linked region. Nevertheless, a statistically significant linkage result limits the search for the predisposing gene to those in the linked region, thus reducing cost and follow-up time. Once a trait gene is mapped by linkage, other strategies such as fine mapping, linkage analysis with additional markers and targeted association analysis can be used to further refine the linked region and identify the gene of interest. These methods include linkage analysis with additional markers, targeted association with SNPs, and genetic sequencing. Using the advances made by the Human Genome Project, reverse genetics has been very effective in identifying genes causing rare Mendelian disorders that result from a single gene.

Linkage analysis has two requirements. These are families with individuals who exhibit a heritable trait

of interest and members of the families that have been genotyped for markers. Thus, the first critical step is to establish that the trait of interest is heritable and to assess its mode of inheritance. The hallmark of a heritable trait is that it is shared to a greater degree by genetically close relatives as compared to distant relatives. Comparison of trait concordance rates in monozygotic and dizygotic twin pairs is the classic approach to assess heritability, but other designs have also been used.

In a linkage analysis, the families can vary in size from nuclear families or sibships with at least two genotyped children measured for the trait to large pedigrees with complicated structures in which a substantial percentage of the members are genotyped and measured for the trait. The trait can be binary, having only two values, such as the absence or presence of a disease, or quantitative, continuous, and possibly normally distributed, such as height. Statistical algorithms are applied to the family structure, marker, and trait data to test if a trait value co-segregates with a particular marker allele within each family more often than one would expect by chance alone. The segregating marker allele may differ among families and the results are combined over all of the families. Chromosome regions where statistically greater than expected marker/trait co-segregation occurs are considered to be linked to the trait. The analytic linkage algorithms can be simple or complex and easy or difficult to apply and interpret. The design of a good linkage study involves the consideration and coordination of all of these factors.

For Mendelian traits that follow a pattern of inheritance consistent with a single gene that is X-linked, autosomal recessive, or autosomal dominant, linkage between the trait and the genetic markers is tested using a parametric linkage analysis that models the mode of inheritance of the trait in the analysis. Application of appropriate computer software results in an estimate of a recombination fraction between the trait gene and a chromosome locus. The linked region is usually relatively large, and a statistically successful linkage result is usually followed by fine mapping of additional markers

in the same pedigrees within the linked region. These marker genotypes are analyzed by additional linkage or association tests to better localize the gene. For genetically complex traits, which are discussed in the section addressing model-free linkage analysis, the initial approach is similar to the one used for Mendelian traits, but the mode of inheritance is not known and thus, not specified.

It should be noted, however, that currently many gene identification studies are bypassing linkage analysis. Pedigrees have been reduced to single individuals, genotyping is done with very dense SNP marker panels, and trait genes are identified by testing individual SNPs using the statistical method of association analysis. Rather than testing co-segregation of marker alleles and trait values of individuals within pedigrees, the current studies capitalize on linkage disequilibrium, which is a reflection of the co-segregation of base-pair alleles over many generations due to their very close proximity.

The important underlying biological concepts and statistical methods for conducting and interpreting a linkage analysis are presented in this chapter. A very detailed discussion of the additional aspects and refinements of linkage analysis is provided in the book *Analysis of Human Genetic Linkage* by Jurg Ott listed in the further reading section.

## 9.2 LINKAGE ANALYSIS: BASIC CONCEPTS

Linkage analysis is a statistical procedure to combine data on family structures, trait values, and genetic markers. Its biological basis is the detection of recombination between markers and traits, and a reduced amount of recombination compared to what is expected is the hallmark of linkage. Interpreting this statistically requires an understanding of likelihood functions, maximum likelihood estimation, and odds ratios. These basic concepts and their integration to produce parametric linkage analysis are discussed below.

### 9.2.1 Recombination: Biological Basis of Linkage Analysis

Linkage analysis is based on the biological phenomenon of genetic recombination, which occurs in the parental gametes during the process of meiosis before the eggs and sperm are produced. In a parental gamete, when a pair of chromosomes, one from each grandparent, aligns in the first metaphase, an exchange of chromosome material often occurs via a crossover event, with the crossover location thought to be determined by chance. This recombination of genetic material results in chromosomes different from those that would be inherited from either parent alone. Thus, each child inherits a unique set of chromosomes that are recombinants of the grandparents'. Linkage analysis is based on identifying

recombination events between genetic markers and trait loci and inferring whether a trait and marker alleles are traveling in close proximity on the same chromosome or are further away or on different chromosomes. The fundamental principle of linkage analysis is that for any two loci on the same chromosome, the closer they are to each other, the less likely it is that they will undergo recombination. Linked genes are those located close enough to each other on a chromosome so that an expected crossover rate within the genetic material separating them at meiosis is less than 50%. Although recombination rates are not uniform across the genome, this principle has provided an effective biological model for linkage analysis.

### 9.2.2 Linkage Analysis Simplified: Inbred Mouse Strains

Families segregating a trait of interest are essential for linkage analysis. The patterns of allele frequencies in human genes and their co-segregation derive from millennia of nonexperimental mating, resulting in our current human population. Thus, unlike experiments with inbred mouse strains, all genetic studies in humans can only be observational. However, to illustrate some basic concepts used in linkage analysis, we first discuss the approach in inbred strains where the ideas are very straightforward. How these principles are applied to analyze linkage in human pedigrees is discussed in greater detail later in the chapter.

In experimental species, controlled crosses can be optimally designed to investigate recombination between loci. For two biallelic markers  $A,a$  and  $B,b$ , homozygous parental inbred strains can be crossed, where a phase known genotype of  $AB/AB$  is crossed with a phase known genotype of  $ab/ab$ , yielding a first generation (F1) with phase known  $AB/ab$  individuals where the alleles inherited from each parent are located on distinct chromosomes divided by the line "/" in our notation here. F1 individuals can be crossed back to either parental line, say,  $AB/ab \times AB/AB$  (backcross), or they can be crossed among themselves,  $AB/ab \times AB/ab$  (intercross). Because the chromosomal origin of each allele is unambiguous, recombination events can be directly identified in each offspring by genotyping markers. Consequently, estimation of the recombination fraction,  $r$ , the observed number of crossovers between the two loci divided by the possible number of crossovers in that interval, is very simple to estimate by counting. If the estimate of  $r$  is close to zero, we can infer that the two markers are very close together; while if it is 50%, we can infer that the two markers are very far apart on the same chromosome or they are on different chromosomes. This is because the expected crossover on the same chromosome or reshuffling of chromosomes during meiosis results in a 50% chance of seeing the parental genotype combination in the child. Linkage is inferred when  $r$  is significantly less than 50%.

The option of counting recombinants is usually not possible in humans. A situation analogous to a murine backcross consists of a nuclear family where the observed parental genotypes for markers at two loci are  $AaBb \times aabb$ . Note, however, that the phase of these genotypes is not known for the double heterozygote: that parent may be either  $AB/ab$  or  $Ab/aB$ . This precludes simple counting as a method for estimating  $r$ . Historically, these two possible phases have been called *coupling*  $AB/ab$  and *repulsion*  $Ab/aB$ . The possibilities of coupling and repulsion are modeled by the statistical methods of parametric linkage analysis. Since there is a 50% chance that the gametes are in coupling and a 50% chance they are in repulsion, the evidence for linkage is examined under both assumptions and the evidence is then combined.

### 9.2.3 Parametric Linkage Analysis: Statistical Concepts

The parametric method to sequentially test for linkage in pedigrees was adapted and applied by Newton Morton in his 1955 paper, listed in further reading section. It is a procedure where  $r$  is the parameter of interest, and each pedigree is analyzed separately, using the same analytic algorithm separately at fixed values of  $r$ . The results are combined sequentially over the families that are tested until a decision regarding linkage is reached. Using Morton's sequential linkage approach, linkage is inferred if the evidence from the tested families results in an LOD score, the test statistic, exceeding 3.0 and is ruled out if the LOD score falls below  $-2.0$ . These values are equivalent to the odds of 1000:1 for linkage or 100:1 against linkage, respectively. They are derived from the prior probability of linkage to a region and the multiple tests that are being conducted. Although many new approaches, algorithms, and extensions to the algorithm have been developed since that time, the method remains in use. Since many of the important concepts and approaches to linkage analysis were developed in relation to this algorithm, those concepts are presented in the context of the algorithm to detect parametric linkage.

**9.2.3.1 Likelihoods, Maximum Likelihood Estimation and Statistical Significance.** We begin by defining the concept of likelihood in the context of linkage analysis. In general, if  $H$  is a hypothesis (e.g. two loci are linked with a recombination fraction of  $r$ ) and  $D$  is the data collected to test the hypothesis (marker and trait information in families), statistical theory tells us that the likelihood of this hypothesis,  $L(H)$ , is proportional to the probability of the data when we assume that hypothesis is true,  $p(D|H)$ . Constructing a likelihood  $L(D|H)$  requires representing in symbols a model of how the data (trait phenotypes and marker genotypes generated in the pedigrees) are expected to co-segregate in the families. The principle of maximum likelihood states that the hypothesis or model with greatest value for the likelihood is that for which the probability of

the data that is observed is maximized. For linkage, we assess the value of  $r$  giving the maximum value for the likelihood for the available data. That is, this maximum is obtained by finding that value of  $r$  which makes the probability of the experimental data in the model containing  $r$ ,  $p(D|r)$ , the largest. Originally the likelihoods were calculated for specific values of  $r$  that are 0.01, 0.05, 0.10, 0.20, 0.30, and 0.40. With the advent of efficient computer programs, the maximum likelihood estimate of  $r$  is obtained currently using a numerical approach, where an algorithm or procedure is used to climb the surface of the likelihood until a maximum is reached. Thus  $r$  may not be estimated at any of the fixed values listed above.

As with all estimates derived from a limited sample of experimental data, a significant sampling error is associated with  $r$ , and linkage cannot be inferred from this value alone, even if it is less than 0.5. Rather, a statistical test that contrasts the likelihoods of linkage and independent segregation is conducted. The likelihood of the latter hypothesis is proportional to the probability of the observations when  $r$  is 0.5, which is the null hypothesis of no linkage. The ratio of the likelihoods is  $p(D|\hat{r})/p(D|0.5)$ , which reflects the odds in favor of linkage at the maximum likelihood estimate of  $r$ . The log of this is referred to as the LOD score, discussed below.

**9.2.3.2 LOD Scores.** The LOD score represents the logarithm in base 10 of the odds of linkage of a trait at a recombination fraction  $r$  with a particular marker locus compared to a recombination fraction of 0.5 between the marker and the trait. The term LOD is derived from the first letters in the log of the odds and the method it represents provides a different way of assessing the significance of the linkage signal, other than a  $p$ -value. The LOD score approach was applied by Newton Morton to the linkage problem in his paper presenting the methods in 1955, and is from the field of sequential methods in statistics, where all the evidence is not assessed in one large analysis, but data are collected and used sequentially to reach a decision. This reflects an efficient and economical method for studying families—once a decision is reached it is not necessary to study additional families. In symbols, the LOD score for linkage takes the form  $\log p(D|\hat{r})/p(D|0.5)$ , where  $D$  is the marker and trait data in the families and  $r$  is the recombination fraction. We infer that there is linkage when the LOD score exceeds 3.0, a value that was calculated by Morton by taking into account the fact that any two loci in the whole genome have a certain prior probability of being on the same chromosome. Other criteria have been proposed for more densely spaced markers, and Nyholt has addressed the issue of significance levels for different statistical methods of linkage analyses more fully. Together with the maximum likelihood estimate of the recombination frequency and its associated LOD score, it remains customary to report a LOD score table, where the LOD



score is computed for a set of predetermined recombination fractions of 0.001, 0.01, 0.05, 0.10, 0.20, 0.30, and 0.40. Through this convention, results from independent studies can be combined at these particular recombination fractions without reanalysis of the combined set of families. Once the LOD score exceeds 3.0 at one of the recombination fractions, linkage is declared, and the distance between the trait gene and the marker is inferred to be that recombination fraction among the ones listed above exhibiting the largest LOD score. If  $r$  is 0.05 at the highest LOD score, it means that the trait and the marker are 5 centiMorgans (cM) apart. A Morgan is defined as the distance along a chromosome where exactly one recombination is expected. A cM is 0.01 of a Morgan. A rough rule is that the map distance of a cM is equivalent to one million base pairs in physical distance. Thus, the recombination fraction of 5 represents a distance of 5 million base pairs. There are likely to be many genes in a region of this size, and follow-up work is required to identify the specific gene involved.

**9.2.3.3 Modeling Traits with Penetrance Functions.** Computer software for parametric linkage analyses requires that the mode of inheritance of the trait of interest be specified in terms of the penetrances of the genotypes at the causal gene. To do this, it is usually assumed that the trait gene is biallelic. Penetrance is the probability that the trait genotype will lead to the phenotype of interest, and the penetrance values vary between 0.0 and 1.0. If we assume that the two alleles are  $d$  and  $D$ , the inheritance pattern is modeled by setting the values of the penetrances of the three possible genotypes,  $DD$ ,  $Dd$ , and  $dd$ . In a simple model of a dominant binary trait, the penetrances of  $DD$  and  $Dd$  are each 1.0 in those with the trait. That is, if one or two copies of the disease allele,  $D$ , is present, it will surely lead to disease. Since the  $dd$  genotype will never lead to disease, its penetrance is 0.0. The penetrance values for those without the disease are 0.0, 0.0, and 1.0 for  $DD$ ,  $Dd$ , and  $dd$  respectively. That is, only those with the  $dd$  genotype will develop the disease. For a recessive disorder, where  $D$  is again the disease allele, the penetrances for  $DD$ ,  $Dd$ , and  $dd$  in those with the disease are 1.0, 0.0, and 0.0 respectively

and in those without it are 0.0, 1.0, and 1.0 respectively. These very simple models ignore the possibility of alternative disease predisposing genes, phenocopies that do not have a genetic basis for the phenotype but exhibit it anyway, and reduced penetrance where the predisposing genotypes result in the disease only some of the time.

**9.2.3.4 Designing and Conducting Parametric Linkage Analyses.** The purpose of linkage analysis is to accrue statistical evidence regarding the co-segregation of a trait and marker alleles within families. It should be noted that the trait can co-segregate with a different marker allele in each family and linkage would be established. Thus, to begin selecting families, each would be chosen because some members have the trait of interest. Mendelian traits that have a known mode of inheritance and are relatively rare are investigated in extended families. This is particularly true if they are recessive conditions in population isolates with few founders or dominant fully penetrant traits where trait and marker inheritances can be followed along extended lineages within the families. In addition, for a rare condition, ascertainment of large pedigrees is usually the only way a substantial number of affected individuals can be obtained for linkage analysis. Linkage analysis of large pedigrees is usually performed with the assistance of computer programs such as LINKAGE and SAGE/LODPAL. Tables 9-1 and 9-2 give some of the most commonly used computer programs that are used to test for parametric linkage. Here, the LINKAGE software is appropriate for the analysis of binary traits in large pedigrees. Other programs, such as Option 2 of Mendel will conduct the same analysis, although the format of the input files and output reports of the programs may differ.

Regarding the selection of families for a study sample, knowledge about the mode of inheritance should have the greatest influence. Families that include individuals who are inbred recessive homozygotes for traits of interest provide a powerful study design when they can be ascertained. It is also important to recognize that informative families are those that are segregating the trait in all branches so that the ability to detect the trait in each branch of the family is an important first step before collecting and

**TABLE 9-1 Linkage Analysis Software for Binary Traits in Large Pedigrees and Nuclear Families Indicating Whether the Analysis Is Parametric and Model Based or Model Free**

Linkage of Binary Traits in	Program Name	Model	Extra Features
Large pedigrees	LINKAGE	Based	HOMOG: locus homogeneity testing
	MENDEL Option 2	Based	Two point and multipoint
	SIMWALK	Both	Uses MCMC algorithm to analyze multiple markers in large pedigrees
	SAGE/LODLINK	Based	Two point
	SAGE/MLOD	Based	Multipoint
Nuclear families	GENEHUNTER/ESTIMATE	Free	Moderate size pedigrees Parent-of-origin
	SAGE/LODPAL	Free	Covariates possible
	MERLIN	Based	Empirical $p$ -values
	SAGE/SIBPAL	Free	Empirical $p$ -values

**TABLE 9-2 Linkage Analysis Software for Quantitative Traits in Large Pedigrees and Nuclear Families Indicating Whether the Analysis Is Parametric and Model Based or Model Free**

Linkage of Quantitative Traits in	Program Name	Model	Extra Features
Large pedigrees	LOKI	MCMC algorithm	Multiple QTL
	SOLAR	Variance components	Bivariate QTL
Nuclear families	GENEHUNTER/MAPMAKERSIBS, NPL, HASEMAN ELSTON	Both	Moderate size pedigrees
	MERLIN/REGRESS	Free	Parent-of-origin effects
	SAGE/SIBPAL	Free	Empirical <i>p</i> -values Empirical <i>p</i> -values

genotyping DNA. It is also important to recognize that a single individual in a sibship where phase is not known will not provide information regarding linkage.

### 9.3 EXTENDING PARAMETRIC LINKAGE ANALYSIS

Since it was developed, methods and computer programs have been included to make linkage analyses more precise and powerful. Their applications are discussed here. First, the methods used to test linkage for a Mendelian binary trait have been extended to model the inheritance of traits that result from a single gene, but the trait genotypes have penetrance values that are neither 0 nor 1. This occurs when the risk genotypes do not always lead to the development of the trait (reduced penetrance) or when there are people who exhibit the trait but do not have the trait genotype (phenocopies). Second, the methods have also been extended to reflect genetic heterogeneity, the existence of genes at other loci leading to the development of the same trait. If it is not recognized, genetic heterogeneity can mask a true linkage signal. Third, incorporating the genetic information from several markers simultaneously can improve the linkage signal and better localize the trait gene. Multipoint analyses accomplish this goal. Each of these important factors is discussed below.

#### 9.3.1 Incomplete Penetrance and Phenocopies

Penetrance specifies the probability that an individual with one of the possible trait genotypes will exhibit the trait. Age of onset or gender-specific trait risks can be incorporated with a penetrance model through multiple liability classes with differing penetrance estimates. For example, disease penetrances for DD, Dd, and dd can be 80%, 40%, and 10% in males and 20%, 10%, and 2% in females. For unaffecteds, the DD, Dd, and dd penetrances would be 20%, 60%, and 90% in males and 80%, 90%, and 98% in females, as the two penetrances within a liability class for a given genotype must always sum to 1.0. Phenocopies reflect the occurrence of a trait undistinguishable from the trait of interest, but resulting from other causes. A phenocopy rate can be

incorporated by assuming that affected individuals with a normal genotype have a small probability of expressing the disease, such as 5%. The power of linkage analysis can be affected significantly by phenocopies when they account for more than a small percentage of the cases. If penetrance values are unknown, the careful investigator will take the precaution of verifying that his or her inference of linkage does not critically depend on the assumed penetrances, by testing linkage over a reasonable range of penetrance estimates.

#### 9.3.2 Genetic Heterogeneity

Defining inherited conditions solely on the basis of their clinical manifestations may obscure the fact that the trait under analysis is the result of distinct genetic etiologies that segregate among the families. Unless the trait is the result of different alleles at a single locus, heterogeneity may drastically reduce the power of a linkage analysis when it is not considered. There are several ways to address this. The first is to make a predefined partition of the families using a particular form of the phenotype or a factor such as their ethnicities. One then conducts a linkage analysis in each group separately and reports the results for each group. At a particular locus, one can establish locus heterogeneity by also testing for linkage in the entire sample. Using these three linkage analyses at a single locus, a likelihood ratio test is constructed, contrasting the product of the maximized likelihoods in each subset to the maximum likelihood obtained for the total sample. The test statistic is represented by  $2 \log_e [\Pi_i p(D_i|r_i)/p(D|r)]$ , where  $i$  indexes the two sets of families. That is twice the natural log of the product of the likelihoods of the two data sets with two different recombination fractions compared to the likelihood of all of the data combined with a single recombination fraction. Under the null hypothesis of homogeneity, a single recombination fraction, this statistic follows a  $\chi^2$  distribution with  $n-1$  df (degree of freedom). Here  $n$  is the number of classes into which the data have been partitioned, which is 2 in this case, resulting in 1 df.

A more formal statistical approach to modeling and estimating the degree of heterogeneity among a sample of families is available through the LINKAGE software referred to as HOMOG. Here a test of heterogeneity can

be used when the alternative to homogeneity is that the families belong to two etiologic classes, one linked and the other unlinked to the marker locus, although they do not have to be divided a priori as with the test described above. Assuming that a proportion,  $m$ , of the families exhibit linkage while  $1 - m$  are unlinked, the likelihood of the observations can be expressed in terms of two parameters, the recombination fraction  $r$  for the linked form and the admixture proportion,  $m$ . A likelihood ratio test can be formulated by contrasting the likelihood obtained when both parameters are estimated to that obtained under the hypothesis of homogeneity, where  $m$  is fixed to unity and only the recombination fraction,  $r$ , is estimated. This follows a  $\chi^2$  distribution with 1 df. The two parameters  $m$  and  $r$  can be estimated simultaneously. For tests of locus heterogeneity involving more than two independent sampling units, greater power is expected for the admixture test than for the predivided sample test, because of the smaller number of degrees of freedom allowed in the test.

### 9.3.3 Multipoint Parametric Linkage Analysis: Location Scores

When conducting a linkage analysis, combining the information from several markers from the same chromosome region simultaneously imparts greater statistical power to detect linkage and provides a more precise localization of the trait gene than when the markers are analyzed separately. The distribution of heterozygosity among the markers will add information regarding linkage of the trait under analysis. Information regarding the location and order of markers used in the analyses is provided by a number of online databases such as the one at National Center for Biotechnology Information. Multipoint analyses are based on more complex probability models than single point. The parameters in the likelihood represent all of the between-locus recombination fractions among markers. Maximum likelihood methods, however, still apply and are used to estimate the recombination fractions between the trait locus and the points along the genetic map of markers in the analysis. The resulting calculations are computationally intensive, but can be carried out using software packages such as MENDEL and SAGE/MLOD. As the trait locus is moved across the marker region, the locus with the largest multipoint LOD score or location score localizes the trait gene to a more refined region than conducting several two point analyses can accomplish.

## 9.4 LINKAGE ANALYSIS FOR COMPLEX AND QUANTITATIVE TRAITS

Linkage analysis has also been extended to include traits that are not Mendelian or binary. Following the completion of the Human Genome Project, the remarkable success in identifying genes for Mendelian disorders

resulted in a gradual shift toward analyzing traits with complex inheritance patterns. Since parametric linkage analysis is not well suited for genetically complex traits, model-free linkage analysis, where a model of inheritance is not known and therefore not included in the analysis, has been conducted. Complexity may derive from something as simple as two disease loci, each exhibiting reduced penetrance, to something as complex as each family with the trait having risk alleles in each 10 possible genes, and requiring one of a large number of environmental triggers. The analyses of both traits are conducted using the same model-free analytic methods. In addition, there has been an interest in the genetics of quantitative traits that may be important on their own or correlate with binary traits of interest. Methods and computer programs have been developed to identify the genetics of these traits as well.

### 9.4.1 Model-Free Linkage Analysis

Model-free methods test if the allele sharing patterns in families are consistent with linkage in a very general way. That is, those in the pedigree who have the trait should display evidence of marker allele sharing to a greater degree than one would expect by chance alone in the linked regions. Most model-free tests only include individuals with the trait of interest. However, genotypes of their relatives are important to make more precise estimates of allele sharing. Using a study sample with the trait reduces the risk of including individuals who do not have the trait but share alleles with those who do. Including them in the analysis would mask evidence of linkage. The most common model-free test statistics is based on allele sharing in sibling pairs. Limiting the analysis to sibling pairs has been effective because it is usually difficult to ascertain larger pedigrees with multiple members having the trait if it is complex. In addition, with multiple common risk alleles, members of large families may develop the trait as the result of several genetic etiologies, thus introducing within pedigree heterogeneity, for which we do not have appropriate analytic approaches. Sibling pairs with a trait are usually available for study and are more likely to have the trait due to a shared genetic etiology. Nuclear families contain less information about linkage than a large pedigree, and thus a larger number of them will have to be studied to find linkage.

The sib-pair allele sharing linkage methods are based on a simple expectation. At a marker, the sibling pairs can inherit four possible alleles from their parents. If there is no gene predisposing to the trait in the region of the marker, the sibling pairs should share 0 alleles 25% of the time, 1 allele 50% of the time, and both alleles 25% of the time. The expected proportion of allele sharing is 50%. A significant deviation from these theoretical values at a marker provides evidence of linkage of the trait to that marker. For model-free analyses, the recombination fraction between the marker and the trait gene is

assumed to be zero.  $R$  is not included in the analysis. For several linkage statistics, the degree of allele sharing for each sibling pair is assessed, and the allele sharing estimates are averaged over the pairs. These individual estimates would be 0,  $\frac{1}{2}$ , or 1 for each pair if the genotypes were fully informative and all parents were completely genotyped. When data are missing or the parental genotypes do not allow for an unambiguous assignment of their inheritance, the marker allele frequencies are used in the estimate of allele sharing. The allele sharing estimates will usually be different from 0,  $\frac{1}{2}$ , or 1 to reflect this. In the simplest case, the average allele sharing value is tested against its theoretical value of  $\frac{1}{2}$ . An additional consideration is that if there are greater than two siblings in a sibship, the pairs will not provide statistically independent allele sharing estimates. A weighting scheme to account for this can be employed. For example, three sibs in a sibship contribute information from 3 pairs, and since the information for the third pair can be derived from the first 2 pairs, allele sharing for these 3 pairs can be weighted by a factor of  $\frac{2}{3}$ . A subset of the programs listed in Table 9-1 implement model-free allele sharing linkage methods for binary traits in nuclear families. They are the SIBPAL program of SAGE, the MLS and NPL options of GENEHUNTER, and the MERLIN REGRESS program. These software packages allow for generation of the allele sharing test statistics at evenly spaced intervals along the entire chromosome, given the marker map, and a multipoint analysis is conducted for each chromosome. The packages provide plots of the statistical significance of the tests across the chromosomes so that the loci with significant evidence for linkage can be identified easily.

### 9.4.2 Linkage Analysis of Quantitative Traits

A quantitative trait has can be dichotomized into one that is binary for a parametric linkage analysis. For example, a systolic blood pressure of greater than 140 is used to classify an individual as hypertensive and hypertension has often been the trait tested for linkage. In fact, the quantitative trait itself may be better suited for linkage analysis than the dichotomized trait, as the extent of variation can be assessed in all family members, thus making it likely to provide increased statistical power to detect genes. In addition, the etiology heterogeneity of genetically complex traits may be reduced by the analysis of a correlated quantitative trait that reflects only one feature of the complex disorder. For example, cardiovascular disease may be better addressed by studying a particular lipid trait, such as cholesterol, rather than the binary trait of a myocardial infarction, which is very likely to have a much broader etiology. Programs to analyze quantitative traits in large pedigrees usually require the assumption of trait normality while those for smaller pedigrees do not require

the rigorous adherence to these assumptions. Programs and their features are given in Table 9-2.

Variation in a quantitative trait usually results from the contributions of multiple genes with small effects modified by environmental influences. If none of the genes contributes a substantial amount to this quantitative variation, loci can be difficult to detect using linkage analysis. However, a gene contributing to a relatively large proportion of the variance of the trait, a major gene, is a good candidate for localization by quantitative trait linkage analysis. When conducting a quantitative trait linkage analysis, it is important to select families for which the trait exhibits marked variation within the pedigree. Those families having multiple members with extreme values of the trait are most likely to provide support for a major gene. Regions that are identified by the linkage analysis of a quantitative trait are usually referred to as quantitative trait loci or QTL.

A list of computer software commonly used for the linkage analysis of quantitative traits is given in Table 9-2. A variance component analysis of a quantitative trait, such as that conducted by the SOLAR software, identifies linked chromosome regions by decomposing the variance of the trait into the components that contribute to it. The log of the ratio of the likelihood of the data with a major gene is compared to the likelihood of the data when there is no major gene modeled at that location. Normality of the trait distribution is an important assumption and if the trait is not normally distributed, transformation to normality is critical to a successful analysis. For non-normal traits in smaller pedigrees, GENEHUNTER/MAPMAKERS-IBS, MERLIN/REGRESS, and SAGE/SIBPAL provide good alternatives. GENEHUNTER/NPL, which uses a nonparametric method that ranks the trait values, is robust against non-normality. The ordered subset analysis approach implemented in OSA can be used to identify QTL for quantitative traits that are correlated with binary traits. The families in the analysis are ordered according to their scores on a quantitative correlated value and the evidence for linkage is assessed as the ordered families are sequentially included in the analysis.

## 9.5 LINKAGE ANALYSIS: FUTURE DIRECTIONS

Linkage analysis has been used to identify chromosome locations of human trait genes for more than 50 years. Its well-developed tools include families, markers, and statistical methods of analysis. The genes for many Mendelian disorders have been identified with these tools. However, as the number of genetic markers increased from 30 to 2.4 million, our ability and enthusiasm to localize and identify genes for traits of increasing genetic complexity has grown proportionally. Such marker



density allows us to capitalize on linkage disequilibrium to identify trait genes, and consequently, during the past 10 years, there have been few modifications to the well-established linkage methods. The primary approach to gene identification has quickly transitioned to genome-wide association studies (GWAS), which capitalizes on the linkage disequilibrium among closely spaced markers in populations. In some sense, analyzing linkage disequilibrium in populations is similar to analyzing linkage in families. To clarify, in the current generation, within a population, SNP alleles are in linkage disequilibrium because they are too close to each other on the chromosomes to have undergone significant recombination over the generations. Thus, one can view GWAS as linkage analyses of a large pedigree that is the current population under analysis. All mapping information is in the current generation, and we test for association of specific marker alleles and the trait of interest.

GWAS are based on association tests of common SNP variants whose frequencies are larger than 5%. The genotyped SNPs have been selected to tag trait variants that may not be tested directly. Although consistent gene associations have been identified for a substantial number of complex traits, their effects have been surprisingly small. Successful GWAS have required very large samples, and the detected effect sizes indicate that the risk is only raised by about 10–20% compared to that of the background genotype. Consequently, the genetics literature has expressed concern that GWAS have not revealed the etiologies of traits with significant heritabilities.

Recently, concern about the small effect sizes of common variants, as well as the dramatic reduction in the cost of whole exome sequencing with “next generation” methods has refocused the interest of many of those studying complex disorders toward the detection of rare genetic variants via sequencing. These variants are expected to have a frequency less than 1%, and may even consist of private mutations. They are also expected to exhibit greater penetrance values and effect sizes than the common variants. To sort through the many variants likely to be uncovered, there has been a renewed interest in the study of large pedigrees. If they exist for a complex trait, analyses will be focused on identifying those pedigrees that segregate the trait where it is acting in a quasi-Mendelian fashion. As with Mendelian disorders, model-based linkage analysis that allows for reduced penetrance and phenocopies may reveal important loci.

Targeted sequencing in linked regions could reveal the genes with the segregating rare variants. With this approach, parametric linkage analysis is likely to again become a natural first step in gene finding efforts over the next few years.

## WEB PAGES FOR LINKAGE ANALYSIS SOFTWARE ARE LISTED IN TABLES 9-1 AND 9-2.

GENEHUNTER. <http://www.broad.mit.edu/ftp/distribution/software/genehunter/>.

LINKAGE. <ftp://linkage.rockefeller.edu/software/linkage>.

LOKI. <http://www.stat.washington.edu/thompson/Genepi/Loki.shtml>.

MENDEL. <http://www.genetics.ucla.edu/software/>.

MERLIN. <http://www.sph.umich.edu/csg/abecasis/Merlin>.

OSA. <http://wwwchg.duhs.duke.edu/research/aplosa.html>.

SAGE. <http://darwin.cwru.edu/sage/>.

SIMWALK. <http://www.genetics.ucla.edu/software/simwalk>.

SOLAR. [http://www.sfbr.org/Departments/genetics\\_detail.aspx?p=37](http://www.sfbr.org/Departments/genetics_detail.aspx?p=37).

## ADDITIONAL WEB PAGES

NCBI. <http://www.ncbi.nlm.nih.gov>.

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### Biography



**Dr Rita Cantor** is a statistical geneticist and a professor of Human Genetics and Psychiatry in the David Geffen School of Medicine at UCLA. With a background in mathematics, statistics, and genetics, her research efforts have been devoted to developing and applying statistical methods in order to reveal the etiologies of genetic disorders. In 1980, she began this work as a postdoctoral fellow in Human Genetics at the Medical College of Virginia by analyzing data from twin studies. At that time, genetic marker data were rare. As the field of genetic epidemiology evolved, she moved to Cedars-Sinai Medical Center and then joined the new Department of Human Genetics at UCLA. During that time she conducted and participated in genome-wide linkage and association studies for both Mendelian and genetically complex disorders. Her current focus is on the genetically complex traits defined by autism spectrum and other psychiatric disorders, coronary artery disease, and systemic lupus erythematosus. The massive amount of data generated by sequencing to identify rare variants will help define her future analytic approaches.

# CHAPTER 10

## Chromosomal Basis of Inheritance

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### 10.1 INTRODUCTION

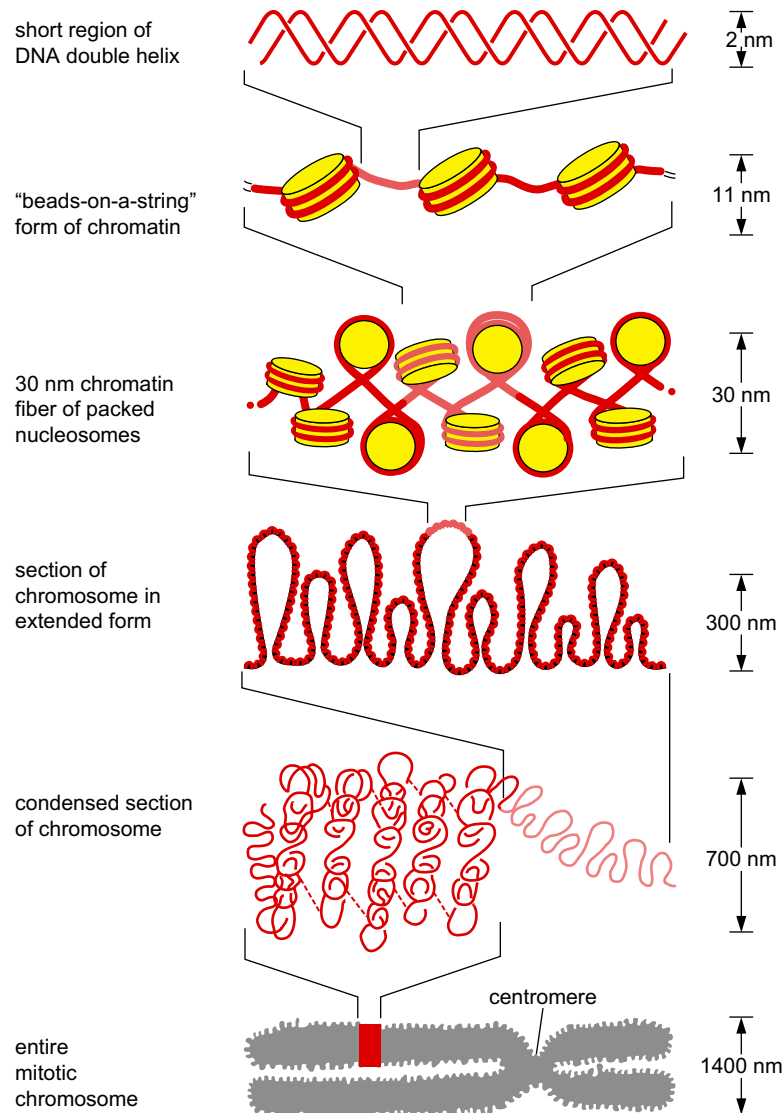
The human genome is packaged into a set of chromosomes as in other eukaryotes. Chromosomes are thus the vehicles of inheritance as they contain virtually the entire cellular DNA with the exception of the small fraction present in the mitochondria. The structure, function, and behavior of chromosomes are therefore of much interest and importance. Chromosomes are derived in equal numbers from the mother and father. Each ovum and sperm contains a set of 23 different chromosomes, which is the haploid number ( $n$ ) of chromosomes in humans. The diploid fertilized egg and virtually every cell of the body arising from it has two haploid sets of chromosomes, resulting in the diploid human chromosome number ( $2n$ ) of 46. The human karyotype consists of 22 pairs of autosomes and a pair of sex chromosomes. The correct chromosome number in humans was determined and confirmed in 1956 (1,2).

The behavior of chromosomes in meiotic cell divisions provides the basis for the Mendelian laws of inheritance, whereas their abnormal behavior in cell division leads to abnormalities of chromosome number. In this chapter, we examine the current understanding of the structure, molecular organization, and behavior of human chromosomes and explore how these features contribute to chromosomal diseases.

### 10.2 CHROMOSOME STRUCTURE

Although the structure of human and other eukaryotic chromosomes is not understood in full detail, recent investigations have provided insights into several aspects of chromosome structure at the molecular level. The haploid human genome consists of about  $3 \times 10^9$  base pairs (bp) of DNA. Since 3000 bp of naked DNA are  $\sim 1 \mu\text{m}$  long, the total length of the diploid human genome is

about 2 m. As the cell nucleus is no more than  $10 \mu\text{m}$  in diameter, it is necessary to fold and compact this DNA, which is accomplished by packaging it in a hierarchy of levels into chromosomes of manageable size (Figure 10-1). Organization of the DNA into chromosomes also maintains the linear order of genes and facilitates faithful replication and segregation of genetic material during cell division. The first level of this packaging, and thus the fundamental unit of chromosome organization, is a regularly repeating protein-DNA complex called the nucleosome. The basic structural features of the nucleosome were established in the early 1970s and have been further confirmed by high-resolution analysis of its crystal structure (3). The nucleosome has the same design in all eukaryotes and consists of a cylindrical core of about 11 nm in diameter and 6 nm in height made up of two molecules each of the four core histones (H2A, H2B, H3, and H4) with 147 bp of DNA wrapped around it. A “linker” DNA connects adjacent nucleosomes. Each nucleosome is also associated with a molecule of histone H1, which changes the path of the DNA as it exits from the nucleosome, and plays a role in further condensation of chromosomal DNA. Formation of the nucleosomes achieves a sevenfold compaction of the DNA double-helix. The next higher level of packaging is the chromatin fiber, visible by standard electron microscopy. This is a superhelix, 30 nm in diameter, composed of nucleosomes and histone H1. The 30 nm fiber is the basic component of interphase chromatin and metaphase chromosomes. Two models have been proposed for the formation of the 30 nm chromatin fiber. In the first model, called the “solenoid,” consecutive nucleosomes are located next to each other in the fiber, folding into a simple one-start helix (4). Subsequently, a second model of the “two-start helix” was proposed on the basis of microscopic observations of isolated nucleosomes (5). Although some variations exist in this model, essentially nucleosomes



**FIGURE 10-1** Various levels of DNA packaging in the cell. (Alberts, B., Johnson, A., Lewis, J., et al. *Part II: Basic Genetic Mechanisms, Chapter 4: DNA and Chromosomes, The Global Structure of Chromosomes, Figure 4-55: Chromatin Packing. In Molecular Biology of the Cell, 4th ed.; Garland Science: New York, 2002.*)

are arranged in a “zigzag” manner, such that a nucleosome in the fiber is bound to the second neighbor, but not the first (6–8). Very recently, it was shown that the two-start zigzag and one-start solenoid models may be present simultaneously in a 30 nm chromatin fiber under certain conditions (9). The structural details of the 30 nm chromatin fiber remain controversial. Formation of the 30 nm chromatin fiber achieves a nearly 50-fold compaction of the DNA double-helix. Short AT-rich regions referred to as matrix attachment regions (MARs) that occur at about every 30–150 kb of DNA anchor the chromatin fiber to the proteins of the nuclear matrix of the interphase nucleus. Topoisomerase II, an enzyme that induces transient double-strand breaks in DNA and permits uncoiling of the two strands of the DNA duplex, is a major matrix protein. At the next level of packaging, the 30 nm chromatin fiber is arranged into loops that radiate from a core or scaffold of the metaphase chromosome. The MARs are also the site for attachment of

the chromatin fiber to the nonhistone protein scaffold of the metaphase chromosome (hence also called scaffold attachment regions) (10). Topoisomerase II is a component of the chromosome scaffold and has been shown to play a role in chromosome condensation. The other major component of the metaphase chromosome scaffold that also plays a key role in chromosome condensation is the condensin complex, a member of the SMC (structural maintenance of chromosomes) family of proteins (11). Other members of the SMC family of proteins mediate chromosomal functions such as sister chromatid cohesion (cohesin complex) and DNA-repair (11). Besides topoisomerase II and condensins, cations are also believed to be essential participants in chromosome condensation (12,13). The details of the higher order structure of chromosomes are not well understood at the molecular level. However, it is clear that each chromosome contains only a single very long duplex of DNA with an estimated packaging ratio of about 1:10,000. At



the highest level of compaction, the metaphase–anaphase chromosomes are most easily movable by the spindle apparatus during cell division.

### 10.3 CHROMOSOMES IN CELL DIVISION

Cell division and proliferation are central to growth and development of multicellular organisms. The major events in the cell cycle are replication and segregation of chromosomes. Cell division also ensures proper segregation and partitioning of the genetic material into daughter cells, thus providing the basis for Mendelian laws of inheritance. The cytologic aspects of mitosis and meiosis, the two forms of cell division in eukaryotes, have been described in great detail in numerous studies in the past. However, the explosive growth of molecular biology in the last 20 years has brought the study of mitosis and meiosis to the forefront again. These investigations have elucidated biochemical aspects of cell cycle biology and chromosome mechanics (14,15). This section describes the essential features of mitosis and meiosis relevant to inheritance. Knowledge of these features is crucial for understanding the Mendelian laws of inheritance, construction of genetic maps, and the origin of chromosome aberrations.

#### 10.3.1 Mitosis

In somatic cells, and in cells of the germline prior to the time they undergo their first specialized meiotic divisions, nuclear division takes place by a process called mitosis. During mitosis, each chromosome divides into two daughter chromosomes (sister chromatids), one of which segregates into each daughter cell. Therefore, the number of chromosomes per nucleus remains unchanged producing daughter cells with identical chromosome constitutions. In cells with a generation time of 18–24 h, mitosis takes about 1–2 h and is divided into five major stages: prophase, prometaphase, metaphase, anaphase, and telophase (Figure 10-2).

In the initial phase of mitosis, prophase, the chromosomes become visible as a result of condensation that continues throughout this phase. Each chromosome has already undergone replication during the preceding interphase, generating two sister chromatids that will become daughter chromosomes. The sister chromatids are closely held together along their length by cohesins until anaphase. The centrioles duplicate during the S phase and move apart to occupy positions at opposite ends of the cell, defining the poles of the mitotic spindle.

During prometaphase, the nuclear membrane begins to disintegrate, allowing the chromosomes to spread around the cell. In metaphase, the chromosomes become attached to microtubules of the mitotic spindle at their centromeres and undergo movements that lead to their alignment in the equatorial plane of the spindle. At this stage, the chromosomes have reached their maximum state of condensation.

Anaphase begins after the chromosomes are fully aligned on the metaphase plate. Each pair of sister

chromatids separates as cohesion is lost, first along the arms and finally at the centromere of each chromosome. The resultant daughter chromosomes move toward opposite poles of the spindle as a result of microtubule dynamics and the action of motor proteins. Recent research has provided insights into the molecular biology of chromosome separation. The two sister chromatids of a chromosome are held together following chromosome replication by cohesins. This is a multi-subunit protein complex that ensures correct segregation of daughter chromosomes at anaphase. At the beginning of anaphase, the cohesin complex is cleaved by a protease called separase, allowing separation of the sister chromatids (16).

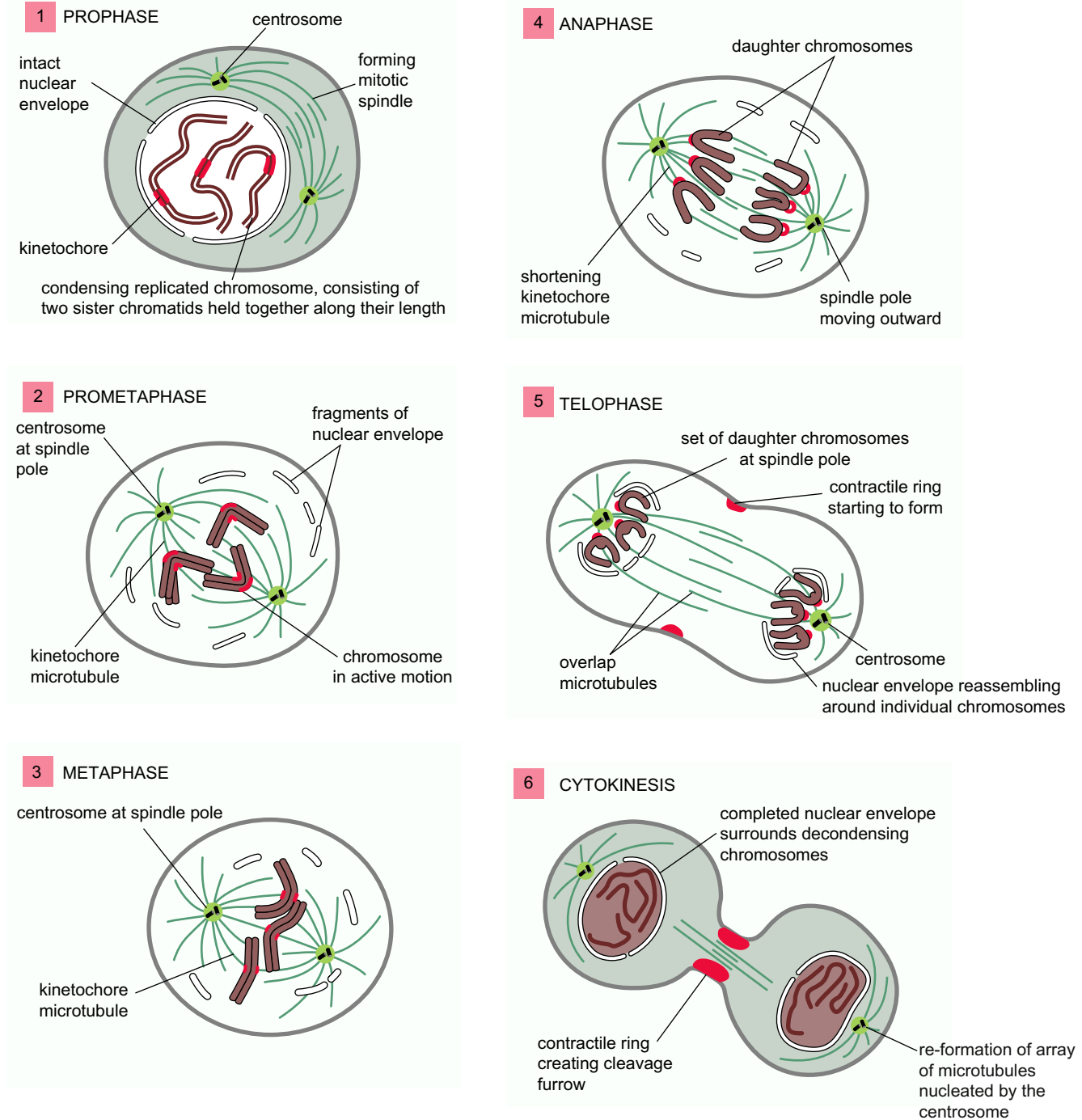
At telophase, each set of daughter chromosomes arrives at the centriole at the ends of the mitotic spindle, and reconstitution of the nuclear membrane begins. Cytokinesis, the division of the cytoplasm, follows telophase and leads to the formation of two genetically identical daughter cells.

#### 10.3.2 Meiosis

Meiosis is a specialized cell division in germ cells that generates gametes with the haploid set of 23 chromosomes. The final gametic set includes single representatives of each of the 23 chromosome pairs selected at random. The details of meiosis and gamete formation are somewhat different in males and females, but the basic features are the same in both and are of fundamental importance. Meiosis accounts for the major principles of Mendelian genetics: segregation, independent assortment, and recombination of linked genes. Recombination or crossing over is the exchange of genetic material between homologous non-sister chromatids, a process that adds to genetic diversity by generating new combinations of genes.

Meiosis consists of two cell divisions (meiosis I and II) and is distinguished from mitosis by the following:

1. Homologous pairing: Maternal and paternal homologs of each chromosome are replicated and then undergo exact pairing along their lengths during prophase of meiosis I. Such a paired unit is called a “bivalent” because there are only two centromeres, although it is composed of four chromatids.
2. Recombination (crossing over): Crossing over occurs at the four-strand stage between non-sister chromatids, that is, chromatids from each of the pair of homologous chromosomes. The probability of recombination increases with the physical distance between two chromosomal sites and therefore provides a basis for the genetic map.
3. Segregation of maternal and paternal homologs: Centromeres do not divide at the first meiotic division (meiosis I). Instead, the members of a homologous pair go to opposite poles at anaphase of the first meiotic division. This accounts for Mendel’s first law, the segregation of homologous genetic units.



**FIGURE 10-2** Diagrammatic representation of the stages of mitosis. (Alberts, B., Johnson, A., Lewis, J., et al. Part IV: Internal Organization of the Cell, Chapter 18: The Mechanics of Cell Division, An Overview of M Phase, Panel 18-1: The Principal Stages of M Phase. In Molecular Biology of the Cell, 4th ed.; Garland Science: New York, 2002.)

The segregation of maternal and paternal homologs in each bivalent chromosome pair occurs independently of the segregation in all the other bivalents. That is, the segregation of chromosome 1 homologs is independent of that of chromosome 2 homologs and so on. This accounts for Mendel's second law of independent assortment of genes. Meiosis I also leads to a reduction in the number of chromosomes from the diploid number ( $2n = 46$ ) to the haploid number ( $n = 23$ ) in the gametes.

4. Division of the haploid set with centromere division: The second meiotic division (meiosis II) occurs without a preceding round of DNA synthesis and chromosome duplication. In meiosis II, the two chromatids of a chromosome move to opposite daughter cells.

Meiotic prophase I is rather prolonged and can be subdivided into five stages on the basis of condensation of chromosomes and the extent of homologous pairing: leptotene, zygotene, pachytene, diplotene, and diakinesis. In

leptotene, the chromosomes start to condense and are visible as long threads but the homologs are still not paired. Chromosomal condensation continues and pairing of homologs (synapsis) begins at zygotene and is completed at pachytene, the stage at which recombination occurs (Figure 10-3). By the pachytene stage, synapsis between homologs is completed and crossing over between non-sister chromatids occurs, during which homologous regions of DNA are exchanged. Synapsis is thought to be mediated and stabilized by the formation of the synaptonemal complex (SC) between homologous chromosomes. The SC is a protein-rich ladder-like structure that has a central element flanked by two lateral elements. The lateral elements and the central element are held together by transverse filaments. The lateral elements are formed of the axial elements of sister chromatids of the paired homologs, and the bulk of the chromosomal DNA is found in chromatin loops emanating from the outer sides of the two lateral elements. Initially, the SC was characterized by ultrastructural analysis. More recent studies have identified several protein components of the SC, although the functions of many of these are unknown. A constituent of the lateral elements is cohesin, consistent with the fact that the sister chromatids of each of the homologs are held together at pachytene. Another interesting feature of SC is the presence of recombination nodules along its length. These are thought to be enzyme complexes that mediate genetic recombination via DNA breakage and repair. Chiasmata or cruciform structures become visible at the more condensed diplotene stage as cohesion is lost along the chromosome arms except at each chiasma, the point of recombination between homologous chromosomes.

Chiasmata are still visible at diakinesis, the stage of maximal condensation, and can be used to determine the frequency as well as location of recombination. Chiasmata, like their underlying recombination events, play an important role in the normal segregation (disjunction) of homologs, and each pair of homologs has at least one chiasma per chromosome arm. Moreover, failure of chiasma formation predisposes to nondisjunction of homologs.

During prophase I in males, pairing and crossing over between the X and Y chromosomes is possible because of a small region of homology at the terminal ends of their arms (i.e. pseudoautosomal regions). The two chromosomes pair and cross over in these regions during prophase I.

In meiotic metaphase I, the nuclear membrane disappears and the chromosomes become aligned on the equatorial plane of the cell where they have become attached to the spindle, as in metaphase of mitosis. Then in anaphase I, the chromosomes now separate to opposite poles of the cell as the spindle contracts. In telophase I, each set of haploid chromosomes has now separated completely to opposite ends of the cell, which cleaves into two daughter gametes, so-called secondary spermatocytes or oocytes.

The meiosis II division resembles an ordinary mitotic division, except for the presence of a single set of 23 duplicated chromosomes, each with two chromatids held together at their centromeres. Also, meiosis II is not strictly a genetically equal division as the two chromatids of a chromosome may not be identical as a result of genetic exchange(s) with a non-sister chromatid. At the end of the two meiotic divisions, each primary spermatocyte or oocyte has given rise to four haploid products (see Figure 10-3). Their fate is rather different in males and females, as discussed later.

### 10.3.3 Spermatogenesis and Oogenesis

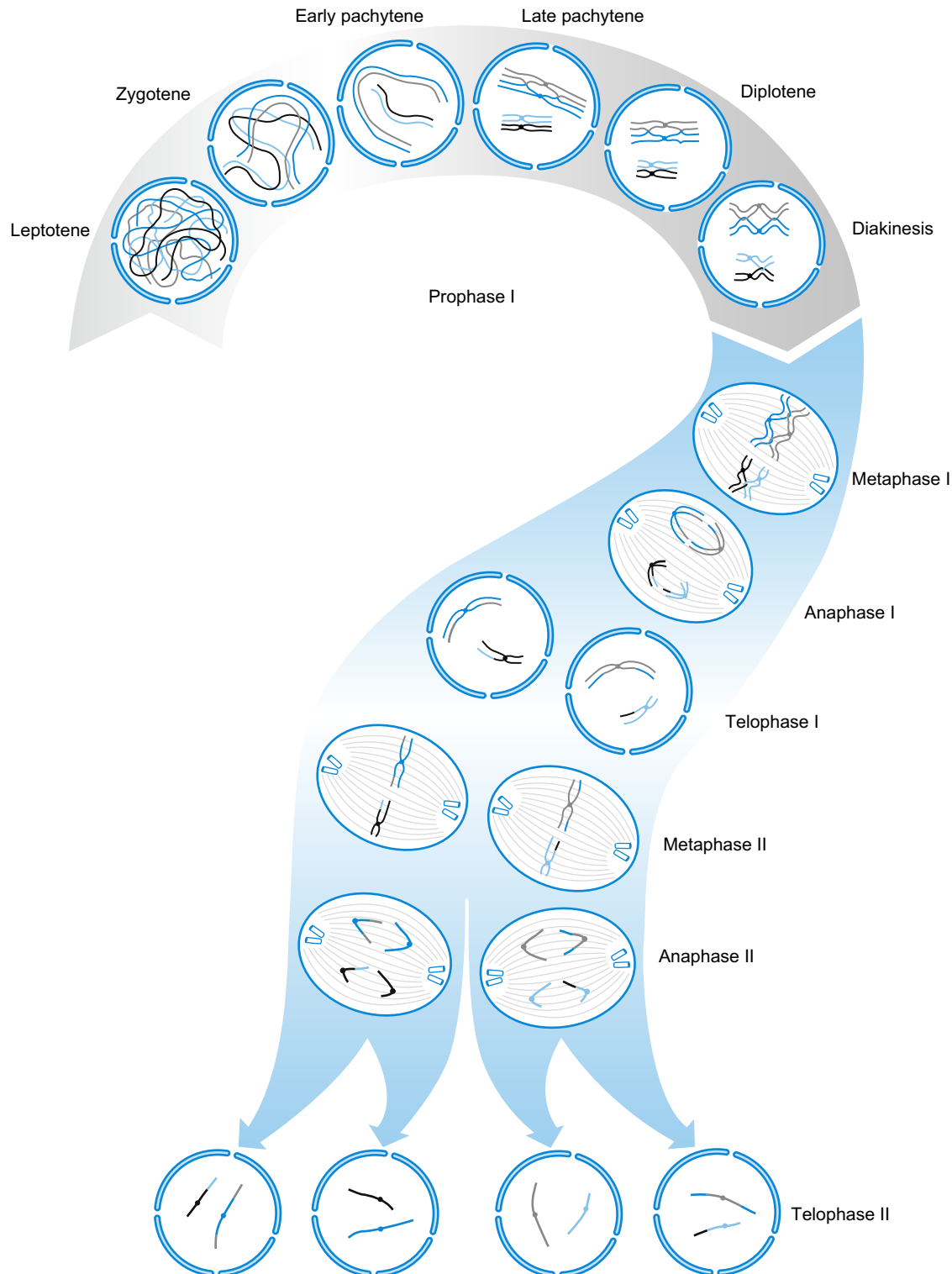
In the human male, the production of sperms begins at puberty and continues throughout life. Undifferentiated stem cells of the germline, the spermatogonia, are abundant in the seminiferous tubules of the testis and show a high rate of mitotic activity throughout the adult life of a normal male. Of the two types of spermatogonia, only type A can differentiate into primary spermatocytes that enter meiosis, whereas type B are the long-lived progenitors that divide to generate daughter cells of both types A and B. In meiosis, each diploid spermatocyte gives rise to four haploid cells, each of which differentiates into a functional sperm. The entire process, from spermatogonium to sperm, takes about 70 days. The rate of sperm production may be as high as 50–100 million per day over many years, and thus the parental spermatogonia undergo many successive mitoses. It is estimated that the number of mitoses before sperm production in a 20-year-old male is about 200, while in a 45-year-old it is about 800 (17). This provides the opportunity for the occurrence of more adverse genetic change with age in males, which is reflected in an increased mutation rate for certain inherited diseases.

The behavior of germline cells in the female is quite different from that in the male. By about the fourth month of prenatal development, about seven million oogonia have begun to develop into primary oocytes and to enter meiosis. Primary oocytes proceed only as far as prophase of meiosis I by the time of birth, where they remain until ovulation. This suspended stage of prophase occurs after pachytene, is referred to as dictyotene, and lasts from birth until after puberty, when small cohorts of the germ cells progress further into meiosis. The first meiotic division is stimulated by ovulation and is an unequal division in that most of the cytoplasm remains in the ovum and very little is pinched off to enter the first polar body, containing one set of homologs. Sperm penetration of the ovum stimulates the second meiotic division, leading to formation of the second polar body that contains a haploid set of chromosomes. On average, one oocyte per ovarian cycle completes the first meiotic division and proceeds to metaphase of the second meiotic division; if fertilized by a sperm, it completes the second division and embryonic development ensues. Thus, over the approximate 30 year reproductive lifetime of a female,

only a few hundred oocytes complete the first meiotic division and few—if any—complete the second (18).

It is of interest to note that the frequency of point mutations and structural chromosomal changes is in general higher in male gametes and increases with age. This increased mutation rate in males is attributed to the much larger number of cell divisions in the male

germline. In contrast, changes of chromosome number increase with age in female gametes. Errors of disjunction seen with advanced maternal age appear to be related to the 13–50 years the oocytes spend in prophase before chromosome segregation. Genetic mapping studies indicate that the number as well as the positioning of crossover events influence meiotic segregation of



**FIGURE 10-3** Diagrammatic representation of the stages of meiosis. (Turnpenny, P. D., Ellard, S., Chapter 3: Chromosomes and Cell Division, Figure 3.19: Stages of Meiosis, In Emery's Elements of Medical Genetics, 12th ed.; Churchill Livingstone: Elsevier, 2005.)



chromosomes (19,20). However, the molecular causes underlying age-dependent nondisjunction are still poorly understood (18).

## 10.4 METHODS FOR STUDYING HUMAN CHROMOSOMES

Technical innovations in the past 60 years have revolutionized the study of human chromosomes. Chromosomes are normally visible only during cell division as they become condensed in preparation for orderly division. Therefore, chromosomes can be studied only in cells that are dividing *in vivo* or *in vitro*. Dividing cells are sufficiently common in some tissues *in vivo* to permit the direct study of chromosomes. This is true of meiotic divisions in the testis and embryonic ovary, and of mitotic divisions in the bone marrow, some epithelia, and tumors. However, cell culture methods have greatly extended the range of tissue and cell types from which dividing cells can be obtained *in vitro*. These include blood lymphocytes, fibroblasts from skin and other tissues, and cells from amniotic fluid or chorionic villi. Viable cells can even be obtained for a number of hours after death of an individual or spontaneous abortion of an embryo. It is thus possible to carry out chromosome studies in a wide range of clinical situations.

The introduction of a short-term peripheral blood culture technique provided a reliable way for obtaining human chromosome preparations of good quality for human cytogenetic investigations and for clinical diagnosis (21). In this widely used technique, T-lymphocytes from a small sample of peripheral blood are stimulated to divide in culture with a mitogen, such as phytohemagglutinin. The blood culture is initiated in a suitable culture medium at 37°C, and within 3 days the stimulated lymphocytes provide very large numbers of dividing cells. These are blocked in metaphase by adding a mitotic spindle poison, such as colchicine, to the culture for a few minutes. Treatment of the cells with a hypotonic solution swells the cells and allows spreading of the chromosomes, which are then fixed and mounted on a glass slide. This makes it possible to prepare well-spread, flattened metaphase chromosome preparations on slides suitable for microscopic analysis using chromosome banding methods and molecular cytogenetic techniques.

### 10.4.1 Human Chromosome Identification

In the early days of human cytogenetic investigations, chromosomes were stained with Giemsa or a similar dye, yielding uniform staining along their lengths. Based on these studies, human chromosomes were classified according to their size and morphology (Figures 10-4 and 10-5).

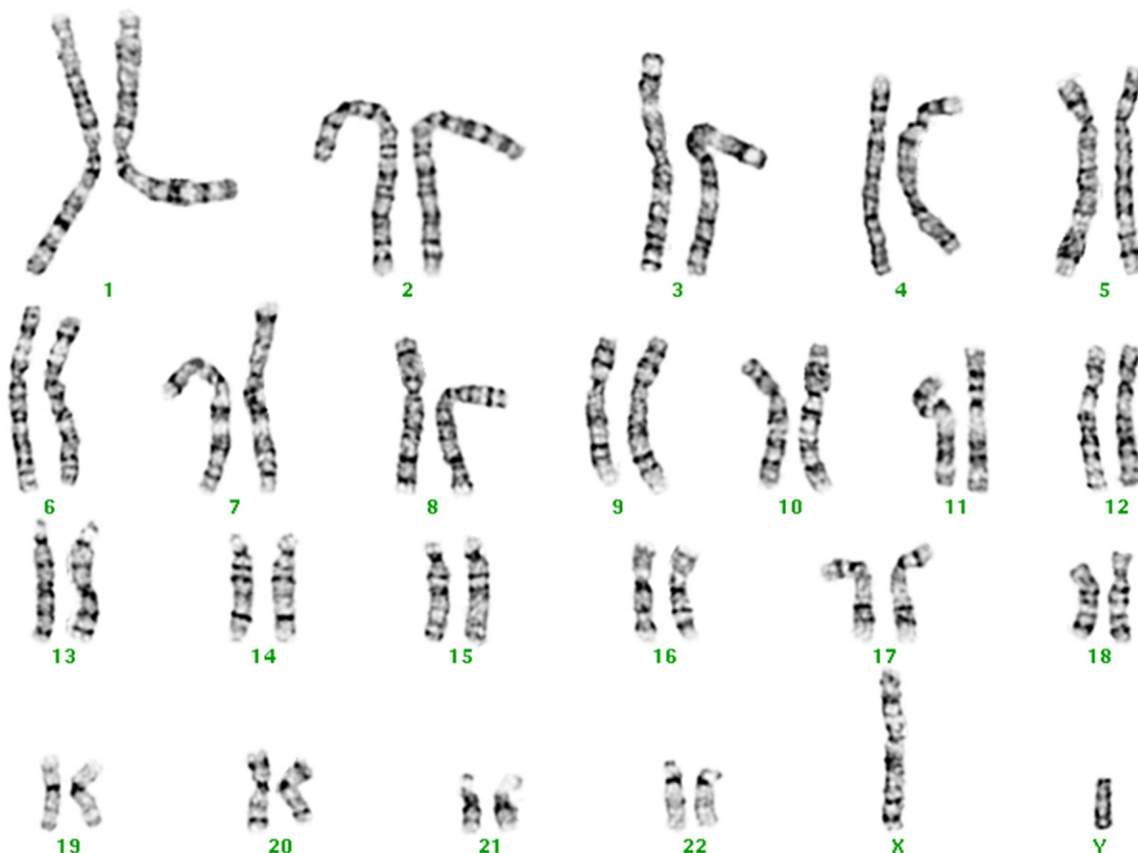
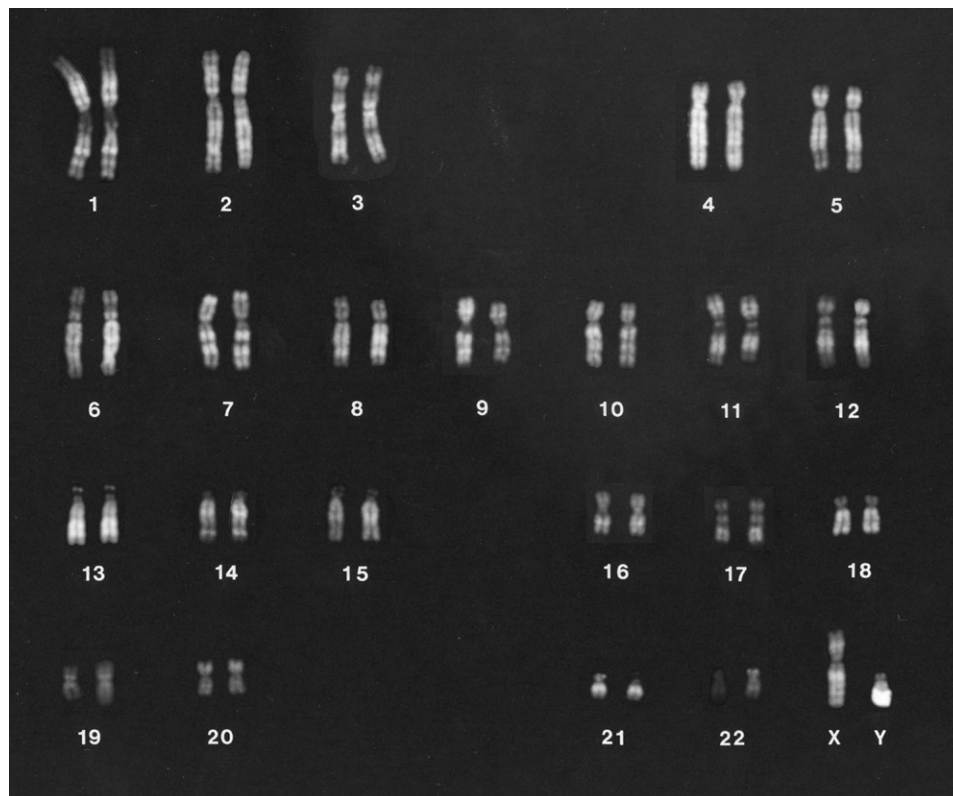


FIGURE 10-4 G-banded karyotype of a male cell.



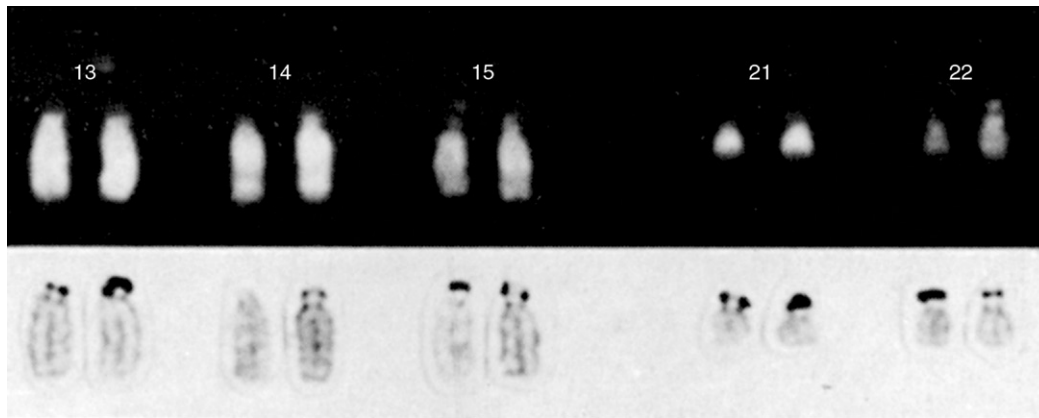
**FIGURE 10-5** Q-banded karyotype of a male cell.

The primary constriction represents the centromere, the chromosomal locus responsible for proper segregation of chromosomes to daughter cells during cell division. Based on the position of their centromeres, human chromosomes are classified as metacentric, in which the centromere is at or near the middle of the chromosome; submetacentric, in which the centromere is located significantly off center; or acrocentric, in which the centromere is very close to one end. For all categories, the short arm of the chromosome is referred to as “p” for petite, and the long arm as “q.” In addition to the centromere or primary constriction, five pairs of the acrocentric chromosomes (numbers 13, 14, 15, 21, and 22) may exhibit secondary constrictions on their short arms (Figure 10-6). These mark the site of each cluster of ribosomal RNA (rRNA) genes and are called nucleolar organizing regions (NORs) because, at telophase, nucleoli are formed at a subset of these sites that are transcriptionally active. The rRNA genes remain in a moderately extended state at metaphase, reflecting the late shutoff of these genes in prophase and the rapid reinitiation of their transcription after the anaphase separation of sister chromatids. Originally, the chromosomes were assigned to groups A through G according to their general size and position of the centromere (Group A = 1–3, Group B = 4–5, Group C = 6–12 + X, Group D = 13–15, Group E = 16–18, Group F = 19–20, Group G = 21–22 + Y) (Figure 10-4).

### 10.4.2 Chromosome Banding

The conventional (Giemsa) staining without pretreatment does not permit precise identification of each chromosome in the human complement. A major technical innovation in human cytogenetics came in 1970, when Caspersson and colleagues discovered that human chromosomes stained with quinacrine mustard, a fluorescent DNA-binding compound, and examined under ultraviolet light show characteristic variation of fluorescence intensity along the length of each chromosome, producing a banded appearance (22). Each chromosome could then be identified by its characteristic quinacrine (Q)-banding pattern (see Figure 10-5). Subsequently, several techniques were developed that reveal banding patterns reflecting the underlying structural features of chromosomes. Techniques such as Giemsa (G)-banding and reverse (R)-banding produce the full range of bands along each chromosome, allowing identification of individual human chromosomes. Other banding techniques produce much more restricted staining of specific subsets of chromosome bands and include centromere (C)-banding and NOR-banding. A technique that differentially stains the two sister chromatids of a chromosome is also of particular interest. Chromosome banding methods of special interest are discussed in the following paragraphs.

Although Q-banding was the method first employed for human chromosome identification, it is rarely used



**FIGURE 10-6** Partial karyotype of the acrocentric chromosomes from a single cell stained first by Q-banding to identify each chromosome (top panel), and then by the AgNOR technique (bottom panel) to show the sites of the rRNA genes (nucleolar organizing regions).

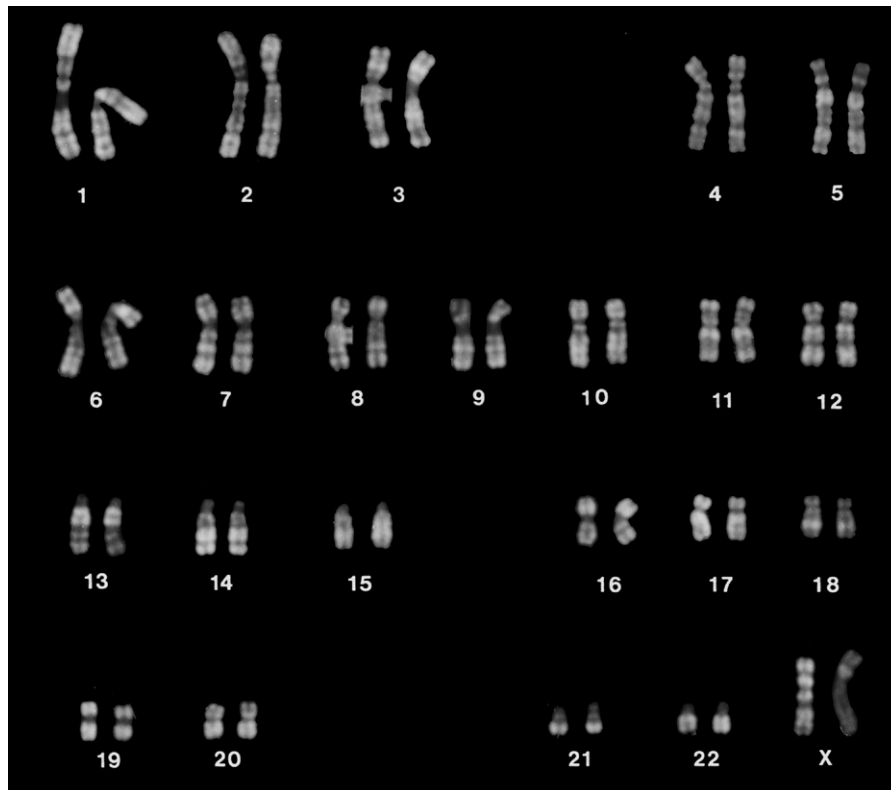
today for routine chromosome analysis in clinical cytogenetics laboratories, as simpler methods have become available that do not require the use of a fluorescence microscope. A banding pattern that is almost identical to the Q-banding pattern can be produced by treatment of chromosomes with a denaturing agent or a proteolysis enzyme, prior to staining them with Giemsa. In the most consistent and commonly used version of this technique, chromosomes are treated with a dilute solution of trypsin followed by staining with Giemsa (23). The resulting G-banding is the most widely used technique for human chromosome identification in clinical cytogenetics laboratories today (see Figure 10-4). The G-banding patterns are also readily captured and analyzed by computerized karyotyping systems used in clinical cytogenetics laboratories. As an extension of the G-banding technique, methods are now in use for obtaining longer, less condensed prometaphase chromosomes that exhibit twice as many G-bands (about 800 bands per haploid set) as the usual metaphase chromosome preparations (about 400–500 bands per haploid set), providing higher resolution to cytogenetic analysis (24).

Another banding technique of interest, although less commonly used for routine analysis, is one that produces an R-banding pattern. Many approaches have been developed to obtain R-banding, in which the staining intensity of each band is the reverse of that seen with Q- or G-banding. A commonly used method to generate R-bands is to subject chromosome preparations to moderate heat (~85 °C in the presence of high salt) before staining them with Giemsa. R-banding of the highest resolution is obtained by a combination of the fluorescent dye chromomycin A3, which emits fluorescence most strongly in the R-bands, and distamycin A, which quenches fluorescence in G-bands. An alternative R-banding technique that also provides some insight into the mechanism of chromosome banding is based on the differential replication timing of chromosome bands (25). In this technique, growing cells are exposed to the thymidine analog bromodeoxyuridine (BrdU) during the S phase of the cell

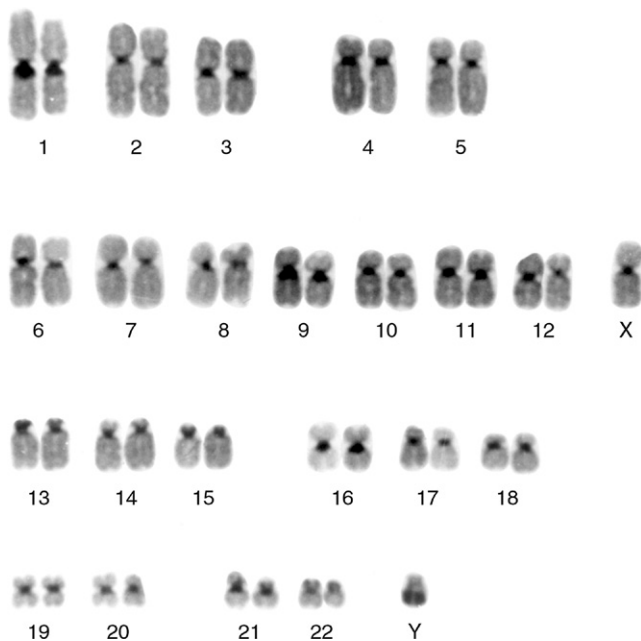
cycle and examined following staining with the fluorochrome acridine orange. Cells that incorporate BrdU into their DNA at the late stage of the S phase are selected for observation. With acridine orange staining, the BrdU-containing chromosomal regions appear dull (G- or Q-bands), whereas the early-replicating regions that have incorporated thymidine fluoresce brightly, giving a reverse or R-banding pattern (Figure 10-7). Thus, the R-bands on chromosomes represent regions that replicate their DNA early in the synthetic (S) phase of the cell cycle. The inactivated, late-replicating X chromosome in females (see later) is also stained differentially (dull) from the active X chromosome following this replication banding protocol (see Figure 10-7).

The banding pattern of each chromosome is specific and can be shown in the form of a continuous series of bands. A standardized map of banded chromosomes is known as an “Idiogram.” Subsequent to the development of banded human karyotypes, a standardized nomenclature for the bands was established by the International Standing Committee on Human Cytogenetic Nomenclature. Updated regularly, this standardized system allows the precise description of chromosome abnormalities (26).

The C-banding method selectively stains the areas located around the centromeres of all chromosomes and on the distal long arm of the Y chromosome (27). The largest C-bands usually occur on chromosomes 1, 9, and 16 and the Y in regions that contain highly repetitive, nontranscribed DNA. To elicit C-bands, metaphase chromosome preparations are treated with sodium hydroxide or barium hydroxide followed by Giemsa staining (Figure 10-8). The size of the C-band on a given chromosome is usually constant in all the cells of an individual but is highly variable from person to person, reflecting variations in the amount of heterochromatic DNA present at the centromeric regions. Such C-band heteromorphisms on chromosomes are transmitted from parent to offspring as simple Mendelian dominant traits. These variations in chromosome morphology are not associated



**FIGURE 10-7** R-banded karyotype of a female cell following incorporation of BrdU into the late-replicating regions of the chromosome.



**FIGURE 10-8** C-banded karyotype of a male cell.

with any known phenotypic effects and are referred to as chromosome polymorphisms. They are, however, useful as heritable chromosome markers in various clinical and epidemiologic studies of chromosome abnormalities.

Silver NOR (AgNOR) staining uses a silver nitrate solution to selectively stain the sites of transcriptionally

active rRNA genes, which are located in the stalk regions on the short arms of human acrocentric chromosomes (28). Silver staining regions are usually present on 6–8 of the 10 acrocentric chromosomes, 13, 14, 15, 21, and 22 (Figure 10-6), although they may be seen on as few as 3 or as many as all 10 of these chromosomes. The sizes of the AgNORs are highly variable in the human population, although the size of each AgNOR in the cells of one individual is quite consistent and usually remains unchanged from one generation to the next. AgNOR staining is useful in characterizing rearrangements involving human acrocentric chromosomes. The mechanism of AgNOR staining is based on the oxidation of nucleolar nonhistone proteins with silver nitrate, by which Ag is reduced to black native silver. Interestingly, the acrocentric chromosomes show association of their satellite stalk regions even in metaphase chromosome preparations, reflecting the functional association of these sites in the formation of the nucleolus in the interphase nucleus. This association of the NORs is considered to be a factor responsible for the high incidence of Robertsonian translocations involving the short arms of acrocentric chromosomes.

Sister chromatid exchange (SCE) is an extension of the replication banding technique using BrdU incorporation to produce differential staining of the two sister chromatids of the metaphase chromosome. This requires incorporation of the thymidine (T) analog BrdU (B) into DNA during two successive rounds of DNA replication. At the end of the first round of DNA replication, the



two newly synthesized strands of DNA in the double-stranded helix will contain BrdU, but not the two template strands. At the end of the second round of DNA replication, two new double-stranded helices will be produced, of which one will have BrdU incorporated on both strands (BB) and the other of which will have BrdU substitution in only one strand of the DNA double helix (TB). When the chromosomes containing singly (TB) and doubly (BB) substituted chromatids are stained with the DNA-binding fluorochrome Hoechst 33258, and exposed to ultraviolet light, they show differential sister chromatid staining, with the bifilarly substituted chromatid exhibiting paler fluorescence (29). Staining of these BrdU-incorporated chromosomes with Giemsa produces darkly stained (TB) and lightly stained (BB) sister chromatids (30) (Figure 10-9). Therefore, exchanges of material between sister chromatids are readily visible at high resolution following this staining protocol. The differential sister chromatid staining observed following the SCE protocol is a remarkable cytologic demonstration of the semiconservative replication of DNA. It also demonstrates that each chromosome is composed of a single very long duplex of DNA. Further, it shows that exchanges between the two sister chromatids take place in somatic cells that could potentially have mutagenic effects. SCE is used to diagnose diseases associated with chromosomal instability in clinical cytogenetics laboratories. For example, SCE analysis is a diagnostic test for Bloom syndrome, a rare autosomal recessive disease caused by mutations in a DNA helicase of the RecQ family that catalyze the unwinding of duplex nucleic acid molecules (31). It is characterized by growth deficiency, predisposition to neoplasia, and chromosomal instability in somatic cells. The frequency of spontaneous SCEs

in cells from patients with Bloom syndrome is markedly increased. SCE analysis is also used to monitor the effects of potentially mutagenic or carcinogenic agents that enhance the rate of SCEs.

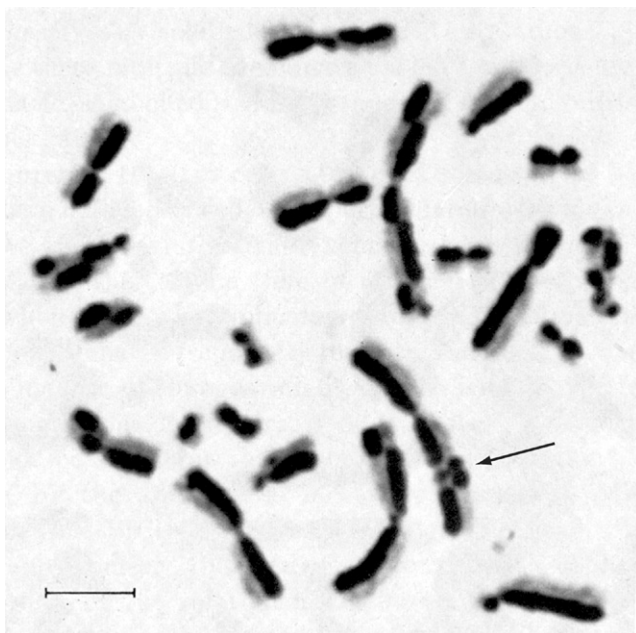
### 10.4.3 Chromosome Banding Reveals Genome Sequence Organization

Quinacrine associates directly with DNA by intercalating between base pairs. Although quinacrine binds equally well to DNA of any base composition, its fluorescence is enhanced in regions containing uninterrupted runs of AT base pairs, and is quenched in regions with more frequent GC base pairs. In the Q-banding pattern of human chromosomes (see Figure 10-5), the intensity of fluorescence is generally proportional to the AT-richness of the DNA (32). However, the highly AT-rich satellite DNA that is concentrated at the C-bands of chromosomes 1, 9, and 16 has interspersed GC base pairs and usually fails to show bright Q-banding. That on the Y, in contrast, has no such GC pairs and is intensely fluorescent. Thus Q-banding is related to both base composition and base interspersion that result in the differential fluorescence or quenching of signals produced by the fluorescent dye. DNA-protein interactions may also be important in the generation of Q-bands.

G-banding is produced most commonly by treatment of chromosomal preparations with the proteolytic enzyme trypsin. Giemsa stains DNA primarily by intercalating between adjacent base pairs in double-stranded regions. G-bands result from the degradation of chromosomal proteins by trypsin, which modifies the interaction of chromosomal DNA with the Giemsa dyes. Since the fixative used in standard chromosome preparation methods, methanol:acetic acid (3:1), removes some of the histone proteins, it is the degradation of the nonhistone proteins that appears to be critical for the production of G-bands. The DNA-protein interactions at the G-band-positive regions apparently render these sites resistant to denaturation by the enzyme.

The commonly used method to generate R-bands is to subject chromosome preparations to moderate heat (~85°C in the presence of high salt) before staining them with Giemsa. The heat pretreatment is thought to selectively denature the more AT-rich DNA sequences, which have a lower thermal stability than GC base pairs, and to result in altered DNA structure on renaturation. Therefore, after chromosomes are exposed to moderate heat, Giemsa stains the unaffected GC-rich double-stranded DNA regions, producing R-banding. R-bands can also be produced by the replication banding technique, which demonstrates that R-band-positive regions contain early-replicating DNA. It also follows that G-band- and Q-band-positive regions contain AT-rich DNA that replicates relatively late in the cell cycle (32).

C-band-positive regions have been found by *in situ* hybridization and DNA sequencing to consist of



**FIGURE 10-9** SCEs shown in Chinese hamster ovary cells.

$\alpha$ -satellite (discussed later) sequences at the centromeres of human chromosomes and of different families of simple sequence satellite DNAs at the large pericentromeric C-band blocks on chromosomes 1, 9, and 16 and distal Yq. Analyses of the completed human genome sequence have defined further families of repetitive DNA (33), but these have not yet been associated with functional or structural landmarks of chromosomes. In contrast, recent studies employing *in situ* hybridization as well as *in silico* analyses of the genome sequence have revealed that the human genome also includes highly homologous duplications of DNA ranging in size from 1 to more than 500 kb. These repeats, called segmental duplications, are located mainly in the pericentromeric and subtelomeric regions of chromosomes, although they are also present as interspersed repeats along the length of the chromosome (33,34). While some of these segmental duplications are known to predispose to genomic deletions and duplications, their significance for chromosomal function is otherwise unknown. A comparison of the characteristics of Q-/G-, R-, and C-bands is presented in Table 10-1.

Also related to simple sequences are chromosomal regions called fragile sites that remain stretched at metaphase after various treatments that limit DNA replication (35). Fragile sites are classified as rare (inherited) or common (constitutional) and are further subdivided according to the conditions under which they are induced (e.g. folate or amphotericin sensitive). Several fragile sites have now been cloned and sequenced. These studies have shown that the expression of rare, inherited fragile sites is associated with repeat expansions (35). The first folate-sensitive rare fragile site to be characterized was the one associated with the fragile X syndrome (FMR1), which was shown to result from the expansion and methylation of a CGG trinucleotide repeat in the 5' UTR of the *FMR1* gene. Other folate-sensitive fragile sites characterized thus far also result from expansion of trinucleotide repeats (36). A distamycin-sensitive rare fragile site on chromosome 16 has been shown to involve the expansion of a 33 bp AT-rich minisatellite (36). In contrast,

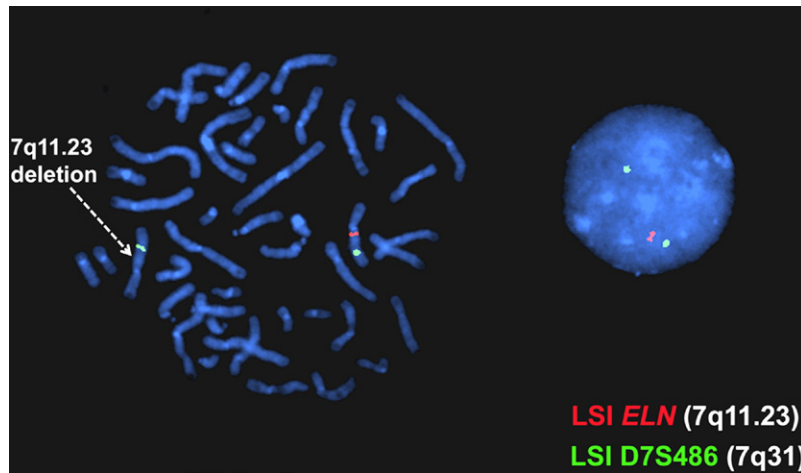
sequencing of constitutional fragile sites has not revealed any characteristic DNA sequences at these sites (37).

#### 10.4.4 Molecular Cytogenetics

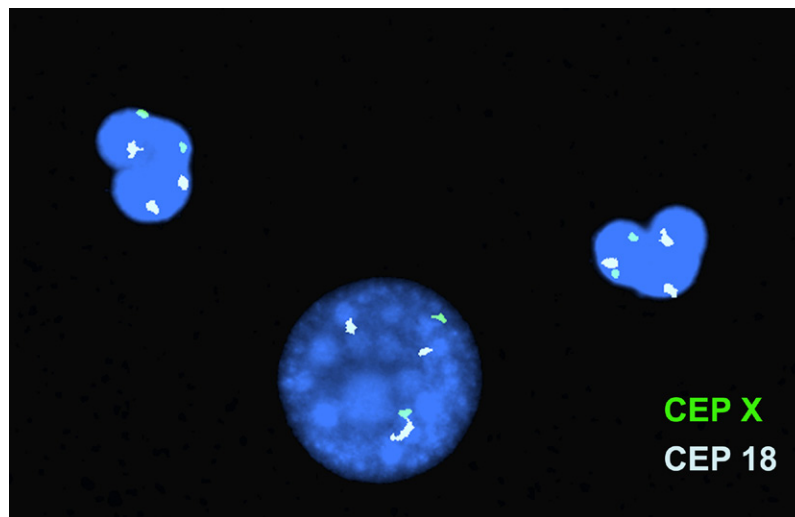
The gap between light microscope resolution of chromosome structure and the gene was bridged by the introduction of several molecular cytogenetic techniques. Fluorescence *in situ* hybridization (FISH) involves hybridizing a fluorescently labeled single-stranded DNA probe to denatured chromosomal DNA on a microscope slide preparation of metaphase chromosomes and/or interphase nuclei prepared from the patient's sample. After overnight hybridization, the slide is washed and counterstained with a nucleic acid dye (e.g. DAPI), allowing the region where hybridization has occurred to be visualized using a fluorescent microscope (38). FISH is now widely used for clinical diagnostic purposes. There are different types of FISH probes, including locus-specific probes, centromeric probes (CEPs), and whole-chromosome paint probes. Locus-specific probes are specific for a particular single locus. They are particularly useful for identifying subtle submicroscopic deletions and duplications (Figure 10-10). CEPs are specific for unique repetitive DNA sequences (e.g.  $\alpha$ -satellite sequences) in the centromere of a specific chromosome. They are suitable for making a rapid diagnosis of one of the common aneuploidy syndromes (trisomies 13, 18, and 21, and sex chromosome aneuploidies) using nondividing interphase nuclei. This is particularly useful in a prenatal setting using amniotic fluid or chorionic villi samples (Figure 10-11). Whole-chromosome paint probes consist of a cocktail of probes obtained from different regions of a particular chromosome. When this cocktail mixture is used in a single hybridization, the entire relevant chromosome fluoresces (is "painted") (Figure 10-12). Whole-chromosome paints are useful for characterizing complex chromosomal rearrangements, and for identifying the origin of additional chromosomal material such as small marker or ring chromosomes.

**TABLE 10-1** Characteristics of Chromosome Bands

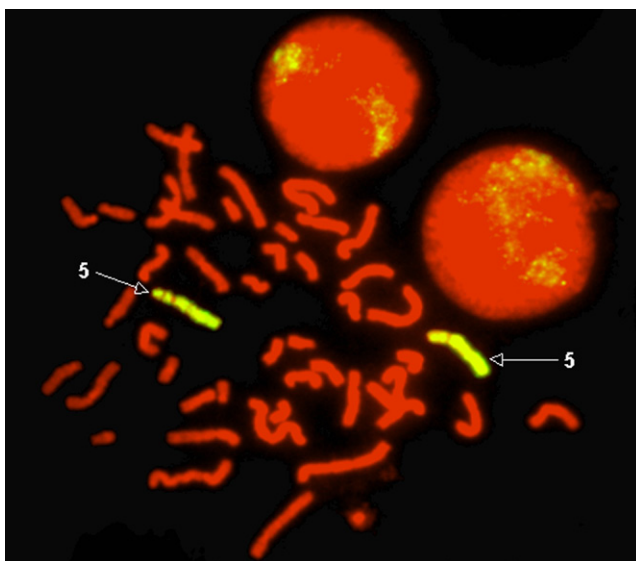
Characteristic	Q- or G-Bands	R-Bands	C-Bands
Location	Chromosome arms	Chromosome arms	Centromeres, distal Yq
Type of DNA sequence	Repetitive, some unique	Unique, some repetitive	Highly repetitive satellite
Base composition	AT-rich	GC-rich	AT-rich, some GC-rich
5-methylcytosine content	Low	Moderate	High
Type of chromatin	Heterochromatin	Euchromatin	Heterochromatin
Replication	Mid to late S phase	Early S phase	Late S phase
Transcription	Low	High	Absent
Gene density	Low	High	Absent
CpG-rich islands	Few	Many	Absent
Repeats	LINE-rich	SINE-rich	—
Acetylated histones	Low	High	Absent



**FIGURE 10-10** Metaphase and interphase FISH analysis in a patient with William syndrome due to deletion on chromosome 7 band q11.23. Note the deletion of the *ELN* gene probe labeled in red.



**FIGURE 10-11** Interphase FISH analysis in a patient with trisomy of chromosome 18. Note the three copies of the chromosome 18 centromeric probe (CEP 18) labeled in aqua.



**FIGURE 10-12** Metaphase FISH analysis using chromosome 5 paint probe.

FISH using locus-specific probes has been extremely useful in the detection of “microdeletion syndromes” resulting from deletions of multiple contiguous genes. These are subtle submicroscopic deletions that are below the resolution of the routine G-banded chromosome analysis. Also, two-color and three-color FISH applications are routinely used to diagnose specific deletions, duplications, or other rearrangements, both in metaphase chromosomes and in interphase nuclei. Use of FISH usually requires that the patient either exhibits features consistent with a well-defined syndrome with known chromosomal etiology or demonstrates an abnormal karyotype. This is because single FISH probes reveal rearrangements only of the segments being interrogated, but do not provide information about the rest of the genome. Another limitation of FISH is the number of probes that can be applied in a simultaneous assay. FISH techniques have been developed utilizing pools of whole-chromosome paint probes for every chromosome

to provide a multicolor human karyotype in which each pair of homologous chromosomes can be identified on the basis of its unique color when studied using special computer-based image analysis software (Spectral karyotyping and M-FISH) (39).

One type of FISH that has the potential to reveal chromosomal imbalances across the genome is comparative genomic hybridization (CGH). In CGH, DNA specimens from patient and normal control are differentially labeled with two different fluorescent dyes and hybridized to normal metaphase chromosome spreads. Difference between the fluorescent intensities of the two dyes along the length of any given chromosome will reveal gains and losses of genomic segments (40). The limitations of this technology include many of the same limitations of G-banded chromosome analysis. Thus, like G-bands, the resolution of CGH is limited to that of metaphase chromosomes, which is approximately 5 Mb for most clinical applications (39).

The latest addition to molecular cytogenetic techniques is array CGH, where CGH is applied to an array of DNA targets (probes) each representing a part of the human genome and fixed to a solid support (usually a glass slide). Like CGH, array CGH directly compares DNA content between two differentially labeled DNA specimens (a test or patient and a reference or normal control), which are labeled and co-hybridized onto the array. Arrays have been constructed with a variety of DNA targets, ranging from bacterial artificial chromosomes (BACs) which are 80–250 kb long to oligonucleotides (oligos), which are 25–80 bp long (41–43). Following hybridization and washing to remove unbound DNA, the array is scanned and analyzed using special computer software to measure the relative ratios of fluorescence of the two dyes and detect gains/losses of genomic regions represented on the array (Figure 10-13). The resolution of array CGH is dependent on the type of probes used (BACs or oligos) and the distance between them. In the past few years, high-resolution whole-genome coverage array CGH platforms have been increasingly used in clinical molecular cytogenetics labs. These provide a relatively quick method to scan the entire genome for gains and/or losses with significantly high resolution and greater clinical abnormality yield than was previously possible. This led to the identification of novel genomic disorders in patients with autism spectrum disorders, developmental delay, mental retardation, and/or multiple congenital anomalies (44).

## 10.5 FUNCTIONAL ORGANIZATION OF CHROMOSOMES

Chromatin is classified into euchromatin and heterochromatin. Euchromatin consists of active genes; however, not all genes in euchromatic regions are active at any given time. Therefore, location in euchromatin is currently thought to be necessary but not sufficient for gene activity.

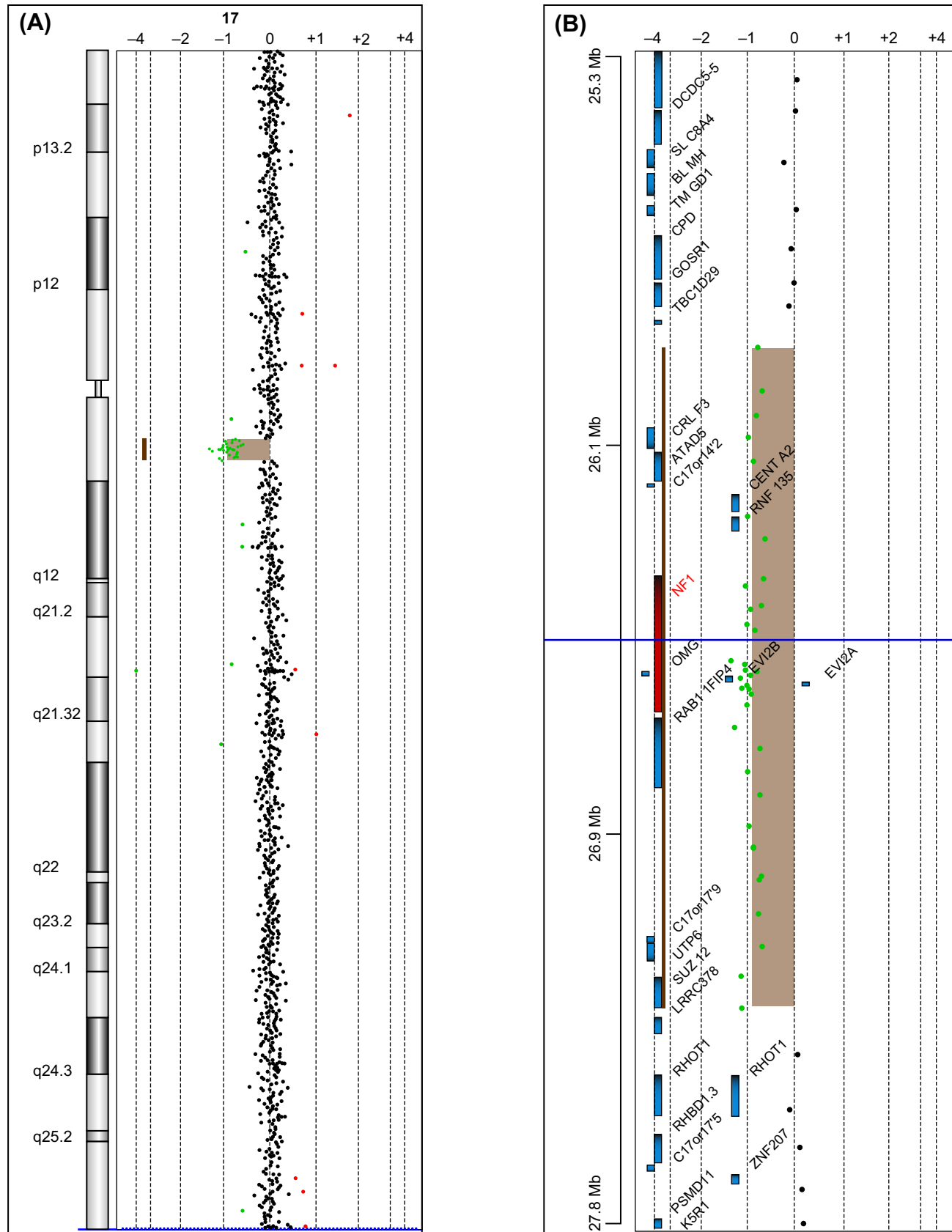
Euchromatin is dispersed in the interphase nucleus and replicates its DNA early in the S phase of the cell cycle. Heterochromatin consists predominantly of inactive genetic material, replicates its DNA late in the S phase, and is condensed in the interphase nucleus. Heterochromatin is further classified into constitutive heterochromatin and facultative heterochromatin. Constitutive heterochromatin consists of highly repetitious simple-sequence DNA, remains transcriptionally inactive, and is located at specific regions of the chromosomes such as the centromere and the distal long arm of the human Y chromosome. Facultative heterochromatin also remains condensed in the interphase nucleus, replicates its DNA late in the S phase, and is largely transcriptionally inactive. However, it is not inactive permanently, does not consist exclusively of repetitious DNA, and can become transcriptionally active. The inactive X chromosome in the human female is a good example of facultative heterochromatin. However, location in facultative heterochromatin does not exclude transcription altogether, as several genes on the inactive X chromosome are expressed (see later). As already noted, the R-band-positive regions of human chromosomes have characteristics of euchromatin in that they replicate their DNA in early S phase and have high transcriptional activity due in part to high gene density (see Table 10-1). The G-band-positive regions, on the other hand, are more heterochromatic as they replicate their DNA in late S phase and are low in transcriptional activity associated with low gene density. Integration of the whole human genome sequence with the cytogenetic map shows a lower density of genes in G-positive bands (45). The C-band-positive regions consist of constitutive heterochromatin with no known functional genes. The facultative heterochromatin of the inactive X chromosome replicates its DNA in late S phase, and forms the condensed Barr body in the interphase nucleus. Consequently, there is a general relationship of functional properties (time of replication during the S phase and transcriptional status or gene density) with chromosome band classes characterized by differential condensation and staining characteristics (46).

Recent investigations have provided insights into the molecular organization of two specialized structures on chromosomes, the centromere and the telomere, which are of particular interest and are summarized below.

### 10.5.1 The Centromere

As already noted, each chromosome has a primary constriction, the centromere, where the sister chromatids of a replicated chromosome are held together until the anaphase stage of cell division. A subdomain of the centromere is the kinetochore, a protein–DNA complex that serves as the attachment site for the spindle fibers essential for chromosome movement and segregation during mitosis and meiosis. The structure of the centromere has been a focus of molecular cytogenetic investigations in recent years. The best characterized eukaryotic





**FIGURE 10-13** Oligo-array CGH analysis in a patient with *NF1* gene deletion on chromosome 17 band q11.2. (A) Whole chromosome 17. (B) Deleted region. Shaded areas show the deleted region.

centromere is that of the budding yeast *Saccharomyces cerevisiae*. In this organism, a short sequence of about 125 bp specifies the centromere of each of the chromosomes. The nucleotide sequence and organization of this centromere DNA is conserved among the different chromosomes in the budding yeast. The search for a similar specific sequence in the larger and more complex centromeres of higher eukaryotes has not been successful. Rather, the centromeres in these organisms consist of large arrays of repeated  $\alpha$ -satellite DNA sequences. In human centromeres, the arrays consist of tandem, head-to-tail repeats of a 171 bp monomer that is further organized into higher order repeats (47). The centromeric chromatin of human chromosomes spans from 0.1 to 4.0 Mb. The sequence of the basic 171 bp unit is sufficiently divergent among human chromosomes that, with very few exceptions, centromere-specific  $\alpha$ -satellite DNA probes can generate fluorescent signals on specific chromosomes in a FISH assay. This is useful from a practical standpoint for identifying and determining the copy number for specific human chromosomes in interphase cells.

Several lines of evidence implicate a critical role for  $\alpha$ -satellite DNA in centromere function. Although there are other repeated sequences in the centromeric heterochromatin,  $\alpha$ -satellite is the only one localized to the centromeres of all normal human chromosomes. Moreover, studies have shown that human artificial chromosome constructs containing  $\alpha$ -satellite DNA are able to form functional centromeres (48). However, independent evidence from rearranged chromosomes suggests that the presence of  $\alpha$ -satellite DNA alone is not sufficient for the formation of an active centromere. Many cases of rearranged human chromosomes containing two centromeric regions have been identified. A true dicentric chromosome with two primary constrictions would be unstable during cell division as spindle fiber attachment occurs independently at the two centromeres, if these are sufficiently far apart. The two centromeres on a single chromatid could then be pulled toward opposite poles of the spindle, breaking the chromosome. However, many dicentric chromosomes with two blocks of  $\alpha$ -satellite DNA and C-band regions are stable and show only one primary constriction, indicating that only one of the two centromeres is active. Such stable dicentric chromosomes, referred to as pseudodicentrics, indicate that the presence of  $\alpha$ -satellite DNA alone is not sufficient for the formation of an active centromere. In addition, several human marker chromosomes have been characterized that originate from normal human chromosomes but lack  $\alpha$ -satellite DNA sequences. These functional centromeres lacking  $\alpha$ -satellite DNA are called neocentromeres (49). As these chromosomes are mitotically stable, presence of  $\alpha$ -satellite DNA is not an absolute requirement for functional centromeres. Thus, although normal human centromeres are composed of  $\alpha$ -satellite DNA, it appears to be neither necessary nor sufficient for centromere formation.

Recent investigations have identified several proteins associated with centromeres that have contributed to our understanding of centromere structure and function (50,51). A group of these proteins are constitutively associated with centromeres while others are associated with centromeres only during a part of the cell cycle and are involved in chromosome movement during cell division. The major constitutive centromere proteins identified are CENP-A, CENP-B, and CENP-C. The location of these proteins at centromeres has been determined by immunofluorescence microscopy using antibodies specific for these proteins. CENP-A is a 17 kDa histone H3-like protein that participates in producing centromere-specific nucleosomes (in place of histone H3) and altered chromatin structure. CENP-A is detected at all functional centromeres, including the neocentromeres. CENP-B is an 80 kDa protein that binds to a specific 17 bp sequence, the CENP-B box, in  $\alpha$ -satellite DNA and is found, as expected, even at the inactive centromere of pseudodicentric chromosomes. CENP-C, a 140 kDa protein, is also found at active centromeres, where it is located in the proteinaceous kinetochore. CENP-C shares homology with a domain of the Mif2 protein of yeast that is essential for normal chromosome segregation. In addition to the CENP-A, -B, and -C proteins that associate with centromeres constitutively, many more that associate transiently during cell division have been identified. An example of the latter class of proteins is CENP-E, a 275 kDa kinesin-related protein that is associated with centromeres and mitotic spindle during mitosis and plays a role in chromosome movement.

## 10.5.2 The Telomere

Telomeres are special DNA-protein structures that are present at the ends of linear chromosomes and prevent fusion of chromosome ends and maintain chromosome integrity. The concept of the telomere was developed from early genetic and cytologic observations that the broken ends of chromosomes are unstable and often fuse with other broken ends. Molecular techniques have now shown that telomeres in eukaryotes exist in a DNA-protein complex consisting of tandem repeats of a simple sequence and a number of proteins. In humans and other vertebrates, the sequence of the basic repeat is 5'-TTAGGG-3' on one strand of the DNA and 5'-CCCTAA-3' on the complementary strand. The G-rich strand runs 5'-3' toward the end of the chromosome, with a short, single-stranded, G-rich overhang (52,53). The human telomeric sequence typically spans about 2–50 kb and is replicated by its own polymerase, called telomerase. In the absence of telomerase, each round of DNA replication leaves 50–200 bp of DNA unreplicated at the 3' end as the DNA replication machinery works only in the 5'-3' direction and requires an RNA primer. This would result in loss of sequences from the ends of chromosomes, ultimately leading to loss of genetic material.

Telomerase is a ribonucleoprotein complex that includes a reverse transcriptase and a short RNA molecule that provides the template for synthesizing the telomeric sequences. By copying the RNA template, telomerase extends the G-rich telomeric DNA strand running 5'–3' toward the distal end of the chromosome. The complementary strand is then synthesized by the cellular DNA replication machinery through lagging strand synthesis. Telomere-associated proteins regulate telomerase activity so that the length of telomere repeat tract is maintained at a level required for maintaining functional telomeres (53,54). Telomerase is present in early embryonic cells and in the majority of immortalized cells, but not in most somatic cells. As a result, somatic cells, but not cancer cells, lose telomeric sequences with each division, leading to dysfunctional telomeres and excessive chromosomal instability. Telomerase activity is therefore considered to be a critical factor contributing to the finite life span of most somatic cells and indefinite growth potential of cancer cells. Studies have shown that telomere sequences can be added to the ends of chromosomes with terminal deletions, thus stabilizing these broken ends. Healing of broken ends can occur through two general pathways, ensuring the acquisition of a new telomeric cap and stabilizing the deleted chromosome. First, direct addition of telomeric sequences onto the broken end can be achieved through a telomerase-mediated *de novo* telomere addition (55,56) or a telomerase-independent recombination-based mechanism (57,58). Second, telomeres can also be retrieved from another location through a mechanism called telomere capture, in which subtelomeres and/or pan-telomeres from another chromosome are translocated at the broken end of the deleted chromosome (59,60).

Adjacent to the human terminal (TTAGGG) $_n$  repeat is a complex region of segmentally duplicated DNA tracts generally referred to as subtelomeric repeat DNA or telomere-associated repeats (TARs). This class of low-copy repeat DNA is characterized by very high sequence similarity (>90%) between duplicated tracts, and variably sized but often very large duplicated segments. Some of the segmental duplications are unique to TARs, some are shared with a subset of pericentromeric repeat regions, and some are shared with one or several interstitial chromosomal loci. These TARs range in size from 100 to 300kb, and just proximal to these regions the unique subtelomeric sequences are encountered (61).

## 10.6 SEX CHROMOSOMES AND SEX DETERMINATION

Sex chromosomes of the human chromosome complement are of special interest, as they determine sex of the human embryo. Also, the sex chromosome pair, the X and Y, is heteromorphic (different in size and morphology) in humans. The Y chromosome is significantly smaller than the X chromosome and contains a

large block of heterochromatin on its q arm comprising noncoding repetitive DNA. This leaves only a short segment of chromosome capable of carrying functional genes. The finding of heteromorphic sex chromosomes in humans, an XX pair in females and an XY pair in males, suggested a chromosomal basis for sex determination in the early twentieth century. However, the dominant role of the Y chromosome in male sex determination became evident only in 1959, when cytogenetic studies showed that individuals with a complete set of autosomes and a single X chromosome developed as females, whereas individuals with two X chromosomes and a Y chromosome developed as males (62,63). We now know that individuals with as many as four X chromosomes and a Y also develop as males. The number of X chromosomes or its ratio to the number of autosomes is not important for human male sex determination. The Y chromosome thus carries a dominant determinant for testis development.

Unlike autosomal pairs of chromosomes, the heteromorphic X and Y are not completely homologous. There are two regions of complete homology between the X and Y chromosomes that reside at the distal ends of their short and long arms, covering approximately 2600 and 320kb of DNA, respectively (64). The X and Y chromosomes pair and cross over in these regions during prophase I. This appears to be essential for correct segregation of the sex chromosomes. As a result of this crossing over, female offspring of males can inherit DNA sequences from the Y chromosome distal to the point of exchange and vice versa. Thus genetic markers in this region of pairing and exchange between the X and Y segregate independently of sexual phenotype, and hence this region is called the pseudoautosomal region.

### 10.6.1 The Y Chromosome and Sex Determination

Identification of the testis-determining factor (TDF) on the human Y chromosome has been of much interest since the role of the Y in male sex determination was established. Early cytogenetic investigations in individuals with structurally abnormal Y chromosomes showed that the TDF resided on the p arm of Y. The isolation and molecular characterization of this gene was made possible by studies of a naturally occurring sex-reversed condition, the XX male. Cytogenetic and molecular investigations of XX males showed that the majority of them resulted from an unequal exchange between X and Y, such that the TDF is transferred from the Y to the X chromosome. By identifying the minimal region of Y necessary for male determination from independent XX males, and searching this region for candidate genes, the SRY (sex-determining region on Y) gene was identified. Later studies confirmed that SRY is the long-sought TDF (65). SRY, which resides just proximal to the pseudoautosomal region on the p arm of the Y chromosome, encodes a protein of 240 amino acids, which is capable of

sequence-specific binding to DNA using a motif known as the HMG box (66,67). This motif is found in several classes of DNA-binding proteins, including several that are known to be transcription factors. Unlike other transcription factors, the SRY protein does not contain any other recognizable motifs, and this has led to the hypothesis that it functions partly as scaffold protein in chromatin (68). SRY induces the differentiation of Sertoli cells in the developing gonad. Sertoli cells produce anti-Müllerian hormone, which causes regression of the female internal genitalia; they also induce Leydig cells to secrete the androgens necessary for the development of male internal and external genitalia (65). Any genetic or environmental factor that prevents testis differentiation in 46,XY embryos leads to the development of a sex-reversed XY female. Recent molecular dissection of other conditions that result in sex reversal has allowed the identification of some of the other genes involved in the sex-determining pathway (69–71). Not surprisingly, many of these are autosomal and not sex-linked genes.

### 10.6.2 The X Chromosome

The heteromorphic nature of the sex chromosome pair in humans immediately raises the question of dosage difference for X-linked genes in the human male and female. The answer to this question was provided by observations on the behavior of the two X chromosomes and the expression patterns of X-linked genes in human and other mammalian females. These findings indicated that there were differences in the functional organization of the two X chromosomes in mammalian females. Early cytologic studies demonstrated that a sex chromatin body (called the Barr body) was present in female interphase cells, but not in male cells. Moreover, the amount of certain X-linked gene products, such as the enzyme glucose-6-phosphate dehydrogenase, was no different in individuals with one, two, or even more X chromosomes. Also, studies on the timing of DNA replication in diploid cells indicated that the DNA in one X chromosome replicated in synchrony with the DNA of the autosomes, while that of any additional X chromosomes replicated late in the S phase. Thus, the number of Barr bodies equals the number of late-replicating X chromosomes.

Based on the observation that female mice heterozygous for X-linked genes show mosaicism for the expression of these genes, Lyon in 1961 proposed the single active X hypothesis (72), which offers an explanation for the gender-specific behavior of the X chromosome and X-linked genes. According to this hypothesis, commonly referred to as the Lyon hypothesis, the somatic cells of all mammals undergo a process of chromosome differentiation early in embryogenesis that leaves a single active X chromosome per cell. All additional X chromosomes are inactivated by a process that renders them heterochromatic and capable of forming a Barr body. Thus diploid somatic cells of individuals with three X chromosomes

have two Barr bodies, while those of individuals with four X chromosomes have three Barr bodies. The initial choice for inactivation of an X is random in a normal female. However, this differentiation is fixed, so that all the descendants of a cell in which the maternal X was inactivated initially will have the maternal X in the inactive state, while the descendants of a cell in which the paternal X was inactivated will have that X in the inactive state. Every XX individual is thus a genetic mosaic consisting of cells in which the maternal X is active and cells in which the paternal X is active.

The phenomenon of X chromosome inactivation has been a subject of much interest and investigation in mammalian biology. This interest in X-inactivation derives from the fact that it is a relatively unique epigenetic process of gene regulation at the level of the chromosome. It is epigenetic because the inactivated X chromosome does not undergo any permanent changes in its DNA sequence and can be reactivated, as it is in female germ cells. Genes from the inactive X chromosome can also be reactivated experimentally in cultured cells (73). Further, attention has been focused on X-inactivation as means of understanding broader aspects of chromatin, specifically the structure and function of facultative heterochromatin. Finally, the burden of human X-linked diseases and X chromosome abnormalities has generated an interest in X-inactivation for a better understanding of the pathogenesis and ultimately the treatment of these conditions.

Investigations in the last two decades have provided insights into the molecular mechanism of X chromosome inactivation. It is now clear that DNA methylation plays a key role in maintaining the X in the inactive state. Studies of several genes have shown that cytosine residues in cytosine-guanine dinucleotides (CpG) in their 5' promoter regions are methylated when they reside on an inactive X, and unmethylated on an active X (74). The binding of proteins that specifically bind methylated DNA and inhibit transcription could account for the transcriptional silencing of genes on the inactivated X. Studies employing immunofluorescent labeling of human metaphase chromosomes with antibodies specific for acetylated isoforms of nucleosome core histones have shown that the inactive X chromosome is hypoacetylated, linking methylation and histone acetylation in the control of gene expression from the inactive X (75,76). Examination of histone H4 acetylation status at the individual gene level has also shown hypoacetylation in the promoter regions of X-inactivated genes (77). Hypermethylation of DNA, hypoacetylation of histones, and methylation of histone H3 at lysine 9 (H3-mK9) are features common to all heterochromatin (78). Initiation of X-inactivation, which must also include counting the number of Xs in a cell and the spreading of inactivation along the X, is still not completely understood. However, these early events in X-inactivation are dependent on the X-inactivation center (XIC), a complex specialized



control locus located in the proximal q arm of the human X chromosome. The XIC is required for the initiation of X-inactivation and is invariably present on all X chromosomes that undergo inactivation, including those with structural rearrangements. A search for candidate genes mapping to the XIC region led to identification of the *XIST* (X-inactive specific transcript) gene. The *XIST* gene, expressed exclusively from the inactive X and not from the active X, is located at the XIC (79). The product of *XIST* is a large noncoding RNA molecule that stays associated with the inactive X (80). Transgenic and knockout experiments indicate that *XIST* is necessary and sufficient for initiating X-inactivation (81–83). While the precise mechanism(s) of X chromosome inactivation remain to be revealed, the process is generally described in four stages: recognition of the number of X chromosomes (also called “counting”); initiation early in development; promulgation whereby the initial signal is spread to the rest of the chromosome; and maintenance of the inactivating signal through successive cell divisions (84).

It is now well established that not all genes on the X chromosome are subject to X-inactivation. Early studies showed that the genes for the Xg blood group and for the enzyme steroid sulfatase (deficiency of which causes X-linked ichthyosis) escape X-inactivation (64,84). More recent studies evaluating an estimated 95% of X-linked genes assayable in cell culture systems show that about 15% of these genes escape inactivation, and an additional 10% show variable levels of expression from the inactive X chromosome (80,85). The majority of the genes that escape inactivation are located on the p arm of the X chromosome, but they are also present on the q arm and are interspersed with genes that undergo inactivation. As expected, the genes in the pseudoautosomal regions escape X-inactivation; these have homologs on the Y chromosome, and dosage compensation is not a requirement for these genes. However, there are genes on the X that escape X-inactivation for which there is no functional homolog on the Y, thus resulting in an increased dose in the female. These differences between the X and Y reflect the evolutionary history of the sex chromosomes. It is thought that the heteromorphic sex chromosomes of today evolved from a homomorphic autosome-like pair with progressive loss of genes from the Y and incorporation of the corresponding genes on the X into the X-inactivation system. The genes that escape inactivation on the X may be essential for normal female development in two doses or they may have simply failed to be incorporated into the X-inactivation system with no adverse consequences. The abnormal development associated with X chromosome aneuploidy is most readily explained by dosage inequities in these genes that escape inactivation. Identification of the specific genes involved in these diseases is, therefore, of great interest and is a focus of ongoing investigations.

## 10.7 UNIPARENTAL DISOMY AND IMPRINTING

It has been appreciated for some time that one paternal and one maternal set of chromosomes are required for the normal development of the embryo. In rare cases, a pregnancy arises in which an ovum undergoes some degree of embryonic development by a process of gynogenesis or androgenesis; that is, the cells are solely of maternal origin (gynogenesis) or of paternal origin (androgenesis). Ovarian teratomas appear to be the result of gynogenetic development of ova that have not undergone the second meiotic division. The cells are thus diploid and XX. In contrast, some pregnancies, which terminate in spontaneous abortion, are associated with the presence of a hydatidiform mole, an abnormal development of extra-embryonic tissue. Many of these moles are diploid and XX (or rarely, XY), with both sets of chromosomes of paternal origin. They may arise as a result of fertilization of an anucleated ovum by two sperms.

One of the most interesting novel concepts to emerge from these and other experimental studies in the mouse is that of genomic imprinting, which provides an explanation for the abnormal development of gynogenetic and androgenetic embryos. Genomic imprinting refers to a process by which maternal and paternal alleles of specific genes or chromosomal regions are differentially marked during gametogenesis such that they are expressed differently in the embryo (86). One allele of the imprinted gene is usually active, while the other is inactive. Thus, the paternal and maternal copies are not functionally equal for all genes, and therefore both a maternal copy and a paternal copy are required for normal development. Like X chromosome inactivation, genomic imprinting is also an epigenetic phenomenon in that the imprinted gene does not undergo any permanent change and the imprint is reversible. Thus a female who begins life with a maternally and paternally imprinted allele at a locus will produce gametes that exhibit only maternal imprint even on her own paternal chromosome. Similarly, males produce only gametes with the male-specific imprint. In other words, during gametogenesis the parental imprint is erased and reset in a sex-specific manner. As in X chromosome inactivation, DNA methylation is a mediator of the maintenance of the imprint in the somatic cells.

Imprinting is known to affect only a small number of genes and chromosomal regions in the human genome. Imprinting thus differs from X-inactivation in that it does not affect a whole or most of a chromosome. Moreover, even within an imprinted chromosomal region, individual genes located within a few hundred kilobases of DNA may show differential imprinting. As a result, one gene may be inactive on the maternal chromosome and active on the paternal chromosome while a neighboring gene exhibits the opposite imprinting, being active on the maternal chromosome and inactive on the paternal chromosome. Imprinting also shows tissue-specific variation

for certain genes. Thus, the Angelman syndrome gene, UBE3A, on chromosome 15 is expressed from both chromosomes (biallelic expression) in somatic cells but is expressed only from the maternal chromosome in the brain. X chromosome inactivation in females is different from imprinting in this regard in that it is presumed to be present in all somatic cells.

Although imprinting affects only a few chromosomal regions, imprinted genes contribute to genetic diseases. The phenotypes exhibited by moles and teratomas are the result of failure to receive either the maternal (mole) or paternal (teratoma) genome. Other phenotypes result from failure to receive specific portions of the maternal or paternal genome, or inappropriately receiving two copies of the same chromosome region from one parent and none from the other parent (uniparental disomy or UPD) (87). A fraction of cases of Prader–Willi syndrome (PWS), Angelman syndrome, and Beckwith–Wiedemann syndrome result from such imbalances in the parental origin-dependent of a chromosome region. In the case of PWS, about 70% of the patients have a deletion in the proximal q arm of the paternally inherited chromosome 15. In normal individuals, the PWS critical gene(s) are transcribed only from the paternal homolog. Therefore, with the deletion of the PWS critical region on the paternal 15, PWS patients are completely deficient for the products of these imprinted genes. The remaining 30% of PWS patients have two chromosome 15s derived from their mother and none from their father. In the absence of a paternal 15, these patients also lack the expression of PWS critical gene(s). A likely mechanism for the origin of this UPD is the conception of a fetus with trisomy for chromosome 15 with two chromosomes from the mother and one from the father. Trisomy 15 is usually lethal and will lead to miscarriage. However, loss of a chromosome 15 in an occasional cell during early embryogenesis will allow that cell line to proliferate and result in a viable fetus. If the sole paternal chromosome is the one that is lost in this trisomy rescue, the resulting infant will have maternal UPD and PWS. Alternatively, UPD could arise from the rescue of a monosomic conceptus, by duplication of the single homolog. Maternal and paternal UPDs for many of the human chromosomes have now been identified. Several of these result in a normal phenotype, presumably because the chromosome does not harbor any imprinted gene(s) (87). However, these individuals may be at risk for being homozygous for recessive genes. The possible role of UPD, a unique form of chromosomal inheritance, in disease states of unknown etiology is being investigated.

## 10.8 CHROMOSOME ABNORMALITIES

Human cytogenetics has advanced during the past four decades because of continuing technical advances and the high incidence of chromosome abnormalities in the human population. It is estimated that the frequency of

significant chromosome abnormalities among live births is about 1 in 150. It is well documented that about 50% of first-trimester pregnancy losses are due to chromosome abnormalities, mostly numerical anomalies. Thus chromosome aberrations have a significant impact as causes of pregnancy wastage, congenital malformations, mental retardation, abnormalities of sex differentiation, and behavior problems. Acquired chromosomal changes play a significant role in carcinogenesis and in tumor progression.

Most chromosomal abnormalities exert their phenotypic effects by increasing or decreasing the quantity of genetic material. Chromosomal abnormalities can be divided into numerical and structural abnormalities. Structural changes such as translocations and inversions pose a much more serious familial recurrence risk for chromosome abnormalities. This is due to aberrant segregation of chromosomes during meiosis in clinically normal carriers of these balanced rearrangements.

### 10.8.1 Numerical Chromosome Abnormalities

The most straightforward of chromosomal abnormalities are alterations of chromosome number. Deviation from the normal diploid complement of 46 chromosomes is referred to as “aneuploidy”; an extra chromosome results in “trisomy,” whereas a missing chromosome results in “monosomy.” Although all the possible chromosomal trisomies have been observed in spontaneous abortions, trisomies 13, 18, and 21 are the only autosomal trisomies to be observed in a nonmosaic state in liveborns, and are discussed in detail in Chapter 43. All autosomal monosomies are lethal. The only viable monosomy involves the X chromosome (45,X resulting in Turner syndrome). Abnormalities associated with sex chromosomes are discussed in detail in Chapter 44. Aneuploidy results from nondisjunction, in which two copies of a chromosome go to the same daughter cell during meiosis or mitosis. Nondisjunction occurs most often in the first meiotic division in the maternal germ line. In meiosis I nondisjunction, both homologs of a chromosome move to the same pole during anaphase I instead of moving to opposite poles, giving rise to one daughter cell with two copies of the chromosome and the other with none. The latter product is never recovered because of lethality associated with monosomy. In the case of meiosis II nondisjunction, the two sister chromatids of a homolog move to the same pole, again giving rise to one daughter cell with two copies of the chromosome and the other with none. Mitotic nondisjunction results in the presence of an aneuploid and a normal cell line—a condition referred to as “mosaicism.” The causes of nondisjunction are unknown. The only well-documented risk factor is advanced maternal age.

The term “polyploidy” on the other hand refers to the presence of a complete extra set of chromosomes;

“triploidy” represents three sets with 69 chromosomes, whereas “tetraploidy” represents four sets with 92 chromosomes. Rarely, a triploid fetus will be liveborn, but in general polyploidy is lethal. In few instances, however, mosaicism for a diploid and a triploid cell line producing congenital anomalies has been compatible with long-term survival.

### 10.8.2 Structural Chromosome Abnormalities

Structural chromosomal rearrangements result from chromosome breakage with subsequent reunion in a different configuration. They can be balanced or unbalanced. In balanced rearrangements, the chromosome complement is complete with no loss or gain of genetic material. Consequently, balanced rearrangements are generally harmless, with the exception of rare cases in which one of the breakpoints disrupts an important functional gene. Carriers of balanced rearrangements are often at risk of having children with an unbalanced chromosome complement. When a chromosome rearrangement is unbalanced, the chromosome complement contains an incorrect amount of genetic material, usually with serious clinical effects.

A deletion involves loss of part of a chromosome and results on monosomy for that segment of the chromosome, whereas duplication represents the doubling of part of a chromosome, resulting in trisomy for that segment. The result is either decrease or increase in gene dosage. In general, duplications appear to be less harmful than deletions. Very large deletions usually are incompatible with survival to term. Deletions or duplications larger than ~5 Mb in size can be visualized under the microscope using G-banded chromosome analysis. Genomic disorders resulting from submicroscopic deletions and duplications (i.e. microdeletions and microduplications) with a size <5 Mb have been identified with the help of molecular cytogenetic techniques. In these syndromes, groups of contiguous genes are either deleted or duplicated, resulting in a defined set of congenital anomalies.

The use of cytogenomic arrays, including array CGH and SNP arrays, to analyze the genomes of normal humans led to the discovery of extensive genomic benign copy number variations (CNVs), both gains and losses, with the majority <500 kb in size (88,89). Benign CNVs have been proposed to be a major factor responsible for human diversity (90). Through genomic rearrangement of rearrangement-prone regions as a result of the genomic architecture, pathogenic CNVs on the other hand can cause genomic disorders due to losses or gains of dosage sensitive gene(s) resulting in a clinical phenotype (91). These pathogenic CNVs include recurrent microdeletions and microduplications flanked by segmental duplication (also called low copy repeats) and mediated by nonallelic homologous recombination, as well as non-recurrent deletions and duplications, varying

in size from a few hundred kilobases to a few megabases, and mediated by other molecular mechanisms (92,93). Well-known genomic disorders can be phenotypically heterogeneous and variable due to incomplete penetrance or variable expression. Clinical variability could also be explained in part by other genetic or environmental determinants, modifying factors of other genes, multigenic inheritance, imprinting, and unmasking of recessive genes. Cytogenomic arrays are proving particularly effective for the investigation of patients with developmental delay, intellectual disability, autistic features, dysmorphic features, and/or multiple congenital anomalies, and are identifying the probable underlying cause of the disease phenotype in approximately 15–20% of previously undiagnosed cases (44,91,94).

Translocations involve the exchange of genetic material between chromosomes. In a balanced reciprocal translocation the exchange is equal, with no loss or gain of genetic material, though it is possible for a gene to be disrupted at one of the breakpoints. More often, the carrier of a balanced translocation is free of clinical signs or symptoms but is at risk for having offspring with unbalanced chromosomes. The phenotype usually is a complex mixture as a result of loss or gain of at least two chromosome segments and therefore can be difficult to predict. One specific type of translocation that is relatively common is the “Robertsonian translocation.” This results from a fusion of two acrocentric chromosomes at the centromere. Carriers of a Robertsonian translocation have 45 chromosomes and are clinically unaffected. The most common clinically significant outcome is trisomy 21, in which a carrier for a Robertsonian translocation involving chromosome 21 produces a gamete with both the translocation chromosome and a normal 21, resulting in trisomy 21 after fertilization.

Inversions occur when there are two breaks in a chromosome and the intervening material flips 180°. Inversions that span the centromere are referred to as “pericentric,” whereas those that do not are called “paracentric.” Inversions generally do not result in added or lost genetic material, and therefore usually are viewed as neutral changes. Disruption of a gene at one of the breakpoints, however, could change the function of that gene. Also, alteration of gene order at the borders of the inversion could affect the function of blocks of genes that are coordinately regulated (“position effect”). If a crossover occurs in the inverted segment of a pericentric inversion during meiosis, two recombinant chromosomes result, one with duplication of one end and deletion of the other end, and the other having the opposite arrangement. Such a crossover event in a paracentric inversion results in dicentric or acentric chromosomes that tend to be unstable.

An insertion occurs when a segment of one chromosome becomes inserted into another chromosome. Because these changes require three chromosomal breakpoints, they are relatively rare. Abnormal segregation in

a balanced insertion carrier can produce offspring with either duplication or deletion of the inserted segment, as well as balanced carriers and normal offspring.

A “marker” chromosome is a rearranged chromosome whose genetic origin is unknown based on its G-banded chromosome morphology. Usually they are present in addition to the normal chromosome complement and are thus called supernumerary marker chromosomes (SMCs). Two-thirds of de novo marker chromosomes can be associated with an abnormal outcome, whereas inherited ones can be passed from generation to generation without apparent clinical effects. Larger markers with more genetically active material are more likely to be of clinical significance. FISH and cytogenomic arrays have proved very helpful in the precise identification of the genetic origin of SMCs. Ring chromosomes are formed when a chromosome undergoes two breaks and the broken ends reunite in a ring structure. Rings encounter difficulties in mitosis and are unstable, resulting in some cells that lose the ring and are therefore monosomic for the chromosome, and others that have multiple copies of the ring. An “isochromosome” is a chromosome in which one arm is missing and the other duplicated in a mirror-image fashion. The most commonly encountered isochromosome is that which consists of two long arms of the X chromosome. This accounts for ~15% of all cases of Turner syndrome (95).

## 10.9 CONCLUDING REMARKS

In this chapter, we have outlined the structural, functional, and behavioral aspects of human chromosomes and their relationship to disease states. Although recent investigations have provided insights into several aspects of chromosome structure, the details of the higher order structure of chromosomes are not well understood at the molecular level in full detail.

We have begun to understand the DNA sequences that are associated with chromosomal landmarks, such as centromeres, telomeres, chromosome bands, and fragile sites. However, we still do not understand the role of such sequences in producing the associated functional correlates. For example, which sequences are critical for centromere function, and how do dicentric chromosomes decide which will function? Most enigmatic, what is the origin of chromosome bands and what is the molecular organization of a band border?

The availability of the finished human genome sequence and cytogenomic arrays has allowed the detection of genomic CNVs on a global scale. It is now appreciated that the underlying genomic architecture plays a crucial role in the origin of these genomic rearrangements in rearrangement-prone regions. Segmental duplications have arisen in the primate genome, driving the process of chromosome evolution. In addition to creating a dynamic, evolvable genome, these segmental duplications result in instability, genomic rearrangement, and disease. We

have begun to understand the organization of segmental duplications, which predisposes the chromosomes that carry them to germline genomic rearrangements such as deletions, duplications, and inversions. However, we do not understand the mechanisms that initially led to the formation of segmental duplications, nor the sequences or structures responsible for their continued instability.

Many new insights have come from understanding the structure and function of the human X chromosome and genomic imprinting. However, we do not know to what extent the remainder of the genome may contain imprinted or partially imprinted genes whose parental origin in part determines tissue-specific expression. Might such “epigenetic” phenomena provide another mechanism for both normal human variation and disease susceptibility?

Much remains to be learned about the molecular aspects of chromosome structure, function, and behavior. It is anticipated that the human genome sequence and its functional characterization will provide the tools with which to approach these problems and define a new frontier for the role of chromosomes in human disease.

## GLOSSARY

- Aneuploidy** – a chromosome constitution with one or more chromosomes extra or missing from a full diploid set.
- Autosome** – any chromosome other than the sex chromosomes X and Y.
- BAC (bacterial artificial chromosome)** – a recombinant plasmid in which up to ~300 kb piece of genomic DNA can be cloned and propagated in bacterial cells.
- Bivalent** – a four-strand structure seen in meiotic prophase I comprising two synapsed homologous chromosomes.
- Centromere** – the primary constriction of a chromosome separating the short (p) arm from the long (q) arm. This is the point at which spindle fibers attach to pull chromatids apart during cell division.
- Chiasma (plural: chiasmata)** – the physical manifestation of meiotic crossover as seen under the microscope.
- Chromatid** – from the end of S phase of the cell cycle until anaphase of cell division, a chromosome consists of two sister chromatids. Each contains a complete double helix and the two are exact copies of each other.
- Chromatin fiber** – the 30 nm coiled coil complex of DNA and histones that is believed to be the basic conformation of chromatin.
- Comparative genomic hybridization (CGH)** – use of competitive fluorescence in situ hybridization to detect chromosomal regions that are gained or lost.
- Constitutional** – a genotype, chromosomal abnormality, or mutation that was present in the fertilized egg and therefore present in all cells of a person. This is distinct from a somatic change.
- Contiguous microdeletion/microduplication syndrome** – a syndrome caused by either deletion or its reciprocal duplication of a group of contiguous genes, some of which are dosage sensitive and contribute to the phenotype.



**Denaturation** – dissociation of complementary strands to give single-stranded DNA.

**Diploid** – have two copies of each chromosome.

**Dosage sensitive** – property of a gene where two copies of that gene are required for its normal function. A change in copy number produces an abnormal phenotype.

**Euchromatin** – the fraction of the nuclear genome which contains transcriptionally active genes and adopts a relatively extended conformation unlike heterochromatin.

**Fluorescence in situ hybridization (FISH)** – in situ hybridization using fluorescently labeled DNA probes.

**Haploid** – describing a cell, typically a gamete, which has only one copy of each chromosome.

**Heterochromatin** – a chromosomal region that remains highly condensed throughout the cell cycle and shows little or no evidence of active gene expression.

**Homologous chromosomes** – the two copies of a chromosome in a diploid cell. Unlike sister chromatids, homologous chromosomes are not copies of each other but one paternal in origin and the other maternal in origin.

**Imprinting** – determination of the expression of a gene by its parental origin.

**Interphase** – all the time in the cell cycle when the cell is not dividing.

**Isochromosome** – an abnormal symmetrical chromosome consisting of two identical arms, which are normally either the short (p) arm or the long (q) arm of a normal chromosome.

**Karyotype** – a summary of the chromosome constitution of a cell or a person.

**Marker chromosome** – an abnormal chromosome of unidentified origin.

**Metaphase** – the stage of cell division when chromosomes are maximally contracted and line up on the equatorial plane of a cell.

**Microdeletion** – a chromosomal deletion that is too small to be seen using conventional chromosome banding techniques (typically <5Mb).

**Monosomy** – having only one copy of a particular chromosome.

**Mosaic** – an individual who has two or more genetically different cell lines derived from a single zygote.

**Nondisjunction** – failure of chromosomes to separate at anaphase and is a major cause of numerical chromosome abnormalities. It can involve sister chromatids in mitosis or meiosis II or paired homologs in meiosis I.

**Nucleolar organizer regions (NORs)** – the stalks of the short (p) arms of chromosomes 13, 14, 15, 21, and 22. NORs contain arrays of ribosomal RNA genes.

**Nucleosome** – the basic structural unit of chromatin.

**Paracentric inversion** – inversion of a chromosomal segment that does not include the centromere.

**Pericentric inversion** – inversion of a chromosomal segment that includes the centromere.

**Polyploid** – having multiple chromosome sets as a result of a genetic event.

**Pseudoautosomal region** – regions at each tip of the X and Y chromosomes and containing X–Y homologous genes.

**Segmental duplication** – a very highly homologous DNA sequence block on different chromosomes or at more than one location within a chromosome.

**Sister chromatid** – two chromatids present within a single chromosome and joined at the centromeres.

**Telomere** – a specialized structure at the tips of chromosomes.

**Translocation** – exchange of genetic material between chromosomes.

**Triploid** – having three copies of the genome.

**Trisomy** – having three copies of a particular chromosome.

**Uniparental disomy (UPD)** – having both copies of one particular chromosome pair derived from one parent.

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### Biography



**Dr Mikhail** graduated from the Faculty of Medicine, University of Alexandria, Egypt in 1990. He completed his clinical pathology residency at the Department of Clinical Pathology in the same University. He received an Egyptian government scholarship to conduct this PhD thesis research work in the United States where he worked as a visiting scholar in the Pathology Department, School of Medicine, University of Illinois at Chicago, IL. He earned his PhD from the Faculty of Medicine, University of Alexandria, Egypt in 2003. Dr Mikhail did his clinical cytogenetics fellowship at the Department of Genetics, School of Medicine, University of Alabama at Birmingham, AL, and was certified by the ABMG in 2007. He joined the faculty in the Department of Genetics, School of Medicine, University of Alabama at Birmingham, AL as an assistant professor in 2006. His research interests include identification of novel constitutional genomic disorders caused by microdeletions and microduplications using cytogenomic array methodologies with special interest in neurodevelopmental disorders, and characterization of the clinical phenotype, molecular breakpoints, mechanism of rearrangement, as well as the functional categorization of the involved genes. Dr Mikhail's research interests also include identification of novel cytogenetic rearrangements in patients with various hematological malignancies that might have a causal role in the oncogenic process using molecular cytogenetic techniques, and identification of the underlying genes.



# CHAPTER 11

## Mitochondrial Medicine: The Mitochondrial Biology and Genetics of Metabolic and Degenerative Diseases, Cancer, and Aging

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### ABBREVIATIONS

AAV – Adeno-associated virus  
 ALA –  $\delta$ -aminolevulinic acid  
 ALS – Amyotrophic lateral sclerosis  
 AML – Acute myeloid leukemia  
 AMPK – Adenosine monophosphate activate protein kinase  
 ANT – Adenine nucleotide translocator  
 ASDs – Autism spectrum disorders  
 BPAD – Bipolar affective disorder  
 CNVs – Copy number variants  
 DCA – Dichloroacetate  
 DSAD – Down syndrome and dementia  
 ER – Endoplasmic reticulum  
 ETC – Electron transport chain  
 ETF – Eelectron transfer flavoprotein  
 FH – Fumarate hydratase  
 FICP – Fatal infantile cardiomyopathy plus  
 FMN – Flavin mononucleotide  
 HSP – Hereditary spastic paraplegia  
 IBM – Inclusion body myopathy  
 IDDM – Insulin-dependent diabetes mellitus  
 LHON – Leber hereditary optic neuropathy  
 MAMs – Mitochondrial-associated membranes  
 MELAS – Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes  
 MERRF – Myoclonic epilepsy with ragged red fibers  
 MM – Mitochondrial myopathy  
 MNGIE – Mitochondrial neurogastrointestinal encephalomyopathy

MODY – Maturity onset of diabetes in the young  
 MPTP – 1-Methyl-4-phenyl-1,2,3,4,-tetrahydropyridine  
 mRNA – Messenger RNA  
 MnSOD – Manganese superoxide dismutase  
 mtDNA – Mitochondrial DNA  
 mtPTP – Mitochondrial permeability transition pore  
 MTS – Mitochondrial targeting sequence  
 NASH – Nonalcoholic steatohepatitis  
 nDNA – Nuclear DNA  
 NMR – Nuclear magnetic resonance  
 NOS – Nitric oxide synthase  
 NRF1, NRF2 – Nuclear respiratory factors 1 and 2  
 OXPHOS – Oxidative phosphorylation  
 PBR – Peripheral benzodiazepine receptor  
 PCR – Polymerase chain reaction  
 PDH – Pyruvate dehydrogenase  
 PDK – Pyruvate dehydrogenase kinase  
 PEO – Progressive external ophthalmoplegia  
 PHDs – Prolyl hydroxylases  
 PNAs – Protein nucleic acids  
 PPAR $\gamma$  – Peroxisome-proliferation-activated receptor  $\gamma$   
 REDOX – Reduction–oxidation  
 ROS – Reactive oxygen species  
 RRFs – Ragged-red muscle fibers  
 SDH – Succinate dehydrogenase  
 SNPs – Single nucleotide polymorphisms  
 tRNA – Transfer RNA  
 UCPs – Uncoupling proteins  
 YBP – Years before present

A wide variety of degenerative and metabolic diseases as well as cancer and aging have now been associated with mitochondrial dysfunction. These can result from mutations in either the mitochondrial DNA (mtDNA) or the nuclear DNA (nDNA) or due to a dysfunctional interaction of the two genomes. Hence, the genetics of mitochondrial diseases can be non-Mendelian and complex.

The mitochondria generate much of the cellular energy by burning hydrocarbons with oxygen via oxidative phosphorylation (OXPHOS), thus generating ATP to do work and produce heat to maintain body temperature. As a result, the mitochondria regulate cellular reduction-oxidation (REDOX) status, produce much of the cellular reactive oxygen species (ROS), control cellular calcium ( $\text{Ca}^{++}$ ) levels, provide subcellular compartmentalization for numerous cellular functions, and can initiate cell death (apoptosis) when mitochondrial energetics is compromised through the activation of the mitochondrial permeability transition pore (mtPTP).

Currently recognized mitochondrial diseases can either be stereotypic in their presentation, as in Leber hereditary optic neuropathy (LHON) or the Leigh syndrome (LS), or result in variable multisystem disorders that generally affect the more oxidative organs of the body, including the central nervous system, skeletal muscle, heart, renal, and/or endocrine systems, though any organ system may be involved. Moreover, as knowledge of the complex biology and genetics of the mitochondrion continue to advance, it is becoming increasingly recognized that mitochondrial dysfunction plays a central role in a broad spectrum of metabolic and degenerative diseases as well as cancer and aging. Hence, the emerging field of Mitochondrial Medicine is impacting virtually every aspect of modern medicine (1).

The eukaryotic cell is the product of an over two billion year old symbiosis between two coequal single-celled organisms, one of which became specialized in encoding the cellular and organismal structure and ultimately gave rise to the nucleus-cytosol while the other became specialized in energy production and led to the modern mitochondrion. The exchange of genes between these two organisms ultimately generated an organism in which all of the genes for structure were all stored in the nDNA while the critical genes for the wiring diagram of mitochondrial energy production were retained in the mtDNA (2).

The energy production capacity of one bacterial cell is to sustain in the order of 2000–3000 genes. Hence, the genome size of all prokaryotic organisms is limited. However, with the advent of the nucleus-cytosol and mitochondrion symbiosis, the number of mitochondria within the cellular cytoplasm could increase. Since many mitochondria could contribute to the energy needed to sustain the nucleus, the energetic limitation of gene number was relieved, permitting the accumulation of over 10 times more genes in the eukaryotic cell nucleus than the prokaryotic nucleoid and thus providing the

information necessary for multicellularity and the elaboration of complex anatomical forms (3). Indeed, genomic energy limitation may have been a major driving force for the transfer of the mtDNA genes to the nucleus. Placing a copy of each essential mitochondrial gene in the nucleus permitted the nuclear gene to supply the protein to all of the mitochondria. The deletion of that gene from all of the mtDNAs then freed up energy previously required for mitochondrial gene replication and expression for use in fostering nuclear gene replication and expression.

Hence, life is the product of the interplay between structure (anatomy), energy (vital force), and information (the information required to encode for both structural and energetic systems) (2). “Western” medicine, however, has traditionally taken an anatomical perspective on health and disease. As a consequence, the central role of bioenergetics and its genetics has been largely overlooked in the health sciences.

The identification and description of specific mtDNA mutations that cause either stereotypic or multisystem diseases such as Leber hereditary optic atrophy (4), myoclonic epilepsy and ragged red fiber (MERRF) disease (5,6), and chronic progressive external ophthalmoplegia (CPEO) (7) have demonstrated the importance of mutations in energy genes to health. The characterization of these and numerous other mitochondrial diseases has since revealed the complex, multisystem, degenerative nature of the clinical phenotypes resulting from chronic energy deficiency. This has set the stage for the reassessment of the pathophysiology of a broad range of metabolic and degenerative diseases, cancer, and aging (1,8,9).

As our understanding of mitochondrial biology and genetics has expanded, several new biomedical paradigms have emerged which are helping the understanding of the pathophysiology of common complex diseases. The first paradigm is that the integrated system of mitochondrial biochemistry and biophysics plays a multidimensional role in human health and disease. Hence, the tradition linear concept of one gene-one polypeptide-one disease is no longer meaningful. Rather, alterations in bioenergetics must be analyzed through systems biology. The second is that “inheritance” is no longer defined exclusively by the rules of Mendelian genetics. Heredity also involves non-Mendelian (maternal) genes, nuclear-cytoplasmic interactions, somatic genetic alterations, and the energetic regulation of cellular metabolism and nDNA gene expression through the cellular signal transduction systems and the epigenome. The third is that different organs and tissues have different energetic demands and energetic functions, the energy anatomy. As a consequence, systemic defects can result in tissue and organ specific symptoms.

In this chapter, we will attempt to summarize the salient features of mitochondrial biology and genetics and then to apply these insights to interpreting the

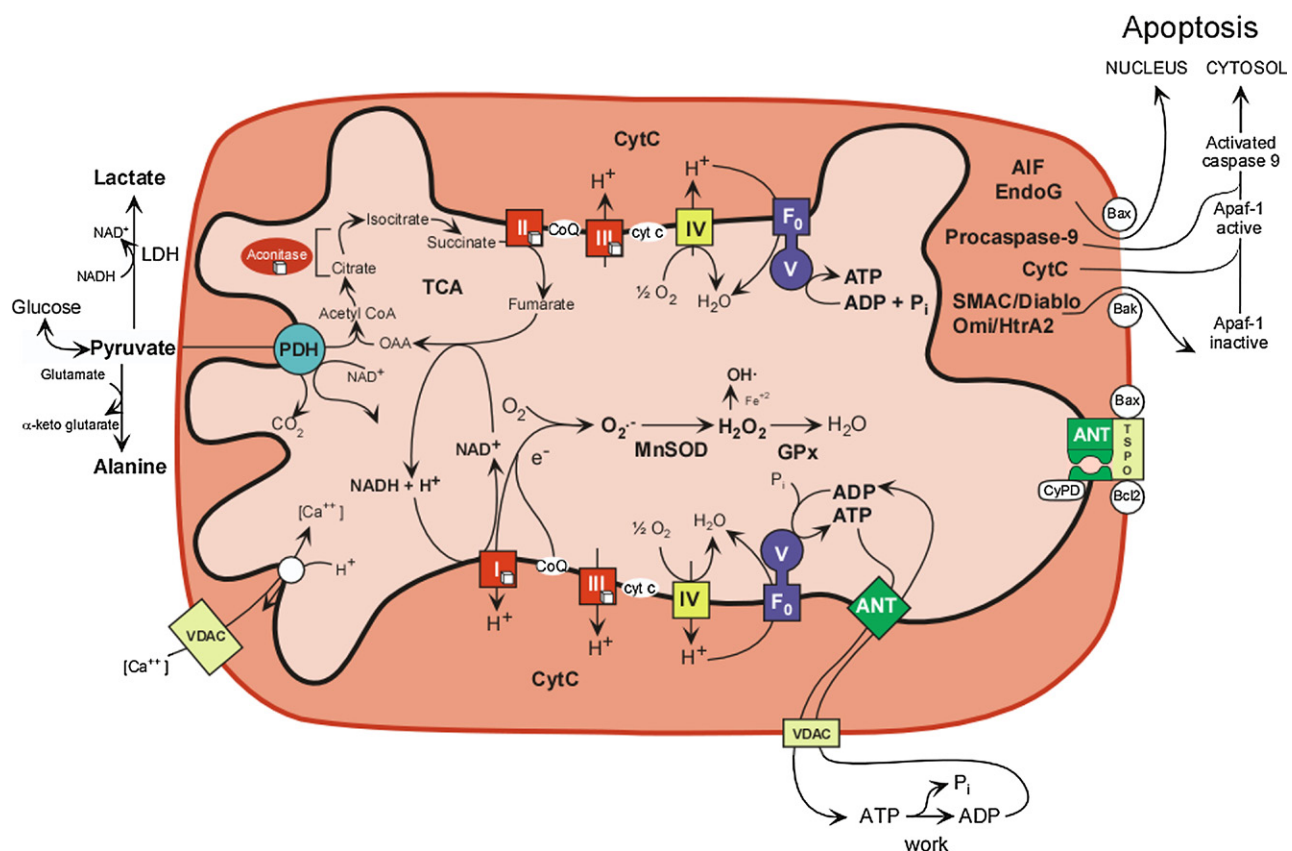
genetics and pathophysiology of both established mitochondrial diseases and common complex disease. In this way, we hope to provide a more comprehensive understanding of genetic disease which has greater predictive power for clinical diagnosis, genetic counseling, and therapeutic development.

## 11.1 MITOCHONDRIAL BIOLOGY AND GENETICS

The mitochondrion has a characteristic double membrane structure (Figure 11-1). The outer membrane is smooth, but the inner membrane is highly folded, forming structures called cristae. The large surface area of the inner mitochondrial membrane accommodates the enzymes of the mitochondrial energy-generating apparatus, OXPHOS.

### 11.1.1 Mitochondrial Biology and the Etiology of Disease: Energy Production, ROS Generation, Apoptosis, and Mitochondrial Dynamics

OXPHOS is composed of five multi-polypeptide enzyme complexes. Complexes I, II, III, and IV make up the electron transport chain (ETC), while complex V is the ATP synthase (ATPsyn) (see Figure 11-1). Dietary carbohydrates and fats are transported to the mitochondria, where they are oxidized by the tricarboxylic acid cycle and the  $\beta$ -oxidation pathways, respectively. This liberates  $\text{CO}_2$  and hydrogen atoms, the latter being transferred to soluble NAD to generate  $\text{NADH} + \text{H}^+$ , or to FAD bound within enzymes to yield  $\text{FADH}_2$ .  $\text{NADH} + \text{H}^+$  is oxidized by complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) and the electrons are transported through flavin



**FIGURE 11-1** Mitochondrial processes involved in energy production, REDOX regulation,  $\text{Ca}^{++}$  homeostasis, RPS production, and regulation of apoptosis through the mtPTP. The OXPHOS complexes, designed I to V, are complex I (NADH: ubiquinone oxidoreductase) encompassing a flavin mononucleotide (FMN) and six Fe-S centers (designated with a cube); complex II (succinate: ubiquinone oxidoreductase) involving a flavin adenine dinucleotide (FAD), three Fe-S centers, and a cytochrome b; complex III (ubiquinol: cytochrome c oxidoreductase) encompassing cytochromes b, c1 and the Rieske Fe-S center; complex IV (cytochrome c oxidase) encompassing cytochromes a+a<sub>3</sub> and CuA and CuB; and complex V (H<sup>+</sup>-translocating ATP synthase). Pyruvate from glucose enters the mitochondria via pyruvate dehydrogenase (PDH), generating acetylCoA which enters the TCA cycle by combining with oxaloacetate (OAA). Cis-aconitase converts citrate to isocitrate and contains a 4Fe-4S center. Lactate dehydrogenase (LDH) converts excess pyruvate plus NADH to lactate. Small molecules diffuse through the outer membrane via the voltage-dependent anion channel (VDAC) or porin. The VDAC together with ANT, Bax, and the cyclophilin D (CD) protein are thought to come together at the mitochondrial inner and outer membrane contact points to create the mtPTP. The pro-apoptotic Bax of the mtPTP is thought to interact with the anti-apoptotic Bcl2 and the benzodiazepine receptor (BD). The opening of the mtPTP is associated with the release of several pro-apoptotic proteins. Cytochrome c (cyt c) interacts with and activates cytosolic Apaf-1, which then binds to and activates procaspase-9. The activated caspase-9 then initiates the proteolytic degradation of cellular proteins. Apoptosis initiating factor (AIF) and endonuclease G (EndoG) have nuclear targeting peptides that are transported to the nucleus and degrade the chromosomal DNA. (Modified from Reference (317).)

mononucleotide (FMN) and multiple iron-sulfur centers until they are transferred to ubiquinone, or coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>). CoQ<sub>10</sub> is subsequently reduced to ubisemiquinone (CoQ<sub>10</sub>H), and then to ubiquinol (CoQ<sub>10</sub>H<sub>2</sub>). CoQ<sub>10</sub> is also reduced to CoQ<sub>10</sub>H<sub>2</sub> by succinate through complex II (succinate:ubiquinone oxidoreductase, EC 1.3.5.1) and by electrons from fatty acid oxidation via the electron transfer flavoprotein (ETF) together with the ETF dehydrogenase (EC 1.5.5.1). Both succinate dehydrogenase (SDH) and ETF dehydrogenase contain an FAD molecule and one or more iron-sulfur centers. CoQ<sub>10</sub>H<sub>2</sub> ferries the electrons through the inner membrane to complex III (ubiquinol:ferrocytochrome c oxidoreductase [EC 1.10.2.2] or the bc<sub>1</sub> complex). Within this complex, the electrons move through the Q cycle, cytochrome b, cytochrome c<sub>1</sub>, and the Rieske iron-sulfur components. The electrons are then transferred from complex III to cytochrome c, which is loosely associated with the exterior of the inner membrane. Cytochrome c transfers the electrons to complex IV (ferrocytochrome c: oxygen oxidoreductase [EC 1.9.3.3.1] or cytochrome c oxidase [COX]). Within this complex, the electrons are transferred through the Cu<sub>A</sub> and Cu<sub>B</sub> centers and cytochromes a and a<sub>3</sub> and ultimately combine with ½O<sub>2</sub> to give H<sub>2</sub>O (actually, four electrons reduce O<sub>2</sub> to 2H<sub>2</sub>O). The energy that is released in this controlled oxidation of the electrons is used to pump protons from inside the mitochondrial matrix across the inner membrane into the intermembrane space through complexes I, III, and IV. The resulting electrochemical gradient, Δp, which combines ΔpH (ΔμH<sup>+</sup>) and an electrostatic potential (Δψ) (i.e. Δp = Δψ + ΔpH), serves as a source of potential energy for synthesizing ATP. ATP is synthesized by complex V (ATP phosphohydrolase [H<sup>+</sup> transporting; EC 3.6.1.34], or ATPsyn), which contains three distinctive components: the base, F<sub>0</sub>, the stalk, and the hexagonal head, F<sub>1</sub>. As protons move back through the proton channel in F<sub>0</sub>, they cause the central axle of the ATPsyn to spin within the hexagonal array of 3α and 3β subunits, causing them to change their conformation. This causes ADP and Pi to bind, be condensed into ATP, and be released into the matrix. Matrix ATP is exchanged for cytosolic ADP by the adenine nucleotide translocator (ANT) (8–10) (see Figure 11-1).

Each of the ETC complexes incorporates multiple electron carriers. Complexes I, II, and III encompass several Fe-S centers, as does aconitase of the tricarboxylic acid cycle. Complexes III and IV encompass the cytochromes (8,9,11).

Since the ETC is coupled to ATP synthesis through Δp, mitochondrial oxygen consumption is regulated by the matrix concentration of ADP. In the absence of ADP, oxygen consumption is slow (state IV respiration). However, when ADP is added, it is transported into the matrix by the ANT, and oxygen consumption goes up as ATPsyn utilizes the proton gradient to phosphorylate ADP back to ATP (state III respiration). The ratio

of state III and state IV respiration is called the respiratory control ratio, and the amount of molecular oxygen consumed relative to the ADP phosphorylated is the P:O ratio. Addition of uncouplers such as 2,4-dinitrophenol and FCCP collapse Δp and permit the ETC and oxygen consumption to run at their maximum rates.

Because OXPHOS is a major sink for reducing equivalents in the cell, it is a major factor in regulating the cellular REDOX state. The REDOX state, in turn mediated a wide range of cellular metabolic and gene expression functions through reversible thiol-disulfide reactions occurring within numerous enzymes and transcription factors (12). Similarly, almost no function within the cell can proceed without the input of energy. As a result, all signal transduction pathways in the cell and the status of the epigenome are modulated by phosphorylation by ATP and/or acetylation by acetyl-CoA, both of which are primarily generated by the mitochondrion (13).

The mitochondrial membrane potential being negative in the matrix provides an electrostatic system for the import of a range of positively charged molecules into the mitochondrion. This includes the cytosolically synthesized polypeptides destined to be imported back into the mitochondrion as well as positively charged ions including calcium (Ca<sup>++</sup>) which is mediated by a specific mitochondrial Ca<sup>++</sup> uniporter (14,15).

The mitochondria are also the primary source of endogenous ROS. The first of the ROS, superoxide anion (O<sub>2</sub><sup>-</sup>) is generated by the transfer of one electron from the ETC to O<sub>2</sub> (see Figure 11-1). Ubisemiquinone, localized at the CoQ binding sites of complexes I, II, and III, appears to be the primary electron donor (16–19).

Mitochondrial O<sub>2</sub><sup>-</sup> is converted to H<sub>2</sub>O<sub>2</sub> by manganese superoxide dismutase (MnSOD) and the resulting H<sub>2</sub>O<sub>2</sub> is reduced to water by glutathione peroxidase-1 in brain, liver, and kidney and possibly by catalase in heart and muscle. However, H<sub>2</sub>O<sub>2</sub>, in the presence of reduced transition metals, is converted to the highly reactive hydroxyl radical (•OH). The Fe-S centers of the tricarboxylic acid cycle and the ETC are the major targets of ROS reactivity. Hence, the mitochondria are particularly sensitive to oxidative stress (11,20–23).

Superoxide production and H<sub>2</sub>O<sub>2</sub> generation are highest when the ETC is more reduced (state IV respiration) and lowest when it is more oxidized (state III respiration) (24–28). Since ubisemiquinone bound to complexes I and III is probably the primary electron donor for O<sub>2</sub><sup>-</sup> generation, ROS production will be maximized under physiologic conditions that maximize the concentration of ubisemiquinone. Hence, mitochondria with primarily oxidized ubiquinone would produce minimal ROS species. However, a predominantly though not completely reduced ETC would have the maximum ubisemiquinone and hence maximal ROS production. This then explains why blocking electron flow through the ETC with drugs such as Antimycin A, which inhibits complex III, stimulates ROS production



and why also adding uncouplers further stimulates ROS production (17,25,26,28).

The mitochondria are also the major regulators of apoptosis, which is initiated through the opening of the mtPTP (see Figure 11-1). The mtPTP is thought to be composed of the peripheral benzodiazepine receptor (PBR), now known as the mitochondrial translocator protein 18kDa (TSPO) (29–32) which may form the outer membrane channel, and an unknown structure that forms the inner membrane channel. This complex interacts with the ANTs (33), cyclophilin D, and members of the pro- and anti-apoptotic Bax and Bcl2 gene family (34–36). When the mtPTP opens,  $\Delta P$  collapses fluid and ions equilibrate between the matrix and cytosol, causing the mitochondria to swell. The mechanism by which the opening of the mtPTP is coupled with the release of the inner membrane space polypeptides is unresolved. However, recent evidence indicates that the pro-apoptotic proteins Bax and Bak aggregate in the outer mitochondrial membrane to form a mega channel that permits egress of the intermembrane space proteins (37–39). The opening of the mega channel in the outer membrane results in the release of a number of cell death-promoting factors including cytochrome *c*, apoptosis initiating factor, the latent form of caspase 9 (procaspases-9), SMAC/Diablo, endonuclease G, and the Omi/HtrA2 serine protease 24. On release, cytochrome *c* activates the cytosolic Apaf-1, which activates the procaspase-9. Caspase 9 then initiates a proteolytic cascade that destroys the proteins of the cytoplasm. Endonuclease G and apoptosis initiating factor are transported to the nucleus, where they degrade the chromatin (11,40–44). Activation of the mtPTP can be mediated by a decline in mitochondrial membrane potential  $\Delta P$ ; increased oxidative stress due to excessive ROS exposure; altered REDOX status, increased matrix  $Ca^{++}$  which binds to cyclophilin D, the action of which can be inhibited by cyclosporin A; and ANT ligands including ADP, bongkrekic acid, and atractyloside (34,41). Thus, disease states that inhibit OXPHOS and increase ROS production also increase the propensity of the cell to undergo apoptosis (34,45,46).

## 11.1.2 Mitochondrial Oxidative Phosphorylation Complexes

**11.1.2.1 Complex I (NADH Dehydrogenase, NADH).** OXPHOS complex I is composed of ~45 polypeptides, 7 (MTND1, 2, 3, 4, 4L, 5, and 6) of which are encoded by the mtDNA. These encompass 3 flavoproteins, 8–9 iron-sulfur proteins (ISPs), and over 24 hydrophobic proteins (47–61).

Complex I oxidizes NADH and reduces  $CoQ_{10}$ , and uses the energy released to pump protons across the mitochondrial inner membrane. The redox differential across complex I is from  $-320\text{ mV}$  for NADH to  $+60\text{ mV}$  for  $CoQ_{10}$ . Electrons from NADH are transferred through FMN and up to eight iron-sulfur centers: N1a (NDUFV2),

N1b (NDUFS1), N2 (NDUFS7), N3 (NDUFV1), N4 (NDUFS1), N5 (NDUFS1), N6a (NDUFS8), and N6b (NDUFS8) (55,62,63). The overall structure of complex I is slipper shaped, with the foot lying in the membrane and the ankle extending into the mitochondrial matrix (64,65).

The bovine complex I consists of a globular hydrophilic region containing the NADH binding site, FMN, and all ISPs; a  $30\text{-}\text{\AA}$  diameter stalk, and a hydrophobic membrane component. The overall molecular mass is  $890\text{ kDa}$ . The hydrophilic globular component and stalk are  $175\text{ \AA}$  and have a mass of about  $520\text{ kDa}$ . The hydrophobic component is  $200\text{ \AA}$  long and has a mass of about  $370\text{ kDa}$  (64). There are 14 core subunits required for its catalytic function, 7 hydrophobic nDNA-encoded subunits (NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS7, and NDUFS8), and 7 hydrophobic mtDNA-encoded subunits (ND1, ND2, ND3, ND4L, ND5, and ND6) (55,66).

Complex I can be split into two major components,  $I\alpha$  and  $I\beta$ , by the detergent lauryl-dimethylamine oxide. The  $I\alpha$  component can oxidize NADH and reduce ubiquinone and contains the NADH binding site, FMN, and all of the known Fe-S centers (60,67–75). The  $I\beta$  component has no enzymatic activity, is hydrophobic, and probably represents the membrane-bound component (60,76,77).

Respiratory complex I can also be subfractionated by treatment with the chaotropic anion perchlorate into three fragments: the flavoprotein fragment, the iron-protein fragment, and the hydrophobic fragment (63). The chaotropic agents *N,N*-dimethyldodecylamine *N*-oxide and  $\beta$ -mercaptoethanol also generate the subcomplexes  $I\alpha$  and  $I\beta$  (55,60,78–81). The  $I\alpha$  subcomplex harbors both hydrophilic and hydrophobic subunits including those containing the FMN and the Fe-S clusters. The  $I\alpha$  subcomplex can be subdivided into a flavoprotein component and the  $I\gamma$  fraction. The  $I\beta$  subcomplex encompasses the hydrophobic mtDNA-encoded proteins (55,66).

The crystal structure of the bacterial (*Thermus thermophilus*) (82) and the yeast (*Yarrowia lipolytica*) complex I (83) have been determined. The *T. thermophilus* enzyme is composed of 14 polypeptides whereas the *Y. lipolytica* enzyme encompasses 35 polypeptides (55). These crystal structures have led to a model as to how the transport of two electrons through complex I can result in the transport of four protons across the mitochondrial inner membrane. The membrane component is composed of two modules  $P_P$  and  $P_D$  which contain the two proton pumping channels. These encompass the ND5 and ND1 + ND6 mtDNA subunits, respectively. They are connected by a horizontal arm that is the mtDNA ND4 subunit. It is envisioned that as electrons are transferred to the bound  $CoQ_{10}$ , they drive the lateral movement of the ND4 lateral arm which then couples proton pumping through the two proton channels. Hence, the flux of two electrons results in the transport of four protons (82–84).

Complex I is affected by a wide variety of inhibitors, the best known of which are rotenone and its derivatives. One notable example of considerable clinical and experimental significance is the activated form of 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridinium. Exposure to MPTP in both humans and other mammals results in the selective destruction of basal ganglia neurons and induction of parkinsonism (85,86).

Assembly and maintenance of active respiratory chain complexes requires the action of additional factors. While mutations have been found in several complex I structural subunit genes encoded by mtDNA or nDNA, the molecular defects that cause more than 60% of complex I patient defects have not been found by sequencing the structural genes (87).

The majority of the defects observed in complex I have proven to be mutations in assembly proteins which do not form the final structure of the complex. The first such assembly mutation was found in a patient with severe childhood-onset progressive encephalopathy leading to death at the age of 13 years. Sequencing the nDNA complex I genes and the known assembly genes revealed that the child harbored a homozygous C182T transition in exon 2 of the *B17.2L* assembly gene, resulting in a premature stop codon at position 45 (R45X) (88). Multiple complex I disease patients have now been found to have mutations in assembly genes. By analyzing the intermediate subcomplexes that accumulate in the various mutations, an assemble pathway has emerged for complex I (55).

### 11.1.2.2 Complex II (Succinate Dehydrogenase).

Complex II oxidizes succinate to fumarate and transfers the electrons to CoQ<sub>10</sub>. The enzyme is composed of four subunits: a 70-kDa flavoprotein, a 27-kDa ISP, a 15-kDa membrane polypeptide, and a 5–7-kDa membrane polypeptide. All four complex II subunits are nuclear encoded. The 70-kDa flavoprotein contains the succinate binding site and a covalently bound FAD moiety (89,90). The 27-kDa ISP contains three iron-sulfur clusters—center 1 ([2Fe-2S]<sup>2+,1+</sup>), center 2 ([4Fe-4S]<sup>2+,1+</sup>), and center 3 ([3Fe-4S]<sup>1+,0</sup>)—that transport electrons to CoQ<sub>10</sub> (91–93). CoQ<sub>10</sub> is bound to the two membrane-intrinsic subunits, CII-3 (94) and CII-4. A *b*<sub>560</sub>-type heme is also associated with CII-3 (SDHD) and CII-4 (SDHC) (95), but its function is unclear (94,96–99). However, in *Caenorhabditis elegans*, the *mev-1* mutant alters the amino acid sequence of the CII-3 (SDHD) subunit, which greatly increases ROS production and decreases life span, suggesting that cytochrome *b*<sub>560</sub> may function to stabilize or dismutate ubisemiquinone (CoQ<sub>10</sub>H) (100).

**11.1.2.3 Complex III (bc<sub>1</sub> Complex).** Complex III is composed of 11 polypeptides, one (cytochrome *b*, MTCYB) encoded by the mtDNA (101–103). The crystal structure of complex III has been determined and revealed that complex III functions as a dimer, with a monomer mass of 240 kDa (104–108). This enzyme

oxidizes ubiquinol (CoQ<sub>10</sub>H<sub>2</sub>), reduces ferricytochrome *c* to ferrocycytochrome *c*, and uses the energy released to pump protons across the mitochondrial inner membrane via the Q cycle. The polypeptides of complex III include two core proteins (subunit I and subunit II), MTCYB (subunit III), cytochrome *c*<sub>1</sub> (subunit IV), the “Rieske” ISP (subunit V), and seven smaller polypeptides, subunits 6–11. Cytochrome *b*, cytochrome *c*<sub>1</sub>, and the Rieske ISP provide the major redox centers of the enzyme (104).

The Rieske ISP and cytochrome *c*<sub>1</sub> are on the intermembrane side of the enzyme, and both contain one membrane-spanning domain. The heme cytochrome *c*<sub>1</sub> is covalently bound to Cys<sup>37</sup> and Cys<sup>40</sup> and its ligands are His<sup>41</sup> and Met<sup>160</sup> (101,109,110). Subunit 8, or the “acidic/hinge protein,” lies above cytochrome *c*<sub>1</sub>, and the two form the docking site for cytochrome *c*. The Rieske ISP encompasses a single 2Fe-2S iron-sulfur center and changes conformation, switching from intermediate (“int”), to “b,” to “c1” forms. In this process, its relationship to the adjacent cytochromes *c*<sub>1</sub> changes, facilitating directional electron flow. In the “c1” state, the iron-sulfur center is adjacent to the heme of cytochrome *c*<sub>1</sub>, in the “int” state the iron-sulfur center is displaced from both cytochromes *c*<sub>1</sub> and *b*, and in the “b” state it interacts with cytochrome *b*.

The central core of the transmembrane domain is composed of cytochrome *b*. Cytochrome *b* has a high-potential heme, *b*<sub>L</sub> (*b*<sub>566</sub>), and a low-potential heme, *b*<sub>H</sub> (*b*<sub>560</sub>). Heme *b*<sub>L</sub> is close to the intermembrane side while heme *b*<sub>H</sub> is on the matrix side. The matrix side of the enzyme is composed of the core 1 and core 2 proteins, together with subunits 6 and 9. Subunit 9 is generated by metalloprotease cleavage of the NH<sub>2</sub>-terminal 78 amino acids of the nascent Rieske ISP. Core 1 shows sequence homology to the β subunit of mitochondrial processing protease; core 2 shows homology to the α subunit. Both core proteins probably catalyze the cleavage (104).

Proton translocation of complex III is linked to electron transport through the intermediates of CoQ<sub>10</sub> oxidation–reduction in the proton-motive Q cycle (71,109,111,112). Complex III has two CoQ<sub>10</sub> binding sites, one on the cytoplasmic side (Q<sub>o</sub> or Q<sub>p</sub> site) and one on the matrix side (Q<sub>i</sub> or Q<sub>n</sub> site) of the inner membrane. Q<sub>p</sub> is on the intermembrane side close to heme *b*<sub>L</sub> and binds the inhibitor myxothiazol, while Q<sub>n</sub> is close to heme *b*<sub>H</sub> on the matrix side and binds the inhibitor Antimycin A (104,109,113–115).

CoQ<sub>10</sub>H<sub>2</sub> binds to the Q<sub>o</sub> site and transfers one electron to the Rieske ISP, which passes it on to cytochrome *c*<sub>1</sub>. The resulting ubisemiquinone donates the other electron to the adjacent *b*<sub>L</sub> heme located on the cytoplasmic side of the membrane. The transfer of the two electrons from CoQ<sub>10</sub>H<sub>2</sub> releases the two ubiquinol protons to the outside of the mitochondrial membrane. The electron at the *b*<sub>L</sub> heme is then transported to the more matrix *b*<sub>H</sub> heme, where it is transferred to CoQ<sub>10</sub> at Q<sub>i</sub> site to generate ubisemiquinone. A second molecule of CoQ<sub>10</sub>H<sub>2</sub> is

oxidized in the same manner, and the resulting  $b_H$  electron is donated to the ubisemiquinone at the  $Q_i$  site. This reduced quinone then combines with two protons from the matrix to generate ubiquinol ( $CoQ_{10}H_2$ ). Hence, two protons are transported through the membrane for each pair of electrons that exits complex III (116).

The Q cycle is driven by the conformational changes of the Rieske ISP. When complex III is oxidized, the Rieske ISP is in the “int” state. When  $CoQ_{10}H_2$  binds to the  $Q_o$  site in cytochrome *b*, the  $CoQ_{10}H_2$  is deprotonated to  $CoQ_{10}H$ , which overcomes the activation barrier. This pulls the ISP to the “b” position. An electron is transferred to the Rieske ISP while it is adjacent to the  $CoQ_{10}H$  (ubisemiquinone) in the “b” conformation. The second electron from  $CoQ_{10}H$  is then transferred to heme  $b_L$ , weakening the interaction between the quinone and the Rieske ISP, permitting the ISP to move to the “c1” state. Here it transfers the electron to cytochrome  $c_1$ , and the ISP moves to the “int” state. The cytochrome  $c_1$  electron moves on to cytochrome *c* and the heme  $b_L$  electron moves on to the heme  $b_H$  site (104).

A gene (*BCS1L*) has been shown to be responsible for a complex III assembly defect in at least four unrelated families. Mutations in this gene give a homogeneous clinical presentation associated with neonatal tubulopathy, liver failure, and encephalopathy (117). A neonatal metabolic disorder with growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, and early death (Gracile syndrome) was also described in Finnish patients due to mutations in the *BCS1L* gene (118).

**11.1.2.4 Cytochrome c.** Cytochrome *c* ferries electrons along the outer mitochondrial membrane from complex III to complex IV. It contains a single covalently bound heme and is encoded by two isoform genes, a systemic cytochrome *c*, and a testes cytochrome *c* [(119–121), Cuticchia, 1995 #823].

**11.1.2.5 Complex IV (Cytochrome c Oxidase).** Complex IV is composed of 13 polypeptides, 3 (COI, COII, and COIII) encoded by the mtDNA. The remaining nuclear polypeptides have been designated IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, and VIII (122). The crystal structure of complex IV (107,123) has revealed that it is a functional dimer with a minimal monomer molecular mass of 204 kDa. Complex IV collects electrons from reduced cytochrome *c* (ferrocytochrome *c*), transfers them to CuA, then to cytochrome *a*, then to the bimetallic cytochrome  $a_3$ /CuB active site, and then to oxygen to give water, concurrently pumping four protons across the mitochondrial inner membrane (47,116,123–127).

Complex IV encompasses two hemes *a* ( $a + a_3$ ); three coppers, two for CuA and one for CuB; one magnesium; and one zinc. The two coppers forming the binuclear center, CuA, are associated with mtDNA subunit II (COII), while the two hemes and CuB (heme *a* and heme  $a_3$ -CuB) form a trinuclear center associated with mtDNA subunit I (COI). Electron flow is from cytochrome *c* to CuA, then to heme *a*, and then to the heme  $a_3$ -CuB binuclear

center, where oxygen is reduced to water (123,127,128). Molecular oxygen ( $O_2$ ) binds to the  $a_3$ -CuB binuclear center, and electron transfer to  $O_2$  is probably linked to proton transport. Complex IV uses four electrons to reduce  $O_2$  and four protons from the matrix to generate  $2H_2O$ . Four additional protons are transferred from the matrix, across the mitochondrial membrane, possibly in the proximity of heme  $a_3$  (129).

The three mtDNA-encoded subunits form the core of the monomer, with the nuclear-encoded subunits surrounding it. COI has 12 membrane-spanning domains (I–XII) that associate in three sets of four transmembrane helices forming three comma-shaped spokes radiating out from the center. COII has two transmembrane domains that lie on one side of COI, while COIII has seven transmembrane domains that lie on the opposite site of COI from COII. Finally, 7 of the 10 nDNA subunits (IV, VIa, VIc, VIIa, VIIb, VIIc, and VIII) have one membrane-spanning domain, which surrounds the core COI, COII, and COIII subunits in the membrane. The remaining three nDNA subunits (Va, Vb, and VIb) do not have transmembrane domains. Subunits Va and Vb lie on the matrix side and subunit VIb on the cytosolic side of complex IV.

Subunits II, VIa, and VIb appear to form the 25-Å diameter cytochrome *c* binding site, composed of acidic residues that interact with the basic residues of cytochrome *c* (123). The CuA coppers form a Cu–Cu bond and lie near the outside of the membrane, at the interface of COII and COI. The heme *a* and heme  $a_3$ -CuB centers are located 13 Å into the membrane. Heme *a* and heme  $a_3$  are close together, perpendicular to the membrane, and at a 104° angle relative to each other. Heme  $a_3$  is 4.5 Å from the magnesium atom and is located close to the trinuclear center, at the interface between COII and COI and ligated to both, directly between CuA and heme  $a_3$ . The zinc atom is associated with the nuclear-encoded subunit Vb on the matrix side of the enzyme, bound to cysteine residues (116,125,127,128).

Complex IV has two hydrophobic proton-conducting channels, D (Asp<sup>91</sup>) and K (Lys<sup>319</sup>), named after conserved amino acids that form the matrix sides of the channels. Channel D ends at the conserved Glu<sup>242</sup> in the middle of the membrane, which resides in a hydrophobic cavity extending toward the binuclear heme–copper center and is required for proton translocation. The K channel also ends at the binuclear center. Protons for both water formation and proton pumping move through these channels.

Proton pumping involves reduction of both metals ( $a_3$ -CuB) at the active site, with two electrons and two protons taken up. Oxygen then reacts, initially forming an oxygen intermediate. An additional electron is acquired, creating the peroxy state. Then three protons are acquired, generating the ferryl intermediate and creating an  $H_2O$  molecule and translocating two protons. A fourth electron is then accepted, resulting in a second water molecule and transport



of two additional protons. The overall reaction involves oxidation of four electrons, generation of two molecules of  $\text{H}_2\text{O}$ , and translocation of four protons (107,130).

While COIII is a universal component of all cytochrome c oxidase enzymes, its function is unclear. It does bind dicyclohexycarbodiimide (131).

The functions of the 10 nuclear-encoded subunits are still unclear. Two of the human subunits (VIa and VIIa) have two isoenzymes, one systemic and the other heart and muscle specific (125,132), that can be regulated at both the transcriptional and post-transcriptional levels (133). cDNAs have been cloned for all of the human COX genes (134–143) and the genes assigned to chromosomes (134,138,144–150).

A number of adenine nucleotide binding sites have been identified in complex IV, at least some of which can alter the kinetics of the enzyme. Five nucleotide binding sites have been found on complex IV: one between subunits I and III on the cytosolic side of the enzyme, two in subunit IV, one each on the cytosolic and matrix ends of the molecule; and two in subunit IVa, again cytosolic and matrix. In addition, three lower affinity binding sites have been detected for ADP (123,151,152). Nucleotide binding to the matrix side of subunit VIa changes the  $\text{H}^+/\text{e}^-$  stoichiometry, while nucleotide binding to the cytosolic domain of subunit IV affects the affinity of cytochrome c (151,153–156).

Complex IV is sensitive to a variety of common toxic chemicals. These include cyanide and azide, which form a bridge between cytochrome  $a_3$  and CuB, and thiocyanate and formate, which bind other locations in the binuclear center (157).

The complex structure of the respiratory complexes suggests that the assembly of active complexes requires the action of additional gene products. This has been shown to be the case for complex IV, where an additional protein, SURF-1, is required for generating normal human complex IV (COX) activity. Mutations in the *SURF1* gene have been found to be the primary cause of COX-deficient LS (158,159). *SURF1* is 25.6% homologous to the yeast gene *SHY1*. While the human *SURF1* mutation gives a specific COX defect, the yeast *SHY1* mutation is more pleiotropic. Hence, it has been suggested that SURF-1 may function in the assembly or maintenance of an active holoenzyme COX complex (158,159).

Mutations in the genes for two additional proteins involved in heme A biosynthesis, *COX10* and *COX15*, have also been found to cause complex IV deficiency. *COX10* mutations can cause LS or neonatal tubulopathy and encephalopathy (160,161), while *COX15* mutations cause fatal infantile hypertrophic cardiomyopathy or LS (162,163). Mutations in the *SCO1* and *SCO2* genes also result in complex IV deficiency. Both of these proteins are involved in inserting the copper atoms into the copper centers of complex IV (164–166).

**11.1.2.6 Complex V ( $\text{H}^+$ -Translocating ATP Synthase).** Complex V encompasses 16 polypeptides, 2 of which (ATPase6 and ATPase8) are encoded by the

mtDNA (60). This  $\text{H}^+$ -translocating ATPsyn utilizes the electrochemical gradient  $\Delta p = \Delta\psi + \Delta \text{pH}$  generated by complexes I, III, and IV to catalyze the condensation of ATP and  $\text{P}_i$  to make ATP. This enzyme uses a rotary catalytic mechanism that is close to 100% efficient.

The  $\text{F}_0$  component of the enzyme is based on the mitochondrial inner membrane, with the enzyme extending into the matrix in a lollipop shape involving a 45-Å long stalk and a 90- to 100-Å diameter barrel ( $\text{F}_1$ ). The  $\text{F}_1$  component, as isolated, is composed of five subunits— $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$ —with amino acid lengths of 510, 482, 272, 146, and 50 amino acids, respectively. The ratio of these subunits in the  $\text{F}_1$  particle is 3:3:1:1:1, with a total mass of about 370 kDa (71). Both the  $\alpha$  and  $\beta$  subunits are highly homologous (20% identical) and form a hexagonal array of alternating  $\alpha$  and  $\beta$  subunits. While both  $\alpha$  and  $\beta$  subunits bind nucleotides, only the  $\beta$  subunits are catalytic. The crystal structure for the  $\text{F}_1$  component shows that the  $\alpha$  and  $\gamma$  subunits form a hollow barrel into which the  $\gamma$  subunit projects up from the stalk to form an axle (167).

Based on the concept of rotary catalysis, complex V has been divided into two functional components: a “rotor” and a “stator” (168–170). The rotor consists of a hydrophobic wheel of 8 “c” (ATP9) subunits lying in the plane of the inner membrane that rotates with its axle extending into the matrix. The axle is composed of the  $\epsilon$  subunit at the base and the  $\gamma$  subunit projecting up into the  $\text{F}_1$  barrel. The stator is composed of a base made by the “a” subunit (ATP6) embedded in the membrane, adjacent to the c subunit wheel. The a (ATP6) subunit anchors a pair of “b” subunits that project out of the membrane and bind to the  $\delta$  subunit of  $\text{F}_1$ , which is attached to an  $\alpha$  subunit of the  $\alpha$ - $\beta$  barrel. Thus, the  $\text{F}_0$  subunit a (ATP6) is linked to the  $3\alpha:3\beta$  barrel in a static structure through the b subunits and  $\delta$ .

The stator subunit a (ATP6) and rotor subunit c (ATP9) interface mediates the proton flux from the intermembrane space to the matrix, thus driving the rotor’s rotation and causing the  $\gamma$  axle to spin within the  $\alpha$  and  $\beta$  subunit barrel. As the c subunit rotor spins past the “a” subunit stator, the rotor’s axle, ending in the  $\gamma$  subunit, rotates inside the hollow  $\alpha$  and  $\beta$  barrel. As it spins, the  $\gamma$  subunit makes two contacts with the  $\beta$  subunit, thus mediating the two components of the catalytic cycle. This generates sequential conformational changes in the  $\beta$  subunit nucleotide binding sites, which bind  $\text{ADP} + \text{P}_i$ , condense it to ATP, and release the ATP into the matrix (168,170).

The mechanism by which the proton motive force drives rotation of the subunit c (ATP9) rotor past the subunit a (ATP6) of the stator is highly conserved. However, the number of c subunit spokes in the wheel differs depending on the kingdom of the organism. All ATP synthases have a key negative charge in the c subunit in the middle of the membrane. This is Asp<sup>61</sup> of the c subunit of the bacterial enzyme and Glu<sup>58</sup> of the ATP6 subunit of the mammalian enzyme. This carboxylate group faces outward



from the wheel adjacent to the opposing face of the stator subunit “a” (ATP6) and also the hydrophobic core of the membrane’s lipid bilayer. The stator subunit a (ATP6) has an opposing positive charge, Arg<sup>210</sup> for the bacterial subunit c and Arg<sup>159</sup> for the mammalian subunit ATP6. This positive charge is offset 0.52 nm from the plane of carboxyl groups, such that it exerts electrostatic attraction against the Asp<sup>61</sup> of c or the Glu<sup>58</sup> of ATP9 without being capable of forming a salt bridge to stop rotation. The stator subunit c has two half-proton channels that penetrate half way into the membrane from opposite sides, but are offset from each other. One half-channel is open to the proton-rich intermembrane space, the other to the proton-poor (alkaline) matrix. In the presence of an electrochemical gradient, the half-channel open to the inner membrane space conducts in a proton to protonate the carboxyl group of subunit c (Asp<sup>61</sup>) or subunit ATP9 (Glu<sup>58</sup>), neutralizing its charge and permitting the subunit to rotate into the hydrophobic lipid bilayer without energetic inhibition. The protonated subunit c rotates all of the way around the wheel until it returns to the subunit a (ATP6) stator, where it encounters the other half-channel opening to the negatively charged and alkaline matrix. In this environment, the proton leaves subunit c (Asp<sup>61</sup>) or ATP9 (Glu<sup>58</sup>) and enters the matrix. The exposed negative charge of the Asp<sup>61</sup> of subunit c or the Glu<sup>58</sup> of ATP9 is then attracted to the displaced positive charge of the Arg<sup>210</sup> of subunit a or the Arg<sup>159</sup> of ATP6, causing the Asp<sup>61</sup> or Glu<sup>58</sup> carboxyl group of subunit c or ATP9 to rotate until it reenters the environment of the half-channel of the proton-rich intermembrane space, where it is again reprotonated (168,170–172). The stoichiometry of proton translocation to ATP synthesis differs between kingdoms because of differences in the number c polypeptides in the F<sub>0</sub> wheel. Animal mtDNAs have eight spokes in the wheel while other organisms can have up to 15 c peptides per wheel. Thus, for animals it takes eight protons to generate an ATP or 2.7 protons per ATP. For other organisms the gear ratio can be as low as 5 protons per ATP (107,173).

In the F<sub>1</sub> barrel, the three  $\alpha$  and three  $\beta$  subunits are capped by  $\beta$ -pleated sheets. Just below this cap is a hydrophobic ring forming a bearing in which the C-terminal end of the  $\gamma$  subunit rotates (167). Below the  $\beta$ -pleated sheet, and in the middle of the  $\alpha$  and  $\beta$  subunits, are the nucleotide binding sites. The conformational change and nucleotide binding sites of the  $\beta$  subunits are dynamic. The three  $\beta$  subunits sequentially go through three states: “O” or open, with very low affinity for ligands and catalytically inactive; “L” or loose, with the capacity to binding the ligands ADP and P<sub>i</sub>, but catalytically inactive; and “T” or tight, with the ligands tightly bound associated with catalysis. These three states are generated as the asymmetrical  $\gamma$  subunit interacts with the interior side of the  $\beta$  subunits (174).

The binding affinity of the  $\beta$  subunit for adenine nucleotides is modified by sequential changes in the  $\beta$  subunit conformation mediated by two “catches” that interact

with the rotating  $\gamma$  subunit. The “major catch” is a loop that protrudes into the interior of the 3 $\alpha$ :3 $\beta$  barrel. This is encountered as the  $\gamma$  subunit rotates from  $\beta$  TP (triphosphate, or tight) to  $\beta$ E (empty, or open). The  $\beta$ E conformation differs from the  $\beta$ TP and  $\beta$ DP (diphosphate, or loose) conformations in that part of the nucleotide binding site is rotated 30°. The “minor catch” separates  $\beta$ DP from  $\beta$ TP (167,175,176). These molecular interactions create a catalytic engine driven by elastic forces rather than thermal effects, which permits the near-100% efficiency (107,170).

A number of the nuclear-encoded complex V subunit cDNAs and genomic clones have been reported. The cDNAs include the ATP synthase  $\alpha$  subunit (ATPsyn $\alpha$ ) (177–179), ATPsyn $\beta$  (180,181), ATPsyn $\delta$  (182), F<sub>0</sub> subunit b (183), F<sub>0</sub> subunit F6 (184), and F<sub>0</sub> subunit 9 or c, which have three isoforms (185–189). The genomic clones include ATPsyn $\alpha$  (190,191), ATPsyn $\beta$  (192–194), and ATPsyn $\gamma$  (191,195,196), which generate two alternative messenger RNAs (mRNAs), heart-muscle and systemic (196). This is accomplished through the loss of exon 9 associated with the synthesis of a *trans*-acting factor that is inducible by high extracellular pH through a protein kinase C signaling mechanism (197).

The assembly of complex V requires the assembly protein ATP12. Mutations in the ATP12 gene have been associated with dysmorphic features, severe developmental delay, and seizures (198).

### 11.1.3 Adenine Nucleotide Translocator and Uncoupling Protein

The mitochondrial anion carrier protein family functions to transport solutes through the mitochondrial inner membrane. This group of related proteins has a tripartite repeating domain, with each domain composed of about 100 amino acids, all of which function as homodimers. This family includes two classes of carriers of particular relevance to bioenergetics, the ADP/ATP carriers or ANTs, and the uncoupling proteins (UCPs).

The ANT proteins are encoded by a small family of nDNA genes. They are multifunctional proteins that are responsible for the export of the ATP synthesized in the mitochondrial matrix by OXPHOS to the cytosol in exchange for ADP. The ANTs constitute up to 10% of the mitochondrial protein. It is thought that, as ATP binds to one of the subunits on the inside of the membrane, ADP binds to the other subunit on the outside, and the two adenine nucleotides are transported to the opposite side of the membrane, driven by the inner membrane electrochemical gradient. Each ANT monomer has six transmembrane helices and contains the consensus sequence RRRMMM, with the three Rs lying at the base of a conical well in which the adenine nucleotides bind (199).

Humans have four distinct ANT isoforms: a heart-muscle specific isoform, ANT1 (200–202); an inducible isoform, ANT2 (201,203); a systemic isoform, ANT3 (200,201); and a testis isoform, which is also expressed

at low levels in liver and brain (204). *ANT1*, which is expressed predominantly in heart and muscle (205), has four exons and a promoter with classical TATA and CCAAT elements (192,206–209). The *ANT1* gene maps to 4q35-qter, close to the FSHMD locus (210–212). *ANT2* is expressed at low levels in mature tissues, but is inducible in situations of metabolic stress and in association with increased cell proliferation (200,205,213–218). *ANT2*, which has been mapped to Xq24-q24, has the same four-exon structure as *ANT1*, a promoter with a canonical TATA and five potential Sp1 sites, but no CCAAT box (219–221). The growth regulation of *ANT2* is controlled by three of the Sp1 transcription factor binding sites: two adjacent CpG sites located together at –79 and –68 that act synergistically to induce expression following demethylation, and a third CpG site located between the TATA box and the transcriptional start site that functions as a negative regulator (222,223). *ANT2* also contains a glycolysis regulated box (GRBOX) at –1.2 kbp, a negative regulator in normal cells that is turned off in glycolytic and transformed cells (200). *ANT3* is expressed in most mature tissues as well as skin fibroblasts (205,218). It maps to Xp22.3 within the pseudoautosomal region, is expressed from both the X and the Y chromosomes, and has the classical four-exon structure. Its promoter lacks TATA and CCAAT elements, though it has potential Sp1 sites (207,224,225). In T cells, *ANT3* is regulated by either interleukin-4 or interferon- $\gamma$  through STAT6 and STAT1 (226). *ANT4* shares only 66–68% amino acid identity with the other three ANTs; yet it still retains the characteristic RRRMMM motif and exchanges ADP and ATP, though at a different kinetics. *ANT4* is located on chromosome 4q28.1 and contains six exons, instead of the usual four, and is expressed primarily in testis, though it is also significantly expressed in liver and brain (204).

In contrast to humans, the mouse has three *Ant* genes (*Ant1*, *Ant2*, and *Ant4*). The mouse *Ant1* gene maps to chromosome 8 (227,228), syntenic with human chromosome 4q35 and the human *Ant1* gene, and has the standard four exons and three introns (227). Mouse *Ant2* has been mapped to the mouse X chromosome outside the pseudoautosomal region, suggesting it is syntenic to human *ANT2* (229). No mouse homolog to the human *ANT3* gene has been isolated (227,229). The mouse homolog to human *ANT4*, designated *Ant4*, is 70.1% homologous to mouse *Ant1* and 69.1% homologous to mouse *Ant2* but 85.9% homologous to human *ANT4*. Moreover, both the mouse and the human *Ant4/ANT4* genes have the distinctive six-exon genomic structure. In adult mice, *Ant4* is expressed exclusively in testis, its mRNA being undetectable in adult heart, brain, spleen, lung, liver, skeletal muscle, or kidney by Northern blot. The *Ant4* promoter contains 47 CpGs that are extensively methylated in brain, kidney, and lung (230).

The ANTs/Ants differ in their ability to regulate the mtPTP and thus to induce apoptosis. Transformation

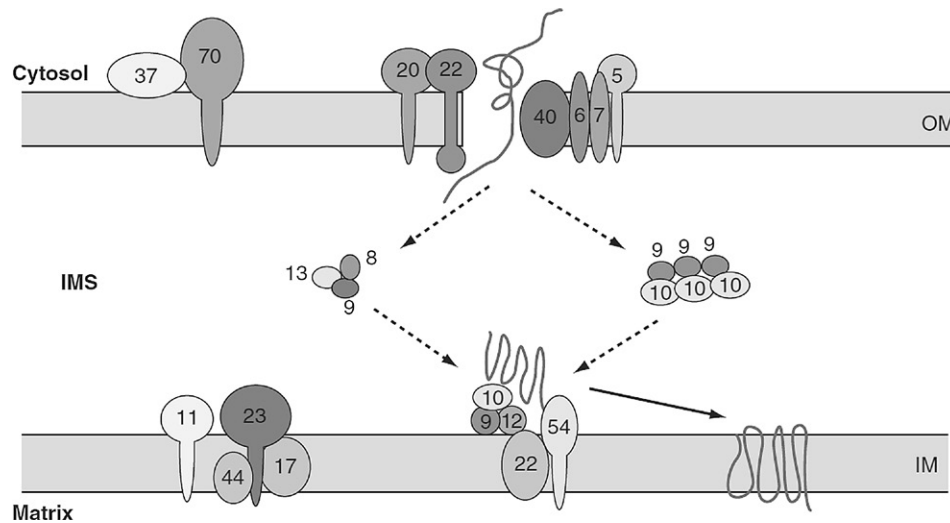
of cells with either the human *ANT1* or *ANT3* cDNA/gene, resulting in overexpression, strongly induces cells to undergo apoptosis. By contrast, transformation with the *ANT2* cDNA/gene does not induce apoptosis (231,232). Mitochondrial fractionation has also revealed that Ant1 is localized to the portion of the mitochondrial inner membrane in direct contact with the outer membrane and in association with hexokinase and thus the complete mtPTP. Ant2 is also distributed in the infolded cristae and is associated with mitochondrial creatine phosphokinase but not hexokinase. Hence, Ant2 is less associated with the mtPTP (233). The importance of the ANTs in regulating apoptosis has been dramatically demonstrated by their being targeted by viral peptides, presumably to regulate apoptosis (234,235). *ANT1* also binds the I $\kappa$ B $\alpha$ –NF- $\kappa$ B complex, which regulates the anti-apoptotic proteins Bcl-x<sub>L</sub>, MnSOD, and c-IAP2. *ANT2* does not interact with I $\kappa$ B $\alpha$ –NF- $\kappa$ B (236). Consequently, the upregulation of *ANT1* would reduce the available I $\kappa$ B $\alpha$ –NF- $\kappa$ B and drive the cell toward apoptosis.

The UCPs form a proton channel through the inner membrane, thus depolarizing the electrochemical gradient and uncoupling electron transport from ATP synthesis. Uncoupling protein-1 (UCP1) is primarily associated with brown adipose tissue, where it functions in thermal regulation. It is strongly induced by cold stress through a  $\beta$ -adrenergic response pathway (237–240). Uncoupling protein-2 (UCP2) has 59% amino acid identity to UCP1 and is widely expressed in adult human tissues, with mRNA levels being highest in skeletal muscle. It is also upregulated in white fat in response to an increased-fat diet. In the mouse, it has been linked to a quantitative trait locus for hyperinsulinemia and obesity (238). Uncoupling protein-3 (UCP3) is 57% identical to UCP1 and 73% identical to UCP2. UCP3 is also widely expressed in adult tissues, and at particularly high levels in skeletal muscle. Moreover, it is hormonally regulated, being induced in skeletal muscle by thyroid hormone and in white fat by  $\beta_3$ -adrenergic agonists, and also regulated by dexamethasone, leptin, and starvation. UCP3 is located adjacent to UCP2 in human chromosome 11q13 and mouse chromosome 7 (241–244).

### 11.1.4 Mitochondrial Protein Import

The mitochondrion is assembled from 13 mtDNA-encoded polypeptides and ~1000–2000 nuclear-encoded polypeptides. These polypeptides are synthesized on cytosolic 80S ribosomes and vectorially transported into the mitochondrion via receptor binding to the outer membrane and transfer through a mitochondrial inner membrane import pore. Once inside the mitochondrial matrix, the proteins are folded and assembled by the chaperone proteins mitochondrial heat shock protein 70 (mtHsp70) and mtHsp60 (245–249) (Figure 11-2).

The mitochondrial protein import apparatus encompasses a set of outer and inner membrane complexes. The Tom complexes function to transport proteins across the



**FIGURE 11-2** Proteins thought to be involved in the import of cytosolically synthesized polypeptides through the outer and inner mitochondrial membranes. The outer membrane Tom (transport across the outer membrane) complexes consist of two main receptor complexes and the general insertion pore (GIP) complex. Receptor complex Tom20 + Tom22 binds proteins with amino-terminal targeting peptides and feeds them through the GIP, of which Tom22 is thought to be a component. Tom37 + Tom70 are thought to bind proteins with internal targeting sequences and to transfer them to Tom20 + Tom22. The central channel protein of the GIP is thought to be Tom40; with Tom5, Tom6, and Tom7 also being important components. Proteins with amino-terminal targeting peptides destined for the matrix are transferred from the intermembrane space domain of Tom22 to the Tim complex. Tim23 is the central channel protein, and Tim44 binds to the preprotein as it emerges from the Tim23 channel on the matrix side. Anion carrier proteins destined for integration into the mitochondrial inner membrane, such as the ANT, follow a different pathway. As they emerge into the intermembrane space they interact with either of two soluble complexes (Tim8 + Tim9 + Tim13) or (3 Tim9 + 3 Tim10). These soluble complexes transport the protein through the intermembrane space to the other Tim complex, which is composed of Tim9 + Tim10 + Tim12 + Tim22 + Tim54. (Reprinted from (258), which was adapted from (255,1440).)

outer membrane, while the Tim complexes function to transport proteins across the inner membrane (250–252) (see Figure 11-2).

Proteins to be imported into the mitochondrion have embedded into their structure-specific mitochondrial targeting sequences. Most proteins to be imported into the mitochondrial matrix have an amino-terminal targeting peptide that is amphiphilic and basic (usually due to an excess of arginine residues). These amino-terminal targeting peptides are cleaved from the protein on import. Other proteins can have internal targeting sequences.

While in the cytosol, proteins destined for mitochondrial import interact with cytosolic chaperones. These include members of the cytosolic 70-kDa Hsp70 family, which bind to non-native proteins. In addition, there is at least one cytosolic chaperone specific for mitochondrial proteins. This is the heterochimeric mitochondrial import stimulating factor, which specifically binds matrix targeting signals. Both Hsp70 and mitochondrial import stimulating factor are ATPases (250–252).

The mitochondrial proteins, presumably maintained in a random conformation by the chaperone proteins, then interact with the receptor complexes on the mitochondrial outer membrane. Two receptor complexes have been identified: Tom20 + Tom22 and Tom37 + Tom70. Current thinking is that the Tom20 + Tom22 complex binds to proteins with amino-terminal targeting peptides. The Tom37 + Tom70 complex is thought to interact with proteins with internal targeting sequences, such as the inner membrane carrier proteins ANT and UCP. The Tom37 + Tom70 complex

then interacts with the Tom20 + Tom22 complex through a 34-amino acid “tetratricopeptide” motifs, and the Tom 20 + Tom 22 complex transfers the protein to the general insertion pore (GIP) (250–252).

The outer membrane GIP is probably composed of Tom40, Tom22, Tom5, Tom6, and Tom7 (253). Tom40 is deeply embedded in the outer membrane and forms a 22-Å hydrophilic transmembrane channel (254). Tom22 functions both as a key component of the receptor complex and an integral part of the GIP channel (250–253).

Once a protein traverses the outer membrane, it can either proceed through the inner membrane to the matrix or be inserted directly into the inner membrane. Proteins destined for the matrix with an amino-terminal targeting sequence are electrostatically attracted to the inner membrane import complex, consisting of Tim23, Tim11, Tim14, and Tim 17, and possibly Tim33. The Tim23 protein is central to this channel and, in the presence of  $\Delta\psi$ , it dimerizes, possibly providing a gating mechanism for the channel. On the matrix side, the Tim23, Tim11, Tim14, and Tim17 complex interacts with an inner membrane complex consisting of Tim44, mtHsp70, and the nucleotide exchange factor (MGE). Tim44 interacts with the preprotein as it emerges from the Tim23 pore and also binds mtHsp70. mtHsp70 then interacts with the preprotein and promotes its import, in conjunction with the hydrolysis of ATP. The interaction of Tim44 and mtHsp70 is regulated by adenine nucleotides and also by MGE, which regulates Tim44-mtHsp70 dissociation (245–252).

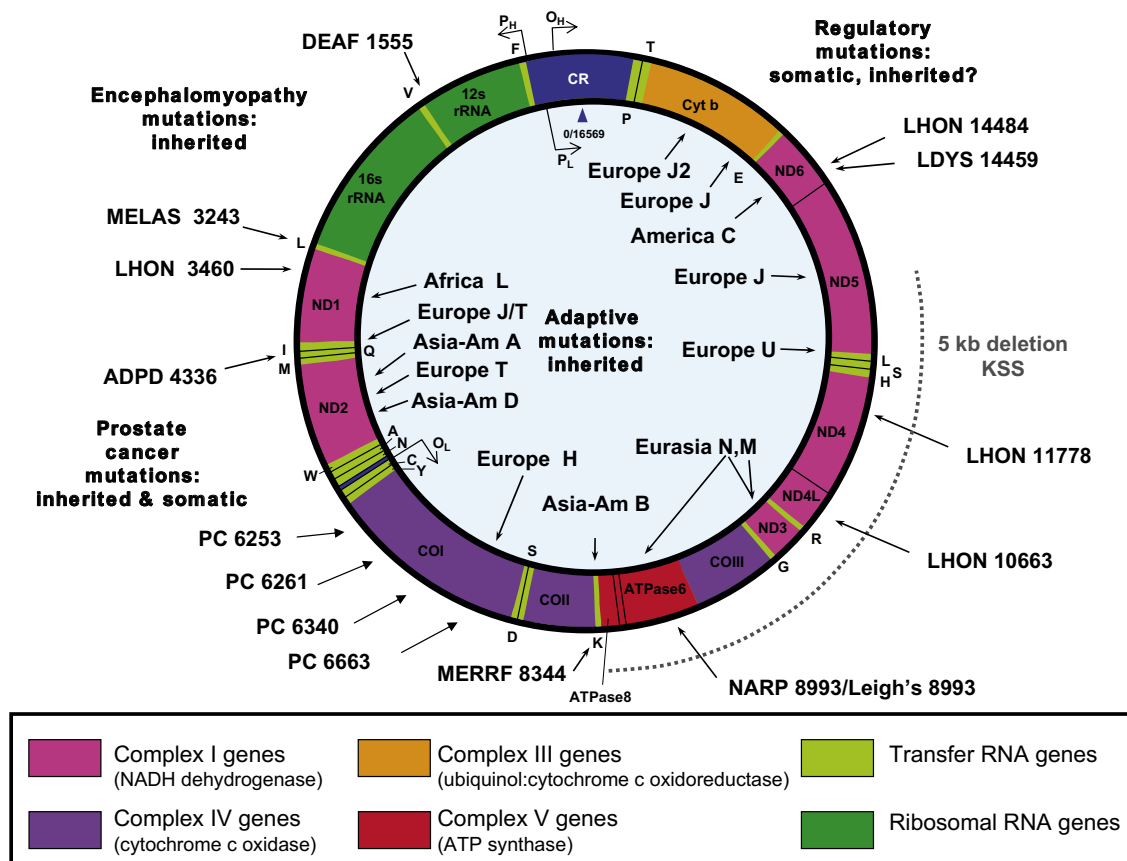
Once in the matrix, the targeting peptides are cleaved off by the mitochondrial processing peptidase, consisting of two subunits: the  $\alpha$  subunit and the catalytic  $\beta$  subunit. A subset of preproteins has a second cleavage by the mitochondrial intermediate peptidase. In the matrix, proteins are infolded by chaperone complexes (mtHsp70, MD5 and MGE, and Hsp60 and Hsp10). Mitochondrial cyclophilins, which are sensitive to cyclosporin A, can also act as folding catalysts (250–252).

Mitochondrial inner membrane transport proteins such as the ANTs and UCPs follow a different pathway. Once they enter the intermembrane space via the Tom complex, they interact with one of the two soluble complexes. One complex involves three Tim9 and three Tim10 subunits, while the other involves one each of Tim8, Tim9, and Tim13 polypeptides. These complexes deliver the carrier protein to a 300-kDa complex composed of Tim54, Tim22, Tim12, Tim10, and Tim9, bound to the outer face of the inner membrane. The polypeptides Tim8, Tim9, Tim10, Tim12, and Tim13 all

belong to the same gene family, which contains a distinctive duplicated C(N3) C motif reminiscent of a zinc finger (255–258).

### 11.1.5 Mitochondrial Genetics

Each human cell contains hundreds of cytoplasmic mitochondria. The mitochondrial genome encompasses 1000–2000 genes, 37 of which are encoded by the mtDNA while the remainder are dispersed across the nDNA chromosomes. While each cell contains one or two copies of each nDNA gene, the cell contains thousands of copies of the mtDNA. Because the mitochondrial genome encompasses two quite different and semiautonomous information storage and retrieval systems, the genetics of the mitochondrion is quite complex. Genetic phenotypes associated with mitochondrial dysfunction can involve Mendelian inheritance, cytoplasmic mtDNA inheritance, or an interaction between the two (Figure 11-3).



**FIGURE 11-3** Human mitochondrial DNA map showing representative pathogenic and adaptive base substitution mutations. D-loop = control region (CR). Letters around the outside perimeter indicate cognate amino acids of the tRNA genes. Other gene symbols are defined in the text. Arrows followed by continental names and associated letters on the inside of the circle indicate the position of defining polymorphisms of selected region-specific mtDNA lineages. Arrows associated with abbreviations followed by numbers around the outside of the circle indicate representative pathogenic mutations, the number being the nucleotide position of the mutation. Abbreviations: DEAF, deafness; MELAS, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; LHON, Leber hereditary optic neuropathy; ADPD, Alzheimer and Parkinson disease; MERRF, myoclonic epilepsy and ragged red fiber disease; NARP, neurogenic muscle weakness, ataxia, retinitis pigmentosum; LDYS, LHON + dystonia; PC, prostate cancer. (Data from (1).)



### 11.1.6 Mitochondrial DNA Genes and Genetics

The cytoplasmic location and high copy number of the mitochondria result in the mtDNA having a unique genetics. The mtDNA is a 16,569-nucleotide pair (np), closed circular molecule, located within the mitochondrial matrix. It codes for 13 key polypeptides of OXPHOS as well as the structural RNAs of mitochondrial protein synthesis, including the small (12S) and large (16S) rRNAs as well as 22 transfer RNAs (tRNAs) (see [Figure 11-3](#)). These 22 tRNAs interpret the mitochondrial genetic code through the use of modified wobble codon rules. Certain mitochondrial tRNAs also have modified anticodons that alter the mitochondrial genetic code. In particular, the anticodon of the tryptophan tRNA is modified so that it reads both the normal tryptophan codon (UGG) and the opal stop codon (UGA). Similarly, the methionine tRNA recognizes both AUG and AUA as methionine. Finally, the “universal” arginine codons AGA and AGG are utilized as stop codons in the mtDNA ([259,260](#)).

The 13 polypeptides of the mtDNA include 7 (ND1, 2, 3, 4, 4L, 5, and 6) of the 45 subunits of complex I, 1 (cytochrome *b*) of the 11 subunits of complex III, 3 (COI, II, and III) of the 13 subunits of complex IV, and 2 (ATP6 and 8) of the 16 subunits of complex V. The mtDNA also contains an approximately 1121-np control region that encompasses the heavy (H)- and light (L)-strand promoters ( $P_H$  and  $P_L$ ) and the H-strand origin of replication ( $O_H$ ). The L-strand origin ( $O_L$ ) is located on the other side of the circle in a cluster of five tRNA genes (WANCY).

The most widely accepted model for mtDNA replication is that DNA replication initiates at  $O_H$  using an RNA primer generated from the L-strand transcript. The L-strand transcript initiates at the adjacent  $P_L$  and is cleaved by the nuclear-encoded mitochondrial RNase processing RNase at runs of G nucleotides in the conserved sequence blocks CSBIII, CSBII, and CSBI, primarily after CSBI ([261–264](#)). The resulting 3'-OH is utilized by DNA polymerase  $\gamma$  as a primer to synthesize a 7S DNA. The 7S DNA ends at the termination associated sequence at the end of the control region. The termination associated sequence binds to a specific factor that may regulate this replication pause site ([265](#)). This newly synthesized region of the H-strand displaces the parental H-strand to create the displacement loop (D-loop) ([265,266](#)). The 7S DNA is subsequently utilized as a primer to synthesize a new H-strand. From the 7S DNA, H-strand synthesis proceeds two-thirds of the way around the mtDNA, displacing the parental H-strand until it reaches the  $O_L$ . Once exposed on the displaced H-strand,  $O_L$  folds into a stem-loop structure and L-strand synthesis initiates and proceeds back along the H-strand template. Consequently, mtDNA replication is bidirectional but

asynchronous ([267](#)). However, recent studies have suggested that the mtDNA may also replicate bidirectionally and thus symmetrically from alternative origins ([268–270](#)).

mtDNA transcription initiates from the two promoters in the control region, one for each strand:  $P_L$  and  $P_H$ .  $P_H$  is responsible for transcribing the 2 rRNA genes, 13 tRNA genes, and 12 of the protein coding genes.  $P_L$  transcribes the ND6 protein gene and nine tRNAs, and also generates the primers for H-strand replication at  $O_H$  ([11](#)). Both promoters are associated with a binding site for a nuclear-encoded mitochondrial transcription factor (Tfam). Tfam is a high-mobility-group DNA-binding protein with two DNA-binding domains and a carboxy-terminal tail essential for transcription ([271–274](#)). It binds with higher affinity to  $P_L$  than  $P_H$ , consistent with their relative transcription frequencies ([275,276](#)). Tfam also binds with a 40- to 50-base periodicity throughout the D-loop, with CSBII and CSBIII being unbound and CSBI being strongly bound. The Tfam phasing downstream from CSBI on the H-strand DNA corresponds to DNA synthesis initiation sites, suggesting that Tfam may help define the transitions between RNA and DNA ([277](#)).

Transcription from both promoters proceeds around the mtDNA circle, creating a polycistronic RNA. The tRNA genes, which punctuate the larger rRNA and mRNA sequences, then fold within the transcript and are cleaved out by an RNaseP-like activity. The freed mRNAs and rRNAs are post-transcriptionally polyadenylated; and the tRNAs are modified and the 3' terminal CCA is added ([278–281](#)).

The rRNAs are present in about a 50:1 ratio with the mRNAs. This differential transcription is accomplished in part by a transcriptional terminator (5'-TGGCAGAGCCCCGG-3') located within the tRNA<sup>Leu(UUR)</sup> gene, immediately downstream from the 16S rRNAs in the direction of H-strand transcription from  $P_H$ . This terminator is bidirectional, thus accounting for the reduction in read-through of the H-strand promoter as well as the termination of the L-strand transcripts prior to its reaching the rRNA genes ([275,282,283](#)).

The mtDNA mRNAs are translated on mitochondrial 55S ribosomes (mitoribosomes) composed of a large 39S and small 28S subunit. These ribosomes have a smaller amount of rRNA than bacterial or eukaryotic ribosomes but a larger number of ribosomal proteins ([284–286](#)). mtDNAs mRNAs lack the traditional Shine-Dalgarno sequence for ribosome binding and generally start with the initiation codon at the 5' end. Translation is thought to initiate with the binding of the small subunit to a 40-base region of the mRNA. The ribosome then moves back to the 5' end to initiate translation. The mRNA has been proposed to wrap around the ribosome, thus limiting polysome formation ([259,287](#)).

The mitoribosomes are sensitive to the bacterial ribosome inhibitor chloramphenicol (CAP), but resistant to the cytosolic 80S ribosome inhibitors cycloheximide and emetine. They are also relatively insensitive to the aminoglycoside antibiotics such as streptomycin and gentamycin except in individuals harboring certain 12S rRNA mutants which markedly increase aminoglycoside sensitivity (260).

### 11.1.7 Mitochondria Are Semiautonomous

Because the mitochondria retain their own self-replicating genome and associated replication, transcription, and translation systems, they behave as semiautonomous organisms within the human cell cytoplasm. This was first demonstrated through studies of cultured mammalian cells selected for resistance to the mitochondrial ribosome inhibitor CAP. CAP-resistant (CAP<sup>R</sup>) cells were enucleated by treatment with cytochalasin B to disaggregate the cytoskeleton and suspension in a centrifugal field to pull the dense nucleus from the cells, thus creating plasma membrane-bound nuclear (karyoplast) and cytoplasmic (cytoplast) fragments, the latter encapsulating the mitochondria and mtDNAs. The CAP<sup>R</sup> cytoplasts were then fused to CAP-sensitive (CAP<sup>S</sup>) cells and the resulting cytoplasmic hybrids, or cybrids, were shown to acquire the CAP<sup>R</sup> genotype and phenotype (288–290). By this cybrid transfer procedure, CAP resistance could be transferred from cell to cell and was later linked to restriction fragment length polymorphisms in the mtDNA (291) as well as to mtDNA-encoded protein polymorphisms (56). Subsequent sequencing of CAP<sup>R</sup> mtDNAs revealed that CAP resistance was due to single nucleotide changes in the peptidyl transferase region of the 16S rRNA (MTRNR1) near the 3' end: a T-to-C transition at np 2991 or a C-to-A transversion at np 2939 (292).

Cybrid transfer of mtDNAs can be facilitated by removing the mtDNAs of the recipient cell prior to fusion. This can be accomplished by fusing the cytoplast to a karyoplast to yield a reconstituted cell (293), by destroying the resident mitochondria by treating the recipient cell with the mitochondrial poison rhodamine-6G (294,295), or by curing the recipient cell of its resident mtDNAs by growth in the mtDNA replication inhibitor ethidium bromide (296). Cells that have been cured of their resident mtDNAs using ethidium bromide are designated  $\rho^0$  cells. They become auxotrophic for uridine (297) and require high glucose levels for energy and pyruvate to maintain redox balance through oxidation of NADH + H<sup>+</sup> via lactate dehydrogenase to give lactate (298). The  $\rho^0$  recipient cells have provided a powerful tool for studying the biochemical basis of mtDNA disease mutations by permitting demonstration that a specific biochemical defect in a patient cell line can be transferred along with the patient mtDNAs to a  $\rho^0$  cell having a different set of nuclear genes (298–302).

### 11.1.8 Mitochondrial DNAs Are Maternally Inherited

The human mtDNA is strictly maternally inherited (303,304). This is due in large measure to the fact that the mammalian egg contains about 100,000 mitochondria and mtDNAs, while the sperm contains on the order of 100 mtDNAs (305,306). The sperm mtDNAs are contributed to the zygote at fertilization and will persist in interspecific crosses (307). However, in intraspecific crosses, the sperm mitochondria are selectively eliminated (308). This has been correlated with the discovery that the sperm mitochondria prohibiting protein are decorated with ubiquitin, which appears to mark them for degradation on entrance into the oocytes (309,310). This destruction of sperm mitochondria and mtDNAs can also be demonstrated by injecting sperm into  $\rho^0$  somatic cells (311).

### 11.1.9 mtDNA's High Mutation Rate

The mtDNA mutation rate is much higher than that of nuclear genes (312). Comparison of the sequence diversity of mtDNA and nDNA genes that function in the same enzymes indicates that mtDNA genes evolve about 10–17 times faster than nDNA genes (181,202,313). However, the mtDNA genes are also highly conserved and critical for life. Therefore, a high mtDNA mutation rate would be expected to create a high genetic load which would ultimately result in the decline in the fecundity of the species. This problem is resolved in that the mammalian ovary has a selective system that eliminates the proto-oocytes with the most severe mitochondrial defects prior to ovulation (314,315). This pre-fertilization selection is possible for the mitochondrion since respiratory defects are manifest at the cellular level so selection can occur before the generation of a complete individual. As a result, mtDNA variants that mildly perturb mitochondrial bioenergetics are continuously being introduced into the population. While most of these still cause significant mitochondrial defects and disease (1), a subset of the variants can be beneficial in certain environments and can become enriched in that context through the action of adaptive selection. Hence, the high mutation rate, tempered by intra-ovarian selection provides a power system for permitting intra-specific adaptation to changing environments. By contrast, since anatomical mutations can only be presented to selection after the individual has been conceived and gone through development, all anatomical mutations are introduced into the population. Hence, nDNA anatomical mutations impart a high genetic load and, thus, nDNA mutation rates must be kept low (316). Therefore, the high mtDNA mutation rate, coupled to pre-fertilization selection, means that the mtDNA is constantly generating potentially adaptive mutations; so mtDNA mutations provide the genetic diversity that allows individuals within a species to adapt to changing environmental conditions (317).

Still, some of the mtDNA mutations are deleterious in particular environmental contexts. New female germline mtDNA mutations can either result in base substitution or rearrangement mutations ([www.mitomap.org](http://www.mitomap.org)) (318). Rearrangement mutations and base substitutions that disrupt an important mitochondrial function are deleterious and are removed by natural selection as disease. Base substitution mutations could also be neutral, which can be retained or lost in the general population by genetic drift, or be advantageous in particular environments, where they can become established in the specific populations by adaptive selection. The high mtDNA mutation rate might explain why animals retain mtDNA recombination (319), while prohibiting inter-mtDNA lineage recombination by strict maternal inheritance. Current evidence suggests that the 13 mtDNA polypeptides are critical for OXPHOS electron and proton transport. Since all of the components of OXPHOS must work together to provide an integrated energy system, strict maternal inheritance ensures that each new mutation is added sequentially to the pre-existing optimized mtDNA genotype. If it is deleterious to mitochondrial energetics, the mutation will be eliminated by selection. However, if advantageous, it may permit the population to survive and expand. Since the high mtDNA mutation rate would frequently result in deleterious mutations occurring on the same mtDNA as advantageous mutations, good mutations would be lost with the bad. The presence of recombination within the female germline would randomly distribute the deleterious and advantageous mtDNA mutations so that they could segregate independently (319).

New mutations in the mtDNA not only accumulate along the female germline, they also arise as mtDNAs replicate within the cells of the body, particularly in the post-mitotic tissues, where the absence of cytokinesis inhibits the segregation of the mutant mtDNAs (320,321). Thus, human mitochondrial genetics involves the interaction between the systemic deleterious, neutral, and adaptive germline mutations and the age-related accumulation of somatic mtDNA mutations.

### 11.1.10 Heteroplasmy and Replicative Segregation

When a mutation arises in a cellular mtDNA, it creates a mixed intracellular population of mutant and normal molecules known as heteroplasmy. When a cell divides, it is a matter of chance whether the mutant mtDNAs will be partitioned into one daughter cell or another. Thus, over time, the percentage of mutant mtDNAs in different cell lineages can drift toward either pure mutant or normal (homoplasmy), a process known as replicative segregation. In somatic cell hybrids between transformed human cell lines, the direction of segregation appears to be random (322). However, in crosses between HeLa cells and either diploid fibroblasts (323–325) or lymphoblastoid cell lines (290), the HeLa mtDNAs are

preferentially lost, even when selected for using CAP resistance. The reason for this directional replicative segregation is unknown. However, it may have clinical significance since it has been reported that, in five of seven  $\rho^0$  cybrids heteroplasmic for the pathogenic tRNA mutation MTTL1\*MELAS3243G (326), the wild-type mtDNAs were selectively lost and the mutant mtDNAs preferentially retained (327). This directional segregation may also involve nuclear genes. In another study, using the same osteosarcoma 143B TK<sup>-</sup>  $\rho^0$  recipient cell, 1 of 14 heteroplasmic cybrids segregated toward mutant mtDNAs, while the others remained stable. By contrast, with a different  $\rho^0$  recipient cell (A549, B2), 5 of 25 heteroplasmic cybrids segregated to wild-type mtDNAs (328). Preferential growth of cells with wild-type mtDNAs has also been observed during cybrid clone isolation (329) and in propagation of heteroplasmic fibroblasts (330). Hence, the rules governing the directionality of mtDNA segregation and the factors that influence it still remain to be elucidated.

### 11.1.11 Threshold Expression

As a result of the energy anatomy of the body, the different tissues and organs rely on mitochondrial energy generation to different extents. This has been well established through studies of respiratory inhibitor toxicity in mammals, including primates. Acute respiratory toxicity can result in unconsciousness, while chronic toxicity can result in optic atrophy, basal ganglia degeneration, and cardiac and renal failure (331). Studies on maternal pedigrees harboring heteroplasmic mtDNA mutations indicate that, as the percentage of mutant mtDNAs increases, energy production declines. Ultimately, the percentage of mutant mtDNAs crosses a threshold resulting in clinical manifestations. Observations from many cases now indicate that the bioenergetic expression thresholds for human organs are, in decreasing order, the central nervous system, heart and skeletal muscle, renal system, endocrine system, and liver (5,6,332,333). The pathophysiologic basis of these organ thresholds is still unclear. However, current data suggest that three major processes of the mitochondria are relevant: decline in energy production, increase in ROS production, and initiation of apoptosis, which eventually removes sufficient cells from the organ that the organ malfunctions, resulting in disease.

While the mechanism for threshold expression may vary in different contexts, genetic studies have repeatedly shown that the difference in mitochondrial function between normal and defective cells can be quite small. In somatic cell hybrids between CAP<sup>R</sup> and CAP<sup>S</sup> cells, as little as 10% CAP<sup>R</sup> mtDNAs are required for the cell to survive and grow in CAP (322). Similarly, in diseases resulting from mtDNA tRNA mutations, the percentage of mutant mtDNAs must be high before the biochemical and clinical expression threshold is exceeded. In families



harboring the MTTK\*MERRF8344G mutation (5), it has been observed that individuals in the 20- to 40-year age range require at least 85% mutant mtDNAs for symptoms to appear (5,6,334). This is consistent with data from cultured somatic cells in which the percentage of MTTK\*MERRF8344G or MTTLI\*MELAS3243G mutant mtDNAs must exceed 90% before both protein synthesis and O<sub>2</sub> consumption decline (335).

Part of the threshold effect is explained by “respiratory control theory.” It has been established that certain of the respiratory complexes can be strongly inhibited without reducing the respiration rate. This results from the fact that the various respiratory complexes have different degrees of excess respiratory capacity, relative to the overall electron flux rate through the respiratory chain. For example, complex I activity must be reduced by more than 70% before oxygen consumption or ATP production is perturbed (300,336,337). Hence, a mutation in an mtDNA complex I subunit would have to increase to a relatively high percentage of the cellular mtDNAs before it would significantly impede mitochondrial respiration and energy production.

### 11.1.12 Mitochondrial Dynamics and mtDNA Complementation

The mitochondria are high dynamic organelles undergoing repeated rounds of fission and fusion. This dynamic process can be linked to the activation of the mtPTP, apoptosis, and the turnover of mitochondrial by autophagy (mitophagy). The first demonstration the mitochondria must fuse came from a complementation experiment between two different mitochondrial when mixed in somatic cell hybrids. Pairs of human cells were fused together in which one harbored an mtDNA in which the mtDNA CAP<sup>R</sup> mutation was linked to an ND3 protein polymorphism (MV-1) and the other cell carried a CAP<sup>S</sup> mtDNA linked to an ND3 allele with a different electrophoretic mobility (MV-2). When the mitochondrial translation products of the hybrids were selectively labeled by growth in <sup>35</sup>S-methionine together with emetine to block cytosolic protein synthesis, both MV-1 and MV-2 were synthesized in proportion to the percentage of the two mtDNAs. Moreover, both proteins continued to be labeled when the hybrids were grown in <sup>35</sup>S-methionine in the presence of both emetine and CAP. This revealed that the CAP<sup>R</sup> mtDNA ribosomes were able to translate the ND3 mRNAs from the CAP<sup>S</sup> mtDNAs in *trans*. Hence, the ribosomes of the CAP<sup>R</sup> mtDNA must occupy the same mitochondrial compartment as the CAP<sup>S</sup> mtDNA-linked MV-2 mRNAs demonstrating the mitochondria of the two parental cells must have coalesced following cell fusion (56).

Intracellular mtDNA complementation has also been demonstrated in cells heteroplasmic for the pathogenic tRNA mutation MTTLI\*MERRF8344G. In cybrids derived from heteroplasmic cells, as little as 10% of normal

mtDNAs were able to restore oxygen consumption and mitochondrial protein synthesis. However, when essentially homoplasmic MTTLI\*MELAS3243G genomes were mixed with homoplasmic MTTK\*MERRF8344G genomes by two-step cybrid selection, complementation was not observed for either oxygen consumption or mitochondrial translation (335).

Mitochondrial complementation is now understood at the cellular level via the processes of fission and fusion. A number of the genes important in these processes have been cloned. Fusion permits the complementation of defects between different mitochondria and mtDNAs (338). Mitochondrial fusion is mediated by mitofusin 1 and 2 (Mfn1 and Mfn2) and OPA1 (optic atrophy 1 protein) while fission is mediated by Fis1 and Drp1 (dynamin-related protein 1) (339). Mitochondrial fusion mediated by Mfn1 and Mfn2 is modulated by the formation of high-molecular-weight GTP-dependent complexes (340), and the genetic inactivation of both Mfn1 and Mfn2 leads to severe mitochondrial defects, including poor growth, heterogeneity in mitochondrial membrane potential, decreased respiration (341), and increased apoptosis (342).

Fission is essential to separate mitochondria for transport throughout large cells like neurons (338,339,343,344). Fission is also modulated by the Bax-Bcl-2 family of proteins (345,346) and permits isolation of mutant nucleoids in mitochondrial vesicles where the mtDNA can be expressed and if defective result in a reduced membrane potential signaling removal by mitochondrial autophagy, mitophagy.

Mitophagy is initiated by the mitochondrial membrane protein PINK1 which is degraded in normal mitochondrial but stabilized in the mitochondrial outer membrane in mitochondria with lowered membrane potential (347). PINK1 phosphorylates the cytosolic protein parkin, attaching it to the mitochondrion (348) where it ubiquitinates mitofusins 1 and 2 (349) and activates a mitochondrial ubiquitin-proteasome system (350). The ubiquitinated mitofusins attract the adaptor protein p62/SQSTM1 (351) which recruits the mitophagy apparatus including the microtubule-associated protein 1 light chain 3 (LC3) protein and Atg autophagy proteins to engulf the mitochondria in an autophagosome preparatory to degradation (345,346,352–354).

### 11.1.13 mtDNA Recombination

It is now clear that mtDNAs within a mitochondrion can recombine. While recombination in cultured mammalian cells is infrequent (355), evidence for the interconversion of duplicated and deleted mtDNA molecules with the same breakpoint junction within patient tissues has suggested that intramolecular recombination may be relatively frequent (356). Analysis of mouse L cell lines has revealed that various sublines of LA9 cells harbored functional mtDNA mutations present at high



percentages of heteroplasmy. Analysis of the mtDNAs of these cell lines has revealed that different cell lines have different combinations of the same mutations. A reconstruction of the chain of events that led to the extant cell line mtDNA genotypes has revealed that the various combinations of alleles must have arisen by at least three independent recombination events (319).

While intracellular mtDNA recombination has been confirmed, extensive analysis of human mtDNAs from populations containing multiple different mtDNA lineages has failed to reveal a bona fide example of recombination between two very different mtDNA lineages (357). Consequently, the mtDNA haplotypes from different populations exhibit a very high degree of linkage disequilibrium throughout the mtDNA (358–361). While some analyses have purported to demonstrate the loss of linkage disequilibrium within mtDNA lineages (362), subsequent analysis has failed to confirm these conclusions (363–365). Since intracellular recombination does occur, the absence of recombination between mtDNA lineages implies that the mixing of mtDNAs between lineages is prohibited. This conclusion has been confirmed by the demonstration that sperm mitochondrial are ubiquitinated (366). These mitochondrial are then degraded by mitophagy on entry into the oocyte (366).

#### 11.1.14 Energy Anatomy and Mitochondrial Seasonal Metabolism

To understand the functional significance of the deleterious and adaptive mtDNA mutations, it is necessary to understand the energetic functions of the different human tissues, the human energy anatomy. Tissues rely on mitochondrial energy to different extents, which markedly affects their responses to mutations in mitochondrial genes. Human and mammalian organs can be divided into four energetic categories:

- The *energy-utilization* tissues, including the brain, which burn carbohydrates and ketone bodies; the skeletal muscle, which metabolizes carbohydrates and fats; the heart, which burns primarily fat; and the kidney
- The *energy-storage* tissues, including white adipose tissue (WAT) for energy storage and brown adipose tissue (BAT) for thermal regulation
- The *energy-homeostasis* tissue: the liver, which maintains serum glucose levels
- The *energy-sensing* tissues: the alpha and beta cells of the pancreatic islets of Langerhans, which monitor the availability of plant calories via serum glucose levels and regulate energy metabolism accordingly, shifting between glycolysis and OXPHOS

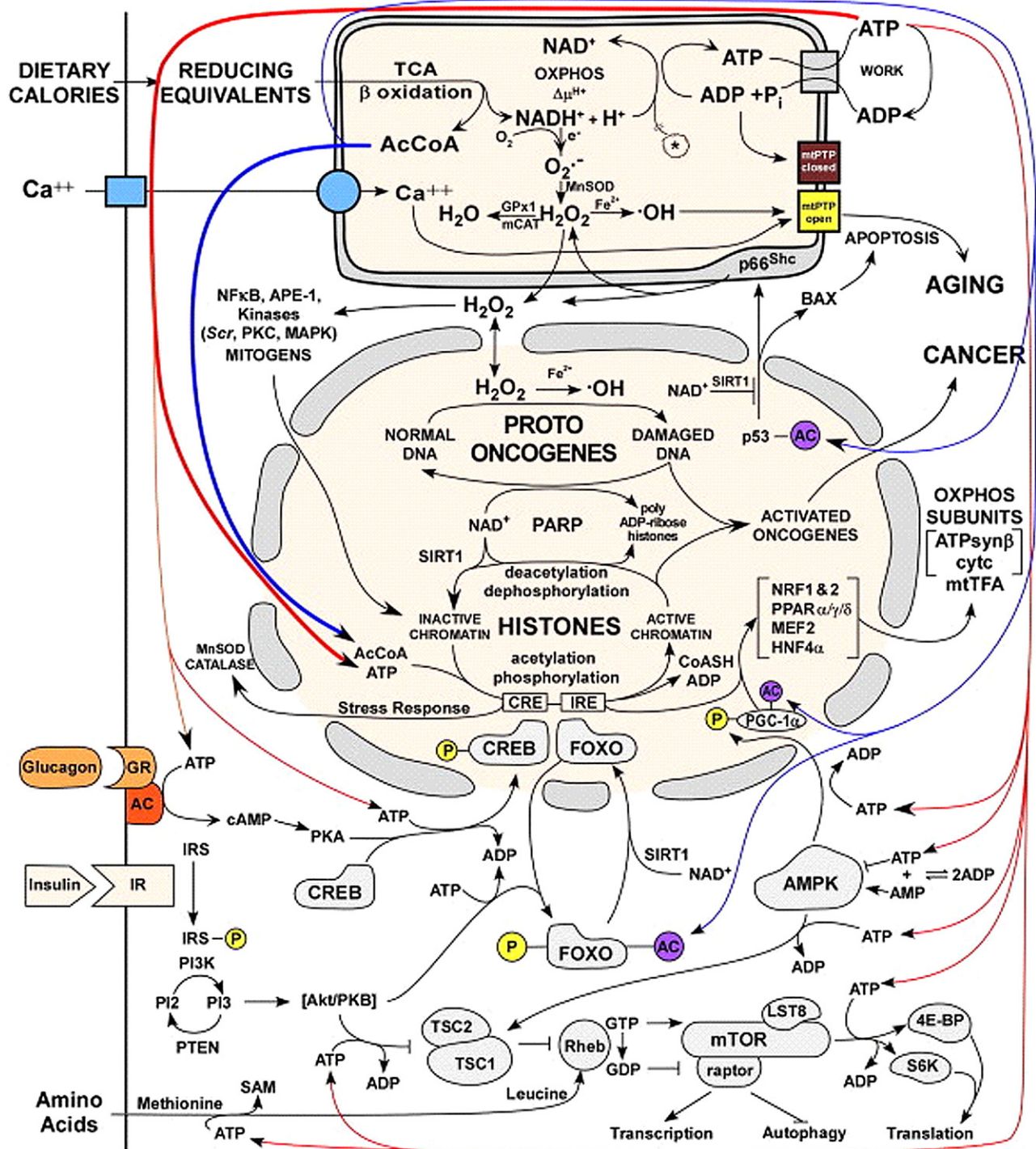
All of these tissues interact to coordinate the utilization and storage of energy, based on the availability of calories in the environment. For our hunter–gatherer

ancestors, the primary variation in available dietary calories was due to the cyclic growing seasons of edible plants caused by either warm versus cold or wet versus dry seasons. During the growing season, plants convert the sun's energy into glucose, which the plants store as starch. When humans ingest these plant tissues, the concentration of glucose in their blood rises.

The elevated serum glucose is detected by the energy-sensing pancreatic beta cells, which respond by secreting insulin into the blood stream. The insulin signal then informs the energy-utilizing tissues to downregulate mitochondrial energy utilization, thus relying more heavily on glycolysis, and to store the excess calories as high-energy fat in the WAT and BAT. As the growing season of the plants ends, the availability of plant carbohydrate calories declines. This results in the decline of animal blood glucose levels. In response, the pancreatic beta cells reduce insulin production and the pancreatic alpha cells increase glucagon production. These hormonal changes signal the upregulation of mitochondrial OXPHOS in the energy-utilizing cells and the mobilization of the fats stored in the WAT to fuel mitochondrial OXPHOS in the energy-utilizing tissues. Low blood glucose also stimulates the liver to synthesize glucose to maintain the basal blood sugar level, which is particularly critical for brain metabolism.

When blood glucose is high and the beta cells secrete insulin, the insulin binds to insulin receptors on target cells throughout the body. Insulin binding to the receptors activates the receptor tyrosine protein kinase to phosphorylate insulin receptor substrates. These activate phosphatidylinositol-3-kinase (PI3K), which phosphorylates phosphatidylinositol 2-phosphate to phosphatidylinositol 3-phosphate (PI3). PI3 then activates the AKT 1/2 kinases (protein kinase B, PKB), which phosphorylate the FOXO forkhead transcription factors FOXO1, FOXO3A, and FOXO4 in the target tissues. Phosphorylation of the FOXOs results in their transport out of the nucleus and the transcriptional inactivation of genes whose promoters contain insulin response elements (IRE) (367,368). The FOXOs can also be removed from the nucleus by acetylation via Cbp or p300 (367) and reactivated by deacetylation via the NAD<sup>+</sup>-dependent SIRT1 (sirtuin 1) (369).

When blood sugar is low, insulin levels decline, the insulin receptor shuts down, AKT 1/2 is inactive, and the FOXOs become dephosphorylated. The unphosphorylated FOXOs enter the nucleus, bind to the IREs, and upregulate transcription of the associated genes. Low blood glucose also stimulates the pancreatic alpha cells to secrete glucagon, which binds to the glucagon receptors in target cells. This stimulates the production of cyclic AMP (cAMP), which activates protein kinase A (PKA). PKA phosphorylates and activates the cAMP response element binding protein (CREB), which activates transcription of genes harboring a cAMP response element (CRE) (370) (Figure 11-4).



**FIGURE 11-4** Mitochondrial regulation of energy flow and its impact on the cellular epigenome and signal transduction pathways mediated by high energy mitochondrial intermediates. Energy is derived from reducing equivalents contained in dietary calories. Carbohydrates and fatty acids are converted to acetyl-CoA, NADH+H<sup>+</sup> and FADH<sub>2</sub> and these are oxidized with oxygen within the mitochondrion. The reducing equivalents of NADH+H<sup>+</sup> and FADH<sub>2</sub> are oxidized by the mitochondrial ETC to generate the mitochondrial electrochemical gradient ( $\Delta P = \Delta \Psi + \Delta \mu^{H^+}$ ) and  $\Delta P$  is utilized by the ATP synthase to generate ATP. Acetyl-CoA and ATP are exported from the mitochondrion and these high energy intermediates are used to modify the proteins chromatin and the cellular signal transduction pathways, thus coupling mitochondrial energy flux to the regulation of the epigenome. The source of the available calories modulates the systems by which the calories are processed based on signals from the pancreatic islets of Langerhans. High carbohydrates activate the  $\beta$  cells to secrete insulin. This binds to the cellular insulin receptors, which act through the PI3K-PTEN-Akt-FOXO pathway to shift energy metabolism away from oxidation and toward glycolysis. By contrast, low carbohydrate diets activate the  $\alpha$  cells to secrete glucagon. This binds to the glucagon receptors which act through the cAMP-PKA-CREB pathway to shift energy metabolism toward oxidative phosphorylation, in significant measure through the induction of PGC-1 $\alpha$ . SIRT1 fine-tunes the interrelationship between energy metabolism and apoptosis through the deacetylation of PGC-1 $\alpha$ , p53, and the histone proteins. Caloric overload or inhibition of OXPHOS perturbs the cellular mitochondrial energetic balance resulting in increased ROS. The increased ROS and decreased mitochondrial energy output sensitizes the mtP, ultimately driving the cell to apoptotic death. The increased ROS also diffuses into the nucleus as H<sub>2</sub>O<sub>2</sub> where it can mutate and activate proto-oncogenes (initiation) and can interact with NF- $\kappa$ B, APE-1 and various kinases to initiate cell division (promotion) leading to neoplastic transformation (cancer). Abbreviations: PARP, poly ADP-ribose polymerase; SIRT1 is the mammalian homolog to Sir2; FOXO3, the most ubiquitous mammalian forkhead transcription factor; P, a phosphorylated protein; Ac, an acetylated protein; IL, insulin ligand; ILL, insulin-like ligand; IR, insulin receptor; ILR, insulin-like growth factor receptor; IRE, insulin response element; PI3K, the PI3 kinase; PI2, the membrane-bound phosphatidylinositol diphosphate; PI3, membrane-bound phosphatidylinositol triphosphate; AKT 1/2, the AKT kinases; ATPsyn $\beta$ , ATP synthase  $\beta$  subunit; cytc, cytochrome c. (Refs. (1,2,13).)



The FOXOs and CREB regulate numerous genes through the IRE and CRE *cis* elements. The promoter of the peroxisome proliferation-activated receptor- $\gamma$  (PPAR- $\gamma$ ) coactivator 1 $\alpha$  (PGC-1 $\alpha$ ) gene contains three IREs that bind unphosphorylated and deacetylated FOXO1. It also contains one CRE that binds phosphorylated CREB (370). PGC-1 $\alpha$ , together with its companion genes PGC-1 $\beta$  (PERC) and PGC-related coactivator (PRC), are transcriptional coactivators that regulate genes for mitochondrial biogenesis, thermogenesis, and fatty acid oxidation (371,372).

The PGC-1 family of transcriptional coactivators interacts with a broad spectrum of tissue-specific transcription factors. This provides the tissue-specific response to the generalized insulin and glucagon hormonal signals. In the energy-utilizing muscle cells (C2C12), PGC-1 $\alpha$  induces mitochondrial biogenesis and UCP-2, acting through the nuclear respiratory factors 1 and 2 (NRF1 and NRF2) (373). NRF1 and NRF2, in turn, activate the transcription of a wide range of nDNA encoded mitochondrial genes, including components of OXPHOS such as the ATPsyn $\beta$  subunit (ATPsyn $\beta$ ) and cytochrome *c* and the mtTFA, which regulates mtDNA transcription (371,372). In muscle, PGC-1 induces mitochondrial biogenesis through the interaction with NRF1, PPAR- $\beta$ , PPAR- $\delta$ , MEF2, ERR2 (estrogen-related receptor 2), HDAC5, and Gabpa/b (GA-repeat binding protein) (374–377). In the heart, PGC-1 $\alpha$  interacts with PPAR- $\alpha$  and NRF1 (375,378). In the brain, the transcriptional partners of PGC-1 $\alpha$  have not yet been identified (379).

In the energy-storage tissue for thermal regulation (BAT), cold stress activates the cAMP-mediated  $\beta$ -adrenergic receptors to upregulate PGC-1 $\alpha$ . PGC-1 $\alpha$  in turn interacts with PPAR- $\gamma$ , PPAR- $\alpha$ , and the thyroid hormone receptor to induce mitochondrial biogenesis and UCP-1 (373,375). The induction of UCP-1 creates a proton channel through the mitochondrial inner membrane, uncoupling OXPHOS. This causes the rapid burning of the stored fats in BAT, generating heat to maintain body temperature (380).

In the energy-storage tissue for fueling work (WAT), PGC-1 $\alpha$  in 3T3-L1 preadipocytes interacts with PPAR- $\alpha$  to stimulate fatty acid oxidation, including induction of the medium-chain acyl CoA dehydrogenase (MCAD) (381). Finally, in the energy-homeostasis tissue (liver), PGC-1 $\alpha$  interacts with FOXO1, hepatocyte nuclear factor-4 $\alpha$  (HNF-4 $\alpha$ ), and the glucocorticoid receptor to induce gluconeogenesis enzymes, including phosphoenolpyruvate carboxylase (PEPCK) and glucose-6-phosphatase (G6Pase), to maintain basal blood glucose levels (375,382–384).

PGC-1 $\beta$  is abundantly expressed in BAT, heart, and kidney and skeletal muscle but also in stomach and in WAT. In 3T3-L1 preadipocytes, PGC-1 $\beta$  is inducible, though PGC-1 $\alpha$  is not, and PGC-1 $\beta$  then interacts with the ERRs to induce MCAD and fatty acid oxidation (385). Thus, both insulin through the FOXOs and glucagon through cAMP modulate mitochondrial energy metabolism in response to the availability of carbohydrates via the regulation of members of the PGC-1 coactivator family.

Mitochondrial energy metabolism is further modulated by PGC-1 $\alpha$  through deacetylation by SIRT1. PC12 cells that overexpress SIRT1 experience a 25% reduction in oxygen utilization (386). Hepatocytes that overexpress SIRT1 induce the gluconeogenesis genes *PEPCK* and *G6Pase* via interaction between PGC-1 $\alpha$  and HNF-4 $\alpha$  (383). Furthermore, on caloric restriction, the SIRT1 in fat cells binds to the nuclear receptor corepressor, blocking its interaction with PPAR- $\gamma$ . This inhibits fat storage and enhances fat mobilization (387,388).

### 11.1.15 Mitochondrial Stress Response Pathways

In addition to responding to differences in the availability and nature of environmental calories to the whole organism, the cell and its mitochondria must respond to the mitochondrial production of ROS with respect to oxidative damage, induction of apoptosis, and signal transduction. While it is well known that mitochondrial ROS damages cellular macromolecules, including mtDNA and nDNA, and can result in apoptosis, it is less well known that mitochondrially generated H<sub>2</sub>O<sub>2</sub> is a vital component in regulating cellular mitosis.

Mitochondrial ROS acts as a mitogen through its interaction with a variety of cytosolic and nuclear signal transduction pathways. Excess mitochondrial H<sub>2</sub>O<sub>2</sub> diffuses out of the mitochondrion and into the cytosol and the nucleus, where it interacts with NF- $\kappa$ B, apurinic/apyrimidinic endonuclease-1 (APE-1), Fos, Jun, and tyrosine kinases (Src kinase, protein kinase C, mitogen-activated protein kinase, and receptor tyrosine kinases) (389). The dual-functional APE-1 is also involved in the redox regulation of Fos, Jun, NF- $\kappa$ B, PAX, hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ), and p53 (390,391).

The activation of the tyrosine kinases and/or Ras activates PI3K, which in turn activates the *Akt*/PKB kinases. The *Akt*/PKB kinases also promote the binding of the hexokinase II to mitochondrial voltage-dependent anion channel, thus coupling mitochondrial ATP production to driving glycolysis. The activated *Akt*/PKB kinases also phosphorylate the FOXO transcription factors. The phosphorylation of the FOXOs removes them from the nucleus so that they do not bind the IREs in gene promoters, including the mitochondrial MnSOD gene and PGC-1 $\alpha$ , thus downregulating mitochondrial antioxidant defenses and mitochondrial OXPHOS. As a consequence, the cells switch from more oxidative to more glycolytic, further compensating for the possible effects of an overabundance of mitochondria.

The mitochondria also contain the p66<sup>Shc</sup> protein attached to the outside of the mitochondrial inner membrane. In response to stress stimuli, p66<sup>Shc</sup> collects electrons directly from cytochrome *c* and passes them to O<sub>2</sub> to generate H<sub>2</sub>O<sub>2</sub> (392). Thus, stress can also act through mitochondrial H<sub>2</sub>O<sub>2</sub> to drive cellular replication.

The mitochondrial modulation of nuclear transcription and replication can also be enhanced by the

expression of cytosolic NADPH oxidases. NADPH oxidases reduce  $O_2$  to generate superoxide anion in the cytosol. The best characterized of the NADPH oxidases is the macrophage “oxidative burst” complex involved in generating the  $O_2^-$  to kill engulfed microorganisms. However, an additional NADPH oxidase, Mox1, is a homolog of the gp91phox catalytic subunit of the phagocyte NADP oxidase. MoxX1-generated  $O_2$  increases mitotic rate, cell transformation, and tumorigenicity when Mox1 is overexpressed in NIH3T3 cells (393). This mitogenic activity of Mox1 is neutralized by overexpression of catalase, indicating that the cell growth signal must be  $H_2O_2$  (394). Hence, the modulation of cellular proliferation through  $H_2O_2$  can be modulated by both mitochondrial number and function and also by expression of the nDNA NADH oxidases.

While at low levels mitochondrial  $H_2O_2$  can serve as a signaling system to the nucleus, at high levels the concentration of  $H_2O_2$  in the nucleus continues to accumulate, where it can be converted to hydroxyl radicals, which will then damage the nDNA. Damage to the nDNA will then activate the nDNA repair system, including poly(ADP-ribose) polymerase (PARP). PARP converts the nuclear  $NAD^+$  into poly(ADP-ribose) linked to nuclear proteins, thus depleting nuclear  $NAD^+$ . The nuclear  $NAD^+$  level is further depleted by its reduction to NADH as a consequence of the inhibition of the mitochondrial ETC. Depletion of nuclear  $NAD^+$  deprives the protein deacetylase SIRT1 of its obligatory substrate  $NAD^+$ , thus inhibiting deacetylation of histones, the FOXOs, and p53. The increased acetylation of p53 increases its activity, thus driving apoptosis.

PARP is a nDNA enzyme that is activated by fragments of DNA resulting from DNA damage. Utilizing  $NAD^+$  as a substrate, it transfers 50 or more ADP-ribose moieties to nuclear proteins such as histones and PARP itself. Massive DNA damage results in excessive activation of PARP, which leads to the depletion of  $NAD^+$ . The resynthesis of  $NAD^+$  from ATP then markedly depletes cellular ATP, leading to death (395). Mice in which the PARP gene has been genetically inactivated show remarkable resistance to cellular stress such as cerebral ischemia (stroke) (396,397) and streptozotocin-induced diabetes (398).

SIRT1 also uses  $NAD^+$  as a cofactor to diacetylate proteins, including FOXOs, p53, and the histones. Diacetylated histones keep silent genes, such as proto-oncogenes, turned off (399). Degradation of  $NAD^+$  would inactivate SIRT1, permit histones to be acetylated and silent genes to be illegitimately expressed, further driving cell division.

The nuclear protein p53 can initiate programmed cell death and is activated by DNA damage and by acetylation. The p53 apoptotic pathway acts through mitochondrial cytochrome c release and requires the intervention of the proapoptotic protein Bax (400). The stress activation

of p66<sup>Shc</sup> can also increase  $H_2O_2$  and contribute to driving cells into apoptosis (392).

The regulation of the nuclear-cytosolic functions by mitochondrial ROS is further modulated by a series of other short- and long-lived small regulatory molecules. One of these is short half-life nitric oxide (NO). NO is generated from arginine by nitric oxide synthase (NOS). There are three cytosolic NOS isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), which is expressed in activated macrophages. NOS has recognition sites for calmodulin, NADPH, FAD, and FMN and uses heme and tetrahydrobiopterin as oxidation–reduction cofactors. nNOS is tethered to N-methyl-D-aspartate (NMDA) receptors in neurons by the PSD-95 protein. Hence, when NMDA receptors are activated by glutamate, permitting  $Ca^{2+}$  to flow into the cytosol, this immediately activates nNOS via calmodulin (395,401). The NO generated by NOS can react with superoxide anion ( $O_2^-$ ) to give the highly reactive peroxynitrite anion ( $ONOO^-$ ). This can be protonated to give peroxynitrous acid ( $ONOOH$ ) (402).

The mitochondrion has its own mitochondria-specific NOS, which may be attached to the mitochondrial inner membrane (403,404). Mitochondrial NO has been shown to reversibly inhibit complex IV (COX), causing a reduction in  $\Delta p$  and  $Ca^{2+}$  release from the mitochondria (405). Mitochondrial NO can also react with  $O_2^-$  to generate  $ONOO^-$ , which can inactivate mitochondrial creatine phosphokinase (406), activate the mitochondrial release cytochrome c (407), inhibit other respiratory complexes (408), and possibly activate PARP (409).

The heme oxygenases (HO) degrade heme by cleaving the porphyrin ring and generating biliverdin and carbon monoxide (CO). Biliverdin is rapidly reduced to bilirubin by biliverdin reductase. The bilirubin-biliverdin system provides a potent antioxidant mechanism for protecting the cell (395). There are two HOs, HO-1 and HO-2. HO-1, the most abundant form, is highly concentrated in the spleen and degrades heme from senescent red blood cells. HO-1 is induced by heme, oxidative stress, and agents that induce heat shock proteins. HO-2 is a noninducible form localized in the brain and testis (395). Both CO and NO function in neuronal transmission of signals to target cells such as the intestine (410), and are involved in endothelial cell-dependent relaxation (411). Interestingly, CO, like NO, is a potent inhibitor of COX. However, NO has a very short half-life and must be produced close to the target, whereas CO is extremely stable and can diffuse over large distances. Hence, the products of HO-2 may interact with mitochondrial energetics through CO and with mitochondrial ROS through the bilirubin-biliverdin antioxidant system.

Acting together, these various enzymes form an integrated metabolic network between the mitochondria and the nucleus-cytosol. This network would appear to be central to maintaining the necessary balance between the two interdependent information storage and retrieval systems.



### 11.1.16 Nature of Inherited mtDNA Variation

Mitochondrial DNA variation falls into two major classes, maternally transmitted variation, which is passed through the female germline, and somatic mtDNA mutations, which accumulate in tissue cells with age due to the continual replication of mtDNA throughout animal life. Maternally transmitted variation can be deleterious and relatively rapidly eliminated by purifying selection, thus causing maternally inherited disease. Neutral or near neutral variants accumulate in human populations by genetic drift and may be beneficial in particular environments and thus enriched by adaptive selection. Deleterious mutations are constantly arising in the population and being removed at the same rate by selection. Hence, all deleterious mutations must have arisen recently. Neutral mutations are constantly arising and are retained or lost by chance. Hence, they can be either recent or ancient. Adaptive (beneficial) mutations are the rarest class, since most functional mutations degrade a gene function rather than enhance it. However, in the case of mtDNA genes that regulate mitochondrial metabolism, functional variants that may be deleterious in one environment can be advantageous in another environment. Thus, adaptive mutations might arise frequently due to the high mtDNA mutation rate, but only become enriched when individuals bearing that mutation are exposed to a new environment such as might result from climatic change or migration. Hence, adaptive mtDNA variants should be ancient, occur at the base of new branches of the mtDNA tree, and correlate with specific geographic domains.

Somatic mtDNA mutations can also be deleterious, neutral, or adaptive, though the majority should be either deleterious or neutral. Since somatic mtDNA mutations would accumulate in post-mitotic cells, the ability of the mitochondria to repair damage would decline as the deleterious mutations accumulate, ultimately resulting in cellular energetic failure and apoptosis. It is now clear that the accumulation of somatic mtDNA mutations is the aging clock for post-mitotic tissues.

### 11.1.17 Three Classes of Inherited mtDNA Variants

Inherited mtDNA variants can be deleterious and cause disease, neutral and accumulate in the population by drift, or beneficial and be enriched by adaptive selection. All three classes have now been discovered in the human mtDNA.

**11.1.17.1 Deleterious mtDNA Mutations.** Of the three classes of inherited mtDNA mutations, the deleterious mutations are the most conspicuous in their impact on the human condition. Deleterious mtDNA mutations fall into two broad classes: rearrangement mutations, including insertions and deletions, and nucleotide

substitution mutations. Most mtDNA rearrangement mutations are relatively large and thus frequently remove one or more of the mtDNA tRNA or rRNA genes. Hence, most mtDNA rearrangement mutations result in partial mitochondrial dysfunction. Since mtDNA deletions are lethal, they are always found in patients in the heteroplasmic state in association with normal mtDNAs and are rarely transmitted through the female germline. Duplications within mtDNA are less deleterious and thus can be maternally transmitted. However, they also have the capacity to undergo internal homologous recombination, resulting in reciprocal deletions that cause progressive, multisystem disease (412).

Base substitution or insertion–deletion mutations affect only one gene. These fall into two classes: mutations that alter 1 of the 13 polypeptide genes, resulting in biochemical defects in specific respiratory complexes (polypeptide mutations) and mutations that alter a tRNA or an rRNA gene, resulting in defects in mitochondrial protein synthesis (protein synthesis mutations) (412). Depending on the severity of the effect of the nucleotide substitution, it either may only survive if heteroplasmic when severe or may be either heteroplasmic or homoplasmic when mild. For polypeptide genes, nucleotide substitutions that change an amino acid are said to be coding or nonsynonymous (NS) mutations, and their frequency in a population for a particular gene sequence is reported as  $K_a$ . The potential phenotype severity of a polypeptide amino acid substitution (missense) mutation can be estimated by the interspecific amino acid conservation index (CI). CI is the ratio of the number of species that have the pre-variant amino acid in the mutant position divided by the total number of species analyzed. Amino acids with high CIs must be functionally important since mutations that arose in these positions must have been repeatedly eliminated by purifying selection.

A large number of deleterious mtDNA rearrangement and base substitution mutations have been identified over the past 24 years since the first diseases resulting from spontaneous rearrangement (7) and inherited nucleotide substitution mutations (4–6) were first reported. The known pathogenic mtDNA mutations are available through the MITOMAP website ([www.mitomap.org](http://www.mitomap.org)) and are discussed in detail later.

**11.1.17.2 Neutral Mutations.** Neutral mutations also fall into two classes: Those that do not alter a gene product and those that do change a gene product but have a minimal effect on the function of the product. Among the mtDNA mutations that alter an mtDNA nucleotide without altering a mitochondrial function, the most common type is the mutations that alter a third codon position and thus do not alter an amino acid. Such mutations are designated silent or synonymous (S), and the frequency of S mutations for a gene within a population is  $K_s$ . Since S mutations are not acted on by selection, they accumulate by chance and thus at a relatively constant rate over time. Thus, the  $K_s$  value provides an estimation of

the time of divergence between two mtDNA sequences. Some mutations in polypeptide genes change an amino acid, but the alteration has little if any deleterious effect on the function of the polypeptide. In polypeptide genes, such mutations generally change amino acids with a low CI.

The level of accumulation of missense mutations along mtDNA lineages is thus a consequence of the time that missense mutations have had to accumulate and the rate at which natural selection removed the mutations. This is calculated by the ratio of  $K_a$  and  $K_s$  ( $K_a:K_s$ ), with  $K_s$  normalizing for the time of divergence.

Since different mtDNA lineages never mix and thus never recombine, base substitution mutations must accumulate as sequential mutations along radiating mtDNA lineages. However, certain neutral mutations might become enriched by selection if they are linked to an advantageous mutation, a process known as “hitchhiking.”

**11.1.17.3 Adaptive Mutations.** Presumably ancient adaptive mutations could occur in any functional element within the mtDNA, including polypeptides, tRNAs, rRNAs, and functional control region (CR) elements. Currently, the most revealing information is available for ancient polypeptide missense mutations. By the process of elimination from deleterious and neutral mutations, adaptive mutations would be expected to change important mitochondrial functions, yet not only have resisted being eliminated by purifying selection but in many cases have been expanded in specific regional human populations. For polypeptide genes, adaptive mutations would change amino acids with a high CI and yet also be ancient and thus found in one of the internal nodes of the mtDNA tree. The adaptive importance of such mutations can be additionally demonstrated by observing that the same mutation appears multiple independent times on different human mtDNA lineages in similar environmental contexts.

### 11.1.18 The mtDNA Tree

Because of the strict maternal inheritance of mtDNA, it is possible to reconstruct the entire mutational history of human mtDNA, which resulted from the sequential accumulation of base substitutions along radiating maternal lineages. As expected, the accumulation of mtDNA mutations results in a progressively branching lineage. What is surprising, however, is that the branches of the mtDNA tree correlate dramatically with the geographic origin of indigenous human populations.

We now understand that the geographic association of specific mtDNA lineages is the product of environmental selection that enriched for specific mtDNA types. This is a direct consequence of the fact that mitochondria generate energy for two purposes: production of ATP to perform work and generation of heat to maintain our body temperature at 37°C. The ratio of ATP production to heat generation is determined by the coupling efficiency of OXPHOS. Sequence variants in mtDNA

that alter the coupling efficiency of OXPHOS can then change the balance from more ATP and less heat in tropical and subtropical Africa to more progressively more heat and less ATP in temperate Eurasia and Arctic Siberia (321,360,361,413).

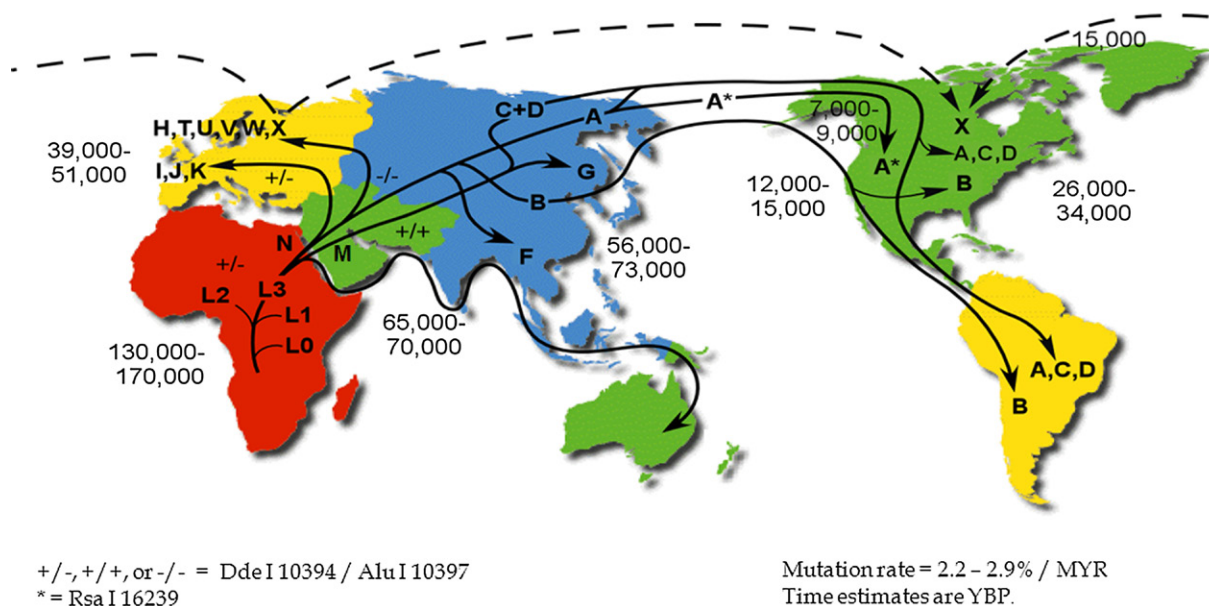
### 11.1.19 Phylogeny of Polymorphic Variation

The unique correlation between mtDNA variation and human geographic origin was first observed when samples from Africans, Asians, and Europeans were analyzed for *HpaI* mtDNA restriction site polymorphisms and the mtDNAs of each population were found to have distinct polymorphisms (414). Further characterization of restriction site polymorphisms revealed that all mtDNA types would fit into a single tree (415), with the greatest mtDNA variation and the root of the tree being in Africa (359,415,416) and with specific branches radiating into the different continents (417). The sequence variation found in a particular mtDNA is designated the mtDNA haplotype, and a group of related haplotypes that form discrete branches of the tree are called “haplogroups.” Haplogroups are defined by ancient sequence polymorphisms that occur at the base of a particular branch of the mtDNA tree (358,360,417).

The most diverse and thus most ancient mtDNAs are found in Africa, with the sequence divergence giving an estimate of African occupation of between 150,000 and 200,000 years before present (YBP). African mtDNAs fall into four major haplogroups: L0 (oldest), L1, L2, and L3 (youngest). L0, L1, and L2 represent about 76% of all sub-Saharan African mtDNAs and are defined by a *HpaI* restriction site at np 3592 (macro-haplogroup L [L0, L1, and L2]) (see Figure 11-5). In northeastern Africa, two mtDNA lineages, M and N, arose from L3 about 65,000 YBP. These were the only mtDNA lineages that succeeded in leaving sub-Saharan Africa and radiating into Eurasia to give all of the Eurasian mtDNAs. In Europe, haplogroup N gave rise to haplogroups H (about 45% of European mtDNAs), T, U, V, W, and X (about 2%) as well as I, J (about 9%), and K (Uk). The European lineages arose about 40,000–50,000 YBP. In Asia, lineages M and N radiated to give rise to a plethora of mtDNA lineages. These include haplogroups A, B, F, and others from N, and haplogroups C, D, G, and others from M (417).

As Asians migrated northeast into Siberia, haplogroups A, C, and D became progressively enriched, such that they became the predominant mtDNA lineages in the indigenous peoples of extreme northeastern Siberia, Chukotka. When the Bering land bridge appeared about 20,000–30,000 YBP, people harboring these mtDNA haplogroups were in a position to migrate into the New World, where they founded the Paleo-Indians. After the land bridge submerged, haplogroup G arose in central Asia and moved into northeastern Siberia to populate the area around the Sea of Okhotsk. About 12,000–15,000 YBP, a migration carrying haplogroup B started from eastern Central Asia

**HUMAN MIGRATIONS: NON-RANDOM DISTRIBUTION OF mtDNA  
VARIATION SUGGESTS SELECTION**  
**Striking Discontinuities:**  
**Tropical Africa to Temperate Eurasia to Arctic Siberia**



**FIGURE 11-5** Diagram outlining the migratory history of the human mtDNA haplogroups. *Homo sapiens* mtDNAs arose in Africa about 130,000–170,000 years before present (YBP), with the first African-specific haplogroup branch being L0, followed by the appearance in Africa of lineages L1, L2, and L3. In northeastern Africa, L3 gave rise to two new lineages M and N. Only M and N mtDNAs successfully left Africa about 65,000 YBP and colonized all of Eurasia and the Americas. The diverse array of mtDNA lineages M and N spawned are clustered together as macrohaplogroups M and N. The founders of macrohaplogroup M moved out of Africa through India and along the Southeast Asian coast down along the Malaysian peninsula and into Australia generating haplogroups Q and M42 around 48,000 YBP. Subsequently, M moved north out of Southeast Asia to produce a diverse array of mtDNA lineages including haplogroups C, D, G and many other M haplogroup lineages. In northeast Asia, haplogroup C gave rise to haplogroup Z. The founders of macrohaplogroup N also moved through Southeast Asia and into Australia generating haplogroup S. In Asia, macrohaplogroup N mtDNAs also moved north to generate central Asian haplogroup A and Siberian haplogroup Y. In western Eurasia, macrohaplogroup N founders also moved north to spawn European haplogroups I, W, and X and in western Eurasia and gave rise to sub-macrohaplogroup R. R moved west to produce the European haplogroups H, J, U, and V and also moved east to generate Australian haplogroup P and eastern Asian haplogroups F and B. By 20,000 YBP, mtDNA haplogroups C and D from M and A from N were enriched in north-eastern Siberia and thus were positioned to migrate across the Bering land bridge (Beringia) to give rise to the first Native American populations, the Paleo-Indians. Haplogroups A, C, and D migrated throughout North America and on through Central America to radiate into South America. Haplogroup X, which is most prevalent in Europe but is also found in Mongolia though not in Siberia, arrived in North America about 15,000 YBP, but remained in northern North America. Haplogroup B, which is not found in Siberia but is prevalent along the coast of Asia arrived in North America about 12,000 to 15,000 YBP and moved through North and Central America and into South America combining with A, C, and D to generate the four dominant Paleo-Indian haplogroups (A-D). A subsequent migration of haplogroup A out of the Chukotka peninsula about 7,000 to 9,000 YBP gave rise to the Na-Déné (Athabaskins, Navajo, Apache, etc.). Subsequent movement across the Bering strait, primarily carrying haplogroups A and D after 6,000 YBP, produced the Eskimo and Aleut populations. Most recently, eastern Asian haplogroup B migrated south along the Asian coast through Micronesia and out into the Pacific to colonize all of the Pacific islands (Reprinted from <http://www.mitomap.org> (1).)

and moved along the coast to the New World, bypassing arctic Siberia. Haplogroup B then mixed with A, C, and D in temperate North and South America and tropical and subtropical Central and South America to generate additional Paleo-Indian groups. About 15,000 YBP, haplogroup X crossed the arctic to the New World in a migration that settled in the Great Lakes region, such that today 25% of the Ojibwa mtDNAs are haplogroup X (417).

Later migrations from northeastern Siberia, carrying a modified lineage of haplogroup A, founded the Na-Déné populations about 9500 YBP. More recently, immigrants from Siberia bearing derived lineages of haplogroups A and D moved along the Arctic Circle and founded the populations of Eskimos and Aleuts (417).

### 11.1.20 Adaptive mtDNA Variants in Human Evolution, Longevity, and Disease

The phylogeographic distribution of mtDNA haplogroups reveals two striking discontinuities in human mtDNA diversity. The first occurs between sub-Saharan Africa and Eurasia. Virtually all of the sub-Saharan African mtDNA diversity remained in Africa, while only derivatives of lineages M and N colonized temperate Eurasia. The second occurs between temperate Central Asia and Arctic Siberia, where the plethora of Asian mtDNA types are markedly reduced to only three ancient mtDNA lineages (A, C, and D). Thus, the major transitions in mtDNA types correlate most strongly with



latitude. This suggests that climatic selection may have enriched for mtDNA variants that changed the coupling efficiency of mtDNA and thus permitted humans to survive in the colder northern climates (321,360,361).

Evidence that climatic adaptation has influenced the geographic distribution of mtDNA diversity was first obtained by analyzing the amino acid replacement (NS) ( $K_a$ ) to silent (S) ( $K_s$ ) mutation ratios ( $K_a:K_s$ ) from the 13 mtDNA open reading frames of mtDNA associated with tropical and subtropical Africa, temperate Europe, and arctic Siberia. This revealed that the amino acid sequence of the *ATP6* gene was highly variable in the arctic, but was strongly conserved in the tropics and temperate zone; *CYTb* was hypervariable in temperate Europe, but conserved in the tropics and arctic; and *COI* was variable in the tropical Africa, but invariant in the temperate and arctic regions. Variation was also observed in multiple ND subunits (360). Such regional gene-specific variation would not be expected if all mtDNA mutations were neutral and thus accumulated purely by chance (genetic drift).

The geographic constraints on mtDNA protein variation were further validated by positioning all of the mtDNA variants from over 1000 complete mtDNA coding sequences collected from around the world. Mutations that were found in a single mtDNA haplotype must be new mutations, while those that were shared by similar mtDNAs must have arisen in a common ancestor. By this logic, all mutations could be arranged into a sequentially mutational tree.

Based on the position of the sequence variant in the tree and the effect that the variant had on the protein function, the mtDNA variants can be divided into three classes: (1) recent mutations many of which alter functionally important amino acids and thus are potentially disease causing, (2) ancient mutations that are either synonymous or alter weakly conserved amino acids and thus are likely to be neutral or near neutral, and (3) ancient mutations that change highly evolutionarily conserved amino acids and must be adaptive. Of the ancient missense mutations, about 74% are weakly conserved with an average CI of  $23.3\% \pm 14.9\%$ , while about 26% alter highly conserved amino acids with a CI of  $85.1\% \pm 9.2\%$ . By comparison, the average interspecific CI of the 22 known pathogenic mtDNA replacement mutations is  $93.3\% \pm 13.3\%$ , which is only slightly higher than that of the conserved ancient variants (361). Thus, the ancient conserved variants are almost as highly conserved as the pathogenic variants. Yet unlike the pathogenic variants, which are eliminated by purifying selection within a few generations, these ancient conserved variants have resisted purifying selection and survived and expanded in the human population over tens of thousands of years. Therefore, they cannot be deleterious. Rather, they must be adaptive and may represent mutations that altered the mtDNA coupling efficiency and permitted people to adapt to different environments (321,360,361).

Confirmation that this interpretation is correct has now come from multiple different observations. First, these

ancient missense mutations frequently initiate geographically constrained branches of the mtDNA tree, meaning that individuals carrying these mutations were able to survive and multiply in new geographic regions. For example, macro-haplogroup N, which left tropical Africa to colonize temperate Eurasia, is founded by two missense mutations, the *ND3* G10398A, which causes an A114T amino acid substitution, plus the *ATP6* G8701A, which causes an A59T substitution. Second, the relative frequency of ancient NS to S mutations (NS:S) increases from south to north, indicating that the change in environment has resulted in an increased rate of fixation of missense mutations. This differential is particularly striking for haplogroups that reside in the arctic and subarctic. The mean internal branch NS:S ratio for northeastern Siberian–North American haplogroups A, C, D, and X was 0.61, much higher than the mean for the nonarctic haplogroups of 0.39, the mean for the African L haplogroups of 0.31, or the mean for the Native American haplogroup B, which bypassed the cold selection of Siberia, of 0.38. Furthermore, the CI of the arctic missense mutations is higher than those of the warmed climates, with the CIs of the arctic variants of haplogroups A, C, D, and X being 51%, that for the remaining global haplogroups being 39%, that of L being 36%, and that of B being 31%. A similar trend is seen for the European branches of the mtDNA tree. The frequency of ancient replacement mutations (NS:S) for haplogroup H is 0.48, J is 0.66, and IWX is 0.63, while that for Africa is 0.31. The NS:S ratio for haplogroup T is only 0.31, but that is because T was founded by a single highly conserved founding replacement mutation in the *ND2* gene (np 4917) with a very high CI of 20.3. Thus, adaptive changes fall into two categories: either the lineage accumulates multiple missense mutations, each changing a reasonably conserved amino acid, or the lineage changes only a few amino acids, but each change alters an amino acid with a high CI.

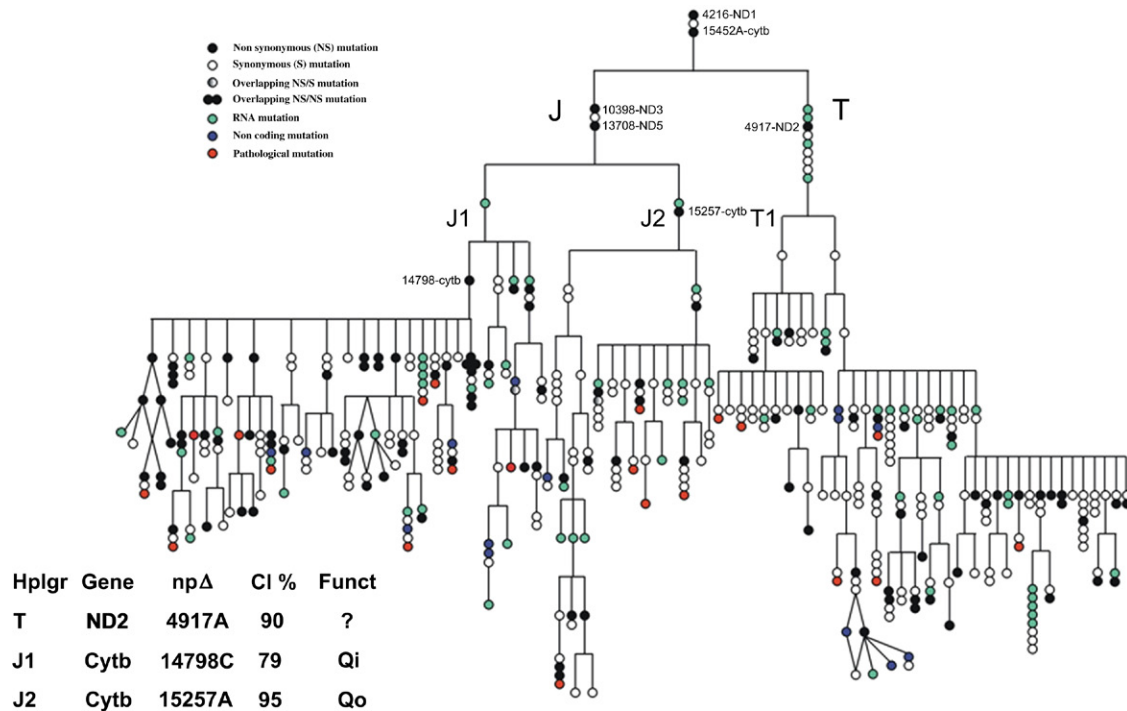
Examples of the adaptive mtDNA mutations that occur in the arctic mtDNAs include two replacement mutations (*ND2* np 4824G [T119A] and *ATP6* np 8794T [H90Y]) for haplogroup A; two replacement variants (*ND4* np 11969A [A404T] and cytochrome *b* np 15204C [I153T]) for haplogroup C; an *ND2* np 5178A (L237M) variant for haplogroup D; and an *ND5* np 13708A (A458T) variant for a sublineage of haplogroup X. This latter variant also appears in European haplogroup J (Figure 11-6).

The European sister haplogroups J and T provide the clearest example of the two classes of adaptive mutation strategies: several less conserved mutations versus a few highly conserved mutations. J and T share a common root involving two amino acid substitutions: *ND1* np 4216C (Y304H) and *CYTb* np 15452A (L236I). J and T then diverge. Haplogroup T is founded by the single nodal adaptive mutation: *ND2* np 4917G (N150D), the most conserved *ND2* polymorphism found (361).

Haplogroup J has two replacement mutations at its root: *ND3* np 10398G (T114A) and *ND5* np 13708A



## BRANCHES OF mtDNA TREE ARE FOUNDED BY VARIANTS IN CONSERVED AMINO ACIDS: EUROPEAN HAPLOGROUPS T & J



**FIGURE 11-6** The European haplogroup J and T branches of the human mtDNA tree shows the nodal mtDNA missense proposed to have been adaptive. The interspecific sequence conservation of the ND2 4917A which defines haplogroup T and the cytb 14798C and 15257A variants that define subhaplogroups J1 and J2 are given in the lower left hand corner of the figure. The sequence variants in ND3 at 10398, ND5 at 13708 and cytb at 14798 have been observed repeatedly in different branches of the human mtDNA tree. Haplogroups J and T have been observed to increase the penetrance of the milder LHON mutations, to be protective of AD and PD and to be enriched in the very old.

(A458T), the second being the same variant found in haplogroup X. Haplogroup J then splits into two subhaplogroups J1 and J2, each defined by a major cytochrome *b* mutation. The J2 variant *CYTB* mutation is at np 15257A (D171N) and the J1 *CYTB* mutation is at np 14798C (F18L). The np 14789C mutation is also found at the root of sub-haplogroup Uk. The np 15,257 and np 14,789 variants alter well-conserved amino acids with CIs of 95% and 79%, respectively. The 15,257 variant alters the outer coenzyme Q binding site ( $Q_o$ ) of complex III, which contacts the Rieske ISP, while the np 14,798 site alters the inner coenzyme Q binding site ( $Q_i$ ) of complex III (361). Since the  $Q_o$  and  $Q_i$  binding sites are essential for complex III proton pumping via the Q cycle, the np 14,798 and 15,257 variants are both likely to have disconnected electron flow through complex III with proton pumping. This would reduce the coupling efficiency of mitochondrial OXPHOS by one-third, thus requiring burning more calories for the same ATP, proportionately increasing heat generation.

That these population variants are clinically relevant has become apparent from studies that correlated mtDNA haplogroups with longevity and degenerative disease. In an Italian study, mtDNA haplogroup J was

found to be overrepresented in centenarians (418,419). Similarly, in an Irish centenarian study, J2 was overrepresented (420), and in a Finnish study of individuals over 90 years, J2, Uk, and WIX were enriched (421). In Japanese centenarians, a sublineage of haplogroup D was enriched (422,423). Hence, specific mtDNA lineages from Europe and Asia are protective against the ravages of aging.

Some of these same mtDNA lineages have also been found to be protective against neurodegenerative diseases. Haplogroups J and Uk are underrepresented in Parkinson disease (PD) (424) and haplogroup T is underrepresented in Alzheimer disease (AD) patients (425,426). The repeated association of haplogroups J1 and Uk with longevity and neuroprotection is particularly illuminating because both haplogroups encompass the same *CYTB* mutation at np 14,798. Such convergent evolution provides strong support for the functional importance of the *CYTB* mutations.

In other studies, haplogroup T has been found to be overrepresented in bipolar affective disorder (427) and haplogroup J has been found to be consistently associated with milder mtDNA missense mutations that are associated with the midlife blindness of LHON (428–431).

Several studies have begun to address the biochemical basis of these associations between mtDNA population polymorphisms and disease. The internal branch mtDNA missense mutations are functionally relevant, which has been demonstrated by comparing the sperm mobility of males harboring the different mtDNA haplogroups. Sperm flagellar motion is driven primarily by ATP generated from the mitochondria in the midpiece. Therefore, sperm with partially uncoupled mitochondria should swim slower than those with coupled mitochondria. As expected, sperm from haplogroup H subjects swam significantly faster than those from T subjects (432), and those of haplogroup U that harbor CYTB missense mutations swim significantly more slowly than those without (433). Moreover, in Europe, individuals who harbor uncoupling CYTB mutations occur in higher frequency in northern Europe than in southern Europe. This is consistent with people who generate on average more heat wishing to live in colder climates (433).

Analysis of somatic cell cybrids harboring mtDNAs from macrohaplogroup L which harbors the ND3 nt 10398 T114A and ATP6 nt 8701 A59T are associated with altered mitochondrial  $\text{Ca}^{++}$  levels and pH (434) and comparison of cybrids harboring haplogroup Uk versus H mtDNAs revealed differences in mtDNA and mtDNA RNA levels, mitochondrial protein synthesis, cytochrome oxidase activity, oxygen consumption, mitochondrial inner membrane potential, and growth capacity (435). Thus, the functional mtDNA variants that founded specific mtDNA lineages affect mitochondrial physiologic functions.

How can the same variants be associated with increased life span and protection against certain diseases on the one hand, yet increase the predilection of developing other degenerative diseases on the other? Additionally, if mtDNA haplogroup variants are deleterious, why do they become enriched in certain populations in apposition of the action of purifying selection? One study has suggested that the reason is that the same variant can be beneficial and thus selectively enriched in one environment but deleterious in another environment. The nt 3394 T>C (Y30H) variant is such a case. This was first observed in association with LHON (430,436–438) and found to reduce complex I activity and cellular respiration between 7% and 28% on the Asian B4c and F1 haplogroup backgrounds. However, complex I activity between B4c and F1 mtDNAs which harbor the common 3394T allele can also differ by 30%. In Asia, the 3394C variant is most commonly associated with the M9 haplogroup which is rare at low elevations but increases in frequency with elevation to an average of 25% of the Tibetan mtDNAs (OR = 23.7). In high altitude Tibetan and Indian populations, the 3394C variant has been observed on three different macrohaplogroup M haplogroup backgrounds, and it is enriched on the M9 background in Tibet and the C4a4 background on the Indian Deccan Plateau (OR = 21.9). When present

on the M9 background, the 3394C variant is associated with a complex I activity that is equal to or higher than that of the 3394T variant on the B4c and F1 backgrounds. Hence, the 3394C variant can either be deleterious or beneficial depending on its haplogroup and environmental context (439).

### 11.1.21 Somatic mtDNA Mutations in Degenerative Diseases, Cancer, and Aging

Since mitochondrially generated ROS would damage mitochondrial macromolecules, including mtDNA, the continual generation of ROS during OXPHOS would be expected to result in the age-related accumulation of somatic mtDNA mutations. As the number of functional mtDNA templates decline with age, the ability of the mitochondria to repair damage to mitochondrial OXPHOS would ultimately decline until the mtPTP is activated and the cells are destroyed by apoptosis. The loss of cells within the tissue would result in organ decline, leading to the age-related accumulation of clinical symptoms, the hallmark of aging. Therefore, it follows that the accumulation of somatic mtDNA mutations may be the aging clock in animals.

Consistent with this hypothesis, both rearrangements and base substitution mutations have been found to accumulate in those mammalian tissues most prone to the effects of aging. Moreover, since most common degenerative diseases also have a delayed onset and progressive course, the accumulation of somatic mtDNA mutations would result in the progressive decline of mitochondrial function, which would push the subclinical phenotype of individuals who had inherited a partial mitochondrial defect across their expression thresholds resulting in disease. Thus, somatic mtDNA mutations may be important in both the onset and the progression of the age-related diseases and mtDNA diseases as well (8–10).

The age-related accumulation of somatic mtDNA mutations in post-mitotic human tissues correlates with the age-related decline in mitochondrial OXPHOS enzymes. OXPHOS enzyme activities have been shown to decline with age in human and primate skeletal muscle (440–442), liver (443), and brain (444). The common 5-kbp (4977-np) deletion has been quantitated in these same tissues (445) and shown to accumulate with age in skeletal muscle (446,447), heart (448–450), extraocular muscle (451), the basal ganglia and cerebral cortex of the brain (452,453), and other tissues (454,455). The highest levels of the common deletion are found in the brain, where deletion levels increase over 10,000-fold from young to old individuals, with maximum levels of the 5-kbp deletion in 80-year-olds exceeding 10% in the basal ganglia and 2–3% in the cortex. By contrast, less than 0.0001% of the 5-kbp deletion accumulates with age in the human cerebellum (452,453,456).

The accumulation of the 5-kbp deletion appears to be but the “tip of the iceberg.” Using long-extension

polymerase chain reaction (PCR) (457) to amplify the entire mtDNA in a single fragment, skeletal muscle mtDNAs from subjects under 40 years of age were found to generate virtually all full-length mtDNA molecules. By contrast, skeletal muscle mtDNAs of subjects older than age 75 years gave very few full-length molecules, but instead gave a wide variety of smaller length PCR products, suggesting extensive mtDNA rearrangement (458). Similar results were obtained in a second less extensive study (459). In an attempt to estimate the amount of rearrangement of mtDNA in skeletal muscle, undigested mtDNA was run on agarose gels and analyzed by Southern blotting and hybridization with a total mtDNA probe. Parallel lanes included chronic progressive external ophthalmoplegia–Kearns–Sayre syndrome (CPEO-KSS) patient DNAs with known levels of the 5-kbp deletion. The Southern blots from younger subjects gave complex patterns, indicating that muscle mtDNA must harbor a wide range of mtDNA conformations including different levels of super-coiled, nicked, and concatenated molecules. In addition, the two CPEO-KSS samples had two extra bands, one migrating slightly faster than the linearized 16.5-kbp mtDNA at 11.5 kbp, consistent with linearized deleted mtDNA; and the other migrating faster than any of the slower migrating super-coiled, nicked, and concatenated molecule bands and probably representing super-coiled or nicked deleted mtDNA. The muscle mtDNAs of the old subjects had all of the same bands as the younger subjects, but in addition all of these samples had an additional diffuse band in the same region as the patient's super-coiled or nicked rearranged mtDNA. This suggests that elderly individuals may accumulate a significant level of heterogeneously rearranged mtDNA molecules (458). This same phenomenon has been observed in the mouse, where long-extension PCR revealed increased mtDNA rearrangements in hearts of old mice but not in those of young mice. Southern blots of undigested mtDNA revealed the accumulation of a novel mtDNA band that is present in old but not young animals. Moreover, this band is absent in calorie-restricted animals (460). Similar age-related accumulation of mtDNA rearrangements has been documented in chimp heart (461).

A more general survey of mtDNA base substitutions in brain using denaturing gradient gel electrophoresis, cloning, and sequencing revealed a spectrum of point mutations, and single base insertions and deletions in the D-loop, with 35–41% of the molecules differing in at least one position. Heteroplasmic mutants were much less common in coding regions. Point mutations were observed to increase 2.5-fold and insertion–deletions 7.7-fold between ages 28 and 96–99 years. Two distinctive CR base substitution mutations were T146C and T195C (477) (462). Using a similar technique to analyze the mtDNAs from diploid fibroblasts cultured from individuals of various ages also revealed distinctive CR variants, including T146C, T152C, T195C, A249G, T285C,

A368G, 383i, and T414G. The most prevalent variant was T414G located at the *Tfam* binding site adjacent to LSP. This variant was found in 50% of the fibroblast cultures derived from patients over 65, but not seen in the cultures of younger individuals. In some individuals, the percentage of mutant mtDNAs exceeded 50% (463). In studies of skeletal muscle, a T408A promoter mutation and a A189G O<sub>H</sub> mutation were found to accumulate with age (464); and a T150C O<sub>H</sub> mutation was found to accumulate with age in white blood cells (465). These mtDNA CR mutations appear to be highly tissue specific. The T414G mutation can be detected at low levels in skeletal muscle by using a highly specific and sensitive protein nucleic acid (PNA)-clamping PCR method. However, this same method has been unable to detect the T414G mutation in the brains of even very elderly people (466).

Analysis of the distribution of somatic mtDNA mutations in aging skeletal muscle indicates that individual mutations are regionally localized and thus may be clonally amplified, just as CPEO-KSS rearrangement mutations are differentially distributed along muscle fibers. Histologic analysis of muscle tissues from old subjects has revealed periodic COX-positive and COX-negative regions (467,468). Each COX-negative region was found through *in situ* hybridization either to contain a specific mtDNA deletion clone or to be deficient in mtDNA (451). Similar analysis of individual cardiomyocytes from human hearts has indicated that individual cells have high levels of specific mtDNA rearrangements, while other cells have only normal mtDNA (469). Furthermore, analysis of intestinal crypt cells has revealed that spontaneous somatic mtDNA mutations arise in the *COI* genes in the stem cells at the base of the intestinal crypt with age. These segregate to homoplasmic mutants, resulting in the intestine becoming a mosaic of COX-negative and COX-positive crypts as the individual ages (470). This implies that individual somatic mtDNA mutations arise spontaneously and randomly in cells, but once they occur, they are selectively replicated in that all result in respiratory deficiency. This replication phase would be exponential and might explain why the frequency of specific mtDNA deletions appears to increase exponentially with time (447–449,452).

The origin of the high rate of mtDNA mutations is unknown. However, one likely source of new mutations is the generation and subsequent resolution of DNA oxidation products, including thymine glycols and 8-hydroxy-2-deoxyguanosine (8-OHdG) (452,453,456,471). 8-OHdG is the most common form of oxidative DNA damage, though it may not be the main mtDNA mutagen, since mtDNA base substitutions show a 20-fold bias toward transitions over transversions (472), while 8-OHdG is thought to increase the transversion rate (473–475). mtDNA oxidation products might also cause replication errors, increasing the rate of mtDNA rearrangement mutations.

mtDNA seems to be particularly prone to oxygen radical damage. The mitochondrial respiratory chain is the major source of ROS generation, and mtDNA is attached to the mitochondrial inner membrane in close proximity to the OXPHOS complexes (476). This preferential mtDNA oxidative damage has been confirmed in several studies. In young rats, the mtDNA has been reported to contain ~16-fold more 8-OHdG than the nDNA, and this increases another 3-fold in older rats (477,478). Current estimates place the differential 8-OHdG levels of the mtDNA at 10-fold above the nDNA (476). Similarly, mtDNA 8-OHdG levels in human brain were 10-fold higher than nDNA levels and increased with age (479). Levels of 8-OHdG have been reported to increase with age in the mtDNAs of human diaphragm, heart muscle (450,480), and brain (481), and 8-OHdG and the 7.4-kbp deletion to increase with age by the same exponential kinetics in human hearts (450,482).

The preferential accumulation of oxidation damage in mtDNA might imply that mtDNA is deficient in DNA repair. While mtDNA cannot repair thymine dimers (483), the mitochondria do contain uracil DNA glycosylase (484), AP endonuclease (485), and enzymes to repair pyrimidine glycols (486). A variety of other repair activities have been detected by treating cultured cells with DNA-damaging agents, purifying genomic DNA, digesting with endonucleases specific for each type of damage, and using Southern blots of alkaline agarose gels to monitor the removal of the endonuclease sensitive sites from post-treatment cells. By this procedure, it has been found that the mitochondria cannot repair pyrimidine dimers, but they can repair 8-OHdG and adducts for 4NQO and monofunctional alkylating agents. The mitochondria cannot repair *cis*-platinum intrastrand cross-links, consistent with an absence of thymidine dimer repair, but can repair *cis*-platinum interstrand cross-links, suggesting recombinational repair (487–493). It is surprising that the mitochondria would retain active repair systems, yet experience such a high degree of preferential damage. Presumably, the level of repair is set to be sufficient to keep the mtDNAs intact until after reproductive age, at which point sustaining the parents is no longer necessary.

Somatic mtDNA mutations have been proven to be important in longevity by regulating the mtDNA mutation rate. The mitochondrial production of hydrogen peroxide, the central mitochondrial ROS molecule, was countered by targeting the peroxisomal enzyme catalase to the mitochondrial matrix. This resulted in the extension of the mean and maximum life span of the mouse in association with reduced damage to mtDNA and increased mitochondrial resistance to exogenous ROS damage (494). Therefore, the lifelong accumulation of oxidative damage to the mitochondria and mtDNA is important in determining the rate of aging, a result consistent with the “free radical theory of aging” (495).

In two independent studies in which the mouse nDNA-encoded mtDNA polymerase  $\gamma$  (POLG) gene was

replaced by a mutant gene copy in which the 3'-to-5' proofreading exonuclease was inactivated by introduction of a D257A substitution. In both sets of POLG-mutator mice, the steady-state mtDNA mutation level was increased severalfold and the animals exhibited an array of progeric phenotypes, including shortened life span, weight loss, reduction of subcutaneous fat, hair loss (alopecia), curvature of the spine (kyphosis), osteoporosis, anemia, reduced fertility, and heart enlargement. These mice also showed an age-related decline in mitochondrial OXPHOS enzymes, respiration rate, and energy production (496,497). This confirms that aging is the product of mitochondrial decline, which in these cases was caused by the destruction of mtDNA, and the mitochondrial biosynthetic capacity. Both studies also found that POLG-mutator mice did not have increased mitochondrial ROS production. This places mtDNA damage downstream of the mitochondrial ROS production in the normal aging process.

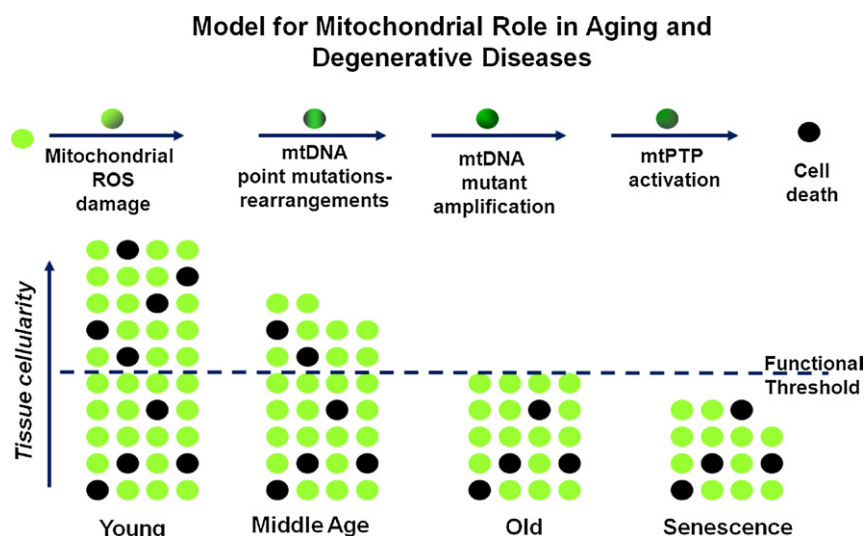
From these observations, we can now refine the pathway involved in the mitochondrial etiology of aging (Figure 11-7). During normal energy production, OXPHOS also generates ROS. This endogenous ROS damages the mitochondrial OXPHOS enzymes, requiring their repair by expression of the genes in the mtDNA. However, in normal animals, ROS also mutagenizes the mtDNA. Ultimately, the percentage of mutant mtDNAs in a cell increases to the point that the remaining mtDNAs are unable to adequately repair the damaged OXPHOS enzymes. At this point, the mitochondrial membrane potential and energy production decline to the point that the mtPTP is activated and the ailing cell is destroyed by apoptosis. Since this progression is the result of random mtDNA mutations, different cells undergo apoptosis at different times. Hence, at any one time only a small fraction of the cells in a tissue are actively undergoing apoptosis (1).

Thus, all of the current evidence supports the hypothesis that the accumulation of mtDNA mutations is the aging clock and that, in most situations, it is the rate of accumulation of oxidative damage that determines the clock rate. Therefore, the longevity of a species will be modulated by the rate of mitochondrial ROS production, the effectiveness of the mitochondrial antioxidant defense systems, the rate of mtDNA mutation, and the effectiveness of mtDNA repair.

## 11.1.22 Inherited mtDNA Diseases

**11.1.22.1 Diseases Resulting from Intragenic Polypeptide Mutations.** Diseases that are caused by mtDNA mutations within a single polypeptide gene are enzyme specific. Consequently, they generally present with more stereotypic clinical phenotypes. Confirmed mtDNA mutations are listed in Tables 11-1–11-3 Panel A, while Panel B refers to reported mtDNA mutations. Confirmed mutations are variants which have been





**FIGURE 11-7** Mitochondrial and cellular model of aging. The upper line of cells shows the mitochondrial role in the energetic life and death of a cell. All post-mitotic cells have a finite probability of dying due to the accumulation of mitochondrial and mtDNA damage associated with OXPHOS ROS production, followed by mutant mtDNA amplification, activation of the mtPTP, and death by apoptosis. The bottom diagram represents the loss of cells in a tissue over the life of an individual through mitochondrial mediated death, black cells. Each individual is born with sufficient extra cells such that even as cells are lost by mitochondrial induced apoptosis, sufficient cells remain to maintain tissue function. Ultimately, however, enough cells are lost that the organ begins to malfunction resulting in the clinical manifestations of old age. The minimum number of cells for the tissue to function normally is indicated by the dashed line (1).

identified in two different laboratories or reports of unrelated families. Additional publications supporting these pathogenic mutations, as well as over 230 other potentially pathogenic mtDNA variants can be found at <http://www.mitomap.org/>.

The first maternally inherited mtDNA polypeptide mutation disease discovered was LHON (4). LHON is generally associated with missense mutations in one of the mtDNA complex I polypeptides (Table 11-1).

#### 11.1.22.2 Leber Hereditary Optic Neuropathy.

LHON is a form of acute or subacute, bilateral, midlife-onset central vision loss associated with the degeneration of the retinal ganglion cell (RGC) layer and optic nerve, resulting in central scotoma. The age of onset is generally in the 20s and 30s, though onset of symptoms can occur from childhood to late adulthood. Typically, the onset and progression of blindness is relatively rapid, with both eyes developing vision loss within a year. The disease is frequently familial, and in all familial cases the affected individuals are related through the maternal lineage. There is a predilection for males to be affected, with the ratio of affected males to females reaching 4:1 in some instances (498–502). While most mtDNA LHON pedigrees manifest primarily LHON, the more severe mutations can exhibit other neurodegenerative disease symptoms. A typical pedigree showing the maternal inheritance of LHON is presented in Figure 11-8

Since the discovery of the MTND4\*LHON11778A mutation in 1988 (4), LHON has been associated with 30 missense mutations. Of these, 15 have been observed in more than one patient or family. Hence, these 15 mutations can be considered to be pathogenic causes of

LHON. These LHON mutations are all complex I gene missense mutations and include MTND1\*LHON 3635A, MTND1\*LHON 3700A, MTND1\*LHON3733A, MTND1\*LHON4171A, MTND4L\*LHON 10663C, MTND6\*LDYT14459A, MTND6\*LHON14482A and 14482G, MTND6\*LHON 14495G, MTND6\*LHON 14502C, and MTND6\*LHON 14568T (see Table 11-1). The remaining 20 sequence variants that have been associated with LHON are considered preliminary since they have only been seen one time, co-occurred with another “pathogenic” mutation, and/or were only found as a homoplasmic variant (see [www.mitomap.org](http://www.mitomap.org) for more information).

Of the top 14 LHON mutations, 3 account for over 95% of cases in Europe (see Table 11-1). These are MTND4\*LHON11778A (69% in European but 95% in Asia) (503), MTND1\*LHON3460A (13%), and MTND6\*LHON14484C (14%). The remaining 11 confirmed mutations are much more rare.

In addition, some of these pathogenic mutations considered as mild pathogenic LHON mutations, including MTND4\*LHON11778A, MTND6\*LHON14484C, and MTND3\*LHON10663C, are commonly associated with the European haplogroup J. This association can now be understood as the result of an interaction between the mild energetic defects associated with the adaptive mutations of this temperate zone haplogroup and the added energetic defect imparted by the recent LHON mutation (428,430,431).

**11.1.22.3 LHON as the Primary Clinical Manifestation.** The three common pathogenic LHON mutations—MTND4\*LHON11778A (4), MTND1\*LHON3460A

TABLE 11-1 Leber's Hereditary Optic Neuropathy (LHON) Disease Mutations									
Top LHON Mutations									
Mutation <sup>a</sup>	Nt Δ	AA Δ	AA Cons <sup>b</sup>	Approximate % Patients	% Ctrls	Het. <sup>c</sup>	Penetrance% Relatives	Penetrance % Males	% R
<b>MTND4*<sup>LHON</sup> 11778A</b>	G-A	R340H	H	69	0	+/-	33-60	82	4
<b>MTND1*<sup>LHON</sup> 3460A</b>	G-A	A52T	M	13	0	+/-	14-75	40-80	22
<b>MTND6*<sup>LHON</sup> 14484C</b>	T-C	M64V	L	14	0	+/-	27-80	68	37-6
MTND1* <sup>LHON</sup> 3635A	G-A	S110N	H	Rare	0	+/-	29 (range 11-64)	54 (range 25-100)	Low
MTND1* <sup>LHON</sup> 3700A	G-A	A112T	H	Rare	0	—	NA	NA	UN
MTND1* <sup>LHON</sup> 3733A	G-A	E143K	H	Rare	0	+/-	24-30	36-44	Yes
MTND1* <sup>LHON</sup> 4171A	C-A	L289M	H	Rare	0	+/-	46	47	Yes
MTND4L* <sup>LHON</sup> 10663C	T-C	V65A	L	Rare	0	-	56	60	UN
MTND6* <sup>LDYT</sup> 14459A	G-A	A72V	M	Rare	0	+	NA	NA	Low
MTND6* <sup>LHON</sup> 14482A	C-A	M64I	L	Rare	0	+/-	NA	89	Yes
MTND6* <sup>LHON</sup> 14482G	C-G	M64I	L	Rare	0	-	NA	NA	UN
MTND6* <sup>LHON</sup> 14495G	A-G	L60S	H	Rare	0	+	NA	NA	Low
MTND6* <sup>LHON</sup> 14502C	T-C	I58V	H	Rare	0	-	14502: 10% 14502+11778: 37%	14502: 11% 14502+11778: 47%	UN
MTND6* <sup>LHON</sup> 14568T	C-T	G36S	M	Rare	0	-	NA	NA	UN

More Publications supporting these pathogenic mutations, as well as over 50 other possibly pathogenic variants in LHON patients, can be found at <http://www.mitomap.org/MITOL>. Penetrance values are rough estimates.

Low, anecdotal low degree of vision recovery; Yes, anecdotal moderate to high degree of vision recovery; UN, unknown; NA, not applicable.

<sup>a</sup>The first three mutations listed (in boldface and shaded in gray) represent ~95% of all cases. The remaining mutations are listed in nucleotide order.

<sup>b</sup>H, high amino acid conservation; M, moderate amino acid conservation; L, low amino acid conservation; NA, not applicable; Ter, termination codon.

<sup>c</sup>Het., heteroplasmy: +, detected; -, not detected.

**TABLE 11-2    Reported Mitochondrial DNA Polypeptide Gene Disease Mutations, Non-LHON**

**Panel A: The Top Pathogenic mtDNA Polypeptide Missense Mutations, Non-LHON**

Syndromes	Locus	Disease	Allele	Nt	AA	Ho
<i>Dystonia/Leigh syndrome</i>						
Leigh syndrome	MTATP6	LS/NARP	T8993C	T-C	L156P	–
Leigh syndrome	MTATP6	NARP	T8993G	T-G	L156R	–
Leigh syndrome	MTATP6	LS	T9176G	T-G	L217R	+
Leigh syndrome	MTATP6	LS/FBSN	T9176C	T-C	L217P	+
Leigh syndrome	MTATP6	LS/Ataxia/NARP-like disease	T9185C	T-C	L220P	+
Leigh syndrome	MTND3	LS	T10158C	T-C	S34P	+
Leigh syndrome	MTND3	LS /LS-like disease/ESOC	T10191C	T-C	S45P	–
Leigh syndrome	MTND3	LS/dystonia/stroke	G10197A	G-A	A-T	+
Leigh syndrome	MTND4	LS	C11777A	C-A	R340S	–
Leigh syndrome	MTND5	LS	T12706C	T-C	F124L	–
Dystonia/Leigh syndrome	MTND6	LDYT/LS	G14459A	G-A	A72V	+
Dystonia/Leigh syndrome	MTND6	LS/dystonia/ataxia	T14487C	T-C	M63V	–
<i>Encephalomyopathy</i>						
Encephalomyopathy, MELAS	MTND1	MELAS/LS	G3697A	G-A	G131S	–
Encephalomyopathy, MELAS	MTND5	MELAS/LS	G13513A	G-A	D393N	–
Encephalomyopathy, MELAS	MTND5	MELAS	A13514G	A-G	D393G	–

**Panel B: Representative Reported Pathogenic Polypeptide Mutations, Non-LHON, For Various Phenotypic Presentations  
Selected from Over 250 Reported Variants**

Syndromes	Locus	Disease	Allele	Nucleotide Change	AA Change	Ho	He	Status
<i>Dystonia/Leigh syndrome</i>								
Dystonia	MTND1	Adult-onset dystonia	A3796G	A-G	T164A	–	+	Rep
Leigh syndrome	MTATP6	LS/FBSN	T9176C	T-C	L217P	+	+	Rep
Leigh syndrome	MTATP6	LS	T9176G	T-G	L217R	–	+	Rep
Leigh syndrome	MTATP6	LS	T9185C	T-C	L220P	–	+	Rep
Leigh syndrome	MTATP6	LS	T9191C	T-C	L222P	–	+	Rep
Leigh syndrome	MTCO3	LS-like	C9537insC	C-CC	Q111frameshift	+	–	Rep
<i>Encephalomyopathy</i>								
Encephalomyopathy MELAS	MTND1	MELAS	T3308C	T-C	M1T	–	+	Rep
Encephalomyopathy MELAS	MTND1	MELAS/LHON	G3376A	G-A	E24K	–	+	Rep
Encephalomyopathy MELAS	MTND1	MELAS	G3697A	G-A	G131S	–	+	Rep
Encephalomyopathy MELAS	MTND1	MELAS	G3946A	G-A	E214K	+	+	Rep
Encephalomyopathy MELAS	MTND1	MELAS	T3949C	T-C	Y215H	–	+	Rep
Encephalomyopathy MELAS	MTND4	MELAS	A11084G	A-G	T109A	+	+	P.M.
Encephalomyopathy MELAS	MTND5	MELAS	A12770G	A-G	E145G	–	+	Rep
Encephalomyopathy MELAS	MTND5	MELAS/LHON/ LS overlap syndrome	A13045C	A-C	M237L	–	+	Rep
Encephalomyopathy MELAS	MTND5	MELAS/LS	A13084T	A-T	S250C	–	+	Rep
Encephalomyopathy MELAS	MTND6	MELAS	G14453A	G-A	A74V	–	+	Rep

TABLE 11-2    Reported Mitochondrial DNA Polypeptide Gene Disease Mutations, Non-LHON—Cont'd								
Panel B: Representative Reported Pathogenic Polypeptide Mutations, Non-HLON, For Various Phenotypic Presentations Selected from Over 250 Reported Variants								
Syndromes	Locus	Disease	Allele	Nucleotide Change	AA Change	Ho	He	Status
Encephalomyopathy MELAS	MTCYB	MELAS/PD	14787del4	TTAA-del	I14frameshift	–	+	Rep
Encephalopathy epilepsy	MTCO1	Therapy-resistant epilepsy	C6489A	C-A	L196I	–	+	Rep
Encephalomyopathy multisystem disorder	MTCO1	Multisystem disorder	G6930A	G-A	G343Ter	–	+	Rep
Encephalomyopathy multisystem disorder	MTCOI	Myopathy and cortical lesions	6015del5	Del 5 bp	Frameshift, 42 peptide		+	Rep
Encephalomyopathy	MTCO2	Encephalomyopathy	T7587C	T-C	M1T	–	+	Rep
Encephalomyopathy multisystem disorder	MTCO2	Multisystem disorder	G7896A	G-A	W104Ter	–	+	Rep
Encephalomyopathy lactic acidosis	MTCO2	Lactic acidosis	8042del2	AT-del	M153Ter	–	+	Rep
Encephalomyopathy	MTCO3	Encephalomyopathy	G9952A	G-A	W248Ter	–	+	Rep
Encephalomyopathy MELAS	MTCO3	MELAS/PEM/NAION	T9957C	T-C	F251L	–	+	Rep
Encephalomyopathy lactic acidosis	MTATP6	Lactic acidosis/seizures	9205del2	TA-del	Ter227M	+	–	Rep
Encephalomyopathy multisystem disorder	MTCYB	Multisystem disorder	A15579G	A-G	Y278C	–	+	Rep
Encephalomyopathy septo-optic dysplasia	MTCYB	Septo–optic dysplasia	T14849C	T-C	S35P	–	+	Rep
<i>Mitochondrial myopathy</i>								
Mitochondrial myopathy exercise intolerance	MTCYB	EXIT	G14846A	G-A	G34S	–	+	Rep
Mitochondrial myopathy	MTCYB	MM	G15059A	G-A	G190Ter	–	+	Rep
Mitochondrial myopathy exercise intolerance	MTCYB	EXIT	G15084A	G-A	W113Ter	–	+	Rep
Mitochondrial myopathy exercise intolerance	MTCYB	EXIT	G15150A	G-A	W135Ter	–	+	Rep
Mitochondrial myopathy exercise intolerance	MTCYB	EXIT	G15168A	G-A	W141Ter	–	+	Rep
Mitochondrial myopathy exercise intolerance	MTCYB	EXIT	T15197C	T-C	S151P	–	+	Rep
Mitochondrial myopathy exercise intolerance	MTCYB	EXIT/Encephalomyopathy	G15242A	G-A	G166Ter	–	+	Rep
Mitochondrial myopathy exercise intolerance	MTCYB	EXIT	G15497A	G-A	G251S	+	–	Rep
Mitochondrial myopathy exercise intolerance	MTCYB	EXIT	15498del24	24 bp deletion-	251GDPDNYTL-del258	–	+	Rep
Mitochondrial myopathy exercise intolerance	MTCYB	EXIT	G15615A	G-A	G290D	–	+	Rep
Mitochondrial myopathy exercise intolerance	MTCYB	EXIT	G15723A	G-A	W326Ter	–	+	Rep



Mitochondrial myopathy	MTCYB	MM	G15762A	G-A	G339E	–	+	Rep
Mitochondrial myopathy CPEO	MTND4	CPEO	T11232C	T-C	L140P	–	+	Rep
Mitochondrial myopathy exercise intolerance	MTND4	EXIT	G11832A	G-A	W358Ter	–	+	Rep
Mitochondrial myopathy exercise intolerance	MTCO1	EXIT/myoglobinuria	G5920A	G-A	W6Ter	–	+	Rep
Mitochondrial myopathy	MTCO1	MM and rhabdomyolysis	G6708A	G-A	G269Ter	–	+	Rep
Mitochondrial myopathy	MTCO2	MM	T7671A	T-A	M29K	–	+	Rep
Mitochondrial myopathy exercise intolerance	MTCO2	EXIT/rhabdomyolysis	T7989C	T-C	L135P	–	+	Rep
Mitochondrial myopathy	MTCO3	Myopathy and myoglobinuria	9487del15	Del 15 bp	Removed 5 aa		+	Rep
<i>Hypertrophic cardiomyopathy</i>								
Hypertrophic cardiomyopathy	MTCYB	HCM	G15243A	G-A	G166E	–	+	Rep
Hypertrophic cardiomyopathy	MTCYB	HCM	G15498A	G-A	G251D	–	+	Rep
<i>Deafness/sensorineural hearing loss</i>								
Deafness	MTCO1	DEAF	A7443G	A-G	Ter514G	+	–	Rep
Deafness	MTCO1	DEAF	A7445C	A-C	Ter514S	+	–	Rep
Deafness sensory neural hearing loss	MTCO1	SNHL/LHON	G7444A	G-A	Ter514K	+	–	Rep
Deafness sensory neural hearing loss	MTCO2	SNHL	A8108G	A-G	I175V	+	–	Rep
Deafness sensory neural hearing loss	MTND6	SNHL	C14340T	C-T	V112M	+	–	Rep
<i>Hypertrophic cardiomyopathy</i>								
Diabetes mellitus	MTND4	DM	A12026G	A-G	I423V	+	–	Rep
<i>Alzheimer and Parkinson diseases</i>								
Alzheimer and Parkinson disease	MTND1	ADPD	A3397G	A-G	M31V	+	–	Rep
Alzheimer and Parkinson disease	MTND2	AD	G5460A	G-A	A331T	+	+	P.M.
Alzheimer and Parkinson disease	MTND2	AD	G5460T	G-T	A331S	+	+	Rep
<i>Hypertrophic cardiomyopathy</i>								
Idiopathic sideroblastic anemia	MTCO1	SIDA	T6721C	T-C	M273T	–	+	Rep
Idiopathic sideroblastic anemia	MTCO1	SIDA	T6742C	T-C	I280T	–	+	Rep

Note: See <http://www.mitomap.org/MITOMAP/MutationsCodingControl> for additional reports.

AD, Alzheimer disease; ADPD, Alzheimer plus Parkinson disease; CPEO, chronic progressive ophthalmoplegia; DEAF, deafness, sensorineural hearing loss; ESOC, epilepsy, strokes; EXIT, exercise intolerance; FBSN, familial bilateral striatal necrosis; HCM, hypertrophic cardiomyopathy; MM, mitochondrial myopathy; MERRF, myoclonic epilepsy and ragged red fibers; NAION, nonarteritic anterior ischemic optic neuropathy; NARP, neurogenic muscle weakness, ataxia, and retinitis pigmentosa; PEO, progressive external ophthalmoplegia; PD, Parkinson disease; SNHL, sensory neural hearing loss; SIDA, sideroblastic anemia; P.M., (point mutation); Rep, reported/provisional mutations that have been reported one or a few times in association with disease but are not confirmed as pathogenic; some published reports have determined the mutation to be a nonpathogenic population variant.

**TABLE 11-3 Pathogenic mtDNA Mutations Resulting from Mutations in the rRNA and tRNA Protein Synthesis Genes**

Panel A: The Top mtDNA Protein Synthesis (rRNA and tRNA) Gene Mutations						
Syndromes	Locus	Disease	Allele	RNA	Ho	He
<b>Cardiomyopathy</b>						
Mitochondrial myopathy and cardiomyopathy	MTTL1	MMC	A3260G	tRNA Leu (UUR)	–	+
Mitochondrial myopathy and cardiomyopathy	MTTL1	MMC	C3303T	tRNA Leu (UUR)	+	+
Maternally inherited cardiomyopathy	MTTI	MICM	A4300G	tRNA Ile	+	+
<b>Deafness/sensorineural hearing loss</b>						
Deafness	MTRNR1	DEAF	C1494T	12S rRNA	+	–
Deafness	MTRNR1	DEAF	A1555G	12S rRNA	+	–
Deafness/sensorineural hearing loss	MTTS1	SNHL	A7445G	tRNA Ser (UCN)	+	+
	precursor			precursor		
Deafness/sensorineural hearing loss	MTTS1	SNHL	T7511C	tRNA Ser (UCN)	+	+
<b>Diabetes mellitus</b>						
Diabetes mellitus and deafness	MTTL1	DM/DMDF/MIDD/SNHL/FSGS/ Cardiac + multi-organ dysfunction	A3243G	tRNA Leu (UUR)	–	+
<b>Encephalomyopathy</b>						
Encephalomyopathy, MELAS	MTTF	MELAS/ MM and EXIT	G583A	tRNA Phe	–	+
Encephalomyopathy, ataxia, myoclonus, and deafness	MTTV	AMDF	G1606A	tRNA Val	–	+
Encephalomyopathy, MELAS	MTTL1	MELAS/LS	A3243G	tRNA Leu (UUR)	–	+
Encephalomyopathy, MELAS	MTTL1	MELAS	C3256T	tRNA Leu (UUR)	–	+
Encephalomyopathy, MELAS	MTTL1	MELAS	T3271C	tRNA Leu (UUR)	–	+
Encephalomyopathy, MELAS	MTTL1	MELAS/myopathy/deafness + cognitive impairment	T3291C	tRNA Leu (UUR)	–	+
Encephalomyopathy, MELAS	MTTQ	MELAS/encephalopathy	G4332A	tRNA Gln	–	+
Encephalomyopathy, Leigh syndrome	MTTW	MILS	A5537insT	tRNA Trp	–	+
Encephalomyopathy	MTTS1	PEM/AMDF/motor neuron disease-like	C7472insC C7471CC (=‘7472insC’)	tRNA Ser (UCN)	+	+
Encephalomyopathy, MERRF	MTTK	MERRF	A8344G	tRNA Lys	–	+
Encephalomyopathy, MERRF	MTTK	MERRF	T8356C	tRNA Lys	–	+
Encephalomyopathy, MERRF	MTTK	MERRF/MICM + DEAF/autism/ LS/ataxia + lipomas	G8363A	tRNA Lys	–	+
Encephalomyopathy	MTTG	PEM	T10010C	tRNA Gly	–	+
Encephalomyopathy, MERRF	MTTH	MERRF-MELAS/cerebral edema	G12147A	tRNA His	–	+
<b>Mitochondrial myopathy</b>						
Mitochondrial myopathy	MTTL1	MM/CPEO	A3243G	tRNA Leu (UUR)	–	+
Mitochondrial myopathy	MTTL1	MM	A3302G	tRNA Leu (UUR)	–	+
Mitochondrial myopathy, CPEO	MTTI	CPEO/MS	G4298A	tRNA Ile	–	+
Mitochondrial myopathy, CPEO	MTTI	CPEO	G4308A	tRNA Ile	–	+
Mitochondrial myopathy	MTTA	Myopathy	G5650A	tRNA Ala	–	+
Mitochondrial myopathy, CPEO	MTTN	CPEO/MM	G5703A	tRNA Asn	–	+
Mitochondrial myopathy	MTTS1	MM / EXIT	G7497A	tRNA Ser (UCN)	+	+
Mitochondrial myopathy, CPEO	MTTL2	CPEO / KSS	G12315A	tRNA Leu (CUN)	–	+
Mitochondrial myopathy	MTTE	Reversible COX deficiency myopathy	T14674C	tRNA Glu	+	–
Mitochondrial myopathy	MTTE	MM+DM/encephalomyopathy	T14709C	tRNA Glu	+	+

Panel B: Representative Reported Pathogenic Protein Synthesis Mutations for Various Phenotypic Presentations Selected from Over							
Syndromes	Locus	Disease	Allele	RNA	Ho	He	Status
<b>Encephalomyopathy</b>							
Encephalomyopathy, Leigh syndrome	MTTV	LS	C1624T	tRNA Val	+	–	Rep
Encephalomyopathy, Leigh syndrome	MTTV	Adult LS	G1644T	tRNA Val	–	+	Rep
Encephalomyopathy Leigh syndrome	MTTW	MILS	A5537insT	tRNA Trp	–	+	Rep
Encephalomyopathy MERRF	MTTF	MERRF	G611A	tRNA Phe	–	+	Rep
Encephalomyopathy MERRF	MTTK	MERRF	G8361A	tRNA Lys	–	+	Rep
Encephalomyopathy MERRF	MTTK	MERRF/MICM+ DEAF/autism	G8363A	tRNA Lys	–	+	Rep
Encephalomyopathy MERRF	MTTL1	MERRF/KSS overlap	G3255A	tRNA Leu (UUR)	–	+	Rep
Encephalomyopathy myoclonus and psychomotor regression	MTTD	MEPR	A7543G	tRNA Asp	–	+	Rep
Encephalomyopathy ataxia, myoclonus and deafness	MTTV	AMDF	G1606A	tRNA Val	–	+	Rep
Encephalomyopathy MELAS	MTTL1	MELAS	G3244A	tRNA Leu (UUR)	–	+	Rep
Encephalomyopathy MELAS	MTTL1	MELAS	A3252G	tRNA Leu (UUR)	–	+	Rep
Encephalomyopathy MELAS	MTTL1	MELAS/myopathy	T3258C	tRNA Leu (UUR)	–	+	Rep
Encephalomyopathy MELAS	MTTL1	MELAS	T3291C	tRNA Leu (UUR)	–	+	Rep
Encephalomyopathy MELAS	MTTV	MELAS	G1642A	tRNA Val	–	+	Rep
Encephalomyopathy MELAS	MTTF	MELAS	G583A	tRNA Phe	–	+	Rep
Encephalomyopathy MELAS	MTRNR2	MELAS	C3093G	16S rRNA	–	+	Rep
Encephalomyopathy	MTTL1	PEM	T3271delT	tRNA Leu (UUR)	–	+	Rep
Encephalopathy	MTTL1	Encephalomyopathy	C3287A	tRNA Leu (UUR)	–	+	Rep
Encephalomyopathy	MTTI	Progressive encephalopathy	T4290C	tRNA Ile	+	+	Rep
Encephalomyopathy	MTTI	Mitochondrial encephalo- cardiomyopathy	C4320T	tRNA Ile	–	+	Rep
Encephalomyopathy	MTTW	Encephalomyopathy	G5540A	tRNA Trp	–	+	Rep
Encephalomyopathy	MTTN	Encephalomyopathy	T5693C	tRNA Asn	+	–	Rep
Encephalomyopathy	MTTC	Encephalopathy	T5814C	tRNA Cys	–	+	Rep
Encephalomyopathy	MTTC	Progressive dystonia	A5816G	tRNA Cys	+	–	Rep
Encephalomyopathy	MTTS1	PEM/MERME	T7512C	tRNA Ser (UCN)	+	+	Rep
Encephalomyopathy	MTTK	Mitochondrial encephalopathy	G8328A	tRNA Lys	–	+	Rep
Encephalomyopathy	MTTK	Dystonia and stroke- like episodes	A8332G	tRNA Lys	+	–	Rep
Encephalomyopathy	MTTG	PEM	T10010C	tRNA Gly	–	+	Rep
Encephalopathy	MTTR	Progressive encephalopathy	A10438G	tRNA Arg	–	+	Rep
Encephalopathy	MTTE	Mitochondrial encephalomy- opathy	C14680A	tRNA Glu	–	+	Rep
Encephalopathy	MTTE	Progressive encephalopathy	A14696G	tRNA Glu	–	+	Rep

**TABLE 11-3 Pathogenic mtDNA Mutations Resulting from Mutations in the rRNA and tRNA Protein Synthesis Genes—C**

**Panel B: Representative Reported Pathogenic Protein Synthesis Mutations for Various Phenotypic Presentations Selected from Over**

Syndromes	Locus	Disease	Allele	RNA	Ho	He	Status
Encephalopathy	MTTE	Mitochondrial leuko-encephalopathy	G14724A	tRNA Glu	–	+	Rep
Encephalopathy	MTTE	Encephalomyopathy + retinopathy	G14740A	tRNA Glu	–	+	Rep
Encephalomyopathy	MTATT	Encephalomyopathy	G15915A	tRNA Thr	–	+	Rep
Encephalopathy	MTTP	MERRF-like disease	G15967A	tRNA Pro	–	+	Rep
Encephalopathy	MTTP	Ataxia+RP+deafness	C15975T	tRNA Pro	–	+	Rep
Encephalomyopathy Rett syndrome	MTRNR2	Rett syndrome	C2835T	rRNA 16S	–	+	Rep
Multisystem disease	MTTI	Varied familial presentation	G4284A	tRNA Ile	–	+	Rep
Encephalomyopathy gastrointestinal reflux and sudden infant death syndrome	MTTG	GER/SIDS	A10044G	tRNA Gly	–	+	Rep
<b>Mitochondrial myopathy</b>							
Mitochondrial myopathy	MTTF	MM	T582C	tRNA Phe	–	+	Rep
Mitochondrial myopathy	MTTF	MM	T618C	tRNA Phe	–	+	Rep
Mitochondrial myopathy	MTTF	EXIT and deafness	G622A	tRNA Phe	–	+	Rep
Mitochondrial myopathy	MTTF	Ataxia, PEO, deafness	T642C	tRNA Phe	–	+	Rep
Mitochondrial myopathy	MTTL1	MM	G3242A	tRNA Leu (UUR)	+	–	Rep
Mitochondrial myopathy	MTTL1	MM/CPEO	T3250C	tRNA Leu (UUR)	–	+	Rep
Mitochondrial myopathy	MTTL1	MM	A3251G	tRNA Leu (UUR)	–	+	Rep
Mitochondrial myopathy	MTTL1	MM	C3254G	tRNA Leu (UUR)	–	+	Rep
Mitochondrial myopathy	MTTL1	Myopathy	A3280G	tRNA Leu (UUR)	–	+	Rep
Mitochondrial myopathy	MTTL1	Myopathy	A3288G	TRNA Leu(UUR)	–	+	Rep
Mitochondrial myopathy	MTTI	MM	A4267G	tRNA Ile	–	+	Rep
Mitochondrial myopathy	MTTI	CPEO	A4302G	tRNA Ile	–	+	Rep
Mitochondrial myopathy	MTTQ	Myopathy	T4370AT	tRNA Gln	–	+	Rep
Mitochondrial myopathy	MTTM	MM	T4409C	tRNA Met	–	+	Rep
Mitochondrial myopathy	MTTM	MM	G4450A	tRNA Met	–	+	Rep
Mitochondrial myopathy	MTTW	MM	G5521A	tRNA Trp	–	+	Rep
Mitochondrial myopathy	MTTW	MM	T5543C	tRNA Trp	–	+	Rep
Mitochondrial myopathy	MTTW	MM	T5543C	tRNA Trp	–	+	Rep
Mitochondrial myopathy	MTTW	Myopathy	T5567C	tRNA Trp	–	+	Rep
Mitochondrial myopathy	MTTA	Myopathy	G5591A	tRNA Ala	–	+	Rep
Mitochondrial myopathy	MTTA	PEO	T5636C	tRNA Ala	–	+	Rep
Mitochondrial myopathy	MTTS1	PEO	G7458A	tRNA Ser (UCN)	–	+	Rep
Mitochondrial myopathy	MTTS1	MM	T7480G	tRNA Ser (UCN)	–	+	Rep
Mitochondrial myopathy	MTTS1	MM	G7497A	tRNA Ser (UCN)	+	+	Rep
Mitochondrial myopathy	MTTD	Mitochondrial myopathy	A7526G	tRNA Asp	–	+	Rep
Mitochondrial myopathy	MTTK	Myopathy	T8355C	tRNA Lys	–	+	Rep



Mitochondrial myopathy	MTTK	Myopathy	T8362G	tRNA Lys	–	+	Rep
Mitochondrial myopathy	MTTR	Mitochondrial myopathy	G10406A	tRNA Arg	–	+	Rep
Mitochondrial myopathy	MTTL2	CPEO	G12316A	tRNA Leu (CUN)	–	+	Rep
Mitochondrial myopathy	MTTL2	MM	A12320G	tRNA Leu (CUN)	–	+	Rep
Mitochondrial myopathy	MTTE	Reversible COX deficiency myopathy	T14674G	tRNA Glu	+	–	Rep
Mitochondrial myopathy	MTTE	Mitochondrial myopathy w respiratory failure	A14687G	tRNA Glu	+	–	Rep
Mitochondrial myopathy	MTTE	CPEO + Myopathy	T14723C	tRNA Glu	–	+	Rep
Mitochondrial myopathy	MTTE	EXIT	G14739A	tRNA Glu	–	+	Rep
Mitochondrial myopathy	MTTT	MM	T15940delT	tRNA Thr	+	–	Rep
Mitochondrial myopathy	MTTP	MM	C15990T	tRNA Pro	–	+	Rep
Mitochondrial myopathy	MTTP	Mitochondrial cytopathy	T16002C	tRNA Pro	–	+	Rep
Mitochondrial myopathy/encephalopathy	MTTS2	Myopathy/encephalopathy	G12207A	tRNA Ser (AGY)	–	+	Rep
Mitochondrial myopathy and cardiomyopathy	MTTL1	MMC	C3303T	tRNA Leu (UUR)	+	+	Rep
Mitochondrial myopathy cytopathy	MTTY	Mitochondrial cytopathy/ FSGS	A5843G	tRNA Tyr	+	–	Rep
Mitochondrial myopathy cytopathy	MTTK	Mitochondrial cytopathy	A8326G	tRNA Lys	–	+	Rep
Mitochondrial myopathy cytopathy	MTTP	Mitochondrial cytopathy	G15995A	tRNA Pro	–	+	Rep
Mitochondrial Myopathy lethal infantile	MTTT	LIMM	A15923G	tRNA Thr	nd	–	Rep
Mitochondrial myopathy Mitochondrial neurogastrointestinal encephalomyopathy	MTTK	MNGIE	G8313A	tRNA Lys	–	+	Rep
Mitochondrial myopathy with chronic intestinal pseudo-obstruction	MTTG	CIPO	A10006G	tRNA Gly	Nd	nd	Rep
Mitochondrial myopathy with chronic intestinal pseudo-obstruction	MTTS2	CIPO	C12246G	tRNA Ser (AGY)	Nd	nd	Rep
Mitochondrial myopathy with myoglobinuria	MTTF	Myoglobinuria	A606G	TRNA Phe	–	+	Rep
Mitochondrial myopathy with renal dysfunction	MTTF	Tubulo-interstitial nephritis	A608G	tRNA Phe	+	–	Rep
Mitochondrial myopathy, CPEO	MTTL1	CPEO	C3254T	tRNA Leu (UUR)	+	–	Rep
Mitochondrial myopathy, CPEO	MTTI	CPEO	T4274C	tRNA Ile	–	+	Rep
Mitochondrial myopathy, CPEO	MTTI	CPEO	T4285C	tRNA Ile	–	+	Rep
Mitochondrial myopathy, CPEO	MTTI	CPEO/MS	G4298A	tRNA Ile	–	+	Rep
Mitochondrial myopathy, CPEO	MTTI	CPEO	G4309A	tRNA Ile	–	+	Rep
Mitochondrial myopathy, CPEO	MTTA	CPEO	T5628C	tRNA Ala	–	+	Rep
Mitochondrial myopathy, CPEO	MTTN	CPEO/MM	T5692C	tRNA Asn	–	+	Rep
Mitochondrial myopathy, CPEO	MTTN	CPEO/MM	G5698A	tRNA Asn	–	+	Rep
Mitochondrial myopathy, CPEO	MTTN	CPEO/MM	G5703G	tRNA Asn	–	+	Rep

**TABLE 11-3 Pathogenic mtDNA Mutations Resulting from Mutations in the rRNA and tRNA Protein Synthesis Genes—C**

**Panel B: Representative Reported Pathogenic Protein Synthesis Mutations For Various Phenotypic Presentations Selected from Over**

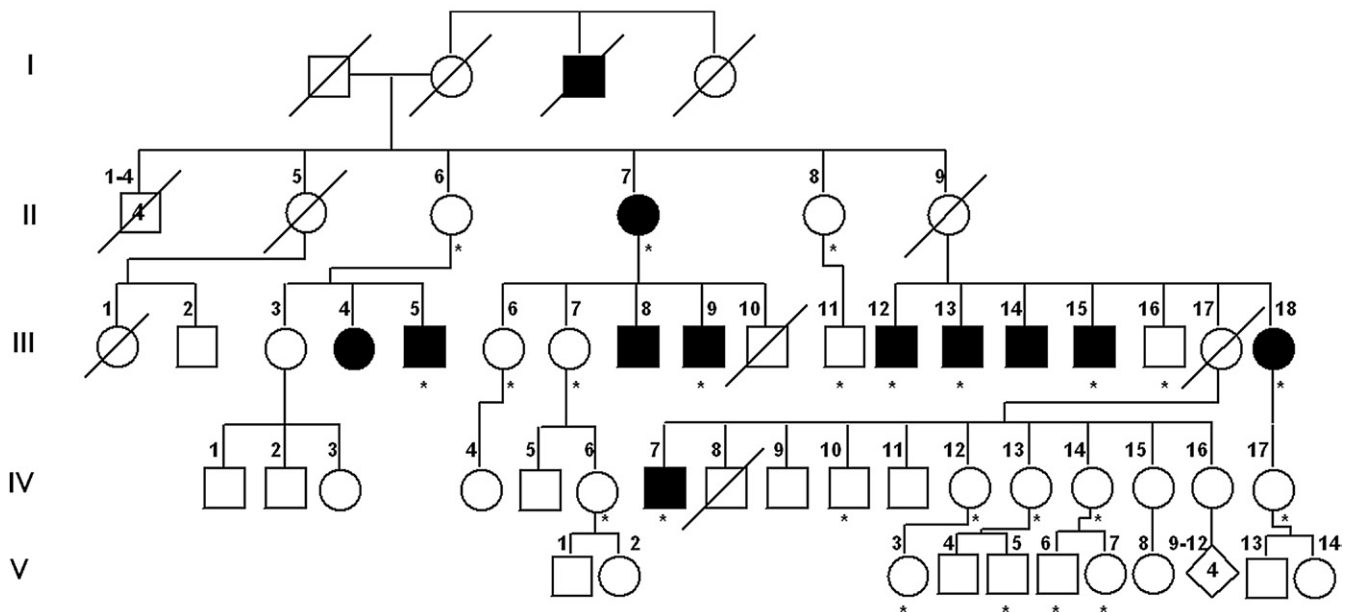
Syndromes	Locus	Disease	Allele	RNA	Ho	He	Status
Mitochondrial myopathy, CPEO	MTTK	CPEO + Myoclonus	G8342A	tRNA Lys	–	+	Rep
Mitochondrial myopathy, CPEO	MTTL2	CPEO	G12294A	tRNA Leu (CUN)	–	+	Rep
Mitochondrial myopathy, CPEO	MTTL2	CPEO	T12311C	tRNA Leu (CUN)	+	+	Rep
Mitochondrial myopathy, CPEO	MTTL2	CPEO	G12315A	tRNA Leu (CUN)	–	+	Rep
Mitochondrial myopathy, exercise intolerance	MTTY	Exercise intolerance	T15940G	tRNA Tyr	–	+	Rep
Mitochondrial myopathy, Gastrointestinal syndrome	MTTW	Gastrointestinal syndrome	G5532A	tRNA Trp	–	+	Rep
Mitochondrial myopathy, KSS	MTTL1	KSS	G3249A	tRNA Leu (UUR)	–	+	Rep
Mitochondrial myopathy, ocular myopathy	MTTL1	Ocular myopathy	T3273C	tRNA Leu (UUR)	–	+	Rep
<b>Cardiomyopathy</b>							
Maternally inherited hypertrophic cardiomyopathy	MTTI	MHCM	A4295G	tRNA Ile	–	+	Rep
Maternally inherited cardiomyopathy	MTTI	MICM	A4300G	tRNA Ile	–	+	Rep
Cardiomyopathy	MTTW	HCM, severe multisystem disorder	C5545T	tRNA Trp	–	+	Rep
Cardiomyopathy	MTTK	Cardiomyopathy	A8348G	tRNA Lys	–	+	Rep
Maternally inherited hypertrophic cardiomyopathy	MTTG	MHCM	T9997C	tRNA Gly	Nd	+	Rep
Maternally inherited cardiomyopathy	MTTH	MICM	G12192A	tRNA His	+	–	Rep
Cardiomyopathy	MTTL2	Dilated cardiomyopathy	T12297C	tRNA Leu (CUN)	–	+	Rep
Fatal infantile cardiomyopathy plus (MELAS)	MTTI	FICP	A4269G	tRNA Ile	–	+	Rep
Fatal infantile cardiomyopathy plus (MELAS)	MTTI	FICP	A4317G	tRNA Ile	Nd	nd	Rep
<b>Deafness</b>							
Deafness	MTTF	DEAF	A636G	tRNA Phe	+	–	Rep
Deafness	MTRNR1	DEAF	A827G	12S rRNA	+	–	Rep
Deafness	MTRNR1	DEAF	T961C	12S rRNA	+	–	Rep
Deafness	MTRNR1	DEAF	T961delT+C(n)ins	12S rRNA	+	+	Rep
Deafness	MTRNR1	DEAF	T961insC	12S rRNA	+	–	Rep
Deafness	MTRNR1	DEAF	T1005C	12S rRNA	+	–	Rep
Deafness	MTRNR1	DEAF	A1116G	12S rRNA	+	–	Rep
Deafness	MTRNR1	DEAF	C1494T	12S rRNA	+	–	Rep
Deafness	MTTC	Myopathy, deafness	G5783A	tRNA Cys	–	+	Rep
Deafness, sensory neural hearing loss	MTTS1	SNHL	T7510C	tRNA Ser (UCN)	–	+	Rep

Deafness, sensory neural hearing loss	MTTS1	SNHL	T7511C	tRNA Ser(UCN)	+	+	Rep
Deafness cerebellar dysfunction	MTTS1	Deafness and cerebellar dysfunction	7472insC	tRNA Ser(UCN)	–	+	Rep
Deafness	MTTH	DEAF + RP	G12183A	tRNA His	–	+	Rep
Deafness, ataxia, and MR	MTTE	Deafness, mental retardation, cerebellar dysfunction	T14709C	tRNA Glu	–	+	Rep
<b>Diabetes mellitus</b>							
Diabetes mellitus	MTRNR1	DM	C1310T	12S	+	–	Rep
Diabetes mellitus	MTRNR1	DM	A1438G	12S	+	–	Rep
Diabetes mellitus	MTTL1	DM	T3264C	tRNA <sup>Leu</sup> (UUR)	–	+	Rep
Diabetes mellitus	MTTL1	DM	T3271C	tRNA <sup>Leu</sup> (UUR)	–	+	Rep
Diabetes mellitus Metabolic syndrome	MTTI	Metabolic syndrome and hypomagnesemia	T4291C	tRNA Ile	+	–	Rep
Diabetes mellitus and deafness and cardiomyopathy	MTTK	DMDF/MERRF/HCM	A8296G	tRNA Lys	–	+	Rep
Diabetes mellitus and deafness	MTTS2	DMDF	C12258A	tRNA Ser (AGY)		+	Rep
<b>Others</b>							
Movement disorder	MTTV	Movement disorder	T1659C	tRNA Val	–	+	Rep
Alzheimer and Parkinson disease	MTRNR2	ADPD	G3196A	rRNA 16S	+	+	Rep
Alzheimer and Parkinson disease	MTTQ	ADPD/hearing loss and migraine	T4336C	tRNA Gln	+	–	Rep
Deafness and migraine							
Dementia and chorea	MTTW	DEMCHO	G5549A	tRNA Trp	–	+	Rep
Multi-organ failure	MTTN	Multi-organ failure	T5728C	tRNA Asn	–	+	Rep

Note: See <http://www.mitomap.org/MITOMAP/MutationsRNA> for additional reports.

ADPD, Alzheimer Disease and Parkinson Disease; AMDF, Ataxia, Myopathy, and Deafness; CPEO, Chronic Progressive Ophthalmoplegia; DEAF/SNHL, Deafness/Sensorineural Hearing Loss; DM, Diabetes Mellitus; DMDF, Diabetes Mellitus and Deafness; FSGS, Focal Segmental Glomerulosclerosis; GER, Gastrointestinal Reflux; MERRF, Myoclonic Epilepsy and Mitochondrial Encephalomyopathy, Lactic acidosis and Stroke-like episodes; MICM, Maternally Inherited Cardiomyopathy; MIDD, Maternally Inherited Diabetes and Deafness Syndrome; MMC, Mitochondrial Myopathy and Cardiomyopathy; MNGIE, Mitochondrial NeuroGastroIntestinal Encephalopathy; PEM, Progressive Encephalomyopathy; RP, Retinitis Pigmentosa; SNHL, Sensorineural Hearing Loss.

**LEBER HEREDITARY OPTIC NEUROPATHY**  
**African-American LHON Pedigree**  
**ND4 np 11778 G>A arginine 340 to histidine**



Shaded symbols indicate affected individuals. Asterisks indicate individuals examined.

**FIGURE 11-8** Maternally inherited pedigree of LHON due to the MTND4\*LHON11778A mutation. Affected individuals (filled symbols) experience acute onset optic atrophy and central vision loss, generally as young adults. Even though the mutation is essentially homoplasmic, penetrance among maternal relatives is highly variable. Moreover, males are about three to four times more likely to lose their vision than females (4,512).

(504–506), and MTND6\*LHON14484C (507,508)—generally present as LHON with few other clinical phenotypes (see Table 11-1). These mutations represent strong risk factors for developing LHON, have been observed in multiple unrelated LHON families, rarely co-occur with each other, and have not been detected in a large number of control mtDNAs (430,509–511) (see Table 11-1).

**MTND4\*LHON11778A.** The MTND4\*LHON 11778A mutation converts the highly conserved ND4 codon 340 from an arginine to a histidine (4). This mutation typically displays variable expression in families, with males being predominantly affected, and is heteroplasmic in about 14% of cases (512). The MTND4\*LHON11778A mutation also has a highly variable penetrance. Among MTND4\*LHON11778A families, about 33–60% of maternal relatives are affected, with 82% of the affected individuals being male and 18% female (313,512). Furthermore, only about 4% of affected individuals experience visual recovery.

Of the affected individuals, the mean age of onset is 27.6 years, with a range of onset from 8 to 60 years. About 58% of patients show additional ophthalmologic features, including peripapillary telangiectasias, microangiopathy, disc pseudoedema, and vascular tortuosity. Fifty-five percent of patients have a simultaneous onset of

vision loss in both eyes, while the overall mean interocular interval is 1.8 months, and the maximum interval is about 9 months. Once vision loss begins, it can progress rapidly or slowly. The mean length of progression for the MTND4\*LHON11778A mutation is 3.7 months, with a range of 0–24 months. In about 98% of cases, the final visual acuity is 20/200 or worse, while only 2% are better than 20/200 (512). Most cases are limited to optic atrophy, although a variety of other mild abnormalities have been associated with this mutation. These include cardiac conduction defects such as abnormal Q–T interval (513), skeletal abnormalities, and other neurologic signs (500).

In one patient, the MTND4\*LHON11778A mutation has been associated with Wolfram syndrome. In addition to optic atrophy, this patient had diabetes mellitus and sensorineural deafness (514).

Some biochemical studies on the MTND4\*LHON 11778A mutation have reported complex I enzymatic defects ranging from 0% to 50%. However, most studies have found no statistically significant reduction in complex I activity (515–520). By contrast, most studies have observed a reduction in mitochondrial respiration using complex I-linked substrates of 30–50%, while respiration rates using succinate are normal (515,518,519,521). When the MTND4\*LHON11778A mtDNAs are transferred to a different nuclear background by cybrid



transfer, the respiration defect is transferred, indicating that the respiration defect is linked to the mtDNA mutation (515,522,523). The MTND4\*LHON11778A mutation has also been reported to have an increased resistance to rotenone, a complex I inhibitor that acts as a ubiquinone antagonist. The mutant enzyme also has an altered affinity for ubiquinone analogs, though other groups have not detected this alteration in rotenone sensitivity (516,517,521). These observations have led to the postulation that the MTND4\*LHON11778A mutation alters the complex I interaction with ubiquinone. Alternatively, the amino acid change has been hypothesized to compromise the enzyme's energy-conserving (proton translocation) function, or to destabilize ubisemiquinone intermediates, promoting the production of ROS. The absence of an enzymologic defect in the presence of a clear respiration defect might be due to a defect in proton translocation or to an alternation of coenzyme Q affinity that is masked in the enzyme assays by the use of coenzyme Q analog at nonphysiologic concentrations as electron acceptor.

**MTND1\*LHON3460A.** The MTND1\*LHON3460A mutation changes a moderately conserved alanine at codon 52 in the *ND1* gene to a threonine and has been observed to be heteroplasmic in a number of families (504–506). Generally, the clinical manifestations of this mutation are confined to LHON, and only occasionally is the MTND1\*LHON3460A mutation associated with other neurologic signs, though patients harboring this mutation can manifest multiple sclerosis (524,525). Virtually every MTND1\*LHON3460A family is due to an independent mutation (430), and a small percentage of the families are heteroplasmic (313). The number of affected maternal relatives has varied widely in different studies, but can approach 75% (526,527), with between 40% and 80% of all affected individuals being male. Approximately 22% of affected individuals with MTND1\*LHON3460A have been reported to experience visual recovery (528).

The MTND1\*LHON3460A mutation is associated with markedly reduced complex I activity, and a reduction in respiration. In multiple studies of patient cells, the MTND1\*LHON3460A mutation has been found to be associated with a 60–80% reduction in the complex I specific activity (504,515,516,519,520). Despite the pronounced reduction in complex I activity, ATP synthesis in MTND1\*LHON3460A mitochondria is not compromised (529). Respiration studies have shown a roughly 30% reduction in maximal respiration rates using complex I-linked substrates (515,521). In cybrid transfer experiments, the complex I defect transfers faithfully (515), although nuclear genetic backgrounds may alter the magnitude of the functional defect found in cybrids (530). When complex I activity has been titrated with ubiquinone analogs/derivatives, a substrate inhibition pattern was observed (530). This suggests that the primary biochemical defect is in altered ubiquinone binding

affinity, which would be in line with the proposal that both the ND1 and ND6 polypeptides are components of the ubiquinone binding site.

**MTND6\*LHON14484C.** Finally, the MTND6\*LHON14484C mutation changes the weakly conserved methionine at codon 64 in the ND6 protein to a valine. This mutation is generally homoplasmic in LHON families (507,508), having been reported to be heteroplasmic in only a few pedigrees (531).

Clinically, the penetrance of the MTND6\*LHON14484C mutation is comparable to that of the other primary LHON mutations, resulting in symptoms in 27–80% of maternal relatives. Also, the male:female ratio of affected individuals is similarly high compared to that for the MTND4\*LHON11778A and MTND1\*LHON3460A mutations (502,526,528). Most MTND6\*LHON14484C families are likely to be independent mutations, but the MTND6\*LHON14484C mutation is only rarely heteroplasmic (531), suggesting that the mutation is sufficiently mild that most of the mtDNAs must be mutant before a phenotype is likely. This is supported by the strong association of the MTND6\*LHON14484C mutation with haplogroup J, indicating that it is less capable of causing LHON alone than are the MTND4\*LHON11778A and MTND1\*LHON3460A mutations (429,431). Occasionally, the MTND6\*LHON14484C mutation has also been found to co-occur with the MTND4\*LHON11778A mutation (502).

The MTND6\*LHON14484C mutation is additionally noteworthy for the tendency of patients to experience visual recovery. Fully 37–50% of patients with this mutation report visual improvement (see Table 11-1) (502,528). Thus, the MTND6\*LHON14484C mutation has the lowest pathogenicity of the primary LHON mutations and frequently requires additional mtDNA variants to augment its biochemical defect sufficiently to cause disease symptoms.

The biochemical defect of the MTND6\*LHON14484C mutation has proven very difficult to detect. Thus the functional defects associated with this mutation may match the milder genetic and clinical signature of this mutation. In enzyme and respiration analyses on patient lymphoblasts and cybrids, the specific activity of complex I was essentially normal, though maximal respiration rates were reduced 15–20% with complex I-linked substrates (515). Similarly, in independent studies, no reduction in complex I activity was found in MTND6\*LHON14484C-positive patient fibroblasts (532,533), although a third study reported a severe (65%) reduction in complex I activity and an associated 20% reduction in complex I-linked ATP synthesis in their patients (534). One study of platelet mitochondria implicated this mutation in complex I–ubiquinone interactions, since the mitochondria had increased sensitivity to the complex I product inhibitors myxothiazol and NBQH2 (532). While additional studies will be

needed to reach a consensus concerning the functional defects of this mutation, it is apparent that the biochemical defect of the MTND6\**LHON*14484C mutation is milder than that of the MTND1\**LHON*3460A and MTND4\**LHON*11778A mutations.

*Additional Potential LHON Mutations.* Of the 50 rare or provisional LHON mutations, all but three alter mtDNA complex I polypeptides. A complete list is available on Mitomap (<http://www.mitomap.org>).

Two of the rare complex I mutants nicely confirm the pathogenicity of the MTND6\**LHON*14484C mutation. These are the MTND6\**LHON*14482G and A mutations, which alter the same codon as the MTND6\**LHON*14484C mutation but convert the methionine at codon 64 to an isoleucine instead of a valine (535).

The remaining 12 rare LHON mutations have been confirmed as pathogenic (MTND1\**LHON* 3635A, MTND1\**LHON* 3700A, MTND1\**LHON*3733A, MTND1\**LHON*4171A, MTND4L\**LHON* 10663C, MTND6\**LDYT*14459A, MTND6\**LHON*14482A and 14482G, MTND6\**LHON* 14495G, MTND6\**LHON* 14502C, and MTND6\**LHON* 14568T). All of these mutants change moderately to highly conserved amino acids, supporting their pathogenicity (see Table 11-1). Despite their relative low frequency, these mutations should now be routinely assayed in all LHON patients lacking the three most common mtDNA mutations (536).

Of the rare LHON mutations, the most frequent and most severe is the MTND6\**LDYT*14459A mutation (537,538). This mutant has been observed in multiple LHON and dystonia pedigrees. It changes a highly conserved amino acid, results in a severe complex I defect, is frequently heteroplasmic in LHON patients, causes dystonia when homoplasmic, and causes LHON regardless of the mtDNA background haplogroup (539).

At the other extreme is the MTND4L\**LHON*10663C mutation which changes a poorly conserved valine to an alanine at amino acid 65 in the ND4L polypeptide (540). This mutation has been found in three independent LHON patients and was not detected in a large number of ethnic groups or haplotype-matched controls, specifically being absent from 254 controls: 51 J and 203 non-J. All of the MTND4L\**LHON*10663C-positive patients also had haplogroup J mtDNAs. Hence, the MTND4L\**LHON*10663C mutation must also require haplogroup J background to cause a clinical phenotype (428,430).

*The Haplogroup J Connection with LHON.* Early studies of LHON patients revealed a number of variants that were commonly associated with LHON, but were also found at low frequencies in the general population (540,541). The most common variant of these is the MTCYB\**LHON*15257A that changes an amino acid with a CI of 95% (540). Subsequent studies of population mtDNA haplogroups revealed that this and other striking mtDNA variants were all associated with regional

haplogroups; in the case of the MTCYB\**LHON*15257A variant, the association is with the haplogroup J, sub-haplogroup J2 (see Figure 11-6).

Moreover, all of the milder pathogenic LHON mutations showed a predilection for haplogroup J. For example, the MTND4\**LHON*11778A mutation causes a moderate complex I respiration defect, and, of 58 LHON patients harboring this mutation, 29% ( $n=17$ ) were also haplogroup J. For the even milder MTND6\**LHON*14484C complex I defect, of 38 LHON patients with this mutation, 79% ( $n=30$ ) were haplogroup J. Finally, for the very mild MTND4L\**LHON*10663C mutation, 100% of the patients (3 of 3) were also haplogroup J (428,429). Thus these data strongly indicate that haplogroup J, which harbors ancient sequence variants that were adaptive for certain temperate zone conditions, can today exacerbate the biochemical defects of the milder causal LHON variants sufficiently to convert them into pathogenic mutations (360,361,413). Because many of the functional variants of these haplogroups are linked together, it is currently unclear which of the various polymorphisms has the greatest impact on the penetrance of the mild causal mutations.

The root of the haplogroup sister J-T lineage involves two missense mutations: MTND1\*4216C (T304H) and MTCYB\*15452A (L236I) (511,540,542) (see Figure 11-6). The T lineage splits off founded by the highly conserved complex I mutation MTND2\*4917A (D150N), with a CI of 90% (430), and the J lineage is separated by complex I missense mutations MTND3\*10398G (T114A) and MTND5\*13708A (A458T) (540,542). At this point haplogroup J subdivides into sub-haplogroup J1, associated with MTCYB\*14798C with a CI of 79%, a sub-branch of which harbors the MTND1\**LHON*3394C variant common to French Canadian MTND6\**LHON*14484C patients; and sub-haplogroup J2, associated with MTCYB\*15257A (D171N) with a CI of 95% (361,428). At the present time, it is unclear which of these variants contributes to the increased penetrance of the milder LHON mutations.

Other lineage-specific mtDNA variants have also been associated with LHON, though the functional significance of these is even less well established. The MTND1\**LHON*4160C mutation was found together with the MTND6\**LHON*14484C mutation in the mtDNA of a large pedigree with acute-onset optic atrophy as well as other neurologic symptoms. Hence, this mutation may augment the pathogenicity of the MTND6\**LHON*14484C mutation. Support for this hypothesis is that an additional variant MTND1\**LHON* 4136G (Y277C) was found in the same pedigree, but appeared to ameliorate the severity of the symptoms of the combined MTND6\**LHON*14484C and MTND1\**LHON*4160C mutations (543).

The MTCO1\**LHON*7444A variant is novel in that it changes the termination codon of the COI polypeptide, extending the polypeptide by three charged

amino acids (544). This variant has been found in association with the MTND6\*LHON14484C and the MTND1\*LHON3460A mutations (430).

The homoplasmic MTCO3\*LHON9438A variant changes a highly conserved glycine to a serine and has been observed in 2.5% of European patients (545), but also at a low frequency in African and African American controls (546). This variant has also been reported in association with the MTND4\*LHON11778A mutation (547).

*Possible Pathophysiologic Mechanisms of LHON.* The pathophysiologic mechanism by which the LHON mutations cause acute-onset vision loss in young adults remains a mystery. One respiratory defect that ties many of the LHON mutations together is an inhibition in the transfer of electrons from complex I to CoQ<sub>10</sub>. A faulty interaction between CoQ<sub>10</sub> and complex I has been suggested for the MTND6\*LHON14484C mutation (532), implying that the region of ND6 around codons 64 through 72 may be involved in CoQ<sub>10</sub> interaction with complex I. Furthermore, eight other confirmed or putative LHON mutations occur in this region of the ND6 gene, which encompasses an evolutionarily conserved transmembrane helix (helix c) that is similar to the ubiquinone-reacting domain of cytochrome *b* in complex III (532). These are the MTND6\*LDYT 14596A (I26M) (548), the MTND6\*LHON14568T (G36S) (549,550), the MTND6\*- LHON14498T (Y59C) (550), the MTND6\*LHON14482G/A (M64I) (551,552), the MTND6\*LDYT14459A (A72V) (537), the MTND6\*LHON14325C (N117D) (553), the MTND6\*LDYT14495G (L60S) (554), and the MTND6\*LHON 14729A (S132L) (555) mutations. Moreover, the biochemical defects of the MTND6\*LDYT14459A and MTND1\*LHON3460A mutations are similar, suggesting a common mechanism, and ND1 is also thought to be important in electron transfer to CoQ<sub>10</sub>. Finally, the MTND4\*LHON11778A mutation has been suggested to alter rotenone binding and thus CoQ<sub>10</sub> binding (516,517,521). Thus, all of the primary LHON mutations have been implicated in problems with CoQ<sub>10</sub> interaction with complex I. Such a defect might be difficult to accurately evaluate given the difficulties with assaying complex I with the physiologic substrate CoQ<sub>10</sub>, necessitating the use of the CoQ<sub>10</sub> analogs such as decyl-ubiquinone. The possibility that all of these mutations impair electron transfer to CoQ<sub>10</sub> is supported by the observation that all of the primary LHON and LHON associated with dystonia (LDYT) mutations that have been studied (MTND6\*LDYT14459A, MTND4\*LHON11778A, MTND1\*LHON3460A, and MTND6\*LHON14484C) show at least a partial defect in complex I-linked respiration, with reductions in maximal respiration rates for the various mutations ranging from 15% to 40% (556). It is possible that even the small reduction in respiration observed for the MTND6\*LHON14484C mutation might reflect a

significant defect in electron transfer within the mitochondria under physiologic conditions.

Assuming that the primary defect of most LHON mutations is the inhibition of the ETC between complex I and CoQ<sub>10</sub>, then the pathophysiologic basis of the disease might involve chronically reduced energy production and/or increased ROS generation. Mitochondrial ATP production was been found to be strikingly reduced in osteosarcoma 143B cybrids harboring the MTND4\*LHON11778A, MTND1\*LHON3460A, and MTND6\*LHON14484C mutations when the cells were switched from glucose to galactose. Cellular ATP concentrations were reduced to 43%, 36%, and 23%, respectively, relative to the glucose control in 3 h and to 15% or less by 16 h (557).

Increased mitochondrial ROS production has also been demonstrated in Ntera2/D1 cell cybrids harboring the MTND4\*LHON11778A and MTND1\*LHON3460A mutations, but only after the cybrids were induced to differentiate into neurons. This ROS production was likely mitochondrial since its production was inhibited by rotenone. Neuronal differentiation also caused a three-fold reduction in the mtDNA:nDNA ratio, suggesting that mitochondrial function and thus energy production might be further reduced in neurons, thus unmasking a latent mitochondrial defect. Consistent with this interpretation, neuronal differentiation was associated with a 30% reduction in cell number of the LHON cybrids relative to control (558). Further confirmation that the LHON mutations are associated with increased ROS production comes from the observation that lymphoblastoid cell lines harboring the MTND4\*LHON11778A mutation have shorter telomeres than controls, and telomere length is affected by oxidative damage (559).

The LHON mutations are also associated with an increased predilection of cybrid cell lines to undergo apoptosis when grown on galactose. The galactose-induced apoptosis is associated with the mitochondrial release of cytochrome *c*, apoptosis initiating factor, and endonuclease G, and the cytosol from galactose-grown LHON cybrids can induce nDNA fragmentation when added to isolated nuclei (557,560,561). Finally, osteosarcoma 143B cybrids harboring the MTND4\*LHON11778A and MTND1\*LHON3460A mutations were found to be more sensitive to anti-FAS induced cell death, and this was associated with the activation of caspase 3, DNA fragmentation, and increased annexin V staining (562).

While these results confirm that the common LHON mutations cause mitochondrial energetic defects, increased mitochondrial ROS production, and increased apoptosis, they still do not provide a clear indication of why the retinal vessels show telangiectasias and distortion in the preclinical phase or why the RGCs cells are preferentially lost in the acute phase of the disease. The tortuous vessels could be the product of ROS-induced activation of the *HIF1a*-vascular endothelial growth factor pathway, or, alternatively, the mitochondrial



ROS could inactivate the NO vasodilator generated by the vascular endothelial cells, resulting in vasoconstriction and transient ischemia. Similarly, the RGCs might be acutely sensitive to energy deficiency or ROS-induced apoptosis.

It will be necessary to generate a mouse model to resolve these questions. An initial effort to generate a mouse model of LHON that supports the ROS toxicity hypothesis was generated by transducing the retina and RGCs with an adeno-associated virus vector carrying a ribozyme against MnSOD. This reduced the MnSOD level to 85% and led to the loss of axons and myelin in the optic nerve and the RGCs (563).

### 11.1.23 LHON, Multiple Sclerosis, and Dystonia

While most LHON cases present with isolated optic atrophy, certain pedigrees manifest additional neurologic symptoms, which have suggested additional diseases that may be of mitochondrial origin. Prominent among the LHON-associated symptoms are a multiple sclerosis-like presentation and movement disorders (502,525,564).

An association between some primary LHON mutations and a multiple sclerosis-like demyelinating disease has been repeatedly reported (565). About 1–2% of female patients from LHON pedigrees with the MTND4\*LHON11778A mutation have been diagnosed with multiple sclerosis-like demyelination disease (512,524,566–569). Eight females with the MTND4\*LHON11778A mutation have been described who presented with LHON but subsequently developed clinical and/or neuroradiologic signs consistent with multiple sclerosis (568). A number of other similar cases exhibited an association between either the MTND4\*LHON11778A or the MTND1\*LHON3460A mutations and multiple sclerosis, mostly in females (524,525), but also in males (525,569). Surprisingly, though the MTND4\*LHON11778A mutation accounts for greater than 90% of Japanese LHON cases, no association has been found between this mutation and multiple sclerosis in the Japanese population (570). LHON mutation associations with multiple sclerosis have been evident only when patients are ascertained through LHON presentation (with or without family history) or through multiple sclerosis presentation with early and prominent optic nerve involvement (524). Given the rare population frequency of both LHON and multiple sclerosis diseases, their frequent coincidence probably reflects a related cause (565). It is possible that neuronal cell lysis resulting from the mitochondrial defect releases mitochondrial antigens (571,572) that initiate the autoimmune response (569,573).

Mitochondrial defects can also cause dystonia. Biochemical analysis of an isolated case of idiopathic dystonia, putaminal lesions, and myopathy revealed a partial complex III defect (574). Moreover, complex I defects

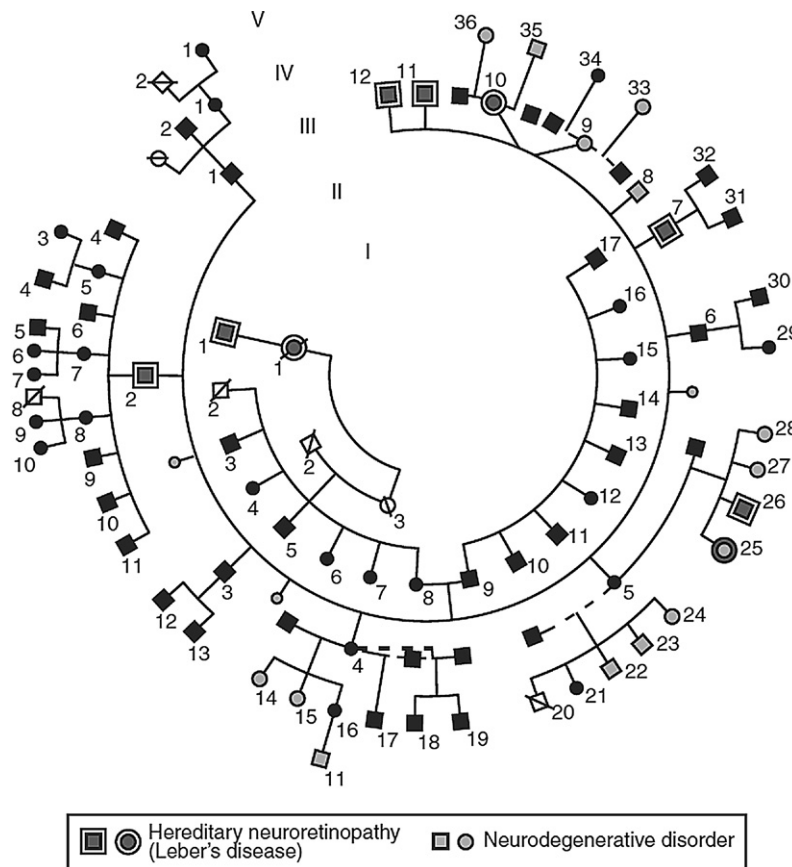
were observed in the platelet mitochondria of a wide variety of dystonia patients, with the average complex I activity of generalized or segmental dystonia patients being reduced 62% while that from focal dystonia patients was reduced 37% (575).

*MTND4\*LHON11778A and Movement Disorders.* A number of LHON pedigrees harboring the MTND4\*LHON11778A mutation include patients which also develop movement disorders. One individual, who lost his vision at 37, developed cerebellar-extra-pyramidal tremor and left-side rigidity associated with bilateral basal ganglia lesions at age 38 (518). A second MTND4\*LHON11778A patient, who suffered visual loss at 23, showed pyramidal signs including spastic paraparesis, inexhaustible patellar and ankle clonus, diffuse muscle weakness, and multiple periventricular and subcortical hyperintensities on magnetic resonance imaging (MRI) compatible with demyelinating disease (523). A third mother and son exhibited ataxia associated with cerebellar and pontine atrophy (576).

*MTND6\*LHON14484C + MTND1\*LHON4160C.* In another large pedigree, the MTND6\*LHON14484C mutation was associated with movement disorders and other neurodegenerative symptoms when present in association with another homoplasmic mutation, MTND1\*LHON4160C, which changes a highly conserved leucine at codon 285 of the ND1 polypeptide to a proline (543). The clinical presentation included sudden-onset optic atrophy with approximately two-fold excess of affected males. Beyond the optic atrophy, there were two neurologic presentations. The first had an onset in the first or second decade of life, starting as a gait abnormality and progressing to include ataxia, spasticity, tremor, dysarthria, posterior column signs, and skeletal deformities. The second had a childhood-onset (5–10 years) severe encephalitic disease occasionally resulting in death; initial signs include headache, convulsions, and abnormal respiration. A novel feature of this later presentation is that some individuals appear to fully recover (577). One branch of the pedigree, with less severe symptoms, was found to have a second homoplasmic mutation, MTND1\*LHON4136G, that was hypothesized to be protective (543). Biochemical analysis of platelet-derived mitochondria from four subjects revealed an 80% average reduction in rotenone-sensitive NADH-CoQ oxidoreductase specific activity (578).

*MTND6\*LDYT14459A.* The MTND6\*LDYT14459A mutation has been shown to cause LHON and/or early-onset generalized dystonia (537,538,564). The MTND6\*LDYT14459A mutation is relatively rare, having been reported in several independent pedigrees (537,538,579). This mutation changes the highly conserved alanine at codon 72 in the ND6 polypeptide to a valine and can manifest as two very different phenotypes: LHON and/or generalized dystonia (Figure 11-9).





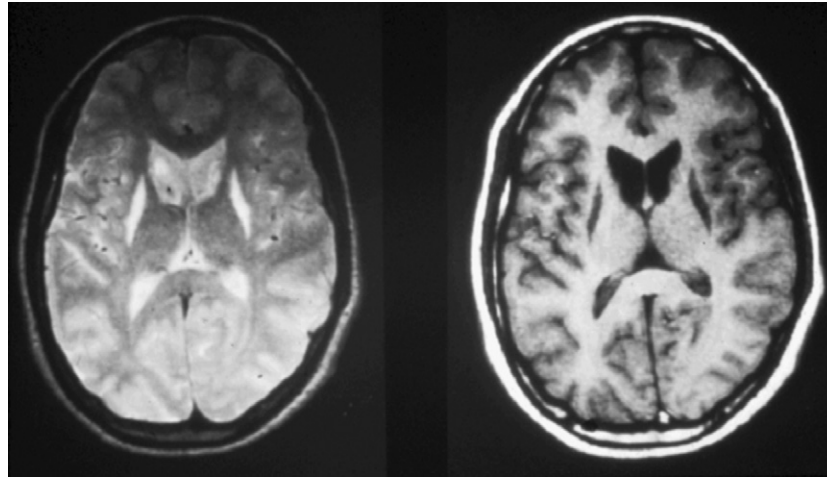
**FIGURE 11-9** Hispanic family with maternally inherited LHON and/or dystonia due to the heteroplasmic mtDNA mutation at MTND6\*LDYT14459A. Individuals with LHON experience acute onset optic atrophy and central vision loss, generally as young adults. Individuals with neurodegenerative disease have a much earlier age on onset and experience gait disturbance, rigidity, pseudobulbar syndrome, impaired intelligence, short stature, and frequently infantile bilateral striatal necrosis (IBS) (564).

This mutation has been found to be heteroplasmic in at least some family members in every pedigree studied (see Table 11-1) (537,538). The dystonia generally has an early onset and includes gait disturbance and rigidity of the lower extremities, which advances with age to include the upper extremities (564). Bilateral basal ganglia degeneration (bilateral striatal necrosis) is also a common finding in the dystonia patients.

Evidence for the independent origin of the different family mutations is clear for the MTND6\*LDYT14459A mutation. The mutation was first identified in a large Hispanic family (Figure 11-10), which proved to harbor a Native American haplogroup D mtDNA (537). Subsequently, the same mutation was found in an African American LHON family with a haplogroup L1 mtDNA and in a European dystonia patient with a haplogroup I mtDNA (537,538). Since mtDNA haplotypes of these three families encompass most of the world's mtDNA sequence diversity, it was not possible that the MTND6\*LDYT14459A mutations are related through a common ancestor. Rather, all three MTND6\*LDYT14459A mutations must have arisen recently and independently, a conclusion consistent with the fact that all three families were heteroplasmic. Hence, this mutation must be the cause of this disease (537,538).

Moreover, two additional complex I mutations, MTND4\*LDYT11696G and MTND6\*LDYT14596A, had similar presentations of large maternal pedigrees with LHON and dystonia, the MTND6\*LDYT14596A mutation also being heteroplasmic (548).

While the MTND6\*LDYT14459A mutation has consistently been associated with neurologic disease, the symptoms have differed among patients with different mtDNA backgrounds. This suggests that genetic background might also influence this mutation. In the five-generation Hispanic pedigree with the background mtDNA haplogroup D, among 42 maternal relatives, 19% had LHON, 31% had dystonia and bilateral striatal necrosis, and 2% had both (564). Its penetrance among maternal relatives is about 61%, with 58% of the affected individuals being male, and there is no record of an affected individual recovering. The mean age of onset of dystonia was 4 years, with a range of 1.5–9 years. The motor system was primarily involved and resulted in gait disturbance and rigidity of the lower extremities, which advanced with age to include the upper extremities. Patients also developed pseudobulbar syndrome (swallowing and speech problems), impaired intelligence, short stature, and myopathic features. These symptoms were often associated with bilateral striatal necrosis



**FIGURE 11-10** Magnetic resonance image of the head of a dystonic patient showing bilateral striatal necrosis. The patient is IV24 in Figure 11-9. The left panel is a T1 image (cellular material light). The right panel is a T2 image (aqueous areas light). The lesions are the symmetrical fusiform lesions below and to the left and right of the ventricles (1,11).

(564), the loss of cells in the striatum, putamen, and caudate. In the African American pedigree with mtDNA haplogroup L1, the mother and daughter both developed LHON, but on MRI scan, the daughter was found to have unilateral basal ganglia degeneration. Finally, the singleton European male with haplogroup I mtDNA had dystonia and bilateral striatal necrosis (537,538).

Analysis of MTND6\*LDYT14459A patient skeletal muscle mitochondria has revealed that complex I activity is essentially eliminated (538). In contrast, biochemical analysis of OXPHOS enzymes and respiration in mitochondria isolated from Epstein–Barr virus-transformed lymphoblastoid cell lines of MTND6\*LDYT14459A patients revealed a 55% reduction in complex I specific activity when normalized to citrate synthase or mitochondrial protein (537). This defect could be transferred along with the mutant mtDNAs to  $\rho^0$  lymphoblastoid cells in cybrid experiments (300). In contrast to the greater than 50% reduction in complex I specific activity, the overall mitochondrial respiration rate using complex I substrates was not significantly reduced. This implies that the complex I defect alone is not sufficient to be rate limiting, consistent with respiratory control theory (336,337).

Kinetic analysis of the MTND6\*LDYT14459A complex I defect revealed that the  $V_{\max}$  for NADH is reduced, but the  $K_M$  is not (300). Furthermore, the enzyme activity with increased decyl-ubiquinone (DB), a CoQ<sub>10</sub> analog, showed a maximum activity at 5 mM and then declined as DB was increased to 10 mM, a result in marked contrast to the steady increase in activity seen in the control enzyme over this same concentration range. Finally, there was a marked product inhibition of the enzyme by 5 mM DBH<sub>2</sub>, with the specific activity being inhibited 71%. These data suggest that the MTND6\*LDYT14459A mutation may alter the CoQ<sub>10</sub> binding site of complex I (300). Hence, the MTND6\*LDYT14459A mutation places MTND6 near the CoQ<sub>10</sub> binding site.

The molecular basis of the variable phenotypes associated with the 14459A mutation is puzzling. In the original report, the mother with LHON was heteroplasmic for the mutation while her two dystonic children were homoplasmic (537). This would suggest that variation in the percentage of mutant might result in variable severity of the disease. However, in another pedigree, variably affected maternal relatives were all found to be homoplasmic for the mutation. This was interpreted as indicating that an nDNA factor or factors were influencing the phenotype (579).

#### 11.1.24 Dystonia and Leigh Syndrome Transition for Nd Mutations

The transition from LHON to dystonia to LS is essentially a continuum for complex I polypeptide mutations. The MTND6\*Ldyt/LS14459A mutations can be manifest as LHON, dystonia, or LS (537,580). The MTND1\*DYT3796C mutation has been associated with adult-onset dystonia in a heteroplasmic family (581). Finally, the MTND6\*LS/DYT14487C mutation can present as either dystonia or LS (582). All of these mutations were found to be heteroplasmic, suggesting that one factor in defining the phenotype is the percentage of mutant mtDNAs and thus the severity of the mitochondrial complex I defect (Table 11-2).

Beyond these mutations, the MTND3\*LS10158C, MTND3\*LS/ESOC10191C, MTND3\*LS/Dystonia10197A, MTND4\*LS11777A, and MTND5\*LS12706C mutations all present as LS. All of these mutations were found to be heteroplasmic and have been confirmed in more than one pedigree. Still, the association with LHON and dystonia mutations is apparent. The MTND4\*LS11777A alters the same amino acid (R340S) as the most prevalent LHON mutation MTND4\*LHON11778A (R340H) and the MTND6\*LS/DYT14487C (M63V) mutation is the adjacent codon to the MTND6\*LHON14484C

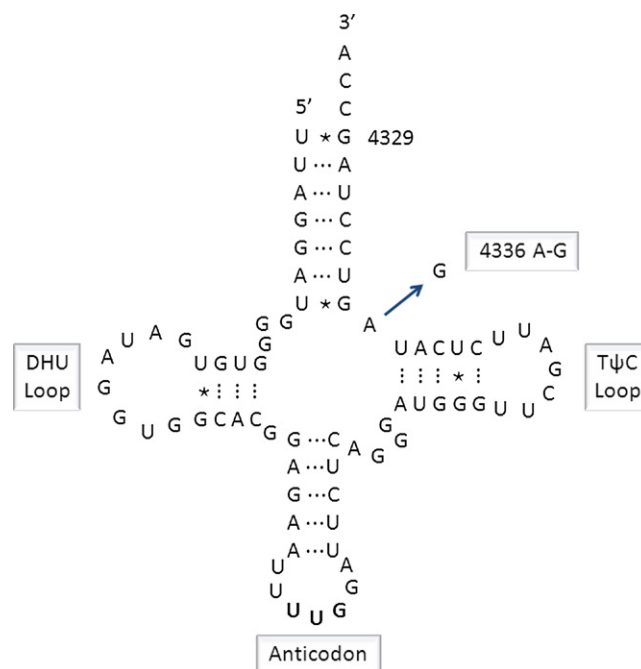
(M64V) mutation. Therefore, it appears that mild complex I mutations such as MTND4\*LHON11778A and MTND6\*LHON14484C result in LHON, more severe mutations such as MTND6\*LDYT14459A can cause LHON but also dystonia with basal ganglia lesions, and severe complex I defects such as MTND3\*LS10158C, MTND3\*LS/ESOC10191C, MTND3\*LS/Dystonia10197A, MTND4\*LS11777A, MTND5\*LS12706C, and MTND6\*LS/DYT14487C cause earlier onset basal ganglia degeneration and LS.

### 11.1.25 Leigh Syndrome and Retinitis Pigmentosa

Pigmentary retinopathy, neurodegeneration, and LS have been associated with mutations in the mtDNA *ATP6* gene, as well as with mutations in several nDNA genes involved in the assembly of the respiratory complexes.

LS (subacute necrotizing encephalomyopathy) has an average age of onset of about 1.5 years, with a mean duration of illness until death of about 5 years. Clinical manifestations include ataxia, hypotonia, spasticity, developmental delay and regression, optic atrophy, nystagmus, respiratory abnormalities, and ophthalmoplegia. The myopathy is generally non-specific and includes fat accumulations. Occasional patients can show liver involvement, cardiomyopathy, and mitochondrial myopathy (MM) including ragged-red muscle fibers (RRFs) and mitochondria with paracrystalline inclusions, although most patients have normal muscle fiber and mitochondrial morphology. A common observation is the abrupt worsening of the patient's clinical and metabolic status with infections or febrile episodes. A common neuroradiologic finding of end-stage patients is the bilateral degeneration of the basal ganglia, readily observed by computed tomography (CT) scan and MRI analysis. This is the same radiologic manifestation seen for the MTND6\*LDYT14459A mutation (see Figure 11-11). Brain pathology classically reveals basal ganglia necrosis associated with marked vascular proliferation in the brain stem (259).

Current estimates of the proportion of LS cases resulting from the known biochemical and molecular defects (583) include (1) about 18% mtDNA mutations; (2) about 10% pyruvate dehydrogenase (PDH) defects (584–586); (3) about 19% complex I defects (586,587); (4) about 18% complex IV defects, including *SURF1* mutations (158,159,324,588); and (5) about 35% other causes, including complex II (589) and pyruvate carboxylase defects (590). The fact that so many different mitochondrial bioenergetic defects can cause the same lethal phenotype indicates that the clinical manifestations of LS represent the common clinical end point for the more severe mitochondrial OXPHOS defects.



**FIGURE 11-11** mtDNA tRNA<sup>Gln</sup> A4336G mutation associated with late-onset AD and PD.

While the clinical presentation for LS is relatively constant, regardless of the molecular defect, the clinical manifestations of other family members can vary greatly depending on the nature of the genetic defect. Generally, in families with LS resulting from a nuclear gene mutation, the clinical presentation of affected family members is similar to that of the proband. Thus, for X-linked LS due to *PDH E1α* mutations, males are primarily affected and have a similarly severe phenotype. Likewise, LS patients resulting from recessive mutations in nuclear genes will have similar phenotypes in affected family members, with consanguineous marriages being more common. By contrast, LS resulting from heteroplasmic mtDNA mutations will be associated with highly variable clinical presentations among maternal relatives, depending on the percentage of mutant mtDNAs inherited by each family member.

In addition to the five mtDNA complex I mutations that can cause LS, six mutations have been identified in the mtDNA *ATP6* gene and one in the *COIII* gene (see Table 11-2 Panels A and B). The *ATP6* mutations include MTATP6\*NARP/LGHS8993G or C, MTATP6\*LS9176C or G, MTATP6\*LS9185C, and MTATP6\*LS9191C. The MTATP6\*NARP8993G (591–595) and MTATP6\*NARP8993C (596,597) mutations have been observed in multiple independent pedigrees and are well-established causes of LS. These two base changes change the leucine at codon 156 from a leucine to either an arginine or a proline. The MTATP6\*LS/FBSN9176C (598) and the MTATP6\*LS/FBSN9176G (599) mutations result in the substitution of the leucine at codon 217 to either an arginine or a proline, the same changes as seen for the NARP8993C

or G mutations. Finally, the MTATP6\*LGH9185C and MTATP6\*LGH9191C mutations both change a leucine to a proline. All six of the ATP6 mutations associated with LS were heteroplasmic in the respective families. Hence, all of these mutations are likely to cause LS.

The best characterized of the mtDNA ATP6 LS mutations are the MTP6\*NARP/LS8993G or C mutations. These are also the most common. The MTATP6\*NARP8993G mutation was the first described, in a pedigree of maternally inherited neurogenic muscle weakness, ataxia, and retinitis pigmentosa, hence the acronym NARP. MTATP6\*NARP8993G pedigrees are invariably heteroplasmic (591). In the initial three-generation pedigree, the severity of the symptoms varied markedly between individuals and generally correlated with the percentage of mutant mtDNAs harbored by the individual. Neurodegenerative symptoms included generalized seizures, axonal sensory neuropathy, dementia, corticospinal tract degeneration, and cerebellar and brain stem atrophy (591). This latter clinical feature can manifest as olivopontocerebellar atrophy on MRI. One individual presented with mental retardation, macular degeneration, and spicular retinitis pigmentosa (592). Subsequently, families harboring the MTATP6\*NARP8993G mutation were also found to encompass individuals presenting with LS (594,595,600–602).

The variability in clinical presentation of the MTATP6\*NARP8993G and C mutations is primarily due to the replicative segregation of the heteroplasmic mtDNA mutation. In a typically MTATP6\*NARP8993G pedigree, symptoms ranged from mild salt-and-pepper retinitis pigmentosa through olivopontocerebellar atrophy to a pediatric cerebellar ataxia, and lethal LS. Generally, adults with retinitis pigmentosa harbor about 75% mutant mtDNAs, while children with lethal LS have in excess of 90% mutant mtDNAs (592).

The pathophysiology of the MTATP6\*NARP8993G and C mutations has been elucidated through the biochemical analysis of patient mitochondria isolated from cultured cells, and through studies of the mechanism of ATP synthesis by ATPsyn. The MTATP6\*NARP8993G and C mutations change the highly conserved leucine 156 in the ATP6 polypeptide to an arginine or a proline, respectively. Biochemical analysis of the MTATP6\*NARP8993G mutation in patient lymphoblasts or fibroblasts has revealed a 30–50% reduction in the rate of ATP synthesis (600,601,603). Moreover, in patient lymphoblasts and in their trans-mitochondrial cybrids, there was a 30–40% reduction in the rate of maximal (state III) respiration and a comparable reduction in ADP:O ratios (301). Finally, the MTATP6\*NARP8993G mutation has been found to significantly increase mitochondrial ROS production (604), which is consistent with other results that demonstrate that inhibition of mitochondrial ATP synthesis results in hyperpolarization of  $\Delta P$  and elevated mitochondrial ROS production (605).

Correlating the position and nature of the amino acid changes caused by the MTATP6\*NARP8993G and C mutations with recent data on the rotor–stator mechanism of ATPsyn indicates that the MTATP6\*NARP8993G and C mutations must compromise the ATPsyn proton channel. The highly conserved leucine at ATP6 amino acid 156 is analogous to the *Escherichia coli* amino acid 207. This forms the base of  $\alpha$ -helix 4 in the bacterial protein subunit a, the homolog of ATP6. Leucine 156 (bacterial 207) is on the same face of the helix as arginine 159 (bacterial 210). Arginine 159 (bacterial 210) is thought to provide the positive charge that pulls the negatively charged, deprotonized glutamate 58 (bacterial aspartate 61) from the matrix-space half-proton channel around to the intermembrane-space half-proton channel. Arginine 159 (bacterial 210) is displaced from the glutamate 58 (aspartate 61) so as to provide attraction, without forming a salt bridge and blocking rotation of the ATP9 (subunit c) protein wheel. However, conversion of leucine 156 to arginine adds a second positive charge on the ATP6 (a)–ATP9 (c) interface, and conversion of leucine 156 to proline disrupts the  $\alpha$ -helix and presumably the interface between ATP6 (a) and ATP9 (c). Hence, both mutations would stall the rotor, block the proton channel, and inhibit ATP synthesis.

The three remaining ATP6 mutant missense mutation sites (MTATP6\*FBSN9176C/G, MTATP6\*LS9185C, and MTATP6\*LS9191C) share similarities with the MTATP6\*LS8993G/C mutations. For example, the MTATP6\*LS/FBSN9176C/G mutation (L217P) is 61 codons away from the MTATP6\*NARP8993G and C mutations. In *Escherichia coli*, mutations in the c subunit (ATP9) that change aspartate 61 to glycine can be suppressed by mutations in the a subunit (ATP6) at Ala 217, Ile 221, and Leu 224. Moreover, mutations at Gly 218 and His 214 of protein a (ATP6) result in uncoupling proton translocation from ATP synthesis, while the double mutant Gly 218 to Lys plus His 245 to Gly restores activity (172). This implies that the region of human ATP6 around amino acid 197 (bacteria 245) may also be important in coupling the proton gradient to ATP synthesis. Thus, the MTATP6\*FBSN9176C mutation affecting ATP6 amino acid 217 (bacterial 256) is adjacent to bacterial His 245 and hence might alter the integrity of the ATPsyn proton channel. Furthermore, since all of the LS mutations convert a leucine to either an arginine or a proline, it seems likely that all of these mutations may affect the ATPsyn proton channel and thus coupling efficiency and ATP synthesis.

Finally, one mtDNA mutation associated with LS was found in the COIII gene, MTCO3\*LS9537insC. This resulted in a frameshift, destroying the protein. Moreover, the mutation was homoplasmic in the patient (606). This case confirms that the final common end point for the most severe missense mutations appears to be LS.



### 11.1.26 Encephalomyopathies and Multisystem Disorders

Mitochondrial encephalopathies associated with mtDNA polypeptide mutations encompass a diverse array of symptoms associated with mitochondrial defects that affect the primary energy-utilizing tissues, including the central nervous system, heart, and muscle. Furthermore, the muscle generally exhibits MM with RRFs. Patients with mitochondrial encephalomyopathies can harbor mitochondrial polypeptide mutations, although these types of symptoms have more commonly been reported in patients harboring mtDNA protein synthesis (tRNA or rRNA) mutations. Several clusters of symptoms occur together in the encephalomyopathies, but the *mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes* (MELAS) syndrome is the cluster most commonly associated with mitochondrial polypeptide mutations (see [Table 11-2](#) Panels A and B).

Patients with a MELAS presentation have been reported to have 12 different mutations in complex I subunits as well as one *COIII* and one *CYTB* mutation. All of the reported mutations have been missense mutations except the *CYTB* mutation, and all have been heteroplasmic, indicating new pathogenic mutations.

Complex I subunit mutations have been reported in the ND1, 4, 5, and 6 subunits (see [Table 11-2](#) Panels A and B). For the MTND1 subunit, the MTND1\*MELAS3308C mutation eliminated the methionine start codon. The MTND1\*MELAS G3376A, G3697A, G3946A, and T3949C mutations affect other amino acids throughout the ND1 polypeptide. The MTND4\*MELAS11084G (T109A) variant has also been associated with a MELAS pedigree ([607](#)), but more recent studies suggest that this may be an Asian polymorphism ([608](#)). However, the MTND5\*MELAS A12770G, A13045C, A13084T, G13513A, and A13514G mutations all make quite significant polypeptide changes and thus are likely to cause the disease. Finally, the MTND6\*MELAS14453A (A74V) mutation ([609](#)) is three codons away and results in the same amino acid change as the well-established LHON and dystonia mutation, MTND6\*LDYT14459A (A72V) ([537,538](#)), which is associated with basal ganglia degeneration. The most common cause of the MELAS syndrome is the tRNA<sup>Leu(UUR)</sup> mutation, MTTTL1\*MELAS3243G, which inhibits mitochondrial protein synthesis and predominantly results in complex I deficiency (see [Section 11.1.32](#)). Hence, MELAS symptoms probably result from a particularly severe complex I defect.

The MTCYB\*MELAS/PD14787del4 ([610](#)) and MTCO3\*MELAS/PEM/NAION9957C (F251L) mutations ([611,612](#)) currently stand alone among the “MELAS” polypeptide mutations since they are the only ones that do not directly affect complex I. Furthermore, the symptoms of these patients are more complex and less stereotyped. For example, the MTCYB\*MELAS/

PD14787del4 boy with a 4-bp mtDNA deletion in the *CYTB* gene first manifested fine motor coordination problems at 6 and showed fatigue and regressive behavior by 14, along with tremor, stimulus-responsive myoclonus, lactic acidosis, cardiac conduction defects, and diffuse cerebral and cerebellar atrophy. At 20, he was experiencing short episodes of disturbed consciousness, episodes of status epilepticus, tonic-clonic seizures, a stroke-like episode associated with cortical blindness, and parieto-occipital cerebral infarcts. Muscle biopsy revealed a complex III defect associated with a single RRF and aggregates of subsarcolemmal mitochondria. Analysis of his mtDNA cytochrome *b* gene revealed that the 14787del4 mutation resulted in a frameshift from codon 13, which terminated the protein at codon 50. In muscle, ~95% of the mtDNAs were mutant, while in blood, hair follicles, oral mucosa, and fibroblasts about 60% of the mtDNAs were mutant ([610](#)).

Multiple severe mtDNA polypeptide mutations have been associated with more complex encephalomyopathies and multiple system disorders. Encephalomyopathies and multiple system disorders associated with COI include MTCO1\*EPLP6489A (L196I), MTCO1\*EM6930A (G343Ter), and MTCOI\*EM6015del5 (frameshift); those associated with COII include MTCO2\*ENMM 7587C (M1T), MTCO2\*MS7896A (W104Ter), and MTCO2\*EM8042del2 (M153Ter); and those associated with COIII include MTCO3\*EM9952A (W248Ter). Encephalomyopathies and multiple system disorders associated with ATP6 include MTATP6\*LA9205del2 (Ter227M), and those associated with the cytochrome *b* mutants include MTCYB\*MS15579G (Y278C) and MTCYB\*SOD14849C (S39P) (see [Table 11-2](#) Panels A and B).

An example of a chain termination mutation that results in a multisystem disorder is the MTCO1\*MSD6930A mutation. This mutation was found in a 21-year-old woman who developed bilateral cataracts at 3 years; sensorineural hearing loss at 7; and muscle weakness with 90% of muscle fibers being COX deficient, myoclonic epilepsy, cerebellar ataxia, optic atrophy, cerebellar atrophy, bilateral basal ganglion degeneration, lactic acidosis, and elevated serum creatine kinase by age 21. Molecular analysis revealed that the G6930A mutation created a stop codon and deleted the last 170 amino acids of COI. The mutation was heteroplasmic in blood (27%), muscle (75%), and myoblasts (33%), but was not detected in the blood of the mother or sister. Cybrids harboring the mutation had reduced growth rates on galactose and diminished COX activity ([613](#)).

The second COI mutation patient had a very different phenotype. She exhibited upper motor neuron disease at 1 year that progressed to spastic paraparesis at 29 years. MRI revealed bilateral corticobulbar track lesions, muscle histochemistry revealed RRFs and mitochondrial COX deficiency, and physiologic studies mild lactic acidosis. Molecular analysis revealed the deletion of one

of two adjacent 5-bp repeats encompassing nps 6015 through 6024. This generated a stop codon six codons downstream from the deletion, resulting in a 42-amino acid product instead of the 513-amino acid product. The patient's muscle was heteroplasmic for the mutation, 47% and 69% on successive muscle biopsies; the muscle of the patient's mother and three sisters lacked the mutation. Single muscle fiber mutation analysis revealed that normal fibers were 29% mutant, COX-deficient fibers 38% mutant, COX-negative fibers 69% mutant, and RRFs 91% mutant (614).

Another patient developed progressive ataxia at 5 years, and was wheelchair bound with mild distal wasting, cognitive impairment, optic atrophy, pigmentary retinopathy, and decreased color vision by age 25 years. A muscle biopsy revealed he had 15% RRF and 80% COX-negative fibers. Molecular analysis revealed a heteroplasmic T7587C mutation in the second base of the initiation codon of the *COII* gene, converting it from a methionine to a threonine. The mutation was present in 91% of muscle, 89% of fibroblast, and 36% of blood samples. In single fiber analysis, COX activity was severely reduced in fibers with over 55–65% mutant mtDNAs (615). In this case, destruction of the methionine initiation codon presumably eliminated the COII protein, thus accounting for the severe phenotype.

Another patient with a COII stop codon mutation collapsed after vigorous exercise at 17 years, experiencing confusion and headache. She developed episodic myalgia by age 21 that progressed to increased fatigue and regular headaches following three pregnancies. This progressed to periodic stuporous states with elevated lactate and creatine kinase and frequent migraine-type headaches. Muscle histology revealed COX-negative fibers but no RRFs, and muscle mitochondrial COX activity was 20% of control while cytochrome *aa<sub>2</sub>* content was 17%. Molecular analysis revealed a G9952A mutation in the *COIII* gene, which changed a tryptophan codon to a stop codon 13 amino acids from the C-terminal end. The mutation was heteroplasmic, being present in 57% of the mtDNAs in two independent tests. In COX-negative fibers the proportion of mutant was 56%, but in COX-positive fibers it was 10% (616).

### 11.1.27 Mitochondrial Myopathy and Ragged Red Muscle Fibers

A wide spectrum of mitochondrial base substitution mutations have been identified that cause MM. MM involves progressive muscle weakness in association with a distinctive muscle pathology detected by Gomori modified trichrome. Trichrome staining reveals turquoise muscle fibers that tear on sectioning and have aggregates of red-staining material, generally in the subsarcolemmal regions. This red material is aggregate of highly abnormal mitochondria, often containing precipitates, membrane whorls, or paracrystalline arrays (see Figure 11-9). The paracrystalline structures contain mitochondrial

creatine phosphokinase (8,617). Such muscle fibers are called *ragged red muscle fibers*.

MM associated with RRFs is most commonly observed in patients harboring pathogenic base substitution mutations in rRNAs or tRNAs, such as in MERRF and MELAS pedigrees. However, MM has also been found to be associated with mutations in the *CYTb* gene (618).

Progressive muscle weakness, exercise intolerance, and MM have been repeatedly linked to deleterious mutations in the mtDNA *CYTb* gene, all of which have been heteroplasmic and many in isolated cases. Thirteen *CYTb* mutations have been reported, including five missense mutations: MTCYB\*EXIT14846A (G34S), MTCYB\*EXIT15197C (S151P), MTCYB\*EXIT15497A (G251S), MTCYB\*EXIT15615A (G290D), and MTCYB\*MM15762A (G339E). Seven additional chain termination mutations have been identified—MTCYB\*MM15059A (G190Ter), MTCYB\*EXIT15084A (W113Ter), MTCYB\*EXIT15150A (W135Ter), MTCYB\*EXIT15168A (W141Ter), MTCYB\*EXIT15242A (G166Ter), and MTCYB\*EXIT15723A (W326Ter)—as well as one 24-np deletion (MTCYB\*EXIT15498del24) (see Table 11-2).

One example of a case harboring a *CYTb* missense mutation is a 29-year-old male with progressive muscle weakness and exercise intolerance associated with complex III deficiency. This individual had a MTCYB\*EXIT15615A mutation, which changed the highly conserved glycine 290 to an aspartate (G290D). The proband had 90% mutant mtDNAs in his muscle, but no mutant in his blood. The blood of his mother and sister also lacked the mutant mtDNA (619).

Two representative examples of MM and exercise intolerance resulting from *CYTb* chain termination mutations are MTCYB\*EXIT15242A and MTCYB\*MM15059A. The MTCYB\*EXIT15242A nonsense mutation case was a 38-year-old female with progressive muscle weakness, severe exercise intolerance, lactic acidosis, and a complex III deficiency. Using <sup>32</sup>P-labeled nuclear magnetic resonance (NMR), she was found to have a marked inhibition of recovery in her muscle PCr:Pi ratio following exercise. The patient's muscle mtDNAs were found to be 85% mutant, while her blood and that of her maternal relatives were homoplasmic wild type (620–623). In an effort to circumvent the block in her ETC, this patient was treated with ascorbate and menadione. This metabolic therapy substantially improved the patient's capacity for PCr:Pi recovery following exercise (622). This patient was reanalyzed and, in addition to exercise intolerance, developed a mitochondrial encephalomyopathy over the years (624).

The MTCYB\*MM15059A mutation occurred in a 27-year-old male with progressive muscle weakness, exercise intolerance, and episodic myoglobinuria. He had a marked decrease in complex III and RRFs, which were SDH and COX positive. His cytochrome *b* protein was truncated by conversion of the glycine 190 codon to

a stop codon, eliminating the terminal 244 amino acids of the protein. The patient was heteroplasmic, with 63% mutant in muscle but no mutant in blood. Furthermore, his RRFs were about 81% mutant while his non-RRFs were about 35% mutant. This mutation could not be detected in his mother or sister (625).

Five cases of MM and/or exercise intolerance have also been linked to mutations in the *MTCOI*, *MTCOII*, and *COIII* genes, all heteroplasmic. Two chain termination mutations have been reported in the *COI* gene associated with MM, rhabdomyolysis, and myoglobinuria. The *MTCO1*\*EXIT5920A mutation truncated the protein at codon 6 (W6Ter) (503), while the *MTCO1*\*MM6708A mutation truncated the protein at codon 269 (G269Ter) (545). Two missense mutations have been reported in *COII* that manifested as MM and exercise intolerance, *MTCO2*\*MM7671A (M29K) (832) and *MTCO2*\*MM7989C (L135P) (652). Finally, an in-frame deletion of 15 bp was reported in the *COIII* genes in a patient with exercise intolerance (626).

An example of the presentation seen in *COII* mutation cases is given by the 4-year-old boy with the *MTCO2*\*MM7671A mutation (M29K). This boy had a history of muscle weakness and fatigue. He was thin and had elevated serum lactate. Histochemical analysis revealed severe COX deficiency in 97% of fibers, though no RRFs on trichrome stain. Other clinical and physiologic findings were normal. Polarographic analysis of muscle mitochondria revealed a COX deficiency, and histochemical studies revealed a severe reduction in COII. Molecular analysis revealed that the T7671A mutation was heteroplasmic in muscle, but was not detected in myoblasts. It was present at 4.5–6% in peripheral blood (627).

The patient with the *COIII* mutation in-frame deletion was a 15-year-old female with periodic muscle cramps, elevated creatine kinase, and myoglobinuria associated with decreased caloric intakes. Histochemical analysis revealed many RRFs with 64% COX-negative fibers. Muscle mitochondrial COX activity was reduced 86% and cytochrome *aa<sub>2</sub>* was also reduced. Molecular analysis revealed a 15-bp deletion in *COIII* removing the intervening sequence between two direct repeats. This mutation was heteroplasmic, with the mutation being present at 92% in muscle and 1% in leukocytes and absent in fibroblasts. In muscle, COX-positive fibers had 25% mutant mtDNAs, COX-deficient fibers 97%, and COX-deficient RRFs 99%. The mutant mtDNA was not detected in the mother's leukocytes (626). These observations demonstrate that heteroplasmic intra-genic polypeptide mutations can result in a wide spectrum of the symptoms associated with mitochondrial encephalomyopathies.

Finally, two complex I subunit mutations have been found to cause MM. The chain termination mutation *MTND4*\*EXIT11832A (W358Ter) was associated with exercise intolerance (628), while the *MTND4*\*CPEO11232C (L140P) missense mutation

caused chronic progressive ophthalmoplegia with MM (629).

In total, these cases suggest that severe polypeptide mutations can cause isolated cases of severe MM. Such mutations are heteroplasmic, suggesting that they arose recently in the female germline.

### 11.1.28 Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy can be a clinical manifestation of a variety of mtDNA mutations, though in some patients it is the presenting feature. Two patients with *CYTB* mutations have been reported with MM and cardiomyopathy. Both of the reported cases have been missense mutations: *MTCYB*\*HCM15243A (G166E) (630) and *MTCYB*\*HCM15498A (G251D) (631).

### 11.1.29 Deafness and Sensorineural Hearing Loss

Sensorineural hearing loss is a common clinical manifestation of mtDNA mutations, both protein synthesis and polypeptide mutations. Six polypeptide mutations have been reported to be associated with deafness, four in *COI*, one in *COII*, and one in *ND6*. The four *COI* mutations are particularly interesting because they all affect the stop codon at the end of the *COI* polypeptide, codon 514. Mutations *MTCO1*\*DEAF7443G (Ter514G), *MTCO1*\*DEAF7445C (Ter514S), and *MTCO1*\*SNHL/LHON7444A (Ter514K) suppress the stop codon and result in the extension of the polypeptide by three amino acids (see Table 11-2 Panel B). The *MTCO1*\*SNHL/LHON7444A (Ter514K) mutation might also increase the risk for developing LHON (544). The fourth *COI* mutation, *MTCO1*\*SNHL7445G (Ter514Ter), changes the sequence of the stop codon and has been associated with deafness in several studies. The pathophysiologic effect of these variants is unclear. However, the fact that a clinical phenotype results even when a stop codon is retained suggests that the effect may not be in the function of the *COI* protein but perhaps in the processing of the polycistronic RNA transcript and thus the adjacent serine tRNA.

The *MTCO2*\*SNHL8108G (I175V) and *MTND6*\*SNHL14340T (V112M) are both missense mutations (632). Their pathophysiologic effect remains to be determined.

### 11.1.30 Movement Disorders and Dementias

Three missense mutations have been reported to be associated with AD and (PD). The *MTND1*\*ADPD3397G mutation was found associated with the *MTTQ*\*ADPD4336C mutation and changes a highly conserved amino acid (633). The *MTND2*\*AD5460A/T mutations have been associated with AD through populations



studies (634), but this association has been questioned (635,636).

### 11.1.31 Diseases Resulting from Intragenic tRNA and rRNA Mutations

Intragenic mutations that alter mtDNA tRNA and rRNA genes and cause disease due to inhibition of mitochondrial protein synthesis are referred to as mtDNA protein synthesis defects. A defect in mitochondrial protein synthesis has the capacity to retard the synthesis of all of the mtDNA-encoded polypeptides, resulting in the inhibition of complexes I, III, IV, and V, but not II. However, since the largest and most numerous mtDNA-encoded subunits belong to complex I (7 subunits) and complex IV (3 subunits), intermediate-severity protein synthesis defects most commonly affect these enzymes.

Unlike mtDNA mutations that inactivate a particular polypeptide and result in more stereotypic clinical presentations, protein synthesis mutations are frequently associated with multisystem disorders. Their variable clinical phenotypes can be additionally complicated by variation in the mutant mtDNA heteroplasmy.

The most common clinical manifestation of mitochondrial protein synthesis mutations is MM involving RRF and abnormal mitochondria, frequently with paracrystalline precipitates. For some mtDNA protein synthesis mutations, this may be the primary clinical presentation. For protein synthesis mutations that result in multisystem encephalomyopathies at high percentages of mutant, patients with lower percentages of mutant mtDNA might only manifest MM.

The variability in clinical presentations of mtDNA protein synthesis mutations was already apparent in the first mtDNA protein synthesis mutation described, the MTTK\*MERRF8344G mutation, causing maternally inherited MERRF syndrome (5,6). Variability in clinical presentation is also the hallmark of the most common mtDNA protein synthesis mutation, the MTTL1\*MELAS3243G mutation, causing MELAS syndrome (326). At 10–30% mutation, the MTTL1\*MELAS3243G mutation causes diabetes mellitus with or without deafness. However, at higher percentages of mutant the same mutation can cause chronic progressive ophthalmoplegia, cardiomyopathy, or the complete MELAS syndrome (412).

In this section, we discuss the mitochondrial protein synthesis mutations classified based on the most common clinical presentation seen when the patients harbor a high percentage of the mutant mtDNA.

### 11.1.32 Mitochondrial Encephalomyopathies and Multisystem Disorders

The mitochondrial encephalomyopathies combine MM with a complex array of neurodegenerative disease

symptoms. A variety of mtDNA tRNA mutations have been reported to result in these combined muscle and brain degenerative diseases (Table 11-3 Panels A and B). These mitochondrial protein synthesis mutations often affect multiple tissues, although the central nervous system, heart, skeletal muscle, kidney, and endocrine system are most often compromised. The nature and extent of the clinical symptoms can vary markedly based on the nature of the mutation and the degree of heteroplasmy in the affected tissues. The more severe protein synthesis mutations are frequently associated with elevated serum, urine, or cerebrospinal fluid lactate, alanine, and/or other organic and amino acids. Mitochondrial encephalomyopathies can either be stereotypical in their presentation, such as Leigh, MERRF, and MELAS syndromes, or they can be associated with a diverse array of symptoms.

Three mtDNA tRNA mutations have been associated with LS (MTTV\*LS1624T, MTTV\*1644T, and MTTW\*LS5537insT). In addition, other mtDNA tRNA mutation pedigrees can occasionally contain a family member who presents with LS. This has been observed in individuals harboring the protein synthesis mutations MTTK\*MERRF8344G (637,638) and MTTL1\*MELAS3243G (259) (see Table 11-3 Panels A and B).

However, LS is a relatively rare presentation for mtDNA protein synthesis gene mutations. By far the most common presentations are MERRF syndrome, most commonly caused by the MTTK\*MERRF8344G mutation (5,6), and MELAS syndrome, most commonly the result of the MTTL1\*MELAS3243G mutation (326). A description of the clinical variability of these common mutations will provide a sense of the complexities in diagnosing mitochondrial protein synthesis diseases.

MERRF-like pedigrees have been associated with nine different tRNA mutations. Classical MERRF syndrome has been observed and has been associated with seven different mtDNA tRNA mutations. By far the most common is the MTTK\*MERRF8344G mutation (5,6). Three other tRNA<sup>Lys</sup> mutations have also been linked to MERRF syndrome, specifically altering positions T8356C, G8361A, and G8363A (see Table 11-3 Panels A and B). Other tRNA mutations associated with MERRF syndrome have been reported in the tRNA<sup>Phe</sup> (611A), tRNA<sup>Leu(UUR)</sup> (3255A), and tRNA<sup>His</sup> (12147A), while related phenotypes have been observed in tRNA<sup>Val</sup> (1606A) and tRNA<sup>Asp</sup> (7543G) (see Table 11-3 Panels A and B).

MERRF syndrome typically presents with myoclonic epilepsy and MM. Myoclonic epilepsy is an uncontrolled periodic jerking, frequently beginning focally but progressing over the course of the disease to generalized cyclic muscle contractions. These are associated with high-amplitude voltage spikes on electromyographic analysis and with marked slowing of electroencephalographic wave activity (4,639).



While a pedigree might first be identified by a patient with MERRF syndrome, careful clinical examination frequently reveals individuals along the maternal lineage with a variety of other less severe clinical presentations. These can include subclinical electrophysiologic changes such as hyperexcitable visual evoked response and clinically related slowing and variability in somatosensory evoked response, sensorineural hearing loss, ataxia, renal dysfunction, MM, diabetes, cardiomyopathy, and dementia (4,259,639,640). Anterior and posterior cervical lipomas are also frequently seen in severely affected MERRF patients (259,638,641).

MERRF syndrome is most frequently associated with mutations in the tRNA<sup>Lys</sup> gene. Approximately 80–90% of cases result from the MTTK\*MERRF8344G mutation (4,642), while many of the remaining cases are due to the MTTK\*MERRF8356C mutation (643,644). Occasionally, other tRNA mutations can also result in MERRF syndrome, most notably the MTTL1\*MELAS3243G mutation (645). The MTTK\*MERRF8344G mutation changes a highly conserved A to a G in the TΨC loop of tRNA<sup>Lys</sup> gene, while the MTTK\*MERRF8356C converts a T to a C, disrupting an A–U base pair at the base of the TΨC loop. MERRF mutations are heteroplasmic in families.

A detailed analysis of the associations between clinical presentation, biochemical defect, and mtDNA genotype in one large MTTK\*MERRF8344G family revealed a strong correlation between genotype and phenotype, when age was taken into account (6,333,642). Among the 19- to 24-year-olds of this family, an individual with 15% normal mtDNAs in skeletal muscle had normal muscle mitochondrial energy capacity as determined by the anaerobic threshold in exercise stress tests ( $\theta_{an}$ ), normal OXPHOS enzyme activities, and normal phenotype, exhibiting only mild electrophysiologic changes. By contrast, 20-year-olds with only about 5% normal muscle mtDNA had reduced mitochondrial energy capacity and more severe symptoms, with the proband having less than 25% normal muscle energy capacity and the complete MERRF syndrome. Similarly, maternal relatives in the 40- to 50-year age range who retained about 10% normal mtDNAs showed higher muscle energy capacity and milder phenotypes than comparably aged individuals with half that level of normal mtDNAs, with the latter exhibiting reduced energy metabolism and more severe symptoms. Finally, among two maternal relatives in the 60- to 70-year range, an individual with 27% normal mtDNAs had significant residual mitochondrial energy capacity and mild to moderate symptoms, while another individual with 16% normal mtDNAs had complete MERRF syndrome. Most striking, however, was the difference between the 19-year-old with 15% normal mtDNAs and no clinical symptoms and the 60-year-old with 16% normal mtDNAs and severe disease. The much more severe clinical problems of the older subject with the sample mtDNA genotype reflect a general feature of

mtDNA diseases: They showed a delayed onset and they progressed with age.

A severe mitochondrial protein synthesis defect has been found to be associated with the MTTK\*MERRF8344G mutation in patient skeletal muscle (646), myoblasts/myotubes (647,648), and fibroblasts (649). The translation defect results in a general reduction in the rate of protein synthesis most affecting the larger mitochondrial polypeptides, together with the generation of abnormal translation products. This results from a 50% to 60% decrease in tRNA<sup>Lys</sup> aminoacylation (650). Accompanying the translation defect is a reduction in mitochondrial O<sub>2</sub> consumption and ETC enzyme activity (6,649). Protein synthesis abnormalities and OXPHOS defects have been unambiguously assigned to both the MTTK\*MERRF8344G and MTTK\*MERRF8356C mutations by cybrid transfer using both patient myoblasts (MTTK\*MERRF8344G only) (648) and fibroblasts (651). It is postulated that the reduction of mitochondrial protein synthesis results in lowering of the steady-state levels of OXPHOS enzyme complexes, perhaps due to premature termination of translation at lysine codons.

MELAS syndrome has now been associated with 13 different mtDNA tRNA mutations, 8 of which are found in the tRNA<sup>Leu(UUR)</sup>. In addition, the MELAS presentation has been associated with tRNA<sup>Phe</sup>, tRNA<sup>Val</sup>, and tRNA<sup>Gln</sup> mutations and one of the rare instances of a heteroplasmic 16S rRNA mutation (see Table 11-3 Panels A and B).

MELAS pedigrees are often ascertained through a patient who has experienced a stroke-like episode. Generally this first occurs between the ages of 5 and 15 years, though it can occur any time from infancy to adulthood. Muscle biopsy of such patients usually reveals MM (652). In addition, maternal relatives can exhibit a wide variety of complex clinical problems involving the central nervous system, muscle, heart, renal, and endocrine systems. MELAS syndrome can be caused by a variety of mtDNA tRNA mutations, most of which are in the tRNA<sup>Leu(UUR)</sup> gene. The MTTL1\*MELAS3243G (653,654) mutation is by far the most common. Additional MELAS mutations include MTTL1\*MELAS3244A (655), MTTL1\*MELAS3252G (656), MTTL1\*MELAS3258C (657), MTTL1\*MELAS3256T (658), MTTL1\*MELAS3271C (659), and MTTL1\*MELAS3291C (660,661). In addition to these mutations, other tRNA mutations, including the MTTK\*MERRF8344G mutation, can give a MELAS-like phenotype. MELAS mutations have also been reported in tRNA<sup>Phe</sup> (G583A), tRNA<sup>Val</sup> (G1642A), and tRNA<sup>Gln</sup> (G4332A) and in the 16S rRNA (C3093G) (see Table 11-3 Panels A and B). Some mtDNA tRNA mutations, such as the MTTL1\*MELAS3256T and the MTTS1 \*MERME7512C mutations, can give rise to a MERRF/MELAS overlap syndrome (658,662,663). The fact that complex I missense mutations give MELAS

syndrome and that defects in mitochondrial protein synthesis that would inhibit the expression of the seven complex I subunits all give MELAS syndrome provides convincing evidence that MELAS syndrome is the product of severe complex I deficiency.

Like the MTTK\*MERRF8344G mutation, both the MTTL1\*MELAS3243G and the MTTL1\*MELAS3271C mutations result in a marked defect in mitochondrial protein synthesis. This is most dramatically demonstrated in cybrid studies performed with patient myoblasts (664,665) and fibroblasts (665,666). Invariably, the MELAS mutations cause a generalized reduction in the rate of mitochondrial protein synthesis of up to 70% (664) and a concomitant reduction in the steady-state levels of mitochondrial proteins when the proportion of mutant mtDNAs is greater than about 94%. Cybrid cell lines with greater than 94% MTTL1\*MELAS3243G and MTTL1\*MELAS3271C mutant mtDNAs can also have oxygen consumption rates reduced up to 70% (664–666), although to a lesser degree in the MTTL1\*MELAS3271C cybrid lines (666). Histologic and immunohistochemical analysis (667) has revealed a reduction in the levels of gene products in patient skeletal muscle harboring the MTTL1\*MELAS3243G mutation. The translation of the larger mitochondrial-encoded gene products appears to be most severely compromised, and preferential defects in complex I and/or complex IV are frequently observed in skeletal muscle (653,668–670) for MTTL1\*MELAS3243G patients.

The actual pathophysiologic mechanism by which the MTTL1\*MELAS3243G mutation results in MELAS remains unclear. Because the 3243 nucleotide lies within the transcriptional terminator sequence for the rRNA genes, it is possible that this mutation perturbs the transcriptional processes. In *in vitro* experiments, diminished affinity for the mitochondrial transcription terminator protein (mTERM) for the mutant terminator sequence has been reported (671). This was related to an impairment of transcript termination for the 16S rRNA gene, and possibly altering the rRNA:mRNA ratio. Some studies have confirmed a reduced affinity of mTERM for the mutant termination sequence (664), and in some cases reduced transcriptional termination (672), but others have found no evidence of abnormal termination, altered rRNA production, or a MELAS-specific perturbation in rRNA:mRNA ratios (664,667,672–674). Moreover, the MTTL1\*MELAS3271C mutation is not found within the transcription. A non-protein coding CR polymorphism at T16189C has also been reported to predispose to type 2 diabetes (675), though this association has been challenged (676).

The primary cause of MELAS syndrome may be altered mitochondrial translation. One proposal is that the MTTL1\*MELAS3243G and MTTL1\*MELAS3271C mutations alter transcript processing. Cybrids harboring these mutants have been reported to accumulate RNA processing intermediate RNA 19, which

corresponds to a transcript containing the contiguous 16S rRNA + tRNA<sup>Leu(UUR)</sup> + ND1 genes (665,666).

The spectrum of clinical manifestations associated with maternal relatives of MELAS patients is best characterized for the MTTL1\*MELAS3243G mutation (259,660,669,677,678). In addition to strokes, patients harboring this mutation can be prone to migraine headaches and focal or generalized seizures, ataxia, myoclonus, sensorineural deafness, retinopathy, and dementia. They can experience fatigability, myopathy, renal failure, myalgia, and ophthalmoplegia, and develop hypertrophic or dilated cardiomyopathy and cardiac conduction defects. In rare cases, this mutation has also been associated with peripheral neuropathy plus rhabdomyolysis (679), demyelinating polyneuropathy (680), and ischemic colitis (681). By far the most dramatic alternative phenotype of the MTTL1\*MELAS3243G mutation is type 2 diabetes mellitus with or without sensorineural hearing loss (682). It is now well established that diabetes mellitus is by far the most common clinical presentation of the MTTL1\*MELAS3243G mutation (see below).

The pathophysiology of mitochondrial stroke is quite different from that of the stroke associated with vascular occlusion. CT and MRI analysis frequently reveals infarcts in the posterior temporal, parietal, and occipital lobes. These frequently overlap several large vessel vascular beds, and can be transitory, often resolving along with the clinical signs. It has been hypothesized that these infarcts are due to transient OXPHOS dysfunction within the brain parenchyma, rather than vascular events (259,652,683). Alternatively, they might be the product of vascular spasm, with the contraction extending along an extended region of the cortical vasculature, inhibiting blood flow and causing ischemia. This hypothesis is supported by extensive postmortem analysis of the cortical vasculature of MTTL1\*MELAS3243G and MTTL1\*MELAS3271C mutation patients (660,674,684,685). Histochemical and electron microscopic analyses of MELAS patient vessels have revealed accumulations of abnormal mitochondria in the vascular endothelial cells and vascular smooth muscle cells. The mitochondria are frequently pycnotic, contain mitochondrial occlusions, and stain extensively for SDH (complex II), which is associated with a large increase in both mutant and normal mtDNAs (653,660,674,686,687). The increased complex II activity implies a compensatory induction in mitochondrial biogenesis in response to a vascular cell OXPHOS deficiency.

Patients with mitochondrial protein synthesis defects resulting from the MTTL1\*MELAS3243G mutation or mtDNA deletions show striking increases in immunohistochemical staining of their RRFs for the mitochondrial MnSOD and weakly increased staining for the cytosolic copper/zinc superoxide dismutase (Cu/ZnSOD). Moreover, the MnSOD staining is strongly positive in the subsarcolemmal region and shows a coarsely granular,

reticular, or diffuse pattern between the myofibrils. Interestingly, the MELAS RRFs that are MnSOD positive are frequently still COX positive, while the CPEO MnSOD-positive fibers are generally COX negative (688). Hence, mitochondrial protein synthesis defects are associated with MnSOD and Cu/ZnSOD induction and hence increased ROS production. Partial defects in respiratory complex I are associated with induction of the mitochondrial MnSOD, suggesting increased oxygen radical production (689).

Vascular blood flow is regulated by the contraction and dilation of the vascular smooth muscle cells, which in turn is regulated by diffusible effectors synthesized and released by the vascular endothelial cells, specifically, the vasodilator NO. NO is rapidly inactivated by superoxide anion ( $O_2^{\cdot-}$ ) and other ROS (690,691). The increased production of ROS should deplete the NO, causing loss of vasodilatory activity. The resulting vasoconstriction could in some instances be sufficient to block blood flow and cause a stroke. Later fluctuations in superoxide production would permit increased NO production and vasodilation, partially restoring blood flow.

After stroke-like episodes, the most common clinical presentation of the MTTL1\*MELAS3243G mutation is ophthalmoplegia, ptosis, and MM. CPEO is also the most common presentation of mtDNA rearrangement syndromes. Since every pathogenic rearrangement that has been studied to date alters or removes one or more tRNA genes (281), mtDNA rearrangement mutations also cause protein synthesis defects. Hence, ophthalmoplegia, ptosis, and MM are common presentations of severe defects in mitochondrial energy production.

Beyond the classical mitochondrial encephalomyopathy phenotypes of LS, MERRF syndrome, and MELAS syndrome, mtDNA protein synthesis mutations have been reported to produce an array of complex phenotypes. These range from a combination of MERRF, MELAS, and progressive encephalopathy symptoms, to ataxia, myoclonus and deafness, to gastrointestinal reflex and sudden infant death syndrome, to Rett syndrome (RS). All of these cases are the result of tRNA mutations except the RS case, which was associated with a 16S rRNA mutation (C2835T) (see Table 11-3 Panels A and B).

One example of this more severe class of mitochondrial encephalomyopathies in the MTTL1\*PEM3271delT mutation is associated with progressive encephalomyopathy (692). This mutation deleted one of the three Ts in the anticodon stem of the tRNA<sup>Leu(UUR)</sup>. The patient harboring the MTTL1\*PEM3271delT initially manifest at 5 years of age with progressive hearing loss leading to deafness by age 18 years. Seizures occurred in childhood, and the disease progressed into young adulthood to include MM (RRFs), retinitis pigmentosa, glaucoma, hypogonadism, dementia, and severe cerebral calcifications (Fahr disease). The patient died at 28 years of tonic-clonic seizures, renal failure, and sepsis. The percentage

of mutant mtDNAs was about 75% in the patient's skeletal muscle, but the mutant was absent in the mother's peripheral blood. Hence, this appears to be a new mutation that arose in the female germline and segregated sufficiently rapidly to give a lethal phenotype.

The more deleterious mtDNA tRNA mutations result in more severe central nervous system defects. These mutations can be so severe that individuals with moderately high percentages of heteroplasmic mutations fail to survive and reproduce. Hence, these are often "spontaneous" cases.

### 11.1.33 Mitochondrial Myopathy

The milder tRNA mutations can present with isolated MM, MM plus CPEO, or MM in association with Kearns-Sayre syndrome (KSS). This class of disease is occasionally referred to as "mitochondrial cytopathy" (see Table 11-3 Panels A and B). To date, more than 45 tRNA mutations have been reported with MM, CPEO, KSS, and mitochondrial cytopathy (see [www.mitomap.org](http://www.mitomap.org)).

Some examples of patients with MM due to a tRNA<sup>Leu(UUR)</sup> mutation are given by the MTTL1\*MM3250C and MTTL1\*MM3302G mutations. The MTTL1\*MM3250C mutation converts a weakly conserved T to a C. This mutation was associated with fatigability and RRFs, with one individual having respiratory muscle weakness and elevated lactate with exercise. The mutation was heteroplasmic, and varying percentages of mutant mtDNAs in skeletal muscle were associated with complex I and IV defects (693). The MTTL1\*MM3302G mutation was heteroplasmic and changed the highly conserved penultimate 3' A to a G. This mutation has been reported in two independent families. The first individual exhibited a progressive muscle weakness as a young adult (694), while the second exhibited an early-onset and rapidly progressive weakness and degeneration of the scapular flexor and extensor muscles resulting in a striking inability to hold the head erect against any lateral pressure (695). Both mutations were heteroplasmic and associated with skeletal muscle RRFs and OXPHOS enzyme defects. However, analysis of mitochondrial protein synthesis in lymphoblast and fibroblast cell lines failed to reveal a mitochondrial protein synthesis defect. Analysis of tRNA<sup>Leu(UUR)</sup> processing by monitoring the RNA 19 (16S rRNA + tRNA<sup>Leu(UUR)</sup> + ND1), 16S rRNA, tRNA<sup>Leu(UUR)</sup>, and ND1 transcripts led to the proposal that this mutation causes a defect in the processing of tRNA<sup>Leu(UUR)</sup> in the skeletal muscle, but not in lymphocytes and fibroblasts, presumably due to a tissue-specific tRNA processing enzyme (694). Additional studies are required to substantiate this hypothesis, but, if true, it provides a novel mechanism for tissue-specific defects in mutations changing highly conserved nucleotides.



An example of an mtDNA protein synthesis mutation in a different tRNA, tRNA<sup>Pro</sup>, is the MTTP\*MM15990A mutation. This mutation converted the proline anticodon UGG (codon CCA) to a serine anticodon UGA (codon UCA), rendering the mutant tRNA nonfunctional. This mutation caused classical MM without extraocular muscle involvement in a 7-year-old patient harboring 85% muscle mutant mtDNA in skeletal muscle. The mutation only affected mitochondrial protein synthesis of cultured cells when present in greater than 90% of the mtDNAs (696,697).

MM together with CPEO can be caused by mtDNA tRNA mutations, mtDNA rearrangement mutations, and nDNA replication enzyme mutations. In a survey of 28 KSS and 109 progressive external ophthalmoplegia (PEO) patients, 28 KSS patients had deletions and 1 harbored the MTTL1\*MELAS3243G mutation, while of the PEO patients, 46 (42%) harbored mtDNA deletions and 15 (14%) harbored the MTTL1\*MELAS3243G mutation. One additional PEO patient had the MTTK\*MERRF8344G mutation. KSS and CPEO patients who harbor mtDNA tRNA mutations are generally members of larger maternal pedigrees with several variously affected family members (678). Among the mtDNA protein synthesis mutations that cause CPEO, by far the most common is the MTTL1\*MELAS3243G mutation. Therefore, about half of the CPEO cases are due to mtDNA rearrangements and half due to mtDNA protein synthesis mutations. Of the those that result from point mutations, the majority of the cases are due to the MTTL1\*MELAS3243G mutation (660,669,677,698,699). Mutations in six other mtDNA tRNA genes have also been reported to cause CPEO.

Two additional tRNA<sup>Leu(UUR)</sup> mutations that have been associated with CPEO and KSS include the np T3273C (700) and G3249A mutations (701).

Ophthalmoplegia is also a major clinical feature of the tRNA<sup>Glu</sup> mutation at MTTG\*CIPOA10006G (702), and the tRNA<sup>Ser(AGY)</sup> mutation at MTTs2\*CIPO12246G (702). Ophthalmoplegia is also an occasional symptom for a variety of other deleterious mtDNA tRNA mutations, including the MTTK\*MERRF8344G mutation (678) and potentially others of the tRNA<sup>Leu(UUR)</sup> MELAS mutations (660). As is the case for mtDNA rearrangement mutations, milder renditions of the CPEO mutations can also cause diabetes mellitus and deafness. This phenotype is a common presentation of patients harboring 5–30% of the MTTL1\*MELAS3243G mutation (660,703).

The clear demonstration that both the MELAS and MERRF tRNA mutations inhibit mitochondrial protein synthesis indicates that this is the underlying mechanism that causes ophthalmoplegia, ptosis, and MM. This would also explain the origin of the symptoms in the CPEO and KSS patients with mtDNA rearrangements, since every pathogenic rearrangement that has been studied to date alters or removes one or more tRNA genes (704). Hence,

the rearrangement mutations would also cause protein synthesis defects, a result confirmed by cybrid studies of CPEO patient deletion cell lines. Hence, ophthalmoplegia, ptosis, and MM must result from primary defects in mitochondrial protein synthesis. Since mtDNA encodes seven subunits of complex I and three complex IV subunits, these would be the OXPHOS enzymes likely to be most affected, as has been observed. Hence, ophthalmoplegia, ptosis, and MM probably reflect severe defects in mitochondrial energy production.

Intestinal dysmotility is a common symptom of patients with mitochondrial encephalomyopathies and myopathies due to tRNA mutations. In some cases, this can be the presenting clinical concern. Five tRNA mutations have been reported in which gastrointestinal problems were the primary complaint. They occurred in tRNA<sup>Phe</sup> A608G, tRNA<sup>Trp</sup> G5532A, tRNA<sup>Lys</sup> G8313A, tRNA<sup>Gly</sup> A10006G, and tRNA<sup>Ser(AGY)</sup> C12246G (see Table 11-3 Panels A and B). However, the stereotypical presentation of intestinal dysmotility is most commonly seen in the autosomal recessive mitochondrial disease, mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), resulting from mutations in the thymidine phosphorylase (TP) gene. MNGIE has been reported in association with a tRNA<sup>Lys</sup> G8313A mutation (705).

### 11.1.34 Cardiomyopathy

Cardiomyopathy is also a common clinical manifestation of mtDNA protein synthesis mutations, frequently found in association with MM. Eight mtDNA tRNA mutations have been found to be associated with cardiomyopathy in association with myopathy. These involve tRNA<sup>Leu(UUR)</sup> A3260G and C3303T, tRNA<sup>Ile</sup> A4295G and A4300G, tRNA<sup>Lys</sup> A8348G, tRNA<sup>Gly</sup> T9997C, tRNA<sup>His</sup> G12192A, and tRNA<sup>Leu(CUN)</sup> T12297C (see Table 11-3 Panels A and B).

Examples of mtDNA protein synthesis mutations that manifest as cardiomyopathy with MM include the MTTL1\*MMC3303T and MTTL1\*MMC3260G mutations. The MTTL1\*MMC3303T mutation was found in two infants who died at 10 weeks and 9 months of age, and was observed to be heteroplasmic (706). The tRNA<sup>Leu(UUR)</sup> mutation MTTL1\*MMC3260G, associated with hypertrophic cardiomyopathy, was been reported in two independent pedigrees (707,708). Individuals harboring the MTTL1\*MMC3260G mutation manifest symptoms of congestive heart failure as young adults, ultimately leading to hypertrophy with disorganized cardiomyocytes, fibrosis, and cardiac conduction defects (Wolff–Parkinson–White syndrome). Other clinical manifestations in the maternal lineage included insulin-dependent diabetes mellitus (IDDM) and cataracts. This heteroplasmic mutation has been shown to segregate along the maternal lineage, and the percentage mutant mtDNAs has been shown to correlate with the level of skeletal muscle complex I deficiency, oxygen



consumption, and clinical severity (708). Transmitochondrial cybrids harboring the MTTL1\*MMC3260G mutation prepared from patient myoblasts have been found to have defective protein synthesis and mitochondrial respiration (709), confirming the pathogenicity of this mutation.

A heteroplasmic tRNA<sup>Gly</sup> mutation, MTTG\*MHCM 9997C, has been reported in associated with maternally transmitted hypertrophic cardiomyopathy in a single, multigeneration family. This mutation changes a highly conserved base, has not been observed in controls, and results in multiple OXPHOS enzyme defects (710).

A heteroplasmic tRNA<sup>Ile</sup> mutation has been associated with adult-onset hypertrophic cardiomyopathy with slow progression. This mutation, MTTI\*MICM4300G, was found in a maternally inherited pedigree in which three maternal relatives were diagnosed with hypertrophic cardiomyopathy and several other maternal relatives exhibited mild clinical and electrocardiographic indications of hypertrophic cardiomyopathy (711). Although RRFs were found in the patient's skeletal muscle, no OXPHOS biochemical defects were detected in cardiac or skeletal muscle.

Cardiomyopathy may also be seen in association with the classical MTTK\*MERRF8344G and MTTL1\*MELAS3243G mutations (660).

Finally, two mutations have been reported to cause fatal infantile cardiomyopathy plus (FICP), the plus including encephalopathy. In the first case, a tRNA<sup>Ile</sup> mutation, MTTI\*FICP4269G, was observed in an 18-year-old who died with hypertrophic cardiomyopathy, MM, renal disease, hearing loss, generalized seizures, and mental retardation. The mutation was heteroplasmic in the mother and proband, was associated with morphologic changes of the mitochondria in cultured cells, and resulted in a protein synthesis defect in cybrids derived from patient fibroblasts (712,713).

The second case of FICP, the MTTI\*FICP4317G mutation, was described in an infant who died at 1 year of severe hypertrophic cardiomyopathy together with skeletal muscle necrosis and a complex I and IV deficiency in the heart (714). While the mutation was proposed to alter the TΨC loop by creating a new G–C base pair, the mutation was not reported to be heteroplasmic, and only 28 controls were screened. Hence, the etiology of this mutation requires confirmation.

### 11.1.35 Deafness, Sensorineural Hearing Loss, and Aminoglycosides

Maternally inherited deafness and/or aminoglycoside-induced deafness has been associated with protein synthesis mutations, eight in the 12S rRNA mutations and five tRNA mutations, three in tRNA<sup>Ser(UCN)</sup>, one in tRNA<sup>His</sup>, and one in tRNA<sup>Glu</sup>. The mutations seen in the 12S rRNA are A827G, T961C, T961delTinsC, T961insC, T1005C, A1116G, C1494T, and A1555G. The three

tRNA<sup>Ser(UCN)</sup> mutations are at T7510C, T7511C, and 7472insC, the tRNA<sup>His</sup> mutation is at G12183A, and the tRNA<sup>Glu</sup> mutation is at T14709C (see Table 11-3 Panels A and B).

The MTRNR1\*DEAF1555G is the best characterized of the mtDNA deafness mutations. The MTRNR1\*DEAF1555G mutation, though consistently homoplasmic, has now been confirmed in multiple independent studies (683,715–718). Individuals harboring this mutation were first observed in a large Middle Eastern pedigree featuring sensorineural hearing loss along the maternal lineage. The maternal transmission with variable penetrance was hypothesized to be the combination of the maternally transmitted MTRNR1\*DEAF1555G mutation acting together with an autosomal recessive nuclear gene (715). Subsequent studies in China and elsewhere have shown that individuals harboring this mutation are acutely sensitive to aminoglycoside inhibition. The mutation occurs at the end of a stem-and-loop structure in the small rRNA subunit, adding a terminal base pair and thus making the mitochondrial ribosome more like a bacterial ribosome at the aminoglycoside binding site (716). Pedigrees harboring this mutation have occasionally been observed to manifest other neurologic signs, including the extrapyramidal signs of tremors and rigidity seen in PD (683). Despite the alteration of rRNA structure, a protein synthesis defect has not been reported for this mutation.

Confirmation that the MTRNR1\*DEAF1555G (nt 1555 A to G) mutation is a pathogenic mutation comes from patients harboring the nt 1494 C-to-T variants in the 12S rRNA gene (see Figure 11-6). The 1494C and 1555A bases are in apposition to each other in the 12S rRNA but do not form a base pair. However, if the C at nt 1494 is mutated to a T or the A at nt 1555 is mutated to a G, then a base pair is formed, extending the adjacent stem by one nucleotide and making the ribosome more bacteria-like (719). The nt 1494T and nt 1555G mutations are relatively innocuous by themselves, the nt 1494T variant having been observed in both a deaf patient (720) and a normal individual from haplogroup A (721). However, these variants greatly increase sensitivity to aminoglycoside antibiotic-induced deafness (716,718,720).

The two tRNA<sup>Ser(UCN)</sup> mutations associated with sensorineural hearing loss have been reported in adjacent nucleotides: T7510C (722) and T7511C (723). Both were heteroplasmic. By contrast, the tRNA<sup>His</sup> mutation at G12183A was homoplasmic (724). In addition to these tRNA mutations associated with deafness, two other tRNA mutations have been reported to be associated with deafness in addition to other clinical manifestations.

Maternally transmitted bilateral sensorineural hearing loss has also been observed associated with a homoplasmic tRNA<sup>Ser</sup> mutation, MTTS1\*DEAF7445G/

MTCO1\*DEAF7445G (725,726). This mutation was subsequently reported to be heteroplasmic within this family (727) and in another (728) (see Table 11-3). Since this mutation occurs in the final base of the COI termination codon and does not change the sense of the codon, it has been proposed that the 7445G mutation impairs the adjacent tRNA<sup>Ser</sup> (729).

### 11.1.36 Diabetes Mellitus with/without Deafness

The genetic linkage of mtDNA tRNA (730) and rearrangement (731,732) mutations to type 2 diabetes has directly implicated mitochondrial protein synthesis defects in the etiology of diabetes and the metabolic syndrome. Evidence that mtDNA defects are a common factor in the etiology of diabetes comes from the observation that, as the age of onset of the proband increases, the probability that the mother was the affected parent also increases, reaching a ratio of 3:1 for patients with a mean age of onset of 46 years (412). These observations have been linked to the larger human metabolic syndrome through the identification of an mtDNA tRNA<sup>Ile</sup> mutation at np T4291C that causes hypertension, hypercholesterolemia, and hypomagnesemia (renal ductal convoluted tubule defect). This mutation was found in a maternal pedigree in association with reduced mitochondrial ATP production and the secondary clinical findings of migraine, hearing loss, hypertrophic cardiomyopathy, and MM (733).

12S rRNA mutations (C1310T and A1438G) and six tRNA mutations (tRNA<sup>Leu(UUR)</sup> A3243G, T3264C, and T3271C; tRNA<sup>Ile</sup> T4291C; tRNA<sup>Lys</sup> A8296G; and tRNA<sup>Ser(AGY)</sup> C12258A) have been associated with diabetes and the metabolic syndrome. The most common mtDNA mutation associated with diabetes mellitus is the MTTL1\*MELAS3243G mutation (703,730,734,735). The MTTL1\*MELAS3243G mutation has been found at about 1.4% in IDDM and NIDDM patients (703). The frequency is higher for NIDDM if the mother is also affected (5.7%), and much higher if the patients have both NIDDM and sensorineural hearing loss, being 60% (3 of 5) in a Japanese study (736) and 10% (2 of 20) in a British study (737). While the great majority of diabetes mellitus MTTL1\*MELAS3243G patients have a primarily NIDDM presentation, many either present with or progress to IDDM (703,736). Furthermore, slowly progressive IDDM has been associated with the MTTL1\*MELAS3243G mutation, and in one study of islet-cell antibody-positive patients with progressive NIDDM, the MTTL1\*MELAS3243G mutation was found in 3 of 27 of the patients (11%), suggesting that islet cell loss and subsequent autoantibody production can also be associated with mitochondrial defects (738,739). These and other observations have led to a delineation of the characteristics of MTTL1\*MELAS3243G diabetes mellitus, which

include maternal transmission; variable clinical phenotype, including NIDDM, slowly progressive IDDM, and IDDM; young to middle-age onset; tendency toward progression; and association with sensorineural hearing loss. Moreover, many of these patients are lean and require insulin, but are less prone to ketoacidosis. They show a delayed insulin response to glucose and impaired glucagon secretion, and they may or may not have autoantibodies to pancreatic islet cells (703,736,740). The MTTL1\*MELAS3243G mutation is invariably heteroplasmic in diabetes mellitus patients, and generally present as a lower percentage mutant than commonly found in MELAS patients, frequently in the range of 5–30% in blood, with levels potentially higher in muscle and fibroblasts (734,735). Indeed, it is not uncommon that blood cells will lack the mutation even though it is present in post-mitotic tissues. Hence, this cause of diabetes mellitus is probably underdiagnosed.

Due to the systemic nature of the MTTL1\*MELAS3243G mutation, it is not uncommon for patients presenting with diabetes mellitus to also exhibit additional clinical manifestations. Examples include diabetes mellitus with diabetic amyotrophy (734), diabetes mellitus with diarrhea and abdominal pain involving 37% mutant mtDNAs in blood and 70% mutant mtDNAs in gastric and rectal mucosa and skeletal muscle (741), and diabetes mellitus with post-insulin treatment neuropathy (742).

In addition, the common tRNA<sup>Lys</sup> MTTK\*MERRF 8344G mutation has been identified in one three-generation pedigree that presented with diabetes mellitus and deafness and harbored the MTTK\*MERRF 8344G mutation, with mutant mtDNA levels ranging from 12% to 28% in peripheral blood cells (640). However, this MERRF mutation is not a common cause of diabetes mellitus since the mutation was not found in a survey of several hundred Japanese diabetes mellitus patients (736).

The MTTT1\*DMDF7472Cins has been found in a maternally inherited pedigree featuring progressive, bilateral hearing loss as the most common clinical manifestation. Ataxia due to cerebellar dysfunction and myoclonus were also prominent in the family (743). This mutation involves the insertion of a C within a homopolymeric stretch of 6 C nucleotides, was found to be heteroplasmic in the family, was not found in nearly 400 controls, resulted a complex I biochemical deficiency, and likely alters the conformation of the TΨC stem-loop. Cybrid studies confirmed the pathogenicity of this mutation, and there was a correlation between proportion of mutant mtDNA and clinical manifestations in blood (743).

Myopathy with diabetes mellitus has been associated with a heteroplasmic MTTE\*MDM14709G mutation in two families (744,745) one of which also presented with mental retardation and cerebellar ataxia (744). Histologic and/or immunohistochemical analysis of patient

skeletal muscle revealed the classical signs of myopathy: RRFs, COX-negative fibers, abnormal mitochondrial morphology, and paracrystalline inclusions. Biochemically, this tRNA<sup>Glu</sup> mutation caused a reduction in complex I and IV specific activities and, in myoblasts, resulted in a mitochondrial protein synthesis defect (744).

Diabetes mellitus is also seen in Wolfram syndrome, which is characterized by diabetes insipidus, diabetes mellitus, optic atrophy, and deafness. Other less common endocrine and neurologic symptoms include hypogonadism, atonic bladder, ataxia, insomnia, seizures, and psychiatric disorders (703). One patient diagnosed with Wolfram syndrome including diabetes mellitus, optic atrophy, and sensorineural deafness was found to harbor the MTND4\*LHON11778A mutation (514), and another case of Wolfram syndrome was found to contain a 7.6-kbp mtDNA deletion (746). However, sequence analysis of the blood cell tRNAs from two other Wolfram patients failed to reveal any disease-specific mutations (682). Hence, the role of mtDNA protein synthesis mutations in Wolfram syndrome remains unclear (703).

### 11.1.37 Movement Disorders and Dementias

In certain instances, mtDNA mutations can present with neuropsychiatric symptoms including depression, movement disorders, and dementias. Movement disorders have been most commonly associated with mtDNA polypeptide gene mutations. Examples discussed previously include the MTND6\*LDYT14459A mutations (537,538), which can present as generalized dystonia, and the report of the MTND4\*LHON11778A mutation associated with Parkinson-like symptoms (518,523). A tRNA<sup>Val</sup> T1659C mutation has also been linked to movement disorders (747).

Patients harboring mtDNA mutations may also develop progressive dementia. One patient with progressive cognitive decline, dementia, deafness, ataxia, and chorea was found to be heteroplasmic for a tRNA<sup>Trp</sup> mutation, MTTW\*DEMCHO5549A. Post-mortem analysis of the brain revealed diffuse and moderate neuronal loss in the cortex and basal ganglia, with gliosis present throughout the brain. RRFs and COX-negative-staining fibers were evident on skeletal muscle analysis, as were morphologically abnormal mitochondria on electron microscopy of skeletal muscle. A complex I defect was detected in mitochondrial respiration assays. Hence, this tRNA<sup>Trp</sup> mutation demonstrates that respiratory defects can cause dementia (748). This has been substantiated by the identification of the tRNA<sup>Gln</sup> gene mutation at np 4336, MTTQ\*ADPD4336G, which has been associated with about 5% of late-onset AD and also linked to hearing loss and migraine (633,749–751). The tRNA<sup>Gln</sup> 4336C mutation has also been associated with a 16S rRNA mutation at G3196A (633).

### 11.1.38 Diseases Resulting from Rearrangement Mutations

A rearrangement mutation is defined in this chapter as a mutation encompassing two or more genes. These can be insertions, deletions, or a combination of the two. Since most of the mtDNA genes are punctuated by tRNAs, rearrangement mutations generally affect one or more tRNAs (752). Therefore, most rearrangement mutations perturb mitochondrial protein synthesis. As a result, patients harboring mtDNA rearrangements frequently have arrays of clinical phenotypes similar to those seen in patients with point mutations in individual tRNA or rRNA genes. Accordingly, the most common rearrangement phenotypes are diabetes, CPEO, and KSS. In addition, mtDNA deletions also cause the more severe Pearson marrow-pancreas syndrome.

#### 11.1.38.1 Diabetes Mellitus with/without Deafness.

As with MTTL1\*MELAS3243G mutations, which can cause CPEO but also diabetes mellitus (703,730,734,735), mtDNA rearrangement mutations can also manifest as diabetes mellitus and deafness (731,732).

Rearrangements in mtDNA have repeatedly been associated with diabetes mellitus. This association was first clearly demonstrated for KSS patients with mtDNA duplications. The smaller the duplication, and larger the reciprocal deletion, the more likely the patient is to develop diabetes mellitus (753–757).

The first proof that type 2 diabetes mellitus could be caused by an mtDNA insertion–deletion mutation came from the study of a large African American pedigree that showed maternal inheritance of diabetes and deafness and harbored an mtDNA duplication and reciprocal deletion (731,732). Since that observation, diabetes mellitus has been found to be a common clinical manifestation in patients with mtDNA duplications, generally in association with KSS (757,758).

In the initial African American family with type 2 diabetes mellitus and sensorineural hearing loss, the maternal relatives developed hearing loss in their 20s and 30s and type 2 diabetes mellitus in their 30s and 40s. Occasional individuals in the rearrangement pedigree experienced stroke-like episodes associated with cortical and brain stem lucencies on MRI examination. None of the patients had ophthalmoplegia or ptosis, nor did they have MM as detected by muscle histology. However, detailed biochemical analysis of several family members revealed a generalized OXPHOS defect in the skeletal muscle. Physiologic analysis indicated that all maternal relatives developed IDDM, with some individuals developing diabetic ketoacidosis. Glucose tolerance tests revealed that severely affected individuals were unable to respond to hyperglycemia with increased insulin production from beta cells or decreased glycogen production from alpha cells (731).

The mtDNA rearrangement in this maternally inherited pedigree consisted of three main types of mtDNA molecules: (1) normal mtDNAs; (2) duplicated mtDNAs



containing an insertion of 6.1 kbp of the mtDNA encompassing from np 4389 in the tRNA<sup>Gln</sup> gene to np 14822 in the *MTCYB* gene and including the ND1, 16S rRNA, 12S rRNA, and part of the *MTCYB* genes, as well as the tRNA<sup>Ile</sup>, tRNA<sup>Leu(UUR)</sup>, tRNA<sup>Val</sup>, tRNA<sup>Phe</sup>, tRNA<sup>Pro</sup>, and tRNA<sup>Thr</sup> genes, and the D-loop including O<sub>H</sub>; and (3) finally, deleted mtDNAs that lack 10.4 kbp of mtDNA, including 11 of the 13 OXPHOS genes, 15 of 22 tRNAs, O<sub>L</sub>, and 75 bp of the 5' end of the *MTCYB* gene. The deleted molecules appear to be present as a dimer of about 12 kbp, and the rearrangement is encompassed by a 10-bp direct repeat (5'-CACCCCATCC-3'). Lymphoblastoid cell lines carrying this rearrangement exhibit a partial protein synthesis defect. Extended propagation results in selective enrichment for the duplicated molecules and loss of the deleted and normal mtDNAs. Moreover, white blood cells from patients have an excess of duplicated molecules, while the post-mitotic muscle had a higher proportion of deleted than duplicated mtDNAs. These results suggest that the duplicated molecules with the extra O<sub>H</sub> are preferentially replicated and hence transmitted through the germline, and that the deleted molecules are generated from the duplicated molecules through a recombinational process in post-mitotic tissues. While the deleted molecules have only one origin, O<sub>H</sub>, it is possible that they could also replicate. If the two components of the dimers were arrayed in a head-to-tail position, then there would be an O<sub>H</sub> for each strand (731,732).

This pedigree is remarkable in that it harbored the same type of duplication and deletion dimer molecular defect that has been documented for diabetes mellitus in association with KSS patients and Pearson syndrome patients who progress to KSS (754,756–759), yet none of the patients exhibited evidence of ptosis, ophthalmoplegia, or MM.

A second family has been reported also with a maternally inherited type 2 diabetes in association with an mtDNA duplication (760).

In another family, a patient was described with dystonia, external ophthalmoplegia, slowly progressive proximal muscle weakness, no RRFs on muscle biopsy (though showing mitochondrial hyperplasia), and diabetes mellitus that developed at age 36 and was treated with insulin. This individual also contained normal and duplicated mtDNAs, with the duplication encompassing nucleotides 13,445 through 3318 and flanked by imperfect direct repeats at nps 3318 through 3337 and 13,445 through 13,462. This duplication duplicated the 12S and 16S rRNAs, ND6 and *MTCYB* genes, O<sub>H</sub>, and the encompassed tRNAs, and was found in 15% of the proband's muscle mtDNA, 80% of his blood mtDNA, and 40% of his mother's blood mtDNA (753). In a second family, the child presented at 8 years with diabetes mellitus and occasional episodes of ketoacidosis, which progressed on to CPEO and MM, with the individual dying at age 20 of cardiac dysrhythmia. The patient harbored a family of mtDNA molecules, including normal, duplicated, deleted, and deletion dimers that

were widely distributed among her tissues, including her pancreas (757). Thus, these two families have duplications similar to those of the maternally inherited diabetes mellitus and deafness family and also had diabetes without or without RRFs, but the patients did have ophthalmoplegia. This implies that mtDNA rearrangement syndromes represent a phenotypic continuum with isolated diabetes mellitus and deafness being the mildest presentation, CPEO and KSS intermediate, and Pearson marrow–pancreas syndrome the most severe.

While diabetes mellitus and deafness can result both from insertion–deletion rearrangements and from the MTLL1\**MELAS*3243G mutation, the phenotypes of the mtDNA rearrangements and the MTLL1\**MELAS*3243G mutations may be somewhat different in that the rearrangement patients are more frequently insulin-requiring diabetics (731,732,757). In one case this has been associated with the loss of pancreatic beta cells (757).

Because of the clinical variability of the mtDNA rearrangement syndromes, diabetes mellitus together with mtDNA rearrangements can be associated with a spectrum of other clinical symptoms. A 5-kbp deletion has been identified in an infant with diabetic ketoacidosis, rickets, and de Toni–Debre–Fanconi syndrome (761), and a 5778-np deletion was detected by PCR in patients with diabetic amyotrophy, diabetic myoatrophy and nephropathy, and diabetic fatty liver, though this deletion was not confirmed by Southern blot and the same deletion was found at lower levels in controls (762). Finally, one patient has been reported with Wolfram syndrome including early-onset diabetes mellitus, optic atrophy, and deafness in association with a 7.6-kbp mtDNA deletion (746), though analysis of another Wolfram patient failed to reveal an mtDNA defect (763).

**11.1.38.2 Ophthalmoplegia, Ptosis, and Mitochondrial Myopathy.** Ophthalmoplegia and ptosis are common manifestations of mtDNA rearrangement syndromes. Patients with ophthalmoplegia, ptosis, and MM resulting from mtDNA rearrangements can have a wide range of additional symptoms. Patients with milder disorders may present with only ophthalmoplegia, ptosis, and MM, with an age of onset ranging from the late 20s up to late adulthood and a relatively mild course. Such patients are designated as having CPEO. By contrast, other patients can present with ophthalmoplegia, ptosis, and MM prior to age 20 and experience multiple additional symptoms, including retinitis pigmentosa and at least one of the following: cardiac conduction defects, cerebellar ataxia, or elevated cerebrospinal fluid protein (>100 mg/dL) (758,764). These patients are said to have KSS. Other symptoms that may be observed in KSS or CPEO patients can include optic atrophy, hearing loss, seizures, dementia, cardiomyopathy, cardiac dysrhythmias, renal failure, endocrine disorders including diabetes mellitus, respiratory failure, lactic acidosis (259,765), chronic diarrhea, and villous atrophy in early childhood (766).



Approximately 83% of KSS and 47% of CPEO patients are the product of mtDNA rearrangements (752). The great majority of these cases are new spontaneous mutations derived from a single clonal event such that all of the affected tissues of the patient are heteroplasmic for normal mtDNAs plus rearranged mtDNAs with a single unique breakpoint junction. Presumably, most of these mutations arose in the oocyte or early in development. A few cases, however, involve rearrangements that can also be detected at low levels in the mother and/or other maternal relatives (767).

Most of the remaining cases are the results of various mtDNA tRNA point mutations, including the tRNA mutation MTTL1\*MELAS3243G (326,660,698,699) (see Table 11-3 Panels A and B). A small percentage of patients with PEO harbor multiple mtDNA rearrangements caused by the inheritance of an autosomal dominant or recessive nuclear mutation that predisposes the mtDNAs to rearrangements (768).

mtDNA rearrangements associated with CPEO and KSS can include deletions, insertions, or a combination of the two (356). The first mtDNA rearrangements were recognized in skeletal muscle of patients with MM (7). Subsequent analysis of a large number of patients revealed that mtDNA rearrangements could be associated with four interrelated phenotypes: KSS, CPEO, Pearson marrow-pancreas syndrome, and maternally inherited diabetes mellitus and deafness.

In addition to ophthalmoplegia and ptosis, patients with CPEO and KSS due to mtDNA rearrangements have a progressive severe MM (see Figure 11-10). Additional histologic analysis of CPEO and KSS muscle has revealed bands of COX-deficient (COX<sup>-</sup>) and SDH-hyperreactive (SDH<sup>+</sup>) activity along the muscle fibers. These COX<sup>-</sup> and SDH<sup>+</sup> fibers generally correspond to regions of mitochondrial proliferation and RRFs, high levels of mutant mtDNA, and induction of nDNA and mtDNA OXPHOS gene expression (216,217,769-771).

The great majority of KSS and CPEO cases are due to spontaneous mtDNA rearrangements (281,699). About 80% of the rearrangement breakpoints are flanked by direct repeats of 4–16 nucleotides, 7% involve direct repeats of 4–6 nucleotides, and 12% do not involve a direct repeat (281). Hence, the majority of rearrangements involve a region of homology, suggesting some type of sequence-associated rearrangement mechanism. Many KSS patients harbor an interrelated family of rearranged molecules, all of which share a common breakpoint junction. These frequently include, in addition to normal mtDNAs, a duplicated mtDNA, a deletion monomer that retains only the duplicated region, and a deletion dimer containing two copies of the inserted sequence (758,759).

Duplications may predispose individuals to the more severe disease, since in one survey, 10 of 10 KSS patients were found to have duplications as well as deletions, while 8 of 8 CPEO patients harbored only deletion

monomers (756). Furthermore, the amount of the duplicated molecule in the KSS patients appeared to correlate with the duplication size: The larger the duplication, the lower the level of duplicated molecules. Moreover, the patients with the smallest duplications (largest deletions) also had deletion dimers. In skeletal muscle, as the disease progresses, the normal and duplicated mtDNAs decline while the deletion dimers increase, and the patients harboring duplications are more likely to develop diabetes mellitus than those with only deletions (758). These observations have led to the speculation that duplicated molecules are propagated more readily than deleted molecules and thus are more widely distributed among the tissues. Hence, patients harboring duplications have more organ systems affected and thus more severe disease (758,772).

While a wide variety of rearrangement breakpoints have been mapped, the great majority of deletions tend to fall into two areas defined by the O<sub>H</sub> and O<sub>L</sub> origins of replication (8). Moreover, deletions are not uniformly distributed. Approximately one-third to one-half of all deletion events occur at the common deletion breakpoint between nps 8469:13,447 joining the mtDNA genes *MTND5* and *MTATP8*, and removing 4977 nps. This “common” 5-kbp deletion occurs between two 13-base pair direct repeats at 8470 through 8482 and 13,447 through 13,459, one repeat of which is lost and the other retained (773–775). Two other frequently observed rearrangements bring together nps 7841 and 13,905 in the *MTCO2* and *MTND5* genes, removing 6063 nps at a 6- to 8-base pair direct repeat; and occur between nps 8648:16,085 in genes *MTATP6* and *MTTP*, removing 7436 nps at a 12-base pair direct repeat (281). The increased frequency of deletions at these breakpoints suggests a predominant rearrangement mechanism. In the case of the common 4977-np deletion, the break occurred in one nucleotide outside the direct repeat. This permitted a demonstration that the upstream 13,447 through 13,459 repeat was retained while the downstream 8470 through 8482 repeat was lost. Since this corresponds to the direction of H-strand replication, it has been proposed that one mechanism for mtDNA rearrangement was slip-mispairing. As replication proceeds, a new H-strand is synthesized along the L-strand template starting from O<sub>H</sub> and moving down in nucleotide numbers from position 16,569, with the parental H-strand being displaced as a single-stranded loop. Direct repeats in the parental H-strand are thus exposed and can base pair with downstream homologous repeats exposed on the L-strand by the replicating fork. Breakage of the single-stranded H-strand on the downstream side of the base-paired repeat would create a 3'-OH, which would permit re-initiation of replication continuing along the template, beginning downstream of the L-strand repeat and skipping the intervening mtDNA (775,776).

Slip-mispairing has been confirmed as an important mutational mechanism in mtDNA through studies of a spontaneous mtDNA mutation that occurs in the D-loop

of a specific European haplogroup I mtDNA lineage. Haplogroup I is defined by the novel restriction site polymorphisms *Hae*II site loss at np 4529, *Ava*II site gain at position 8249, *Alu*I site gain at 10028, and *Bam*HI site gain at 16389 (777). Individuals who harbor this mtDNA haplotype are prone to develop a heteroplasmic 270-np duplication in the D-loop between np 302 and np 567. This tandem duplication duplicates P<sub>H</sub> (nps 545 through 567), P<sub>L</sub> (nps 392 through 445), CSBIII (nps 346 through 363), and part of CSBII (nps 299 through 315) (777,778). While the original report proposed that this duplication predisposed mtDNAs to subsequent large-scale deletions (778), this has not been confirmed (758,777,779). Subsequent analysis revealed that haplogroup I mtDNAs harbored a germline mutation that inserted a run of C nucleotides at nps 568 through 573. This created a tandem repeat of the sequence 5'-ACCCCCCCC=CCCC-3' on each side of duplicated region. Every individual harboring this germline mutation was found to be heteroplasmic for the duplication in all somatic tissues tested. Since the duplicated regions are tandemly arrayed, the simplest explanation for this self-generating D-loop duplication is slip-mispairing (777). While predisposition to this duplication does not appear to be pathogenic per se, it has been found to be present at relatively high levels (32%) in one patient with MM including RRFs and partial COX deficiency. This patient also had a small percentage of mtDNAs with this region triplicated (779). This observation could suggest that either the duplication can be toxic at high levels or, alternatively, that the duplicated mtDNA is preferentially amplified in MM due to the presence of the additional promoters and O<sub>H</sub>.

Alternative deletion mechanisms that have been proposed include topoisomerase II cleavage (776) and homologous recombination. Putative topoisomerase II recognition sequences have been observed in the vicinity of certain mtDNA deletions and proposed to play a role in removal of sequences (780,781). Recombination is likely to play an important role since it is now established that many rearrangement patients harbor a complex array of related molecules, including duplication molecules, deletion monomers, and deletion dimers, suggesting that they may be interconverted by a recombinational process (356,756,759). mtDNA recombination has now been confirmed in mice harboring two mtDNAs that differ by 91 nucleotides (782) and has been reported in somatic cell hybrids between two cell lines harboring non-overlapping CPEO deletions, both of which retained OH and OL. In the hybrids, the mtDNAs complemented each other to give respiratory competence, and a few cell lines have been found to contain recombinant molecules involving the coalescence of the two deleted molecules (783). Hence, mtDNA recombination does occur in cultured human cells, though in this experiment its frequency was very low.

The pathophysiology of KSS and CPEO resulting from spontaneous mtDNA rearrangements appears to be the result of a protein synthesis defect combined with the

stochastic distribution of the mutant molecules during development. All KSS- and CPEO-associated deletions studied to date remove at least one structural RNA (tRNA or rRNA) essential for mitochondrial protein synthesis. Cytoplasmic transfer of deleted molecules from patient cells to  $\rho^0$  HeLa cells confirmed that the deleted molecules caused a defect in mitochondrial protein synthesis. In cybrids with up to 60% deleted mtDNAs, the normal mtDNAs complemented in *trans* permitting high respiration and synthesis of the mtDNA-encoded polypeptides. However, above the 60% mutant mtDNA threshold, mitochondrial respiration, protein synthesis, and the level of the individual mitochondrial polypeptides declined precipitously, ultimately falling to zero (784). This threshold effect on protein synthesis for deletion mutations explains why KSS and CPEO, like the pathogenic tRNA mutations, are frequently associated with MM and RRFs. Analysis of the tissue distribution of mtDNA deletions in KSS and CPEO patients at autopsy has revealed that the rearranged molecules are broadly distributed throughout the tissues of the body (757,772,785). The interesting exception is blood, which is commonly devoid of the mtDNA deletions (7,757,772,775), though duplications have been found (758). Hence, molecular diagnosis of KSS and CPEO generally requires a muscle biopsy.

The time in development when pathogenic mtDNA deletions arise is not yet known. However, their broad tissue distribution would imply a mutation in the oocyte or very early in development. This is supported by surveys of the mtDNAs of human oocytes left over from in vitro fertilization clinics, which have revealed that many oocytes do harbor deletions. Eight of the 15 oocytes harbored the common 4977-np deletion, confirming that deletions must occur early in development (786,787). The maximum number of molecules containing the 5-kbp deletion was 171 molecules, or about 0.1% (786). Moreover, a survey of oocytes from women of different ages revealed that, of women less than 38 years, 28% of the oocytes had the common deletion, while for women older than 38, 93% of the oocytes had this deletion. Consequently, the mean age of women without oocyte deletions was 31.4 years, while that of women with deletions was 37.7 years (787). This correlates with a marked increase in ovarian mtDNA deletions at menopause, about age 45 (788). Thus, it appears that mtDNA rearrangements may arise in the oocyte or early in development, and the frequency of mtDNA rearrangement syndromes may increase with maternal age at conception.

Molecular histologic analysis of the distribution of deleted mtDNAs in skeletal muscle of KSS and CPEO patients has revealed that the levels of mtDNA deletions increase with age (758,789,790), and that the deleted molecules are not uniformly distributed along the length of the muscle fiber. Staining muscle fibers for COX and SDH have revealed that the COX levels vary from normal to absent and that the SDH staining is normal where COX

is normal, but greatly increased in COX-negative regions (467). In situ hybridization and regional PCR amplification have shown that regions that are COX negative have increased levels of the deleted mtDNAs and a coordinate induction of mtDNA transcripts from the undeleted genes. By contrast, the COX-positive regions have predominantly normal mtDNAs (770,771,791). These observations imply that the deleted mtDNAs are initially scattered along the muscle fiber, but that, as the individual ages, there is a selective amplification of the deleted molecules, ultimately reaching a high enough concentration to inhibit mitochondrial protein synthesis and cause COX deficiency.

The mechanism by which the deleted mtDNAs are selectively amplified remains unknown, though two hypotheses have been put forward. The first hypothesis is that the deleted molecules are shorter and thus have a replicative advantage (8). Mitochondrial proteins and mtDNAs are known to be continuously, albeit slowly, turned over in post-mitotic tissues (792–795). Hence repeated replication cycles might favor shorter deleted mtDNAs or duplicated molecules with extra origins. The alternative hypothesis is that the nuclei adjacent to mutant mtDNAs and mitochondria sense the OXPHOS defect of the mutant mitochondria and attempt to compensate by inducing mtDNA replication and mitochondrial biogenesis in the surrounding mitochondria. Coordinate induction of nuclear and mtDNA OXPHOS gene expression has been documented in skeletal muscle of patients with both mtDNA rearrangement and tRNA mutations (216,217,769), and since skeletal muscles are syncytia, each nucleus controls a particular cytosolic domain (796). If mtDNA OXPHOS gene expression is modulated by a mitochondrial substrate, such as NADH, then inhibition of the ETC by an mtDNA mutation would cause a local rise in NADH and concomitant increase in mitochondrial gene expression in the adjacent nucleus. Such a process could be mediated via a nuclear transcription factor that activates OXPHOS gene expression. The factor has been identified binding to the REBOX sequence element located in a variety of nuclear bioenergetic genes and possibly also in the D-loop of mtDNA. The binding of the REBOX binding factor is stimulated by a reducing environment (192,206). Thus, in cells with partial respiratory deficiency, increased NADH could activate REBOX binding factor to bind to the REBOX element and thus stimulate the nucleus to induce the biogenesis of the surrounding mitochondria, including the associated mutant mtDNA. The resulting increase in number of mutant mtDNAs would further increase NADH and stimulate nuclear gene expression, creating a self-perpetuating feedback loop that would preferentially amplify the mutant mtDNAs (493,797). Whatever the mechanism, it would appear that it is the regional bioenergetic defect caused by the selective amplification of the mutant mtDNAs that causes the progressive decline in muscle function in KSS and CPEO.

**11.1.38.3 Pearson Marrow–Pancreas Syndrome.** A more severe form of the CPEO-KSS mtDNA rearrangement syndrome presents in the first 5 years of life with pancytopenia (loss of all blood cells). This condition is known as the Pearson marrow–pancreas syndrome. Pearson patients develop a severe transfusion-dependent, macrocytic anemia with varying degrees of neutropenia and thrombocytopenia. The bone marrow of Pearson patients shows extensive vacuolization of erythroid and myeloid precursors, hemosiderosis, and ringed sideroblasts. Pearson patients generally die due to complications of the bone marrow dysfunction or transfusions. In addition to pancytopenia, many Pearson patients develop exocrine pancreatic insufficiency, hepatic failure, renal failure, and other neuromuscular problems (259,798–802). Analysis of circulating white blood cells has revealed a generalized respiratory defect in association with mtDNA rearrangements, both deletions and duplications. As with most CPEO and KSS patients, Pearson patients are generally isolated cases, suggesting mutational events early in development (746,803–805). Moreover, autopsy analysis of Pearson patients has revealed that the mtDNA rearrangements are not simply localized to the bone marrow, but are systemic (806,807), and the rare Pearson patients who spontaneously recover the ability to make blood cells and survive ultimately progress to a KSS-like phenotype (758,801,808). These observations suggest that the Pearson marrow–pancreas syndrome and CPEO-KSS are the same disease, with the phenotypic differences resulting from the distribution of the rearranged molecules in the different tissues. This interpretation is supported by the observation that some Pearson patients, including those who have progressed to KSS, harbor duplications as well as deletions (756,758).

One explanation for the difference between Pearson syndrome and KSS-CPEO is that, in Pearson syndrome, the rearranged mtDNAs are widely distributed and include all of the bone marrow precursor cells. As the bone marrow stem cells replicate, the rearranged mtDNAs accumulate until there is insufficient energy for further proliferation and/or maturation. At this point, the rearranged molecules are prevalent in the peripheral blood and blood cell production declines, leading to pancytopenia. By contrast, in KSS-CPEO patients—or in Pearson syndrome patients who survive and progress to KSS—the rearranged molecules may be distributed such that a portion of the bone marrow precursor cells are free of mutant mtDNAs. As the stem cells containing rearranged molecules decline in their replicative potential, the cells having only normal mtDNAs continue to proliferate and ultimately supplant the mutant cells and repopulate the bone marrow. At this point, the patient loses the rearranged mtDNAs in the circulating white blood cells, but still retains rearranged mtDNAs in other organs. These later mutant mtDNAs exert their effects later in life, leading to the multisystem diseases of KSS and CPEO.



## 11.2 INHERITED NDNA MITOCHONDRIAL DISEASES

A recent count reported that mutations in 228 nuclear genes have been identified that cause non-genic mitochondrial diseases (809). These can affect structural and assembly genes of OXPHOS, mitochondrial biogenesis, maintenance, protein import, fusion–fission, and mobility (412,810).

### 11.2.1 Mutations In OXPHOS Structural Genes

Mutations in the nDNA-encoded structural genes of OXPHOS enzyme complexes generally result in LS, a frequently lethal childhood disease involving ophthalmoplegia and ptosis, hypotonia, progressive loss of motor and intellectual milestones, and bilateral lesions in the basal ganglia frequently leading to death (see Table 11-4). LS is a common presentation for boys with mutations in the X-linked  $E1\alpha$  subunit of PDH (585). Moreover, LS has been reported to result from mutations in the complex I *NDUFS1*, *NDUFS3*, *NDUFS4*, *NDUFS7*, *NDUFS8*, *NDUFV1*, *NDUFA1*, *NDUFA2* and *NDUFA10* genes (55,58,587,811–814) (see Table 11-4). Both *NDUFS6* and *NDUFA11* mutations have been reported associated with fatal infantile lactic acidosis (815,816). More recently, a homozygous mutation in the *NDUFB3* gene was identified in a patient with a severe complex I deficiency and lactic acidosis resulting in early death at 4 months of age (817). Moreover, mutations in the *NDUFS2* and *NDUFV2* genes have been reported to cause cardiomyopathy, hypotonia, and encephalomyopathy (818,819).

LS was also the phenotype of two brothers harboring mutations in the complex II SDH-A subunit (589). However, mutations in the other complex II subunits do not give LS but instead result in paraganglioma and pheochromocytoma. Reasons for this unexpected outcome are discussed in Section 11.5.

Finally, mutations in two of the subunits of complex III, UQCRB, have been linked with hypoglycemia and lactic acidosis (820) and UQCRCQ linked with a severe neurological phenotype (821). All of the nDNA-encoded diseases are Mendelian recessives, indicating that the phenotype is the result of loss of function mutations.

Examples of LS patients resulting from complex I gene mutations include a patient who was a compound heterozygote for mutations in the *NDUFS8* gene (TYKY or 23-kDa subunit). *NDUFS8* contains two 4Fe–4S ferredoxin consensus motifs, potentially forming the binding site for the N-2 iron–sulfur cluster. The missense mutations in the two alleles were Pro79Leu and Arg102His, resulting in a severe clinical phenotype (587). Finally, one patient presented with late-onset LS. This patient was found to be a compound heterozygote (P85L and R138H) for a mutation in the *NDUFS8* gene (822) (see Table 11-4).

Two other LS cases resulting from complex I mutations were homozygous for a missense mutation changing a Val122Met in the *NDUFS7* gene (58).

Four additional pathogenic mutations have been reported in nDNA complex I subunits in children with progressive degenerative disease. These children had a variety of symptoms, many reminiscent of LS, including hypotonia, feeding problems, vomiting, strabismus, myoclonic epilepsy, and MRI findings including basal ganglia abnormalities and atrophy. The complex I genes affected are for the *NDUFS4* 18-kDa protein (5-bp duplication) (823), the *NDUFV1* gene (two compound heterozygotes for Arg59Stop + Thr423Met), and the *NDUFV1* gene (Ala341Val) (58).

The most detailed report of an nDNA complex I mutation patient involved the *NDUFS4* gene (18-kDa subunit) resulting from a 5-bp duplication. This child presented with hypotonia, mental retardation, convulsions, and brain and basal ganglia degeneration. The patient was found to be homozygous for a 5-bp duplication that resulted in a frameshift at codon Lys158, causing a change in the amino acid sequence of the protein from amino acid 178 to the end. This frameshift destroyed a consensus phosphorylation site and extended the protein by 14 amino acids. The *NDUFS4* complex I gene was mapped to chromosome 5 (823) (see Table 11-4).

The LS complex II mutation altered the SDH-A gene on chromosome 5p15 and was observed in two siblings. The proband had isolated complex II deficiency in skeletal muscle, cultured skin fibroblasts, and circulating lymphocytes, with the enzyme showing a heightened sensitivity to oxaloacetate, the physiologic inhibitor. The causal mutation was a C-to-T transition at nucleotide 1684 in the cDNA, resulting in an Arg544Trp substitution in a highly conserved domain of the protein. Both siblings were homozygous mutants, while the two parents were heterozygotes (589) (see Table 11-4).

A homozygous mutation in *COX6B1* a complex IV structural subunit was identified in a consanguineous family with progressive neurologic phenotype, lactic acidosis, and leukodystrophic brain changes, resulting in a significant reduction of activity and assembly of complex IV (824). Complex V deficiency was also associated with a mutation in *ATP5E* gene and responsible for neonatal-onset lactic acidosis, peripheral neuropathy, and a mild mental retardation (825).

### 11.2.2 Mutations in Assembly Genes

Mutation in nDNA genes affecting the OXPHOS complexes have also been discovered in assembly genes for all complexes of the respiratory chain (see Table 11-5). Mutations in complex I assembly genes *NDUFAF1*, *NDUFAF2*, *NDUFAF3*, *NDUFAF4*, *NUBPL*, *ACAD9*, *C20orf7*, and *FOXRED1* have been found to be associated with early-onset encephalomyopathy, hypertrophic cardiomyopathy, and LS (88,826–832).



TABLE 11-4 Structural Nuclear Genes						
Complex	Name	OMIM	Function	Chromosome	Inheritance	Clinical Phenotype
Complex I	<i>NDUFS1</i>	157655	IP fraction	2q33-q34	AR	LS
	<i>NDUFS2</i>	602985	IP fraction	1q23	AR	Encephalopathy, cardiomyopa
	<i>NDUFS3</i>	603846	IP fraction	11p11.11	AR	LS
	<i>NDUFS4</i>	602694	IP fraction	5q11.1	AR	LS
	<i>NDUFS6</i>	603848	IP fraction	5pter-p15.33	AR	Fatal infantile lactic acidosis
	<i>NDUFS7</i>	601825	HP fraction	19p13.3	AR	LS
	<i>NDUFS8</i>	602141	HP fraction	11q13	AR	LS
	<i>NDUFB3</i>	603839	HP fraction	2q31.3	AR	Fatal infantile lactic acidosis
	<i>NDUFV1</i>	161015	FP fraction	11q13	AR	LS
	<i>NDUFV2</i>	600532	FP fraction	18p11	AR	Cardiomyopathy, hypotonia, encephalopathy
	<i>NDUFA1</i>	300078	HP fraction	Xq24	X	LS progressive neurodegenera disorder
	<i>NDUFA2</i>	602137	HP fraction	5q31.2	AR	LS
Complex II	<i>NDUFA10</i>	603835	HP fraction	2q37.3	AR	LS
	<i>NDUFA11</i>	612638	IP fraction	19p13.3	AR	Fatal infantile lactic acidosis
						Encephalocardiomyopathy
	<i>SDH-A</i>	600857	FP subunit	5p15	AR	LS
	<i>SDH-B</i>	185470	IP subunit	1p36.1-p35	AD	Phaeochromocytoma and paraganglioma
	<i>SDH-C</i>	602413	Membrane subunit	1q21	AD	Autosomal dominant paragang type 3
	<i>SDH-D</i>	602690	Membrane subunit	11q23	AD	Autosomal dominant paragang type 1, pheochromocytoma
Complex III	<i>UQCRRB</i>	191330	Electron transfer	8q22	AR	Hypoglycemia, lactic acidosis
	<i>UQCRRQ</i>	612080	Electron transfer	5q31.1	AR	Severe neurological phenotype
Complex IV	<i>COX6B1</i>	124089	Cytochrome oxidase activity	19q13.1	AR	Encephalomyopathy
Complex V	<i>ATP5E</i>	606153	ATPase activity	20q13.3	AR	Lactic acidosis, mental retardation, peripheral neuropathy

LS, Leigh syndrome.

TABLE 11-5 Nonstructural Nuclear Genes						
Complex	Name	OMIM	Function	Chromosome	Inheritance	Clinical Phenotype
Assembly Complex I	<i>NDUFAF1 (CIA30)</i>	606934	Assembly	15q13.3	AR	Cardioencephalomyopathy
	<i>NDUFAF2 (B17.2L)</i>	609653	Assembly	5q12.1	AR	Early onset progressive encephalopathy
	<i>NDUFAF3</i>	612911	Assembly	3p21.31	AR	Neonatal encephalopathy
	<i>NDUFAF4 (HRPAP20)</i>	611776	Assembly	6q16.1	AR	Infantile encephalopathy
	<i>C20orf7</i>	612360	Assembly	20p12.1	AR	LS
	<i>NUBPL</i>	613621	Assembly	14q12	AR	Encephalomyopathy
	<i>FOXRED1</i>	613622	Unknown	11q24.2	AR	LS
	<i>ACAD9</i>	611103	Assembly and activity	3q26	AR	Hypertrophic cardiomyopathy, encephalopathy
Complex II	<i>SDHAF1</i>	612848	Assembly	19q12-q13.2	AR	Leukoencephalopathy
	<i>SDHAF2</i>	613019	Assembly	11q12.2	AD	Autosomal dominant paraganglioma type 1
Complex III	<i>BCS1L</i>	603647	Assembly	2q33	AR	Encephalopathy, hepatic and tubulopathy, Björnstad syndrome
Complex IV	<i>SURF1</i>	185620	Assembly	9q34	AR	LS
	<i>SCO1</i>	603644	Copper transport	17p13-p12	AR	Neonatal hepatic failure, encephalopathy
	<i>SCO2</i>	604272	Copper transport	22q13	AR	Neonatal cardioencephalopathy
	<i>COX10</i>	602125	Heme A farnesyl transferase	17p12-p11.2	AR	Neonatal tubulopathy and encephalopathy, LS, cardiomyopathy
	<i>COX15</i>	603646	Heme A synthesis	10q24	AR	Early-onset hypertrophic cardiomyopathy, LS
	<i>LRPPRC</i>	220111	Assembly	2p21-p16	AR	French-Canadian LS
	<i>FASTKD2</i>	612322	Role in apoptosis	2q33.3	AR	Encephalomyopathy
	<i>TACO1</i>	612958	Translational activator of COX1	17q22-q24.2	AR	LS
Complex V	<i>ATPAF2</i>	608918	Assembly	17p11.2	AR	Early-onset encephalopathy, acidosis
	<i>TMEM70</i>	604273	Assembly	8q21.11	AR	Neonatal encephalopathy, cardiomyopathy

MtDNA stability	<i>POLG (PEOA1)</i>	174763	Polymerase gamma mtDNA replication	15q25	AD-AR	Alpers syndrome, AD-PEO AR-PEO, male infertility SANDO* syndrome, SANDO AD-PEO
	<i>POLG2 (PEOA4)</i>	610131	Catalytic subunit of DNA polymerase gamma	17q23-q24	AD	AD-PEO
	<i>ANT1 (PEOA2)</i>	609283	Adenine nucleotide translocator isoform 1	4q35	AD	AD-PEO, multiple mtDNA deletions
	<i>MPV17</i>	137960	Regulation of mtDNA copy number	2p23-p21	AR	Hepatocerebral MDDS
	<i>C10ORF2 (PEOA3)</i>	609286	Twinkle helicase	10q24	AD	AD-PEO, SANDO syndrome
	<i>TYMP (ECGF1)</i>	603041	Thymidine phosphorylase	22q13.32-qter	AR	MNGIE, mtDNA depletion
	<i>DGUOK</i>	601465	Deoxyguanosine kinase	2p13	AR	Hepatocerebral mtDNA depletion syndrome
	<i>RRM2B (PEOA5)</i>	604712	mitochondrial dNTP pool maintenance Ribonucleotide reductase M2 B dNTP pool	8q23.1	AR	Encephalomyopathic renal tubulopathy MNGIE,
	<i>SUCLA2</i>	603921	Succinate-CoA ligase, ADP-forming, beta subunit	13q12.2-q13	AR	Encephalomyopathy with methylmalonic aciduria
	<i>SUCLG1</i>	611224	Succinate-CoA ligase, alpha subunit	2p11.2	AR	Encephalomyopathy with methylmalonic aciduria
	<i>TK2</i>	188250	Thymidine kinase Mitochondrial dNTP pool maintenance	16q22	AR	Myopathic mtDNA depletion
Mitochondrial import	<i>DDP</i>	304700	Protein import	Xq22	X-linked	Deafness–dystonia or Mohr–Tranebjærg syndrome
	<i>DNAJC19</i>	608977	Protein import	3q26.3	AR	Cardiomyopathy, ataxia
Mitochondrial protein synthesis	<i>EFG1</i>	609060	Elongation factor G1; mitochondrial translation defect	3q25	AR	Severe hepatocerebral and lactic acidosis
	<i>YARS2</i>	610957	Tyrosyl-tRNA synthetase	12p11.21	AR	Myopathy, lactic acidosis, sideroblastic anemia
	<i>SARS2</i>	612804	Seryl-tRNA synthetase	19q13.2	AR	Hyperuricemia, pulmonary hypertension, renal failure
	<i>DARS2</i>	611105	Aspartyl-tRNA synthetase	1q25.1	AR	Leukoencephalopathy and acidosis
	<i>RARS2</i>	611523	Arginyl-tRNA synthetase	6q16.1	AR	Pontocerebellar hypoplasia
	<i>MRPS16</i>	609204	Mitochondrial translation	10q22.1	AR	Neonatal lactic acidosis, callosum agenesis, ataxia
	<i>MRPS22</i>	605810	Mitochondrial translation	3q23	AR	Cardiomyopathy, tubulopathy
	<i>TSFM</i>	604723	Mitochondrial translation elongation	12q13-q14	AR	Encephalomyopathy, hypomyelination, hypomyelinating cardiomyopathy
	<i>TUFM</i>	602389	Mitochondrial translation elongation	16p11.2	AR	Leukodystrophy with micropolygyria

TABLE 11-5		Nonstructural Nuclear Genes—Cont'd				
Complex	Name	OMIM	Function	Chromosome	Inheritance	Clinical Phenotype
Iron homeostasis	<i>FRDA</i>	606829	Frataxin Trinuc. repeat	9q13	AR	Friedreich ataxia, neuropathy, cardiomyopathy, diabetes
	<i>ABCB7</i>	301310	Iron transport	Xq13.1-q13.3	X-linked	X-linked sideroblastic anemia with ataxia
	<i>GLRX5</i>	205950	Iron–sulfur cluster biosynthesis	3p22.1	AR	Sideroblastic anemia
	<i>ISCU</i>	255125	Iron–sulfur cluster biosynthesis	12q23.3	AR	Myopathy, lactic acidosis, intolerance
	<i>BOLA3</i>	613183	Iron–sulfur cluster biosynthesis	2p13.1	AR	Encephalomyopathy, cardiomyopathy
	<i>NFU1</i>	608100	Iron–sulfur cluster biosynthesis	2p13.3	AR	Lactic acidosis, multiple respiratory chain deficiency
Coenzyme Q10 biogenesis	<i>COQ2</i>	609825	CoQ10 deficiency	4q21-q22	AR	Encephalomyopathy, neuropathy
	<i>COQ4</i>	612898	CoQ10 deficiency	9q34.13	AR	Encephalomyopathy, mental retardation
	<i>COQ9</i>	612837	CoQ10 deficiency	16q13	AR	Neonatal lactic acidosis, cardiomyopathy
	<i>APTX</i>	606350	CoQ10 deficiency	9p13.3	AR	Cerebellar ataxia, oculomotor apraxia
	<i>PDSS1</i>	607429	CoQ10 deficiency	10p12.1	AR	Deafness, valvulopathy, mental retardation
	<i>PDSS2</i>	610564	CoQ10 deficiency	6q21	AR	LS, nephrotic syndrome
	<i>CABC1</i>	606980	CoQ10 deficiency	1q42.2	AR	Cerebellar ataxia, lactic acidosis
Chaperone function	<i>SPG7</i>	607259	Paraplegin ATPase protease	16q24.3	AR	Spastic paraplegia
	<i>HSPD1</i>	118190	Mitochondrial chaperone	2q33.1	AR	Spastic paraplegia, leukodystrophy
Mitochondrial integrity	<i>OPA1</i>	165500	Dynamin-related protein	3q28-q29	AD	AD–optic atrophy, multiple deletions
	<i>MFN2</i>	609260	Mitofusin Mitochondrial fusion	1p36-p35	AD	Charcot–Marie–Tooth disease-2A2 (CMT2A)
	<i>DLP1</i>	603850	Mitochondrial and peroxisomal fission	12p11.21	AD	multiple deletions, microcephaly
	<i>G4.5 (Tafazzin)</i>	302060	Cardiolipin defect	Xq28	X-linked	Microcephaly, abnormal development, optic atrophy, lactic acidosis
	<i>RMRP</i>	250250	RNAse Mitochondrial RNA processing	9p13-p12	AR	Barth syndrome, X-linked cardiomyopathy
Mitochondrial metabolism	<i>PDHA1</i>	308930	Pyruvate dehydrogenase E1- $\alpha$ subunit	Xp22.2-p22.1	X-linked	Metaphyseal chondrodysplasia–hair hypoplasia
	<i>ETHE1</i>	602473	Ethylmalonic acid metabolism	19q13	AR	LS
	<i>PUS1</i>	600462	Pseudouridine synthase	12q24.33	AR	Encephalopathy, ethylmalonic aciduria
						Myopathy, lactic acidosis, sideroblastic anemia

LS, Leigh syndrome; SANDO, Sensory Ataxic Neuropathy, Dysarthria, and Ophthalmoparesis; SCAE, Spinocerebellar Ataxia with Epilepsy; GRACILE syndrome, Growth Retardation, renal overgrowth, Lactic acidosis, and Early death; MNGIE, Mitochondrial NeuroGastroIntestinal Encephalopathy; MDDS, Mitochondrial DNA Depletion Syndrome.



Mutations in the *SDHAF1* assembly gene were identified responsible for a progressive neurologic deterioration during the first year of patient life associated with leukodystrophic brain abnormalities (833). Similar to SDH-B, -C, and -D, mutation in *SDHAF2* another complex II assembly subunit results in paraganglioma (834).

Similarly, a mutation in the *BCS1L* gene on chromosome 2q33 resulted in different phenotypes such as encephalomyopathy, hepatic failure, renal dysfunction, LS, and GRACILE or Björnstad syndromes (117,118,835).

The eight complex IV assembly genes that have been found to be mutant in patients fall into four categories: assembly, copper transport, heme a synthesis, and complex IV regulation. Mutation in the complex IV assembly gene *SURF1* has been observed in multiple LS families with complex IV deficiency. The *SURF1* gene is located on chromosome 9q34. The reported *SURF1* mutations included nonsense mutations, missense mutations, and frameshift mutations resulting from small insertions and deletions (158,159,251,836).

Defects in two copper transport protein genes (*SCO1* and *SCO2*) have also been found to result in complex IV deficiency. Infants with mutations in the *SCO2* gene present with lethal hypertrophic cardiomyopathy, lactic acidosis, neurodegeneration and atrophy, and hepatomegaly associated with a complex IV defect (see Table 11-5). To date, patients with pathogenic *SCO2* mutations always have one allele with the E140K missense mutation. The other allele has been either a null allele or another missense mutation (e.g. S225F or R171W) (165). Interestingly, in the yeast model system, the equivalent of the E140K mutation does not significantly reduce complex IV activity, while the S225F mutation results in respiratory deficiency. Yeast with the S225F mutation assembles complex IV without the mtDNA COII subunit. This suggested that *SCO2* is involved in the insertion of Cu<sup>2+</sup> into the Cu<sub>A</sub> site of the mtDNA COII subunit (164).

Infants with mutations in the *SCO1* gene develop hepatic failure and encephalomyopathy with a complex IV defect due to faulty Cu<sup>2+</sup> incorporation (see Table 11-5). Patients have been found to have compound heterozygous mutations including frameshift and missense mutations. Two identified missense mutations are C250T and P174L, the latter mutation being thought to affect the copper-binding domain (CXXXC) of the enzyme (166).

Mutations in two other nDNA genes (*COX10* and *COX15*) inhibit the synthesis of complex IV heme a. The index case harboring mutant *COX10* genes presented with ataxia, severe hypotonia, ptosis, pyramidal syndrome, status epilepticus, and proximal tubulopathy and died at 2 years (see Table 11-5). He had an older sister who died at 5 years of mitochondrial encephalopathy associated with complex IV deficiency, and a younger sister who had progressive neurologic deterioration

and died at 3 years. Genetic analysis revealed that these patients were homozygous for a deleterious missense mutation (N204K) in the *COX10* gene. The parents were both heterozygous for this mutation. *COX10* encodes the heme a:farnesyltransferase enzyme, and the patients have a 50% reduction in complex IV subunits III and IVc and more than 97% reduction in subunit II (166).

Mutations in the *COX15* gene on chromosome 10q24 result in the inhibition of heme a synthesis. Patients with defects in this gene develop early-onset hypertrophic cardiomyopathy or LS (837). Mutations in the *LRP-PRC* gene on chromosome 2p21-p16 were described in a large series of French Canadian patients associated with a cytochrome oxidase assembly deficiency and LS (838). The LS phenotype was also linked to mutations in *TACO1* required for efficient translation of COX1 an mtDNA-encoded subunit, critical for complex IV assembly (839). A mutation in *FASTKD2* which was located on chromosome 2q33.3 was found to be responsible for an encephalomyopathy and suggested to regulate mitochondrial apoptosis (840).

Finally, a complex V assembly gene, *ATPAF2*, has been identified on chromosome 17p11.2. Defects in this gene are associated with early-onset encephalomyopathy with lactic acidosis (198). Another complex V assembly gene *TMEM70* was found to be defective in a large series of patients affected with neonatal mitochondrial encephalocardiomyopathy and severe complex IV deficiency (841).

### 11.2.3 Mutations in Mitochondrial Protein Metabolism and Assembly

Mutations in nDNA-encoded genes involved in mitochondrial biogenesis, assembly, and turnover have also been identified. The first-discovered pathologic mutation in an nDNA protein biogenesis gene occurred in the mitochondrial protein synthesis elongation factor G1. Inactivation of this gene in chromosome 3q25 results in lactic acidosis in association with severe liver and central nervous system disease (198).

The translational machinery requires a large variety of nuclear-encoded subunits including aminoacyl-tRNA synthetases, initiation, elongation, and termination factors. Mutations in the genes encoding mitochondrial aminoacyl-tRNA synthetases have been described for *YARS2*, *SARS2*, *DARS2*, *RARS2* linked to a variety of phenotypes such as myopathy, pulmonary hypertension, or pontocerebellar hypoplasia (842–845).

A homozygous nonsense mutation in the *MRPS16* gene was discovered in a patient affected with fatal neonatal lactic acidosis and agenesis of the corpus callosum, resulting in a defect in mitochondrial translation. Another mutation in the *MRPS22* gene was associated with cardiomyopathy and tubulopathy and a combined OXPHOS defect (846).

Mitochondrial translation elongation factors have been also associated with encephalomyopathy, hypertrophic cardiomyopathy, leukodystrophy with micropolygyria due to mutations in *TSM* and *TUFM* genes, respectively (847,848).

The first nDNA mutation that affects mitochondrial protein import has been localized to the *DFN-1* gene and causes the Mohr–Tranebjaerg syndrome, encompassing dystonia and deafness. The Mohr–Tranebjaerg syndrome presents in early childhood with sensorineural hearing loss that can progress to dystonia, spasticity, mental deterioration, paranoia, and cortical blindness. Those patients who develop the movement disorder characteristically exhibit progressive degeneration of the basal ganglia, corticospinal tract, and brain stem. The Mohr–Tranebjaerg syndrome gene is located on the X chromosome, and the index case was found to have a 10-bp deletion in exon 2 of the *DFN1* gene. The identity of the gene was confirmed in a second family found to harbor a 1-np deletion in exon 1. *DFN1* generates a 1167-np cDNA encoding a 97-amino acid, 11-kDa polypeptide designated DDP1p. The predicted polypeptide has a high homology to a *Schizosaccharomyces pombe* gene of unknown function (849) (see Table 11-5).

A role for proteins of the DDP1 structure in mitochondrial protein import was demonstrated by studies of the yeast mitochondrial import proteins, while studying the *Saccharomyces cerevisiae* import pathway for mitochondrial inner membrane carrier proteins such as the ANT (yeast ACC) (256). DDP1 is the mammalian counterpart to the yeast protein Tim8p. Tim8p is a member of a family of proteins located in the mitochondrial intermembrane space that includes Tim8p, Tim9p, Tim10p, Tim12p, and Tim13p. All of these polypeptides are similar in size and contain a distinctive duplicated C(N)<sub>3</sub>C motif, reminiscent of a zinc finger protein. Surprisingly, the DDP1 protein of the Mohr–Tranebjaerg syndrome was found to have the same duplicated C(N)<sub>3</sub>C motif as the Tim8p family protein. Moreover, synthetic DDP1 is incorporated into the mitochondrial intermembrane space in yeast, and haptin-tagged DDP1 is specifically localized to yeast and mammalian mitochondria (256).

The Tim proteins form two similar 70-kDa complexes in the mitochondrial inner membrane. The Tim9p–Tim10p complex is important for the import of most of the inner membrane carriers including the ANT, phosphate carriers, and dicarboxylate carrier. However, the Tim8p–Tim13 (DDP1–TIMM13) complex is important in the import of the Tim23p (TIMM23) and in the early steps of the biogenesis of Tom40p of the TOM complex. In addition, this complex is involved in the import into the mitochondria of two calcium-responsive aspartate/glutamate carriers, citrin and aralar1. These are components of the malate-aspartate NADH shuttle. Moreover, DDP1 and TIMM13 are preferentially expressed in the

brain and particularly in large neurons such as Purkinje cells. Thus, it would appear that the pathophysiology of Mohr–Tranebjaerg syndrome is impaired NADH transport between the cytosol and the mitochondria (850).

The demonstration that inactivation of the DDP1 protein would compromise mitochondrial biogenesis and in turn bioenergetics of neurons, resulting in deafness and dystonia, is consistent with the fact that the mtDNA mutation MTND6\*LDYT14459A, which impedes mitochondrial complex I (NADH dehydrogenase), also results in dystonia. Thus, both nDNA and mtDNA mutations that affect NADH metabolism in the brain can cause the same phenotype.

The DNAJC19 protein shares similarity with the yeast TIM14 protein, a component of the mitochondrial protein import system. Defective *DNAJC19* gene has been linked to an autosomal recessive disorder with early onset dilated cardiomyopathy, ataxia, and significant increases of 3-methylglutaconic acid and 3-methylglutaric acid in urine organic acids (851).

The first nDNA mutations associated with a defect in mitochondrial protein turnover result in hereditary spastic paraplegia (HSP). HSP, in the “pure” form, presents primarily with progressive weakness and spasticity of the lower limbs. However, in the “complicated” form, symptoms can include mental retardation, peripheral neuropathy, amyotrophy, ataxia, retinitis pigmentosa, optic atrophy, deafness, and ichthyosis. Muscle histology of patients in one mutant family revealed RRFs, which were SDH hyperreactive and contained packed abnormal mitochondria. HSP was initially found to be linked to the paraplegin gene (SPG7) on chromosome 16q24.3 in a large Italian family (852). The paraplegin gene consists of five exons extending over 10.8 kbp of genomic sequence. The index family was found to harbor a complex rearrangement in the gene, possibly associated with the interactions of multiple Alu repeat elements. Paraplegin mutations have also been observed in two additional families: a homozygous 2-bp deletion causing a frameshift that abolished 60% of the protein, and a single A insertion that created a frameshift and stop codon two amino acids downstream, thus removing the C-terminal 57 amino acids. The paraplegin protein is an 88-kDa polypeptide with a 40- to 45-amino acid targeting peptide. It shows homology to a subfamily of adenosine triphosphatases (AAA proteins) that are yeast mitochondrial metalloproteases. In yeast, such proteins have both protease and chaperone-like activities (852). Thus, the paraplegin protein may function in the assembly and degradation of mitochondrial enzyme complexes. Spastic paraplegia and leukodystrophy were also linked to mutations in *HSPD1* gene in a large consanguineous family (853).

Primary CoQ deficiency resulting from defects in one of the several steps involved in the CoQ biosynthesis is rare. Primary CoQ deficiency syndromes are also heterogeneous, presenting with encephalomyopathy, LS,

nephritic syndrome, severe multisystem infantile disease, cerebellar ataxia, or pure myopathies (854–860). However, when the diagnosis of primary CoQ deficiency is made, the patient can benefit from CoQ10 supplementation.

### 11.2.4 Mutations that Alter Mitochondrial Physiology

Three nDNA gene mutations that alter mitochondrial metabolism are particularly relevant: Barth syndrome, ethylmalonic aciduria, and Friedreich ataxia. Barth syndrome is an X-linked disease associated with dilated cardiomyopathy. The mutant protein Tafazzin is involved in cardiolipin synthesis, and cardiolipin is a specific and essential component of the mitochondrial inner membrane. Presumably loss of Tafazzin then inhibits mitochondrial function in the heart, resulting in the phenotype (861,862).

Ethylmalonic aciduria together with encephalopathy has been discovered to be due to mutations in a gene located on chromosome 19q13, and all types of mutations—missense, stop, frameshift, and splice mutations—have been identified in patients affected by ethylmalonic encephalopathy. This *ETHE1* gene results in strikingly elevated ethylmalonate in the urine. This elevation might be related to the gene defect altering glutathione metabolism. If so, then this may be a disease resulting from increased oxidative stress (863,864).

Mitochondria are also centrally involved in iron metabolism. Diseases those are associated with a dysregulation of mitochondrial iron, as in the case of Friedreich's ataxia, which is caused by a deficiency in frataxin, a regulator of mitochondrial iron processing. Patients with Friedreich ataxia manifest cerebellar ataxia, peripheral neuropathy, hypertrophic cardiomyopathy, and diabetes as the result of inactivation of the frataxin gene on chromosome 9q13. The frataxin gene contains an intron trinucleotide repeat that, when expanded, inhibits gene expression. Repeat expansion of both alleles lowers frataxin protein levels sufficiently to result in the disease. Frataxin binds iron in the mitochondrial matrix, thus minimizing mitochondrial OH production. The loss of the frataxin protein results in excessive ROS generation, which inactivates all mitochondrial iron–sulfur-containing enzymes, including complexes I, II, III, and aconitase. Thus, Friedreich ataxia appears to be the result of increased mitochondrial ROS production and decreased mitochondrial OXPHOS (22,865,866).

In addition to frataxin, there are other proteins that govern the tightly regulated processes of mitochondrial iron utilization. Mutations in *ABCB7*, *GLRX5*, *ISCU*, *BOLA3*, and *NFU1* genes involved in iron transport or Fe-S cluster biogenesis cause distinctive human diseases from sideroblastic anemia, myopathy, encephalopathy, cardiomyopathy, and multiple respiratory chain deficiency (867–870).

### 11.2.5 Mutations that Affect mtDNA Fusion and Fission

Autosomal dominant optic atrophy and a form of Charcot–Marie–Tooth disease have been found to result from defects in mitochondrial fission and fusion (see Table 11-5). Dominant optic atrophy is phenotypically very similar to LHON, with a midlife sudden onset of central vision loss. However, instead of being maternally inherited, it is inherited as an autosomal dominant. The majority of dominant optic atrophy cases are due to mutations in the *OPA1* gene, a dynamin-related GTPase located on chromosome 3q28–q29. This protein appears to be important in mitochondrial fission (871,872). It was demonstrated that *OPA1* mutations can be responsible for syndromic forms of autosomal dominant optic atrophy associated with sensorineural deafness, ataxia, axonal sensory motor polyneuropathy, CPEO, and MM with cytochrome c oxidase (COX)-negative and ragged red fibers, called dominant optic atrophy “plus” phenotypes. These phenotypes are related to mtDNA instability resulting in multiple mtDNA deletions emphasizing the role of *OPA1* in mtDNA maintenance (873).

The peripheral neuropathy Charcot–Marie–Tooth disease can result from mutations in a variety of genes. However, Charcot–Marie–Tooth type 2A2 results from inactivation of the mitofusin 2 gene (*MFN2*). Mitofusin, which is located on chromosome 1p35–p36, is essential for mitochondrial fusion (874,875). Presumably, the loss of the appropriate mitochondrial fission–fusion cycle in the neurons alters the ability of the mitochondria to be appropriately distributed throughout the neuron, resulting in localized energy deficiency and impaired neuronal function.

Recently, a mutation in *MFN2* in a large three generational family with optic atrophy was associated with axonal neuropathy and MM. Similar to *OPA1* mutations, the “plus” phenotype was linked to mtDNA instability resulting in multiple mtDNA deletions in patient skeletal muscle (876).

Mutation in another dynamin *DLP1* has been associated with defective mitochondrial and peroxisomal fission. A heterozygous dominant-negative mutation in the *DLP1* gene was responsible for microcephaly, abnormal brain development, optic atrophy, and lactic acidosis in a newborn that died after 3 weeks of life (877).

### 11.2.6 Mutations Affecting mtDNA Stability and Expression

A variety of nDNA defects have now been identified, which affect the replication of mtDNA. These can be grouped into five phenotypic categories: chondrodysplasia, intestinal dysmotility, brain and liver dysfunction, multiple mtDNA deletions, and mtDNA depletion.

Metaphyseal chondrodysplasia, or cartilage-hair hypoplasia, has been discovered to be due to mutations

in the MRP RNase. Cartilage-hair hypoplasia is an autosomal recessive disorder associated with disproportionate short stature, hypoplastic hair, ligamentous laxity, defective immunity, hypoplastic anemia, and neuronal dysplasia of the intestine. These problems can be manifested as congenital megacolon (Hirschsprung disease) and predisposition to lymphomas and other cancers. The disease is remarkable in its clinical variability within and between pedigrees. The RNase MRP has been proposed to cleave the nascent mtDNA L-strand transcript to generate a primer for initiating H-strand replication, though the protein is also present in the nucleus. The enzyme includes an RNA component that is located on chromosome 9p13 and transcribed by RNA polymerase III. Two classes of mutations have been identified: deletions between the promoter and the transcription start site, which result in null mutants, and base substitutions in the structural RNA. Four structural mutations have been identified, two of which alter bases in single-stranded loops and a base substitution and a two-base insertion that affect double-stranded components of the RNA (878). While the pathophysiology of this disease is currently unknown, it is interesting that many of the symptoms associated with this disease are also seen in other mitochondrial diseases. Hence, it is possible that mitochondrial deficiency may play an important role in this disease process.

Intestinal dysmotility is the hallmark of MNGIE, which is associated with MM, including RRFs and abnormal mitochondria; decreased respiratory chain activity; and multiple mtDNA deletions, mtDNA depletion, or both. This autosomal recessive disease has been linked to multiple mutations in the nuclear TP gene. While the disease is caused by TP mutations, the clinical symptoms are probably the result of the destruction of mtDNA. Hence, it has been hypothesized that inactivation of TP alters cellular thymidine pools, which are important in mtDNA maintenance (879) (see Table 11-5).

Alpers disease is associated with generally lethal encephalopathy associated with white matter lesions and liver dysfunction. This autosomal recessive disorder is now known to result from null mutations in the mtDNA POLG gene, located on chromosome 15q25 (880). However, mutations in the POLG gene have also been shown to result in a variety of other clinical phenotypes, including autosomal dominant or recessive PEO (881,882), sensory ataxic neuropathy, dysarthria, and ophthalmoparesis (SANDO), and spinocerebellar ataxia with epilepsy (883). Of the PEO cases seen in the clinic, ~6% have been found to harbor multiple deletions and show a clear autosomal dominant inheritance pattern. Such families are described as having autosomal dominant-PEO (AD-PEO) (678,768,884,885) AD-PEO with multiple deletions can result from POLG, ANT1, and Twinkle helicase gene mutations (see Table 11-5).

In AD-PEO, each affected individual has a different array of mtDNA deletions. This demonstrates that it is

a nuclear mutation that increases the tendency toward deletion and that this tendency is inherited, not the deletion itself (768,884,885). Clinically, the mean age of onset of AD-PEO is 26 years, which is older than the average spontaneous CPEO case. In addition to PEO, these patients experience proximal muscle atrophy and weakness in about 62% of cases, hearing loss in 25%, and ataxia in 17% of cases. Cardiac and retinal involvements are rare, and the most common cause of death is respiratory failure (886). Multiple deletions have also been found in four patients with RRFs but atypical neurologic symptoms. Two individuals presented with parkinsonism, one with ptosis, a third with exercise intolerance and limb weakness, and a fourth with elevated blood creatine phosphokinase (887). The nature of the deletions in multiple deletion syndrome is similar to that found in spontaneous deletion patients. Among the 81 deletions reported from multiple deletion syndrome patients, 96% are encompassed by direct repeats of 4–13 nucleotides, while 4% involve indirect repeats of 4 base pairs (281). Moreover, patients show multiple deletions in tissues throughout the body (888).

Mutation in POLG appears to be the most common cause of AD-PEO with multiple mtDNA deletions. POLG maps to chromosome 15q25, and a wide variety of POLG mutations have now been identified with various disease phenotypes, as discussed in W. Copeland's website (<http://tools.niehs.nih.gov/polg/>). In all of these cases of AD-PEO, the damaged POLG results in instability of the mtDNA template leading to multiple mtDNA rearrangement mutations, known as multiple deletions syndromes. The clinical phenotype of AD-PEO patients resulting from POLG mutations primarily involves MM. The POLG gene also encompasses a trinucleotide repeat, and alteration in the repeat length has been implicated in male sterility (889).

A mouse has been generated in which the proofreading function has been inactivated by knocking into the chromosomal POLG locus the D257A mutation in the 3'-to-5' exonuclease. These mice had a shortened life span and developed a premature aging phenotype involving weight loss, reduction of subcutaneous fat, hair loss (alopecia), curvature of the spine (kyphosis), osteoporosis, anemia, reduced fertility, and heart enlargement. This was associated with an age-related decline in respiratory complexes I and IV and in mitochondrial ATP production rates in the heart. Analysis of the mtDNA revealed that the knock-in animals had a three- to fivefold increase in mtDNA base substitution mutations in brain, heart, and liver, with a higher number of mutations in the CR cytochrome b gene than in the mtDNA CR (497). Thus, increased mtDNA mutations can directly increase the aging rate, implying that mtDNA is one of the most important targets for mitochondrial ROS damage in aging.

The DNA polymerase gamma is also composed of two different subunits: a 140-kDa catalytic subunit (POLG1)



and a 55-kDa accessory subunit (POLG2). A patient with typical late-onset PEO carried a heterozygous mutation changing a glycine in glutamate at position 451 in the POLG2 gene (890).

The second major locus causing a multiple deletions syndrome was mapped in a Finnish study at chromosome 10q23.3-24.3 (891,892). This was subsequently shown to be the result of mutations in the mtDNA Twinkle helicase, giving AD-PEO and SANDO (893,894). Twinkle is homologous to the bacteriophage T7-type pg4 hexameric primase-helicase. It interacts with POLG from the core of the mtDNA replicasome. Patients with Twinkle mutants develop AD-PEO, but can also develop major depression (895) (see Table 11-5).

Transgenic mouse models of AD-PEO caused by Twinkle mutations were prepared by pronuclear injection of a transgene with a milder A360T mutation and a more severe in-frame duplication of amino acids 353 through 365. Both transgenic mouse models accumulated multiple mtDNA deletions, and the duplication mouse model also showed mtDNA depletion, though the mice did not have heightened mtDNA base substitution mutations. The Twinkle duplication mutation developed COX<sup>-</sup> and SDH<sup>+</sup> muscle fibers by 18 months, with abnormal mitochondria containing spiral cristae, though not paracrystalline precipitates. Moreover, the Twinkle duplication mice showed that, by 18 months, about 1% of the Purkinje cells were COX<sup>-</sup>, SDH<sup>+</sup> and that occasional COX<sup>-</sup>, SDH<sup>+</sup> cells were seen in the olfactory bulbs, substantia nigra, and hypothalamus. Moreover, numerous COX<sup>-</sup>, SDH<sup>+</sup> cells were epithelial cells of the choroids plexus. None of the mice developed the muscle weakness seen in humans, but the neurologic pathology provides a possible explanation for the depression seen in the Twinkle mutant patients (896).

A third AD-PEO locus, which mapped to chromosome 4q34-35 (897,898), proved to be ANT1 (see Table 11-5). Two missense mutations have been identified. One was found in five Italian families with a common background haplotype, suggesting that they are related by descent. This mutation changes a highly conserved alanine at codon 114 to a proline. The second mutation was identified in a single individual and changed the valine at codon 289 to a methionine (899). The various ANT1 mutations associated with AD-PEO have proven to be missense mutations (A114P, V289M, L98P, D104G, and A90D). Furthermore, modeling of these mutations in yeast has revealed that they are acting as dominant-negative mutants (899–902).

On the other hand, a single patient has been identified who was homozygous for a null missense mutation. This individual manifested cardiomyopathy and MM with multiple deletions, but did not exhibit ophthalmoplegia (903). Interestingly, this is the same phenotype that was obtained when the Ant1 locus was knocked out in mouse (227) in two ways: by homologous recombination knockout with a neomycin gene driven by the PGK

promoter (PGK-neo) and by knock-in with a promoterless  $\beta$ geo cassette. Staining of adult tissues revealed strong expression in heart, muscle, and brain. Pathologic analyses of the Ant1<sup>-/-</sup> mice revealed the presence of cardiomyopathy and MM, the latter with classical RRFs and markedly increased COX and SDH staining in the type I oxidative fibers, and a massive proliferation of giant, swollen mitochondria. When subjected to an exercise stress test, the Ant1<sup>-/-</sup> animals were found to be highly fatigable, collapsing after half the exercise regimen of the Ant1<sup>+/+</sup> animals (227). Ant1 expression was confirmed in the extraocular eye muscles using the Ant1  $\beta$ geo mice, and Ant1 deficiency was associated with MM with increased COX and SDH staining and enlarged abnormal mitochondria. However, the Ant1 defect did not affect extraocular eye movement (904). This is the same phenotype seen in the ANT1-null patient (903).

Analysis of the hearts of 4- to 6-month-old Ant1<sup>-/-</sup> mice revealed a classical hypertrophic cardiomyopathy. Ultrastructural analysis revealed a partial reduction in contractile elements and a substantial induction of mitochondrial proliferation. As Ant1<sup>-/-</sup> mice age, they progress from hypertrophic cardiomyopathy at 3 months to dilated cardiomyopathy after 15 months (905).

Physiologic analysis of the Ant1<sup>-/-</sup> animals revealed elevated urine lactic acid, alanine, citrate, and succinate. Furthermore, ADP normally stimulated respiration in Ant1<sup>-/-</sup> liver mitochondria, partially stimulated respiration in the heart mitochondria, but had no effect of respiration in muscle mitochondria, consistent with the tissue distribution of Ant1. Analysis of mitochondrial H<sub>2</sub>O<sub>2</sub> production in the Ant1<sup>-/-</sup> tissue mitochondria revealed substantial ROS production in skeletal muscle, heart, and brain mitochondria, thus confirming that Ant1 deficiency is metabolically affecting brain, muscle, and heart. Consistent with the increased ROS production, glutathione peroxidase expression was increased in heart and muscle and MnSOD was increased in muscle. Moreover, the level of mtDNA rearrangements was greatly increased in the hearts of 15-month-old Ant1<sup>-/-</sup> mice (227).

As in the case of MNGIE-associated thymidine phosphorylase (TYMP) mutations that cause both mtDNA multiple deletions and mtDNA depletion, nDNA mutations have been identified in several other mitochondrial enzymes—ANT1 (899), thymidine kinase (TK2) (837), deoxyguanosine kinase (DGUOK) (906) and MPV17 (907), ribonucleotide reductase M2B (908) succinyl-CoA ligase subunits (909,910)—that are involved in mtDNA substrate provision through the maintenance of mitochondrial deoxyribonucleotide pool for mtDNA synthesis resulting in mtDNA depletion (see Table 11-5).

Mitochondrial depletion is associated with early childhood, frequently lethal, respiratory failure, lactic acidosis, and selective organ failure involving muscle (MM), heart, liver, or kidney (911–919).

Hence, in mtDNA depletion syndrome, organ failure is associated with a severe diminution of the level

of mtDNA relative to nDNA in the affected tissue. This has been documented by molecular hybridization of mtDNA and 18S rRNA sequences to Southern blots of PvuII digests followed by determining the ratio of hybridization by densitometry (915,918) or by dot-blot hybridization using a  $^{35}\text{S}$ -dCTP-labeled mtDNA probe and a  $^{32}\text{P}$ -dCTP-labeled arginosuccinate synthetase gene probe (916). Depletion has been confirmed by demonstrating the loss of mtDNA gene products and loss of cytoplasmic mtDNA by immunohistochemistry (915,918).

The age of onset and severity of symptoms vary among patients, and these variations have been loosely correlated with the severity of the depletion. In one series of 10 patients, individuals exhibiting symptoms shortly after birth and dying in the first year of life had between 2% and 17% residual mtDNA in the affected organ, while individuals who exhibited symptoms after the first year and lived for three or more years had between 14% and 34% of the normal mtDNA (914,915,918). This association is less clear in another series of patients, although one complexity is the rapid rise in the mtDNA:nDNA ratio during the first 2–3 years of life (916).

## 11.3 MITOCHONDRIAL ETIOLOGY FOR COMMON METABOLIC AND VISCERAL DISEASES

### 11.3.1 Diabetes and Metabolic Syndrome

The linkage of mtDNA rearrangement (731,732) and tRNA mutations (730) to type 2 diabetes directly linked mitochondrial defects to common diseases. Evidence that mtDNA defects are a common factor in the etiology of diabetes comes from the observation that as the age-of-onset of the proband increases, the probability that the mother was the affected parent also increases, reaching a ratio of 3:1 for patients with a mean age-of-onset of 46 years (412). These observations can be linked to the larger human “metabolic syndrome” through the identification of a mtDNA tRNA<sup>Leu</sup> mutation at np 4291 (T>C) that causes hypertension, hypercholesterolemia, and hypomagnesemia (renal ductal convoluted tubule defect) in a maternal pedigree in association with reduced mitochondrial ATP production and the secondary clinical findings of migraine, hearing loss, hypertrophic cardiomyopathy, and MM (733).

Insights into how mitochondrial defects cause type 2 diabetes have been obtained from the analysis of the mitochondrial gene expression profiles of cells harboring different percentages of the tRNA<sup>Leu(UUR)</sup> 3243 mutation generated using transmitochondrial cybrids (288,289). The cybrids were generated from cells of a patient that was heteroplasmic for the tRNA<sup>Leu(UUR)</sup> A3243G mutation which were enucleated and the cytoplasts fused to a recipient 143B(TK-) osteosarcoma cell which lacked mtDNA ( $p^0$ ) (296). Cybrid clones were then selected for those with different percentages of mutant A3243G and

wild-type A3243A mtDNAs, and tested for the effect of the level of mutant heteroplasmy on the expression of nDNA and mtDNA mitochondrial genes using the MITOCHIP. The MITOCHIP is a custom cDNA microarray encompassing ~600 genes involved in mitochondrial bioenergetics, biogenesis, antioxidant defense systems, and apoptosis. Those cybrids with less than about 60% A3243G mtDNAs were found to have significantly downregulated about 40 nDNA-encoded mitochondrial genes, slightly downregulated selected mtDNA-encoded genes, and upregulation of a dozen nDNA genes. This experiment linked a partial mitochondrial protein synthesis defect with type 2 diabetes and with a downregulation of mitochondrial gene expression, suggesting that diabetes was a mitochondrial disease.

Consistent with these cellular results, studies of patients with type 2 diabetes have also revealed the downregulation of mitochondrial function and gene expression. Insulin-resistant offspring of type 2 diabetic patients have been found to have impaired mitochondrial energetics, as assessed by  $^{31}\text{P}$ -MR Spectroscopy (920). Furthermore, type 2 diabetes patients consistently show a downregulation of nDNA encoded mitochondrial gene expression, in association with alterations in the levels of the PPAR $\gamma$ -coactivator 1 (PGC-1) (921,922). The PPAR $\gamma$ -PGC-1 is a major regulator of mitochondrial biogenesis and fat oxidation (373,923).

Diabetes mellitus is also seen in Friedreich ataxia, caused by increased mitochondrial ROS production. Friedreich patients manifest cerebellar ataxia, peripheral neuropathy, hypertrophic cardiomyopathy, and diabetes as the result of inactivation of the frataxin gene on chromosome 9q13. Frataxin binds iron in the mitochondrial matrix thus minimizing mitochondrial OH production. The loss of the frataxin protein results in the ROS inactivation of all mitochondrial iron-sulfur containing enzymes including complexes I, II, III, and aconitase. Thus, increased mitochondrial ROS production and decreased mitochondrial OXPHOS have been linked to diabetes (22,865,866).

Type 2 diabetes has also been associated with a Pro121A polymorphism in the PPAR $\gamma$  (924). PPAR $\gamma$  is thought to play a role in the regulation of peroxisome and mitochondrial number and structure. Type 2 diabetes has also been associated with a Gly482Ser polymorphism in the PGC-1 in Danish populations (925) and in the Pima Indians (926).

Maturity onset of diabetes in the young (MODY) is an early-onset autosomal dominant form of type 2 diabetes that might also be linked to mitochondrial dysfunction. The molecular defects of four forms of MODY have been identified. MODY 2 is the result of mutations in glucokinase, MODY 1 due to mutations in the hepatocyte nuclear factor (HNF)-1 $\alpha$ , MODY 3 due to mutations in HNF-4 $\alpha$ , and MODY 4 due to mutations in insulin promoter factor (IPF)-1. Early indications are that these genes may modulate  $\beta$  cell mitochondrial

function. Glucokinase has a very high  $K_m$  for glucose and is thought to be the glucose sensor. Glucokinase may bind to mitochondrial VDAC such that the ATP binding site is fed mitochondrial ATP through the ANT (927–929). After a meal, when the serum concentration of glucose is high, glucose binds to glucokinase and is phosphorylated to glucose-6-phosphate (G-6-P). G-6-P is then converted to pyruvate for use in mitochondrial ATP generation (Figure 11-4).

Mutations in HNF-1 $\alpha$  are associated with post-pubertal diabetes, obesity, dyslipidemia, and arterial hypertension, all features of mitochondrial diseases. Moreover, HNF-1 $\alpha$  is also important in regulating nDNA encoded mitochondrial gene expression and the expression of GLUT 2 glucose transporters (930). HNF-4 $\alpha$ , a member of the steroid/thyroid hormone receptor super-family, acts as an upstream regulator of HNF-1 $\alpha$  (931). Therefore, the MODY 1, 2, and 3 genes appear to adversely affect mitochondrial function in the pancreatic  $\beta$  cells.

The importance of mitochondrial deficiency in defects in  $\beta$  cell insulin secretion has been confirmed in two mouse models. In the first, the mitochondrial transcription factor, Tfam, was inactivated in the pancreatic  $\beta$  cells. This resulted in increased blood glucose in both fasting and non-fasting states, and the progressive decline in  $\beta$ -cell mass by apoptosis (932). In the second, the ATP-dependent K<sup>+</sup>-channel ( $K_{ATP}$ ) affinity for ATP was reduced resulting in a severe reduction in serum insulin, severe hyperglycemic with hypoinsulinemia, and elevated D-3-hydroxybutyrate levels (933). These models demonstrate that mitochondrial ATP production is critical in the signaling system of the  $\beta$  cell to permit insulin release (934). Thus, pancreatic  $\beta$  cell mitochondrial defects are important in both glucose sensing through glucokinase and insulin releases through the  $K_{ATP}$  channel.

These observations lead to the following model of mitochondrial involvement in type 2 diabetes. On ingestion of a meal, the glucose concentration in the blood rises until it exceeds the  $K_m$  of the  $\beta$  cell glucokinase. Since glucokinase is bound to the mitochondrial VDAC, its ATP binding site is continuously occupied by mitochondria-derived ATP provided by ANT. The binding of glucose by glucokinase results in the production of G-6-P, which is then converted to pyruvate via the glycolytic pathway. Pyruvate is oxidized by the mitochondria to generate ATP. The elevated ATP production increases the ATP/ADP ratio which results in the closing of the  $K_{ATP}$  channel, depolarizing the  $\beta$  cell plasma membrane. The depolarized plasma membrane opens the voltage sensitive Ca<sup>++</sup> channel. The influx of Ca<sup>++</sup> stimulates the transport of insulin containing vesicles to the plasma membrane and thus the release of insulin (Figure 11-4).

These observations lead to the hypothesis that type 2 diabetes is a mitochondrial disease. In individuals who inherit mutations in nDNA or mtDNA mitochondrial genes, the partial defect in OXPHOS reduces the

capacity in their post-mitotic cells to oxidize carbohydrates and fats to make ATP. Given a high caloric diet, these individuals overload their mitochondrial capacity to oxidize the glucose associated reducing equivalents. The high circulating glucose signals the  $\beta$  cells to secrete insulin, resulting in concurrent elevated glucose and insulin, “insulin resistance.” The sustained high serum glucose and lipids result in the deposition of fats in storage tissues including liver resulting in nonalcoholic steatohepatitis (NASH), NASH being observed in over 30% of individuals with indications of insulin-resistance, hypertriglyceridemia, and hypercholesterolemia (935).

The high circulating glucose stimulates the  $\beta$  cells to secrete insulin. Insulin interacts with the insulin receptor of different target tissues to stimulate the storage of fats and glycogen and to downregulate mitochondrial energy metabolism, presumably through PPAR $\gamma$  and PGC-1. Moreover, the excess of reducing equivalents overloads the ETC elevating the NADH/NAD<sup>+</sup> ratio and driving mitochondrial ROS production. In the peripheral tissues, this further erodes mitochondrial function and increases insulin resistance. In the  $\beta$  cells, this erodes the mitochondrial ATP production capacity eventually leading to a decline in insulin secretion due to inadequate ATP for glucokinase and low ATP/ADP ratio which cannot activate the  $K_{ATP}$  channel. The high glucose but reduced insulin levels is termed “insulin-independent diabetes.”

Continued calorie overload in the  $\beta$  cells causing chronic mitochondrial ROS production ultimately activates the mtPTP which results in the death of the  $\beta$  cells by apoptosis, resulting in “insulin-dependent diabetes.” The chronic mitochondrial oxidative stress on the peripheral tissues ultimately results in damage to the retina, vascular endothelial cells, peripheral neurons, and nephrons leading to end stage diabetes. Therefore, these studies suggest that mild, chronic mitochondrial defects may be a primary cause of type 2 diabetes.

Haplogroup J has been associated with increased risk of diabetes in certain European descent populations (936–938), while haplogroup N9a is protective of diabetes, metabolic syndrome, and myocardial infarction in Asians (939–941). Haplogroups N9a and F have been shown to affect mitochondrial function through alterations in expression array studies confirming that the mtDNA haplogroups do contribute to the pathophysiology of diabetes and metabolic syndrome (942).

### 11.3.2 Ischemic Heart Disease and Dilated Cardiomyopathy

Ischemic heart disease results from the development of atherosclerotic plaques on the coronary arteries of the heart. When the arteries constrict, the plaques occlude the artery, blocking blood flow to the heart and starving the heart mitochondria of oxygen (ischemia). In the absence of oxygen, the ETC stops and, since fatty acids and hence reducing equivalents are abundant, the

electron carriers become fully reduced. On dilation of the coronary artery, blood flows back into the heart, supplying oxygen (reperfusion). At this point, electrons contained in reduced ubiquinone can be donated directly to oxygen to give a burst of superoxide anion, which is rapidly converted to  $H_2O_2$  and OH. Within 1 minute of reperfusion in experimental systems, 95% of the OH is located in the mitochondria (943). These cyclic bursts of oxygen radicals damage heart mitochondrial membranes, proteins, and DNA.

A high degree of mtDNA damage in chronically ischemic hearts has been confirmed by quantitating the common 5-kbp deletion. Individuals with dilated cardiomyopathy due to chronic ischemia–reperfusion were found to have between 8- and 2200-fold more mtDNA deletion than age-matched controls. Moreover, the levels of the 7436-bp and 10,423-bp deletions went up concurrently with the 5-kbp deletion, and the ischemic hearts showed a coordinate induction of the nuclear OXPHOS genes for ANT1, ANT3, and ATPsyn $\beta$  and the mtDNA genes for MTCYB, MTRNR (12S rRNA), and MTRNR2 (16S rRNA) (448,449). These data suggest that chronic cardiac ischemia and reperfusion result in severe destruction of the cardiac mtDNAs and probably a decline in bioenergetic capacity. This in turn is compensated for by the induction of mitochondrial biogenesis. Elevated mtDNA 5-kbp deletion has also been observed in patients with idiopathic dilated cardiomyopathy (449), and multiple different rearrangements were detected by whole-genome PCR amplification in 18 of 40 dilated cardiomyopathy endomyocardial biopsies (944) and in myocardium (459). Similarly, increased rearrangement and base substitution mutations have been reported in the hearts of children with sudden infant death syndrome (945), and 212 different deletions have been found in the heart of a child with familial dilated cardiomyopathy, versus only five in a control heart. Furthermore, both this patient and a patient with hypertrophic cardiomyopathy were found to have a 12S rRNA A-to-G transition at np 827 that alters a conserved nucleotide (471). Consequently, it is possible that mitochondrial energy depletion may be an important factor in cardiac failure.

Autosomal dominant hypertrophic cardiomyopathy has been linked to mutations in a variety of cardiac contractile protein genes (946–948). However, the pedigrees exhibit variable expressivity, suggesting that other genetic factors may influence the cardiac phenotype. One modifying factor might be mtDNA mutations. Analysis of several hypertrophic cardiomyopathy patients has revealed not only mutations in contractile proteins, but also mutations in the mtDNA, including the tRNA mutations at positions T4314G and A4315G in the MT-TI or at positions A15902G and A15935G in the MT-TT (949).

Mitochondrial defects have been established to cause MM and hypertrophic cardiomyopathy in mice by genetic inactivation of the mouse Ant1 gene (227,905).

These animals also develop multiple mtDNA deletions in their heart mtDNAs associated with increased mitochondrial ROS production (605). Hence, mitochondrial dysfunction can lead directly to cardiomyopathy.

A patient with a null mutation in the ANT1 gene has also been found to have MM together with hypertrophic cardiomyopathy associated with multiple mtDNA deletions (903).

The mouse ANT1 deficiency has been partially ameliorated by transfecting the mouse muscle with the Ant1 cDNA, carried on an adeno-associated virus (AAV) vector. Transduction of the muscle reintroduced functional ANT1 into the muscle, which could transport ATP, resulting in a production of ATP up to 40% of normal. This was accompanied by the restoration of nearly normal muscle histology (950).

### 11.3.3 Idiopathic Sideroblastic Anemia

Idiopathic sideroblastic anemia has also been associated with the appearance of somatic mtDNA mutations in the mtDNA COI gene (951,952). Sideroblastic anemia is characterized by the inadequate formation of heme and the excessive accumulation of iron in erythroblastoid mitochondria. A common finding is anemia associated with ring sideroblasts, which are also seen in Pearson marrow–pancreas syndrome. Sideroblastic anemia is associated with the accumulation of protoporphyrin IX and the deposition of iron in the mitochondria. To make heme, iron must be in the reduced  $Fe^{2+}$  form to be inserted in protoporphyrin IX by ferrochelatase, and the reduction of iron occurs via the ETC. Hence, it has been proposed that sideroblastic anemia may be the result of mitochondrial defects (952). This concept has been supported by the discovery of two novel mtDNA COI missense mutations in patients with sideroblastic anemia. The first patient was found to have a T6721C mutation that changed a highly conserved methionine 273 to a threonine. This mutation was heteroplasmic in the myeloid lineage, but was not present in other tissues of the patient or in the mother or daughter. The second patient was found to have a T6742C mutation that converted the conserved isoleucine 280 to a threonine. This mutation was also heteroplasmic. Hence, it would appear that these somatic mutations arose in the bone marrow and resulted in the physiologic malfunction of the descendant cells (952).

## 11.4 MITOCHONDRIAL ETIOLOGY FOR NEURODEGENERATIVE DISEASES

Evidence is rapidly accumulating that mitochondrial dysfunction lies at the core of many of the neurodegenerative diseases such as Alzheimer disease (AD), Parkinson disease (PD), Huntington disease (HD), amyotrophic lateral sclerosis (ALS), and so on.



## 11.4.1 Alzheimer Disease

**11.4.1.1 The Biology and Genetics of Alzheimer Disease.** Mitochondrial defects have repeatedly been reported for AD brains. AD has been associated with mtDNA haplogroups, aggregates of amyloid  $\beta$  peptide ( $A\beta$ ) have been observed to enter the mitochondrion and inhibit mitochondrial function, the amyloid precursor protein (APP) and the presenilin (PS)  $\gamma$ -secretase which process APP into  $A\beta$  have been associated with the mitochondrion and mitochondrial-endoplasmic protein (ER) complex, and the apolipoprotein E (ApoE) e4 variant associated with late-onset AD correlates has been found to generate a fragment that is imported into the mitochondrion.

AD is the most commonly recognized form of late-onset dementia (953). AD is a progressive neurodegenerative disease resulting in dementia that has been associated with the deposition of  $A\beta$  amyloid peptide plaques and neurofibrillary tangles in the brain (954).

Clinically, AD has been split into early-onset and late-onset forms, divided roughly at age 65. Early-onset AD has been associated with mutations in several autosomal dominant loci, including the APP gene on chromosome 21, presenilin 1 (PS1) on chromosome 14, and PS2 on chromosome 1. APP is processed by “secretase” proteolytic cleavage. A number of APP mutations have been identified which cause the inappropriate processing of APP into  $A\beta_{40}$  and  $A\beta_{42}$  peptides,  $A\beta_{1-40}$  and  $A\beta_{1-42}$ . The  $A\beta$  peptides have been assumed to be toxic, with  $A\beta_{1-42}$  being the more so. The  $A\beta$  peptides have a tendency to aggregate, and it has been found that the toxic forms of  $A\beta$  are oligomers and to a lesser extent filamentous aggregates (955,956). The PSs are required for the secretase processing of APP, with defects in these proteins favoring the accumulation of the  $A\beta$  peptides. Current evidence indicates that PS1 is a component of the  $\beta$ -secretase enzyme complex (957). Multiple pathogenic PS1 missense mutations have been identified that account for the majority of early-onset cases and 2% of the total AD cases (958–960). In addition, two homologous genes encoding  $\beta$ -secretase activity have been cloned, BACE 1 and BACE 2 (961). Tissue-specific transcription levels (962,963) and genetic mapping studies (961) also support a correlation between  $A\beta$  production in AD.

The excessive deposition of  $A\beta$  in AD brains has generated the “amyloid cascade hypothesis” of AD.  $A\beta$  is envisioned as perturbing cellular homeostasis, possibly through altering  $Ca^{2+}$  metabolism or generating toxic ROS. The increased ROS stimulates neurofibrillary tangle formation, which fills and ultimately kills the cell (964). Support for this hypothesis comes from observations that all three autosomal dominant AD genes increase the production of  $A\beta$  peptides. Considerable evidence has implicated aggregates of the  $A\beta$  peptide in increasing cellular calcium permeability, either directly or indirectly through  $Ca^{2+}$  or  $K^+$  channels (965–967).

$A\beta$  might also increase the sensitivity of AD cell mitochondria to oxygen radical generation and  $Ca^{2+}$  toxicity (968). Moreover,  $A\beta$  has been shown to bind  $Zn^{2+}$  and  $Cu^{2+}$  and in that form to aggregate and actually generate ROS, particularly  $H_2O_2$  (969–972). Since increased oxidative damage in AD brains is well documented (973,974), it is very possible that the pathophysiology of amyloid accumulation could be the result of increased neuronal oxidative stress.

Late-onset AD is also genetically heterogeneous, with several loci now implicated in this disease. Although the molecular defects that cause the late-onset, sporadic AD cases have not been determined (975), the inheritance of the ApoE e4 allele has been identified as a major risk factor for developing late-onset AD (975–978). Multiple other loci have been associated with late-onset AD (979–981), although these associations can be controversial. In addition, several groups have associated the interleukin-1 gene with AD (981–986). Another late-onset AD locus on chromosome 10 appears to be related to the insulin degrading enzyme (IDE), which may be involved in the degradation of  $A\beta$  (987,988). A related enzyme, neprilysin, has recently been directly implicated in  $A\beta$  degradation. Suppression of neprilysin in normal rat brain elevates  $A\beta$  deposition (989), and the human neprilysin gene is polymorphic (990).

**11.4.1.2 Mitochondrial Defects in AD.** Multiple studies have implicated mitochondrial OXPHOS defects in both AD. Histologic analysis of AD brains has revealed alterations in mitochondrial morphology in apparently normal dendrites from the superficial cortical layers, which include increased matrix density and paracrystalline inclusions in the intercrystal space (991). Positron emission tomography studies of AD patients have observed decreased glucose transport, suggesting a bioenergetic defect (992), and analysis of brain sections from AD patients has revealed a 30% reduction in PDH activity (993,994) and a 42% reduction in the ADP: $O_2$  ratio, suggesting the partial uncoupling of OXPHOS (995). Chronic inhibition of complex IV in rats by infusion of sodium azide impairs both spatial and nonspatial learning in rats (996), and significant reductions in glucose and glutamine oxidation and calcium accumulation have been reported for AD skin fibroblasts (997). Normal skin fibroblasts treated with the OXPHOS uncoupler carbonyl cyanide *m*-chlorophenylhydrazine show a 10-fold increase in epitopes recognized by antibodies to paired bilateral filaments and a 157-fold increase in the protein detected by the AD-specific monoclonal antibody 50 (998). Finally, ROS have been implicated in AD, both in damaging neurons and in catalyzing the aggregation of the  $A\beta$  peptide (999,1000).

AD patient materials have repeatedly been reported to have partial defects in respiratory complex IV (COX) in both platelet and brain mitochondria (836,1001–1004). These complex IV defects have been proposed to be associated with mtDNA defects, based on their

apparent ability to be transferred from AD patients to cultured cells by fusion of patient platelets to  $p^0$  Ntera/D1 neurons or the neuroblastoma SH-SY5Y. AD platelet cybrids have been reported to have reduced complex IV activity, increased ROS production, and reduced  $Ca^{2+}$  capacity (1003,1005–1015), though the validity of these experiments has been questioned (1016).

Histochemical staining of COX in AD brains revealed marked reduction in activities in the dentate gyrus and the hippocampal subfields (CA1 > CA2 > CA4) (1017). Defects in mitochondrial respiration (995) and in PDH (993,994) have also been reported. Consistent with these biochemical defects, levels for several, but not all, nDNA and mtDNA OXPHOS gene transcripts are reduced in AD brain (1018–1021). This downregulation of specific mitochondrial mRNA levels is paralleled by the downregulation of a variety of genes encoding proteins relevant to AD pathology, as detected by DNA microarray (chip) analysis. Downregulated genes included phosphatases/kinases, cytoskeletal proteins, synaptic proteins, and glutamate and dopamine receptors. By contrast, the cathepsin D mRNA is upregulated in tangle-containing cells (1022).

The potential importance of the mitochondria and oxidative stress in AD has been demonstrated by treating brain synaptosomes and cultured astrocytes with A $\beta$  peptides. Treatment of nucleate- and mitochondria-rich cortical synaptosomes with A $\beta$ ,  $Fe^{2+}$ , or the complex II inhibitor 3-nitropropionic acid reduced glucose and glutamate uptake, reduced mitochondrial  $\Delta\psi$ , increased mitochondrial ROS generation, and stimulated expression of the stress proteins HAP70, HSP60, and GRP78. Moreover, the deleterious effects of these agents were substantially reduced in diet-restricted animals (1023). APP binds to heme oxygenase-2 (HO-2). The heme oxygenases degrade bilirubin to generate an antioxidant and CO. The binding of APP to HO-2 inhibits its antioxidant effects, and this inhibition is greatly accentuated if the APP harbors familial AD mutations (1024,1025).

HO-1 is a stress response protein that degrades heme into CO plus biliverdin. Biliverdin, in turn, is converted to bilirubin, which has free radical scavenging properties. CO is thought to bind and inhibit mitochondria, blocking COX in the ETC and increasing mitochondrial ROS production. The degradation of the heme releases iron, which appears to be taken up by the mitochondria. The excess intramitochondrial iron then stimulates OH production via the Fenton reaction, and the increased mitochondrial oxidative stress is compensated by the induction of MnSOD.

Treatment of cortical astrocytes with A $\beta$  peptides induces HO-1 which results in the sequestration of nonheme iron by the mitochondria and the activation of the mtPTP. These astrocyte effects can be inhibited by concurrent treatment with dexamethasone, which suppresses HO-1 induction, and cyclosporin A, which stabilizes the mtPTP (1026). The induction of HO-1

subsequently results in the induction of MnSOD, and this induction can be blocked by antioxidants including ascorbic acid, melatonin, and resveratrol (1027). These results directly link A $\beta$  peptide toxicity to mitochondrial oxidative stress and the activation of the mtPTP, at least in part through the mediation of HO-1.

While the reason for the specific toxicity of the A $\beta$  peptides for the CA1 layer of the hippocampus is not yet clear, one possibility could lie with the cell- and tissue-specific modulation of the mtPTP in both iron uptake and the induction of apoptosis. Such regulation would require the tissue-specific expression of a protein that interacts with the mtPTP. Expression of the PRAX-1 gene is tissue specific, being expressed at high levels in the CA1 neurons, specifically binding to the mitochondrial benzodiazepine receptor (TSPO) of the mtPTP. PRAX-1 is a 1857-amino acid protein that links two mitochondrial benzodiazepine receptors, and thus mtPTPs, together through their C-terminal peptides. Moreover, PRAX-1 has a long N-terminal end that contains three proline-rich domains, two glutamate-rich domains, an Src homology 3 domain, mitochondrial targeting sequence, and three nuclear targeting signals. Thus PRAX-1 has all of the characteristics that might be expected for a protein coordinating the actions of the mtPTP with other structures in CA1 cells (1028).

A mitochondrial association with AD might also explain the association between the ApoE e4 allele and AD risk, since the presence of the ApoE e4 allele has been associated with increased brain oxidative damage (1029–1031).

A $\beta$  has been reported to enter the mitochondrion through the mitochondrial outer membrane “TOMM” (Translocase of Outer Mitochondrial Membrane) complex, become localized in the mitochondrial cristae, and alter mitochondrial function (1032–1035). A $\beta$  increases mitochondrial membrane viscosity, causing a decrease in ATP/O ratio, reducing ETC activity, increasing ROS production, and facilitating cytochrome c release (1036). Incubation of cultured neurons with A $\beta$  oligomers results in an 89% reduction in mitochondrial membrane potential within 2 hours of exposure, followed by mitochondrial release of cytochrome c and apoptosis initiating factor (AIF) within 8 h (955). Synaptic mitochondria are more sensitive to A $\beta$  mitochondrial toxicity than are neuronal mitochondria, and synaptic mitochondrial dysfunction is an early manifestation in mouse models of AD (1037,1038).

A $\beta$  has been shown to specifically inhibit cytochrome c oxidase (COX or complex IV) (1032,1039). One possible mechanism for this inhibition is that A $\beta$  can bind heme, including the heme-a of COX, creating an A $\beta$ -heme complex, which is also a peroxidase (1040). Chronic exposure of rats to the COX inhibitor  $NaN_3$  results in cognitive deficits and neuronal morphological changes (1041).

A $\beta$  has also been reported to inhibit mitochondrial MnSOD, increasing oxidative stress, and overexpression

of MnSOD is protective of AD (1042). Similarly, A $\beta$  has been reported to inhibit cyclophilin D, a component of the mtPTP (1043). Early stage AD brains also show lipoxidation of the  $\alpha$ -subunit of the ATP synthase, in association with reduced ATP synthase activity (1044). In addition, A $\beta$  perturbs the mtPTP, inhibits OXPHOS complexes III and IV, and also binds and inhibits mitochondrial alcohol dehydrogenase, designated A $\beta$  alcohol dehydrogenase (ABAD) (1033,1035). The interaction between A $\beta$  and ABAD has been associated with mitochondrial dysfunction and dementia, and one hypothesis argues that alteration of ABAD increases mitochondrial aldehyde toxicity (1043,1045–1047).

APP has also been found to be located in the outer mitochondrial membrane (867,1048). Similarly, PS1 has been located in the mitochondrial inner membrane of rat brain and liver (1049). The other components of the presenilin  $\gamma$ -secretase, including nicastrin, APH-1, and PEN-2, have also been found in a high-molecular-weight complex within the mitochondrial inner membrane. Although the import mechanism for these proteins is not fully understood, the nicastrin protein has been found to harbor both ER and mitochondrial N-terminal targeting signals (1050). The processing of APP bound to the mitochondrial outer membrane by mitochondrial  $\gamma$ -secretase would release the APP intracellular domain (AICD) within the mitochondrion (1051). Consistent with the possibility that A $\beta$  enters the mitochondrion, IDE has been discovered to have a mitochondrially directed form generated by initiating translation at an AUG codon 41 amino acids upstream from the canonical IDE peptide start codon. Since the IDE is thought to degrade both cerebral A $\beta$ -peptide and plasma insulin, it could function in mitochondrial A $\beta$  turnover (1052). Mitochondrial A $\beta$  is also turned over within the mitochondrion by presequence protease (1053).

Given that  $\gamma$ -secretase is unique in its ability to cleave peptide bonds within membranes, the PS1 complex might also be involved in processing mitochondrial inner membrane proteins such as those of the respiratory complexes. Hence mutations in the presenilin complex associated with early-onset AD might also cause mitochondrial defects.

PS1 and PS2 have been reported to interact with mitochondrial proteins. They form a complex with the outer mitochondrial membrane protein FKBP38 and Bcl-2 thus modulating mitochondrial mtPTP-mediated apoptosis (1054). PS1 also interacts with Om1/HtrA2, a pro-apoptotic protein stored in the mitochondrial intermembrane space. The C-terminus of PS1 activates the proteolytic activity of Om1/HtrA2, which degrades anti-apoptotic proteins (1055).

The PS1 and PS2 complexes have recently been associated with “mitochondrial-associated membranes” (MAMs). MAMs are thought to be specialized compartments of the endoplasmic reticulum (ER), which connect the ER with the mitochondrion and are important in

lipid and calcium (Ca<sup>++</sup>) metabolism. Altered PS metabolism could thus alter Ca<sup>++</sup> flux into the mitochondrion, initially activating rate-limiting mitochondrial dehydrogenases, then increasing mitochondrial ROS production, and ultimately activating the mtPTP. Activation of the mtPTP would lead to caspase activation and synaptic loss (1056,1057). Thus, the localization of PS1 and 2 in the MAMs is consistent with the observed association between ER stress, Ca<sup>++</sup> dysregulation, and mitochondrial dysfunction in AD (1058,1059).

Late-onset AD cases are commonly associated with the apolipoprotein E gene  $\epsilon$ 4 allele (ApoE  $\epsilon$ 4) (1). This association has been linked to mitochondrial dysfunction by demonstrating that transgenic mice which overexpress ApoE  $\epsilon$ 4 have reduced levels of complexes I, IV, and V (1060). The special neuronal toxicity of ApoE  $\epsilon$ 4 has been proposed to result from ApoE  $\epsilon$ 4's unique ability to undergo an interaction between the N- and C-terminal domains, creating a compact structure. ApoE  $\epsilon$ 4 is then subject to neuron-specific cleavage and the N-terminal cleavage product becomes associated with the mitochondrion and inhibits mitochondrial function (1061,1062).

The ApoE  $\epsilon$ 4 allele is linked to the mitochondrial outer membrane protein import gene, TOMM40. Recently, an association has been observed between a TOMM40 polymorphism and AD, which is independent of the ApoE  $\epsilon$ 4 association, thus further supporting a direct link between mitochondrial function and AD (1063,1064).

A mitochondrial etiology of AD could also explain the near-universal association of A $\beta$  amyloid plaques with AD. At the concentrations found in biologic fluids, A $\beta$ 1-40 and A $\beta$ 1-42 have been proposed to act as antioxidant and antiapoptotic polypeptides, presumably because of their tendency to chelate transition metals, particularly copper. However, when the A $\beta$ s aggregate, they become pro-oxidants, possibly through placing the transition metal in the presence of the redox-active Met 35 (1065–1067). Given this perspective on the A $\beta$  peptides, it could follow that genetic variants which chronically increase mitochondrial ROS production would overwhelm the antioxidant capacity of basal levels of A $\beta$ . To compensate, the brain would increase A $\beta$  production. However, this would eventually lead to excess A $\beta$ , which would then aggregate into a pro-oxidant form (972) and thereby exacerbate the problem. Consistent with this scenario, the brains of late-onset sporadic AD patients have been observed to have increased oxidative damage, increased activated caspase activity (1068,1069), and increased numbers of terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling-(TUNEL) positive cells (1008). Increased mitochondrial ROS would also increase the mtDNA somatic mutation rate and exacerbate mitochondrial oxidative stress, mtPTP activation, and synaptic loss.

AD is associated with neurofibrillary tangles, which are composed of hyperphosphorylated Tau. The N-terminal 20–22 kDa of the Tau protein is enriched in the



synaptosome mitochondria of AD patients and correlates with A $\beta$  multimeric species. Therefore, A $\beta$  interacts with 20–22 kDa N-terminal Tau and the complex may impair mitochondrial function (1070).

Dysfunctional autophagy may also contribute to the decline in mitochondrial function in AD (1071). This may be attributed in part to altered mitochondrial dynamics (fission and fusion) (1072).

Therefore, early-onset AD might also be explained via a mitochondrial pathophysiology. Mutations in the A $\beta$  processing pathway could increase the rate of A $\beta$  production and aggregation, creating an active pro-oxidant that would increase synaptic oxidative stress, damage the mitochondria, and destroy the synapses. Alternatively, mutations in the PS complex could alter the processing of proteins in the mitochondrial inner membrane, resulting in alterations in mitochondrial function and pathology.

**11.4.1.3 mtDNA Mutations in AD.** Genetic evidence that mitochondrial dysfunction may be the underlying cause of AD comes from epidemiologic data that demonstrated that inheriting AD from the mother is 1.7–3.6 times more likely than inheriting the disease from the father (1073,1074). This strong maternal bias in transmission of AD suggests that some mtDNA variants may be risk factors for AD. This concept has been supported by showing that the mitochondrial OXPHOS defects of AD patients can be transferred with the cytoplasm in transmitochondrial cybrids (1075).

Several mtDNA mutations and variants have also been linked to late-onset dementia and AD. One patient with progressive cognitive decline, dementia, deafness, ataxia, and chorea was found to be heteroplasmic for a tRNA<sup>Trp</sup> mutation, MTTW\*DEMCHO5549A. Postmortem analysis of the brain revealed diffuse and moderate neuronal loss in the cortex and basal ganglia with gliosis present throughout the brain. RRFs and COX-negative-staining fibers were evident on skeletal muscle analysis, as were morphologically abnormal mitochondria on electron microscopy of skeletal muscle. A complex I defect was detected in mitochondrial respiration assays. Hence, this tRNA<sup>Trp</sup> mutation demonstrates that mitochondrial defects can cause dementia (748).

Furthermore, analysis of the mtDNAs of late-onset AD patients revealed a significantly increased frequency of the tRNA<sup>Gln</sup> gene polymorphism at np 4336, MTTQ\*ADPD4336G (633,749,751). This A4336G population polymorphism in the tRNA<sup>Gln</sup> gene was found in 3.2% of AD patients but in only 0.4% of controls (633). In one confirmatory study, the np 4336 mutant was found in 6% of AD patients and 0.3% of controls (1076), and in the other this mutation was found in 3.6% of AD but in 0 of 100 controls (749). An Italian study has recently confirmed this association, identifying the A4336G in 2% of AD patients and 0.8% of controls (1077). While the association between the A4336G variant and AD and PD has been confirmed

multiple independent times, the association was not confirmed in two studies (1078,1079).

The MTTQ\*ADPD4336G variant arose in Europe about 8500–17,000 years ago creating a European mtDNA lineage predisposed to neurodegenerative disease, now designated haplogroup H5a (633). Since this was a European-specific variant, it is not surprising that this variant was not found in Japanese AD patients (1080). In the initial survey of AD and PD patient mtDNAs, several additional variants were found, including a missense mutation in ND1 at np 3397, a 16S rRNA mutation, and an insertion in the 12S rRNA gene at np 956 through 965 (633).

An mtDNA A-to-G transition in the ND2 gene at np 5460 variants has also been reported to be associated with AD (634), but was subsequently found to be a polymorphism (635,636,1081). Regional sequencing of the mtDNAs of French Canadian AD patients and controls found one mtDNA lineage that was at increased risk for AD. This lineage harbored the cytochrome b 15,812–amino acid polymorphism (425), which has been shown to be a marker for the Caucasian mtDNA haplogroup J (777,1082). The complete sequence of two other mtDNAs of AD patients were analyzed, one harboring this mtDNA haplotype and another harboring the np 4336 mutation (1083).

Since this first association, multiple studies have shown that certain mtDNA haplogroups either increase or decrease the probability of neurodegenerative disease. Haplogroups U and T have been associated with decreased risk of developing AD in most contexts (425,426,633,1084), though Uk was found to increase AD risk in a brain imaging study (1085).

A mitochondrial role in AD has been further corroborated by the demonstration that in the brains of AD patients who died prior to age 75, the 5-kbp deletion is increased on average 15-fold whereas in the brains of patients who died after age 75, the 5-kbp deletion is present at one-fifth the level of that in control brains (1086). Consistent with this hypothesis, the mtDNAs of AD brains have been found to have 20-fold higher 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels than nDNA, and there is a significant threefold increase in 8-OHdG levels in the mtDNAs of AD parietal cortex relative to controls (479). The sudden drop in the level of mtDNA deletions in older AD brains might be the result of loss of the brain cells containing the most mutated mtDNA through apoptosis. This is consistent with the increased neuronal apoptosis observed in AD brains (1087). However, this elevation of somatic mtDNA rearrangements has not been confirmed in some other studies (1088,1089) one of which only examined patients over age 70 years at which the mutant mtDNAs are lost (1089).

An association between mitochondrial deficiency and AD has been further strengthened by analyzing mtDNA somatic base substitution mutations. Using



protein nucleic acid-clamping PCR, the somatic mtDNA CR T414G mutation in the P<sub>L</sub> mtTFA binding site was detected in 65% of all AD brains and in 57% of Down syndrome (DS) patients which developed dementia (Down syndrome and dementia (DSAD)) but not in any control brains. Moreover, the level of somatic mtDNA CR mutations in AD brains is increased by 63% overall, with the elevation of somatic mtDNA CR mutations in AD patients over 80 years being 120%. Furthermore, in the AD and DSAD brains, the somatic mtDNA mutations preferentially occur within the known mtDNA transcription and replication regulatory elements, whereas very few of the mtDNA CR mutations found in the control brains occur in functional elements. The increased mtDNA somatic mutation rate is systemic, since analysis of brain and blood mtDNAs from autopsy samples of the same individuals revealed that the somatic mtDNA mutations were elevated in both tissues. Furthermore, in some cases, the same somatic mtDNA control region mutation was found at high levels in brain and blood tissues of the same individual indicating that the mutation must have arisen early in development. Elevated somatic mtDNA mutations were also observed in lymphoblastoid cell lines of AD and DSAD derived cell line. Consistent with the accumulation of somatic mutations in association with dementia, the quantification of the ratio of brain ND6:ND2 mRNA levels revealed approximately a 50% reduction in the ND6 levels in AD, DS, and DSAD brains relative to controls. Moreover, the mtDNA:nDNA ratio was decreased in AD and DSAD brains relative to age matched controls, even though the mtDNA:nDNA ratio declined with age in normal brains. Interestingly, the mtDNA:nDNA ratio was increased in DS brains, suggesting a compensatory upregulation of mitochondrial biogenesis prior to the onset of dementia (1090,1091).

These data suggest that the accumulation of somatic mtDNA CR mutations in AD brains that damage key mtDNA regulatory elements may be a central feature in the etiology of the disease. The accumulation of these mutations with age would result in a progressive decline in mitochondrial energy production and an associated increase in mitochondrial ROS production. The increased ROS would ultimately lead to the activation of the mtPTP and the loss of neuronal synapses through mitochondrial-induced cytochrome c release and caspase activation.

By this logic, individuals harboring mtDNAs that generate tightly coupled mitochondria would experience chronically increased ROS production on a high-calorie diet. This would increase the probability of mtDNA CR mutations and thus the probability of developing AD. By contrast, individuals who harbor mtDNAs with uncoupling polymorphisms, such as haplogroup J, would have reduced mitochondrial ROS and be less prone to somatic mtDNA CR mutations and AD. Population studies have confirmed these predictions (425).

### 11.4.2 Parkinson Disease

About 20% of AD patient brains also show the neuro-pathologic features of PD (AD-PD). Idiopathic PD is a progressive movement disorder characterized by bradykinesia, rigidity, and tremor associated with the death of dopaminergic neurons in the substantia nigra. Clinical symptoms generally become apparent when ~80% of the dopaminergic neurons are lost. Neuropathologically, PD patients' neurons contain characteristic cellular inclusions known as Lewy bodies composed of  $\alpha$ -synuclein and ubiquitin. (1092–1094). The link between AD and PD is also supported by inclusion body myositis, which is associated with intermyofiber accumulations of A $\beta$ . Parkin is expressed in the brain, skeletal muscle, and other tissues, and muscle and cells lacking parkin are more sensitive to intracellular A $\beta$  toxicity. Increased levels of parkin are protective of both mitochondrial toxins and A $\beta$  (1095).

Genetically, PD is heterogeneous (1096) and classical twin studies have been unsuccessful at defining the extent of the hereditary component (1097). The first clear evidence that mitochondrial dysfunction might contribute to PD came from investigating the induction of PD by a contaminant of illicitly synthesized meperidine, MPTP (1098). MPTP is oxidized by monoamine oxidase B into its active form, 1-methyl-4-phenylpyridinium (MPP). MPP is actively taken up by the dopaminergic neurons of the substantia nigra through the dopamine transporter and electrostatically attracted into the mitochondrial matrix, like other lipophilic cationic compounds (1093,1099,1100). The selective concentration of MPP in dopaminergic neurons and their mitochondria increases the mitochondrial matrix concentration of MPP several hundredfold, into the millimolar range. At this concentration, MPP binds to complex I and inhibits respiration by NADH-linked substrates. The inhibition of complex I occurs between the highest potential iron-sulfur cluster and CoQ, the same site that binds rotenone (1093,1099,1100). The inhibition of complex I by MPP is thought to increase the complex I production of ROS, which is supported by the fact that MnSOD-deficient mice are more sensitive to MPTP (1101).

Chronic rotenone exposure has also been shown to induce parkinsonism in rats and to reproduce the anatomic, neuropathologic, and behavioral changes seen in PD (1102,1103). This is also associated with the induction of complex I ROS production, which can be blocked by treatment with the antioxidant  $\alpha$ -tocopherol (vitamin E) (1104). Finally, annonacin, a product of the tropical plant soursop, whose leaves are used to make a tea in Guadeloupe in the French West Indies, is a complex I inhibitor and is associated with an atypical form of PD (1105). Hence, environmental inhibition of mitochondrial complex I in the brain would appear to be one important cause of PD (1102).

The analysis of mitochondrial OXPHOS enzymes in the brain, blood, and muscle cells have also frequently

revealed complex I deficiencies in PD patients. Initial studies of the substantia nigra of autopsy patients revealed a 30–40% reduction in complex I activity (1106–1108). Skeletal muscle mitochondrial from PD patients has also been found to have mitochondrial complex I defects and in some cases other OXPHOS defects as well (1109–1111). Complex I deficiencies have also been reported in the blood platelets and isolated mitochondria from PD patients (1093,1112–1115). That some of the mitochondrial defects found in PD might have an mtDNA etiology has been suggested by cybrid experiments in which the patient's platelets are fused to cells that lack mtDNA and the cybrids biochemically characterized (1075,1116,1117). Hence, considerable data have accumulated indicating that PD patients harbor OXPHOS defects.

While the above studies implicated mitochondrial defects in PD, the fact that about 10% of PD cases showed a classical Mendelian mode of inheritance was originally taken as evidence that the mitochondrial defects were secondary to some other brain-specific nDNA genetic defect. This perspective has changed radically as the various nDNA PD genes have been cloned and their functions assessed. In the great majority of cases, the mutant nDNA gene has been found to affect mitochondrial function and integrity.

Both autosomal dominant and autosomal recessive nDNA gene mutations have been identified. The two dominant PD genes encode  $\alpha$ -synuclein (PARK1) and LRRK2 (PARK8). The  $\alpha$ -synuclein gene was the first Mendelian PD gene to be cloned (1118) and its deficiency shown to be associated with mitochondrial function (1119–1121). Inactivation of  $\alpha$ -synuclein results in a 28% reduction in the molar concentration of brain cardiolipin, reduction in the polyunsaturated side chain levels of mitochondrial cardiolipin, alteration in the mitochondrial inner membrane fluidity, and reduction in the electron flux rate between the mitochondrial respiratory complexes (1122). Recently,  $\alpha$ -synuclein has been shown to heal the distortions that arise in lipid bilayers when they are subjected to a high degree of bending, a common characteristic of mitochondrial inner and outer membranes. Since the disruption of the lipid bilayer creates the substrate for the fusion of two membranes, increased  $\alpha$ -synuclein decreases mitochondrial fission (1123).

The second autosomal dominant locus encodes the Leucine-rich repeat kinase 2 (LRRK2). Mutations in this gene account for about 1–2% of spontaneous cases. LRRK2 is a serine-threonine protein kinase as well as a GTPase, and the most common PD mutation causes a G2019S codon change. Patient fibroblasts harboring a mutant LRRK2 have reduced mitochondrial membrane potential; ATP production using complexes I, II, or IV-lined substrates; and increased mitochondrial elongation and interconnectivity (1124). Since the G2019S mutation increases the kinase activity and the  $\alpha$ -synuclein in Lewy Bodies is phosphorylated, it has also been

proposed that LRRK2 might also be the  $\alpha$ -synuclein kinase (1125).

Among the recessive Mendelian PD genes, four have been linked to the mitochondrion. The Omi/HtrA2 protein gene (PARK13) is a serine-protease which resides in the mitochondrial intermembrane space. When released from damaged mitochondria it can activate the caspase cascade of apoptosis. Its serine-protease activity is activated by phosphorylation by the PD-associated PINK1 kinase (352).

DJ-1 (PARK7) plays a role in protecting mitochondria from oxidative stress. ROS converts the DJ-1 cysteine 106 to sulfinic acid and this protects neurons from cell death (136) (1126). DJ-1 is associated with complex I defects, increased mitochondrial ROS production, reduced mitochondrial membrane potential, and altered mitochondrial morphology including reduced mitochondrial branching and disintegration of the mitochondrial network but with increased mitochondrial mass. These effects are associated with decreased autophagy levels and reduced lysosomal activity (1127,1128). The *Drosophila* homolog of DJ-1 is predominantly localized to the mitochondrion, and its genetic inactivation results in reduced locomotor function and increased sensitivity to paraquat-induced oxidative stress. The loss of the DJ-1 homolog in *Drosophila* also reduces mitochondrial ATP production in association with uncoupled mitochondria (1129,1130).

Studies of DJ-1-deficient mice have revealed that the reason that the SNc dopaminergic neurons die but the adjacent ventral tegmental neurons do not is due to differential mitochondrial oxidative stress in the SNc neurons. During repetitive pace-making neuronal discharge,  $\text{Ca}^{++}$  enters the SNc neurons through L-type channels. This results in oscillating mitochondrial oxidative stress as the mitochondria drive OXPHOS to maintain the membrane potential decreased by the uptake of cytosolic  $\text{Ca}^{++}$  into the mitochondrion. The increased mitochondrial ROS production is partially mitigated by a 20–30 mV decrease in the mitochondrial membrane potential achieved by increased expression of the UCPs, UCP4 and UCP5. DJ-1 is a redox sensitive participant in a signaling cascade activated by mitochondrial superoxide which helps to sustain the expression of UCP4 and UCP5. Therefore, loss of DJ-1 reduces the uncoupler protein levels, increases the SNc neuron oxidative stress, and predisposes these neurons to death. Since the ventral tegmental neurons do not undergo the  $\text{Ca}^{++}$  oscillations and concomitant oxidative stress, they are more resistant to the mitochondrial oxidative stress toxicity (1131).

The recessive parkin (PARK2) (1132) and PTEN-induced kinase 1 (PINK1, PARK6) (1133) protein genes are also related to the mitochondrion, since they have been found to be central components of the mitochondrial quality control system mediating the turnover of defective mitochondria. PINK1 is targeted to normal mitochondria but is cleaved and degraded. However,

in damaged mitochondria, it remains intact in the outer mitochondrial membrane (347) where it phosphorylates the cytosolic protein parkin. The phosphorylated parkin is attracted to the mitochondrion (348), and since it is an E3 ubiquitin ligase it ubiquitinates mitofusins 1 and 2 (349). The ubiquitinated mitofusins attract the adaptor protein p62/SQSTM1 (351) which recruits the mitophagy apparatus including microtubule-associated protein 1 light chain 3 (LC3) protein and the Atg autophagy proteins to engulf the mitochondria in an autophagosome which subsequently fuses with the lysosome to degrade the mitochondria. Mutations in PINK1, parkin, and the lysosomal ATPase, ATP13A2 (PARK9) can all inhibit this process (353). Since PINK1 is inactivated in normal mitochondria, but remains active in mitochondria with a reduced membrane potential, this system selectively removes defective mitochondria (352–354).

Mitochondria are continually undergoing fusion and fission within the cell (339). This permits the sharing of mtDNA gene products and thus the complementation in *trans* of mutant mtDNAs within the cell (341,344). However, as the load of mutated mtDNAs increases in the cell, the overall energetic capacity declines. At this point, it becomes essential to reduce the proportion of mutant mtDNAs. mtDNAs are packaged in nucleoids which appear to be composed of a clone of one type of mtDNA. Therefore, nucleoids with mutant mtDNAs need to be isolated in a surrounding mitochondrial membrane vesicle so that their defect can be recognized by PINK1 and removed by parkin and ATP13A2. This compartmentalization of mutant mtDNA nucleoids is accomplished by mitochondrial fission. Therefore, mitochondrial fission is essential for PINK1 and parkin activation (1134).

Since overexpression of  $\alpha$ -synuclein inhibits fission, overexpression or triplication of the  $\alpha$ -synuclein would result in long mitochondria which would inhibit the separation and removal of mutant mtDNAs. This effect can be only be compensated by the overexpression of PINK1, parkin, or DJ-1 (1123).

Thus, many of the chromosomal PD genes are part of an integrated pathway for maintaining the integrity of the mitochondria. This includes the mitochondrial fusion and fission system to isolate mutant nucleoids, the decline of OXPHOS and the mitochondrial membrane potential of the mitochondrial fragments harboring mutant mitochondria, the stabilization of PINK1 in the fragmented-mtDNA mutant mitochondria, the recruitment of parkin to damaged mitochondria, and the degradation of the defected mitochondrion and its mtDNA through mitophagy.

Consistent with this model, overexpression of the PINK1-parkin pathway in cultured cells harboring a heteroplasmic mtDNA mutation has been found to reduce the percentage of mutant mtDNAs. In cells heteroplasmic for a mtDNA complex IV COI, gene mutations (COXICA65) were studied at a high percentage mutant, the mitochondrial membrane potential declined to 45%

of normal and the PINK1-parkin pathway was activated to reduce the mutant mtDNA levels. By contrast, a cell line heteroplasmic for a cytochrome b mutation (Cytb3.0) with a 63% or normal mitochondrial membrane potential the PINK1-parkin mitophagy pathway was not activated and the mutant mtDNA load was not reduced. This suggests that the critical membrane potential threshold must be about 50% of normal. The rate and extent of removal of the COI mutant mtDNAs can be accelerated by inhibiting mitochondrial fusion with vMIA or blocking the ATP synthase from sustaining the inner membrane potential by hydrolyzing ATP (1134).

The demonstration that nDNA-encoded PD loci are involved in maintaining mtDNA and mitochondrial integrity implicate the accumulation of mtDNA damage and mutations in PD. Direct evidence that mtDNA mutations can influence the predisposition to PD has come from the report that a pedigree showing the maternal transmission of PD was found to harbor the common LHON pathogenic mutation MTND4\**LHON11778A* (1135), and the MTND6\**LDYT14459A* mutation has been repeatedly linked to generalized dystonia (537,538). Furthermore, the 12S rRNA np 1555 (*MTRNR1\*DEAF1555G*) mutation was observed in a family with sensorineural hearing loss, tremor, and Parkinsonism (1136). The mtDNA tRNAGln np 4336 (*MTTQ\*ADPD4336C*) variant has been found 5.3% of late-onset in PD and 6.8% in AD + PD versus 0.4% in controls (633) and in other studies this mutation has been found in 8.7% of PD cases but in 0 of 100 controls (749). Consistent with an mtDNA role in PD, haplogroup H has been associated with increased risk, and haplogroups J and Uk with decreased risk, for developing PD (424,1137,1138).

The accumulation of somatic mtDNA mutations has also been demonstrated in PD. The common 5 kb mtDNA deletion accumulates with age in many tissues, but it reaches the highest levels in the cerebral cortex and the basal ganglion of the brain, the tissues affected by AD and PD (452,453). Deletions in the mtDNA of dopaminergic neurons have also been observed in the SNc of patients and aged-controls. The mtDNA deletions have also been associated with neuronal OXPHOS enzyme defects by histochemical staining (1139,1140). Base substitution mutations in the mtDNA control region have also been found to be elevated in PD brains (1141).

The increase in somatic mtDNA mutation would appear to be systemic as has been found in AD. Analysis of blood platelet mitochondria has revealed mitochondrial complex I and complex IV defects (1112) and when platelets are fused to cultured human neuroblastoma cells lacking mtDNA ( $\rho^0$ ) mitochondrial defects are introduced. The resulting trans-mitochondrial cybrids have been found to harbor OXPHOS complex I defects, increased mitochondrial ROS production, altered mitochondrial  $\text{Ca}^{++}$  regulation, altered mitochondrial fission–fusion dynamics, Lewy Bodies, and altered neurological signaling (1013,1116,1117,1142–1145).



### 11.4.3 Huntington Disease

Studies on HD have implicated mitochondrial dysfunction in its etiology as well (412,1146–1148). HD is inherited as an autosomal dominant mutation resulting from the expansion of a CAG repeat in the first exon in the IT15 gene creating an extended polyglutamine (polyQ) stretch of amino acids of the huntingtin protein (Htt). Expansion of more than 36 repeats results in predilection to symptoms which is associated with the appearance of nuclear aggregates of mutant Htt complexed with ubiquitin (1149). The Htt protein is ubiquitously expressed, but the neurological symptoms correlate with the loss of  $\gamma$ -aminobutyric acid (GABA) medium spiny neurons of the striatum. The Htt contains 36 HEAT helix-loop-helix domains that mediate its interaction with multiple proteins involved in gene expression, endocytosis, vesicle trafficking, intracellular signaling, and metabolism. The age of onset can vary from 30 to 50 years and once initiated the disease progresses over the next 12–50 years leading to death (1146). The age of onset correlated with the length of the polyQ expansion, with accounts for about 73% of the variance in motor functions (1150).

While the precise function of the Htt protein remains unknown, considerable evidence has accumulated indicating that HD patients have a major defect in energy metabolism. Patients have elevated lactate in their basal ganglia and occipital cortex; reduced brain ATP; reduced phosphocreatine to phosphate ratios as assessed by  $^{31}\text{P}$ -NMR spectroscopy; reduced  $^{18}\text{F}$ -2-deoxyglucose ( $^{18}\text{F}$ -2-DOG) uptake in HD cortex and striatum by PET, increased sensitivity of the mtPTP to  $\text{Ca}^{++}$  activation possibly resulting for mutant Htt interaction with the ER InsP3R1  $\text{Ca}^{++}$  channel; reductions in OXPHOS complexes II and III in the caudate and putamen; and a 90% reduction in mitochondrial aconitase in the caudate, a 70% reduction in the putamen, and a 50% reduction in the cortex (1146,1151). HD patients also show a reduced anaerobic threshold during exercise stress tests (1152) and treatment of rodents with the mitochondrial complex I (SDH) inhibitor 3-nitropropionic acid (3-NP) mimics the features of the diseases (1153). Sensitization of the mtPTP and inactivation of the complexes II and III and mitochondrial aconitase suggest that mutant Htt increases mitochondrial oxidative stress since the mtPTP is sensitized by ROS (33,1154,1155) and complexes II and III and mitochondrial aconitase all incorporate iron-sulfur centers which are readily inactivated by ROS (460,1156–1158). This conclusion is supported by the demonstration that HD patients have increased levels of the lipid peroxidation product 4-hydroxy-2-nonenal (4-HNE) as do the striatum of the R6/2 mouse model of HD and treatment of R6/2 striatal neurons of R6/2 mice with the antioxidant nordihydroguaiaretic acid (NDGA) restored mitochondrial structure and function (1159). The importance of energy metabolism in HD has

been confirmed in a *Drosophila* in which the Htt mutant protein, Httex1pQ93, was expressed in glial and neuronal cells. When Httex1pQ93 was expressed in glia, the motor dysfunction of the flies could be improved and life span extended by the systemic expression of *Drosophila* UCP (DmUCP5). This protection was also obtained when human UCP2 was expressed in glial cells alone. However, DmUCP5 overexpression failed to protect the flies when Httex1pQ93 was expressed in neurons. By contrast when *Drosophila* MnSOD and mitochondrially targeted catalase (mCAT) were expressed in flies they showed protection for expression of Httex1pQ93 in neurons but not in glia. *Drosophila* in which Httex1pQ93 was overexpressed in glia could also be rescued by the overexpression of the *Drosophila* glucose transporter (DmGluT1) (1149). These data confirm that the motor symptoms in this *Drosophila* model system of HD are the results of defects in energy metabolism, primarily mediated by increased mitochondrial oxidative stress in neurons but by energy deficiency in the more glycolytic glia.

Abberations in mitochondrial morphology have also been documented in HD and linked to excess mitochondrial fission over fusion leading to mitochondrial fragmentation and the loss of mitochondrial cristae organization. HeLa cells transformed with an N-terminal fragment of Htt containing 17, 28, 74, and 138 polyQ were examined for mitochondrial function and mitochondrial morphology. In cells harboring the Htt fragment with 74 polyQ, the mitochondria were observed to be clustered around the nucleus and were of smaller length. Cells expressing Htt fragments with 74 and 138 repeats were found to be sensitized to oxidative stress (25 mM  $\text{H}_2\text{O}_2$ )-induced mitochondrial fragmentation with highly disorganized cristae. The 74Q Htt cells had 30% less ATP than cells expressing 28Q Htt. Cells with the 74 polyQ Htt had reduced mitochondrial movement, fusion, and ATP levels which could be compensated by addition of a dominant negative Drp-1 or normal Mfn2 protein. In a *Caenorhabditis elegans* expressing Htt28Q and Htt74Q in muscle, the longer repeat lengths were associated with reduced worm mobility which could be offset by knockdown of Drp-1 (1160). The relevance of these in vitro to HD patients was demonstrated by the analysis of mitochondrial fission and fusion, mRNA, and protein levels in HD brains versus control brains. This revealed that the mRNA levels for the mitochondrial fission proteins Drp1 and Fis1 were increased while those of the mitochondrial fusion proteins, Mfn1, Mfn2, and Opa1 were decreased. Cyclophilin D (CypD) was upregulated in HD brains and the level of CypD mRNA increased as the disease progressed. Drp1 was increased by 2.8- to 10.9-fold and Fis1 6.9- to 36.2-fold while Opa1 mRNA was reduced by 2.6- to 4.3-fold, Mfn1 2.5- to 4.0-fold while Mfn2 mRNA was reduced 3.4- to 50-fold. CypD mRNA was increased by 9.8- to 50-fold. These changes were reflected in relative levels of protein. OXPHOS mRNAs were increased about twofold along



with increased levels of cytochrome b and COI in HD brains. Mutant Htt oligomers were found to be associated with both the nucleus and the mitochondria (1161).

The anatomical relevance of this to HD mitochondrial morphology was confirmed by analysis of the mitochondrial morphology of lymphoblastoid cell lines derived from HD patients. Lymphoblastoid lines heterozygous for a 48Q allele had striking abnormalities in mitochondrial ultrastructure including mitochondrial fragmentation and clustering with deranged mitochondrial cristae. The prop-fission protein, Drp-1 is normally phosphorylated and inactive in mitochondrial fission. However, when dephosphorylated by calcineurin, Drp-1 binds to the mitochondrion and initiated fission. The calcineurin phosphatase is activated by  $\text{Ca}^{++}$  which has been observed to be elevated in HD tissues. Calcineurin activity was also found to be elevated in cells with 111Q Htt in association with increased releasable ER stores of  $\text{Ca}^{++}$ . Mitochondrial fragmentation could be corrected by increased expression of OpaI, Mfn1, or dominant-negative Drp-1. HD lymphoblasts as well as striatal neurons from the YAC128 mouse model of HD were more sensitive to intrinsic mitochondrial cell death initiated by staurosporine,  $\text{H}_2\text{O}_2$ , and etoposide. This was associated with a greater tendency of the polyQ cell mitochondria to release cytochrome c which correlates with the disruption of the OpaI oligomers that are responsible for maintaining the cristae junctions and is consistent with cristae remodeling being activated by Drp-1. Calcineurin inhibition by FK506 prevented translocation of Drp-1 to the mitochondrion and protected polyQ cells from cell death. Expression of OpaI protected the mitochondrial cristae from disruption by pro-apoptotic stimuli (1162).

These observations lead to a scenario of how mitochondrial functional morphology might be modulated in HD. Mutant Htt interacts with the plasma membrane  $\text{Ca}^{++}$  channels VGCC and NMDAR to increase  $\text{Ca}^{++}$  influx into the cell and with the InsP3R1 receptors in the ER to release its  $\text{Ca}^{++}$  stores (1146,1151). The increased cytosolic  $\text{Ca}^{++}$  then activates the calcineurin phosphatase to dephosphorylate phospho-Drp-1. The dephosphorylated Drp-1 then migrates to the mitochondrion where it initiates fragmentation. This destabilized the OpaI cristae junction structures and increases the sensitivity of the mitochondria to stress-induced cytochrome c release thus increasing the predilection of the striatal neurons to undergo apoptosis (1163). Hence, according to this model, the tendency of mutant Htt causes mitochondrial dysfunction through altered mitochondrial dynamics.

Another model of how the polyQ expansions of mutant Htt impinged on mitochondrial function is through transcriptional regulation of the nDNA-encoded mitochondrial genes (1146). This follows from the discovery that the mutant Htt protein interferes with the expression of PGC-1 $\alpha$ , the nDNA-encoded mitochondrial gene transcriptional coactivator. Initially, it was observed that HD model R6/2 mice were cold intolerant

and since adaptation to cold stress in the mouse is mediated by PGC-1 $\alpha$  induction of UCP1 in brown fat, this implied a defect in PGC-1 $\alpha$ . This was confirmed by showing a reduction in PGC-1 $\alpha$ mRNA as well as the reduction in the mRNAs of multiple mitochondrial PGC-1 $\alpha$  target genes. It was corroborated by demonstrating reduced respiration on site I substrates and reduced membrane potential, defects that could be ameliorated by overexpression of PGC-1 $\alpha$  (1164). Analysis of muscle of HD patients confirmed the systemic reduction in PGC-1 $\alpha$  and revealed reduction of PGC-1 $\beta$ , oxygen consumption, and oxidative fibers. In HD myoblasts, knockdown of mutant Htt increased PGC-1 $\alpha$  expression. Reduction in the muscle expression of PGC-1 $\alpha$  and its target genes was also confirmed in HD transgenic mice (1165). In parallel studies, PGC-1 $\alpha$  expression was found to be reduced 30% in the caudate of HD patients, though not in the hippocampus or cerebellum. Moreover, expression was reduced 5.6-fold in the medium spiny neurons but increased 47-fold in the interneurons. In striatal neuronal cultures of STHdh<sup>Q111</sup> mice, cAMP, ATP, cytochrome c, and COXIV levels were decreased and PGC-1 $\alpha$  mRNA was reduced 10-fold. These effects could be partially reversed with the cAMP analog 8-bromo-cAMP, PGC-1 $\alpha$ , or by increased levels of TAF4 plus CREB. In R6/2 mouse brains, PGC-1 $\alpha$  mRNA was reduced. Moreover, mutant Htt was found associated with the CREB/TAF4 complex on the PGC-1 $\alpha$  promoter, which contrasts with normal Htt's association with the dopamine D2 receptor gene. Striatal necrosis associated with mutant Htt expression could also be ameliorated by Lentivirus transduction of PGC-1 $\alpha$  (1166). Mutant Htt has been found to interact with the cAMP responsive element (CRE) binding protein (CREB) through interaction with the CREB-binding protein (CBP) and with the TAT box-binding protein (TBP)-associated factor TAF4/TAFII130. Since this complex regulates the expression of PGC-1 $\alpha$ , this could be the mechanism for inhibition of PGC-1 $\alpha$  by mutant Htt (1151,1167–1169).

Transglutaminase 2 (TG2), which is the predominant transglutaminase in the brain, has also been proposed to play an important role in the pathophysiology of HD. TG2 has multiple functions, though the primary enzymatic function is the  $\text{Ca}^{++}$ -activated transglutaminase reaction in which a peptide-bound glutamine forms a thioester intermediate with a TG2 cysteine which then reacts with a suitable nucleophile such as a lysine within another polypeptide to form glutamate  $\epsilon$ ( $\gamma$ -glutamyl) lysine cross-links. TG2 can also mediate the reaction of polyamines to proteins. TG2 is important in forming cross-links for extracellular matrix proteins but has also been found to translocate to the nucleus under certain conditions. In the absence of  $\text{Ca}^{++}$  TG2 becomes a GTPase (1170).

While TG2 levels do not increase in the presence of mutant Htt, the presence of increased polyQ tracks stimulates TG2 cross-linking activity. Furthermore,

increased cytosolic  $\text{Ca}^{++}$ , in part due to the mHtt activation of NMDA receptors should stimulate the activity of TG2. Genetic inactivation of TG2 in R6/2 mice provides substantial protection against cortical and striatal brain neuron death, a 12% increased in life span, and a significant increase in motor activity. Interestingly, reduction of TG2 increases the extent of neuronal internuclear inclusions supporting the idea that these aggregates are protective rather than deleterious (1171).

In striatal neurons cultured from HdhQ111 knock-in mice, STHdh<sup>Q111</sup> cells, expression array studies have revealed that 461 genes are dysregulated, 289 upregulated, and 172 downregulated. The TG2 protein has two nuclear localization signals and treatment of STHdh<sup>Q111</sup> cells with the TG2 inhibitor peptide inhibitor, Z-QVPL (ZDON) returned 42% of the dysregulated genes toward normal suggesting that TG2 has a role in transcriptional regulation. Direct analysis of the PGC-1 $\alpha$  and cytc mRNAs in mutant cells confirmed that PGC-1 $\alpha$  mRNA was reduced 80% and that cytc mRNA was reduced 40% but inhibition of TG2 increased PGC-1 $\alpha$  mRNA 3- to 4.5-fold and cytc mRNA 3-fold. Transformation of the cells with a cytc promoter luciferase reporter construct confirmed that mHtt decreased transcription and that inhibition of TG2 increased promoter activity 250%. Using chromatin immunoprecipitation (ChIP) it was found that STHdh<sup>Q111</sup> cells had 10X more TG2 at the cytc promoter than did control STHdh<sup>Q7</sup> cells. Similarly, TG2 was 5X increased within the PGC-1 $\alpha$  gene at the boundary region of intron 2 and exon 3, and the TG2 inhibitor, ZDON, reduced the TG2 occupancy of this site back to that of STHdh<sup>Q7</sup> cells. Consistent with the inhibition of PGC-1 $\alpha$ , the total mitochondrial mass, citrate synthetase activity, ND6 protein level, and mitochondrial membrane potential were all decreased in STHdh<sup>Q111</sup> cells and these reductions were partially restored by treating the cells with ZDON. Another mRNA that was downregulated in the STHdh<sup>Q111</sup> cells was the HSP40 chaperone family member DNAJB10 which was also normalized by ZDON. While HDAC inhibitors have been shown to partially reverse the transcriptional inhibition associated with HD including the upregulation of PGC-1 $\alpha$ , TG2 regulation proved to be independent of the HDACs, though a clear interaction has been seen between TG3 and histone 3 (H3) (1172,1173).

The N-terminal amino acid tails of histones are positively charged and bind to the negatively charged sugar-phosphate backbone of DNA shutting down transcription. Modification of the histone tails by phosphorylation using ATP, acetylation using acetyl-CoA, of methylation by S-adenosylmethionine (SAM), all of which are reliant on energy flux through the mitochondria, results in the repulsion of the tails and the opening of the chromatin to transcription; thus when there is sufficient energy to support biogenesis the epigenome is modified and turns on nDNA gene expression (13). The observation that TG2 regulates transcription has

led to the hypothesis that TG2 becomes activated by mHtt which modulates particular transcriptional circuits including those of transcription factors that incorporate glutamine-rich activation domains including Sp1, CREB, and NRF-1. These, in turn, regulate the transcription of nDNA-encoded mitochondrial genes. This effect seems to be enhanced by the polyQ expansion of mutant Htt. Activated TG2 reacts with glutamines 5 and 19 in H3 and perhaps glutamine 22 in histone 2B to attach a polyamine. The increased positive charge increases the affinity of the histone tails to the DNA thus inhibiting transcription of the target genes, one of which is PGC-1 $\alpha$ . This, in turn, downregulates mitochondrial biogenesis and probably the distribution of calorie flow from glycolysis and fatty acid oxidation into the mitochondrion. Reduced mitochondrial energy flux renders the GABAergic medium spiny neurons less resistant to stress ultimately leading to their death (1173).

Consistent with an important role of mitochondrial genes in HD, in a German cohort mtDNA haplogroups were found to contribute to the age of onset of HD. Patients harboring mtDNA haplogroup H, as assessed by the COI SNP nt C7028T, were found to have a significantly lower age of onset and higher ATP levels in PHA-stimulated CD4<sup>+</sup> cells (1174). HD patient age of onset was also associated with a polymorphism in intron 2 of PGC-1 $\alpha$  (1175) as well as polymorphisms in nuclear respiratory factor 1 gene (NRF-1) and in mtDNA transcription factor A gene (TFAM) (1150). The combination of the mtDNA haplogroup H polymorphism plus the PPARGC1A, NRF1, and TFAM polymorphisms have been proposed to account for about 4.8% of the age of onset variance (1150). Given that mtDNA and nDNA mitochondrial genetic variation can modulate the age of onset of HD, it follows that the delayed onset and progressive course of the disease should be associated with an elevated mtDNA somatic mutation rate. Analysis of the level of the common mtDNA 5 kb deletion in HD patient's cerebral cortex has confirmed that somatic mtDNA mutations are indeed elevated (1176).

The importance of somatic mtDNA mutations in HD have been confirmed by generating cybrids via fusion of patient blood platelets from HD patients to cultured human cells which lack mtDNA ( $\rho^0$ ). The resulting cybrids have been found to have defects in glycolytic and mitochondrial function (1177). Since the inherited defect in HD is a nuclear trinucleotide repeat mutation and platelets lack nDNA, the only way that these observations can be connected is if the nDNA HD mutation caused secondary mutations in the blood cell mtDNAs which were then transferred to somatic cell cybrids.

Since in AD somatic mtDNA mutations are elevated in both brain and blood cells (1091) and somatic mtDNA mutations are elevated in AD, PD, and HD, it seems likely that the pathophysiology and genetics of all three of these diseases has the same basis of mitochondrial dysfunction.

### 11.4.4 Amyotrophic Lateral Sclerosis

ALS is a fatal neurodegenerative disease associated with upper and lower motor neuron dysfunction. It typically manifests between ages 50 and 60 years, resulting in paralysis and death within 2–5 years. Neuropathological studies reveal pallor of the corticospinal tract due to loss of motor neurons and the presence of ubiquitin-positive inclusion within the surviving motor neurons and the presence of TDP-43 positive aggregates containing the TAR-DNA binding protein (TARDBP or TDP-43). Approximately 5% of ALS is familial and of these, about 15–20% of the cases are caused by mutations in the Cu/Zn superoxide dismutase (Cu/ZnSOD) gene, *Sod1*. A significant proportion of the remaining familial cases are the result of mutations in the TARDBP, *FUS* (fused in sarcoma, a RNA-binding protein), *OPTN* (optineun), vesicle-associated membrane protein B, valosin-containing protein (VCP) gene, and C9ORF72 genes (1178–1182).

The most extensive studies on the pathophysiology of ALS have been conducted on ALS-associated mutations in the *SOD1* gene. Transgenic mice that express a human mutant Cu/ZnSOD develop motor neuron disease associated with vacuolated mitochondria containing high concentrations of Cu/ZnSOD (1183). The mutant Cu/ZnSOD mice also accumulate 8-OHdG in cytoplasmic granules, possibly representing mtDNA nucleoids (1184), and in cultured neuroblastoma cells, the toxicity of a mutant Cu/ZnSOD can be ameliorated by the increased expression of MnSOD (1185). ALS linked mutations in *Sod1* can either inactivate or leave active the enzymatic activity but all result in increased misfolding of the protein. The mitochondrial uptake of the three common familial ALS Cu/ZnSOD mutants (G37R, G41D, and G93A) is blocked by the heat shock proteins Hsp70, Hsp27, and Hsp25 (1186). Analysis of the spinal cord mitochondria from transgenic rats harboring the *Sod1*<sup>G93A</sup> mutation revealed the association of the misfolded protein to the cytoplasmic face of the mitochondria in association with an increase in the levels of 33 mitochondrial proteins and a decrease in 21 proteins, the proteins of complex I being particularly affected, in association with a 30% reduction in mitochondrial protein import (1187). In addition, binding of misfolded SOD1 was preferentially associated with VDAC1 resulting in reduced conductance for these outer mitochondrial membrane channels and association with reduced ADP transport across the outer membrane (1179). Mutant SOD1 is also associated with altered mitochondrial morphology in motor neurons. This involved the decline of the cylindrical network morphology of the mitochondria and their substitution by more spherical forms (1188). Hence, studies of the familial ALS SOD1 mutants strongly implicated mitochondrial dysfunction in the etiology of ALS.

While ALS has traditionally been viewed as a specific motor neuron neurodegenerative disease, identification

of additional ALS-associated genes has revealed that gene mutations that cause a variety of other tissue defects can also cause ALS. This implies that systemic cellular defects can result in motor neuron-specific disease. Mutations in the VCP gene had previously been shown to cause inclusion body myopathy (IBM) with Paget's disease of the bone (PDB) and frontotemporal dementia (FTD) or IBMPFD and more recently PD (1189). The IBMPFD knock-in mouse shows progressive vacuolization of mutant muscle myofibrils, centrally located nuclei, cytoplasmic accumulation of TDP-43, ubiquitin-positive inclusion bodies in quadriceps myofibrils and brain, increased number of autophagosomes suggesting impaired autophagy, and increased apoptosis as indicated by elevated caspase-3 activity and increased TUNEL-positive nuclei (1190). Since mutations in the VCP gene have now been found in 1–2% of familial ALS patients (1180,1181), this directly links mitochondrial dysfunction to ALS.

An expanded hexanucleotide repeat (GGGGCC) in the noncoding region of the functionally unknown open reading frame (C9ORF72) at chromosome 9p21 has also been linked to ALS and found to account for 46% of familial ALS and 21% of sporadic ALS in the Finnish population and 24% of familial cases in other populations. The normal allele has between 2 and 23 repeats while the mutant alleles may be in the range of 700–1600 repeats. While antibodies are still poorly characterized, one study suggests that the C9ORF72 protein has a predominantly cytoplasmic and synaptic localization. In addition to accounting for a significant proportion of familial ALS cases, expansion of the C9ORF72 hexanucleotide repeat has also been found in 12% FTD (1178,1182,1191). Hence, the C9ORF72 hexanucleotide repeat mutation, like the VCP mutations, must have a multi-tissue function, impairment of which can cause motor neuron dysfunction.

### 11.4.5 Autism Spectrum Disorders

Autism and autism spectrum disorders (ASDs) are defined as neurobehavioral syndromes associated with deficits in social interaction, impaired communication skills, and repetitive stereotypic behaviors (1192–1194). Neuropathological studies demonstrate that ASD patients have alterations in the synaptic and dendritic organization of the brain (1195).

The prevalence of autism and ASD is currently estimated at 6.7 per 1000 with a male to female ratio of 4:1. Chromosomal mapping studies have identified autism susceptibility loci at 1p, 2q, 5q, 7q, 15q, 16q, 17q, 19p, and Xq, but no one locus has been found to make a predominate contribution. While monozygotic twins are 70% concordant for autism and 90% concordant for ASD, dizygotic twins are only 5% concordant for autism and 10% concordant for ASD (1192,1196–1200). If autism were due to simple Mendelian variation,



we might expect 40–45% of dizygotic twins to be concordant for ASD. That this is not the case suggests that autism involves the interaction of a number of interrelated genetic factors.

ASDs can either be idiopathic or syndromic. Syndromes associated with a predilection to autism include RS, tuberous sclerosis, neurofibromatosis, Angelman syndrome (AS), fragile X syndrome, and Timothy syndrome.

**11.4.5.1 Autism and Chromosomal Genetics.** Chromosomal linkage and candidate gene studies have identified a number of gene loci associated with autism which are important in the structure of the dendrite and synapse. These include the neuroligins (NLGL1-5), neurexins (NRXN1-3), contactins, and contactin-associated proteins (CNTNAP2), and the SHANK3 protein (1196,1201–1205).

Neuroligin interacts with the scaffolding protein, PSD-95, which is associated with glutamate receptor expression. Accordingly, some autistic cases have been linked to glutamate 6 receptor (GRIK2) mutations (1206), and GRIK2 is deleted in a mental retardation family (1207). Autism cases have also been linked to single nucleotide polymorphisms (SNPs) surrounding the glutamate receptor ionotropic NMDA 2A gene (GRIN2A) and the 4-aminoglutarate transferase (ABAT) gene of GABA metabolism (1208).

Homozygosity mapping in inbred families has revealed a number of autism susceptibility loci. Homozygous deletions were observed in the velocardiofacial syndrome region, and 6.4% (5/78) of probands from consanguineous families have been found to harbor homozygous deletions ranging from 18 kb to >880 kb (1209–1211). The association of autism with regional chromosomal copy number variants (CNVs) has been greatly extended using comparative genomic hybridization (CGH). Using an oligonucleotide array with probes separated by 35 kb, Wigler and collaborators examined 118 sporadic cases, 77 multiplex families, and 196 controls. Among the sporadic autism patients, 10% (12/118) were found to have CNVs; in the multiplex families, 3% (2/77) had CNVs; and among the controls only 1% (2/196) had CNVs (1199). These investigators hypothesized that there are two genetic origins for autism CNVs. The majority of CNV cases are de novo mutations affecting only one patient. However, the minority are inherited. Wigler then hypothesized that women may be less prone to be affected, thus accounting for the male bias, though no rationale was given for his assumption (1199,1200). What is clear is that autism can result from a plethora of different gene mutations and thus is oligogenic. Some autism mutations affect structural elements of synapses, excitatory neuronal receptors, and ion channels. However, mutations in Mendelian structural genes for synapse formation cannot explain the plethora of autism susceptibility loci, the discrepancy in concordance between monozygotic and dizygotic twins, or the strong male bias among patients.

#### 11.4.5.2 Autism and Mitochondrial Dysfunction.

Alterations in mitochondrial structure and function have been repeatedly observed in autistic patients (1192,1212,1213). One nDNA-encoded mitochondrial transport enzyme that has been associated with autism is the mitochondrial inner membrane Ca-regulated aspartate/glutamate carrier (AGC). The human genome harbors two AGCs; AGC1 or aralar1 encoded by the SLC25A12 gene on chromosome 2q24, and AGC2 or citrin encoded by the SLC25A13 gene on 7q21.3. AGC1 is expressed in brain, heart, and skeletal muscle and is the only isoform expressed in neurons. AGC2 is the predominant isoform in liver and kidney and is expressed in glia along with AGC1. Both AGCs have four EF-hand  $\text{Ca}^{++}$  binding motifs and are activated by cytosolic  $\text{Ca}^{++}$ .

Initially, ASG1 was linked to autism through two SLC25A12 SNPs (1198) with the higher activity alleles associated with autism (1197). More recently, a three-fold increased AGC1 activity has been reported in the brains of autism patients in association with a 2.7-fold elevation in brain  $\text{Ca}^{++}$  levels (1214).

Alteration in brain AGC1 activity should affect the transport of aspartate into the cytosol. Aspartate is the substrate for N-acetyl-aspartate (NAA), a precursor for myelin. SLC25A12-deficient mice show impaired myelination (1215). However, the AGCs also function in conjunction with the malate/ $\alpha$ -ketoglutarate carrier (MaKC) to permit the exchange across the mitochondrial inner membrane of mitochondrial and cytosolic reducing equivalents ( $\text{NADH} + \text{H}^+$ ). Upregulation of AGC1 would increase the flux of cytosolic  $\text{NADH} + \text{H}^+$  into the mitochondrion, funneling extra reducing equivalents (electrons) into the mitochondrial ETC. This would transiently increase respiration and ATP production, but ultimately overload the ETC, resulting in increased production of toxic ROS and loss of synaptic integrity (1214). Consistent with this scenario, when expression of AGC1 is enhanced in cultured neurons, neurite outgrowth is initially enhanced, but the neurites subsequently died off (1197).

Since cytosolic  $\text{Ca}^{++}$  levels are buffered by the mitochondrial energy-linked uptake of  $\text{Ca}^{++}$ , a mild defect in mitochondrial energy production could result in a chronically elevated brain  $\text{Ca}^{++}$  level. The elevated cytosolic  $\text{Ca}^{++}$  would activate AGC1, increase ROS production, activate the mtPTP, and destroy dendritic connections. Consistent with there being a chronic mitochondrial energy defect, cytochrome c oxidase (COX, complex IV) activity is elevated in autistic brains, a common compensatory response to OXPHOS defects (33,314,1214). Mitochondria from autistic brains also show elevated oxidative damage (1214).

Energetic dysfunction in autistic brains has been detected by functional MRI (1216) and metabolites associated with both methylation capacity (plasma methionine and S-adenosylmethionine/S-adenosylhomocysteine) and antioxidant pathways (plasma cysteine



and glutathione and reduced/oxidized glutathione) are reduced in autistic patients (1217). Screening of autistic patient blood and urine has revealed that free and total carnitines were reduced in blood of a quarter to a third of subjects; urine pyruvate levels were elevated in 4/5 of cases, plasma alanine was elevated in 4/5 of cases, and ammonemia in 3/4 of cases (1218). In another study, 8% of ASD subjects were found to have evidence of mitochondrial dysfunction, including elevated plasma lactate and urine lactate and organic acids (1219). Similarly, plasma lactate was found elevated in 17% of cases, lactate:pyruvate ratios were elevated in 26%, and muscle mitochondrial OXPHOS defects were found in 23% (7/30) of cases (1220). In a Portuguese study, plasma lactates were elevated in 20% of autistic cases, the lactate to pyruvate ratios were increased in 85% of cases, and muscle mitochondrial defects were found in 55% (6/11) of cases (1221). One child that experienced autistic regression following immunization was reported to have an underlying muscle OXPHOS complex I and complex III defect (1222). Finally, in lymphoblastoid cell lines from autism patients and controls, OXPHOS complex I respiration was found to be reduced 40–50 (1223).

Three to five percent of autistic patients harbor an inverted duplication of the chromosome 15q11-q13 region. These patients have elevated urine lactate and serum lactate, pyruvate, and alanine, and their muscle mitochondrial complex III is reduced in association with mitochondrial hyperproliferation (1212,1224).

One autism locus located to chromosome 7q31 was found to have reductions in two candidate genes, IMMP2L and DOCK4. The IMMP2L or IMP2-LIKE (MIM 612323) gene is a component of the mitochondrial inner membrane peptidase (IMP) complex that generates mature, active, mitochondrial proteins by proteolytically removing the targeting presequence of nDNA-encoded proteins. Mutations in this gene were also found in a Tourette patient (1225).

A subset of autistic patients have also been reported to harbor mtDNA mutations. One family harbored a heteroplasmic tRNA<sup>Lys</sup> nt G8363A mutation (1226). Of 12 children who presented with hypotonia, epilepsy, autism, and developmental delay (HEADD), three quarters were found to have abnormal mitochondria, 7/8 OXPHOS defects, and five elevated levels of large mtDNA deletions (1227). Three pedigrees with autism patients were found to harbor the pathogenic tRNA<sup>Leu(UUR)</sup> nt A3243G mutation and the proband of one pedigree was found to have a 75% depletion in muscle mtDNAs (1228).

Autistic symptoms can occur in RS, tuberous sclerosis complex (TSC), neurofibromatosis-1 (NF1), AS, and fragile X mental retardation (FMR), and Timothy syndrome. RS is caused by mutations in the nDNA methyl CpG binding protein (MeCP2) gene (MeCP2) (1229–1231). These patients have been reported to harbor mitochondrial structure aberrations and reductions in muscle OXPHOS complexes I, III, and IV. Mice lacking MeCP2

have increased complex III and core protein 1 gene (Ugcr1) expression, together with reduced OXPHOS coupling (1232–1234).

TSC is a cell overgrowth disorder caused by mutations in either the hamartin TSC1 gene or the tuberin TSC2 gene. Twenty-five percent to 50% of TSC cases exhibit ASD. TSC1 + 2, act through Rheb to regulate TORC1, and TOR has been implicated in regulation of mitochondrial OXPHOS (1235,1236) (Figure 11-1).

NF1 is another overgrowth syndrome associated with ASD (1237). The mtDNAs of NF1 neurofibromas have been found to contain somatic control region mutations (1238) and NF1 has been shown to regulate *Drosophila* mitochondrial ROS production and longevity through a cAMP mediated mechanism (1155).

AS and Prader–Willie syndrome (PWS) result from alterations in the imprinted loci on chromosome 15q11-13, AS resulting from a maternal deficiency and PWS from paternal deficiency. Current evidence suggests that loss of expression of UBE3A is responsible for the AS phenotypes and of SNRPN for the PWS phenotype (1239). We have found that mice lacking UBE3A harbor small and dense hippocampal mitochondria and have reduced OXPHOS complexes II + III (1240).

The FMR protein (FMRP) has recently been shown to be required for the expression of the mitochondrial and cytosolic Cu/Zn superoxide dismutase (SOD1) (1241). SOD1 and MnSOD (SOD2) function to eliminate mitochondrial superoxide generated on the outside and inside of the mitochondrial inner membrane, respectively (1).

Timothy syndrome presents with Long QT syndrome and autism, and has been linked to mutations in the T-type voltage-gated Ca<sub>v</sub>1.2 channel encoded by the CACNA1C gene, most commonly involving the G406R allele (1242). Ca<sub>v</sub>1.2 shows its highest expression in the hippocampus, amygdale, and putamen. A survey of the CACNA1H gene, a close paralog of the Timothy syndrome channel, in 461 autism DNAs revealed six new mutations. Functional studies of two of these, R212C and R902W, revealed extended Ca<sup>++</sup> influx into the cytosol (1243). Cytosolic Ca<sup>++</sup> levels are regulated by mitochondrial Ca<sup>++</sup> uptake, driven by the mitochondrial inner membrane potential ( $\Delta\psi$ ). Excessive Ca<sup>++</sup> uptake and chronically increased oxidative stress can activate the mtPTP and initiate cellular destruction. Therefore, defects in a variety of systems that alter Ca<sup>++</sup> can lead to neuronal impairment.

**11.4.5.3 Mitochondria and Synaptogenesis.** Mitochondria play a central role in neurite outgrowth, axonal polarity, and synaptic plasticity (1244–1247). In Purkinje cells, the mitochondrial anti-apoptosis protein, Bcl-W, interacts with the glutamate receptor  $\delta$  (Grid2). These regulate Ca<sup>++</sup> flux into the cytosol and mitochondria, mitochondrial fission, function, autophagy, and neuronal structure. Inactivating Bcl-2 and Grid2 in mice inhibits mitochondrial fission, blocking the movement of mitochondria into dendritic spines (1248). Therefore,

mitochondrial dysfunction has been implicated in both non-syndromic and syndromic ASD.

**11.4.5.4 Mitochondrial Physiology and Autism.** Mitochondria generate much of the ROS in cells. Of these,  $H_2O_2$  is the most stable and an important second messenger. It can diffuse out of the mitochondrion and into the nucleus-cytosol where it can act on a broad spectrum of kinases and redox sensitive proteins that regulate cell growth, differentiation, inflammation, and death (Figure 11-4). Mitochondrial biogenesis is also regulated by the availability of reducing equivalents in the form of glucose via insulin, the insulin receptor, PI3K, Akt (PKB), and the FOXO pathway and by the glucagon, cAMP, phosphor-CREB pathway. Both the FOXOs and CREB regulate PGC-1 $\alpha$  which induces the expression of OXPHOS genes (1,2) (Figure 11-4). The FOXOs and PGC-1 $\alpha$  are also regulated by acetylation, controlled by SIRT1 and NAD<sup>+</sup> (Figure 11-4). SIRT3 also regulates OXPHOS enzymes activity through acetylation (1249,1250).

The mitochondrial redox status and ROS production directly activate NF- $\kappa$ B which upregulates the inflammatory cytokine genes thus generating inflammation. Hence, dysregulation of AGC in autistic brains would increase ROS, activate NF- $\kappa$ B, and induce inflammation. Bacterial lipopolysaccharide (LPS) stimulates the liver to release the pro-inflammatory cytokines, TNF $\alpha$  and IL-1 $\beta$ . TNF $\alpha$  stimulates oxidation of mitochondrial thioredoxin 2 (Trx2) but not cytosolic Trx1, resulting in the elevation of cellular ROS within an hour of exposure (1251). The increased ROS induces the transcription of HIF-1 $\alpha$ , activates Akt, and thus mTOR, and mTOR increases the protein synthesis of HIF-1 $\alpha$ . HIF-1 then increases inflammatory gene expression. Autistic children have altered antigenic responses (1252,1253), altered immune cell function, and increased pro-inflammatory cytokine levels (TNF- $\alpha$ , interferon (IFN)- $\gamma$ , interleukin (IL)-1 $\beta$ , IL-6, and IL-12) (1252,1254–1265). Therefore, it follows that if autistic children have partial mitochondrial defects, then infections and other immunological challenges could alter their precarious redox balance, precipitating dendritic and synaptic spine withdrawal, and autistic regression. Environmental challenges could also trigger for Ca<sup>++</sup> signaling through the IP3 receptors (1266) which could stimulate the mitochondrial tricarboxylic acid cycle and OXPHOS and ultimately lead to Ca<sup>++</sup> overload and apoptosis (Figure 11-4).

**11.4.5.5 Mitochondrial Explanations of Autism.** Therefore, some of the complexities of autism might then be understood by hypothesizing that mitochondrial defects are a common contribution to autism. Mild mitochondrial defects would preferentially affect the brain, due to its high energy demand and the importance of neuronal Ca<sup>++</sup> regulation. The large number of mitochondrial genes means that many different gene alterations as well as environmental toxins can perturb mitochondrial homeostasis. Since mild mitochondrial diseases such as LHON show a strong male bias in penetrance

(1267–1271), mild mitochondrial defects causing autism would also be expected to show a strong male bias. The low concordance in fraternal twins and the extreme variability in penetrance of autism genes could be the result of variable interactions between polymorphisms in the hundreds of nDNA- and mtDNA-encoded mitochondrial genes. The variability in clinical courses of autistic patients is characteristic of mitochondrial disease, and reflects the many different types of mitochondrial genes and variable severity of their defects. The loss of milestones associated with regressive autism in response to febrile events is also seen in LS and is consistent with the mitochondrial regulation of Ca<sup>++</sup>, ROS, and redox state and their effects on gene regulation and inflammation. Finally, the mild mitochondrial defects such as those proposed to be associated with autism are often too subtle to be detected by current mitochondrial diagnostic tools, explaining why mitochondrial defects have not been consistently found in ASD patients using current mitochondrial diagnostic tools.

## 11.4.6 Psychiatric Disorders

There is growing evidence that psychiatric disorders may have an mtDNA component. An extensive genetic epidemiologic analysis of bipolar affective disorder (BPAD) pedigrees revealed that there was a higher than expected frequency of affected mothers of probands, a 2.3- to 2.8-fold increased risk of illness for maternal relatives, and a 1.3- to 2.5-fold increased risk of illness for offspring of affected mothers. Moreover, 10 pedigrees were observed in which disease transmission was almost exclusively matrilineal, though sequencing of the mtDNA from these pedigrees did not reveal a common mtDNA mutation to which BPAD could be ascribed (427,1272). Patients with PEO associated with multiple deletion syndrome have been reported to have affective disorders including depression, apathy, fatigue, and insomnia (1273), and severe psychiatric disturbance principally involving depression and irritability was observed in several members of the large LHON plus neurologic disease pedigree (577) subsequently found to harbor the MTND6\*LHON14484C and MTND1\*LHON4160C mutations (543). Analysis of the acidification of brains at autopsy has revealed a significant association between increased brain pH and haplogroups (U and Uk). There are suggestions of haplogroup bias in affected patients, and the rate of nucleotide substitutions in the mtDNA was found to be 22% higher in dorsolateral prefrontal cortex of patients with schizophrenia than controls (1274).

## 11.5 MITOCHONDRIAL DEFECTS IN CANCER

Interest in the role of mitochondrial functional alterations in cancer dates back almost 70 years to when Otto Warburg observed that many cancer cells produce excessive lactate while still respiring, a state he called

“aerobic-glycolysis” (1275). Contrary to previous expectations, it is now clear that the mitochondria of cancer cells are not defective (1276). Multiple studies have demonstrated that cells that have been cured of their resident mtDNA ( $\rho^0$  cells) (296,297,1277) have markedly reduced tumorigenicity relative to their mtDNA containing parental counterparts (1278–1281).

Rather than eliminate mitochondrial function, the cancer cell must alter its bioenergetics to shift its metabolic state from that of quiescent and oxidative cells in which most calories are oxidized to generate energy to that of an actively replicating cell in which much of the calories must be directed to cellular biogenesis frequently in low oxygen environments (1282). Thus, most cancer cells acquire mutations that alter the PI3K-PTEN-Akt pathway to increase glycolysis and biogenesis at the expense of oxidative metabolism (1283), the HIF-1 $\alpha$  system to optimize growth at low oxygen levels (1284), and the myc gene to increase glutamine metabolism to generate metabolic intermediates for growth and redox control (1282).

While the mitochondria of cancer cells are not defective, they are altered and this commonly involves mutations in the mtDNA. This is clear since aging is the most significant risk factor for most solid tumors and caloric restriction in rodents markedly reduces cancer risk (1276,1285–1289), and it is the accumulation of somatic mtDNA mutations that has been repeatedly associated with aging (1,452).

The importance of mutations in mitochondrial genes for cancer was clearly established by the discovery that mutations in certain nDNA-encoded mitochondrial genes results in specific types of tumors (296,297,1277–1281). Mutations in SDH have been associated with hereditary paragangliomas and pheochromocytomas. SDH is composed of four subunits. SDHA binds the substrates succinate and fumarate and contains an FAD cofactor. SDHB contains the iron sulfur center electron carriers. SDHC and D constitute the inner membrane cytochrome b and coenzyme Q binding sites, SDHD being the smaller cytochrome b subunit (cybS) while SDHC is the larger cytochrome b subunit (cybL). In addition, SDH assembly involves two factors, SDH assembly factors 1 (SDHAF1) and 2 (SDHAF2). All familial paragangliomas are associated with germline heterozygous mutations in SDH subunits or SDHAF2 which are homozygous mutant in the tumors (1290). Mutations in SDHD (1291), SDHC (1292), and SDHB (1293,1294) have repeatedly been observed, but only one cancer patient has been reported with a mutation in SDHA and this resulted in an abdominal catecholamine-secreting paragangliomas (1290). SDHAF2 (SDH5) encodes a conserved protein necessary for incorporation of the FAD cofactor into SDHA. SDHAF2 mutations are associated with benign multifocal head and neck paragangliomas (1290).

The pathophysiology of SDH mutation cancer formation has been proposed to be the result of excessive succinate secretion by mitochondria via the dicarboxylate carrier. The succinate is thought to inhibit the

$\alpha$ -ketoglutarate-dependent prolyl hydroxylases (PHDs 1-3) which, together with the von Hippel-Landau (VHL) gene product, degrade the HIF-1 $\alpha$  transcription factor (1295). The HIF transcription factors play a central role in the shift from oxidative to glycolytic metabolism of cancer cells (1284,1290,1296–1299).

Mutations in the TCA cycle enzyme fumarate hydratase (FH) are associated with leiomyomatosis (multiple cutaneous uterine leiomyomata) and renal cell cancer (1300,1301). Cultured cells harboring FH mutations can produce up to a 100-fold increase in fumarate, 7-fold increase in succinate, and marked decrease in malate and citrate (1302). As with SDH deficiency, the dominant pathophysiological hypothesis for tumor formation is that fumarate inhibits the PHD enzymes thus stabilizing and activating the HIF-1 pathway (1303). An alternative hypothesis is that the excessive fumarate results in the succination of KEAP1 and the activation of the Nrf2 stress response pathways. Among other genes, activation of Nrf2 results in the induction of heme oxygenase 1 (HMOX1). HMOX1 degrades heme and since heme is synthesized by the condensation of glycine with succinyl-CoA to yield  $\delta$ -aminolevulinic acid (ALA) via mitochondrial ALA synthase, this would remove the excessive succinyl-CoA that would accumulate from the FH blockade (1302). Since many tumors become dependent on glutaminolysis for generation of the TCA cycle intermediates through the activation of myc (1304,1305), the induction of HMOX1 helps maintain the carbon flux through the mitochondrion thus promoting cellular biogenesis.

While tumors associated with SDH and FH mutations are homozygous mutants, the mutations in isocitrate dehydrogenase isoforms 1 and 2 (IDH1 and IDH2) found in gliomas, secondary glioblastomas, and acute myeloid leukemia (AML) are heterozygous mutations. The IDHs reversibly convert isocitrate to  $\alpha$ -ketoglutarate coupled with the reduction of NADP<sup>+</sup> to NADPH. IDH1 is located in the cytosol while IDH2 is found in the mitochondrion. In gliomas, the IDH1 neoplastic mutations are R132C and R132G while IDH2 mutations involve R132 and R172. In AML, the mutations are found in IDH1 at R132 and IDH2 at R172K and R140Q. All of these mutations change the activity of IDH to one which uses NADPH to reduce  $\alpha$ -ketoglutarate to R(-)-2-hydroxyglutarate (2HG). It has again been proposed that 2HG inactivates PHDs and activates HIF-1 $\alpha$ . As a result, 2HG has been called an “oncometabolite” (1306,1307).

While SDH, FH, and IDH1 and 2 perturb mitochondrial metabolic pathways, reductions in the activity of the mitochondrial RNA helicase protein SUV3 as a result of mutations in the Suv3 gene result in the destabilization of the mtDNA. Partial reduction in SUV3, which is seen in 60% of breast cancers (1308), is associated with the accumulation of shortened mitochondrial RNA species, a shift in mitochondrial morphology from tubular to granular, impaired mitochondrial protein synthesis, decreased mtDNA copy number, down-regulation of OXPHOS enzymes, increased ROS generation, reduced



membrane potential, and reduced ATP production (1309). Mice heterozygous for a Suv3 null mutation also exhibit reduced OXPHOS enzyme activity and mtDNA copy number, increased somatic mtDNA mutation rates, and markedly elevated tumor rates. When Suv3+/- female mice are mated with wild-type males over successive generations, the life span of the mice progressively decreases until the F4 generation when the mice become infertile. This reduction in life span and increase in tumor rate is retained in descendants of Suv3+/- females that have an Suv3+/+ genotype. Hence, the partial reduction in SUV3 destabilizes the mtDNA, but it is the presence of the mutations in the mtDNA that cause the decreased life span and predisposition to cancer (1308).

The discovery that Suv3+/- induced mtDNA damage is oncogenic now makes the observations of mtDNA mutations in cancer cells relevant. The first clear evidence that mtDNA mutations might play an important role in neoplastic transformation came with the report of a renal adenocarcinoma that was heteroplasmic for a 294-np in-frame deletion in the mtDNA ND1 gene. This deletion generated a truncated mRNA, confirming that the mutant mtDNA resulted in mitochondrial functional defect (1310). Subsequently, a variety of mtDNA coding region and control region mutations have been reported cancer cells including colon cancer cells (1311–1313), head and neck tumors (1314), astrocytic tumors (1315), thyroid tumors (1316,1317), breast tumors (1318), and prostate tumors (1319–1322). Comparison of the reported cancer cell mtDNA mutations with those that have been identified as markers for the various mtDNA haplogroups has revealed that more than half of the cancer mutations are the same variants that have been found to be haplogroup specific variants. Since many of these variants have been proposed to be adaptive (360,361), it is possible that cancer mtDNA mutations may confer at least two advantages to cancer cells, contributing to the neoplastic transformation process and permitting cancer cells to adapt to their varying environmental challenges (1323).

Because of the large number of critical bioenergetic and metabolic functions of the mitochondrion, mutations in mitochondrial genes might contribute to neoplastic transformation in a number of different manners. By analogy to the SDH, FH, and IDH mutations, mtDNA mutations might alter mitochondrial metabolism to redirect metabolites from terminal oxidation into biosynthetic pathways permitting stimulation of cell growth. Alternatively, alteration of mitochondrial OXPHOS might alter mitochondrial ROS production and thus alter signal transduction pathways. Partial increases in ROS have long been known to act as a potent cellular mitogen. Only when exposed to excessive levels of ROS will cells undergo apoptosis (12,1324–1326). Low levels of ROS are thought to be mitogenic through their interaction with various kinases (Src kinase, protein kinase C, mitogen-activated protein kinase, and receptor tyrosine kinases), as well as with different transcription factors

(Fos, Jun, NF- $\kappa$ B) (389). Furthermore, the bifunctional protein apurinic/apyrimidinic endonuclease/redox factor-1 (APE/Ref1Red/Ox) plays a role in the DNA base excision pathway but also in the redox regulation of the transcription factors Fos, Jun, NF- $\kappa$ B, PAX, HIF-1 $\alpha$ , and p53 (390,391,1327,1328). The role of mitochondrial ROS in cancer is supported by the observations that mice heterozygous for the MnSOD knockout locus have a 100% increase in cancer incidence (1329), many types of tumors have reduced MnSOD, transformation of certain tumors with the MnSOD cDNA can reverse the malignant phenotype, and a cluster of three mutations in the MnSOD gene promoter region that alter AP-2 binding and promoter efficiency are found in a number of tumors (389,1330).

Further evidence of the importance of mtDNA mutations and mitochondrial ROS production in neoplastic transformation comes from studies of complex IV (COX) mutations in prostate cancer. A proteomic survey of prostate cancer epithelium cells isolated by laser capture microscopy revealed that the ratio of nDNA-encoded subunits (COX IV, Vb, and VIc) to mtDNA-encoded subunits (COI and II) was increased in most prostate tumors (1331). This alteration in the ratio of nDNA to mtDNA COX subunits in prostate cancer can result from mutations in the mtDNA COI gene. Both germline and somatic COI gene mutations have been identified in prostate cancer (1322). An analysis of prostate cancer specimens revealed that 11% harbored germline COI missense mutations, 4 of which were found in multiple independent patient tumors, often on different background mtDNA haplotypes (T6253C, C6340T, G6261A, and A6663G) and these changed highly conserved amino acids (conservation index CI=69–97%) (1322). A COI missense mutation that caused a partial complex I defect would be expected to increase cellular ROS production (314,319). Three other prostate cancer COI mutations were heteroplasmic and thus either recent germline mutations or somatic mutations. These included G5949A-G16X (Stop), T6124C-M74T (CI = 95%), and C6924T-A341S (CI = 100%) (1322). The G16X mutation was particularly informative since it was found to be homoplasmic mutant in the tumorigenic epithelial cells, but homoplasmic wild type in the adjacent normal epithelial cells, which was confirmed by immunohistochemical staining for the COI protein. Therefore, this G16X mutation must have arisen de novo at the time of neoplastic transformation and been strongly selected for in the cancerous cells (1322).

The physiologic significance of mtDNA mutations in cancer cells was demonstrated by introducing a known pathogenic mtDNA mutation into the prostate cancer cell line PC3 via transmitochondrial cybrid fusions (294). PC3 prostate cancer cells were cured of their resident mtDNAs by treatment with the mitochondrial poison rhodamine 6G and then fused with cytoplasts from either homoplasmic mutant (T8993G) or homoplasmic



wild-type (T8993T) cell lines (1322) derived from a patient who was heteroplasmic for the pathogenic ATP6 np T8993G (L156R) mutation (591). This mutation reduces mitochondrial ADP production (591) but also increases ROS production (604). When injected into immune compromised mice, the PC3 cells harboring the normal T8993T base barely grew while the PC3 cybrids with the mutant T8993G base generated rapidly growing tumors that killed the mice (1322). Staining the cellular nodules from the T8993T and T8993G cybrid tumors with dihydroethidium revealed that the mutant T8993G tumors were making much more ROS than the wild-type T8993T nodules (1322). Since MnSOD has been found to be reduced in precancerous intraepithelial neoplastic lesions and in prostate cancer by immunohistochemical and in situ hybridization studies (1332,1333) and prostate cancer cells have a 42% increase in cytoplasmic staining for APE/Ref1<sup>Red/Ox</sup> (1334), these data support the hypothesis that mitochondrial ROS production contributes to the etiology of prostate cancer.

The relevance of increased mitochondrial ROS production to the etiology of other forms of cancer is demonstrated by a tumorigenic mouse cell line found harboring an mtDNA ND6 missense mutation at nt G13997A and frameshift mutation at 13885insC. These cells exhibited a substantially increased ROS production and growth rate, traits that could be transferred from one cell to another by cybrid fusions and resulted in the conversion of low tumorigenic cells to high tumorigenicity (1335). A human cell line harboring a homoplasmic mtDNA ND5 frameshift mutant grew less rapidly and had a lower neoplastic potential than cells that were heteroplasmic for the frameshift mtDNA and a normal mtDNA, the increased growth rate and tumorigenicity of the heteroplasmic cells correlating with increased mitochondrial ROS (1336). Direct proof of the importance of mitochondrial ROS in tumorigenesis has been demonstrated in mice expressing the polyoma middle T oncoprotein which predisposes to lung and breast tumors. Introducing the mitochondrially targeted catalase (mCAT) transgene which removes matrix H<sub>2</sub>O<sub>2</sub> reduced tumor formation (1337). Similarly, mice heterozygous for the adenomatous polyposis coli multiple intestinal neoplasia gene (APC<sup>Min/+</sup>) combined with the heterozygous Tfam allele (Tfam<sup>+/-</sup>) had an increased frequency of intestinal tumors in association with reduced levels of TFAM and mtDNA and increased antimycin A-accentuated ROS production. The frequency of tumors in these mice declined when combined with the mCAT transgene (1338).

In addition to the initiation of tumorigenesis, mitochondrial dysfunction has been shown to contribute to tumor invasiveness through mitochondrial stress activation of mitochondrial retrograde signaling. Ethidium bromide induced 50–80% reduction of mtDNA copy number in the noninvasive, established, mouse myoblast cell line, C2C12, which resulted in cells showing the features of the epithelial–mesenchymal transition together

with a vastly increased invasiveness in a xenotransplantation assay (1339). The reduced mtDNA copy number resulted in reduction in the mitochondrial membrane potential and increased cytosolic Ca<sup>++</sup>. The Ca<sup>++</sup> acts through the Ca<sup>++</sup> responsive phosphatase calcineurin to activate NF-κB via an IκBβ-dependent pathway. This activates an enhanceosome and upregulates the expression of cathepsin L, the ryanodine receptor 1, Glut4 and IGF-1R, and so on leading to altered cell morphology, metabolic shift, resistance to apoptosis, and increased invasive behavior (1340,–1343).

## 11.6 THERAPEUTIC APPROACHES TO MITOCHONDRIAL DISEASE

As the importance of bioenergetic dysfunction to a broad spectrum of human disease becomes apparent, the need for effective therapeutics for mitochondrial dysfunction is becoming acute. Deficiencies in mitochondrial function might be treated by either metabolic or genetic therapies.

### 11.6.1 Metabolic Therapeutics

Metabolic therapeutic strategies could focus on three potential mitochondrial drug targets: energy generation, ROS production, and apoptosis modulation through the mtPTP.

**11.6.1.1 Modulating Mitochondrial Energy Production.** A variety of metabolic strategies have been used to increase mitochondrial energy production. Various combinations of vitamins have also been used to treat mitochondrial disease patients with varying levels of success (683) though metabolic supplementation to bypass metabolic blocks has been successful in selected cases. One CPEO patient with respiratory failure due to a predominantly complex I defect was successfully treated with succinate plus CoQ. The succinate introduced reducing equivalents into the ETC via complex II, thus bypassing the complex I block, and the CoQ<sub>10</sub> would enhance the transfer of the electrons from complex II to complex III. This treatment permitted the patient to be respirator independent as long as the therapy continued (775). In a patient with complex III deficiency due to a CYTB chain termination mutation (623), the complex III block was bypassed by giving ascorbate and menadione. This treatment significantly improved her muscle strength and mitochondrial energy production, as assessed by <sup>31</sup>P-MRS (622,1344).

Since dihydroorotate CoQ oxidoreductase (dihydroorotate dehydrogenase) uses the ETC for an electron sink for reduced CoQ<sub>10</sub>, the ETC is required for de novo pyrimidine biosynthesis. Consequently, it has been proposed that providing uridine might be beneficial to mitochondrial disease patients. Preliminary studies have suggested that supplementation with triacetyluridine, bioavailable form of uridine, may be beneficial for some patients (1345).

L-arginine has been proposed as a treatment for the stroke-like episodes associated with the MELAS syndrome.

Current trials are focusing on patients harboring the tRNA<sup>Leu(UUR)</sup> 3243A>G mutation. These stroke-like episodes in MELAS show ischemia across vascular beds which has led to the hypothesis that they are the results of more global vasoconstriction, perhaps due to the inactivation of the vascular endothelial cell vasodilator NO by mitochondrially generated ROS. By providing L-arginine either orally or intravenously, it is envisioned that the NOS will use the L-arginine substrate to generate NO and relieve the vasoconstriction. Preliminary reports look promising (1346–1348), though additional clinical trials are required to determine the reliability and generality of the therapy.

Dichloroacetate (DCA) has been used to stimulate PDH and reduce lactate production. DCA inhibits pyruvate dehydrogenase kinase (PDK) and PDK inactivates PDH through phosphorylation of the E1 $\alpha$  subunit. Therefore, DCA treatment activates PDH driving pyruvate into the mitochondrion to generate acetylCoA and NADH. While DCA can reduce lactate in patients with severe neonatal lactic acidosis due to partial PDH deficiency, when patients harboring the 3243A>G mutation were treated with DCA, limited success was observed in ameliorating clinical symptoms (1349–1351) and in one DCA trial of 3243A>G patients the trial was suspended due to a high incidence of drug-induced peripheral neuropathy (1350).

Mitochondrial energy output could be increased by upregulation of the mitochondrial mass. Caloric restriction has been shown to increase mitochondrial biogenesis and longevity in a variety of animal models, acting in part through the sirtuins (1352,1353). Therefore, mitochondrial energy output might be enhanced in patients through activation of Sirt1 by using resveratrol or its analogs (SRT1720, SRT2183, etc.). The success of this approach has already been reported for several age-related diseases including type 2 diabetes (1354). Activation of SIRT1 can stimulate mitochondrial biogenesis and aerobic capacity in mice through deacetylation and activation of PGC-1 $\alpha$  (1353) and thus could provide therapeutic benefits for mtDNA disorders (1355).

Overexpression of PGC-1 $\alpha$  was found to delayed onset of symptoms in a COX10 knockout mouse model of COX deficiency muscle disease. Similar benefit was obtained by activating the PPAR/PGC1 $\alpha$  pathway using the PPAR agonist bezafibrate (1356). Bezafibrate has been shown to increase several mitochondrial functions in mitochondrial patient cell lines (1357,1358) and to partially ameliorate symptoms in a mouse model of HD (1359). The PPAR $\gamma$  agonists such as rosiglitazone and pioglitazone have been found to increase mitochondrial biogenesis in mouse brain (1360) and reported to show cognitive and functional improvement in ApoE  $\epsilon$ 4 noncarriers (1361). However, conflicting reports on the efficacy of rosiglitazone and pioglitazone in treating AD mean that this approach to therapy requires additional exploration (1362).

The PPARs are members of the nuclear receptor gene family which also interacts with the energy monitoring

adenosine monophosphate activate protein kinase (AMPK) to form a regulatory network (1363). PPAR $\delta$  agonist GW1516, in association with exercise, increases oxidative muscle fibers in mice. AMPK, acting through PPAR $\delta$ , can also increase endurance exercise capacity. Treatment of sedentary mice for 4 weeks with the AMPK agonist AICAR induced 32 genes linked to oxidative metabolism, 30 of which are induced in PPAR $\delta$  transgenic mice. The AICAR treated mice ran 23% longer and 44% farther than untreated mice (1364). Therefore, activation of the AMPK-PPAR $\delta$  pathway has the potential to increase mitochondrial muscle function and thus treating MM.

**11.6.1.2 Modulating Mitochondrial ROS and REDOX.** While antioxidants such as vitamin E, ascorbate, and lipoic acid are commonly administered to mitochondrial disease patients, their efficacy remains variable (683,1365). CoQ<sub>10</sub> has been given to mitochondrial disease patients based on the logic that the CoQ<sub>10</sub> would enhance electron transfer into the ETC and also act as antioxidants. While routinely provided the success of CoQ<sub>10</sub> in treating mitochondrial disease patients has been variable (1214,1366). CoQ<sub>10</sub> supplementation is beneficial for that subset of mitochondrial disease patients that have mutations in one of the steps in CoQ<sub>10</sub> biosynthesis. Patients with primary CoQ<sub>10</sub> defects are relatively rare and can present with a spectrum of clinical manifestations including encephalomyopathy, LS, severe multisystem infantile disease, cerebellar ataxia, or pure myopathy (858,859,1367,1368).

The antioxidant therapeutic potential of vitamin E and CoQ have been increased by linking them to the positively charged alkyltriphenylphosphonium ion (TPP<sup>+</sup>), thus targeting the compounds to the mitochondrial matrix (1369–1371). The combination of CoQ<sub>10</sub> with TPP<sup>+</sup> resulted in the compound MitoQ (mitoquinone mesylate: [10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadienyl) decyl triphenylphosphonium methanesulfonate]) (1372–1375). MitoQ has been found to collect electrons from complex II and glycerol-3-phosphate dehydrogenase, but is a poor substrate for complex I, complex III, and the electron-transferring flavoprotein (ETF): quinone oxidoreductase. Therefore, it is thought that MitoQ acts primarily as an antioxidant and does not participate in the ETC (1376,1377). MitoQ has been used to treat a number of animal disease models. Among these, MitoQ was recently used to treat the 3xTg-AD model of AD. A 5-month MitoQ regime resulted in prevention of cognitive decline, oxidative stress, A $\beta$  accumulation, astrogliosis, caspase activation, and synaptic loss (1378). This indicates that AD is associated with mitochondrial oxidative stress and that mitochondrially targeted antioxidants may be beneficial for a variety of complex diseases.

Other derivatives of CoQ<sub>10</sub> have altered the structures of the hydrophobic side chain to permit greater bioavailability or altered the quinone and side chain to alter the redox potential of the quinone. One CoQ<sub>10</sub> analog, idebenone

(2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone), has received particular attention, since its shorter side chain permits the quinone to more readily cross the blood–brain barrier (1366). The most extensive studies on idebenone have been applied to Friedreich's ataxia, an autosomal recessive disease which manifests as diabetes mellitus and hypertrophic cardiomyopathy due to a defect in frataxin, a mitochondrial protein important in iron homeostasis loss of which results in increased mitochondrial oxidative stress (22). Treatment of Friedreich's patients with idebenone have been reported to ameliorate the cardiomyopathy (1379,1380) and high doses have been reported to provide some neurological protection (1381). However, early treatment of patients is likely to be most beneficial to minimize accumulated mitochondrial and tissue damage.

Idebenone has been reported to be beneficial in treating LHON in both open label (1382,1383) and a double-blind trial (1384) studies. Idebenone has also been reported to increase complex I activity in fibroblasts from patients harboring the ND4 11778 and ND1 3460 LHON mutations (1385).

Another CoQ analog, EPI-743, has been used to treat both a range of mitochondrial disease patients (1386) and LHON patients (1387). In both open label studies, most patients reported beneficial effects and in the general mitochondrial disease patient study, the redox status of the patients' brains detected by technetium-99m-hexamethylpropyleneamine oxime (HMPAO) single photo emission computer tomography (SPECT) improved in parallel with their well-being (1386).

Even more effective antioxidant therapy might be achieved by using mitochondrially targeted catalytic antioxidants. These include the superoxide dismutase mimetics which catalytically destroy the mitochondrial ROS. One of these, manganese-5,10,15,20-tetrakis-(4-benzoic acid) porphyrin (MnTBAP), has proven particularly effective in treating the dilated cardiomyopathy that develops in mice genetically deficient in MnSOD (Sod2  $-/-$ ) which die at a mean age of 8 days. Death is secondary to the ROS inactivation of the mitochondrial iron–sulfur center enzymes. Unfortunately, MnTBAP does not readily cross the blood–brain barrier, and the MnTBAP-treated animals proceed to develop movement disorders in association with a spongiform encephalopathy of the motor centers (460). Treatment of the MnSOD-deficient animals with the salen manganese complexes EUK-8, -134, and -189, which do cross the blood–brain barrier, protected these animals from both the cardiomyopathy and the spongiform encephalomyopathy (1158). Finally, growing *C. elegans* in these salen manganese complexes extended its life span (1388). Hence, mitochondrially targeted antioxidant enzymes may have promise in treating certain aspects of mitochondrial disease.

**11.6.1.3 Regulation of Mitochondrially Induced Apoptosis via mtPTP Ligands.** A third approach to treating mitochondrial disease would be to inhibit the

mtPTP and thus delay cell loss due to apoptosis. This is still a new area.

Since apoptosis can be initiated by the activation of the mtPTP, the manifestations of mitochondrial disease might be ameliorated by treatment with inhibitors of mtPTP activation (1). Cyclosporin A is a specific inhibitor of cyclophilin D which regulates the mtPTP and could potentially be used in treatment (33). However, cyclosporine A is also immunosuppressant acting through calcineurin. Hence, cyclosporin A is not appropriate for use by mitochondrial disease patients. Recently, analogs of cyclosporin A have been developed which still bind to cyclophilin D but not to calcineurin, thus inhibiting the mtPTP with affecting immune function. An example is Debio 025 (1389). The potential application of this strategy has been demonstrated to be beneficial on patients and mice with mutations in the extracellular matrix protein gene for collagen VI, specifically Bethlem myopathy and Ullrich's congenital muscular dystrophy. Surprisingly, the clinical features of these diseases can be markedly reduced simply by treating with mtPTP inhibitors (1390–1393).

Nortriptyline, a tricyclic depressant, has been shown to inhibit the activation of the mtPTP. In a mouse model of ALS it delayed the onset of symptoms and increased life span (1394). These observations support the hypothesis that modulating the mtPTP may be relevant to a much wider range of clinical problems than simple mitochondrial disorders.

## 11.6.2 Mitochondrial Gene Therapy

Diseases resulting from mtDNA mutations can also be treated by gene therapy. Mitochondrial gene therapy can include both somatic therapy to ameliorate symptoms and germline therapy to eliminate maternally inherited pathogenic mutations.

### 11.6.2.1 Somatic Mitochondrial Gene Therapy.

Somatic mitochondrial gene therapy could take three approaches: direct genetic modification of an nDNA-encoded mutant mitochondrial gene, insertion of a modified mtDNA protein gene into the nucleus and redirection of the normal polypeptide back to the mitochondrion, and direct modification of the mtDNA.

Somatic gene therapy approaches include efforts to modulate the proportion of mutant and wild-type mtDNAs in heteroplasmic cells (heteroplasmy shifting), efforts to directly alter the mtDNA genotype of transcriptional products, and efforts to introduce a corrected gene copy into the nucleus and have the product redirected back to the mitochondrion. Heteroplasmy shifting can be accomplished by either selecting for increased percentages of the normal mtDNA or by using approaches to selectively destroy the mutant mtDNAs. Metabolic selection to enrich for the normal mtDNAs has been accomplished by treatment of cell heteroplasmic for the NARP/MILS ATP6 T8993G mutation in medium containing galactose and oligomycin and observing enrichment for the



wild-type leucine at codon 156 containing mtDNA over the mutant arginine mtDNA (1395). Similarly, growth of cells heteroplasmic for an mtDNA 1.9kb deletion in ketogenic metabolites (acetoacetate,  $\beta$ -hydroxybutyrate, or both) resulted in the enrichment of the wild-type mtDNA from 13% to 20–23% after 5 days (1396).

Heteroplasmic shifting has also been achieved by targeting site-specific restriction enzymes to the mitochondrial which either selectively digest the mtDNA harboring the mutant mtDNA or enrich for the desired mtDNA (1397). In NARP/MILS, the wild-type ATP6 gene sequence 8993T was enriched over the mutant by inserting a mitochondrially targeted transgene for the SmaI restriction endonuclease into the nucleus and having the protein imported into the mitochondrion. There the SmaI selectively cleaved the mutant mtDNAs (1398). Although not all mtDNA mutants create a usable restriction site, a more generalizable approach could be achieved using the sequence specificity of zinc finger DNA-binding protein which when appended to a restriction endonuclease creates highly sequence-specific zinc finger nucleases (ZFNs) (1399). These approaches have been utilized to shift the percentage of two different mtDNAs in cells and animals and have been delivered by AAV (1400–1402).

Efforts to directly alter the replication of mutant mtDNAs have utilized mitochondrially targeted PNAs which have a *N*-(2-aminoethyl)-glycine peptide backbone connected to standard purine and pyrimidine bases which can base pair with nucleic acids. Since the peptide backbone is uncharged, PNAs bind tightly to nucleic acids. In *in vitro* mtDNA replication studies, a PNA synthesized complementary to the common 4977-np deletion breakpoint inhibited the replication of the deletion template 80% without affecting the replication of the normal template. Similarly, a PNA complementary to the MERRF A8344G variant inhibited replication of the mutant mtDNA by about 75% but did not inhibit the normal mtDNA. PNAs have also been observed to be taken up by mammalian cells (1403). This system has been further elaborated by coupling the PNA to the 4-thiobutyltriphenylphosphonium ion through a disulfide bond. This construct was rapidly taken up by the 143B osteosarcoma cells studied. However, the disulfide bond was then reduced in the cytosol, releasing the PNA. Consequently, the PNA remained in the cytosol while the 4-thiobutyltriphenylphosphonium ion continued into the mitochondrion (1404).

Further modification was able to stabilize the PNA–TPP linkage with the construct delivered to the mitochondrion. Unfortunately, the 11 base MERRF 8344G PNA was unable to shift the percentage of the 8344G mutant mtDNAs in cultured cells (1405). To increase the mtDNA binding capacity of the PNA–TPP constructs, the controls have been linked to benzoylbenzoic acid. This benzophenone system, once bound to the mtDNA through the PNA, can then be cross-linked to the mtDNA by UV photoactivation (1406).

An alternative approach would be to target DNA directly to the mitochondrion. A fluorescein-labeled

oligonucleotide has been introduced into the mitochondria of cells by covalently linking the amines at the 3' end of the oligonucleotide to those of the mitochondrial targeting peptide from ornithine transcarbamylase using glutaraldehyde. When this construct was encapsulated into liposomes and the complex exposed to skin fibroblasts, the oligonucleotide-targeting peptide was deposited in the cytosol and subsequently taken up into the mitochondria, with the mitochondrial fluorescence persisting up to 8 days (1407). While this procedure targeted the oligonucleotide to the mitochondria, the covalent linkage of the oligonucleotide to the protein would limit its bioavailability. As an alternative, oligonucleotides have been hybridized to PNAs which are in turn covalently linked to mitochondrial targeting peptides. The mt targeting peptide-PNA:homologous oligonucleotide-labeled complex was then introduced into myoblast and myotube cells using either branched-chain polyethylenimine or streptolysin O permeabilization. Under these circumstances, the “targeting peptide-PNA:oligonucleotide-labeled” constructs were delivered to the cytosol and then rapidly taken up by the mitochondria. Moreover, colloidal gold-labeled oligonucleotides could be observed in the mitochondrial matrix by electron microscopy (1408). While all of these approaches suggest that it may be possible to modify the mtDNA via exposure of cells to exogenous DNA or PNA, no successful alteration of the mtDNA within a living cell has yet been documented.

The third approach that is being actively pursued is to introduce mtDNA gene derivatives into the nDNA, have the gene transcribed, and the resulting protein returned to the mitochondrion. This allotopic mtDNA gene expression has been used for gene products that are derived from very different species or relatively similar mammalian species. Defects in mitochondrial complex I genes have been complemented by introducing the yeast NDI1 (NADH dehydrogenase) gene which can oxidize NADH and transfer the electrons to CoQ via a single polypeptide, though in the absence of proton pumping.

An OXPHOS defect in CHO cells deficient in an nDNA-encoded complex I has been treated by transfection with a plasmid carrying the yeast NDI1 gene, which encodes a single polypeptide. NDI1 transformation of the gene restored the NADH oxidase activity, resulting in complex I that was insensitive to rotenone but sensitive to flavone, characteristics of the yeast but not mammalian NADH dehydrogenase (1409). A high-expression nDNA NDI1 transformant was found to reconstitute the NADH-dependent respiration; the P:O ratio on malate/glutamate substrates to two-thirds that of the wild-type human cells, which would be expected since the yeast NADH dehydrogenase lacks a coupling site; and the ability to grow on galactose (1410). The efficacy of transfer of the yeast NDI1 gene was greatly increased by its introduction into an AAV vector (1411). This AAV-NDI1 transduction system was then used to transduce the yeast NDI1 gene into mouse and rat neuronal cells (1412). Moreover, this AAV-NDI1 vector was used to



transduce the brain substantia nigra and striatum as well as the skeletal muscle of rats (1413).

As a complement to the yeast Nid1 NADH dehydrogenase gene complementation, the ascidian *Ciona intestinalis* cyanide-insensitive and SHAM sensitive alternative oxidase (AOX) has been used to oxidize the reducing equivalents of reduced CoQ to water. This can complement complex III and complex IV deficiency when incorporated into mammalian and *Drosophila* cells (1414–1419). Furthermore, in a *Drosophila* of neurodegenerative disease in which mitochondrial dysfunction was generated by downregulating a subunit of mtDNA polymerase in cholinergic, serotonergic, and dopaminergic neurons, mitochondrial function can be restored by expression of AOX (1418).

Moreover, a combination of the Ndi1 NADH dehydrogenase gene as well as the AOX CoQ/oxidase gene is able to restore respiratory function and uridine independence in mouse cells lacking mtDNA ( $\rho^0$  cells) (1419). Therefore, complementation of mitochondrial respiratory defects by introduction of heterologous genes is potentially promising for the treatment of severe mitochondrial disease.

Defects in nDNA-encoded mitochondrial genes can potentially be treated by reintroduction of a normal copy of the gene into the nucleus. The feasibility of this approach has been demonstrated in the treatment of the muscle mitochondrial energy deficiency associated with null mutations in the heart-muscle isoform of the ANT1. AAV carrying the Ant1 cDNA have been used to transduce mouse Ant1-deficient fibroblasts, myoblasts, and skeletal muscle derived from an Ant1 knockout mouse 227. Like human ANT1 mutant patients, the Ant1 knockout mouse was found to harbor multiple cardiac mtDNA deletions (903). AAV–Ant1 cDNA transduction partially restored the mitochondrial ANT1 activity and mitochondrial export of ATP, and significantly reduced the muscle histopathology associated with the Ant1 defect (950).

Nuclear DNA transformation and transduction is also being explored as a system for treating mtDNA polypeptide gene mutations such as NARP/MILS ATP6 T8993G L156R mutation (591) and LHON ND4 G11778A R340H (4). The logic involves the cloning of the mtDNA gene that harbors the pathological mutations, correcting the genetic code of the mtDNA gene to be compatible with cytosolic translation, adding an N-terminal mitochondrial targeting sequence (MTS) to return the protein to the mitochondrion, adding the necessary nuclear transcription and translation signals, and delivering the construct to the nucleus so that it can be transcribed, translated on cytosolic ribosomes, and targeted back to the mitochondrion. This process is known as allotopic expression. The first application of this strategy was an attempt to correct the complex V defect associated with the ATP6 gene T8993G mutation which reduces mitochondrial ATP production about 70% (301). Transformation of ATP6 T8993G cells with a FLAG-tagged ATP6 gene modified

for the universal code and fused to the COX8 targeting peptide resulted in the import of the nDNA ATP6 polypeptide into the mitochondrion, assembly into complex V, increased mitochondrial ATP production, and improved growth on galactose medium (1420).

This same strategy is being developed for the allotopic treatment of LHON. In LHON, the affected cells are the RGCs which lie at the surface of the retina adjacent to the vitreous. Therefore, viral transduction can be achieved by delivering the viral particles into the vitreous. Two groups are actively pursuing this approach, Dr Guy and Dr Corral-Debrinski (1421,1422). The first objective of the Guy team was to develop a mouse model of LHON. In one experiment, they delivered a ribozyme to the RGC that degraded the mRNA for the complex I NDUFA1 gene resulting in destruction of RGC and optic nerve axons (1423). In the second model, they transduced a hammerhead ribozyme to destroy the mRNA for the mitochondrial MnSOD which also destroyed the RGCs and optic nerve (563). These experiments confirmed that the RGCs and associated optic nerve fibers are sensitive to reduced complex I function and increased mitochondrial oxidative stress. They then showed that transduction of MnSOD cDNA into the retinas of NUDFA1 ribozyme treated mice protected the RGCs and the optic nerve (1424). Next, the Guy group prepared allotopic construct for the mtDNA ND4 gene with the normal sequence 11778G (340R) fused to the ATP synthase “c” polypeptide MTS and flag tagged. The mRNA was linked through an IRES GFP and inserted into the AAV pTR-UF11 transcribed from CMV enhancer–chicken  $\beta$ -actin promoter construct. This construct complemented the mitochondrial defects in 11778A (340H) mtDNA-containing cybrids (1425). MnSOD transduction also improved mitochondrial function of 11778A cybrids (1426). They then created an allotopic ND4 11778A (340H) construct and used AAV to transduce the RGCs resulting in disruption of the mitochondrial structure, increased ROS production, swelling of the optic nerve head, and increased apoptosis. The wild-type ND4 gene did not cause retinal pathology (1427). While concern has been expressed about inappropriate import of allotopic mitochondrial proteins (1419,1428), Guy’s group was able to demonstrate that the ND4 polypeptide entered the mitochondrial matrix and was incorporated into complex I (1429). They have further increased AAV transduction efficiency by using self-complementary AAV (scAAV) introduced into both mice and non-human primates (1430,1431).

Dr Corral-Debrinski and associates discovered a 3′-UTR that causes the allotopic gene mRNA to be translated on mitochondrially bound polysomes thus increasing the import of long-hydrophobic proteins into the mitochondrion (1421,1422). Large, hydrophobic proteins such as COX10 use this system to be imported into the mitochondrion through the TOMM and TIMM complexes concurrent with their translation. For the allotopic expression on the mtDNA-encoded proteins, they use the

COX10 MTS (21 amino acids) and 3'-UTR (1425 nt) to bracket the mitochondrial cDNA transcribed from the CMV enhancer-promoter (1421,1432,1433). With this and related constructs they have used allotopic ATP6 and ND4 genes to complement the mitochondrial defects of NARP ATP6 T8993G and LHON ND4 G11778A mutations in cultured cells (1434) and allotopic ND1 to complement the LHON ND1 3460A mutation (1435). They have also used allotopic gene plasmid electroporation of the RGCs to introduce the 11778A mutant ND4 gene into rat retinas and have produced a 30–38% reduction in the rat RGCs which were partially ameliorated when they also electroporated the allotopic wild-type ND4 11778G gene (1436). Finally, they have prepared an AAV2 vector harboring the apoptosis inducing factor (AIF) cDNA with its MTS and mitochondrial 3'-UTR and used intravitreal injection of this vector to complement the AIF genetic defect and associated complex I defect and retinal degeneration of the Harlequin mutant mouse (1437).

#### 11.6.2.2 Germline Mitochondrial Gene Therapy.

While somatic mtDNA gene therapy may hold promise for treating the symptoms of patients with mtDNA disease, these approaches will not help the woman who harbors a pathogenic mtDNA mutation, and would like to have children that are free of disease. Because maternal inheritance is absolute, all of her children will inherit her mtDNA and thus be at risk for the disease. To assure the future mother that her children will have normal health, it is necessary to break the maternal transmission of the mutant mtDNA. One procedure proposed in the 1980s that could accomplish this is nuclear transplantation. In this scenario, the nucleus of the mother's egg is removed by micropipette and injected into an enucleated oocyte from a woman with normal mtDNA. Subsequent fertilization of the modified oocyte with the father's sperm should result in mtDNA mutant-free offspring (331).

This has now been achieved in primates by the nuclear transplantation of the spindle of one *Macaca* oocyte into another oocyte. Subsequent fertilization and development has resulted in the normal development and the birth of normal offspring with less than 3% of the mtDNA carried over from the original oocyte (1438). This advance is now moving toward the clinic. Scientists in Newcastle, England have reported successful transfer of pronuclei between abnormally fertilized human zygotes in vitro with minimal carryover of donor mtDNA (1439).

## 11.7 A MITOCHONDRIAL PARADIGM FOR COMPLEX DISEASE

The pursuit of diseases resulting from mitochondrial gene mutations has revealed that energy deficiency can result in every symptom that has been attributed to the common metabolic and degenerative diseases. From a Mendelian genetic perspective, these common clinical problems appear to be “complex” in that they do not conform to standard chromosomal inheritance patterns. Hence, they have been assumed to be the results of the interaction of multiple

chromosomal loci plus the environment. However, there is another possibility and that is that the common metabolic and generative diseases are the results of systemic partial energy deficiencies and that the complexity in the genetics results from the assembly of the bioenergetic systems of the cell between 1000 and 2000 nDNA-encoded genes, the thousands of copies of the mtDNA genes, and the energetic regulation of the expression of the genes through the epigenome and the signal transduction systems.

Once we reorient our thinking about disease from the anatomical perspective of the current clinical subspecialties (neurology, ophthalmology, nephrology, etc.) to an energetic perspective, clinical problems that had been assumed to be separate entities suddenly become manifestations of the same pathophysiological mechanism, bioenergetic decline. Thus, as in the progressive failure of the electrical systems during a metropolitan brown out, as the energy production of the body declines the tissues with the highest energy demand will be the first to be affected.

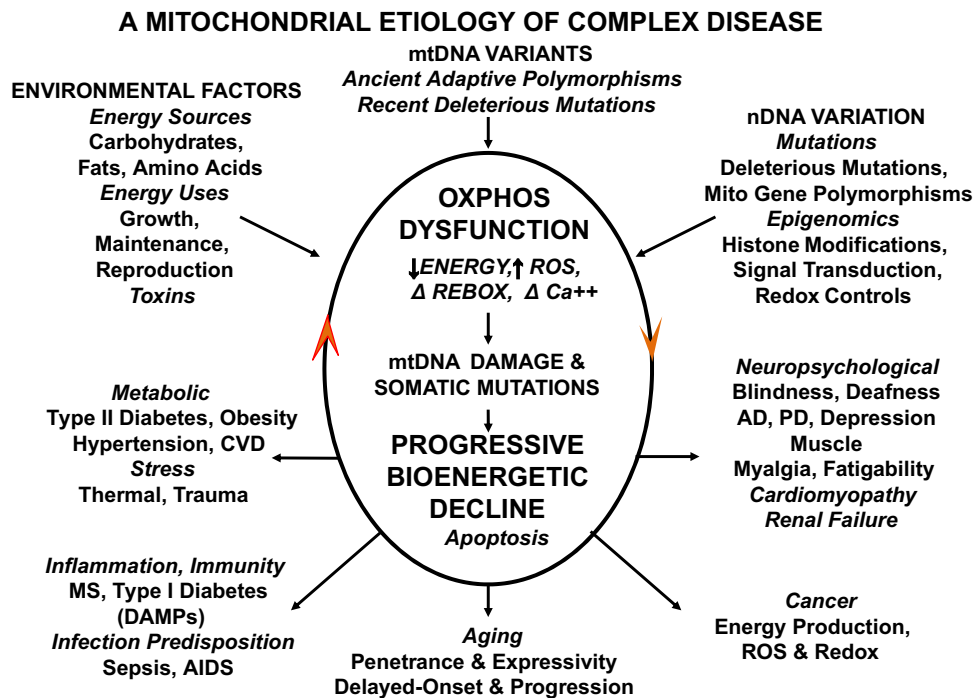
The assembly and regulation of the systemic energy system then provides the nexus for understanding the genetics and pathophysiology of the “complex” diseases. The central element for health and disease becomes mitochondrial energy production via OXPHOS. The capacity of OXPHOS to generate energy can be compromised in multiple ways. Alterations in the hundreds to thousands of nDNA genes involved in assembly and regulation of mitochondrial OXPHOS can occur through inherited mutations and/or polymorphic variants. The expression of the nDNA bioenergetic genes can also be altered by changes in the epigenome and the cellular signal transduction systems. OXPHOS can also be altered by either recent deleterious mtDNA mutations or ancient adaptive polymorphisms. Finally, mitochondrial OXPHOS is fueled by the availability of calories in the environment and impinged on by the demands made on energy flow for growth, maintenance, and reproduction. OXPHOS is also the most sensitive system in the body to effects of environmental toxins including pesticides and herbicides.

When mitochondrial OXPHOS is inhibited, this reduces energy production, alters the cellular redox state, alters  $\text{Ca}^{++}$  homeostasis, and increases ROS production. These impinge on mtDNA replication, repair, and turnover and result in the progressive accumulation of somatic mtDNA mutations. As the mutations accumulate, they erode mitochondrial energetic further, causing an age-related decline in mitochondrial function thus generating the aging-clock. When mitochondrial energy production becomes sufficiently impaired, the mtPTP can be activated and the energy impaired cell removed by apoptosis. The progressive loss of cellular function and ultimate loss of cellularity due to apoptosis results in organ and tissue functional decline which we call aging. This progressive stochastic energetic decline exacerbates inherited energetic defects until one or more tissues fall below the minimum energy output for them to function and symptoms ensue. This explains the delayed-onset and progressive course of metabolic and degenerative disease,

and the statistical variability seen in all complex diseases is referred to as their variable penetrance and expressivity.

The organs that are most prone to dysfunction resulting from partial energy deficiency are the central nervous system, cardiovascular system, skeletal muscles, renal, and endocrine systems. Hence, partial energy defects cause the most severe symptoms in these organs and result in forms of blindness and deafness as well as the clinical manifestations that we call LS, AD, PD, cardiomyopathy, renal failure, and so on. Moreover, since the mitochondria lie at the interface between the consumption of calories and the use of the energy generated, alterations in mitochondrial function are central to metabolic diseases such as diabetes, obesity, hypertension, and cardiovascular disease. The mitochondria are also the most prevalent bacteria in our bodies, with  $\sim 10^{17}$  bacteria in our cells but only  $10^{14}$  bacteria in our guts. The mitochondrial bacteria have naked DNA and N-formyl-methionine containing

peptides which are highly antigenic (572). Hence, apoptosis which is initiated by mitochondrial energy failure, excessive mitochondrial ROS damage, or  $\text{Ca}^{++}$  results in the destruction of the intracellular mitochondria before they enter the blood stream and can activate the innate immune system. However, apoptosis requires energy and severe mitochondrial energy failure leads to necrosis and the release of the mitochondria into the intracellular space and circulation. This then initiates the inflammatory response which can initiate the autoimmunity response. Hence, mitochondrial dysfunction may be an important primer of the immune response that leads to autoimmune diseases such as type 1 diabetes, multiple sclerosis, lupus, and so on. Finally, cancer cells are growing at a high rate and hence are continuously limited for both energy and substrates for cellular biogenesis. Hence, alterations in mitochondrial energy are central to neoplastic transformation and cancer cell growth as well (Figure 11–12).



**FIGURE 11-12** Mitochondrial etiology of “complex” diseases. An integrated model for the genetics and pathophysiology of complex diseases, aging, and cancer. The top of the figure indicates the three types of variations that impact on individual mitochondrial OXPHOS robustness and hence risk for developing disease symptoms. These include nuclear DNA (nDNA) variation encompassing DNA sequence changes and epigenomic modification of gene regulation and signal transduction pathways; mitochondrial DNA (mtDNA) variation including recent deleterious mutations and ancient adaptive polymorphisms, and environmental influences encompassing the availability and demand for calories and inhibition of mitochondrial function by environmental insults. The central oval encompasses the pathophysiological basis of disease processes and the basis of disease progression. The primary defect is reduction in the energy transformation capacity of OXPHOS. This can result in reduced energy output, increased reactive oxygen species (ROS) production, alter redox status, and altered calcium homeostasis. The decline in OXPHOS efficiency can in turn perturb mitochondrial biogenesis, increase ROS production, impair mitophagy, and so on resulting in the progressive increase in mtDNA damage and somatic mutations and further decline in mitochondrial function. Once mitochondrial function falls below the bioenergetic threshold of a tissue symptoms ensue. Continued energetic failure can initiate cell destruction by apoptosis or necrosis. The lower five derived disease categories summarize the phenotypic outcomes of perturbed mitochondrial energy transformation. The bottom arrow shows the effect of the stochastic accumulation of somatic mtDNA mutations resulting in delay-onset and progressive course of diseases and aging. The right arrow indicates clinical problems that can result from reduced energy production in the most energetic tissues, the brain, heart, muscle, and kidney. The number and severity of symptoms in these organs reflect the degree and specific nature of the mitochondrial defect. The arrow to the left indicates the metabolic effects of mitochondrial dysfunction which result in the perturbation of the body’s energy balance. This results in the symptoms of the metabolic syndrome. The arrow toward the lower right corner indicates the mitochondrial alterations that are critical for cancer initiation, promotion, and metastasis. The arrow to the lower right corner outlines the hypothesized inflammatory and autoimmune responses that may result from the chronic introduced into the blood stream of the mitochondria’s bacteria-like DNA and N-formyl methionine proteins.



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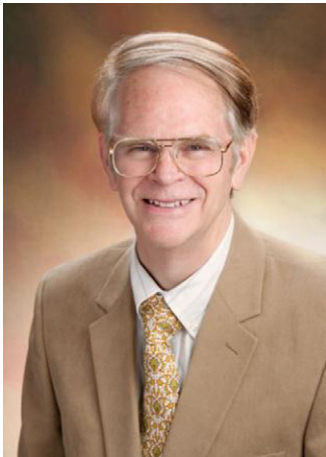
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## Biographies



**Doug Wallace, PhD** has been working on human and mammalian mitochondrial genetics for 40 years. He was the first to demonstrate that mammalian cells harbored cytoplasmically inherited genes by inventing the cybrid transfer technique in the early 1970s and used this system to demonstrate that mammalian chloramphenicol resistance could be transferred from cell to cell by fusing cytoplasmic fragments in the absence of a nucleus. He then proceeded to define the rules of mammalian mitochondrial genetics, culminating in his demonstration of the maternal inheritance of the human mitochondrial DNA (mtDNA) in 1980. His research then followed two paths: the investigation of the nature and extent of human mtDNA variation in aboriginal populations and the quest for diseases resulting from mutations in the mtDNA. The population studies revealed that mtDNA variation was unique in that it correlated highly with the ethnic and geographic origins of indigenous peoples. The quest for mtDNA diseases culminated with his 1988 report that Leber hereditary optic neuropathy was caused by an mtDNA missense mutation, making it the first maternally inherited mtDNA disease to be identified. Since that time, Wallace has shown that mtDNA variation is central to both rare and common multisystem diseases and aging.



**Vincent Procaccio, MD, PhD**, is Professor of Medical Genetics in the Department of Genetics and Biochemistry at the School of Medicine, University of Angers, France. He obtained his MD from Grenoble School of Medicine, his PhD from Grenoble University (France), and he is Board Certified Clinical Molecular Geneticist. From 2000 to 2003, he was a Post-Doctoral Fellow and worked on mitochondrial research at Emory University (Atlanta, USA). From 2003 to 2007 he was Assistant Professor and promoted Associate Professor in 2007 at the Department of Pediatrics, Center for Molecular and Mitochondrial Medicine and Genetics, University of California Irvine. Since 2008, he has been Professor of Medical Genetics and runs the Diagnostic Molecular Laboratory of Angers hospital, focused on genetic testing for mitochondrial and other genetic disorders. His research interests center on investigating the role of mitochondria in neurodegenerative diseases and mitochondrial disorders, with special emphasis on pharmacological and therapeutic aspects such as mechanisms of regulation of mitochondria by hormones.



**Marie Lott, MA**, is a bioinformatics specialist at the Children's Hospital of Philadelphia. She has 30 years of experience studying human mitochondrial DNA variation and disease with the Wallace group. She was part of the pioneering team that discovered the first LHON mutation at 11778, the MERRF mutation at 8344, and the "common deletion" in Kearns Sayre Syndrome. She is co-founder of the mitochondrial database Mitomap. She has been curator of Mitomap since its inception in 1995 with the goal of making available all information about the human mtDNA including its sequence, encoded functions, population variation, and clinical mutations.

# CHAPTER 12

## Multifactorial Inheritance and Complex Diseases

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### 12.1 INTRODUCTION

If a disease or condition is caused by a single locus of large effect, it is called a single-gene or *monogenic* disease, disorder, or more generically, condition. There are over 10,000 such examples, which include cystic fibrosis, Huntington disease, Duchenne muscular dystrophy, and Marfan syndrome. It is possible that a single-gene disease has *locus heterogeneity* if that disease is caused by single mutations in different genes, but this is more properly considered a special case of an oligogenic disorder. For example, osteogenesis imperfecta is caused by single mutation in genes on either chromosome 7 or chromosome 17. *Oligogenic* disorders are explained by a few loci with large effects (for examples, see Reference (1)). In contrast to oligogenic traits, *polygenic* inheritance is due to many loci with small effects at each locus. Thus, the term *polygenic* is generally used to describe multiple factors that are exclusively genetic. Any of these genetic effects, with or without the combination of an environmental effect, can give rise to a *multifactorial* disorder. Multifactorial diseases are caused by the simultaneous action of multiple genetic and/or environmental factors.

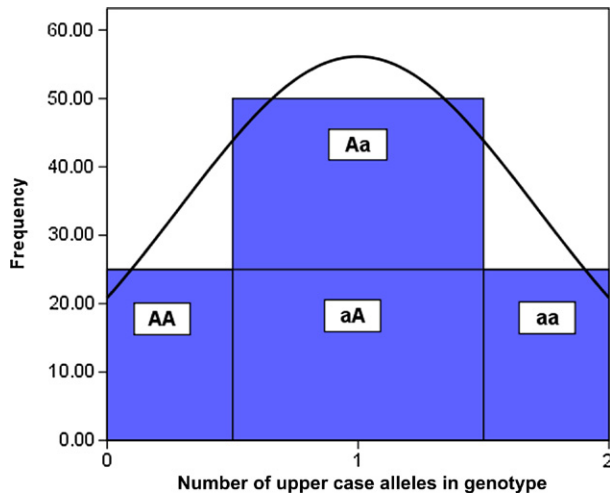
In contrast to dichotomous traits (i.e. affected versus unaffected), quantitative traits are measured on a continuous scale, most of which are thought to be multifactorial (e.g. blood pressure and body mass index). Some quantitative traits may be due to major gene effects with a multifactorial background. Multifactorial inheritance is responsible for the majority of modern deleterious health conditions such as heart disease and diabetes. Atopic syndrome, diabetes, cancer, spina bifida/anencephaly, pyloric stenosis, cleft palate, congenital hip dysplasia, club foot, and many other disorders and complex phenotypes result from multifactorial inheritance.

### 12.2 DEFINITIONS AND TERMINOLOGY

The *polygenic model* has its origins from Fisher's seminal work (2), which showed that “many small, equal and additive loci” would result in a Gaussian (or normal) distribution for a phenotype. Similarly, the combined additive effects of many genetic and environmental factors will also produce an approximately Gaussian phenotypic distribution. To illustrate, suppose (naïvely) that a quantitative trait such as percent body fat is determined by a single gene with two codominant<sup>1</sup> alleles, *A* and *a*, which have equal frequency ( $p=0.50$ ). Assume individuals with an *A* allele tend to have a higher value of the trait, and individuals with an *a* allele tend to have a lower value of the trait. If *A* has an additive effect, then there are three distinct phenotypic groups, namely, high (2), intermediate (1), and low (0). If the allele frequencies of *A* and *a* are both 0.50, then 25% of individuals would be expected to be *aa* and of low-percent fat, 50% would be expected to be *Aa* and of moderate-percent fat, and 25% would be expected to be *AA* and of high-percent fat. Figure 12-1 gives the distribution of the trait in a population.

Now, suppose that the trait is determined by two loci. The second locus also has two codominant alleles, *B* for high and *b* for low expression of the trait, with *B* having an allele frequency of 0.50 and the same effect magnitude as the *A* allele. There are now nine possible genotypes (see Table 12-1).

<sup>1</sup>“An allele *a* is said to be codominant with respect to the wild-type allele *A* if the *A/a* heterozygote fully expresses both the phenotypes associated with the *a/a* and *A/A* homozygotes.”—from <http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=glossaryTerm&key=codominant>.



**FIGURE 12-1** Expected phenotype distribution for a trait with a single causal locus with an allele frequency of 50% and in Hardy-Weinberg equilibrium.

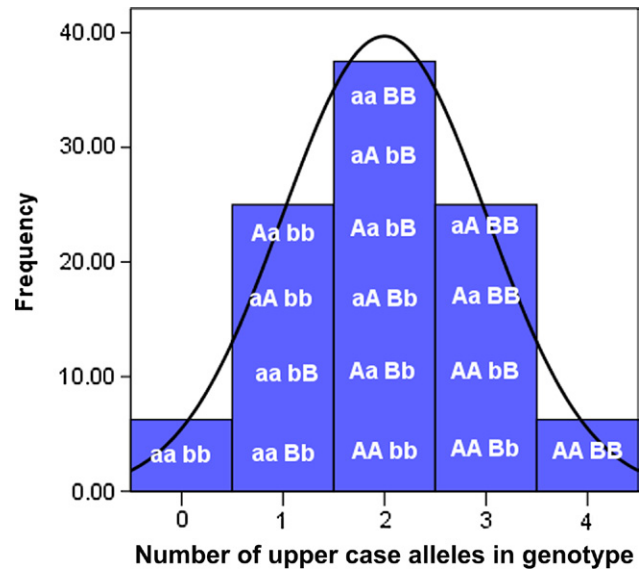
TABLE 12-1 Frequency Distribution of Genotypic Values for Two Loci with No Linkage Disequilibrium			
	AA	Aa	aa
BB	0.0625	0.1250	0.1250
Bb	0.1250	0.2500	0.1250
bB	0.0625	0.1250	0.0625

An individual can possess 0, 1, 2, 3, or 4 “high”-trait alleles. Assuming that the combined effects of the two loci are also additive,<sup>2</sup> there are five distinct phenotypes with respect to the number of high-trait alleles (see Table 12-2).

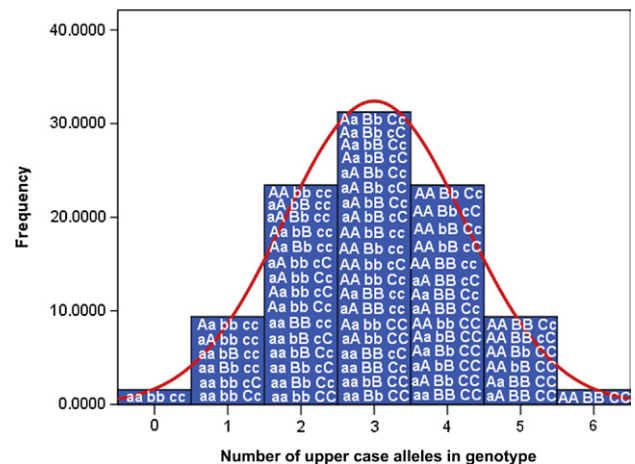
The trait distribution with respect to genotypic value distribution is shown in Figure 12-2. As can be seen in Figure 12-2, even with two loci, the distribution of the phenotype starts to look Gaussian. An example of a three-locus system with equal allele frequencies, no linkage disequilibrium,<sup>3</sup> and equal additive effects, is shown in Figure 12-3. It can be shown that six diallelic loci are enough to produce population frequencies virtually indistinguishable from a normal curve.

Many traits (or diseases) are treated as dichotomous variables because they appear to be either present or absent (e.g. cancer). By definition, dichotomous variables do not approximate a Gaussian distribution. However, these diseases may still be polygenic or multifactorial because

TABLE 12-2 Genotypic Values of Two Loci Genotypes			
	AA	Aa	aa
BB	4	3	2
Bb	3	2	1
bb	2	1	0



**FIGURE 12-2** Expected phenotype distribution for a trait with two independently segregating causal loci of equal effect and allele frequency.



**FIGURE 12-3** Expected phenotype distribution for a trait with three independently segregating causal loci of equal effect and allele frequency.

they do not follow the patterns expected of Mendelian (single-gene) diseases. A common explanation is that an underlying *liability* distribution exists for multifactorial diseases (3). Individuals on the low end of the distribution have little chance of developing the disease because they possess few of the alleles or environmental factors that jointly cause the disease. By contrast, individuals on the high end of the liability distribution have a greater chance

<sup>2</sup>That is, not epistatic, where *epistatic* refers to an interaction (in the statistical, not necessarily biochemical sense) between two different loci, such that the effect of genotype at one locus depends on the genotype at another locus.

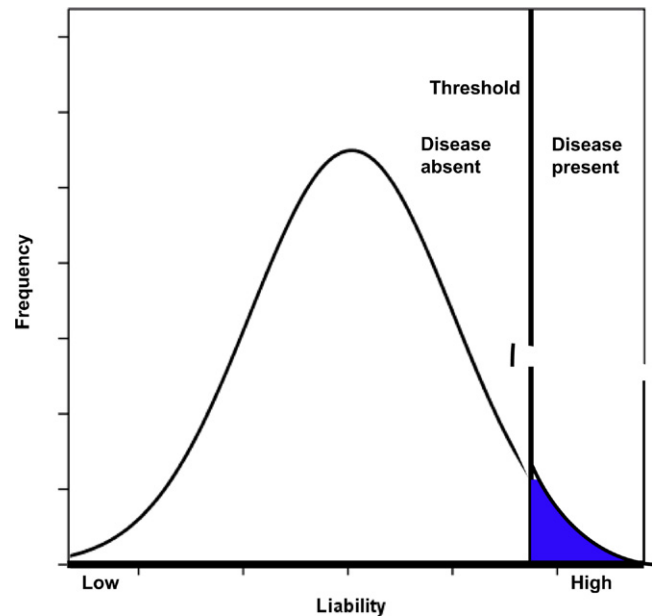
<sup>3</sup>*Linkage disequilibrium* is defined as the nonrandom association between alleles at *linked* (or adjacent) loci (133). Two loci are said to be *linked* if they are close sufficiently close on the same chromosome such that they do not segregate independently.

of developing the disease because they possess many of the alleles and/or environmental factors that jointly cause the disease. The liability distribution is assumed to be continuous (representing the sum of a large number of independent genetic and environmental factors) and normally distributed within the population. It is also a commonplace to assume that all correlations between relatives are due to shared genes but not shared environment. For multifactorial diseases that are either present or absent, there is a hypothesized *threshold of liability* that must be crossed before the disease is manifest (3).

For example, consider the development of the cleft palate. Early in embryonic development, the palatal arches are in a vertical position. Through embryonic and fetal development, the head grows larger, making the arches farther apart, and the tongue increases in size, making it more difficult to move. In addition, the arches themselves are growing and turning horizontally. There is a critical stage in development by which the two arches must meet and fuse. Head growth, tongue growth, and palatal arch growth are all subject to many genetic and environmental factors. If the two arches start to grow in time, grow at the proper rate, and begin to move soon enough to the horizontal, they will meet and fuse in spite of head size and tongue growth. The result is no cleft palate. They may fuse well ahead of the critical developmental stage or just barely make it in time; it is impossible to know. However, if they don't meet at the critical stage, a cleft palate results. If they are close together at the critical stage, a small cleft will result, perhaps only a bifurcated uvula. If they are far apart, a more severe cleft will result. That critical difference in liability is called the *threshold*. Beyond the threshold, disease results. Below the threshold, normal development is observed. Thus the underlying liability is distributed as the normal curve shown in Figure 12-4.

Some diseases may have more than one threshold, and commonly two liability thresholds are present as defined by factors such as gender, race, age of onset, and so on, causing different levels of severity (4). Examples include pyloric stenosis (sex dimorphism for liability) (5) and orofacial cleft syndrome/cleft lip and palate (two thresholds for fetal mortality and disease) (6,7). The latter model proposes a lower threshold level of liability resulting in a cleft formation and a higher level causing a fetal death (preferentially in males).

It should be emphasized that, similar to any other loci, the individual loci underlying a polygenic or multifactorial trait are generally assumed to follow the Mendelian principles of random segregation and independent assortment.<sup>4</sup> The difference is that they act together to influence the trait. Thus, the multifactorial model assumes:



**FIGURE 12-4** Liability distribution for a multifactorial disease. To be affected with the disease, an individual must exceed the threshold.

1. several, but not an unlimited number, of loci are involved in the expression of the trait;
2. the loci act in concert in an additive manner, each adding or subtracting a small amount from the phenotype; and
3. the environment interacts with the genotype to produce the final phenotype.

## 12.3 DETERMINING THE GENETIC COMPONENT OF A TRAIT

Historically, the genetic study of any trait can be divided into four broad categories: familial aggregation, segregation analysis, linkage analysis, and association studies. This paradigm was useful in discovering genes for many monogenic disorders.

### 12.3.1 Familial Aggregation

The first step of any genetic analysis is to establish a genetic component to the disease. In addition, one must establish the relative size of the genetic effect in comparison to other sources of variation, such as common household effect and random environmental effect.

*Familial aggregation* can be established using family-based or twin/adoption studies. Family members share genes and environment; therefore, familial aggregation of the trait could be due to genetic and environment together. In general, very few traits are influenced only by genes or only by the environment. Detection and estimation of familial aggregation is the first step in the genetic analysis of any multifactorial trait. Twin and adoption studies are traditionally used to determine the genetic component of the trait (8–11). As monozygotic

<sup>4</sup>Good descriptions of these principles can be found in the following web pages: <http://biology.about.com/library/weekly/aa110603a.htm> and <http://biology.about.com/library/weekly/aa100903a.htm>.



**TABLE 12-3** Using Twin Concordance and Discordance Rates to Test for a Genetic Component of a Disease

Twins	Concordant Pair	Discordant Pair	Total Pairs
MZ	$n_{11}$	$n_{12}$	$n_{MZ}$
DZ	$n_{21}$	$n_{22}$	$n_{DZ}$
	$n_C$	$n_D$	$n$

$$\chi^2_1 = \frac{n(n_{11}n_{22} - n_{12}n_{21})^2}{n_C n_D n_{MZ} n_{DZ}}$$

(MZ) twins share all their nuclear genes, any difference between them regarding a particular trait should be due solely to environmental effects. If the trait is completely influenced by genes, then MZ twins should have essentially identical expression of the trait. This is not true for dizygotic (DZ) twins because, on average, they share only 50% of their genes.

Twin studies to determine the genetic component of the threshold character are based on comparing *concordance rates* of MZ and DZ twins. If both members of a twin pair have the same status of a dichotomous trait (i.e. either both have the disease or both do not have the disease), they are *concordant*. If they do not share the trait status, they are *discordant*. The concordance rate is the proportion of concordant twin pairs among all those with the trait. Significantly higher concordance rates in MZ twins compared with DZ twins is considered an evidence for a significant genetic component of the disease. The significance of the difference can be easily tested by a  $2 \times 2$  contingency table using a chi-square test (see Table 12-3).

However, concordance rates are not appropriate for continuous traits, so correlation coefficients can be used instead of concordance rates (12).

The method to determine the degree of genetic component of a continuous trait is based on a comparison of the variance of the differences between MZ twins and differences between DZ twins. As MZ twins share all their genes, the variance of the trait between MZ twins ( $V_{MZ}$ ) must be due to environmental variance ( $V_E$ ), so in this case, we have  $V_{MZ} = V_E$ . However, the variance of the trait between the DZ twins ( $V_{DZ}$ ) could be due to both environment ( $V_E$ ) and shared genes ( $V_G$ ). So, genetic variance is  $V_G = V_{DZ} - V_{MZ}$ , and therefore, the *heritability*,  $h^2$ , is defined as

$$h^2 = \frac{V_{DZ} - V_{MZ}}{V_{DZ}}$$

Heritability ranges between 0 and 1, with 0 meaning a solely environmentally determined trait and 1 meaning a completely genetically determined trait.

Adoption studies provide a second familial aggregation strategy for estimating the influence of genes on

multifactorial traits. The strategy consists of comparing disease rates among the adopted offspring of affected parents with the rates among adopted offspring of unaffected parents. Certain biases can influence these studies, namely, (1) parental environment could have long-lasting effects on an adopted child; (2) adoption agencies attempt to match the adoptive parents with natural parents in terms of socioeconomic status; (3) children might be several years old when adopted, introducing the potential for many environmental confounds. Moreover, these studies are reasonably good at estimating additive genetic effects that are not age specific, but poor at estimating nonadditive genetic effects or genetic effects that are expressed differently across the age span.

For continuous traits, the familial aggregation is usually measured by heritability, which is the proportion of variability of the trait explained by genetic variation. Heritability can be defined as either using total genetic effects (sum of additive, dominant, and epistatic effects) or using only additive effects. The former quantity is called *heritability in the broad sense* and is given by

$$h^2 = \frac{\text{Var}(G)}{\text{Var}(T)},$$

where  $\text{Var}(G)$  and  $\text{Var}(T)$  are genetic and total variance, respectively. The latter quantity is called *heritability in the narrow sense* and is given by

$$h^2 = \frac{\text{Var}(A)}{\text{Var}(T)},$$

where  $\text{Var}(A)$  is the additive genetic variance.

One cannot conclude the number of genes or which genes are involved in the etiology of the trait from a heritability estimate. Although the absence of familial aggregation is generally thought to rule out a genetic contribution the trait, there are some unlikely yet plausible scenarios where this is not so, including but not limited to phenotypic competition within families (13) that counters genetic effects, and an extreme form of epistasis referred to by some as *emergence* (14). It is also important to emphasize that heritability is a population-specific estimate, and thus can vary from population to population.

There are many other methods to detect and estimate familial aggregation using family data. For example, the recurrence risk is often used to determine the strength of familial aggregation for a discrete trait. The recurrence risk is the probability that a relative of an affected individual is also affected. The most commonly used measure is the sibling recurrence risk, i.e. the probability that a sibling of an affected individual is also affected. The ratio of the sibling recurrence risk and the overall disease prevalence is called a *sibling relative risk*. It is one of the measures of the magnitude of the genetic contribution to susceptibility for a dichotomous trait (affected versus unaffected). Examination of relative recurrence risk values for various classes of relatives could suggest that

the trait is influenced by multiple loci (15). For a single ascertainment scheme, the sibling recurrence risk can be calculated from sibling data as follows (16):

$$K_s = \frac{\sum_{s=1} \sum_{a=1} (a-1)n_{s(a)}}{\sum_{s=1} \sum_{a=1} (s-1)n_{s(a)}}$$

where  $a$  = number of affected sibs in a sibship,  $s$  = number of siblings in the sibship, and  $n_{s(a)}$  = number of sibships of size  $s$  with  $a$  affected sibs.

Note that the familial aggregation methods given above use only trait information from the sample. Owing to the availability of genome-wide single nucleotide polymorphism (SNP) data, it is now feasible to calculate the heritability using genome-wide SNP markers. One such method was proposed by Visscher et al. (17), who used genome-wide identity-by-descent (IBD) sharing probability between full sibs using genome-wide SNPs data.

### 12.3.2 Segregation Analysis

Once a genetic basis of the trait has been established, traditionally, the next step has been to determine the genetic models that explain the segregation of a phenotype (continuous, dichotomous, or ordinal) in a given familial data set via segregation analysis. Segregation analysis requires phenotypic data on related individuals and does not require any molecular data. Segregation analysis is the statistical methodology to determine whether a model with one or more major genes and/or polygenes (i.e. a set of genes each with small quantitative effect that together produce a phenotype) is consistent with the observed pattern of phenotypic inheritance, and to estimate the parameters of the best-fitting genetic model. It entails determining the mode of inheritance (additive, recessive, or dominant), estimating “disease” allele frequency, and estimating penetrances (probability of being affected given genotype). At one time, segregation analysis was one of the most important tools for genetic analysis of familial data. In the late 1980s, large numbers of DNA markers became available, which rendered segregation analysis less popular.

If the trait is monogenic and thus owing to single major gene effect, segregation analysis has proven to be a very effective tool in determining the parameters for mode of inheritance. Subsequently, these parameters have been used in model-based linkage analysis (see below for more detail) to find the location of putative disease-causing genes. This paradigm has been used successfully for the simple Mendelian traits, in which only one gene is segregating. For multifactorial traits, which may be due to the effects of many genes and environmental effects, estimation of the genetic model may be virtually impossible using segregation analysis.

In order to determine the parameters of the genetic model using segregation analysis, the likelihood of a particular mode of inheritance can be formulated using three types of probability functions. First, there is a

probability distribution for segregation of genotypes among the founders (individuals whose parents are not included in the observed pedigree data), where genotypes of the founders are independently drawn from the population based on the prevalence of the disease and mode of inheritance. Second, there is a probability distribution of the segregating genotypes of the non-founders (individuals with both parents in the pedigree) conditional on their parental genotypes. Third, there are penetrance functions (probability of being affected given a particular genotype). To test whether there is a segregation of a single gene, the likelihood under the assumed genetic model is compared with the likelihood under the null model of segregation with no genetic effect. The more complicated or general model could be included for testing a particular mode of inheritance including polygenic or multifactorial components in modeling the disease. However, the number of possible genetic models with a given mode of inheritance may be too large to make any meaningful inference about the disease model.

### 12.3.3 Linkage Analysis

Genetic linkage analysis is based on the observation that any two loci, which are in close proximity to each other on the same chromosome, will tend to cosegregate among related individuals more often than two random loci in the genome. Thus, the affected individuals sharing a genotype at a putative disease locus would be more likely to share a genotype at linked marker loci. In the absence of linkage, the recombination fraction (i.e.  $\theta$  = the proportion of gametes in which two genes on the same parental chromosome are separated; for more details, see [http://en.wikipedia.org/wiki/Genetic\\_linkage](http://en.wikipedia.org/wiki/Genetic_linkage)) is  $\frac{1}{2}$ ; however, if there is a linkage, the recombination fraction is  $< \frac{1}{2}$ . If we cover the entire human genome using evenly spaced markers across the chromosomes, it will be possible to find marker loci associated with a given trait of interest.

The methods of linkage analysis can be divided into two broad classes: *model-based* (parametric) and *model-free* (sometimes referred to as *nonparametric*) linkage analysis.

**12.3.3.1 Model-Based Analysis.** Model-based methods assume a specific genetic model underlying the trait, and the statistical evidence in favor of linkage with a marker locus is summarized by the maximum value of the *lod score* (18). The lod score is the logarithm of the likelihood ratio of observing a particular set of family data under a specific alternative hypothesis of linkage relative to the null hypothesis of no linkage between disease and marker loci ( $H_0: \theta = \frac{1}{2}$ ). For details of how the likelihood is formulated, see Elston and Stewart (19), Elston and Rao (20), and Lander and Green (21).

This approach has been remarkably successful in identifying genes responsible for Mendelian disorders. In order to calculate such likelihood for families, we must specify a probability model. Several assumptions

are usually made in calculating lod scores: the mode of inheritance underlying the marker and the trait is known, the parameters such as penetrances and allele frequencies at both marker and trait loci are known without error, and all founders are unrelated to each other. Misspecification of any of these assumptions can affect the validity or power of the analysis and can result in an inconsistent estimate of the recombination fraction. Thus, the models used in model-based analysis must approximate the complexity of the disease being investigated. It is noteworthy that incorrect specification of a legitimate model-based linkage test may reduce power but generally does not lead to an inflated Type I error rate (false-positive rate).

**12.3.3.2 Model-Free Linkage Analysis.** The genetic mechanism underlying a complex disease is often unknown, and it is impossible to specify the correct genetic parameters such as mode of inheritance, disease allele frequency, and penetrance in complex diseases. Under these circumstances, model-free linkage analysis, which makes no assumption about the mode of inheritance of the trait, is usually preferred. If a disease susceptibility locus and a marker locus are linked, and, by definition, cosegregating in a family, pairs of relatives who are concordant for the disease (i.e. both affected or neither affected) should share more alleles identical-by-descent (IBD<sup>5</sup>) than will an average pair of relatives with the same degree of kinship. Similarly, discordant (affected–unaffected) relative pairs should share fewer alleles IBD than will an average pair of relatives with the same degree of kinship at the disease locus or marker linked to the disease locus. Model-free methods were first derived for sib pairs (22,23), but were extended to other relative pairs (24,25). The Haseman–Elston method consists of regressing the squared phenotypic difference among siblings within sib pairs on the estimated proportion of alleles the sibs share IBD at a marker locus. A negative slope suggests linkage because it indicates that greater similarity at a trait locus tends to occur with greater similarity at a marker locus. There have been a number of extensions to the Haseman–Elston method to increase its power (26–35). In 2003, *Human Heredity* published special topic issue titled *Recent Advances in the Analysis of Genetic Traits* celebrating the thirtieth anniversary of the seminal paper by Haseman and Elston for quantitative trait linkage analysis (*Human Heredity* 2003, Vol. 55, No. 2–3). Several software programs are available to perform linkage analyses, including S.A.G.E. (36), GENEHUNTER (37), LINKAGE (38–40), Mx (<http://www.vcu.edu/mx/>) and MERLIN (41).

The model-free methods described above mostly utilize relative pairs such as sib pairs. However, large extended families could provide more linkage information than

these relative pairs. Methods based on the variance components framework have become a popular choice for linkage analysis because of ease of modeling covariates and gene–gene or gene–environment interactions, and can utilize large extended families in the model-free environment (42–46). There are several programs available to perform linkage analyses based on variance components methodology such as ACT (43), SOLAR (42), and Mx (<http://www.vcu.edu/mx/>). Mx uses a structural equation modeling approach, which is equivalent to the other types of variance components approaches under most circumstances. There are many more statistical genetics packages freely available for public use, and a complete list along with download information for each package can be found at <http://linkage.rockefeller.edu/soft/> and <http://www.soph.uab.edu/ssg/linkage/lddac>.

### 12.3.4 Transmission Disequilibrium Test and Association Analysis Using Familial Data

In classical genetic studies, the identification of a chromosomal region with linkage analysis is the first step in the gene mapping process. As linkage analysis provides information of a genomic region, a typical quantitative trait locus (QTL) may cover several millions of base pairs and may contain hundreds of genes. The initial detection of the QTL is followed by addition of more markers within the QTL to narrow down the region as much as possible. Once the resolution limit of the linkage approach is reached, the most commonly employed follow-up is to fine map using association analyses with SNPs.

The rationale for association analyses is to confirm the involvement of a putative allele involved in a trait of interest. The rationale of fine mapping is that the greater number of SNPs and the greater sensitivity of the association tests provide more detailed information of the target region. The SNPs located close to a disease locus may cosegregate because of linkage disequilibrium, i.e. allelic association due to linkage. The allelic association forms the theoretical basis for association mapping. Allelic association that is not due to linkage disequilibrium is of no interest in mapping disease genes. The simplest way to test for association is to perform a case–control study, where the cases are the individuals with the disease and the controls are without the disease. Association is then tested by ascertaining whether a particular marker allele is more frequent among the cases than the controls. A significant result will be observed if the marker is in linkage disequilibrium with the disease locus, or from a variety of confounding reasons such as population stratification. Therefore, case–control association studies without controlling for stratification are prone to false-positive results with no biological significance, and for this reason, association studies were not popular until the mid-1990s when methods to account for this stratification were established using familial data (47).

<sup>5</sup>“IBD alleles in an individual or in two people are defined as alleles that are identical because they have both been inherited from the same common ancestor, as opposed to identity-by-state.”—from <http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=hmg>.

To control for population stratification, Spielman et al. (47) proposed the transmission disequilibrium test (TDT), which is a family-based association test (FBAT) in the presence of linkage that controls for population stratification by comparing the allele frequencies among alleles transmitted to an affected offspring to those that are not transmitted to an affected offspring from informative parental matings (i.e. matings with at least one heterozygous parent). This study design requires the collection of family trios that include two parents and an affected offspring. More than 225 extensions and variations of the original TDT have been proposed (see the exhaustive review of TDT procedures in Tiwari et al. (48)). There are a number of software programs available for TDT and/or association analyses using family data such as FBAT (<http://www.biostat.harvard.edu/~fbat/fbat.htm>), ASSOC (<http://darwin.cwru.edu/sage/>), and GASSOC ([http://mayoresearch.mayo.edu/schaid\\_lab/software.cfm](http://mayoresearch.mayo.edu/schaid_lab/software.cfm)). A complete list of association programs can be found at <http://linkage.rockefeller.edu/> and <http://www.soph.uab.edu/ssg.asp?id=1087>.

Once the results from the association analyses are deemed adequate, the next step is to screen the candidate genes for DNA sequence variation by direct sequencing. The relevance of the detected mutations is confirmed with additional association studies in the original and other populations, as well as functional assays in vitro (expression studies in different cell lines) and in vivo (transgenic and knock-out animal models) (49).

## 12.4 THE INTERNATIONAL HapMap PROJECT

In the context presented above, studies progress from estimates of heritability to segregation analysis to linkage and then finally familial association analysis to determine candidate genes for a trait of interest. However, this paradigm has changed recently. With the advent of high-dimensional genotyping technologies using microarrays, the approach for discovering new genetic variants for a disease or trait have changed drastically. In 1996, Lander proposed the common-disease common-variant (CDCV) hypothesis (50). The HapMap project was initiated to create dense set of genetic markers to test the CDCV hypothesis. The CDCV is based on the idea that the genetic component of common diseases is attributable in part to common allelic variants (i.e. alleles with frequency at least 5%). The draft of the complete human genome sequence was completed in 2001 and had strong effect in advances in the genome sequencing technology (51). The International HapMap Project ([www.hapmap.org](http://www.hapmap.org)) was an international partnership that was formed in 2002 to help researchers find genes associated with human disease by providing a public database of common genome-wide human variation across populations (52–54). The first stage of the HapMap project focused on four diverse populations: 30 trios (two parents and adult child) from Yoruba in Ibadan

(90 individuals), Nigeria, 30 trios from the Centre d'Etude du Polymorphisme Humain (CEPH) collection of Utah residents of Northern and Western European ancestry, 45 unrelated individuals from the Han Chinese in Beijing, and 45 unrelated individuals from Japanese in Tokyo. This project genotyped over 1 million SNPs in Phase I and additional 2.1 million in Phase II in the HapMap samples (53,54). It helped initiate advances in SNP array technologies to make genome-wide association studies (GWAS) feasible and affordable. Affymetrix and Illumina SNP arrays became available to researchers, which initially surveyed approximately 100,000 SNPs, and which now survey 2.5 million SNPs. During the most recent phase, HapMap 3, 1184 individuals representing 11 global populations were genotyped for approximately 1.6 million common SNPs (55; <http://www.sanger.ac.uk/resources/downloads/human/hapmap3.html>; <http://www.broadinstitute.org/~debakker/p3.html>; <http://hapmap.ncbi.nlm.nih.gov/>).

As a complement, the 1000 Genomes Project, which began in 2008, was initiated to provide a catalog of low-frequency SNPs and structural and sequence variants in the human genome (56; <http://www.1000genomes.org/>).

## 12.5 GENOME-WIDE ASSOCIATION STUDIES

GWAS is an approach that involves scanning thousands to a few million SNPs across the whole genome on many individuals to find association with a disease or trait. As mentioned earlier, GWAS became a popular choice of genetic studies to detect putative loci associated with a disease or trait because of availability of high-throughput SNP arrays, decreased cost of genotyping, and methods to correct for population stratification (i.e. systematic differences in allele frequencies between subpopulations in a given population possibly because of different ancestry). Before the HapMap era, investigators were reluctant to conduct association studies using population data because of concerns about population stratification. For example, in case-control studies, we usually test association of a particular SNP by comparing allele frequency between cases and controls. Allele frequencies are known to vary within and between populations depending on genetic ancestry (53,57). Genetic ancestry becomes a confounding variable leading to spurious associations if allele frequencies are different within or between race/ethnic groups. Methods for correcting population substructure are described later.

### 12.5.1 Study Designs

Any type of data set such as pedigree data, case-control data, or population data are all appropriate choices for GWAS, but one has to adjust for familial correlations in pedigree data and population stratification in population or case-control data sets to control for the confounding



due to relatedness or population substructure. Case-control and population data have been commonly used for GWAS because of availability and convenience of ascertainment. There are some issues associated with the case-control design. If the disease is heterogenous, extra attention should be paid to minimize heterogeneity in case selection, e.g. selecting the most extreme cases or selecting individuals from a familial disease cohort. There has been a controversy in how to select optimal controls. Usually, controls from the same population and residing in same geographic area are preferred, but these can be difficult to ascertain. The Wellcome Trust Case Control Consortium used 3000 UK controls and 2000 cases from each of seven different diseases and showed that using common controls was effective, had minimal impact on genotypic distributions, and did not lead to excess false positives (58,59). Misclassification error in control selection could affect the power of the association analysis. Specifically, this is true for late-onset diseases because controls have not yet reached the age to develop the disease; this issue can be resolved by increasing the sample size (58). Population stratification and cryptic relatedness (i.e. relatedness among the individuals in the study that is not known to the investigator) can also increase the false-positive findings as previously discussed. The family-based association studies are robust to population stratification, but it is difficult to ascertain all pedigree members, which leads to missing data within families and loss of power compared to case-control designs (60). There are other issues with study design selection; an excellent review is provided by McCarthy et al. (58).

### 12.5.2 Quality Control

The first step of GWAS analysis is the QC of the genotypic and phenotypic data. There are a number of procedures needed to ensure the quality of genotype data both at the genotyping laboratory and after calling genotypes using statistical approaches. Here, we assume that the genotyping laboratory has used best practices to remove technical variation, and we present only the statistical methods that are used after completion of the genotyping. The QC and association analysis of GWAS data can be performed using the robust, freely available, and open source software PLINK developed by Purcell et al. (61). Two recent publications provide excellent reviews of the QC protocol for GWAS data (62,63). Here, we provide few important steps of the QC in GWAS in similar guidelines as in Laurie et al. (62) and Turner et al. (63). Note that the current genotyping technology is very reliable, but there are still some possibilities of errors when genotyping large number of SNPs.

### 12.5.3 Sex Inconsistency

It is possible that self-reported sex of the individual is incorrect. Sex inconsistency can be checked by comparing

the reported sex of each individual with predicted sex by using X-chromosome markers' heterozygosity to determine the sex of the individual empirically.

### 12.5.4 Relatedness and Mendelian Errors

Another kind of error that can occur in genotyping is due to sample mix-up, cryptic relatedness, duplications, and pedigree errors such as self-reported relationships that are not accurate. To detect sample relatedness, one can calculate three IBD probabilities of sharing 0, 1, and 2 alleles that are identical-by-descent for each pair of individuals using software such as PLINK and a kinship coefficient matrix. Individuals sharing zero alleles at every locus are unrelated, individuals sharing one allele IBD at every locus are parent-offspring pairs, individuals sharing two alleles IBD at every locus are MZ twins or a duplicated sample, and on average, sib pairs share 0, 1, and 2 alleles IBD with sharing probabilities 0.25, 0.5, and 0.25, respectively. The relationship errors can be corrected by consulting with the self-reported relationships and/or using inferred genetic relationships. Cryptic relatedness can inflate the variance of the test statistic (e.g. if the test statistic is the difference in the overall allele counts between case and control samples in a trend test (64)). The presence of cryptic relatedness in case-control studies increases the false positives in association analysis. Devlin and Roeder provided a method to correct for the variance inflation (see References (64) and (65) for details).

### 12.5.5 Batch Effects

For GWAS, samples are processed together for genotyping in a batch. The size and composition of the sample batch depends on the type of the commercial array; for example, an affymetrix array can genotype up to 96 samples, and an Illumina array can genotype up to 24 samples. To minimize batch effects, samples should be randomly assigned to plates with different phenotypes, sex, race, and ethnicity. The downstream association study can be confounded by the batch effects. There are several methods available to detect any batch effects. The most commonly used method is to compare the average minor allele frequencies (MAFs) and average genotyping call rates across all SNPs for each plate. Most genotyping laboratories perform batch effect detection and usually re genotype the data if there is a batch effect or a plate is discarded when there is a large amount of missing data.

### 12.5.6 Marker and Sample Genotyping Efficiency or Call Rate

*Marker genotyping efficiency* is defined as the proportion of samples with a genotype call for each marker. If large numbers of samples is not called for a particular marker, that is, an indication of a poor assay and the marker

should be removed from further analysis. A threshold for removing markers varies from study to study depending on the sample size of the study. Usually recommended call rates are 98–99%. If the quality of the DNA sample is poor, it leads to low call rate of genotypes for the individual, i.e. the number of missing genotypes will be large and the sample should be excluded from further analysis. Before performing the association analysis, one should filter out the samples and markers using some threshold for marker and sample call rates.

### 12.5.7 Population Stratification

There are a number of methods proposed to correct for population substructure. Three commonly used methods to correct for the underlying variation in allele frequencies that leads to confounding due to population stratification are genomic control (4,64–71), structured association testing (72–74), and principal components (PCs) (75,76). The genomic control method controls for confounding due to substructure by estimating an inflation factor (ratio of the variance of the test statistic to the variance under the null hypothesis), then adjusting the test statistics for all markers in GWAS downward by the inflation factor. Usually, the inflation factor is calculated using a few hundred loci. Structure association testing (72,77) (1) estimates ancestry proportions of each individual from the founding population using markers with different allele frequencies in the founder population; and (2) uses these proportions to cluster individuals to create homogenous groups with similar ancestry profile for the association analysis. Principal components analysis (PCA) uses thousands of markers to detect population stratification and PCs then can be used to correct for stratification by modeling PCs as covariates in the model (75,76). PCs can be calculated using a program Eigenstrat (75,76). There are two issues with using PCA: (1) how many SNPs to use, and (2) how many PCs should be included as covariates in the association analysis.

### 12.5.8 Marker Allele Frequency and Hardy–Weinberg Equilibrium Filter

The Hardy–Weinberg equilibrium (HWE) test compares the observed genotypic proportion at the marker versus the expected proportion. Deviation from HWE at a marker locus can be due to population stratification, inbreeding, selection, nonrandom mating, genotyping error, actual association to the disease or trait under study, or a deletion or duplication polymorphism. However, HWE is typically used to detect genotyping errors. SNPs that do not meet HWE at a certain threshold of significance are usually excluded from further association analysis. It is also important to discard SNPs based on MAF. Most GWAS studies are powered to detect a disease association with common SNPs ( $MAF \geq 0.05$ ).

The rare SNPs may lead to spurious results because of the small number of homozygotes for the minor allele, genotyping errors, or population stratification.

## 12.6 ASSOCIATION METHODS/ STATISTICAL ANALYSIS

### 12.6.1 Discovery Phase of the GWAS

Discovery phase consists of scanning the genome with at least 1000 cases and 1000 controls with an appropriate statistical test. The choice of the statistical test for association depends on the study design and the phenotype under consideration. In the case-control design, the goal is to compare the allele or genotypic frequencies between cases (affected) and controls (normal). This can be tested with Pearson's chi-square test, Fisher's exact test, or the Cochran–Armitage test. Pearson's chi-square tests the null hypothesis of no association between rows and columns of the  $2 \times 3$  contingency table consisting of the counts of the three genotypes among cases and controls (78). Fisher's exact test is similar to the Pearson's test, but the deviation from the null hypothesis is calculated exactly from all possible permutations of the data, and thus, does not assume the asymptotic property as Pearson's test (79). The Cochran–Armitage test for trend is a test of proportions of cases versus controls (80–82) and assumes an additive mode of inheritance that is a linear trend. There is a loss of power if the trend is not linear. Freidlin et al. (83) recommended using a maximum of the test statistics obtained from additive, dominant, or recessive effects models. Note that in the above statistical procedures, one cannot model covariates such as sex, age, race, age of onset, PCs (PCs from admixture), and so on. To accommodate any relevant covariates in the analysis, one can use logistic regression. Logistic regression is more flexible in that it can model covariates, multiple SNPs as main effects, SNP by SNP interactions, SNP by environment interactions, and so on. If the phenotype is continuous, analysis of variance and general linear model approaches can be employed. One can also use a linear regression framework if extremes of the distribution are used to define case and control status. Huang and Lin have given an efficient association method using extreme phenotypes (84). The analysis of familial data requires correcting for the dependency of observations. The notable methods include linear mixed model (85–87), FBAT (see review by Laird and Lange (88)), or ASSOC (a module of S.A.G.E. software suite (36)). After scanning 1–2 million SNPs (i.e. 1–2 million statistical tests) to determine significant associations, appropriate multiple testing correction is required to control for false positives and also in choosing SNPs for follow-up studies. The guideline for significant association for GWAS is generally a  $p$  value of  $\sim 5 \times 10^{-8}$  (60,89), but, it is common practice to use a higher  $p$  value threshold for follow-up study or replication. Balding (90) provides a

comprehensive discussion of the advantages and disadvantages of these methods pertaining to GWAS.

### 12.6.2 Validation and Replication Phase

Some investigators have recommended reanalysis of the original discovery phase GWAS data using a different genotype platform for validation, which has been termed *technical validation* (58). Technical validation allows detection of technical errors in genotyping that might give rise to spurious association signals or false positives, but given limitations of the resources available to investigator, it may not be feasible. The replication phase or follow-up study is one of the most challenging aspects of the GWAS and is required to control for false positives. Replication in an independent data set with similar genetic background and phenotype is warranted. Usually several hundreds or a few thousands of SNPs are tested in a replication set, depending on the threshold used for significant association  $p$  value. The statistical methods are the same as in the discovery phase, depending on the study design and type of phenotype.

## 12.7 ANALYSIS OF RARE VARIANTS USING NEW TECHNOLOGIES

Introduction of the HapMap project and large-scale GWAS studies were driven by the “common disease, common variant” (CVCD) hypothesis that was first introduced in the 1990s (50,91). The CDCV is based on the idea that the genetic component of common diseases is attributable in part to allelic variants that are present in more than 5% of the population. An extension of this hypothesis is that the same variants will be responsible for the disease across multiple populations (92). The early success of GWAS (age-related macular degeneration) seemed to support the theory that a large proportion of the genetic variants underlying complex disease could be explained by the CDCV. It is now becoming apparent that many common variants confer only a small portion of risk individually and also explain a small portion of the heritability of common complex diseases (93). While GWAS have been successful in many ways, identifying hundreds of variants for a large number of traits (<http://www.genome.gov/26525384>), there still remains a large proportion of heritability that has yet to be explained.

When the CDCV hypothesis was first introduced, it was not without contention (94). One of the strongest counter arguments was based on the hypothesis of “common disease, rare variant,” (CDRV) which is in essence the antithesis of the CDCV hypothesis (77,95). The rare variant hypothesis proposes that common complex diseases are due to the combined effect of multiple rare variants with moderate to low individual risk. Unlike CVCD, it is generally thought that owing to population history, these rare variants will be population specific (96). It is only recently, with the availability of affordable large-scale sequencing

technology and advances in analytical methods (discussed below), that scientists have gained the ability to address the role of CDRV in human disease. In reality, it is likely that the genetic basis of complex disease is somewhere between the two extremes, with multiple genes interacting together with a variety of common and rare variants and other genetic and environmental factors (97).

As new high-throughput, massively parallel sequencing technologies emerged in 2005, direct sequencing became commonly used to directly interrogate whole genomic sequences for association with disease without prior specification of SNPs currently available on commercial SNP chips (98). Such technologies overcome some of the shortcomings of GWAS methods, such as ascertainment bias in the set of currently available SNPs, and the ability to assay rare or private variants. In addition, greater flexibility exists in the search for variants other than SNPs, such as copy number variants, insertions or deletions or indels, inversions, and so on. Whole exome sequencing, in which only the sequence of exons are assayed, has been used to discover causal mutations in a number of Mendelian disorders such as Miller’s syndrome (99) and Hereditary Spastic Paraparesis (100). Because of the enormous number of variants introduced from new sequencing technologies and the small sample sizes typically present, new bioinformatic and statistical methods have been developed to reduce the dimensionality and improve the probability of detection of causal variants. Prior bioinformatic processing may include filtering by IBD methods if family data is present (101), or filtering based on the expected mode of inheritance (99). In addition, if only rare variants are desired, then common variants can be filtered out using dbSNP (102), and in addition, predicted functional variants (nonsense, missense, splice site variants, indels, frameshift mutations, etc.) can be discerned using tools such as SIFT and PolyPhen (102,103). Once likely non-functional variants have been filtered out, new statistical methods for summarizing the effect of multiple rare variants at a single gene can be applied. Some examples of these methods include the cohort allelic sums test method, which compares the number of individuals with mutations within a gene between cases and controls (104), the combined multivariate and collapsing (105), which collapses multiple rare variants in conjunction with common variants using multivariate analysis, methods that weight the counts of each variant using the estimated standard deviation of the total number of mutations (106,107), or a method that models these weights in a flexible Bayesian framework (108). A review of recent methods is given in reference (109). Whole-exome and whole-genome studies (in which the contribution of noncoding regions to disease can be assayed) are currently underway for complex (multifactorial) diseases. The next couple of years will show if these technologies can help to fill in the gaps from GWAS studies, termed *missing heritability* (93), and in identifying causal variants underlying multifactorial diseases. In addition, new sequencing technologies

offer opportunities for functional characterization studies such as gene expression profiling using next generation sequencing (110), epigenetic profiling (111), and in identifying somatic mutations occurring in cancer (112–114).

## 12.8 INTEGRATION OF GENETIC, GENOMIC, AND FUNCTIONAL DATA FOR ANALYSIS OF MULTIFACTORIAL DISEASES

Genes do not work in isolation, but instead work in concert to carry out complex biological functions (115). The field of “Systems Biology” is an emerging area of research that aims to study these complex interactions (116–118). Following the Central Dogma of molecular biology (DNA→RNA→Protein), the ability to integrate genetic data with other types of information (e.g. gene expression, protein interaction, etc.) is essential to our ability to understand the underlying complex etiology. A systems biology approach to complex diseases is possible in part due to the ever increasing amount of omics data (e.g. genomics, proteomics, metabolomics, etc.) that is becoming available in public databases, the scientific literature, and elsewhere. There are a wealth of methods that have been developed, or are being developed, that are designed to utilize this wealth of data to help prioritize and interpret results of genetic association studies. One of the most popular types of methods is *pathway analysis*, often referred to as *gene set analysis*, which can be broadly defined as methods that use preexisting information about SNPs, genes, transcripts, or other genomic information to interrogate subsets (e.g. pathways) of genomic variables as they relate to a complex trait or disease. These methods were first developed for genome-wide gene expression studies, but have recently been applied to GWAS, and will have great utility in sequencing-based studies. These methods typically examine test statistics to determine if the members of a group of genes are enriched for association with a trait (e.g. gene set enrichment analysis (119,120)), or to test if the group itself is associated with the trait (e.g. gene set ridge regression in association studies, (121,122)). For additional information on these methods, see references (123–129). Inclusion of prior network knowledge or de novo network discovery in conjunction with pathway methods has also been shown to further improve genomic data discoveries, with significant examples in cancer as exemplified in (130–132).

## 12.9 CONCLUSIONS

Genetic modeling is a challenging art and science. Advances in molecular technology, statistical methodology,<sup>6</sup> and increasing availability of large

samples allow many new investigations to be undertaken on unprecedented scales. Interpretation of the resulting findings remains both difficult and one of the more exciting challenges facing today’s biomedical researchers.

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<sup>6</sup>A steady stream of videos offering tutelage on these advances can be freely seen at: <http://www.soph.uab.edu/ssg/courses/ssgseminars>.



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**T Mark Beasley, PhD.** Dr Beasley is an associate professor and has been a faculty member of the UAB Department of Biostatistics and the Section on Statistical Genetics (SSG) since 2001. Dr Beasley has published over 30 articles in the area of statistical methodology, focused in five major areas: (1) methodological problems in statistical genetics; (2) nonparametric statistics; (3) simulation studies; (4) the use of linear models; (5) longitudinal analysis, and (6) mediation analysis. He has also coauthored over 30 applied research articles in a variety of disciplines including education, psychology, medicine, genetics, and gerontology, pharmacology.



**Hemant K Tiwari, PhD.** Dr Tiwari is William “Student” Sealy Gosset Professor in Biostatistics and Interim Head of the Section on Statistical Genetics in the School of Public Health at the University of Alabama at Birmingham. His research interests include genetic linkage analysis, disequilibrium mapping, genome-wide association studies (GWAS), population genetics, structural variations in the genome, pharmacogenetics/pharmacogenomics, and bioinformatics. At present, he is involved in gene mapping studies of rheumatoid arthritis, SLE, cardiovascular diseases, and obesity-related traits.



# CHAPTER 13

## Population Genetics

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### 13.1 INTRODUCTION

With the monumental scientific advances that have resulted from the Human Genome Project, the genetic composition of populations can now be examined in detail. Thousands of rare alleles are known to be disease-causing mutations and for most of these diseases (e.g. cystic fibrosis, Tay–Sachs, phenylketonuria (PKU), hemophilia A, and familial hypercholesterolemia), many different mutations are found within the same gene. In general, the frequencies of these rare disease alleles differ among populations as do the frequencies of common alleles (>1%), many of which are associated with common diseases such as Crohn's disease, diabetes, coronary disease, celiac disease, multiple sclerosis and macular degeneration. The principles of population genetics attempt to explain the genetic diversity in present populations and the changes in allele and genotype frequencies over time. Population genetic studies facilitate the identification of alleles associated with disease risk and provide insight into the effect of medical intervention on the population frequency of a disease. Allele and genotype frequencies depend on factors such as mating patterns, population size and distribution, mutation, migration, and selection. By making specific assumptions about these factors, the Hardy–Weinberg law, a fundamental principle of population genetics, provides a model for calculating genotype frequencies from allele frequencies for a random mating population in equilibrium.

### 13.2 HARDY–WEINBERG LAW

The allele frequencies at a locus can always be calculated from the genotype frequencies, but the converse is not necessarily true. The Hardy–Weinberg law states that for a single autosomal locus in a large population in which (1) mating takes place at random with respect to genotype, (2) allele frequencies are the same in males and females, and (3) mutation, selection, and migration are negligible, genotype frequencies can be calculated from

allele frequencies after one generation regardless of the allele and genotype frequencies in the initial population. This is not true for a single X-linked locus or for any set of loci considered jointly; for these loci, the establishment of this relationship between allele and genotype frequencies takes more than one generation.

#### 13.2.1 Autosomal Locus

Consider a locus with two alleles,  $A_1$  and  $A_2$ , and suppose the population frequencies of the three genotypes  $A_1A_1$ ,  $A_1A_2$ ,  $A_2A_2$  are  $p_{11}$ ,  $p_{12}$ ,  $p_{22}$ , respectively, where  $p_{11} + p_{12} + p_{22} = 1$ , then, in this initial population, the frequency of  $A_1$  is  $p_{11} + \frac{1}{2}p_{12}$  and the frequency of  $A_2$  is  $p_{22} + \frac{1}{2}p_{12}$ . Random mating is approximately equivalent to random union of gametes. Thus, random mating within this initial population results in the following genotype frequencies in the next generation:

$$\text{Frequency of } A_1A_1 = \left(p_{11} + \frac{1}{2}p_{12}\right)^2$$

$$\text{Frequency of } A_1A_2 = 2\left(p_{11} + \frac{1}{2}p_{12}\right)\left(p_{22} + \frac{1}{2}p_{12}\right)$$

$$\text{Frequency of } A_2A_2 = \left(p_{22} + \frac{1}{2}p_{12}\right)^2$$

The genotype frequencies in this second generation may be different from those in the first generation. However, calculation of the allele frequencies from the genotype frequencies in the second generation gives

$$\begin{aligned} \text{Frequency of } A_1 &= \left(p_{11} + \frac{1}{2}p_{12}\right)^2 + \left(p_{11} + \frac{1}{2}p_{12}\right)\left(p_{22} + \frac{1}{2}p_{12}\right) \\ &= p_{11}\left(p_{11} + p_{12} + p_{22}\right) + \frac{1}{2}p_{12}\left(p_{11} + p_{12} + p_{22}\right) \\ &= p_{11} + \frac{1}{2}p_{12} \end{aligned}$$

Similarly, the frequency of  $A_2$  is  $p_{22} + \frac{1}{2}p_{12}$ , which is equal to  $1 - (p_{11} + \frac{1}{2}p_{12})$ . These allele frequencies

are identical to those in the first generation. In other words, if the allele frequencies are  $p = p_{11} + \frac{1}{2}p_{12}$  and  $q = 1 - p = p_{22} + \frac{1}{2}p_{12}$ , then after one generation of random mating, the genotype frequencies are  $p^2$ ,  $2pq$ , and  $q^2$ . These frequencies are the Hardy–Weinberg proportions, and the population is said to be in Hardy–Weinberg equilibrium.

Table 13-1 presents a numerical example, in which the initial population comprises 20, 40, and 140 individuals with genotypes  $A_1A_1$ ,  $A_1A_2$ , and  $A_2A_2$ , respectively. The genotype frequencies are

$$\text{Frequency } A_1A_1 = p_{11} = 20/200 = 0.10$$

$$\text{Frequency } A_1A_2 = p_{12} = 40/200 = 0.20$$

$$\text{Frequency } A_2A_2 = p_{22} = 140/200 = 0.70$$

and, therefore, the allele frequencies are

$$\text{Frequency } A_1 = 0.10 + \frac{1}{2}(0.20) = 0.2$$

$$\text{Frequency } A_2 = 0.70 + \frac{1}{2}(0.20) = 0.8$$

Random union of gametes results in the following genotype frequencies in the next generation:

$$\text{Frequency } A_1A_1 = 0.2^2 = 0.04$$

$$\text{Frequency } A_1A_2 = 2(0.2)(0.8) = 0.32$$

$$\text{Frequency } A_2A_2 = 0.8^2 = 0.64$$

Note that these genotype frequencies are different from those in the initial population. To confirm that these results are correct, Table 13-1 shows the genotype frequencies in the offspring that result from each of the six possible mating types. For example, all the offspring of the mating  $A_1A_1 \times A_1A_1$  must be  $A_1A_1$ , while for the mating type  $A_1A_1 \times A_1A_2$ , each of the two offspring genotypes,  $A_1A_1$  and  $A_1A_2$ , has a probability of one half. (Table 13-1) Summing-up the columns in Table 13-1 gives the frequencies of each of the genotypes in the second generation. These frequencies are the same as those obtained by random union of gametes, and the allele frequencies calculated from these genotype frequencies are

$$\text{Frequency } A_1 = 0.04 + \frac{1}{2}(0.32) = 0.2$$

$$\text{Frequency } A_2 = 0.64 + \frac{1}{2}(0.32) = 0.8$$

Repeating these steps will give identical genotype and allele frequencies in the third generation to those in the second generation. Note that only the genotype frequencies change in the establishment of equilibrium; the allele frequencies in the initial population remain the same in subsequent generations.

The chi-square goodness-of-fit test may be used to determine whether the observed numbers of each genotype are significantly different from those expected under Hardy–Weinberg equilibrium. The total number of individuals is 200, so the expected numbers for the three genotypes are  $(0.2)^2 200 = 8$ ,  $2(0.2)(0.8) 200 = 64$ , and  $(0.8)^2 200 = 128$ , compared with the observed numbers of 20, 40, and 140. The test value is  $(20 - 8)^2/8 + (40 - 64)^2/64 + (140 - 128)^2/128 = 28$ . This value is compared to the chi-square distribution with one degree of freedom. (There are three classes, but the total number of individuals is known and also the allele frequencies are known. Thus, there is only one independent class and one degree of freedom.) In general, the number of degrees of freedom is equal to the number of genotypes minus the number of alleles. The 99.9th percentile of the chi-square distribution with one degree of freedom is 10.83.

Thus, the observed numbers of each genotype in the initial population are significantly different at the 1% level from those expected under Hardy–Weinberg equilibrium. However, after one generation of random mating, the observed and expected numbers are the same.

Calculation of allele frequencies from genotype frequencies is straightforward when all three genotypes are observable, but, in the case of recessive diseases, such as cystic fibrosis, only two phenotype classes are observed. However, if equilibrium is assumed, the frequency of affected individuals is  $q^2$ ; thus, the square root of this frequency is the frequency of the disease allele. The frequency of heterozygotes (carriers) is  $2(1 - q)q$ , and the proportion of carriers among unaffected individuals in the population is

$$[2(1 - q)q] / (1 - q^2) = 2q / (1 + q)$$

**TABLE 13-1** Table Establishment of Equilibrium in One Generation for an Autosomal Locus

	Mating Frequency	Offspring Genotype Frequencies		
		$A_1A_1$	$A_1A_2$	$A_2A_2$
$A_1A_1 \times A_1A_1$	$(0.1)^2$	$(0.1)^2$	0	0
$A_1A_1 \times A_1A_2$	$2(0.1)(0.2)$	$(0.1)(0.2)$	$(0.1)(0.2)$	0
$A_1A_1 \times A_2A_2$	$2(0.1)(0.7)$	0	$2(0.1)(0.7)$	0
$A_1A_2 \times A_1A_2$	$(0.2)^2$	$1/4(0.2)^2$	$1/2(0.2)^2$	$1/4(0.2)^2$
$A_1A_2 \times A_2A_2$	$2(0.2)(0.7)$	0	$(0.2)(0.7)$	$(0.2)(0.7)$
$A_2A_2 \times A_2A_2$	$(0.7)^2$	0	0	$(0.7)^2$
Total	1	0.04	0.32	.64

For example, in populations of European ancestry, the frequency of cystic fibrosis is estimated to be 1/2000; thus, the frequency of the abnormal allele is 0.022 and the normal allele is 0.978. The frequency of heterozygotes is therefore  $2 \times 0.022 \times 0.978$ , which is about 1/23. That is, approximately 4% of the populations are carriers, but less than 0.1% are affected. Several different mutations have been described in the cystic fibrosis gene. Each one of these is a disease allele; thus, the frequency of 0.022 is actually the sum of the frequencies of all the disease alleles in the cystic fibrosis gene.

The Hardy–Weinberg principle may be extended to more than two alleles. In general, for  $n$  alleles,  $A_1, A_2, \dots, A_n$ , with frequencies  $p_1, p_2, \dots, p_n$ , the genotype frequencies are  $p_i^2$  for homozygotes  $A_iA_i$  and  $2p_i p_j$  for heterozygotes  $A_iA_j$ . The heterozygosity value ( $H$ ) for a locus is the total frequency of heterozygotes, and it may be written as

$$H = \sum 2p_i p_j = 1 - \sum p_i^2$$

For two alleles, the maximum heterozygosity is 0.5, for five alleles it is 0.8, and for 10 alleles it is 0.9. In other words, for a locus to have a heterozygosity of 80%, it must have at least five alleles. (The maximum heterozygosity is reached when the alleles have equal frequencies.)

### Example

Suppose a locus has five alleles (designated 1, 2, 3, 4, 5) with frequencies 0.5, 0.3, 0.1, 0.08, 0.02. What are the genotype frequencies when Hardy–Weinberg equilibrium is established? What is the heterozygosity value ( $H$ ) at this locus?

With  $n$  alleles, there are  $n(n+1)/2$  genotypes. Thus, for five alleles there are 15 genotypes. The frequencies of the five homozygotes, 1–1, 2–2, 3–3, 4–4, 5–5, are 0.25, 0.09, 0.01, 0.0064, 0.0004, respectively. The frequencies of the 10 heterozygotes, 1–2, 1–3, 1–4, 1–5, 2–3, 2–4, 2–5, 3–4, 3–5, 4–5, are 0.3, 0.1, 0.08, 0.02, 0.06, 0.048, 0.012, 0.016, 0.004, 0.0032, respectively.

Heterozygosity ( $H$ )

$$= 1 - (0.25 + 0.09 + 0.01 + 0.0064 + 0.0004) \\ = 0.6432$$

## 13.2.2 X-Linked Locus

The genotype frequencies at a locus on the X chromosome differ in the two sexes because males have only one X chromosome, whereas females have two X chromosomes. Thus, in males the genotype frequency is equal to the allele frequency. For Hardy–Weinberg equilibrium, the allele frequencies in males must be equal to those in females. Suppose the frequency of the  $A_1$  allele is  $p_m$  in males and  $p_f$  in females in the first generation. By the principles of X-linked inheritance, the frequency of this allele in males in the second generation must be  $p_f$  because males get their X chromosomes from their mothers. By contrast, for females in the second generation the frequency of the  $A_1$  allele is  $\frac{1}{2}(p_m + p_f)$  because females

get one X chromosome from each parent. The difference between the male and female frequencies in this generation is  $p_f - \frac{1}{2}(p_m + p_f) = \frac{1}{2}(p_f - p_m)$ , which is one half of the difference in the first generation. Similarly, in the third generation, the male allele frequency is  $\frac{1}{2}(p_m + p_f)$ , while the female frequency is  $\frac{1}{2}p_m + \frac{3}{4}p_f$ , and the difference is  $\frac{1}{4}(p_f - p_m)$ . With each generation, the difference between the male and female frequencies becomes smaller, and equilibrium is reached when they are the same. The equilibrium allele frequency of  $A_1$  is equal to  $\frac{2}{3}p_f + \frac{1}{3}p_m$  in both sexes. Table 13-2 shows the approach to equilibrium for an X-linked locus when the initial allele frequencies are 0.33 in males and 0.57 in females. With each generation, the difference between the frequencies in males and females is reduced, and they approach the equilibrium frequency of  $\frac{2}{3}(0.33) + \frac{1}{3}(0.57) = 0.49$ . If the frequencies of the two alleles at the locus ( $A_1$  and  $A_2$ ) are  $p = \frac{1}{3}p_m + \frac{2}{3}p_f$  and  $q = 1 - p$ , the equilibrium genotype frequencies are  $p$  and  $q$  in males and  $p^2$ ,  $2pq$ , and  $q^2$  in females. Table 13-3 gives the frequency of each possible mating type and the expected offspring genotype frequencies for males and females. Summing-up these genotype frequencies shows that the equilibrium frequencies are maintained in the next generation (Table 13-3).

### Example

Suppose the frequency of an allele at an X-linked locus is 0.03 in males and 0.06 in females. What is the equilibrium allele frequency? Furthermore, suppose that this allele is responsible for a recessive trait. What are the equilibrium frequencies of this trait in males and females, and what is the frequency of heterozygous (carrier) females?

The equilibrium frequency of the allele is  $\frac{2}{3}(0.03) + \frac{1}{3}(0.06) = 0.05$ . Thus, the frequency of males with the trait is 0.05 and the frequency of females with the trait is  $(0.05)^2 = 0.0025$ . The frequency of carrier females is  $2(0.05)(0.95) = 0.095$ .

**TABLE 13-2** Approach to Equilibrium for a Locus on the X Chromosome

Generation	$p_m$	$p_f$
0	0.33	0.57
1	0.57	0.45
2	0.45	0.51
3	0.51	0.48
4	0.48	0.495
5	0.495	0.4875
6	0.4875	0.49125
7	0.49125	0.489375
8	0.489375	0.4903125
9	0.4903125	0.48984375
10	0.48984375	0.490078125
11	0.490078125	0.4899609375
12	0.4899609375	0.49001953125
·		
·		
·		
Equilibrium	0.49	0.49

**TABLE 13-3** Genotype Equilibrium Frequencies for an X-Linked Locus

Offspring Genotype Frequencies						
	Mating Frequency	Male		Female		
		$A_1$	$A_2$	$A_1A_1$	$A_1A_2$	$A_2A_2$
$A_1 \times A_1A_1$	$p^3$	$p^3$	0	$p^3$	0	0
$A_1 \times A_1A_2$	$2p^2q$	$p^2q$	$p^2q$	$p^2q$	$p^2q$	0
$A_1 \times A_2A_2$	$pq^2$	0	$pq^2$	0	$pq^2$	0
$A_2 \times A_1A_1$	$p^2q$	$p^2q$	0	0	$p^2q$	0
$A_2 \times A_1A_2$	$2pq^2$	$pq^2$	0	0	$pq^2$	$pq^2$
$A_2 \times A_2A_2$	$q^3$	0	$q^3$	0	0	$q^3$
Total		$p$	$q$	$p^2$	$2pq$	$q^2$

**TABLE 13-4** Joint Genotype Frequencies for Two Loci

Genotype	Frequency	Equilibrium Frequency
$A_1A_1B_1B_1$	$g_{11}^2$	$p_1^2q_1^2$
$A_1A_1B_1B_2$	$2g_{11}g_{12}$	$2p_1^2q_1q_2$
$A_1A_1B_2B_2$	$g_{12}^2$	$p_1^2q_2^2$
$A_1A_2B_1B_1$	$2g_{11}g_{21}$	$2p_1p_2q_1^2$
$A_1A_2B_1B_2$	$2g_{11}g_{22} + 2g_{12}g_{21}$	$4p_1p_2q_1q_2$
$A_1A_2B_2B_2$	$2g_{12}g_{22}$	$2p_1p_2q_2^2$
$A_2A_2B_1B_1$	$g_{21}^2$	$p_2^2q_1^2$
$A_2A_2B_1B_2$	$2g_{21}g_{22}$	$2p_2^2q_1q_2$
$A_2A_2B_2B_2$	$g_{22}^2$	$p_2^2q_2^2$

### 13.2.3 Two Loci

Equilibrium is reached after one generation of random mating for a single autosomal locus and over several generations for an X-linked locus. However, the approach to equilibrium may be much longer for two loci considered jointly, and the number of generations depends on the recombination fraction. Suppose the first locus has alleles  $A_1$  and  $A_2$ , with frequencies  $p_1$  and  $p_2$ , and the second locus has alleles  $B_1$  and  $B_2$ , with frequencies  $q_1$  and  $q_2$ , respectively. The four possible gametes are  $A_1B_1$ ,  $A_1B_2$ ,  $A_2B_1$ ,  $A_2B_2$ ; let their frequencies in the population be  $g_{11}$ ,  $g_{12}$ ,  $g_{21}$ ,  $g_{22}$ , where  $p_1 = g_{11} + g_{12}$ ,  $p_2 = g_{21} + g_{22}$ ,  $q_1 = g_{11} + g_{21}$ , and  $q_2 = g_{12} + g_{22}$ . Allowing these gametes to unite at random gives the genotype frequencies in the next generation (Table 13-4). Now consider the gametic output of this population. In doing so, we must take into account the fact that the frequency of gametes produced by the double heterozygote ( $A_1A_2B_1B_2$ ) depends on the recombination fraction,  $\theta$  (Table 13-4). If the phase is  $A_1B_1/A_2B_2$ , then  $A_1B_1$  and  $A_2B_2$  are nonrecombinants, and  $A_1B_2$  and  $A_2B_1$  are recombinants. Conversely, if phase is  $A_1B_2/A_2B_1$ , then  $A_1B_2$  and  $A_2B_1$  are nonrecombinants, and  $A_1B_1$  and  $A_2B_2$  are recombinants. Therefore, the frequency of  $A_1B_1$  gametes from double heterozygotes

is  $g_{11}g_{22}(1 - \theta) + g_{12}g_{21}\theta$ . In addition, all the gametes produced by individuals with the genotype  $A_1A_1B_1B_1$ , and one half of those produced by individuals with the genotypes  $A_1A_1B_1B_2$  and  $A_1A_2B_1B_1$  will be  $A_1B_1$ . Thus, the total frequency of  $A_1B_1$  gametes in this generation is  $g_{11}^2 + g_{11}g_{12} + g_{11}g_{21} + g_{11}g_{22}(1 - \theta) + g_{12}g_{21}\theta$ , which may be written as  $g_{11} - \theta D$ , where  $D = g_{11}g_{22} - g_{12}g_{21}$ .  $D$  is called the coefficient of linkage disequilibrium (LD) and is a measure of allelic association. Similar calculations may be done for each of the gametic types, and the frequencies obtained are  $g_{12} + \theta D$ ,  $g_{21} + \theta D$ , and  $g_{22} - \theta D$  for  $A_1B_2$ ,  $A_2B_1$ , and  $A_2B_2$ , respectively.

If the loci are unlinked,  $\theta = 1/2$ , and the change in gametic frequency from one generation to the next is  $1/2D$ . For linked loci the change is  $\theta D$ . Thus, the more closely two loci are linked, the slower is the approach to equilibrium. The coefficient of LD after  $t$  generations may be written as

$$D_t = (1 - \theta) D_{t-1} = (1 - \theta)^t D_0$$

which approaches zero as  $t$  tends to infinity.

At equilibrium,  $D$  is equal to zero and the genotype and gametic frequencies are products of the allele frequencies (see Table 13-4). The gametic frequencies may be written as  $g_{11} = p_1q_1 + D$ ,  $g_{12} = p_1q_2 - D$ ,  $g_{21} = p_2q_1 - D$ , and  $g_{22} = p_2q_2 + D$ . Each of these gametic frequencies must be greater than or equal to zero. Thus,  $D$  must be greater than or equal to both  $-p_1q_1$  and  $-p_2q_2$ , and  $D$  must be less than or equal to both  $p_1q_2$  and  $p_2q_1$ . These results may be written as

$$D_{\min} = \max(-p_1q_1, -p_2q_2)$$

$$D_{\max} = \min(p_1q_2, p_2q_1)$$

For two loci each with two alleles,  $D$  must lie between  $-0.25$  and  $0.25$ , and it can reach these extreme values only if the frequencies of the four alleles are  $0.5$ . Thus, the value of  $D$  is dependent on allele frequencies, meaning that  $D$  values for different pairs of loci are not comparable. The value of the standardized measure,  $D' = D/D_{\text{extreme}}$ , where  $D_{\text{extreme}} = -D_{\min}$  if  $D < 0$  and  $D_{\max}$  if  $D > 0$ , is less dependent on the allele frequencies and lies between  $-1$  and  $1$ .



The statistic,  $\delta$ , is another measure of LD that is useful for estimating the location of a disease locus if a single mutation is likely. The formula is

$$\delta = (p_D - p_N) / (1 - p_N)$$

where  $p_D$  is the frequency of the associated allele on disease chromosomes and  $p_N$  is the frequency of this allele on normal chromosomes. This value represents an estimate of the proportion of disease chromosomes bearing the original associated allele. If there is a single mutation, the proportion of chromosomes carrying this mutation is the same for all marker loci, so differences in  $\delta$  across loci should largely represent effects of recombination. Thus,  $\delta$  can be used to determine the most likely location of the disease locus among a set of tightly linked marker loci.

The effectiveness of LD in locating disease mutations was demonstrated with the identification of the cystic fibrosis  $\Delta F508$  mutation in 1989. Over the next decade, it was used successfully in the identification of ethnic-specific disease mutations in the Ashkenazi Jewish, Finnish, Acadian, Roma, and other isolated and endogamous populations.

Note that LD is one possible explanation for association between a phenotype and a marker allele in a population. In this case, the disease locus is tightly linked to the marker locus. However, population association does not necessarily mean tight linkage, and vice versa. Other possible reasons for association are pleiotropy (multiple effects of the same gene), such as the association between stomach cancer and the A allele of the ABO blood group and departures from random mating due to events such as racial admixture, stratification, inbreeding, and assortative mating.

### Examples

1. Suppose the frequencies of the gametes  $A_1B_1$ ,  $A_1B_2$ ,  $A_2B_1$ ,  $A_2B_2$  are 0.5, 0.1, 0.3, 0.1, respectively. What is the value of  $D$  after one generation of random mating if (1) the two loci are unlinked and (2) the recombination fraction between the two loci is 0.01?

The value of  $D$  in the original population is  $(0.5)(0.1) - (0.1)(0.3) = 0.02$ . After one generation,  $D = (1 - 0.5)(0.02) = 0.01$  if the two loci are unlinked, and  $D = (1 - 0.01)(0.02) = 0.0198$  if the recombination fraction is 0.01.

2. How many generations are required for the value of  $D$  to be one-half its initial value?

$D_t/D_0 = (1 - \theta)^t = 1/2$ ; therefore,  $t = \log(1/2)/\log(1 - \theta)$ . Thus, for  $\theta$  equal to 0.3, 0.1, 0.01, and 0.001, the numbers of generations required are approximately 2, 7, 69, and 693, respectively. Note that for unlinked loci,  $D$  is halved in one generation as seen in the first example.

## 13.3 FACTORS THAT AFFECT HARDY-WEINBERG EQUILIBRIUM

The assumption of a large, random mating population is fundamental to Hardy-Weinberg equilibrium. If mating is not at random, the allele frequencies at a locus (say,  $p$

and  $q$ ) in the population do not change from one generation to the next, but the genotype frequencies are not  $p^2$ ,  $2pq$ , and  $q^2$ . Evolutionary forces such as random genetic drift, mutation, selection, and migration, however, will change allele frequencies (and consequently genotype frequencies) from one generation to the next.

### 13.3.1 Factors that Affect Genotype Frequencies but Not Allele Frequencies

Random mating has been assumed so far in all the derivations. If gametes do not unite at random, the genotype frequencies are not in Hardy-Weinberg proportions and cannot be derived simply from allele frequencies. Consanguinity (inbreeding), assortative mating, and stratification (e.g. ethnic subgroups within a population) are examples of nonrandom mating. In these situations, the frequency of homozygotes is increased at the expense of heterozygotes, and the genotype frequencies may be significantly different from Hardy-Weinberg expectations. However, allele frequencies do not change.

**13.3.1.1 Consanguinity and Inbreeding.** Individuals who are related genetically are termed consanguineous, and the offspring of mating between such individuals are said to be inbred. Inbreeding increases the frequency of homozygous genotypes and decreases the frequency of heterozygous genotypes in the population. The offspring of consanguineous marriages have an increased risk over that of the general population of having recessive disorders. The increase in risk depends on the population frequency of the disease allele and the degree of relationship between the parents. In cultures in which uncle-niece and first- and second-cousin marriages are encouraged, recessive disorders that are rare in most randomly mating populations may be relatively common. The coefficient of inbreeding ( $F$ ) for a child of a consanguineous marriage is the probability that the child receives two alleles at a given locus that are both from the same ancestor and are, thus, identical by descent (autozygous). For example, half first cousins share a grandparent in common. The probability that a child of half first cousins is homozygous by descent at a locus is  $F = (1/2)^5 = 1/32$ . In general, for autosomal loci, the inbreeding coefficient for an individual is  $F = (1/2)^{n_1 + n_2 + 1}$ , where  $n_1$  and  $n_2$  are the numbers of generations separating the individuals in the consanguineous mating from their common ancestor. (This formula assumes that the common ancestor is not inbred.) Half first cousins are separated from their common grandparent by two generations. Thus, the exponent is  $2 + 2 + 1 = 5$ . Table 13-5 gives the estimated proportion of alleles shared by consanguineous individuals that are identical by descent as well as the coefficient of inbreeding for the offspring of these consanguineous matings (Table 13-5). If a child is inbred through more than one line of descent, the total coefficient of inbreeding is the sum of each of the separate coefficients. For example, first cousins are related through two grandparents. Thus,

**TABLE 13-5** Proportion of Alleles Shared by Related Individuals that Are Identical by Descent and the Inbreeding Coefficient ( $F$ ) in the Offspring of Various Types of Consanguineous Mating

Type of Mating	Proportion of Shared Alleles	$F$
Parent–offspring	1/2	1/4
Brother–sister	1/2	1/4
Half sibs	1/4	1/8
Uncle–niece, aunt–nephew	1/4	1/8
First cousins	1/8	1/16
Double first cousins	1/4	1/8
Half first cousins	1/16	1/32
First cousins once removed	1/16	1/32
Second cousins	1/32	1/64
Second cousins once removed	1/64	1/128
Third cousins	1/128	1/256

the inbreeding coefficient for the offspring of first cousins is  $F = (\frac{1}{2})^5 + (\frac{1}{2})^5 = (\frac{1}{2})^4 = \frac{1}{16}$ . The coefficient of inbreeding is also an estimate of the proportion of loci at which an individual is autozygous.

The coefficient of inbreeding for X-linked loci depends on the number of males in the lines of descent and is always zero for male offspring, because they have only one X chromosome. In order to calculate the inbreeding coefficient for daughters of first cousins, four possibilities need to be considered for the first cousins: their fathers are brothers, their mothers are sisters, the father of the male cousin and the mother of the female cousin are siblings, or vice versa. If the fathers are brothers, the first cousins cannot share any X-linked alleles in common because the male first cousin did not inherit an X chromosome from his father. Thus, female offspring of this type of first-cousin mating are not inbred for X-linked loci and have an inbreeding coefficient of zero. Similarly, if the first cousins are offspring of a brother and a sister with the father being the son of the brother and the mother being the daughter of the sister, the inbreeding coefficient for their daughters is zero because the first cousins cannot share any X-linked alleles in common. On the other hand, if the mothers of the first cousins are sisters, then the inbreeding coefficient for X-linked loci in their daughters is greater than that for autosomal loci because a male transmits the X chromosome he received from his mother to all his daughters. Thus, the inbreeding coefficient in this situation is  $(\frac{1}{2})^3 + (\frac{1}{2})^4 = \frac{3}{16}$ . The fourth possibility is that the first cousins are offspring of a brother and sister, with the sister being the mother of the male and the brother being the father of the female. In this case, the inbreeding coefficient for X-linked loci in female offspring is  $(\frac{1}{2})^3 = \frac{1}{8}$ .

Genotype frequencies in inbred populations cannot be calculated from the allele frequencies alone, but they

can be obtained if the average inbreeding coefficient in the population is known. The amount of inbreeding in the population may be measured in terms of the decrease in heterozygosity relative to a random mating population. If the allele frequencies at a locus are  $p$  and  $q$ , then under random mating the frequency of heterozygotes is  $2pq$ . Suppose the frequency of heterozygotes in the inbred population is  $H$ . Then the inbreeding coefficient for the population is  $F = (2pq - H)/2pq$ . Therefore,  $H = 2pq - 2pqF$ . The frequencies of the two types of homozygotes in the inbred population can then be calculated to be  $p^2 + pqF$  and  $q^2 + pqF$ . If the inbreeding coefficient is zero (i.e. random mating), the genotype frequencies are those expected for Hardy–Weinberg equilibrium. On the other hand, if there is complete inbreeding ( $F = 1$ ), the frequency of heterozygotes is zero, and the population consists only of homozygotes with frequencies of  $p$  and  $q$ . However, note that the allele frequencies will not change from one generation to the next, regardless of the value of the inbreeding coefficient in the population.

### Example

Suppose the frequency of an autosomal recessive disease is 1/40,000 in the general population. What is the expected frequency of the disease among the offspring of first cousins?

The frequency of the deleterious allele is 1/200, the square root of the frequency of the disease. The inbreeding coefficient for offspring of first-cousin marriages is 1/16. Thus, the frequency of the disease among the offspring of first cousins is  $1/40,000 + (199/200)(1/200)(1/16) = 1/2977$ .

**13.3.1.2 Assortative Mating.** Assortative mating is the tendency for people to choose mates who are more similar (positive) or dissimilar (negative) to themselves in phenotype characteristics than would be expected by chance. If these characteristics are genetically determined, positive assortative mating may increase homozygosity in the population. An important difference between inbreeding and positive assortative mating is that inbreeding affects all loci, while assortative mating affects only those that play a role in the phenotype characteristics that are similar. Clinical examples of positive assortative mating are those between individuals who are profoundly hearing impaired or blind, which in some cases may be attributable to the same genotypes.

**13.3.1.3 Stratification.** A stratified population is one in which mating occurs within subgroups, and thus mating is not random in the population (even though random mating may occur in each subgroup). Suppose there are two subgroups ( $A_1$  and  $A_2$ ) with allele frequencies of  $p_1$  and  $q_1$ , and  $p_2$  and  $q_2$ . Then the frequency of heterozygotes in the combined population ( $A_1 + A_2$ ) is  $p_1q_1 + p_2q_2$ . If there was random mating in  $A_1 + A_2$ , then after one generation the frequency of heterozygotes would be  $p_1q_1 + p_2q_2 + \frac{1}{2}(p_1 - p_2)^2$ , which is always

greater than  $p_1q_1 + p_2q_2$ , while the decrease in each of the two homozygote frequencies is  $1/4(p_1 - p_2)^2$ . Thus, if  $A_1$  and  $A_2$  remain as separate subgroups, the frequency of heterozygotes in the combined population will always be lower (and the frequency of homozygotes greater) than expected under Hardy–Weinberg equilibrium.

### 13.3.2 Factors That Affect Allele Frequencies

Evolutionary forces such as random genetic drift, mutation, selection, and migration change the allele frequencies in a population. Important examples of each of these forces have been documented in human populations, and their effects are becoming better understood as knowledge of the genetic structure of populations at the DNA level increases.

**13.3.2.1 Random Genetic Drift.** The Hardy–Weinberg principle assumes that population size is large, and this assumption is probably valid for many present-day populations. However, if the population size is small, allele frequencies may change from one generation to the next by chance alone. This change is a consequence of sampling in small populations and is called random genetic drift. The sample is the set of gametes that contributes to the next generation. Suppose this sample consists of  $2N$  gametes ( $N$  individuals) and consider a locus with two alleles,  $A_1$  and  $A_2$ . The  $2N+1$  possible values of the frequency of  $A_1$  are

$$0, 1/2N, 2/2N, 3/2N, \dots, (2N-1)/2N, 2N/2N$$

The probability that the number of  $A_1$  alleles in the population is  $k$  ( $0 \leq k \leq 2N$ ) depends on the population size and the frequencies,  $p$  and  $q$ , of  $A_1$  and  $A_2$ , respectively, in the previous generation. It may be written as

$$Pr(k) = \binom{2N}{k} p^k q^{2N-k}$$

Thus, if  $N$  and  $p$  are known, the probability of a particular frequency of  $A_1$  in the next generation may be calculated. For example, if  $N=50$  and  $p=0.5$ , the probability that the frequency of  $A_1$  in the next generation is less than 0.4 or greater than 0.6 is 0.023, while the probability that it is between 0.45 and 0.55 is 0.682. The probability that  $A_1$  will either be lost or become fixed in the population in the next generation is extremely small, but is greater than zero. If  $N=50$  and  $p=0.01$ , the probability that  $A_1$  will be lost in the next generation is 0.37, and the probability that it will have a frequency of greater than 0.05 is 0.002. The precise change in allele frequency from one generation to the next cannot be predicted because drift is a random process. However, over a number of generations, drift can lead to the loss of some alleles from the population, with others becoming fixed. If a large number of populations are considered, the average behavior of allele

frequencies can be predicted. The probability that a new allele in a population will eventually become fixed is  $1/2N$ , the frequency of the allele in the population at the time it arose. If the allele is to become fixed in the population, the average time to fixation is approximately  $4N$  generations. After a large enough number of generations of random genetic drift, every allele in a population can be traced back to a single allele in the initial ancestral population. All other alleles in the initial population will have been lost. This concept is known as coalescence, and it has been used to model DNA sequence variation in populations.

Random genetic drift in a population is similar to inbreeding and stratification, in that its effect on the population is a reduction in the number of heterozygotes and an increase in the number of homozygotes. When the population size is drastically reduced (a bottleneck), the genetic drift is known as a founder effect. Examples of this effect (e.g. new colonization by a small subset of a population or environmental disasters such as plague and famine) abound in history. Founder effect is likely to explain the relatively high frequency of certain diseases in some ethnic groups (e.g. Tay–Sachs disease in the Ashkenazi Jewish population).

#### Example

Suppose a new mutation arises in a population of size 500. What is the probability that this allele will be lost in the next generation? What is the probability that it will eventually become fixed in the population?

The total number of gametes in the population is 1000. Thus, the frequency of the new allele is 0.001, and the probability that it will be lost in the next generation is  $[1000!/(0!)(999!)](0.999)^{1000} (0.001)^0 = 0.37$ . The probability that this new allele will eventually become fixed in the population is  $1/1000$ .

**13.3.2.2 Mutation.** When mutations occur in the germ cells, they may be passed on to the next generation. The change in the DNA may be a single nucleotide substitution or it may involve many nucleotides, such as in the case of an insertion or deletion. Many hemoglobinopathies are due to point mutations that cause the replacement of an amino acid (missense) and consequently an abnormal protein product. The most common mutation causing Tay–Sachs disease is a 4-base-pair (bp) insertion (frameshift), while the  $\Delta F508$  mutation in the cystic fibrosis gene is a 3-bp deletion.

The source of genetic variation in a population is mutation. Mutation rates in humans have been estimated to be of the order  $10^{-4}$ – $10^{-6}$  per gene per generation. The rate of nucleotide substitutions is estimated to be 1 in  $10^8$  per generation, implying that 30 nucleotide mutations would be expected in each human gamete.

Most new mutations are lost due to chance. However, new mutations arise in each generation, and some become

**TABLE 13-6** Selection against the  $A_2A_2$  Genotype at an Autosomal Locus

Genotype	$A_1A_1$	$A_1A_2$	$A_2A_2$
Frequency before selection	$p^2$	$2pq$	$q^2$
Relative fitness	1	1	$1-s$
Frequency after selection	$p^2$	$2pq$	$q^2(1-s)$
After one generation of selection			
Frequency $A_1 = (p^2 + pq)/[p^2 + 2pq + q^2(1-s)] = p/(1-sq^2)$			
Frequency $A_2 = [pq + (1-s)q^2]/(1-sq^2)$			
If $s = 1$ (i.e. complete selection against the $A_2A_2$ genotype), then			
After one generation of selection			
Frequency $A_1 = (p^2 + pq)/(p^2 + 2pq) = 1/(1+q)$			
Frequency $A_2 = pq/(p^2 + 2pq) = q/(1+q)$			
After $t$ generations of selection			
Frequency $A_1 = [1 + (t-1)q]/(1+q)$			
Frequency $A_2 = q/(1+q)$			

established in the population. Suppose  $\mu$  is the mutation rate from  $A_1$  to  $A_2$  per generation. If the frequencies of  $A_1$  and  $A_2$  are  $p_t$  and  $q_t$ , respectively, in generation  $t$ , then in the  $(t+1)$ th generation the frequency of  $A_2$  is

$$q_{t+1} = q_t + \mu p_t = q_t + \mu(1 - q_t) = \mu + (1 - \mu)q_t$$

assuming no back mutation.

Similarly,  $q_t = \mu + (1 - \mu)q_{t-1}$ ,  $q_{t-1} = \mu + (1 - \mu)q_{t-2}$ , and so forth. By substitution,  $q_t$  may be written in terms of  $q_0$ , the frequency of  $A_2$  in the initial generation:

$$q_t = 1 - (1 - \mu)^t(1 - q_0)$$

$$\text{or } (1 - \mu)^t = (1 - q_t)/(1 - q_0) = p_t/p_0$$

Because  $\mu$  is very small,  $(1 - \mu)^t$  is approximately equal to  $e^{-t\mu}$ . Thus, the number of generations required to change the frequency of  $A_2$  from  $q_0$  to  $q_t$  is inversely proportional to the mutation rate. Also note that as  $t$  gets larger and larger,  $q_t$  gets closer and closer to 1. In other words, if mutation from  $A_1$  to  $A_2$  is the only force acting to change the allele frequencies, then  $A_2$  will eventually become fixed in the population. The change in allele frequency from one generation to the next is  $q_{t+1} - q_t = \mu(1 - q_t)$ , meaning that the change in allele frequency is greater for smaller frequencies of  $A_2$ .

So far we have considered mutation in only one direction. Now suppose the mutation rate from  $A_1$  to  $A_2$  is  $\mu$  and the reverse rate from  $A_2$  to  $A_1$  is  $\nu$ . Then the change in the frequency of  $A_2$  per generation is  $\mu p - \nu q$ , and equilibrium is reached when this change is equal to zero. Thus, the equilibrium frequencies are  $p = \nu/(\mu + \nu)$  and  $q = \mu/(\mu + \nu)$ . This equilibrium is stable, meaning that if the frequencies are disturbed, they will eventually return to their equilibrium values as long as no other forces are affecting them.

Mutation rates have been estimated for a number of autosomal dominant disorders, such as neurofibromatosis

type I, which has the high rate of  $10^{-4}$ , and tuberous sclerosis, with a rate of about  $10^{-5}$ . Some of these disorders (e.g. achondroplasia, for which the mutation rate is estimated to be  $10^{-5}$ ) have reduced fitness, which is discussed in the next section.

### Examples

1. How many generations will be required to change the frequency of  $A_2$  (a) from 0.1 to 0.2, (b) from 0.8 to 0.9, if the mutation rate from  $A_1$  to  $A_2$  is  $10^{-4}$ ?

The number of generations is

$$\begin{aligned} t &= 1/\mu [\ln(1 - q_0) - \ln(1 - q_t)] \\ &= 1/\mu [\ln(0.9) - \ln(0.8)] \\ &= 1/\mu (0.1178) \end{aligned}$$

Therefore, for a mutation rate of  $10^{-4}$ , 1178 generations are required, whereas for a mutation rate of  $10^{-5}$ , 11,780 generations are required to change the frequency of  $A_2$  from 0.1 to 0.2. On the other hand, to change the frequency from 0.8 to 0.9 requires 6932 generations if the mutation rate is  $10^{-4}$  and 69,315 generations if the mutation rate is  $10^{-5}$ .

2. Suppose the mutation rate from  $A_1$  to  $A_2$  is  $10^{-4}$  and the reverse rate is  $10^{-5}$ . What is the equilibrium frequency of  $A_1$ ?

The equilibrium frequency of  $A_1$  is  $10^{-5}/(10^{-4} + 10^{-5}) = 0.091$ . However, to reach this equilibrium frequency may take tens of thousands of generations, depending on the initial allele frequencies.

**13.3.2.3 Selection.** The fitness of an individual is defined as an ability to survive and reproduce. The process by which the frequencies of genotypes in individuals with greater fitness increase in the population is natural selection. It acts to decrease the frequencies of the less-fit genotypes. The relative fitness is defined as  $1 - s$ , where  $s$  is the selection coefficient against the deleterious genotype. Thus, the most-fit genotype has a relative fitness of 1 (and a selection coefficient of 0).

Consider the situation where there are three genotypes,  $A_1A_1$ ,  $A_1A_2$ ,  $A_2A_2$ , at a locus with relative fitnesses of 1, 1,  $1 - s$ , respectively. That is, there is selection against the  $A_2A_2$  homozygote. (If  $s = 1$ , the selection is complete, meaning that individuals with the  $A_2A_2$  genotype do not reproduce.) Table 13-6 shows the change in allele frequencies from one generation to the next. In the case in which  $s = 1$ , the frequencies after  $t$  generations can be written in terms of the initial allele frequencies (Table 13-6). Substituting in the formula given in Table 13-6 shows that when the  $A_2A_2$  homozygote does not reproduce, the number of generations required to reduce the frequency of  $A_2$  to one half its initial value is equal to the reciprocal of its initial value. Thus, if the frequency of  $A_2$  is 0.01, it will take 100 generations of complete selection against the  $A_2A_2$  homozygote to reduce the frequency of  $A_2$  to 0.005. In other words, lack of reproduction of individuals with a rare recessive disease does not lead to a



**TABLE 13-7** Selection Favoring the Heterozygous Genotype at an Autosomal Locus

Genotype	$A_1A_1$	$A_1A_2$	$A_2A_2$
Frequency before selection	$p^2$	$2pq$	$q^2$
Relative fitness	$1-s_1$	1	$1-s_2$
Frequency after selection	$p^2(1-s_1)$	$2pq$	$q^2(1-s_2)$
After one generation of selection			
Frequency $A_1 = (p-s_1p^2)/(1-s_1p^2-s_2q^2)$			
Frequency $A_2 = (q-s_2q^2)/(1-s_1p^2-s_2q^2)$			
The change in the frequency of $A_2$ from one generation to the next is $pq(s_1p-s_2q)/(1-s_1p^2-s_2q^2)$ . Equating this quantity to zero gives the equilibrium allele frequencies, which are			
Frequency $A_1 = s_2/(s_1+s_2)$			
Frequency $A_2 = s_1/(s_1+s_2)$			

rapid reduction in the frequency of the deleterious allele from one generation to the next.

Now consider the situation in which there is partial selection against the  $A_2A_2$  genotype. The allele frequencies after  $t$  generations cannot be written in terms of the initial frequencies, but the decrease in the frequency of the  $A_2$  allele from one generation to the next can be calculated. This decrease is equal to  $sq^2(1-q)/(1-sq^2)$ , and the number of generations required to change the frequency of  $A_2$  from its initial value to a new value can be approximated. For example, if  $s=0.001$  and the initial frequency of  $A_2$  is 0.01, more than 100,000 generations will be required to reduce the frequency to 0.005. This example makes the point that even if the selective disadvantage of a genotype is very small, the allele frequencies in the population will gradually change. For the same selection coefficient ( $s=0.001$ ), 11,665 generations are required to reduce the frequency of  $A_2$  from 0.7 to 0.1. If there is selection against the heterozygous genotype ( $A_1A_2$ ) as well as the  $A_2A_2$  genotype, with  $s=0.001$  for  $A_2A_2$  and  $s=0.0005$  for  $A_1A_2$ , then 6156 generations are required to reduce the frequency of  $A_2$  from 0.7 to 0.1.

In the case in which there is selection favoring the heterozygote over both homozygotes, an equilibrium state is reached for the allele frequencies. Table 13-7 shows the change in allele frequencies from one generation to the next. At equilibrium,  $s_1p = s_2q$ , so that  $p = s_2/(s_1 + s_2)$  and  $q = s_1/(s_1 + s_2)$  (Table 13-7).

This equilibrium is stable and is called a balanced polymorphism. This type of selection is known as overdominance. If, on the other hand, selection is against the heterozygote, the equilibrium is unstable, and the selection is known as underdominance. The equilibrium frequencies are the same, but if a disturbance occurs such that  $q > s_1/(s_1 + s_2)$ ,  $q$  will increase further rather than returning to its equilibrium value. The reverse is also true, so eventually one allele or the other will be eliminated.

Let us now consider a balance between mutation and selection. Suppose the mutation rate from  $A_1$  to  $A_2$  is  $\mu$ , and the relative fitnesses of the genotypes  $A_1A_1$ ,  $A_1A_2$ ,

**TABLE 13-8** X-Linked Locus: Selection against the  $A_2A_2$  Genotype in Females and the  $A_2$  Genotype in Males

Females			
Genotype	$A_1A_1$	$A_1A_2$	$A_2A_2$
Frequency before selection	$p^2$	$2pq$	$q^2$
Relative fitness	1	1	$1-s$
Frequency after selection	$p^2$	$2pq$	$q^2(1-s)$
Males			
Genotype	$A_1$	$A_2$	
Frequency before selection	$p$	$q$	
Relative fitness	1	$1-s$	
Frequency after selection	$p$	$q(1-s)$	
After one generation of selection in males			
Frequency $A_1 = p/(1-sq)$			
Frequency $A_2 = (q-sq)/(1-sq)$			

$A_2A_2$ , are 1, 1,  $1-s$ , respectively. As shown in Table 13-6, the frequency of  $A_1$  after selection is  $p/(1-sq^2)$ . Thus, the increase in frequency of  $A_2$  due to mutation from  $A_1$  to  $A_2$  is  $\mu p/(1-sq^2)$ , while the decrease due to selection is  $sq^2(1-q)/(1-sq^2)$ . At equilibrium,  $\mu p/(1-sq^2) = sq^2(1-q)/(1-sq^2)$ , which simplifies to  $q = \sqrt{\mu/s}$ . This equilibrium is stable and  $q = \sqrt{\mu}$  when  $s=1$ . Thus, for a lethal recessive disease and a mutation rate of  $10^{-6}$ , the equilibrium frequency of the deleterious allele is 1/1000.

In the case of a deleterious dominant phenotype, the fitness of both the homozygote and the heterozygote is reduced. With selection coefficients of  $1-s$ ,  $1-s$ , 1, the increase in the frequency of  $A_1$  due to mutation is equal to the decrease due to selection when  $q = \mu/s$ , which reduces to  $q = \mu$  for  $s=1$ . If individuals with a dominant disease do not reproduce, the frequency of the deleterious allele in the next generation is equal to the mutation rate. Examples of such disorders are atelosteogenesis and thanatophoric dysplasia, which are both lethal forms of short-limbed dwarfism. In the case of achondroplasia, fitness is not zero, but it is considerably lower than one, and is estimated to be about 0.2. Thus, the equilibrium frequency of the deleterious allele is  $10^{-5}/0.8 = 1.25 \times 10^{-5}$ , or slightly higher than the mutation rate.

Selection against genotypes at loci on the X chromosome needs to be tabulated separately for males and females because males have only one allele at an X-linked locus. Table 13-8 shows the case in which the  $A_2A_2$  genotype and the  $A_2$  genotype are selected against in females and males, respectively. The decrease in frequency of  $A_2$  due to reduced fitness in females is extremely small compared with the decrease due to reduced fitness in males. Thus, we will only consider males (Table 13-8). The loss of  $A_2$  alleles is equal to  $sq(1-q)/(1-sq)$ , which is  $q$  if  $s=1$ . In other words, if selection is complete, all male  $A_2$  alleles are lost in each generation. Because males have only one allele and females have two, this loss represents one third of the  $A_2$  alleles in the population. If

the mutation rate from  $A_1$  to  $A_2$  is  $\mu$ , the increase in frequency of  $A_2$  due to mutation in males is  $\mu p/(1-sq)$ . But mutation in males represents only one third of the mutations that are occurring in the population. Thus, increase in frequency due to mutation balances decrease due to selection when  $3\mu p/(1-sq) = sq(1-q)/(1-sq)$ , which reduces to  $q = 3\mu/s$ . For an X-linked recessive lethal,  $s = 1$ , and  $\mu = q/3$ . In other words, one third of the deleterious alleles in the population, and, thus, one third of cases of diseases such as Duchenne muscular dystrophy, are new mutations. In less severe X-linked disorders, the proportion of cases that are new mutations is not as high; for example, the relative fitness of individuals with hemophilia A is about 70%. Therefore, the proportion of new mutations is  $0.3q/3$ , meaning that about 10% of cases are new mutations. Of course, during the initial years of the AIDS epidemic when blood was not being screened for HIV, the relative fitness of hemophiliacs was considerably lower than 70%. The effect of this transient reduction on the frequency of hemophilia A will be seen in future generations.

### Example

Suppose the relative fitnesses of the genotypes  $A_1A_1$ ,  $A_1A_2$ ,  $A_2A_2$  are 0.8, 1, 0.1, respectively. What are the equilibrium frequencies of the two alleles?

The heterozygote has a selective advantage in this population. At equilibrium, the frequencies of  $A_1$  and  $A_2$  are  $0.9/1.1 = 0.82$  and  $0.2/1.1 = 0.18$ , respectively. Even though selection against the  $A_2A_2$  homozygote is quite extreme, the equilibrium frequency of  $A_2$  is relatively high because of overdominance. If the relative fitnesses were 0.9, 1, 0.7, the frequencies of  $A_1$  and  $A_2$  would be 0.75 and 0.25, respectively.

**13.3.2.4 Migration (Gene Flow).** Migration introduces alleles into the population and, like mutation, increases heterozygosity. In general, migration rates are higher than mutation rates, so migration is more effective than mutation in counterbalancing the effects of genetic drift.

Comparison of alleles in different ethnic groups demonstrates the contribution of gene flow to the current population gene pools. For example, the most common mutations in phenylalanine hydroxylase that cause PKU are likely to be of Celtic origin. These same mutations have been found in many different populations throughout the world, reflecting the migrations of the Celts.

## 13.4 APPLICATIONS IN POPULATION GENETICS

The evolutionary forces that govern the frequencies of genotypes and alleles provide us with the tools to understand the genetic structure of populations. Such tools can help deduce why some disease mutations are relatively common in some ethnic groups, but not in others. Ethnic variation in allele frequencies is found throughout the

genome, and by examining this genetic diversity, evolutionary patterns can be inferred, and variants contributing to the cause of common diseases can be identified.

### 13.4.1 Ethnic Diversity of Rare Disease Alleles

The existence of different disease alleles among ethnic groups is significant both for understanding the origins of the disease in a population and for estimating recurrence risks that will depend on ethnicity. Several examples show the benefit of applying population history to medical genetics.

The thalassemias have relatively high frequencies in many different populations, presumably due to selective advantage (increased relative fitness) of the heterozygotes over the homozygotes against malaria. Mutations in the genes encoding both the  $\alpha$ -chain (chromosome 16) and the  $\beta$ -chain (chromosome 11) of hemoglobin cause thalassemia. Most of the  $\beta$ -thalassemia mutations are single bp substitutions as opposed to the  $\alpha$ -thalassemias, in which complete genes are deleted. More than 80  $\beta$ -chain point mutations that cause  $\beta$ -thalassemia have been described. These mutations have a wide ethnic distribution, with several different common alleles found in Mediterranean, African, and Southeast Asian populations.

Tay–Sachs disease provides another excellent example of ethnic-specific mutations. The most common mutation in the hexosaminidase-A  $\alpha$ -subunit gene (chromosome 15) causing Tay–Sachs disease in the Ashkenazi Jewish population is a 4-bp insertion in exon 11. It is found in 80% of mutant alleles in this population, but in less than 20% in other populations. Three alleles account for 99% of mutations in the Ashkenazi Jewish population. The frequency of Tay–Sachs alleles is also relatively high in the French Canadian population, in which two different mutant alleles have been described. Members of several Acadian families in southwestern Louisiana were found with Tay–Sachs disease; in 11 of 12 disease alleles, the mutation was the 4-bp insertion that is the most frequent mutant allele in the Ashkenazi Jewish population.

Other diseases, such as gyrate atrophy and familial hypercholesterolemia, show similar ethnic diversity in the distribution of mutant alleles. Founder effect (random genetic drift), selection, and gene flow determine the frequencies of mutations in different populations.

### 13.4.2 Evolutionary Patterns

Various types of DNA marker alleles have been analyzed in studies of population structure and evolution, and before the introduction of DNA markers, similar studies were done using blood group and protein polymorphisms. The evolutionary tree derived from these studies suggested four major groupings, consisting of Africa, Europe/Asia, Americas, and Australia/New Guinea, with the most likely origin being the

African branch. Detailed analyses of mitochondrial DNA (mtDNA) and Y-chromosome haplogroups have extended these findings and confirmed that contemporary populations are largely the descendants of people who migrated out of Africa about 50,000 years ago. For example, the mtDNA and Y haplogroups found in southeastern Asia and Australia are distinct from those in the rest of Asia and Europe. This variation is most likely the result of random genetic drift and northern and southern migrations at different times. The Americas were the last continents to be colonized, and as would be anticipated, most native American Y chromosomes belong to a single haplogroup. Interestingly, the results of voyages by Europeans to the Americas and Oceania in the past 500 years are clearly revealed through mtDNA and Y-chromosome analyses. In these populations, European Y-chromosome haplogroups are relatively common, while the mtDNA haplogroups are those of the indigenous population.

Because of sex-specific gene flow and the small amount of the genome represented, studies of historical migration based only on mtDNA and Y chromosome are biased (1). With the availability of next-generation sequencing, entire human genomes can be compared. In fact, a draft sequence of the Neandertal genome has now been completed (2), revealing gene flow from Neandertals to modern humans after moving out of Africa. There is also an extensive amount of genetic variation among African populations (3), with most African-Americans having a mixed ancestry that is not specific to any one African group (4). This high level of admixture can be quite helpful for identifying genetic variants associated with disease or response to drugs.

### 13.4.3 Genome Variation

The advances of the human genome project have renewed appreciation and interest in the study of naturally occurring variation in the human genome. About 90% of human DNA variation is due to single nucleotide base changes. On average, a single bp difference between two human genomes is observed for every 1000 bp. But the odds of finding a difference may be as much as 100-fold greater in some regions of the genome than in others. Single nucleotide polymorphisms (SNPs) are defined as loci with alleles that differ at a single base, with the rarer allele having a frequency of at least 1% in a random set of individuals in a population. In general, the likelihood of finding an SNP is much higher in noncoding regions than in coding regions. (An SNP in a coding region is sometimes called a cSNP.) Most SNPs found in the human genome are thought to have originated long after speciation, but before the separation into different human populations. This explains the observation that human SNPs are usually not shared with primates, but are common to all populations; only about 15% are thought to be “private.” Also, only a

few of the SNP alleles that were present when humans moved out of Africa have become fixed (either 0% or 100%) at this point in time.

As a result of major international initiatives, approximately 10 million SNPs, both common and rare, have been identified in the human genome, and more than 1.5 million have been genotyped in over a thousand individuals from 11 global populations (5). In addition, the detection and characterization of copy number variants (CNVs), which tend to map to duplicated segments, has provided access to important variation, particularly in highly duplicated gene families, which is likely to contribute to some common diseases (6). And recently, the 1000 Genomes Project has generated and cataloged several million more SNPs and CNVs, bringing the total to nearly 20 million. The goal of this project is to discover, genotype, and provide accurate haplotype information on DNA polymorphisms in multiple human populations (7). These extensive datasets provide the population geneticist with a huge set of densely mapped polymorphisms for reconciling genome variation with population histories of bottlenecks, admixture, and migration, and for revealing evidence of natural selection. They also enable informative studies such as pinpointing functional elements affecting gene expression in noncoding DNA, which tend to be in regions of reduced variation (8). Moreover, the knowledge gained from these studies is applicable to many disciplines including forensics, pharmacogenomics, and complex disease research.

### 13.4.4 Identification of Causal Variants for Common Diseases

Earlier in this chapter, the concept of LD was described, and it was mentioned that studies exploiting this phenomenon have been helpful in defining the precise location of disease genes. The complexity of patterns of LD and the extent and explanation of variability among populations are critical factors that are only now becoming understood (9).

A large number of genome-wide association studies have been performed using the millions of SNPs that are now available. These studies have identified genomic regions containing genetic risk factors for many complex diseases, for example, Parkinson disease (10). However, for the majority of such diseases that have been studied so far, much of the genetic influence on them remains to be explained (11).

The availability of massive databases of genetic variation and automated technology for genotyping, sequencing and bioinformatic analysis is significantly enhancing collaborative efforts between population geneticists and molecular geneticists and advancing understanding of many diseases. As we would anticipate, thousands of interesting new questions are arising and the tools to answer many of them are now at our fingertips.

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## RELEVANT WEBSITES

International HapMap Project <http://hapmap.ncbi.nlm.nih.gov/>.  
A Catalog of Published Genome-Wide Association Studies <http://www.genome.gov/gwastudies>.

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## Biographies



**Bronya J B Keats** is currently a professor in the Research School of Biology at the Australian National University (ANU). From 1999 to 2008, she was professor and chair of the Department of Genetics at Louisiana State University Health Sciences Center (LSUHSC) in New Orleans. Dr Keats received her PhD in human population genetics from ANU in 1976, and spent several years at the University of Hawaii before moving to LSUHSC. Much of her research has focused on linkage and LD mapping, as well as the identification and characterization of genes for hearing loss and neurodegenerative disorders in the Acadian population of south Louisiana. A particular research interest has been genome variation and instability in microsatellites, especially repeat expansions associated with neurodegenerative disorders. She has worked on Usher syndrome and Friedreich ataxia, and is presently involved in promising preclinical studies using cellular and mouse models for these diseases.



**Stephanie L Sherman** obtained her PhD in Human Genetics from Indiana University School of Medicine in 1981 and is currently a professor in the Department of Human Genetics at Emory University in Atlanta, GA. Her training is in the area of genetic epidemiology and she has been involved in coordination of multisite projects to ascertain families for gene mapping studies of complex traits and genotyping/phenotype correlation studies. Currently, she is involved in research to understand the genetic and environmental risk factors associated with human chromosome nondisjunction, particularly trisomy 21. In addition, she and her colleagues have worked on strategies to identify susceptibility genes related to specific birth defects associated with Down syndrome. She also focuses her research on identifying genetic factors that increase the risk for fragile X premutation-associated disorders including primary ovarian failure (FXPOI) and tremor/ataxia syndrome (FXTAS).



# CHAPTER

# 14

## Pathogenetics of Disease

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*To wrest from nature the secrets which have perplexed philosophers in all ages, to track to their sources the causes of disease, to correlate the vast stores of knowledge, that they may be quickly available for the prevention and cure of disease—these are our ambitions.*

Sir William Osler, 1902 (1)

*The great ease with which molecular information can be collected on the genomes of higher organisms will tempt many. We can inevitably expect vast compendia of sequences but, without functional reference, these compendia will be uninterpretable, like an undeciphered ancient language. Many people and many computers will play games with these sequences, but we will have to find out by experiment what the sequences do and how the products they make participate in the physiology and development of the organism. Thus, although the analysis of the genotype has been taken care of, we still need better ways of analyzing phenotypes. Many of us are ultimately interested in the causal analysis of development and the reduction of the complex phenotypes of higher organisms to the level of gene products. This is still the major problem of biology. We must understand what cells can do because all of what we are is generated by cells growing, moving and differentiating.*

Sydney Brenner, 1973 (2)

*The effort spent on the identification of genes is likely to prove only a small fraction of that required to work out their normal function in the tissues in which they are expressed. Yet that is where clues to the treatment and prophylaxis of disease are most likely to arise.*

John Maddox, 1994 (3)

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### 14.1 INTRODUCTION

The foregoing quotations emphasize that the general theme of this chapter—all that occurs between the gene and the bedside—is not a new one. The true promise of the Human Genome Project only began to be realized when our genome was sequenced, and the really hard work remains (4,5).

At the outset, the definitions of four terms are fundamental to everything that follows. All deal with the causation of phenotypes, and they are distinguished as to method and scope of enquiry.

Etiology is the study of the causes of a phenomenon and, in the medical context, of disease. Its method is to discover the association between factors that are thought to be causes and certain features that we wish to explain.

The goal and method of the discipline are strictly empirical (“black box”), with at best minor interest in discerning the actual mechanisms involved. For instance, there is a heavily documented etiologic relationship between the total cholesterol level in plasma and the incidence of atherosclerosis. However, whether the former directly causes the latter or the relationship is more oblique is as yet unresolved.

Pathogenesis is the study of the mechanisms by which the etiologic factors are converted into disease states. For instance, the etiologic role of cholesterol in atherosclerosis has been ascribed to the infiltration of oxidized low-density lipoprotein into the arterial wall (the insudation theory); to the stimulation of organization and repair of small arterial thrombi (the encrustation theory); to the promotion of cellular and humoral immune processes

within the arterial wall; and to the secondary accumulation of cholesterol in areas of minor initial damage. Study of these rival, or perhaps complementary, explanations falls within the domain of pathogenesis.

Genetic etiology is a more specialized topic that deals with the properties of the genetic causal factors of disease and how they behave. Mendel's laws, which were formulated before anything was known of the genes and their mechanisms, are arguably the high point of genetic etiology. A positive family history for early coronary artery disease is widely recognized as an important risk factor in the cause of myocardial infarction, with no appeal to explicit mechanisms, even genetic (see Chapter 20). Yet there are undoubted genetic versions, such as the Werner syndrome (OMIM\*277700), an autosomal recessive disorder with premature aging and accelerated atherosclerosis (see Chapter 18).

Pathogenetics, a condensation of "genetic pathogenesis," is the study of how anomalies in the genome are converted into phenotypic disorders. Atherosclerosis has many genetic and nongenetic causes (see Chapter 55). Its pathogenesis has occupied decades of intensive study costing billions of dollars, and it can be fairly stated that the pathogenesis is still uncertain (6–10). One explicit instance of progress in an etiologic component is familial hypercholesterolemia, an autosomal dominant disorder (OMIM\*143890) associated with precocious atherosclerosis. A pathologic mutation in the *LDLR* gene causes one of at least five types of defects in the cellular receptor for low-density lipoprotein (Table 14-1); the result of any such mutation is disruption of cholesterol homeostasis and overt disease of early onset in nearly all instances. This understanding was the first major insight into the pathogenetics of this complex phenotype, and was recognized with the Nobel Prize in Physiology or Medicine to Brown and Goldstein in 1985.

In this chapter, the emphasis is on disease, while stressing the relationship of genetic processes to ordinary developmental mechanisms and maintenance of the healthy body, known as orthogenetics. One such concern is preserving an intact vascular system. Minor leaks

in the vascular tree are mended by the hemostatic plug, comprising platelets and fibrin. At times, this mechanism may become exuberant, resulting in thrombosis, which may occlude a strategic blood vessel. In turn, the thrombus may be covered by endothelium, with its remains, sealed in the arterial wall, perhaps converted into an atheromatous lesion. This gradation points out the perils of drawing sharp boundaries between the so-called normal and abnormal states, boundaries that are neither necessary nor illuminating. Precise definition of a disorder, although an important question in its own right, is a luxury that we cannot afford here (10; see Chapter 2). For this reason, orthogenetics and pathogenetics are best seen as parts of a single continuous field of enquiry (11). Understanding the phenotype comes from the interactions among the insights from many disciplines.

## 14.2 THE SCOPE OF ABNORMAL PHENOTYPES: DISEASE AND MALFORMATION

Conventionally, we distinguish malformations from diseases. Both terms are indefinite, but that need not concern us unduly. However, other challenges cannot be escaped so easily. It is a truism among pathologists that disease occurs only in the living. On the one hand, a cadaver cannot undergo poisoning, suppuration, or neoplasia. On the other hand, instantaneous death by catastrophe of a healthy person may occur without any disease even being started. Disease comprises both a disruption and a reaction to it: homeostasis; inflammation and its sequelae (resolution, repair, and regrowth); curtailment of activities, whether voluntary or by invalidism; and diverse concealing devices ranging from epistasis to psychoneurosis.

There are various ways of exploring these devices, but they call for some circumspection about topics that we shall now address.

## 14.3 PHYSIOLOGIC HOMEOSTASIS

Homeostasis, a mainstay of physiology, is a concept attributed to Bernard (12), studied and named by Cannon (13), and cast in formal terms by Wiener (14). It involves cybernetic devices that maintain the inner environment of the organism in a state that favors normal functioning. Despite its cardinal role in both normal and disturbed physiology, homeostasis has been astonishingly neglected in medical genetics. Its characteristic pattern is to offset departures of a measurable characteristic, such as body temperature, glucose concentration, or blood pressure, from a steady state that is in some sense optimal (the homing value). The size and direction of the response vary with the perturbation, features that put it beyond the scope of the methods most quantitative geneticists use, which are founded on Galton–Fisher theory (15).

**TABLE 14-1** Effects of Mutations in *LDLR* on the Gene Product

Mutation Class	Consequence
Null	No receptor protein
Transport defective	No or reduced receptor at cell surface
Binding defective	Normal receptor number; absent or reduced binding of LDL particles
Internalization defective	Normal receptor number and LDL binding; absent or decreased endocytosis of LDL
Recycling defective	Normal receptor number, LDL binding, and endocytosis; no or reduced release of LDL and recycling of receptor to cell surface

LDL, low-density lipoprotein.

Two main structures characterize the response of a homeostatic system to a perturbation (16). One structure is the precise disturbance to which it is responding. Some models suppose that the response is to the current information only. Others suppose the system to be responding as well to data recorded indefinitely far into the past. The second structure is the form of the response. It will always be of opposite sign to the displacement. If its size is some multiple,  $b$ , times the displacement raised to some power,  $k$ , it is of order  $k$ . If  $k=1$ , the process is linear; if  $k=0$ , it is of zero order. Intermediate values of  $k$  are processes of fractional order, and higher values are quadratic, cubic, quartic, or of noninteger or mixed orders, and so forth. The zero-order process is the simplest and resembles a simple domestic thermostat.

What lends special interest to biologic processes lies in that innocuous phrase “current information.” Messages travel at finite velocities. In cosmic terms, transmission through nerves is slow, and considerably slower through hormones. So the information is at best always somewhat out of date: a lag time ( $L$ ) may or may not be fixed and homogeneous. For instance, the sensors monitoring blood gases lie some way downstream from the apparatus that modifies them, the lungs. The latest information will be out of date by at least 10s (the transit time). Sometimes delay is gross: The response of red cell production to high altitude takes weeks. Lag introduces new and important features not present where the response is instantaneous. For example, in a linear system, if the product  $bL$  exceeds  $\pi/2$ , a wild (ever-amplifying) oscillation results that, left unchecked, would destroy the whole organism. If  $bL$  is negative, feedback is positive, which would also be fatal in most physiologic systems. Thus, both extremes are harmful. Darwinian selection will constrain the actual value well between these limits, but that state is not proof against pathologic lengthening of  $L$  during life, such as happens in some forms of type 2 diabetes mellitus.

Systems of fractional order are metastable: Minute departures from the homing value set up perpetual oscillation, the amplitude of which depends on  $b$ ,  $L$ , and  $k$ , but in no way on the initial displacement. While oscillation vitiates the primary stabilizing goal of homeostasis, it is a simple, reliable generator for rhythmic processes. It may explain how success in various physiologic maneuvers (e.g. breathing of the newborn, menarche and menopause, electric shock in ventricular fibrillation) stands or falls by and whether the first step can be provoked. Much the same argument would explain bipolar mood disorder and particularly those puzzling, so-called endogenous depressions in which the initiating factor may be so trivial as to be totally overlooked (17).

Processes with  $k>1$  have a property that is very rare, perhaps unique, in biologic models. If not large enough to cause wild oscillation, a perturbation causes

a finite number of oscillations only, such as occurs in unsustained clonus (18).

Much of the detailed workings of homeostatic systems depend on enzymes, receptors, and ligands, all of which exhibit genetic variation. Perturbations are a fact of life, and physiologic homeostasis to correct them is ubiquitous. No reasonable system can correct them fully and instantaneously. Furthermore, as we have seen in the control of periodic functions, other competing benefits of homeostasis are to be considered. We attempt to give a brief summary of the advantages and disadvantages of the various types of functions in Table 14-2. Wherever there is an inescapable lag, there will be added difficulties in achieving a prompt response without undesirable overshoot and perhaps even total loss of control. Optimal states may be very complicated, which we consider difficult to develop by wholly random evolution. This fruitful field in the genetic exploration of disease has been largely neglected.

For many bodily characteristics, health is the avoidance of extremes. Diseases may then result from excess or deficiency, and the dynamic reactive component will aim to restore the optimal. Now, in Galton–Fisher theory, any variable genetic component becomes fixed in magnitude at conception. By contrast, the responses of health and disease are perpetually exercised in a fashion variable in both degree and direction, in accordance with the size and sign of each perturbation. It is scarcely surprising that Galton–Fisher theory, so useful in static traits, is irrelevant to most diseases. From time to time, Galton–Fisher theory has furnished more refined descriptors such as Pearson’s threshold model in congenital heart disease. But it is difficult to cite any instance in which these maneuverings have led to yet deeper questions and understanding of disease, its cause, its pathogenesis, or its genotypic fate at large (genetic population dynamics). History demands that clinical geneticists not discard Galton–Fisher theory

**TABLE 14-2** Some Broad Patterns of Response in Physiologic Homeostasis

Generating Specifications			
Pattern of System	$L$	$b$	$k$
Monotonic amplification	Any	$<0$	Any (positive feedback)
Pure drift	Any	0	Any
Monotonic decay	0	0	Any
Fast decay for big perturbations	Small	Large	Large
Fast decay for small perturbations	Small	Large	Fractional
Perpetual steady oscillation	$>0$	Any	$<1$
Damped oscillation <sup>a</sup>	$\pi/2b$	$>0$	1
Temporary oscillation	$1 \pi/eb$ to	Moderate	$1 \pi/eb$
Wild oscillation	Moderate	Otherwise	$>1$ , then decay

<sup>a</sup> $L$  may assume values only in the open interval shown.

altogether. However, we believe this theory to be too restricted to be of much interest in pathogenetics or orthogenetics, and it often obfuscates enquiry.

### 14.3.1 Limited Scope of the Galton–Fisher Theory

The original conflict between the Pearsonians (in the Galtonian tradition) and the Batesonians (in the Mendelian tradition) was at first thought to be irreconcilable. Fisher's reformulation recasts them in more unified terms. Two serious gaps were left that we have been slowly and painfully discarding since the time of the epochal papers on the specific chemistry of the gene. Fisher's argument was centered on the mean and variance of binomial and multinomial variables, which are strictly not genes but statistical properties of genes. It solved the problem, but the questions resulting were much more intransigent. Linkage analysis and its analytic difficulties notwithstanding, it is an analogous mathematical descriptor. The elaborate formal theories for it have never had a rationale with a literal meaning. Linkage is now so refined and reconciled with detailed gene assignment that the false character of linkage anatomy scarcely matters any more. But we can make allowance for the fact that, even in combining small linkage intervals, Fisher's binomial assumption may yet prove misleading (because of non-additivity, etc. in particular cases) and require special handling.

## 14.4 MULTIVARIATE NORMAL DISTRIBUTIONS AND THE THRESHOLD MODEL

Galton's reconciliation with Mendel was based on multivariate normal theory, and we know of many instances—arguably the great number of instances—in which the assumptions are in fact sound. Fisher recognized this fact very well and specified it in assumptions. But many papers in clinical medicine on “the inheritance” of quantitative aspects of disease have fallen into methodological disrepute without having ever been repudiated formally. We suggest that (like linkage analysis) Pearsonian quantitation of disease be treated as a descriptor of statistical properties of genetic disease and no longer as either an explanation or an account of it. It is only fair to Pearson's explicit warning (which has been widely ignored) that “quantitative genetics” should not be applied to putative “threshold traits” when no threshold has been identified, has been the object of sound surmise, or both. Even so, the assumption of approximate Gaussian normality calls for some serious justification. As understanding of disorders gradually improves, the need for approximations becomes harder and harder to justify. We suggest that the now outmoded practice should be abandoned.

### Example

Many details suggest that most cases of type 2 diabetes mellitus at their outset are due to neither defective nor deficient insulin, but to impaired insulin regulation. Arguably, hyperglycemia and hypoglycemia are the same condition in different phases, as illustrated by potentially rapid oscillations between the two states in “brittle” diabetes. The interpretation of two apparently opposite disorders as a manifestation of instability of a single trait is not so startling as it may first appear. Besides the obvious precedent of bipolar mood disorder, there are many analogs, such as postural hypotension, dysautonomia, and anorexia–bulimia (19), but all such conditions would be inaccessible to Galton–Fisher theory, centered as it is on the first two moments of the Gaussian distribution as a gauge of the variation of means. Galton–Fisher theory is not sensitive to variation in tolerances as a segregating trait. Purely technical use of Galton–Fisher theory in almost purely genetic bipolar disease may yield heritability at or close to zero and lead to the mistaken conclusion that the disease is nongenetic. Diabetes occurring early in life is usually type 1 and insulin dependent, and its etiology has long been recognized to have both environmental and multiple genetic components (see Chapter 86). One of these genes is the insulin locus, at which variation in tandemly repeated sequences 5' to the coding sequence affects regulation of insulin transcription (20,21).

## 14.5 ONTOGENESIS OF ANATOMIC STRUCTURES: ANGULAR HOMEOSTASIS

A colleague from the humanities identifies a fundamental problem: “A novel is half a million words long and one line thick. How can a novel be coherently written?” This evocative image is the very nub of the pathogenetic challenge for large and complex structures. How is a linear (single-dimensional) code “one nucleotide thick” in the genome to be translated into the fetus? The objective involves three dimensions in space and one in time. That the whole must be carefully orchestrated is self-evident. (The linear genetic code certainly has spatial orientation; however, this levorotatory optical asymmetry cannot convey any genetic information, since the essence of a code is the possibility of an alternative, and no dextrorotatory alternative is known.)

Perfect assembly of a spatial structure is rarely enough: it must commonly be adequately matured by a specified ontogenic timeline. Development of the lips and palate calls for exquisite timing. Even in genetic etiology, the ontogenic phenomenon of “time windows” indicates some state of minimal precision. This complex feat of organizing form and timing may perhaps be achieved by “dead reckoning,” by rigorous specifications and a relentless timetable. However, there is evidence that the system is more robust than that, because of what Waddington (22) called homeorrhexis. This process in ontogeny, which we call angular homeostasis, is akin to Bernard–Cannon homeostasis (23,24). In it, discrepancies between the current and the ideal states of ontogeny are discerned and corrected. There is plenty of biologic evidence from age-old observations of tropisms and taxisms and from



modern studies of target tracking and predation to make the whole strategy highly plausible. What exactly the cybernetic details are and how they operate are emerging in expressly and explicitly quantitative chemical terms (25,26). But the greater efficiency that continuous sensing and correction enjoys over unbending protocol is evident to anyone who has experience both of the minutely directed process of firing a gun at a moving target, where the path of the bullet, once fired, is beyond correction.

If usual development is difficult to reconcile with a linear code of instruction, so too is maldevelopment. In this “postgenome” era, a variety of approaches will be required to address both the normal and the abnormal (4,5).

### Example

Phenotypes associated with additions or deletions of many genes, such as in aneuploidy, or deletions or duplications of contiguous genes (“genomic disorders”) have stimulated considerable debate about pathogenesis (see Chapters 43 and 45). On the one hand are those who hold to the reductionist position that the phenotype reflects, in essence, mass action of too much or too little of the products of the affected genes. One result is the attempt to identify, for example, the gene or genes on chromosome 21 responsible for the cardiovascular malformations in the Down syndrome. On the other hand are those who see the phenotypes as the result of complex interplays among multiple genes, which are being expressed in a local environment that is asynchronous with normal development.

### Example

Coding the laterality of the brain is a more perplexing problem than ever. For the choice now does not seem to be an anatomic one at all, but a question of which information and which kinds of mental process shall be assigned to the left side and to the right. Wholly indifferent, random assignment is theoretically possible (27). However, although the facts have been distorted in the past by social prejudice, there still seems to be a higher rate of “left brain dominance,” and although the patterns are not altogether clear, there is evidence that genetic factors are at work. The genetics of handedness, as a surrogate for cerebral dominance, has been studied for decades. According to one hypothesis, mothers tend to support infants using their left arm, perhaps so to soothe them with the sound of the maternal heart. Thus, mothers who were by nature right-handed (dextral) would find some evolutionary favor (28). Another hypothesis suggests that left-handed warriors were more successful (29). What is clear, however, is that left-handedness is less common in all human populations, despite some geographic variation. Thus, whatever selective pressures favor left-handedness must be balanced by some negative ones for the trait to remain less common. The prevalence of left-handedness decreases with age (29). Whether sinistrality itself reduces life span, or serves as a marker for underlying neuropathology (perhaps related to cerebral dominance), remains unclear.

As to the vastly more complex problem of how the brain is constructed out of linearly arranged genetic instructions, the only mechanical model that comes to mind—and it is a feeble one—is the process of simultaneously loading and configuring a language onto a

computer. Present general theory of the construction and operation of the brain taxes the imagination to the limit. But one, especially a geneticist, cannot help but be impressed by the reliability of the system.

### 14.5.1 Elaborateness of Repair

One is struck by the reciprocal relationship between ontogenic robustness and recuperative power. The brain is the most highly organized structure but, while having some functional capacity to recuperate from damage by the fluidity in allocating space to functions, only recently has it been shown to have any regenerative power of parenchymal cells at all. The structure of the kidney may be less critical than that of the heart but, like the heart, it has little capacity to repair damage to its architecture. Anatomically at least, the liver is both less critically structured and more robust, but has much greater forces of recuperation. Tissues such as skin, bone marrow, spermatogonia, and intestinal endothelium are still less elaborate and are therefore ready for regeneration as to be notorious sites of sensitivity to mutagens.

### 14.5.2 Life History

A natural feature of the impact of a disorder is how it affects well-being, fertility, and length of life. These three, although distinct, are obviously connected; yet the traditional methods of analysis pay little attention to this fact. Discussions on fitness make much of the fact that clinical, genetic, evolutionary, athletic, and moral fitness are so different that they must be discussed separately. Indeed, excellence in one may go with mediocrity, even total incompetence in another. The superathlete may be sterile and morally bankrupt. The puny may live a long life free of disability. The fertile may be negligent in the care of their progeny. But it is absurd to suppose the several types of fitness to be totally unconnected. The issues are too large to deal with here, and we consider only the relationship between clinical fitness and length of life.

It is useful to distinguish between the unfolding of a disease and the impact of its complications. The wholly static disorders are mostly trivial: red–green color blindness, tone deafness, pentosuria, synophrys, and the like. The usual pattern of deterioration may vary greatly. In severe cardiac malformations, the disability is evident at birth or soon after. Even so, major complications such as pulmonary hypertension and reversals of flow through a patent ductus arteriosus or an atrial septal defect may occur late. In hereditary polyposis coli, it is the course of the disease itself—the long latent phase, progressive polyposis, and malignancy—that is the chief concern, while other complications (except those due to therapy) are minor. By contrast, Alzheimer disease (OMIM\*104300) often appears so late that it is frequently obscured by intercurrent and competing diseases. Indeed, until recently “senile dementia” was viewed by some as a

concomitant of aging, and not as a disease. But while the duration of Alzheimer disease averages perhaps 5 years depending on diagnostic finesse and management, it is surprisingly difficult to find evidence that the patients ever have neurologic deaths. They seem to die of the complications (e.g. trauma, intercurrent infections, malnutrition) that occur at much increased risk.

The notion that well-being is eroded by overt catastrophes (e.g. strokes) or those imperceptible insults (e.g. chronic pyelonephritis) that we identify as “wear and tear” is so appealing that we are led, almost unconsciously, to accept multistage models (belonging to the broad Erlangian class). Where the genetic disorder becomes manifest early (as in Duchenne muscular dystrophy), the competing risks are small, and the pattern of deterioration is dominated by a single class of insults, with the typical survivorship curve positively skewed (i.e. with a long tail to the right). When the onset of disease is late, the patient shows the characteristic multiplex pathology so familiar to the geriatrician: damage in several body systems, so that it is often difficult to say which is the final cause of death. The survivorship is then often negatively skewed. That class of survivorship in which death is due to whichever of several partially damaged systems fails first is a “bingo” model, usually given some qualifying term denoting the structure of the decay in the individual systems (e.g. “bingo-gamma,” “bingo-logistic”). It is a common state that flagrantly violates the assumptions underlying Galton–Fisher theory.

### 14.5.3 Nature and Nurture Reconsidered

Part of the notorious “nature–nurture” vision of medicine (i.e. the image that disease is in origin either genetic or environmental or between them) was that it was suggested (we presume as a pun) for making sure that in the consequences nothing is overlooked. We have serious doubts that even at its best it was ever an illuminating axis. One merely has to think of whether scurvy is a vitamin deficiency or (so far as we know) an inborn error of metabolism. In a fair-skinned population at the equator, sunburn is almost purely environmental. In a mixed population of pure fair-skinned and pure black-skinned individuals, it will be almost purely genetic. That radical and basically meaningless anomaly is well known. There is also the hidden and unsatisfactory notion of anomalous division into classes that are exclusive and exhaustive. This dichotomy is not only logically incoherent but also practically unsound. The difficulties lie in the uneven logic. An environmental disease requires at the very least a black box empirical proof. Empirically, a Mendelian trait either must powerfully follow from birth the pattern of Mendelian inheritance or must be shown to be due to an inherited polypeptide anomaly or various combinations of them. Again, a chromosomal trait must at some scale be cytologically evident from birth. Where a “multifactorial” trait is concerned, however, scarcely

any proof at all is called, and only the feeblest criteria. Inevitably, then, anything that is discarded from lists of empirical causes, Mendelian traits, and chromosomal anomalies will too often, sometimes alarmingly often, be treated as some nonspecific assembly of disorders likely (especially if occurring in early life) due to “genes,” a claim that is almost disprovable (31). Consider a few telling consequences:

- Homosexuality has been declared a Mendelian trait, although its frequency is continually being identified as high, even preposterously high because it must severely impede reproduction and play havoc with the most fundamental principles of linkage.
- It has long been called into question whether hysteria exists at all (32). Yet, though its very existence is still equivocal, “hysteria” remains very common.

## 14.6 PATHOGENETICS OF REFINED TRAITS

The most important process known in genetics is generating the primary sequence of the polypeptide in the proper cell at the right time. This is attained by the elaborate apparatus of genetic coding, transcription and its regulation, and translation, which is highly conserved in evolutionary time, but the high organization largely ends at that stage. Once formed, the polypeptide assumes its secondary and higher structure by processes that are little understood; aside from posttranslational modifications catalyzed by enzymes, there seems to be little need to direct these processes. The polypeptide quickly assumes a stable low-energy state. Whether it ever becomes completely fixed is not readily established. But in or near that state, it functions most efficiently. The subsequent fate of the polypeptide may be largely random.

### Example

The theory of red cell survival suggests that the cell is eventually destroyed by random wear and tear, and the hemoglobin with it. However, survival of the whole is still shortened by some mutant forms of the primary structure of hemoglobin or of components of the erythrocyte wall.

The speed at which the polypeptide is made is certainly important. For instance, sickle hemoglobin is manufactured more slowly than the wild-type, such as to lead to a representation in the heterozygote in a ratio of 2:1 to 3:1. Furthermore, in heterozygotes, A and S hemoglobins tend to be concentrated in particular cells. However, one does not ordinarily regard translation (as opposed to transcription) primarily as a timed or quantitative process. Posttranslational modification is also sensitive to time. A mutation that results in substitution of a glycine in the triple-helical domain of type I procollagen results in slower winding

of the helix. This in turn exposes for a longer time critical amino acids to the enzymes that catalyze modifications, such as glycosylation. The net result is a much more “damaged” molecule than a simple amino acid substitution might predict. But there is even a higher order effect possible when a protein is malformed or otherwise damaged as it traverses the cellular machinery. The process of translocating proteins across membranes is being elucidated (33). A key component is the endoplasmic reticulum (ER). When the ER encounters a malformed protein, processing slows; if severe, a situation of “ER stress” ensues, which can lead to marked cellular dysfunction, even cell death (34–36). Interestingly, the cellular phenotype may be the same for different mutations that affect entirely separate proteins. Understanding the importance of ER stress to the overall phenotype may afford a generic approach to therapy, whereby refolding of the mutant protein is facilitated (37).

Where the components are interchangeable (e.g.  $\beta^A$ - and  $\beta^S$ -globins), systems are appropriately described by their corporate properties. Where the numbers are large (e.g. numbers of erythrocytes), the usual device is the probabilistic model; and where the numbers are even larger (e.g. molecules), deterministic methods greatly simplify the analysis with negligible loss of accuracy. However, whatever the value of deterministic models in microbial populations, they have little place in studies of human beings; even in molecular studies, they must be handled with circumspection. This is a major difference between classic population genetics and the highly individualized character of medical genetics.

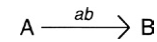
## 14.7 PATHWAYS AND MULTIPLE-STAGE PROCESSES

Two highly refined approaches have much in common and may, in certain circumstances, be united by a single theory. There is much to be gained by dealing with them interchangeably in stochastic and chemical terms, by seeing the usual dynamics as probabilistic. However, we shall first treat them separately.

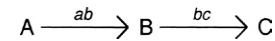
### 14.7.1 Simple Pathways

The simplest possible process involves synthesis of B from A by enzyme ab (Figure 14-1). There are three potential deleterious consequences:

- Precursor toxicity: Because ab fails, A accumulates and proves harmful. Alkaptonuria (OMIM\*203500) is such a disorder, as are most enzymopathies in catabolic pathways, such as lysosomal storage disorders.
- Product deficit: Because ab fails, B is reduced or absent. Examples include the various forms of albinism due to failure to produce pigment (e.g.



**FIGURE 14-1** An enzyme, ab, catalyzes conversion of substrate A to product B.



**FIGURE 14-2** An enzyme, ab, catalyzes conversion of substrate A to intermediate B, which is converted to final product C by enzyme bc. A defect in ab impairs production of C. The gene specifying ab is epistatic to that encoding bc.

OMIM\*203100) and most enzymopathies involving posttranslational processing of proteins. In a few mammalian species, the inability due to deficiency of one enzyme to synthesize ascorbic acid is another example. This example raises the fundamental issue of whether deficiency of this enzyme in all humans excludes it from the category of “disease.”

- Combined product deficit and precursor excess: The glycogen storage disorders are examples (e.g. OMIM\*232200). The glycogen that accumulates disrupts cellular and tissue processes, while failure to release glucose from glycogen leads to hypoglycemia. Phenylketonuria (OMIM\*261600) is another such example; phenylalanine is toxic in excess, and synthesis of tyrosine is impaired, resulting in the pleiotropic manifestations of phenylalanine hydroxylase deficiency.

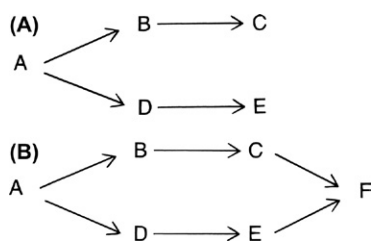
This elemental pattern extends to the three-step process:  $A \rightarrow B \rightarrow C$  (Figure 14-2). If A is absent, then B is lacking, and C cannot be synthesized. This suppression is epistasis; the gene governing the first step is epistatic to that governing the second. The classic example in humans is the rather trivial Bombay blood group phenomenon (OMIM\*211100) in which the failure to generate H substance destroys all means of expressing the ABO blood group phenotype.

### Example

Consider a typical multistage metabolic pathway, such as the synthesis of cholesterol or thyroid hormone. Each step is under the control of an enzyme. It will be at once evident that total failure at one or more steps means total blockade of later stages and that substrate accumulates before the first failed step. The gene for the enzyme at any step is therefore epistatic to all subsequent steps. The combined effect of defects in all genes will be the same as that of any (non-empty) subset of defective genes. In this it is quite different from the usual additivity of traits in Galton–Fisher theory.

### 14.7.2 Branching Pathways

We distinguish two kinds of branching pathways: the open and the closed. In the open type (Figure 14-3A), the branches do not rejoin and pool their products; thus, they compete for substrate, and the flow through each is correspondingly



**FIGURE 14-3** Metabolic pathways with branches. (A) Open branched pathway. (B) Closed branched pathway.

decremented. In the closed type (Figure 14-3B), the paths rejoin, and the result is a parastasis; two or more pathways run in parallel, which accelerates the entire process and acts as a fail-safe device should any of them fail. This scheme can be used as an advantage in treatment, such as by promoting remethylation of homocysteine to methionine through an alternative pathway dependent on the cofactor betaine (see Chapter 92). Those classic inborn errors of metabolism that lack adequate alternate pathways are the most severe clinically. Their rarity argues that metabolic processes without auxiliary paths are the exception, and the selective disadvantages may explain why.

On the other hand, a defect in one branch of a pathway may generate all or most of its pathology by leading to overflow through the alternative branch. For example, a defect in the enzyme hypoxanthine-guanine phosphoribosyltransferase (OMIM\*308000) leads to overproduction of phosphoribosylpyrophosphate. This in turn drives overproduction of purines, which leads to hyperuricemia, hyperuricosuria, and gout (see Chapter 95).

### 14.7.3 Pathways with Feedback

Metabolic pathways may be actively regulated in some cases by demands downstream. Negative feedback, positive feedback, or both can achieve a desired rate of processing or level of synthesis. This represents a form of physiologic homeostasis. Production of most hormones involves feedback at multiple levels. For example, estrogen is secreted by ovarian follicular cells in response to the anterior pituitary hormone and follicle-stimulating hormone (FSH); estrogen in turn feeds back on both the hypothalamus, to inhibit production of gonadotropin-releasing hormone, and on the anterior pituitary, to inhibit release of FSH, thereby modulating estrogen production and preparing the endometrium for implantation. Once an embryo implants in the endometrium, synthesis of chorionic gonadotropin signals the ovary to continue production of progesterone, which maintains the endometrium as a nurturing environment for continuing the pregnancy.

### 14.7.4 Multiple-Hit Processes

A metabolic process involving several steps may, where necessary, be viewed in more quantitative terms. In the

synthesis of insulin in response to a carbohydrate load,  $(n+p)$  steps are involved, but physiologic impact occurs only in the final  $p$  (potent) steps where physiologically active forms reside. The lag time for the response, then, is that for the transit through the system, and the first  $n$  (neutral) components, the inert precursors are neither here nor there. In this sense, what matters is only the process as a whole; permutations of the components do not matter.

#### Example

At a more clinical level, one may cite the proposals that the onset of Alzheimer disease (38) and aortic dissection in Marfan syndrome (39) are multiple-hit processes.

### 14.7.5 Multiple-Compartment Models

To deal with chemical processes in the foregoing fashion has certain uses, notably in understanding steady-state processes. Where changes need to be more rapid, however, we must take a more quantitative approach. Many of the processes of converting one class of compound into another are of so-called zero-order kinetics, that is, other things being equal, the rate of transfer equals the concentration of the substrate multiplied by the Michaelis constant,  $m$ . In a system without replenishment of substrate, these conditions define the negative exponential process, and the mean time for conversion, the waiting time, is precisely  $1/m$ . This pattern may be viewed from two perspectives: first, as a chemist sees it, deterministically conforming to the law of mass action; and second, as a probabilist sees it, a random exponential (one-hit) process in which every eligible molecule is at a fixed instantaneous hazard of change. As long as the number of molecules remains large, the distinction hardly matters. But the probabilistic model is more appealing because of its wider relevance in biology (e.g. when the number of decaying items—molecules, body cells, recurrent bodily insults—is small, and random uncertainty may no longer be safely ignored). Assuming that each step operates independently, the transit time through the metabolic chain is then the sum (or, strictly, convolution) of the waiting times of each step. The whole is termed a multiple-hit process. Its mean value is the sum of the waiting times, that is, the sum of the reciprocals of the Michaelis constants. (Throughout this discussion, the reader may be struck by the precise analogy to the corporate properties of the resistances in an electrical circuit.)

It will be clear that, the smaller any particular Michaelis constant compared to constants for steps elsewhere in the chain, the larger is its reciprocal and the greater its impact on the whole transit time. Moreover, other things being equal, for any given variation in  $m$ , the smaller the mean, the larger the variation in its reciprocal, and the more sensitively the impact of variations in it will be detected. At some, quite arbitrarily small, value of  $m$

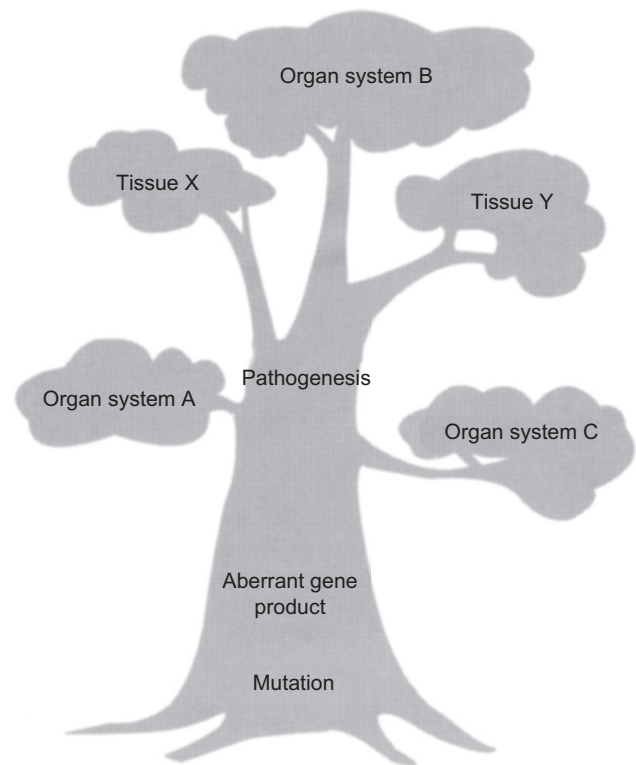


and large value for its reciprocal, this dominating step is termed the rate-limiting step; viewed genetically, if this step is itself Mendelian, the whole will be termed Mendelian. It will be evident that the conventional distinction between Mendelian and Galtonian (“multifactorial”) traits is both vague and arbitrary.

## 14.8 MOLECULAR PATHOGENETICS

When the defect in a genetic disorder is described at the level of the mutation, the object is at, or very close to, the genotype, and the field of inquiry is etiology. Anything more remote from the mutation represents phenotype and at least the first layer of complexity in the pathogenetics (Table 14-3). Thus, the resolution or sensitivity of the methods being brought to bear on the investigation of how the disorder arises determines how closely the mutation can be approached. The clinician has long had to deal with crude tools—stethoscope, tape measure, electrocardiogram, radiograph, urinalysis—to define the phenotype; what generally results is a perception of pathogenesis that is shallow, often complex, even confused. At the bedside, and even in the clinical laboratory, one usually sees only the leaves on the pathogenetic tree. Advances in clinical chemistry, biochemical genetics, cytogenetics, immunology, noninvasive and invasive imaging of many types, and pathology have all led to a more radical, hence sensitive, discernment of what is wrong with the patient and elucidation of pathogenesis. All these advances have facilitated in brushing aside the leaves and clambering part way down branches toward the trunk of the pathogenetic tree (Figure 14-4).

This figure also illustrates a fundamental characteristic of many human phenotypes termed pleiotropy. This word encompasses several concepts in biology; here it refers to multiple, even seemingly unrelated, aspects of the same syndrome. Indeed, syndrome embodies this notion of several clinical properties “running together.” Each of the leaves on the pathogenetic tree represents an aspect of the phenotype, connected through the limbs of pathogenesis. The analogy breaks down in that, while all leaves appear similar, the clinical details of the phenotype may be quite diverse. For example, dislocated lens, elongated digits, dural ectasia, and aortic root aneurysm are cardinal manifestations of Marfan syndrome, but outwardly bear no connection to each other (see Chapter 153). These features are all rooted in mutations in the gene encoding a large structural protein (*FBNI*), and at the first level of pathogenesis, share defects in an extracellular structure, the microfibril (corresponding to the trunk in the figure). However, microfibrils vary structurally and functionally in different organs and tissues, so each of the limbs of the tree heads in its own direction. The molecular bases of pleiotropy are as diverse as the features of some syndromes (40). For example, the complexity of some Mendelian inborn errors of metabolism is due to the mutant enzyme having functional roles



**FIGURE 14-4** Pathogenetic tree for a Mendelian condition. Leaves correspond to the phenotypic features, detectable by bedside investigation. Branches represent the pathogenetic pathways leading to organ- and tissue-specific pathology. The trunk corresponds to the gene product. Roots indicate the cause, in this case, the mutation.

**TABLE 14-3** Exploration of the Phenotype by Increasing Level of Complexity

mRNA
Translated protein
Posttranslationally modified protein
Localization of gene product
Macromolecular aggregate
Cellular metabolism
Tissue/organ function or structure
Clinical manifestations

beyond that in the specific metabolic pathway at issue. The extent of such “moonlighting enzymes” in human biology is uncertain (41).

The phenotype can be explored systematically, beginning with the first product of the mutant gene, messenger RNA (mRNA) (Table 14-3). Various defects in the structure and amount of a given mRNA can be described, albeit only with considerable effort and sophistication in techniques. The thalassemias (see Chapter 71) constitute one group of diseases that beautifully illustrate the molecular pathology of mRNA.

It is more feasible and instructive to focus on the stable product of most genes, the protein, and describe the types of molecular pathology that arises from mutation. In the most fundamental terms, a mutation can affect

the quantity of a protein, the quality of a protein, and occasionally both aspects. The quantity of a protein synthesized by a gene is regulated at the level of transcription, by promoters, enhancers, and other locus control elements, and at the level of translation (see Chapter 5). Mutations in any of a number of sites, *cis* and *trans*, to the gene of interest can affect the amount of protein produced. Usually, but not always, production from mutant alleles is decreased. One class of mutation that has garnered considerable attention is the expansion of a trinucleotide repeat within or, more commonly, outside the actual coding sequence (see Chapter 7). The number of repeats is inversely proportional to transcription of the gene. Furthermore, the more the repeats, the more severe the phenotypic change.

A change in the primary structure, the amino acid sequence, of a protein may alter its function (i.e. the quality of the protein). The study of diverse variants of the same protein has greatly advanced the understanding of molecular pathogenetics and given a certain sophistication to the “new” field of endeavor, investigating authentic relationships between genotype and phenotype. This inquiry calls for a new commitment to meaning and the authenticity of the descriptors, matters that are much more sophisticated than the correlations and coefficients that have dominated classic Galton–Fisher theory. The latter may lead to such paradoxical results as that, a nearly perfect correspondence between genotype and phenotype, may nevertheless yield a zero correlation and hence zero heritability. How the quality of a protein can be affected depends, in the first instance, on its normal function.

A mutation can change both quality and quantity of a protein. For example, a change in primary sequence might affect the stability of the protein and lead to enhanced (or retarded) degradation. In some situations, the amount of the mutant protein is crucial to the severity of the phenotype, especially in the dominant-negative scenario detailed later.

Proteins can be classified into three classes based on function: (1) those whose essential functions involve interactions with small molecules, such as enzymes, receptors, and transporters; (2) those that perform regulatory roles, such as transcription factors and hormones; and (3) those that function in complex systems, often in a structural role, and often in association with other proteins. Table 14-4 lists some examples.

Most proteins have one or more domains associated with specific functions. Not surprisingly, proteins in the same class often have domains in common, and there is remarkable conservation of sequences among domains. For example, transcription factors all have one or more amino acid sequence motifs (leucine zipper, zinc finger) that facilitate binding of the protein to DNA sequences. Cellular receptor molecules have domains that enable interaction with the lipid bilayer of the cell membrane, an extracellular domain that binds a ligand, and often

**TABLE 14-4** Classification of Proteins Based on Function

Function	Examples
Interaction with small	Enzymes, channel proteins, molecules transport proteins
Regulation	Peptide hormones, transcriptional regulators, RNA-binding proteins
Structure	Collagens, elastin

a domain that resides in the cytoplasm, perhaps exhibiting kinase activity. Some molecules have many domains, some of which are composed of dozens of repeated motifs—witness fibrillin (see Chapter 153), lipoprotein(a) (see Chapter 96), and plasminogen (see Chapter 54). The conservation of domains has facilitated discovery of the cause of numerous diseases through positional cloning. Thus, when a newly identified open reading frame is sequenced and found to be a strong candidate for the cause of a disorder, generally through identification of a mutation, the logical question is what the function of the protein encoded by the gene might be. If the coding sequence specifies an amino acid motif typical of a zinc finger, the protein is likely to be a transcription factor. This process is aided considerably by large databases that incorporate knowledge of genetic sequences and protein structure and function from both humans and all other organisms.

Proteins that interact with themselves (to form multimers) or with other proteins are subject to enhancement of a pathologic effect when one copy of their gene is mutant. Even though the patient is heterozygous for the mutation, the defective protein, by interacting with the product of its normal allele, or the products of other nonmutant genes, consumes these normal proteins; the result is a much more severe phenotype than would be expected from having half-normal levels of the normal protein. This is termed the dominant-negative effect, and is rather common. The irony is that a “more severe” mutation, such as one that eliminates transcription from the mutant allele altogether (i.e. a null allele), has less effect on phenotype than does a missense mutation that leads to normal transcription and translation of a mutant protein.

Within each of the three classes of proteins, a mutation can have one of four consequences: quantitative increase or decrease in function and qualitative gain or loss of function. Each of these consequences can have a number of molecular explanations (37).

A quantitative increase in function can be due to a regulatory mutation. An example is loss of sensitivity to inhibition, such as by a repressor molecule. A mutation could also affect the active site of an enzyme, such that its  $V_{\max}$  was increased, or the binding site of a hormone, such that the  $K_M$  was lowered. Many disorders associated with expansion of unstable trinucleotide repeats,

such as Huntington disease, share gain-of-function pathogenesis (42).

A quantitative decrease in function could operate by the converse of any of the mechanisms in the previous paragraph. The extreme of the spectrum of decreased function is loss of function, perhaps the easiest to conceptualize, and certainly the most prevalent consequence. For example, most inborn errors of metabolic pathways result from an enzymatic failure. The enzymopathy can be due to a mutation in or around the locus encoding that enzyme, resulting in a qualitative or quantitative defect as described earlier; to abnormal posttranslational processing of the nascent enzyme; to abnormal subcellular localization or extracellular trafficking; to altered affinities for substrates or cofactors; or to altered responsiveness to allosteric regulators of activity. Other examples of loss-of-function phenotypes include familial hypercholesterolemia due to many of the defects in the low-density lipoprotein receptor and cancers due to defects in tumor suppressor genes such as the retinoblastoma or neurofibromatosis type 1 genes. Strains of mice bearing gene “knockouts” represent specified loss-of-function mutations; these are especially popular tools for studying development and neoplasia.

Quantitative and qualitative loss of function clearly overlap. A mutation that reduces the ability of an enzyme to bind substrate also might lead to enhanced degradation and a reduced steady-state amount of the protein molecule.

Mutations that cause a gain in function, that is, a function not intrinsic to the wild-type protein, are less common. The diverse familial amyloidoses are examples, in which a change in amino acid sequence of one or another protein (e.g. transthyretin) results in enhanced stability of the protein and abnormal tissue deposition (see Chapter 79).

The least commonly recognized molecular phenotype, also qualitative, is a change in function. One example is the product of the fusion of the BCR and the ABL genes in chronic myelogenous leukemia (see Chapter 75). Another example is the *p53* protein, which when mutated in some ways, assumes regulatory capabilities foreign to the normal product.

As useful as these protein phenotypes are for classification (and education), there are limitations in making the intellectual leap to the next level of pathogenetic complexity. For example, gene knockout mutations are relatively easy to generate in mice, and increasingly in other species. Many investigators see this technique as a facile way to isolate the physiologic role of a particular gene product, to generate an animal model of a given disease, or to serve as the background strain into which a defined mutation is introduced (43). There is no question that the approach has been brilliantly successful in a number of instances. However, the pitfalls have been underemphasized. For example, some mice homozygous for the absence of transforming growth factor- $\beta_1$

(TGF- $\beta_1$ ) were born normal in appearance and survived, both unexpected results given the prominent role of this cytokine in many aspects of development (44). The reason is “rescue” of the embryo by maternal TGF- $\beta_1$  that presumably crosses the placenta in sufficient quantity to replace the deficient fetal sources. But at a more fundamental level, the “null” mutant animal cannot be viewed as an artificial isolated system focused on that deficient gene product. Rather, the mutant strain is a complex homeostatic system capable of responding to loss of a specific protein, even compensating for it. Thus, if the null strain shows no phenotype, it would be inappropriate to conclude that the missing protein is not important to the physiology of a given system (physiologic or developmental) (45).

The actual effect of the mutation may be loss of function at the protein level, but gain of function at the cellular level. For example, Rett syndrome (OMIM\*312750) is a pleiotropic, severe neurologic disorder that primarily affects girls. The cause is mutation of the *MECP2* locus, which encodes a protein that represses transcription of other genes (46). Mutations that inactivate the *MECP2* protein result in enhanced or inappropriate production of proteins in various tissues, most obviously the brain.

## 14.9 CONCLUSIONS

The prognosis of a disease is largely a matter of pathogenesis. For instance, its age of onset, the rapidity of its course, and the vulnerable points at which disease and complications may occur all depend on details that in principle, as much as in fact, may be difficult to infer even from the most detailed knowledge of the basic defect. Some knowledge of the prognosis may come from “black box” empirical inquiries—the natural history of myotonic dystrophy, for instance—but this course calls for extensive data, and there may be disturbing discrepancies between one study and another that are not readily reconciled. If the pathogenesis is understood, even partially, more incisive methods may be available, including direct measurements of the progress of components of the disease. For instance, the pathogenesis of familial polyposis coli is not clearly established, but currently the course of this disease and its response to treatment are easier to study than Alzheimer dementia. Refined studies at the molecular level make for very precise statements about etiology. It is tempting, but rather treacherous, to view pathogenesis in the same way. But where the concern lies in either the assessment of morbidity or the study of the population and eugenic behavior of the mutant, to attach too much weight to refined biochemistry may push the precision of the statement at the expense of its significance. For the overt clinical pattern and the target of selection are very coarse matters; the many modifying factors, which to the basic scientist are largely a nuisance, may have important attenuating effects on the course of the disorder.

Many advances in therapeutics have resulted from largely empirical reasoning as to choosing an approach, but from an understanding of natural history in judging whether the therapy was successful. A more rational approach to targeting therapy is based on an understanding of pathogenesis. Some fondly held the hope of circumventing “indirect” therapies for genetic disorders by simply replacing the defective gene. But considerable experience has amply shown the general fallacy in this approach. Until the molecular pathogenesis of a disorder is elucidated, the effects of simply adding back, or even replacing, a gene that should have been functioning perhaps from conception will be as empirical as anything physicians had available in the eighteenth century.

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## CROSS REFERENCES

Aging; Atherosclerosis; Amino acid disorders; Amyloidosis; Autosomal aneuploidies; Coagulation; Diabetes; Gene structure; Hemoglobinopathies; Leukemia; Lipoproteins; Mutation; Nature of genetic disease; Marfan; Purines and pyrimidines.

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### Biography



**Reed Pyeritz** completed his PhD and MD at Harvard and trained in internal medicine at the Peter Bent Brigham Hospital in Boston and medical genetics at the Johns Hopkins Hospital in Baltimore. He is a professor of Medicine and Genetics at the Raymond and Ruth Perelman School of Medicine of the University of Pennsylvania in Philadelphia, where he directs the Center for the Integration of Genetic Healthcare Technologies and serves as vice-chair for academic affairs of the Department of Medicine. He is a senior fellow in both the Center for Bioethics and the Leonard Davis Institute for Health Economics.

His research has focused on several areas, including clinical investigations of heritable disorders of connective tissue and cardiovascular diseases, especially those affecting the thoracic aorta. Currently he is studying how more refined methods of investigating genetic variation, such as cytogenomic arrays and whole genome sequencing, actually increase the level of uncertainty in applying the results clinically.

# CHAPTER 15

## Human Developmental Genetics

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### 15.1 INTRODUCTION AND OVERVIEW

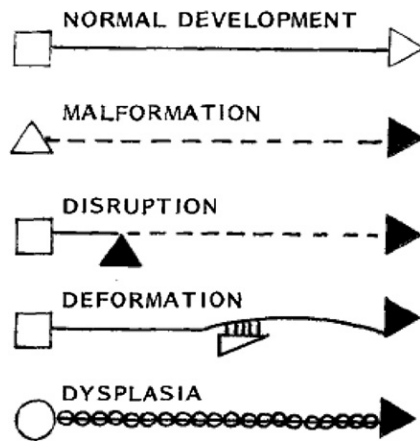
Congenital anomalies in humans can be classified into the following categories, which are defined as follows (1–3) (see Chapter 35 for details):

1. **Malformation:** A defect resulting from an intrinsic abnormality in the development of an organ or region of the body, that is, an error in morphogenesis from the earliest stages of embryogenesis or organogenesis.
2. **Disruption:** A structural defect in an organ, part of an organ, or a region of the body caused by extrinsic interference with, or breakdown of, a normally developing organ during organogenesis; the causes of disruption may be vascular (e.g. interruption of blood supply to developing organ), mechanical (e.g. amniotic bands), or infectious in origin, and the resulting defect does not conform to developmental fields.
3. **Deformation:** An “abnormal form, shape, or position of a part of the body” (1) due to either an extrinsic mechanical force (e.g. intrauterine compression from uterine leiomyomas) or an intrinsic developmental anomaly such as congenital myopathies leading to reduced limb movements in utero and consequently arthrogryposis.
4. **Dysplasia** is the “abnormal organization of cells into tissue(s)” (1) and therefore reflects “dyshistogenesis.” It may affect multiple organ systems that share the same tissue type, and unlike malformations, disruptions, and deformations, the resulting abnormality may change overtime with physical growth in the postnatal period. Examples of dysplasias include skeletal dysplasias and neurocutaneous melanosis sequence (OMIM# 249400) (Figure 15-1).

Malformations may be due to (1) incomplete morphogenesis in which there is either a complete lack

of development of an organ/tissue, or an incomplete developmental process, that is, a “developmental arrest”; (2) aberrant morphogenesis resulting in the presence of a tissue that does not appear in normal morphogenesis; (3) redundant morphogenesis resulting in the presence of accessory or duplicated structures such as polydactyly and preauricular skin tags (3,4).

Much of what we have learned about human development has come from studies in animal models. This is possible because the genes involved in development are highly conserved across species. The most common animal models that have been used are *Caenorhabditis elegans* (roundworm), *Drosophila melanogaster* (fruit fly), *Danio rerio* (zebrafish), *Xenopus laevis* (frog), *Gallus gallus* (chicken), and *Mus musculus* (mouse), although *Xenopus laevis* is tetraploid which renders genetic experiments challenging. However, there are differences in the functions of developmental genes among different animal classes, and even within the same class such as mammals. Recently, it has been shown that Oct4, a transcription factor that is critical for the induction and maintenance of pluripotency in stem cells, is expressed only in the inner cell mass (ICM) in the mouse blastocyst, but it is expressed in both the ICM and the trophectoderm (TE) in the cow blastocyst (5). Differences in patterns of gene expression between human and mouse pre-implantation embryos have also been reported (6). These data suggest that early embryonic development may differ even among placental mammals. Moreover, the phenotypic resemblance of a mutant animal model to the human phenotype depends partly on the genetic background and the presence of modifier genes in the animal model.



**FIGURE 15-1** Schema depicting different types of errors of morphogenesis. The broken line in malformation, disruption, and dysplasia symbolizes the developmental potential, not the time of manifestation of the defect, which may be late in embryogenesis. A dysplasia can be the result of an intrinsic or an extrinsic (disruptive) event. (Spranger, J.; Benirschke, K.; Hall, J. G.; Lenz, W.; Lowry, R. B.; Opitz, J. M.; Pinsky, L.; Schwarzacher, H. G.; Smith, D. W. *Errors of Morphogenesis: Concepts and Terms. Recommendations of an international working group*. J. Pediatr 1982, 100 (1), 160–165.)

## 15.2 TIMING OF NORMAL HUMAN DEVELOPMENT

The development of the human embryo (i.e. embryogenesis), which occurs in the first 8–9 weeks from fertilization to the beginning of the fetal period can be divided into 23 “Carnegie stages” based on work by the embryologist Franklin P. Mall (1862–1917) in the Department of Embryology at the Carnegie Institution of Washington. Carnegie staging of human embryos is based primarily on the “morphologic characteristics” rather than the age or size of the embryo, although each stage corresponds to an approximate post-fertilization/postconception age (NOT gestation age, which begins from the last menstrual period, approximately 2 weeks prior to fertilization in women with regular menstrual cycles) and size in terms of crown-rump length. The Carnegie stages, with the key events at each stage and the approximate post-conception age in days, are shown in Table 15-1 (7).

The first 4 weeks of development, from fertilization until the end of gastrulation (*vide infra*) (i.e. Carnegie stage 13), is also known as *blastogenesis*. Organogenesis begins at the end of blastogenesis and is characterized by morphogenesis (formation of organs and other body parts) and histogenesis (differentiation of cells and tissues) (8). The fetal period, which begins after Carnegie stage 23 and continues through delivery, is the period of continuing growth and differentiation of the organs formed during embryogenesis; the fetus also grows rapidly in length and weight during this period.

Knowledge of the timing of organ development helps us understand the latest gestational age at which a genetic or teratogenic “insult” could have occurred.







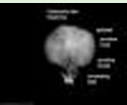


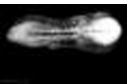



Table 15-2 lists some of the malformations that might result from incomplete morphogenesis and the timing at which those malformations could have occurred (9). In general, errors in morphogenesis that occur earlier in development result in more severe malformations than those that occur later in development.

## 15.3 THE CONCEPT OF DEVELOPMENTAL FIELDS AND FIELD DEFECTS

Morphogenesis is believed to occur within developmental fields, defined as “a region or a part of an embryo which responds as a coordinated unit to embryonic interaction and results in complex or multiple anatomic structures” (1). The *primary field*, which comprises the entire early embryo, is established through blastogenesis. The primary field is then subdivided through the process of pattern formation into *progenitor fields*, which give rise to the primordia of all morphologic structures. Subsequent differentiation results in *secondary* or *epimorphic fields*, which form the “irreversibly determined final structure(s).” Histogenesis occurs much later in development, usually after pattern formation. As such, most organs that are malformed are histologically normal and not at risk of developing malignancies; on the other hand, abnormal histogenesis in a dysmorphogenetic organ might result in malignancy.

Abnormal differentiation during organogenesis usually leads to a *monotopic field defect* and hence an anomaly in a single structure or organ. In contrast, abnormal blastogenesis usually results in a *polytopic field defect* in which multiple structures/organs in different parts of the body that share the same primary developmental field are malformed. Multiple congenital anomalies may also be part of a *sequence*, defined as “a pattern of multiple anomalies derived from a single known or presumed prior anomaly or mechanical factor”—it refers to a “cascade of consequences with known pathogenetic mechanisms”; a *syndrome*, defined as “a pattern of multiple anomalies thought to be pathogenetically related and not known to represent a single sequence or a polytopic field defect”; or an *association*, defined as “a non-random occurrence in two or more individuals of multiple anomalies not known to be a polytopic field defect, sequence, or syndrome [...] refers solely to statistically, not pathogenetically or causally related anomalies” (1,10). It has been suggested that the term “association” be limited to the “the idiopathic statistical occurrence of multiple congenital anomalies apparently of non-blastogenetic origin.” Hence, the VATER “association” (OMIM# 192350), which is postulated to be due to a blastogenetic defect, should be considered a polytopic field defect instead of an association (10). Similarly, the CHARGE “association” became the CHARGE syndrome (OMIM# 214800) when *CHD7* was identified as the causative gene in this condition.











TABLE 15-1 Carnegie Stage Table

Stage	Days (approx.)	Size (mm)	Images (Not to Scale)	Events
1	1 (week 1)	0.1–0.15		Fertilized oocyte, zygote, pronuclei
2	2–3	0.1–0.2		Morula cell division with reduction in cytoplasmic volume, blastocyst formation of inner and outer cell mass
3	4–5	0.1–0.2		Loss of zona pellucida, free blastocyst
4	5–6	0.1–0.2		Attaching blastocyst
5	7–12 (week 2)	0.1–0.2		Implantation
6	13–15	0.2		Extraembryonic mesoderm, primitive streak, gastrulation
7	15–17 (week 3)	0.4		Gastrulation, notochordal process
8	17–19	1.0–1.5		Primitive pit, notochordal canal
9	19–21	1.5–2.5		Somitogenesis <b>Somite Number 1–3</b> neural folds, cardiac primordium, head fold
10	22–23 (week 4)	2–3.5		<b>Somite Number 4–12</b> neural fold fuses
11	23–26	2.5–4.5		<b>Somite Number 13–20</b> rostral neuropore closes
12	26–30	3–5		<b>Somite Number 21–29</b> caudal neuropore closes
13	28–32 (week 5)	4–6		<b>Somite Number 30</b> leg buds, lens placode, pharyngeal arches (also known as branchial arches)

Continued



TABLE 15-1 Carnegie Stage Table—Cont'd

Stage	Days (approx.)	Size (mm)	Images (Not to Scale)	Events
14	31–35	5–7		Lens pit, optic cup
15	35–38	7–9		Lens vesicle, nasal pit, hand plate
16	37–42 (week 6)	8–11		Nasal pits moved ventrally, auricular hillocks, foot plate
17	42–44	11–14		Finger rays
18	44–48 (week 7)	13–17		Ossification commences
19	48–51	16–18		Straightening of trunk
20	51–53 (week 8)	18–22		Upper limbs longer and bent at elbow
21	53–54	22–24		Hands and feet turned inward
22	54–56	23–28		Eyelids, external ears
23	56–60	27–31		Rounded head, body and limbs

Source: <http://php.med.unsw.edu.au/embryology/>

## 15.4 CELLULAR SIGNALING IN DEVELOPMENT: THE CONCEPTS OF INDUCTION AND COMPETENCE

Cellular differentiation and organ formation are dependent on interactions between cells and tissues of different origins that are in close proximity to each other. This type of interaction is known as *induction*. The tissue that releases the signal is known as the inducer, while the “target tissue” is known as the responder. For a tissue to be a responder, it has to possess the appropriate receptor for the signaling molecule, as well as *competence*, which is defined as the ability of the tissue to respond to a specific signal; not all tissues are competent to respond to a specific signal from a specific inducer. For example, in the development of the lens in *Xenopus*, only the anterior ectoderm (i.e. ectoderm in

the head) is competent to be induced by the optic vesicle. However, an initial induction may result in a specific tissue becoming competent to respond to signals from a subsequent inducer. Moreover, once induction has occurred, the responder may then induce its own inducer, a phenomenon known as *reciprocal induction*. Examples of induction include the interactions between the epidermis of the skin or epithelium of the intestines (both derived from ectoderm) and the underlying dermis or mesenchymal tissue (both derived from mesoderm) respectively. Induction may be “region-specific” such that the structures derived from the responder may be dependent on the region from which the inducer was derived. For example, the cutaneous structures that develop from the epidermis (responder) depend partly on the source of the dermal mesenchyme (inducer) (11).

**TABLE 15-2 Developmental Pathology of Certain Malformations Which Represent Incomplete Development**

Tissues	Malformation	Defect in—	Cause prior to—	Comment
Central nervous system	Anencephalus	Closure of anterior neural tube	24 days	Possibility also that origin may be after closure 80% lumbosacral
	Meningomyelocele	Closure in a portion of posterior neural tube	26 days	
Face	Cleft lip	Fusion of lateral and median nasal processes	36 days	42% associated with cleft palate
	Cleft maxillary palate	Fusion of maxillary palatal shelves	8 weeks	Preauricular and along line of anterior sternocleidomastoid
	Branchial sinus and/or cyst	Resolution of branchial cleft	8 weeks	
Gut	Esophageal atresia + tracheo-esophageal fistula	Lateral septation of foregut into trachea and foregut	30 days	Associated incomplete or aberrant mesenteric attachments
	Rectal atresia with fistula	Lateral septation of cloaca into rectum and urogenital sinus	6 weeks	
	Duodenal atresia	Recanalization of duodenum	7–8 weeks	
	Malrotation of gut	Rotation of intestinal loop so that cecum lies to right	10 weeks	
	Omphalocele	Return of midgut from yolk sac to abdomen	10 weeks	May contain gastric and/or pancreatic tissue
	Meckel's diverticulum	Obliteration of vitelline duct	10 weeks	
	Diaphragmatic hernia	Closure of pleuro-peritoneal canal	6 weeks	
Genital-urinary system	Extroversion of bladder	Migration of infraumbilical mesenchyme	30 days	Associated Müllerian and Wolffian duct defects
	Renal agenesis	Genesis of ureter from mesonephric duct	30 days	
	Bicornuate uterus	Fusion of lower portion of Müllerian ducts	10 weeks	
	Hypospadias	Fusion of urethra (labia minora) folds	12 weeks	
	Cryptorchidism (of testicle)	Descent of testicle into scrotum	7–9 months	
Heart	Transposition of great vessels	Directional development of bulbus cordis septum	34 days	
	Ventricular septal defect	Closure of ventricular septum	38 days	
	Patent ductus arteriosus	Closure of ductus arteriosus	9–10 months	
Limb	Aplasia of radius	Genesis of radial bone	38 days	Often accompanied by other defects of radial side or distal limb
Complex	Syndactyly	Separation of digital rays	42 days	Secondary defects of mid-face and forebrain Associated defects of cloacal development
	Cyclopia, holoprosencephaly	Prechordal mesoderm development	26 days	
	Sirenomelus, symphodia	Development of posterior axis	26 days	

Induction is often mediated by *paracrine factors*, which are signaling proteins that are secreted by a group of cells (i.e. the inducer) and diffuse across relatively short distances in the intercellular space to alter the differentiation or “behavior” of neighboring cells (i.e. the responder); this is known as a paracrine interaction. In contrast, *endocrine factors* (i.e. hormones) have to travel through the blood stream across relatively long distances to their receptors. Induction

may also be mediated through *juxtacrine signaling* or interactions in which cell membrane proteins on the inducing cell interact directly with the receptors on the adjacent responding cell. Almost all of these interactions lead to a series of enzymatic reactions in the responding cell that result in either the regulation of transcription factors and hence gene expression, or the regulation of cytoskeletal structures and hence cell shape or motility (11).

## 15.5 A LIMITED REPERTOIRE OF DEVELOPMENTAL GENES AND PATHWAYS

Embryogenesis and the development of various organ systems utilize a common set of genes and developmental pathways. The same gene may be expressed in different tissues at different developmental stages or regulate different developmental pathways and hence affect the development of different organ systems. Some developmental pathways interact with one another. There are also parallel pathways leading to “redundancy” due to the presence of paralogous genes (i.e. genes with very similar nucleotide sequences and are presumably derived from the same ancestral gene by gene/chromosome duplication) (3). For example, the Hox (homeobox) gene families are responsible for the determination of the anterior–posterior axis and patterning of the axial skeleton in all vertebrates. All mammals have four Hox gene clusters (*Hoxa*, *Hoxb*, *Hoxc*, *Hoxd*) on different chromosomes that arose from duplications of each ancestral Hox gene cluster such that the equivalent Hox gene in each cluster is paralogous with one another, although not every Hox gene is present in each cluster (e.g. the Hox1 paralogous group comprises *Hoxa1*, *Hoxb1*, and *Hoxd1*—*Hoxc1* does not exist in mammals; the Hox4 paralogous group comprises *Hoxa4*, *Hoxb4*, *Hoxc4*, and *Hoxd4*) (11). It was found that mutations in all six alleles of the three Hox10 paralogous genes in the mice (*Hoxa10*, *Hoxc10*, *Hoxd10*) resulted in a complete lack of lumbar vertebrae, abnormal sacral vertebrae, and ectopic ribs, but mutations in any five of the six alleles resulted in a far less severe phenotype with the preservation of the lumbar vertebrae. More importantly, the phenotypes of these “five allele mutants” mice were similar regardless of the allele that was mutated, demonstrating the redundancy among the Hox10 paralogous genes. Similar findings were reported for the Hox11 paralogous genes when those complete “triple mutants” mice were compared with mice harboring mutations in five out of the six Hox11 paralogous alleles (12).

## 15.6 STEPS AND CONCEPTS IN EMBRYONIC DEVELOPMENT

Following the fertilization of the mammalian oocyte by the spermatozoa in the ampulla of the oviduct, meiosis II in the oocyte is completed, the pronuclei of the oocyte and spermatozoa fuse, and about 24–27 hours later, the diploid *zygote* undergoes the first *cleavage* (i.e. mitosis without synthesis of cytoplasm and hence without an increase cellular mass) to form two *blastomeres* (i.e. early embryonic cells formed by through mitosis of the zygote). Subsequent cleavage results in the formation of a “solid” *morula* with 12–15 cells by day 4 post-fertilization, comprising a collection of cells that will form the inner cell mass *ICM* surrounded by an external layer of cells (*trophoblast*) that will form the trophectoderm *TE*. The ICM will give rise

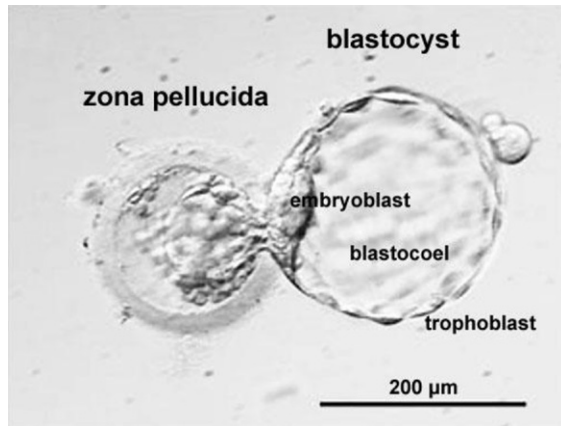
to the embryo while most of the cells of the TE will form the chorion, which is the embryonic component of the placenta (11,13). The TE is the first type of cells to differentiate in mammalian development, and it is required for the subsequent formation of the blastocoel (*vide infra*) (14).

Mammalian cleavage is asynchronous in that not all blastomeres undergo mitosis at the same time. Consequently, there can be an odd number of blastomeres in the embryo. Maternal mRNA that is present in the oocyte at the time of fertilization may be translated either before or after fertilization, and these maternally derived proteins regulate the development of the zygote in the earliest stages. In the human embryo, the zygotic genes are first expressed at around day 3 post-fertilization between the four-cell and eight-cell stages (3). It is believed that this maternal–zygotic transition is regulated by microRNAs miR-302, miR-372, and miR-516–520, which are the human orthologs of the zebrafish miR-430 cluster (15). Cleavage and development is therefore regulated from a relatively early stage by zygotic rather than maternally inherited proteins.

Following the third cleavage (eight-cell embryo) at approximately day 4 post-fertilization just prior to the formation of the morula, the blastomeres which had hitherto had a “loose configuration,” aggregate tightly together in a process known as *compaction* due to the expression of cell adhesion proteins such as E-cadherin (*CDH1*). Homozygous *Cdh1* mutations in mouse embryonic stem cells resulted in embryos that failed to maintain compaction and were incompatible with life, but embryonic stem cells with heterozygous *Cdh1* mutations appeared to develop normally (16).

The earliest blastomeres, which have the potential to become either the ICM or the trophoblast, are *totipotent* (i.e. capable of everything), while the cells of the ICM can form any tissue in the fetus but not those of the TE such as the chorion, and hence are considered *pluripotent* (i.e. capable of many things). *Cell fate* refers to the type of cell that an undifferentiated embryonic cell would normally develop into. Commitment of a cell to a specific cell fate is initiated by the potentially reversible process of specification followed by the generally irreversible process of determination. Cells in most vertebrates undergo *conditional specification* in which the fate of a given cell is dependent on paracrine factors secreted by the neighboring cells. The fate of a cell may also be specified by the concentration of morphogens, which may be paracrine factors secreted by a group of cells externally or transcription factors within the cell; a given cell may have different fates depending on the concentration of the morphogen it is exposed to. *Determination* is said to have occurred when a cell differentiates into its original (intended) cell fate even when it is experimentally placed in another region of the embryo (11).

The timing of when the blastomeres are first determined to become the ICM and the trophoblasts remains somewhat controversial, but it is generally believed to occur after the formation of the morula. Cavitation, the process



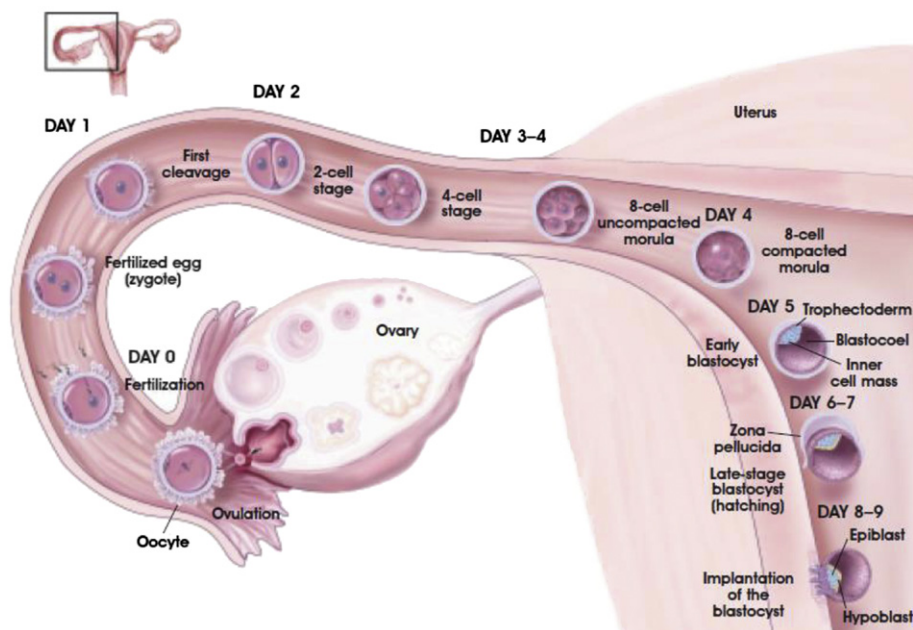
**FIGURE 15-2** Human blastocyst hatching from zona pellucida (embryoblast=ICM). (Hill, M. A. *UNSW Embryology*. <http://php.med.unsw.edu.au/embryology/> (Accessed March 18, 2012).)

in which the TE cells secrete fluid into the morula itself to form a blastocoel, occurs at approximately day 5 post-fertilization (about a day after compaction) and results in the formation of the blastocyst. As the blastocoel expands, the ICM is “pushed” to one side of the blastocyst and becomes a separate layer from the TE by the 64-cell stage, although it continues to secrete proteins that stimulate the trophoblasts to divide (11). Meanwhile, at approximately days 6–7, the ICM differentiates into the epiblast and the hypoblast (also known as the primitive endoderm); the hypoblast is the outermost layer of cells that overlies the epiblast (Figure 15-2). The epiblast gives rise to all embryonic lineages and the extraembryonic mesoderm, and is hence considered pluripotent; the hypoblast gives rise to the parietal

endoderm and visceral endoderm (VE) (14). The epiblast and hypoblast together form the bilaminar embryonic disc (also known as bilaminar blastoderm), thereby establishing the dorsal–ventral axis of the embryo with the epiblast being the dorsal surface and the hypoblast being the ventral. As these two layers differentiate, a basement membrane is formed between them (18).

### 15.6.1 Hatching and Implantation

The unfertilized oocyte is surrounded by the zona pellucida, an extracellular glycoprotein matrix that is critical for sperm binding during fertilization. After fertilization, the zona pellucida continues to encase the early developing embryo to prevent it from adhering to the wall of the oviduct, which could result in an ectopic pregnancy. However, when the blastocyst reaches the uterine cavity, it has to “hatch” from the zona pellucida, a process that may be mediated by the secretion of a trypsin-like protease by the trophoblasts (see Figure 15-2). The hatched blastocyst attaches itself to the uterine endometrium, initially through L-selectin molecules on the trophoblast cells and subsequently through other adhesion systems involving integrins, P-cadherins, heparan sulfate proteoglycans, and many other cell adhesion molecules (11). Some of the genes and adhesion proteins involved in implantation are reviewed in (19) and (20). The endometrium is receptive to implantation by the blastocyst only within a 48-hour window, about 7–10 days after ovulation (not fertilization). This whole process from fertilization to implantation is illustrated in Figure 15-3.



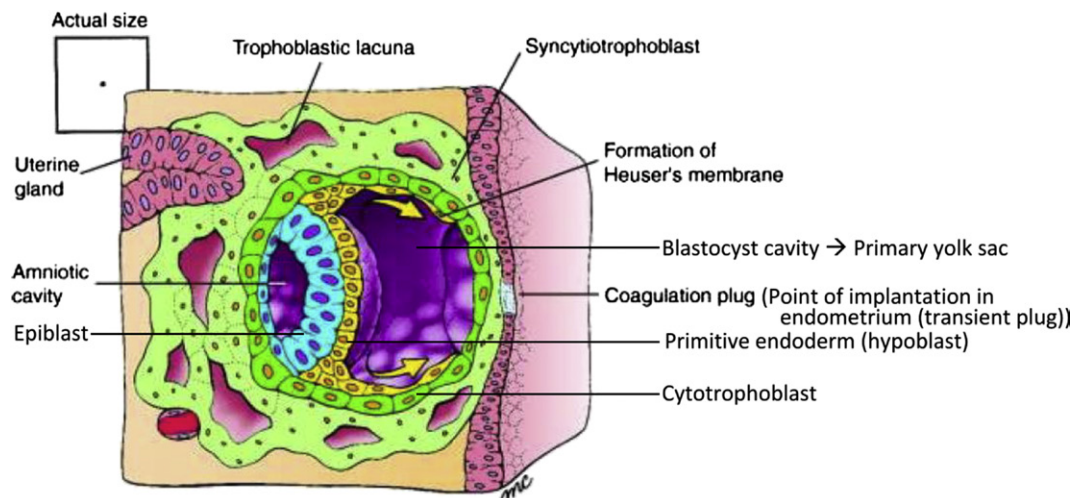
**FIGURE 15-3** Development of the preimplantation blastocyst in humans. Adapted from Reference (17) figure copyright: Terese Winslow (<http://teresewinslow.com/>).



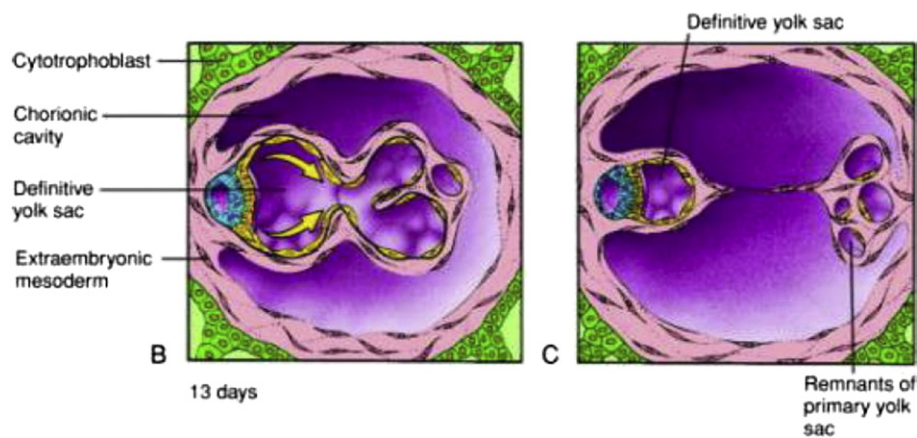
### 15.6.2 Formation of the Amniotic Cavity and Yolk Sac

At approximately day 8 post-fertilization, an amniotic cavity forms within the epiblast, resulting in the bilaminar embryonic disc becoming sandwiched between the amniotic cavity dorsally and the blastocyst cavity ventrally. The epiblast cells surrounding the amniotic cavity form an extraembryonic membrane known as the amnion. Cells from the hypoblast then migrate to form an exocoelomic (Heuser's) membrane around the blastocyst cavity, which becomes the primary yolk sac. Meanwhile, the trophoblast differentiates into a cellular cytotrophoblast and an expanding syncytiotrophoblast (a multinucleate cytoplasmic mass that arises from a loss of plasma membrane and mitosis without division of cytoplasm), both of which contribute to the eventual formation of the placenta (18) (Figure 15-4).

The extraembryonic mesoderm forms around day 10 and fills the space between the exocoelomic membrane and the cytotrophoblast; it is believed that the extraembryonic mesoderm is derived from cells in the hypoblast and primary yolk sac. The extraembryonic mesoderm continues to expand and fills the space between the amnion and the cytotrophoblast by day 12. The extraembryonic mesoderm overlying the blastocyst cavity then “splits” to form the chorionic cavity (also known as extraembryonic coelom), which expands to surround the primary yolk sac, the bilaminar embryonic disc, and the amniotic cavity, except at the connecting stalk, which is the part of the extraembryonic mesoderm that suspends the developing embryo in the chorionic cavity. A second wave of migration of hypoblast cells displaces the primary yolk sac to form the secondary (definitive) yolk sac; the primary yolk sac eventually degenerates by day 13 (18) (Figure 15-5).



**FIGURE 15-4** Day 9 embryo implanted in endometrium (18). (Schoenwolf, G. C.; Bleyl, S. B.; Brauer, P. R.; Francis-West, P. H., Larsen's Human Embryology, 4th ed., Churchill Livingstone: Philadelphia, 2008.)



**FIGURE 15-5** On days 12 and 13, a second wave of migration of hypoblast cells produces a new membrane that migrates out over the inside of the extraembryonic mesoderm, pushing the primary yolk sac in front of it. This new layer becomes the endodermal lining of the secondary (definitive) yolk sac. (B and C) As the definitive yolk sac develops on day 13, the primary yolk sac breaks up and is reduced to a collection of vesicles at the abembryonic end of the chorionic cavity. (Schoenwolf, G. C.; Bleyl, S. B.; Brauer, P. R.; Francis-West, P. H. Larsen's Human Embryology, 4th ed., Churchill Livingstone: Philadelphia, 2008.)

### 15.6.3 Gastrulation, Segmentation, and Formation of Body Axes

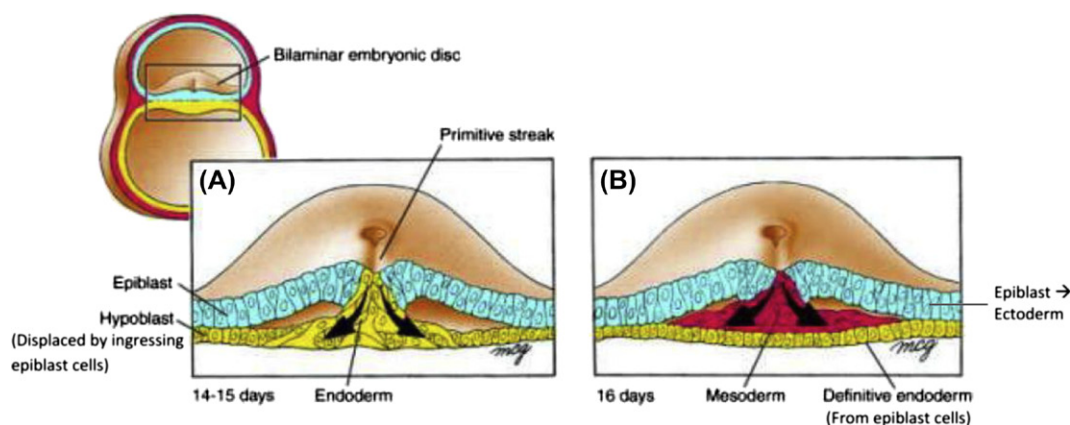
At approximately day 15, epiblast cells proliferate and migrate to the mid-sagittal plane in what will become the more caudal part of the of the bilaminar embryonic disc to form the primitive streak. The primitive streak then elongates along the sagittal plane and a narrow primitive groove develops within the primitive streak. The cranial end of the primitive streak expands to form the primitive node, within which is a small depression known as the primitive pit. Epiblast cells then migrate toward the primitive streak where *epithelial-to-mesenchymal transformation (or transition)* (EMT) occurs, a process in which the epiblast cells become less regularly shaped (often “flask-shaped”) and less tightly connected with one another, i.e., becoming mesenchymal in nature. These transformed epiblast cells invade and displace the hypoblast, eventually replacing it with a new layer of cells known as the definitive (embryonic) endoderm. Beginning around day 16, the invaginating (or ingressing) mesenchymal epiblast cells enter the space between the epiblast and the hypoblast/endoderm to form the intraembryonic mesoderm (Figure 15-6). Once the definitive endoderm and intraembryonic mesoderm have been formed, the remaining epiblast cells become the ectoderm. The formation of all three germ layers from epiblast cells, resulting in the trilaminar embryonic disc, constitutes gastrulation (18).

In an area just cranial to the primitive streak, the ectoderm forms a mild depression and fuses with the endoderm without the mesoderm to form a bilaminar membrane known as the oropharyngeal membrane, which eventually ruptures during the fourth week of development to form the opening of the mouth; a similar bilaminar membrane—the cloacal membrane, which eventually gives rise to the openings of the anus, the urinary and genital tracts—is formed caudal to the primitive streak. At around day 17, a subpopulation of mesodermal cells migrates cranially from the primitive

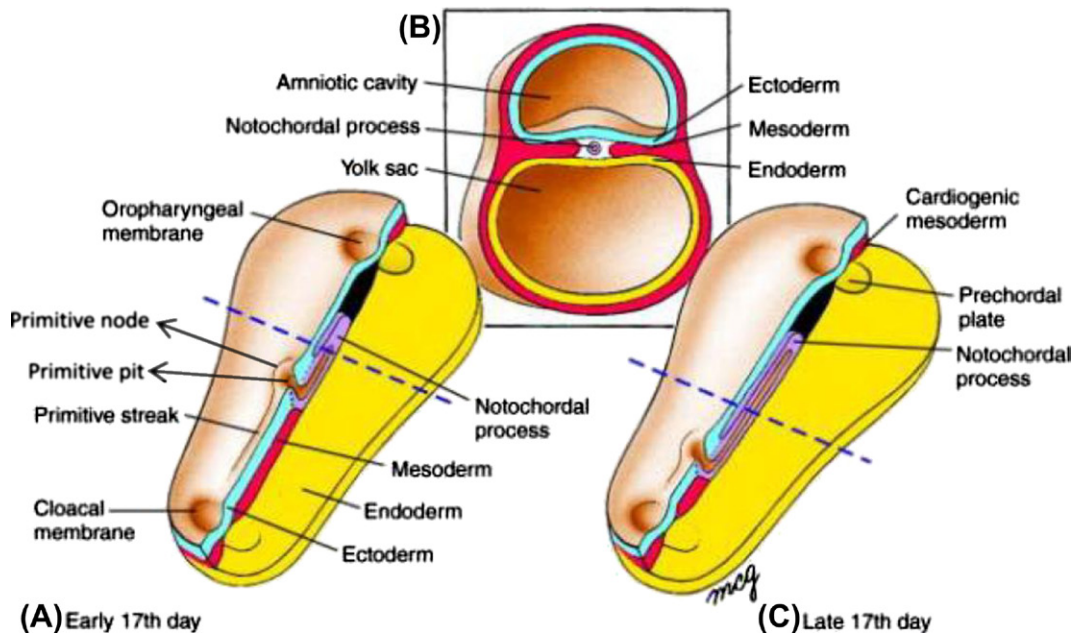
node to form the hollow notochordal process, which is subsequently transformed into a solid notochord by day 22, as illustrated in Figures 15-7 and 15-8. The notochord is responsible for the induction of vertebral bodies (18).

A subpopulation of mesodermal cells becomes the paraxial mesoderm and flanks the notochord on each side (Figure 15-8). In the region that will eventually become the head, these mesodermal cells form “bands of cells” that remain unsegmented and form the head mesoderm; in the region that will become the trunk, the column of paraxial mesoderm on each side of the notochord, known as the presomitic mesoderm, forms “bands of cells” that segment into blocks known as somites in a cranial–caudal direction, starting at the head–trunk junction on day 20 and lasting through day 30 when about 42–44 pairs of somites are eventually formed. The most caudal somites subsequently regress, leaving about 37 pairs of somites. These somites give rise to the occipital bone of the skull, the spine, and the skeletal muscles of the neck, trunk, and limbs. The first four pairs of somites contribute to the formation of the occipital skull bone, the bones of the mid-face and the inner ear, and the muscles of the tongue. The remaining somites form the vertebrae, skeletal muscles, and dermis of the cervical, thoracic, lumbar, and sacral spine; the three most caudal somites are the coccygeal somites that form the coccyx. Immediately lateral to the somites is the intermediate mesoderm, which also forms segments, and lateral to the intermediate mesoderm is the unsegmented lateral plate mesoderm (18) (Figure 15-9).

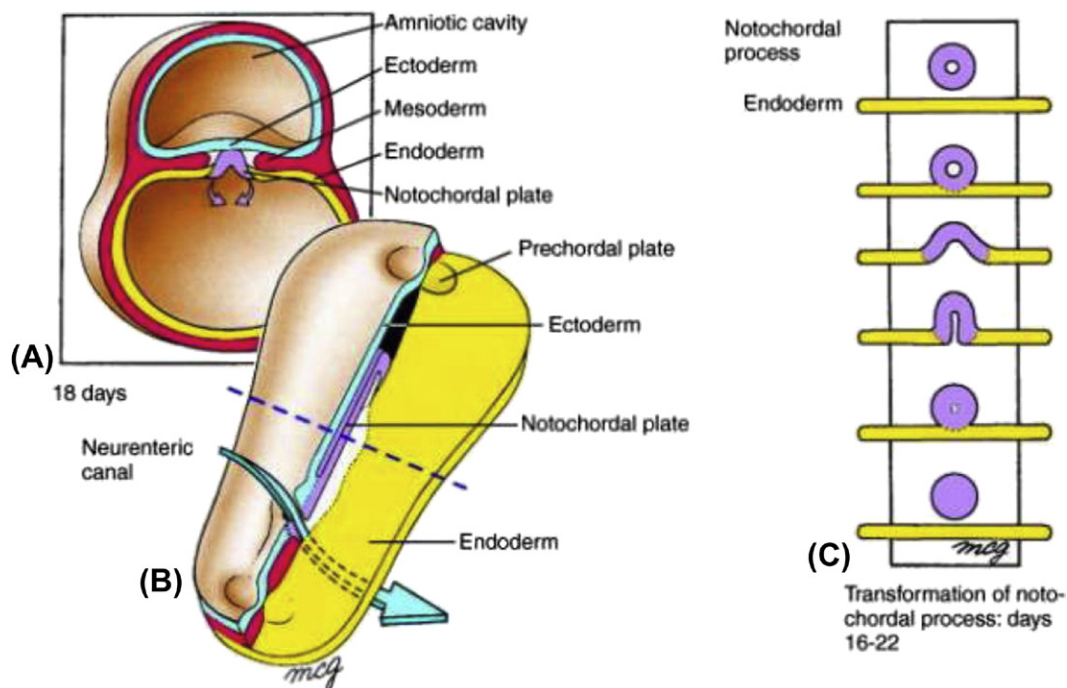
The formation of the primitive streak establishes the major body axes. Since the primitive node/pit lies at the cranial end of the primitive streak, the primitive streak defines the cranial–caudal axis. With the primitive streak being in the midline (i.e. most medial), the medial–lateral axis is thereby defined. When viewed from within the amniotic cavity, the midline primitive streak divides the embryo into the right and the left sides, hence defining



**FIGURE 15-6** (A) On days 14 and 15, ingressing epiblast cells displace hypoblast and form definitive endoderm. (B) Epiblast that ingresses on day 16 migrates between endoderm and epiblast layers to form intraembryonic mesoderm. (Schoenwolf, G. C.; Bleyl, S. B.; Brauer, P. R.; Francis-West, P. H. *Larsen's Human Embryology*, 4th ed., Churchill Livingstone: Philadelphia, 2008.)

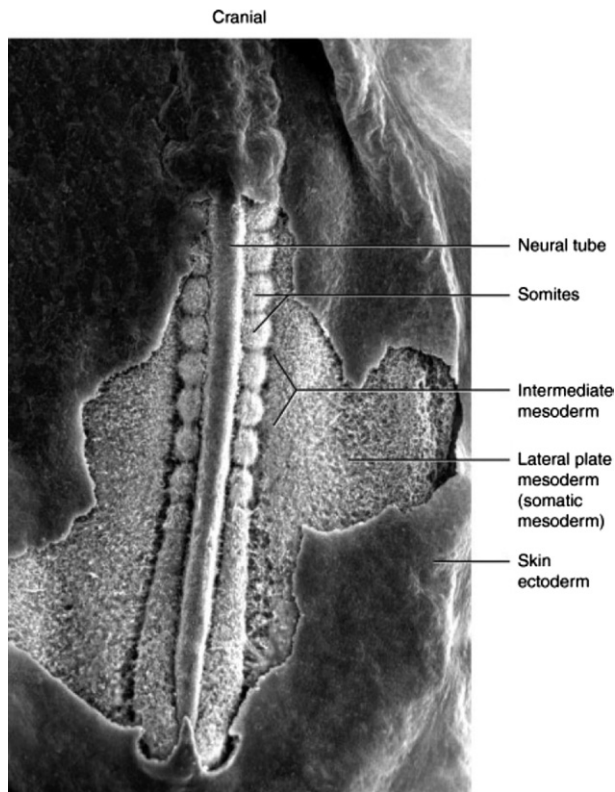


**FIGURE 15-7** Formation of the notochordal process. (A and C) Stages showing hollow notochordal process growing cranially from the primitive node. Note changes in relative length of the notochordal process and primitive streak as the embryo grows. Also note, fusion of ectoderm and endoderm in the oropharyngeal and cloacal membranes. (B) Cross section of the embryonic disc at the level indicated by the dotted lines. (Schoenwolf, G. C.; Bleyl, S. B.; Brauer, P. R.; Francis-West, P. H. *Larsen's Human Embryology*, 4th ed., Churchill Livingstone: Philadelphia, 2008.)



**FIGURE 15-8** The process by which the hollow notochordal process is transformed into a solid notochord between days 16 and 22. (A and B) First, the ventral wall of the notochordal process fuses with the endoderm and the two layers break down, leaving behind the flattened notochordal plate. As shown in (B), this process commences at the caudal end of the notochordal process and proceeds cranially (the dotted line marks the level of (A)). An open neurenteric canal is briefly created between the amniotic cavity and the yolk sac cavity. (C) Series of events by which the notochordal process becomes the notochordal plate and then the notochord. (Schoenwolf, G. C.; Bleyl, S. B.; Brauer, P. R.; Francis-West, P. H. *Larsen's Human Embryology*, 4th ed., Churchill Livingstone: Philadelphia, 2008.)



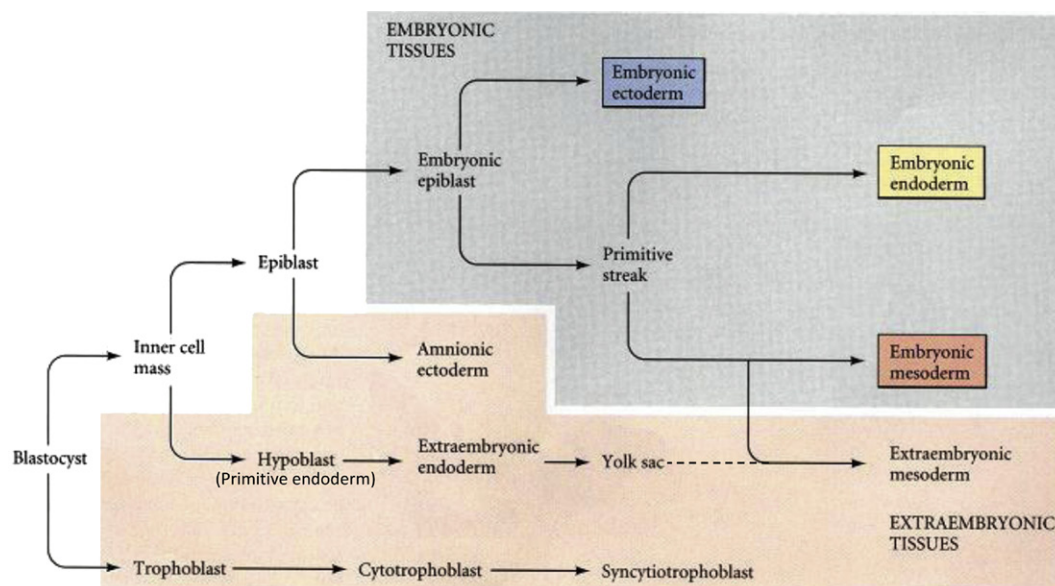


**FIGURE 15-9** Scanning electron micrograph of the trunk region of a chick embryo with the surface ectoderm partially removed to show the underlying neural tube and mesoderm (cranial is toward the top). Note the somites and, more caudally, the paraxial mesoderm that have not yet segmented. Lateral to the somites, the mesoderm has been subdivided into the intermediate mesoderm and lateral plate mesoderm (somatic mesoderm, the layer just deep to the surface ectoderm, is visible). (Schoenwolf, G. C.; Bleyl, S. B.; Brauer, P. R.; Francis-West, P. H. *Larsen's Human Embryology*, 4th ed., Churchill Livingstone: Philadelphia, 2008.)

the left–right axis. The early ectoderm–endoderm axis that is established with gastrulation before body folding defines the future dorsal–ventral axis (Figure 15-10).

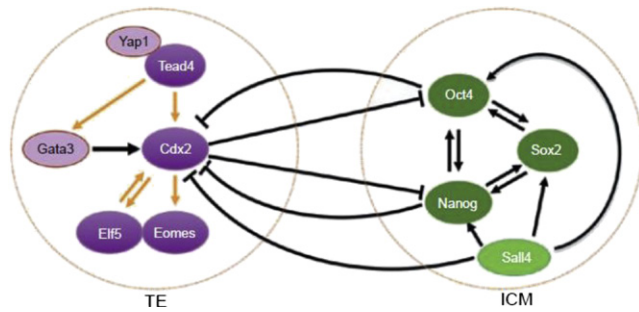
### 15.6.4 Genes and Transcription Factors in Embryogenesis

The homeodomain (also known as homeobox) transcription factors Oct4 (also known as either Pou5f1 [UniProt recommended name] or Oct3) and Nanog that confer pluripotency are initially expressed in all early blastomeres but are subsequently expressed only in the ICM. The transcription factors Cdx2 and Tead4 are required for the initial commitment of the blastomeres to trophoblasts, while Eomes (Eomesodermin homolog) and Elf5 strengthen the commitment of the cells to becoming the trophoblasts. It is now thought that maternally derived Cdx2 that was present in the early zygote is also required for the initial commitment and polarization of the blastomeres into TE. These transcription factors, in turn, are regulated by several other factors, and they cross-regulate each other as well (Figures 15-11 and 15-12). Yap1 is a Tead4 cofactor that, when unphosphorylated, localizes to the nuclei of the outer cells, and the binding of Yap1 to Tead4 in those cells results in the transcription of zygotic *Cdx2* and other trophoblastic genes, thereby committing those cells to the TE lineage. Oct4 is required for the determination of the ICM; Nanog is critical for pluripotency and is required for the formation of the epiblast; Sall4 is required for the differentiation of the ICM into the epiblast and the primitive ectoderm. An HMG-box transcription factor, Sox2, heterodimerizes with Oct4 and promotes the binding of Oct4 to its target genes. Oct4 and Cdx2 bind to each other's promoter to

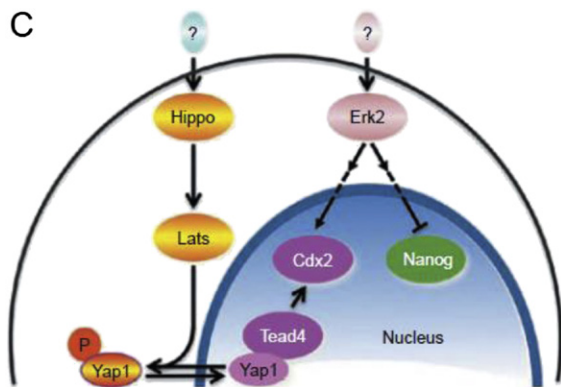


**FIGURE 15-10** Schematic diagram showing the derivation of tissues in human and rhesus monkey embryos. The dashed line indicates a possible dual origin of the extraembryonic mesoderm. (Gilbert, S. F. *Developmental Biology*, 9th ed., Sinauer Associates, Inc.: Sunderland, MA, 2010.)





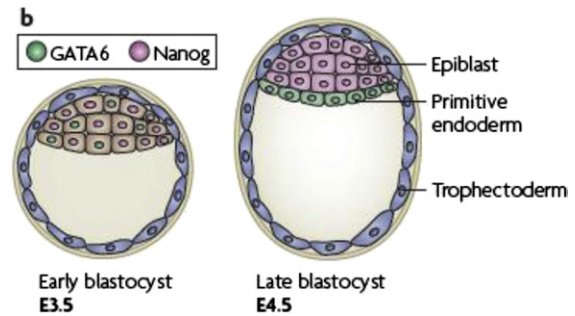
**FIGURE 15-11** Molecular mechanisms for the ICM/TE segregation: The regulatory network of key transcription factors in ICM/TE specification. Cdx2, Tead4, Eomes, and Elf5 are the major TE-specific transcription factors. Tead4 cooperates with Yap1 to activate Cdx2, which in turn upregulates Eomes and Elf5. Conversely, Elf5 is able to enhance Cdx2 expression. In addition, GATA3 directly binds to the intron 1 region of the Cdx2 locus and activates Cdx2 expression. In contrast, Oct4, Nanog, and Sox2 form a core regulatory circuitry to promote ICM cell fate. Sall4 not only activates Oct4, Nanog, and Sox2 but also suppresses Cdx2 expression, and this is critical to the differentiation of the ICM and the TE. (Chen, L.; Wang, D.; Wu, Z.; Ma, L.; Daley, G. Q. *Molecular Basis of the First Cell Fate Determination in Mouse Embryogenesis*. *Cell Res.* 2010, 20 (9), 982–993.)



**FIGURE 15-12** Two signaling pathways, the Hippo and MAPK pathways, are involved in the ICM/TE cell fate determination. In inside cells, Lats in the Hippo pathway phosphorylates Yap1, resulting in cytoplasmic distribution of Yap1. Without nuclear Yap1, Tead4 is unable to activate Cdx2. Thus, these cells develop to the ICM. In contrast, due to the inactive Hippo pathway in outer cell, Yap1 remains unphosphorylated and is localized in the nucleus. Yap1 cooperates with Tead4 to activate Cdx2, promoting the TE fate. Erk2 in the MAPK pathway activates Cdx2 and suppresses Nanog, facilitating the TE formation. The mechanisms for Erk2 regulating Cdx2 and Nanog, and the events upstream of Hippo and Erk2, remain unknown. (Chen, L.; Wang, D.; Wu, Z.; Ma, L.; Daley, G. Q. *Molecular Basis of the First Cell Fate Determination in Mouse Embryogenesis*. *Cell Res.* 2010, 20 (9), 982–993.)

suppress each other's transcription, so the cells will differentiate into either the ICM or trophoblasts (11,21).

By the time the ICM has differentiated into the epiblast and the primitive ectoderm, Nanog is expressed exclusively (22) in the epiblast, while the zinc finger transcription factor Gata6 is expressed specifically in, and is required for the development of, the hypoblast (Figure 15-13). Of note, recent studies suggest that signals from properly specified epiblasts are also required



**FIGURE 15-13** The hypoblast and the epiblast lineages in the mouse embryo segregate from the ICM at the blastocyst stage. At E3.5, the ICM shows mosaic and random “salt and pepper” expression of the transcription factors Nanog and GATA6. GATA6-positive cells are subsequently sorted to the distal surface of the ICM, where they give rise to the hypoblast. Nanog-positive cells exclusively give rise to the pluripotent epiblast, the founder tissue of the embryo proper. (Arnold, S. J.; Robertson, E. J. *Making A Commitment: Cell Lineage Allocation and Axis Patterning in the Early Mouse Embryo*. *Nat. Rev. Mol. Cell Biol.* 2009, 10 (2), 91–103.)

for the specification of the hypoblast; hence, Nanog is also indirectly involved in the specification of the hypoblast. It has been suggested that the expression of Gata6 is induced by the activation of the RTK–MAPK (mitogen-activated protein kinase) pathway (*vide infra*) via FGF (fibroblast growth factor) and Grb2; this process also appears to inhibit the expression of Nanog. Recently, it has been found that Sox17 promotes the expression of Gata6 and Gata4, and all three proteins are required for the differentiation of the ICM into the hypoblast. In addition, microRNAs have been found to suppress the transcription of specific pluripotency factors such as OCT4, SOX2, and KLF4 in human embryonic stem cells, which suggests that microRNAs may be important in regulating the differentiation of the ICM (14,23,24).

### 15.6.5 Epigenetics in Early Embryogenesis

Epigenetic changes regulate the pluripotency of the ICM and the epiblast and prevent their differentiation into TE. Soon after fertilization, the paternal genome is actively and rapidly demethylated before the first cell division while the maternal genome undergoes a much slower passive demethylation that lasts through at least the four-cell stage; however, the differentially methylated imprinting control regions are not demethylated. This demethylation leads to the transcription of zygotic Oct4 and Nanog. “Epigenetic modifiers” including various methyltransferases and demethylases then maintain the pluripotent-inducing genes, Oct4, Nanog, Sall4, and Sox2 in an active state, which ensures that the ICM and epiblast remain pluripotent. However, for subsequent differentiation to occur, these pluripotent-inducing genes have to be silenced by various repressors such as transcription factors and methyltransferases (e.g. repression of Oct4 by the EMHT2-EMHT1 methyltransferase

complex). Similarly, *Elf5* is methylated in the ICM and the epiblast, leading to a reduction in the levels of *Cdx2* and *Eomes*, thereby preventing their differentiation into TE (14,23).

### 15.6.6 Genes Involved in Gastrulation, Segmentation, and Axes Formation

Activation of the Wnt signaling pathway leads to competence in the posterior part of the embryo that enables the primitive streak to be formed. Gastrulation is initiated by members of the TGF- $\beta$  superfamily including Nodal. Signaling by Nodal and specific FGFs such as FGF8 induce the epiblast cells to “de-epithelialize” to form the primitive streak and to ingress so as to form the mesoderm and endoderm. There are four FGF8 isoforms in humans (FGF8a, FGF8b, FGF8e, and FGF8f), generated by alternative splicing of exon 1, each of which has a different role in development, but some isoforms may have overlapping roles. For example, experiments in mouse models suggest that although *Fgf8b* is responsible for both gastrulation and development of the midbrain and hindbrain, the presence of *Fgf8a* can partially compensate for the loss of *Fgf8b* in the gastrulation process but not in the development of the midbrain and hindbrain (25). This may explain the diversity of clinical phenotypes arising from loss-of-function mutations in *FGF8* such as gonadotropin-releasing hormone (GnRH) deficiency leading to either idiopathic hypogonadotropic hypogonadism (OMIM# 146110) or Kallman syndrome (hypogonadotropic hypogonadism with anosmia) (OMIM# 612702) (26,27), holoprosencephaly (28), and at least in mouse models, abnormal lung development (29).

Gastrulation also requires *SNAI1*, *SNAI2*, and *SNAI3*; the expression of *SNAI1*, *SNAI2*, and probably *SNAI3*, are induced by members of the TGF- $\beta$  superfamily and maintained by FGF signaling (30). The *SNAI* proteins are zinc finger transcription factors that are the human orthologs of the *Drosophila* Snail protein family. *SNAI1* represses the expression of E-cadherin, which is one of the cell adhesion proteins responsible for compaction of the blastomeres (*vide supra*); *SNAI2* promotes EMT (Figure 15-14); the functions of *SNAI3* have not been well characterized but its amino acids share more than 40% identity with *SNAI1* and *SNAI2*. Many other transcription factors such as the T-box transcription factor, *EOMES*, and the basic helix-loop-helix transcription factors, *MESP1* and *MESP2*, are required for the various cellular processes in gastrulation. Some of these processes and genes are reviewed in (30) and (22).

*SNAI2* is required for the migration and survival of melanoblasts, and as expected, *Snai2*-null mice have been found to have areas of depigmentation on their skin as well as a “white forehead blaze” among other characteristics consistent with the expression of *Snai2* in the developing mesenchymal tissues of the skull/face, lungs, kidneys, and intestines (31). Homozygous deletions in *SNAI2* have been

reported in 2 out of 38 unrelated individuals with Waardenburg syndrome type 2 (OMIM# 608890), characterized by sensorineural hearing loss and heterochromia irides (32). Heterozygous deletions in *SNAI2* have been reported in 3 out of 17 unrelated individuals with piebaldism (OMIM# 172800) who were negative for *KIT* mutations; all three of these individuals had patchy hypopigmentation in all of their bodies (33). However, in a more recent study with 30 Waardenburg syndrome type 2 subjects, no *SNAI2* mutations or deletions were identified (31).

The “clock and wavefront” model underlies the molecular basis of somitogenesis. In this model, the transcription of specific genes in the Notch signaling pathway such as *HES7* (hairly and enhancer of split 7 (*Drosophila*)), *LFNG* (lunatic fringe), and *DLL3* (delta-like 3) in the presomitic mesoderm oscillates (i.e. cycles) due to negative feedback loops between the Notch and the Wnt signaling pathways, and these cells are competent to form somites only when the expression of these genes is in a specific phase; this is the “segmentation clock,” and the oscillating expression of these genes recycles and spreads continually from the caudal to cranial direction (Figure 15-15). Meanwhile, *FGF8* is expressed by cells in the tail bud as the embryo elongates caudally but *FGF8* expression decays overtime such that its expression is lowest in the most cranial, and highest in the most caudal, presomitic mesoderm; in essence, this creates a “wavefront” of *FGF8* expression that moves from the cranial to caudal direction. In addition, expression of *ALDH1A2* (also known as *RALDH2* (retinaldehyde dehydrogenase 2)), a gene that is required for the synthesis of retinoic acid (RA), is higher in the newly formed somites. This thereby creates an increasing gradient of RA, which antagonizes *FGF8* signaling and activates genes required for somitogenesis such as *MESP1* and *MESP2*, from the previously formed somites into the more caudal presomitic mesoderm. High levels of *FGF8* inhibit somitogenesis. Therefore, somites are formed only when both the expression of the segmentation clock genes are in a specific phase and *FGF8* expression is below a certain threshold—this forms the “determination front” in somitogenesis (18,34,35). Recently, it has been suggested that the wavefront includes the expression of both *FGF8* and *FGF4* (36).

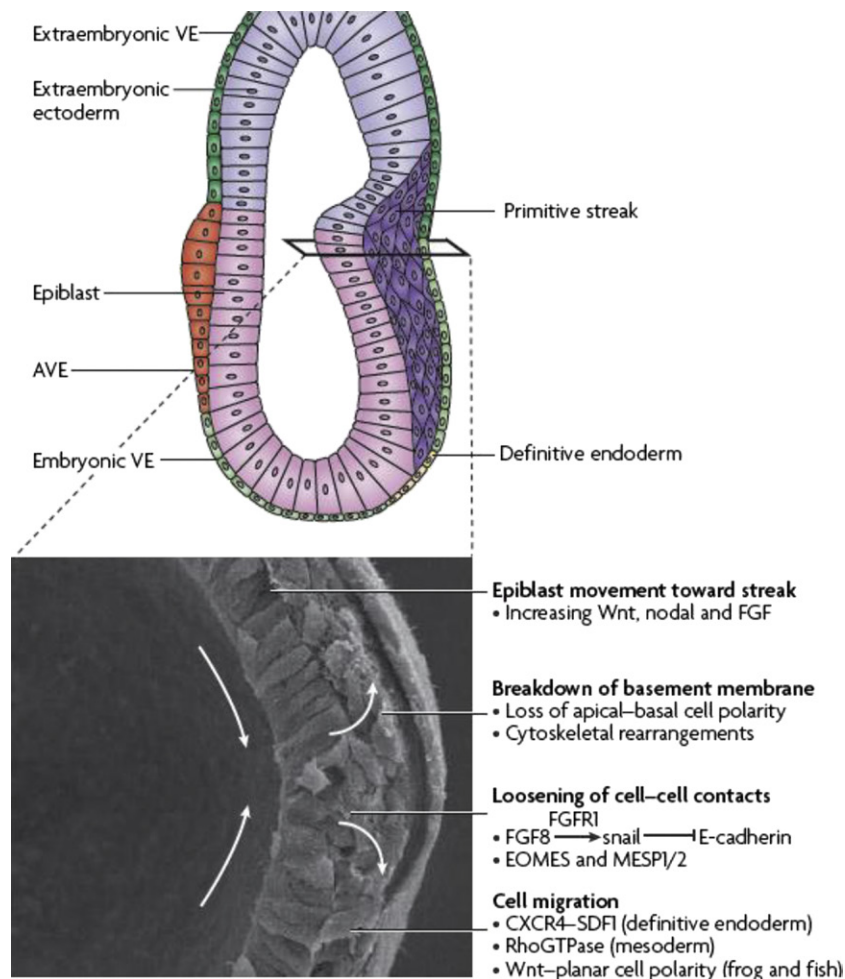
It is interesting that homozygous or compound heterozygous loss-of-function mutations in *DLL3*, *MESP2*, *LFNG*, and *HES7* cause autosomal recessive spondylocostal dysostosis types 1 to 4 (OMIM# 277300, 608681, 609813, 613686), respectively. However, most *MESP2* mutations have resulted in spondylothoracic dysostosis, instead of spondylocostal dysostosis, in which a homozygous *MESP2* mutation has been reported in only one family (37). It is notable that as discussed above, *DLL3*, *LFNG*, and *HES7* are all part of the segmentation clock, while *MESP2* is critical for somitogenesis, including determining the polarity of somites. Spondylocostal

dysostosis is characterized by the presence of multiple contiguous segmentation defects of the vertebrae resulting in a short neck and short trunk, rib anomalies, and small thoracic cavity especially in neonates. Spondylo-thoracic dysostosis has a similar but more severe phenotype resulting in a shorter spine and greater risk for respiratory insufficiency in infancy (38,39).

Studies on the genetics and development of left–right asymmetry have been complicated by the findings that the homologous left–right organizers in the different vertebrate species have different ultrastructures, initiate left–right determination differently, and mutations in homologous genes can result in vastly different phenotypes in different species, even among mammals. Our understanding of left–right determination has been based

on studies in vertebrate animal models including mice, rabbits, chickens, frogs (*Xenopus*), and zebrafish, but how well these models reflect the mechanism of left–right determination in the human embryo is unclear. Nevertheless, some basic developmental principles appear to be common to many species.

It is believed that left–right asymmetry is first established in the primitive node, which is homologous to the ventral node in the mouse, the Hensen's node in the chicken, and the dorsal lip of the blastopore in *Xenopus*. The cells at the center of the node have a motile cilium (known as monocilium) each. Each monocilium is made up of a ring of nine microtubule doublets, each with an inner and an outer dynein arm; unlike cilia in other cells, there are no central microtubules, so these monocilia are



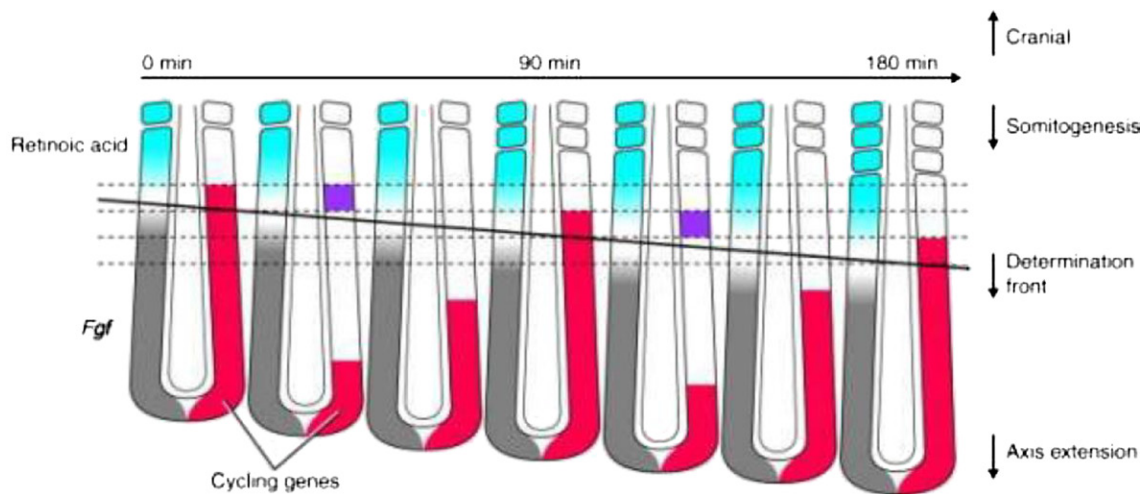
**FIGURE 15-14** EMT in the primitive streak. Formation of nascent mesoderm during gastrulation is a result of an EMT and tissue migration. Epithelial cells of the epiblast sheet converge toward the primitive streak, where increasing concentrations of signaling molecules, such as WNT3, fibroblast growth factor 8 (FGF8) and nodal, influence cell behavior. Cells in the primitive streak detach from the basement membrane, lose their characteristic apical–basal cell polarity, and undergo rapid and drastic cytoskeletal rearrangements that enable them to delaminate and migrate. A signaling cascade that involves FGF8 and the zinc-finger transcription factor snail causes the downregulation of the epithelial cell-adhesion molecule E-cadherin from adherens junctions, allowing mesodermal cells to migrate away from the streak. Additional activities of the transcription factors eomesodermin (EOMES), mesoderm posterior 1 (MESP1), and MESP2 are required for CDH1 downregulation and EMT, respectively. Nascent mesoderm cells migrate laterally and anteriorly between the epiblast and the overlying VE. In lower vertebrates, chemoattractant–receptor interactions, such as stromal-derived factor 1 (SDF1)–C-X-C-chemokine receptor 4 (CXCR4), cytoskeletal rearrangements regulated by RhoGTPases, or convergence–extension movements that are governed by the Wnt or planar cell polarity pathway, orchestrate these complex cell movements. AVE, anterior VE. (Arnold, S. J.; Robertson, E. J. *Making A Commitment: Cell Lineage Allocation and Axis Patterning in the Early Mouse Embryo*. Nat. Rev. Mol. Cell Biol. 2009, 10 (2), 91–103.)



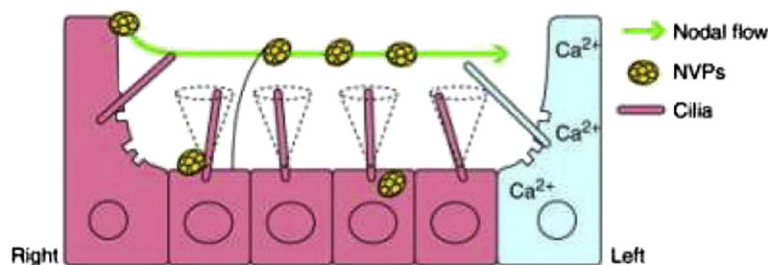
referred to as “9+0 motile cilia.” In contrast, cells at the periphery of the node have non-motile sensory cilia that lack dynein arms. The motile cilia rotate in a vortical fashion to create a leftward flow of extracellular fluid across the node. This is the so-called “nodal flow model” of left–right determination (Figure 15-16). It has been postulated that this nodal flow carries nodal vesicular parcels (NVPs), which are vesicles that contain morphogens including sonic hedgehog protein (Shh) and RA, across to the left side of the node. This flow of morphogens is detected by the non-motile sensory cilia and leads to a rise in intracellular calcium in the cells on the left side of the node. The secretion of NVPs, release of Shh and RA, and calcium influx into the left-sided cells, are all mediated by signaling through the Fgf pathway. The genes that were expressed only on the left side of the node are also expressed in the left lateral plate mesoderm, resulting in asymmetric gene expression (and hence left–right asymmetry) in the lateral plate mesoderm (18,40,41).

However, using electron microscopy and by studying (i) the expression of the Goosecoid (*Gsc*), which is a gene thought to be responsible for the “organizer” activity

of the node and hence the development of the body axes, and (ii) the expression of *Nodal*, which is closely associated with the node and is one of the genes that is expressed in the left lateral plate mesoderm, a group of authors found that in the rabbit, cells in Hensen’s node are not ciliated, but a single monocilium was identified on each cell posterior (i.e. caudal) notochordal process, which is anterior (i.e. cranial) to the anatomic primitive node. While expression of *Gsc* was present in Hensen’s node and the cranial part of the primitive streak in early stage embryos, *Gsc* expression in the later stage embryos was confined to the prechordal plate. Similarly, *Nodal* expression in the primitive node was transient and had faded by the time the notochordal plate was being formed. However, *Nodal* expression was subsequently detected on both sides of the posterior notochordal plate, and when the embryo was at the 2–3 somite stage, *Nodal* expression became asymmetric and concentrated in the left lateral plate mesoderm. The authors suggested that the posterior notochord is the site of the cilia-mediated nodal flow for the establishment of left–right asymmetry in mammals. They further suggested that the longitudinal



**FIGURE 15-15** Diagrams of the caudal end of chick embryos during two rounds of somitogenesis. Retinoic acid (blue) and Fgf8 (gray) gradients move caudally as the embryo elongates (axis extension) during somitogenesis. In chick, a somite pair forms every 90 min, which constitutes the length of the clock cycle. Expression of cycling genes (red) extends from caudal to cranial, and when expression of these genes spreads cranially to cross the threshold level of *Fgf8* signaling (called the determination wavefront; diagonal line), somites are established (indicated by expression of *Mesp* genes; purple). (Schoenwolf, G. C.; Bleyl, S. B.; Brauer, P. R.; Francis-West, P. H. *Larsen’s Human Embryology*, 4th ed., Churchill Livingstone: Philadelphia, 2008.)



**FIGURE 15-16** Model showing the transport of NVPs by motile cilia and the stimulation of calcium signaling (blue) at the left side of the node by nonmotile cilia. (Schoenwolf, G. C.; Bleyl, S. B.; Brauer, P. R.; Francis-West, P. H. *Larsen’s Human Embryology*, 4th ed., Churchill Livingstone: Philadelphia, 2008.)



body axes are determined through the “organizer activity” of the anatomic primitive node (41).

The dynein arms in the motile monocilia contain the left–right dynein protein, encoded by *DNAH11* (axonemal dynein, heavy chain 11), which is the human homolog of the mouse *Lrd* (left–right dynein), and other dynein proteins encoded by various axonemal dynein genes such as *DNAL1*, *DNAI1*, *DNAI2*, and *DNAH5*. The proper assembly and function of dynein require the medaka kintoun protein encoded by *KTU*, and the coiled-coil domain containing proteins 39 and 40 encoded by *CCDC39* and *CCDC40*. Homozygous or compound heterozygous loss-of-function mutations in any one of these genes result in primary ciliary dyskinesia with or without situs inversus (OMIM phenotypic series# 244400) (40). It should be noted that when the cilia are nonfunctional (e.g. immotile), the fluid flow around the functional left–right node is random and there is random expression of the “left-specific” genes on either side. Hence, not all individuals with mutations in these genes will have situs inversus (42).

In the mouse and some other vertebrate models, the nodal flow results in *Nodal* being asymmetrically expressed in the left lateral plate mesoderm. *Nodal* then induces the expression of *Lefty2* and *Pitx2* in the left lateral plate mesoderm, as well as *Lefty1* in the left floor plate of the neural tube. In these animal models, *Lefty1* constrains the expression of *Nodal* to a limited area, while *Lefty2* prevents the spread of the *Nodal* signal from left to right; *Pitx2* is involved in the maintenance of left–right asymmetry during organogenesis. Heterozygous loss-of-function mutations in *NODAL* have been found to cause heterotaxy (situs ambiguus), which is a clinically and genetically heterogeneous group of conditions characterized by abnormal left–right placement of thoracic and/or abdominal organs and is often associated with congenital heart malformations and asplenia (in the case of *NODAL* mutations) or polysplenia (OMIM# 270100) (43). Mutations in many other genes have been associated with heterotaxy including *ZIC3* (OMIM# 306955), an X-linked gene that encodes a protein hypothesized to act upstream of *NODAL*, *CFC1* (OMIM# 605376, 613853), which encodes a co-receptor for *NODAL*, and *LEFTY2* (OMIM# 601877). However, mutations in *LEFTY1* have not been reported to be associated with any heterotaxy or situs inversus syndromes, while mutations in *PITX2* (OMIM# 601542) have only been reported to cause syndromes associated with ocular anomalies but without heterotaxy or situs inversus such as Axenfeld–Rieger syndrome, iridogoniodygenesis, Peters anomaly, and ring dermoid of cornea. This suggests that caution should be exercised in extrapolating animal experimental data to humans.

**15.6.6.1 Spatial Context and Functional Redundancy of Developmental Genes.** Many genes that are expressed in early embryonic development are also involved in other developmental processes at the later stages in embryonic and fetal development. Moreover,

there is some functional redundancy among some of these genes such that mutations in one or more of these genes may not lead to embryonic lethality. For example, although *SOX2* is one of the four pluripotent-inducing genes, it is also involved in the maintenance of neural stem cells and hence neurogenesis, development of the optic cup by maintaining the neurogenic fate of the retinal neuroepithelium, generation of peripheral sensory and autonomic neurons through interactions with the Notch signaling pathway, and at least in mouse neural stem cells, regulation of *Jag1*, *Gli3*, and *Mycn* through interactions with the *Chd7* cofactor (44–47). Individuals with heterozygous loss-of-function *SOX2* mutations survive to adulthood and have syndromic microphthalmia type 3 (sometimes known as anophthalmia–esophageal–genital syndrome) (OMIM# 206900), which is associated with structural brain malformations, renal and genitalia malformations, and tracheoesophageal fistula or esophageal atresia; the ocular manifestations vary from bilateral microphthalmia or anophthalmia to anterior segment dysgenesis, coloboma, or even a complete lack of ocular phenotype (48). In view of the recently identified interactions between *Sox2* and *Chd7* as discussed above, it is not surprising that these phenotypic features overlap with those seen in CHARGE syndrome (OMIM# 214800) caused by heterozygous mutations in *CHD7*.

Another pluripotent-inducing gene involved in early embryonic development is *SALL4*. Heterozygous loss-of-function mutations in *SALL4* result in either Duane–radial ray syndrome (also known as Okihiro syndrome) (OMIM# 607323) or IVIC syndrome (OMIM# 147750); IVIC is the abbreviation for Instituto Venezolano de Investigaciones Científicas in Caracas, Venezuela, where the first family with this syndrome was reported (49). Duane–radial ray syndrome is characterized by Duane anomaly, radial ray malformations that may include hypoplasia/aplasia of the thumbs and radii or preaxial polydactyly, and sensorineural hearing loss; the allelic acro–renal–ocular syndrome is also associated with structural renal malformations. Duane anomaly is the absence of the abducens nerve (cranial nerve (CN) VI) and aberrant innervation of the lateral rectus muscle by the oculomotor nerve (CN III) leading to limited abduction or adduction of the eye, associated with retraction of the globe and narrowing of the palpebral fissure on adduction (50). IVIC syndrome is another allelic disorder that can present with imperforate anus, mild thrombocytopenia and mild leukocytosis, in addition to the other clinical findings described above (49). Recently, it was shown that *Sall1* upregulates the expression of *Nanog* in mouse embryonic stem cells and interacts physically with both *Nanog* and *Sox2*, and it also suppresses the differentiation of ectoderm and mesoderm. These data suggest that *Sall1* is directly involved in the regulation of pluripotency and tissue differentiation in early embryonic development (51). Heterozygous mutations in *SALL1* result in Townes–Brocks syndrome (OMIM# 107480),

characterized by imperforate anus, dysplastic ears with associated sensorineural or conductive hearing loss, and thumb malformations (typically preaxial polydactyly, triphalangeal or hypoplastic thumbs); renal anomalies are also commonly associated with this syndrome (52). It was thought that Townes–Brocks syndrome was caused by dominant negative mutations in *SALL1*, but with the identification of *SALL1* deletions in patients with very similar phenotypes, it is now believed that haploinsufficiency of *SALL1* may result in the same condition (53). Thus, it is clear that mutations in at least two *Sall* family genes, *SALL1* and *SALL4*, are completely compatible with life despite their roles in early embryonic development, and both genes are involved in the development of a number of different organ systems beyond the early embryonic period. It is also notable that both Duane–radial ray syndrome and IVIC syndrome have phenotypic features that closely resemble those seen in Townes–Brocks syndrome, which suggests that these two genes may be involved in the regulation of similar, but not necessarily identical, developmental pathways in the same organ systems, consistent with the finding that *Sall1* and *Sall4* share structural and functional similarities (51).

As blastocysts mature and become “attachment competent” for implantation, one of the heparan sulfate proteoglycans that they express on their surfaces is HSPG2 (basement membrane-specific heparan sulfate proteoglycan core protein; also known as perlecan). However, homozygous or compound heterozygous hypomorphic mutations in HSPG2 do not result in failure of implantation, presumably because there is functional redundancy with other heparan sulfate proteoglycans. Instead, these mutations result in Schwartz–Jampel syndrome type 1 (OMIM# 255800) or the more severe Silverman–Handmaker type of dyssegmental dysplasia (DDSH) (OMIM# 224410). Schwartz–Jampel syndrome is characterized

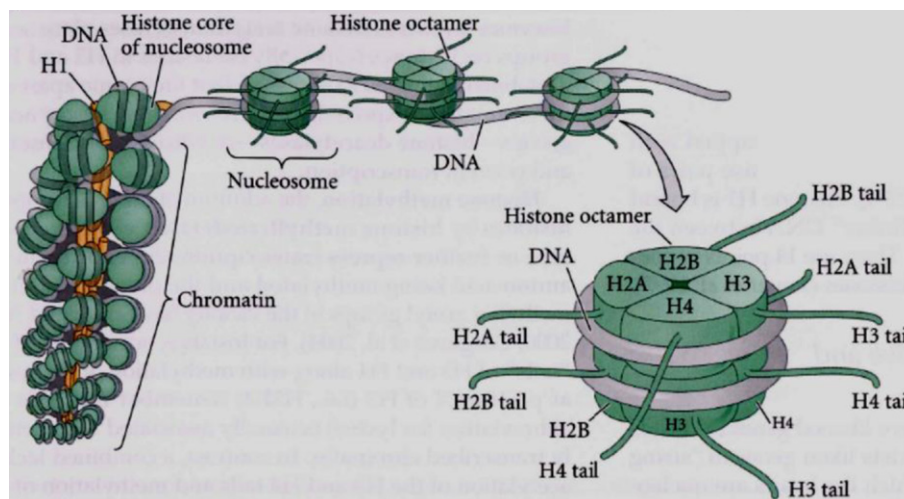
by myotonia with sustained skeletal muscle contractions on electromyography, joint stiffness, and chondrodysplasia with short stature, kyphoscoliosis, and bowing of the long bones; DDSH is a neonatal lethal form of short-limbed dwarfism with anisodondyly (i.e. variations in size and shape of vertebral bodies), micromelia, and micrognathia. The functions of HSPG2 include cell adhesion, maintenance of the basement membrane and cartilage, and anchoring of acetylcholinesterase at neuromuscular junctions, failure in which may account for the myotonia (54,55).

## 15.7 REGULATION OF GENE EXPRESSION IN DEVELOPMENT

Almost all somatic cells in the body contain an identical set of genes—the concept of *genomic equivalence*. Therefore, for each cell to differentiate into specialized tissues and organs, differential gene expression must occur. Such regulation of gene expression can occur at different steps in the process from transcription of the genes to posttranslational modifications of the proteins encoded by the genes.

### 15.7.1 Transcriptional Regulation

**15.7.1.1 Regulation by Histone Modification.** Eukaryotic chromosomes are composed of chromatin, a complex of DNA, proteins (primarily histones), and small amounts of RNA. Every 145–147 base pairs of DNA wrap in 1.65–1.67 loops around an octamer of histones comprising two molecules each of histones H2A, H2B, H3, and H4, forming the nucleosome, which is the basic unit of chromatin (Figure 15-17). The nucleosomes are linked to each other by about 60 bp of DNA, attached to which are histone H1. These nucleosomes usually form a compact solenoid structure, held together by histone



**FIGURE 15-17** Model for the arrangement of nucleosomes in the highly compacted solenoidal chromatin structure. Histone “tails” protruding from the nucleosome subunits allow for the attachment of chemical groups. (Gilbert, S. F. *Developmental Biology*, 9th ed., Sinauer Associates, Inc.: Sunderland, MA, 2010.)

H1, that prevents transcription factors and enzymes from binding to the DNA, thereby inhibiting transcription. Extending from each histone in the nucleosome is an amino acid “tail” that can be acetylated or methylated to regulate gene expression (11).

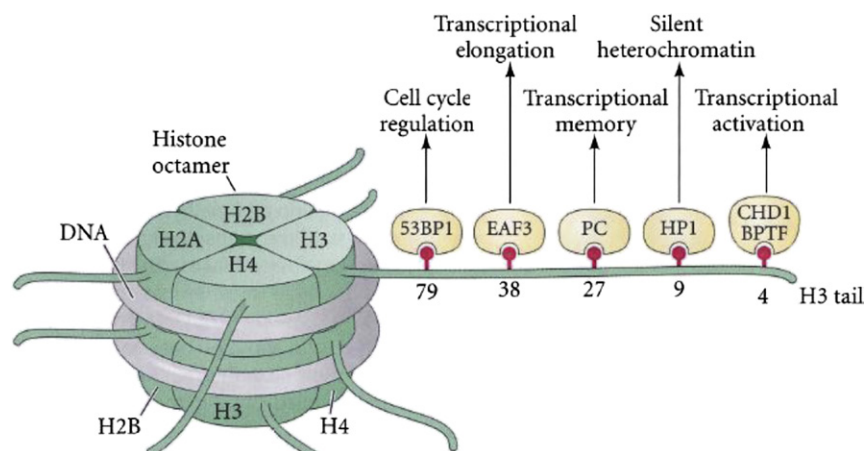
The acetylation of lysine residues on the tails of histones H3 and H4 by histone acetyltransferases results in destabilization of the nucleosomes, activating transcription (Figure 15-18); histone deacetylases stabilize the nucleosome and repress transcription. Methylation of histone tails by histone methyltransferases may result in either activation or repression of transcription, depending on the specific amino acid residue and the acetylation or methylation states of the surrounding amino acids.

Heterozygous loss-of-function mutations in the histone acetyltransferases CREBBP and EP300, both of which interact with each other and acetylate the lysine residue at amino acid position 56 of histone H3 (H3K56), result in Rubinstein-Taybi syndrome types 1 and 2 (OMIM# 180849 and 613684), respectively. Heterozygous loss-of-function mutations in euchromatin histone methyltransferase 1 (EHMT1), which forms part of the E2F-6 transcription repressor complex and methylates H3K9, result in Kleeftstra syndrome (OMIM# 610253). Heterozygous loss-of-function mutations in *MLL2*, which encodes a H3K4 methyltransferase, result in Kabuki syndrome (OMIM# 147920). All of these syndromes are characterized by intellectual disabilities, specific dysmorphic features, and multiple congenital anomalies, perhaps reflecting the ubiquitous role that histone modifiers play in the development of various organ systems. However, mutations in some histone-modifying genes lead to more limited phenotypic manifestations. For example, SIRT6 is a H3K9 and H3K56 histone deacetylase that associates with chromatin at the telomeres and is required for the stable association of the WRN protein with telomeric chromatin, together with which telomere function

and replication is maintained; in human cell cultures, SIRT6 deficiency leads to premature cellular senescence. Since homozygous loss-of-function mutations in *WRN* (also known as *RECQL2*) result in Werner syndrome (OMIM# 277700), an autosomal recessive premature aging syndrome that is not associated with intellectual disability nor with multiple congenital anomalies, it has been hypothesized that mutations in *SIRT6* could lead to a similar premature aging syndrome, although *SIRT6* mutations have not been reported in human patients (56,57).

Differentiated cells in tissues and organs have to retain identical sets of expressed and repressed genes through each mitotic generation. At the chromatin level, this is achieved through the Polycomb group (PcG) proteins that maintain genes in an inactive state and the Trithorax group (TrxG) proteins that keep genes active. PcG proteins function as part of large multi-protein complexes, including different versions of Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2); the specificity of each version may depend on the subunits within the complexes. PRC2 can di- and trimethylate H3K27 (H3K27me<sub>2/3</sub>) through the histone methyltransferases Ezh1 and Ezh2. PRC1 can monoubiquitylate (also known as monoubiquitinate) the lysine residue at amino acid position 119 of histone H2A (H2AK119ub) through the ubiquitin ligases Ring1A and Ring1B; one component of PRC1 can also bind specifically to H3K27me<sub>2/3</sub>. For some genes, there may also be another set of PcG that binds to the methylated tail of histone H3 to keep the histone in a methylated state. In contrast, some TrxG proteins can reverse the effects of PcG enabling the binding of transcription factors, or maintain H3K4 in an active, trimethylated state (11,58).

**15.7.1.2 Regulation by Cohesin.** Although the cohesin complex is one of the three “structural maintenance of chromosome” (SMC) protein complexes that are responsible for chromosome condensation and segregation during mitosis and meiosis, it is now known that cohesin also



**FIGURE 15-18** The tail of histone H3 sticks out from the nucleosome and is capable of being methylated or acetylated. Here, lysines can be methylated and recognized by particular proteins. Methylated lysine residues at positions 4, 38, and 79 are associated with gene activation, whereas methylated lysines at positions 9 and 27 are associated with repression. The proteins binding these sites are represented above the methyl group. (Gilbert, S. F. *Developmental Biology*, 9th ed., Sinauer Associates, Inc.: Sunderland, MA, 2010.)

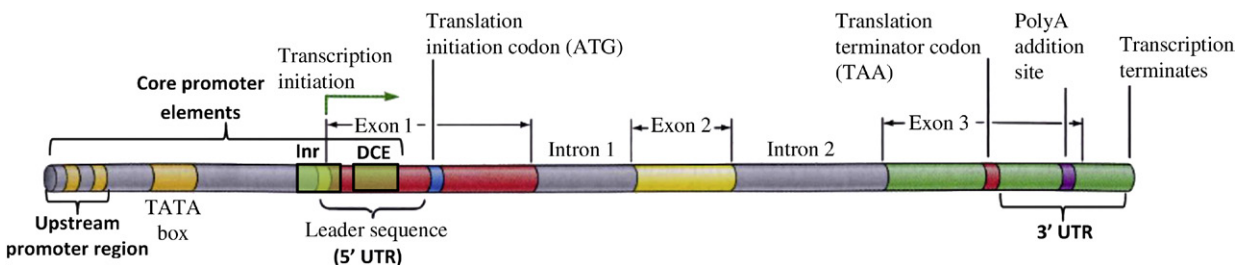


regulates gene transcription independent of its SMC functions. Binding of the cohesin complex to chromosomes is dependent on the presence of the Nipped-B-Mau-2 complex (encoded in humans by *NIPBL*) and ATPase of SMC1 and SMC3, both of which are proteins in the cohesin complex. It is thought that mediation of sister chromatid cohesion requires relatively small amounts of cohesin bound to chromosomes while regulation of gene transcription requires larger amounts of cohesin to be bound to chromosomes. Cohesin appears to bind close to the transcription start sites of specific genes that are active, but it also binds to CCCTC-binding factor (CTCF) binding sites, which are sites that block enhancer–promoter interactions and repress transcription. However, with some exceptions, cohesin does not usually bind to genes that have been silenced by PcG through H3K27me3 (*vide supra*). One of the genes that cohesin upregulates is *myc*, a protooncogene which itself encodes a factor that regulates the expression of genes involved in cellular growth and proliferation. Cohesin also downregulates certain genes that promote cellular differentiation (11). Heterozygous mutations in *NIPBL* result in Cornelia de Lange syndrome type 1 (OMIM# 122470), hemizygous mutations in X-linked *SMC1A* result in Cornelia de Lange syndrome type 2 (OMIM# 300590), and heterozygous mutations in *SMC3* result in Cornelia de Lange syndrome type 3 (OMIM# 610759). All three types of Cornelia de Lange syndrome are characterized by intellectual disability and distinctive facial features; in addition, individuals with *NIPBL* mutations usually have hypertrichosis, micro-melia or other upper limb skeletal defects, and in some instances, multiple congenital anomalies.

**15.7.1.3 Regulation via DNA Sequences: Promoters, Enhancers, Silencers, and Insulators.** Starting from the most upstream (5') end, the DNA sequence of a protein-coding gene comprises in order, the promoter region, the transcription initiation site (also known as the cap sequence), the 5' untranslated region (5' UTR) (also known as the leader sequence), the translation initiation site (ATG), exons and introns, a translation termination codon, the 3' untranslated region (3' UTR), and the transcription termination sequence (Figure 15-19) (11).

In mammals, the core promoter region overlaps with exon 1 of the gene and contains one or more core promoter

elements such as the “TATA box” (i.e., containing the consensus nucleotide sequence TATAWAAR) [*per International Union of Pure and Applied Chemistry (IUPAC) nomenclature*: W=nucleotide A or T; R=nucleotide A or G] typically about 30–31bp upstream of the transcription initiation site, the initiator element (Inr) with the consensus sequence YYANWYY [*IUPAC nomenclature*—Y=nucleotide C or T; N=any nucleotide] about 2bp upstream to 5bp downstream of the transcription initiation site and the A nucleotide being designated as the +1 nucleotide regardless of whether transcription is predominantly initiated at this nucleotide, and downstream core elements (three subunits that lie 6–34bp downstream of the transcription initiation site). Some TATA boxes in core promoters are flanked by an upstream TFIIB (transcription factor II B) recognition element (BREu) with the consensus sequence SSRGCGC and a downstream TFIIB recognition element (BREd) with the consensus sequence RTDKKKK [*IUPAC nomenclature*: S=nucleotide G or C; D=nucleotide A, G, or T; K=nucleotide G or T]. There are two classes of core promoters—those that regulate one or a small cluster of transcription initiation sites (the “focused core promoter”) and those that regulate a broad range of potential transcription initiation sites over a 50–100bp region (the “dispersed core promoter”), with the TATA box being more common among the focused core promoters (59). In addition, there are proximal promoter elements (also known as upstream promoter region) in the DNA sequence that are thought to promote interactions between the enhancers and the core promoter region (60). Within the 3' UTR is the “polyA addition site” where polyadenylation of the RNA transcript occurs, that is, the addition of a long stretch of adenine nucleotides to the RNA transcript. Polyadenylation helps to stabilize the RNA, enables the RNA to enter the cytoplasm, and allows the resultant mRNA in the cytoplasm to be translated. On the same chromosome are DNA sequences, known as enhancers, that bind transcription factors and regulate the efficiency of transcription from a given promoter. Due to the compact structure of the chromatin (*vide supra*), these enhancers may regulate the expression of genes thousands of base pairs away, but always on the same chromosome (hence classified as *cis*-regulatory elements); moreover, enhancers may lie upstream (5'), downstream (3'), or within the introns



**FIGURE 15-19** Schematic representation of the locations of the core promoter elements, 5' untranslated region (leader sequence), exons, introns, and 3' untranslated region. (Gilbert, S. F. *Developmental Biology*, 9th ed., Sinauer Associates, Inc.: Sunderland, MA, 2010.)



of the genes that they regulate. Each enhancer may bind several different transcription factors, and it is the specific enhancer-transcription factors combination that determines whether the promoter of a given gene is activated. A gene can have multiple enhancers, each of which can bind to different, and sometimes multiple, transcription factors. For example, *PAX6* has a specific enhancer for each of the cell type or tissue in which it is expressed (retina, lens/cornea, neural tube, and pancreas) and each enhancer binds to a specific set of transcription factors, regulating the expression of *PAX6* in the different tissues. Moreover, each transcription factor may bind to the enhancers and promoters of different genes. The requirement for a specific set of transcription factors to bind to an enhancer before a given gene is activated enables coordinated gene expression in a developing tissue or organ. For example, many genes involved in the development of the lens contain an enhancer that promotes gene expression only when *PAX6*, in addition to other transcription factors, is bound to it, so none of these genes will be activated until *PAX6* is present (11). Mutations in enhancers can lead to syndromes associated with dysfunction of the genes that they regulate in specific tissues or organs. For example, expression of the sonic hedgehog gene (*SHH*) in the developing limb bud is regulated by the ZRS (ZPA (zone of polarizing activity) Regulatory Sequence), which lies within intron 5 of *LMBR1*, about 1 Mb away from *SHH* on chromosome 7q36.3; gain-of-function mutations in ZRS result in preaxial polydactyly type II, triphalangeal thumb-polysyndactyly syndrome, and isolated triphalangeal thumbs (OMIM# 174500) (61–63), whereas complete loss-of-function mutations in ZRS appear to result in acheiropody (OMIM# 200500), which is characterized by congenital “amputations” of all four limbs and aplasia of hands and feet (64). Of note, none of these syndromes are associated with the brain or ocular malformations typically seen in germ line *SHH* mutations, which could be due to the tissue specificity of this enhancer. *LMBR1* itself is thought to encode a transmembrane receptor and does not appear to be involved in limb development, but it is most strongly expressed in the heart and the pancreas instead (65).

Once a set of transcription factors has bound to an enhancer, a histone-modifying enzyme such as a histone acetyltransferase or histone methyltransferase may be recruited by the transcription factors to activate transcription. In addition to their roles in “dissociating” or activating the nucleosomes, transcription factors can also stabilize the transcription preinitiation complex, thereby allowing RNA polymerase in the complex to bind to the core promoter region to initiate transcription (11). Transcription factors may be categorized into different families based on their molecular structures, as shown in Table 15-3.

Mutations in genes encoding transcription factors can lead to human developmental disorders. For example, MITF is a transcription factor that activates the promoter of the tyrosinase (*TYR*) gene, which in turn

catalyzes the conversion of tyrosine to melanin in pigment production. MITF also activates the promoter of *GPR143* (previously known as *OA1*), a gene that encodes a G protein-coupled receptor expressed only in melanocytes and retinal pigment epithelium. Heterozygous mutations in *MITF* result in Waardenburg syndrome type 2A (OMIM# 193510), characterized by a white forelock, leukoderma, and sensorineural hearing loss at birth (melanocytes in the cochlea may affect the function of inner ear hair cells), as well as an allelic condition, Tietz syndrome (OMIM# 103500), characterized by congenital sensorineural hearing loss, white-blond hair, and light-colored skin.

Silencers are DNA elements (sequences) that repress gene transcription. There are two main classes of silencers—silencer elements that interfere with the formation of the transcription preinitiation complex via transcription factors that repress transcription, and negative regulatory elements that prevent the binding of transcription factors to their *cis*-regulatory DNA sequences. Silencer elements are located upstream of the genes that they regulate, while negative regulatory elements may

**TABLE 15-3** Some Major Transcription Factor Families and Subfamilies

Family	Representative Transcription Factors	Some Functions
Homeodomain		
Hox	Hoxa1, Hoxb2, etc.	Axis formation
POU	Pit2, Unc-86, Oct-2	Pituitary development; neural fate
LIM	Lim1, Forkhead	Head development
Pax	Pax1, 2, 3, 6, etc.	Neural specification; eye development
Basic helix-loop-helix (bHLH)	MyoD, MITF, daughterless	Muscle and nerve specification; <i>Drosophila</i> sex determination; pigmentation
Basic leucine zipper (bZip)	C/EBP, AP1	Liver differentiation; fat cell specification
Zinc finger		
Standard	WT1, Krüppel, Engrailed	Kidney, gonad, and macrophage development;
Nuclear hormone receptors	Glucocorticoid receptor, estrogen receptor, testosterone receptor, retinoic acid receptors	Secondary sex determination; craniofacial development; limb development
Sry-Sox	Sry, SoxD, Sox2	Bend DNA; mammalian primary sex determination; ectoderm differentiation

Reproduced from Reference (11).

be located upstream, downstream, or within introns or exons of the genes that they regulate (60). Some silencers restrict the expression of genes to highly specific tissues, such as the neural restrictive silencer element found in certain genes that prevent the activation of the promoters of those genes in tissues other than neurons (11).

Insulators are *cis*-regulatory DNA sequences that prevent the interactions between enhancers or silencers and one or more of the genes that they regulate. Enhancer-blocking insulators, which often contain binding sites for the CTCF, prevent enhancer–promoter interactions, thereby preventing gene expression. Barrier insulators lie between heterochromatin and euchromatin domains, and serve to prevent chromatin-mediated gene silencing (60).

**15.7.1.4 Regulation by DNA Methylation and Demethylation.** Histone modifications, as described above, confer short-term “epigenetic memory” to the cells in that those modifications can be altered before or during mitosis. Long-term, stable and heritable epigenetic regulation is usually achieved through methylation of DNA itself at CpG sites (i.e. a cytosine–guanine doublet linked by a phosphodiester bond as in a strand of DNA) by converting cytosine to 5-methylcytosine, although methylation also occurs at non-CpG sites in embryonic stem cells. The patterns of DNA methylation across the genome are first established in the blastocyst stage by the *de novo* DNA methyltransferases, DNMT3A and DNMT3B. This is subsequently maintained during DNA replication by the maintenance or perpetuating DNA methyltransferase, DNMT1, which recognizes the methylcytosine residue one strand (a hemimethylated CpG site) and methylates the cytosine residue in the CpG site on the complementary strand, although it can also methylate CpG sites. Lack of DNMT1 results in absence of methylation on the daughter strands, leading to loss of DNA methylation after multiple rounds of cell division. Methylated cytosines in the promoter regions repress transcription from specific genes, either by preventing the binding of transcription factors to enhancers or facilitating the binding of specific proteins that then methylate or deacetylate histones. For example, when MeCP2 is bound to the methyl group in methylated cytosine, it recruits histone deacetylases and histone methyltransferases leading to the stabilization of the nucleosomes and preventing gene transcription (11,66,67). Homozygous or compound heterozygous mutations in DNMT3B, typically in the methyltransferase domain, result in immunodeficiency–centromeric instability–facial anomalies (ICF) syndrome type 1 (OMIM# 242860). The immunodeficiency is characterized by variably low IgA, IgG, and IgM, and in some patients, low T cells, but B cells are usually present. The centromeric instability is manifested as chromosomal rearrangements (such as deletions of whole chromosome arms and multibranched (multi-radial) chromosomes) in the heterochromatic regions adjacent to the centromeres of chromosome 1, 16, and less frequently, chromosome

9, in phytohemagglutinin-stimulated lymphocytes; these regions are hypomethylated, at least in these specific chromosomes, consistent with the deficiency in DNMT3B. It has been hypothesized that hypomethylation of the heterochromatin in those pericentromeric regions affect the expression of genes elsewhere in the genome. Other manifestations of ICF syndrome type 1 include facial dysmorphism and variable degrees of developmental delays or intellectual disability (68). Recently, heterozygous mutations in *DNMT1* have been shown to cause hereditary sensory neuropathy type 1E (also known as hereditary sensory neuropathy with dementia and hearing loss) (OMIM# 614116), manifested as sensory ataxia, early-onset dementia before 50 years old, sensorineural hearing loss in the 20s and 30s, and death by early mid-50s in most patients. These mutations impair the folding of DNMT1 and hence its enzymatic activity, leading to global hypomethylation but local hypermethylation at CpG sites of specific promoters (69).

## 15.7.2 Posttranscriptional Regulation: Before Translation of mRNA

**15.7.2.1 RNA Editing.** In some cells, after the pre-mRNA (also known as the primary transcript) has been transcribed from DNA, it undergoes RNA editing, which in humans involves the substitution of bases that have been transcribed, specifically amination of uridine to cytidine (U-to-C), deamination of cytosine to uracil (C-to-U), or deamination of adenosine to inosine (A-to-I). Such editing of pre-mRNA can result in alterations of the splice donor/acceptor sites, regulatory sequences, and start/stop codons, thereby contributing to the variety of different proteins that a single gene can encode for. The carboxyl-terminal domain of RNA polymerase II is involved in coordinating pre-mRNA editing and splicing to ensure that RNA editing, which requires the presence of specific introns, occurs before splicing (70).

The commonest form of RNA editing in humans, A-to-I conversion, is mediated by the ADARs (adenosine deaminases that act on RNA) family of enzymes, which bind to double-stranded RNA formed by a duplex comprising an exon and an intron downstream of the exon. If this conversion occurs in the coding region of the transcript, the inosine base will be read as guanosine during translation; if this conversion occurs around a splice site, it could lead to the gain or loss of that splice site. Heterozygous mutations in *ADAR1*, a ubiquitously expressed member of ADARs, result in dyschromatosis symmetrica hereditaria (DSH) (OMIM# 127400), characterized by hyperpigmented and hypopigmented macules on the dorsum of the hands and feet, and to a lesser extent, on the face that first appear in infancy or early childhood; the affected patients are otherwise healthy. The exact pathogenesis of DSH and the reason for the specific distribution of the lesions are unknown. Another member of the ADARs family, *ADAR2*, converts glutamine to arginine

at amino acid position 607 in the primary transcript of *GRIA2*, which encodes the beta subunit of the glutamate receptor in the central nervous system. Although *ADAR2* is expressed in multiple tissues, its substrate appears to be restricted to a single primary transcript in the central nervous system, and it is partly responsible for neuronal calcium homeostasis through proper assembly of the glutamate receptors. Mutations in *ADAR2* have not been reported in humans, perhaps because such mutations would lead to neuronal death and not be compatible with life, although homozygous null *Adar2* mice live to the postnatal day 20, albeit with seizures. Primary transcripts from a number of other neurotransmitter receptors in the central nervous system are also edited by various members of the ADARs family (70,71).

The apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (APOBEC1) mediates the C-to-U deamination in the pre-mRNA of *APOB* in intestinal cells, resulting in the conversion of a codon for glutamine into a stop codon. The unedited *APOB* transcript encodes ApoB-100, which is synthesized in the liver and forms part of very low-density lipoproteins, while the edited transcript encodes ApoB-48, which is synthesized in the small intestines and forms part of chylomicrons and chylomicron remnants (71).

**15.7.2.2 Alternative Splicing.** The introns and in some cases, selected exons, from pre-mRNA are spliced out by spliceosomes to form mature mRNA, typically before the mRNA is exported from the nucleus to the cytoplasm. Different exons from the same pre-mRNA may be spliced out in different cells or tissues or at different developmental stages, a phenomenon known as alternative splicing, which contributes to the diversity of proteins that can be encoded by a single gene; the different proteins that arise from alternative splicing are known as splicing isoforms. Sometimes, different splicing isoforms can have very different functions. For example, depending on how an alternative 5' splice site in the first coding exon of *BCL2L1* (also known as *BCLX*) is used, either a large isoform, BCLXL, that inhibits apoptosis or a smaller (shorter) isoform without exon 2, BCLXS, that promotes apoptosis will be produced. It has been estimated that more than 90% of human protein-coding genes undergo alternative splicing (11).

The spliceosome is made up of small nuclear RNAs (snRNAs) including the small ribonucleoproteins (snRNPs) U1, U2, U4, U5, and U6, as well as proteins known as splicing factors. The U1 snRNP recognizes and binds to the consensus sequence at the 5' splice site (i.e. start of intron) while the U2 snRNP binds to a "branch point" sequence within the intron close to the 3' splice site; the binding of U2 is facilitated by the binding of the U2 auxiliary factor (U2AF) to the polypyrimidine tract close to the branch point sequence. The triple snRNP complex with U4, U5, and U6, then interacts with U1 and U2, and through two sequential transesterification reactions, the intron is cleaved and the two exons are ligated.

The different splicing factors in a spliceosome recognize different sequences as introns; hence, a sequence recognized as an intron in one cell type may be recognized as an exon in another cell type, which forms the basis of alternative splicing. DNA sequences known as splicing enhancers and silencers both in exons and introns close to the 5' and 3' splice sites bind to various splicing factors including the "serine- and arginine-rich" protein family (SR proteins) and the heterogeneous nuclear ribonucleoproteins (hnRNPs). These splicing enhancers and silencers, along with the splice site and branch point sequences, help to identify a specific DNA sequence as an exon versus an intron. Binding of the splicing factors to the splicing enhancers increases the use of those splice sites, while binding to the splicing silencers decreases the use of those sites. Small nucleolar RNAs (snoRNAs) can also regulate the use of specific splice sites (72,73). For example, in the processing of the serotonin receptor 2C gene (*HTR2C*) mRNA, the snoRNA SNORD115 (HBII-52) binds to and promotes the inclusion of exon 5b, which is crucial for the function of the receptor, instead of exon 5a (74). More recently, the mouse ortholog of HBII-52 has also been found to regulate splicing of other genes, including *TAF1* (associated with X-linked dystonia-parkinsonism (OMIM# 314250)) and *CRHR1* (corticotropin-releasing hormone receptor 1) (74). However, human diseases secondary to SNORD115 deficiency have not been reported.

The survival motor neuron (SMN) protein, encoded by *SMN1*, forms a complex with spliceosomal snRNP and Gem-associated protein 2 (encoded by *GEMIN2*, previously known as *SIP1* (survival of motor neuron protein-interacting protein 1)). SMN is critical for the assembly and nuclear import of spliceosomal snRNP and hence pre-mRNA splicing. Mutations in *SMN1* result in spinal muscular atrophy (SMA), and characterized by hypotonia and muscle weakness. It has been suggested that SMA may be partly due to the inability of motor neurons to splice and process specific pre-mRNAs, although it may also be due to disruptions in other roles that SMN may play in the survival of motor neurons such as transportation of substances down the axons. *SMN2* is an ancestral inverted duplication of *SMN1* that lies centromeric to *SMN1*. Each individual has between 0 and 5 copies of *SMN2*. One of the key differences in the sequences between *SMN1* and *SMN2* is a silent cytosine-to-thymine transition in exon 7 of *SMN2* that results in the conversion of an exonic splicing enhancer to an exonic splicing silencer, thereby producing a shorter, unstable SMN protein that lacks exon 7 but is still partially functional. About 85% of the mRNA from *SMN2* is thought to lack exon 7 (73,75). The severity of the SMA phenotype depends on the number of copies of *SMN2*—most children with SMA type 1 (Werdnig-Hoffmann disease) (OMIM# 253300), which is the most severe form and usually lethal by age 2 years, have one or two copies of *SMN2*; in contrast, most children with

SMA type 3 (juvenile SMA or Kugelberg–Wielander disease) (OMIM# 253400) have three or four copies of *SMN2*, manifest weakness after 18 months of age and have a normal life span (76).

The U12-dependent spliceosome is a minor spliceosome that recognizes the 5' splice site with the sequence (G/A)TATCCT and a specific branch site in a relatively small number of genes. In contrast to the more common U2-dependent spliceosome described above, the U12-dependent spliceosome comprises the U11, U12, U4atac, U5, and U6atac snRNPs subunits (the U5 snRNP being common to both the U2 and the U12-dependent spliceosomes). Recently, it was found that homozygous or compound heterozygous mutations in *RNU4ATAC*, which encodes U4atac, result in microcephalic osteodysplastic primordial dwarfism type 1 (MOPD1) (also known as Taybi–Linder syndrome) (OMIM# 210710). The key clinical features of MOPD1 include severe intrauterine growth retardation, microcephaly with structural brain malformations, intellectual disability, and skeletal dysplasia. Of note, *RNU4ATAC* lies within intron 2 of *CLASP1*, which encodes a microtubule-associated protein that regulates microtubule dynamics and stabilizes the mitotic spindle during cell division. However, mutations in *RNU4ATAC* do not affect splicing or expression of *CLASP1* (77,78).

Heterozygous mutations in some members of the precursor mRNA-processing (splicing) factor family including *PRPF3*, *PRPF6*, *PRPF8*, and *PRPF31* result in non-syndromic retinitis pigmentosa, not typically associated with other clinical manifestations.

Myotonic dystrophy type 1 (OMIM# 160900), characterized by muscle weakness, myotonia, cataracts, cardiac conduction defects, and diabetes mellitus in some patients, is caused by expansion of the CTG trinucleotide repeats in the 3' UTR of *DMPK*. The transcribed long CUG repeats in the mRNA have a high binding affinity for the splicing factor MBNL1 (muscleblind-like protein 1), leading to a depletion of this splicing factor and effectively a loss of its activity in the nucleoplasm. At the same time, the abnormally long CUG repeats also lead to upregulation of *CELF1*, which encodes CUGBP Elav-like family member 1 (previously known as CUG-binding protein). CELF-1 regulates the alternative splicing of *CLCN1* (which encodes a chloride channel in skeletal muscle), *TNNT2* (human cardiac troponin T), and *INSR* (insulin receptor), among other genes. It is believed that the phenotypic manifestations of myotonic dystrophy type 1 are due to aberrant alternative splicing as a result of the lack of MBNL1 and excess CELF-1 (72). Hence, although the causative mutation in myotonic dystrophy type 1 is in *DMPK*, it may be the disruption in alternative splicing due to an imbalance in those two splicing factors that underlie the pathogenesis and pleiotropy in this condition. Myotonic dystrophy type 2 (OMIM# 602668), due to an expansion of the CCTG nucleotide repeats in intron 1 of *CNBP* (previously known as *ZNF9*), has

similar but possibly milder manifestations compared to myotonic dystrophy type 1; its pathogenesis is due to the sequestration of MBNL1, which also has a high affinity for the long CCTG repeats.

It should be noted that abnormal splicing of genes does not always result in an undesirable phenotype. Myostatin, encoded by *MSTN*, is a negative regulator of skeletal muscle growth. A child with muscle hypertrophy and unusual strength, but no other clinical manifestations up to age 4½ years (OMIM# 614160), was found to have a homozygous splice donor (5') site mutation that resulted in an aberrantly spliced mRNA with a premature termination codon and hence a lack of myostatin. His mother, who was described as “muscular” and was a former professional athlete, was a heterozygous carrier for this mutation (79).

### 15.7.3 Translational Regulation

The regulation of protein synthesis (i.e. translation of mRNA) in eukaryotes typically occurs during the initiation stage rather than the subsequent elongation and chain termination stages. Translation begins with the assembly of the 43S preinitiation complex comprising the 40S ribosomal subunit and a set of eukaryotic initiation factors (eIFs) including eIF3, eIF1, eIF1A, and the eIF2-GTP-Met-tRNA<sup>Met</sup><sub>i</sub> (eIF2-TC) complex [Met = methionine]. The 43S complex attaches to and scans the mRNA from the 5' UTR until it locates the translation initiation codon, which is usually the first AUG within the consensus sequence GCC(A/G)CCAUGG and is recognized by the anticodon of eIF2-TC. eIF1 and eIF1A facilitate this process by unwinding the secondary structures in the 5' UTR. eIF1 also helps to ensure that eIF2-TC binds only to AUG within the consensus sequence, thereby ensuring that the 43S complex binds to a translation initiation site. The binding of the 43S complex to the translation initiation codon leads to the formation of the 48S initiation complex and the subsequent hydrolysis of eIF2 from GTP resulting in the release of both eIF2-GDP and inorganic phosphate (GTP → GDP + P<sub>i</sub>). The 60S ribosomal subunit then combines with the 48S complex to form the 80S initiation complex. The remaining eIFs then dissociate from the 80S subunit and chain elongation begins. Termination of translation leads to the recycling of the ribosomal subunits that will be used in the next round of translation. The recycling of eIF2-GDP (which will form part of the eIF2-TC complex) is mediated by eIF2B, which functions as a “guanosine nucleotide exchange factor that promotes GDP-GTP exchange” (80). Homozygous or compound heterozygous mutations in the genes that encode any one of the five eIF2B subunits (*EIF2B1*, *EIF2B2*, *EIF2B3*, *EIF2B4*, *EIF2B5*) result in leukoencephalopathy with vanishing white matter (VWM) (OMIM# 603896); mutations in *EIF2B2*, *EIF2B4*, and *EIF2B5* also result in ovarioleukodystrophy, which is the same condition with ovarian failure as



an additional manifestation. These mutations reduce, but almost never completely eliminate, the activity of EIF2B. VWM is characterized by the widespread disappearance of white matter and replacement by cerebrospinal fluid in the central nervous system, resulting in progressive ataxia and spasticity. Other clinical manifestations depend on the type of VWM, as classified by the age of onset. For example, the antenatal form can be associated with congenital hypotonia, seizures, and multiple congenital anomalies including cataracts, renal hypoplasia, and hepatosplenomegaly. The early childhood-onset form is characterized by onset of ataxia between the ages of 1 and 5 years, followed by progressive spasticity with hyperreflexia, tremor, dysarthria, seizures, and optic atrophy. The adult-onset form can present with behavioral issues, cognitive decline, and ovarian dysgenesis in the females. In most postnatal forms of VWM, episodic neurologic deterioration may occur with stresses such as minor head trauma and minor infections (81). VWM appears to selectively affect oligodendrocytes and astrocytes. It has been suggested that these cells are hyperre-active to physiological stress in VWM, possibly due to activation of the “unfolded protein response,” a pathway that is typically activated when there is accumulation of unfolded or denatured proteins in the endoplasmic reticulum (ER). However, the precise pathophysiology of VWM remains unclear (82).

### 15.7.4 Posttranslational Regulation

As the primary polypeptide chain elongates during translation, it is translocated into the lumen of the ER through the binding of the ribosome-primary polypeptide-signal recognition particle (SRP) to the SRP receptor on the ER membrane. Once it enters the lumen of the ER, the primary polypeptide chain begins to fold into its secondary and tertiary structures while translation is still occurring. Within the ER lumen are enzymes that catalyze the posttranslational modifications of the protein. As such, protein folding can influence posttranslational modifications, and vice versa. Once translation is completed and the entire primary polypeptide chain has translocated into the ER, protein folding will be completed and the tertiary protein structure will be assembled into its final quaternary structure, during which further posttranslational modification may occur (83). Posttranslational modification regulates the properties and hence, functions, of a protein by “the addition of a modifying chemical group or another protein to one or more of its amino acid residues” (84). A given protein or amino acid residue may be modified by different types of posttranslational modification at different times. The different types of posttranslational modification are discussed in the following sections.

**15.7.4.1 Phosphorylation.** Serine, threonine, and tyrosine residues are phosphorylated and dephosphorylated by protein kinases and phosphatases, respectively. The

phosphoryl group results in a conformational change in the protein and affects the activity of the protein or protein–protein interactions; some protein domains such as SH2 (Src homology domain 2) and PTB (phosphotyrosine-binding domain) will only recognize phosphorylated tyrosine, while others such as forkhead-associated domain will only recognize phosphorylated serine. Many of these phosphorylated proteins are involved in control of cellular growth, mitosis, and response to DNA damage. The use of phosphorylation to amplify signals involved in these processes is common across multiple species.

**15.7.4.2 Ubiquitination (Also Known as Ubiquitylation).** Ubiquitin is a small protein that can be covalently linked to lysine residues of proteins targeted for intracellular degradation by proteasomes. Ubiquitin is first activated by ubiquitin-activating enzyme 1 (UBE1), followed by conjugation to ubiquitin-conjugating enzyme E2, and ligation to lysine residues of specific proteins by ubiquitin protein ligase E3. The target site can be modified by either a single ubiquitin molecule (monoubiquitination), which regulates endocytosis of receptors and repair of DNA damage, or a chain of ubiquitin molecules (polyubiquitination), which is found in proteins targeted for intracellular proteolysis (84).

Mutations in the X-linked ubiquitin-like modifier-activating enzyme 1 (*UBA1*), previously known as ubiquitin-activating enzyme E1 (*UBE1*), result in X-linked infantile spinal muscular dystrophy (OMIM# 301830). Also known as X-linked distal arthrogryposis multiplex congenita, it is characterized by severe congenital hypotonia, areflexia, and contractures due to poor intra-uterine movements, with loss of anterior horn cells in the spinal cord. It has been postulated that amino acid changes in the interaction domain of *UBA1* result in altered interactions between *UBA1* and gigaxonin, which mediate the degradation of microtubule-associated protein 1B (*MAP1B*). This could then lead to accumulation of *MAP1B* and consequent neuronal cell death (85). Mutations in the X-linked ubiquitin-conjugating enzyme E2A (*UBE2A*) lead to a form of X-linked intellectual disability syndrome (OMIM# 300860) with lack of speech, seizures, widely spaced nipples, a small penis, and generalized hypertrichosis. Mutations in the maternally inherited copy of ubiquitin protein ligase E3A (*UBE3A*), a gene that is imprinted in the brain such that only the maternally inherited copy is normally active, result in Angelman syndrome (OMIM# 105830), which is characterized by intellectual disabilities, minimal speech, seizures, and a generally happy personality. The exact pathogenesis of both syndromes caused by mutations in *UBE2A* and *UBE3A* remains unclear, but they share some overlapping phenotypic features and it is possible that it may be related to excess protein accumulation in neurons due to disruptions in ubiquitination.

**15.7.4.3 Sumoylation.** The small ubiquitin-related modifier (SUMO) proteins are structurally similar to

ubiquitin and conjugate to lysine residues of target proteins through a series of reactions catalyzed by an E1-activating enzyme, a specific E2-conjugating enzyme (ubiquitin-conjugating enzyme E2I (UBE2I); also known as UBC9), and a small number of E3 protein ligases. Sumoylation can change the activity of the sumoylated protein, alter the intracellular localization of the protein, or affect protein–protein interactions. There are four SUMO isoforms, encoded by *SUMO1* to *SUMO4* (86). Haploinsufficiency of *SUMO1* was identified in a girl with non-syndromic unilateral cleft lip and palate (OMIM# 613705) (87), but there have been no other reports of *SUMO1* mutations in patients with cleft lip/palate, so the direct association remains controversial and it has been hypothesized that this association may be modified by environmental factors (88,89).

**15.7.4.4 Acetylation.** Acetylation occurs with the transfer of acetyl groups from acetyl coenzyme A (acetyl CoA) to lysine residues by acetyltransferase leading to neutralization of their positive charge. As discussed in Section 15.7.1.1, acetylation can alter gene expression epigenetically. Acetylated lysines are recognized by a specific bromodomain found in some transcription factors and coactivators. These acetylated transcription factors are able to bind more strongly to DNA. Acetylation also alters protein–protein interactions and the stability of proteins. For example, acetylated E2F transcription factor 1 (E2F1) is more stable than the unacetylated form.

**15.7.4.5 Methylation.** In addition to the methylation of histones and DNA as described above, gene expression can also be regulated by methylation of lysine and arginine residues in specific proteins. Methylated lysine residues interact with the chromodomain while methylated arginine residues interact with the “Tudor domain.” A protein may be methylated to different degrees (mono-, di-, or trimethylation). An example of a protein whose activity is regulated by methylation is p53; methylation can either increase or decrease its activity depending on the site and degree of methylation (84).

**15.7.4.6 Hydroxylation.** The proline residue in proteins that are secreted and those that are involved in intracellular oxygen sensing can be hydroxylated by prolyl hydroxylases. EGLN1 is one of the prolyl hydroxylase domain-containing enzymes that hydroxylates the  $\alpha$ -subunit of hypoxia-inducible factor (HIF), a transcription factor, and targets it for proteolysis when oxygen tension is normal. When the cell is hypoxic, hydroxylation of HIF is diminished, leading to accumulation and increased binding of HIF to an enhancer for erythropoietin, and hence a rise in erythropoietin concentration (84,90). Heterozygous loss-of-function mutations in *ELGN1* lead to familial erythrocytosis-3 (OMIM# 609820) in which hemoglobin concentration and hematocrit are increased but erythropoietin is inappropriately normal.

**15.7.4.7 Glycosylation.** The addition of oligosaccharide chains to the surfaces of some secreted and

membrane-bound proteins is required for proper protein folding and localization. There are two main types of glycosylation: (i) N-linked glycosylation in which an oligosaccharide chain is attached to the amide group of an asparagine residue via *N*-acetylglucosamine; and (ii) O-linked glycosylation in which an oligosaccharide chain is attached to the oxygen in the hydroxyl group of serine or threonine, and less often hydroxyproline and hydroxylysine residues. N-linked glycosylation begins in the ER and is completed in the Golgi apparatus. In contrast, O-linked glycosylation occurs primarily in the Golgi apparatus after protein folding and N-linked glycosylation (if applicable) has been completed (83,91).

Defects in N-glycosylation result in a category of disorders known as congenital disorders of glycosylation (CDG), which are divided into two main groups: CDG-I are due to defects in the assembly and attachment of the oligosaccharide chain to the asparagine residue, while CDG-II are due to defects in the processing of the oligosaccharide chain whereby three glucose and one mannose residues are cleaved from the nascent chain, transported to the Golgi apparatus where other sugars are added to the final structure. Many, but not all, types of CDG are associated with neurological complications (e.g. developmental delay, hypotonia, seizures, cerebellar hypoplasia) and involvement of other organ systems particularly the heart (cardiomyopathy), gastrointestinal (GI) tract (protein-losing enteropathy), liver, and eyes (e.g. coloboma, optic nerve hypoplasia, cataracts) (91,92).

Defects in O-glycosylation result in a number of different disorders with varying phenotypic features. Homozygous or compound heterozygous mutations in *B4GALT7* (xylosylprotein beta 1,4-galactosyltransferase, polypeptide 7), which is involved in synthesis of O-xylosylglycans, result in the progeroid form of Ehlers–Danlos syndrome (OMIM# 130070) characterized by wrinkled skin on the face, osteopenia, hypotonia, and developmental delay, in addition to joint laxity and thin, atrophic scars (91). The polypeptide *N*-acetylgalactosaminyltransferase 3, encoded by *GALNT3*, is involved in the synthesis of O-*N*-acetylgalactosaminyl glycans. Homozygous or compound heterozygous mutations in *GALNT3* are a cause of hyperphosphatemic familial tumoral calcinosis (OMIM# 211900), which presents with hyperphosphatemia, ectopic deposition of calcium phosphate crystals in soft tissues around the large joints, as well as vascular calcifications, retinal angioid streaks, and dental anomalies in some patients. Intracellular proteolysis of the FGF23 is normally prevented by glycosylation mediated by *GALNT3*. In the absence of *GALNT3*, FGF23 is cleaved before secretion, resulting in reduction of intact circulating FGF23, which usually inhibits reabsorption of phosphate by the kidneys (93). Defects in the synthesis of O-mannosylglycans may be due to mutations in protein-O-mannosyltransferase 1 (*POMT1*) or protein-O-mannosyltransferase 2 (*POMT2*), both of which catalyze the first step in the synthesis of O-mannosylglycans,

or in protein O-linked-mannose beta-1,2-*N*-acetylglucosaminyltransferase 1 (*POMGNT1*), which catalyzes the second step in the synthesis of O-mannosylglycans. Mutations in all three of these genes result in a group of congenital muscular dystrophies known as muscular dystrophy–dystroglycanopathy due to abnormal glycosylation of alpha-dystroglycan; O-mannosylated alpha-dystroglycan is found in greatest amounts in the brain and muscle, but it may also be found in other tissues. The mildest forms dystroglycanopathies are the limb-girdle muscular dystrophy–dystroglycanopathies (OMIM# 609308, 613158, 613157) associated with mild to moderate intellectual disability and without structural brain abnormalities that are identifiable on brain MRI. At the other end of the spectrum are those muscular dystrophies associated with structural brain and eye abnormalities in the “muscle-eye-brain” group of disorders (OMIM# 236670, 613150, 253280) such as cobblestone lissencephaly, cerebellar hypoplasia, Dandy–Walker malformations, microphthalmia, retinal dysplasia, and optic nerve hypoplasia.

## 15.8 DEVELOPMENTAL GENE PATHWAYS AND FAMILIES

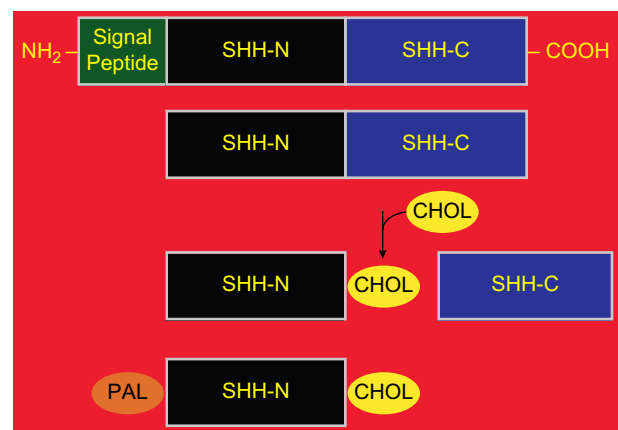
Intercellular signaling through either paracrine or juxtacrine interactions results in the activation of intracellular proteins and factors through a well-defined cascade that eventually initiates or represses the transcription of specific genes. Many of the paracrine factors can be classified into one of four families based on their shared molecular structures, viz. the Hedgehog, Wnt, FGF, and transforming growth factor- $\beta$  families. These factors typically bind to cell surface receptors that comprise three interconnected components: an extracellular ligand-binding domain, a transmembrane region, and a cytoplasmic region with enzymatic domains that remain inactive in the absence of ligand binding; the binding of a paracrine factor to the ligand-binding domain induces a conformational change in the receptor that is transmitted through the transmembrane region to the cytoplasmic region, activating the enzymatic domains of the receptor, thereby catalyzing other intracellular reactions (11). In this section, we will highlight some of the paracrine and juxtacrine signaling pathways that result in a recognizable phenotype in humans when they are perturbed during development.

### 15.8.1 Hedgehog Signaling Pathway

The Hedgehog signaling pathway is involved in the patterning and differentiation of the neural tube, various facial structures including the eyes and the teeth, the limb buds, the heart, and the GI tract. It is therefore critical for the formation of the brain, the distal limbs (fingers and toes), and other organ systems. Inappropriate inactivation of the Hedgehog pathway results in congenital malformations while inappropriate ectopic activation of the

same pathway results in malignancies that may involve the central nervous system, the skin, and skeletal muscles (11,94). There are three Hedgehog paracrine signaling molecules in vertebrates—the Desert Hedgehog (DHH), Indian Hedgehog (IHH), and SHH (Figure 15-20).

Each of the three Hedgehog paracrine signaling ligands (i.e. DHH, IHH, and SHH) is synthesized as a full-length Hedgehog (HH) protein with two components of similar sizes: a “Hedge” domain at the amino-terminal and the “Hog” domain at the carboxyl-terminal. The “Hog” domain has autocatalytic activity and cleaves the full-length Hedgehog protein into the amino-terminal “Hedge” polypeptide (HH-N) and the carboxyl-terminal “Hog” polypeptide (HH-C). This autocleavage reaction requires cholesterol and results in a cholesterol moiety being covalently linked to the carboxyl-terminal end of the HH-N polypeptide. The amino-terminal of HH-N then undergoes palmitoylation, catalyzed by hedgehog acyltransferase (HHAT), resulting in the covalent attachment of an amide-linked palmitate. Thus, HH-N is now “double lipid-modified” (i.e. bilipidated) with palmitate at the amino-terminal and cholesterol at the carboxyl-terminal. HH-N is the active signal-transducing component of HH. The bilipidated HH-N molecule is secreted from the cell in which it was synthesized; HH-C diffuses away. The secretion of HH-N into the extracellular space requires the transmembrane protein, DISP1 (protein dispatched homolog 1), which may be involved in the transportation of HH-N across the plasma membrane and perhaps the processing of HH-N into lipoprotein multimers; it is possible that DISP2 (protein dispatched homolog 2) may also be involved in these processes. Both DISP1



**FIGURE 15-20** SHH has an N-terminal signaling domain [SHH-N] and a C-terminal catalytic domain [SHH-C] that causes autocleavage of the protein, resulting in an ester-linked cholesterol moiety [CHOL] at the carboxy-terminal end of the signaling domain; the catalytic portion diffuses away. Following the addition of the cholesterol adduct, the action of Skinny hedgehog [Ski] acyltransferase adds an amide-linked palmitate [PAL] to the N-terminal end of the signaling domain. In the responding cell, this bilipidated sonic hedgehog [PAL-SHH-CHOL] becomes the ligand for PATCHED 1 [PTCH 1]. (Cohen, M. M., Jr. *Hedgehog Signaling Update*. Am. J. Med. Genet. A 2010, 152A (8), 1875–1914.)

and DISP2 are the human homologs of the *Drosophila* DISP (dispatched) protein. The newly secreted HH-N remains closely associated with the plasma membrane through interactions with glypicans, a form of heparan sulfate proteoglycans. The HH-N is eventually released from the cell surface following cleavage of the glypican anchoring molecule by Notum, and it is transported to the target cells in lipoprotein particles; the transport of HH-N to the target cells is also dependent on the biliarypidation of HH-N. This model is based largely on our understanding of the process in *Drosophila*. Details of the secretion and transport of HH-N in vertebrates are still being elucidated (94,95).

In general, PTC1 (protein patched homolog 1), encoded by *PTCH1*, is the receptor for HH-N on the surface of the target cells, although in some cells, it is thought that SHH-N in particular binds to PTC2 (encoded by *PTCH2*) instead of PTC1. CDON (cell adhesion molecule-related/downregulated by oncogenes) and BOC (Brother of CDON) are co-receptors that facilitate the binding of HH-N to PTC1, and presumably PTC2. Recently, GAS1, a membrane-bound HH-N binding protein, was shown to be an essential HH-N co-receptor for SHH-mediated neural and limb patterning, and the authors hypothesized that CDON, BOC, and GAS1 interact physically with PTC1 (96).

When HH-N is not bound to PTC1, PTC1 inhibits SMO (smoothened), the signal transducer in the HH pathway, which is normally located across the membrane of intracellular vesicles within the target cells, possibly via vitamin D3. Transcription of genes in the HH signaling pathway is mediated by the GLI family proteins (GLI1, GLI2 and GLI3), which are transcription factors that contain a zinc finger domain. GLI1 and GLI2 are transcriptional activators. The full-length GLI3 is a transcriptional activator, but cleavage of its C-terminal results in a repressor form of GLI3. Of note, *GLI1* is a target of the SHH pathway and is transcribed after GLI2 is activated (97). When SMO is inhibited (i.e. when HH-N is not bound to PTC1), GLI3 is phosphorylated by protein kinase A, glycogen synthase kinase 3, and casein kinase 1. The phosphorylated GLI3 associates with SUFU (suppressor of fused homolog) and is recognized by BTRC (beta-transducin repeat-containing protein), leading to the ubiquitination and degradation of the C-terminal portion of GLI3, leaving the N-terminal component that is a transcription repressor. A similar process leads to the degradation of the entire GLI1 and GLI2 proteins (94,98). Some authors have suggested that GLI2 has a similar N-terminal transcription repressor component and can be similarly cleaved to form a repressor too, although the GLI3 repressor is more potent than the GLI2 repressor (97,99). Therefore, genes that are regulated by HH-N are not transcribed in its absence.

When HH-N binds to PTC1 and its co-receptors CDON/BOC, SMO is activated, possibly via oxysterols. Activated SMO translocates from the membrane of the

intracellular vesicles to the axoneme of the primary cilium (typically one nonmotile primary cilium per mammalian cell). Of note, the primary cilium is present only when the cells are not in mitosis; the cilium is reabsorbed just before mitosis and re-assembled after mitosis. The SUFU–GLI complexes accumulate at the tip of the cilium where SMO activity leads to the dissociation of these SUFU–GLI complexes. This, in turn, prevents the degradation of GLI1 and GLI2, and the ubiquitination of full-length GLI3, thereby promoting the transcription of genes regulated by the HH-N pathways. The accumulation of the SUFU–GLI complexes at the tip of the cilium is facilitated by the kinesin protein KIF7, which interacts with the GLI proteins (94,98).

Hedgehog signaling can be modulated by other proteins, including HHIP (Hedgehog-interacting protein), the Ras-associated protein RAB23, and the serine/threonine kinase ULK3. HHIP, which can be either attached to the cell surface or secreted, binds to HH-N and inhibits the Hedgehog signaling pathway (100). RAB23 inhibits GLI2 and promotes the processing (cleavage) of GLI3 into its repressor form, and is hence a negative regulator of the pathway. It is believed that RAB23 acts intracellularly downstream of SMO, although the exact targets have not been clearly identified (101). In contrast, ULK3 binds to the SUFU–GLI2 complex, and it has been hypothesized that in the absence of SHH-N, ULK3 promotes the phosphorylation of full-length GLI2 by protein kinase A, glycogen synthase kinase 3, and casein kinase 1, leading to cleavage of GLI2 into the repressor form. However, in the presence of SHH-N, ULK3 dissociates from the complex and by itself phosphorylates and activates full-length GLI2, thereby promoting the translocation of active GLI2 into the nucleus. Therefore, ULK3 may be both a negative and positive regulator of the pathway, depending on the absence or presence of SHH-N (97).

An overview of the birth defects, congenital syndromes, and malignancies associated with mutations in the various genes in the Hedgehog signaling pathway is provided by Cohen (94). The neurological phenotypes that result from a disruption in the SHH pathway have been reviewed by Murdoch and Copp (99).

Mutations in several genes, including *SHH*, *PTCH1*, *CDON*, *GAS1*, and *GLI2*, that lead to decreased signaling through the SHH pathway can result in the holoprosencephaly spectrum (OMIM# 236100) in humans and in some animal (mainly mouse) models (102). These are usually heterozygous (autosomal dominant) mutations in humans but homozygous (null) mutations in the animal models; heterozygous mutations of these genes in mouse models do not usually result in any observable neurological phenotype. Note that almost all the reported mutations in *PTCH1* that result in holoprosencephaly are missense mutations that either alter the ability of PTC1 to bind to SHH-N or possibly increased signaling through the Hedgehog signaling pathway in the target cells since PTC1 normally inhibits the pathway in



the absence of HH-N. The expressivity of many of these genes is highly variable, ranging from cyclopia with alobar holoprosencephaly at the severe end of the spectrum to hypotelorism or single maxillary incisor alone without any observable brain malformations at the mildest end; the penetrance is also incomplete even within a single family (103,104). Although it has been suggested that mutations affecting the signaling of all HH-N proteins (i.e. receptors and signal transducers in the target cells) tend to result in a more severe phenotype than mutations in *SHH*, this has not been consistently demonstrated in the animal models, nor in humans (99). However, it is possible the mutations that result in the most severe phenotypes may be lethal in the embryonic or fetal period and hence have never been reported.

In contrast, mutations that result in increased signaling through the Hedgehog pathway may lead to the development of tumors or cancer-predisposition syndromes. Heterozygous loss-of-function mutations in *PTCH1* can result in the nevoid basal cell carcinoma (also known as basal cell nevus) syndrome (OMIM# 109400), which is characterized by multiple basal cell carcinoma in childhood or young adulthood, relative macrocephaly, ectopic calcification of the falx cerebri, jaw keratocysts, palmar or plantar pits, and in about 5% of individuals, medulloblastoma in childhood; cardiac and ovarian fibromas have also been reported in nevoid basal cell carcinoma syndrome (105). Somatic *PTCH1* mutations have also been identified in basal cell carcinoma samples from non-syndromic individuals who are otherwise healthy (OMIM# 605462). Germ line heterozygous loss-of-function mutations in *SUFU* that lead to inappropriate activation of the SHH pathway greatly increase the risk of desmoplastic medulloblastoma (OMIM# 155255). On the other hand, homozygous loss-of-function mutations in *RAB23* result in Carpenter syndrome (acrocephalopolysyndactyly type II) (OMIM# 201000), which is characterized by craniosynostosis (usually sagittal, coronal, and metopic sutures), obesity, brachydactyly, postaxial polydactyly of the hands, and insertional or preaxial polydactyly of the feet, cutaneous syndactyly in hands and feet, and congenital heart disease; cryptorchidism and hypoplastic external genitalia have been reported in males. There is no known increased risk for any cancers in Carpenter syndrome. It is believed that this syndrome is caused by inappropriate activation of GLI2 (*RAB23* usually inhibits GLI2) (94,106).

Heterozygous mutations in *GLI3* result primarily in two phenotypically distinct syndromes: Greig cephalopolysyndactyly syndrome (GCPS) (OMIM# 175700) and Pallister–Hall syndrome (OMIM# 146510); in addition, a small number of individuals with *GLI3* mutations have clinical features suggestive of orofaciocigital syndrome in which the clinical manifestations overlap with those of Pallister–Hall syndrome. GCPS is characterized by macrocephaly, hypertelorism, postaxial polydactyly of the hands, preaxial polydactyly of

the feet, and cutaneous syndactyly. Pallister–Hall syndrome is characterized by hypothalamic hamartoma, bifid epiglottis, laryngeal cleft, mesoaxial (insertional) polydactyly, anal atresia, and other visceral anomalies including adrenal insufficiency. This phenotypic dichotomy reflects the bifunctional role of GLI3 as a transcription activator and transcription repressor. Almost all the *GLI3* mutations that result in GCPS are loss-of-function mutations that occur mainly in either the 5′ or 3′ ends of the gene (5′ of cDNA nucleotide position 1998 or 3′ of cDNA nucleotide position 3481), with a smaller number of mutations in the middle part of the gene between nucleotide positions 1998 and 3481; the zinc finger DNA-binding domain is encoded by the nucleotides 5′ of position 1998 (i.e. in the amino-terminal of GLI3) while the transactivation domain is encoded by the nucleotides 3′ of position 3481 (i.e. in the carboxyl-terminal of GLI3). Recent data suggest that mutations 3′ of position 3481 may result in a milder GCPS (“sub-GCPS”) phenotype. The transcription activator function of GLI3 is therefore abrogated by all the mutations that lead to GCPS or sub-GCPS. In contrast, almost all *GLI3* mutations that result in Pallister–Hall syndrome are truncating mutations that occur in the middle part of the gene between cDNA nucleotide positions 1998 and 3481, which encode the normal GLI3 proteolytic processing site where full-length (activator) GLI3 is cleaved to form the repressor form of GLI3. Therefore, the *GLI3* mutations that lead to Pallister–Hall syndrome are those that result in the formation of a repressor form of GLI3 independent of HH-N binding to PTC1 (essentially a gain-of-function mutation for a repressor *GLI3*) (94,107,108). In contrast, isolated postaxial polydactyly type A (i.e. with a well-formed digit) and type B (i.e. poorly formed “pedunculated postminimi”) (OMIM# 174200) can also be caused by heterozygous *GLI3* mutations although the effects of these mutations on the GLI3 protein remain unclear (107,109).

It is believed that another member of the Hedgehog protein family, *DHH*, is expressed primarily in the gonads, although there is some expression in the neural sheaths as well. Homozygous loss-of-function mutations in *DHH* can result in 46,XY complete gonadal dysgenesis in which individuals with a 46,XY karyotype present as phenotypic females with bilateral streak gonads and in some individuals, bilateral gonadoblastoma (OMIM# 233420) (110,111). There has also been a report of a 46,XY female with partial gonadal dysgenesis (female external genitalia, immature uterus, and unilateral streak testicle) and minifascicular polyneuropathy who was found to have a homozygous mutation in *DHH* (112). However, no other patient with partial gonadal dysgenesis and minifascicular polyneuropathy has been found to have mutations in *DHH*.

Primarily expressed in prehypertrophic chondrocytes, *IHH* regulates the differentiation of chondrocytes, development of the growth plates, and endochondral

ossification, at least partly by inducing the expression of parathyroid hormone-related protein (PTHrP) (also known as parathyroid hormone-like hormone (PTHrH)). Heterozygous missense mutations in *IHH* that lead to reduced binding with its receptor (PTC1) and enhanced degradation within the lysosomes, but increased interactions with heparan sulfate proteoglycans and increased diffusion of the molecule away from the secreting cells (i.e. having both gain-of-function and loss-of-function effects), result in brachydactyly type A1 (OMIM# 112500) in which there is hypoplasia or absence of the middle phalanges of the digits, and short proximal phalanges of the thumbs and halluces (113–115). Homozygous mutations in *IHH* have been identified in two consanguineous families with acrocapitofemoral dysplasia (OMIM# 607778) in which there is brachydactyly, cone-shaped epiphyses in the middle and distal phalanges in the hands, premature fusion of the proximal femoral epiphyses leading to a short femoral neck, and short stature (116,117). One of these two homozygous mutations is close to the amino-terminal end while the other is close to the carboxyl-terminal end of the active *IHH* amino acid sequence (i.e. the amino-terminal domain of unprocessed *IHH*), in contrast to the heterozygous mutations that result in brachydactyly type A1, all of which occur in the middle part of the active *IHH* amino acid sequence (118). This suggests that these two different disorders are due to mutations in different domains (or subdomains) within the active *IHH* protein. Recently, it was shown that duplication of a conserved noncoding element upstream of *IHH* resulting in the overexpression of *IHH* can lead to syndactyly type 1 (OMIM# 185900), which is characterized by cutaneous syndactyly and synostosis of the distal phalanges in the hands and feet, or to Philadelphia-type craniosynostosis (OMIM# 601222), which is characterized by sagittal craniosynostosis and complete cutaneous syndactyly of the hands and feet with synostosis of the distal phalanges of the hands in a few of the affected individuals (119,120).

As discussed above, cholesterol is essential for the processing of the full-length Hedgehog proteins into the active signal-transducing HH-N ligands, and it has recently been shown that sterols are required for the activation of Smo in a mouse model, which suggests that defects in cholesterol synthesis and metabolism may affect the Hedgehog signal transduction pathway in the target cells (121). As such, the phenotypic manifestations of Smith–Lemli–Opitz syndrome (OMIM# 270400) in which there is a deficiency in 7-dehydrocholesterol reductase, the enzyme that catalyzes the last step in the cholesterol synthesis pathway, may be partly attributed to a secondary disruption in the Hedgehog signaling pathway (122).

### 15.8.2 Wnt Signaling Pathways

The Wnt signaling pathways regulate the transcription of specific developmental genes and are involved in

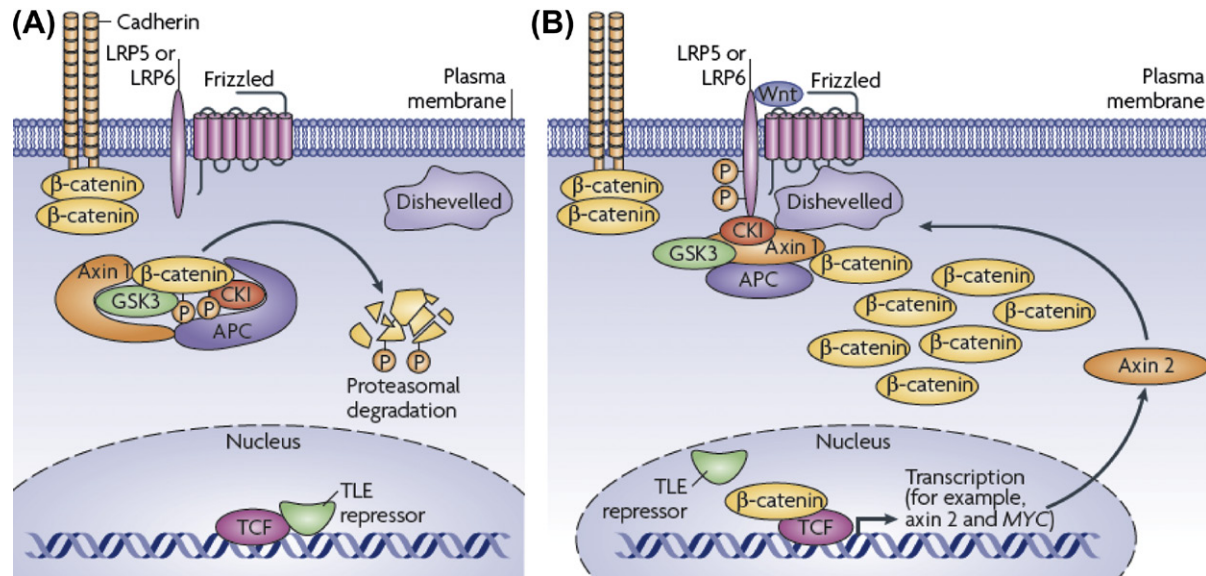
the development of the urogenital organs, limbs, skin, and other organ systems. There are two sets of pathways: the canonical Wnt pathway in which  $\beta$ -catenin is the key transcription co-regulator, and a number of different non-canonical Wnt pathways in which transcription is regulated by modulators such as RHOA (transforming protein RhoA) and calcineurin instead of  $\beta$ -catenin (11).

The Wnt ligands are cysteine-rich glycolipoproteins that undergo N-linked glycosylation after synthesis, which is required for the secretion of the ligand. Some Wnt molecules are palmitoylated (i.e. addition of palmitate) at specific cysteine residues, and in Wnt3a, palmitoleic acid is added to the serine residue at amino acid position 209 toward the C-terminal of the molecule. It has been suggested that the addition of palmitate facilitates binding of the ligand to its receptor while the addition of palmitoleic acid in Wnt3a is specifically required for its secretion. The palmitoleoylation of Wnt3a at serine 209 is believed to be mediated by PORCN (protein-cysteine N-palmitoyltransferase porcupine). PORCN may also be involved in the lipidation and secretion of other Wnt ligands (123,124).

The canonical Wnt pathway was the first Wnt signaling pathway to be elucidated and remains the best understood. Figure 15-21 illustrates the canonical Wnt pathway.

As illustrated in Figure 15-21, when a Wnt ligand is not bound to its specific receptor, excess  $\beta$ -catenin that is synthesized in the receptor cell is phosphorylated by the “destruction complex,” polyubiquitinated via the SCF-class E3 ubiquitin ligase BTRCP (beta-transducin repeat-containing protein) that only recognizes correctly phosphorylated  $\beta$ -catenin, and is degraded by 26S proteasomes in the cell. Transcription of the Wnt-regulated genes is normally repressed by the formation of a complex comprising either T-cell factor (TCF) or lymphoid enhancer-binding factor 1 (LEF1) together with TLE1 (the human homolog of *Drosophila groucho*) that binds to DNA, promoting the deacetylation of histones and compaction of chromatin. Note that there are at least four different TCF genes and multiple splicing variants in humans (123–125).

Binding of a specific Wnt ligand to its specific frizzled (Fz) transmembrane G protein-coupled receptor leads to the formation of a receptor complex with the low-density lipoprotein receptor-related protein LRP5 or LRP6 co-receptor. Disheveled (DVL1, and probably DVL2 and DVL3) is recruited to the Wnt–Fz–LRP5/6 complex. The cytoplasmic tail of LRP5 or LRP6 is then phosphorylated, resulting in the formation of an Axin 1-binding site. The destruction complex is thereby disrupted and free (unphosphorylated)  $\beta$ -catenin accumulates and enters the nucleus where it displaces TLE1, forms a complex with one of the TCF proteins or with LEF1, and recruits other transcription co-activators. Therefore, TCF proteins have



**FIGURE 15-21** The canonical Wnt signaling pathway. (A) In the absence of a signal, the destruction complex adenomatous polyposis coli (APC)-axin 1-glycogen synthase kinase 3 (GSK3)-casein kinase 1 (CK1) binds and phosphorylates non-cadherin-associated  $\beta$ -catenin, targeting it for destruction by the proteasome. In the nucleus, DNA-binding proteins of the TCF and lymphoid enhancer-binding factor 1 (LEF1) family are bound by transcriptional repressors (such as the transducin-like enhancer proteins (TLEs)). (B) The binding of a Wnt ligand to its frizzled receptor and lipoprotein receptor-related protein 5 (LRP5) or LRP6 co-receptor induces a change in conformation that results in phosphorylation of the co-receptor. This creates a high-affinity binding site for axin 1, causing disruption of the destruction complex.  $\beta$ -Catenin can then accumulate and associate with the TCF or LEF1 proteins, dislodging the TLE repressors and hence promoting transcriptional activation of a program of genes, including *MYC* and *axin 2*. *Axin 2* feeds back to inhibit the pathway by promoting the assembly of more destruction complexes. (McNeill, H.; Woodgett, J. R. *When Pathways Collide: Collaboration and Connivance among Signalling Proteins in Development*. Nat. Rev. Mol. Cell Biol. 2010, 11 (6), 404–413.)

the ability to both activate and repress transcription of Wnt-regulated genes, depending on the presence or absence of the Wnt ligands and hence  $\beta$ -catenin binding (123–125).

Table 15-4 lists some human diseases resulting from the gain (GOF) or loss (LOF)-of-function mutations in the canonical Wnt signaling pathway genes, as summarized by MacDonald et al. (124).

In the non-canonical Wnt pathway, Wnt ligands may bind to either the frizzled receptor (in what is also known as the frizzled-PCP (planar cell polarity) pathway), or other receptors including the tyrosine-protein kinase receptors such as ROR2 and RYK. Some Wnt ligands that activate the non-canonical Wnt pathway can inhibit the canonical Wnt pathway. For example, WNT5A is primarily a non-canonical Wnt ligand that binds to ROR2 and RYK; however, WNT5A also inhibits the canonical Wnt pathway by various mechanisms such as activating kinases that phosphorylate and inactivate TCF transcription factors, and competing with other Wnt ligands for binding to frizzled receptors (125). Heterozygous loss-of-function mutations in WNT5A have been found to be associated with autosomal dominant Robinow syndrome (OMIM# 180700), which is characterized by primarily mesomelic dwarfism, distinctive facial features such as hypertelorism, short nose with broad nasal bridge and anteverted nares, genitalia abnormalities such as short penis, clitoris, or

labia majora. In contrast, homozygous or compound heterozygous loss-of-function mutations in ROR2 result in autosomal recessive Robinow syndrome (OMIM# 268310) which is similar to, but phenotypically more severe than the autosomal dominant Robinow syndrome; rib anomalies including rib fusion and vertebral anomalies such as hemivertebrae are commonly seen in autosomal recessive Robinow syndrome. Of note, heterozygous gain-of-function mutation in ROR2 results in brachydactyly type B1 (OMIM# 113000) in which there is shortened middle phalanges and absent or severely hypoplastic distal phalanges and nails affecting particularly the fourth and fifth digits; mild distinctive facial features including hypertelorism may be observed (126).

### 15.8.3 Receptor Tyrosine Kinase Signaling Pathways

The receptor tyrosine kinases (RTKs) are a superfamily of transmembrane receptors that bind various growth factors, hormones, and other intercellular signaling molecules. There are at least 58 different RTKs in humans, subclassified into 20 subfamilies, as shown in Figure 15-22 (127). Many of the signaling pathways mediated by RTKs are involved in tissue morphogenesis and embryonic or fetal development. As such, germ line mutations in the genes that regulate these pathways can result in congenital malformation syndromes while



**TABLE 15-4 Human Diseases Associated with Mutations of the Wnt Signaling Components**

Gene	Function	Human Disease
PORCN	+ Wnt lipid modification/processing	LOF X-linked focal dermal hypoplasia
WNT3	+ Ligand for Wnt/ $\beta$ -catenin signaling	LOF tetra-amelia
WNT4	+ Ligand for Wnt/ $\beta$ -catenin signaling	LOF Müllerian duct regression and virilization
WNT5B	+ Ligand for Wnt/ $\beta$ -catenin signaling	(?) type II diabetes
WNT7A	+ Ligand for Wnt/ $\beta$ -catenin signaling	LOF Fuhrmann syndrome
WNT10A	+ Ligand for Wnt/ $\beta$ -catenin signaling	LOF odonto-onycho-dermal dysplasia
WNT10B	+ Ligand for Wnt/ $\beta$ -catenin signaling	LOF obesity
RSP01	+ Wnt agonist	LOF XX sex reversal with palmoplantar hyperkeratosis
RSP04	+ Wnt agonist	LOF autosomal recessive anonychia and hyponychia congenita
SOST	– LRP5/6 antagonist predominantly expressed in osteocytes	LOF high bone mass, sclerosteosis, Van Buchem disease
Norrin (NDP)	+ Specific ligand for FZD4 and LRP5 during eye development	LOF familial exudative vitreoretinopathy
LRP5	+ Wnt co-receptor	GOF hyperparathyroid tumors (alt. splicing), GOF high bone mass, LOF osteoporosis-pseudoglioma, LOF FEVR eye vascular defects
LRP6	+ Wnt co-receptor	LOF early coronary disease and osteoporosis
FZD4	+ Wnt receptor	LOF familial exudative vitreoretinopathy
Axin1	– Facilitates $\beta$ -catenin degradation; acts as a tumor suppressor	LOF caudal duplication, cancer
Axin2	– Facilitates $\beta$ -catenin degradation; acts as a tumor suppressor	LOF tooth agenesis, cancer
APC	– Facilitates $\beta$ -catenin degradation; acts as a tumor suppressor	LOF familial adenomatous polyposis, cancer
WTX	– Facilitates $\beta$ -catenin degradation; acts as a tumor suppressor	LOF Wilms tumor
$\beta$ -catenin (CTNNB1)	+ Primary Wnt effector; acts as an oncogene	GOF cancer
TCF4 (TCF7L2)	+ $\beta$ -catenin transcriptional partner	(?) type II diabetes

LOF, loss-of-function; GOF, gain-of-function.

Reproduced from Reference (124).

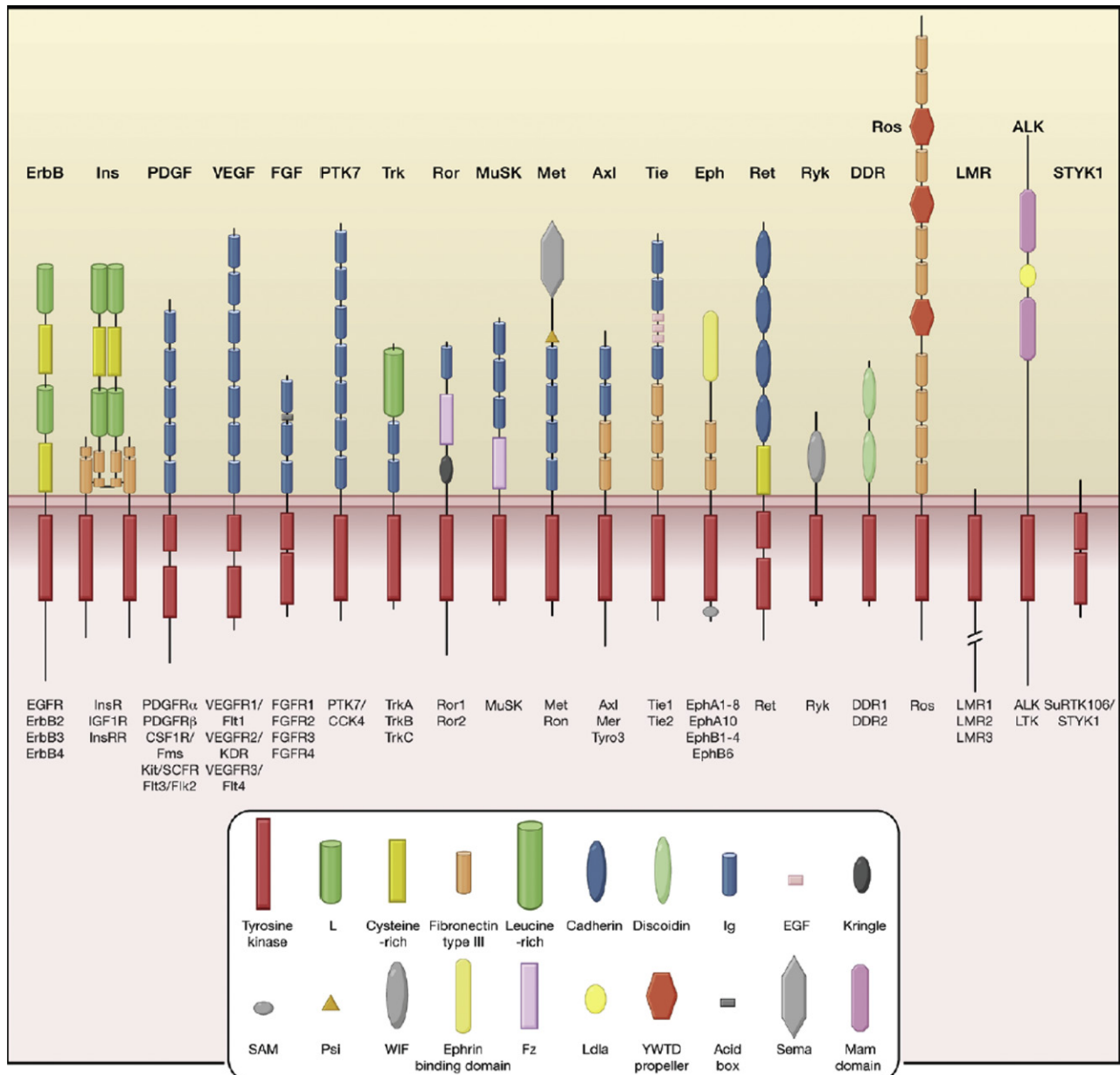
somatic mutations in some of these genes result in the development of various malignancies.

Each RTK comprises an extracellular ligand-binding domain, a single transmembrane helix, and a cytoplasmic catalytic domain with tyrosine kinase activity. Traditionally, it had been thought that the binding of the ligand to the receptor induces dimerization of the receptor and subsequent activation of the tyrosine kinase domains. However, more recent studies suggest that some RTKs form dimers and oligomers on cell membranes even in the absence of ligand binding, but it is the binding of specific ligands to the specific dimeric or oligomeric receptors that results in tyrosine kinase activity; some RTKs form homodimers (i.e. with the same type of RTK receptor) but others form heterodimers between different RTKs within the same subfamily. Monomeric RTKs are almost always inactive due to intramolecular (i.e. *cis*) autoinhibition of the tyrosine kinase domains; dimerization or oligomerization along with ligand binding releases this autoinhibition by various mechanisms including (i) trans-autophosphorylation of the activation loop that blocks the enzymatic site and prevents substrate binding as well (i.e. phosphorylation of specific tyrosine residues in the activation loop of the “partner” co-receptor), (ii)

phosphorylation of specific tyrosine residues in the juxta-membrane region (i.e. between the transmembrane helix and the intracellular tyrosine kinase domain), and (iii) direct allosteric activation through contact between the dimers or oligomers. Signal transduction through RTKs is therefore regulated by their distribution and density on the cell surface. Moreover, each RTK usually binds only one or a small subset of ligands (127,128).

When the tyrosine kinase domains have been activated, specific tyrosine residues in the cytoplasmic domains are phosphorylated. These phosphotyrosine residues can recruit and provide docking sites for adaptor (also known as docking) proteins that contain Src homology-2 (SH2) or PTB domains; these adaptor proteins can, in turn, be phosphorylated at specific tyrosine residues by the tyrosine kinase in the RTK on which they have docked. Each adaptor protein bound to an RTK can activate a variety of intracellular signaling cascades including the RAS–MAPK, PI3K–AKT, PLC $\gamma$ –PKC, and JAK–STAT pathways. Some of these signaling cascades can also be activated by the direct binding of specific molecules to the phosphotyrosine residues in the cytoplasmic domains directly. Activation of these pathways usually results in alteration in gene transcription (127,128).



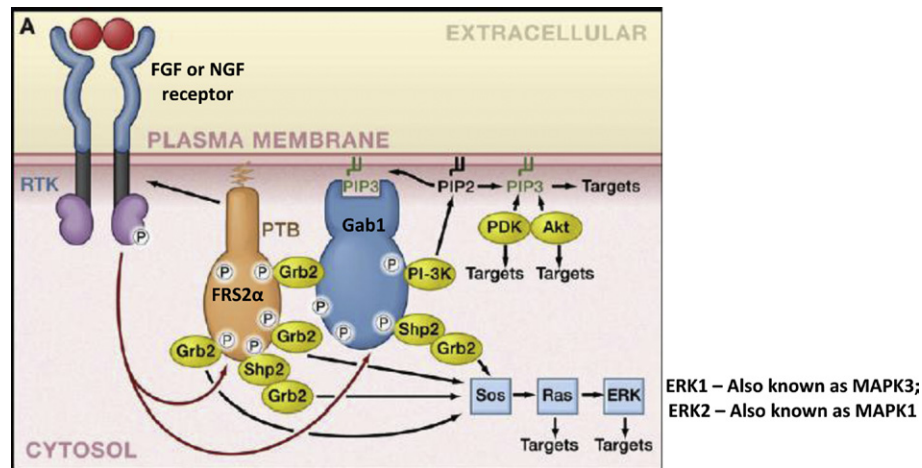


**FIGURE 15-22** The 20 subfamilies of human RTKs, shown schematically with the family members listed beneath each receptor. Structural domains in the extracellular regions, identified by structure determination or sequence analysis, are indicated according to the key. The intracellular domains are shown as red rectangles. (Lemmon, M. A.; Schlessinger, J. *Cell Signaling by Receptor Tyrosine Kinases*. Cell 2010, 141 (7), 1117–1134.)

(Note abbreviations: RAS, “Rat sarcoma,” a family of GTP/GDP-binding GTPases whose members include HRAS, KRAS, NRAS; MAPK, mitogen-activated protein kinase, which is a family of serine/threonine protein kinase; PI3K, phosphoinositide 3-kinase; AKT, not an abbreviation but named after a thymoma cell line derived from the AKR mouse strain—it is a serine/threonine protein kinase, also known as protein kinase B; PLC $\gamma$ , phospholipase C- $\gamma$ ; PKC, protein kinase C; JAK, Janus kinase; STAT, signal transducer and activator of transcription.)

A simplified view of RTK signal transduction through adaptor proteins is illustrated in Figure 15-23.

The activity of the RTK-mediated signaling pathways is partly regulated by a series of positive and negative feedback loops. Positive feedback resulting in sustained or increased tyrosine kinase activity may be achieved through either stimulation of kinase activity or inhibition of protein tyrosine phosphatases (PTPs) that normally reverse autophosphorylation of the cytoplasmic domains, both of which processes may be induced by ligand binding. Negative feedback may be accomplished by direct activation of PTPs. For example, SHP1 (encoded by *PTPN6*) and SHP2 (encoded by *PTPN11*) are SH2 domain-containing PTPs that are recruited to dephosphorylate activated epidermal growth factor



**FIGURE 15-23** The docking (i.e. adaptor) protein FGF receptor substrate-2 (FRS2 $\alpha$ ) forms a complex with activated FGF or nerve growth factor (NGF) receptors via its PTB. The activated RTK phosphorylates FRS2 $\alpha$  on multiple tyrosines, and the resulting phosphotyrosines recruit multiple growth factor receptor-bound protein 2 (Grb2) and Shp2 (also known as tyrosine-protein phosphatase non-receptor type 11, encoded by *PTPN11*) molecules. This brings a second docking protein, GRB2-associated-binding protein 1 (Gab1), into the complex. Gab1 is tyrosine phosphorylated and recruits additional signaling proteins, including PI3K. PI3K initiates a positive feedback loop in which phosphatidylinositol (3,4,5)-triphosphate (PIP3), generated by PI3K, recruits more Gab1, leading to further PI3K activation. (Abbreviations: PIP2, phosphatidylinositol (4,5)-biphosphate, phosphorylation of which by PI3K results in PIP3; PDK, 3-phosphoinositide-dependent protein kinase; SOS, son of sevenless; ERK, extracellular signal-regulated kinase, a member of the mitogen-activated protein kinase (MAPK) family.) (Lemmon, M. A.; Schlessinger, J. *Cell Signaling by Receptor Tyrosine Kinases*. Cell **2010**, 141 (7), 1117–1134.)

receptor (EGFR). In the RAS-MAPK/ERK (extracellular signal-regulated kinase) cascade, MAPK/ERK directly phosphorylates SOS, impeding its interactions with GRB2; MAPK/ERK also directly phosphorylates and reduces the activity of the RAF serine/threonine protein kinase that activates MAPK/ERK via an MAPK kinase (MAPKK), forming negative feedback loops. Some activated RTKs such as EGFR are degraded intracellularly via ubiquitination, which is an important mechanism that prevents excessive signaling through those RTKs (127).

It should also be noted that some RTKs such as EGFR and the neurotrophic tyrosine kinase receptor subfamily can be internalized into endosomes and the signaling pathways that they activate depend on whether the receptors are bound to the plasma membrane or to the intracellular endosomes (128). In some RTKs such as MET, trafficking of the receptor to a perinuclear endosome is required only for the phosphorylation of specific, but not all, proteins (127).

#### 15.8.4 RAS-MAPK/ERK Pathway

It has now been established that heterozygous mutations in genes that regulate the RAS-MAPK/ERK pathway, which is one of the intracellular signaling pathways mediated through the RTKs, result in a group of congenital malformation syndromes known as the “RASopathies,” some of which are listed in [Table 15-5](#). Almost all of these mutations result in prolonged or overactivation of the RAS-MAPK/ERK pathway; gain-of-function mutations were identified in the genes that encode proteins that promote the activation of the pathway while loss-of-function

TABLE 15-5		Syndromes Associated with Mutations in the RAS-MAPK/ERK pathway genes
Genes	Protein	Syndromes
NRAS	RAS	Costello syndrome
KRAS	RAS	Costello syndrome
HRAS	RAS	Costello syndrome
RAF1	RAF	Costello syndrome
MEK1	MEK	Costello syndrome
MEK2	MEK	Costello syndrome
ERK1	ERK	Costello syndrome
ERK2	ERK	Costello syndrome
SHC1	SHC	Costello syndrome
SHC2	SHC	Costello syndrome
GRB2	GRB	Costello syndrome
SOS1	SOS	Costello syndrome
SOS2	SOS	Costello syndrome
PI3K	PI3K	Costello syndrome
AKT1	AKT	Costello syndrome
AKT2	AKT	Costello syndrome
AKT3	AKT	Costello syndrome
MTOR	MTOR	Costello syndrome
PTEN	PTEN	Costello syndrome
FOXO1	FOXO	Costello syndrome
FOXO2	FOXO	Costello syndrome
FOXO3	FOXO	Costello syndrome
FOXO4	FOXO	Costello syndrome
FOXO5	FOXO	Costello syndrome
FOXO6	FOXO	Costello syndrome
FOXO7	FOXO	Costello syndrome
FOXO8	FOXO	Costello syndrome
FOXO9	FOXO	Costello syndrome
FOXO10	FOXO	Costello syndrome
FOXO11	FOXO	Costello syndrome
FOXO12	FOXO	Costello syndrome
FOXO13	FOXO	Costello syndrome
FOXO14	FOXO	Costello syndrome
FOXO15	FOXO	Costello syndrome
FOXO16	FOXO	Costello syndrome
FOXO17	FOXO	Costello syndrome
FOXO18	FOXO	Costello syndrome
FOXO19	FOXO	Costello syndrome
FOXO20	FOXO	Costello syndrome
FOXO21	FOXO	Costello syndrome
FOXO22	FOXO	Costello syndrome
FOXO23	FOXO	Costello syndrome
FOXO24	FOXO	Costello syndrome
FOXO25	FOXO	Costello syndrome
FOXO26	FOXO	Costello syndrome
FOXO27	FOXO	Costello syndrome
FOXO28	FOXO	Costello syndrome
FOXO29	FOXO	Costello syndrome
FOXO30	FOXO	Costello syndrome
FOXO31	FOXO	Costello syndrome
FOXO32	FOXO	Costello syndrome
FOXO33	FOXO	Costello syndrome
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FOXO46	FOXO	Costello syndrome
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FOXO50	FOXO	Costello syndrome
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FOXO52	FOXO	Costello syndrome
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FOXO55	FOXO	Costello syndrome
FOXO56	FOXO	Costello syndrome
FOXO57	FOXO	Costello syndrome
FOXO58	FOXO	Costello syndrome
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FOXO61	FOXO	Costello syndrome
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FOXO63	FOXO	Costello syndrome
FOXO64	FOXO	Costello syndrome
FOXO65	FOXO	Costello syndrome
FOXO66	FOXO	Costello syndrome
FOXO67	FOXO	Costello syndrome
FOXO68	FOXO	Costello syndrome
FOXO69	FOXO	Costello syndrome
FOXO70	FOXO	Costello syndrome
FOXO71	FOXO	Costello syndrome
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FOXO81	FOXO	Costello syndrome
FOXO82	FOXO	Costello syndrome
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FOXO93	FOXO	Costello syndrome
FOXO94	FOXO	Costello syndrome
FOXO95	FOXO	Costello syndrome
FOXO96	FOXO	Costello syndrome
FOXO97	FOXO	Costello syndrome
FOXO98	FOXO	Costello syndrome
FOXO99	FOXO	Costello syndrome
FOXO100	FOXO	Costello syndrome

Syndrome	OMIM#	Gene(s) Mutated <sup>a</sup>
Noonan syndrome	163950, 609942, 613706, 611553, 610733, 613224	<i>PTPN11</i> , <i>SOS1</i> , <i>RAF1</i> , <i>KRAS</i> , <i>NRAS</i> , <i>MEK1</i> , <i>BRAF</i> , <i>CBL</i>
Noonan syndrome-like disorder with loose anagen hair	607721	<i>SHOC2</i>
Cardiofaciocutaneous (CFC) syndrome	115150	<i>BRAF</i> , <i>MEK1</i> , <i>MEK2</i> , <i>KRAS</i> , <i>SOS1</i>
Costello syndrome	218040	<i>HRAS</i>
Gingival fibromatosis type 1	135300	<i>SOS1</i>
Neurofibromatosis type 1	162200	<i>NF1</i>
Legius syndrome	611431	<i>SPRED1</i>

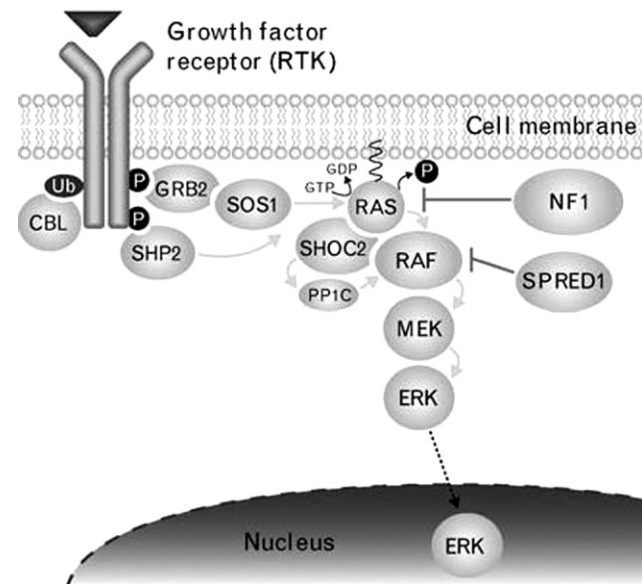
<sup>a</sup>**Bold:** Genes that are more frequently mutated in those syndromes.

Table adapted and modified from Zenker, M. Clinical Manifestations of Mutations in RAS and Related Intracellular Signal Transduction Factors. *Curr. Opin. Pediatr.* 2011, 23 (4), 443–451.

mutations were found in genes that encode proteins that repress the activity of the pathway. Many of these syndromes share some common but variable clinical features such as postnatal growth retardation, relative macrocephaly, learning or intellectual disabilities, congenital structural heart defects, skin lesions, and specific malignancies. Of note, germ line mutations that result in these syndromes appear to be “less activating” than somatic mutations found in tumors. The clinical manifestations

of these syndromes were recently reviewed by Tartaglia and Gelb (129) and Zenker (130).

MAPKs is a group of at least four distinct serine/threonine kinases: ERK1/ERK2 (also known as MAPK3/MAPK1 respectively), Jun amino-terminal kinases (JNK1/2/3—also known as MAPK8/9/10 respectively), p38 proteins (p38 $\alpha$ /p38 $\beta$ /p38 $\gamma$ /p38 $\delta$ —also known as MAPK14/11/12/13), and ERK5 (also known as MAPK7), each of which phosphorylates one or more distinct transcription factors; some transcription factors can be phosphorylated by different MAPKs. Each



**FIGURE 15-24** On recruitment to activated growth factor receptors, guanine nucleotide exchange factors such as SOS1 activate RAS through facilitating the exchange of GDP by GTP. Active GTP-bound RAS initiates the RAS-MAPK/ERK pathway, leading to the activation of ERK. RAS is converted back to its inactive state by hydrolysis of GTP to GDP through its intrinsic GTPase activity, which is augmented by GTPase-activating proteins such as neurofibromin (NF1). NF1 is thus a negative regulator of RAS signaling. SHOC2 and SPRED1 are positive and negative modulators, respectively, at the level of the RAS–RAF interaction. CBL protein is an E3 ubiquitin (Ub) ligase that can recognize tyrosine-phosphorylated substrates and hence negatively modulates RTK signal transduction. (Zenker, M. *Clinical Manifestations of Mutations in RAS and Related Intracellular Signal Transduction Factors*. *Curr. Opin. Pediatr.* 2011, 23 (4), 443–451.)

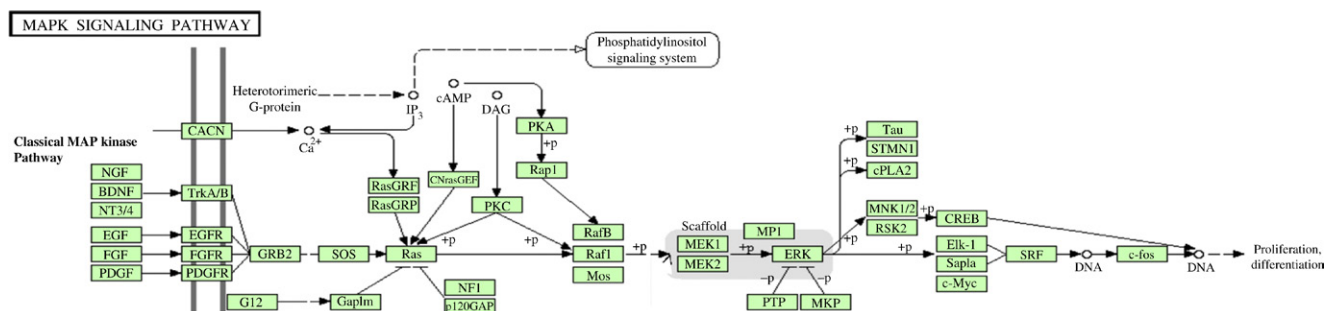
group of MAPKs has to be activated via phosphorylation by a specific MAPKK: MAPK/ERK kinases 1 and 2 (MEK1 and MEK2, official symbols: MAP2K1 and MAP2K2) both activate ERK1/ERK2, MAPKKs 3/6 (MKK3/6, official symbols: MAP2K3/6 respectively) activate the p38 proteins, MAPKKs 4/7 (MKK4/7, official symbols: MAP2K4/7 respectively) activate the JNK proteins, and MAPK/ERK kinase 5 (MKK5, official symbol: MAP2K5) activates ERK5. Each of these MAPKKs may be activated by one or more distinct MAPK kinase kinases; in the RAS-MAPK/ERK pathway, these would be the RAF family kinases such as BRAF and RAF1 (131–134).

The RAS-MAPK/ERK pathway is activated when specific ligands bind to one of the RTKs that mediates this pathway such as the neurotrophic tyrosine kinase receptor types 1 and 2 (also known as the “high-affinity nerve growth factor receptor” and “BDNF/NT-3 growth factors receptor,” respectively), EGFR, FGFR (fibroblast growth factor receptor), and PDGFR (platelet-derived growth factor receptor) (132). GRB2 then binds to specific tyrosine residues in the cytoplasmic component of these RTKs that have been phosphorylated by the activated tyrosine kinase domains. This leads to the recruitment and activation of SOS1, a guanine nucleotide-exchange factor. SOS1 catalyzes the conversion of GDP to GTP, thereby activating the RAS family of GTPases. Activated GTP-bound RAS then activates the RAF family of kinases that are the MAPKs in the RAS-MAPK/ERK signaling pathway. The activity of RAS is moderated by its own intrinsic GTPase activity which converts it back to the inactive GDP-bound form, and by repressors such as neurofibromin encoded by *NF1* (129,130). A simplified diagram of this pathway is shown in Figure 15-24.

A more comprehensive diagram illustrating the various ligands and receptors in the RAS-MAPK/ERK pathway is shown in Figure 15-25.

### 15.8.5 Homeobox Genes and Anterior–Posterior Patterning

The HOX gene families are members of the ANTP class in the homeobox superfamily of genes. The genes



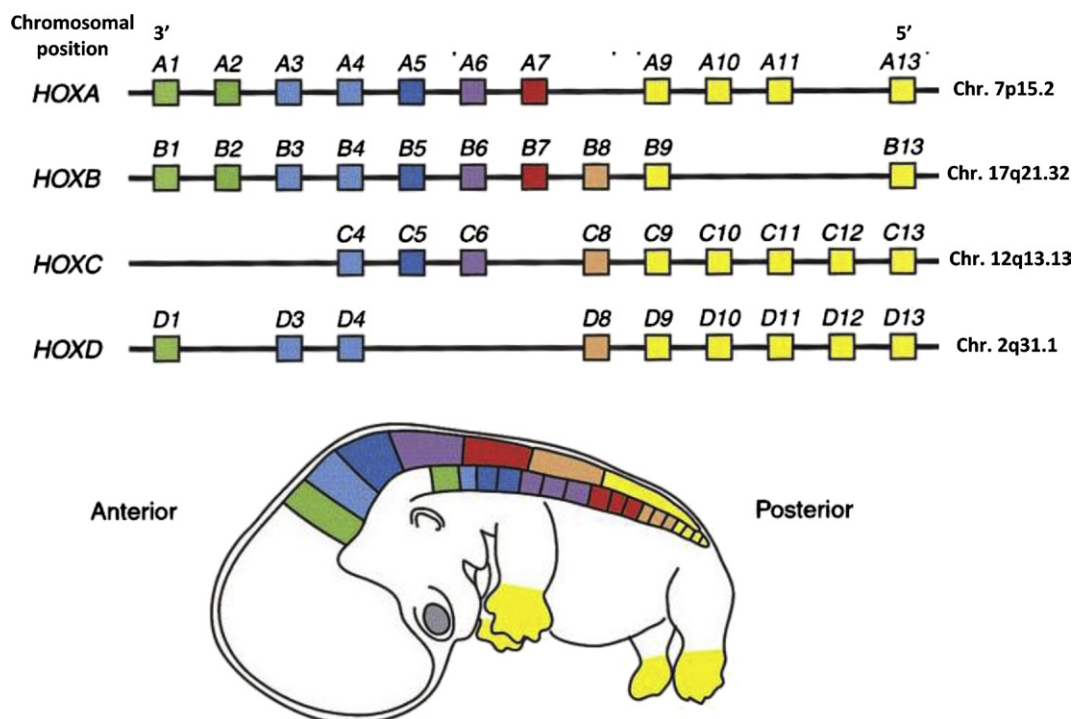
**FIGURE 15-25** Note: Interactions with the JNK and p38 MAPK pathways have been omitted for simplicity. (Kanehisa Laboratories (KEGG: Kyoto Encyclopedia of Genes and Genomes) MAPK signaling pathway: *Homo sapiens* (human), <http://www.genome.jp/kegg/pathway/hsa/hsa04010.html>, (accessed Jun 2, 2012).)



in the homeobox superfamily are characterized by the presence of a highly conserved 180bp sequence close to the 3' end of these genes; this sequence, known as the homeobox, encodes the homeodomain, which is a 60-amino acid DNA or mRNA-binding helix-turn-helix motif that can regulate transcription and translation by binding to homeobox responsive elements in DNA or mRNA (11,135). There are four clusters of HOX genes in humans (HOXA, HOXB, HOXC, and HOXD) due to genome duplication during evolution, resulting in a total of 39 HOX genes. The genes within each cluster lie close to one another within a given chromosomal segment. As described in Section 15.5 above, genes in the equivalent positions within each cluster are derived from the same common ancestral gene and therefore share very similar structures and functions (i.e. paralogous with one another across all four clusters), although none of the clusters contains every paralogous gene. There is some functional redundancy among members within each paralogous group. In humans, there are 13 paralogous HOX groups, classified into seven HOX gene families, namely HOX1, HOX2, HOX3, HOX4, HOX5, HOX6-8, and HOX9-13 (136,137). The HOX genes are partly responsible for determining the anterior–posterior axis in the developing embryo and regulate the formation of musculoskeletal structures in each body segment. Within each cluster, the HOX genes are arranged sequentially

such that the genes that are expressed earliest in development (e.g. HOXA1, HOXB1, and HOXD1) are at the 3' end while those that are expressed later in development (e.g. HOXA13, HOXB13, HOXC13, and HOXD13) are at the 5' end of the chromosomal strands. The HOX genes are shown in Figure 15-26, depicted in the order in which they are transcribed within each cluster and their chromosomal loci.

While the hindbrain is specified by the most anterior (and hence earliest expressing) HOX genes such as *HOXA1*, *HOXA2*, and *HOXB2*, the anterior–posterior and dorsal–ventral axes of the forebrain and midbrain are specified by the OTX (orthodenticle homolog) gene family, which is a member of the PRD class in the homeobox superfamily, and by the EMX (empty spiracles homolog) gene family, which is a member of the ANTP class in the homeobox superfamily, just like the HOX gene families. The OTX genes are expressed in both the forebrain and the midbrain before the EMX genes are expressed, with *OTX2* being the first of these four genes to be expressed, followed by *OTX1*; expression of these two genes is restricted to the forebrain and the midbrain. Both OTX genes are also expressed in the developing eye and possibly other sensory organs in the head. *EMX2* and *EMX1* are then expressed but only in the forebrain and in that order. In addition, *VAX1* and *VAX2* in the VAX (ventral anterior homeobox) gene family interact with *EMX2*



**FIGURE 15-26** The four clusters of HOX genes in mammals and the expression patterns (inferred from mouse expression studies) of the orthologous genes in a stage 19 human embryo. The colored fields depict the anterior-most domains of expression. The posterior boundaries of the expression domains may overlap in more caudal regions. Note the shift in the anterior expression boundaries between the nervous system and the segmented mesoderm, which nevertheless preserves the relative order of gene expression. Several of the posterior *HOXA* and *HOXD* genes are also expressed in the limb primordia, as indicated by the yellow coloration. (Veraksa, A.; Del Campo, M.; McGinnis, W. *Developmental Patterning Genes and their Conserved Functions: From Model Organisms to Humans*. Mol. Genet. Metab. 2000, 69 (2), 85–100.)



and *EMX1*, respectively; the *VAX* gene family is also a member of the ANTP class in the homeobox superfamily (139,140). Details of the other homeobox genes involved in the patterning of the forebrain have been reviewed by Wigle and Eisenstat (140).

Some HOX paralogous groups regulate the development of entire sclerotomal regions in the axial domain such as vertebrae, ribs, and muscles defined during somitogenesis. For example, the HOX paralogous group 9 (i.e. *HOXA9*, *HOXB9*, *HOXC9*, and *HOXD9*) is required for the development of “floating ribs”; the HOX paralogous groups 10 and 11 (i.e. *HOXA10*, *HOXC10*, *HOXD10* and *HOXA11*, *HOXC11*, *HOXD11*) are required for the proper formation of the sacrum. Some HOX genes in the anterior and central paralogous groups in conjunction with members of the CDX (caudal type homeobox) gene family comprising *CDX1*, *CDX2*, and *CDX4*, may stimulate the growth and hence length of the axial skeleton. This process may be mediated through the canonical Wnt signaling pathway. In contrast, members of the HOX paralogous group 13 (i.e. *HOXA13*, *HOXB13*, *HOXC13*, and *HOXD13*) can inhibit axial growth (141).

Some of the syndromes that result from mutations in the various homeobox genes described above are shown in Table 15-6, all of which are due to heterozygous mutations in the associated genes.

It should be noted that regulation of gene expression by homeobox genes is not the only mechanism for the determination of the anterior–posterior axis during embryonic development. A concentration gradient of specific molecules including FGF8 and RA that is highest in the posterior region and lowest in the anterior region

of the embryo also influences the proper development of the anterior–posterior axis (11).

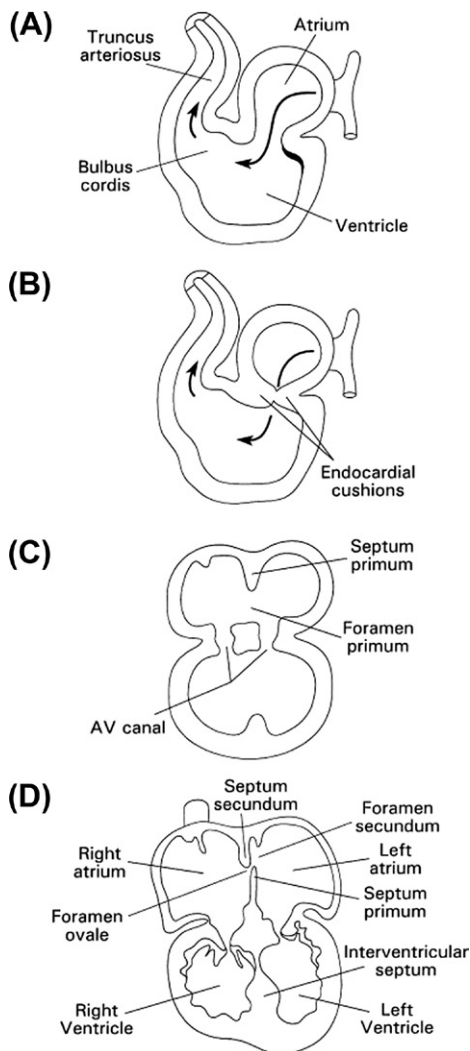
## 15.9 ORGANOGENESIS

### 15.9.1 Heart

A functioning cardiovascular system is required once the oxygenation needs of the embryo cannot be met because of the increasing size of the developing embryo. Although the neural crest provides some cells to the heart, the majority of the tissues of the heart are derived from mesoderm (142,143). The first step in the formation of the embryonic heart is the fusion of a pair of endocardial tubes derived from the splanchnic mesoderm, which starts to beat at around 22–23 days even before it has fully formed, making it the first functional organ in the embryo. As the tube enlarges, constrictions mark the division into the truncus arteriosus, bulbus cordis, ventricle, atrium, and sinus venosus from rostral to caudal. The bulbus cordis is incorporated into the mature right ventricle. The conus cordis (derived from the bulbus cordis), along with the truncus arteriosus, forms the outflow tracts of the ventricles. The sinus venosus receives venous blood. As the heart tube grows, it bends and forms the cardiac loop. The looping results in left–right asymmetry and in the different parts of the heart assuming their correct relative spatial orientations. The heart tube loops such that the cranial portion moves ventrally and to the right, while the atria move dorsally and to the left. The result is that the atrium and sinus venosus lie dorsal to the truncus arteriosus, bulbus cordis, and ventricle.

**TABLE 15-6 Some of the Syndromes Associated with Mutations in Homeobox Genes**

	OMIM# (Syndrome)	Gene Mutated	Major Tissues/Organs Involved
Bosley–Salih–Alorainy syndrome	601536	<i>HOXA1</i>	CN (Duane syndrome), ears in both syndromes; brain (developmental delay, central hypoventilation (hindbrain)) and heart especially in ABDS
Athabaskan brain stem dysgenesis syndrome (ABDS)			
Microtia, hearing impairment, and cleft palate	612290	<i>HOXA2</i>	Ears, mouth
Congenital vertical talus	192950	<i>HOXD10</i>	Feet
Radioulnar synostosis with amegakaryocytic thrombocytopenia	605432	<i>HOXA11</i>	Forearms, fingers, pelvis, bone marrow
Hand–Foot–Uterus (also known as Hand–Foot–Genital) syndrome	140000	<i>HOXA13</i>	Hands and feet (thumbs and big toes), external and internal genitalia, kidneys
Brachydactyly type D	113200	<i>HOXD13</i>	Thumbs and big toes
Brachydactyly type E1	113300	<i>HOXD13</i>	Fingers and toes
Brachydactyly–Syndactyly	610713	<i>HOXD13</i>	Hands and feet
Syndactyly type V	186300	<i>HOXD13</i>	Hands and feet
Synpolydactyly-1 (syndactyly type II) with or without foot anomalies	186000	<i>HOXD13</i>	Hands and feet
Microphthalmia with retinal dystrophy, optic nerve hypoplasia, pituitary dysfunction, developmental delay	610125	<i>OTX2</i>	Brain, CNs, eyes
Combined pituitary hormone deficiency	613986	<i>OTX2</i>	Pituitary gland
Schizencephaly	269160	<i>EMX2</i>	Brain



**FIGURE 15-27** Development and septation of the heart. (A and B) Sagittal sections of the heart. (C and D) Coronal sections of the heart. Arrows indicate the flow of blood through the heart.

The atrioventricular (AV) canal connects the common atrium and the early embryonic ventricle. The AV canal is divided by endocardial cushions that arise at the superior and inferior borders of the canal in the fourth week. The cushions also form the AV valves. The endocardial cushions grow across the AV canal and fuse, creating left and right AV canals. Regions of the endocardial cushions then fuse with both atrial and ventricular septa. If both atrial and ventricular septa and the medial AV valve leaflets are incomplete due to failure of fusion of the cushions, a persistent AV canal can result, as may sometimes be seen in association with Down syndrome.

In the atria, the septum primum, a thin membrane, grows from the dorsal roof of the common atrium toward the AV canal. Its forward edge creates an opening between the septum and the endocardial cushions, the ostium primum. As the ostium primum is being closed by fusion of the septum primum and the fused endocardial cushion tissue, perforations form in the septum primum by programmed cell death. The coalescence

of these holes forms the ostium secundum. A septum secundum then forms to the right of the septum primum, growing from the roof of the atrium, covering the ostium secundum. However, the septum secundum forms an incomplete division of the atria. The opening between the two septa is the foramen ovale. Defects in closure of the ostium primum or ostium secundum result in atrial septal defects. Ostium secundum defects are relatively common and can be due to defects in formation of the septum primum or the septum secundum.

The muscular interventricular septum is formed for the most part as a result of cavitation of the right and left ventricular chambers. Final closure of the ventricular septum is brought about by fusion of the muscular septum with endocardial cushion tissue. This tissue also fuses with the aorticopulmonary septum. This final region of ventricular septation is the membranous part of the interventricular septum. Ventricular septal defects are the most common congenital heart defect, and membranous defects are the most common form.

The truncus arteriosus divides to form the proximal portion of the aorta and pulmonary artery. The septum between these great vessels forms by fusion of bilateral ridges. Neural crest cells migrate from the pharyngeal arches to participate in the formation of this septum. Failure of the ridges to fuse results in persistent truncus arteriosus. This is usually associated with a ventricular septal defect. Unequal division of the outflow vessels can lead to tetralogy of Fallot, which is characterized by (i) right ventricular outflow obstruction, resulting from a narrow pulmonary orifice, (ii) ventricular septal defect, (iii) overriding aorta, and (iv) hypertrophy of the right ventricle (which is secondary to the right ventricular outflow obstruction). The genetic and epigenetic regulations of cardiogenesis have recently been reviewed by López-Sánchez et al. (144), van Weerd et al. (145), and Miquerol and Kelly (146). The development of the heart is shown in Figure 15-27.

### 15.9.2 GI Tract

The epithelial lining of the GI tract is derived from the endoderm, the connective tissue and smooth muscle are derived from the mesoderm, and the enteric nervous system is derived from the ectoderm. The primitive gut is defined by the oropharyngeal membrane rostrally and the cloacal membrane caudally. The mesodermally derived and endodermally derived components induce each other's development; the different functional portions of the GI tract (e.g. stomach, small intestine, large intestine) are partly due to regional differences in these interactions.

The foregut, midgut, and hindgut are each supplied by different blood vessels. The foregut gives rise to the pharynx, respiratory system, liver, esophagus, stomach, and proximal duodenum. The respiratory diverticulum forms from the ventral surface of the endodermal tube. A division forms between the developing esophagus and the trachea. An error in normal development of the tracheoesophageal

septum can lead to esophageal atresia. Diverticula that will form the liver, biliary apparatus, and pancreas arise from the distal portion of the foregut (147–149).

The midgut forms the distal part of the duodenum, the jejunum, the ileum, the cecum and appendix, the ascending colon, and the proximal two thirds of the transverse colon. The connection between the midgut and the yolk sac, the vitelline duct, is located within the umbilical cord. Rapid growth of the midgut forms a loop of gut. At the sixth week, the midgut then grows outside of the abdominal cavity into the umbilical cord and the yolk stalk. This physiologic herniation of the midgut is required for normal growth of the intestines as there is insufficient space in the abdominal cavity at this time to contain the growing intestines. During the 10th week, the midgut returns to the embryonic abdomen. Abnormalities in the retraction process resulting in persistence of the herniation of the midgut into the umbilical cord can result in omphalocele.

The hindgut gives rise to the distal one third of the transverse colon, the descending colon, the rectum, and the proximal portion of the anal canal. It also gives rise to the epithelium of the bladder and most of the urethra. The caudal part of the hindgut is continuous with the cloaca, which is divided by the urogenital septum into the urogenital sinus (which gives rise to the bladder and urethra) and the rectum. Incomplete septation of the hindgut can cause a persistent cloaca.

The endodermal tube is initially hollow. Many parts of it then become solid as the endodermal epithelium rapidly proliferates. Recanalization reopens the lumen by weeks 8–10. Stenosis or atresia of the GI tract can result from failure to recanalize, especially in the proximal duodenum. More caudal atresias or stenosis may be due to compromise of the vascular supply, resulting in narrowing of the lumen.

The distal foregut and the midgut undergo a series of rotations to reach their adult orientation. The stomach rotates such that the dorsal side forms the greater curvature while the ventral side forms the lesser curvature. Thus the original left side becomes ventral while the original right side becomes dorsal. Rotation of the stomach causes the duodenum to adopt a C-shaped loop. While the midgut loop is in the umbilical cord and outside of the abdominal cavity, it begins to rotate around the axis of the superior mesenteric artery. A total rotation of 270° counterclockwise occurs, and this rotation fixes the placement of the stomach, duodenum, and intestines. Abnormal rotation can result in twisting of the intestines (volvulus) and may compromise the blood supply to that region. The genes involved in the development of the intestines have been reviewed by Noah et al. (150).

### 15.9.3 Genitourinary System

The urinary and internal genital systems have a common origin, being derived primarily from the intermediate mesoderm (151). Growth of the mesoderm creates the urogenital ridge, which forms on either side of the dorsal aorta. It gives rise to the nephrogenic cord and

the genital ridge. Three successive pairs of kidneys form from this mesoderm. They are termed the pronephros, mesonephros, and metanephros. The pronephros is rudimentary. In addition to the mesonephric duct, the mesonephric kidney gives rise to the mesonephric tubules, which open into the mesonephric duct. The metanephros gives rise to the adult kidney. The permanent kidney begins to form at 5 weeks gestation and becomes functional toward the end of the first trimester. It arises from inductive interactions between the ureteric bud (a mesonephric duct diverticulum) and the metanephric cap (metanephric mesoderm). The cells of the cap form small vesicles, which give rise eventually to the nephron, the excretory unit of the kidney. The ureteric bud forms the collecting system of the kidney, as well as the ureters. The kidneys initially develop in the pelvic region ventral to the sacrum but assume a position in the abdomen by the ninth week. This “migration” is primarily due to growth of the abdomen and pelvis.

Between 4 and 7 weeks, the urorectal septum divides the cloaca into the anorectal canal and the urogenital sinus. The urogenital sinus gives rise to the urinary bladder and prostatic and membranous portions of the urethra and becomes connected to the rest of the urinary system.

The kidneys may be united at their lower poles, forming a single horseshoe kidney. Horseshoe kidneys do not properly ascend (pelvic kidney). Renal agenesis is due to degeneration of the ureteric bud. Without the bud, the metanephric cap does not develop and no nephrons are formed. The genes involved in the development of the kidneys have been reviewed by Potter et al. (152), and Song and Yosypiv recently reviewed the genetic bases of congenital renal malformations (153).

### 15.9.4 Central Nervous System

The human brain develops from a uniform-appearing pseudostratified neuroepithelium that is present after initial induction of the neural plate/tube. The understanding of brain development while extremely rudimentary, illustrates lessons that can be useful in diagnosing human genetic diseases. Brain development can be broken down to five basic steps: tissue/cellular specification, proliferation followed by final differentiation, neuronal migration, axonal outgrowth/target finding and finally synaptogenesis/network modification. Each of these processes will be discussed briefly and illustrated by examples in human disease when the processes go awry. Disorders that lead to structural disruption of the brain are easier to recognize and categorize clinically and hence it is easier to identify their underlying genetic causes. Genetic disorders that affect only cognition or behavior are generally more difficult to definitively categorize due to the relative lack of phenotypic heterogeneity. It is also more difficult to determine the pathophysiology of cognitive dysfunction because of the complexities of modeling these diseases in animals.

The nervous system can be divided into three categories, central, peripheral, and enteric nervous systems. Only

the central nervous system that includes the brain and the spinal cord will be discussed here (see (154,155) for more details on the peripheral nervous system and (156–158) for enteric nervous system). The understanding of human brain development is greatly facilitated by the study of model organisms, including mouse, rat, chick, *Drosophila*, *C. elegans*, and zebrafish. There are many similarities between the development of model organisms and the human brain, but there are key differences as well. Therefore, one should be wary of being too dogmatic when applying lessons from animal studies to human diseases.

The purpose of the brain is to allow an individual to perceive stimuli, interpret the information, and interact with the environment. Environmental information is received by sensory organs in the peripheral nervous system (except the retina, which is part of the central nervous system), relayed to the central nervous system, processed, and either a reflex action is made or information is interpreted and action taken. At a cellular and highly simplified level, information flows into a neuron through dendrites or the cell body at synapses as either a change in voltage potential via flow of ions across the plasma membrane or as a biochemical signal via second messengers. This information is summed in the cell body of a neuron and if a critical electrical depolarization threshold is reached, an electrical action potential is transmitted down the axon via flow of ions through voltage-sensitive channels. Neurons generally communicate from an axonal terminal to its target via chemical synapses. In addition to neurons, many other cells types in the brain play critical functions as well. Oligodendrocytes help increase the speed of an action potential down an axon and form the white matter of the brain. Astrocytes perform a myriad of critical support roles. Pathogenic oligodendrocytic and astrocytic functions result in numerous human diseases (see (159) for more details on basic concepts of neuronal activity and cellular function).

The brain is organized into different structures that can be separated into divisions along the neuroaxis from rostral (beak or nose) to caudal (tail) regions and the proximate timing of developmental events can be seen in Table 15-7 (160–164). The most rostral region includes the forebrain (prosencephalon, which is further subdivided into telencephalon and diencephalon), followed by the midbrain (mesencephalon), the hindbrain (rhombencephalon, which is composed of metencephalon and myelencephalon), and finally the spinal cord. Each anatomic region will be used to illustrate important lessons to understand the basic neuronal development as well as genetic diseases. Many developmental principals are used in more than one region. However, some developmental patterns or mechanisms appear to be unique to a single region.

**15.9.4.1 Spinal Cord.** The spinal cord is one the simplest structures within the central nervous system but understanding its development offers insights into the complexity of understanding brain development. Critically, it has the same basic dorsal–ventral pattern for its entire length, with motor neurons located toward and their axons

**TABLE 15-7** Timing of Human Brain Development

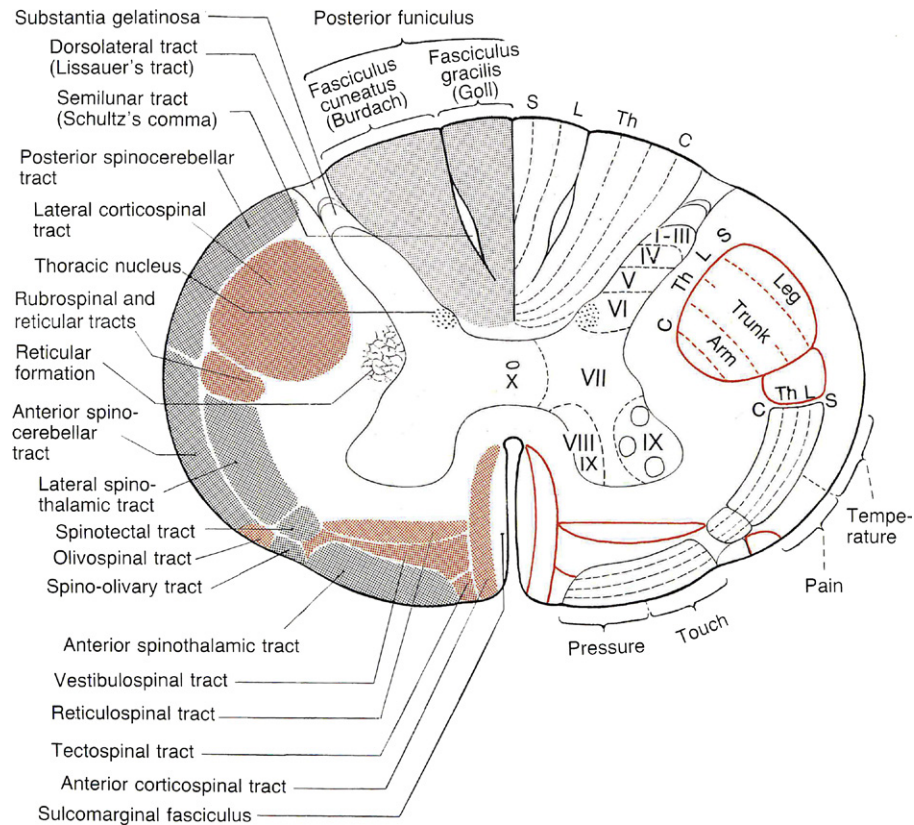
Event	Gestation Age
Neural plate forms	16th day
Longitudinal groove forms	20th day
Neural tube closure start at second to seventh somite	22nd day
Neuromeres start to form in hindbrain	22nd day
Forebrain induction	Third week
Cerebellum formation starts	Fourth week
Purkinje cells are born	5–6 weeks
Cerebral cortex start to expand	6 weeks
Ganglionic eminences form	6–7 weeks
Cerebral cortex preplate forms	6–7 weeks
Cortical plate (CP) forms	7–10 weeks
Cerebral cortical neurogenesis	7–18 weeks
Neuronal migration into CP (including GABAergic neurons)	7–28 weeks
Corpus colossal fiber start to cross midline	12 weeks
Myelination	
Most of cerebral cortex	First year of life
Cerebral commissures	First half of first decade
Intracortical association	Through second decade
Synapse dynamics (visual cortex)	
Formation	Third trimester to 5 years
Pruning	5 years to second decade

Adapted from Sidman, R. L.; Rakic, P. Development of the human central nervous system. In *Histology and histopathology of the nervous system*, Haymaker, W.; Adams, R. D., Eds.; Charles C Thomas: Springfield, IL, 1982; Vol. 1, pp 3–145; Huttenlocher, P. R., Morphometric study of human cerebral cortex development. *Neuropsychologia* 1990, 28 (6), 517–527; Rabinowicz, T.; de Courten-Myers, G. M.; Petetot, J. M.; Xi, G.; de los Reyes, E., Human cortex development: estimates of neuronal numbers indicate major loss late during gestation. *J. Neuropathol. Exp. Neurol.* 1996, 55 (3), 320–328; Yakovlev, P. I.; Lecours, A. R., The Myelogenic cycles of regional maturation of the brain. In *Regional Development of the Brain in Early Life*, Minkowski, A., Ed. Blackwell Scientific Publications: Oxford, 1967; pp 3–70; ten Donkelaar, H. J.; Lammens, M.; Wesseling, P.; Thijssen, H. O.; Renier, W. O., Development and developmental disorders of the human cerebellum. *J. Neurol.* 2003, 250 (9), 1025–1036.

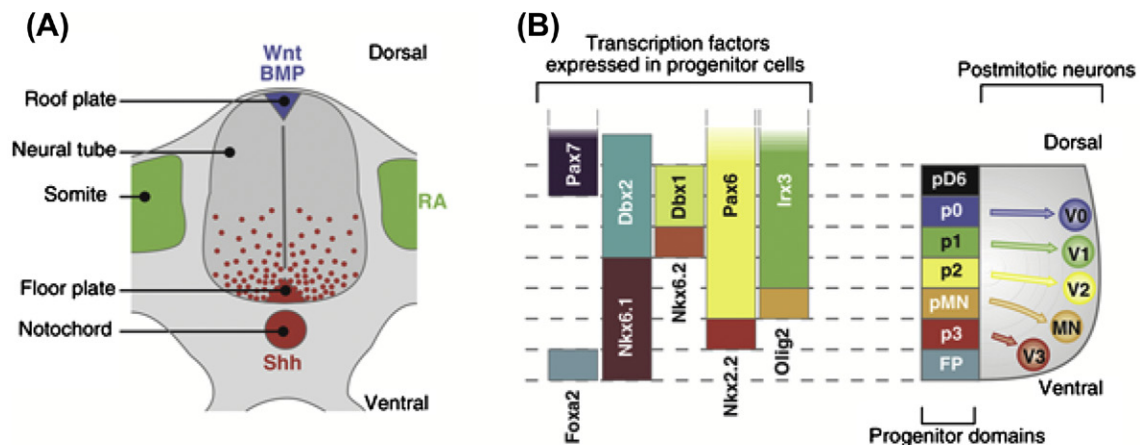
exiting through the ventral surface while sensory nerves enter the dorsal regions; a series of interneurons are present throughout the dorsal–ventral axis (Figure 15-28). SHH (see Section 15.8.1) is involved in the induction of the neural tube establishes a gradient within the developing spinal cord that forms a combinatorial code of transcription factors that produce the various cell types in the ventral region (Figure 15-29) (167–169). The factors produced in the roof plate (Wnt and BMP) may play a similar role for the dorsal progenitor cell types (169). It has also become clear that motor neurons at specific spinal cord levels along the rostral–caudal axis are predestined to target specific muscle groups, demonstrating neuronal specification in the rostral–caudal (170,171).

**15.9.4.2 Hindbrain.** Within the hindbrain resides the control of many basic functions including CN nuclei that help to control eye movements (CN III, IV, VI), facial sensation and muscles of mastication (CN V) derived from the first pharyngeal arch, muscles of facial movements





**FIGURE 15-28** A diagram of spinal cord showing localization of white matter tracts (not addressed here) and regions of neurons within the gray matter. The gray matter is subdivided into different regions (I–X) that have different functions. For instance, the motor neurons localized in IX innervate skeletal muscle. (DeArmond, S. J.; Fusco, M. M.; Dewey, M. M. *Structure of the Human Brain, a Photographic Atlas*. Oxford University Press: New York, 1974; Duus, P. *Topical Diagnosis in Neurology*, 3rd ed., Thieme: New York, 1998.)

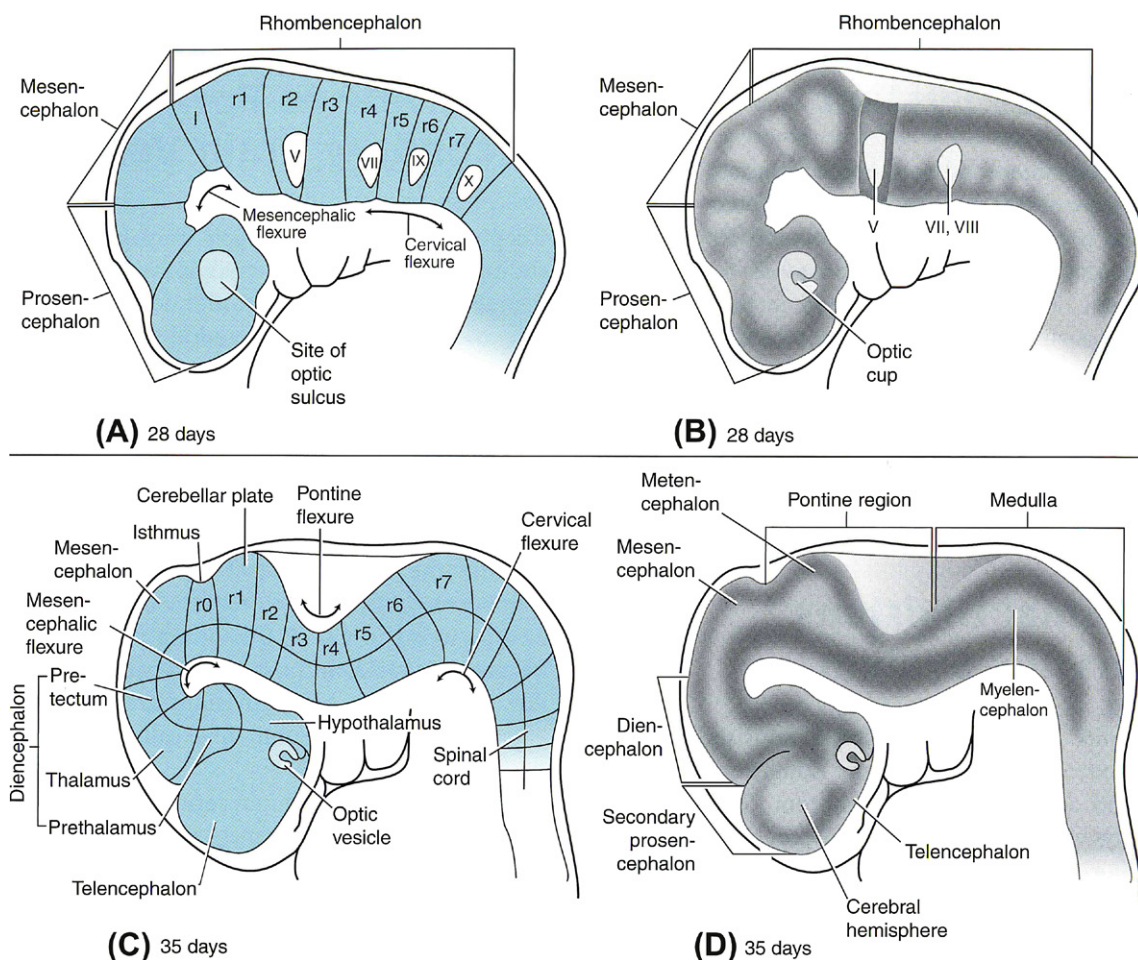


**FIGURE 15-29** (A) Schematic of spinal cord induction. Shh (red) is secreted by the notochord and floor plate (i.e. ventrally), retinoic acid (RA) (green) is secreted by the somites that flank the neural tube, and bone morphogenetic proteins (BMP) and Wnt gene family (blue) are produced at the roof plate dorsally. Shh establishes a gradient from ventral to dorsal within the ventral neural tube (red dots). This Shh gradient regulates the differential expression of specific transcription factors in neural progenitor cells within the ventral region of the developing neural tube, as shown in (B): *Pax7*, *Pax6* and *Irx3* are repressed by Shh signaling while *Dbx1*, *Dbx2*, *Nkx6.1*, *Olig2*, *Nkx2.2* and *Foxa2* require Shh signaling for their expression. (B) This combinatorial code of transcription factor expression establishes progenitor domains in the ventral region that will produce various interneuron cell types, V0–V3 or motor neurons (MN). A similar mechanism for regulating the expression of genes in the dorsal region may be mediated via Wnt and BMP (not shown). FP, floor plate. (Dessaud, E.; McMahon, A. P.; Briscoe, J. *Pattern Formation in the Vertebrate Neural Tube: A Sonic Hedgehog Morphogen-Regulated Transcriptional Network*. *Development* 2008, 135 (15), 2489–2503.)

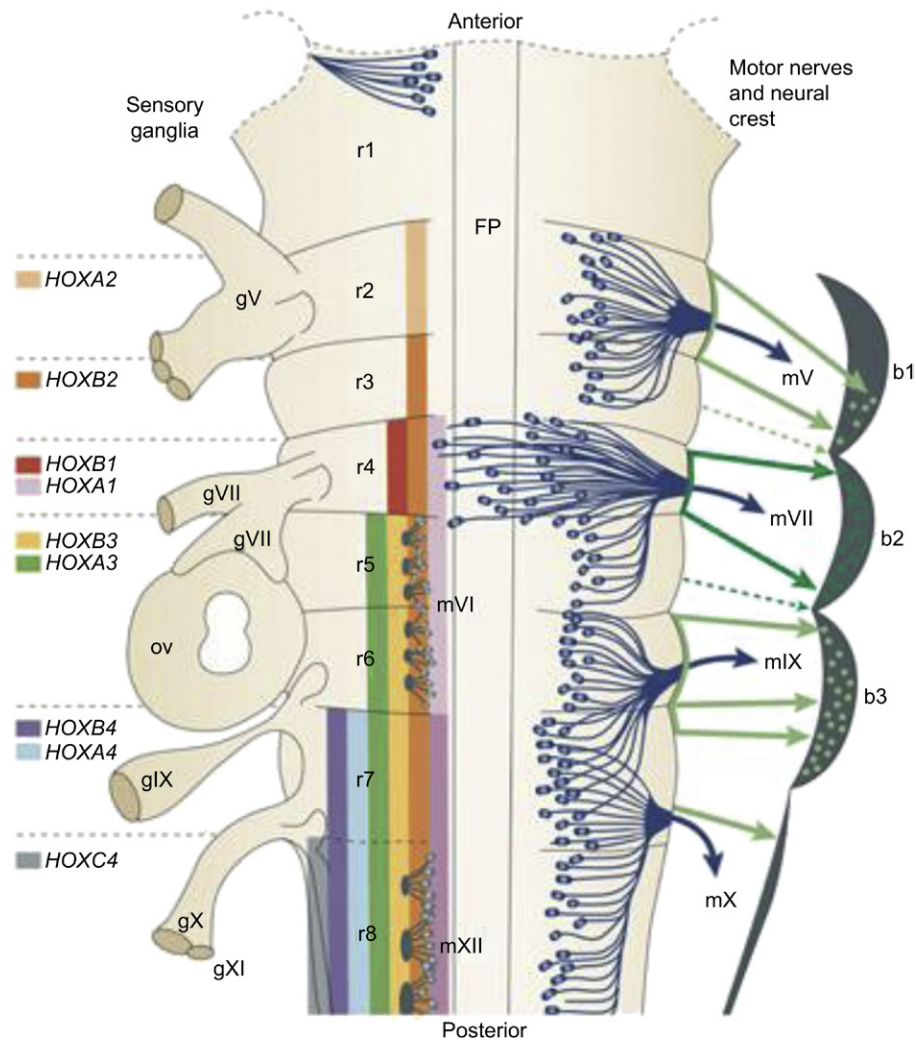
(CN VII) derived from the second pharyngeal arch, hearing and equilibrium (CN VIII), salivation and muscles of the pharynx (CN IX) derived from the third pharyngeal arch, pharynx and larynx (CN X) derived from the fourth pharyngeal arch, and tongue movements (CN XII). The hindbrain also regulates control of breathing, coordination of movements through cerebellar-related nuclei and many other functions (166). The earliest visible specification of the hindbrain is the formation of rhombomeres (neuromeres) (Figure 15-30), which is regulated by many processes including segmentation genes such as members of the Hox gene families (see Section 15.8.5). These transcription factors are expressed prior to the transient morphological appearance of rhombomeres. Instead of discussing the development of each brain stem nucleus, we will illustrate principles of brain stem development (see (172,173) for review of hindbrain development). Rhombomeres define anatomical segments within which

different cell types are born (Figure 15-31). Like the spinal cord, there are also ventral–dorsal differences. The most ventral region, known as the basal plate and nearest to the inducing notochord, produces motor neurons while the dorsal neural tube, known as the alar plate, produces sensory neurons. Not surprisingly, mutations of individual Hox genes such as HOXA1 can result in specific brain stem abnormalities (OMIM# 601536) (174). It is critical to note that while the Hox genes establish the initial basic patterning within the hindbrain, a network of transcription factors is required to define individual cell types (329).

**15.9.4.3 Cerebellum.** The adult cerebellum is three-layered structure comprising the molecular layer, Purkinje cell layer (PCL), and granule cell (i.e. granular) layer. (See Figure 15-32 for details.) It is derived from rhombomere 1 (r1 in Figure 15-30) by signals produced by the isthmus organizer at the midbrain–hindbrain junction (175). Purkinje cells are produced within the ventricular zone,



**FIGURE 15-30** Early embryonic brain development. The three primary brain vesicles: prosencephalon, mesencephalon, and rhombencephalon. (A) Shows rhombomeres (r) and where specific CNs will exit; “I” is the isthmus at the mesencephalon–rhombencephalon border. Note that the rhombomeres are only structurally visible temporarily, but within them gene expression and the cells they produce have long-lasting effects. (B) Morphological appearance of illustrations in (A). Note the presence of neuromeres corresponding to rhombomeres. The primary brain vesicles develop into five secondary vesicles: the diencephalon and telencephalon (from the prosencephalon), the enlarged mesencephalon, and the metencephalon and myelencephalon (from the rhombencephalon). The cerebral hemispheres appear and undergo rapid expansion. (D) Morphological appearance of illustration in (C). Note the neuromeres are no longer present, but functional segmentation of function has already occurred. (Schoenwolf, G. C.; Bleyl, S. B.; Brauer, P. R.; Francis-West, P. H. *Larsen’s Human Embryology*, 4th ed., Churchill Livingstone: Philadelphia, 2008.)



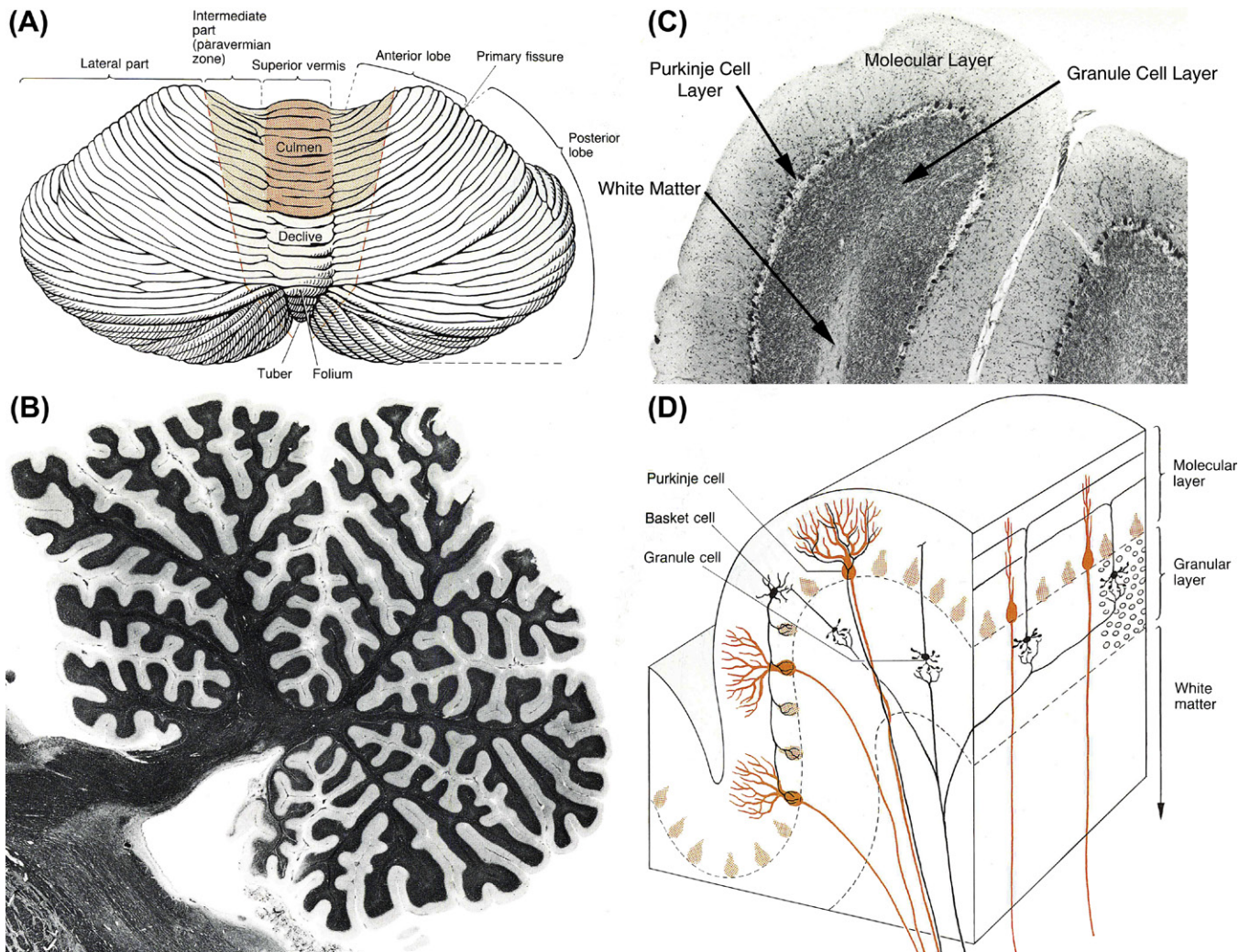
**FIGURE 15-31** Hindbrain and Hox gene expression. Illustration of segmental but overlapping Hox gene expression in the vertebrate (chick) hindbrain along with individual motor CN nuclei (prefixed by “m”) on the right and sensory CN nuclei (prefixed by “g”) on the left emerging from their respective rhombomeres (prefixed by “r”); “ov”—optic vesicle. Also shown are the projections of the motor CNs V, VII, and IX into the first, second, and third pharyngeal arches (b1, b2, and b3), respectively; CN X (vagus nerve) innervates a larger segment of the body. The neural crest derivatives (represented by green arrows) migrate and incorporate into specific pharyngeal arches. (Kiecker, C.; Lumsden, A. *Compartments and their Boundaries in Vertebrate Brain Development*. Nat. Rev. Neurosci. 2005, 6 (7), 553–564.)

likely in the fifth and sixth weeks of gestation. During development, r1 and the plane of the Purkinje cells that derive from it appear to turn 90° (Figure 15-33) (176). Granule cells are derived from the dorsal region known as the rhombic lip, grow over the top of the primordial cerebellum and proliferate through the first postnatal year in humans. Granule cell proliferation is driven by SHH derived from Purkinje cells and is required for the cerebellum to achieve its typical folia growth pattern. Interestingly, as discussed in the section on hedgehog signaling pathway, activating mutations in *PTCH1*, which encodes the receptor for the active hedgehog molecule HH-N, may result in medulloblastomas (178). The genetics of cerebellar development was recently reviewed by White and Stillitoe (179).

Studies of genetic conditions in both animal models and humans have provided specific insights into aspects

of cerebellar development and provided a basis to categorize cerebellar malformation diseases (180). Abnormal migration of Purkinje cells from the ventricular zone to the more dorsal regions disrupts the typical Purkinje cell monolayer and prevents SHH delivery to granule cells for proper proliferation (164,175,176). Mutations in the *reelin* gene (*RELN*), its receptors, or downstream signaling molecules can result in Purkinje cell migration phenotypes such as lissencephaly (OMIM# 257320) or cerebellar ataxia, mental retardation, and dysequilibrium syndrome (OMIM# 224050) (181–187). Mutations that induce the death of Purkinje cells produce a proportional loss of granule cells (188) not only due to the lack of Purkinje cell-produced SHH required for granule cell proliferation but because granule cells require synaptic connections to Purkinje cells for survival. Of note, the midline cerebellum (vermis) during development appears





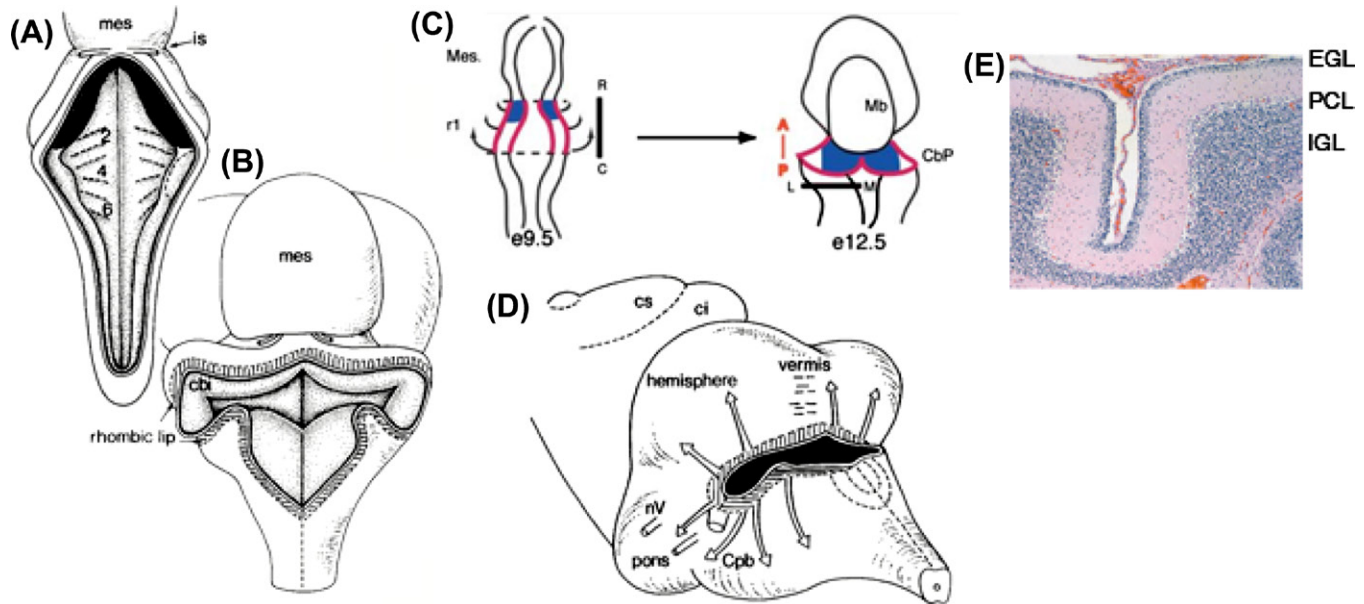
**FIGURE 15-32** (A) A schematic of the human cerebellum as looking at the back of the brain. In the horizontal plane, the cerebellum is divided into the vermis at midline, the lateral lobes more laterally and the paravermian region in between. The midline structures are more critical for control of the axial body and the lateral lobes control the limbs. (B) A sagittal section from the lateral lobes of the cerebellum. The darkly stained areas are both the cell-dense granular layer and the white matter tracts. The molecular layer is the light gray area on the surface of the cerebellum. (C) Higher resolution of individual folia with a cell body stain. The molecular layer contains the unmyelinated axons of the granule cells known as parallel fibers as well as Purkinje cell dendrites that parallel fibers synapse upon. Under the molecular layer is the monolayer of Purkinje cells (large cell bodies). (D) A schematic showing the axis of the Purkinje cell dendrites and the other layers of the cerebellum. (DeArmond, S. J.; Fusco, M. M.; Dewey, M. M. *Structure of the Human Brain, a Photographic Atlas*. Oxford University Press: New York, 1974; Duus, P. *Topical Diagnosis in Neurology*, 3rd ed., Thieme: New York, 1998.)

smaller in size than other regions of the cerebellum (Figure 15-33) but it eventually becomes prominent (Figure 15-32). It has been speculated that either Purkinje cells must migrate more medially or midline Purkinje cells proliferate more to account for the prominence of the vermis. Regardless of the mechanism, vermal hypoplasia with relative sparing of cerebellar hemispheres is found in multiple syndromes including Joubert syndrome (OMIM# 213300) and Dandy-Walker syndrome (OMIM# 220200). General cerebellar hypoplasia is a common feature of many syndromes as well (OMIM# 300486, 300749, 607596, 277470, 604382, 601427). Since granule cell proliferation and cerebellar development continue through the first years of life, it is sometimes difficult to determine if a small cerebellum is due

to developmental abnormalities or from degeneration after normal development of the cerebellum has been completed.

**15.9.4.4 Forebrain.** The forebrain is induced by a complex interaction of multiple factors (189–192). The anterior border of the neuroectoderm and ectoderm, termed the anterior neural ridge (ANR), secretes multiple FGFs, with FGF8 being the best studied (Figure 15-34). These factors induce expression of SHH (see Section 15.8.1) from the non-neuronal axial midline structures and the prechordal plate (ventral to the neuroectoderm). In mice, Shh is required to induce ventral forebrain components and helps to establish the expression of key transcription factors (193–195). Disruption of SHH or many of the downstream SHH signaling molecules result in





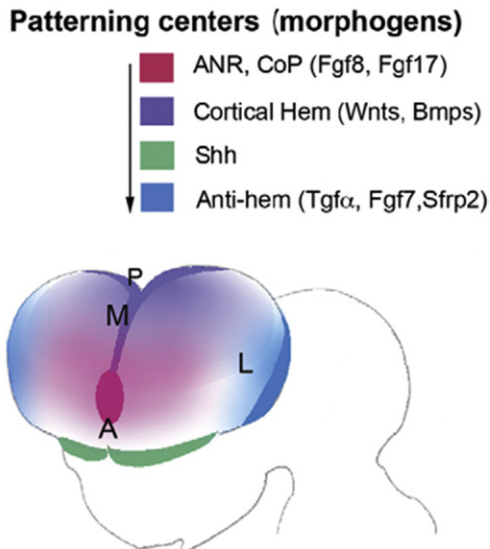
**FIGURE 15-33** Cerebellar morphogenesis. (A) The cerebellum is derived from r1 (shown in black). Also shown are the mesencephalon (mes), isthmus (is), and r2, r4 and r6. Note the V-shaped primordial cerebellum. (B) The cerebellum (cb) is a horizontally oriented structure by about 13 weeks gestation. (C) Fate mapping studies show that r1 rotates 90° on its axis with the lateral portions moving more than medial portions (blue engrailed 2 transcription factors as a marker). (D) The rhombic lip produces multiple cell types including cerebellar granule cells, several pontine nuclei and the inferior olive. The granule cells migrate over the surface of the primordial cerebellum (arrows) while the other cell types migrate to their proper locations. (E) Section from postnatal human cerebellum. Granule cells proliferate in the external granule cell layer (EGL). As they stop dividing and differentiate into neurons in the EGL, granule cells extend axons to synapse with Purkinje cell dendrites. Granule cell bodies migrate through the molecular layer and PCL into the internal granule cell layer (IGL). (ten Donkelaar, H. J.; Lammens, M.; Wesseling, P.; Thijssen, H. O.; Renier, W. O. *Development and developmental disorders of the human cerebellum*. J. Neurol. 2003, 250 (9), 1025–1036; Sgaier, S. K.; Millet, S.; Villanueva, M. P.; Berenshteyn, F.; Song, C.; Joyner, A. L. *Morphogenetic and Cellular Movements that Shape the Mouse Cerebellum; Insights from Genetic Fate Mapping*. Neuron 2005, 45 (1), 27–40; Prayson, R. A., *Neuropathology*; Elsevier: Philadelphia, PA, 2005.)

holoprosencephaly (192). Holoprosencephaly is a failure of forebrain induction that results in incomplete separation of the forebrain with variable severity and may be associated with multiple craniofacial abnormalities, most dramatically cyclopia. It can be caused by both genetic and environmental factors that likely reflect multiple pathways associated with forebrain development (192,196).

The cerebral cortex has a highly ordered structure (see Figure 15-35, adult). A small column of cells in cerebral cortex is thought to form a functional unit. The cerebral cortex has two major classes of neurons, named after their predominate neurotransmitters. Glutamatergic neurons (glutamate as the neurotransmitter) are excitatory and many project long distances whereas GABAergic (GABA as the neurotransmitter) neurons are inhibitory and project locally. The cerebral cortical glutamatergic neurons are all produced within the ventricular region of the cerebral cortex. However, in mice, cerebral cortical GABAergic neurons migrate tangentially from the ganglionic eminences (future basal ganglia) (Figure 15-35) (197). In contrast, human GABAergic neurons of the cerebral cortex are derived from both the ganglionic eminences and the cerebral cortex (198,199).

Our understanding of the developing brain is generally based on animals model, rodents being the most

studied. However, the rodent cerebral cortex lacks gyri and is much smaller than other mammals. It is increasingly clear that there appears to be critical differences between mouse and human development and some of these differences will be illustrated below. The cerebral cortex, like the rest of the neural axis, starts as a single layer of pseudostratified epithelium (Figure 15-35, panel 1). Initially, cells proliferate symmetrically with every cell division producing two additional progenitors, increasing the number of cells exponentially. The nuclei move up and down within the pseudostratified epithelium replicating their DNA toward the pial surface and mitosis occurring at the ventricular surface (200). The preplate contains the first neurons of the cerebral cortex and lies between the neuroepithelium (ventricular zone) and the pial surface (Figure 15-35, panel 2) (199). Interestingly, preplate cells are critical for development but later die or play little to no role in the function of the mature cerebral cortex. The Cajal–Retzius cells of the preplate express a large extracellular protein, reelin (encoded by *RELN*), that is critical for proper neuronal migration, the loss of which can result in a form of lissencephaly with microcephaly and thickened cortex (OMIM# 257320) (181,185); these cells subsequently form the marginal zone (MZ). Subplate cells play a role in axonal guidance (201). Within the ventricular zone,



**FIGURE 15-34** There is a complex balance between factors that induce the forebrain and cause specific regions to develop properly. Fgf8 and other FGF proteins are expressed by the ANR. FGF causes the induction of Shh in the prechordal floor plate that later induces Shh in the ventral forebrain. SHH is required for the continued expression of FGFs from the ANR. In the dorsal forebrain, Wnts and Bmps induce dorsal regionalization in a structure called the cortical hem. The anti-hem is a region near the border of the lateral cerebral cortex and the lateral ganglion eminence (border between dorsal and ventral telencephalon). This region expresses several proteins including Tgfa, Fgf7, and Sfrp2 that are functionally antagonistic to the hem-expressed proteins. In mice, the balance of secreted factors leads to the expression gradients that appear to be required for regionalization of functional domains of the cerebral cortex. (O’Leary, D. D.; Sahara, S. *Genetic Regulation of a Realization of the Neocortex*. *Curr. Opin. Neurobiol.* 2008, 18 (1), 90–100.)

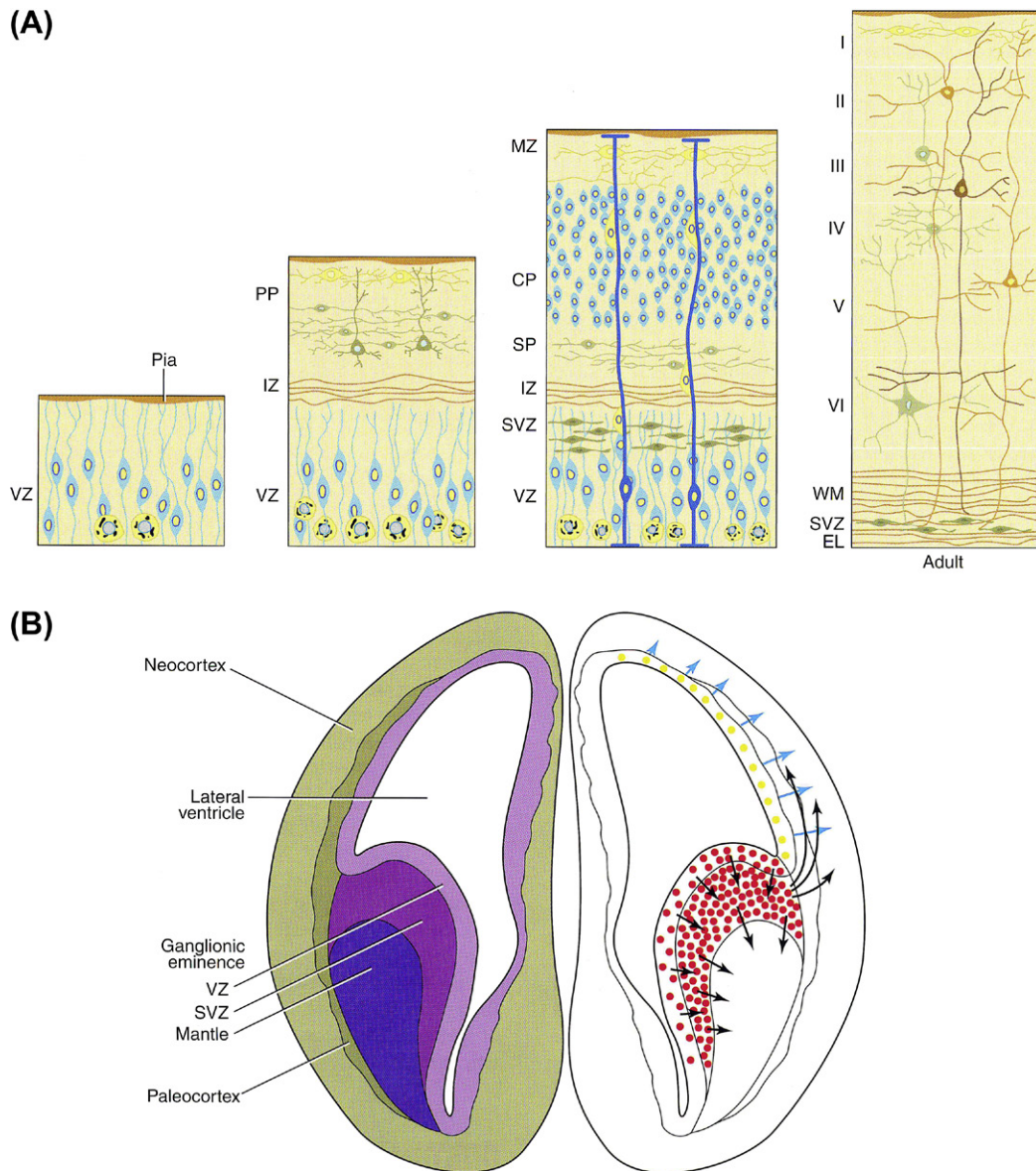
after a period of symmetric cell division and exponential increase in the number of cells, some cells will begin to differentiate into neurons. While many cells continue to expand the ventricular zone by dividing symmetrically, some divide asymmetrically to produce one progenitor and one differentiated neuron (202–204). Cells that differentiate into neurons migrate away from the ventricular zone and split the preplate into the MZ (future layer I) that contains the Cajal–Retzius cells and the subplate with the subplate neurons (Figure 15-35, panel 3). In addition, some neuronally committed cells migrate away from the ventricular surface to form the (inner) subventricular (SV) layer and begin to change their expression profile (205,206). These cells then generally go through one additional round of proliferation to produce differentiated neurons. In addition, it now appears that mammals with gyri and larger brains have additional sources of dividing cells that are uncommon in mice. The outer SV zone appears to have extensive proliferative capacity and is thought to be an important source of cells in mammals with larger brains (207–209) in contrast to the inner SV zone cells that appear to divide only once before terminally differentiating into neurons. After multiple cell symmetric divisions producing more precursors,

outer SV zone cells differentiate, but the extent of their role for producing neurons in gyric mammals remains to be definitively determined (Figure 15-36).

**15.9.4.5 Congenital Structural Brain Malformations.** Microcephaly may be caused by developmental or degenerative genetic diseases, or injuries to the brain from vascular insults, infection, trauma, or other causes. Only developmental microcephalies are addressed in this chapter. Developmental microcephaly has many different genetic etiologies (210–212). Since the cerebral cortex is the largest structure in the human brain, it is almost always affected in these conditions. Microcephaly vera (true microcephaly) is defined as microcephaly in the absence of other neurological findings except intellectual disability. Mutations in eight different genes have been found to be associated with microcephaly vera: *MCPH1* (213), *ASPM* (214), *CDK5RAP2* (215), *CENPJ* (215), *STIL* (216), *WDR62* (217–219), *CEP152* (220), and *CEP63* (221). All of these genes encode proteins associated with the centrosome, but they do not appear to have the same role in centriole duplication or centrosome function (222,223).

Since microcephaly vera has a “small brain” as its only phenotype, it is a useful model for understanding developmental microcephaly (Figure 15-37) (330). Moreover, microcephaly vera is associated with a reasonably normal body size, suggesting that the centrosomal-related mechanism leading to deficient production of neurons is largely brain specific. Some microcephaly vera genes have been shown to increase the amount of asymmetric cell divisions in mouse models within the ventricular zone producing more neurons prematurely as opposed to symmetric cell divisions which produce more precursors (226–228). Excessive asymmetric cell division early in development can result in a deficiency of precursors in late neurogenesis (204). This mechanism may also be responsible for conditions with neuronal migration abnormalities since the centrosome is also important for neuronal migration (229,230). Of note, the effects of mutations in these microcephaly genes on the outer SV zone is unknown since mice, the most common genetic model organism for cerebral cortical development, are largely deficient in this cell type (207–209).

Microcephaly is also associated with abnormalities in DNA repair (231). The phenotypes for these conditions, in addition to microcephaly, include somatic growth retardation (Seckel syndrome 1 and 2: OMIM# 210600, 606744) (232), somatic growth retardation and immunodeficiency (Nijmegen breakage syndrome: OMIM# 251260 (233–235) and LIG4 syndrome: OMIM# 606593 (236)), and microcephaly with seizures (early infantile epileptic encephalopathy-10: OMIM# 613402) (237). Studies in mouse models suggest that microcephaly in these syndromes is likely due to neuronal progenitors cell death shortly after differentiation (231,238–240). The reasons for developing neurons to be so sensitive to abnormalities in DNA repair remains a mystery.

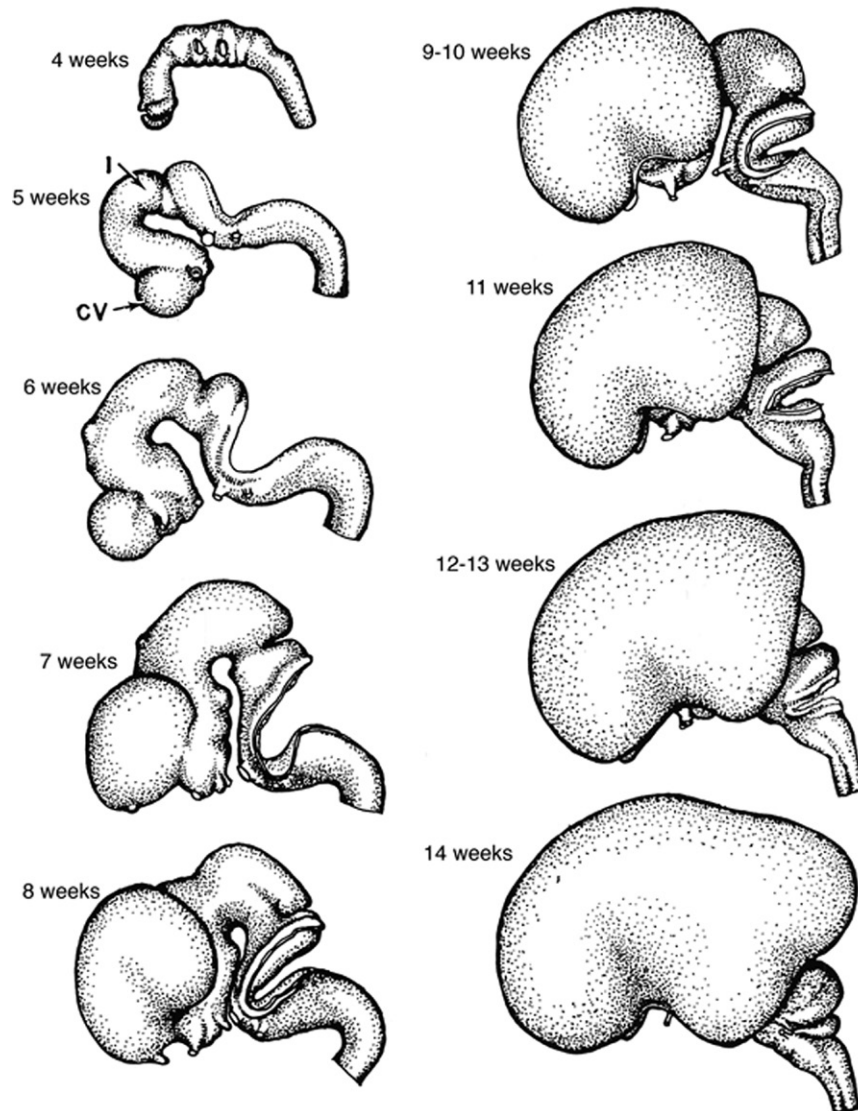


**FIGURE 15-35** (A) Different stages of cerebral cortical development. *Panel 1:* Pseudostratified neuroepithelium with cells having connections at the pial and ventricular surfaces; this structure can be considered the ventricular zone (VZ). *Panel 2:* Preplate (PP) is formed from both Cajal–Retzius and subplate neurons. Intermediate zone (IZ) is in between preplate and ventricular zone. *Panel 3:* The SV zone contains two different types of precursors, cells with either limited (inner SV zone cells) or extensive (outer SV zone cells) proliferation capacity. Differentiated neurons form the CP, from which the future neurons of the cerebral cortex arise, by splitting the preplate into the MZ containing Cajal–Retzius cells and the subplate (SP). Most neurons migrating from the ventricular region to the CP use radial glial (long dark blue cells) processes, many of which are processes of ventricular zone cells connected to the pial surface. *Panel 4 (Adult):* The layers of the cerebral cortex are distinguished by the glutamatergic neurons. The top layer closest to the meninges or pia surface (layer I) is cell sparse and includes dendrites and axons. Layers II and III contain small- to medium-sized neurons that generally send axons to different parts of the cerebral cortex. Layer IV receives axonal projections from the thalamus and send axons to other layers generally within its own functional unit. Layer V consists of very large neurons and projects to distant structures such as the brain stem and the spinal cord while layer VI projects to the thalamus. Below layer VI are myelinated white matter tracts containing axons projecting both in and out cerebral cortex as well as intracortical projections. (B) Diagram of coronal section of embryonic mammalian forebrain during development. Glutamatergic neurons of the cerebral cortex are derived from cerebral cortical ventricular and migrate radially (light blue arrows). Many GABAergic neurons are derived from the ganglionic eminences and migrate tangentially into the cerebral cortex. (Schoenwolf, G. C.; Bleyl, S. B.; Brauer, P. R.; Francis-West, P. H. *Larsen's Human Embryology*, 4th ed., Churchill Livingstone: Philadelphia, 2008.)

As stated above, glutamatergic neurons are born within both the ventricular and the SV zones of the developing cerebral cortex while GABAergic neurons migrate in from the ganglionic eminences or from the SV zone. This illustrates that the final destination of differentiated

neurons is not generally where they proliferate and begin their differentiation, so neuronal migration is an important process in brain development. There are two broad categories of neuronal migration, radial and tangential. Radial migration is in the direction of orientation of the





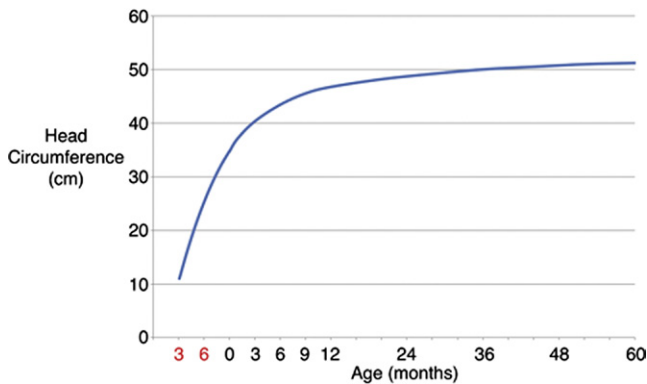
**FIGURE 15-36** Drawings of human brain development from 4 weeks to 14 weeks gestation ("I" is the isthmus and "CV" is the cerebral vesicle). Note that the cerebral vesicle starts at the most rostral end of the neural axis. However, it grows and eventually covers the more caudal segments including the diencephalon (thalamus and hypothalamus), mesencephalon (midbrain), and occipital regions, and it eventually rests above of the hindbrain. The images are not scaled to each other (160). (F. Hochstetter, *Beitrage Zur Entwicklungsgeschichte Des Menschlichen Gehirns I*, Deuticke, Vienna, 1919.)

undifferentiated precursors and often uses processes of those precursors (radial glia, long blue cells in panel 3 [Figure 15-35A](#) and light blue arrows in [Figure 15-35B](#)) as a substrate to "climb" on ([241,242](#)); tangential migration occurs as neurons migrate perpendicular to the plane of the progenitors ([Figure 15-35B](#): black arrows into developing neocortex) ([197](#)). Differentiating glutamatergic neurons migrate from where they were "born," which is close to the ventricular zone, toward the pial surface, arresting in the cortical plate (CP) forming the future neurons of the cerebral cortex ([243,244](#)). As neurons continue to differentiate, newly "born" neurons migrate away from the ventricular zone regions past all previously born and reside below layer I ([Figure 15-38](#)). This means that cerebral cortex is "born" inside out,

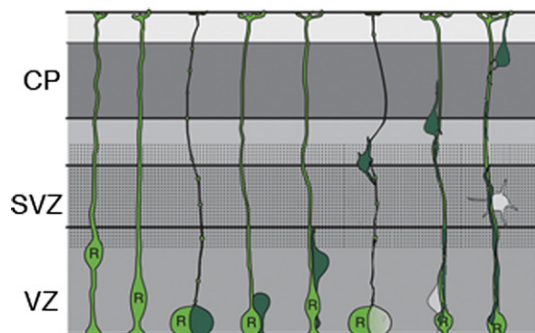
with the deepest neurons of layer VI being born first and the latest born neurons in Layer II.

There are many different recognizable abnormalities in neuronal migration that are frequently associated with epilepsy ([246,247](#)). These abnormalities are seen on neuroimaging as gray matter (neurons) where they do not belong or abnormalities in the structure of the cortex. Neuronal migration abnormalities can occur because of genetic or environmental insults during critical aspects of neuronal migration ([248](#)). Neurons can be found at the ventricular surface where they are born in the form of periventricular heterotopia (OMIM# [300049](#), [608097](#)) ([249,250](#)). Periventricular heterotopia is caused by abnormalities in the initiation of neuronal migration, which may be due to a disruption of the ventricular surface





**FIGURE 15-37** Pre- and postnatal brain growth from about 13 weeks post-fertilization to 60 months after being born at full term (prenatal ages in red and postnatal ages in black). Note that while the microcephaly vera phenotype is very likely to be secondary to deficiencies in proliferation of neurons of the cerebral cortex, these neurons have completed proliferation at the start of the curve above (Table 15-7). Therefore, all the brain growth that is seen on this curve is due to other processes such as glia proliferation, synapse formation, myelination, etc. However, these processes are dependent on the number of neurons generated by 18 weeks gestation. The growth curve is a mathematic composite of fetal (224) and childhood (225) data and is intended for illustrative purposes, not for clinical use.



**FIGURE 15-38** Detailed schematic of neuronal migration: Nuclei move up and down within the ventricular zone with precursors (radial glial cells, light green). Asymmetric cell division occurs when a differentiated neuron (dark green cell) initiates migration to CP utilizing radial glia. Asymmetric cell division occurs again and a white cell is produced that moves to the SV zone and assumes a different morphology before dividing again. The dark green cell releases itself from the radial glial process after receiving the putative stop signal at or near the pial surface. (Kriegstein, A. R.; Noctor, S. C. *Patterns of Neuronal Migration in the Embryonic Cortex*. Trends Neurosci 2004, 27 (7), 392–399.)

close to where neuroblasts proliferate (251). A disruption in neuronal migration can also result in lissencephaly (smooth brain) in which neurons lack their normal organization within the cerebral cortex (185,252–257). An interesting example is *DCX*, an X-linked gene that causes lissencephaly in males and a double cortex (i.e. a normally formed cortex at the proper location and a subcortical band of abnormally migrated neurons) in females (OMIM# 300067) (253–255). This gender difference in phenotype arose because disruption of the only copy of

their *DCX* gene on their single X chromosome in males causes all neurons to migrate improperly, while random X-inactivation in females results in properly migrating neurons when the X chromosome carrying the mutant gene is inactivated and improper migration only if the non-mutant X chromosome is inactivated. Many genes associated with lissencephaly syndromes encode for proteins involved in centrosomal or microtubule activity, and as discussed above, are often associated with microcephaly as well (258,259). Cobblestone lissencephaly, that is, a smooth brain with some bumps upon the surface, is due to over-migration of neurons past their normal arresting point and onto the pial surface of the cerebral cortex (259). This is caused by mutations in genes that result in glycosylation defects (260,261). In addition to cobblestone lissencephaly, these mutations also result in congenital muscular dystrophy and various structural ophthalmologic abnormalities. This group of conditions is now known as “congenital muscular dystrophy–dystroglycanopathy with brain and eye anomalies” (OMIM# 236670, 613150, 253280, 253800, 613153, 613154, 614643). Another recognizable pattern of brain malformation is polymicrogyria, which can be due to a variety of genetic and environmental etiologies (262). One genetic form of polymicrogyria that is associated with an over-migration phenomenon resulting in a cobblestone-like lissencephaly in a mouse model is bilateral frontoparietal polymicrogyria (OMIM# 606854) due to mutations in *GPR56* (263). Since schizencephaly is often associated with polymicrogyria, it is possible that these two phenomena share similar pathogenesis, but schizencephaly is also associated with holoprosencephaly (264).

**15.9.4.6 Development of Synapses and Axonal Path Finding.** The remaining aspects of neuronal development will only be addressed in a cursory manner. This is not because they are not important, but because their disruption is often only associated with intellectual disability or other cognitive dysfunction, which makes clinical classification and therefore genetic characterization challenging. Furthermore, establishing the cellular mechanism of cognitive dysfunction in animal models is generally more difficult for intellectual than it is for structural abnormalities because structural pathophysiological correlates between human and mouse brains often appear more convincing than behavioral correlates of cognitive dysfunction. Shortly after differentiation and sometime during migration, neurons extend their axons to reach their desired target. There are many different cues that each neuron must take to find their proper target (159). Once axons reach their targets, synapses are formed. There is considerable communication between pre- and post-synaptic structures that strengthen or weaken synapses based on the firing characteristics of each cell and synapse type. Ion channels determine the electrical characteristics of a neuron. Most neurons are also thought to require a functional connection with an appropriate target from which they will receive trophic factors

that are required for their survival, as seen in granule cells and Purkinje cells in the cerebellum. This has been hypothesized to foster a Darwinian-like competition between neurons for the best connections. Neurons that are unable to make proper connections to targets during development due to missing targeting cues, less efficient connections with targets, or lack of targets die through programmed cell death (265). Many synapses are made early in childhood; however, these synapses continue to be created, lost, strengthened, or weakened overtime as more experience is accumulated (all these processes are reviewed in more detail in Purves et al. 2012 (159)).

Axonal path finding, neuronal firing characteristics, and synaptic plasticity are thought to be associated with various cognitive defects. However, due to the dearth of definitive genetic evidence for these mechanisms in conditions such as autism or psychiatric illnesses and the difficulties in modeling human cognitive impairments in animal models, these hypotheses are generally difficult to prove conclusively. However, some epilepsy syndromes have been found to be associated with mutations in a single gene (266–269). Since ion channel genes regulate action potentials, it should not be surprising that epilepsy is frequently associated with mutations in these genes, although mutations in genes that control the formation of brain structures often result in epilepsy as well (269). An example of a gene associated with intellectual disability and neuronal path finding abnormalities is MASA (mental retardation, aphasia, shuffling gait, and adducted thumbs) syndrome, also known as spastic paraplegia-1 due to mutations in an X-linked gene, *L1CAM* (OMIM# 303350) (270). This is allelic with X-linked hydrocephalus (OMIM# 307000) and the acronym CRASH (corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraplegia, and hydrocephalus) syndrome describes the range of anomalies that can be seen in individuals with *L1CAM* mutations. The identification of the underlying pathophysiology in cognitive disorders can be critical and potentially fruitful. For example, Fragile X syndrome (OMIM# 300624) is a syndromic form of intellectual disability that is not associated with gross structural brain abnormalities but has been shown to have synaptic changes (271). This has led to the investigation of potential therapeutic interventions based on our knowledge of its neuropathophysiology (272). There is hope that as we learn more about the genetic etiology and developmental pathways in various neurodevelopmental disorders, we will be able to develop targeted therapies for these disorders.

### 15.9.5 Limbs

Higher vertebrates have two sets of distinguishable paired limbs: forelimbs and hindlimbs. Although the precise form of the limbs varies between species, they share a common morphologic pattern (273,274). Each limb is composed of three major components, from proximal to

distal: the stylopod, zeugopod, and autopod. In humans, the stylopod is a single proximal long bone (the humerus in the arm and the femur in the leg), the zeugopod is a middle pair of long bones (ulna and radius in the arm, tibia and fibula in the leg), and the autopod are the carpal (hand) or tarsal (foot) bones, with the phalanges of the digits being most distal. The ordered development of the limb must produce correct orientation in three planes: proximal–distal (shoulder to fingers), anterior–posterior (thumb to little finger), and dorsal–ventral (back of hand to palm). How these axes are established and maintained depends on the precise coordination of multiple signaling molecules and transcription factors. The lateral plate mesoderm gives rise to the bone, cartilage, and tendons while the somitic mesoderm gives rise to the muscle and neurovascular bundle (nerve and blood vessels) of the vertebrate limb.

Initiation of the outgrowth of limb buds involves members of the FGF family. *Fgf10* is expressed in the lateral plate mesoderm prior to the onset of limb budding. As the bud arises, an apical ridge that expresses *Fgf8* forms in the ectoderm overlying the bud (the apical ectodermal ridge (AER)) (275–277). The forelimb and hindlimb are morphologically quite distinct. The signals specifying whether a forelimb or a hindlimb forms and the position of the limb relative to the rest of the body appear to correlate with the expression of the genes in the Hox9 paralogous group (278). In addition, the T-box (*Tbx*) genes, which encode another family of transcription factors, are differentially regulated in forelimb versus hindlimb (279–282). *Tbx4* is expressed in the hindlimb, while *Tbx5* is expressed in the forelimb. *Pitx1*, which can induce *Tbx4* expression, encodes a bicoid-like transcription factor and is also expressed in the early hindlimb. Expression of *Pitx1* ectopically in the forelimb can induce hindlimb identity while the *Pitx1* knockout mouse has abnormalities of the hindlimb (283–285).

**15.9.5.1 Longitudinal Growth of the Limb: The Proximal–Distal Axis.** The AER, a specialized region of ectoderm, is essential for the outgrowth of the limb from the bud. It forms at the tip of the limb bud along its dorsal–ventral boundary. The position and maintenance of the AER appear to depend on signals from the underlying mesenchyme. The AER itself is also the source of secreted factors that allow for longitudinal outgrowth of the limb. Excision of the AER causes the limb to stop growing distally, resulting in a truncation defect (i.e. loss of distal structures) (286,287). The structures that are lost depend on the timing of the removal of the AER. If the AER is removed early, the limb is severely truncated, while later removal results in loss only of more distal structures. At least three FGFs are known to be expressed in the AER: *FGF4* posteriorly, and *FGF2* and *FGF8* throughout the ridge (288–291). The progress zone is a region of rapidly proliferating undifferentiated mesenchyme that lies beneath the AER. This region specifies the proximal–distal axis in the developing limb.

Cells in the AER differentiate into their final cell type while cells in the ZPA are kept undifferentiated.

The exact mechanisms through which the proximal–distal axis in the limbs is established have remained controversial and many models have been suggested (292). In the “Progress Zone Model,” the proximal–distal identity of the bone is determined by the amount of time that a given cell has spent in the progress zone under the influence of permissive FGF (293,294). If a cell has spent only a short amount of time in the zone, a proximal bone (e.g. humerus) will form; conversely, cells that exit from the zone last and have spent the most time in the zone will form distal elements (e.g. distal phalanges). This model assumes that cells somehow measure the time spent in the progress zone and thus obtain their positional identity, but this model has been challenged (295). A second model is the “Early Specification Model” in which the expression of genes that regulate limb specification early in development result in the formation of “small stripes across the early limb bud” (292), each with its unique specific limb identity. With proliferation, these stripes would subsequently increase in size and form the appropriate limb component that had been specified (294). However, neither of these models is supported by experimental evidence and many other models have been suggested, one of which is a “dynamic expression” approach that attempts to integrate the various signals that arise from the flank, the AER, and the developing limb itself (292).

In chick models of limb development, it has been shown that dissociated cells from the distal limb bud that are exposed to RA can be “reprogrammed” to develop proximal limb elements, that is, adopt a “proximal identity” (296). Moreover, this proximal re-specification can be inhibited by RA antagonists but can be promoted by FGF receptor antagonists (297), which suggests that RA and FGF have diametrically opposite effects on proximal specification in the developing limb. However, some recent studies have casted doubts on the role of RA in proximal–distal limb patterning and some authors have suggested that RA facilitates “remote signaling” but is not required for the local patterning of the limb bud (297).

FGFs from the AER regulate the expression of many genes involved in the outgrowth of the developing limb bud. We will discuss the functions of four of these genes as examples of the genetic control of limb development: *MEIS1*, *HOXA11*, and *HOXA13* in the stylopod, zeugopod, and autopod, respectively, and *AP2* in the undifferentiated zone that is responsive to AER/FGF signaling. The spatial and temporal expression of these genes is illustrated in Figure 15-39.

The marker for stylopod (*MEIS1*) and the marker for the undifferentiated zone (*AP2*) are initially co-expressed in the earliest developing limb bud, which in the chick would be at developmental stage 17. It is believed that *MEIS1* expression is induced by RA signaling from the

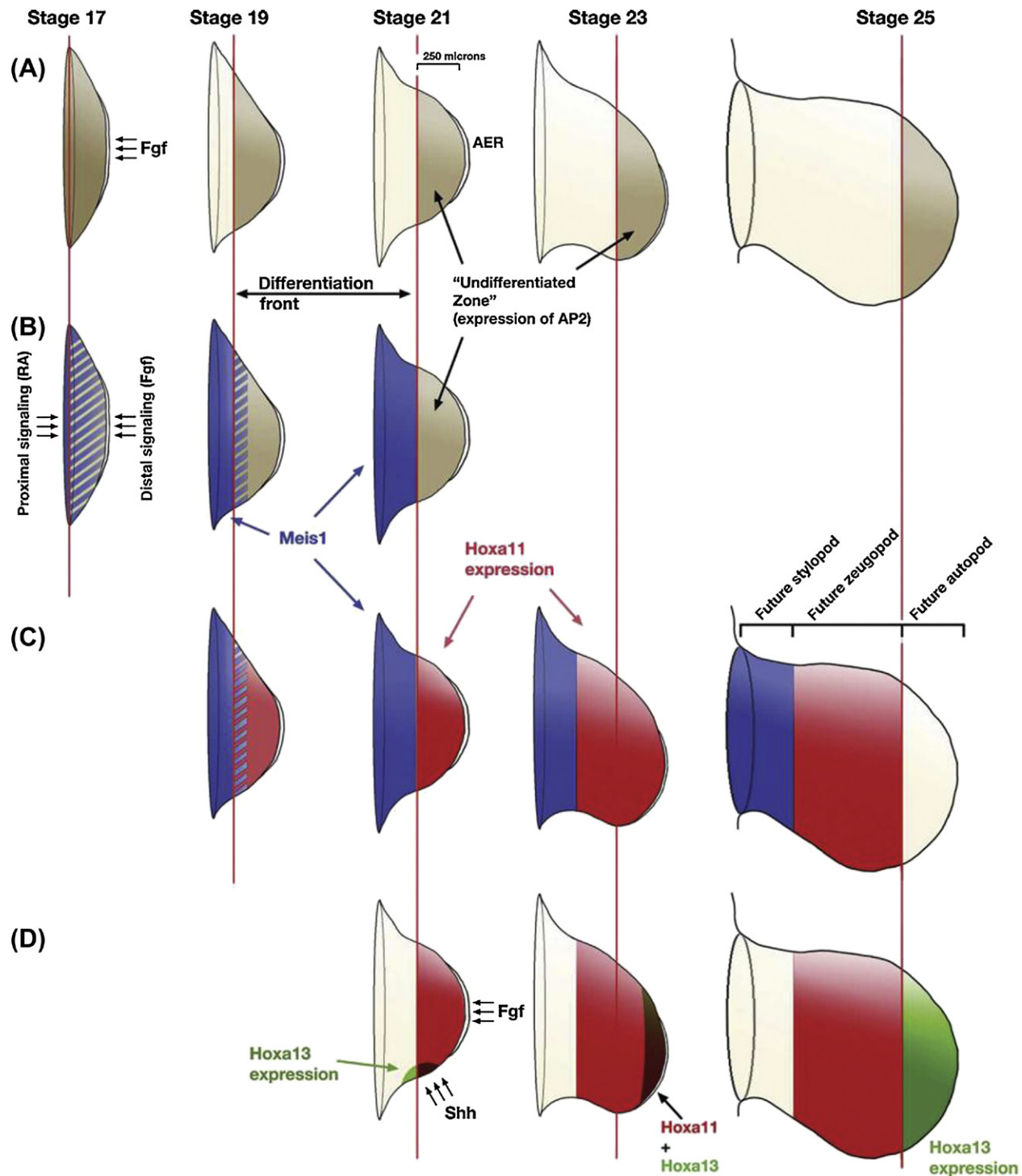
flank (i.e. proximal signaling) while *AP2* expression is induced by FGF signaling from the AER (i.e. distal signaling). Due to the tiny size of the limb bud at this early stage of development, it is conceivable that all the cells in the limb bud are exposed to both the proximal and the distal signals. As the limb bud increases in size, expression of *MEIS1* becomes restricted to the proximal region while expression of *AP2* becomes restricted to the most distal region of the limb bud. Expression of *HOXA11* begins in the chick embryo at developmental stage 19 as the regions that express *MEIS1* and *AP2* become increasingly distinct; *HOXA11* begins to fill the void created by this separation even before the *MEIS1* and *AP2*-expressing regions are completely distinct. Subsequently, *HOXA13* is expressed in the region distal to the *HOXA11*-expressing region. Although there is initially overlap between the adjacent regions, these regions are distinct by the time of differentiation (292).

Differentiation of the different regions of the limb bud to their corresponding musculoskeletal structure is partly dependent on the proper spatial and temporal expression of the *HOXD* cluster of genes, particularly *HOXD9* to *HOXD13*, as shown in Figures 15-40 and 15-41.

The specification of forelimbs versus hindlimbs is determined partly by the expression of specific T-box (*Tbx*) genes, which encode transcription factors—*TBX4* is expressed only in the hindlimbs while *TBX5* is expressed only in the forelimbs (298). As such, mutations in *TBX4* result in the small patella syndrome (OMIM# 147891), which is associated with hypoplastic or absent patella as well as structural pelvic abnormalities and mild anomalies in the feet (increased gap between the first and second toes). In contrast, mutations in *TBX5* result in Holt–Oram syndrome (OMIM# 142900), which is associated with structural heart defects (usually atrial or ventricular septal defects), cardiac conduction defects (usually AV blocks), and malformations of the upper limbs.

**15.9.5.2 Dorsal–Ventral Specification.** Signaling by the dorsal and ventral ectoderm of the early limb bud regulates dorsal–ventral axis specification (299). Dorsal identity is controlled at least in part by *Wnt7a*, and null mutations in the mouse result in ventralization of distal dorsal aspects of the limb (300). *Wnt7a* induces expression of *Lmx1* in the underlying mesenchyme. *Lmx1b* mutation results in partial loss of dorsal structures (301). Ectopic expression of either *Wnt7a* or the LIM homeobox gene *Lmx1* in the chick can induce ventral tissues to become dorsal, resulting in a mirror-image dorsal limb (302,303). The homeobox gene *Engrailed-1* (*En1*) appears to be important for ventral specification. Loss of *En1* expression causes dorsal specification of the ventral limb with ventral expansion of the dorsal markers *Wnt7a* and *Lmx1*, resulting in a double dorsal limb (304,305). However, in the *Wnt7a* knockout, *En1* expression is not altered (300). This implies that limb cells have a ventral identity unless signaled by *Wnt7a*



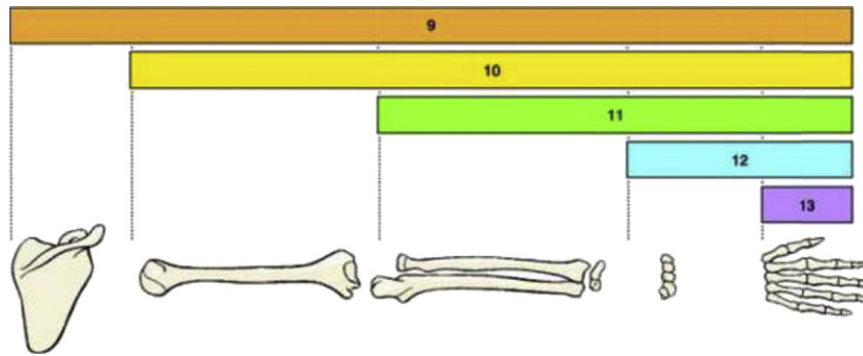


**FIGURE 15-39** (A) Approximate map of the undifferentiated zone in the distal 250  $\mu\text{m}$  of the limb bud (tan) at various stages of limb development in the chick. (B) Comparison of the domains of expression of *MEIS1* (a stylopod marker; blue) and *AP2* (an undifferentiated zone marker; tan) at different stages of limb development. (C) Comparison of the domains of expression of *MEIS1* (a stylopod marker; blue) and *HOXA11* (a zeugopod marker; red) at different stages of limb development. (D) Comparison of the domains of expression of *HOXA11* (a zeugopod marker; red) and *HOXA13* (an autopod marker; green) at different stages of limb development. *HOXA11* and *HOXA13* expression overlap at early stages of their expression (brown). (Tabin, C.; Wolpert, L. Rethinking the Proximodistal Axis of the Vertebrate Limb in the Molecular Era. *Genes. Dev.* 2007, 21 (12), 1433–1442.)

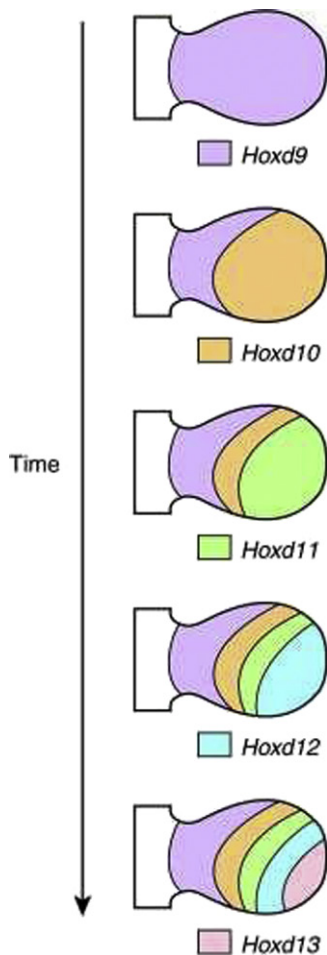
or *Lmx1* to acquire a dorsal fate. *En1* preserves ventral identity by suppression of *Wnt7a* expression in the ectoderm and subsequently *Lmx1* expression is confined to the dorsal limb mesenchyme. In addition, *En1* seems

to play a role in restricting the AER to the apex of the bud (305,306). Haploinsufficiency of *LMX1B* results in nail-patella syndrome (OMIM# 602575), characterized by abnormalities in the nails, knees, elbows, and pelvis





**FIGURE 15-40** Expression of HOXD9 to HOXD13 regulate the development of specific bones in the upper limbs. (Schoenwolf, G. C.; Bleyl, S. B.; Brauer, P. R.; Francis-West, P. H. *Larsen's Human Embryology*, 4th ed., Churchill Livingstone: Philadelphia, 2008.)



**FIGURE 15-41** Diagram showing the expression of HOXD9 to HOXD13 in the developing limb bud overtime—proximal end on the left and distal end of the limb bud on the right. (Schoenwolf, G. C.; Bleyl, S. B.; Brauer, P. R.; Francis-West, P. H. *Larsen's Human Embryology*, 4th ed., Churchill Livingstone: Philadelphia, 2008.)

(iliac horns). These clinical features are consistent with the findings described above since the nail and patella are dorsal structures in the limbs.

**15.9.5.3 Anterior–Posterior Patterning.** In humans, the anterior digit is the first digit, or thumb, and the posterior digit is the fifth digit. Anterior–posterior patterning

is controlled by the ZPA. The ZPA confers posterior identity on limb mesenchyme cells, with the cells closest to the ZPA acquiring the most posterior fate. Transplantation of an ectopic ZPA to the anterior margin of the chick limb bud results in mirror-image duplication of the digits. That is, the normal 2-3-4 pattern of digits is replaced by a 4-3-2-2-3-4 pattern. This pattern is presumably the result of cells at a greater distance from the ZPA being exposed to a lower concentration of a morphogen than those closer to the ZPA. The concentration of the morphogen reflects the distance from the ZPA. The implication is that the specification of which particular digit will form at a given site is dependent on a certain threshold for activation. This classical model explains why mirror-image duplications would result from application of a second ectopic ZPA, as there would be two sources of the morphogen. This model would also predict that application of an increased amount of the morphogen at a given position would induce more posterior development. When two ZPAs are grafted onto a wing bud, one at the apex and one at the anterior portion, only the more posterior digits are formed (307).

As described earlier, the properties of the ZPA suggest the presence of a morphogen gradient, and the soluble factor SHH has been shown to play a role. The effect of the ZPA in determining digit identity in the chick can be mimicked by the application of Shh (308). Shh signaling is dose dependent, as would be predicted for a morphogen. Chick limbs exposed to beads soaked in high concentrations of Shh specify a more posterior digit, while progressively lower concentrations induce correspondingly more anterior digits. However, in that series of experiments, it was found that no digit was induced for 12–16 h after the initial exposure to SHH, but three wing digits (d2, d3 d4) were subsequently induced over a period of 8 h (309). This led to a revision of the classical model of anterior–posterior patterning known as the promotion-morphogen gradient model in which both the concentration and the duration of exposure to a morphogen are critical for the specification of digits (310). Other models of anterior–posterior patterning (specifically digit determination) that have been proposed based

on mouse and chick models include the temporal-spatial gradient model, which incorporates the cumulative amount of SHH that the target cells are exposed to and the temporal gradient of the exposure; and the biphasic model in which SHH plays an early and “transitory” role in determining digit identities but is required throughout the development of the limb to ensure that there are sufficient “digital precursor cells” (310). The failure to establish digit identity is often associated with oligodactyly (i.e. missing digits). Hence, the generation of digital identity and the expansion of the digital plate are closely related.

Application of RA to the anterior margin can also induce mirror-image duplication and mimic ZPA signaling (311–313). It has subsequently been shown that RA activates Shh (308). Application of RA inhibitors results in loss of Shh expression in the posterior limb bud (314,315). Thus, the anterior-posterior axis patterning effects of RA could be mediated by Shh, and Shh may be the morphogen that specifies position across that axis of the limb. RA can also induce expression of *Hoxb8* in the limb (316), the ectopic expression of which can result in ectopic Shh expression in the mouse (317). *Hoxb8* is normally expressed in the lateral plate mesoderm, with an anterior expression boundary at the posterior region of the forelimb bud in chick and mouse (316–318). Thus, it appears that *Hoxb8* may specify the position of the ZPA in the lateral plate mesoderm. Activation of expression of regulatory genes at the 5' region of the *Hoxa* and *Hoxd* clusters is controlled by patterning cues, and the patterns of expression map out different regions of the limb bud (319). At least 23 different Hox genes are expressed during chick limb development (320). *Hoxa* and *Hoxd* are expressed in both the forelimb and the hindlimb. Expression of the Hox genes is very dynamic.

Maintenance of Shh expression and the ZPA can be regulated by *Fgf4* from the AER (321,322). Shh also maintains *Fgf4* expression in the AER. In this way, proximal-distal patterning and anterior-posterior patterning are linked. Mutant mice null for *Shh* have proximal structures in the limbs but not distal ones, indicating that Shh plays a role in maintaining proximal-distal outgrowth but not in initiating limb growth. Although *Fgf4* can maintain *Shh* expression in the AER, *Fgf4* may not be required as evidenced by a conditional knockout of *Fgf4* that still had *Shh* expression in the AER (323). It is possible that there is functional redundancy between different Fgf family members.

The bone morphogenetic protein-2 gene (*Bmp2*) is expressed in a pattern overlapping *Shh* expression (308,324). *Bmp2* can induce *Fgf4* expression anteriorly in the AER (325,326). Shh can induce *Bmp2* expression, and *Bmp7* expression is induced in a dose-dependent fashion (309). BMP signaling may be quite complex as at least *Bmp2*, *Bmp7*, and *Bmp4* are expressed in the limb, and the molecules may also form heterodimers.

The genetics of human limb malformations was recently reviewed by Zuniga et al. (327). A recent review by Biesecker (328) listed 310 genetic conditions known to be associated with polydactyly, 290 of which were considered “syndromic” (i.e. associated with other malformations). Of these 310 genetic conditions, the causative genes have been identified in only 80 of these conditions, which suggest that much remains to be learned about the genetics of limb development and malformation.

## 15.10 CONCLUSION

Human development requires the precise regulation of a large but finite number of genes in the different developing tissues and organs, both in terms of where the genes are expressed and when they are expressed. Malformations in one or more organ systems occur when these genes are mutated and thereby disrupting the evolutionary-conserved sequence of development.

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### Biographies



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# CHAPTER

# 16

## Twins and Twinning

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### 16.1 INTRODUCTION

Humans have always been fascinated by twins. They are the makings of legends and “old wives’ tales.” Mythology is full of tales of twins. Romulus and Remus, said to be the founders of Rome, were twins who were supposedly nursed by a she-wolf. The two brightest stars in the Gemini constellation, Castor and Pollux, are named after a pair of mythologic twins. Conjoined or “Siamese” twins have also fascinated humans and used to be part of circuses and sideshows, where they were displayed as oddities or “freaks of nature.” The most famous pair of conjoined twins were Chang and Eng from Siam (hence the term Siamese twins), who were exhibited in an American circus for many years.

Throughout history, different cultures have had different attitudes regarding twins. In some, the birth of twins was thought to be good luck and to predict times of abundance, giving the family a special place in the community. In other cultures, the birth of twins was thought to be a bad omen, and both the twins and the mother were often rejected by the community or even killed. With time, as scientific and medical knowledge have increased, the opportunity to study similarities and differences within twin pairs has led to numerous scientific advances (1–3).

### 16.2 DETERMINING ZYGOSITY

Twins can be either dizygotic (DZ) (also called “fraternal”) or monozygotic (“identical”). DZ twins are the result of two different ova fertilized by two different sperm growing and developing at the same time in the same uterus. MZ twins are the result of one ovum fertilized by one sperm that divides to form two embryos (4,5). In the past, the only way of differentiating between

MZ and DZ twins at birth was their sex and appearance. If twins were of unlike sex, they were said to be DZ, whereas if they were like-sexed and looked identical, they were said to be MZ (5). Although not always definitive, placentation was another means of establishing zygosity (4). Other methods that have been used to differentiate between MZ and DZ twins include blood types, blood protein polymorphisms, human leukocyte antigen (HLA) typing, and dermatoglyphic studies that document fingerprints, palm prints and creases. More recently, DNA analyses have been used to establish zygosity. Notably, zygosity assessment based on physical similarity has been shown to be approximately 95% accurate (6).

A statistical approach to establish zygosity for population studies was proposed by Weinberg (7), who used the Weinberg differential method to estimate the number of MZ and DZ twin pairs. This method assumes that all MZ twins are like-sexed. It also assumes that in DZ pairs the sex of twin pairs occurs at random, so that one half is like-sexed and one half is unlike-sexed (i.e. in one-fourth of DZ twin pairs both are female, in one half there will be male/female pairs, and in one-fourth both twins are male). Thus, if A is the number of like-sexed twins and B is the number of unlike-sexed twin pairs observed, the estimated proportion of MZ twin pairs would be  $(A - B)/(A + B)$  and the estimated proportion of DZ twin pairs would be  $2B/(A + B)$ . The reliability of the Weinberg method has been questioned by new evidence. For instance, unlike-sexed twins are not always DZ, as when one is 46XY and the other is 45X (8–10).

Today, sex, placentation, cord blood type, HLA antigens, and DNA analysis, including DNA fingerprinting (11–13), DNA microsatellites (14) and, recently, single nucleotide polymorphisms (15), are all used to

differentiate between MZ and DZ twin pairs, with DNA analysis now being the most accurate method for determining zygosity. The analysis of DNA from cells such as fibroblasts or buccal cells may be more accurate than analyzing DNA from blood cells, as chimerism of blood supply is known to occur in both DZ and MZ twins (16,17).

### 16.3 INCIDENCE OF TWINS

The incidence of MZ twins had been thought to be constant throughout the world. In contrast, the incidence of DZ twins was known to vary from population to population (18), with a higher prevalence in some areas, such as Nigeria (19,20), and a lower prevalence in other areas, such as Japan (21,22). The prevalence of MZ twins had been reported to be remarkably constant and was not observed to be affected by environmental or maternal factors until recently. The incidence of twins (the combination of MZ and DZ) in North America was estimated at 10–15 in every 1000 births, that is, roughly one in every 80 births (23). This meant that approximately one in every 40 people in North America was born a twin. There does seem to be seasonal variation, with peaks in March to June and September in the Northern Hemisphere (24). However, the incidence of twins has increased over the past few decades, to almost one in 36 live births. This mostly represents an increase in DZ twins, due to the increase in older mothers and the use of assisted reproductive technologies (ARTs) (25–28); however, MZ twinning also increases with the use of ARTs (29,30).

Data from several countries suggest that the spontaneous rate of MZ births may be increasing as well (31–33). A number of studies have suggested an increase in twin births because of periconceptual vitamin intake (34), particularly increased levels of folic acid (35,36), but other studies have failed to replicate such findings (37,38). The mechanism(s) for the increase in live births of twins is unclear but may reflect improvement in the living environment and better nutrition in the general population (39). The number of twin conceptions is hard to assess, as studies have shown that at least 70% of twin pregnancies diagnosed by ultrasound examination before the tenth week undergo miscarriage or convert to singleton pregnancies (40,41). The disappearance of a co-twin has been termed the vanishing twin.

### 16.4 VANISHING TWIN

Up to 70% of all conceptions, including twin conceptions, are lost prior to live birth, mostly because of either implantation failure or early pregnancy loss (42,43). Several studies have shown that the number of twins in spontaneous abortuses is three times more frequent than that in live births (44,45). Estimates of vanishing twin rates vary, depending on the time (i.e. how early)

and the number of ultrasound scans performed. Several investigators have confirmed that the number of twins at delivery is considerably less than the number of twin conceptions seen on ultrasound examination in early pregnancy (40,46–48).

Twin pregnancies resulting from in vitro fertilization have also shown that the number of twins seen at birth is lower than that seen on ultrasound in early pregnancy (49). However, studies of corpora lutea in spontaneous twins suggest that DZ twins do not seem to have an increased spontaneous miscarriage rate, so the observed excess loss may well be primarily among MZ twin conceptions (50).

The greatest “disappearance” of the co-twin recognized by ultrasound examination seems to occur during the first trimester. The earliest loss may not be detectable by current methods. Some of the mechanisms that have been suggested for the vanishing twin include vascular compromise, life-threatening malformations, spontaneous mutations incompatible with life (40), and cord entanglement (48), all of which might be expected to be more common in MZ twins.

### 16.5 STRUCTURAL DEFECTS IN TWINS

Both MZ and DZ twins are known to have an increased risk for structural defects compared to singletons (51,52). Deformations are particularly increased presumably because of the external pressure due to two growing fetuses sharing the space usually meant for one. However, all anatomic sites appear to be involved in the overall increase rather than just those expected to be increased by external compression. Structural defects in MZ twins are three times more frequent than among DZ twins and two to three times more frequent than seen in singletons (53–55). The structural defects seen in MZ twins can be divided into three groups (Table 16-1) based on the type of process producing them. Because of the high rate of structural anomalies in twins, and the high rate of conversion of a twin pregnancy to a singleton (see below), many singletons born with congenital anomalies may have started as part of a twinning process.

### 16.6 TWINS IN GENETIC STUDIES

Classical twin study methods (56) rely on the assumption that MZ twins are genetically identical; therefore, any discordance between them would be expected to be due to environmental influences, whereas differences within DZ twin pairs are thought to be due to a combination of both genetic and environmental factors. For the most part, the assumption that MZ twins are identical genetically is probably correct, but there are increasing numbers of reports of discordance, for disease (see Table 16-5) and for epigenetic factors such as DNA imprinting and methylation (57). However, the extent to which epigenetic discordance will influence a given trait is unclear.

**TABLE 16-1 Structural Defects in Monozygotic Twins**

Associated with the Twinning Process Due to Incomplete Splitting of the Embryo	Due to Shared Vascular Connections or to <i>in Utero</i> Death of Second Twin	Due to Fetal Constraint or Crowding <i>in Utero</i>
Conjoined twins Fetus <i>in fetu</i> Fetus papyraceous	Acardia (TRAP sequence) Asplenia Microcephaly, hydrocephaly Intestinal atresia Aplasia cutis Terminal limb defects Gastroschisis Disseminated intravascular coagulation	Craniosynostosis Positional defects of the foot Bowing of the limbs Some contractures

There are many types of studies in which twins can provide valuable information. The approach is particularly useful because it can deal with many variables simultaneously. Many twin registries have been set up for this purpose (58). Usually the purpose of twin studies is to obtain results that are applicable not only to twins but also to the whole human population. However, when using twin methodologies, it is necessary to determine whether twin and singleton populations differ in the mean or variance for the trait under study, because a background difference may alter the interpretation of the conclusions (59). It is assumed that MZ and DZ twins are exposed to the same pre- and postnatal environmental factors, which may not be true. Machin and colleagues (60) suggest that the intra-uterine blood supplies to each individual of an MZ twin pair may be markedly different and that the two twins in an MZ pair may start with very different cell numbers (61,62). Studies of MZ twins reared apart have been helpful in establishing the significance of genetic and environmental factors regarding susceptibility to complex psychiatric disorders such as schizophrenia (63), nightmares (64), and manic depressive disorders (64), as well as to measure the extent of inherited versus environmental contribution in disorders with multifactorial inheritance. Thus, twin studies may help to sort out the genetic aspects of a variety of phenotypic traits and disorders with multifactorial inheritance (termed complex traits/diseases) (2,65–67).

## 16.7 DIZYGOTIC TWINS

DZ twins are derived from the fertilization of two ova by two sperm and may be of the same or different sex (68). Their genetic contribution is different because it comes from two different ova and two different sperm (69) (e.g. two siblings who happen to be in the same uterus at the same time). DZ twinning is a common occurrence in animals. Many mammals are known to have sizable litters, almost always due to polyovulation, making every member of a litter a DZ twin.

Multiple ovulation, the release of more than one ovum from the ovaries, is necessary for DZ twinning. Women with a history of DZ twinning have an increased

incidence of multiple follicle growth (70,71), and two corpora lutea can be seen on ultrasound in DZ twin pregnancies (72), with twin ovulations occurring from different ovaries about half the time (50). Women who have given birth spontaneously (i.e. without the use of fertility drugs) to DZ twins are known to have higher levels of follicle-stimulating hormone (FSH) and luteinizing hormone than are found in those who have delivered singletons (73,74). Increased DZ twinning in older women is thought to result from depletion of the ovarian follicle pool and subsequent rise in concentrations of FSH (75,76).

### 16.7.1 Types

DZ twins produced by the fertilization of multiple ova may also be the result of superfecundation. Superfecundation occurs when two different ova are fertilized by two different sperm in more than one act of coitus. In a case reported by Archer (77), one twin was white and the other black; in another case (78), the twins were significantly discordant in size and development at birth. DNA-based paternity testing has revealed additional cases (79,80). DZ twins may also arise from superfetation. Superfetation occurs when a second fertilized ovum implants in a uterus already containing a pregnancy of at least 1 month (69). Superfetation has been suggested in some cases in which the twins are markedly discordant for birth weight, supposedly due to different gestational ages (69,81). A case of superfetation was recently detected following ART in a woman with an undiagnosed ectopic pregnancy (82).

Other types of DZ twinning have been proposed, but in reality these are exceptionally rare and may not be compatible with life. Polar body twins are thought to arise from the simultaneous fertilization of the meiotic products of the same primary oocyte, the oocyte and a polar body, by two different sperm (81). Polar body twinning has not yet been reported in live-born DZ twins but has been suggested in one case as a cause for acardia (83) that was triploid. “Semi-identical” twins, where twins are identical for their mother’s genotypes but not

for their father's, have also been reported; they are the result of two sperm from the same father fertilizing one ovum that split either just prior or subsequent to fertilization (84,85).

### 16.7.2 Placentation

By definition, DZ twins would be expected to have two placentas, with two chorions and two amnions, that is, they would be diamniotic dichorionic (60). However, placentas in DZ conceptions may fuse and look like one placenta. In cases of fused placentas, DZ twins often have vascular connections (86). van Dijk and colleagues (87) have demonstrated chimerism in 8% of DZ twins using sensitive immunological techniques, suggesting that these DZ twins will have immunologic tolerance and be good transplantation donors for each other (88). Bianchi suggests that direct placental connections may not be necessary for microchimerism in DZ twins (16). Careful pathologic and histologic examination of the placenta and other membranes will help in establishing the zygosity of a twin pregnancy (60,89), although nowadays zygosity is most accurately established by DNA analysis. Cases of monochorionic diamniotic DZ twins have been reported associated with ARTs (90,91) and spontaneous DZ twin pregnancies (92). These cases can be particularly confusing because chimerism can occur so that DNA studies based on blood samples appear to predict MZ twinning (93,94).

### 16.7.3 Incidence

The spontaneous incidence of DZ twins varies from country to country (19). However, because of the lack of accurate distinction between MZ and DZ twins, most countries report combined twinning rates. If the MZ twinning rate is assumed to be constant, the variation is thought to reflect DZ twinning rates. Asia, particularly Japan, has the lowest reported twinning rate (2–7 per 1000 births) (21). A slightly higher twinning rate has been reported in Europe (9–20 per 1000 births). The highest twinning rate is seen in the black African population, where it is estimated to be as high as 45–50 per 1000 births (20). The DZ twinning rate in North America is generally estimated at 7–11 per 1000 births (18). The DZ twinning rate is closely related to increasing maternal age, particularly over the age of 30 years (95,96), and peaks around the age of 35–39 years; higher parity is also independently associated with a higher DZ twinning rate (97,98). Tall heavy women are more likely to give birth to DZ twins than short thin women (99–101). DZ twinning has been associated with relatively high levels of gonadotrophin (leading to excess ovulation). Maternal smoking has been suggested to play a role in DZ twinning (100,102).

The DZ twinning rate has increased steeply since the lowest recorded rates in the 1970s (103), coincident with the increased use and improvements in ARTs. In North America, approximately one in 100 births has been of DZ twins and one in 50 individuals was born as a DZ twin: now 1%–2% of pregnancies are achieved through ARTs (as many as 3%–4% in Europe and Australia), and one-third of these result in multiple births. However, most of the increase has been attributed to older maternal reproductive age, both among mothers who conceive twins spontaneously (responsible for 50%–60% of the increase) and those undergoing ART procedures (28,104,105).

Lewis and colleagues (106), using the Australian twin registry, have shown a major genetic contribution to the occurrence of spontaneous DZ twinning. They point out that multiple ovulation does not always result in a live-born DZ twin because of the high spontaneous background loss rate that occurs in one of the twins; nevertheless, increased multiple ovulation is likely to lead to increased fertility. A gene for increased fertility therefore could be expected to have a selective advantage. They suggest that a single gene responsible for DZ twinning would be expected to be quite frequent in the general population if it confers a selective advantage (e.g. more individuals who survive born with the trait). Meulemans and colleagues (107), analyzing European twin data, also suggested a single autosomal dominant gene model for DZ twinning. The total evidence accumulated to date, however, suggests that inherited DZ twinning is likely to be a multifactorial complex genetic trait, influenced by a number of genes.

### 16.7.4 Familial Dizygotic Twinning

There are many reports of familial DZ twinning (108). The female members of these families are thought to have an inherited predisposition to multiple ovulation and in turn have a higher number of DZ twin pairs when compared to the general population (107). The risk of having twins is up to 2.5 times higher for a woman with a sister with DZ twins than it is for the general population (69,106). An established association between higher gonadotrophin levels and higher incidence of DZ twins in certain families is thought to be the basis for familial DZ twinning. While there appears to be some controversy whether this is an autosomal maternal or paternal effect (106,109), in reality a twinning gene could be inherited through either the maternal or paternal side, although it will only be expressed in females. It is possible that some genetic disorders may also predispose to DZ twinning (110).

### 16.7.5 Genetics of Dizygotic Twinning

Studies of species other than humans have revealed a number of genes that contribute to DZ twinning. The



study of sheep has proved particularly informative, as sheep typically give birth to a single offspring at a time but some strains have high incidences of multiple births (111). To date, three genes have been confirmed to influence DZ twinning rates: the growth differentiation factor 9 (*GDF9*) and bone morphogenetic protein 15 (*BMP15*) genes, both of which are expressed in the oocyte and essential for follicle development, and the bone morphogenetic protein receptor 1B (*BMPR1B*), the receptor for *BMP15* and expressed in multiple cell types in the ovary. Interestingly, while mutations in *GDF9* and *BMP15* can increase twinning rates when one copy is present (i.e. when the mother is heterozygous for the mutation), they can also cause female *infertility* when two copies are present in one individual (i.e. in homozygous form) (112,113).

At present, only mutations in *GDF9* appear to influence human DZ twinning, although such mutations are rare. Screening these genes in large numbers of DZ twinning families has revealed a loss-of-function mutation and a two-base deletion in *GDF9* in heterozygous form in three families (114,115), and it appears that overall genetic variation in *GDF9* is more common in mothers of DZ twins than it is in controls (115). No such effect has been found for *BMP15* (116) or *BMPR1B* (117). Interestingly, both *GDF9* and *BMP15* have been implicated in premature ovarian failure (118).

There are a number of additional genes known to have roles in ovulation but as yet there is little evidence that these have anything more than a minor, if any, role in human DZ twinning (25). Genetic variants that result in changes in amino acids in the follicle-stimulating hormone receptor (FSHR) protein were suggested to contribute to DZ twinning (119) but further studies found no evidence for the involvement of this gene (120). Likewise, evidence for the involvement of serine proteinase inhibitor clade A member 1 (*SERPINA1*, commonly known as alpha-1-antitrypsin; (121,122)), peroxisome proliferator-activated receptor gamma (*PPARG*; 46) and the fragile X (FRAXA) “premutation” (123,124) has not been borne out in later studies (25). Family-based “linkage” studies have found no evidence for increased levels of genetic sharing among family members over chromosomal regions in which such candidate genes are located (125–127).

Linkage studies have, however, indicated chromosomal locations that may harbor new candidate genes for DZ twinning (125,127,128). A recent study including 525 DZ twinning families suggests the presence of

such genes on a number of chromosomes, most notably chromosomes 6, 12 and 20, in Australian and Dutch DZ twinning families and confirmed that DZ twinning is a complex trait likely to be influenced by multiple genes (127). Interestingly, a variant with a known functional effect located in the promoter of the *FSHR* gene was found to segregate with the DZ twinning phenotype in one large family, although whether this variant influenced DZ twinning in this family remains to be investigated. Much work remains to be done to find the genes underlying the tendency to human DZ twinning.

## 16.8 MONOZYGOTIC TWINS

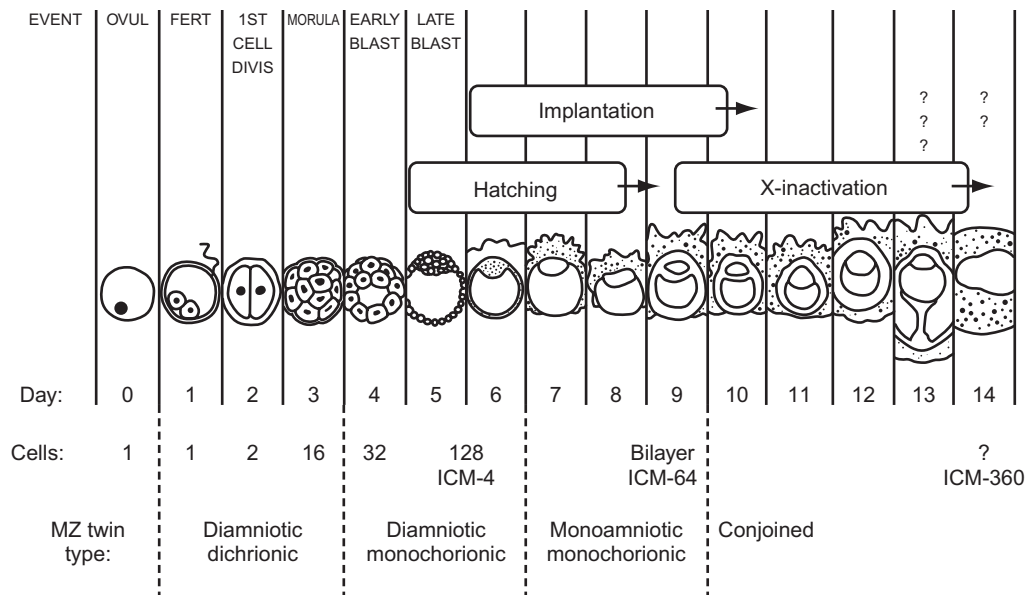
MZ twins are the result of the fertilization of one ovum by one sperm. The single fertilized ovum (zygote) then divides into two embryos; both embryos are thought to initially have the same genetic contribution and in the past have been expected to be genetically identical. However, with new molecular genetic techniques, it has become clear that some MZ twins are not completely genetically identical (57,129,130). MZ twins have been described as natural clones; however, this is incorrect. In the course of the development of every large multicellular organism, somatic mutations as well as epigenetic and stochastic processes lead to distinct genetic differences between MZ twins (131). MZ twinning (as compared to DZ twinning) is rare among nonhumans, where most multiple births represent polyovulation rather than splitting of a zygote. For instance, McLaren and colleagues (132) found no evidence of MZ twinning among 1247 pups born following spontaneous or induced ovulation, suggesting that MZ twinning in mice is either extremely rare or that both members of an MZ twin pair rarely survive to weaning.

### 16.8.1 Types of Placentation

The type of placentation in MZ twins is thought to correspond to the stage in embryonic life during which the twinning event occurred (Table 16-2; Figure 16-1); the later the timing of the event, the more likely the twins are to be monochorionic, monoamniotic, or even conjoined (60,68). There are a number of different types of placentation according to the number of chorionic membranes and amniotic sacs. MZ twins may have separate or contiguous placentas and may be monochorionic monoamniotic (extremely rare with a poor outcome because of tangled umbilical cords; <1% at birth), monochorionic

**TABLE 16-2** Types of Monozygotic Twins According to Placentation

Types of Placentation in MZ Twins	Time of Division after Fertilization (Days)	MZ Twin Pregnancies Surviving at Birth (%)
Dichorionic diamniotic	<3	25
Monochorionic diamniotic	4–6	70–75
Monoamniotic monochorionic	7–13	1–2



**FIGURE 16-1** Process of monozygotic twinning during postfertilization. Ovul, ovulation; fert, fertilization; divis, division; blast, blastulation.

diamniotic (almost uniformly with vascular connections, about 70% at birth), or dichorionic diamniotic (with separate or contiguous placentas, about 30% at birth) (see Table 16-2). There is likely to be increased loss (of the pregnancy or of one twin) among the types of placentas with vascular connections (48,133), and the frequency of various types of MZ chorionicity at conception is unknown. In the case of contiguous placentas, MZ twins often share vascular connections and circulation. About 75% of MZ twins surviving to birth are thought to have had vascular connections in utero.

## 16.8.2 Incidence

The incidence of MZ twins in humans had been thought to be constant throughout the world, at 3–4 in every 1000 births (20,21). This meant that approximately one in 300 births has been an MZ twin birth and one in 150 individuals has been born as an MZ twin. The rate of MZ twinning appears to be unaffected by maternal age, parity, height, or weight (31). Although temperature, delay from the time of ovulation until fertilization or implantation, oxygen supply, and various teratogenic agents have been shown to affect MZ twinning rates in other animals (134), no such factor has been associated with MZ twinning rates in humans. A recent association between an increase of twin births (both MZ and DZ) and periconceptual vitamin supplementation, specifically folic acid, has been reported by Czeizel and colleagues (34), suggesting that adequate maternal nutrition is important for survival to birth for human twins (e.g. loss or conversion to singleton may occur with inadequate nutrition). The population rate of spontaneous MZ twins seems to be increasing (31) and a 3–5 times increase in MZ twin births has been seen with in vitro fertilization (29,135), perhaps related to ovarian stimulation, disturbance of the zona pellucida, or

culturing conditions or handling (e.g. blastocyst transfer) during ART procedures (29). MZ twins and MZ triplets are frequent among spontaneous triplets (136).

## 16.8.3 Familial Monozygotic Twinning

There have been a number of reports of families in which MZ twinning occurs more frequently than expected (137–141), which has been termed familial MZ twinning. Interestingly, there does not seem to be an increase in congenital anomalies among the MZ twins in these families. Because familial MZ twinning has been reported on both the maternal and paternal sides of the family, it has also been suggested that it may be caused by a single gene effect that is unaffected by the sex of the parent transmitting the gene (140,142). However, data from Lichtenstein and colleagues (109) have suggested that there is no paternal effect on familial MZ twinning. Familial MZ twinning could be related to an inherited defect in the zona pellucida leading to early hatching—a mechanism similar to that proposed for the increased rate of MZ twinning seen following ARTs. Alternatively, familial MZ twinning may be related to environmental or epigenetic factors. However, studies on familial MZ twinning are hampered by the possibility of ascertainment bias as such studies typically only include families containing multiple MZ twin pairs.

## 16.8.4 Anomalies Associated with Monozygotic Twin Conceptions and Observed Differences between the Members of an MZ Pair of Twins

Twin pregnancies and twins are at risk for a variety of obstetrical, perinatal, and neonatal medical problems not covered in this chapter. However, MZ twins are

known to have a higher incidence of all types of congenital anomalies (see Table 16-1), and there are also some anomalies that are unique to the MZ twinning process itself. Most of these unique anomalies are thought to be due to defects in the completion of the development of the embryo, such as conjoined twins (2,71,143–145) and fetus in fetu (146,147); vascular aberrations such as acardia (148–151); vascular accidents such as terminal limb defects and death of the co-twin (152); and fetal constraint (which occurs in both types of twins) (Table 16-3). The intrauterine environment may be different for the two MZ twins of a pair because of the number of cells allocated to each twin, the timing of the twinning event, and the placental vascular supply to each twin (60,89). Interestingly, in utero studies of MZ twin behavior have recently shown clear differences in activity and responsiveness before 20 weeks of the pregnancy (153).

It is becoming clear by studying MZ twin pairs that a number of neurologic, immunologic, and structural aspects of embryogenesis appear to be independent of genetic factors. Steinmetz and colleagues (154) have described the discordance in brain surface anatomy in MZ twins. Wolf and colleagues (155) and Trejo and colleagues (156) have shown differences in the immune response between MZ twins in spite of exposure to very similar in utero and postbirth environments. Somatic changes have been observed to occur in the course of development of the MZ twins, leading to differences between the MZ twins in telomere length (157), the fragile X triplet repeat expansion (158), and Alu band variants (159).

### 16.8.5 Mirror-Image Twins

Some MZ twins have features that appear to be mirror-imaged. The most common differences are minor

asymmetries of facial features, such as the side of upsweep of the head hair or eyebrow, one-sided ptosis, or the side of eruption of the first tooth (160–162). A very interesting hypothesis regarding mirror-image twins was made by Boklage (163–165), suggesting that MZ twinning sometimes requires readjustment of the body axis plan. Sperber and colleagues (162) suggest that mirror-image twinning may occur relatively late in embryonic development after the orientation of axis of the zygote has been established but early enough to avoid conjoined twins.

### 16.8.6 Sex Ratio in Monozygotic Twins

The sex ratio (the proportion of males compared to the combination of males and females) among MZ twins is lower than that among DZ twins or singletons (166,167) (Table 16-4). Conjoined twins have an even lower sex ratio than MZ twins (68,168), meaning that there is an excess of female conjoined twins. Tsunoda and colleagues (169) showed in mice that female embryos are behind male embryos in the number of cells present at a specific stage in early development, suggesting that female conceptions may be at higher risk, because of their delayed embryonic development, for late splitting of the embryo, leading to the observed excess of conjoined and MZ twins. Interestingly, there is an excess of same-sex twins among DZ twins.

### 16.8.7 Phenotypic and Epigenetic Discordance in Monozygotic Twins

Although typically considered genetically identical, there are increasing numbers of publications reporting discordance for phenotypic and genetic factors between the twins of an MZ pair. This is most clearly seen when twins are discordant for disease (Table 16-5),

**TABLE 16-3 Anomalies Exclusive to Monozygotic Twins**

Anomaly	Description	Reference
Fetus <i>in fetu</i>	Small parasitic dead twin attached to a normal twin. Often confused with a tumor. Generally located at the origin of the superior mesenteric vessels. Other sites have been reported.	(146,147) MacGillivray et al.
Fetus papyraceous	Mummified dead fetus usually attached to the placenta and present with a normal or more viable twin.	(133,152)
Acardia	Twin with an absent or rudimentary or nonfunctioning heart and whose circulation has been sustained by a normal twin. Associated with a higher rate of chromosomal anomalies (1 in 35,000 births with excess of females).	(55,151)
Conjoined twins	Incomplete twins resulting from an abnormality of the twinning process. They are derived from a single zygote and are always of the same sex. Incidence varies from one to 20,000 to one to 100,000. Females make up 80% of conjoined twins.	(69,143,144,210)

**TABLE 16-4 Sex Ratio in Twins**

	DZ Twins and Singletons	All MZ Twins	Conjoined Twins	Sacral Teratomas
Sex ratio (M/(M + F))	0.514	0.496	0.23	0.25

Data from James (167).

**TABLE 16-5 Examples of Reported Discordance in Monozygotic Twins**

Acardiac	(211)
Adrenal hyperplasia	(212)
Aging	(213)
Aglossia-adactylia	(214)
Aicardi syndrome	(215)
Alagille syndrome	(216)
Alzheimer disease	(217–220)
Amniotic bands	(221)
Amyoplasia	(222)
Amyotrophic lateral sclerosis	(223)
Anorchia	(224)
Asplenia	(225)
Asthma	(226)
Attention deficit disorder	(205,227)
Autism	(173,228,229)
Basal cell carcinoma	(230)
Beckwith–Wiedemann syndrome	(182,209,231–236)
Behçet's syndrome	(237)
Biliary atresia	(238,239)
Body mass index/overweight	(240)
Body stalk	(241)
Breast cancer	(242)
Cavum septum pellucidum	(243)
Cerebral hemisphere	(244,245)
Cerebral palsy	(246)
Choanal atresia	(247)
Chromosomes 1 and 4	(248)
Chronic fatigue syndrome	(249,250)
Chronic periodontitis	(251)
Cleft lip ± cleft palate	(252–255)
Cloverleaf skull	(256)
Coeliac disease	(257,258)
Congenital heart disease	(259)
Copy number variation	(260)
Corpus callosum	(261)
Costello syndrome	(262)
Cutaneous mastocytosis	(263)
Cutis laxa	(264)
Cystitis	(265)
Deletion 22q	(204)
Developmental coordination disorder	(266)
Diabetes	(267,268)
Duane retraction syndrome	(269)
Duchenne muscular dystrophy	(170,270,271)
Endocardial fibroelastosis	(272)
Epilepsy	(273)
Factor IX deficiency	(274)
Fibular aplasia	(275)
Fragile X syndrome	Krayer et al. (1993)
Frontonasal dysplasia	(276)
G syndrome	(277)
Genital anomalies	(278)
Gerstmann–Sträussler–Scheinker disease	(279)
Goldenhar syndrome	(280)
Gonadal dysgenesis	(281)
Hair whorls	(282)
Handedness	(163)
Hirschsprung disease	(283,284)
Hunter disease	(285)
Huntington disease	(286)
Hydranencephaly	(287)
Hypertrophic cardiomyopathy	(288)
Hypothyroidism	(289)
Infantile spasms	(206)
Inflammatory bowel disease (Crohn's Disease, ulcerative colitis)	(59,290,291)

**TABLE 16-5 Examples of Reported Discordance in Monozygotic Twins—cont'd**

Joint mobility	(292)
Kabuki syndrome	(293)
Kallmann syndrome	(294)
Kleeblattschädel anomaly	(295)
Leukemia	(296–298)
Lipodystrophy	(299)
McCune–Albright syndrome	(198)
Macular degeneration	(300)
Megacystis–microcolon–intestinal hypoperistalsis syndrome	(301)
Mental retardation	(302)
Migraines	(303)
Monosomy 11p	(180)
Multiple sclerosis	(177,304,305)
Myasthenia gravis	(306,307)
Neural tube defects	(55,308,309)
Neuroblastoma	(310)
Oculo–oto–radial syndrome	(311)
Oral–facial–digital syndrome, type 1	(312)
Parkinson disease	(313)
Polydactyly	(314)
Primary biliary cirrhosis	(239)
Proteus syndrome	(315)
Pyloric stenosis	(316)
Renal agenesis	(317)
Retinitis pigmentosa	(318)
Rhabdoid tumor	(319)
Rheumatoid arthritis	(320,321)
Ring chromosome 19 mosaicism	(322)
Rubinstein–Taybi syndrome	(323)
Russell–Silver syndrome	(324)
Sex	(325–327)
Schimmelpenning–Feuerstein–Mims syndrome	(328,329)
Schizophrenia/bipolar disorder	(63,330–333)
Scheuermann disease	(334)
Scleroderma	(335)
Seizures	(336,337)
Sirenomelia	(338)
Skeletal dysplasia	(259)
Sotos syndrome	(339)
Spinocerebellar ataxia	(340)
Strabismus	(341)
Sudden infant death syndrome	(342)
Suicide	(343,344)
Symbrachydactyly	(345)
Synaesthesia	(346)
Systemic sclerosis	Nelson et al. (2003); (93)
Teratoma	(347)
Testicular cancer	(348)
Thyroid dysgenesis	(349)
Toxoplasmosis infection	(350)
Trisomy 1	(351)
Trisomy 13 (Patua syndrome)	(352,353)
Trisomy 18 (Edward syndrome)	(354–356)
Trisomy 21 (Down syndrome)	(92,179,181)
Tuberous sclerosis	(357–359)
Turner syndrome	(325,327,360–364)
Urinary tract anomalies	(365,366)
Vaginal dysgenesis	(367)
van der Woude syndrome	(185)
VATER association	(368)
Vitiligo	(369)
von Hippel–Lindau syndrome	(370)



for example X-linked Duchenne muscular dystrophy (170,171), autosomal Mendelian diseases such as familial amyloid polyneuropathy (172), and complex diseases such as autism (173). Alternatively, both twins may be diagnosed with the same disease but manifest different symptoms or timing of disease onset (174–176). Magnetic resonance imaging has recently shown discordances in brain development and anatomy (177,178). Underlying causes may include discordance for chromosomal aneuploidy (179–181), uniparental disomy (182), chromosomal rearrangement (183), triplet expansion (184) or nuclear (185,186) or mitochondrial (187,188) point mutations that have occurred postzygotically (57).

Recent advances in genetic technology have revealed further areas of MZ twin discordance, at the level of the DNA sequence (for example copy number variants (189)), gene expression (190), and epigenetics, particularly DNA methylation (191). Epigenetic phenomena, now known to be key regulators of gene expression, change dynamically over time and under different environmental conditions, and studies have now shown increasing discordance between MZ twins with increasing age and lifestyle differences (192,193). Discordance in telomere length has also been shown to increase with age (194).

### 16.8.8 Etiology of Monozygotic Twinning

The etiology of MZ twinning in humans is unknown, but several mechanisms have been proposed. Stockard (145) suggested that MZ twinning may be due to lack of oxygen prior to implantation, which caused developmental arrest and splitting in the zygote. His work was supported by the finding that the implantation of the ovum is delayed in the armadillo, which results in MZ quadruplets or octuplets (195), and by studies in rabbit and roe deer showing that twinning in these animals is also associated with delayed implantation (69). These findings suggested that MZ twinning is associated with disturbance of development clocks or thresholds and that delayed fertilization or delayed implantation may play a role in MZ twinning.

On the basis of observations of a higher-than-expected incidence of MZ twins after *in vitro* fertilization (12,196), Edwards and colleagues (197) suggested that abnormalities or rupture of the zona pellucida may lead to herniation of the blastocyst and predispose to MZ twinning (198). Boklage (160,164,165) pointed out that differentiation of the chorion occurs at approximately the fourth day after fertilization and that, in monochorionic MZ twins, the physical separation of two embryos is unlikely if the zona is still intact when the chorion begins to develop. Boklage suggested that if the zona is intact, rather than a physical separation of the MZ twins, there may be “developmental” separation, rendering two groups of cells within a morula that organize themselves separately and continue with embryogenesis separately.

Other investigators have suggested that twinning itself may be a type of congenital anomaly or an abnormality of development, with the “twinning” fertilized egg (i.e. a fertilized egg resulting in twins) developing at a different rate and in a different way, as compared to a “normal” fertilized egg (i.e. an egg resulting in a singleton). There must be a relatively narrow window during which MZ twinning can occur (normally only up until 11–13 days postfertilization, when the primitive streak forms), and there are a number of different events taking place during postfertilization, including hatching, implantation, genomic imprinting, and X-inactivation (see Figure 16-1). If the twinning zygote is maturing at a different rate than the normal zygote, the timing for all these events may be shifted and may even occur in an order different from the predicted normal timing for singletons.

The finding that mammalian female embryos are somewhat behind male embryos in the number of cells present at a certain stage during early stages of embryonic development (169) and the fact that there is a slightly higher incidence of female MZ twins, particularly among conjoined twins, in which the twinning process is assumed to occur relatively late in the very early embryonic developmental process support the suggestion that MZ twinning is somehow related to delayed implantation and that the timing of different developmental clocks plays a critical role in MZ twinning.

Several authors (4) have suggested that skewed X-chromosomal inactivation may play a role in female MZ twinning if, during embryogenesis, two different foci were to arise: one expressing the maternal X and the other expressing the paternal X. A number of female MZ twins have been discordant for a variety of X-linked recessive diseases (199), suggesting, and often demonstrating, nonrandom X-inactivation (Table 16-5). Goodship and colleagues (171) have tested the hypothesis that skewed X-inactivation can trigger MZ twinning in females by studying umbilical cord tissue in female MZ twins. They observed random X-inactivation in most pairs of female MZ twins, but some showed marked skewing. Thus, it would appear that skewed X-inactivation does not explain all female MZ twinning but could be responsible for the excess of MZ female twins. Interestingly, Tan and colleagues (200) have shown that X-inactivation occurs at different times in different tissues postimplantation in the mouse embryo. These findings suggest that since X-inactivation occurs at the time of tissue differentiation, X-inactivation in blood and skin may not be representative of the rest of the tissue in an organism. To properly determine the exact role of X-inactivation in female MZ twinning, it would be necessary to study many different tissues. Bamforth and colleagues (201) have studied the parent of origin of X-inactivation in placental membranes and umbilical cords in twins and triplets. The chorion did show asymmetric X-inactivation in MZ dichorionic

twins. Of course, the chorion is not representative of the whole embryo but may represent processes that occurred early in development. The study also suggested that monochorionic MZ twins may react differently from dichorionic ones, reflecting the importance of timing in the MZ twinning process.

Observations of discordance in the expression of genetic material in MZ twins (see Table 16-5) have suggested the intriguing possibility that some cases of MZ twinning may occur because of epigenetic events (105,202), such as a discordance in the expression of genetic information, not only by discordance of X-inactivation but also by many other mechanisms, including genomic imprinting (203,204), loss of imprinting, the development of uniparental disomy (182), changes in chromosome number, mitochondrial mutations (202,205,206), and telomere crossover (207) and length (208). Weksberg and colleagues (209) found differential imprinting in female MZ twins discordant for Beckwith–Wiedemann syndrome and proposed that in such cases either unequal splitting of the inner cell mass or, alternatively, a lack of maintenance of DNA methylation, leads to a loss of imprinting that predisposes to MZ twinning. Such discordance would be expected to arise early in development among cells from a single zygote. A discordance of expression of genetic information could then lead to division of the zygote into two separate embryos during a specific period, early in development, perhaps from the stage of eight cells to approximately 360 cells in the inner cell mass, when differentiation and primitive streak formation begins. After birth, this discordance of genetic information could be mosaic in each twin, but it could be present to different degrees in the two different twins sufficient to cause observable phenotypical discordance. In other words, once measurement error and environmental influences are accounted for, genetic discordance or differences in the expression of genetic information should be suspected in cases of discordant MZ twins.

## 16.9 CONCLUSIONS

In the field of twins and twinning, there is still a great deal to be learned. The development of new DNA molecular and cytogenetic techniques, the use of prenatal diagnosis such as chorionic villi sampling, amniocentesis, and ultrasound examination in humans, as well as embryopathology, histology and genetic advances all give clues to the increased understanding of MZ and DZ twins and the twinning process itself.

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## CROSS REFERENCES

Nature and Frequency of Genetic Disease; Epigenetics; Mutations in Human Disease: Nature and Consequences; Mendelian Inheritance; Analysis of Genetic Linkage; Chromosomal Basis of Inheritance; Multifactorial inheritance and complex traits; Genetic Epidemiology; Human Developmental Genetics; Genetic Risk Assessment for Common Disease; The Genetic Basis of Female Infertility; Fetal Loss; Transplantation Genetics.

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# CHAPTER 17

## The Molecular Biology of Cancer

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### 17.1 INTRODUCTION

Cancer is a genetic disease, arising from the progressive accumulation of mutations that promote the clonal selection of cells with increasingly deregulated growth. Studies from many different fields, including tumor virology, chemical carcinogenesis, molecular biology, somatic cell genetics, and genetic epidemiology, have all provided fundamental insights into the genetic basis of cancer. Most of these alterations are somatically acquired, but some are inherited. During the last four decades, many of the genetic targets of the mutations have been identified. The cancer phenotype, however, is a result of complex interactions, not only involving the known cancer gene products but also a multitude of other proteins.

An increasing number of “modifier” genes and low-penetrance cancer susceptibility gene variants are being discovered, such as the breast cancer risk modifier genes, *FGFR2*, *CASP8*, *MAP3K1*, *TOX3/TNRC9* and *RAD51* genes (Milne and Antoniou, 2011), and the colorectal cancer (CRC) risk modifier variants in the *APC*, *TP53*, *IGFBP-3*, *TCF7L2* and *TGFBR1* genes (Jasperson et al., 2010). The relative risk attributable to any one of these genes is generally small compared to that of the known high-penetrance cancer genes, but collectively their variant alleles may strongly influence an individual’s cancer risk. Additionally, all these interactions are subject to modification by incompletely understood environmental factors. The disentangling of all such interrelations poses a major challenge to cancer biologists.

Most known cancer genes fall into three broad classes: proto-oncogenes that are positive regulators of cell proliferation and survival; tumor suppressor genes (TSGs) that are negative regulators; and DNA repair genes that are responsible for the detection and repair of genetic damage. The coordinated regulation of these gene classes is responsible for maintaining tissue homeostasis. Cancer reflects a fundamental breakdown in this control. The types of alteration that occur in proto-oncogenes and TSGs display intrinsic differences. Point mutations in proto-oncogenes are usually missense

and tend to activate—or give new functions to—the gene product. Alternatively, proto-oncogenes can be amplified or can undergo translocations that either place them under the control of active promoter sequences or generate a fusion product with a new or constitutive function. Oncogenes are thus proto-oncogenes that have undergone gain-of-function alterations that act in a positive fashion to promote tumorigenesis. TSGs, on the other hand, are subject to loss-of-function alterations, commonly through point mutations that truncate the protein product (or alter a crucial functional domain) or through deletions. These differences partially account for the dominant tumorigenic activity of oncogenes and the usual recessiveness (at the cellular level) of tumor suppressors. Many of the cellular genes affected by human cancer-associated mutations (particularly those that are inherited) are discussed in greater detail below.

Many studies have highlighted the critical role of mutations involving the third class of genes in the cancer process; that is, the DNA repair genes, also termed the “stability genes” or “caretaker genes” (Vogelstein and Kinzler, 2004). As with the TSGs, the loss-of-function mutations in these genes are the basis for their role in tumorigenesis. They can be considered as a subset of TSGs, but they differ from other TSGs in critical ways. The protein products of some TSGs may, while functioning normally, provide growth-inhibitory signals or activate cell differentiation pathways. Other tumor suppressor proteins may mediate programmed cell death (apoptosis) following DNA damage or cell cycle perturbations. In contrast, the stability genes encode proteins that either (a) repair (by nucleotide or base excision repair, or mismatch repair) small-scale aberrations in the DNA following DNA replication or the action of mutagens or (b) maintain the normal chromosomal structure such as in mitotic recombination or segregation (Vogelstein and Kinzler, 2004). Therefore, the principal effect of the *inactivation* of DNA repair genes is genomic instability and an increased rate of mutations in a variety of cellular genes, including proto-oncogenes and the TSGs, in affected cells. Because the accumulation of mutations

in these latter two classes of growth-regulating genes appears to be an important rate-limiting step in tumorigenesis, the inactivation of genes that are normally involved in DNA damage recognition and repair greatly accelerates the process of tumor progression.

Given the enormous progress that has been made in furthering our understanding of the genetic alterations present in cancer cells, this chapter does not attempt to review all of the thousands of mutations in oncogenes and the TSGs that have been identified to date in human cancers. Rather, the primary aims of this chapter are (1) to review the historical basis underlying the search for genetic alterations in human cancers; (2) to review the roles of a number of TSGs and a few relevant proto-oncogenes that are associated with inherited cancer syndromes; and (3) to address the molecular biology of some of the more common inherited cancer syndromes resulting from defects in DNA repair genes. An extremely useful continuously updated online database of germline mutations in human disease-associated genes, with multiple links to other similar worldwide databases, is maintained at the Institute of Medical Genetics in Cardiff (<http://www.hgmd.cf.ac.uk>) and at the Diagnostic Mutation Database that is maintained at the Manchester National Genetic Reference Laboratory. Access to these databases requires registration. In addition, online lists of mutations are provided within the appropriate gene-specific OMIM article (within the “Allelic variants” section). In addition, there are several disease-specific online databases of mutations, such as the Breast Information Core (BIC) database of BRCA1 and BRCA2 mutations, the database of HNPCC and FAP-related mutations (freely accessible at the InSiGHT International Society for Gastrointestinal Hereditary Tumors) website, and the Birt Hogg Dube mutation database (see Table 17-1).

## 17.2 A GENETIC BASIS OF CANCER

An inherited basis of some cancers in humans and other animals has been hypothesized for over a century. Broca was perhaps the first to describe an inherited cancer syndrome. In 1866, he described a family with many members affected by breast and liver cancers and suggested that some inherited aberration within the affected tissue allowed tumors to form. Subsequent studies of mammary tumor formation in inbred strains of mice led Haaland, in 1911, to propose that tumorigenesis behaved in a formal sense as a Mendelian genetic trait. Similarly, Warthin’s studies of four families with markedly increased cancer rates led him to propose in 1913 that susceptibility to several cancer types appeared to be transmitted as an autosomal dominant Mendelian trait. Although these and other studies were consistent with the proposal that a genetic basis of cancer might account for familial clustering of cancer, other explanations of these observations were possible (e.g. clustered exposure

**TABLE 17-1** Examples of Cancer-Related Online Mutation Databases

Name	URL
HGMD (Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff)	<a href="http://www.hgmd.cf.ac.uk">http://www.hgmd.cf.ac.uk</a>
Breast Cancer Information Core (BIC) database (requires password)	<a href="http://research.nhgri.nih.gov/bic/">http://research.nhgri.nih.gov/bic/</a>
Diagnostic Mutation Database (at Manchester National Genetics Reference Laboratory)	<a href="http://www.ngri.org.uk/Manchester/dmudb.html">http://www.ngri.org.uk/Manchester/dmudb.html</a>
InSiGHT (International Society for Gastrointestinal Hereditary Tumours)	<a href="http://www.insight-group.org/mutations/">http://www.insight-group.org/mutations/</a>
Birt Hogg Dube mutation database	<a href="https://grenada.lumc.nl/LOVD2/shared1/home.php?select_db=FLCN">https://grenada.lumc.nl/LOVD2/shared1/home.php?select_db=FLCN</a>

to environmental or dietary agents). Moreover, it was argued that the majority of cancers arose as sporadic or isolated cases.

The proposal that cancer might result from somatic alterations in the genetic material of cells was first advanced by Boveri in 1929. He had previously noted that abnormal mitotic divisions and atypical cell masses could be seen in sea urchin eggs fertilized by two sperms. He reasoned that the abnormal masses bore significant similarity to tumors, and thus he hypothesized that cancer might result from cellular aberrations that produced abnormal mitotic figures. Nevertheless, Boveri’s studies provided some insights into the genetic or cellular mechanisms that might give rise to abnormalities in the regulation of cell division.

The studies of Peyton Rous can therefore be viewed as a particularly critical turning point in the search for a genetic basis of cancer development because they provided the first mechanistic insights into the pathogenesis of cancer. In 1911, he demonstrated that sarcomas in chicken (*Galus galus*) could be induced following inoculation with a cell-free filtrate obtained from an independent chicken sarcoma. Nearly 70 years later, it was conclusively demonstrated (by Stehelin et al. in 1976) that the Rous sarcoma virus (RSV) is a retrovirus that induces cancers in chickens because it harbors an altered version of a cellular proto-oncogene known as the *src* gene. Though retroviruses have thus far been implicated in the causation of a limited number of forms of human cancer (e.g. acute T-cell leukemia), their study acquired a much larger significance by providing experimental access to the genetic underpinnings of tumorigenesis.

More recently, it became clear that tumorigenesis in humans is a multistep process, with these steps reflecting underlying genetic alterations that drive the progression into malignancy. It was suggested—in a seminal

publication by Hanahan and Weinberg—that the enormous range of cancer-cell genetic alterations is, in effect, a manifestation of six essential alterations in cellular physiology or functional capabilities (acquired through differing mechanisms) that collectively permit malignant growth. These comprise (1) self-sufficiency in growth signals, (2) insensitivity to growth-inhibitory signals, (3) evasion of apoptosis, (4) limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion and metastasis (Hanahan and Weinberg, 2000).

## 17.3 VIRAL ONCOGENES

### 17.3.1 RNA Tumor Viruses and the Identification of Proto-Oncogenes

RNA tumor viruses (also known as retroviral tumor viruses) can be divided into two classes: acute and chronic transforming viruses. The identification of oncogenes began with the studies of acutely transforming retroviruses. Although not oncogenic in humans, these can rapidly induce tumors in animals and transform cells in vitro. In contrast to the chronic transforming viruses, the genomes of the acute transforming viruses contain nucleic acid sequences that have been acquired or transduced from the host cell as a result of genetic recombination. The altered host-derived sequences are termed *viral oncogenes* (*v-onc* genes), and these sequences, although not necessary for the replication of the virus, are directly responsible for its rapid transforming activity. Characterization of the *v-onc* genes present in various acute transforming viruses has led to the identification of many different cellular genes (known as *proto-oncogenes* when in the unaltered state) that have been transduced by various tumor viruses (Table 17-2).

There is no reason to believe that proto-oncogenes are unique in their capacity to be transduced by retroviruses; rather, the proto-oncogenes represent a unique class of genes because of their biological properties. Transduction of other classes of genes does not give rise to acute transforming viruses. Hence, because of their vital role in the generation of acute transforming viruses, the proto-oncogenes might be presumed to represent at least part of the genetic repertoire that might underlie cancer. Moreover, because the *v-onc* genes harbored by the acute transforming viruses represent mutated versions of their corresponding cellular proto-oncogenes, comparison of the two sequences might be expected to provide insights into the means by which oncogenic variant alleles are generated by somatic mutations, such as point mutation, chromosomal translocation, or gene amplification. Subsequent to the discovery that *v-src* has a counterpart in the cellular gene *c-src*, it has transpired that nearly all retroviral oncogenes are transduced cellular proto-oncogenes whose expression and/or activity is increased as a consequence of the transduction process.

**TABLE 17-2 Retroviral Oncogenes and Tumors**

V- <i>ONC</i> Gene	RNA Tumor Virus	Species of Origin	Major Disease
Growth factor family			
V- <i>SIS</i>	SSV	Woolly monkey	Glioma/sarcoma
	PI-FeSV	Cat	Sarcoma
Integral membrane tyrosine kinases			
V- <i>FMS</i>	SM-FeSV	Cat	Sarcoma
V- <i>ERBB</i>	AEV-H, AEV-ES4	Chicken	Erythroleukemia, sarcoma
V- <i>KIT</i>	HZ4-FeSV	Cat	Sarcoma
V- <i>ROS</i>	UR2	Chicken	Sarcoma
Membrane-associated tyrosine kinases			
V- <i>SRC</i>	RSV	Chicken	Sarcoma
V- <i>FGR</i>	Gr-FeSV	Cat	Sarcoma
V- <i>YES</i>	Y73/ESV	Chicken	Sarcoma
F- <i>FPS</i>	FuSV/PRCII	Chicken	Sarcoma
V- <i>ABL</i>	Ab-MLV	Mouse	Leukemia
V- <i>ABL</i>	HZ2-FeSV	Cat	Sarcoma
Serine–threonine kinases			
V- <i>MOS</i>	Mo-MSV	Mouse	Sarcoma
V- <i>RAF</i>	MSV-3611	Mouse	Sarcoma
RAS family			
V- <i>H-RAS</i>	Ha-MSV	Rat	Sarcoma
V- <i>K-RAS</i>	Ki-MSV	Rat	Sarcoma
Nuclear proteins			
V- <i>MYC</i>	MC29	Chicken	Carcinoma, myeloid leukemia
V- <i>MYB</i>	E26	Chicken	Erythroleukemia
V- <i>FOS</i>	FBJ-MSV	Mouse	Osteosarcoma
V- <i>SKI</i>	SKV770	Chicken	Carcinoma
V- <i>REL</i>	REV-T	Turkey	Lymphatic leukemia
V- <i>ETS</i>	E26	Chicken	Erythroleukemia
V- <i>ERBA</i>	AEV-ES4	Chicken	Erythroblastosis

Modified from Weinberg, (1994); with permission.

The acute transforming viruses cause tumors at the site of inoculation after short latency periods (e.g. a few weeks in newborn animals), and they are also able to transform cells in culture. The chronic transforming viruses are capable of producing tumors only in hosts in which the virus can replicate. The tumors arise after relatively long latency periods and at specific tissue sites, irrespective of the inoculation site. The differences in the properties of the two classes of viruses can be traced to differences in their genetic content. Chronic transforming retroviruses (such as HTLV1, mouse mammary tumor virus and avian leukosis virus) possess no cellular sequences and instead transform cells by the effects of the random integration of a DNA copy of the virus, termed the *provirus*, into the host cell genome. The provirus can affect the genes in the region of the host chromosome where it integrates. If a host proto-oncogene is contained in the region, provirus integration can alter the structure and/or expression of the proto-oncogene and thus contribute to tumorigenesis. The relatively long latency of the tumorigenic process for the chronic transforming viruses reflects

the low probability that any one integration event will specifically affect a proto-oncogene. Nevertheless, given the infection of the sufficient cells, transformation will be likely to occur, with leukemias and lymphomas being the most prominent tumor types generated by these chronic transforming viruses. Several of the proto-oncogenes that are altered by chronic transforming viruses in animal tumors are also affected in a subset of human tumors by somatic mutations (Table 17-3).

As mentioned above, these chronic transforming retroviruses induce neoplastic transformation after a latency period of months or years, via the integration of the proviral DNA in such a way as to activate specific host target genes: Cellular genes are either brought under the control of the viral LTR or are fused to viral sequences with the consequent production of novel proteins. Knowledge of this led to the prospective use of insertional mutagenesis as a means of identifying novel proto-oncogenes that are consistent targets for proviral insertion. It was suggested that *spontaneous* alteration of any of these genes might account for tumors—including most human tumors—whose initiation is virus independent. The finding that around one in five tumors possesses genomic sequences with intrinsic transforming ability supports this hypothesis. Indeed, tumor gene transfer experiments (see “Oncogenes Identified by DNA Transfection”) identified oncogenes already known to be the cellular counterparts of various retroviral oncogenes, in addition to several novel genes (many of which belonged to the *src* and *ras* superfamilies). The presence of activating alterations in many of these cellular genes was, in fact, subsequently demonstrated. Other oncogenes were identified directly through their association with translocations, which caused activation either by the generation of new fusion products or by bringing the proto-oncogene under the transcriptional control of nearby enhancer elements. Probes and primers derived

from the oncogenes identified in these various ways were then used to isolate further, homologous, genes by cross-hybridization or by the use of PCR.

### 17.3.2 DNA Tumor Viruses

Many DNA viruses—of the adenovirus, herpesvirus, poxvirus and papovavirus families—also encode oncoproteins; however, the transforming genes rarely have proto-oncogene homologs within the normal genome. In general, while the RNA tumor viruses promote tumor development by harboring or generating oncogenic alleles, several of the DNA tumor viruses, such as the simian type 40 virus (*SV40*), human papillomaviruses and adenoviruses, primarily contribute to tumorigenesis by inactivating or inhibiting the activities of tumor suppressor proteins. Several of the specific interactions of the DNA tumor virus proteins with tumor suppressor proteins are addressed below in detail in the discussion of TSG products.

## 17.4 ONCOGENIC ALLELES IN HUMAN CANCERS

The precise location of oncogenic mutations often provided the first indication of the locations of important functional domains of the protein. Genetic, biochemical and cellular studies in both humans and model organisms have further illuminated the functions of these genes. The majority of proto-oncogenes are now known to be the components of signal transduction pathways, which are responsible for translating extracellular signals into nuclear changes in gene expression. Activation of proto-oncogenes (and inactivation of TSGs) results in the deregulation of these pathways, such that the aspects of cellular behavior—for example, proliferation and survival—become autonomous. Proto-oncogenes have been discovered at most stages of the signal transduction pathways, acting as (among others) ligands, receptors, membrane-associated tyrosine kinases, lipid kinases, cytoplasmic regulators, G proteins, kinases, transcription factors and transcriptional coactivators. A small subset of proto-oncogenes is more directly involved in cellular proliferation and survival, and functions as components of the cell cycle or anti-apoptotic machinery. For example, *CCND1* and *CDK4*, encoding cyclin D1 and cyclin dependent kinase 4 (CDK4), respectively, have both been found to be activated in tumors. The anti-apoptotic gene *Bcl-2* is another target for activation, and was first identified at the breakpoint of translocations in follicular lymphomas. An important consequence of oncogenic activation, therefore, is the uncoupling of cell proliferation and survival from regulation by extrinsic factors. This can be achieved either by constitutive activation of mitogenic signaling pathways or through direct stimulation of cell cycle progression or cellular survival.

Certain oncogenes (and also TSGs) are important in the advanced stages of tumorigenesis. A variety of

**TABLE 17-3 Cellular Genes Activated by Chronic Transforming Retroviruses**

Gene	Disease	Virus	Animal
<i>myc</i>	Bursal lymphoma	ALV, CSF, REV	Chicken
	T-cell lymphoma	MLV	Mouse
	T-cell lymphoma	FeLV	Cat
<i>erbB (egfr)</i>	Erythroleukemia	ALV	Chicken
<i>myb</i>	Lymphosarcoma	MLV	Mouse
<i>h-ras</i>	Nephroblastoma	MAV	Chicken
<i>mos</i>	Plasmacytoma	IAP	Mouse
<i>il2</i>	T-cell lymphoma	GalV	Ape
<i>il3</i>	Myelomonocytic leukemia	IAP	Mouse
<i>Int1</i>	Mammary cancer	MMTV	Mouse
<i>int2</i>	Mammary cancer	MMTV	Mouse
<i>evi1</i>	Myeloid lymphoma	MCF-MLV	Mouse
<i>evi2</i>	Myeloid lymphoma	MCF-MLV	Mouse
<i>pim1</i>	T-cell lymphoma	M-MLV	Mouse



proto-oncogenes are involved in the regulation of the actin cytoskeleton, cell adhesion, and extracellular matrix. Deregulation of these facets via oncogene activation promotes the cell migration and substrate independence required for tumor cell invasion and metastasis. Other oncogenes are thought to have a role in the process of angiogenesis; the induction of new blood vessel growth from the existing vessels. This process is probably essential if solid tumors are to enlarge beyond around 1 mm<sup>3</sup>.

Proto-oncogene activation probably contributes to the development of all sporadic cancers. Activating germline mutations in proto-oncogenes is likely, in general, to be lethal, but mutations in a limited number of proto-oncogenes are heritable and give rise to familial cancer syndromes. Germline mutations in *MET*, *RET* and *CDK4*, for example, are respectively responsible for hereditary papillary renal cancer, multiple endocrine neoplasia type 2 (MEN2), and some cases of familial melanoma. The majority of cancer syndromes are caused by germline mutations in TSGs, where, in contrast to proto-oncogene mutations, loss of the remaining wild-type allele is the initiating transforming event. Even here, the progression to full tumorigenicity requires further mutations in additional, probably multiple, proto-oncogenes and tumor suppressors. It should be stressed that although the study of the oncogenes carried by acute transforming retroviruses has led to the identification of many genes with critical roles in cell growth, only a subset of these genes is frequently affected by somatic mutations in human cancer and, as mentioned previously, most of these occur as somatic rather than germline mutations. In addition, as

reviewed below, several oncogenes that are frequently activated in human cancer involve gene sequences that are not known to be harbored by acute transforming viruses. Many of the oncogenic alleles often observed in human cancers are summarized in Table 17-4. A more complete list is available online at <http://www.sanger.ac.uk/genetics/CGP/Census/>.

### 17.4.1 Oncogenes Identified by DNA Transfection

DNA transfection techniques were first used to study and identify the transforming genes of RNA and DNA tumor viruses. In these assays, DNA from primary human tumors or tumor-derived cell lines is transferred into non-tumorigenic recipient cells, and cells that acquire morphologic changes and lose contact inhibition of their growth (so-called “focus formation”) are studied further (Figure 17-1). The human tumor-derived DNA sequences that are sufficient for mediating transformation can ultimately be recovered and characterized by serial passaging of DNA from a focus (Table 17-5).

In seminal studies using the DNA transfection approach, the research groups of Robert Weinberg, Jeffrey Cooper, and Michael Wigler identified the first oncogenic allele in human cancer. This oncogenic allele was a mutated *H-RAS* gene in the human bladder cancer cell line known as EJ or T24. Their observation was particularly notable because *RAS* genes had previously been identified by the characterization of the cellular homologues of the *v-onc* genes in the Harvey and Kirsten rat sarcoma viruses (*h-ras* and *k-ras*, respectively).

**TABLE 17-4 Representative Oncogenic Alleles in Human Cancers**

Gene	Activation Mechanism	Protein Properties	Tumor Type
K-RAS	Point mutation	p21 GTPase other cancers, leukemia	Pancreatic, colorectal, lung (adeno) and
N-RAS	Point mutation	p21 GTPase	Myeloid leukemia
H-RAS	Point mutation	p21 GTPase	Bladder and other cancers
<i>EGFR (ErbB-1)</i>	Amplification, rearrangement	Growth factor receptor	Gliomas, carcinomas
<i>NEU (ErbB-2)</i>	Amplification	Growth factor receptor	Breast, ovarian, and other carcinomas
<i>MYC</i>	Chromosome translocation amplification	Transcription factor	Burkitt's lymphoma; SSCL; other cancers
<i>N-MYC</i>	Amplification	Transcription factor	Neuroblastoma, SCCL
<i>L-MYC</i>	Amplification	Transcription factor	SCCL
<i>BCL2</i>	Chromosome translocation	Anti-apoptosis protein	B-cell lymphoma (follicular type)
<i>CYCD1</i>	Amplification, chromosome translocation	Cyclin D	Breast cancer, B-cell lymphoma, various carcinomas
<i>BCR-ABL</i>	Chromosome translocation	Chimeric nonreceptor tyrosine kinase	CML, ALL (T cell)
<i>RET</i>	Rearrangement	Chimeric receptor tyrosine kinase	Thyroid cancer (papillary)
<i>TRK</i>	Rearrangement	Chimeric receptor tyrosine kinase	Colorectal cancer
<i>HST</i>	Amplification	Growth factor (FGF-like)	Gastric cancer
<i>APL-RARA</i>	Chromosome translocation	Chimeric transcription factor	Acute promyelocytic leukemia
<i>E2A-PBX1</i>	Chromosome translocation	Chimeric transcription factor	Pre-B-ALL
<i>MDM2</i>	Amplification	p53 binding protein (nuclear)	Sarcomas
<i>GLI</i>	Amplification	Transcription factor	Sarcomas, gliomas
<i>CDK4</i>	Amplification	Cyclin-dependent kinase	Sarcomas, gliomas

Abbreviations: ALL, acute lymphoid leukemia; CML, chronic myeloid leukemia; SCCL, small cell lung cancer.

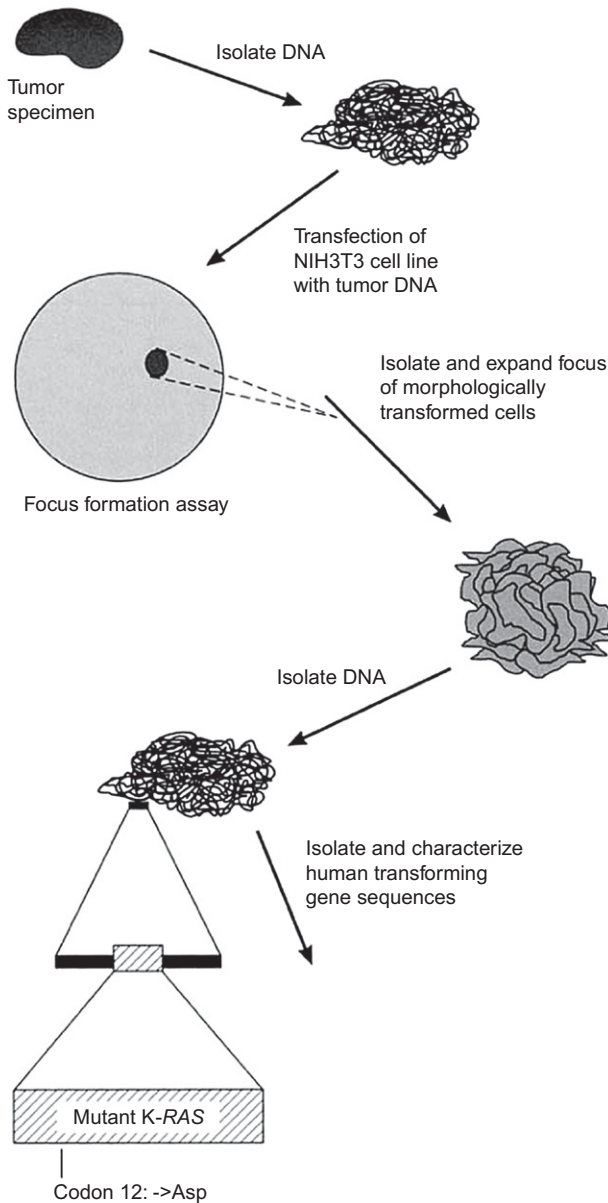


FIGURE 17-1

Subsequent studies established that the “activated” *H-RAS* allele in the bladder cancer cell line differed from a normal copy of the gene by a single nucleotide substitution. This nucleotide substitution, from G to T, resulted in a single amino acid change from glycine to valine at codon 12. Additional DNA transfection studies established that about 10–15% of human tumors and tumor cell lines possessed *H-RAS*, *K-RAS*, or *N-RAS* alleles with mutations. *N-RAS* is the third closely related *RAS* gene family member that was first identified as a transforming allele in a human neuroblastoma. *RAS* gene mutations are particularly prominent in several common cancer types (Downward, 2003), and the mutational spectrum and functional significance of *RAS* gene mutations in several types of cancer are addressed below.

Several other oncogenic alleles that are not members of the *RAS* gene family have also been identified by the DNA transfection approach. Moreover, several of these genes identified are not related to the *v-onc* genes of the acute transforming viruses. Among the genes identified are *NEU*, *TRK*, *RET*, *MET*, *MAS*, *HST*, and *KS3*. While the *MAS* oncogene appears to be an integral membrane protein, the others are members of the tyrosine kinase growth factor receptor family (*NEU*, *MET*, *RET*, or *TRK*) or the fibroblast growth factor family (*HST*, *KS3*). Despite the successes of the DNA transfection approach in the identification of novel oncogenic alleles in human cancer, the approach is very labor intensive and has failed to detect a number of oncogenic alleles present in human cancers, in large part because the mutant alleles failed to cause morphologic transformation of rodent fibroblasts, the recipient cells typically used in the assays. Moreover, the approach often identified mutant alleles that were generated during the in vitro manipulations of the tumor-derived DNA and did not actually exist in the cancers from which the DNA was prepared.

**17.4.1.1 The *RET* Gene and Multiple Endocrine Neoplasia Type 2.** Unlike most inherited cancer predisposition syndromes, multiple endocrine neoplasia type 2 (MEN 2) results from the activation of a proto-oncogene, *RET*, rather than the inactivation of a TSG. *RET*, which maps to 10q11.2 and comprises 21 exons spanning >60kb, encodes a transmembrane tyrosine kinase. The gene was identified by the transfection method using DNA from a human monocytic leukemia cell line and a standard NIH-3T3 transformation assay. The extracellular portion of the *RET* protein comprises a cadherin-like domain in addition to a cysteine-rich motif, while its intracellular region contains two tyrosine kinase subdomains. The protein forms part of a receptor complex whose ligands include glial cell line-derived neurotrophic factor (GDNF), neurturin, artemin and persephin (Marx, 2005). The ligand specificity is dictated by the presence in the complex of GDNF family receptor (GFR) alpha proteins 1–4 that act as co-receptors. Ligand binding by the receptor induces its dimerization and the activation of its tyrosine kinase. This, in turn, leads to its auto-phosphorylation on tyrosine residues and the subsequent activation of signaling cascades, including the *RAS*/mitogen-activated protein kinase pathway. The receptor plays an important role during the development of the kidney and the enteric nervous system with GDNF acting to promote the growth of the ureteric bud and to protect the enteric neurons from apoptotic cell death.

Germline gain-of-function mutations in *RET* are linked to three dominantly inherited related cancer predisposition syndromes: multiple endocrine neoplasia (MEN) type 2A and 2B (MEN 2A and MEN 2B) and familial medullary thyroid cancer (FMTC) (Marx, 2005). Individuals affected by FMTC simply show familial clustering of medullary thyroid cancer (MTC). In those with

TABLE 17-5 Summary of Selected Familial Cancer Syndromes						
Syndrome	Inheritance in Familial Cases	Tumor	Associated Cancers/Trails	Chromosome Location	Responsible Gene	Principal Pathogenesis
Neurofibromatosis type 1 (NF1)	AD	Neurofibromas	Neurofibrosarcoma, brain tumors	17q11	<i>NF1</i>	Negative regulation of cell growth
Hereditary non-polyposis colorectal cancer (HNPCC)	AD	Colorectal cancer	Endometrial, ovarian, hepato-biliary, and urinary tract cancer	2p16 3p21 Others	<i>MSH2</i> <i>MSH2</i> <i>Others</i>	DNA mismatch repair
<i>MutYH</i> (or <i>MYH</i> ) associated polyposis (MAP)	AR	Colorectal cancer	Intestinal polyposis	1p34.1	<i>MUTYH</i>	DNA glycosylase
Familial adenomatous polyposis (FAP)	AD	Colorectal cancer	Intestinal polyposis Duodenal tumors Desmoid tumors Jaw osteomas Medulloblastoma	5q21	<i>APC</i>	Regulation of actin
Familial breast cancer	AD	Breast cancer	Ovarian cancer (especially with <i>BRCA1</i> )	17q21 13q12 17q22	<i>BRCA1</i> <i>BRCA2</i> <i>RAD51C</i>	Double-strand break repair
Familial melanoma	AD	Melanoma	Pancreatic cancer	9p21	<i>CDKN2A</i>	Encoding a regulatory protein
von Hippel–Lindau disease	AD	Renal cell carcinoma pheochromocytoma	Retinal angiomas, cerebellar hemangioblastomas	3p25	<i>VHL</i>	Indirect regulation of cell growth
Multiple endocrine neoplasia type 1 (MEN1)	AD	Parathyroid hyperplasia Pituitary adenomas Pancreatic islet cell tumours		11q13	<i>MEN1</i>	Probable regulation of cell growth
Multiple endocrine neoplasia type 2 (MEN2)	AD	Medullary thyroid cancer Pheochromocytoma	Parathyroid hyperplasia (in MEN2A) Mucosal neuroma (in MEN 2B)	10q11	<i>RET</i> proto-oncogene	Receptor tyrosine kinase
Cowden syndrome	AD	Breast & thyroid cancer	Endometrial cancer macrocephaly, mucocutaneous lesions	10q23	<i>PTEN</i>	Lipid metabolism
Hereditary diffuse gastric cancer	AD	Diffuse gastric cancer	Possibly lobular breast cancer	16q22	<i>CDH1</i>	Calcium and regulation of cell growth
Neurofibromatosis type 2	AD	Acoustic neuromas	Meningioma, gliomas, ependymomas	22q12	<i>NF2</i>	Linker protein in cell adhesion
Li-Fraumeni syndrome	AD	Sarcomas, breast cancer	Brain tumors, leukemia, adrenocortical carcinoma, others	17q13	<i>TP53</i>	Response to cell growth
Familial retinoblastoma	AD	Retinoblastoma	Osteosarcoma	13q14	<i>RB1</i>	Transcription control

MEN 2A, however, MTCs are often accompanied by pheochromocytomas and parathyroid hyperplasia. The most severe syndrome (MEN 2B) is characterized by the features of MEN 2A in addition to ganglioneuromas of the lips, tongue, and colon. In contrast to the gain-of-function point mutations in *RET* associated with these conditions, the loss-of-function *RET* mutations cause Hirschprung's disease. Rarely, Hirschprung's disease has even occurred in families with MEN 2, but the pathogenic mechanism is not yet fully understood.

The different MEN 2 and FMTC phenotypes are associated with different spectra of predisposing *RET* mutations. Missense mutations affecting a conserved cysteine residues (Cys 609, 611, 618, 620, or 634) in the cysteine-rich region of the extracellular domain of the RET have been found, by the International *RET* Mutation Consortium, in 98% of 203 MEN 2A families and in 79% of 30 FMTC families tested. It is presumed that these cysteine residues normally form intramolecular disulfide bonds and that the mutation of such a residue leaves an unpaired cysteine, which then forms an intermolecular disulfide bond with an adjacent mutated RET molecule. It is envisaged that the resulting homodimerization of the receptor leads to constitutive receptor activation. Cysteine 634 is far more commonly mutated in MEN 2A than in FMTC. The basis of the phenotypic difference between FMTC and MEN 2A patients is not yet clear, although it may result from differing levels of receptor activation.

In contrast, the mutations found in those with MEN 2B (and in some FMTC families) occur in one of the intracellular tyrosine kinase domains (Marx, 2005). In particular, a germline mutation involving the replacement of methionine 918 by a threonine residue is found in almost all MEN 2B cases (Marx, 2005). This methionine residue is situated in the substrate recognition pocket within the catalytic core of the tyrosine kinase. Its substitution leads to altered substrate specificity, with, for example, reduced binding to GRB2, and, presumably, aberrant cellular signaling. As might be expected, MEN 2B tumors have been found to have a different gene expression pattern from those in MEN 2A (Santoro et al., 2004). The intracellular FMTC-associated mutations, at codons 768, 790, 791, 804, 844 and 891, cause receptor activation in the absence of ligand binding and dimerization.

Finally, somatic *RET* mutations are uncommon in sporadic pheochromocytoma but somatic codon 918 *RET* mutations have been detected in 25% of sporadic MTC, and germline *RET* mutations have been reported in 6–23% of apparently sporadic cases of MTC. Following the success of the small molecule inhibitor STI571 (imatinib) in targeting tyrosine kinases, testing is now underway in several possible further targeted therapies. For instance, hereditary MTCs have been reported to be highly responsive to the tyrosine kinase inhibitor, vandetanib, which

appears to have specific activity for the mutated *RET* receptor (Imyanitov and Moiseyenko, 2011).

### 17.4.2 Oncogenes Identified by the Study of Chromosomal Translocations

The nonrandom chromosomal alterations observed in cancer cells have proven particularly valuable for the identification of oncogenic alleles. In many cases, a strong correlation has been established between a particular chromosomal abnormality and the type of tumor, and even its specific histopathological subtype. Over the past two decades, standard karyotypic analyses have identified an enormous number of specific chromosomal abnormalities in leukemias and lymphomas, perhaps in large part, because these malignancies can be induced to divide in culture more readily than many solid tumors (Messahel et al., 2005) and obtaining good quality metaphase chromosome preparations is considerably easier. Nevertheless, nonrandom chromosomal aberrations are increasingly being detected in solid tumors using either standard karyotypic analysis or fluorescence in situ hybridization-based approaches, and several oncogenic alleles generated by specific chromosomal abnormalities in solid tumors have been characterized (Messahel et al., 2005).

Many of the chromosomal abnormalities in lymphomas and lymphoid leukemias involve recombination between an immunoglobulin (Ig) or T-cell receptor (TCR) locus and a novel gene. Sequence analysis of the translocation joint sequences has revealed that the canonical heptamer–nonamer sequences used by the recombinase system during normal somatic rearrangement of the *Ig* and *TCR* genes have probably generated the translocation, as a result of a recombination error. The translocation juxtaposes the gene sequences present at the chromosomal break point with a TCR or an *Ig* locus, thus altering the normal structure and/or the expression pattern of the gene(s) present near the break point. For example, a gene that is normally expressed only in neural tissues or in developing embryonic tissues may be abnormally activated. The altered gene expression consequently leads to abnormalities in the proliferation and/or differentiation of the affected cells. In addition, because many of the translocations in lymphomas or leukemias involve *Ig* or *TCR* locus sequences, the subsequent proximity of the novel gene to the well-characterized *Ig* or *TCR* locus sequences has often allowed a novel gene to be identified rapidly by standard molecular cloning approaches. Among the genes that have been found to be commonly activated in lymphoid tumors by juxtaposition with *Ig* or *TCR* locus sequences are *MYC* and *BCL2*, both of which are discussed in greater detail below. Other genes that have been found to be activated in lymphoid tumors by juxtaposition with the *Ig* and *TCR* loci are genes encoding transcription factors, such as *TTG1*, *LYL1*, and *SCL1*, and



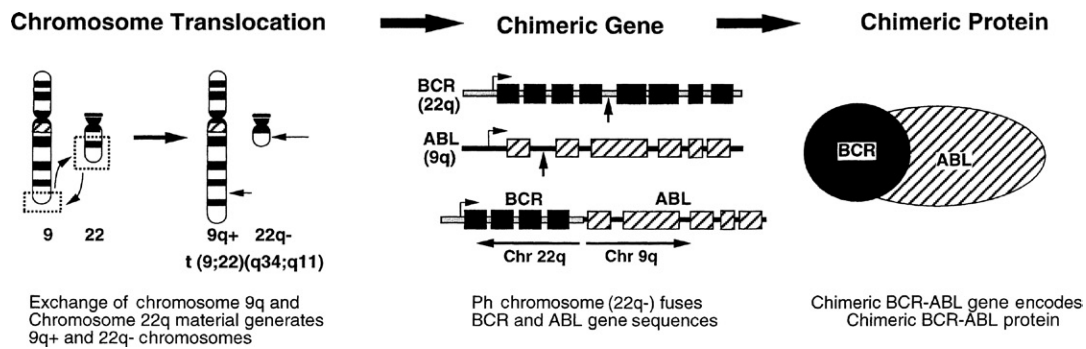


FIGURE 17-2

those encoding cell-cycle regulatory proteins, including *PRAD1*, *BCL1*, and *CCND1*.

A number of other translocation break points that do not involve the Ig or TCR locus have also been characterized in hematopoietic malignancies. Many of these other translocations, while deregulating gene expression, also generate novel chimeric proteins by fusing gene sequences from one side of the break point with the gene sequences on the other side of the break point. The first example of such an oncogenic allele encoding a chimeric protein was provided by the elucidation of the molecular basis of the Philadelphia chromosome. The Philadelphia chromosome was first described by Nowell and Hungerford in 1960, as a result of their karyotypic analysis of bone marrow cells from a patient with chronic myelogenous leukemia (CML). Subsequent studies in the 1970s established that the Philadelphia chromosome was generated by a translocation involving chromosomes 9 and 22. The Philadelphia chromosome can be identified by cytogenetic studies in the vast majority of patients with CML, as well as in some adult and pediatric patients with lymphoid leukemia. The translocation fuses chromosome 9q sequences from the *ABL* gene, a cellular homolog of a *v-onc* gene, with sequences from chromosome 22q (Figure 17-2). The novel sequences on chromosome 22q are termed the *BCR* (for break point cluster region) gene. The resultant chimeric BCR-ABL protein retains the active protein tyrosine kinase sequences from the *ABL* gene, but replaces the *ABL* regulatory sequences with *BCR* sequences. The net result is the generation of a protein kinase with altered structure and function.

**17.4.2.1 Imatinib Mesylate.** One of the most exciting developments in medicine in recent years has been the development of the clinically effective selective tyrosine kinase inhibitor, imatinib mesylate (Gleevec or STI-571, Novartis Pharmaceuticals). It has revolutionized the treatment of CML through its ability to inhibit BCR-ABL, inducing remissions with almost complete elimination of the signs and symptoms (Hehlmann et al., 2005). It was probably the first neoplasia for which the elucidation of the genotype led to the rational design of a targeted therapy and it now serves as a model for the development of molecularly targeted therapies. The drug may not, however, be able to eradicate leukemic

stem cells and resistance to imatinib can eventually arise through mutations in the *ABL* kinase domain (Komarova and Wodarz, 2005; Michor et al., 2005). On account of its ability to inhibit another tyrosine kinase, C-KIT, imatinib is also effective as a therapy for metastatic gastrointestinal stromal tumors (GISTs), which are generally difficult to treat with conventional chemotherapy (Chen et al., 2005). The recent review of targeted therapies by Stegmeier et al. describes the current uses of imatinib and discusses the importance of precise dosage in effecting optimal responses in treating CML and GIST (Stegmeier et al., 2011). In addition, the importance of molecular drug transporters in relation to the effectiveness of imatinib has now been determined (Eechoute et al., 2011). The success of imatinib and the subsequent generations of the constitutively activated *ABL* kinase have paved way for the development of several further small molecule inhibitors of mutated gene products. These include inhibitors of mutated *EGFR*, *ERBB2*, *KIT*, *PDGFRA*, *PML-RARA*, *MET*, and *ALK*, which are either in clinical trials or are already in clinical use (Stratton, 2011).

### 17.4.3 Oncogenes Identified by DNA Amplification

Several oncogenic alleles have been identified because they are contained within DNA sequences that become sequentially (tandem) repeated or amplified in tumor cells. In some cases, the amplified DNA sequences were first revealed in karyotypic analyses as extrachromosomal elements termed *double minute chromosomes* (DMs) or as novel banding regions on chromosomes termed *homogeneously staining regions* (HSRs). DMs and HSRs contain from 20 to several hundred copies of a chromosomal region of several hundred thousand base pairs. Biological selection for the generation and maintenance of the amplified DNA sequences in the tumor cells is thought to be driven by the increased copy number and increased expression of a target gene or genes within the larger region of the amplified DNA. Further detailed molecular genetic characterization of the amplified sequences is necessary to identify the specific target(s) genes in the larger region of the amplified DNA. In some

cases, a cellular homolog of a known *v-onc* gene is thought to be the target gene present in the amplified DNA. In other cases, characterization of the amplified DNA sequences has led to the identification of novel oncogenic alleles.

Among the cellular homologs of *v-onc* genes affected by DNA amplification in human tumors are the *MYC*, *MYB*, epidermal growth factor receptor (*EGFR*), and *ERBB2* (also known as *HER2* or *NEU*) genes. The *MYC* and *MYB* genes encode transcription factors, and the *EGFR* and *ERBB2* genes encode transmembrane receptor tyrosine kinase proteins. Other novel cellular proto-oncogenes that have been identified because they are present within the amplified DNA sequences include two genes, *N-MYC* and *L-MYC*, that are closely related to *MYC*, the *GLI* gene, the *MDM2* gene (see the *p53* tumor suppressor gene section), the *cyclin D1/PRAD1* gene (also activated by chromosomal translocation as noted above), and several genes encoding growth factors (e.g. *HST*, *INT2*). Because the DNA amplifications involve several hundred thousand base pairs, in some tumors more than one cellular proto-oncogene may be contained in the amplified sequences. One example of DNA amplifications affecting several proto-oncogenes is the amplification of chromosome 12q sequences in a subset of sarcomas. These amplifications often involve and activate the expression of the *MDM2*, *GLI*, and *CDK4* (see the section on p105-RB function below) genes.

Many candidate oncogenes have been identified by comparative genome hybridization and by studying changes in global gene expression in human solid tumors. Additionally, candidate oncogenes have been found to be amplified in certain tumor types. For example, telomerase activity has been linked to cellular immortality and tumor progression and is a potential target for chemotherapy. The human telomerase RNA gene (*hTERC*) was mapped to chromosome 3q26.2–q27, a region frequently subject to copy number gains in human tumors. Moreover, mutations in *hTERC* are associated with the rare, autosomal dominant form of dyskeratosis congenita, while the most common, X-linked, form is associated with mutations in the *DKC1* gene, encoding DYSKERIN, another protein closely involved in telomerase function (Bessler et al., 2004; Marrone et al., 2005).

#### 17.4.4 Oncogenes Identified by DNA Sequencing

A major program of cancer genome sequencing is currently underway at the Wellcome Trust Sanger Institute in Cambridge, United Kingdom, and elsewhere worldwide. The objective is to sequence the genomes of thousands of individual cancers. The data have revealed that these genomes usually contain between 1000 and 10,000 somatically acquired mutations (Stratton, 2011). The systematic sequencing of cancer genomes has led to

the discovery of new proto-oncogenes, including *IDH1*, which encodes isocitrate dehydrogenase 1 (a Krebs cycle component), *EZH2*, *JAK2* and *FOXL2* (encoding a tissue-specific transcription factor) and *BRAF* (Stratton, 2011). Somatic mutations in *BRAF* (which encodes a serine–threonine kinase) are particularly frequent, occurring in 50–70% of malignant melanomas, 10–15% of CRCs and in 50% of papillary thyroid cancers and one specific mutation V600E is especially common, facilitating molecularly targeted drug development. In fact, since the identification of *BRAF* as a mutated cancer gene in an early cancer genome sequencing screen in 2002, orally available small molecule inhibitors have been developed and tested in clinical trials and the mechanisms of emerging drug resistance have even been identified. The current catalog of identified cancer genes can be viewed at the Cancer Genome website (<http://www.sanger.ac.uk/genetics/CGP/Census/>) and shows that more than 1% of human genes are mutated in cancer (mostly somatically but 20% of these contain germline mutations).

### 17.5 TUMOR SUPPRESSOR GENES

As reviewed above, the identification of oncogenic alleles in human tumors has been facilitated by the prior identification of the *v-onc* genes, the characterization of sequences present at translocation break points, and the ability of the oncogenes to generate tumorigenic growth properties when transferred to nontumorigenic recipient cells. In contrast, the direct identification of TSGs has proven far more difficult because functional strategies for their identification have a number of practical problems. For example, although the successful transfer of a TSG to a tumor cell would be expected to suppress at least some of the altered growth properties of the tumor cell, such as anchorage-independent growth, lack of contact inhibition, unlimited life span, and tumorigenicity, the identification of suppressed cells in a background of fully transformed cells has proven to be a particularly difficult experimental task. Thus, the strategies to identify TSGs and the specific alterations in these genes in human cancers have been, by necessity, more circuitous.

#### 17.5.1 Historical Evidence for the Existence of Tumor Suppressor Genes

The studies of Henry Harris and his colleagues in 1988 formed the basis of the concept that the ability of cells to generate a tumor is a recessive trait, resulting, at least in part, from the inactivation of a class of cellular genes termed TSGs. These investigators first established that hybrids generated from the fusion of fully malignant murine tumor cells with nontumorigenic murine cells were often nontumorigenic when injected into mice. The hybrid cells, however, often reverted to tumorigenicity when passaged in culture, and the tumorigenic revertants

had chromosome losses detected when compared to the nontumorigenic hybrids. The interpretation of Harris and his colleagues, that is, that malignancy could be suppressed in somatic cell hybrids between tumorigenic and nontumorigenic cells, subsequently received support from many additional studies of somatic cell hybrids generated from the fusion of normal and tumorigenic rodent cells, as well as normal and malignant human cells. Hybrids retaining both sets of parental chromosomes were suppressed, with tumorigenic segregants arising most commonly after the loss of specific chromosomes from the normal parent. Moreover, it was found that tumorigenicity could be suppressed even if activated oncogenes, such as mutated *RAS* genes, were expressed in the hybrids.

The observation that the reversion to malignancy of the hybrids was associated with the loss of specific chromosomes contributed by the nontumorigenic cells suggested that specific chromosomes might be sufficient for suppression of tumorigenicity. Furthermore, the findings suggested that tumors must arise, in part, through the inactivation of a class of cellular genes termed TSGs. Evidence supporting the proposal that specific TSGs might be sufficient for the suppression of the tumorigenic phenotype was initially provided by studies in which single chromosomes were transferred using a technique known as microcell-mediated chromosome transfer. For example, the tumorigenicity of some cervical cancer cells or rhabdomyosarcoma cells was shown to be suppressed following transfer of human chromosome 11. There was a degree of cell-type specificity in relation to the ability of a particular chromosome to suppress tumorigenicity because chromosome 11 failed to suppress the tumorigenicity of some neuroblastoma and renal cell cancer cells. Furthermore, although the ability of the cells to form tumors in immunocompromised (“nude”) mice and to grow in an anchorage-independent fashion could be suppressed following single-chromosome transfer, other traits characteristic of the parental tumor cells (e.g. unlimited life span in culture) were still retained in the suppressed hybrids. The observation that not all properties of the parental tumor cells could be suppressed by a single chromosome (and perhaps a single TSG), however, is clearly consistent with the proposal that tumors arise as a result of multiple genetic alterations.

While the somatic cell genetic studies of tumorigenesis reviewed above did not lead directly to the identification of TSGs, they did provide persuasive evidence that a subset of the mutations in human cancers must be in genes of this type. In addition, concurrent with the initial cell fusion experiments of Harris and his colleagues, other epidemiological and genetic studies also suggested an important role for the inactivation of cellular genes in cancer development. Many of these studies were first carried out on retinoblastoma, and some of the critical observations from these studies are reviewed below.

## 17.5.2 The Retinoblastoma Gene

**17.5.2.1 Retinoblastoma: A Paradigm for Tumor Suppressor Gene Identification.** The relative rarity of retinoblastoma belies the importance of observations gleaned through the study of this tumor. Indeed, many of the concepts and techniques used to identify and characterize TSGs and inherited and somatic genetic alterations in cancer were first established through the studies of the genetic basis of retinoblastoma. As is true of a number of cancer genes, however, the precise functions of the protein that the underlying gene encodes are currently still being elucidated (see below).

Retinoblastoma is a pediatric tumor that occurs sporadically in many patients but also occurs in some families in a pattern consistent with autosomal dominant inheritance. Knudson’s analysis of the age-specific incidence of retinoblastoma led him to propose that two distinct mutagenic events were necessary for the development of retinoblastoma in all patients. Knudson’s hypothesis is thus often termed “the two-hit hypothesis.” He proposed that in those patients with the inherited form of the disease, one mutation was present in the germline and in all cells of the body. A second mutational event occurring in any one of the developing retinoblasts would then lead to the generation of a retinoblastoma. Thus, in a patient with the inherited form of the disease, one or more retinoblastomas might likely develop; however, the number of retinoblastomas arising in an inherited case might well be expected to vary from none to three or four, depending on the frequency of second mutations. With this model, Knudson could account for the absence of disease in some patients known to harbor the predisposing mutation (i.e. skipped generations), as well as the presence of bilateral or multifocal disease in some affected family members. In those with the sporadic form of retinoblastoma, Knudson proposed that both the mutational events were somatic and arose in the same developing retinoblast. The least probability that the two somatic mutations would occur in the same retinoblast was consistent with the low prevalence of retinoblastoma in the general population. Although each of the two mutational events could have been predicted to affect different genes, Knudson subsequently proposed that the two mutational events affect the two parental copies of a retinoblastoma TSG (subsequently referred to here as the *RB1* gene). Thus, while retinoblastoma could be inherited as a dominant trait at the phenotypic level, at the cellular level, mutations in the *RB1* gene were predicted to be recessive, such that both parental copies of the gene would need to be inactivated in a cell for a retinoblastoma to develop.

The significance of Knudson’s hypothesis should not be underestimated. The two-hit hypothesis not only served as a framework for considering the mechanisms through which inherited and somatic genetic mutations might interact in tumor development, but it also linked

the notion of recessive genetic determinants for human cancer development to the somatic cell genetic observations demonstrating that the ability to form tumors is often a recessive trait.

Cytogenetic studies of peripheral blood lymphocytes from retinoblastoma patients provided the first clues to the location of the retinoblastoma predisposition gene. In about 5% of patients with retinoblastoma, interstitial deletions involving band q14 of chromosome 13 were identified in peripheral blood lymphocytes or normal skin fibroblasts. It was thus hypothesized that a gene predisposing to retinoblastoma development must reside at 13q14. Subsequent studies noted that the levels of esterase D, an enzyme of unknown physiological function, were reduced in normal cells from some patients with germline chromosomal deletions involving 13q14, as compared to karyotypically normal family members. Therefore, in at least a subset of patients with deletions of chromosome band 13q14, the deletions were likely to affect not only one copy of the *RB1* gene but also the esterase D gene and perhaps several other genes.

Further examination of the esterase D locus in those with retinoblastoma yielded several other critical insights into the genetics of retinoblastoma. Analysis of the segregation patterns of polymorphic esterase D isozymes in several retinoblastoma kindreds in which the affected members lacked cytogenetic alterations of 13q14 provided strong evidence for genetic linkage between the esterase D locus and the retinoblastoma predisposition gene. In addition, studies of esterase D proved particularly illustrative in a child with inherited retinoblastoma who had no detectable cytogenetic alteration in her normal cells. Though a cytogenetic alteration was not apparent, this child was noted to have an ~50% reduction of esterase D levels in peripheral blood cells. Of even greater interest, tumor cells from this patient had no detectable esterase D activity, despite having one copy of chromosome 13. Based on these observations, Benedict, Sparkes, and their colleagues proposed that in all cells of the patient, one copy of chromosome 13 was affected by a submicroscopic deletion. This submicroscopic deletion inactivated one copy of the *RB1* gene and one copy of the esterase D gene. During tumor development, the normal copy of chromosome 13 was lost, leaving only the defective chromosome 13 in the tumor cells. Based on their findings in this patient, these investigators concluded that the predisposing mutation in the patient, the submicroscopic 13q deletion involving the *RB1* gene and the esterase D gene, was recessive at the cellular level. The effect of the predisposing mutation could be observed, however, by the inactivation of the remaining normal copy of the *RB1* gene, such as by its loss in the tumor cells. These studies thus provided the first direct experimental evidence supporting Knudson's two-hit hypothesis for retinoblastoma development.

To establish the generality of this two-hit model for retinoblastoma, Cavenee, White, and their colleagues undertook the studies of additional cases of inherited retinoblastoma as well as sporadic cases, using DNA probes from chromosome 13. The probes detected DNA sequence polymorphisms and thus allowed the two parental copies of chromosome 13 to be distinguished from one another in normal and tumor tissues from each patient. Following the comparison of the parental alleles present in the paired normal and tumor samples from each patient, Cavenee et al. demonstrated that the loss of heterozygosity (LOH, also termed allelic loss; i.e. the loss of one parental chromosome) for chromosome 13 had occurred during the development of more than 60% of retinoblastomas studied. They also demonstrated that LOH could occur by a number of different mechanisms (Figure 17-3). The unmasking of the recessive predisposing mutations at the retinoblastoma locus, whether the mutation had been inherited or had arisen somatically, occurred through the same chromosomal mechanisms. Furthermore, the study of inherited cases confirmed that the chromosome 13 homolog retained in tumor cells that had suffered chromosome 13 LOH was always derived from the affected parent.

Previous epidemiological studies had established that patients with the inherited form of retinoblastoma were also predisposed to the development of osteosarcomas, with these lesions most often arising in areas that had been exposed to radiotherapy. Study of chromosome 13q LOH in osteosarcomas arising in patients with retinoblastoma, as well as in apparently sporadic cases of osteosarcoma, revealed that the majority of osteosarcomas studied had undergone LOH for the 13q region containing the *RB1* gene. Such findings established a genetic link between retinoblastoma and osteosarcoma.

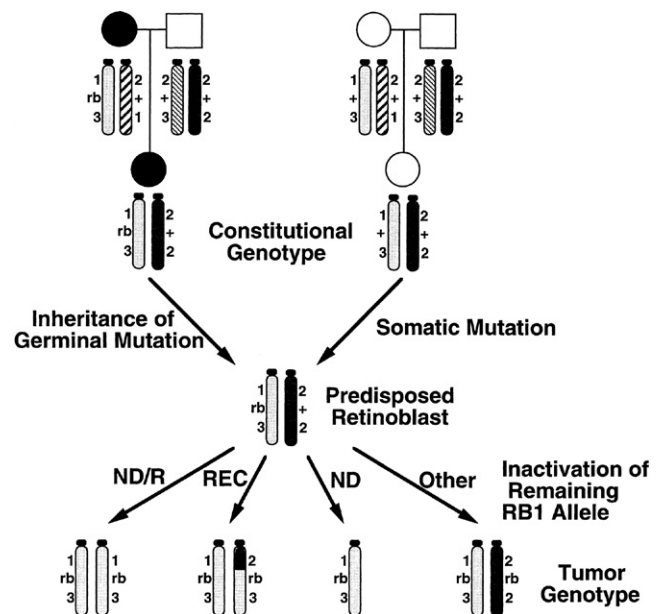


FIGURE 17-3



The studies of chromosome 13q LOH in retinoblastomas and osteosarcomas were therefore particularly instructive. They provided definitive evidence in support of Knudson's hypothesis and illustrated the multiple chromosomal mechanisms that could unmask the pre-existing recessive mutations at a tumor suppressor locus. The studies also established that similar genetic mechanisms could account for both inherited and sporadic cases of a particular type of cancer. Finally, the DNA-based studies provided genetic evidence that inactivation of the same TSG was responsible, at least in part, for the development of two distinct tumor types.

**17.5.2.2 Molecular Cloning and Mutational Analysis of the Retinoblastoma Gene.** The molecular cloning of the *RB1* gene was greatly facilitated by the identification of a chromosome 13q14 DNA marker that detected rearrangements in Southern blot analysis of two retinoblastomas and the complete loss of both retinoblastoma alleles in a third case. Following its identification, it was established that *RB1* is a large gene with 27 exons, spanning more than 200 kb of genomic DNA. The penetrance of *RB1* germline mutations is high and it has been estimated that 90% of individuals with a germline *RB1* mutation would develop retinoblastoma.

The cloning of the *RB1* gene allowed definitive studies of the inherited and somatic mutations present in the gene. Although gross deletions, of either inherited or somatic origin, affect the *RB1* gene in a subset of retinoblastomas and osteosarcomas, most of these tumors do not have gross alterations of the gene. In fact, most mutations inactivating the *RB1* gene are point mutations or small insertions and deletions that result in premature truncation of the protein product. Several missense and splicing mutations have also been identified in primary retinoblastomas and osteosarcomas. Almost all *RB1* tumorigenic mutations result in the disruption or loss of the "pocket" domain of the retinoblastoma protein (pRB) that is required for interaction with E2F protein and viral oncoproteins (see below). In general, no clear genotype–phenotype association has yet emerged. As predicted by Knudson's hypothesis and the studies of 13q LOH, both *RB1* alleles are inactivated in retinoblastomas and osteosarcomas.

In addition to the identification of germline and somatic mutations that inactivate the *RB1* gene in retinoblastomas and osteosarcomas, functional evidence indicates that the *RB1* gene can suppress aspects of the tumorigenic phenotype when a cloned copy is introduced into retinoblastoma or osteosarcoma cells growing in culture. At least in some cases, successful transfer of a normal copy of the *RB1* gene to retinoblastoma or osteosarcoma cells results in changes in cell morphology and the suppression of growth in culture or in nude (immunocompromised) mice. In fact, there is evidence that *RB1*, which is often regarded as a prototypical tumor suppressor, can regulate not only cellular proliferation but also differentiation (Nguyen and McCance, 2005).

Since its initial cloning in 1986, *RB1* mutations have been extensively analyzed in many tumor types other than retinoblastoma and osteosarcoma. Some of these studies were motivated by observations that 13q LOH was prevalent in other tumor types. The mutations inactivating the *RB1* gene in other tumor types are similar to those in retinoblastomas and osteosarcomas and include frameshift and nonsense mutations that result in a truncated and often unstable protein; however, the prevalence of mutations in the *RB1* gene varies markedly from one tumor type to another. For example, *RB1* mutations have been observed in nearly 100% of small cell carcinomas of the lung and in about 10–20% of breast, bladder, pancreatic, and prostate carcinomas, while less than 5% of primary colorectal carcinomas contain *RB1* mutations.

Inactivation of the pRB protein in tumors can also occur indirectly, by altered expression or activity of its upstream regulators (see below), such as p16/INK4A in melanomas and pancreatic carcinomas and CDK4 in glioblastomas (Liu et al., 2004a, 2004b).

At least at first glance, the identification of somatic *RB1* mutations in tumor types other than retinoblastoma and osteosarcoma is somewhat puzzling. Patients with germline mutations in the *RB1* gene are at significantly elevated risk (relative to the general population) of developing a rather limited spectrum of tumors, including retinoblastoma, osteosarcoma, and occasionally soft tissue sarcoma and melanoma. Patients with germline mutations in the *RB1* gene do not, however, appear to be at elevated risk of the development of other tumor types in which somatic *RB1* mutations are relatively common. Given that the *RB1* gene is ubiquitously expressed in adult tissues and that its inactivation apparently contributes to the development of a number of common human cancer types, it is not yet clear why those individuals possessing germline *RB1* mutations are not predisposed to a greater variety of cancer types. Nevertheless, the data suggest that in some cell types, such as retinoblasts, the *RB1* gene is one of the primary controlling elements in growth regulation, while in other cell types, such as mammary and prostatic epithelial cells, the growth regulatory pathways in which the *RB1* gene plays a role may be more redundant.

The cloning of the *RB1* gene ushered in a new era in the management of patients and families with inherited cancer syndromes. Those individuals who carried germline mutations could now be identified and closely followed, while those who had not inherited a specific *RB1* mutation possessed by a parent could be spared anxiety and frequent clinical examinations.

**17.5.2.3 Function of the Retinoblastoma Protein.** The protein product of the *RB1* gene, pRB (also termed RB or p105-RB), is a nuclear phosphoprotein of 928 amino acids residues with a molecular mass of about 105–110 kDa. The first critical insights into the function of pRB were provided by the studies of Ed Harlow

and his colleagues in 1988. They demonstrated that the E1A oncoprotein of the murine DNA tumor virus adenovirus type 5 complexed with pRB. Prior studies had established that E1A had many functions, including cell immortalization, induction of DNA synthesis, cooperation with other oncoproteins in transformation, and regulation of viral and host gene transcription. The studies of the Harlow group suggested that the complexing of E1A with pRB might account for some of E1A's effects on cells. Furthermore, they provided compelling evidence that the binding of E1A to pRB not only was physiologically significant but was absolutely critical to E1A's ability to mediate neoplastic transformation. In particular, they demonstrated that mutant E1A proteins that were unable to bind pRB were also unable to transform cells.

The significance of this physical interaction between a TSG product and a DNA tumor virus oncogene product was reinforced by the subsequent demonstration that several other DNA tumor virus proteins could bind to pRB, including simian virus type 40 (SV40), large T antigen and the E7 protein of human papillomavirus (HPV) type 16 (Figure 17-4). Mutations that inactivated the transforming activities of these proteins were found to inactivate their ability to complex with pRB. In addition, while HPV E7 proteins from the “high-risk” viruses (i.e. those strongly associated with cancer development), such as HPV types 16 and 18, were shown to complex tightly with pRB, HPV E7 proteins from “low-risk” viruses (e.g. HPV types 6 and 11) showed reduced binding to pRB. It is now recognized that these viral proteins interact, via their LXCXE motif, with the so-called “pocket” domain of pRB, the 3-D structure of which has now been solved.

Although the DNA tumor virus proteins inactivate the function of pRB by directly complexing with it, pRB is physiologically inactivated by protein phosphorylation during the normal progression through the cell cycle. Indeed, pRB appears to exist in a predominantly unphosphorylated or hypophosphorylated state in the G1 phase of the cell cycle and maximally phosphorylated in G2 (Figure 17-5). The critical phosphorylation events regulating the function of pRB are mediated, at the boundary between the G1 and S (DNA synthesis) phases of the cell cycle, by protein complexes that consist of a cyclin (especially cyclins D1 and E) and also a cyclin-dependent kinase (CDK). There are 16 potential CDK phosphorylation sites on pRB, and it appears that different cyclin/CDK complexes phosphorylate distinct subsets of sites, possibly in response to different growth factor signaling pathways. These phosphorylation events at the G1-S boundary reduce the ability of pRB to bind to certain cellular proteins, including members of a family of at least eight transcriptional regulatory proteins known as the E2F proteins (Tsantoulis and Gorgoulis, 2005). When it is not phosphorylated, pRB complexes with the E2F protein and prevents it from activating gene expression,

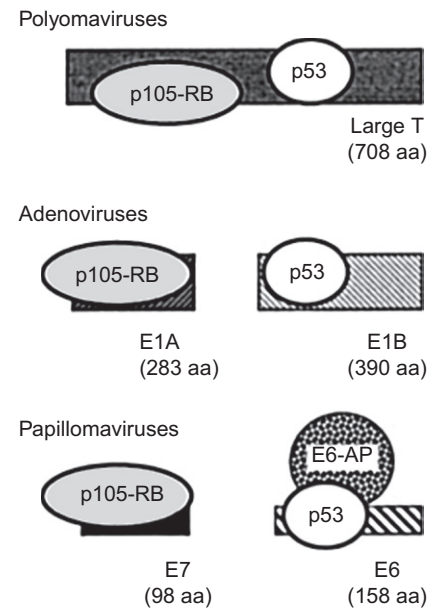


FIGURE 17-4

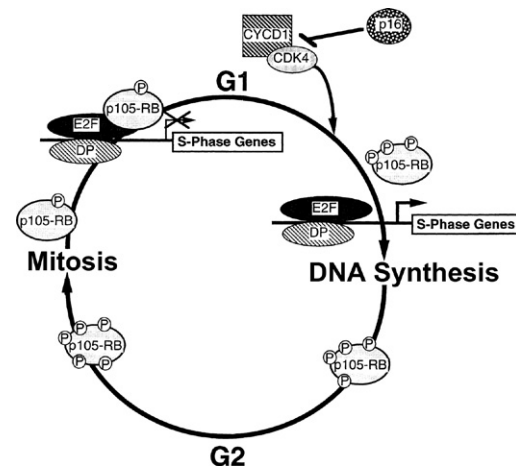


FIGURE 17-5

although the latter may remain bound to the promoter of its target gene. This inhibition of E2F-mediated transcriptional activation is not simply due to masking by pRB of the activation domain of E2F. Rather, pRB, bound to E2F, shuts off transcription in an active way by recruiting a histone deacetylase (HDAC1) protein (Sherr, 2004), leading to local changes in chromatin structure that result in the transcriptional repression of the gene to which the E2F protein is bound (Tsantoulis and Gorgoulis, 2005).

The transcriptional repressor complex of E2F–pRB–HDAC is believed to be disrupted by binding of a viral oncoprotein to pRB, mutation of pRB, or, physiologically, by the phosphorylation of pRB. In the resulting nonrepressed state, the E2F protein, heterodimerized with one of at least two members of the DP protein family, can activate the expression of its target genes (Figure 17-5). Further support for the role of pRB in

regulating transcription through chromatin remodeling has been provided by the detection of complexes containing pRB together with HDAC and the nucleosome disrupting proteins, hSWI/SNF. The *E2F* target genes include a number of genes that are likely to play a critical role in the cell's transition into S phase, including DNA polymerase alpha, thymidine kinase, dihydrofolate reductase and alternative reading frame (ARF). In fact, it may be the increased expression of the latter and the consequent stabilization of p53 protein (Harris and Levine, 2005) (see below), which causes the apoptosis that normally follows the phosphorylation of pRB.

Increasing evidence suggests that pRB may also bind to other cellular proteins. For instance, the C-domain of pRB, located at the C-terminal to the pocket domain, binds to C-ABL (the function of which it inhibits) and to MDM2. In fact, given that MDM2 is a critical regulator of *TP53* (see below) and that a trimeric complex of pRB–MDM2–p53 has been detected, it is likely that pRB and *TP53* act together in regulating apoptosis. In addition, pRB binds to and inhibits TAF250 kinase, a component of the important TFIID transcription factor complex, and this interaction may contribute to the transcriptional repression by pRB discussed above. Phosphorylation of the A-domain within the pocket region of pRB blocks its interaction with many proteins, including the E2F1, E2F2, E2F3 and BRG1 proteins, while the phosphorylation of the C-terminus of pRB blocks the interaction with HDAC (Cobrinik, 2005).

In addition to the repression of the expression of E2F-regulated genes, it now appears that pRB plays an important role in genomic stability (Amato et al., 2009; Manning et al., 2009; Coschi et al., 2009). In particular, loss of pRB function can cause different types of genomic change. These include changes in ploidy at the chromosomal and the subchromosomal levels. For instance, pRB inactivation leads to local amplifications, chromosome arm gains and losses and even an increased frequency of whole chromosome missegregation with losses or gains of whole chromosomes (chromosome instability or CIN). The latter is thought to result from abnormalities in the mechanism by which chromosome segregation takes place or is regulated. Such abnormalities include so-called merotelic kinetochore attachments; that is, the interaction of microtubules from both spindle poles to a single kinetochore. Currently, however, pRB inactivation appears to play a predominantly indirect role by, for instance, blocking the normal recruitment and the localization of CAP-D3/condensin II and cohesin (Manning et al., 2009; Manning and Dyson, 2011).

**17.5.2.4 Other Pocket Proteins.** Several other nuclear phosphoproteins with important roles in growth regulation share significant similarity with the retinoblastoma protein: In particular, the proteins known as p107 and p130, which, together with pRB, are known as the “pocket proteins.” These pRB-related proteins may possess physiological functions that are closely related to

those of pRB and have, in fact, also been found to complex with the E1A, SV40 large T antigen, and HPV E7 DNA tumor virus proteins. In addition, p107 has been found to substitute partly for pRB in the negative regulation of E2F1–E2F3 (Cobrinik, 2005). All three pocket proteins can regulate cell cycle progression and induce cell cycle arrest in G1 when they are overexpressed (Tsantoulis and Gorgoulis, 2005). There seems to be a certain degree of redundancy, however, between p107 and p130. In addition, mice deficient in either p107 or p130 develop normally while mice deficient in pRB die as embryos. Thus, these two pocket proteins are not yet fully regarded as classical tumor suppressors (Tsantoulis and Gorgoulis, 2005).

### 17.5.3 The *TP53* Tumor Suppressor Gene

The p53 protein was first identified in the late 1970s because it formed a tight complex with the SV40 large T antigen. It was named p53 because it is a phosphoprotein with a relative molecular mass of about 53,000 Da. Subsequent studies established that the p53 protein formed complexes with other polyomavirus large T antigens and the adenovirus E1B oncoprotein (Figure 17-4). While the p53 protein was present in small amounts in normal cells and was metabolically labile, high levels of p53 protein were present in many tumors and tumor cell lines. In addition, transfection of *TP53* expression constructs was shown to immortalize some cells. Furthermore, *TP53* appeared to cooperate with some oncogenes, such as mutated *H-RAS*, to transform primary rat embryonic fibroblasts cells to immortal, tumorigenic cells. The initial interpretation of these observations was that p53 functioned in a positive fashion to participate in tumorigenesis and that *TP53* appeared to be an oncogene.

Many subsequent studies, however, indicated that *TP53* was a TSG rather than an oncogene. For example, in Friend virus-induced mouse erythroleukaemias, the *TP53* gene was found to be a frequent target site for viral integration, and many of the integration events led to *TP53* inactivation. Rearrangements and deletions that appeared to completely inactivate *TP53* were also observed in the HL60 human promyelocytic leukemia line and in several human osteosarcomas. Furthermore, reexamination of the cellular transformation studies revealed that the cloned murine *TP53* genes used in the transformation assays were not wild type, but contained point mutations in their coding sequences. Both the wild-type murine and human *TP53* genes were shown to be incapable of mediating transformation in collaboration with *RAS* oncogenes, and the wild-type *TP53* gene was found to inhibit the transforming ability of mutated *TP53* and other oncogenes. Moreover, it was realized that the p53 protein that was found to be abnormally abundant in many tumors was usually transcriptionally inactive.

**17.5.3.1 Inactivation of the *TP53* Gene in Human Cancer.** Evidence that the *TP53* gene might be frequently inactivated in human cancers was initially provided by studies demonstrating that the LOH for chromosome 17p alleles was common in a number of tumor types, particularly CRC. Because the *TP53* gene was contained in the 17p region frequently affected by LOH, sequence analysis of the *TP53* alleles retained in colorectal tumors with 17p LOH was carried out. This analysis identified point mutations in the great majority of cases. Subsequent analysis of other tumor types with 17p LOH, including breast, lung, bladder, and brain tumors also revealed frequent mutations in the *TP53* coding region.

Mutations in *TP53* are now recognized to be very prevalent in a wide spectrum of human cancers. Indeed, *TP53* is believed to be one of the most commonly mutated genes in human cancer and loss of functional *TP53* occurs in over 50% of human tumors. The overwhelming majority of the mutations identified are located in the central region of the *TP53* coding sequences and result in the synthesis of a p53 protein with a missense substitution that usually abrogates p53's DNA-binding activity (Figure 17-6). Although *TP53* is inactivated in around 50% of human cancers by somatic mutations, in a further 20% of cancers the function of p53 is inactivated by other mechanisms. For example, in cervical cancers, the main known risk factor is the infection with the so-called "high-risk" or cancer-associated HPVs (i.e. HPV types 16 and 18). The E6 proteins encoded by the high-risk HPVs bind a cellular protein called E6-AP (for E6-associated protein). The resulting complex binds p53 and mediates its degradation via the ubiquitin pathway (Hengstermann et al., 2005) (Figure 17-4). Cancer-associated HPVs are present in the vast majority of cervical cancers, and only a small subset of cervical cancers has somatic *TP53* mutations. In the rare instances when a cancer-associated HPV E6 protein and a cellular *TP53* mutation are both present, the cancers may behave more aggressively.

In a subset of soft tissue sarcomas, a cellular protein known as MDM2 is overexpressed as a result of gene amplification involving sequences on chromosome 12q. When expressed at high levels, the *MDM2* gene functions as an oncogene. It is now well established that the MDM2 protein can bind to and inactivate the p53 protein and that MDM2 is a key physiological regulator of p53. It achieves this by masking p53's transactivation domain, and also by ensuring that p53 is a short-lived protein. MDM2 accomplishes the former by interacting with p53's N-terminus and the latter by acting as an E3 ubiquitin ligase, thus promoting p53's destruction in the proteasome. Responses of p53 in vivo are believed generally to require the stabilization of the protein, principally through reduced MDM2 binding. This occurs, for example, following (a) phosphorylation of serine 15 or serine 20 in the N terminus of p53 following the activation of the checkpoint kinase CHK2, DNA-dependent protein kinase (DNA-PK) and ataxia-telangiectasia mutated (ATM) in response to DNA damage (e.g. by gamma irradiation); or (b) transcriptional activation of p14/19ARF caused by oncogene activation. It is by binding via its amino terminus to MDM2, that p14/19ARF, (which is encoded by an ARF) at the same locus as the p16INK4A CDK inhibitor, prevents MDM2 from inducing degradation of p53. In addition to the stabilization of the protein, full activation of p53 appears to require the acetylation of lysine residues 320 and 382 at its C terminus by PCAF and p300, respectively.

**17.5.3.2 Li-Fraumeni Syndrome.** Li-Fraumeni syndrome (LFS) is a rare autosomal dominantly inherited and highly penetrant condition with a predisposition to breast and brain tumors, soft tissue sarcomas, osteosarcomas, adrenocortical carcinomas and leukemia. In addition to the somatic mutations of the *TP53* gene in a variety of different tumor types, inherited *TP53* gene mutations have been identified in 60–77% of individuals with LFS (as strictly defined) and in 20–40% of patients

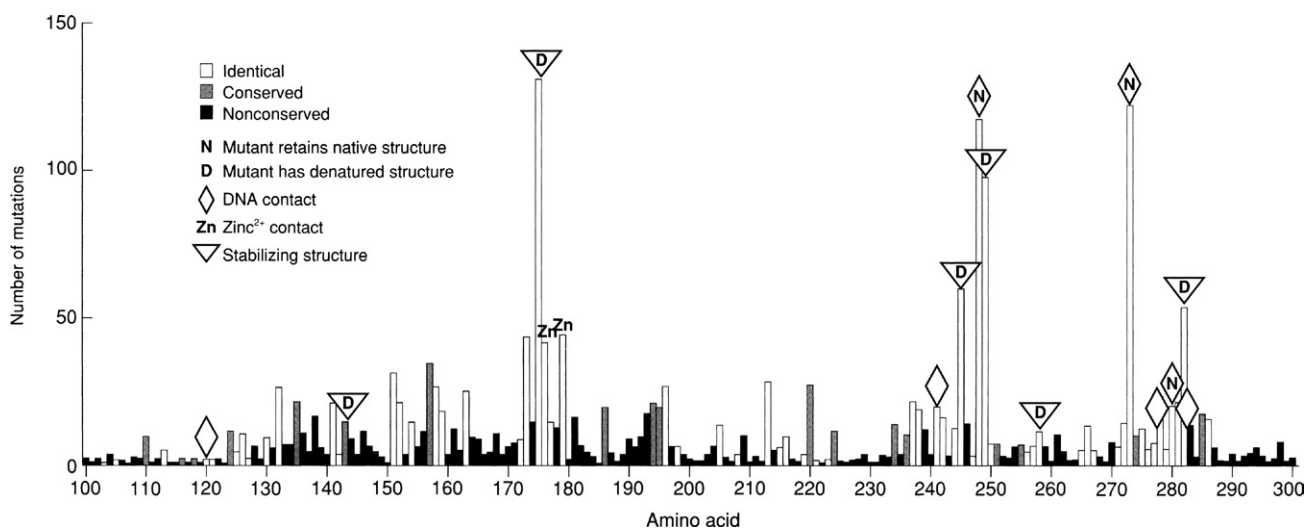


FIGURE 17-6



with Li–Fraumeni-like syndrome (Varley, 2003; Barlow et al., 2004), as well as in a subset of pediatric patients with tumors such as osteosarcomas, who do not meet more strict criteria for LFS. A high proportion of children with adrenocortical carcinomas, in particular, possess germline mutations in *TP53* (Varley, 2003). LFS has been strictly defined as a proband with any childhood tumor, or a sarcoma, brain tumor, or adrenocortical tumor in those aged under 45, plus a first- or second-degree relative in the same lineage with a typical LFS tumor at any age and also an additional first- or second-degree relative in the same lineage with any cancer under the age of 60 years.

The best method for screening patients in LFS families or those with germline *TP53* mutations is still uncertain, but may include MRI for women at risk of breast cancer and abdominal ultrasound for children. In view of the radiation sensitivity of *TP53* mutant cells, radiotherapy should be avoided if possible (Varley, 2003). Given the ubiquitous expression of *TP53* in adult tissues, the basis of the tissue specificity of *TP53*-related tumorigenesis remains to be elucidated. It does not simply correspond to those tumor types in which somatic mutations are common. Mutations located within the central core (DNA-binding) domain of the gene tend to be associated with a gain of function and, unlike many TSGs, do not require a subsequent inactivation of the wild-type allele for tumorigenesis (Varley, 2003). This dominant-negative effect may result from the ability of the p53 protein monomers to bind to other similar proteins, forming homotetramers. In LFS families possessing wild-type *TP53* genes, heterozygous germline mutations have, albeit rarely, been found in the *CHK2* gene (Barlow et al., 2004) but several other candidate LFS genes have now been excluded, including *MDM2*, *PTEN*, *CDKN2A*, *BCL10*, *CHK1*, *TP63* and *BAX* (Barlow et al., 2004; Varley, 2003).

**17.5.3.3 *TP53* Function.** The p53 nuclear phosphoprotein functions primarily in transcriptional regulation, though it may also have other functions (Yee and Vousden, 2005). The central core domain of the 393 amino acid protein binds to specific DNA sequences,

and its aminoterminal sequences have a transcriptional activation function (Figure 17-7). The carboxy-terminal sequences appear to be critical to the ability of the p53 protein to form homodimers and tetramers with itself. The p53 protein has been implicated in the positive regulation of several genes with critical roles in the control of the cell cycle, including the *p21WAF1/CIP1* gene, which regulates CDK activity, and the *GADD45* (for growth arrest DNA damage inducible) gene (Figure 17-8). Hundreds of other p53-induced genes have now been identified, including those encoding 14–3–3 sigma, which, like p53-induced *GADD45*, is reported to lead to cell cycle arrest in G2 and proapoptotic proteins such as PUMA and NOXA that now appear to represent critical p53 targets in triggering apoptosis (Hemann et al., 2004), and BAX. The transcription of *MDM2* is also positively regulated by p53, while that of ARF is reported to be repressed by p53, providing negative feedback control. This explains the detectable increase in p53 protein levels in tissues possessing mutated *TP53* that is transcriptionally inactive. The vast majority of the mutations identified in *TP53* alleles of human tumors inactivate the DNA-binding activity of the p53 protein and its ability to induce target gene expression. Based on its 3-D structure, many of the mutations affect amino acids that mediate p53's contact with DNA. In other cases, the mutations generate misfolded p53 proteins that can no longer bind to the specific DNA recognition sequence (Figure 17-6).

Other studies suggest that p53 may also function as a repressor of transcription and that this may contribute to the biological effects of p53. For example, p53 has been reported to repress expression of (a) the growth-promoting genes *C-FOS*, *C-MYC*, *IL2*, *IL4*, *IL6* and the insulin receptor; (b) the genes encoding the transcription factors, TBP, SP1, estrogen receptor, and hypoxia-inducible factor; and (c) the genes encoding the anti-apoptotic proteins, BCL-2, MAP4 and survivin (Slee et al., 2004). This repressive effect is believed to be mediated by the C-terminal region of p53 but, unlike p53's ability to activate transcription, it may not be sequence specific since many of the promoters of these

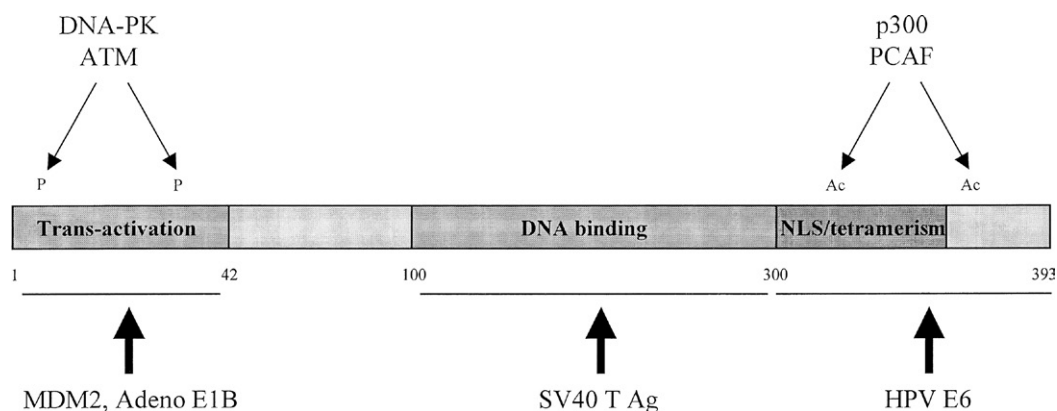


FIGURE 17-7

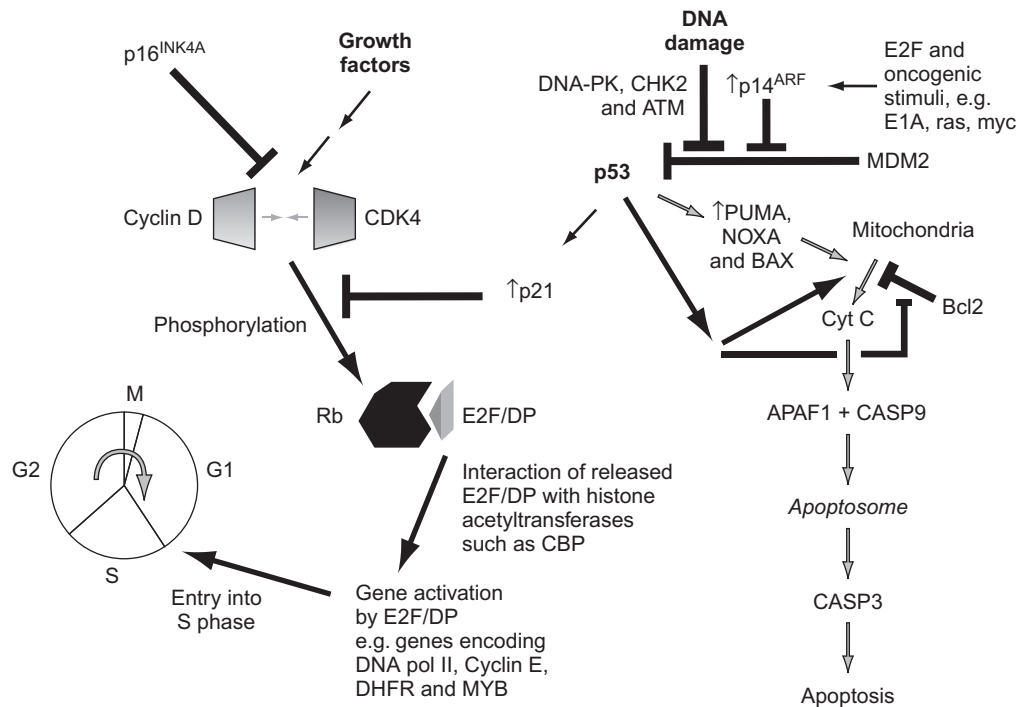


FIGURE 17-8

p53 transrepressed genes do not possess p53 binding sites (Slee et al., 2004).

Several cellular functions for p53 have been identified (Yee and Vousden, 2005). These include the regulation by p53 of the G1/S checkpoint of the cell cycle, preventing the cell from entering the DNA synthesis phase and also the ability of p53 to induce programmed cell death (apoptosis). Of great additional interest, particularly with regard to cancer pathogenesis and therapy, is that several studies have demonstrated that p53 possesses a critical role in the cellular response to DNA damage. Specifically, cells with wild-type p53 function appear to arrest in the G1 phase of the cell cycle in response to DNA damage thereby allowing the damaged DNA to be repaired before new DNA is synthesized. Cells lacking functional p53 fail to arrest and thus may replicate a damaged DNA template.

The ability of p53 to promote apoptosis appears to involve not only the transcriptional activation of proapoptotic activators such as PUMA and NOXA, but also an extra-nuclear p53 function. It is now recognized that p53 functions like the “BH3-only” proteins at mitochondria. It thus activates, by protein–protein interactions, the proapoptotic proteins BAX and BAK, leading to mitochondrial membrane permeabilization (Chipuk et al., 2004; Leu et al., 2004). The role of p53 in inducing apoptosis is crucial in preventing carcinogenesis. Mouse studies have indicated that the ability of p53 to induce a proliferative block, contributing to the maintenance of genomic stability, may be as important as the induction of apoptosis (Liu et al., 2004a, 2004b).

One of the goals of a number of ongoing drug-discovery programs is to identify small-molecule chemotherapeutic

agents that might restore or enhance wild-type p53 function in cancer cells. These agents might then be used either by themselves or in combination with conventional cytotoxic agents or radiotherapy. An apparently very promising approach is to inhibit the normal MDM–p53 interaction for instance by using small molecules to either inhibit the E3 ubiquitin ligase of MDM2 (Yang et al., 2005) or to block specific MDM2 interaction sites on p53 (Vassilev et al., 2004). Other future opportunities have arisen through the recognition of molecules other than ubiquitin that can modify and thus regulate p53, such as SUMO and NEDD8 (Hock and Vousden, 2010).

Somewhat surprisingly, there is growing evidence that the p53 protein possesses important roles that are distinct from the elimination (by inducing apoptosis or replicative senescence) of cells that have already acquired tumorigenic changes. In fact, it is now clear that p53 can act earlier, preventing oncogenic transformation of cells. It can achieve this by two principal mechanisms (Vousden, 2010). The first is by hindering the cancer-associated metabolic switch from oxidative phosphorylation to glycolysis, by either reducing the expression of genes necessary for glycolysis such as *GLUT1*, *GLUT4* and *PGM*, or by activating genes that drive mitochondrial respiration, such as *SCO2*. The second mechanism involves limiting oxidative damage by increasing the expression of particular genes, such as those encoding the antioxidant sestrin proteins—*SOD2*, *GPX1* and *ALDH4*—resulting in less reactive oxygen species (ROS)-induced DNA damage. Recent studies describing the p53-mediated activation of *GLS2* provide another example of the second mechanism, since *GLS2*, in turn,

leads to an increased availability of glutathione, which is an important intracellular scavenger of ROS (Hu et al., 2010; Suzuki et al., 2010). Moreover, it appears that the role of p53 in promoting antioxidant activities occurs under normal growth conditions, even without acute stress to the cell.

**17.5.3.4 Homologs and Isoforms of p53.** Two homologs of p53 have been identified: p73 and p63 (also known as Ket, p40, p51 and p73L). At least when overexpressed, both proteins mimic the ability of p53 to bind DNA and to induce gene transcription and apoptosis and several studies have detected p63 overexpression in many human cancers (Pruneri et al., 2005). Both *TP73* and *TP63*, the two corresponding genes, are rarely mutated in human cancer, with mutations occurring in <1% of tumors (Dotsch et al., 2010). Moreover, unlike p53-deficient mice, mice lacking p73 or p63 are not generally prone to tumors. Mutations in *TP63*, which is located at chromosome 3q27, have, however, been identified in several autosomal dominant human developmental syndromes. In general, these syndromes involve abnormal limb development and/or ectodermal dysplasia. They include ectrodactyly, ectodermal dysplasia, and clefting (EEC) syndrome; ankyloblepharon, ectodermal dysplasia, clefting (AEC or Hay–Wells) syndrome; acrodermato–ungual–lacrima–tooth (ADULT) syndrome; limb–mammary syndrome (LMS); and nonsyndromic split hand/foot malformation (SHFM). Interestingly, mutations associated with EEC and AEC syndromes tend to cluster within the DNA-binding domain and sterile-alpha-motif (SAM) domains, respectively. The SAM domain, which is located at the C-terminus, mediates protein–protein interactions. Many p63-regulated genes have now been identified in keratinocytes, including *IKKα*, *BPAG1* and *p21*; these genes have roles in differentiation, cell adhesion and proliferation, respectively (Pozzi et al., 2009).

Evidence has emerged that *TP73* may, too, play a role in normal development, with defects in neuronal development being reported in *p73* knockout animals (Dotsch et al., 2010). Interestingly, p73 induces apoptosis by not only transactivating *BAX* and *PUMA* but also through inducing ER stress by altering scotin expression and subcellular localization (Rossi et al., 2004).

Like *TP63* and *TP73*, which each have dual promoters, *TP53* has been shown, by David Lane's group, to possess an internal promoter, which is located within intron 4 (Bourdon et al., 2005). This results in an amino-terminal truncated p53 isoform, which is detected in most tissues. In addition, alternative splicing results in the absence of the exon 10-encoded amino acids in p53 beta and gamma. These isoforms consequently lack the oligomerization domain, cannot bind to certain normally p53-responsive promoters, and differ in proapoptotic activity. The beta isoforms have reduced proapoptotic activity and the beta isoform of amino-terminal truncated p53, in particular, is not only apoptosis defective,

but has a dominant-negative effect on the activity of the full-length isoform. The pattern of p53 isoform expression is complex, tissue specific, and often abnormal in tumors, with, for instance, reduced p53 gamma and upregulation of the N-terminal truncated p53. Interestingly, tumors that retain wild-type p53 often express an abnormal balance of p53 isoforms.

## 17.5.4 The Adenomatous Polyposis Coli Tumor Suppressor Gene

Hereditary CRC syndromes have long been recognized. The syndromes are usually divided into polyposis and nonpolyposis types. The polyposis types are those in which hundreds and sometimes even a thousand or more benign tumors (polyps) arise before the development of cancer. In the nonpolyposis types, very few polyps are noted, despite the very elevated risk of cancer. The nonpolyposis CRC syndromes are addressed later in this chapter. The well-documented polyposis syndrome, familial adenomatous polyposis (FAP), is an autosomal dominant disorder characterized by the development of hundreds of adenomatous polyps in the colon and rectum of the affected individuals by early adulthood together with congenital hypertrophy of the retinal pigment epithelium (CHRPE). The term Gardner syndrome has previously been used to refer to the occurrence of benign extracolonic tumors (including desmoid tumors, osteomas and dental abnormalities) in addition to the manifestations of FAP. But Gardner syndrome and FAP are now regarded as representing a disease continuum, caused by mutations in the same gene (and even by the same mutation within a single family) (Strate and Syngal, 2005).

The initial observation that led to the localization of the gene underlying adenomatous polyposis coli, *APC*, was the demonstration by Herrera and Sandberg and coworkers in 1986 of an interstitial deletion on chromosome 5q in a patient with hundreds of adenomatous polyps. Subsequent linkage analysis studies confirmed that in multiple kindreds with FAP or the related Gardner syndrome, the polyposis phenotype segregated with markers near 5q21. An intense research effort over the next 4 years identified the specific gene responsible for predisposition to polyposis: the *APC* gene. *APC* is a relatively large gene with the main transcript containing 15 exons and encoding a very large protein of 2843 amino acid residues (~300 kDa) that is expressed in many adult tissues. The predicted protein product shows little similarity to other proteins and is localized in the cytoplasm. It binds to beta-catenin, an abundant protein, much of which is normally bound to the cell–cell adhesion protein, E-cadherin. The beta-catenin–*APC* interaction occurs via two motifs on *APC*: One consists of three 15-amino acid repeats located between residues 1020 and 1169, while the other consists of seven 20-amino acid repeats located between residues 1324 and 2075, which can be

phosphorylated by the serine kinase, glycogen synthase kinase 3 (GSK3).

**17.5.4.1 APC Function and WNT Signaling.** In normal cells, as a member of a cytoplasmic complex that also contains constitutively active GSK3 and the scaffolding protein, AXIN, APC promotes the phosphorylation and subsequent proteosomal degradation of beta-catenin. However, if this phosphorylation of beta-catenin by GSK3 is inhibited, free beta-catenin accumulates (Figure 17-9). Such inhibition can occur either as a result of Wingless (WNT) signaling via the Frizzled receptor and the Dishevelled (DSH) protein, or through the loss of functional APC, as occurs in more than 80% of sporadic CRCs. Some of this excess beta-catenin moves into the nucleus where it forms a complex with T-cell factor 4 (TCF4), which then induces the transcription of growth and apoptosis regulating genes, including those encoding c-MYC, cyclin D, ephrins and caspases. The APC gene has thus been proposed to act as a “gatekeeper,” a gene which normally functions to maintain correct cell numbers by directly inhibiting abnormal cell proliferation or by promoting cell death. Mutations in *AXIN* and in *CTNNB* (encoding beta-catenin) have been detected in some hepatocellular carcinomas and in APC-wild-type colon tumors, respectively.

The beta-catenin/TCF4 complex also induces the expression of the nuclear hormone receptor named peroxisome-proliferator-activated receptor delta (PPAR-delta). This protein, following its activation by a ligand, can then induce the expression of other, unidentified, genes. Interestingly, nonsteroidal anti-inflammatory drugs (NSAIDs), which can, to an extent, reduce the size and number of polyps in FAP (Strate and Syngal, 2005),

appear to reduce the binding of PPAR-delta to DNA. This may occur by a cyclooxygenase-2 (COX-2)-independent mechanism, or by the inhibition of the COX-2-mediated production of PPAR-delta ligands such as eicosanoids, or by a combination of COX-2-dependent and -independent mechanisms. Importantly, this inhibition of PPAR-delta function appears to promote apoptosis of colon cancer cells.

Although the best-characterized function of APC is its role in regulating beta-catenin levels in the WNT signaling pathway, evidence is accumulating that interactions of APC with cytoskeletal proteins are also important to cancer development. For instance, lack of APC leads to chromosome missegregation and to mitotic spindle defects that can be rescued only by the addition of APC containing the direct microtubule-binding site (Dikovskaya et al., 2004). In addition, APC is now known to shuttle between the cytoplasm and the nucleus and the N-terminal region of the protein contains two nuclear export signals (NES). In early mitosis, APC is found to be localized to spindle microtubules, centrosomes and the ends of microtubules that are embedded in kinetochores (Nathke, 2004).

**17.5.4.2 APC Gene Mutations.** Mutations in the *MutYH* gene (see later section) should be considered in polyposis patients without family history evidence of vertical transmission, especially in those with an attenuated phenotype (i.e. less than 100 polyps) or a negative APC mutation screen. In most individuals affected by FAP or Gardner syndrome, however, germline mutations can be identified in one of the two APC alleles. The germline mutations in APC studied thus far generally appear to inactivate the protein. The mutations

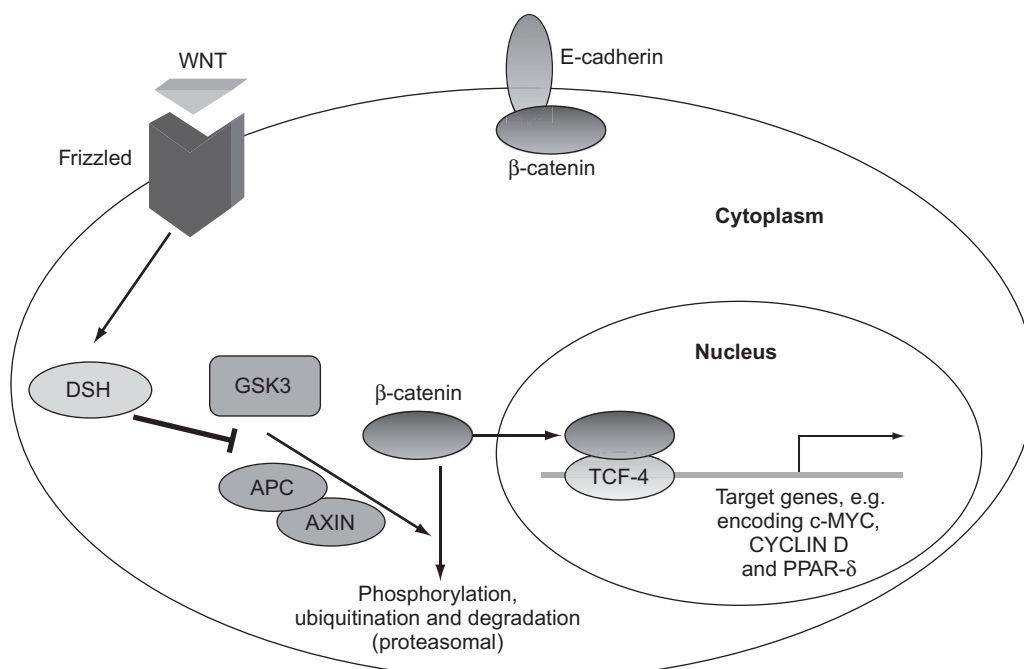


FIGURE 17-9



include gross deletions of the gene in a minority of cases, but, in the majority of cases, cause truncation by creating frameshifts or premature stop codons and result in the expression of N-terminal fragments of the protein (Nathke, 2004). An *APC* gene mutation database is currently available online at <http://www.insight-group.org/mutations/>. The second hit, present in most colorectal adenomas in FAP patients, is generally another truncating mutation, though, more rarely, is allele loss or LOH. Interestingly, in both sporadic and FAP colorectal tumors, the nature of the second *APC* gene hit appears to be dependent on that of the first. Thus, LOH tends to occur as the second hit when the first hit is a mutation lying close to codon 1300, or between codons 1285 and 1378. In contrast, truncating second hit mutations are preferentially selected when the first mutation lies outside the MCR. This association may reflect an “optimal” level of beta-catenin signaling for the development of colorectal tumors.

A genotype–phenotype correlation has emerged regarding the location of germline mutations within the *APC* gene. The amino terminal region of *APC* contains the putative oligomerization domains, two nuclear export signals and the armadillo repeats, which are reported to bind to various proteins, including the regulatory subunit of protein phosphatase 2A (PP2A) and the *APC*-stimulated guanine nucleotide exchange factor (ASEF) for Rho family proteins. The middle section of *APC* contains the binding sites for beta-catenin and axin. The less well conserved C-terminus contains the basic stretch that binds microtubules and is similar to the microtubule-binding site of TAU. Finally, the C-terminal 15 residues represent a PDZ-binding site.

Classical polyposis is generally associated with mutations located between codons 169 and 1403 with the occurrence of CHRPE being associated with mutations situated between codons 463 and 1387. Particularly profuse polyposis in *APC* may occur with germline mutations lying between codons 1285 and 1465, including the most common germline *APC* mutation, at codon 1309. In contrast, mutations located proximal to codon 157 of *APC* appear to correlate with an attenuated phenotype in which there are fewer polyps and a later age of onset of CRC. An explanation of the attenuated phenotype may be that these proximal mutations lead to the reinitiation of translation using an internal ribosome entry site and an in-frame ATG at codon 184 with the consequent production of an N-terminal truncated protein.

A predisposition to extracolonic tumors, which is especially evident in those said to have “Gardner syndrome,” appears to be associated typically with mutations located between codons 1403 and 1578 of *APC* (de la Chapelle, 2004). The studies reviewed above have provided definite support for the proposal that the loss of function mutations in the *APC* gene lead to predisposition to adenomatous polyps of the colon and

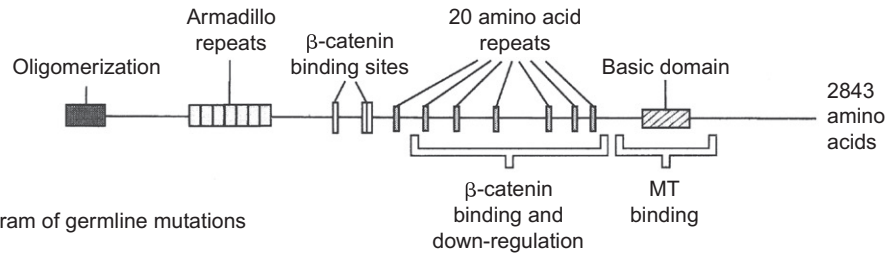
rectum. In addition, a valuable mouse model of intestinal tumorigenesis resulting from the inactivation of one allele of the murine homolog of the *APC* gene has also been described. This model is known as the Min (for multiple intestinal neoplasia) mouse. However, while FAP patients develop adenomatous polyps mostly of the colon and rectum, the Min mouse develops adenomatous polyps predominantly in the small intestine.

Currently, screening protocols for *APC* mutations generally involve DNA sequencing of the full gene to detect point mutations and small insertions or deletions, plus the use of multiplex ligation dependent probe amplification (MLPA) in order to detect larger duplications or deletions (i.e. leading to gene dosage alterations) (Necker et al., 2011). Two unrelated patients with classical polyposis coli (who did not have a significant family history and in whom no *APC* gene abnormality had been detected by these methods) were found by the protein truncation test (PTT) to possess a low level (15% or less) of mosaic pathogenic mutation, leading the authors to suggest that PTT should be retained as a diagnostic test (Necker et al., 2011).

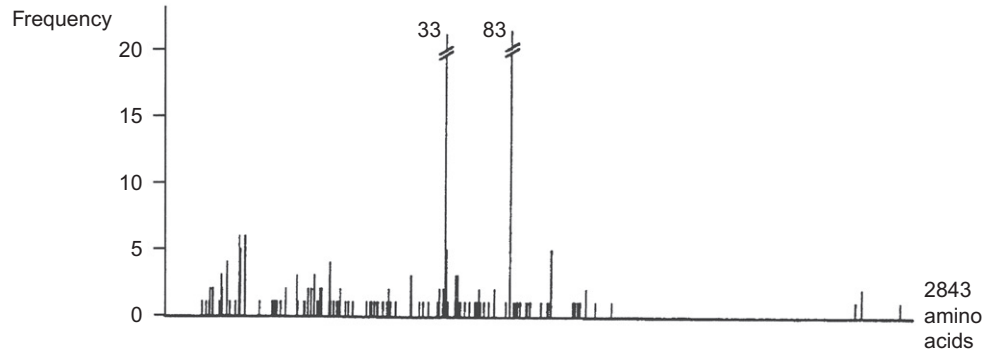
Transcriptional silencing of the *APC* gene can occur by promoter inactivation such as by deletion. In this respect, it is important to note that the gene is governed by two promoters: 1A and 1B (Rohlin et al., 2011). The transcriptional activity of 1B has recently been reported to be higher than that of 1A and promoter 1B deletions can result in familial polyposis (Rohlin et al., 2011).

While germline inactivation of the *APC* gene is an infrequent predisposing factor of CRC, somatic inactivation of the *APC* gene appears to be critical in the development of the majority of sporadic colorectal adenomas and carcinomas. The chromosome 5q region containing the *APC* gene is affected by LOH in the majority of colorectal adenomas and carcinomas from patients without polyposis. Since the identification of the *APC* gene in 1991, detailed analyses of somatic *APC* mutations have been carried out (Figure 17-10). The somatic mutations identified in sporadic tumors have a similar spectrum and distribution to the germline mutations observed in those with FAP or Gardner syndromes. Present findings suggest that more than 70% of colorectal tumors and adenomatous polyps, regardless of their size or histopathological features, harbor a specific somatic mutation in one or both of their *APC* alleles. Gastric cancers are among the other tumor types that have been found to harbor mutations in the *APC* gene. In addition, *APC* gene inactivation may also be involved in the genesis of other tumor types. For example, patients with Turcot syndrome (in most cases, an allelic variant of FAP) are predisposed to the development of intestinal polyps and, also, primary brain tumors. Finally, a low-penetrance mutation in the *APC* gene, I1307K, has been identified, that is found almost exclusively in Ashkenazi Jews (see Section “Predisposition to Colorectal Cancer by Low Penetrance Genes”).

## (A) APC protein



## (B) Histogram of germline mutations



## (C) Histogram of somatic mutations

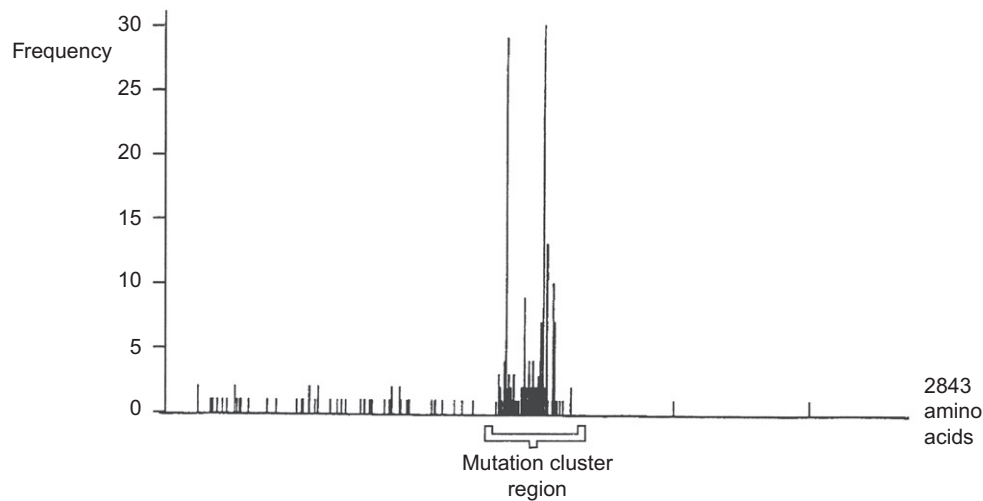


FIGURE 17-10

### 17.5.5 The Neurofibromatosis Type 1 Tumor Suppressor Gene

Type 1 neurofibromatosis (NF1) or von Recklinghausen disease has an incidence of ~1 in 4000 live births (Castle et al., 2003). It is a dominantly inherited syndrome whose principal manifestations involve tissues derived from the neural crest. In addition to neurofibromas and café-au-lait spots, NF1 patients are at increased risk of developing pheochromocytomas, optic gliomas, neurofibrosarcomas, and primary brain tumors. The *NF1* gene was localized to the pericentromeric region of chromosome 17q by linkage analyses carried out on several large kindreds with NF1. Subsequently, karyotype analysis led to the identification of two patients with NF1 and constitutional chromosomal translocations involving

band 17q11.2. Intensive molecular genetic analyses then focused on this region, and in 1990, the *NF1* gene was identified. Although recognized as the predominant underlying gene, *NF1* is not the only gene that causes this type of disorder as *SPRED1* is now known to cause an NF1-like disorder with multiple café-au-lait macules and axillary freckling but microcephaly rather than macrocephaly (Brems et al., 2007).

*NF1* is a large gene comprising 60 exons, spanning over 350 kb, and encoding a very large protein product with an estimated mass of 327 kDa. Around a half of affected individuals possess a de novo *NF1* mutation. The estimated new mutation rate of 1 in 10,000 is, in fact, one of the highest for a human disorder and most mutations are paternal in origin, although there is no

significant paternal age effect. Comprehensive mutation analysis of the gene is currently laborious on account of the gene's size and the large number of exons, the presence of several *NF1* pseudogenes and the wide spectrum of genetic lesions, including gross rearrangements and distinct point mutations. Many mutations have been described, of which around 80% are predicted to disrupt the encoded protein. For example, one mutation analysis study identified mutations in 64 (95%) of 67 unrelated *NF1* patients, of whom 37 had nonsense or frameshift mutations and 19 possessed splice-site mutations.

There are a few known genotype–phenotype correlations at present. In particular, a 1.4Mb recurring genomic deletion, encompassing 14 protein-encoding genes (including *NF1* gene) is associated with dysmorphic features, increased numbers of early-onset neurofibromas, and significant developmental delay (in addition to the typical *NF1* manifestations) (Castle et al., 2003). In addition, patients with deletions may be more predisposed to malignant peripheral nerve sheath tumors (MPNSTs) (De Raedt et al., 2003). In contrast, an exon 17 single codon deletion causes an unusually mild *NF1* phenotype without cutaneous neurofibromas (Upadhyaya et al., 2007). Furthermore, *NF1* patients who present with bilateral spinal neurofibromas have more missense and splice-site mutations than patients with classical *NF1* (Upadhyaya et al., 2009). It is likely that a number of modifier genes contribute to the phenotypic expression of the condition. Large investigations of over 1000 patients with *NF1* are currently underway to identify these genes by, for instance, genome-wide association studies (Huson et al., 2011).

In addition to germline *NF1* gene mutations in those with neurofibromatosis, somatic mutations inactivating the *NF1* gene have been found in a subset of other solid tumor types, including CRC, melanoma, neuroblastoma, and in some bone marrow specimens from those with the myelodysplastic syndrome. In benign neurofibromas, in accordance with the Knudson model of TSG function, the normal copy of the *NF1* gene often undergoes LOH or point mutation. The same is also true in *NF1*-associated MPNSTs, pheochromocytomas and astrocytomas (Frahm et al., 2004; Upadhyaya et al., 2004). Moreover, in benign neurofibromas, the Schwann cells, but not the fibroblasts, generally exhibit the loss of *NF1* transcript and neurofibromin, indicating that the abnormal cell in neurofibromas is the Schwann cell.

The 2818 amino acid protein encoded by the *NF1* gene is termed neurofibromin. It is localized in the cytoplasm of the cell and bears considerable similarity to the catalytic domains of GTPase-activating proteins (GAPs). One of the best studied GAPs is RAS-GAP, which has been shown to stimulate the (regulatory) GTPase activity of wild-type K-RAS, H-RAS, and N-RAS proteins. In addition to the predicted similarity of the neurofibromin protein to GAPs, studies have shown that neurofibromin

can, indeed, stimulate the GTPase activity of both yeast and mammalian RAS proteins (Arun and Gutmann, 2004). This activity of neurofibromin is envisaged, in vivo, to help prevent the overactivity of the RAS signaling proteins in the pathway which promotes cell proliferation. Thus, the loss of neurofibromin results in the abnormal persistence of the GTP-bound (i.e. active) form of RAS (Arun and Gutmann, 2004). The mechanisms by which *NF1* mutations alter cell growth are still not completely understood, however. They probably also involve interactions of neurofibromin with components of the cytoskeleton such as microtubules and intermediate filaments and a role of neurofibromin in positively regulating intracellular cAMP levels. Like *NF1*, *SPRED1* encodes a protein that negatively regulates signaling through the mitogen-activated protein kinase (MAPK) pathway. It does so by inhibiting RAS–RAF interaction (Brems et al., 2007).

The pathogenesis of MPNSTs in *NF1* patients is not yet well understood. It is known, however, to involve the deregulation of several key regulatory genes, such as *TP53*, *RB1*, *CDKN2A*, *EGFR*, *PDGFR*, *PDGFRA*, *HGF*, *MET* and *SOX9* and the constitutive activation of multiple cell signaling pathways (Upadhyaya, 2011).

### 17.5.6 The *CDKN2A* Gene

Loss of heterozygosity affecting chromosome 9p has been seen frequently in a wide spectrum of tumors, including melanomas, gliomas and leukemias as well as bladder, head and neck and non-small cell lung cancers. A subset of tumors was found to have homozygous deletions affecting the 9p21 region. In addition to the frequent somatic alterations on 9p in cancers, linkage studies of some families with inherited melanoma established that a melanoma predisposition gene mapped to 9p.

These findings motivated efforts to define a more limited region of chromosome 9p that was presumed to contain a TSG affected by both germline and somatic mutations. One of the genes identified in this region of 9p as a result of positional cloning efforts was termed *MTS1* (multiple tumor suppressor 1). Sequence analysis of the *MTS1* gene revealed that it was identical to a previously identified gene *p16*. The *p16* (now named *CDKN2A*) gene was originally identified because it encoded a protein (now termed p16INK4A) that functions as a specific inhibitor of cyclin-dependent kinases CDK4 and CDK6. The *CDKN2A* gene encodes not only the p16INK4A but also, as the result of an alternative reading frame, the p14<sup>ARF</sup> protein. The *CDKN2A* gene has been found to be mutated in the germline DNA of around 25–40% of individuals with familial cutaneous malignant melanoma (CMM). Mutations in this gene result in a lifetime risk of CMM of between 50 and 80% depending on the level of ambient sun exposure (Bishop et al., 2002). As in other disease-related genes, certain mutations are far more commonly found than others,

depending on the population. The M53I mutation, for instance, is a recurrent mutation that is pathogenic and, from haplotype analyses, appears to have a Scottish origin (Lang et al., 2007).

In familial CMM, there have been, in addition, at least two reports of germline *CDKN2A* exon 1 beta (which encodes p14<sup>ARF</sup>), and even *CDKN2A* intronic mutations (Harland et al., 2005a, 2005b). Somatic mutations in *CDKN2A* have, in fact, been found in a significant fraction of many different cancer types, including but not limited to melanomas, gliomas, pancreatic and bladder cancers, and leukemias. In some of these tumors, deletions of the *CDKN2A* gene were also found to involve a nearby gene, *p15*, that encodes a protein closely related to *CDKN2A*. In other tumors, mutations have been found that appear to inactivate *p15*, but not *CDKN2A*.

The *CDKN2A* gene, as mentioned above, has been shown to encode a second unique protein (in addition to the p16 protein). This protein is termed p14<sup>ARF</sup> in humans and p19<sup>ARF</sup> in mouse. Both the p16INK4A and the p14<sup>ARF</sup> proteins have been found to possess growth suppressive functions. Hence, there may be strong selection for simultaneous inactivation of both proteins during tumor development.

The prevalence and nature of *CDKN2A* mutations appear to vary markedly from one tumor type to another, and the gene appears to be more frequently mutated in tumor cell lines adapted for culture than in primary tumors. This finding may reflect the fact that *CDKN2A* inactivation may provide an additional growth advantage to cultured tumor cells. Homozygous deletion is a particularly common mechanism by which *CDKN2A* has been found to be inactivated in some primary human tumors and tumor cell lines. Other studies suggest that p16INK4A expression is lost in some tumor types as a result of methylation of the gene's regulatory sequences (i.e. promoter/enhancer elements), a situation that in several other genes often results in the loss of gene expression. In fact, the finding of *CDKN2A* inactivation by DNA methylation led to the investigation and subsequent widespread acceptance of DNA methylation as a general mechanism for the inactivation of TSG function during tumorigenesis. As discussed above for the *RB1* and *TP53* genes, the frequent inactivation of the *CDKN2A* gene in a wide spectrum of human cancers underscores the importance of the physiological control of the cell cycle in normal cell growth and in the suppression of the neoplastic phenotype.

Mutations in another cell cycle control gene, *CDK4*, are much less frequently found in familial CMM although there is a specific mutational hot spot at codon 24. As mentioned previously, *CDK4* is one of the few examples of a familial cancer gene that is a proto-oncogene rather than a TSG. It is unlikely, however, that *CDKN2A* and *CDK4* are the only genes that cause familial CMM. A locus that is believed to harbor another pathogenic gene

is 1p36. At this locus, a possible CMM TSG is *CHD5* (Lang et al., 2011) although further studies are required before this can be confirmed. Other melanoma susceptibility genes are likely to be identified in the future.

### 17.5.7 P16INK4a and P14<sup>ARF</sup>/P19<sup>ARF</sup> Functions

The p16INK4A protein is a CDK inhibitor that maintains pRB in its growth-suppressive, hypophosphorylated state. In contrast to p21<sup>WAF1</sup>, whose expression in replicating fibroblasts increases sharply just before the onset of replicative senescence but then declines, p16INK4A expression increases gradually to a sustained, greatly elevated level in the later stages of senescence. Thus, while p21<sup>WAF1</sup> may be responsible for the induction of senescence, p16INK4A may be essential for its maintenance. While inactivation of the p19/p53 pathway is sufficient for murine cells to escape senescence, human cells generally need to disrupt, at least, both the p53/p21 and the p16INK4A/pRB pathways (Figure 17-8). Perhaps as a consequence, human cells spontaneously immortalize in culture much more rarely than rodent cells.

### 17.5.8 *WT1*, the Wilms' Tumor Suppressor Gene at Chromosome 11P13

Similar to retinoblastoma, and despite its relative rarity compared to common adult tumors, Wilms' tumor has provided a number of critical insights into TSG identification and function. The first findings to suggest the contribution of a gene at 11p13 to the development of Wilms' tumor were those of Miller and coworkers in 1964. They noted an association between aniridia (a congenital absence of the iris) and Wilms' tumor. Further studies confirmed that a rare complex of developmental abnormalities, including aniridia, genitourinary malformations, and mental retardation, was associated with a high probability of developing Wilms' tumor, hence the acronym WAGR (Wilms' tumor with aniridia, genitourinary malformations, and retardation) syndrome. Similar to the situation observed in some patients with inherited retinoblastoma and deletion of chromosome 13q14 sequences, some patients with WAGR syndrome were found to have constitutional deletions involving part of one copy of chromosome 11, at band p13. Based on these data, it was inferred that one of the genes involved in Wilms' tumorigenesis must reside at 11p13 and the *WT1* gene was identified in 1990. In fact, the hemizygous chromosomal deletion, often cytogenetically visible, in children with WAGR syndrome encompasses not only *WT1* but also generally at least the *PAX6* gene, and WAGR is therefore regarded as a "contiguous gene syndrome."

The *WT1* protein is a zinc-finger transcription factor of 55 kDa that is encoded by 10 exons. The mRNA produced is subject to a complex pattern of alternative splicing involving exons 5 and 9. The significance of exon 5



alternative splicing is currently unknown as mice lacking this exon develop normally and are fertile. In contrast, alternative splicing of exon 9 results in either WT1 “-KTS” or “+KTS” isoforms in which three amino acids (lysine, threonine and serine) are either absent or present, respectively, between zinc fingers three and four. This three-residue insertion results in different DNA-binding properties of WT1 and in altered kidney and gonad development in mice, and in humans affected by Frasier syndrome (Rivera and Haber, 2005). WT1 (+KTS) is the most abundant form and colocalizes with splicing factors and small nuclear ribonucleoproteins (snRNPs) within nuclear “speckles” (Rivera and Haber, 2005).

Based on the predicted amino acid sequence, the WT1 proteins appear to function in transcriptional regulation, and evidence has been obtained that the WT1 protein may suppress the expression of several growth-inducing genes, including the early growth response (*EGR1*), insulin-like growth factor II (*IGF2*), and platelet-derived growth factor A (*PDGFA*) genes. WT1 has also been shown to induce the expression of the pro-apoptotic factors, BAK and BIK, and may also repress the expression of the anti-apoptotic factor, BCL2 (Morrison et al., 2005). It appears likely therefore that inactivation of WT1 results in Wilms’ tumor development as a result of not only increased cellular proliferation but also failed apoptosis during kidney development.

Although Wilms’ tumor is a relatively common childhood tumor, true familial Wilms’ tumor is very rare (Rivera and Haber, 2005). Mutations in the *WT1* gene are present in just 10–15% of sporadic cases of Wilms’ tumor. It is now recognized that Wilms’ tumor can occasionally occur as part of a number of syndromes, including not only WAGR but also Denys-Drash, Frasier, Beckwith-Wiedemann, Simpson-Golabi-Behmel, Sotos, and Perlman and Familial Wilms’ Tumor 1 and 2 syndromes (Rivera and Haber, 2005). In patients with Denys-Drash syndrome (DDS), there is typically a germline missense mutation, causing a single amino acid substitution most often at Arg 394 of WT1, rather than the hemizygosity associated with WAGR syndrome (Royer-Pokora et al., 2004). In fact, in DDS, the phenotypic severity is greater than that of in WAGR syndrome, possibly reflecting a dominant negative effect of the mutant WT1.

### 17.5.9 The *NF2* Gene and Merlin

Neurofibromatosis type 2 (NF2), also known as central neurofibromatosis, is an autosomal dominant disorder that is genetically and clinically distinct from NF1 and is ~10 times less common. The hallmark of NF2 is the occurrence of bilateral schwannomas affecting the vestibular branch of the eighth cranial nerve (acoustic neuromas). In addition, NF2 patients are also at risk of the development of other tumors, including cranial and spinal meningiomas, spinal schwannomas, and ependymomas. The *NF2* gene was localized to chromosome 22q in the late 1980s

by a combination of linkage analyses and LOH studies. The gene was identified in 1993 using a positional cloning approach. It spans 110kb and comprises 16 constitutive and one alternatively spliced exon. The gene is widely expressed both in terms of tissues and individual cell types. Germline mutations inactivating the *NF2* gene have been observed in those with NF2, and patients with truncating mutations generally have an earlier onset and an increased number of tumors. In addition, large deletions have been reported to account for as many as 33% of *NF2* gene alterations. Somatic mutations in the gene have been detected in up to 60% of sporadic schwannomas and also in 70% and 83% of sporadic fibroblastic and transitional meningiomas, respectively. Mutations of the *NF2* gene have also been detected in up to 30% of melanomas and 41% of malignant mesotheliomas.

The *NF2* gene encodes a protein of 595 amino acids named merlin that bears strong similarity to a family of cytoskeletal proteins that are thought to act as linker proteins that connect integral membrane proteins to cytoskeletal scaffolding proteins of the filamentous submembrane lattice. These “ERM” linker proteins, ezrin, radixin and moesin, each possess an N-terminal FERM domain, a long alpha-helical central region, and a charged C-terminal domain that binds to F-actin and can interact intramolecularly with the N-terminal region. The intramolecular interaction but not the F-actin binding is a property of schwannomin and appears to be necessary for the protein’s tumor suppressor function. The presence of the alpha-helical central region may permit merlin to form a stable interaction, as a coiled-coil, with another ERM protein or with other proteins such as the coiled-coil protein, SCHIP-1. The homology of merlin and ERM proteins suggests a number of possible mechanisms by which *NF2* gene alterations might affect the cell shape, cell-cell interactions, and tumor growth. Several proteins have been reported to interact with merlin but such studies have been hampered by technical difficulties relating, for instance, to merlin solubility. The tumor suppressor activity of merlin relates to its role in both the coordination of growth factor receptor signaling and cell adhesion (McClatchey and Giovannini, 2005). For instance, merlin binds to GRB2, an adaptor protein that coordinates receptor tyrosine kinase and RAS signaling (Wiederhold et al., 2004) and to Lin-7/Pals1, which, in *Caenorhabditis elegans*, controls Erb-B2 membrane distribution (Cao et al., 2005). In addition, several studies suggest that merlin is essential for contact-dependent inhibition of proliferation.

Merlin is phosphorylated on several sites but the best studied is serine 518. Phosphorylation on S518 inactivates merlin’s growth suppressing activity and inhibits its self-association. Kinases that promote this phosphorylation include the major RAC effector p21-activated kinase (PAK) and also protein kinase A (PKA) (Alfthan et al., 2004). Therefore, the activity of merlin is likely to be intracellularly regulated by several routes. Merlin is,

in fact, now believed to play an important tumor-suppressive role within the nucleus (such as the regulation of mitogenic signaling via inhibition of the MAPK cascade or via an effect on the hippo protein), as well as at the cell surface (Huson et al., 2011; Yi and Kissil, 2010). Its relative contributions at each location, however, remain to be determined (Huson et al., 2011).

### 17.5.10 The *VHL*, *SDH* and *TMEM127* Genes

VHL syndrome is a rare, dominantly inherited disorder that predisposes individuals to the development of hemangioblastomas of the central nervous system and retina, as well as renal cell carcinomas and pheochromocytomas. The *VHL* gene was assigned to chromosome 3p by linkage analysis, and LOH studies were used to establish that the gene behaves as a typical tumor suppressor, as defined by Knudson's model, wherein both the alleles are inactivated during tumorigenesis. A positional cloning approach was successfully employed to identify the *VHL* gene and germline inactivating mutations that are responsible for VHL. Somatic mutations in the *VHL* gene have also been detected in most sporadic renal cell carcinomas of the clear cell type but not in other histological subtypes of renal cell carcinoma. Interestingly, in many of the sporadic clear cell renal cancers that do not harbor a detectable mutation in the *VHL* gene, the *VHL* gene is inactivated by methylation of its transcriptional regulatory sequences, as occurs with other TSGs such as the *CDKN2A*, *MLH1*, *CDH1* and *BRCA1* genes. Therefore, the *VHL* gene plays a critical role in the pathogenesis of the most common form of adult renal cancer. In other tumor types, however, inactivation of the *VHL* gene by somatic mutations or methylation appears to be uncommon.

Initial studies demonstrated that the *VHL* gene encodes a 213 amino-acid residue protein, pVHL, that complexes with the B and C subunits of the elongin transcriptional elongation factor, forming a protein complex that appeared to regulate transcriptional elongation by RNA polymerase II; however, no regulation of transcriptional elongation by pVHL has been shown in vivo. Rather, pVHL appears to participate in a regulatory mechanism that normally prevents inappropriate accumulation of a transcription factor—hypoxia-inducible factor (HIF) 1 alpha—that stimulates the transcription of hypoxia-responsive genes such as vascular endothelial growth factor (*VEGF*) and *EPO* (Kim and Kaelin, 2004). The VHL protein comprises two domains. Its alpha domain functions as a platform on which a complex is assembled, including elongin C, elongin B, Cul2 and Rbx1 (known, collectively, as the VCB–Cul2 complex). The pVHL beta domain has been shown to bind to the oxygen-dependent degradation domain of the alpha subunit of HIF1. By structural comparisons of the VCB–Cul2 complex with the yeast SCF complex, and

by extensive studies of pVHL activity, it is now recognized that (1) HIF1-alpha, under normoxic conditions, undergoes ubiquitin-mediated degradation; (2) the ubiquitination of HIF1-alpha is dependent on its interaction with the ubiquitination machinery of the VCB–Cul2 (or VCBC) complex (which functions as an E3 ubiquitin ligase) via the beta domain of pVHL; (3) this ubiquitination is disabled either in hypoxic conditions or as a consequence of most *VHL* mutations; and (4) this disabling leads to HIF1-alpha accumulation and the activation of hypoxia-responsive genes such as *VEGF*, which is an important factor in tumor angiogenesis.

The oxygen sensitivity of the degradation of HIF1-alpha is due to the fact that the transcription factor undergoes hydroxylation only under normoxic conditions and the hydroxyl groups then form the binding site for pVHL (Kim and Kaelin, 2004). The hydroxylation takes place on evolutionarily conserved proline residues (Pro-402 and Pro-564) and is carried out by prolyl hydroxylase enzymes of the so-called EGLN (Egl-nine) family. In addition, X-ray crystallography has revealed that the prolyl hydroxyl groups can then form hydrogen bonds with two hydrophilic residues of pVHL.

The prolyl hydroxylases split molecular oxygen and not only hydroxylate the prolyl substrate but also form succinate. The latter—a TCA cycle intermediate—is converted to fumarate by succinate dehydrogenase (SDH). Inherited and somatic mutations in *SDH* genes (particularly *SDH-B* and *SDH-D*) have been shown to be associated with pheochromocytomas and paragangliomas. The *SDH-B* mutations tend to be associated with a more aggressive disease and a poorer prognosis. Intriguingly, tumorigenesis associated with germline *SDH-D* mutations, specifically, has been reported to result exclusively from paternal transmission of the mutations and this may be explained by imprinting of a chromosome 11 gene other than *SDHD* itself (Hensen et al., 2004). There is accumulating evidence, in fact, that this gene lies on the short arm of chromosome 11 and that tumor formation can, rarely, occur after maternal transmission. In addition, in view of the biochemistry of prolyl hydroxylases, it was hypothesized and then demonstrated in an elegant study, that a crucial link between *SDH* mutations and tumor formation might be that succinate, having accumulated as a result of reduced SDH activity, might inhibit prolyl hydroxylation of HIF1-alpha by product inhibition of the prolyl hydroxylase, leading to HIF1-alpha stabilization and activation (Selak et al., 2005). The EGLN prolyl hydroxylases have now become a focus of much research into pheochromocytomas and other tumors (Maxwell, 2005).

Recently, mutations in another gene, *TMEM127* (which encodes a negative regulator of mTOR), and (albeit rarely) in another chromosome 11 gene, *SDHAF2* (encoding a cofactor of the SDH complex), have been reported to be associated with familial

phaeochromocytomas and paragangliomas, respectively (Qin et al., 2010; Bayley et al., 2010).

### 17.5.11 E-cadherin

E-cadherin is a calcium-dependent, epithelial cell–cell adhesion molecule whose function is critical for normal epithelial development and is lost during the growth of most, if not all, human epithelial cancers. In particular, somatic loss of its expression is regarded as being a characteristic feature of both diffuse gastric cancer and lobular breast cancer (Brooks-Wilson et al., 2004). It is a transmembrane glycoprotein and binds to (and thus sequesters at the cell membrane) beta-catenin, a protein which, when released, can, together with the TCF4 transcription factor, activate growth-promoting genes such as c-MYC (Figure 17-9). Beta-catenin is also negatively regulated by the APC/AXIN/GSK3 complex (see Section “APC Function and WNT Signaling”). Inactivation of the alleles of the *CDH1* tumor suppressor gene (which encodes E-cadherin) may occur by deletion, point mutation, or promoter hypermethylation. Germline *CDH1* mutations were originally identified in three diffuse gastric cancer kindreds of Maori origin and are now a well recognized cause of familial gastric cancer (of diffuse but not intestinal type) and are generally detected in around a third of families with two or more cases of diffuse gastric cancer; that is, hereditary diffuse gastric cancer (HDGC). *CDH1* mutations were, however, detected in as many as 48% of such families when an additional selection criterion (of age at diagnosis of 50 or less) was used (Brooks-Wilson et al., 2004). The authors of that study concluded that mutation analysis is unlikely to yield a *CDH1* mutation unless the family history includes two or more individuals with gastric cancer of whom at least one represents a documented case of diffuse gastric cancer (DGC) diagnosed at the age of 50 or younger (Brooks-Wilson et al., 2004). The association of *CDH1* mutations with early onset diffuse gastric cancer has been confirmed by a more recent analysis (Corso et al., 2011). Carriers of pathogenic germline *CDH1* mutations are estimated to possess a cumulative risk by age 80 of gastric cancer of ~67% for men and 83% for women, who may also have a risk of breast cancer of almost 40% (Brooks-Wilson et al., 2004).

Inactivation of the wild-type allele in germline *CDH1* mutation carriers generally occurs by transcriptional silencing due to promoter methylation. Interestingly, *CDH1* promoter hypermethylation has been detected on both alleles of the gene in two patients with sporadic leukemia, as has been previously observed for *MLH1* in sporadic CRCs. In many sporadic human cancers, loss of E-cadherin expression occurs late in the process of tumorigenesis and is associated with metastasis. This fact, together with its role as a cell–cell specific recognition receptor, suggests that the protein functions as an “invasion suppressor.” Loss of E-cadherin is believed to

promote invasion through the loss of cell adhesion but also to promote mitogenesis via increased beta-catenin signaling (Hayashida et al., 2005). The cell-to-cell adhesion that is mediated by E-cadherin is inhibited by the overactivity of the oncogene product, Src tyrosine kinase (Swaminathan and Cartwright, 2011).

### 17.5.12 The *PTEN* Gene

An extensive linkage analysis involving a genome scan of 12 classical Cowden syndrome (CS) families localized the gene to 10q23. In addition, frequent LOH at this locus was found in follicular thyroid tumors, suggesting that the putative CS gene was a tumor suppressor. Subsequently, two independent groups isolated a novel gene at this locus. Sequence analysis predicted that the gene would encode a protein with homology to the cytoskeletal proteins, tensin and auxilin, and that the protein would contain a protein tyrosine and serine/threonine phosphatase domain. The new gene was named *PTEN*, for phosphatase and tensin homolog on chromosome 10.

An increasing number of disorders are being associated with *PTEN* (Pilarski and Eng, 2004). These disorders constitute a group named the *PTEN* hamartoma tumor syndromes (PHTS) (Eng, 2010). For instance, germline mutations in the *PTEN* gene are now being found in around 80% of individuals with CS, in 50–60% of those with another related dominantly inherited disorder, Bannayan–Zonana (or Bannayan–Riley–Ruvalcaba syndrome (BRRS)) syndrome, in 20% of those with Proteus syndrome, and 50% of those with a Proteus-like syndrome (Nagy et al., 2004). Germline mutations in *PTEN* are now also known to cause Lhermitte–Duclos disease, or dysplastic gangliocytoma of the cerebellum. It is not clear whether the CS cancer risks (e.g. for breast, follicular thyroid, and endometrial cancers) are applicable also to those non-CS patients who possess *PTEN* mutations, but it has been suggested that they should be screened like those with CS (Nagy et al., 2004). On the basis of a study of over 3000 probands, a new clinical scoring method for the selection of patients for *PTEN* analysis has been proposed by Charis Eng and colleagues (Tan et al., 2011).

In common with many other TSGs (although not *TP53*), the majority of mutations result in protein truncation. Although the mutations are scattered over the entire gene, clustering is observed in the phosphatase core motif in exon 5 and in the phosphorylation sites in exons 7 and 8. Specific germline *PTEN* mutations may give rise to either CS or BRRS, even within the same family, but there is a tendency for CS-associated mutations to be located within the 5′ five exons and for the BRRS-related mutations to be sited within the 3′ exons.

The tumor suppressor function of the *PTEN* protein, the first TSG to be identified in the phosphatase family, appears to depend on its lipid phosphatase, rather than its protein phosphatase, activity. In particular, its

critical activity is the dephosphorylation of the lipid second messengers phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (PIP3), at position 3 on the inositol ring, thus antagonizing the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway and resulting in either cell cycle arrest in G1 and/or apoptosis depending on the tissue type (Nagy et al., 2004). Cells derived from the embryos of homozygous PTEN knockout mice do show elevated levels of PIP3 and constitutively increased activity of the PKB/Akt enzyme, consistent with the role of PTEN in the inhibition of the PI3K pathway. Moreover, they exhibit a reduced sensitivity to apoptosis, consistent with a role of PTEN in the negative regulation of cell survival.

When PTEN is inactive, Akt (also known as protein kinase B or PKB) promotes cell cycle entry, cell survival and cell growth by phosphorylating several key substrates. These include NF-kappaB, forkhead transcription factors, GSK3, p27, caspase 9, BAD, MDM2 and TSC2. Phosphorylation of tuberous sclerosis complex 2 (TSC2) results in the degradation of the TSC1–TSC2 complex, resulting in the increased activity of mTOR and consequently that of the cell size regulator, S6K. In addition, evidence suggests that mTOR also mediates a feedback loop as it can, itself, phosphorylate and activate Akt (Guertin and Sabatini, 2005). Although a great deal has thus been revealed regarding the importance of the lipid phosphatase activity of PTEN, the precise role of the protein phosphatase activity of PTEN remains much less clear.

## 17.6 THE ROLE OF DNA DAMAGE REPAIR GENES IN INHERITED CANCER SYNDROMES

Tumor suppressor genes have been defined strictly in this chapter as the class of genes that are inactivated by germline and somatic mutations in cancers and that normally have active roles in the regulation of cell growth in response to growth-inhibitory or differentiation signals. Though they are also inactivated in cancer cells, the DNA damage repair genes can be viewed as having more indirect roles in the processes controlling growth. Loss-of-function mutations in these genes are presumed to have little direct effect on cell growth. Rather, their inactivation appears, in affected cells, to increase the rate of mutations in cellular genes, including proto-oncogenes and tumor suppressor genes. The DNA damage repair genes may, however, for simplification, be regarded as a subset of TSGs in view of the requirement for their mutational *inactivation* for tumorigenesis.

Several cancer predisposition syndromes have now been described that result from germline mutations inactivating genes involved in DNA damage repair. These include hereditary breast/ovarian cancer, xeroderma pigmentosum, Werner syndrome (WS), Bloom syndrome (BS), Nijmegen breakage syndrome (NBS), ataxia

telangiectasia, MutYH-associated polyposis (MAP) and the hereditary nonpolyposis colorectal cancer (HNPCC) syndromes. The principal mechanisms of DNA repair include (a) mismatch repair of mispaired bases (mainly from errors arising in DNA replication), (b) repair by homologous recombination (or by end joining) of double-strand breaks (DSBs) (induced, e.g. by X-rays), (c) nucleotide excision repair of intrastrand pyrimidine dimers, induced by UV light, and (d) base excision repair (BER) of small-base modifications caused, for example, by oxygen radicals and alkylating agents.

### 17.6.1 Hereditary Breast Cancer and the *BRCA1* and *BRCA2* Genes

It has long been known that family history is a major contributor to breast cancer risk and that the risk is greatest in those who have a history of breast cancer in one or more first-degree relatives. However, it was not until the late 1980s that definitive evidence was obtained that the predisposition to premenopausal breast cancer in some families could be attributed to a highly penetrant autosomal dominant allele. Subsequently, Hall, King, and their coworkers reported in 1990 that one such gene, now termed *BRCA1* (for breast cancer predisposition gene 1), mapped to chromosome 17q21. Other investigators subsequently provided evidence that the *BRCA1* locus was associated with susceptibility to both breast and ovarian cancers. On account of the intensive research efforts that were focused on the chromosome 17q region thought to contain the gene, *BRCA1* was ultimately identified in 1994.

While germline mutations in the *BRCA1* gene account for around 50% of the families in which four or more members are affected by breast cancer at an early age, ~30% of such kindreds have been attributed to mutations of another highly penetrant autosomal dominant susceptibility gene termed *BRCA2*. The *BRCA2* gene was mapped to chromosome 13q12–13 in 1994 and was subsequently identified. Mutations in *BRCA1* and *BRCA2* appear to confer, in the high-risk families studied, similar “lifetime” risks of female breast cancer, of up to around 80% (Narod and Offit, 2005). The risk of ovarian cancer appears considerably higher in those with *BRCA1* mutations than in those with *BRCA2* mutations, at 54% and 23%, respectively (King et al., 2003). Conversely, the risk of breast cancer in males with a *BRCA2* mutation (around 6%) is substantially higher than in males with a *BRCA1* mutation. The risks of other cancers are not yet clear, but may include prostate and colon cancers for *BRCA1*. For *BRCA2* mutation carriers, statistically significant increases in risk were observed, by the Breast Cancer Linkage Consortium, for malignant melanoma and for prostate, pancreatic, gall-bladder, bile duct and stomach cancers. Perhaps not surprisingly, in population-based studies, the penetrances of *BRCA1* and *BRCA2* mutations appear to be significantly lower than in high-risk families, probably on account of the presence or absence



of risk-modifying gene variants (see Section “*BRCA1* and *BRCA2* Mutations and Cancer Predisposition”).

**17.6.1.1 Structure and Function of the *BRCA1* Protein.** The *BRCA1* gene contains 24 exons, of which the first is noncoding, with exon 11 encompassing about half of the coding sequence. It encodes a 220 kDa nuclear protein, BRCA1-p220, of 1863 amino acid residues, which appears to interact with a variety of proteins that have diverse cellular functions (Figure 17-11).

At its amino terminus is a zinc-binding RING finger domain. Ring fingers are evolutionarily conserved structures consisting of two loops held together at their base by eight histidine or cysteine residues and two zinc ions. They can function, rather like the pVHL protein complex, as ubiquitin–protein ligases or E3s. In addition, the RING finger of BRCA1 can bind to the RING finger of another protein-designated, BRCA1-associated RING domain 1 (BARD1), thus forming a heterodimeric BRCA1–BARD1 complex. The BRCA1–BARD1 complex inhibits polyadenylation by binding to the polyadenylation factor CStF-50 and may thus prevent inappropriate RNA processing at the sites of DNA repair. The heterodimer may also ubiquitinate topoisomerase II alpha and may thus possibly play a role in chromatin decatenation (or disentangling), for instance of sister chromatids at the onset of anaphase (Ashworth, 2005).

Located in the central portion of BRCA1 are three putative nuclear localization signals (NLS). Two of these regions of highly charged basic residues were found to interact with a component of the NLS receptor complex, importin- $\alpha$ , in a yeast two-hybrid screen using BRCA1 as the bait. Located at the extreme C terminus are two BRCT (BRCA1 C-terminal) repeats. BRCT domains are evolutionarily conserved regions, of ~95 amino acids residues, whose 3-D structure suggests a role in protein–protein interactions and which are found in many proteins involved in DNA repair and cell cycle control. While almost all identified mutations in *BRCA1* result in a truncated product, yielding little functional information, many missense mutations have been found.

These are located predominantly within either the RING finger domain or the BRCT repeats, providing support for the functional importance of these regions.

The role of BRCA1 in tumor suppression may involve a combination of DNA repair, transcriptional regulation, cell-cycle control and possibly apoptosis. Evidence that it is involved in DNA repair includes the observation that (a) BRCA1-deficient embryonic stem (ES) cells are hypersensitive to both ionizing radiation and hydrogen peroxide, and also that (b) such cells have impaired transcription-coupled DNA repair and homology-directed chromosomal DSB repair. In addition, following DNA DSBs BRCA1 appears to be phosphorylated by ATM, ATR, or CHK2 (Turner et al., 2005). ATM phosphorylates BRCA1 at its SQ cluster domains (SCDs), which represent clusters of serine and threonine sequences (Narod and Foulkes, 2004). Moreover, the BRCT domains of BRCA1 have been reported to bind to the ends of linear DNA fragments, independent of the DNA sequence, although, as mentioned above, they may also participate in protein–protein interactions.

Previous studies have found that BRCA1 interacts, through the C-terminal end of the region encoded by exon 11, with both RAD51 and RAD50. RAD51 is the human homolog of the yeast RecA protein, which functions in homologous recombination and DNA damage repair. Significantly, RAD51 is known to form a complex with both BRCA1 and BRCA2 during DNA repair. Similarly, on induction of DNA damage, RAD50 forms a complex with BRCA1, MRE11 and NBS1/p95/NIBRIN (the protein encoded by the gene mutated in NBS). Through these interactions, BRCA1 is believed to assist in the maintenance of genomic integrity, principally by promoting the potentially error-free homologous-directed DNA DSB repair in preference to the error-prone, potentially mutagenic, mechanisms such as nonhomologous end joining (NHEJ) and single-strand annealing (SSA) (Turner et al., 2005).

With regard to transcriptional regulation by BRCA1, a region between amino acids 1142 and 1646 and a

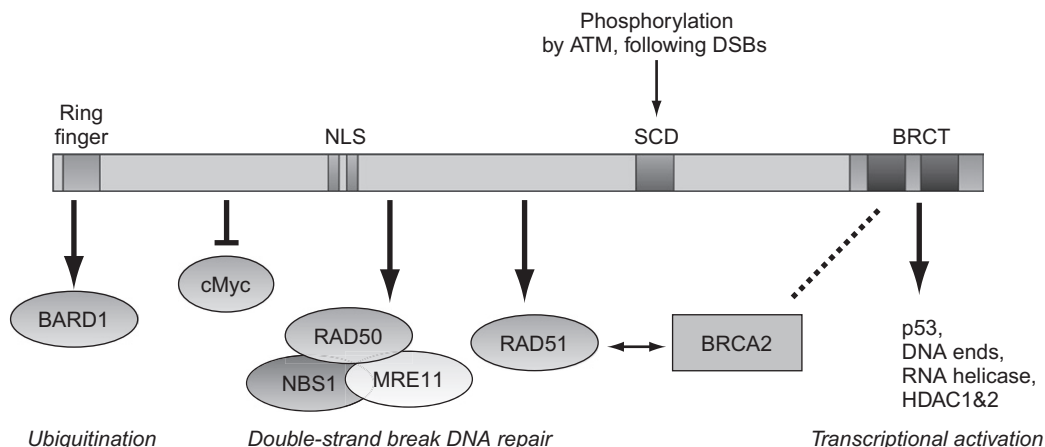


FIGURE 17-11

domain encompassing the carboxy-terminal residues 1760–1863 have been shown to possess transactivation activity. Additionally, evidence is accumulating that BRCA1 functions as a transcriptional regulator by interacting with other proteins, including sequence-specific binding proteins. For instance, BRCA1 interacts with both the transcriptional transactivator, c-MYC, whose activity it inhibits, and the RNA helicase A. Furthermore, the BRCT domain, located between amino acids 1536 and 1863 of BRCA1, can also bind to components of the histone deacetylase complex (HDAC). The implied function of BRCA1 in the regulation of chromatin remodeling could, theoretically, contribute to transcription, replication, recombination, or DNA repair. The BRCA1 C-terminal region also interacts with p53, appearing to stimulate its transcriptional activation of the *p21* gene, and with the RNA polymerase II holoenzyme itself. In fact, in human cells the cell cycle arrest that occurs following ectopic expression of BRCA1 appears to be mediated by transactivation of the *p21* promoter. Another gene that is involved in cell cycle regulation, *GADD45*, is also transcriptionally upregulated in response to BRCA1. Finally, BRCA1 also appears to function as a negative transcriptional regulator as it represses estrogen-induced transcriptional activation mediated by the estrogen receptor, ER $\alpha$ .

In summary, the regulation of gene transcription by BRCA1 probably contributes to most or all of its biological activities. This transcriptional regulation occurs through BRCA1 functioning as a coregulator, either by (a) binding to sequence-specific DNA binding transcription factors and stimulating (e.g. p53) or inhibiting (e.g. ER- $\alpha$ ) their activity or (b) through its role as a component of the RNA Pol II complex (modulating basal transcription and/or mediating interactions between sequence-specific transcription factors and the basal transcription factors) (Rosen et al., 2005).

**17.6.1.2 Another Splice Variant of BRCA1.** BRCA1 encodes not only the well-recognized BRCA1-p200 protein, but also a variant that results from alternative splicing—BRCA1-IRIS (“in-frame reading of BRCA1 intron 11 splice variant”) (ElShamy and Livingston, 2004). It is encoded by an alternative exon 1, exons 2–11 and an additional 34 amino acids derived from part of the intron that separates exons 11 and 12. It has a molecular mass of 150kDa and, with a presumably distinct promoter, is expressed in both quiescent and replicating cells, unlike BRCA1-p220, which is expressed only in the latter. BRCA1-IRIS protein is found to be associated with DNA replication origins and is likely to be involved in DNA replication.

**17.6.1.3 Structure and Function of the BRCA2 Protein.** The *BRCA2* gene, which comprises 27 exons, encodes a nuclear protein of 3418 amino acid residues and 384kDa, which is almost twice the size of BRCA1. Like in *BRCA1*, exon 1 is noncoding and exon 11 is

particularly large. With regard to the role of the BRCA2 protein in tumorigenesis, a function in DSB DNA repair appears particularly important. Like the BRCA1 protein, BRCA2 can bind to the DNA recombinase RAD51. Again, the region involved is encoded by exon 11, though in BRCA2 the RAD51-interacting region contains four of the eight copies of a motif of 30–80 amino acids known as the BRC repeat. Furthermore, cells of BRCA2 knockout mice exhibit an increased sensitivity to genotoxic agents but normal apoptotic and cell cycle checkpoint activation, thus further implicating BRCA2 in DNA repair. In the presence of DSBs, BRCA2 binds to RAD51 and it is now known that the complex interacts with the sites of DNA damage with the assistance of a small acidic protein named DSS1 (Gudmundsdottir et al., 2004). Error-free repair takes place by homologous recombination, as is also promoted by BRCA1. The function of RAD51 is to catalyze homologous pairing and DNA strand exchange. BRCA2 is necessary for the delivery of RAD51 protein to the site where it can affect DNA repair. BRCA2 achieves this by physically interacting with both RAD51 and single-stranded DNA, which is exposed following DNA damage (or stalled replication) (Holloman, 2011).

Located at the carboxyl terminus of BRCA2 are three nuclear localization signals, suggesting that disease-causing mutations, which tend to cause truncation upstream of this position, would all result in an abnormal cytoplasmic localization of the mutant protein. At the amino terminus of BRCA2 is a region that interacts with the transcriptional coactivator protein PCAF (p300/CBP-associated factor), which possesses histone acetylase activity. In addition, studies in yeast indicate that the BRCA2 exon 3 product can activate transcription when fused to a DNA-binding domain. Thus, although less well supported than for BRCA1, the crucial normal function of BRCA2 may involve transcriptional regulation in addition to DNA repair and maintenance of genomic integrity.

An interesting paradox is that homozygous BRCA2 truncation, alone, in mice leads to proliferative arrest rather than tumorigenicity. In fact, studies in mice suggested that BRCA2-induced tumorigenesis may require the co-inactivation of one or more cell cycle regulators that normally respond to mitotic spindle disruption, such as the mitotic checkpoint kinases—Bub1 and Mad3L. *BRCA2* is a chromosome instability (*CIN*) gene and it may also regulate cell cleavage and separation (Rudkin and Foulkes, 2005). An important finding in this regard was that cells deficient for BRCA2 (for instance resulting from RNA interference) cannot complete cell division properly, leading to binucleated cells (Daniels et al., 2004). An interesting further observation was that the normal accumulation of myosin II necessary for the cleavage-inducing actinomyosin “contractile ring” was defective. In addition, it colocalizes with the cytokinetic protein, AURORA-B, suggesting that BRCA2 plays an active role in cytokinesis itself. This might explain

the aneuploidy that is frequently detected in *BRCA2*-deficient cells.

**17.6.1.4 *BRCA1* and *BRCA2* Mutations and Cancer Predisposition.** From studies of *BRCA1* mutations in breast cancer patients, many important findings have emerged. By linkage and mutation analyses of nearly 237 families with four or more cases of breast cancer diagnosed before age 60 (or at any age in male cases), germline mutations of *BRCA1* and *BRCA2* were implicated in 52% and 32%, respectively, overall. Moreover, germline *BRCA1* mutations were thought to account for the cancer predisposition in as many as 81% of those families affected by both breast and ovarian cancers (Figure 17-12). In contrast, *BRCA2* mutations were thought to be responsible for ~76% of families in which there is at least one case of male breast cancer.

A sizeable variety of germline mutations in the *BRCA1* gene have been identified and around 95% are predicted to result in the synthesis of a truncated *BRCA1* protein. Most mutations have been identified in only one or two families, but a limited number have been seen recurrently. Of particular note, a population survey of Ashkenazi Jews, selected without regard to family history of cancer, demonstrated that about 1% carry the *BRCA1* 185delAG frameshift mutation. This mutation, or one of the two other Ashkenazi founder mutations, the *BRCA1* 5382insC and the *BRCA2* 6174delT, is found in 2.5% of individuals in this population group. Similarly, 0.6% of Icelanders carry the 999del5 *BRCA2* mutation, which accounts for almost all hereditary breast cancers in Iceland.

Notably, population-based studies of the penetrance of the Ashkenazi founder *BRCA1* mutation 185delAG and the Icelandic founder *BRCA2* mutation 999del5 suggested lower breast cancer penetrances (of 56% and 37% by age 70, respectively) than the previous pedigree-based studies. The differences were likely to be due to the presence or absence of important risk-modifying gene variants, which may well serve to increase the risk when a gene carrier is a member of a family with several affected members. In *BRCA2* mutation carriers, the

ovarian cancer risk is highest for those with mutations located in the “ovarian cancer cluster region” (at nucleotides 3035–6629) of exon 11 (Lubinski et al., 2004).

The use of candidate gene studies and particularly genome-wide association studies (GWAS) has now identified many SNPs that are associated with increased or reduced risk of breast cancer in the general population (~20 SNPs) and act as risk modifiers in *BRCA1* or *BRCA2* mutation carriers (at least 5 and 7 SNPs, respectively). These include SNPs in the *FGFR2*, *CASP8*, *MAP3K1*, *TOX3/TNRC9* and *RAD51* genes (Milne and Antoniou, 2011). Although, generally the SNPs are individually of small effect, they may of course, in combination, exert larger effects.

Surprisingly, very few sporadic tumors have been found to harbor detectable somatic pathogenic mutations in *BRCA1* or *BRCA2*. This has raised questions regarding the role of these genes in sporadic breast and ovarian cancers. Promoter hypermethylation, rather than somatic mutation, may represent an important mechanism by which at least *BRCA1* is inactivated in certain types of sporadic tumors. *BRCA1* promoter hypermethylation has been observed in 67%, 55% and 12% of sporadic medullary, mucinous and ductal breast carcinomas, respectively. In addition, *BRCA1* hypermethylation has been detected in 20% of sporadic ductal breast tumors showing *BRCA1* LOH, suggesting that in these tumors, one allele might be lost by deletion, while the other is silenced by aberrant methylation.

Study of the pathology of sporadic and hereditary breast cancers has revealed some intriguing differences. For instance, the histology of breast cancers in *BRCA1* germline mutation carriers is more often of higher-grade malignancy and more frequently of medullary type than in sporadic cases. In addition, these tumors are more frequently negative for estrogen receptor; progesterone receptors, *ERBB2* (HER2/NEU) (i.e. “triple negative”), are more often positive for p53 overexpression (Narod and Offit, 2005), and often show a “basal-like” gene expression profile (Foulkes et al., 2010). So-called

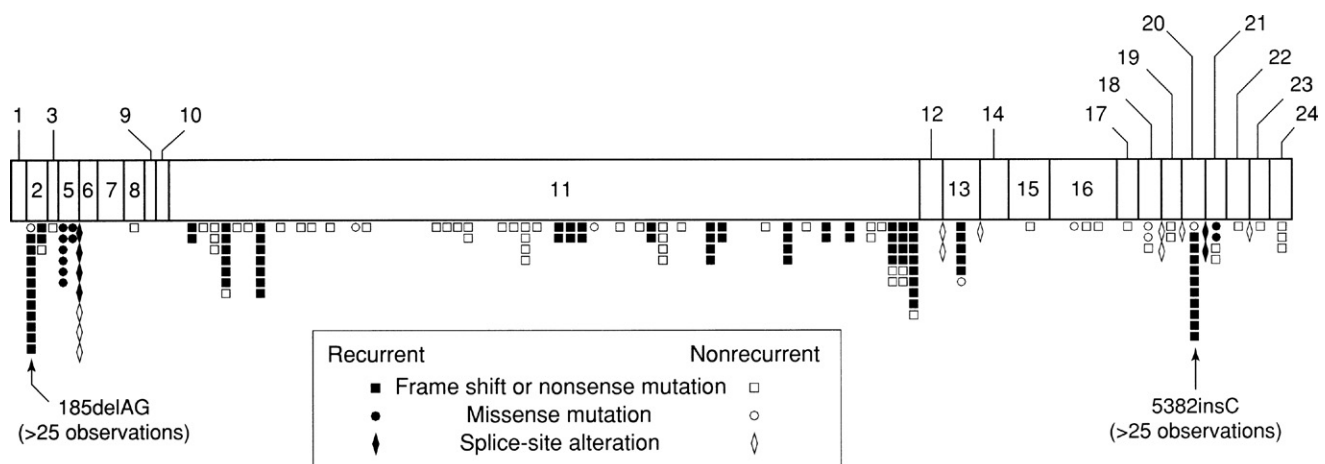


FIGURE 17-12

“basal-like” phenotype refers to the expression of high molecular weight (i.e. basal) cytokeratins (cytokeratins 5, 14 and 17), lower levels of p27, and higher levels of cyclin E, caveolins 1 and 2 and caspase 3.

Triple negative breast tumors and basal-like breast cancers generally have a poorer prognosis (Foulkes et al., 2010). No specific biological therapy is currently available and chemotherapy is usually chosen. Nevertheless, some of these tumors will be deficient in BRCA1 pathway function. This is important as these tumors may be sensitive to poly (adenosine diphosphate-ribose) polymerase (PARP) inhibitors. These inhibitors target cells that lack normally functioning homologous recombination DNA repair by inhibiting the enzyme that mediates base-excision repair. Blocking the latter type of repair leads to unrepaired single-strand breaks, stalled DNA replication forks and ultimately, possibly the production of DSBs that are then lethal to these cells (Hewish et al., 2010). PARP inhibitors (such as olaparib) have in fact been found to yield encouraging results when used in trials to treat tumors arising in BRCA mutation carriers as well as sporadic triple-negative cancers (Foulkes et al., 2010).

### 17.6.2 FANCD1/BRCA2

It is known that *BRCA2* is the Fanconi anemia complementation group D1 gene. Thus, biallelic *BRCA2* mutations have been detected in the cells of patients with this autosomal recessive condition. Monoallelic mutations in other Fanconi anemia genes, however, appear from mutation analyses in familial breast cancer patients of *FANCA*, *FANCC*, *FANCD2*, *FANCE*, *FANCF*, and *FANCG*, unlikely to frequently underlie high-penetrance breast cancer predisposition. Together with RAD51, *BRCA2* probably acts downstream of the other Fanconi anemia proteins in the Fanconi anemia DNA repair pathway since, in cells deficient in *BRCA2*, the core Fanconi anemia protein complex is unaffected and still results in *FANCD2* ubiquitination.

### 17.6.3 Hereditary Non-Polyposis Colorectal Cancer and Mismatch Repair

Familial clustering of CRC has long been noted. In fact, a genetic component is likely to be a primary factor in at least 10% of CRC cases. HNPCC probably accounts for about 5–8% of all CRC patients. At least 10-fold less frequent than HNPCC is the familial adenomatous polyposis, which, as reviewed above, is a rare syndrome (with an approximate incidence of 1 in 10,000) that results from germline mutations in the *APC* gene. MAP is a more recently described autosomal recessive syndrome with a phenotype similar to that of attenuated familial adenomatous polyposis.

The diagnosis of the HNPCC syndromes on a clinical basis alone is problematic for two reasons. First, for

a common malignancy like CRC, there is a likelihood of clustering within families by chance. Second, there is a lack of overt premalignant clinical characteristics, such as polyposis. Nevertheless, strict diagnostic criteria for HNPCC have been published, the Amsterdam criteria II: (1) exclusion of familial polyposis in the CRC cases, if any, and verification of tumor types by pathological examination; (2) HNPCC-associated cancer in at least three relatives, one of them being a first-degree relative of the other two; (3) two or more successive generations affected; and, in addition, (4) at least one of the affected individuals should be less than 50 years of age at the time of diagnosis. For these strict criteria, the “HNPCC-associated cancer” was defined as cancer of the colon or rectum, the endometrium, small bowel, ureter, or renal pelvis. These tumors were considered to be the most specific for HNPCC, having the highest relative risks. It was acknowledged, however, that gastric, ovarian, brain, hepatobiliary and sebaceous skin tumors are also associated with the syndrome, despite not being included in the Amsterdam criteria II. These criteria represented a modification (by the International Collaborative Group on HNPCC) of the original “Amsterdam” criteria, in which only CRCs were included. Nevertheless, the modified criteria remain relatively strict and are designed primarily as selection criteria for collaborative studies. They were not intended to serve as a guide for the exclusion of suspected families from genetic counseling and mutation analysis. In addition to the Amsterdam criteria and their modifications, the Revised Bethesda Guidelines (Umar et al., 2004a, 2004b) were developed, though principally with the aim of selecting tumors for testing for mismatch repair deficiency. The attempts that have been made to improve on these guidelines included mutation analysis for the *BRAF* V600E mutation and *MLH1* methylation analysis (Hewish et al., 2010). A fairly comprehensive practical review of clinical management guidelines for CRC screening in moderate and high risk groups has been published recently (Cairns et al., 2010).

The HNPCC kindreds were often previously categorized as either Lynch syndrome I (CRC only) or Lynch syndrome II (colorectal and other tumors). The more inclusive term HNPCC is often used now to encompass both syndromes and distinguishes them from the polyposis syndromes. In HNPCC, compared to sporadic cases, the CRCs show a predilection for the proximal colon, a tendency to be of mucinous type with a lymphoid aggregation at the tumor margin, and, on immunohistochemistry, to exhibit loss of *MLH1*, *MSH2*, or *MSH6* protein expression. Furthermore, the adenomas that occur in HNPCC have been reported to be larger, have a villous growth pattern, and tend to be more dysplastic than those arising in the general population.

**17.6.3.1 Genes Responsible for HNPCC.** Studies from the mid-1980s attempted to localize the genes responsible for HNPCC. No evidence was obtained to link the



HNPCC syndrome to any known TSGs (such as *APC*, *TP53*, or *RB1*). Thus, a search of the entire genome for the HNPCC gene(s) was carried out. Several different genes responsible for the HNPCC syndromes have since been identified, predominantly by linkage analysis of large kindreds, including a gene at chromosome 2p16 (*MSH2* or *hMSH2*) and one at chromosome 3p21 (*MLH1* or *hMLH1*) that together account for the majority of HNPCC cases. Based on studies of highly related genes from bacteria and yeast, the protein products of the *MSH2* and *MLH1* genes (homologs of the bacterial mismatch repair enzymes MutS and MutL, respectively) were predicted to be involved in the recognition and repair of DNA mismatches in humans. Additional mismatch repair (*MMR*) genes have been identified, the MutL homologs, *PMS1* and *PMS2*, and two MutS homologs, *MSH6* and *MSH3*, which both encode proteins that heterodimerize with *MSH2*.

Approximately 50% of those families who satisfy the original Amsterdam criteria have mutations in either *MLH1* or *MSH2* genes with mutations in *PMS1*, *PMS2*, or *MSH6* (also known as *GTBP*) being detected much less frequently. In contrast to germline mutations in *MLH1*, 49% of which are nonsense or frameshift, 83% of germline mutations in *MSH2* are truncating. Moreover, large genomic deletions are common in *MSH2* (10–20% of all mutations) but are less common in *MLH1* and rare in *MSH6* (de la Chapelle, 2004). They generally arise through *Alu* repeat-mediated homologous recombination (Van der Klift et al., 2005). Mutations in *MMR* genes are listed in the database of the International Society for Gastrointestinal Hereditary Tumors (InSiGHT), which is accessible online at <http://www.insight-group.org>.

It is estimated that a deficiency of mismatch repair is present in 15–17% of all primary CRCs (Hewish et al., 2010). In fact, for very early-onset CRC (<30 years), unselected for family history, as many as 28% of patients possess a germline mutation in an *MMR* gene. In these early onset cases, the determination of replication error (RER) status by screening for microsatellite instability (MSI) (the accumulation of different-sized alleles of microsatellite sequences in tumor DNA) appears to be useful in predicting the presence of a germline mutation in an *MMR* gene. The positive predictive value of the analysis in one study was 63%, with a sensitivity of 12/14 cases (86%).

Germline deletions affecting the 3' end of the *EpcAM* gene that result in transcriptional read-through and consequent hypermethylation (and transcriptional silencing) of *MSH2* have been observed. In fact, these deletions and incidences of germline *MLH1* hypermethylation together account for as many as 16% of “genetically proven Lynch syndrome cases” (Niessen et al., 2009a, 2009b). The *EpcAM* gene encodes the epithelial cell adhesion molecule.

Interestingly, mutations in *PMS2* can cause an autosomal recessive childhood cancer syndrome consisting of supratentorial primitive neuroectodermal tumor (PNET),

together with café-au-lait patches, rather than classical early-onset HNPCC (De Vos et al., 2004). Moreover, as a consequence of the discovery of a transcribed close paralog of the *PMS2* gene, *PMS2CL*, in addition to the nontranscribed *PMS2* pseudogenes, there is a growing possibility that mutations in *PMS2* are less rare than previously suggested, on account of detection difficulties resulting from the presence of these other sequences (De Vos et al., 2004; Nakagawa et al., 2004).

Dominantly inherited CRC phenotypes resembling HNPCC have also been found to be associated with germline mutations in a few non-*MMR* genes such as *AXIN2* (Lammi et al., 2004), *TGFBetaR2* and *POLD*. Interestingly, *AXIN2* encodes a protein involved in the WNT/APC signaling pathway and is somatically mutated in some sporadic CRCs.

A few founder mutations associated with HNPCC have now been described. These include an *MSH2* intron 5 splice donor-site mutation that leads to transcriptional skipping of exon 5 and the loss of *MSH2* expression, and is believed to account for 5–10% of all HNPCC-associated germline mutations worldwide (de la Chapelle, 2004). This mutation, a founder mutation in Newfoundland, in fact arises de novo in most cases and occurs in a stretch of 26 adenines, probably creating a DNA polymerase slippage hotspot (de la Chapelle, 2004). Other founder mutations include an *MLH1* exon 16 deletion that accounts for over 50% of HNPCC cases in Finns, an *MSH2* Ala636Pro substitution in Ashkenazi Jews (Foulkes et al., 2002), and an *MSH2* exons 1–6 deletion in North Americans (Lynch et al., 2004) believed to have been introduced into North America in 1727 by an immigrant from Germany (de la Chapelle, 2004).

Although MSI is present in cancers in nearly all patients with HNPCC, in sporadic CRC cases, in general, MSI is observed in only 10–15%. In these sporadic tumors with MSI, however, somatic mutations in *MMR* genes are found in only around 25%. The most common mechanism of somatic *MMR* gene inactivation in at least sporadic endometrial and gastric cancers with MSI, in contrast, is hypermethylation of the CpG island at the *MLH1* promoter, followed by the loss or hypermethylation of the second allele.

**17.6.3.2 The Mismatch Repair System.** The roles of the protein products of the genes involved in HNPCC have been determined. Mismatched bases, usually arising from replication errors, are initially recognized by a complex of two MutS-related proteins. Generally, single-base mismatches are recognized by a MutS alpha heterodimer consisting of *MSH2*/*MSH6*, while insertion/deletion loops are recognized by *MSH2*/*MSH3* (MutS beta). The DNA-bound MutS heterodimer subsequently interacts with a heterodimeric complex of MutL-related proteins (*MLH1*/*PMS1* or *MLH1*/*PMS2*). Following the assembly of this large protein complex, a portion of the DNA strand containing the mismatched base is excised

by an exonuclease that proceeds from a “nick” made up to 2kb from the mismatch. Finally, the excised tract is repaired with correctly pairing bases by DNA polymerase delta.

Interestingly, patients with HNPCC whose tumors show no or mild MSI (i.e. “MSI-low”) often possess germline *MSH6* mutations. This finding presumably reflects the predominant involvement of *MSH6* in repair of mismatches, rather than of the insertion/deletion events that give rise to MSI. An important implication of this finding is that when HNPCC is suspected, absence of MSI does not completely exclude the possibility that an MMR gene mutation is present.

In cells with one normal and one mutant allele of a DNA mismatch repair gene, DNA repair is not, for the most part, impaired. Inactivation of the remaining normal allele can occur as a result of a somatic event, usually allelic loss, in an initiated cell population during tumor development. Once the cell loses this vital component of its self-protection mechanism, it acquires a so-called “mutator phenotype.” Many errors may then arise and fail to be repaired during each cell division. Complete inactivation of an MMR gene may, therefore, promote tumorigenesis through a failure to recognize and repair the multiple point mutations that arise subsequently. Although many of these mutations may be detrimental to cell growth and survival, a subset of the mutations is likely to activate oncogenes or inactivate other TSGs. For instance, genes that have been identified as being frequently mutated in tumors with an MMR defect include those encoding the transforming growth factor beta type II receptor, the E2F4 transcription factor the IGF-II receptor, PTEN and the BAX proapoptotic protein.

**17.6.3.3 Cancer Predisposition in MMR Gene Mutation Carriers.** As mentioned above, in addition to CRC, HNPCC mutation carriers are at a significantly increased risk of other cancers. A study of 360 Finnish mutation carriers found them to have a significantly increased risk of colorectal (standardized incidence ratio (SIR) of 68, with a cumulative incidence of 82% by age 70), endometrial (SIR 62, 60% in women), ovarian (SIR 13, 12% in women), biliary tract (SIR 9.1, 2.0%), bladder/ureter/urethra (SIR 7.6, 4.0%), gastric (SIR 6.9, 13%), kidney (SIR 4.7, 3.3%) and CNS (SIR 4.5, 3.7%) cancers. The ovarian cancers were predominantly cystadenocarcinomas, while the CNS tumors were most commonly glioblastoma multiforme. Only four breast cancers occurred, with an SIR of 1.4 that was not statistically significant. The 360 carriers were from 50 families, most of whom possessed the same *MLH1* mutation and only three had an *MSH2* mutation. Interestingly in females, by age 70, the incidence of endometrial cancer (60%) exceeded that of CRC (54%), while in men, the incidence of CRC was said to be 100%. These figures were thus higher than those of Dunlop et al. (1997) who, studying 67 mutation carriers (predominantly of

*MSH2*), found that the incidences of endometrial cancer and CRC in females (by age 70) were 42% and 30%, respectively, while the incidence of the latter in males was 74%.

**17.6.3.4 Predisposition to Colorectal Cancer by Low Penetrance Genes.** Many apparently familial CRCs that are not due to FAP or HNPCC, may be predisposed to by low-penetrance gene mutations or polymorphisms. An example of the latter is the polymorphism in the *APC* gene, the T to A transversion at nucleotide 3920, which results in the substitution of a lysine in place of an isoleucine at codon 1307 (I1307K). More importantly, it creates a small hypermutable region of the gene, by converting an AAATAAAA sequence to an AAAAAAAA sequence, which is then susceptible to a single adenine insertion or deletion. This founder polymorphism, which is found mainly in Ashkenazi Jews (with a heterozygote frequency of 5–7%), appears to be associated with a relative risk of CRC of between 1.5 and 2.2 with wide confidence limits, and corresponding attributable risk in this population of between 3 and 7% (de la Chapelle, 2004).

It is now apparent that in Caucasian populations, an Ala-repeat polymorphism (6Ala) in the coding region of the antiproliferative *TGFbetaR1* gene is present in ~14% of individuals and is associated (in heterozygotes) with a relative risk of 1.14 of colon cancer, as calculated in a meta-analysis (Pasche et al., 2004). This polymorphism was also calculated to predispose to breast and ovarian cancers with relative risks in heterozygotes of 1.34 and 1.29, respectively (Pasche et al., 2004).

In a large study aiming to identify modifier genes for HNPCC, 6 SNPs originally identified in a large GWAS of unselected CRCs were analyzed in CRC families. Only two of them (at 8q23.3 and at 11q23.1) proved to be significantly associated with tumor development in HNPCC mutation carriers (Antoniou and Chenevix-Trench, 2010).

## 17.6.4 Emerging Therapeutic Possibilities for MMR Deficiency

Several different therapeutic approaches are being investigated as ways of selectively targeting MMR deficient cells. Drugs that cause oxidative damage, including methotrexate, have been observed to be selectively lethal to *MSH2*-deficient tumor cells. This was found to be accompanied by an accumulation of the oxidative stress-associated DNA lesion, 8-OHdG (Martin et al., 2009). Clinical studies of methotrexate in patients with *MSH2*-deficient metastatic CRC are currently underway.

Other approaches include the targeted inhibition of the protein products of oncogenes that frequently acquire secondary (activating) mutations as a consequence of the mutator phenotype (that occurs as a result of MMR deficiency). For instance, inhibitors of the PI3K/mTOR pathway are being investigated (Vilar et al., 2009).

Inhibition of PARP has been found to be a promising new therapy for the treatment of BRCA1- and BRCA2-deficient breast and ovarian cancers. PARP inhibition may also prove useful for some MMR-deficient tumors in the future, in view of the secondary mutations that occur in genes (such as *MRE11* and *hRAD50*) that encode components of the DSB repair mechanism (Hewish et al., 2010).

It is likely that, in the future, the selection of a therapy for MMR-deficient tumors will depend on the precise primary and secondary tumorigenic mutations that have occurred.

### 17.6.5 *MutYH/MYH*-Associated Polyposis

MAP is an autosomal recessive colorectal adenoma and carcinoma predisposition syndrome with phenotypes very similar to FAP and attenuated FAP. This important novel syndrome results from biallelic inherited mutations in a BER gene *MutYH* (human MutY homolog, also commonly known as *MYH*) in the absence of inherited mutations in the *APC* gene.

The condition was initially identified as a result of the investigation of a family in which three out of seven siblings appeared to have attenuated FAP, but without any detectable inherited truncating mutation in *APC* (Al-Tassan et al., 2002). Further analyses revealed somatic mutations in the *APC* gene in the colorectal tumors, which were mostly G:C to T:A transversions. This type of mutation was already known to result from defects in the specific BER pathway that normally corrects mutations that are related to 7,8-dihydro-8-oxo-guanine (8-oxoG). This, the most stable product of oxidative DNA damage, is highly mutagenic on account of its ability to mispair with adenine residues, therefore resulting in spontaneous G:C to T:A transversion mutations in cells deficient in the necessary BER mechanism. Consequently, mutation analysis was carried out on the genes, *MutYH* (*MYH*), *OGG1* and *MTH1*, which encode the proteins involved in this type of DNA repair. This subsequently led to the discovery of missense germline mutations in both alleles of *MutYH* in each of the affected siblings. Colonoscopies of the siblings that were heterozygous for either of the mutations were normal. *MutYH* is a DNA glycosylase that normally removes adenines that have been misincorporated into DNA as a result of pairing with an 8-oxoG residue.

A variety of different mutations have been identified in individuals with MAP. The two mutations detected in the affected siblings were Y165C and G382D, which are the most common mutations in the *MutYH* gene in northern Europeans, by far. In fact, these mutations, which are now known as Y165C and G396D, are present in 1–2% of North Americans and northern European populations (Jones et al., 2009). The functional consequence of these mutations has been verified in vitro and they have been shown to result in reduced DNA glycosylase activity. A

few other recurrent mutations have been detected in specific populations such as the 1395delGGA mutation in Italians (Gismondi et al., 2004). Truncating mutations have been found throughout the coding sequence while non-truncating mutations tend to cluster in the functional domains (Sampson et al., 2005).

In general, biallelic *MutYH* mutations have been detected in around 25% of patients with phenotypes like FAP or attenuate FAP but in whom no germline *APC* mutations have been found (Gismondi et al., 2004; Sampson et al., 2005; Isidro et al., 2004; Venesio et al., 2004; Eliason et al., 2005). Biallelic *MutYH* mutations appear to impart an excess risk of CRC of 93-fold with almost complete penetrance by the age of 60 (Farrington et al., 2005). The number of adenomas can be less than 10 and in several cases CRC has even developed in the apparent absence of any obvious polyposis (Sampson et al., 2005; Croitoru et al., 2004). The phenotype has been found to be more severe in those patients with homozygosity for the Y179C mutation than those who are homozygous for the G396D mutation (or who are compound heterozygotes) (Nielsen et al., 2009a, 2009b).

Identification of those patients with MAP and biallelic *MutYH* mutations facilitates the appropriate testing and surveillance of the appropriate family members, particularly the siblings of MAP patients. A very recently reported longitudinal study of 492 patients on the Manchester Polyposis Registry has found that those individuals who possess biallelic *MutYH* mutations have a median survival of 69.7 years (Newton et al., in press). Duodenal adenomas have been reported in MAP patients (Sampson et al., 2005) and a European study has found that individuals with MAP have a lifetime risk of duodenal cancer of 4% (Vogt et al., 2009). In addition, a significantly increased incidence of ovarian, bladder, and skin cancers (standardized incidence ratios of 5.7, 7.2, and 2.8, respectively) was observed (Vogt et al., 2009).

An additional important question has been whether there is any clinically significant increase in the risk of CRC in individuals who are *MutYH* heterozygotes. Early data from a very large study (2239 cases and 1845 controls) suggested that if there is such an increased risk it is likely to be relatively small and possibly only related to late-onset disease (Farrington et al., 2005). A subsequent, retrospective, study of ~350 parents of patients with MAP (and matched controls) has found that there is a modest increase in risk of CRC of approximately 2-fold but with no significantly increased CRC-related mortality (Jones et al., 2004). The authors noted that this increased risk was comparable to the relative risk observed in the individuals in the general population who are first-degree relatives of patients who have sporadic CRC. They therefore suggest that colorectal screening in heterozygous carriers of a *MutYH* mutation need not be more intensive than in that group (Jones et al., 2004). There is some evidence that the mechanism by



which CRC may occasionally occur in *MutYH* carriers may involve loss of heterozygosity.

With regard to the molecular changes within the tumors developing in MAP, MSI is not usually present (Al-Tassan et al., 2002). There is, in addition, no evidence of mutations in *BRAF*, *SMAD4*, or *TGF-beta* but in some MAP adenomas there is an unusual activating missense mutation of K-RAS, G12C, that results from a G to T transversion (Jones et al., 2004). In addition, in contrast to the MMR genes and particularly the *APC* gene, *MutYH* is not often somatically inactivated by mutation or epigenetic silencing in sporadic colorectal tumors. There is some evidence, however, that BER cooperates with the MMR system in preventing 8-oxoG-mediated mutagenesis. In fact, earlier biochemical analyses established that *MutYH* can bind directly to MSH6, resulting in increased glycosylase activity of *MutYH*.

The study by Fleischmann et al. evaluated whether germline *MutYH* mutations accounted for a significant proportion of early-onset CRCs in general (Fleischmann et al., 2004). Peripheral blood lymphocyte DNA samples from a total of 358 CRC patients in whom CRC had been diagnosed before the age of 56 (but otherwise unselected for phenotype or family history) were screened for germline changes in the gene. Only two cases (0.6%), however, were found to have biallelic *MutYH* mutations and it was concluded that biallelic *MutYH* mutations were unlikely to account for more than 3% of early-onset CRC. Additional data, consistent with this, have been reported (Farrington et al., 2005) indicating that biallelic pathogenic *MutYH* mutations were detected in only 1 out of 50 patients who were diagnosed with CRC aged under 40 years and in just 1 out of 150 patients diagnosed under 55 years of age.

A recent report suggests that *MutYH* polyposis can, in some cases, result from the additive effects of mutations in different genes that encode components of the same (base excision) DNA repair pathway (Morak et al., 2011). Specifically, a patient with CRC and adenomas at age 36 was found to be a double heterozygote for a *MutYH* missense mutation and an *OGG1* mutation that affects splicing of exon 1. The *OGG1* gene encodes 8-oxo-guanine DNA glycosylase. The *MutYH* mutation, p.Ile223Val, alters an amino acid located in the helix-hairpin-helix domain, and was classified by the authors as possibly pathogenic despite the structural similarity of Ile and Val. The splicing effect of the novel *OGG1* (p.Arg46Gln) mutation was confirmed by cDNA analysis (Morak et al., 2011). Further studies will be required to confirm the conclusions from this study.

### 17.6.6 Ataxia Telangiectasia (and the Role of ATM in DNA Double-Strand Break Repair)

Ataxia telangiectasia (AT) is an autosomal recessive disorder primarily affecting the central nervous and

immune systems. The condition is characterized by cerebellar degeneration, immunodeficiency, radiation sensitivity, cell cycle abnormalities, chromosomal instability, and a striking predisposition to leukaemias and lymphomas. For many years, there have been conflicting findings regarding whether heterozygosity for an AT mutation confers an increased risk of breast cancer. A large study, of 1160 relatives of patients with AT, did indicate a moderately increased risk of breast cancer in AT mutation carriers. The relative risk, compared to the general population, of breast cancer in carriers was 2.23 overall and 4.94 in those younger than age 50 years (Thompson et al., 2005). Truncating and non-truncating mutations were not found to be associated with significantly different relative risks (Thompson et al., 2005). More recently, in silico analyses of *ATM* missense mutations have been used to evaluate the frequency of occurrence of such mutations in evolutionarily conserved regions of the gene in breast cancer patients and in controls (Tavtigian et al., 2009). They found that those missense mutations that are predicted by algorithms to be deleterious to the protein's function were associated with breast cancer risk, which was even higher than the risk found to be associated with truncating or splice-site mutations. This finding would suggest a dominant negative effect. It is now regarded that the *ATM* gene, like *CHEK2*, *PALB2* (Partner and localizer of BRCA2) and *BRIP1* (BRCA1-interacting protein 1), is an intermediate penetrance gene for breast cancer, conferring, overall, a 2–3-fold increased risk of breast cancer (Shuen and Foulkes, 2011).

Originally, AT was thought to be genetically heterogeneous, with four complementation groups attributed to different genes. Using positional cloning strategies, *ATM* (for AT, mutated), the gene on chromosome 11q responsible for AT, was identified. Surprisingly, *ATM* was found to be mutated in the germline of patients from all four complementation groups, indicating that it was the sole gene responsible for this disorder. The *ATM* gene, which possesses 66 exons and is located at 11q22-23, is widely expressed and has a transcript size of 12 kb. It encodes a protein of 350 kDa exhibiting similarity, through a kinase domain located in its C-terminal region, to several yeast and mammalian phosphatidylinositol-3 kinases involved in DNA damage recognition and cell cycle control. Its kinase domain, however, possesses protein, rather than phospholipid, kinase activity. *ATM*'s activity increases dramatically within minutes following DSBs such as those induced by ionizing radiation and its activation is believed to be associated with its conversion to an active monomer from an inactive homodimeric state in which the kinase domain of each *ATM* molecule is blocked by the FAT domain of another (Ball and Xiao, 2005). The activation of *ATM* is only partially understood, but it involves auto- or trans-phosphorylation of serine-1981 in response to DNA DSBs and may require protein phosphatase 5 activity (Kurose et al., 2005; Ali et al., 2004). The intriguing



question of how a few breaks in the genome can lead rapidly to the activation of the entire nuclear ATM pool might therefore be answered if a chain reaction occurs, involving released active ATM monomers phosphorylating inactive ATM dimers.

Phosphorylation of *TP53* directly by ATM occurs on Ser15. However, although this direct phosphorylation is critical for the apoptotic activity of *TP53*, the mechanism by which stabilization of *TP53* is induced by ATM is indirect. It occurs by ATM's activation of CHK2/hCds1, which, in turn, phosphorylates *TP53* on Ser20 preventing *TP53*'s degradation by MDM2 (Figure 17-8). In addition to this role in inducing cell-cycle arrest following DNA damage, ATM is implicated in signaling to the repair machinery. For instance, in response to DSBs, it appears to be required for the activation of both the BRCA1–RAD51 complex and the NIBRIN–MRE11–RAD50 complex (see Section “Nijmegen breakage syndrome”). Finally, the autophosphorylation of ATM may be necessary but not sufficient to activate the cellular DNA damage response, merely priming the cells to respond to DNA damage.

### 17.6.7 Bloom Syndrome, Werner Syndrome and Rec-q Helicases

The disorders (BS, WS and Rothmund–Thomson syndrome) that result from defects in the genes encoding the RecQ helicase proteins generally cause premature aging and a predisposition to cancer.

BS is a progressive autosomal recessive disorder characterized by small stature, sun sensitivity, immunodeficiency, chromosome instability, and a striking predisposition to cancer. Cells from patients with BS show an approximate 10-fold increase in the frequency of sister chromatid exchanges (SCEs), which arise by crossing over of chromatid arms during homologous recombination. *BLM*, the gene responsible for BS, was identified by “somatic crossover point mapping” and encodes a 1417 amino acid residue protein with homology to the bacterial RecQ helicases, proteins that can unwind double-stranded DNA into single-stranded DNAs and are believed to possess a role in DNA repair and/or replication. The BLM protein contains seven conserved helicase motifs and appears to exist as a hexameric ring structure surrounding a central hollow. It interacts with RAD51 recombinase and together with topoisomerase III $\alpha$  can resolve double Holliday junctions. Like the WS protein (see below), it thus recognizes and unwinds a wide variety of different DNA structures that arise during homologous recombination DNA repair. Although crossing over is required in meiosis and in the repair of DSBs, in mitotic cells it can be associated with detrimental loss of heterozygosity. BLM is believed to prevent excessive crossing over with BLM inactivation therefore resulting in hyper-recombination and thus SCEs and genomic instability. It also catalyzes the annealing of DNA single strands and it may be involved in the

resolution of DNA structures occurring in the late stages of DNA replication; for example, where two replication forks converge (Cheok et al., 2005). The physiological role of BLM in human cells remains to be fully determined but it is widely believed that the protein plays an important part in restarting blocked DNA replication forks (Chabosseau et al., 2011).

Werner syndrome is a rare autosomal recessive disorder characterized by premature aging, with an early onset of age-related diseases such as atherosclerosis and, in addition, tumors such as soft-tissue sarcomas. The causative gene, *WRN*, has been identified and its function studied. Like the BS gene, it encodes an ATP-dependent DNA helicase, with 3'–5' directionality, homologous to the *E. coli* protein RecQ and which unwinds double-stranded DNA. The WRN protein has been found to interact with numerous proteins including DNA replication proteins, such as the multifunctional replication protein A (RPA), PCNA, topoisomerase I, DNA polymerase delta and FEN1 (Ozgenç and Loeb, 2005). It also interacts with DNA repair proteins such as *TP53*, PARP-1, DNA polymerase beta, APE-1 and the Ku-DNA-PK complex (Ozgenç and Loeb, 2005). In addition, consistent with the observed premature replicative senescence and increased loss of telomeres in WS cells, WRN has been found to associate with the telomere repeat binding factors: TRF1 and TRF2 (Opresko et al., 2004; Machwe et al., 2004). In addition to helicase and ATPase activities, the WRN protein, unlike other RecQ helicases, possesses a 3'–5' exonuclease function. Cells from WS patients, unlike those from BS patients, do not typically show increased SCEs, though both groups of cells do exhibit genomic instability. WS cells also exhibit defects in DNA replication, recombination, repair and transcription (Kyng and Bohr, 2005). The importance of WRN in vivo, however, especially in relation to WS, has not yet been defined precisely, but most likely involves the participation of WRN in the resolution of alternative DNA structures thought to exist as intermediates in a variety of DNA synthetic processes. These processes include DNA replication (forked DNA), repair (e.g. partial duplexes with a single-stranded bubble), recombination (triplex DNA, G4 tetraplexes and Holliday junctions) and telomere maintenance (D-loops). Recently, a small molecule inhibitor of WRN helicase activity, without inhibiting the other DNA helicases, has been identified from the US National Cancer Institute's Diversity Set (Aggarwal et al., 2011).

In addition, patients with Rothmund–Thomson syndrome, a further rare autosomal recessive disorder associated with genomic instability, premature aging and a predisposition to neoplasia, have been found to have mutations in another RecQ helicase gene, *RECQL4*. The RECQL4 protein has been found to localize to distinct nuclear foci and to associate with RAD51, thus implicating it in the repair of DNA DSBs by homologous recombination (Petkovic et al., 2005). Although the

precise function of RECQL4 is not yet fully understood, a general consensus is that it is involved in DNA repair and the most recent data indicate that it is the N-terminal region so the protein that is most important for its normal function (Abe et al., 2011).

### 17.6.8 Nijmegen Breakage Syndrome

Nijmegen breakage syndrome is another rare autosomal recessive DNA repair disorder. Like in both AT and BS, there is chromosomal instability, immunodeficiency, and a predisposition to cancer. In NBS, there is a highly increased risk of lymphoreticular malignancy (Seemanova et al., 2005). Unlike in AT, however, specific neurological features are rare, and the serum alpha-fetoprotein is not raised. In addition to those features mentioned above, microcephaly with relatively preserved mental development, typical facial appearance, growth retardation and X-ray hypersensitivity are characteristic, with café-au-lait spots, vitiligo, clinodactyly and syndactyly being additional important features. The SCEs, characteristic of BS, are not, however, a feature of either AT or NBS. The first patient with NBS was not described in 1981 and the defective gene, *NBS1*, was identified in 1998. *NBS1* is located on chromosome 8q21 and encodes a protein named NIBRIN. Sequence analysis revealed two amino terminal domains, a forkhead associated domain (FHA) and a breast cancer carboxy terminal domain (BRCT), both of which had previously been found in DNA damage responsive cell cycle checkpoint proteins. Further evidence for the role of NIBRIN in the repair of DNA damage was provided by the finding that it forms a heterotrimeric complex with MRE11 and RAD50, which participate in DSB DNA repair. This complex is recruited to DSB sites to process the DNA in preparation for repair. It appears that MRE11, by virtue of its exonuclease activity, prepares the ends of the DNA for repair and that RAD50 holds the ends together (Stracker et al., 2004).

The similarity between the chromosomal instability observed in at least AT and NBS can now be partly explained at the protein level. The protein kinase, ATM, which is activated by DSBs, is now known to phosphorylate not only p53, CHK2, and BRCA1, but also NIBRIN. Phosphorylation of NIBRIN by ATM occurs at four sites—serine residues 278, 343, 397 and 615—and the phosphorylation of all four appears necessary for the cellular response to DNA damage. Thus, in the presence of damaged DNA, ATM normally functions not only by activating cell-cycle checkpoints (by phosphorylating CHK2 and p53) and thus preventing duplication of damaged DNA but also by activating the repair of the damage. Studies using Nbs1 mouse models indicated that nibrin acts not only downstream of ATM but also upstream of ATM, forming a positive feedback or amplification loop that permits a small number of DNA DSBs

to generate a potent checkpoint response (Diflippantonio et al., 2005).

Over 90% of patients with NBS have been found to possess a founder truncating 5bp *NBS1* deletion, 657–661 delACAAA, which results in the production of a 26 kDa protein that lacks the MRE11-binding domain (Ball and Xiao, 2005). Most patients are of Slavic origin and are homozygous for this 657del5 mutation (Seemanova et al., 2005). Monozygotic twin brothers, compound heterozygous for 657del5 and a 643C>T(R215W) mutation, were reported to be more severely affected than the majority of NBS patients, with severe developmental delay, that is unusual in NBS patients (Seemanova et al., 2005). Accordingly, their cells displayed strongly reduced phosphorylation of ATM and p53.

### 17.6.9 Xeroderma Pigmentosum and Nucleotide Excision Repair

Xeroderma pigmentosum (XP) is one of three rare recessive syndromes with significant cutaneous photosensitivity (XP, Cockayne syndrome and the photosensitive form of trichothiodystrophy) that results from a defect in the nucleotide excision repair (NER) DNA repair mechanism. The role of NER is to remove helix-distorting bulky adducts, such as the UV-induced cyclobutane pyrimidine dimer (CPD) formed between adjacent pyrimidines in the same DNA strand. The cutaneous hallmarks of XP are “parchment skin” and prominent freckles on the sun-exposed areas of skin. Moreover, XP patients have an ~1000-fold increased risk of skin cancer, predominantly basal cell and squamous cell carcinomas, with melanomas occurring less frequently. The mean age of onset of skin tumors is 8 years. Patients with CS or trichothiodystrophy (TTD), in contrast, have not been demonstrated to possess an increased risk of skin cancer. Cockayne syndrome (CS), in addition to cutaneous photosensitivity, comprises a growth defect, cataracts, progressive neurological degeneration, microcephaly, sensorineural hearing loss, pigmentary retinopathy and dental caries. TTD is a complex neuroectodermal disorder that involves a deficiency in the synthesis of high-sulfur matrix proteins and comprises sulfur-deficient brittle hair, cutaneous photosensitivity, growth retardation, intellectual impairment and other features such as ichthyosis and neutropenia.

Cell fusion and molecular studies have defined eight genes (initially differentiated as complementation groups) for XP (*XPA* to *XPG*; *XPV*), two for CS (*CSA* and *CSB*), and three for TTD (*XPB*, *XPD* and *TFB5*) that have been cloned and the function of their products has been partly determined. There are two tightly regulated NER pathways, together involving a total of around 40 proteins, and both can be affected in XP: global genome repair (GGR) and the less widespread (but often more rapid) transcription-coupled repair (TCR). In general, NER

proceeds by the following steps: (a) initial DNA damage recognition (involving XP-C and XP-E in GGR, and CS-A and CS-B together with a stalled RNA polymerase in TCR); (b) local chromatin unwinding (involving the zinc-finger protein, XP-A, and the two helicase subunits of the transcription initiation factor TFIIH, XP-B and XP-D); (c) excision of 24–32 nucleotides of the damaged strand by incisions made at sites flanking the lesion (by the 5' and 3' endonucleases XP-F and XP-G); and finally (d) gap filling by DNA polymerase delta, PCNA and DNA ligase (Cleaver, 2005).

Many patients in the XP-A, XP-B, XP-D, and XP-G groups (in which, typically, both TCR and GGR NER activities are deficient) tend to have more severe NER deficiencies, while patients in the XP-C and XP-E groups (who have deficient GGR but not TCR) and in the XP-F group tend to have either absent or late-onset neurological symptoms. The absence of cancer predisposition in patients with CS might be related to the role of CS-A and CS-B in mediating only the initial damage recognition step of transcription coupled repair. It has been suggested that the failure to carry out TCR, specifically, would lead to increased apoptosis and consequent neuronal loss rather than a high degree of genomic instability, copy number changes and cancer predisposition. Genomic instability would instead be envisaged to be a feature in patients with mutations affecting the XP genes encoding proteins involved in either GGR initiation specifically or even within the common part of the pathway (i.e. steps subsequent to the initial recognition step). Inactivation of XP proteins within the common part of the pathway would also be predicted to predominantly affect GGR rather than TCR, since only around 1–2% of total genomic DNA is estimated to be actively transcribed (Cleaver, 2005). Similarly, those patients whose mutations affect proteins that are not involved in TCR (i.e. XP-C, XP-E and XP-V) have fewest non-cancer disease manifestations and rarely have significant neurodegenerative disorders. Patients with mutations in XPB, XPD, or XPG have symptoms representing combinations of XP and either TTD or CS, depending on the particular mutations. Finally, patients with XP variant (XP-V) possess mutations in the gene encoding DNA polymerase  $\epsilon$ , which carries out bypass replication past unrepaired CPDs, and have a wide variability in disease severity that does not relate to the mutation type or position.

In the NER pathway, the same set of core protein factors essentially have to be able to recognize an enormous range of different possible abnormalities in the DNA, many of which are remarkably subtle structural changes. It appears that this is achieved by a complex but elegant step-by-step process rather like a “decision tree analysis” that involves the sequential screening of the DNA structure by the multiple XP subunits, each analyzing the DNA for a different abnormal characteristic (Naegeli and Sugawara, 2011).

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### Biography



Edward S Tobias

Qualifications: BSc (1st class Hons), MBChB (Commendation), MRCP, PhD (Molecular Biology), FRCP.

Dr Tobias' research group aims to improve the understanding of a variety of medical genetic disorders. The group is closely linked with the West of Scotland Regional Genetics service with several projects arising from the molecular investigation of patients with clinical genetic conditions that range from single gene disorders to complex diseases. The group has strong interests in the genetic basis of long QT syndrome (in collaboration with the FANS network and Generation Scotland) and congenital Mullerian abnormalities, in addition to the role of tumor suppressor genes in human cancer.

Dr Tobias has multiple collaborators within and outside the United Kingdom. Please note that the Publications and Grants pages are currently being updated. Dr Tobias had previously gained an MRC Training Fellowship and won a prestigious GlaxoWellcome/GlaxoSmithKline Senior Clinical Fellowship. He had previously discovered a new cancer gene (submitted to the international Genbank database and leading to papers in *Oncogene* and in *Nature Genetics*). Recent molecular genetic work identifying a new candidate tumor suppressor gene was graded "Excellent/clearly outstanding" by the Scientific Committee of the Chief Scientist Office. In addition, very recent work in elucidating the parent-of-origin effect in SDHD-related tumorigenesis led to the award of a prize to Dr Tobias from the British Society of Human Genetics (September 2011) and to a paper now in press in the *Journal of Clinical Endocrinology and Metabolism*. Two other projects have led to the award of prizes.



# CHAPTER 18

## The Biological Basis of Aging: Implications for Medical Genetics

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### 18.1 INTRODUCTION

Evolutionary biology has provided a robust theory to explain why we age, but we have much less confidence that we understand how we age, by which we mean the proximal molecular mechanisms of aging. Medical geneticists are in a good position to advance our knowledge of such mechanisms. The first strategy is the time-honored approach of mapping, cloning, and characterizing the relevant gene actions underlying important late-onset disorders of aging, such as dementias of the Alzheimer type, atherosclerosis, ocular cataracts, type 2 diabetes mellitus, osteoporosis, osteoarthritis, and cancer. Significant progress has been made using this strategy, including major advances in our understanding of segmental progeroid syndromes such as the Werner and Hutchinson–Gilford syndromes. The second strategy is to investigate the genetic basis for unusually well-preserved structure and function during the latter half of the usual life span. Unfortunately, medical geneticists have been too preoccupied with disease and, with some notable exceptions (e.g. studies of centenarians), physicians and geneticists have not shown a strong inclination to investigate exceptional well-preserved late-life phenotypes. The new statistical and molecular tools at our disposal are impressive, but they have not been matched by the application of sensitive functional assays.

### 18.2 WHAT IS AGING?

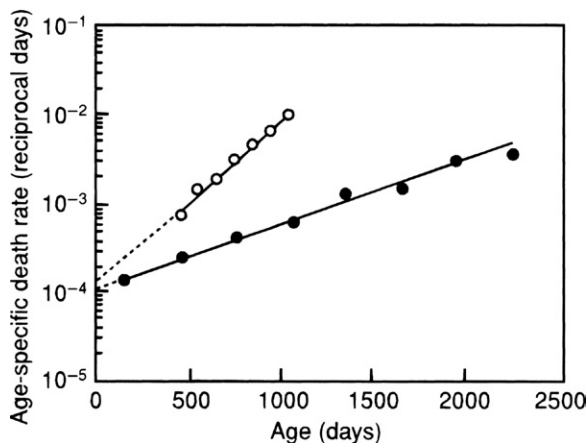
Some gerontologists, particularly those interested in the aging of plants, sharply differentiate between the terms aging and senescing. Senescent changes, they would

argue, are those structural and functional changes that occur near the end of the life cycle of a cell, tissue, organ, or organism, and are associated with the impending death of the tissue or organism. By contrast, the term aging would be used for any change in structure or function throughout the life cycle. In other words, some would argue that “aging begins at birth.” Most gerontologists who work with mammals and human subjects, however, use the two terms more or less interchangeably. While different scholars define human aging in various ways, most include exponential increases of age-specific mortality rate and declines of the physiological functions as general characteristics of human aging processes. These parameters were primarily derived from cross-sectional population studies. Researchers of basic biology of human aging at the organismal level have a major difficulty that stems from the fact that, unlike model animals, humans are genetically heterogeneous and undergo behavioral and environmental changes throughout their lifetime. Moreover, due to the relatively long life span of humans the cohort studies of longevity, for example, take many years to complete. One approach to testing the validity of the population studies of human longevity and aging is to compare the findings of other model organisms such as yeast (*Saccharomyces cerevisiae*), fruit fly (*Drosophila melanogaster*), worm (*Caenorhabditis elegans*) and mice, with those from *Homo sapiens* (1). They created a large segment of the foundations for the progress we have seen in human biology. Moreover, their contributions have typically been more incisive, in part because of the experimental tractability of their materials.

While none would deny the importance of development in determining the subsequent life history of an organism, most gerontologists are concerned with declines in structure and function that gradually and insidiously unfold after the organism has achieved the young, mature adult phenotype. At the level of populations, these functional declines translate into an exponential increase in the force of mortality over unit time—the hazard function or instantaneous mortality rate (2,3). This is the famous Gompertz relationship (4). This was modified by Makeham (5), who included a constant,  $A$ , to account for kinetic departures presumed to have resulted from causes of death during the early life history that were age-independent. The Gompertz–Makeham equation can thus be given as the sum of two types of mortalities, age-independent and age-dependent, the latter exhibiting exponential kinetics over the adult life span:

$$\mu_x = A + Re^{ax}$$

where  $\mu_x$  is the force of mortality at a given age,  $x$ ;  $A$  is the Makeham constant;  $R$  is the hypothetical value for the force of mortality at birth, the lowest force of mortality, or the  $Y$  intercept in a graphic plot of age ( $X$ -axis) versus force of mortality ( $Y$ -axis) (Figure 18-1);  $e$  is an exponent; and  $\mu$  is a constant representing the slope of the graphical plot (Figure 18-1). Figure 18-1 illustrates differing rates of exponential increases in the force of mortality for two non-inbred wild-type murine species

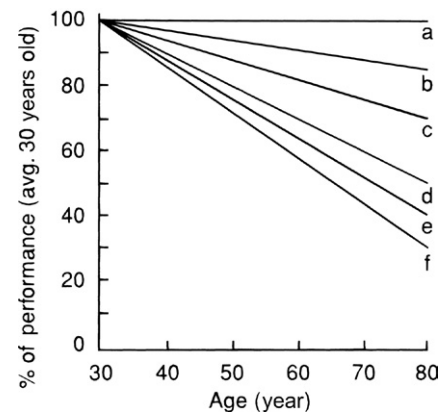


**FIGURE 18-1** Gompertz function plot of the age-specific mortality rates for combined sexes of two different murine species of contrasting maximum life-span potentials but of comparable size. Both species were wild-type and randomly bred from small cohorts captured near the Argonne National Laboratories (Argonne, IL) by the late George A. Sacher. They were housed under essentially identical conditions (caging, bedding, humidity, temperature, diet) in adjacent animal rooms with no special efforts to establish specific pathogen-free conditions (G. A. Sacher, personal communication to G. M. Martin). The longer lived species (●), *P. leucopus*, was found to have a maximum life span of about 8 years, approximately twice that of *M. musculus* (m). (From Sacher, G.A. *Evolution of Longevity and Survival Characteristics in Mammals*. In *The Genetics of Aging*; Schneider, E.L., Ed.; Plenum Press: New York, 1978.)

despite comparable values for  $R$ . These two species, *Peromyscus leucopus* and *Mus musculus*, are of approximately the same size and were housed and fed throughout their lifetimes under identical conditions (6). The maximum life span of *Peromyscus* sp. was found to be about 8 years, about twice that of *Mus* sp. These data illustrate the importance of genetic factors in the determination of approximate life potential. Given the considerable evolutionary distance between these two species (at least 15 million years), this is not a surprising result. Mortality rates of different human populations in the twentieth century also followed Gompertz–Makeham relationship until they reach to very old age (2,3,7).

Experiments employing very large populations of aging cohorts of fruit flies and medflies have been reported as showing dramatic departures from Gompertz kinetics within the oldest cohorts, with apparent decreases in the force of mortality at very advanced ages (8). Very aged flies, however, may become virtually immobilized and may therefore be protected from environmental hazards related, for example, to attempts at flight. Declines in age-specific mortality rates have also been seen for very aged individuals in human populations (9). Perhaps this observation might also be explained by behavioral changes (e.g. a more protective environment) in extreme old age. It will nevertheless be prudent to explore various non-Gompertzian models of mortality in human populations that adequately describe mortality at extreme ages. The existence and estimate of the upper limit of the human longevity or maximum life span of human has been a focus of debates, although it is generally believed to be around 125 years based on the verified longest lived human (10).

Functional declines can be documented in virtually every organ systems starting shortly after sexual maturation. Most physiologic declines, at least in cross-sectional



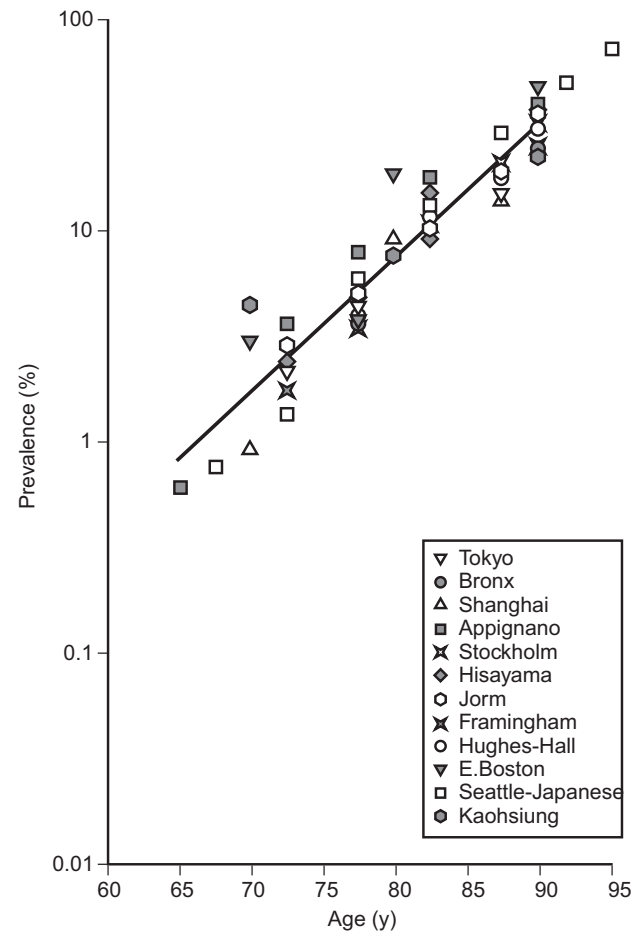
**FIGURE 18-2** Linear declines of functional assays for several different human physiological parameters as studied cross-sectionally by the late Nathan W. Shock and colleagues. The values are expressed as percentages of the average performances of healthy 20- to 35-year-old male subjects. (a) Fasting blood glucose. (b) Nerve conduction velocity. (c) Cardiac index (resting). (d) Vital capacity and renal blood flow. (e) Maximum breathing capacity. (f) Maximum work rate and maximum oxygen uptake. (Adapted from Shock, N. W. *Systems Integration*. In *Handbook of the Biology of Aging*; Finch, C. E., Hayflick, L. Eds.; Van Nostrand-Reinhold: New York, 1977.)

studies, exhibit linear declines, the slopes of which are variable (Figure 18-2) (11). Declines in the various physiological processes (and underlying molecular and biochemical processes) that maintain optimum functions are likely to “set the stage” for the plethora of late-life disorders and diseases, some 87 of which have recently been tabulated, all of which are subject to both genetic and environmental modulations (1). However, observations of exponential increases in the force of mortality within populations should not lead one to conclude that underlying processes of aging or incidences of geriatric diseases necessarily exhibit exponential kinetics. Consider, for example, the world records of marathon runners, which select for the most robust, physically fit members of our population. This is an attractive assay, as it tests for fitness of multiple organ systems and one’s ability to maintain metabolic homeostasis. Declines are observable during the fourth decade, later than what is the case for sprinters. This probably occurs, in part, because it takes considerably more training and experience in perfecting one’s optimal pacing for a marathon. It also takes years for the gradual development of such compensatory processes as cardiac muscle hypertrophy. For a remarkably wide range of sports, peak activity occurs during the third decade (12).

Many major diseases of late life, however, show exponential increases in age-specific incidence and prevalence, although there may be slight declines in age-specific incidence at very advanced ages, raising the question of selection for genotypic resistance against specific late-life disorders. Alzheimer disease serves as a good example (13,14). Figure 18-3 summarizes the results of several community-based studies of the age-specific incidence of late-life dementias, most of which are due to dementias of the Alzheimer type (15).

Longitudinal studies of physiologic parameters may exhibit striking variation among individuals (16). Figure 18-4 illustrates the case of a measure of renal function. By this measure, some individuals show no evidence of a decline in renal function; some may have superior compensations for structural alterations (17). Are any of these varied patterns of functional decline (or lack of decline) in apparently normal aging human subjects determined, in part, by constitutional differences in the genotype? Essentially no research has been carried out to address this important question. Medical geneticists have an obvious bias in favor of the discovery of mutations and deleterious allelic variants. There is a great need to define allelic variants that, in ordinary environments, are associated with the maintenance of enhanced structure and function during aging. One such example may be the ApoE2 allele, the prevalence of which is significantly increased in centenarians (18). The APOE2 results are understandable in that there is evidence that carriers are provided with some protection against Alzheimer disease (19).

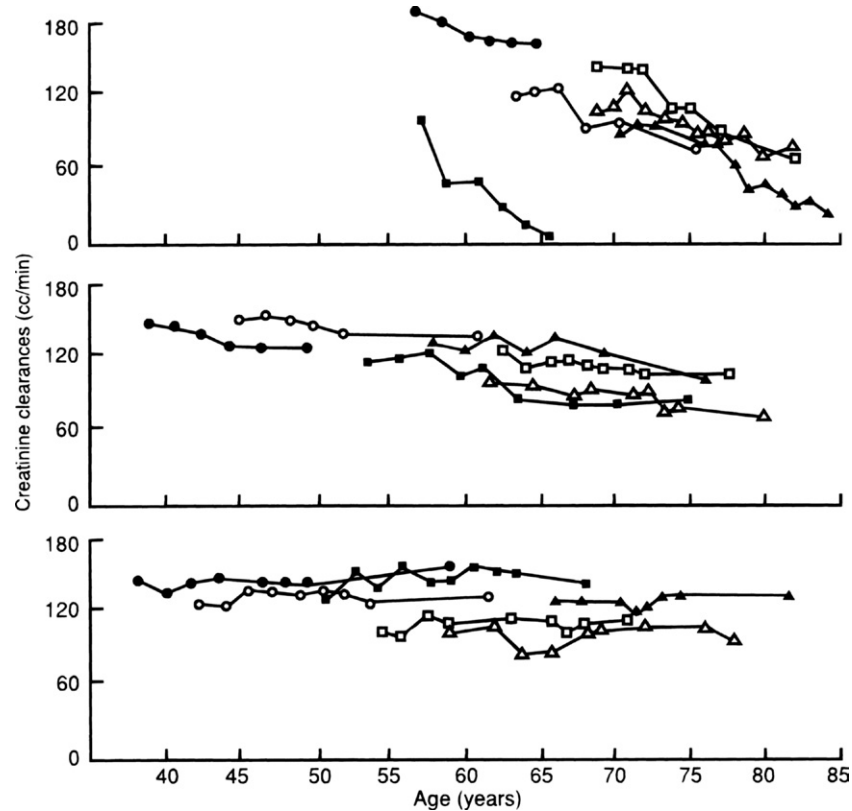
There is very strong evidence indicating a major role for the constitutional genotype in the susceptibility to various familial and “sporadic” forms of Alzheimer disease. In addition to the apparent protection by the E2



**FIGURE 18-3** Evidence for exponential increases in the age-specific incidence of probable Alzheimer disease in six different community-based studies. (From Breteler, M. M. B. et al. *Epidemiology of Alzheimer Disease*. Epidemiol. Rev., 1992, 14, 59–82.)

allele of ApoE noted above, individuals carrying the E4 allele, particularly homozygotes, are clearly at elevated risk to develop the disease (20). ApoE4 may act as an age-of-onset modifier for the common relatively late-onset forms of the disorder. There may be multiple mechanisms underlying the effects of the e4 allele (21). The lay perception that aging is accompanied by a global loss of cognitive function is certainly incorrect. Only selected regions of the nervous system appear to be particularly susceptible.

The other type of terminally differentiated cell receiving special attention from gerontologists is the multinucleated skeletal muscle cell. Structural and functional declines in skeletal muscle vary from muscle to muscle, with weight-bearing muscles more susceptible; the rates of these declines accelerate after about age 70 (22). At least some proportion of the pathology is likely to be related to denervation atrophy. Disuse atrophy is also an important component, as evidenced by the significant gains in muscle strength in even very aged subjects following a modest conditioning regimen (23). Old skeletal muscle is also more susceptible to contraction-induced injury and is less subject to repair via satellite cell-mediated regeneration (24).



**FIGURE 18-4** Longitudinal studies of creatinine clearance (an approximate measure of the glomerular filtration rate) for a representative sample of a subset of 446 clinically normal male volunteers in the Baltimore Longitudinal Study of Aging of the National Institute on Aging followed between 1958 and 1981. The results could be classified in one of three major patterns. The top panel illustrates substantial rates of decline in this measure of renal function for six representative subjects who were followed for 8–14 years. The middle panel illustrates a pattern of slight, but significant decline for six representative subjects followed for 11–22 years. For the six representative subjects in the bottom panel, who were followed for periods of 15–21 years, there were no apparent declines in this measure of renal function. (From Lindeman, R. D. et al. *Longitudinal Studies on the Rate of Decline in Renal Function with Age*. J. Am. Geriatr. Soc. **1985**, 33, 278–285.)

Many postreplicative aging cell types gradually accumulate a mixture of complex fluorescent pigments called lipofuscins. These are likely to vary in composition from tissue to tissue. Most investigators believe that all lipofuscins are the products of lipid peroxidation reactions. They could therefore be regarded as evidence in support of theories of aging that invoke oxidative alterations of macromolecules. That lipofuscins are markers of some underlying aging process is a theory supported by three lines of evidence. First, they appear to be almost invariable features of aging in an amazing variety of organisms, including certain strains of fungi under certain growth conditions (e.g. *Podospora anserina* and *Neurospora crassa*), paramecia, nematodes, snails, fruit flies, houseflies, frogs, parrots, house mice, rats, guinea pigs, cats, dogs, pigs, monkeys, and humans (25,26). Second, quantitative studies of lipofuscin rates of accumulation in the hearts of dogs and humans indicate appropriate correlations with the life-span potentials of those species (27). (No such correlations have been observed, however, among cardiac tissues from a group of primates of contrasting life spans (28).) Third, age-related increases in concentrations of at least some classes of lipofuscins are blunted by caloric restriction, an intervention known to increase life span in mammals (29,30).

The importance of extracellular aging has been emphasized by the late Robert R Kohn (31). Long-lived proteins, such as lens crystallins and collagens, are particularly susceptible to a variety of posttranslational alterations. Diabetics, who have many progeroid features, are particularly susceptible to glycation of proteins (32). Advanced glycation end products may also play an important role in the genesis of osteoarthritis (33). Modified matrix components could perturb cell–matrix interactions and hence change cell function. Such a scenario has been suggested to play a role in the genesis of atherosclerosis (34). It remains to be seen which of the numerous human mutations involving matrix proteins can inform us as to pathogenetic mechanisms in the normative aging of the extracellular environment.

### 18.3 WHY DO WE AGE?

Evolutionary biologists believe that they have an answer to the ultimate cause of aging in age-structured populations (i.e. populations that consist, at any given time, of cohorts of varying chronological ages) (35). Simply put, we age because senescent phenotypes escape the force of natural selection (36). This theory was developed for the



case of species with age-structured populations, a situation that occurs when there are serial episodes of reproduction in an individual's lifetime, as opposed to one massive “big-bang” production of progeny in short-lived animals (37). For human populations, the late William Hamilton showed that the force of natural selection for or against alleles that do not reach phenotypic expression until about the age around 45 years is essentially nil (38,39). More recently, a mathematically rigorous challenge to Hamilton's theory has been published (40). It describes a scenario whereby the force of natural selection can actually increase during aging. There are in fact some species of fish that continue to grow and, as such, become more like predators than prey. Under those circumstances, it is easy to imagine declines in the force of natural selection with age.

August Weissmann, one of the giants of nineteenth century biology, postulated a limited life span of somatic cells, from which he proposed programmed death theory with the idea that aging is good for the species in that it results in enhanced resources for the young. While a finite replicative capacity of somatic cells was later confirmed experimentally (41), there has been no good evidence that aging evolved because it was adaptive for the species or the individual. Essentially all population geneticists who have considered this issue have concluded that aging is nonadaptive. A striking demonstration that single gene mutations can extend the life span of nematodes, fruit flies and mice—sometimes dramatically (42)—provides evidence against the classical evolutionary biologic theory of aging, which would predict a highly polygenic modulation. Moreover, we have known for many decades that a single environmental manipulation, caloric restriction (or, more conservatively, dietary restriction), can substantially enhance life span in a remarkably wide range of species (reviewed by Masoro (43)). Some would argue that these observations support a “programmed” mechanisms of aging—that is something more in the line of sequential, determinative changes in gene expression that actively produce aging. One interpretation of both the single gene mutation and caloric restriction experiments is that all or many of them are examples of *diapauses*—time-outs from the business of reproduction during “bad times”—be they nutritional, climatic, or other environmental challenges.

In terms of genetic mechanisms that form the basis of the classical evolutionary theory of aging, two ideas currently dominate the field. The first, championed by the late Peter Medawar, is generally referred to as “mutation accumulation” (44). This is an unfortunate name, as the mutations in question are not somatic mutations developing during the life span, but germ line mutations that do not reach phenotypic expression until late in the life course, when the force of natural selection would be attenuated. Huntington disease is the prototypical example. Haldane was puzzled by the surprisingly

high prevalence of this disorder, which exceeds 15 per 100,000 in some western European populations (45), while germ line mutations typically have frequencies of about one in a million. Haldane suggested that the reason the mutation survived in the population was because of its delayed manifestations, thus escaping the force of natural selection (46). If that were the case, there would be selection for “suppressor alleles” that progressively delayed the age of phenotypic expression. Medawar concluded that many such suppressors might only delay these deleterious effects (44); eventually, however, the delayed age of expression would be such that there could be little or no influence of natural selection. This scenario, especially when coupled with the other mechanisms discussed below, would result in an enormous degree of heterogeneity in patterns of aging among individuals in out-breeding populations. Thus, each of us may be essentially unique in precisely how we age.

The second dominant idea was first clearly enunciated by George C Williams (47), and has been referred to as antagonistic pleiotropy; it has been elaborated by Michael R Rose (36). By this view, some varieties of genes might have been selected because of good effects early in the life span, but may also have deleterious effects late in the life span, thus contributing to aging phenotypes. As one potential example, Williams speculated that alleles selected because of enhanced incorporation of calcium into bones might be responsible for forms of calcific arteriosclerosis, when acting over long periods. There have been many suggested examples in the literature, but they have been hard to definitively establish. Potential examples include atherosclerosis (48), the role of the apolipoprotein E4 allele in Alzheimer disease (49), common late-life cancers (50), and immunosenescence (51). Medical geneticists are in a good position to suggest a number of other examples, and perhaps to provide compelling supportive evidence. Such research has the potential to illuminate the most basic aspects of the aging problem.

Another conceptual formulation, one that overlaps with what has been discussed above, is that there is, inevitably, a trade-off of energetic resources expended by an organism for purposes of reproduction and resources devoted to the maintenance of the macromolecular integrity of the organism. Examples include repair of DNA, scavenging of abnormal proteins, and replacement of effete somatic cells; this is the disposable soma theory of Tom Kirkwood (52). These ideas can be generalized as life history “optimization” theories of aging (53). Experimental evidence in *Drosophila* sp. supports both optimization theories and the mutation accumulation theory of aging (53,54). The relative quantitative contributions of each theory, however, particularly in *H. sapiens*, are completely unknown. As pointed out by Partridge and Barton (53), the resolution of this issue has potentially profound implications for the future life history of our species. If optimization mechanisms predominate, any life-span extensions may be offset by the trade-off

of lower early fertility, delayed maturation, and potential increases in early life history morbidity and mortality. If mutation accumulation mechanisms prevail, enhanced life span attributable to the elimination of such constitutional mutations would presumably have few effects on early life-span structure and function. Given a major role for optimization theories, a continuation of the present secular trends of elective delays in the ages of reproduction in the developed societies would predict the emergence, by indirect selection, of increased life spans and related declines in early fertility after several centuries of continued evolution of our species. However, it has been well documented that advanced parental age is associated with increases in germ line mutations. Of particular relevance to our interest in late-life disorders is the evidence that there is a large paternal age effect for point mutations (55). Such secular trends could therefore be associated with increases in germ line mutations, with potential deleterious effects in subsequent generations. It has been difficult, however, to confirm a relationship of paternal age and the occurrence of nonfamilial varieties of such common polygenic late-life disorders such as Alzheimer disease or prostate cancer (56).

At a more fundamental level, one can ask why one observes such striking variations in the life spans of various mammalian species. While such variation is obviously related to the constitutional genotype, it does not necessarily follow that aging is “programmed”—at least in the sense of concerted, determinative, sequential gene action comparable to what one observes in development. The most satisfying idea invokes differential impacts of environmental hazards (e.g. accidents, predation, drought, starvation, infectious diseases) during the emergence and maintenance of various species. This is nicely articulated in a popular book on aging (57). Species with comparatively high hazard functions would be expected to evolve life history strategies that emphasize rapid maturation, high fecundity, early fecundity, and short life spans. An attenuation of those hazards could set the stage for the emergence of sibling species with a more leisurely rate of maturation, lower early fecundity and longer life spans. One of the few field biology studies to examine this idea has in fact provided strong support for that hypothesis (58). Contrary findings have been reported, however, for different species in different ecologies (59).

The evolutionary formulations of the nature of aging have a number of interesting and important implications, in addition to those noted above. Let us summarize some of these propositions:

1. Stochastic processes: These are likely to play a major role in senescence. This follows from the conclusion that one is not dealing with a determinative sequence of concerted gene action but rather with an epiphenomenon of selection for gene action designed for reproductive fitness. Consider the analogy with a spacecraft engineered to function for a given period of time in order to complete a specific mission. Engineering

specifications for indefinite maintenance of the craft would be prohibitively expensive or impossible. One would therefore anticipate an element of chance as to which components will initially exhibit structural and functional failures and when such failures will be detectable with the available diagnostic facilities. Many major geriatric diseases of humans (e.g. cancer, strokes, coronary thrombosis) are surely based on stochastic events. In the case of malignant neoplasms, selection for a series of random somatic mutations is the key to the understanding of the pathogenesis. Overall longevity also is subject, in part, to stochastic laws. There are numerous examples in which investigators have rigorously controlled both environment and genotype, yet have observed marked variations in longevity. The most convincing example comes from studies of *C. elegans*, which can be grown in chemically defined medium in suspension cultures, thus ensuring rigorous control of the environment (60).

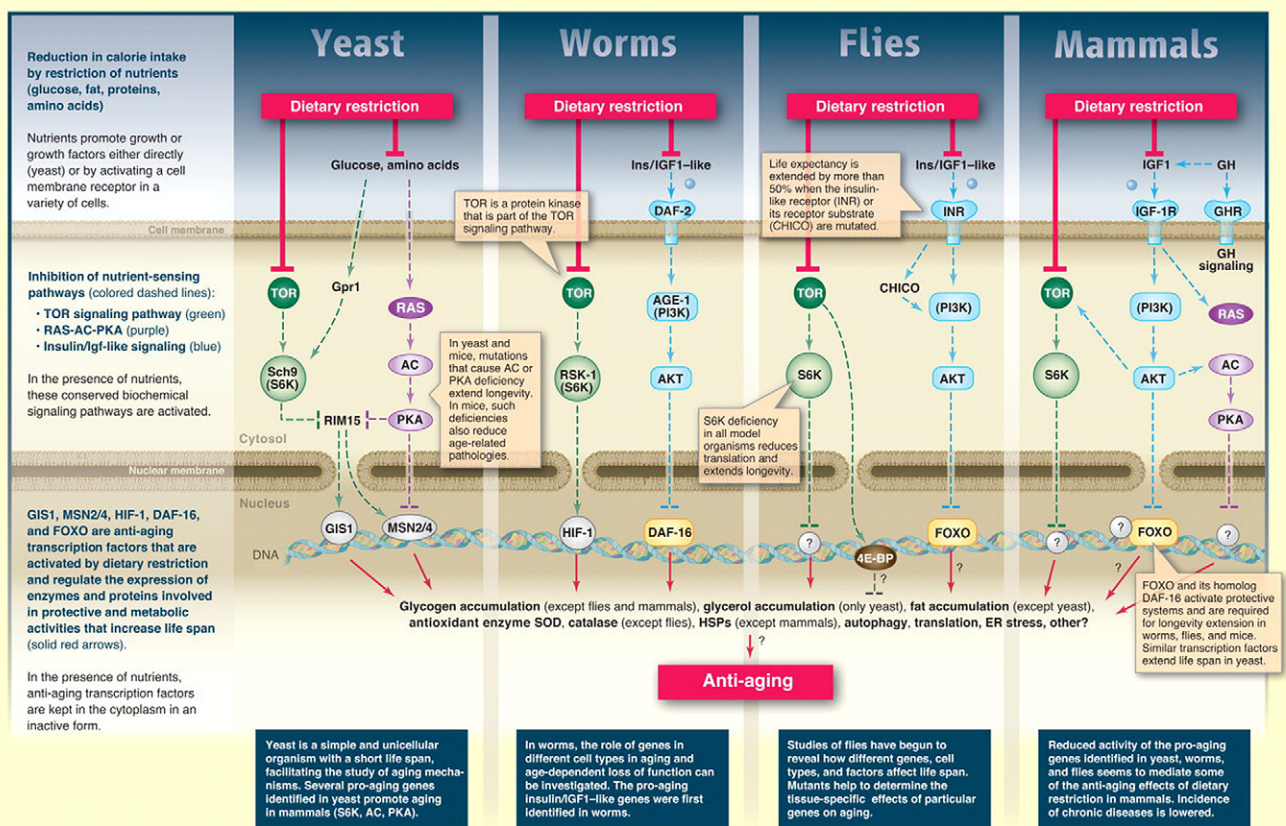
2. Polygenic basis: There is a polygenic basis for aging. There is no reason to believe that the optimization theories or mutation accumulation theories involve only a few genes. Indeed, for the case of the successful experiments involving indirect selection for increased life spans in genetically heterogeneous wild-type stocks of *D. melanogaster*, genetic analysis indicated genes on all of the major chromosomes (61). Martin (62) estimated (as an upper limit) that allelic variation or mutation at close to 7% of loci of the human genome has the potential to modulate varying aspects of the senescent phenotype. A different and more conservative estimate—the number of genes likely to have evolved in the hominoid lineage leading to humans (and thus could be associated with the increased life span of *H. sapiens*)—gave a figure of 0.6% of functional genes (63). Neither estimate can be characterized as oligogenic.
3. Multiple mechanisms: There are likely to be multiple mechanisms of aging, although there would be selective pressure toward some degree of synchronization of the ages of expression of phenotypic effects resulting from independent mechanisms. This proposition follows from the randomness of the accumulated constitutional mutations and from the great variety of types of gene action that could be involved in “trade-off” types of gene action. Against this proposition, however, is the fact that a single environmental manipulation—caloric restriction—regularly leads to life-span extension in rodents, or at least those that have been selected for the easy life of the laboratory setting (reviewed by Masoro (43)). We have little information on the effects of caloric restriction in the wild, however. The life course histories of organisms, whose evolutionary history reflected exceptionally high environmental hazard functions, such as the mice and rats used for most of the calorie-restriction experiments, are quite distinct from those of the higher primates (57). It is therefore not at all clear that caloric restriction would

make a significant impact on the life-span potentials of human subjects. Evidence is pointing toward the conclusion that caloric restriction may delay the onset of age-associated pathologies and reduce the incidence of common age-related disorders and age-related deaths (64). We shall have to await the final outcome of current research in rhesus and squirrel monkeys (reviewed by Roth et al. (65)) to know how likely such an effect will obtain for our species. Meanwhile, common sense tells us that we should avoid gluttony!

4. Species specificity: There is likely to be a degree of species specificity in relevant gene actions. We have already developed certain of these arguments, but let us consider an extended argument. If aging is in fact an epiphenomenon—a by-product of selection for alleles ensuring an optimal degree of reproductive fitness in a given environment—there is no a priori reason to expect identical scenarios of gene action among very different species. Consider the striking differences in the behavioral patterns among different species that lead to

successful matings. There are surely a wide variety of different loci involved, and those that are operative in fruit flies must surely differ from those that are relevant for man! Nevertheless, it is quite possible that there are a number of common mechanisms among groups of related species (including all mammals), and it is even conceivable that such global mechanisms as oxidative damage to macromolecules underlay the aging of all or most organisms (see, e.g. the previous discussion on lipofuscins). This is the rationale for carrying out comparative gerontologic research. Recent research in that direction has in fact revealed evidence for at least one “public” mechanism of aging (54). Remarkably, “leaky” mutations in comparable neuroendocrine signal transduction pathways involving insulin-like growth factor (IGF) receptors and the nuclear translocation of a transcription factor can lead to substantial extensions of life spans of such diverse organisms as *C. elegans*, *D. melanogaster* and *M. musculus domesticus* (Figure 18-5) (42). At first blush, these observations

## Conserved Nutrient Signaling Pathways Regulating Longevity



**FIGURE 18-5** Evidence for a partially conserved “public” mechanism for the modulation of aging in yeast, worms, fruit flies, laboratory mice, and, possibly humans. These signal transduction pathways evolved to modulate metabolism under transient adverse environmental conditions, during which gene actions that enhance the protection of somatic cells are up-regulated while further growth, development, or reproduction is postponed. Certain mutations in these pathways were found to substantially enhance the life spans of laboratory strains of model organisms amenable to genetic analysis. Mutations so far studied in this general pathway in humans result in pathology. The possible effects of a wider spectrum of such mutations upon the longevity of humans are not yet understood. (From Fontana, L.; Partridge, L.; Longo, V.D. *Extending Healthy Life Span from Yeast to Humans*. Science 2010, 328, 321–326.)



would appear to contradict the conclusion, discussed above, that aging is under polygenic controls and that multiple mechanisms are at work. The authors' interpretation of these important discoveries, however, is that they are examples of diapause—nature's “time-outs” from the business of reproduction when faced with conditions of nutritional, climatic, or other environmental challenges (1). These can best be considered as “reprieves”; they will be eventually trumped by other gene actions that, unlike diapause, have escaped the force of natural selection.

5. Intraspecific variations: There are likely to be significant intraspecific variations in phenotypic patterns of aging, particularly in humans. This also follows from many of the arguments discussed above. Given the polygenic nature of aging, the likelihood of a variety of mechanisms, a strong stochastic component, the realization that one is dealing with alterations in all body systems, and the enormous genetic and environmental heterogeneity in our species, one would certainly predict substantial differences in the way it plays out in individual subjects. Every clinician has witnessed this phenomenon first-hand. While differential impacts of the environment are likely to be partially responsible for such variations, the challenge for medical geneticists is to dissect out specific major and minor genetic factors responsible for particularly favorable or unfavorable nature–nurture interactions.
6. Plasticity: The life span of a species should exhibit a degree of plasticity. This follows directly from the arguments on the nature of gene action in aging discussed above and from the experimental results in *Drosophila* sp. Nonetheless, there are likely to be some severe constraints on such plasticity—constraints related to the basic architecture of the organism. We do not expect a fruit fly to live as long as a mouse, without essentially creating a new species.

## 18.4 HOW DO WE AGE?

We now turn to a more systematic consideration of the present state of our knowledge concerning the underlying molecular mechanisms of aging. In contrast to the reasonably satisfying evolutionary explanations for why we age, there is no consensus as to how we age, although the research programs of a growing number of investigators appear to be motivated by the theory that oxidative damage to macromolecules, including those mediated by chemical-free radicals (the “free radical theory of aging”) (reviewed by Muller et al. (66)), are of paramount importance.

### 18.4.1 Alterations in Proteins

In 1963, Orgel introduced the protein synthesis error catastrophe theory of aging (67,68). It was proposed

that transcriptional and/or translational errors in the synthesis of proteins that were themselves used for the synthesis of proteins (e.g. DNA-dependent RNA polymerases, ribosomal proteins) could result in an exponential cascade of errors involving essentially all proteins, leading to cell and organismal death. Biosynthetic errors in protein synthesis appear to be rare, however, even in old organisms (69). Although most gerontologists have abandoned this theory, very few tests of the theory have been carried out with postreplicative cells in vivo (70). By contrast, there is a growing body of evidence indicating the prevalence of posttranslational modifications in proteins in aging tissues; although, of the more than 140 major and minor known modifications of proteins, only a few have been studied in aging cells, tissues and organisms (71). Beginning with a classic paper on senescent nematodes by Gershon and Gershon (72), many studies have demonstrated an accumulation of immunologically detectable, but enzymatically inactive, enzyme molecules in various mammalian tissues. These may result from a variety of posttranslational modifications, including subtle conformational changes (69). There is currently a great deal of interest in oxidative alterations (73). Metal-catalyzed oxidation systems have the potential to inactivate enzymes oxidatively via attacks on the side chains of certain amino acids, with the formation of carbonyl derivatives. The side chains of histidine, arginine, lysine, and proline are particularly susceptible. The sulfhydryl groups of methionine are also susceptible to oxidation. Other posttranslational changes that can be observed in aging cells include racemization, deamidation, isomerization, phosphorylation, and glycation.

Many gerontologists believe that glycation, the spontaneous nonenzymatic reaction of glucose with proteins and nucleic acids, may be a major factor in the development of certain age decrements, as well as complications of diabetes mellitus. Glycation is the slow, spontaneous reaction of the aldehydic form of glucose with free amino groups to form a Schiff base, which subsequently rearranges to form a stable Amadori product. Subsequent reactions, possibly involving oxygen radicals, generate more complex products referred to as advanced glycosylation end (AGE) products. Some of these compounds, including pentosidine, have been characterized. Antibodies to the AGE products have been generated and used to map their distribution to neuritic plaques and tangles and to other sites (32). Because the levels of AGE products increase with age and with elevated blood glucose, cross-link proteins, and change their physical and biologic properties, they are thought to underlie the development of atherosclerosis, cataracts, and peripheral neuropathies. In addition, macrophage receptors bind to the AGE products and initiate the secretion of inflammatory cytokines such as the tumor necrosis factor (74). Thus, glycation represents a progressive age change linked to age-associated disabilities. Support for these ideas has come from experiments in aging dogs, in



which it was possible to reverse myocardial stiffness and improve cardiac function by the administration of an experimental compound known to break the cross-links associated with the formation of advanced glycation end products (75).

Calorically restricted rodents, which have substantially increased life spans, exhibit evidence of both enhanced defenses against reactive oxygen species and reduced levels of protein glycation (associated with decreased levels of plasma glucose). Such results suggest that both the free radical theory of aging and the glycation theory of aging may be operative and potentially synergistic (76). A number of different types of amyloids accumulate in mammalian tissues during aging (77). In their advanced states, they are detected extracellularly as protein aggregates associated with proteoglycans and other proteins. Each type is derived from a different precursor protein. These include the beta-amyloid protein of Alzheimer disease and the aging brain a transthyretin-derived amyloid in peripheral nerve tissues, autonomic nervous system, choroid plexus, cardiovascular system, and kidneys; atrial amyloid derived from the atrial natriuretic peptide the amylin-derived amyloid in the pancreatic islets of Langerhans (78); systemic amyloid AA derived from apolipoprotein A-II (79); and possibly unique types of amyloid in the anterior pituitary gland, intervertebral discs, the aortic intima and media, aortic heart valves and the adrenal cortex. In certain of these conditions, mutations or polymorphic variants in the precursor protein greatly accelerate the rates of deposition of the derivative amyloids.

It is a challenge for the future to discover common denominators underlying this remarkable propensity of mammalian tissues to accumulate these different types of abnormal proteins. Obvious approaches would include more detailed studies of alterations in protein turnover with age (including the turnover of amyloid deposits) and how such turnover might be modulated by endocrine and neuroendocrine factors. Another promising and relatively new area of research seeks to define gene products that function in the repair of altered proteins. An example is the catalysis of the transfer of a methyl group from *S*-adenosylmethionine to L-aspartyl and D-aspartyl residues by protein carboxyl methyltransferases (ED 2.1.1.77). These enzymes have the potential to repair abnormal proteins via the conversion of L-isoaspartyl residues to L-aspartyl residues (80). This enzyme is polymorphic in humans, raising the question of the differential repair of such classes of altered proteins during aging in human populations (81).

## 18.4.2 Alterations in DNA

**18.4.2.1 Nuclear DNA—Epigenetic Events.** Given the fact that, for most genetic loci, only two alleles are present, nuclear DNA would appear to be a particularly vulnerable target for damage during aging. Historically,

the first specific type of somatic “mutational” theories of aging was proposed by a physicist, Leo Szilard (82). He envisioned random “hits” that would inactivate entire chromosomes or chromosome arms. In modern terms, such inactivations could conceivably be associated with epigenetic events, as for the case of the random inactivation of one of the two X chromosomes of the human female during embryonic development and the processes of parental genomic imprinting. There is no good evidence of widespread heterochromatizations or inactivation of large chromatin domains during aging. In fact, at least for the case of mice, there is evidence of a reactivation of certain gene loci on a previously inactive X chromosome during aging (83–85). No such reactivation could be demonstrated for the case of the *HPRT* locus of heterozygous human females (86). Reactivation has also been demonstrated for a genomically imprinted autosomal locus in mice (83). Global losses of 5-methyl cytosine have been demonstrated in aging fibroblast cultures (87) and in tissues of two species of aging rodents (88), but there have been few studies of altered methylation in specific domains of specific genes during aging. In one such study, hypermethylation was mapped to the proximal 5′ spacer domain of ribosomal DNA genes of aging mice; silver stains of cytogenetic preparations revealed that the ribosomal gene cluster on chromosome 16 was preferentially inactivated (89). It remains to be seen, however, whether this remarkable result reflects some developmental, adaptive process in laboratory mice or in the particular strain of mice investigated, as the biochemical changes were observed as early as 6 months. A form of gene-specific methylation of CpG islands has clearly been established to progress steadily into old age in human subjects. It is associated with the silencing of the estrogen receptor gene of a subset of cells of the colonic mucosa (90). A striking finding in that study was that, of a set of 45 colorectal human tumors examined, including those in very early stages of oncogenesis, estrogen receptor expression was either diminished or absent. Moreover, the introduction and expression of an estrogen receptor gene in a line of colon carcinoma cells resulted in marked growth suppression. This important paper therefore demonstrates a link between a presumably epigenetically based progressive repression of a specific gene during aging and the susceptibility to the development of a common type of cancer of aging.

Using the yeast model of replicative aging, Lenny Guarente and his colleagues highlighted a key role of NAD-dependent histone deacetylation in the regulation of energy metabolism, genomic silencing and aging (reviewed by Imai and Guarente (91)). There is a great deal of current research in various organisms on homologs (sirtuins) of the yeast Sir2 gene responsible for the histone deacetylation (91,92). A variety of other changes in gene expression occur throughout the life span, but it remains to be seen which of these alterations are of

primary significance to one or more aging processes and which are merely epiphenomena. One such approach is to explore the effects of caloric restriction (93). A marked transcriptional stress response, with lowered expression of metabolic and biosynthetic genes, is found in aging mouse tissues. These alterations are ameliorated in calorically restricted mice.

**18.4.2.2 Nuclear DNA—Mutational Events.** In 1961, a now classic paper appeared, casting doubt on the validity of somatic mutational theories of aging (94). Taking advantage of the occurrence of a species of wasp in nature—the males of which exist as either haploid or diploid organisms—it was found that there was no difference in the life spans of these organisms. As expected, however, the haploid wasps were much more susceptible to the effects of ionizing radiation. These results were strong evidence against a role for recessive mutations in insects. They did not rule out, however, some role for a combination of dominant and recessive mutations in the aging of such organisms. Moreover, the interpretations are complicated by the occurrence of polyploidy cell types. Finally, those experiments told nothing about the role of somatic mutations of replicating populations of cells in the limitation of life span, since wasps and other insects, with the exception of gonadal tissues and, in *Drosophila*, certain intestinal cells, consist of postreplicative cells. For the case of such replicating populations of cells, there is now compelling evidence that somatic mutations constitute a link between the biology of aging and the biology of cancer. Thus, while much more data are required, there are reasonable correlations of species-specific life spans and rates of development of various neoplasms (e.g. (95)). Clearly, among those genes that evolved in association with the relatively long life spans of *H. sapiens* (the longest lived of all mammalian species), there must be loci conferring enhanced genomic stability in comparison, for example, with those of *M. musculus domesticus*. Moreover, there may be considerable species differences in the patterns of somatic mutation. For mice, for example, there is evidence of a marked susceptibility to cytologically detectible chromosomal mutations during aging (96), while in comparable cell types (renal tubular epithelial cells), there is little evidence for the accumulation of mutations (presumably intragenic) at the *HPRT* locus, a target chosen because of the lack of evidence for selection against such mutations in renal tissue (97). Mutations have been shown to accumulate at the *HPRT* locus of T lymphocytes and in the renal tubular epithelial cells of aging human subjects, however, with higher frequencies of mutation being observed in the epithelial cell type (98,99). The lymphocyte study showed that deletions were relatively common (100). Such accumulations could be attributable to chronology rather than to intrinsic biologic aging. We will require additional research in mammals of contrasting life-span potentials to address this question; an approach

using comparable transgenic reporter constructs may be promising, if these could be comparably buffered from position effects (101).

**18.4.2.3 Nuclear DNA—Molecular Misreading.** What originally appeared to be the accumulation of frameshift mutations in DNA in aging mammals now appears to be the result of transcriptional errors at particularly vulnerable sites, especially those with runs of GAGAG. van Leeuwen has named this phenomenon “molecular misreading.” The process can impact upon the fidelity of transcription of such important loci as the beta-amyloid precursor protein and ubiquitin B (102).

**18.4.2.4 Telomeric DNA.** Perhaps the most robust age changes noted in the nuclear genome of normal somatic cells are alterations in telomere length, leading to replicative senescence (reviewed by de Lange (103)). Telomeres have a highly repetitive structure (TTAGGG in humans and mice) that extends for many thousand of nucleotides at the ends of chromosomes. The telomeres stabilize chromosomal structure and their loss leads to various cytologic aberrations and the arrest of cell division. Current concepts suppose that telomeres in cells of the germ line and in many neoplastic cells are added to the ends of chromosomes *de novo* by a unique enzyme referred to as telomerase, which uses an associated RNA to code for the hexanucleotide repeats. It appears that telomerase is lost or its concentration greatly diminished in the progeny of somatic stem cells (but not in cells of the germ line). Somatic cell telomeres are then duplicated during cell division by DNA polymerases without the assistance of telomerase. It is characteristic of DNA polymerases that they fail to copy some 50–200 terminal bases of the trailing strand and the telomeres are shortened by this amount with every cell division. The shortening of telomeres is strikingly apparent when one examines the telomeric/subtelomeric DNA isolated by appropriate restriction enzyme cleavage from normal human fibroblasts that have undergone large numbers of cell divisions in culture. Exit from the cell cycle may occur as when only a few chromosome arms reach a critical level of shortening, thus activating cell cycle checkpoints (104).

The development of a PCR-based method for the assay of telomere lengths has led to a remarkable association of short telomeres in DNA from peripheral blood of elderly human subjects with mortality; the results were largely attributable to earlier deaths from cardiovascular and infectious diseases (105). Shorter telomere lengths have also been reported in mothers of chronically ill children; the authors surmised that life stress could shorten life span (106). An alternative interpretation, however, is that the mothers of many chronically ill children are more frequently exposed to infectious agents, thus driving proliferation of lymphocytes, resulting in shorter telomeres.

**18.4.2.5 Mitochondrial DNA.** Considerable attention is now being directed to changes in mitochondrial DNA (mtDNA) that might underlie age deficits in metabolism in various mammalian tissues, especially skeletal muscle myocardium and brain (reviewed by Wallace et al. (107)). This is based in part on a growing number of observations that various types of mtDNA mutations accumulate, to varying degrees, in multiple tissues of aging human subjects and in the tissues of other aging mammals. Most of the assays were for large deletions, but tandem duplications and point mutations have also been reported.

mtDNA is closed circular DNA of some 16,569 nucleotides that codes for some of the mitochondrial proteins plus the tRNAs and rRNAs used for mitochondrial protein synthesis. Other components of the mitochondria are coded for by nuclear genes and are transported to the mitochondria. Essentially, all mtDNA molecules are maternal in origin; thus, mtDNA genetic diseases are maternally transmitted.

Based on the frequency and consistent location of common specific age-related deletions, one can postulate that the sequences between the direct repeats are looped out following damage to the DNA by a slip replication mechanism. The damage to mtDNA molecules may be initiated by oxygen radicals generated as a by-product of the oxidative phosphorylation reactions carried out by the mitochondria. The proximity of mtDNA to the sources of oxygen radicals, plus the lack of associated histones, would make mtDNA more vulnerable than nuclear DNA. While certain forms of DNA repair are lacking in mitochondria, other lesions appear to be repaired (108). Thus, the age changes observed could be due to increased damage and/or reduced repair. One important mechanism for repair is the proofreading domain of DNA polymerase gamma, the enzyme that replicates mtDNA. Support for the importance of this function has come from the synthesis of mice with knock-in mutations in that domain; these transgenic mice exhibited progeroid features (109).

In spite of the fact that there are substantial increases in mtDNA damage with age, only a small proportion of the total mtDNA molecules are affected. Indeed, no clear consensus exists as to whether oxidative phosphorylation declines with age. Thus, some investigators doubt that the alterations occurring in mtDNA are a significant factor underlying aging and/or age-associated diseases. Alternatively, it has been proposed that damaged mtDNA molecules preferentially replicate as a local adaptive response to energetic deficits created by impaired mitochondria. An alternative hypothesis is that damaged mtDNA molecules accumulate because they generate fewer altered proteins and are thus less likely to be targeted for turnover; this theory has been referred to as the “survival of the slowest” (110). Thus, as in the genetic mtDNA diseases, it is proposed that the altered DNA molecules are concentrated by a focal expansion

in individual cells, nerves, and muscle fibers, and lead to the death of such cells, perhaps through apoptosis. An increase in mtDNA damage may therefore cause the progressive depletion of single cells without causing major alterations in the remaining cells in the tissue, which would be affected individually and later. Clearly, such a mechanism could play an important role in the decline of CNS function in the development of frailty, impaired wound healing, and other age-associated declines by depleting cells from these tissues.

**18.4.2.6 Germ Line Mutations.** Medical geneticists are well aware of the increased risk to the conceptus of chromosomal types of mutations (mainly aneuploidies) as functions of maternal age. This is, of course, the basis for the clinical practice of counseling women of the availability of prenatal diagnosis. The relationship of paternal age to the increased risk of certain types of mutations has also been well documented (111) and has been mentioned above in a different context. Suffice it to say that, from the point of view of the pathobiology of aging, we remain ignorant of the underlying mechanisms. These important subjects have in fact received substantially less attention by the gerontologic community than the question of somatic mutation and aging.

### 18.4.3 Alterations in Lipids

Given the seminal importance of membranes in cell biology, alterations in the structure of membrane lipids could constitute a primary mechanism of age-related cellular dysfunction and cell death. Most research in this field has addressed the issue of lipid peroxidation, an integral component of the free radical theory of aging. Aspects of this idea have been discussed above, including the ubiquitous nature of lipofuscin pigments as a biologic marker of aging. A second line of research in this field has emphasized age-related increases, in various cell types, of the cholesterol-to-phospholipid ratios of plasma cell membranes, with a consequent decrease in membrane fluidity. However, at least in some cell types, such as neurons of the dorsal root ganglia, the decline in membrane fluidity, as measured by lateral diffusion coefficients, is related to development rather than to post-maturational aging (112).

## 18.5 PROGEROID SYNDROMES OF HUMANS

Having reviewed the present state of our knowledge of the biology and pathobiology of aging, we can now consider spontaneous mutations in man that may modulate the aging phenotype. As we have seen, however, evolutionary theory would argue that no single mutation or polymorphism is likely to modulate all aspects of the senescent phenotype. In a systematic survey of several editions of McKusick's catalog of the Mendelian



inheritance of humans, one of us (GMM) indeed concluded that no single mutation has yet been identified that could be characterized as a global progeria (62). A number of mutations, however, could be characterized as “segmental progeroid mutations,” in that multiple segments of the complex senescent phenotype of man appear to have been affected, whereas unimodal syndromes predominantly impact a single organ (e.g. dementias of the Alzheimer type) (62,113). The responsible mutations include those that impact genomic stability, nuclear structure, numbers of triplet-repeats, and alterations in lipid and carbohydrate metabolism. Some chromosomal aneuploidies (e.g. trisomy 21) also exhibit segmental progeroid features (62). Two best-known examples of segmental progeroid syndromes are Werner syndrome (WS) and Hutchinson–Gilford progeria syndrome (HGPS), which are discussed in more detail in the following.

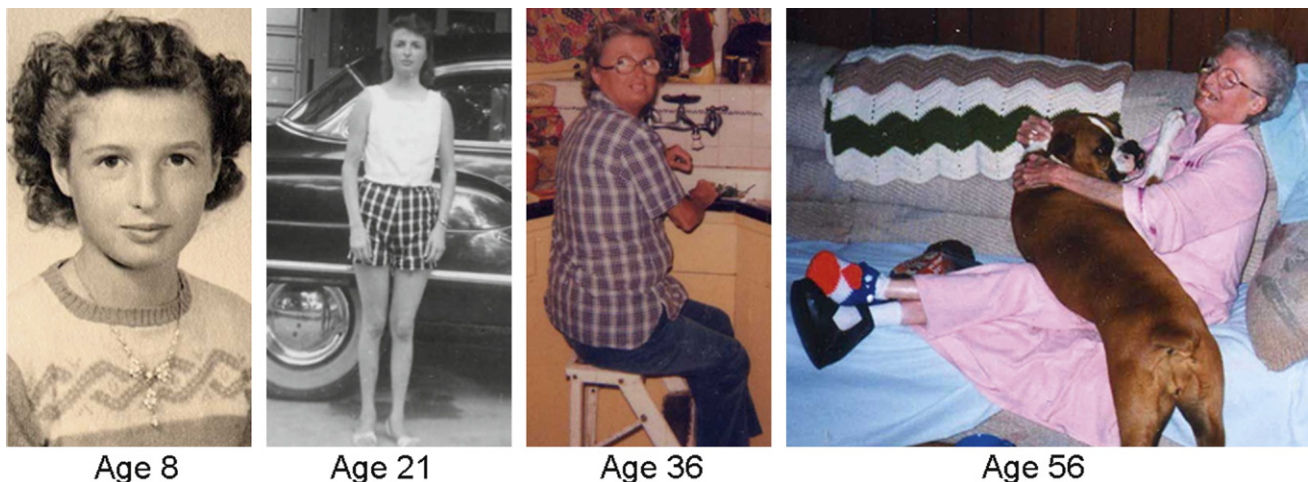
### 18.5.1 Werner Syndrome

The clinical phenotype of WS (OMIM# 277700) has been succinctly summarized as a “caricature of aging” (Figure 18-6) (114,115). WS patients usually develop normally until they reach the second decade of life. The first clinical sign is a lack of the pubertal growth spurt during the teen years. In their 20s and 30s, patients begin to exhibit a general appearance of accelerated aging with skin atrophy, loss of subcutaneous fat, and loss and graying of hair. They also develop common age-related disorders including type 2 diabetes mellitus, bilateral ocular cataracts (requiring surgery at a median age of 30), osteoporosis; gonadal atrophy (with early loss of fertility), premature and severe forms of arteriosclerosis (including atherosclerosis, arteriolosclerosis and medial calcinosis); and peripheral neuropathy. Multiple cancers can be observed by middle age (116). Our recent survey of WS patients with a molecularly confirmed

diagnosis revealed that the prevalence of cataracts was 100% (87/87) (117). The prevalence of osteoporosis was 91%, hypogonadism 80%, diabetes mellitus 71%, and atherosclerosis 40% at the time of diagnosis. Median age of death in the most recent study was 54 years, a significant increase over what had been observed several decades ago (114,115), perhaps the result of improved medical management. The most common causes of death are myocardial infarction and cancers (117).

Although many clinical features of WS are similar to those observed during “normal” aging, there are significant distinctions. There are a disproportionate number of sarcomas in WS patients: the ratio of mesenchymal cancers to epithelial cancers in WS is approximately 1:1 as compared to 1:10 in general population (116). Alzheimer-type dementia is not common in WS (118). The long bones of the limbs, especially of the lower limbs, are particularly vulnerable to osteoporosis, whereas in ordinary aging the vertebral column is particularly vulnerable, especially in females (119). There is also a peculiar osteosclerosis of the distal phalanges that is not seen during ordinary aging (120). Necrotic skin ulcers and necrosis around ankles and occasionally around elbows, which eventually may require amputation, are characteristic to WS, but rarely seen during normal aging.

Classical WS is caused by mutations of the *WRN* gene on chromosome 8. The locus spans approximately 250 kb and consists of 35 exons, 34 of which are protein coding (121). *WRN* encodes a 180 kDa multifunctional nuclear protein that belongs to the RecQ family of helicases (122). A structural study revealed a unique interaction between the RecQC-terminal domain of *WRN* protein and the DNA substrates during base separation (123). In contrast to other members of the RecQ family, *WRN* protein includes an N-terminal domain that codes for exonuclease activity (124). A single strand-DNA annealing activity in the C-terminal region has also been reported (125). Its preferred substrates resemble various



**FIGURE 18-6** Werner syndrome patients with homozygous *WRN* mutations. (From Hisama, F.M.; Bohr, V.A.; Oshima, J. *WRN's Tenth Anniversary*. Sci Aging Knowledge Environ. 2006, 28, pe18.)



DNA metabolic intermediates, substrates for which its helicase and exonuclease activities function in a coordinated manner, suggestive of roles in DNA repair, recombination and replication (126). The WRN protein is also involved in telomere maintenance (127), which explains the accelerated telomere shortening of fibroblasts derived from WS patients (128). Interestingly, epigenetic inactivation of WRN was observed in sporadic cancers found in general population, supporting a role of WRN as a tumor suppressor by providing genomic stability (129).

To date, more than 70 different WRN mutations have been reported, some of which appear to be specific to certain ethnic groups (130). The majority of these disease mutations result in the truncation of the nuclear localization signal at the C-terminus of the WRN protein (131), which makes mutant WRN proteins unable to enter the nuclei. This seems to be a satisfactory explanation of why we do not observe noticeable phenotypic differences among various common WRN mutations (117,130). WS cases are most frequently reported in Japan, where the prevalence of heterozygotic carriers, as estimated from the most common Japanese mutation, was approximately 1/167 (132). Another region with high incidence of WS is Sardinia, where the prevalence of heterozygous carriers was estimated to be of the order of 1/120 (133). Frequencies of WRN mutations in other population are unknown, as WS may often escape diagnosis.

Some evolutionary biologists would argue that the WS is a poor model of aging, in that it is clear that it would not fit the definition of a set of phenotypes that have escaped the force of natural selection (36).

### 18.5.2 Hutchinson–Gilford Progeria Syndrome

HGPS (OMIM# 176670) is a childhood-onset progeria (Figure 18-7). It was first described by Jonathan

Hutchinson (134) in a boy with baldness and atrophic skin. Hastings Gilford (135) then described a patient with accelerated aging and ateleiosis who died with symptoms of angina pectoris at age 18. HGPS patients are typically normal at birth, but exhibit growth retardation within the first 3–6 months of life along with accelerated degenerative changes of the cutaneous, musculoskeletal and cardiovascular systems (136,137). Baldness and a characteristic “plucked bird appearance” generally develop by age 2. Median age of death in HGPS patients is 13.5 years, and virtually all the patient succumb to myocardial infarction or congestive cardiac failure (138). This is in contrast to patients with the adult-onset progeroid syndrome, WS, whose onset is after puberty (114,136). Unlike normal old individuals, Alzheimer-type dementia and ocular cataracts are usually not seen. Malignancies are also not commonly seen in HGPS, perhaps because they die at such early ages.

HGPS is caused by mutations in *LMNA*, which encodes nuclear intermediate filaments, lamin A and C (139,140). Lamin A and C, generated by alternative splicing of *LMNA*, undergo dimerization and head-to-tail assembly to form nuclear lamina that lies on the inner surface of the inner nuclear membrane (141). Point mutations within *LMNA* exon 11 found in HGPS create a cryptic splicing site and generate a 50 amino acid in-frame deletion that includes the proteolytic site required for the maturation of prelamin A to lamin A (139,140). Unlike wild-type lamin A, this in-frame deletion mutant, termed progerin, retains the farnesyl moiety at its C-terminus. The resulting accumulation of progerin is thought to be responsible for the phenotypic presentation of HGPS (142). At a cellular level, the presence of progerin is shown to cause structural abnormalities and/or fragility of nuclei (143), aberrant reorganization of the heterochromatin and epigenetic changes (144), genomic instability (145),

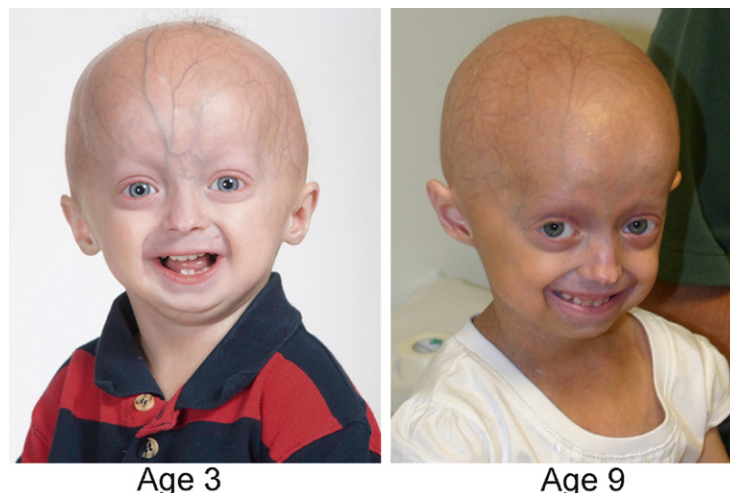


FIGURE 18-7 Hutchinson–Gilford progeria syndrome. (Courtesy of the Progeria Research Foundation.)

and impaired telomere maintenance (146). Age-associated accumulation of very small amount of progerin has been demonstrated in human, suggesting the possibility that progerin may be, in part, involved in development of the age-related pathologies in normal individuals (138,147).

LMNA mutations are also responsible for a group of disorders, termed laminopathies, including Emery–Dreifuss muscular dystrophy, dilated cardiomyopathy type 1A (DCM1A) with or without atrio-ventricular conduction disturbance, limb-girdle muscular dystrophy type 1B (LGMD1B), Charcot–Marie–Tooth disease type 2 (CMT2), Dunnigan-type familial partial lipodystrophy, mandibuloacral dysplasia, atypical forms of WS, and restrictive dermopathy (RD) (148). With the exception of RD and HGPS, the majority of LMNA mutations found in these disorders are amino acid substitutions instead of in-frame deletions.

Farnesyltransferase inhibitors have been shown to ameliorate HGPS phenotypes in cell cultures and in mouse models (149,150). Clinical trials are ongoing. The rationale for this approach has been challenged, however, by the finding that non-farnesylated progerin can elicit HGPS-like phenotypes in mice (151).

### 18.5.3 HUMAN ALLELIC VARIANTS HOMOLOGOUS TO PRO-LONGEVITY GENES IN MODEL ORGANISMS

There has been a surge of interest in testing the hypothesis that the ability to achieve remarkable longevity in centenarians is due to the inheritance of alleles at a few loci of major relevance. A priori, one would predict that such research would be quite risky, given the arguments made earlier in this chapter that life span is under highly polygenic modulations and that it is also determined, in part, by stochastic events. It is the case, however, also noted above, that atherosclerosis (and the associated heart attacks and strokes) is a major limitation of human life span in the developed societies. Therefore, it is perhaps not surprising that an association of unusual longevity with variant alleles for lipoprotein metabolism has been observed (152). More recent studies demonstrated the association of polymorphisms in the forkhead box class O (FOXO) family of transcription factors among several independent centenarian populations (153). The FOXO genes are key regulators of the insulin-IGF1 signalling pathway (Figure 18-5).

There is also considerable interest lately in Laron dwarfism because of their mutations in the growth hormone pathway (154,155). People with Laron dwarfism in the Ecuadorian villages are resistant to cancer and diabetes and are somewhat protected against aging. This is consistent with findings in mice with a defective growth hormone receptor gene, suggesting this “public mechanism” of aging may apply to our species (156).

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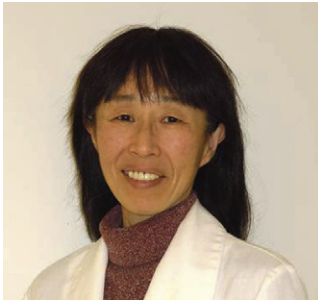
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# CHAPTER 19

## Pharmacogenetics and Pharmacogenomics

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### 19.1 INTRODUCTION

*Pharmacogenetics* is defined as “the study of heritable variability in drug response (168),” or simply, “gene–drug interactions.” Responses to drugs often differ among individuals. Each person has his own unique “pharmacogenetic profile”—just as each of us has our own distinct pattern of microsatellite differences, single nucleotide polymorphisms (SNPs), or thumbprint. Our genetic makeup largely determines our drug response. Drug response is very complex, however, and includes (a) numerous events associated with genomic differences (some known, others not yet understood), (b) many environmental effects (e.g. diet, cigarette smoking, drug–drug interactions, and exposure to occupational chemicals and other environmental pollutants), and (c) endogenous factors (e.g. age, gender, exercise, various disease states, status of renal function, function of other organs, etc.).

*Pharmacogenomics* is defined (23) as “the study of how drugs interact with the *total genome*, to influence biological pathways and processes.” This field, a direct by-product of The Human Genome Project, might help in identifying new drug targets and, thus, in designing new drugs. The terms pharmacogenetics and pharmacogenomics are often used interchangeably, but they should not be.

Recently, pharmacogenetics/pharmacogenomics has become synonymous with “*individualized drug therapy*,” a major subset within the broader field of “*personalized medicine*.”

The field of pharmacogenomics has expanded enormously in the past two decades; therefore, this chapter represents “a snapshot in time.” The breakneck speed

of publications in this area of research includes results of high-throughput large-cohort studies, new data from genome-wide association (GWA) studies, and the very recent “1000 Genomes Project,” whole exome sequencing, and “next-generation deep resequencing” studies. As our knowledge of the human genome expands, our comprehension of pharmacogenomics will continue to grow.

#### 19.1.1 Definition of Adverse Drug Reactions

*Adverse drug reactions (ADRs)* rank as approximately the fifth leading cause of death in the United States (85). ADRs include dose-dependent reactions, dose-independent reactions (including idiosyncratic drug reactions and allergic reactions), dose- and time-dependent reactions (cumulative), time-related reactions, and withdrawal reactions (31). Advances in pharmacogenetics and pharmacogenomics should help to reduce the morbidity and mortality caused by *idiosyncratic dose-independent drug reactions*.

#### 19.1.2 Definition of Allelic Variants

Each gene has two alleles on a chromosome pair—one from each parent. A minor allele having a frequency of  $\geq 0.05$  (5%) is termed a *common variant*. A minor allele having a frequency of  $\geq 0.01$  is called a *polymorphic variant*, and a minor allele with a frequency of  $< 0.01$  is termed a *rare variant*. The Hardy–Weinberg equilibrium (HWE), “ $p^2 + 2pq + q^2 = 1$ ” was originally developed to describe the frequencies of  $p$ , the wild-type trait, and  $q$ , the mutant



(or variant) trait. From the 1860s until the late 1980s, it was thought that there would be (for any gene) only one wild-type allele and only a few variant alleles.

If  $q = 0.01$ , the percentage of individuals homozygous for an autosomal recessive trait will be  $q^2$ , i.e. 1 in 10,000. Owing to previous difficulties in studying populations of 10,000 or greater, for most clinical studies, the lowest frequency of a variant allele usually investigated has been 0.05, i.e. common variants. During the past several years, cohorts have become larger, and interest has now been focused on alleles with frequencies between 0.01 and 0.05. At present, one cohort of 500,000 subjects, with height as the phenotype, is being studied. After it became appreciated in the late 1980s that multiple alleles exist for every gene (124),  $q$  is now considered the sum of all variant alleles.

The term  $q$  has been replaced by MAF (minor allele frequency), referring to “the frequency at which all biallelic SNP alleles other than the major allele occur in a given population.” An additional complexity is that the so-called wild-type (or “major,” or “consensus”) allele in one ethnic population might be a minor allele in ethnically different populations. For example, when 313 genes in 82 unrelated individuals of five geographically isolated subgroups were examined, more than one-third of the selected genes had no major allele with a frequency exceeding 50% (149).

### 19.1.3 Individualized Drug Therapy

Can we design DNA tests that will predict how a patient will respond to any particular drug—with close to 100% degree of certainty? This is what the physician wishes to know: which patient will benefit from drug efficacy vs which patient will develop an ADR or therapeutic failure? This approach has become very popular during the past two decades, and, indeed, some scientists still believe in this lofty goal. Innumerable publications have predicted that such DNA tests are here or will very soon become mainstream. However, the purpose of this chapter is to describe some of the complexities now known to exist in the human genome. We can then use that knowledge to make a distinction between “predictions of plasma or urine levels of a drug or metabolite or therapeutic failure” vs “predictions of ADRs or drug efficacy”; the former reflects a high-penetrance predominantly monogenic (hPpM) trait (124), whereas the latter (vide infra) is usually as complicated and heterogeneous as any human “complex disease” (e.g. obesity, schizophrenia, pancreatitis, seizures, stroke, coronary artery disease, or type 2 diabetes).

The US Food and Drug Administration (FDA) recommends that all physicians genotype their patients for specific biomarkers—before prescribing currently more than 70 different medications. The FDA suggests that these genetic biomarkers might help physicians identify patients in whom these commonly used drugs would be “less efficacious, insufficiently metabolized, or toxic.” In recent surveys, most physicians agree that genetic profiles of patients can affect drug therapy, but only approximately 10% of physicians believe they have been adequately

informed about using such genetic biomarkers in patients; accordingly, few physicians actually order these tests.

Furthermore, few physicians appreciate the actual range of increased risk associated with positive results of these tests—a range of approximately only 1.8- to 2-fold. Several reasons account for this lack of genomics testing usage and implementation; these include the need to demonstrate more clearly and unequivocally clinical utility as well as bombardment of physicians with a continuous, bewildering, and indigestible volume of new genomics information. Often genomics testing results are irreproducible or they vary between different ethnic populations and make insurance reimbursements problematic. Therefore, individualized drug therapy or personalized medicine based entirely on genomic testing is now far from being a clinical reality, or even becoming so soon.

### 19.1.4 Organization of this Chapter

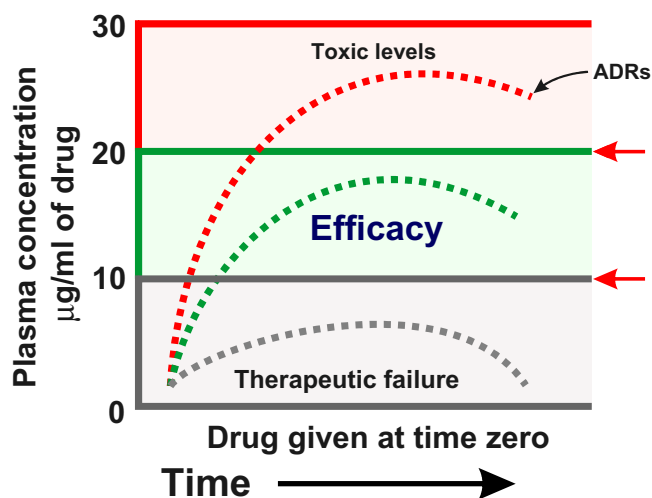
First, several fundamental aspects of clinical pharmacology are reviewed, followed by a brief summary of classical *human monogenic diseases* and then several classical *human hPpM* pharmacogenetic disorders. We then point out the importance of *ethnic differences* in these hPpM traits and the likelihood that interethnic variability is a feature that reflects human migration and geographic isolation over tens of thousands of years.

The latter half of the chapter is devoted to advances in genomics during the past two decades, which are relevant to the field of pharmacogenomics. We propose that drug efficacy, as well as ADRs involving drug toxicity, in most cases are conceptually the same as *human complex diseases*. We now realize that to understand drug efficacy and ADRs, we can apply all the same novel human genomics methodologies—now being carried out in the study of complex diseases—to the field of pharmacogenomics.

## 19.2 FUNDAMENTAL ASPECTS OF CLINICAL PHARMACOLOGY

The clinical pharmacologist’s objective of treating a patient with a drug is to maintain optimal plasma levels of the active principle (drug) in the *therapeutic range* (Figure 19-1). If the dose of the drug is too small, the interval of administration inadequate, bioavailability of the drug product low, or the active principle extensively metabolized and quickly excreted—the plasma drug level might not reach the minimal effective concentration. This would lead to the absence of the expected response (*therapeutic failure*). On the contrary, if the dose of drug is too large, its interval of administration too short, or the active principle poorly metabolized or excreted, the drug can reach *toxic levels*. Levels that cause toxicity will lead to ADRs; these *multiplex phenotypes* will occur due to drug accumulation at toxic levels in blood as well as in one or more critical target organs.

These scenarios exist if the *parent drug* is the one responsible for efficacy as well as toxicity. If a *metabolite* is the active principle (i.e. that which causes efficacy) and



**FIGURE 19-1** Theoretical plasma concentration curves for any drug, as a function of time after oral administration of the dose. In this hypothetical case, the horizontal line (at 10 µg/mL) is the minimum effective concentration and the horizontal line (at 20 µg/mL) is the minimum toxic concentration.

also the toxicant, then one can replace the word “drug” with “metabolite” and describe the same events leading to *efficacy vs therapeutic failure vs toxic levels* (124).

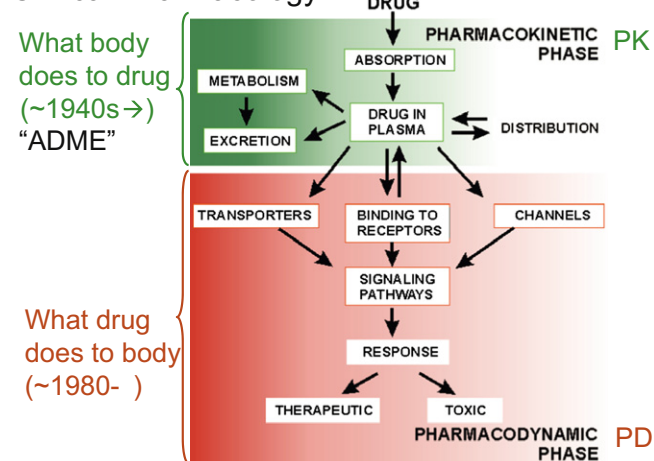
### 19.2.1 Pharmacokinetics and Pharmacodynamics

Pharmacokinetics (PK) represents what the body does to the drug, whereas pharmacodynamics (PD) reflects what the drug does to the target tissues (Figure 19-2). The end result of all PK and PD processes is therapeutic failure, drug efficacy, or toxicity (ADRs); the latter two outcomes represent processes usually conceptually the same as complex diseases.

Variations in the drug’s PK phase encompass the processes of *absorption, distribution, metabolism, and excretion*, which pharmaceutical companies commonly describe as ADME. PK differences are generally detected by determinations of the active drug (or metabolites) in blood and less often by urinary (or saliva, sweat, fecal) determinations. For most systemic drugs, the concentration of the (unbound or free) active principle in blood is proportional to the concentration of active principle in the target tissue. Most of the pharmacogenetic differences that had been initially reported involved genes participating in drug metabolism. More recently, pharmacogenetic differences were reported in genes participating in distribution (binding, transport), absorption, and excretion.

Variations in the PD phase occur “downstream” of the PK phase (Figure 19-2). Thus, gene differences might be seen in transporters and channels across the different membranes of the cell; receptors at the cell surface, in other intracellular membranes, and in the cytosol and the nucleus; tissue- and cell-type-specific transcription factors (TFs) and signal transduction pathways; nucleic acid and protein repair processes;

### Clinical Pharmacology:



**FIGURE 19-2** Basis of clinical pharmacology. The processes involve the *pharmacokinetic* (PK) phase and the *pharmacodynamic* (PD) phase—of any drug or over-the-counter preparation.

chaperones; and cell infrastructure (e.g. nuclear matrix, membranes, and subcellular organelles such as Golgi bodies, melanosomes, or peroxisomes). Until the 1980s, what happened in the PD phase was largely considered a “black box.” Owing to rapid advances in molecular biology, downstream drug signaling pathways have now become better understood and the field of PD has exploded (124).

Historically, drugs or chemicals were given to human volunteers or laboratory animals and differences in urinary metabolite profiles were determined (e.g. (12,175)). Next, variations in laboratory animals (164) and in clinical PK (166)—reflected as differences in plasma drug levels or clearance—led to the elucidation of genetic variability in drug metabolizing enzymes (DMEs). This occurred in human volunteers or patients because blood samples were easier to obtain than tissue biopsies. Also, this happened at the laboratory bench because enzyme assays were especially popular between the 1940s and 1980s.

With advances in molecular biology methodologies and then the explosion in knowledge derived from The Human Genome Project, elucidation of genetic differences in measurements other than DME assays has become more commonplace. For example, receptor assays began in the late 1970s and developed during the 1980s, transporter and ion channel assays were initiated in the 1980s and became popular in the 1990s, and analyses of signal transduction pathways, post-translational modifications, and many other subcellular processes have greatly expanded during the past two decades. Another consideration in the 1980s and 1990s was that DME genes are in general smaller, spanning approximately 5–20 kb, whereas receptor and transporter genes are usually approximately 50–>100 kb in length; the cloning, sequencing and characterization of DME genes therefore was technically easier than for the much larger genes.

### Therapeutic “Window”

$$\frac{\text{Toxic dose (TD}_{50}\text{)}}{\text{Effective dose (ED}_{50}\text{)}} = 20 \text{ [large window] A}$$

$$\frac{\text{Toxic dose (TD}_{50}\text{)}}{\text{Effective dose (ED}_{50}\text{)}} = 2 \text{ [narrow window] B}$$

- If genetic differences in drug response are 10-fold then drug A → no problem; drug B → ADRs

**FIGURE 19-3** Simple equations illustrating what represents a large vs a small therapeutic “window” or index.

### 19.2.2 Therapeutic Index (or “Window”)

Most pharmacogenetic studies owe their success to the selection of study drugs having narrow therapeutic indices. If a drug shows a wide therapeutic window, it is unlikely to cause toxicity in a significant portion of any human population; therefore, this would be of little concern to public health, and the need to prevent ADRs would be small. For example, if the dose causing toxicity is 20 times greater than the dose needed to be effective (Figure 19-3), and genetic differences in handling this drug are never greater than 10-fold across human populations, this drug would be of little concern to a pharmacogeneticist. On the other hand, if the dose causing toxicity is only twice the dose for efficacy, and pharmacogenetic differences in handling this drug are 10-fold, this drug could be an important candidate to study in order to understand ADRs that might lead to morbidity and mortality.

## 19.3 CLASSICAL GENETICS AND PHARMACOGENETICS: 1900 TO APPROXIMATELY 1990

### 19.3.1 Human Monogenic Diseases

In human genetics, from Sir Archibald Garrod in the first decade of the twentieth century through the late 1980s, it was believed that humans—like insects, plants, or yeast—had only one “wild-type” allele and only a few mutant (or variant) alleles (116). Cloning and sequencing of the phenylalanine hydroxylase consensus gene (*PAH*) in a heterozygous carrier of phenylketonuria (PKU) constituted a major landmark because it identified the first mutation (first nucleotide of intron 12) responsible for a human monogenic disease (27). Following additional sequencing studies, it became apparent that different mutations in the same gene (genotype) caused the same disease (phenotype); this phenomenon is termed *allelic heterogeneity*. At present more than 500 *PAH* mutations have been recorded that result in “the PKU phenotype.”

However, virtually no disease reflects a truly monogenic trait; from the earliest autosomal recessive *monogenic disorders* described by Garrod (e.g. alkaptonuria and cystinuria) to cystic fibrosis caused by mutations

in the *CFTR* gene, the onset and severity of symptoms were realized to be affected by *modifier genes* (52,182) as well as environmental factors (116). For example, severe forms of PKU become expressed during early infancy, whereas a milder form of PKU might present in late childhood or adolescence. Also, the same missense mutation in the coding region of the acid-β-glucosidase gene (*GBA*) can cause debilitating Gaucher disease—presenting as massive hepatosplenomegaly, mental retardation, and death before the age of 10 years in the proband—while the grandfather of the proband might show only mild signs of the disease such as slight hepatosplenomegaly, and yet live into his 70s.

### 19.3.2 hPpM Traits

Variations among individuals in PK and sometimes PD (Figure 19-2) usually represent hPpM traits. These genetic differences can range from approximately 2- to more than 100-fold. Examples of hPpM traits include dramatic differences in metabolism or transporter function, e.g. *N*-acetyltransferase, debrisoquine oxidation, thiopurine methyltransferase (*TPMT*), and glutathione *S*-transferase activities; ATP-binding cassette (ABC) and solute-carrier (SLC) transporters; and G-protein-coupled receptor (GPR) functions. Table 19-1 includes a list of various hPpM traits and disorders. Note that most early discoveries reflected metabolism gene deficiencies, whereas the latter ones began to represent transporter gene defects.

### 19.3.3 Drug Metabolism Enzymes and DME-Related Transporters

The human genome contains hundreds of metabolism genes. Virtually all drugs are metabolized by Phase I DMEs (*functionalization*), followed by Phase II (*conjugation*) DMEs (Figure 19-4). Phase I DMEs insert or activate a functional (generally polar) group on the drug. One major class of Phase I DMEs comprise the cytochromes P450 (CYPs), probably the most thoroughly studied of all pharmacologically relevant enzymes. There are 57 human *CYP* genes, compared with, for example, 103 mouse and 89 rat *Cyp* protein-coding genes [<http://drnelson.uthsc.edu/>]. Five human P450 enzymes (CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5) metabolize more than 80% of all commonly prescribed and over-the-counter medications. Each of five additional P450 enzymes also metabolize another few dozen drugs: CYP1A2, CYP2A6, CYP2B6, CYP2E1, and CYP4A11 [<http://medicine.iupui.edu/clin-pharm/ddis/table.asp>]. Seven P450 enzymes are primarily responsible for detoxifying (in some cases) or metabolically activating (in other cases) environmental carcinogens: CYP1A1, CYP1A2, CYP1B1, CYP2B6, CYP2E1, CYP3A4, and CYP3A5 (134). Other Phase I DMEs include flavin-containing monooxygenases (FMOs), hydroxylases, hydrolases, esterases, galactosidases, glycosidases, peroxidases, lipooxygenases, cyclooxygenases, monoamine oxidases, dioxygenases, reductases, and dehydrogenases.

**TABLE 19-1** Predominantly Monogenic Pharmacogenetic Disorders That Have Been Characterized<sup>a</sup>

Disorder	Major Gene Known to be Responsible	Pivotal Reference(s)
Phenylthiourea–nontaster	<i>TAS2R1</i>	(77,146)
Hypocatalasemia	<i>CAT</i>	(154)
Atypical serum cholinesterase	<i>BCHE</i>	(72)
Glucose-6-phosphate dehydrogenase deficiency	<i>G6PD</i>	(102)
Isoniazid slow <i>N</i> -acetylation	<i>NAT2</i>	(9,36)
Fish odor syndrome (trimethylaminuria)	<i>FMO3</i>	(58,62)
Debrisoquine/sparteine oxidation poor metabolizer	<i>CYP2D6</i>	(32,47,97)
Serum paraoxonase low activity	<i>PON1</i>	(63,170)
Thiopurine methyltransferase deficiency	<i>TPMT</i>	(177)
Sensitivity to alcohol	<i>ALDH2</i>	(158)
<i>S</i> -mephenytoin oxidation deficiency	<i>CYP2C19</i>	(25,81)
Sulfotransferase deficiency	<i>SULT1A1</i> , <i>SULT1A2</i>	(176)
Coumarin, nicotine oxidase deficiency	<i>CYP2A6</i>	(184)
P-glycoprotein transporter defect	<i>ABCB1</i>	(78)
Malignant hyperthermia	<i>RYR1</i>	(96)
Quinone oxidoreductase defect	<i>NQO1</i>	(161)
Peptide transporter defect	<i>TAP2</i>	(132)
Phenytoin, warfarin oxidation defect	<i>CYP2C9</i>	(24)
Debrisoquine ultrametabolizers	<i>CYP2D6</i> *1XN	(69)
Epoxide hydrolase deficiency	<i>EPHX1</i>	(51)
Glutathione <i>S</i> -transferase null alleles	<i>GSTM1</i> *0, <i>GSTT1</i> *0	(76,178)
Long-QT syndrome	<i>KCNH2</i>	(19)
Dihydropyrimidine dehydrogenase deficiency	<i>DPYD</i>	(105)
Chlorzoxazone hydroxylation defect	<i>CYP2E1</i>	(60)
Peptide transporter defect	<i>TAP1</i>	(133)
Sulfonylurea receptor defect	<i>ABCC8</i>	(177)
Calcium channel defect	<i>CACNA1A</i>	(187)
Androstane glucuronosyl conjugation	<i>UGT2B4</i>	(88)
Congenital long-QT syndrome	<i>SCN5A</i>	(174)
<i>S</i> -oxazepam glucuronosyl conjugation	<i>UGT2B7</i>	(150)
Paclitaxel hydroxylase deficiency	<i>CYP2C8</i>	(21)
Chlorpyrifos oxidation deficiency	<i>CYP3A4</i>	(20)
Acrodermatitis enteropathica	<i>SLC39A4</i>	(171)
Nifedipine oxidation deficiency	<i>CYP3A5</i>	(86)
Cyclophosphamide metabolism deficiency	<i>CYP2B6</i>	(82)
Hyperinsulinemic hypoglycemia	<i>SLC16A1</i>	(129)
Hereditary folate malabsorption	<i>SLC46A1</i>	(189)

<sup>a</sup>This list is not meant to be all-inclusive. In each case, compared with the consensus allele, one or more variant alleles lead to a defective gene product, resulting in decreased metabolism, transporter or receptor activity, or channel function. The clinical consequence in most homozygous affected subjects is toxicity, because of drug accumulation with enhanced drug activity. Occasionally, decreased drug activity (*therapeutic failure*) ensues if the variant reflects ultrarapid drug metabolism or if, for activity, the drug requires metabolic conversion to an active form and this conversion is decreased in the variant. We realize that some of the traits listed here might concern primarily environmental toxicants (e.g. *TAS2R1*, *CAT*, *FMO3*, and *PON1*) rather than prescribed drugs.

Phase II DMEs conjugate the Phase I products, usually inserting a highly polar conjugation moiety on a somewhat polar moiety produced by Phase I enzymes (Figure 19-4). Examples of Phase II DMEs include glucuronosyl, sulfate, glutathione, glycosyl, acyl, glycine, amine, acetyl, and methyl transferases. Drug metabolism research for the past six decades focused largely on these Phase I and Phase II DMEs.

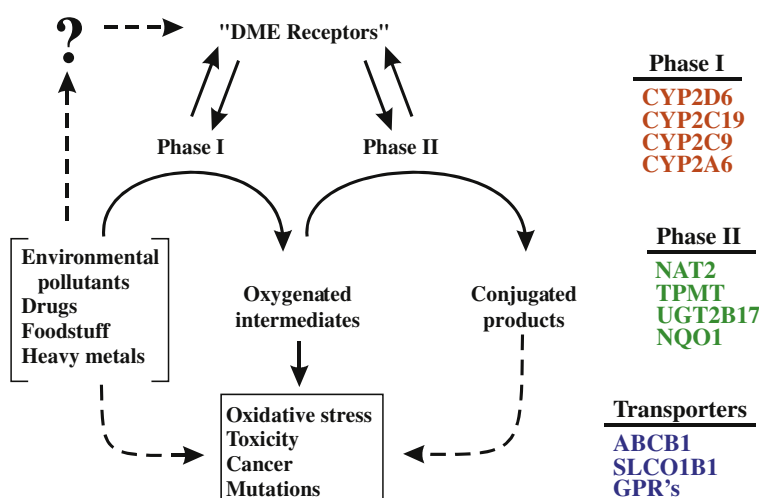
### 19.3.4 Plasma Clearance of Drug

In the late 1960s, comparisons of drug clearances in monozygotic vs dizygotic twins were reported. The “heritability index” was calculated as the variance within pairs

of dizygotic twins ( $V_d$ ) minus the variance within pairs of monozygotic twins ( $V_m$ ) divided by  $V_d$ . A heritability index of 1.0 would represent “purely genetic,” whereas a heritability index of less than 0.50 would indicate “predominantly environmental” factors. From twin studies (of dicoumarol, phenylbutazone, desipramine, halothane, nortryptiline, oxyphenylbutazone, and antipyrine), the main conclusion was that large variations among healthy subjects in drug clearance rates reflect a strong genetic component (165). Four decades later, we can appreciate that these observations reveal, principally, allelic differences in a few DME and/or DME-Related Transporters (DRTs) genes. Thus, “rapid” and “slow” Phase I and Phase II DME metabolism, respectively, plus “efficient”



### hPpM genes encode DMEs or drug-related transporters



**FIGURE 19-4** Scheme of Phase I and Phase II drug metabolizing enzymes (DMEs), the DME Receptors that Up- and Downregulate DME Levels, and DME-related transporters (DRTs) that move drugs and metabolites in and out of the cells, as well as across internal cell membranes. Certain environmental pollutants, drugs, foodstuff, and heavy metals interact with DME receptors, or other (reception) pathways, to up- or downregulate DME gene expression. (Modified from Reference (120)).

and “defective” DRT function, are among the principal effectors of plasma drug and metabolite clearance and therapeutic failure. These results underscore one of the themes of this chapter: that some pharmacogenetic differences—including large variability among normal subjects in plasma and urine drug and metabolite levels—can reflect polymorphisms in one or a few hPpM genes.

### 19.3.5 Extrahepatic Pharmacogenetic Differences and Endogenous Functions of DME

A common misconception is that DMEs exist almost exclusively in the liver. A corollary is that only drugs, and not endogenous compounds, are substrates for DMEs. Both statements are incorrect (111,114). CYP3A4, the most abundant P450 in liver, is also present in large concentrations in the gastrointestinal tract. Many DMEs are located in the lung and kidney. Many Phase I and Phase II DMEs exist in all vascular endothelial cells and contribute to the expression of the arachidonic acid cascade and thus eicosanoid synthesis and degradation; the result is effects on cell division, inflammatory response, cell migration, bronchoconstriction, vasodilation, and numerous other homeostatic mechanisms (119). Because DMEs exist in the choroid plexus, substantia nigra, vasculature, and basically any other cell type of the brain, they play pivotal roles in neuroendocrine functions. CYP2D6, encoding a major P450 that metabolizes drugs in the liver, is responsible for serotonin synthesis in the brain (186). Phenytoin oxidation (principally due to CYP2C9 and CYP2C19) is as much as 50-fold greater in human oral mucosa than liver (191). Some DMEs exist at high concentrations in nasal mucosa (48). Clinical geneticists

must recognize that variability in drug response can occur in any tissue and any cell type, not solely in the liver.

It is doubtful whether any DME exists that metabolizes only drugs without endogenous function (111,121). When an hPpM variant leads to an absent DME activity in a human, the clinical equivalent of a “knockout mouse,” one might ask—if the DME carries out, in addition to drug metabolism, one or more “critical life functions”—why is there usually no phenotype of illness or lethality? Actually, the same result (i.e. no striking change in phenotype) has been observed in numerous hPpM knockout mouse lines after genes encoding DMEs, receptors, DRTs, or other moieties have been disrupted. The answer is that Mother Nature most likely has incorporated a great deal of *redundancy*, beneficial for survival of the species.

### 19.3.6 Examples of hPpM Pharmacogenetic Polymorphisms

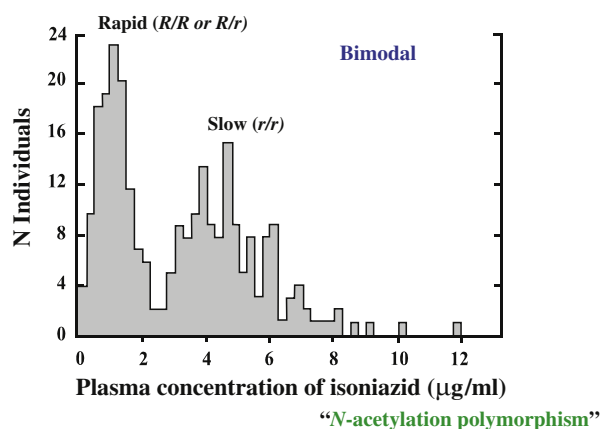
What follows are several examples of hPpM variants. Owing to space limitations, only a few cases will be described, but most remaining examples in Table 19-1 have been reviewed extensively [(117,118) and references therein].

**19.3.6.1 N-Acetylation Polymorphism (NAT2 Gene).** Originally called the “isoniazid acetylation polymorphism,” this defect was identified in the late 1940s when patients who converted to a positive tuberculin test routinely received isoniazid. A high incidence of peripheral neuropathy was noted. This is an example of the active principle (parent drug) reaching toxic levels (Figure 19-1) when the major detoxication enzyme in the isoniazid metabolic pathway is defective.

After isoniazid was administered to different individuals and their plasma isoniazid levels measured (Figure 19-5),

## Isoniazid 9.8 mg/kg, plasma levels 6 h later

---Br Med J 1960; 2: 485

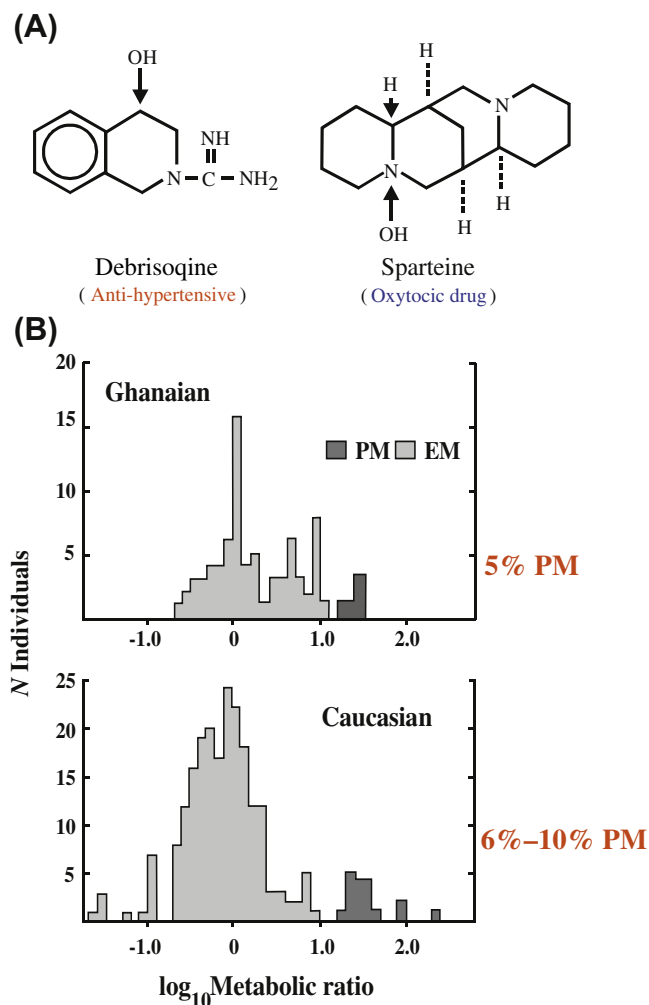


**FIGURE 19-5** Plasma isoniazid concentrations 6h after the drug was given. Results were obtained in 267 members of 53 complete family units. All subjects received 9.8mg isoniazid per kilogram body weight. (Redrawn from Reference (36)).

a *bimodal distribution* was described (36). Individuals were phenotyped as “slow-acetylators” or “rapid acetylators” (slow vs rapid plasma clearance). Parents of slow-acetylator children were also slow acetylators—indicating that slow acetylators are homozygous for the “slow-acetylator” allele (*r*), whereas rapid acetylators are either heterozygous or homozygous for the “rapid” (*R*) allele. Hence, the *slow phenotype* is inherited as an *autosomal recessive* trait. Frequency of the *r* allele was approximately 0.72 in the 1959 United States population (36); using the Hardy–Weinberg Equilibrium equation, this means that about one in every two individuals in this population is homozygous for *r/r*, reflecting the slow-acetylator trait.

Isoniazid *N*-acetyltransferase is an example of a Phase II DME polymorphism (Figure 19-4). Two human *N*-acetyltransferase protein-coding genes (*NAT1*, *NAT2*) are located in tandem on chromosome (Chr) 8p22. Rapid- and slow-acetylator phenotypes reflect the *NAT2* gene, encoding the *NAT2* enzyme, which has a 10-times-lower  $K_m$  than *NAT1*, using isoniazid and other arylamine substrates. Several *NAT2* slow-acetylator variant alleles encode a stable protein having little or no enzymatic activity or an unstable protein (9). A consensus nomenclature for the *NAT1* and *NAT2* alleles was developed in 1995 and is still updated online [<http://louisville.edu/medschool/pharmacology/consensus-human-arylamine-n-acetyltransferase-gene-nomenclature/>]. The (rapid-acetylator) consensus allele is *NAT2*\*4; presently, there are 63 alleles identified.

**19.3.6.2 Debrisoquine/Sparteine Oxidation Polymorphism (CYP2D6 Gene).** In the mid-1970s, the debrisoquine/sparteine polymorphism (Figure 19-6A) was discovered independently by two groups. The Robert L Smith laboratory in England (4) studied oxidative metabolism of the antihypertensive agent, debrisoquine. Smith noticed that, soon after its release in the United Kingdom,



**FIGURE 19-6** Illustration of the *CYP2D6* polymorphism. (A) Structures and major metabolites of debrisoquine and sparteine. (B) Frequency of the efficient (EM)- and poor (PM)-metabolizer phenotypes in populations from Ghana and Caucasians in the United Kingdom. Urinary “metabolic ratio” (MR) = parent drug debrisoquine divided by hydroxylated debrisoquine metabolites; because PM individuals show less metabolism, this ratio places PM subjects to the *right* of EM subjects. (Redrawn from Reference (193)).

debrisoquine caused a high incidence of ADRs; he reasoned that the combination of a narrow therapeutic index (described earlier, Figure 19-3) with an underlying genetic variation in metabolism might be responsible. Smith and three laboratory colleagues took the “recommended prescribed” dose of debrisoquine. Smith himself became hypotensive; his urinary 4-hydroxy metabolite was about 20-fold lower than that of his three colleagues who appeared unaffected by that dose. This is another example of a pharmacologically active drug reaching toxic levels because of insufficient metabolism to an inactive metabolite (Figure 19-1). A larger population was screened, and a *bimodal distribution*, separating “poor-metabolizer” (PM) subjects from “extensive-metabolizer” (EM) subjects.

Michel Eichelbaum for his 1975 thesis work in the Dengler laboratory in Germany studied human metabolism of the oxytocic drug, sparteine. This drug was

known to cause erratic and excessive uterine contractions in women; the urinary ratio of sparteine to dehydrosparteines showed a bimodal distribution (33). This variability in debrisoquine/sparteine metabolism is another example of a Phase I DME polymorphism.

The PM phenotype for debrisoquine occurs in 6–10% of people of European descent (Figure 19-6B, *bottom right*), compared with EM phenotype subjects who metabolize the drug 10–50 times more effectively (64). Incidence of the PM phenotype is approximately 5% in African populations (Figure 19-6B, *upper right*) and <1% in Asians. An “ultrarapid metabolizer” (UM) phenotype was also described; this phenotype was found to be due to amplification of the *CYP2D6* gene from 2, to as many as 13, copies (107). Incidence of the UM phenotype is about 0.8% in Northern Europeans, 21% in Saudi Arabians, and 29% in Ethiopians (65); the reason for these ethnic differences is not known but is likely to be the result of some selective pressure (probably dietary) or of a genetic bottleneck.

The P450 enzyme responsible for the debrisoquine/sparteine polymorphism is *CYP2D6*, a Phase I enzyme. Cloning of the *CYP2D6* genes and characterization of several mutant alleles (47) represented a landmark study providing for the first time a molecular mechanism to explain a descriptive pharmacogenetics disorder. PM alleles can code for an inactive enzyme because of mutation, unstable protein, incorrect splicing of the gene transcript, or even complete deletion of the gene—resulting in lowered, or completely absent, enzyme activity.

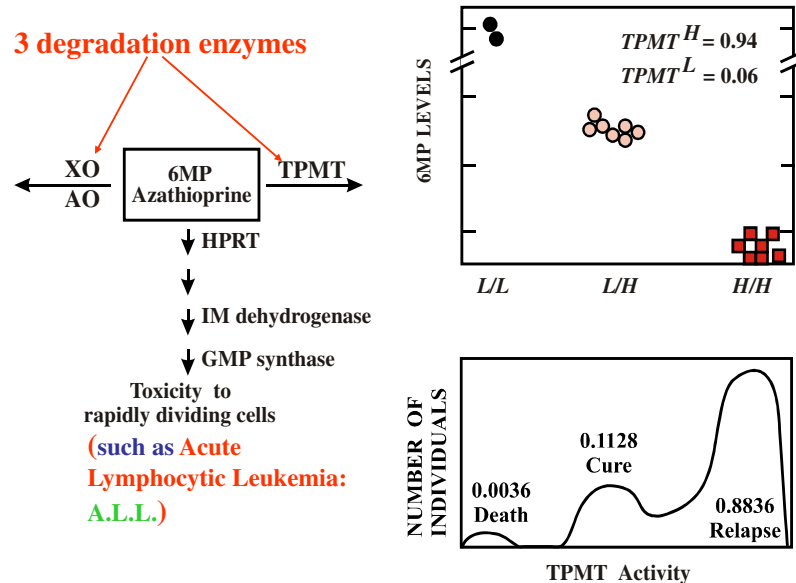
A unified system for naming human *CYP2D6* alleles is now used for allelic nomenclature of many human genes. The *CYP2D6*\*1 allele is the consensus, or reference, sequence (wild-type, EM); and currently, 126

allelic variants or haplotypes, plus 11 additional variants in which the haplotype is not yet understood, have been reported [<http://www.cypalleles.ki.se/cyp2d6.htm>].

The *CYP2D6* polymorphism is important in elimination of >20% of all commonly prescribed drugs, as well as over-the-counter drugs. The debrisoquine “panel” now comprises more than eight dozen drugs: tricyclics and other antidepressants including serotonin reuptake inhibitors and monoamine oxidase inhibitors, neuroleptics, antiarrhythmics and antihypertensives (including  $\beta$ -blockers), and opiates [<http://medicine.iupui.edu/clin-pharm/ddis/table.asp>].

**19.3.6.3 TPMT Polymorphism.** TPMT is a Phase II enzyme with a major role in the detoxication of 6-mercaptopurine (6MP), commonly used in chemotherapy for childhood acute lymphocytic leukemia. In the original study of a Caucasian cohort (177), frequencies of *high/high*, *high/low*, and *low/low* phenotypes were reported as approximately 88%, approximately 11%, and approximately 0.4%, respectively. This means that, when the “commonly recommended prescribed dose” of 6MP is given, 11% of patients would have a high probability of being cured of their disease, 88% would have relapses in their leukemia due to undertreatment, and one of approximately 300 patients might die from 6MP toxicity (Figure 19-7). In other words, if metabolism of 6MP is too extensive in 88% of patients, *therapeutic failure* (illustrated in Figure 19-1) may ensue.

This pharmacogenetic disorder is very dramatic, because it can lead to life-or-death clinical situations. Accordingly, in 1994, the *TPMT* polymorphism was presented to the US Congress as “the quintessential pharmacogenetic disorder” on the basis of which more federal funding was



**FIGURE 19-7** Diagram of 6-mercaptopurine (6MP), azathioprine or 6-thioguanine toxicity (which occurs in all cells but more so in rapidly dividing cells such as acute lymphocytic leukemia (ALL) white cells, due to disruption of purine biosynthesis), and the response of ALL patients given the “commonly recommended” prescribed dose of 6MP. Xanthine dehydrogenase (XO encoded by the *XDH* gene), adenine oxidases-1 and -2 (AO encoded by the *DUOX1* and *DUOX2* genes), and TPMT (thiopurine methyltransferase) are all enzymes that detoxify these chemotherapeutic drugs. Toxicity of these agents occurs more readily in *TPMT*<sup>L/L</sup> individuals than in *TPMT*<sup>H/H</sup> individuals. About 3 in 1000 Caucasians are homozygous for the L/L phenotype, 13% are heterozygotes, and 88% are homozygous for the H/H phenotype.

requested for pharmacogenetics research. Because the TPMT defect can lead to dire consequences, patients with acute lymphocytic leukemia (ALL) are now routinely phenotyped for red cell TPMT activity prior to initiation of 6MP chemotherapy. *TPMT<sup>HH</sup>* patients are usually given a four times larger dose, and *TPMT<sup>LL</sup>* patients are given a 10- to 15-times smaller dose—leading to a much better cure rate and survival rate for childhood leukemia.

The *TPMT* gene has nine exons, spanning 26.8kb. A total of 29 allelic variants have been identified (5), in each case resulting in very low or negligible catalytic activity.

Azathioprine and 6-thioguanine are other TPMT substrates (Figure 19-7). Azathioprine is widely used as an immunosuppressant in conditions as diverse as systemic lupus erythematosus and organ transplantation. Thioguanine is one of the agents used in treating chronic myelocytic leukemia. As with 6MP, azathioprine or 6-thioguanine can be lethal to the one-in-300 patient with homozygous *TPMT<sup>LL</sup>* if that subject receives the “commonly recommended prescribed” dose.

**19.3.6.4 Glutathione S-Transferase Polymorphisms (GST Genes).** GSTs are Phase II enzymes that add glutathione to many drugs and chemical metabolites. High GST activity can lead to rapid detoxication rates of antibiotics and chemotherapeutic agents (160). Usually considered Phase II detoxication enzymes (Figure 19-4), GSTs can also be involved in metabolic activation (109). The *GST* gene family comprises 16 genes in six subfamilies: *GSTA*, *GSTM*, *GSTO*, *GSTP*, *GSTT*, and *GSTZ* (122). Humans show high frequencies for the total deletion of the *GSTM1* or *GSTT1* gene (the so-called null alleles *GSTM1\*0*, *GSTT1\*0*); the incidence of GST-null individuals ranges between 20% and 50%, varying among different ethnic populations.

During the past two decades, dozens of associations have been reported of the NAT2 or NAT1 acetylation phenotypes with toxicity or cancer (56,110); many epidemiological studies suggested associations between *CYP2D6* allelic differences and toxicity or cancer (113). Dozens of publications proposed associations between the *GSTM1\*0* or *GSTT1\*0* allele and either enhanced or diminished risk of cancer or toxicity, depending on the etiologic agent (115). Clearly, without glutathione conjugation, it seems reasonable to expect more drugs or environmental toxicants to be redirected toward more pathways that include highly reactive metabolites. However, as discussed later, recognition has increased that most studies of one or a few SNPs associated with a multiplex phenotype in relatively small cohorts represent statistically underpowered false-positive data (113,124).

## 19.4 ETHNIC DIFFERENCES IN GENE-DRUG INTERACTIONS

Virtually every pharmacogenetic disorder investigated has shown substantial ethnic variability. For example, large ethnic differences exist in frequencies of the rapid-acetylator consensus allele (*NAT2\*4*) vs slow-acetylator

variant alleles (Table 19-2); frequencies of the slow-acetylator allele range worldwide from less than 10% in Japanese populations to more than 90% in some Mediterranean people.

This polymorphism was the first of many that presented a dilemma for the “pure” geneticist: the predominant allele in one ethnic group often is not the predominant allele in another. Which of these alleles should be regarded as the “wild-type?” In the case of the *N*-acetyltransferase polymorphism, the rapid-acetylator allele (inherited as autosomal dominant) was arbitrarily decided to be the “consensus” allele.

Ethnic differences in *CYP2D6* alleles [<http://www.cypalleles.ki.se/>] and many other P450 genes exist. One of the most pharmacogenetically important examples of ethnic variability is *CYP2C19*; among many substrates, this enzyme metabolizes *S*-mephenytoin, omeprazole, diazepam, propranolol, citalopram, indomethacin, proguanil/chloroguanil, imipramine, amitriptyline, mephobarbital, and hexobarbital [<http://medicine.iupui.edu/clinpharm/ddis/table.asp>]. The *CYP2C19* PM patient requires a lower dose of these drugs to achieve efficacy than the *CYP2C19* EM patient. The PM patient is more prone to toxicity than the EM person when both receive the “recommended” commonly prescribed doses of such drugs, especially if the drug has a narrow therapeutic index (Figure 19-3). At least 42 *CYP2C19* mutant alleles have been described [<http://www.cypalleles.ki.se/>], many exhibiting little or no enzyme activity. The incidence of the *CYP2C19\*2A* and *\*2B* alleles (responsible for splicing defects) is 2–5% in Caucasians, yet 20–30% in Asians. This is an excellent example in which the physician must be more careful in prescribing any drug in “the *CYP2C19* repertoire” for an Asian than for a Caucasian patient. Similarly, East Asian drug companies must recognize the importance of the *CYP2C19* polymorphism in their population more so than companies in predominantly non-Asian countries.

Mitochondrial aldehyde dehydrogenase-2 (ALDH2) deficiency is another example of ethnic differences

**TABLE 19-2** Frequency of N-acetylator NAT2 PM Phenotypes in Different Ethnic Populations

Ethnic Population	No. of Studies	Frequency of PM Phenotypes
Japanese	7	0.09
Eskimo	4	0.23
South Pacific Islands	5	0.35
Korean/Chinese	14	0.37
North and South Amerindian	10	0.50
African <sup>a</sup>	19	0.71
Central and West Asian	22	0.74
European	50	0.75
Egyptian	2	0.96

<sup>a</sup>Excluding the Kung Bushman of southern Africa, in which the PM frequency is 0.18. Data modified and condensed from (71).



**TABLE 19-3** Distribution of the ALDH2 Deficiency Phenotype in Different Ethnic Populations

Ethnic Population	Percent Having ALDH2 Deficiency <sup>a</sup>
Japanese	44
Central, East, and Southeast Asian	25–50
South Amerindian	40–45 <sup>b</sup>
North Amerindian	2–5
European, Mideast, and African	<0.1

<sup>a</sup>The mutation in Asians and North American Amerindians appears to be exclusively Glu504Lys, which causes a complete loss of ALDH2 activity in that subunit. The enzyme is encoded by four subunits; if one or more subunits are encoded by the *ALDH2*\*2 allele, then the entire tetramer is inactive. Thus, the *ALDH2*\*1/\*2 heterozygote has  $(1/2)^5 = 1/16$ , or 6.25%, of activity of the *ALDH2*\*1/\*1 subject.

<sup>b</sup>Mutation in South American Amerindians is purportedly different from that in Asians and North Amerindians. Data modified and condensed from (44).

(Table 19-3). The incidence of the *ALDH2* Glu504Lys mutation ranges between 50% and 75% in most Asian populations but is virtually never seen among Africans or Caucasians. A putatively different *ALDH2* allele, causing the same lack of ALDH activity, occurs in South American Amerindians (71), probably due to a founder effect or genetic bottleneck. These data led to speculation that *ALDH2* deficiency arose only in populations traditionally not using ethanol or spices (i.e. Asians have boiled water for at least the past 30 centuries), compared with populations for many centuries using alcohol and spices for enjoyment and preservation of foods.

Why do we see ethnic variability, especially in hPpM genes? Drugs are largely plant metabolites and derivatives thereof; hence, variations in diet of specific ethnic groups for thousands of years have led to important differences in hPpM genes. Examining *NAT2* variants in approximately 15,000 subjects from 128 populations (139) revealed a higher prevalence of the slow-acetylator *NAT2* phenotype in populations practicing farming and herding compared to those relying mostly on hunting and gathering. Perpetuation of mutant alleles resulting in monogenic disorders, as well as in some hPpM traits, can also result from enhanced resistance in the heterozygote to certain infections (114).

How long might it take for the human genome to adapt to dietary selective pressures? By means of genetic drift and natural selection (random mutations, inversions, insertions, deletions, gene duplication, crossing-over events, etc.), new genes will become fixed and passed on to the next generation—if the new configuration confers an ecological advantage to the species. The response of the genome to environmental pressures, over a minimal number of generations, has been described as *molecular drive* (29), *meiotic drive* (157), cryptic genetic variation (CGV) (43), and *decanalization* (42). This response is also likely to influence the processes of drug- or plant-induced efficacy or toxicity.

Whereas mutational changes happen slowly over many dozens of generations, epigenetic changes (i.e. no alteration in DNA sequence) can occur rapidly—in response to severe selective pressures. If the population of a species decreases dramatically so that rare variants are more likely to reproduce, the distribution in that population shifts even more rapidly. Within a population, emergence of individuals resistant to environmental changes has been demonstrated experimentally in various organisms from prokaryotes to insects and vertebrates, but mechanisms involved remain basically obscure. For example, Atlantic tomcod fish developed resistance to polychlorinated biphenyls (PCBs) living in the polluted Hudson River for 50–100 years (50–100 generations); a 6-nucleotide deletion in the *AHR2* gene, resulting in aryl hydrocarbon receptor-2 having poor affinity for planar PCBs, was the basis for the PCB resistance (179). Depending on the organism studied, between nine and 45 generations are usually required (112). For humans, nine to 45 generations require approximately 200 and approximately 1000 years, respectively.

The five major *Homo sapiens sapiens* geographically isolated subgroups diverged from one another between 45,000 and 60,000 years ago (190). Based on animal studies alone, 10,000 years suffice for striking differences in pharmacogenetically relevant mutations to have arisen from selective pressures due to tribal differences in diet or exposure to other environmental signals such as altered climate and altitude. For example, it seems likely that a 10,000-year diet principally of goat meat and milk products on a high desert—compared with that of tropical fruit and fish at the seashore—could cause striking allelic frequency differences in hPpM genes.

## 19.5 PHARMACOGENOMICS

So far, we have described two classes of genes: those contributing to *monogenic* diseases and hPpM traits. The former is a result of mutations in a crucial nonredundant gene, which, when malfunctioning, leads to a serious well-defined disease (e.g. PKU, cystic fibrosis, Gaucher disease, and cystinuria). The latter is a result of mutation in a highly penetrant but noncritical and usually somewhat redundant gene (e.g. *CYP2D6*, *NAT2*, *TPMT*, *CYP2C19*, *SLCO1B1*, *ABCB1*), which, when malfunctioning, can lead to altered plasma or urine drug or metabolite levels or therapeutic failure. Because other redundant enzymes and transporters participate in the same drug pathway, the defect in any one particular hPpM gene is usually only approximately 10–25% accurate in predicting a patient's drug response.

For example, one large retrospective study that correlated thiopurine-related ADRs with the *TPMT* genotype by DNA testing (163) noted that 78% of ADRs were *not* associated with the *TPMT* gene polymorphism, but rather “attributable to factors other than mutations in the *TPMT* gene.” Thus, perhaps the *TPMT* polymorphism

**TABLE 19-4 Partial List of ADRs That Can Occur in Patients Receiving Commonly Prescribed Drugs**

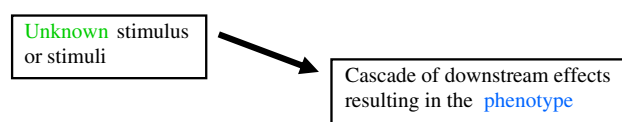
Organ or System	Possible ADRs (Multiplex Phenotypes)
Central nervous system	Headache, fainting, hallucinations, stroke, mood or mental changes (e.g. new or worsening anxiety, nervousness, agitation, confusion, depression, restlessness, sleeplessness, inability to concentrate), memory loss, new or worsening nightmares, seizures, suicidal thoughts or actions, brain damage and psychotic episodes, toxic psychosis, insomnia, anxiety attacks, ataxia, cogwheel rigidity, speech disorder, irritability, panic attacks, postural hypotension
Eyes	Glaucoma, changes in vision, blurred vision, loss of vision, photosensitivity, phototoxicity, dry eyes, periorbital edema, increased tearing, keratitis, cataracts
Gastrointestinal tract	Constipation, severe or persistent diarrhea, gas, nausea, vomiting, severe or persistent stomach pain/cramps, difficulty in swallowing, abdominal cramps, bloody or tarry stools, gastrointestinal disorders, heartburn (dyspepsia), indigestion, ulcers of mouth/esophagus/colon, gingival overgrowth
Heart	Chest pain, shortness of breath, angina, heart failure, heart block, cardiac arrhythmias, sinus tachycardia, palpitations, atrial fibrillation, ventricular fibrillation, postural hypotension
Hematological system	Anemia, unusual bruising or bleeding, excessive bleeding (sometimes fatal), clotting disorders, bone marrow suppression
Immune system	Severe allergic reactions (rash; hives; itching; difficulty breathing; tightness in the chest; swelling of the mouth, face, lips, or tongue; unusual hoarseness), immunosuppression, autoimmune diseases
Inner ear	Tinnitus, dizziness, light headedness, hearing loss
Kidney	Decreased or painful urination, renal insufficiency or failure, hypertension, hypotension
Liver	Symptoms of liver problems (e.g. dark urine, loss of appetite, pale stools, jaundice), chemical hepatitis, liver failure
Musculoskeletal system	Pain, soreness, redness, swelling, weakness, or bruising of a tendon or joint area; muscle pain or weakness; inability to move or bear weight on a joint or tendon area, irreversible tendon damage, spontaneous tendon ruptures, tremor, Ankylosing spondylitis, rhabdomyolysis, bone pain, bone loss, leg cramps
Pancreas	Symptoms similar to diabetes (e.g. high or low blood sugar, dizziness; fainting; fast breathing; flushing; increased thirst, hunger, or urination; increased sweating; vision changes), hypoglycemia, chemical pancreatitis
Peripheral nervous system	Symptoms of nerve problems (e.g. changes in perception of heat or cold, decreased sensation of touch, unusual burning, numbness, tingling, pain, or weakness of the arms, hands, legs, or feet), tremors, irreversible peripheral neuropathy
Reproductive system	Vaginal discharge, irritation, or odor; erectile dysfunction, loss of libido; perpetual erection, painful menstruation
Respiratory tract, lower	Shortness of breath, wheezing, pulmonary edema, pulmonary thrombosis, lower respiratory infection, cough
Respiratory tract, upper	Fever, chills, sore throat, or unusual cough; runny nose; dry nose and throat; stuffy nose and congestion; upper respiratory infection
Skin	Moderate or severe sunburn; red, swollen, blistered, or peeling skin; irreversible skin damage; Stephen-Johnson syndrome; toxic epidermal necrolysis; hives; eczema; edema
"Systemic" (?)	Any type of cancer, facial flushing, obesity, anorexia, decreased levels of sodium, decreased levels of potassium, drug–drug interactions, food–drug interactions, death
DNA changes	DNA damage, mutations, increased risk of cancer

itself might reflect 20–25% of thiopurine-related ADRs. It also should be noted that the red blood cell TPMT enzyme assay is a phenotype reflecting the combination of an unknown number of genotypic variants plus environmental factors (age, illness, renal clearance, recent blood transfusion, etc.). Hence, the TPMT phenotyping assay remains more accurate in predicting drug response than the *TPMT* genotyping assay (116).

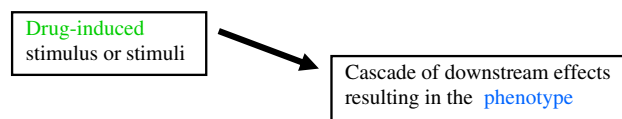
The third class of genes represents those contributing to complex (polygenic) diseases (e.g. asthma, schizophrenia, dementia, stroke, obesity, type 2 diabetes, etc.). Understanding this class of genes has changed greatly over the past two decades—due to The Human Genome Project (initiated in October 1990) and the incredible advances in human genomics since then.

Why should consideration of complex diseases concern pharmacogenomics? Because we believe that drug efficacy and ADRs caused by drug toxic levels are similar neither to monogenic diseases nor to hPpM traits; rather, drug efficacy and drug toxicity represent multiplex phenotypes: traits reflecting many genes plus epistatic (gene–gene) effects plus epigenetic effects (gene–environment

### Human complex diseases:



### Drug-induced ADRs:



**FIGURE 19-8** Similarities between complex diseases and drug-induced ADRs, both in genetic origin and phenotypic manifestation.

interactions that accumulate with each passing decade of life). Table 19-4 lists some ADRs in patients who receive commonly used drugs.

These ADRs are analogous to complex diseases (Figure 19-8). Table 19-5 lists examples of FDA-approved prescribed drugs given to a large population, following

**TABLE 19-5** Examples of Prescribed Drugs That Cause ADRs to a Limited Subset of Patients<sup>a</sup>

Name of Drug	Recommended for Treatment of	Number of Patients Treated	Number of Patients Affected	ADR Seen in a Subset of Patients Treated
Januvia (sitagliptin)	Diabetes type 2	Hundreds	88	Acute pancreatitis
Bisphosphonates	Osteoporosis	Unknown	1.93-fold increased risk	Esophageal cancer
Gadolinium-based contrast agents	Medical imaging of kidney disease	Unknown	Unknown	Nephrogenic systemic fibrosis
Meridia (sibutramine)	Obesity	Unknown	Unknown	Heart attacks, stroke
Flibanserin	Depression	Unknown	Unknown	Increased libido in women
Multaq (dronedarone)	Heart disease	Unknown	2	Liver failure
Psychotropic drugs	Schizophrenia, posttraumatic stress disorder, mood disorders, dementia	Unknown	79%, 72% 61%, and 11% increased 10-year risk, respectively	Chronic heart disease
Antidepressants	Depression	5000	Unknown	Acute-angle-closure glaucoma over the age of 66 years
Cladribine	Hairy cell leukemia	Hundreds	1	Progressive multifocal leukoencephalopathy
Pioglitazone	Diabetes type 2	30,173	1.4-fold increased risk	Urinary bladder cancer

<sup>a</sup>This information was taken from the Drug Information Agency daily news over the past 3 years.

which a small subset then develops an ADR appearing to be no different from a complex disease. How does this happen (Figure 19-8)? How does a small-molecular-weight drug, given to a few patients, cause an ADR that resembles a complex disease, whereas the vast majority experience efficacy?

### 19.5.1 How Many Genes Contribute to Each Type of Trait?

A single nonredundant gene is primarily responsible (90–98%) for a monogenic disease (Figure 19-9, *top*). However, additional genes are able to modify the phenotype (onset, severity, etc.), thus contributing the remaining 2–10% to that disorder. As stated above, an hPpM trait (Figure 19-9, *middle*) usually reflects a major gene contributing approximately 10–25% to such traits as plasma or urine drug or metabolite levels or therapeutic failure; however, additional dozens of hPpM genes plus perhaps hundreds of “downstream” genes contribute the remaining 75–90% to the trait.

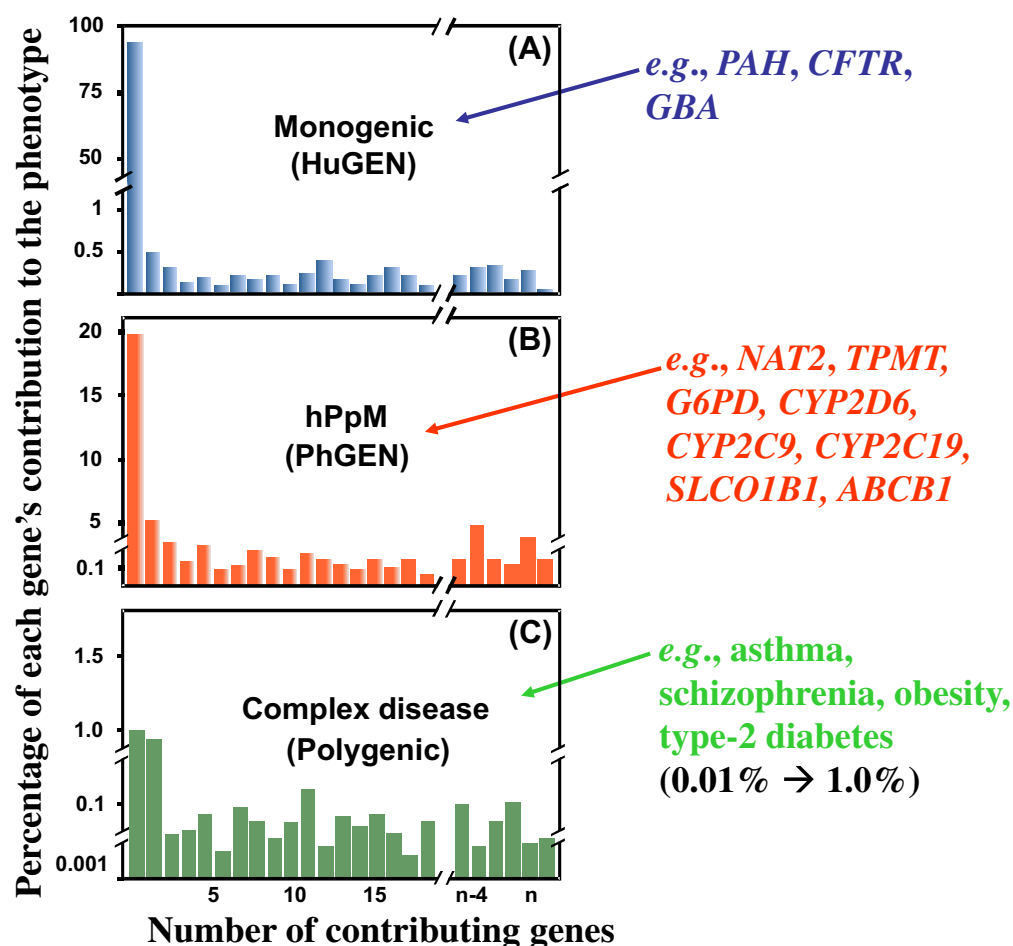
Recent advances in human genomics have now made us realize that hundreds, perhaps thousands, of genes contribute to the *multifactorial trait* that we call *complex diseases* (Figure 19-9, *bottom*). Moreover, it is rare to find any one particular gene contributing any more than 0.001–1.0% (94,162,83); this small amount of individual gene contribution is usually within the “background noise” of most genetic studies—unless large cohorts of 250,000 or 1 million are studied to sufficiently increase statistical power. Indeed, this is why the latest genotype–phenotype association studies are trending toward using larger cohort sizes.

### 19.5.2 Combined 2-Tier PK Plus PD System to Explain Pharmacogenomic Response

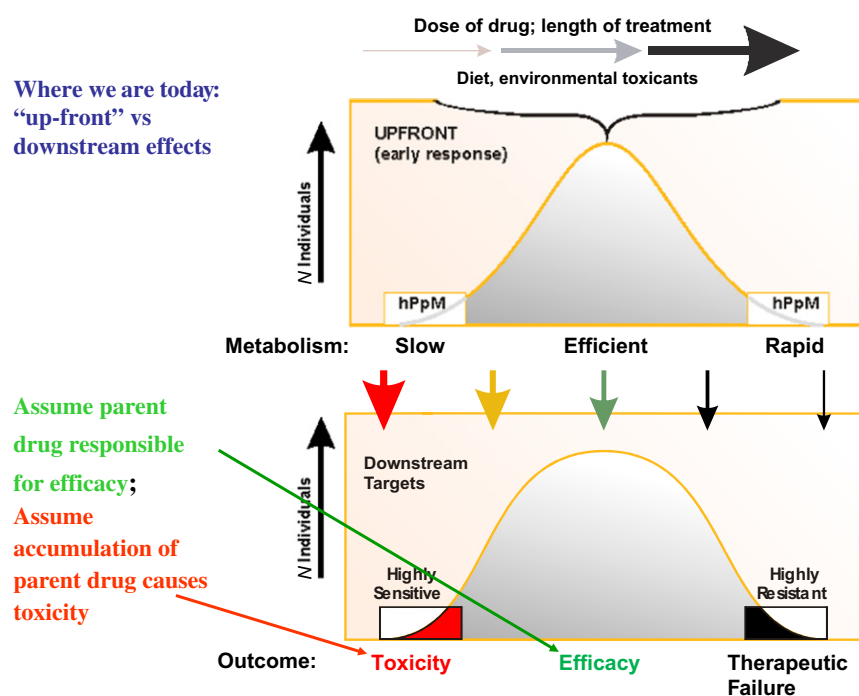
We previously proposed (124) the following 2-tier system (Figure 19-10) that still holds true: (a) front-end differences in hPpM gene polymorphisms are associated with PK processes and (b) downstream genes contributing to the phenotype (drug efficacy as well as ADRs caused by toxic levels of a particular drug) are mostly associated with PD processes. Because of hPpM variability in inheritance, the up-front genes can provide a substantial impact on the PK of any incoming drug or combination of drugs. Because the downstream targets are not usually encoded by hPpM genes, the ultimate outcome (efficacy or toxicity) becomes problematical, i.e. it more closely reflects genes that contribute to virtually any human complex disease.

In clinical pharmacology, most of the upfront gene products are encoded by DME or DRT genes (which have also been termed xenobiotic metabolizing enzyme (XRE) and XRE-related transporter (XRT) genes (113)). In any population, when these genes initially respond to a drug, one can expect slow vs rapid drug metabolism (Figure 19-10). The early-response hPpM genes (Table 19-1) might predict, in part, those individuals at the extreme low and extreme high ends of the spectrum. Responses to the drug by downstream genes will reflect much more of a gradient—from one extreme of toxicity (ADRs), to efficacy, to therapeutic failure (Figure 19-10).

Downstream targets include the class of genes contributing to complex diseases. In the case illustrated, we have assumed that the parent drug is the active form and causes the ADRs, but a similar scenario can be made for a metabolite as the active principle.



**FIGURE 19-9** Percentage of each gene's contributions to the phenotype: (A) monogenic diseases, (B) hPpM traits, and (C) complex diseases. The number of contributing genes (X-axis) will total in the dozens or hundreds. (Modified from Reference (124)).



**FIGURE 19-10** Two-tier system to explain drug toxicity vs efficacy vs therapeutic failure. "Upfront" early responses include the hPpM genes and gene products, which are PK processes. Downstream targets include dozens or hundreds of other genes and gene products, which are PD processes. (Modified from Reference (124)).



Patients having the genetic predisposition for slowest drug metabolism, due to the up-front early-response network, combined with having downstream targets most sensitive to the parent drug, would have the highest risk for accumulation of the drug, leading to *toxicity*. Subjects having predisposition for rapid metabolism, due to the up-front early-response network, combined with having downstream targets most resistant to damage by the specific drug, would clear the drug most rapidly and be at greatest risk for therapeutic failure. Those with intermediate metabolism by the up-front genes would provide the optimum amount of drug to the downstream targets, resulting in the best chance at efficacy. In addition, the dose, drug accumulation, and length of treatment time might all affect this balance (as well as all physiological and environmental factors) and, hence, could ultimately lead to drug toxicity or therapeutic failure, even after the drug had demonstrated efficacy for some period (Figure 19-10). Only a few DNA tests can provide 100% accuracy even for diagnosing monogenic diseases. To date, DNA tests generally cannot provide any more than 10–25% accuracy in predicting an hPpM disorder—in any individual patient.

### 19.5.3 Spin-Offs from the Human Genome Project

We now know that approximately 7% of the human genome has been under purifying selection during the past 100 million years (1,8,83): about 1.5% represents the approximately 21,000 protein-coding genes; regulatory elements controlling gene expression occupy about another 0.5%; and approximately 5% corresponds to functional conserved noncoding elements that produce non-protein-coding RNAs (ncRNAs). The remaining 93% of the genome is likely to have functions; we simply do not know at this time.

Because mouse, rat, and nonhuman primates have approximately the same number of protein-coding genes as humans, clearly we must look elsewhere to explain why all these species differ so much. The diploid genome (two haploid genome sequences from the same individual) was first reported in a Caucasian (89), and now, hundreds of diploid genomes from other ethnic groups have been sequenced. All comparisons between the two haploid genomes in each person have found only about 99.5% nucleotide similarity. These findings are most remarkable in that the nucleotide similarity between a human and a chimpanzee haploid genome is approximately 98.7%! Indeed, *Science* magazine's "2007 Scientific Breakthrough of the Year" (130) was our "newly appreciated recognition of human genetic variability"—i.e. variation in our genome is far greater than most of us could ever have imagined, even just 1 year earlier.

**19.5.3.1 "SNP Fever".** In the mid-1990s, what had been commonly known as "nucleotide substitutions" (by yeast, worm, and fly geneticists for several decades)

was described as SNPs, pronounced "snips". What followed—starting in 1995 and continuing even today—is what we call the "SNP fever." Hundreds of publications sought statistically significant ( $P < 0.05$ ) associations between a few SNPs in one or a few complex disease genes and traits such as hypertension, coronary artery disease, stroke, and even various types of cancer. Not far behind, others sought statistically significant ( $P < 0.05$ ) associations between a few SNPs in one or a few hPpM genes and either drug efficacy or drug-associated toxicity. Almost always, these statistically underpowered, false-positive, uninformative reports could not be corroborated in additional studies (124). Before acceptance of a manuscript, some journals now request confirmation of a genotype–phenotype association in a second ethnic group and/or experimental proof-of-biological-function.

**19.5.3.2 Results from Encyclopedia of DNA Elements.** The Encyclopedia of DNA Elements (ENCODE) Project—comprising many investigators located in at least 80 institutes worldwide—selected 1% of the human genome and examined this in great detail (7). A large variety of parameters was dissected: genes (protein-coding and noncoding exons), promoters, enhancers, repressor/silencer sequences, origin-of-replication and termination sites, conserved sequences, RNA transcripts, transcription-factor-binding sites, methylation sites, DNase I hypersensitivity sites, and chromatin modifications. The human DNA sequence was also compared with those of chimpanzee, mouse, or pufferfish (<http://genome.ucsc.edu/ENCODE>).

Breakthroughs from ENCODE included a greater understanding of: the extensive overlap of gene transcripts and many non-protein-coding regions; complex networks of transcripts (this challenges the concept of "lone transcription units"); many new transcription start sites; an arrangement of "far more complex regulatory sequences and binding of TFs than heretofore imagined"; regulation of genes in *cis* that can be governed by motifs millions of bases away; regulation of genes in *trans* that can be controlled by enhancers even on other chromosomes; interactions among chromatin structure, regulation of transcription, and replication; and finally, 60% of all DNA appears to be under significant evolutionary constraint (7), including the 7% under purifying selection.

These unexpected findings by ENCODE have thrown into disarray previous concepts of "what constitutes a gene" (41). By the end of the twentieth century, "the gene unit" was defined as "a segment of heritable DNA giving rise to a functional product (protein, small peptide, or any of the classes of regulatory RNAs) and including DNA from the 5'-most to the 3'-most regulatory elements." Since 2007, the ENCODE-proposed definition of "the gene unit" has been "a union of genomic sequences coding for a coherent set of potentially overlapping functional products" (7). This definition sidesteps the complexities of regulation and transcription (41).

The complexity revealed by ENCODE (35) has now led to changes in our thinking from “gene expression” as only a local (*cis*-regulated) transcriptional activation to a three-dimensional transcriptional regulatory network that operates in the dimensions of time and space and is cell-type specific. This new appreciation of complexity has now led to thinking in terms of gene expression networks for describing “how the genotype influences the phenotype” of a complex disease. We believe that this same complexity holds true for the many genes involved in drug efficacy and toxicity (ADRs).

**19.5.3.3 GWA Studies.** GWA studies are experiments in which numerous SNPs (usually 1 or 2 million or more, currently across approximately 90% of the genome) are genotyped in large populations. *Multiple locus testing* for statistical associations of each SNP with a phenotype are then performed (137). Phenotypes—including those as diverse as height, skin color, alcohol or caffeine addiction, schizophrenia, neurodegenerative disease, obesity, susceptibility to diabetes or asthma, blood pressure, renal or lung function, bone density, any type of cancer, and fasting glucose or blood lipid levels—can be chosen. GWA studies of drug toxicity, efficacy, or therapeutic failure have also begun to be reported. The power to detect significance (usually  $P < 5 \times 10^{-8}$ ) depends in part on the number of SNPs examined, plus the cohort size (141).

The National Human Genome Research Institute keeps a catalog of all GWA publications in which at least 100,000 SNPs have been studied; associations of genotype with more than 210 phenotypes are currently listed (<http://www.genome.gov/gwastudies/>). At the time of this writing, more than 1300 GWA studies, plus several dozen reviews, have been published. GWA studies have clearly provided numerous putative associations, which by and large can be regarded as “helpful” in understanding complex diseases and hopefully identifying new potential drug targets (46,83). Since 2006, more than 1100 loci affecting more than 210 phenotypes have been associated with common traits and diseases (83).

However, GWA studies have not helped significantly in predicting the risk of common diseases or in predicting drug efficacy or toxicity. The conclusions reached with GWA studies are not that different from the scenario described earlier, i.e. “associations of SNPs in or near one or a few genes with a multiplex phenotype are highly unlikely to be truly statistically significant.”

The biggest problem in taking advantage of all the GWA study information and using it for personalized medicine and individualized drug therapy—is our limited understanding of genomic functional significance. Moreover, we also do not understand the genome’s relationship to epigenetic factors, including drug and other environmental contributions to health and disease. Another problem in interpreting bona fide GWA studies is that causative variants are often located at relatively large distances from the SNP—as much as 2 Mb—and

still contribute to that association (136). This distance can include dozens of genes.

**19.5.3.4 Copy-Number Variants.** Copy-number variants (CNVs) represent segments of DNA—from less than 500 bp to more than 2 Mb—that can be duplicated in one’s genome up to 48 times or deleted. In a map constructed from 185 human genomes (108), more than 28,000 CNVs were identified; thus, several hundred CNVs generally occur in one genome. CNVs comprise as much as 15% (approximately 450 Mb) of our genome, and CNV patterns can vary from one cell to the next in the same tissue, i.e. mosaicism (91). In twin studies, some CNVs were even found to differ between copairs of identical twins (13,98).

Significant effects of CNVs on complex disease, drug efficacy, and ADRs remain unclear. Some studies have suggested that CNVs are associated with certain complex diseases including cancer and neurological disorders, whereas others are not. However, if CNVs encompass hPpM genes, they are highly likely to influence drug or metabolite levels in plasma or urine and/or the phenotype of therapeutic failure (53).

**19.5.3.5 Examples of Pharmacogenomics GWA Studies.** Recent advances in pharmacogenomics and drugs have been made through the use of candidate gene and genome-wide studies (Table 19-6). At present, one highly popular association is the combination of mutations in the *CYP2C9*, *VKORC1*, and *CYP4F2* genes, which might “contribute as much as 35–50% of the patient’s total variation” in the optimal dose of warfarin anticoagulation (101). This level of “certainty” in the individual patient, however, is insufficiently high for the physician to treat the patient with confidence.

Perhaps the two most striking influences seen clinically in drug-treated patients, because of major changes in plasma drug levels, are the effects of (a) poor- vs rapid-metabolizer-*CYP2C19* phenotypes and outcome of clopidogrel treatment and (b) ultrarapid metabolizer *CYP2D6* phenotypes following codeine therapy (68).

In a GWA study of “habitual caffeine intake,” more than 47,000 individuals of European descent within the United States were compared by meta-analysis adjusted for age, sex, and smoking history (18); two SNPs achieved genome-wide significance—one near the *AHR* gene and the other near the *CYP1A2* gene. The study makes sense functionally, because *CYP1A2* metabolizes caffeine and *AHR* regulates *CYP1A2* expression. However, this observation does not contradict our main message: poor predictability of genetic variants for a multiplex phenotype such as “caffeine addiction.” This study represents an issue of effect size (predictability and clinical utility) instead of just the *P*-value (analytical validity); one can establish the association with highly significant *P*-values by meta-analysis of almost 50,000 samples, but the effect sizes remain extremely low (<1% contribution to the trait). Therefore, this finding has no clinical utility whatsoever. The result, however,

does increase our knowledge about caffeine intake and metabolism.

A similar GWA study of metabolic traits in nearly 2000 subjects from two cohorts found an SNP near the *NAT2* gene to be highly significantly associated with coronary artery disease and response to drug toxicity (151). In a meta-analysis of 23 studies evaluating *GSTM1* and 19 studies evaluating *GSTT1* (39), a *GSTM1*–*GSTT1* interaction in patients having the dual null genotype was strongly associated with increased risk of uterine cervical cancer. Meta-analysis of two populations totaling approximately 40,000 subjects detected a strong association between an SNP near the *ALDH2* gene and elevated blood pressure (75). However, the effect size is far too small in each of these large-population studies ever to consider testing individual patients.

In each of the pharmacogenomics examples in Table 19-6, some patients with the mutation were not associated with the phenotype and other patients without the mutation were associated with the phenotype. As originally described by Fisher (38), *statistical interaction* is a *population-level concept*, whereas for the *individual* (patient), we need to understand the *biological interaction* or *function*. In other words, when testing for statistical interaction, the resulting model can be far more complex than is justified by the power that the sample design permits (172).

This problem also underscores the complexity of calculating the “cost–benefit” ratio: after the patient pays for a test and if the physician remains unconvinced by the result, what benefit is this test? Most importantly, this is why the use of genotyping to make informed clinical decisions about individualized drug therapy is not widely practiced. This “degree of uncertainty” reinforces our contention as to the difficulty in achieving any DNA test acceptable to the physician for *predicting* risk of a multiplex phenotype, such as complex diseases, drug efficacy, or ADRs.

### 19.5.4 The Dilemma: “Missing Heritability” in Multiplex Phenotypes

Concerning GWA studies, what has changed scientifically in the last few years is the accumulating confusion concerning GWA studies and their ability to identify unequivocally important genes for complex diseases in human populations. In study after study, applying GWA to every common disease, the results have been remarkably consistent: only genes having very minor effects have been uncovered (2,26,100). In other words, are causative alleles common or rare? Synthetic associations created by rare variants have not explained most GWA study data (3,142,181). The genetic variation—confidently expected by medical geneticists initially to explain common diseases—cannot be found. Medical geneticists do not dispute the GWA results themselves but are now assuming that genes predisposing to common diseases

**TABLE 19-6** List of Recent Associations between Pharmacogenomics Traits and One or Several Genes

Gene (or Allele)	Drug	References <sup>a</sup>
<i>TPMT</i>	Mercaptopurine- and thioguanine-induced toxicity	(28,177)
<i>ESR1</i>	Tamoxifen resistance	(74)
<i>BCR-ABL</i>	Imatinib, dasatinib, nilotinib resistance	(30,80)
<i>UGT1A1</i>	Irinotecan, nilotinib resistance	(66)
<i>ERBB2</i>	Lapatinib, Trastuzumab resistance	(34,145)
<i>HLA-B*5701, HLA-DR7, HLA-DQ3</i>	Abacavir-induced hypersensitivity	(99)
<i>EGFR</i>	Cetuximab, Erlotinib, Gefitinib, Panitumumab resistance	(95,128)
<i>CYP2C9, VKORC1, CYP4F2</i>	Warfarin relative resistance	(135,155)
<i>HLA-B*1502</i>	Carbamazepine-induced SJS-TEN <sup>b</sup> in Asians	(93)
<i>KRAS</i>	Cetuximab, Panitumumab resistance	(92)
<i>KIT</i>	Imatinib resistance	(59)
<i>CYP2D6</i>	Codeine toxicity in ultrarapid metabolizers	(192)
<i>HLA-B*5701</i>	Floxacin-induced hepatic injury	(22)
<i>IL28B</i>	Response to HCV infection by peg-Interferon- $\alpha$ -2b or $\alpha$ -2a combined with Ribavirin	(40,152,156)
<i>CYP2C19</i>	Clopidogrel response	(104,143)
<i>HLA-DRB1*1501, HLA-DQB1*0602, HLA-DRB5*0101, HLA-DQA1*0102</i>	Lumiracoxib-induced hepatic injury	(144)
<i>HLA-A*3101</i>	Carbamazepine-induced hypersensitivity, SJS-TEN in Europeans	(103)
<i>CYP1A2, AHR</i>	Caffeine addiction	(18)

<sup>a</sup>The list of references is arranged roughly chronologically but is not exhaustive.

<sup>b</sup>SJS-TEN, Stevens–Johnson syndrome-toxic epidermal necrolysis.

must somehow have been missed by the GWA methodology. There is a big problem, however, in that geneticists have been unable to agree upon where this “dark matter of DNA” might be hiding.

In addition, we have seen large GWA studies of particular multiplex phenotypes in which the same ethnic cohort has been analyzed by several laboratories—with distinctly different results (67,125). In some instances (Table 19-7), a locus is identified by all three laboratories; in other cases, only two of the three laboratories found the highly significant locus; and on still other occasions, only one of the three laboratory found a highly

**TABLE 19-7** Example of Three Laboratories, Each Performing a GWA Study in Different Cohorts of Caucasians, Yet Coming Up with Different Findings as to Which Loci Are Associated with Basal Metabolic Index (BMI)

Gene or Locus	Reported by Laboratory		
	A	B	
<i>NEGR1</i>			
<i>SEC16B, RASAL2</i>		B	
<i>TMEM18</i>	A	B	
<i>ETV5</i>		B	
(Gene desert) near <i>GNPDA2</i>	A		
<i>PRL</i>			C
<i>NCR3, AIF1, BAT2</i>		B	
<i>PTER</i>			C
<i>BDNF</i>		B	
<i>MTCH2</i>	A		
<i>FAIM2, BCDIN3D</i>		B	
<i>SH2B1</i>	A	B	
<i>MAF</i>			C
<i>FTO</i>	A	B	C
<i>NPC1</i>			C
<i>MC4R</i>	A	B	C
<i>KCTD15</i>	A	B	

For example, see (54,131,188).

significant gene or locus. It has been posited (42) that all three publications might, in fact, be correct and that these data might be explainable on the basis of epigenetic effects, to be discussed later.

Recently it has become possible to determine the amount of contribution by all identified genes toward any multiplex phenotype—addressing the question as to how much of the heritability can be attributed to each locus identified by GWA studies. A list of percent contribution of SNPs identified by GWA studies contributing to several traits is summarized in Table 19-8. The recent concern about GWA studies (57) is the fact that the total contribution of all genes “identified as being significantly associated (having *P*-values between  $5 \times 10^{-8}$  and more than  $10^{-250}$ ) with a *multifactorial trait*”—is surprisingly small (45,83).

Although 20 loci were initially highly associated with height (49,87,167,173), all variants combined could not explain even 3% of the population variation in height. In a cohort of more than 183,000 subjects (84), at least 180 loci could explain only approximately 10% heritability of the phenotypic variation in height. In fact, in a study of 500,000 unrelated individuals, although 100% of those at the effect size (detected so far with existing power) might be found, this would still amount to no more than approximately 30% of all height heritability; one inference is that “the other 70% would participate in even smaller effects.” To fit the observed distribution of variants explained by genetic markers from GWA studies, real-disease models (e.g. lipid profiles, diabetes type 2, and Crohn disease) were examined (147). Estimates

**TABLE 19-8** Several GWA Studies Showing Contribution of SNPs to That Trait<sup>a</sup>

Phenotype	Number of Loci Found	Proportion of Heritability Explained (%)
Diabetes type 1	41	~60
Fetal hemoglobin levels	3	~50
Macular degeneration	3	~50
Diabetes type 2	39	20–25
Crohn disease	71	20–25
LDL and HDL levels	95	20–25
Height	180	~12
Body mass index	At least 35	6–11
Prolonged QT interval	14	~5.0–6.5

<sup>a</sup>Taken, in part, from (83).

indicate that probably hundreds to nearly a thousand variants underlie these multiplex phenotypes.

Currently, a consensus explanation for this puzzle of “missing heritability” in complex diseases has not yet emerged. Genetic studies might fail because (1) interrogative methods cannot identify the causal genetics (i.e. “genome-wide” measurements with SNP chips currently cover no more than 90% of the entire genome), (2) phenotyping is incorrect, or (3) the trait is not heritable (e.g. *de novo* mutation not found in either parent) (26).

In conclusion, there appears to be a serious “missing heritability” problem that demands attention be directed to other sources of variance. This unexplained heritability could be partly due to gene–environment interactions or more complex pathways involving multiple genes, gene–gene interactions (epistasis), and exposures (159).

It should be acknowledged that some (185) still “have no reason to believe that any heritability is missing”; some propose that consistent effects of epigenetic variants, gene–environment interactions, and nonadditive traits (of combinations of variants) be excluded from consideration, because these factors are taken into account when defining “heritable genetic variance.” Consistent with this argument, a comparison of 29 whole genome sequences (193) suggested that “the rare variants are significantly more likely to be functional than the more common variants.”

### 19.5.5 Increasing Appreciation of the Epigenome

Epigenetics and the epigenome are among many the reasons for explaining the above-discussed dilemma of missing heritability. Examples of epigenetic effects include *DNA methylation* patterns, covalent *modifications of histones*, *chromatin remodeling*, *RNA interference* (RNAi) processes, and *decanalization*. Some of these categories are currently better understood than others. Epigenetics might provide a new framework for our search of etiological factors contributing to complex diseases, drug efficacy, and ADRs.



We are certain that there will also be “genetic differences” in epigenetic response: monozygotic co-twins are expected to exhibit more similarities in epigenetics than dizygotic co-twins. In support of this, on comparing DNA methylation in co-pairs of monozygotic with that in copairs of dizygotic twins (73), there was significantly less epigenetic variability in buccal cells from monozygotic than dizygotic copairs of twins ( $P = 1.2 \times 10^{-294}$ ).

During the past decade, there has been an explosion in appreciation of epigenetics. Some epigenetic variants can be inherited by offspring (or the offspring’s offspring)—indicating the existence of a *transgenerational* mechanism (23) for biological heredity not based on DNA sequence. For example, because of the Dutch Famine of 1945, neurodevelopmental disorders increased in grandchildren whose grandparents were exposed prenatally (153). Famine in a small Swedish village, at the time a male enters puberty, sends an unknown message to his grandson, resulting in *less risk* of diabetes type 2; famine in the same population, when a female’s oocytes are forming during the third trimester in utero, *increases the risk* of obesity and diabetes type 2 in that baby’s granddaughter (129). Cigarette smoking during pregnancy by a grandmother increases the risk of asthma in the granddaughter by four-fold (90). Germ cells can carry epigenetic effects from the grandmother’s diet (17). Individuals whose grandparents suffered malnutrition in utero during the 1959–1961 Chinese famine have increased risk of schizophrenia (183); however, another analysis of these same data suggests that this result might reflect population stratification (148). Even chronic stress in the pregnant mother (70) or dietary effects in the father’s sperm (126) are believed to be sufficient to induce changes in the *epigenetic landscape* of the developing embryo and fetus in utero.

It is extremely likely that epigenetic changes are involved in various complex diseases including cancer and asthma (37). We believe that the epigenome is also likely to play a role in drug efficacy and ADR phenotypes. Epigenetic effects increase with each passing decade of life, because of constant bombardment of environmental stimuli (which include drugs—as well as diet and chemical or metal toxicants). Within the next several years, there should be many more examples of epigenetic-mediated effects on gene–drug interactions.

**19.5.5.1 DNA Methylation.** Genome-wide DNA methylation occurs as a function of time in a highly specific cell-type manner. DNA hypermethylation is known to reflect environmental impact, as a function of each increasing decade of life. Promoter hypermethylation usually results in turning genes “off,” whereas promoter hypomethylation usually turns genes “on.” Vast differences exist between individual DNA methylation loci and are sometimes under genetic control, as well as related to age and gender (10). DNA methylation can modify the regulation of hPpM genes—thus changing drug or metabolite profiles in blood or urine or altering the degree of therapeutic failure.

Individuals exposed in utero during the Dutch famine of 1945, when examined six decades later, showed less DNA methylation of the imprinted *IGF2* gene, compared with their unexposed same-sex siblings (55); this study demonstrates that early-life environmental conditions can cause transgenerational epigenetic changes in humans that persist throughout life of that individual. Genome-wide analysis of DNA methylation in peripheral blood was compared among current smokers, former smokers, and those who had never smoked (11); significant differences were found between smokers and the other two groups. Looking at DNA methylation changes in a specific gene, or even a group of genes, faces the same difficulties (*vide supra*) as looking at SNPs in one or a few genes, i.e. multiplex phenotypes might reflect hundreds of genome-wide DNA methylation changes, each contributing 0.001–1.0% to variability in the trait.

**19.5.5.2 RNA Interference : MicroRNAs.** The human genome encodes more than 2000 MicroRNAs (miRNAs) in approximately 100 evolutionarily conserved families. The miRNAs are 20- to 25-nucleotide-long ncRNAs that regulate gene expression posttranscriptionally by mediating translational repression or promoting degradation of their target mRNAs (15,79,169). Each typical conserved miRNA targets, on an average, approximately 200 mRNAs. Clearly, genetic variation in one’s miRNA profile can be pivotal not only in hPpM disorders but also in monogenic diseases and multiplex phenotypes, i.e. complex diseases, drug efficacy, and ADRs.

**19.5.5.3 Chromatin Remodeling.** Transcription in mammals is regulated by TFs that associate with the genome in a cell-type-, temporal-, spatial-, and condition-specific manner. Chromatin organization forms part of the basis for this cell-type specificity by allowing or denying TF access to regulatory DNA regions. The basic units of chromatin structure are the nucleosomes. Heritable chromatin status and TF binding can differ because of genetic variation, which in turn may underlie phenotypic variation in humans. This field has just begun to be appreciated, but there are implications for effects on all the traits mentioned above.

**19.5.5.4 Histone Modifications.** Histones form the protein core around which eukaryotic genomic DNA is wrapped in chromatin. The structure and the transcriptional state of chromatin are closely associated with histone posttranslational modifications, which can affect gene expression. The highly heterogeneous nature of histone modifications in the intact cell or animal poses a daunting challenge for the future. Again, we can expect to find histone modifications that will affect all the traits mentioned above.

**19.5.5.5 Decanalization (“Cryptic Genetic Variation”; “Molecular Drive”).** *Canalization* pertains to “the evolution of robustness to genetic or environmental perturbation in populations.” Environmental perturbation includes the effects of diet, stress, or drug usage; because canalization can also be inherited transgenerationally,

there is likely to be an interrelationship with DNA methylation and other epigenetic processes. Most of these (virtually unknown) canalization mechanisms have been delineated in the fruit fly, but we predict that this process in humans will affect some or all of the traits mentioned above. In canalized populations, most individuals tend to cluster around the robust optimal phenotype. A decanalized individual would be significantly outside this robust optimum and, hence, more susceptible to an altered condition—including certain complex diseases, ADRs, or degree of drug efficacy.

Consider the rapid “telescoping” of the human genome during the past few hundred years, compared with the previous 5–6 million years since hominids diverged from apes. For 5 million years, evolution was very gradual and able to accommodate comfortably to environmental or genetic alterations. In contrast, during the past 6000 years, humans have gone from hunter-gatherer, to farmer, to villager, to living in towns and polluted cities. Dramatic cultural changes in the past few centuries (striking dietary shifts, tobacco smoking, air pollution, altered pathogen exposures, psychological stress, and consuming prescribed vs over-the-counter vs recreational drugs) have dramatically pushed physiology away from the evolved robust mechanisms normally preventing disease. Thus, our genome has undergone tremendous “telescoping,” on comparing the past one to two dozen generations with the previous 3000 generations (42).

If we consider these striking environmental and cultural perturbations during recent generations, this might lead to the uncovering of CGV (140) as a major source of complex disease susceptibility. Processes of drug efficacy and risk of ADRs might similarly reflect decanalization. For example, the increases that we see in rates of obesity and autism spectrum disorder, as well as psychotropic drug-induced obesity—during these past few decades in western societies—might mirror decanalization that has occurred very recently. Moreover, the “missing heritability” from GWA studies (*vide supra*) might well correspond, at least in part, to decanalization.

## 19.5.6 Beyond DNA Sequence Assays

**19.5.6.1 Transcriptomics.** Transcriptomics (61,106) and laser-microdissection transcriptomics (14) refer to the studies of gene transcripts—generally analyzed by cDNA expression microarrays. These arrays measure the amount of steady-state mRNA levels in the tissue being studied, which of course can sometimes differ substantially from the amount of functional protein in that tissue. Such cDNA expression studies have led to a number of exciting breakthroughs in basic science, as well as in cancer biology, in which prognosis and treatment can sometimes be more accurately directed by observing an expression profile of a tumor (6).

**19.5.6.2 Proteomics.** Proteomics is the study of all proteins encoded by the genome; protein or metabolite

profiles can be studied in urine, tissues, or even single cells (138). Although an average of 3.0 proteins per gene has been estimated, others suggest that the true number of proteins per gene might be much higher. In theory, protein profiles from various sources should represent the outcome of all genetic plus epigenetic effects; from this standpoint, it might be more comprehensive than transcriptomics.

Why is transcriptomics or proteomics unlikely to play a major role in individualized drug therapy? The answer is related to the major limitation of microarray expression in patients receiving drugs: i.e. easy access of the appropriate source of sampling—blood, excreta (urine, saliva, sweat, breast milk, ejaculate, menses, feces), or tissue in which relevant cDNAs exist (dissected tumor, tissue biopsies, placenta, foreskin). It would not be ethical or reasonable, for example, to attempt to predict individual psychotropic drug response outcomes from brain biopsies in healthy patients.

It is conceivable that proteomics in the future might identify certain protein profiles—similar to the ways in which metabolomics can identify certain metabolite profiles. At present, it is not known whether this will become useful in predicting complex diseases, drug efficacy, or risk of ADRs.

**19.5.6.3 Metabolomics.** The “metabolome” represents an integrated response, in real time, to all endogenous processes plus all exogenous stimuli (drugs, chemical exposures, occupation, lifestyle, nutrition, disease state, age, and gender). The metabolome should reflect all genetic plus epigenetic variability. Metabolomics therefore might provide an exquisitely sensitive means to follow an individual’s total phenotype—as a function of age, nutrition, course of disease, or therapy. Accordingly, in principle, this technique offers great promise to individualized drug therapy and personalized medicine but will also be subject to some of the pitfalls identified above for pharmacogenomics.

Metabolites measured include not only those from drugs but also thousands of small-molecular-weight compounds that exist in every metabolic pathway in our bodies. The patient’s intestinal flora interacts extensively with the host, resulting in metabolic exchange in the gastrointestinal tract and shared metabolism of substrates—which can be detected by metabolomic analysis (16).

This method of analysis is so sensitive that one can detect diurnal rhythm, menstrual cycle, and foodstuff recently consumed. Accordingly, metabolomic analyses will be required to pay particular attention to the time of day, time of month for menstruating females, recent dietary intake, and recent exercise history, as well as exposures to perfume, hairspray, cosmetics, cigarette smoke, occupational chemicals, pollen, urban vs rural air, and perhaps new furniture and rugs.

Urinary metabolite profiles of the individual patient are expected to reflect, at that particular moment in

time, the combination of all genetic and epigenetic influences. Metabolomics might be regarded as being similar to a “liver profile” test in clinical pathology, except that metabolomics includes measurement of metabolites present at much lower concentrations and, accordingly, provides several orders of magnitude greater sensitivity (femtomolar to attomolar range). Even though metabolite profiling can be performed only on available samples, such as blood, urine or other excreta, dissected tumor, or biopsy tissues, this method still holds promise of clinical success and might be regarded as an extension of the present practice of clinical pharmacology (123,136,138).

Clinical pharmacologists primarily have been using blood or urine to follow therapeutic responses for the past 60 years. This new form of metabolite profiling would resemble what clinical pharmacology has been doing previously, but would be several orders of magnitude more sensitive in detecting drug efficacy or risk of ADRs, perhaps long before these become clinically overt. Ultimately, it is anticipated that the combination of metabolomics, proteomics, and transcriptomics will complement genomics and epigenomics in revolutionizing individualized drug therapy.

## 19.6 CONCLUSIONS

1. The human genome is incredibly complex, far more than most of us could ever have imagined.
2. Phenotyping assays therefore remain superior to (DNA) genotyping assays in assessing most pharmacogenetic and pharmacogenomic disorders.
3. Statistical interaction is a population-level concept, whereas for the individual, we need to understand biological interaction or function; therefore, DNA variants showing statistical significance in a large cohort often have too small an effect size to predict in, or provide benefit to, the individual patient.
4. We categorize genes into three types—those associated with monogenic traits, hPpM disorders, and complex diseases.
5. The hPpM genes are principally responsible for changes in urine or plasma drug or metabolite levels, as well as therapeutic failure.
6. Drug efficacy and ADRs usually represent multifactorial traits that are conceptually not different from complex diseases.
7. One gene generally contributes 90–98% to a monogenic disease, whereas one hPpM gene might contribute 10–25% to an hPpM trait; in contrast, one gene might contribute 0.001–1.0% to multiplex phenotypes such as complex diseases, drug efficacy, or risk of ADRs.
8. GWA studies have been extremely useful in identifying genes participating in multifactorial traits; these might lead to the discovery of novel drug targets.

9. After more than 1300 GWA publications, we realize that the contribution of the total number of genes associated with any multiplex phenotype is between 5% and perhaps 60%. The remaining 40–95% is referred to as “missing heritability.”
10. It is likely that epigenetics is the main contributor to this missing heritability.
11. Epigenetics includes DNA methylation, RNAi processes, histone modifications, chromatin remodeling, and decanalization (also called CGV).
12. Urinary metabolite profiles in the individual patient are expected to reflect, at a particular moment, the combination of all genetic and epigenetic influences.
13. Transcriptomics, proteomics, and metabolomics might complement genomic (DNA sequence) assays in achieving improved success in personalized medicine, a large subset of which is individualized drug therapy.
14. Given all the complexity of the human genome, it is now easy to realize how difficult it would ever be to predict a common disease (*personalized medicine*) or to predict in advance “which drug and dose to give” (*individualized drug therapy*) to an individual patient.

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## FURTHER READING

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### Biographies



After college at Wesleyan University; Middletown, CT, Daniel W Nebert earned his combined MS degree (Biochemistry) and MD degree at the University Oregon Medical School in Portland. He then did his internship and residency in clinical pediatrics at the UCLA Medical Center (Los Angeles), before spending more than 20 years at the National Institute of Child Health & Human Development (NICHD), National Institutes of Health (NIH, Bethesda), where he was Chief of the Lab of Developmental Pharmacology (1975–1989). Joining the University Cincinnati Medical Center in December 1989, he became Professor in the Department of Environmental Health, with a joint appointment since 1991 as Professor of Pediatrics and Molecular & Developmental Biology, Division of Human Genetics. Nebert is board qualified in both Pediatrics and Human Genetics and has maintained a medical license in the states of California and Ohio. He has received many national and international awards including the coveted Bernard B. Brodie Award on Drug Metabolism given by the American Society of Pharmacology and Experimental Therapeutics (1986) and the Society of Toxicology's "Distinguished Lifetime Toxicology Scholar" Award (2005). He is currently the author and coauthor of more than 600 basic and clinical science publications, invited reviews, and book chapters.



Elliot S Vesell, MD, ScD, received a BA from Harvard College, magna cum laude, Phi Beta Kappa (1955); received his MD from Harvard Medical School, magna cum laude (1959); and interned at Massachusetts General Hospital (1959–1960). Dr Vesell was a Research Associate and Assistant Physician at Rockefeller University (1960–1962), and Assistant Resident in Medicine at Harvard's Peter Bent Brigham Hospital in Boston, MA (1962–1963). He was a Clinical Associate at NIH's NIAMD in Bethesda, MD (1963–1965), and then Head, Section on Pharmacogenetics, NHLI, NIH (1965–1968). In 1968, Dr Vesell was named the Founding Chair and Professor of Pharmacology of The Penn State University's new medical school, at the Penn State College of Medicine and Milton S. Hershey Medical Center, in Hershey, PA, his tenure as Chair lasted until 2000. He also served as Assistant Dean of Graduate Education from 1973 to 1995. Dr Vesell was selected in 1981 as Evan Pugh Professor, Penn State's highest honor bestowed upon a faculty member. He remains as Evan Pugh Professor Emeritus. Dr Vesell has received four national awards in clinical pharmacology and two honorary doctorates from major universities. He has published 335 peer-reviewed articles in the medical literature.

# CHAPTER

# 20

## Genetic Evaluation for Common Diseases of Adulthood

Maren T Scheuner and Shannon Rhodes

### 20.1 BACKGROUND

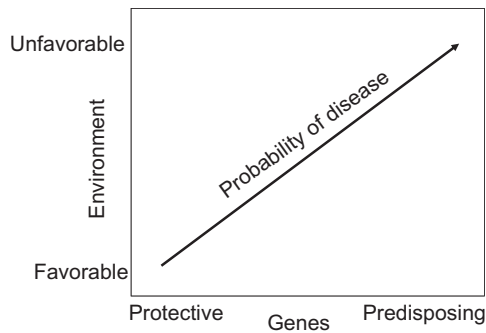
Common diseases such as coronary heart disease (CHD), stroke, cancer, and diabetes are high-prevalence diseases that have considerable impact on public health. Common diseases are typically chronic conditions that develop over decades, usually occurring in adulthood because of genetic and environmental risk factors, including exposures, infectious agents, and cultural and behavioral factors such as diet and exercise (i.e. multifactorial etiology) (Figure 20-1). Rarely, a common chronic disease of adulthood can occur as the manifestation of a Mendelian disorder, and *variable expressivity* and *reduced penetrance* are typical (1). *Genetic heterogeneity* is also characteristic of genes contributing to Mendelian forms of common diseases. For example, there are at least four genes known to cause Lynch syndrome, the most common form of hereditary colon cancer (2) and 12 genes are known that cause hereditary hypertrophic cardiomyopathy (3). *Pleiotropic effects* of genotypes contributing to common diseases can exist and must be considered when offering genetic testing. For example, methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms predispose to cardiovascular disease (4), neural tube defects (5), and colorectal cancer (6), and the apolipoprotein E genotypes contribute to serum cholesterol differences in the population and have been associated with cardiovascular disease (7) and Alzheimer disease (8).

Genetic tests are increasingly available for both Mendelian and multifactorial forms of common diseases. Genetic test results have the potential to impact health outcomes by improving our ability to diagnosis, treat, and prevent common diseases. To date, *multiplex testing* for common diseases has limited proven utility (9) and family history remains the best strategy for assessing genetic risk for these disorders (10). Family history is also key to identifying individuals at risk for Mendelian forms of common diseases (1).

Early disease detection and prevention are possible because of the chronic nature of common diseases, whether inheritance is Mendelian or multifactorial. Outcomes relating to genetic evaluation for common diseases can be characterized as psychological, cognitive, behavioral, or clinical (11). Results of genetic tests can be used to inform the shared clinical decision-making process between health care providers and patients and reproductive and personal decisions made by patients, such as decisions about career, finances, and marriage.

#### 20.1.1 Psychological and Cognitive Outcomes

Generally, psychological and cognitive outcomes related to genetic counseling and testing for common diseases are positive. Many studies have shown that genetic counseling for inherited susceptibility to breast cancer among women affected with breast cancer, and those at risk because of family history, did not increase psychological distress and resulted in some improvements in decreasing worry, anxiety, and depression (12,13). Studies assessing psychological or affective outcomes before and after genetic counseling in patients at risk for hereditary colon cancer reported improvements in satisfaction, knowledge, and reductions in worry (14,15). A randomized controlled trial on the disclosure of apolipoprotein E (ApoE) genotype to participants with a parent diagnosed with Alzheimer's disease found no difference in depression, anxiety, or distress in those who received genotype information compared to those who did not (16). Generally, even 1 year after ApoE genotype disclosure, participants in the study considered that the benefits of genetic testing outweighed the risks (17). A randomized controlled trial in adult non-diabetic offspring of diabetic parents reported that education and personalized prevention recommendations



**FIGURE 20-1** Both genetic and environmental factors contribute to the development of common diseases such as CHD, diabetes and cancers. Disease is least likely for individuals who have genotypes that are protective and who have favorable environmental exposures. Conversely, disease is most likely for individuals who have predisposing genotypes and unfavorable environmental risk factors. In industrialized cultures, unfavorable environmental factors such as inactivity, excess calories, processed foods, tobacco, alcohol, radiation and pollution are common with nearly everyone encountering them. Therefore, individuals who develop common chronic diseases, especially at young ages, are generally those who have the greatest genetic predisposition.

increased the accuracy of risk perception for diabetes without increasing worry (18).

### 20.1.2 Behavioral and Clinical Outcomes

A survey of family practice physicians found that they expect their patients to be more likely to get screened for cancer and make lifestyle and behavioral changes if genetic testing identified them as being at increased risk (19). However, a systematic review of 11 studies assessing behavioral responses to genetic risk information among individuals at risk for cancer found mixed results, with some studies showing no change in behavioral outcomes, whereas others reporting increased participation in cancer screening (20). A few studies have documented lifestyle changes after receiving genetic test results. Reduction in smoking was observed (although results were short lived) after receiving genetic test results that revealed information about cancer susceptibility related to smoking, (21,22) and a significant increase in dietary supplement use was observed in ApoE4 allele carriers at risk for Alzheimer's disease compared to noncarriers (23) despite the absence of scientific support for dietary supplements in preventing Alzheimer's disease (24).

Clinical outcomes, such as reduced incidence of disease after a recommended intervention, are less well studied. A study that assessed clinical outcomes in 251 women with *BRCA1* or *BRCA2* mutations reported that after counseling 15% and 50% underwent risk-reducing surgery for breast cancer and ovarian cancer, respectively, and four early-stage cancers were identified at surgery. Among women who did not undergo prophylactic surgery, recommended cancer screening increased (25).

## 20.2 THE PROCESS OF GENETIC EVALUATION FOR COMMON DISEASES

Clinical genetic evaluation for common disease should be considered for individuals with a strong familial risk or when a Mendelian disorder is suspected. Genetic evaluation is composed of several components including (1) genetic risk assessment and diagnosis through clinical assessment, family history assessment, and genetic testing; (2) appropriate recommendations for management and prevention given a genetic risk or diagnosis; and (3) genetic counseling and education.

### 20.2.1 Clinical Assessment

Assessment of signs and symptoms of the disease of concern should be performed to more accurately assess risk for the patient. For example, when evaluating a genetic risk for heart disease, review of systems should include questions regarding angina, shortness of breath, dyspnea on exertion, paroxysmal nocturnal dyspnea, pedal edema, palpitations, claudication, and exercise tolerance. In the case of risk assessment for colorectal cancer, questions should be asked regarding frequency of bowel movements, caliber and color of the stool, and presence of blood in the bowel movement. If symptoms are present, follow-up confirmatory testing should be recommended, for example, stress testing or echocardiogram to evaluate cardiovascular symptoms or colonoscopy to assess change in bowel habits or blood in the stool.

Physical examination should be performed to identify signs of the disease of concern as well as characteristic manifestations of Mendelian forms of a disease. For example, an evaluation for cardiovascular risk should include auscultation of the heart, lungs, and major vessels in the neck, abdomen, and groin and palpation of the aorta and distal pulses. Any abnormality can be followed up with additional studies, such as ultrasound. Blood pressure in the upper and lower extremities can identify hypertension, and these measurements can be used to calculate the ankle-brachial blood pressure index (ABI). Values  $<0.9$  are correlated with atherosclerosis. Weight and height should be obtained, and body mass index calculated to identify overweight and obese patients; follow-up measurements can help monitor diet and exercise interventions. Waist circumference should be obtained, as increased values are associated with the metabolic syndrome, a common cause of cardiovascular disease (26). Evaluation of possible lipid disorders should include examination of the eyes, assessing corneal arcus and lipemia retinalis, and examination of the skin for xanthelasma and tendinous xanthomas. Physical signs of Mendelian disorders that feature cardiovascular disease should be assessed, such as dolichostenomelia and arachnodactyly associated with Marfan's syndrome, abnormal scarring and translucent skin associated with Ehlers-Danlos syndrome type IV,



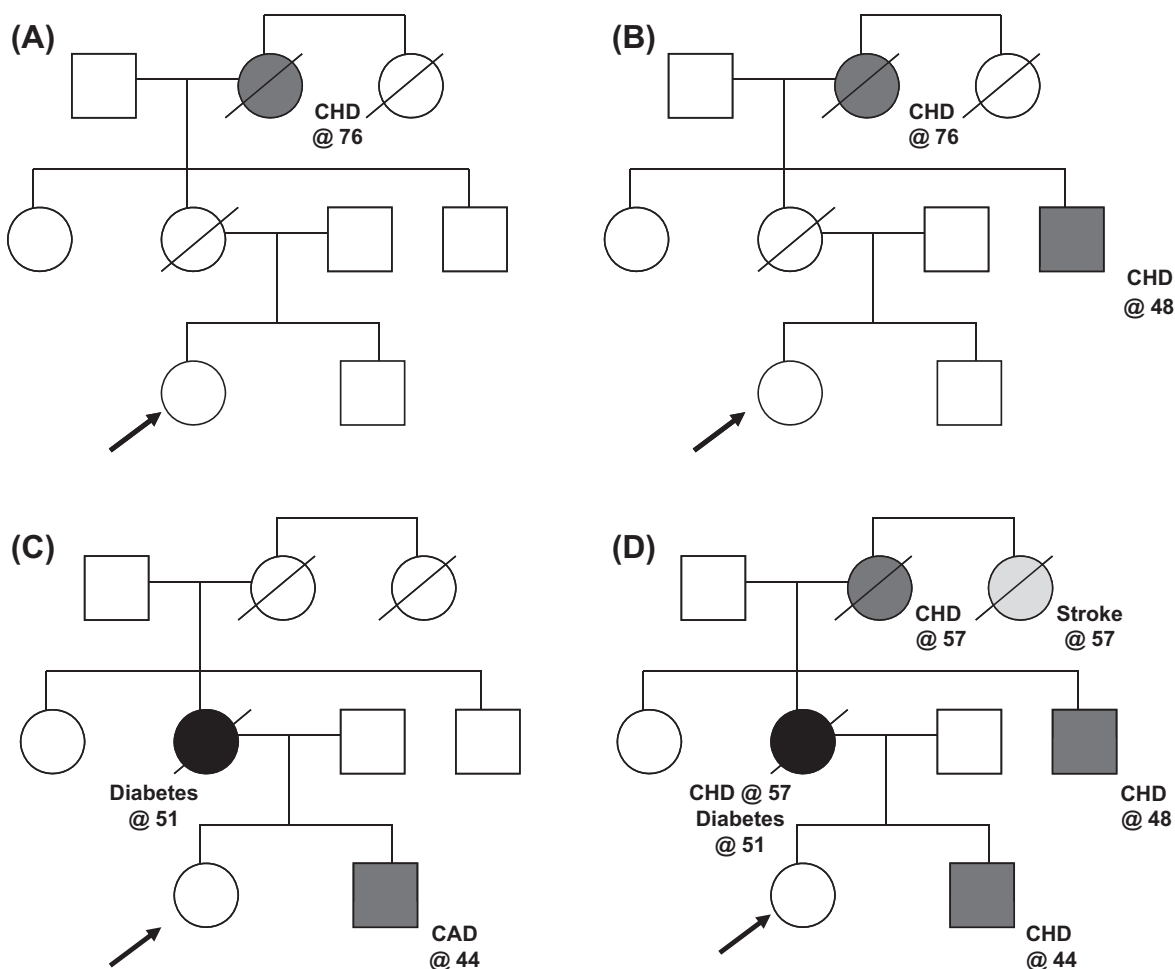
popular skin lesions and plaques in flexural creases and angioid streaks on the retina associated with pseudo-xanthoma elasticum, and angiokeratomas (vascular cutaneous lesions) and corneal and lenticular opacities associated with Fabry disease.

### 20.2.2 Family History Assessment

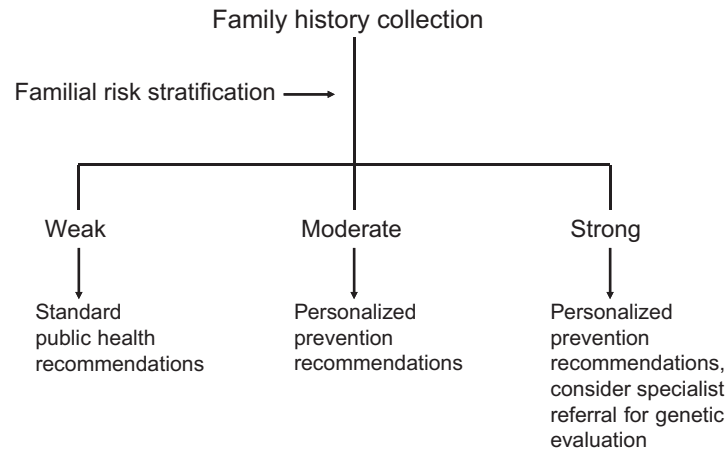
Currently, family history collection and interpretation is the most practical strategy for identifying individuals with a genetic susceptibility to many common chronic diseases (10,27). Family history represents complex interactions of genetic and nongenetic factors (e.g. exposures, diet, and behaviors) shared by family members. For many common diseases, a positive family history is quantitatively significant, with relative risks ranging from two to five times those of the general population, and this risk generally increases with an increasing number of affected relatives and earlier ages of disease onset (28–36). Family history characteristics that suggest

a possible Mendelian disorder include early age at diagnosis, two or more closely related relatives affected by a disease or a related condition, a single family member with two or more related diagnoses, multifocal or bilateral disease, and occurrence of disease in the less-often-affected sex (e.g. male breast cancer). By recognizing the magnitude of risk associated with these familial characteristics and patterns of disease consistent with known Mendelian disorders, stratification into different familial risk groups (e.g. weak, moderate, and strong) is possible (Figure 20-2), which can guide risk-specific recommendations for management and prevention (Figure 20-3). Referral for genetic evaluation by a geneticist or other specialist is appropriate for individuals with strong familial risk or a suspected Mendelian disorder.

An important step in family history assessment for common chronic diseases is pedigree analysis, which begins with the creation of the pedigree structure. This usually includes all first- and second-degree relatives and spans three to four generations. Demographic



**FIGURE 20-2** Each of the pedigrees are “positive” for a family history of CHD. However, the level of familial risk is different for each. In pedigree A, the consultand reports a maternal grandmother with CHD at age 76; her family history is a weak risk factor. In pedigree B, there are two second-degree maternal relatives affected with CHD; one diagnosed at an early age (48) and the other at a later age. This family history is more significant, but is likely only a moderate risk factor. Pedigree C is more concerning and consistent with a strong familial risk since a first-degree relative is affected with CHD at a young age. The report of diabetes in a first-degree relative in pedigree C may be related and is suggestive of the metabolic syndrome. The highest familial risk is seen with Pedigree D, which has three generations of early-onset CHD.



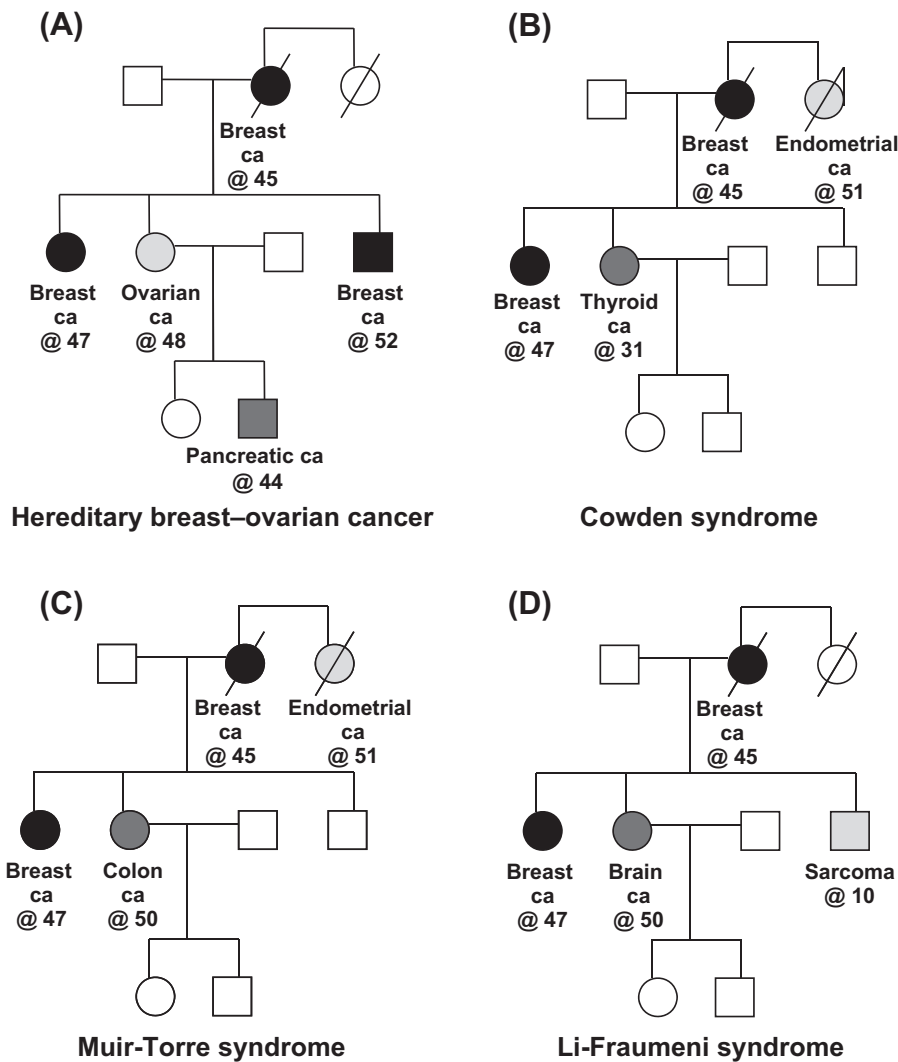
**FIGURE 20-3** Family history collection followed by risk stratification that recognizes characteristics that increase disease risk (e.g. early age at diagnosis, two or more close relatives affected by a disease or a related condition, a single family member with two or more related diagnoses, multifocal or bilateral disease, and occurrence of disease in the less-often-affected sex) can guide risk-specific recommendations for disease management and prevention. Standard public health messages would be appropriate for individuals with a weak familial risk and the absence of significant personal risk factors. Personalized prevention recommendations should be provided to individuals with moderate or strong familial risk, which should include emphasis of public health messages, consideration of earlier and more frequent screening, and use of chemoprevention when available. Referral for genetic evaluation by a geneticist or other specialist should be considered for individuals with strong familial risk or when a Mendelian disorder is suspected.

information for each family member is documented, which typically includes each relative's current age or age at death. Medical history is documented for each family member including age at diagnosis, cause of death if deceased, and known interventions or procedures, which can help clarify a diagnosis. For example, questioning regarding coronary artery bypass surgery, angioplasty, heart transplant, or pacemaker placement may help clarify a relative's diagnosis of heart disease. Information is also collected regarding important risk factors for a disease, such as the use of hormone replacement therapy in the case of endometrial cancer, chest irradiation in the case of breast cancer, and smoking, asbestos exposure, and coal mining in the case of lung cancer. Medical records are reviewed when possible to verify the medical history of each family member or at least those who are critical to the genetic risk assessment and diagnosis. The family history should include ethnicity and country of origin of grandparents, as certain conditions might be more prevalent in certain groups. For example, the prevalence of insulin resistance is high among individuals of Native American admixture (37) and Asian Indian origin (38) and there are common *BRCA* gene founder mutations in Ashkenazi Jewish families with breast and ovarian cancer (39).

Once family history is collected, the most likely mode of inheritance (i.e. Mendelian vs multifactorial) and the risk of disease to the patient and to the unaffected relatives is determined based on their position in the pedigree. When a Mendelian disorder is suspected, this analysis also helps to elucidate a differential diagnosis through pattern recognition (1). For example, when considering an inherited form of breast cancer, there are at least five different Mendelian disorders to consider,

including hereditary breast–ovarian cancer syndrome, Cowden syndrome, Peutz–Jeghers syndrome, Li–Fraumeni syndrome, and Muir–Torre syndrome. The types of cancers and other conditions reported in the family help to distinguish each of these syndromes (Figure 20-4). Mutations in different genes underlie the genetic susceptibility of these syndromes, and genetic testing can help to confirm a suspected diagnosis. For certain hereditary cancers, models are available to help predict the probability of an inherited gene mutation (40–42). For pedigrees that lack convincing evidence of Mendelian inheritance and are more consistent with multifactorial inheritance, quantitative risk information can be provided for some conditions through the use of mathematical models or published estimates that consider the family history (43–48).

Accuracy of the family history data is paramount particularly if clinical decisions will depend on the information. Many studies have shown that self-reports of family health history are relatively accurate for many common chronic conditions, such as CHD, stroke, diabetes, and many forms of cancer. Most positive predictive values for self-reports of a family history of these conditions in a first-degree relative range from 70 to 90% and negative predictive values are usually 90% or greater (49–51). These values depend on the type of disease, age of the historian, and distance of the relative from the *consultand*. Therefore, before clinical decisions are based on such information, confirmation of family health histories is advisable. Currently, such confirmation is handled by health professionals who request and review medical records, pathology reports, and death certificates of family members to verify self-reports. This can be time consuming and costly (52).



**FIGURE 20-4** Each pedigree depicts a strong familial risk of breast cancer. However, by recognition of the patterns of cancer in the family, a more accurate diagnosis can be made. Pedigree A features early-onset breast and ovarian cancer and is most consistent with hereditary breast-ovarian cancer syndrome, which is almost always due to *BRCA1* or *BRCA2* gene mutations. In this case, a *BRCA2* gene mutation is likely given the family history of male breast cancer and pancreatic cancer. Pedigree B features early-onset breast, thyroid and endometrial cancer and is most consistent with Cowden syndrome due to *PTEN* gene mutations. Pedigree C features early-onset breast, colon and endometrial cancer and suggests the possibility of Muir-Torre syndrome, a variant of hereditary nonpolyposis colon cancer due to mutations in mismatch repair genes. The early-onset breast cancer, brain tumor and childhood sarcoma in pedigree D is consistent with Li-Fraumeni syndrome due to *TP53* gene mutations. Multiple primary cancers are common among individuals with Li-Fraumeni syndrome.

### 20.2.3 Genetic Testing

Genetic testing for common diseases in clinical practice is generally limited to testing for Mendelian disorders (53). Indications for genetic testing of Mendelian disorders that feature common chronic diseases can be characterized as *diagnostic*, *prognostic*, and *predictive* of disease risk and therapeutic response (i.e. *pharmacogenetic* testing). A particular indication for genetic testing can correspond to multiple reasons for testing (Table 20-1). For example, diagnostic testing in a patient with signs and symptoms of disease may be performed primarily for medical decision making, yet it may also inform a patient's reproductive or life-planning decisions.

Diagnostic tests are typically performed to confirm a diagnosis suspected on the basis of signs or

symptoms of the condition. Diagnostic tests can also include those used for *prenatal* and *preimplantation diagnosis*. Testing for Mendelian disorders can sometimes provide prognostic information on the basis of known genotype-phenotype correlations. For example, *SOD1* gene testing can reveal information about disease progression in familial amyotrophic lateral sclerosis (54) and certain *APC* gene mutations are associated with colon polyp number and extracolonic manifestations of familial adenomatous polyposis (55). Testing for somatic genetic changes in tumor tissue is another type of prognostic genetic test; results can inform risk for recurrence and treatment options (56,57). Predictive genetic testing for common diseases is performed in asymptomatic individuals often at risk because of

a family history of Mendelian disorders of high penetrance (*presymptomatic testing*) or reduced penetrance (*predisposition testing*). Pharmacogenetic testing is another type of predictive genetic testing; it is done to predict an individual's response to a drug, including adverse reactions. Predictive testing for multifactorial conditions consisting of multiplex testing of low-penetrance alleles (*susceptibility testing*) is generally provided through direct-to-consumer marketing (58). This is likely due to limited progress in the discovery of genes for multifactorial forms of common diseases that have meaningful clinical relevance (9). Very little is understood regarding the interactions between genetic and nongenetic risk factors underlying multifactorial diseases, and attempts to build genetic risk models have failed to show that additional genetic information can substantially improve the prediction of risk for common diseases, such as diabetes or cardiovascular disease

(59,60), and evidence for improved health outcomes is lacking (61).

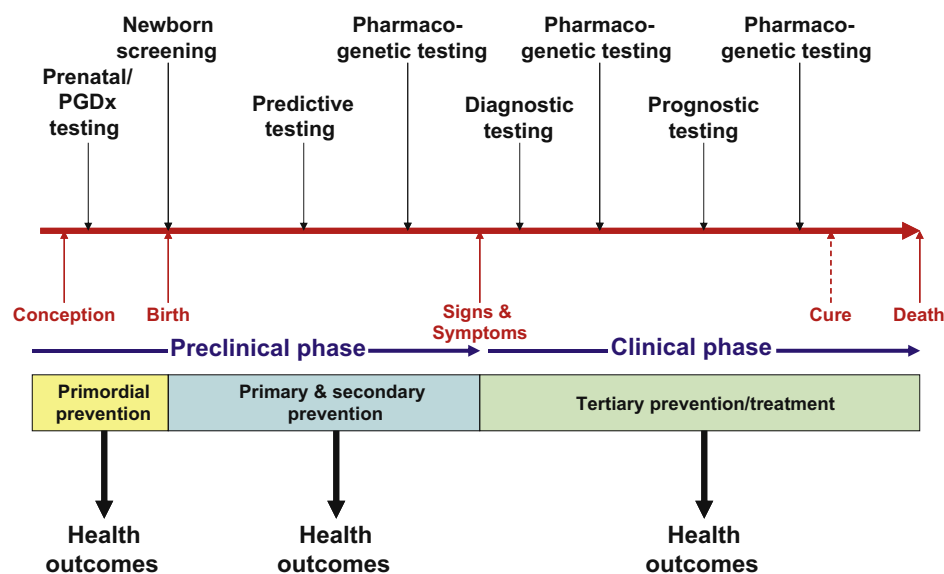
Aside from *newborn screening*, currently, genetic testing for common diseases of adulthood has a role throughout the lifespan (Figure 20-5). Most individuals undergo genetic testing for common diseases to diagnose Mendelian disorders or to learn of a risk of Mendelian disorders. Prenatal and preimplantation genetic diagnosis is less often performed for Mendelian disorders that feature common chronic diseases, mostly because of reduced penetrance, variable expressivity, adult onset, and the opportunities for prevention. Pharmacogenetic testing may be performed before or after signs and symptoms of disease occur. Carrier testing is usually performed for autosomal recessive and X-linked recessive disorders, which are not typical modes of inheritance for most common diseases (1).

The ACCE (analytic validity, clinical validity, clinical utility and ethical, legal, and social issues) framework is

**TABLE 20-1** Intended Uses of Genetic Testing Performed for Various Indications

	Medical Decision Making	Reproductive Decision Making	Personal Decision Making
Diagnostic (confirmation)	X	X	X
Presymptomatic/predisposition	X	X	X
Susceptibility	X		
Pharmacogenetic	X		
Prognostic	X		X
Carrier testing		X	
Prenatal	X	X	
Preimplantation		X	

There may be multiple intended uses of genetic testing performed for various indications, including medical, reproductive, and personal decision making. Medical decision making includes decisions regarding disease management (in fetus too), screening for early detection or prevention. Reproductive decision making includes decisions regarding conception/timing of conception, adoption, gamete donor, and pregnancy termination. Personal decision making includes decisions regarding life, career, and financial planning (does not meet the "medical necessity" criteria of most insurers).



**FIGURE 20-5** Currently, genetic testing for common diseases of adulthood has a role throughout the lifespan, including testing that can impact primordial, primary, secondary, and tertiary disease prevention. Predictive testing includes presymptomatic testing for highly penetrant Mendelian disorders, predisposition testing for Mendelian disorders with reduced penetrance, and susceptibility testing for common diseases due to multifactorial inheritance.



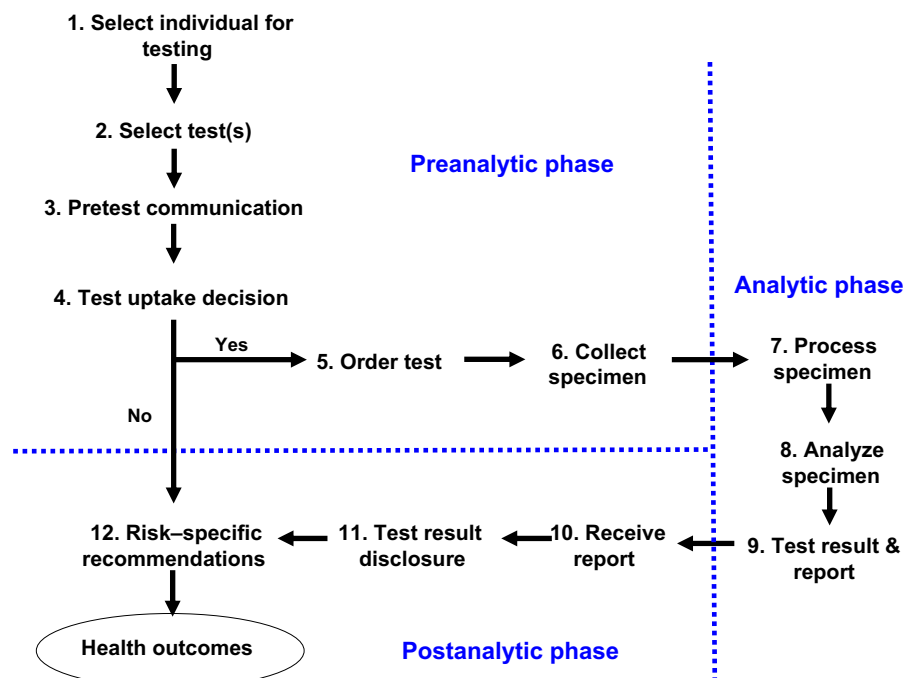
commonly used to evaluate genetic tests for common diseases (62). *Analytic validity* refers to how accurately and reliably the test measures the genotype of interest, *clinical validity* measures how consistently and accurately the test detects or predicts the intermediate or final outcomes of interest related to a genotype, *clinical utility* is a measure of how likely the test result will influence health outcomes, and the *ethical, legal, and social implications* that should be addressed are those that may arise as a result of genetic testing, such as loss of privacy, confidentiality, stigmatization, and discrimination. The disorder, setting, and clinical scenario are important considerations when applying the ACCE framework. The clinical scenario should include the purpose and indication for testing.

A framework depicting the steps involved in the delivery of genetic tests according to the preanalytic, analytic, and postanalytic phases of the genetic testing process is shown in Figure 20-6. In general, all steps should occur for any clinical scenario. The actions performed for each step may vary, depending on the genetic testing indication. The process begins with patient selection (Step 1), which will often depend on personal and family history characteristics. With this information, a differential diagnosis is developed, and in the case of predictive testing, risk assessment is provided usually based on the suspected mode of inheritance and position in the pedigree. Thus, use of family history is central to providing access to genetic testing services that are currently available, and it is likely that the paradigm of familial risk assessment will inform future genetic testing of less-penetrant susceptibility alleles. Therefore,

family history will likely remain a relevant genetic tool for many years to come (63).

Step 2 consists of identification of appropriate tests that may be helpful in confirming a genetic diagnosis and refining genetic risk. When considering genetic testing, clinicians should choose the testing strategy that would be most informative for patients and their family members. Ideally, genetic testing should begin in an affected family member to identify the specific genetic determinant(s) of disease. If an abnormality or abnormalities are identified, then at-risk relatives can be tested for those familial factors. Excluding these familial factors in at-risk relatives can provide reassurance regarding disease risk due to the family history; however, there will always be a background or population risk for disease development. It is not always possible to test an affected family member. In this case, unaffected family members may participate in testing. A normal test result can exclude the genetic risk factors that have been tested but not the possibility of an inherited susceptibility. In such a case, medical management would typically depend on the empirical risks associated with the personal and family histories. To further clarify the situation, testing additional family members, both affected and unaffected, may be helpful.

Pretest communication about testing follows in Step 3 and often includes an informed consent process that describes the potential benefits, risks, and limitations of genetic testing, as well as the alternatives to testing (see Section 20.2.5). In Step 4, a shared decision between patient and provider is made regarding genetic testing. The provider then identifies a laboratory that



**FIGURE 20-6** A framework depicting the delivery of genetic tests according to the preanalytic, analytic and postanalytic phases of the genetic testing process is shown. The specific actions of each step will vary, depending in large part on the indication for genetic testing. Yet in general, all steps should occur for any given clinical scenario.

can perform the testing and orders the test in Step 5, providing necessary information to the laboratory on the test requisition to facilitate selection of the appropriate test method and interpretation of results. Most genetic tests are performed in reference laboratories, therefore sample processing and shipping is often necessary (Step 6). Issues to consider when choosing a laboratory include methodology, analytic sensitivity and specificity, technical support, cost, and turnaround time. Understanding which methodology is used is important because standards for many genetic tests currently do not exist. There is great variability in testing procedures for many conditions. As a result, clinicians need to be familiar with a given laboratory's protocol and the test limitations.

The analytic phase includes Steps 7–9. Genetic tests for assessing common diseases are typically DNA-based tests. Most commercial laboratories performing DNA-based testing use polymerase chain reaction (PCR)-based methods to amplify the patient's DNA to be followed by sequencing or other methodologies. There are limitations to each method, and the method chosen often depends on the testing indication and purpose. Rarely, errors may occur with sample handling, contamination by airborne particles in the laboratory, or failure of the PCR technique. In most cases, sequencing is considered the gold standard for identification of an unknown mutation. However, sequencing can miss rearrangements or large deletions or duplications of DNA (64,65) or mutations affecting gene expression (66). Although successes of whole genome or whole exome sequencing are being documented (67,68), the application of these technologies to common disease is complex even in the presence of a recognized family history (69). The key challenges for bringing these technologies to the clinic are the collection and characterization of phenotype information, the incorporation of the influences of environmental and behavioral factors, and the creation of standards for utilizing this information in clinical practice (70–72).

When the genetic test results are available, the laboratory provides the ordering clinician with a test report (Step 10). A posttest consultation typically follows to review the implications of the test result for both the patient and the family members (Step 11). The genetic test results should be interpreted within the context of the individual's personal and family histories and the indication and purpose for testing, and recommendations for disease management and prevention should be specific to the risk assessment and diagnosis (Step 12).

### 20.2.4 Management and Prevention Strategies

Knowledge of genetic susceptibility to common diseases may identify important biologic differences that could lead to better disease management and prevention through the use of targeted therapies and enhanced

screening and prevention strategies. Prevention strategies for common chronic diseases include targeted lifestyle changes; screening at earlier ages, more frequently and with more intensive methods than used for average risk individuals; use of chemoprevention; and, for those at highest risk, prophylactic procedures and surgeries.

For many Mendelian disorders that feature common chronic conditions, specific guidelines for initial evaluation, management, and follow-up for affected persons and surveillance for at-risk relatives are available. However, most of these guidelines are based on clinical observation and expert opinion, and outcomes research that assesses their clinical utility is needed. Recommendations for screening and prevention based on evidence derived from clinical studies exist only for a minority of Mendelian disorders, including hereditary breast and ovarian cancer (73–75) and colon cancer (76).

In the absence of clinical guidelines for common chronic diseases associated with Mendelian disorders, clinicians can suggest management and prevention strategies that have been proved to be effective for the general population. Such guidelines exist for CHD (77), stroke (78), diabetes (79), and cancer (80,81). However, clinicians must proceed with caution, because interventions that are effective for the general population might not be effective for individuals with a genetic predisposition. For example, tamoxifen is associated with a significant reduction in the occurrence of breast cancer (82); however, women with *BRCA1* gene mutations do not appear to benefit from the use of tamoxifen for primary prevention of breast cancer (74).

Recommendations for management and prevention options for an individual with genetic risk should be communicated in writing so that the patient and the referring clinician can incorporate these recommendations into a plan for future health management. Family members who might benefit from risk assessment and genetic testing should be identified, and the clinician should facilitate communication between the index case and at-risk relatives and provide referrals to genetic professionals or other specialists for relatives. Plans for follow-up with a genetic professional may be appropriate to review the individualized plan for management of genetic risk, and updated personal and family history information can be obtained with revision of the management and prevention plan as indicated. New information and technology available for genetic risk assessment or management and prevention of disease can also be discussed.

### 20.2.5 Genetic Counseling and Education

Genetic counseling is critical for delineating a patient's motivation for genetic evaluation and likely responses to learning of a genetic risk or diagnosis. An important goal of genetic evaluation for common chronic diseases is the development of individualized management and preventive strategies based on genetic risk assessment and the patient's personal medical history, lifestyle, and

preferences. Through genetic counseling, patients will be educated about the role of genetic and nongenetic risk factors for disease; basic concepts of genetics, such as inheritance patterns, penetrance, and variable expressivity; and the options for treatment, prevention, or risk factor modification tailored to the genetic risk or diagnosis.

How genetic risk factor information may influence behavior is complex and may depend on the condition of concern, the contribution of the genetic factor to risk or severity of disease, and personal beliefs about the role of genetics (83). There is evidence that awareness of increased risk due to family history does not automatically translate to spontaneous improvement in lifestyle choices. For example, in young adults, the occurrence of a heart attack or stroke in an immediate family member did not lead to self-initiated sustained change in modifiable risk factors (84) and among low-income, rural African-American women who had not had a recent mammogram, knowledge of family history of breast cancer was not associated with perceived risk or screening (85). These results suggest that consumers may not appreciate how genetic and familial risk factors relate to their disease risk, or they may not be aware that despite a genetic predisposition there are actions that they can take to lower their risk. Genetic counseling and education may be necessary to actively intervene in people with a genetic susceptibility to common chronic diseases.

Genetic counseling also ensures the opportunity to provide informed consent, including discussion of the potential benefits, risks, and limitations of testing; implications for family members; and the alternative of not testing. A benefit in knowing of a genetic predisposition through genetic testing is the potential to improve diagnosis, management, and prevention efforts for individuals and their family, and there may be psychological and cognitive benefits as well (12–15). Potential risks relate to family issues that may arise with genetic testing for common chronic diseases (86). Family members may experience loss of privacy when asked to share their medical history and medical records, and if labeled as having a genetic predisposition to disease, family, friends, or society may stigmatize them. Family dynamics may change from the knowledge of a genetic risk for disease. For example, a parent may feel guilt about passing on a disease predisposition or a sibling may experience survivor guilt if a genetic susceptibility is excluded for them but identified in another sibling. There is also the concern that third parties such as employers, educators, and insurers could use genetic information to exclude individuals from employment or education opportunities or from obtaining health, life, long-term care, or disability insurance, and this concern has had a negative impact on the utilization of genetic services (87) despite the lack of evidence of genetic discrimination against otherwise healthy individuals (11). It is hoped that the Genetic Information Nondiscrimination Act (GINA) of 2008, (88) which provides new legal protections from

genetic discrimination by employers and health insurers, will mitigate some of these concerns.

### 20.3 INTEGRATING GENETIC INFORMATION INTO ROUTINE CLINICAL PRACTICE

Attempts to integrate genetic/genomic knowledge of common chronic conditions into routine clinical practice are still in the early stages. The primary care setting is ideal for initial genetic risk assessment. Primary care clinicians play a major role in assessing risk factors and providing preventive services. The role of primary care providers in providing genetic services may vary depending on their knowledge and comfort with the steps involved (Figure 20-6). But in general, primary care should be prepared to identify patients who may benefit from genetic services, provide basic genetic information to facilitate the referral process for patients with complex genetic services needs, recognize the special psychosocial issues for a family with a genetic condition and coordinate care and monitor the health of patients with genetic conditions (89).

Unfortunately, primary care clinicians lack knowledge and confidence relating to provision of genetic services, including assessment of familial risk, ordering and interpreting genetic tests, and recommending referral and risk-specific interventions (90). Significant barriers relate to the use of the family history, including clinicians' lack of time and perceived lack of knowledge and skills to collect and interpret family history and lack of sufficient evidence on how to perform familial risk assessment and the effects of familial risk on clinical outcomes (91,92). These barriers could be overcome in large part through the use of family history tools (e.g. stand-alone tools or applications integrated into electronic health record and personal health record systems) that have the capabilities to facilitate collection of relevant personal and family health history in a structured format, organize the data in a usable form such as a graphic display following pedigree drawing standards, interpret the familial risk and recognize patterns of familial disease suggestive of inherited susceptibilities (i.e. pedigree analysis), and recommend interventions that are tailored to the familial risk and personal factors. However, most electronic health records have not integrated family history in such a way as to meet the needs of clinicians (93). Data elements and functionality requirements that clinicians would like to see include pedigree drawing, clinical decision support for familial risk assessment and genetic testing indications, a patient portal for patient-entered data, and standards for data elements, terminology, structure, interoperability, and clinical decision support rules.

Results of a national survey show that most Americans believe that knowing family history is very (73%) or somewhat (24%) important to their personal health, yet only a minority actively documents their family history (94). Efforts such as the Surgeon General's Family History

Initiative have begun to raise awareness among the public and health professionals about the value of family history (95). In 2004, the US Surgeon General declared Thanksgiving as the National Family History Day and encouraged Americans to collect their family members' health histories and share the information with their health care providers. A web site was created with educational materials as well as a family history data collection tool. A validation study found that the Surgeon General's family history tool has high sensitivity and specificity for family history documentation, with values ranging from 67 to 100% and 92 to 100%, respectively (96). In addition, familial risk assessment conducted by experts using the data obtained from the tool vs data obtained by the experts were identical in 94–99% of the pedigrees with a history of diabetes and colon, breast, and ovarian cancers but were lower for pedigrees with coronary artery disease (68%) and stroke (83%). Family Healthware is another web-based tool developed by the Centers for Disease Control and Prevention (97). Family Healthware collects family health history and information about preventive behaviors, estimates familial risk for six common diseases, and provides users with individualized prevention messages that incorporate their familial risk assessment. This tool was assessed for impact on health behaviors in a randomized trial and demonstrated modest beneficial effects on dietary and physical activity behaviors (98).

## 20.4 SUMMARY

Genetic factors play an important role in the etiology, natural history, and response to therapy for many common diseases. Knowledge of the genetic factors contributing to common diseases can provide the basis for recommending alternative disease management and enhanced preventive strategies. Currently, the family history is the best initial approach for genetic risk assessment of most common chronic diseases. Genetic testing can then be used to further refine the genetic risk assessment, diagnosis, and management decisions. Primary care clinicians are best suited to identify patients with increased familial risk, with referral of high-risk cases or those suspected of having a Mendelian disorder. However, barriers for primary care include the lack of time and the perceived lack of knowledge and skills to collect and interpret family history. Electronic family history tools have the potential to mitigate these barriers. More translation research is needed to develop the evidence base necessary for integration of genomic medicine into the routine health care for common diseases.

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# CHAPTER

# 21

## Genetic Counseling and Clinical Risk Assessment

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### 21.1 WHAT IS GENETIC COUNSELING?

#### 21.1.1 Evolving Definitions

A discussion of what constitutes genetic counseling quite naturally begins with the definition posed by the person who coined the term in 1947, Dr Sheldon Reed. Dr Reed, who held a PhD in human genetics, stated that “The primary function of genetic counseling is to provide people with an understanding of the genetic problems in their family” (1,2). As the director of a hereditary disease clinic at a time when being able to test for or treat a genetic disease was the exception rather than the rule, it makes sense that his definition focused on the social rather than medical aspects of the encounter (3). He recognized the importance of providing families with accurate information to promote understanding. However, he also appreciated the myriad of emotions generated by genetic diagnoses, and the necessity of addressing these (4). Furthermore, the definition, with its focus on people, rather than populations, distanced genetic counseling from both eugenics and a public health model of service provision.

Since this first description of genetic counseling, a number of definitions have been developed by individual practitioners and professional organizations to reflect the evolution of genetic services. In 1975, the American Society of Human Genetics (ASHG), whose members include researchers, medical geneticists, clinical geneticists and genetic counselors, defined genetic counseling as “...a communication process which deals with the human problems associated with the occurrence or risk of occurrence of a genetic disorder in a family.” The definition states that “appropriately trained persons” help individuals or families “comprehend medical facts,” “appreciate

the way heredity contributes to the disorder and the risk of recurrence,” “understand the alternatives for dealing with the risk,” choose their own course of action, and “make the best possible adjustment” (5). This definition, similar to Reed’s, emphasizes the importance of information in helping clients understand and cope with a genetic condition or risk. The definition adds a statement about the importance of informing families about their options for managing genetic risk. These options, including prenatal diagnosis and the legal right to terminate a pregnancy, were largely not available in 1940s and 1950s. The definition highlights the importance of letting clients make their own decisions regarding these alternatives, further distancing clinical genetics from its eugenic roots.

In the years following the publication of the ASHG definition, genetic counseling services have expanded into new arenas. Genetic counseling is occurring in settings beyond reproductive and pediatric genetics clinics. Increasingly, family medical history and genetic testing can be used to identify individuals at increased risk for common, adult-onset diseases. For some of these, identification of genetic risk guides clinical management strategies that can reduce morbidity and mortality. Such situations require that the clinician takes a more active role in promoting informed decision making. In response to these changes in practice and an identified need to clarify the goals of genetic counseling, in 2003, the National Society of Genetic Counselors (NSGC) convened a Definition Task Force. After a thorough review of existing definitions, the Task Force concluded that a new definition of genetic counseling was needed. The following definition, which was developed with input from the NSGC membership and a wide variety of stakeholders, was adopted by the NSGC Board of Directors in 2005 (6).



“Genetic counseling is the process of helping people understand and adapt to the medical, psychological, and familial implications of genetic contributions to disease. This process integrates the following:

- Interpretation of family and medical histories to assess the chance of disease occurrence or reoccurrence.
- Education about inheritance, testing, management, prevention, resources, and research.
- Counseling to promote informed choices and adaptation to the risk or condition (6).”

The NSGC definition adds to the previous definitions in important ways. First, it uses the phrase “genetic contributions to disease” in recognition of the expanded role of genetic counseling in complex (multifactorial) diseases (6). Second, it relates that education in genetics extends to prevention. Prevention is a word that is appropriately avoided when genetic counseling is discussed in the context of reproductive decision-making; however, it is a critical component of the educational process in cardiovascular genetics, cancer genetics, and other preventable adult-onset diseases. Despite these additions, the Reed, ASHG, and NSGC definitions are similar in two important ways. They all emphasize the importance of helping people understand and adapt to the genetic information.

### 21.1.2 Goals and Approaches to Genetic Counseling

As the above review of key definitions has shown, despite big changes in the scope of medical genetics services over the past 60 years, promoting understanding and adaptation remain fundamental goals of genetic counseling. All three definitions allude to or specifically state the importance of education in achieving these goals. The NSGC definition is the only one that explicitly states the role of counseling (6). However, its role is implied in Reed’s description of genetic counseling as a “kind of genetic

social work” (1). Furthermore, it may be implied in ASHG’s description of genetic counseling as a “communication process” dealing with “the human problems” associated with genetic conditions (5).

Joan Marks, director of the first genetic counseling program at Sarah Lawrence College, recognized early in the program’s history that “The hallmark of training genetic counselors is based on the premise that understanding the emotional component of genetic risk is central to providing good genetics services” (7). To prepare her students to accomplish this task, she developed a counseling curriculum to complement the science one. She used Carl Rogers’ Client-Centered Therapy, adopted from mental health counseling, with its emphasis on the counseling relationship, facilitative conditions (empathy, unconditional positive regard, genuineness), and a non-directive approach as theoretical basis for a course on interviewing (7,8). As such, elements of Client-Centered Therapy, later named Person-Centered Therapy, were incorporated into genetic counseling practice.

Others have since proposed models specific to genetic counseling. Seymour Kessler described the education (teaching) and the counseling models of genetic counseling as two distinct approaches with differing goals (9). Table 21-1 describes the goals, assumptions, tasks and relationships in each model. His impression was that genetic counselors “... attempt to combine aspects of both approaches with, I suggest, limited success, since the fundamental philosophies and professional demands of each seldom mesh ...” (9).

The counseling model, as described by Kessler, seems more flexible and to provide a more personalized approach to genetic counseling. In addition, counseling patterns of interaction may be associated with greater client satisfaction (10). Nonetheless, studies investigating the use of both approaches show that the teaching model predominates (10,11). There can be significant variation between genetic counselors in terms of the proportion of teaching

**TABLE 21-1 Comparison of the Genetic Counseling Teaching Model and Counseling Model**

	Teaching Model	Counseling Model
Goals	Educated clients	Understand the client, bolster sense of competence, promote greater sense of control, support, relieve psychological distress, help client find solutions
Perception	Clients seek information	Clients come for genetic counseling for complex reasons (information is only one of these)
Assumptions	Informed clients should be able to make own decisions	Complex assumptions about human behavior and psychology
Priorities	Cognitive and rational processes emphasized over human behavior and psychology	
Counseling task	Provide balanced, impartial information; correct misinformation	Counseling tasks are complex and require assessing client situation, using a range of counseling skills, individualizing sessions, and self-awareness/health
Relationship with client	Based on authority, counselor as teacher	Mutuality
Role of education	Education is an end in itself	Education is used as a means to achieve goals

Adapted from Kessler S (9).

versus counseling strategies used in a session. Furthermore, individual counselors may have a preferred style, which they use independent of client characteristics (12).

Having a clearly defined model of practice is important because it describes how a service is delivered and why it is delivered in this way. It incorporates the commonly held principles and beliefs of its practitioners (tenets), the goals of the clinical activity, and the strategies and behaviors (techniques) used to meet those goals (13). Importantly, it also provides a framework for evaluating the effectiveness of the activity through research. Many have advocated for the genetic counseling profession to develop its own specific model or models of practice. With the increased emphasis on evidence-based medicine, having a testable model (or models) that can be used to evaluate what works, what does not work, which strategies are essential and which may be more situation-dependent is of critical importance to the field.

Recognizing the value of a practice model, McCarthy Veach et al. (13) convened a group of North American genetic counseling program directors for the purpose of describing explicitly the current model of genetic counseling practice. The group was charged with describing the tenets, goals, strategies and behaviors for addressing clients' genetic concerns and to identify variations in the model related to genetic counseling indications or client cultural background. Using a consensus building process, the group identified five tenets. For each tenet, 3–4 goals were described. Some goals were related to the process of genetic counseling—the conditions that must be met for successful outcomes. Some goals were related to the outcomes (end results). The five tenets are as follows: information is key; relationship is integral to genetic counseling; patient autonomy must be supported; patients are resilient; patient emotions make a difference.

The tenets and goals identified through the above activity were developed into a model of practice called the reciprocal engagement model (13). The genetic counselor–patient relationship is the central component of the model because it is through this relationship that education occurs, individual attributes (supporting patient autonomy, patient resilience and emotions) are considered, and genetic counseling goals are met. The model is reciprocal because each component affects the others. The word engagement is used to stress the importance of both the patient and the genetic counselor participating in the process.

There are limits to the proposed reciprocal engagement model and it is still a work in progress. However, the development and implementation of such a model is a critical step in evaluating the effectiveness of genetic counseling strategies for all who provide genetic counseling.

### 21.1.3 What About Nondirectiveness?

Nondirectiveness has been a component of genetic counseling for several decades and in the past, it has been

described as its central ethos or guiding principle. The purpose of nondirectiveness was to distance genetic counseling from eugenics and to put decision-making authority into clients' rather than practitioners' hands. However, over the years, there have been numerous critiques of nondirectiveness. One critique is that there are a number of different definitions, some broad and some narrow, the use of which may have different practice implications. Another is that a nondirective approach may result in providers failing to adequately address the moral and ethical issues that clients face in making decisions about genetic information. Furthermore, the approach might deter a counselor from actively engaging with clients in exploring their feelings, perceptions, values and beliefs and how these factors affect the decisions to be made. The third critique is that although aspects of nondirectiveness may be applicable to reproductive genetic decision making, they may not apply in areas such as cancer or cardiovascular genetics. Fourth, some consider nondirectiveness as a model of practice, which it is not, and this may hinder the development of real practice models (14). Such critiques and a growing body of literature have led genetic counselors to question the role of nondirectiveness in the genetic counseling process.

In 2003, at the Annual Education Conference of the NSGC, a workshop was held to discuss the relationship of nondirectiveness to genetic counseling (15). Participants identified the value of nondirectiveness including its historical relevance, value as an ethical stance, and its use to promote client autonomy. They also identified several limitations, similar in scope to the ones listed above. On the basis of the discussion, the workshop leaders made several suggestions. First, they indicated that it is important to clarify what the role of nondirectiveness is and what it is not. It may be a component of the ethical basis of practice that promotes client autonomy and encourages counselors to pay attention to the myriad of factors that have an impact on client decision making. However, it is not a theory of practice. Second, genetic counselors must use "... flexible, interactive, skill-based counseling ..." to be effective (15). They should not let narrow definitions of nondirectiveness prevent them from doing so. Third, as genetic counseling expands into new practice settings, it will be important to identify whether traditional genetic counseling approaches work or whether new approaches are needed. The workshop leaders closed with encouraging genetic counselors to evaluate practice models that other professions have used to successfully integrate psychosocial and medical information.

In summary, there are still elements of nondirectiveness that remain important to genetic counseling, namely promoting patient autonomy and attaching importance to clients' values over genetic counselors'. These elements are particularly important in settings such as reproductive genetics. However, the term nondirectiveness, without a clear definition, should not be the guiding

principle of genetic counseling. Furthermore, it is not a model of practice; it may deter genetic counselors from fully engaging with clients, and may not work well in some practice settings. Efforts to better define the guiding principle(s) and models of practice of genetic counseling are underway. These efforts have the potential of enhancing the effectiveness of genetic counseling in a way that nondirective approaches cannot.

### 21.1.4 Indications for Genetic Counseling

With the expansion of genetic testing and the understanding of genetic factors in common diseases, the indications for genetic counseling services have grown. Genetic counseling remains important for single-gene disorders such as cystic fibrosis (CF) and Huntington's disease but it has become increasingly important in communicating genetic information that can influence the expression of multifactorial and adult-onset conditions such as cardiovascular disease. One hope is that with an understanding of genetic factors that influence the expression of multifactorial conditions, individuals will be empowered to make wise health choices to reduce their disease risk and health care providers will be able to provide individualized medical management that considers an individual's physical health and genetic risk factors to a disease to optimize an individual's health and medical management.

The three primary indications for referral for a genetic evaluation fall into the general categories of reproductive genetics, pediatric genetics and adult onset disorders. Referral patterns are also rapidly evolving to include many more adult-onset disorders in addition to cancer and other multifactorial conditions, in which the genetic basis is better understood utilizing the advances in molecular genetics. Common goals of many of these evaluations are to establish if an individual is affected with a single-gene disorder or determine the genetic risk factors, provide recurrence risk counseling for themselves and family members, to assist in medical management of the disorder and to provide education about the condition and related issues.

In many cases, it is the general practitioners such as a pediatrician, internal medicine physician or family medicine physician who identify a patient in their practice with a potential genetic concern and then refer them for a genetic evaluation. Therefore, it is important for all health care professionals to be knowledgeable about the conditions and findings that are common to their discipline that might suggest an individual has a genetic condition or risk factor. There are numerous recommendations and guidelines for appropriate referrals for genetic counseling and genetic evaluation published by genetics organizations from around the globe (Table 21-2). Providers are advised to be familiar with any genetic practice guidelines or policy statements related to their specific discipline published by their own professional organization.

**TABLE 21-2 International Medical Genetic Organizations**

Organization	Website
National Society of Genetic Counseling	<a href="http://www.nsgc.org/">http://www.nsgc.org/</a>
American College of Medical Genetics	<a href="http://www.acmg.net/">http://www.acmg.net/</a>
American Society of Human Genetics	<a href="http://www.ashg.org/">http://www.ashg.org/</a>
National Coalition for Health Education in Genetics	<a href="http://www.nchpeg.org/">http://www.nchpeg.org/</a>
International Society of Nurses in Genetics	<a href="http://www.isong.org/index.php">http://www.isong.org/index.php</a>
Canadian College of Medical Genetics	<a href="http://www.ccmg-ccgm.org/index2.html">http://www.ccmg-ccgm.org/index2.html</a>
Canadian Association of Genetic Counselors	<a href="https://cagc-accg.ca/">https://cagc-accg.ca/</a>
European Society of Human Genetics	<a href="https://www.eshg.org/">https://www.eshg.org/</a>
British Society of Human Genetics	<a href="http://www.bshg.org.uk/">http://www.bshg.org.uk/</a>
Clinical Genetics Society	<a href="http://www.clingensoc.org/">http://www.clingensoc.org/</a>

### 21.1.5 Who Provides Genetic Counseling?

Genetic counseling services are provided by a variety of health care professionals in a number of different settings. In some settings, such as to pediatric genetics, metabolic disease, and specialty disease clinics, services are often provided by the genetics team. This team is composed of professionals trained in various aspects of genetics including clinical (MD) geneticists, genetic counselors, genetic nurses, and medical (PhD) geneticists with laboratory support from cytogeneticists, molecular geneticists, and biochemical geneticists. Other key professionals such as nurses, dietitians, social workers, and/or psychologists can also be members of the team. Each team member has his/her own set of skills and corresponding role in the team although there can be some overlapping functions. Various team members may contribute to the interpretation/risk assessment, education, and counseling functions of genetic counseling. This role is not limited to genetic counselors.

In reproductive genetics, genetic counseling services are often provided by genetic counselors or genetic nurses working with geneticists or with physicians boarded in obstetrics or maternal fetal medicine specialists. Similarly, in adult onset disorder clinics, genetic counselors may work with a variety of specialists including but not limited to oncologists, surgeons, gastroenterologists, cardiologists, or neurologists.

In the examples above, at least one health care professional has formal training in a genetics specialty. However, as the availability of and demand for genetic testing have increased, and as applications of genetic testing have expanded (e.g. population-based carrier screening programs, pharmacogenetics testing), more

pressure has been exerted on all health care professionals to provide some level of genetics services. Deficits in medical genetics knowledge have been identified as barriers to provision of appropriate genetics services (16). Some health professions and specialty groups are working to overcome the knowledge deficits. For instance, physician assistants have developed genomics competencies (17) to incorporate into their training programs. Nursing groups have defined genomics competencies for different levels of nurses (18). The National Coalition of Health Professional Education in Genetics has developed core competencies in genetics, now in their third edition, for all health care professionals (19). Organizations such as the American Society of Clinical Oncologists have stated their commitment to providing educational opportunities in genetics (20).

In the face of such efforts, the question becomes where does the role of the primary health care provider end and where does the role of the genetics professional begin? What situations should be managed in primary care and when should a patient be referred for genetics services? Some organizations, such as the American College of Obstetrics and Gynecology (ACOG) and the American Academy of Pediatrics, provide guidance to their membership regarding these questions through policy statements, practice and education bulletins and committee opinions. But this is an area where genetics professionals should also have a strong voice. Working collaborations between genetics professionals and other health care providers are required if the goal of ensuring that patients have adequate and appropriate access to genomics services is to be achieved.

## 21.2 PROCESS OF GENETIC COUNSELING

As the NSGC' definition delineates (6), the process of genetic counseling involves interpretation of family and medical history to assess risk, education, and counseling. This is accomplished through five basic tasks: information gathering, risk assessment, information giving, psychosocial assessment and counseling, and management/follow-up. The following sections describe how these components are integrated throughout the genetic counseling process.

### 21.2.1 Information Gathering

**21.2.1.1 Presession Information.** Information gathering often begins before the genetic counseling session takes place. In some cases, the genetic counselor (this term will be used to denote any professional involved in genetic counseling) may have the opportunity to speak to the client before the session to request records, to inquire about the client's goals, to collect preliminary family history information, or to answer questions about the visit. In others, the genetic counselor

gathers information indirectly, from details collected by an administrative staff person upon scheduling the appointment, from medical records, from a completed family medical history questionnaire, or from a conversation with a referring physician. Such information can help the genetic counselor to begin assessing the nature of the genetic risk, the client's experience with the condition or concern, and potential client goals for the genetic counseling session. However, as pressures to work more efficiently increase, genetic counselors may no longer have the luxury to gather and review this information in depth precounseling.

Whether first contact occurs by telephone presession or at the time of the encounter, it is important to remember that, with perhaps the exception of prenatal diagnosis, most people are not familiar with genetics services. As such, a referral can generate feelings of anxiety, fear, shame, or anger. Furthermore, if the client feels coerced into the referral by his/her primary care provider or family, he/she may also be resentful. The referral may confirm fears that a family already has about a specific diagnosis, such as muscular dystrophy, or introduce the possibility of a previously unheard-of condition or risk. Unlike other types of referrals, a genetics referral raises fears not only for the potentially affected individual but also for other family members—born and unborn. Even a person who has self-referred may have taken years to work up the courage to call and request testing or counseling. Thus, it is critical that the basis for future open communication and trust be established at this point.

**21.2.1.2 Contracting: Setting a Mutually Agreeable Agenda.** Once initial introductions have been made, the next step of the genetic counseling session is contracting. Contracting is the process of identifying the client's main questions, concerns, and goals for the session for the purpose of setting a mutually agreeable agenda. The process begins by determining what the client or family understands regarding the reason for referral. What were they told regarding the purpose of the appointment? What has their health care provider shared about his or her concerns? Does the individual or family share these concerns or do they feel the appointment is unwarranted? Was a specific diagnosis mentioned? If so, what were they told about the likelihood of this diagnosis or about its implications? What information has the individual or family gathered about the condition or risk before the appointment? This question becomes increasingly important given the easy accessibility of both high- and low-quality information on the Internet.

Such questions help the genetic counselor ascertain the client or family's grasp of the situation, motivations for genetic services, and expectations. They also set the stage for developing the session goals and agenda and for providing realistic expectations. For instance, a woman who is referred for a positive maternal serum screen may indicate that she was told her baby has Down syndrome.



In this case, the first priority becomes explaining that the test indicated an increased chance but not a certainty of the diagnosis. A family may present to pediatric genetics thinking that their child finally has an established diagnosis and they will simply be learning about the condition when in reality, the purpose of the session is to determine if the diagnosis can be made. In this case, the genetics professional has to clarify session goals and potentially manage the family's emotional reactions to the possibility of no diagnosis. A woman may present for cancer genetics services indicating that her physician told her she had to have counseling before having genetic testing for hereditary breast cancer. In this situation, it is important to clarify the purpose of counseling in the context of accurate risk assessment and genetic test selection. It may also be beneficial to the working relationship to help the woman recognize that she has some control in setting the session goals.

Once the counselor has ascertained the client or family's perceptions and expectations, the next step is to develop specific and realistic goals and a road map for achieving those goals (agenda). In outlining the goals and agenda, it is important to include what the consultation will entail, who will be involved and the various roles of these team members, and what types of additional investigations (e.g. genetic tests, medical tests, information from other relatives) may be necessary. It is also important to let the individual or family know what is expected of them. Informing them of their role in providing information puts some ownership of the session outcome in their hands and can make the session more collaborative. Inviting them to ask questions and explaining that questions are expected also set the stage for collaborative, client-focused genetic counseling. The goals and agenda should be offered tentatively to give the client license to provide input. Once the counselor and client have reached mutually agreeable session goals and agenda, the work of the session can begin. The counselor should check in with the client periodically to assess whether the session goals remain the same and the agenda still makes sense. If goals or objectives change because of newly identified information or concerns, the counselor should be flexible and modify the agenda accordingly.

Is contracting really important? From a practical standpoint, it is. Even when individuals or families present for genetic counseling with the same indication, their goals and expectations can be quite different. For instance, in one pediatric genetics clinic day, two families presented for evaluation and counseling because they each recently had a baby diagnosed with Down syndrome. One couple received the diagnosis prenatally. They had already identified the early intervention services available to them, their child had had much of the recommended medical follow-up, and they had made contact with other families who have children with Down syndrome. Their primary goal was to make sure their child was obtaining all the medical services indicated. In

contrast, the second couple received the diagnosis after birth. The pregnancy was unplanned and the mother was working on her graduate dissertation when the baby was born. This couple was overwhelmed by the unexpected diagnosis, the demands of a new baby (the couple's first), multiple appointments with various health care professionals, and graduate school responsibilities. They were unable to articulate session goals but it was clear that one goal would be to help them identify sources of support. By gathering the necessary information, the counselor was able to identify each couple's major concerns and manage the cases accordingly. Case examples aside, there is also evidence in the medical literature that contracting is important. For instance, one study found that genetic counselors were not always accurate in assessing client's concerns and that client satisfaction with genetic counseling was lower in these cases (21).

In summary, contracting, which involves gathering information about clients' questions, concerns, and goals, and then using the information to develop a mutually agreeable agenda, is an important part of the genetic counseling process. Failure to tailor genetic counseling sessions may result in failing to meet clients' needs and expectations. It may also result in decreased client satisfaction.

**21.2.1.3 Collecting Family and Personal Medical History.** The family medical history, as captured in the genetic pedigree, is considered the cornerstone of genetic counseling. In addition, it has been recognized as an important part of personalized medicine approaches (22). The pedigree serves many functions. It can be used to establish a diagnosis, identify a pattern of inheritance, calculate genetic risks, distinguish between genetic and nongenetic risk factors, and identify healthy relatives who could benefit from medical screening tests (23). The pedigree also provides an excellent snapshot of how both genes and environment interact in a given family to cause disease. Furthermore, identifying a client's risk for disease through pedigree analysis can provide valuable information about the positive predictive value of a screening testing or genetic test for that disease in the individual (24).

The pedigree is also a valuable tool for establishing rapport with clients. It puts the client in the role of expert and encourages participation in the genetic counseling process. In addition, collecting family history information provides clients an opportunity to explore and share their unique perspectives on what it has been like to have a medical condition in the family. Does the family view the condition as mild or severe? What are the family's opinions regarding identification of those at high versus low risk of the condition? What are the family's opinions about acceptable versus unacceptable ways to manage risk? How are the client's views similar or dissimilar to other relatives' views? This information can help the counselor understand what having a genetic condition has meant to the family. It can help the counselor identify accurate conceptions and misconceptions. Such

information is beneficial to the counselor in tailoring the counseling session and in assessing whether the decisions clients are making about genetic risks are consistent with their experiences, perceptions, voiced opinions, values, and beliefs. It can also help the genetic counselor provide anticipatory guidance should the client choose to manage his or her risk in a way that is different from how other relatives have done so.

The pedigree can also be useful as a tool for educating the client about the condition in the family (23). The graphical representation of the family history that a pedigree provides can help the genetic counselor explain the pattern of inheritance in the family. Concepts such as variable expressivity and reduced penetrance can be demonstrated with examples taken directly from the history. When relevant, the impact of environmental risk factors can be pointed out and discussed.

A detailed account regarding how to collect family history information, including what questions to ask, how to ask them, and how many generations to include is available (23). Standardized pedigree nomenclature has been adopted and should be used (25). Depending on the purpose of the pedigree, the types of questions asked and the amount of detail sought will vary. Targeted questioning—asking questions directly related to the indication for genetic services or symptoms of the condition in question—may enhance the quality of information received about the family and may aid in establishing a diagnosis or providing an accurate risk assessment.

Despite the many potential benefits of collecting family history and developing pedigrees, there are some limitations. A pedigree is only as good as the information provided. Some clients will be poor historians. These individuals may not be familiar with their family medical history, may not have contact with a portion of the family, may not understand what type of information the counselor is trying to collect, or may be hesitant to share what they view as sensitive information. Some clients may be willing and able to collect extensive family history information but because they were unaware that they would be asked to provide it, are unprepared. Even with good historians, the accuracy of the medical information provided tends to be highest for close relatives and lower for those more distantly related. Therefore, medical record confirmation of key diagnoses can be a helpful and, in some cases, an essential part of risk assessment. It is also important to keep in mind that family history information is dynamic. As such, risk assessments may change over time. For instance, what appeared to be a low risk history can become high risk as more relatives are diagnosed with a condition. This is especially relevant when family history is being used to assess risk of adult-onset disorders.

There are several ways in which a genetic counselor can improve the quality of family medical histories. Explaining the relevance of the pedigree and why certain information is being collected, especially when this information is construed as sensitive, is important. Informing

clients in advance that family history questions will be asked can be helpful. Sending family history questionnaires or links to web-based questionnaires to clients before their appointment and encouraging them to reach out to their relatives to collect medical histories may enhance the quality of the information received. Using client-friendly language in inquiring about conditions or symptoms and adopting the language, the client uses to describe these can also help. Addressing concerns about the confidentiality of the family history information may also be important in promoting client disclosure of such information. Finally, given the dynamic nature of family history, it is good practice to encourage clients to update their histories regularly and to recontact their health care professional if notable changes occur.

### 21.2.2 Psychosocial Assessment and Counseling

Kessler stated that the counseling component of genetic counseling is primarily concerned with three tasks: understanding the client, communicating that understanding back to the client, and when possible, empowering the client feel better about herself or the situation (26). But how is this achieved in genetic counseling and what skills are needed? A genetic counseling practice analysis, conducted by the American Board of Genetic Counseling for the purpose of delineating the skills and activities associated with genetic counseling, provided a detailed description of how genetic counselors in the United States and Canada currently achieve this (27). Twenty-one tasks were identified under the major content domain of psychosocial assessment and support (27) (Table 21-3).

These activities and the skills needed to accomplish them do not appear to be specific to North American genetic counseling services. In reviewing 56 international genetic testing guidelines to determine what constitutes ideal genetic counseling, Rantanen et al. (28) found that a majority mentioned the importance of psychological support. Having appropriate education and training not only in genetics but also in communication and counseling skills was deemed valuable in that such training provides the basis for relationship building between the counselor and client, enhances the counselor's ability to understand the client's needs, and supports the counselor's role in facilitating decision making (28).

Why is the psychosocial aspect of genetic counseling important? Referrals for genetic counseling are often associated with a variety of emotions including anger, anxiety, guilt, shame, or grief/loss. Furthermore, some people who present for genetic counseling are in crisis, especially when the situation that precipitated the genetic counseling referral is unexpected and/or requires that a difficult decision be made in a short period of time. Strong emotional reactions can preclude clients from being able to listen to and fully understand the genetic education provided. In such cases, identifying and acknowledging

**TABLE 21-3 Psychosocial Assessment and Support Tasks in Genetic Counseling**

Psychosocial Assessment	Psychosocial Support/Counseling
<ol style="list-style-type: none"> <li>1. Recognize factors that may affect the counseling interaction</li> <li>2. Assess client and/or family               <ol style="list-style-type: none"> <li>a. Emotional reactions</li> <li>b. Support systems</li> <li>c. Defense mechanisms and coping strategies</li> <li>d. Cultural/religious beliefs and values</li> </ol> </li> <li>3. Evaluate social and psychological histories</li> <li>4. Assess client's psychosocial needs and recognize need for referral</li> </ol>	<ol style="list-style-type: none"> <li>1. Address client emotion and or behavior using:               <ol style="list-style-type: none"> <li>a. Primary empathy</li> <li>b. Advanced empathy</li> <li>c. Direct statements</li> <li>d. Questions</li> <li>e. Emotion-specific techniques</li> </ol> </li> <li>2. Utilize reframing to broaden client's perceptions</li> <li>3. Employ anticipatory guidance</li> <li>4. Utilize cross-cultural counseling techniques</li> <li>5. Promote competence and autonomy with direct, supportive statements</li> <li>6. Address family communication issues</li> <li>7. Facilitate decision making</li> <li>8. Promote coping and adjustment</li> </ol>

Adapted from Hampel et al. (27).

the client or family's emotional responses to genetic information is the first step to promoting informed consent, facilitating decision-making and promoting adaptation.

Psychosocial aspects are also important because referrals are often related to emotionally charged subjects including illness or disability, loss (of health, life, or desired outcome), and childbearing. To achieve the goals of genetic counseling, the counselor must understand what a diagnosis or risk means to the individual and family based on their experiences, worldview, values and goals. It is important to consider both the individual and family because genetic conditions can have an impact on both. Furthermore, the family itself and its place in the life cycle can have a significant impact on how an individual perceives and adapts to a condition or risk (29). For instance, the diagnosis of a disabling condition in the person who is the primary breadwinner in a family has different implications than when the diagnosis occurs in a young child. In both situations, the family faces significant change, but the challenges are different.

How much the counseling aspects of genetic counseling should be emphasized? This question is a source of considerable debate. In reality, the degree of emphasis is usually determined by the training of the professional involved and his or her level of comfort and perceived competence in this area. The clinical situation itself may also dictate the practicality and necessity of counseling- versus teaching-based approach. For example, employing a counseling-oriented model may be very beneficial for some couples who have elected pregnancy termination for a fetal abnormality. In contrast, there may be less need or opportunity for this approach in the preliminary counseling for prenatal diagnosis related to advanced maternal age.

The needs of the individual client can also have an impact on to what extent the counselor uses a teaching- versus counseling-oriented approach. Most clients who present for genetic counseling are psychologically not different from people in the general population. They usually are seeking information about genetic issues, not

psychotherapy. In fact, some will very clearly state up front that they do not need "counseling." On the other hand, there are many unique challenges to a person's psyche that are inherent in the issues raised by genetic counseling, the decisions clients are asked to make, and the risk or occurrence of a genetic condition or birth defect. Some of these are unlike anything most individuals have ever encountered. It is incumbent on the genetic counselor to recognize and address the common emotional reactions to these psychic challenges, even if at face value, the client is not asking for such an intervention.

A genetic counselor is typically able to manage the psychological reactions of most of his or her clients. However, when issues arise that are beyond the counselor's counseling expertise, he or she must be able to recognize this and make an effective referral to the appropriate mental health professional. It is worthwhile to understand the differences between therapeutic approaches and to cultivate good working relationships with therapists, including licensed counselors, psychologists, and psychiatrists, who have demonstrated interest in and sensitivity to genetic issues. Among these are also clergy, who, through pastoral counseling, can provide a spiritual dimension that some clients will find more comforting than other counseling approaches.

**21.2.2.1 The Process of Psychosocial Assessment.** A psychosocial assessment begins at intake. The genetic counselor can start to hypothesize potential factors that may affect the counseling interaction based on the indication for services. Reviewing medical records, when relevant, can provide insight into the client's state of health, what has transpired before the referral, and clients' experiences with the health care system, any of which can have an impact on genetic counseling. Medical records, a referral letter, or the genetic clinic's own medical/family history questionnaires can help the counselor begin to identify provider and/or client expectations for the session.

First contact is an opportunity to further assess client goals and expectations through contracting. As a part of this process, the counselor can assess the client's

willingness to participate in genetic counseling. Whereas some individuals are eager to take part, others are resistant or reluctant (30). Resistant clients are not completely invested in genetic counseling services. They may feel as if they were pushed into genetic counseling (by a health care provider or a family member), may not completely understand the reason for referral, may be scared about what they will find out, or may see the genetic counselor as a gatekeeper of, and hence impediment to, something they want such as genetic testing. They may show their resentment or may refuse to take part in important aspects of the genetic counseling process such as agenda setting or providing complete family history information. However, at some level, they are willing to be present at the session. Reluctant clients have been distinguished from resistant ones in that they do not want to take part in genetic counseling at all (30). Since resistance or reluctance can impede the work of genetic counseling, identifying and addressing these client characteristics is an important early component of psychosocial assessment.

A key component of the psychosocial assessment process is ascertaining the client or family's knowledge and experiences related to the condition or risk in question. An effective way to collect this information is by eliciting their story. Determining how the client learned about the genetic condition or genetic risk, what he or she knows and has been told about it, how serious he or she views it to be, and how he or she has managed the condition or risk in the past helps the counselor begin to understand the potential impact of genetic information. If the client is affected with a condition, obtaining his or her personal story, from first learning about risk through diagnosis, treatment, and follow-up, is important. Information gathered as part of the family history, including who has been affected, how they were diagnosed, the severity of the condition in these individuals, and who is thought to be affected (even without an established diagnosis) is another source of psychosocial information. Through hearing the story, attending to nonverbal messages, using open-ended questioning, and using primary empathy, the counselor is often able to identify the client's emotional and cognitive reactions to the genetic condition or risk. Identifying whether the client's views are similar or dissimilar to those of their spouse, family, cultural group, religious group, or other possible sources of support is another important component of the assessment process.

Some clients may be hesitant to share their story out of fear of having a strong emotional reaction, concerns about confidentiality, or because of feelings of distrust, guilt or shame. Alternatively, hesitance may be a function of not understanding the scope of the genetic counseling process. It is contingent on the counselor to demonstrate his or her willingness to fully explore the client's experience. In addition, demonstrating unconditional positive regard, empathy, and genuineness (Carl Rogers' facilitative conditions) can help the counselor develop rapport, build trust, and promote client disclosure.

**21.2.2.2 Psychosocial Assessment of Defenses.** Part of the psychosocial assessment process can involve identifying a client's defense mechanisms and coping strategies. Defense mechanisms are a person's way of trying to dampen the emotional effects and maintain control of a potentially threatening situation. They can provide the client with a temporary way of distancing him/herself from the full impact of the situation while he/she (ideally) garners his/her resources to cope with it in a more effective way. McCarthy Veach, LeRoy and Bartels identified nine different defense mechanisms that may be encountered in genetic counseling: denial, displacement, identification, intellectualization, projection, rationalization, regression, repression, and undoing (30).

Coping strategies are the way that people approach to solving a problem or dealing with a difficult situation. Djurdinovic identified eight coping strategies in genetic counseling clients: confrontative, distancing, self-controlling, escape-avoidance, accept responsibility, seek social support, plan, and positive reappraisal (31). Whereas the first five function more as defense mechanisms, clients who seek support, plan, or use positive appraisal are more likely to adapt positively to the situation. Recognizing where the client is in the process of adapting to genetic information, the short-term value of defense mechanisms at points in the process, and when it is important to encourage clients to seek more productive ways of coping is an important part of promoting adaptation.

**21.2.2.3 Psychosocial Assessment and Cultural Competency.** A client or family's cultural identification can have an effect on how they view a genetic condition or risk and how they participate in genetic counseling. Culture has been defined as the beliefs, behaviors, attitudes, values and expectations held by a social group (32). Ethnicity has been defined as membership in such a group based on shared kinship, language, experience, and/or religion (32). Culture goes beyond race or ethnicity to include other social groups such as the Deaf community or the intellectual disability community. In genetic counseling, culture and ethnicity can have an impact on beliefs about causality, the social implications of a condition, and acceptable ways to manage genetic risk. It can also have an impact on expectations and perceptions of genetic counseling, communication within the family and with health care providers, and views and practices related to health, illness, disability, marriage, and reproduction. For these reasons, identifying whether a client identifies with a particular cultural group and then managing the genetic counseling session in a culturally sensitive way should be part of the psychosocial assessment and counseling process. This requires that the provider strive for cultural competence.

Cultural competence is the ability to recognize the impact of a client's culture on perceptions, beliefs, attitudes and values and then to effectively tailor services in response to these cultural considerations. Three major



components of cultural competence in health care have been described: knowledge of the culture, the skills to work with clients from different cultures, and awareness of one's own culture and the potential biases it imposes (32). In genetic counseling, knowledge should extend to factors such as racism and history of persecution, both of which can have an impact on the counselor's ability to build a trusting relationship with clients. Recognizing different decision-making styles and who has the authority to make decisions is particularly important. So is identifying the value that the client/family places on individual autonomy versus collectivism or familism.

Skills include being able to use culturally sensitive communication approaches based on awareness of factors such as proxemics, client's use of eye contact when speaking and listening, and the appropriateness of touch (for example, hand shaking). Being able to navigate differences between a client's culturally determined beliefs about causality and biomedical explanations is another necessary skill. Recognizing and responding to differences in how emotions are expressed and receptiveness to answering routine (but potentially sensitive) questions are two additional skills a counselor should cultivate in striving for cultural competence. It is important to keep in mind that immigrants from any country are highly diverse, often representing many ethnic, religious, socioeconomic, or linguistic groups, and varying levels of education and medical sophistication. Thinking that all people of a specific ethnicity are the same can impede the work of genetic counseling. Knowledge of a culture should serve as a jumping-off point for assessing a client or family's beliefs and values. Having a way to assess the impact of acculturation on a family's cultural identity is particularly important to prevent stereotyping.

Language barriers can significantly influence the quality of communication in genetic counseling sessions. Having access to skilled interpreters can enhance communication between health care providers and clients who speak a different language. However, access is not always available. Although sometimes unavoidable, using a family member—especially a child—or expecting one member of a couple to translate for the other can compromise the interaction. It can create awkward situations when sensitive information must be obtained or discussed. In addition, the relative may choose to share only a subset of the information provided by the counselor, thus jeopardizing informed consent. Ideally, the genetic counselor should cultivate relationships with one or more professional medical interpreters and familiarize them with the goals and processes of genetic counseling. Asking an interpreter about relevant cultural issues can also help the counselor identify appropriate strategies for working with a client.

The third component of cultural competency is awareness. All genetics providers should be aware that they have their own cultural views and that these views may have a conscious or subconscious effect on genetic

counseling interactions. These views may be related to ethnicity and/or religion but also to being a health care professional. For instance, by virtue of the services they provide, prenatal genetic counselors believe in the value of prenatal screening and diagnosis. Cancer genetic counselors believe in the value of risk identification, screening and prevention. These values may not be shared by a subset of reproductive genetics or cancer genetics clients. The counselor should also strive to recognize her attitudes regarding individuals from various cultural groups and assess the validity of these attitudes and their impact on genetic counseling. Finally, the counselor should be aware of cultural biases that are built into the health care system and, when possible, work to address those that prevent culturally different clients from having access to services.

In summary, culture and ethnicity can have a significant influence on all aspects of the genetic counseling process. As client bases become more ethnically diverse, it is even more critical for genetic health care providers to recognize and address this influence in a culturally sensitive way. As such, assessing the impact of culture and ethnicity is a necessary component of the psychosocial assessment.

**21.2.2.4 Psychosocial Counseling and Support.** As delineated in the genetic counseling practice analysis (27), counseling and support, as it is currently practiced, involves addressing clients' emotions and behaviors, using reframing to help them think about a problem in a different way, using knowledge gained during the psychosocial assessment to provide anticipatory guidance, helping them recognize that they have the ability and authority to make their own decisions (within their cultural framework), and facilitating decision making, communication within the family, and adjustment. Different techniques adapted from various theoretical approaches are used to accomplish these counseling objectives. The sections below provide additional detail about elements of this process.

**21.2.2.5 Addressing Client Emotional and Cognitive Responses.** Referral for genetic services can trigger a number of emotional and cognitive responses, which unless acknowledged and addressed, can block the work (e.g. education, informed decision making, coping) of genetic counseling. Communications skills such as attending, empathy, and open-ended questioning serve a number of functions. The counselor can use these skills to bring clients' emotions and thoughts out in the open, to give them permission to discuss their reactions, and to build rapport and establish a trusting relationship. Use of such skills can help the counselor determine when a surface reaction, such as anger, might be masking a different feeling, such as fear, the approaches to managing which would be different. A counselor can also determine the intensity of the client's emotional reaction (e.g. concern versus despair) and context in which the reaction is occurring. For instance, many clients who are referred for positive maternal serum screen results are anxious,

but the level of and source of anxiety can vary. Some are mildly anxious and others are overwrought. For some, the anxiety comes because the result was unexpected, whereas for others, it is because they are under the misconception that the baby has already been diagnosed with the condition. In contrast, others may be more anxious about the possibility of having a diagnostic procedure. Through skilled use of communication techniques, the genetic counselor can assess the quality of and primary reasons for clients' reactions and address it accordingly.

Two client reactions that are prevalent in genetics services and warrant special consideration are guilt and shame. Guilt is feeling responsible for a situation, i.e. not meeting one's own standards or living up to one's own expectations. The typical defense mechanisms associated with guilt include trying to forget the source of one's bad feelings (repression), finding somewhere else to place blame for the situation (intellectualization) or trying to consider the positive aspects of it (rationalization) to reduce personal responsibility (33). All of these serve to reduce a person's self-recriminations. In contrast, shame is the feeling that a person is not meeting someone else's standards or societal expectations. With shame, the defense mechanisms often serve to reduce the cause of a person's loss of self-esteem through denial, relabeling a loss as a gain (reaction formation), compensating or overcompensating in some other area (for instance, being the "best" parent to an affected child), or highlighting others faults to diminish attention on one's own (33).

Because the psychological implications of guilt and shame are different, they require different counseling interventions. In both cases, the process starts by providing the client with an environment where she can openly admit her feelings (confession of guilt or shame). Then interventions that use authority, normalization, reframing (thinking about responsibility in a different way), or limiting liability can be used for addressing guilt reactions (33). Those that seek to develop a nonjudgmental working alliance promote sharing of feelings, highlight the positive aspects of the client, and bolster her ego are more appropriate for shame (33). Being able to distinguish between guilt and shame and use the related counseling interventions is thus important in managing these reactions effectively. Simply telling someone that it is not their fault without further exploration is probably not effective and represents a missed opportunity to help manage what can be significant reactions.

**21.2.2.6 Reframing.** Sometimes, clients are not fully aware of their emotional or cognitive reactions. For instance, a client may not realize that her fear of amniocentesis is more related to the potential results of the test than the procedure itself. Or, sometimes, clients need help thinking about their genetic risk or condition in a different way. For example, a woman presenting for cancer genetic risk assessment and testing may think that inheriting the mutation in her family means she will die of breast cancer similar to her mother rather than seeing

it as an opportunity to reduce her chance of this outcome. In these situations, the tasks of the genetic counselor are to generate ideas about potential gaps in the client's awareness and then fill in these gaps with the goal of broadening the client's perspective (reframing or advanced empathy).

Reframing can bring unrecognized emotions to the surface or challenge longstanding beliefs. Therefore, counselors need to have built rapport and established trust before using this technique. An advanced empathy statement should be clear, specific, nonjudgmental, and suitable for the client. It should also be tentative, so that the client has a chance to agree or disagree with all or parts of the message (30). Reframing is not used as often as other techniques in genetic counseling. However, when used appropriately it can enhance a client's insight. This in turn could result in more informed decision making and/or better adaptation to genetic information or risk.

**21.2.2.7 Facilitating Decision Making and Promoting Adaptation.** Genetic counseling clients are faced with a lot of different decisions. These include whether to have carrier testing to learn about reproductive risk; whether to have prenatal diagnosis for a genetic condition; whether to continue, terminate, or place for adoption an affected pregnancy; whether to use assisted reproductive technology (ART) to prevent the conception of an affected pregnancy; whether to have a diagnostic test or have a child tested; and whether to have presymptomatic or predictive genetic testing. In such situations, the goal of genetic counseling is to ensure that the client makes an informed decision, without coercion, that is consistent with his/his family or community's values and beliefs and is in his/their best interests.

What is the role of the clinician in these types of decisions? Traditionally, in genetic counseling, the role has been to provide the information about the conditions to facilitate informed decision making. The clinician outlines the potential decisions to be made and provides unbiased (ideally) information about each, so that client can make his own decision. Additionally, some clinicians may explore the client's perceptions regarding the benefits, risks, or limitations of each decision as part of the decision-making process. However, in keeping in line with the nondirective roots of genetic counseling, clinicians typically have not shared their own impressions about which decision is best. An exception is made, though, when the decision involves medical care such as screening recommendations for a preventable adult-onset condition such as cancer or cardiovascular disease.

What is the role of the client? The client is expected to use the information in genetic counseling to make the best possible decision for the given situation. However, despite the value genetics professionals place in a planful (rational) decision-making style, this is only one of the possible ways a client may approach a decision. Other decision-making styles include intuitive, agonizing, delaying, impulsive,

fatalistic, compliant, and paralytic (30). Although these styles make work in some situations, they are often not ideal when a client is faced with making a decision that has significant long-term consequences. Moreover, there can be factors that affect the client's willingness or ability to use the information presented to formulate a decision. These can include factors specific to the client such as educational level, motivations to learn about genetic risk, and perception of disease severity. They can also include factors specific to the clinician such as willingness to provide information in an understandable way, supportiveness, and awareness of relevant cultural factors. For these reasons, it is incumbent on the clinician to carefully assess the quality of the decisions clients are making and to work further with clients when the quality is in question. The goal is not to impose the clinician's decision on the client but to make sure the client's decision is as well informed by his circumstances as possible.

Whereas many genetic counseling clients, once presented with the needed information, are able to make a decision, others may become overwhelmed by the process. In such cases, using a rational decision-making model (30) may help the clinician facilitate decision making. In this model, the clinician works with the client to lay out all the possible decisions that can be made (options) and to list the relevant factors associated with each option. Relevant factors can include but are not limited to medical facts, financial issues, cultural influences, and emotional or cognitive reactions. Once all the options and factors have been listed, the client can determine which option is the best based on which one includes the most important of his relevant factors. This model provides clients with a systematic way to identify which factors they value the most and which decisions are most affected by these factors. It also helps clinicians better understand the context in which the decision is being made.

### 21.2.3 Risk Communication and Assessment

Genetic counseling involves the calculation and communication of numerous risks and risk factors. Accurate risk and recurrence risk assessment are viewed as critical for clients to make informed choices about reproduction, pregnancy management, health screening and surveillance. There are many nuances related to the communication of risk or probabilities of an event in the context of the clinical setting and client's worldview.

Jehannine Austin suggests that:

*"... we tend to use the word "risk" as a synonym for "probability"... what we are actually measuring and discussing is not the accuracy of our clients' risk perception, but rather the relationship between our clients' subjective perception of numeric probability and the objective numeric probability of a given outcome." (34)*

This quote highlights that while numbers, facts and figures are useful decision-making tools for many health care practitioners and some clients, they may also be confusing and at times irrelevant to a client whose views are shaded by their personal experiences with the condition and own perception of a "high" risk or a "low" risk (35). Furthermore, the concepts of percentages and probabilities are difficult concepts to explain and for many people to apply in a meaningful way when making a health care decision. There is a growing body of literature that examines the role of risk communication in the genetic counseling process and its effectiveness. In many cases, clients are unable to accurately recall the risks provided by the health care provider or may be confused by the implications of the risk provided (34,36).

A client's subjective risk perception may vary significantly from the objective risk provided oftentimes because of their personal context. An individual's context is defined by the client's personal experience with a condition, their perception of the severity of the condition, their knowledge of the disorder and the perceived impact that the disease or condition will have on them and their family. Many clients' views are shaded by having taken care of a family member affected with a particular condition, the loss of loved ones to a genetic disorder and concern for their own children or other family members if they themselves were to become sick or disabled. This information is utilized by the client to establish their personal concept of risk in context of their life before a genetic counseling session. It may be helpful to ask the client early in the session what their perceived risk is for a particular event and how they have determined their risk. This will allow the counselor insight into the contextual considerations of the client that may cause them to under- or overestimate their risk. For example, two sisters whose mother is BRCA positive have the same objective risk to be a carrier. However, one woman may perceive her risk to be higher than her sister's risk because she looks or acts similar to her mother. In this case, the objective risk assessment is modified by the client's "likeness" to her mother in appearance and personality that is actually irrelevant to her Mendelian risk of 50% to be a carrier.

Genetic counselors strive to present risk in a nondirective, value neutral manner that allows a client to utilize the information for informed decision making. As previously mentioned, numerical information is difficult for many people to evaluate and compare. Table 21-4 lists several evidence-based principles for effectively providing risk information. Risk may be presented to a client in numerical terms such as probabilities, percentages and frequencies or in verbal terms such as "high," "very high" or "minimal." Visual aids such as bar graphs and pie charts can be utilized to explain risk in a visual manner. Verbal descriptors are very subjective in value and should be minimized in order to prevent the counselor or provider's personal risk perception from unduly influencing the client's perception of the risk (34).

**TABLE 21-4** Principles for Effective Communication of Risk Information

- Use of ratios (e.g. 4 in 1000) not proportions (e.g. 1 in 250) when comparing risk
- Use of frequencies not percentages for single event probabilities
- Avoid using percentages if they indicate a chance of less than one (e.g. 0.15%)
- Round up percentages (i.e. do not use decimals)
- Avoid explaining risks in purely verbal terms (such as “low risk”) because of the vagueness and directive nature: elaborate by providing numbers
- Use similar presentations when comparing the chances of different outcomes (e.g. do not use “50%” for risk A and “4 in 100” for risk B)
- Use the same denominator when comparing risks (e.g. do not use “1 in 100” for risk A and “1 in 10” for risk B)
- Present absolute risks alongside relative risks
- Offer outcomes in both a negative (undesired) and positive (desired) framing (e.g. risk of having an affected child versus risk of not having an affected child)

Adapted from Henneman et al. (83).

Framing of risk can be a powerful way of presenting risk information. Framing of risk involves the presentation of an outcome in a positive or negative manner. A risk that is framed in a negative sense reports the chance of an adverse outcome such as 60–80% chance of breast cancer. A risk framed in a positive context highlights the likelihood that an adverse outcome will not happen; a 20–40% chance of not developing breast cancer. One cannot assume that a client will be able to determine the positive frame if only the negative frame is provided or vice versa; therefore, it is valuable to explain risk in the terms of a positive and negative frame.

Checking a client’s understanding of the risk information that is provided in the session by giving them the opportunity to ask questions or repeat the information back can help in determining if the client has understood the information provided.

Risk communication is dependent on accurate and individualized risk assessment. Some of the most challenging risks are the ones that a genetic counselor or geneticists must calculate to incorporate one or more factors that will modify a person’s genetic risk for a condition. Because of this, accurate risk calculation is at times a complex process that some individuals may find daunting. It is recommended that any multipart risk calculation be reviewed by other team members to ensure the accuracy of the calculation and that all considerations have been included (37). A more detailed discussion of this topic is included at the end of this chapter.

## 21.2.4 Client Education

Genetic education is an ongoing piece of the genetic counseling and can be found in every step of the process. The

importance of education is highlighted in the definition of the profession, “Education about inheritance, testing, management, prevention, resources and research.” (6). It is positioned between the medical interpretation portion of the definition and the counseling portion of the definition, which signifies the value of education to bind the genetic assessment to the usefulness and assimilation of the information into the client’s worldview. In many ways, a correct diagnosis or risk assessment is of little value to a client if it is not provided in a way that allows the client to derive personal meaning from the information. It is also important to consider that the manner in which the client education piece of the genetic counseling experience is provided because it can influence the reaction and adjustment of the client to the information.

Genetic counselors must consider numerous variables when providing education to a client. They must quickly assess an individual client’s educational background, cognitive abilities, learning style, prior knowledge of the condition, and personal experience with the condition. Each of these factors will shape the type of information that a client would like to know about the clinical diagnosis and the best way for an individual to receive and assimilate this information (38). Psychological factors such as grief, stress, and depression can also influence the reception and interpretation of the educational component of the genetic counseling process. In many situations, genetic counselors are also obligated to provide education on topics such as pregnancy termination or genetic discrimination that a client may be resistant to hearing about or completely unaware.

The public’s general awareness of genetics continues to increase because of the immense amount of information available on the Internet to anyone with access to a computer. The available information includes both primary scientific literature and individual accounts of a genetic condition, some of which is reputable and some of which is not. As a result, many genetic counseling clients have done a significant amount of self-education about genetics; however, there also exists a client population that presents with very limited knowledge of genetics. This challenges the counselor to be well versed in explaining genetic information to anyone along a wide continuum of knowledge and experience.

## 21.2.5 Genetic Testing and Screening

Genetic testing may be recommended as a part of genetic evaluation or a client may seek genetic counseling about the result of a screening or diagnostic genetic test that was ordered by another health care provider. There are many different types of genetic tests available and they each serve a distinct role in the evaluation of an individual for an inherited condition or predisposition to an inherited condition.

The specific application of a diagnostic clinical genetic test is dependent on the client’s needs. When there is a



known or suspected genetic condition in an individual, the diagnostic test is used to confirm or rule out the condition in an individual with clinical symptoms of the disorder. A presymptomatic or predictive test is utilized in an asymptomatic individual with a family history of a genetic disorder. In this case, a positive test would confirm the condition in the individual and a negative test would exclude it.

Clinical genetic testing is ordered in the health care setting by a qualified health care provider and the results are included in the client's medical record. Laboratories that provide clinical testing must meet the standards for quality and proficiency established by the Clinical Laboratory Improvement Act/Amendment (CLIA) (14). GeneTests, which has recently been redesigned and renamed the Genetic Testing Registry, is a database hosted by the National Center for Biotechnology Information (NCBI), which provides a voluntary, but fairly comprehensive, searchable listing of clinical and research genetic testing.

Genetic screening is offered in several settings to identify genetic disease or risk for disease in the general population or in specific high-risk ethnic groups. Some screening tests do not test the specific gene/genetic defect responsible for a condition; instead, they test for other analytes that suggest the disease and then the individual is referred for additional diagnostic testing. Or, a screening test may only analyze a portion of commonly known disease-causing alleles as in general population screening for cystic fibrosis. Screening tests are generally not as sensitive or specific as diagnostic tests and clients should be fully informed of the limitations of a screening test.

Newborn screening is another application of genetic screening for rare, but oftentimes catastrophic conditions on a population level. In this situation, newborns are determined to be at risk for certain hereditary inborn errors of metabolism and other genetic or not genetic disorders that can require intervention in the newborn period. Further diagnostic testing can proceed while the infant is being treated for the disorder and hopefully avoid many of the devastating effects of the disease.

Prenatal testing can include both screening and diagnostic testing. In this setting, maternal serum screening and invasive diagnostic testing should be available to all women, regardless of age, for chromosome aneuploidies (39). Maternal serum Alpha-fetoprotein (AFP) screening is used to identify pregnancies that may have an open neural tube defect. The screening test identifies a cohort of women that are at a relative increased risk for these conditions. These women receive additional counseling and are offered a prenatal diagnostic procedure to confirm or exclude the potential condition in the fetus. Prenatal diagnosis and preimplantation diagnosis (PGD) can be utilized by clients with a history of a known genetic condition or for common aneuploidies to identify a fetus or embryos affected with the familial condition or aneuploidy.

For some syndromes and conditions, there may not be a clinical test available and one must consider research

testing options. Research tests are usually done as part of a study (e.g. gene mapping or disease characterization) or when the lab is in the investigational stage of developing a clinical test. Research testing is conducted under institutional review board-approved protocols that usually specify that participants will not be given their results and any results obtained should not be used for clinical management. In order to use these results for management, they must be replicated in a CLIA approved laboratory.

The genetic counselor is often responsible for investigating testing options and identifying the most appropriate testing laboratory for the individual client. In addition to the above mentioned issues to consider when selecting a test, one must also consider several other factors. The methodology to evaluate a specific gene can be very different between laboratories and certain strategies are more appropriate for some clients than others. A laboratory must be selected based on the use of the best methodology for the particular client. Cost and insurance coverage for a test are also a consideration when ordering for genetic testing. Genetic testing can range from a few hundred of dollars to thousands of dollars. Third party payers such as Medicare, Medicaid and private insurance have very specific policies about the circumstances in which they will pay for testing and for what portion of the cost of the test they will pay. Additionally, there may be requirements that a certain laboratory be utilized for specific testing.

Many times, a genetic counselor will write a Letter of Medical Necessity (LMN) on behalf of the client to determine if a test will be paid for by the insurance company and to obtain preauthorization if needed for a test. Within the letter, there should be information about the indication for testing, application of the results, and the CPT (Current Procedural Terminology) codes used for billing. The turnaround time for test results and sample requirements, are also important considerations to ensure that the testing can be completed on the sample available and in a time frame that meets the clients needs.

Pretest counseling before any genetic screening or diagnostic test should include a complete disclosure of the potential risk, benefits and limitations of the genetic testing and screening. The counselor should be prepared to discuss the testing methodology, clinical utility, costs, expected results and possible unexpected results such as variants of unknown significance, unanticipated results (e.g., identification of a risk for adult onset disease in a child having whole exome sequencing for pediatric indication), or false paternity and any other issues raised by the client before testing. In addition to technical issues related to the test, pretest counseling should include a broader discussion of the potential for genetic discrimination in the context of insurance coverage or employment. Providers should be well versed in both federal and state legislation regarding genetic discrimination in order to provide clients with a full disclosure of additional implications of genetic testing. As part of the pretest

counseling session, any required laboratory or institutional informed consent documentation for the testing should be reviewed and completed. Clinicians should also be aware of any state legislation requiring informed consent and comply with such regulations. Some clients will participate in pretest counseling and elect to decline the testing or procedure. In this case, the client's decision should be supported and the reasons for the client's decision should be documented as part of the medical record.

Posttest counseling may be in the form of a brief phone call to let the client know that a result was normal and to answer any additional questions or it may be a part of an additional counseling session. During a posttest counseling session, a client is typically provided a full disclosure of the results as well as the implications of a positive, negative and a variant of uncertain significance to the individual. Some exceptions may be made with whole exome sequencing where clients may be given the option of different levels of disclosure or some information may be withheld depending on the nature of the genetic risks identified. Ideally, posttest counseling is done in person in order for the provider to have a significant amount of time to discuss the result and gauge the client's understanding based on verbal and nonverbal communication. However, this is not always possible because of a multitude of issues including the client's access to the clinical setting, missing time from school or work or scheduling conflicts.

Direct to consumer (DTC) genetic testing is now available from numerous private companies to anyone that would like to submit a sample and a payment for the test. This testing falls outside of the traditional medical model and has the potential to increase access to genetic testing for virtually anyone. The purchaser receives an evaluation of their genome with information about pharmacogenetics, carrier status of certain conditions and relative risks for some common disease. Genetic counseling may or may not be available from the company that provides the test. Currently, there are limited regulations of companies that provide DTC testing (40). Several studies have been done to assess how consumers respond to this information (41,42). It remains unclear at this time how testing ordered outside of the medical model can be integrated in to our current medical record system. A study of the NSGC membership revealed that genetic counselors have had few requests for counseling related to the results obtained from DTC testing and that they would consider recommending DTC testing in certain clinical scenarios (43).

### 21.2.6 Follow-up

At the conclusion of a genetic evaluation or genetic counseling session, it is important to develop a mutually agreed upon follow-up plan with the client. The follow-up plan should include documentation of the encounter to the referring physician and all specialists involved in the client's care, documentation of the visit in the medical record and ideally written communication back to the

client that summarizes the evaluation and management plan. The genetic counselor is oftentimes also responsible for coordinating any testing recommended during the evaluation, for developing a plan with the client regarding results disclosure, for scheduling any recommended referrals to other specialists, and for coordinating return appointments to the genetics center as needed.

The follow-up communication with the referring physician, specialists and the client serves as a way to ensure that outcome of the genetic evaluation is available for future use. These communications may take the form of dictations to the client's medical record, letters to a referring physician or a "patient letter" that summarizes the genetics assessment and counseling in laymen's terms. Medical dictations and letters to a referring physician may also be reviewed by the client's insurance company to justify the billing and reimbursement for a genetics visit and justification for genetic testing that was recommended during the consultation. It is important that the letter or note captures the full breadth of the evaluation, the level of service, the counseling provided to the client and the follow-up plan. These documents may also serve as an educational tool to provide a referring physician and future physicians with information about diagnosis that is important for an individual's health management plan. For example, a letter or note for a woman that has been evaluated for a family history of breast cancer could include her family history of cancer, personal medical history, genetic risk assessment to be a *BRCA 1* or *2* gene carrier, a brief description of hereditary breast and ovarian cancer syndrome, recommendations for testing for the *BRCA 1* and *2* genes, suggestions regarding appropriate breast and ovarian cancer surveillance should she have a positive or negative test, the client's desires for testing and surveillance at the time of the appointment, and/or recommendations for testing of other family members. This information would then help the client's referring physician manage the client's health surveillance plan to best address her potential increased risk for breast and ovarian cancer. It could also be used to help support the need for genetic testing to her insurance company when requesting payment for the test.

Many centers send client letters at the conclusion of the genetic evaluation and counseling to summarize historical data and test results that were relevant to the diagnosis and to review the information about etiology, prognosis, or genetic risk that was provided. Such a letter gives clients a record of the consultation that they can retain as part of their personal medical information and may make it easier for them to share relevant findings with other family members and future health care providers. The letter may also include information about support groups, advocacy groups and other psychosocial resources that the client may find helpful based on their particular situation.

Letters to the client can also provide an additional opportunity to identify any misperceptions that the client may have had during the evaluation and to emphasize the importance of seeking another consultation should their

family situation or reproductive plans change. If there is no intention to see the family back, the letter should point out that because of rapid advances in medical genetics, which may affect future methods of diagnosis or treatment, the family may wish to stay abreast of progress relevant to their condition via newsletters from advocacy groups, online resources, and/or by recontacting the genetics center if they have questions. The client can also be encouraged to recontact the center if there are changes in the family history such as new babies born with a birth defect or a family member diagnosed with cancer that might influence the client's genetic risk assessment. If a client letter is not provided to the client, a copy of the dictation or letter to the referring physician can be sent to the client for their records. While these documents may have language that is unfamiliar to the client, it will provide them with a record of their evaluation that they can share with others as needed.

### 21.2.7 Genetic Counseling Across the Lifespan

Genetics and genetic factors influence human growth and development from before our conception until our ultimate death. The scientific communities' ability to identify and test for contributing genetic factors has significantly increased with advances in genetic technology. In some cases, the advances in technology have proceeded faster than the ability to incorporate them into the current medical practice models. Below is a description of common indications for genetic counseling throughout the life-cycle and the counseling considerations for each as well as possible future directions. As it is impossible to capture the full scope of any single area of genetic counseling within this chapter, readers are encouraged to review other chapters within this text and outside resources that have more in-depth reviews of many of these topics.

### 21.2.8 Reproductive Genetic Counseling Overview

Reproductive genetics includes not only prenatal screening and diagnosis but also encompasses preconception genetic counseling, Assisted Reproductive Technologies (ART) and preimplantation genetic diagnosis (PGD). Information about prenatal diagnosis should also be considered in almost any genetic counseling situation, as many clients are interested in learning about the chance of passing on a particular condition to their children. Therefore, it is important to be aware of the reproductive options currently available for genetic conditions regardless of an individuals' practice area.

According to the 2010 National Society of Genetic Counseling's Professional Status Survey, 32% genetic counselors provide prenatal counseling (44). This represents the largest single practice area for genetic counselors. Initially, much of the growth in demand for genetic counseling services came with the dawn of prenatal

diagnosis in the late 1960s and early 1970s. At that time, women were referred for prenatal diagnosis via an amniocentesis because of the increased risk for chromosomal trisomies associated with advanced maternal age or because of a risk of a specific genetic condition due to a history of a previously affected child or family member. Testing at that time was limited to the identification of large structural cytogenetic abnormalities and aneuploidies and testing for a few single-gene disorders. Now prenatal testing, and in many cases, preimplantation genetic diagnosis is possible for most single-gene disorders in which there is a known mutation. Enhanced cytogenetic testing with florescence in situ hybridization (FISH) and array comparative genomic hybridization (aCGH) is also being utilized and developed for prenatal diagnosis to identify small gains and losses of genetic as well as the large cytogenetic abnormalities (45).

Owing to the advances in maternal serum screening and prenatal ultrasound, a greater number of pregnancies at risk for neural tube defects, Trisomy 21 and 18 are identified by noninvasive measures. There have also been changes in current standard of care that now make these screening tests and diagnostic tests (amniocentesis, chorionic villus sampling (CVS)) available to all women, regardless of age (39). The improvements in the sensitivity of noninvasive prenatal screening, and possibly society's changing and more inclusive view of individuals with disabilities, have resulted in a significant decrease in the utilization of prenatal diagnostic procedures since the mid-1990s (46). Emerging directions in prenatal screening include the utilization of noninvasive prenatal diagnosis utilizing fetal DNA analysis from maternal serum may. This may further impact the use of the traditional prenatal diagnostic procedures of CVS and amniocentesis (47).

### 21.2.9 Preconception Genetic Counseling

Women and couples are referred or seek genetic counseling before pregnancy for numerous reasons, some of which are listed in Table 21-5. This is an ideal setting for clients to establish a relationship with their genetics provider and fully explore their available options and

**TABLE 21-5 Common Indications for Preconception Genetic Counseling**

- Recurrent pregnancy loss or prior stillborn child
- One parent with a known genetic disease, congenital anomaly or developmental disorder
- Prior child with a known genetic disease, congenital anomaly or developmental disorder
- Family history of genetic disease
- Teratogen exposure (e.g. medications for maternal illness that may put a fetus at risk)
- Ethnic based carrier screening
- Consanguinity
- Infertility

personal feelings about each alternative. These clients benefit from the opportunity to gain accurate information about their individualized risk in future pregnancies, plan any testing that would be needed before a future pregnancy to enable informative genetic diagnosis if desired without being under the pressure and time constraints of an ongoing pregnancy.

Common goals of these sessions are to identify the potential genetic risk factors for the client based on personal, medical and family histories, provide education about the etiology and recurrence risks for the specified condition(s), discuss the prenatal screening and diagnosis options for the specific condition(s), review alternative options such as preimplantation genetic diagnosis, gamete donation or adoption, recommend and facilitate any genetic testing that needs to be done before pregnancy in order to complete prenatal diagnosis, make recommendations to the referring physician for the management of future pregnancies and to provide psychosocial support and resources that could be helpful to the client depending on the indication for the referral.

### 21.2.10 Genetic Applications of ARTs

Assisted reproductive technologies (ART) are available to women and couples that experience infertility in addition to fertile couples that seek out ART as part of PGD for familial single-gene disorders or chromosome conditions such as translocations (Table 21-6). There are a wide variety of ART procedures that range from minimally invasive procedures and hormone stimulation to others that are multistep medical procedures. Artificial insemination and intrauterine insemination deposit a woman's partner's sperm or donor sperm directly into the uterus at the time of optimal fertility. In vitro fertilization (IVF) requires hormonal stimulation of the female to produce oocytes. The oocytes are retrieved and fertilized by sperm in the laboratory. The embryos that are produced divide to a multicell stage and are then returned to the female's uterus for implantation or frozen for use in the future. IVF can be used for male or female infertility and can use the client's gametes or donor gametes. Couples may elect to use donor gametes to reduce their risk for a known single-gene disorder or for women of advanced maternal

age to decrease their aneuploidy risk and to increase the chance of a successful pregnancy. When donor gametes are used because of a known single-gene disorder, it is important to screen the donor for the known condition. When counseling women who have used donor oocytes, the aneuploidy risk to the fetus should be based on the donor's age but not the client's age.

Intracytoplasmic sperm injection (ICSI) is used to address male infertility. Common genetic causes of male infertility are congenital absence of the vas deferens (CBAVD) in men that have cystic fibrosis (CF) or are CF carriers with a 5T allele, Y-chromosome deletions, sex-chromosome disorders such as Klinefelter syndrome (47,XXY) and Kallmann syndrome (48). For this procedure, spermatozoa are retrieved from the testes and a single spermatozoa is selected and manually injected into the oocyte with a micropipette. Couples that elect to undergo this procedure for Y-chromosome deletions and other forms of inherited male infertility should be informed that their male children will also be at risk for future infertility.

Women and couples that are carriers of known autosomal genetic conditions and balanced structural chromosome rearrangements can also consider preimplantation genetic diagnosis. PGD involves IVF combined with genetic testing of the embryos. The embryos that are predicted to be unaffected are returned the uterus for implantation before day 6 or cryopreserved for future use. Women are offered prenatal diagnosis to confirm that the resulting pregnancy is truly unaffected.

Each procedure has its own risks, benefits, limitations and costs. These considerations should be disclosed to clients before proceeding with anyone of these procedures. ART pregnancies have a reported 30% increased risk for birth defects over the general population risk (49). Pregnancies conceived utilizing ART may also have altered second-trimester maternal serum screen results (50). A recent prospective study of children born after PGD and ICSI at 2 years of age did not report any significant difference in their growth development or health between the two groups (51).

### 21.2.11 Indications for Referral and Prenatal Genetic Counseling

Common indications for a prenatal genetic counseling are listed in Table 21-7. Prenatal diagnosis is an option for families with known genetic risk factors such as single-gene disorders and familial chromosome structural anomalies that would increase a couple's chance to have a child with the identified condition. In the 1960s, amniocentesis was introduced and recommended for women of age 35 and older and prenatal screening and diagnosis became a part of routine prenatal care for women, regardless of family history. Genetics in prenatal care has continued to expand to the current recommendations by the American College of Obstetricians and Gynecologists for all women, regardless of age, to be offered prenatal screening

**TABLE 21-6** Indications for Preimplantation Genetic Diagnosis

- Avoiding clinical termination of an abnormal fetus
- Nondisclosure of parental genotype
- Cancer and other adult-onset disorders
- Selecting HLA-compatible embryos
- Mendelian disorders
- Chromosomal rearrangements
- Recurrent pregnancy loss due to aneuploidy
- Aneuploidy testing to improve pregnancy rate



**TABLE 21-7**   **Indications for Prenatal Genetics Referral**

Abnormal first, second, or integrated maternal serum screen
Ultrasound anomaly
Advanced maternal age ( $\geq 35$ at the age of delivery)
Parent with single gene disorder
Both parents are carriers of an autosomal recessive condition
Mother is a carrier of an X-linked recessive condition
Parent with balanced structural chromosome abnormality
Teratogen exposure (e.g. prescription medications, illegal drugs, alcohol, occupational exposure, radiation exposure)
Maternal disease (e.g. diabetes)
Prior child with single-gene disorder
Prior child birth defect or learning disability or mental retardation
Family history of a single-gene disorder
Family history of a learning disability or mental retardation
First trimester serum screening and Nuchal translucency
Ethnic-based carrier screening

for aneuploidies before 20 weeks of pregnancy (39). In addition to maternal serum screening, ethnic-based carrier screening is recommended for women and couples that are pregnant or planning pregnancy. Maternal screening is routinely offered by the client's obstetrician and the client may or may not understand the purpose and limitations of a maternal serum screening test.

Many women are referred for prenatal genetic counseling after they have been informed of an "abnormal" maternal screening result with little additional information about the implications of the result. As a result, these individuals and couples who arrive for prenatal genetic counseling may have a heightened level of concern due to the unexpected potential risk to their unborn child in an otherwise uneventful pregnancy, misconceptions about the reason for their referral and great concern about the possible outcome of their prenatal genetic evaluation. It is important to be mindful of the emotional issues that clients face while they are asked to comprehend the risk figures and procedures that are discussed during their consultation.

### 21.2.12 Overview of the Prenatal Genetic Counseling Process

Many clients are only seen one time for prenatal genetic counseling unless a problem is detected in the fetus. The session is usually before the client's ultrasound, prenatal diagnostic procedure and consultation with a maternal fetal medicine specialists or perinatologist. During the session, the counselor will explain the client's indication for referral, discuss her screening test results or other risk factors, review the mother's medical and prior pregnancy history, obtain and review the family history to identify additional risk factors to the pregnancy, discuss the client's prenatal testing options if indicated, offer population- and ethnic-based carrier screening when appropriate, discuss pregnancy management options if a

problem is discovered or confirmed in the fetus and provide psychosocial support for issues related to concerns about the pregnancy and possible outcomes of testing. For women who elect to undergo genetic testing, the genetic counselor is usually the one who contacts the client with the results. If a client has an affected fetus, the genetic counselor is often responsible for disclosing the results and providing follow-up counseling that includes education about the diagnosis, implications of the diagnosis, resources for more information about the condition or others with the same diagnosis, information about pregnancy management if she chooses to continue the pregnancy and pregnancy termination if she elects this option.

### 21.2.13 Prenatal Diagnostic Procedures

The two most common prenatal diagnostic procedures are CVS and amniocentesis. Both procedures can be used for molecular, cytogenetic and biochemical testing. Both procedures also have associated risks for miscarriage and pregnancy loss.

CVS is offered in the first trimester at 10–13 weeks gestation. During the procedure, a portion of the placenta is obtained by a transabdominal or transcervical approach for genetic testing. The placenta can be utilized because it has the same genetic complement as the fetus. Mosaic chromosome results are more common in a CVS than an amniocentesis and in this case, an amniocentesis in the second trimester may be required. CVS during the first trimester allows many women to undergo prenatal diagnosis before anyone is aware of their pregnancy. It is limited in that it cannot evaluate the fetus for neural tube defects and other structural abnormalities that are not obvious until the second trimester. Therefore, a detailed ultrasound in the second trimester is indicated for women who have undergone CVS as is a second trimester maternal serum alpha fetoprotein (AFP) test to screen for open neural tube defects. The amniocentesis is performed in the second trimester after the fourteenth week. During this transabdominal procedure, a small amount of amniotic fluid is removed and fetal cells are isolated from the fluid for cytogenetic, molecular and biochemical studies if needed. AFP and acetylcholinesterase can also be measured to evaluate the fetus for an open neural tube defect.

Prenatal ultrasound plays an important role in the evaluation of the fetus. Advances in technology allow qualified providers to obtain detailed views of a fetus. Nuchal translucency measurements in the first trimester combined with maternal serum screening results are utilized to improve the detection rate maternal serum screening for fetal aneuploidies. Ultrasound can also routinely identify open neural tube defects, ventral wall defects, some structural changes in the brain and heart, limb defects and overall disorders in growth and development. One challenge presented in counseling clients that have a fetus with one or more ultrasound anomalies with or without a

chromosome disorder is the limited ability to predict the degree of physical impairment the child may experience and, in many cases, the cognitive abilities of the child.

### 21.2.14 Genetic Testing in Prenatal Diagnosis

There are several important points to consider and discuss with clients to assist them in understanding the different options available to them and the potential implications of each option. Counselors should be prepared to discuss each test's diagnostic accuracy for the condition(s) for which they are at risk, further testing that might be needed in the case of inconclusive results, the fetal risk or maternal discomfort associated with each test, timing for testing during pregnancy, turnaround time for results and the expense and insurance coverage for the test. Asking an individual or couple to rank these factors as most and least important can help them choose the option that is best for them. A reflective summary by the counselor of their decision-making process helps to ensure that they have understood and weighed all the various alternatives and their implications.

In clients for whom the primary indication for prenatal diagnosis is the risk of fetal aneuploidy due to an abnormal serum screen, ultrasound findings, or maternal age, an FISH interphase study for the most common aneuploidies (13, 18 and 21) and chromosomes X and Y can be done on the sample. These results are often available 24–48 h after the procedure and can provide some rapid reassurance to many women about their pregnancy. It is important when providing these results to explain that it is only a limited evaluation of the fetal chromosomes and it does not evaluate all chromosomes or the structure of the individual chromosomes. The complete cytogenetic studies are usually available within 2 weeks of the procedure following culturing and full karyotyping.

There is a debate in the current literature and clinical genetics community about the applications of microarray-based aCGH to enhance and possibly replace cytogenetics for prenatal diagnosis. aCGH has the ability to detect copy-number alterations and very small duplications and deletions of genetic information that are not detectable with cytogenetic studies and can have an associated abnormal phenotype. The technology also reveals copy-number variants (CNVs) that may be benign or are of uncertain significance. One limit of this technology is that it does not detect balanced chromosome rearrangements (those that do not result in a net gain or loss of genetic information), which can be seen with conventional cytogenetic studies. These balanced rearrangements can have clinical consequences (52). One of the primary genetic counseling considerations with this technology is how to provide clients with this information during pregnancy in a way that will enable them to utilize it for pregnancy management and not increase anxiety unnecessarily. Van den Veyver et al. (45) reported the clinical use of aCGH in 300 prenatal cases and reported the identification of CNVs in 19.3%

of samples. Forty were classified as likely benign, 15 clinically significant and 3 were of uncertain clinical significance. The authors concluded that this technology did not present major genetic counseling challenges, however, they did not assess the client's perceptions or anxiety related to the results or the genetic counseling provided for the clients.

For women who seek prenatal diagnosis for single-gene disorders, it is prudent to explore the testing options for the condition before proceeding with an invasive procedure. One must consider numerous issues when working with a family that requests prenatal diagnosis for a specific condition. Some considerations are as follows: Has there been informative genetic testing in the family that can be used for prenatal diagnosis? Is there a laboratory that provides prenatal diagnosis for the condition in the family? Can the results be obtained in a timely manner for a client to use them for pregnancy management or termination if desired? What is the cost of the test and the insurance coverage for the testing? Owing to the complexity of arranging prenatal diagnosis for familial conditions, it is prudent for women with a personal or family history of a single-gene disorder that would like prenatal diagnosis to be referred for preconception genetic counseling or early in their pregnancy in order to provide them with as many prenatal options as possible.

### 21.2.15 Population-Based Carrier Screening

Specific genetic conditions such as Tay–Sachs disease and CF are known to be more frequent in certain ethnic populations. The genes for many of these conditions have been identified and carrier screening is now available and recommended on a population basis. Some women will be offered screening in their physician's office and others will be counseled and offered testing as part of a prenatal genetic evaluation. Ethnicity is usually self-reported by the client as part of the medical history. The health care provider should be cautious about assuming an individual's ethnic background based on outward physical appearances.

The American College of Obstetrics and Gynecology (ACOG) and the American College of Medical Genetics (ACMG) have published carrier screening recommendations (53–55). In most cases, clients will have no family history of the conditions for which screening is recommended and limited knowledge, if any, about these conditions. When a woman and/or her partner is identified to be a carrier for a particular condition, the risk to the current pregnancy or future children should be discussed as well as the possibility of prenatal diagnosis for the condition.

### 21.2.16 Communication of Risk in Prenatal Genetic Counseling

A great deal of the informational content of prenatal counseling pertains to risk: the estimated chance that a

fetal condition is present, the likelihood that it can be diagnosed with available techniques, the procedural risks, and the “background” incidence of various problems in the general population. Obviously, these factors are central to decision making, and care must be taken to ensure not only that they are understood but also that their emotional impact is addressed. Just the word “risk” elicits a negative response, and although it is difficult to avoid using it, words such as “chance” or “likelihood” are more emotionally neutral. Talking about background risks can also be alarming; few people embark on a pregnancy with awareness that even without any identifiable risk factors, there is a 3% chance that their baby will have a significant abnormality and a 2% chance that they could lose the pregnancy in the second half of gestation.

Helping clients understand how first- or second-trimester screening changes their chances for certain aneuploidies and neural tube defects can be especially challenging. Many primary care providers do not have a good understanding of the limits of certain maternal serum screening tests and will not be able to answer all of the client’s initial questions about the results. The genetic counselor oftentimes is the first person to have the opportunity to help clients understand that an “abnormal” test result suggests an increased risk for a particular condition and it does not necessarily indicate a fetal abnormality or chromosome disorder.

### 21.2.17 Discussion of Possible Diagnosis in the Fetus

The vast majority of clients who come for prenatal genetic counseling because of an abnormal maternal serum screen are at an increased chance for a specific chromosome aneuploidy or birth defect such as neural tube defect. The genetic counselor or professional working with the client should provide counseling regarding the screening results and should be well versed in the serum screening patterns, fetal and maternal factors that influence serum screen results and the phenotype of each of the possible conditions that can be identified with a maternal screen. The counselor should be able to provide the client with an accurate description of the conditions and the spectrum of the outcomes with each condition. Important things to discuss include any physical or cognitive impairments and special needs a child affected with a particular condition might have, any anticipated delivery considerations such as delivery at a different hospital or surgery after delivery, if a child with a particular condition would be more likely to die in infancy or childhood because of the condition and the genetic basis of the condition. These discussions can be very stressful for an individual or couple and it is essential that a counselor, be mindful of the impact of this discussion can have, anticipates certain reactions to the information such as distress, denial or fear and addresses any obvious psychosocial needs that a client or couple may express.

### 21.2.18 Exploration of Feelings about Testing and Possible Outcomes of Prenatal Diagnosis

At the time of referral, some individuals and couples may unwaveringly state their desire for a particular mode of prenatal diagnosis and their intentions in case of certain outcomes. Others may remain ambivalent and unsure of what to do about testing even after extensive counseling. Clients need to understand from the outset that the role of the counselor is to provide information about the tests and to help them make decisions they are comfortable with. This is particularly important in follow-up of an abnormal screening result, because the urgency of the referral and the anxiety surrounding it may make clients feel as though they have been thrust into a cascade of events over which they have little control. For some clients, the counselor’s nondirective message may not seem to match what they have heard from their primary practitioner. They may say that they are there only because their “doctor said I should have this test” or may even fear that their prenatal care will be jeopardized if they do not follow what they perceive as their prenatal care provider’s recommendation.

It is important that the counseling session not become so focused on the diagnostic methods and the diagnosable conditions that little attention is paid to the reasons for offering testing. Unfortunately, there is still a widespread perception that the sole purpose of prenatal diagnosis is to identify anomalous fetuses, so that their birth can be prevented by pregnancy termination. Other valid reasons that should be reviewed (according to the couple’s situation) are as follows: (1) the likelihood that testing will provide reassurance; (2) the fact that knowing more about the baby’s condition may change how the pregnancy and delivery are managed; (3) the ability to plan for the baby’s treatment in the neonatal period (or in rare cases, prenatally); and (4) the opportunity for the family to adjust, make plans, and marshal resources before the baby’s birth.

Families should also be informed that adoption is another alternative to continuing the pregnancy and caring for the child or pregnancy termination. There are specific adoption services that specialize in the placement of children with special needs with families that have requested the opportunity to adopt and care for a child with special needs.

Before a final decision is made about testing, the counselor should discuss with the client and her partner or support person if available, if they have considered what they would do in the event of an abnormal outcome. Although this is an uncomfortable question for both counselor and counselee, it does acknowledge that they may not be reassured by testing and could face a painful decision as a result. It also provides an opportunity for client to ask about the particulars of pregnancy termination if they wish and gather more information. This is a

good time for the counselor to mention that individuals and couples do not always end up making the decisions they thought they would, to normalize the fact that the wait for test results is always difficult, to invite the client to contact the counselor if they have questions in the interim, and to establish at what time and where they would like to be called with results.

### 21.2.19 Pregnancy Management of a Fetus with an Abnormality

The pregnancy management options that are available to a woman with a fetus with an abnormality or suspected abnormality will vary based on what point during the pregnancy the diagnosis is made. In general, people are offered the option to continue the pregnancy or pregnancy termination. Planned adoption and perinatal palliative care (56) are also options that should be made available when appropriate and available.

For women who elect to continue a pregnancy following the diagnosis of an anomaly or genetic disorder, the general focus of management is optimizing the delivery of the infant and neonatal care. In some cases, a woman will need to transfer her prenatal care to a perinatologist or maternal–fetal medicine specialist, has serial ultrasounds and delivers her baby at a different hospital. If the child will have immediate surgical or medical needs at birth, the family should be given the opportunity to meet with the specialists that will be involved in the neonatal care. The intervening time between the diagnosis and birth is also an important time for the family to plan for the needs of the child, contact support groups and early intervention specialists if needed and prepare their other children, if any, for the additional changes of having a new baby with special needs may bring. Women and couples also begin the active grieving process involved with the loss of the “normal child” and the adaption to the concept of parenting a child with a genetic condition or special needs.

Pregnancy termination options for women are to some degree regulated by state laws regarding the legal gestational age limit for a termination. Providers should be aware of the limits imposed by the state in which they provide care, how the gestational age is calculated and any time constraints that are imposed related to counseling about the termination procedure. In some cases of diagnosis at an advanced gestation age, a woman may have to travel to a different state to receive termination services if they are not legally available in their community.

Pregnancy termination via induction will result in the birth of a stillborn baby. This option provides the opportunity for clinical examination and fetal pathology. Also, additional genetic testing may be possible to determine a diagnosis if one was not established during the pregnancy. This information is very important, especially if a baby had ultrasound anomalies and normal prenatal genetic studies. Information gathered following the

birth of the baby is valuable to provide the parents with answers to why the baby had anomalies and the potential recurrence risk for future pregnancies.

Having an induction procedure also allows for the family to see and hold the baby. While many clients may be hesitant to take advantage of the option, the counselor should discuss with them the unique and limited opportunity that they will have with their baby. At a minimum, pictures should be taken of the baby for the parents to view at a later date. If an anomaly or genetic condition is identified early in a pregnancy, a woman may undergo a dilatation and evacuation (D&E). This procedure does not retain the fetus intact and viewing of the fetus and additional clinical examination will not be possible.

The psychosocial needs for couples who choose pregnancy termination include all of the grief processes that surround a miscarriage, stillbirth or infant loss with the additional feelings of shame and/or guilt for having elected to end a pregnancy (57). There are numerous resources for families that have been faced with this difficult decision that should be provided to the client. An extensive discussion of bereavement counseling is beyond the scope of this chapter, but there are many good resources for this special area of counseling. There is also a growing body of lay literature appropriate for couples that have had a miscarriage, stillbirth, or neonatal death. Numerous local and international support groups exist for parents who have lost a baby. Social services associated with obstetrics and pediatric units can often be of help in identifying resources for patient referral.

### 21.2.20 Pediatric Genetic Counseling Overview

Genetic counseling is provided to families of children with known genetic conditions or at risk for a familial condition, congenital anomalies, dysmorphic features, learning disabilities and mental retardation. ACMG has published recommendations for appropriate indications for referral for a genetic evaluation (58). When families are seen within a genetics center, they often meet with a genetic counselor as well as the medical geneticists. One of the primary goals of a genetics consultation is determining if a child has a genetic condition. Some children have a syndrome that is obvious to the geneticists at the time of the appointment, while in other cases, an etiology, genetic or otherwise, is never determined. It can be challenging to distinguish between environmental, multifactorial and single-gene disorders, which all need to be considered during an evaluation. The accurate diagnosis for a child is essential in order to provide the family with physical, developmental and health expectations for the child and to accurately provide them with recurrence risk for the parents, child and other family members.

Owing to the multisystem involvement of most genetic conditions, families also see a wide variety of other medical specialist, allied health care providers such as



occupational therapist and physical therapists in addition to their pediatrician. They may also interact with numerous educational specialists in order to develop adaptive education plans when needed. Families may ask genetic questions to any member of the health care and educational team, so in a sense, each team member may provide genetic counseling. For this discussion, the focus will be on genetic counseling within the context of the genetics center. Yet, it is important for any professional who works with individuals and families with a genetic condition to become knowledgeable about the genetic etiologies and full spectrum of the condition to better understand the patient or client. Genetics professionals are available for consultation to help all members of the team optimize the care and treatment of individuals with a genetic condition.

### 21.2.21 Overview of the Genetic Counselor in the Pediatric Setting

It is beyond the scope of this chapter to outline in detail the medical evaluation of a child that has been referred for a genetic evaluation; therefore, the emphasis will be on the goals of genetic counseling and the role of the genetic counselor in the evaluation. Other chapters in this book and other resources will provide a full discussion of a genetics medical examination because it is unique in the range of considerations. The genetic counselor plays a central role in the evaluation and care of a child by the genetics team. Many counselors work with medical geneticists or other pediatric specialists such as neurologist and orthopedists that routinely care for children with genetic disease. While the genetic counselor's interaction with the child is very important, it is essential for the counselor to establish a relationship with the parent(s) because he or she will likely interact with the family on numerous occasions. Many children and families are seen on a routine basis of every 6 months, yearly or every 2–3 years to monitor the development of the child.

Before a visit, a genetic counselor may confirm the accurate scheduling of an individual based on indication for referral, collect and review medical records, and contact the family by phone to collect preliminary medical and family history information. During the consultation, the genetic counselor will greet the family and establish the goals and agenda for the visit, collect the medical and family history and explore the family's expectations for the visit. The counselor will then review this information with the medical geneticists or other physician(s) who will see the patient. During the physical exam provided by the physician, the counselor may assist in the exam or assist in the documentation of the exam. The physician will often discuss and educate the family about the diagnosis or differential diagnosis, recommendations for testing, imaging or consultations with other specialists.

The genetic counselor will provide additional education for the family about the information provided by the physician. This may include the inheritance pattern or etiology of a diagnosis, full explanation of any recommended testing including the risks, benefits and limitations of the testing, recurrence risk of the condition for the parents and the child, management guidelines for individuals with specific diagnosis, psychosocial support for issues related to having a child with a medical or genetic condition, identification of resources related to the diagnosis and support groups and the establishment of a plan for follow-up and result disclosure if indicated. Genetic counselors also ensure that the consultation is appropriately documented and billed to the family's insurance company.

Routine follow-up is important in pediatric genetics. A large amount of medical information is provided to a family in the context of an often stressful situation and this can limit the amount of information that can be processed at any one time. Return appointments provide the opportunity to discuss test results and to review information and clarify any misconceptions or questions a family may have. Owing to the rapid advances in genetics, return appointments enable families to be informed of any developments and changes that could be important to their care. Some children are seen during infancy and it may not be until several years later that their diagnosis is obvious based on their growth and development or changes in the family history over time may reveal a diagnosis.

### 21.2.22 Counseling Considerations in a Child with a Birth Defect or Genetic Condition

For many people, the birth of a new baby brings with it their hopes and dreams for the future. Parents name their children, plan their future and set goals for their children before they are even born. These dreams are often devastated when a child is born with a birth defect or genetic disorder. Providing care and counseling for families in this emotionally charged setting can be challenging. In most cases, information about the condition and suspected diagnosis should be shared with the new parents as soon as possible. Some families will be seen in the hospital on the consult service and others will be seen in the office after the child is born. Similar to providing an abnormal prenatal test result, the counselor should be prepared to discuss the diagnosis in detail, including the short- and long-term consequences, assess the parent(s) support and resources, and have plenty of time for the parent(s) to ask questions and process the information and emotional reaction. The counselor should also have written information (pamphlets, websites, suggested lay reading) about the diagnosis or condition at the time of the disclosure to enable them

to identify trusted resources for good information. It is unlikely that the parents will hear all of what is said at the initial appointment or ask all of their questions, therefore, it is important for the counselor to contact the family after the visit to see if they have other questions and to provide contact information to the family, so they can call back or schedule an appointment for additional counseling and education.

One way to assess the couple's support network and their understanding of what they have been told is to ask them with whom they intend to share the information and what they will tell them. It is also important to ask the couple about their other children (if any), for they are sure to sense the parents' distress and may not only be alarmed about the baby but also fearful that something has happened to their mother. The counselor can help the couple decide how much the other children need to know at this point and suggest age-appropriate ways of discussing the baby's problems with them.

With confirmation of the diagnosis, the parents must begin to accept their new reality. This can be accompanied by feelings of responsibility, guilt over real or imagined past misdeeds, anger at the unjustness of the situation, disorientation, and overwhelming sadness. By preparing the couple for these reactions, the counselor can provide reassurance that the emotions are normal and appropriate and that they are not "losing control." They should also be cautioned that two of them may grieve in different ways and at different times and be admonished to keep the lines of communication open.

In later consultations or conversations with a child's parents, the counselor should specifically inquire about how the child's diagnosis has affected other family members, especially the grandparents and siblings, and about how their relationships with each other and with their family and friends have changed. It is also important to find out what lifestyle adjustments they have had to make (childcare, employment, recreation) and to help them differentiate between changes they have made because of the child's condition and those that would have been necessary with any new baby. Referrals to an early intervention program or support group should be made when appropriate. In some cases, one or both members of the couple will not progress in their grieving process, or maladaptive behavior patterns will emerge. At this point, the possibility of individual, marital, or family counseling with another professional should be raised.

Some families are unprepared for the challenges of caring for a child with special needs and need access to alternatives to caring for the child themselves. In these cases, the possibility of placing the child outside of the family, either temporary or permanent, can be raised. Parents should be connected with agencies that can assist with adoption of a child with a handicap or to provide good short- or long-term foster care. For parents who

place their child with an adoptive or foster family, their sense of grief at the loss of their "normal" child can be compounded by their perception that they have also failed in parenting the child. There may be guilt because they see themselves as selfish and the adoptive or foster family as unselfish. The counselor should not minimize the couple's feelings but can remind them of the considerations they took into account in making a decision that they felt was best for them and their child.

### 21.2.23 Evaluation and Counseling for an Older Child or Child with a Chronic Condition

For some children that have a genetic condition it may not be apparent during infancy and it is only when the child fails to meet developmental milestones, develops chronic health problems or has a specific pattern of health problems that the possibility of a genetic condition is addressed. The genetic counselor's role in the evaluation of an older child is very similar to the role of evaluating a newborn or infant but they will have the opportunity to build rapport with the child and the parent(s).

If a child's developmental or health problems have become increasingly apparent over time, much expense and emotional energy may have been devoted to finding a cause, and the family may actually be relieved to have a diagnosis at last. On the other hand, such a diagnosis can bring with it new and distressing information about additional threats that may lie ahead, and in some cases, removes any hope that the child will ever be "well." When a diagnosis such as CF or muscular dystrophy is made, a youngster who has been thought by the family only to have frequent infections, or just to be clumsy and tire easily, now has a known syndrome and the implications for the family as a whole are impacted. The family will understandably be thrown into crisis as they try to learn about the condition and initiate treatment amid fears for what lies ahead—emotionally, socially, and financially. Some families will be expecting another child or already had other children by the time the first child is diagnosed with a genetic condition and are faced with the possibility of having more than one child with a specific condition.

It is perhaps even harder for families to have a possible diagnosis raised without warning. This may occur, for instance, when a pediatrician expresses concern about mild dysmorphism, short stature, delayed development, a suspicious number of café-au-lait spots, or some other finding in a child whom the family perceives as entirely normal. In terms of the family's shock, denial, and anxiety over the implications of the finding, this situation is not unlike having a genetic diagnosis suspected in a newborn and warrants expediting the genetic evaluation and providing the same ongoing counseling. This situation

again raises concerns not only for the child but also for the parents and siblings.

### 21.2.24 Adults with Pediatric Genetic Conditions

An increasing number of individuals with conditions that were previously lethal in childhood are surviving to adulthood. The average life expectancy of a person with CF has increased into the mid-30s (59) and the life expectancy of an individual with Down syndrome is now 60 (60). These individuals face unique challenges in finding “adult” doctors who can help manage their genetic condition as well as other health issues common in adulthood. These adults oftentimes have the same desires for an education, a meaningful employment and a family as other adults. However, they are faced with a chronic inherited health condition that can impact these aspirations. For individuals with significant cognitive impairment, the identification of vocational resources and support services is challenging after they age out of the public education system. As the medical field continues to be successful in increasing the life expectancy for a growing number of persons with previously lethal childhood conditions, it also has to work to meet the needs of this unique adult population.

## 21.3 ADULT-ONSET DISORDERS

While the historical foundation of genetic counseling resides with prenatal and pediatric genetics, it has rapidly expanded into the area of adult-onset disorders. Genetic counseling for adults has been available for many years for certain single-gene genetic syndromes such as Huntington disease and other neurological conditions. Since the mid-1990s, there has been a large expansion into the field of cancer genetics with the discovery of genes responsible for familial breast, ovary, colon and other cancers. Most recently, the genetic counseling field has begun to branch out into the area of multifactorial adult-onset diseases such as cardiovascular disease. Below is a description of genetic counseling services in cancer genetics and adult-onset neurodegenerative disorders (Huntington disease) as two important and representative areas of adult genetics.

### 21.3.1 Counseling Considerations in Adult-Onset Disorders

The adult population that is evaluated for a genetic condition or predisposition to a disease such as cancer has many of the same concerns and needs as those previously addressed, but also some new ones. Many times, individuals who have been healthy their whole lives and have the expectation of good health well into late age are suddenly faced with an unexpected genetic disease

associated with significant morbidity or mortality. Alternatively, the disease may be known in the family and they have assumed that they too will be affected. These individuals may have also had children of their own with or without the knowledge that they had a condition that could be transmitted to them. In this case, there may be competing concerns; concern for their personal health and well-being and the additional concern about the consequences of passing on the condition to their children. This can lead to significant distress as well as difficulty in determining the best course of action with regard to identifying and managing genetic risk.

### 21.3.2 Cancer Genetic Risk Assessment and Counseling

At least 55 genetic syndromes associated with an increased risk of cancer have been described (61). Some are solely associated with elevated risks of cancer and/or benign tumors (e.g. hereditary breast ovarian cancer syndrome), whereas others involve specific physical features in addition to cancer risks (e.g. Beckwith–Wiedemann syndrome). Some are primarily adult-onset disorders (e.g. Lynch syndrome), whereas with others, onset can occur in childhood and/or adulthood (e.g. von Hippel–Lindau syndrome). Clients may be referred to evaluate their chances of having any of these hereditary cancer syndromes. However, the most common indications for referral are to rule out a hereditary breast cancer or hereditary colon cancer syndrome.

Clients are referred for cancer genetics services for a number of reasons including to assess personal cancer risks, to assess risks to other family members, to guide screening recommendations, to make decisions about preventive measures, and/or to have genetic testing to further clarify risks. Some individuals who present for services have already had a cancer diagnosis and others are unaffected. For those who have had cancer, some present at the time of their diagnosis, some are in the middle of adjuvant therapies and others are months or years out from their diagnosis and treatment. Some are healthy, some are in remission, and others are in the terminal stages of cancer. Many clients will have extensive family histories of cancer but a subset will be the first to be diagnosed in the family. The informational and psychological needs and the goals of cancer genetic counseling clients will vary based on these different variables.

Typically, cancer genetic risk assessment begins with a three–four generations family medical history. An accurate family history is critical in assessing whether an individual has an increased risk of developing specific types of cancer and in syndrome identification. Family history is also helpful in determining which other relatives may be at increased risk and in whom genetic testing would be most informative. For those relatives with cancer, the family history should include information about what type of cancer was diagnosed, at what

age the diagnosis was made, how it was diagnosed and treated, the relative's current age or age of death, and whether any relatives have had multiple primary tumors. For those without cancer, current age or age at death and information regarding screening practices should be collected. For both affected and unaffected individuals, information about relevant environmental exposures and prophylactic surgeries should also be gathered. Some syndromes are associated with benign tumors, dysmorphic features, or other medical conditions. When such syndromes are part of the differential diagnosis in the family, appropriate questions targeted to the symptoms of these conditions should be asked of both those affected and unaffected with cancer. Failure to ask such questions can impede accurate syndrome identification.

At times, medical record confirmation of reported cancer diagnoses is important for syndrome identification and risk assessment. For example, Schneider et al. (62) found that cancer diagnoses in hereditary breast ovarian cancer families were more accurately reported than in Li-Fraumeni syndrome families. Whereas relying on verbal reports alone would not have changed recommendations for genetic testing in the hereditary breast ovarian cancer families, underreporting of diagnoses in Li-Fraumeni families would have resulted in fewer than half of the families being offered testing (62).

An accurate family history sets the stage for risk assessment. There are two aspects of risk that are routinely addressed in cancer genetics—an individual's risk of developing cancer and his/her risk of harboring a mutation in a cancer predisposition gene. These risks are estimated by looking for characteristics of hereditary cancer in the family. The characteristics include early age of onset, multiple relatives in a single lineage affected with the same or related cancers, evidence of Mendelian inheritance, individuals with multiple primary cancers or bilateral cancers in paired organs, and/or the presence of rare cancers or benign tumors. On the basis of the presence or absence of these features, a family history can be classified as low (population) risk, moderate risk (probable multifactorial etiology), or high risk (suspected hereditary cancer syndrome).

Risk assessment models that quantify a person's risk of developing cancer are available for some cancers (e.g. breast and ovarian cancer) as are models that estimate mutation probabilities in some genes (e.g. *BRCA1/2*, *MSH2/MLH1*). Clinicians need to be familiar with the limits of these models in order to use them appropriately. In the absence of an existing model, assessing risk involves looking for characteristics of hereditary cancers and symptoms of syndromes that are part of the differential diagnosis in the family history. A physical examination may be part of the risk assessment process when the clinician is trying to rule out a condition with associated physical findings.

Factors including genetic heterogeneity, reduced penetrance, age-related penetrance, variable expressivity, and environmental modifiers (e.g. relatives who have had

prophylactic surgery) can complicate pedigree-based risk assessment. Underreporting of cancer diagnoses, inaccurate reporting and limited family structure can also have an impact. Finally, family history is dynamic and as such, a risk assessment may change over time. Clinicians need to be cognizant of these factors and take them into consideration whenever performing a pedigree-based risk assessment.

Once risks have been estimated, the next step is to communicate them to clients in the most effective way as possible. One part of communicating risk involves discussing the chance that a person will develop certain types of cancer. For some cancers, empiric estimates of absolute risk are available for those with moderate- and high-risk family histories. When possible, it is important to provide interval risks (over a certain period of time) in addition to lifetime risks. For example, the National Cancer Institute's breast cancer risk assessment tool (Gail model) provides 5-year breast cancer risks in addition to lifetime risks (63,64). Lifetime risks should be adjusted in consideration of the current age of the client. For instance, an 80-year-old woman, whose mother had breast cancer at 40, may have started out with a 20% lifetime risk of cancer but she has lived through a majority of this risk at her current age. Absolute risk estimates should be used whenever possible over relative risks as the latter are hard for clients to put into context. For instance, a threefold increase in risk may sound like a high risk, but could translate into a 1 in 1000 risks of cancer or a 30% risk of cancer, depending on the incidence of the cancer in the population studied.

When a hereditary cancer syndrome is suspected or has been identified, syndrome-specific cancer risks should be provided in the context of the likelihood that the client has familial mutation/predisposition to cancer. Most cancer risk estimates available in the medical literature represent the maximal risk of developing cancer and clients need to understand that there is variation in risk between and within families. In addition, some clients may not recognize that the syndrome in their family places them at risk for more than one type of cancer. For instance, a woman who has an *MSH2* mutation but a personal and family history of colon cancer only may not consider herself at risk for uterine cancer. The clinician will have to help her understand, in the context of variable expressivity and reduced penetrance, why she is at risk for the full spectrum of Lynch syndrome cancers even in the absence of a family history of these.

The second part of communicating risk is discussing the probability that a client has a mutation in a cancer predisposition gene. When a mutation has already been identified in a family, determining this risk is generally straightforward and the likelihood that the client will have the mutation can be presented with relative certainty. In contrast, when testing has not yet been done in a family, the limitations and variation in estimates of risks drawn from risk assessment models or clinical



criteria should be presented. Factors that could improve the validity of the estimate, such as verifying cancer diagnoses, should be discussed.

Before communicating risk information, the clinician should inquire about the client's perception of their own risk and knowledge of general population risks. If a client's perception of her own risk is significantly different than her actual risk, the clinician should anticipate that she may have a difficult time accepting the new risk information. Likewise, a client whose view of general population risks of cancer is very different from actual risks that may have a skewed perspective regarding the significance of her own risks. For instance, a client who is given a 7% risk of colon cancer may feel that this is a very high risk if she does not realize the population risk is 5–6%.

As illustrated above, a portion of the educational component of a cancer genetic counseling session involves educating the client about risk. Other components include any or all of the following: describing the different classifications of cancer in families—sporadic (low or population risk), familial (moderate risk) and hereditary (high risk, Mendelian inheritance) and how family history information is used to distinguish between the different classifications; reviewing the genetics of cancer and the role of somatic versus germline mutations in carcinogenesis; discussing the natural history (associated cancers, risks, and other physical features) of relevant cancer genetic syndromes; reviewing screening recommendations and preventative measures based on the client's risk; discussing the option of genetic testing, when applicable, to further assess cancer risk; and providing informed consent for genetic testing. Important elements of informed consent in genetic testing for cancer predisposition include who is/are the best candidate(s) in the family for testing, the likelihood of a positive result, the significance of a positive versus negative (normal) test result, the possibility of a variant of uncertain significance, how the results of genetic testing may affect clinical management, timing and readiness for genetic testing, current protections against genetic discrimination, psychological risks of testing including survivor's guilt, and the importance of informing other relatives when a mutation is identified (65).

The information shared during a cancer genetic counseling session most commonly triggers decisions about genetic testing and clinical management. Clients may be considering whether to pursue genetic testing or whether to ask another (more informative) relative to consider testing first. In some cases, the client may feel that the decision to test is not theirs alone. For instance unaffected family members may pressure an affected relative to undergo testing to provide genetic information for the rest of the family. In such cases, the genetics professional can help the client weigh the benefits and risks of testing for her and her family and discuss how to communicate her testing decision to relatives.

Sometimes parents are faced with making a decision about testing a child. Several organizations have policies or position statements that support deferring predictive testing for late-onset disorders, such as cancer until adulthood, and/or the child can participate in decision making when there is no medical benefit of testing (66). However, for some hereditary cancer syndromes, such as von Hippel–Lindau syndrome and familial adenomatous polyposis, screening for symptoms is initiated in childhood. In such situations, parents have to weigh the benefits, costs, and risks of screening a child who may or may not have inherited a familial mutation against definitively determining risk in childhood through genetic testing. The genetics professional can be instrumental in helping the parents consider all the consequences of such decisions.

With regard to clinical management decisions, clients may be deciding whether to follow through with screening guidelines or whether to pursue preventative measures such as chemoprevention or prophylactic surgeries. Alternatively, they may be making treatment decisions based on genetic status. For instance, a woman recently diagnosed with breast cancer who has a *BRCA1* mutation may choose bilateral mastectomy over lumpectomy in light of her elevated risk of developing a second breast cancer. When clinical evidence dictates that a specific course of action (such as colonoscopy) is in the best interest of the client, the genetics professional has a responsibility to recommend this course of action. This is very different than the role genetics professionals have in other decisions such as genetic testing or prenatal diagnosis; however, it is an accepted role in cancer genetic counseling.

The primary goal of cancer genetic risk assessment and counseling is to identify individuals at increased risk for the purpose of promoting early detection and prevention. However, a number of psychosocial factors can have an impact on a client's willingness to learn about cancer risk and follow through with clinical management recommendations. Living at increased risk of cancer can trigger a number of emotional responses including anger, anxiety, fear of disfigurement, fear of becoming a burden, fear of dying, grief, guilt, loss of control, negative body image, sadness, sense of isolation and shame (67). These responses can in part be related to personal experiences with cancer, outcomes in relatives with cancer, timing of cancer diagnoses (for instance, during significant life transitions), health belief model, experiences with medical care, familial beliefs about cancer etiology and familial screening/preventative practices. Such factors and experiences may motivate a person to take every precaution necessary to reduce the risk of developing or dying from cancer. On the other end of the spectrum, they may cause a person to deny cancer risks and avoid appropriate medical care. Therefore, identifying, determining the impact of, and discussing a client's psychological reactions to cancer risk are essential elements of effective cancer genetic counseling and risk assessment.

The need for follow-up after an initial cancer genetics consultation varies. Sometimes a cancer genetics consult is limited to one visit, as might be the case for a client at low or moderate risk whose medical management will be coordinated by a primary care physician. Sometimes additional family history information is needed for accurate risk assessment and a second consultation is scheduled to review this new information. When the first consult results in genetic testing, a follow-up consultation for results disclosure is often scheduled, typically in person but in some clinics by telephone. When at the first consult, the client is informed that another relative would be a better candidate for genetic testing, sometimes the client comes back for counseling with that relative or presents a second time independently once the relative has had testing.

When a mutation/syndrome has been identified in a client, follow-up includes a discussion of interval (when available) and lifetime cancer risks, current screening guidelines and available preventative measures, as well as referrals to other health care professionals as needed. The consultation also includes a discussion about risks to other relatives and the importance of notifying them of risk (duty to warn). Some clients also benefit from brainstorming strategies for contacting relatives. The follow-up consultation also provides the clinician an opportunity to assess how well the client is adjusting to the risk information and the likelihood of compliance with medical management. For those in whom noncompliance is a concern, additional follow-up may be warranted.

In summary, cancer genetic risk assessment and counseling is a growing area of genetics services. It is an area of genetics where early recognition of elevated risk can potentially reduce morbidity and mortality and as such significantly change the course of disease in families with generations of early cancer diagnoses and death. As such, the stakes can be high. Therefore, clinicians providing these services need to be well informed about the intricacies of cancer risk assessment, need to stay abreast of evolving phenotypes and changing management guidelines, and need to be willing to identify and address client resistance to optimal medical care.

### 21.3.3 Genetic Testing for Adult-Onset Neurodegenerative Disorders—Huntington Disease

Huntington disease was the first inherited adult-onset disorder for which predictive molecular testing was offered, initially by linkage analysis and then by direct analysis once the causative CAG expansion in the *HD* (*IT15*) gene was identified (68). Guidelines for predictive testing of this autosomal-dominant condition have been available for almost two decades and support a multidisciplinary approach that includes a neurological examination, psychological consultation, and genetic counseling (69). These guidelines have served as a model for predictive and presymptomatic testing protocols for other adult-onset

neurodegenerative diseases such as the spinocerebellar ataxias, early-onset autosomal-dominant Alzheimer disease, and hereditary amyotrophic lateral sclerosis.

Huntington disease is associated with typically adult onset of progressive motor symptoms including chorea, progressive cognitive impairment, and psychiatric disturbances. Symptoms begin on average between the ages of 35–44 years but with a range of 2–80 years. Most die from the disease 15–18 years after the onset of symptoms (70). There is no cure for Huntington disease and predictive testing does not influence clinical management. For this reason, when genetic testing by linked markers became available in the early 1980s, there was considerable debate about whether predictive testing was ethical. However, extensive discussions between clinicians, advocates, and families at risk led to the development of the first predictive testing protocol in 1986 (71).

Benefits of predictive testing for Huntington disease include that it can relieve the uncertainty of not knowing whether one will develop the disease and may be used for life planning, including reproductive decision making. However, uptake of testing has been relatively low, ranging from 3% to 21% (72). As such, it appears that many at risk do not want to know their status. In addition, about 40% of individuals who enter into a testing protocol do not proceed with testing (71). This suggests that pretest counseling plays a critical role in helping those at risk weigh the personal benefits, risks, and limitations of testing. The decision to test should be the client's decision alone. The role of the multidisciplinary team is to make sure that the client is adequately and completely informed; that he has considered the full set of consequences, both positive and negative, of genetic testing in light of his personal circumstances; that he is psychologically ready for the results; and that testing will not lead to adverse outcomes such as suicide.

Smith et al. (73) developed a set of 11 guidelines for genetic counseling and for managing the ethical situations that can arise from Huntington disease predictive testing based on their review of 29 cases. The guidelines tackle a broad array of issues and the counseling conditions necessary to address them appropriately. The issues include confidentiality, (the clinician) refusing or postponing testing, (the client's) freedom to reject disclosure, others preferences not to know genetic status, reproductive choices, and testing children. For instance, the guideline on confidentiality addresses a counselor's ethical obligation to maintain a client's genetic information as private, yet recognizes that an exception may have to be made when there is a "real, pressing, and demonstrable" public safety concern (73). The guideline on "others preferences not to know" addresses the situation where one relative's genetic test will reveal the status of another relative who does not want to know (e.g. child's positive test result revealing parental status) and states that this is not a sufficient reason to deny or delay testing (73).

In terms of counseling conditions, the guidelines developed by Smith et al. speak to the necessity of skilled counseling, the importance of providing appropriate information (anticipatory guidance), and the value of pre- and posttest counseling. These guidelines direct clinicians to learn about a client's experiences with and perspectives on Huntington disease, and the impact that the condition has had the family. They encourage clinicians to use this information to anticipate potential ethical dilemmas or adverse reactions. The guidelines describe the necessary components of pretest counseling and that informed consent goes beyond education regarding natural history and risk to include a discussion of potential psychological sequelae, insurance and/or employment discrimination, and familial repercussions. They also address those circumstances under which the client's request for testing may be refused by the clinician, such as when a client is suicidal or emotionally not able to cope with predictive information (73).

There are a number of questions that the clinicians involved in the predictive testing protocol might ask in assessing a client's experiences, motivations, and readiness for predictive testing. What has it been like having a relative with Huntington disease? Did/does the client have close contact and/or serve as a caregiver for an affected relative or was/is there little contact? Who in the family is considered to be affected, even if she or he has not yet developed symptoms? What does the client think his genetic status is—is he expecting to develop Huntington disease or does he think that he is not at risk? What changes would the client make in his life, plans, or health care if he knew for certain that he would (or would not) develop the Huntington disease? Has the client made any life decisions already based on his anticipated genetic status? How would the information influence reproductive decisions, including possible use of prenatal or preimplantation diagnosis? What are possible economic consequences of learning genetic status, such as loss of health or life insurance? With whom would the client share his results and why? How would having or sharing his genetic status change his relationships with his relatives, spouse, or friends? How would he feel if he was genetically at risk but another close relative was not, or vice versa? Is this the best time to have testing? Why now?

It is also important for the psychologist or other clinicians involved in the predictive testing protocol to specifically ask about the client's mental health history. Does the client have a history of depression or other psychiatric illness? Has he ever required medication or hospitalization for this or had counseling or psychotherapy? Does the client have an ongoing relationship with a therapist, and if so, has he discussed his risk and thoughts about genetic testing with the therapist? Does the client currently feel anxious, depressed, or hopeless? Although prior mental illness is not by itself a contraindication to predictive testing, it may serve as a risk factor for an adverse reaction. Several studies have shown that fewer than 10% of people

undergoing predictive testing for Huntington disease have had a significant adverse event (suicide, suicide attempt or plan, diagnosis of clinical depression, or psychiatric hospitalization) after testing. However, those who did were likely to have had poorer psychological functioning before testing and were more likely to drop out of follow-up studies (74,75). The critical periods for suicide risk are just before receiving a diagnosis and later, when affected individuals experience a loss of independence (76).

For those who proceed with testing, face-to-face results disclosure with the client and at least one support person is most desirable. This provides the predictive testing team an opportunity to assess the client's reactions and to make an appropriate and tailored plan for follow-up. It is important to recognize that carrier status alone (carrier versus noncarrier) does not determine how a client will respond to results. A noncarrier may be relieved that neither he nor his children are at risk. Alternatively, a noncarrier who has lived her life thinking she is a carrier and who has made major life decisions based on this supposition may be unprepared for her new status. A noncarrier may also feel survivor's guilt or be distressed by her newly defined role as family caregiver rather than patient. As such, follow-up for both carriers and noncarriers may be warranted.

In summary, Huntington disease is an incurable, adult-onset neurodegenerative disease, which by virtue of its autosomal-dominant inheritance pattern, can have a significant impact on the families affected. Although identifying risk presymptomatically does not change medical management or the course of the disease at this time, a subset of at-risk individuals find value in learning their status. For those interested, testing should ideally be done in the context of a multidisciplinary approach that includes a neurological examination, psychological evaluation and genetic counseling. Skilled pre- and post-test genetic counseling that helps the client in identifying and taking into consideration his experiences, perceptions, motivations, and readiness for predictive testing is essential. Once counseling has been provided, the client should have the right to make his own decision about predictive testing, without pressure or coercion. For those who have testing, adequate posttest support, particularly at critical periods in the natural history of the disorder, is important in preventing adverse outcomes.

The Huntington disease predictive testing protocol has served as the model for predictive testing in other adult-onset conditions. Some of these conditions are similar to Huntington disease in prognosis and outcomes and some are different in important ways. Clients and clinicians invest (out of necessity) a lot of time in this protocol. Given the limited number of experienced professionals available, this has the potential of limiting access to services. Thus, as more predictive genetic tests become available, it will be important to evaluate for which conditions this protocol is vital and when alternative methods can achieve informed consent and positive counseling outcomes.

## 21.4 GENETIC RISK ASSESSMENT AND CALCULATION IN THE CLINICAL SETTING

As previously mentioned, accurate risk calculations are an important part of the genetics assessment. Genetic counselors are called upon to provide a variety of risk calculations and estimates for known single-gene disorders, multifactorial conditions and teratogens. In order to provide the most accurate and individualized risk, one must be able to collect and interpret client pedigrees, consider limiting factors within a pedigree and be aware of genetic factors that can modify gene expression. Below is a discussion of the risk assessment process and considerations in the genetic counseling practice.

### 21.4.1 Mathematical Methods of Risk Calculation

Risk calculation relies on the application of Mendelian genetic principles of inheritance patterns that may then be further modified or conditioned using mathematical principles such as Bayesian analysis and the laws of addition and multiplication for individualized risk assessment. The Unified Approach to Bayesian analysis is useful when there are numerous variables to consider modifying a risk. This method relies on the “Rule of All Configurations” and “The Rule of Fundamental Probabilities” (77). The appropriate applications of these mathematical principles result in a quantitative risk assessment that can incorporate information such as genetic test results, physical findings, and the number of unaffected individuals in a family to adjust a Mendelian risk derived from known inheritance patterns for individualized risk assessment.

The laws of addition and multiplication are important concepts to understand in order to apply quantitative risk assessment. The law of addition (“or”) states that if two (or more) events are mutually exclusive, and if the probability of event 1 occurring is  $P_1$  and that of event 2 occurring is  $P_2$ , then the probability of either the first event or the second event occurring equals  $P_1 + P_2$ . For example, twins can be either monozygotic (MZ) or dizygotic (DZ) but they cannot be MZ and DZ. Therefore, the probability that they will be either MZ or DZ equals 1. The law of multiplication (“and”) is applied when two or more events are independent. For example, for DZ twins born to parents who are carriers for CF, the probability that both the first and second twins will have CF is  $1/4 \times 1/4 = 1/16$ .

Example 1: Healthy parents have had a child with a severe autosomal-recessive disorder (Figure 21-1). They are counseled about autosomal-recessive inheritance and are informed that they have a 1/4 chance to have a child that is affected, a 1/2 chance to have a child that is an unaffected carrier and a 1/4 chance to have a child that is an unaffected noncarrier for the condition. Their next pregnancy is determined to be a twin pregnancy. The parents now wish to know the probability that at

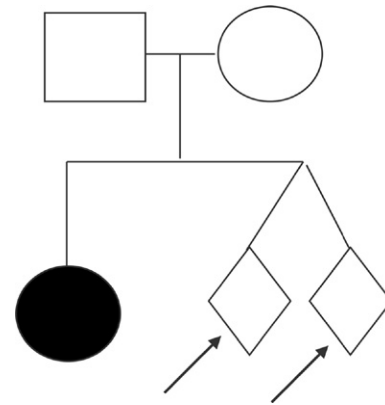


FIGURE 21-1 Pedigree for example 1.

least one twin will be affected by the autosomal-recessive disorder. Zygosity has not yet been established.

To answer this question, the risks are first calculated for the two mutually exclusive possibilities that the twins are either (1) MZ ( $P = 1/3$ ) or (2) DZ ( $P = 2/3$ ).

1. *Monozygotic*. In this situation, the probability that both twins will be affected equals 1/4, that only one will be affected equals 0, and that both will be unaffected equals 3/4.
2. *Dizygotic*. If the twins are DZ, then the genotype of one twin does not influence the genotype of the other twin (i.e. these events are independent). Therefore, the probability that both twins will be affected equals 1/16 (i.e.  $1/4 \times 1/4$ ), the probability that only one will be affected equals 3/8 [i.e.  $(1/4 \times 3/4) (3/4 \times 1/4)$ ], and the probability that both will be unaffected equals 9/16 (i.e.  $3/4 \times 3/4$ ).

Using this combination of mutually exclusive and independent events, the parents' question can now be answered. The overall probability that

- (a) both twins will be affected equals  $1/3 \times 1/4$  (MZ) plus  $2/3 \times 1/16$  (DZ), giving a total probability of 1/8;
- (b) only one twin will be affected equals  $1/3 \times 0$  (MZ) plus  $2/3 \times 3/8$  (DZ), giving a total probability of 1/4;
- (c) both twins will be unaffected equals  $1/3 \times 3/4$  (MZ) plus  $2/3 \times 9/16$  (DZ), giving a total probability of 5/8.

With this information, the parents can be reliably informed that there is a probability of  $1/8 + 1/4 = 3/8$  that at least one of their unborn babies will be affected. Another way to approach answering the same question is to subtract the chance that neither baby will be affected from 1.

### 21.4.2 Bayes Theorem

The application of Bayes theorem enables one to calculate a conditional probability or risk considering one or more conditions or events (such as carrier or



noncarriers, affected or unaffected) and then weighing or “conditioning” these by incorporating relevant information. The incorporation of all possible conditions will enable one to derive a risk for a specific individual in a specific family and avoid generalized estimates that may be gross over- or underestimates of risk. It is important to consult with colleagues when calculating complex cases with multiple factors.

The initial probability of each event is known as its prior probability and is based on “anterior” information such as the phenotypic findings and/or family history. The observations that modify these prior probabilities allow conditional probabilities to be derived from “posterior” information such as the results of a carrier tests. The resulting probability for each possibility or event is known as its joint probability (prior probability  $\times$  joint probability). The Unified Approach’s Rule of All Configurations requires one to determine all possible genetic configurations in family and calculate the joint probabilities for each configuration and then use this information to determine the posterior probabilities (77). The overall final probability of each event, which is known as its posterior or relative probability, is obtained by dividing its joint probability by the sum of all the possible joint probabilities. A table (Table 21-8) can be utilized to apply Bayes theorem to clinical situations and the table should be drawn up that include all relevant possibilities and that all informative observations should be used once and only once. The following example utilizes this theorem in a clinical setting.

Example 2: The consultant, II<sub>1</sub> in Figure 21-2, wishes to know the probability that her male fetus will have X-linked hemophilia. She has one previously unaffected son. She had a brother and two maternal uncles with a clinical diagnosis of the condition but no molecular testing is available. In order to answer this question for her, one must calculate her chance to be a carrier considering the family history information and then her chance to pass on the gene for hemophilia if she is a carrier.

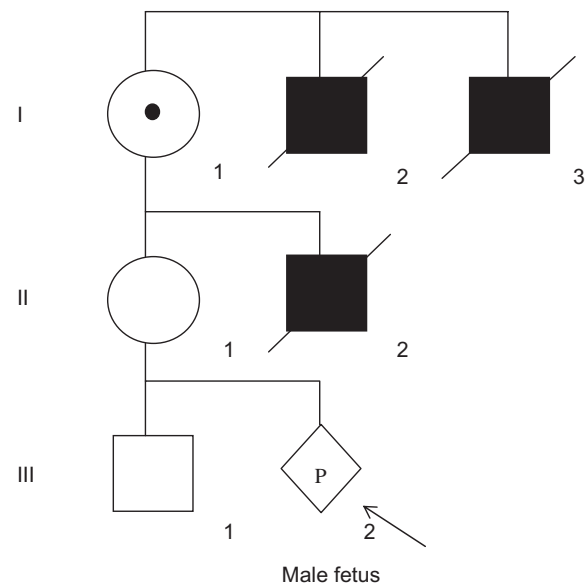
On the basis of the family history information, her mother is an obligate carrier for the condition. This anterior information allows us to assign II<sub>1</sub>, a prior probability of being a carrier as 1 in 2. Similarly her prior probability of not being a carrier also equals 1 in 2. These are mutually exclusive events, and the sum of their probabilities equals 1.

This consultant already has one unaffected son. This information decreases her chance to be a carrier. The conditional probability that a carrier would have an unaffected son is 1/2. The conditional probability that II<sub>1</sub> would have one unaffected son if she is not a carrier equals 1, that is,  $1 \times 1 = 1$ .

All this information is now incorporated in a Bayesian table (Table 21-9). The posterior probability that II<sub>1</sub> is a carrier equals 1/3 and the chance she is not a carrier equals 2/3. Considering that this is an X-linked condition, there is a 50% chance she would pass on the genetic

**TABLE 21-8 Basic Bayesian Calculation Table for One Condition**

	Outcome A (Example: Carrier or Affected)	Outcome B (Example: Noncarrier or Unaffected)
Prior	a	b
Conditional	c	d
Joint	ac	bd
Posterior	$\frac{ac}{ac + bd}$	$\frac{bd}{ac + bd}$



**FIGURE 21-2** Pedigree for example 2.

**TABLE 21-9 Example 2 Bayesian Table**

	II <sub>1</sub> Carrier	II <sub>1</sub> Noncarrier
Prior	1/2	1/2
Conditional (1 prior unaffected male)	1/2	1
Joint	$1/2 \times 1/2 = 1/4$	$1 \times 1/2 = 1/2$
Posterior	$\frac{1/4}{(1/2 + 1/4)} = 1/3$	$\frac{1/2}{(1/2 + 1/4)} = 2/3$

mutation to her male fetus if she was a carrier; therefore, the chance for an affected male fetus equals 1/3 (II<sub>1</sub> carrier risk)  $\times$  1/2 (chance to pass on the affected X) = 1/6 and the chance of an unaffected male fetus is 5/6.

### 21.4.3 Application of the Hardy–Weinberg Law in Risk Assessment

The Hardy–Weinberg law is a fundamental principle of population genetics that can be applied to determine the carrier frequency in a population of an

autosomal-recessive condition. The Hardy–Weinberg law states that if the frequency of the normal (wild-type) gene equals  $p$ , and the frequency of the abnormal (mutant) gene equals  $q$  so that  $p + q = 1$ , then the incidence of noncarriers, carriers, and affected will be  $p^2$ ,  $2pq$ , and  $q^2$ , respectively. This conclusion applies if the population is in Hardy–Weinberg equilibrium and involves several assumptions:

1. A large, randomly mating stable population.
2. Either a constant mutation rate, which balances out those genes lost because of death of affected homozygotes, or no new mutations and no selection against affected homozygotes.
3. No selection for or against carriers.

These assumptions are not always valid, and special care should be taken not to calculate carrier frequencies when the disease incidence has been derived from a small consanguineous community. For practical counseling purposes, these limitations can usually be ignored.

If the Hardy–Weinberg law applies, then the incidence ( $I$ ) of carriers in the general population, which will equal the probability that a healthy member of that population is a carrier, will approximate to twice the square root of the disease incidence ( $q^2$ ), that is

$$I = 2 \times p \times q (= 2q \text{ as } p \text{ is very close to } 1)$$

Therefore, for conditions such as cystic fibrosis and phenylketonuria, which have incidences of approximately 1 in 2500 and 1 in 10,000, respectively, the respective carrier incidences will be

$$2 \times \sqrt{(1/2500)} = 1/25$$

$$2 \times \sqrt{(1/10,000)} = 1/50$$

The ability to apply the Hardy–Weinberg law will enable one to calculate the carrier frequency of a recessive disease with a known disease incidence. This is helpful when calculating a risk for an affected child when one parent has a family history of a specific autosomal-recessive condition and the partner's carrier risk is equal to the general population.

#### 21.4.4 Calculating Risk for At-Risk Family Members and Autosomal-Recessive Conditions

Determining the recurrence risk for a couple that has had a child with a recessive condition is generally straightforward based on Mendelian inheritance. The assumption is that both parents are carriers and there is a 1/4 chance with each additional pregnancy to have another affected child. It is important to remind clients that each pregnancy is a new event and that one affected child does not translate to three subsequent unaffected children. Informative molecular testing on the affected

individual can allow for carrier testing in family members. There are genetic mechanisms that may make this untrue such as a new mutation of a recessive allele as seen in spinal muscular atrophy or uniparental disomy of the recessive allele as reported in cystic fibrosis, but these are very rare. One must also consider possible false paternity if the reported father does not share a known allele with the affected child.

The probability that the healthy full sibling of an affected individual is a carrier equals 2/3 rather than 2/4, as the fact that the sibling is unaffected means that the denominator is 3 and not 4. It is important to document full siblings and half siblings as their risk to be a carrier will be different. The unaffected half sibling to an individual with an autosomal-recessive condition will have a 1 in 2 chance to be a carrier for the condition. The risks to extended family members of an affected individual are smaller than siblings' risk but usually increased over the general population and can be calculated by tracing the relationship through the pedigree.

Example 3: For example, the female first cousin of an individual with cystic fibrosis (CF) will have a 1 in 4 chance to be a CF carrier as compared to the approximate general population risk of 1 in 25 in the non-Hispanic Caucasian population (Figure 21-3). This information can then be used to determine her chance to have a child with CF.

In this case, if one assumes that both members of the couple are non-Hispanic Caucasian and there is no family history of CF on the father's side of the family, the chance of an affected pregnancy would be calculated by multiplying the mother's carrier risk (1 in 4) by the father's general population carrier risk (1 in 25) by 1 in 4 (chance of two carrier parents to have a child with an autosomal-recessive condition). Their chance to have a child with CF is 1 in 400, which is significantly increased over the general population chance for a non-Hispanic Caucasian couple with no family history of 1 in 2500. If there is consanguinity in the family, the risk for an autosomal-recessive disease may be significantly increased depending on the degree of the relationship between the parents.

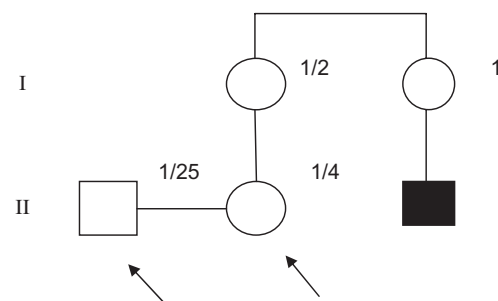


FIGURE 21-3 Pedigree for example 3.

### 21.4.5 Modifying Factors in Mendelian Conditions and Accurate Risk Assessment

Numerous genetic factors may influence the expression of a condition within an individual or family and these factors should also be considered when determining the risk of a condition for an individual. These factors include reduced penetrance, variable age of onset, somatic or germline mosaicism, and new mutations. These concepts are reviewed here related to risk assessment in the clinical setting and detailed discussions of these principles in other places within this text.

### 21.4.6 Reduced Penetrance

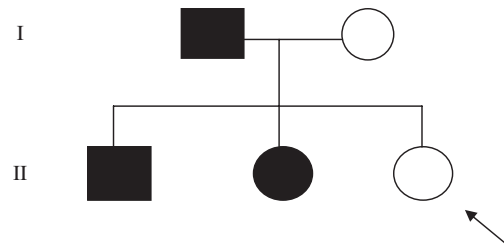
Reduced penetrance is observed in conditions in which only a portion of individuals with a specific genotype express the phenotype associated with the mutation. Individuals who fail to express the condition but carry the mutation can pass on the condition to their offspring, who may express the condition. Penetrance is an all-or-none concept and it remains unclear the factors that influence penetrance. It is presumed that other genes, allelic or nonallelic, somehow influence expression of the relevant allele. Alternatively, expression of an allele may require a second and somatic mutational event, which must occur within a specific organ and time frame. This concept presents a challenging counseling dilemma and a great deal of uncertainty for the client. It is important to explain the concept clearly when discussing the implications of a condition that is known to have reduced penetrance. Retinoblastoma and split-hand deformity are two autosomal-dominant conditions with known reduced penetrance. In these families, it may appear to the family that the condition is “skipping a generation” when in fact the genetic mutation is present, but not expressed. Knowledge of the expression of a condition can be applied to Bayesian analysis to calculate the risk for an individual to have an affected offspring or to be a carrier him or herself.

**Example 4:** Consider the pedigree below (Figure 21-4). This is a known autosomal-dominant trait in which 70% of individuals with the mutation will express the condition and 30% will be nonexpressing carriers with a 50% chance to pass the allele on to each child. In this case, the penetrance ( $P$ ) is calculated as 0.7. The consultant would like to know her risk to be an unaffected carrier.

This result estimates her chance to be a nonexpressing carrier of the mutation for the disease in question is approximately 3/13 or 23% (Table 21-10).

Furthermore, her chance to have an affected child would be:

$3/13$  (her chance to be a carrier)  $\times 1/2$  (chance to pass on dominant allele)  $\times 0.7$  (penetrance) = 0.08 or 8%.



**FIGURE 21-4** Pedigree for example 4.

**TABLE 21-10** Bayesian Analysis for Example 4

	Carrier	Noncarrier
Prior	1/2	1/2
Conditional	3/10	1
Joint	$1/2 \times 3/10 = 3/20$	1/2
Posterior	$\frac{3/20}{3/20 + 10/20}$	$\frac{10/20}{3/20 + 10/20}$

### 21.4.7 Variable Age of Onset and Late-Onset Disorders

While a germline mutation for a genetic condition is present from the time of conception, when the mutation expresses itself can be variable based on the condition. In some conditions, the expression of that mutation may be so significant that it is lethal in utero or a prenatal ultrasound will reveal anomalies suggestive of the condition. In others, it may not express until childhood or well into adulthood. For the parents of children at risk for conditions that express in later childhood such as some forms of spinal muscular atrophy or Duchenne Muscular Dystrophy (DMD), the watch and wait period can be agonizing. For conditions that do not express until adulthood, there are unique counseling considerations because the at-risk individual may have already had children of their own and unknowingly passed on the condition to their children.

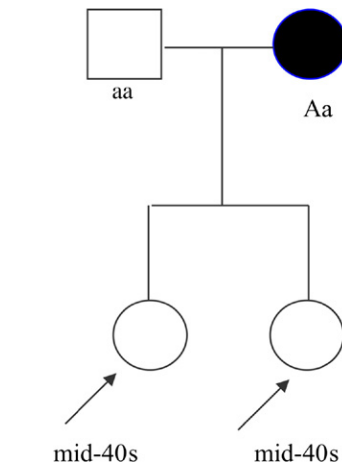
It is important to be knowledgeable of the typical age of onset for a particular condition in order to accurately evaluate and interpret a medical exam and/or family history. For example, a normal neurological exam in an 18-year-old male who has two siblings ages 16 and 24 and a 55-year-old parent with Huntington's disease is of little clinical value to reassure him that he did not inherit the condition because he is younger than the mean age on onset (35–44 years). However, if the consultant is 78 and his siblings are 76 and 84 and none of them have developed neurological symptoms of the condition, it is much less likely that anyone of them inherited the condition from their parent.

Increasingly, genetic testing is available for many conditions with variable or late onset; however, an

unaffected at-risk individual may or may not choose to be tested for the condition due to a multitude of concerns about the implications for their own health, their children and other family members and concerns related to genetic discrimination. In this situation, the clinical information and family history information can be used applying Bayesian analysis to modify an individual's genetic risk. Accurate risk assessment is also helpful in deciding if a test is worth the financial costs based on a client's risk to carry the condition.

**Example 5:** Two sisters request counseling for an autosomal adult-onset disorder that is affecting their mother (Figure 21-5). Both women are in their 40s. The penetrance for the disorder at their age is 44% and the sporadic risk is 4%.

Using Bayesian analysis, their chance of carrying the disease-causing allele has been reduced from 50% to 37% (Table 21-11).



**FIGURE 21-5** Pedigree for example 5.

### 21.4.8 Germline Mosaicism and New Mutations

When providing recurrence risk counseling for a couple that has had a child with an autosomal-dominant condition and the family history is negative for the condition, one must consider the proband as having a new mutation in the family or germline mosaicism in one of the parents. The potential recurrence risk in each case will likely be significantly less than the expected 50% if one parent were affected, but should be established for the client. A new mutation expresses when a proband has a mutant allele on one locus that is not inherited from either parent. For conditions with variable expression such as neurofibromatosis, a careful examination of the parents for any previously unrecognized medical symptoms of the condition should be done to confirm that both of the parents is truly unaffected.

Germline mosaicism is a result of postzygotic mutation in an individual's gametes that results in all or a portion of their gametes harboring the mutation for a specific condition that is not otherwise phenotypically expresses in the individual. Germline mosaicism has been reported in numerous conditions (OI, NF1, retinoblastoma) (78) and clients should be counseled about potential for recurrence in a family that has been previously unaffected.

### 21.4.9 Direct Mutation Analysis and Multiple Alleles

While genetic testing is available for a growing number of conditions, in some instances, the testing does not identify all mutated alleles in individuals with a specific genetic condition or as a part of general population screening for a common condition. CF is one

TABLE 21-11	Bayesian Analysis for Example 5	
	Woman is Aa	Woman is aa
Prior	0.50	0.50
Conditional (unaffected)	$1 - 0.44 = 0.56$	$1 - 0.44 = 0.96$
Joint	$0.50 \times 0.56 = 0.28$	$0.50 \times 0.96 = 0.48$
Posterior	$0.28 = 0.37$	$0.48 = 0.63$
	$0.28 + 0.48$	$0.28 + 0.48$

example of a relatively common genetic condition with over 1000 disease-causing alleles but only a fraction of those are part of the population screening program based on the frequency of specific mutations in certain ethnic groups. ACMG recommends testing for only 23 of these mutations for general population screening (79). Routine carrier screening of the non-Hispanic Caucasian population will identify approximately 90% of gene carriers and it will identify 97% of carriers in the Ashkenazi Jewish population. Therefore, in this condition, a person who receives a negative carrier test for CF will not have a risk equal to "0" but they will have a significantly decreased risk over their prior risk of the general population. This is an important counseling point when explaining to clients that they can have a negative test but still have a relatively small, but real risk to have an affected child.

**Example 6:** The review of a client's family history reveals that she had a first cousin with CF. Molecular testing was not completed on the affected individual. Both members of the couple are non-Hispanic Caucasians and there is no known history of consanguinity. Both members of the couple have had negative carrier screening. Their risk would be reduced independently based on their prior risk to be a carrier. The mother's risk is shown in Table 21-12 and the father's risk is shown in Table 21-13.

This couple's revised risk to have a child with CF is  $3.2\% \times 0.4\% \times 25\% = 0.0032\%$  (1/33604).



**TABLE 21-12** Example 6 Mother's Bayesian Analysis

Mother's Revised Carrier Risk	Carrier	Noncarrier
Prior	$\frac{1}{4}$	$\frac{3}{4}$
Condition negative carrier test	$\frac{1}{10}$	1
Joint	$\frac{1}{40}$	$\frac{3}{4}$ (30/40)
Posterior	$\frac{1/40}{1/40 + 30/40} = \frac{1}{31}$ (3.2%)	$\frac{3/4}{1/40 + 30/40}$

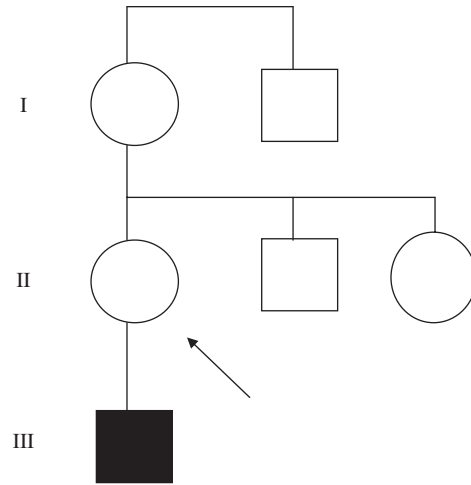
**TABLE 21-13** Example 6 Father's Bayesian Analysis

Father's Revised Carrier Risk	Carrier	Noncarrier
Prior	$\frac{1}{28}$	$\frac{27}{28}$
Condition negative carrier test	$\frac{1}{10}$	1
Joint	$\frac{1}{280}$	$\frac{27}{28}$
Posterior	$\frac{1/280}{1/280 + 270/280} = \frac{1}{271}$ (0.4%)	$\frac{270/280}{1/280 + 270/280}$

### 21.4.10 Risk Assessment in X-Linked Conditions

When reviewing family histories for X-linked conditions, it is important to appreciate the difference in risk assessment for an isolated case of the condition in a family or if there are multiple affected family members. For a woman who has a brother and a son with DMD, it is clear based on Mendelian inheritance that she is an obligate carrier for the condition and her risk to have another affected son is 50% and each of her daughters will have a 50% chance to be a DMD carrier.

However, determining the risk associated with X-linked recessive lethal conditions becomes more challenging when it is the first incidence of the condition in the family (Figure 21-6). Because severe X-linked conditions have a rate of de novo or new mutations, the challenge in these cases lies in determining in whom the pathogenic mutation began in a family. They may be the result of a new mutation in the affected male, a new mutation in the mother passed on to the affected male, or a mutation in the maternal grandmother passed on to the mother of the affected male. Specifically to calculate the prior probability of a carrier state in female relatives of an isolated case of an X-linked lethal disorder, one must consider (1) whether the mother of an affected son inherited the mutation from her mother; (2) whether the mother has a newly mutated allele on the X chromosome she inherited from her mother or (3) whether the mother has a newly mutated allele on the X chromosome she inherited from her father. Historically, the assumption

**FIGURE 21-6** Pedigree for X-linked recessive lethal condition.**TABLE 21-14** X-Linked Lethal New Mutation

	Mother Carrier	Mother Noncarrier
Prior	$4\mu$	$1-4\mu$ (approximately 1)
Conditional (affected son)	$\frac{1}{2}$	$\mu$ (new mutation in the X her son inherited from her)
Joint	$2\mu$	$\mu$
Posterior	$\frac{2\mu}{2\mu + \mu} = \frac{2}{3}$	$\frac{\mu}{2\mu + \mu} = \frac{1}{3}$

has been that there are equal mutation rates per locus per meiosis in males ( $\nu$ ) and females ( $\mu$ ). The mother in the first generation is not a carrier, and affected males are unable to reproduce (genetic fitness = 0). In the second generation, the probability that a female will be a carrier equals  $2\mu$ . In the third generation, this value raises to  $3\mu$ , that is, half of the mother's probability of  $2\mu$  plus  $2\mu$ . By generation  $n$ , this probability of heterozygosity will have reached  $4\mu$ . This then is the value for the prior probability that any female who does not have a positive family history will be a carrier of a sex-linked recessive disorder with zero fitness in affected males when mutation rates are equal in the sexes.

The mathematical result in this situation results in 1/3 of cases the male with the condition is the result of a new mutation and in 2/3 of cases the mother is the carrier (Table 21-14).

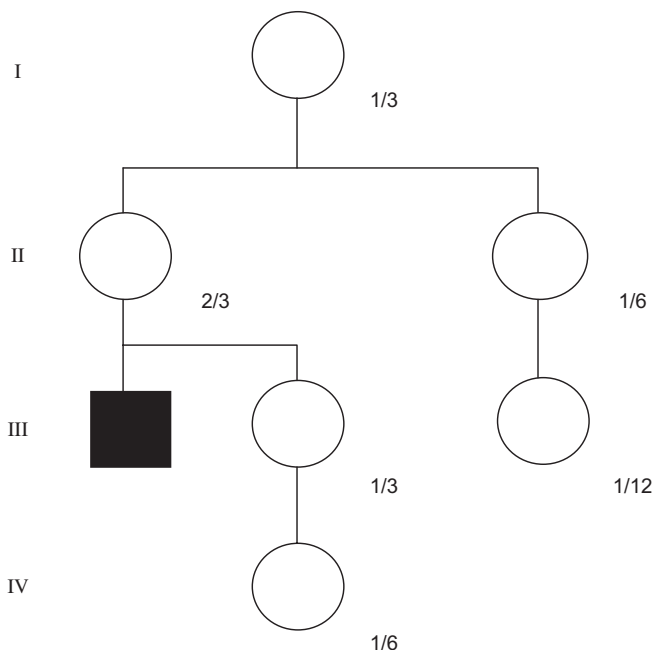
Using Bayesian analysis (Table 21-15), the risk that the maternal grandmother of an isolated case is a carrier is 1/3 (Figure 21-7). Knowledge of this information makes it easy to calculate the carrier risk for extended female relatives.

Interestingly, there are now studies that suggest that the new mutation rates for certain conditions such as X-linked myotubular myopathy and DMD differ in male and female germ lines (80), which would have an impact on calculating the carrier probabilities in X-linked disorders.

**TABLE 21-15** Bayesian Analysis for X-Linked Lethal Condition

	Maternal Grandmother Carrier	Maternal Grandmother Noncarrier
Prior	$4\mu$	$1-4\mu$ (approximately 1)
Conditional (affected grandson)	$1/4$	$2\mu^a$
Joint	$4\mu \times 1/4 = \mu$	$1 \times 2\mu$
Posterior	$\mu = 1/3$	$2\mu = 2/3$
	$2\mu + \mu$	$2\mu + \mu$

<sup>a</sup> $\mu$  (new mutation in affected grandson) +  $1/2 \times 2\mu$  (chance that grandson inherited a mutation from his mother that was a new mutation in her).

**FIGURE 21-7** Pedigree for isolated case of X-linked lethal condition.

### 21.4.11 Incorporating Laboratory Results and Clinical Findings

In some cases, the estimate of an individual risk may be modified based on certain laboratory and molecular test results or clinical findings that can modify the risk for a genetic condition. The incorporation of these factors by

Bayesian analysis will provide a client more accurate and individualized risk estimate that then can be used to help them in understanding the true chances of a condition occurring and their next course of action. The individual providing genetic counseling should be well versed in the sensitivity and specificity of the result and/or clinical finding and the scientific literature available to support the modification of an individual's risk.

**Example 7:** A non-Hispanic Caucasian woman is seen for a prenatal ultrasound and echogenic bowel is discovered. Echogenic bowel can be a clinical ultrasound finding in a fetus with CF. She is a known CF carrier. Her partner has a full sibling with CF but has not pursued carrier testing because of insurance issues. His prior risk of being a carrier is  $2/3$ . The counselor must make several assumptions based on the current literature discussing echogenic bowel and CF in order to modify the chance, the fetus is affected. The example below assumes that the probability that a fetus has echogenic bowel if it has CF is 0.11 and the probability that a fetus has an echogenic bowel is a carrier for CF is 0.00098 and the probability that the fetus has an echogenic bowel and is not affected and not a carrier is 0.00035 (81).

The probability of an affected fetus in this case is 97% (Table 21-16). On the basis of this significant increased risk, prenatal diagnosis could be considered. The client should be counseled about the limitations of prenatal diagnosis if only her mutated allele is identified. Full sequencing of the *CFTR* gene could be considered if the father of the baby remained unavailable for testing.

### 21.4.12 Family History Considerations in Risk Assessment

Accurate family history collection is invaluable for risk assessment purposes. The recurrence risk for Mendelian single-gene disorders should be determined based on the known mode of inheritance for the condition. This assumes that the diagnosis in the proband is correct and the family member is knowledgeable about the family history. Information related to a diagnosis is easily confirmed if one is providing risk estimates for the proband or close family members, however, it can become more difficult for extended family members if they have incorrect or incomplete information about the condition in the family. Collection and review of medical records on

**TABLE 21-16** Bayesian Analysis to Incorporate a Clinical Finding

Father	Carrier	Noncarrier
Prior	$2/3$	$1/3$
Fetus (given carrier mother)	Affected 0.25	Carrier 0.5
Cond.-EB present	0.11	0.00089
Joint	0.0183	0.0002963
Posterior	0.97	0.016

NC = Noncarrier; EB = Echogenic bowel.

the proband, when possible, will enable one to more confidently provide assessment.

Several factors may make the interpretation of a client's pedigree challenging and should be considered when collecting and interpreting a family history for risk assessment. A small family size may not provide the counselor with a lot of information to determine inheritance patterns and to differentiate between a sporadic or familial condition. Individuals who are adopted often have little or no information about their biological family to base risk assessment on. Some clients may withhold essential information such as false paternity or illegal drug use for fear of being stigmatized. False paternity can lead to the collection of information that is irrelevant to an individual resulting in an over- or underestimation of their risk for a genetic condition. And certain pregnancy exposures such as illegal drugs, medications or alcohol may not be disclosed by a client but can have associated phenotypes that might mimic a single-gene disorder as well as carry a specific teratogenic risk.

### 21.4.13 Consanguinity

Consanguinity and inbreeding may significantly impact the occurrence and recurrence of autosomal-recessive conditions and congenital anomalies. In some instances, couples may request genetic counseling because of consanguinity or it may be revealed after the birth of a child with a genetic condition or birth defect. It is prudent to ask about consanguinity in all genetic counseling sessions, either in relation to the client couple or in some past relationship in the family.

Determining the degree of relationship between the proband and the affected individual is also important. For example, many individuals are unaware of the difference between a first cousin, second cousin and first cousin once removed. While this may seem trivial to a client, it has significant implications to the amount of genetic information that they share in common with a proband and the risk for a recessive condition in a consanguineous mating (Table 21-17). Accurate degrees of

relationship are required to provide counseling for carrier status of autosomal-recessive and X-linked recessive conditions. This is also important information when counseling consanguineous couples. The degree of relationship is used to calculate the coefficient of inbreeding (F) for consanguineous couples. The coefficient of inbreeding is defined as the probability that homozygote for a condition received both alleles at a locus from a common ancestor (identical by descent).

### 21.4.14 Empiric Risk for Chromosome Aneuploidies and Multifactorial Conditions

Empiric data is used to assess the risk for presumably multifactorial conditions, when available. These data are derived from observations of many apparently similar cases that may actually have varying etiologies, and although they might be the best risk figures that exist, they may not be accurate for a given family. Given the roles of modifying genes and environmental factors, this may also be true of the data we have on the likelihood of specific complications occurring in variably expressed Mendelian disorders. In these types of situations, providing specific risk figures may imply a level of precision that is misleading and clients should be informed of the limited utility of this generalized information to a specific individual.

The risks for occurrence and recurrence of chromosome aneuploidies are also based on empirical data collected on pregnancy outcomes from large populations of women. These data are collected based on the pregnancy outcome at the woman's age at delivery, not her age at the conception of the child. These studies have revealed that the primary risk factor for having a child with a chromosome aneuploidy is maternal age (82). Providing this information to clients allows each individual to know their specific age-related risk and compare that risk with other factors such as risks for aneuploidies derived from serum screen results and the risk of invasive prenatal diagnostic testing.

### 21.4.15 Risk for Disease Expression

As a part of comprehensive risk assessment during genetic counseling, it is important to discuss the risk of the potential manifestations of a condition with known genetic factors. This may include the risk for specific cancers over a lifetime in individuals with a known *BRCA* or Lynch syndrome associated mutation or the chance of developing a deep vein thrombosis for an individual that is homozygous for a Factor V Leiden mutation. For example, after an absolute genetic risk is determined, such as a 50% risk for a *BRCA1* mutation in the daughter of an individual with a known *BRCA* mutation, risk figures are also provided for the risk of breast and ovarian cancer as compared to the general population. These figures are provided in an attempt to demonstrate an increased risk, and to assist a client in making medical decisions. In most

TABLE 21-17

Degree of Relationship	Proportion of Genes in Common	Coefficient of Inbreeding (F)
First (parent–child; brother–sister)	1/2	1/4
Second (brother–half sister; uncle–niece; aunt–nephew; double first cousins)	1/4	1/8
Third (half-uncle–niece; first cousins)	1/8	1/16
Forth (First cousins once removed)	1/16	1/32
Fifth (Second cousins)	1/32	1/64

cases, these risk numbers are based on cohort data from other individuals with a *BRCA 1* or 2 mutations. It can be difficult to quantify for clients other factors such as ethnic background, lifestyle choice and modifying genetic variants that determine why one person develops cancer and another family member with the same genetic change will live to an advanced age and never develop cancer.

### 21.4.16 Closing Thoughts

Summarizing the field of genetic counseling, the genetic counseling process and clinical risk assessment in one coherent chapter is a challenge because of the breadth of the field and the rapid advances in medical genetics. This chapter aims to provide readers with an awareness of the fundamental applications of the field and encourage further readings on topics of interest. The process of genetic counseling is unique in many ways from other areas of medicine because of the sometimes nondirective nature, holistic medical evaluation of the client and their family history, and the integration of psychosocial assessment and support. Individuals who provide genetic counseling are able to analyze large volumes of analytical information yet communicate it on the most basic level if needed to assist a client in understanding and utilize the information. This skill set is no doubt shared by many in health care and will continue to be important as genomic medicine becomes integrated into all areas of medicine.

### ACKNOWLEDGMENTS

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### CROSS REFERENCES

History of Medical Genetics; Medicine in a Genetic Context; Nature and Frequency of Genetic Disease; Mendelian Inheritance; Analysis of Genetic Linkage; Chromosomal Basis of Inheritance; Mitochondrial Genes in Degenerative Diseases, Cancer and Aging; Multifactorial Inheritance and Complex Traits; Population Genetics; Genetic Epidemiology; Human Developmental Genetics; Twins and Twinning; The Molecular Biology of Cancer; Genetic Assessment and Pedigree Analysis (Patterns of inheritance); Genetic Risk Assessment for Common Disease; Diagnostic Molecular Genetics; Heterozygote Testing and Carrier Screening; Prenatal Screening for Neural Tube Defects and Aneuploidy; Techniques for Prenatal Diagnosis; Neonatal Screening; Ethical and Social Issues in Clinical Genetics; Legal Issues in Genetics in

Medicine; The Genetic Basis of Female Infertility; Male Infertility; Fetal Loss; A Clinical Approach to the Dysmorphic Child; Clinical Teratology; Abnormal Mental Development; Down Syndrome and Other Autosomal Trisomies; Sex Chromosome Abnormalities; Deletions and Other Structural Abnormalities of the Autosomes; Cancer of the Colon and Gastrointestinal Tract; Hemoglobinopathies and Thalassemias; Other Hereditary Red Blood Cell Disorders; Hemophilias and Other Disorders of Hemostasis; Cancer of the Breast and Female Reproductive Tract; Fragile X Syndrome and Other Causes of X-linked Mental Handicap; Duchenne, Congenital, and Autosomally Inherited Muscular Dystrophies; Hereditary Motor and Sensory Neuropathies.

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## Biographies



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# CHAPTER

# 22

## Cytogenetic Analysis

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### 22.1 INTRODUCTION

Cytogenetics traditionally refers to the study of chromosomes by microscopy following the application of banding techniques, permitting identification of abnormalities of chromosome number, loss or gain of chromosomal material or positional changes. Advances over the past 30 years have resulted in an increased reliance on molecular techniques, such that the current field is a hybrid of microscopic and molecular based technologies. Because chromosomes carry the genetic material, an understanding of their behavior, structure, and function is essential to an understanding of genetics. Visible or submicroscopic changes in the number and structure of chromosomes have been associated with a broad spectrum of disease, and so the analysis of chromosomes has been an important tool in medical genetics for decades. Key technical advances in chromosome analysis include fluorescence in situ hybridization (FISH), first introduced in the mid-1980s, and then array-based genomic analysis or chromosome microarray analysis (CMA), introduced in the 1990s and brought into clinical practice beginning around 2003 (1–3). This chapter describes the most useful techniques of those now available for chromosome analysis. In recent years, array-based techniques have become the first line of investigation for analysis of chromosome abnormalities, with cytogenetic and FISH tools utilized to help interpret the results of imbalances diagnosed by array. Array-based techniques generally only identify imbalances resulting in deletions or duplications; there are cases of balanced translocations, inversions or ring chromosomes that can only be identified currently by standard chromosome analysis (4).

### 22.2 MILESTONES IN HUMAN CYTOGENETICS

Human chromosomes were probably first observed by Arnold in 1879 by dividing tumor cells. Hanseman in 1891 and Flemming in 1898 attempted to count the number of chromosomes in serial sections of dividing cells and obtained crude estimates of about 24. Quite different results were reported by de Winiwarter in 1912. He seems to be the first to study sections of gonadal tissue, and he found counts of 47 in testes and 48 in ovary. He interpreted these results by indicating that, like some invertebrates, males had a single X chromosome and females had two. In 1921, Painter demonstrated in testis sections the presence of an additional small Y chromosome that was overlooked by de Winiwarter. Although he assumed that 48 was the correct chromosome number in both sexes, it is interesting to note that in his 1921 paper he states that he could count only 46 chromosomes in the clearest mitotic figures. In a paper published in 1923, Painter predicted the existence of individuals with unusual combinations of sex chromosomes, in particular intersexes with an XXY sex chromosome complement. No one seems to have tested this idea until 1942, when Severinghaus described an XY sex chromosome constitution in an XY female. Largely as a result of the unfavorable material available for studying human chromosomes, there were few reports on this subject until the early 1950s. An important exception was a paper by Koller (1937) in which the X–Y pairing segment in human meiosis is described for the first time and the possibility of partial sex linkage raised. The possibility that pathologic disorders might be due to abnormalities of



chromosome number and structure seems to have been suggested first by Theodor Boveri. He described his theory on the origin of cancer from chromosomal aberrations in a classic monograph published in 1914. Then, 46 years later, the first specific chromosome abnormality associated with malignancy was described, namely, the Philadelphia chromosome in chronic myeloid leukemia (5). Subsequent developments in cancer cytogenetics have fully confirmed the role of chromosome aberrations in the pathogenesis of cancer and established cytogenetic analysis as an essential component in classification and prognosis. With regard to constitutional chromosome abnormalities, Waardenburg in 1932 was one of the first to suggest that Down syndrome might be due to a numeric chromosome aberration resulting from non-disjunction. This may have prompted Mittwoch (1952) to study testis material from a case of Down syndrome. She counted an average of 24 bivalents in diakinesis and about 48 in spermatogonial mitoses and concluded that the chromosome number was normal. Had hypotonic pretreatment and control material from a normal individual been available, she might have appreciated that the number at diakinesis in Down syndrome was abnormal, with 23 bivalents plus one univalent.

Human cytogenetics became a practical proposition with the discovery by Tjio and Levan (1956) that the correct chromosome number was 46 and not 48 (6). Both of them were experienced cytologists who exploited the availability in their laboratory of fetal tissue culture from abortus material. Levan had earlier introduced into plant cytogenetics the use of colchicine to arrest and accumulate mitoses at metaphase, and he knew about the effect of hypotonic solutions to separate individual chromosomes from one another by pretreatment before fixation. The hypotonic technique had been discovered independently by three scientists, Hsu (1952) in the United States, Makino and Nishimura (1952) in Japan, and Hughes (1952) in England. Apparently, both Hsu and Makino made the discovery fortuitously, after mistakenly adding hypotonic instead of isotonic salt solution during the washing stage before fixation. After fixation, the technique current at that time was to squash the dividing cells between the slide and coverslip so that the chromosomes could be spread in one optical plane. A variety of nuclear stains including aceto-orcein, Giemsa, and Feulgen were used to stain the chromosome preparations. All these methods, especially tissue culture, contributed to the rapid developments in human cytogenetics in the following years.

The chromosome number of 46 was soon confirmed in testis material by Ford and Hamerton (1956). Two years later, Ford et al. (1958) introduced a method for chromosome analysis using bone marrow samples that was suitable for clinical studies in that it exploited the presence of actively dividing cells in that material. At about the same time, Lejeune et al. were reinvestigating the chromosomes in Down syndrome from fibroblast cultures (7).

Their discovery of trisomy for one of the smallest autosomes was announced in 1958, although the description in nine patients was not published until early in 1959. Simultaneously, interest in the chromosomes of the Turner and Klinefelter syndromes was prompted by the paradoxical nuclear sex chromatin (Barr body) findings in 1954 suggesting that amenorrheic women with Turner syndrome might be genetic males, and that infertile men with Klinefelter syndrome might be genetic females. The latter seemed to be confirmed by Ford et al. (1958), who found one Klinefelter patient to have an apparently normal female chromosome constitution on bone marrow preparations (8). However, Ford reported in 1959 that Turner syndrome was usually associated with a 45, X chromosome complement and Jacobs and Strong found that Klinefelter syndrome had a 47, XXY complement (9). The important conclusion from these studies was that human sex differentiation was determined by the Y chromosome and not by the number of X chromosomes.

It came as a surprise to clinicians that such gross numeric chromosome abnormalities could be associated with viability. There followed intense activity worldwide to determine whether other dysmorphic conditions were due to chromosomal abnormalities visible under the microscope. Trisomies 13 and 18 were quickly identified, followed by several instances of sex chromosomal mosaicism, translocation Down syndrome, and the deletion of the short arm of chromosome 5, which causes the cri du chat syndrome (10).

The search for new examples of conditions that are due to chromosome aberrations was accompanied by strenuous attempts to simplify techniques and improve the identification of individual chromosomes so that smaller structural changes could be detected. The most important of these early technical developments was the introduction of phytohemagglutinin, which allowed chromosome preparations to be made within 2–3 days from peripheral blood samples (11). This reagent was originally used to clear red cells from preparations of lymphocytes, but it was found that T lymphocytes underwent transformation and division under its influence. When colchicine was used to accumulate lymphocytes in metaphase during short-term culture, air-dried drop preparations of metaphase chromosomes could be made far superior to any previous method. The simplicity of the technique, which is still in use almost unchanged, has undoubtedly been responsible for the widespread application of chromosome analysis throughout the world and for the growth of human cytogenetics as a diagnostic procedure in clinical medicine. In the 1960s, individual chromosomes were identified by characteristics such as total length, centromere index (length of short arm divided by total length), the presence of heterochromatic regions, and the pattern of DNA replication as revealed by pulse labeling with tritiated thymidine. These studies revealed considerable normal variation in chromosome size and centromere position, much of which was

heritable and of no clinical significance. Only chromosomes 1, 2, 3, 9, 16, and the Y chromosome could be identified with certainty in any one metaphase by standard techniques. Chromosomal heteromorphisms mainly involved the centromeres of chromosomes 1, 9, 16 (and occasionally chromosomes 3 and 4); the short arms and satellites of chromosomes 13, 14, 15, 21, and 22; and the distal heterochromatic region of the long arm of the Y chromosome.

Because of the limited availability of testicular biopsies and the virtual lack of access to ovarian tissue, meiotic chromosome analysis has contributed comparatively little to human cytogenetics and to the diagnosis of chromosome aberrations. However, much has been learned about the behavior of human chromosomes in meiosis, about the identification of bivalents at pachytene (especially the nucleolar chromosomes), about the pairing of chromosomal rearrangements, about the number and location of chiasmata, and about the extent of homologous and nonhomologous pairing of the X and Y chromosomes. Although the morphologic aspect of meiosis has been well studied, much has still to be learned about the biochemistry of synapsis, recombination and chromosome segregation.

In 1970 two new techniques were introduced that have had a major impact on modern cytogenetic analysis. The first was the demonstration by Pardue and Gall (12) that isotopically labeled DNA probes could be annealed to complementary DNA sequences in cytologic preparations of chromosomes made by standard techniques, a procedure referred to as *in situ* hybridization (ISH). Pardue and Gall also noted that when the denatured chromosomes were stained by Giemsa, the paracentric regions were preferentially stained (C bands, see later). Various modifications of the denaturing and staining process by other workers following Pardue and Gall yielded chromosomes that showed patterns of differential staining along their length, which appeared specific for each chromosome. These experiments were extended and led to the second major development, the establishment of a standard chromosome banding pattern, and this has become the criterion for chromosome identification and classification in many species.

Even before chromosome denaturation was being used to produce various banding patterns, Caspersson et al. independently discovered that quinacrine compounds that intercalate in DNA could produce bright fluorescent bands visible along the chromosome using fluorescence microscopy (13). The quinacrine bands (Q bands) were at first more reproducible than those produced by denaturing and Giemsa staining but yielded virtually the same banding pattern and were equally useful for chromosome identification.

Cytogenetic analysis has evolved from these beginnings, the main ingredients being cell culture, colchicine treatment to accumulate metaphases, manipulations of the cell cycle to enhance chromosome morphology,

hypotonic treatment to separate the chromosomes from one another, fixation in suspension followed by air drying, banding methods for chromosome identification, and *in situ* hybridization for the localization of specific DNA sequences. The growing emphasis on timely management of patients and the detection of chromosome abnormalities beyond the resolution of the light microscopy in recent years has led to the development of targeted molecular cytogenetic techniques freed from the cell culturing and lengthy protocols used for the preparation of high-quality metaphase spreads. These new technological advances allow quantitative evaluation of the chromosomal content and include methods such as quantitative FISH and polymerase chain reaction (Q-PCR), comparative genomic hybridization (CGH) and array-CGH, multiplex amplifiable probe hybridization (MAPH) and multiplex ligation-dependent probe amplification (MLPA). These methods allow higher resolution chromosome analysis and at the same time are more amenable to automation and high throughput of the samples than traditional methods.

FISH-based techniques provided a bridge between the chromosomes, as viewed under the microscope, and the DNA that comprises the chromosomes. FISH vastly improved our ability to diagnose small aberrations and characterize marker chromosomes not identifiable by banding. FISH was introduced to cytogenetics in the mid-1980s, just as the field of molecular biology was being revolutionized by advances in DNA analysis, most spectacularly, the PCR. As the human genome project progressed through the 1990s, more and more FISH probes became available, increasing our power to diagnose clinically recognizable syndromes. In general, FISH studies are limited by the need to know which region of the genome should be analyzed (disease-related critical regions, specific chromosomes) and cannot be used to survey the entire genome at high resolution. A partial exception to this was the development of a subtelomere assay, in which the subtelomeric regions of all of the human chromosomes were analyzed sequentially in one assay. The subtelomeric probes were developed in response to the hypothesis that polymorphisms in human telomeric region are more prone to breakage and reunion and could play a role in cryptic translocation events that underlie a significant percent of human pathology (14). As probe sets containing probes for each of the human subtelomeric regions became available, they were used in screening cohorts with varying criteria (mental retardation, with and without dysmorphic features, other congenital anomalies, family history of related abnormalities), and varying but significant percentages of patients with sub-telomeric imbalances were identified, ranging from 3% to 29% (15,16).

CGH was the next major advance in genomic analysis, and provided a tool to determine the amount of DNA in specific genomic regions (thereby diagnosing deletions or duplications) on a molecular level. Initial methods

allowed comparison of DNA content from a patient, with a normal control, by fragmenting the two genomes, labeling test subject and control with fluorescent dyes of different wavelengths, and co-hybridizing to chromosomes from a normal individual. The ratio of fluorescence of the two colors is measured along the length of each chromosome, and relative gains or losses were recorded as deviations from the 50:50 mixture anticipated if the test subject is normal (17). Since this technique utilizes DNA, there is no need to culture cells, which is an advantage in analysis of tumors or in any case where living tissue is not available. The resolution of CGH done in this fashion, however, was not higher than that of analysis of metaphase chromosomes when applied to constitutional cytogenetics. Nevertheless, CGH paved the way for array-based CGH, in which DNA from patient and control is co-hybridized against DNA that has been spotted on an array (2). With array-CGH, the choice of which clones to place on the array lies in the hands of the investigator and can range from selected clones covering specific regions of the genome to a tiling path of the entire genome. The array can utilize large pieces of DNA such as inserts of human DNA into bacterial artificial chromosomes (BACs), smaller DNA fragments (oligonucleotides) or can utilize polymorphic regions of the genome such as single nucleotide polymorphisms (SNPs). The use of high-resolution arrays is presenting unique opportunities to explore changes in DNA copy number in both health and disease. Both array-CGH and SNP array analysis have surprisingly identified a significant fraction of the genome that can vary in copy number between apparently healthy individuals (3,18–22). The current challenge in the cytogenetics/cytogenomics laboratory is to incorporate these technological advances to identify clinically significant copy number alterations, while avoiding the copy number alterations that are true polymorphisms.

### 22.3 THE INDICATIONS FOR CYTOGENETIC ANALYSIS

The history of human cytogenetics over the past 55 years has been punctuated by the introduction of new technology followed by the identification of increasing numbers of aberrations, smaller in extent and often associated with less striking phenotypic changes. The indications for cytogenetic analysis have thus widened over the years. For example, the occurrence of a monogenic disorder associated with mild mental handicap may indicate the presence of a microdeletion detectable by array-based analysis or of DNA disruption at one of the breakage sites of a reciprocal translocation. A list of general indications for cytogenetic analysis includes the following:

1. Confirmation or exclusion of the diagnosis for known chromosomal syndromes.
2. Unexplained intellectual disability or developmental delay with or without dysmorphic features.

3. Autism spectrum disorders.
4. Congenital anomalies.
5. Abnormalities of sexual differentiation and development.
6. Infertility/subfertility.
7. Recurrent miscarriages or stillbirth.
8. Pregnancies shown to be at risk of aneuploidy from the results of maternal serum screening or fetal ultrasound scanning.
9. Neoplastic conditions for which the identification of specific chromosomal aberrations may be valuable in diagnosis and management.

Full descriptions of the main chromosomal syndromes are given in Chapters 43–45.

### 22.4 TISSUE SAMPLES AND CELL CULTURE

Chromosome preparations for cytogenetic analysis are made from dividing cells, either directly from tissue samples (e.g. bone marrow, testis, chorionic villi, neoplastic tissue) or after cell culture (biopsy of skin or almost any other living tissue including amniotic fluid cells). Tissue preserved in a fixative is not suitable for making conventional chromosome preparations. It is imperative to use the correct sterile containers when sending biopsy and other tissues to the cytogenetics laboratory to ensure that the cells remain viable and suitable for cell culture. Paraffin-embedded tumor tissue sections can be “FISHed” for deletions/amplification of tumor suppressor genes and oncogenes to assist in diagnosis, prognosis, and management after therapy.

For array-based techniques, dividing cells are not necessary, and all that’s needed is a source of DNA. This can come from peripheral blood or tissue samples and techniques are improving to obtain the DNA from paraffin-embedded tissue sections.

### 22.5 CHROMOSOME BANDING

For identification of normal chromosomes and detection of aberrations, the air-dried chromosome preparations must be stained appropriately. Chromosome banding refers to alternating light and dark regions along the length of a chromosome, produced after staining with a dye. A band is defined as the part of a chromosome that is clearly distinguishable from its adjacent segments by appearing darker or lighter with the use of one or more banding techniques. The fluorescent dye quinacrine was used to produce the first banding patterns as mentioned above (Q-banding), and through the years several general banding stains, as well as several techniques for staining specific parts of the genome have been used. Generalized banding techniques have included Q-banding, Reverse (R) banding and G-banding. Specific staining protocols include C-banding and staining

of the nucleolar organizing regions (NORs) using a silver stain (Ag-NOR). G-banding is the benchmark for the routine analysis of human chromosomes, producing a characteristic light and dark banding pattern along the chromosomes (Figure 22-1). Each chromosome has a unique sequence of bar code-like stripes, allowing identification of individual homolog and the analysis of abnormalities of their structure by disruption of the normal banding pattern. Many methods have been published for the production of G bands, but most of them rely on proteolytic digestion of the chromosomes by trypsin followed by staining with either Giemsa or Leishman stain. Little is known of the precise nature of the mechanism of G-banding despite much conjecture. The dark bands contain mainly A–T rich DNA, and the light bands are G–C rich. Manipulation of the cell cycle to produce prometaphase chromosomes with resolution of >550 G-bands per haploid set provides a mechanism for high-resolution analysis of the structure of the chromosomes.

## 22.6 THE NORMAL HUMAN KARYOTYPE

Each species has a chromosome complement, characteristic in number and form. This is known as the karyotype, and the name is also used for a photographic preparation in which the stained chromosomes are arranged approximately in order of decreasing length. Conventional banding allows each normal human chromosome to be given its specific identity number. To make analysis easier, the banding pattern can be diagrammatically represented in the form of an idiogram (Figure 22-2). About 400 distinct bands per haploid complement usually may be visible in any one metaphase preparation. In prometaphase cells, more than 550 bands may be resolved because the chromosomes are more extended. The International System for Human Cytogenetic Nomenclature (ISCN) was established in 1978 by a standing committee to provide a simple shorthand, based on the human banded

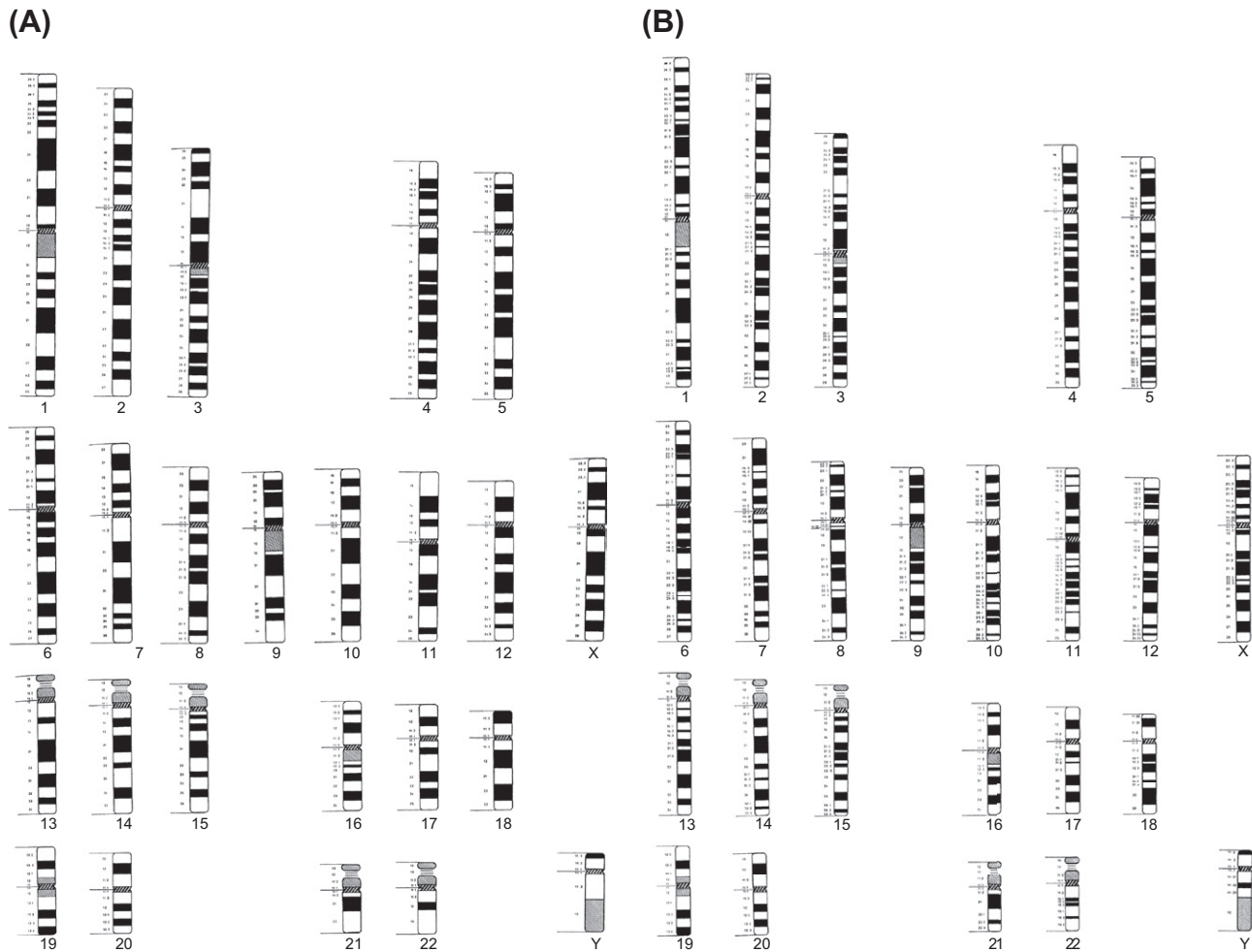
idiogram, to describe any given chromosome aberration and the karyotype of any individual (24). In this nomenclature, which has been updated regularly, the number of chromosomes in the karyotype is given first, then the sex chromosome constitution, followed by a shorthand description for any abnormal chromosome. Thus, 46, XY is a normal male karyotype, and 47, XX, +21 is the karyotype of a female with Down syndrome. Parts of chromosomes or sites of chromosome rearrangement are identified using a simple numbering system specific for each chromosome band or sub-band. In this description, the chromosome identifier is given, followed by the designation for the chromosome arm, p (petit) for the short arm and q for the long arm, and then the number of the chromosome band involved in the rearrangement. The main landmarks of each chromosome are the centromere, cen, and the end of the arm, pter for the short arm and qter for the long arm. The most striking of the bands are the remaining landmarks, and these divide the arm into distinct regions. Each region is further subdivided into bands and sub-bands. Thus, band Xp21.2 is to be found in the short arm of the X chromosome in region 2, band 1, and sub-band 2. The shorthand for the exchange of chromosome fragments between 7p21.2 and, for example, 9q34.1 in a female individual would be given as 46, XX, t (7;9) (p21.2;q34.1), where t, translocation and the semicolon is used to separate the chromosomes and break points. These and other symbols used are listed in Table 22-1. The ISCN provides nomenclature to describe constitutional and acquired chromosome abnormalities and fluorescence in situ hybridization studies (further details of ISH nomenclature are given in the FISH section) (25). Examples of the use of the shorthand are given in the following section where the classification of chromosome abnormalities is considered.

Chromosome analysis in normal individuals has revealed substantial variation in the karyotype that is without phenotypic effect. It is important to recognize and distinguish this normal variation from the abnormal chromosomal rearrangements that are clinically significant. The most striking of these variations, or heteromorphisms, occur at the centromeric regions of chromosomes 1, 9, and 16, at the short arms of chromosomes 13, 14, 15, 21, and 22, and at the distal end of the long arm of the Y chromosome. All these regions contain variable amounts of highly repetitive DNA (satellite DNA) composed largely of tandemly arranged repeats that are not transcribed. Unequal crossing-over within these repeats during meiosis may account for the variation, but this must occur rarely, because extreme examples of these heteromorphisms are invariably transmitted unchanged in pedigrees. Several euchromatin heteromorphisms have also been described that may present a specific diagnostic concern, particularly when detected at prenatal diagnosis or in an individual with an abnormal phenotype. Euchromatic variants at chromosomes 8p23.1, 9p12 and qh, 15q11.2, and 16p11.2 have been characterized on



**FIGURE 22-1** G-banded metaphase with three marker chromosomes (whose origin from chromosomes 22, 11, and 14 cannot be identified by this technique).





**FIGURE 22-2** Human idiogram based on G-banding pattern and showing the banding nomenclature according to the International System for Human Cytogenetic Nomenclature (23). (A) Approximately 400 of the main G bands per haploid complement are shown, as seen in most banded metaphase preparations suitable for cytogenetic analysis. (B) Approximately 550 G-bands per haploid complement are shown, as seen in early metaphase preparations.

a molecular level and found to comprise region-specific pseudogene cassettes containing processed paralogous sequences frequently dispersed/transposed to/from pericentromeric or protelomeric sites (26–29).

## 22.7 CHROMOSOME ABNORMALITIES

Genetic mutations involve duplication, deletion, or rearrangement of DNA. The extent of the change varies from the gain or loss of a single nucleotide (a point mutation) to gain or loss of whole chromosomes. When the field of genetic analysis relied on either chromosome analysis or DNA analysis, those changes that were large enough to be detectable under the light microscope were classified as chromosome aberrations, but they differed from molecular mutations involving individual genes only in terms of scale. Molecular techniques generally involve analysis of single genes, and focus on mutations that are intragenic, while cytogenetic methods revolve larger genomic alterations affecting multiple genes. With the introduction of increasingly

sensitive techniques into the cytogenetics laboratory, we are able to diagnose single gene deletions, or intragenic deletions or duplications, and the divisions between the cytogenetic and molecular diagnostics are blurring. Historically, it has been possible to identify genomic deletions or duplications that are a minimum of 5–10 Mb by cytogenetic techniques, although there are many cases where even deletions or duplications slightly greater than 10 Mb were missed by analysis of banded chromosomes. Array-based techniques provide resolution that is much finer, and some arrays can diagnose single exon changes (30). Currently, many laboratories use standard cut offs of 100–200 kb for diagnosis of deletions, and 500 kb to 1 Mb for duplications. There are several chapters in this book that deal with the specifics of chromosome abnormalities including Trisomy and triploidy (Chapter 43), structural chromosome abnormalities such as translocation (balanced or unbalanced), Robertsonian translocations, isochromosomes, insertional translocations, deletions, duplications, inversions, marker chromosomes and

**TABLE 22-1 The International System for Human Cytogenetic Nomenclature (ISCN) Nomenclature: Symbols and Abbreviations Used for Describing Chromosome Aberrations**

Symbol	Definition
p	Short arm
q	Long arm
pter	Terminal of short arm
qter	Terminal of long arm
cen	Centromere
h	Heterochromation
add	Additional material of unknown origin
del	Deletion
der	Derivative of a translocation
dic	Dicentric
dup	Duplication
fra	Fragile site
i	Isochromosome
isodic	Isodicentric
inv	Inversion or inverted
r	Ring chromosome
rec	Recombinant from inversion or insertion
t	Translocation
upd	Uniparental disomy
mat	Maternal origin
pat	Paternal origin
mar	Marker chromosome of unknown origin
dir	Direct as opposed to inverted
::	Breakage with reunion
/	Mosaicism (separates clones)
+/-	Before a chromosome number, indicates gain or loss of that chromosome
;	Separates altered chromosomes and break points involved in structural rearrangements—indicates from and to

ring chromosomes (see Chapter 45). As techniques for cytogenetic analysis have evolved, the types of chromosome abnormalities that are detectable have changed as well. Initially, only larger abnormalities such as gain or loss of an entire chromosome or large alterations visible on banding could be diagnosed.

## 22.8 IN SITU HYBRIDIZATION

The ISH technique is based on the principle that when double-stranded DNA is heated, it denatures into single-stranded DNA. On cooling, the single-stranded DNA reanneals with its complementary sequence into double-stranded DNA. If an appropriately labeled DNA sequence (probe) is denatured and added to denatured nuclei, or chromosomes during the process of reannealing, some of that labeled DNA will hybridize to its complementary sequence in the chromosomal DNA. Detection of the labeled DNA will identify the site of hybridization and thus the region of chromosomal DNA complementary to the DNA sequence in the labeled probe. Thus, ISH

may be used to map and order genes and other DNA sequences along the length of a chromosome.

In the early days of ISH, DNA probes were labeled with radioisotopes, such as tritium ( $^3\text{H}$ ) or radioactive iodine ( $^{125}\text{I}$ ), and detected by autoradiography using photographic emulsion applied directly to the microscope slide. The scatter of radioactive disintegrations, revealed by silver grains in the developed emulsion, meant that the resolution was poor. The autoradiographs often had to be exposed for several weeks to achieve an adequate signal; therefore it was not possible to tell whether the probe had hybridized satisfactorily until long after the experiment had been set up. These disadvantages, coupled with the hazards of radioisotopes and the need to count silver grains in a large number of cells to establish significant counts above background levels, led to the search for nonisotopic methods of labeling.

In situ hybridization was used first to map the chromosomal location of highly repetitive (satellite) DNA to the centromeric regions of mouse chromosomes and the moderately repetitive ribosomal DNA to the nucleolus organizing regions in the polytene chromosomes of *Diptera* (12). Similar studies soon located these repetitive DNAs to the equivalent sites in human chromosomes (31). However, it was not possible to incorporate sufficient radioactivity in probes derived from single-copy DNA sequences to permit the more extensive application of ISH in gene mapping. This had to await the introduction of recombinant DNA techniques and the development of DNA sequences cloned in plasmids that could be heavily labeled. The early 1980s saw the first chromosomal localizations of single-copy DNA sequences by ISH, and ISH soon became the method of choice for assigning a cloned DNA sequence to its position in the chromosomal map (32–34). It was quickly appreciated that ISH also had application in diagnostic cytogenetics, particularly for the detection of structural abnormalities beyond the resolution of conventional banding techniques.

The search for nonisotopic alternatives for labeling probes for ISH led first to the introduction of biotin and then other haptens including acetylaminofluorene and digoxigenin (23,35–38). Biotin and digoxigenin have proved the most useful, and a variety of methods have been developed to detect both these labels in cytologic preparations using fluorescence or enzyme-linked reactions.

Almost all types of DNA probe used in FISH applications in diagnostic cytogenetics contain interspersed repetitive elements that will hybridize to complementary sequences throughout the entire genome leading to a high level of background signals. This background can be suppressed effectively by preannealing unlabeled genomic or Cot-1 (enriched for highly repetitive sequences) DNA to the labeled probe before hybridizing the probe to the target DNA (39). The unlabeled DNA acts as a competitor and will form duplexes rapidly with the repetitive DNA sequences within the probe. The low-copy DNA remaining in the probe will then be available to hybridize with

complementary sequences in the target DNA. With many DNA probes, the use of Cot-1 DNA may be avoided by allowing the probe to preanneal with itself before application to the target.

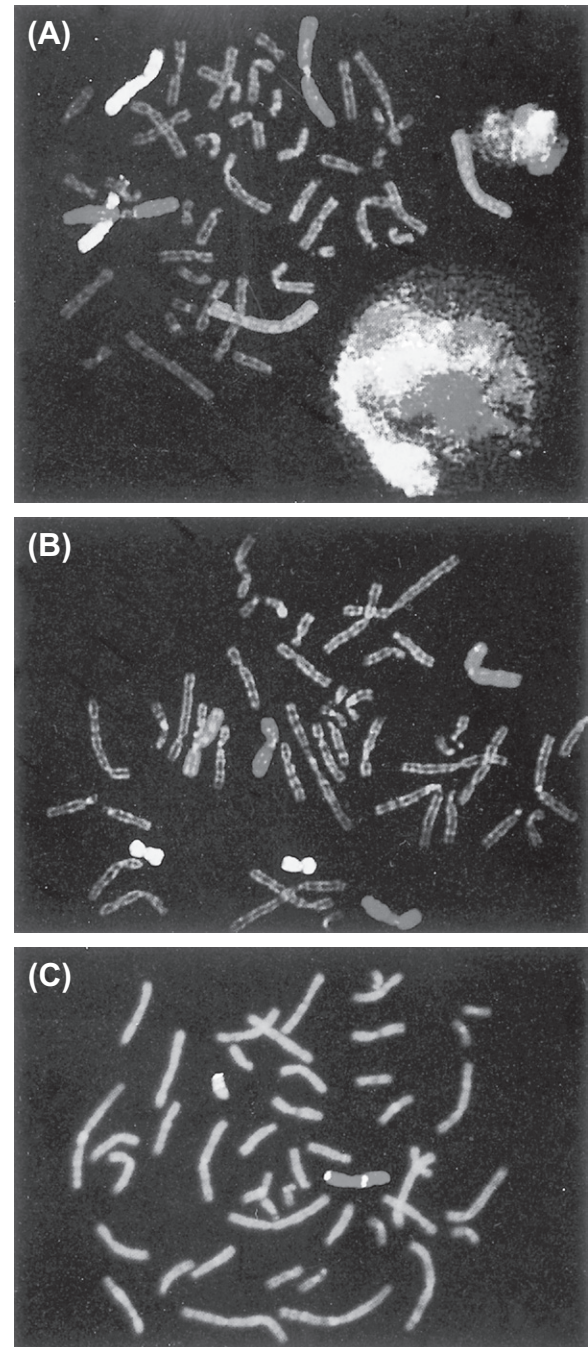
### 22.8.1 DNA Probes and Their Applications

The various classes of DNA probe have wide applications in diagnostic cytogenetics to augment conventional banding analyses of chromosomal rearrangements. When genomic analysis starts with array-based testing, FISH is often needed to obtain positional information, such as the chromosomal location of a duplicated region of the genome.

Collections of chromosome-specific painting probes (Figure 22-3) derived from each chromosome, usually by flow-sorting and less frequently by microdissection, and labeled end-to-end by nick translation or DOP-PCR, are routinely used as a screening tool in the characterization of an abnormal chromosome (40). Centromeric probes on their own and in combination with single-copy DNA sequence probes are widely used in the determination of chromosome copy number in interphase nuclei or as control probes in interphase FISH analysis (Figure 22-4). The main applications are in rapid aneuploidy tests for prenatal and preimplantation diagnosis, in characterization of marker chromosomes, and in the diagnosis of hematological cancers.

Thanks to the International Human Genome Project, a physical map of the human genome composed of a series of overlapping cloned DNA sequences along each chromosome is available in BAC vectors. These clones act as reference markers along the chromosome and are also suitable for FISH. Many other DNA sequences of interest, including sequences from known genes, have been cloned in yeast artificial chromosome (YAC) and cosmid vectors; both are suitable for FISH. YAC clones can accept inserts of up to 2 million base pairs and have the disadvantage that they may contain sequences from more than one chromosomal region (due to colligation) and seem more liable to lose parts of the insert through deletion. Cosmid clones can accept smaller inserts of up to 40kb and do not have these disadvantages. Both types of clone can be used as markers for gene loci, for the delineation of break points and for the identification of deletions and duplications associated with chromosomal syndromes. For example, cosmid clones are widely used for the diagnosis of microdeletion syndromes (see Table 22-2 and Figure 22-5) and in screening for subtelomeric chromosomal imbalances (Figure 22-6) found in 5–10% of patients with dysmorphic features and developmental delay (16,41–43). Today, many large cytogenetic laboratories have access to a bank of clones that represent sequences covering the whole genome with a resolution of up to 1Mb to allow more precise characterization of different chromosomal abnormalities.

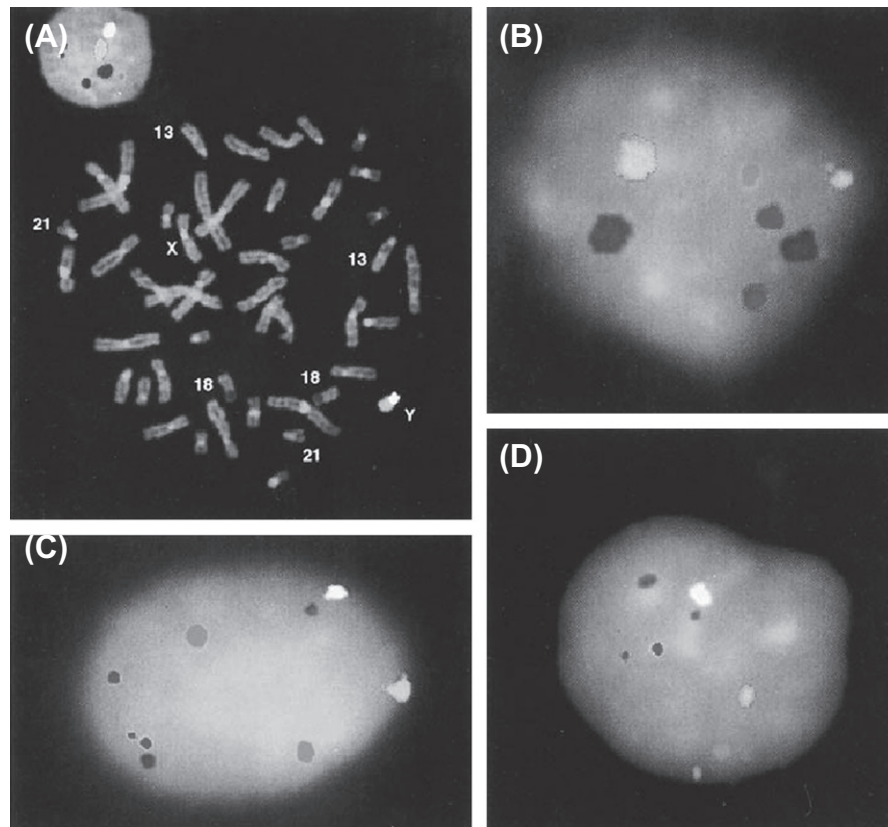
Multicolor clone sets for the detection and monitoring of critical genetic aberrations associated with



**FIGURE 22-3** Chromosome painting using chromosome-specific probes (CAMBIO Ltd., Cambridge, England) from flow-sorted chromosomes (DAPI counterstain). (A) Chromosome 1, chromosome 2, and chromosome 6. Interphase nucleus reveals chromosome domains within nucleus. (B) Chromosome 7, chromosome 11, and chromosome 20. (C) X chromosome, Y chromosome. Note Y signal on XY homologous regions of Xp (tip) and Xq (proximal third) and X signal on XY homologous region of Yp (tip).

hematopoietic disorders are widely used for the analysis of uncultured bone marrow samples and tumor tissue biopsies. Hybridization targets of these DNA probes have been designed to flank or span common translocation break points on one or both rearranged chromosomes, thus resulting in characteristic signal





**FIGURE 22-4** FISH probes useful for determining chromosome copy number in interphase nuclei. (A) Lymphocyte metaphase showing centromeric probes for X chromosome, Y chromosome, and chromosome 18; a YAC clone marks chromosome 13 and a contig of two overlapping cosmids marks chromosome 21. (B) Uncultured amniotic fluid cell nucleus from a female fetus hybridized with the above probes revealing normal copy number of each chromosome. (C) As above, from male fetus with trisomy 21 (Down syndrome). (D) As above, from normal male fetus. (From Divane, A.; Carter, N. P.; Spathas, D.H.; Ferguson-Smith, M. A. *Rapid Prenatal Diagnosis of Aneuploidy from Uncultured Amniotic Fluid Cells Using Five-Color Fluorescence in situ Hybridization*. *Prenat. Diagn.* **1994**, 14, 1061–1069, with permission.)

pattern in normal and abnormal cell populations. The probes most commonly used in the diagnosis and monitoring of hematological malignancies are centromeric probes, dual and single fusion probes (Figure 22-7A), extra signal probes, and break-apart probes. In solid tumor diagnostic enumeration, probes complementary to unique sequences of known oncogenes and cancer suppressor genes are being widely used on imprinted fresh tumor samples and paraffin-embedded tissue sections (Figure 22-7B). Recent developments in automated scanning and image capturing systems together with automated spot counting and image analysis software allow high throughput, automated interphase FISH analysis of many different sample types as well as simultaneous FISH and conventional immunofluorescence staining.

### 22.8.2 Molecular Substitutes for Multiprobe Fish Tests

Faster, cheaper, and often more precise molecular techniques are adding to an expanding set of tools in the diagnostic cytogenetic laboratories. MAPH is a method that measures copy number (dosage) variation of DNA

of almost any size. The method combines hybridization of DNA probes to target genomic DNA immobilized on a solid support. After washing, each specifically bound probe is present in an amount proportional to its target DNA copy number. All probes are then simultaneously amplified using a single primer pair and quantified after electrophoretic separation (44). MLPA shares the basic principles of primary probe hybridization with subsequent quantitative probe amplification with MAPH. Instead of probe hybridization to a filter-bound target DNA, in MLPA two adjacent complementary oligonucleotides hybridize to their target sequence in solution. One of the two oligonucleotides is cloned in an M13-derived vector that contains an inserted stuffer sequence size-specific for each probe. In the subsequent ligation step, only the adjacent oligonucleotides hybridized to their target DNA sequence are ligated. As in MAPH, the ligation-generated template is suitable for PCR amplification using a single primer pair. After the electrophoretic separation that identifies each probe, the PCR products are quantified by comparing the peak areas of the amplification products to a set of amplified controls or to neighboring peaks (45).



**TABLE 22-2 Recurrent Segmental Deletions/Duplications Associated Syndromes**

Chromosome Band	Syndrome	Del/Dup	Assayed Genes/Loci
1p36	Monosomy 1p36	del	DVL1
2q27.3	Albright hereditary osteodystrophy like	del	GPR35
4p16.3	Wolf–Hirschhorn syndrome	del	WHS
5p15.2	Cri du chat syndrome	del	CDC/D5S23/D5S721
5q35	Sotos syndrome	del	NSD1
7p13	Greig cephalopolysyndactyly	del	GLI3
7q11.23	Williams syndrome	del	ELN
8p23.1	8p23.1 deletion	del	GATA4
8q24	Langer–Giedion syndrome	del	EXT1
8q24	Trichorhinophalangeal syndrome	del	TRPS1
11p15.5	Beckwith–Wiedemann syndrome	dup	IGF2
11p13	WAGR syndrome	del	PAX6/WT1
11p11.2	Potocki–Shaffer syndrome 11q deletion syndrome/Jacobsen/ Paris-Trousseau	del	EXT2/ALX4
11q23.3-q24.1	syndrome	del	FLI1
12q24.1	Noonan syndrome	del	PTPN11
13q14.11	Retinoblastoma	del	RB1
15q12	Prader–Willi syndrome	del	SNRPN/D15S10
15q12	Angelman syndrome	del	UBE3A/D15S10
16p13.3	$\alpha$ -Thalassemia	del	16ptel
16p13.3	Rubinstein–Taybi syndrome	del	CREBBP
16p13.3	Tuberous sclerosis 2	del	TSC2
16p13.3	Polycystic kidney disease	del	PKD1
17p13.3	Miller–Dieker syndrome	del	LIS1
17p12	Charcot–Marie–Tooth disease, 1A	dup	PMP22
17p12	Hereditary neuropathy with liability to pressure palsies	del	PMP22
17p11.2	17p11.2 duplication syndrome	dup	SMS
17p11.2	Smith–Magenis syndrome	del	SMS
17q11.2	Neurofibromatosis 1	del	NF1
20p11.23	Alagille syndrome	del	JAG1
22q11.2	DiGeorge syndrome/velocardiofacial syndrome	del	TBX1/TUPLE1/D22S75
22q11.2	22q11.2 duplication	dup	TBX1/TUPLE1/D22S75
22q13.3	22q13 deletion syndrome/Phelan–McDermid syndrome	del	SHAK3/PROSAP2
Xp22.3	Steroid sulfatase deficiency	del	STS
Xp22.3	Kallmann syndrome	del	KAL1
Xp21.2	DMD/glycerol kinase deficiency/adrenal hypoplasia congenita	del	DMD/GK/DAX1
Yq11.2	Male infertility	del	AZF <sub>a</sub> , AZF <sub>b</sub> , AZF <sub>c</sub>

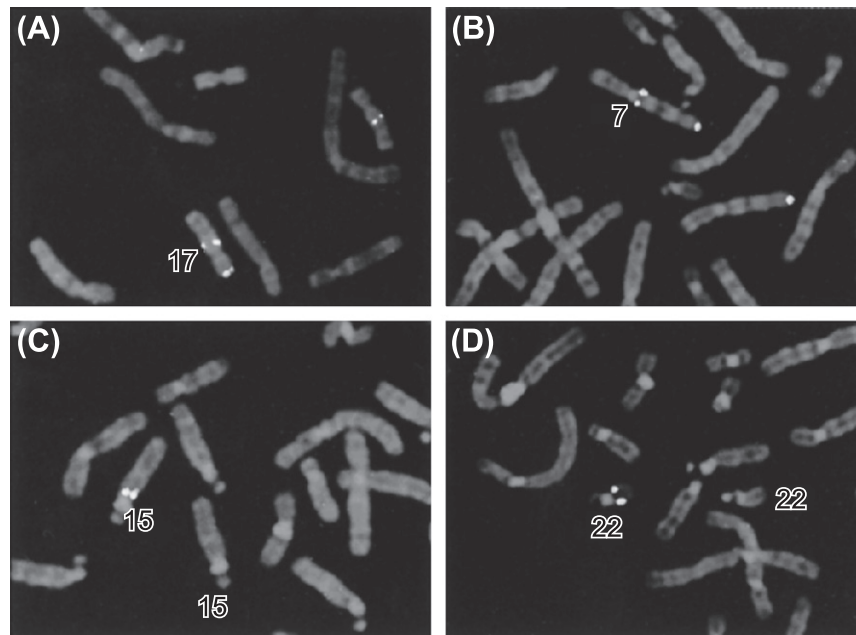
DMD, Duchenne muscular dystrophy; WAGR, Wilms' tumor, aniridia, genitourinary abnormalities and mental retardation.

Both methods allow reliable screening for DNA copy number changes at ~40 loci simultaneously, and both methods are being used in various diagnostic applications, including screening for subtelomeric deletions/duplications and unbalanced cryptic telomeric translocations, aneuploidies, whole or partial gene duplications, or deletions (29,45–48).

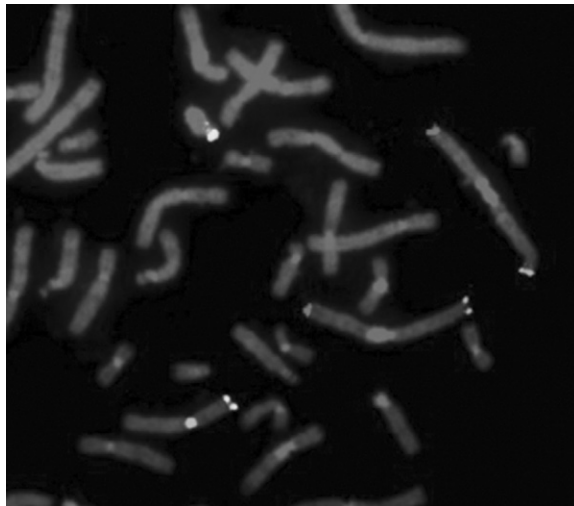
### 22.8.3 DNA Fiber-FISH

Because of the condensation of the chromosome fiber at metaphase, the fluorescent signals from two cosmid clones hybridized to the same chromosome can be resolved only if they are more than 2–3 Mb apart. Because the chromosomes are 10 times more extended in hypotonic preparations of interphase nuclei, the resolution at interphase is much better, and two cosmids

more than 50kb apart can usually be distinguished from one another (49). The order of several closely linked cosmids may be determined at interphase, provided they are more than 50kb and less than 1 Mb apart (50). The latter restriction is due to the tendency of a chromosome to coil back on itself and because the chromosome fiber forms loops radiating in all directions from the central chromosome scaffold. Various techniques have now been developed that release the chromosome fiber from its associated protein within the chromosome scaffold (51–53). This permits DNA sequences to be hybridized directly onto extended chromosome fibers fixed on a microscope slide and analyzed by FISH (Figure 22-8). The resolution of this technique is astonishing; sequences less than 5 kb apart can be separated readily, and distances down to 1 kb have been claimed (54).



**FIGURE 22-5** The diagnosis of microdeletion syndromes using labeled cosmid clones (ONCOR, Gaithersburg, MD) that map into the region involved in the deletion. (A) Miller–Dieker syndrome. Chromosome 17 identified by cosmid clone in 17q. Note loss of signal at 17q13 in one homolog (right). (B) Williams syndrome. Chromosome 7 identified by cosmid clone at distal end of 7q. Note loss of signal at the elastin locus on 7q11.23 in one homolog (lower). (C) Prader–Willi syndrome. One homolog of chromosome 15 shows loss of signal at 15q12. (D) DiGeorge syndrome. One homolog of chromosome 22 shows loss of signal at 22q11.



**FIGURE 22-6** Human metaphase showing telomeric probes for chromosome 1p, 1q, and pairing regions of Xp and Yp. X alpha centromeric probe identifies the X centromere.

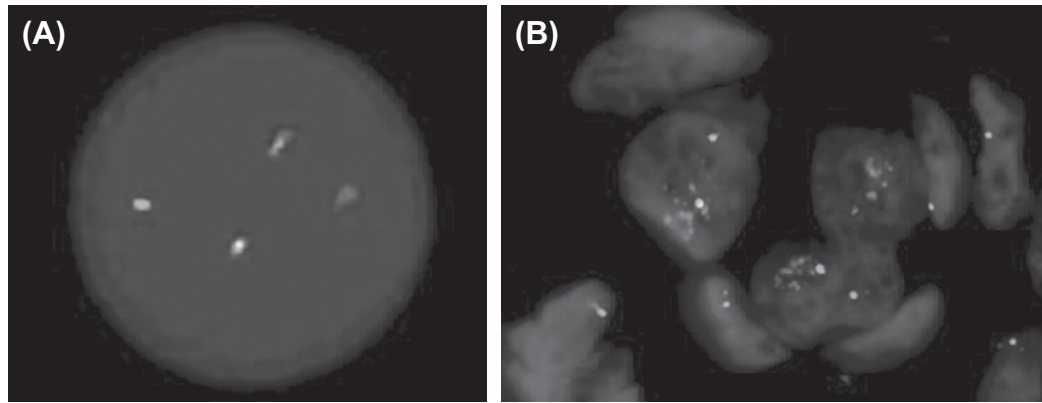
Decondensation of chromatin by detergent and salt or alkaline solutions results in the release of loops of chromatin fibers of more than 200nm in length from nuclei in air-dried fixed preparations. Typically, the chromatin loops out from the nucleus and forms a halo of DNA fibers around the nucleus. Where the loops are broken, the fibers are more extended and are seen as long thin filaments that radiate away from the nucleus. Fibers up to 10Mb long have been achieved by using agarose-embedded cells treated with proteinase K and ribonuclease. The agarose is then melted and spread onto

poly-l-lysine-coated microscope slides (55). This method seems more robust than others used for fiber-FISH.

The analysis of extended DNA fibers by FISH has application both in the mapping and ordering of contiguous DNA sequences and has been used in the detection of carriers of intragenic gene deletions in X-linked Duchenne muscular dystrophy (56).

#### 22.8.4 FISH Nomenclature

The Standing Committee for the ISCN has developed nomenclature to describe investigations and abnormalities detected by DNA probes. The observed chromosome banding is described by the standard nomenclature. This is followed by a period (.) and the abbreviation “ish,” which precedes the description of the in situ hybridization findings. The abnormality identified by ISH is described starting with the abbreviation for the type of abnormality followed, in separate parenthesis, by the chromosome(s), break point(s), and the loci for which probes were used, designated according to the Genome Database (GDB) and ordered from pter to qter. If no GDB locus is available, the name of the probe can be given instead. Capital letters are used for the locus, and “+” or “–” given immediately afterward indicates whether the locus has been identified as being present or absent, respectively. Thus, ish del (22) (q11.2q11.2) (D22S75–) indicates a deletion in the DiGeorge chromosome region, and ish dup (17) (p11.2p11.2) (CMT1A++) indicates a duplication at the Charcot–Marie–Tooth locus. If no abnormality is detected, the chromosomal location follows ish,



**FIGURE 22-7** (A) Chronic myeloid leukemia showing detection of the Philadelphia translocation using BCR/ABL dual color dual fusion probe. The ABL gene and the BCR region map to chromosomes 9 and 22, respectively, and the translocation regions are revealed by the combined signals on each derivative chromosome. (B) Amplification of HER2 (ERBB2) oncogene in paraffin-embedded section of breast cancer tissue. Centromeres of chromosome 17 are shown.



10  $\mu$ m

**FIGURE 22-8** Fiber-FISH. Decondensation of chromatin by alkali treatment of fixed nuclei and hybridization of closely linked labeled cosmid clones. Three cosmids from the HLA region (lower), FITC (middle), and a 50:50 mixture of FITC and HLA (upper), respectively. Each cosmid measures 35kb and there is a 5–10 kb gap between the different cosmids.

and the usual two copies of the probe are confirmed; for example, ish 22q11.2 (D22S75x2). The origin of a cryptic balanced translocation identified by forward painting is given as ish t(4;11) (p16.3;p15) (wcp4+;wcp11+), where wcp is whole chromosome paint. Similarly, the origin of an extra marker chromosome revealed by reverse painting is given as 47, XY, +mar.rev ish der (18) t(12;18) (p13.3;p11.2). The ish nomenclature includes symbols appropriate for interphase FISH (nuc ish) and fiber-FISH (fib ish) (25).

### 22.8.5 Comparative Genomic Hybridization

An ingenious FISH technique was developed for the cytogenetic analysis of tumor samples that is capable of

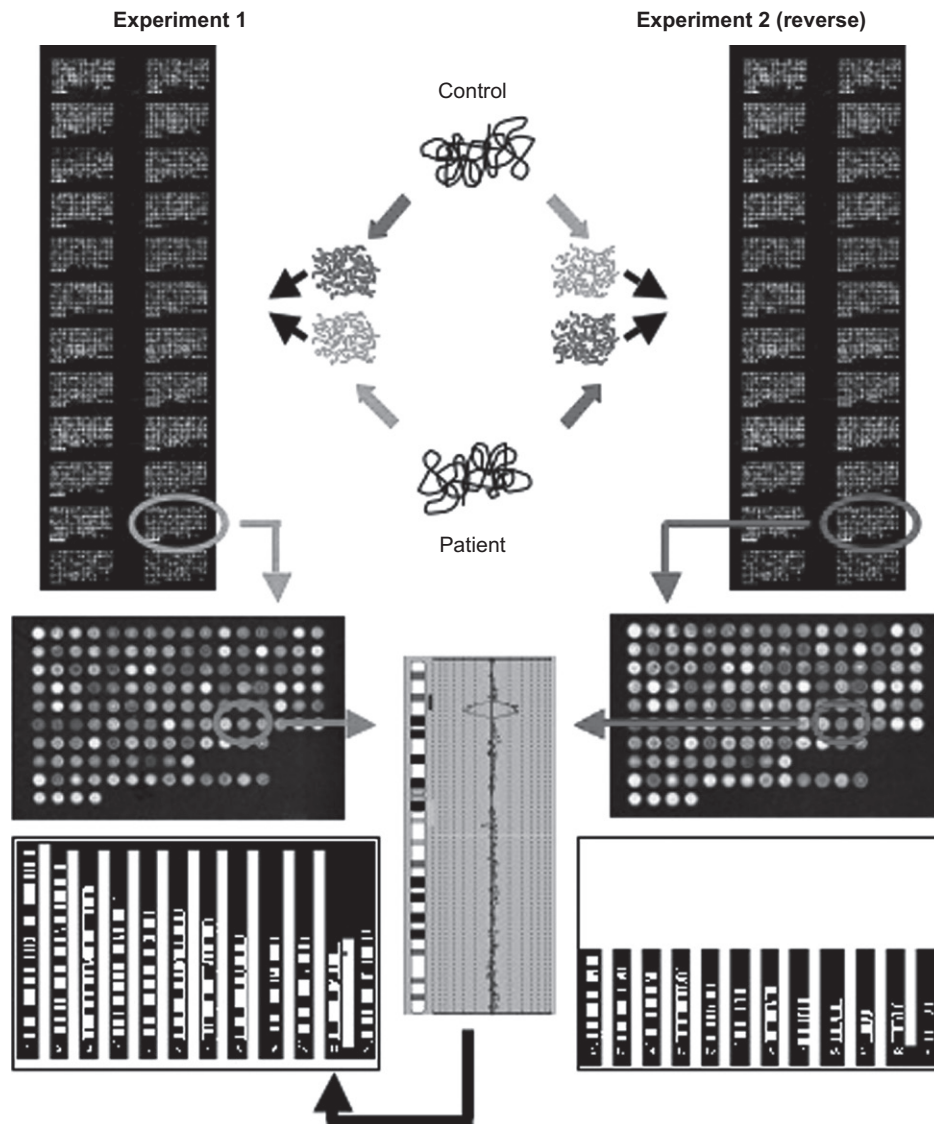
identifying DNA amplifications and deletions of around 5 Mb and the threefold to fivefold amplification of oncogenes in a single sample of tumor material (17). The method, CGH, is a development of reverse painting in which test DNA and normal genomic reference DNA are isolated, fragmented, differentially labeled (for example, in green and red, respectively), mixed and allowed to compete for hybridization sites in sets of normal high-resolution metaphase spreads. The relative amounts of test and reference DNA that anneal to a particular chromosome region will depend on the number of copies of DNA complementary to that region in the test sample. If the test sample contains relatively more of a particular DNA than the reference sample, this will be revealed by an increased green/red fluorescence ratio in the complementary region; similarly, chromosomal deletion in the test sample will be revealed by a decreased green/red fluorescence ratio. Significantly, this method uses DNA from the individual to be tested, with no need to culture cells prior to the analysis. CGH requires digital fluorescence microscopy in which the relative amounts of green and red fluorescence are measured along the length of the chromosome.

In addition to confirming the results of conventional chromosome analysis in neoplastic conditions, the CGH technique has been used to identify chromosomal regions that are recurrently lost or gained in specific tumors. CGH has also been successfully applied in constitutional cytogenetics, and in fact its use has contributed to a revolution in cytogenetic diagnostics.

### 22.8.6 Array-Based Genomic Analysis

The replacement of metaphase spreads by an array of cloned DNA sequences or PCR products offer much higher resolution and more precise information on the break points of rearranged chromosomes than does conventional metaphase CGH (2,57). Moreover, each clone and PCR product spotted on an array has known

## ARRAY-COMPARATIVE GENOMIC HYBRIDIZATION

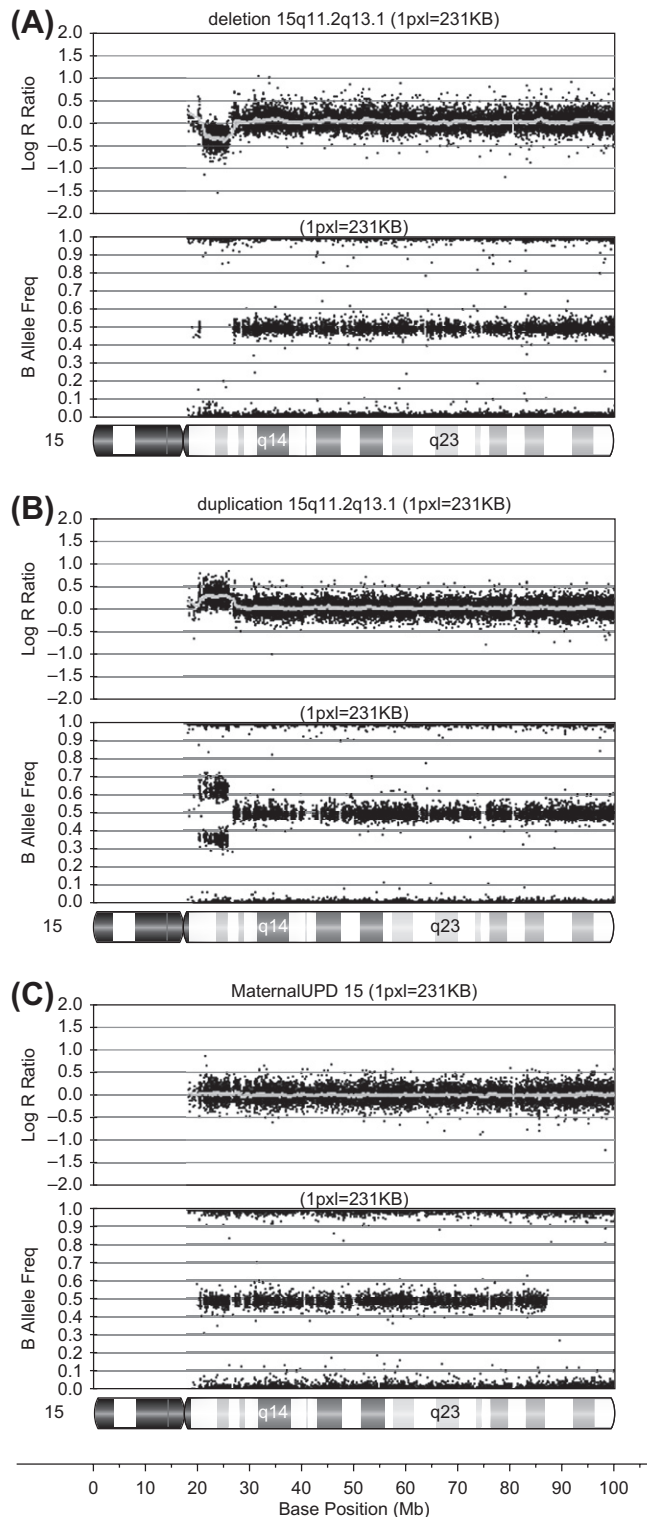


**FIGURE 22-9** Array-CGH analysis in a patient with epilepsy and moderate mental deficiency revealing a cryptic duplication of chromosome 11 short arm. Total labeled genomic DNA from patient is co-hybridized with labeled control DNA and unlabeled Cot-1 DNA to an array platform containing approximately 3000 characterized fragments selected from across the human genome. In two experiments using alternative labels for patient and control DNA, the fluorescence ratio is measured for each DNA spot on the array and plotted on chromosome-specific ratio profiles. Significant deviations from the expected 1:1 for a given DNA fragment indicate a change in copy number for that particular sequence in the patient with respect to the control. True copy number changes are seen as symmetrical deviations from the 1:1 axis in both experiments. The remaining chromosome profiles appear normal with the exception of occasional large-scale copy number polymorphisms (35).

sequence and contains known genes and other expressed sequences, facilitating precise genotype–phenotype correlations and consequently targeted patient management. Initially CMA was performed using relatively large insert DNAs from BACs, but the technology has evolved with initial utilization of shorter inserts from cosmids or fosmids to utilization of single-stranded oligonucleotides, ranging in size from 20 to 70 bp (Figure 22-9). Currently, the most commonly used platforms utilize oligonucleotides, SNPs or a combination of the two (58).

SNP arrays differ from oligonucleotide-based arrays in that they capture genotyping information in addition to copy number information. SNP arrays were originally developed for genotyping applications, but it subsequently became clear that they could also reveal genomic copy number information. The genotyping information provided by the SNP array expands the types of abnormalities that can be detected to include detection of regions of homozygosity (which could indicate either uniparental disomy or regions that are identical by descent). This allows diagnosis of imprinting disorders,





**FIGURE 22-10** Genome-wide SNP array results for individuals with three different abnormalities of chromosome 15 (deletion, duplication and uniparental disomy). Each panel shows the log *R* ratio (demonstrating SNP signal intensity) and B allele frequency. The B allele frequency indicates genotypes for the bi-allelic SNPs on the array at each locus, with one allele labeled as A and the other as B. Therefore BB has a B allele frequency of 1 (2/2 alleles are B); AB has a frequency of 1/2, and AA has a frequency of 0. Chromosome 15 ideogram lies below each patient result. Panel A shows a patient with a deletion of proximal 15q showing the decreased Log *R* ratio and the loss of heterozygosity within the deletion. Panel

which were previously undetectable in the cytogenetics laboratory. In addition, utilization of SNP arrays permits diagnosis of low-level mosaicism, chimerism and incest (59) (Figure 22-10).

CMA began to be used by clinical laboratories in 2003 and over the next few years it became clear that the technology could identify genomic alterations that were likely to be causative in from 5% to 15% of individuals who had previously had a normal karyotype (60). This huge success has resulted in almost universal adaptation of CMA and in 2010 it was recommended that CMA be used as the first tier in clinical diagnostic testing, based on its increased sensitivity (4).

The primary challenge of the clinical utilization of CMA emerged in 2004, with the discovery that deletions and duplications including genes were found in individuals without any known clinical problems (controls). These deletions and duplications have been called copy number variants or CNVs. In fact the total number of genes in a healthy individual can vary by more than 100 genes. Therefore it is crucial to distinguish benign CNVs (deletions or duplications) from pathogenic. Pathogenic variants are those that are not seen in the normal, control population, and are expected to contain gene or DNA sequences that cause disease when deleted or duplicated. Conversely, benign variants are genes that are not dosage sensitive, and therefore are found to vary in the normal population. To aid in this, data from a wide variety of studies has been collected and curated in public databases, such as the Database of Genomic Variants (<http://projects.tcag.ca/variation/>). As our collective experience with CMA increases, so will our ability to interpret the data that is generated. But despite the challenges, the success of CMA in diagnosis of clinically significant genomic alterations has been spectacular. Many new syndromes have been described that are caused by deletions or duplications (e.g. 16p11.2 17q21.3 deletion) that were previously undetectable by cytogenetics or FISH, and the more common of these are described in Chapter 45.

### 22.8.7 Array Nomenclature

The Standing Committee for the ISCN has developed nomenclature to describe investigations and abnormalities detected by chromosomal microarray analysis. A normal female characterized by array testing is indicated

B shows a patient with a duplication of proximal 15q demonstrating the increased log *R* ratio and the additional genotypes in the duplication (BBB, ABB, AAB and AAA). Panel C shows a patient with uniparental disomy for chromosome 15, with heterodisomy across much of the long arm and isodisomy toward the telomere, from base position ~85 Mb (at band 15q26.1) to qter. Note, the region of heterodisomy was confirmed by comparison of the patient with parental genotypes, as it cannot be proven by the SNP array alone, although the region of isodisomy at the telomeric end raises suspicion for UPD of the whole chromosome.

arr(1–22),(X)x2, while a male is arr(1–22)x2,(XY)x1, the difference being the relative number and type of sex chromosomes. In the case of deletions or duplications, the aberration can be specified precisely, by presentation of the cytogenetic band location, followed by specification of the genomic nucleotides. For example, an interstitial deletion of the short arm of chromosome 20 might be arr 20p12.2(10,454,698–10,818,327)x1. This specifies a 323,630 bp (324 kb) deletion within 20p12.2. It is important to include information on which build of the human genome is being referenced, as the coordinates change from build to build. This should be included in the body of the report. Note that array studies by themselves do not provide any further positional information regarding the imbalance. This deletion could be associated with a translocation, for example and the only way to determine this would be to carry out accompanying chromosome or FISH analysis. SNP arrays can also identify uniparental disomy, and this can be indicated using the abbreviation upd, and homozygosity can be indicated using hmz. Inclusion of the genomic coordinates for each of these abnormalities allows the laboratory or physician to go to the genomic databases to determine the precise gene content of the deletion or duplication.

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Chromosomal basis of inheritance; Deletions and other structural abnormalities of the autosomes.

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### Biographies



**Nancy B Spinner, PhD** is the Evelyn Willing Bromley Professor of Pediatric Pathology and Director of the Clinical Cytogenomics Laboratory at The Children's Hospital of Philadelphia, and a Professor of Pathology and Laboratory Medicine at the Perelman School of Medicine at The University of Pennsylvania School of Medicine. She received her PhD training at The University of California at Berkeley, and her Fellowship Training in Cytogenetics at The Children's Hospital of Philadelphia. Her research interests are in the identification of the genetic etiology of Pediatric Disease, and her laboratory has made contributions to understanding the genetics of Alagille syndrome, Biliary Atresia and the Ring Chromosome 20 syndrome in particular. She serves on the Scientific Advisory Board of the Alagille Syndrome Alliance, and is on the Editorial Board of Human Mutation and PLoS Genetics. She has received multiple teaching awards at The University of Pennsylvania, and a Faculty Mentoring Award from the Children's Hospital of Philadelphia.



**Malcolm Ferguson-Smith, PhD** is currently a Research Professor in the Department of Veterinary Medicine, University of Cambridge, UK. He was a Fellow in Medicine at Johns Hopkins in 1959 and he remained there working on chromosomes for nearly 3 years. During this time he established the first chromosome diagnostic service in the USA and undertook cytogenetic research into the Turner syndrome. Returning to Glasgow University in late 1961, he was appointed successively as Lecturer, Senior Lecturer and Reader before becoming Burton Professor of Medical Genetics in 1973. In 1987, he was appointed as Professor and Head of Pathology at Cambridge University and Director of the East Anglia Regional Genetics Service. On retiring from the headship of Pathology in 1998, Professor Ferguson-Smith and his research team moved to the Department of Veterinary Medicine, to establish the Cambridge Resource Centre for Comparative Genomics. He was elected Fellow of the Royal Society of Edinburgh in 1978 and the Royal Society of London in 1983. In 1980, he became the founding editor of Prenatal Diagnosis. With JM Connor his undergraduate textbook "Essential Medical Genetics" has run to five editions.



**David H Ledbetter, PhD, FACMG**, is Executive Vice President and Chief Scientific Officer at Geisinger Health System. He came to Geisinger from Atlanta's Emory University School of Medicine where he was the Robert W Woodruff Professor and Director of the Division of Medical Genetics in the Department of Human Genetics. Dr Ledbetter previously held academic and leadership positions at the University of Chicago, the National Center for Human Genome Research (now NHGRI) at NIH and Baylor College of Medicine. He is a graduate of Tulane University and earned his doctorate at the University of Texas-Austin. After his early discovery of the genetic cause of Prader-Willi syndrome and Miller-Dieker syndrome, Dr Ledbetter has focused his research efforts on discovering the underlying etiology of childhood developmental disabilities such as autism, and the translation of new genomics technologies into clinically useful genetic tests for early diagnosis and intervention. His current research interest includes leveraging the massive amount of genomics data generated during routine patient care for knowledge generation and integration of this information into electronic health records in a clinically useful manner.

# CHAPTER

# 23

## Diagnostic Molecular Genetics

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### 23.1 INTRODUCTION

There can be no question that we are in the midst of a revolution in clinical medicine, as profound in its own way as the early revelations of human anatomy and physiology and the germ theory of disease. The twenty-first century has already been christened the age of molecular medicine—the molecule in question being, of course, DNA. Molecular biology, which came to dominate basic life sciences research over the second half of the twentieth century, has now become firmly ensconced in clinical medicine as well. The development of robust technologies such as the polymerase chain reaction (PCR) and massively parallel DNA sequencing and the extraordinary productivity of the Human Genome Project in elucidating medically relevant targets for these techniques have firmly established patient DNA as a powerful “analyte” in the clinical laboratory and a valid substrate for therapeutic manipulation in the clinic.

While all areas of medicine have been impacted by these developments, it is medical genetics, the specialty most directly connected to the human genome, that is the first discipline to be entirely transformed by them. If the promise of gene therapy continues to remain frustratingly elusive through successive editions of this book, such is not the case for gene-based diagnostics. In no other sector of medical practice does at least the question, if not always the actual execution, of molecular testing arise with virtually every patient seen. Not only is medical genetics by definition concerned with inherited alterations in the patient’s genome that are detectable in this way but much of its attention is also directed at ascertaining recessive carrier states, fetal conditions that will not be expressed until after birth, and predisposition to adult-onset disorders before they are symptomatic; such diagnoses often exhibit no other evidence of their presence than at the DNA level. For these reasons, medical geneticists and genetic counselors, as a group, tend to be significantly more facile and familiar with the applications and interpretation of molecular genetic tests than other clinicians. Nevertheless, keeping up with a field as rapidly evolving as this one, in which new techniques

and disease genes (not to mention their associated ethical and societal dilemmas) are reported every week, can be a challenge for even the most dedicated specialist. That is why close two-way communication between the clinic and the laboratory is so essential, more so in medical genetics practice than in any other.

### 23.2 INDICATIONS FOR MOLECULAR GENETIC TESTING

Molecular genetics has a unique range of indications, most of which are quite different from the uses of traditional clinical laboratory testing and even molecular biologic testing in other disease classes (e.g. infectious disease, cancer). Most notably, as mentioned earlier, these procedures are often performed on healthy people who have no other signs or symptoms of the disease being tested. Even if they are symptomatic, the assay is not directed at a discrete anatomic site, as would be the case with a DNA test for an infection or a tumor. Since genetic disorders reflect heritable mutations in the germline, the test in most cases can be performed on any accessible body fluid or tissue. The most common specimen is whole blood, although saliva, buccal swabs, dried blood spots, urine, and other specimens can be used, while amniocytes or chorionic villus samples (CVS) and, most recently, fetal DNA in maternal blood are collected for prenatal diagnosis.

The technical approaches as well as the psychosocial and ethical implications of molecular genetic tests may vary substantially depending on the reason for testing. The major indications are considered individually below.

#### 23.2.1 Diagnostic Testing

Most straightforward and least foreign to other areas of laboratory medicine are diagnostic molecular genetic tests performed on symptomatic patients. These are most often performed for diagnostic confirmation of a clinical impression, although they may also assist in differential diagnosis. In the latter regard, however, it should be kept

in mind that gene-based tests are specific for the disease gene being tested, and even closely related disorders will not be detected when testing for a single one (e.g. the DNA test for spinocerebellar ataxia (SCA) type 1 will not detect the mutations of SCA type 2). In addition, while diagnostic tests do not typically carry the heavy ethical concerns of predictive tests, the uniqueness of genetic disease comes into play here as well, such that a positive test in the proband places other blood relatives at risk.

### 23.2.2 Newborn Screening

Newborn screening, despite the name, is in a sense actually diagnostic testing performed very early in life, on large populations of infants who are not (yet) symptomatic, although the affected ones may well exhibit at least biochemical signs of the disease, which are the metabolite targets detected by the screening technology. The goal, of course, is to identify affected babies early in life so that treatment (dietary or pharmaceutical) can be initiated before irreversible damage occurs. Unfortunately, most of the classic autosomal recessive disorders screened in this setting, such as phenylketonuria and galactosemia, have so many causative mutations that molecular genetic testing would be less sensitive and far less cost-effective than testing by biochemical or enzymatic means, at least given the current state of DNA sequencing technology. For most such disorders, the molecular tests, if available, are reserved for backup confirmation of indeterminate results, for further genotype–phenotype correlation, or for identification of DNA markers that could be used by the family for prenatal diagnosis in a subsequent pregnancy. However, as DNA-based methods become more efficient with the continued evolution of microarray technologies and massively parallel sequencing, they may eventually become the first-tier approach for certain newborn screens.

### 23.2.3 Prenatal Diagnosis

While classical cytogenetic analysis remains the most commonly performed mode of prenatal diagnosis, the advent of reliable molecular techniques has opened a new world of prenatal testing for single gene defects. Even though some inborn errors of metabolism can be diagnosed by biochemical analysis of amniotic fluid, other disorders, such as muscular dystrophies, do not produce such easily accessible analytes and would require a far more invasive biopsy of the target tissue (e.g. fetal muscle) for examination of the gene product. Molecular genetic techniques in most situations obviate this need, since DNA containing the mutation of interest is readily obtainable from amniocytes or CVS specimens. However, given the time constraints of the prenatal setting, such an approach is usually not attempted unless there is prior knowledge of the identity of the parental mutation(s), at least in the case of disorders with extensive mutational heterogeneity. Needless to say, prenatal

molecular genetic testing demands meticulous technique because of the irreversible nature of the intervention provoked by the test results. A key aspect unique to this setting is the detection and avoidance of maternal cell contamination, which is especially crucial in CVS. Laboratory guidelines for addressing this phenomenon using tandem repeat polymorphisms have been published (1).

A more specialized variant of prenatal diagnosis available in certain situations is preimplantation diagnosis, involving single-cell genetic analysis (using powerful PCR techniques) performed on a blastomere biopsy of embryos produced by in vitro fertilization (IVF). Because of the difficulty and expense of the procedure, it is usually reserved for those couples who would be averse to abortion of an in utero pregnancy or who are already undergoing IVF for other obstetric or infertility indications. Even more so than for traditional prenatal diagnosis, the exact identity of the mutation in the parent (for a dominant disease) or in both parents (for a recessive disease) must be known before embarking on this involved procedure. Still, despite its formidable prerequisites, preimplantation diagnosis has been used many times for pregnancies at risk for cystic fibrosis (CF), thalassemia, and other disorders (2).

Most recently, the prospect of routine noninvasive prenatal diagnosis has emerged. After many years of trial and error at detecting fetal cells or DNA circulating in the maternal blood during pregnancy by cell sorting and PCR, the advent of massively parallel sequencing, which affords far greater sensitivity for analyzing small minority sequences within a much larger (maternal) DNA pool, has made this approach a reality. By measuring the relative amounts of sequenced fragments from the various chromosomes, Down syndrome and other trisomies can be diagnosed, and it is possible to assess the entire fetal genome in the maternal blood to test for monogenic mutations (3,4).

### 23.2.4 Carrier Testing and Screening

Carrier screening is another type of laboratory testing unique to the genetics setting. The term is most properly used to denote testing for recessive mutations in the heterozygous state in otherwise healthy individuals. It may be indicated in two quite different situations: one when an individual is at risk because of a positive family history for the disorder in question; the other when the individual is at risk by virtue of belonging to an ethnic group in which the population frequency of one or more mutant alleles is particularly high. The first application is more accurately termed carrier testing rather than carrier screening, especially when the causative mutation in the family is already known. The second application is true screening, since it is performed on an entire population without respect to family history, and the likely mutation in any one individual being tested will not be known (unless the disorder has only a single mutation, as in sickle cell disease).

To be considered a candidate for population carrier screening, a number of criteria must be met by both the disease and the testing technology. The disorder must be

frequent enough in the target population to justify the effort, expense, and attendant complexities. The natural history of the disorder must be severe and predictable enough to enable couples to make informed decisions about prenatal diagnosis and pregnancy termination. The available technology must be robust, relatively inexpensive, and capable of detecting a reasonable proportion of the possible disease-causing mutations. The options for intervention in those who test positive must be available, effective, and acceptable to the target population. In addition, the overall screening program must be of sufficient interest and acceptance to the target population to ensure cost-effective uptake. As will be seen, these criteria, or a subset of them, are not met by some of the gene targets that would otherwise appear to be the most obvious. CF carrier screening, although now in widespread practice, is still hampered by mutational complexity and phenotypic variability. Hereditary hemochromatosis, a possible target of adult presymptomatic screening, is currently in limbo because of confusion over the true penetrance of the mutations. Screening for fragile X syndrome mutations is controversial because of questions about prevalence and cutoff ranges and phenotypic implications of premutation trinucleotide expansions (5,6).

Because screening tests must be kept inexpensive in order to be cost-effective, screening strategies for disorders with mutational heterogeneity will often entail complex decisions regarding the number and type of mutations to be included in the test panel. Such debates have been waged for years in the case of CF (7). A decision must also be made as to whether molecular genetic testing, with its narrow focus on specific mutations, casts a wide enough net within the target population to justify using it in preference to biochemical or enzymatic screening methods, which are often less expensive. In the case of CF, the carriers have no biochemical abnormalities that can be ascertained, so direct mutation testing is the only alternative. In the case of Tay-Sachs disease, even though there are a limited number of hexosaminidase A mutations that account for most carriers in the Ashkenazi Jewish target population, enzymatic testing has long been preferred for population screening because of its ease of use, low cost, and ability to capture carriers with uncommon mutations. However, as screening in this ethnic group has expanded to encompass as many as 15 or more additional recessive disorders, it has become easier to perform all of them at once in a multiplexed molecular assay (8). Since recessive mutations are clinically significant only in the setting of reproduction, it follows that, whatever the approach, the ultimate goal of carrier testing and screening is to identify couples at risk so they can be offered the opportunity for prenatal testing.

### 23.2.5 Presymptomatic/Predisposition Testing

Predictive genetic testing is, in some ways, the most problematic of molecular genetic applications. It is

applied primarily to adult- or late childhood-onset autosomal dominant disorders. It is never appropriate as a screening test but is strictly reserved for individuals with a strong, usually parental, family history of the disorder, conferring on them a 50% a priori risk of having inherited the mutant gene. Depending on the penetrance of the mutations being tested, predictive testing is further subdivided into presymptomatic testing and predisposition testing. The former is applied to those disorders with virtually complete penetrance, such as Huntington disease, in which a positive DNA test result is fully predictive of eventual symptomatology (although not absolutely indicative of severity or age of onset). The latter is applied to diseases with reduced penetrance, such as familial breast/ovarian cancer due to mutations in the *BRCA1* and *BRCA2* genes, in which a positive test result confers increased risk of eventual disease compared to the baseline population risk, but does not allow prediction that any particular patient will actually experience the disease. This situation makes genetic counseling and clinical decision making much more difficult, especially for diseases like familial breast/ovarian cancer, in which risky or irreversible medical or surgical interventions may be initiated based on a positive DNA test result. With both types of predictive testing, there are significant psychosocial risks since otherwise healthy adults are being given potentially devastating news about future disease. There are also theoretical risks of insurance or employment discrimination (supposedly barred by the Genetic Information Nondiscrimination Act in the United States and similar laws in other countries) that beg the question of whether a nucleotide change predictive of future disease in a presently healthy person is to be considered a “preexisting condition.” For all these reasons, predictive genetic testing has been the area most scrutinized regarding standards for informed consent, pre- and posttest genetic counseling, and psychosocial supportive services. Because the offspring of patients with autosomal dominant disorders are aware of their risk, many such requests for testing are self-referred, and some of these individuals prefer not to involve their primary care provider or insurance carrier in the testing process. These special situations may place additional demands on genetic counseling services and on the testing laboratory.

## 23.3 TECHNICAL APPROACHES TO MOLECULAR GENETIC TESTING

### 23.3.1 Specimen Collection

Just as many of the applications are unique, so too the types of patient samples collected for molecular genetic testing may be different from those obtained for other types of clinical laboratory testing. Since germline mutations are present in every cell of the body, site-specific biopsy is not required and simple phlebotomy will suffice for most purposes (this is in contrast to the approach



for laboratory diagnosis of infectious and neoplastic diseases, where a specific lesion must be sampled). Alternatively, the great sensitivity of PCR allows for genetic testing to be performed on minute samples of any readily accessible tissue or body fluid, including saliva and urine. Newborn DNA screening can easily be performed on the same blood spots collected for biochemically based screening. For prenatal diagnosis, either amniocytes or CVS can be used, since both contain the full complement of fetal DNA. In addition, PCR-based single-cell genetic analysis now allows for preimplantation diagnosis on individual embryonic blastomeres, as discussed earlier, and most recently prenatal diagnosis targeting fetal DNA circulating in the maternal blood has become practicable (4), thus potentially obviating the need for any sort of invasive fetal sampling.

### 23.3.2 Selection of Technique

Once in hand, the specimen can be subjected to any of the molecular genetic techniques in the current armamentarium. The choice of technique will depend on the nature of the disease gene being studied (especially its size and mutational heterogeneity), the purpose of the test, and to some extent the condition of the specimen. Specimens that are extremely small, fixed, or degraded will require some sort of PCR-based analysis, since that technique is the most tolerant of suboptimal samples. Once amplified to an abundant amount, the DNA is then amenable to further analysis by other techniques such as allele-specific oligonucleotide (ASO) hybridization or DNA sequencing. Southern blotting, on the other hand, which is still used for certain tests involving large genomic DNA targets (such as the fragile X full mutation, although PCR-based methods have recently been developed (9)), requires intact DNA of high molecular weight in pristine enough condition to be digested efficiently with restriction endonucleases and to produce reproducible patterns on gel electrophoresis. This usually means at least a few milliliters of whole blood or one or two dishes of cultured amniocytes transported to the laboratory in a timely fashion. In either case, the first step is typically the isolation of DNA from the specimen, for which a variety of commonly used methods and commercial reagents are available.

**23.3.2.1 Detection of Known Point Mutations.** For detection of a known point mutation or microdeletion/insertion, the patient DNA sample can be hybridized with ASO probes that contain either the wild-type or mutant sequence within them. Since hybridization under high stringency can distinguish a single nucleotide change by mismatch of a probe with its target, using these ASO probes in pairs provides an internal control for accurately genotyping both heterozygotes and homozygotes. An alternative strategy capitalizes on the availability of a wide variety of bacterial restriction endonucleases with many different target recognition sequences. With

a little investigation (made easy these days through the use of computer DNA sequence analysis programs), it will often be found that the mutation of interest either disrupts a preexisting restriction enzyme cleavage site or creates a new one in the target DNA. In such cases, the mutation can be detected by exposing the patient's PCR-amplified DNA to the specific restriction enzyme and analyzing the cleavage products (sometimes called amplified restriction fragment polymorphisms, or AmpFLPs) by agarose gel electrophoresis. The same thing can sometimes be done by Southern blotting, since it too involves a restriction enzyme digestion step, although that approach is more laborious. PCR primers can also be designed to hybridize directly at the site of the mutation, in which case a mismatch between a wild-type primer and a mutant target sample will block amplification and produce a blank result. This technique has been called the amplification-refractory mutation detection system (ARMS).

The technical approaches just described are usually developed "in house" by the testing laboratory, often designated in the vernacular of the trade as "home brew" assays or "laboratory-developed tests (LDTs)." With the increase in test volume in molecular genetics in recent years, driven in large part by the advent of population carrier screening for CF and testing for common disease variants such as factor V Leiden, there has been a continuing movement by the laboratories toward more standardized, commercially prepared testing platforms. A few of these have gone through formal US Food and Drug Administration (FDA) review and can then be called "kits." Those that have not are sold as components of an assay that the laboratory must complete from some of its own sources and are designated "analyte-specific reagents" (10). In either case, these systems have proven robust and reliable and have generally been accepted as a boon to the field. Examples currently available for detection of point mutations include paper strip arrays of ASO hybridization probes, microarrays composed of chips or beads or a combination of the two, real-time PCR systems incorporating ASO probes with differential melting curves, oligonucleotide ligation with ASO probes analyzed by capillary electrophoresis, the Invader assay using specific cleavage of ASO hybrids to produce signal amplification by fluorescent resonance energy transfer, and mass spectrometry (11).

**23.3.2.2 Detection of Large Deletions.** Large deletions, while sometimes detectable by these methods, are usually more easily seen by Southern blot hybridization (signaled by the absence of one or more expected hybridization bands). Alternatively, one can use differential PCR amplification, in which the failure of one or both of a pair of PCR primers to hybridize to the target DNA because of the deletion will result in absence of an expected PCR product. Many primer pairs can be multiplexed to scan for unknown deletions across a gene, as

is done for the diagnosis of Duchenne muscular dystrophy (DMD). The main pitfall of this technique is that there are many other technical reasons for PCR failure (of artifactual origin), and so appropriate internal PCR amplification controls must be included in the assay. A more refined method now in common use is multiplex ligation-dependent probe amplification (MLPA) (12).

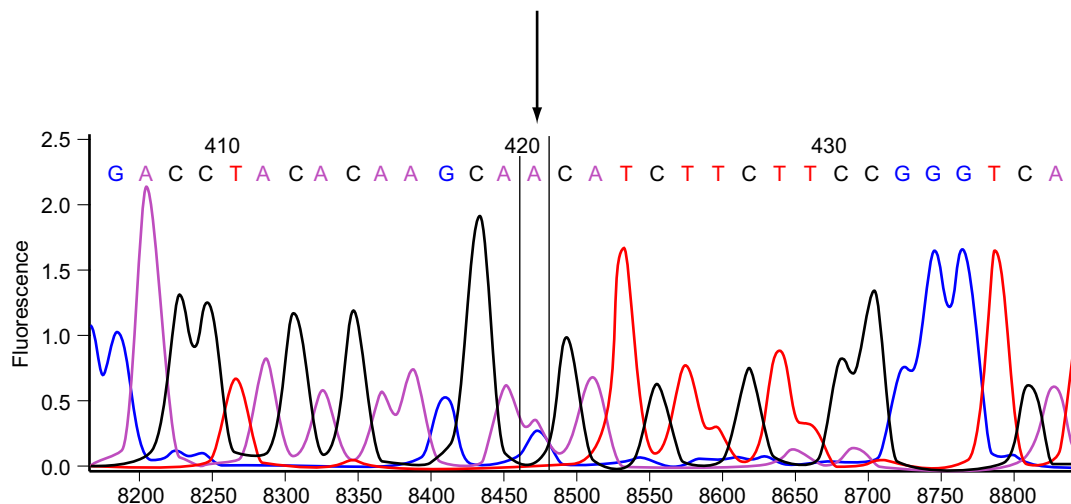
**23.3.2.3 Detection of Trinucleotide Repeat Expansions.** To detect the trinucleotide repeat expansion mutations characteristic of fragile X syndrome, Huntington disease, and other neuromuscular disorders, accurate sizing becomes important. Indeed, this is the most visible area of molecular genetic testing that approaches quantitative assay criteria, as opposed to the more qualitative observational assays for point mutations and deletions. If the expansion size is of moderate length, as in Huntington disease and the SCAs, amplification and sizing of PCR products suffices. This is typically done by capillary electrophoresis or automated DNA analysis instruments. For larger expansions that are difficult or impossible to amplify, such as in fragile X syndrome, sizing by Southern blot analysis or newer long-PCR methods is usually required.

**23.3.2.4 Detection of Heterogeneous or Unknown Mutations.** What about those disorders in which a wide range of point mutations has been reported, often spanning a very large gene? Such mutationally heterogeneous disorders present special problems to the laboratory in attempting to assure detection of a majority of the mutations; detection of all potential mutations is virtually impossible, even by complete DNA sequencing of the gene (since mutations may lie in noncoding regions, far upstream enhancers, etc.). Sequencing does cast the widest net and is therefore considered the “gold standard,” but it is not easily adaptable to high-throughput screening tests. As a compromise, one can select a more limited test panel comprising a few of the more commonly

recurring mutations in the gene, recognizing at the outset that some fraction of carriers will go undetected. These then become amenable to any of the targeted approaches discussed previously. These multiplex assays may be constructed at various density levels, depending on the number of sequence variants targeted. At highest density (up to a million or more probes) is the microarray or “DNA chip.”

DNA sequencing, because of its effort and expense, is usually reserved for situations in which it is not clinically acceptable to overlook a pathologic mutation if one is present; an example would be predictive testing for *BRCA1/2* mutations of familial breast/ovarian cancer. In other cases, sequencing may be used for detection of even a limited number of mutations in a gene, provided they are localized to hot spots within one or a few exons. These days most laboratories use automated DNA sequencer instruments, usually built on capillary electrophoresis. To confirm heterozygous single-nucleotide substitutions, which can sometimes be difficult to discern on these instruments (Figure 23-1), sequencing in both directions (i.e. sequencing both strands of the double helix) is recommended.

Finally, there are the so-called mutation scanning techniques. These can be used as a first-pass screen of a gene or gene fragment for the presence of point mutations, without the effort and expense of meticulous gene sequencing. These techniques capitalize on the altered three-dimensional topology of a mutant DNA fragment compared to the corresponding wild-type fragment, an alteration that can be detected by differential migration on electrophoresis. Popular techniques include single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), and denaturing high-performance liquid chromatography (dHPLC). However, because they are qualitative assays relying on poorly understood physicochemical



**FIGURE 23-1** Example of automated DNA sequence readout on a capillary electrophoresis instrument, showing a heterozygous G-to-A substitution at position 416 of the connexin-26 (*GJB2*) gene. Any interpretive difficulty in discerning the heterozygous nature of the superimposed peaks can be confirmed by reverse sequencing.

behaviors of DNA fragments, they are never 100% sensitive. Furthermore, any abnormality detected must then be confirmed by DNA sequencing of the fragment for precise mutation location, identification, and interpretation. A rather different and more predictable mutation scanning technique is the protein truncation test (PTT), in which a gene or exon is used as substrate for an *in vitro* transcription/translation assay and the polypeptide products are analyzed by acrylamide gel electrophoresis. Presence of a nonsense mutation (or a frameshift mutation leading to a stop codon downstream) will produce a shortened (truncated) polypeptide as compared to a wild-type control. Of course, this technique will not pick up missense mutations, so a substantial proportion of pathologic alterations in the gene under study will go undetected. For all these reasons, and because DNA sequencing has become so much more rapid and less expensive in recent years, mutation scanning techniques are not used as much as they once were.

#### 23.3.2.5 Detection of Unknown Disease Genes.

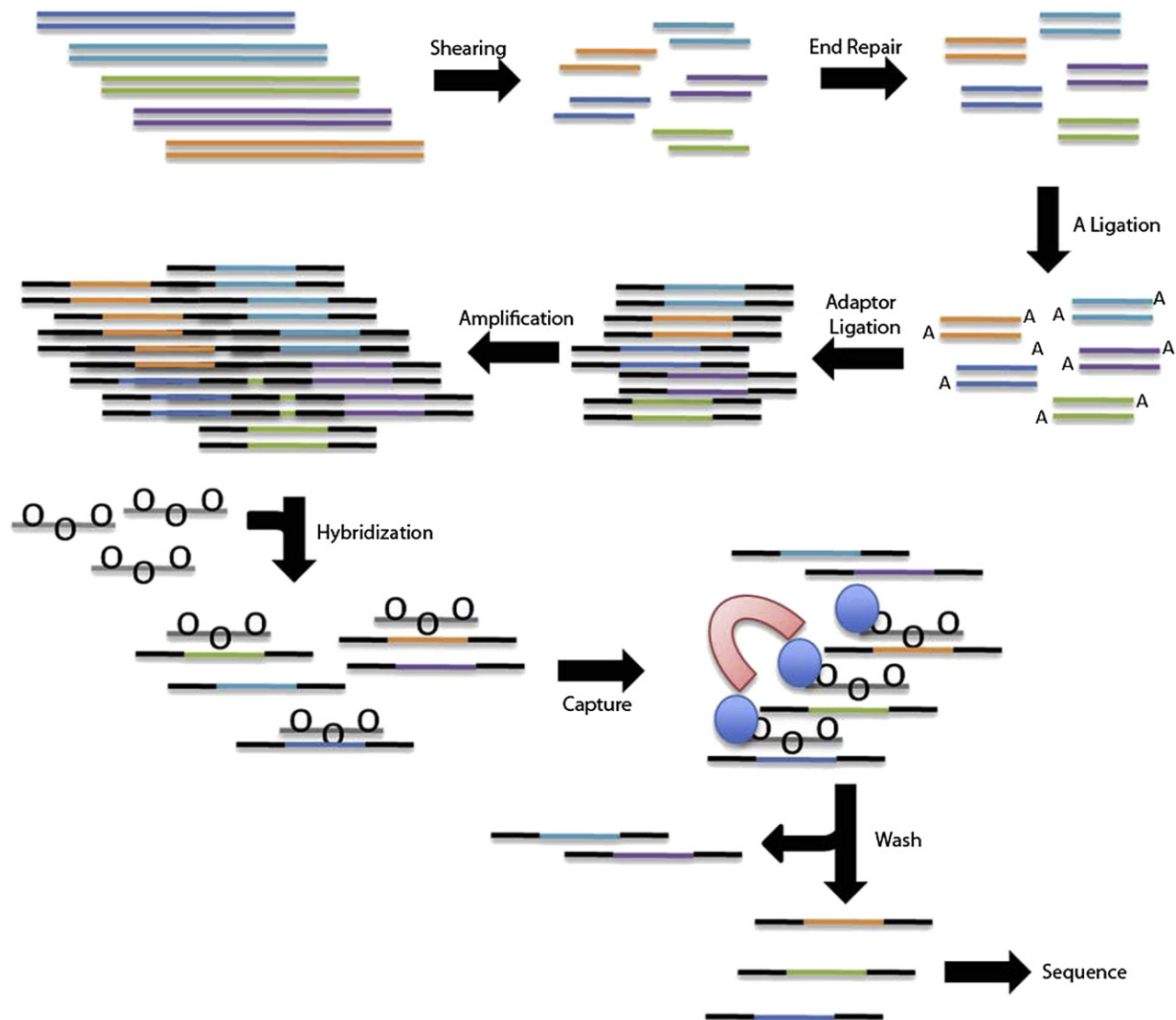
All the approaches listed earlier are direct mutation detection methods, which can only be used for disorders whose genes have been identified and cloned. Testing for diseases whose genes have not yet been identified but have at least been mapped to a particular chromosome requires linkage analysis, the use of polymorphic DNA markers to follow segregation of the mutant allele in a family group. This sort of testing requires collection of DNA specimens not only from the proband but also from multiple other affected and unaffected family members over two or three generations. Also required are polymorphic markers located sufficiently close to (or even inside) the disease gene so that false results due to crossing over are minimized. Although these are now available for most loci in the form of short tandem repeat markers, linkage analysis is not especially favored by diagnostic laboratories and instead is kept in reserve as a last resort for special situations involving diseases with unknown or very large genes. Linkage analysis is always more cumbersome and involved than direct mutation analysis; it has a higher risk of misleading results and requires family cooperation, which may not always be feasible. It is also typically more expensive, and reimbursement becomes awkward when third-party payers see multiple noncovered individuals being tested under the same billable procedure. Most laboratories are only too happy to leave it behind the instant the identification of the disease gene is reported.

Furthermore, the advent of massively parallel (next generation) DNA sequencing has placed linkage analysis even lower on the priority list, since it is now possible to accomplish both gene discovery and mutation identification by whole-exome sequencing of the proband and parents. Currently available next-generation sequencing platforms offer sequencing throughput

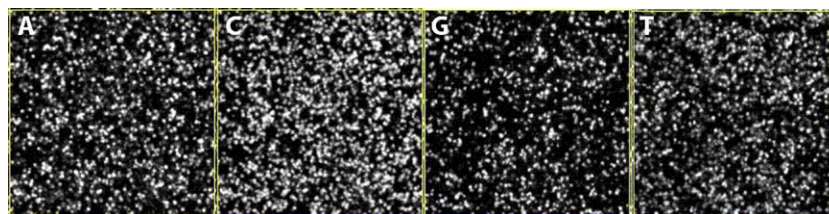
many orders of magnitude above that which is possible using traditional Sanger (dideoxy chain-termination) sequencing (13). The technology is based on the ability to perform many parallel DNA synthesis reactions in a manner that allows for the individual products to be analyzed. This parallelization is achieved through the addition of universal primers to DNA fragments that have been immobilized onto a solid support at a dilute concentration (Figure 23-2). Each DNA molecule is amplified, producing a colony of distinct fragments, and then the colonies are sequenced through the capture of images that reflect the base-by-base addition of nucleotides (Figure 23-3). Differences in sequencing chemistries of the various platforms result in differences in sequencing capacity, read length, and run time. When sequencing is complete, the resulting sequence reads are aligned against the consensus human genome sequence and all variants are listed, filtered, and annotated. It goes without saying that the computer informatics steps in this form of testing are just as important as the “wet bench” analytical steps.

**23.3.2.6 Interpretation of Novel Sequence Variants.** Because sequencing will detect any nucleotide change present—not just the previously known mutations in the gene—it becomes important to distinguish benign nucleotide sequence variants (polymorphisms) from pathologic ones. This is not as easy or straightforward as it may seem. While nonsense, frameshift, and splice-site mutations are usually quite obviously pathologic, especially if they occur prior to the 3′ terminus of the gene, missense mutations can bend the rules: even a mutation leading to an apparently conservative or synonymous amino acid substitution may somehow disrupt transcription or somatic stability down the line, and a major amino acid substitution may not be deleterious if it lies within a static or nonessential domain of the protein.

Given that roughly one in every thousand nucleotides in the genome is polymorphic and that we have not yet begun to scratch the surface in our sequence analysis of most genes, any laboratory performing whole-gene sequencing or whole-exome sequencing is going to have to deal with this conundrum. Both the College of American Pathologists (CAP) and American College of Medical Genetics (ACMG) guidelines stipulate that it is the laboratory’s responsibility to at least attempt an interpretation of the likely clinical significance of any novel variants discovered, rather than simply passing off the burden to the ordering physician. This interpretive discussion, even if admittedly somewhat speculative, must be part of the laboratory test report. Parameters that enter into this analysis include the nature of the amino acid substitution (conservative, nonconservative, and synonymous), the position of the substitution in the gene and protein product, phenotypic data on the variant if available in the medical literature or mutation databases, degree of evolutionary conservation of



**FIGURE 23-2** Schematic overview of next-generation sequencing methodology. Adapter ligation to short DNA fragments is followed by amplification, hybridization, and capture of select genomic regions prior to sequencing. The simultaneous capture of specific genomic regions is utilized when intending to test for only a panel of genes (e.g. for all the known genes associated with hypertrophic cardiomyopathy or hereditary hearing loss), as well as for sequencing all of the coding exons in the genome (also known as whole-exome sequencing). Whole-genome sequencing does not require any hybridization and capture steps to be performed. (Photo courtesy of Dr. Hane Lee, UCLA)



**FIGURE 23-3** Images from one of the commonly used next-generation sequencing platforms (Illumina). With this particular sequencing chemistry, the four nucleotides are each labeled with a different fluorescent dye. After addition of all four nucleotides to the chamber and excitation with a laser, a high-resolution image is captured to identify the newly incorporated nucleotide in a one-base extension step at all of the DNA fragment clusters. The reversible terminators are then removed, and a new cycle of sequencing is initiated. (Photo courtesy of Dr. Hane Lee, UCLA)

the nucleotide and amino acid in question, presence or absence of the variant in affected and unaffected relatives of the patient or in the general control population, in vitro functional studies (if available), and prediction of likely impact of the single nucleotide polymorphism

(SNP) on protein function using available programs such as PolyPhen and SIFT (14)—recognizing up front that none of these criteria or programs is absolutely predictive. ACMG has published guidelines for interpretation of sequence variants (15).



## 23.4 MOLECULAR GENETIC DIAGNOSIS OF PARTICULAR DISEASES

### 23.4.1 Cystic Fibrosis

It seems appropriate to begin the discussion of specific disease examples of molecular genetic testing with CF, since during the 1990s and 2000s it has come to be viewed as the prototypic challenge in DNA testing for mutationally heterogeneous disorders. Because it has one of the highest carrier frequencies of any serious autosomal recessive disease, it was considered a high-priority target for population screening ever since the causative gene was identified in 1989 (16,17). Because CF carriers have no detectable signs or symptoms to distinguish them from other members of the population, such screening could only be accomplished at the DNA level, making CF a “pure” molecular genetic disease from this standpoint. In addition, DNA testing for CF mutations allows for reliable prenatal diagnosis (provided the identity of both carrier mutations has been established in the parents) and for clinical diagnosis in cases in which the manifestations or the sweat chloride levels are atypical or borderline.

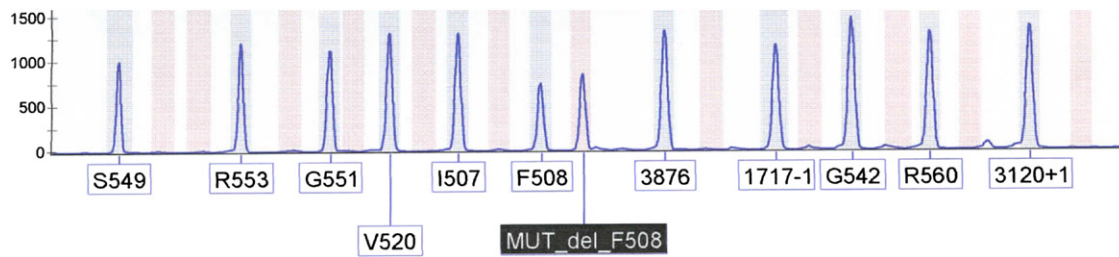
However, as is now widely known, the CF gene and in some sense the disease itself have proven resistant to simplistic approaches. While approximately 70% of Caucasian carriers have the common  $\Delta F508$  mutation, the other 30% (and an even greater proportion in other racial and ethnic groups) may have any of over 1800 other, much less common, mutations. The second most common mutation in non-Jewish Caucasians, G542X, has a frequency of only about 2%, and beyond the first six mutations, the individual carrier frequencies fall below 1% (18). Additional problems are raised by the extreme ethnic heterogeneity of the US target population, ethical issues surrounding prenatal testing for a disease of considerable clinical variability, the notoriously poor correlation between individual CF mutations and phenotypic expression (19), and a perception of inadequate genetic counseling resources to handle the caseload of nationwide screening (20).

As discussed earlier, the most assured way to pick up the greatest number of possible mutations in a large gene like *CFTR* (which spans 230kb and 27 exons (21)) is DNA sequencing. However, that procedure is too expensive for general population screening, and too comprehensive as well, in that missense variants of unknown significance will be found in an appreciable proportion of individuals. Screening requires a more targeted procedure such as allele-specific PCR or microarray hybridization with ASO probes. But which probes to use? Choosing a consensus panel of prevalent CF mutations was not a straightforward task, since all but one are rare, and the prevalence of individual mutations varies greatly according to ethnic group. Over the years, laboratories have employed panels of 6, 16, 30, or over 100 mutations, with remarkable diversity in practice from laboratory to

laboratory (22). After much deliberation, a core, panethnic panel of 25 mutations (along with some associated polymorphisms) was recommended by a subcommittee of the ACMG in order to bring some uniformity to the field and to offer the most efficient strategy for screening in an ethnically diverse population like that of the United States (23). The criterion used for inclusion in the panel was greater than 0.1% carrier frequency in the affected population as gauged by a large survey of CF patients (24); in addition, some mutations especially prevalent in ethnic groups likely to be screened, such as the W1282X mutation in the Ashkenazi Jewish population and the 3120+1G→A mutation in African-Americans, were included. This core panel produces carrier detectability rates of about 97% in Ashkenazi Jews, 80% in non-Jewish Caucasians, 69% in African-Americans, 57% in Hispanic Americans, and an uncertain, although very low, percentage in Asian-Americans.

As in many programs that quickly expanded from a limited pilot cohort to large-scale application, the early years of CF carrier screening revealed a few surprises. One mutation in the core panel, 1078delT, was found to be more rare in the general population than initially thought and would not have reached the 0.1% threshold for inclusion. Another one, I148T, was discovered to be a benign polymorphism and an incidental finding in affected patients who invariably had another, more rare, mutation in the same allele. Both of these variants have been removed in a revision of the panel announced in 2004 (25). Conversely, laboratories offering screening with larger panels discovered a number of other mutations with frequencies above the 0.1% threshold, but for reasons of uniformity and parsimony it was decided that these would not be added to the core panel for the time being. Also surprising was the relatively low uptake initially by obstetricians, despite the fact that offering this screening test to all pregnant couples (and those planning a pregnancy) was determined to be the standard of care. However, this situation has improved steadily over the years. Another, perhaps predictable, trend has been the tendency among some laboratories, especially on the commercial side, to offer ever larger mutation screening panels, even though the official guidelines discouraged the use of so-called extended panels beyond the core 25 (now 23), and it remains debatable how much added benefit such panels provide, especially at the cost of including some variants of dubious or poorly characterized clinical significance (7,26,27).

Laboratories and manufacturers have developed a variety of platforms to encompass whatever mutation panel is chosen. Some CF mutations can be differentiated by electrophoretic sizing and/or restriction endonuclease digestion of PCR products encompassing the codon in question, although this approach becomes somewhat laborious when targeting more than a handful of mutations. Reagent and equipment manufacturers have come forward with platforms to handle the much



**FIGURE 23-4** Results of oligonucleotide ligation assay (Abbott) on a specimen heterozygous for the *CFTR*  $\Delta$ F508 mutation. Following PCR amplification, allele-specific wild-type and mutant probes are hybridized to the corresponding DNA templates. Ligation to fluorescently labeled common probes followed by separation and detection by capillary electrophoresis allows for the simultaneous discrimination of a core panel of *CFTR* mutations.

higher sample throughput in CF carrier screening, in the form of ASO probe hybridization on paper strips, microarrays, bead arrays, oligonucleotide ligation assay (Figure 23-4), and single nucleotide variant analysis on automated DNA sequencers. A few laboratories now offer complete *CFTR* gene sequencing (28) and/or mutation scanning (29), but these approaches are more applicable to diagnostic testing than to carrier screening. They can also be helpful in identifying rare parental mutations in an affected child, which can then serve as targets for prenatal diagnosis in a subsequent pregnancy.

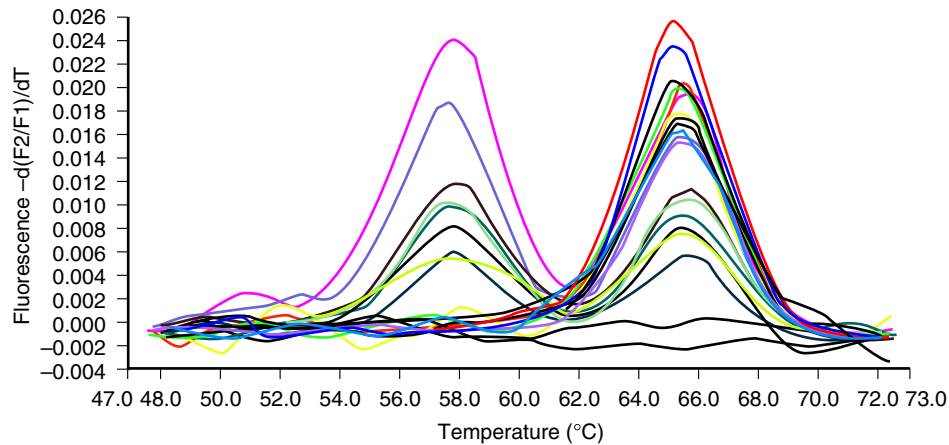
Another lesson from the CF gene story, since repeated in other diseases as well, is the broadening of the clinical phenotype as novel mutations are detected in increasing numbers and varieties of patients. It was already known that some CF patients may have minimal lung disease (30) or normal sweat chloride levels (31), but it later became apparent that there were individuals with mutations in the *CFTR* gene presenting with phenotypes not resembling classical CF at all. The gene has been implicated in such conditions as pancreatitis (32) and sinusitis (33), but most important has been the association of certain *CFTR* mutation and polymorphism combinations with an isolated congenital malformation, bilateral absence of the vas deferens (34). Especially associated with this condition are the R117H mutation and an intronic polymorphic tandem repeat of either five thymidines in *trans* or seven thymidines in *cis* (35); five thymidines in *trans* with other mutations can also produce the same malformation (36). The variable expressivity of the R117H mutation, requiring reflex testing for the poly T tract, has proved to be one of the more problematic aspects of the CF carrier screening program (37). However, CF is certainly not unique in this regard, and that is why the lessons we learn from the widespread molecular testing in this disease will be so relevant to many other applications in diagnostic molecular genetics.

### 23.4.2 Factor V Leiden and the Hereditary Thrombophilias

Similar to the CF carrier state, the Leiden mutation in the clotting factor V gene is a common enough allele (up to 7% in Caucasians (38)) that its DNA assay has been

considered as a screening test, if not in the general population then perhaps at least in those individuals already at risk for thrombosis due to environmental factors (surgery, paralysis, trauma, malignancy, and oral contraceptive use). Indeed, the DNA test itself is very easy, since the Leiden mutation (R506Q) disrupts an *MnlI* restriction endonuclease site present in the wild-type gene (39). There are also a variety of automated methods, microarray hybridization, and real-time PCR coupled with melt-curve analysis (14). Yet, also analogous to *CFTR*, the biology of this gene in vivo and in large populations is somewhat more complex.

The R506Q substitution renders factor V resistant to cleavage by activated protein C (APC), a key mechanism for keeping the coagulation cascade in check. It is the major (but not the only) cause of thrombophilia due to APC resistance. It is best to think of factor V Leiden as a genotype and APC resistance as the phenotype. As such, the latter can be diagnosed by functional coagulation assays on patient plasma, and some authorities believe that the functional assay is preferable to the DNA test as an initial diagnostic screen since it casts a wider net. On the other hand, technical modifications of the functional assay have made it almost entirely specific for the factor V Leiden etiology (40). In any event, the DNA test is certainly useful because of its lack of interference by anticoagulant therapy and its ability to reliably distinguish homozygotes from heterozygotes (Figure 23-5). The latter is important because heterozygotes have a 7- to 10-fold increased risk of venous thrombosis, while homozygotes have about an 80-fold increased risk (41). While these relative risk estimates may seem high, it is important to remember that idiopathic thrombosis in otherwise healthy adults, especially young adults, is quite rare; so the absolute risk, especially in heterozygotes, is not particularly great. In fact, the lifetime risk of an adverse event in a carrier is much less than the risk of complications from long-term anticoagulant therapy, which is the intervention presumably being considered in those testing positive. A similar risk differential can be stated for female carriers taking oral contraceptives who, as a result of DNA testing, might be obligated to seek other, less effective methods of birth control, leading to the risk of adverse events in unintended pregnancies



**FIGURE 23-5** Detection of factor V Leiden mutation by real-time PCR and melt curves with allele-specific probes (Roche Molecular Diagnostics). Each colored curve represents the genotype of a particular patient tested, illustrating a selection of wild-type (right peak), homozygous factor V Leiden (left peak), and heterozygous (both peaks).

(73)—although this notion remains somewhat controversial and varies depending on the hormonal makeup of contemporary birth control pills. In view of these low-magnitude relative probabilities, it is understandable that the concept of population screening of asymptomatic individuals for the factor V Leiden mutation has not been endorsed by either of two consensus statements issued by the ACMG (42) and CAP (43). The test remains useful, however, in differential diagnosis of otherwise unexplained thromboembolic events in patients under age 50 and in recurrent pregnancy losses. Because its main application is thus diagnosis rather than prediction, factor V Leiden analysis differs from many of the other genetic tests mentioned in this chapter. It is ordered primarily by internists, hematologists, and obstetricians rather than by geneticists and is widely viewed, rightly or wrongly, as a routine blood test rather than a genetic test. The consensus statements recognized this and did not require any pretest informed consent or genetic counseling (42).

Factor V Leiden is but one of a group of disorders classified as hereditary thrombophilias. While some of the others, such as protein S, protein C, and antithrombin III deficiencies, as well as variants in the factor VIII, fibrinogen, thrombomodulin, and platelet glycoprotein genes, are too rare and mutationally heterogeneous to become candidates for testing at the molecular level (44), there is one that approaches factor V Leiden in its indications and ease of testing. The prothrombin 20210A mutation (located in the 3'-untranslated region of the gene) leads to increased levels of prothrombin in the circulation and a phenotype similar to that of factor V Leiden (45). It too can be detected by restriction enzyme digestion of PCR products or by automated methods, including multiplex analysis with the factor V Leiden test (46,150). In addition, since it is also relatively common (1–2% carrier frequency), it makes some sense to test for it at the same time as factor V Leiden, provided the same indications are present. Some laboratories also include

in their thrombophilia DNA panel the 677C→T variant of the methylenetetrahydrofolate reductase (*MTHFR*) gene, which leads to elevated plasma homocysteine levels through inhibition of the folate-mediated remethylation of that compound. This allele is very common, found in 30–40% of the general population (47,152). Another variant, 1298A→C, has also been associated with elevated homocysteine levels when in compound heterozygosity with 677C→T. However, the phenotype is different from that for factor V Leiden and prothrombin 20210A, since elevated homocysteine is also associated with arterial (including coronary) thrombosis. Moreover, not everyone with an *MTHFR* variant exhibits elevated homocysteine, and not all cases of elevated homocysteine are caused by *MTHFR* variants. Furthermore, recent studies have called into question the fundamental association of modestly elevated homocysteine levels with thrombotic risk (48), and such elevation, even if present, is readily lowered by folate ingestion, either as a vitamin supplement or via widespread fortification in foods. For purposes of clinical decision making, therefore, there does not seem to be a compelling reason to lump this test in with the other two. On the other hand, since all three of these carrier states are quite common, it is not unusual to see double (or even triple) mutants, with documented synergistic effects on thrombotic risk (47,49). It should also be kept in mind that the prothrombin 20210A mutation has itself been implicated in myocardial infarction in women (50).

The anticoagulant system is extremely complex, and no doubt there are other hereditary factors yet to be discovered. Even though factor V Leiden is a single, simple mutant allele, the thrombophilic state behaves more like a complex, multifactorial disease, with numerous gene products interacting with one another and with environmental stresses. Sometime in the future, we might anticipate the advent of a “thrombophilia DNA chip” for screening, but at present one would be hard pressed to determine which of the many mutations and

polymorphisms that have been reported in hemostasis genes should be placed on such an array. For such a technology to be truly useful, we will need to achieve a much more thorough understanding of the biology of this complex system.

### 23.4.3 Hereditary Hemochromatosis

Despite the greater attention paid to CF, hemochromatosis is actually the most common inherited single gene disorder in people of northwest European ancestry. It is an autosomal recessive disease estimated to affect about 1 in 300 individuals in this population (51,153). The gene, *HFE*, is located 4.5 megabases telomeric to the HLA-A locus and encodes a major histocompatibility complex class I-like protein that regulates iron absorption from the intestine.

Two missense mutations, C282Y and H63D, have been identified within the gene (52). About 70–100% of hereditary hemochromatosis cases in various studies are homozygous for the C282Y mutation, which is carried by about 10% of Caucasian individuals (53). The other mutation, H63D, is found in a compound heterozygous form with C282Y in 3–5% of patients. These two mutations are in linkage disequilibrium and have not been found together on the same haplotype. However, considering the high carrier frequency of the H63D mutation in the general population (about 25%), its involvement in the pathogenesis of the disease remains controversial; it might represent a minor mutation of low penetrance or even a polymorphism (54). One recent large study of over 3500 affected patients found homozygosity for C282Y in 81%, homozygosity for H63D in about 1%, compound heterozygosity for C282Y and H63D in 6.7%, and combinations with another variant, S65C, in fewer than 0.5% (55).

Most laboratories offering *HFE* mutation testing assay for both mutations, while a few also include S65C. Molecular diagnostic testing for these mutations can be used to confirm the diagnosis of hemochromatosis in individuals with symptomatic disease, and potentially to detect those with presymptomatic iron overload in whom future disease manifestations may then be prevented by simple measures (regular phlebotomy). Because of the high carrier frequency, easy therapeutic intervention, and irreversibility of organ damage if not diagnosed early, hereditary hemochromatosis has been considered as a target for population screening. It remains controversial, however, because of the reduced and variable penetrance, even in C282Y homozygotes (56). While the penetrance of this genotype has commonly been quoted as about 70% (57), more recent surveys have suggested that it may be much lower, possibly less than 10% (58,59). It is also not clear whether, analogous to the situation with factor V Leiden, the functional assay (in this case, transferrin saturation and serum ferritin levels) might be the more appropriate initial screening test. In

a large survey of 100,000 individuals, assessed for both serum iron measurements and *HFE* genotype, biochemical differences between C282Y homozygotes and controls were variable, and no consistent deleterious effects of iron overload between the two groups could be demonstrated (60). It should also be noted that there are less common types of hereditary hemochromatosis caused by mutations in other genes such as *hepcidin*, *hemojuvelin*, and *ferroportin* (61).

### 23.4.4 Hereditary Hemoglobinopathies

Hereditary hemoglobinopathies encompass a vastly heterogeneous group of genetic disorders affecting globin protein structure and/or synthesis. Therefore, while the globin genes are relatively small compared to those of other important medical genetic disorders, the approach to their molecular testing is quite varied.

**23.4.4.1 Structural Globin Disorders.** While the greatest incidence of sickle cell disease occurs in sub-Saharan Africa, the carrier frequency in the African-American population is 8%, making it potentially a significant target for DNA testing and screening, although socioeconomic factors have impeded such efforts in the past. This is ironic considering that sickle cell disease was the first human genetic disorder for which the molecular basis was described. Later, the  $\beta$ -globin gene was the first human gene to be cloned and its various mutations demonstrated. Over 400 point mutations in the  $\beta$ -globin gene have now been identified, with the sickle cell mutation being the most common and most severe. While many of these can be identified by nonmolecular methods as well, it is important to remember that the number of structural variants that can be identified by standard hemoglobin (Hb) electrophoresis represents a minority of the total number in existence, because only approximately one-third of them produce an altered charge in the Hb molecule. In addition, in hyper-unstable Hb syndromes, the protein may be so labile or short-lived that electrophoretic identification is hampered. In these cases, DNA analysis will be preferable. For the same reasons, molecular testing can serve as a useful second-tier backup to newborn sickle cell disease screening by biochemical methods, even using the same blood spots (51).

The original DNA-based method for prenatal diagnosis of sickle cell disease used linkage analysis. An *HpaI* restriction fragment length polymorphism (RFLP) downstream of the  $\beta$ -globin gene was linked to the sickle cell mutation in 87% of African-American Hb S genes (62). Now, of course, direct mutation detection strategies are preferred. This can be accomplished by hybridizing labeled ASO probes complementary to either the  $\beta^A$  or  $\beta^S$  allele to genomic DNA blotted or spotted onto a membrane or array (63). Alternatively, one can use a PCR approach that capitalizes on disruption of a restriction endonuclease cleavage site by the HbS mutation. The mutation ablates an *MstII* or *DdeI* restriction site in



the gene, producing a larger fragment after enzyme digestion, as compared to normal DNA (64) (Figure 23-6).

Other structurally abnormal Hb disorders that may be detected in this manner include HbE, HbD-Los Angeles, and several rarer variants (65). The alternate mutation in codon six of  $\beta$ -globin causing HbC disease does not affect a restriction endonuclease recognition site and therefore must be detected with ASO probes (66) or ARMS. Multiplexing of ARMS primers to detect several globin mutations at once has been developed (67), as has competitive priming of fluorescently labeled primers (68).

**23.4.4.2 Disorders of Globin Synthesis—The Thalassemias.** Widespread population-specific screening of carriers for autosomal recessive disorders was initiated first with the thalassemias. Successful thalassemia screening programs have resulted in a marked reduction in the birth rate of affected children in Greece, Cyprus, continental Italy, Sardinia, Taiwan, China (Guangzhou), and South-east Asia (69). The thalassemias are a large and heterogeneous group of disorders of imbalanced Hb synthesis with widely varied phenotypic expression. From the laboratory perspective, the most important ones are  $\alpha$ - and  $\beta$ -thalassemia (and their respective intermediate subtypes).

**23.4.4.2.1  $\alpha$ -Thalassemia.**  $\alpha$ -Thalassemia is most commonly due to a deletion of one or more of the four  $\alpha$ -globin genes ( $\alpha_1\alpha_2/\alpha_1\alpha_2$ ). Deficits relate closely to gene dosage effects, and therefore dosage, and sometimes phase analysis, is an important component of the molecular diagnostic effort. Deletion of one  $\alpha$ -globin gene, usually the  $\alpha_2$ , is most common. These occur predominantly in two types, a 3.7kb deletion ( $-\alpha^{3.7}$ ) and a 4.2 kb deletion ( $-\alpha^{4.2}$ ). The  $-\alpha^{3.7}$  deletion is seen in individuals from Africa, the Mediterranean, the Middle East, and Oceania. The  $-\alpha^{4.2}$  deletion is seen in individuals from South-east Asia and the Pacific Islands (70). A subset of patients in certain ethnic groups may have point mutations in the  $\alpha$ -globin genes.

Since most  $\alpha$ -thalassemias are thus due to sizable deletions, Southern blotting of endonuclease-digested genomic DNA was traditionally used for

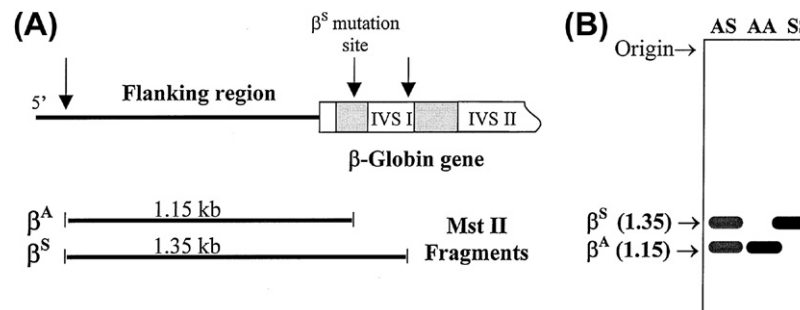
diagnosis, although now more modern techniques such as dHPLC and MLPA are usually used (12). The two  $\alpha$ -globin genes have divergence in their sequences in the 110-nucleotide-long 3'-untranslated region, allowing the presence of one or the other or both to be distinguished.

**23.4.4.2.2  $\beta$ -Thalassemia.** More than 200 different defects in the  $\beta$ -globin gene that result in  $\beta$ -thalassemia have been described. Molecular diagnosis of the  $\beta$ -thalassemias is made complicated by this heterogeneity. However, a unique spectrum of mutations is usually prevalent in a given population at risk for  $\beta$ -thalassemia. Fewer than 10 mutations usually encompass the majority of molecular lesions in the target population, making screening and prenatal diagnosis manageable (71). In fact, approximately 20 total mutations account for 80% of all the  $\beta$ -thalassemia alleles in the world.

Most  $\beta$ -globin gene defects (about 95%) that result in  $\beta$ -thalassemia syndromes are due to point mutations, including small deletions (up to 17bp) and insertions. About half these mutations completely inactivate the  $\beta$ -globin gene ( $\beta^0$ -thalassemia). This portion is caused by mutations in the initiation codon or the splice junction by or nonsense or frameshift mutations. The  $\beta^+$ -thalassemia mutations mainly affect transcription or messenger RNA (mRNA) processing, usually resulting from mutations located in the promoter region. Gross deletion (>24 bp) of the  $\beta$ -globin gene alone is quite rare, with fewer than 20 types seen (72). The deletion more often involves large segments of the  $\beta$ -globin gene cluster, resulting in  $\delta\beta^0$ - and  $\delta\beta^+$ -thalassemias. Depending on the number and variety of mutations expected, laboratories can choose any of a number of ASO or mutation scanning or sequencing strategies.

### 23.4.5 Trinucleotide Repeat Expansion Disorders

Trinucleotide repeats are members of the tandem repeat families found throughout the genome. However, unlike the more ubiquitous di- and tetranucleotide repeat



**FIGURE 23-6** Detection of the sickle cell disease missense mutation by restriction endonuclease digestion. (A) Schematic diagram of the 5' portion of the  $\beta$ -globin gene containing the sickle cell disease mutation site in codon 6. *Mst*II restriction endonuclease cleavage sites are indicated by the arrows. The abolition of one *Mst*II site by the  $\beta^S$  mutation results in an increase in size of the digest fragment from 1.15 to 1.35 kbp. (B) Schematic diagram of an electrophoretic gel or Southern blot in which the two fragments have been separated: AS, heterozygote HbS carrier; AA, normal control; and SS, HbS homozygote. (From Chang, J. C.; Kan, Y. W. A sensitive new prenatal test for sickle-cell anemia. *N. Engl. J. Med.* **1982**, 307, 30–32.)

polymorphisms, expansion of these triplet repeats to larger size can have a pathologic effect. Such expansion has been identified as a novel mutation mechanism in at least 15 neuromuscular disorders that result from instability of the expanded repeat once it has reached a certain length. The expansions are unstable mutations that tend to increase in size through successive generations, in contrast to repeat lengths in the normal range that are usually transmitted stably despite their polymorphic nature. The subsequent expansion of the repeat in descending generations does not adhere strictly to the rules of Mendelian inheritance because of the dynamic nature of the mutation and its variable expressivity. Anticipation is the hallmark of the trinucleotide repeat disorders, where increasing severity and earlier onset occur in successive generations and are correlated directly with the length of the repeat for some of the disorders. Parent-of-origin effect is also a feature.

Despite their shared features, trinucleotide repeat disorders can be separated into two distinct groups, type I and type II (74). Type I is composed of neurodegenerative disorders that are associated with neuronal loss in the brain, brainstem, and spinal cord. The members of this group are Huntington disease (HD), spinobulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA), and the SCAs, SCA1, SCA2, SCA3 (Machado-Joseph disease), SCA6, SCA7, SCA8, SCA12 and SCA17. Type I disorders are all autosomal dominant, except for SBMA, which is X-linked. These diseases are caused by CAG (or CTG in SCA8) repeat expansions in the coding regions of the genes, resulting in the insertion of abnormally long polyglutamine tracts in the mutant protein. This change in the protein leads to a gain-of-function defect that is ultimately damaging to neurons in which it is expressed. The magnitude of

the intergenerational expansion is usually greater in male than female transmissions; the most severely affected individuals inherit the disease gene from their father. The type II disorders, in contrast, show a variety of clinical features other than neurodegeneration and involve repeat sequences other than CAG that are not in the coding regions of the genes. The sequence and expansion ranges of the repeats in the major trinucleotide disorders are summarized in Table 23-1.

#### 23.4.5.1 Type I Disorders.

**23.4.5.1.1 Spinobulbar Muscular Atrophy.** SBMA, known also as Kennedy disease, is a motor neuron disorder characterized by progressive muscle weakness and atrophy in males. The disease results from an expanded CAG repeat in the androgen receptor gene located at Xq12. The number of CAG repeats ranges from 9 to 34 in normal alleles. Affected males and carrier females have an allele with greater than 35 CAG repeats, detectable by PCR.

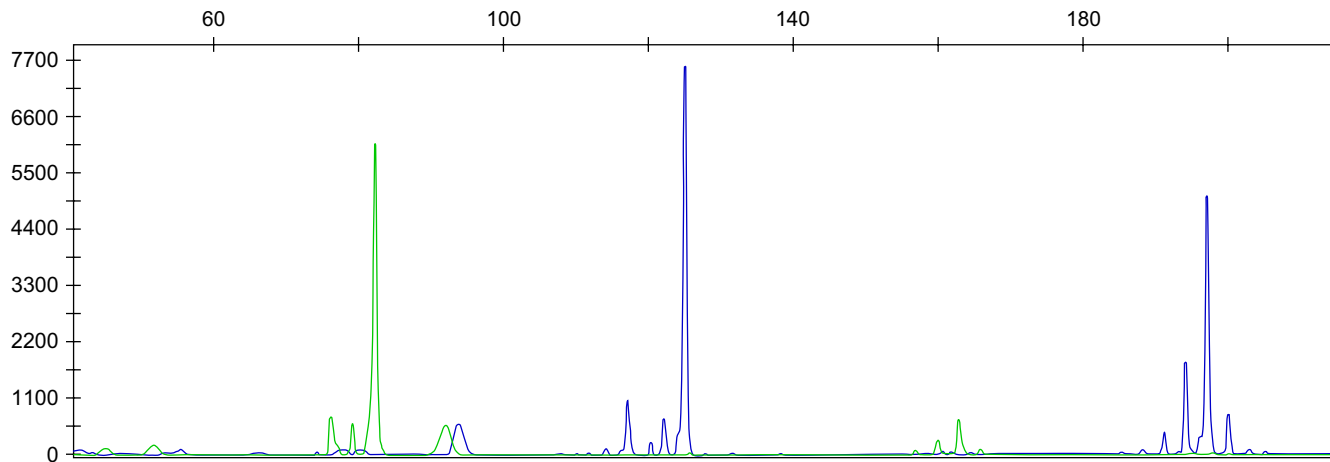
**23.4.5.1.2 Huntington Disease.** The unstable CAG repeat in HD patients lies in exon 1 of the *HTT* gene on chromosome 4p16.3. The CAG repeat length at this locus in the normal population ranges from 10 to 35, whereas in HD patients it ranges from 36 to 121, with a reduced penetrance at repeat sizes of 36–39. Strong inverse correlations between repeat length and age of onset have been observed. Adult-onset patients usually have an expansion in the range of 40–55, whereas juvenile-onset patients have expansions greater than 60 that are often inherited from the father. Sizing of repeat lengths is readily accomplished by PCR amplification and electrophoresis, best done on a capillary instrument for greatest accuracy (Figure 23-7).

Earlier PCR primer sets encompassed a neighboring nonpathologic CAG repeat, itself polymorphic, which

**TABLE 23-1 Mutation Ranges for the Trinucleotide Repeat Disorders**

Disease	Repeat Unit	Normal Range	Premutation or Partial Penetrance Range <sup>a</sup>	Affected Range
SBMA	CAG	9–34	—	36–62
HD	CAG	10–35	36–39	40–121
DRPLA	CAG	6–35	—	49–88
SCA1	CAG	6–34	—	39–81
SCA2	CAG	14–31	32–35	36–64
SCA3	CAG	12–37	—	64–84
SCA6	CAG	4–18	—	21–33
SCA7	CAG	4–35	28–35	37–200+
SCA8	CTG	16–92	—	110–130
SCA12	CAG	7–28	—	65–78
SCA17	CAG	25–42	—	45–63
Fragile X	CGG	5–54	55–230	230–1000+
DM	CTG	5–37	38–49	50–3000+
Friedreich ataxia	GAA	6–34	—	67–1700

<sup>a</sup>Absence of a listing in this column does not necessarily mean that the disorder has no premutation range, only that it has not yet been documented or well defined.



**FIGURE 23-7** Positive test result for HD trinucleotide repeat expansion. Fluorescently labeled primers (green) are used to amplify the HD CAG repeat region by PCR, and the fragments are separated and sized using capillary electrophoresis. One normal allele (<27 repeats) and one full mutation allele (>40 repeats) are evident on the above example. A second set of fluorescently labeled primers that encompass a neighboring polymorphic short-tandem repeat (blue) are run simultaneously in order to confirm the successful amplification of both alleles in cases when only a single peak is seen with the original set of green primers.

led to inaccurate sizing. Current PCR strategies do not include this repeat in the amplicon. However, the older primer set is still sometimes helpful for confirming two normal alleles when only a single peak is seen on the primary screen, which could indicate either homozygosity for a normal allele or failure to amplify a very large expansion. Southern blotting is another way to distinguish these two possibilities. Another reported reason for observing only a single band is PCR failure due to a “null allele” at the primer hybridization site on the opposite chromosome (75). Needless to say, accurate sizing of the repeat number in this disease is extremely important, not only because of the emotionally charged nature of the test but also since the difference in size between affected and intermediate or normal alleles is as little as three nucleotides.

HD was the first genetic disorder to be a target of presymptomatic molecular testing. Because of the virtually 100% penetrance of the full CAG expansion and the lack of any preventive therapy, a positive result in this test represents particularly devastating news, carrying risks of depression, suicide, and discrimination (76). For these reasons, standard practice for predictive testing emphasizes pre- and posttest psychosocial support and genetic counseling.

#### **23.4.5.1.3 Dentatorubral-Pallidoluysian Atrophy.**

DRPLA results in both ataxia and choreoathetosis, along with myoclonus, epilepsy, and dementia, so it is sometimes considered in the differential diagnosis of HD. The gene encodes a cytoplasmic protein with a polyglutamine tract in the middle. The number of CAG repeats in unaffected individuals ranges from 6 to 35, whereas in patients with DRPLA it ranges from 49 to 88. Molecular sizing is done as for HD.

#### **23.4.5.1.4 Spinocerebellar Ataxias.**

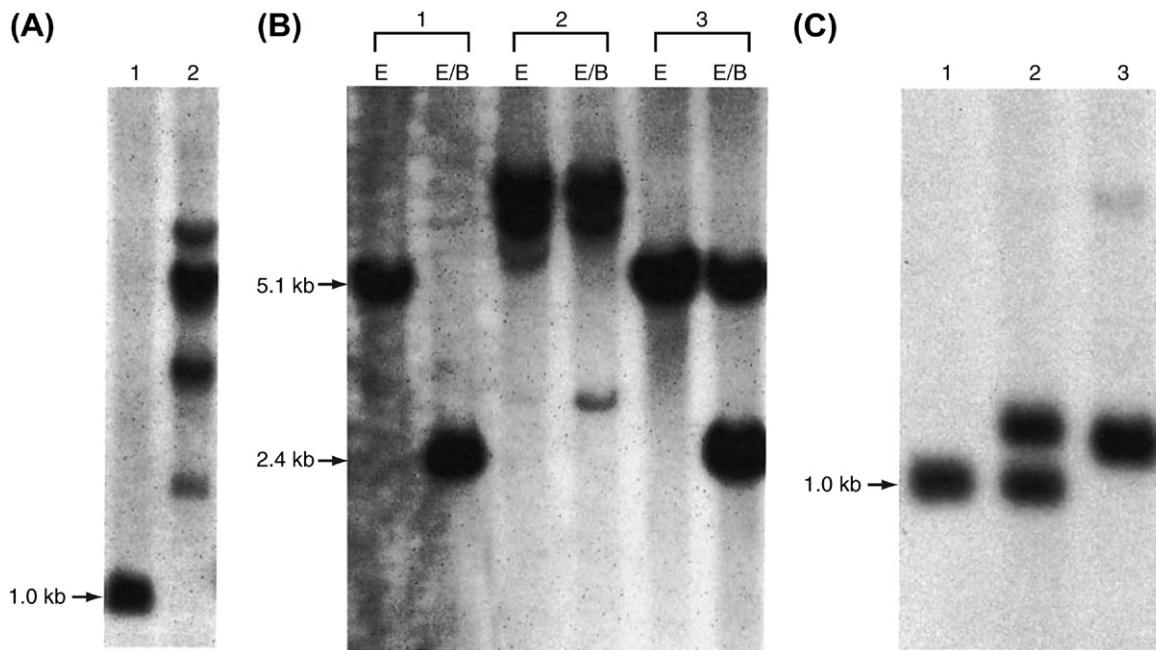
The SCAs are a group of genetically diverse neurologic conditions that

share progressive deterioration in balance and coordination due to degeneration of the cerebellum and its afferent and efferent pathways. Linkage studies identified multiple subtypes, eight of which have thus far been found to have unstable CAG (or CTG) expansions (types 1, 2, 3, 6, 7, 8, 12 and 17). The relatively small size of the SCA expansions (Table 23-1) allows for PCR-based testing as for HD.

**23.4.5.2 Type II Disorders.** The type II triplet repeat disorders include myotonic dystrophy (DM), fragile X syndrome, and Friedreich ataxia. In contrast to the type I group, these are multisystem disorders that are characterized by a constellation of symptoms involving many tissues. The repeat sequences are different in each, located in noncoding regions of the genes, and characterized by much larger expansions that may require Southern blot analysis for sizing. The expanding triplet repeats are located in the 5′-untranslated region of the *FMR1* gene in fragile X, in the 3′-untranslated region of the *DMPK* gene in DM, and, remarkably, in the first intron of the frataxin gene in Friedreich ataxia. The underlying molecular mechanism does not alter protein structure but rather alters the expression of the gene.

**23.4.5.2.1 Fragile X Syndrome.** Fragile X syndrome is caused by a large expansion of CGG repeats in the 5′-untranslated region of the fragile X mental retardation (*FMR1*) gene. The larger expansions are associated with increased methylation of both the repeat and the adjacent CpG island, leading to transcriptional silencing of the gene. The expansion and the subsequent methylation account for the presence of a fragile site (FRAXA) at chromosome Xq27.3 that results from a failure of normal chromatin condensation during mitosis.

Alleles of the CGG repeat can be classified as normal, premutation, or full mutation based on the number of the repeats. In the normal population, this repeat



**FIGURE 23-8** Southern blot analysis of the *FMR1* gene of fragile X syndrome. (A) *PstI* digestion and probing of genomic DNA: 1, normal control showing unexpanded allelic band at 1.0kbp and 2, patient with full CGG expansion mutation showing a spread of high-molecular-weight bands. (B) Southern blot methylation analysis by *EcoRI* and *EcoRI/BssHII* double digestion (*BssHII* is a methylation-sensitive restriction enzyme): 1, normal male showing single unexpanded, unmethylated allele; 2, affected male showing a single expanded and almost completely methylated allele; and 3, normal female showing two unexpanded alleles, one of which is methylated due to X-inactivation. (C) *PstI* digestion and probing of genomic DNA: 1, normal male; 2, female with one normal allele and one premutation allele; and 3, premutation male.

is highly polymorphic, ranging from 5 to 54 repeats, while in affected individuals the expansions range from about 200 to 230 to over 1000 repeats and are referred to as full mutations. The full mutation alleles are usually methylated and fail to express the *FMR1* mRNA and protein. Southern blot sizing of the *FMR1* expansion also includes use of a methylation-sensitive restriction enzyme to determine methylation status (Figure 23-8), which is especially important since some males with full but unmethylated expansion and no clinical symptoms have been reported (77). In addition, use of the methylation-sensitive enzyme allows separation of the two *FMR1* alleles in females, since the one located on the inactive X chromosome will be resistant to digestion because of intrinsic (nonpathologic) methylation. This approach helps to avoid overlooking a very large expansion in a female when only a single normal band is observed on the first-pass Southern blot (using a methylation-insensitive enzyme) or PCR. On the other hand, PCR is preferred for accurate sizing of premutations in carrier females, since the risk of expansion in offspring increases incrementally with increasing premutation lengths (78). Difficulty in amplifying the GC-rich premutation alleles of fragile X patients can be overcome by the addition of dimethylsulfoxide, 7-deaza-dGTP, and betaine to the PCR reaction, and recent innovations in primer design now allow for amplification and even methylation analysis of full mutations (9).

Large expansions display mitotic instability, resulting in a mixture of allele sizes within a single individual.

Premutation alleles ranging from about 55 to 200 are not typically associated with methylation, but they display instability in subsequent generations. These alleles may change in size when transmitted from either sex, invariably remaining within the premutation size range in male transmission but expanding to full mutation size in female transmission. (Alleles in the so-called “gray zone” of 45–54 repeats have a slight chance of expanding incrementally into the premutation range but not into the full mutation range.) The presence and location of AGG triplets interspersed within the CGG repeats in the *FMR1* gene also play an important role in the stability of the repeat. An uninterrupted pure CGG repeat of greater than about 33–39 triplets appears to increase the instability of maternal alleles (79,80). While it was long assumed that premutation carriers have a normal phenotype, we now know that they can cause late-onset effects, including premature ovarian failure in women and a middle-aged tremor-ataxia-dementia syndrome in men (81). Needless to say, the awareness of these phenomena now raises ethical concerns in the reporting of premutation results in fetuses and children, particularly if discovered as a consequence of population screening (5).

Identification of some families that were cytogenetically positive for the fragile site on chromosome Xq27-28 but negative for *FMR1* expansion led to the discovery of two more fragile sites, FRAXE and FRAXF, both located distal to FRAXA (82). Both involve GCC repeat expansion, with FRAXE exhibiting mild mental retardation in the absence of the characteristic physical stigmata

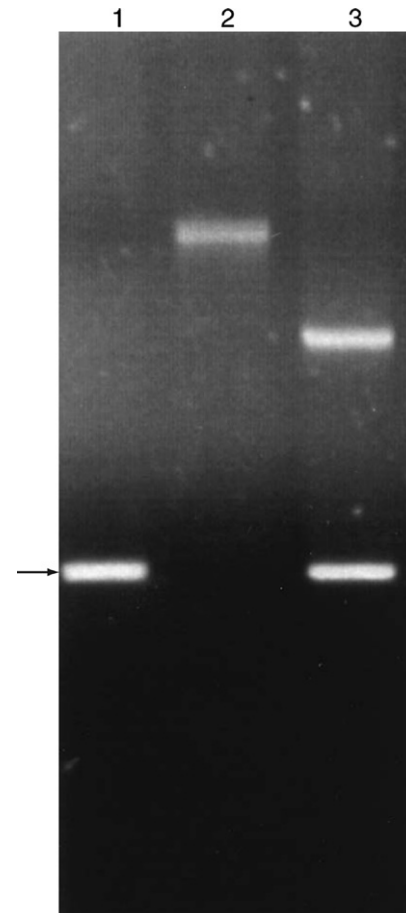


of FRAXA and FRAXF showing no apparent clinical phenotype. In practice, these two genes are not often investigated.

**23.4.5.2.2 Myotonic Dystrophy.** DM is the most common inherited muscle disorder in adults, with effects in many other organ systems as well. The genetic basis for DM is an expanded CTG repeat in the 3'-untranslated region of a protein kinase gene (*DMPK*). The size of the expanded repeat and the severity of the disease tend to increase in successive generations. The normal repeat size is 5–37 copies, while expansions range from 50 to more than 3000 in affected individuals. CTG repeat lengths in the range of 38–49 are considered “premutations.” In the range of 50–150 repeats, patients develop mild DM with cataract, mild myotonia, or diabetes mellitus, and they may have fully active lives and a normal or minimally shortened life span. In the range of 100–1500 repeats, patients usually develop classic DM with muscle weakness and wasting, myotonia, cataracts, and often cardiac conduction abnormalities, with the age of onset typically in the 20s and 30s. Most infants with congenital DM have more than 1000 CTG repeats, with mental retardation (in 50–60% of cases) and early death. Infants with congenital DM nearly always inherit the expanded *DMPK* allele from the mother, but inheritance from the father is possible. Because of the large size of some DM expansions, “long PCR” or Southern blotting may be required for diagnosis.

**23.4.5.2.3 Friedreich Ataxia.** Friedreich ataxia, the only autosomal recessive among the triplet repeat disorders, is caused in 97% of patients by the expansion of a GAA repeat in the first intron of the frataxin gene. The level of frataxin protein expression is decreased in affected individuals with expanded repeats, presumably by an inhibitory effect of the intronic repeat on transcription (83). The larger the repeat, the greater the effect on mRNA and protein levels. Normal individuals have 6–34 uninterrupted GAA repeats, and affected patients have 67–1700 repeats. Sizing can be accomplished by either PCR or Southern blotting (Figure 23-9). Compound heterozygotes have been identified in a small percentage of cases with a triplet expansion in one allele and either a nonsense mutation, missense point mutation, or initiation codon mutation in the other allele. This makes the results reporting rather complicated, since the methods used for repeat sizing will not pick up point mutations, and referral to a specialized laboratory offering full gene sequencing may be required.

**23.4.5.3 Duchenne and Becker Type Muscular Dystrophies.** Duchenne (DMD) and Becker (BMD) muscular dystrophies are allelic forms of an X-linked neuromuscular disorder that affects about 1 in 3500 live male births. Both are caused by mutations arising in the gene encoding dystrophin, a cytoskeletal protein that underlies the plasma membrane of normal skeletal muscle. The dystrophin gene is located on the short arm of the X chromosome (Xp21) and is one of the largest



**FIGURE 23-9** PCR analysis of Friedreich ataxia GAA trinucleotide repeat expansion. Arrow indicates the size of PCR products generated from normal alleles. The samples shown are 1, normal; 2, homozygous expanded; and 3, heterozygous.

genes ever characterized, spanning 2.4 Mbp and encoding 79 exons (84). The gene has an unusually high rate of intragenic recombination of about 10–12% in normal pedigrees (85). Recombination events appear to occur mostly in two hotspots located between exons 1–8 and exons 44–51 (86). The majority of the mutations are intragenic deletions (~65%) or duplications (5%) (87,88). The remaining one-third of cases are due to point mutations such as microdeletions, insertions, and substitutions. According to the frameshift hypothesis proposed by Monaco et al. (89), a patient most likely develops a DMD phenotype if the mutation disrupts the reading frame, whereas a BMD phenotype results if the reading frame is maintained. This would explain the apparent paradox of even fairly large BMD deletions producing a mild phenotype. At the protein level (e.g. on Western blot), DMD is seen to be caused by the absence of detectable dystrophin, whereas muscle from BMD patients shows a protein of reduced size at less than 40% of normal levels.

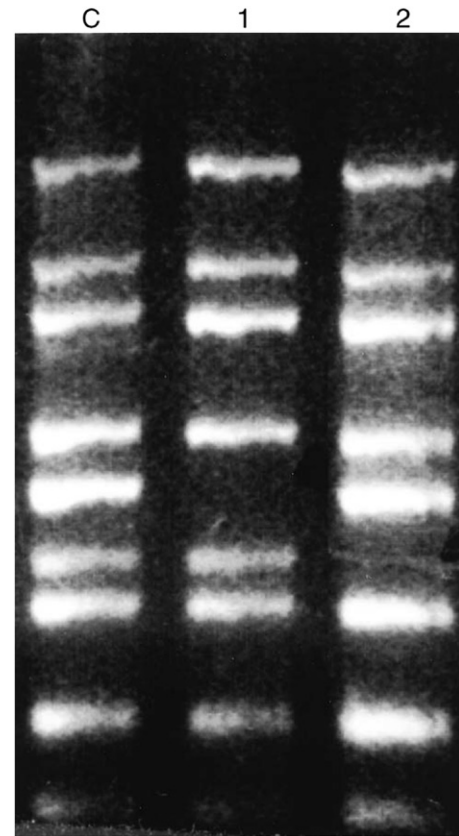
Since DMD is a serious disorder for which at present there is no effective treatment, much emphasis has been given to prevention. This involves the ascertainment of

women at risk of giving birth to affected sons and the provision of genetic counseling and prenatal diagnosis. Accurate carrier detection and genetic counseling depend on first identifying the mutation itself in the proband. The current strategy for DNA testing in this disorder was developed to attempt to circumvent problems arising from the unusual features of the gene, such as its very large size and the great variety of mutations. The approach will vary somewhat depending on the intent and indications for testing, as described in the following paragraphs.

**23.4.5.3.1 Detection of Large Deletions in Patients.** The original method for detecting large deletions was the Southern blot. This approach uses full-length or partial complementary DNA probes to detect deletions by the absence of hybridization or appearance of novel hybridizing bands (junction fragments) (90). This is a laborious method, which may take several weeks to complete if multiple probes have to be tested sequentially. It has now largely been supplanted by a PCR method that uses two sets of multiplex primers, each covering deletion hotspots in various exons of the dystrophin gene (46,91). Deleted exons fail to be amplified and are recognized as absent bands on the electrophoretic gel of the PCR products (Figure 23-10). Because the PCR method screens many regions of the gene simultaneously, it is dramatically faster than the Southern blot approach, with results possible in one day.

**23.4.5.3.2 Detection of Large Deletions in Carriers.** Once a deletion is identified in an affected patient, DNA samples from women at risk in the family can be tested for carrier status. The assay is complicated, however, by the presence of two X chromosomes, so the particular deletion must be detected by dosage analysis. The woman's DNA is subjected to Southern analysis using the same probe that detects the deletion in the related patients. If done carefully, a deletion in one of her two dystrophin alleles results in autoradiographic bands with half the normal intensity (92). Dosage analysis can also be performed by combining the multiplex PCR assay with capillary electrophoresis to measure the height and/or integrated area of each product peak. Alternatively, the deletion can be identified directly if a junction fragment is observed on the Southern blot autoradiogram (93). A junction fragment arises as the result of fusion of the two DNA fragments flanking the deletion, producing a hybridizing band of abnormal size. Unfortunately, however, junction fragments are found in less than 5% of patients tested. As it has for diagnosis, carrier detection has now moved into the PCR era, and a number of quantitative and multiplex approaches are available that can readily detect deletions and duplications, even in the carrier state, and cover all 79 exons of the gene (94,95).

**23.4.5.3.3 Detection of Microlesions in Patients and Carriers.** One-third of mutations in the dystrophin gene are base substitutions or microdeletions/insertions. Several methods have been used to screen the gene for these microlesions, including the



**FIGURE 23-10** Multiplex PCR for detection of dystrophin deletions in Duchenne/Becker type muscular dystrophy, by the method of Beggs et al. (149). The samples shown are C, normal control showing PCR products corresponding to (from top to bottom of electrophoretic gel) promoter and exons 3, 43, 50, 13, 6, 47, 60, and 52; 1, patient positive for a deletion involving exon 13; and 2, patient positive for a deletion involving exon 6.

mutation scanning techniques introduced earlier: SSCP, heteroduplex analysis, chemical mismatch cleavage, DGGE, and PTT (96,97). More recently, the advent of next-generation DNA sequencing has rendered the huge dystrophin gene amenable to complete sequencing and direct detection of point mutations (98). Once the mutation is found in a patient, reliable carrier detection is possible by sequencing only the region containing that particular mutation using the Sanger technique.

In families in which the mutation cannot be detected, carrier risk can be assessed by serum creatine kinase (CK) levels in female relatives and linkage analysis using flanking and intragenic markers. These markers can also support the identification of deletion mutations, exclusion of maternal cell contamination in CVS, confirmation of paternity, and mapping of dystrophin gene recombinations.

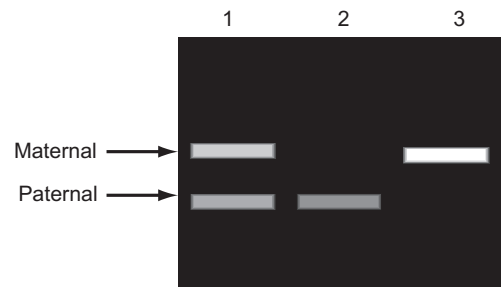
Female relatives of DMD and BMD patients often request genetic counseling, genetic testing, and prenatal diagnosis. As a first step, a pedigree analysis is helpful in evaluating the carrier status in a female. A woman with an affected son and affected relatives from her maternal side is an obligate carrier. A woman with one or more

affected brothers but no affected offspring is a possible carrier. A woman with more than one affected son and no family history of muscular dystrophy is a full carrier, a germline mosaic, or a somatic mosaic including her germline (99). Subsequent brothers and sisters of a patient with a “new” mutation have an estimated risk of 5–10% of inheriting the same germline mutation. Prenatal diagnosis is readily achieved by molecular detection of mutations using chorionic villi or amniocytes.

### 23.4.6 Prader–Willi and Angelman Syndromes

Despite their disparate clinical phenotypes and completely independent genes, these two disorders are forever linked in cytogenetics and molecular genetics laboratories because of their coincident deletion sites on chromosome 15q11–q13 and mirror-image imprinting mechanisms. About 70% of cases of both disorders are due to deletions at this locus, some of which are too small to be visible by chromosome banding, although they may be detected by fluorescence in situ hybridization. Most of the remainder are due to uniparental disomy (UPD) in Prader–Willi syndrome (PWS) and point mutations in Angelman syndrome (AS). About 10% of AS cases are due to an imprinting defect, while a smaller proportion (3–5%) are due to UPD. The small fraction of PWS cases not due to deletion or UPD apparently involve an imprinting defect. Owing to the imprinting phenomenon active at this locus, the AS gene (*UBE3A*) is expressed only on the maternally inherited chromosome 15, while the PWS gene (which is still not identified) is expressed only on the paternally inherited chromosome. Thus, loss of maternal *UBE3A* gene expression by either deletion or UPD for the paternal allele will produce AS, while loss of the paternal PWS gene by either deletion or UPD for the maternal allele will produce AS. As mentioned, loss of *UBE3A* function through point mutation is also seen in AS, but whether or not it occurs in PWS will not be known until the gene is identified. Either disorder can also be produced by aberrant parental gene expression due to a primary imprinting defect.

Molecular genetic testing for these disorders is aimed at tracking the maternal and paternal alleles and determining whether one or the other is either missing (deletion) or duplicated (UPD); obviously, the UPD mechanism results in both duplication of one allele and loss of the other. The two parental alleles can be distinguished from one another because of the differential methylation that occurs at this locus, using Southern blotting following digestion of patient DNA with a methylation-sensitive restriction enzyme (100), methylation-sensitive PCR, or real-time PCR (101). The PCR technique capitalizes on the property that exposure of DNA to sodium bisulfite converts cytosine to uracil except when it is methylated. Primer or restriction endonuclease digestion can be designed to distinguish this difference at a site within



**FIGURE 23-11** PCR-based methylation analysis of the Prader–Willi/Angelman region on chromosome 15, using the sodium bisulfite method. The upper band corresponds to the maternal allele and the lower band to the paternal allele. The samples shown are 1, normal; 2, AS; and 3, Prader–Willi syndrome.

the critical locus, such as the *SNRPN* gene. A missing maternal allele is diagnostic of AS, while a missing paternal allele indicates PWS (Figure 23-11). The real-time PCR technique relies on differential melting curves of methylated vs. nonmethylated CpG sequences. None of these approaches can distinguish between the deletion and UPD mechanisms, however, although in actual practice the difference is not critical since both have very low recurrence risks. Documentation of UPD requires microsatellite analysis of chromosome 15 in the patient and parents, demonstrating homozygosity for the polymorphic alleles, or observation of long stretches of homozygosity on chromosomal microarray analysis. PWS or AS caused by imprinting defects (presumably involving aberrant methylation) will also not be distinguished by these methods (although the tests will still be positive), which is unfortunate because those cases do have a much higher recurrence risk (102). This mechanism is usually established by exclusion of the other molecular defects and is otherwise difficult to prove. However, a microdeletion of the putative imprinting center can be demonstrated in some cases (103).

### 23.4.7 Familial Cancer Syndromes

The advent of counseling for and diagnosis of hereditary cancers represented something of a paradigm shift for the specialty of medical genetics, and one with which it has yet to come completely to terms. Indeed, it is safe to say that it would not even be part of the specialty, remaining instead within the exclusive domain of oncologists, were it not for the advent of molecular diagnostic testing with all its risks and complexities. After all, familial cancers have always been with us, but they have come to the forefront of public consciousness only in the last 15–20 years because there are now predisposition tests for some of the major malignancies. In most cases these are not simple tests with clear-cut results; extreme mutational heterogeneity and variable penetrance make their performance and interpretation quite difficult. These factors, and the theoretical risks of insurance discrimination and adverse psychosocial impact, have led to



practice guidelines mandating pre- and posttest genetic counseling for some of them. Indeed, there are documented cases of serious misinformation ensuing when these procedures are not followed (104).

There are at least 35 strongly heritable syndromes in which various cancers are a primary manifestation (105). This section considers only those autosomal dominant forms for which DNA testing has become prevalent. All of them have in common a pathogenetic mechanism fitting the classical tumor suppressor gene model.

**23.4.7.1 Familial Breast/Ovarian Cancer.** While it was the retinoblastoma gene many years before that first defined the “two hit” model of dominantly inherited cancer, it was the discovery of the *BRCA1* and *BRCA2* genes that really put familial cancer on the map for the medical genetics community and led to only the second routinely offered presymptomatic genetic test after HD. In fact, the two tests are often discussed as instructive analogies for one another, yet they could hardly be more different. Mutations in the HD gene show virtually 100% penetrance, giving the DNA test an extraordinarily high predictive value, while *BRCA1/2* mutations may only be 50–85% penetrant for breast cancer and 25–45% penetrant for ovarian cancer in various families (106). HD has no prevention or treatment (yet), while breast cancer (and to a lesser extent ovarian cancer) can be prevented in some cases by prophylactic surgery and/or medication and screened for early detection. Breast cancer is one of the most common serious diseases in medicine, affecting roughly 1 in 9 women, although it is important to keep in mind that the *BRCA1/2* genes account for only 10% of this incidence at best. Finally, and most important from the perspective of this chapter, HD shows only a single species of easily detectable mutation (the CAG triplet repeat expansion), while *BRCA1/2* mutations and variants are extremely heterogeneous and still not yet completely characterized.

Because of its difficulty and expense, the potential risk of stigmatization or other adverse psychosocial reactions, and the not uncommon finding of nucleotide changes of unknown significance within the gene imparting even more uncertainty to the patient than she had before the test, *BRCA* testing should never be undertaken lightly. Initially there was a general consensus that testing not be encouraged unless the a priori risk of the proband testing positive exceeds 10% (although this strict numerical cutoff has since been relaxed somewhat (107)), and there are several formulas for making this estimation based on personal and family history, ethnicity, and other factors (108,109). Of course, testing of minors should not be considered outside of exceptional circumstances, since there is a risk of misunderstanding and stigmatization for a condition that has no symptomatic onset or clinical intervention until adulthood.

The *BRCA1/2* genes are very large, and hundreds of mutations have been reported, spread diffusely across the length of both genes. In this sense they are similar,

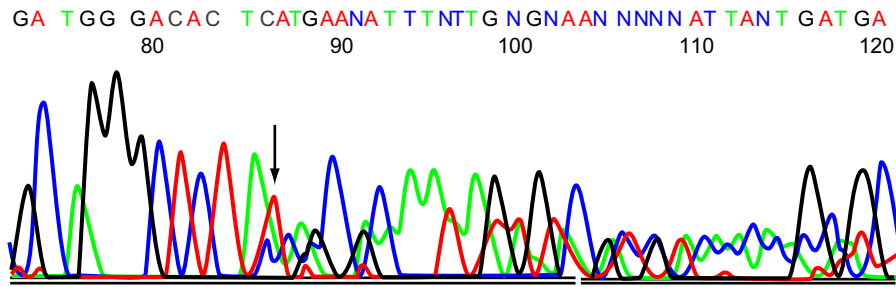
from a molecular testing point of view, to the *CFTR* gene. However, while the clinical impact of missing a carrier of a rare *CFTR* mutation in a population screening program does not justify the cost of sequencing the gene, the adverse consequences of missing a *BRCA* mutation in a presymptomatic test are great enough that full sequencing of both genes is justified despite the cost (assuming there was a strong enough personal or family history to consider testing in the first place). Patients testing negative must be cautioned that even complete sequencing could potentially overlook a mutation in an unusual location (outside the coding region), and also that it is possible the cancer in their family was due to a non-*BRCA* etiology. One class of mutations known to be missed by DNA sequencing are large deletions. Since it is now recognized that these constitute a significant minority of *BRCA* mutations, screening for exon dosage by either Southern blotting or quantitative amplification has become an important component of the testing process (110).

There are two major exceptions to the need for complete gene sequencing in *BRCA* testing. One is the situation in which the mutation in the family is already known from prior testing of an index case (something that should always be sought if the affected relative is available). The other is in the Ashkenazi Jewish population, in which three recurring mutations account for the vast majority (at least 90%) of *BRCA* carriers: 185delAG and 5382insC in *BRCA1* and 6174delT in *BRCA2* (111). Individuals in this ethnic group can be offered a limited screening panel (Figure 23-12) at much reduced cost, although if they test negative in the absence of a known mutation in the index case, the difficult question of pursuing more comprehensive testing of lower yield will arise.

While clinical follow-up is continuing, initial outcomes data indicate dramatic, although not absolute, reduction in breast and ovarian cancer incidence in women testing positive for *BRCA* mutations presymptotically, who then opted for prophylactic surgery. Importantly, in premenopausal women, oophorectomy reduces not only the incidence of ovarian cancer but that of breast cancer as well (112,154).

**23.4.7.2 Hereditary Nonpolyposis Colon Cancer.** Colon cancer, like breast cancer, is a common disease, and similarly, about 10% of cases have a strong familial component. The dominant form of adult-onset colon cancer, as distinguished from familial adenomatous polyposis, is hereditary nonpolyposis colon cancer (HNPCC, also known as Lynch syndrome), characterized by predisposition to early-onset (average age 44 years) tumors mostly proximal to the splenic flexure. Some forms (Lynch II) are associated with other adenocarcinomas of the endometrium, ovary, small bowel, stomach, pancreas, and other organs. Ascertainment of HNPCC families based on history is done using the Amsterdam criteria or their subsequent modifications (113,114),



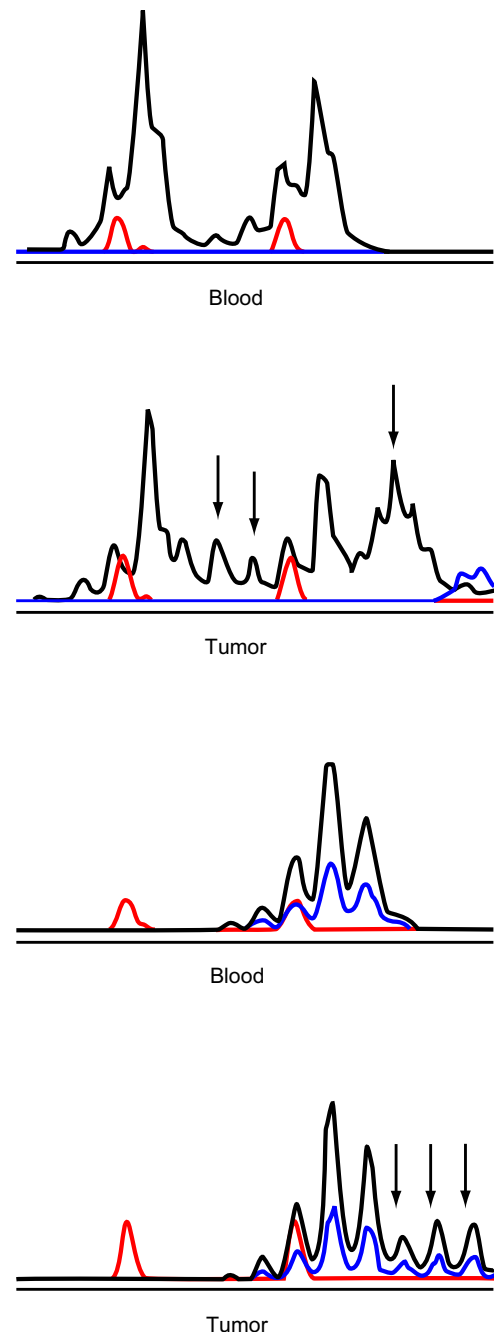


**FIGURE 23-12** Targeted detection of the Ashkenazi Jewish *BRCA1* founder mutation 185delAG by automated sequencing analysis. Because this is a heterozygous two-nucleotide deletion, it results in a heterozygous frameshift on the sequencing readout, causing mismatched nucleotides and overlapping peaks following the mutation site (arrow).

again recognizing, just as in hereditary breast/ovarian cancer, that overly strict adherence to such guidelines results in falsely excluding some affected families (115).

At least five genes are known to be responsible for HNPCC: *MSH2*, *MLH1*, *PMS1*, *PMS2*, and *MSH6*; the first two account for about 70% of the cases (116). The protein products of these genes are involved in DNA mismatch repair, producing the so-called replication error or mutator phenotype in tumor cells. The latter can be assayed in the molecular diagnostic laboratory by observing novel, nongermline bands in electrophoretic analysis of short tandem repeat loci (Figure 23-13), a phenomenon called microsatellite instability (MSI). Indeed, this is an easier test than searching for the many possible mutations in the five causative genes, although it is not entirely sensitive or specific for familial colon cancer (about 15% of sporadic colon cancers also exhibit the phenomenon). However, it may be helpful in estimating whether a colon tumor is due to HNPCC when the family history is incomplete or ambiguous (117). The presence of MSI also has some prognostic and management value, as these tumors tend to be more responsive to standard therapies (118). Direct mutation testing of the five dominant genes by the same approaches used for the *BRCA* genes is available, although expensive. It can be offered to patients and relatives meeting the family history criteria and to those whose tumors demonstrate MSI. Analysis usually starts first with *MSH2* and *MLH1* and then proceeds to the other genes if those are negative. As for *BRCA* testing, the possibility of large deletions invisible to standard sequencing, and missense variants of uncertain clinical significance, must be considered. In index cases from whom tumor tissue is available, analysis of the mismatch repair gene products by immunohistochemistry may offer a less expensive initial screen to allow selection of the particular impaired mismatch repair gene for sequencing.

**23.4.7.3 Familial Adenomatous Polyposis.** This autosomal dominant syndrome is characterized by early onset (childhood or young adulthood) of large numbers of colonic polyps with high malignant potential, along with benign and malignant lesions in a number of other tissues. It is due to mutations in the *APC* gene, which are quite numerous and heterogeneous, although the majority are of the nonsense or frameshift class. Individuals



**FIGURE 23-13** Polymorphic short tandem repeat analysis of paired blood and colonic tumor DNA samples, illustrating extra, nongermline alleles (arrows) in the tumor DNA indicative of MSI. (Red peaks are internal size markers.)

with the “attenuated” form of familial adenomatous polyposis (<100 polyps) tend to have mutations near the 5′ or 3′ ends of the gene (119). This is one of the few predictive genetic tests acceptable for performance in children, because of the early onset of polyps and the onerous clinical surveillance procedures.

**23.4.7.4 Multiple Endocrine Neoplasia.** Multiple endocrine neoplasia (MEN) type 1 is characterized predominantly by neoplasms in the parathyroid, pancreas, adrenals, and pituitary. MEN type 2 features medullary thyroid cancer, pheochromocytoma, and hyperparathyroidism. A subtype, MEN2B, is further characterized by marfanoid habitus, ganglioneuromas, and mucosal neuromas.

The gene for MEN2, the *RET* proto-oncogene, was discovered first, and DNA testing has proved quite valuable for at-risk family members. Indeed, the *RET* gene seems to be everything the *BRCA* genes are not: it contains a limited number of mutations localized to a few exons, and the penetrance is virtually 100%. In fact, most of the mutations in MEN2A are found in exons 10 and 11, with a single cystine codon (number 634) most commonly affected with every possible missense substitution of its three nucleotides. Less common mutations have also been found in exons 13 and 14 (120). Furthermore, almost all cases of MEN2B, which have a unique presentation, show a single missense mutation at codon 918 in exon 16. Thus, molecular genetic testing for MEN2 is very straightforward, typically involving ASO probes or limited sequencing of the mutable exons to identify almost all carriers. Because the penetrance is so high and the highly aggressive medullary thyroid cancers can occur in childhood, *RET* gene analysis is another predictive genetic test indicated for use in presymptomatic children.

The MEN1 gene (designated simply *MEN1*) was identified more recently and has been less subject to testing because of the large number of mutations (>400) described. Most of them are of the nonsense, frameshift, or deletion variety (121) and are typically detected by sequencing.

**23.4.7.5 Other Dominantly Inherited Cancer Syndromes.** Molecular tests are available on a more limited basis for the less prevalent familial cancers. Just a few of the more prominent examples will be mentioned here.

*Li–Fraumeni syndrome*, characterized by a wide variety of tumors in affected families (breast, brain, sarcoma, leukemia, lung, pancreas, etc.), is caused by germline mutations in the *p53* (also designated *TP53*) tumor suppressor gene. The mutations are diverse and must be distinguished from the somatic *p53* mutations that are found in many nongenetic tumors (122).

*Familial melanoma* accounts for about 10% of all melanomas and may be associated with the dysplastic nevus syndrome. Sequencing of the implicated *CDK4* and *CDKN2A* (also designated *p16*) genes is available (123).

*Von Hippel–Lindau disease* exhibits a variety of benign and malignant tumors, most prominently renal clear cell carcinoma. The causative *VHL* gene contains only three exons and so can be sequenced readily, but about a fourth of the mutations are large deletions that must be detected by another method, such as Southern blot or MLPA (124).

*Retinoblastoma*, the cancer that set the stage for our understanding of tumor suppressor gene action, remains one of the more difficult to test for, owing to the large size of the *RB1* gene, the complexity of the mutations, and the rarity of the disorder. However, sequencing and mutation scanning approaches are available in a few laboratories (125).

## 23.5 MITOCHONDRIAL DNA DISORDERS

Lastly, we must not forget about “the other human genome,” the one that exists outside the nucleus, in those energy-generating organelles called mitochondria. Notwithstanding the emphasis of the Human Genome Project on identifying disease-associated genes in the nuclear genome, the mitochondrial genome is subject to pathogenic mutations as well and thus is fair game for molecular diagnostic attention. In fact, the mutation or replication error rate of mitochondrial DNA is substantially higher than that of nuclear DNA, making the mitochondrial genome highly polymorphic between individuals and heteroplasmic within different cells and tissues of the same individual. This latter property in particular makes DNA-based diagnosis of mitochondrial disorders tricky and subtle. Because polymorphisms are usually homoplasmic and mutations are usually heteroplasmic, mitochondrial DNA diagnostic techniques must be of sufficient sensitivity to detect alterations in a portion, or even a minority, of cells (similar to infectious disease and oncology testing), yet of sufficient specificity and clinical predictive value to distinguish them from benign polymorphisms. Next-generation DNA sequencing, because of its ability to sample even minor subpopulations in parallel with the majority, without being diluted out, may be particularly amenable to this application (126).

The 16.5 kbp mitochondrial genome carries genes for 13 polypeptides (components of the respiratory chain), 22 transfer RNAs, and two ribosomal RNAs, and mutations may be found in any one of them (127). Since a number of these mutations are recurrent in particular disorders, a common approach is to test first for some of these as a panel, such as the G11778A mutation, which is found in 50–70% of patients with Leber’s hereditary optic atrophy, or the A3243G mutation, found in a large proportion of patients with myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). Laboratories may offer one or more core mutation panels to be employed as a first-tier test depending on the clinical presentation of the patient, to be followed by another panel or a wider mutation

scanning method if the first test is negative (128,129). The protocol requires close communication with the referring physician in order to decide which panel to utilize and just how far to pursue a mitochondrial etiology that may in fact never be found. In embarking on such testing, the phenomenon of heteroplasmy must always be at the top of one's mind, even from the point of specimen collection: mitochondrial DNA testing may require sampling of the affected tissue, rather than simple phlebotomy, in order to increase the chances of finding the causative mutation.

### 23.6 OTHER TARGETS OF MOLECULAR GENETIC SCREENING

As of 2012, GeneTests, the online directory of molecular genetic testing laboratories (<http://www.ncbi.nlm.nih.gov/sites/GeneTests/?db=GeneTests>), lists over 2500 different disorders for which DNA-based tests are available. Many of these disorders are quite rare, and 11% are offered only on a “research” basis by the investigating laboratories that are studying the genes in question. These are true “orphan” diseases, lacking the test demand to be of interest to mainstream clinical molecular genetics laboratories, and thus the research laboratories fulfill a vital service in making this testing available to families at risk. At the same time, it must be recognized that they are operating outside the legally accepted domain of clinical laboratory testing (at least in the United States), since most of them do not have certification or licensure under the Clinical Laboratory Improvement Amendments (CLIA) guidelines or by other regulatory bodies. Given that most research laboratories would not find it practical or desirable to pursue such certification, while the established clinical laboratories are not interested in assuming the burden of such low-volume and esoteric tests, the best solution seems to lie in the establishment of a network of orphan testing dedicated to covering the aggregate of these rare disorders (130).

At the other end of the spectrum are diseases resembling CF in that they have high carrier frequencies but for one reason or another they have not yet been taken up as targets for large-scale screening. One such example is **spinal muscular atrophy**, with a carrier frequency of about 1 in 50 for mutation in the *SMN1* gene. Given the severity of the disorder and a mutation carrier frequency that approaches that of CF, one might think it an obvious target for population screening. But molecular testing, especially for carriers, is quite challenging because of the presence of a closely homologous adjacent gene, *SMN2*, which can be present in multiple copies and can have a mitigating effect on the phenotype (131). Despite a recommendation from ACMG that population carrier screening be implemented (155), uptake has been slow, and there has been some pushback from the obstetric providers (132).

Another example is **congenital hearing loss**, which is now a target of universal phenotypic screening of newborns in the United States. Because the audiologic screening techniques are not uniformly reliable for the desired early diagnosis and intervention, ancillary molecular genetic testing has been proposed (133). The most obvious target for such screening is the *GJB2* gene, encoding the cochlear hair cell protein connexin-26; mutations in this gene are responsible for about half the cases of nonsyndromic autosomal recessive hearing loss. The mutation carrier frequency in the general population is about 3%, and there are predominant European Caucasian, Ashkenazi Jewish, and Asian mutant alleles. Molecular screening can be done using allele-specific techniques for these three high-frequency mutations or by complete gene sequencing for the more than 100 other variants, since the entire gene is only two exons. Some laboratories also test for a deletion in the neighboring *GJB6* (connexin-30) gene, which can interact with mutations in *GJB2* to cause hearing loss, even in the double heterozygote form. However, there are at least 100 other genes associated with hereditary hearing loss, and to develop a test encompassing them all requires either high-density microarrays or comprehensive next-generation sequencing of the entire gene panel (134,135).

Because of the presence of a founder mutation, 167delT, in the *GJB2* gene carried in as many as 10% of individuals of Ashkenazi Jewish descent, connexin mutation testing could also be added to the **Ashkenazi Jewish carrier screening panels** offered by a number of laboratories. These panels typically contain anywhere from 3 to 13 diseases, the most common ones being CF (carrier frequency in this population, 1/29), Tay–Sachs disease (1/27), Gaucher disease (1/15), Canavan disease (1/36), and familial disautonomia (1/30) (136). As in *GJB2*, the genes for these disorders feature between two and five predominant Ashkenazi mutations each, making screening technically easy and producing an aggregate yield of at least one positive carrier per six individuals tested (137). However, there are a few wrinkles that render the selected composition of the screening panel less than obvious. The most common Gaucher mutation, N370S, produces a fairly mild and late-onset phenotype (138), making decisions about pregnancy termination difficult. Tay–Sachs screening is successfully done by biochemical methods, so there is less impetus to incorporate it into a multiplex DNA panel. Canavan disease seems to be less common in some areas than its carrier frequency would suggest, and CF carrier screening is offered to all pregnant couples anyway, regardless of ethnic background (23).

If screening for connexin mutations becomes widely accepted, it would be the first example of a primary molecular approach in the **newborn screening** setting, since the other first-tier assays in newborn screening are biochemical in nature. However, DNA-based tests

have served as second-tier or follow-up tests for infants showing positive primary screens for CF or sickle cell disease in some states, and increasingly for other disorders in the expanded newborn screening panel. Still, any stand-alone molecular newborn screening test would have to meet the criteria of well-defined predictive value, a manageable number of predominant mutations, availability of a relatively inexpensive test methodology to pick up a significant proportion of them, and effective clinical interventions for those testing positive (136).

### 23.7 PHARMACOGENETIC TESTING

Another application of molecular genetic testing that could potentially involve large segments of the population is pharmacogenetic screening. At its broadest definition, the goal of pharmacogenetic testing is to identify mutations and polymorphisms throughout the genome that determine response and reaction to drugs. Presumably by their effects on drug metabolism and excretion, these genetic variants will identify those patients likely to respond or not respond to varying doses of the drug or to manifest adverse reactions to standard doses. Characterization of these genotypes prior to drug administration could hopefully enable individualized therapeutic approaches to maximize efficacy at the lowest possible dosage while avoiding side effects and toxicity. Pharmacogenetic tests may target germline variants, delineating heritable differences in drug response, or acquired, somatic mutations in tumors to predict response to chemotherapy; only the former application falls within the domain of medical genetics and is considered here.

The largest single class of enzymes involved in drug metabolism is the cytochrome P450 family of microsomal enzymes. SNPs in various members of this family, such as CYP2D6, CYP2C9, and CYP2C19, have been associated with impaired metabolism and hence toxicity of many important drugs, including codeine, haloperidol, propranolol, omeprazole, diazepam, amitriptyline, tamoxifen, clopidogrel, and warfarin (139). Testing for these variants is now possible on a variety of multiplex platforms, some of which are FDA approved. However, uptake by treating physicians has been quite low, owing in part to unfamiliarity but more importantly to the low predictive value of many of these genotypes (140).

Other potential pharmacogenetic targets are more narrowly drug specific. For example, the mitochondrial DNA mutation A1555G is known to confer increased sensitivity to the ototoxic side effects of aminoglycoside antibiotics (141). While not in routine practice because of other considerations surrounding the emergent use of these agents in the critical care setting, one might imagine pretreatment mitochondrial DNA testing for this variant if a rapid assay and alternative therapeutic strategies were available. A similar case could be made

for preoperative testing of patients for mutations in the ryanodine receptor gene *RYR1* as a screen for potential risk of malignant hyperthermia in response to common surgical anesthetics. The practicality of this approach is limited by the genetic heterogeneity of the disorder and evolution of anesthesiology practice beyond the former gas anesthetics like halothane. Still, a panel of 15 mutations has been recommended by a European consortium for testing at-risk family members (142).

The conceptualization of pharmacogenetic testing may even be expanded beyond traditional drug response to encompass other forms of therapy, such as radiotherapy. Heterozygote carriers of mutations in the *ATM* and related genes have increased sensitivity to therapeutic radiation, mirroring the more extreme radiosensitivity that is a hallmark of the affected patients with ataxia telangiectasia (112). With a carrier frequency of at least 1% in the general population, and the possibility that these carriers also have increased susceptibility to cancer, many of these individuals may find themselves facing radiotherapy sometime in their lives. A pretreatment screen for *ATM* and related mutations could identify those whose radiation dosage should be decreased in order to avoid severe tissue damage.

### 23.8 QUALITY ASSURANCE, REIMBURSEMENT, AND REGULATORY ISSUES

All clinical laboratories in the United States must adhere to the CLIA guidelines as a minimal level of quality assurance. Since all molecular genetic tests are considered “high complexity,” the regulations for that category must be followed. A problematic loophole has existed from the fact that molecular genetic tests had not been listed specifically in the existing CLIA regulations when written. For this reason, and because the CLIA regulations are seen as minimal standards, professional organizations have developed more detailed and rigorous practice guidelines. The most important are those of the ACMG, CAP, and the Clinical Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards), along with more general policy statements by the Association for Molecular Pathology (AMP). The CAP Molecular Pathology Checklist is particularly crucial since it is used for inspection and accreditation of those laboratories that choose to submit to this agency, which has been granted “deemed” status under CLIA. This checklist and the ACMG Standards and Guidelines for Clinical Genetics Laboratories are continually updated to reflect changes in techniques and maturation of the discipline, and the most recent versions are available on the respective organizations’ Websites (see next section). They contain many guidelines unique to molecular diagnostic laboratories, addressing issues such as PCR contamination, communication with genetic counselors, informed consent, interpretation



of mutational heterogeneity and sequence variants, molecular genetics personnel qualifications, technical procedures, appropriateness of test requests, and so on. While some have argued that it is not within the purview of laboratories to serve as gatekeepers for such matters as informed consent and appropriate test ordering, in practice it often falls to them since primary care providers may not be aware of such criteria.

Quality assurance (QA) encompasses all aspects of testing, from requisitions to specimen receipt through performance, interpretation, and reporting. In addition, recent initiatives by governmental and professional organizations have focused increasing attention on the need to include pre- and postanalytic factors within overall QA for genetic tests, especially those of a predictive nature. Tests must be validated both analytically and clinically prior to introduction, although that is often easier said than done, particularly for predisposition tests that may take decades to establish clinical predictive value. On the other hand, documentation of analytic validity has been assisted greatly by a continuing effort in partnership with the Center for Disease Control and Prevention (CDC), AMP, and the Coriell Cell Repositories to develop, characterize, and make generally available genomic DNA reference materials representing the most important alleles for genetic diseases and pharmacogenetic testing (143).

Another important component of QA is proficiency testing. This involves the regular shipment to laboratories of specimen unknowns that are analyzed, reported, and sometimes graded. CLIA mandates that laboratories participate in proficiency testing programs for each analyte they test, and if an organized formal program is not available, they must revert to other means such as informal sample exchanges with another laboratory or blinded retesting of previous samples. A successful and ever-expanding national program exists, codeveloped by CAP and ACMG and evolving in scope to address newer technologies like next-generation DNA sequencing. The performance of all participating laboratories is aggregated and provides a useful snapshot of the state of the art at any given time (144).

Reimbursement for molecular genetic testing has been a particularly frustrating issue for the laboratories. These tests tend to be relatively expensive and labor-intensive to perform and are often considered esoteric or experimental by third-party payers. Furthermore, since they are frequently performed on healthy individuals (e.g. in carrier screening for recessive mutations), the usual diagnostic/symptomatic indications for clinical laboratory testing are often absent. The acceptance and updating of essential procedural billing codes that more realistically represent the range and workload effort of modern molecular techniques was a step in the right direction, beginning with the addition of codes for such techniques as DNA sequencing, multiplex PCR, and mutation scanning some years ago (145), but it is a slow process. As

of 2012 there is a move to convert the method-specific codes like these that have always been used in molecular genetics (often “bundled” to reflex the ingredients of multiplex assays) to disease-specific codes (e.g. “CF”) as has always been the practice in molecular microbiology. There are both potential advantages and disadvantages to this approach.

Lastly, there is great concern that the whole field might be priced out of existence if mandatory royalties for use of patented genes and mutations in clinical laboratory tests inordinately add to the cost. Many of the genes discussed in this chapter have been patented, and various levels of exclusivity or sublicensing royalties are being enforced. Many laboratories of limited means have been forced to cease offering important molecular genetic tests because of restrictive gene patent conditions, and the situation can only get worse as next-generation sequencing technologies place all genes within reach of a single test. A number of lawsuits on this matter have been brought in US and European courts, perhaps the most publicized one contesting the constitutionality of the patents on the *BRCA1* and *BRCA2* genes (146). Even if these cases do not result in the global invalidation of gene patents, a program of reasonable royalties to allow access to clinically important gene targets would benefit both patients and laboratories by keeping prices down and allowing for peer comparison in performance across the genetic testing community.

## 23.9 INTERNET RESOURCES FOR MOLECULAR GENETIC TESTING

With the Human Genome Project producing an overwhelming information load and new genetic tests appearing almost every week, print sources in this field can never be up to date, and even if they were, it is difficult and time-consuming to extract the information needed, especially to answer an urgent clinical question. Fortunately, the genetics revolution has occurred at the same time as the informatics revolution, so computer technology can be used for rapid searching of relevant information that is constantly updated. The Internet offers many sites with useful medical genetics content; listed here are some of the key resources available to assist those ordering or performing molecular genetic tests.

### 23.9.1 Online Mendelian Inheritance in Man (OMIM)

(<http://www.ncbi.nlm.nih.gov/omim/>)—Most readers of this book will already be familiar with OMIM, the most comprehensive and up-to-date catalog of human genetic disorders available, currently boasting over 21,000 entries. Each disease entry includes, in addition to phenotypic features, information on the mapping and structure of the involved gene (where known) and its mutations.

### 23.9.2 GeneTests

(<http://www.ncbi.nlm.nih.gov/sites/GeneTests?db=GeneTests>)—GeneTests is the primary listing of genetic testing laboratories, searchable by disease (>2500 currently listed) or gene symbol. It is extremely useful for those facing a patient in need of an “orphan” genetic test for a rare disease, when one does not know where in the country (or the world) it might be offered. It is also an excellent referral source for more routine molecular genetic tests. Contact information for each laboratory is provided in order to address indications and requirements for testing prior to sending a specimen.

### 23.9.3 GeneReviews

(<http://www.ncbi.nlm.nih.gov/sites/GeneTests/review?db=GeneTests>)—GeneReviews is the sister site of GeneTests, providing detailed background material on the major disorders subject to molecular genetic testing, including information on clinical manifestations, the molecular pathology of the gene, and interpretation of DNA test results.

### 23.9.4 GeneCards

(<http://www.genecards.org/>)—GeneCards is structured like an encyclopedia, with entries on human genes, their protein products, variants and mutations, and their associated genetic disorders.

### 23.9.5 Human Gene Mutation Database

(<http://www.hgmd.cf.ac.uk/ac/index.php>)—Providing even finer detail, this database is the most comprehensive, continually updated listing of molecular alterations, currently cataloging over 85,000 reported mutations in over 3000 human genes. This database has become an even more essential reference for evaluating the thousands of nucleotide variants detected by whole-exome and whole-genome sequencing.

The Websites of professional and governmental organizations can also be useful, both as starting points for additional links and as sources of educational programs.

National Coalition for Health Professional Education in Genetics (<http://www.nchpeg.org/>)—This Website serves as a clearinghouse for a number of educationally oriented genetics information Websites as well as its own instructional material aimed for students and for non-genetic practitioners.

### 23.9.6 The Genetic Alliance

(<http://www.geneticalliance.org/>)—This is the best source for listings of patient support groups for genetic diseases, including many rare diseases (over 1000 currently represented); it is searchable by disease. The

Alliance is heavily involved in both advocacy and education.

### 23.9.7 College of American Pathologists

(<http://www.cap.org/apps/cap.portal>)—This Website contains the most up-to-date version of the Molecular Pathology Checklist for laboratory inspection, along with many other products and services relevant to QA and accreditation. Some content is open only to members.

### 23.9.8 American College of Medical Genetics

(<http://www.acmg.net//AM/Template.cfm?Section=Home3>)—This Website features its own updated Standards and Guidelines document and many policy statements related to molecular genetic testing, along with educational resources in various media. These cover both general testing issues and guidelines specific for individual diseases and include detailed descriptions of the available methodologies, appropriate use of the test, results interpretation, risk calculations, and reporting protocols.

### 23.9.10 Association for Molecular Pathology

(<http://www.amp.org/>)—The only organization devoted exclusively to molecular diagnostics, it is a source of information on technical and regulatory issues in the field. It also features its own laboratory test directory, focusing on non-genetic molecular tests not covered by GeneTests.

### 23.9.11 The DNA Learning Center

(<http://www.dnalc.org/>)—For those desiring a more basic introduction to the field, the DNA Learning Center, based at Cold Spring Harbor Laboratories, contains multimedia tutorials on introductory molecular biology concepts and history of the discipline.

## 23.10 SOCIETAL IMPACT OF THE NEW GENETIC TECHNOLOGY

The dawn of the twenty-first century has been called the beginning of the age of molecular medicine. Surely there can be no doubt that virtually every patient undergoing diagnosis or treatment in the coming decades will be involved in some sort of molecular genetic procedure. Even if it is on the treatment end (gene therapy), it will have been preceded by a molecular diagnostic procedure, since no patient can become a candidate for gene therapy until the precise molecular defect is known. Also certain is the fact that the diagnostic methods outlined in this chapter will become increasingly automated. As

DNA microarrays and other multiplex platforms become available as completely closed systems, free of complex hands-on manipulations and expert troubleshooting, they could just as easily move into the automated clinical chemistry laboratory as stay in the molecular genetics laboratory. Such a development would not necessarily serve the patients well, however, for even as the technical aspects of DNA tests become easier, their interpretation becomes more complex. Professional organizations have emphasized repeatedly that pre- and postanalytic aspects of genetic tests are just as important as the technical procedures themselves. Even if the technical portion is highly accurate and robust, much harm can ensue if the test was ordered inappropriately or the results information conveyed improperly.

With PCR, microarray, massively parallel sequencing, and other powerful technologies allowing ever more comprehensive genetic analyses on ever smaller and less invasive specimens, many complex issues surrounding genetic privacy and informed consent have arisen. Both governmental and professional organizations are attempting to address these concerns through safeguards in the testing process as stipulated in professional practice guidelines and legislation. Especially thorny have been the concerns surrounding predictive genetic testing in presently healthy individuals, since a positive test result carries risks of stigmatization, discrimination, anxiety, and depression. For these reasons, most professionals believe that specific informed consent and strict confidentiality are prudent for predictive testing. Furthermore, to assure appropriate ordering and results counseling for complex predictive tests, a case can be made that the laboratory should accept such orders primarily from genetics professionals or others with specific expertise in the field.

In the United States, more so than in other Western countries, the specter of insurance discrimination, which has been documented in limited numbers of patients (102,110,151), is often raised, impelling some patients to pay out of pocket for expensive tests so that their insurance carrier will not be aware of the result or even the fact that the patient was being tested. Yet, this raises other ethical issues such as inequitable access to testing, since only the economically well-off can afford to pay the cost themselves. Sometimes, however, these concerns become so blown out of proportion as to appear slightly irrational. Despite the hypothetical risk, most medical geneticists and genetic counselors would have a difficult time recalling even a single case in their experience of such blatant discrimination as loss of health insurance because of a genetic test result. Furthermore, we (and our patients) must always keep in mind the motivation for the test in the first place. For example, a woman may wish to pay for a predictive BRCA test out of pocket to keep the results secret from the insurance carrier, but if she tests positive and then desires a prophylactic mastectomy or oophorectomy, is she intending to pay for

the costs of surgery out of pocket also? Probably not, and yet she cannot expect the insurance company to pay unless it is aware of the indication for surgery, which is the positive test result itself. Such patients can be somewhat reassured by the passage of the Genetic Information Nondiscrimination Act by the US Congress in 2008 (147), although the actual extent of protection from this legislation remains to be tested in actual cases.

All these dilemmas remain moving targets because of the continuing march ahead of technology. The advent of next-generation sequencing, placing the entire genome or exome of a patient within reach for less than the price of sequencing of two or three individual genes, probably represents the greatest sea-change in molecular diagnostics since the innovation of PCR in the mid-1980s. Will comprehensive sequencing soon supplant all other targeted gene tests and screens? Will a drop of blood from every newborn be used for whole-genome sequencing in addition to or instead of the traditional biochemical screens for metabolic disorders? Or, taking it one step further, will pan-genomic screening be done in utero, or noninvasively using free fetal DNA circulating in the maternal blood as has recently been shown feasible (3,4,148)? Could the availability of these less physically invasive methods of prenatal diagnosis, as well as less emotionally invasive methods such as preimplantation genetic diagnosis, lead to a more cavalier attitude toward the use of molecular genetic technology to select for traits not directly related to disease?

Aside from the obvious ethical concerns in such scenarios, we should always remain aware of the limitations of molecular genetic testing, no matter how sophisticated the technology. Single genes and mutations rarely act entirely alone, and complex disorders clearly involve hundreds or perhaps thousands of genes all interacting at the DNA, RNA, and protein levels and with the outside environment. Thus, no DNA test, no matter how comprehensive, can ever tell the whole story. As the initial phase of the Human Genome Project is completed and we move on to the “postgenomic” or “proteomics” era, we will need to begin to think of genetic disease pathogenesis on a more “three-dimensional” level, taking into account the many interacting gene products in the nucleus and cytoplasm, as opposed to merely the “one-dimensional” string of nucleotides on a stretch of DNA. Whether tests of such phenomena, once they arrive, will be part of the domain of the molecular genetics laboratory or will move into a new “proteomics” laboratory section is anyone’s guess. However, as we await such advances, we can remain confident that the molecular genetic tests of today, and those added to the menu with each passing month, already offer a tremendous and irreplaceable service to many patients and their families, providing information and choices for their own lives and those of their offspring that had never been available before, in any form, in the entire history of medicine.

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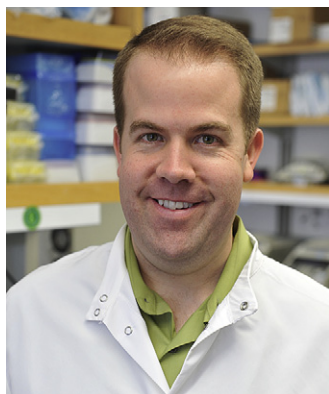
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# CHAPTER

# 24

## Heterozygote Testing and Carrier Screening

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### 24.1 INTRODUCTION

The specific biochemical or genetic abnormality in nearly 3000 genetic diseases in man has been determined (1). Most of these are transmitted with simple either autosomal- or X-linked-recessive patterns of inheritance. The ability to identify such conditions from affected individuals (probands) permits the rapid and accurate diagnosis of such disorders. Many of these methods have also been applied to permit detection of such disorders in the fetus during early gestation. Such approaches can provide vital options in genetic counseling for many families. The delineation of the underlying metabolic defect has also enabled investigators to develop rational and effective therapies for a variety of these disorders (2–4). In addition to the diagnostic and possible therapeutic implications of such discoveries, comparable methodologies have been employed for the detection of heterozygous carriers of many of the recessive traits involved. Accordingly, not only is a striking deficiency of enzymatic activity, gene dosage, or metabolic dysfunction evident in the diagnosis of the homozygous or hemizygous state (the affected male with an X-linked-recessive disorder) but also a distinct quantitative decrease of the same function can be demonstrated in the otherwise normal individual carrying the recessive allele. This capability to quantify gene dosage, and thereby to identify carriers of recessive traits, has been reported for many of the inborn errors of metabolism where the primary defect is known.

Advances in the applications of molecular genetic techniques have made it possible to identify (either directly or indirectly) heterozygous carriers of an increasing number of mutant alleles. In some instances this may involve direct detection of the mutation (e.g. sickle-cell trait or carriers of mutations for  $\beta$ -thalassemia, cystic fibrosis (CF), Tay–Sachs disease (TSD), and fragile X syndrome). In some conditions where the gene has eluded isolation

to date, or where extensive allelic heterogeneity still limits direct mutation detection, carrier identification has been achieved with the use of very highly polymorphic genetic markers (e.g. short tandem repeats (STRs)) segregating in specific families linked very closely to the disease-associated gene (e.g. Duchenne muscular dystrophy (DMD), hemophilia A).

It is not our intent in this chapter to review all known disorders and their current status regarding carrier detection. Rather, specific disorders will be cited as illustrative examples of the issue being discussed. Other chapters in this text more fully elaborate the fundamental inborn errors associated with such disorders or deal specifically with issues of prenatal and neonatal screening as well as heterozygote detection. It is also important to make the distinction between individual heterozygote testing and carrier screening. Heterozygote testing is the use of specific assays to determine the genetic status of individuals already suspected to be at higher risk for an inherited disorder because of a family history or clinical symptoms (5). In contrast, population-based carrier screening is the use of genetic tests to evaluate specific populations or groups of individuals independent of a family history of a disorder and without clinical symptoms.

### 24.2 CARRIER SCREENING IN CLINICAL PRACTICE

#### 24.2.1 To Prevent Anxiety in Family Members

When a patient is identified with a genetic defect or the family history reveals such a disorder in a close blood relative, the question of whether to test that individual and other family members for heterozygosity should be considered. In many instances where such tests are available and accurate, this can serve to reduce expressed or

hidden anxieties in other family members. Since most of these disorders are individually rare, it is relatively unlikely that other family members need be too fearful of producing affected offspring. For example, taking a maximum risk situation, an African-American individual whose brother or sister is a patient with sickle-cell anemia may be concerned about having affected children. Assuming they reproduce with another African-American person with no known sickle-cell anemia in the family, what are the actual risks? The unaffected sib (obviously not SS) of a person with sickle-cell anemia has two chances in three of having sickle trait (if born of the same parents as the patient with sickle-cell anemia). The approximate overall sickle trait frequency in African-Americans is about 1 in 10. Therefore, the likelihood that this person will be at risk for sickle-cell anemia in his or her offspring is  $2/3 \times 1/10 = 1/15$ . The likelihood that any given pregnancy will result in a child with sickle-cell anemia is  $1/4$  of that risk, or 1 in 60.

### 24.2.2 To Prevent Subsequent Intrafamilial Cases of Disease

Although these statistics alone can be somewhat comforting, simple carrier detection studies in the appropriate persons can put this question out of the realm of “calculation” and definitively establish if one or both individuals are carriers. If both prospective parents are found to be carriers, then comprehensive genetic counseling with a complete discussion of all available options can be initiated, including prenatal diagnosis, gamete donation (e.g. sperm or ovum from an established non-carrier donor), preimplantation testing, etc. Where at-risk couples are identified, this can lead to prevention of subsequent intrafamilial cases of disease—a second major reason to consider carrier testing in such families.

### 24.2.3 Marital Counseling

A third rationale for carrier screening relates to marital counseling, particularly where consanguinity between prospective parents may exist. Because of religious, moral, or other concerns, individuals might also use information about their carrier status in their decision regarding mate selection. This is the practice in certain Hassidic Jewish communities where strong religious prescriptions against abortion exist (6). Since marriages are “arranged” (often in childhood) and require rabbinical approval, some rabbis have opted for TSD carrier testing among the children or adolescents of their community (7). Approval for marriage is withheld by the rabbi (to whom solely the results of carrier testing are conveyed) if two carriers are “matched.” Alternative “arrangements” are then made without specifying the reason, thus avoiding family or individual stigmatization. While appropriate for individuals of this autocratic socioreligious belief system, generalization of this approach to other

individuals or groups is likely to be highly problematic. However, this paradigm has been expanded with the inclusion of carrier tests for several other diseases that are also found more frequently among the Ashkenazi Jewish population including Canavan disease, CF, and type I Gaucher disease, to name a few (8,9).

Currently, the American College of Obstetricians and Gynecologists (ACOG) (10) recommends that individuals of eastern European Jewish ancestry be offered carrier screening for CF, TSD, Canavan disease, and familial dysautonomia. In addition, individuals of Ashkenazi Jewish ancestry may inquire about screening for mucopolidosis IV, Niemann–Pick disease type A, Fanconi anemia group C, Bloom syndrome, and Gaucher disease.

### 24.2.4 Artificial Insemination or Ovum Donation

Another important consideration for carrier testing is where assisted reproductive technology is being considered by a couple as an alternative reproductive strategy to avoid the birth of a child affected with an autosomal-recessive or X-linked disorder. Nearly 3 in every 100 births in the United States are the result of some form of assisted conception (11). Potential sperm or ovum donors should be evaluated (where feasible and practical) by appropriate carrier screening tests to avoid what otherwise could be a most unfortunate tragedy. The authors are aware of two children with TSD, conceived by artificial insemination, born to two unrelated families who were attempting to have an unaffected child after they had had a previous child with this fatal condition. In another instance, screening of three medical students as potential sperm donors for a woman who had been previously identified as a TSD heterozygote showed one of the three to be a TSD carrier also. Of course, he was excluded as a potential donor. A recent survey of public sperm donor banks across the United States showed significant variation in the type of genetic testing performed on sperm donors (12). For example, CF carrier screening, hemoglobin evaluations, and chromosome analyses were performed on the majority of sperm donors. However, none of these sperm banks offered carrier screening for spinal muscular atrophy (SMA). Finally, less than half the surveyed sperm banks (37.5%) had a genetics professional on their staff. Such screening, in addition to a thorough family history on all potential gamete donors for other relevant issues, can help avert tragedy.

## 24.3 CARRIER SCREENING IN INDIVIDUALS OF DEFINED SUBPOPULATION GROUPS

A list of relatively “common” autosomal-recessive disorders (Table 24-1) seen in defined subpopulations in the United States is presented with data indicating the respective carrier frequency and newborn disease incidence in

**TABLE 24-1 Frequency and Incidence for Selected Autosomal-Recessive Disorders in Defined Ethnic Groups in the United States**

Disease	Ethnic Group	Gene Frequency	Carrier Frequency	"At-Risk" Couple Frequency <sup>a</sup>	Disease Incidence in Newborns
Sickle-cell anemia	African-Americans	0.040	0.080	1:150	1:600
Tay-Sachs disease	Ashkenazi Jews	0.016	0.032	1:900	1:3600
$\beta$ -Thalassemia	Greeks, Italians	0.016	0.032	1:900	1:3600
$\alpha$ -Thalassemia	Southeast Asians and Chinese	0.020	0.040	1:625	1:2500
Cystic fibrosis	Northern Europeans	0.020	0.040	1:625	1:2500
Spinal muscular atrophy	Asian	0.010	0.020	1:2500	1:10,000
Phenylketonuria	Northern Europeans	0.008	0.016	1:4000	1:16,000

<sup>a</sup>Likelihood that both members of a couple are heterozygous for the same recessive allele (assuming nonconsanguinity and that both are of the same ethnic group).

those ethnic groups. Gene frequencies and incidence of the disorder may vary considerably in the same ethnic groups in other parts of the world. The fact that selected genetic diseases occur predominantly in certain ethnic, religious, or racial groups is not surprising when one considers the relatively high degree of inbreeding seen in defined subpopulations (13,14). In addition to inbreeding, in some situations, selective environmental factors may have existed at some point in history that provided a biological (reproductive) advantage to carriers of the recessive gene (e.g. relative resistance to malaria in individuals who are heterozygous for sickle-cell hemoglobin,  $\beta$ -thalassemia, or glucose-6-phosphate dehydrogenase (G6PD) deficiency). Because of this selective effect, the gene becomes "enriched" from one generation to the next in that population.

It should be emphasized that while many of these diseases are relatively rare, the frequency of carriers can be quite high. For example, the disease incidence of CF among Caucasians of Northern European ancestry is approximately 1 in 2500 newborns, yet nearly 1 in 25 such individuals are carriers of a CF mutation. Similarly, the disease incidence for TSD among Ashkenazi Jewish newborns is about 1 in 3600, while the carrier rate in this population is approximately 1 in 30. Because of these population distributions and the availability of relatively simple, accurate, and inexpensive carrier detection methods, it is possible to screen individuals in these groups and identify persons and, more critically, couples at risk for homozygous disease in their offspring before affected children are born. With comprehensive genetic counseling, and the important options that prenatal diagnosis can provide, many "at-risk" families, identified through screening such subpopulations, might choose to have only children unaffected with the disorder for which they are found at risk.

For those relatively "common" autosomal disorders that occur within defined ethnic groups, there is usually no known prior history of the disease on either side of the family. Such a positive history may be present in only 20% of instances where a child with such a disorder is

diagnosed. For this reason, clinicians should consider carrier screening for all individuals in these subpopulation groups where heterozygote detection is readily available. Certainly, such testing and its implications should be thoroughly discussed with the patients, or they can be referred to appropriate regional agencies for such services. This is not only considered optimal preventive medicine but also has become the standard of care in many cases (10,15).

### 24.3.1 Tay-Sachs Disease

Perhaps the most effective effort of this nature has been the experience with TSD carrier screening and prenatal diagnosis (16–18). From 1970 (when serum carrier detection methods were first described) to 1999, community-based TSD education–screening–counseling programs have been initiated in Jewish communities throughout North America, as well as in Israel, South Africa, Europe, South America, and Australia. More than 1.4 million adults have been screened voluntarily and over 51,000 heterozygotes detected. Most critically, nearly 1400 couples—none of whom had an affected child previously—have been identified as being at risk for this fatal disorder in their offspring. Over 3200 pregnancies at risk for TSD have been monitored by amniocentesis or chorionic villus sampling, and the births of more than 625 affected infants destined to die with this disorder have been prevented. More importantly, this approach has been associated with the birth of over 2550 healthy infants. Overall, these efforts have contributed to more than a 95% decline since 1970 in the incidence of this disorder in Jewish infants throughout North America (6,17,19).

### 24.3.2 Beta-Thalassemia

Similar efforts directed at the prevention of  $\beta$ -thalassemia through education, carrier screening, and prenatal diagnosis have been initiated in several European countries and certain areas of North America. These programs

have dramatically reduced the newborn incidence of  $\beta$ -thalassemia in several Mediterranean countries (20,21). The highest incidences are reported in Cyprus (14%) and Sardinia (12%) (22). In Sardinia, for example, these preventative measures have reduced the incidence of disease by more than 94% of the prescreening levels (23).

### 24.3.3 Sickle-Cell Anemia

Screening for sickle-cell trait has been initiated in several parts of the world (including the United States). Although the capability to diagnose homozygous sickle-cell anemia in early fetal life is well established, through either amniocentesis or chorionic villus sampling, this alternative has not been widely adopted. This may reflect (at least in the United States) some of the complex sociopolitical issues associated with genetic screening of minority populations. However, in the United Kingdom, universal screening (both antenatal and neonatal) is recommended in regions where the populations comprise 15% or greater of the ethnic groups that are at risk for sickle-cell disorders (24).

### 24.3.4 Cystic Fibrosis

The American College of Medical Genetics (ACMG) and the ACOG published guidelines in 2001 for the screening of CF in the general population using a panethnic panel of 25 cystic fibrosis transmembrane conductance regulator (CFTR) mutations and several polymorphisms (25,26). These particular mutations were included in the panel because they had a  $>0.1\%$  frequency in CF patients. Another round of data review was initiated in 2002 because the initial results of large-scale screening with this panel revealed that some of the mutations initially included on this panel either were less frequent than originally thought (1078delT) (27) or were in fact polymorphic variants (I148T) when not coupled with a rare disease-causing mutation (3199del6) (28). Because of these initial shortcomings in the CF carrier screening panel, the ACMG just updated the panel (15), and this points out the value in designing and continually monitoring a large-scale carrier screening program from the very outset. Strom et al. (29) reviewed the performance of the ACMG/ACOG core CF panel in a clinical database of approximately 3 million carrier screen results and more than 2000 extensive sequencing analyses and suggest that this panel is performing as expected. These data show detection of approximately 90% of non-Hispanic white CF carriers and 77% of CF carriers in a US panethnic population (consisting of 64% non-Hispanic whites, 16% Hispanic Americans, 14% African-Americans, 5% Asian Americans, and 1% Ashkenazi Jews).

### 24.3.5 Spinal Muscular Atrophy

SMA is the most common fatal hereditary disease among newborns and infants worldwide and affects about 1 in

10,000 live births (30). This disease is caused by mutations in the survival motor neuron (*SMN1*) gene and affected patients demonstrate the homozygous deletion of *SMN1* exon 7 in most cases (31). Carrier frequency is on the order of 1:40 to 1:60, with the highest carrier frequency seen in Caucasians (1:35) and the lowest in Hispanic populations (1:117) (32). Although no treatment is currently available for SMA, carrier detection is feasible and is estimated to have a detection rate of about 90% within most populations (33). Universal carrier screening for SMA was recommended in 2008 by the ACMG (30).

## 24.4 THERAPEUTIC IMPLICATIONS FOR HETEROZYGOTES

Although in most instances heterozygosity for a recessive trait is of no known health consequence to the individual, there are conditions in which the heterozygous state may impart certain health hazards. These concerns discussed below pertain primarily to carriers of autosomal-recessive genes and heterozygotes of X-linked disorders, which may well have therapeutic issues depending on the disorder and lyonization. Accordingly, for these individuals, knowledge of their carrier status may have therapeutic or preventive health implications. For example, persons with AS hemoglobin (sickle-cell trait) should be aware of the possible hazards of exposure to reduced ambient oxygen concentrations (e.g. mountain climbing at high altitude, flying in an unpressurized aircraft above 8000 feet) (34). In addition, alerting the anesthetist of the AS trait of the patient prior to gaseous anesthesia could avert inadvertent hypoxia, which might be particularly hazardous to such an individual. Heterozygous individuals for type II hypercholesterolemia may be predisposed to premature atherosclerotic degeneration and coronary artery insufficiency. Appropriate therapy (diet, weight control and/or specific medication) may reduce the risk for early myocardial infarction in individuals carrying this dominantly expressed disorder. In a similar fashion, persons heterozygous for alpha-1-antitrypsin deficiency (MZ) may be predisposed to chronic obstructive pulmonary disease in early adulthood. Avoidance of tobacco smoke and other noxious inhalants may greatly reduce this risk. Having identified an individual as heterozygous for this mutation, it might be of great practical value for this person to be counseled and guided into appropriate job selection and/or environmentally safe areas, as well as informed of the particular importance of not smoking.

## 24.5 METHODS AND TISSUES USED IN CARRIER IDENTIFICATION

Depending on the genetic nature of the condition, its expression in different organ systems, and the availability of appropriate material for examination, a variety of approaches have been used historically for heterozygote detection. These approaches ranged from physiological



studies to direct mutation analysis. For example, somatic cell methods assessing the enzymatic (HPRTase) or physiological ( $^3\text{H}$ -hypoxanthine incorporation) properties of clones of skin fibroblast cells were useful in the past for carrier detection in Lesch–Nyhan syndrome. However, molecular genetic techniques including direct mutation detection have replaced this approach to carrier identification for this disorder (35). Newer methods for DMD carrier testing include quantitative polymerase chain reaction (PCR) methods followed by DNA sequencing of the dystrophin gene, and these methods offer advantages over the more classical approach using a combination of pedigree analysis, serum creatine kinase levels and linkage analyses to assess an individual's carrier status (36).

### 24.5.1 Molecular Genetic Techniques

A variety of molecular genetic techniques are used for direct mutation detection in the heterozygote (Table 24-2). In general, these are PCR-based methods to scan or screen small exon-sized DNA fragments for point mutations or small (e.g. 3–5 base pair) insertions or deletions. Direct sequencing methods are commonly used for direct mutation detection in CF,  $\beta$ -thalassemia, and other disorders. Extremely large insertions, deletions, or other rearrangements are still best detected with Southern blotting, as is the case for the common deletions in  $\alpha$ -thalassemia or the larger premutations and full expansion mutations in fragile X syndrome. In some instances a combination of DNA-based methods may be used to optimize carrier detection. For example, carrier identification for fragile X syndrome may involve

a combination of Southern blotting and PCR to reliably detect the full spectrum of expansion mutations observed within the *FMR-1* gene (37). In many laboratories, mutation detection for relatively small genes such as beta-globin has moved from PCR and allele-specific oligonucleotide hybridization to direct sequence-based methods using semiautomated capillary electrophoresis systems. In some laboratories, the sequencing of large genes including CFTR is now becoming routine.

### 24.5.2 Tissues Used for Heterozygote Detection

Historically, tissues used for heterozygote detection in representative recessive disorders are presented in Table 24-3. In considering carrier detection (either testing an individual or screening a subpopulation), the accessibility of appropriate tissue or material for testing influences the feasibility and cost of such procedures. In some instances, accurate heterozygote identification can be achieved with readily available tissues such as serum, circulating blood cells, buccal epithelial cells, or tears. In other cases, optimal carrier detection may require cultured skin fibroblasts or even biopsied liver or muscle tissue. Clearly, whether to conduct such studies in individuals related to an affected person, or more generally, will be strongly influenced by such considerations.

### 24.5.3 DNA-Based Techniques

The application of DNA-based techniques in the detection of mutant genes has had great impact on this issue. Since adequate DNA samples are readily obtained from a routine blood sample, the aforementioned reservations are in large part obviated if “DNA methods” can be employed. In some instances, DNA from buccal cells rinsed from the mouth (or even the amount extracted from a single somatic cell) in conjunction with PCR-based methods may provide sufficient material for carrier testing purposes. These PCR-based procedures can be performed more quickly and easily than Southern blot hybridization methods. In addition, PCR methods can be used on small amounts of genomic DNA present on dried blood spots. Accordingly, ease of testing as well as ultimate cost (particularly since such methods are readily automated) should allow for greatly expanded carrier testing for these disorders.

TABLE 24-2 Selected Molecular Genetic Techniques Used for Direct Mutation Detection and Heterozygote Identification	
Method	Representative Disorders
Restriction enzyme digestion of PCR products	CF, FV Leiden TSD
Allele-specific oligonucleotide hybridization	CF, $\beta$ -thalassemia
Southern blotting	$\alpha$ -thalassemia, hemophilia A, fragile X syndrome
Reverse dot blot hybridization, oligonucleotide	CF
Ligation assay (OLA)	
Denaturing gradient gel electrophoresis (DGGE)	Hemophilia A, CF
Single strand conformation polymorphism (SSCP) analysis	Hemophilia B
Multiplex ligation-dependent probe amplification (MLPA)	DMD
Direct DNA sequencing of PCR-amplified DNA	$\beta$ -thalassemia, CF, DMD

CF, cystic fibrosis; TSD, Tay–Sachs disease; DMD, Duchenne muscular dystrophy.

## 24.6 PROBLEMS IN HETEROZYGOTE DETECTION

### 24.6.1 Statistical Constraints

The great majority of the inborn errors of metabolism are relatively rare conditions. Although in the aggregate it is estimated that perhaps 1% of all live-born infants

**TABLE 24-3** Tissues Historically Used for Carrier Detection in Representative Autosomal-Recessive Disorders

Tissue	Methods	Disorder
Serum, plasma	Enzyme assay, immunoquantitation, functional assay	TSD, hemophilias
Erythrocytes	Enzyme assay, electrophoresis	G6PD deficiency, hemoglobinopathies
Leucocytes	Enzyme assay, histology, functional tests	Gaucher, Batten, CGD, Lesch–Nyhan
Skin fibroblasts	Enzyme assay, cell cloning	MLD, Hunter
Hair follicles	Enzyme assay/ratio	Lesch–Nyhan, Fabry disease
Tears	Enzyme assay	TSD
Teeth	Vertical banding	Amelogenesis imperfecta-XR
Eyes	Fundoscopy	XR-fundal dystrophies, RP
Liver, muscle biopsy	Enzyme assay, histology	OTC, DMD
DNA from somatic cells	Direct mutation detection, linkage	Hemoglobinopathies, CF, DMD, hemophilias, CF

TSD, Tay–Sachs disease; CGD, chronic granulomatous disease; OTC, ornithine transcarbamylase deficiency; CF, cystic fibrosis; MLD, metachromatic leukodystrophy; DMD, Duchenne muscular dystrophy; RP, retinitis pigmentosa; G6PD, glucose-6-phosphate dehydrogenase.

will, at some time in life, manifest such a single-gene disorder, there are thousands of such disorders now recognized (1). This poses critical problems for carrier identification. The only individuals who are obligatory carriers of an autosomal-recessive genetic trait are the biological parents of an affected individual (discounting the  $10^{-5}$ – $10^{-6}$  possibility of new mutation). Therefore, in the establishment of a carrier identification method, it is critical that a “significant number” of such obligatory heterozygotes be studied (and control individuals as well) before statistical validity can be assigned to the testing method. In this regard, one must be careful in interpreting research publications in which only small numbers of obligate heterozygotes have been tested and where results suggest that the methods are applicable to carrier detection. Obviously, the larger number of samples of obligate carriers and controls, and the greater the separation observed in the test results between the two groups, the greater the likelihood for significant applications of the method to heterozygote identification.

It is a very different matter to study persons who the investigator knows must be carriers for a particular trait than individuals who are complete unknowns. Essentially all prior experience with nonmolecular methods

used in carrier detection reveals a variable distribution of test results both for carriers and controls. The narrowness of each distribution and the degree of separation between them are critical in assigning a statistical probability that any given test result falls into one or the other distribution.

With molecular methods, however, these constraints are obviated in large part. Certainly, where direct mutation detection is possible, carrier detection is evident without the need for massive numbers in control studies or expansive statistical determinations. With the use of restriction fragment length polymorphisms or STRs, on the other hand, the proximity of the polymorphic marker to the disease locus is critical to interpretation, since possible meiotic crossover events can confound apparent conclusions drawn by linkage analysis.

### 24.6.2 Why Variability?

One would expect that individuals who are heterozygotes for an autosomal-recessive mutation would reflect 50% of the value (whatever the measurement happens to be) of that found in homozygous normal persons. This is clearly not the case. Not only is there variability of test values in heterozygotes but considerable variation may also be evident in data obtained from normals. This may reflect the limitations of the methods employed and/or the inherent biological variability of such functions. Other genetic and environmental factors may influence any given biological parameter such that a range of results is seen in carriers and noncarriers. In some instances where, for example, an enzymatic activity measurement is the test employed, there may be levels of activity in heterozygotes distinctly less than 50% of normal. Where the relevant enzyme is composed of multiple subunits—only one of which is under control of the gene in question—random aggregation of normal and abnormal subunits can result in a wide range of activities in the multimeric enzyme. Other mutations may result in only partial reduction in activity of the respective polypeptide. In this instance, the heterozygote may have near-normal activity or activity that overlaps considerably with the range of measurements found in noncarriers.

With X-linked conditions, the carrier female reflects a broad range of test results extending from clearly normal levels to those seen in affected males. This is predominantly a manifestation of the well-known lyonization effect with X chromosome-linked genes. This biological phenomenon can make carrier determination for such X-linked genes difficult. In fact, heterozygous females who carry X-linked-recessive genes are mosaic in the expression of most of the genes in question, with a certain proportion of their somatic cells expressing the normal gene and the remainder reflecting the mutant state. The reader is referred to other chapters in this volume concerning specific

X-linked disorders for further discussion of carrier-state identification.

### 24.6.3 Other Factors Influencing the Carrier Test

In addition to the consideration that other genes in an individual's constitution may modify the expression of a distant specific gene locus, other biological factors may influence gene expression as well. Factors such as age, pregnancy, drugs and medications and certain illnesses might influence the parameter in question, thereby altering the ability to distinguish carriers from noncarriers. Such issues need to be addressed before wide-scale application of a carrier detection method is made.

**24.6.3.1 Genetic Heterogeneity.** Genetic heterogeneity is an important factor in identifying carriers of mutant genes. Two types of heterogeneity are recognized: genetic heterogeneity and allelic diversity. Genetic heterogeneity can be defined as mutations at two or more genetic loci that produce the same or similar phenotypes (either biochemical or clinical). This is relevant since genetic heterogeneity can present problems for heterozygote detection. For example, forms of Ehlers–Danlos syndrome, a disease of the connective tissue, are known to result from alterations at multiple genes inherited with autosomal-dominant, autosomal-recessive, and X-linked-recessive patterns of inheritance. Similarly, the multiple complementation groups in methylmalonic acidemia also indicate genetic heterogeneity for this condition. Many other examples exist as well. Linkage analysis for the purposes of carrier detection and prenatal diagnosis should be used with extreme caution in disorders where evidence exists for genetic heterogeneity.

Allelic diversity is the presence of more than one mutation within the same gene that may be associated with highly similar, or very different, biochemical or clinical phenotypes. This has obvious relevance for heterozygote detection. For example, if a standard hemoglobin electrophoresis result (biochemical phenotype) is the only parameter used for sickle-cell trait identification, then persons carrying the mutation for hemoglobin-D will be incorrectly identified as carriers of the sickle-cell trait since the S and D hemoglobins electrophorese similarly under standard conditions. In this instance, of course, the adjunct use of other methods, such as hemoglobin solubility studies or sickling on deoxygenation, will clarify this possible discrepancy. Similarly, one must be able to differentiate the mutations associated with hemoglobins S and C when performing DNA-based diagnosis of the sickle-cell trait. Allelic diversity is also an important consideration in disorders such as CF, hemophilia A, and  $\beta$ -thalassemia. Direct mutation analysis in these disorders for only a subset of specific mutations may fail to detect a different disease-causing mutation in a particular family, which may have critical genetic counseling implications for many individuals within that pedigree.

**24.6.3.2 Benign or Pseudodeficient Mutant Alleles.** Benign or pseudodeficient mutant alleles are additional examples of allelic diversity with important implications for carrier detection and genetic counseling. These are mutations that mimic the true disease-related carrier state but are not associated with abnormal clinical phenotypes. Such mutations are relatively common in the genes directing the synthesis of various lysosomal hydrolyses (38). In these disorders, enzyme-based screening tests will fail to distinguish carriers of a disease-causing mutation from carriers of a benign or pseudodeficient allele. For example, in TSD screening, the enzymatic test employs an artificial substrate in carrier identification. Deficient enzymatic activity is observed both in carriers of pseudodeficient mutations as well as in carriers of disease-related mutations (19). These pseudodeficient mutations, however, are not associated with TSD or any known clinical abnormalities. Two such pseudodeficient alleles are the most common mutations found among the enzyme-defined “carriers” of TSD in non-Jewish populations. To distinguish true carriers from carriers of pseudodeficient mutations, DNA-based testing is indicated.

Accordingly, DNA testing should be employed after the enzymatic determination of the carrier state in all non-Jewish individuals and all couples who are identified as at risk (both carriers), regardless of religion. This has obvious implications for accurate genetic counseling and prenatal diagnostic decisions (19,39). To conduct primary screening with DNA-based testing alone would miss a significant number of carriers and would not be cost effective. Rather, sequential use of gene product and molecular methods in this way optimizes genetic counseling and prenatal diagnostic interpretations, as well as optimizing cost-effectiveness.

**24.6.3.3 Environmental Factors.** In some instances where nonmolecular methods for carrier detection are employed, certain environmental factors such as drugs, diet, or other agents could affect biological functions and thereby influence their applicability to carrier detection. Iron deficiency can cause hematological changes that mimic the findings of heterozygotes for  $\beta$ -thalassemia. Birth control medications and pregnancy have been shown to result in a relative reduction in serum hexosaminidase A levels, making many of these women appear as carriers for TSD (40). These are only two examples where such determinations can be influenced by external factors, resulting in inaccurate carrier identification studies.

## 24.7 SENSITIVITY AND SPECIFICITY

With all the above considerations in mind, and having assessed reasonable numbers of obligate heterozygotes and controls with the recommended method(s), significant overlap in the distribution of carriers and noncarriers may still remain. Capabilities of any one laboratory may not be comparable with those of others reflecting,

perhaps, differences in preferred methodologies or other inherent variables. Thus the ability to identify all true carriers (sensitivity) is reflected in the false negative frequency of the test employed. The identification of only true carriers (specificity), and not other persons with a false-positive test, is also of importance. The greater the overlap in distributions of values between carrier and noncarrier, the greater the likelihood for either or both types of misclassification.

The advantage of mutation-specific DNA-based testing is that extremely high levels of specificity are achieved. However, since such methods will identify only the specific mutations examined, a decrease in sensitivity is inherent because other mutations in the same gene will not be identified. Since most mutations within a gene affect the gene product, carrier test sensitivities are highest when gene product analysis is the testing method employed. Ideally, carrier screening should be done initially by gene product assay (e.g. enzyme, protein, messenger RNA) with its high sensitivity. High-specificity DNA-based methods can then be employed to confirm abnormal results, to rule out pseudodeficient states and to identify specific mutations that may have obvious implications for diagnosis or genetic counseling.

With nonmolecular carrier detection methods, even where a defined level of overlap is known to exist, carrier detection studies may still be appropriate. In this context, a test result clearly in the carrier range may indicate, with great likelihood, that the person is heterozygous, while a result in the overlap area would be less definitive and leave the carrier status indeterminate. Similarly, a result at the upper levels of the noncarrier distribution might make the probability of heterozygosity exceedingly small. Where such difficulties exist, carrier screening is best restricted to use only in high-risk individuals (close relatives of probands), rather than in more general or subpopulation screening. Only those approaches whose statistical reliability and accuracy and relative ease of applicability have been proved by prior studies should be candidates for more general use.

## 24.8 COST AND FEASIBILITY

Where significant morbidity or cost would be involved in performing carrier detection studies (even where the accuracy is optimal), these issues should be considered and discussed with the individual before proceeding. DNA-based methods usually can be employed with DNA extracted from readily available somatic cells (leukocytes, skin cells, buccal epithelial cells in saliva, etc.) and at reasonable cost.

## 24.9 AGE FOR CARRIER TESTING

Heterozygosity for most recessive traits has little if any health consequences for the individual and it is only a matter relevant to reproduction. For this reason, carrier

testing is best instituted just prior to, or during, the reproductive age. This has added benefits in that the person's ability to comprehend the meaning of such information is much more likely to be adequate at such an age. Likewise, carrier testing among children or young teenagers should not be undertaken routinely and should be considered only under special circumstances. One's level of maturity and background education may be important factors in obviating any possible stigmatization that carrier identification could entail. A parental request to determine the possible carrier status of their child(ren) need not be a sufficient basis to proceed. Rather, a full discussion with the parents as to the lack of health implications and possible psychosocial hazards of testing youngsters may lead to deferral of testing to a more appropriate time. With regard to adult-onset disorders, a committee of the American Academy of Pediatrics has recommended that persons 18 years of age or younger only be tested if testing will offer immediate benefits to the person being tested or another family member, and if there is no harm anticipated to the individual being tested (41).

## 24.10 CONCLUSIONS

From its very outset a number of complex and important social and ethical issues have been identified with genetic screening (5,42). Issues such as possible personal, familial, or even more general stigmatization of the identified carrier; maintenance of utmost confidentiality of test results; rigorous protection of individual privacy; and informed consent of the tested individual are but a few of the more important concerns raised. Clearly, the physician must consider all these matters in their interaction with the patient and families where possible genetic testing is anticipated.

The implementation of carrier screening programs must rely upon education and standardization. Both the affected population and health care providers need education about genetic screening programs. This is amply illustrated by the cumulative experiences of the TSD,  $\beta$ -thalassemia and CF carrier screening programs. Even if the individual tested receives a negative screening result, it will still be appropriate for the physician to order a diagnostic test if warranted by the clinical circumstances. Subpopulations affected by targeted carrier screening programs also need to be reliably informed about the mutation detection rates and overall clinical utility to ensure that genetic counseling is effective (5,18). Standardization will play an increasingly important role as carrier screening programs expand and new ones develop. Some have suggested that technologies could be standardized to just a few analytical testing platforms (5). In addition, carrier screening programs can borrow from the successes of newborn screening programs that exist in the United States and those around the world.



Heterozygote detection in the future will continue to rely heavily upon DNA-based methods including direct sequencing methods. In 2012, there were over 20,000 entries in the Online Mendelian Inheritance in Man database (1). We expect that novel methods will be developed that may involve the analysis of metabolite (e.g. peptide fragments) and gene products (e.g. enzyme). Furthermore, a variety of analytical testing platforms that have recently been developed may gain even more widespread use including but not limited to microarray and resequencing chips and massively parallel next-generation sequencing methods. Eventually, screening may involve the simultaneous analysis of multiple disease loci by almost completely automated methods. With appropriate technical, medical, and educational expertise, the expanded use of these new approaches in the future will serve to reduce the individual, familial, and societal burdens associated with many severe, and currently untreatable, hereditary disorders.

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# CHAPTER

# 25

## Prenatal Screening for Neural Tube Defects and Aneuploidy

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### 25.1 INTRODUCTION

Neural tube defects (NTDs) and aneuploidies are major causes of perinatal death and childhood morbidity. Routine screening for these anomalies has become a standard part of prenatal care in many countries throughout the world. As with all screening programs, the screening tests seek to identify women at highest risk for the particular disorders of interest, who are in turn offered a more specific and frequently more expensive or invasive diagnostic test. Recent improvements in screening tests for aneuploidy has led to a dramatic shift in the practice patterns and a decline in the number of diagnostic tests, such as chorionic villus sampling (CVS) or amniocentesis, which can be associated with fetal loss. A combination of folate supplementation (both on an individual level and at a national level in grain products) for primary and secondary prevention and screening with maternal serum alpha-fetoprotein (AFP) followed by comprehensive ultrasound evaluation for screen-positive women, and termination of affected fetuses, has dramatically reduced the birth incidence of NTDs. The impact of prenatal screening and diagnosis on the incidence at birth of aneuploidy has been less profound and likely reflects changes in the maternal age distribution and social mores. Recent advances in combined first- and second-trimester screening strategies, utilizing both biochemical and ultrasound evaluation, have increased the detection rates of chromosomal abnormalities to such levels that many women elect to forego prenatal diagnostic procedures, resulting in reduction of rates of these procedures by more than 50% in many countries.

### 25.2 NEURAL TUBE DEFECTS

#### 25.2.1 Classification

Anencephaly and spina bifida comprise 90% of NTDs. All anencephaly cases and most spina bifida cases are

open lesions, classified by exposure of neural tissue and meninges, detectable by biochemical testing due to leakage of AFP from the fetal circulation. In contrast, closed lesions, in which skin covers the defect, are not detectable by biochemical assays. Only 15–20% of spina bifida cases are closed defects but, in general, the prognosis is more favorable. The remaining 10% of NTDs consists of encephalocele, in which there is herniation of neural tissue through a cranial defect, and iniencephaly, a condition characterized by severe head retroflexion, absent occiput, and fusion of the spinal and cranial cavities.

#### 25.2.2 Frequency and Occurrence

Worldwide, the incidence of NTDs is approximately 1–10 per 1000 births, with some geographic variation (1–3) (Table 25-1). Multiple studies in the 1990s established that adequate maternal folate levels are essential for normal fetal neural tube development and closure. After widespread dietary supplementation and fortification of food staples with folate in the United States, there was a 23% reduction in spina bifida in less than a decade (4). Despite these efforts, the incidence of myelomeningocele remains significant at 3.4 per 10,000 live births (5).

Approximately 90–95% of NTDs occur in women with no family history of the condition (6). There are several lines of evidence that support an underlying genetic, albeit heterogeneous, cause in NTD development. First, NTDs are associated with trisomy 13 and 18 among other chromosomal abnormalities (7). Second, in mothers with a previous NTD-affected pregnancy, the risk of recurrence in a subsequent pregnancy is 3%, representing more than 40-fold increase over the general population, with risks increasing further with each additional affected offspring or first-degree relative (8). Finally, more than 200 mouse models of NTDs have been developed with mutations in various genes (9).

**TABLE 25-1** Prevalence of Neural Tube Defects in Pregnancy

Country	Period	Crude Pregnancy Prevalence/10,000 <sup>a</sup>			
		Anencephaly	Spina Bifida	Encephalocele	Total
<i>Argentina</i>	1994	9.5	9.1	1.9	20.5
<i>Australia</i>	1994	5.8	8.5	2.9	17.2
<i>Belarus</i>	1994	8.8	17.7	—	26.5
<i>Belgium</i>	1993–1994	2	4.8	1.6	8.4
<i>Brazil</i>	1994	7.6	8.2	2.5	18.3
<i>Chile</i>	1994	8.5	8.5	1.8	18.8
<i>Czech Republic</i>	1994	3.2	4	0.6	7.8
<i>Denmark</i>	1993–1994	1.7	3.3	1.7	6.7
<i>France</i>	1993–1994	3.5	5.1	1.6	10.2
<i>Eire</i>	1993–1994	4.5	3.7	1.3	9.5
<i>Italy</i>	1993–1994	2.7	3.4	1.1	7.2
<i>Japan</i>	1994	3.4	3.3	0.7	7.4
<i>Mexico</i>	1994	15.8	16.1	2.9	34.8
<i>Netherlands</i>	1993–1994	2.3	3.6	1	6.9
<i>Norway</i>	1994	4.1	4.6	0.8	9.5
<i>UK</i>	1993–1994	4.8	4.7	1	10.5
<i>USA (Atlanta)</i>	1994	1	3.8	0.5	5.3
<i>USA (Hawaii)</i>	1994	4.4	4.9	2.9	12.2
<i>USA (California)<sup>b</sup></i>	1990–1994	4.9	4.2	0.8	9.9
<i>Uruguay</i>	1992–1993	5.6	3.3	1.4	10.3
<i>Venezuela</i>	1993–1994	8.5	10.7	1.1	20.3
<b>Mean rates</b>	—	<b>4.8</b>	<b>6.2</b>	<b>1.3</b>	<b>12.3</b>

All data from Ref. 2 except as noted.

<sup>a</sup>Includes live births, stillbirths, and induced abortions.

<sup>b</sup>Data from Ref. 1.

### 25.2.3 Prevention and Treatment

Regardless of treatment, the prognosis is poor for most large open NTDs. Neonates with anencephaly typically die within the first few days of birth and severe cases of myelomeningocele frequently end in spontaneous abortion. Neurologic function below the level of the lesion is typically impaired and survivors with myelomeningoceles usually experience paralysis, bowel and bladder dysfunction, and cognitive and developmental abnormalities. Multiple surgeries to correct the defect and to treat the consequent hydrocephalus with shunting typically are required. Cognitive abnormalities may be related to complications of shunting (e.g. ventriculitis due to shunt infections) or may be related to the underlying neurologic developmental abnormalities that lead to the defect in the first place. The IQ of children with repaired myelomeningocele is significantly lower than unaffected controls (10). NTDs are associated with alterations in the circulation of cerebrospinal fluid, resulting in ventriculomegaly and hindbrain herniation, which may be either secondary to the altered fluid dynamics or related to a primary defect in the development of the posterior fossa (11). Owing to morbidities associated with hydrocephaly and Arnold–Chiari malformations, ways of reducing postnatal complications have been investigated. The first in utero surgical correction of myelomeningocele was via an endoscopic technique in 1994 (12). All fetuses died in

this study, and so the technique was abandoned. Further efforts exploring the endoscopic approach were also met with disappointing results (13–15). Open prenatal correction of myelomeningocele was first performed in 1998 (16) and multiple observational studies reported salutary results with regard to neurologic function (17–22). Recently, a large multicenter randomized trial evaluating the efficacy and safety of prenatal surgery compared to standard postnatal surgery was reported (23). This study showed several favorable outcomes, including decreased risk of death or shunting at 1 year of age, lesser degrees of hindbrain herniation, and improved cognitive and motor function at 30 months of age. Prenatal surgery was associated with higher rates of preterm delivery, operative complications, and uterine dehiscence.

Dietary supplementation with folic acid is the primary means of preventing NTDs. A large multicenter randomized controlled trial demonstrated that periconceptional folate supplementation decreased the recurrence of NTDs in women with a prior affected pregnancy by approximately 70% (24). Studies have demonstrated that folic acid intake can also prevent primary occurrences of NTDs (25,26). The Centers of Disease Control recommends that women with a previously affected pregnancy take 4mg of folic acid daily before conception and throughout the first trimester (27) while others suggest that up to 5mg per day might be more effective (28).



The U.S. Public Health Service and the ACOG recommend that all women of reproductive age take 0.4 mg of folic acid daily (6). The timing of folate supplementation is critical as the neural tube is closed by 4–5 weeks of development (6–7 weeks menstrual age); thus, folic acid must be taken before conception and through the first trimester.

## 25.2.4 Prenatal Diagnosis of Neural Tube Defects

Identification of pregnancies affected by NTDs allows termination of the pregnancy, planning for postnatal care and surgery, or if appropriate, prenatal correction of the defect. Maternal serum alpha-fetoprotein (MSAFP) levels are elevated in most pregnancies affected by NTDs. Traditionally, women with elevated MSAFP were offered genetic amniocenteses with measurement of AFP and acetylcholinesterase in the amniotic fluid. Recent advances in ultrasound technology and imaging have dramatically changed the evaluation of screen-positive gestations, with diagnostic amniocentesis generally restricted to a subset of pregnancies.

**25.2.4.1 AFP in the Fetal Circulation.** AFP is produced by the fetal yolk sac and liver and is a major protein in the fetal circulation, with concentrations  $10^6$ -fold higher in the fetal circulation compared to the maternal circulation (Figure 25-1). The protein is freely filtered through fetal glomeruli, is present in fetal urine, and accumulates in the amniotic fluid with advancing gestation. AFP reaches the maternal serum by a combination of transplacental and transamniotic diffusion.

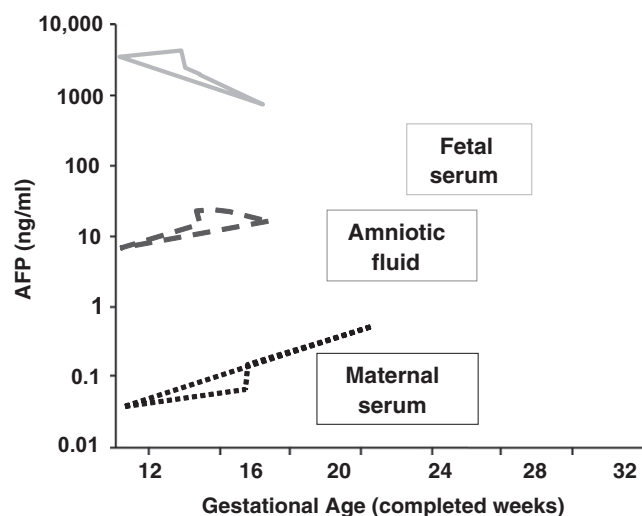
AFP can be easily and accurately measured by quantitative immunoassays. The serum concentration of AFP is gestational, age-dependent and peaks in the third

trimester; therefore, an accurate estimation of gestational age is essential for interpretation of the concentration (29). To account for variance between laboratories and the timing of specimens, concentrations are usually expressed as multiples of the median (MoM) of unaffected pregnancies at the specific gestational age and comparisons are then made using MoMs.

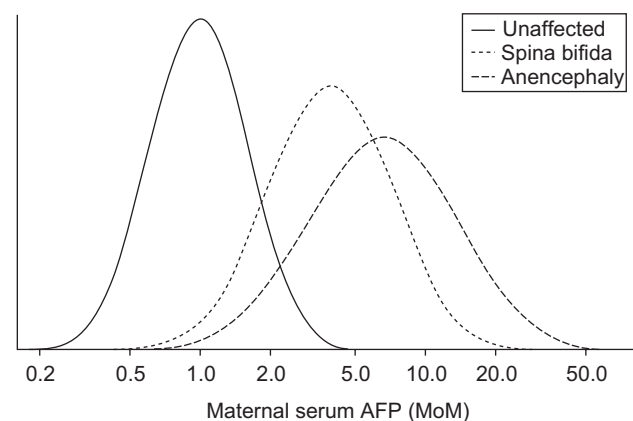
**25.2.4.2 Prenatal Screening for NTDs by MSAFP.** In the early 1970s, the relationship between elevated amniotic fluid levels of AFP and open NTDs was discovered (30). Later it was shown that maternal serum levels of AFP were also increased in affected pregnancies (31). Defects in fetal skin, including open NTDs and ventral wall abnormalities such as gastroschisis and omphalocele, cause increased amniotic fluid AFP concentrations by a direct transudative process (29). Fetal renal disease, intrauterine demise, and contamination of the amniotic fluid with fetal blood also result in elevated AFP concentrations (29).

Multiple large prospective trials have demonstrated that MSAFP testing represents an effective screening strategy (32–35). In a large study including over 18,000 unaffected and 300 affected pregnancies and MSAFP screening at 16–18 weeks, MSAFP levels of 2.5 MoM or greater detected 88% of anencephaly cases and 79% of spina bifida cases (35). Although there is considerable overlap between affected and unaffected pregnancies, a cutoff of 2.5 MoM was associated with just a 3% false-positive rate. Detection is enhanced when pregnancy dating criteria are based on a biparietal diameter measurement because fetal head size is smaller in the setting of an NTD. This results in a slight underestimation of the GA and the expected median AFP will be lower (36) (Figure 25-2).

Serum screening for NTDs using MSAFP is usually not effective until after 14 weeks of gestation (37). The widest separation of MSAFP values between affected and unaffected pregnancies occurs at 17 weeks (35). MSAFP screening performance is acceptable over the broader range of 15–20 weeks typically utilized in screening programs for aneuploidy. Serum marker levels can be affected



**FIGURE 25-1** Mean concentrations of AFP in maternal serum, amniotic fluid, and fetal serum at various stages of pregnancy. From Haddow, J. E. *Prenatal Screening for Open Neural Tube Defects, Down's Syndrome, and Other Major Fetal Disorders*. Semin. Perinatol. 1990, 14, 488–503, with permission.



**FIGURE 25-2** Distribution of maternal serum AFP (in MoM) in anencephalic, open spina bifida, and unaffected pregnancies.

by multiple factors, including maternal weight, ethnicity, and the presence of insulin-dependent diabetes (38). All fetuses produce AFP at a fairly constant rate determined by the size and age of the fetus and placenta. AFP entering the maternal circulation is then diluted in the intravascular space, and so the concentration is lower in larger women because of a greater volume of distribution and higher in thin women, who have a smaller volume of distribution (39). Concentrations, therefore, must be corrected for maternal weight for the most accurate evaluation. For uncertain reasons, race also affects maternal serum AFP levels. African-American women have AFP concentrations 10–15% higher than white women (38). Most screening programs typically employ race-specific AFP medians. Insulin-dependent diabetes mellitus is associated with a 20% reduction in maternal serum AFP levels, and hence, adjustment is also required for accurate interpretation of AFP levels in diabetic women (40,41).

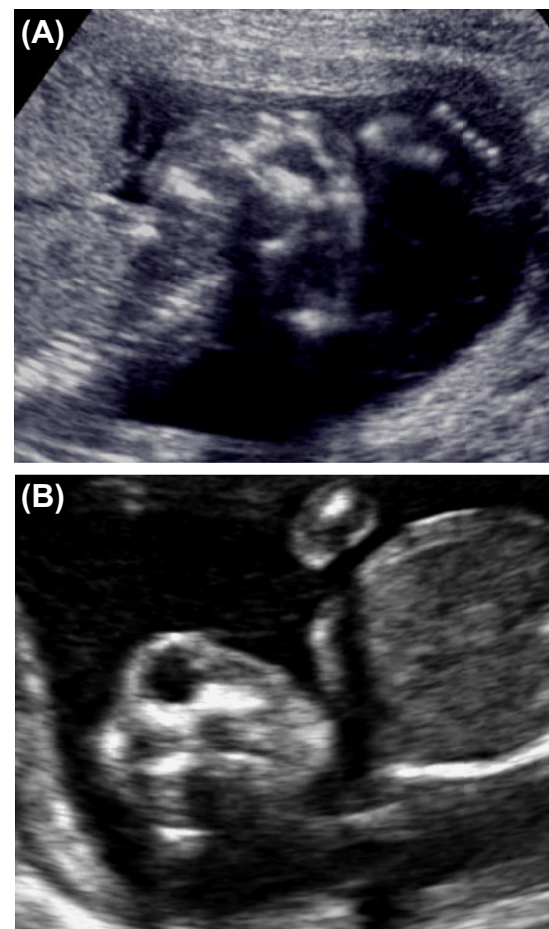
Multiple gestations pose a diagnostic dilemma in serum screening for NTDs. In general, serum biomarkers, including AFP, in twin pregnancies are approximately twice those of singletons (42,43). Detection rates of NTDs are significantly lower in twins than in singletons. In a series of 46 twin pregnancies, using a cutoff of 5.0 MoM, the detection rate of anencephaly was 83% and open spina bifida was only 39% with a false-positive rate of 3.3% (42). Lower cutoffs, such as 3.0–3.5 MoM, provided higher detection rates (96–98% for anencephaly and 69–80% for open spina bifida) but were associated with a 12–19% false-positive rate. Given the evolution in ultrasound and its importance in the evaluation of screen-positive pregnancies as opposed to amniocentesis, utilization of lower cutoffs may be warranted in order to preserve sensitivity.

**25.2.4.3 Prenatal Screening and Diagnosis of NTDs by Ultrasound.** In addition to the important role that ultrasound plays in accurate dating and appropriate interpretation of amniotic fluid and maternal serum AFP levels, ultrasound screening for major fetal anomalies in the second trimester has become a routine part of prenatal care in many countries (44). Detailed evaluation of most fetal anatomy is best accomplished at 18–20 weeks of gestation as major anomalies can be diagnosed and time allowed for the option of pregnancy termination. While many anomalies can be visualized earlier, this time also allows for better evaluation of the cardiac anatomy than possible at earlier gestational ages. The detection rates of NTDs by ultrasound vary considerably with the quality of the equipment, the fetal position, the maternal habitus and the experience of the operator. In expert hands, ultrasonography alone has 97% sensitivity and 100% specificity in the detection of NTDs (45).

Anencephaly results from failed closure of the cephalic portion of the neural tube and disrupted cranial development resulting in exencephaly, the precursor of anencephaly. Ultrasound findings include decreased crown-rump length (CRL), absent calvaria, extruding lobulated cerebral tissue (exencephaly) or absent neural tissue, abnormal

head shape with the eyes delineating the upper portion of the fetal face in the coronal plane, and, later in gestation, polyhydramnios due to impaired fetal swallowing (Figure 25-3) (46). Given the marked abnormalities evident on ultrasound, the detection rates for anencephaly approach 100% (47). As ossification of the skull is not visualized until after 12 weeks of gestation, care should be taken in diagnosing anencephaly before this time. The abnormal contour of the head and amorphous appearing cerebral tissue are often visible in the first trimester.

Open spina bifida is detected by both cranial and spinal abnormalities on ultrasound. The two major cranial anomalies, the so-called “lemon” and “banana” signs, are typically easily recognized (48). The lemon sign describes the flattening or concavity of the frontal bones of the skull visible in the transverse plane (Figure 25-4). Decreased intraspinal pressure with concomitant downward displacement of the brain and decreased intracranial pressure is thought to cause this abnormality (49). This theory is supported by the fact that the lemon sign is visible in 98% of fetuses with open spina bifida before 24 weeks but in only 13% after 24 weeks when ossification of the cranium has progressed (50). The banana sign



**FIGURE 25-3** (A) Sagittal and (B) coronal ultrasound images of a mid-trimester fetus with anencephaly. Note the absence of the cranium above the level of the orbits and the presence of only amorphous cerebral tissue.

signifies the caudal displacement of the cerebellum with alignment of the cerebellar hemispheres as part of type II Arnold–Chiari malformation (Figure 25-4). In contrast to the lemon sign, this abnormality persists throughout gestation although visualization with ultrasound becomes more difficult in the third trimester (46).

Approximately 99% of fetuses with open spina bifida lesions exhibit at least one cranial finding on ultrasound (51). Both lemon and banana (or absent cerebellum) signs were observed in 97% of fetuses with spina bifida, ventriculomegaly in 75%, cisterna magna obliteration in 68%, and diminished biparietal diameter in 61% of fetuses. These findings, including an effaced cisterna magna, a small posterior fossa, and a small cerebellum, are strongly (>90%) associated with spina bifida (52). This study found that ventriculomegaly and the lemon sign were less frequently (81% and 53%, respectively) associated with spina bifida. As nearly all fetuses with closed spina bifida will have normal cranial anatomy, this can be used to differentiate between open and closed lesions (53).

Sonographic identification of the area of spinal dysraphism is often more difficult than detecting cranial abnormalities in spina bifida. The length of the spine must be examined in the axial, coronal, and sagittal planes (46). The axial view is most useful for visualization of all three ossification centers and will show splaying of the posterior lamina in fetuses with spina bifida (54). If the protruding sac can be visualized, the thickness of the sac wall may indicate if the defect is open or closed (54). Additionally, the approximate level of the lesion can be identified, which can provide some prognostic data regarding future neurological function (55). Three-dimensional ultrasound has garnered recent attention as an adjuvant imaging tool for fetal anatomy (56,57). This is a particularly useful tool in imaging the

fetal spine as its entirety cannot be visualized in a single plane. Furthermore, three-dimensional ultrasound is less dependent on the technical skills of the operator and may uncover spinal anomalies that would otherwise go unrecognized (58).

Recent studies have examined first-trimester sonographic markers of spina bifida. At 11–13 weeks, the nasal bone is visualized and the nuchal translucency (NT) is measured to evaluate for aneuploidy (see Section 25.3.9.5). In the same midsagittal view, the fourth cerebral ventricle can be visualized as an intracranial translucency. Preliminary studies indicate that absence of intracranial translucency is a specific marker of spina bifida (59,60) but this screen is only 50% sensitive for the anomaly (60). This has not been evaluated in large-scale trials.

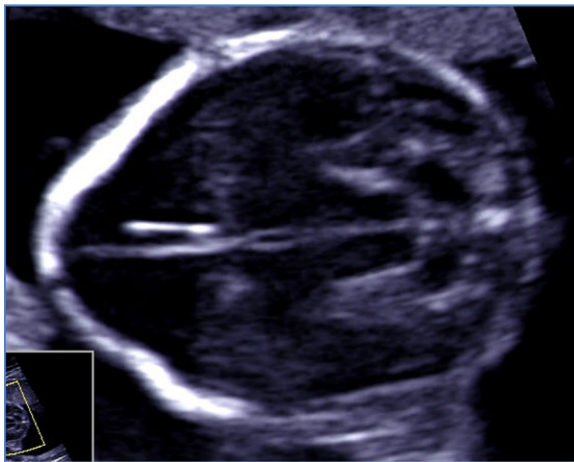
**25.2.4.4 Prenatal Diagnosis of NTDs by Amniotic Fluid Biochemistry.** Traditionally, women with elevated MSAFP levels were further evaluated by amniocentesis, so that amniotic fluid levels of AFP and acetylcholinesterase were measured to confirm the diagnosis of an NTD. In a large study of nearly 10,000 women, elevated amniotic fluid acetylcholinesterase, defined as an amniotic fluid AFP concentration >2.0 MoM for the gestational age, detected 100% of anencephaly and spina bifida cases, with a false-positive rate of only 0.08% (61). The additional advantage of this approach is that the fetal karyotype can be determined from the same amniocentesis sample. As previously noted, ultrasound technology has advanced significantly since the introduction of MSAFP screening and carries detection rates for NTDs comparable to amniocentesis. Additionally, amniocentesis is an invasive procedure and is associated with a risk of pregnancy loss of 1 in 200 (6).

## 25.3 ANEUPLOIDY

### 25.3.1 Classification, Frequency, and Occurrence

Studies in the 1970s showed that chromosomal abnormalities affect approximately 1 in 160 live births (62). A more recent European study of second-trimester amniocenteses suggests that this incidence may be higher when minor alterations, such as mosaicism, are included (63). The majority of abnormalities are sex chromosome alterations or autosomal trisomies, most frequently trisomy 21, 18, or 13. Approximately 1 in 800 children is born with trisomy 21 (Down syndrome), 1 in 6000 is born with trisomy 18 (Edwards syndrome), and 1 in 10,000 is born with trisomy 13 (Patau syndrome) (64). Most autosomal trisomies are caused by nondisjunction during maternal meiosis, a process that is more frequent with advancing maternal age (see Section 25.3.2).

There are limited preventative measures that have been shown to decrease the risk of aneuploidy in pregnancy. First, conception at a younger age is associated with a decreased rate of chromosomal aberrations. Second, couples with



**FIGURE 25-4** Axial view of fetal head demonstrating findings associated with a neural tube defect. The normal contour of the articulation of the frontal bones is altered so that there is an overlap and scalloping to generate the “lemon sign.” In the posterior fossa, the cerebellum is inferiorly displaced and elongated consistent with a Type II Arnold–Chiari malformation giving the appearance of the “banana sign.”



known genetic abnormalities, including balanced translocations, may utilize preimplantation genetic diagnosis to select for unaffected embryos. While some studies have suggested that folate supplementation may decrease the risk of Down syndrome (65), others have shown no effect (66).

### 25.3.2 Maternal Age Risks

The association between advanced maternal age and an increased risk of Down syndrome was first described in the 1930s (67). A multitude of subsequent studies have confirmed this association and defined its magnitude. The risk of Down syndrome and other aneuploidies increases with maternal age, so that a 40-year-old woman has a more than 13-fold higher risk of having a pregnancy affected by Down syndrome than does a 20-year-old woman (68).

The molecular basis for the association between maternal age and aneuploidy is an increased rate of meiotic non-disjunction in aging oocytes (69). Oocytes are suspended in the dictyotene stage of prophase I from the time they are formed during fetal development until they are fertilized in adulthood. During this protracted time in prophase, the chromosomes are kept aligned on the equatorial plate by chiasmata, the sites of recombination. In the majority of cases of maternal non-disjunction it is thought that aging causes deterioration of the chiasmata and subsequent misalignment of sister chromatids. This results in missegregation of chromosome pairs to the daughter oocytes (69).

### 25.3.3 Spontaneous Fetal Loss

Pregnancies affected by aneuploidy have a greater risk of spontaneous abortion than unaffected pregnancies. True estimates of this rate are difficult to ascertain as a proportion of affected pregnancies that are detected by prenatal screening programs will be terminated and some may occur so early in development that the woman does not recognize them as a pregnancy loss. A large-scale study showed that 43% of pregnancies with Down syndrome detected by the first-trimester CVS and 23% of pregnancies with Down syndrome detected by the second-trimester amniocenteses will end in miscarriage or stillbirth (70). Fetal loss rates in pregnancies affected by trisomy 13 or 18 are even higher. Forty-nine percentage of pregnancies diagnosed with trisomy 13 in the first trimester and 42% diagnosed with trisomy 13 in the second trimester will end in miscarriage or stillbirth. Seventy-two percentage of pregnancies diagnosed with trisomy 18 in the first trimester and 65% of pregnancies diagnosed with trisomy 18 in the second-trimester will end in miscarriage or stillbirth (71). This information is vital for counseling women regarding prognoses for their affected pregnancies.

### 25.3.4 Previous Affected Pregnancy

Women with a prior pregnancy with aneuploidy carry a significant risk of recurrence in subsequent pregnancies.

For Down syndrome, the relative risk of recurrence among all women is approximately 2 (72,73). The risk is higher in women who carry the first affected pregnancy before age 35 with a relative risk of 3.5 (73). The risk of recurrence for trisomy 18 is over 3, whereas the relative risk of recurrence for trisomy 13 is the highest at over 9 (73). Again, these risks are higher in women who are under age 35 during their first affected pregnancy. Women with any offspring with trisomy are at increased risk for a different trisomy in a subsequent pregnancy (72,73). These recurrences can be explained by possible gonadal mosaicism, in cases of the homotrisonomy recurrence, or by increased rates of meiotic error in women with homo- or heterotrisonomy recurrences. These numbers may be used to alter the *a priori* risk when counseling women with a prior affected pregnancy and allow better risk estimation than the traditional 1% risk estimate provided to most women after a prior trisomic conception.

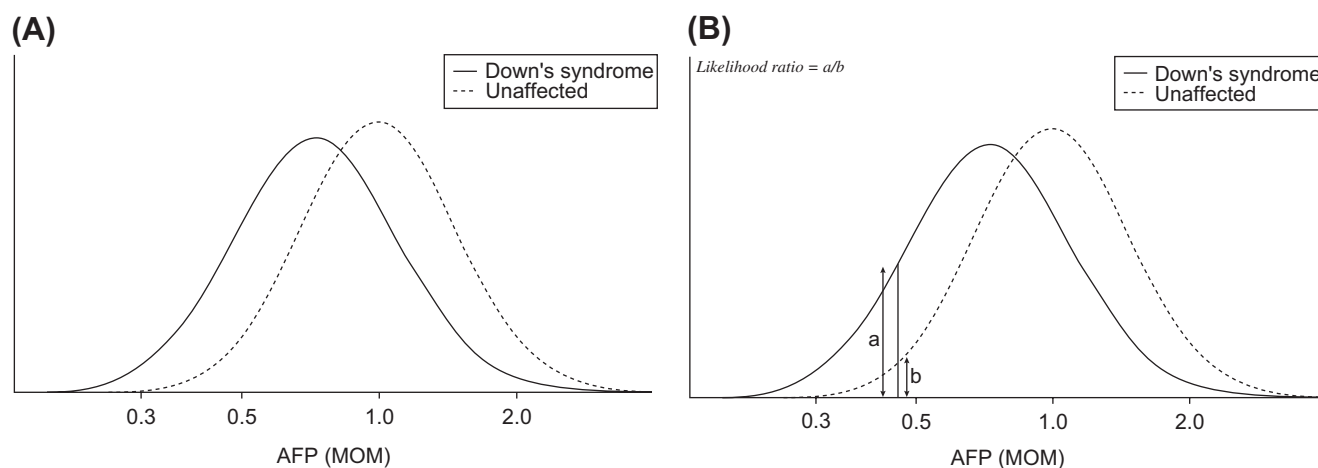
### 25.3.5 Prenatal Screening for Aneuploidy

**25.3.5.1 Maternal Age Screening.** In the early years of prenatal screening, maternal age was primarily used to select pregnancies at high risk for aneuploidy. Diagnostic testing, by amniocentesis or CVS, was offered to all women over a specific age cutoff, typically age 35. Age 35 was chosen as a cutoff as this represented the age at which the risk of Down syndrome equaled the risk of procedure-related pregnancy loss (74). Evidence has emerged that maternal age is not adequate to screen for aneuploidy. The majority of pregnancies affected by aneuploidy occur in women under 35. The American College of Obstetricians and Gynecologists (ACOG) recommends offering prenatal screening for aneuploidy to women of all ages (75). ACOG further recommends that all women should have the option to undergo invasive testing with either CVS or amniocentesis, irrespective of screening results, for chromosomal abnormalities (75).

**25.3.5.2 Second-Trimester Biochemical Screening for Aneuploidy.** Before the mid-1980s, the only options available for prenatal screening were the invasive procedures—CVS or amniocentesis. At this time, Merkatz and colleagues discovered the association between low MSAFP and trisomy 21 (76). Cuckle and colleagues confirmed this finding (77). As there is a considerable overlap between AFP levels in unaffected and affected pregnancies, a Gaussian model (Figure 25-5) was proposed to estimate the probability that an AFP level is correlated to an affected pregnancy (77).

As noted previously, AFP levels vary with gestational age. Therefore, precise estimation of gestational age is a prerequisite for valid screening with MSAFP and other serum markers and these analyte levels are expressed as MoM. A likelihood ratio for AFP, or any other serum marker, may be calculated by dividing the height of the affected pregnancy frequency distribution curve at a given MoM value by the height of the distribution





**FIGURE 25-5** (A) Distribution of maternal serum AFP (in MoM) in unaffected and Down syndrome pregnancies. (B) Estimation of the likelihood of an affected pregnancy associated with a specific AFP level.

curve of unaffected pregnancies at the same MoM (Figure 25-5b). This likelihood ratio can then be multiplied by the age-specific risk to estimate the adjusted risk. For example, a 30-year-old woman with an *a priori* age-related risk of Down syndrome of 1 in 415 and a likelihood ratio of 2.0 derived from serum screening would have an adjusted risk of 1 in 207. As the detection rate of MSAFP screening for Down syndrome and other aneuploidy is relatively low (77), expanded screening strategies, as described in the following sections, have been developed to increase detection and decrease false positives.

### 25.3.6 Serum Markers in Aneuploid Pregnancies in the Second Trimester

**25.3.6.1 Trisomy 21.** Soon after the association between low MSAFP levels and Down syndrome was discovered, Bogart and colleagues showed that high serum human chorionic gonadotropin (hCG) levels correlated with aneuploid fetuses (78,79). While several studies indicate a higher detection rate for Down syndrome when measuring the free b-subunit of hCG as compared to the intact dimeric hCG (80–83), other studies have showed no difference in the two assays (84,85). As the free b-subunit is unstable at ambient temperatures (86–89), most prenatal screening programs measure the intact hCG in the second trimester.

Unconjugated estriol (uE<sub>3</sub>), a steroid hormone derivative of dehydroepiandrosterone produced by trophoblasts, emerged next as an additional marker in the second-trimester screening. Canick and colleagues showed that uE<sub>3</sub> levels were reduced by 20% in pregnancies affected by Down syndrome (90). Initial studies showed that the combination of uE<sub>3</sub>, MSAFP, hCG (the so-called “triple screen”) and maternal age resulted in a detection rate of nearly 60% and the false-positive rate of about 3–5% for Down syndrome (91–93). Later, larger studies demonstrated detection rates of greater

than 75% with similar false-positive rates (94–96). The test is even more sensitive in women over 35, with studies indicating a 75–94% detection rate, depending on the defined cutoff (96–98).

Dimeric inhibin A (InhA) was the fourth marker added to the second-trimester serum screen, the modern version of which is now referred to as the “quadruple screen.” Multiple investigators have found elevated levels of InhA in pregnancies with Down syndrome (99–102). Subsequent studies showed that incorporation of InhA into two- or three-biomarker screening panels increased Down syndrome detection rate by 15–20% while maintaining a similar screen-positive rate (103–107). In contrast, a recent California-wide study of over 550,000 women showed that the detection rates for Down syndrome were similar between the triple screen and quadruple screen (77.4% vs 75.7%) but the screen-positive rate was 5.4% for the triple screen and just 3.8% for the quadruple screen (108). Most studies indicate that addition of InhA improves the performance of mid-trimester serum screening.

**25.3.6.2 Trisomy 18.** The discovery that all three analytes—AFP, hCG, and uE<sub>3</sub>—are decreased in pregnancies affected by trisomy 18 led to the development of screening algorithms with initial detection rates 58–80% and false-positive rates of just 0.3–0.6% (109,110). Other studies have indicated higher detection rates of over 80% with these three analytes, with similar low false-positive rates (96,111–113). Of the three biomarkers, reduced uE<sub>3</sub> levels are most predictive of trisomy 18 (114). InhA levels are also reduced in pregnancies with trisomy 18 although inclusion of InhA in the algorithm for trisomy 18 detection has not been as well studied (103,115,116). One study reported that the quadruple screen achieved a detection rate of 100% with a false-positive rate of 0.3% for detection of trisomy 18 (117).

**25.3.6.3 Trisomy 13.** Second-trimester biomarker screening is largely unsuccessful in detecting cases of trisomy 13 as there is no specific analyte pattern associated with this condition (98,117–119). Examination of

the analytes separately showed significantly lower levels of  $uE_3$  in pregnancies with trisomy 13 (119). Reduced levels of second-trimester pregnancy-associated plasma protein-A (PAPP-A) are found in pregnancies affected by trisomy 13 (120); however, this finding is of little clinical utility as PAPP-A is typically used as a first-trimester biomarker (see Section 25.3.7). Several small studies have suggested that InhA may be useful in screening for trisomy 13, but data are conflicting and limited by small sample sizes (113,116,121,122). Additionally, trisomy 13 may be detected by elevated AFP as many, but fewer than 50% (123), affected fetuses have open NTDs or ventral wall defects. Fortunately, careful ultrasound examination can identify most fetuses suspected of having trisomy 13.

**25.3.6.4 Sex Chromosome Aneuploidies.** Pregnancies affected by Turner syndrome (45,X) have significantly decreased levels of  $uE_3$  (124). Subsequent studies confirmed this observation and also showed an increase in AFP in many affected pregnancies (125). Furthermore, hCG levels are increased in pregnancies with hydropic Turner syndrome fetuses and decreased in pregnancies with nonhydropic fetuses (124). InhA is also elevated in Turner syndrome cases with hydrops and decreased in cases without hydrops (115,126).

**25.3.6.5 Triploidy.** Triploidy is defined as presence of three full sets of chromosomes. The additional set of chromosomes may be maternally derived (digynic) or paternally derived (diandric). Diandric triploid gestations typically have normal fetal growth, although there may be gross fetal malformations, and placentomegaly with partial molar changes. Digynic triploid pregnancies usually display significant fetal growth restriction and relatively small placentas. Triploidy can be incidentally detected with algorithms designed to detect Down syndrome or trisomy 18 (127,128). Depending on the parental origin of the additional set of chromosomes, second-trimester hCG may be high or low. Presumed diandric triploid pregnancies with partial molar changes are associated with high hCG levels, whereas presumed triploid digynic pregnancies are associated with low hCG levels (127). Similarly, diandric triploid gestations have high AFP levels and digynic pregnancies have normal AFP levels (129).

### 25.3.7 Serum Markers in Aneuploid Pregnancies in the First Trimester

Serum screening during the second trimester results in diagnosis of aneuploidy later in gestation, at a time when the mother may feel fetal movements, others may be aware of the pregnancy, and pregnancy termination may be more emotionally difficult for the family. Therefore, investigation has focused on earlier screening and diagnosis during the first trimester, when termination may be safer and less risky for the mother and her family.

**25.3.7.1 Trisomy 21.** Similar to the second trimester, hCG levels are elevated in the first trimester in pregnancies with Down syndrome (130,131). The detection rate

for trisomy 21 of hCG alone at a false-positive rate of 5% is 42–46% (132,133). To enhance the performance of the test, other markers were explored. Brambati and colleagues were the first to demonstrate that pregnancies affected by aneuploidy were associated with decreased levels of PAPP-A (134). This was confirmed in subsequent studies (135–139). In combination with maternal age, hCG and PAPP-A have a detection rate of approximately 67% (133). When these markers are combined with sonographically measured fetal NT (see Section 25.3.9.2.1) detection rates are even higher.

**25.3.7.2 Trisomy 18.** First-trimester maternal serum hCG and PAPP-A are decreased in pregnancies affected by trisomy 18 (136,140–142). When combined with NT, detection rates approach 90% with a false-positive rate of 1% (142).

**25.3.7.3 Trisomy 13.** Pregnancies with trisomy 13 are also associated with reduced serum levels of hCG and PAPP-A in the first trimester. Biochemical screening and NT measurement results in a detection rate of 90% with a false-positive rate of 0.5% (143).

**25.3.7.4 Sex Chromosome Aneuploidies.** A series of 46 cases of Turner syndrome showed significantly lower levels of PAPP-A and unchanged levels of hCG (144). Other sex chromosome abnormalities, such as 47XXX, XXY, XYY, were not associated with significant differences in PAPP-A or hCG levels (144).

**25.3.7.5 Triploidy.** In diandric triploidy, first-trimester maternal serum hCG is markedly increased and PAPP-A is slightly decreased. Digynic triploid pregnancies are associated with significantly reduced levels of both hCG and PAPP-A (145).

### 25.3.8 Maternal and Pregnancy Variables

**25.3.8.1 Gestational Age.** As several biomarkers vary significantly during gestation, a precise estimate of gestational age is vital for appropriate interpretation of screening results. Pregnancy dating by self-report of last menstrual period is highly variable, leading to inaccurate approximations of gestational age. Early first-trimester ultrasounds provide the most reliable dating criteria. Wald and colleagues showed that the addition of ultrasonographic measurements of fetal biometry increased the detection rate of the serum screen (including AFP, hCG, and  $uE_3$ ) by 15% for a given false-positive rate and decreased the false-positive rate by 46% at a given detection rate (146). With accurate dating, first-trimester screening is most effective between 10 and 14 weeks, and second-trimester serum screening is most effective between 15 and 22 weeks.

**25.3.8.2 Maternal Weight.** Heavier women have larger volumes of distribution and, therefore, decreased concentrations of serum biomarkers. Hence, all three analytes are adjusted based on maternal weight (39,146–148).

**25.3.8.3 Multiple Pregnancy.** As expected, hCG levels are elevated in twin pregnancies, with studies showing

free b-hCG levels approximately twice that of singleton pregnancies (149). InhA levels are also approximately doubled in twin gestations (150). In affected twin pregnancies, however, analyte levels are not consistent. The overall detection rate for aneuploidy in twin gestations is only 50–55% with second-trimester serum screening (151,152). First-trimester screening with serum biochemistry and NT measurement performs better with a detection rate of 80% and 5% false-positive rate (153), which was confirmed in prospective analyses (154,155).

Chorionicity also appears to affect first-trimester markers. Serum levels of hCG and PAPP-A are decreased in monochorionic twin gestations as compared to dichorionic gestations (156–158). By incorporating chorionicity-specific medians, the performance first-trimester screening algorithms approach that of singletons (158).

**25.3.8.4 Smoking.** In a recent study of more than 45,000 women, smoking was associated with reduced levels of first-trimester PAPP-A and hCG, reduced levels of second-trimester uE<sub>3</sub> and hCG, and increased levels of second-trimester AFP and InhA (159). These findings were in agreement with previous studies (160–170). Additionally, smoking is associated with elevated InhA (171). These altered analyte levels lead to increased screen-positive rates for Down syndrome in the second-trimester serum screen and for trisomy 18 in both the first-trimester combined and integrated tests (159).

**25.3.8.5 Ethnicity.** Several studies have demonstrated that hCG levels are higher in black women as compared to Caucasian women, but uE<sub>3</sub> levels are similar among the two groups (172). Furthermore, AFP levels are also higher in black women (173). Thus, race-specific medians have been developed for Caucasian and African-American women (174,175). These race-specific medians result in improved detection rates and lower screen-positive rates for these groups (176). A recent study showed that analyte levels also differ between Caucasian and Hispanic women resulting in lower screen-positive rates for trisomy 21 and NTDs in Hispanics (177). Ethnic-specific medians reduce this disparity (177).

**25.3.8.6 Fetal Gender.** Multiple studies have shown that pregnancies with female fetuses have reduced second-trimester AFP levels and higher hCG levels than those with male fetuses (178–185). This results in a higher false-positive rate for Down syndrome in female fetuses (185). This disparity does not result in significant differences in detection rates between the two sexes (185,186). First trimester analytes follow a similar pattern in that hCG levels are higher and PAPP-A levels are lower in women carrying female fetuses (187,188). Again, these differences result in high false-positive rates in pregnancies with female fetuses but no significant differences in detection rates (188).

**25.3.8.7 Diabetes Mellitus.** AFP levels are significantly reduced in women with pre-existing diabetes as compared to healthy controls (189,190), and this reduction

appears to be correlated with glycosylated hemoglobin, an indication of glycemic control (41). Additionally, uE<sub>3</sub> levels are slightly lower (191) and InhA levels are higher (192,193) in diabetic women. Most programs make adjustments in the standard risk calculations in diabetic women. A study examining first-trimester screening showed that women with insulin-dependent diabetes had a 15% reduction in PAPP-A, while hCG and NT measurements were unaffected (194). The authors propose that adjustments should be made to account for this difference in first-trimester screening as well.

**25.3.8.8 Renal Disease.** Women with renal disease have significantly higher levels of free b-hCG in the second trimester as compared to women with normal renal function (195). Similarly, one study found that high levels of hCG in the first trimester are correlated with potentially undiagnosed renal impairment (196).

**25.3.8.9 Medication Effects.** Numerous studies have shown that some medications may alter serum marker levels. Methadone and its metabolites inhibit the conversion of androgens to estrogens by placental aromatase (197), and methadone-addicted women have reduced uE<sub>3</sub> levels (198,199). Other opiates, such as morphine, heroin, hydromorphone, and hydrocodone, increase uE<sub>3</sub> levels (200). In a large cohort study, Pekarek and colleagues showed that multiple classes of medications, including methadone, immunosuppressants, antidepressants, antiemetics, antihypertensives, antiepileptics, and asthma medication, alter multiple serum biomarker levels (199). Further analysis showed that screen-positive rates for NTDs were higher in women taking immunosuppressants, antibiotics, and antidepressants and the screen-positive rate for trisomy 18 was higher in women taking methadone (199).

**25.3.8.10 Assisted Reproduction.** Analyte levels are altered in pregnancies conceived by assisted reproductive technologies. Second-trimester levels of InhA are increased in patients undergoing all types of assisted reproductive technologies (ART), while uE<sub>3</sub>, hCG, and AFP are altered only in women undergoing certain treatments (201). In contrast, first-trimester analytes are not significantly altered in patients undergoing ART (202).

**25.3.8.11 Vaginal Bleeding.** Cuckle and colleagues showed in a meta-analysis of second-trimester serum screens that vaginal bleeding was associated with a 10% increase in MSAFP and no change in uE<sub>3</sub> or hCG (203). The authors speculated that if adjustments were made for vaginal bleeding, there would be <1% gain in the detection rate of Down syndrome. A retrospective study of nearly 50,000 women undergoing first-trimester screening demonstrated no difference in hCG, PAPP-A, or NT measurements in women with vaginal bleeding in early pregnancy (204).

**25.3.8.12 Other Pregnancy Complications.** False-positive second-trimester screens for Down syndrome are associated with multiple adverse outcomes including growth restriction, low birth weight, small for gestational

age, preterm delivery, preeclampsia, and stillbirth (205–207). Low second-trimester uE<sub>3</sub> is associated with fetal death, miscarriage, and anencephaly (208). Reduced uE<sub>3</sub> is also associated with several genetic syndromes, such as Meckel–Gruber syndrome, characterized by encephalocele, postaxial polydactyly, and cystic kidneys, Smith–Lemli–Opitz syndrome, a defect in cholesterol biosynthesis resulting in multiple congenital anomalies and intellectual disability, and X-linked ichthyosis, a disease with altered steroid metabolism and characteristic scaly skin (208). The utility of low estriol for the detection of Smith–Lemli–Opitz syndrome has led some screening programs to report a specific risk for this disorder and recommend diagnostic evaluation when the risk exceeds a specific threshold. Implementation of such a program increases the screen-positive rate by <0.1%. (209) Similarly, low levels of first-trimester PAPP-A are associated with a number of obstetrical complications, including fetal loss, low birth weight, preeclampsia, gestational hypertension, preterm birth, preterm premature rupture of membranes, abruption, and stillbirth (210,211). Increased NT and low levels of hCG are also correlated with increased spontaneous fetal loss (211). Consequently, patients with abnormal serum screening results and normal fetal karyotypes may warrant monitoring for subsequent adverse pregnancy outcomes.

## 25.3.9 Ultrasound Screening for Fetal Chromosome Abnormalities

### 25.3.9.1 Ultrasound Screening in the Second Trimester.

**25.3.9.1.1 Nuchal Fold.** In the 1980s, mid-trimester thickening of the nuchal fold was first described as sonographic marker of Down syndrome. (212) Using a cutoff of 6 mm or greater for the nuchal fold has been demonstrated to allow detection of approximately 40% of fetuses with trisomy 21 (213,214) while the finding of a nuchal fold of <6 mm reduces the risk of Down syndrome to <1% (215). As an isolated marker, a thickened nuchal fold has a likelihood ratio of 9.8 for trisomy 21 (216).

**25.3.9.1.2 Femur and Humerus.** The second sonographic marker of Down syndrome that emerged was a relatively short femur length (217). The ratio of the femur length to the biparietal diameter is used to detect the relatively shortened femurs (218,219). The combination of a thickened nuchal fold and relatively short femur demonstrated a sensitivity of 75% and specificity of 98% (220). A short humerus appears to be a more sensitive and specific marker for Down syndrome (221). When combined with nuchal fold thickness, the sensitivity of a short humerus was 75% (222). Multiple subsequent studies have confirmed this association and showed a marked increase in the risk of Down syndrome when both humerus and femur length were short (223–226).

**25.3.9.1.3 Pyelectasis.** Although there is a high prevalence in euploid fetuses, pyelectasis or renal pelvic

dilation occurs more commonly in fetuses with trisomy 21 (227). Up to 25% of fetuses with Down syndrome have this minor abnormality, whereas only 2–3% of euploid fetuses demonstrate this finding (227–231). Despite this disparity in prevalence, however, when noted as an isolated finding, renal pelvic dilation is not associated with an increased likelihood of aneuploidy (216).

**25.3.9.1.4 Intracardiac Echogenic Foci.** The pathology literature previously reported an increased incidence of calcifications in the papillary muscle of infants with Down syndrome (232). Subsequent studies showed that an intraventricular echogenic focus (Figure 25-6) was associated with Down syndrome (233–235). Although this association has been confirmed in multiple studies (236–238), its clinical significance in isolation remains controversial and the likelihood ratio associated with this finding is 1.1 (216). Several studies have noted an increase in the frequency of this finding in certain racial groups (239). This finding, as well as advances in ultrasound technology, have enabled better visualization and have likely contributed to the poor specificity of this marker for aneuploidy.

**25.3.9.1.5 Choroid Plexus Cysts.** Choroid plexus cysts (Figure 25-7) are also associated with aneuploidy, especially trisomy 18 (240,241). Approximately 50% of fetuses with trisomy 18 will have a choroid plexus cyst (242). However, only 1% of fetuses with isolated choroid plexus cysts will have aneuploidy (242).

**25.3.9.1.6 Combined Markers.** Given the high proportion of fetuses with isolated sonographic markers of aneuploidy that have a normal karyotype, multiple scoring systems have been developed to quantify the risk of aneuploidy with a combination of markers (243,244). By integrating maternal age into the scoring system, further refinement of the method can be used for patient counseling to modify the *a priori* risk of aneuploidy (245,246).



**FIGURE 25-6** Four chamber view of the fetal heart. Note bright, echogenic focus in the left (posterior) ventricle which represents calcium deposition at the point of attachment of the chordae tendinae to the papillary muscle.



The combination of more than one sonographic marker is associated with over a 5-fold increase in the risk of Down syndrome, whereas a normal ultrasound decreases the chance of Down syndrome by approximately one-half (247).

**25.3.9.2 Ultrasound Screening in the First Trimester.** With improvements in ultrasound imaging technology and increased interest in earlier prenatal diagnosis, there have been significant advances in first-trimester ultrasound for aneuploidy screening such that first-trimester genetic sonograms are valuable adjuncts to biochemical analysis in prenatal screening.

**25.3.9.2.1 Nuchal Translucency.** NT is the first-trimester analog of the second-trimester nuchal fold (Figure 25-8). NT is the term used to describe a collection of fluid behind the fetal neck recognized during the first trimester (248). While all fetuses have some degree of measurable NT, fetuses with trisomy 21, as well as other chromosome abnormalities, have measurements that are two- to three-fold larger than that of unaffected fetuses (248), making it a powerful marker for estimation of the risk of fetal aneuploidy. In addition to its role in aneuploidy screening, an increased NT

has also been identified in a multitude of single-gene disorders and structural malformations (e.g. congenital cardiac defects, renal malformations, neuromuscular abnormalities) (249,250). For this reason, any fetus with an NT  $\geq 3$ –3.5 mm should have a targeted ultrasound evaluation for careful assessment of anatomy.

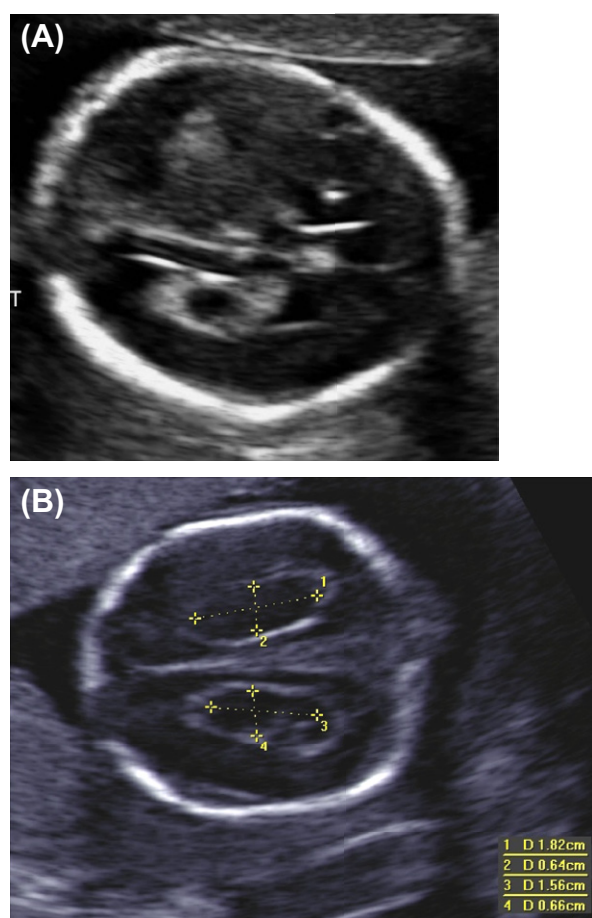
The optimal time measure in the NT is between 11 and 13 6/7 weeks, at a corresponding CRL of 45–84 mm (131). The NT should be measured in the sagittal plane at its maximal thickness. The NT measurement may be expressed in multiple ways. One way is to report the measurement is the delta NT, which is the difference in millimeters between the normal median for the CRL and the measured NT (251). The second approach is to derive the MoM by dividing the measured NT by the normal median at the gestational age (252). This allows for an age-adjusted risk assessment similar to biochemical screening. The third method, termed *the mixture model*, involves analyzing the NT in a CRL-dependent and CRL-independent manner as a higher proportion of aneuploid fetuses have CRL-independent increases in NT (253). Regardless of the analysis methodology, several prospective studies have shown that detection rates for Down syndrome and other aneuploidies approach 80% with NT measurement (131,254).

Multiple studies have shown that increased NT is associated with aneuploidy (251,255–257). Using an increased NT as the sole criteria for fetal aneuploidy screening detects approximately 70–75% of fetuses with trisomy 21, and when combined with the maternal age-associated *a priori* risk, a detection rate in excess of 80% is achievable (254,258–261).

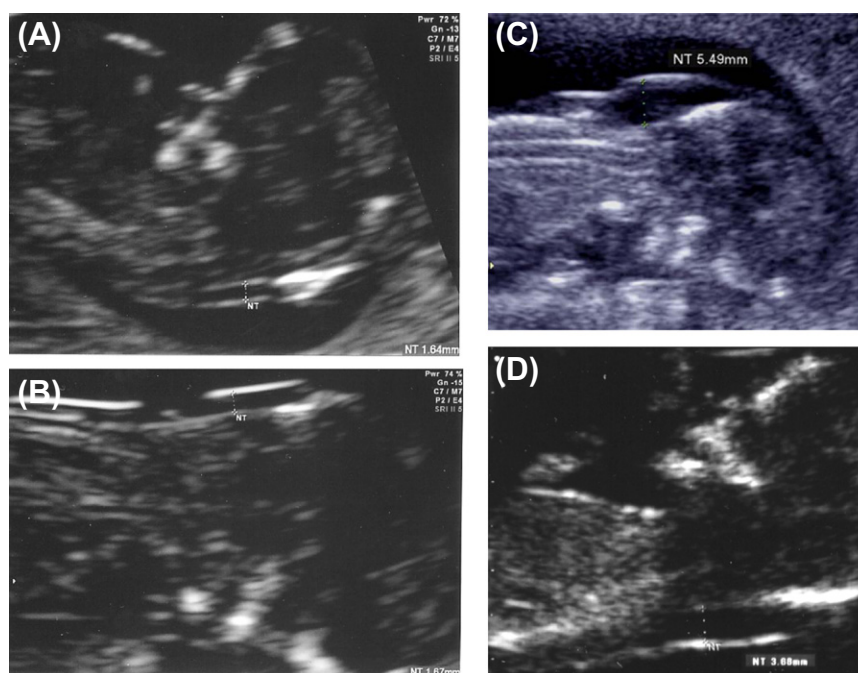
Fetal NT measurements are independent of maternal serum levels of hCG and PAPP-A; therefore, these markers can be combined into a single screening test. The combination of free  $\beta$ -hCG or hCG, PAPP-A, and NT with maternal age is able to detect approximately 80–85% of fetuses with trisomy 21 at a false-positive rate of 5% (262–269).

**25.3.9.2.2 Fetal Heart Rate.** Studies of fetal heart rate (FHR) in association with aneuploidy have reported conflicting results. Aneuploidy has been associated with both bradycardia and tachycardia (270–272). While some studies have shown significant gains in detection rates by adding FHR to other first-trimester markers (270), other studies have suggested negligible effects on detection rate (272).

**25.3.9.2.3 Ductus Venosus.** A significant proportion of fetuses with chromosomal aberrations will have abnormal high-impedance flow through the ductus venosus due to structural or functional cardiac abnormalities (273,274). Analysis of pooled data from previous studies showed that while only 5% of karyotypically normal fetuses will display abnormal ductus flow, approximately 70–90% of fetuses with trisomy 21, 28, 13 or Turner syndrome will have this finding (275). Addition of ductus flow to a combined screening



**FIGURE 25-7** Axial views of the fetal head demonstrating the presence of hypoechoogenic areas within the lateral ventricle consistent with a diagnosis of choroid plexus cysts. (A) Unilateral choroid plexus cyst. (B) Large bilateral choroid plexus cysts in a fetus that proved to have trisomy 18.



**FIGURE 25-8** Nuchal translucency measurement in the first trimester. NT within average range: (A) fetal prone position and (B) Fetal supine position. Increased NT: (C) prone and (D) supine.

included maternal age, NT, FHR, hCG, and PAPP-A resulted in a detection of 96%, 92%, 100%, and 100% of trisomy 21, 18, 13, and Turner syndrome, respectively (275). Abnormal ductus flow in twin pregnancies was also associated with an increased rate of aneuploidy or other adverse outcomes, such as twin-twin transfusion syndrome, but 75% of dichorionic and 40% of monochorionic twins with this finding had normal outcomes (276). Furthermore, recent study showed that an altogether absent ductus venosus is associated with aneuploidy or other genetic syndromes in a high proportion of cases when the NT is increased, whereas this abnormality is associated with a favorable prognosis in the setting of a normal NT (277).

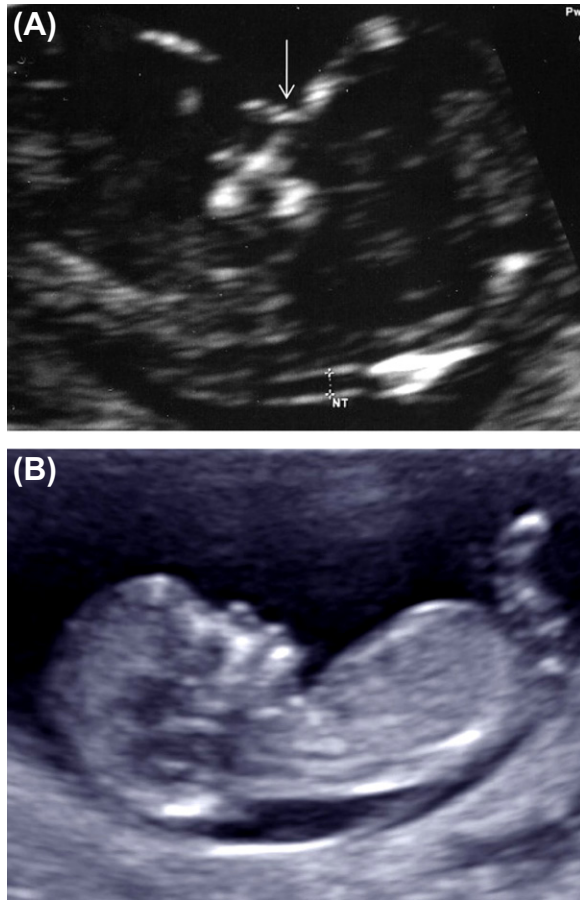
**25.3.9.2.4 Tricuspid Regurgitation.** Tricuspid regurgitation, detected by pulsed wave Doppler flow, has been associated with Down syndrome (278,279). This finding is believed to be associated with the high incidence of valvular structural abnormalities in the setting of aneuploidy and/or the decreased cardiac compliance associated with myocardial dysfunction. By adding tricuspid regurgitation assessment to a program based on maternal age, NT, FHR, hCG, and PAPP-A, Down syndrome detection rose from 91% to 96% (280).

**25.3.9.2.5 Nasal Bone.** A high proportion of aneuploidy fetuses demonstrate hypoplasia or failure to visualize the nasal bone in the first trimester (Figure 25-9). Interrogation of the fetal facial profile in the first trimester is an extension of the physical examination performed in the neonatal period, in which a flattened nasal bridge and profile are often noted in aneuploidy infants, especially those with trisomy 21 (281). Although dependent

somewhat on ethnicity, up to 75% of fetuses with trisomy 21 will have nonvisualization of the nasal bone in the first trimester compared to fewer than 1% of euploid fetuses (281,282). By adding the nasal bone to other first-trimester screening, including hCG, PAPP-A, NT, and FHR, this strategy decreased the false-positive rate for trisomy 13, 18, 21, and Turner syndrome and further increased the detection rate for trisomy 21 (282,283).

## 25.3.10 Integrated Screening

The implementation of first-trimester programs has shifted the paradigm of prenatal screening and diagnosis. By combining ultrasound with biochemical analysis, high detection rates with low false-positive rates can be achieved. In the largest study of first-trimester screening of over 8500 patients, a screening program based on maternal age, serum hCG and PAPP-A levels, and NT measurement showed a 79% detection rate for Down syndrome with a false-positive rate of 5% (284). These rates are comparable with second-trimester biochemical screening, as shown in the Serum, Urine and Ultrasound Screening Study (SURUSS) (267). SURUSS indicated that using a fully integrated screening program, combining NT, first-trimester PAPP-A, and second-trimester AFP, uE3, hCG, and InhA, provides a low false-positive rate at 1.2% with an 85% detection rate (285). The First- and Second-Trimester Evaluation of Risk (FASTER) trial was a landmark prospective study of nearly 40,000 women that compared multiple combinations of screening strategies, including sequential (first- and second-trimester results reported independently) vs integrated



**FIGURE 25-9** Middle sagittal image of fetus in supine position for assessment of fetal nasal bone. (A) Nasal bone present—note the echogenic area (arrow) below the skin echo to give appearance of an equal sign. (B) Absent nasal bone.

(first- and second-trimester results reported as single risk assessment) screening (269). FASTER sequential screening resulted in a 95% detection rate and fully integrated screening resulted in a 96% detection rate. However, sequential screening results in higher false-positive rates, possibly resulting in unnecessary invasive diagnostic tests (269,286).

Despite its high detection rates and low false-positive rates, exclusive use of integrated screening eliminates the option of first-trimester risk assessment and early prenatal diagnosis. Hybrid screening models are often employed in clinical practice. One model is contingent sequential screening in which first-trimester results are used to triage patients to have no further screening, proceed with second-trimester screening, or undergo an immediate CVS. This model has not been evaluated prospectively, but decision analysis modeling has shown that this is the most cost-effective approach (287,288). Using the stepwise sequential model, a preliminary risk may be quoted in the first trimester for women at highest risk. This provides the option of immediate CVS or awaiting the fully integrated screening results during the second trimester while minimizing the chance of additive false-positive results (75).

### 25.3.11 Frontiers in Prenatal Screening and Diagnosis

While significant efforts have focused on improving the sensitivity and specificity for serum and ultrasound techniques for prenatal screening, definitive diagnosis still requires an invasive procedure such as CVS or amniocentesis. Noninvasive techniques for obtaining fetal genetic material are the newest developments in prenatal screening and diagnosis (289).

Early in pregnancy, small erosions in the decidua capsularis allow trophoblast cells enter the uterine cavity and shed into the cervix. In 1971, Shettles and colleagues first reported retrieval of trophoblasts from the endocervix in the first trimester for sex determination (290). Later studies showed that these cells could be obtained for aneuploidy screening by FISH and hemoglobin genotyping (291–293). Depending on the exact retrieval technique, success rates vary widely from 40% to 90% (294); thus, further studies are required to optimize this potentially valuable noninvasive method.

The presence of fetal cells in the maternal circulation has been known for over a century. As the concentration is very low, at approximately 1 per 10 million (295), the development of efficient isolation and analysis techniques remain challenging. A multicenter trial using this technique showed that the correct fetal gender was detected in <50% of cases (296). More refined techniques have been investigated (297,298) but this methodology remains experimental.

The most promising technique involves isolation of cell-free fetal nucleic acids. In 1997, Lo and colleagues showed that fetal DNA could be amplified from maternal blood (299). The cell-free fraction of blood likely represents a better source as the concentration of fetal DNA is nearly 1000-fold higher in plasma than in the cellular component (300). However, fetal DNA still represents a low (3–6%) proportion of the total circulating free DNA (300), making detection of alterations in fetal chromosome dosage still challenging. One answer to this problem is use of massively parallel genomic sequencing, a type of shot-gun sequencing in which short tags across the genome are amplified, sequenced, and quantified (301,302). Evaluation of over 700 pregnancies with this technique demonstrated a 100% sensitivity and 98% specificity in the detection of Down syndrome (303). Studies are ongoing to assess the utility of this technology in a variety of patient populations.

## 25.4 SUMMARY

Prenatal aneuploidy screening of all women with a combination of biochemical and sonographic analysis has become standard of care in the United States, among a multitude of other countries. Advances in serum screening and ultrasound technology has improved detection rates and decreased false-positive rates, reducing the need for invasive diagnostic procedures. In particular, integrated



screening, combining first- and second-trimester serum and ultrasound analysis, has revolutionized prenatal screening with detection rates of >95% (269). Newer methodologies involving fetal nucleic acid extraction and analysis from maternal serum are currently under investigation. If proven effective, these techniques could supplant the current prenatal aneuploidy screening paradigm and make invasive procedures, such as CVS and amniocentesis, obsolete.

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# CHAPTER

# 26

## Techniques for Prenatal Diagnosis

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### 26.1 INTRODUCTION

The cornerstone of prenatal diagnosis has been the direct evaluation of fetal tissue. Advances resulting from the Human Genome Project have increased the number of fetal abnormalities amenable to invasive prenatal diagnosis. In addition, in the past few decades, there has been an expansion of our capabilities to assess risk for fetal abnormalities by noninvasive screening modalities including high-resolution ultrasonography and maternal serum analyte screening. Effective screening protocols reduce the number of invasive procedures in women carrying unaffected fetuses. Recent data showing improved safety of chorionic villus sampling (CVS) and amniocentesis now raise the possibility of offering invasive prenatal diagnosis to all women in the first and second trimesters. Indeed, the American College of Obstetricians and Gynecologists has recommended that invasive diagnostic testing for aneuploidy should be available to all women, regardless of maternal age (American College of Obstetricians and Gynecologists (2007)).

This chapter reviews the techniques used for invasive prenatal diagnosis and their safety. Recent advances in noninvasive screening protocols, including new approaches combining first-trimester ultrasonography and maternal serum analytes to provide a risk assessment for fetal Down syndrome and other aneuploidies. Recent developments in the isolation and analysis of fetal DNA from maternal blood are briefly addressed.

### 26.2 AMNIOCENTESIS

#### 26.2.1 Traditional Amniocentesis: 15 Weeks Gestation and Greater

**26.2.1.1 Technique.** Amniocentesis, the aspiration of amniotic fluid, has traditionally been performed at

and after 15–17 weeks gestation (menstrual weeks). At this stage of gestation, the volume of amniotic fluid is approximately 200 mL. The ratio of viable to nonviable cells in the amniotic fluid of second-trimester fetuses is relatively high (1), thus allowing timely culture and diagnosis of fetal cytogenetic abnormalities. This allows women the option of pregnancy termination when a fetal abnormality is detected.

Amniocentesis is routinely performed in an outpatient facility. An ultrasound examination should be done immediately before the procedure to evaluate fetal number and viability, perform fetal biometric measurements to confirm gestational age, establish placental location, and estimate amniotic fluid volume. In our center, a fetal anatomic survey to screen for major anomalies is standard at the time of amniocentesis. In addition, ultrasonography may be useful in discovering maternal anatomic conditions (e.g. uterine fibroids, overlying bowel) that could influence the performance of the amniocentesis.

Once the preoperative ultrasound examination is completed, a needle insertion site is chosen. We prefer to insert the needle as close to the midline fundal area of the uterus as is possible, but this is not always the site at which the optimal pocket of amniotic fluid is located. Not infrequently, a lower uterine segment or lateral approach is required. We seek to avoid the placenta if possible; however, transplacental amniocentesis has not been shown to reduce the safety of amniocentesis (1–3). If tapping the optimal pocket of fluid requires traversing the placenta, we select the thinnest and most peripheral portion of the placenta possible through which the needle can be directed. If a transplacental approach is selected, the umbilical cord insertion site should be identified and avoided. Maternal bowel and bladder should also be located as these should likewise be avoided. A

local subcutaneous anesthetic (e.g., 2–3 mL of 1% xylocaine) may be used, but we usually find this unnecessary as many women complain of mild discomfort when the needle traverses the parietal peritoneum of the uterus, and this cannot be anesthetized with a local agent.

After the maternal skin is cleaned with an iodine-based solution, sterile drapes are placed around the needle-insertion site to help maintain an aseptic field. We prefer a 22- or 23-gauge spinal needle and recommend no larger than a 20-gauge. It has been suggested that use of a 20-gauge needle is associated with lower risk of intrauterine bleeding in case of transplacental needle insertion for faster fluid retrieval when compared with a 22-gauge needle; however, use of a 20-gauge needle is associated with more immediate discomfort during the procedure (4). Ultrasonographic monitoring of the amniocentesis with continuous visualization of the needle should be performed throughout the procedure. Although three-dimensional (3D) and 4-dimensional real-time sonography represent a rapidly developing area of medical imaging, and they have been used recently to guide prenatal invasive procedures, these modalities do not appear to offer any advantages over 2-dimensional real-time sonography, at least as determined by reducing the number of needle insertions (5). Ultrasound gel is applied adjacent to the insertion site, and a real-time ultrasound transducer is held in position such that the ultrasound beam is directed parallel to the planned needle track. A local anesthetic (e.g., 2–3 mL of 1% xylocaine) may or may not be used; however, local anesthesia does not appear to affect the level of pain of the procedure (6,7). Neither do other techniques such as lidocaine–prilocaine cream applied to the skin (8). Counseling before amniocentesis should emphasize that the actual pain and anxiety experienced during the procedure are significantly lower than expected (9). Needle insertion should be performed with one smooth continuous motion until the needle tip is within the amniotic cavity (Figure 26-1A–C). Some practitioners locate the needle tip within the subcutaneous fat and assess the distance from tip to amniotic cavity as well as the appropriate needle angle required to successfully enter the amnion and avoid maternal structures and the fetus. Amniotic membrane “tenting” is rarely encountered in procedures after 15 weeks gestation; in such cases, resolution usually occurs when the needle is rotated upon its axis or advanced further into the cavity. If such maneuvers fail to obtain amniotic fluid, needles should not be directed away from the axis of insertion and arbitrarily moved within the cavity as such actions can lead to lacerations of the amniotic membrane. In these cases, consideration of needle removal and choosing an alternative insertion site is warranted.

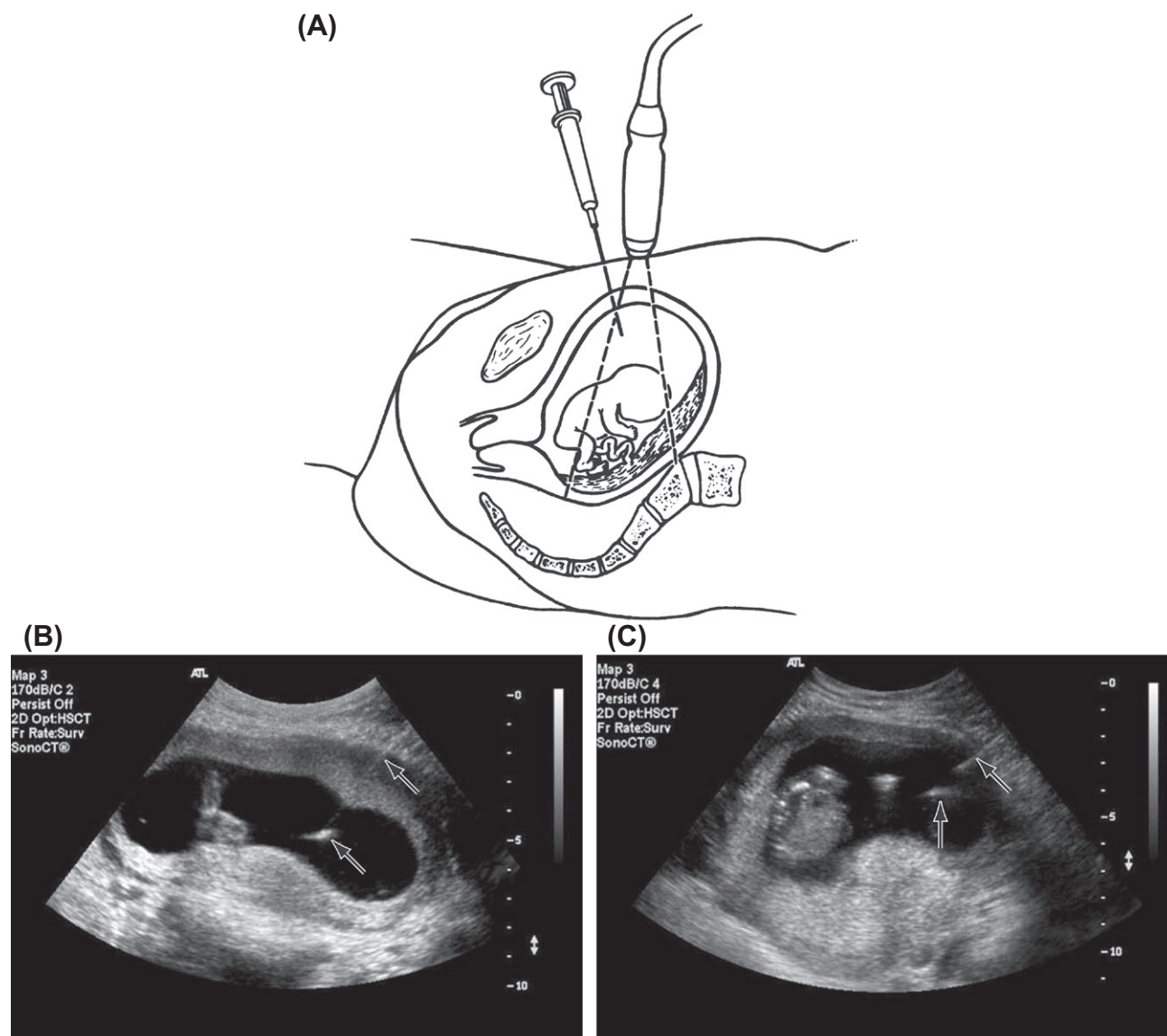
The initial 2–3 mL of amniotic fluid is aspirated into a syringe. These first few milliliters are theoretically the most likely to contain maternal cells from blood vessels, the abdominal wall, or the myometrium; therefore, this initial sample is usually discarded or set aside for

$\alpha$ -fetoprotein (AFP) analysis. For a second-trimester amniocentesis performed at 15–20 weeks inclusive, 20–30 mL of amniotic fluid is usually aspirated. Some operators recommend aspirating amniotic fluid directly into vacuum tubes, eliminating some manipulation with the needle (10,11). We do not find use of vacuum tubes to be particularly helpful. Once the amniotic fluid is obtained, the specimen is clearly labeled and transported at ambient temperature to the laboratory.

Amniotic fluid and urine are often indistinguishable in appearance. Analysis of cells derived from maternal urine could obviously lead to erroneous interpretations of fetal status. Inadvertent aspiration of maternal urine is a particular risk when a suprapubic needle insertion site is chosen. If the origin of aspirated fluid is in doubt, tests should be performed to determine its origin. Our group found that the crystalline arborization pattern characteristic of amniotic fluid is observed if the fluid is allowed to dry on an acid-cleaned slide and examined under low power (~100 magnification) (12). This test readily differentiates amniotic fluid from urine. However, with the widespread use of concurrent ultrasound, such tests should only be rarely necessary.

Bloody amniotic fluid is aspirated in perhaps 1–2% of amniocenteses. The blood, which is almost always maternal in origin, does not adversely affect amniotic cell growth. Indeed, the performance of a transplacental amniocentesis has been shown not to increase the risk for fetal loss compared to non-placental procedures but may increase the risk for a bloody tap. By contrast, brown- or dark red- or wine-colored amniotic fluid is associated with an increased likelihood of poor pregnancy outcome. This color indicates prior intra-amniotic bleeding, with hemoglobin breakdown products accounting for the fluid color. Pregnancy loss eventually may occur in about one-third of such cases (13). If the abnormally colored fluid is also characterized by an elevated AFP level, the outcome is usually unfavorable (fetal death, anencephaly, spontaneous abortion, or fetal abnormality). Green-colored amniotic fluid, presumably due to meconium staining, is apparently not associated with poor pregnancy outcome (14–16). By contrast, brown amniotic fluid has been associated with an increased risk of fetal aneuploidy (15).

The propriety of administering Rh immunoglobulin (RhIG) to prevent Rh immunization in unsensitized women with Rh-positive fetuses remains controversial. Fetomaternal transfusion by disruption of the fetoplacental circulation logically might have an immunizing effect; however, the magnitude of the risk has not been determined. The task is difficult because one must consider such variables as ABO compatibility, number of needle insertions, placental location, and the amount of fetal blood transfused into the maternal circulation. Early investigators opined that prophylactic RhIG should not be administered after genetic amniocentesis (17), but almost all now advocate its routine use (18–21). The dose to be administered is also controversial. The American



**FIGURE 26-1** (A) Amniocentesis performed concurrently with ultrasound. (From Simpson, J. L.; Elias, S. *Prenatal Diagnosis of Genetic Disorders*. In *Maternal-Fetal Medicine: Principles and Practice*; Creasy, R. K.; Resnik, R., Eds.; WB Saunders: Philadelphia, 1994, with permission.) (B) Ultrasonographic visualization of a transplacental amniocentesis. Thin arrow points to the needle shaft within the placenta and the wall of the uterus, and the thick arrow points to the needle tip within the amniotic cavity. (Courtesy of Leeber Cohen, MD.) (C) Ultrasonographic visualization of non-transplacental amniocentesis. Thin arrow points to the needle shaft within the wall of the uterus, and the thick arrow points to the needle tip within the amniotic cavity. (Courtesy of Leeber Cohen, MD.)

College of Obstetricians and Gynecologists recommends that 300  $\mu$ g of RhIG be administered for an exposure of 30 mL of fetal blood (22). In the United Kingdom, the recommended dose of RhIG is 50  $\mu$ g before 20 weeks gestation and 100  $\mu$ g thereafter (23). We routinely administer 300  $\mu$ g of RhIG after genetic amniocentesis, regardless of whether the needle has traversed the placenta.

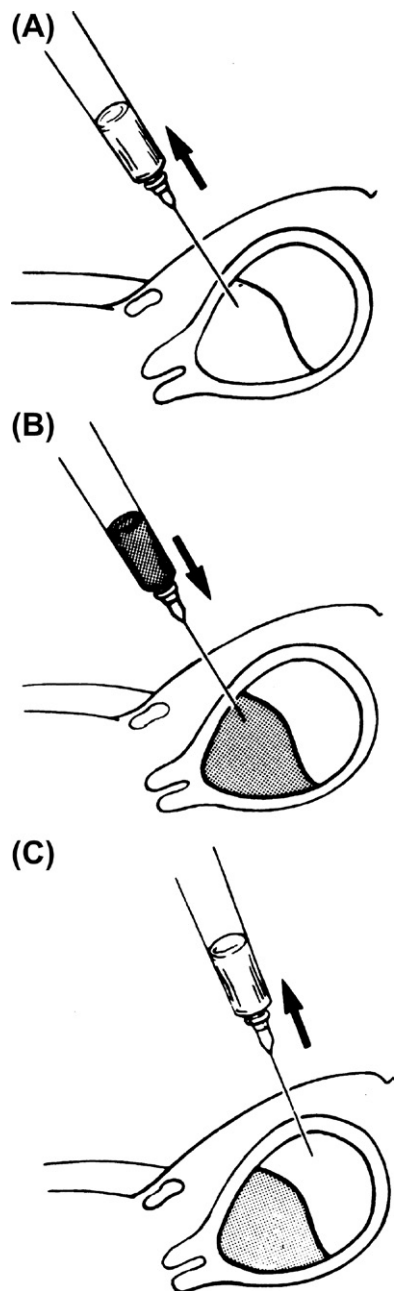
Following amniocentesis, the fetal heart motion should be documented by ultrasonographic visualization. The patient is observed briefly after the procedure and is instructed to report any fluid loss or bleeding per vagina, uterine cramping, or fever. Although antibiotic prophylaxis after amniocentesis has been recommended by one group, we and others believe that the

risks outweigh the benefits (24). Reasonably normal activities may be resumed after the procedure; however, we recommend that strenuous exercise (e.g. jogging or aerobic exercises) and coitus be avoided for at least a day.

**26.2.1.2 Multiple Gestations.** In multiple gestations, amniocentesis can usually be performed on all fetuses, provided amniotic fluid volume is adequate (25). After aspiration of amniotic fluid from the first sac, 2–3 mL of indigo carmine, diluted 1:10 in bacteriostatic water, is injected before needle withdrawal. The second amniocentesis is then performed. The second needle is then inserted into the sac of the second fetus, preferably determined after visualizing the membranes separating



the two sacs. Aspiration of clear fluid confirms that the second sac has truly been entered (Figure 26-2). In experienced hands, amniocentesis is performed successfully in more than 95% of twin pregnancies with ostensibly no increased risks over that of amniocentesis in singleton pregnancies (25–27). Anderson and Goldberg (26) observed a postprocedure twin-loss rate of 3.57% up to 28 weeks, a rate interpreted as not increased over the sum



**FIGURE 26-2** Technique for amniocentesis in twin gestations. (A) Fluid is aspirated from the first amniotic sac. (B) Blue indigo carmine dye is injected into the first amniotic sac. (C) A second tap is made in the ultrasonographically determined location of the second fetus. Clear fluid confirms that the second amniotic sac was successfully aspirated. (From Elias, S.; Gerbie, A. B.; Simpson, J. L., et al. *Genetic Amniocentesis in Twin Gestations*. Am. J. Obstet. Gynecol. 1980, 138, 169, with permission.)

of background twin-loss rate plus the loss rate associated with singleton amniocentesis; a similar rate of pregnancy loss (3.87%) was reported by Toth-Pal and colleagues among women with multiple gestations undergoing amniocentesis before 24 weeks gestation (27).

Most recently, Cahill and colleagues reported a 16-year retrospective cohort of all twin pregnancies that underwent ultrasound evaluation at the Washington University School of Medicine, St. Louis, MO, who were followed for pregnancy outcomes. Women who underwent amniocentesis were compared with those who had not. Fetal loss was defined as loss before 24 weeks gestation. Of 1934 twin pregnancies, 311 women elected amniocentesis. Women who elected amniocentesis were more likely to experience a pregnancy loss than those who did not (3.2% vs 1.4%; risk difference, 1.80%; 95% CI, 0.24–3.84%, which was significant after adjustment for advanced maternal age, chorionicity, sonographic findings, alcohol exposure and race (adjusted odds ratio, 2.9; 95% CI, 1.2–6.9). Thus, the attributable risk of pregnancy loss before 24 weeks gestation after mid-trimester amniocentesis in twin pregnancies was found to be 1 in 56 (1.8%) (28).

Triplets and higher order multiple gestations can be managed similarly by sequentially injecting dye into successive sacs. As long as clear fluid is aspirated, one can be reassured that a new amniotic sac has been entered. However, the overall safety of amniocentesis in triplets and higher order multiple gestations has not yet been formally determined.

Other techniques for sampling multiple gestations have been reported, including a single-puncture technique and an “air-bubble” infusion procedure. Despite advances in ultrasonography, we have concerns that single-puncture techniques could lead to cross-contamination between sacs, resulting in diagnostic inaccuracies. In addition, we believe it is unwise to instill any substance into the amniotic cavity unless the instillation is performed in a strict, aseptic manner and serves to considerably and consistently improve safety or accuracy. More recently, some operators have not used dye instillation to distinguish separate sacs, instead relying on ultrasonographic visualization alone (29–31). In view of the considerable positive experience with dye instillation techniques over the past 25 years with regard to safety and accuracy, we still prefer the dye instillation procedure described above.

**26.2.1.3 Safety.** Any procedure that involves passing a device into an organ, especially the pregnant uterus, carries a risk. Amniocentesis is no exception. Amniocentesis carries potential danger to both mother and fetus. Maternal risks are quite low, with symptomatic amnionitis occurring only rarely. Minor maternal complications such as transient vaginal spotting and minimal amniotic fluid leakage occur in 1% or less of cases, but these are almost always self-limited in nature. Other very rare complications include intra-abdominal organic injury or hemorrhage.

The safety of traditional amniocentesis has been addressed by several large collaborative studies. The U.S.

National Institute of Child Health and Human Development (NICHD) conducted the first major prospective study of genetic amniocentesis that comprised 1040 subjects and 992 matched controls. Of the 1040 women undergoing amniocentesis, 950 (91.3%) had the procedure performed for cytogenetic analysis and 90 (8.7%) to evaluate for the possible presence of an inborn error of metabolism. Of all women who underwent amniocentesis, 3.5% experienced fetal loss between the time of the procedure and delivery compared with 3.2% of controls; the slight difference was not statistically significant and disappeared completely when corrected for maternal age. In Canada, a collaborative group conducted a cohort study but did not include a concurrent control group (32,33). Analysis was based on 1223 amniocenteses performed during 1020 pregnancies in 900 women. The pregnancy loss rate was 3.2%, a frequency similar to that reported in the U.S. collaborative study.

A later British collaborative study found that the rate of fetal loss after amniocentesis was significantly greater than in controls (2.6% vs 1%) (34). In the British study, however, one common indication for amniocentesis was elevated maternal serum  $\alpha$ -fetoprotein (MSAFP), now recognized itself as a factor associated with fetal loss and adverse perinatal outcome. Analysis after excluding subjects undergoing amniocentesis for that indication lowered the loss rates between subject and control groups to <1%, albeit still a significant difference (35).

None of the collaborative studies cited above were conducted with high-quality ultrasonography as defined by today's standards, nor was concurrent ultrasonography universally applied. More relevant data are from a more recent Danish randomized controlled study of amniocentesis that involved 4606 women aged 25–34 years who were without known risk factors for fetal genetic abnormalities (36). Women with three or more previous spontaneous abortions, diabetes mellitus, multiple gestation, uterine anomalies, or intrauterine contraceptive devices were excluded. Maternal age, social group, smoking history, number of previous induced and spontaneous abortions, stillbirths, live births, and low-birth-weight infants were comparable in the study and control groups, as was gestational age at the time of entry into the study. Amniocentesis was performed under real-time ultrasound guidance with a 20-gauge needle by experienced operators. Follow-up evaluation was available for all but three women. The spontaneous abortion rate after 16 weeks was 1.7% in amniocentesis patients compared with 0.7% in controls ( $P < 0.01$ ), with a 2.6-fold relative risk of spontaneously aborting if the placenta was traversed. Transplacental needle passage is a risk factor that has more recently been shown not to increase the risk for postprocedure loss (see previous). The frequency of postural malformations in the infants in the two groups did not differ. However, respiratory distress syndrome was diagnosed more often (relative risk 2.1) in the study group and more infants were treated for

pneumonia (relative risk 2.5). An assessment of the safety of second-trimester amniocentesis can also be found in the CEMAT study that compared conventional second-trimester amniocentesis to early amniocentesis (37). In this multicenter trial performed by experienced operators using concurrent ultrasonography, the total loss rate in the second-trimester amniocentesis cohort was 5.9%.

More recent studies evaluating the safety of amniocentesis continue to confirm the safety of the second-trimester procedure. In a study from British Columbia, Baird and colleagues (38) considered the question of whether children delivered of women who had mid-trimester amniocentesis can be identified by a population-based database of congenital anomalies and disabilities at a different rate from that of matched controls (i.e. offspring of women who had not undergone amniocentesis). The authors studied 1296 cases (651 males and 645 females) and 3704 matched controls (1867 males and 1837 females) among live births (1972–1983) from the Health Surveillance Registry with data collected to 1990 to allow a follow-up of 7–18 years. Cases were children of mothers who had mid-trimester amniocentesis for advanced maternal age (35 years or older) and whose results were normal for chromosomal disorders and neural tube defects. When possible, three controls per case were matched for age of mother, sex, date of birth, and health from provincial birth records. One hundred and twenty-eight (9.9%) of the cases and 308 (8.3%) of the controls were registered (relative risk 1.23); this relative risk was not significantly different from one. The likelihood of having disabilities was examined for cases as compared with controls, and no difference was found except for an increased ABO isoimmunization associated with amniocentesis. Overall, this study provides reassuring data for patients considering mid-trimester amniocentesis with respect to long-term outcome. Tongsong and colleagues (39) reported a large-scale cohort study among 2256 women carrying singleton pregnancies undergoing amniocentesis between 15 and 24 weeks gestation and a control group matched on a one-to-one basis for maternal age, parity, and socioeconomic status. The authors found no significant differences in fetal loss rates, premature deliveries, or placental abruptions between the two groups; however, the study was not powered to identify differences less than 1%.

Using the database from the National Institute of Child Health and Human Development-sponsored multicenter First and Second-Trimester Evaluation of Risk (FASTER) trial designed to compare first-trimester Down syndrome screening with nuchal translucency, pregnancy-associated plasma protein A (PAPP-A), and free  $\beta$ -human chorionic gonadotropin ( $\beta$ -hCG) to second-trimester screening with AFP, hCG, unconjugated estriol and inhibin A, Eddleman and colleagues analyzed the procedure-related fetal loss rate after mid-trimester amniocentesis (40). Among those enrolled in the FASTER trial, 3096 women underwent mid-trimester amniocentesis (study group) and 31,907 did not (control group). The spontaneous

fetal loss rates <24 weeks gestation were 1.0% in the amniocentesis group and 0.94% in the control group. Multiple logistic regression analysis was used to adjust for potential confounders. The difference between these two groups was not significant ( $P=0.74$ , 95% CI—0.26–0.49%). Thus, these investigations concluded that there was no significant difference in loss rates between those undergoing amniocentesis and those not undergoing this procedure. However, the safety of amniocentesis was not a primary endpoint of the FASTER trial, and the authors' interpretation of their data was controversial (41–45).

Two literature reviews corroborate the relatively low frequency of pregnancy loss after second-trimester amniocentesis. A Cochrane review assessed the increased risk for fetal loss after second-trimester amniocentesis to be <1% (3). Seeds (46) reviewed studies of more than 1000 amniocenteses, each found in the world literature from over three decades. On the basis of data from more than 68,000 procedures, he concluded the following:

- (1) Amniocentesis with concurrent ultrasound guidance in controlled studies appears to be associated with a procedure-related (excess pregnancy) loss of 0.6% (95% CI, 0.31, 0.90).
- (2) The use of concurrent ultrasound guidance appears to reduce the number of punctures and the incidence of bloody fluid. Concurrent ultrasound guidance was associated with a reduced rate of loss when all studies were compared but not among controlled studies.
- (3) Direct fetal needle trauma is rare, and rarely proved, but may occur more frequently than reported because of failure to diagnose and failure of consistent adverse sequelae.
- (4) An increased rate of pregnancy loss in cases of placental puncture is not demonstrated.

Large-scale randomized trials with skilled operators using modern ultrasonographic equipment are needed to better delineate current risks for mid-trimester amniocentesis as the often quoted increased risk of pregnancy loss of 0.5% after amniocentesis may be high.

In conclusion, we believe it is wise to continue to counsel that the risk of pregnancy loss secondary to amniocentesis is 0.5% over baseline, or perhaps slightly less at centers with experienced operators. At our center, we counsel patients that the risks of serious maternal complications and fetal injuries are “remote,” but are not zero (47,48).

### 26.2.2 Early Amniocentesis: 14 Weeks Gestation or Less

With the advent of high-resolution ultrasound equipment, some physicians began offering amniocentesis before 15 weeks gestation. Some programs not offering CVS viewed early amniocentesis as an attractive alternative for those women who desired prenatal diagnosis before the time in pregnancy when traditional amniocentesis is performed

(i.e. 15 weeks gestation or greater). In other medical centers, early amniocentesis was explored to obviate the inconvenience of patients having to be rescheduled if they came in for CVS and were determined to be beyond 12 weeks gestation but under 15 weeks gestation.

A number of programs, including our own center, had reported experiences suggesting early amniocentesis to be a promising technique. In a series of 936 amniocenteses at 12.8 weeks gestation or less reported by Hanson and colleagues (49), loss rates were 0.7% (7 of 936) within 2 weeks of amniocentesis, with an additional 2.2% before 28 weeks and an additional 0.5% stillbirths or neonatal deaths. Total losses (32 of 936, or 3.4%) were considered comparable with the 2.1–3.2% in ultrasonographically normal pregnancies not undergoing a procedure; however, lack of corrections for maternal age and gestational age render comparisons less than exact. Other series reported include those conducted by Benacerraf and colleagues (50), Elejalde and colleagues (51), Penso and colleagues (52), Strippario and colleagues (53), Hackett and colleagues (54), Assel and colleagues (55), Djalali and colleagues (56), Henry and Miller (57), Yang and colleagues (58), Eiben and colleagues (59), and Kerber and Held (60). Our group, then at the University of Tennessee, Memphis (61), compared our initial experience with 250 early amniocenteses (14 weeks or less) to that of our first 250 cases of transabdominal CVS (9.5–12.9 weeks), finding loss rates for early amniocentesis and transabdominal CVS to be 3.8% and 2.1%, respectively. Our group also reported early amniocentesis in six twin gestations (mean 11.9 weeks; range 10.5–13.6 weeks), using a similar dye injection technique as described for traditional amniocentesis (see previous) (62). We successfully tapped both amniotic sacs in each of six cases (five requiring two needle insertions, one requiring three needle insertions); all cultures yielded normal cytogenetic results, and all six pregnancies resulted in the delivery of healthy infants.

However, these studies mostly represented observational reports of amniocentesis with most of the procedures being performed at or after 13 weeks gestation. Comparative studies evaluating the safety and efficacy of early amniocentesis have failed to corroborate the generally favorable outcomes reported in the observational studies. Nicolaides and colleagues (63) reported a comparison of amniocentesis and CVS at 10–13 weeks gestation. Early amniocentesis was performed in 731 patients (493 by choice and 238 by randomization) and CVS in 570 (320 by choice and 250 by randomization). Both procedures were performed by transabdominal ultrasound-guided insertion of a 20-gauge needle. The spontaneous loss (intrauterine or neonatal death) was significantly higher after early amniocentesis (total group mean, 5.3%; CI, 3.8–7.2; randomized subgroup mean, 5.9%; CI, 3.3–9.7) than after CVS (total group mean, 2.3%; CI 1.2–3.9; randomized subgroup mean, 1.2%,

CI 0.3–3.5). Subsequently, Nicolaides and colleagues (64) reported further findings of this study. Again, post-procedure loss rates were significantly higher in the total early amniocentesis group (4.9%) and the randomized early amniocentesis subgroup (5.8%) than in the total transabdominal CVS group (2.1%) and the randomized transabdominal CVS subgroup (1.8%). The frequency of talipes equinovarus was also higher in the early amniocentesis group, but this difference did not attain statistical significance.

Sundberg and colleagues (65) performed a similar type of randomized trial comparing early amniocentesis and CVS. In this study, the rate of talipes equinovarus was significantly increased in the early amniocentesis group, despite the fact that the investigators used a filter system to reduce the amount of fluid removed (66). This finding led the authors to prematurely terminate their study for safety concerns.

The results of the Canadian Early Amniocentesis versus Mid-Trimester Amniocentesis Trial (37,67)—a multicenter, randomized trial of early amniocentesis and conventional second-trimester amniocentesis—have been most revealing. This multicenter trial randomized 4374 women into an early amniocentesis cohort ( $n=2183$ ) and a conventional mid-trimester amniocentesis cohort ( $n=2185$ ). In the early amniocentesis cohort, 1916 women (87.8%) underwent amniocentesis before 13 weeks gestation.

Loss rates were 7.6% for the early amniocentesis cohort and 5.9% for the mid-trimester cohort ( $P=0.012$ ). Talipes equinovarus occurred in 1.3% of infants delivered of women in the early amniocentesis group compared with 0.1% in the mid-trimester cohort ( $P=0.0001$ ). In addition, postprocedure amniotic fluid leakage occurred more frequently in the early amniocentesis group (3.5%) than in the mid-trimester group (1.7%;  $P=0.0007$ ). Failed procedure, multiple needle insertions, and culture failure also occurred more frequently in the early amniocentesis group (37,67).

A recent publication of the US National Institutes of Health-sponsored multinational multicenter trial showed somewhat different findings from that of the Canadian trial (68). This prospective randomized trial of amniocentesis and CVS performed between 11 and 14 weeks gestation found a significant fourfold increase in talipes equinovarus among women undergoing amniocentesis compared with women undergoing transabdominal CVS but demonstrated a nonsignificant increase in fetal loss in the amniocentesis cohort. Although there appears to be a modest association between talipes equinovarus and amniocentesis at  $\geq 15$  weeks' gestation, this mainly seems to be confined to children with non-isolated talipes equinovarus. Overall, second-trimester amniocentesis is unlikely to contribute to the development of talipes equinovarus (69).

In summary, the preponderance of data from rigorously performed studies support the concept that

amniocentesis before 14 weeks carries risks that are increased in comparison with first-trimester CVS or second-trimester amniocentesis. Accordingly, most have abandoned the use of early amniocentesis for conventional prenatal diagnosis.

## 26.3 CHORIONIC VILLUS SAMPLING

Because amniocentesis is most commonly performed in the mid-second trimester (15–16 weeks), fetal diagnosis cannot usually be established before 17–18 weeks gestation. A technique that could be performed during the first trimester would be highly desirable to reduce the psychological stress of awaiting results until mid-pregnancy and to allow a safer method of pregnancy termination, should an abnormality be detected. CVS is such a technique.

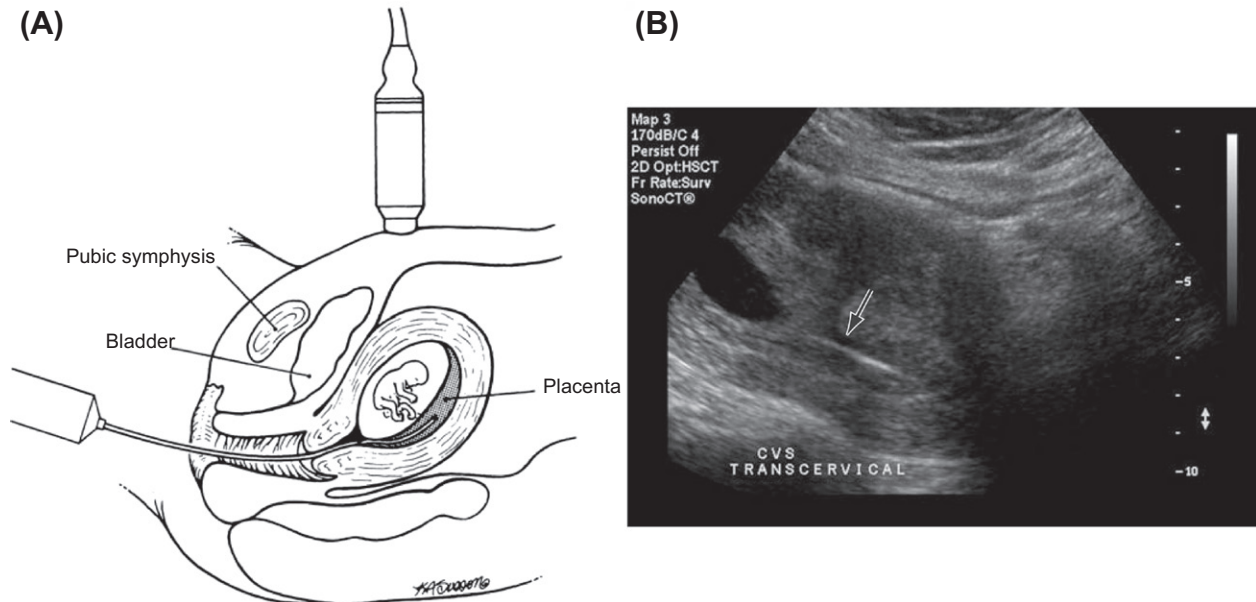
After fertilization, the zygote differentiates first into the blastocyst, which contains an inner cell mass that develops into the fetus and an outer trophoblastic layer that develops into non-fetal structures such as amnion, chorion, and placenta. The genetic complement of the outer cell mass nearly always reflects the genetic constitution of the inner cell mass (i.e. the fetus) because both are derived from the same zygote. It follows that cytogenetic, DNA, or biochemical analysis on trophoblastic cells should provide information comparable with that obtained from cultured amniotic fluid cells. The one major exception is that assays requiring amniotic fluid, specifically AFP, require amniocentesis.

### 26.3.1 Techniques for CVS

**26.3.1.1 Transcervical CVS.** Transcervical CVS is now usually performed at 10–12 completed gestational weeks. Absolute contraindications to transcervical CVS include active cervical or vaginal pathology (e.g. herpes, chlamydia, or gonorrhea infection) or maternal blood group sensitization. Relative contraindications include leiomyoma obstructing the cervical canal, bleeding from the vagina within 2 weeks of planned CVS, and a markedly retroverted, retroflexed uterus (70). Before CVS, fetal viability and normal fetal growth must be confirmed by ultrasound. The procedure is performed with a device that consists of a plastic cannula enclosing a metal obturator extending just beyond the catheter tip; the diameter of most catheters is approximately 1.5 mm.

At our center, CVS is performed in an ultrasound suite. The patient is positioned in the dorsal lithotomy position. The vagina is cleaned with an iodine preparation and the perineum draped with sterile towels. After insertion of a sterile vaginal speculum, placement of a tenaculum on the anterior lip of the cervix may occasionally be needed to help correct uterine anteversion or retroflexion. The CVS catheter is introduced transcervically under simultaneous ultrasonographic visualization, with optimal catheter placement being parallel to the long axis of the placenta (Figure 26-3A,B). The obturator is





**FIGURE 26-3** (A) Transcervical CVS. (From Elias, S.; Simpson, J. L. *Techniques and Safety of Genetic Amniocentesis and Chorionic Villus Sampling*. In *Diagnostic Ultrasound Applied to Obstetrics and Gynecology*, 3rd ed.; Sabbagha, R. E., Eds; JB Lippincott: Philadelphia, 1994, with permission.) (B) Ultrasonographic visualization of transcervical CVS. Catheter is coursing through the cervix (arrow), with catheter tip in the substance of the placenta. (Courtesy of Leeber Cohen, MD.)

then withdrawn and the catheter Luer-lock attached to a 20- or 30-mL syringe. Chorionic villi are then aspirated by multiple, rapid aspirations of the syringe plunger to 20- to 30-mL negative pressure. The catheter is withdrawn under continuous maximum negative pressure. An adequate sample is at least 5mg of villi, but a sample of 10–25 mg is preferred.

After the procedure, fetal heart activity is verified by ultrasonography. Patients are monitored for any untoward effects for approximately 30 min. Unsensitized Rh-negative patients are given RhIg. Maternal serum AFP screening for fetal neural tube defects is recommended at 16–18 weeks gestation.

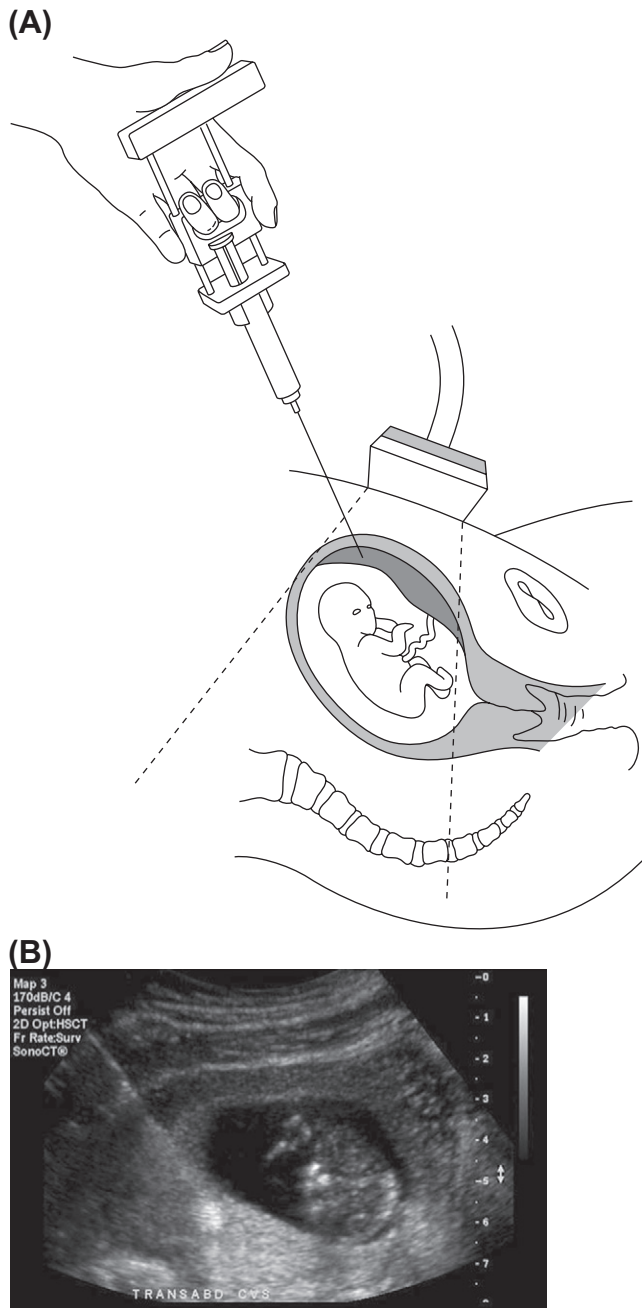
**26.3.1.2 Transabdominal CVS.** Transabdominal CVS can be used to evaluate pregnancies at the same gestational age as transcervical CVS at 10–12 weeks; however, this procedure also can be performed later in pregnancy (71–73), particularly when fetal abnormalities are visualized at ultrasound and a rapid diagnosis may influence pregnancy management. Placentas, especially amenable to the transabdominal approach, include those located in the fundus or those located anteriorly in an anteфлекed uterus. Transabdominal CVS is also an option in certain circumstances when transcervical sampling is contraindicated (e.g. active genital herpes or cervical lesions).

After selection of a needle-insertion site based on ultrasound findings, the overlying skin is infiltrated with a local anesthetic, cleaned with an iodine preparation, and draped with sterile towels. As performed at our centers, a 19-gauge spinal needle is inserted percutaneously through the maternal abdominal wall and myometrium under continuous ultrasound guidance.

The tip is then guided into the long axis of the placenta (Figure 26-4A,B). The needle stylet is withdrawn, and next a syringe housed in an aspiration device (Cameco syringe pistol, Precision Dynamics, San Fernando, CA) is connected to the Luer-lock of the needle. Chorionic villi are obtained by repeated (15–20) rapid aspirations of the syringe plunger to 20 mL of negative pressure. The needle is then withdrawn under continuous negative pressure. The same postoperative protocol is used as for transcervical CVS.

Most physicians performing transabdominal CVS employ the aforementioned “free-hand” technique. Alternatively, some operators still use a guide-needle or double-needle system device, which punctures the uterine wall once but permits multiple attempts at villus aspiration (74).

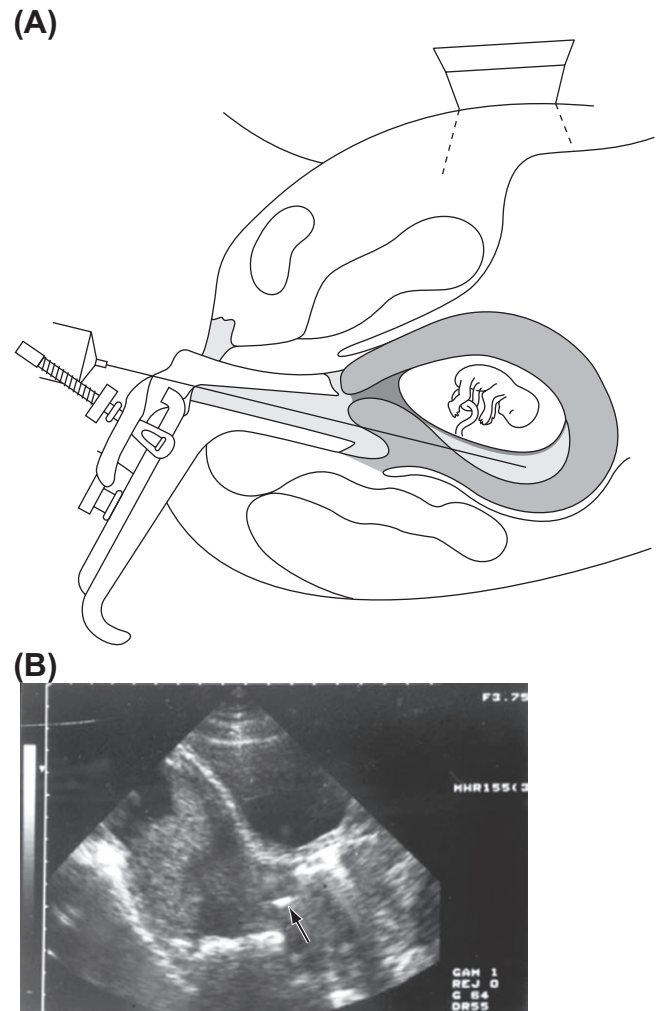
**26.3.1.3 Transvaginal CVS.** Although not commonly necessary, transvaginal CVS may be the only option in women having a retroverted, retroflexed uterus with a posterior placenta. Patients are prepared in a manner similar to transcervical CVS. We use a 35-cm 18-gauge aspiration needle to obtain chorionic villi. The wall of the vagina posterior to the cervix is infiltrated with a local anesthetic. Transabdominal ultrasound is used for needle guidance through the vaginal mucosa and myometrium into the placenta. Once within the placenta, the needle stylet is removed, and villi are aspirated in a similar manner as with transabdominal CVS (Figure 26-5A,B; (75)). Although anecdotal assessment of the safety of transvaginal CVS does not suggest an increased risk of fetal loss or other adverse outcomes, a rigorous assessment of the safety and efficacy of this procedure has not been performed.



**FIGURE 26-4** (A) Transabdominal CVS. (From Elias, S.; Simpson, J. L. *Techniques and Safety of Genetic Amniocentesis and Chorionic Villus Sampling*. In *Diagnostic Ultrasound Applied to Obstetrics and Gynecology*, 3rd ed.; Sabbagha, R. E., Eds.; JB Lippincott: Philadelphia, 1994, with permission.) (B) Ultrasonographic visualization of transabdominal CVS. Thin arrow points to the needle shaft within the substance of the placenta, and the thick arrow points to the needle tip within the placenta. (Courtesy of Leeber Cohen, MD.)

### 26.3.2 Safety of CVS

**26.3.2.1 Pregnancy Loss after CVS.** The U.S. Cooperative Clinical Comparison of CVS and Amniocentesis study and the Canadian Collaborative



**FIGURE 26-5** (A) Transvaginal CVS. (B) Ultrasonographic visualization of transvaginal CVS. Arrow points to needle tip about to enter uterine wall. (Figure 26-5A,B originally published in "Shulman, L.P. et al. *Prenat. Diagn.* 1992, 12, 229–234".)

CVS–Amniocentesis Trial Group study initially reported the pregnancy loss rate of CVS to be 0.8% and 0.6% higher than traditional amniocentesis, respectively (76,77). Neither figure was statistically significant. In the US study, 2278 women self-selected transcervical CVS; 671 women similarly recruited in the first-trimester-selected amniocentesis. Randomization did not prove possible. The Canadian study was randomized, with 1391 subjects assigned to transcervical CVS and 1396 to amniocentesis. Variables shown to influence fetal loss rates adversely included fundal location of the placenta, number of catheter passages, small sample size, and prior bleeding during the current pregnancy (77,78). Almost all the above invariably reflect technical difficulty. Other obstetric complications (e.g. intrauterine growth restriction (IUGR), placental abruption, premature delivery) did not exceed those in women not undergoing CVS.

Transcervical CVS and transabdominal CVS appear to be equally safe procedures for first-trimester diagnosis when the approach is chosen based on placental location and ease of procedure. In a later US NICHD collaborative study, in which 1844 patients were randomized to transcervical CVS and 1929 patients to transabdominal CVS, the loss rates of cytogenetically normal pregnancies through 28 weeks were 2.5% and 2.3%, respectively (79). Of note, the overall loss rate (i.e. background plus procedure related) after CVS decreased by about 0.8% during this 1988–1990 randomization trial, in comparison with rates observed during the transcervical versus amniocentesis self-selection study (1985–1987). This decrease in procedure-related loss rate probably reflects increasing operator experience, as well as availability of both transcervical and transabdominal approaches. In a small Italian randomized trial, Brambati and colleagues (80) also found no difference between transabdominal and transcervical CVS. By contrast, in a randomized comparison of amniocentesis, transabdominal CVS, and transcervical CVS in Denmark, Smidt-Jensen and colleagues (81) found similar fetal loss rates after transabdominal CVS and amniocentesis but a significantly increased loss rate associated with transcervical CVS. These results are not surprising, however, as Danish experience with transabdominal CVS was far greater than with transcervical CVS.

One major study that substantively differs from the United States, Canadian, and Italian collaborative trials is the Medical Research Council Study (82). In this multicenter randomized comparison between first-trimester CVS performed by any approach deemed suitable by the obstetrician and second-trimester amniocentesis, the ultimate variable measured was completed pregnancies. The 4.4% fewer completed pregnancies in the CVS cohort reflected both unintended and intended pregnancy terminations. The latter, in turn, probably reflected inexperience in cytogenetic interpretation, given some terminations seemingly arguable in retrospect (i.e. confined placental mosaicism). Experience with CVS by the MRC study operators was also considerably less than in the US operators. For example, the only requirement for participation in the MRC study was 30 “practice” CVS procedures. Some centers also contributed very few cases.

No formal attempts to assess the safety of transvaginal CVS have as yet been attempted. In our own experience (75), as well as that of Sidransky and colleagues (83), neither major complications nor obvious excessive fetal loss rates have been observed.

There have now been a number of assessments of the safety of CVS come from randomized trials evaluating the safety of CVS. Sundberg and colleagues (65) found a total risk of loss among 579 women randomized to transabdominal CVS to be 4.8%. In a more recent study sponsored by the US NICHD, 2.1% of cytogenetically normal pregnancies exposed to transabdominal CVS at

11–14 weeks inclusive resulted in fetal loss or preterm delivery before 28 weeks gestation (84). Such information confirms earlier studies of the risks of transabdominal CVS.

More recently, Caughey and Colleagues (85) reported a retrospective cohort study of all amniocentesis and CVS procedures resulting in a normal karyotype from 1983 to 2003 at the University of California, San Francisco. In a comparison of 9886 CVS and 30,893 amniocentesis procedures performed during the study period, the overall loss rates were 3.12% for CVS and 0.83% for amniocentesis ( $P < 0.001$ ). However, in the most recent time period, 1998–2003, there was no difference between the two procedures (adjusted odds ratio 1.03, 95% CI 0.23–4.52). The authors concluded that “...there is no longer a statistically significant difference between the two (procedures).” In reviewing the extant data, the American College of Obstetricians and Gynecologists has similarly concluded that “In experienced individuals and centers, CVS and amniocentesis procedure-related loss rates may be the same as those for amniocentesis.” (86).

We conclude that clinical judgment and patient individualization in choosing the optimal approach for CVS increases safety. For example, some technically difficult transcervical CVS procedures (e.g. fundal placentas) should be avoided in favor of a technically more facile transabdominal approach. The converse is also true.

**26.3.2.2 CVS in Multifetal Pregnancy.** CVS in multifetal pregnancies has become more widely used with increasing use of fetal reduction resulting from assisted reproductive technologies (ART). Many women requiring ART to become pregnant are also at increased risk for fetal chromosome or Mendelian abnormalities, primarily because of advanced maternal age. CVS before fetal reduction allows for the detection of chromosome or DNA abnormalities, thus allowing for the reduction of only abnormal gestations (87–90).

Few data exist for the safety of CVS in multiple gestations. In a major US study involving four centers, the total loss rate of chromosomally normal fetuses (spontaneous abortions, stillborns, neonatal deaths) was 5.0% (91), only slightly higher than the 4.0% observed for singleton pregnancies (77). Brambati and colleagues (88) reported no differences in fetal and perinatal loss rates among 198 sets of twins undergoing CVS compared with a matched cohort of twin pregnancies not undergoing invasive prenatal diagnosis. Brambati and colleagues (92) reported their ongoing experience with CVS before multifetal reduction. Prenatal diagnostic and clinical outcomes of 424 multiple pregnancies reduced to twins ( $n = 255$ ) or singletons ( $n = 169$ ) were compared with 147 twin- and 885 singleton-matched controls, in which reduction procedures were not performed. Transabdominal CVS was successfully performed in 100% of cases with an accuracy of 99.2%. The overall loss rate after reduction was 3.3%; no significant differences in overall pregnancy loss, low birth weight, severe prematurity, or neonatal death



were observed in the two study groups. However, mean gestational age at delivery and mean birth weight were significantly lower in the reduction cohort. The authors concluded that the safety and efficacy of transabdominal CVS before multifetal reduction warrants its consideration for women who desire prenatal diagnosis in such situations. Most recently, Simonazzi and colleagues (93) reported a retrospective cohort study of 204 twin pregnancies who underwent amniocentesis ( $n=100$ ) or CVS ( $n=104$ ). The fetal loss rate <24 weeks was 3.85% in the CVS group and 4.00% in the amniocentesis group ( $P=0.95$ ).

We perform CVS in multiple gestations when the placentas can be reliably sampled by any technique as determined by ultrasonography. When placental locations preclude the reasonable obtainment of separate and distinct samples, second-trimester amniocentesis is preferred.

**26.3.2.3 Limb-Reduction Deformities.** Evaluation of the safety of CVS has recently shifted focus from concerns about the risk of fetal loss to its being the possible cause for congenital abnormalities. In 1991, Firth and colleagues (94,95) reported that 5 of 289 (1.7%, or 17 in 1000) infants exposed to CVS at 56–66 days of gestation (i.e. 42–50 days after fertilization) had severe limb-reduction deformities (LRDs). Four of the five infants had oromandibular-limb hypogenesis (all transabdominal CVS); the fifth had a terminal transverse limb reduction alone (transcervical CVS). Subsequently, a number of reports followed, both supporting and refuting such an association (96–101). In the United States, Burton and colleagues (102) reported a second cluster among 394 infants, whose mothers had undergone CVS. Thirteen (3.3%) had major congenital abnormalities, including four with transverse LRD (10 of 1000, or 1%). All four LRDs were transverse distal defects involving hypoplasia or absence of the fingers and toes. Three of these cases followed transcervical sampling, using a device that, in the hands of the reporting physicians, was associated with an 11% fetal loss rate.

Teratogenic mechanisms whereby CVS might cause LRDs have been hypothesized (103). These include (1) hypoperfusion due to fetomaternal hemorrhage or pressor substances released by disturbance of villi or the chorion; (2) embolization of chorionic villus material or maternal clots into the fetal circulation; and (3) amniotic puncture and limb entrapment in exocoelomic gel.

The potential association of LRDs with CVS has been explored through various registries. On the basis of data from the Italian Multicenter Birth Defects Registry, Mastroiacovo and colleagues (104) reported that eight cases of oromandibular–limb hypogenesis complex were entered into the registry from January 1988 through December 1991, four of which had been exposed to CVS, compared with 36 exposed subjects among 8445 controls. There were 166 cases of transverse limb defects alone, four having been exposed to CVS, compared

with 36 cases among 8445 controls. A 1994 update of this study, based on 11 CVS-exposed cases, continued to indicate an association with transverse limb defects (105). The highest risk was associated with procedures performed at <70 days gestation (OR 23.2; 95% CI 1.31–41.0); a lower, but still increased risk with procedures at 70–76 days (OR 17.1; 95% CI 6.7–44.0); over 44 days, there were no exposed cases and the risk interpreted as considerably lower. By contrast, analysis of other European registries in aggregate involving more than 600,000 births showed that only 4 of 336 cases (1.2%) with limb-reduction abnormalities had been exposed to CVS, compared with 78 of 11,883 (0.66%) cases with other malformations (OR 1.8, 95% CI 0.7–5.0) (106).

Firth and colleagues (107) summarized LRDs in 75 infants exposed to CVS by combining their own cases with those reported in the literature. The median gestational age at CVS ranged from 56 (range, 49–65) postmenstrual days for the most severe defects to 72 (range, 51–98) for the least severe. They concluded that there was a correlation between the severity of the defects and the duration of gestation when CVS was performed.

In 1994, the US Centers for Disease Control and Prevention (CDC) held an open forum in Atlanta where data were presented from a US multistate case-control study in an effort to quantify the risk of LDR associated with CVS (108). Case subjects were 131 infants with non-syndromic limb deficiency from seven population-based birth defects surveillance programs born to women 34 years or older from 1988 through 1992. Controls were 131 infants with other birth defects matched to case subjects by the infant's year of birth, mother's age, race, and state of residence. Overall, the odds ratio for limb deficiency after CVS from 8 to 12 weeks gestation was 1.7 (95% CI 0.4–6.3). However, when analyzed for specific anatomic subtypes, there was an association for transverse digital deficiency (OR 6.4; 95% CI 1.1–38.6). They concluded that the absolute risk for such defects was approximately 1 in 3000. It should be noted that such transverse digital deficiencies occurred in only seven exposed cases.

Finally, the World Health Organization Committee on CVS analyzed data collected through an international voluntary registry (109,110). A total of 77 cases with LRDs among 138,996 infants born after CVS was reported from 63 registering centers. Pattern analysis of the types of limb defects and overall frequencies of specific LRDs were compared with a background population study from British Columbia (111). The pattern of defects showed upper limb to be affected in 65%, 13% in the lower limb, and 23% in both upper and lower limbs compared with frequencies in the general population of 68%, 23%, and 9%, respectively. Transverse limb defects occurred in 41% of infants in the cohort exposed to CVS, compared with 43% in the general population, and longitudinal limb deficiencies were found in 59% of cases, compared with 57% in the



general population. It was concluded that the pattern analysis of the types of limb defects and calculation of overall incidences failed to find a difference between the CVS and background populations.

A review of CVS safety sponsored by the World Health Organization (112) evaluated the clinical outcomes of 216,381 CVS cases performed worldwide. In this review, a total of 115 LRDs were observed with an incidence of 1/1881, similar to the rate of 1/1642 observed in the general population. The distribution of LRD (upper limbs 71.3%, lower limbs 11.3%, both limbs 17.4%) was also similar to that observed in the general population. The authors concluded the data demonstrate that "...CVS carries no increased risk for fetal loss or congenital malformation, including limb reduction defect..." compared with conventional mid-trimester amniocentesis.

A recent controversy has arisen as to a possible association between CVS and preeclampsia (113). Our group at Northwestern University, Chicago, IL, reported 653 women who underwent prenatal diagnosis (501 by amniocentesis and 152 by CVS) who were matched by age with 653 women who did not undergo invasive prenatal diagnosis. In multivariate analysis, the factors that remained significantly associated with preeclampsia were (1) maternal age <25 years old (OR 7.4, 95% CI 23.3–23.6); nulliparity (OR 2.7, 95% CI 1.1–6.9); and (3) having had a CVS as a nulliparous woman (OR 4.2, 95% CI 1.4–12.6) (114). Others have failed to show an association between gestational hypertension and preeclampsia with DVS (115,116).

In summary, CVS is the only established method for first-trimester prenatal diagnosis and carries minimal, if any, increased risk for adverse pregnancy outcomes when performed by experienced operators. To maximize the safety of CVS, we avoid performing CVS before 10 weeks gestation except in cases of profound risk for fetal abnormalities (e.g. fetal hydrops). We continue to counsel patients about the LRD controversy and inform them that the absolute risk at 10–12 weeks is believed to be very low, approximately 1 in 2000, a rate similar to that observed in the general population (112). We also stress that this issue be placed in proper perspective and weighed against the substantial advantages that CVS offers first-trimester prenatal diagnosis.

## 26.4 FETAL BLOOD SAMPLING

Access to the fetal circulation was initially accomplished by fetoscopy, a method of directly visualizing the fetus, umbilical cord, and chorionic surface of the placenta, using endoscopic instruments (25,117). Fetoscopy for this purpose has now been replaced by ultrasound-directed percutaneous umbilical blood sampling (PUBS), also termed *cordocentesis* or *funipuncture*.

Fetal blood chromosome analysis has been used to help clarify purported chromosome mosaicism detected

in cultured amniotic fluid cells (118) or chorionic villi. Rapid assessment of fetal chromosome complement has been accomplished by "direct" cytogenetic analysis of uncultured nucleated blood cells (119). Short-term fetal lymphocyte cultures can provide a cytogenetic result within 72 h; direct analysis of spontaneously dividing fetal cells (probably nucleated red blood cells) can provide a karyotype result within 24 h. This proves particularly useful for patients presenting late in the second trimester, when results from amniocentesis would be available only after pregnancy termination would no longer be available. In addition, in cases of fetal structural abnormalities or intrauterine growth retardation (IUGR) presenting in the third trimester, rapid results may prove useful for decision making concerning the mode of delivery (120,121). More recently, fluorescent in situ hybridization (FISH) with chromosome-specific DNA probes has also been used for rapid prenatal diagnosis of aneuploidy using nucleated fetal blood cells from umbilical cord blood, as well as chorionic villus and amniotic fluid cells (122).

Fetal blood sampling is used in the prenatal evaluation of many fetal hematological abnormalities (123). Daffos (124) reported normal hematological values for second-trimester fetuses, and Forestier and colleagues (125,126) reported normal blood chemistry values for second-trimester fetuses. Fetal blood hematocrit can be directly measured to assess hemolysis resulting from Rh or other blood antigen isoimmunization states (127). Before this, obstetricians had to rely on indirect evidence of fetal hemolysis, such as maternal antibody titers, past obstetric history, ultrasound findings, and spectrophotometry of bilirubin in amniotic fluid; the need for subsequent transfusions was based on somewhat arbitrary guidelines. Now the decisions about who, when, how much, and how often to transfuse can be made more rationally on the basis of actual fetal blood analyses such as hemoglobin level, hematocrit level, blood group, direct antibody titer, and reticulocyte count. Fetal hemoglobin can be directly evaluated to diagnose sickle cell disease,  $\alpha$ - or  $\beta$ -thalassemia, or other hemoglobinopathies (117) although these disorders are now usually diagnosed by DNA analysis of chorionic villi or amniotic fluid cells. Fetal blood sampling can also be used to directly assess both the quantity of platelets and the quality of function (128). With regard to maternal PLA2 (platelet antigen) alloimmunization, PUBS is useful not only for diagnosis but also for access to the fetal circulation allowing therapeutic alternatives including in utero platelet transfusion or maternal immunotherapy with gamma globulin or steroids (129).

Fetal blood has been used for the diagnosis of blood factor abnormalities such as hemophilia A, hemophilia B, or von Willebrand disease (126,130). In addition to hematologic studies, fetal blood samples can be used to diagnose autosomal-recessive or X-linked immunologic deficiencies, including severe combined immunodeficiency,

Chédiak–Higashi syndrome, Wiskott–Aldrich syndrome, and chronic granulomatous disease (131–133).

Recovery of fetal blood permits assessment of viral, bacterial, or parasitic infections of the fetus. Detection of fetal viral or parasitic infection is usually made on the basis of maternal antibody titers or ultrasound-detected fetal structural abnormalities. Serum studies of fetal blood allows for quantification of antibody titers (134,135). In addition to antibody studies, PUBS can be used for direct analysis of viral, bacterial, and parasitic infections by culture of fetal blood (134,136,137).

Although many of the indications for detecting fetal abnormalities that previously required fetal blood sampling are now performed by amniocentesis or CVS using DNA analysis (122,138,139), the continuing value of fetal blood sampling lies not only with those few remaining diagnostic indications but also in providing the potential for drug therapy. For example, fetal arrhythmias have been treated with direct administration of antiarrhythmic medications, and fetal paralysis may be induced to facilitate invasive procedures such as fetal transfusions or for magnetic resonance imaging (MRI) (140).

### 26.4.1 Technique

Today, the technique most commonly used for fetal blood sampling is ultrasound-directed PUBS. The procedure can be safely undertaken from 18 weeks onward although successful procedures have been reported as early as 12 weeks (141,142).

PUBS can be performed as an outpatient procedure. Maternal sedation is usually unnecessary, but oral benzodiazepine 1 or 2 h before the procedure may be of benefit to the anxious patient and usually results in a transient decrease in fetal movement that can facilitate the procedure. A preliminary ultrasonographic examination of the fetus is performed to assess fetal viability, placental and umbilical cord location, fetal or placental anomalies, and fetal position. A suitable site for needle insertion is then selected; the skin over this site is anesthetized with 5 mL of 1% xylocaine. A sterile field is established; the skin is cleansed with an iodine-based solution and sterile drapes applied. The ultrasound transducer is placed on the abdomen away from the sterile insertion site but at a location that permits visualization of the complete path of the needle from skin to fetal blood vessel.

There are several potential sampling sites. Owing to its fixed position, the placental cord root is usually the site of choice whenever it is clearly visible. Alternatively, free loops of cord or the intrahepatic vein are possibilities (123,143,144). After percutaneous insertion of the spinal needle into the fetal blood vessel under direct ultrasound guidance, a small amount of blood is aspirated. The presence of fetal blood in this initial sample is confirmed using a model ZBI Coulter counter and channelizer to differentiate fetal from maternal blood on the basis of erythrocyte volume. The amount of blood

aspirated for diagnosis depends on the indication for PUBS; rarely more than 5 mL is required.

Upon completion of the fetal blood sampling, the spinal needle is withdrawn, and an ultrasound examination is performed to evaluate fetal status. The woman and her fetus are monitored for about 1 h after PUBS. At our centers, all women at risk of Rh-isoimmunization receive 300 µg of Rh immunoglobulin after the procedure.

### 26.4.2 Safety

Fetal blood sampling appears to be a relatively safe procedure when performed by experienced physicians, albeit carrying more risk than CVS or amniocentesis. Maternal complications are rare but include amnionitis and transplacental hemorrhage (130,145). Data from large perinatal centers estimate the risk of in utero death or spontaneous abortion to be 3% or less after PUBS (124,135,136,146–152). Collaborative data from 14 North American centers, sampling 1600 patients at varying gestational ages and for a variety of indications, revealed an uncorrected fetal loss rate of 1.6% (153).

A more recent assessment of fetal blood sampling loss risk was performed by Antsaklis and colleagues (154), who reviewed their clinical outcomes based on indication for fetal blood sampling. They divided their cohort into five main indication subgroups and found highly significant differences in procedure-related loss rates between the five groups. The highest loss rates were observed in the two groups characterized by fetal abnormalities and growth restriction, thus demonstrating that indication for fetal blood sampling has a major impact on risk for the procedure. In addition to the indication for fetal blood sampling, the technique used to obtain fetal blood (e.g. arterial puncture) (150), and prolonged postprocedure fetal bradycardia (155) may presage an increased risk for adverse clinical outcomes. However, performing cordocentesis in a pregnancy characterized by a single umbilical artery does not appear to increase the risk for procedure-related loss (156,157).

Unfortunately, no studies directly comparing loss rates in control and treated groups have yet been published. Nonetheless, the genetic delineation of an increasing number of Mendelian disorders has and will continue to decrease the need for fetal blood sampling to evaluate pregnancies at increased risk for a wide variety of congenital and acquired conditions.

## 26.5 FETAL TISSUE SAMPLING

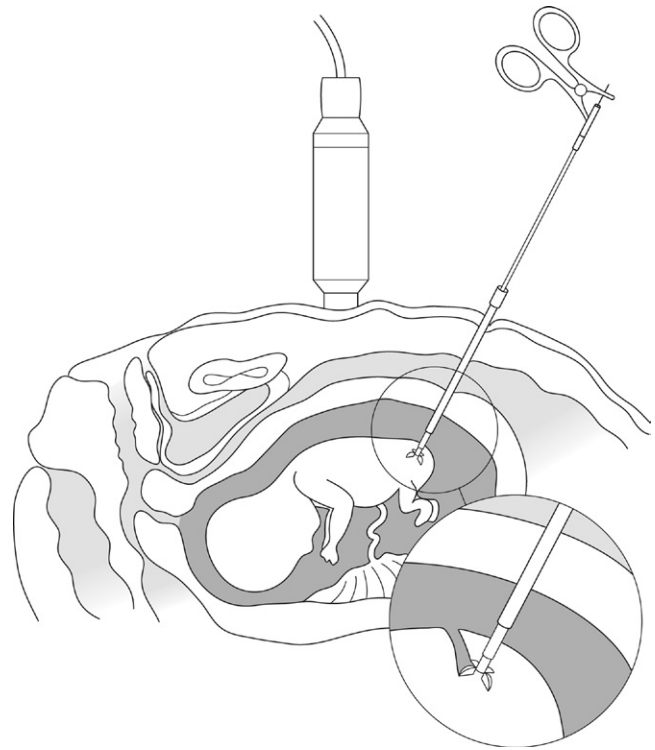
Advances in gene and protein identification as a result of the Human Genome Project and other public and private genetic initiatives has considerably reduced the need for fetal tissue biopsy for the prenatal detection of genetic disorders. Conditions such as fetal genodermatoses and Duchenne muscular dystrophy once required fetal skin and muscle biopsy, respectively, for direct pathological

evaluation. However, most of these conditions have been associated with specific gene mutations and are now amenable to diagnosis by CVS, amniocentesis preimplantation genetic diagnosis (PGD) (158,159).

With regard to congenital genodermatoses, fetal skin biopsies were required for ultrastructural or immunohistochemical analysis. Genodermatoses in which prenatal diagnosis has been established by fetal skin sampling have included anhidrotic ectodermal dysplasia (160), bullous congenital ichthyosiform dysplasia (epidermolytic hyperkeratosis) (161–163), epidermolysis bullosa dystrophica (Hallopeau–Siemens) (164), harlequin ichthyosis (165,166), hypohidrotic ectodermal dysplasia (167), epidermolysis bullosa lethalis (168,169), nonbullous ichthyosiform erythroderma (170), and Sjögren–Larsson syndrome (171). One of the more common lethal genodermatoses, Herlitz junctional epidermolysis bullosa, is now amenable to prenatal diagnosis by DNA analysis (172). However, the prenatal diagnosis of harlequin ichthyosis still requires fetal skin sampling, although Akiyama and colleagues (173) have reported that the diagnosis can now be made earlier than 21–22 weeks gestation and recent attempts to use 3D ultrasound to detect affected fetuses have generally been successful (174,175).

On rare occasions, fetal skin sampling is still required and is best performed at 17–20 weeks gestation. The procedure was initially performed under direct visualization using fetoscopy, a procedure associated with a total fetal loss rate of 4–7% (176,177), or a procedure-related loss rate of perhaps 2%. From a safety standpoint, it seemed reasonable to assume that the smaller the caliber of the instrument introduced into the uterus, the safer the procedure. For this reason, we began to use ultrasound-directed biopsy forceps for fetal skin sampling in the 1980s (Figure 26-6; (178)). We reported the clinical outcomes of 17 ultrasound-guided fetal skin sampling procedures (178). In five cases, a fetal skin disorder was diagnosed, and these pregnancies were terminated. In the remaining 12 cases, all infants were delivered without complications at 37 weeks gestation or later. Despite the use of biopsy forceps that take smaller fetal skin samples than the biopsy forceps used for fetoscopically directed fetal skin sampling, superficial scarring lesions have occasionally been noted.

Prenatal diagnosis of Becker–Duchenne muscular dystrophy is usually possible by DNA analysis. However, in a few families, meiotic recombination or homozygosity of multiple restriction fragment length polymorphisms precludes a DNA-based diagnosis (179–181). In these cases, fetal muscle biopsies can be used for immunohistochemical analysis with a fluorescent antidystrophin antibody. Males with Duchenne muscular dystrophy lack dystrophin and with Becker muscular dystrophy lack or demonstrate a variable dystrophin pattern (182), whereas muscle biopsies from unaffected male fetuses show normal amounts of dystrophin.



**FIGURE 26-6** Fetal skin sampling. (From Elias, S.; Emerson, D. S.; Simpson, J. L., et al. *Ultrasound-Directed Fetal Skin Sampling for Prenatal Diagnosis of Genodermatoses*. *Obstet. Gynecol.* 1994, 83, 337, with permission.)

Fetal muscle sampling is usually performed at around 18 weeks gestation using a technique analogous to fetal skin sampling or fetal liver sampling. Muscle biopsies are taken from the gluteal region by directing a Klear Kut (Baxter, Los Angeles, CA) kidney forceps gun toward the fetal buttock. As with fetal skin and liver biopsy procedures, only a small number of fetal muscle sampling procedures have been reported (179,183,184). Thus, statements cannot be made concerning the safety of this procedure.

## 26.6 COELOCENTESIS

Coelocentesis involves the transcervical or transabdominal aspiration of fluid from the extraembryonic celom. This procedure has been suggested as a possible method for early first-trimester prenatal diagnosis. Jurkovic and colleagues (185) were successful in aspirating fluid from 48 to 50 (96%) pregnancies between 6 and 10 weeks gestation. Although the authors were unable to obtain metaphase analyses from these specimens, subsequent work by Cruger and colleagues (186,187) demonstrated success in culturing and karyotyping. In addition, Makrydimas and colleagues (188) reported successful prenatal detection of  $\beta$ -thalassemia by coelocentesis. Among coelocentesis procedures performed on 14 women performed before pregnancy terminations at 8–9 weeks' gestation, Jourannic and colleagues (189) found that coelomic fluid–DNA PCR analysis without marked

maternal contamination was only achieved in 8 of the 14 (58%) of samples.

However, Ross and colleagues (190) reported that short-term pregnancy loss rates associated with coelocentesis were markedly increased. In a comparative study of women undergoing pregnancy termination, 25% of women spontaneously aborted before their termination compared with 5% of women not undergoing coelocentesis ( $P < 0.01$ ). These high loss rates suggest that coelocentesis may not be appropriate as a routine prenatal diagnostic test.

Of interest is the work of Santolaya-Forgas and colleagues. This group has investigated coelocentesis in baboons to determine its potential applicability for fetal therapy. In one series, coelocentesis was performed in nine baboons; fluid aspiration (1–5 mL) was successful in eight cases, and seven of the eight pregnancies were continuing 140 days after the procedure (191). Coelocentesis was subsequently performed in six other baboons; assessment of the extracoelomic fluid osmometry and electrolyte composition in these six samples found that the chorion laeve behaves as a semipermeable membrane at 40 days gestation (192), suggesting that it may be useful for maternal–fetal transfer of some substances that could be used for fetal therapy.

## 26.7 EMBRYOSCOPY

Embryoscopy remains an investigational technique that permits direct visualization of the fetus as early as the first trimester (193–195). Initially, a rigid fiberoptic endoscope was passed transcervically into the extracoelomic cavity, permitting inspection of fetal anatomic structures; fetal blood sampling was also feasible by this method (194). However, improvements and advancements in fiberoptic technology have led to the performance of thin-gauge transabdominal and transcervical embryoscopy (196), allowing visualization as early as 4 weeks after conception (197).

Initial procedures were performed only on women who had elected pregnancy termination; however, embryoscopy has since been performed on continuing pregnancies (194). Ville and colleagues (198) reported a procedure-related loss rate of 12% when the procedure was performed in the first trimester.

Further and larger studies of the safety, accuracy, and applications of this new modality will be needed before embryoscopy is used as a routine prenatal diagnostic tool. However, the ability to access the embryonic circulation may have important application for therapeutic interventions such as drug, gene, and cell therapy.

## 26.8 POLAR BODY BIOPSY

Polar body biopsy involves micromanipulation to remove either the first or the second polar body, and subsequent genetic analysis. This approach allows the oocyte to

remain viable for embryo development. Verlinsky and colleagues (Verlinsky et al., 1990) pioneered polar body biopsy genetic diagnosis using PCR to identify single-gene disorders in first polar bodies and transfer of unaffected embryos. For example, if a woman is heterozygous for an autosomal-recessive disease, a polar body having the mutant allele should be complemented by a primary oocyte presumed to have the normal allele. Thus, the oocyte could be used for in vitro fertilization (IVF) and transfer. Conversely, if the polar body was determined not to carry the mutant allele, the oocyte would contain the mutant allele and not be fertilized. A disadvantage is recombination, the meiotic phenomenon that occurs routinely between homologous chromosomes. Diagnostic difficulties resulting from recombination are a greater problem for genes located nearer the telomeres because these genes display recombination approximately 50% of the time. To detect hemizygous normal oocytes resulting after the second meiotic division, analysis of the second polar body is needed. The most accurate diagnosis can be achieved when the first polar body is heterozygous, so that detection of the normal or mutant gene in the second polar body indicates the opposite mutant or normal genotype of the resulting maternal contribution to the embryo. Similar to single-gene disorders, aneuploidy can be deduced. For example, if the first polar body failed to show a chromosome 21, the oocyte would be presumed to have two 21 chromosomes, and hence, the zygote would have trisomy 21. However, sequential testing of blastomere(s) is still required to exclude rarer paternally derived abnormalities (199).

## 26.9 PREIMPLANTATION GENETIC DIAGNOSIS

PGD is a technique that combines IVF with genetic analysis of the embryo. As only unaffected embryos are transferred to the uterus for implantation, PGD provides an alternative to invasive postconception procedures, i.e. CVS or amniocentesis.

PGD has been performed for a large number of single-gene disorders over the past two decades, and today the basic requirement for PGD is identification of the specific gene mutation in the family. These include X-linked, autosomal recessive, and autosomal dominant disorders (200). However, the most common indication for PGD detection of chromosomal abnormalities, numerical or structural, either by FISH, or genome-wide molecular approaches such as single cell array comparative genome hybridization testing for genome-wide single nucleotide polymorphisms (201–204), chromosomal aberrations, particularly aneuploidy, is known to be associated with early spontaneous abortions, it was believed that success rates for IVF and other forms of ART could be improved by so-called preimplantation genetic screening (PGS). The intent of such screening is to improve live birth rates by excluding chromosomally abnormal



embryos for transfer in IVF. The main indications for PGS are advanced maternal age, recurrent implantation failures, and recurrent spontaneous abortions. Initial studies suggested favorable effects on pregnancy success with PGS, but randomized controlled trials have not shown that PGS increases the delivery rate (205). The American College of Obstetricians and Gynecologists has indicated that there are no data to support use of PGS for recurrent spontaneous abortions and recurrent implantation failures, and its use for these indications be restricted to research studies (206). Finally, PGD has been used to determine the human leukocyte antigen (HLA) of embryos to produce a child that can serve as an HLA-matched hematopoietic stem cell donor for transplantation to a sibling, who is affected with a condition such as leukemia or lymphoma (207).

The most widely used method for PGD is blastomere biopsy at the cleavage (6–8 cell) stage of embryonic development. This technique involves disruption of the zona pellucida with a laser drilling or chemical means followed by insertion of a micropipette and aspiration of one or two blastomeres. Alternatively, the blastocyst stage is reached on day 5 or 6 after fertilization and allows the removal of multiple cells from the early trophoblast; however, this approach has not been widely applied.

Removal of one or more blastomeres does not appear to convert a significant risk of congenital anomalies to subsequent infants as reviewed recently by Simpson (208).

## 26.10 ULTRASONOGRAPHY

Ultrasonography has been one of the most important advances in the practice of obstetrics. At intensities usually produced by diagnostic equipment, ultrasound has not been found to cause any harmful effects on pregnant women, fetuses, other patients, or operators (209–211). Indications for ultrasonography during pregnancy are multiple and diverse and include estimation of gestational age by biometric measurements, estimation of fetal growth, bleeding from the vagina, determination of fetal growth, suspected multiple gestation, adjunct to invasive procedure (e.g. CVS or amniocentesis), suspected uterine or adnexal abnormality, suspected fetal demise, and many others (212,213).

In the context of prenatal diagnosis, many major, and even some minor, fetal structural anomalies can now be reliably detected. It is unrealistic, however, to expect 100% accuracy in detecting fetal anomalies, even with the most expert and thorough scanning. Some anomalies are more readily diagnosed than others. For example, anencephaly and marked hydrocephaly are rarely misdiagnosed, whereas others are more difficult to diagnose and may be overlooked, such as heart defects, facial clefts, diaphragmatic hernias, skeletal abnormalities, and neural tube defects (212–214).

Although there is no question that high-resolution ultrasonography is invaluable in detecting fetal anomalies (213–215), accuracy of diagnosis will vary depending on the experience of the sonographer, as well as on equipment, gestational age at time of scanning, and the *a priori* risk of the abnormality in question (216–219). As such, the limitations of diagnostic ultrasonography must be recognized. For example, in an admittedly dated study, Platt and colleagues (220) reviewed the ultrasound findings in 161 fetuses with spina bifida identified in the California MSAFP screening program. Before information regarding elevated MSAFP was made available to the examiner, in 13 of the 161 cases, spina bifida was not initially visualized. Subsequently, the defect was found in 10 of the 13 fetuses; in the remaining three cases, amniocentesis was declined, and the spina bifida was only diagnosed at birth. In screening for fetal anomalies, data concerning the sensitivity, specificity, and predictive values of diagnostic ultrasound are usually not available (68).

Although most women today receive one ultrasound examination during pregnancy, controversy still persists as to whether ultrasound monitoring of all obstetric patients should be routine to screen for fetal structural anomalies. These issues were first addressed in a report from the NICHD-sponsored Routine Antenatal Diagnostic Imaging with Ultrasound (RADIUS) study (221). Low-risk pregnant women with no indication for ultrasonography were randomly assigned to have either two screening sonograms (15–22 weeks and 31–35 weeks) or conventional obstetric care with ultrasonography used only on a selective basis as determined by the clinical judgment of the patient's physician. Major congenital anomalies occurred in 2.3% of the 15,281 fetuses and infants. Antenatal ultrasonography detected 35% of the anomalous fetuses in the screened group vs only 11% of the control population (relative detection rate 3.1; 95% CI 2.0–5.1). Surprisingly, ultrasonography did not significantly influence the management or outcome of pregnancies complicated by fetuses with congenital malformations. Moreover, ultrasonography screening had no significant impact on survival rates among infants with potentially treatable, life-threatening anomalies despite the opportunity to take precautionary measures such as delivery at a tertiary center. The RADIUS study also provided some insight into the content and limitations of obstetric ultrasound examinations. For example, a four-chamber view of the heart permitted detection of only 43% of fetuses with complex heart disease, and only 30% of fetuses with cleft lip and palate were detected. Finally, it was estimated that a public health policy of routine ultrasonographic screening would increase US health care costs by at least \$500 million. This study concluded that “given this extraordinary cost and the lack of measurable benefit, ultrasonographic screening for fetal anomaly detection cannot be justified.”

Many groups and centers subsequently reevaluated the RADIUS findings as well as their own experience and came to the opposite conclusion. DeVore (222) reported that the RADIUS study demonstrated that second-trimester ultrasonography could be provided in a cost-effective manner to low-risk women. Skupski and colleagues (223) reported on their experience with ultrasound in a low-risk population and found that the detection of major and minor malformations had a profoundly positive impact. The Eurofetus Study (224) combined the ultrasound and clinical outcomes of 61 European centers over a 3-year period and found that systematic ultrasound pregnancy detected a large proportion of fetal malformations. In 2009, the American College of Obstetricians and Gynecologists published a Practice Bulletin on the topic *Ultrasonography in Pregnancy* (206). Included in the clinical considerations and recommendations, it was pointed out that ultrasonography can be used to diagnose many major fetal anomalies, and that it has been suggested that all patients be offered routine ultrasound screening, given that 90% of infants with congenital anomalies are born to women with no risk factors. However, patients should be counseled about the limitations of ultrasonography and the sensitivity of the examination for the detection of abnormalities and potential false-positive findings. Ideally, all women should be offered aneuploidy screening before 20 weeks gestation, regardless of maternal age. For women presenting before 14 weeks gestation, the option for first-trimester screening is available, which includes measurement of nuchal translucency as a component for Down syndrome and trisomy 18 screening (discussed below). A second-trimester ultrasound examination may show indications of fetal chromosome abnormalities, including echogenic bowel, intracardiac echogenic focus, short femur or humerus, and dilated renal pelvis; these markers have a low sensitivity and specificity for Down syndrome, particularly in a low-risk population. However, ultrasound evaluation is not recommended as a primary screening modality for Down syndrome and other chromosomal abnormalities.

As mentioned above, new applications of ultrasound have made a considerable impact on our ability to provide prenatal diagnosis. Work in the early 1990s showed that first-trimester ultrasonography could be used to detect fetuses at increased risk for fetal chromosome abnormalities (225–227). However, further work showed that ultrasound screening, specifically the measurement of the nuchal translucency (Figure 26-7), could be applied to the screening of high- and low-risk women for fetal chromosome and structural abnormalities (225,228–231). Similar to the second-trimester ultrasonography, operator, gestational, and mechanical factors can alter diagnostic ultrasonographic outcomes (232,233). The measurement of certain maternal serum



**FIGURE 26-7** Prominent nuchal translucency (0.29 cm between asterisks) measured in fetus at 11.6 weeks gestation. Fetus found to have trisomy 21 by CVS. (Courtesy of Leiber Cohen, MD.)

markers, including free  $\beta$ -hCG, PAPP-A, AFP, hCG, and unconjugated estriol in various combinations, has been shown to provide an adequate, though not optimal, first-trimester screening protocol for fetal Down syndrome (233,234). It is the combination of first-trimester ultrasound and maternal serum analyte analysis that has profoundly altered the approach to screening for fetal Down syndrome and trisomy 18.

### 26.10.1 Ultrasound Screening for Fetal Down Syndrome: Second Trimester

Analysis of maternal serum analytes has been the mainstay of screening for fetal chromosome abnormalities for almost two decades (235). In 1987, Benacerraf and colleagues (236) reported that second-trimester fetuses with Down syndrome were more likely than normal fetuses to have a thickened nuchal skin fold and shortened femurs. Among more than 5500 fetuses evaluated, the detection rate was 75% with a specificity of 98%. When other structural abnormalities were added (e.g. atrioventricular canal), the sensitivity rose to 82%. Although such ultrasound markers have been confirmed by others, such correlations have not been universally observed (237–239). Although Vintzileos and Egan (240) suggested that second-trimester ultrasound can be used to modify second-trimester serum analyte screening outcomes for Down syndrome, Palomaki and Haddow (241) provided multiple screening and ultrasound concerns that would preclude the application of ultrasound in this manner. Indeed, a meta-analysis by Smith-Bindman and colleagues (242) showed that the various ultrasound markers, except for nuchal thickness, showed low sensitivities for fetal Down syndrome detection in the absence of overt structural anomalies. Although a meta-analysis may or may not be appropriate in assessing the utility of ultrasound markers, the conclusions were consistent with those of most individual reports (Figure 26-8).



**FIGURE 26-8** Arrow points to fetal nasal bone at 11.7 weeks gestation. Fetus with normal karyotype by CVS. (Courtesy of Leeber Cohen, MD.)

### 26.10.2 Ultrasound Screening for Down Syndrome: First Trimester

The use of ultrasound for first-trimester screening for fetal Down syndrome and trisomy 18 has begun to gain wide acceptance in Europe and the United States. The association of prominent nuchal translucency and increased risk for fetal chromosome abnormalities has been well documented (225–227,243); however, it was Nicolaides and colleagues (230) who proposed the use of first-trimester nuchal measurement and first-trimester maternal serum analytes in a screening paradigm for fetal Down syndrome. Among 1273 women carrying singleton pregnancies who were undergoing first-trimester CVS, a nuchal measurement of  $\geq 3$  mm could identify more than 85% of trisomy fetuses with a false-positive rate of approximately 5%.

Further studies have shown that the incorporation of two serum analytes, PAPP-A and free  $\beta$ -hCG, can improve the screening of pregnancies for Down syndrome and trisomy 18. The detection rate of first-trimester biochemical screening is similar to second-trimester screening (244). However, a recent large multicenter trial (245) incorporating biochemicals (PAPP-A and free  $\beta$ -hCG) showed a detection rate for Down syndrome of 79% at a positive screening rate of 5% and for trisomy 18, a detection rate of 91% at a positive screening rate of 2%. A study from the United Kingdom demonstrated a 93% detection rate for fetal Down syndrome at a positive screening rate of 5.9% and a detection rate of 96% for all chromosome abnormalities at a 6.3% positive screening rate (246).

In addition to the aforementioned screening protocol, an integrated screening involving first- and second-trimester screening protocols is now available and has been reported to yield higher detection rates with lower positive screening rates (247,248). In addition, ultrasound and maternal serum analyte analyses from the first trimester have been correlated with adverse obstetrical outcomes including PAPP-A ( $\leq 5$ th percentile) associated

with an increased risk for spontaneous pregnancy loss before 24 weeks gestation, low birth weight, preeclampsia, gestational hypertension, preterm birth, stillbirth, preterm premature rupture of the membranes, and placenta abruption. In addition, nuchal translucency ( $\geq 99$ th percentile) and free-beta subunit hCG ( $\leq 1$ st percentile) were associated with an increased risk of spontaneous loss at or before 24 weeks gestation (249).

The FASTER trial was a large US multicenter study comparing first- and second-trimester combined screening and second-trimester quadruple screening (MSAFP, hCG, unconjugated estriol, and inhibin A), first-trimester combined (PAPP-A, hCG and nuchal translucency) resulted in a slightly higher detection rate of Down syndrome than second-trimester serum screening (83% vs 81%) (250).

To achieve higher rates of Down syndrome detection and a reduction in the false-positive rate (i.e. reduce the need for invasive testing), the *integrated* approach to screening can be used, which incorporates both the first- and second-trimester markers to adjust a woman's age-related risk of having a child with Down syndrome. These results are reported only after both first- and second-trimester screening tests are completed. In this integrated approach, the detection rate for Down syndrome (94–96% in the FASTER trial and 94% in the SUPUSS trial) is higher than either first- or second-trimester screening alone (248,250). A concern in using the integrated approach is that first-trimester results are withheld from the patient until completion of the second blood test. Thus, the patient does not have the opportunity of early (i.e. first-trimester) diagnostic testing, as well as compliance with a two-step screening process.

To allow the results of the first-trimester combined screening to be disclosed to the patient, two sequential screening strategies have been introduced, namely, *stepwise* and *contingent* screening. If the stepwise strategy is used, the patient is offered a diagnostic test (CVS or amniocentesis) if the risk of aneuploidy is greater than a predetermined cutoff point; if not, she proceeds with the quadruple screening and receives a recalculated final risk figure based on the first- and second-trimester measurements. In contingent sequential screening, women are stratified according to the initial adjusted Down syndrome risk; only women with an intermediate Down syndrome risk (e.g. between 1 in 30 and 1 in 1500) would undergo the second-trimester screening. Finally, some have used a “default” approach called *independent sequential screening*, which involves carrying out a combined test (PAPP-A, hCG and nuchal translucency) in the first trimester, followed by a quadruple screening in the second trimester, and calculating separate risks from each. This approach is considered invalid since the second-trimester test does not incorporate all the available risk-related information, and is therefore not recommended (251).



### 26.11 FETAL CELLS AND FETAL DNA IN MATERNAL BLOOD

The concept of identifying and isolating fetal cells or fetal DNA in maternal blood is one that has been evaluated by numerous groups around the world for the past three decades. Such technology would ostensibly permit more effective screening for a wide variety of chromosome and Mendelian disorders and allow for the noninvasive diagnosis of certain detectable fetal abnormalities. The presence of fetal cells in maternal blood was first documented by Walknowska and colleagues (252) when they found XY metaphases in the maternal blood of pregnant women carrying male fetuses. De Grouchy and Trubuchet (253) also reported male metaphases from pregnant women carrying male fetuses, and other groups followed (254–256). During the late 1970s, Herzenberg and colleagues applied flow sorting technology to enrich for fetal cells (257,258). Their experimental design used HLA-A2-negative women who had an HLA-A2-positive spouse (heterozygote or homozygote). Any A2-positive cells recovered by flow sorting presumably must be fetal in origin that antigen having been inherited from the father.

Subsequently, a number of centers around the world have investigated isolation and analysis of intact fetal cells from mothers' blood for prenatal diagnosis. Our group was successful in detecting fetal aneuploidies, including trisomies 18 and 21, by sorting for CD 71, glycophorin-A positivity, cell size and cell granularity followed by interphase FISH with chromosome-specific probes (259–264).

However, subsequent work by our and other centers failed to develop techniques or processes that would provide a consistent number of fetal cells amenable to successful diagnostic protocols. Indeed, an NICHD conference reviewed the current status of work in the field of fetal cells in maternal blood and provided a clear consensus of the inadequacy of the methods used to date (265). The rarity of fetal cells in maternal blood is likely an important factor in the inability to develop an effective screening program. However, this could be overcome by fetal cell culture, which if successful, could obviously increase the absolute number of fetal cells present.

In contrast to diagnosis of fetal aneuploidy using intact fetal cells isolated from maternal blood, detection of fetal Mendelian disorders by analysis of fetal cells does not necessarily require enrichment procedures (266). Polymerase chain reaction (PCR)-based technology alone may suffice because DNA from any fetal cell should be reflective of fetal status. A circumstance permitting detection of a Mendelian disorder through analysis of fetal cells or fetal DNA in maternal blood arises when the father is heterozygous (Aa) and the mother is homozygously abnormal (aa) for an autosomal-recessive trait. The normal allele, which may

or may not be transmitted by the heterozygous father, should be readily detectable. If blood from the homozygous mother reveals DNA of the normal paternal allele (A), the fetus could be deduced to be heterozygous. An example of PCR in maternal blood to detect autosomal-recessive disorder is identifying pregnancies at risk for fetal Rh(D) disease. The molecular basis for an individual's being Rh(D)-negative (dd) is usually a gene deletion, d representing lack of the DNA sequence that, if present, encodes D. If the mother is Rh-negative and the father is homozygous for Rh(D) (Rh-positive), all fetuses must be heterozygous (Dd); every pregnancy would then be a risk for RhD-isoimmunization. If the father is heterozygous, however, the likelihood is 50% that the fetus would inherit his RhD gene and, hence, be affected; the other 50% of pregnancies would not be at risk for Rh-isoimmunization. Nested primers can be constructed so that the CE sequence is concurrently amplified, allowing an "internal control" that assures lack of D is not the result of primers failing to anneal or absence of cellular DNA in the sample tested. Using such a strategy, Lo and colleagues (267,268) studied 57 RhD-negative women throughout pregnancy. All RhD-positive fetuses in the second and third trimesters were correctly identified; 10 of 12 in the first trimester were also detected.

Initially, the discussion regarding RhD and other Mendelian conditions implied that the fetal DNA to be analyzed is present in the nucleus. Actually, cell-free DNA is also present in maternal plasma and serum (268,269). Our group (270) as well as others (268) have shown Rh(D) DNA in maternal serum of Rh-negative women (dd) carrying heterozygous (Dd) fetuses. Indeed, the detection of free fetal DNA in maternal serum for prenatal RhD genotyping in cases of maternal alloimmunization is now beginning to be used clinically in Europe (271–273) and is actively being studied in the United States (274).

Most recently, noninvasive detection of trisomy 21 has been reported using cell-free fetal DNA in maternal blood. Chui and colleagues (275) used two protocols of multiplexed massively parallel sequencing of DNA molecules in maternal plasma. In their "8-plex sequencing" protocol, the detection rate among trisomy 21 cases was 68/86 (79.1%) and the false-positive rate among non-trisomy 21 cases was 6/571 (1.1%). In their "2-plex sequencing" protocol, the detection rate among trisomy 21 cases was 86/86 (100%) and the false-positive rate among non-trisomy 21 cases was 3/146 (2.1%). They concluded that multiplexed maternal plasma DNA sequencing could be used to rule out fetal trisomy 21 among high-risk pregnancies, and about 98% invasive diagnostic tests could be avoided in patients referred for amniocentesis or CVS for increased risk for fetal trisomy 21.

Ehrich and colleagues (276) reported a blinded study of 480 prospectively collected maternal blood samples from women undergoing CVS, second-trimester amniocentesis or pregnancy termination. After exclusion of 31



samples for insufficient DNA quantity or quality, or failed assay quality control parameters, 449 samples remained for analysis. Using multiplexed massively parallel shotgun sequence of cell-free fetal DNA, 39 trisomy 21 samples were correctly identified; one sample from an unaffected fetus was classified as trisomy 21. Thus, the test showed a 100% sensitivity (95% CI: 89–100%) and a 99.7% specificity (95% CI: 98.5–99.9%). In an accompanying editorial, Shulman (277) cautions that although the report of Chiu and colleagues (275) is encouraging, further studies, including economic feasibility studies, portability studies and larger studies to better assess the detection rate of this assay for fetal trisomy 21 are required before such technology is even incorporated into screening algorithms, let alone replace current screening protocols.

## 26.12 FUTURE DIRECTIONS

As our ability to use molecular technologies to delineate fetal abnormalities increases, the need for invasive and highly morbid procedures will decrease. However, as technology advances increase our ability to safely evaluate and access the embryo and early fetus, we will face daunting social and economic issues that will need to be addressed by the medical and lay communities.

With the increasing age of the obstetrical community (278), advanced maternal age as a screening strategy for Down syndrome is considerably inferior to combined serum and sonographic screening protocols (279). In view of the overall improvement in the safety of invasive testing in combination with the increasing complexity of community-based screening protocols for Mendelian disorders (e.g. cystic fibrosis) and emerging first- and second-trimester screening protocols for fetal chromosomal abnormalities, consideration for universal offering of invasive testing to all women, regardless of age or risk, is warranted and should be further explored. In fact, Harris and colleagues (57) showed that the universal offering of invasive prenatal diagnosis would be cost-effective.

The increasing number of fetal disorders that are amenable to prenatal screening and diagnosis alone will necessitate a change in our methods for patient counseling. Elias and Annas (168) first proposed an innovative paradigm, “generic consent for genetic screening,” to restructure the informed consent process in the post-Human Genome Project era. This model recognizes that screening for numerous genetic disorders will require concepts that avoid information overload and promote patient autonomy. The effective performance of prenatal diagnosis is limited as much by our ability to effectively communicate with our patients as it is by our technology.

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## Biographies



**Sherman Elias, MD** is a Professor and past John J Sciarra Professor and Chair of the Department of Obstetrics and Gynecology of the Feinberg School of Medicine at Northwestern University. Previously, he was a Professor and Henry and Emma Meyer Chair in Obstetrics and Gynecology and Professor of Molecular and Human Genetics at Baylor College of Medicine in Houston (1994–1998), and the William G Arends Chair and Phillip and Beverly Goldstick Professor of the Department of Obstetrics and Gynecology and Professor of Molecular Genetics at the University of Illinois at Chicago (1998–2003).

Dr Elias has served as President of the Society of Gynecologic Investigation, Director of the American Board of Obstetrics and Gynecology, Secretary of the International Society for Prenatal Diagnosis, President of the Central Association of Obstetricians and Gynecologists, Vice President for Clinical Practice of the American College of Medical Genetics, and President of the American Association of Obstetricians and Gynecologists Foundation. Dr Elias has had continuous funding from the National Institutes of Health and other sources since 1987. He has been the recipient of the Basil O'Connor Award and the Jonas Salk Health Leadership Award in Research from the March of Dimes Birth Defects Foundation, the W K Kellogg National Fellowship Award, the Distinguished Alumnus Award from the University of Kentucky, and named a University of Illinois Scholar. He has authored over 375 articles, reviews and chapters and six books. Dr Elias' research focuses on prenatal genetic diagnosis, reproductive genetics and medical ethics.

Dr Elias is a native of Louisville, Kentucky. He earned Bachelor of Science from the University of Louisville and his Doctor of Medicine from the University of Kentucky. He served his internship in Obstetrics and Gynecology at Michael Reese Hospital in Chicago, and residency in Obstetrics and Gynecology at the University of Louisville, where he served as chief resident. He served two postdoctoral fellowships in Medical Genetics, the first in at Yale University and at the second at Northwestern University. He is a Diplomate of the American Board of Obstetrics and Gynecology and a Diplomate of the American Board of Medical Genetics.



**Lee P Shulman MD** is the Anna Ross Lapham Professor in Obstetrics and Gynecology and Chief of the Division of Clinical Genetics at the Feinberg School of Medicine at Northwestern University in Chicago, Illinois. He also serves as the Director of the Cancer Genetics Program of the Robert H Lurie Comprehensive Cancer Center of Northwestern University and the Co-Director of the Northwestern Ovarian Cancer Early Detection and Prevention Program. Dr Shulman is an Adjunct Professor in the Department of Medicinal Chemistry and Pharmacognosy at the University of Illinois at Chicago College of Pharmacy. He is a Fellow of the American College of Obstetricians and Gynecologists and a Founding Fellow of the American College of Medical Genetics.

Dr Shulman was graduated from Cornell University in 1979 with a BA degree in the College Scholar program. He then attended Cornell University Medical College where he received his Doctor of Medicine degree in 1983. Dr Shulman completed an internship and residency in Obstetrics and Gynecology at North Shore University Hospital—Cornell University Medical College in 1987 and served as Chief Resident during his final year. From there he completed a fellowship in Reproductive Genetics at the University of Tennessee, Memphis and then joined the Ob/Gyn faculty of the University of Tennessee, Memphis, becoming the Director of Reproductive Genetics in 1994. In 1999, he relocated to the University of Illinois at Chicago where he served as Deputy Head of the Department of Obstetrics and Gynecology, Director of the Divisions of Reproductive Genetics and Ambulatory Care Services and as Medical Director of the Center of Excellence in Women's Health.

Dr Shulman is a member of numerous regional, national and international organizations that pertain to the health and care of women. His work has been recognized regionally and nationally; most recently, he was included in the list of "Top Doctors" in Chicago (2007–2010) and the United States (2006–2010). He is the Editor-in-Chief of the *Yearbook of Obstetrics and Gynecology and Reproductive Medicine*, is a Contributing Editor for *The Journal of Reproductive Medicine* and the Executive Editor of the *Journal of Gynecologic Surgery*. He also serves on the editorial boards of *Prenatal Diagnosis*, *Contraception*, *Menopause*, and as a peer-reviewer for 32 other journals. Dr Shulman served at the Chair of the Board of Trustees of the Association of Reproductive Health Professionals from 2006 to 2008. A frequent contributor to the peer-reviewed and informational literature with over 130 peer-reviewed articles and over 50 book chapters, Dr Shulman's major research interests are in reproductive and cancer genetics, contraception, menopause and botanical interventions in women's health.

# CHAPTER

# 27

## Neonatal Screening

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### 27.1 INTRODUCTION

Genetic screening is a search in a population for persons who possess genotypes that (i) are associated with disease or predispose to disease, (ii) may lead to disease in their descendants, or (iii) produce other variations not known to be associated with disease. The primary focus of this chapter is newborn genetic screening for inborn errors of metabolism. Neonatal screening provides a means of early recognition of disorders in order to initiate treatment where available and to reduce confusion, anxiety, and delay associated with late ascertainment. Genetic screening can be a source of epidemiological data regarding birth defects and can also lead to a substantial increase in our knowledge of the natural history and variability in the genetic disorder. In the past, the nature and number of newborn screening tests varied widely in different localities. Recent technological advances and increased public awareness, however, have led to a much greater standardization of the tests used, as well as the number and types of disorders screened. At the request of the Maternal and Child Health Bureau of HRSA (Health Resources and Services Administration), the American College of Medical Genetics (ACMG) developed a uniform panel of 29 disorders that should be included in all state screening panels [Web resource 1]. Since this recommendation was adopted in September 2005, all states have implemented the uniform panel, increasing coverage substantially [Web resource 2]. Based on cumulative data from state newborn screening programs, about 1 in 800 newborns is born with a condition for which screening and treatment are available.

### 27.2 HISTORICAL ASPECTS

Genetic screening as we know it dates from the 1960s. As early as 1908, however, Archibald Garrod stated that inborn errors of metabolism could be recognized "... by some strikingly unusual appearance of surface tissues or of excreta, by the excretion of some substance

which responds to a test habitually applied in the routine of clinical work, or by giving rise to obvious morbid symptoms." This observation was first applied on a population-wide basis to phenylketonuria (PKU), but only after two important advances. The first occurred in 1934 when Fölling described the association between PKU and mental retardation. The second occurred when Bickel and coworkers developed a phenylalanine-restricted diet that was shown to prevent the mental retardation seen regularly in untreated PKU. The remaining challenge was to identify PKU in the presymptomatic newborn infant so that the dietary intervention could be optimally effective. This challenge was met when Guthrie developed a bacterial assay for phenylalanine that required only a few drops of blood, easily obtainable from the heel of newborn infants and dried on filter paper. Routine newborn screening for PKU using this method began in Massachusetts in 1962 and, after a successful field trial, expanded widely in the United States and abroad. Guthrie subsequently introduced microbiological assays to screen for maple syrup urine disease (MSUD), homocystinuria, tyrosinemia, histidinemia, galactosemia, and other disorders. Paper chromatographic methods for general newborn screening of blood and urine amino acids were also introduced at this time. The frequencies of some of these traditionally-screened disorders are shown in Table 27-1. Citations for these historical statements can be found in the previous edition of this book (1).

The great advantage of Guthrie's newborn screening system resided in the specimen of blood dried on filter paper, now often referred to as the Guthrie specimen or Guthrie card. This specimen is easily collected, readily transported to a central testing laboratory, and can be used in many different tests. In the 1970s, for instance, Dussault and colleagues introduced newborn screening for congenital hypothyroidism by developing a radioimmunoassay for thyroxine (T4) that could be applied to the Guthrie specimen (2). More recently, DNA extracted from the dried blood has been used for molecular testing.

In the 1980s, many newborn screening programs added tests for the sickle-cell diseases (viz. sickle-cell anemia, hemoglobin SC disease, and  $\beta$ -thalassemia). This occurred after demonstration in a large clinical trial that prophylactic treatment with penicillin reduced by 84% the mortality caused by infections with *Streptococcus pneumoniae* that otherwise killed many infants with sickle-cell disease. In the 1990s, screening was added for biotinidase deficiency by assay of biotinidase activity and for congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency, which accounts for more than 90% of all cases, by immunoassay of 17-hydroxyprogesterone (17-OHP). In 2010, programs have expanded further to include screening for cystic fibrosis (CF) by immunoreactive trypsinogen (IRT)-based methods and for many disorders affecting amino acids, organic acids, and fatty acid oxidation using the newly introduced technology of tandem mass spectrometry (MS/MS) (3).

Newborn screening for PKU and other inborn errors using dried blood has spread widely throughout the developed world. Not only has the practice of newborn screening increased but also the number of disorders covered has increased dramatically. In the United States this coverage has extended from the traditional PKU congenital hypothyroidism, galactosemia, MSUD and homocystinuria to CAH, sickle-cell disease and other hemoglobinopathies and additional amino acid disorders, as well as the organic and fatty acid oxidation disorders. Most of this expansion has been possible because of the introduction of MS/MS into newborn screening. Unlike traditional blood spot tests measuring a single analyte to detect individual disorders, MS/MS technology can screen for 20 or more disorders in a single blood spot. As a result of this new technology, the ACMG has proposed that all newborn screening programs include at least 29 “core” disorders (4,5) and all programs in the United States now adhere to this recommendation (Table 27-2). In addition, 25 “secondary target” disorders were recommended and many programs include all or most of these as well. Still other new tests continue to be proposed, with much recent interest focused on those that are DNA based (4). In view of the continuing changes in neonatal screening, the reader should consult an appropriate Web site for the latest information substantially [Web resource 2].

## 27.3 COMPONENTS OF SCREENING PROGRAMS

### 27.3.1 Collection of Specimens

The newborn Guthrie blood specimen is currently used for newborn screening in the United States and, with few exceptions, all other countries. One exception is the Canadian province of Quebec, which still collects and tests newborn urine specimens in addition to the newborn blood specimen. Several programs outside the

**TABLE 27-1** Frequencies of Some Metabolic Disorders and Conditions Detected by Screening Newborn Blood

Disorder or Condition	Frequency
Hyperphenylalaninemias	
Phenylketonuria	1:13,000
Other hyperphenylalaninemia	1:20,000
Galactosemia	1:50,000
Biotinidase deficiency	1:72,000
Maple syrup urine disease	1:70,000
Hypermethioninemia (homocystinuria)	1:200,000
Hereditary tyrosinemia	Very low

United States collect cord blood for congenital hypothyroidism screening.

The Guthrie specimen is usually collected in the nursery from the lanced heel of the infant by allowing drops of blood to saturate the filter paper within designated circles. The specimen is air dried in the nursery and then placed in an envelope or a plastic bag for transport to the central testing laboratory. Occasionally, the blood specimen is transferred to the filter paper from a capillary tube or a venous specimen. In Quebec, parents collect the newborn urine specimen when the infant is 3 weeks old using a kit supplied at the time of nursery discharge. The parent collects the specimen by placing the filter paper between folds of a wet diaper and pressing the diaper to push urine into the specimen card (6). Newborn urine screening was discontinued in Australia and the United States because of uncertainties regarding medical significance of many of the findings (e.g. histidinemia, Hartnup disorder, cystathioninemia, hyperlysinemia, and sarcosinemia). It has largely been supplanted by MS/MS technology, which detects some disorders previously screened in urine (e.g. methylmalonic acidemia, propionic acidemia, and argininosuccinic acidemia) and others (e.g. fatty acid oxidation disorders (FAODs)) that were not previously covered.

### 27.3.2 Organization of Screening

All screening programs in the United States are organized on a state-by-state basis, in that the state determines the disorders to be screened. Specimens are sent to the state program, and the program conducts the follow-up. In most states, the specimen is tested by the state laboratory or in a contract laboratory designated by the state. Several regional programs have been formed within which each participating state sends specimens to a testing laboratory within the region yet maintains within the originating state all other elements of the screening program. Newborn screening was fully funded by the states when first established, whereas most programs now charge a fee that may or may not include follow-up, confirmatory testing, and/or genetic counseling. Screening outside the United States has usually been organized in a manner similar to that in the United States. Nevertheless,

**TABLE 27-2 Core Disorders Recommended for Screening by American College of Medical Genetics**

Disorder	Acronym	Primary Marker
Metabolic disorders detected using tandem mass spectrometry		
<b>Organic acid disorders</b>		
Beta-ketothiolase deficiency (mitochondrial acetoacetyl CoA thiolase deficiency)	BKT	C5:1/C5OH
Cobalamin defects A,B,C,D,E,F	CBL (A-F)	C3
Isovaleric acidemia*	IVA	C5
Glutaric aciduria I	GA-I	C5DC
3-Hydroxy 3-methylglutaryl-CoA lyase deficiency*	HMG	C5OH/C6DC
Multiple carboxylase deficiency*	MCD	C3/C5OH
3-Methylcrotonyl-CoA carboxylase deficiency	3MCC	C5OH
Methylmalonic aciduria (mutase)*	MMA	C3
Propionic acidemia*	PA	C3
<b>Fatty acid oxidation defects</b>		
Carnitine uptake defect (carnitine transporter defect)	CUD	C0
Long-chain hydroxyacyl-CoA dehydrogenase deficiency*	LCHAD/D	C16OH/C18:1OH
Medium-chain acyl-CoA dehydrogenase deficiency	MCAD/D	C8
Trifunctional protein deficiency*	TFP	C16OH/C18:1OH
Very-long-chain acyl-CoA dehydrogenase deficiency	VLCAD/D	C14:1/C14
<b>Amino acid disorders</b>		
Argininosuccinic aciduria (argininosuccinate lyase deficiency)*	ASA	Citrulline
Citrullinemia I (argininocuccinate synthetase deficiency)*	CIT-1	Citrulline
Phenylketonuria	PKU	Phenylalanine
Maple syrup urine disease*	MSUD	Leucine
Homocystinuria	HCY	Methionine
Tyrosinemia type I	TYR-I	Tyrosine
<b>Other metabolic disorders</b>		
Biotinidase deficiency	BIOT	Biotinidase activity
Galactosemia*	GALT	Total galactose, GALT activity
<b>Endocrine disorders</b>		
Congenital adrenal hyperplasia*	CAH	17-Hydroxyprogesterone
Congenital hypothyroidism	CH	T <sub>4</sub> , TSH
<b>Hemoglobin disorders</b>		
Sickle cell anemia	HbSS	Hb variants
Sickle cell disorder	HbS/C	Hb variants
Hemoglobin S/ $\beta$ -thalassemia	HbS/betaTh	Hb variants
<b>Other disorders</b>		
Cystic fibrosis	CF	Immunoreactive trypsinogen
Hearing	HEAR	Hearing test

GALT = Galactose-1-phosphate uridylyltransferase; T<sub>4</sub> = thyroxine; TSH = thyroid-stimulating hormone.

\*Can manifest acutely in the first week of life.

there are a few national programs, notably the national screening program in Japan. These programs are usually included in national health services.

### 27.3.3 Analysis of Specimens

The formerly widely used Guthrie-type assays have been replaced by MS/MS, which allows for coverage of far more disorders. The details of the bacterial inhibition assays are of historical interest (1). Galactosemia is identified by either an enzyme assay for galactose-1-phosphate uridylyltransferase (GALT) activity or, less often, a metabolite assay for total blood galactose, the combination of galactose and galactose-1-phosphate. The enzyme assay is limited to the detection of galactosemia due to GALT activity, whereas a metabolite

assay can identify galactosemia and the two other galactose metabolic disorders, galactokinase deficiency and uridine diphosphogalactose-4-epimerase deficiency.

Biotinidase activity is also measured by an enzyme assay in the dried blood specimen. In the semiquantitative assay used in screening (7,8), biotinidase releases *p*-aminobenzoate from the artificial substrate, biotinyl-*p*-aminobenzoate. Color-developing reagents added subsequently cause samples with biotinidase activity to become purple, whereas samples lacking biotinidase activity remain clear or straw colored.

Congenital hypothyroidism, CAH, and CF are screened by immunoassays for, respectively, T<sub>4</sub> and/or thyroid stimulating hormone (TSH), 17-OHP, and trypsinogen (9). Sickle-cell diseases are screened by



examination of hemoglobin mobilities using either isoelectric focusing or hemoglobin electrophoresis (9).

MS/MS has been adapted for newborn screening. Use of this method is increasingly widespread in screening programs throughout the world. It allows the simultaneous screening for most amino acid disorders as well as for many organic acid and fatty acid oxidation disorders in a single assay (10,11). The technology employs two mass spectrometers in tandem separated by a collision chamber (Figure 27-1). The mass of the ionized molecules is determined in the first spectrometer and, after fragmentation in the collision chamber, the molecules are identified on the basis of the fragmentation profiles in the second spectrometer (12). The data are analyzed by a computer program and electrically translated into a graph with representative peaks for each metabolite. MS/MS is replacing the single amino acid bacterial assays used previously to screen for amino acidopathies and is expanding coverage to include many organic acidopathies and fatty acid oxidation defects (Tables 27-2 and 27-3).

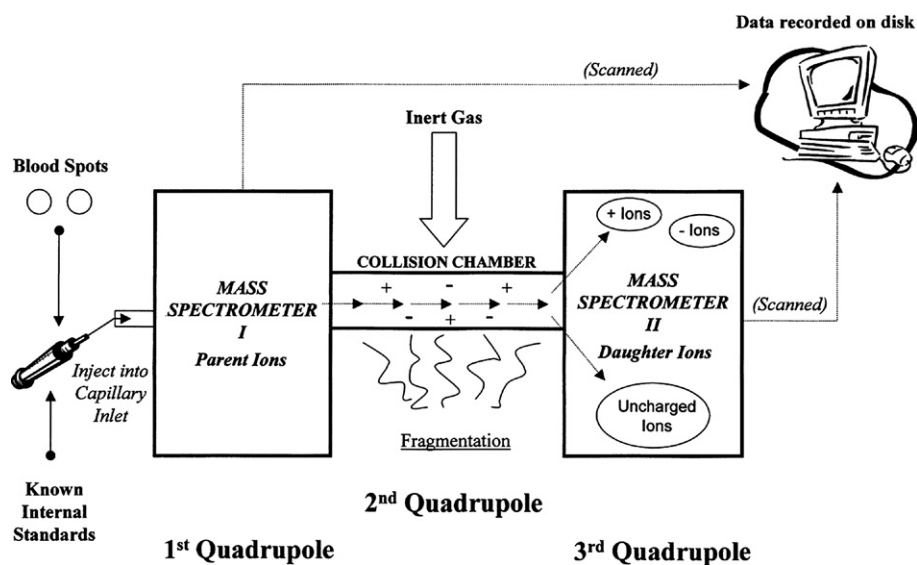
Almost all newborn screening has focused on an increase in an analyte, with notable exceptions being decreased T4, indicating congenital hypothyroidism, and enzyme assays for galactosemia and biotinidase deficiency, in which reduced activity is the indicator. Newborn screening programs led by the Mayo Clinic laboratory are considering identifying reduced analyte levels to indicate disorders in which the increase of an analyte is not currently identifiable. Examples are reduced methionine to indicate defects in methionine remethylation (13) and reduced citrulline to indicate proximal urea cycle disorders (N-acetylglutamate synthase (NAGS) deficiency, carbamylphosphate synthetase

(CPS) deficiency, or ornithine transcarbamylase (OTC) deficiency (14)).

Screening by direct analysis of DNA from Guthrie specimens has to date been limited to second-tier testing of specimens found to be positive by a primary, non-DNA screening method. Second-tier DNA screening by PCR analysis and detection of mutant alleles is currently in use for CF, medium-chain acyl-CoA dehydrogenase deficiency (MCADD), and galactosemia. A much more expansive application of DNA technology in routine newborn screening (e.g. exomic or whole genome screening) is a topic of much current discussion.

### 27.3.4 Interpretation of the Screening Results

Table 27-2 lists the core disorders identifiable by newborn screening, the acronym for each disorder, and the newborn screening abnormality, or primary marker, that might indicate the disorders as usually reported to the health care provider by the newborn screening program. All newborn screening reports indicating an abnormality should be followed up either by a repeat newborn screen, by obtaining specific confirmatory tests, or by referral to a metabolic center. The type of follow-up will probably be recommended by the newborn screening program coordinator and/or be indicated in the report. The recommendation is usually based on the magnitude of the abnormality or the potential immediate impact of the suspected disorder. Specifically, when the screening abnormality markedly deviates from the cutoff or the potential disorder can have a life-threatening neonatal effect (e.g. galactosemia or MSUD) the recommendation will be for immediate investigation or immediate referral to a metabolic center.



**FIGURE 27-1** Tandem mass spectrometry (MS/MS) in neonatal screening for inherited metabolic disorders. The mass of ionized molecules is determined in the first spectrometer and, following fragmentation in the collision chamber, the molecules are identified on the basis of the fragmentation profiles in the second spectrometer. The data are analyzed by means of a computer program and electrically translated into a graph with representative peaks for each metabolite.

**TABLE 27-3 Major Acylcarnitine Elevation in Expanded Newborn Screening and the Identified Organic Acid and Fatty Acid Oxidation Disorders**

Acylcarnitine	Abbreviation	Disorder
<i>Organic Acid Disorders</i>		
Propionylcarnitine	C3	Propionic acidemia Methylmalonic acidemia Cobalamin defect
Isovalerylcarnitine	C5	Isovaleric acidemia 2-Methylbutyryl-CoA dehydrogenase deficiency
3-Hydroxyisovalerylcarnitine	C5OH	3-Ketothiolase deficiency 3-Hydroxy-3-methylglutaryl-CoA lyase deficiency 3-Methylcrotonyl-CoA carboxylase deficiency
Glutaryl carnitine	C5DC	Glutaric acidemia type I
<i>Fatty Acid Oxidation Disorders</i>		
Butyrylcarnitine	C4	Short-chain acyl-CoA dehydrogenase deficiency Isobutyryl-CoA dehydrogenase deficiency
Octanoylcarnitine	C8	Medium-chain acyl-CoA dehydrogenase deficiency Multiple acyl-CoA dehydrogenase deficiency
Tetradecanoylcarnitine	C14:1	Very-long-chain acyl-CoA dehydrogenase deficiency
Palmitoylcarnitine	C16	Carnitine palmitoyl transferase II deficiency Carnitine-acylcarnitine translocase deficiency Multiple acyl-CoA dehydrogenase deficiency
Hydroxypalmitoylcarnitine	C16:OH	Long-chain hydroxy acyl-CoA dehydrogenase deficiency
Carnitine (reduced)	C0	Carnitine transport defect

Conversely, an abnormality that is less deviant from the cutoff and that suggests a disorder without a neonatal phenotype might initially require only a repeat newborn screen.

To aid the physician in understanding these complex newborn screening findings and in deciding what to do when a report of an abnormality is received, the ACMG has developed “ACT Sheets,” 1–2 page explanations of newborn screening abnormalities and recommendations for follow-up. Accompanying each ACT Sheet is an algorithm to guide the follow-up testing and interpretation of the test results. The ACT Sheets and algorithms can be accessed through ACT Sheets and Confirmatory Algorithms [Web resource 3]. On the left of the site is “Short

cuts” and under that is a link to the ACT Sheet indicator. Click on that and you enter the site with an option for each type of abnormality. In addition, a full explanation of the metabolic disorders can be accessed at the GeneReviews web site [Web resource 4].

The majority of positive screening results is not due to a metabolic or other disorder. False positives frequently result from mild transient elevations in blood amino acids or acylcarnitines, which suggest the possibility of an amino acid, organic acid or fatty acid oxidation disorder in otherwise normal newborns. Premature infants receiving hyperalimentation or infants who are very ill often have multiple amino acid elevations, especially in the presence of severe liver dysfunction. Transient deficiencies of GALT produce false-positive results in galactosemia screening, especially during summer months because of inactivation of the enzyme by humidity and/or heat. Transient deficiencies of biotinidase activity, particularly in premature infants, may also be encountered in screening for biotinidase deficiency. Artifacts also account for many false-positive results. Multiple applications of blood to one area of the filter paper or the presence of blood clots can produce “caking” and falsely elevate the apparent concentrations of amino acids in screening assays. The frequency of false-positive results for some disorders such as PKU has decreased substantially with the introduction of MS/MS analyses, although the number of false-positive results overall has probably increased because of the much greater number of metabolites tested in the newborn specimen, more in some programs than in others. In view of the implications of false-positive results on the need for further testing and medical evaluation, and on parental anxiety, the establishment of appropriate cutoffs and care in the collection of the specimen are of great importance.

### 27.3.5 Confirmatory Tests

Importantly, an abnormal screening result, even when present on repeat testing, does not establish a specific diagnosis. An abnormality reflected by a positive screening test may have more than one possible genetic or nongenetic cause, and the etiology in each particular case must be ascertained. Confirmation of PKU, galactosemia, and other inborn errors of metabolism requires not only additional diagnostic tests but also rigorous clinical diagnostic evaluation before an appropriate plan of management can be formulated. DNA testing for confirmation of diagnosis and identification of mutant alleles is increasingly available for screened disorders. Direct DNA testing for diagnosis and genetic counseling is available for PKU and galactosemia among the “traditional” metabolic disorders, as well as for CAH, CF, MCADD, and the hemoglobinopathies, among the other disorders detectable by neonatal screening. A clear genotype/phenotype relationship has been described in some of the traditional metabolic disorders (e.g. galactosemia)

and has been identified in CAH, biotinidase deficiency, and the hemoglobinopathies. The ACMG ACT Sheets and algorithms indicated earlier provide guidance for confirmatory testing and interpretation.

## 27.4 POTENTIAL PROBLEMS IN NEWBORN SCREENING

Several important potential problems can affect the interpretation of the screening results and should be considered when deciding whether an infant with clinical evidence of disease should have additional metabolic tests and, if so, which tests to order.

### 27.4.1 False-Positive Results

Most positive results in newborn screening are transient aberrations not caused by a metabolic disorder or any other pathologic state. In some instances, the transient abnormality is associated with an identifiable temporary influence, such as antibiotic therapy, prematurity, or postprandial status, but often no cause is identified. These transient abnormalities are usually of mild degree. The cutoff or threshold level selected for the purpose of suspecting the presence of a metabolic disorder is usually a level that presumably will be exceeded by all affected infants, reflecting the high priority set on achieving maximum test sensitivity. The cutoffs, along with test sensitivity (i.e. the probability that a person with the disorder will have a positive test result) and specificity (i.e. the probability that a person without the disorder will have a negative test result), and positive predictive values (PPVs) (i.e. the proportion of persons with positive test results who actually have the disorder) are currently being analyzed in extensive national and international collaborations (14). These data will also be of great value for reducing the frequencies of false-positive and false-negative results.

False-positive results not only add to the expense and workload of newborn screening but also produce great anxiety in the parents of infants thus identified. Parents deserve simple and clear explanations of the reasons that the additional specimens are requested. Some newborn screening programs distribute specially prepared written explanations in advance to physicians or include them with requests for follow-up specimens. A study of expanded newborn screening for biochemical genetic disorders evaluated 50 children identified with inborn errors, 94 screened children with false-positive results, 81 screened children with normal results, and their respective parents. False-positive findings in newborn screening generated anxiety in parents, as indicated by a twofold greater likelihood that their children would have an emergency department visit or hospitalization compared with screened children with normal results. Mothers of children in the false-positive group compared with mothers of children with normal screening results

attained higher scores on the Parental Stress Index and the Parent–Child Dysfunction scale (6,15).

A major advantage of new technologies is the potential for reducing false-positive results. MS/MS provides a metabolite profile rather than only the single metabolite levels measured by the bacterial assays. In screening for PKU, for instance, it is the ratio of phenylalanine to tyrosine that best distinguishes the infant with PKU from the much larger number of infants with only transient hyperphenylalaninemia. MS/MS also provides a leucine/isoleucine:alanine ratio that distinguishes infants with MSUD from those with a transient increase in the blood leucine concentration. False-positive rates for MCADD screening can be reduced using an MS/MS profile that includes C6 and C10:1 acylcarnitines along with the C8 analyte.

Second-tier testing of newborn specimens exhibiting abnormal primary screens is increasingly being performed in screening laboratories in order to distinguish the infant with a true disorder from infants who have transient increases or who have a benign variant of the disorder. Second-tier screening has traditionally been practiced for galactosemia and congenital hypothyroidism. A specimen with an increased galactose concentration can be retested by performing a spot enzyme assay for GALT activity to distinguish the infant with classical galactosemia from infants with benign variants or transient increases in galactose as well as the infant with another galactose metabolic defect. Infants with low T4 levels on primary screening are encountered frequently, and their specimens can then be assayed for TSH to identify those with congenital hypothyroidism who will have both a low T4 and a high TSH. DNA analysis is increasingly being used now for second-tier testing. This began with screening for CF to distinguish infants with CF from those with only transient increases in IRT. A secondary molecular assay for F508del, the mutant allele present on 70% of CF chromosomes, along with a panel of the most frequent CF alleles, eliminates most of the false-positive results. IRT/DNA algorithm assays detect the 23 mutations currently recommended by ACMG (16). This approach usually involves referral of newborns with IRT values above the cutoff and 1 or 2 CFTR mutations for sweat testing in a CF center. More recently, second-tier DNA testing has been extended to galactosemia screening (17) by some programs. The Duarte mutation (N314D), for example, is a frequent source of benign initial positive screens. Screening begins with quantitative GALT enzyme analysis. If the quantitative GALT enzyme value is consistent with a diagnosis of pronounced deficiency or carrier status for galactosemia, DNA analysis of the *GALT* gene 6-mutation panel is performed to detect the four most frequently encountered classic galactosemia alleles (Q188R, S135L, K285N, and L195P) in addition to the N314D (Duarte) and L218L (Los Angeles) variants. Second-tier DNA testing has also proved useful in biotinidase screening (18). For specimens that show increased 17-OHP in the primary screen for CAH, second-tier testing by MS/MS has recently been

introduced (19). The infant with CAH is likely to have a low cortisol level determined by this method. Second-tier testing methods for isovaleric acidemia and methylmalonic acidemia have also been developed (20).

### 27.4.2 Early Newborn Screening

All newborn screening programs require or strongly recommend collection of the Guthrie specimen before the neonate is discharged from the hospital. This essentially guarantees that the newborn will be screened. Traditionally, the specimen would be obtained on the second or third day of life, but the discharge of newborns at or before 24 h of age means that many neonates are being screened “early” relative to the traditional parameters of newborn metabolic screening. Early discharge has revived concerns about missing affected infants by screening them prior to the time when their metabolic abnormality becomes detectable and has led to the recommendation for collection of a second blood specimen from infants initially tested at or before 24 h. Most disorders, including PKU, can be identified before 24 h of age. Early identification of isovaleric acidemia has been demonstrated (21) and suggests that other organic acid disorders, as well as fatty acid oxidation defects detected by elevations of acylcarnitine derivatives, could also be identified early. Biotinidase deficiency and galactosemia can be detected in Guthrie specimens collected before 24 h of age because the corresponding enzyme assays are not affected by the timing of specimen collection. In contrast, detection of some disorders is adversely affected by early specimen collection. The hypermethioninemia required to detect homocystinuria due to cystathionine  $\beta$ -synthase (CBS) deficiency usually does not develop until at least 24 h of age or later (22). Detection of congenital hypothyroidism may also be compromised in the early specimen. The current trend is to insist on collection of the initial specimen at discharge regardless of age and to recommend the collection of a second Guthrie specimen at no later than 7 days of age from infants discharged “early,” that is, prior to 24 h of age (Table 27-4).

### 27.4.3 Transferred and Transfused Infants

Failure to collect the newborn screening specimen is not uncommon among infants transferred to a special care

nursery, especially when this transfer is to another hospital. Ironically, the transfer may be necessitated by the symptoms of a disease identifiable in routine screening, such as galactosemia or MSUD. To guard against this omission, the initial screening specimen should be collected before transfer and a second screening specimen collected at discharge (Table 27-4).

The infant who receives a blood transfusion is also vulnerable to missed diagnosis in newborn screening. Notably at risk are the diagnoses of galactosemia and sickle-cell disease, both of which depend on identifiers in the red blood cell (viz. lack of GALT enzyme activity and the presence of hemoglobin S, respectively). Donor erythrocytes in the transfused blood will mask these abnormalities by providing normal red blood cells to the affected infant. The initial screening specimen should be collected prior to any transfusion and a second screening specimen collected at about 2 months of age when the infant has replaced most of the donor erythrocytes with endogenous red blood cells (Table 27-4).

### 27.4.4 Missed and Uncovered Disorders

A common misconception is that all infants with metabolic disorders for which newborn screening is conducted are detected in the screening process. Although most are detected, an occasional infant with PKU, congenital hypothyroidism, or another screened disorder is missed (23). There are a number of reasons why these infants can be missed, including delay in appearance of the identifier (e.g. lack of elevated blood methionine in homocystinuria) (22). FAODs are among the most likely to be missed, either because the initial screen is normal (24) or because the repeat specimen to evaluate an abnormal initial result is normal (25). Laboratory error must also be considered. A recent addition to the causes of not identifying an infant in newborn screening is the “opting out” of expanded (MS/MS) newborn screening by a parent (26). Based on the presumption of a normal newborn screen and even after receiving a normal newborn screening report, it is important not to assume that an infant with a clinical phenotype compatible with one of the screened metabolic disorders is unaffected. Evaluation of these patients should include tests for all relevant metabolic disorders, without excluding the ones covered by newborn screening, in order to avoid serious delays in diagnosis and treatment.

Misconceptions about newborn screening also extend to the range of coverage for metabolic disorders. Knowledge that newborn screening includes metabolic disorders can be misinterpreted as indicating that all metabolic disorders are covered. This misinterpretation can lead to the omission of metabolic testing in a clinically abnormal infant who otherwise would receive these tests, resulting in delays in diagnosis and therapy.

**TABLE 27-4 Specimen Collection in Newborn Screening**

Neonate	Initial Specimen	Repeat Specimen
Normal	$\geq 24$ h <24 h	No $\leq 7$ days
Low birth weight	3 days	No
Transferred	At transfer	Discharge
Transfused	Before transfusion	2 months



## 27.5 DISORDERS AND CONDITIONS DETECTED BY NEWBORN BLOOD SCREENING

Although detailed descriptions of these disorders are beyond the scope of this chapter, aspects especially pertinent to screening are considered below.

### 27.5.1 Phenylketonuria and Hyperphenylalaninemia

The conversion of phenylalanine to tyrosine is catalyzed by the enzyme phenylalanine hydroxylase (PAH). This enzyme requires a cofactor known as tetrahydrobiopterin ( $\text{BH}_4$ ). Confirmed hyperphenylalaninemia in the infant is due to a primary or secondary ( $\text{BH}_4$  deficiency) reduction in PAH activity. As in many inherited disorders, the detection of a primary metabolite abnormality, in this case hyperphenylalaninemia, requires the consideration of a spectrum of possible genetic causes before proper diagnosis and management are possible. The failure to appreciate that several disorders as well as benign metabolic conditions can cause hyperphenylalaninemia was a serious early problem in newborn metabolic screening (1).

The causes of hyperphenylalaninemia (27) include

1. Transient hyperphenylalaninemia
2. Persistent non-PKU hyperphenylalaninemia
3. PKU (PAH deficiency)
4. Three disorders causing defective  $\text{BH}_4$  synthesis
5. Impaired recycling of  $\text{BH}_4$  due to dihydropteridine reductase (DHPR) deficiency

The phenylalanine concentration in newborn blood is normally less than  $120\ \mu\text{M}$  ( $2\ \text{mg/dL}$ ). The screening result is considered to be positive when the phenylalanine concentration exceeds  $120$  or  $240\ \mu\text{M}$  ( $4\ \text{mg/dL}$ ), depending on which cutoff the screening laboratory uses. With MS/MS technology, many laboratories also consider the phenylalanine:tyrosine ratio, which is increased to five-fold or greater in PKU. Positive screening results should be promptly followed by confirmatory tests of blood and urine. Complete plasma amino acid analysis can confirm the elevated phenylalanine and reduced tyrosine concentrations and rule out the presence of other amino acid abnormalities. The diagnosis of PKU is generally assigned when (i) the plasma phenylalanine concentration is  $>400\ \mu\text{M}$  ( $>6.5\ \text{mg/dL}$ ) in confirmatory follow-up tests while the infant is ingesting a normal diet, (ii) the tyrosine concentration is normal or reduced, and (iii) analyses of pterins in urine and DHPR activity in blood yield normal results (see later). When these criteria are met, a phenylalanine-restricted diet should be instituted promptly in order to prevent the cognitive impairment seen in untreated PKU. If the confirmatory phenylalanine level is  $120\text{--}360\ \mu\text{M}$  ( $2\text{--}6\ \text{mg/dL}$ ), the infant is likely to have mild hyperphenylalaninemia and a normal diet may be continued (28).

$\text{BH}_4$  is the cofactor for PAH. About 30–40% of patients with PKU, usually the mild form, are responsive to pharmacologic amounts of  $\text{BH}_4$  in that, when treated with this cofactor (available as the drug “Kuvan”), the phenylalanine level decreases by 30% or more. In rare instances this therapy may be sufficient to control the blood phenylalanine level without diet. Much more frequently diet is still required but with a reduction in stringency (29). Certain PAH mutations seem especially likely to be associated with  $\text{BH}_4$  responsiveness (30,31).

Primary hyperphenylalaninemia is due to PKU produced by a deficiency of PAH, whereas secondary hyperphenylalaninemia is caused by a deficiency of  $\text{BH}_4$ . The  $\text{BH}_4$ -deficiency disorders are characterized by progressive neurological deterioration despite dietary phenylalanine restriction and control of the hyperphenylalaninemia (32). Thus, they must be distinguished from PKU.  $\text{BH}_4$  is required as a cofactor not only for PAH but also for tyrosine and tryptophan hydroxylases. Consequently, in  $\text{BH}_4$  deficiency the conversions of tyrosine to dihydroxyphenylalanine (DOPA) and tryptophan to 5-hydroxytryptophan are also impaired, disrupting synthesis of the neurotransmitters dopamine and serotonin, respectively. A number of patients and families with  $\text{BH}_4$  deficiency have been collected and studied. The genes for these enzymes have been cloned and several mutations characterized (32). Therapy consists of supplementation with  $\text{BH}_4$  and the neurotransmitter precursors DOPA and 5-hydroxytryptophan. Because the degrees of hyperphenylalaninemia in PKU and  $\text{BH}_4$  deficiency are similar during the newborn period, accurate diagnosis requires an *in vivo* or *in vitro* test or a combination of these two methods (32,33). In the United States, the usual method for distinguishing PKU from  $\text{BH}_4$  deficiency is to collect filter-paper specimens of blood and urine at confirmatory examination. The blood is assayed for DHPR, the recycling enzyme for  $\text{BH}_4$ , and the urine is tested for pterins. Reduction in DHPR suggests the DHPR-deficiency form of  $\text{BH}_4$  deficiency, while an abnormal urine pterin pattern suggests a biopterin synthesis defect. In Europe, loading the hyperphenylalaninemic neonate with  $\text{BH}_4$  at a dose of  $20\ \text{mg/kg}$  is used to distinguish  $\text{BH}_4$  deficiency and  $\text{BH}_4$ -responsive PKU from PKU that is nonresponsive to  $\text{BH}_4$  (28). In  $\text{BH}_4$  deficiency,  $\text{BH}_4$  administration results in a reduction in the phenylalanine level to almost normality and in  $\text{BH}_4$ -responsive PKU the phenylalanine level decreases significantly, whereas in PKU that does not respond to  $\text{BH}_4$ , the phenylalanine level is unchanged. In the United States, the  $\text{BH}_4$  challenge is usually not given until or close to 2 years of age.

Hyperphenylalaninemia not due to either PKU or  $\text{BH}_4$  deficiency may be transient and disappear within the first months of life (33). This requires no specific therapy and is usually mild. In some instances, the hyperphenylalaninemia is accompanied by transient hyper-tyrosinemia. Other infants, however, will have mild hyperphenylalaninemia that persists and is accompanied

by a normal tyrosine concentration. This non-PKU mild hyperphenylalaninemia seems to be benign. A study from Germany evaluated 31 adolescent and young adult individuals with non-PKU mild hyperphenylalaninemia whose natural blood phenylalanine levels were no greater than 600  $\mu\text{M}$  (10 mg/dL) and who were never treated. All had normal IQs, normal educational experiences, and no changes in the cerebral white matter on examination by MRI (34).

Maternal PKU is not identified by neonatal screening but is an unintended consequence of screening and the prevention of mental retardation from PKU. When these women later reproduce, their pregnancies are at high risk to the nonphenylketonuric fetus, with potential consequences that include mental retardation, microcephaly, congenital heart disease, and intrauterine growth retardation (35). Data from the Maternal PKU Collaborative Study, however, indicate that diet begun before conception or within the first six gestational weeks with maintenance of good metabolic control, specifically blood phenylalanine concentrations in the range of 120–360  $\mu\text{M}$  (2–6 mg/dL), results in good offspring outcome (36). In contrast with maternal PKU, data from the same study indicated that women with mild hyperphenylalaninemia whose natural blood phenylalanine concentrations were no greater than 600  $\mu\text{M}$  (10 mg/dL) and who remained untreated during pregnancy also had normal offspring, suggesting that maternal non-PKU mild hyperphenylalaninemia is benign (37). Offspring from mothers with PKU rarely have PKU themselves but all such offspring must be carefully tested for PKU to be certain that they are non-hyperphenylalaninemic. Breast feeding can be practiced by the mother with PKU and will not raise the phenylalanine level of the baby even if the mother discontinues the diet after giving birth (38).

## 27.5.2 Galactosemia

At least three disorders of galactose metabolism are detectable in newborn screening. They are appropriate targets for newborn screening because of the need for early intervention. The most frequent of these disorders is classical galactosemia due to deficiency of GALT, an enzyme that is normally present in most tissues, including liver, skin fibroblasts, and red blood cells (39). The untreated clinical course is characterized in the neonate by failure to thrive, jaundice, hepatomegaly, and often death from sepsis, usually due to *Escherichia coli*. Untreated infants who survive suffer from developmental retardation, cirrhosis, and cataracts. Early diagnosis can lead to treatment that prevents neonatal death. Even when the infant is obviously ill, the correct clinical diagnosis of this rare inborn error of metabolism is often not made. Although newborn screening and early treatment are clearly needed, they have not prevented later complications that include reduced cognition, dyspraxia, neurological abnormalities, and ovarian failure (40).

Several variants of galactosemia have been described (41,42). In the African-American variant, associated with the S135L mutant GALT allele, low but detectable levels of GALT activity are present in the liver and intestinal mucosa but not in erythrocytes, and the clinical course is usually relatively mild. Variants such as Rennes and Indiana may be associated with some or all of the serious clinical abnormalities. The Duarte variant, associated with the N314D mutant allele, is characterized by diminished erythrocyte GALT activity and altered electrophoretic mobility of the enzyme without clinical abnormalities. This variant is probably benign (43).

The two other galactose metabolic disorders are galactokinase deficiency and uridine diphosphate galactose-4-epimerase deficiency. These are much less frequent than galactosemia. Galactokinase deficiency leads to cataract formation in older untreated patients without other evidence of galactose toxicity, whereas epimerase deficiency is usually benign. Treatment of galactosemia or galactokinase deficiency aims at rigorous exclusion of galactose from the diet and, if instituted early, is completely effective in preventing or reversing the potentially lethal neonatal abnormalities (44). Despite successful treatment in infancy, the majority of classic galactosemia patients in all countries nonetheless go on to experience serious long-term complications in cognitive, neurologic, and in females, ovarian functions (41).

## 27.5.3 Homocystinuria (Cystathionine $\beta$ -Synthase Deficiency)

Increased methionine in newborn screening leads to identification of homocystinuria due to CBS deficiency. This can result in early treatment that prevents serious phenotypic abnormalities. CBS is responsible for the condensation of L-homocysteine and L-serine to form cystathionine, using pyridoxal-5-phosphate (vitamin B<sub>6</sub>) as cofactor. A deficiency of CBS produces increased homocysteine and secondary hypermethioninemia. Homocystinuria is inherited as an autosomal recessive condition and may result in clinical abnormalities that include mental retardation, ectopia lentis, thromboembolism affecting large and small arteries, and skeletal abnormalities. Early treatment has been effective in preventing the clinical consequences (45). Treatment includes a diet that is restricted in methionine and supplemented with cystine and, in those patients responsive to pyridoxine, pharmacological doses of the vitamin. Treatment with orally administered betaine has also proved effective in controlling the level of homocystinemia in many patients (46). Some cases of homocystinuria have escaped detection by newborn screening. This has raised concerns about a possible slow rise in blood methionine (22). The blood methionine concentration may not exceed normal (67  $\mu\text{M}$ ) until after the first week of life in affected infants. Lowering the cutoff level for methionine in screening from the usual 134  $\mu\text{M}$

to 67  $\mu\text{M}$  has resulted in detecting cases screened before 48 h of age who otherwise would have been missed (22). Conversely, later in the neonatal period normal infants may have methionine elevations to 170  $\mu\text{M}$  or greater that are usually transient and related to a high-protein diet. Other causes of hypermethioninemia include liver disease, tyrosinemia (see later), and methionine adenosyltransferase I/III deficiency (47), as well as glycine N-methyltransferase deficiency (48,49). Hyperhomocysteinemia and increased urinary homocystine also result from defects in the metabolism of folates and cobalamins that are required for the methylation of homocysteine to methionine (50). In these conditions, the concentration of methionine is low or normal, even though the concentrations of homocysteine and related compounds are increased. Consequently, these remethylation disorders escape detection in newborn screening that is limited to detecting increased methionine.

#### 27.5.4 Maple Syrup Urine Disease

Increased leucine in newborn screening is the marker for MSUD. This disorder results from deficient activity of one of the enzymes involved in the oxidative decarboxylation of the  $\alpha$ -keto acid derivatives of the branched-chain amino acids leucine, isoleucine, and valine (51). The newborn with MSUD is clinically normal at birth but during the first week begins to feed poorly, rapidly followed by vomiting, lethargy, muscular hypertonicity, seizures, coma, and death. Laboratory features include metabolic acidosis and, often, hypoglycemia. The well-known urinary odor of maple syrup may not be present in the newborn. Acute therapy includes elimination of protein intake, high-calorie intravenous fluids, and enteral feeding of a special amino acid elemental formula that does not include leucine (52,53). This anabolic regimen after identification by newborn screening has resulted in the prevention of clinical disease (52) or rapid recovery once clinical symptoms occur (53) and good clinical outcome. Hemodialysis to quickly reduce blood leucine may be required in infants who are critically ill. Several variants of MSUD with milder clinical and biochemical abnormalities have been described (51). A transient elevation of the blood leucine concentration (rarely greater than 6 mg/dL) occurs in 0.1–0.2% of newborns screened.

#### 27.5.5 Tyrosinemia

Tyrosine is measured in all newborn screening programs. The major reason for including the measurement of tyrosine was to identify tyrosinemia I (hepatorenal tyrosinemia), the most frequent of the tyrosinemias. It is now apparent that neonates with tyrosinemia I almost always have normal or near-normal levels of tyrosine in newborn screening and therefore cannot be reliably detected in this manner. In 2002, a method for measuring the

metabolite succinylacetone in the screening blood specimen was developed (54). An elevation of this metabolite is pathognomonic of tyrosinemia I. Consequently, many newborn screening programs have added a succinylacetone test to the screening profile. The inclusion of succinylacetone analysis into routine analysis of acylcarnitines and amino acids allows for rapid and cost-effective screening for tyrosinemia I, with the potential to eliminate the risk of false-negative results. Most screening programs continue to measure tyrosine, however, in order to detect the two other tyrosinemia metabolic disorders, tyrosinemia II and tyrosinemia III. Transient tyrosinemia is prevalent in newborn screening. In at least 1–2% of infants, the blood tyrosine concentration exceeds 5 mg/dL at some time during the first 3 months of life. The frequency is even higher in premature babies. Transient tyrosinemia usually disappears spontaneously within a few weeks; its disappearance can often be promoted by administering vitamin C or with a protein-restricted diet (55).

Tyrosinemia I is an autosomal recessively inherited disorder in which the primary enzyme defect is deficiency of fumarylacetoacetate hydrolase, the final enzyme in the degradation of tyrosine (55). The prevalence of this disorder is unusually high in the Quebec province of Canada. The clinical abnormalities are usually evident within the first 6 months of life but can be detected in a neonate. These abnormalities include hepatomegaly with liver disease, often accompanied by ascites, and kidney disease characterized by the renal Fanconi syndrome. Confirmatory plasma amino acid analysis usually reveals increased tyrosine and methionine, although the amino acid levels in the presymptomatic neonate may be normal. Confirmatory testing should include the measurement of succinylacetone in blood and urine. The presence of elevated succinylacetone confirms the diagnosis. Dietary restriction of phenylalanine and tyrosine has not prevented progression of liver disease, and approximately one-third of dietary-treated patients develop hepatocellular carcinoma, often in childhood. Thus, liver transplantation was previously the only effective option for these patients. Fortunately, a specific therapy known as Orfadin (NTBC) is now available and is the therapy of choice. This therapy has been highly effective in controlling the accumulation of succinylacetone and reversing or preventing the liver disease (56). Liver transplantation is still an option, however, for patients with chronic liver disease in whom the therapy may be too late to prevent hepatocellular carcinoma.

Tyrosinemia II, also called the Richner–Hanhart syndrome, results from deficiency of hepatic tyrosine aminotransferase and has been reported in fewer than 100 cases (55). Blood tyrosine concentrations are 20–50 mg/dL on an unrestricted diet. Mental retardation is usually present, while hepatic and renal involvement is absent. Other clinical abnormalities include keratitis and palmar hyperkeratosis. This disorder does produce elevated blood tyrosine within the first days of life and has been detected by

routine newborn screening using MS/MS (57). Tyrosinemia III may also be detected by elevated tyrosine in newborn screening. This appears to be a benign condition (55).

Other inherited disorders may lead to a secondary elevation of the blood tyrosine concentration when the infant is ill. Notable among these are galactosemia and hereditary fructose intolerance. Both of these disorders elevate blood tyrosine concentration by deleterious effects on hepatic metabolism. Because liver disease of any cause can elevate the blood tyrosine concentration, detecting either tyrosinemia or hypermethioninemia may simply signal the presence of liver disease.

### 27.5.6 Biotinidase Deficiency

Biotinidase deficiency can be detected in newborn screening, and if treated with pharmacological doses of biotin, the otherwise serious abnormalities are prevented. Biotin is a water-soluble vitamin that acts as a coenzyme for four carboxylases required for the catabolism of two intermediary organic acids in branched-chain amino acid metabolism, for the first step of gluconeogenesis, and for the synthesis of fatty acids (7). These carboxylases are normally activated by covalent attachment of biotin. Proteolytic degradation of these carboxylases releases a biotin-containing product known as biocytin (biotinyl-lysine). Normally, biotinidase hydrolytically releases biotin from biocytin, allowing some of the biotin to be recycled. In biotinidase deficiency, the biotin is not released, leading to intracellular deficiency of biotin.

Biotinidase deficiency is inherited in an autosomal recessive pattern (7). Most symptomatic children have profound biotinidase deficiency (less than 10% of the normal mean for serum activity). The frequency of the deficiency in newborns has varied among reported studies of newborn screening results, but the incidence of the profound deficiency is estimated at about 1:112,000 and the incidence of partial deficiency at about 1:129,000, for a combined incidence of 1:60,000 (7). Confirmation of diagnosis is by assay of serum biotinidase activity. Mutant alleles of the biotinidase gene have been identified. Although genotype–phenotype correlations have not been firmly established (7), one mutation, c.1489C>T (p.Pro497Ser), is frequent in profound deficiency and another mutation, c.1330G>C (p.Asp444His), has always linked to partial deficiency (57). Biotinidase-deficient persons may become biotin deficient during infancy or early childhood (8). Signs and symptoms are mainly neurological and cutaneous and include seizures, ataxia, hypotonia, developmental delay, hearing loss, skin rash, and alopecia. Symptomatic individuals with profound deficiency usually also manifest an organic aciduria, metabolic acidosis, and mild hyperammonemia (57). The reversible symptoms of biotinidase deficiency (seizures, rash, and alopecia) have consistently responded to treatment with biotin, and newborn screening identification and treatment prevent all of the symptoms. Importantly,

biotinidase deficiency must be differentiated from holocarboxylase deficiency with which it shares many clinical and biochemical features (8).

### 27.5.7 Urea Cycle Disorders

Routine neonatal screening currently can identify three of the six urea cycle disorders. These include citrullinemia and argininosuccinic acidemia, both identified by increased citrulline in newborn screening, and arginase deficiency, identified by increased arginine. Detailed discussion of these disorders can be found elsewhere in this book (Chapter 92). The urea cycle is responsible for metabolizing ammonia released during the turnover of amino acids, which results from the ingestion and endogenous turnover of protein. A deficiency in any of the six urea cycle enzymes blocks this metabolic pathway and produces hyperammonemia as well as a reduction in urea. The hyperammonemia is extremely toxic to the brain and can produce life-threatening cerebral edema. Citrullinemia (argininosuccinic synthetase deficiency) and argininosuccinic acidemia (argininosuccinic lyase deficiency) may present acutely in the neonate or have a later onset with a chronic course. Mild citrullinemia has been reported as benign (59), and argininosuccinic acidemia may also have a relatively benign or completely benign phenotype (60,61). Arginase deficiency usually has a clinical phenotype very different from the other urea cycle disorders in that the hyperammonemia is milder and the patients have a chronic neurologic picture characterized by spastic diplegia and developmental delay. A rare patient with arginase deficiency, however, has been described with an acute lethal neonatal course (62).

A second form of citrullinemia has recently been reported. This form, called citrullinemia type II or citrin deficiency, is also identified in neonatal screening by increased citrulline in the newborn specimen. The primary defect is in citrin, a mitochondrial carrier protein primarily located in the liver. The most frequent neonatal feature has been intrahepatic cholestasis that often results in a secondary increase in galactose, methionine, phenylalanine, and/or tyrosine, elevations that may also be detected in newborn screening (63).

Several newborn screening programs also screen for decreased citrulline. This allows for the identification of the proximal urea cycle disorders NAGS deficiency, CPS deficiency, and OTC deficiency (14). These three disorders can have a life-threatening neonatal presentation with profound hyperammonemia and, if so, require immediate and dramatic therapy.

Treatment of the urea cycle disorders requires immediate measures for the acute hyperammonemic neonatal course. These include discontinuation of protein intake and administration of intravenous fluids with very high caloric value. In addition, “scavenger” medication such as sodium benzoate and sodium phenylacetate are administered to aid in waste nitrogen removal. Hemodialysis



may be required. The chronic therapeutic regimen consists of a low-protein diet supplemented by the organic acid medications.

### 27.5.8 Organic Acid Disorders

Many of the organic acid disorders may also be detected by expanded neonatal screening using MS/MS technology. Identification is on the basis of an increased concentration of an acylcarnitine. The organic acidemias are a heterogeneous group of disorders and are described in greater detail in Chapter 97 of this book. Most are defects in amino acid metabolism but, in contrast with the amino acid disorders, are at a step in the metabolic pathway where an organic acid derivative of the amino acid is normally converted to another organic acid. The defect results in an increased concentration of the unconverted organic acid, some of which conjugates with free carnitine to produce the increased concentration of an acylcarnitine.

At least 11 organic acid disorders have been identified by expanded newborn screening, most of which are included in [Tables 27-2 and 27-3](#), with the acylcarnitine elevation by which they are detected. The clinical phenotype of the organic acid disorders includes an acute neonatal presentation with metabolic acidosis, hyperammonemia, hepatomegaly and liver dysfunction, perhaps hypoglycemia, and leukopenia or pancytopenia. Such infants are profoundly ill and require emergency measures that include cessation of protein intake and intravenous fluids to provide calories and correct the metabolic imbalance. The most frequent of the organic acid disorders detected by newborn screening that are most likely to have this presentation are propionic acidemia, methylmalonic acidemia, and isovaleric acidemia. Once the infant is stabilized, chronic therapy includes a special diet reduced in the precursor amino acids that lead to the organic acid accumulation and a high caloric intake. These organic acidemias may also have a later onset with a chronic course characterized by developmental delay and other neurologic abnormalities. In addition, many if not most infants with isovaleric acidemia identified by newborn screening have had a mutation that seems to confer a milder or perhaps benign form of the disorder ([65](#)).

Glutaric acidemia type I (GA I) has a more chronic presentation. Affected infants appear to be normal during their first months of life but sometime in the first or second year develop a severe dystonia. Often the onset of dystonia closely follows immunization or an acute febrile illness. Other organic acidemias in [Table 27-3](#) have a variable expression, ranging from a benign course to episodic hypoglycemia with metabolic acidosis and hypotonia, usually not occurring until late infancy or childhood. 3-Methylcrotonyl-CoA carboxylase (3-MCC) deficiency seems to be especially frequent in newborn screening and to often have a benign course ([65](#)). Moreover, in a number of instances in which the infant is screen positive for possible 3-MCC deficiency, the abnormality in the newborn screen proves to be transient and follow-up testing

shows that the mother, not the infant, has the disorder (maternal 3-MCC deficiency).

### 27.5.9 Fatty Acid Oxidation Disorders

Fatty acid oxidation primarily occurs in mitochondria and is critical for energy generation in the fasting state or during exercise. The sequence of fatty acid oxidation includes linkage of the fatty acids to carnitine for transport into the mitochondria, decoupling from carnitine within the mitochondria, and  $\beta$ -oxidation to yield ketones. Each step involves one or more transport or enzyme reactions. Notably, several enzymes, including chain length specific dehydrogenases, are involved in  $\beta$ -oxidation. Chapter 97 in this book describes the pathways of fatty acid oxidation and the FAODs. All the known disorders can be detected by expanded newborn screening ([Tables 27-2 and 27-3](#)). The cardinal feature of FAOD is hypoketotic hypoglycemia. This may present in the neonate and the most likely to do so is MCADD. When MCADD is expressed in the neonate, the hypoglycemia is usually accompanied by metabolic acidosis, hyperammonemia, and hepatomegaly with some degree of liver dysfunction. Neonatal expression has also been described with very-long-chain acyl-CoA dehydrogenase deficiency (VLCADD). The latter is usually associated with hypertrophic cardiomyopathy that can cause an arrhythmia in the neonate but is more frequently not recognized until later in infancy. Long-chain hydroxyacyl-CoA dehydrogenase deficiency (LCHADD) and mitochondrial trifunctional protein deficiency usually have a chronic course that includes liver disease and retinopathy. Carnitine transport defect may produce severe cardiomyopathy and general myopathy in the neonate and can be life threatening. The neonatal expression of carnitine palmitoyltransferase II (CPT II) deficiency is associated with multiple congenital defects and is usually lethal. Short-chain acyl-CoA dehydrogenase deficiency (SCADD) has been reported to have a neonatal expression but current evidence indicates that it is most likely a benign disorder ([66,67](#)).

Despite the possibility of a neonatal presentation, the most important reason to include FAOD in neonatal screening is the high risk of sudden death. This may happen in infancy or in the second or third year of life. The factor that leads to sudden death is almost always vomiting and reduced intake, usually due to an acute illness such as gastroenteritis or upper respiratory infection ([68](#)). The vomiting with the accompanying reduced food intake (“fasting”) provokes hypoglycemia. MCADD is the most frequent of the FAODs and certainly the FAOD that is most prone to sudden death. Nevertheless, many if not most infants identified with MCADD seem to have a mild form of the disorder that may be benign. Evidence suggests that the level of C8 in the initial newborn screen might serve to differentiate infants with clinically significant disease from those with perhaps an insignificant form ([68](#)).

The major treatment of an identified FAOD is prompt attention to any illness in which there is repeated vomiting and lack of intake as well as fasting for more than 10–12 h. Treatment requires intravenous glucose (D10) and hospitalization until vomiting ceases and food intake commences. Chronic treatment includes carnitine supplementation to treat or prevent secondary carnitine deficiency. VLCADD and LCHADD treatment also includes medium-chain triglycerides (MCT) to provide the fatty acids distal to the metabolic block and allow ketones to be formed. For carnitine uptake defect, the treatment is carnitine supplementation. Notably, as in 3-MCC deficiency, maternal carnitine uptake disorder characterized by a low free carnitine level in the mother has been identified in a number of families wherein the carnitine deficiency in the neonate has been transient (69).

## 27.6 OTHER NEWBORN SCREENING

Newborn screening programs in varying numbers of states screen for additional inherited and acquired disorders including congenital hypothyroidism, CAH, CF, and hemoglobinopathies.

### 27.6.1 Congenital Adrenal Hyperplasia

Screening for CAH became technically feasible in 1977 when Pang and colleagues (1) introduced an immunoassay using Guthrie specimens to measure 17-OHP in order to detect CAH due to 21-hydroxylase deficiency, which accounts for more than 90% of all cases. Nationwide and regional screening programs for CAH were subsequently introduced in several countries. Worldwide incidence of CAH was estimated at 1:15,000 live births, with the salt-wasting form comprising 75% of the total and often causing hypovolemic shock and death in newborns and the simple-virilizing form, which does not result in spontaneous hypovolemia, comprising 25%. Molecular testing suggests that the proportion of affected newborns that have the salt-wasting form is closer to 60% (19). Initial screening is by immunoassay for 17-OHP; a second screen is performed in some programs. Most programs have established cutoff levels among programs related to prematurity or low birth weight. Follow-up is often by referral to a medical consultant. Neonatal screening aims to prevent deaths from delayed recognition and treatment of the salt-wasting form, to prevent sex misassignment of affected female newborns that can occur with either the salt-wasting or simple-virilizing forms, and possibly to prevent premature epiphyseal closure in children with simple-virilizing CAH (see also Chapter 87, Genetic Disorders of the Adrenal Gland).

### 27.6.2 Cystic Fibrosis

Newborn screening for CF by measuring the IRT in dried blood followed by DNA analysis for varying number of CF mutations has been widely implemented in the

United States and some other countries. Newborns in all 50 US states are now screened for CF. CF was included among the 29 core disorders in the national uniform panel (4) following endorsements of the screening by the US Centers for Disease Control and Prevention (CDC) and the recommendation of the US Cystic Fibrosis Foundation. Several longer term studies from Wisconsin provided evidence of nutritional and developmental benefits, and improved survival in CF newborns (70). Newborns in Australia have been screened for CF for many years. The most convincing evidence of a short-term benefit was the demonstration that the number of hospital days during the first 2 years of life required by children with CF identified by screening 400,000 newborns in Australia was lower for the screened patients than in affected children born prior to screening. Long-term benefits have also been demonstrated in Australia (71).

Based on detailed analysis, the CDC concluded that the net balance of benefits and risks was favorable if state programs were of high quality and carefully monitored (72). Current CF newborn screening in North America and Australia, and in some European countries, begins with the measurement of IRT in the Guthrie specimen. Most US programs use an IRT/DNA algorithm (73). Specimens with IRT values exceeding the cutoff are analyzed for either the common F508del *CFTR* mutant allele alone or, more often, a panel of up to 400 *CFTR* mutations. The mutations used may include *CFTR* alleles from the ACMG 23 mutation panel or others based on the allele frequencies in local populations. Some programs follow up with *CFTR* gene scanning and selective sequencing (74). Based on the magnitude of the IRT elevation, along with the number and identity of the *CFTR* mutations, screen-positive infants are referred for sweat testing, medical management and genetic counseling (see also Chapter 58, Cystic Fibrosis).

### 27.6.3 Hemoglobinopathies and Thalassemias

Most programs in the United States screen newborns for hemoglobinopathies, particularly those involving sickle hemoglobin, with the primary objective of detecting the sickle-cell diseases. The initial screen is most frequently by means of isoelectric focusing, less often by high-performance liquid chromatography or cellulose acetate electrophoresis. Confirmatory testing also uses these methods, and programs are increasingly adding genotyping by DNA analysis for this purpose. The early identification of infants with one of the sickling hemoglobinopathies coupled with aggressive follow-up has been shown to result in a significant reduction of mortality during the early years when the risks are especially great. Moreover, the investigators in the multicenter oral penicillin prophylaxis trial achieved an 84% reduction in the incidence of pneumococcal disease, a major cause of early morbidity and mortality in the sickling disorders, by means of inexpensive and safe treatment with

penicillin. In addition to treatment of affected newborns and genetic counseling of their parents, some programs attempt to follow up the parents of carrier newborns in order to identify and counsel those couples in which both are carriers. Ascertainment of 28 at-risk pregnant women as a result of parent testing in a large newborn hemoglobinopathy screening program led to amniocentesis in 14 and elective termination of three of the four affected pregnancies. In contrast, only a small percentage of those couples ascertained by means of prenatal carrier screening in a large program with pregnancies at risk for a serious globin disorder chose to use prenatal diagnosis (1). Screening programs outside major metropolitan areas have addressed the issue of whether to screen all newborns and have accepted the additional costs and problems that accompany increasing program size rather than attempt to identify and screen only those newborns that, based on racial or other criteria, are considered to be at risk (see also Chapter 71, Hemoglobinopathies and Thalassemias).

### 27.6.4 Congenital Hypothyroidism

Congenital hypothyroidism was once a prominent cause of mental retardation but identification by newborn screening followed by therapy with T4 has virtually eliminated retardation from this disorder. Screening for congenital hypothyroidism began in 1974 with the introduction of a radioimmunoassay for T4 applicable to the newborn filter paper blood specimens (2). Screening for congenital hypothyroidism is now part of many programs and has had the interesting effect of promoting regionalization of screening because the immunoassay used for hypothyroidism was technically more complicated than the bacterial assays used for inborn error screening at that time. Many programs now use elevated TSH as the marker for identifying congenital hypothyroidism. A T4 value two standard deviations or more below the mean or a markedly increased level of TSH or both suggest the presence of congenital hypothyroidism in the infant and the parents and physician are advised to obtain additional testing and possible treatment. This approach identifies congenital hypothyroidism in approximately 1:3600 to 1:5000 newborns (75). Although commonly combined with newborn screening for principally genetic disorders, only a few of the etiologies of congenital hypothyroidism are Mendelian (76) (see also Chapter 84, Thyroid Disorders).

### 27.6.5 Other Disorders

Other disorders that were included in some newborn screening programs but are no longer included are  $\alpha_1$ -antitrypsin deficiency, Duchenne muscular dystrophy, and adenosine deaminase deficiency. The hyperlipidemias have been considered for newborn screening. To date, only severe combined immune deficiency (SCID) has

been recommended for addition to the uniform screening panel. SCID newborn screening has been initiated in a number of states using the circles assay described by Puck (77).

### 27.6.6 Lysosomal Disorders

The potential role for and importance of newborn screening has been heightened by other developments. Specific enzyme therapy for Gaucher disease became available in 1994. Specific enzyme therapies have become available for mucopolysaccharidosis type I (MPS I—Hurler, Hurler–Scheie and Scheie syndromes), mucopolysaccharidosis type II (MPS II—Hunter syndrome), glycogen storage disease type II (Pompe disease), and mucopolysaccharidosis type VI (MPS VI—Maroteaux–Lamy syndrome). Substrate-diverting medications have also been developed for several of these. Experience with enzyme therapies in already diagnosed adults and older children has shown that, in general, the course of the pathologic changes in these disorders can be slowed but not substantially reversed, except perhaps for hepatosplenomegaly. Hematopoietic stem cell transplantation (HSCT), involving the intravenous infusion of autologous or allogeneic stem cells collected from bone marrow, peripheral blood, or umbilical cord blood, has also been used successfully in several of these disorders. In most cases, HSCT must be done in the earliest stages of the disease to be successful. Detection at the earliest possible time has thus seemed to offer the greatest opportunity for successful treatment.

In response to strong and organized advocacy, in August 2006, New York became the first state in the United States to initiate population-wide screening for a lysosomal disorder. Scientists at the Wadsworth Center in Albany (78) successfully adapted previous high-throughput methods (79,80) for the measurement of galactocerebrosidase (GALC) by MS/MS in blood spots. The initial analysis of the galactocerebrosidase activity of 139,074 anonymous newborns, 56 known carriers, and 16 Krabbe patients using a MS/MS method provided data to establish cutoffs and a diagnostic algorithm. Blood specimens with reproducibly low GALC activity were analyzed for GALC mutations, particularly for homozygosity for the 30-kb deletion because of its strong association with early infantile Krabbe disease. Directed primarily toward the early ascertainment of the severe infantile form of Krabbe disease for the purpose of prompt stem cell transplantation, the complexities in this disorder posed obstacles. Initial discussions involving the directors of the regional Metabolic Disease Treatment Centers to whom screen-positive newborns would be referred for confirmation and management raised concerns about many unresolved issues. Uncertainties were apparent regarding the methods for establishing the diagnosis of the infantile form as well as for

ruling it out. Facilities with the expertise needed for confirmatory laboratory and clinical testing, and for HSCT, were limited. The discussions were broadened with the formation of the Krabbe Disease Consortium, the composition and initial deliberations of which have been described (81). The participants included the directors of the eight metabolic disease specialty centers of New York and scientists from the New York State (NYS) screening laboratory along with child neurologists at all the participating institutions, neuroradiologists, neurophysiologists, transplant physicians, neurodevelopmental pediatricians, among others. The Consortium developed an algorithm for classifying screen-positive newborns into high-, moderate- and low-risk categories, each associated with a specific plan for diagnostic and clinical follow-up. Positive screens proved to be more frequent than was initially expected from the estimated population frequency for infantile Krabbe disease of 1 in 100,000 births. Screening of more than 1.45 million newborns since its inception five and one-half years ago has led to referral of 248 infants for neurodiagnostic evaluation in the regional centers. Follow-up laboratory measurements and neurodiagnostic studies have for many of these infants indicated no further concerns or placed them in the low-risk category. Experience during the past few years has shown that a substantial number of newborns who had GALC activities in the moderate- or high-risk range did not develop the infantile-onset phenotype, although concerns remain about the possible development of later onset forms of Krabbe disease. Four newborns were originally identified as affected. One family declined transplant and the child is currently in a palliative care facility. Three have been transplanted. One died in transplant of multiorgan failure. Another developed chronic autoimmune hemolytic anemia and the steroid treatment has led to cardiomyopathy and steroid myopathy. The third was already symptomatic when transplanted at three weeks of age and reportedly has severe neurologic impairments. None of the remaining high-risk newborns and none of the moderate- or low-risk children has developed clinical signs of disease. The Krabbe Disease Consortium has continued to meet to discuss further developments and the many remaining issues and uncertainties. Pending legislation in New York would require screening of newborns for Fabry, Gaucher, Pompe and Niemann–Pick types A and B diseases. Similar bills have been passed in Illinois, Missouri and New Mexico.

Addition of Krabbe disease screening has been proposed under the established formal process for individuals or organizations to nominate a heritable disorder to be considered for inclusion in the recommended uniform screening panel. As indicated on the Web site of the Advisory Committee on Heritable Disorders in Newborns and Children [Web resource 5], six conditions have been proposed to the Advisory Committee: Krabbe disease, Fabry disease, Niemann–Pick disease, Pompe disease, SCID,

and (SMA). The considerations underlying the decision not to approve at this time the requests for the addition of Krabbe disease corresponded to many of the concerns expressed by the Krabbe Disease Consortium (82).

## 27.7 ISSUES AND CONCERNS IN SCREENING

Most screening tests are designed to be sufficiently sensitive to minimize false-negative results, but this reduces specificity and renders them seldom diagnostic. Accurate and definitive diagnosis requires additional testing in nearly all instances. Regardless of sensitivity, false-negative results occur with every screening procedure, so a negative result from screening should not preclude appropriate evaluation for a disorder suspected clinically.

Most newborn screening programs store the newborn screening specimen after the testing has been completed. The original purpose was to have the specimen available for retesting should the child be clinically identified with a disorder but have had a presumably normal newborn screening result. This might identify flaws in the screening process, either an incorrect cutoff level or a laboratory error, or show that the newborn screening marker for a disorder is not always present in an affected baby. In fact, the stored specimen has been a very valuable source of information for all three of these purposes. The metabolites have been found to be surprisingly stable in the stored specimen, especially under optimal storage conditions (78). The duration of storage varies widely among programs, ranging from a few weeks to decades. Because it is possible to perform extensive DNA analysis in the specimen, the policy of storage has been included in the category of “biobanking,” provoking controversy and, in some instances, a legal challenge to the practice. Procedures are being developed to require consent for the use of the stored specimen and to ensure strict confidentiality of testing results (84).

Expanded newborn screening by MS/MS has resulted in substantially higher frequencies of certain disorders than expected on the basis of previous clinical ascertainment. While there is no doubt that expanded screening has substantially improved the short-term outcome of many metabolic disorders (85,86), the greater number of cases has led to uncertainty of clinical significance in many of the identified infants (87,88). This uncertainty as well as the need to assess long-term outcome in all disorders has led to plans for the development of a national long-term follow-up study within the structure of the Newborn Screening Translational Research Network organized by the ACMG (89). Within regional screening programs, several long-term follow-up efforts are underway (90–94).

Screening programs and procedures should meet a variety of criteria both before they are implemented and continually during their operation. These criteria include technical, educational, and organizational aspects. A high signal-to-noise ratio is needed to maximize effectiveness



and minimize adverse effects. The noise in the system includes the true biological variation of the character being measured in the screened population and the variation within the testing procedure itself. In addition to normal biological variation and transient abnormalities, confusing artifacts are produced by contamination of blood and urine specimens with microorganisms, drugs used systemically or locally, diaper powder, food supplements, and the like. Disentangling these sources of confusion and delay requires great expertise. As noted earlier, positive results in a screening test necessitate repeat testing with follow-up and additional testing, possibly over a period of weeks or months. These can be expensive in terms of personnel, materials, and parental anxiety and inconvenience (6). The rarer the trait being sought by screening, the higher will be the proportion of false positives.

Finally, screening programs should be organized and administered in such a way that they are constantly updated to incorporate the latest technical and medical advances. They must also be responsive to changes in society. After several decades with few fundamental changes in neonatal screening programs, the recent discussions centered on the potential advantages and limitations of expanded screening based on MS/MS technology has stimulated much debate and discussion. Advocacy groups consisting of professionals, parents of affected children, and others have increasingly appealed to screening programs themselves and to state legislatures for the addition of this screening that detects a number of disorders not detected by other screening methods. Interest has focused particularly on disorders such as GA I and MCADD. In both disorders, early presymptomatic therapy and anticipation of acute intercurrent illness can prevent the otherwise irreversible brain damage or sudden death. Moreover, the interval between birth and the first episode is sufficiently long to allow an appropriate metabolic diagnosis. These considerations would seem to strongly favor inclusion of these disorders in routine newborn screening.

The introduction of MS/MS technologies in several of the larger screening programs has stimulated a major reevaluation of newborn metabolic screening after many years of relative constancy (95). The widespread inclusion of this or other new technologies in screening programs requires that the traditional questions and issues be addressed. Is the frequency of the disorder detected sufficiently great to justify screening? Are the genetic heterogeneity, expression, and natural history of the disorder well characterized? Is an effective treatment available and, if so, must it begin shortly after birth or will it be equally effective if started later? What is the detection rate, and what are the frequencies of false-positive and false-negative screening test results? Is the screen cost-effective? Discussions and debates regarding the claims, issues, and concerns about disorders and conditions to be screened will likely produce major and extensive changes in screening programs over the next few years.

## CROSS REFERENCES

Cystic Fibrosis; Hemoglobinopathies and Thalassemias; Immunodeficiency Disorders; Genetic Disorders of the Adrenal Gland; Amino Acid Metabolism; Disorders of Carbohydrate Metabolism; Organic Acidemias and Disorders of Fatty Acid Oxidation.

## GLOSSARY

**Algorithm** – a scheme for sequential testing to confirm or reject the diagnosis of a disorder.

**Amino acid disorder** – a metabolic abnormality in the catabolism of an amino acid resulting in an increase in the amino acid and clinical problems that often include mental retardation.

**Bacterial (inhibition) assay** – a test on a bacterial plate that responds to an increased level of an amino acid by growth around a disc from the Guthrie newborn screening specimen.

**Conditions, core and secondary** – core conditions are those disorders that the American College of Medical Genetics recommended as primary targets for newborn screening in all states. Secondary conditions are those disorders identified in newborn screening as a result of screening for the core conditions.

**DNA-based gene tests** – in newborn screening this refers to tests of genes in the DNA of the dried blood specimen for the purpose of either primary newborn screening (e.g. screening for SCID) or as second-tier screening of the newborn specimen in which an abnormality was identified in the initial newborn screen (e.g. cystic fibrosis).

**Exome and genome sequencing** – exome sequencing is a strategy to selectively sequence the coding regions of the genome to identify novel genes associated with rare and common disorders. Whole genome sequencing refers to sequencing all of the bases in the genome of an individual.

**Expanded newborn screening** – expansion of newborn screening using MS/MS to include many metabolic disorders not previously identifiable by newborn screening.

**False-positive result** – concluding that after additional testing an individual initially identified in screening as possibly having a disorder does not have the disorder.

**Fatty acid oxidation defect** – a metabolic abnormality in the mitochondrial oxidation of fatty acids resulting in the lack of ketone production, especially harmful when glucose is limiting and ketones are required as a source of energy.

**Genotype–phenotype correlation** – the degree to which a genotype predicts the clinical severity of a genetic disorder.

**Guthrie specimen or card** – the dried blood on filter paper collected from the heel of the infant and sent for newborn screening to a newborn screening laboratory.

**Lysosomal storage disease** – any one of a large number of disorders caused by a defect in one of the lysosomal enzymes, resulting in the accumulation of the molecular substrate for that enzymes. The accumulation is usually in neurons and/or reticuloendothelial cells.

**Organic academia** – a metabolic abnormality in the catabolism of an organic acid resulting in an increase in the organic acid as well as related organic acid metabolites, which produce metabolic acidosis as well as secondary clinical problems.

**Phenylalanine-restricted diet** – a special diet markedly reduced in phenylalanine for the treatment of PKU. It is composed of foods very low in protein (e.g. fruits and vegetables) and an elemental formula consisting of required amino acids except for phenylalanine with added vitamins, minerals, a carbohydrate source and, often, fat.

**Positive predictive value (PPV)** – the likelihood that an individual has the disorder for which the screening is conducted after identification in population screening.

**Screening test and diagnostic test** – a screening test is a test applied to a population (i.e. all newborns) to identify the rare individual in that population who has an abnormality on that test while eliminating from consideration almost all (but not all) other members of that population. The test should have high sensitivity but low specificity. A diagnostic test is a test with both high sensitivity and high specificity that distinguishes the individual identified by newborn screening who has the disorder from those who screened positive but are not affected.

**Second-tier testing** – additional testing of the Guthrie newborn screening specimen by a technique not used in routine newborn screening to increase specificity for a disorder identified by initial newborn screening.

**Sickle-cell disease** – an inherited hematological disorder in which the hemoglobin is abnormal and tends to form the shape of a sickle, especially under conditions of low oxygen. Includes sickle-cell anemia (hemoglobin SS), SC disease (hemoglobin SC) and beta-thalassemia (hemoglobin S<sup>thal</sup>).

**Tandem mass spectrometry (MS/MS)** – a test method that measures amino acids and carnitine-linked organic and fatty acids used in newborn screening as well as to confirm metabolic disorders identified in newborn screening.

**Urea cycle disorder** – a metabolic abnormality in the urea cycle resulting in hyperammonemia and reduced urea synthesis.

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## WEB RESOURCES

1. Recommended Uniform Screening Panel of the Secretary's Advisory Committee on Heritable Disorders in Newborns and Children. <http://www.acmg.net/resources/policies/ACT/condition-analyte-links.htm>.
2. National Newborn Screening and Genetics Resource Center. <http://genes-r-us.uthscsa.edu/>.
3. ACMG Actsheets and Confirmatory Algorithms. <http://www.acmg.net/resources/policies/ACT/condition-analyte-links.htm>.
4. GeneTests. <http://www.genetests.org> or <http://ncbi.nlm.nih.gov/sites/GeneTests>.
5. Advisory Committee on Heritable Disorders in Newborns and Children. <http://www.hrsa.gov/heritabledisorderscommittee/nominate.htm>.

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### Biographies



**Richard W Erbe** received his MD degree in 1964 from the University of Michigan Medical School in Ann Arbor. He completed Residency in Medicine at the Peter Bent Brigham Hospital and Harvard Medical School in Boston. Thereafter, he was a research associate with Dr Philip Leder at the National Cancer Institute, then research fellow in the Department of Biological Chemistry at Harvard Medical School. In 1970, he joined Dr John W. Littlefield in the Genetics Unit at the Massachusetts General Hospital, later becoming Chief as well as associate professor of Pediatrics and Genetics at Harvard Medical School. During this time he was Director of the NIGMS Genetics Training Grant and Program (postdoctoral) at Harvard Medical School; Founding Codirector with Dr Allen Crocker of the New England Genetics Group; Chair of the American Society of Human Genetics Program Committee (1979–1982); and Member, Editorial Board, New England Journal of Medicine (1976–1979). He is certified by the American Board of Internal Medicine (1974) and the American Board of Medical Genetics (Clinical Genetics 1982, Clinical Cytogenetics 1993, and Clinical Molecular Genetics 1993). In 1989, he moved to the Children's Hospital of Buffalo and SUNY Buffalo, where he is Chief of the Division of Genetics and Director of the Metabolism Program. His research interests focus on inborn errors of folate metabolism, and biochemical and molecular genetics.



**Harvey L Levy, MD**, is senior physician in Medicine and Genetics at Children's Hospital Boston and professor of Pediatrics at Harvard Medical School. He has been involved in metabolic disorders for over 40 years. This involvement includes newborn screening, diagnosing and treating children and adults, and research. Dr Levy attended Emory University in Atlanta and obtained his medical degree from the Medical College of Georgia. He trained in pediatrics in Boston, New York, and Baltimore, then concentrated in metabolic disorders under one of the pioneers, Dr Mary Efron, at the Massachusetts General Hospital. Following this training he served on the faculty at the Massachusetts General Hospital, Director of the Massachusetts Metabolic Disorders Program, and Chief of Biochemical Genetics in the New England Newborn Screening Program. For the past 33 years he has been at the Children's Hospital, Boston, and has served as director of the Metabolic Program, Director of Metabolic Research and the Maternal PKU Program and, currently, attending in metabolism. He has authored over 400 publications on metabolic disorders, including journal articles, books, chapters and reviews. A major interest has been the process and follow-up of newborn screening, particularly the study of outcome in metabolic disorders identified by screening.

# CHAPTER

# 28

## Therapies for Lysosomal Storage Diseases

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### 28.1 INTRODUCTION

There has been remarkable progress in the ability to treat lysosomal storage diseases. Enzyme replacement therapy (ERT), bone marrow transplantation, and substrate deprivation are currently being used to treat these disorders, and the potential of pharmacologic chaperones, gene augmentation, and stem cell therapies is being investigated (1–7). This review focuses on the current status and the lessons learned from treatment of lysosomal storage diseases by ERT and describes the potential of emerging therapeutic strategies for the treatment of the visceral as well as the central nervous system (CNS) manifestations of these diseases.

About 40 years ago, Nobel Laureate Christian de Duve first suggested that lysosomal diseases could be treated by replacing the defective enzyme with its normal counterpart (8). Subsequent experiments demonstrated that when the appropriate active normal enzyme was added to the media of enzyme-deficient cultured fibroblasts from individuals with various lysosomal storage diseases, the exogenous enzyme gained access to and degraded the substrates accumulated in the lysosomes (9–11). Of note, only 1–5% of normal intracellular enzyme activity was required to correct the metabolic defects in the enzyme-deficient cells. The subsequent discovery that lysosomal enzyme glycoproteins are targeted to the lysosome by the mannose 6-phosphate residues on their oligosaccharide chains provided further rationale for the treatment of lysosomal disorders by ERT (12,13). The pathway mediated by mannose 6-phosphate receptor is

responsible for the intracellular delivery of newly synthesized lysosomal enzymes to the lysosomes, as well as for the delivery of exogenously supplied normal lysosomal enzymes to the lysosomes via the mannose 6-phosphate receptors on the plasma membranes of most cell types (for reviews, see Kornfeld (12) and Sabatini and Adesnik (13)). These studies indicated the feasibility of ERT and, in particular, that exogenous lysosomal enzymes could gain access to intracellular lysosomal sites and that low levels of enzyme activity could metabolize the accumulated substrate(s) in cellular lysosomes. Thus, these findings provided the rationale for the early clinical studies of ERT in lysosomal storage diseases. However, it was not until the early 1990s that ERT became a reality with the demonstration of its safety and effectiveness in patients with type 1 Gaucher disease (14,15). Subsequently, ERT was approved for Fabry disease, Pompe disease and mucopolysaccharidoses I, II and VI, and clinical testing of ERT for Niemann–Pick disease (NPD) type B and mucopolysaccharidosis IVA are in progress. Table 28-1 lists the lysosomal storage diseases treatable by ERT.

The fact that ERT does not correct all the manifestations of lysosomal storage diseases or does not effectively address neuropathic diseases has prompted investigations into the potential use of small molecule drugs (for recent review, see Smid (16)). These alternate therapies could be deployed either as stand-alone or adjuvant therapies to ERT. One of the more developed concepts is referred to as substrate reduction therapy (SRT), which seeks to reduce the rate of biosynthesis of the substrate (17–20).

TABLE 28-1    Lysosomal Diseases Treatable by ERT						
Disease/Subtypes	Deficient Enzyme	Inheritance	Residual Activity	CNS Involvement	Primary Site of Pathology	Major Manifestations
<b>Gaucher disease type 1</b>	Acid $\beta$ -glucocerebrosidase	AR	+	–	RES	Hepatosplenomegaly, skeletal disease
<b>Fabry disease</b>						
Classic	$\alpha$ -Galactosidase A	XLR	–	–	Vascular endothelium	Renal failure, pain, skin lesions, stroke
Later-onset cardiac variant	$\alpha$ -Galactosidase A	XLR	+	–	Cardiomyocytes	Cardiomegaly, arrhythmias & failure
<b>Pompe disease</b>						
Infantile	$\alpha$ -Glucosidase	AR	–	–	Cardiomyocytes	Cardiomegaly, muscular weakness
Later-onset type	$\alpha$ -Glucosidase	AR	+	–	Myocytes	Muscular weakness
<b>MPS I</b>						
Hurler	$\alpha$ -L-Iduronidase	AR	–	+	CTC, RES, neurons	Skeletal disease, organomegaly, hearing loss
Hurler–Scheie	$\alpha$ -L-Iduronidase	AR	+	–	CTC, RES	Intermediate phenotype between Hurler and Scheie
Scheie	$\alpha$ -L-Iduronidase	AR	+	–	CTC	Corneal clouding, joint contractures, normal intelligence
<b>MPS II</b>						
Severe	Iduronate sulfatase	XLR	–	+	CTC, RES	Skeletal disease, neurons organomegaly, early demise
Mild	Iduronate sulfatase	XLR	+	–	CTC, RES	Normal intelligence, short stature, normal life span
<b>MPS VI</b>	Arylsulfatase B	AR	–	+	RES, CTC	Corneal clouding, normal intelligence
<b>MPS VII</b>						
Infantile	$\beta$ -Glucuronidase	AR	–	–	CTC, RES neurons	Skeletal disease, hepatosplenomegaly, normal intelligence
Adult	$\beta$ -Glucuronidase	AR	+	–	CTC, RES	Skeletal disease, hepatosplenomegaly, normal intelligence
<b>Nieman–Pick disease type B</b>	Acid sphingomyelinase	AR	+	–	RES	Hepatosplenomegaly, pulmonary insufficiency, normal intelligence
<b>Wolman disease</b>	Acid lipase	AR	–	–	Liver	Hepatosplenomegaly, steatorrhea, death before 1 year of life
<b>Cholesteryl ester storage disease</b>	Acid lipase	AR	+	–		Hepatosplenomegaly, widespread lipid storage

AR, autosomal recessive; CTC, connective tissue cells; RES, reticuloendothelial system; XLR, X-linked recessive.



The intent is to reduce the burden of accumulation of the offending substrates, thereby offsetting the catabolic deficiency and restoring the balance between the rate of biosynthesis and catabolism. At present, an inhibitor of glucosylceramide synthase (miglustat), the enzyme that catalyzes the first step in the synthesis of glycosphingolipids, is approved for treating patients with mild type I Gaucher disease (21). As miglustat is reportedly able to traverse the blood–brain barrier, it has also been approved in Europe for treating NPD type C (22).

The use of small molecules that act as pharmacologic chaperones to rescue misfolded/mistrafficked mutant proteins provides a novel and attractive approach for the treatment of protein misfolding disorders (for recent reviews, see Perlmutter (23), Cohen and Kelly (8), Desnick (24), and Ulloa-Aguirre and colleagues (25)). For lysosomal enzymopathies, this therapeutic strategy involves the use of reversible competitive enzyme inhibitors that can rescue misfolded/mistrafficked enzymes that would otherwise be transported to the proteasome for degradation by the endoplasmic reticulum-associated degradation (ERAD) machinery (for reviews, see Hampton (26), Ulloa-Aguirre and colleagues (25), and Meusser and coworkers (27)). Pharmacologic chaperones are attractive therapeutic agents as they can be orally administered, may cross the blood–brain barrier, and presumably can gain access to most or all cell types. These small molecule chaperones have entered clinical testing for Fabry, Pompe, and Gaucher diseases.

An emerging technology platform for treating lysosomal storage disorders is gene augmentation therapy (for review, see Chapter 29). This approach seeks to genetically modify a subset of patients' cells *in situ* to produce and secrete the deficient enzymes into systemic circulation. As the enzymes are expressed continuously, the higher bioavailability might facilitate improved efficacy, particularly for those organs that are not well served by periodic infusions of exogenous enzymes. This modification can be realized using a number of gene transfer systems of viral and nonviral origin (for reviews, see Cheng (28), Biffi (29), Sands (30)). The most advanced of these delivery systems is that based on adeno-associated viral (AAV) vectors. While systemic gene therapy with recombinant AAV vectors for lysosomal storage diseases is still in preclinical research, this technology is in clinical development for other genetic diseases. Similar vector systems have also been employed to deliver the therapy directly to the CNS to address lysosomal storage diseases with neuropathic disease. Although the procedure of intracerebral delivery is invasive, reports that expression of the enzymes can persist for a very protracted period provides continued optimism.

In this chapter we review the current status of ERT for lysosomal storage diseases and emphasize the principles for effective treatment. We also provide an overview of emerging therapies for lysosomal diseases and describe recent *in vitro* and *in vivo* studies that highlight the potential of these therapeutic strategies.

## 28.2 ERT FOR LYSOSOMAL STORAGE DISEASES

### 28.2.1 Early Clinical Studies

Beginning in the early 1970s, pilot clinical studies of ERT were undertaken in several lysosomal disorders (Fabry, Gaucher, Pompe, and Sandhoff diseases) by intravenous infusion of the respective normal human enzyme. In each case, the partially purified human enzyme was rapidly cleared from the circulation ( $t_{1/2}$  approximately 10–20 min), and there was evidence for clearance of the respective accumulated substrate(s) (for reviews, see Desnick and coworkers (31,32), Tager and colleagues (33), and Desnick (34)).

These early and encouraging studies supported the feasibility of enzyme replacement with highly purified human enzymes. However, they clearly indicated that the treatment of disorders with primary neuronal involvement was not feasible by this approach, since intravenously administered enzymes did not cross the blood–brain barrier (35). Thus, investigators realized that ERT for disorders with severe neurologic involvement, such as Tay–Sachs disease, Sandhoff disease, and type A NPD, was not feasible and focused their efforts on disorders without significant neurologic involvement.

In 1972, and again in 1979, the March of Dimes Birth Defects Foundation sponsored international workshops on “Enzyme Therapy in Genetic Diseases,” in which basic and clinical scientists reviewed the developments in the area (31,34). In addition, these workshops identified the major obstacles confronting successful ERT in lysosomal diseases, which included (1) the inability to produce and purify sufficient quantities of lysosomal enzymes, including specific glycoforms; (2) the inability to target exogenously administered enzymes to specific tissues and cellular sites of pathology, particularly to the CNS; and (3) the lack of animal models of human lysosomal diseases to evaluate the pharmacokinetic and pharmacodynamic effects of enzyme administration.

### 28.2.2 ERT Proven Safe and Effective in Type 1 Gaucher Disease

In the 1980s, many researchers found the obstacles to ERT too formidable to pursue and focused their research on more basic studies of lysosomal biology and disease. Only a few researchers continued to pursue clinical studies of ERT. Among these, Dr Roscoe Brady and colleagues at the National Institutes of Health were determined to develop ERT for type 1 Gaucher disease, which is caused by the deficient activity of  $\beta$ -glucocerebrosidase (36). The primary cellular site of pathology in Gaucher disease is the macrophage/monocyte system, and the bone marrow and reticuloendothelial organs of affected individuals become infiltrated with lipid-laden “foam” cells, known as “Gaucher” cells. Patients develop massive enlargement of their

livers and spleens, pancytopenia, and severe skeletal disease causing severe bone pain and pathologic fractures.

Brady's group sought to reverse, or at least halt, the disease progression by ERT with  $\beta$ -glucocerebrosidase purified from human placenta. Although most lysosomal glycoproteins are targeted to the lysosome via the trafficking system mediated by mannose 6-phosphate receptor,  $\beta$ -glucocerebrosidase was not. It was shown that  $\beta$ -glucocerebrosidase was targeted to the mannose-specific receptors on macrophages, the primary cellular site of pathology in type 1 Gaucher disease. This was accomplished by modifying the enzyme's *N*-linked oligosaccharide chains by sequentially removing the sialic acid,  $\beta$ -galactosyl, and  $\beta$ -*N*-acetylglucosaminyl residues, thus exposing the mannose residues (37). This "mannose-terminated" form of the enzyme was efficiently recognized by the abundant mannose receptors on macrophage membranes and then delivered to macrophage lysosomes for substrate catabolism (37). Early results of enzyme replacement using the mannose-terminated enzyme had encouraging but limited clinical effects, presumably because of the small doses administered (38).

Many investigators were skeptical about the prospects for enzyme therapy until Brady's group reported in the early 1990s that weekly or biweekly intravenous infusions of large doses (2–3 mg/kg) of the mannose-terminated enzyme markedly reduced the hepatosplenomegaly, improved the hematologic values, and led to substantial improvements in bone density and other clinical manifestations in these patients (14,15,39). Their demonstration that biweekly ERT was safe and well tolerated, and that the enzyme could reverse years of substrate accumulation in these patients, provided the first clinical "proof of principle" for ERT in lysosomal disorders without primary neurologic involvement.

Initially,  $\beta$ -glucocerebrosidase was purified from human placenta by industrial-scale techniques; later, the recombinant human enzyme produced in Chinese hamster ovary (CHO) cells was shown to be equally effective (40). Since a viable murine or naturally occurring animal model for Gaucher disease did not exist, investigators experimented with the dose and dose schedule to determine the minimum effective dose of the enzyme in order to decrease the cost of therapy. Eventually, it was appreciated that clinical response was dose dependent and that the maintenance dose was not significantly different from the dose originally used to reverse years of substrate accumulation. During the past 15 years, worldwide experience with ERT in over 4000 patients with type 1 Gaucher disease has clearly documented its safety and effectiveness (for reviews, see Grabowski and associates (41) and Weinreb and colleagues (42)).

Table 28-2 lists the principles learned from over a decade of treating type 1 Gaucher disease by ERT. Of note, the reversal of lysosomal storage by ERT was unexpected, as most investigators presumed that the storage was irreversible and that, at best, stabilization of the disease process would be obtainable. The principles learned

**TABLE 28-2 Principles of ERT Learned from Gaucher Disease**

<ul style="list-style-type: none"> <li>• Proof of concept: ERT works</li> <li>• Safe and generally well tolerated               <ul style="list-style-type: none"> <li>– Minimal immunologic complications (cross-reactive immunologic material positive (CRIM+) patients)</li> </ul> </li> <li>• Dose-dependent enzyme delivery               <ul style="list-style-type: none"> <li>– Mannose receptor-mediated enzyme uptake</li> <li>– Inability to cross blood–brain barrier</li> </ul> </li> <li>• Dose-dependent substrate clearance</li> <li>• Unexpected reversal of lysosomal storage in organs</li> <li>• Rapid reaccumulation off therapy: lifetime therapy</li> <li>• Need to treat early to prevent irreversible disease</li> </ul>
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included (1) enzyme delivery was receptor mediated and dose dependent, (2) substrate clearance was dose dependent, (3) decreased dose or cessation of ERT resulted in rapid substrate reaccumulation, and (4) patients experienced clinical benefit. In addition, it soon became apparent that the earlier the ERT was initiated, the more effective it was—even preventive, since irreversible damage (e.g. bone disease, fibrosis) could not be altered.

Over the past few years, alternate sources of recombinant  $\beta$ -glucocerebrosidase have been proposed for use in treating type I Gaucher patients. These so-called biosimilars are made in human fibroblasts (43) through gene activation and transgenic carrot root cells (44). Although all versions contain minor differences in amino acid sequences, comparison of the forms made in CHO cells and human fibroblasts showed no major differences with respect to pharmacokinetics and efficacy in pre-clinical and clinical studies (45,46). It is anticipated that longer term studies in the clinic will be required to fully understand the safety profile of the biosimilars, particularly those that are derived from plant cells.

### 28.2.3 ERT for Other Lysosomal Diseases

The success of ERT in type 1 Gaucher disease stimulated investigators to develop and evaluate enzyme replacement for other lysosomal storage diseases, and the past and current clinical trials of ERT in these diseases are listed in Table 28-3. As discussed in this section, these efforts were facilitated by the cloning of the complementary DNAs (cDNAs) and genes encoding most of the human lysosomal enzymes, the development of eukaryotic expression systems to produce large quantities of recombinant enzyme (i.e. Ioannou and colleagues (47)), and the use of gene-targeting techniques to generate "knockout" murine models for preclinical studies. These advances abrogated two of the major obstacles to ERT: the lack of sufficient amounts of human enzyme and the need for animal models for preclinical studies.

**28.2.3.1 Production of Recombinant Human Enzymes.** Researchers turned to CHO cells to manufacture these enzymes since these cells were easy to grow and

<b>Disease</b>	<b>Human Enzyme Replaced/Source<sup>a</sup></b>	<b>Trial<sup>b</sup></b>	<b>Sponsor<sup>c</sup></b>	<b>No. of Patients</b>	<b>Monthly Dose (mg/kg)</b>	<b>Status</b>
Gaucher type 1	Acid $\beta$ -glucocerebrosidase/CHO	Phase 1/2	GEN	12	1.6	Approved
Fabry	$\alpha$ -Galactosidase A/HF	Phase 1	TKT	10	0.007–0.1 single doses	Approved
	$\alpha$ -Galactosidase A/CHO	Phase 2; R, DB, PC	TKT	26	0.4	Approved
		Phase 3; R, DB, PC	TKT	80	0.4	
		Phase 1/2	GEN	15	0.6, 2.0, 6.0	
		Phase 3; R, DB, PC, MS	GEN	58	2.0	
		Phase 4; R, DB, PC, MS, OL	GEN	70	2.0	
MPS I	$\alpha$ -L-Iduronidase/CHO	Phase 1/2	BM/GEN	10	2.0?	Approved
		Phase 3; R, DB, PC, MS	BM/GEN	45	2.3	Approved
MPS VI	Arylsulfatase B/CHO	Phase 1/2; R, DB, PC, MS	BM	6	0.8, 4.0	Approved
		Phase 2; OL, MS	BM	10	4.0	Approved
		Phase 3; R, DB, PC	BM	39	4.0	
MPS II	$\alpha$ -L-Iduronidate sulfate/CHO	Phase 1/2; R, DB, PC	TKT	12	0.3, 1.0, 3.0	Pending
		Phase 3; R, DB, PC	TKT	96	2.0	
Pompe, infantile	Acid $\alpha$ -glucosidase/TR	Phase 1	GEN	4	60, 80, 160	Approved
		Phase 2; OL	—	2	160	
	Acid $\alpha$ -glucosidase/CHO	Phase 1/2	GEN	3	40	
		Phase 2	GEN	8	40	
		Phase 3	GEN	16	40	
Pompe, late-onset	Acid $\alpha$ -glucosidase/TR	Phase 1/2	GEN	3	40–80	
	Acid $\alpha$ -glucosidase/CHO	Phase 3; PC, R	GEN	72		

<sup>a</sup>CHO, Chinese hamster cells; HF, human fibroblasts; TR, transgenic rabbit.

<sup>b</sup>DB, double-blind; MS, multisite; OL, open label; PC, placebo-controlled; R, randomized.

<sup>c</sup>BM, BioMarin Pharmaceutical Inc., Novato, CA, USA; GEN, Genzyme Corporation, Cambridge, MA, USA; TKT, Transkaryotic Therapies, Inc., Cambridge, MA, USA (Shire Pharmaceuticals Inc.).

performed posttranslational modifications nearly identical to those of human cells. Importantly, it was discovered that the overexpression of a lysosomal cDNA in CHO cells resulted in the delivery of its glycosylated enzyme to the lysosomes, and, more importantly, that the majority of the recombinant human enzyme was selectively secreted into the culture media (47), thereby facilitating large-scale production of the critical highly glycosylated human enzyme. Of note, the secreted enzymes retained their terminal mannose 6-phosphate and sialic acid residues, whereas the enzyme's oligosaccharide chains were trimmed by glycosidases in the lysosome. Other expression systems using human fibroblasts, transgenic animals, and even plant systems have been used to produce therapeutic enzymes.

**28.2.3.2 Generation of Mouse Models for Lysosomal Storage Diseases and Preclinical Studies of ERT.** The development of gene-targeting technology in the 1990s led investigators to generate mouse models for lysosomal storage diseases, most of which had the biochemical, pathologic, and/or clinical manifestations of their human counterparts. Thus, by the mid-1990s, lysosomal disease researchers had available large quantities of recombinant enzymes and disease-specific animal models to vigorously pursue the development of ERT. Preclinical studies in animal models permitted evaluation of the pharmacokinetics and pharmacodynamics of ERT for lysosomal storage diseases. Since the Gaucher disease knockout mouse was not viable, the first preclinical studies of ERT to demonstrate proof of concept were conducted in Fabry knockout mice in the mid-1990s and reported later (48) (see below).

**28.2.3.3 Factors Affecting Selection of Candidate Lysosomal Disorders for ERT.** Several factors influence the selection of candidate lysosomal disorders for ERT. These include the target site(s) of pathology, the likelihood of reversing certain manifestations (e.g. skeletal, connective tissue, and neural diseases), and the presence or absence of residual enzymatic activity. For example, in type 1 Gaucher disease, the major pathologic cell type is the easily targeted macrophage; however, treatment must begin early to prevent the progressive bone disease as severe bone complications appear to be irreversible. In addition, ERT did not reverse the severe neurologic manifestations in patients with types 2 and 3 Gaucher disease. These results further support the initial findings that infused enzymes could not cross the blood-brain barrier (35) and that CNS manifestations were not treatable by ERT. In Fabry disease, the major site of pathology is the vascular endothelium, which is readily accessed by exogenous enzyme, whereas the dramatic bone and joint abnormalities in mucopolysaccharidoses are due to substrate accumulation in connective tissue matrix and cells (e.g. chondrocytes), which take up little, if any, exogenous enzyme. Thus, the effectiveness of ERT in lysosomal disorders depends on the delivery of sufficient amounts of the infused enzyme to the target sites of pathology and the reversibility of certain clinical manifestations.

Animal model studies have also revealed organ-specific variations in response to ERT, which are primarily due to the biodistribution of the infused enzyme. For example, in Fabry and NPD mice, the tissue distribution of intravenously infused enzyme and the amount and duration of substrate clearance (i.e. pharmacodynamics) from target sites of pathology were clearly dose dependent (48,49). Thus, for each disease, the infused enzymes must be administered in high-enough doses to be delivered to specific and unique cell types.

In addition, therapeutic responsiveness is related to disease subtype and disease-specific mutations. For most lysosomal disorders, patients who inherit mutations that render the gene product absent or nonfunctional manifest the severest form of the disease, often causing a rapid neurodegenerative course (for review, see Chapter 7). Alternatively, mutations that alter the protein's stability or kinetics, but retain residual function, have a milder phenotype. Thus, certain disease subtypes may be more amenable to ERT (or SRT and pharmacologic chaperone therapy (PCT); see later). For example, the mucopolysaccharidosis (MPS) I H subtype (Hurler disease) results in early onset of disease manifestations, including mental retardation, whereas types 1 H/S and I S (Hurler–Scheie and Scheie subtypes, respectively) are characterized by later onset and less-severe disease manifestations, including the absence of mental retardation.

The presence or absence of residual enzymatic activity, even if less than 1% of normal, primarily determines the immunologic response to ERT. In type 1 Gaucher disease, all patients have residual  $\beta$ -glucocerebrosidase activity, and experience with over 4000 type 1 patients has documented that less than 15% raise nonneutralizing immunoglobulin G (IgG) antibodies against the normal enzyme (40,50,51). These antibodies have no effect on efficacy and rarely cause infusion-associated reactions (52). In contrast, up to 90% of patients with classical Fabry disease, who have no or very little residual  $\alpha$ -galactosidase A activity, develop nonneutralizing IgG antibodies after four to six infusions (3,53). Up to half of these patients may experience infusion-associated reactions, including chills, rigors, and/or fevers, which do not affect efficacy and can be managed conservatively by premedication with nonsedating antihistamines and antipyretics and by slowing the infusion rate, since these reactions are directly related to protein load. Importantly, patients who seroconvert decrease their antibody titers with time and may eventually become tolerant. Of note, even in patients with no residual enzyme, the intravenous infusion of the recombinant normal enzyme has been well tolerated and has not precluded treatment.

Clinical trials of ERT using recombinant human enzymes have led to approval of this treatment for patients with Fabry disease (3,30), MPS I (54,55), MPS VI (56), Pompe disease (57–59), and MPS II (60,61) (Table 28-4). These clinical trials are discussed in greater detail later. In addition, clinical trials have begun in NPD



**TABLE 28-4 FDA and EMEA Approval of ERT for Lysosomal Storage Diseases**

Disease	Status (January 2006)
Gaucher disease	Approved 1991 (US) <sup>a</sup> ; approved in >80 countries
Fabry disease	Approved 2001 (EU), <sup>b</sup> 2003 (US); approved in >40 countries
Mucopolysaccharidosis I	Approved 2003 (EU & US)
Mucopolysaccharidosis VI	Approved 2005 (US), approved 2006 (EU)
Mucopolysaccharidosis II	Approved 2006 (US), approved 2007 (EU)
Pompe disease	Approved 2006 (EU & US)
Niemann–Pick type B disease	Phase 1b trial to begin in 2012

<sup>a</sup>Approval by U.S. Federal Drug Administration (FDA).

<sup>b</sup>Approval by European Union (EU), European Agency for Evaluation of Medicinal Products (EMA).

type B. Preclinical studies in knockout mice or naturally occurring animal analogs are underway for several other disorders, including galactosialidosis (62), Wolman disease/cholesteryl ester storage disease (63), and MPS VII (64,65). Most recently, studies in the MPS VII mouse model have documented uptake of recombinant enzyme into the brain when intravenously administered at doses greater than 4 mg/kg/week (66). These findings suggest that high-dose delivery of lysosomal enzymes may result in neural uptake and possible benefit for patients with neurologic involvement. For reviews of ERT and clinical trials, see Desnick and Schuchman (1) and Desnick (24).

**28.2.3.4 Fabry Disease.** Fabry disease is an X-linked recessive disorder resulting from the deficient activity of  $\alpha$ -galactosidase A ( $\alpha$ -Gal A) and the progressive accumulation of primarily globotriaosylceramide (GL-3) in the plasma and in tissue lysosomes throughout the body. In classically affected males, who have no detectable  $\alpha$ -Gal A activity, GL-3 accumulation in the vascular endothelium causes the major disease manifestations (67). Clinical onset in childhood is characterized by severe acroparesthesias, angiokeratoma, hypohidrosis, and corneal/lenticular opacities. With advancing age, the progressive lysosomal GL-3 accumulation, particularly in the microvasculature, leads to renal failure, vascular disease of the heart and brain, and premature demise, typically in the fourth or fifth decade of life. Atypical variants, who have residual  $\alpha$ -Gal A activity and no vascular endothelial involvement, have later-onset manifestations, usually limited to the heart or kidneys (i.e. the “cardiac variants”).

The studies of ERT for Fabry disease included the first preclinical studies of various recombinant enzyme glycoforms in the Fabry mouse, which determined the pharmacokinetics and pharmacodynamics for ERT in lysosomal diseases; a Phase 1/2 clinical trial that evaluated dose and dose schedule; a large multicenter, multinational, randomized double-blind placebo-controlled

Phase 3 clinical trial that demonstrated clearance of the accumulated GL-3; and a Phase 4 clinical trial that demonstrated clinical benefit, even in patients with advanced disease.

**28.2.3.4.1 Early Clinical Trials.** Early pilot trials of enzyme replacement in Fabry disease indicated the feasibility of this therapeutic approach as early as 1970 (68–70). However, the rate-limiting obstacles were the inability to produce sufficient amounts of purified normal enzyme and the lack of an animal model to evaluate the pharmacokinetics and pharmacodynamics required for the design of clinical trials.

**28.2.3.4.2 Preclinical Studies in Fabry Mice: Proof of Concept.** These obstacles were overcome by the isolation of the human  $\alpha$ -Gal A cDNA (71) and its high-level expression in CHO cells (47), which provided a source of selectively secreted recombinant human enzyme that was highly sialylated and had mannose 6-phosphate residues for lysosomal targeting. In addition, the generation of an  $\alpha$ -Gal A knockout mouse permitted the evaluation of enzyme replacement in an animal model of Fabry disease (48,72). This was the first lysosomal mouse model in which ERT was evaluated. These studies established the pharmacokinetics (i.e. plasma clearance), biodistribution, and pharmacodynamics (i.e. plasma and tissue glycosphingolipid substrate clearance) of the intravenously administered enzyme (48). Of note, subsequent ERT studies in other lysosomal storage disease mouse models resulted in similar findings (49,66,73).

With these advances, preclinical studies of four recombinant human  $\alpha$ -Gal A glycoforms were performed in the enzyme-deficient mice. The pharmacokinetics and biodistributions were determined for each glycoform, which differed in sialic acid and mannose 6-phosphate content (48). The plasma half-lives of the glycoforms ranged from approximately 2–5 min, with the more sialylated glycoforms circulating longer. The tissue distributions of the glycoforms were dose dependent. Following intravenous doses of 0.3–10 mg/kg, each glycoform was primarily recovered in the liver (approximately 30% of dose), with low but increasing levels of each detected in the heart, lung, spleen, and kidney, but not in the brain. Importantly, the accumulated GL-3 was cleared from the liver, heart, spleen, kidney, and plasma in a dose-dependent manner. These studies showed not only that enzyme delivery and GL-3 clearance were dose dependent but also that they determined the rate of GL-3 reaccumulation, which indicated an every-2-week dosing schedule. These preclinical studies provided proof of concept and the pharmacokinetic and pharmacodynamic data critical for the design of clinical trials of enzyme replacement. The optimal  $\alpha$ -Gal A glycoform, AGA-1, was subsequently made by Genzyme Corporation and designated agalsidase beta or Fabrazyme.

**28.2.3.4.3 Clinical Trials of ERT in Fabry Disease.** Trials of ERT in patients with classic Fabry disease have been carried out using recombinant human

$\alpha$ -Gal A produced by two different companies (3,53,74–77). Recent studies comparing the structural and kinetic properties as well as the cell uptake and in vivo pharmacokinetics and biodistribution of the two preparations—agalsidase alfa (Replagal; Shire Pharmaceuticals) and agalsidase beta (Fabrazyme; Genzyme Corporation)—found that the enzyme glycoproteins were similar in their physical and kinetic properties and in their in vivo pharmacokinetics and biodistributions in the mouse model ((78,79); M. Elleder, personal communication, 2005). The only notable differences were the findings that Fabrazyme had three times more mannose 6-phosphate and higher sialylation and that its uptake in the kidney and heart was greater (80,81). A review of published evidence for clinical efficacy of the two enzyme preparations revealed no clear differences in the patients' responses to treatment (82). The clinical trials that provided the evidence-based efficacy for Fabrazyme are described in the following section. These included two multicenter, multinational, randomized, double-blind placebo-controlled studies.

**28.2.3.4.3.1 Phase 1/2 Clinical Trial.** A Phase 1/2 open-label dose-escalation trial involving 15 classically affected males evaluated the safety and effectiveness of five doses of Fabrazyme in dose regimens of 0.3, 1.0, or 3.0 mg/kg every 14 days or 1.0 or 3.0 mg/kg every 48 h (75). The enzyme was well tolerated, and rapid marked reductions in plasma and tissue GL-3 were observed biochemically, histologically, and ultrastructurally. Notably, the clearance of plasma GL-3 was dose dependent (75). Mean GL-3 content decreased by 84% in liver ( $n=13$ ) and was markedly reduced in the kidney in four of five patients who had pre- and posttreatment renal biopsies. Importantly, GL-3 deposits also were reduced in the vascular endothelium of the kidney, heart, skin, and liver by light and electron microscopic evaluations. The trial demonstrated that the dose-dependent GL-3 clearance seen in the preclinical studies was also seen in patients with Fabry disease. In addition, patients reported decreased acroparesthesias, decreased gastrointestinal problems, and increased sweating (75). Thus, these results provided dose-response data and explored the possible primary efficacy end points for a Phase 3 pivotal trial.

**28.2.3.4.3.2 Phase 3 Clinical Trial and Extension Study.** A Phase 3 multinational, multicenter, randomized, double-blind placebo-controlled clinical trial, sponsored by the Genzyme Corporation, evaluated the safety and effectiveness of Fabrazyme in 58 patients who received 1 mg/kg of the enzyme or placebo every 2 weeks for 20 weeks (11 doses) (3). The primary efficacy end point was the percentage of patients whose renal capillary endothelial GL-3 deposits cleared to normal or near normal. In this study, 20 of 29 Fabrazyme-treated patients (69%) cleared the accumulated GL-3 from the renal capillary endothelium versus 0 of 29 placebo-treated patients ( $P<0.001$ ). Compared to the

placebo group, enzyme-treated patients also had markedly decreased microvascular endothelial GL-3 in the skin ( $P<0.001$ ) and heart ( $P<0.001$ ). Patients receiving the enzyme cleared the accumulated GL-3 in plasma to nondetectable levels. Pain and quality-of-life assessments improved in both treatment groups compared to the baseline, indistinguishable from a placebo effect. Note that patients in this study were neither selected for pain nor were pain medications discontinued during the treatment.

All 58 patients completed the Phase 3 trial and received Fabrazyme in an extension study (77). After 6 months of the open-label therapy, 98% of both former placebo-treated and enzyme-treated patients who had biopsies achieved or maintained normal or near-normal renal capillary endothelial histology. Importantly, the GL-3 was cleared to normal or near-normal levels ("0" scores by three independent renal pathologists) in the renal interstitial, glomerular, and nonglomerular endothelial cells, mesangial cells, and interstitial cells after 6 or 12 months of therapy (83). Reduced GL-3 deposits were also histologically documented in the podocytes and tubular epithelial cells (83).

Similar results (i.e. "0" scores) were found for GL-3 clearance in the skin (96%) and heart (75%) in the extension study. Thus, the study confirmed the results of the double-blind Phase 3 trial and demonstrated that the patients treated with enzyme for 12 months had continued GL-3 clearance from the vascular endothelium in the kidney, heart, and skin, the key sites of pathology in this disease. Also, the accumulated plasma GL-3 in the former placebo group decreased to nondetectable levels after 6 months of enzyme treatment. Of note, the mean serum creatinine concentration remained normal (0.8–0.9 mg/dL) without changing from baseline after 12 months of treatment. However, 3 of the 58 patients who were older (42–48 years), had more than 50% glomerulosclerosis (57–100%) and had urinary protein:urinary creatinine ratios greater than 2.0 at baseline exhibited increased their serum creatinine levels. These findings were instructive, as they indicated those patients who already had significant renal disease and whose renal disease would progress, presumably more slowly when treated with adequate doses of ERT (77). Furthermore, these data indicated that ERT was most effective when treatment was started earlier in the disease process, before irreversible damage occurred.

Treatment with Fabrazyme was well tolerated; the adverse event incidence and profiles were similar for both treatment groups in the Phase 3 trial and extension study, except for mild to moderate infusion reactions to the recombinant enzyme, which were managed conservatively. While IgG seroconversion occurred in 88% of enzyme-treated patients, GL-3 clearance was not impaired, and titers decreased with continued treatment. For example, in the Fabrazyme Phase 3 extension study, after 3 years of treatment at 1 mg/kg, in over 50% of

the 58 patients the IgG titers had decreased more than fourfold, and 7 had tolerized. Based on the results of the preclinical and Phase 1/2 and Phase 3 clinical trials, Fabrazyme was approved by the European Agency for Evaluation of Medical Products in 2001 and by the US Food and Drug Administration (FDA) in 2003 (74). Fabrazyme is now approved in over 40 countries worldwide.

**28.2.3.4.4 Clinical Studies of ERT with Fabrazyme.** Since the approval in Europe (2001) and the United States (2003), there have been numerous reports of the clinical benefits of Fabrazyme treatment. These studies have demonstrated the effectiveness of Fabrazyme at 1 mg/kg in stabilizing renal function (84) and improving cardiac function (85–87), gastrointestinal manifestations (88), the Fabry neuropathy (89), and quality of life (90). Of note, Fabrazyme has been administered during hemodialysis without loss of the recombinant enzyme in the dialyate (91). Moreover, Fabrazyme treatment has been shown to improve the quality of life of Fabry patients on dialysis by decreasing pain, improving gastrointestinal symptoms, and slowing or reversing the progressive Fabry cardiomyopathy (92).

**28.2.3.4.4.1 Highlights of the Phase 4 Clinical Trial.** Since the FDA approved Fabrazyme on the basis of a biologic marker, GL-3 clearance from the kidney and other sites, a Phase 4 clinical trial was required by the FDA's Accelerated Approval Program to further confirm the clinical benefit of the treatment. This study was a multinational, multicenter, double-blind placebo-controlled trial involving 82 patients with classical Fabry disease who had mild to moderate renal disease. Patients were randomized 2:1 (Fabrazyme:placebo) at each study site, and the median study time was 18.5 months (35 months total). The primary end point compared the time to the first clinical event (renal, cardiac, or cerebrovascular event or death) between the two treatment groups. After a clinical event, a patient could be switched to open-label enzyme.

This trial showed that, compared to placebo, Fabrazyme at 1 mg/kg every 2 weeks slowed the rate of progression of Fabry disease and substantially reduced the risk of renal, cardiac, and cerebrovascular events together and individually. After the proscribed adjustment for the baseline imbalance in proteinuria between the two treatment groups, patients randomized to Fabrazyme were 53% less likely than the placebo-treated patients to experience a clinically significant renal, cardiac, or cerebrovascular event. Similar to other renal diseases, baseline proteinuria was the most important determinant of outcome. Among the 74 patients who were compliant with the study protocol (the “Per-Protocol” population), after the prespecified adjustment for the proteinuria baseline imbalance between the two treatment groups, the patients who received Fabrazyme were 61% less likely to experience a clinically significant event ( $P=0.034$ ). The most pronounced benefits of Fabrazyme were seen when therapy was started earlier in the course of the

disease (i.e. with less renal dysfunction). These findings emphasize the importance of early treatment with 1 mg/kg of Fabrazyme. In addition, the results of this study were reviewed by the Committee for Medical Products for Human Use of the European Agency for Evaluation of Medical Products (EMA), and based on their recommendation, the label for Fabrazyme in the European Union was changed in August 2005 to include the statement “the results of these studies indicate that Fabrazyme treatment at 1 mg/kg every other week provides clinical benefit on key clinical outcomes in patients with early and advanced Fabry disease. Because this condition is slowly progressive, early detection and treatment may be critical to achieve the best outcomes.”

**28.2.3.4.4.2 Recommendations for Treatment with Fabrazyme.** A group of physicians expert in Fabry disease established consensus recommendations for the diagnosis and treatment of the disease (93). These experts recommended that all males with Fabry disease (including those with end-stage renal disease) and heterozygous females with substantial disease manifestations should be treated with ERT and that the treatment should be initiated as early as possible, particularly in boys with the classical phenotype.

**28.2.3.5 Mucopolysaccharidosis I.** MPS I is an autosomal recessive disorder that results from the absent or deficient activity of  $\alpha$ -L-iduronidase and the accumulation of its glycosaminoglycan substrates, dermatan sulfate and heparan sulfate (for review, see Chapter 102). There are three major phenotypic subtypes, designated Hurler, Hurler–Scheie, and Scheie diseases, whose clinical manifestations range from the infantile onset of severe skeletal, organ, and neurologic involvement and demise in childhood (Hurler) to a later-onset attenuated disorder primarily affecting the liver, spleen, heart, skeleton, and connective tissue, but with no neurologic manifestations or premature demise (Scheie).

**28.2.3.5.1 Phase 1/2 Clinical Trial.** Based on preclinical studies in hound dogs with  $\alpha$ -L-iduronidase deficiency (94,95), an open-label Phase 1 clinical trial of recombinant human  $\alpha$ -L-iduronidase produced in CHO cells (Aldurazyme; BioMarin Pharmaceutical Inc., and Genzyme Corporation) was undertaken in 10 patients (96). After 26 weeks of treatment at a dose of 0.58 mg/kg/week, marked decreases in liver volume and urinary glycosaminoglycan excretion were observed, range of motion improved, and the incidence of sleep apnea and hypopnea decreased. Adverse events included infusion-associated reactions with skin rash, headache, and hives. All patients continued treatment for over 3 years in an open-label extension trial.

**28.2.3.5.2 Phase 3 Clinical Trial.** A multicenter, multinational, double-blind placebo-controlled Phase 3 trial was undertaken with 45 patients (23 patients received enzyme and 22 placebo) who received weekly infusions of either 0.58 mg/kg of enzyme or placebo for 26 weeks (55). The coprimary efficacy end points were pulmonary



capacity (percentage predicted forced vital capacity (FVC)) and a 6-minute walk test (6MWT). Enzyme-treated patients had a mean FVC that was significantly improved over baseline values compared to the placebo group ( $P=0.009$ ), and the 6MWT end point showed a positive trend with an increase of 38.1 m distance in enzyme-treated patients ( $P=0.066$ ). In addition, enzyme-treated patients showed significantly reduced hepatomegaly and urinary glycosaminoglycans. Infusion-associated reactions were managed conservatively. More severely affected patients experienced improved sleep apnea and shoulder flexion. Most patients (91%) developed IgG antibodies to the recombinant  $\alpha$ -L-iduronidase, with no apparent effect on safety or efficacy. In all 45 patients enrolled in an open-label extension study long-term treatment with Aldurazyme led to sustained improvement in FVC and 6MWT (97). Enzyme replacement for MPS I was approved by the FDA in April 2003 (details of the FDA reviews are available at <http://www.fda.gov/ohrms/dockets/ac/cder03.html#EndocrinologicMetabolicDrugs> [Endocrinologic and Metabolic Drugs Advisory Committee of January 15, 2003]) and by the EMEA in June 2003.

**28.2.3.6 Mucopolysaccharidosis VI.** MPS VI (Maroteaux-Lamy syndrome) is an autosomal recessive disorder resulting from the deficiency of arylsulfatase B and the accumulation of dermatan sulfate (for review, see Chapter 102). The disease is characterized by severe skeletal involvement, hepatosplenomegaly, cloudy cornea, and cardiac disease, but no neurologic manifestations.

**28.2.3.6.1 Phase 1/2 and 2 Clinical Trials.** Preclinical studies of ERT were carried out in cats with MPS VI, which demonstrated its effectiveness (98). The development of ERT for MPS VI was sponsored by BioMarin Pharmaceutical Inc. A Phase 1/2 randomized, two-dose double-blind trial of enzyme replacement was undertaken in six patients who received either 0.2 or 1.0 mg/kg/week for 24 weeks and then entered an open-label study (56). All six patients completed 24 weeks of treatment, and five completed 48 weeks. Patients receiving the higher dose had the greatest improvement. There were no infusion-related reactions, presumably because these patients had residual enzyme activity. After 48 weeks of treatment, urinary glycosaminoglycan levels decreased by 51% and 63% in patients who received 0.2 and 1.0 mg/kg, respectively. Five patients who continued in the study had improvement in the 6MWT. One of the three patients with a tracheotomy had an improved FVC, and two had four- to sevenfold decreases in their apnea-hypopnea index. Range of motion improved in all five patients after 48 weeks of treatment.

An open-label multinational Phase 2 trial was conducted with 10 patients who received 48 weekly infusions of 1.0 mg/kg of recombinant enzyme (99). After the 48 weeks of treatment, the mean distance walked in 12 min increased by 138% and the 3-min stair climb showed an average increase of 147% over baseline

values. Joint Pain and Stiffness Questionnaire scores improved by at least 50% by week 24 and were maintained after 48 weeks of treatment. In three of the six patients, improvement in pulmonary function in the absence of growth was observed. In addition, mean urinary glycosaminoglycan levels were decreased by 76% at 48 months (99). Adverse events attributed to the enzyme were generally mild and did not require treatment. IgG antibodies to the enzyme were present in six patients, but were not correlated with adverse events or other safety measures.

**28.2.3.6.2 Phase 3.** A double-blind, randomized placebo-controlled Phase 3 trial involved 39 patients receiving either 1 mg/kg of recombinant enzyme or placebo for 24 weeks. The patients' ages ranged from 5 to 29 years. The primary end point was the change in endurance compared to placebo as measured by the distance achieved in a 12-min walk test. The enzyme-treated patients showed greater increases in the 12-min walk and 3-min stair climbing tests compared to the placebo-treated patients. Based on these results, the drug (Naglazyme) was approved by the FDA in August 2005 and by the EMEA in the European Union in January 2006.

**28.2.3.7 Mucopolysaccharidosis II.** MPS II (Hunter syndrome) is an X-linked recessive disease due to the deficient activity of  $\alpha$ -L-iduronidate sulfatase and the resultant lysosomal accumulation of heparan sulfate and dermatan sulfate (for review, see Chapter 102). There are mild and severe subtypes, both having significant skeletal, organ, and connective tissue manifestations, but differing in the degree of neurologic involvement.

**28.2.3.7.1 Phase 1/2 Clinical Trial.** Based on preclinical studies of ERT in a mouse model of MPS II (100), a Phase 1/2 randomized, double-blind placebo-controlled clinical trial was conducted with 12 patients aged 6–20 years (101,102). The development of ERT for MPS II was sponsored by Transkaryotic Therapies, Inc. (Shire Pharmaceuticals). Doses of 0.15, 0.5, and 1.5 mg/kg were infused every 2 weeks for 6 months, and then patients continued in an open-label extension. In each dose group, three patients received the enzyme, whereas one received placebo. Enzyme-treated patients had reduced liver and spleen volumes and decreased urinary glycosaminoglycan excretions (by 51%). Infusion-related reactions occurred in patients treated with the 0.5- and 1.5-mg/kg doses but were managed with premedication and by reducing the infusion rate. All 12 patients entered the open-label extension study and received the same doses (0.15, 0.5, and 1.5 mg/kg) as in the Phase 1/2 trial. Urinary glycosaminoglycan excretion decreased by 50% and 45% after 6 months and 1 year, respectively, of enzyme therapy in the extension study. Liver and spleen volumes decreased by 27% and 26%, respectively, after 1 year of ERT (61). The 6MWT, a test that integrates the function of the respiratory, cardiovascular, and musculoskeletal systems, increased an average of 48 m after 48 weeks.



**28.2.3.7.2 Phase 3 Clinical Trial.** A double-blind, placebo-controlled multinational Phase 3 clinical trial enrolled 96 patients who received either 0.5 mg/kg of recombinant enzyme or placebo weekly or every other week for 52 weeks (103). A total of 94 patients finished the trial, and all patients enrolled in the open-label extension. The primary end point combined measurements of endurance (the 6MWT) and pulmonary function (FVC). Patients treated with the recombinant enzyme weekly and every other week showed a statistically significant increase in the combined end points over baseline values compared to patients in the placebo group. The group treated weekly with enzyme showed a 37m increase in the 6MWT and a 2.7% increase in percentage of predicted FVC. Treatment was well tolerated, but infusion reactions occurred and antibodies were detected in 46.9% of the patients. Based on these results, a Biologics License Application (BLA) for the drug (Elaprase) was approved by the FDA in July 2006 and in the European Union in January 2007.

**28.2.3.8 Pompe Disease.** This autosomal recessive disease is caused by a glycogenosis resulting from the deficiency of acid  $\alpha$ -glucosidase and the lysosomal accumulation of glycogen primarily in smooth and skeletal muscle. The infantile form of the disease is characterized by massive cardiomegaly, weakness and hypotonia, and death due to cardiorespiratory failure in the first year of life. In contrast, later-onset forms present with progressive muscle weakness and involvement of the respiratory muscles, which can lead to severe respiratory difficulty. Two groups have performed trials of ERT using enzymes produced in the milk of transgenic rabbits or in CHO cells (73,104) (see Table 28-3).

**28.2.3.8.1 Infantile Pompe Disease: Phase 1 and 2 Clinical Trials.** Preclinical trials conducted in acid  $\alpha$ -glucosidase knockout mice that have the myocardial phenotype (73) and the quail model (105) demonstrated proof of concept. Several clinical trials have been performed in infantile Pompe disease and later-onset patients, sponsored by the Genzyme Corporation. The initial pilot studies were conducted in Europe with recombinant human acid  $\alpha$ -glucosidase produced in transgenic rabbit milk (106,107) and in the United States with recombinant human enzyme produced in CHO cells (108). The European study was an open-label Phase 1 trial involving four infantile-onset patients with severe cardiomyopathy. Initially, doses of 15 or 20 mg/kg, and later 40 mg/kg, were infused weekly. At the 40 mg/kg dose, enzyme activity was normalized in skeletal muscle, lysosomal glycogen was reduced and tissue morphology improved in all patients. Cardiac function improved, and left ventricular wall thickness and mass decreased significantly. Patients developed IgG antibodies, and infusion-associated reactions were manageable by slowing the infusion rate. All patients survived beyond the critical age of 1 year, with one patient walking independently and remaining ventilator free as of February 2006.

In a parallel Phase 1/2 open-label study conducted in the United States, three infants received twice-weekly infusions of 5 mg/kg of the recombinant human acid produced in CHO cells for 1 year (108). The enzyme infusions were well tolerated, although two patients were pretreated with antihistamine. Treatment resulted in decreased heart size (left ventricular mass) with maintenance of normal cardiac function. In addition, improved skeletal muscle function was noted. One of the three patients had a good skeletal muscle response, is currently 6.5 years old (as of February 2006), walks independently, and remains ventilator free. Other patients in both studies showed lesser degrees of motor improvement, and most of them eventually became ventilator dependent.

Two additional infantile-onset patients were treated for 22 months with a dose of 40 mg/kg weekly of recombinant human enzyme purified from transgenic rabbit milk (57,109). The improvements in cardiac size and function as well as muscle strength observed in the initial 48 weeks of treatment continued during the 10-month follow-up period (57,109).

Subsequently, an open-label, multicenter trial of eight infantile-onset patients who had cardiomegaly and cardiomyopathy by age 6 months was initiated with the CHO cell-derived recombinant enzyme infused weekly at 10 mg/kg (110). All patients developed nonneutralizing IgG antibodies after 2 months. Three patients died of non-ERT causes. Of the remaining five, all had Alberta Infant Motor Scale scores greater than the third percentile for age, and four were ventilator free. Three patients achieved ambulatory status. All treated patients also had reduced left ventricular mass, and none had signs of cardiac failure. As of February 2006, 8 of the 17 patients in these pilot studies survived past the age of 4 years (111).

**28.2.3.8.2 Infantile Pompe Disease: Pivotal Clinical Trial.** A pivotal clinical trial of recombinant human acid  $\alpha$ -glucosidase produced in CHO cells was conducted in 18 infantile-onset patients under 6 months of age (100). About 83% of the enzyme-treated patients met the primary end point of being alive and ventilator free at 18 months, compared to the historical cohort (only 2%). This higher-than-anticipated positive response when compared with the response of earlier studies was attributed to the initiation of therapy before 6 months of age. However, in a subsequent clinical trial, 21 infants who started at a more advanced age (median age of 13 months) also showed prolonged survival and invasive ventilator-free survival compared with the untreated reference group (112). Based on the results of these successful clinical trials for the infantile-onset form, a BLA for ERT with this enzyme (Myozyme) was approved by the FDA and EMA in April 2006.

**28.2.3.8.3 Later-Onset Pompe Disease: Phases 1/2 and 3 Clinical Trials.** A Phase 1/2 open-label trial was conducted in three later-onset patients (ages 11, 16, and 32 years; two of them being ventilator dependent) with recombinant human acid  $\alpha$ -glucosidase purified from

transgenic rabbit milk (59). Initially, patients received weekly infusions of 10 mg/kg, and then the dose was increased to 20 mg/kg. After 3 years of treatment, their pulmonary functions improved or stabilized. The youngest and least-affected patient, who had been wheelchair dependent for 4 years, started to walk, suggesting that ERT could improve muscle strength, particularly when started early. The other two patients remained wheelchair dependent, but showed improvement in quality of life. No significant infusion reactions were reported.

Another randomized controlled trial in 90 later-onset patients (8 years and older) studied the effect of biweekly infusions of 20 mg/kg of recombinant human acid  $\alpha$ -glucosidase produced in CHO cells (58). After 78 weeks, treatment with the enzyme was associated with improved walking distance (increase of 28 m) in the 6MWT compared to the placebo group and stabilization of pulmonary function (FVC). An independent study of 44 patients with late-onset Pompe disease administered 20 mg/kg enzyme over 12 months showed a similar modest stabilization of neuromuscular deficits (113). Based on these findings, a drug (Lumizyme) was approved by the FDA in May 2010 for Pompe patients who are 8 years and older and who do not have evidence of cardiac hypertrophy. Lumizyme is produced in the 4000-L bioreactor scale and harbors minor differences in glycosylation to Myozyme, which is produced at the 160 L scale.

**28.2.3.9 Niemann–Pick B Disease.** Types A and B NPD result from deficient activity of acid sphingomyelinase (ASM) (114). Type A is the infantile form of the disease and is characterized by severe neurodegeneration that generally leads to death before the age of 5 years. In contrast, most type B patients have little or no neurologic involvement and many survive into adulthood. In these patients, the disease primarily manifests in reticulo-endothelial organs. Intermediate forms of ASM-deficient NPD also have been described.

Recombinant human ASM was produced in CHO cells (115) and used to evaluate ERT in ASM knockout mice (49). These studies revealed that intravenous ASM administration led to the significant depletion of accumulated sphingomyelin and markedly improved pathology in the major pathologic sites, including the spleen, liver, and lung. There was no effect on the progression of brain disease. Preclinical studies also revealed a novel ASM-associated toxicity noted only in ASM knockout mice following high initial doses (>10 mg/kg). Based on these findings, the Genzyme Corporation undertook the clinical development of this therapy for type B NPD. To date, industrial-scale, recombinant human ASM has been produced and characterized, and a large natural history in type B patients has recently been completed. An Investigational New Drug application was submitted to the FDA in 2005, and a Phase 1 clinical trial was completed in 2009. A total of 11 adult patients were administered single ascending doses of intravenous ASM in 5 dose cohorts (0.03, 0.1, 0.3, 0.6 and 1 mg/kg). Dose-related

hyperbilirubinemia, acute phase response and constitutional symptoms were noted between 1 and 3 days post-treatment. The maximum tolerated starting dose was determined to be 0.6 mg/kg. Within-patient dose escalation is proposed as an option for higher repeat doses of the enzyme.

## 28.2.4 ERT: Principles for Effective Treatment

In summary, clinical experience and clinical trials in six lysosomal disorders have demonstrated or indicated the therapeutic effectiveness of ERT. From these studies, certain general principles for effective treatment by ERT have become evident (Table 28-5):

- (1) Intravenously administered recombinant lysosomal enzymes are cleared from the circulation primarily by the pathway mediated by mannose 6-phosphate (mannose in Gaucher disease) receptor for cellular uptake and lysosomal delivery. Thus, the administered enzymes must have their full complement of mannose 6-phosphate residues and be fully sialylated for maximal lysosomal delivery to organs other than the liver, where the hepatocytes will rapidly take up asialo- and galactose-terminated glycoproteins via the asialofetuin receptor (116).
- (2) Dose is important. The higher the dose, the greater is the biodistribution, especially to cell types or sites that have limited uptake, such as the heart and kidney. The recent demonstration of neural uptake and neuronal substrate clearance in the MPS VII adult mice by high-dose enzyme administration clearly emphasizes this principle (66). However, consideration should be afforded to the potential of generating toxic metabolites, as noted in the nonclinical studies in the ASM knockout mice.

**TABLE 28-5 Principles for Effective Treatment for Lysosomal Storage Diseases**

- Enzyme delivery is receptor mediated and dependent on the number of receptors on plasma membrane
  - Mannose 6-phosphate receptor for most lysosomal enzymes
  - Mannose receptor for  $\beta$ -glucocerebrosidase uptake (Gaucher disease)
  - Enzyme: Available mannose 6-phosphate residues
- Avoid galactose residues for asialoglycoprotein receptor
- Inability to cross the blood–brain barrier
- Enzyme delivery is dose dependent
- Substrate clearance is dose dependent
- Adequate dose required for delivery to critical organs
- Off ERT, rapid substrate reaccumulation and clinical exacerbation
- Immune reactions depends on cross-reactive immunologic material (CRIM) status; infusion-associated reactions are manageable
- Treat early to prevent irreversible damage; even in advanced disease, treat to slow/prevent progression

- (3) Substrate clearance is dose dependent.
- (4) There is rapid reaccumulation of substrate if ERT is interrupted or stopped. This was surprising since ERT cleared decades of substrate accumulation, but in the absence of continued treatment, the substrate rapidly reaccumulates. This may be related in part to cell turnover or the proclivity of enlarged “hungry” lysosomes.
- (5) Infusion-associated reactions occur, presumably when patients develop IgG antibodies against the infused recombinant enzyme, but these reactions are transient, can be managed conservatively, and are more frequent in patients with little or no residual enzyme activity. With time, patients may reduce their antibody titers and become tolerized to these enzymes.

Until gene therapy, or another therapy, proves effective in these diseases, ERT provides a safe and effective means to reverse years of substrate accumulation and to control further deposition. A clear need for each of these diseases will be the identification and monitoring of biomarkers that will assess disease progression or improvement, analogous to the use of chitotriosidase activity in type 1 Gaucher disease (78,117,118). Chitotriosidase is expressed by activated macrophages and has been proved to be useful in determining dose requirements in type 1 Gaucher patients (118). Such biomarkers may determine if the initial dose of the enzyme used to debulk years of substrate accumulation can be adjusted to a maintenance dose in individual patients.

## 28.3 SUBSTRATE REDUCTION THERAPY

The aim of SRT is to reduce the amount of noncatabolized substrates in the lysosomes by inhibiting the synthesis of their precursors (for review, see Platt and Jayakumar (18)). The concept, first proposed by Norman Radin for type 1 Gaucher disease, seeks to inhibit the enzyme glucosylceramide synthase that catalyzes the first committed step in the synthesis of glycosphingolipids (17,20). Since its inception, significant progress has been made in identifying small molecule inhibitors of this enzyme with the appropriate safety and efficacy profiles for use in patients with Gaucher disease as well as other glycosphingolipidoses. The potential to deploy small molecule drugs offers the convenience of oral delivery and the potential to have a pharmacodynamic response that is different and potentially complementary to ERT.

As SRT seeks to restore the balance between the rate of biosynthesis and catabolism of the substrates, this therapy is ideally suited for disease populations that retain some residual enzymatic activity. Subjects with type 1 Gaucher disease as well as others with lysosomal storage disorders with a milder disease course typically exhibit this feature. In these patients, SRT can be deployed as a monotherapy; indeed, SRT using miglustat (Actelion Pharmaceuticals) is in clinical practice for treating mild

to moderate type 1 Gaucher disease patients unwilling or unable to receive ERT (119). In subjects who harbor mutations that extinguish enzymatic activity altogether, SRT could be considered in the context of an adjunctive therapy to ERT (120). As miglustat reportedly can traverse the blood–brain barrier into the CNS, it has also been tested in neuropathic type 3 Gaucher disease and NPD type C (22,121).

### 28.3.1 Inhibitors of Glucosylceramide Synthase

Two chemical classes of inhibitor of glucosylceramide synthase are undergoing extensive testing; one is based on the prototypical imino sugar, *N*-butyldeoxynojirimycin (NB-DNJ, miglustat) and the other on *D*-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (eliglustat, Genzyme Corporation) (122).

Early indications that miglustat could effect substrate reduction came from cell culture studies in the presence of conduritol- $\beta$ -epoxide, an agent that inhibits glucocerebrosidase activity and induces the accumulation of glucosylceramide. Treatment of these cells with miglustat abrogated lysosomal storage of the substrate (123). Subsequent studies in mouse models of lysosomal storage disease confirmed the utility of this inhibitor in treating a variety of glycosphingolipidoses including those with CNS involvement. Initial preclinical studies with miglustat were performed in mouse models of Tay–Sachs (19) and Sandhoff diseases (124). Administration of high doses of the drug reduced the rate of accumulation of glycosphingolipids in the visceral organs and brains of these animals. Importantly, in the Sandhoff mice, treatment delayed the onset of behavioral deficits and motor function and extended their longevity by approximately 40%. Efficacy has also been reported in mouse models of Fabry disease (125), GM<sub>1</sub> gangliosidosis (126) and NPD type C (127) that had been treated with the imino sugar. These preclinical studies with miglustat, combined with the earlier clinical experience of this drug in humans with HIV infection (128), encouraged its clinical testing in patients with glycosphingolipidoses.

Characterization of the inhibitory activity of eliglustat on glycosphingolipid synthesis *in vitro* showed that it was more potent and selective than miglustat (79). However, as it is a substrate for the P-glycoprotein multidrug transporter, it does not accumulate in brain tissue and therefore is unlikely to be useful for treating neuropathic lysosomal storage diseases. Testing of eliglustat in a mouse model of type 1 Gaucher disease showed that it reduced the number of Gaucher cells in the liver and the accumulation of glucocerebroside in the liver, spleen and lung. Moreover, Gaucher mice treated sequentially by ERT (Cerezyme) and eliglustat exhibited significantly lower accumulation of glucocerebroside than animals treated with either agent alone (129). Testing of eliglustat either as a monotherapy or combination therapy with

ERT (Fabrazyme) in Fabry mice showed similar benefits (120). These data argue that SRT could function as an adjunctive therapy to ERT for these glycosphingolipidoses. In subjects whose disease has been stabilized by ERT, SRT could potentially be used as a convenient maintenance therapy. In subjects naïve to treatment, ERT followed by SRT could potentially accelerate clearance of the accumulated substrates.

### 28.3.2 SRT for Type 1 Gaucher Disease

The preclinical studies with NB-DNJ in Tay–Sachs and Sandhoff diseases combined with the experience of this drug in human subjects with late-stage HIV infection and AIDS encouraged its clinical testing (miglustat) in patients with type 1 Gaucher disease (21). In a multicenter Phase 1/2 trial, 28 patients with Gaucher disease who had been previously stabilized with ERT were treated with doses of 100 mg three times daily for 1 year. Significant improvements were noted after 12 months, which improved further in patients who elected to enroll in an extension study. Target plasma concentrations of approximately 6  $\mu$ M miglustat were attained, and results showed a decrease in liver and spleen volumes and a progressive decline in the biomarker chitotriosidase. In the extended study period, a clinically significant increase in hemoglobin and platelet counts was also observed (130). These salutary findings were recapitulated in an independent study at another center (131). An additional clinical study that evaluated the use of a lower dose (50 mg three times daily) resulted in a slower therapeutic effect, suggesting a dose-dependent clinical outcome (132). Studies were also conducted to evaluate the merits of switching type I Gaucher patients who have been on ERT to miglustat (133). In this study, 36 patients who were clinically stable on ERT were switched to SRT, maintained on ERT or subjected to a combination of SRT and ERT. After 6 months, no differences in hematological parameters or organ volumes were noted between subjects treated with miglustat or ERT. Based on these observations, it was suggested that SRT was effective for the long-term maintenance of type I Gaucher patients who had previously received enzyme therapy.

The most frequent adverse event was diarrhea due to inhibition of intestinal disaccharidases, which occurred in 79% of the patients. Tremor was relatively common (25% of patients), but this tended to resolve spontaneously or became attenuated with lower doses. Peripheral neuropathy was noted in a small number of patients in the initial trial, which resolved slowly upon withdrawal of the drug, and this feature was absent in subsequent independent trials. Although instances of disturbed cognitive function were also reported, it has not been observed in follow-up monitoring, subsequent clinical trials and pharmacovigilance reports. As preclinical studies in some strains of mice showed that miglustat altered the morphology and mobility of spermatozoa,

these effects have also been recently studied in healthy men (134). No abnormalities in sperm concentration, motility or sperm morphology were noted after 6 weeks of therapy with 100 mg/day of miglustat suggesting the effects on male fertility were species specific. Based on the safety and efficacy profile of miglustat, the drug was approved by the EMA in November 2002 and the FDA in July 2003 for the treatment of adult type I Gaucher patients with mild or moderate disease who are unwilling or unable to receive or to continue ERT.

Based on preclinical studies showing that eliglustat has a higher specificity for glucosylceramide synthase than miglustat and that it does not inhibit the gut glycosidases or glucocerebrosidase, this oral inhibitor has also entered clinical testing in type 1 Gaucher patients. Initial Phase 1 clinical testing of eliglustat in 99 healthy volunteers showed that the drug was well tolerated at doses of 50–200 mg administered twice daily, inducing plasma concentrations within the predicted therapeutic range (135). Plasma glucocerebroside levels were reduced after dosing for 4 days. Mild increases in electrocardiogram PR, QRS and QT/QTc intervals were noted, and inhibition of CYP2D6 is suspected for the nondose-proportional increase in drug levels.

An uncontrolled Phase 2 clinical testing of eliglustat in 26 adult type 1 Gaucher patients showed significant improvements in spleen and liver volumes, hemoglobin concentrations, platelet counts and bone mineral density (136,137). Patients were administered 50 mg or 100 mg of eliglustat twice daily based on plasma drug concentrations, the latter dose to ensure that rapid metabolizers attained concentrations of approximately 10 ng/mL. A composite primary efficacy end point requiring improvement in at least two of three disease manifestations (spleen volume, hemoglobin concentration and platelet counts) was met by 77% of all patients and 91% of the 22 patients who completed the trial at 52 weeks. The magnitude of the hematologic and visceral responses was within the ranges observed with ERT over the same period. However, the increase in lumbar spine bone mineral density was more rapid than with ERT suggesting that cortical bone might have greater access to eliglustat, presumably because of its smaller size. Plasma glucocerebroside and GM<sub>3</sub> levels were normalized and chitotriosidase, CCL18, angiotensin-converting enzyme and tartrate-resistant acid phosphatase levels were decreased by 35–50% relative to baseline. Treatment was generally well tolerated; seven mild transient adverse events in six patients were considered treatment related. Unlike miglustat, gastrointestinal events were low with eliglustat, which is consistent with its lack of inhibition of intestinal disaccharidases, and there was no treatment-related tremor. One patient exhibited asymptomatic nonsustained ventricular tachycardia that was considered possibly treatment related and requiring hospitalization for detailed cardiac telemetry. An analysis of all patients for QTcF interval showed no effect, although minor changes in the PR and QRS intervals were noted.



All 20 eligible patients from the Phase 2 trial elected to continue in the extension period and subsequently into the third year of the trial. Treatment responses observed during the first 52 weeks of eliglustat were confirmed and extended during the second year. Hematological, visceral and skeletal manifestations of type 1 Gaucher disease continued to improve, with no further safety trends emerging over the latter period. Three Phase 3 randomized controlled clinical trials of eliglustat have been initiated. One is in patients who have a confirmed diagnosis and who have not been treated with ERT in the previous 9 months or with an oral therapy in the previous 6 months. Another is designed to compare the efficacy and safety of eliglustat as a maintenance therapy in type 1 Gaucher patients previously stabilized with ERT for at least 3 years. The third trial seeks to compare the effects of a once daily dosing of eliglustat with twice daily administrations.

### 28.3.3 SRT for Other Lysosomal Storage Disorders

As glucosylceramide synthase catalyzes the first committed step in the synthesis of glycosphingolipids; inhibitors of this enzyme may also be considered for treating other glycosphingolipidoses, such as Fabry disease. Preclinical studies in murine models of Fabry disease showed that a dose-dependent reduction in globotriaosylceramide could be attained in several visceral organs (120,138). Moreover, when used in combination with ERT, the effects were shown to be additive. However, an early clinical trial using miglustat (100 mg daily or twice daily) in Fabry disease was stopped as a result of untoward neurological toxicity. Of the 16 males who received the drug 13 developed tremor and 2 complained of paresthesia (139).

As miglustat is reportedly able to traverse the blood-brain barrier into the CNS, it has also been considered for treating lysosomal storage diseases with neurological involvement (140). While initial clinical testing in neuropathic Gaucher type III disease patients suggested a positive effect of miglustat (141), a randomized controlled trial in 30 Gaucher type III patients receiving ERT showed no effect on neurological manifestations (121). Measurements of the aberrant saccadic eye movement that is characteristic of this group of patients as well as neurological and neuropsychological evaluations were not significantly different between the drug-treated and untreated groups. It has been suggested that this failure may be due to the limited potency of miglustat or due to the irreversible nature of the neurological disease in these subjects. Moreover, despite encouraging preclinical data obtained with miglustat in animal models of GM<sub>2</sub> gangliosidosis, an open-label study in 5 patients with juvenile GM<sub>2</sub> gangliosidosis showed that treatment did not alter the progression of neurological disease over a 24-month period (142). However, there was evidence

of reduction of intracytoplasmic inclusions in peripheral blood white cells. A separate study of miglustat in two patients with the infantile form of Tay-Sachs disease there was failure to arrest neurological deterioration over 12 months (143).

Interestingly, SRT with miglustat is reportedly modestly effective in patients with NPD type C. The disorder is characterized by neuronal accumulation of complex gangliosides and neutral glycosphingolipids resulting from a defect in cholesterol and ganglioside trafficking. Treatment of murine and feline models of the disease with miglustat reduced ganglioside accumulation and neuropathology, delayed neuronal dysfunction and increased their lifespan (127). A randomized controlled clinical trial conducted in 29 patients who were 12 years or older showed an improvement in the horizontal saccadic eye movement velocity in patients who received 200 mg miglustat three times daily when compared to those receiving standard care (22). Improvements in swallowing capacity, stable auditory acuity and a slower deterioration in ambulatory index were also noted at 12 months, which were sustained in the majority of patients when analyzed after 24 months (144). Two additional clinical studies in NPD type C patients also showed a stabilization of the neurological disease although the effects were more modest among younger patients who were at a more advanced stage of disease (145,146). Based on these findings, miglustat was approved by the European Medicines Agency (EMA) for treating NPD type C in January 2009. As of March 2010, approval by the US FDA for a supplemental New Drug Application is awaiting additional requests for preclinical and clinical information.

### 28.3.4 Conclusions

It is apparent that SRT is emerging as a competitive alternate therapeutic to ERT. The convenience that this oral therapy offers coupled with the encouraging observations in clinical studies indicate that SRT can represent an advancement in the standard of care for patients with lysosomal storage disorders (LSD). The promise of this technology lies in the development of molecular entities that are able to traverse the blood-brain barrier in quantities that are therapeutic for subjects with neuropathic LSD. This will require an investment either in medicinal chemistry to improve the characteristics of the existing entities or in additional high-throughput screens for new entities with the appropriate drug profile. Now that the concept has been validated, it is also imperative that efforts are expanded to identify drugs for SRT of additional LSD that extend beyond the glycosphingolipidoses.

## 28.4 PHARMACOLOGIC CHAPERONE THERAPY

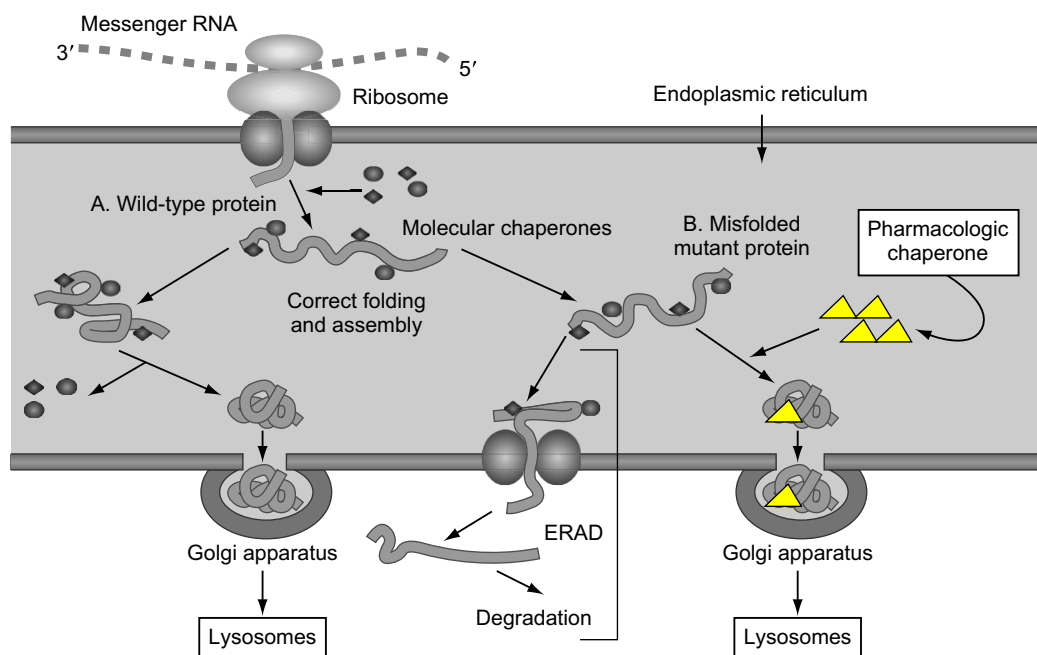
“Pharmacologic chaperones” are specific, preferably low-molecular-weight, hydrophobic ligands that bind to and

rescue misfolded and/or mistargeted proteins and augment the cell's molecular chaperones to enhance correct folding and organelle targeting of the misfolded mutant conformers. By stabilizing the corrected conformation of the mutant protein, they prevent its degradation. These chaperones may also function by stabilizing a specific conformation of the misfolded protein, reducing aggregation, and/or preventing nonproductive interactions with other resident proteins (for reviews, see Hampton and colleagues (26), Ulloa-Aguirre and associates (25), and Meusser and colleagues (27)).

As proposed for enzymes by Fan and coworkers (147,148), pharmacologic chaperones can diffuse into the cell and bind site specifically to folding intermediates of a mutant enzyme protein, thereby stabilizing the intermediate that is rate limiting for the folding and/or trafficking of the mutant enzyme and rescuing a portion of it from degradation. Competitive reversible enzyme inhibitors can be effective pharmacologic chaperones because of their high affinity to the catalytic domain. Such inhibitors serve as a folding template for those mutant proteins with fragile conformational structures during the protein folding process and induce proper folding. In this way, the pharmacologic chaperone prevents excessive degradation of the misfolded mutant enzyme, which may retain full or partial activity if it can be properly folded, processed, and transported to its normal site of action.

For lysosomal storage diseases, pharmacologic chaperones (active-site-directed reversible enzyme inhibitors) would rescue the misfolded/mistrafficked enzyme that would otherwise be transported by the ERAD machinery to the proteasome for degradation. If even a small percentage of the mutant misfolded/mistrafficked enzymes are rescued, they can be transported to the Golgi for oligosaccharide processing and then delivered to the lysosomes, where the chaperone is displaced by the high concentration of the accumulated substrate(s) (Figure 28-1). It is anticipated that even a small increment in enzymatic activity can markedly attenuate a severe phenotype, as illustrated by the fact that the less-severe subtypes of Gaucher disease and other lysosomal disorders are due to the presence of low levels of mutant enzyme that have very low levels of residual activity (even <1% of wild type). Thus, this therapeutic strategy offers the potential to prevent or effectively reverse the disease manifestations in lysosomal diseases resulting from mutant enzyme misfolding and/or mistrafficking.

The first studies of PCT for lysosomal diseases, carried out in Fabry disease (deficient  $\alpha$ -Gal A activity), demonstrated that mutant  $\alpha$ -Gal A enzymes encoded by various missense mutations could be effectively rescued by various  $\alpha$ -Gal A inhibitors, including 1-deoxygalactonojirimycin (DGJ) (148,149). Subsequently, specific pharmacologic chaperones have been identified for Gaucher,



**FIGURE 28-1** (A) Schematic of the processing, correct folding, and assembly of newly synthesized lysosomal enzymes. The newly synthesized enzyme is translocated into the endoplasmic reticulum (ER), where molecular chaperones facilitate its proper folding and subunit assembly. The molecular chaperones then dissociate from the folded assembled enzyme, which moves to the Golgi apparatus and then to lysosomes, where the enzyme is stable and active in the acidic environment of these organelles. (B) Misfolding and misassembly of mutant lysosomal enzymes. The mutant proteins are retained in the ER and are eventually degraded by the ERAD machinery. Pharmacologic chaperones (yellow triangles) bind to the active site of the enzyme, promote folding, and stabilize the mutant enzyme for proper subunit assembly. This promotes transport of the mutant enzymes to the Golgi apparatus and the lysosomes, thereby increasing the concentration of the mutant enzyme and its residual enzyme activity. (From Fan, J. Q. A Contradictory Treatment for Lysosomal Storage Disorders: Inhibitors Enhance Mutant Enzyme Activity. *Trends Pharmacol. Sci.* 2003, 24, 355.)

Tay–Sachs, and Sandhoff diseases and GM<sub>1</sub> gangliosidosis (150–154). In a murine model of GM<sub>1</sub> gangliosidosis, studies have shown that a pharmacologic chaperone can cross the blood–brain barrier and decrease the neural lysosomal substrate accumulation (152). Recognizing the potential of therapeutic effectiveness of PCT, investigators are evaluating potential compounds as chaperones for other lysosomal storage diseases. In addition, clinical proof of concept has been demonstrated in a patient with later-onset Fabry disease (155). These findings have been translated into the first FDA-approved clinical trial of PCT for a lysosomal disease. A Phase 1 clinical trial of PCT for Fabry disease has been successfully completed (Amicus Therapeutics; <http://www.amicustherapeutics.com>) and a Phase 2 clinical trial is underway (<http://www.clinicaltrials.gov/ct/show/NCT00214500?order=1>).

In the following sections, we provide an overview of the molecular basis of PCT for lysosomal diseases and describe recent *in vitro* and *in vivo* studies that highlight the potential of this therapeutic strategy for lysosomal diseases in which the mutant lysosomal enzyme can be rescued and its stability, delivery to the lysosome, and function increased.

### 28.4.1 Lysosomal Enzyme Biosynthesis and Degradation

Lysosomal proteins are synthesized in the cytoplasm and then secreted into the lumen of the endoplasmic reticulum (ER) in a largely unfolded state. In general, protein folding is governed by the principle of self-assembly (156). Newly synthesized polypeptides and glycopolypeptides fold into their native (active) conformation based on their amino acid sequences in a thermodynamic manner with help from the resident molecular chaperones (binding protein (BiP), calnexin, heat shock proteins (HSPs), etc.). In order to monitor the *in vivo* folding process, the ER has evolved a “quality-control” mechanism, termed ERAD, which uses molecular chaperones to bind and rescue unstable misfolded conformers to facilitate their proper folding and assembly and to prevent the aggregation of nonnative forms through binding and release cycles (13,25,157,158). This machinery ensures that only properly folded and assembled proteins are transported to the Golgi complex for further maturation. Improperly folded proteins are retained in the ER by molecular chaperones and then transported to the cytosol for ubiquitination and degradation within the cytosolic proteosomes (158). In this way, misfolded or unstable (normal or mutant) proteins are eliminated from the cell (158–162). However, even after interaction with molecular chaperones, it is estimated that up to 30% of normal proteins do not achieve their functional state, misfold and/or aggregate, and are rapidly degraded within minutes of their synthesis by the cell’s quality-control machinery (118).

### 28.4.2 Mutations Causing Lysosomal Diseases

Most lysosomal disorders result from a variety of mutations in their respective disease gene that render the encoded enzyme or protein nonfunctional (for review, see Chapter 7). Nonsense and frameshift mutations encode truncated and markedly altered polypeptide sequences that cannot function and are rapidly transported, presumably by the ERAD machinery, to the cytosol for proteosome degradation. Splicing defects result either in no enzyme protein or in an insufficient amount of normal enzyme, which results in substrate accumulation. Certain missense mutations and some small in-frame deletions may not (or minimally) impair the functional domains of the mutant protein (i.e. active site, receptor binding site, etc.) but may cause polypeptide misfolding, aggregation, instability, and/or altered trafficking to the lysosome. Presumably, mutations that cause misfolding slow the normal folding process, thereby resulting in higher concentrations of “folding intermediates,” which can self-aggregate (25,163). Such mutant lysosomal proteins are retained in the ER, where they become associated with molecular chaperones (e.g. calnexin, BiP) that attempt to restore the native conformation or, having failed to form a functional state, undergo rapid degradation by the quality-control system.

In many lysosomal disorders, certain missense mutations produce mutant enzymes that retain a small amount of residual enzymatic activity (even <1%). These missense mutations are associated with the less-severe disease phenotypes. The presence of residual activity presumably results from the small amount of the mutant glycopeptide that was properly folded, assembled, posttranslationally modified, and trafficked to the lysosome. Such mutations are excellent candidates for PCT (see Section 28.4.5). Indeed, even mutations that totally misfold are potentially rescuable by pharmacologic chaperones.

### 28.4.3 Criteria for Rescuable Mutations by PCT

Ulloa-Aguirre and associates (25) have proposed general guidelines to identify rescuable and nonrescuable missense mutations:

- (1) The missense mutation should not alter critical residues essential for ligand binding or substrate/cofactor binding in the case of enzymes that are, predictably, nonfunctional.
- (2) Loss or gain of a cysteine residue may potentially disrupt required sulfuryl bridges or form inappropriate bridges that may be so significantly disruptive to the protein’s structure that rescue cannot occur.
- (3) Loss or gain of a proline residue may limit or even impede pharmacologic rescue, since this amino acid typically causes forced turns in the protein sequence

that may dramatically alter the structure, rendering it nonrescuable. In some proteins, an abrupt turn is likely a requisite for correct structure and cannot be corrected by pharmacologic chaperones.

- (4) Substitutions that impede or promote hydrogen bond formation may reduce the ability of the pharmacologic chaperone to rescue because of the inability of the mutant protein to establish correct interactions between its different domains.
- (5) Substitutions by larger amino acid residues (valine, tryptophan, threonine, and cysteine) may or may not be destabilizing, depending on their position, whereas replacements with smaller residues (glycine or alanine) may allow for more steric freedom and may be accommodated in the folding process, and, therefore, are potentially strong candidates for pharmacologic chaperone rescue.
- (6) If the enzyme or protein has been crystallized, molecular modeling studies can be used to predict the effect of the amino acid substitution on the conformation of the mutant enzyme and the impact on its active site.

For lysosomal enzymes, these pharmacologic chaperones must bind reversibly, so that, when the rescued protein arrives in the lysosomes, the chaperone will be displaced by the high concentration and greater affinity of the already accumulated natural substrate(s). In contrast to ERT with recombinant lysosomal enzymes, the small hydrophobic molecules given orally may cross the blood–brain barrier, diffuse through connective tissue matrices, and reach target sites of pathology that infused lysosomal enzymes cannot, or only can when administered at very high doses. Thus, PCT is particularly attractive for the treatment of neurodegenerative lysosomal diseases.

#### 28.4.4 Experimental Studies of Pharmacologic Chaperone Therapy in Lysosomal Disorders

Most lysosomal storage diseases have less-severe subtypes, resulting from mutations encoding altered enzymes with a small amount of residual activity. Such mutant enzymes are targets for PCT. To identify potential pharmacologic chaperones for lysosomal disorders, investigators have evaluated known or chemically modified substrate inhibitors and analogs or have performed high-throughput screens of chemical libraries (164). These compounds can be evaluated in tissue culture systems or transgenic mice to determine their *in vitro* and *in vivo* ability to rescue certain mutant enzymes (see examples later). PCT may be particularly useful in disorders resulting from common missense mutations that are rescuable, such as  $\beta$ -glucocerebrosidase N370S in type 1 Gaucher disease (153,165),  $\beta$ -hexosaminidase  $\alpha$  chain G269S in chronic Tay–Sachs disease (154), and  $\alpha$ -Gal A N215S in Fabry disease. However, the use of a specific enzyme

**TABLE 28-6** Studies of PCT in Lysosomal Storage Diseases

Disease/Deficient Enzyme	System	Chaperone <sup>a</sup>	Reference
Fabry: $\alpha$ -galactosidase A	Cells	DGJ	Fan et al. (148)
	Mouse	DGJ	Fan et al. (148)
	Human	Galactose	Frustaci et al. (155)
Gaucher type 1: $\beta$ -glucocerebrosidase	Cells	NN-DNJ	Sawkar et al. (153)
	Cells	Various	Sawkar et al. (165)
	Cells	NOV	Lin et al. (151)
GM <sub>1</sub> gangliosidosis: $\beta$ -galactosidase	Cells	NOEV	Matsuda et al. (152)
	Mouse	NOEV	Matsuda et al. (152)
Tay–Sachs/ Sandhoff: $\beta$ -hexosaminidase	Cells	NGT	Tropak et al. (154)

<sup>a</sup>DGJ, 1-deoxygalactonojirimycin; NGT, *N*-acetylglucosamine-thiazoline; NN-DNJ, *N*-(*n*-nonyl)deoxynojirimycin; NOEV, *N*-octyl-4-epi- $\beta$ -valienamine; NOV, *N*-octyl- $\beta$ -valienamine.

inhibitor that binds to the active site has the potential to rescue a variety of missense mutations whose mutant proteins retain residual activity, even less than 1% of the wild type. Experimental studies of PCT in lysosomal diseases are described in this section (Table 28-6). Extension of these studies to mutant enzymes encoded by other missense mutations and to missense mutations encoding mutant proteins in other lysosomal diseases is anticipated.

**28.4.4.1 Fabry Disease.** As noted previously, this X-linked disorder due to deficient  $\alpha$ -Gal A activity has two major subtypes, the severe childhood-onset “classical phenotype” and the later-onset phenotypes, which include the cardiac and renal variants (67,166–169). To date, over 400  $\alpha$ -Gal A mutations have been identified, including missense, nonsense, splicing, and frameshift mutations due to small and large deletions and insertions (Human Gene Mutation Database) (170). In males affected with the classical phenotype, all types of mutations have been reported (67). However, most patients with the later-onset phenotype had missense mutations that encoded mutant enzymes with low levels of residual  $\alpha$ -Gal A activity (167–169).

The crystal structure of human  $\alpha$ -Gal A has been determined, and the location and predicted effect of various missense mutations have been correlated with the observed clinical phenotypes (171,172). Molecular modeling (173) and expression studies (174,175) have been used to characterize the missense mutations and to predict or assess their residual activities.



Previously, galactose, a weak  $\alpha$ -Gal A inhibitor, was shown to stabilize the residual activity of certain  $\alpha$ -Gal A missense mutations, but not others, when these mutant alleles were overexpressed in COS-1 cells (176). Fan and colleagues evaluated a series of  $\alpha$ -Gal A substrate analogs and identified the imino sugar DGJ, a compound that bound to several mutant  $\alpha$ -Gal A enzymes at sub-inhibitory concentrations and most effectively increased their residual activities (147–149). For example, the residual  $\alpha$ -Gal A activities in cultured lymphoblasts from patients with the Q279E and R301Q mutations were enhanced seven- to eightfold after incubation with 20  $\mu$ M DGJ for 4 days. In addition, these investigators generated transgenic mice carrying the rescuable R301Q mutation and demonstrated that the oral administration of DGJ caused a dose-dependent increase in  $\alpha$ -Gal A activity in the tissues of the mice. No toxic effect was observed in transgenic mice treated with DGJ for 140 days (148). Independent studies in R301Q transgenic mouse fibroblasts demonstrated that DGJ treatment released the rescuable R301Q mutant enzyme from the molecular chaperone BiP in the ER. The rescued mutant enzyme was then trafficked via the pathway mediated by mannose 6-phosphate receptor to the lysosome, where it cleared the accumulated GL-3 (177). Thus, these “in vitro” findings indicated that DGJ stabilized the mutant  $\alpha$ -Gal A glycoprotein such that more of the enzyme was transported to the lysosome where it functions.

**28.4.4.1.1 Clinical Proof of Concept: PCT for Fabry Disease.** The clinical efficacy of pharmacologic chaperones for lysosomal disorders has been investigated in the “cardiac variant” of Fabry disease. As noted earlier, patients have residual  $\alpha$ -Gal A activity and an attenuated later-onset phenotype (67,168,169). As proof of concept, a patient with the cardiac variant of Fabry disease who had severe heart disease and was a candidate for cardiac transplantation was treated with galactose, a reversible competitive inhibitor of  $\alpha$ -Gal A. Since galactose is not as effective an inhibitor as DGJ, 1 gm/kg was administered intravenously three times weekly. The infusions were well tolerated, and there was no evidence of hepatic, ophthalmologic, or other side effects. The biochemical, histologic, and clinical effects of the infusions were monitored at 3 months and 2 years of therapy (155). After 3 months of treatment, there was evidence of improvement, and after 2 years of continuous treatment, there was marked improvement in cardiac contractility (an increase in the left ventricular ejection fraction), a moderate reduction in ventricular wall thickness, and a reduction in cardiac mass (Table 28-7). These improvements, which persisted for more than 3 years, were confirmed by the findings of independent observers, by two-dimensional echocardiography, and by cardiac magnetic resonance imaging studies. Cardiac transplantation was no longer required in this patient, because of the clinical improvement (from New York Heart Association functional class IV to class I).

**TABLE 28-7 Clinical Effect of Chaperone Therapy in Fabry Disease**

	Treatment		
	Before	3 Months	2 Years
<b>Echocardiographic data</b>			
LV end-diastolic diameter (mm)	68	59	58
LV end-systolic diameter (mm)	53	45	44
Interventricular septum thickness (mm)	20	16	16
LV posterior wall thickness (mm)	16	14	14
Shortening fraction (%)	22	24	24
LV ejection fraction (%)	33	55	55
<b>Hemodynamic data</b>			
LV end-diastolic pressure (mm Hg)	25	14	—
Cardiac index (L/min/m <sup>2</sup> )	1.7	2.9	—
<b>Cardiac MRI data</b>			
Mean LV wall thickness (mm)	18	15	14
Mean RV wall thickness (mm)	10	7	6
LV mass (g)	293	235	228
LV ejection fraction (%)	32	51	55

LV, left ventricular; RV, right ventricular.

From Frustaci, A.; Chimenti, C.; Ricci, R., et al. Improvement in Cardiac Function in the Cardiac Variant of Fabry's Disease with Galactose-Infusion Therapy. *N. Engl. J. Med.* 2001, 345, 25.

Galactose served as a reversible inhibitor that could bind to the active site and rescue the mutant  $\alpha$ -Gal A protein, thereby promoting the proper folding, dimerization, and processing of the mutant enzyme and preventing the proteasomal degradation of misfolded mutant enzyme glycopeptides (see Figure 28-1). Thus, for patients with the later-onset cardiac variant of Fabry disease whose residual  $\alpha$ -Gal A activity can be enhanced in vitro, active-site-specific PCT may prove safe and therapeutically effective.

**28.4.4.1.2 Clinical Trials of PCT for Fabry Disease.** Based on the preclinical studies in Fabry cultured cells (148), and in transgenic R301Q mice (178), and the clinical proof of concept in the later-onset cardiac variant of Fabry disease (155), efforts are underway to develop PCT for Fabry disease. Under the sponsorship of Amicus Therapeutics, Inc., a Phase 1 safety study was conducted in normal individuals that established the pharmacologic chaperone AT1001's (DGJ) safety in humans and also demonstrated that the rescue of normal  $\alpha$ -Gal A activity was dose responsive (<http://www.amicustherapeutics.com>). Phase 2 clinical

testing of AT1001 in 26 Fabry patients showed a measurable increase in enzyme activity in the leukocytes of 24 patients. In subjects with the greatest increase of enzyme activity, a reduction in substrate load was noted in kidney biopsies. A double-blind, randomized placebo-controlled Phase 3 trial is ongoing to evaluate the efficacy, safety and pharmacodynamics of AT1001 (Amigal) in males and females with Fabry disease. The trial will enroll 60 subjects who are naïve to ERT or who have not received ERT for at least six months prior to the start of therapy with Amigal (150 mg every other day). The primary end point is kidney GL3 levels; secondary end points are urinary GL3 levels, glomerular filtration rate, and safety parameters.

If PCT proves clinically effective for patients with Fabry disease who have residual  $\alpha$ -Gal A activity, investigators will be stimulated to carry out clinical trials of PCT in other lysosomal diseases, particularly those with neurologic manifestations, as described here for Gaucher disease, GM<sub>1</sub> gangliosidosis, and Tay–Sachs/Sandhoff diseases.

**28.4.4.2 Gaucher Disease.** There are three major subtypes of Gaucher disease due to mutations in the approximately 7-kb  $\beta$ -glucocerebrosidase gene that result in absent or markedly reduced enzyme activity (179): type 1, a nonneurologic disorder with clinical onset from childhood to late adult life; type 2, a severe neurodegenerative disorder of infancy; and type 3, a neurologic form with juvenile or late-juvenile onset (179). To date, over 190  $\beta$ -glucocerebrosidase mutations have been identified, including missense, nonsense, splicing, small deletions and insertions, and complex mutations due to crossovers and/or gene conversions involving a pseudogene that is 16 kb downstream from the functional gene (Human Gene Mutation Database) (170,179). Genotype/phenotype studies have revealed that certain mutations have residual activity and are responsible for the nonneurologic type 1 phenotype, whereas others are more severe and occur in the neuropathic type 2 and 3 patients. Several of the  $\beta$ -glucocerebrosidase mutations are common in various populations. For example, the “neuroprotective” N370S mutation occurs in about 70% of alleles in the Ashkenazi Jewish type 1 patients and is also common in European type 1 patients. In addition, the common L444P missense mutation is panethnic and when homoallelic, causes type 3 disease. The crystal structure of  $\beta$ -glucocerebrosidase has been recently determined (180), and studies have attempted to correlate various genotypes with the disease phenotypes (179,181,182).

Recent studies in cultured cells from patients with various  $\beta$ -glucocerebrosidase genotypes have shown that certain mutant enzymes encoded by missense mutations, such as K157Q, D409H, P415R, and L444P, are retained in the ER, where they become bound to calnexin, and are then transported by the ERAD pathway to the cytosol for proteasomal degradation (183). Several of these misfolded enzymes have been the subject of

pharmacologic chaperone rescue by active-site-directed substrate analogs. Lin and colleagues demonstrated that *N*-octyl- $\beta$ -valienamine (NOV) rescued the residual F213I  $\beta$ -glucocerebrosidase activity in cultured cells from type 1 Gaucher patients (151). However, NOV did not rescue other  $\beta$ -glucocerebrosidase mutant enzymes encoded by N370S, L444P, and 84GG.

In contrast, Kelly and colleagues evaluated several substrate analogs of  $\beta$ -glucocerebrosidase and found that *N*-(*n*-nonyl)deoxynojirimycin (NN-DNJ) at subinhibitory concentrations (10  $\mu$ M) led to a twofold increase in the residual activity of the common N370S mutant enzyme (153). Of note, NN-DNJ did not rescue the mutant enzyme encoded by the common more severe L444P missense mutation. The L444P glycoprotein was not rescuable with NN-DNJ either because of the intrinsic nature of the mutant enzyme (less stable, more rapidly degraded, etc.) or because of the alteration of its active site such that the substrate analog bound poorly. Thus, certain mutant proteins for a given enzyme may require different pharmacologic chaperones. However, it is more likely—and attractive—that a particular active-site-directed low-molecular-weight chaperone could bind most, if not all, the mutant proteins with residual activity encoded by the same gene, as has been shown for Fabry disease (J.-Q. Fan, personal communication, 2005). This logic should hold for other mutant proteins with residual receptor, effector, or epitope function. More recently, Kelly and colleagues evaluated 34 potential  $\beta$ -glucocerebrosidase inhibitors to determine if they could rescue the activity of the N370S, L444P, and G202R mutant enzymes in cultured skin fibroblasts. Of these, several compounds increased the activity of N370S and G202R, but not L444P (165).

It is of interest to note that miglustat (NB-DNJ), an inhibitor of ceramide glucosyltransferase used in type 1 Gaucher patients for substrate deprivation, has been studied to determine if it is also a pharmacologic chaperone for  $\beta$ -glucocerebrosidase (150,165). Studies of transfected COS cells with constructs expressing several mutant  $\beta$ -glucocerebrosidase enzymes, including N370S, S364R, V15M, and M123T, had 1.5- to 9.9-fold increases in activity when incubated with 10  $\mu$ M NB-DNJ, whereas the imino sugar did not rescue the activities of the mutant L444P, L336P, and S465del  $\beta$ -glucocerebrosidases, suggesting that NB-DNJ was both a substrate-deprivation molecule for glycolipid synthesis and a pharmacologic chaperone for  $\beta$ -glucocerebrosidase. However, the Kelly group did not find NB-DNJ to have an enhancing or inhibiting effect on the N370S or G202R mutant enzymes in cultured fibroblasts (165).

Thus, several substrate analogs for  $\beta$ -glucocerebrosidase have been shown to serve as pharmacologic chaperones for various mutant enzymes. The prevalence of the type 1 subtype, and particularly the frequency of the N370S mutation, makes Gaucher disease an excellent candidate disease for PCT. Moreover, if a pharmacologic chaperone

can be identified to rescue the L444P mutant enzymes, this therapeutic approach might stabilize or reverse the neurologic manifestations in patients with type 3 disease.

Based on the encouraging preclinical data with isofagamine, an active site inhibitor of glucocerebrosidase, Amicus Therapeutics initiated a Phase 1 clinical trial for Gaucher type 1 disease in 2006. Administration of isofagamine (Plicera) to 54 healthy volunteers was well tolerated and a dose-dependent increase in glucocerebrosidase levels was observed in white blood cells. However, an uncontrolled Phase 2 study in 19 treatment-naïve adult Gaucher type I disease patients failed to demonstrate clinically meaningful improvements in key measures of the disease. Two dosing regimens were studied over a 6-month period. All patients enrolled experienced an increase in the level of glucocerebrosidase activity in their white blood cells, whereas clinical improvements were noted in just 1 of the 18 patients who completed the study. In 2009, Amicus Therapeutics announced that it does not plan to advance Plicera into Phase 3 development.

**28.4.4.3 GM<sub>1</sub> Gangliosidosis.** This autosomal recessive disorder results from the deficient activity of the lysosomal exogalactosidase, acid  $\beta$ -galactosidase (for review, see chapter 104). There are at least three major subtypes, all of which have neurologic involvement because of the accumulation of GM<sub>1</sub>, GA<sub>1</sub>, and related ganglioside substrates (184). The infantile form is a rapidly progressive neurodegenerative disease with demise in the first years of life. The juvenile- and adult-onset forms have residual  $\beta$ -galactosidase activity, but at such low levels that the substrate(s) accumulate, although to a lesser degree in the adult-onset form. To date, over 50 mutations causing GM<sub>1</sub> gangliosidosis have been identified in the  $\beta$ -galactosidase gene (Human Gene Mutation Database) (170), and genotype/phenotype correlations have been proposed (184). The crystal structure of the human enzyme has not been solved.

Suzuki and colleagues reported the pharmacologic chaperone rescue of mutant  $\beta$ -galactosidase activity in deficient human cultured fibroblasts and in a transgenic mouse model of GM<sub>1</sub> gangliosidosis (152). Using the galactose derivative *N*-octyl-4-epi- $\beta$ -valienamine (NOEV), they demonstrated increased  $\beta$ -galactosidase activity in cultured fibroblasts from unrelated patients with GM<sub>1</sub> gangliosidosis. Based on these *in vitro* studies, they introduced a rescuable mutation, R201C, into  $\beta$ -galactosidase-null mice. These transgenic mice were given NOEV *ad libitum* in their drinking water for 1 week (approximately 1.4 mg/day) and then sacrificed. Increased  $\beta$ -galactosidase activity was observed in all tissues, including the brain, as determined by activity assays and histochemical staining. In addition, the accumulated ganglioside substrates GM<sub>1</sub> and GA<sub>1</sub> were decreased in the brain, as shown by immunostaining. These studies indicated that the orally administered pharmacologic chaperone gained access to the brain, rescued the

newly synthesized  $\beta$ -galactosidase mutant enzyme, and decreased the already accumulated substrates in brain lysosomes.

These studies demonstrate that small molecular ligands can rescue a variety of missense mutations and that pharmacologic chaperones can be administered orally and may readily cross the blood-brain barrier, making this approach particularly attractive.

**28.4.4.4 Tay-Sachs and Sandhoff Diseases.** Tay-Sachs disease results from the deficient activity of the  $\beta$ -hexosaminidase A isozyme, and Sandhoff disease results from the deficient activities of both  $\beta$ -hexosaminidase A and B isozymes (185). Both diseases are autosomal recessive disorders (for review, see chapter 104).  $\beta$ -Hexosaminidase A is a heterodimer with  $\alpha$  and  $\beta$ -subunits, each encoded by a different gene.  $\beta$ -Hexosaminidase B is a homodimer of the  $\beta$ -subunit. Mutations in the  $\alpha$ -subunit gene cause  $\beta$ -hexosaminidase A deficiency in Tay-Sachs disease, whereas mutations in the common  $\beta$ -subunit gene result in the deficient activity of both isozymes (185). There are three major subtypes of Tay-Sachs disease: an infantile form that is a severe neurodegenerative disorder with demise typically by 5 years, a juvenile-onset neurodegenerative disease, and a chronic form characterized primarily by progressive ataxia and muscle weakness beginning in childhood or adolescence and later onset of mild to moderate intellectual impairment. The infantile-onset subtype is prevalent in the Ashkenazi Jewish population, with a carrier frequency of about 1 in 25. The less-frequent later-onset form also occurs primarily in individuals of Ashkenazi Jewish descent.

To date, over 100  $\alpha$ -subunit mutations have been identified that cause Tay-Sachs disease (Human Gene Mutation Database) (170). Two common Jewish mutations cause the infantile form: a 4-base exonic insertion and a splicing defect, both of which result in no enzyme protein and the infantile phenotype. In contrast, most later-onset forms result from a common missense mutation, G269S (185,186).

Sandhoff disease also has three major subtypes, the infantile-, juvenile- and adult-onset phenotypes (185). The disease is rare and is not common to any particular population. To date, over 30  $\beta$ -subunit mutations have been identified, and like the respective Tay-Sachs phenotypes, infantile-onset Sandhoff disease results from mutations that produce no stable enzyme protein or activity. In contrast, the juvenile- and adult-onset phenotypes are caused by mutations that have residual activity of less than 5% of wild type (185).

Tropak and colleagues evaluated various known  $\beta$ -hexosaminidase inhibitors to identify a pharmacologic chaperone that could increase  $\beta$ -hexosaminidase  $\alpha$ -chain mutant G269S residual enzymatic activity (7). Of the seven inhibitors tested, *N*-acetylglucosamine-thiazoline (NGT) specifically, and most effectively, increased the amount of mature  $\alpha$ -chain glycopolyptide and

$\beta$ -hexosaminidase activity in the lysosomal subcellular fraction of cultured fibroblasts from a later-onset patient who was homoallelic for the G269S  $\alpha$ -chain mutation. Increasing concentrations of NGT in the growth media resulted in higher residual activity with days in culture and was not toxic to the cells. Similarly, NGT was able to enhance the  $\beta$ -hexosaminidase  $\beta$ -chain P504S mutant enzymatic activity in fibroblasts from a patient with adult-onset Sandhoff disease. Thus, these studies demonstrated that NGT bound to and stabilized the mutant enzymes in the ER, resulting in increased amounts of mutant enzymes that exited the ER and were transported to the lysosomes, where they could function at their preferred acidic pH.

### 28.4.5 Criteria for Selection of Pharmacologic Chaperones

Implicit in the selection of a pharmacologic chaperone for the treatment of patients with a lysosomal or other disease is the demonstration that the compound is nontoxic, is readily eliminated in the urine so it or its metabolites do not accumulate, and optimally rescues a variety of disease-causing mutations. In addition, molecules that could function as pharmacologic chaperones should be cell permeable and have the ability to reach the ER and remain undegraded/unmetabolized long enough to stabilize the target mutant protein and transport it to the lysosome (25). For neurodegenerative disorders, the compound must be able to reach sufficient concentration in the brain. Finally, clinical studies must demonstrate long-term safety, biochemical effectiveness (increased enzymatic activity and/or substrate clearance), and clinical benefit without adverse reactions.

### 28.4.6 Pharmacologic Chaperone Therapy for Nonlysosomal Diseases

Pharmacologic chaperones have been sought and/or evaluated for a variety of nonlysosomal diseases (Table 28-8). These include disorders such as Alzheimer disease (187) and retinitis pigmentosa (188), in which there is mutant protein misfolding and aggregation, and other diseases such as cystic fibrosis (188) and nephrogenic diabetes insipidus (189), in which there are both protein misfolding and trafficking defects. Pharmacologic chaperones have been the subject of recent reviews (1,23,25,147,190–194).

## 28.5 CONCLUSIONS AND FUTURE DIRECTIONS

The past 15 years have seen remarkable changes for scientists and clinicians working on lysosomal diseases. The era of lysosomal disease treatment has led to new awareness of these disorders and renewed excitement among researchers and patients. ERT is available for

**TABLE 28-8 Pharmacologic Chaperones: Rescue of Misfolded Proteins in Genetic Diseases**

Disease	Defective Protein
<b>Lysosomal enzymes</b>	
Fabry disease	$\alpha$ -Galactosidase A
Gaucher disease	Acid $\beta$ -glucosidase
<b>Channel proteins</b>	
Cystic fibrosis	CFTR
Long QT syndrome	HERG K <sup>+</sup> channel
<b>Receptor defects</b>	
Nephrogenic diabetes insipidus	Vasopressin V <sub>2</sub> receptor
Retinitis pigmentosa (autosomal dominant)	Rhodopsin
Familial hypercholesterolemia	LDL receptor
Hypogonadotropic hypogonadism	Gonadotropin-releasing hormone receptor
<b>DNA transcriptional protein</b>	
Cancer	p53
<b>Conformational diseases</b>	
ATT deficiency	$\alpha_1$ -Antitrypsin
Amyloidoses	Transthyretin

Based on Fan, J. Q. A Contradictory Treatment for Lysosomal Storage Disorders: Inhibitors Enhance Mutant Enzyme Activity. *Trends Pharmacol. Sci.* 2003, 24, 355.

six disorders (Gaucher, Fabry and Pompe diseases and MPS I, II and VI), and is under development for several others. Experimental and clinical experience with other approaches, including bone marrow transplantation, substrate deprivation, and gene and stem cell therapies, have identified their useful applications and limitations (2,3,6,7,28). However, despite these remarkable advances, significant hurdles still remain before the goal of treating all or most lysosomal diseases can be realized. Primary among them is the need to treat the neurodegenerative lysosomal diseases.

PCT represents an attractive new strategy that uses small molecules that can be administered orally to rescue misfolded/mistargeted lysosomal enzymes (or other proteins), thereby resulting in their increased activity and therapeutic benefit. In addition, PCT offers the possibility of treating disorders with neurologic involvement, since low-molecular-weight pharmacologic chaperones may be designed to cross the blood–brain barrier. It is likely that PCT strategies may have a significant therapeutic effect; however, the treatment will be limited to patients with rescuable mutations. It is important to note that pharmacologic chaperones may have markedly different biodistributions than replaced recombinant human enzymes, whose tissue delivery is primarily dependent on their respective receptor-mediated delivery and that do not cross the blood–brain barrier. Thus, for certain diseases, combined PCT and ERT may be beneficial, particularly for the treatment of neurodegenerative lysosomal and other diseases of the CNS. In summary,



it is anticipated that the early years of the twenty-first century will witness the development of safe and effective ERT and PCT for patients with lysosomal and other inborn errors of metabolism.

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## CROSS-REFERENCES

Human Gene Mutation in Inherited Disease: Molecular Mechanisms and Clinical Consequences; Gene Therapy: From Theoretical Potential to Clinical Implementation; Mucopolysaccharidoses; Sphingolipid Disorders and the Neuronal Ceroid Lipofuscinoses or Batten Disease (Wolman Disease, Cholesteryl Ester Storage Disease, and Cerebrotendinous Xanthomatosis).

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## RELEVANT WEBSITES

- <http://www.fda.gov/ohrms/dockets/ac/cder03.html#endocrinologycmetabolicdrugs>.
- <http://www.amicustherapeutics.com>.
- <http://www.clinicaltrials.gov/ct/show/nct00214500?order=1>.

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# CHAPTER

# 29

## Gene Therapy: From Theoretical Potential to Clinical Implementation

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*It would be the height of folly—and self-defeating—  
to think that things never heretofore done  
can be accomplished without means never heretofore tried.  
Sir Francis Bacon, Novum Organum*

### 29.1 GENES AS MEDICINES— THE ORIGINS OF GENE THERAPY

The identification of most genes that makeup the human genome makes it easy to think about the potential medical applications of genomics. Cutting a gene out and using it to replace a missing one does not sound like science fiction anymore. However, the idea of using genes as medicines was born too early. In fact, it was proposed before it was straightforward to identify individual genes within genomes and before the discovery of restriction enzymes, i.e. before cutting and pasting in the genome was possible. Thus, the first experiments were primitive by today's standards, and literally stopped while the technologies of molecular biology were being discovered.

In the early 1970s RNA oncogenic viruses were shown to cause cellular transformation through the insertion of viral genomic sequences into the infected host cell genome. Around the same time, scientists were discovering that many inherited diseases were caused by mutations in single genes. They thus reasoned that if genes could be isolated from normal nonmutated human genomic sources and inserted into RNA viruses in lieu of their wild-type oncogenes, and these modified viruses

then used to transfer the normal genes into the cells and tissues of affected patients, inherited diseases could be treated directly by gene transfer. Thus, gene therapy was envisioned.

Realizing these ideas had to wait many years before restriction enzymes, routine sequencing and the manipulation of viral genomes became easily accessible. Interestingly, during this incubation stage, scientists discussed the ethical boundaries of gene therapy as well as the foundations for the ethical and scientific review of potential future clinical trials in gene therapy. The Food and Drug Administration established a Cell and Gene Therapy section, and the National Institutes of Health created the Recombinant DNA advisory committee to review the science and ethics of future clinical trials in gene therapy.

The establishment of the function of the regulatory bodies was crucial because earlier attempts at gene therapy were marred by unclear overview powers. Early attempts by Martin Cline from UCLA to transfer a globin gene and thymidine kinase into blood and/or bone marrow cells from patients suffering from sickle cell anemia were carried out abroad, in Italy and Israel, before the existence of either of the regulatory agencies. Although this trial occurred outside the United States and had local ethical approval, the use of reagents produced under US federal oversight and funding and the absence of federal US approval for this trial effectively ended the scientific career of the Chair of Haematology-Oncology at UCLA.

From then onward guidelines were developed to usher the era of genetic medicine in the United States, and thus, the rest of the world Figure 29-1 for the evolution of gene therapy, and Figures 29-2 and 29-3 for the most common vectors in clinical use and the diseases being treated.

## 29.2 THE BASIC SCIENCE: GENE TRANSFER (TABLES 29-1–29-3)

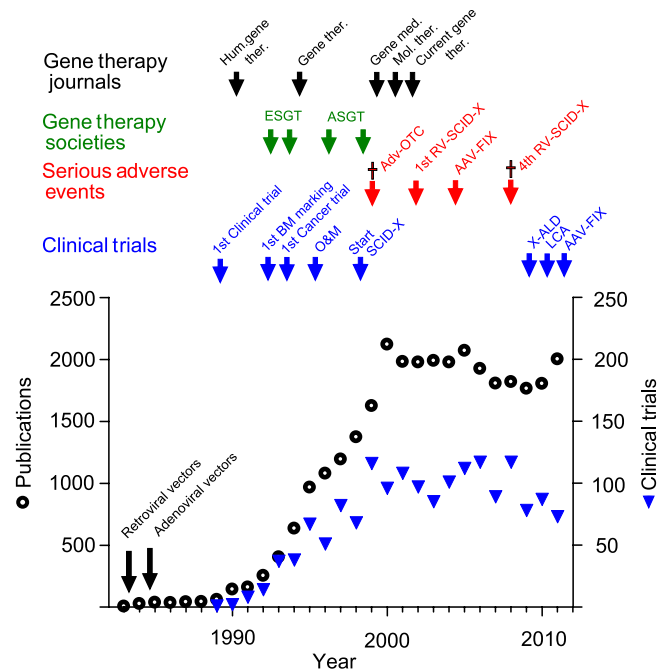
### 29.2.1 Retroviral and Lentiviral Vectors

Viruses are very effective in transferring their own genes into eukaryotic cells and have been adapted as Trojan horses to deliver therapeutic DNA sequences into affected cells. Retroviruses were the first vectors developed. Vector genomes were engineered to remove sequences encoding for pathogenic functions and generally to abolish the replication of the parent wild-type viruses (1–3).

Retroviruses are enveloped single-stranded RNA viruses and have been widely used in gene transfer and gene therapy. Retroviruses have a genome of about 7–10 kb, composed of three gene regions termed *gag*, *pol* and *env*. These gene regions encode structural capsid proteins, the integrase and viral reverse transcriptase, and envelope glycoproteins, respectively. The genome also has a packaging signal ( $\Psi$ ) and cis-acting sequences, termed long terminal repeats (LTRs), at each end, which have a role in transcriptional control and integration of the viral DNA into the infected cell's genome (4–6).

The retrovirus life cycle is initiated by the interaction of the viral envelope protein with one or more specific receptor molecules located on the plasma membrane of target cells (7). Following entry into target cells, the RNA is reverse transcribed into linear double-stranded (ds) DNA by the viral reverse transcriptase and subsequently integrated into the cell chromatin by the viral integrase. One of the most important properties of retroviruses is their ability to integrate into the genome of target cells, ensuring stable transduction and potential long-term gene expression. Integration of the simpler retroviruses only occurs in dividing cells, since retroviruses use the mitotic breakdown of the nuclear membrane to access the host cell chromosomes. Lentiviruses, a group of more complex retroviruses, have evolved the capacity to cross the intact nuclear membrane and thus integrate into nondividing cells. This is particularly useful for transducing the neurons of the central nervous system (CNS), which cannot be transduced by retroviral vectors but can be transduced well with lentiviral ones (8).

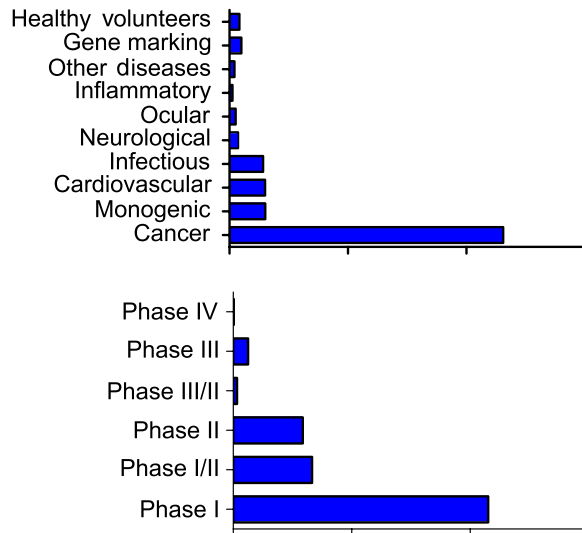
In order to make retroviral vectors carrying therapeutic genes the *gag*, *pol* and *env* genes are removed. This provides a space of up to 8 kb for exogenous DNA to be inserted. The products of the deleted genes are required for the production of functional retroviral vectors,



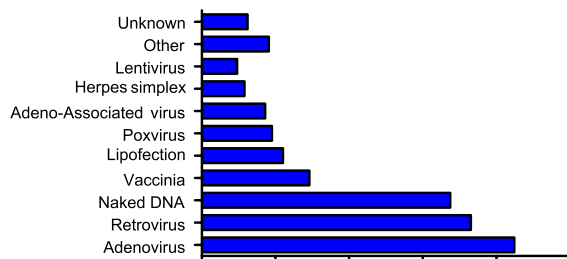
**FIGURE 29-1** Illustration of the yearly number of publications that mention “gene therapy” in the title or abstract from the PubMed database compared with the number of clinical gene therapy trials, taken from the Journal of Gene Medicine database. Above the figure, starting dates of the first and subsequent significant clinical trials are indicated in blue and reports of the most serious adverse effects are given in red. In addition, we have shown the appearance of International Gene Therapy Societies, and the most important journals in the field. The first clinical trials in gene therapy were those performed by Malcolm Brenner and collaborators in Memphis on bone marrow marking and the treatment of ADA-deficiency. “O&M” indicates the publication of the Orkin and Motulsky (256) report on the status of gene therapy that recommended a return to the basic science. “Start SCID-X” refers to the trial conducted by Alain Fischer and collaborators in Paris, accepted as the first-ever conclusive “cure” achieved by gene therapy (257). “X-ALD” indicates the successful treatment of X-linked adrenoleukodystrophy (133). “LCA” indicates the successful treatment of Leber’s congenital amaurosis (134) and “AAV-FIX” in blue indicates the successful treatment of hemophilia (258) “+Adv-OTC” in red indicates the death of J. Gelsinger during the clinical trial for ornithine transcarbamylase deficiency at the University of Pennsylvania (259). “1st RV-SCID-X” indicates the first of four times that leukemia was diagnosed in each of the affected patients treated for SCID-X in Paris (A fifth patient was diagnosed with leukemia during a trial in London). “AAV-FIX” in red indicates the description of the loss of transgene expression and transient hepatitis in one patient treated with AAV-FIX for hemophilia in the trial led by Kay and High in Stanford and Philadelphia (245). “4th RV-SCID-X” indicates the report of the death from refractory leukemia of one of the patients in the Paris SCID-X trial (By this time the remaining three patients diagnosed with leukemia in this trial and the single patient who developed leukemia in the London trial have all been successfully treated and have been in remission for several years).

however. Therefore, these proteins are provided in *trans* by packaging cell lines expressing the *gag*, *pol* and *env* genes but lacking most viral cis-acting regions. The use of packaging cell lines reduces the risk of generating replication-competent retroviruses (RCR) by homologous recombination events between the viral plasmid

and the packaging cell line. The current generation of “safe” packaging cell lines involves them carrying two to three separate constructs, one of which expresses the



**FIGURE 29-2** This figure divides the clinical trial described in Figure 29-1 by diseases treated and whether they were early Phase I or more advanced Phase III trials. Notice that most trials are for cancer and most are Phase I toxicity trials.



**FIGURE 29-3** This figure indicates the vectors used for the clinical trials represented in Figure 29-1. Notice that retrovirus, the very first viral vectors developed, and adenoviruses continue to be the most common vectors in clinical use.

gag and pol protein and the second that expresses the env protein. Alternatively, each gene may be carried by separate plasmids. Expressing each gene from a different plasmid improves the biosafety by increasing the number of recombination events required for the generation of RCR (9–11).

Further developments to improve the efficacy of retroviral vectors have been the generation of “pseudotyped” vectors. Pseudotyped vectors carry the genetic information, the core of one virus, and an envelope containing the env protein of a different one. Pseudotyped viruses have thus a different host range. Use of the envelope protein of viruses with a very narrow host range, such as HIV, allows to target gene delivery to particular cell types, but for other applications, a broader cell tropism is desirable. For example, vectors pseudotyped with the G glycoprotein of the vesicular stomatitis virus (VSV-G) can infect most cell types, are particularly stable and can be concentrated to titers exceeding  $1 \times 10^8$  TU/mL. VSV-pseudotyped lentiviral vectors efficiently transduce neurons and glial cells of the CNS of rodents. Stable long-term transgene expression was observed without any major vector-associated toxicity (8,12).

One of the newer retroviruses used for gene therapy vectors are the lentivirus. Lentiviral vectors are derived from human immunodeficiency virus type-1 (HIV-1), feline immunodeficiency virus (FIV), or the equine infectious anemia virus (EIAV). Vectors derived from viruses that are nonpathogenic in humans, such as EIAV, have an obvious safety advantage. Lentiviral vectors transduce and integrate into dividing and nondividing cells in vitro and in vivo (8,13,14). Lentiviral vectors can integrate into the genome of postmitotic cells, such as neural cells, monocytes and hematopoietic bone marrow, and stem cells (15,16).

To develop HIV-derived vectors, initially, accessory genes (vif, vpr, vpu and nef) that are not essential for

**TABLE 29-1** Gene Transfer Vehicles Used in Gene Therapy Applications

	Ad	HC-Ad	HSV-1/r	HSV-1/a	AAV	Retrovirus	Vaccinia Virus	Microinjection	Transfection
Size	36	30–36	152	10–30	4.68	3.5–9.2	186	Unlimited	Unlimited
Cloning capacity (kbp)	7.5	~30	30	10–30	2–4.5	~8	30	Unlimited	Unlimited
Transduction									
In vivo?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
In vitro?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Long-term expression	No	Yes	Yes	Yes	Yes	Yes	No	?	No
Vaccination	Yes	Yes	Yes	Yes	Yes	Yes	Yes	—	—
Vector titers (pfu/mL)	$10^{12}$	$10^{11}$	$10^8$	$10^8$	$10^9$	$10^7$	$10^6$ – $10^8$	—	—

HC-Ad, helper-dependent adenovirus vector; HSV-1/a, herpes simplex type 1 amplicon; HSV-1/r, herpes simplex type 1 recombinant vector; pfu/mL, plaque-forming units per milliliter.

**TABLE 29–2 Advantages of the Most Common Viral Vectors for Gene Therapy**

Virus	Maximum Cloning Capacity	Advantages
Ad	8 kpb	<ul style="list-style-type: none"> <li>• Broad cell tropism</li> <li>• Infection of dividing and nondividing cells</li> <li>• Easy to produce at high titer</li> </ul>
HC-Ad	~30 kbp	<ul style="list-style-type: none"> <li>• Broad cell tropism</li> <li>• Less inflammatory and cellular immune response</li> <li>• Longer term transgene expression</li> </ul>
AAV	>5 kbp, 10 kbp (new-generation vectors)	<ul style="list-style-type: none"> <li>• Broad cell tropism</li> <li>• Infection of dividing and nondividing cells</li> <li>• Integration into host genome</li> </ul>
HSV	30 kbp (HSV-1/r) 150 kbp (HSV-1/a)	<ul style="list-style-type: none"> <li>• Broad cell tropism</li> <li>• Latency in neurons</li> <li>• Very stable</li> </ul>
Lentivirus	10 kbp	<ul style="list-style-type: none"> <li>• Infection of dividing and nondividing cells</li> <li>• Integration into host genome</li> </ul>

**TABLE 29–3 Possible Disadvantages among Gene Transfer Vehicles in Gene Therapy**

Vector	Disadvantages
Ad	<ul style="list-style-type: none"> <li>• Host immune responses: inflammatory and cytotoxic reactions in patients and depletion of transduced cells</li> <li>• Host's humoral immune responses may neutralize adenoviral vector particles during, or even before, the gene transfer process</li> <li>• Not suitable for long-term expression of the transgene due to the lack of integration into host genome</li> <li>• Complex vector genome</li> </ul>
HD-Av	<ul style="list-style-type: none"> <li>• Can only be grown in the presence of a helper virus</li> <li>• Immune responses against capsid antigens are still possible</li> </ul>
AAV	<ul style="list-style-type: none"> <li>• High titers of pure virus are difficult to obtain</li> <li>• Requires a helper adeno- or herpesvirus</li> <li>• Vector system is still not well characterized</li> <li>• Limited capacity for foreign genes (about 2–4.5 kbp)</li> <li>• Lack of specific integration for recombinant AAV vectors, which may result in cell mutagenesis</li> </ul>
HSV-1	<ul style="list-style-type: none"> <li>• Host immune responses: inflammatory cytopathogenicity and neurotoxicity reactions in patients</li> <li>• Complicated vector genome</li> <li>• Difficult to produce</li> <li>• HSV-1-derived vectors could potentially reactivate latent wild-type HSV-1</li> </ul>
Retrovirus	<ul style="list-style-type: none"> <li>• Random insertion of viral genome, which may result in mutagenesis and activate oncogenes</li> <li>• Possibility of replication-competent virus formation by homologous recombination; possible recombination with human endogenous retroviruses (HERVs)</li> <li>• Retroviral vector particles are rapidly degraded by the complement</li> <li>• Infects only dividing cells, small insert capacity (6.5 kbp)</li> </ul>
Vaccinia virus	<ul style="list-style-type: none"> <li>• Widespread use of vaccinia as a live vaccine requires improving safety while achieving an even higher immune response to the recombinant protein</li> </ul>
Microinjection Transfection <sup>a</sup>	<ul style="list-style-type: none"> <li>• Difficult to introduce DNA on a scale large enough for biochemical analysis</li> <li>• Targeting is not specific</li> <li>• Low transfection efficiency</li> <li>• Only transient expression</li> <li>• Difficult in vivo applications</li> <li>• Host immune responses: inflammatory reactions in patients if vectors express chimeral cell receptors on their surface, or in the presence of unmethylated CpG sequences of bacterial plasmid DNA</li> </ul>

<sup>a</sup>Cationic liposomes or DNA-protein complexes.

transduction were eliminated (17). Second, a three-plasmid expression system was used to minimize the possibility of generating replication-competent virus through recombination (8). Third, self-inactivating (SIN) vectors

were engineered by deletions in the 3' LTRs of the HIV-1 vectors. SIN vectors also facilitate regulated expression of genes from internal promoters by reducing cis-acting effects of the HIV-1 LTR (18,19).



Retroviruses are the vectors of choice for *ex vivo* gene therapy, such as diseases that can be treated by replacing a gene within bone marrow cells, such as the blood disorder ADA deficiency, various leukodystrophies, and SCID-X (see below). For *ex vivo* gene therapy, bone marrow hematopoietic precursor cells are removed and infected *in vitro* with retroviral vectors. Clones will be selected and those expressing the highest levels of transgene expression will be used to transplant back into the patients. This procedure selects for clones with a clear growth advantage resulting in the transplantation of those clones with the highest growth rates. The functional implications of this will become clearer when reviewing some of the side effects due to the integration sites of the retroviral genomes.

For a long time it was thought that retroviruses integrated randomly into the host cell's genome. Recent detailed analysis of integration sites, using the knowledge gained from the human and mouse genome projects, has shown that although there is no site-specific integration of retroviral vectors, they do integrate preferentially into areas of the genome containing highly active or transcribed genes. This results in the existence of hotspots for genomic integration. How these integration sites affect the expression of the genes located nearby has only been studied in detail in the case of the patients who developed leukemia post gene therapy during the treatment of SCID-X.

Newer developments concern targeted vectors that will selectively infect predetermined cell types and the use of cell-type-specific and inducible promoter systems, advances that are now being applied to most available vector systems. Also, a number of groups have recently described the use of replication-competent retroviral vectors. These have been applied particularly to the treatment of brain tumors. Brain is made up of a majority of nondividing or very slowly dividing populations of cells; therefore, brain tumors constitute a population of highly mitotically active cells within an organ built essentially from nondividing ones. Therefore, replication-competent retroviral vectors, in principle, would be able to replicate specifically throughout the brain tumors, without damaging healthy brain cells such as the neurons and the astrocytes. In this case the replication competency of the vectors is what allows them to specifically reach the intended target, namely, the brain tumors (20,21).

Tai et al., (22) reported that a replication-competent retroviral vector derived from murine leukemia virus encoding the suicide gene yeast significantly extended the survival of mice with experimental glioma, and this treatment is currently the subject of a Phase I clinical trial.

### 29.2.2 Herpes Simplex Virus

Herpes simplex virus (HSV) can infect and express genes in both dividing and nondividing cells (23–25). It is a

large neurotropic human virus containing 152kb of linear ds DNA that naturally establishes lifelong asymptomatic infections of the nervous system with periodic epidermal manifestations, without the need of integrating the viral chromosome into the host genome. It can cause a virulent lytic infection or stay latent in the CNS (26). Episomal HSV genomes can reactivate, causing dermal lesions. The neurotropism of this virus has prompted the development of vectors for gene delivery to the nervous system (for a review of the use of HSV-derived vectors in brain disease, see Marconi et al., (27)). The HSV viral genome consists of unique long (UL) and unique short (US) regions, which encode gene products that regulate gene expression, DNA replication and structural proteins that form the viral capsid and the tegument. The genome also contains several cis-acting regions, including origins of replication and sequences within the terminal repeats, which are essential for packaging of genomes into virions (24). After infection of animal cells, a cascade of viral gene products is expressed that leads to the production of progeny virus. In neurons, HSV vectors are delivered by rapid retrograde transport along neurites to the cell body. The viral DNA is deposited in the nucleus, initially in a circularized episomal form, and eventually replicates and enters latency. During latency no proteins are expressed; however, a few mRNAs are expressed under the control of the latency (LAT) promoter. The expression of these LAT transcripts is driven by two adjacent promoters known as LATP1 and LATP2. Long-term transgene expression (up to 6 months) has been shown when the LATP2 promoter is used (28). The ability of viral particles to move along neural processes provides an indirect approach for targeting gene transfer to cells that are difficult to reach directly, but whose terminal fields are accessible, such as dorsal root ganglion neurons that can be reached from their axonal terminals in the skin.

Two types of vectors are derived from HSV: recombinant viral vectors (rHSV) and amplicon vectors. rHSV contain the full viral genome mutated in one or more viral genes to reduce toxicity and provide space for transgenes in the range of 30–50kb. In order to generate rHSV it is important to remove virally encoded cytotoxic functions produced during the lytic cycle and promote the establishment of latency. Early rHSV vectors involved the deletions of ICP4 and ICP27, which effectively block viral replication and promote the entry of the HSV genome into latency (29,30). These viruses are grown on specially constructed cell lines that encode the genes that are missing *in trans*. Unfortunately, these early rHSV were shown to be highly toxic because of the continued synthesis and accumulation of other IE gene products (29). rHSV vectors can enter a stable, nontoxic, episomal latent state in neurons but with consequent down-regulation of most viral and cellular promoters (31). The use of various latency promoters to drive

transgene expression has allowed the maintenance of transgene expression during latency.

It has been shown that rHSV vectors deleted of all viral genes are essentially silent, except for transgene expression (32). An important modification of rHSV vectors for in vivo gene transfer into postmitotic cells has been the generation of vectors lacking the ICP34.5 gene. These viruses are incapable of growing on nondividing cells, although they can be grown in culture on dividing cells (33). These vectors were effectively utilized in the very first clinical trials of HSV-derived vectors in clinical gene therapy. HSV-1716, an ICP34.5-deleted HSV1-derived vector, has been evaluated in three Phase I trials for glioma (34), and a Phase II trial is currently being developed. G207, which, in addition to ICP34.5 deletion, has an additional deactivating insertion, lacZ insertion in UL39, has been used in two clinical trials for glioma (35).

An alternative HSV vector system is the “HSV amplicon.” The amplicon system relies on introducing the gene of interest into a plasmid, which has an HSV origin of replication and packaging signal. This construct is then introduced into cells by transfection. The cells are then infected with a helper HSV virus resulting in the amplicon becoming packaged into the virus particle (36). The advantages of these vectors are that they are essentially nontoxic or antigenic, as they express no virus proteins, although they generally contain low levels of contaminating recombinant replication-competent virus during packaging. They also have

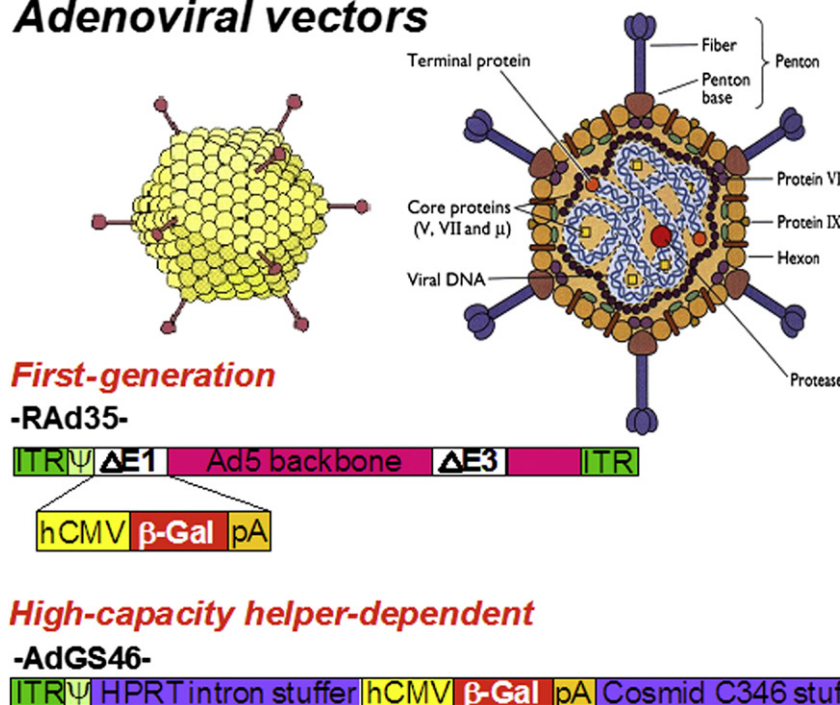
a large transgene capacity (theoretically up to 150 kb), relatively high titers (up to  $10^8$  ti/mL with the current packaging modalities), high infectivity for cells of the nervous system and retention for several months in nondividing cells (31). Amplicon vectors have yet to be tried in humans but have shown some promising preclinical results (37).

### 29.2.3 Adenovirus Vectors

The major clinical interest in adenovirus vectors (AdVs) stems from their broad host range, high infectivity for most cells in the human body and the ability to infect quiescent and dividing cells. Adenoviruses belong to the family Adenoviridae, and to date human adenoviruses are found as 47 different serotypes. The viruses are grouped together into six subgroups termed A–F according to physical and pathological properties. The most commonly studied serotypes are 2 and 5, and they have been widely studied to determine adenovirus biology and have also been exploited for the development of gene transfer vectors.

Adenoviruses are medium-sized, nonenveloped viruses having 36-kb ds DNA and icosahedral particles that are approximately 80 nm in diameter (Figure 29-4). The adenovirus consists of a protein capsid surrounding a core containing the ds DNA genome. The protein capsid is composed of 252 protein subunits (capsomeres), of which 240 are hexons and 12 are pentons. As suggested by their names, penton and hexon subunits are

## Adenoviral vectors



**FIGURE 29-4** Schematic view of AdVs. The top left image illustrates an adenoviral capsid in three dimensions and the constituents are indicated in the top right figure. Below, the genomes of a first-generation vector (e.g. RAD35, only lacking the E1 and E3 region is illustrated) and a novel high-capacity helper-dependent vector (AdGS46), lacking all wild-type adenovirus genomic sequences, are shown.

surrounded by five and six neighbors, respectively. Each penton is composed of a penton base and a projecting fiber located at the vertices of the icosahedral capsid (Figure 29-4). The Adenovirus genome is functionally divided into two major non-contiguous overlapping regions, early and late, defined by the onset time of transcription after infection. The viral chromosome is further divided into five early coding regions (E1a, E1b, E2, E3, and E4), two intermediate transcription units (pIX and Iva2) and one major late region (MLR) that consists of five principal coding units (L1–L5). The MLR encodes the information for capsid and internal core structural proteins. An additional region of the chromosome is transcribed by RNA polymerase III and encodes two virus associated RNAs, one of which acts to prevent inhibitory phosphorylation of the cellular elongation factor eIF-2 and thus maintain protein translation in spite of a cellular interferon response (38). Other features of the genome include the inverted terminal repeats (ITRs) located at the end of the viral chromosome, which act as the origin of replication. Preceding the ITR is a  $\Psi$  sequence, which encodes the packaging signal (39).

Adenovirus infects cells by binding to cell surface receptors, mediated by the interaction of the penton fiber protein with the coxsackie adenovirus receptor (CAR) (40) and the binding of the penton base with cellular integrins,  $\alpha_v\beta_5$ . Binding to the cell surface receptors leads to the internalization of the virus via clathrin-coated pits (39), followed by internalization and endosome exit. The nucleocapsid is transported to the nucleus via a microtubule-dependent process, followed by the translocation of the Ad genome across the nuclear membrane by an ATP-dependent process where viral DNA synthesis, or transgene expression in the case of vectors, proceeds (41,42).

Once in the nucleus, the E1a gene is the first viral gene that is expressed. Because the E1a products have a key role in controlling viral replication, early transcription and DNA synthesis, the earliest adenoviral vectors had deletions in their E1 region (43). Premature cell death is regulated by the expression of E1b products, E1B-19K. Furthermore, E1 proteins protect the infected cell from the action of interferons  $\alpha$  and  $\beta$ . The E3 gene products are also important in preventing an immune response; they serve to protect infected cells by preventing cytolysis by cytotoxic T lymphocytes (CTLs) or tumor necrosis factor  $\alpha$ . Cells expressing the E3gp-19K are protected from CTLs because the protein binds directly to the peptide-binding domain of MHC I, causing its retention and thereby preventing presentation of viral antigen by infected cells.

Following the onset of DNA replication, the major late promoter becomes active, enabling transcription of the late genes, which produce the capsid and internal structural proteins. The capsid proteins are then transported from the cytoplasm to the nucleus, initiating viral assembly. Ad genomes of the correct size together with

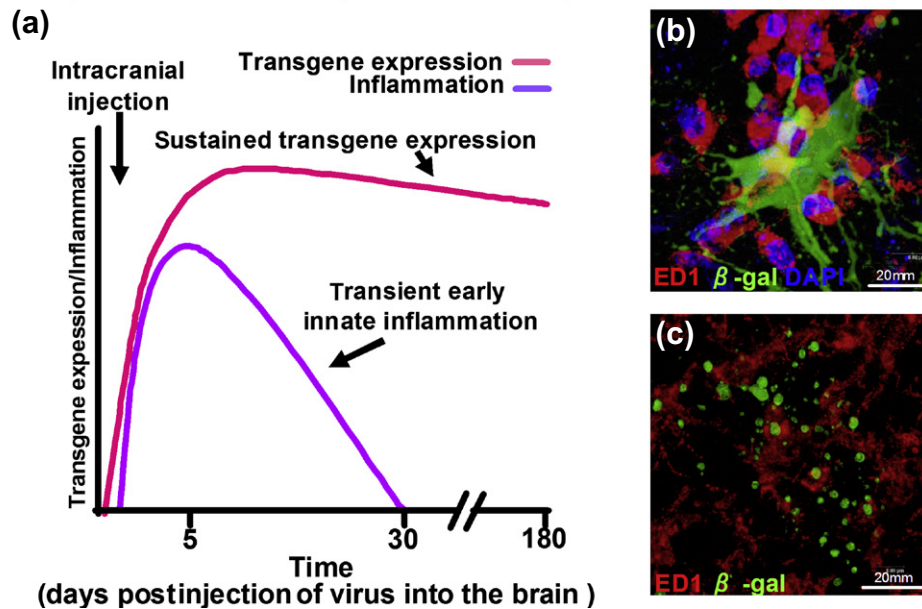
an appropriate packaging signal are packaged into virions. The Ad life cycle is terminated by cell lysis, and the release of 10,000 viral particles per cell (44).

**29.2.3.1 First-Generation AdV.** The most common first-generation AdVs developed for gene therapy are based on the Ad2 and Ad5 serotypes, since the genetics and biology of these viruses had been extensively investigated. Initial recombinant AdVs were made replication defective through deletions in the E1 region, where the transcriptional cassette could be inserted; most also contained small deletions in E3 to increase cloning capacity. A human embryonic kidney-derived 293 cell line was used to provide the E1A and E1B functions in trans. Recombinant AdV expressing a transgene of interest could then be generated by homologous recombination between the backbone of the virus genome and a shuttle plasmid containing the expression cassette flanked by a region of viral DNA. As a result, 90% of the wild-type Ad genome is retained in these first-generation vectors (45).

First-generation E1-deleted AdVs have a number of positive characteristics. One of the most important is their relative ease for scale-up of titers reaching above 1012 IU/mL (infectious units/milliliter). Other attractive features include the ability to infect many different cell types, both dividing and non-dividing, and having an extremely low probability of random integration into the host chromosomes (46). The majority of first-generation AdVs are also deleted for the E3 region, which is not required for virus replication in cell culture. E1, E3-deleted Ad vectors theoretically allow for DNA insertion of around 8 kb.

First-generation AdVs have been used to successfully deliver genes to a wide variety of tissues and organs in animal models of human disease and in the majority of the more than 400 human Phase I clinical trials worldwide using adenoviral vectors. In general, Ad-mediated gene therapies have been well tolerated, with only minor, transient side effects (47–49). In spite of the early region deletions, first-generation AdVs have residual expression of viral genes that lead to a strong host-adaptive immune response, resulting in generation of high titer neutralizing anticapsid antibodies that inhibit reinfection with the same serotype of AdV (50), as well as a CTL response directed against various proteins expressed from the wild-type genome sequences. After systemic delivery of vector, the Ad capsid proteins appear to immediately activate chemokine expression from infected cells, commonly referred to as the innate immune response (51). In addition, at high viral doses this residual virus gene expression leads to cellular cytotoxicity, which can result in an immune-mediated loss of the transduced cells (52). Although injection of first-generation recombinant AdV into the brain parenchyma causes acute cellular- and cytokine-mediated inflammatory responses, this does not affect transgene expression. In the presence of adenoviral immune responses, transgene expression for

### Adenovirus-mediated acute inflammation is transient and does not affect transgene expression long-term from first-generation or HC-Adv vectors



**FIGURE 29-5** (a) Schematic illustration of the time course of the acute adenovirus-mediated innate inflammatory response. (b, c) Innate immune responses in the brain in vivo. Injection of  $1 \times 10^8$  i.u. of RAd36 into the rat brain. (b) Macrophages/microglia immunoreactive for ED1 (in red) contact  $\beta$ -gal expressing cells (green). (c) Presence of  $\beta$ -gal immunoreactivity within macrophages, indicating that ED1-immunoreactive brain macrophages/microglia have phagocytosed dead transduced brain cells.

first-generation adenovirus is rapidly ablated (53,54). Adenovirus-induced cytotoxicity is only seen when high vector doses of  $>10^8$  i.u. are used to transduce the target tissue (55) (Figures 29-5 and 29-6).

**29.2.3.2 Second-Generation AdVs.** To overcome the immunological problems caused by proteins expressed from the wild-type sequences in adenoviral vector genomes, a series of Ad vectors with further deletions were developed. In order to propagate multiple deleted Ad vectors packaging cell lines were developed that trans-complement the growth and packaging of these vectors. To date, packaging cell lines coexpressing the Ad E1 and E4 genes, the E1 and E2a (single-strand DNA-binding protein, ssDBP) genes, the E1 and preterminal protein genes, and the E1 and protease genes have been generated and used to package the E1, E4-deleted Ad vectors (56), the E1, E2a-deleted Ad vectors (57), the E1, E2b-deleted Ad vectors (58), and the E1- and protease-deleted Ad vectors (59), respectively.

**29.2.3.3 E1,E4-Deleted AdVs.** The E4 region is located at the right end of the Ad genome and encodes seven open reading frames. The E4 protein has several functions, including regulation of Ad replication and late gene expression (60). Upon administration of the E1,E4-deleted Ad vectors, the transgene expression was extremely transient, likely due to the lack of an E4-encoded trans-acting factor that can influence expression from some promoters (61). Comparing the toxicity with first-generation Ad vectors, E1,E4-deleted Ad vectors demonstrated prolonged transgene expression and

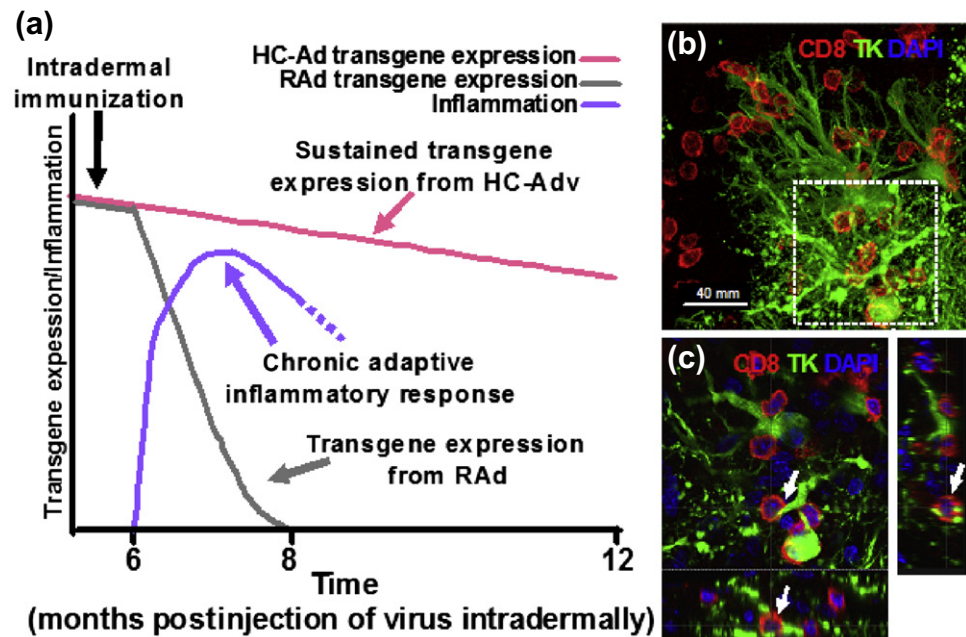
decreased evidence of hepatotoxicity (62). The cloning capacity of this vector is up to 10 kb.

**29.2.3.4 E1,E2a- or E1,E2b-Deleted AdVs.** The E2 region can be subdivided into E2a and E2b. E2a encodes a 72-kDa DBP that is essential for the initiation of viral DNA synthesis and for the activation of the major late gene expression. E2b encodes a 140-kDa DNA polymerase and an 80-kDa precursor terminal protein, both of which are required for viral DNA replication. Intravenous infusion of an E1,E2a-deleted AdV encoding human factor VIII into primates resulted in physiological plasma levels of hFVIII, facilitated by widespread hepatic transduction (57). E1,E2a-deleted Ad vectors have shown improved decreases in inflammatory responses but are difficult to grow at high titers. Recently, an E1,E2a-deleted AdV has been utilized as helper virus for propagation of helper-dependent AdVs (discussed below) in order to further minimize any toxicity that may be attributable to helper virus contamination (63). The main advantages of E1,E2b-deleted AdVs are reduced acute toxicity, extended persistence of the transgene, and ease of scale-up.

**29.2.3.5 100K, Protease-Deleted AdVs.** The 100K gene and protease functions are normally expressed only after Ad replication occurs. The 100K protein is necessary for Ad capsid assembly, hexon monomer protein transport from the cytoplasm into the nucleus and hexon trimerization. The protease is required for full maturation of the capsid. AdVs deleted for 100K or protease are not affected in their ability to replicate their genomes,



### Systemic immunization against adenovirus eliminates transgene expression from first-generation adenovirus and causes prolonged brain inflammation, but does not abolish expression from HC-Adv



**FIGURE 29-6** Careful injections of vectors into the brain parenchyma, in the absence of systemic delivery of vector antigens, lead to long-term expression; (a) the time course of the adaptive adenovirus-mediated immune response that occurs following a systemic priming of the immune system. (b, c) Adaptive immune responses in the brain in vivo. Presence of CD8<sup>+</sup> T cells within the brain parenchyma of a systemically immunized rat, following the injection into the brain of RAAd-TK. In (a) we illustrate triple labeling with CD8 (red), thymidine-kinase-positive cells (green) and nuclei (DAPI, blue). Note the presence of CD8-positive cells in the striatum of a rat injected intracranially with RAAdTK and immunized intradermally with RAAdHPRT, and killed 14 days after the immunization. CD8 cells usually were close to TK-positive brain cells (a). We could detect frequent contacts between CD8 cells and TK-positive cells (c, arrows). The analysis of the contacts can be observed in 0.5- $\mu$ m-thick optical layers examined by confocal microscopy, illustrated by the views from the x- and y-axes as shown in (b). Notice that whereas activation of the innate immune response does not eliminate transgene expression the adaptive immune response does. The mechanisms by which the adaptive immune response achieves this remain to be demonstrated.

but packaging of the replicated vector genomes is abolished. The 100K or protease-deleted AdVs enable higher transgene expression levels at much lower dosages of vector, resulting in decreased toxicity (59,64).

**29.2.3.6 Helper-Dependent Adenoviral Vectors.** In spite of statistical improvements to toxicity and longevity of expression with second/third-generation adenoviral vectors, immune responses were not abolished and toxicity from remaining wild-type sequences could not be eliminated. Thus, Frank Graham and collaborators aimed to eliminate completely all vector-encoding sequences from adenoviruses vectors. A number of strategies were initially shown to be possible. The biggest challenge was to eliminate any first-generation vector used as a helper virus from the final vector preparations. Eventually, the strategy based on the removal of the packaging signal from the helper first generation through recombinase-mediated removal, turned out to be the most efficient. Similarly, this strategy is also the most effective in generating completely deleted amplicon HSV1 vectors.

Helper-dependent adenoviral vectors (also known as high-capacity “gutless” vectors (HD-Ad)) have been developed that are devoid of all viral coding sequences

(65–68). These vectors have a minimum requirement for the ITR of the linear adenovirus genome, containing the cis-acting elements for viral DNA replication and packaging, and the packaging signal. Since these elements are contained approximately 500 bp from the ends of the genome (69), helper-dependent vectors have the potential to range in size from a few hundred base pairs to carry up to approximately 36kb of the foreign DNA, which is close to the size of the native Ad genome. HD-Ads are copropagated with an E1-deleted helper virus, which provides in trans all proteins required for the propagation of the vector.

Several systems have been developed to prevent packaging of the helper viral genomes during the HD-Ad vector rescue/amplification process in order to minimize the helper virus contamination. The Cre/loxP-based system for the generation of HD-Ad involves the use of a first-generation helper virus where the packaging signal is flanked by loxP recognition sites (70). Infection of Cre-expressing 293 cells with the helper virus results in excision of the viral packaging signal, rendering the helper virus DNA unpackageable but still able to replicate and provide helper functions for HD-Ad vector propagation (71). Purification by cesium chloride centrifugation is

necessary to reduce the titer of the helper virus to negligible levels, typically ranging from 0.1% to 0.01% of the HD-Ad vector titer. Recently, another Flp/rtt-based system has been developed. The Flp recombinase was used in place of Cre and has shown to excise the rtt-flanked packaging signal in helper virus efficiently (72,73). The recent improvement to this system is the development of a new Cre-expressing cell line based on E2T, an E1 and E2a complementary cell line. Thus, an E1 and E2a double-deleted helper virus can be used with the new cell line to produce HD-Ad vector with low helper contamination and further improving the HD vector safety (63). Another major obstacle currently hindering the progress of HD vectors is the difficulty of large-scale vector production. Recently an improved producer cell line was generated along with protocols that have successfully addressed common problems. With this system,  $>1 \times 10^{13}$  viral particles can be easily produced from 3L of cells within 2 weeks of vector rescue, with specific yields of  $>10,000$  vp/cell and with exceedingly low helper virus contamination of 0.4%–0.1%. This new system represents a major improvement over the original method in terms of simplicity, speed, vector yield, and purity and it will significantly improve our ability to assess this promising gene therapy technology, especially in large animal models and ultimately for clinical applications (74).

Owing to the large cloning capacity of HD-Ad vectors, there is enough cloning space to contain large regulatory regions or to produce hybrid vectors that combine the advantage of a two-vector system. Regulatory gene expression by HD-Ad vectors can be achieved by using a tissue-specific promoter, so that the transgene is only expressed in a specific organ or tissue (75). Another option for regulating gene expression is to use a promoter that is only active after addition of an exogenous compound (76). This regulatable HD-Ad vector system represents an important tool for transgene regulation that can be used for potentially diverse applications, ranging from tissue-specific gene expression in transgenic animals to human gene therapy.

Compared with first-generation AdVs, the HD-Ad vector can also efficiently transduce a wide variety of cell types from numerous species in a cell-cycle-independent manner. The HD-Ad vectors have the added advantage of increased cloning capacity, reduced toxicity and immune responses and prolonged stable transgene expression *in vivo* (55,77–80). The limitations of HD-Ad vectors consist of difficulties in large-scale production and helper virus contamination. If these shortcomings can be overcome by improved viral vector production technique, then the HD-Ad vector will become one of the key viral vectors for gene therapy. To date, no peer-reviewed publication reports their use in human clinical trials, although one such trial has apparently been conducted.

As with other vectors discussed, replication-competent adenoviruses are also being developed for the clinic.

The first oncolytic virus to be used in human trials was Onxx-015, an E1B 55-kDa gene-deleted adenovirus that is expected to replicate in and lyse cells that are deficient in the tumor suppressor gene p53. This vector appears to be more effective in combination with chemotherapy (81). The utility of replication-competent oncolytic adenoviruses against cancer has been recently reviewed (82).

### 29.2.4 Adeno-Associated Vectors

Adeno-associated virus (AAV) is a small human parvovirus having a 4.7kb linear single-stranded DNA genome. It has not been associated with any human disease and its genome is integrated in a site-specific manner, thereby allowing stable transgene expression without the risk of mutation caused by random integration, which makes this virus a good candidate for a gene therapy vector (12). AAVs are capable of infecting a wide variety of hosts but this usually depends on coinfection with a helper virus (either adenovirus (Ad) or HSV) for a productive infection (83). A total of 11 different serotypes have been cloned (84,85). Most studies to date have focused on AAV-2 and AAV-5. Serotype 2 of AAV (AAV2) was cloned first and is most commonly used to derive vectors. Various regions of the brain have successfully been transduced by rAAV vectors (86–89), with apparently no toxicity. In the striatum, rAAV2-mediated gene expression under the control of the cytomegalovirus (CMV) promoter was shown to be maintained up to 1 year (89,90). Little is known about the mechanism underlying the stability of rAAV2-mediated gene expression in the brain.

The AAV genome is composed of two open reading frames called rep and cap, which are bounded on either side by ITRs. The ITRs contain the viral origins of replication and are the only cis-acting elements, which are required for efficient encapsidation and participate in viral DNA integration (91). The rep region encodes for proteins, which mediate AAV replication, viral DNA transcription and endonuclease functions used in host genome integration. The rep genes are the only AAV genes that are required for viral replication. The cap sequences encode structural proteins that form the viral capsid.

All vectors are derived from a plasmid, which retains only the AAV 145-bp ITRs flanking the transgene cassette of choice. The deleted viral coding sequences are present on a separate template, referred to as an AAV helper or packaging plasmid. The current method used to generate defective AAV vectors takes advantage of the fact that the ITRs are the only cis-acting elements required in a plasmid vector for rescue, replication and packaging. All other elements, including the rep/cap gene products and helper virus proteins (Ad E1a, E1b, E2a, E4 and VA), can be supplied *in trans* by co-transfecting the plasmids simultaneously in the human embryonic 293 cell line (91). This three-component plasmid results

in the generation of mixed stocks of rAAV and Ad. Stable rAAV is usually purified by inactivating the contaminating Ad by heating at 56°C for 45 minutes followed by CsCl centrifugation. This allows rAAV to be purified to titers between  $10^7$  and  $10^{10}$ . This production of large-scale viral vectors is labor intensive. Recently, the development of better packaging cell lines and column chromatographic methods of vector purification have improved the rescue method (92).

Generation of rAAV requires transfection of the vector and packaging constructs into Ad-infected cells. Owing to the lack of homology between vector and helper sequences, rAAV produced in this system is essentially free of wild-type AAV; however, the primary concern is the need to remove the contaminating Ad particles. In addition, the inherent competition between AAV and Ad for critical viral gene functions affects the final yield of vectors generated. Complete removal of Ad has relied on physical techniques such as CsCl<sub>2</sub> gradients, column chromatography and a heat-denaturing step to inactivate any residual Ad particles that may still be present. Although most of these procedures have succeeded to various degrees, the potential for Ad contamination is an unwanted risk and the presence of Ad denatured proteins is unacceptable for clinical use (93). Recent improvements have eliminated the need for Ad helper virus. In this system the Ad helper functions are delivered from a plasmid, pXX6, which contains the essential helper genes but lacks the Ad structural and replication genes. Furthermore, combination of these two new plasmids increases rAAV vector yields by 40-fold and also increases the infectivity and transduction compared to conventional procedures that use Ad particles as the helper (94).

The advantages of rAAV are that (1) it is an integrating vector, with the potential of persistent transgene expression following integration, (2) it efficiently transduces a wide range of host cells, and most importantly, (3) wtAAV is nonpathogenic. Initial experiments investigating the immune response against rAAV vectors in the muscle of mice showed only mild and transient inflammation. However, neutralizing antibodies were generated and could significantly reduce the efficacy of vector readministration (95). Other studies have looked at the CTL and antigen-presenting cell (APC) responses to rAAV vectors. rAAV delivery to mouse muscle showed the development of high titers of neutralizing antibodies, and no cell-mediated immune responses were detected. The observation that APCs present in some tissue, such as muscle, are not efficiently transduced by rAAV, do not express transgene products and do not proliferate may explain the lack of cellular immune responses (96,97). This view is controversial, however, and it has been shown that CTL and humoral responses to a delivered transgene depend on a number of variables. These variables include the nature of the transgene, route and site of injection, the immunological background of the recipient, the degree of

contamination with helper virus and the maturation state of APCs exposed to rAAV administration (98).

The main drawbacks of rAAV are (1) limited cloning capacity (4.5 kb) available for the transgene, (2) difficulty producing high titers, (3) presence of helper virus in purified stocks and (4) the fact that total elimination of the helper virus may modify some AAV properties, such as the infection of nondividing cells or integration into the host genome. Several strategies are being explored to expand the cloning capacity of rAAV, including the development of hybrid viral capsid structures, the use of very small promoters and heterodimerization of separate rAAV vectors. So far these manipulations have resulted in the doubling of the cloning capacity of rAAV (99,100).

Overall, rAAV, which is a relatively new vector for gene therapy, appears to be a promising tool for the future. These vectors are able to transduce a large variety of cells, including non-dividing cells, and they are associated with minimal toxicity, thereby providing a high degree of safety. The vector has been successfully delivered *in vivo* to many different organs (for example, CNS, liver, lung and muscle). Moreover, there have been reports of preclinical efficacy in different animal models of genetic and acquired diseases. Clinical trials using rAAV for the treatment of cystic fibrosis are underway with early evidence of gene transfer and expression of the human clotting factor IX in haemophilia B patients (12).

It has been reported that AAV vectors enter the cell rapidly but uncoat very slowly. This was found to be especially the case for AAV2 vectors, which can uncoat over approximately 8 weeks. This has particular immune consequences. Although AAV2 vector genomes do not express any viral capsid proteins, the slow uncoating would make viral capsid proteins available to the immune system over a very long time, potentially long enough to either prime the immune system or to make available antigenic epitopes over the time it takes the vector to uncoat. This slow uncoating is thought to explain, at least in part, the slow increase in AAV-mediated expression, as well as some untoward immune responses. Interestingly, other AAV serotypes, such as AAV1, 6, and 8 uncoat much faster than AAV2 and also have individual patterns of tissue distribution, cell-type-specific transgene expression and immune responses, which are currently being examined in detail. With particular reference to gene therapy for the brain, AAV9 has recently been reported to have an unusual ability to cross the blood–brain barrier and thus transduce cells of the CNS after intravenous administration (101).

## 29.2.5 Other Vectors

**29.2.5.1 Measles Virus.** Six Phase I clinical trials have been completed or are under way using vectors derived from the enveloped, single-stranded negative-sense RNA paramyxovirus that causes measles. Five are anticancer treatments and one is an HIV vaccine trial. Results have



been reported for a clinical trial using a measles-derived vector to treat ovarian cancer (102), and preclinical studies leading to the first clinical trial of a measles virus-derived vector for glioblastoma have been described (103).

**29.2.5.2 Newcastle Disease Virus.** Another member of the Paramixoviridae that shows promise as an oncolytic virus is the causative agent of Newcastle disease. While extremely dangerous for birds, particularly domestic poultry, this virus poses little danger to humans but appears to proliferate preferentially in tumor cells (104). It was administered therapeutically to cancer patients as early as 1999 (105), and a Phase I/II trial examining the feasibility of treating glioblastoma with Newcastle disease virus has been completed in Israel (106).

**29.2.5.3 SV40.** Louboutin et al. (107) report that a vector derived from the simian polyoma virus SV40, deleted for the oncogenic large T antigen, could be injected intravenously in mice and transduce large numbers of mature neurons, particularly if mannitol administration was used to increase blood–brain barrier permeability.

## 29.3 DEVELOPING CELL-TYPE-SPECIFIC AND REGULATABLE GENE DELIVERY VECTORS

Gene therapy is emerging as an effective tool and will soon be a major consideration in therapeutic medicine. The next challenge for the field is to develop techniques that will target the therapeutic gene specifically to the damaged or “sick” tissue and to have control of when the gene is turned on and off. Developments in gene regulatory systems have produced effective regulatory switches to tightly activate and silence therapeutic genes in order to alleviate and control the recurring symptoms of a neurodegenerative disease without causing harmful side effects from constant expression of the transgene. The use of exogenous substances to facilitate the regulatable switch is becoming the preferred method for controlling the presence and absence of the transgene.

For a transcriptional regulatory model to be effective for clinical gene therapy applications, it has to satisfy five criteria. These are (1) zero or negligible basal activity and expression of the therapeutic gene in the absence of the transcriptional inducer, (2) good induction kinetics, (3) high sensitivity to the inducer on administration, (4) tight transgene regulation, and (5) negligible or minimal immune-mediated cytotoxicity or inflammation associated with the transactivator of the regulatory switch system. Consequently, administration of lower inducer concentrations to the patient in the clinical perspective will be safer. In adjunct to levels of therapeutic vector titers and extent of their readministration within the brain, the quantity, concentration and frequency of inducer administration to the patient to attain the required levels of therapeutic product are crucial factors that require careful evaluation in preclinical studies on testing and employing antibiotic-based gene control systems (Table 29-1). A regulatory

switch with substantial promise for somatic gene therapy is the tetracycline (tet)-dependant regulatory system. The system is essentially composed of two types of switches that are categorized as the tet-off and tet-on models. A plethora of literature reviews on these regulatory systems has been published over the years to illustrate their successes in achieving regulated gene therapy (Figure 29-7).

### 29.3.1 Molecular Mechanisms of Tet-On- and Tet-Off-Based Transcriptional Regulatory Systems

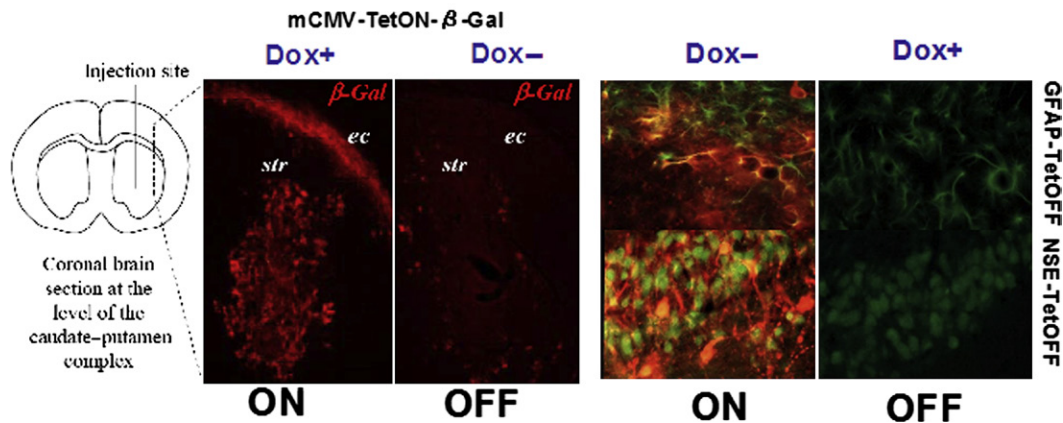
The discovery of mammalian regulatory transcriptional expression systems initially arose from experimental research done on the bacteria *Escherichia coli* (108). The use of tet-based inducible switches to regulate transcriptional genes has demonstrated an enormous benefit in physiological and clinical medicine. The tet-on and tet-off regulatory systems have been repeatedly upgraded over the years to achieve enhanced induction kinetics and regulation of gene expression. The core switch of the conventional tet-off system functions with the synchronized interaction of two basic components. These are the tet repressor protein (TetR) and the tetracycline response element (TRE). The TetR was initially discovered in the bacterial strain *E. coli*, in which the TetR negatively regulates genes of the tetracycline-resistance operon on the Tn10 transposon. In bacteria, the TetR hinders transcription of these genes by binding to tet operator sequences (tetO) in the absence of tetracycline. The TetR is a 37-kDa protein made up of 207 amino acids. Fusion of TetR to a viral protein called VP16, a eukaryotic transactivator derived from HSV type I, transforms the TetR from a transcriptional repressor to an activator called transactivator (tTA). The tTA, equipped with its own promoter and poly(A) sequence, is independent of the TRE-driven transgene cassette.

The TRE unit is composed of seven recurring tetO and is situated upstream of the immediate transgene initiation codon sequence of the minimal human CMV (hminCMV) promoter, driving the transgene encoded cassette. Gene regulation is achieved by the synthesis of transactivators and their resulting interaction with the inducer and TRE. In the tet-off system, gene expression is switched “on” and turned “off” in the absence and presence of the inducer (tetracycline or an analog, i.e., doxycycline), respectively. When the gene is switched off upon delivery of doxycycline (dox), dox becomes bound to the constitutively synthesized tTA. The dox-bound tTA hinders the ability of the tTA to become docked to its tetO sequences within the TRE and thereby impedes subsequent gene expression. When the gene is switched on, due to the absence of the dox inducer, the tTA binds freely to its operator sequences (tetO sequences) within the TRE, triggering TRE and its promoter to induce gene expression.

Gossen and Bujard (109) created an altered form of the tTA by mutating the original TetR fused to the



## Turning transgenes (medicines) on and off



**FIGURE 29-7** In vivo transgene expression from HC-Ad-mTetON- $\beta$ -Gal (left-hand panels) in the rat brain, using the Tet-ON system. Rats were injected with  $1 \times 10^7$  blue forming units (BFU) of HC-Ad-mTetON- $\beta$ -Gal and received drinking water with 1% sucrose plus 2.0 mg/mL Dox (DOX +) or drinking water with 1% sucrose alone (DOX-) for 6 days. Transgene expression was determined by  $\beta$ -galactosidase immunocytochemistry. Images show  $\beta$ -Gal-immunopositive cells in striatal sections in the ON condition (Dox+) but not in the off condition (Dox-). In vivo transgene expression from GFAP-Tet-Off- $\beta$ -gal, and NSE-Tet-Off- $\beta$ -gal in the hippocampus of rats is shown in the right-hand figure. This demonstrates the astrocyte specific expression from the GFAP promoter, in the top right panels, and the neuronal specific expression from the NSE promoter in the lower right panels. In red, expression of  $\beta$ -galactosidase was detected by immunocytochemistry, and in green, the expression of the cell type specific markers was assessed (GFAP in the top right panels, and NeuN in the lower right-hand panels). Double labeling renders the appropriate cells yellow indicating that the right cell type expresses the transgene in the ON situation. Because of the use of the Tet-Off system in these experiments, the ON condition is Dox-, whereas gene expression is turned OFF by providing Dox to the animals. This figure illustrates that cell-type-specific and inducible transgene regulation can be achieved both from first-generation adenoviral vectors (right-hand panels), and high-capacity adenoviral vectors (left-hand panels).

VP16 by four amino acid changes within the protein core, where the inducer becomes bound, resulting in the tTA exhibiting reverse functions. This mutant, termed rtTA, triggers transcription in the presence of the inducer instead of repressing transcription in the presence of the inducer, resulting in the development of the tet-on system. This reverse transactivator has been utilized successfully in achieving the desired regulated gene expression in a number of experimental studies but had some limitations in achieving tight gene regulation. Despite the absence of the dox inducer, tet-on-based rtTA was found to exhibit some level of affinity to the tetO that makeup the TRE, thus activating the target promoter and inducing a basal level of transcription in the off state. Urlinger and colleagues (110) induced random and site-directed mutagenesis in rtTA seeking novel forms that can enhance tight regulation and therefore minimizing background expression in the absence of the inducer to negligible levels. Using *Saccharomyces cerevisiae* for screening assays, studies revealed five rtTA mutants, one of which produced negligible basal expression in the absence of the inducer and required a 10-fold-lower doxycycline concentration for TRE activation. This rtTA mutant transactivator was named rtTA2S-M2. Quantitative analysis of luciferase assays with rtTA2S-M2 transactivator revealed tight regulation of  $\beta$ -galactosidase gene expression in transfected HeLa X1/6 cells (110).

Despite extensive characterization in animal models, including non-human primates (111) and dogs (112), no

use of a vector with tetracycline regulatable transgene expression has yet been reported in humans.

### 29.3.2 Latest “Tet-On”-Based Tetracycline-Dependent Transactivators

Tight regulation of therapeutic gene expression is of critical importance when considering implementation of gene therapy to the human trials. Tet-on-based regulatable gene expression systems for clinical gene therapeutics are better than the tet-off system in theory because less antibiotic needs to be used. The tet-off system requires more antibiotic in order to sustain gene expression in the off state. This constant presence of antibiotics in circulation could lead to complications because of a growing resistance to the antibiotic or unwanted side effects. Therefore, novel tet-on transactivators of tetracycline-dependent regulatable systems are more appropriate, as they do not require constant administration of the drug and gene expression is switched on only when needed.

In contrast to the conventional rtTA-based tet-on systems, the latest generated regulatory switch composed of a tetracycline response promoter coengineered with mutant rtTA2SM2 transactivator and a tTSkid repressor, via an internal ribosome entry site (IRES), has recently shown to be effective in producing tight regulation of transgene expression in mice and nonhuman primates (113,114). Lamartina and coworkers developed and tested a bicistronic vector ex vivo containing a single regulatory cassette, encompassing rtTA2S-M2

transactivator and tTS-Kid tet repressor separated via an IRES. The rtTA2S-M2/IRES/tTS-Kid complex produced negligible basal activity, had 1000-fold inducibility of serum alkaline phosphatase gene expression and exhibited elevated sensitivity to doxycycline (113). This novel regulatory switch has the characteristics of an ideal tet-on regulatory model for gene therapy applications.

Applying the tet-on regulatory switch system containing the rtTA2S-M2/IRES/tTS-Kid complex to the HC-AdV provides a strong gene therapeutic tool. This vector will express high levels of the transgene while being tightly regulated to produce the therapeutic gene at specific times and therefore minimize side effects caused by overexpression of the transgene. Excellent disease candidates for which this regulatable switch system can be utilized are neurodegenerative diseases such as Parkinson's disease (PD) that produce recurring symptoms. This disease is a consequence of a defect in the dopaminergic circuit of the basal ganglia that sustains and controls motor activity. It has been shown that using first-generation adenoviral vectors in the 6-OHDA animal model will reduce disease symptoms (115). Using HC-AdVs coengineered with specific neurotropic factors under the control of the rtTA2S-M2/IRES/tTS-Kid tet-on regulatory switch, tight regulation can be achievable, and hopefully similar, if not better, therapeutic results can be achieved compared to the first-generation vectors. This approach can be ideal in the clinical setting for the treatment of PD, since the tet-on transactivator facilitates tight regulation of gene expression with negligible background expression in the uninduced state. Preclinical testing of neurotropic-gene-encoded HC-AdVs coengineered with the regulatory switch will allow us to further improve and examine these gene therapeutic strategies for PD (Table 29-1).

The well-established tetracycline derivative doxycycline, principally due to its nontoxic effects, is a broadly employed antibiotic in treatment medicine. A recent study by Chtarto et al. (116) has discovered an additional tetracycline derivative that was revealed to be effective and less cytotoxic than doxycycline in vivo. Minocycline, an antibiotic that is considered to exhibit antiapoptotic and antiinflammatory properties, exhibited reduced cytotoxicity and a faster elimination time of minocycline withdrawal compared to doxycycline. Although emerging doxycycline-based tet-on regulatory systems show promising results, studies on regulation kinetics are important to further optimize this and other tet-on systems to initiate and promote safe gene therapy treatments in the clinical milieu.

### 29.3.3 Other Transcriptional Gene Regulatory Systems

Before the emergence of the widely employed tetracycline-based regulatory systems over a decade ago, earlier developments on other gene regulatory systems demonstrated both successes and failures in inducing tight

transgene regulation. Initial efforts in constructing these systems relied primarily on the use of endogenous promoters that induce gene expression. Such endogenous promoters included heat shock (117), heavy metals (118), interferons or ds RNA (119), or steroids (120). Some primary drawbacks of these methods included the heavy dependence on endogenous transcription factors and second, complications in generating consistent in vivo results comparable to results from in vitro studies.

Although the bacterial lac operator–repressor system does not function in vivo, the lac system has shown successful gene regulation in vitro. It produces minimal levels of basal activity and high induction levels of the transgene in the presence of the inducer isopropyl- $\beta$ -D-thiogalactoside (121). In contrast to the lac system, the RU 486 regulatory system produced successful tightly regulated transgene gene expression both in vivo and in vitro. This system fundamentally operates on the progesterone regulatory system that is dependent on the inducer RU 486 to regulate transcription of genes (122). Other notable regulatory systems developed that generated efficient and tight regulation of gene expression both in vivo and in vitro were the *Drosophila melanogaster* receptor-hormone-dependent ecdysone system (123) and the rapamycin system based on the human FK506-binding protein/FKBP12-rapamycin associated protein (FKBP/FRAP) protein interaction (124).

### 29.3.4 Cell-Type-Specific and Regulated Gene Expression in the CNS

In addition to the therapeutic-gene- and regulatory-switch-encoded viral vector for gene transfer, a fundamental element needed to achieve localized transgene expression within specific cell types is the use of a specific promoter driving expression of the therapeutic transgene within localized cells. Excellent examples of such cell-type-specific targeted gene therapy have been demonstrated in animal models with induced pituitary tumors, illustrating the feasibility and effectiveness of this approach (95, 125–127). With the application of cell type specificity and regulatory switches coengineered within the adenoviral vector and direct stereotactic injection of adenoviral vectors to the affected brain anatomy in animal studies, gene therapy has become a more powerful approach for the treatment of neurological diseases. Smith-Arica et al. (95) have demonstrated that effective antibiotic-based transcriptional gene regulation and cell-type-specific transcriptional targeting produced successful regulatable transgenes in experimental cell lines and targeted localized regions within the rat brain. A first-generation adenoviral vector encoded with a glial fibrillary acidic protein (GFAP) promoter, which drives the expression of GFAP under the control of the tet-off regulatory switch, produced localized doxycycline-dependent GFAP expression within glial cells in vitro and in vivo in a dose-dependent manner.

The use of a neuronal-specific enolase (NSE) promoter to induce expression within neurons in the absence of doxycycline, however, did not exhibit neuronal-restricted transgene expression in cell lines *in vitro* but produced successful transgene specificity *in vivo* (95). Taking into perspective the results of these studies, the choice of a specific promoter does not always produce the expected localized transgene expression. For the development of human neurological gene therapy, careful testing of the promoter through experimental studies will allow us to verify its specificity *in vitro* and *in vivo*. In a comparable study by the same group, specific promoters restricted to the anterior pituitary-hormone-synthesizing cells in a first-generation adenoviral vector encoded with the tet-off tetracycline-responsive transcriptional cassette expressed the predicted regulatable transgenes within targeted cell populations in both *in vitro* and *in vivo* studies (95).

Compared to the strong  $\beta$ -actin/human nonspecific (CAG) promoter, using first-generation recombinant adenoviral vectors engineered with TRE and the human prolactin promoter led to transgene expression only within GH3 tumor and lactotrophic cells (125). A fundamental idea to be learned from *in vivo* and *in vitro* studies is that a conscientious choice of promoter is crucial for achieving and maintaining appropriate transgene expression.

A promising advance in cell-type-specific control of transgene expression has been the recent development of microRNA-regulated systems (128). These systems exploit the ability of microRNAs—small non-coding RNA sequences—to interfere with the translation of mRNA containing sequences of partial complementarity to the microRNA in their 3' UTR. The endogenous regulatory microRNAs are expressed in a cell-type-specific pattern, and if the cell type of interest expresses a microRNA with a known target sequence, this sequence can be included in the 3' UTR of the transgene. An interesting application of this technology was described by Gentner et al. (129) for the gene therapy of globoid cell leukodystrophy. This disease is caused by defective production of the enzyme galactocerebrosidase (GALC) and can be corrected by transfer of hematopoietic stem cells expressing the wild-type protein. Among the cell types that develop from these stem cells are microglia that migrate into the brain and, if sufficient enzyme is secreted, they can compensate for the lack of endogenous expression. The level of expression that is required from the differentiated microglia is toxic to the stem cells in their predifferentiation stage, precluding the use of a simple strong promoter. To overcome this problem, Gentner et al. included the target sequence for microRNA-126 in the GALC-encoding vector with which the hematopoietic stem cells were transduced. This microRNA is expressed in the stem cells and suppresses expression of the transgene but then is downregulated in the differentiated progeny, allowing robust transgene expression.

## 29.4 THE CLINICAL SCIENCE: TOWARD GENE THERAPY OF HUMAN DISEASE

### 29.4.1 The Beginnings of Clinical Use of Gene Transfer and Gene Therapy

The first vectors that were tested in human patients were actually used in a trial of cell marking, rather than treatment of disease, by the team of Malcolm Brenner at St. Jude Children's Research Hospital in Memphis. At that time an important question was whether relapses following stem cell rescue as therapy for malignant disease were due to insufficient killing of tumor cells remaining in the patients or due to tumor cells remaining in the transplanted bone marrow. This was an ideal question to be answered with the new tools of gene therapy. The question was whether malignant cells at the time of relapse originated from residual malignant cells in autologous marrow. The trials involved transducing bone marrow *in vitro* and reinfusion of marked cells into patients. These studies demonstrated that relapse in acute myeloblastic leukemia and neuroblastoma patients subjected to high-dose chemotherapy and stem cell rescue were due to tumor cells remaining in the infused bone marrow.

The first officially sanctioned and reviewed clinical gene therapy trial for the treatment of disease was conducted by Michael Blaese, W. French Anderson, Kenneth Culver, and colleagues at the Blood and Heart Institute at the National Institutes of Health in Bethesda. Two girls suffering from severe combined immunodeficiency (SCID) due to adenosine deaminase (ADA) deficiency were treated. Their T cells were collected, transduced with an ADA-encoding retroviral vector *in vitro*, expanded and reinfused. Indices of both humoral and cellular immunity showed a tendency to correction, and the two girls have been able to continue with a normal life. These patients continued to be treated, as before gene therapy, with PEG-ADA. The seriousness of the disease, and the fact that it affects children, has impeded the removal of systemically administered PEG-ADA. Removal of PEG-ADA would be the final test to demonstrate the clinical effectiveness of gene therapy beyond reasonable doubt. A subsequent trial in Italy using retroviral transduction of CD34+ hematopoietic stem cells, rather than peripheral T cells, and mild conditioning of the bone marrow with busulfan treatment, has yielded more unconditionally positive results (130). After 2–8 years of follow-up, patients' peripheral blood contained large numbers of various cell types expressing the transgene, numerous immunological parameters were improved, and eight of 10 patients treated no longer required enzyme replacement therapy. Similar successes have been reported from a trial in England (131) for ADA-SCID and also from a trial in France of gene therapy for X-linked SCID (132).

Other notable successes of gene therapy include treatments for X-linked adrenoleukodystrophy (X-ALD) and for Leber's congenital amaurosis. X-ALD is a monogenic

disease cause by mutations in the *ABCD1* gene encoding a peroxisomal membrane transporter protein called ALDP. The disorder is manifested as a devastating progressive demyelination of the CNS, and, as described above for globoid cell leukodystrophy, it can be treated by hematopoietic stem cell transfer because cells expressing the wild-type protein traffic into the CNS and compensate for the endogenous defect. A group in France (133) has reported treating the disease in two patients by transducing autologous CD34<sup>+</sup> cells with a replication-defective, self-inactivated, VSV-pseudotyped lentiviral vector encoding wild-type *ABCD1* and then reinfusing the cells into the patients. Patients' bone marrow was myeloablated to encourage engraftment of the transduced cells. Over 24–30 months of follow-up, cells of several lymphoid and myeloid lineages expressing the transgene were detected in the periphery, confirming successful engraftment, and 14–16 months after treatment, progressive demyelination was halted.

Leber's congenital amaurosis is a group of congenital retinal disorders of which the cause may be a mutation in one of a number of genes. One comparatively common mutation is in *RPE65*, which encodes a protein necessary for retinal pigment epithelium function. Maguire et al. (134) treated three patients with severe and worsening vision loss due to *RPE65* mutations by injecting subretinally an AAV vector encoding human wild-type *RPE65*. Patients were injected unilaterally in their least functional eye, and within 1–4 months of the injection, visual function in this eye was substantially improved. Improvements were reported to be stable without additional adverse events at follow-up more than a year later (135). The basic idea of gene therapy was initially targeted to the treatment of inherited genetic disease, in which inheritance was either recessive or dominant. The very first trials in gene therapy thus addressed the treatment of mostly children suffering from inherited diseases. Once the potential broader applicability of the “genes as medicines” concept was visualized, gene therapy was quickly diversified to be applied to other non-inherited diseases, such as neurodegenerative disorders, cancer and others. Below, we will examine the utilization of gene therapies for the treatment of both inherited and sporadic degenerative disorders and tumors, focusing on the brain as the main target for these new treatments.

## 29.4.2 Applications to Neurodegenerative Disorders

Neurodegenerative disorders are progressive brain diseases, each targeting specific regions of the brain. The diagnoses of the neurodegenerations characteristic of Alzheimer's disease (AD), PD or Huntington's disease are based on clinical, laboratory, genetic and neuro-radiological criteria. AD cell loss targets the neocortex and hippocampus; in Parkinson's, it affects mostly the basal ganglia; and in Huntington's, the basal ganglia

and neocortex undergo progressive massive degenerative changes.

Treatments are symptomatic and vary in their clinical efficiency. Although no treatment delays the progressive nature of brain degeneration, PD symptoms can be treated for many years and the recent use of ACHE inhibitors in AD has somewhat delayed serious memory loss. No treatment has shown any efficacy in Huntington's disease. It is thought that new treatment strategies including gene- and cell-based therapies for the transduction of growth factors and modulation of the immune system offer new opportunities to prevent further neuronal loss.

In addition, transplantation of neuronal precursors has now been tested in two double-blind controlled clinical trials. In spite of some positive effects in some patients, in both clinical trials approximately 15% of patients developed significant side effects that had not been identified in the previous uncontrolled trials from which the results were more promising. As a result of the undesirable side effects, the limited therapeutic effects and the development of other more effective methods such as deep brain stimulation, the upshot of the controlled clinical trials has been reduced enthusiasm for the transplantation of neuronal precursors (Brundin et al. (136)), although many involved with the uncontrolled trials both in the United States and Europe have suggested that they would continue implementing this strategy in human patients. As an alternative, it has been proposed to replace the transplantation of neural progenitors with neural stem cells. This does not solve the issue of how to direct the differentiation of stem cells down the pathway required (137) and to avoid the formations of teratomas. The use of induced pluripotent stem cells derived from patients' own somatic cells also holds great promise (138), particularly since it would obviate the problem of immune-mediated rejection of grafted stem cells, which is currently thought to be a major problem for stem cell transplant therapy (136). It may be more likely in the future to combine stem cells with gene transfer methods; by expressing the necessary transcription factors, gene therapy tools will be able to direct stem cell differentiation in the desired direction in a specific, controllable, and clinically tested manner (139,140). For example Sanchez-Danes et al. (141) report that transduction of human embryonic or induced pluripotent stem cells with a lentiviral vector encoding the ventral midbrain-specifying transcription factor increased the efficiency with which these cells developed the phenotype of ventral midbrain dopaminergic neurons.

**29.4.2.1 Alzheimer's Disease.** AD is characterized by the deposition of extracellular amyloid plaques, intracellular neurofibrillary tangles, synaptic loss and neurodegeneration. Amyloid plaques are composed of insoluble amyloid-beta (A $\beta$ ) fibril fragments of the high-molecular-weight amyloid precursor protein (APP). In familial AD, mutations in presenilin and APP alter the



proteolytic cleavage of APP by secretases, leading to an extracellular accumulation of amyloid plaques. Neurofibrillary tangles are composed of insoluble hyperphosphorylated tau. Normally, phosphorylated tau stabilizes neuronal microtubules. Tangles are thought to lead to neuronal dysfunction, neuronal loss and synaptic loss. These neurodegenerative changes lead to a disruption of the connectivity among various brain regions (142).

Nevertheless, there is still discussion on whether the extracellular amyloid plaques or the intracellular tau neurofibrillary tangles are the primary lesion leading to the symptomatology of AD. Apart from mutations in APP, proteins related to APP processing, and tau, apolipoprotein E (apoE) alleles have been shown to be a risk factor for AD. The apoE4 allele increases, whereas the apoE2 allele decreases the risk of developing AD. The apoE4 allele increases the accumulation of A $\beta$ 42 and its binding properties to tau and decreases the age of onset in a dose-dependent manner (143). Although the noradrenergic and cholinergic innervation of the neocortex is also compromised, it is unclear whether these changes are primary or secondary, and they have not been linked to specific mutations. Given the partial and limited efficiency of AChE inhibitor, it has been proposed that cholinergic neurons arising in the nucleus basalis Meynert and innervating the hippocampus and neocortex may play a central role in disease pathogenesis (144,145).

NGF promotes survival of basal forebrain cholinergic neurons (146). This has led to the use of NGF to attempt to rescue degenerating basal forebrain cholinergic neurons (147–149); however, the causes for the decrease in markers of cholinergic function remain unknown. Cholinergic dysfunction could be a primary defect, or they could be secondary to neocortical degeneration. Understanding the disease pathophysiology will be important in determining the potential therapeutic benefit of cholinergic drugs in AD.

Infusion of NGF into the ventricles of patients with AD had serious side effects such as pain and weight loss (150). As an alternative delivery, transplantation of primary rat fibroblasts producing human NGF were first investigated in fimbria-fornix-lesioned rats (147). After safety and feasibility had been confirmed in primates (151–153), a Phase-I trial was initiated (153).

Eight patients with mild AD showed no adverse effects 22 months after transplantation of autologous fibroblasts obtained from skin biopsies infected with an NGF-expressing MoMLV-derived retroviral vector and stereotactically implanted unilaterally ( $n=2$ ) or bilaterally ( $n=6$ ) into the nucleus basalis of Meynert. Clinical follow-up indicated an apparent slowing in the rate of cognitive decline, and 18F-FDG PET performed in four patients showed a significant increase in cortical glucose consumption after treatment. Developing this paradigm, Ceregene has completed a Phase I/II trial and is currently conducting Phase II clinical trials of CERE-110,

an AAV-derived vector encoding NGF, which is injected into the basal forebrain. The current perspective on this vector is discussed by Mandel (154).

Other gene therapy strategies for AD include transduction of apoE2 (155) to reduce the A $\beta$  burden and the subsequent development of neuritic plaques in AD mice. Most recently there has been much work on immunization approaches to reduce intraparenchymal levels of A $\beta$ . Vaccination with a plasmid that encodes A $\beta$ 42 (156) or by intranasal administration of replication-incompetent AdV carrying both A $\beta$  and GM-CSF genes (157) have been tested. These alternative immunization strategies are currently being developed, as the active immunization with synthetic A $\beta$ 1–42 has been shown to be effective in mouse models to significantly reduce the A $\beta$  burden accompanied by improved cognitive performance, but clinical trials had to be stopped after enrollment of about 300 patients because of the development of a T-cell-mediated aseptic meningoencephalitis in 6% of treated patients (158,159). The interpretation of the results is complicated by the fact that only very few brains were available for pathological analysis. Those that were published indicated that inflammation correlated with reduced plaque load. In the absence of larger samples, it remains difficult to know whether these anecdotal data are significant.

Immunization techniques are clearly double-edged swords. In the trials that proceeded, patients were immunized with endogenous brain proteins. Immunization against brain proteins is known to lead to brain autoimmune disease. These experiments have shown that immunization against A $\beta$  amyloid is no exception. Although a number of strategies are now being tested in the hope that immunogens will be found that do not cause brain inflammation, it is difficult to predict whether this strategy will succeed clinically. The expectation is that immunogens can be tailored to induce a humoral but not a cellular immune response. Antibodies to A $\beta$  amyloid, by acting either directly in the brain or systemically, should reduce the levels of A $\beta$  and thus restore neuronal function. For example, Ryan (160) used an AAV-1-derived vector to induce hippocampal expression of an amyloid-beta binding fragment of a human antibody in a mouse model of AD and achieved some degree of cognitive improvement; however, both T cells and antibodies have been shown to cause neuroinflammatory disease. Monitoring microglial activation in vivo noninvasively before any clinical symptoms develop may be needed if these clinical trials are allowed to proceed. Initial immunizations were tested in mice and in mouse models of AD. However, none of these developed significant brain inflammatory disease. The variation in human MHC haplotypes, compared to the homogeneous H2 of transgenic mice, could explain why inflammation was finally only detected in humans, although it would have been predictable based on our knowledge on how to induce brain inflammation by immunizing against brain proteins.

More recently, neprilysin, a major extracellular enzyme that degrades A $\beta$  has been proposed as an alternative approach for gene therapy, and as such, has been tested in a number of different experimental models (161). Also, IGF-1 has been shown to be effective in experimental models of neuronal degenerations in another neurodegeneration, namely, in mouse models of amyotrophic lateral sclerosis (162). Even though questions remain on how IGF-1 may be delaying the death of affected mice in this model, the strong experimental results in a disease that is otherwise untreatable has led to the accelerated development of significant clinical trials. Should IGF-1 prove effective in protecting spinal cord motor neurons from progressive ALS, it may also be tried as a neuro-protectant in AD. Equally, siRNA has now been shown to be an effective manner of reducing the overexpression of pathogenic proteins in the case of experimental models of Huntington's disease (e.g. huntingtin), dominant inherited ataxias, and torsion dystonia (163–166).

**29.4.2.2 Parkinson's Disease.** The second most common neurodegenerative disorder is PD in which a progressive loss of dopaminergic neurons in the substantia nigra and other brain stem nuclei occurs. This neuronal loss is associated with the formation of intracellular Lewy inclusion bodies and leads to dopamine depletion from the striatum, with projections to the putamen being most affected. Later in the disease, other transmitter systems involving serotonergic cells in the median raphe, noradrenergic cells in the locus ceruleus and cholinergic cells in the nucleus basalis Meynert get involved in the neurodegenerative process. Therefore, patients with PD not only have the typical motor impairment with resting tremor, bradykinesia and rigidity but also balance problems and autonomic nervous dysfunction and show cognitive and psychiatric features.

The exact mechanisms of dopaminergic neuron degeneration are not fully understood. Genetic factors include mutations in the alpha-synuclein (PARK1) and parkin (PARK2) genes (167,168). Parkin functions as an E3 ubiquitin-protein ligase, and a loss of function results in the failure of intracellular protein processing with consecutive accumulation of various proteins to toxic levels (169). Although sporadic and inherited PD have different causes, they likely will intersect in common pathways (170,171). The central cause of sporadic PD seems to be a mitochondrial complex I inhibition, and complex I deficiency may cause alpha-synuclein aggregation, contributing to the degeneration of dopaminergic neurons (170,171).

Gene therapy for PD was first developed in rat models using transduction of a single gene encoding tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis (86,172). Limitations of this approach included the side effects of the helper-dependent HSV1 amplicon vector used at that time, limited expression and expression of TH as the only gene. In the past 10 years, gene therapy approaches for PD have been further developed

in the following three directions: (1) transduction of multiple genes essential for the synthesis of dopamine to restore dopamine levels; (2) transduction of genes encoding growth factors, differentiation factors, transcription factors and antiapoptotic proteins to prevent ongoing neurodegeneration of nigrostriatal dopamine neurons; and (3) improvements and further developments of vector and promoter systems to reduce toxicity and immune responses, increase longevity of expression, and regulate transgene expression.

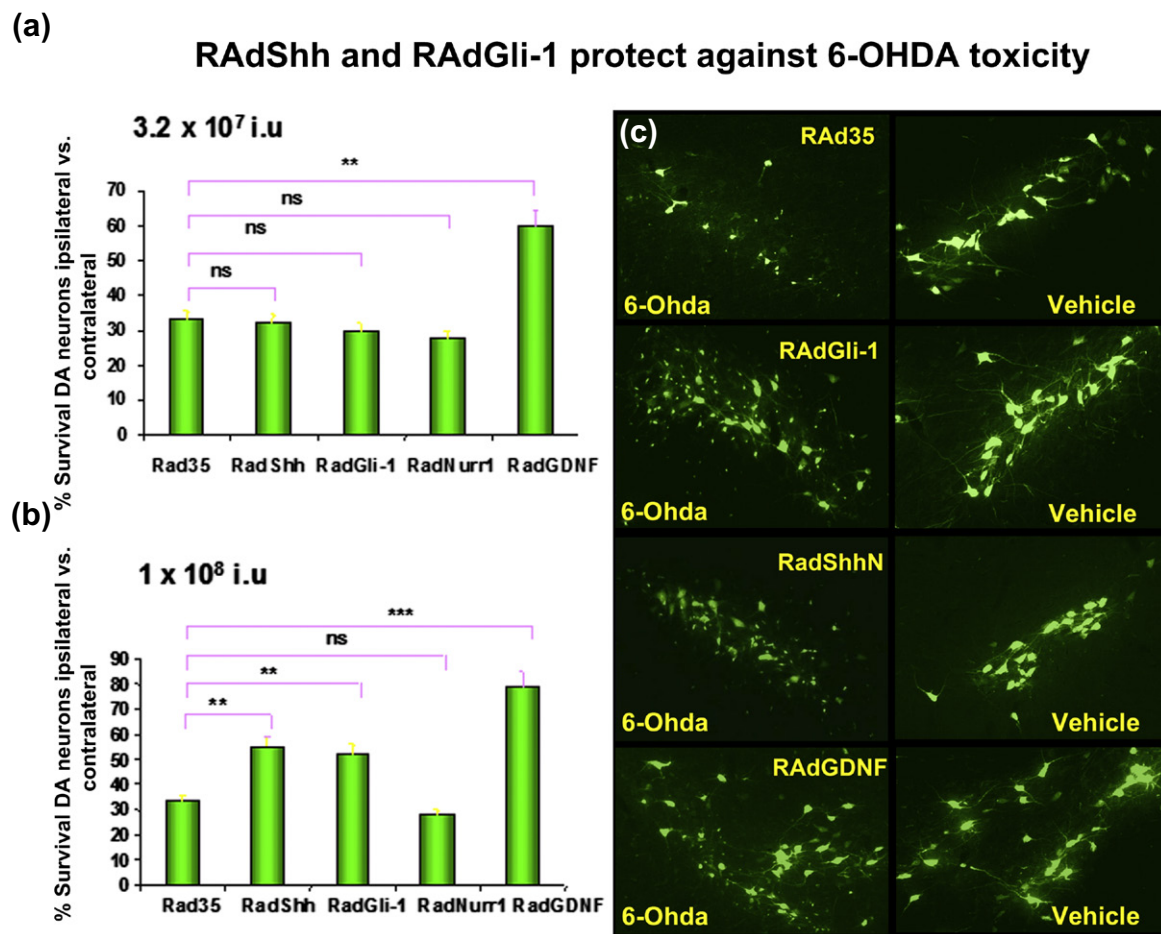
Coexpression of multiple proteins are required for the efficient production and release of dopamine, and recent attempts have been made to express these to improve the levels, synthesis and regulation of dopamine production in gene therapy trials. These enzymes and their functions are as follows: (1) TH converts tyrosine to L-DOPA in the presence of tetrahydrobiopterin (BH<sub>4</sub>), (2) GTP cyclohydroxylase 1 (GCH1) is the rate-limiting enzyme in the biosynthesis of BH<sub>4</sub>, (3) aromatic amino decarboxylase (AADC) converts L-DOPA to dopamine, and (4) vesicular monoamine transporter type 2 (VMAT-2) concentrates dopamine into synaptic vesicles. The following gene combinations have been successfully used in rat and primate models of PD using AAV, HSV1 amplicon or lentiviral vectors: TH and GHI; TH, GCHI and AADC (173–175); TH, GCHI, AADC and VMAT-2 (176). To date, clinical trials have been completed or are under way evaluating vectors encoding five transgenes (of which one is a combination of three transgenes), glutamic acid dehydroxylase (GAD); AADC; the combination of TH, AADC and GCH1; neurturin; and glial-cell-derived neurotrophic factor (GDNF). The mechanistic rationales for using each of these transgenes are explained below. Neuroprotective gene therapy should be especially useful in early PD stages, when sufficient nigral neurons remain and could be protected from further degeneration. It has been shown that transduction of GDNF (177) and brain-cell-derived neurotrophic factor (178), as well as other neurotrophic factors related to GDNF, such as neurturin, protect nigrostriatal neurons from neurotoxic insults in rat and primate models of PD (115,179–181). For potential clinical application uncertain consequences of long-term growth factor expression and questions regarding timing and regulation of therapy (182) need to be addressed; however, it will be difficult to mimic the very long-term expression that will occur in humans. Preclinical experiments have shown that convection-enhanced delivery of an AAV-derived vector encoding GDNF leads to widespread long-term expression of the neurotrophic factor in primate striatum and some functional recovery in a monkey model of PD (183). This approach is currently the subject of a Phase I clinical trial that commenced in 2009. The results of a Phase II, sham-surgery-controlled double-blind clinical trial using AAV to induce neurturin expression in the putamen were reported in 2010 (184). No significant differences in primary end point between controls and AAV neurturin

were observed at 12 months after vector administration, although some modest improvements were seen in a subset of patients at 18 months after administration.

Alternative growth-factor-based gene therapies are being developed based on the protective properties of sonic hedgehog, a secreted neurodifferentiation factor (185,186). Sonic hedgehog has many functions during brain development, one of which is to ventralize the developing brain, including the production of midbrain dopamine neurons. Thus, potentially, the administration of sonic hedgehog could act on local precursor neurons to differentiate them in the direction of nigrostriatal dopamine cells (Figure 29-8). Other paradigms of gene therapy for PD that are currently being tested in animals models include the transduction of dopaminergic neurons with JNK-interacting protein-1, apoptotic protease activating factor-1 (187); dominant negative inhibitor, neuronal apoptosis inhibitor protein (NAIP; (188)); Hsp70 (189), and Parkin (190,191) and are reviewed elsewhere (192).

Owing to the existence of potentially unknown side effects of the gene therapies, especially in the very long-term, it will be important that novel strategies include safety procedures for regulating and inhibiting gene expression. This raises further complexities, given that regulatory requirements are easier to fulfill the simpler the novel genetic constructs are. Paradoxically, it is easier and cheaper for the biotech industry to get approval for clinical trials utilizing less-safe unregulated genetic constructs! Owing to a large number of current considerations, it will be unlikely that either of the trials that will go ahead in the near future will be done using regulatory systems.

Another proposed gene therapy paradigm is the conversion of excitatory to inhibitory output neurons arising from the subthalamic nucleus (STN). To do so it has been proposed to transduce excitatory glutamatergic neurons of the STN with AAV vectors expressing glutamic acid decarboxylase (GAD), the enzyme that synthesizes the inhibitory transmitter GABA (193). Inhibition of



**FIGURE 29-8** Effects of gene transfer on substantia nigra dopaminergic neurons. Quantitative results from experiments on adenovirus-mediated gene transfer of  $3.2 \times 10^7$  IU (a) or  $1 \times 10^8$  IU (b) of RAAd-35, RAAd-GDNF, RAAd-ShhN, RAAd-Gli-1, or RAAd-Nurr-1; all vectors were tested against 6-OHDA-induced neurodegeneration of nigrostriatal cells retrogradely labeled with fluorogold. The morphological analysis is shown in (c). The side injected with 6-OHDA is shown on the left, and the control side is shown on the right. Injection of RAAd-GDNF, RAAd-ShhN and RAAd-Gli-1 protected a significant amount of nigrostriatal neurons compared to animals injected with the negative control vector RAAd-35. Note the survival of large fluorogold+ neurons in the ipsilateral site of animals injected with RAAd-ShhN, RAAd-Gli-1 and RAAd-GDNF compared with RAAd-35. Survival of nigrostriatal neurons was expressed as a percentage of unlesioned contralateral neurons.



the STN through other approaches such as deep brain stimulation is known to be effective clinically. This paradigm has already been implemented in a clinical trial, the first clinical gene therapy trial in humans suffering from PD. The results of this open-label Phase I trial were reported in 2007 (194) and suggested that the treatment was safe and offered significant benefits. The results of a Phase II sham-surgery-controlled double-blind trial using the same approach were published in 2011 (195) and confirmed a therapeutic benefit of the gene therapy. The benefit is modest and arguably not better than what would be expected from deep brain stimulation, but this nonetheless can be considered a landmark study since it is the first well-controlled clinical trial to show a benefit of gene therapy for PD, as assessed by the primary end point defined in the design (196). An alternative to using neurotrophic factors to encourage the survival of neurons is to prevent their death by interfering with apoptosis. The apoptotic protease activation factor-1 (Apaf-1), a gene in the apoptosis cascade, has been used to prevent dopaminergic cell death in a mouse model for PD.

The rationale for using AADC as a therapeutic transgene is that its expression in striatum will lower the doses of L-DOPA needed, thus allowing patients to remain for longer on safe, and effective, doses of this drug. A Phase I trial using AAV to induce AADC expression in the putamen has been completed and results suggest that the treatment successfully increases AADC activity in the target region and produces modest clinical benefits (197). A large Phase I/II trial is going on in England and France to evaluate a lentiviral vector derived from EIAV encoding a combination of TH, AADC and GCH1, but thus far no results have been reported in peer-reviewed publications.

#### 29.4.2.3 Brain Tumors.

**29.4.2.3.1 Definition.** Gliomas are the most common primary intracranial neoplasms and are divided into astrocytomas, oligodendrogliomas, oligoastrocytomas and glioblastomas (198). Glioblastoma is the most fatal primary brain neoplasm with an incidence of three to six in 100,000. Molecular lesions in glioma cells include deregulation of the cell cycle, alterations of apoptosis and cell differentiation, and histopathology shows endothelial proliferation, neovascularization and tumor cell migration and invasion. During progression from low-grade astrocytoma (WHO grade II) to anaplastic astrocytoma (WHO grade III) to glioblastoma multiforme (WHO grade IV) genetic alterations accumulate; however, a clear-cut accumulation of identical lesions across patients typical of each grade of glioblastoma, as has been described for colon cancer, has not yet been described.

**29.4.2.3.2 Gene Therapy.** Originally, the concept of gene therapy for glioblastoma was to transplant fibroblasts genetically engineered to secrete retrovirus vectors carrying a prodrug-activating enzyme gene (199). As retroviruses would only be able to integrate their genetic

material into dividing cells, this concept seemed to be safe for the selective transduction of highly proliferating tumor cells; however, clinical application of this approach did not show a clinical benefit for patients (200–202). The most important limitation of gene therapy for glioblastoma is the heterogeneity of the tumor tissue, with highly proliferative tumor areas alongside areas of necrosis and nondividing tumor cells migrating into the surrounding parenchyma. Subsequent development of gene therapy for glioblastoma has concentrated on

1. combination of different therapeutic genes for synergistic action
2. combination of drug therapy with gene and immunotherapy
3. improved methods of vector administration based on convection-enhanced delivery
4. imaging-based control of vector application and therapy read-out.

The various gene therapeutic strategies that have been studied to treat glioma models have been reviewed recently (203).

- Vectors encoding prodrug-activating enzymes (e.g. thymidine kinase, cytosine deaminase from various bacteria or yeast, guanine phosphoribosyl transferase, cytochrome P450 deoxycytidine kinase, folylpolyglutamyl synthetase, carboxylesterase)
- Vectors designed to correct genetic defects, e.g. by replacing defective cell cycle regulating proteins (p53, p16, p21, PTEN, Rb, p300 etc)
- Vectors encoding factors inhibiting angiogenesis (endostatin, angiostatin, antisense VEGF, dominant negative VEGF receptors, antisense EGF, dominant negative EGF receptors, antisense basic FGF and IGF1)
- Vectors immunomodulation (e.g. IL2, IL4, IL6, IL12, IL13, GM-CSF, TNF- $\alpha$ , interferon- $\gamma$ , antisense TGF- $\beta$ , TGF- $\beta$  soluble receptors, Flt3L)
- Conditionally replicating “oncolytic” viruses based on HSV1, AdV and other viruses.

#### 29.4.2.3.3 Enzyme/Prodrug Combinations: HSV

**Type 1 Thymidine Kinase/Ganciclovir.** This constitutes the most classical gene therapy approach for GBMs, namely, the use of the thymidine kinase from HSV1, which has been extensively employed experimentally and clinically. On administration of ganciclovir, phosphorylated ganciclovir is the toxic final product that mainly kills dividing cells; thus its great potential in treating brain tumors.

The HSV1-TK/GCV system displays significant bystander effects that are thought to amplify its cytotoxic activity. The bystander effect has various components that either stimulate the antitumor immune response or need cell-to-cell contacts to transfer GCV triphosphate to untransduced cells.



HSV1-TK has been delivered using cationic liposomes, HSV, adenovirus, retrovirus vectors, and even replicating vectors. When HSV1-TK is delivered using replicating HSV vectors, GCV itself inhibits the propagation of the vector. This has allowed replicative virus to be used, with the aim of increasing the percentage of tumor cells, which can be transduced, using GCV as a useful safety mechanism to prevent uncontrollable viral spread.

Exciting preclinical data for HSV1-TK/GCV resulted in various Phase I–II clinical trials of HSV1-TK gene therapy mediated by retrovirus, used immediately following surgical resection in patients with recurrent GB. Retrovirally transduced fibroblasts expressing TK were administered by intracerebral injection immediately after tumor resection. The data were suggestive, but the fact that they originated from small uncontrolled trial meant that their real significance still needed to be tested in larger Phase III trials.

Preliminary data highlighted that gene transfer was low, and that, therefore, insufficient numbers of tumor cells would have been transduced. Nevertheless, pharmaceutical interests led to the conduct of a large multicenter Phase III trial with hundreds of patients. In spite of substantial funds devoted to this trial, when patients on the gene therapy arm were doing worse than those on the control arm, the trial had to be stopped. The full extent of the lessons to be extracted from this trial for both the ethics of clinical trials of novel complex technologies and the role of the biotechnological industry in the advance of such techniques remains to be fully explored. The failure of the large Phase III trial has now consigned the use of retroviral vectors expressing TK to the archives of medical experimental history. In doing so, it has also compromised an otherwise powerful experimental approach, namely, HSV1-TK itself.

An intelligent alternative to these approaches has been provided by Seppo Ylä-Herttuala, who compared side by side the effectiveness of TK when expressed from retroviral vectors and from first-generation adenoviral vectors. These studies showed in controlled trials that the AdV were more effective than the retroviruses (204–206). This research has now been expanded and a Phase III trial is now in the final planning and implementation stages. Larger Phase II trials have demonstrated a statistically significant effect of AdV expressing TK+GCV in patients suffering from GBM. Other alternative conditional cytotoxic approaches, such as cytosine deaminase and 5-fluorouracil and carboxypeptidase G2 and proalkylating agents, are also being developed for the treatment of gliomas.

**29.4.2.3.4 Correction of Genetic Defects in GBMs.** Although the genetic alterations that give rise to a cancer cell are numerous, there are some frequently occurring gene mutations in glioblastoma multiforme. Perhaps, the most common genetic alteration is in the p53 tumor suppressor gene and upregulation of the EGF-R, which are mutated in a large percentage of the

human tumors. Tumor suppressor genes encode for proteins that suppress cell division; therefore, by introducing an unmutated tumor suppressor gene into cancer cells, apoptosis can be induced and tumor growth inhibited.

Inactivation of p53 occurs early in glial tumorigenesis, and thus, replacement of mutated p53 gene has been described for many tumor models. Adenoviral delivery of wt p53 into glioblastoma cell lines was attempted, and p53 inhibited proliferation and mutant p53 induced apoptotic cell death. Mutations of the retinoblastoma (Rb) gene and/or p16 gene are also common. Restoration of p16 expression into p16-deficient glioma cell lines, D-54M, U-251MG and U-87MG produced growth arrest. In addition, abnormalities in the apoptotic cascade are almost always present in GBMs, and activation of apoptosis promoting pathways such as the Fas-FasL system have been used to induce glioma cell killing.

#### **29.4.2.3.5 Inhibiting Blood Vessel Formation.**

Important and highly active angiogenic factors were initially discovered through tumor-resection-induced growth of distant tumor sites. Angiostatin and endostatin have been utilized in models of GBM. Growth inhibition was observed and encouraged clinical trials. The turnover time of these small peptides is very fast and it has been difficult to deliver enough of these proteins during long-term periods in humans, and therefore, long-term high-level delivery using gene therapy vectors has been proposed. Gene therapy applications of these ideas are now being tried in clinical trials (see below).

**29.4.2.3.6 Activation of the Immune Response.** Although it is difficult, if not impossible, to stimulate an immune response from the CNS itself, once an immune response has been stimulated systemically activated T lymphocytes have relatively few limitations in finding and destroying tumors located within the brain. The blood–brain barrier, the absence of classical lymphatic drainage from the CNS, and the lack of antigen-presenting dendritic cells (DCs) from the naïve CNS are barriers to the priming but not of the effector phase of the immune response. Tumor cells can indeed be targets of the immune system. The appearance of progressive multifocal leukoencephalopathy in immune-manipulated human patients suffering from multiple sclerosis and Crohn disease demonstrates that the immune system is constantly monitoring the endogenous brain milieu. Therefore, stimulation of the brain immune response is being attempted either through systemic vaccination, systemic stimulation of the immune system or even direct intracranial injection of DCs. A particularly promising approach is the stimulation of the immune system from within the brain proper. Ali et al. (207,208) have recently demonstrated that administration of the powerful differentiator of DCs Flt3L injected directly into the brain in combination with HSV1-TK is able to eliminate rather large brain tumors from the CNS of rodents.

**29.4.2.3.7 Enhancement of the Immune Response Using Cytokines.** The use of cytokines, such as TNF- $\alpha$ , has been attempted but without encouraging data of either preclinical or clinical outcomes. Whether TNF- $\alpha$  might be beneficial in conjunction with other tumor-killing strategies remains to be explored. IL-4 has also been used for glioma treatment because of its induction of very strong immune response in the brain parenchyma and antiproliferative effects on glioma cells. Again, combination therapies may be more effective, although in trials of IL-4 in experimental models it has been shown to be effective. IL-2, IL-12 and IFN- $\gamma$  have also been tested as potential adjuvants for the treatment of brain tumors. More interesting are the novel approaches in which IL-4 has been expressed fused to the translocation and enzymatic domains of pseudomonas exotoxin. This has now been tested in a number of clinical trials and has been touted to demonstrate positive effects in humans. This is now leading to larger clinical trials, and the results thereof are eagerly expected, as they are the only ones that may indicate the real long-term value of these novel experimental approaches. IFN- $\beta$  has now been tested on its own in a number of clinical trials. Lack of striking results has impeded the progression of these therapies into Phase III trials, which would be needed to demonstrate the real value of these novel approaches.

**29.4.2.3.8 Manipulation of Dendritic Cells as a Tool for Brain Cancer Immunotherapy.** An important approach is to increase the number and activity of primary APCs, e.g. the DCs. GM-CSF and Flt3 ligand differentiate hematopoietic precursors into DCs and attract them to the tumor site, also activating the DCs to process the tumor antigens, migrate to the local lymph nodes, and present antigen to naïve T lymphocytes to induce antitumor effects.

Antigen-pulsed autologous DCs are also being used to stimulate antitumor cytotoxic T-cell responses, an approach successfully used experimentally and also in Phase I/II human trials.

Given our still poor understanding of the brain's immune system, the challenge of immune stimulation against brain tumors is whether clinically effective responses can be induced in the absence of brain autoimmune disease (209). Importantly, induction of immune responses against melanomas, even in cases causing vitiligo, have never induced brain disease. Vitiligo destroys skin melanocytes that contain melanin, a protein that is also expressed within the nigrostriatal human neurons. On the other hand, non-small cell lung cancer that develops antitumor immune responses is many times accompanied by immune-mediated cerebellar degeneration, which is evidenced clinically as a paraneoplastic disorder.

Various trials of DC vaccination, in which gene therapy is used to deliver cytokine genes, or antigens, are currently progressing at various centers around the world. Recent resurgence in the interest in regulatory T cells, that are potentially inhibitory to the antitumor immune

responses are also forcing a rethinking on how to prime the subtype of DC that will stimulate cytotoxic T cells without the simultaneous induction of T regulatory cells.

**29.4.2.3.9 Conditional Replication Oncolytic Viruses.** The idea behind oncolytic virotherapy is a simple one. In the course of their natural life cycles, viruses often kill the cells they infect, and this is often cell-type specific. If viruses could be found or engineered that would replicate specifically in cancer cells, this would presumably be of therapeutic benefit. In fact several viruses with a naturally greater ability to kill cancer cells have been identified and several more have been developed by genetic engineering. Progress in using oncolytic viruses to treat glioma is discussed by Zemp et al. (210). Uses of the ICP34.5 mutant HSV vector G207 and the E1B-55 kDa mutant Adenovirus ONYX-015 are discussed above in the sections on those vectors. In addition to these, clinical trials have been performed using reovirus (211), and Newcastle disease virus (106), and a clinical trial using measles virus is in preparation (103).

Some of these systems are currently being evaluated in clinical gene therapy protocols (<http://www.gemcris.od.nih.gov>). The 27 protocols currently described as targeting glioma can be broken down as follows:

- Prodrug therapy (5 trials)
  - HSV-1 thymidine kinase gene mediated by an AdV with subsequent ganciclovir or valaciclovir (2 trials)
  - HSV-1 thymidine kinase gene mediated by a retroviral vector with subsequent ganciclovir or valaciclovir (1 trial)
  - HSV-1 thymidine kinase expression mediated by producer cells that are stereotactically implanted with subsequent ganciclovir treatment (1 trial)
  - E. coli* cytosine deaminase expression mediated by producer cells that are stereotactically implanted with subsequent 5-fluorocytosine treatment (1 trial)
- Cell cycle regulation (1 trial)
  - p53 gene mediated by a recombinant AdV vector
- Antiangiogenesis (2 trials)
  - (a) Episome-based antisense cDNA transcription of insulin-like growth factor I (1 trial)
  - (b) AdV-mediated expression of EGFR-CD533, a dominant-negative EGFR (1 trial)
- Immunomodulation (12 trials)
  - (a) Autologous tumor cells transduced with IL-4 using retrovirus and injected peripherally as vaccine (1 trial)
  - (b) Irradiated autologous tumor cells expressing TGF- $\beta$ 2 antisense RNA from plasmid injected peripherally as vaccine (1 trial)
  - (c) Human interferon- $\beta$ -mediated by AdV (2 trials)
  - (d) Allogeneic or autologous glioblastoma tumor cell lines (IR850) mixed with allogeneic cells

genetically modified with plasmid encoding GM-CSF (IR851) (2 trials)

- (e) Autologous DCs loaded with apoptotic autologous glioma cell fragments, coinjected peripherally as vaccine with autologous fibroblasts transduced with a retroviral vector encoding IL-4 (1 trial)
- (f) Autologous or allogeneic CD8<sup>+</sup> T cells genetically modified with a plasmid encoding a modified T-cell receptor targeting the mutant IL-13 receptor often upregulated on glioma cells (2 trials)
- (g) Expanded autologous bone-marrow-derived stromal cells expressing IL-12
- (h) Cytomegalovirus-specific DCs and T cells (2 trials)
- Replication-competent oncolytic viruses (7 trials)
  - (a) G207 (5 trials)
  - (b) Delta-24-RGD (replication-competent AdV) (1 trial)
  - (c) Recombinant Poliovirus (1 trial)
- Protection of autologous hematopoietic cells
  - (a) Autologous hematopoietic stem cells retrovirally transduced with a mutant methylguanine-DNA methyltransferase to protect them against an otherwise toxic oncolytic drug regimen including high-dose temozolomide and an MGMT inhibitor (2 trials).

**29.4.2.4 Silencing Gene Expression: An Approach to Treat Dominantly Inherited Diseases.** The technologies developed by the gene therapy scientific community mainly concern the expression of genes in target tissues. Thus, as discussed in detail above, various types of vectors have been optimized for expressing genes in any predetermined target tissue. The push for expression of proteins not expressed endogenously has led to the optimization of expression levels. Furthermore, great strides have also been made in the control of tissue-specific expression through the choice of promoters and the regulation of expression through the engineering of inducible expression controls; however, the reduction of endogenous gene expression has been more difficult to obtain. This has left dominantly inherited diseases where the expression of a mutated protein causes the disease without being developed as targets for gene therapy. This includes diseases such as Huntington's disease, dominantly inherited ataxias and dominantly inherited spinal cord degenerations such as the familial cases of amyotrophic lateral sclerosis.

The recent discovery of endogenous cellular mechanisms that regulate precisely and effectively the levels of endogenous mRNA in cells has opened up the possibility of using these methods, generally known as siRNA, to block expression of endogenous genes (212–217). These techniques have now been used experimentally to study the role of individual genes in physiological processes

and more recently as an effective way to inhibit gene expression in a therapeutic context.

Various groups have now demonstrated that siRNAs can be expressed from various types of vectors, and thus, these have been tested in the potential treatment of models of brain (164,166,217), liver diseases (218,219), infectious diseases (220–231) and cancer (199,232–234). The already mature nature of the gene therapy field is likely to allow the rapid translation of these findings into clinical trials. The treatment of inherited dominant diseases has been limited to palliative treatments. Recent successes in experimental models of Huntington's disease (157) (235) are thus likely to be made into treatments in shorter time frames, than gene therapies for those diseases that already have effective, even though not curative, treatments available, such as PD.

### 29.4.3 The Future Challenges of Gene Therapy

During the past 25 years major strides have been made in making the potential of gene therapy into a clinical reality. Vectors have been engineered to accept transgenic cassettes, in some cases up to 30kbp (adenoviral HC-AdV), or even 150kbp capable of encoding large genomic sequences (i.e. HSV-1 amplicons (236–241)), to target vectors to predetermined cell types and to engineer the expression of the therapeutic transgenes by placing these under the transcriptional control of regulatory elements.

Something that has continued to challenge the field is the innate and adaptive immune response to the vectors (209,242,243). Although a few years ago it was thought that the main culprits responsible for immune responses had been identified, recent surprises sometimes coming from the clinical arena have forced a reconsideration of this field. Although it was thought that the use of vectors that do not encode any viral protein, such as vectors derived from lentivirus, AAV, HSV1 amplicons and HC-AdV, would be absolutely safe, it was found that immune responses against capsid proteins could still be induced (244), not only in experimental systems but also potentially in human patients. The seemingly unavoidable clashes with the immune system have now prompted calls for the use of short-term immune suppression during the early phases of vector delivery (245) or until the vectors uncoat and deliver their genomes to the nuclei of transduced cells.

Even if the challenge of immune responses against viral vector capsids is eventually resolved, the potential immunogenicity of transgenes may force the development of tolerance induction in gene therapy (246–250). So far, the issue of immune responses against the therapeutic proteins or their regulatory elements has only been addressed by a handful of papers and constitutes a problem whose magnitude is yet to be determined. However, the development of immune responses against

the transgene in a retroviral vector in immunosuppressed HIV-infected patients (251) clearly highlights that the immune responses against transgenes cannot be ignored. Joint efforts with transplant immunologists are being made to develop strategies that could be applied to humans treated with gene therapy and ways to help patients that might develop deleterious immune responses to vectors and/or transgenes.

The field also needs to develop ways to measure success. A case in point is the treatment of boys suffering from the “bubble boy syndrome” (SCID-X1) due to a deficiency in the gene encoding for the  $\gamma$ c-cytokine receptor subunit. Originally pioneered by the group of A. Fischer in Paris, this gene therapy has now been repeated by investigators in London, Australia and the United States. The results that have been made available so far indicate the treatment to be effective. Indeed, the treatment of SCID-X represents one of the greatest successes for gene therapy, having demonstrably achieved the cures promised more than 25 years ago.

The development of clonal T-cell lymphoproliferations in three patients treated in Paris raised serious concerns. The discovery of leukemia in some of the patients reignited a search for retroviral integration sites in the human genome. Although these had been reported to be “random,” a reexamination of this issue with new techniques demonstrated that most integrations occurred into genes that were transcriptionally active and that in patients who developed lymphoproliferations, insertions were detected upstream of LMO2, a gene that had previously been linked to this disease. So, what went wrong, and could this have been avoided?

Although it may turn out impossible to reconstruct precisely what happened, there are some scenarios that are probable. In SCID-X1, in the absence of the  $\gamma$ c-cytokine receptor subunit, the receptors for the following cytokines are deficient: IL2, IL4, IL7, IL9, and IL15. This leads to a block in T- and NK-cell differentiation. Therefore, in the bone marrow of SCID-X1 patients the lymphoid progenitors lack the receptor for five different cytokines. Gene-therapy-mediated replacement of the  $\gamma$ c-cytokine receptor subunit provides transduced cells with the receptor for five different cytokines involved in the growth, survival and differentiation of lymphoid progenitors. Thus, the engineered cells have an enormous growth advantage against a bone marrow from which lymphoid cells are absent. This is not the case in ADA deficiency, in which the patient’s endogenous bone marrow maintains a low number of endogenous lymphocytes. And before transplantation, by the nature of the *in vitro* transduction protocol, rapidly growing clones will of course be preferentially selected for transplantation. Should the insertion of the retroviral vector into the LMO2 locus provide a growth advantage to those clones, which is highly probable, such clones would be preferentially transplanted. Finally, upon delivery into a patient with a bone marrow devoid of these cells, the

transplanted clones could grow unhindered and those with a growth advantage could eventually outgrow all others.

It is unlikely that this outcome could have been avoided, since previous experiments in mice, rats and non-human primates never gave any indications that this could happen. It is only by reengineering the bone marrow of the SCID-X1 patients that all conditions were met for the development of T-cell lymphoproliferations. Nevertheless, it seems to be the case that this outcome is less likely the earlier the gene therapy proceeds, and thus, through the identification of such factors, gene therapists will be able to avoid these problems in the future. In any case, even for those patients who eventually developed leukemia, the treatment was a success that allowed them a substantial amount of normal life outside the sterile bubble they would have otherwise have been confined to.

Finally, the majority of clinical trials are Phase I trials. These are toxicity trials, rather than therapeutic trials. The lack of a double-blind design, adequate control groups and small numbers of patients involved in Phase I trials makes it generally statistically impossible to draw therapeutic conclusions. Given the history of the field of gene therapy, it is important to use a large dose of patience, detachment and caution in extracting therapeutic efficacy conclusions from these early trials; however, press releases and Internet postings continue to draw therapeutic conclusions from such Phase I trials.

Equally, it will be important to utilize novel molecular pathological assessments of patients being included in clinical trials. For example, global molecular markers of disease (e.g., patterns of genes that segregate disease subtypes by microarray gene expression) should be used in performing Phase I clinical trials for diseases such as glioblastoma multiforme. In glioblastoma recent microarray studies have shown that there are two populations of patients, one with a rapid “typical” progression and a second long-term survivor group that survives for up to 3 years; the impossibility of differentiating tumors from either group by any other means indicates that tests such as these need to be used in the choice of patients for clinical trials (252–254). The increasing availability of molecular markers allowing identification of patients with particular signal transduction amplifications will enable the exclusive enrollment of patients who are likely to benefit from particular interventions. This will increase the rate of success for the appropriate patients and eliminate useless trials for others (252–255).

## 29.5 CODA

Gene therapy has moved from a potential therapy at its inception in the late 1970s to a clinically effective therapy in the second decade of the twenty-first century. Diseases for which there were no treatments available 40 years ago are now treated with gene therapy in experimental settings. In the next decade gene therapy will become



part of the “routine” treatments offered to patients—not as an experimental therapy but as one regular therapeutic option available. New gene therapies are increasingly being tested in advanced clinical trials, leading to a rise of its clinical indications. Gene therapy is now becoming transformed into an additional therapeutic option, and combination with other therapies such as immunomodulation, chemotherapy, radiotherapy, etc., will continue to improve patients’ outcomes. As the field reaches its maturity and the original dreams become therapeutic realities for patients, the visionary imagination of the pioneers’ can only be quietly admired. We have finally acquired the necessary hindsight to confirm their intuitions to realize that indeed, there was “light at the end of the tunnel.”

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History of Medical Genetics; Medicine in a Genetic Context; Nature and Frequency of Genetic Disease; Genomics and Proteomics; Genome and Gene Structure; Epigenetics; Human Gene Mutation in Inherited Disease: Molecular Mechanisms and Clinical Consequences; Genes in Families; Analysis of Genetic Linkage; Chromosomal Basis of Inheritance; Mitochondrial Medicine: The Mitochondrial Biology and Genetics of Metabolic and Degenerative Diseases, Cancer, and Aging; Multifactorial Inheritance and Complex Diseases; Population Genetics; Pathogenetics of Disease; Human Developmental Genetics; The Molecular Biology of Cancer; Genetic Evaluation for Common Diseases of Adulthood; Enzyme Replacement and Pharmacologic Chaperone Therapies for Lysosomal Storage Disease; Ethical and Social Issues in Clinical Genetics; Leukemias, Lymphomas, and Other Related Disorders; Disorders of Leukocyte Function; Mucopolysaccharidoses; Genetics of Alzheimer Disease; Basal Ganglia Disorders; Primary Tumors of the Nervous System; Optic Atrophy; Hereditary Retinal and Choroidal Dystrophies; Retinoblastoma and the RB1 Cancer Syndrome; Epidermolysis Bullosa.

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# CHAPTER

# 30

## Ethical and Social Issues in Clinical Genetics

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### 30.1 INTRODUCTION

Ethical issues abound in all areas of health care. These can relate to the medical management of individual patients or to broader social and political issues, such as the access to care for disadvantaged groups within society and the just deployment of the available resources. Ethical discussions in some clinical areas focus on “end-of-life” or “beginning-of-life” issues, including euthanasia, the withdrawal of futile treatments, and the status of the embryo, while in genetic services there has been an unusually broad range of contentious topics. Important areas have included reproductive issues such as prenatal diagnosis and the selective termination of pregnancies, the proper communication of information within families, the making of decisions on behalf of children or incompetent adults, professional issues such as the role of the clinical geneticist or genetic counselor and the scope of their potentially competing obligations to their patients or clients, their patients’ families and to society more broadly, and the interrogation of common assumptions and attitudes concerning the just treatment of individuals within society.

Two additional factors make any reflection upon ethical issues in the practice of clinical genetics particularly complex. First, there are ethical issues that confront our clients as individuals and families. In most of medicine there are issues that challenge the professional, and there may also be complex issues where society has to declare its view when there are limited resources to be allocated or where the scope of professional practice may need to be regulated. In genetics, however, there are numerous occasions where it is our clients who are confronted by the ethical difficulties. Indeed, it is sometimes our role to formulate the issues for our clients to consider. We therefore need to distinguish among the challenges that we face as professionals, those that are faced by our clients, and those faced by society at large.

Second, our discipline is characterized by the rapid growth of genetic knowledge and, simultaneously, by the rapid development of technologies for generating genetic information and for applying the new knowledge. The sequence of the human genome was first reported only a decade ago but it is already feasible for the genome sequence of individual patients to be determined to achieve a full diagnosis of their condition. In addition to diagnosis, however, the applications of genomics in medical practice now include (i) informing decisions about disease prevention or the surveillance for complications of disease in those at risk from recognized, familial disorders, (ii) assisting in reproductive decisions either by identifying conceptions (after implantation or before) that are affected by specific genetic disorders or by identifying the healthy carriers of recessive disorders, and (iii) guidance on the choice of therapies, especially in the treatment of malignancy.

The challenge for practitioners has shifted from the generation of the DNA sequence of important single genes to the interpretation of vast quantities of data—full exome sequencing or even a whole genome sequence. This new situation of abundant data of which we can make only limited sense may well persist for a decade, or two, or longer. These technical developments are changing the shape of the ethical and social issues confronting us in clinical genetics. The underlying principles and the issues at stake may not be new but the contexts within which clinicians and clients/patients/families are confronting these difficulties are, for the moment, unfamiliar. We have yet to develop common professional habits of practice that allow all parties to feel comfortable and safe. Accordingly, the questions arising in practice repay particular care and attention.

It is my aim here to sketch out the principal social and ethical issues that arise in our work in clinical genetics, considering in particular the impact of recent developments; it is in these areas that practitioners are experiencing particular challenges.

In addition to promoting discussion and debate within our profession, I will be particularly pleased if this chapter contributes to the flow of ideas and discussions across the conventional boundaries between health professionals and patients/clients on the one hand and, on the other, between health professionals and scholars from the social sciences and humanities. Faced with the challenges that confront us all, we can only benefit from the sympathetic understanding and questioning of those with particular skills in these other disciplines.

### 30.2 THE HISTORICAL CONTEXT

Clinical genetics developed after the Second World War in response to the concerns of families and clinicians about the risk of genetic disease occurring within a family. Pediatricians were consulted about the risk of recurrence of congenital malformations or childhood genetic disorders and neurologists were consulted about the risk of healthy individuals developing the neurodegenerative or neuromuscular disorders that affected other members of their families, while some biologists and physical anthropologists collected information that could help to answer such questions. Clinical genetics as we know it arose from the convergence of clinicians tackling these questions with nonmedical scientists interested in human genetics and sometimes caught up in the very human dimension of their studies (1). Genetic counseling emerged as an activity alongside clinical genetics and it has slowly become a profession in its own right (2,3). Human genetics, however, has a longer history than this, which cannot be ignored.

The study of heredity and its influence on human variation, especially on “natural ability,” was developed in nineteenth century Britain by Sir Francis Galton. His focus on quantitative traits was most productive, eventually merging with Mendelian genetics, but he applied this focus with particular enthusiasm to intelligence, social virtues (or their lack), and social achievement. He regarded these traits as primarily genetic and, like many before him, had the goal of improving the genetic constitution of future generations. He coined the term eugenics to describe “the study of agencies under social control that may improve or impair the racial qualities of future generations either physically or mentally.” The term was soon applied to the social movement promoting the improvement of such qualities. The perennial concern about the moral decay of society, widespread among the elders in societies ancient and modern, latched onto theories of heredity in late Victorian Britain as offering solutions to the vices of alcoholism, pauperism, homelessness, and feeble-mindedness. The same set of ideas also reinforced assumptions about the relative evolutionary development of different populations, reassuring the citizens of the major imperial powers that they were justified, indeed destined, to rule over their biologically inferior colonial subjects. Such ideas flourished in literature and the arts, as well as the sciences, and were integral to the mentality of the society at large in

much of Europe and North America. Indeed, the belief or claim that one’s nation is “superior” may lead directly to the need to identify other, less favored peoples over whom to rule, and hence to expansionist policies that would justify the “rightful” exploitation of subject peoples whether in Africa or Eastern Europe. Eugenics may have played a part in fostering the mind-set that led to the disasters and evils of the 1930s and 1940s.

The notion that “Biology Is Destiny” sometimes has a rather narrow set of connotations, relating primarily to gender, but when applied retrospectively to the nineteenth century it can encapsulate the then tacit, taken-for-granted, realities of race and social class as well as of gender. From these common assumptions developed the theories and practices that we now condemn as the eugenic abuses of human genetics. In the early twentieth century, racist immigration policies were introduced in the United States in the guise of intelligence tests; testing to ensure the “quality” of immigrants was then acceptable, whereas overtly racist legislation was not. Legislation to enable the forced sterilization of those with mental handicap, neurodegenerative disorders (4), and a range of other medical and social disorders was introduced in North America, Sweden, Germany, and other countries. Such legislation was not introduced in Britain, but the segregation of the sexes in institutions and the involuntary sterilization of many with mental handicap, especially women, was widespread over many years because the assumptions that led this to appear appropriate, “normal” practice, were so pervasive. Even a full awareness of the horrors of the Nazi campaign of racial hygiene, in which many physicians, anthropologists, geneticists, and other scientists actively participated, and the murderous consequences of the related belief in Aryan racial superiority, did not lead to the discontinuation of eugenicist practices in Sweden and the United States, for example, until the 1970s.

It is unhelpful for contemporary geneticists to be perpetually apologizing for the sins committed by their intellectual forebears, but neither can we afford to forget those horrific events that happened so very recently. As the scientific validity of contemporary genetics and its potential impact on society are now much greater than was the case 70 years ago, it is essential that we identify and make explicit our current taken-for-granted attitudes and our deeply held but often unstated, and perhaps unexamined, convictions. How might these be leading us into errors that we or our descendants will only recognize too late, with hindsight, once great damage has been done to our profession, to our patients, or to society as a whole? This is the hard lesson we should learn from considering the past abuses of human genetics.

### 30.3 GENETIC COUNSELING, TESTING AND SCREENING

A crucial distinction, familiar to every practitioner, is the contrast between the clinical genetic services made

available in response to the preexisting concerns of particular individuals and families, and the population screening programs offered proactively to large groups of the population, who have usually not sought the test that is being offered.

In the context of a specific family, genetics professionals will respond to the particular questions or concerns of family members. For example, (i) parents may be seeking an explanation for a serious developmental problem affecting their child, (ii) an individual at risk of a familial neurodegenerative condition or malignancy may wish to know his or her chance of developing the family's disorder, or (iii) a couple may wish to know the chance of their future children being affected by an inherited condition that has previously affected others in the family. Such a consultation will usually begin with the professional listening to the family's account of their concerns, then performing any relevant clinical assessments or investigations, and finally providing whatever information is appropriate back to the family. This process may be extended in time—perhaps occupying several consultations separated by weeks or months, especially if the precise diagnosis is not readily apparent. Great sensitivity is often required to indications of the client's understanding and emotional responses, whether spoken or unspoken, if the professional is to respond adequately to their needs. It will often be appropriate to provide emotional support, drawing upon the complementary skills of different members of the clinical genetics team, and occasionally referral for more formal psychotherapy may be helpful. In all these cases, however, the professional is responding as best as they can to the client's preexisting concerns.

In the context of population screening, the encounter between professional and client is very different. The professional is actively promoting a specific course of action—that the client/patient should undergo an investigation. The client may never have had any questions or concerns to which the screening test could be understood as a helpful response, so the professional's suggestion of the screening test may itself generate concerns rather than allaying them; it imposes the burden of an unsought decision when one course of action—compliance with the offer of testing—is being heavily recommended in the very act of making the test available at all. Although there are some parallels, this differs from the commercial marketing of a genetic test in several ways; there is an implied professional approval in both settings but this is stronger if no charge is made for screening provided through public mechanisms and the question of cost is not there to serve as a warning cry of “caveat emptor.”

There most certainly are circumstances in which it is thoroughly appropriate for health services to promote a particular screening test or other health intervention—as with the immunization of infants, the measurement of blood pressure in adults, or cervical cytology and mammography in women of specific age groups. In the sphere of reproduction and genetics, however, the promotion of

screening can be regarded as especially contentious; in this context, individual choice is highly valued. Newborn screening for the remediable disorders, phenylketonuria (PKU) and congenital hypothyroidism is universally applauded, whereas there is dissent in relation to reproductive genetic screening, including antenatal screening for Down syndrome or congenital malformation and carrier screening for autosomal recessive diseases. It is unarguable, however, that the context of proactive population screening for genetic disease is very different from that of genetic counseling as a response to preexisting family concerns. This has important ethical implications for professional practice in these areas.

### 30.4 GOALS AND OUTCOMES OF GENETIC SERVICES

In the bad old eugenic past, the principal aim of reproductive genetics was to improve the biological quality of the next generation. This meant encouraging the gifted, successful and virtuous to have more children and discouraging or preventing those with medical or social disadvantages (whether inherited or acquired) from having so many. This would help to overcome what was perceived as the unhelpfully low fertility of the higher social classes and of the “more advanced races.” Such concerns about differential fertility still resonate around the globe; for example, they are reflected, in different ways, in contemporary state policies in Singapore and China and are whipped up from time to time by those politicians in many countries (including developed European and North American countries) who think they stand to benefit from intercommunity strife.

Western society today pays little overt attention to the antiquated concepts of “racial hygiene.” In the contemporary rhetoric of clinical genetics and genetic counseling, our professional goals are therefore quite different from former ideas of “the health of the people” (i.e. of the race). Respect for the autonomy of the individual takes precedence over measures of impact of genetic services on the population as a whole, such as the incidence of genetic disorders at birth or the number of terminations of pregnancy for specific conditions. To describe our goals in terms of such crude population outcomes would now be regarded as frankly and unacceptably eugenic.

However, there is a contemporary context within which comparable assumptions about the effectiveness of genetic services could be seen by some as natural and defensible. This is the economic frame, in which the primary goal of genetic services would be seen as the reduction of expenditure on caring for those with genetic disease or disability, achieved through a reduction in the birth incidence of conditions for which prenatal or carrier screening has been made available. From this perspective, those affected by Down syndrome or  $\beta$ -thalassaemia (for example) would be regarded as a net burden to society.

Despite the contested nature of these claims, over the last 30 years many studies of prenatal or carrier population screening, including prenatal screening for Down syndrome or carrier screening for cystic fibrosis (CF), have essentially argued for the implementation of such programs on the grounds that screening, and the termination of affected pregnancies, is less costly than caring for affected individuals. Although many clinicians may regard these arguments as ethically unacceptable, they are still employed *sotto voce*. Some of those who use these arguments have no moral qualms about doing so. Others may value the offer of reproductive choices per se and therefore support the existence of prenatal screening programs while rejecting the monetary, neo-eugenicist (or “consumer eugenics”) arguments. They feel that screening should be available to all pregnant women and that the absence of such a screening program would, for many, remove the possibility of making important reproductive decisions. The adoption of particular views on this issue by different social groups, including political parties, religious communities and ethnic groups, is heavily influenced by national political alignments so that the political right in the United States, for example, has a very different perspective from much of the political right in Europe. We will turn later to the different context in developing countries.

Crude monetary arguments are not generally used to justify the provision of family-based clinical genetic and genetic counseling services. Such an analysis might show a net cost savings to society, because genetic counseling does sometimes have the consequence of reducing the number of infants born with serious (costly) genetic disorders, through parental decisions not to have children or through the selective termination of affected pregnancies, although clinical genetic professionals may and (I would argue) should refuse to use these arguments to justify their services. To adopt such arguments would concede that families that make other choices, those who decide to have affected children, are making wrong choices. Furthermore, such economic analyses depend on numerous assumptions about the future pattern of health and social care provision and the most appropriate way of discounting the likely future costs of care to be traded against current actual costs of providing the clinical genetics services. These analyses would therefore be complex and contentious even within a purely economic frame.

How, then, can the outcomes of clinical genetics services be measured to permit a comparison of the worth of different models of clinical genetics services or their comparison with expenditure on altogether different areas of health service activity? A number of outcome measures have been proposed and employed in research studies, but it remains unclear whether any of them is suitable for the ongoing evaluation of a clinical service.

- The recall of risk information, concerning the risk of a disease or the risk of its recurrence in a future child, can be assessed after a lapse of months or years, but

recall is the result of many psychological processes including the tendency to polarize probabilities toward 0 or 1, that is, No (it will not happen=0) or Yes (it is certain to happen=1). Further, the significance attached to recurrence risks by clients is itself influenced by the way in which the information is framed in the counseling process (5).

- Reproductive plans can be assessed before and after genetic counseling. This approach prejudices reproduction as the primary concern of our clients, many of whom are not specifically seeking information about reproduction. Furthermore, such plans are merely hypothetical so that their expression will be heavily dependent on the context and framing of the issues by the professional.
- Reproductive behavior has been assessed in some studies but requires prolonged follow-up and a consensus on what would count as a satisfactory outcome for both the clients and the health service professionals.
- Client satisfaction with the service, and levels of anxiety or concern, can be assessed, but may be heavily influenced by the environment in which a service is provided. Clients may not be aware of how the service they have experienced compares with other services, and their views are likely to be influenced by the information they are given in the clinic. For example, clients may be distressed if given an item of “bad news” or they may be resentful if even the most skillful diagnostic process has failed to identify the cause of some serious disorder in their child.
- For the same reasons, questionnaire-based measures of healthy physical and mental functioning are unsuitable outcome measures for genetic services, whereas they may be entirely appropriate for assessing care for osteoarthritis of the hip or ischemic heart disease.
- A more client-focused measure, “perceived personal control,” has been developed as a major improvement on other measures of outcome, avoiding many of the previously identified pitfalls, and can contribute to the assessment of the quality of services provided (6). A similar measure has emerged independently from studies on the outcomes of clinical genetics services in the United Kingdom, encapsulated in the concept of “empowerment” (7). Both approaches can be applied across many different contexts and are not restricted to reproductive genetics.

The ethical importance of the choice of outcome measure is especially grave within a large population-based health service because the professionals in national health care systems or large insurance-based schemes (including health maintenance organizations) can be driven to comply with population-based or corporate goals rather more readily and directly than in a system of truly individual health care arranged between the physician and the patient. If a health service decided to reduce the birth incidence of Down syndrome, CF, or Duchenne



muscular dystrophy (DMD), for example, and to judge the performance of health service staff according to their “achievements” in these respects—perhaps with implications for their rates of pay or job security—then it would be no surprise to see service goals systematically distorting the pattern of health care provided to large numbers of individual patients (8). This is not an argument for the privatization or fragmentation of health care, which brings other problems, but for great vigilance in any large health care system; professionals must watch to ensure that their responsibilities are not subverted, perhaps rather subtly, by the imposition of institutional (state or corporate) policies.

I am arguing here that there is no utilitarian calculus that can determine the worth of clinical genetic services in a neutral, detached or objective manner. Despite these difficulties, there are ways of using research evidence to help decide what services should be made available, and then there are ways of assessing the quality of the services that are provided (9). The decisions about what services to provide will draw on research evidence about the practical consequences of providing particular patterns of service. These decisions are inevitably the product of a political process reflecting both the value that society places upon autonomy in the sphere of reproduction and society’s concern for the welfare of individuals with genetic disorders and their families. Once decisions have been made about what services to provide, their quality can be assessed as a proxy for their outcomes. The provision of specific services to a population may also be assessed as an important dimension of that outcome (10).

### 30.5 NONDIRECTIVENESS IN GENETIC COUNSELING

Respect for client autonomy is one of the core values of the genetic counseling profession. One of the manifestations of this respect for autonomy is the claim that genetic counseling is, or at least should be, “nondirective”: it should not lead the client to make specific choices predetermined by the counselor or the health care system. In addition to respect for client autonomy, however, there are several other important factors that contribute to the cult of “nondirectiveness,” and it is necessary to understand these other factors if we are to use this concept appropriately.

The claim that genetic counseling is nondirective allows the professional to distance herself or himself from emotional involvement in the decision. This may be very helpful, in so far as emotional over-involvement can be damaging. The claim that genetic counseling is nondirective can also function well for professionals by emphasizing that the legal responsibility for their clients’ decisions lies firmly with the clients—an important clarification. Another function of the claim to be nondirective is that it helps professionals reject suggestions that they

may be contaminated by any taint of Nazi eugenics. In all these respects, then, the claim of adherence to the doctrine of nondirectiveness may be attractive and helpful to genetic counseling professionals.

There are several other respects, however, in which the concept of “nondirectiveness” may be problematic and has been challenged. First, at least some elements of clinical genetic services may inevitably be “directive,” leading clients toward specific decisions and outcomes. This applies particularly to population genetic screening programs, most especially in the context of reproductive genetic screening, where the existence of a screening program must in itself carry the implicit message that screening is worthwhile, indeed recommended. This can be referred to as “structural” directiveness and does not imply that the individual professionals involved are actively recommending one course of action or another, although they may be doing so, or the screening program may be so routinized that neither professionals nor clients give active consideration to the issues involved and both parties merely accept screening as standard practice (11,12). In these settings, professionals may have to work hard to help clients appreciate that the decision really is one for them to make.

Second, there are contexts in clinical genetics where it is entirely proper for a professional to recommend a specific course of action to clients (13). This may involve the recommendation that a client at risk of a specific disorder should undergo surveillance for complications of that disorder, as with colonoscopy for those at increased risk of bowel cancer or echocardiography for those with Marfan syndrome. This in turn may involve recommending a predictive genetic test, so that, for example, colonoscopic surveillance for bowel cancer can be targeted at those family members with a high risk, and those who are at population risk can avoid the inconvenience, costs, and hazards of unnecessary investigations.

Genetic counseling professionals may also actively recommend to their clients to share genetic information within their family. When important information about a client’s genetic condition could be relevant to other members of the client’s family, the professional may very reasonably point this out. It could be relevant to the future health of the client’s relatives or to their reproductive decisions. In general, genetic counselors will promote open family communication about any relevant genetic disorder and will indicate the type of problems that can arise if communication is blocked. This is generally acknowledged to be good professional conduct, although it clearly conflicts with “nondirectiveness” if that is interpreted in a narrow and rigid manner.

There is another sense in which an exaggerated respect for nondirectiveness can be unhelpful: when compliance with “nondirectiveness” is taken to require that genetic counselors accept without challenge every statement expressed by their clients, even mere preferences that they may not have considered with much care. This approach

could so constrain the counselor that their ability to help the client is severely restricted. Effective counseling, as in scenario decision counseling, helps the client to think through the consequences of any particular decision or course of action that is planned. This inevitably entails the use of authoritative counselor interventions, such as direct questioning to gather information or confrontation of the client with unwelcome information or with the need to consider some previously unanticipated potential consequences of planned action. Not to make use of such interventions would greatly limit the worth of the genetic counseling (14–16). An inappropriately passive understanding of “nondirectiveness” will undermine the authority and effectiveness of the genetic counselor and reflects a shallow understanding of “counseling” in general, but it is all too easy for such misunderstandings to arise unless professionals monitor their practice with the help of regular supervision.

Finally, there is the danger that nondirectiveness in relation to a complex practical question with extensive moral implications may be felt as abandonment by the client. This has been particularly problematic for those patients recently diagnosed with cancer, who are faced by choices between therapeutic options where the evidence is unclear and difficult to interpret. An impersonal and detached presentation of the options can then, understandably, be experienced as an emotional withdrawal from the client, as abandonment. In other contexts, as within clinical genetics, the practice of nondirectiveness could also be perceived by clients as a lack of full personal engagement unless the professionals take care to make this concept function on behalf of the clients rather than on behalf of their professional selves.

Any claim that genetic counseling should always be “nondirective” would be unhelpful, and any claim that it is so would clearly be false. Respect for “nondirectiveness” is appropriate but only when it is understood as a rich and complex concept that usefully sustains the tension within practitioners between respect for the client’s autonomy and other competing priorities. If this appropriate sense of “nondirectiveness” is to be recognized in practice, we will require sensitive tools for the analysis of language in the specific interactional context of genetic counseling—within a sophisticated methodological framework for the understanding of language in its more general social context. Mechanical or formulaic definitions of what constitutes “nondirectiveness” will be unhelpful.

### 30.6 DIAGNOSTIC GENETIC TESTING

When an individual presents for medical attention because of a clear health problem, there are usually few ethical issues for the physician to consider in striving for a diagnosis. This will usually have to be the first step in meeting the broader needs of the patient and the family for prognostic information and advice about treatment.

However, there are at least two types of problems that may arise: the difficulty of handling diagnostic information about the patient when it has important implications for others and the difficulties that may arise when investigations reveal information of uncertain significance.

Communicable disease, especially sexually transmitted disease, provides another context in which a diagnosis may have important implications for others; health professionals will work with a patient in an attempt to pass on the relevant information to their sexual partner/contacts and there may be legal sanctions when important information is withheld. This situation may parallel transmissible genetic disorders in some respects, although, clearly, not all. Legal force is not usually applied to compel a patient to disclose sensitive personal information in the context of genetic conditions, although legal protection may sometimes be available to health professionals who disclose such information to the at risk relatives of the reluctant patient.

When a physician is investigating the cause of a patient’s symptoms, the differential diagnosis will often include the possibility that the problem may have a genetic basis, although the chance of this will often be small. The physician may well not mention this possibility of a genetic basis for the problem in discussion with the patient or family unless investigations make it likely that this is the case. What chance of the diagnosis being genetic should prompt the investigating physician to raise it as a possibility? How would this differ for different possible diagnoses? Does it change with the particular prognosis, response to treatment, family implications, etc.? Two examples may assist reflection:

- (1) When the diagnosis of DMD is being considered in a young boy with delay of motor and perhaps language skills, telling the parents that such a diagnosis is a possibility will cause distress but may help the parents prepare themselves and the wider family to deal with the implications if the suspected diagnosis is confirmed. Should it be discussed at the initial presentation or only once first-line investigations make it more likely, such as the finding of a greatly raised serum creatine kinase? The answer will often depend on contextual circumstances that cannot be prescribed as a general rule but it would be unusual not to mention the possibility of an inherited disease before either proceeding to analysis of the dystrophin gene or to a muscle biopsy.
- (2) If an adult has presented with dementia, this clinical picture will occasionally be the result of an underlying genetic condition, such as Huntington disease (HD) or one of the less common inherited dementias. This is more likely if the onset of disease is at a younger age than usual, if there is a relevant family history or if the patient has some additional neurological manifestations. Depending on these factors, the physician will need to consider (a) *whether* the

possibility of an inherited disorder should be mentioned, (b) *how* it should be mentioned, (c) *with what emphasis* and (d) *to whom*. The possibility of an inherited basis would often be discussed with the patient (and perhaps one or more relatives) if the diagnosis of HD was being considered as quite a distinct possibility but might not be mentioned if the molecular test for HD was being performed as the least likely of a long list of possible conditions under consideration. Even if mentioned, the patient's anxiety, cognitive impairment or both may limit whether or how this information could be passed to relatives. In practice a spouse or other relatives may also be informed of the suspected nature of the condition as a transmissible disorder, perhaps through being present with the patient in the consultation. This can open up an opportunity to help the family manage communication about the condition more effectively, if investigations subsequently confirm that it is genetic.

While the genetic nature of a condition remains uncertain, it may be difficult for the family to communicate effectively about it: those aware of the possibility may be uncertain what to say, or to whom, and how much concern would be appropriate to share with others when it may all come to nothing. This leaves open the question of how the professional strikes the right balance between backing away altogether from giving a warning in any family or creating needless anxiety in too many families, while of course always respecting each patient's confidentiality.

The other problem area is of investigations yielding information of uncertain significance. This difficulty has always been there in genetics since the earliest days of chromosome studies, which may be regarded as the first genome scanning technology, albeit of very low resolution. As laboratory techniques have progressed, so that the resolution of the available genome scan has improved, both scientific knowledge and clinical experience have accumulated. After the counting of chromosomes, the development of chromosome banding techniques was the next major step forward. It has taken years, sometimes many years, for the medical implications of each anomaly to be appreciated and there can still be real difficulty in predicting or interpreting the reproductive consequences of complex chromosomal rearrangements.

The interpretation of diagnostic results from the molecular genetics laboratory has passed through phases in which uncertainty has been more or less prominent. The early application of genetic linkage analysis to families gave physicians good practice in explaining the limitations of molecular diagnostics and estimating the frequency of misleading conclusions (e.g. double recombinant events). The use of linkage disequilibrium to estimate the probability that an individual carried

CF trained us to attend to a person's population of origin. As mutation testing then became possible for some important loci, it seemed that the ease of interpretation was steadily increasing. Even difficulties such as the distinction between Duchenne and Becker muscular dystrophy caused by mutation in the same gene (the dystrophin gene) could usually be accounted for by the frameshift hypothesis. However, the interpretation of sequence information then became more complex in the clinical context, most prominently in some of the Mendelian cancer-predisposing genes. When a novel sequence variant was found in an individual with a strong family history of cancer, how should it be interpreted? This remains a problem today in the context of familial breast cancer and some of the novel variants found in *BRCA1* and *BRCA2* in those with breast cancer and/or a family history of breast or ovarian cancer; such variants are often known as VUSs (variants of uncertain significance).

Comparable difficulties arise in relation to autosomal recessive disorders. Sequence changes in the CF locus *CFTR*, for example, may be entirely innocent or they may be weakly pathogenic when accompanied by a definitely pathogenic allele on the other chromosome (leading to a late-onset and rather mild predisposition to chest infection), or they may predispose to a very mild phenotype, such as "congenital bilateral absence of the vas deferens" (CBAVD), resulting in male infertility. This gives rise to the need to distinguish between the clinical condition and the gene: simply having two mutations in two *CFTR* alleles is not necessarily equivalent to having the clinical disorder known as CF; this emphasizes the problems of talking about the "gene for" something (whether that something is musicality, sexual orientation, Viking ancestry, a character trait or a disease, such as CF).

With the switch from mutation searching in Mendelian genes to high-throughput genetic technologies, some other major changes have occurred. First, while genome-wide association studies (GWAS) have proved useful in some research applications, their clinical utility in the common, complex degenerative diseases of developed societies has not been demonstrated. While many highly significant associations have been found between single nucleotide polymorphisms (SNPs) and disease, the relative risks involved have generally been very small and the proportion of the genetic contribution to the risk of disease that can be accounted for (i.e. used in risk "prediction") is generally in the range of 10–20%. The problems of accounting for the remaining 80–90% of heritability is known as the "missing heritability" (17). In addition, there are unresolved questions about (i) how to demonstrate (how to avoid overlooking) important gene–gene interactions (i.e. departures from the simple multiplicative combination of relative risks) and (ii) the appropriate models of selection that must operate to account for the high levels of genetic polymorphism observed within human populations.

Moving beyond SNP-based association studies to the direct detection of abnormalities in high-throughput studies, the first technology to enter regular clinical practice has been comparative genomic hybridization (CGH) arrays to detect copy number variants (CNVs). This has shown not only the high frequency of CNVs of varying sizes that are compatible with normal development and functioning but also the contribution of specific deletions and duplications to disorders of physical and mental development and vulnerability to the acquisition of psychiatric disease (18). Particular CNVs that appear compatible with normal development may contribute to severe developmental problems in the presence of environmental triggers or other specific CNVs (the “two-hit” model of developmental disorders) (19). The caution required in interpreting these investigations has recently been emphasized by the American College of Medical Genetics (ACMG) (20).

The next technology to impact on genetic diagnostic services is that of whole genome sequencing (WGS), or at least whole exome sequencing (WES) of protein-encoding genes. This is not yet widespread in regular clinical practice but experience is being gained through research-led studies both of healthy individuals and of those with overt disease (21–23). The “Thousand Genomes Project” and other studies are revealing very high levels of rare variants, some of which are likely to make a substantial contribution to disease or risk of disease (24). However, the sheer number of rare or completely novel variants identified in each human genome sequenced is such that the interpretation of WGS data is difficult and demanding; the generation of sequence data is no longer the rate-limiting step in diagnostics but rather its interpretation (25). Bioinformatic techniques make such interpretations feasible, although they depend, for now, on numerous assumptions that will not always be correct. Computer modeling of the functional consequences of “mutations” can give helpful results but is not infallible. It will take years, perhaps decades, of data accumulation and analysis and of functional molecular studies for the interpretation of most DNA sequence variants to be interpretable in a clinical context with reasonable confidence. The interpretation of WGS data is likely to shift frequently over the next few years as new knowledge accumulates, so it is highly unstable (26). In the meantime, we have two major problems: (i) how do we organize high-throughput sequencing and the associated data storage and analysis, conducted to answer clinical questions? (ii) what do we say to patients?

One possible approach to the clinical use of WGS—we could call this a “minimalist” or “focused” approach—would be to carry out the best analysis possible for the clinical question at hand and to ignore any findings likely to be incidental to this, however important for other reasons. Data would not be stored, so the problems of refined future analyses, of data attrition and of completely incidental results would disappear. For

example, it may become cheaper and easier to perform WGS (or exome) analysis when there are many genes that need to be sequenced for diagnostic purposes. Once one or more gene variants are found that may plausibly be contributing to the disease, it may be regarded as the achievement of the desired output. There would be no need for further genetic analysis; the sequence changes could be confirmed (e.g. by Sanger sequencing), the report produced, and then the data could be deleted. If further testing came to be required for some other purpose (in relation to another disease, or to produce information about carrier status for reproduction or pharmacogenetic information for “therapeutic guidance”) then WGS would have to be repeated. However, it may soon be inexpensive enough not to be the rate-limiting step.

A very different approach would be (i) to report on all the incidental findings unrelated to the reason for testing, in addition to answering the questions asked; (ii) to keep the full WGS (actively maintaining the data over time to prevent its degradation) so that it remains available for interrogation for any future health questions; (iii) to conduct functional studies on rare variants in the patient’s WGS that bioinformatic studies or published literature suggest may be relevant to the risk of a disease; and (iv) to produce updated WGS interpretations from time to time, as knowledge develops year by year. This approach clearly involves a much more active, open-ended and costly commitment on the part of the laboratory team performing the genomic analysis.

Comparison between these two approaches makes clear some of the ethical problems that are beginning to declare themselves in the clinical practice of this new world of genomic medicine. They can be summarized in the set of questions below. As yet we have no clear consensus on how to handle these difficulties, although the ACMG has given helpful guidance on the parallel difficulties from array CGH studies (20).

1. Should care be taken to avoid generating any additional information beyond that required to answer the specific clinical questions being asked?
2. Is there an obligation to interpret all sequence information generated or only what is thought to be relevant to the clinical question asked? Would this obligation lie with the diagnostic laboratory or with the clinician requesting the test?
3. When information is generated about a child’s genome sequence, should there be additional controls on access to the data and its interpretation?
4. Should sequence information generated in a diagnostic laboratory be stored for the long term, released to the patient/client, or destroyed once it has been reported?
5. When a laboratory stores sequence information, should it undertake and report periodic reanalyses as the accuracy of sequence interpretation improves?



6. When a laboratory generates and stores sequence information, should this be managed actively to prevent its degradation?
7. In addition to an individual's medical team, who, if anyone, should be able to access their stored sequence data?

The second question is what to say to the patient or at risk individual. It will often be impossible to say nothing, especially if further family studies—samples from other relatives—may be helpful in resolving the uncertainties. There is already some experience of these issues in practice (27–30) but the scale of the difficulty is altogether new. It will be necessary for the diagnostic laboratory to make decisions as to what information, which variants, to pass on to the patient's clinical team, and then the clinical team will need to decide which of these to work on actively (e.g. sampling other relatives), which to declare as being of uncertain significance but with the possibility that their interpretation may become possible, and which to file away without mention. This will be an important area to watch as practices evolve.

The use of genomic information to guide clinical decisions about treatment is well established in oncology and is likely to increase and broaden in scope. Such practices raise social and ethical issues somewhat different from traditional diagnostic practices because the wider family implications may not be fully appreciated (31) and the imperative to provide the best treatment can mean that the decision process has to be rapid and unwelcome prognostic information about the individual may be generated, as well as difficult issues to discuss with relatives (32,33).

### 30.7 PREDICTIVE GENETIC TESTING

Individuals at risk of developing a serious inherited disorder will sometimes wish to find out if they have inherited the relevant gene. For the autosomal dominant neurodegenerative disorders, such as HD and some of the rare familial forms of early onset Alzheimer disease, highly accurate predictive testing is possible. Uncertainty as to whether the individual will develop the disease is replaced by uncertainty as to when (and how) it will manifest. In the context of the autosomal dominant familial cancer disorders, testing may also be possible, although there are limitations to the predictive power of these tests, because some individuals with their family's gene mutation, predisposing, for example, to breast or colon cancer, will escape the relevant cancer, whereas others, without the relevant mutation, may nevertheless develop the cancer. Inheritance of the predisposing mutation increases the statistical risk of developing the cancer from the population risk (of more than zero) to a high risk (but still less than 100%). In *BRCA1*-associated familial breast cancer, the increase in (lifetime) risk may be from approximately 10% up to 70–80%.

The ethical issues that can arise in the course of predictive genetic testing are numerous, and many are considered elsewhere in this chapter. Here, we will discuss just two particular topics. First, it is instructive to consider what has been learned from the numerous studies performed on the process of genetic counseling and predictive testing. Clients seeking predictive genetic testing and recruited to research studies have been asked to complete psychometric and open-ended questionnaires; in some studies, they have also been interviewed by a researcher. In general, it has been found that the process of genetic testing for those at risk of a familial disorder has been well tolerated, even when unfavorable results have been generated (34,35). Surprising, originally perhaps counterintuitive, findings have also emerged, however, such as the distress and social dislocation sometimes found in those given a favorable predictive test result. A favorable result can lead to feelings of guilt in young adults with siblings who are already affected or who are shown to be at high risk of disease—a form of survivor guilt. Family ties, perhaps strengthened from sharing the impact of the disease, may weaken for those no longer directly involved with the condition (36–38). Those who have made important life decisions, perhaps about career, relationships, or reproduction, may wish they had acted differently when they find that the basis on which the decisions were made—their genetic risk—has been removed.

It is usually recommended that those going through the process of “counseling plus testing” are supported by a companion, who is often their partner and who is, usually, not himself or herself also at risk of the disease in question. It has become clear that such support persons may themselves benefit from support and from at least some exploration of their thoughts and feelings in the pretest counseling sessions. If the person at risk is destined to develop HD, there may be serious implications for the future life of their partner too.

Another finding has been the inadequacy of the understanding of predictive genetic testing to emerge from psychometric assessments of its impact. Any simple interpretation of anxiety as a negative, unhelpful, or undesirable response to testing or test results has been challenged by qualitative evidence that such measures of anxiety may reflect an active process of confronting and engaging with potentially unwelcome facts (39). To use evidence of increased anxiety after testing to make a policy decision not to provide such testing services would be completely inappropriate. Furthermore, some of those in whom the measures of anxiety are increased before receiving the test result may subsequently be in less need of emotional support than others in whom the pretest measures of anxiety had been lower.

The second issue to consider is the family dimension of predictive tests, sometimes amounting to a conflict of interest between family members. The genetic testing of individuals at 25% prior risk of an autosomal dominant

disease provides the starkest examples of such conflicts of interest. What if a young man, whose grandparent has HD, wants to know if he will develop the disease too? He wants to marry and start a family but wishes to clarify his genetic status first. His at risk parent appears healthy but has made it clear to all concerned that she or he does not want to undergo testing. If the young man is found to carry the HD mutation, then his parent must carry the mutation, too. There are ethical issues here for the young man, his at risk parent, and the genetics professional. In most families, a resolution can be found to the potential conflict if the professionals encourage discussion within the family and are available to discuss the issues with the various parties—a solution through facilitating communication. Occasionally, however, real conflict persists and professionals have to decide whether to make testing available to one family member if doing so may have adverse consequences for others. The process of resolving such family disagreements can be particularly difficult when a couple unsure of their genetic situation have a pregnancy and wish to make a decision about prenatal diagnosis—the need for haste in making decisions then adds to the difficulties.

Professionals also face the difficulty, in counseling for predictive tests, as to how to challenge those going into tests with the possible outcomes. Our experience is that it is helpful to anticipate problems by thinking through various possible scenarios, but not everyone wishes to engage in this process. How hard should we try to encourage them (40,41)?

Additional difficulties can arise in families with diseases that show marked anticipation, such as myotonic dystrophy. In this case, a diagnostic test (of an infant with possible congenital myotonic dystrophy) may effectively be a predictive test when considered from the perspective of the apparently healthy mother who has not yet recognized her minor symptoms (42). This makes the counseling issues especially difficult if the child is to be given the appropriate diagnosis while the mother—and her parents—are led gently to consider testing for themselves. Similarly, complex, transgenerational issues arise in fragile X syndrome families, where testing of the affected child's maternal grandparents is a form both of carrier testing and, for the possibly premutation-carrying grandfather, of predictive testing for late-onset disease.

## 30.8 CONFIDENTIALITY

Respect for confidentiality is one of the oldest principles of medical ethics—at least as old as Hippocrates and certainly more ancient than the contemporary attachment to autonomy. My own view is that confidentiality must continue to be respected in medical ethics at (almost) all costs. In relation to genetics, however, it is subject to challenge on several grounds. First, genetic information might be sought about individuals by their employers or insurance companies or by the state. Second, the fact that we share

(almost) all our genes with other members of our families has been used to justify the claim that genetic information about one individual “really” belongs to his or her relatives, too, so that respect for confidentiality (genetic privacy) need not apply so strongly within families. However, that argument may be said to beg the question.

### 30.8.1 Third Parties

Protecting the individual's right to genetic privacy from third parties, such as employers, insurers, and the state, has been robustly defended. Although insurance corporations have generally accepted that they should not require genetic testing before making life insurance available to applicants, they have been less ready to concede that prospective clients should be able to have genetic tests performed without passing on that information to the insurance company, as with any other medical information. The counter-argument runs that genetic information is not like other medical information because if the individual is currently in good health genetic information about propensity to disease is essentially predictive or risk modifying and not at all like information about current clinical problems. The danger for the insurance companies is that of “adverse selection” in which their clients will be better informed than they are, and so those at high risk of serious problems may take out more insurance while paying only standard premiums, so that the company, and ultimately its other clients, lose out, and higher premiums have to be charged.

When applying for health or life insurance, most individuals at high risk of serious genetic disease will in any case have to declare their family history and so the insurance corporation will be aware of some of their pretest risks. If they have a favorable predictive test result, they may inform the company, but otherwise the individual seeking insurance may prefer not to disclose the result. The number of adults at high risk of a serious genetic disorder but who are in good health when seeking life insurance is small, however, and the number who are likely to die from the disorder during the term of insurance is smaller, so that the industry is unlikely to suffer much from adverse selection in this context. The potential for adverse selection is probably greater for health insurance, especially in the context of a predominantly commercial health care sector as in the United States, although there is no evidence that such adverse selection has occurred to any great extent.

This problem can be used as a powerful argument for the organization of health services by the state, whether as a single scheme or a few regional schemes (as in the United Kingdom), or through a largely private, but closely regulated, insurance scheme (as in many European countries and Canada).

Employers may wish to gain access to genetic information about their employees, or potential employees, for several reasons. Genetic information might be used

to alter the selection of job applicants or the promotion prospects of staff in post, in favor of those less likely to develop serious disease before retirement. The scope for genetic screening of the workforce for susceptibility to disease is small because DNA-based screening tests for susceptibility to the common, complex diseases are insufficiently sensitive for this to make commercial sense, but that situation may change, especially if ways are found to integrate genetic results with clinical findings on examination, biochemical measurements or imaging. Screening the workforce for susceptibility to specific occupational disorders is also not feasible at present, although a case has been made for screening workers for  $\alpha_1$ -antitrypsin deficiency (A1ATD) in industries where workers are exposed to smoke or dust, as in quarries, mines, building sites, because this condition predisposes individuals to severe, chronic chest disease after such exposure. An argument for caution in genetic testing of a workforce is that the exclusion of susceptible workers from hazardous sites could be used as a cheap but inappropriate substitute for the proper environmental protection that would in fact benefit the whole workforce and the local environment; perhaps both approaches could be adopted.

Those at risk of HD, or in the early stages, report discrimination in the context of insurance much more commonly than in relation to employment (43). Several countries in Europe and North America have developed national agreements to restrict the gathering and application of genetic information by insurance companies or have introduced legislation to prevent discrimination against those affected by, or at risk of, genetic disease in insurance or employment. Predictive assessments of life insurance applicants on the basis of lifestyle information becomes in practice a moral judgment; genetic medicine fits into the same process but the question of blame is more difficult to justify as the individual has no control over their inheritance; this area will repay further detailed work (44). Without proper legal protection, there is the fear that individuals at above average risk of specific disorders may become unable to find insurance cover, and in some jurisdictions this will make it much harder for them also to find employment if health insurance is provided by the employer. This could lead to the formation of a class of people identified by molecular genetic testing who would be excluded from full participation in society—an underclass reminiscent of George Orwell's 1984 and of real societies that practice slavery.

### 30.8.2 Privacy within the Family

Although conventional medical ethics has insisted on respecting the confidentiality of patients except in the most extreme and exceptional cases, this view has been challenged in the context of genetic disease. Information about one member of a family can be regarded as information about their relatives, too (45,46). Harm could result, it is argued, if genetic information is not shared

with relatives—an individual at risk of a disorder may develop complications that could have been avoided if they had been given foreknowledge of the risk, or a child may be born with a serious disorder when the pregnancy could have been terminated if the parents had been warned of this possibility (47). To what extent these “harms” could be avoided, and whether the birth of a child with a genetic condition can be regarded as a “harm,” are complex issues that require further analysis, but most of us can at least agree that family members have a moral obligation to pass on potentially relevant genetic information to their relatives (48,49), even if we might dispute the legal force of this obligation or whether the obligation extends also to professionals, and we might contest the notion of genetic harm implicit in this discourse when it leads toward the notion of a “wrongful life.”

The topic of debate then shifts to professionals and how they can best promote the appropriate transmission of information within family networks, accepting that it may be difficult to contact some family members, that others will be distressed by genetic information that may well turn out not to be relevant to them, and that the duty to contact relatives can prove burdensome to our clients. There may be no easy solution to the problems arising from the duty to transmit information. Perhaps a client has just found out about a sex-linked disorder present in the family; his niece is to be married next Saturday afternoon and will then be going on honeymoon for 3 weeks: what should our client do (i.e. when should he talk with her about the genetic risk)? Perhaps another client has just been told by his physician that his kidney complaint is inherited as an autosomal dominant trait and his four (adult) children are at risk—but Sally has just become engaged, Manfred is sitting his university final examinations in 2 months, Mark's wife is 6 months pregnant, and Maureen has just been admitted to hospital with her second bout of severe depression in 2 years. Whom should he tell about the polycystic kidney disease? Or our client may suffer from male infertility but find it difficult to discuss this with his wider family, even though they may be carriers of the CF mutations identified in him, or could even themselves be (mildly) affected. There may be no “right” time to pass on such information but it usually should be transmitted at some point in the not-too-distant future.

One approach is to view these problems from a family systems perspective and for the genetic counselor to work on managing the situation on behalf of the whole family but through the individuals with whom he or she already has contact. This counseling-based approach is usually successful, in that the blocks to the flow of important information usually dissolve over time and with sympathetic discussion and support from the genetic counselor. But what should the professional do if an individual persists in refusing to transmit the information or breaks off contact with the genetics team?

There are those who argue that the professionals should then be prepared to pass on information about one family member to others without their consent, although always informing them of this decision before implementing it. The circumstances in which such a breach of confidence would be justified have been considered by several learned bodies, although there may still be no consensus about what to do in specific cases or even about how to resolve any differences (50,51).

This could lead us to discuss in detail the various ethical frameworks within which such issues could be addressed but here we have space merely to outline four approaches to ethical deliberation. One approach would be to reflect on and discuss the competing principles at stake, with the principle of autonomy usually trumping the others in our contemporary, individualistic, Western societies. Another approach that is also founded on a positivistic individualism is that of utilitarianism, attempting to weigh, in a notional calculus, the likely outcomes of particular courses of action, and perhaps considering the concrete particulars of the case in its analysis as a bioethical case discussion (for a critical view, see (52)). A third approach is that of “common morality” (53), respecting rationality and impartiality in decision making within an open, public framework of moral rules. The final approach is that of narrative ethics, in which the professionals do not seek to abstract the issues of principle from the particulars of the case, the context, but to focus on the experience of their clients and encourage them to reflect on the future paths they can envisage taking (54). This approach can draw together a professional ethic derived from MacIntyre’s virtue-based morality (55) with the professional skills of humanistic counseling, making use of narrative approaches to promote the personal growth, development, and healing of their clients. It is hoped that this may thereby resolve (or dissolve) many of the ethical difficulties that could otherwise persist and become problematic if a confrontational approach is adopted.

Studies have examined how professionals say they respond in practice to situations in which a client fails to disclose important information to his relatives, once the professional has recognized the problem. It seems that professionals only rarely become seriously concerned and hardly ever in practice breach confidentiality (56). While the obligation to inform relatives carries weight (57,58), empirical research has demonstrated the multiple barriers that operate to hinder family communication and the factors that promote it (59,60). One approach to such problems, and which can be adopted when there are clear health benefits at stake so that the maximization of information flow through families is a worthy goal, is a very proactive professional orientation to passing on information to relatives. This encourages, supports and normalizes the contacting of individuals directly by a health screening program, as in some programs of cascade testing for familial hypercholesterolemia (61).

Some professionals fear that the development of a formalized framework for breaching confidences will inevitably make such practices a mere routine, even an expected or obligatory component of the professional activity of genetic counseling. Although it would be inappropriate to announce that there are no circumstances in which a professional could legitimately breach confidences, many problems would be generated by any regular, systematic policy of transmitting confidential genetic information about one individual to their relatives without consent. Professionals could become less inclined to persist with alternative approaches to resolving these communication problems. Clients, aware that confidentiality could be jeopardized by their counselor or clinician, could tend to be less than frank about their disease or their family structure from their first contact with the clinical genetics team. These potential consequences of altering the rules of professional conduct would be difficult to demonstrate in a quantifiable fashion but would be nonetheless real for that.

### 30.9 GENETIC TESTING IN CHILDHOOD

When is it appropriate to carry out genetic tests on children? There are few issues on which there is so much disagreement among health professionals or between professionals and families and so much variation in practice.

There are circumstances in which all agree that genetic testing is entirely appropriate. If a child has a clinical disorder that requires a diagnosis and which may be genetic in origin, then genetic testing may be the most appropriate investigation. For example, a young boy with delayed motor and speech development and an elevation of the serum creatine kinase may have DMD. A molecular genetic investigation, searching for deletions and duplications within the DMD gene or sequencing for point mutations, may well yield a diagnosis and thereby perhaps avoid the need for the invasive procedure of muscle biopsy. More complicated to consider are the “diagnostic” tests whose results may be difficult to interpret, as with array CGH tests for CNVs and whole exome or whole genome sequencing (see earlier, Diagnostic Genetic Testing).

Predictive testing for a disorder with onset usually in childhood is another context in which childhood testing can be appropriate. Thus, in the aforementioned family, if the boy does have DMD, it may be appropriate to test his younger, apparently healthy, brother. The family is likely to be highly anxious about him—will he develop the same disease—and it may take months or even years before either the diagnosis becomes apparent or the family is reassured. Some might prefer to wait and watch but many families wish to know one way or the other. There may be contexts, however, where parents may find an unfavorable result especially difficult to live with, as when a test indicates that the child has an increased risk of psychosis (62) or sudden death (63).



Another context in which a child might very reasonably have predictive testing for a genetic disorder, even where the condition will often not manifest until adult life, is where the child is at risk of complications for which surveillance can usefully begin in childhood, as in familial adenomatous polyposis coli.

Predictive testing for disorders that usually have an adult onset and that are untreatable is another matter altogether. To test a child, who is too young to participate in the decision, prevents them from making their own decision later. This may be especially important where, as in HD, most at risk adults choose not to be tested, preferring to live in a state of uncertainty. To carry out a test to resolve the anxieties of the parents would then be quite inappropriate. As well as abrogating the child's future autonomy, it also breaches the principle of respect for confidentiality—if tested as an adult, a person has complete control over who else is told about the test result. In addition, there is the possibility that a child's upbringing could be damaged by the result of the genetic testing. Might altered parental expectations of the child's future career prospects, educational attainments or personal relationships be damaging and perhaps self-fulfilling? There is no firm evidence on this point, and it would be difficult to gather "evidence" that would resolve this—what type of evidence would in fact settle this question? So we have strong grounds for caution without the prospect of readily establishing "the truth." Fortunately, most professional bodies and lay disease support groups share this perspective and would not be prepared to carry out predictive tests for HD or similar disorders on healthy children, at least in northwestern Europe and North America. Professionals in other (especially Mediterranean and non-Western) cultures do not all share this perspective, however, and some would be willing to perform predictive tests for HD on even very young children (64). The role of the family in less individualistic societies certainly creates a different context to which we should not transfer our culture-bound judgments without great care, although that does not necessarily mean that our value systems have to be discarded in the face of a traditional medical paternalism just because it is based in a different culture.

Considering the impact of both a disease and information about the risk of disease on the family as a system is helpful in discussing the genetic testing of children (65,66). One major influence on children's responses to a risk of inherited disease is the manner in which they learn about the condition, and whether this happens gradually in childhood or abruptly in adult life (67–71). It is important for families and professionals to consider their decisions over time and not to see the making of a decision as a single event (72).

Carrier testing in childhood is the most hotly contested issue in relation to the genetic testing of children, and both professionals and families differ widely in their approaches (73). Parents of children with autosomal

recessive disorders may wish to know which of their healthy (unaffected) children are genetic carriers. In the context of chromosomal rearrangements, such as balanced translocations, it used to be standard practice to test a whole sibship when the family presented clinically, and a similar approach has been adopted by some pediatricians for Mendelian disorders, notably CF, as molecular testing has become possible. Given that the young children tested for chromosome translocations in the past have not suffered obvious harm, is there any reason why professionals should not comply with parental requests to test young children to determine their carrier status?

One response is to point out that the children themselves will not benefit from the testing until adulthood; the results are not relevant to them until they come to make their own relationship and reproductive decisions in the future. Being identified as a carrier can have effects on an individual's sense of well-being, and we do not know how this could affect a child's developing sense of self. Carrier testing presents the same challenge to the ethical principles of autonomy and confidentiality as does predictive testing, although there will usually be rather less at stake because the child's own health is not in question, but there is still the concern that parental expectations could exert an undue influence on future relationships and reproduction. That may apply particularly in the context of sex-linked disorders or chromosome rearrangements, where the child's own future children may be affected irrespective of the genetic constitution of their partner. There are, therefore, considerations that weigh in favor of deferring a test until the child can participate in the decision (74). Furthermore, performing a test in childhood does not ensure that the results will ever be passed to the child in a comprehensible and relevant fashion in the future, whether the child does or does not turn out to carry the condition. It is the experience of several studies that genetic carrier test results are not always transmitted to the children tested even by the time they are adolescents or young adults, and there are often misunderstandings about the inheritance of the disorder in the family and about the meaning of the test results (75,76). Carrier testing of the young child, then, is not a complete solution to family concerns, and the offer of genetic counseling will often be helpful to the child when older even when the test has been carried out years before.

Discussing these issues with parents who are seeking to have carrier testing for a young child will often lead to an agreement that the family will talk with the child about the genetic issues in the family as he or she grows up. An arrangement can be made with the family either to keep in very occasional contact or to defer future contact until the children decide to seek information and testing for themselves.

It cannot be emphasized too strongly that not testing for a condition is not the same as not talking about it! If

the family has very strong feelings about testing, wanting the test to go ahead when a child is too young to contribute to the discussion, the counselor can prompt a careful discussion of the issues, including the parents' strong feelings, and an exploration of their expectations from the test, but they need not completely block testing if the parents are heavily focused on this. Although it may be appropriate to defer testing to allow some further reflection, a complete refusal to perform carrier tests in young children can lead to anger and resentment that may be very unhelpful in the future. It may be better to avoid rigid positions in such a sensitive area, to advocate caution but then leave the final decision with the parents.

An approach that can be presented to families as often being the best way forward through the tangled issues and feelings may be the encouragement of discussion and openness within a family but the deferral of testing so as deliberately to leave that decision to the child when he or she is older, in adolescence or later. Several studies have suggested that openness about the family's inherited disorder is helpful to the children's future adjustment (68–71). While an imposed solution may result in rejection and defiance, this may help these adolescents and young adults to arrive at reasonable and responsible decisions with which they can live more happily in the future (77–79).

These issues and guiding principles can work out rather differently in the setting of a child being considered for adoption (80). On the one hand, it can be seen as important not to subject a child to genetic testing that would not usually take place if the child had been able to remain in his/her birth family. The idea of testing a child so that he/she can be adopted if the result is favorable and otherwise left in the care system seems repugnant; surely the best adoptive family would be one that is willing to adopt while being aware of the genetic issues and willing to face the possibility that the child may have inherited a genetic problem of some sort. On the other hand, families adopting a child already face numerous uncertainties and the possibility of resolving one of these uncertainties may seem very helpful. In practice, it is usually possible to defer testing and for a serious potential adopting family to meet with the clinical genetics team for discussion. This can be more helpful than simply having a test result and will often lead to their willingness to defer testing until the "usual time."

The final topic to be considered in this section is the handling of requests from a child or adolescent for a predictive or carrier test. The question is whether the legal minor is sufficiently mature to understand the considerations involved in making a decision about testing. Gaining sufficient experience of life to arrive at a wise decision must take time, but it can be argued that living through particular experiences that have given the child an intimate knowledge of the family's genetic disease accelerates this process. Living in a family touched by genetic disease may give a child both the wish to find out

their carrier status and the capacity to make decisions about carrier testing. What might be inappropriate for a minor of the same age in the general population may be very reasonable within a particular family context (81). There are parallels between children being involved in decisions about genetic testing and the involvement of children with short stature in decisions about leg-lengthening surgery (82). It is important to recognize that a child can be involved in a decision without necessarily making the decision alone; children can be brought into the process of making a decision, making a contribution from an earlier stage than has often been appreciated, but this is not an all-or-nothing involvement.

The question as to when children can make their own decisions about genetic testing without parental involvement or even knowledge is separate. An assessment approach has been found helpful in the context of requests from adolescents for predictive testing for HD (83); a similarly careful approach can also be important in counseling young adults as maturity is not attained overnight (84).

### 30.10 POPULATION GENETIC SCREENING

The important differences between clinical genetic services made available to individual families and those provided as screening programs to a whole population have been outlined. There are three ethical aspects to genetic screening programs that need to be considered further here.

First, there is the issue of consent, or, as it is usually known, informed consent. It is always important to ensure that participation in a population screening program is conditional upon consent, but it may be difficult to define "informed consent" so that it can be readily operationalized (i.e. applied in practice in such a way that it can be readily assessed in any evaluation of the screening program: was this particular act of participation in a screening program enacted on the basis of consent, or not? Yes or No?). As a minimum, the potential participant needs to recognize that they have been given a choice and to understand what the choice is about. There may be potential disadvantages to participation as well as potential advantages, and this would have to be recognized. Probably the most serious limitations to informed consent for genetic screening arise from the routinization of the offer of testing—its incorporation into standard clinical practice, perhaps with the subtle discouragement of questioning, and the active promotion of testing by committed and enthusiastic staff.

The second issue to be considered here is the decision-making process whereby any particular screening program is made available to, or provided for, a population. In a national health care system, this will be decided by the health service, at regional or national level. Under a private insurance scheme, such a decision could be

imposed by legislation or determined by commercial factors, the “free” market. What is important from the perspective of ethics is how the decision is made: what evidence is taken into account, being regarded as relevant to the decision? Which interest groups have a say in the decision? On what grounds is the decision made and justified? Are these judgments defensible in open, public debate? This perspective amounts to a procedural ethics, appropriate to the democratic bureaucracies of the early twenty-first century. From this perspective, it becomes especially important to examine the processes by which such decisions are made in practice, as has been done in the case of two genetic screening and testing programs (85,86).

Finally, there is the distinction between maximal and optimal rates of uptake of a screening test. There will often be tension between the conventional goal of maximizing uptake for a screening test that is considered to be “a good thing” and the goal of good clinical practice. A high uptake may be seen as necessary to achieve maximal efficiency both in the clinic and the laboratory, but a very high uptake may be regarded as presumptive evidence of routinization and hence a poor quality of consent. There is certainly no point in using scarce resources to provide a screening test that is not wanted by most members of the public, nor is it adequate to argue that a screening program is worthwhile because it achieves a high rate of uptake. In the context of screening for the health benefit of the individual, it may be assumed that uptake has to be maximized, at least until the marginal cost of promoting further uptake becomes too great, but, especially in the context of reproductive decisions, it may be more helpful to recognize that screening will be valued by some individuals and not by others. It then becomes more appropriate to optimize the uptake of screening rather than maximizing it. This involves making the offer of screening in such a way that those who are likely to find it helpful to participate in screening do so and those who would not find it helpful do not participate. This topic is explored further later.

### 30.11 NEWBORN SCREENING

The first population screening program to be introduced for genetic disease was the newborn screening program for PKU, which transformed the prognosis for affected infants. There is no doubt that this, and the subsequent introduction of screening for congenital hypothyroidism, has been a great success. There is also good evidence that the identification of infants with sickle-cell disease (SCD), and their early prophylactic treatment with penicillin or other antibiotic, is to their benefit. What ethical issues arise in these flagship programs, the “acceptable face” of genetic screening?

First, there is the issue of whether newborn screening for these treatable disorders should be mandatory, as it is in some countries and some states of the United States, or

whether it should be voluntary, on the basis of assumed or explicit parental consent, as elsewhere. The uptake of testing is scarcely influenced in Western countries by legal compulsion—it is universally high—and parental consent must be the preferred route to implementation on all other grounds, so there seems to be little reason to justify compulsion. Operating on a basis of assumed parental consent does not have the explicit advantages of informed parental consent but may be acceptable in this context, where a degree of routinization is likely in practice. If “real” informed consent were preferred, then there would be no basis for the attempt by professionals to persuade reluctant parents to agree to testing for their infants: the parents’ views should be accepted. However, responsible professionals will try to persuade parents to permit screening to go ahead and will regard parental refusal as indicating that the parents have misunderstood the program. In different health care systems, where primary care might be delivered in a fragmented manner by multiple, poorly coordinated providers of variable skill and experience, the case for mandatory newborn screening for treatable disorders would be stronger.

The other issues around newborn screening relate to screening for disorders where the child is unlikely to benefit directly from any clinical intervention. There are reasoned arguments both in favor of and against newborn screening for DMD, for example, and it is not possible to resolve this issue “in principle”; that is, by reflection in the abstract. This is a good example of a situation where practical experience of both systems may be required so that a judgment can be made on the basis of how the two situations differ overall, whether, on balance, screening is worthwhile. For that reason, this is worth discussing at greater length.

There are two principal arguments for screening. First, many families with a boy affected by DMD have noticed a problem in the second or third year of life, but they are usually reassured by friends and professionals, and it may take several more years before a diagnosis is made. The parents in these families are often bitter about the diagnostic delay and the impact this has had on their treatment of the boy’s complaints. They often state that they would like to have known the diagnosis earlier. Second, in some of these families another affected child is born before the elder affected boy has been diagnosed and the family has two (or three) affected sons. If the diagnosis had been made earlier, then the parents could have been offered genetic counseling in advance of another pregnancy, perhaps thereby averting the birth of the second affected child. Although newborn screening would not have the goal of reducing the birth incidence of DMD, it could have a modest effect in this direction.

Arguments against the program include the distress caused to the family when their relationship with the boy is just beginning; it could spoil the first couple of years of the child’s life, and the family’s pleasure in him, without improving the outlook for his health and with

the possibility of damage to parent–child relationships. Practical experience with newborn screening for DMD in Wales, where the program has been running on the basis of informed parental consent for 10 years, has shown that most families are pleased to have the diagnosis early and do use this knowledge in practical planning for the future, including for their reproductive decisions. There has, inevitably, been distress at the diagnosis, but most families have been pleased to know about the condition from this early stage. Parental satisfaction with the diagnostic process, adhering to a protocol refined in the early months of the program, was greater than in a group of families where the diagnosis was made after a clinical presentation with symptoms (87).

Two important issues surround this program: the adequacy of the informed consent process at entry to screening and the adequacy of the support made available to families once an infant has been identified as affected but before clear symptoms, and standard clinical care, are appropriate. First, the uptake rate of >90% suggests an element of routinization, which could undermine the basis of informed consent and would be inappropriate in this disease context. Alterations to the process of testing, marking this test as different from the therapeutically helpful PKU test, reduce uptake to what may be a more appropriate figure of ~70%. This emphasizes the influence of structural and social factors on individual decision making about genetic tests (88). In addition, if infants are going to be diagnosed early then good information, early physiotherapy assessments and some practical and emotional support should be available in addition to genetic counseling services.

Newborn screening for other diseases is feasible, as with CF, SCD and A1ATD, and for many inborn errors of metabolism that can be detected through tandem mass spectrometry (TMS), including medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. Which of these tests should be made available? The case for introducing newborn screening for CF is strong in that active management of nutrition and infection can commence earlier after newborn screening, and it is plausible to suggest that this may improve the long-term prognosis, although this has not been proved. Screening for SCD is also of clear benefit to affected infants, so that a program is worthwhile where the disease incidence is more than minimal. Experience with screening for A1ATD is limited, but a program in Sweden was discontinued because it seemed to result in parental distress and increased parental smoking, a specific hazard for affected children.

Considering newborn screening for CF and SCD introduces another issue, that of detecting carriers incidentally but unavoidably through the methods used to detect affected infants. A small number of child carriers of CF are detected because a biochemical test is used to identify those with raised serum trypsin (IRT) levels;

molecular genetic testing performed on those samples then identified the common disease-associated *CFTR* alleles. Those with raised IRT and a single CF-causing allele then have a sweat test performed to see if they are affected or not. Overall, roughly equal numbers of affected infants and carriers are identified (as the program operates in Wales) (89). In contrast, population screening to identify infants affected by SCD identifies all carriers of SCD, not just a small proportion, because the laboratory test identifies all those with sickle hemoglobin (HbS), whether they are homozygous or heterozygous. This is useful in that those compound heterozygotes with HbS and  $\beta$ -thalassemia are recognized but it means that many more carriers than affected infants are detected. This makes more complicated the process of seeking parental consent and may greatly increase the work of community health professionals to inform and support these carriers and perhaps to refer them for genetic counseling. It can also raise questions about the child's paternity.

In relation to metabolic disorders that can be detected by TMS, an important question is whether consent should be obtained for testing each disease individually (with some parents choosing to have screening for some diseases but not for others), or whether consent should be given to “testing by TMS” as a single test, either to be performed or not (this is an instance of the question of “generic” consent, in contrast to specific consent). Because testing for PKU is now performed by TMS, this superficially attractive and simplifying approach to consent would fail to give any real choice about testing children for many of the metabolic disorders for which early diagnosis fails to confer any substantial benefit to the child, and which parents might wish to decline, while accepting the test for PKU. This approach would oblige professionals not to act on indications of a possible problem in some children; this would therefore be unpopular among practitioners.

An example to consider, MCAD is a disease where there can be not only real benefits from screening but also some difficulties. There is still some uncertainty about the natural history of the condition and about the likely balance between the improved prognosis for some of the young children, who are protected from hazardous episodes of metabolic decompensation, and the imposition of unhelpful medicalization on others. Some children who would have suffered no ill effects from the condition itself may be recognized and then admitted to hospital with every minor childhood illness, if their carers lack confidence in recognizing when the child is “sufficiently unwell” to warrant this precaution. Any process of evaluation of such screening programs must be broad based; they must examine not only the clinical medical outcomes but also the social impact on the families caught up in screening and the costs imposed on both the health services and the families involved. The ethical issue in this and some other disorders may



become a practical one—at what threshold of biochemical malfunction should a child be regarded as having a positive screening result—and experience is accumulating to guide practice.

Another important point to note is that clinical diseases must not be subjected to redefinition as cases of mutation in the relevant gene. Thus, in screening for CF, it is important to recall that the aim is to commence early treatment of infants who would otherwise be malnourished or have suffered serious pulmonary infections before the diagnosis was discovered; the goal is not to identify all cases of disease associated with mutations in *CFTR*, including late-onset respiratory problems or male infertility, where diagnosis in early infancy is most unlikely to be helpful. Similarly in MCAD deficiency, if screening is introduced, the goal must be to identify those destined to develop serious problems in early childhood, not all those with mild biochemical abnormalities but without an appreciable risk of severe metabolic decompensation, who would become “patients in waiting” (90). Those involved in making decisions about the implementation of such screening programs must take a broad view and not just reflect a coalition of vested interest groups: enthusiastic professionals, the manufacturers of the technology, and medical administrators interested primarily in containing health care costs.

There are currently discussions about the future shape of newborn screening in many countries. These often focus on the question of which disorders to screen for but, in the United States, there is active debate about broader issues that threaten public acceptance of screening, including the long-term retention of newborn blood spots and their potential forensic use by the state (91). In addition, there are discussions about the acceptability of mounting a newborn screening program to recruit infants to a therapeutic trial (e.g. for spinal muscular atrophy (92)), and there are grounds for doubting the overall wisdom of screening for fragile X syndrome despite some possible advantages (93). Can the advantages be sufficient to outweigh the distressing predictive information for premutation carriers of a risk of midlife neurodegeneration (Fragile X-associated Tremor and Ataxia Syndrome (FXTAS)) or premature menopause and the difficulties of consent and counseling for mildly affected young women?

## 30.12 ANTENATAL SCREENING

There are several issues around antenatal screening, in addition to those discussed earlier about nondirectiveness and screening programs in general. First, there is the question of the goals of antenatal screening. Then there are the unanticipated consequences of screening, especially the impact of antenatal screening on women and couples who become caught up in the clinical process but may not have really thought through the issues in advance. Finally, there is the impact of antenatal

screening on those individuals already affected by the conditions for which screening is made available.

### 30.12.1 Goals

Is the primary purpose of antenatal screening to reduce suffering, to provide choice, or to reduce the costs of health care? Perhaps the best way to address this question is to examine particular screening programs in detail and test out their rhetoric against their operation in practice. If we do that, what might we find? That is the type of empirical ethics research that is needed alongside cost-benefit analyses, which may drive decisions inappropriately if the only evidence drawn upon relates to the economic aspects of screening.

It is almost incontestably good to reduce “suffering,” but whose suffering do we mean? For the present, at least, prenatal screening is essentially targeted at Down syndrome and at structural malformations of the fetus. It utilizes maternal serum screening and fetal ultrasound scans to detect fetuses affected by (or at high risk of) structural malformations and chromosomal anomalies. Fetal anomaly ultrasound scans can reduce perinatal mortality, but most of this effect consists of bringing forward inevitable neonatal deaths so that they are no longer counted in either perinatal or neonatal mortality figures. Such reclassifications of inevitable deaths need not reflect any improvement in the clinical reality for the mother or family, because a midtrimester termination of pregnancy takes the place of a sad but inevitable neonatal death, which might have left the family with fewer regrets, less guilt, and greater emotional support.

Neural tube defects (NTDs) certainly can cause physical suffering in the child, although it may be difficult to argue that anencephaly causes the neonate more suffering than a termination of pregnancy without feticide, but there is no consensus among adults with NTD about the rights and wrongs of selective termination. Many take a “permissive” view, arguing that screening and termination should not be prohibited, but often maintaining that their lives are worthwhile despite their physical problems and that they must be acknowledged and respected as full members of society. There is a strong tension in this position, discussed further later, between the freedom of pregnant women to make their own reproductive decisions on the one hand and the respect due to our fellow human beings, whatever their physical or intellectual capacities, on the other.

Although Down syndrome can cause serious physical problems for affected individuals, it is the intellectual limitations and the stigmatizing physical features that are usually thought of as the major problems it leads to. So the suffering to be avoided through selective terminations of pregnancy is in large part a combination of the sadness likely to be experienced by the parents and the wider family and the social stigma experienced by the affected individual, along with the “courtesy” stigma of those

around him or her, rather than any direct physical suffering. The parents' feelings will be complicated, including sadness and disappointment at the child's physical frailty and health problems and at the limitations imposed on him or her by their genetic constitution, and irrational but often powerful feelings of guilt, of social stigmatization, and of worry about the child's future happiness in society outside the home once the parents are no longer able to care for the child. However, there will often be positive feelings, too. To claim that antenatal screening is motivated by a desire to reduce "suffering" is therefore a complex claim, difficult to justify in any simple and unqualified sense. This claim is made more difficult to accept in practice by the reluctance of many practitioners to accept a mother's decision not to terminate a pregnancy affected by Down syndrome or a similar condition, so that those who decline the offer of pregnancy termination may need real strength to resist the pressure they are placed under and to continue with the pregnancy (94,95).

The offer of informed reproductive choice is also given as a possible goal of prenatal screening (96), but this begs many questions. Given that the imposition of decisions about testing and (dis)continuing wanted pregnancies is burdensome, with its own social and emotional costs, what decision-making task is appropriate to impose on every pregnant woman (97)? Who makes this decision and on what grounds? It is not possible to sustain the view that a simple maximization of the number of decisions made is an end in itself. We must examine the context within which these decisions are made available.

### 30.12.2 Decisions in Social Context

If the termination of a pregnancy on the grounds of fetal Down syndrome is motivated by the desire to avoid suffering, it could be argued that there are alternative approaches to this problem that are often not proposed by public health physicians, health economists, or genetics professionals. These other approaches may have major advantages over the process of prenatal screening and the termination of wanted pregnancies, with the pain and suffering felt by the pregnant woman and the distress, guilt, and depression that so commonly ensue for her and her partner. Society could respond to the challenge of Down syndrome, and mental handicap or intellectual disability more generally, by ensuring that children with such problems are actively welcomed by society and integrated effectively into schooling and work, that parents are provided with a high standard of social support, and that older individuals with Down syndrome are offered well-supported, sheltered housing near the homes of their friends and families. The decisions made about prenatal screening and the termination of pregnancies might be different: (i) if parents were confident that, if their child were to have Down syndrome or a similar condition, neither they nor their child would

experience overt hostility or more subtle forms of stigmatization and (ii) if they could have a realistically optimistic view about the future for their child once they are themselves insolvent, infirm, or dead.

This idyllic state of affairs would require major changes in public attitudes and in the provision of health and social services in most societies, but without such developments any promotion of prenatal screening can be understood as (at least implicitly) coercive. Some families may feel that they have little real choice about prenatal screening because the support they can expect to receive from society for a baby with special problems and needs would simply be inadequate. Altering the social context in this broad way, at the "macro" level, would also be likely to improve "micro" aspects of social functioning, the subtle, intimate hostility that can all too frequently be displayed toward individuals with disability and their parents.

In contemporary society, the notion that "reproductive choice" is a "good thing," good enough to justify the systematic promotion of prenatal genetic screening, is therefore highly questionable. The emphasis on individual choice and autonomy that is so strong in contemporary medical ethics, including ethical discussions of prenatal screening, can be seen as a mirage (98). To consider ethical issues in the abstract, divorced from the social context of those confronting them, is to inhabit a fantasy (99). This is not to argue that individual choice is a "bad thing" or that individuals should be prohibited from using prenatal screening, but the active promotion of screening on the grounds of respecting individual choice is disingenuous if the social framework within which the choice is made available remains unexamined. Such a policy deflects attention away from the processes through which society arrives at decisions about health and social care. These decisions inevitably reflect the interests of different social groups, and to present them as neutral and based solely on objective considerations is actively misleading, making it harder for those with little power and few resources to contribute their views in an effective manner that will be "heard" and acknowledged.

### 30.12.3 Entry to Antenatal Screening

Consent for entry to prenatal screening is often not based on carefully considered and well-informed decision-making, as is known from many studies of the antenatal clinic process and its routinization (100–104). It is perhaps the fetal anomaly scan where "consent" is most problematic in that this is often approached as a happy social occasion rather than a serious search for fetal abnormality (105). Indeed, the way in which the test is offered is often routinized, and this leads to further problems for those women and couples in whom the screening test indicates a possible problem. If they have complied with screening in the expectation that the test will "make sure the baby is alright," such an ambiguous phrase, then

they can find it very difficult when a real or possible problem is identified. Such women can feel swept along on a diagnostic conveyor belt that often leads to amniocentesis and sometimes toward termination of pregnancy; it can be very difficult to slow this process sufficiently to permit reflection or stop the conveyor belt and get off.

Such difficult experiences would be minimized if it were made very clear to all potential screening participants:

- that the primary purpose of screening is to identify pregnancies in which the fetus has a problem, not to provide reassurance or photographs for the baby album or to act as an emotional glue to “promote bonding”;
- that participation is entirely voluntary;
- that professionals are likely to raise the question of terminating the pregnancy if the tests do indicate a problem;
- that the reassurance gained through a normal screening test result is always incomplete;
- that some test results may be of uncertain significance, being neither completely “normal” nor definitely “abnormal,” and these would require further investigation and/or consideration before any decision could be made, but even with further investigation or discussion, confidence in the interpretation of the test results may be impossible;
- that there are services available locally to support families who have a child with Down syndrome, spina bifida, or other conditions likely to be detected by the screening program.

Some pregnancies are terminated in haste when the couple learns about a problem detected on the fetal ultrasound scan or on fetal chromosome analysis, such as a sex chromosome anomaly. Then, when the parents learn more about the diagnosis, especially if the child might have enjoyed a very reasonable quality of life, they can bitterly regret their decision. Such problems are less likely to arise when women are informed about the possibility of “gray” results before deciding to go ahead with screening, but it is emotionally difficult and demanding for both staff and pregnant women to confront these issues in every pregnancy. The issues are therefore often evaded until there is a serious decision to be made in a hurry, and of course these are not the best circumstances in which to make such decisions.

The ethical challenges in this setting are faced by our clients, the society at large, and us professionals. They can be categorized thus:

- Under what circumstances, and with what goals and ethos, are we prepared to make prenatal screening and diagnosis available?
- Pregnant women have to decide whether or not to participate in the prenatal screening programs on offer. Some will wish to take any available measures to avoid having a child with problems, whereas others

will want to welcome their child unconditionally into this world. Yet others may wish to spare their child what they fear might be a life of misery and would prefer to take any suffering upon themselves, suffering the distress of terminating a wanted pregnancy so as to spare their child from physical suffering or from life in a hostile society. These considerations are frequently encountered in families having prenatal diagnosis for a condition they know well, and these circumstances are very different from those that arise in the course of population screening.

- Society has to decide how to support pregnant women and vulnerable children. Should it set out to welcome every infant unconditionally and generate confidence in the willingness of society to care lifelong for those with special needs? Or should it adopt a more hard-nosed, instrumentalist approach to its future citizenry by discouraging the birth of those likely to require more than the average from its health and social services?
- If the system of prenatal screening within which we work is unsatisfactory, to what extent does our compliance with it serve to bolster it and perpetuate these problems?

### 30.12.4 Making Decisions and the Outcomes of Antenatal Screening

Entry to antenatal screening may in practice often be routinized but it is to be hoped that any further decisions, such as whether to accept the offer of an amniocentesis, whether to terminate the pregnancy, will be made consciously and deliberately. Some accounts suggest that, once any screening test gives a positive result, some women feel pressure to take the next step as inevitability. Ethicists sometimes write as if pregnant women should weigh up dispassionately the considerations for or against an invasive test, for or against a termination. They may even propose that there is an ethically correct decision to be made and that they know what it is (106). Research examining the decision-making process suggests that life is more complicated, that women often consider the interests of the fetus alongside those of the family overall (107) and that they usually make decisions in small steps, as they are obliged to, rather than as a grand, strategic plan (108).

It is certainly of crucial ethical importance that our evaluations of prenatal screening programs develop more sophisticated ways of weighing the costs and benefits of screening. At present, it is all too easy for a family tragedy to be recorded, for the purposes of clinical audit or health services research, as the successful identification and termination of an abnormal fetus (e.g. with Turner syndrome). At present, most research and audit on prenatal screening avoids the active search for these sometimes disastrous outcomes, so the evidence base is systematically distorted. This is not merely an issue of

research methodology but a serious ethical issue about the framing of research questions that have received insufficient attention. In this era of “evidence-based medicine,” what types of evidence “count” as such? How are these decisions made?

### 30.12.5 Unanticipated Consequences

Quite apart from the rare miscarriages caused by invasive fetal diagnostic procedures, antenatal screening has other undesirable consequences. One of these is the altered experience of pregnancy known, in the phrase coined by Barbara Katz Rothman, as “the tentative pregnancy” (109); until the antenatal screening results are known, a woman may regard her pregnancy as not really established and not fully (unreservedly) welcomed. This is a different approach to pregnancy that contrasts with the unconditional welcoming of any child born (110). It is this decision, as to when in the pregnancy (or after birth) to welcome the fetus or infant unconditionally, that recognizes the fetus (or infant) as belonging to the human community.

### 30.12.6 Disability Issues, Cost-Benefit Analysis and Sex Selection

The persistent focus of research studies investigating population genetic screening programs on crude cost comparisons, the cost to society of either screening and terminating affected pregnancies or caring for affected individuals, causes great offense to many individuals with disability. It may horrify other citizens, too, but it is especially offensive to those of us who have the conditions that society seems determined to “prevent” on the grounds that affected individuals cost too much to look after. This really does amount to putting a price on a person’s head! Such cost-savings arguments, essentially, that it would be cheaper to stop your patients being born than to look after them, would be totally unacceptable in other medical contexts. Indeed, it is generally accepted that medical care costs money and that society should be prepared to pay for it. The simplistic, narrowly economic approach to prenatal screening reduces human value to a cash price and reflects a deeply amoral cynicism.

There are other reasons why terminations of pregnancy may be justified, but the widespread use of crude cost calculations to justify prenatal screening for Down syndrome suggests that society and the professions have, perhaps by default, decided to promote prenatal screening on those grounds. Such policies have helped to generate considerable hostility toward genetics within the disability movement. This is most unfortunate because genetics may have a lot to offer society, including those with disease and disabilities, and a rejection of genetics *tout court* could obstruct useful progress on several fronts. Such narrowly instrumentalist and simplistically utilitarian attitudes fail to consider the duties we

owe other humans, including our future selves when we become unwell or infirm, and fail to recognize the important virtue of human solidarity.

One obstacle to mutual understanding is that “genetics” covers many different activities. The term can be used to describe prenatal screening programs and their apparent contempt for the worth of people with disabilities. It can also be used to describe research into the causes of disability, which may result in a more sophisticated diagnostic taxonomy and give insights into the causation of progressive and sometimes fatal disorders that cause disabilities; such research may eventually lead to the improved treatment of affected individuals. Hostility to one aspect of genetics may carry over to other areas also labeled as “genetics.” Furthermore, while effective, rational, gene-based treatments, if not gene therapy as such, may be years from widespread application in many areas, even a distant prospect of such therapy generates conflicting emotions within affected individuals and their families. On the one hand there can be surges of hope that individuals already born will be helped by new, rationally designed treatments to lead better (healthier, more able, fuller) lives, while waves of resentment can also arise when anyone suggests that therapy would be wanted; this can be taken to imply a lack of respect for the daily achievements of affected individuals, who live their lives creatively and with enjoyment and who seek recognition as valued individuals, just as everyone strives to do. Neither the (bio)medical model of disability (“you have a problem with your body and we professionals are doing what we can to fix it”) nor the social model (“society is to blame for all the difficulties confronted by individuals with disability”) does full justice to the complex relationship among the elements here (111,112): the cause of a disability, its practical manifestations in the life of an affected person, the broader social impact of their disability, and the emotional consequences for the individual and those around them.

Those with disabilities may fear that genetics will be used by society to eradicate either their disabilities or the people themselves. Of course, the complete eradication of disabilities is never going to be possible because so many acquire disabilities for other (not genetic) reasons, but there may still be great resentment that anyone would wish to do so (113,114). It is likely that for many years it will remain technically much easier to eradicate people with inherited disabilities prenatally, sometimes, and rather inappropriately, regarded as a prevention of the disease, than to prevent the loss of these abilities in those with progressive disorders or to restore them to someone who has lost them or never had them. Suspicion of genetics will therefore persist but it is possible for the disabled and their health professionals to develop a dialog that slowly softens and removes these misunderstandings. This is an important activity from which both groups can learn; the professionals can learn more about the experiences and aspirations of the disabled,



and the disabled can learn that “genetics” is not a single, monolithic entity that aims to wipe them out but refers to a range of different activities, some of which will help at least to understand the causes of inherited disabilities and of progressive, disabling diseases, and may one day provide useful therapies.

One particular disabled community that deserves specific mention here is that of the deaf, especially the Deaf, those with prelingual deafness who use sign language. This group sees itself as a threatened cultural minority, and genetic research into deafness is perceived by some as a direct threat to their continued existence as a distinct minority culture (115). This group of the Deaf often does not see deafness as a problem, in contrast to those with later-onset hearing impairments. Is such prelingual deafness, then, a disease, a disability, a condition, an innocent trait, like red hair, or a marker of belonging to a specific cultural community? They can feel threatened by the twin assault of genetic technologies with the potential for prenatal diagnosis and the treatment of deafness by cochlear implants in infants diagnosed through newborn screening. The source of recruits to the social world of the Deaf may diminish and so the culture may indeed be threatened with extinction.

Mention must also be made of fetal sex selection. Many cultures have a preference for sons; this was true until recently of the ruling and the laboring classes of Western societies. The pressure to have sons has powerful socioeconomic roots and relates to cultural practices such as the dowry and the need for sons to fight, work in the fields, join the family business, or inherit property and names. In some societies, newborn daughters may be exposed or killed. Furthermore, women who have daughters are sometimes abused, assaulted, divorced, or murdered, so that the women can be as eager as their men folk to have a son. In this cultural setting, those who can afford prenatal diagnosis sometimes use it to ensure that a pregnancy does produce a son, especially if they already have a daughter (116). Sex selection, now largely prenatal and based on the social termination of pregnancies in which fetal ultrasound scanning suggests a female fetus, is so common in some areas that the sex ratio in parts of India and China has declined dramatically and is approaching 0.7 female infants per live born male. Indian immigrants to the United States utilize the prenatal sex selection that is legal there, whereas it is illegal in India (117).

Compliance with requests for fetal sex determination as a means to achieve a son can be regarded in the West as collusion with an offensive, patriarchal system that oppresses women and is hugely disrespectful to them. British medicine has decided not to permit prenatal diagnosis for such purposes; to do so would be seen as lending support to an unacceptable system of discrimination. This raises two important questions. First, if a woman seeks fetal sex selection out of fear that she will be assaulted or killed if she has a daughter, then is that

not at least as powerful a justification for prenatal diagnosis and pregnancy termination as many of the “social” terminations carried out every day? Second, if we Western professionals adopt this stance because we hold women in such high esteem, what message is conveyed by our collective willingness to promote prenatal screening for Down syndrome? If the disability movement had a higher profile, would we discontinue that type of prenatal selection, too?

### 30.12.7 The Future of Antenatal Screening

One important technical advance of the last few years is beginning to enter clinical practice. Its impact has so far been modest but it is likely to transform the practice of antenatal screening and prenatal diagnosis, and the associated ethical issues. This is the analysis of free fetal (or embryonic) DNA in the maternal blood, known as *ffDNA*. This is already in widespread use to look for paternally derived alleles of interest (such as the Y chromosome for fetal sexing and the Rhesus antigen for hemolytic disease) but can also be used for prenatal diagnosis for paternally derived autosomal dominant disorders for which the fetus is at risk. These methods allow noninvasive prenatal diagnosis (NIPD) from ~7 weeks of gestation, which will remove the risk of miscarriage from invasive techniques (amniocentesis or chorionic villus biopsy (CVB)), permit definitive diagnostic testing from much earlier in pregnancy than is possible now even with CVB, and shape women’s experience of pregnancies and the place of disability in developed societies (118).

The ability to offer prenatal diagnosis early will be welcomed and will be a great benefit in many circumstances. However, where women currently use the risk of miscarriage to justify their decision not to accept antenatal screening or prenatal diagnosis, they will become less able to resist persuasion or pressure, whether from professionals or family, to accept prenatal screening or, further along the conveyor belt, to terminate the pregnancy. The very advantages of NIPD contain this unlooked-for consequence.

The risk-free availability of fetal DNA from early in pregnancy allows the determination (i.e. the resequencing) of the fetal genome, if the free DNA in the maternal blood is sequenced to sufficient depth (119,120). Less technically complex than this, however, and likely to be realized much sooner in clinical practice and population screening, is the determination of relative dosage of fetal chromosomes allowing the detection of fetal trisomy for chromosomes 13, 18, 21 and the sex chromosomes (and fetal X chromosome monosomy) (121). This opens up the possibility of testing every pregnancy in developed countries for fetal trisomies, with a technology that will before long yield a definitive diagnosis, rather than the relative risk figures generated by current screening methods. This in turn raises some important questions. There is the question of consent for tests whose power is not yet fully

appreciated even by professionals (122); there are also important questions about the prenatal elimination of some major categories of chromosome anomaly, not only the autosomal but also the sex chromosome trisomies and Turner syndrome (123). This major change in the composition of society may be welcomed without reservation by some but will generate both disquiet and frank opposition from others. The advent of next generation sequencing (NGS) in diagnostic laboratories will have many positive uses; the application of NGS to antenatal screening will have important consequences but these have not yet been fully thought through and discussed.

Finally in this section, we should consider the prenatal determination of fetal genome structure (i.e. CNVs) or complete sequence. As in the section on Diagnostic Genetic Testing, we can see that this will present serious challenges for families and professionals. Where there remains a substantial degree of uncertainty about the meaning of WGS results, however, the lack of clarity will be worse because we will not have the phenotype of the child available for direct physical examination or developmental assessment, which simplifies the interpretation of WGS results on a live born child or an adult. Furthermore, there will be major concerns about access to the future child's genome sequence and its interpretation. Will the data and the interpretation be stored? Will the parents be given only the clear items of information about the specific questions being asked in the pregnancy, but not about other health issues, or will the "full" results (as far as that is an intelligible concept) be made available to the parents, even when they have not asked the relevant questions?

### 30.13 CARRIER SCREENING

Screening to identify unaffected, presumably healthy carriers of autosomal recessive diseases in the general population is another form of reproductive genetic screening, and it can be made available to a couple contemplating reproduction, or less ideally in pregnancy, when it may be represented as being "most relevant." Similar issues arise to those in the field of prenatal genetic screening because the simplest outcome measures are the numbers of individuals tested, the number of carriers and of carrier couples identified, the number of pregnancies tested, and the number of affected pregnancies terminated. To attempt simply to maximize any of these figures could lead health professionals to direct their patients into testing, so it is important that health services do not impose crude testing targets that could encourage unacceptably directive clinical practices.

There are potential benefits from being identified as a carrier, especially the ability to make reproductive decisions, such as asking a partner to be tested, but there are disadvantages, too. The negative consequences of being identified as a carrier include the emotional impact of this unwelcome information, the burden of future reproductive decisions, possible lingering concerns about one's

own health and the potential for stigmatization and discrimination in personal relationships. Granted that there will always be many more individuals identified as carriers than there will be carrier couples who could have an affected child, how does one weigh these minor burdens on many people with the more serious positive and negative effects on carrier couples, who will not only be given important personal information but will also have the burden of making decisions about prenatal testing, and perhaps termination, in their pregnancies?

Pilot carrier screening programs for a number of disorders have been set up over the past three decades, especially for CF, the hemoglobin disorders, and Tay-Sachs disease. Such pilot schemes have shown that the general public has little interest in carrier screening but will often comply if screening is actively offered by enthusiastic health professionals (124). Indeed, some studies have shown that the rate of uptake is critically dependent on the way in which testing is made available, varying from <10% to >70%, with higher rates if testing is offered actively and is available on the spot but much lower if a separate appointment is required. It would seem that the mode of offer of the test could be selected to generate whatever rate of uptake of the test was desired. In addition, if screening is made available to women or couples in early pregnancy, then the uptake is substantially higher again (>95%). How is this to be interpreted? Does it indicate a real interest in testing or compliance with an actively promoted test because of the vulnerability of pregnant women to suggestions that they should have a test "to make sure baby is alright?" One's answer to this question may reflect one's attitude to the suggestion that CF carrier screening should be introduced widely into the antenatal setting. If uncomfortable with the policy of the National Institutes of Health (NIH) Consensus Conference, one may well ask, *cui bono*? How was the consensus arrived at? What evidence was considered, and how was it weighed? What (vested) interests were represented or promoted in the decision-making process?

Further issues arise in antenatal carrier screening as to the precise offer to be made—testing just the pregnant woman, or the couple as individuals or the couple as a unit—because the nature of the offer made reflects the goals of the program and will have implications for how these are perceived. What about adding in extra conditions and obtaining generic consent to the whole process rather than to each individual test (125)? What about screening in pregnancy for female carriers of fragile X syndrome, with the problems outlined earlier (126)?

When testing is actively promoted by the dominant social institutions, as with screening for  $\beta$ -thalassaemia in Cyprus, promoted by the Orthodox Church, it can be very difficult for outsiders to distinguish popular consent from institutional coercion. Societies certainly differ in the degree to which individuals are willing to comply with decisions made by authority figures within the family or society at large.

The variation in frequency of autosomal recessive diseases between populations and ethnic groups means that the frequency of the carrier state varies widely. So a carrier screening program that could be appropriate in one population may be inappropriate in another. Problems may arise where racial disharmony and discrimination exist in a mixed population, with two (or more) ethnic groups. The promotion of genetic carrier screening could then be used by one ethnic group to stigmatize and discriminate against the other. This indeed occurred in the 1970s in the United States, when screening for sickle-cell trait (carrier state) was actively promoted and exacerbated discrimination against Afro-Americans in employment. So the social context within which carrier screening is made available can be a highly charged political issue; clinicians and scientists should beware.

The development of new sequencing technologies means that the earlier approach to the introduction of carrier screening, considering one disease at a time, is being superseded by genomic approaches in which carrier status for many recessive disorders is made available simultaneously (i.e. in parallel) as a single investigation (127,128). This is comparable to the introduction of TMS as a screening test for pediatric metabolic disease. In these settings, the process of making a policy decision about the introduction of the test and the information-and-consent process for those being offered the test are both very different when a single investigation gives information about many conditions. The generic nature of the consent is bound to alter the type of discussion that takes place between practitioner and the client or patient (126), although there has been little opportunity yet for experience with this to accumulate. Is it a problem that some may wish to be much more selective about the conditions for which they are screened, only wanting to be given some of the results generated? Or the comprehensive nature of carrier detection may make it seem more worthwhile as a population program, perhaps to be introduced into schools (129). This will be an area to watch in the future to ensure that testing does not become so routinized as to undermine the voluntariness of testing.

In less developed countries, the challenges may be somewhat different as older technologies will continue in use for some years. Once infant mortality rates have improved, so that a country passes through the demographic transition, inherited diseases and congenital malformations become much more apparent, contributing to a higher proportion of the country's mortality and morbidity in childhood. As has been appreciated in some Mediterranean and Middle Eastern countries, the survival of large numbers of children with diseases that can be treated successfully but at a substantial cost raises ethical challenges for a country's health services. As a cohort of survivors appears with  $\beta$ -thalassemia, for example, providing the best current therapy will lead to an ever-growing number of patients requiring

expensive care, in this case, either blood transfusion and iron chelation or bone marrow transplantation. Blood transfusion and iron chelation would then consume an ever-increasing proportion of the resources available for health care and could compromise a country's ability to provide services for many other conditions. Some decades ago, as discussed earlier, Cyprus confronted this by setting up a carrier screening program with the support of the Orthodox Church; this has led to a dramatic decline in the births of affected infants so that the country can afford to care for those individuals who are affected. Several countries in the Middle East (including Iran, Saudi Arabia and Qatar) have their own schemes that encourage carrier screening. Other countries are likely to adopt comparable policies. This raises the question of how active the promotion of carrier screening should be when "too enthusiastic" a program could become coercive, and whether the program should seek to influence the choice of partner (as with premarital screening in a country with arranged marriages) or to promote prenatal diagnosis and the selective termination of affected pregnancies. These are difficult issues that developed countries need not confront in the same way because of their greater ability to afford treatment for those affected. How will the use of carrier screening work out in the context of arranged marriages, in developing or developed countries (130)?

## 30.14 OTHER CHALLENGES IN GENETIC COUNSELING

Several other ethical issues arise in genetic counseling, to which we must refer before passing on to some broader topics.

### 30.14.1 Cross-Cultural Genetic Counseling

**30.14.1.1 Language.** The process of communication in genetic counseling is obviously made much more difficult if the counselor and client do not share a common language in which both can discuss the full range of technical topics and the intimate issues that so frequently also arise. The use of an interpreter may provide a solution but may also generate its own set of problems. If the interpreter is a member of the family, a young child, perhaps, or the male or female partner of the client, then it may be difficult for the client or the counselor to talk freely, and the counselor may lack confidence that the discussion is being reported fairly to the client. Issues of power between husband and wife may intrude, and the counselor may find it difficult to walk the tightrope between collusion with the more powerful partner and causing him (or her) offense, leading to the complete withdrawal of the family from genetic services.

**30.14.1.2 Religion or Culture?** A language barrier is only the most obvious obstacle to communication in genetic counseling. Other cultural issues may arise even

more frequently, although they may not always be recognized as such. Religious beliefs may be given as an explanation by the client to the counselor in accounting for their decision not to make use of genetic testing, especially in the context of prenatal diagnosis and a possible termination of pregnancy. Religious traditions, however, do not always give a clear ruling on the “correct” decision for an adherent to make; differences exist within many traditions on such matters, and it may be the cultural customs and assumptions surrounding a religion that influence client decisions and understandings more than the details of religious doctrine per se. Patients’ and clients’ appeals to “religion” may often be used as a signal by the patient/client that she or he is unwilling to pursue the topic under consideration, having deep-seated objections to the whole process although these objections need not necessarily be theological in nature. This signal is usually respected by professionals in a way that the mere expression of an opinion by the client may not.

**30.14.1.3 Decision Making.** Cross-cultural genetic counseling may also raise the question of different patterns of decision making in families. Who would make the decision about predictive genetic testing for a young adult at risk of HD, for example, or polyposis coli? Would it be the individual at risk, a patriarch or matriarch, or a wider group within the family? When a couple are discussing prenatal diagnosis in a pregnancy, who makes the decisions? (In the United Kingdom, it would usually be the couple making a joint decision but, in the face of a disagreement, it is always the woman who makes the decision). If parents request predictive or carrier testing for a young child, they may assume that they have the right to do that without any need to explain their reasoning to professionals. These “cultural” questions come down to issues of power. To what extent can we Western professionals use our power in the clinical setting to steer family decisions and communication practices, i.e. family power relations, along a path that we find acceptable?

**30.14.1.4 Access.** Access to clinical genetic services within multicultural societies may vary between different communities for a number of reasons, including not only patterns of language use and religion but also broader social and cultural factors. The place of an ethnic minority group in relation to the dominant society may have a crucial role here. Access to all health services may be restricted, and low uptake of genetic services may reflect this as opposed to any specific cultural factor making genetics unacceptable. Access of certain communities in Britain to health care may be restricted not only by the reluctance of women to be seen by male health professionals, or the reluctance of their men folk to permit this, but also by the same influences that restrict uptake of health services by members of the same social class drawn from the dominant (white) majority; these economic and class factors will be especially relevant when recently arrived immigrants are still in the process of establishing themselves economically.

It is important that health professionals do not seize upon either the social or the biological differences between ethnic groups and communities to explain differences in health-related behaviors or in the patterns of disease between the communities without giving very full consideration to other possible factors, such as the material circumstances experienced by the communities. For example, differences in perinatal mortality between groups may reflect differences in socioeconomic class as much as genetic differences or differences in social customs.

#### **30.14.1.5 Customary Consanguineous Marriage.**

Customary consanguineous marriage is especially prevalent in certain South Asian and Middle Eastern groups and may be more pronounced in Britain than is usual in their countries of origin because of the smaller number of potential partners in the relevant community in Britain. Although this does increase the risk of autosomal recessive disorders appearing in children born into these communities (131), it would be all too easy for Western health professionals (or politicians) to attribute blame and responsibility for the disorders to the minority culture and its representatives. Such provocative attributions of blame ignore the stabilizing social functions of consanguinity in many communities, this marriage pattern being widely practiced around the globe, and are not likely to promote any useful mutual understanding or change in customs. High-profile interventions by politicians and public health agencies calling for the communities to end their traditional approach to marriage are likely to antagonize rather than help. If not provoked by such moral assaults, communities that place social value on consanguineous marriages may be able to express concern because of a high perinatal mortality or the frequency of serious genetic problems in young children. It then becomes possible to develop an education program that would be acceptable to the community, and perhaps to incorporate genetic testing as part of an effort to counter the genetic risks of consanguinity (132). Perinatal mortality may also be tackled by attention to maternal nutrition and the provision of effective antenatal care, without an exclusive focus on genetic issues.

**30.14.1.6 Stereotyping.** The final problem of cross-cultural genetic counseling to be mentioned here is that of stereotyping. The counselor must not make assumptions about the likely response of an individual to a genetic problem in their family on the basis of how she or he thinks that a client from that community or ethnic group (or social class) would typically respond. Our inevitably patchy understandings of other cultures could all too easily lead us to imagine a spurious homogeneity within these other communities, especially in the case of disadvantaged ethnic minority groups. There is evidence that health professionals are less likely to offer some services to those from minority groups on the assumption that “they” would not want to consider genetic testing or a termination of pregnancy, falling into this trap of inappropriate



cultural stereotyping. One aspect of the stereotyping is the fatalism that is often expressed by the powerless when facing difficult and distressing circumstances; health professionals should be cautious about accepting it as a complete rejection of western medicine (133).

### 30.14.2 The Naming of Syndromes

Many families arrive in the genetic clinic with the hope that the condition affecting their child will be diagnosed. The process of labeling, however, can have substantial repercussions for the family, which they may not always have foreseen (134).

Where a diagnostic label is available, it may have unforeseen negative as well as positive consequences. Although it may facilitate access to support services, it may also provide an unwelcome insight into the likely future for their child that a family may not be prepared to confront immediately. There may also be problems when a diagnosis is not available, as happens quite frequently. The family may then feel bitter and resentful, especially if the lack of a diagnostic label makes it more difficult for them to access appropriate social, educational, or health services for their child. Where a diagnostic label can be applied, this too may have adverse consequences if educational provision for the child is altered inappropriately—if the child is treated as the diagnosis and not primarily as an individual. There is also much scope for the self-fulfilling prophecies of low expectations on the part of parent or teacher.

A very real problem for some individuals affected by genetic disorders and their families is the name given to their diagnosis, their syndrome. This applies particularly to the field of dysmorphology. It can be difficult enough for a family to accept that one (or more) of them has a disorder manifest in their physical appearance, without insult being added to injury by the offensive nature of the name given to their condition. Although some names are unfortunate but understandable, others are the expression of a brand of medical humor that should not be paraded before the families in our care; this should have no place in clinical practice (135). Given that families now routinely have access to the Internet, they can readily find out all the names given to “their” syndrome, so it is not possible for clinicians to use one name with families and another name for the same condition when talking with their colleagues.

The name “cri du chat” syndrome can be unfortunate when it causes a family to flinch whenever they hear their child’s cry, the cry having caused no concern before they were given the name of the condition. This name was coined in good faith as a clinically useful description and not as a thoughtless witticism, but it does have this unanticipated impact on families. This name, however, need not be used by professionals—5p minus syndrome is a useful alternative—although I suspect that the more evocative name will linger. There are many other syndrome

names, however, especially acronyms, which have surely been devised for their verbal felicity and humorous effect with complete disregard for the impact of the name on those affected and on their families. Examples include CATCH 22 syndrome, LEOPARD syndrome, DEFECT syndrome, and CRASH syndrome. The giving of a name to a syndrome can be as serious a matter as the giving of a diagnostic label to a child.

Another reason for taking great care in the naming of genetic conditions has been explored by Peter Harper in relation to the eponymous Hallervorden–Spatz disease (136). These two research neuropathologists made use of the brains of children with neurological and mental disorders who were exterminated in Nazi death camps as part of the German policy of race hygiene, and Hallervorden (at least) was closely and knowingly involved in selecting children to be murdered so as to provide material for the neuropathology. Harper and others have called for this condition to be given a different name and a preferred term is “pantothenate kinase-associated neurodegeneration (PKAN).”

A final topic to be mentioned under this heading is the process of giving a diagnostic label to a patient or family (137). This process can be very delicate, and information about the diagnosis, even the name itself, may need to be passed slowly to the family as they become ready to accept it. A full and abrupt disclosure of information about a distressing problem in a young child may be unhelpful, especially if the family has not yet recognized the severity of the problem affecting their child, and may block the process of confronting reality. On the other hand, the professional’s fear of triggering a strongly emotional reaction should not prevent them from delivering “bad news” when this is necessary. Professionals should keep their practice in this area under review in a supportive process of group or individual supervision.

### 30.14.3 A Duty to Recontact?

When a family attends genetic counseling, the clinician and counselor provide the best answers they can to the family’s questions. When new information comes to light in the future, especially if it involves treatments or the possibility of prenatal diagnosis, is it reasonable to expect the responsible clinician to recontact the family to pass on this information? If the family is still under review, then no special problem arises, but what if the family has not attended for some years, or follow-up was never planned, or they have changed address? How far does the obligation to recontact such families extend?

A strong duty to recontact could impose such burdens on individual clinicians and counselors, and on clinical genetic services more broadly, that clinical genetics practice could cease to be a viable specialty (138,139). There has to be a balance of responsibility, with the physician updating families with whom he or she has ongoing contact but families being obliged to keep the genetics service

informed of changes of address and to request occasional review consultations if they think that new information may become available that could be of interest to them.

#### 30.14.4 Tensions in Genetic Counseling

At several points in this chapter, I have expressed the notion that there are ethical tensions inherent in genetic counseling practice, such as that between the reproductive autonomy of pregnant women and the support and respect due to individuals with genetic disorders. Another example is that between the “right” of the adult at one in four risk of HD (the grandchild of an affected individual) to find out his genetic status as opposed to the right of his at risk parent not to have his or her genetic status determined. This approach, identifying points of tension where ethical principles may be said to conflict, has both rhetorical and heuristic value and can be most helpful. It has one major drawback, however, in that it suggests that neither pole of the tension is “correct” and that there will be a middle way to be reached by an appropriate compromise. This thought is itself an ethical danger to be avoided; it could lead to a complete ethical paralysis because any position to be advocated could be portrayed as being at one pole of a tension, and the desire for a “middle way” could undermine it. The contemporary, postmodern condition of human life already makes it difficult to sustain and justify any coherent process of ethical reasoning; the use of rhetorical maneuvers to demolish any conclusions emerging from an ethical discussion could easily amount to a form of nihilism.

#### 30.14.5 Pharmacogenetics, Commercialization and the Common, Complex Diseases

The promise of “the new genetics” is that an improved understanding of the genetic basis of the major diseases of Western society will give new insights into their pathogenesis; this, in turn, will lead to improved, indeed truly rational, therapies. From an understanding of the contribution of genetic variation to disease susceptibility, it will be possible, so goes the implicit promise, to calculate each individual’s personal profile of disease susceptibilities and hence define a suitable set of lifestyle measures, nutritional recommendations or preventive medications that will keep the individual in the best possible health. This goal sounds laudable but, as soon as it is examined closely, numerous difficulties and objections appear.

First, there are problems with gathering and analyzing the data that would be required to dissect the genetic basis of the common, complex diseases such as diabetes, hypertension, rheumatoid disease, osteoporosis, cerebrovascular disease, coronary artery disease, Alzheimer disease, and the common cancers. Working with genome-wide panels of SNPs in linkage studies, it has proved possible to define many of the gene loci important in a small subset

of those affected by such conditions, the 5% or so of cases where there is an effectively Mendelian predisposition to disease, but there has been much less progress through GWAS toward defining the risk-modifying genetic factors of lesser effect. For most complex conditions, only 10–20% of the genetic contribution to risk of disease has been assigned. Given the realities of human population history, the nonrandom association of specific sequences with each other and with disease may result from the operation of several different factors other than simple linear processes of disease causation. This has (predictably (140)) made it highly problematic to establish the causal nature of any associations that are identified, even if they have been replicated in independent studies. Not only are there problems of linkage disequilibrium but also the nonrandom mating of humans throughout their history and prehistory for both geographical and cultural reasons, the varied and fluctuating nature of selective processes, and the present rapid mixing of populations, conspire to make it immensely difficult to assess the influence of specific “genetic backgrounds.” The dissection of quantitative trait loci in the mouse has only been possible because of the imposed pattern of breeding of defined laboratory strains and the close control of environmental variables. The feasibility of either collecting sufficient human data or conducting an interpretable analysis capable of identifying disease-related polygenic (small effect) molecular variation is poor. While the problems of linkage disequilibrium, using SNPs as proxies for the true causal factors, will diminish as more complete human genome sequence data accumulate, the problems of analysis will remain and even become more difficult as the mass of data in need of interpretation increases dramatically.

Second, where molecular variation associated causally with disease susceptibility is identified, it still might not be amenable to effective therapeutic intervention. In the context of genetic disorders, there has been a long lag period between improved understanding of pathogenesis and improved treatments for patients. It would be irresponsible to raise public expectations beyond the point where we can be confident that progress in knowledge will lead to progress in therapy.

Third, there has been a strong temptation for commercial laboratories to make available, to sell, molecular genetic testing for disease susceptibility, no matter how poor the evidence base. Indeed, several providers of such SNP panels have achieved high visibility on the Internet. The principal weakness is that the SNPs tested account for such a small proportion of the causal factors, while the marketing of the tests often claims, misleadingly but sometimes only implicitly, that they can be used to guide health-related decisions. Such claims are especially worrying if someone with a significant family history of disease relies on such tests instead of seeking a formal clinical genetics assessment and mutation testing in any relevant Mendelian genes. The legal status of such GWAS-based SNP tests has been under review in

the United States and several companies have recently ceased these activities. There have also been discussions in European countries about the need for regulation of this “direct-to-consumer” marketing of genetic tests.

For the present, the use of GWAS-based risk estimates for cardiovascular disease is no improvement on measuring blood pressure and cholesterol and using standard clinical scores (141,142).

Another set of concerns about genetic testing for disease susceptibility relates to the behavioral responses to risk information. Although the rhetoric emphasizes the positive behavioral application of risk information, experience so far does not suggest that it works out that way (143,144).

- It could cause great stress and anxiety, through identifying those at increased risk of disease without offering adequate countermeasures.
- It could consume limited health care resources without improving health.
- It could lead those given increased risks of disease to place inappropriate demands for intervention upon their regular health care providers, posing a threat to these systems.
- It could distract attention from environmental and lifestyle measures that are already known to be helpful to the whole population (the shaping of national food, employment, education, transport, and leisure policies to promote healthy eating and exercise).
- It could distract attention from the group of individuals at very substantially increased risk of disease, for whom more effective interventions are appropriate and already available (e.g. LDL-receptor disorders, Lynch syndrome, etc.).
- It could lead to paradoxical behavioral consequences for those at high and at low risk of disease, through inducing a sense of either fatalistic inevitability or inappropriate invulnerability.

An adequate evaluation of any screening test for susceptibility would include assessments of the clinical, psychological, social, and economic consequences of introducing the test, for those given decreased as well as increased risks, and for society as a whole. There will be a temptation to examine only the clinical consequences for those at high risk of disease, and this would be much too narrow an assessment. A full evaluation should preferably be conducted before any such disease susceptibility screening test could be introduced to either a state or a private medical care system. Where both types of systems operate, applying restrictions to the state health care but not the private system could undermine the effectiveness of the state system, where such testing does turn out to have a useful clinical role. There is a danger that restrictions on the introduction of clinically helpful susceptibility tests could be exploited as a means of limiting pressures on the health care budget, using appeals to “evidence-based medicine” as a veil.

Perhaps the single most important ethical issue in relation to population screening for susceptibility to disease is the nature of the evidence that would be required to justify and permit screening of this type. Crucially, what evidence would be required and who makes that decision (145–148)?

Although there are clearly many potential problems with genetic susceptibility screening, not all of which have been discussed here, it must not be forgotten that there have been useful outcomes of genetic research in the area of the complex disorders through GWAS, principally in opening up an understanding of disease mechanisms rather than any established clinical utility from risk stratification. Such insights may lead over time to the development of radically new therapeutic approaches, although it will be many years before any such therapy could be validated for general clinical application. More likely in the short term, however, and certainly more likely to reach fruition in the short-to-medium term, is the possible use of genetic tests to guide pharmacological therapy. There are several important clinical entities that may represent a common end point of rather different pathogenic processes, including hypertension, depression, and seizures. It is widely recognized that the selection of the most appropriate therapy for patients with these problems is immensely difficult and is currently a fairly hit-and-miss affair. At present, all affected individuals are lumped together for therapeutic trials, which then recognize the medicines most effective in the majority of patients. What might become possible is the selection of the most appropriate therapy for each individual, given the pattern of molecular genetic variation (i) that has predisposed them to develop the disorder or (ii) that influences their likely response to a range of different therapies. This tailoring of the treatment to the individual represents the Holy Grail of therapeutics. We must not expect such developments to work out quickly, because the complexities of human biology may simply make this goal unattainable, but it is reasonable to entertain the modest hope that progress in this direction will accumulate over the next few decades. While cautious hope is appropriate, promoting exaggerated expectations will not be helpful and could be damaging.

Another possible benefit of identifying genetic variation associated with disease susceptibility, where this becomes feasible, could be its application to clinical trials of new therapies. If only those at increased risk of a disease are recruited, it may require fewer trial participants to be followed for fewer years before an effect of the therapy should be apparent. A possible problem here is that this high-risk subgroup, while important in its own right, may not respond to therapies or preventive measures in the same way as the rest of the population, so extrapolation from them may be problematic. Studies correlating DNA sequence variation with adverse drug reactions are also planned and would be an appropriate use of the

technology. If individuals susceptible to adverse drug reactions could be identified, then their suffering and the substantial health care costs associated with iatrogenic disease could both be reduced.

### 30.15 RESEARCH IN HUMAN GENETICS

The principal issues to be considered are consent, confidentiality, and control over the process and application of the research. The safety of gene-based therapies is not yet a major issue as there have been few trials of gene therapies and these early trials have been handled with care through conventional safety assessment routes. The few casualties have resulted from either unfortunate effects of the proposed therapeutic agent or from poor compliance with procedures. We will also consider questions relating to the study of human diversity and “race.”

#### 30.15.1 Consent and Feedback

At recruitment into a research project, it is important that participants appreciate that the genetic sample being obtained for study is going to be examined in a research laboratory—the analytic process may not be subjected to the same quality control measures as in a regular diagnostic laboratory, and the applicability of the results to any health care decisions may not have been established. It may be the simplest and cause the least confusion if the research studies are thoroughly separated from the clinical services being provided to the patient (149), although this is not always feasible. It is obligatory, however, to make clear to the participant who is also a patient how, if at all, the research results may alter their prognosis or treatment. If participation will make no difference to their condition or if the results will not be revealed to the participant, then this must be clear at the point of recruitment to the study.

With banked samples of DNA or tissue, it is seen as good practice to return to the sample donor for consent to carry out new investigations for which specific consent had not previously been obtained (150–152). This also allows the donor to be given fresh information about the progress of the research. If the donor has died, then it might be appropriate to contact his or her family if information to be generated in the research could have implications for them. Such recommendations for the practice of research are often feasible in relation to uncommon disorders, such as malformations or Mendelian diseases, where small numbers of patients or families are involved and the research team may have a continuing relationship with the participants. In the context of the common, complex diseases, however, where data on large numbers of individuals are being examined, the imposition of these requirements may make it impractical for some types of research (e.g. genetic epidemiology) to proceed at all.

With banked tissue samples that were obtained for diagnostic purposes, perhaps at surgery for the removal

of a malignancy, and for which consent for research was not obtained, should researchers have to contact the patient and gain their consent before carrying out the research? Surely yes, if the investigation might generate clinically important information about the patient’s genetic constitution, but otherwise perhaps no, if the research is only intended to characterize the tumor tissue. If the patient has died, should the family be asked to give consent before the researchers proceed with their studies? Again, yes, if the results could be relevant to other members of the family, but perhaps no if the study is essentially epidemiological with no direct consequences for family members.

It is important to distinguish on the one hand between research investigations carried out on individuals or groups of individuals who have a specific genetic condition or disease, such as a Mendelian or chromosomal disorder, and on the other hand those studies of large groups or populations that seek to identify the genetic influences upon the more complex, multifactorial disorders. These population studies either seek to correlate clinical information available at the time of the genetic sample collection with genotypic data generated in the study or continue to gather clinical information about those who have contributed samples to compare susceptibilities to disease and responses to treatment that become apparent over time. There are very substantial differences between these types of research that must be made clear to participants at recruitment.

When molecular genetic studies are carried out on individuals with Mendelian or chromosomal disorders, this will generate information about the underlying basis of their condition and may provide insight into the pathogenesis or prognosis of their disorder. Although such information should not be used as an inducement to persuade individuals to contribute to research, it may be made available within a suitable clinical framework if that is the wish of the participants. In some studies, this may be inappropriate but it will often be thoroughly reasonable to provide feedback from the study to participants. In population-based research, however, this is much more problematic.

In studies seeking associations between the common, complex diseases and individuals’ genotypes, the research, if successful, could provide information that would be relevant to third parties such as insurers or employers as well as to the individuals and their families. This would need to be explained in counseling before recruitment unless either the samples and the relevant clinical information were anonymized or the results of the analysis were provided to participants in the form of general conclusions and not as the particular results applicable to each individual. It is of course impossible to provide individual results when anonymization of the samples and their accompanying clinical information has taken place, but anonymization cannot be performed when clinical information is to be gathered prospectively



for use in the analysis. There are good grounds for avoiding the feedback to individual participants in this type of research of their personal results, however, even when it would be a possibility.

First, agreement in advance to the principle of being given individual research results at the end of the project will usually be invalid, because the nature of the results will usually not be at all clear at recruitment. If feedback is left to the discretion of the researchers, when they consider the results to be “relevant” or “important” for the individual, then the inevitable enthusiasm of the researcher is likely to exaggerate the weight of the findings. It would be far more appropriate for the ethics committee approving each population study of a complex disorder to expect the researchers to feed the general results of the study back to participants but not to provide individual results. Interested research participants could then seek appropriate counseling and testing (i.e. effectively retesting) in a clinical environment if they so chose, if and when the study results were validated and an accredited diagnostic laboratory were able to conduct the analysis. In this model, participants in anonymized studies would have the same access to the research findings as participants in the studies with ongoing collection of clinical data.

In recruitment to open-ended research, whose future uses cannot be predicted, it may be more important to provide assurances that future investigations will be subject to independent ethics review than requiring researchers to recontact participants to approve each proposed new analysis. The early issues raised by “gene databanks,” especially the instructional problems of the Icelandic deCode databank (153,154), have evolved as the focus is moving toward assuring proper process rather than explicit consent for each application of the sample or data. As it becomes more difficult to guarantee complete privacy, which occurs as greater quantities of genome sequence data are produced and released, it may be misleading to guarantee privacy but instead the need is to focus on clarity (transparency) about this along with ensuring independent ethics review of new projects and the prevention of genetic discrimination on the basis of any information that is released or reconstituted inappropriately (155–157). This accords well with the perspective of Manson and O’Neill (158).

“Informed consent” as a concept has been asked to carry such a weight of responsibility that informed consent as an interactional process in the clinic cannot support it (159). At times, this has to be put to one side if important research is to be feasible (e.g. some types of newborn screening research (160)). On other occasions, informed consent may need to be modified (“tailored”) to make it useful in practice as well as in theory (161).

There are additional ethical problems with obtaining consent for the recruitment of children or incompetent adults (162). Although parents can give valid consent for the recruitment of their children to research, research

should in general not be performed on children where it could be conducted adequately on adults. Furthermore, children should be involved in discussions about research to the extent that they can understand the issues and contribute their views. If children withdraw their assent for research, then this must be respected, whereas, in contrast, important therapies may be administered against a young child’s wishes as long as the parent consents. Particular concerns might be raised by research into the predictive testing of children for later onset (usually adult-onset) disease for which no useful health intervention is available in childhood. Similar concerns apply to newborn screening programs where the natural history of the disorder is unclear, or where the purpose of recognizing a high-risk subgroup of children would be to recruit them into a trial of possible preventive strategies (e.g. for juvenile-onset diabetes mellitus). In such a case, the goal of recruitment into a trial must be transparent to the consenting parents at the point of entry; otherwise they could agree to participate in screening on the assumption that it would benefit their child and then find that there was no established treatment for a child shown to be at risk.

The important considerations relating to the recruitment of incompetent adults into research are not specific to genetics research and will be passed over here, suffice it to say that any decisions made must take the subject’s best interests into account and must not be enforced against their will (i.e. at least the passive assent of the subject is required). The context is very similar to conducting diagnostic investigations on an incompetent adult at the request of a concerned relative, anxious to know if they might have a child affected by the same (so far undiagnosed) disorder.

### 30.15.2 Confidentiality

Medical, personal, and demographic information contributed to a research project must of course be kept securely, as for their medical records, and the privacy of research participants must be protected. Third parties should not be given personal genetic information about individuals without their specific consent. In studies where the samples and data are anonymized, the protection of genetic privacy is simpler, but elaborate precautions, including encryption and tightly restricted access, may be needed to ensure the confidentiality of subjects in population studies with continuing data collection.

There is pressure from research funders to obtain the maximum benefit from the research undertaken and this has led some funders to insist that raw data be made available at the end of a project for other researchers to analyze. This carries dangers in that the identity of individual research participants could become known and their very personal genome sequence data might in effect become public. In addition, the practical burdens that this policy of the NIH imposes upon researchers from outside

the United States are such that it may in practice restrict research collaboration instead of opening it up (163).

The attachment of research results to patients' medical records without their knowledge is a potentially serious problem, and this danger is one of the reasons for the recommendation that genetic research should be carried out in a different laboratory, and the results recorded in a separate format, to be kept distinct from the individual's regular medical records. Otherwise, a member of staff unaware of the background context could inadvertently pass on research results that are highly sensitive or of uncertain significance, or where the participant had chosen not to be told, or staff could act on the results in a clinically inappropriate fashion.

### 30.15.3 Control and Ownership

In most jurisdictions, patients who have donated a sample of blood or tissue for research, or who have had blood or tissue removed as part of their medical care, thereby lose rights in that tissue. Ownership passes to the relevant clinical or research institution. This used not to cause many difficulties but the commercialization of biotechnology and of biomedical research, with potentially large sums accruing to researchers developing the right molecular technologies, to their institutions, and to their investors, has partially undermined the former "gift relationship," which (in many countries) used to motivate patients and members of the public to give blood and tissue samples for research, much as for blood transfusion services. Although individual patients and research subjects are expected to treat their genetic material as a part of their person, and therefore not a commercial resource to be bought, sold, or otherwise exploited, the very same genetic material can be treated as property by researchers, institutions, and corporations. This contrast is explicit in many of the research consent forms used in genetics research, in which patients contributing samples have to sign away any right to commercial exploitation or benefit with respect to the sample before it will be accepted into research.

Patients and clinicians will generally acknowledge that the practical, clinical application of advances in human molecular genetics to the welfare of individuals with genetic and other diseases requires financial investment and therefore the involvement of biotechnology and pharmaceutical corporations. Many, however, feel revulsion at the way this is working in practice to the advantage of corporations or laboratories that have contributed only to the very last stage of an arduous struggle to identify the genetic basis of the relevant diseases. It offends the sense of justice of many individuals that patients and families with genetic disorders, who have contributed biological samples to research and often also supported the research by fundraising activities over many years, now find that they, or the health services that care for them, are being charged

license fees for applying the discoveries that "their" research led to.

The question of whether human (or any other organism's) DNA sequences can be patented has roused similar passions. Patenting in general is conditional upon three criteria: novelty, an inventive step, and an industrial application. Discoveries, therefore, should be clearly distinct from inventions, and so naturally occurring DNA sequences, genes, and organisms would seem not to be patentable, but patents that give effective control over certain genetic sequences have been awarded. A landmark legal decision was the granting of a patent on a modified organism, an oil-eating bacterium, by the US Supreme Court in 1980, following which precedent further patents on organisms and DNA sequences have been granted. This heralded, perhaps enabled, the spate of investment in medical biotechnology over the past few decades (164). A rush for patents on sequences of human DNA led to competition between research groups eager to patent specific disease genes and to the race between the public and private human genome sequencing projects. The debates about patenting human gene sequences continue within the United States and internationally.

Openness and a collaborative approach among scientists would be preferable to commercial secrecy on any acceptable understanding of the scientific endeavor, even if this would entail slower progress in understanding the mechanisms of the human genome. It seems likely that commercial secrecy has distorted the process of research and has perhaps slowed the overall pace of scientific progress in this field. Whether a more collaborative spirit can be reestablished in the next phase of research is uncertain but this must be an important goal for scientists concerned for the ethical conduct of their craft.

### 30.15.4 Human Genome Diversity Project, Human (Pre)History, "Race," Community Consent, and Sharing of Benefits

One area of human biology that is being illuminated by genetic research is that of the history of human populations. By defining the human genetic variation present in populations around the globe, the movement of peoples over the millennia may be traced and compared with the understandings reached in folklore and through other tools such as archeology and comparative linguistics. The making of artifacts and the speaking of languages, however, can be learned, can be transmitted culturally, so that a comparison of the spread of a style of pottery across Europe with molecular data from surviving populations may give an indication of the extent to which the spread of the artifacts reflected the movement of people and how much resulted from the social learning of new customs. One could even imagine tackling classical myths, such as those of the Aeneid: Do modern Romans have Trojan Y chromosomes but Sabine mitochondria?

What problems lurk in this apparently delightful backwater of molecular archeology? One issue is the potential misapplication of molecular results to make “race” a superficially more valid concept. Although human populations differ in their physical characteristics and their genetic constitutions, there is no clear demarcation between population groups. We belong to communities socially, but these communities are not biologically distinct entities that correlate with specific genetic differences at the level of the individual. Population genetics describes the variation in gene frequencies between groups in a statistical manner, and its application to individuals is fraught with scientific problems as well as cultural confusion. Even the drawing of geographical boundaries around populations is arbitrary, likely to misrepresent a cline in gene frequency as a biological discontinuity when the mean frequencies of specific genetic variants in the two constructed groups are found to differ. That is why the term “race” has been discredited in the biological sense, and the term “ethnicity,” indicating a social group to which an individual chooses to be affiliated, is used instead in the social sciences.

We must remember that social tensions between ethnic groups, sometimes associated with real differences in biological characteristics, have always been used to justify discrimination against minorities and against neighboring communities or countries. Although scientists may have no such intentions, there is every possibility that genetic studies that focus on the differences between population groups will be systematically misused. Molecular reconstructions of population history are likely to support some popular or traditional “origin myths” and to disconfirm others. How might such evidence be used by those antagonistic to their neighbors? If the myth of a group’s distinct origin is supported by a research finding, then discrimination against that group, where it is in the minority, may be strengthened. Where a research finding disputes the oral or written tradition, the group may be subject to mockery or abuse. However fascinating, we must be very cautious in pursuing such studies without the full involvement of the groups concerned. Fortunately, the HapMap project seems to have learned from earlier projects and has largely avoided these problems (165–167).

The potential application of samples and results from the human genome diversity project to other fields, especially those relating to socially important characteristics, such as intelligence or other valued traits, raises the frightening prospect of major social conflicts and political disputes. If a site of genetic variation, such as a SNP, were found to be correlated with variation in IQ in one population, the frequencies of these SNP alleles would be assessed in other populations and spurious claims could easily be made about differences between the populations in IQ. The “Race and Intelligence” debates of the 1970s would appear tame in comparison with what could emerge. There are many problems that can be foreseen as likely consequences of such research, including

an increased emphasis on nationalism in politics, higher levels of interethnic conflict within multicultural societies, and a greater reluctance of the public or the state to support special educational interventions for children from disadvantaged ethnic minority groups. To pursue the research without attending to the likely social consequences would be irresponsible (168), and there seems to be no prospect of neutralizing the potential disasters.

One issue that has attracted much discussion is the process through which valid consent for participation in this research could be gained. When dealing with indigenous, aboriginal communities who have had little exposure to external influences, and no science education, it may be effectively impossible for the research team to educate each member of the community to the same understanding of the project and of their rights as in genetics research conducted within a Western society. Is there a place for a different type of consent in this setting? Can a community give its consent to participate, through its own processes of discussion, in the light of information from, and discussions with, project representatives? Does each individual still need to give their personal consent, and what would that mean in societies with little concept of individual autonomy (169,170)? Furthermore, is there a place for community inducements to participate in research, such as genetic testing for a prevalent disease, or provision of medical care for a defined period, or would that be improper leverage? What if the project were to identify some important disease resistance factor, which could yield enormous profits when commercially exploited—(how) would the community benefit from this in practice?

### 30.16 GENETICS, GENETICIZATION AND SOCIETY

Genetics has received increasing levels of public attention since the start of the Human Genome Project in 1990, and this trend seems set to continue as more findings emerge. Although gratifying to geneticists, there are problems with this focus on genetics, especially when society looks to genetics to explain, and even perhaps intervene to “solve,” social problems. The list of social problems for which a gene is being sought now is long and strikingly similar to the list of social vices that concerned the eugenics movement 70–120 years ago. The list of traits of interest, or concern, to the traditional eugenicists included homelessness, addiction to drugs and alcohol, learning difficulties, sexual orientation, violence, impulsivity, and criminality.

When the phrase “gene for X” is translated into something like scientific language, then the absurdity of some of the public expectations becomes clear. This phrase becomes “a site in the genome, not necessarily a gene, at which DNA sequence variation is associated with variation in the propensity to develop X as a problem, although this association need not be causal and

may only be found in one or a few population groups.” The links mediating the social consequences of genetic variation are so long and so complex that, even in principle, it could never be possible to predict these consequences from the molecular facts, proceeding from the DNA sequence through cell biology to neuronal excitation and then the behavioral phenotype in its full social context. The genetic sites at which molecular variation correlates with behavioral consequences, for example, will not be genes “for” the social problems. Rather, the genes will have distinct biological functions that evolved over millennia and that may be elucidated by the relevant neurosciences. The brain is far too complex an organ for geneticists to be able to make detailed predictions about behavior, although certain statistical associations between molecular variation and the probabilities of specific behaviors may be established.

The legal system is one arena in which disputes about the contribution of genetics to behavior may well be argued out in the future. Leaving aside the biological validity of claims that a crime was committed “because” the guilty individual was predisposed to this action by his genetic constitution, there are two possible responses to this claim: either the genetic predisposition can count in mitigation because the person’s responsibility was indeed limited or the likelihood that the criminal will reoffend should ensure a heavier sentence than usual, perhaps even an indefinite sentence, and perhaps even in advance of any specific crime, a form of “preventive detention.” A case can be made for both arguments, and the legal consequences of following either course would be hugely disruptive. Legal systems might be best advised to ignore the evidence presented of a genetic predisposition to criminal behavior except in the most unusual circumstances.

Genetic determinism is the attempt to reduce the whole of biology to the physical sciences, with the behavior of organisms being shaped largely by their genetic constitution. This is a powerful but destructive concept and in reality is a doomed enterprise. Given the inevitable limitations of the physical sciences in dissecting the functions of the brain, and *a fortiori* our limitations in tackling societal characteristics and problems, how should clinical geneticists respond to the widespread misunderstandings about the relevance of genetics to social issues?

- We should ensure that we do not inadvertently exacerbate these misunderstandings through the inappropriate use of pseudoscientific terminology—“genes for X.”
- We should dissociate ourselves from, and refuse to participate in, research predicated upon such misleading premises or likely to promote such misunderstandings.
- We should speak out, as individuals or, preferably, collectively, when we see genetics being hijacked and abused by those with damaging social or political motivations, especially if we see that vulnerable and

inarticulate groups of our patients are likely to suffer as a result.

- We should take care not to attribute health problems to genetic causes when social and political factors are likely to be as heavily involved and probably more open to remedy. This could, of course, be to our professional disadvantage, because we might benefit from research funds if the principal cause of a problem is seen as being largely genetic, but that would be intellectually dishonest and would, eventually, return to haunt us as our failure to resolve the problem was recognized.
- We should combat inappropriate talk about genetics to help minimize the harm done to the families in our care by some of the wildly exaggerated expectations current in the media.

The tendency to attribute causation of health or social problems to genetic factors is an example of “geneticization,” a term coined by Abby Lippman to describe the more general practice of accounting for observed differences between individuals and populations in terms of underlying genetic differences (171). This process allows vested political interests to obscure the remediable, environmental causes of social inequalities in health and individual attainment by focusing attention on the fixed and unalterable genetic constitutions of individuals. This, of course, fits well with the conservative view of human nature as often propagated under the label of sociobiology or evolutionary psychology; from such a perspective, social problems are framed as biological inevitabilities determined by each individual’s genetic constitution. This can lead those frustrated by the problems they see in society to advocate technological solutions when collective political action may be much more appropriate and effective. The process of geneticization therefore distracts energy and resources from the social and environmental strategies that could lead to real solutions.

One aspect of genetics research that is especially alarming, because of the ease with which it could be misapplied, especially in the context of “race” and human diversity, is the genetic dissection of nondisease traits, particularly intelligence and personality traits, and including variation in intelligence within the normal range. The apparent rationale for pursuing this research is that it may teach us about mild learning difficulties and about fundamental brain processes involved in learning. Given the barriers to interpreting any associations that may be found between intelligence and molecular variation, however, I would argue that the short-term results of such research are more likely to be social mayhem than a productive understanding of cognitive processes. The results are likely to be distorted, deliberately, by those intent on mobilizing political support behind racist or nationalist campaigns.

One final topic to consider here is the “thrifty gene hypothesis,” as an example of geneticization distorting



the research agenda. This hypothesis was proposed by James Neel to account for the high frequency of type 2 diabetes mellitus in aboriginal societies undergoing rapid development. A “diabetogenic” genotype would be favored as promoting maximal efficiency in food use when food supply is limited. When food becomes abundant, the efficient use of food becomes unnecessary and perhaps a disadvantage, with type 2 diabetes (T2D) developing as a consequence. On this understanding, T2D will remain prevalent in societies emerging from a harsh subsistence because of their genetic constitution, which will change over some generations as new selective forces operate. The emergence of this hypothesis gave an apparently adequate account for the very high frequencies of T2D found in some aboriginal communities; other possible causal factors, including socioeconomic factors, received little attention.

Research within a different paradigm has shown that the risk of developing T2D (and other chronic diseases) in adult life is greater for infants born with fetal growth retardation. This research has supported other hypotheses: either “fetal programming” (172) or perhaps the common, genetic basis of both fetal growth retardation and a propensity to T2D (173). The fetus may be programmed by the intrauterine environment to respond throughout postnatal life to nourishment and other environmental factors in a manner reflecting its growth in utero—the Barker hypothesis of the fetal origins of adult cardiovascular and related morbidity. This challenges the thrifty gene hypothesis, although both explanations could have some validity, and an extreme fetal programming model that discounts genetic factors is untenable. Given this, how should we respond to evidence that the incidence of T2D in some aboriginal populations is falling faster than would be expected by selection against a genetic predisposition to diabetes? Public health officials should be delighted, because the postwar improvement in fetal and infant nutrition may now be taking effect, supporting the fetal programming hypothesis. There would be every reason then to hope that attention to the nutrition and general health of the young aboriginal women should reduce this epidemic further in future generations.

With hindsight, it can be appreciated that the genetic explanation for an epidemic of morbidity in a deprived ethnic minority population led health professionals to discount further efforts at preventing T2D by attention to environmental measures. With hindsight, we can see that public health would have been better served by appropriate efforts in nutrition and antenatal care (172) and perhaps by more general attention to deprivation and social status (174).

### 30.17 REPRODUCTIVE TECHNOLOGIES AND CLONING: “REPROGENETICS”

Three reproductive technologies need to be discussed here. These are preimplantation genetic diagnosis (PGD),

human reproductive cloning, and NIPD by the sequencing of free fetal DNA (ffDNA) in maternal plasma.

PGD entails all the procedures of in vitro fertilization (IVF)—superovulation, oocyte harvesting and sperm collection—as in the treatment of infertility, along with the strict obligation to use reliable contraception alongside the IVF procedures. The zygotes are allowed to develop to six to eight cells, when one or two cells are removed from each morula for analysis, employing either fluorescent in situ hybridization (FISH) techniques, to look for chromosome anomalies or microdeletions, or polymerase chain reaction (PCR) methods, to identify specific disease-associated mutations or the number of repeat units in a triplet repeat or a microsatellite sequence. Then, an embryo (or perhaps two) without evidence of the family’s genetic disorder is implanted into the woman’s hormonally prepared uterus.

PGD can be the perfect solution for couples with infertility who also have a risk of transmitting a serious genetic disorder to any child they might have. For couples without a fertility problem, but who are concerned about transmitting their genetic disorder to a child and who wish to avoid the procedures of prenatal diagnosis and pregnancy termination, PGD may also appear an attractive option. The process of PGD is inconvenient and expensive, however, and is emotionally and physically draining.

For those who wish to avoid having a child with their family’s genetic disorder, weighing up the potential advantages and disadvantages of PGD against conventional prenatal diagnosis and the selective termination of an affected pregnancy entails weighing up the practical difficulties and costs of PGD against the fear of “needing” a pregnancy termination. Included in this equation may be the welfare of any children the couple already have and disruption to the wider social networks of both partners, including their employment. In discussing this with a couple, it may be helpful to encourage them to assess the strength of their objections to a pregnancy termination; this may clarify their thoughts and feelings.

Objections have been made to PGD because it is a technology that could be applied to choose between embryos on the basis of nondisease traits, including sex. If there are six healthy embryos and two are to be implanted, then it may seem very reasonable to allow the couple to choose the sex of the two embryos. There are good grounds for rejecting fetal sex selection in general, as discussed earlier, but are there any valid reasons for not permitting a couple in these circumstances to choose between the healthy embryos on the basis of sex or other nondisease traits for which testing might become possible, such as physical or even mental characteristics? If it were permitted for those using PGD because of an infertility problem to select between embryos on the basis of such traits, would the same be permitted for those who had no fertility problem but simply wished to select the characteristics of their child?

PGD can be seen as being located part of the way down the slippery slope to one type of “designer baby.” Although the burdensome nature of IVF technology and its costliness make it unlikely that many couples would wish to use it now, testing for nondisease traits may become available and the PGD technology is likely to improve, to be less burdensome. It is probably worth trying to arrive at a resolution of these problems before that occurs. There is no reason why a couple wishing to use such services would consult a clinical geneticist, so in that sense it is not a problem for “us,” but this area of “reprogenetics” lies so close to clinical genetics that we are bound to be affected by these developments.

One temporary resolution is to treat as distinct PGD for disease, for sex selection, and for selection on the basis of nondisease traits.

- PGD for disease presents fewer ethical problems than prenatal diagnosis and selective pregnancy termination and appears a reasonable option for those undergoing infertility treatment or with strong objections to pregnancy termination.
- PGD for sex selection is disrespectful to women and should not be performed except as a way of avoiding sex-linked disease (see the earlier discussion about prenatal diagnosis and sex selection).
- PGD for nondisease traits is not available at present because of technical limitations, and testing for socially important characteristics may not become available for many years, if ever. PGD on these grounds would not be part of the work of a clinical geneticist and should probably not be practiced at all. Such a development would be widely understood as a step toward a brave new world of genetically manipulated children.

The cloning of Dolly the sheep, announced in 1997, attracted acclaim and simultaneously also enormous concern. Human reproductive cloning has not yet been performed, but the world was compelled to acknowledge that it may be technically possible. In the context of clinical genetics, when might our patients seek reproductive cloning? What should our response be if/when such cloning becomes feasible?

Individuals who cannot have their own children because of infertility may find cloning an attractive option. Couples in which one partner has a dominant genetic disorder, or carries a sex-linked disorder or a chromosome rearrangement that could lead to problems with the child, may choose to clone the fertile, healthy, or noncarrier partner rather than run the risk of transmitting the condition to an affected child. It is difficult to envisage how the technology, or its public acceptance, will develop, but let us hope that biotechnology will be able to assist in such family scenarios before human cloning becomes available, so that the incentive to work for human reproductive cloning is lessened.

There would be very real concerns about the biological welfare of children born after cloning of an adult human because of cell aging effects, including telomere shortening and the accumulation of mitochondrial and other mutations. They may also suffer from infertility, and this could be a special problem for infertile males with Y-chromosome microdeletions. There would also be major concerns about the psychological welfare of the child clones of an adult “parent”; such a child would find itself uncannily similar to its mother or father when he or she was a child. The climate of expectations likely to surround these children in at least some families could well cause serious disturbances in their emotional development. If such children are born, however, it will be of the greatest importance to monitor their health and their emotional state, but without excessive intrusion into their family lives. Concerns about the privacy of families in which children were conceived by IVF prevented the systematic assessment of the long-term safety of such practices; such an opportunity must not be missed again, especially when the hazards appear to be much greater.

Finally, we turn to the analysis of ffDNA in maternal plasma as a means of performing prenatal genetic diagnosis without putting the pregnancy at risk by CVS or amniocentesis. This has been discussed earlier but here we consider the further development of its application in practice. This technique was initially used to detect paternally derived sequences that would not usually be present in the pregnant woman’s blood. This allowed fetal sexing (looking for Y chromosome sequences), identifying maternofetal Rhesus incompatibility or the transmission of a paternally derived autosomal dominant disorder. Recurrence of a recessive disorder can be excluded by showing that the father has transmitted his low-risk (nondisease) allele, or not excluded if his disease allele has been transmitted.

By comparing the quantitative yield of sequences from the different chromosomes, pregnancies carrying a fetus with a trisomy or other chromosome disorder can be identified; indeed *de novo* or inherited fetal CNVs can readily be detected.

The recent application of high-throughput genome sequencing technologies to this context is greatly extending the range of what can be discovered about the fetus (or embryo) from ~7 weeks’ gestation. By performing sufficiently “deep” sequencing, and especially if both parental genome sequences are available, it is possible to determine most of the fetal genome sequence, although this is still a research application and not readily available.

The immediate application of the new molecular sequencing technologies to reproduction then includes (i) carrier screening for autosomal recessive diseases and (ii) NIPD for Down syndrome and other chromosomal aneuploidies. For the first time, these two applications make realistic the prospect of drastically reducing, almost to nil, the incidence of autosomal recessive disease, Down

syndrome and other chromosomal anomalies. This raises profound questions about the type of society we want to live in, the extent to which we should regulate or control the biological composition of our communities, and whom we are prepared to welcome among us as fellow citizens. Would we want to change the nature of society so much? While one may feel a reluctance to take that road, the alternative may entail deliberately turning our backs on innovations that would prevent much suffering; is that not too high a price to pay for the nostalgic feeling of a now unattainably innocent virtue? There has been talk of “eugenics” for a long time; we now have the capacity to implement such policies, which used to be science fiction or mere “hot air.” We therefore need to be correspondingly more thoughtful about how we implement these developing technologies in practice.

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 American Society of Human Genetics (Policy Overview): [http://www.ashg.org/pages/policy\\_overview.shtml](http://www.ashg.org/pages/policy_overview.shtml)  
 British Society of Human Genetics (BSHG): <http://www.bshg.org.uk>  
 Center for Genetics and Society (Oakland, California): <http://www.geneticsandsociety.org>  
 Council of Europe Bioethics: [http://www.coe.int/t/dg3/healthbio-ethic/default\\_en.asp](http://www.coe.int/t/dg3/healthbio-ethic/default_en.asp)



Council for Responsible Genetics (a US-based Critical Voice): <http://www.councilforresponsiblegenetics.org/>

Eubios Journal of Asian and International Bioethics (EJAIB): <http://www.unescobkk.org/index.php?id=2434>

European Society of Human Genetics (Policy Statements): <https://www.eshg.org/eshgdocs.0.html>

Genéthique (France): A Useful Portal for Accessing Bioethics Policies and Debate in French: <http://www.genethique.org/>

GeneWatch (a pressure group critical of many industrial and governmental applications of genetic technologies): <http://www.genewatch.org/>

Human Genetics Commission (UK) (This Government Advisory Body is to be Reconfigured or Discontinued): <http://www.hgc.gov.uk/Client/index.asp?ContentId=1>

HumGenInternational. A Project Established by Bartha Maria Knoppers at the Centre of Genomics and Policy at the University of Montreal. In English, French and Spanish. <http://www.humgen.org/int/>

UNESCO (Bioethics Division), UNESCO (Bioethics Division): <http://www.unesco.org/new/en/social-and-human-sciences/themes/bioethics/>

US Government: Office of Science Policy, National Institutes of Health: Office of Biotechnology Activities, Secretary's Advisory Committee on Genetics, Health, and Society (SACGHS). This Committee has been Discontinued (from 2011) but Previous Papers have been Archived and Remain Available: [http://oba.od.nih.gov/SACGHS/sacghs\\_home.html](http://oba.od.nih.gov/SACGHS/sacghs_home.html)

University of Toronto Joint Centre for Bioethics: <http://www.joint-centreforbioethics.ca/>

WHO Genomic Resource Centre: <http://www.who.int/genomics/en/>

WHO ELSI section: <http://www.who.int/genomics/elsi/en/>

### Biography



**Professor Angus Clarke** studied Medical and Natural Sciences at Cambridge, taking his BA Part II in Genetics, and qualified in Medicine from Oxford University. After registration, he worked in General Medicine and then in Paediatrics. He studied the clinical and molecular genetics of ectodermal dysplasia in Cardiff and then worked in clinical genetics and pediatric neurology in Newcastle upon Tyne, developing an interest in Rett syndrome and neuromuscular disorders. He returned to Cardiff in 1989 as Senior Lecturer in Clinical Genetics and is now Professor in Clinical Genetics. He has maintained his interests in Rett syndrome and ectodermal dysplasia and has developed further interests in genetic screening, the genetic counseling process and the social and ethical issues around human genetics. He represents the Chief Medical Officer for Wales on the UK Department of Health's Emerging Science and Bioethics Advisory Committee. He has (co)authored and edited seven books, including "Genetic Testing" (2011—jointly with Michael Arribas-Ayllon and Srikant Sarangi) and "Living with the Genome" (2006—with Flo Ticehurst). He established and directs the Cardiff MSc course in Genetic Counselling.



# CHAPTER

# 31

## Legal Issues in Genetic Medicine

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### 31.1 INTRODUCTION

In this chapter, I address aspects of genetic medicine that raise important ethical and legal questions that have been or will be addressed by judicial decisions, legislation, or regulatory action by executive agencies. Years of debate, changes in relevant technologies, conflicting judicial opinions, inharmonious laws, and contrasting policy positions suggest how difficult it is to resolve key issues. For some topics, notably the laws concerning abortion, public policy seems to forever teeter on a shaky foundation. The main topics I shall consider are tort liability (malpractice issues) in clinical genetics, including the duty to warn; genetic counseling; abortion; genetic testing and adoption; newborn screening; regulatory oversight of new genetic diagnostic tests; regulation of direct to consumer genetic testing; genetic discrimination; surrogacy motherhood contracts; the regulation of human genetic research (especially informed consent); the regulation of human embryonic stem cell research; patenting genetic information and the Orphan Drug Act. The United States is far more litigious than most other nations, so some of the aforementioned topics are less relevant, for example, in Europe. Where possible, I will attempt to provide an international perspective.

No clear boundary demarcates legal issues in genetic medicine. Some topics, such as regulatory oversight of reproductive medicine and the impact of genetic testing on adoption, I will address only briefly. Other topics, such as the constitutionality of human cloning, the regulation of biobanks, reimbursement for the cost of medical foods, insurance coverage for genetic tests, and the use of DNA testing in immigration law, paternity disputes and forensics, I will not discuss.

The core theme to the legal issues that I address is that genetic data—information that can be ever more efficiently generated about each of us—holds significant potential for both good and harm, and for this reason, it is appropriate to craft societal rules that aim to maximize the former and minimize the latter.

A historical note is appropriate. One remarkable aspect of human genetics is how often advances (or, in some cases, apparent advances) in science have evoked a significant and sustained response from society expressed through the law. Perhaps the most infamous example was the widespread enactment during the first three decades of the twentieth century, both in the United States and internationally, of involuntary sterilization laws targeting the mentally retarded and the mentally ill. These laws reflected public reaction to naïve scientific conceptions that most such conditions had monogenic causes that were highly heritable and that therefore were in part preventable. In 1927, the United States Supreme Court upheld Virginia's involuntary sterilization law (1), and in 1934, the German government, significantly influenced by American eugenicists, enacted a similar Racial Hygiene Law. Involuntary sterilization programs flourished in more than a dozen countries, most notably the United States and Germany, until World War II; hundreds of thousands were sterilized (2). Although muted, programs of forced sterilization continue. For example, in 2010 a group of Uzbek women claimed that they were sterilized without their knowledge or consent as part of a government-backed population control program (3).

In the early 1970s, almost certainly in a well-intentioned effort to do good, legislators in 13 states enacted mandatory testing for sickle cell trait, often making proof of having been tested a condition of enrollment in the public schools. Such testing was not conducted as part of a comprehensive education and counseling program, and it outraged many in the African-American community. The resulting political hue and cry led to the enactment of a federal law that conditioned access to federal grants to states for sickle cell screening upon evidence that they were operating comprehensive voluntary programs (4).

Soon after it became apparent in the late 1980s that the ability to sequence the human genome was within

our grasp, concern about the possibility of abuse of personal genetic data became widespread. In the United States, the threat of genetic discrimination has been much discussed for the past 20 years, although somewhat less so since the enactment of the federal Genetic Information Nondiscrimination Act (5), which I discuss later. This sustained concern arose in no small part due to the patchwork nature of health care coverage in the United States. In virtually all other developed nations, citizens do not fear that a preexisting genetic condition could be grounds for denial or loss of coverage.

### 31.2 GENETIC MALPRACTICE AND THE DUTY TO WARN

From the perspective of tort law (the centuries-old, ever-evolving common law that defines what constitutes an actionable injury by one party to another), concerns about negligent behavior by health professionals who hold themselves out as able to practice genetic medicine fit relatively well within the broad rubric of medical malpractice. To establish negligence, the law requires that the injured party prove that he or she suffered an injury, that the harm occurred because of a breach of a recognized duty or standard of care, that the breach was the proximate cause of the injury, and that the damage should be actionable. There are other elements to this complex social equation—such as absence of contributory negligence—but for our purposes, the elements of a tort described above are sufficient.

The domain of malpractice law that is most relevant to genetic medicine is a diagnostic error, typically the failure to make a timely diagnosis. For example, each year, there are hundreds of lawsuits filed claiming that by failing to properly perform a breast examination, a physician delayed making the diagnosis of breast cancer, thus putting the plaintiff in a much more dangerous clinical situation than she would have been if the lump had been palpated at an earlier time. In genetic medicine, a comparable situation arises if a clinical geneticist, after completing his or her workup of a child, fails to make the correct diagnosis of a monogenic disorder and/or fails to properly counsel about recurrence risk. Diagnostic delay could in turn limit the benefits of proper treatment and exacerbate the ongoing injury caused by the genetic condition. In addition (and unlike most diagnostic challenges faced in pediatrics), however, a diagnostic error could indirectly result in the birth of a second affected child. For example, I know of several cases (most tort lawsuits are settled and very few are appealed, so it is difficult to collect good data) in which plaintiffs sued clinical geneticists alleging that negligence in failing to diagnose Fragile X syndrome was the proximate cause of the birth of a second affected child.

When they were first filed, now more than three decades ago, plaintiffs like these had to persuade the courts of the validity of either one of the two novel

causes of action: wrongful birth and wrongful life (6). Essentially, in wrongful birth lawsuits, the plaintiff must prove that had she known of a risk that should have been communicated to her, she would have avoided the birth of the affected child who, now in existence, is in need of special care. Over the years, supreme courts in more than one-half of the states have considered arguments favoring this novel cause of action. Although troubled by how to calculate appropriate damages, the courts have almost unanimously recognized this new tort. For example, in 2003, a Minnesota Court of Appeals held that physicians had a duty to warn a woman of the genetic diagnosis of her child and that if they failed to do so and she gave birth to a second affected child (with Fragile X syndrome) they could be liable (7). However, when such lawsuits included a cause of action by the child with the genetic disease, appellate courts have not recognized a right to sue. This is largely because the courts were unwilling to recognize a claim (often called “wrongful life”) premised on the argument that if a physician had properly performed his or her duty, the plaintiff would not ever have come into existence (8).

How does tort law articulate the duty that a clinical geneticist owes to the patient and his family? The clinician has the duty to use the knowledge available to him to attempt to diagnose the condition in a manner that other similarly trained professionals would conclude was reasonable. Such a vague standard may seem dissatisfying at first, but because it relies heavily on the (often unstated) rules of conduct within the specialty and because the plaintiff is unlikely to convince a jury of negligence if he cannot produce an expert in the field who will testify that a standard of care was breached, plaintiffs face significant hurdles in establishing a breach of conduct. In general, in medical malpractice law, most cases are dismissed in favor of the defendant or eventually settled within the coverage limits of the malpractice insurance policy. Of the relatively few cases that go to trial, the defense wins about 75–80%; this is probably also true when the issues involve genetic medicine. There are only a handful of appellate court decisions involving misdiagnosis of genetic disease. Of course, with the rapid growth of DNA-based testing, it is possible that litigation in this area will become more common.

Genetic medicine has generated significant debate about a core principle: confidentiality. For more than a century, tort law has held that physicians (and now all health professionals) must hold in confidence the information that they learn from and about their patients. There are of course certain exceptions to this—such as the recognized need to disclose facts to other physicians to assist in the proper care of the patient and the legislatively defined duty to report persons with contagious diseases. But, the discovery of a genetic fact about an individual—such as that his early-onset colon cancer was probably driven by a germline mutation—could be of immense clinical importance to his siblings, children,

and parents. This challenges us to reconsider the near-absolute nature of the duty of confidentiality. Should there be an exception for certain kinds of genetic information? If so, what should be the boundaries of that exception? Should the physician merely have a duty to warn his patient that he should communicate a genetic risk to his relatives? Should the physician have a right to reach out to first-degree relatives? What criteria determine when it is proper to do so? Should the physician ever be required to reach out to parties who are not his patients? If so, what constitutes appropriate discharge of that duty?

Discussion of a possible legal duty to warn third parties of risk despite an unavoidable breach of confidentiality derives mainly from a landmark California case, *Tarasoff v. Regents of the University of California* (9), in which the state's highest court held that a psychotherapist could have a duty to warn an individual if during the course of treatment a patient stated that he intended to kill that person and that there was a reasonable basis to conclude that the threat was real. The *Tarasoff* decision had a major impact on psychiatrists and other therapists, and various professional bodies spent years studying the feasibility of assessing risk to third parties and trying to outline the dimensions of a duty to warn. The boundaries of that duty remain uncertain.

An analogous problem arises in genetic counseling if the physician or counselor has discovered health information that is of potentially great importance to blood relatives of the patient. In clinical genetics, there is no clearly established legal obligation to warn persons who are not one's patients. This topic has been addressed repeatedly by blue-ribbon committees, by bioethicists and by legal scholars, but very few lawsuits have focused on them. In the United States, in the 1990s, two appellate courts considered the dimensions of the duty to warn nonpatient third parties about genetic risk. The Florida Supreme Court held that a physician who treated a woman with a dominantly inherited condition (medullary thyroid cancer) had a duty to warn her children about their risk for the disorder, but that the duty was discharged when the physician informed his patient of the risk to her children (10). In contrast, a New Jersey court held that a physician who treated a man for multiple polyposis had a duty to warn those known to be at risk for this (dominant) disorder of "avoidable harm," reversing a lower court decision that the physician had no duty to the children of his patient (11). Since courts in only a few states have ruled in this area, there is not yet a sufficiently large body of common law from which to discern a rule.

In general, professional bodies and academics have advocated that a health care professional should have a limited right to make a selective disclosure (a technical breach of confidentiality) to a few third parties when he or she has reason to believe that the index patient will not or cannot warn first-degree relatives. Practically speaking, the decision by the Florida Supreme Court that a physician discharges his duty to warn when he

informs his patient of risks to close relatives seems the best. It is unrealistic to impose a duty on physicians to reach out to people who are not their patients. For now, in the United States, the duty to warn appears satisfied by clearly informing one's patient of the possible risks to other family members. Perhaps the best way to resolve the issue of providing genetic risk information to third parties is to enact legislation. This has been done in a few countries, including Australia and Germany (12).

### 31.3 GENETIC COUNSELING

Although the practice of genetic counseling was introduced by a handful of physicians and research geneticists in the United States more than 60 years ago, it was not until the mid-1970s that it began to evolve into a health profession. With a few exceptions, persons who work as genetic counselors in the United States have earned an undergraduate degree and a master's degree from a genetic counseling program. Most practicing genetic counselors have also passed an examination that is developed and managed by the National Society of Genetic Counselors. It should be noted that certification is different from licensure, a term generally reserved for recognition by the state that an individual has demonstrated a specific level of professional training and skill. For well over a decade in the United States, genetic counselors, often supported by clinical geneticists, have lobbied the states to provide formal licensure of their profession. In the United States, state licensure is a key moment in the development of a profession. It is essential if genetic counselors wish to practice independently and be empowered to seek insurance reimbursement for their services. The movement for universal state licensure has proceeded slowly. At the time of this writing, eight states have enacted laws that confer licensure upon genetic counselors who meet the statutory requirements, and laws are being actively considered in six other states (13). It is likely that this trend will continue and that the majority of states will eventually adopt such laws; however, it may take another decade.

Genetic counseling is slowly evolving into a mature profession in Europe as well. Article 12 of the European Convention on Human Rights and Biomedicine requires that there be adequate genetic counseling before providing predictive or carrier testing. The Association of Genetic Nurses and Counselors, which represents counselors in the United Kingdom and Ireland, has convened a Working Group to develop a set of best practice recommendations (14). Genetic counseling is now selectively available in 38 European countries, but a survey of the practitioners in Europe found that the profession is still largely unregulated. In 13 of these nations, there were, as recently as 2006, not even any published professional guidelines. Many professionals working in Europe have called for more uniform training and favored more government regulation (15).

Lawsuits filed against genetic counselors proceed in the same manner and under the same legal rubric as those filed against physicians. It is uncommon, however, for a genetic counselor to be named as the main defendant in a malpractice lawsuit. This is because the vast majority of counselors work as the agents (under the legal oversight) of physicians. In most instances, the distinction is unimportant because the counselor and the physician work at the same institution and are covered by the same insurance plan.

Among the more common lawsuits in which genetic counselors are named as defendants are those that allege improper prenatal diagnosis, especially failure to offer an amniocentesis or failure to conduct certain tests (such as for carrier status in a couple at ethnic-based risk of one or more autosomal recessive diseases). Less common are lawsuits alleging failure to take a proper family history (that arguably would have uncovered a risk). A typical case, filed by parents of a child born with cri du chat syndrome against personnel employed at the University of Massachusetts/Memorial Medical Center in 2008, named two doctors, a nurse practitioner, and a genetic counselor as defendants. The key factual question was whether or not the team had properly informed the plaintiffs about the availability of amniocentesis. The case was settled out of court for \$7 million (16). One of the most important ways to reduce the risk of malpractice litigation over the subsequent birth of a child with a genetic disorder is to place a comprehensive note in the record that contemporaneously documents the actions taken and the information that was provided to the patient or the couple.

### 31.4 ABORTION

Although some states had enacted statutes that protected a woman's right to terminate her pregnancy before then, it was *Roe v. Wade* (17), a decision by the United States Supreme Court in January 1973 that recognized that the constitutionally protected right to privacy encompassed a woman's right to terminate her pregnancy under reasonable safety constraints until a fetus had developed to the point when it was viable (capable of life outside the womb). Since the *Roe* decision, the dimensions of the right to terminate a pregnancy have been repeatedly before the state legislatures and unendingly before the federal courts. Time and again it has appeared that the privacy right advanced by *Roe* would be lost, but (sometimes by the narrowest of votes) the US Supreme Court has never reversed the fundamental holding. Efforts by fervent opponents of abortion have succeeded in curtailing the reach of *Roe*—for example, securing laws that require parental consent for minors to obtain abortions, that limit state funding to pay for such procedures, and that require a woman to review “educational” material before she may proceed with a pregnancy termination. In addition, abortion opponents have successfully

blocked one method of late (and rarely used) abortion. A full review of abortion law is far beyond the scope of this chapter. I shall note a few of the more than 20 Supreme Court decisions that have addressed this bitterly contested issue.

Efforts to limit the impact of *Roe v. Wade* began immediately; during the 1970s, abortion opponents filed hundreds of such bills in state legislatures. Prophecies groups often filed lawsuits challenging the constitutionality of such laws on the day they were enacted. The next major case arrived before the US Supreme just 3 years later. In 1976, the high court struck down a Missouri law that required the consent of spouses and parents (of patients under age 18) before a woman could terminate her pregnancy (18). In 1979, the US Supreme Court (by an 8-1 vote) again invalidated state laws that required parental consent and held that the states must recognize a minor's right to demonstrate that she is mature enough to (in consultation with her physician) make a decision about termination independent of her parents (19).

Between 1977 and 1980, the US Supreme Court issued several opinions upholding the right of states to forbid the use of state Medicaid funds to pay for abortions for indigent women, decisions viewed as a victory for opponents of abortion (19–21), as they forced more poor women to continue unwanted pregnancies. In 1983, the Supreme Court struck down an Ohio law that required that second trimester abortions be performed in hospital (but upheld a provision that required parental or judicial consent to abortion for a minor) (22). In 1986, the high court struck down a law that required that a woman listen to a state-scripted speech designed to deter her from obtaining an abortion. In 1989, the Supreme Court upheld a Missouri law that forbid the use of public employees and facilities to perform abortions. In 1991, the high court upheld a federal regulation barring abortion counseling and referrals in family planning clinics that received any federal funds (23). In 1992, (by a 5-4 vote) the court upheld a Pennsylvania law that required patients to receive an “informed consent” booklet and imposed a 24-hour waiting period and a parental consent requirement. The only provision of that law that the court struck down was the spousal consent requirement. In 2000, the high court overturned a Nebraska law that banned the so-called partial birth abortions (24). However, in 2007 (in effect, reversing itself), the high court upheld a similar federal Partial Birth Abortion Ban of 2003, the first time the high court recognized the right of the government to forbid physicians from using a specific medical procedure. This closely decided case was the first in which the court had been realigned by the departure of Sandra Day O'Connor and the arrival of Samuel Alito. It has been nearly 5 years since the US Supreme Court issued an opinion involving the contours of *Roe*, and, at the time of this writing, no case is on its docket.

Over the past few years, there has been a sustained effort by abortion opponents in many states to enact



laws that interpret the protections provided to women by *Roe* as narrowly as possible. For example, as of April 2011, four states had adopted laws prohibiting abortions after 20 weeks of pregnancy (on the grounds that fetuses at that stage of development can feel pain) and similar bills had been introduced in more than 20 other states. Several states have enacted laws that forbid insurers from covering abortion services in their standard policies (women can still purchase coverage under more expensive supplemental policies). Activists in other states, such as Colorado and Montana, have sought to amend the state Constitution to define a person to include fertilized human eggs. For the foreseeable future, states will pass laws that challenge the limits of *Roe*; a few of them will make their way to the US Supreme Court. Based on the makeup of the court, it would not be surprising to see these restrictive state laws upheld. However, even though the US Supreme Court currently includes four justices whose view of constitutional law would favor letting each state decide the boundaries of abortion rights, I doubt the core holding in *Roe v. Wade* will be overturned.

### 31.5 ADOPTION

During the mid-1990s, some prospective adoptive parents began to inquire about the role of genetic testing in assessing the present and future health of an infant being placed for adoption (25). This caused concern that testing might be inappropriately used to choose among available children. In 2000, the American Society of Human Genetics (approving work of the Social Issues Committee) issued recommendations on this topic. Its three key points were that (1) all genetic testing of children in the adoption process should be consistent with testing that would be done on other children of similar age and condition, (2) that genetic testing should be limited to those conditions that manifest in childhood or for which preventive measures could be implemented in childhood, and (3) that in the adoption process, genetic testing should not be used to detect “predispositions to physical, mental, or behavioral traits within the normal range” (26).

A legal action for “wrongful adoption” was first recognized in the United States in Ohio in 1986. Since then this cause of action has evolved to include suits for fraudulently (knowingly) withholding important information material to the decision to adopt and negligently failing to learn or convey such information about the child. It is, for example, fraudulent to hide the fact that the birth mother abused alcohol throughout her pregnancy. Although there have been few cases turning on the disclosure of genetic data, in states where this issue can be raised, it is likely that courts will regard withholding clinical information that demonstrates or suggests that the child is burdened with a significant medical problem as material to the adoption decision. This could

also include medical facts about the birth parents. For example, it could be material that an infant was born to a woman with a late onset, serious, dominantly inherited condition.

In the past decade, lawsuits for “wrongful adoption” have become more common, especially with regard to international adoptions. In one prominent case in 2010, an American couple sued an agency in Russia when it discovered that the adopted child who they had been told had a good prognosis for normal development was actually the biological child of a woman who had abused alcohol during her pregnancy and who had fetal alcohol syndrome and an IQ of 53 (27). In about one-half of the states, it requires a court order to unseal an original birth certificate once an adoption is complete. But, beginning in 2000, a growing number of states (currently seven) enacted laws that permit adults who were adopted to have access routinely to their birth certificates. Similar bills have been introduced in New Jersey and New York (28).

The immense impact that DNA testing has had on paternity litigation—virtually ending what was once a common battle in the courts—is now only of historical interest. Because it can be used to rapidly and definitively establish parentage, in the United States, DNA testing, which is now universally recognized by the courts as a powerful evidentiary tool, helps women obtain child support for tens of thousands of children each year.

DNA testing to establish relationships is also used in the resolution of immigration disputes. In many nations (Denmark, Canada, Germany, and Switzerland, for example), DNA testing is used in programs that attempt to unite persons who seek entry as refugees and who claim they are the relatives of earlier immigrants (family reunification projects). The United States briefly operated a DNA testing program for similar purposes but suspended it in 2008 after finding that so many applicants were not related to their putative relatives. The United Kingdom also briefly used DNA testing in an attempt to establish the country of origin for African immigrants, but that project was suspended, in part due to criticism of the accuracy that the program claimed (29).

### 31.6 SURROGACY

Since the Supreme Court of New Jersey decided *In Re Baby M* case (30), legal issues involving surrogacy contracts have been frequently litigated. The famous New Jersey case arose after a physician whose wife had multiple sclerosis answered an advertisement offering surrogacy services. Mary Beth Whitehead agreed to become pregnant by artificial insemination with Dr. Stern’s sperm and to give up the child to him and his wife promptly after she gave birth. Upon delivery, the birth mother decided she wished to keep the child. When the ensuing custody battle reached the New Jersey Supreme Court, the justices, using an analysis based on the “best

interests of the child,” awarded custody to Dr. Stern but gave visitation rights to Mary Beth Whitehead. (When the child turned 18 years, she terminated her relationship with her birth mother).

In the past two decades, there has been a significant amount of litigation over the enforceability of surrogacy agreements, largely because few jurisdictions have enacted comprehensive legislation. In the United States, the law is in conflict; some state courts have refused to uphold agreements pursuant to the woman who agreed to become pregnant with the promise that she would give up the child at birth, whereas others have accorded a superior interest to the couple who engaged the woman by contract to act as a surrogate. California law routinely recognizes surrogacy agreements, whereas while in New York State, they are illegal. At least seven states are in accord with California. The legality of surrogacy agreements is emerging as an important issue for gay men who increasingly rely on them to have children. In this regard, Arkansas, which has one of the most permissive surrogacy laws, has attracted a substantial number of gay male couples.

In Europe, the practice of commercial surrogacy is in general tightly regulated, with some states forbidding payment of the gestational mother for the service she provides (31). In Ukraine, enabling legislation is quite liberal. Surrogacy contracts are recognized in India where the practice is widespread, raising ethical questions about the economic disparity between the parties to the agreement. Full discussion of this topic, the regulatory response to which remains in flux and about which there is a plethora of review articles in the legal literature, is beyond the scope of this chapter.

### 31.7 FROZEN EMBRYOS

Another subject of growing importance that has generated disparate holdings in different jurisdictions in the United States concerns the legal status and disposition of human embryos. Currently, there are more than 400,000 frozen human embryos in storage in the United States. Until a decade or so ago, many contractual agreements involving assisted reproductive technologies did not clearly delineate the process for the disposition of stored human embryos after the death of the biological parents or, more importantly, if they divorced. This was further complicated by the fact that neither the courts nor the legislatures had ever defined the legal status of stored human embryos. Over the past 20 years, a few court decisions have generated a majority view, but some legal issues remain unsettled. Neither has any appellate court accorded personhood to frozen embryos (a position that would be incompatible with *Roe v. Wade*) nor do the courts think of them as mere property. The leading case in this area, *Davis v. Davis* (30), decided by the Supreme Court of Tennessee, developed the idea that stored human embryos must be accorded “special respect,”

although it is unclear as to what that designation means and how it should shape disposition.

Embryo custody cases usually arise in the context of a divorce, and the key issue is to determine which of the biological parents shall have the right to control future use of the embryos. Generally speaking, in the absence of clear prior agreements to the contrary, the majority view is that the party wishing to avoid procreation should prevail, but some courts have noted that there might be circumstances in which this rule should not be followed (32). As access to the frozen embryos for reproductive purposes is generally more important to the woman, some have argued that she should have the primary legal interest. What then of the man, who no longer wishes to have a child with the woman? Has she the right to bear his biological child over his objection? Does a duty to support that child attach to him, even though the marriage ended before the pregnancy began? What are the implications of such child bearing for the laws of inheritance? Fortunately, most of these intractable problems will soon be viewed historically. Today, most legal agreements covering treatment of infertility anticipate the problems related to divorce and address such issues in a manner most courts would regard as definitive (33).

A vexing ethical problem concerns the proper disposition of frozen human embryos not destined to be brought to personhood by their genetic parents. In the United States, unlike a number of European countries, such as the United Kingdom and Italy (which limits the number of frozen embryos one can create), in vitro fertilization and embryo storage are largely unregulated. Most of the frozen embryos will never be used to attempt pregnancy. In a large survey conducted in 2008, more than half of the couples who did not want to attempt to have more general opposed donating their embryos to other infertile couples, and 43% did not want to destroy the embryos. About 66% were willing to donate them for research (which can be difficult to accomplish, as it often requires the direct engagement of the genetic parents with the research team). About one in five couples apparently prefer to hold the embryos indefinitely in storage (34).

The unresolved issues surrounding custody and/or disposal of frozen human embryos are just a few in a skein of difficult problems involving assisted human reproduction. Another important set of issues surrounds sperm donation, a process that has been widespread and largely unregulated in the United States for more than five decades. Most of the problems that have arisen pursuant to that practice will also complicate egg donation as it becomes more common. Among the more obvious concerns about sperm donation are inadequate health and genetic screening of the donor, limiting the number of donations, and guaranteeing the privacy of the donor. Perhaps the most notorious example of the failure of the regulatory process in sperm donation is a case in Michigan in which a donor contributed 11 samples, all of which were used and five of which resulted in babies

with the same serious genetic cause of neutropenia. Some nations (such as The Netherlands) limit the number of times a man can donate sperm, but most do not (35).

In recent years, coincident with the growth of the Internet, a large percentage of individuals who learn they were conceived with sperm donation are seeking to learn about, and often to meet, their genetic father. This has put great pressure on infertility clinics that are usually bound by a legal agreement not to disclose the identity of the donor. In Britain (and several other European countries), sperm donors must register and the offspring now have the right to learn the donor's identity on reaching the age of 18 years (35). I think the interest that children have in learning their biological heritage outweighs the privacy interest of the donor.

### 31.8 NEWBORN SCREENING

From its inception in the United States in 1962, newborn screening for genetic disorders has been implemented with state-based mandatory laws. In less than a decade, all 50 states enacted laws that required children to be tested for phenylketonuria. The laws were usually crafted in a manner that delegated power to the state department of public health to add tests to the screening program as it determined to be appropriate. Over the ensuing three decades, newborn screening in the United States became a set of regional programs; typically, a single state laboratory would serve a region (such as Massachusetts for most of New England), funded with state and federal funds and (in some cases) user fees. From the outset, some state laws provided exemptions to compulsory testing (typically, for claims based on religious beliefs), but little attention was paid to the consent process. The advent of tandem mass spectrometry in the late 1990s, a technology that significantly expanded the number of disorders that could be screened, led some jurisdictions to reconsider program operations. In Massachusetts, for example, when it was determined that screening for certain additional disorders should be considered experimental, a program was introduced to permit parents to “opt out” for certain tests. Still, the overall approach to newborn screening was that it was a valid exercise of the state's police power to require newborn screening on behalf of the public health. To my knowledge, the right of a government to operate compulsory newborn screening programs has been legally challenged only once. In 2001, a couple in Ireland challenged that nation's mandatory newborn test for phenylketonuria, arguing that the risk of the disease to their child was too remote to override their rights as parents to make an informed decision. The Supreme Court of Ireland found in their favor.

Recently, in the United States, there have been some important challenges to the operation of newborn screening programs. In 2006, the Texas Civil Rights Project, acting on behalf of five parents, sued the state to end the practice of the department of health of collecting and

indefinitely storing blood samples obtained for newborn genetic screening without having obtained informed consent. The plaintiffs prevailed, and the unconsented samples were destroyed (36). Texas subsequently enacted a new law that requires the provision of detailed information about newborn screening before taking a child's sample and that gives the parent the right to “opt out” of the testing. The law also provides that once they have become adults, individuals whose parents consented to the storage of a sample may, nevertheless, request that it be destroyed. In 2009, the Citizens' Council of Health Care and nine families sued the Minnesota Department of Public Health, alleging that it was collecting, storing, and using DNA samples from newborns without obtaining adequate consent, in violation of the state's 2006 genetic privacy law. At trial, the district court granted summary judgment in favor of the department of health, essentially ruling that the privacy law did not override the newborn screening law (which does include a parental opt-out provision). On appeal, the Minnesota Court of Appeals affirmed. Because only two state courts have considered challenges to newborn screening laws, it is not possible to discern a trend. It seems likely that “opt-out” provisions that avoid the expensive time-consuming process of obtaining pretest consent, but retain a mechanism through which parents can decline, will emerge as a compromise policy (37).

An emerging issue in newborn screening is to define under what circumstances, if any, screening samples may be used anonymously to conduct epidemiological research (38). Some experts have argued that these retained samples constitute an invaluable resource for improving human health; over 160 research questions have already been investigated by studying aliquots of samples (39). For example, the retained blood samples could be used over time to monitor levels of maternal exposure to teratogenic drugs or to certain widespread environmental pollutants. As of May 2010, 18 states had laws that addressed some aspects of this topic, but the laws vary widely in their approach (40). It would be beneficial to build a uniform approach to managing this issue, as is suggested in a recent helpful study done under the auspices of the Institute of Medicine.

State-based newborn screening has always been hobbled by inadequate funding. In 2008, the Congress enacted and the President signed the Newborn Screening Saves Lives Act of 2007, essentially an amendment to the Public Health Service Act to provide grants to the states to expand newborn screening, but funds to support the law were not appropriated. Until federal grant support is adequate, disparities among state and regional programs will continue. Although the states are becoming ever more similar in regard to the number of disorders for which they screen, the process is not likely to ever be completely uniform. For this reason, some children will be fortunate enough to be born in a state that screens for the disorder with which they are afflicted, whereas others will not be so fortunate.

### 31.9 PRENATAL AND CARRIER SCREENING

Prenatal screening is not conducted pursuant to state or federal law. Rather the standard practices—such as offering to test for fetal aneuploidies and to screen for neural tube defects—arise from policies advocated by recognized professional bodies such as the American College of Obstetricians and Gynecologists and the American Society of Human Genetics. Such promulgations have significant legal impact. They become a *de facto* standard of care that physicians ignore at their peril.

Similarly, with rare exceptions, carrier screening is not conducted pursuant to legislative fiat. Rather, from time to time—either because of promulgations by professional societies or as the result of malpractice litigation—there emerge new duties that must be incorporated into practice. Examples include the presumed duties to inform (1) an Ashkenazi Jewish couple of their risk for conceiving fetuses with Tay–Sachs or other recessive disorders that are comparatively prevalent in that ethnic group and (2) an African-American couple about their increased risk of bearing a child with sickle cell anemia. A duty arguably exists to take a genetic history of all couples, including questions related to consanguinity. For example, the discovery that a patient has a cousin with cystic fibrosis arguably triggers a legal duty to offer carrier testing.

The most famous of the few examples in which government bodies have aggressively pursued carrier screening involve screening for  $\beta$ -thalassemia. In Sardinia, a program initiated in 1978 has dramatically reduced the birth of children with this serious blood disorder in a single generation (41).

### 31.10 GENETIC DISCRIMINATION

Beginning in the mid-1980s, coincident with the growing awareness that we would gradually develop technologies to enable the collection of virtually unlimited amounts of genetic data from individuals, understandable concerns arose about the potential for misuse of this information. Early on (in public discourse, not by governmental regulation), genetic discrimination was defined broadly as the loss or denial of some desired goal (insurance, employment) because genetic information or family history suggested an increased risk of a disease, a reproductive risk, or a disability with that loss or denial creating an actual or potential economic harm. Note that the term did not include discrimination based on the presence of a disease state; for example, it did not include the refusal of a life insurer to offer a policy to a person with Huntington's disease.

Certainly, there are numerous lessons from recent history that support such fears, the most powerful being the ghoulish use of a pseudoscientific eugenics by the Nazis to justify the murder of countless Jews, gypsies, and mentally retarded and mentally ill persons. In the early 1970s, a number of states in the United States developed

compulsory programs to identify persons who carried an allele for sickle cell anemia. These short-lived programs (which failed to provide access to genetic counseling or adequate pretest education) did much to reenforce historical suspicions among African-Americans about the clinical and research communities.

The possibility that genetic data would be used by insurers, especially health insurers, to deny access to coverage was a major political topic throughout the 1990s. In the late 1990s, literally hundreds of bills were filed in virtually all the 50 states to address this fear. Many fewer, but still a substantial number of, states also regularly considered bills to address genetic discrimination in life insurance underwriting and in employment. By 2000, more than one-half of the states had enacted laws to prevent health insurers from labeling genetic risk as a preexisting condition and, thus, being able to deny or limit coverage should it manifest. Enactment of the federal Health Insurance Portability and Accountability Act in 1996, which included a provision that forbade the use of genetic information to deny coverage under the preexisting condition clause, complemented the state laws and probably helped to reshape underwriting behaviors.

A few states also enacted laws aimed at life insurance underwriting, but these for the most part only required that persons with various genetic risks be treated in the same manner and that the insurers be able to actuarially justify premium ratings. Early on, the American Council of Life Insurance took the position that genetic information was simply yet another form of medical information and that it had the right to review all such data as part of the process of deciding whether to issue a policy and at what rates.

Concern about genetic discrimination in the workplace was provoked in part by a long battle between women who worked in environments that could threaten a developing fetus. In the 1980s, several companies with manufacturing processes that exposed workers to high levels of lead made proof of infertility a condition of employment. These “fetal exclusion” policies were designed to protect the employers from lawsuits brought by women working in such settings whose babies evidenced the stigmata of lead poisoning. In 1985, women at Johnson Controls sued their employer alleging a violation of Title VII of the Civil Rights of 1964 as amended by the Pregnancy Discrimination Act of 1978. After 6 years, the US Supreme Court, reversing the lower courts, held that employers could not ban nonpregnant women from a workplace simply because they were exposed to agents that could cause birth defects (42).

By 1999, about 20 states had enacted laws to forbid employers from using genetic information in decisions about hiring or promotion. In 2000, President Clinton issued an Executive Order banning similar behaviors in the federal workplace. By 2004, about 32 states had laws to forbid or limit genetic discrimination in the workplace. There is little evidence to suggest that these state laws had



much impact. The federal Americans with Disabilities Act (1990), which forbids the use of most health information in determinations about hiring, had already accomplished most of what the state laws aimed to achieve. Furthermore, there were then relatively few genetic tests that could provide important information about one's capabilities of performing a particular job. With very rare exceptions, there have been virtually no complaints filed or no lawsuits initiated pursuant to these state laws.

One much discussed exception is an action taken in 2001 by the Equal Employment Opportunity Commission against the Burlington Northern Santa Fe Railway. The complaint alleged that the company was violating the Americans with Disabilities Act by forcing some employees (about 125 of a total of 40,000) who had filed disability claims for carpal tunnel syndrome to undergo a genetic test for hereditary neuropathy with liability to pressure palsies (HNPP). The case was settled in May 2002 through voluntary mediation; the company agreed to desist from testing and to pay damages to those that it had coercively tested. As part of the settlement, it was also permitted to state that it did not believe that it had violated the law (43).

After considering bills for more than a decade, the Congress passed and President Bush signed The Genetic Information and Nondiscrimination Act of 2008, popularly known as GINA (5). In essence, GINA prohibits group and individual health insurers from using a person's genetic information in determining eligibility and premiums; prohibits an insurer from requiring or requesting that a person undergo a genetic test; prohibits employers from using a person's genetic information in making employment decisions such as hiring, firing, or job assignments; and prohibits employers from requesting, requiring, or purchasing genetic information about individuals or their family members. GINA does not prevent health care providers from recommending genetic tests to their patients; does not mandate coverage for any particular test or treatment; does not prohibit medical underwriting based on current health status; does not address life, disability, or long-term care insurance; and does not apply to members of the military services. GINA should help to smooth the uneven nature of the protections offered by myriad state laws; at least 35 states now have employment laws and 47 have laws addressing aspects of health insurance (44).

As of May 2011, only one case, that of a Connecticut woman who claims she was fired after she disclosed that she underwent a double mastectomy because of a family history of breast cancer had been filed before the Equal Employment Opportunity Commission. Based on the history of litigation pursuant to similar state laws, some enacted more than a decade ago, I expect that litigation based on GINA will be small.

A new controversy over the use of genetic testing may be emerging in college sports. Effective August 1, 2010, the NCAA Division I legislation began to require that student-athletes provide as part of the medical

examination the results of a test for sickle cell trait. The individual may decline to be tested, but must sign a written release from liability should he be injured and turn out to be a carrier. The policy grows out of the settlement of a lawsuit against Rice University in 2006 after a student-athlete who turned out to have sickle cell trait died during a practice. Since 2000, the deaths of nine college athletes have been associated with sickle cell trait. In 2012, the American Society of Hematology sharply criticized the NCAA testing rules, calling them both overbroad and incomplete (45). It will not be surprising if over time such requirements expand to include testing for long QT syndrome, hypertrophic cardiomyopathy, and other genetically driven conditions.

### 31.11 REGULATION OF GENETIC DIAGNOSTIC TESTS

For nearly two decades, various groups, particularly genetic professionals and consumer advocacy groups, have expressed concern that under the existing regulatory framework in the United States, a laboratory may develop and commercially deploy a new genetic diagnostic test without first seeking some level of Food and Drug Administration (FDA) approval of that test. At the time of this writing, there is minimal oversight of genetic testing at the federal level and with a few notable exceptions (New York, California) in most states. There are signs that this situation may change. At the federal level, the FDA is evaluating whether or not to require prior approval of such tests. In 2008, the Secretary's Advisory Committee on Genetics, Health and Society (SACGHS) recommended that the DHHS create a mandatory public registry for genetic tests and genetic testing laboratories. This would constitute a welcome first step and would provide consumers with at least some data with which to decide about undergoing tests. In 2009, in the United Kingdom, the Science and Society Committee of the House of Lords issued a report on Genomic Medicine. It also advocated the creation of registry that would help consumers evaluate genetic tests. Probably because of the guaranteed health care coverage provided through the National Health Service, the Report specifically rejected the need for a genetic discrimination law such as GINA. It did, however, support the extension of the existing moratorium on the use of genetic testing in life insurance underwriting that has been in place in the United Kingdom since 1999 and is scheduled for review in 2011.

### 31.12 DIRECT TO CONSUMER GENETIC TESTING

In the United States, federal oversight of clinical laboratories is exercised through the Clinical Laboratories Improvement Amendments (CLIA) of 1988, the implementation of which falls within the jurisdiction of the FDA. Although FDA officials have asserted jurisdiction

over the regulation of genetic testing, they have not exercised it. Currently, many clinical genetic tests are offered pursuant to a provision of the law that permits a clinical laboratory that has developed a test to offer it from a single site. Under CLIA, certification and oversight are essentially delegated to state departments of public health. It is important to understand that, in general, this regulatory framework focuses on the operation of the laboratory and does not evaluate individual tests. The regulatory requirements that must be satisfied to gain CLIA certification are not particularly onerous. New York has exercised its right under CLIA to demand a higher standard for certification. In practice, many smaller states regard a laboratory that has been certified by New York as having met their standards. This is particularly true for genetic testing. Currently, New York requires prior review of every genetic test that a clinical laboratory proposes to offer. Furthermore, it has exercised jurisdiction over direct to consumer genetic testing laboratories, refusing to permit them to collect samples in the state unless they have met regulatory requirements. In 2010, the NIH, in an effort to improve public knowledge of genetic tests, launched a Genetic Testing Registry, a voluntary program that invites all laboratories to submit information about the tests that they offer.

In Europe, some states that are signatories to the Convention on Human Rights and Biomedicine have also signed the “The Additional Protocol on Genetic Testing.” Article 7 of the protocol requires that genetic testing for health purposes “may only be performed under individualized medical supervision.” Some European states have acted outside the Convention. For example, Germany has banned direct to consumer genetic testing.

Much has been written about the lack of regulatory oversight of direct to consumer genetic testing (30,46). A significant problem in developing adequate regulatory oversight is the unavailability of funds. Faced with this reality, what might be the key element of a simple yet effective oversight strategy? One thoughtful article identified the following five issues that must be addressed: (1) before testing adequate education must be provided and consent obtained, (2) laboratories must undergo an accreditation process and participate in quality assurance programs, (3) the scientific validity of the association between the disease state or liability and the genetic test result must be established, (4) laboratories must ensure that consumers have access to qualified professionals to help them understand the test results, and (5) claims about the value of the tests must satisfy consumer protection guidelines (47).

Direct to consumer genetic testing is a new service industry, operating in dramatic opposition to the decades-old model that required all test requests to be ordered by a physician. As the cost of testing continues to drop and the value of the tests continues to rise, it is likely that the direct to consumer testing will grow

substantially. Carrier testing for autosomal recessive disorders and pharmacogenetic testing are two areas that are poised for growth. Although the cost of testing for carrier status for perhaps 500 recessive conditions may soon be available for less than \$500, there is no evidence that most people will use the service.

### 31.13 REGULATION OF HUMAN GENETIC RESEARCH

In the United States, research involving human subjects is regulated by a body of rules embodied in the Federal Code of Regulations in 1978 (48). This relatively brief document provides the legal infrastructure for the Institutional Review Boards (IRBs) that dominate the local oversight of research involving human subjects, sets forth the elements of adequate informed consent, and provides special rules for vulnerable populations such as pregnant women, children, human embryos, and prisoners. From a regulatory perspective, oversight of human genetic research is covered by the same guidelines that cover other kinds of research involving human subjects. Although its opinions do not have the impact of regulatory authority, the NIH has for many years operated the Recombinant DNA Advisory Committee (the RAC), which provides an independent review of research proposals involving gene therapy. Its main concerns are the safety of the proposed study and the adequacy of the consent process.

The explosive growth during the 1990s of genetic research involving human subjects posed new challenges for IRBs, in part because the regulatory framework developed in the 1970s contemplated physical risks such as exposure to novel drugs, while genetic research posed informational risks such as wrongful disclosure of personal information. Novel questions emerged. What are the possible consequences of disseminating genetic data about individuals? Do relatives of subjects who are participating in clinical trials have a right to know about genetic discoveries that might be important to their health? Do researchers have a duty to make such disclosures? Do they have a right to do so? Who owns the DNA samples that are collected in the research setting? Should the consent process address ownership of a retained DNA sample? Does the donor of the sample retain any property rights in it? Should the team conducting the research disclose any economic ties that they have with a sponsoring company?

A decision by the Supreme Court of California in 1990 (49) remains the leading case on the topic of ownership of tissue. The court ruled that Moore did not retain an ownership on cells taken from his spleen that were used to start a cell line of value in leukemia research. It did, however, assert that the investigator has a duty to disclose to the prospective human subject any financial ties from which he may benefit should the research be successful.

Legal commentary (there have been very few lawsuits and even fewer appellate decisions to provide guidance)

concerning the regulation of human genetic research focus on risk of informational harm (such as discrimination in the workplace), disclosure of genetic data to others, adequacy of consent, ownership of tissue and the right, if any, to share in economic gains flowing from research, participation of children as research subjects, and use of human embryos.

One relatively new legal issue involving informed consent concerns the boundaries of the researcher's right to use samples obtained specifically for one study in other studies. Recently, the Havasupai Indians, who inhabit the deep crevices of the Grand Canyon, settled a lawsuit with Arizona State University growing out of a research project to study the genetics of the diabetes, which is common among the tribal members. One of the researchers involved also wished to use the samples to study the genetics of schizophrenia. In 2003, the tribe sued the university, alleging improper (beyond the scope of that delineated in the consent form) use of the samples. The parties disagreed over the clarity of the consent form on this point. In settlement, the university paid \$700,000 to be shared by 41 members of the tribe and returned all the samples to them (50).

### 31.14 REGULATION OF RESEARCH WITH STEM CELLS DERIVED FROM HUMAN EMBRYOS

Throughout the presidency of George W. Bush, it was federal policy to forbid the use of funds to support any research involving cells derived from human embryos (with the exception of 21 cell lines that were established prior to the implementation of the ban and which were suboptimal for research purposes). Although this certainly slowed progress in stem cell research, actions taken by a number of states, notably California, wherein 2004 voters approved a 10-year \$3 billion dollar effort to fund such work, may have more than compensated for the lack of federal funding. Shortly after becoming President, on March 9, 2009, Barack Obama lifted the executive order that had banned the use of funds for this purpose. Soon thereafter, two NIH scientists who oppose using human embryos in research sued to reinstate the ban, arguing that a federal law protecting embryos from research applied to the creation of cell lines. To the consternation of most scientists working in the field, a federal district court judge reinstated the ban. On appeal, the United States Court of Appeals of the District of Columbia overturned the lower court decision. As of the spring of 2011, the NIH has the right to fund research involving stem cells derived from human embryos.

### 31.15 GENES AND PATENTS

In brief, a patent is a governmental guarantee to market exclusivity for a defined period of time that is provided in response to a full disclosure of a discovery or

invention that satisfies certain tests. The right to seek protection of novel intellectual property is embodied in Article I, Section 8, of the United States Constitution. For more than two centuries, statutes, litigation, and evolving United States Patent and Trademark Office (USPTO) policy have shaped the definitions of what discoveries or inventions may be patentable. Similar bodies of law have developed in most, but not all, countries. The dimensions of patent policy in the United States and Europe are ever evolving. The first patent granted for a chemical composition in the United States was for adrenaline in 1906. The first patent involving a genetically engineered microorganism (a bacterium that degraded hydrocarbons) was issued in 1980 (51). In 1998, the USPTO issued a patent on the first transgenic animal, a mouse made susceptible to breast cancer. In the early 1990s, advances in genomic technology more or less marked the commencement of the rush to patent DNA sequences. As of 2010, about 40,000 patents have been issued in the United States that relate to about 2000 genes. Some single genes or proteins coded by them are the object of more than 100 patents. At one time, the NIH was a major filer of patents involving DNA sequences that suggested than unstudied genes (ESTs). Universities collectively own many patents covering aspects of human genes and often outlicense access to them (52).

United States policy encourages the creation of patents growing out of research conducted in part with federal funds. This position, the core principal of the Bayh–Dole Act (35 US Code Sections 200-212), is based on the premise that technology transfer to the private sector is most likely to benefit the general public. If the licensee does not develop that which has been licensed to it, the law permits the government to “march in” and offer licenses to others (a right that is rarely utilized).

The development of technologies to enable the detailed investigation of genomes and genes has posed several ongoing and challenging issues for national patent offices. This discussion will focus on US patent law, but it is important to note, especially in regard to genetics, that there are issues about which the policies of European nations and the United States diverge. A brief, but excellent, overview of possible solutions to current problems in regard to patenting DNA sequences in both the United States and Europe is available (53).

In general, academic laboratories do not engage in drug development beyond the preclinical stage; however, several medical schools operate genetic testing laboratories, those that sometimes constitute an important source of revenue. The controversy over gene patents is at the bottom a debate about whether newly discovered correlations between sequence variations and the presence of or risk for disease should be granted intellectual property protection. If such a patent is granted in the United States, the patentee has for a period of 20 years (from the date the patent was filed) exclusive control over the use

of that test, including the right to refuse licenses to other clinical laboratories to offer the test. During the past decade, several controversies have erupted over the right of a company or university laboratory to limit access to or charge high fees for access to a test. These have each been settled.

It is common for multiple patents to be issued concerning discoveries pertaining to a single genetic test. For example, at least 8 issued patents are relevant to testing for cystic fibrosis, at least 17 are issued in regard to testing for long QT syndrome, and 22 that pertain to testing for hereditary breast cancer. One concern has been that conflicting patents will create an intellectual property morass (often called a “patent thicket”) with one patent blocking another. Generally speaking, patent holders regularly solve these issues through cross-licensing or, sometimes, purchase of rights (54).

Over the past 15 years, there has been a tightening of the interpretation by the courts of key elements of patent policy as they apply to DNA sequences. For example, in 2009, a federal appellate court upheld the decision by the patent office to reject a patent application on the nucleotide sequence that governs activation proteins in natural killer cells on the grounds that it was an “obvious” topic to study (55).

Currently, the most watched lawsuit involving US patent policy in regard to genetics is a challenge brought by the American Civil Liberties Union (ACLU) and others against Myriad Genetics and the USPTO concerning its decision to issue patents that gave Myriad Genetics virtually exclusive commercial control over DNA diagnostic testing of the breast and ovarian cancer risk genes, *BRCA1* and *BRCA2*. Although the lawsuit attacked the issued patents on several grounds, the central argument was that a DNA sequence is an “unpatentable product of nature” (one of the exclusions from patentability recognized in patent law). A federal district (lower) court agreed with the plaintiffs, and Myriad appealed. The US Department of Justice intervened, filing a friend of the court brief that argued that it should be permissible to obtain patents on DNA sequences that have been altered for a beneficial purpose, but not on newly discovered sequence variations, such as those with mutations associated with a risk for breast cancer (essentially siding with the ACLU position). A federal appellate court reversed much of the district court’s decision in favor of Myriad (56), and the case was appealed to the United States Supreme Court. In 2012, the Supreme Court issued a decision in another case that in effect denied the patentability of a test based on a pharmacogenetic treatment algorithm (57). In so doing, it also returned the Myriad case to the appellate court for reconsideration in light of its new opinion.

Should the Myriad patent be invalidated, it will constitute a major revision in intellectual property policy, potentially threatening hundreds of other issued patents. Normally, a shift in policy of this magnitude is

implemented by federal legislation. Should it be implemented through litigation, it could have a chilling effect on commercial investment in genetic research. Regardless of whether Myriad Genetics prevails and the current rules on patenting DNA sequences for diagnostic purposes remain in place, there is sure to be much discussion about amending existing statutes to make such patents less restrictive. Recently, Belgium, France, and Switzerland enacted laws that can force foreign patent holders to grant use for public health needs. (53). In the United States, the SACGHS has recommended that there be a diagnostic use exemption for patents involving clinically relevant sequence variants. Of course, if such amendments to patent policy are enacted, industry may devote fewer resources to developing new tests.

Although bills proposing to amend patent laws are perennially before the United States Congress, there were few major changes to patent policy over the past half century. In 2012, the Congress passed and the President signed the Patent Reform Act of 2012. Its main impact is to adopt the “first to file” system, replacing the current “first to invent” system and bringing United States law in line with that of the rest of the industrialized nations. In addition, revisions to the concepts of novelty and nonobviousness may ultimately have some impact on patents involving DNA sequences. Part of the price of a patent is the disclosure of trade secrets. The new law includes provisions stating that failure to disclose the “best mode” cannot be used to invalidate or cancel a patent. This could be commercially beneficial to biotechnology companies by permitting them to maintain a competitive advantage even after a patent expires.

Although patent policy is determined by federal law, some states, perhaps concerned that GINA does not provide adequate protection against discrimination, are considering bills that would, if enacted, assert that genetic information is the property of the individual, “the unauthorized use of which interferes with both privacy rights and property interests of the individual.” This language is from a “Genetic Bill of Rights” introduced into the Massachusetts legislature in 2011 (58). If such laws are enacted, to the extent that they contradict federal intellectual property law, they will probably be overturned.

### 31.16 THE ORPHAN DRUG ACT

In 1983, the Congress passed and the President signed Public Law 97-414, popularly called the Orphan Drug Act (59). Few, if any, other laws have had such an important impact on patients with rare genetic disorders and their families. Essentially, the law offers a set of incentives to industry to pursue the development of drugs for comparatively small numbers of patients with uncommon disorders. It defines a “rare disease condition” as one that “affects less than 200,000 persons in the United States.” If a drug is approved by the FDA that has earned the designation as treating such a disorder, it is provided



with a substantial set of competitive advantages, the most important of which is blocking the approval of similar drugs for the same condition for 7 years. In Europe, analogous laws provide 10 years of market protection.

The ever-growing biotechnology industry makes extensive use of the Orphan Drug Act. For example, every drug approved in the United States in recent years for the lysosomal storage disorders, as well as many others, has been marketed with orphan drug status. In the 5-year period from 2007 through 2011, nearly one-third of all newly approved drugs were for orphan disorders. This protection is a positive incentive to the flow of capital into the biotechnology industry. Some, however, have argued that the law has anticompetitive effects and chills innovation (60).

### 31.17 CONCLUSION

For the most part, genetic information is being integrated into health care under a long-established medicolegal framework. Despite a growing stream of genetic data that is of potentially great importance to patients, few lawsuits have arisen from negligence in regard to the proper use or interpretation thereof. Concern over the duty to warn in genetic counseling has not emerged as a serious problem and is not likely to. The right to terminate a pregnancy before the point of fetal viability is under constant attack by those who oppose abortion. In the nearly 40 years since *Roe v. Wade* was decided, never has the membership of the United States Supreme Court been composed of a group that is in aggregate so unfriendly to *Roe*. Still, I think that the fundamental right provided by *Roe* will weather the storm. Genetic testing could with time become a regular feature of the adoption process, but it will not be used as a screening tool with which to select children. I think, instead it will be more akin to newborn screening—used to help the child. Virtually all aspects of assisted reproductive technology remain inadequately regulated in the United States (but better overseen and managed in Europe). Our society needs to craft relatively uniform social policies concerning the creation, storage, and disposition of frozen human embryos, sperm donation, and egg donation. Newborn genetic screening will continue to operate by consortia of state agencies, but with the rise of very low cost, high-throughput DNA sequencing, in time, a new legal model for newborn screening could emerge, including a private market. Prenatal screening, carrier testing, and direct to consumer genetic testing will continue to operate in a free market subject to the constraints imposed by the law of torts and contracts. Gradually, more regulatory structure will be imposed, eventually from a central authority such as the FDA. The long-standing and widespread fear of genetic discrimination has not materialized and, thanks to local and national laws that forbid it, it quite probably never will. The extant framework governing research with human subjects has demonstrated that it can properly

review and govern human genetic research (although it would be valuable to carefully reexamine the function of IRBs given that the rules that govern them have not been reconsidered in nearly 40 years). The vitriolic debate over the use of human embryos in research will fade away as the study of induced pluripotent cells becomes widespread. Existing patent rules can be effectively applied to intellectual property arising from DNA sequences. Recent litigation may be too disruptive of the system to be helpful. Legislative change is often more thoughtful and comprehensive. The Orphan Drug Act and related laws continue to constitute an important driver of large-scale commercial investment in genetic research.

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## RELEVANT WEBSITES

[www.genomicslawreport.com](http://www.genomicslawreport.com)  
[www.nsgc.org](http://www.nsgc.org)  
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### Biography

**Philip R Reilly, JD, MD**, is a Venture Partner at Third Rock Ventures in Boston, where he works to start companies to develop treatments for rare genetic disorders. From 2000 to 2006, he was the CEO and Chairman of the Board of Interleukin Genetics, Inc. Before joining Interleukin Genetics, Dr Reilly was the Executive Director of the Eunice Kennedy Shriver Center for Mental Retardation, Inc., a not-for-profit organization affiliated with the Massachusetts General Hospital. He has held numerous teaching positions, including Assistant Professor of Neurology at the Harvard Medical School and Adjunct Professor of Legal Studies at Brandeis University. Dr Reilly has served on many national committees chartered to explore public policy issues raised by advances in genetics. He is the author of six books and has published more than 100 articles in scholarly journals. Dr Reilly's most recent book, *The Strongest Boy in the World: How Genetic Information is Reshaping Our Lives*, was published by Cold Spring Harbor Laboratory Press in 2006. Dr Reilly has twice (2000 and 2003) been President of the American Society of Law, Medicine, and Ethics. From 1994 to 1997, he served on the Board of Directors of the American Society of Human Genetics. He is a founding fellow of the American College of Medical Genetics.

# CHAPTER

# 32

## Genetics of Female Infertility in Humans

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### 32.1 THE HYPOTHALAMIC–PITUITARY–GONADAL AXIS

Pubertal development and reproductive competence in mammals depend upon the coordinated efforts of the hypothalamic–pituitary–gonadal (HPG) axis. Gonadotropin releasing hormone (GnRH) neurons migrate from the nasal placode region into the hypothalamus along olfactory neurons. These neurons are dispersed throughout different regions of the brain although most affecting reproduction reside within the arcuate nucleus of the hypothalamus. In the arcuate nucleus, GnRH is synthesized and then secreted into the hypophyseal-portal vessels, where it then is delivered to the anterior pituitary gland (3). At the pituitary, GnRH binds to its G-protein-coupled transmembrane receptor in gonadotrope cells, which then synthesize and secrete the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH). The gonadotropins stimulate the synthesis and secretion of sex steroids in the gonads (testosterone in males; estradiol and progesterone in females). Sex steroid negative feedback in both sexes results in inhibition at both the hypothalamus and the pituitary (3). Numerous growth factors including inhibins (A and B), activins, and insulin-like growth factors also play a role in normal HPG function. In addition, numerous neuropeptides, as well as altered function of the adrenal or thyroid glands, affect GnRH pulsatility that can impair function. It should also be recognized that gonadotropin-inhibitory releasing hormone (GnIH) has been identified, and initial findings suggest that this peptide inhibits gonadal function at each compartment of the HPG axis (4).

Aberrations of the HPG axis may manifest a wide range of clinical disorders in both males and females (Table 32-1). Somewhat arbitrarily, they have been

categorized into hypothalamic, pituitary, gonadal, and outflow tract abnormalities. First, a reasonable, systematic approach to the diagnosis of reproductive dysfunction in females is outlined (Figure 32-1). The genetic basis for disorders that comprise each diagnostic category will then be reviewed. Patients with reproductive dysfunction may be classified according to their gonadal status—as eugonadal or hypogonadal. Predictably, hypogonadism normally results in a more severe phenotype such as delayed puberty and infertility compared with patients who underwent normal pubertal development but subsequently manifest infertility.

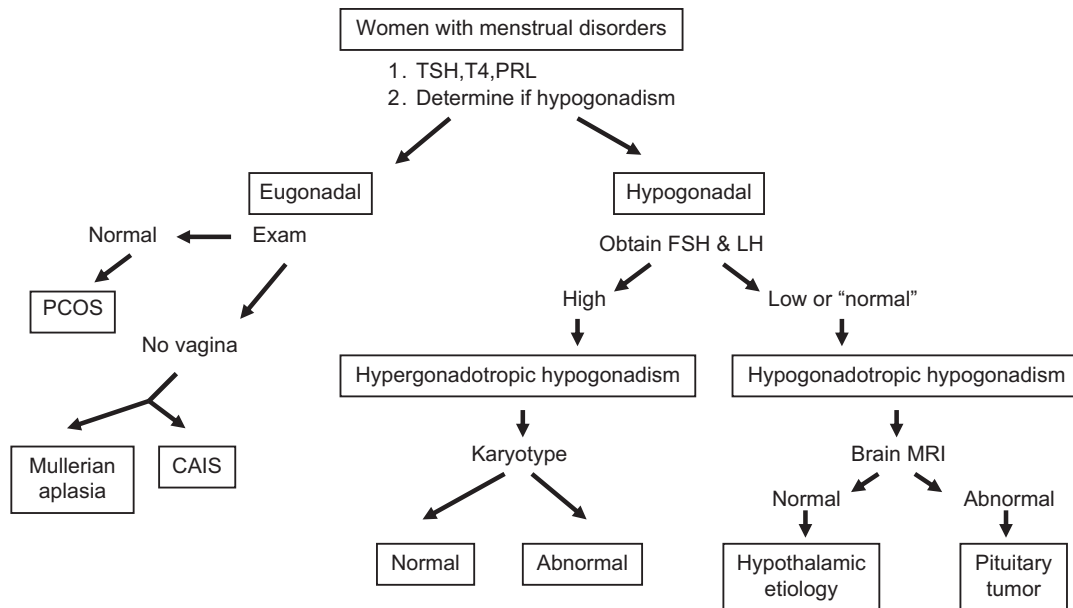
### 32.2 THE DIAGNOSIS OF HYPOGONADISM

The first step in identifying the underlying mechanism of reproductive dysfunction in females with menstrual dysfunction is to determine if hypogonadism is present (5).

**TABLE 32-1** Diagnostic Categories of Female Infertility

I. Hypogonadism
A. Hypogonadotropic hypogonadism
B. Hypergonadotropic hypogonadism
1. Abnormal chromosomes
a. Females: 45, X (with or without mosaicism); 46,XY
b. Males: 47,XXY; 46,XY
2. Normal chromosomes
II. Eugonadism
1. Ovulatory disorders—PCOS
2. Endometriosis
3. Disorders of the genital tract





**FIGURE 32-1** An overview of the diagnostic steps in females with reproductive dysfunction. The two most common causes of outflow obstruction causing primary amenorrhea are CAIS (complete androgen insensitivity syndrome) and Müllerian aplasia (Mayer–Rokitansky–Kuster–Hauser syndrome). PCOS=polycystic ovary syndrome; T4=thyroxin; TSH=thyroid stimulating hormone; PRL=prolactin. CDP=constitutional delay of puberty; IHH=idiopathic hypogonadotropic hypogonadism.

In females, serum estradiol levels are typically not accurate in the lower range, so it may be difficult to distinguish hypoestrogenism (<25 pg/mL) from normal early follicular phase levels (30–50 pg/mL). In the preovulatory period, the serum estradiol levels are usually about 200–300 pg/mL per mature follicle. If the patient completely lacks breast development, this is an important clinical indicator of hypoestrogenism (hypogonadism).

In females with breast development who have never menstruated (primary amenorrhea) and in young women with normal breast development who had menarche but have ceased menstruating (secondary amenorrhea), the determination of estrogen status is quite important (5). Both these clinical conditions indicate that estrogen was present at some time in the past, but this does not signify that estrogen status is now normal. Several different methods to assess estrogen status may be employed to determine estrogen status—the vaginal maturation index and a progestin challenge test. The presence of small parabasal cells (with a high nuclear/cytoplasmic ratio) obtained from a vaginal swab suggests hypoestrogenism, while the existence of superficial cells (large cytoplasm and small pyknotic nucleus) indicates normal estrogen status. Similarly, a reasonably normal 3–5-day menstrual withdrawal bleed following the administration of a progestogen indicates estrogen presence, while no bleed (or minimal spotting) suggests hypogonadism. Of course neither test is necessary if breast development is absent—this indicates a hypogonadal state. Importantly, the demonstration of hypoestrogenism does not define the specific etiology of the hypogonadism, it only indicates that hypogonadism is present (5).

**TABLE 32-2** Prevalence of Diagnostic Categories of Both Primary Amenorrhea (6) and Secondary Amenorrhea (7) in Females Are Shown

	Primary Amenorrhea (%) (6)	Secondary Amenorrhea (%) (7)
I. Hypogonadism		
A. Hypergonadotropic	43	11
1. Abnormal chromosomes	27	0.5
2. Normal chromosomes	16	10
B. Hypogonadotropic	31	42
1. Reversible	19	39
2. Irreversible	12	3
II. Eugonadism	26	46
1. Ovulation disorder	8	39
2. Genital tract obstruction	18	7

If the insult resulting in hypogonadism occurs before or during puberty, pubertal development may be absent or arrested. Delayed puberty in females is usually defined as either the absence of breast development (thelarche) by age 13 or the absence of menses (menarche) by age 15, both of which are 2.5 standard deviations above the mean for North American adolescents. However, hypogonadism may instead ensue after pubertal development has been completed. The most common diagnoses of hypogonadism are shown in Table 32-2. For patients with evidence of estrogen production, most patients with menstrual dysfunction will have anovulation—usually

caused by polycystic ovary syndrome (PCOS) (in 70%), but also by thyroid dysfunction or hyperprolactinemia. A small percentage of patients will present with uterine or vaginal outflow obstructive disorders, which will be determined by pelvic exam.

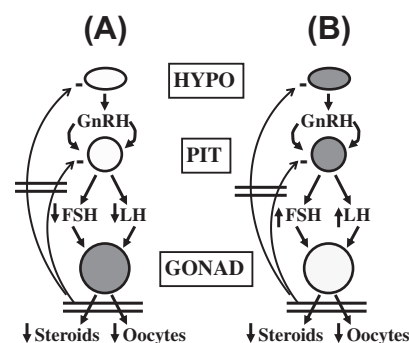
### 32.3 CATEGORIES OF HYPOGONADISM

Once the diagnosis of hypogonadism is established, the precise etiology should be determined. The hypoestrogenic state results in removal of gonadal steroid negative feedback, so the performance of serum FSH and LH levels will discern if the defect is gonadal or hypothalamic/pituitary in origin (Figure 32-2) (5). Elevated serum gonadotropins on at least two occasions several weeks apart indicate a diagnosis of *hypergonadotropic hypogonadism* (or gonadal failure). A karyotype should be performed in females with hypergonadotropic hypogonadism who fail to undergo normal puberty to rule out a chromosomal abnormality—most commonly, a 45,X cell line with or without mosaicism or a pure 46,XY cell line (6). As might be expected, these karyotypic abnormalities in hypergonadotropic hypogonadism are much more common in patients with primary amenorrhea rather than secondary amenorrhea (Table 32-2) (6,7).

When serum gonadotropins are inappropriately low (or normal) in the hypogonadal female, hypothalamic/pituitary dysfunction is present indicating *hypogonadotropic hypogonadism*. Once extreme stress, eating disorders, or excessive exercise are excluded and there is no central nervous system (CNS) tumor by MRI, the most common cause is normosmic idiopathic hypogonadotropic hypogonadism (nIHH) or Kallmann syndrome (KS) if idiopathic hypogonadotropic hypogonadism (IHH) is accompanied by anosmia (or hyposmia). Although the prevalence of karyotype abnormalities is much less frequent than those with hypergonadotropic hypogonadism, approximately 2–3% of patients with hypogonadotropic hypogonadism could have chromosomal rearrangements (8). In fact, one of these balanced translocations resulted in the discovery of a new gene (*WDR11*) involved in hypogonadotropic hypogonadism (9). In patients with extreme short stature and hypogonadotropic hypogonadism, other conditions such as growth hormone (GH) deficiency, hypothyroidism, or combined pituitary hormone deficiency (CPHD) could be present (3).

### 32.4 EUGONADAL INFERTILITY

If the patient with menstrual irregularity has evidence of adequate endogenous estrogen production, then anovulation, usually from PCOS, will be present in most patients if thyroid disease and hyperprolactinemia have been excluded. Less commonly in routine infertility patients, obstruction of the genital tract (uterus, vagina or both)



**FIGURE 32-2** The hypothalamus–pituitary–gonadal axis is shown. (A) Hypogonadotropic hypogonadism is depicted. Low estradiol fails to trigger a rise in GnRH and FSH indicating that the defect is at the hypothalamus or pituitary (the hypothalamus and pituitary are shaded lighter to reflect hypofunction). (B) Hypergonadotropic hypogonadism is shown. Low estradiol elicits an appropriate rise in hypothalamic GnRH and pituitary gonadotropins indicating the defect is the lack of a gonadal response (the ovary is shaded lighter to represent hypofunction).

could be present. However, in women with primary amenorrhea, the two most common eugonadal disorders include congenital absence of the uterus and vagina (CAUV) and complete androgen insensitivity syndrome (CAIS) (6). These disorders would be ascertained by the finding of an absent vagina on pelvic exam.

### 32.5 SPECIFIC DISORDERS IN EACH DIAGNOSTIC CATEGORY

The molecular basis for female infertility will now be detailed according to the diagnostic categories discussed above. Rarer but more serious hypogonadal causes of infertility will be considered—first hypogonadotropic hypogonadism and then hypergonadotropic hypogonadism. This will be followed by the more commonly encountered disorders in eugonadal individuals; unfortunately, the pathophysiologic basis for eugonadal causes of infertility (other than secondary to infection) is not as well understood as for the hypogonadal category.

### 32.6 HYPOGONADOTROPIC HYPOGONADISM

Patients with IHH who do not exhibit CNS tumors by MRI of the brain (with and without gadolinium) have a high likelihood of having a genetic form of hypogonadism. Hypoplasia or absent olfactory tracts and bulb suggests the diagnosis of KS, which pairs IHH with olfactory dysfunction (anosmia or hyposmia) (3). Other congenital anomalies may accompany IHH/KS, which could provide a clue to the etiologic gene. These include: unilateral renal agenesis, midline facial defects, dental aplasia, neurologic deficits such as synkinesia, hearing loss, or visual abnormalities as these may be associated with IHH/KS (3).

### 32.6.1 Genetic Causes of Hypogonadotropic Hypogonadism

Hypothalamic causes are discussed first (Table 32-3), followed by pituitary etiologies.

**32.6.1.1 KAL1.** Men and women with KS demonstrate IHH and anosmia, further supporting the common developmental pathway of GnRH and olfactory neurons, which migrate from the olfactory placode region into the brain. *KAL1* mutations on chromosome Xp22.3 have been described in males with X-linked recessive KS (10,11). *KAL1* encodes anosmin-1, a putative neural cell adhesion molecule is involved in the migration of GnRH and olfactory neurons during embryologic development. *KAL1* mutations account for ~33–70% of familial cases of KS and 3.1–27.8% of apparently sporadic forms of IHH with anosmia (12,13). There is currently no convincing evidence that *KAL1* mutations are pathogenic in females.

**32.6.1.2 FGFR1 and FGF8.** Mutations in the gene encoding the fibroblast growth factor receptor-1 (*FGFR1*) cause a form of KS designated *KAL2*. This gene was identified by positional cloning aided by the availability of several patients with contiguous gene deletion syndromes on chromosome 8p11.2–p12 who also had KS (14). No *FGFR1* gene deletions were identified in 43 additional KS patients, but 12/129 (9%) had heterozygous *FGFR1* point mutations (14). Subsequent studies of both males and females with autosomal dominant IHH/KS indicated that *FGFR1* mutations may cause either anosmic/hyposmic KS or nIHH (14–18), as well as in patients with subsequent reversal of their hypogonadism (19). Current evidence indicates that *FGFR1* mutations

occur in up to 10% of either KS or nIHH (17). Some patients with *FGFR1* mutations may also have associated neurologic abnormalities and midfacial defects similar to those with X-linked KS. As is true in many autosomal dominant diseases, a complicating and challenging feature among families with inactivating *FGFR1* mutations is variable expressivity. A number of mutations leading to nIHH or KS have been described, many of which impair fibroblast growth factor (FGF) signaling in vitro (14,16,17,20,21).

The human *FGFR1* gene is composed of 18 exons contained within 56 kb of genomic DNA encoding a single transmembrane receptor. The receptor consists of three immunoglobulin (Ig)-like domains, a heparin-binding domain, and two tyrosine kinase domains. *FGFR1* binds ligands FGF1 and FGF2 with high affinity, involving a K18K sequence essential for receptor activity and interaction with heparin (22). As both *FGFR1* and anosmin-1 utilize heparin sulfate proteoglycans, anosmin-1 could be a ligand for *FGFR1*. The importance of the FGF pathway in GnRH neuron development has been demonstrated by a number of studies. *FGFR1*–3 are expressed in the nasal placode about E10.5 in the mouse when GnRH neurons first appear. *FGFR1* and *FGFR3* are also expressed in GnRH neurons at E15.5 and postnatally in the mouse (23). In addition, an *FGFR1*-dominant negative mutant results in an attenuation of neurite outgrowth following stable transfection. These experimental findings indicate that FGF signaling is involved in GnRH neuron specification, migration, and axonal targeting (23).

Both activating and inactivating *FGFR1* mutations have been described. In fact, the first *FGFR1* mutations described were activating ones that cause craniosynostosis and skeletal anomalies such as craniofacial-skeletal dysplasia (Jackson–Weiss syndrome), Pfeiffer syndrome, trigonocephaly, osteoglophonic dysplasia, and Antley–Bixler syndrome (24–27). However, it is the inactivating *FGFR1* mutations that cause IHH/KS although skeletal effects may occur in both types of mutations.

Fortuitously, mutation in *FGFR1* was known to occur in a region where ligand FGF8 binds. Therefore, *FGF8* became a plausible physiologic candidate gene, and heterozygous mutations have been identified in about 1% of nIHH/KS patients (28). These findings suggested that *FGF8* mutations lead to autosomal dominant inheritance although one additional patient had biallelic mutations. Nevertheless, the finding that both *FGF8* and *FGFR1* genes possess mutations in nIHH/KS patients indicates the importance of FGF signaling in GnRH neuronal development and function.

**32.6.1.3 KISS1R.** Even though kisspeptins, which are ligands for G-protein-coupled receptor 54 (GPR54), were originally isolated from melanomas (the first kisspeptin was denoted as metastin), the significance of this pathway in reproduction did not become appreciated until the positional cloning of the *GPR54* (now known as *KISS1R*) gene in nIHH patients. Independently, two

**TABLE 32-3** Gene Mutations Affecting Hypothalamic Function in Females

Gene	Localization	Phenotype	Inheritance
<i>KAL1</i>	Xp22.3	KS	X-LR (males only)
<i>FGFR1</i> ( <i>KAL2</i> )	8p11.2–p11.1	IHH & KS	AD
<i>FGF8</i>	10q24	IHH & KS	AD
<i>KISS1R</i>	19p13.3	nIHH	AR
<i>LEP</i>	7q31.3	nIHH & obesity	AR
<i>LEPR</i>	1p31	nIHH & obesity	AR
<i>NROB1</i>	Xp21	AHC & nIHH	X-LR (females reported)
<i>PCSK1</i>	5q15–q23	Obesity, n IHH	AR
<i>PROKR2</i> ( <i>KAL3</i> )	20p13	KS	AR
<i>PROK2</i> ( <i>KAL4</i> )	3p21	KS & nIHH	AR
<i>CHD7</i> ( <i>KAL5</i> )	8q12	nIHH & KS	AD or sporadic
<i>NELF</i>	9q34	KS & nIHH	AR
<i>TACR3</i>	4q25	nIHH	AR
<i>TAC3</i>	12q13–q21	nIHH	AR
<i>GNRH1</i>	8p21–p11	nIHH	AR
<i>WDR11</i>	10q26	KS & nIHH	AD or sporadic

groups studied consanguineous families by linkage analysis and demonstrated highly significant LOD scores for chromosome 19p13, where *KISS1R* resides (29,30). In a Saudi Arabian family, DNA sequencing revealed a homozygous missense mutation, while another unrelated patient had compound heterozygosity for a nonsense mutation (R331X) and a read through mutation (X399R) (30). All three mutations demonstrated impaired signal transduction (reduced inositol phosphate production) in vitro (30). A French group identified a homozygous missense mutation and a homozygous 155 bp deletion in two different nIHH patients (29). The missense mutation was later found to also impair GPR54 signaling (31). No definitive mutations in the *KISS1* ligand have yet been identified in humans but they are likely to exist.

*KISS1R* mutations that were studied in vitro demonstrated reduced GnRH secretion, but had some impaired gonadotropin response to pulsatile GnRH (29,30). Affected females successfully responded to gonadotropin ovulation induction with achievement of normal pregnancy, delivery, postpartum recovery and lactation (32). As expected in an autosomal recessive disease, heterozygous offspring were normal (32). Further support for the role of GPR54 in normal puberty comes from the *KISS1R* knockout (KO) mouse, which was hypogonadal. Homozygous KO mice demonstrated a normal anatomic location of GnRH neurons and normal GnRH content in the brain, suggesting that *GPR54* signaling is important in GnRH release (30). Several additional rare sequence variants of *KISS1R* have been described, some of which are likely to be functional, but their consequences are not completely understood (33).

Kisspeptins are most highly expressed in the placenta, but they are also found in the anteroventralperiventricular (AVPV) nucleus and the arcuate nucleus of the hypothalamus. Neurons from these nuclei send projections to the medial preoptic area of the hypothalamus, where GnRH neurons reside and secrete GnRH into the hypophyseal-portal system. It is known that GPR54 is present in 50–75% of GnRH neurons (34–36). The transition from the prepubertal state to puberty is marked by kisspeptin activation of GPR54 (37). Kisspeptin infusions result in increased pituitary gonadotropin secretion, an effect that can be abolished by GnRH antagonist administration, indicating a hypothalamic effect of kisspeptin upon GnRH release (37). Gonadectomy increases and sex steroid replacement decreases kisspeptin expression in the arcuate nucleus, whereas the opposite is true in the AVPV nucleus (38). These findings suggest that kisspeptin signaling may be important in steroid feedback and the LH surge. Mutations in *KISS1R* nicely exemplify how the identification of even rare mutations in a gene (<1% of nIHH patients) can lead to profound improvement in our understanding of human physiologic processes.

**32.6.1.4 *LEP* and *LEPR*.** Leptin is a protein secreted by white adipose tissue that participates in body weight regulation by decreasing food intake and increasing energy

expenditure. Leptin is also involved in the modulation of neuroendocrine, reproductive, immune, and cardiovascular systems, particularly in energy deficient states. Its role in regulating the HPG axis in humans is supported by a study in which leptin administration resulted in ovulation in three of eight women with hypothalamic amenorrhea (39). Leptin binds to the leptin receptor (LEPR), a member of the cytokine family of receptors. Leptin signaling involves activation of the STAT3 pathway intracellularly as well as interaction with the melanocortin 4 receptor (MC4R) to produce anorectic effects (40).

Further support for leptin's role in reproduction has come from the identification of human mutations in individuals with severe, early-onset obesity. Mutations in leptin (*LEP*) (41–44) or *LEPR* (45,46) lead to hyperphagia and morbid obesity in early childhood. The first human *LEP* gene mutation was characterized in a family with two obese prepubertal children who had extremely low serum leptin levels, but all subjects were prepubertal (42). Subsequently, a second *LEP* mutation was identified in a consanguineous family containing three affected obese individuals (41). A 14-year-old female presented with primary amenorrhea and a 22-year-old male had irreversible pubertal delay, low testosterone, low gonadotropins, but a normal response to exogenous hCG and GnRH. Both affected family members demonstrated homozygosity for a missense *LEP* gene mutation impairing leptin secretion in vitro (41). The phenotype of humans with *LEP* mutations is similar to leptin-deficient *ob/ob* mice as they manifest extreme obesity, hyperinsulinemia, and hypogonadotropic hypogonadism, but unlike the mice, affected humans do not have hyperglycemia, hypercortisolemia, or stunted height (41). At least 12 *LEP* mutations have been identified to date in individuals with extreme, early-onset obesity, indicating that the prevalence in all IHH/KS patients is very low (47). The possibility of *LEP* mutations should be considered in nIHH patients with extreme obesity and low serum levels of leptin.

Leptin-resistant *db/db* mice have mutations of the *Lepr* gene, but until recently (47), only one human *LEPR* mutation was described (46). Leptin resistance was observed in a family of severely obese females with nIHH and elevated serum leptin levels. Homozygosity for an *LEPR* splice mutant resulted in exon skipping, thereby truncating the receptor so that it lacked both the transmembrane and intracellular domains (46). In addition to IHH, this patient also had mildly reduced secretion of GH and thyroid stimulating hormone (TSH) (46). Subsequently, a subset ( $n=300$ ) of a large cohort of 2100 early-onset obese subjects who were negative for mutations in known obesity genes was screened for *LEPR* mutations (47). Nearly 3% of subjects had *LEPR* mutations (missense or nonsense), all of which impaired *LEPR* signaling in vitro. Affected individuals also demonstrated hyperphagia, altered immune function, and delayed puberty secondary to nIHH. Of interest, serum leptin levels were not different from similarly obese patients



without *LEPR* mutations, and somewhat surprisingly, the clinical features of patients with *LEPR* mutations were less severe than those with *LEP* mutations (47).

**32.6.1.5 *NR0B1*.** Mutations in *NR0B1* result in an X-linked recessive form of IHH coupled with adrenal hypoplasia congenita (AHC) (48). This gene was cloned from the dosage sensitive sex (DSS) reversal region of chromosome Xp21 in humans, which when duplicated was reported to cause undermasculinization in 46,XY males (48,49). The identification of *NR0B1* was aided by the findings from patients with a contiguous gene deletion sequence on Xp21 manifesting Duchenne muscular dystrophy, glycerol kinase deficiency, as well as AHC/IHH. The human *NR0B1* gene encodes for the DAX1 (DSS-AHC critical region of the X chromosome, gene 1) protein, which is a member of the steroid hormone receptor superfamily that has no known ligand (and therefore is an orphan receptor). DAX1 is a transcription factor necessary for normal development of the hypothalamus, pituitary gonadotropes, and adrenal cortex (48,49).

Males with *NR0B1* mutations usually present with adrenal failure in infancy or childhood due to an underdeveloped permanent zone of the adrenal gland (48,49). Affected children who are adequately treated with adrenal steroids manifest delayed puberty due to IHH. Occasionally, patients develop adrenal insufficiency later in life or only after targeted testing of the adrenal gland (50–52). Unfortunately, patients with *NR0B1* mutations do not respond well to gonadotropins and may show minimal response to pulsatile GnRH, implicating an inherent defect in the gonads (53,54). A large variety of different *NR0B1* mutations, including deletions and point mutations, have been reported in AHC patients, which have been almost completely confined to males (55). However, a homozygous *NR0B1* mutation was reported in an IHH female who did not have adrenal failure but had two males with AHC in her family. Her homozygosity was thought to be due to a gene conversion event. Female carriers with *NR0B1* mutations rarely may manifest delayed puberty (56).

DAX1 regulates gonadotropin secretion at both hypothalamic and pituitary levels (55,57). It may also inhibit transcription of another important steroid receptor, Steroidogenic factor-1 (SF1), which is expressed in the same tissues and is important in sexual differentiation (55). Although initial data suggested that *NR0B1* might be an ovarian determinant gene because of its localization within the DSS region on Xp, a conditional KO of the mouse ortholog of *NR0B1* (*Nr0b1*) did not confirm this hypothesis (58). Instead, these mice demonstrated normal ovarian development and function; however, male mice displayed degeneration of testicular germinal epithelium independent of gonadotropin and testosterone abnormalities (58). These findings suggested that *Nr0b1* may be more important in spermatogenesis than in ovarian development.

**32.6.1.6 *PCSK1*.** We are only aware of only one case of IHH caused by a mutation in the prohormone convertase, subtilisin/kexin type 1 (*PCSK1*) gene (59–61). The affected female patient presented with extreme early-onset obesity, IHH, hypocortisolism, and abnormal glucose homeostasis. She had elevated plasma proinsulin and pro-opiomelanocortin (POMC) concentrations, and extremely low insulin levels (59–61). Compound heterozygous *PCSK1* mutations were detrimental: a frameshift mutation predicted protein truncation, while the missense mutation prevented processing and lead to its retention in the endoplasmic reticulum (59–61). Because of the similarity of this proband to that of the *fat/fat* mouse, it appears that molecular defects in prohormone conversion could constitute a mechanism for obesity and endocrine disease common to humans and rodents.

**32.6.1.7 *PROKR2* and *PROK2* (KAL3 & 4).** The genes for prokineticin-2 (*PROK2*) and its receptor (*PROKR2*) were first suggested as candidate genes for IHH/KS because the *Prokr2* KO mice, lacking a 7-transmembrane GPR, demonstrated impaired olfactory development and decreased numbers of GnRH neurons (62). Similarly, KO of the gene for the ligand, *Prok2*, impaired normal olfactory bulb development (63). When 192 KS patients underwent DNA sequencing for both genes, 10 mutations in *PROKR2* (5.2%) and 4 (2.1%) mutations in *PROK2* were identified (64). However, the mode of inheritance was unclear as *PROKR2* mutations were either heterozygous, homozygous, or compound heterozygous while the *PROK2* were heterozygous. Since these studies, it now appears that mutations in both genes are likely to be autosomal recessive as segregation analyses in informative families, in which heterozygous parents are unaffected, and only the homozygous offspring are affected (65). Therefore, it is possible that the reported prevalence of these mutations is somewhat overestimated as heterozygous carriers are included. However, the finding of either heterozygous *PROK2* or *PROKR2* mutations in IHH/KS patients could indicate that they possess a mutation in the second gene (which has been reported—see below).

**32.6.1.8 *CHD7* (KAL5).** Mutations in the human chromodomain helicase DNA-binding protein 7 (*CHD7*) gene cause CHARGE syndrome, an autosomal dominant or sporadic disorder consisting of Coloboma of the eye, Hear defects, choanal Atresia, Retardation of growth and development, Genitourinary anomalies, and Ear abnormalities (vestibular and auditory) (66). The gene was initially identified by studying two CHARGE patients with microdeletions on chromosome 8q12. The nine genes within this region were sequenced in 17 CHARGE syndrome patients without deletions, and 10 heterozygous *CHD7* mutations (7 nonsense, 2 missense, and 1 potential splice mutant) were identified (66). *CHD7* mutations occur in 60–80% of patients with CHARGE syndrome and span across the entire gene, but there is currently no correlation between genotype and phenotype and most

are de novo (66,67). *CHD7* appears to play an important role in the nucleus in binding to chromatin and regulating gene expression (68,69).

Mutations of *Chd7* generated by ENU mutagenesis techniques in mice corroborated human CHARGE syndrome. Phenotypic effects observed in these heterozygous mice included cleft palate, choanal atresia, cardiac septal defects, hemorrhage, prenatal death, vulvar and clitoral defects, and keratoconjunctivitis sicca (70). *Chd7* is ubiquitously expressed by semiquantitative RT-PCR in fetal and adult tissue, including the olfactory epithelium, eye, inner ear, and vascular system (66). At E10.5 in the mouse, *Chd7* is selectively expressed in the forebrain, olfactory pit, optic vesicle, hindbrain, cardiac outflow tract, facio-acoustic preganglion, and mandibular portion of the first branchial arch (71). Additionally, loss of function in gene-trapped reporter mice resulted in embryonic lethality at E10.5 (72), which suggests that homozygous mutations might also be lethal. *Chd7* expression in this relevant animal model was observed in the brain, retina, ear, craniofacial structures, pituitary, heart, and kidney at the expected time of endogenous *Chd7* mRNA expression (E10.5) (72).

Several studies implicated the *CHD7* gene in IHH/KS (73). The phenotypic findings of CHARGE syndrome and KS overlap as some CHARGE patients have anosmia and hypogonadotropic hypogonadism. However, many of the CHARGE patients were not yet pubertal, so the absolute diagnosis of IHH was not able to be satisfied. It was hypothesized that KS might represent a mild allelic variant of CHARGE syndrome, which was supported by the identification of sporadic heterozygous *CHD7* mutations in ~6% of nIHH and 6% in KS patients who did not fulfill criteria for CHARGE syndrome (73). Developmental expression within the hypothalamus and within the GnRH neuronal migratory pathway along with the presence of human mutations indicates that *CHD7* has an important role in puberty and reproduction (73).

**32.6.1.9 NELF.** The gene for nasal embryonic LHRH factor (*Nelf*) was cloned and characterized from migrating GnRH neurons using differential screening techniques in mouse (74). High levels of mRNA and protein expression were identified in the forebrain, olfactory epithelium, and olfactory pit of embryos with maximal expression between E12.5 and E14.5 in the olfactory epithelium and olfactory pit (74). These authors suggested that NELF may serve as a common guidance cue for olfactory axon projections and subsequent migration of GnRH neurons. The human ortholog was cloned, and one heterozygous missense mutation without functional analysis was reported (75). The first *NELF* mutation supported by in vitro analysis was reported to cause only KS when a coexistent *FGFR1* mutation was present (20). These findings suggested that digenic disease could be present in some patients (see below). Recently, the first human *NELF* biallelic mutations (with no mutation in any of 12 other IHH/KS genes) were identified in KS

(76). Interestingly, heterozygous *NELF* mutations were only found if there was a heterozygous mutation in a second gene. These findings in mice and humans indicate that NELF plays an important role in mammalian pubertal development (76).

**32.6.1.10 TAC3/TACR3.** Utilizing genome-wide SNP arrays in consanguineous Turkish families with nIHH, linkage was demonstrated to chromosome 4q25. Of the genes within this region, *TACR3*, encoding the GPCR-neurokinin B receptor (NK3R), appeared to be the most likely candidate and homozygous mutations were demonstrated in all affected individuals (77). NK3R is known to be highly expressed in hypothalamic neurons that also express kisspeptin. In other nIHH families, linkage to chromosome 12q13 was observed where the gene *TAC3* encoding the ligand neurokinin B (NKB) was located. Homozygous inactivating mutations were demonstrated in *TAC3* as well (77). Therefore, these studies demonstrated the importance of the neurokinin B system in the regulation of human puberty. Since this study, *TACR3* and *TAC3* mutations were studied in a large cohort of nIHH patients. Biallelic *TACR3* mutations were identified in 5.5% of patients, whereas *TAC3* mutations were rare and found only in two sisters from a consanguineous family (78).

The phenotype of females with *TAC3* and *TACR3* mutations is remarkably similar in that they all had primary amenorrhea and absent breast development (78). In addition, several patients showed spontaneous activity of the HPG axis upon discontinuation of hormonal treatment in adulthood demonstrating a reversible nature of the dysfunction. One patient with homozygous *TAC3* mutations experienced spontaneous puberty and pregnancy, whereas her sister with the same mutation had amenorrhea (78). This reversibility of hypogonadotropic hypogonadism with *TAC3/TACR3* mutations as opposed to the irreversibility of many other gene mutations such as in *KISS1R* is very interesting and requires further study. The exact mechanism of action of NKB and its receptor in mammalian puberty is not yet fully understood. It is postulated that they interact with kisspeptin as they colocalize in the same neurons and their action is expected to regulate release of GnRH from the hypothalamus (78).

**32.6.1.11 GNRH1.** The most obvious candidate gene for mutations in nIHH has been the GnRH ligand (*GNRH1*), particularly because the naturally occurring hypogonadal mouse with an intragenic deletion manifested hypogonadotropic hypogonadism (79), which was rescued by gene therapy with the wild-type *Gnrh1* (80). These studies were performed in 1986, and although a number of small studies in humans subsequently failed to demonstrate *GNRH1* mutations, it was not until 2009 that human mutations were finally discovered. Two different groups found biallelic *GNRH1* mutations in 1/146 (0.7%) (81) and 1/310 (0.3%) (82) nIHH patients, respectively in an autosomal recessive manner. This low prevalence explains

the previous negative studies. One of the more interesting facets of these findings is that GnRH is the first (and only at the time of this writing) hypothalamic releasing factor found to possess mutations in humans—none have been identified in the genes encoding GHRH, CRH, TRH, or somatostatin. These findings suggest that perhaps rarely, mutations will be discovered in these genes as well.

**32.6.1.12 WDR11.** Recently, the *WDR11* gene was identified by positional cloning in a KS patient with a 46,XY,t(10;12)(q26.12;q13.11) balanced chromosomal translocation (8,9). Chromosome 10q26 was hypothesized to represent the most likely region to contain the gene based on phenotypic overlap of IHH/KS compared to those with cytogenetic abnormalities of 10q26. As the breakpoint at 10q26 did not directly disrupt a gene, nearby genes expressed in IHH/KS relevant tissues were tested as positional candidates for mutations (9). Although *FGFR2* represented the most likely candidate based on similarity to known IHH/KS gene *FGFR1*, no mutations were found in this gene or in several others nearby. However, heterozygous missense *WDR11* mutations were identified in ~3% of IHH/KS patients (9).

The protein possesses 12 predicted WD (tryptophan/aspartic acid) domains that form two  $\beta$ -propellers, which are likely to be involved in protein/protein interactions. Confirmation of the human mutations was performed by in silico protein modeling and in vitro studies. Murine *Wdr11* was expressed in the developing olfactory and GnRH migratory pathway and in the adult hypothalamus. In addition, wild-type *WDR11* protein was found to colocalize with EMX1 in vivo and in vitro, and three of the human mutations had impaired EMX1 binding. Zebrafish *wdr11* was expressed throughout the brain at 24 h post-fertilization, which partially overlapped *emx1* expression. This expression was particularly noteworthy in the region of diencephalic GnRH3 neurons at 30–36 h (9). The characterization of human *WDR11* mutations in IHH/KS patients, which were absent in controls and supported by animal studies, and in vitro analysis indicate that *WDR11* likely plays an important role in human puberty. However, the precise function of *WDR11* in puberty will require additional study.

### 32.6.2 Digenic Mutations in IHH/KS

Mutations in more than one gene have been increasingly reported in IHH/KS. In fact, there are at least 12 different combinations described in 18 patients: (1) *PROKR2/KAL1*; (2) *FGFR1/NELF*; (3) *FGFR1/GNRHR*; (4) *FGFR1/FGF8*; (5) *PROK2/PROKR2*; (6) *FGFR1/PROKR2*; (7) *NELF/KAL1*; (8) *NELF/TACR3*; (9) *WDR11/KAL1*; (10) *WDR11/GNRHR*, (11) *KAL1/TACR3*, and (12) the oligogenic pattern of *KAL1/NELF/PROKR2* (83).

Interestingly, for most of these described digenic cases, a mutation in one of the genes would be sufficient to cause IHH/KS based on known inheritance of

monoallelic *FGFR1*, *FGF8*, and *WDR11* (autosomal dominant), monoallelic *KAL1* (X-linked recessive), or biallelic *GNRHR* (autosomal recessive) mutations (83). The prevalence of digenic mutations in IHH/KS has not been extensively reported, as most were reported as single cases. However, one large series in which eight genes (*FGFR1*, *KAL1*, *PROKR2*, *GNRHR*, *FGF8*, *KISS1R*, *NELF* and *PROK2*) were sequenced in 397 IHH/KS patients indicated that digenic disease occurred in 10/88 (11%) of patients who had one known mutation in one gene and 10/397 (2.5%) of all patients (84). When the 13 most common IHH/KS genes (*KAL1*, *GNRHR*, *FGFR1*, *KISS1R*, *TAC3*, *TACR3*, *FGF8*, *PROKR2*, *PROK2*, *CHD7*, *NELF*, *GNRH1*, and *WDR11*) were studied by another group, 6/48 (12.5%) of all IHH/KS patients studied had digenic disease (83). Therefore, most IHH/KS patients appear to have monogenic IHH/KS, given the current number of genes being studied. It has been suspected that digenic gene mutations could cause IHH/KS by synergistic heterozygosity (76). It is certainly feasible that many of the abovementioned genes are likely to function within the same pathway.

### 32.6.3 Adult-Onset Forms of IHH

Mutations in some genes such as the *GNRHR* may result in a phenotype that is mild—adult-onset IHH (see below), while others such as *FGFR1* and *TACR3* may predispose to spontaneous reversal of the phenotype. The female counterpart to male adult-onset IHH is hypothalamic amenorrhea, which is known to be associated with eating disorders, excessive exercise or extreme stress. Recently, a series of 55 females with hypothalamic amenorrhea were studied for mutations in IHH/KS genes, and two had heterozygous *FGFR1* loss-of-function mutations, of which could cause the phenotype (85).

### 32.6.4 Pituitary Causes of Hypogonadism

Mutations in at least seven genes result in impaired pituitary action. Some (*GNRHR*, *LHB*, and *FSHB*) affect only gonadotropin function, whereas the others may affect other pituitary functions (thyroid, adrenal, GH, and prolactin). The molecular basis for those with a known cause is detailed here (Table 32-4).

**32.6.4.1 GNRHR.** The pituitary-expressed GnRH receptor (*GNRHR*) gene represented the first identified gene to be involved in autosomal recessive nIHH in humans (86,87). The *GNRHR* belongs to the GPCR class of receptors, most of which possess an extracellular ligand-binding domain, a seven-transmembrane domain, three extracellular loops, three intracellular loops, and an intracellular carboxy terminal tail (although the *GNRHR* has no C-terminal tail).

To date, all *GNRHR* mutations have been identified in nIHH patients, most of whom have compound



**TABLE 32-4** Gene Mutations Affecting Pituitary Function

Gene	Localization	Phenotype	Inheritance
<i>GNRHR</i>	4q21.2	Normosmic HH	AR
<i>LHB</i>	19q13.3	Isolated LH deficiency	AR
<i>FSHB</i>	11p13	Isolated FSH deficiency	AR
<i>PROP1</i>	5q	CPHD2—short stature, hypothyroid, HH	AR
<i>HESX1</i>	3p21.1–21.2	Septo-optic dysplasia	AR or AD
<i>LHX3</i>	9q34.3	CPHD3	AR
<i>LHX4</i>	1q25	CPHD4	AD
<i>SOX2</i>	3q26.3–q27	Anophthalmia/microphthalmia; CPHD	AD
<i>SOX3</i>	Xq26	CPHD	X-LR

AR = autosomal recessive; AD = autosomal dominant; CPHD = combined pituitary hormone deficiency.

Note that CPHD1 due to *POU1F1* mutations is not shown here as it does not affect gonadotropins.

heterozygous missense mutations (88,89), while intra-genic deletions are uncommon (90). Human *GNRHR* mutations may affect ligand binding, signal transduction or both (88,89). Some *GNRHR* mutations also adversely affect activation of gonadotropin subunit or *Gnrhr* gene promoters in vitro (89). A number of the mutant *GNRHR*s can be rescued in vitro from misfolding and degradation within the cell by the addition of *GNRHR* antagonist IN3 (91).

The prevalence of *GNRHR* gene mutations has been estimated to be 3–5% of all normosmic IHH patients (88,89). Associated somatic anomalies seen with *FGFR1*, *CHD7*, or *KAL1* are uncommon. Of interest, two different missense mutations comprise about half of the reported mutant alleles. These mutations cause either complete IHH (no evidence of puberty) or incomplete IHH (partial evidence of puberty) although some genotypes are associated with mild disease in some families and severe disease in others. *GNRHR* mutations have also been reported to cause constitutional delay of puberty, and may be identified in patients with reversible IHH (88,89).

**32.6.4.2 LHB.** Isolated deficiencies of LH and FSH are exceedingly rare. The pituitary dimeric glycoprotein hormones consist of a common  $\alpha$ -subunit encoded by the chorionic gonadotropin- $\alpha$  (*CGA*) gene and a specific  $\beta$ -subunit gene that confers specificity for the four proteins—hCG, LH, FSH, and TSH. No human *CGA* mutations have been reported, but findings of the KO mouse indicate that the expected phenotype would include nIHH and hypothyroidism due to gonadotropin and TSH deficiency. Homozygous *Cga* KO mice have normal appearing neonatal gonads, but prepubertal adult gonads, which suggests that gonadotropins are not necessary for prenatal sexual differentiation (92).

The *LHB/CGB* gene complex in humans consists of six *CGB* genes with one *LHB* gene, which are highly homologous but polymorphic. However, there have only been several human *LHB* mutations, which are inherited in an autosomal recessive manner. At least five males have now been reported and all had small testes, oligospermia, and low testosterone (93). The first reported patient with an *LHB* mutation had elevated immunoreactive, but reduced biologically active, serum LH levels (94), whereas the others had low/undetectable LH levels and measurable to elevated FSH levels. Two reported females with *LHB* mutations demonstrated normal pubertal development probably because of normal FSH secretion, but became amenorrheic and had low LH with elevated FSH levels (93). It is likely that *CGB* mutations could be lethal in humans as placental production would be predicted to be deficient.

**32.6.4.3 FSHB.** Mutations in *FSHB* have been described in autosomal recessive isolated FSH deficiency (95,96). All mutations studied have been shown in vitro to result in low immunoreactive and low bioactive FSH levels (95,97,98). Most females completely lack sexual development, but some have had incomplete breast development. All females have presented with primary amenorrhea with low serum estradiol levels, very low or unmeasurable serum FSH, and elevated LH levels. This endocrinologic profile indicates that in the face of hypogonadism, GnRH appropriately rises and stimulates LH, which results in elevated serum LH levels. However, as there is an *FSHB* mutation, dimeric FSH is reduced, resulting in isolated FSH deficiency. Several males with *FSHB* mutations have had either normal pubertal development or delayed puberty, but all have been azoospermic.

Females with isolated FSH deficiency have elevated serum LH and exaggerated LH pulses, but somewhat surprisingly, they do not exhibit hirsutism or hyperandrogenism (99,100). Women with PCOS, who have similar LH pulses and elevated LH/FSH ratios, demonstrate hyperandrogenism. These findings challenge the tenants of the two-gonadotropin two-cell hypothesis, which states that LH stimulates ovarian thecal cells to produce androgens, which serve as precursors for estrogens in granulosa cells where FSH induces aromatization to estrogens (99,100). In fact, one woman with isolated FSH deficiency only had a rise in serum testosterone level after FSH followed by LH administration (not LH alone). The fact that isolated FSH deficiency is associated with low androgens suggests that FSH could play an important role in ovarian androgen production, perhaps by increasing LH receptors, or by inducing CYP17 enzyme activity (an androgen dependent enzyme), inhibin, or growth factors (99,100). Ovarian follicles typically show primordial, primary, and antral follicles, and normal fertility may be restored with FSH treatment.



### 32.6.5 Combined Pituitary Hormone Deficiency

CPHD is defined as the deficiency of GH plus at least one additional pituitary hormone (ACTH, TSH, prolactin, FSH, or LH). Mendelian Inheritance in Man (MIM) lists CPHD1 (MIM 613038), CPHD2 (MIM 262600), CPHD3 (MIM 221750), and CPHD4 (MIM 262700); and all except CPHD1 (*POU1F1*) affect gonadotropins. If all pituitary hormones are deficient, the patient has panhypopituitarism, which may be fatal unless treated, especially because of subsequent adrenal insufficiency. If this CPHD is congenital, the patient should display extremely short stature due to deficiency of TSH and GH. The genetic basis for forms of CPHD that affect the reproductive axis is now discussed (Table 32-5).

**32.6.5.1 *PROP1* (CPHD2).** Mutations in pituitary transcription factors have been shown to cause isolated hypogonadotropic hypogonadism in association with deficiencies of other pituitary hormones. *PROP1* is important for early pituitary development as mutations of *PROP1* result in CPHD in humans. *PROP1*-deficient patients have an autosomal recessive form of TSH, GH, prolactin, and gonadotropin deficiency (101,102). Affected individuals manifest extremely short stature secondary to deficient TSH and GH levels and may have a reduced ability of their pituitary to respond to the corresponding releasing factors. Many different mutations have been characterized including missense mutations and small deletions (101,102) and mutations of the mouse ortholog—*Prop1*—cause a similar phenotype in the Ames dwarf mouse. *PROP1* mutations are

rare in IHH patients without failure of other pituitary hormones.

**32.6.5.2 *HESX1*.** Homozygous and heterozygous mutations in *HESX1*, which encodes a transcription factor, have been identified in families with affected individuals with septo-optic dysplasia, a disorder characterized by panhypopituitarism, optic nerve atrophy, and other midline CNS abnormalities including agenesis of the corpus callosum and septum pellucidum (Table 32-5) (104,105). The mouse ortholog (*Hesx1*) is expressed in early development of the forebrain. Later, *Hesx1* expression is restricted to Rathke's pouch, which ultimately becomes the anterior pituitary gland. Since panhypopituitarism occurs, IHH is a common feature of septo-optic dysplasia.

**32.6.5.3 *LHX3* (CPHD3) and *LHX4* (CPHD4).** Two other genes contribute to the pathophysiology of autosomal CPHD. *LHX3* mutations cause autosomal recessive CPHD (106), whereas *LHX4* mutations cause an autosomal dominant form. The phenotype of patients with *LHX3* mutations consists of severe growth retardation (secondary to GH and TSH deficiency) and hypogonadotropic hypogonadism. All pituitary hormones (GH, TSH, prolactin, FSH, and LH) except ACTH may be deficient (106). Interestingly, there is severe restriction of cervical spine rotation, which results in elevation and anteversion of the shoulders similar to what is seen with mutation in the mouse ortholog. Although a family with TSH, GH, and ACTH deficiency due to a heterozygous *LHX4* mutation has been described, the child was prepubertal, so gonadotropin levels were not investigated (107). *LHX4* should also result in gonadotropin

**TABLE 32-5 Single-Gene Disorders in Hypergonadotropic Hypogonadism in Females**

Gene	Localization	Phenotype	Inheritance
<i>SRY</i>	Yp11.3	Swyer syndrome (genetic males)	Sporadic, Y-linked
<i>POF1</i>	Xq26q28	Ovarian failure (F)	Sporadic or XLD
<i>POF2</i>	Xq13.3–q22	Ovarian failure (F)	Sporadic; or XLD
<i>DIAPH2</i>	Xq22	Ovarian failure (F)	Disruption in X-auto-some translocation
<i>FMR1</i>	Xq27.3	Fragile X syndrome (M); ovarian failure (F)	XLD
<i>BMP15</i>	Xp11.2	Ovarian failure (F)	XLR
<i>FSHR</i>	2p21p16	Primary amenorrhea (F); oligospermia (M)	AR
<i>AIRE</i>	21q22.3	APECED	AR
<i>POF3 (FOXL2)</i>	3q23	BPES (M & F); with ovarian failure (M)	AD
<i>NOBOX</i>	7q35	Ovarian failure	AD, sporadic
<i>FIGLA</i>	2p12	Ovarian failure	Sporadic; possible AD
<i>NR5A1</i>	9q33	Adrenal failure/sex reversal (M); POF (F)	AR or AD
<i>GALT</i>	9p13	Galactosemia (with ovarian failure)	AR
<i>EIFB2</i>	14q24	Ovarioleukodystrophy	AR
<i>EIFB4</i>	2p23.3	Ovarioleukodystrophy	AR
<i>EIFB5</i>	3q27	Ovarioleukodystrophy	AR
<i>CYP17A1</i>	10q24.3	17-hydroxylase deficiency	AR
<i>CYP19A1</i>	15q21.1	Aromatase deficiency	AR

XLD = X-linked dominant; XLR = X-linked recessive; AR = autosomal recessive; AD = autosomal dominant.

LHR mutations can also cause gonadal failure in males with varying degrees of sexual ambiguity; in females, it causes anovulation and is included in Table 32-6.

deficiency, and this was recently described as affected children were followed into pubertal age (108,109).

**32.6.5.4 SOX2 and SOX3.** Mutations in *SOX2* and *SOX3*, both of which are high-mobility group (HMG) box transcription factors, have been described in humans with hypogonadotropic hypogonadism. Heterozygous de novo *SOX2* mutations were identified in patients with developmental delay, bilateral anophthalmia or microphthalmia, short stature, and male genital tract abnormalities. Recently, mice heterozygous for a targeted *Sox2* disruption did not have eye defects, but did demonstrate abnormal anterior pituitary development with GH, TSH, and LH deficiency (110). In a large series of patients with “congenital hypothalamic-pituitary disorders,” 8/235 (3.4%) had heterozygous *SOX2* sequence variations, most of which were de novo (110). Mutations were nonsense, frameshift deletions/insertions, or missense mutations that impaired function (DNA binding, nuclear translocation, or transactivation). Patients with *SOX2* mutations had eye defects, along with anterior pituitary hypofunction including IHH. However, additional defects of the corpus callosum, hypothalamic hamartomas, sensorineural hearing loss, and esophageal atresia were also observed (110). These findings in both mouse and human suggest that *SOX2* is necessary for normal reproductive development and function.

*SOX3* mutations cause an X-linked recessive form of pituitary deficiency. Several *SOX3* mutations have been reported, the first being an in-frame 33-bp duplication in a family with X-linked mental retardation, facial abnormalities, and GH deficiency (111). Another ~686 kb duplication was identified in two siblings with variable degrees of hypopituitarism, corpus callosum abnormalities, anterior pituitary hypoplasia, an ectopic posterior pituitary, and an absent infundibulum (112). Mental retardation, however, was not present in these siblings. Corroborative findings have been identified in mice with targeted deletion of *SOX3*. The phenotype consisted of impaired pituitary development and midline structures, and these pituitary and hypothalamic defects persisted postnatally (113).

## 32.7 HYPERGONADOTROPIC HYPOGONADISM

Women with hypergonadotropic hypogonadism may either have a normal 46,XX karyotype or have aneuploidy or a structurally abnormal X chromosome (Table 32-5). Not unexpectedly, the greatest risk for a chromosome abnormality is for females with primary amenorrhea, rather than those with secondary amenorrhea. The results from several very large clinical studies of women with primary and secondary amenorrhea are shown in Table 32-2 (6,7). More than two-thirds of females with primary amenorrhea had a karyotypic abnormality vs ~1% of women with secondary amenorrhea (6–7).

### 32.7.1 X Chromosome Abnormalities

Females completely lacking an X chromosome include those with pure 45,X karyotypes as well as those with another cell line (46,XY, 46,XX, 47,XXX, or 46,X iXq). About 90% of females with a 45,X cell line with or without mosaicism present with primary amenorrhea, the complete lack of sexual development, and irreversible ovarian failure (6,114). About 5–10% of 45,X females have normal puberty and menarche, but these menses are usually short-lived and often cease before age 40. Women with a 45,X cell line who menstruate may be fertile, but reproductive loss may occur in the form of spontaneous abortions, stillbirths, and chromosomal abnormalities (including 45,X and Down syndrome) and is common (114). Patients with a 45,X cell line (Turner syndrome) manifest phenotypic features such as short stature, widely spaced nipples, webbed neck, shield chest, multiple skin nevi, and a short fourth metacarpal. However, the most consistent feature is short stature with heights under 5 ft. The most serious associated somatic anomalies include cardiac (in about 50%) and renal abnormalities (114). A dilated aorta has a several percentage estimated chance of rupturing during pregnancy, suggesting that donor egg in vitro fertilization should be discouraged (115). If there is a coexistent Y chromosome with a 45,X cell line, gonadoblastomas may occur within the streak gonads, which may give rise to more serious germ cell tumors. Therefore, the gonads should be removed in these patients with a Y cell line.

Individuals with a 45,X/46,XY karyotype may manifest any of several different phenotypes. If bilateral abdominal streak gonads are present, the phenotype resembles other 45,X females who do not have a Y cell line with short stature, absent breast development, but with an intact vagina and Müllerian system. If an intra-abdominal streak and a contralateral testis either in the abdomen or in the labioscrotum are present, sexual ambiguity will result. Much less commonly, 45,X/46,XY patients have bilateral scrotal testes, which renders them a male phenotype (116).

Turner syndrome is thought to result from haploinsufficiency of multiple genes on the X chromosome that affect embryologic development, stature, and ovarian function. The short stature likely results from the deletion of one allele of *SHOX* (short stature homeobox gene), a transcription factor on Xp22 that is primarily expressed in osteogenic cells (117). Similarly, patients with idiopathic short stature (without Turner syndrome) may harbor mutations in *SHOX*. The phenotype of patients possessing *SHOX* mutations varies depending upon the mutation type—either Langer mesomelic dysplasia resulting from deletions or Leri-Weill dyschondrosteosis, a skeletal dysplasia with disproportionate short stature, mesomelic limbs, and the Madelung deformity (a radial bone anomaly also sometimes seen in Turner syndrome) caused by nonsense mutations (117,118).

Partial deletions of the X chromosome may also impair ovarian function. In general, deletions affecting Xp11 result in ovarian failure in about half of the patients, while the other half have menstrual function (119). Even if menstrual function is normal, fertility is typically impaired. With more distal deletions, such as at Xp21, the phenotype is usually less severe. Most women with Xp deletions are short, regardless of ovarian function, further supporting that other statural determinant genes could reside within these regions. Several families with Xp deletions have also been reported (119).

Deletions of Xq may also result in ovarian failure. Similar to Xp deletions, proximal Xq (such as Xq13) deletions are usually more severe, and these patients have absent breast development, primary amenorrhea, and gonadal failure. If the deletion involves more distal Xq, menarche may occur with or without ovarian failure. Familial forms of Xq deletions manifesting as ovarian failure have also been reported. The mechanism of ovarian failure in patients with X chromosome abnormalities has not been clearly elucidated, but could involve a dosage phenomenon, especially if the involved gene/genes do not escape X-inactivation. Deleted/disrupted ovarian determinant genes probably increase follicular atresia of the ovary similar to that seen in patients deleted of an entire X chromosome. It is also possible that the Xq deletion contains genes that might affect mitosis or meiosis, which could result in enhanced follicular atresia (see specific genes below). Chromosomal rearrangements involving the X chromosome have also been reported to disrupt ovarian gene function (119).

### 32.7.2 Y Chromosome Disorder—Swyer Syndrome

Although patients with Swyer syndrome have a pure 46,XY cell line, their phenotype is that of a sexually delayed female. These patients will have bilateral streak gonads, normal stature, and a normal uterus and vagina without sexual ambiguity. They appear to have the highest risk of developing tumors in their dysgenetic gonads (up to 25%); therefore, extirpation of the gonads should be performed after the diagnosis (120).

The gene for the sex-determining region of the Y chromosome (*SRY*) resides on distal Yp. When *SRY* is present, the undifferentiated gonad will develop into a testis, which will elaborate anti-Müllerian hormone (AMH) to remove the Müllerian system, followed by the secretion of testosterone for sexual differentiation. In Swyer syndrome patients, the gonad is dysfunctional (and replaced by fibrous streaks), so that AMH and testosterone are not produced. The lack of AMH leads to the retention of the Müllerian system, while the reduction in normal Leydig cell function results in low testosterone and absent male sexual development. Therefore, the patient presents as a phenotypic female without breast development. Most cases of Swyer syndrome are sporadic and only about

15% are caused by *SRY* mutations, which is a single-exon gene encoding a transcription factor with a conserved HMG domain. Therefore, other yet to be discovered genes must be involved in the pathogenesis (120,121). Other genes may cause disorders of sexual development resulting in sexual ambiguity, but they will be covered elsewhere. Swyer syndrome is included here because it results in gonadal failure in phenotypic females.

### 32.7.3 46,XX Ovarian Failure

Women with hypergonadotropic hypogonadism are said to have premature ovarian failure (POF) when this occurs in about 1% of women before age 40. Ovarian function may wax and wane; and pregnancy may occasionally occur spontaneously. These patients may sometimes bleed when the progestin withdrawal test is performed if there is some residual ovarian function. This has prompted some investigators to suggest that it should be called hypergonadotropic amenorrhea or primary ovarian insufficiency (122). Nevertheless, most patients with hypergonadotropic hypogonadism demonstrate ovarian failure. For practical purposes, the molecular basis for most patients with amenorrhea, elevated gonadotropins on two occasions, and a 46,XX karyotype is unknown if known causes such as pelvic radiation, chemotherapy, or surgical extirpation are excluded. Clinical studies have suggested that many of these women may have an autoimmune basis for their hypogonadism as other associated endocrinopathies such as hypothyroidism, adrenal insufficiency, and diabetes may be present. However, a small percentage of patients have been found to have single-gene disorders (see below) (122).

### 32.7.4 Single-Gene Disorders Causing Ovarian Failure and Infertility

The Xq13–Xq26 region has been previously suggested to contain ovarian determinant genes, but surprisingly few have been identified. On the basis of these findings, the Xq26–q28 region has been designated POF1, whereas Xq13.3–q21.1 has been identified as POF2. As detailed in MIM, there are currently eight forms of POF—POF1 (MIM 311360); POF2A (MIM 300511); POF2B (MIM 300604); POF3 (MIM 608996); POF4 (300510); POF5 (MIM 611548); POF6 (MIM 612310); and POF7 (MIM 612964). The *FMR1* gene, which causes fragile X syndrome, resides within POF1 region, whereas *DIAHP2* and *POF1B* genes reside within the POF2 region. The POF3–POF7 regions are found on autosomes (except POF4, which is located on the X chromosome) and contain genes possessing human mutations backed by functional studies (Table 32-5). The X chromosome genes will first be reviewed followed by the autosomal genes. If the gene is relevant to males, this will only be briefly mentioned as this will be covered elsewhere.

### 32.7.5 Single-Gene Disorders of the X Chromosome Associated with Ovarian Failure

The prevalence of mutations in known genes for patients with 46,XX ovarian failure except fragile X syndrome is rare. Nevertheless, even rare-mutation-containing genes can provide insight into mechanisms of disease.

### 32.7.6 Diaphanous 2 (*DIAPH2*) within the POF2 Region

*DIAPH2* was the first gene suggested to be an ovarian determinant gene. A woman with POF had a balanced t(X;12)(q21;p1.3) that appeared to disrupt diaphanous 2 (*DIAPH2*) gene. However, the importance of this gene remains speculative as no point mutations have yet been described (123). Disruption of *DIAPH2* was proposed to be causative in this patient as this gene has high homology to *dia* in *Drosophila*, which is expressed in the testes and ovary, and results in sterility when mutated (123). Of interest, the other gene within this region (*POF1B*) has not conclusively been shown to possess mutations that are not seen in controls with normal ovarian function.

### 32.7.7 *FMR1* within the POF1 Region

Fragile X syndrome is the most frequently recognized single-gene disorder that includes POF. Fragile X syndrome is an X-linked dominant disorder with incomplete penetrance characterized by large ears and jaws, varying degrees of mental deficiency, and macro-orchidism in affected males. The mental deficiency may be extremely variable being subtle or manifested solely as autism. *FMR1* resides at a fragile site of Xq27 (POF1 region) and contains a triplet repeat of CGG nucleotides ranging from 6 to 50 copies in unaffected individuals. Affected males have a full expansion to  $\geq 200$  repeats, while carrier females have 50–200 repeats (the premutation allele). Premutation alleles are unstable and may expand in meiosis in carrier females such that the full expansion results, which gives rise to an affected male. Some females who possess premutation alleles may also manifest a mild degree of mental deficiency or a learning disability.

Fragile X males have macro-orchidism and normal testicular histology, but ~15% of females with premutation alleles ascertained through fragile X families may have POF (124). If large cohorts of females with POF are analyzed (rather than Fragile X families), the risk of carrying the *FMR1* premutation allele is approximately 3–4% if there are no other family members with POF, but up to 12–15% if there are at least two affected females within the family (125). It is interesting that no women with full *FMR1* mutations had POF—it was only the carrier females. The fragile X full mutation is a

null allele with impaired methylation and protein inactivation. However, the premutation allele is associated with mRNA overexpression, and has been found to be expressed in the ovary. These considerations must be taken into account when genetic counseling is provided to women with POF who possess an *FMR1* premutation allele. Should pregnancy occur, half of her sons will have fragile X syndrome and half of her daughters will be carriers. Even if the identified premutation carrier female does not conceive, other family members could potentially be carriers and be at risk for fragile X syndrome. It is also known that males with premutation alleles could later manifest an adult-onset tremor ataxia syndrome (122).

### 32.7.8 Bone Morphogenetic Protein-15 (*BMP15*) Gene in POF4

*BMP15* mutations were first described in a family with two young women (ages 23 and 18), who had primary amenorrhea, hypoplastic ovaries, and elevated gonadotropin levels. A heterozygous missense *BMP15* mutation was identified in both of the affected females transmitted from the father. The mutation was not observed in 210 controls, and in vitro analysis supported a functional effect upon protein action (abnormal processing, impaired granulosa cell growth, and antagonism of wild-type *BMP15* stimulated granulosa cell growth) (126). Subsequent studies of large cohorts of POF women suggest that the prevalence of *BMP15* mutations is <1% (127). Most mutations are missense mutations, which appear to impair secretion.

### 32.7.9 Single-Gene Disorders of the Autosomes Associated with Ovarian Failure

Interestingly, some of the autosomal genes possessing mutations in POF women, which were identified before POF 3, 5, 6 and 7, are not designated in such a manner in MIM. Known human autosomal genes resulting in ovarian failure are discussed.

**32.7.9.1 Follicle Stimulating Hormone Receptor (*FSHR*).** *FSHR* gene mutations have been identified to cause autosomal recessive POF (128). The FSH ligand binds to this heptohelical GPCR located on the cell surface of ovarian granulosa cells, which then results in estradiol production. Most affected females described present with primary amenorrhea, and about half have breast development, while the others have not initiated thelarche. Some of the women with normal breast development may have menstrual periods before becoming amenorrheic and hypogonadal. Ovarian follicles range from primordial to mature, indicating that the defect is not as severe as mutations of the *FSHB* ligand (see *FSHB* gene in pituitary disorders). Interestingly, males with *FSHR* mutations are fertile. Identified *FSHR* mutations



may affect binding, signal transduction, or both. Mutations of the other gonadotropin (LH) receptor (*LHR*) usually cause anovulation in females (and are discussed under eugonadism).

**32.7.9.2 Autoimmune Regulator (*AIRE*).** The autoimmune polyglandular syndrome type 1, also known as autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED), is an autosomal recessive multisystem autoimmune disease. APECED is more frequent in Finnish individuals and Iranian Jews. Moniliasis is a common initial presenting feature in over half of the patients, but can occur at anytime. Hypoparathyroidism (80%) and adrenal failure (70%) are the most frequent endocrinopathies of the disorder, but ovarian failure (60%) and testicular failure (14%) may also occur (129,130).

Mutations in the *AIRE* (autoimmune regulator) gene, which encodes a transcriptional factor with two PHD-type zinc-finger motifs, have been identified in APECED patients (129,130). The elucidation and characterization of *AIRE* mutations represented the first instance of a single-gene defect causing systemic autoimmune disease in humans. Approximately 80% of *AIRE* mutations in Finnish patients consist of a single nonsense mutation (Arg257X), while a 13-bp deletion accounts for over half of the mutations in North American patients. A single nonsense mutation (Arg139X) occurs in about 80% of Sardinian *AIRE* alleles (129,130). Currently, it is not clear whether specific mutations increase the likelihood of ovarian failure vs other endocrinopathies.

**32.7.9.3 Forkhead Transcription Factor (*FOXL2*) in POF3.** In a fascinating biological phenomenon, a syndrome comprised of blepharophimosis (narrowing of horizontal opening of the eyelids), ptosis, and epicanthus inversus syndrome (BPES) may include POF (BPES type I) or not (BPES type II). In both types of diseases, which are inherited in an autosomal dominant manner, heterozygous *FOXL2* mutations have been identified. Why mutations of this gene affect eye lid development and normal ovarian function, while sparing testicular function (gonadal failure is sex-limited) is perplexing. Most patients with type-I and -II BPES have mutations in one of the two hotspots of the *FOXL2* gene on chromosome 3q23 (131). Thirty percentage of mutations lead to polyalanine expansions and 13% consist of frameshift duplications. The prevalence of *FOXL2* mutations in isolated POF without BPES is likely to be rare, but has been described (132).

**32.7.9.4 Newborn Ovary Homeobox, Mouse, Homolog of (*NOBOX*).** *NOBOX* is a homeobox gene localized to chromosome 7q35 expressed predominantly in the ovary and testis. Of interest, *Nobox* KO male mice were fertile, but females had atrophic ovaries. *Nobox* is preferentially expressed in the oocyte and has a role in folliculogenesis and regulation of oocyte-specific genes in the mouse. One of the 96 Caucasian POF patients had a heterozygous missense mutation not present in 278 ethnically matched controls. Its role in POF is currently unclear, but is probably uncommon (133).

**32.7.9.5 Factor in Germline Alpha (*FIGLA*) in POF6.** Two of the 100 Chinese POF women were recently described to possess small deletions (3bp and 22bp) in the *FIGLA* gene on chromosome 2p12 that were absent in 340 female controls with regular menses and no history of infertility (134). *FIGLA* encodes a germ-cell-specific transcription factor, which regulates expression of genes in the oocyte. Both women with the mutations had secondary amenorrhea and hypergonadotropic hypogonadism. One of these deletions disrupted *FIGLA* binding to the TCF3 helix-loop-helix domain, while the other resulted in a frameshift (134).

**32.7.9.6 Nuclear Receptor Subfamily 5, Group A, Member 1 (*NR5A1*) in POF7.** SF1 is a vital transcription factor regulating steroidogenesis in the gonad and adrenal glands. It is encoded by the *NR5A1* gene, which has been known as *FTZF1*. Similar to the targeted disruption of the mouse ortholog, a mutation in the *NR5A1* gene caused undermasculinization of 46,XY males and adrenal failure (135). However, *NR5A1* mutations are now known to cause a wide range of disorders of sexual differentiation with and without adrenal failure that may be inherited as autosomal recessive or autosomal dominant traits (136).

The phenotype in genetic females was predicted to consist of adrenal failure and delayed puberty with absent breast development and primary amenorrhea, with elevated serum gonadotropins. It does now appear that mutations do cause 46,XX ovarian failure with or without adrenal failure. Interestingly, these individuals with POF can be noted in families with affected males. In fact, two of 25 (8%) POF women with normal adrenal function were found to harbor heterozygous *NR5A1* mutations consistent with autosomal dominant inheritance (136).

**32.7.9.7 Galactose-1-Phosphate Uridyltransferase (*GALT*).** Galactosemia is an autosomal recessive disease in which galactose cannot be properly metabolized to glucose. The phenotype usually consists of failure to thrive, nausea, vomiting, hepatomegaly, cataracts, mental retardation, speech abnormalities, and hemolytic anemia. If treated by a galactose-free diet, the prognosis for liver function and mental capacity is improved but not to the normal level. Several different enzymes are involved in galactosemia, but *GALT* mutations have been shown to have a sex-specific effect upon reproduction in that females, but not males, develop gonadal failure (119). The enzyme encoded by *GALT* converts galactose-1-phosphate and UDP-glucose to UDP-galactose and glucose-1-phosphate.

About two-thirds of women with galactosemia had POF, while none of the eight men had testicular failure (137). These females had normal pubertal development, but half presented with primary amenorrhea, and the remainder had secondary amenorrhea. However, LH and FSH levels were elevated in both groups. The precise etiology of ovarian failure in galactosemia is unknown, but a detrimental metabolic developmental defect could be caused by galactose-1-phosphate or by abnormal

glycosylation of gonadotropin glycoproteins or their receptors. More than several hundred *GALT* mutations have been described, many of which are missense mutations. Heterozygotes do not develop gonadal failure. Interestingly, the *Galt* KO mouse has a less-severe phenotype, with no gonadal developmental defect or dysfunction (138).

### 32.7.10 Eukaryotic Translation Initiation Factors 2, 4, and 5

Leukoencephalopathy with vanishing white matter is caused by mutations in any of the five subunits of the translation initiation factor eIF2B. It has also been reported that patients with leukoencephalopathy with vanishing white matter and ovarian failure (also known as ovarioleukodystrophy) may possess mutations in three of the subunits (2, 4, or 5) (139). All are inherited in an autosomal recessive manner. Females may present with primary or secondary amenorrhea, elevated levels of serum gonadotropins, and a variety of neurologic abnormalities including gait and speech problems, spasticity, the need for a walker, dysarthria, optic atrophy, and reduced cognitive function. When 93 women with isolated POF (and no neurologic abnormalities) were screened for mutations in the *EIF2B2* gene, none were identified, suggesting that they are not a common cause of POF (139).

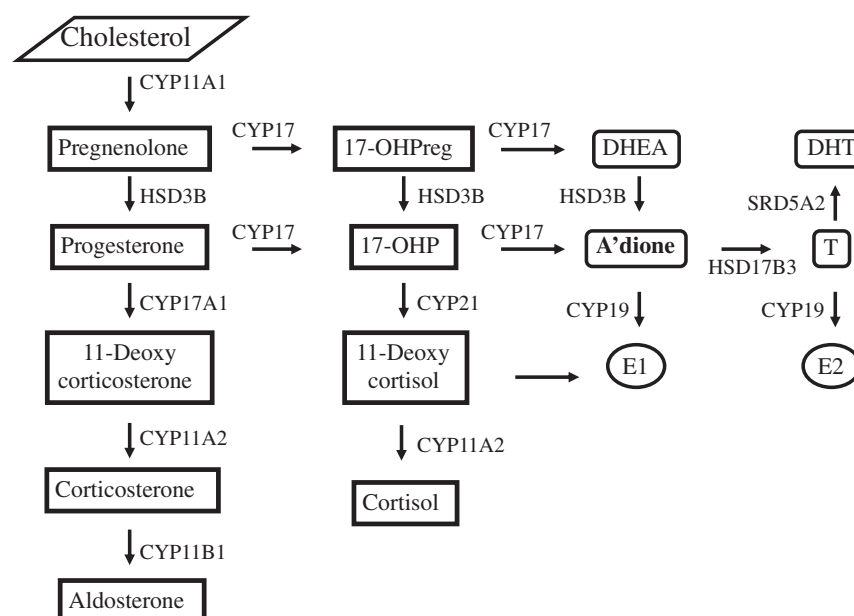
### 32.7.11 Genes in Steroid Enzyme Pathway that Result in Gonadal Failure

The biochemical pathway utilizes 27-carbon cholesterol as a precursor for the production of sex steroids, adrenal

mineralocorticoids, and glucocorticoids (Figure 32-3). Mutations in some of these enzymes cause disorders affecting both adrenal function and disorders of sexual development (congenital adrenal hyperplasia); but these are not discussed here. However, selected genes of the steroid enzyme pathway, which may result in gonadal failure in females, are discussed.

**32.7.11.1 Cytochrome P450, Family 17, Subfamily A, Polypeptide 1 (*CYP17A1*) Gene.** Cytochrome P450, encoded by *CYP17A1*, is an enzyme with at least two functions: (1) 17-hydroxylase activity (converting progesterone to 17-hydroxyprogesterone and pregnenolone to 17-hydroxypregnenolone) and (2) 17–20 desmolase activity (converting 17-hydroxypregnenolone to dehydroepiandrosterone [DHEA] and 17-hydroxyprogesterone to androstenedione) (140). *CYP17A1* mutations cause an autosomal recessive deficiency in the production of androgens, progestins, and estrogens. Cortisol is deficient as it is derived from 17-hydroxyprogesterone, but mineralocorticoids such as 11-deoxycorticosterone and corticosterone may be elevated, with associated hypertension and hypokalemic alkalosis (Figure 32-3).

The phenotype of *CYP17* deficiency in 46,XX females includes delayed puberty with absent breast development, primary amenorrhea, and elevated gonadotropins. The vagina, uterus, and ovaries are present, but they are prepubertal. 46,XY males have a similar phenotype to females except that they will not have a uterus or upper vagina as AMH is elaborated from their normal testes. Some males with partial deficiency may have sexual ambiguity. A variety of mutations in *CYP17* have been described, consisting predominantly of deletions and insertions (140).



**FIGURE 32-3** The steroid enzyme pathway is shown. The steroids are indicated within boxes while the enzymes are shown adjacent to arrows. E1 = estrone; E2 = estradiol; A'dione = androstenedione; T = testosterone; DHT = dihydrotestosterone; 17OHP = 17-hydroxyprogesterone. Relevant mutations—*CYP17A1* and *CYP19A1*—are discussed in the text.

**32.7.11.2 Cytochrome P450, Family 19, Subfamily A, Polypeptide 1 (CYP19A1) Gene Encoding Aromatase.** Although rare, mutations in *CYP19A1*, which encodes for the cytochrome P450 enzyme aromatase, cause autosomal recessive aromatase deficiency. Affected females with aromatase deficiency cannot convert the androgens (testosterone and androstenedione) to the estrogens (estradiol and estrone), respectively (141). At birth, females have sexual ambiguity with clitoromegaly, but because they have ovaries, they do not demonstrate labioscrotal gonads. When these children approach pubertal age, FSH rises, and multicystic ovaries develop (141). However, the increased FSH cannot elicit the production of sufficient ovarian estradiol so that breast development and menstruation do not occur. Serum LH levels are also usually elevated in affected patients.

An interesting effect of *CYP19A1* mutations is that the heterozygous mother carrying an affected female fetus can also develop hyperandrogenism and hirsutism (141). This occurs because the fetal placenta (derived from the fetus and with the same genotype as the fetus) cannot adequately convert androgens to estrogens. Normally, the placenta has quite robust aromatase activity, which serves to protect the mother from hyperandrogenemia.

### 32.7.12 Eugonadal Causes of Infertility

Most women with menstrual disorders are eugonadal. Genetic aspects of eugonadal infertility are now discussed (Table 32-6). These have been divided into those with non-obstructive eugonadism, which is discussed first, and obstruction.

### 32.7.13 Polycystic Ovary Syndrome

PCOS is the most common cause of anovulation, comprising more than two-thirds of all ovulation disorders affecting about 5–8% of all women (142). In general, PCOS is defined as hyperandrogenic anovulation with or without polycystic-appearing ovaries on ultrasound (143,144). The phenotype of PCOS women includes menstrual abnormalities (amenorrhea, oligomenorrhea, and/or menometrorrhagia), variable degrees hirsutism or elevated androgens (testosterone and/or dehydroepiandrosterone sulfate), obesity, and hyperinsulinemia with an increased risk for developing type-2 diabetes mellitus. These patients may have hypercholesterolemia and hypertriglyceridemia. The combination of hypertension, increased waist/hip ratio, impaired glucose tolerance, and hyperlipidemia comprise the metabolic syndrome, which has profound risks for future cardiovascular disease (142).

The pathophysiology of PCOS is quite complex and not well understood, but chronic anovulation results. In PCOS, tonic (rather than cyclic) estrogen production is present, which fails to elicit regular LH surges. Instead of an increased estradiol (E2)/estrone (E1) ratio, PCOS

is characterized by a reduced E2/E1 ratio. Sex-hormone-binding globulin (SHBG) is also reduced in PCOS, and several factors may contribute to this—hyperandrogenemia, hyperinsulinemia, and obesity (because about three-quarters of PCOS women are obese). Decreased SHBG results in increased free estrogens and androgens. The unopposed estrogen stimulation of the endometrium increases the risk of endometrial cancer (142). The increased free estrogens tonically stimulate LH further inciting hyperandrogenemia, which results in follicular atresia and the clinical signs and symptoms of hirsutism. Insulin also increases the production of androgens, further exacerbating hirsutism and predisposing to diabetes (142).

The pathophysiology and the molecular basis of PCOS are currently unknown. Evidence suggests that PCOS is a complex disease, which has largely been investigated through the use of association studies (145). Genes including insulin (*INS*), the insulin receptor (*INSR*), steroid enzymes, and follistatin (*FST*) have been implicated,

**TABLE 32-6 Gene Mutations in Eugonadism**

Gene	Localization	Phenotype	Inheritance
<i>Non-Obstructive Infertility</i>			
<i>POMC</i>	2p23.3	PCOS-strong association/multiple comparisons	Association only
<i>FBN3</i>	19p13	PCOS-strong association/multiple comparisons	Association only
<i>ACVR2A</i>	2q22.2–q23.3	PCOS	Association only
<i>FEM1B</i>	5q23.1	PCOS	Association only
<i>SGTA</i>	19p13	PCOS	Association only
<i>LHR</i>	2p21	Anovulation (F); undermasculinization (M) <sup>a</sup>	AR
<i>NFE2L3</i>	7p15	Endometriosis	Association only
<i>HOXA10</i>	7p15	Endometriosis	Association only
<i>HOXA13</i>	7p15–p14.2	Hand–foot–uterus syndrome (also hypospadias in males)	AD
<i>Obstructive Infertility</i>			
<i>AR</i> <sup>a</sup>	Xq11q12	Complete androgen insensitivity syndrome (M)	XLR
<i>WNT4</i>	1p35	Müllerian aplasia & hyperandrogenism	Sporadic, possible AD
<i>HNF1B</i>	17q12	MODY & Müllerian aplasia	AD

<sup>a</sup>Incomplete forms cause sexual ambiguity in males (not discussed here). XLR = X-linked recessive; AR = autosomal recessive; AD = autosomal dominant; YL = Y-linked.

but currently, a causative molecular defect is unknown. More recent evidence suggests the most significant association of PCOS is with fibrillin 3 (*FBN3*) on chromosome 19p13.2 and POMC on chromosome on 2p23.3 with lesser degrees to several other genes (Table 32-6), but causative mechanisms have not been determined (145).

### 32.7.14 Luteinizing Hormone Receptor Gene

A very rare cause of anovulation is the mutation of the gene for the LH receptor (*LHR*), a GPCR expressed in the thecal cells of the ovary and the Leydig cells of the testis. The phenotype of patients with *LHR* gene mutations depends on the karyotype (146–148). Genetic females with *LHR* gene mutations develop normal puberty and have menarche. However, they then become anovulatory with usually elevated (or normal) levels of LH and FSH (146–148).

46,XY males with severe, loss-of-function *LHR* mutations also usually present as phenotypic females, but they have absent breast development and can demonstrate normal appearing external genitalia except they have a blind vaginal pouch (146). As the testes are normal and produce AMH, no uterus or vagina is present. Therefore, these patients will appear as phenotypic females with no breast development. Depending on the severity of the *LHR* mutation, the external genitalia ranges from normal appearing female (severe mutations) to sexual ambiguity to bilaterally descended testes (mild mutations). Serum gonadotropins are usually elevated, and the testes are small and devoid of Leydig cells. Inactivating *LHR* mutations are inherited in an autosomal recessive manner, in contrast to activating *LHR* mutations, which cause autosomal dominant familial male precocious puberty.

### 32.7.15 Endometriosis

Endometriosis can be a debilitating disease estimated to affect 8–10% of reproductive age women and up to 50% of women presenting with infertility. Endometriosis is defined as the presence of glands and stromal tissue of the endometrium found in locations other than the uterine cavity. Furthermore, endometrial tissue found in the ovary is called an endometrioma and that found in the myometrium is termed adenomyosis. Women with endometriosis may present with pelvic pain, dysmenorrhea, infertility, menorrhagia or bleeding per rectum/urethra.

Evidence linking endometriosis to infertility is strong but not conclusive. Distorted pelvic anatomy, altered peritoneal function, altered hormonal and cell-mediated function, endocrine and ovulatory abnormalities as well as impaired implantation have been proposed but not proven as potential mechanisms by which endometriosis

may affect fertility. Decreased fecundity has been shown in some but not all studies and improvement in fecundity with treatment of endometriosis has not been universal in all studies (149). Treatment generally consists of anti-estrogenic agents.

The most widely accepted theory for pathogenesis of endometriosis is retrograde menstruation. Although up to 90% of women are estimated to have retrograde menstruation, a combination of eutopic endometrial load, inherent invasive nature of endometrial cells, inflammatory capacity of the peritoneum and deficiencies in the immune system is thought to result in the relatively lower incidence of the condition in the general population. A single endometriotic lesion originates from a single progenitor cell (150).

Evidence of genetic contribution to the development of endometriosis is conflicting (151). Using a candidate gene approach, at least 76 genes have been implicated. However, replication of these studies for confirmation of the association has more often than not failed to confirm association. Linkage analysis studies have implicated regions in chromosomes 7 and 10 but convincing evidence for genes from these regions being causative for endometriosis is lacking (151).

Recently, a genome-wide association study of ~3200 individuals with surgically proven endometriosis and ~7000 controls from Australia and the United Kingdom was performed. The strongest association signal was on 7p15.2 (rs12700667) for all endometriosis, which was again found in a replication study from a cohort in the USA. The genome-wide significant *P* value was  $1.4 \times 10^{-9}$  (OR=1.20, 95% CI 1.13–1.27) for all endometriosis in the combined datasets. This marker on 7p is located nearby to candidate genes *NFE2L3* and *HOXA10*. In addition, epigenetic alterations in *HOXA10*, *NR5A1*, *CYP19A1*, *PGR*, *ESR2*, and *CDH1* genes have been implicated in the development of endometriosis (152).

### 32.7.16 HOXA13

Heterozygous *HOXA13* homeobox gene mutations have been identified in women with the hand–foot–uterus syndrome (153). These females may not have infertility despite developmental abnormalities of the urogenital system. The phenotypic findings in individuals with this autosomal dominant disorder typically include small hands and feet along with a duplicated uterus. The uterine anomalies consist of either bicornuate or didelphic abnormalities—defects of midline Müllerian fusion. Associated urinary anomalies, such as displaced urethral or ureteral openings may also be present in females (males may have hypospadias) (153). Hand developmental anomalies include delayed ossification or fusion of wrist bones, short middle phalanges of the fifth fingers, short first metacarpals, and small distal phalanges of the thumbs. The great toe may demonstrate a short first metatarsal and a small pointed distal phalanx. Only



a small number of *HOXA13* mutations have been characterized (154).

### 32.7.17 Obstructive Disorders of the Genital Tract

The molecular basis is only known for several disorders of the genital tract. The two most common are CAIS and Müllerian aplasia (MA).

**32.7.17.1 AR Mutations in Complete Androgen Insensitivity Syndrome.** Testosterone synthesized in the steroid enzyme pathway interacts with its receptor (Figure 32-3). The androgen receptor (AR) belongs to the steroid superfamily of nuclear hormone receptors, and is encoded by the *AR* gene. The protein consists of an amino-terminus, a DNA-binding domain, and a carboxy terminal androgen-binding domain (155). 46,XY males with CAIS who have *AR* mutations present as phenotypic females with normal breast development, minimal or no axillary and pubic hair, and a blind vaginal pouch. The phenotype results from aberrant interaction of normal androgen levels with the receptor at the target. Since testes are normal, AMH is produced, which inhibits the formation of the uterus and upper vagina. The vagina is absent or appears as a blind vaginal pouch without a cervix. The testes, which may be intra-abdominal or inguinal, are capable of making testosterone, and normal adult male levels are produced. Incomplete forms of AIS cause sexual ambiguity in males.

More than 300 different mutations, most commonly missense mutations, in *AR* have been reported to cause AIS (156,157). Interestingly, nearly all of the exon 1 mutations cause CAIS or incomplete AIS, and most of these produce a premature stop codon. However, most *AR* mutations occur in exons 2–8, despite the fact that exon 1 encodes for more than half of the protein (156).

**32.7.17.2 *WNT4* and *HNF1B* in Müllerian Aplasia.** Mayer–Rokitansky–Kuster–Hauser syndrome (MRKH), also known as MA, is a severe reproductive developmental disorder that results in the CAUV. It affects approximately 1/5000 females and is the second most common cause (10%) of primary amenorrhea in girls (158). During embryologic development, both Müllerian ducts migrate into proper anatomic position and come into contact with the urogenital sinus, which gives rise to the midline uterus as well as both fallopian tubes and the upper vagina. Renal development is intimately related to Müllerian development. Therefore, disruption of either system can affect the development of the other.

Patients with MRKH are 46,XX females that undergo normal puberty since they have normal ovarian function. They demonstrate normal adult breast development and pubic hair (which distinguishes MRKH from CAIS), but have either an absent vagina or a small blind vaginal pouch (a urogenital sinus derivative). Typically, the uterus is either absent or very hypoplastic although

fallopian tubes may be present, suggesting that the development of the caudal portions of the Müllerian ducts are more adversely affected. Rarely, the endometrium may be functional, enough to cause pelvic pain due to menstruation and genital tract obstruction. Pregnancy is only possible in these patients if oocytes are retrieved for in vitro fertilization with embryo transfer into a surrogate.

If only MA is present, which occurs in about two-thirds of patients, it is classified as type I (158). Approximately one-third of the patients with MRKH may have unilateral renal agenesis, and another 10–15% may have skeletal abnormalities, including the Klippel–Feil sequence (fusion of cervical vertebrae). This form has been classified as type II or under the acronym MURCS (Müllerian duct aplasia, unilateral renal agenesis, and cervical somite anomalies), which may also include deafness (158). Cardiac anomalies have also been reported in 10–12% of all cases (type III). In fact, MRKH has been associated with other syndromes including Holt–Oram syndrome (autosomal dominant due to *TBX5* mutations) and thrombocytopenia-absent radius (TAR) syndrome (autosomal recessive), both of which consist of associated cardiac and skeletal abnormalities. It has been suggested that perhaps CAUV, Holt–Oram syndrome, TAR syndrome, and Klippel–Feil could have a common pathway of defective T-box transcription (159).

The molecular basis of Müllerian development is largely unknown as it is difficult to identify families as pregnancy and vertical transmission will not occur (unless transmitted by a surrogate). Although a number of genes have been implicated and studied (*CFTR*, *GALT*, *HOXA7*, *HOXA13*, *PBX1*, *HOXA10*, *AMH*, *AMHR*, *RARG*, *RXRA*), only mutations in *WNT4* appear to be causative in humans (160). These patients also have mild excess androgen production resulting in hirsutism. It is also interesting that some patients with maturity onset diabetes of the young (*MODY5*) due to *HNF1B* mutations have MA (161). Several chromosomal translocations and genomic imbalances have been reported, which could be useful in uncovering the molecular basis.

## 32.8 Summary and Conclusions

Disruption of the reproductive axis may result in human disease at the level of the hypothalamus, pituitary, gonad, or outflow tracts. Many of the mutations in genes affect fertility in females. Additional genes will continue to be identified, and this will no doubt be enhanced by next generation DNA sequencing and probably by genome wide association studies. The identification of new genes in humans continues to improve our understanding of the pathophysiology of reproductive dysfunction, which will assist in genetic counseling and ultimately in treatment.

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### Biographies



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**Dr Layman** went to medical school at the University of Cincinnati and completed a residency in Obstetrics & Gynecology at the University of Louisville followed by a fellowship in Reproductive Endocrinology and Genetics at the Medical College of Georgia. He is a physician–scientist who has been a principle investigator on four NIH grants, including continuous funding from 1997–2015 for the “Genetics of Delayed Puberty.” He was among the first to identify and characterize mutations in the *FSHB*, *GNRHR*, *CHD7*, *NELF*, and *WDR11* genes in human hypogonadotropic hypogonadism. He has nearly 100 publications and 16 book chapters.

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**Bala Bhagavath** completed his medical education at Madras Medical College in India. He then completed his training in Obstetrics and Gynecology in the United Kingdom and is a fellow of the Royal College of Obstetricians and Gynaecologists. He spent four years as a registrar in obstetrics and gynecology at the National University Hospital in Singapore before moving to the United States to complete a residency in Obstetrics and Gynecology at the Medical College of Georgia and a fellowship in Reproductive Endocrinology and Infertility at the UT Southwestern Medical Center. He is board certified in the USA in Obstetrics and Gynecology and subspecialty certified in Reproductive Endocrinology and Infertility. He developed an interest in the genetics of female infertility early in his career and has published many peer reviewed articles. He is currently a Clinical Assistant Professor in Obstetrics and Gynecology at Warren Alpert School of Medicine at Brown University, Providence, RI. He has just relocated to his new position as Associate Professor of Obstetrics and Gynecology, Director, Fellowship in Minimally Invasive Gynecologic Surgery, University of Rochester.

# CHAPTER

# 33

## Genetics of Male Infertility

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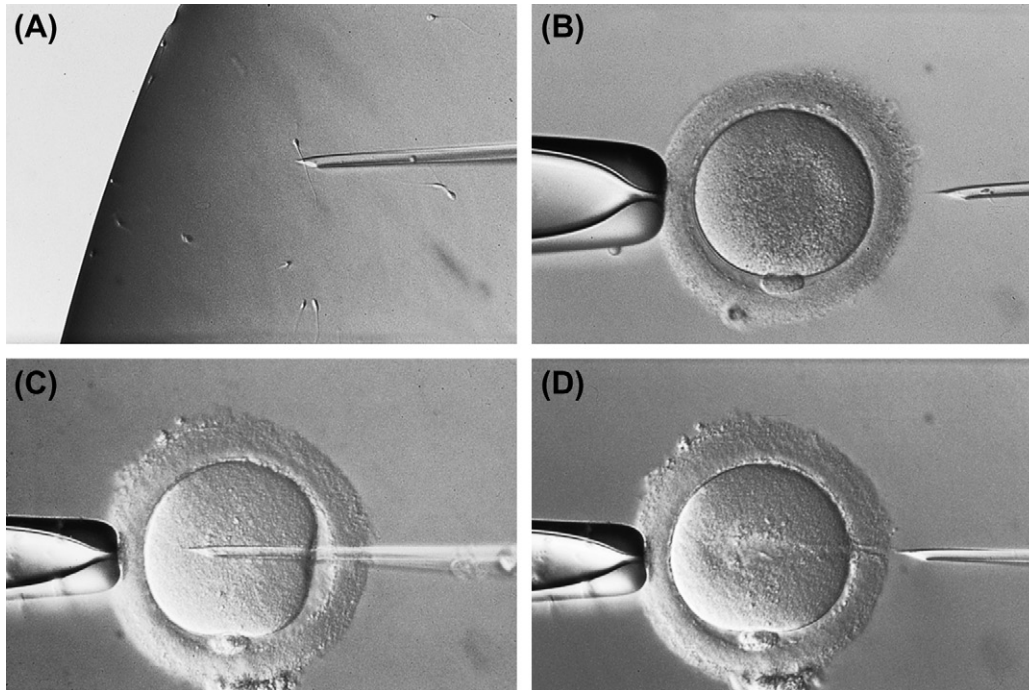
### 33.1 MALE INFERTILITY— INTRODUCTION

About one out of seven couples suffer from reproductive health disorders in the form of infertility. Male factor is an important, sometimes the only, contributory cause in about half of these involuntary childless couples. Therefore, it can be assumed that approximately 7% of all men are confronted with fertility problems. The etiology of impaired sperm production and function can be ascribed to different factors including genetic anomalies (1). With advances in diagnostic techniques, many of the men previously called “idiopathic” are now reclassified into one of the genetic disorders of male infertility. With the introduction and dissemination throughout the world of assisted reproductive techniques (ART) for the treatment of spermatogenesis defect in the male partner, many previous infertile or subfertile men can now father biological children. While males with impaired sperm production due to a genetic factor may now benefit from the wide availability and utilization of ART, the potential risk of transmitting genetic defects to the offspring deserves thoughtful consideration. At the beginning of the 1990s, the introduction of intracytoplasmic sperm injection (ICSI) gave rise to new possibilities for the treatment of severe male factor infertility, including azoospermia (no spermatozoa in the ejaculate) (2). In contrast to conventional *in vitro* fertilization (IVF), ICSI performed using sperm of poor quality is associated with considerably better fertilization rates as this technique involves the injection of a single spermatozoon directly into the oocyte (Figure 33-1). ICSI can also be applied in azoospermic patients by using either epididymal (obstructive cases) or testicular spermatozoa (obstructive or non-obstructive azoospermia). Concerns have been raised about ICSI itself because of the invasiveness of this procedure and the possibilities of adverse effects on the offspring; a number of

follow-up studies on children born after ART (including ICSI) have addressed these topics. Studies comparing children born from ICSI to those born after the less-invasive IVF failed to find a significant difference between the two techniques in terms of incidence of both malformations and genetic aberrations. In contrast, the comparison between ICSI babies and naturally conceived children provide evidence of a higher *de novo* aneuploidy rate (three-fold increase), mostly sex chromosomal aneuploidies (3). The question whether the congenital malformation rate is higher in ART babies remains debatable. In fact, some authors do not report any significant difference, whereas others assess that the risk of a major birth defect is twice as high in children born after ART (3,4). ART pregnancies are associated with low- and very-low-birth-weight babies, even in singleton pregnancies, compared to naturally conceived babies (5). Small gestational weight babies are associated with childhood and adult diseases, such as obesity, and metabolic disturbances with increased coronary heart disease risk (6–8). A correlation between semen parameters and a higher incidence of chromosomal anomalies in ICSI-derived fetuses has also been reported (9) indicating that the morbidity associated with ART does not depend on the technique used but on the underlying health risks of being subfertile, that is, more likely a carrier of constitutive or *de novo* chromosomal anomalies in the gametes. Studies to date have only followed ART children into early teenage years; therefore, there are no data on certain illnesses manifesting only later in life (10). It is therefore essential to perform longer follow-up studies in the future, with special focus on the fertility status of boys born from severely oligo/azoospermic men carrying genetic defects in their genomic and/or gamete DNA.

With the advancement and widespread use of molecular genetic tools, a number of genetic factors can now





**FIGURE 33-1** Different steps in the ICSI procedure. (A) The injection pipette presses the tail of the spermatozoon against the bottom of the Petri dish until it stops moving. The spermatozoon is then aspirated, tail first, into the injection pipette. (B) The metaphase II oocyte is immobilized by slight negative pressure exerted on the holding pipette at 9 o'clock. The polar body is held at 6 o'clock. (C) The micropipette containing a single spermatozoon is pushed through the zona pellucida and the oolemma into the ooplasm at 3 o'clock. (D) The injection pipette is withdrawn gently, and the injected oocyte is released from the holding pipette.

be easily identified and some of them are currently part of the diagnostic work-up of selected groups of subfertile patients. These factors can be transmitted as autosomal recessive, autosomal dominant, X-linked, and Y-linked traits. This chapter aims to describe known monogenic and chromosomal causes of infertility. In some instances, the genetic defects occur in infertile but nonetheless healthy males, whereas in others, the infertility is associated with additional minor or major clinical symptoms; all these aspects are discussed in the following sections.

## 33.2 CHROMOSOME ANOMALIES

### 33.2.1 Karyotype Anomalies

After the first description of the presence of an extra X chromosome in association with azoospermia (11), many surveys have been conducted to determine the contribution of chromosomal anomalies to male infertility. One of the largest reviews dealing with chromosomal aberrations in 8000 infertile males showed a 27-fold increase (3.8% vs 0.14%) of sex chromosome anomalies and a 5-fold increase (1.3% vs 0.25%) for autosomes with a combined 13-fold higher frequency (5.1% vs 0.38%) when compared to the newborns born to couples without subfertility (12). The general conclusion from these studies is that the more severe

the testicular phenotype, the higher is the frequency of chromosomal abnormalities. Patients with <10 million spermatozoa/ml ejaculate show 10 times higher incidence (4%) of mainly autosomal structural abnormalities compared to the general population. Among severe oligozoospermic men (<5 million spermatozoa/ml), this frequency is doubled to 8%, whereas in non-obstructive azoospermic men, it reaches the highest values (15–16%) and it is mainly related to sex chromosome abnormalities.

In the group of numerical sex chromosomal aberrations (sex chromosomal aneuploidy), patients with Klinefelter syndrome (KS) (47, XXY or mosaics 46, XY/47, XXY or higher grade sex chromosomal aneuploidy, i.e. XXXY, XXXXY, etc.) are the most frequent. The clinical spectrum of the phenotype of adult KS patients is very broad with the reproductive system abnormalities including androgen deficiency and infertility. Testosterone deficiency occurs in about 50% of affected KS but elevated LH and FSH levels are almost universal. The clinical phenotype of androgen deficiency in KS ranges from clinically overt hypogonadism to normally virilized males. The classical symptoms include small firm testes with hyalinization of seminiferous tubules, azoospermia or extremely severe oligozoospermia, hypergonadotropic hypogonadism with or without gynecomastia, and eunuchoid proportions (13). These patients also have

non-reproductive problems including dyslexia, executive dysfunction, behavioral problems and predilection to other systemic diseases such as diabetes mellitus and autoimmune disorders (14,15). The large majority of subjects affected by this syndrome are azoospermic. However, testicular sperm extraction (TESE) and especially microsurgical TESE (micro-TESE) followed by ICSI with an average of 30–50% of testicular sperm recovery rate may allow KS patients to generate their own genetic children (for review, see References (13) and (16)). Moreover, occasional spermatozoa can be found in the ejaculate of some mosaic patients or of non-mosaic but younger patients (17). As germ cell loss is progressive with age, the potential importance of an early diagnosis of KS might allow preventive cryopreservation of ejaculated spermatozoa in some cases and extraction of intratesticular spermatozoa in many patients to preserve fertility. According to recent reviews, children born from KS fathers are healthy and only one 47, XXY fetus has been reported so far (13,18). Despite these encouraging data, owing to the significant increase of sex chromosomal and autosomal abnormalities in the embryos of KS patients, ICSI followed by preimplantation genetic diagnosis (PGD) should be considered as an appropriate preventive option (18,19).

Other sex chromosomal anomalies include 47, XYY or 46, XX males. The 47, XYY karyotype is observed among infertile men more frequently than in newborn males (0.26% and 0.07%, respectively). However, their spermatogenic picture can range from a severe impairment to an apparent normality. The XX-male condition denotes a disorder of sex determination and occurs in about 1:20,000 newborns. In about 80% of cases, XX-male phenotype can be explained by the translocation of the SRY gene (encoding the testis determining factor) to the X chromosome. The cause of SRY-negative XX-male phenotype remains to be elucidated. Similar to KS, the phenotypic features of this syndrome include gynecomastia, female hair pattern and small testes with azoospermia. Unlike XXY males, these men are not tall. Genital malformations such as hypospadias are rare. In SRY-negative 46, XX patients, ambiguous genitalia are a frequent finding. Structural alterations of the Y chromosome are mainly found in severe male factor infertility, and cytogenetically visible deletions of the Yq are a relatively frequent cause of azoospermia. Section 33.2.2 covers the latter issue in more detail.

Robertsonian translocations, reciprocal translocations, paracentric inversions and marker chromosomes are the most frequently found abnormalities in oligozoospermic men. According to Johannisson et al. (20), a correlation exists between the increased frequency of the XY bivalent and the Robertsonian trivalent association, present during the pachytene stage of meiosis, and the extent of germ-cell impairment. The importance of the detection of these structural chromosomal anomalies

is related to the increased risk of aneuploidy or unbalanced chromosomal complements in the fetus. In cases of Robertsonian translocations, a special risk is represented by uniparental disomies, which are generated through a mechanism called “trisomy rescue” (repairing the trisomic status) during the first division of the zygote. For chromosomes 14 (the most frequently involved chromosome) and 15, both paternal and maternal uniparental disomies are pathological and give rise to severe diseases such as Angelman or Prader–Willi syndromes, despite an apparently normal or balanced karyotype.

The causal relation between chromosomal anomalies (both numerical and structural) and impaired sperm production has been suggested to be a structural effect related to alterations in the process of chromosome synapsis during meiosis. In mice, asynapsed regions may trigger the meiotic checkpoint machinery to eliminate spermatocytes (21). A similar mechanism might explain why some chromosomal abnormalities in humans are associated with deficient spermatogenesis. Another mechanism might be related to the multitude of genes involved in spermatogenesis, some of which may be dosage-sensitive and potential mutational targets for chromosomal breakpoints.

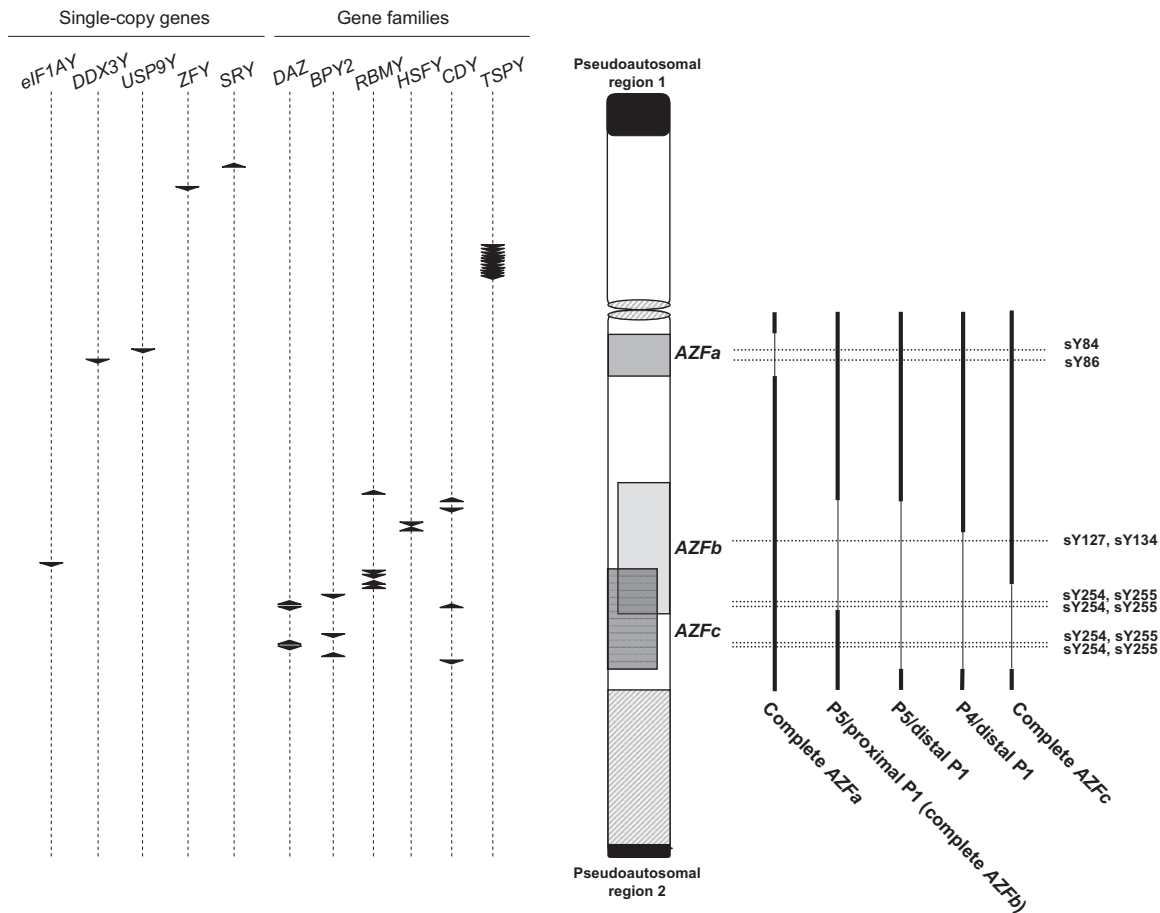
In conclusion, on the basis of the frequencies of chromosomal aberrations in patients with different sperm concentration, karyotype analysis should be strongly considered in the diagnostic workup in all oligozoospermic men with <10 million spermatozoa/ml and in all non-obstructive azoospermic men. In case there is a family history of recurrent abortions, malformations, or mental retardation, karyotype analysis should be requested regardless of the sperm concentration. The diagnosis of a karyotype anomaly has multiple implications with both diagnostic and predictive value for the health consequences of the future offspring.

### 33.2.2 Microdeletions of the Y Chromosome

The pivotal role of the Y chromosome in spermatogenesis is supported by the presence of Y-linked genes specifically expressed in germ cells and by pathological phenotypes deriving from the deletion of regions containing the abovementioned genes.

The Y chromosome is an acrocentric chromosome and consequently contains a short arm (Yp) and long arm (Yq), demarcated by a centromeric region essential for chromosome segregation (Figure 33-2). The human Y chromosome is classically divided into two functionally distinct regions: (i) the pseudoautosomal regions (PAR1 and PAR2), which are homologous with X chromosome sequences and are responsible for correct pairing between the two sex chromosomes during male meiosis; (ii) the male specific region Y (MSY).

This chromosome is singular for its haploid nature, which precludes recombination with the X chromosome for most of its length, resulting in the accumulation of



**FIGURE 33-2** Schematic representation of the Y chromosome showing the localization of the three AZF regions as well as the two pseudoautosomal regions (PAR1 and PAR2). The Y chromosome is enriched with a number of both single-copy genes and multicopy gene families, which are indicated on the left side of the figure. On the right side, a model of the five recurrent deletions occurring on the Y chromosome is displayed. The dotted lines indicate the position of the STS primers used as region-specific deletion markers for the routine diagnostic testing.

a high proportion of segmental duplications that are arranged in direct or inverted repeats that may include palindromes (22). The presence of duplicated sequences within the chromosome allows two mechanisms to occur: gene conversion and nonallelic homologous recombination (NAHR). The first is a unidirectional conversion-based system of gene copy “correction” which permits the preservation of a certain number of Y genes from the gradual accumulation of deleterious mutations ensuring their continuity in time; on the other hand, NAHR produces recurrent deletions/duplications affecting the dosage of different Y genes.

The first description of *de novo* deletions on the Yq is in 1976 (23). In six azoospermic males, deletions were found in part of the nonfluorescent (euchromatic) region at band q11 but such deletions were not present in the fertile fathers and brothers of the patients. On the basis of this finding, Tiepolo and Zuffardi predicted the presence of an azoospermia factor (AZF) in the distal region of the Y chromosome. With the development of molecular genetics tools, it became possible to circumscribe the AZF region, in which microdeletions arise, and to highlight a certain deletion pattern with three recurrently deleted subregions in proximal, middle and distal Yq11,

designated AZFa, AZFb and AZFc, respectively (24). AZF deletions are likely to occur in germ cells during meiosis when NAHR between sister chromatids may take place. From the molecular characterization of the AZF regions, in each region, several putative Y-linked spermatogenesis genes and gene families are present.

**33.2.2.1 The AZF Deletions.** The AZFa region is 792 kb long and contains two single-copy genes *USP9Y* and *DDX3Y* (formerly *DBY*), which are ubiquitously expressed. Complete AZFa deletions occur after homologous recombination between identical sequence blocks within the retroviral sequences in the same orientation *HERVYq1* and *HERVYq2* (25). Such deletions are associated with sertoli cells only syndrome (SCOS), characterized by the total absence of germ cells in the testis.

Complete deletions of the AZFb region are caused by homologous recombination between the palindromes P5/proximal P1, which removes also part of the AZFc region belonging to P1 (26). This deletion removes 6.2 Mb (including 32 copies of genes and transcription units). The associated phenotype is azoospermia with spermatogenic arrest, resulting in the absence of mature germ cells in the testis. The AZFc region includes 12 genes

and transcription units, each present in a variable number of copies making a total of 32 copies. The complete deletion of AZFc region originates from the homologous recombination between amplicons b2 and b4 in palindromes P3 and P1, respectively (27). Contrasted with complete AZFa and AZFb deletions, a direct genotype/phenotype correlation is lacking for complete AZFc deletions, which are associated with a semen phenotype varying from oligozoospermia to azoospermia and with different testis histology. This variable phenotype might be due to a progressive regression of the germinal epithelium over time leading from oligozoospermia to azoospermia, or could also be influenced by other factors, such as the genetic background (i.e. compensation for the absence of Yq genes, by autosomal or X-linked factors), the presence of 45X lines (a more severe phenotype) and environmental factors in different individuals (28). Deletions of both AZFb and AZFc together can occur in two breakpoints between P4/distal P1 (7.0 Mb, 38 gene copies removed) or between P5/distal P1 (7.7 Mb and 42 gene copies removed). Complete AZF deletions are generally *de novo* mutations although exceptional cases of transmission have been reported and pertain uniquely to complete AZFc deletion. As described above, AZF deletions are specific for spermatogenic failure as no deletions have been reported in the genomic DNA (derived from lymphocytes) of normozoospermic men (29). Indications for complete AZF deletion screening are based on sperm count because clinical parameters such as hormone levels, testicular volume, varicocele, maldescended testis, and infections do not have any predictive value (28,29). The test is currently performed in all infertile men with <5 million spermatozoa/ml during the routine diagnostic workup. The highest deletion frequency is found in idiopathic azoospermic men (10%) who are more likely to be carrying this genetic anomaly. AZF deletions are less frequent in severe oligozoospermic men (2–5%) and have been exceptionally reported in mild oligozoospermic men. As AZF genes are mainly expressed in the testis, a number of studies have been undertaken in order to clarify if AZF deletions may cause testis-related pathologies other than spermatogenic failure. No final evidence for a cause–effect relationship was observed for varicocele, cryptorchidism and testis cancer (for review, see Reference (28)). Apart from the diagnostic value, Yq deletion screening provides additional prognostic information for testicular biopsy (TESE) in azoospermic men. In fact, deletions removing the entire AZFa or AZFb regions (“complete” deletions) represent a negative predictive factor for TESE as mature spermatozoa are unlikely to be found in the carrier testis. In case the deletion is found in a man undergoing ICSI or TESE/ICSI, genetic counseling is mandatory in order to provide information about the obligatory transmission of the AZF deletions, and therefore, the risk of giving birth to a son with impaired spermatogenesis. The diagnostic testing of Yq deletions should follow the procedure described in the European Academy of Andrology/European Molecular genetics

Quality Network guidelines (30), which is based on PCR amplification of AZF specific STS primers and control markers.

**33.2.2.2 Partial AZFc Deletions/Duplications.** In the past few years, new types of rearrangement, namely partial AZFc deletions and duplications, have been described in association with spermatogenic impairment (31). The AZFc region is organized in several amplicons containing multicopy testis-specific genes and, owing to this structure full of homologous sequences, it is particularly susceptible to NAHR events that lead to the formation of both partial deletions and duplications. Although a number of different partial AZFc deletions have been described, only one of them has been shown to be clinically relevant. This is the gr/gr deletion (named after the “green” and “red” probes used when it was detected for the first time) that results in the removal of half of the AZFc region gene content (31). This type of deletion is a significant risk factor in some populations but apparently not in others. In fact, the frequency and phenotypic expression may vary in different ethnic groups, depending on the Y chromosome background (in specific Y haplogroups, such as D2b and Q3, common in Japan and certain parts of China, the deletion is fixed and apparently does not have any negative effect on spermatogenesis). Despite controversies, it is undeniable that the gr/gr deletion has a significant effect on spermatogenesis; indeed, normozoospermic carriers have a lower sperm count compared to men with an intact Y chromosome (32). According to the largest study population published to date in Caucasians, gr/gr deletion is significantly more frequent among oligozoospermic men (3.4%) compared to normozoospermic men (0.4%) and gr/gr deletion carriers are at a 7.9-fold increased risk for spermatogenic impairment (OR=7.9, 95% CI 1.8–33.8) (33). These data, together with recent meta-analyses (32,34,35), indicate that the gr/gr deletion is a unique example of an important risk factor for impaired sperm production in Caucasian populations. Consequently, there are mainly two reasons for gr/gr deletion testing in infertile men. The deletion contributes to the etiopathogenesis of impaired sperm production as it is able to influence the spermatogenic potential of the carrier. The couple should be aware that the deletion (i.e. a genetic risk factor for impaired sperm production) will be obligatorily transmitted to their male offspring and the deletion may become complete AZFc deletion (i.e. a clear cut causative factor for spermatogenic impairment) in the next generation.

**33.2.2.3 Y Chromosome Gene-Specific Deletions.** Owing to the extent of the deletions, several genes or (part of the copies of) gene families are absent simultaneously. This makes it difficult to estimate the importance of these genes, and their proteins, in the process of normal fertility and infertility because of absence, reduction in copy number, or loss of function. The only gene-specific mutation reported to date was found in the AZFa region that contains two widely expressed genes,



*USP9Y* and *DDX3Y* (36). Both *USP9Y* and *DDX3Y* (former *DBY*) are ubiquitously expressed and have functional homologs on the X chromosome. In the first place, after sequencing both genes, the loss of *USP9Y* was believed to have a direct effect on spermatogenesis, causing azoospermia, whereas no mutation was found in the *DDX3Y* gene (37). However, azoospermia was just one of the possible phenotypes related to *USP9Y* deletion and it is also associated to mild oligo- or normozoospermia. Therefore, *USP9Y* has been proposed as a fine spermatogenic modulator, the absence of which is compatible with a highly variable phenotype probably linked to the genetic or other background of the carrier (38). Given the extreme rarity of AZF gene-specific deletions and the heterogeneous phenotype of the *USP9Y* deletion, the routine screening for AZF gene-specific deletions is not advised.

### 33.3 GENE DEFECTS INVOLVED IN ENDOCRINE FORMS OF INFERTILITY

#### 33.3.1 Mutations and Polymorphisms Affecting the Androgen Receptor

**33.3.1.1 Mutations of the AR Gene.** The androgen receptor (AR) gene is located in the long arm of the X chromosome (Xq11–12). The AR gene has four major domains: the transactivation domain; the DNA binding domain; the hinge region; and the hormone binding domain. The transactivation region of AR encloses two segments of glutamine (CAG) and glycine (GCN) repeats. The repeat segments are polymorphic (see later). Mutations of the AR gene are uncommon and occur in 1/20,000 to 1/64,000 males; about 300 mutations have been observed. Mutations of the AR gene result in a variable phenotype from a phenotypic female in the complete androgen insensitivity syndrome to an under-androgenized male with ambiguous genitalia in the partial androgen insensitivity syndrome. In addition, subtle mutations of AR can be associated with a normal male phenotype with defective spermatogenesis. Mutations in AR associated with impaired spermatogenesis and male infertility can occur in the transactivation domain or ligand-binding domain of AR (39–42). Androgens are essential for the post-meiotic development of male germ cells, differentiation of spermatids to spermatozoa and release of sperm from the germinal epithelium. Complete and selective knockout AR mouse models help to delineate the specific actions of AR in the development of the male phenotype and the regulation of normal spermatogenesis (43). AR mutations causing severe oligo- and azoospermia were first reported by Aimen et al. (44) whose study found three phenotypically normal infertile men having high production rates of testosterone with normal or elevating serum luteinizing hormone (LH) levels. AR binding in the genital fibroblasts was about half or less compared to normal men or women. The low amount of

AR with high testosterone and high-serum LH suggests that the defective spermatogenesis in these infertile men was the consequence of androgen insensitivity due to AR mutations (45). However, not all mutations of AR result in reduced androgen action and male infertility. In addition, mutations of AR at residues 727 and 866 in the ligand-binding domain interfere with coactivator interactions and are associated with severe oligozoospermia (42). Other examples include mutations in residues 798 and 712 that are associated with severe oligozoospermia. In vitro studies suggest that the mutated AR responds normally to 5- $\alpha$  dihydrotestosterone and not to testosterone. Thus, treatment with dihydrotestosterone in some mutations of AR with milder defects of spermatogenesis may be able to stimulate mutant AR function and increase the chances of fertility (42).

**33.3.1.2 Expansion of the Polyglutamine Repeats of Exon 1 of the Androgen Receptor.** Gross expansion of the polyglutamine repeats (CAG) in exon 1 within the transactivation domain of the AR results in spinal bulbar atrophy associated with decreased virilization and oligo- or azoospermia. In vitro studies have shown that the expansion of the polyglutamine tract in the AR results in a decreased AR transactivation and androgen insensitivity. Multiple reports have shown the association of longer CAG repeats in men with “idiopathic infertility” compared with fertile men in some ethnic groups (Asians in Singapore, Caucasians in Australia, United States and France) but this association was not identified in others studies (Sweden, Germany, Netherlands, Denmark). A recent combined analysis of the published studies showed that there was a small difference in CAG repeat length between infertile and fertile men and the difference appears to be more marked in Asians than Caucasians of European descent (42). This suggests that the polymorphism of the AR CAG repeat length may only play a minor role as a cause of male infertility.

#### 33.3.2 Congenital Hypogonadotropic Hypogonadism

Patients with congenital hypogonadotropic hypogonadism (CHH) have reduced or defective secretion of gonadotropin-releasing hormone (GnRH), which manifests as pubertal delay and low levels of gonadotropins (MIM \*146110). CHH in association with decreased sense of smell (anosmia or hypo-osmia) is known as Kallmann syndrome. CHH and Kallmann syndrome both present as sporadic and inherited cases, with autosomal recessive, autosomal dominant, and X-linked modes of inheritance. The distinction between CHH and Kallmann syndrome is not always obvious because GnRH deficiency with and without anosmia often occurs in different members of the same family. Genes associated with CHH can be classified according to whether the proteins they encode are involved in the development and

**TABLE 33-1 Classification of Genes and Phenotypes Associated with Congenital Hypogonadotropic Hypogonadism**

Gene	Locus	OMIM	Product	Inheritance	Associated Phenotype
Development and Migration of GnRH Neurons					
<i>KAL1</i>	Xp22.3	308700	Anosmin-1	X-linked	Hyposmia/anosmia, unilateral renal agenesis, midline facial defects (cleft lip/palate), synkinesia, oculomotor abnormalities, short metacarpals, sensorineural hearing loss, cerebellar ataxia, gut malrotations
<i>FGFR1</i> ( <i>KAL-2</i> )	8q11.2-p11.1	136350	Fibroblast growth factor receptor-1	AD, Digenic	From anosmia to normosmia, variable severity of hypogonadism, cleft lip/palate, synkinesia, dental agenesis
<i>FGF8</i>	10q24	600483	Fibroblast growth factor 8	AD, AR, Digenic	Hyposmia/anosmia Cleft lip/palate, synkinesia
<i>PROK2</i>	3p21.1	607002	Prokineticin-2	AD/AR	Sleep disorders, obesity
<i>PROKR2</i>	20p13	244200	Prokineticin-2 receptor	AD, AR, Digenic	From anosmia to normosmia
<i>CHD7</i>	8q12.1	608892	Chromodomain-helicase-DNA-binding protein 7	AD	Part of CHARGE syndrome (coloboma, heart disease, choanal atresia, retarded growth and development and/or central nervous system anomalies, genital anomalies and/or hypogonadism, ear anomalies and/or deafness), from anosmia to normosmia
<i>NELF</i>	9q34.3	608137	Nasal embryonic LHRH factor (for neurons and axonal outgrowth)	Digenic	Hyposmia/anosmia
<i>WDR11</i>	10q26	606417	Member of the WD repeat-containing protein family	AD?	None reported
GnRH Secretion					
<i>KISS1</i> <sup>a</sup>	1q32	603286	Kisspeptin		None reported
<i>KISS1R</i>	19p13.3	604161	KISS1 receptor	AR	Normosmia
<i>LEP</i>	7q31.2	164160	Leptin	AR	Severe Obesity
<i>LEPR</i>	1p31	601007	Leptin receptor	AR	
<i>TAC3</i>	12q13-q21	162330	Neurokinin B	AD, AR	Microphallus, cryptorchidism
<i>TACR3</i>	4q25	162332	Receptor for TAC3		
<i>PCSK1</i>	15q15-q21	162150	Protein convertase subtilisin/kexin-type 1	AR	Obesity, variably impaired processing of several pro-hormones including those of LH, FSH, proinsulin and pro-opiomelanocortin, hormone levels either low or immunologically measurable with low bioactivity
<i>NROB1</i>	Xp21.3-p21.2	300473	Dosage-sensitive sex reversal-adrenal hypoplasia congenita (DAX1)		Adrenal failure
<i>GNRH1</i>	8p21-p11.2	152760	Gonadotropin-releasing hormone	AR	None reported
GnRH Action					
<i>GNRHR</i>	4q21.2	138850	GnRH receptor	AR, Digenic	Microphallus, cryptorchidism

<sup>a</sup>*KISS1* gene mutations have been described only in association with central precocious puberty (CPP).

migration of GnRH neurons from the nasal placode to the hypothalamus, regulation of GnRH secretion, or GnRH action (see Table 33-1 for details).

Mutations in genes implicated in the development and migration of GnRH neurons cause Kallmann syndrome (KS), a condition of cHH with olfactory

abnormalities. The incidence of Kallmann syndrome has been estimated at about 1 in 10,000 males and at 5–7 times lower frequency in females. The degree of both hypogonadism and smell deficiency can vary significantly not only among unrelated patients but also within affected families, even between monozygotic twins. In

**TABLE 33-2** Genes associated with cHH

	Gene	Locus	OMIM	Product	Inheritance	Associated phenotype
Development and Migration of GnRH neurons	KAL1	Xp22.3	308700	Anosmin-1	X-linked	Hyposmia/anosmia, unilateral renal agenesis, midline facial defects (cleft lip/palate), synkinesia, oculomotor abnormalities, short metacarpals, sensorineural hearing loss, cerebellar ataxia, gut malrotations
	FGFR1 (KAL-2)	8q11.2-p11.1	136350	Fibroblast growth factor receptor-1	AD, Digenic	From anosmia to normosmia, variable severity of hypogonadism, cleft lip/palate, synkinesia, dental agenesis
	FGF8	10q24	600483	Fibroblast growth factor 8	AD,AR, Digenic	Hyposmia/anosmia Cleft lip/palate, synkinesia
	PROK2	3p21.1	607002	Prokineticin 2	AD/AR	Sleep disorders, obesity
	PROKR2	20p13	244200	Prokineticin-2 receptor	AD,AR, Digenic	From anosmia to normosmia
	CHD7	8q12.1	608892	Chromodomain helicase DNA-binding protein-7	AD	Part of CHARGE syndrome (coloboma, heart disease, choanal atresia, retarded growth and development and/or central nervous system anomalies, genital anomalies and/or hypogonadism, ear anomalies and/or deafness), from anosmia to normosmia
	NELF	9q34.3	608137	Nasal embryonic LHRH factor (for neurons and axonal outgrowth)	Digenic	Hyposmia/anosmia
	WDR11	10q26	606417	member of the WD repeat-containing protein family	AD ?	None reported
GnRH secretion	KISS1*	1q32	603286	Kisspeptin		None reported
	KISS1R	19p13.3	604161	KISS1 receptor	AR	Normosmia
	LEP	7q31.2	164160	Leptin	AR	Severe Obesity
	LEPR	1p31	601007	Leptin receptor	AR	
	TAC3	12q13-q21	162330	Neurokinin B	AD,AR	Microphallus, cryptorchidism
	TACR3	4q25	162332	Receptor for TAC3		
	PCSK1	15q15-q21	162150	Protein convertase subtilisin/kexin-type 1	AR	Obesity, variably impaired processing of several pro-hormones including those of LH, FSH, pro-insulin and pro-opiomelanocortin, hormone levels either low or immunologically measurable with low bioactivity
	NROB1	Xp21.3-p21.2	300473	Dosage-sensitive sex reversal-adrenal hypoplasia congenita (DAX1)		Adrenal failure
	GNRH1	8p21-p11.2	152760	Gonadotropin releasing hormone	AR	None reported
GnRH action	GNRHR	4q21.2	138850	GnRH receptor	AR, Digenic	Microphallus, cryptorchidism

\**KISS1* gene mutations have been described only in association with central precocious puberty (CPP).

some families, both typical KS phenotypes and dissociated phenotypes with either hypogonadism or anosmia occur. In addition, a variety of nonreproductive, non-olfactory anomalies can be present in KS patients (46). The type of anomalies as well as their incidence depends on the gene involved. For example, renal agenesis has been described only in association with *KAL1* mutations, while cleft lip and/or palate is more common in *KAL-2* mutation carriers. To date, seven causal genes have been identified (see Table 33-2).

Because of the over-representation of males with Kallmann syndrome, a predominant X-linked form was first suspected and the gene, designated *KAL1*, was isolated from the pseudoautosomal region (Xp22.3) (MIM \*308700) (47,48). *KAL1* encodes anosmin-1, a protein showing similarities with neural adhesion molecules and proteins involved in neuronal migration and axonal path finding; it interferes with the migration of GnRH neurons and olfactory nerves to their position in the hypothalamus (49). Although this dual function of anosmin-1 may

explain its involvement in both cHH and anosmia, only approximately 50% of X-linked families have mutations in *KAL1*. In sporadic patients with Kallmann syndrome, this frequency is even lower (5%). Subsequently, other genes responsible for variably transmittable forms of Kallmann syndrome have been identified (46). To begin with, *FGFR1* (or *KAL2*; MIM \*136350) mutations cause an autosomal dominant form of Kallmann syndrome (50). *FGFR1* encodes a tyrosine kinase receptor whose signaling pathway regulates neuronal migration, differentiation and survival, as well as cell proliferation during embryonic development. Mutations in *FGFR1* account for approximately 10% of Kallmann cases. The severity of the hypogonadism and the presence of associated phenotypes have variable expressivity including incomplete penetrance. Differently from *KAL1*, mutations in *FGFR1* were found in patients with normosmic cHH. Mutations in *FGF8*, which encodes one of the many ligands of *FGFR1*, are linked to Kallmann syndrome (51). *FGF8*-related Kallmann syndrome can be transmitted either in an autosomal dominant or in an autosomal recessive manner. Another autosomal dominant form of Kallmann syndrome is caused by mutations in the *CHD7* gene, which encodes the chromodomain-helicase-DNA-binding protein 7. These mutations predict to account for 6% of all cHH cases and are part of CHARGE syndrome, which can be present at different degrees of severity depending on the type of mutation. However, mutations in *CHD7* were also identified in patients with sporadic, normosmic cHH and Kallmann syndrome who were not diagnosed with the CHARGE syndrome. Two other genes implicated in both autosomal dominant and recessive forms of cHH are *PROKR2*, encoding a G-protein-coupled receptor (PK-R2), and *PROK2*, encoding a PK-R2 ligand, prokineticin-2 (PK-2). The prokineticin–receptor interaction primes a number of signaling cascades that are important for the development of the olfactory system as well as the initiation of GnRH neuronal progenitors. Mutations in either *PROKR2* or *PROK2* are estimated to account for 5–10% of all Kallmann syndrome cases. Lately, mutations in the *NELF* gene have been described in association with Kallman syndrome. *NELF* encodes the nasal embryonic LHRH factor, which is a guidance factor for olfactory and GnRH neurons and axonal outgrowth. Recently, one case of a de novo balanced chromosome translocation form a Kallmann syndrome patient with karyotype 46, XY, t(10;12) (q26.12; q13.11) has been described and the consequent detection of multiple independent missense variants in the *WDR11* gene in cHH patients argues strongly for a causative role for *WDR11* in this disorder. As all *WDR11* mutations were heterozygous, the cHH phenotype is likely to be transmitted in an autosomal dominant manner (52).

For each genetic form of Kallmann syndrome characterized so far, the clinical heterogeneity of the disease clearly indicates that the manifestation of different

Kallmann syndrome phenotypes is dependent not only on the gene involved but also on other factors—such as epigenetic modifications or modulator genes—that remain to be identified. Moreover, the incomplete penetrance of the disease might be partly due to a digenic or oligogenic inheritance pattern. For instance, some studies show that digenic mutations (e.g. in *FGFR1* and *NELF*) can synergize to produce a given phenotype (53).

Mutations in genes implicated in GnRH secretion at a hypothalamic level are associated with normosmic cHH (54). The most potent regulator of GnRH secretion and puberty onset is represented by Kisspeptin-1, encoded by the *KISS1* gene, which operates through interaction with the G-protein-coupled *KISS-1* receptor (also known as GPR54), encoded by *KISS1R*. While *KISS1* mutations have been described only in association with central precocious puberty (55), inactivating mutations in *KISS1R* account for about 5% of normosmic cHH cases, showing an autosomal recessive pattern of transmission. The same inheritance pattern is typical of mutations in the *LEP* gene, encoding the fat-derived hormone leptin, and the *LEPR* gene, which encodes the leptin receptor. Such gene alterations have been found in patients with severe obesity and hypogonadotropic hypogonadism and account for less than 5% of normosmic cHH. Another critical central regulator of GnRH secretion is neurokinin B (NK3R), a member of the substance P-related tachykinin family encoded by the *TAC3* gene. Loss-of-function mutations in *TAC3* or *TACR3* (encoding the neurokinin B receptor, NK3R) lead to severe congenital gonadotropin deficiency and pubertal failure (56). Only three cases of hypogonadotropic hypogonadism have been described in association with mutations in *PCSK1*, which encodes a neuroendocrine convertase (also known as prohormone convertase) that processes precursor peptides to release bioactive fragments (e.g. proinsulin). Although no reports have been published on the molecular mechanisms by which *PCSK1* mutations cause hypogonadism, the impairment of GnRH prohormone precursor processing is probably involved in these cases. The few *PCSK1* mutation carriers reported to date suffer from obesity as carriers of *LEP/LEPR* mutations. cHH can also be part of the adrenal hypoplasia congenita when the *NROB1* gene is mutated. *NROB1* encodes the orphan nuclear receptor DAX-1, which is crucial for the development and function of the adrenal gland and hypothalamic–pituitary–gonadal axis. Consequently, *NROB1* mutations are uncommon in hypogonadism without clinical evidence or a family history of adrenal failure. However, some cases of *NROB1* mutations leading only to reproductive disorders with normal adrenal function or mild/compensated adrenal failure have been described. An important feature of patients with these mutations is the resistance of azoospermia to gonadotropin stimulation. This is possibly due to the fact that *NROB1* is expressed in sertoli cells, and thus plays an important role in spermatogenesis.



An obvious candidate gene for normosmic cHH is *GNRH1*, which encodes gonadotropin-releasing hormone 1. Nevertheless, only one example of *GNRH1* mutation causing a familial HH has been reported to date (57).

Mutations in *GNRHR* are established to cause normosmic cHH. Encoding the GnRH receptor, *GNRHR* is the only gene implicated in GnRH action. Germline mutations in *GNRHR* were the first to be identified in hypogonadotropic patients and compound heterozygosity is frequently found for mutations with partial effects on *GNRHR* signaling. The Gln106Arg substitution is one of the most frequent mutations occurring in *GNRHR*. Large-scale screenings indicate that *GNRHR* mutations account for 3.5–16% of sporadic cases of normosmic cHH and up to 40% of familial cases of normosmic cases, which are transmitted in an autosomal recessive manner (54).

cHH associated with either anosmia (Kallmann syndrome) or a normal sense of smell is a treatable condition. Exogenous therapy with pulsatile GnRH or gonadotropin therapy usually restores normal pubertal development and fertility, whereas androgen therapy induces virilization. In about 80% of azoospermic men affected by cHH spermatogenesis can be induced by GnRH or gonadotropin therapy within 2 years and spontaneous pregnancy is highly likely during gonadotropin treatment. Although cHH is widely thought to require a lifelong therapy, reports on the sustained reversal of this disorder are available (58–60). These studies indicate that sustained reversal of cHH includes Kallmann syndrome patients and normosmic cHH, and this phenomenon has been described in association with mutations in a number of genes: *GNRHR*, *FGFR*, *KAL1*, *KISS1*, *TAC3* and *PROK2R*. To date, no clinical or laboratory data are available for the prediction of future reversibility; therefore, it is advised that all patients affected by cHH should be reassessed for recovery of the hypothalamo–pituitary–gonadal axis after a certain period of hormonal replacement therapy.

### 33.3.3 Persistent Müllerian Duct Syndrome

Persistent Müllerian duct syndrome (PMDS; MIM \*261550) is characterized by the persistence of the Müllerian duct derivatives (i.e. uterus, cervix, fallopian tubes and upper two-thirds of vagina) in a 46, XY patient with perfectly virilized external genitalia. PMDS leads to an uncommon (<200 cases described) autosomal recessive form of male pseudohermaphroditism with cryptorchidism (and associated azoospermia) and/or inguinal hernia as most prominent clinical features. In the absence of long-standing cryptorchidism, the testes usually contain germ cells (61).

In normal males, regression of the Müllerian duct is conducted by the action of the anti-Müllerian hormone (AMH), a member of the transforming growth factor- $\beta$

(TGF- $\beta$ ) family, and two receptors necessary for AMH ligand binding (AMH type II receptor) and signal transduction (AMH type I receptor) (62,63). Mutations leading to PMDS have been identified in the *AMH* gene (localized at chromosome 19p13.3) and in the AMH type II receptor (chromosome 12q13) (64,65). Depending on the affected gene, either normal quantities of AMH are present in the testis (AMH type II receptor mutations; MIM \*600956) or no AMH is produced (AMH gene mutations; MIM \*600957). Some of these mutations do not affect the AMH secretion rate (normal serum levels were detected), suggesting that they might only compromise the function and the bioactivity of the hormone (64). However, some PMDS patients have no mutations in either of these two genes, indicating that other genes are involved in PMDS. The AMH type I receptor would be a likely candidate, but mutations in this receptor have not yet been identified.

### 33.3.4 Luteinizing Hormone, Luteinizing Hormone Receptor and FSH and FSH Receptor

Gonadotropins—LH, follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG)—have a common  $\alpha$ -subunit and a hormone-specific  $\beta$ -subunit (66). So far, mutations in the  $\alpha$ -subunit have not been described but on the basis of a knockout mouse model, the phenotype would probably include hypogonadotropic hypogonadism, and hypothyroidism due to associated thyroid-stimulating hormone (TSH) deficiency (67).

The crucial role of LH in the maintenance of Leydig cell function is demonstrated by the description of a single patient carrying the same mutation in both alleles of the LH  $\beta$ -subunit (*LH- $\beta$* ; MIM \*152780). This patient is presented with hypogonadism (small, bilaterally descended testes) and pubertal delay (68). Immunoreactive LH was higher than normal but bioactive LH was lower, explaining the delayed puberty. A testicular biopsy revealed spermatogenic arrest and a complete absence of Leydig cells, indicating the absolute need of LH in Leydig cell formation. Molecular analysis of his *LH- $\beta$*  gene demonstrated the presence in both alleles of an arginine substitution for a glutamine (Q34R), which impaired hormone-receptor binding. As no other patients have been described, LH- $\beta$  deficiency is probably a very rare cause of autosomal recessive hypogonadism.

Both inactivating and activating mutations have been described for the LH receptor (MIM \*152790) (69–71).

Inactivating mutations lead to an impaired testosterone response to LH and hCG as both these gonadotropins bind to the LH receptor. Inactivating mutations were first reported in two siblings with 46, XY karyotype who presented with female external genitalia and primary amenorrhea (around 40 years of age) (72). Depending on the residual function of the receptor, patients (46,

XY) carrying such mutations can present with phenotypes ranging from complete failure of virilization, through hypospadias, to micropenis, absence of puberty and infertility. Several other patients of male genetic sex with inactivating mutations in the LH receptor have since been described, and the mutations impair either signal transduction or ligand binding, or both (73,74). Women carrying inactivating homozygous mutations in the LH receptor might suffer from oligomenorrhea or amenorrhea although they undergo a normal pubertal development (69).

Activating mutations in the LH receptor lead to a constitutively activated receptor without stimulation by a gonadotropin ligand (69). These mutations are associated with familial precocious male puberty, an autosomal dominant condition in which puberty in patients is initiated at the age of 1–4 years (MIM \*176410). The constitutively activated LH receptor does not induce puberty in females, probably because both LH and FSH are required for the activation of ovarian steroidogenesis.

The exact role of FSH in spermatogenesis is less clear. In human studies, FSH is necessary for quantitatively normal spermatogenesis (75). In fact, mutations of *FSHβ* (FSH  $\beta$ -subunit) and *FSHR* (FSH receptor) have been reported mainly in women (76,77). However, azoospermia has been reported in two men carrying mutations on the so-called “seatbelt” region of *FSHB*, while milder phenotypes could result from mutations in other regions of the glycoprotein (78).

### 33.4 MONOGENIC DEFECTS IN POST-TESTICULAR AND PRIMARY TESTICULAR FORMS OF MALE INFERTILITY

#### 33.4.1 Post-testicular Form: Congenital Absence of the Vas Deferens

Congenital bilateral absence of the vas deferens (CBAVD; MIM \*277180) is a post-testicular disease characterized by the absence of the scrotal vasa, which results in a blockade of the transport of the spermatozoa from the testis or the epididymis to the distal genital tract. This form of obstructive azoospermia is responsible for 1–2% of male infertility and for up to 6% of all patients with obstructive azoospermia (79). The congenital unilateral absence of the vas deferens (CUAVD) is associated with oligozoospermia. CBAVD is also present in most males with cystic fibrosis (CF; MIM \*219700), the most common autosomal recessive disease in populations of Caucasian origin. The similarity between infertility in CF males and in males with CBAVD without other clinical characteristics of CF led to the hypothesis that isolated CBAVD is an incomplete form of CF (80). CF occurs with a frequency of about 1 in 2500 live births (carrier frequency 1 in 25) and is caused by mutations in *CFTR*, which encodes the

CF transmembrane conductance regulator, a protein involved in chloride conduction across epithelial cell membranes (81–83). The most widely diffused mutation in *CFTR* is delta F508 ( $\Delta F508$ ), which accounts for about 70% of the total CF mutations in Caucasians. This 3-bp deletion in exon 10 leads to the deletion of a phenylalanine at amino acid position 508 of the protein (p.Phe508del) and is responsible for the majority of defects found in CF and in congenital absence of the vas deferens (CAVD) patients. *CFTR* is one of the most studied genes in the human genome: more than 1400 mutations have been identified in *CFTR* and most of these occur with a low frequency or are even unique to single CF families. A wide variation in phenotype occurs with *CFTR* mutations. Patients with the gravest form of CF have mutations in both alleles of *CFTR* resulting in no residual *CFTR* activity. Patients with milder clinical manifestations such as CAVD, instead, might present with two scenarios: a severe mutation in one allele and a mild mutation, which leaves some residual *CFTR* activity, in the other allele; or, two mutant alleles with mild effect on the protein.

In the majority of males with CBAVD (about 80%), *CFTR* mutations can be found (84). Many of these mutations are unique to CBAVD and are not found in CF patients. Moreover, a role of intron 8 variants (IVS8-5 T, IVS8-7 T, and IVS8-9 T) in the phenotypic expression of mutations has also been established. The three variants include different numbers of thymidine within the acceptor splice site of intron 8, i.e. 5, 7 and 9 thymidines, respectively. The length of the thymidine (T) tract affects the splicing efficiency of exon 9 and thus the percentage of normal *CFTR* mRNA. The 5 T tract is the least efficient and allows about 8–10% of *CFTR* mRNA to be completed with exon 9. The splicing efficiency of the 5 T variant, probably influenced by other polymorphisms in *CFTR* (84,85), is variable between different individuals and different tissues of the same individual, but in the epididymal epithelium and the vas deferens, it is consistently lower than in other tissues (84,86). The 5 T variant of *CFTR* appears to occur at a much higher frequency in CBAVD than in the general population (21% vs 5%) (87). For all these reasons, the 5 T variant has been classified as a mutation associated with CBAVD but with incomplete penetrance. In about 20% of patients, CBAVD does not seem to represent a mild form of CF (88,89). These patients have CBAVD-associated urinary tract malformations, and no *CFTR* mutations are found in this situation. The etiology of this form of CBAVD remains unknown but is probably caused by damage to the Wolffian duct before its split into the reproductive and the ureteral part at about 7 weeks gestation. Evidence is provided by the fact that neither CF nor CF-associated CBAVD patients present alterations in the ureteral ducts. In fact, *CFTR* dysfunction probably leads to damage of the genital ducts, either through a morphogenetic defect

or through a progressive obstruction due to excessive mucus secretion, once the split of the Wolffian duct has occurred (90).

The condition CUAVD is also associated with mutations in *CFTR* when contralateral genitourinary anomalies are present (91). This is not the case when anatomically complete and patent vasa are present on the side of the palpable vas. These patients often present with renal abnormalities at the ipsilateral side of the absent vas.

Involvement *CFTR* in other conditions of male infertility has also been suggested (92–94). An increased frequency of *CFTR* mutations was described in patients with obstructive azoospermia (non-CBAVD) and reduced sperm quality and quantity; these data have not been confirmed by subsequent studies and currently, the *CFTR* mutation screening should be limited to obstructive defects, which can be correlated to the reduction of functional *CFTR* protein (i.e. CAVD on at least one side) and idiopathic epididymal obstruction. Given that the frequency of a particular *CFTR* mutation is variable among different geographic areas and shows important ethnic differences, the routine mutation screening is based on a panel of mutations (normally 30–80), which are the most common for a given population. Since the 5 T tract variant is now considered a mild *CFTR* mutation rather than a polymorphism, it should be analyzed in each CAVD patient. If no mutations are found, a full-sequence analysis is performed.

Patients affected by CAVD may have sperm in their ejaculate (unilateral forms, CUAVD) or are azoospermic (bilateral forms, CBAVD). By combining the application of testis biopsy with ICSI, both CF and CBAVD patients may now generate their own biological children although the risk of transmitting their *CFTR* mutations to their descendents has to be considered. As the carrier frequency of *CFTR* mutations in people with Northern European descent is high (1:25), the screening for CF gene mutations in the female partners of men with CAVD without congenital kidney anomalies or with CF should be recommended before assisted reproduction. If mutations are detected in both partners (possibly by sequencing *CFTR*), the risk of an offspring with CF or mild forms of CF is very high and PGD should be offered to the couple. However, in many cases, it remains difficult to make precise risk estimates due to different degrees of penetrance of the same genotype between different individuals.

Given its association with obstructive azoospermia in combination with chronic sinopulmonary disease, a condition referred to as Young syndrome (MIM \*279000) was thought to be related to CF. However, no increased frequency of *CFTR* mutations is found in these patients, indicating that Young syndrome is a CF-independent disorder. Instead, it might be related to childhood mercury poisoning and thus be an acquired disease (95).

### 33.4.2 Monogenic Defects in Primary Testicular Failure

On the basis of data obtained from animal models, expression studies, linkage analyses and cytogenetic findings, a number of candidate spermatogenesis genes have been identified and represent the most obvious targets for mutation screening in men with spermatogenic failure (96). These genes can be classified according to whether they are involved in the endocrine regulation of spermatogenesis (discussed in detail in Section 33.3), common cell functions, and specific spermatogenic functions.

Studies on genes involved in common cell functions that are relevant also for normal spermatogenesis are relatively few and are mainly single studies. An exception to this statement is *MTHFR*, which encodes methylenetetrahydrofolate reductase, one of the key enzymes of folate metabolism, which reduces 5,10-methylenetetrahydrofolate to its biologically active form 5-methyltetrahydrofolate. In particular, the C677 T polymorphism of *MTHFR* correlates with decreased *MTHFR* activity. Altered folic acid metabolism influences DNA methylation, and subjects with homozygous C677 T mutations have a lower level of genomic DNA methylation compared to wild-type controls (97). As aberrant DNA and protein methylation are likely to affect spermatogenesis, C677 T has been extensively analyzed in infertile patients from different populations (98–102) with highly discordant results. The most likely explanation is that the polymorphism is relevant only in specific environmental conditions in combination with low dietary intake of folates (34).

Regarding those genes involved in specific spermatogenic functions, not only Y-linked genes but also an increasing number of autosomal and X-linked candidate genes are described to play a role in the complex process of spermatogenesis (103). In particular, the X chromosome appears to be of special interest as men are hemizygous and de novo mutations will have an immediate impact because compensation by a second normal allele is not possible. Furthermore, this chromosome contains an unexpectedly high number of X-linked genes expressed in the testis that might have an important role in spermatogenesis (104). However, human data are extremely scarce in this regard and only seven selected X-linked genes have been screened in infertile men (*AR*, *SOX3*, *USP26*, *NXF2*, *TAF7L*, *FATE* and *AKAP4*). Except for *AR*, no infertility causing mutations have been described thus far (105).

A large number of genetic variants, mainly in autosomes, have been proposed as risk factors for male infertility, but in many cases, only sporadic data are available and when more studies are published on the same polymorphism, results are often contradictory (34,103). The only clinically relevant polymorphism confirmed by meta-analyses is the gr/gr deletion, about which more

detail is given in Sections 33.1 and 33.2.2. Whether the paucity of results is due to a real rarity of causative gene mutations/genetic risk factors or to an inappropriateness of the currently used approaches is yet to be established.

## 33.5 SYNDROMIC MONOGENIC DEFECTS

### 33.5.1 Bardet–Biedl Syndrome

Bardet–Biedl syndrome (BBS; MIM \*209900) is characterized by obesity, polydactyly, mental retardation, renal anomalies, retinal pigmentary dystrophy, and hypogenitalism (106,107). Additional characteristics include deafness, diabetes mellitus, dental anomalies, and small stature. In females, vaginal atresia and delayed puberty are observed. In males, hypogenitalism includes cryptorchidism, micropenis, and various degrees of hypospadias.

BBS is primarily inherited as an autosomal recessive disease but more complex forms of inheritance might exist (108,109). The incidence is less than 1 in 100,000 live births. The syndrome seems to be associated with *BBS1* (MIM \*209901) in most of the families (36–55%). However, the condition is genetically very heterogeneous, and 14 genes have so far been implicated (110).

### 33.5.2 Prader–Willi Syndrome

Prader–Willi syndrome (PWS; MIM \*176270) is a neurobehavioral disease that affects both males and females and is characterized by infantile hypotonia, developmental delay and mental retardation, obesity, and hypogonadism (111). In males, the hypogonadism manifests as cryptorchidism and scrotal hypoplasia. The prevalence of PWS is from 1/10,000 to 1/15,000. PWS is caused by a disturbance in the parent-of-origin pattern of inheritance at chromosome region 15q11–q13 (imprinting) and results from the absence of expressed paternal genes from this imprinted region. Several genes at 15q11–q13 are expressed only from the paternal chromosome, and PWS results from paternal interstitial deletion of the region (about 70% of patients), maternal disomy (29%), or a defect in the imprinting mechanism (1%). The mechanism underlying imprinting is not well understood, but parent-of-origin-specific DNA methylation is important in the regulation of the expression of imprinted genes.

### 33.5.3 Primary Ciliary Dyskinesia

Primary ciliary dyskinesia (PCD, also known as immotile cilia syndrome; MIM \*242650) constitutes a genetically heterogeneous group of disorders resulting from the absence of mucociliary clearance of the airways and other ciliated structures because of severe or complete immotility of the cilia (112). The major clinical features are

chronic respiratory tract disease, rhinitis, and sinusitis. Most males are also infertile because of immotility or poor mobility of spermatozoa as a consequence of structural and functional abnormalities in the central part of the sperm flagella. About half of the patients with PCD also display situs inversus, a condition known as Kartagener syndrome (MIM \*244400). The prevalence of PCD is around 1/25,000 and that of Kartagener syndrome 1/50,000. The mode of inheritance is autosomal recessive in the majority of cases.

The diagnosis of PCD can be made only by electron microscopy to identify the defective components in the cilia. The common defect in PCD is the absence of dynein arms, the structures necessary for the generation of movement of cilia and sperm tails. At the molecular level, mutations in three genes have been identified as causing PCD. Two of these genes, *DNAH5* and *DNAH11*, encode axonemal heavy dyneins while the third gene, *DNAI1*, encodes an intermediate dynein. Several other genes involved in cilia formation have been identified, but no mutations have been reported (99).

### 33.5.4 Noonan Syndrome

Noonan syndrome (MIM \*163950) is an autosomal dominant disorder characterized by short stature, congestive heart failure (most often pulmonary valve stenosis or hypertrophic cardiomyopathy), and a typical facial dysmorphism with posteriorly rotated ears. About half of the male patients with Noonan syndrome have azoospermia or oligozoospermia resulting from bilateral cryptorchidism. Affected females and males with normally descended testes are fertile (113). The incidence of Noonan syndrome is estimated at 1/1000–1/5000 live births. Mutations in *PTPN11*, a gene encoding the non-receptor protein tyrosine phosphatase SHP-2, cause Noonan syndrome in approximately 50% of patients. A significant proportion of Noonan syndrome cases (almost 50%) are the result of new mutations (sporadic cases) that probably originate most of the time in the paternal germline, at least when associated with *PTPN11* mutations (114).

In some Noonan syndrome-like conditions such as cardio-facio-cutaneous, Noonan/neurofibromatosis and Costello syndromes, no mutations in *PTPN11* were found (115). In contrast, mutations in *PTPN11* are found in LEOPARD syndrome (116).

### 33.5.5 Myotonic Dystrophy

Myotonic dystrophy (DM; MIM \*160900) is an autosomal dominant disease caused by a trinucleotide (CTG) repeat expansion in *DMPK* at chromosome 19p13.3 (117–120). It is the most common form of adult muscular dystrophy (prevalence 1/7000–1/8000 births)



characterized by myotonia and muscle weakness and wasting. Additional anomalies include cardiac conduction disturbances, cataracts, and progressive testicular tubular atrophy in 60–80% of males.

The number of CTG repeats in the 3′ untranslated region of *DMPK* is polymorphic in normal individuals and ranges from 5 to 35. Genes with 50–80 repeats are unstable and the number of repeats is likely to expand on transmission to the offspring. The severity and time of onset of the disease will, in general, be related to the number of repeats. The progressive earlier appearance of the disease in successive generations with an increase of disease severity is called anticipation (121,122). In its severest form, a few thousand repeats are present. A gene for a less-frequent form of myotonic dystrophy (DM2, proximal myotonic myopathy or PROMM, MIM \*602668) is clinically very similar to classic DM and is caused by a CCTG expansion in intron 1 of *ZNF9* (123).

### 33.6 CONCLUSION

In this chapter, most of the more common genetic defects associated with male infertility are described in detail. With the advent of testing for Y chromosome microdeletions, about 7% of those patients previously designated “idiopathic” now have a defined cause. Testing for Y chromosome microdeletions is necessary before performing TESE/ICSI because deletions of the AZFa and AZFb regions of the Y chromosome will lead to absence of spermatogenesis in male offspring. With the advancement of ART including ICSI and TESE, previously infertile men can now generate their own progeny. Patients include those with KS and those with CBAVD and CF. However, with the wider use of these sophisticated but effective forms of ART, the couple has to be counseled about the potential transmission of the gene defect to the progeny. In cases where infertility may be associated with a severe medical condition, the option of PGD needs to be discussed with the couple. It should be noted that ART babies have smaller birth rates and the long-term effects of low birth weight in population-based studies are not known. Considering the high number of genes involved in spermatogenesis (more than 2000) and the fact that only a fraction of them has been analyzed so far, we expect that new genetic factors will be discovered in the near future. In the era of ICSI, the definition of genetic causes is urged, considering that still about 50% of infertile men have an unknown etiology and are likely to be carriers of unknown genetic anomalies.

Besides genetic factors, it is expected that altered epigenetic modifications of the male gamete may also play a role in male infertility. Some studies suggest that there is an increased incidence of rare imprinting disorders in babies conceived by ART, however, to date, only few studies have focused on the analysis of imprinting abnormalities in spermatozoa derived from infertile men

(for review, see Reference (124) and references therein). Recently, it has been reported that the repertoire of epigenetic modifications in the mature sperm may have a potential role in the developing embryo (125). These epigenetic changes include DNA demethylation and the retention of modified histones at important developmental, signaling and micro-RNA genes. Alterations of the sperm epigenome may therefore reduce the fertilizing potential of spermatozoa and thus become a new cause of male factor infertility. However, it is also predicted that epigenetic alterations may not necessarily abolish the sperm fertilizing ability although they would still occur with a higher frequency in infertile men. Future epigenetic studies are warranted, especially if we consider that methylation alterations in the gametes of infertile patients may pose a risk for transgenerational epigenetic inheritance.

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# CHAPTER

# 34

## Fetal Loss

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### 34.1 BACKGROUND

The loss of a desired pregnancy, while sad, is not an uncommon event. It is well established that 15% of recognized pregnancies are lost (1), usually during the first 13 weeks of pregnancy (2), with the incidence of early pregnancy loss increasing with maternal age and a history of prior pregnancy loss. An additional 22% of conceptions are lost between implantation and the clinical recognition of pregnancy (preclinical losses), bringing the total rate of early pregnancy loss to nearly 40% of conceptions (3). The vast majority of fetal loss (4) occurs during the first trimester of pregnancy, with the loss rate dropping dramatically after 12 weeks' gestation. The incidence of these early losses is directly correlated with the age of the mother, as the incidence of pregnancy loss increases threefold over the mean in women who are between 34 and 39 years of age, and sixfold in women over 40 years of age. As early as 1984, Hassold and Chiu (5) demonstrated that the pattern of age-related fetal loss closely mirrors the increased incidence of aneuploidy associated with advanced maternal age, suggesting a relation between these two phenomena. Yet, the obstetrical literature is replete with investigations of a variety of other causes of pregnancy loss, with little consensus on the factors responsible for the limited viability of human conceptions, and many reviews reiterating that in 50% of couples experiencing pregnancy loss, the causative factor cannot be determined. This chapter will review the data on the etiology of pregnancy loss, focusing on embryonic and fetal chromosome abnormalities and other established genetic causes that are usually associated with fetal loss early in gestation, and discuss other

factors that contribute to later pregnancy loss, as well as recurrent loss.

### 34.2 DEFINITION OF TERMS

Pregnancy loss can be defined as the unplanned, spontaneous loss of a pregnancy before the fetus is capable of extra uterine survival. In the United States, the term spontaneous abortion or miscarriage is applied to a pregnancy lost prior to 20 weeks' gestation, after which time the loss is defined as either an intrauterine fetal death or stillbirth. Preclinical abortion (before 6 menstrual weeks' gestation) occurs when the conceptus fails to implant or aborts shortly after implantation. The term missed abortion is used when the fetus has died but is retained in the uterus, often for several weeks. A pregnancy is classified as an inevitable abortion when uterine contractions cause dilation of the cervix, leading to either a complete abortion (all products of conception (POC) are expelled and the cervix subsequently closes) or an incomplete abortion (only a portion of the POC are passed through the cervix). Recurrent abortion has typically been defined as two to three or more losses. Recurrent fetal loss is less common, involving only 1–2% of couples, and recurrent fetal loss at or beyond 14 weeks of gestation is a very rare event.

The majority of spontaneous abortions occur in pregnancies with incomplete fetal development (6). However, abnormal development does not always end in spontaneous abortion, as is evident from the 3–4% population incidence of sporadic congenital anomalies in the newborn population.

### 34.3 EARLY PREGNANCY LOSS

#### 34.3.1 Cytogenetic Abnormalities in Human Conception

It has been known since the mid-1960s that chromosome abnormalities are found by cytogenetic evaluation of cultured tissue from spontaneous abortions in at least 60% of first-trimester losses. More recent data suggests that as high as 80% of first-trimester losses are the result of an unbalanced karyotype in the fetus, making chromosome anomalies the single most common reason for the loss of a pregnancy during the first trimester. An early epidemiologic study (7) on 1500 spontaneous abortion samples collected from 1966 to 1972 reported that 61.5% of pregnancy losses in which the embryo was less than 12 weeks of age demonstrated a chromosome abnormality by culturing tissue collected at the time of the loss. Historically, most laboratories culturing this type of “products of conception” material find a significant excess of 46,XX karyotypes among the samples without a chromosome error, and this excess of 46,XX normal female results has been ascribed to nonviable fetal material with the growth of maternal tissue that was present in the sample collected. In such cases, it is the maternal, rather than the fetal karyotype that is being described, and this falsely inflates the number of “normal” fetal karyotypes reported. In addition, the early studies were done in the “pre-banding era,” when the techniques available would fail to recognize more subtle genomic abnormalities (such as deletions or duplications). Other complications limiting the evaluation of the true incidence of abnormal fetal genomes by doing tissue culture and routine chromosome analysis on abortus specimens include the rate of culture failure due to nonviable specimens (either due to missed abortions or samples with nonviable genetic alterations) and contaminated samples that cannot be grown and evaluated. Hence, the value of approximately 60% aneuploid fetuses reported by this and other early studies should be considered a minimum estimate of the incidence of chromosome abnormalities in miscarriages. Thus it is not surprising that a recent study, using semidirect analysis of chorionic villi (CV) from pregnancies that arrested in the first trimester (8) found that 80% of early pregnancy losses demonstrated a chromosome abnormality. As clinical evaluation of ongoing pregnancies have shown confined placental mosaicism, which is the presence of a chromosome abnormality in extraembryonic tissue that is not present in the fetus, in only 1–2% of CV studies, this work of Morales et al. (8) suggests that upwards of 80% of early pregnancy losses actually are the result of a chromosome anomaly in the fetus.

The knowledge that 15% of recognized human pregnancies are lost and that upwards of 80% of these are aneuploid, coupled with the frequency of unbalanced chromosome abnormalities in stillborns and live borns (5% and 0.4%, respectively), suggests that chromosome

abnormalities occur fairly frequently at conception in humans. Yet the “order of magnitude” decrease in the incidence of chromosome abnormalities from the first trimester of pregnancy, to the last trimester of pregnancy, and then again to birth, suggests that there is very strong selection against human fetuses with unbalanced chromosome complements. A variety of methodologies, designed to improve preimplantation genetic diagnosis of embryos fertilized in vitro, have provided information addressing the incidence of abnormal karyotypes close to the time of conception. It is important to remember when evaluating these studies that they are complicated by the use of embryos created in vitro and the very manipulation of gametes and embryos in an artificial setting may affect genomic stability (9). In addition, besides discrepancies between the techniques themselves, the populations from which the embryos were obtained are not always equivalent, with varying ages and pregnancy histories, which as shown later, will influence the incidence of chromosome abnormalities. But these studies provide previously unrecognized information about the incidence and complexity of embryonic chromosome abnormalities, and show an unanticipated very high frequency of chromosomal abnormalities in preimplantation embryos, which may be correlated with the high incidence of early loss in both in vivo and in vitro embryos.

Fluorescence in situ hybridization (FISH) analysis of fertilized oocytes, early embryos and individual cells from such embryos obtained during the in vitro fertilization (IVF) process has provided new insights into the complexity of human reproduction. This approach uses differentially colored fluorescent probes, directed to single regions, often the centromere, of a chromosome, to assess the chromosome content of interphase cells. While this technique can be applied to single cells, or nonviable samples, it is limited by the number of chromosome-specific probes that can be evaluated in a single study and thus cannot provide a complete chromosome analysis. These studies, depending on the probe set used, have confirmed a high level of aneuploidy (30–83%) in early embryos (10–15). In addition, these studies have revealed the presence of both autosomal monosomy and nullosomy (10), which are virtually never observed in live borns or cell culture of miscarriage samples, but may contribute to the incidence of preclinical pregnancy loss. As seen in both prenatal diagnosis and newborn surveys, these studies revealed that the incidence of fetal aneuploidy is highly correlated with advancing maternal age (11), so that the incidence of aneuploid embryos observed is directly proportional to the mean maternal age in the group being studied. FISH done on multiple cells from early embryos also has shown a high rate of embryonic mosaicism, with up to 83% of 4-day embryos showing more than one cell population (12). The same study demonstrated that this mosaicism, which by definition is a postfertilization event, declines with further development of the embryo, possibly because of the death of abnormal cells, or an aneuploid rescue mechanism, so that only 42% of

8-day embryos showed multiple cell lines. An even more unexpected observation was the presence in several multi-cell FISH studies of “chaotic embryos” showing extreme mosaicism with different chromosome abnormalities in almost every cell evaluated from the same embryo (12). All the aberrations detected by FISH contribute to the lack of viability of IVF embryos and, if also present in vivo, could explain the lower fertility rates seen in humans when compared to other mammals (10).

Molecular karyotyping by comparative genomic hybridization (CGH) has furthered the ability to assess the frequency and origin of chromosome abnormalities in both oocytes and embryos at various stages of development. This technology permits the assessment of both whole chromosome as well as segmental aneuploidy. However, the need to amplify the DNA from a single cell prior to array comparative genomic hybridization (aCGH) may introduce artifacts. Array-based CGH on human oocytes has demonstrated aneuploidy in 3–65%, depending on the study, age of the mother, and level of maturity (13). CGH following degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) amplification of the DNA from single blastomeres of 12 human cleavage embryos (12) revealed only 3 to be normal (25%), with 3 (25%) having evidence of meiotically derived aneuploidy (all cells with the same abnormality), and the other 50% showing mosaicism, a postfertilization event. Similarly, Wells and Delhanty (18) reported 75% of 3-day embryos to have chromosome abnormalities with a high percentage of chromosomal mosaicism. Both these early studies reported some “chaotic embryos” in which different chromosome abnormalities were observed in different cells from the same embryo. Using aCGH, Vanneste et al. (19) showed that while only 12.5% of fertilized human oocytes were chromosomally unbalanced, 83% of 3-day embryos showed whole-chromosome aneuploidy. Only 3 of 19 (15.8%) of these embryos showed consistent abnormalities in all blastocysts, compatible with a meiotic origin of the abnormality, while the rest were mosaic. Healthy, cleavage-stage embryos obtained by IVF (for evaluation of X-linked disorders, BRCA2 mutations, or familial microdeletion syndromes, from woman less than 35 years of age, whose partners had a normal semen analysis, and without parental chromosome rearrangements) showed mosaicism in 91% of embryos. Whole-chromosome aneuploidy was observed in 83%, terminal segmental aneuploidy (deletions, duplications or amplifications) in 70% and uniparental disomy (UPD) in 9% (20). A comprehensive series of aCGH studies on 1290 human embryos from women aged 29–50 years (10) demonstrated that 58% of these blastocysts contained chromosome abnormalities. Subsequent single nucleotide polymorphism (SNP) analysis of these embryos revealed that only 43% of these abnormalities (representing 25% of all blastocysts) were due to meiotic errors. Overall, the range of genetic imbalance detected in embryos

by aCGH varies significantly from 12.5% to 83% (15,16,18,19,21,22), possibly a reflection of differences in the maternal populations from whom the embryos were obtained. Analysis of multiple single cells from a single embryo demonstrates a high level of postfertilization instability leading to both mosaicism and the chaotic embryos reported by FISH. Of note, in a majority of cases, embryos with mitotically derived errors (mosaic or chaotic embryos) were less likely to be viable than those with meiotic errors suggesting that many of the mitotic, postfertilization chromosome errors may contribute to infertility and preclinical loss, while the meiotic errors may be more commonly associated with the loss of recognized pregnancies. In addition, aCGH has found more subtle chromosome imbalances in 2 of 11 fetuses (18%) reported to have a normal banded karyotype (21), indicating that even pregnancies reported to be normal and balanced by routine cytogenetics may harbor subtle segmental aneuploidies that contribute to their nonviability.

SNP arrays have been used to evaluate early embryos, and the limited reports using this methodology do not always support the high abnormality rates identified by FISH. A group specifically addressing this question by evaluating the same embryos with both FISH and SNP microarrays (22,23) reported significant discrepancies. In one study on 13 cleavage-stage embryos, nine-probe FISH demonstrated mosaic aneuploidy in 77%, while only 38.5% were abnormal on microarray analysis. In another study, the same group found consistency between FISH and microarray results in less than 50% of samples, with most of the discrepancies being in embryos reported to show monosomy or complex aneuploidy. Another group (24), using a different genome-wide genotyping SNP microarray and software that makes use of parental genotypes to minimize error-prone single-cell microarray data, found aneuploidy rates in early embryos of 20–70% depending on the age of the women from whom the embryos were obtained. Additional use of this approach may also be of value in determining the impact of UPD on pregnancy loss, which to date, has been reported to be infrequent (25).

### 34.3.2 Pattern of Chromosome Abnormalities Seen in Aborted Pregnancies

The vast majority of chromosomal abnormalities observed in aborted fetuses evaluated by G-banding are numerical, including autosomal trisomies, polyploidy, sex chromosome monosomy and double trisomies (26). A 2009 study, combining G-banding with MLPA and aCGH (27) on 115 first-trimester miscarriages, found 69 (60%) to be chromosomally abnormal. Of these, 69% had autosomal trisomy (including 2% with double trisomies), 12% were polyploid (primarily triploidy), and 10% had sex chromosome monosomy (45,X), with only 1% showing structural abnormalities and the rest

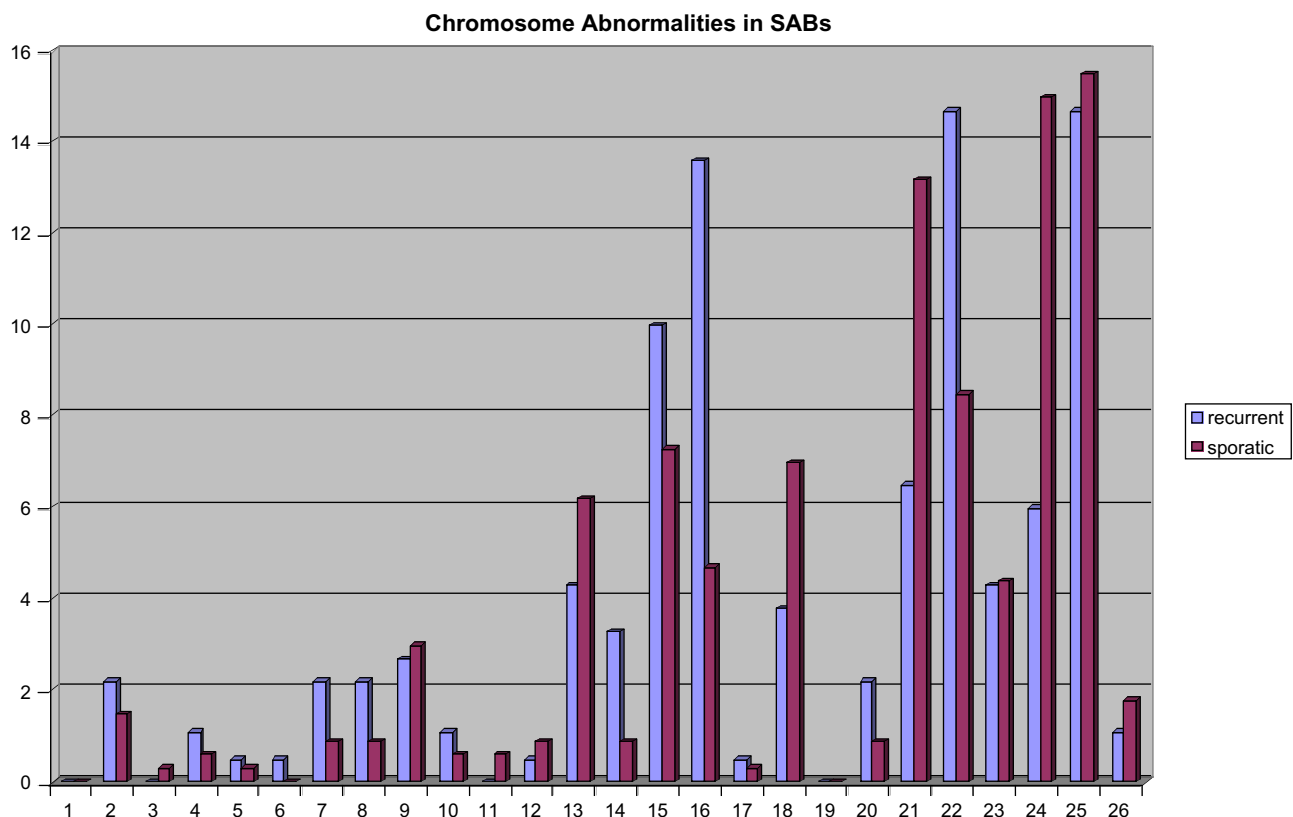


showing errors not involving entire chromosomes, such as duplications or deletions. Similar results were reported by combining karyotype analysis with reflex FISH (28), which observed 61% trisomy, 15% polyploidy (primarily triploidy), 14% sex chromosome monosomy and 7% structural abnormalities. It is not surprising that the most common abnormalities seen are autosomal trisomies, as it was recognized as early as 1984 by Hassold and Chiu (5) that the risk of both pregnancy loss and the incidence trisomy as the result of maternal nondisjunction increase with maternal age, and thus are likely to occur concurrently. Our own data (unpublished) shows the most frequent chromosome abnormalities in presumably sporadic fetal losses to be triploidy, sex chromosome monosomy, and trisomies (21, 22, 15, 18, 13 and 16 in descending order (Figure 34-1)). A slightly different pattern was observed among losses from women with a history of pregnancy loss, with the most prevalent abnormalities being triploidy, and trisomies 22, 16, 15 and 21. Interestingly, the pattern associated with sporadic loss is similar to that due to meiotic errors (9), while the pattern seen in the women with recurrent loss has been associated with mitotic errors seen in mosaic IVF embryos. The relative paucity of sex chromosome monosomy among the recurrent losses might be related to the

slightly advanced age (37.3 vs 36.2 years) in this group, as sex chromosome monosomy is most often due to non-disjunction in males, and thus would not necessarily be related to maternal age. Double trisomies, which, except in very rare instances involving the presence of an extra sex chromosome, are not viable, are not uncommon in abortus samples, representing about 1–2% of these cases (29). They are almost always a result of maternal nondisjunction (30) and are also associated with older maternal age. It should be noted that while studies on preimplantation embryos (see earlier) often report autosomal monosomy, peaking at about the eight-cell stage, such karyotypes are inviable, and autosomal monosomy has not been reported in abortus specimens.

### 34.3.3 Association of Advanced Maternal Age and Chromosome Abnormalities in Miscarriage

It is well established that increasing maternal age is the most important etiological factor associated with trisomy in humans. The National Down Syndrome Project (31) showed a significant association between advanced maternal age and trisomy 21 due to both meiosis I and meiosis II errors. This age-related increase in nondisjunction may be



**FIGURE 34-1** Relative frequency of chromosome abnormalities observed in cytogenetically abnormal POCs from women with a reported history of recurrent pregnancy loss compared to those with reported sporadic pregnancy loss. Mean maternal age was 37.3 years in the recurrent group and 36.2 years in the sporadic group. Number of chromosomes involved presented across the X axis with 23=double trisomy, 24=monosomy X, 25=triploidy, 26=tetraploidy. The abnormalities that are considered viable (trisomy 13, 18 and 21 and monosomy X) are all more frequent in the group with sporadic losses, with trisomies 15, 16 and 22 being more prevalent among those with recurrent loss.

due to alterations in the cohesin complex (32,33) or differences in the patterns of recombination (34) during meiosis. Since the incidence of miscarriage also increases with maternal age, going from 10% in women 20–24 years of age to over 90% in women over 45 years of age (35), most pregnancy losses, especially those in older women, are associated with errors due to maternal nondisjunction. Boue and colleagues (36) reported that the mean maternal age of woman whose abortuses demonstrated a chromosome anomaly was increased compared to those with normal fetal results. This is consistent with the increased incidence of autosomal trisomy with advancing maternal age. Hassold and Chui (5) confirm this association, reporting that the rate of trisomies seen in spontaneous abortions is 20% in women under 25 years, 33% for those 30–35 years of age, and at least 67% in women who are 40 or older. Similarly, Fragouli and Wells (10) found that older women had a tendency to generate more chromosomally abnormal blastocysts, with an aneuploidy range of 46.5% in women 34 years of age or younger and 60.6% in women over age 35. In one set of SNP microarray studies cited earlier (22,23), the incidence of fetal aneuploidy increased from 19.6% when the mean maternal age was 31 (range 22–37 years) to 76.9% with a mean maternal age of 38.8 (range 32–44 years). Thus, it can be inferred that most pregnancy losses result from a fetal chromosome abnormality, and that this is particularly true in older women, who comprise a disproportionate number of those experiencing pregnancy loss.

### 34.3.4 Relative Incidence of Chromosome Abnormalities in Sporadic vs Recurrent Pregnancy Loss

Women who experience pregnancy loss usually take longer to achieve their desired family size, and so are often attempting pregnancy at older ages. Thus, the age-related incidence of nondisjunction and associated fetal chromosome abnormalities (see earlier) could be contributing to their unsuccessful pregnancy history. In a case-control study (37) women having experienced two consecutive miscarriages were found to be older than controls, with 42.6% being older than 35 years of age, while in the control group the percentage of women over 35 years was only 13.9. In addition, the risk of repeated miscarriage was threefold higher in women over 35 as compared to those 25–29 years of age. Rubio et al. (38) performed PGD on 71 couples with a history of recurrent miscarriage and a age-matched control group of 28 couples undergoing PGD because of the risk of sex-linked diseases (without other infertility problems) and observed that the couples with recurrent miscarriage produced chromosomally abnormal embryos at a higher rate than those not having this reproductive problem. Marquand et al. (39) evaluated first-trimester losses in 180 women who were over 35 years of age. Of these, 24% had experienced at least three prior losses. In this recurrent loss

group, the incidence of aneuploidy was 78%, which was not statistically different from the 70% aneuploidy rate in the sporadic group. Both groups had similar mean maternal ages. Bianco et al. (40) observed that, the more pregnancy losses a woman had experienced, the greater the likelihood that she would be found to have a fetus with a chromosome abnormality at prenatal diagnosis. In their evaluation of nearly 47,000 women undergoing prenatal diagnosis, the incidence of an abnormal fetal karyotype increased from 1.39% in those with no history of pregnancy loss to 2.18% in those who had three or more losses. Our own data (unpublished) on POC shows the incidence of at least one chromosomally abnormal sample from women where we received at least two POCs was 73.9% compared to the overall abnormality rate of 49.5%. These observations would tend to refute the concept that recurrent pregnancy loss is primarily due to factors other than chromosome abnormalities in the fetus.

### 34.3.5 Parental Chromosome Abnormalities

Although fetal chromosome abnormalities are extremely common in miscarriage samples, most of the abnormalities observed are numerical and *de novo*, and do not necessitate parental chromosome analysis. However, there are situations in which parental chromosome analysis is indicated, as parental balanced chromosome rearrangements can lead to abnormalities in meiosis and the generation of unbalanced gametes.

**34.3.5.1 Structural Rearrangements.** Couples experiencing two to three pregnancy losses are often referred for chromosome analysis, as a vast literature (2,41–46) suggests that in about 5% of these couples, one of the partners will have a balanced chromosome rearrangement (either a translocation or an inversion). Mau-Holzman (47) in a study of the somatic karyotype of infertile men and women found chromosome rearrangements in 4% of men with azoospermia, 24% of men with oligospermia, and 20% of women who were candidates for ISCI, confirming the principle that meiosis in individuals with balanced chromosome rearrangements often generates unbalanced gametes leading to nonviable conceptions associated with either infertility or pregnancy loss. The incidence of parental chromosome abnormalities is lower than this in couples achieving, but subsequently losing pregnancies (48–53), being in the range of 2.7–7.6% (for comparison, the incidence of balanced chromosome rearrangements detected in 269,371 prenatal diagnosis studies was only 0.09% (54) and only 0.2% in a population of phenotypically normal, fertile adult males being tested as sperm donors (52)). Couples with one partner who has a balanced chromosome rearrangement tend to have a very poor pregnancy history, because of the production of genetically unbalanced gametes and fetuses. Sugiura-Ogasawara et al. (42) evaluated couples in which one partner was known to carry a translocation

and observed a pregnancy loss rate of 61% if the translocation was carried by a male and 72% if carried by a female. Thus, some clinicians recommend that these couples undergo IVF with PGD in the hopes of achieving a pregnancy with a balanced karyotype.

**34.3.5.2 Mosaicism.** The empirical risk for recurrence of autosomal trisomy, after a couple has experienced one such pregnancy, is routinely quoted as 1%. Several older studies demonstrated that this 1% recurrence rate may be explained by the presence of parental mosaicism for an autosomal trisomy, including gonadal mosaicism, and that 1% of couples who give birth to a child with an autosomal trisomy demonstrate such mosaicism. Since fetal autosomal trisomy, even those trisomies that are considered viable, is often associated with pregnancy loss, parental mosaicism might contribute to the occurrence of chromosomally abnormal pregnancy losses, especially in couples with recurrent losses. Although there are several isolated reports of the finding of parental mosaicism in couples with a history of pregnancy loss (55–58), Kuo (56) reviewed karyotypes from 1010 couples with a history of recurrent spontaneous abortion (blood lymphocytes and skin fibroblasts) and found only two women (0.2% of couples) who demonstrated mosaicism for chromosome 21. An earlier study, showing that the recurrence of the same chromosome abnormality in subsequent losses (60) did not occur more often than expected by chance, also concluded that the incidence of gonadal mosaicism was low in couples with recurrent

abortion. However, Warburton et al. (61) did find a higher recurrence risk for trisomy 21 mosaicism only among younger mothers with repeat losses, suggesting that in this population, gonadal mosaicism may explain some recurrent losses.

### 34.3.6 Other Causes of Pregnancy Loss

While the single most common reason for the loss of a recognized pregnancy during the first trimester is the presence of a genetically unbalanced fetal genotype associated with an abnormal karyotype, many other factors have also been investigated (Table 34-1). Some women, who experience pregnancy losses, may do so because of underlying maternal diseases, which may be amenable to treatment. Any disease that can potentially decrease uterine-placental blood flow profoundly may be implicated in causing a fetal demise. These could include maternal hypertension, systemic lupus erythematosus (SLE), insulin-dependent diabetes mellitus, sickle-cell disease, renal disease with hypertension, severe maternal trauma, and drug exposure. Women who were themselves exposed to diethylstilbestrol in utero also show an increased incidence of spontaneous abortion, ectopic pregnancies and preterm births, presumably as a result of congenital tubal or uterine abnormalities induced by their exposure to the drug during embryogenesis (62). Unfortunately, many of the studies involving these potential causative factors have investigated the couples

**TABLE 34-1 Evaluation and Management of Recurrent Early Pregnancy Loss**

Etiology	Prevalence (%)	Diagnostic Studies	Treatment
Parental chromosome rearrangement	5–8	Karyotype of both partners	Genetic counseling PGD? Donor gametes?
Uterine anatomy	15–20	HysteroGRAPHY Hysterosalpingography Saline infusion sonohysteroGRAPHY	Hysteroscopic metroplasty Hysteroscopic myomectomy Lysis of adhesions
Immunologic	15–20	Lupus anticoagulant Anticardiolipin IgG/IgM B <sub>2</sub> -glycoprotein-1 IgG/IgM Phosphatidylserine IgG/IgM	Low-dose aspirin Heparin/enoxaparin
Thrombophilia	8–12	Factor V Leiden mutation Prothrombin gene mutation Fasting homocysteine level Antithrombin III activity Protein C activity Protein S activity	Heparin/enoxaparin Folic acid
Endocrinologic	8–12	Midluteal phase endometrial biopsy Midluteal phase progesterone Thyroid stimulating hormone Prolactin level Fasting glucose and insulin	Progesterone Levothyroxine Cabergoline/bromocriptine Metformin/insulin
Microbiologic	8–10	Endometrial biopsy Vaginal/cervical cultures	Appropriate antibiotics
Environmental	5	Review exposure to alcohol, tobacco and caffeine Review exposure to environmental chemicals and toxins	Eliminate exposure

experiencing losses, without appropriate control populations or taking into consideration the chromosomal status of the fetus. Thus, some of the possible causes of pregnancy loss addressed in the prior version of this text (e.g. HLA similarities between parents and skewing of X-inactivation) have since been discredited (63–66), and will not be addressed here. However, in the minority of couples experiencing the loss of chromosomally normal fetuses, these factors can be explored, as potential remedies exist for some.

### 34.3.7 Other Genetic Factors

**34.3.7.1 Single Gene Disorders.** A few single gene disorders have been associated with both early and late pregnancy loss including stillbirth. These include hemoglobinopathies, inborn errors of metabolism and inherited thrombophilias. Most of these disorders are inherited in an autosomal recessive fashion. Thus, consanguinity increases the risk for these disorders in that the likelihood that both parents carry the same autosomal recessive mutation is increased, as a result of “identity by descent.” Alpha thalassemia major is an example of a single gene disorder that can cause pregnancy loss (67,68). Alpha thalassemia causes pregnancy loss when both parents carry complete deletions (null alleles) that are in the cis configuration. This results in deletion of both  $\alpha$ -thalassemia genes on the same chromosome. If the fetus inherits the deleted chromosome from both parents, it will be deleted of all four  $\alpha$ -globin genes, resulting in hemoglobin Barts and hydrops fetalis leading to fetal death in the second and third trimesters. Individuals of Southeast Asian ancestry are at greater risk to carry the cis configuration and to have affected offspring. However, the majority of autosomal recessive disorders are not associated with increased risk for early pregnancy loss.

Most genetics textbooks include a discussion of X-linked dominant disorders, the majority of which are lethal in males, including pedigrees depicting multiple losses of male pregnancies. However, the list of disorders in this group is small (Table 34-2) and the incidence of carriers is very low, so that this is a very infrequent cause of pregnancy loss. For the rare families in this category, PGD may help to achieve a desired family size.

There are limited data to suggest that other familial mutations might increase the risk of pregnancy loss in certain families. Transmission rate distortion has been reported for spinal muscular atrophy (69,70), which could be explained by the early loss of some fetuses homozygous for the mutant allele. A study investigating the incidence of variants of *BRCA2* in newborns (71) found a deviation from the Hardy–Weinberg equilibrium, with reduced fitness for homozygotes with variant alleles suggesting reduced prenatal viability for these genotypes. For individual families, heterozygosity for a common allele in the parents may contribute to recurrent loss, as has been reported in some consanguineous populations (72).

**TABLE 34-2 X-Linked Dominant, Male Lethal Disorders Are Rare**

Disorder/MIM	Incidence
Aicardi 304050	1:93,000–1:167,000
Chondrodysplasia punctata 302960	1–9/1,000,000
Congenital hemidysplasia with ichthyosiform erythroderma and limb defects (CHILD) 308050	<1/1,000,000
Goltz syndrome (focal dermal hypoplasia) 305600	<1/1,000,000 95% new mutations
Incontinentia pigmenti 308300	1–9/1,000,000
Microphthalmia with linear skin defects 309801	<1/1,000,000
Oculo–facio–cardio–dental 300166	<1/1,000,000
Oral–facial–digital type I 311200	1:250,000–1:50,000
Rett 312750	1:8500
Terminal osseous dysplasia 300244	<1/1,000,000
Wildervanck syndrome 314600	?

**34.3.7.2 Thrombophilias.** The thrombophilias are a group of disorders in which there is a propensity to develop venous or arterial thrombosis. Both inherited and acquired thrombophilias and the associated thrombosis of placental vessels have been proposed to influence pregnancy loss (73–75). Mutations in the genes for both factor V and prothrombin have also been associated with unexplained late fetal loss and other thrombosis-related pregnancy complications (76,77). Factor V Leiden (FVL) mutations and the associated resistance to activated protein C are reported to be more prevalent in woman with a history of pregnancy loss in several studies (76,79–86), with an incidence of 7–44% in those with losses as compared to 1.6–6% in controls. Studies on mice, made deficient for factor V through gene targeting, show that half the embryos die during the first half of gestation, probably as a result of abnormalities in yolk-sac vasculature (87). Earlier reports suggested that FVL deficiency is associated with preeclampsia, abruptio placentae, fetal growth restriction, late pregnancy loss and stillbirth. Yet, a number of large collaborative studies have not confirmed this association (88–90). An additional hereditary thrombophilic tendency implicated in recurrent pregnancy loss is hyperhomocysteinemia, which has also been associated with other thrombotic morbidities, such as adult myocardial infarction (91–93). Some reports have shown an association between a homozygous C→T polymorphism at nucleotide 677 in the gene coding for methylenetetrahydrofolate reductase (MTHFR), which leads to a thermolabile variant of the enzyme and low levels of serum folate, hyperhomocysteinemia and early pregnancy loss (91,94,95). The combination of serum folate deficiency and hyperhomocysteinemia has been reported to result in defective chorionic villus vascularization (93) and would be amenable to treatment with relatively high doses of folic acid (5 mg/day) along with pyridoxine supplementation (91). Two meta-analyses



and related studies of thrombophilic disorders and fetal loss showed an association between FVL, activated protein C resistance or the prothrombin G20210A (PGM) mutation and recurrent early fetal loss, but demonstrated no association between the MTHFR mutation and fetal loss (96–98). A combination of heparin or enoxaparin and low-dose aspirin is recommended for treatment of pregnant women with FVL and prothrombin gene mutations (98). This is the current protocol followed at our center.

**34.3.7.3 Autoimmune Disorders.** The presence of autoimmune antibodies, with or without other stigmata of autoimmune disease, has been described as a risk factor for recurrent pregnancy loss. The role of autoimmune antibodies as the cause or result of the pregnancy loss has been influenced by studies of the effect of thrombophilia on pregnancy loss rates (Table 34-3). During pregnancy and the postpartum period, SLE, with a prevalence of

1–1.6% in the female population (99,100), can be associated with a series of remissions and exacerbations with an increased risk of “flares” during pregnancy (101). If the disease is in remission at the time of conception, the fetal survival rate is quite good, about 85% (102), but still below that seen in unaffected women. However, if SLE is active during the pregnancy, particularly if a lupus flare occurs in the first trimester, the fetal survival rate decreases to 50–75% because of complications of placental thrombosis and hypertension (103) associated with lupus flares. Similarly, several connective tissue diseases that produce circulating antibodies, such as Ro (SSA), can lead in utero to complete heart block (104) and pregnancy loss.

**34.3.7.4 Antiphospholipid Syndrome.** The antiphospholipid syndrome involves the presence of a spectrum of antibodies directed against cellular phospholipid components. This syndrome has been associated with arterial and venous thrombosis, recurrent pregnancy loss, and immune thrombocytopenia in the absence of rheumatologic disease. The antiphospholipid syndrome is defined on the basis of clinical and laboratory criteria. The criteria proposed by Laskin et al. (105) are shown in Figure 34-2.

The obstetrical literature reports that certain antiphospholipid antibodies, particularly lupus anticoagulant and anticardiolipin IgG and IgM, are associated with recurrent early pregnancy loss. Elevated levels of both antiphospholipid antibodies (lupus anticoagulant) and anticardiolipin IgG and IgM have been reported in women with a history of pregnancy loss (106). Both these classes of antibodies lead to a higher incidence of thrombotic events (lupus anticoagulant interferes with the conversion of prothrombin to thrombin), causing placental thrombosis and infarction (107). Thus, while the presence of these antibodies has been associated with

**TABLE 34-3 Factors that Contribute to Vascular Problems in the Placenta**

Autoantibodies	Anticardiolipin Lupus anticoagulant Antiphospholipid syndrome Systemic lupus erythematosus
Clotting factors	Factor V Leiden Factor XII Protein C Prothrombin
Metabolic problems	Hyperhomocyst(e)inemia Methylenetetrahydrofolate reductase deficiency
Other	45,X karyotype

#### Clinical Criteria

Vascular thrombosis

Adverse pregnancy outcome

- At least one unexplained pregnancy loss after 10 weeks GA
- Three or more unexplained, consecutive pregnancy losses before 10 weeks GA
- One or more preterm births of a normal neonate at or before 34 weeks GA because of preeclampsia, eclampsia or placental insufficiency

#### Laboratory Criteria

Moderate or high titer IgG or IgM anticardiolipin antibody measured on two or more occasions at least 6 weeks apart

Circulating anticoagulant measured on two or more occasions at least 6 weeks apart

**FIGURE 34-2** Criteria for classification of antiphospholipid syndrome.

pregnancy loss in any trimester, most of the losses occur later in pregnancy (108). Antiphospholipid antibodies were detectable in 16% of patients with three or more pregnancy losses as compared with 7% of normal controls, and in only 3% of women who had never reported a pregnancy (109). Creagh et al. (110) reported the incidence of women with lupus anticoagulant or anticardiolipin as 7/35 and 6/35, respectively, in a group with more than two losses, as compared to 1/31 and 0/31 in a group with two or fewer losses. However, low-positive IgG fractions of antiphospholipid antibodies or isolated IgM fractions do not establish a diagnosis of the antiphospholipid syndrome. Women with prior fetal losses and high levels of anticardiolipin IgG antibodies appear to be at the highest risk of fetal loss in subsequent pregnancies and may benefit the most from preventive therapies (111). An elevation of antinuclear antibodies occurred in the sera of a woman with previous pregnancy loss, even when the loss had been explained by other causes such as anatomic or luteal phase defects (LPD) (112). However, antinuclear antibodies alone appear to be nonspecific markers in women with pregnancy loss, with other investigators demonstrating no difference in the proportion of positive antinuclear antibody tests between women with recurrent losses and appropriate controls (112). Takakuwa et al. (113) observed that the incidence of chromosomally abnormal abortuses was lower in woman with elevated anticardiolipin antibodies (20% as compared to 60% in controls), suggesting that some of their losses were not due to chromosome abnormalities, but associated with this autoimmune phenomenon. Whether some of these autoimmune antibodies are generated in response to fetal demise in susceptible women rather than being the cause of the pregnancy loss has been cause for speculation. However, immunoglobulin isolated from women with elevated anticardiolipin levels can induce abortion in pregnant mice (114), suggesting that the antibodies themselves may be the underlying etiology of the loss. In addition, several studies have suggested the use of corticosteroids, heparin, aspirin, and immunoglobulin therapy in preventing pregnancy loss in women with antiphospholipid antibodies (108–118). Controlled trials have confirmed the efficacy of low doses of aspirin and/or heparin (119–121). Interestingly, the efficacy of heparin may be due to its inhibition of complement activation rather than its anticoagulant effects (122). Other antiphospholipid antibodies such as antiphosphatidylserine and  $\beta_2$ -glycoprotein-1 may be associated with recurrent pregnancy loss. However, the evidence for this association is weak (123).

**34.3.7.5 Endocrine Disorders.** A number of maternal endocrine disorders have been linked to pregnancy loss.

**34.3.7.5.1 Diabetes Mellitus.** In nonpregnant women of child-bearing age, the term type I diabetes is used to include all insulin-dependent diabetes, while type II diabetes implies non-insulin-dependent disease. In pregnancy, classification is based on whether or not

a woman has had diabetes prior to pregnancy. Pregestational diabetes is then subdivided into alphabetical classes depending on the age of onset, duration of the disease, or both and whether any diabetic vascular complications occurred. Gestational diabetics have diabetes only during pregnancy and are classified as A1 if controlled with diet only, and A2 if insulin is needed (124). Even prepregnancy obesity, possibly through its association with insulin resistance and the metabolic syndrome, has been associated with an increase in stillbirths and neonatal deaths (125). Poorly controlled diabetes mellitus is known to cause miscarriage. Elevated glycosylated hemoglobin levels associated with poor glycemic control in early pregnancy are associated with spontaneous abortion. In contrast, the risk for pregnancy loss is not increased in women with well-controlled diabetes (126). Metabolic control of diabetes prior to conception and during the first trimester dramatically influences both the risk of spontaneous abortion and the incidence of major congenital anomalies (126,127). The perinatal mortality rate of about 3% in well-controlled diabetic women has been stable for over 30 years (128), with associated elevated risks for preeclampsia and preterm delivery. Poorly controlled diabetes is associated with an even higher perinatal death rate (129). Excluding lethal fetal anomalies, maternal complications of ketoacidosis, pregnancy induced hypertension (PIH), pyelonephritis, or neglect increase the perinatal mortality rate to as high as 17% (130). Preeclampsia was diagnosed in about 12.7% of diabetic women and preterm labor in 32% (125). Major congenital anomalies were increased threefold in diabetic pregnancies, but excellent prepregnancy control can markedly reduce the incidence of malformations (126,128). In a recent outcome survey of 273 women with type I diabetes, although the congenital abnormality rate was twice that seen in the general population, the rate of miscarriage was 14.7%, which is within the background rate (125,131). Even elevated maternal weight, which may be associated with type II diabetes, has been implicated as a risk factor for pregnancy loss (132). Early detection, excellent control, fetal surveillance, and blood pressure monitoring are key factors in lowering the perinatal mortality rate associated with maternal diabetes.

**34.3.7.5.2 Luteal Phase Defect.** During the luteal phase of the menstrual cycle, the corpus luteum produces progesterone, which induces the secretory changes in the endometrium that are necessary for implantation and maintenance of pregnancy. Once pregnancy occurs, the corpus luteum continues to secrete progesterone until the placenta produces enough progesterone to maintain the pregnancy.

LPD is defined as a lag of more than two days in the histological development of the endometrium compared with the day of the menstrual cycle. This retardation in endometrial development in the peri-implantation period may be associated with recurrent miscarriage. However,

many studies of this phenomenon have not included concurrent controls, and normal women can have endometrial histology suggestive of LPD in up to 50% of menstrual cycles, suggesting that the association between LPD and recurrent pregnancy loss remains speculative.

A midluteal phase serum progesterone of <10 ng/mL is considered to be diagnostic of an inadequate luteal phase. Uncontrolled studies suggested that treatment with progesterone improves pregnancy outcome in women with recurrent miscarriage. However, several meta-analyses of controlled trials revealed no statistically significant difference in miscarriage rate between progestins and placebo or no treatment (133). Well-designed randomized trials are needed to establish the efficacy of progesterone supplementation in the treatment of recurrent early pregnancy loss.

**34.3.7.5.3 Thyroid Disorders.** Clinical hypothyroidism and hyperthyroidism have both been associated with decreased fertility and increased pregnancy loss rates. In particular, untreated or inadequately treated hypothyroidism has been associated with an increased risk for spontaneous abortion (134). Subclinical thyroid dysfunction, however, has not been linked to pregnancy loss (134,135). Antithyroid antibodies are more frequent in women with recurrent pregnancy loss, although, if the patient is clinically euthyroid, the presence of antithyroid antibodies does not appear to influence pregnancy outcome (136). In patients with clinical hypothyroidism, treatment with thyroxine, and subsequent restoration of normal thyroxine levels, reduces the risk for pregnancy loss.

**34.3.7.5.4 Hyperprolactinemia.** Hyperprolactinemia has also been suggested as a cause of miscarriage by affecting the hypothalamic–pituitary axis and causing inadequate oocyte maturation and luteal phase deficiency. However, evidence from controlled studies to support this theory is lacking. In an observational study involving 54 hyperprolactinemic women who underwent 64 pregnancies, Crosignani et al. (137) reported a miscarriage rate of 25%. In a series of 64 women with hyperprolactinemia who underwent 103 pregnancies (78 treated with bromocriptine and 25 untreated), Rossi et al. (138) reported an increased rate of ectopic pregnancy in the untreated group (24% vs 5%,  $p < 0.02$ ) but no increase in rate of spontaneous abortion. However, in one study involving 64 women with hyperprolactinemia among 352 women with recurrent pregnancy loss, treatment of hyperprolactinemia with bromocriptine was associated with a decrease in the rate of recurrent pregnancy loss (139).

**34.3.7.5.5 Polycystic Ovarian Syndrome.** Polycystic ovarian syndrome has also been investigated in women with recurrent spontaneous abortions (140). Over 40% of women with recurrent loss demonstrated features consistent with polycystic ovarian syndrome. However, as the successful pregnancy rate in these women was similar to that of normal controls, it is

unlikely that this disorder is related to pregnancy loss in ovulatory women.

**34.3.7.6 Nongenetic Factors.** Pregnancy losses beyond the first or early second trimester have been associated with nongenetic factors such as maternal structural anomalies, infection or trauma.

**34.3.7.6.1 Uterine Anomalies.** Defects caused by abnormal Müllerian fusion are a recognized cause of pregnancy loss. The presence of an intrauterine septum has been associated with recurrent early pregnancy loss. However, other Müllerian anomalies such as bicornuate and unicornuate uterus have been associated with second-trimester losses or preterm delivery rather than first-trimester losses (141). Observational studies suggest that surgical resection of intrauterine septae is associated with improved pregnancy outcome. Lin et al. (141) reported a study involving 36 women who had complete uterine septae with double cervix and vaginas. Twenty-one women who underwent hysteroscopic metroplasty were compared with 15 women who had no treatment. The rate of spontaneous abortion prior to the study was similar in both groups. The spontaneous abortion rate in the subsequent pregnancy was 11.1% in the metroplasty group and 87% in the no treatment group ( $p = 0.03$ ) (141). Women with uterine leiomyomata have been reported to be at increased risk for second-trimester spontaneous abortion (142,143). Submucous myomata can result in distortion of the uterine cavity and have been associated with recurrent pregnancy loss in observational studies. A recent meta-analysis showed no significant benefit from hysteroscopic myomectomy in women with submucous myomas (relative risk (RR) = 1.6; 95% confidence interval (CI) = 0.7–3.6) (144). Endometrial polyps and intrauterine adhesions or synechiae (Asherman syndrome) secondary to uterine curettage, particularly in the presence of endometritis, have been associated with recurrent pregnancy loss in uncontrolled studies. Hysteroscopic polypectomy and lysis of adhesions has been reported to be associated with improvement of fertility in patients undergoing IVF (144,145). However, to date, there are no controlled studies demonstrating a reduction in recurrent early pregnancy loss rates with hysteroscopic polypectomy or lysis of adhesions. Structural defects of the uterus have been reported in 15–30% of women who habitually abort as compared to 0.5–2% of normal controls (146), and sonohysterography of woman with at least two prior losses revealed intrauterine abnormalities in 50% (147), suggesting a causal relationship. Surgical correction of uterine abnormalities may be a reasonable option if other causes of recurrent pregnancy loss have been excluded. It is reported that 82% of patients with histories of recurrent loss, who underwent surgical correction of a septate uterus, delivered viable infants in their subsequent pregnancies (148). Cervical insufficiency (incompetent cervix) has been associated with preterm labor and pregnancy loss. Classically, cervical insufficiency presents as painless dilation

without labor, resulting in bulging of the fetal membranes into the vagina. Subsequently, the membranes can rupture and cause expulsion of a previable fetus. This condition, when diagnosed, is amenable to treatment by cerclage. Historically, the necessity of this procedure has been controversial (149). A recent meta-analysis of five randomized trials found that composite perinatal mortality and morbidity were significantly reduced (15.6% vs 24.8%) in women with previous spontaneous preterm birth and short cervix (<25-mm length by vaginal ultrasound) who were treated with cerclage (150). The recognition of a treatable anatomic abnormality often terminates the search for other causes of pregnancy loss.

**34.3.7.6.2 Infectious Agents.** Pregnancy-related infections are uncommon, but have been reported to be a cause of fetal loss. Mumps and measles, when acquired during pregnancy, have been associated with increased rates of spontaneous abortion (151). Other maternal infections, such as cytomegalovirus (CMV), parvovirus B-19 and syphilis, can result in second- or third-trimester fetal death (152,153) because of the sequelae of overwhelming fetal infection. A few other bacterial and protozoan microorganisms have been associated with spontaneous abortion including *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Brucella abortus*, *Salmonella typhi*, *Vibrio fetus*, *Chlamydia trachomatis*, and *Toxoplasma gondii*. Transplacental infection has been reported with each of the above agents, making them a possible cause of miscarriage. However, in a study of 818 women enrolled in the early first trimester, Simpson et al. (154) found no difference in clinical infection among 112 women who had pregnancy losses compared with 702 women who had successful pregnancy outcomes. In addition, in a study of 54 chromosomally normal abortuses, PCR of CV showed no evidence of *U. urealyticum*, *M. hominis*, CMV or adenovirus, while eight specimens revealed human papilloma virus and one specimen showed *C. trachomatis* (155).

Other infectious diseases, such as rubella and varicella (156,157), may produce a substantially increased risk of structural fetal abnormalities, or developmental abnormalities, such as CMV, without intrinsically increasing risk of fetal loss (158).

**34.3.7.6.3 Teratogens.** Exposure to teratogenic agents during pregnancy is known to produce abnormalities of form or function, including fetal wastage. The pathogenetic mechanisms leading to fetal death may occur through disturbance of one or more developmental processes. These include hypoplasia or hyperplasia of developing tissues, failure of cell differentiation, interaction or migration, or mechanical disruption of cells. Often, the end result of teratogenic action is an organ with too few cells. Subsequently, the organ system may fail to develop fully because of a lack of critical mass required for differentiation. Pregnancy loss due to teratogenetic agents is often difficult to prove conclusively, and many relatively safe agents have been incorrectly implicated from anecdotal experience. Gardella and Hill (159)

reported that the only teratogens that are convincingly associated with pregnancy loss are ionizing radiation (the Hiroshima experience), organic solvents, alcohol, mercury and lead, with some additional data implicating cigarettes. However, the majority of known teratogens are not associated with an outcome as severe as fetal death. Table 34-4 lists agents that include pregnancy loss as part of their related effects. Certain classes of drugs, such as folic acid antagonists (aminopterin and methotrexate) are well-documented abortifacients and have been specifically studied in terms of the timing and dose required for subsequent fetal wastage (160). Cancer during pregnancy is a rare occurrence, with an incidence of about 0.04% (161). The most common malignancies include those of the breast, cervix, thyroid, ovary, and lymphoma. Pregnancy outcome appears to be extremely good in women with hematological malignancies (162). Chemotherapy in the first trimester has been associated with an increased incidence of abortion and fetal abnormalities (163). Antineoplastic agents in general raise serious concerns regarding the mutagenic, teratogenic, and abortifacient effects on the human embryo. However, with the exception of aminopterin and methotrexate (160), individual agents have not been sufficiently studied to determine whether a clear risk for pregnancy loss exists.

**34.3.7.6.4 Maternal Stress.** Maternal psychological stress has been considered to be a risk factor for early pregnancy loss. However, scientific evidence to support the association between stress and miscarriage is sparse. In a study comparing urinary cortisol levels during the first 3 weeks post conception between 9 women who carried to term and 13 women who miscarried, Nepomnaschy et al. (164) found higher mean cortisol levels in the women who miscarried (RR=2.7 (95% CI=1.2–6.2)). They concluded that increased levels of

**TABLE 34-4** Teratogenic Agents Associated with Fetal Wastage in Human Pregnancy

Anticoagulants	Coumarin derivatives
Anticonvulsants	Paramethadione Trimethadione
Antineoplastic agents	Aminopterin Methotrexate Ionizing radiation
Sedatives	Ethyl alcohol
Illicit drugs	Cocaine
Vaccines	Measles Mumps Smallpox
Vitamin retinoids	Etretinate Isotretinate
Gastrointestinal agents	Misoprostol
Other	Organic solvents Mercury Lead



cortisol serve as a stress marker for higher risk for pregnancy loss. Several studies have reported that supportive care in early pregnancy for women with a history of recurrent spontaneous abortion resulted in a reduction in risk for subsequent pregnancy loss (165,166). For women whose losses were not attributable to identifiable pathology, Clifford et al. (166) reported a rate of successful outcome in the next pregnancy of 69%. This was compared with a successful pregnancy rate of 49% for the women who did not attend the clinic for early supportive care. In the subgroup of women with favorable prognostic features (age less than 40; less than six prior losses), the success rate in the next pregnancy was 79%. Medical therapy and other interventions are often prescribed for women with recurrent losses even in the absence of underlying causes in an attempt to improve outcomes of subsequent pregnancies.

Based on the above findings, women without underlying pathology can be counseled that the outcome for the next pregnancy is likely to be favorable with supportive care alone.

**34.3.7.6.5 Maternal Trauma.** Trauma during pregnancy is not an uncommon occurrence. Approximately 6–7% of pregnancies are affected by some degree of traumatic injury. These injuries are usually accidental but may be a result of intentional violence (167). Trauma is one of the leading causes of maternal death, accounting for up to 46% of cases (168). Factors that predict fetal loss in the face of maternal trauma include the severity of injury, maternal acidosis, hypoxia, shock, severe head injury, direct uteroplacental injury, placental abruption, coagulopathy and maternal death (169). Most studies reporting pregnancy loss rates following maternal trauma have involved perinatal losses in the late second and the third trimesters. There are very limited data regarding maternal trauma and risk for early pregnancy loss. With respect to intentional trauma, Nelson et al. (170) reported the results of a nested case-control study involving 392 women who experienced spontaneous abortion prior to 22 weeks and 807 controls who carried their pregnancies beyond 22 weeks. The authors were unable to demonstrate a relationship between any measure of physical violence and spontaneous abortion. Despite these findings, domestic violence is an important problem. More than 40% of women of reproductive age have experienced at least one episode of violence in their lifetime. As such, domestic violence is a very prevalent and alarming public health issue.

## 34.4 LATE PREGNANCY LOSS

Second- and third-trimester pregnancy loss, although much less common than first-trimester miscarriage, is still a very important event in a woman's reproductive history. A number of retrospective studies have shown an association between prior second-trimester loss and

increased risk for spontaneous preterm birth and recurrent second-trimester loss in subsequent pregnancies (171–173). In some studies, second-trimester losses are classified along with those that occur in the first trimester. However, it is important to recognize that the causes for loss in later pregnancy are often distinctly different from those observed in the first trimester. Factors associated with late pregnancy loss include fetal structural anomalies (including cytogenetic abnormalities), maternal anatomic abnormalities, abnormal first- or second-trimester serum screening results, infection, and inherited thrombophilias. Late losses generally include those occurring after 14 weeks of gestational age because miscarriages that occur before 14 weeks frequently reflect a fetal death that happened a week or two earlier (174).

### 34.4.1 Chromosome Abnormalities

Although 60–80% of first-trimester losses are the result of a chromosome abnormality, only 10–24% of second-trimester losses and 5% of third-trimester stillbirths are associated with chromosome abnormalities (175,176). In contrast, the incidence of chromosome abnormalities in live births is 0.5–0.6% (177). The types of cytogenetic abnormalities found in second- and third-trimester losses are similar to those seen in live births. The most common chromosome abnormalities observed in late pregnancy losses are trisomy 13, 18, 21, and monosomy X and other sex chromosome aneuploidy.

### 34.4.2 Maternal Anatomic Abnormalities

As stated earlier, uterine abnormalities have been associated with an increased rate of pregnancy loss. These losses generally occur in the second trimester or result in preterm delivery (141). These abnormalities, including Müllerian anomalies, cervical insufficiency and uterine leiomyomata, are discussed in greater detail earlier in this chapter.

### 34.4.3 Abnormal Serum Markers

First- and second-trimester serum biochemical markers are widely used to screen for fetal aneuploidy. In the first trimester, low levels of pregnancy-associated plasma protein A (PAPP-A) and free beta-human chorionic gonadotropin ( $\beta$ -hCG) with or without increased nuchal translucency (NT) thickness in the presence of a normal fetal karyotype, have been associated with increased risk for second- and third-trimester pregnancy loss (132,178–180). In a series of 7932 patients undergoing first-trimester screening, Goetzl et al. (178) observed a pregnancy loss rate prior to 20 weeks gestational age of 1.4% for women with multiple of the median (MoM) values for PAPP-A below the fifth percentile compared with 0.36% for MoM values in the normal range (adjusted

odds ratio (OR)=2.8). With respect to second-trimester serum analyte screening, a study involving over 77,000 women showed that those with very low (<0.25 MoM) maternal serum alphafetoprotein (MSAFP) levels had increased risk for spontaneous abortion (RR=12.5), preterm birth (RR=4.8), low birth weight (RR=5.8) and neonatal death (RR=1.9). Likewise, women with very high MSAFP levels (>2.5 MoM) also had increased risk for spontaneous abortion (RR=15.1), preterm birth (RR=2.2) and stillbirth (RR=4.0) (181). There are no proven interventions to prevent adverse outcomes for women who are at increased risk based on abnormal maternal serum screening values or NT measurements. Current data suggest that women with serum analyte and NT values in the screen-negative range can be reassured that the risks for adverse pregnancy outcome are low (178,180,181).

#### 34.4.4 Thrombophilic Disorders

As discussed earlier, in a number of observational and retrospective case-control studies, the thrombophilias and antiphospholipid syndrome have been associated with placental insufficiency and increased risk for adverse pregnancy outcome. Several of these studies have shown an association between FVL and PGM mutations and second- and third-trimester stillbirth (98,182–184). In a prospective cohort study involving 67 women with antenatal fetal death, Simchen et al. found that 33 women (49.3%) had evidence of a placental cause for fetal death (fetal growth restriction, oligohydramnios, placental abruption, and/or histological placental abnormality). Thirty-six of the 67 women (53.7%) tested positive for at least one of the following thrombophilias: FVL, PGM or C677T MTHFR (182). In a retrospective study of a cohort of 363 women with three or more consecutive early or late pregnancy losses, Lund et al. (185) found that the unadjusted live birth rate in the subsequent pregnancy was 46% in carriers of FVL or PGM mutations versus 63% in noncarriers of these mutations ( $p=0.04$ ). However, after adjusting for significant covariables, they found that this difference was no longer significant (185). In a systematic review and meta-analysis of 10 prospective cohort studies, Rodger et al. (184) found that women with FVL had an absolute risk of 4.2% for late pregnancy loss (OR=1.52). Thus, it appears that women with FVL or PGM mutations have an increased relative risk for adverse pregnancy outcome including fetal death. However, the absolute magnitude of this increased risk is small.

#### 34.4.5 Infection

Although infection does not appear to be a common cause of early pregnancy loss, there are data that suggest that maternal–fetal infection is associated with pregnancy

loss in the second and third trimesters. A number of studies implicate bacterial colonization of the lower and upper genital tract as a cause of a significant proportion of preterm births (186–189). Approximately 10–12% of all births in the United States are preterm (<37 weeks gestation). Nearly half of all preterm births are associated with spontaneous preterm labor and approximately a third result from premature rupture of membranes (186). An inverse relationship between the percentage of positive chorioamnion bacterial cultures and gestational age at delivery has been observed (190). In addition, positive chorioamnion cultures have been reported in 73% of women with spontaneous delivery prior to 30 weeks compared with 21% in women with clinically indicated delivery prior to 30 weeks (190). Amniotic fluid levels of proinflammatory cytokines, particularly interleukin-6 (IL-6), have been shown to be higher in women with spontaneous preterm delivery than in those with indicated preterm delivery (191,192).

Bacterial vaginosis (BV) is a condition characterized by overgrowth of gram-negative and anaerobic bacteria resulting in a decrease in the number of beneficial hydrogen peroxide producing *Lactobacillus* species. The most common organisms seen in BV include *Gardnerella vaginalis*, *Bacteroides* sp., *M. hominis*, and *U. urealyticum*. Studies have demonstrated a twofold increased risk of spontaneous preterm birth among women with BV (186,193). In a prospective cohort study involving 1916 pregnant women, Nelson et al. (189) found a twofold increased risk of second-trimester pregnancy loss in women with high levels of BV-associated organisms and low levels or absence of vaginal *Lactobacillus* sp. in the first trimester. Although the presence of BV is a risk factor for late pregnancy loss, it is important to recognize that not all women with BV have upper genital tract infection. In fact, the majority of pregnant women with BV actually deliver at term and clinical trials involving treatment for BV in women with this condition have not shown a decrease in the rate of preterm labor or delivery (194).

Viral infection, particularly with CMV and parvovirus B-19, has been associated with increased risk for second-trimester pregnancy loss (151,195,196). Johansson et al. (196) analyzed blood samples from women taken in early pregnancy for the presence of parvovirus B-19 and herpes viruses. They found that 11 of 234 women (4.7%) who had second-trimester losses and 10 of 270 women (3.7%) who had preterm birth (prior to 32 weeks) had parvovirus B-19 viremia. This was compared with parvovirus B-19 viremia in 5 of 294 (1.7%) women who delivered at term. These findings corresponded to adjusted odds ratio of 3.76 for second-trimester miscarriage and 2.66 for preterm birth <32 weeks. Data such as these suggest that women with viremia in early pregnancy have increased risk for late pregnancy loss. However, more data are needed to confirm this association (196).

### 34.4.6 Recurrent Loss

While pregnancy loss itself is not uncommon, recurrent pregnancy loss, often defined as three or more losses, only affects 1–2% of couples (197), but may suggest some underlying mechanism. These are the families that most often seek medical intervention and make up a large proportion of those being studied to determine underlying causes of pregnancy loss. Empirical data shows that a history of pregnancy loss increases the risk of miscarriage at subsequent pregnancies (1,132), with the risk increasing from 12% without any prior history to 24% after one loss, 32% after three losses and 53% after six or more losses. This supports the idea that in some couples, it is not chance alone that is responsible for an unsuccessful pregnancy history. Although the likelihood of a successful pregnancy for such couples is generally better than 60% (197,198), many seek medical assistance to identify the issues involved, in the hopes of determining a strategy to alleviate the problem. There have been numerous possible causes of recurrent pregnancy loss investigated, and while the literature on this subject is very extensive, many of the proposed causes of recurrent miscarriage have not been substantiated by carefully controlled research, including some of those that have become part of clinical practice in some arenas.

The majority of studies of couples with recurrent pregnancy loss have reported the same risk factors as demonstrated for sporadic losses. As mentioned earlier, the incidence of fetal chromosome abnormalities and the types of abnormalities observed are also very similar, although several studies have shown a higher incidence of chromosome abnormalities in abortuses from women with a history of pregnancy loss, possibly correlated with the older maternal ages in this group. Parental chromosome rearrangements and other genetic factors (discussed earlier) can lead to recurrent fetal loss in individual families, while parental mosaicism is likely to be more of a factor for young women with recurrent loss. Yet, the most consistent difference observed between women with sporadic vs recurrent pregnancy loss appears to be maternal age and a history of prior loss.

Factors that do seem to influence recurrence risks include loss of a chromosomally normal fetus (199), loss after the first trimester, a history of preterm deliveries, infertility problems (200), and younger maternal age (165,200,201). In a Danish study of all reported pregnancies between 1978 and 1992, the overall rate of spontaneous pregnancy loss was reported to be 13.5%, similar to most other studies (201). Within this study, the rate of spontaneous loss in women aged 20–24 years was only 8.9%, while the rate of loss at age 42 was 50%, with nearly 75% of pregnancies in woman 45 years of age and older resulting in a spontaneous loss. A high rate of loss in older women, about 50% in women over 40, was confirmed in another study (165). Ironically, as a woman's age at each subsequent pregnancy is more advanced, failure

to achieve successful pregnancies, and the delay involved, can contribute to the risk of loss. As seen earlier, increasing maternal age is accompanied by a higher likelihood of a chromosomally abnormal fetus, which may then be the explanation for subsequent losses. It is significant that younger women (<35 years of age) with recurrent pregnancy loss tend to have a lower frequency of chromosomally abnormal fetuses than age-matched peers with a single pregnancy loss (35). Thus, in these younger women with recurrent spontaneous abortions the investigation of other explanations of pregnancy loss is warranted. Other possibilities include endocrine/endometrial abnormalities, uterine structural abnormalities, thrombophilic disorders, immune disorders, and occasionally parental chromosome rearrangements or other genetic disorders.

## 34.5 EVALUATION AND MANAGEMENT OF RECURRENT ABORTION

Evaluation of a couple with recurrent pregnancy loss begins with a detailed history, physical examination and appropriate diagnostic studies. The history should include the pattern and gestational age at the time of each prior loss. Gestational age at the time of loss should be confirmed, if possible, by ultrasound, serum hCG results and embryo/fetal pathology. If available, prior cytogenetic results are useful to determine whether the previous losses were due to a structural or numerical chromosome abnormality. The review of systems should include an assessment of endocrinologic and immunologic disorders. Physical examination and laboratory evaluation should be directed toward detection of parental balanced structural chromosome abnormalities, uterine anatomical abnormalities, endocrinologic disorders, immunologic disorders, microbial infection, and inherited thrombophilias. Once etiologic factors are identified, a plan of management should be implemented based on current scientific evidence. A suggested plan for evaluation and management of recurrent early pregnancy loss is shown in Table 34-1.

Psychologic support is often needed for couples with recurrent pregnancy loss. Recurrent miscarriage may lead to feelings of anger, guilt and depression. It may be helpful to refer couples for grief counseling or to suggest that they participate in support groups before attempting a subsequent pregnancy. As noted earlier, supportive care and close monitoring of subsequent pregnancies has been reported to be associated with a greater likelihood of successful outcome.

## 34.6 CONCLUSIONS

Pregnancy loss, particularly when it is recurrent, is an important problem for patients and their clinicians, largely because of the variety of factors that have been proposed to be involved, although only a few of these have been confirmed by evidenced-based investigation (Table 34-5). The single most common cause of

**TABLE 34-5 Factors Definitively Associated with Early Pregnancy Loss**

Unbalanced karyotype in fetus	>60%
Parental chromosome rearrangement	5%
Mendelian disorder in fetus	Rare—family dependent
Uterine abnormalities	~20–75%
Diabetes mellitus (poorly controlled)	RR = 1.9–5.8

pregnancy loss is the presence of a chromosome abnormality in the fetus, particularly if the loss occurs early in the pregnancy. As these abnormalities are the result of random events that occur during meiosis and are highly correlated with maternal age, little follow-up or intervention may be indicated for these women, other than the reminder that as a woman ages, her risk of both chromosomally abnormal fetuses and pregnancy loss will increase. Therefore, karyotype analysis of POC should be a critical part of the evaluation of pregnancy loss, as the observation of a fetal chromosome abnormality would usually preclude the need for additional investigation. It would also serve to identify that population of woman with chromosomally normal fetal losses, in whom other factors may be contributing to recurrent pregnancy loss and in whom additional studies are indicated. These could include investigation of uterine abnormalities and evaluation of antiphospholipid antibodies or conditions that predispose to thrombosis, where potential therapies exist. Limiting clinical research studies to women in this population group should facilitate the evaluation of the efficacy of various therapeutic interventions. In the small population of couples in which parental chromosome abnormalities or other genetic disorders are detected, IVF and preimplantation prenatal diagnosis may help the couple achieve a successful pregnancy outcome. Predicting the likelihood of a successful pregnancy in a couple with recurrent pregnancy loss is complex. Factors to consider are the number of prior losses, the gestational age at the time of loss, cytogenetic findings, the patient's medical history, and results of appropriate diagnostic studies. As noted earlier, if no underlying factors are discovered, many couples will eventually have a successful pregnancy with supportive care alone. The temptation to prescribe unproven treatments should be resisted, even if the couple is highly motivated and willing to "try anything." Careful monitoring of the patient's emotional status is important because patients with recurrent pregnancy loss often experience anxiety, anger and depression. Referral for grief counseling or psychiatric care should be individualized based on the patient's level of well-being.

## CROSS REFERENCES

Chromosomal Basis of Inheritance; Cytogenetic Analysis; Prenatal Screening for Neural Tube Defects and

Aneuploidy; The Genetic Basis of Female Infertility; Clinical Teratology; Down Syndrome and other Autosomal Trisomies; Sex Chromosome Abnormalities; Deletions and Other Structural Abnormalities of the Autosomes; Preeclampsia; Common Genetic Determinants of Coagulation and Fibrinolysis; Hemoglobinopathies and Thalassemias; Autoimmunity: Genetics and Immunological Mechanisms; Systemic Lupus Erythematosus; Thyroid Disorders; Diabetes Mellitus; Disorders of the Gonads, Genital Tract and Genitalia.

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## RELEVANT WEB SITES

OMIM On Line Mendelian Inheritance in Man: <http://www.ncbi.nlm.nih.gov/omim>.  
 GeneTests: Reviews: <http://www.ncbi.nlm.nih.gov/sites/GeneTests/review?db=GeneTests>.

# CHAPTER

# 35

## A Clinical Approach to the Dysmorphic Child

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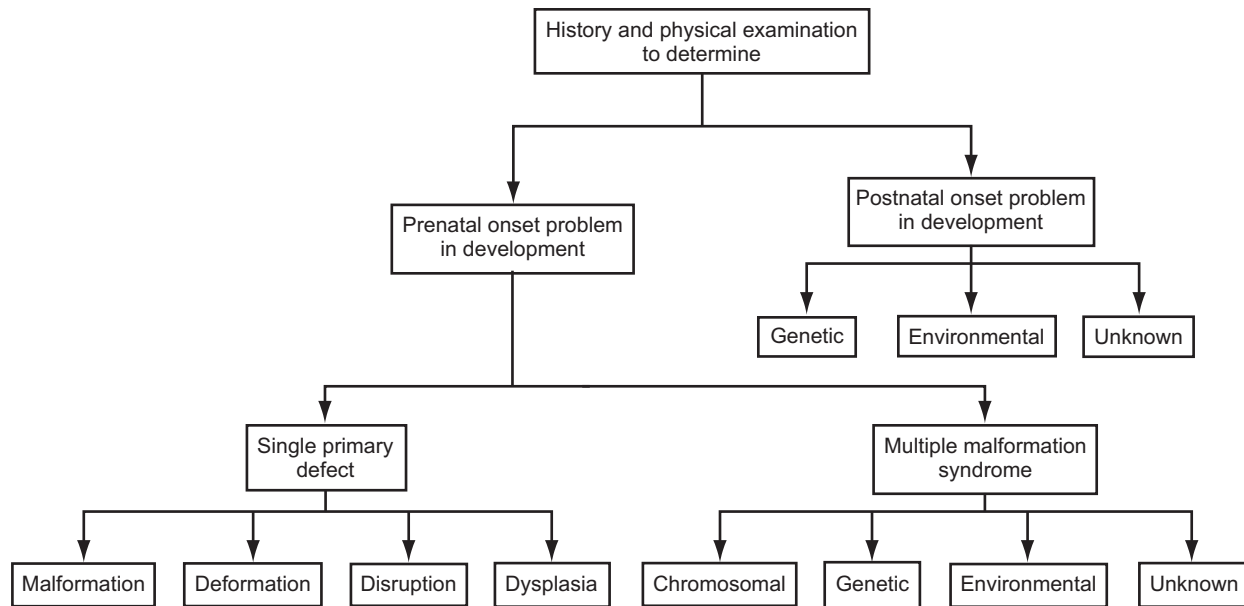
### 35.1 INTRODUCTION

The purpose of this chapter is to present a clinical approach to the child with structural defects. The approach is predicated upon the concept that the nature of the structural defects presents clues to the time of onset, mechanism of injury, and probable etiology of the problem, all of which determine the direction of the evaluation. It presumes that the dysmorphic child represents an experiment in human development, which, if interpreted correctly, can provide answers regarding the etiology of various structural defects, as well as permit insights into mechanisms of normal and abnormal morphogenesis. The method upon which this approach is based has been most articulately set forth by Sir Arthur Conan Doyle's fictional character Sherlock Holmes, who showed "how much an observant man might learn by accurate and systematic examination of all that came within his way" (1). This chapter adapts this method to the evaluation of the child with structural defects. By sharpening the faculties of observation, the clinician can narrow systematically the diagnostic possibilities so that the laboratory and the literature can be consulted in a rational fashion to arrive at an accurate diagnosis. The precise cause of many malformations and malformation syndromes is not known. However, careful clinical evaluation in combination with an expanded range of cytogenetic and molecular testing has allowed the elucidation of the mechanism underlying a growing list of clinical disorders. The separation between genetic and environmental factors as well as cytogenetic and single-gene abnormalities is somewhat arbitrary. However, the approach is intended to be practical and to facilitate detection and prevention of human malformations. *Gorlin's Syndromes of the Head and Neck* (2) and *Smith's Recognizable Patterns of Human Malformation* (3) are particularly useful. In recent years, computerized databases available online and on CD ROM have become useful adjuncts to diagnosis (London Dysmorphology Database (4); Possum Web (5); Online Mendelian Inheritance in Man (OMIM) (6)).

### 35.2 PRENATAL VERSUS POSTNATAL ONSET OF DEVELOPMENTAL PROBLEMS

A method of approach to children with structural defects is set forth diagrammatically in [Figure 35-1](#). Although the lists of exceptions is growing, a history and physical examination usually make it possible to determine if the structural abnormality is of prenatal or postnatal onset. In this chapter, "prenatal onset" designates structural abnormalities that are present at birth, and "postnatal onset" designates structures that have previously developed and differentiated normally. Although the genetic alteration responsible for many of the disorders included under postnatal-onset structural defects is present at the time of conception, the structural manifestations of that genetic alteration do not become obvious until postnatal life. On the basis of this distinction, a more rational approach to the problem can be developed, as this determination narrows considerably the diagnostic probabilities and, it is hoped, permits a more judicious selection of adjunctive laboratory tests.

Generally speaking, prenatal-onset problems in development are a consequence of genetic or chromosomal alterations that cause programming problems in the development and/or differentiation of structure or are the result of factors unique to the pregnancy itself, such as environmental agents, abnormalities of placentation, or mechanical constraint. Although always evident at birth, most prenatal-onset problems remain static or improve postnatally without evidence of neurologic deterioration. By contrast, postnatal-onset problems in development usually result in deterioration in structure or function that has previously been normal. Deterioration may reflect postnatal accumulation of a toxic metabolic product (as in phenylketonuria), progressive storage of a metabolite (as in Hurler syndrome), deteriorating energy production (as in mitochondrial myopathies), or ongoing infection (as in deafness from cytomegalovirus). Children with postnatal problems usually appear to have thrived in utero. The structural



**FIGURE 35-1** Approach to a child with structural defects.

and functional consequences of the problem manifest after the newborn period.

Certain historical information can be particularly helpful in determining the onset of the problem. Structural defects of prenatal onset are frequently associated with the following abnormalities noted by the mother during pregnancy and at the time of delivery, whereas, by contrast, with postnatal-onset structural defects, the pregnancy and delivery usually are normal.

Alterations of pregnancy associated with prenatal onset of developmental problems are as follows:

1. Alterations in gestational timing (prematurity or postmaturity). As discussed in several other chapters, the majority of conceptuses do not survive to be born at 40 weeks gestation. Much of this loss occurs in the very early part of pregnancy and is the result of gross chromosomal abnormalities and/or malformation. Numerous studies have documented an increased frequency of chromosomal and genetic abnormalities in losses from the second and third trimesters. Thus, premature delivery may reflect late fetal wastage rather than maternal disease. Postmaturity rarely occurs today because of improved fetal monitoring techniques. In years before the widespread use of the ultrasound, anencephaly typically presented in pregnancies that continued well beyond the due date since the fetal pituitary–adrenal axis is involved in the triggering of labor.
2. Alterations in the onset of fetal activity, nature of fetal activity, or both. Although it is clear that fetal activity begins much earlier, it is usually not felt by the mother until about 18 weeks of gestation. Fetal activity increases in amount and intensity from that time, reaches a maximum between 29 and 38 weeks,

and then decreases somewhat until delivery. Discussion with mothers who have given birth to babies with structural defects suggests that certain structural defects are often associated with delayed onset and/or decreased intensity of fetal activity. Moreover, fetal movement may be localized to one particular quadrant of the abdomen, for example, when the defect represents deformation due to intrauterine compression in a previously normally formed structure. Other examples are defects in brain development and meningomyelocele, conditions in which the decreased fetal activity is secondary to neurologic impairment.

3. Abnormalities in the amount of amniotic fluid, for example, polyhydramnios or oligohydramnios. During the latter part of gestation, amniotic fluid is maintained in equilibrium by fetal urination and fetal swallowing. Polyhydramnios occurs when the fetus has difficulty swallowing amniotic fluid, for example, because of early problems in central nervous system development or upper gastrointestinal obstruction. Oligohydramnios is usually present after chronic leakage of amniotic fluid or whenever fetal urinary excretion is decreased, for example, because of renal agenesis, infantile polycystic kidney disease, or urethral obstruction.

Alterations noted at delivery associated with prenatal onset of developmental problems are as follows:

1. Increased incidence of breech presentation. Breech presentation occurs in 3.1 percent of normal deliveries at 40 weeks gestation. However, it occurs much more frequently in some disorders that adversely affect the form and/or function of the fetus. Defects of form include structural abnormalities such as hydrocephalus, which would be less compatible with the



vertex position because of the large head and joint dislocations, which may limit the capacity of the fetus to alter its position. Defects of function include some conditions associated with neuromuscular dysfunction, for example, the trisomy 18 syndrome and Smith–Lemli–Opitz syndrome associated with hypertonemia and the Prader–Willi syndrome and Zellweger syndrome associated with hypotonia.

2. Prenatal-onset growth deficiency. Drillen (7) studied the incidence of malformations, mental retardation, and/or neurologic defects in 180 1-year-old children whose birth weight was 2000 g or less and who were small for gestational age. She documented an increased incidence of prenatal-onset malformations as weight-for-gestational age decreased. In addition, she showed a marked increase in suspected mental and neurologic defects in those small-for-gestational-age children who had some structural anomaly.
3. Difficulty with neonatal adaptation. Children with prenatal-onset structural defects frequently have problems with neonatal respiratory adaptation, probably secondary to malformations of brain structure. Therefore, one should always be cautious when attributing mental retardation to a perinatal insult in a child who has associated prenatal-onset structural malformations. Mental retardation in such patients may well be related to a problem in brain development of prenatal onset.

Other historical information that will be useful in determining etiology includes the following:

1. Family history with attention to any health, developmental, or functional issues in first-, second-, or third-degree relatives as well as the presence or absence of consanguinity;
2. Past obstetrical history with attention to unexplained fetal losses;
3. Maternal health and exposure history in mothers with diabetes, epilepsy, and certain immunological conditions may have a higher risk for adverse outcomes. Certain drugs, chemicals, and infections are known to increase risk.

The most helpful way to determine whether a structural defect is of prenatal or postnatal onset is a careful physical examination (8). In the vast majority of situations, the nature of the problem will determine the direction the diagnostic evaluation should take. The physical examination should focus on delineating the pattern of major and particularly minor malformations. Major malformations are birth defects that have significant cosmetic and/or functional consequences to the individual concerned. About 15–20% of stillborn babies and 2–3% of all live-borns have a major malformation, and an additional 2% have occult cardiac, renal, and nervous system malformations that become manifest by age 5 years. Major anomalies are often what bring an

individual to clinical attention, but they rarely lead to an etiologic diagnosis.

Minor malformations, on the other hand, have no functional or cosmetic consequences for the affected individual, and include, for example, complete two to three syndactyly of the toes or a single transverse palmar crease. By definition, minor malformations occur in less than 4% of the general population. Although these anomalies themselves have few adverse implications, the presence of two or more minor anomalies greatly increases the likelihood of a major anomaly. In addition, certain minor anomalies (such as a preauricular tag) may be associated with specific major anomalies (such as hearing loss). Moreover, most syndromes are diagnosed based on the pattern of minor malformations rather than the major malformations with which they occur. For example, a diagnosis of Down syndrome may be suspected in an infant with upslanting palpebral fissures, epicanthal folds, small ears, a flat face, loose nuchal skin, a single transverse palmar crease, and wide spaces between toes one and two regardless of the presence of a cardiac defect. Lastly, minor malformations may provide clues as to the timing of the insult in development. For example, the interphalangeal flexion creases develop at about 9 weeks gestation in response to movement across the joint. Hand contractures associated with absent palmar creases suggest that the fetal hand was not moving at 9 weeks gestation, whereas, contractures in the face of normal creases suggest that early hand movement was normal.

In addition to structure, posture, tone, and behavior may all provide diagnostic clues.

### 35.3 PRENATAL-ONSET PROBLEMS IN DEVELOPMENT

Once a given problem has been determined to be of prenatal onset, a distinction should be made between those that are single primary defects in development and those that are multiple malformation syndromes. Although the concepts are not totally analogous, separation of prenatal problems into these two categories permits some practical generalizations that can be extremely helpful in counseling about recurrence risk.

Conceptually, “single primary defect in development” is an anatomic or morphogenetic designation. In most cases, the defect involves only a single structure, and the child is otherwise completely normal. Table 35-1 sets

**TABLE 35-1 Common Single Primary Defects in Development**

Malformation: Cleft lip ± cleft palate, cleft palate, cardiac septal defects, defects in neural tube closure
Deformation: congenital hip dislocation, talipes equinovarus
Dysplasia: pyloric stenosis

forth the seven most common single primary defects in development. For most of them, the specific etiology is unknown, making definitive recurrence risk counseling difficult. However, from a practical standpoint, most single primary defects are explained on the basis of multifactorial inheritance, which is thought to carry a recurrence risk for first-degree relatives of between 2% and 5%. Thus, recognition that a child's structural defect represents a single primary defect in development usually enables the clinician to suggest recurrence risk percentages between 2% and 5% for unaffected parents with one affected child.

The multifactorial model is a theoretical construct that was developed to explain the observed 2–5% risk for first-degree relatives, the twin discordance and gender inequality observed for many common single defects. The model stipulates that expression of a given characteristic represents the interaction between genetic susceptibility to a given biologic error and a threshold beyond which a given characteristic is expressed. Susceptibility is depicted as a normal distribution in the population. The threshold may be manipulated by environmental factors, which may either increase or decrease the likelihood that a defect will be evident. The extent to which multifactorial inheritance contributes to the etiology of some of the less common single defects in development is at present unclear. The fact that single primary defects are etiologically heterogeneous implies that some will have a clearly environmental etiology, whereas others will result from dominantly or recessively inherited single altered genes. Craniosynostosis secondary to in utero constraint is an example of the former, and postaxial polydactyly illustrates the latter. Before multifactorial risk figures are used for counseling, references such as OMIM (6), or Practical Genetic Counseling (9) should be consulted to determine if other risk figures have been reported.

In contrast to the anatomic concept of the single primary defect in development, the designation “multiple malformation syndrome” indicates that the observed structural defects all have the same cause. The defects themselves usually include a number of anatomically unrelated errors in morphogenesis. Multiple malformation syndromes are caused by gross chromosomal abnormalities, chromosomal microdeletions and duplications, teratogens, and single-gene defects usually inherited in Mendelian patterns. Recurrence risk depends on an accurate diagnosis and ranges from zero in cases that represent fresh gene mutations or are caused by one-time teratogen exposures to 100% for the unusual case of a child with the Down syndrome in which one parent is a balanced 21/21 translocation carrier. To review, recognition that a child has a prenatal-onset single primary defect in development suggests a 2–5% risk; recognition that a child has a multiple malformation syndrome is not helpful with respect to recurrence risk counseling unless a specific diagnosis can be made.

### 35.3.1 Single Primary Defect in Development

Single primary defects can be subcategorized, as shown in Table 35-1, according to the nature of the error in morphogenesis that has produced the observed structural defect (10). Thus, single primary defects involve malformation, deformation, disruption, or dysplasia of the developing structure. A malformation implies a primary structural defect arising from a localized error in morphogenesis. A deformation should be thought of as an alteration, usually through compression, in shape and/or structure of a part that has differentiated normally; the term disruption is used for a structural defect resulting from destruction of a previously normally formed part. The term dysplasia refers to an abnormal organization of cells and the structural consequences. Dysplasias may be localized or generalized. Localized dysplasias (e.g. hemangiomas) are generally single primary defects in development. However, generalized dysplasias such as connective tissue disorders usually present as multiple malformation syndromes in that a variety of structures are involved because of the widespread distribution of the dysplastic tissue.

Table 35-1 sets forth the most common single primary defects in development. Four are malformations, the result of a localized error in morphogenesis. Two (congenital hip dislocation and talipes equinovarus) are the result of intrauterine molding and thus represent deformation of previously normally formed structures. One (pyloric stenosis) is a dysplasia resulting from abnormal muscular hypertrophy at the gastric outlet. Each of these seven anomalies occurs with a frequency of 0.5 to 1 per 1000 in live-born infants. The major reason for separating single defects in development into malformation, deformation, disruption, and dysplasia is to gain information that can be helpful relative to prognosis. Of the deformations noted at birth, 90% will correct spontaneously; of those that do not, the vast majority can be corrected with early postural interventions, such as casting or bracing. Conversely, spontaneous correction of both malformations and disruptions almost never occurs, and, when correction is possible, surgery is virtually always necessary. Because dysplasias involve abnormal organization and localized deregulation of growth in affected structures, many change over time with involution occurring in some and malignant transformation taking place in others.

### 35.3.2 Malformations

Most children with a localized malformation in development, such as a cardiac septal defect or cleft lip and palate, are otherwise completely normal. After appropriate reconstruction, prognosis is excellent. In those cases in which Mendelian inheritance has not been previously documented, multifactorial recurrence risk figures

(2–5%) can be given to unaffected parents. If the malformation in development involves a structure that is not amenable to surgical correction, such as the brain, the long-term prognosis may be poor. The environmental factors that modify the threshold for expression of most malformations are for the most part unknown. For neural tube malformations, however, folic acid supplementation before conception substantially reduces the risk for recurrence (11).

### 35.3.3 Deformations

Most deformations involve the musculoskeletal system and are believed to be caused by intrauterine molding (12). The pressure required to produce such molding may be intrinsic (e.g. neuromuscular imbalance within the fetus) or may be extrinsic (e.g. fetal crowding). In either case, the ability of the fetus to kick is impaired, resulting in decreased fetal movement, an important factor in the development of a normal musculoskeletal system. This is particularly true with respect to joint development because motion is essential for normal development of the joints. In addition, because of fetal plasticity, marked positional deformation of any body part can occur when the fetus is unable to change position and thus alter the direction along which potentially deforming extrinsic forces are being directed.

**35.3.3.1 Intrinsically Derived Prenatal-Onset Deformations.** Disorders involving muscle degeneration, such as Steinert myotonic dystrophy, and disorders involving motor neurons, such as Werdnig–Hoffmann disease, are uncommon causes of positional deformations. Early defects in development of the central nervous system are more common causes and should be seriously considered whenever a structural defect is thought to be an intrinsically derived prenatal-onset deformation.

**35.3.3.2 Extrinsically Derived Prenatal-Onset Deformations.** Fetal crowding, the common pathway in extrinsically derived postural deformations, is usually due to a decreased volume of amniotic fluid, a situation that occurs normally during the later weeks of gestation when the fetus undergoes extremely rapid growth. However, it also occurs abnormally when fetal urinary output is diminished and in cases of chronic leakage of amniotic fluid. Other extrinsic factors associated with the development of deformations include breech presentation and the shape of the amniotic cavity. When a fetus is held in the breech position, the legs may be trapped between the body and the uterine wall. In that position, the fetus is unable to kick optimally and therefore is immobilized and more susceptible to molding and deformation. Breech presentation is associated with a 10-fold increase in the incidence of deformations. The shape of the amniotic cavity, which has a profound influence on the shape of the fetus that lies within it, is influenced by many factors, among which are the following: uterine shape; volume of amniotic fluid; size and shape of the

fetus; presence of more than one fetus; site of placental implantation; presence of uterine tumors; shape of the abdominal cavity, which is influenced by the pelvis, sacral promontory; and neighboring abdominal organs; and tightness of the abdominal musculature.

The various forms of talipes and congenital hip dislocation are the most frequently observed congenital postural deformities. Most children with these deformations are otherwise completely normal, and their prognosis is excellent. Correction usually occurs spontaneously. However, recognition that a structural defect represents a deformation does not always imply fetal crowding and should lead to careful consideration of other etiologic possibilities that might have far greater importance to the child. For example, because decreased fetal movement can be secondary to serious neurologic abnormalities, multiple joint contractures should always alert the clinician to the possibility of a malformation in central nervous system development. Although the most common deformational single primary defects—congenital hip dislocation and talipes—have a 2–5% recurrence risk, most deformations are the result of physiologic crowding and have virtually no recurrence risk. Deformations that are due to pathologic crowding (e.g. uterine tumors or malformation) have a much higher recurrence risk unless the factors leading to crowding are altered before subsequent pregnancies. Deformations that are the result of an underlying malformation (e.g. renal agenesis) have a recurrence risk similar to that for the underlying malformation.

### 35.3.4 Disruptive Defects

Disruptive defects occur when there is destruction of a previously normally formed part. There are at least two basic mechanisms believed to produce disruption. One involves entanglement followed by renting, amputation, or both of a normally developed structure, usually a digit, arm, or leg, by strands of amnion floating within amniotic fluid (“amniotic bands”) (13).

The second mechanism through which disruption occurs involves the interruption of blood supply to a developing part, leading to infarction, necrosis, and resorption of structures distal to the insult. If interruption of blood supply occurs early in gestation, the disruptive defect seen at term usually involves atresia or absence of a particular part. If the infarction occurs later, necrosis is more likely to be present. Examples of disruptive single primary defects for which infarctive mechanisms have been implicated include nonduodenal intestinal atresia (14), gastroschisis (15), pencephaly, and transverse limb reduction defects (16). The extent to which disruption of developing structures plays a role in dysmorphogenesis is unknown (17).

Because disruptions do not involve programming errors intrinsic to the fetus, genetic factors appear to play a minor role in their pathogenesis. Thus, most disruptive

defects are sporadic events in otherwise normal families. Cocaine is an environmental agent whose mechanism of action is vascular disruption. Multiple disruptive defects have been seen in the offspring of women who abuse this agent in pregnancy (18). The prognosis for a disruptive defect is determined entirely by the extent and location of the tissue loss. Thus, a child with a limb amputation has an excellent prognosis for normal function but a child with porencephaly does not.

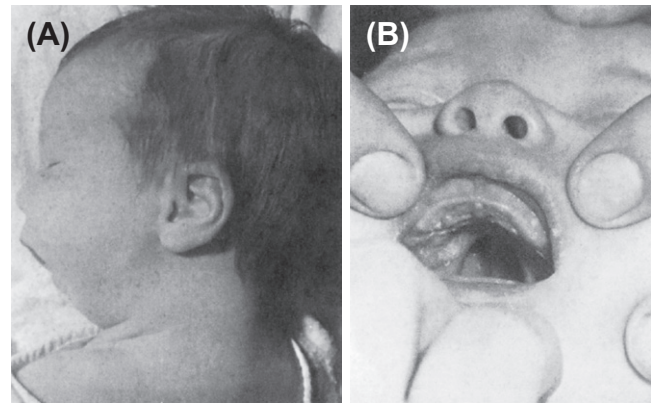
### 35.3.5 Dysplasia

The causes of the vast majority of localized dysplasias are unknown. Because many generalized dysplasias are the result of abnormal genes, it is probable that localized dysplasias will reflect somatic mutation in specific tissues. This hypothesis is consistent with the observation that empirical recurrence risks for localized dysplasias are low. The process of dysplasia appears to involve deregulation of growth; hence most dysplasias change over time. Capillary hemangiomas become involuted (the bathing trunk nevus illustrated in Figure 35-5 carries a risk for malignant transformation). Knowledge of the natural history of a lesion is critical in the long-term follow-up of children with localized dysplasias.

### 35.3.6 Sequence

“Sequence” describes the pattern of multiple anomalies that occurs when a single primary defect in early morphogenesis produces multiple abnormalities through a cascading process of secondary and tertiary errors in morphogenesis. When evaluating a child with multiple anomalies, it is extremely important from the standpoint of recurrence risk counseling to differentiate between multiple anomalies secondary to a single localized error in morphogenesis (a sequence) and a multiple malformation syndrome. In a sequence, recurrence risk counseling for the multiple anomalies depends entirely on the recurrence risk for the initiating, localized error. The words malformation, deformation, disruption, and dysplasia sequence are used if the nature of the initiating error in morphogenesis is known.

The patient depicted in Figure 35-2 has mandibular hypoplasia, glossoptosis, and cleft palate, an example of multiple anomalies secondary to a single localized error in morphogenesis. The primary defect in this case is mandibular hypoplasia, a malformation. Because the tongue is relatively large for the oral cavity, it drops back (glossoptosis), blocking closure of the posterior palatal shelves, resulting in a U-shaped cleft palate. This condition has previously been referred to as the Pierre Robin syndrome. However, because both glossoptosis and cleft palate are secondary to mandibular hypoplasia, the disorder is now referred to as the Robin malformation sequence. Recognition that all of the observed defects are due to a single localized error in morphogenesis (mandibular



**FIGURE 35-2** Infant with the Robin malformation sequence. (A) Micrognathia. (B) U-shaped palatal cleft.

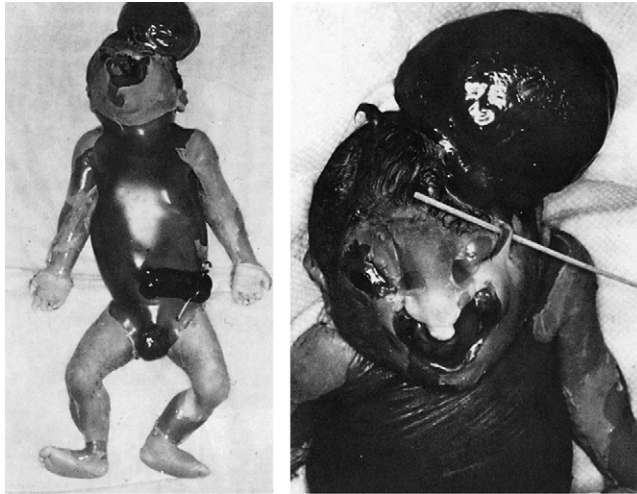


**FIGURE 35-3** Newborn infant with breech deformation sequence. Note the deformed cranial shape and positional deformities at the hips and feet.

hypoplasia) permits recurrence risk counseling based on the etiology of the single defect. The patient depicted in Figure 35-3 has bathrocephaly, torticollis, facial asymmetry, a dislocated right hip, and valgus anomalies of both feet. All of the structural defects are the result of compression of developing fetal parts. The pattern of abnormalities in this patient is referred to as the breech deformation sequence. Intrauterine crowding in this situation was the result of a large infant with a birth length of 54 cm, birth weight of 3.9 kg, and head circumference of 36 cm, delivered from a breech position to a small, primigravida mother. Recurrence risk is therefore negligible. Recognition of the deformational nature of the abnormalities is helpful with respect to prognosis. All the problems should resolve spontaneously or with postural therapy.

The patient depicted in Figure 35-4 has the amnion rupture sequence. All of the craniofacial and limb defects



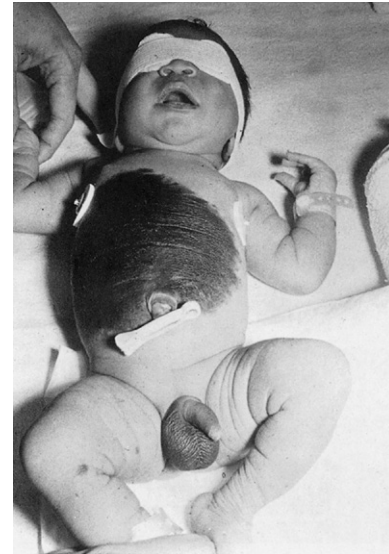


**FIGURE 35-4** Infant with amniotic band disruption sequence. Note the asymmetrical encephalocele, severe disruption of facial development, and digital anomalies.

are secondary to multiple fibrous strands of amnion extending from the placental insertion of the umbilical cord to the surface of the amnion-denuded chorion or floating freely within the chorionic sac. These strands of amnion, which result from disruption of the normally formed membrane, cause secondary defects through any one or more of the following mechanisms. Malformations occur if a strand of amnion interferes with the normal sequence of embryologic development. For example, a strand of amnion could interrupt fusion of the facial processes so that a cleft lip would result. Disruptions, on the other hand, are secondary to tearing apart of structures that have previously developed normally. As such, an amniotic band might act to cleave areas in the developing craniofacies along a line not conforming to the normal planes of facial closure. Deformations due to fetal compression occur secondary to oligohydramnios, tethering of a fetal part, or both. The former situation may result from rupture of both amnion and chorion, leading to chronic leakage of amniotic fluid. Tethering occurs when the fetus or one of its parts becomes immobilized by the constraining effect of an amniotic band such that it is unable to change position and thus alters the direction along which potentially deforming forces are being directed.

When used in conjunction with the word sequence, malformation, deformation, disruption, and dysplasia describe only the initiating error in morphogenesis of the sequence. The nature of the individual secondary defects that ensue from the initiating event depend on the manner in which the initiating error alters subsequent morphogenesis. In the case of the amnion rupture sequence, the initiating event, disruption, can lead to multiple structural defects through three of the mechanisms listed earlier.

The child depicted in [Figure 35-5](#) has a neurocutaneous melanosis sequence. In this dysplasia sequence,



**FIGURE 35-5** Infant with neurocutaneous melanosis. Note the bathing trunk nevus. This infant also had seizures, presumably from melanocytic infiltration of the pia and arachnoid.

melanocytic hamartosis of the skin occurs in conjunction with similar changes in the pia and arachnoid. Affected individuals are at risk for malignant degeneration within the hamartoses and are also at risk for neurologic sequelae, including seizures and mental retardation. Although the etiology of this sporadic condition is unknown, the single defect in development is presumed to involve melanoblastic precursors migrating from the neural crest.

Finally, a sequence, like any other single defect in development, can occur by itself in an otherwise normal individual or may be one feature in a multiple malformation syndrome. Stickler syndrome, cerebrocostomandibular syndrome, and spondyloepiphyseal dysplasia congenita are examples of multiple malformation syndromes in which the Robin malformation sequence represents one feature. In this situation, recurrence risk counseling is based on the etiology of the overall condition.

### 35.3.7 Multiple Malformation Syndromes

The category of multiple malformation syndromes includes patients in whom a primary developmental anomaly of two or more systems has occurred, all of which are thought to be due to a common etiology. Other than Down syndrome, which has an incidence of 1:660, and XXY syndrome (1:500 males), few of these disorders occur more frequently than 1 in 3000 live births.

As shown in [Figure 35-1](#), multiple malformation syndromes can be categorized on the basis of etiology.

### 35.3.8 Chromosomal Abnormalities

The ability to perform chromosomal studies has led to the recognition of a number of multiple malformation syndromes due to chromosomal abnormalities. Two classic

references delineate the features of the most common of these (19,20). Certain generalizations are important to consider when deciding whether or not a chromosome study should be performed. First, because chromosomes are present in most cells of the body, a chromosome aberration may be expected to affect adversely many parts of the body. Consequently, a person with only an incurved fifth finger and a heart defect is very unlikely to have those features on the basis of a chromosome problem. Second, some sex-chromosome disorders (e.g. XXX, XXY, and XYY) have few, if any, defects recognizable at birth and may present as postnatal problems in development associated with learning difficulties and behavioral challenges. In addition, as more experience is gained with chromosomal abnormalities involving very small deletions and duplications (currently referred to as copy number abnormalities), it has become increasingly clear that prenatal growth deficiency and mental retardation should not be the only requirements for chromosome studies.

The most common disorder associated with a chromosomal abnormality is Down syndrome (trisomy 21). The principal features of the disorder (flat facies with upward slant to the palpebral fissures, hypotonicity, and small ears) are usually present at birth, making a clinical diagnosis possible in the newborn period. Ear length is measured by the maximum distance from the superior aspect to the inferior aspect of the ear. Aase and colleagues (21) documented decreased ear length as a consistent feature in newborn infants with Down syndrome. In their series, no full-term white infant with Down syndrome had an ear length greater than 3.4 cm (mean 3 cm), and no normal full-term white infant had an ear length less than 3.2 cm (mean 3.8 cm).

Until recently, the most commonly performed screening test to define the etiology of an unrecognized, prenatal-onset multiple malformation syndrome was a high-resolution chromosome analysis (>550 band resolution).

Another technique that may be useful in certain circumstances is fluorescent in situ hybridization (FISH) analysis. Because the probes used to perform FISH are specific to defined areas of the genome, FISH analysis is not a screening technique, but rather is best applied when there is high clinical suspicion of a recognized pattern of malformation for which FISH analysis is available (i.e. velocardiofacial syndrome or Williams syndrome).

Comparative genomic hybridization (CGH) and single-nucleotide polymorphism (SNP) arrays are technologies that have moved into the arena of constitutional chromosomal abnormalities. Array-based CGH identifies copy number variation (either duplication or deletion) across the entire genome at high resolution. The most broadly available arrays use fluorescence techniques to compare DNA content in two differentially labeled genomes. Thousands of individual DNA sequences can be simultaneously interrogated, providing precise

information about copy number at specific genomic locations. Several platforms are commercially available for testing. Most use oligonucleotides. In addition to detecting copy number changes, SNP arrays have the capacity to detect areas of homozygosity that could suggest unknown or undisclosed consanguinity or uniparental disomy (UPD). Arrays vary in resolution and coverage. Most currently provide <1 Mb resolution and dense coverage in areas of known clinical significance. Recent meta-analysis of the diagnostic yield of array CGH in the evaluation of individuals with learning disabilities and congenital anomalies has suggested a 10% rate of causal copy number abnormalities. The down side of the technology is that it also detects copy number abnormalities that will eventually be determined to be noncausal (usually based on parental studies) at almost the same rate, 7%, the false-positive rate (22). Some have advocated that array CGH should be a first-line diagnostic test in the evaluation of a child with a multiple malformation syndrome, given the ability of the technology to identify causal copy number changes that might not be specifically suspected on clinical grounds alone as well as all of the aneuploid anomalies identified on routine chromosome analysis and diagnostic FISH testing (23,24). Arguments against the routine use of array CGH focus primarily on cost, the false-positive rate (which adds cost if parental studies are required), and the inability of the technology to detect balanced rearrangements. If using array CGH as a first-line test, it must be borne in mind that the technology cannot detect balanced rearrangements such as translocations and inversions that may relate directly to the phenotype or represent a predisposing factor to rearrangement.

Recurrence risk counseling for chromosome abnormalities depends on the nature of the cytogenetic abnormality identified. Risk is usually low in aneuploidy; however, the risk for certain trisomies (21, 18, XXX, and XXY) may increase with increasing maternal age. In case of an unbalanced translocation, parental karyotypes are warranted before specific risks are cited. In cases of de novo abnormalities, the risk is usually low except for the rare circumstance of gonadal mosaicism in a parent. Most microdeletion and microduplication syndromes are mediated by low copy repeats and carry a low risk for recurrence.

### 35.3.9 Disorders with Known Genetic Etiology

A single mutant allele or a pair of mutant alleles has been implicated as the cause of some recognizable multiple malformation syndromes of prenatal onset. Correct diagnosis in most of these disorders depends on clinical recognition, because in the majority of cases there is no testing available for the abnormal gene, although this is changing at a very rapid rate. A family history of a similarly affected individual can be extremely helpful in

suspecting that a single gene might be operative. However, many patients with multiple malformation syndromes of genetic etiology represent simplex events as a result of fresh gene mutation or the first presentation of an autosomal recessive disorder in a family. In such situations, family history will be noncontributory, although older paternal age might suggest fresh gene mutation or parental consanguinity the effects of an autosomal recessive gene. Diagnosis of most single-gene disorders depends entirely on the evaluation of the patient's phenotype. The type of laboratory evaluation that may be helpful as an adjunct to clinical recognition is usually an extension of the physical examination, for example, radiographic assessment of bony structure or tissue histopathology. As the genetic basis for many single-gene disorders is elucidated, diagnosis by mutation analysis has become available. However, the cost for some genetic tests is still quite high. Thus, most diagnoses still rest on clinical assessment.

In addition to Mendelian patterns of inheritance, some multiple malformation syndromes may arise as a consequence of dosage imbalance of a gene. At some loci in the genome, only one copy of a gene is active even though two copies are normally present. Inactivation of one member of a gene pair involves the process of imprinting. Parent of origin effects are evident at imprinted sites where it is possible in a normal individual to document that either the maternally inherited copy of the gene or the paternally inherited copy of the gene is active. Prenatal-onset multiple malformation syndromes may occur if an abnormality in the imprinting process causes both the maternal and paternal genes to be active (or inactive) at a specific locus or if an affected individual inherits two copies of a gene from one parent and none from the other. The latter situation is termed UPD. Each of these mechanisms accounts for some cases of the Beckwith–Wiedemann syndrome. The extent to which imprinting and UPD play a role in the etiology of genetically determined multiple malformation syndromes is at present unknown.

### 35.3.10 Disorders Caused by Teratogens

Disorders caused by teratogens include multiple malformation syndromes due to the effect of specific infections and drugs or chemical agents with which the embryo or fetus has come into contact. These disorders take on special importance because they represent the only group of dysmorphic conditions in which prevention before conception may be feasible. This is particularly true in the case of drugs and chemicals if the mother is aware that the agent in question can affect her baby. It is difficult, on the other hand, for a pregnant woman to avoid contact with all infectious agents. Immunization of at-risk individuals may assist in prevention of birth defects caused by specific viral infections (e.g. rubella or varicella-zoster virus).

A careful history of drug intake and chemical exposure should be obtained from the parents of all children with multiple malformation syndromes. This is particularly true when the etiology of the disorder is unknown. Two excellent references in determining the teratogenic potential of any environmental factor with which the mother has had contact are available (25,26). In addition, several online databases are available through subscription, including Reprotox (27) and Teris (28) (which includes Shepard).

Although a specific and easily distinguishable phenotype does not exist for each of the infectious agents that are commonly associated with altered fetal development, intrauterine infection can be suspected, based on the overall pattern of malformation. Any small-for-gestational-age patient with microcephaly or hydrocephalus; ocular findings including microphthalmia, chorioretinitis, cataracts and/or glaucoma; hepatosplenomegaly; and thrombocytopenia and who is developmentally delayed may be suspected of having had an intrauterine infection. It should be emphasized that each of these intrauterine infections has a wide spectrum, from fetal death, to the severely affected newborn infant with multiple malformations, to the child with no malformation disabilities. The latter situation is illustrated by a study by Hanshaw and coworkers (29) indicating a significant increase in school failure and deafness after clinically inapparent congenital cytomegalovirus infection (another exception to the prenatal–postnatal distinction set forth at the beginning of the chapter).

### 35.3.11 Recognized Patterns

Most multiple malformation syndromes are diagnosed based on the pattern of malformation in the affected individual, specifically the pattern of minor malformations. As the molecular basis for many syndromes is elucidated, etiologic heterogeneity has become the rule rather than the exception. A case in point is Rubenstein–Taybi syndrome (RTS). This disorder is a well-recognized pattern of malformation associated with pre- and postnatal growth deficiency, mental retardation, typical craniofacial features that change over time, and distinctive limb anomalies. Most cases of RTS are produced by functional loss of one copy of the gene encoding the transcriptional coactivator CREB-binding protein on chromosome 16p13.3 (30). Microdeletions of this region, first thought to be causal in the majority of cases, actually account for only 11% of affected cases and produce a more severe phenotype than point mutations in the gene, which account for the majority of cases. Moreover, mutations in a second gene, *EP300* on chromosome 22q13, also produce a similar phenotype. Thus, RTS is both a chromosomal and a single-gene disorder. RTS is typical of a growing number of recognized patterns of malformation in which different genetic mechanisms may produce the same phenotype. As a note of caution, array CGH testing

will identify the deletion cases but not those due to point mutation in the gene.

Array CGH has allowed delineation of the molecular basis of previously unrecognized patterns of malformation, such as the Potocki–Lupski syndrome (31). It is interesting that, in many cases, the distinguishing phenotype has not been delineated until after the molecular cause was known.

The molecular basis of many multiple malformation syndromes remains unknown, although most will likely be determined to be a consequence of altered copy number, single-gene mutation, epigenetic factors that alter expression or function of existing genes, or as yet unrecognized environmental exposure.

## 35.4 POSTNATAL-ONSET PROBLEMS IN DEVELOPMENT

Most children with postnatal-onset malformation problems are normal at birth, having appeared to thrive in utero. Neurologic problems frequently begin within the first week of life, and deterioration is often rapid. A specific pattern of malformation is typically not present at birth; structural abnormalities develop as the result of neurologic deterioration, storage of metabolites, or progressive loss of function in a specific tissue. In disorders with a known metabolic aberration, other manifestations of the metabolic defect such as cataracts, sparse hair, coarse facies, unusual skin pigmentation, and hepatosplenomegaly are frequently present. As set forth in Figure 35-1, these disorders can be categorized on the basis of etiology.

### 35.4.1 Genetic Factors

**35.4.1.1 Metabolic Conditions.** Most metabolic conditions are the result of deficiency of a specific enzyme, transporter, or cofactor. Because of the possibility that early institution of dietary or enzyme replacement therapy may help to prevent mental retardation, these disorders are of particular interest, and newborn screening is offered for an expanding list of these conditions throughout the United States. Because the placenta is able to compensate for the metabolic deficiency, affected infants are typically normal at birth (e.g. aminoacidurias and organic acidurias). Most of these conditions have an autosomal recessive mode of inheritance. Their incidence is extremely low. Phenylketonuria (1:14,000) is the most common and represented, in its untreated state, about 1% of most institutionalized populations surveyed before newborn screening programs were instituted. A second group of metabolic conditions produces a phenotype through abnormal accumulation or storage of material in various tissues in the body. Although the placenta does not compensate for the enzymatic deficiency, the phenotype does not manifest until a

period of time has passed during which accumulation of metabolites occurs (e.g. glycogen storage diseases and mucopolysaccharidoses). Consequently, there is usually a postnatal presentation.

**35.4.1.2 Central Nervous System Degenerative States.** A genetic etiology is increasingly known for many of these disorders. However, at present, the clinical diagnosis relies on imaging and histopathology.

**35.4.1.3 Myopathies and Connective Tissue Disorders.** Myopathies and connective tissue disorders represent a group of conditions in which structures deteriorate with wear and tear over time—hence the typically postnatal presentation. A diagnosis may be suspected by the communality of the involved tissue (i.e. wound-healing problems and ligamentous laxity for connective tissue disorders or cardiomyopathy and weakness for muscle disorders).

### 35.4.2 Environmental Factors

Trauma, infection, hypoxia, and metabolic derangements can result in severe neurologic impairment. Progressive joint immobility, abnormal positioning, and paralysis secondary to central nervous system deterioration are the most frequent structural anomalies resulting from this type of injury. Deafness and cataract may also be seen.

## 35.5 CONCLUSION

A clinical approach to children with structural defects has been set forth. It is based on the concept that the diagnostic evaluation should be directed by the nature of the structural defects. The ultimate goal of this approach is a specific overall diagnosis. The purpose of a diagnosis is to address the two questions facing all parents when a child with a birth defect is born: what does this condition mean to my child and what are my chances of having another child with a similar problem? An accurate overall diagnosis is critical to understanding the natural history of the disorder such that associated complications may be anticipated through screening, appropriate interventions offered, and precise reproductive counseling be provided. The birth of a child with congenital anomalies usually places severe stress on a family eliciting all the stages of a grief response. Understanding the cause and implications of the condition is often helpful in mitigating this stress. When an overall diagnosis is lacking, a better understanding of the nature and onset of the problem is commonly possible. That in itself can often be helpful to parents and to all others dealing with children who have structural defects. Lastly, reaching an overall diagnosis may take time as some characteristic developmental and behavioral findings may not manifest until an individual is older. Reevaluation is a critical component of this approach.



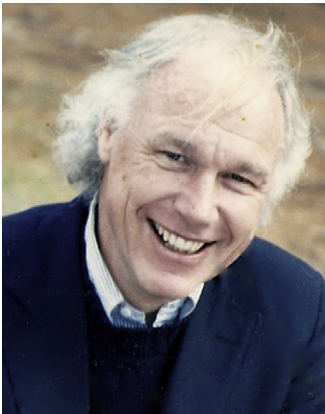
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### Biographies



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# CHAPTER

# 36

## Clinical Teratology

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### 36.1 INTRODUCTION

#### 36.1.1 Historical Overview

Pregnant patients who are undergoing genetic evaluation or prenatal diagnosis often have concerns about the possible adverse effects of nongenetic factors, such as exposures to drugs or occupational agents. Less than one-third of families seen in most genetics clinics have conditions that are principally genetic in origin, and the differential diagnosis often includes disorders that have a predominant nongenetic cause. Accordingly, it is incumbent on practitioners of clinical genetics to be aware of the non-heritable sources of human variability, their potential interactions with genetic factors, and their implications for human health, growth, and development. Teratology is the branch of medical science devoted to the study of the causes of abnormal prenatal growth and development, and this chapter focuses on the nongenetic causes. Teratological effects include structural congenital anomalies, growth disturbances, and functional deficits such as behavioral and cognitive abnormalities that may not be apparent until some time after birth.

The term “teratogen” has been used to denote an agent that can cause abnormalities of form, function, or both in an exposed embryo or fetus, but this usage is somewhat misleading. It implies that any given agent either is or is not teratogenic and that clinical teratology consists merely of memorizing a list of “human teratogens.” In reality, teratogenicity is a property of an *exposure*, which involves not only the physical and chemical nature of the agent but also the dose, route, and gestational timing involved. The occurrence of other concurrent exposures as well as the biological susceptibility of the mother and embryo or fetus are also factors that can determine whether a given exposure produces damage in a particular instance.

Although interest in teratogenic effects has been recorded in surviving fragments of tablets from ancient

Middle Eastern cultures, much of the information available in this field before 1950 can hardly be classified as more than folklore (1). Despite the pioneering experimental teratology studies of Warkany and Nelson (1940) and others, the rekindling of serious scientific and medical interest in the teratology of chemical and other exposures resulted from the successive tragedies produced by the thalidomide treatment of pregnant women and the rubella pandemics in the early 1960s.

Succeeding decades have seen significant growth of basic, clinical, and epidemiologic investigations into problems of teratogenesis. Regulatory agencies have erected barriers against the introduction of teratogenic exposures into our environment. Furthermore, the past three decades have seen the development of teratogen information services (2,3). These services, supported by computer-based information resources such as *TERIS* (<http://depts.washington.edu/~terisweb/teris/index.html>) and *REPROTOX* (<http://www.reprottox.org>), have improved the access of pregnant women and their health care providers to available information and have fostered a systematic approach to risk assessment and risk management. Unfortunately, significant public health barriers remain, and our goal of primary prevention through the avoidance of hazardous prenatal exposures remains substantially unrealized. Although progress is being made in our understanding of normal and abnormal embryonic development, the mechanisms by which most teratogenic exposures produce their pathogenic effects are still unknown.

#### 36.1.2 Mechanisms of Teratogenesis

James Wilson summarized our understanding of the biological basis of teratogenesis in the magisterial four-volume *Handbook of Teratology* he published with Clarke Fraser in 1977 (4) in terms that are still

remarkably insightful (5). Wilson formulated six principles of teratology, the third of which is, “Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate abnormal embryogenesis.” Wilson used the term “mechanisms” in a very precise manner to mean the earliest, if not the first, event in the pathogenic pathway between a teratogenic exposure and its effect. He explained that later events in the pathogenic process might be expressed as one or more higher-level effects, such as excessive or reduced cell death, abnormal cellular interactions, reduced biosynthesis of important products, aberrant morphogenic movement, or mechanical disruption of tissues, and that these, in turn, might result in a few final common pathways of abnormal development. He explicitly recognized that little was then known about the earliest teratogenic mechanisms, which were difficult or impossible to study experimentally with the techniques available at that time (4).

Table 36-1 lists some of the mechanisms that we now know to be important in mammalian embryonic development. Of these, epigenetic control of gene expression has been very intensively studied in recent years (6–9). Epigenetic mechanisms provide a possible link between the complex genetic program that underlies early development and teratogenic exposures (see Chapter 6). For the various mutagenic mechanisms listed in the table, it is important to point out that teratogenesis and mutagenesis are not the same thing (10). Many teratogenic exposures, including those involving retinoids and thalidomide, are not mutagenic, and many chemicals that have mutagenic potential do not appear to be teratogenic when administered to pregnant women in therapeutic doses.

**TABLE 36-1 Mechanisms of Teratogenesis**

- Alterations of the cytoskeleton
- Alterations of the integrity of intracellular organelles
- Altered energy sources<sup>a</sup>
- Altered membrane characteristics<sup>a</sup>
- Altered nucleic acid integrity or function<sup>a</sup>
- Chromosomal nondisjunction and breaks<sup>a</sup>
- Disturbances of intracellular or intercellular signaling
- Dysfunction of molecular chaperones
- Effects of mechanical forces on embryogenesis
- Effects of small regulatory RNAs
- Effects on the distribution of molecules into subcellular compartments
- Enzyme inhibitions<sup>a</sup>
- Epigenetic control of gene expression
- Genomic imbalance resulting from copy number changes
- Lack of precursors and substrates needed for biosynthesis<sup>a</sup>
- Mitotic interference<sup>a</sup>
- Mutation<sup>a</sup>
- Osmolar imbalance<sup>a</sup>
- Perturbations of the extracellular matrix

<sup>a</sup>Included among the mechanisms presented by Wilson (4).

### 36.1.3 Genetic Susceptibility to Teratogenic Effects

It is no surprise that genetic factors influence susceptibility to teratogenic effects, given that maternal absorption, maternal and fetal metabolism, and maternal and fetal excretion of potentially teratogenic agents, as well as embryonic development itself, are under genetic control. This has been unequivocally demonstrated in experimental animals, although the direct evidence in humans is more limited (11). Nevertheless, clinical observers have repeatedly shown that the risk for recurrence of a teratogenic syndrome (assuming the exposure continues) is much higher in the subsequent children of a woman who has had one affected child than the risk of occurrence in exposed pregnancies in general (12,13).

Genetic susceptibility to teratogenic effects has also been assessed through case-control studies of common complex congenital anomalies that focus on gene–environment interactions. This approach has been used extensively for orofacial clefts in relationship to candidate genes such as *TGFA* and *NOS3* and maternal cigarette smoking and for neural tube defects in relationship to candidate genes such as *MTHFR* and *MTRR* involved in folic acid metabolism (11,14). Although statistically significant gene–environment interactions have been demonstrated in some of these studies, no such association has been found in others.

While there is no clear consensus regarding the relative contributions of genetic and environmental factors to these particular congenital anomalies or, indeed, to the total load of birth defects in general, it is clear that genetic factors by themselves do not account for many such abnormalities. With this realization has come a renewed emphasis on the prevention of teratogenic exposures. A few generalizations have emerged that help us to characterize and recognize teratogenic exposures.

### 36.1.4 Characterization of Teratogenic Exposures

Teratogenic exposures act by a relatively limited number of pathogenetic processes (5,15) that may produce cellular death, alter tissue growth (hyperplasia, hypoplasia, or asynchronous growth), or interfere with cellular differentiation or other basic morphogenetic processes, including mechanical ones. Some agents may also act by destroying (“disrupting”) normally developing structures.

Similar general effects may be produced by different teratogenic exposures. For instance, many exposures may stunt growth, resulting in a neonate who is small for gestational age. Thus, certain characteristics are common to a wide range of teratogenic exposures and may be used as general indicators of potential teratogenicity. Indicators of the fact that a teratogenic effect may have occurred include:

1. Infertility or fetal wastage
2. Prenatal-onset growth deficiency



3. Alterations of morphogenesis, i.e. congenital anomalies
4. Alterations of central nervous system function.

Exposures that have been reproducibly associated with one or more of these effects should be considered possibly teratogenic. Fetal wastage and prenatal-onset growth deficiency seem to be especially frequent indicators of teratogenicity.

These general indicators of teratogenic activity reflect disturbances of basic processes occurring in many tissues. Such processes affect critical events in growing cells and developing organisms and commonly manifest in more than one tissue or organ in the developing embryo or fetus at any one time. It is not surprising, therefore, to find that teratogenic exposures usually have the capability of producing abnormalities in more than one tissue or organ system and that teratogenic exposures tend to produce characteristic patterns of abnormal growth and morphogenesis. For this reason, while individual abnormalities of morphogenesis are not specific for a particular teratogenic exposure, certain patterns of abnormal growth and development may be distinctive.

Agents are teratogenic only under certain conditions of exposure. This is why classifying some agents as “teratogens” and others as “nonteratogens” is misleading (16). One critical factor is the developmental stage of the embryo at the time of exposure. The most sensitive period to alter embryonic development appears to be from roughly two weeks after conception to the eighth week after conception for most teratogenic exposures. Data from animal experiments suggest that earlier adverse exposures are usually either lethal to the embryo or produce no demonstrable effect on morphogenesis. Exposures occurring after the period of embryogenesis may produce problems of cell depletion or organ function and could, therefore, be related to such effects as growth retardation or renal failure. Agents that lead to fetal constraint and consequent deformations are likely to have their most significant effect in the third trimester of pregnancy, during the phase of most rapid fetal growth. Exposures to infectious agents that produce cell death or tissue necrosis may cause disruption at any stage of gestation.

Thalidomide and angiotensin-converting enzyme (ACE) inhibitors provide especially vivid illustrations of the importance of timing to teratogenesis. The pattern of limb reduction defects, facial hemangioma, microtia, ocular abnormalities, renal malformations, and congenital heart disease that characterizes the thalidomide embryopathy only occurs in children whose mothers are treated between 27 and 40 days of gestation (17). In contrast, maternal treatment with the ACE inhibitors captopril and enalapril only produces fetal renal failure and oligohydramnios during the later stages of pregnancy (18). These effects appear to result from the exquisite sensitivity of the fetus to the pharmacological

hypotensive action of ACE inhibitors during the second and third trimesters of gestation (19).

Dose is a critical feature of any teratogenic exposure. Teratogenic effects occur only when the dose exceeds a certain threshold (20). Agents that are generally considered to be safe may have adverse effects on the embryo or fetus if given in doses high enough to produce maternal toxicity. This is an especially important consideration in exposures associated with suicide attempts, drugs of abuse (e.g. toluene inhalation), or agents encountered occupationally. Chronic exposure is usually of more concern than a single exposure, given similar doses.

The route of exposure is also of importance—there is unlikely to be a risk associated with any agent when the exposure occurs by a route that does not permit systemic absorption. This is the case with many dermal exposures. Exposure to methylene blue illustrates the importance of route of exposure to teratogenicity. Several studies have found a strong association between the occurrence of intestinal atresia and the instillation of methylene blue into the amniotic sac during midtrimester genetic amniocentesis in twin pregnancies (21). The risk of intestinal atresia in an infant born after this procedure is about 20% (22). Neither oral nor topical administration of methylene blue to the mother has been associated with a similar teratogenic risk.

Some agents, such as ionizing radiation, have direct access to the embryo, whereas others do not reach the embryo until after extensive metabolism by the mother. The teratogenicity of agents that are metabolized by the mother may depend on whether the teratogenic metabolites reach the embryo or fetus in sufficient quantities to produce adverse effects. This, in turn, depends on a number of factors including the route of entry, physical properties of the agent, maternal dose, amount of systemic absorption, and maternal metabolic capacity.

Another factor that influences teratogenicity is the chemical and/or physical nature of the agent itself. Some agents are inherently more risky than others. Maternal thalidomide treatment during embryogenesis is the classic example of an exposure that usually presents little direct risk to the mother but has strong developmental toxicity. There are only a few other examples of exposures that exhibit such selective developmental toxicity.

The teratogenicity of an exposure is also influenced by both the maternal and fetal genotypes, which may result in differences in cell sensitivity, placental transport, metabolism, receptor binding, and distribution. These differences explain why only some of the children of women who have exposures that are similar with respect to agent, dose, and gestational timing exhibit adverse effects. The importance of the genetic susceptibility of the fetus to teratogenesis is clearly illustrated by the higher rate of concordance for fetal alcohol syndrome among monozygotic twins than dizygotic twins of mothers who heavily abuse alcohol during pregnancy (23).

Together, these factors probably account for most variation encountered among patients adversely affected by prenatal exposures. It must be recognized that the teratogenic potential of an exposure is commonly expressed over a wide spectrum when all exposed individuals are considered. That is to say, when dealing with affected individuals, variability of effect is the rule, not the exception.

These generalizations lead to the following conclusions:

1. Teratogenic exposures are most easily recognized (that is, the effects are most specific) at the severe end of the spectrum where a clear-cut pattern of abnormalities of growth and development often emerges.
2. As such patterns are clear only at the severe end of the spectrum, “milder” or partial effects, including single major abnormalities of morphogenesis, may be more frequent among exposed individuals and less specific to individual teratogenic exposures. Such effects may sometimes be viewed as being “consistent with a prenatal teratogenic exposure” even though they are not specific.
3. Certain general features of teratogenic exposures may be sought in screening for potentially hazardous agents, and the use of agents displaying some of these characteristics would be most prudently avoided during pregnancy.
4. All the above features should be taken into account for complete clinical characterization of the effects of a teratogenic exposure and in order to interpret this information to patients in a meaningful way. A checklist of the types of information needed for the clinical characterization of a teratogenic exposure is presented in Table 36-2. Table 36-3 presents a list of features used to characterize effects that may occur in the child as a result of such exposures. Much more information on each of these points is needed for virtually every known or suspected teratogenic exposure in humans.

### 36.1.5 Risk Assessment and Counseling for Teratogenic Effects

**36.1.5.1 Clinical Settings.** Clinical geneticists and genetic counselors need to consider the possibility of teratogenic effects in three clinical settings. The first is during the evaluation of a patient, usually a child, in whom the medical history and/or physical findings suggest the possibility of a teratogenic effect. Both a high index of suspicion and considerable skepticism are required in such cases. Careful review of the prenatal history for the nature and circumstances of any potentially teratogenic exposure is essential, and the child should be examined for major and minor abnormalities known to be associated with such exposures. Except in the case of some infectious agents, laboratory confirmation of the diagnosis is not possible and recognition of a teratogenic cause may

**TABLE 36-2 Characterization of Teratogenic Exposures**

<i>Agent</i>
Nature of the chemical, physical, or infectious agent
Inherent developmental toxicity
Capacity to produce other kinds of toxicity in the mother
<i>Dosage to embryo or fetus</i>
Single, repeated, or chronic exposure
Duration of exposure
Maternal dose
Maternal route of exposure
Maternal absorption
Maternal metabolism and clearance
Placental transfer
Fetal metabolism and clearance
<i>Time of exposure in pregnancy expressed in gestational weeks (or days)</i>
Between conception and onset of embryogenesis
Embryogenesis
Fetal period
<i>Other factors</i>
Genetic susceptibility of mother
Genetic susceptibility of the fetus
Other concurrent exposures
Maternal illness or other conditions associated with exposure
Availability of tests to quantify the magnitude of maternal exposure

**TABLE 36-3 Characterization of Teratogenic Effects for Counseling**

<i>General effects</i>
Alterations of morphogenesis
Alterations of CNS function
Other functional impairments
Death of the conceptus, embryo, or fetus
Prenatal-onset growth deficiency
Carcinogenesis
<i>Specific effects</i>
Recognizable syndrome
Other distinctive features
<i>Magnitude of risk</i>
Absolute
Relative
<i>Prenatal diagnosis</i>
Detailed ultrasound examination
Amniocentesis or other invasive method
Availability
Reliability
Utility

require the skills of an expert dysmorphologist (24–26). In many instances, a firm diagnosis can only be made by exclusion of alternative explanations for any abnormality identified and by long-term follow-up of the patient.

Concern is often voiced that informing a couple that a child’s birth defects have resulted from the adverse effects of a drug or other potential teratogenic exposure may create serious psychological problems, particularly

in situations that involve maternal drug abuse or excessive consumption of ethanol. However, only through such a frank approach will there be an opportunity to provide optimal care for the affected child and the potential for prevention of similar problems in future children. It is clear, however, that the sharing of such information may indeed produce psychological problems. Under these circumstances, long-term support for affected families through community and health care agencies is extremely important.

A second setting in which the geneticist or genetic counselor must consider a teratogenic effect is when a patient who is not currently pregnant but wishes to have children is concerned about possible teratogenic effects of a current or future medical treatment or occupational exposure. The most certain way to prevent birth defects from teratogenic exposures is exclusion of the exposure from the prenatal environment. The approach in this instance involves determining whether the exposure is of concern and, if so, whether it can be avoided, replaced by a safer alternative, completed before pregnancy, or deferred until the pregnancy is over. Although this is not always possible, increased attention should be paid by physicians to patient education to help avoid unnecessary exposures to potentially teratogenic agents during pregnancy, particularly during critical periods of embryonic or fetal development. When a woman of reproductive age requires drug therapy, the prescribing physician's discussion of risks and benefits should include the potential for teratogenic risks. Failure to inform a woman of reproductive age of teratogenic hazards relating to medical treatment or procedures may place the physician in legal jeopardy.

Sometimes it is impossible or unwise for a woman to avoid a potentially teratogenic exposure, e.g. when failure to use a potentially teratogenic treatment poses a greater risk to the woman than the treatment does, even if she is pregnant. When it is not possible or prudent to avoid such treatment, it may still be possible to minimize the magnitude of the exposure or to avoid it during the most sensitive period of embryonic development. In the final analysis, decisions regarding the use of potentially teratogenic treatments during pregnancy are best left to an informed couple, supported through a comprehensive pregnancy risk assessment, risk communication, and management process by a sensitive and knowledgeable physician or counselor.

A third circumstance in which a geneticist or genetic counselor must consider a teratogenic effect is when a patient is concerned about the possible adverse effect of an exposure during her current pregnancy. For example, half of all pregnancies in the United States are unintended, and a woman may have abused alcohol or "recreational drugs" before she realized that she was pregnant. Alternatively, she may have become pregnant while taking a particular medication that she has now stopped. In these instances, any benefit of the exposure is no longer relevant and her concern may be whether

she should terminate the pregnancy, consider prenatal diagnosis, or accept the additional risk (if any) and continue the pregnancy. Providing appropriate teratogen risk counseling in such situations requires careful evaluation of the woman, her fetus, and the exposure, as well as a review of relevant scientific literature regarding the risk and nature of potential adverse outcomes, their ability to be diagnosed prenatally, and the effectiveness with which they can be treated if they do occur.

**36.1.5.2 What Is Risk?** Three kinds of risk are used in the medical literature to describe a teratogenic effect: absolute risk, relative risk (or odds ratio), and population attributable risk. *Absolute risk* is the chance that a woman who has had a particular exposure during pregnancy will have an affected baby. Absolute risk is useful in counseling because it answers the question that most pregnant women ask about an exposure: "What is the risk of birth defects in my baby?" In addition, absolute risk can be compared directly to other familiar risks such as the risk of miscarriage following prenatal diagnosis or the risk of nontreatment of the mother's disease.

*Relative risk* is a statement of how much more likely a woman who has had a particular exposure during pregnancy is to have an affected baby than a woman who has not had that exposure. Relative risks (or odds ratios, which are numerically equivalent for uncommon events) are usually reported in epidemiologic studies of birth defects because they are easy to calculate and easy to interpret. In counseling, it often is helpful to convert a relative risk (or odds ratio) to an absolute risk, but this requires knowledge of the incidence of the birth defect(s) of interest in the population that was studied.

Population attributable risk is the proportion of adverse outcomes of a given type in the population as a whole that are caused by a particular treatment during pregnancy. Population attributable risk is generally not relevant for counseling an individual patient, but it is useful for public health officials as an estimate of the amount by which the overall rate of a birth defect could be reduced by prevention of the teratogenic exposure.

The term "high risk" may mean two different things to patients. A teratogenic risk may be considered to be high if the severity of the effect is great, e.g. a severe brain anomaly. Thus, the risk for Ebstein anomaly, a severe congenital heart defect associated with maternal lithium treatment during pregnancy, may be considered to be great, even though the frequency of this malformation among the children of women treated with lithium during pregnancy is small (27–29). Alternatively, a risk may be considered to be great if it is numerically large, even if the severity is mild. For example, maternal tetracycline treatment very frequently causes staining of the primary dentition in fetuses exposed during the second or third trimester of gestation, but this staining is only of cosmetic significance (30). Maternal treatment with thalidomide or isotretinoin during critical times of pregnancy are examples of teratogenic risks that are great in both severity and

frequency, but fortunately not many other teratogenic exposures of this kind are known to exist in humans.

**36.1.5.3 Teratogen Risk Counseling.** Providing teratogen risk counseling involves more than just identifying and estimating the magnitude of risk. The information must be communicated to the patient in a way that allows her to make informed decisions about the management of her pregnancy. The approach varies from patient to patient and depends on many factors, including the patient's cultural and social background, her understanding of the counselor's language, her level of general and scientific knowledge, and her commitment to the pregnancy. The uncertainty that usually exists complicates teratogen counseling and requires that information be provided as risks, which are difficult for many people to understand. Counselors need to be aware of the information and misinformation patients bring with them to the counseling session and how it affects their perception of risk (31,32). Finally, the importance most women place on having healthy children and the emotional circumstances that often surround teratogen risk counseling require that the counselor have considerable skill as well as a thorough knowledge of clinical teratology.

**36.1.5.4 Dealing with Uncertainty.** One of the most difficult aspects of counseling pregnant women about teratogenic risks associated with various exposures during pregnancy is the fact that there are very few exposures for which the available information is sufficient to estimate the magnitude and severity of risk with any confidence. Available data are insufficient to determine the teratogenic risk associated with conventional treatment of pregnant women with most prescription medications (33,34), and the same is true for treatment with many over-the-counter drugs or herbal remedies and occupational or environmental exposures. Nevertheless, geneticists, genetic counselors, and other health professionals who take care of pregnant women must advise them about these risks.

It is important for health professionals to admit the limitations of their knowledge to themselves and to their patients. When counseling pregnant women, risks should be presented as best estimates and couched in appropriate uncertainty. Admitting the limitations of one's knowledge in this way may be unsatisfactory for some patients and is certainly unsatisfying for the counselor, but it is better than assuming that a lack of information means a lack of risk or, alternatively, that any maternal exposure during pregnancy may pose a significant risk to the developing embryo.

## 36.2 EVALUATING THE PATIENT AND HER EXPOSURE

### 36.2.1 Evaluation of the Pregnant Patient for Teratogenic Exposures

In order to provide a context for counseling, the counselor should review the patient's general medical, obstetrical,

and family history. The purpose of teratogenic risk assessment is to determine whether a pregnant woman's exposure increases her risk of having a child with congenital anomalies above the risk that she would have if she were unexposed. The "background" risk of serious congenital anomalies usually quoted is 3–5% for the general population, but the risk for a particular woman may be much greater because of her age, family history, medical condition, or other exposures. It is common for the risk of congenital anomalies associated with these other factors to equal or exceed the risk associated with a particular exposure of concern to the patient, and it is important for any teratogenic risk to be presented in the context of these other risks for adverse pregnancy outcomes when they are present.

The dose, route, duration, and timing for each exposure of concern should be determined as precisely as possible. For example, one can usually obtain the name, amount, frequency, and length of time that a medication was taken. This is much more informative than just knowing what was prescribed. In some cases, blood levels of a drug or occupational chemical to which the patient was exposed can be used to define the exposure very precisely. In other instances, measurements of a chemical or its metabolite in the urine or an occupational hygiene assessment can help to quantify an exposure of particular concern. One should always establish the reason for the exposure (e.g. medical treatment of a particular disease) and whether the woman experienced any toxic effects herself as a result of the exposure. If toxic effects did occur, they should be described as fully as possible. The evaluation should also include information about any relevant occupational exposures and the patient's use of other medications, alcohol, tobacco, and other "recreational" drugs.

### 36.2.2 Assessing the Scientific Literature

Clinical assessment of human teratogenic risk requires the careful interpretation of data obtained from several kinds of studies (35). Fortunately, the problem of collecting and analyzing the available data has been greatly simplified by the advent of online clinical teratology knowledgebases (<http://www.reprotox.org/>; <http://depts.washington.edu/terisweb/teris/>). In some places, health professionals and patients can also obtain information on teratogenic risks over the telephone from dedicated teratogen information services (36,37).

**36.2.2.1 Animal Studies.** For many exposures, the only data available on the effects of exposure during pregnancy are the results of studies done in laboratory animals. Such studies are valuable because they provide a means of identifying exposures with teratogenic potential before humans have been harmed. Unfortunately, however, it is usually impossible to extrapolate findings directly from animal experiments to a clinical situation involving an individual pregnant woman. Comparisons



between species are confounded by differences in placentation, pharmacodynamics, embryonic development, and other factors that may influence the likelihood of teratogenic effects. In addition, the consistent response seen in a controlled experiment in a genetically inbred strain of laboratory animals often contrasts with the highly variable response seen in the outbred human population, and the doses and routes of exposure used in animal teratology experiments often are not comparable to those that usually occur in humans.

**36.2.2.2 Case Reports.** Anecdotal descriptions of individual cases in which a child with birth defects was born to a mother who was exposed to a particular drug, other chemical, or physical agent during pregnancy are often reported in the medical literature. These associations are usually coincidental rather than causal because both birth defects and maternal exposures during pregnancy are relatively frequent. Case reports may be useful for suggesting particular outcomes that require further investigation and for raising causal hypotheses, but other kinds of studies are needed to determine whether these hypotheses are true.

**36.2.2.3 Case Series.** Teratogenic exposures typically produce qualitatively distinct patterns of congenital anomalies in affected children. Most of the exposures that are currently known to be teratogenic in humans were initially identified in clinical series on the basis of such characteristic patterns of anomalies (38). The evidence of a causal relationship can be compelling when a highly characteristic pattern of congenital anomalies is recognized in children whose mothers experienced similar well-defined exposures at similar times in pregnancy, especially if both the pattern of anomalies and the exposure are otherwise rare. Fetal alcohol syndrome provides the best known example of this (39,40), but the embryopathies associated with maternal exposure to rubella virus (41), thalidomide (17,42), and isotretinoin (43) were also first recognized by astute clinicians in case series.

Because clinical series can include a very thorough assessment of the circumstances of maternal exposure and the phenotype of each affected child, they are well suited to the recognition of such syndromes. Unfortunately, however, case series are subject to extremely biased ascertainment and multiple sources of confounding. Because they do not include controls, clinical series cannot be used to provide quantitative estimates of the strength or statistical significance of a teratogenic effect. Properly conducted epidemiological studies are needed to determine the magnitude of a teratogenic risk.

**36.2.2.4 Pregnancy Registries.** Pregnancy registries are an increasingly popular method of collecting information on outcomes among women who have taken a particular drug or group of drugs during pregnancy. These registries usually depend on voluntary identification of exposed pregnancies by the women themselves or by physicians who are treating them. Pregnancy registries are most useful if they identify women for inclusion “prospectively”, i.e. during or after the exposure

but before the outcome of the pregnancy is known, to avoid the bias toward reporting adverse outcomes that occurs when cases are voluntarily submitted following delivery. A major limitation of most pregnancy registries is the lack of an appropriate control group. Comparisons are often made to “expected” rates of congenital anomalies obtained from dedicated birth defects registries with active ascertainment and rigorous standards for diagnosis and classification, usually the Metropolitan Atlanta Congenital Defects Program (44). Such comparisons are inappropriate because data are collected in a very different way for pregnancy registries. Pregnancy registries are often also limited by the quality of available data regarding the birth defect outcomes and exposures, the diagnostic methods employed, the reliability and consistency of the outcome assessments, and the length of follow-up. These limitations can be overcome if appropriate control groups are available and if the exposure and outcome data are collected in a rigorous manner. If these conditions are met, pregnancy registries can be used to ascertain patients for high-quality-exposure cohort studies (45).

**36.2.2.5 Randomized Controlled Trials.** Randomized controlled trials are generally considered to be the optimal epidemiological approach to assessing the effects of a treatment, but they are rarely used in clinical teratology. It would be unethical to conduct a trial in humans to determine if a particular maternal treatment during pregnancy caused birth defects in the infants, so trial data on fetal outcomes is usually collected in the assessment of adverse effects of treatments for maternal conditions such as hypertension or premature labor. The treatment in these studies almost always occurs after the period of embryogenesis, so available clinical trial data provide little or no information on teratogenic risks associated with first-trimester exposure.

**36.2.2.6 Cohort Studies.** Cohort studies are used in clinical teratology research to compare the frequency of birth defects among children born to women treated with an agent during pregnancy to the frequency among children whose mothers were not so treated. There are two different ways that these studies are performed: as population-based cohorts of all newborns (or all pregnancies) and as exposure cohort studies through teratogen information services.

Population-based cohort studies can provide information on many different outcomes, including ones that do not become apparent until later in childhood, if appropriate follow-up data are available. However, population-based cohort studies must be very large to be useful for clinical teratology studies because both the exposures and the outcomes of interest are infrequent. As a consequence, population-based cohort studies are very expensive to perform, and only a few have been done to assess teratogenic risks.

Exposure cohort studies provide a more practical approach because data only need to be collected on the outcomes of pregnancies in women who were exposed

to a particular agent and the outcomes of appropriate control pregnancies. Women with the exposures of interest often call a teratogen information service for counseling, so subjects can be ascertained for exposure cohort studies in the course of regular service provision. This strength is also a limitation because studies performed through teratogen information services are not representative of the population as a whole but only of women who call these services.

Both population-based and exposure cohort studies can be used to estimate relative risk and statistical significance of associations that are observed between maternal teratogenic exposures and birth defects in the children. Either type of study may be subject to serious biases and confounding if not well designed and if the effects of covariates are not appropriately considered. Both depend greatly on the quality of the information regarding exposures as well as on the quality of the outcome data. Insufficient statistical power is frequently a concern with cohort studies, especially if the exposures, the outcomes being assessed, or both are rare.

**36.2.2.7 Case-Control Studies.** Case-control studies are used in clinical teratology research to compare the frequency of a maternal exposure, such as treatment with a particular drug, during pregnancy among children with or without birth defects. Case-control studies are often population based, an important factor in avoiding many kinds of ascertainment bias. Because case-control studies focus on women who have given birth to a baby with birth defects, case-control studies are usually far more statistically powerful than population-based cohort studies of an equivalent size. A major limitation of case-control studies is that they only provide information regarding the outcome or outcomes selected for study, so that an association with birth defects of an unanticipated kind, such as a previously unrecognized pattern of minor anomalies, cannot be identified. In this regard, case definition and the system used to classify birth defects into the group(s) chosen for inclusion in the study may be of particular importance, and selection of a group of anomalies that are thought to have similar pathogenesis—for example, vascular disruption—may be more relevant than conventional anatomic classifications.

Case-control studies can be used to estimate the odds ratio and statistical significance of an association observed between birth defects in children and maternal teratogenic exposures. Like cohort studies, case-control studies depend on the quality of both the outcome data (e.g. case ascertainment) and the exposure data (e.g. exposure characterization and timing) and may be subject to serious biases and confounding. Another frequent concern with large population-based case-control studies is that many case groups involving different kinds of birth defects may be analyzed for associations with several different maternal exposures simultaneously, creating a “multiple comparisons” problem that may not be resolvable without additional investigations.

Sample size and consequent statistical power are, of course, important considerations in any epidemiological study, but the very high power of the case-control design when used with birth defect groups that are rare in the general population may raise a different issue: identification of a statistically significant risk that is real but clinically irrelevant, or nearly so. An exposure that doubles the risk of, say, sphenomelia to 2 of 100,000 from 1 of 100,000 in unexposed pregnancies but does not affect the risk of any other congenital anomaly might be of great interest in terms of pathogenesis but would be of little clinical consequence to an individual pregnant woman who seeks counseling.

**36.2.2.8 Record Linkage Studies.** Record linkage studies provide a means of performing cohort studies or case-control studies or both in a very cost-effective manner. Record linkage studies are done by connecting information on exposures during pregnancy in the mother to information on birth defect outcomes in the infant through existing electronic medical records or administrative databases. These data systems may be very large, providing excellent sample sizes. A major limitation of most record linkage studies is that the exposure and outcome data are collected for other purposes and may be of less-than-ideal quality for epidemiological research. In addition, information on important covariates is usually limited, so these factors cannot be managed in the statistical analysis.

**36.2.2.9 Ecological Studies.** Ecological studies differ from all other epidemiological studies used for clinical teratology research in that the unit of analysis is groups of people rather than individuals. Information on exposure and outcomes is collected on the group as a whole, and exposure metrics for the group, which generally includes men, nonpregnant women, and children, as well as pregnant women, are used to estimate the level of exposure during pregnancy among the mothers of children with birth defects. An advantage of ecological studies is that they can usually be performed with data that are collected for other purposes, making them much less expensive than population-based cohort or case-control studies.

Ecological studies are usually done to investigate the effects of environmental or occupational exposures to toxic chemicals or radiation. Statistical tests for association are performed between a summary measure of exposure (e.g. average concentration of a particular chemical in drinking water) and a summary measure of disease (e.g. the frequency of miscarriage) in a group. Associations observed in ecological studies must be interpreted with great caution because they are subject to the “ecological fallacy”: attributing correlations observed in groups to individual members of those groups. Because the analyses in ecological studies are performed on populations rather than individuals, consideration of confounding factors is problematic at best and often impossible.

**36.2.2.10 Data Synthesis.** Meta-analysis provides a systematic approach to identifying, evaluating, synthesizing, and combining the results of epidemiological studies. Meta-analysis is useful because it may permit quantitative conclusions to emerge from the joint assessment of several studies, which cannot be drawn from the analysis of any individual study. Meta-analysis also provides a way to assess the effects of biases and the limitations of the individual studies. However, a conclusion reached by meta-analysis is no better than the original studies on which it is based and is also subject to limitations inherent in the joint analysis. Of particular concern is combining studies that use fundamentally different definitions of exposure or outcome. Such differences are often encountered, and ignoring them may confound rather than inform interpretation of the available data.

Publication bias—the greater likelihood for studies that show an effect, especially a large effect, to be published in comparison to those that do not show a significant effect—has been found to occur in clinical teratology studies (3,46). This “file drawer problem” can be assessed in a meta-analysis if many studies are available but may not be detectable if there are only a few published studies.

An alternative way of interpreting multiple clinical teratology studies available on a particular exposure is through expert consensus. Expert consensus is a qualitative, rather than rigorously quantitative, approach that can provide a summary assessment of studies of widely varying types, sizes, and quality, including nonepidemiological studies such as clinical series. The quality and value of an expert consensus depends on the thoroughness and rigor of the assessment and, quite critically, on who is making it.

Determining whether an exposure is teratogenic in humans requires careful assessment of all relevant available information, and especially data obtained directly by study of the outcomes of human pregnancies. A statistically significant association in one or more epidemiological studies is not sufficient to establish causality without other evidence to support such a conclusion. It is critically important that observed associations make biological sense: an association between the occurrence of birth defects in a child and an exposure during pregnancy in the mother that is not biologically plausible is almost certainly not indicative of a teratogenic effect. A chemical exposure cannot be teratogenic unless it is systemically absorbed by the mother and it or its metabolic products reach susceptible sites in the embryo or placenta. Exposures that produce congenital anomalies do so only during times in which the involved structures in the embryo or fetus exhibit appropriate sensitivity. In most cases, exposure to a greater quantity of the agent can be expected to increase the likelihood of abnormalities. The existence of a reasonable pathogenic mechanism for the observed effect in animal or in vitro experiments may provide further support for a causal inference.

## 36.3 RECOGNIZED TERATOGENIC EXPOSURES

Teratogenic exposures may be conveniently grouped into four major categories on the basis of the kind of agent involved: infectious agents, physical agents, drug and chemical agents, and maternal metabolic factors. Because current information on specific agents is incomplete, in this chapter we present only general summaries for several of the more important teratogenic exposures in each group. The reader is cautioned that considerable disagreement still exists over the role of many of these exposures in the production of human birth defects. Thus, the following discussion should be used as a guide and standard clinical teratology resources and the current literature should be consulted for a more thorough consideration of any particular agent. One should not use the information included in this chapter for counseling pregnant patients regarding the teratogenic potential of particular exposures without consulting up-to-date information resources such as *TERIS* (<http://depts.washington.edu/~terisweb/teris/index.html>) or *REPROTOX* (<http://www.reprottox.org/Default.aspx>) that are designed for this purpose.

### 36.3.1 Infectious Agents

For many years, clinicians have been aware of infectious agents that can attack the fetus in utero. Recognized effects on the fetus include death, intrauterine growth retardation, congenital defects, and intellectual disability. The pathogenesis of these abnormalities can generally be ascribed to direct fetal infection, which may be associated with inflammation of fetal tissues and cellular death. Many, if not all, of these defects represent the “disruption” pathogenetic category (see Chapter 35).

Certain signs and symptoms characterize prenatal infections of the fetus and can therefore be used as indicators of a potential infectious etiology for a child’s congenital abnormalities. Direct invasion of the nervous system may result in microcephaly, often associated with cerebral calcifications, intellectual disability, disorders of movement and muscle tone (sometimes mischaracterized as “cerebral palsy,” see Chapter 37), seizures, and central auditory and visual deficits. As the eye represents a direct developmental extension of the central nervous system, it is not surprising to find such defects as chorioretinitis, cataracts, and microphthalmia. Furthermore, as the central nervous system controls limb movement, contractures and other fetal positional limb deformations are sometimes encountered in cases in which severe central nervous system damage has occurred. However, major intercalary limb reduction malformations, polydactyly, and syndactyly are not typically associated with teratogenic infections. Other general abnormalities associated with prenatal infections include prematurity, low birth weight for gestational age, and failure to thrive. Affected

infants may exhibit evidence of widespread sepsis such as pneumonitis, hepatitis with hepatosplenomegaly and jaundice, and bleeding disorders manifested by petechiae and purpura. Chronic skin rashes and certain categories of congenital heart disease, such as stenotic vascular anomalies and defects due to disturbed blood flow, may also be associated with congenital infections (47).

Ophthalmologic examination and imaging studies in the infant may reveal additional signs of congenital infection. Serological studies in the mother and infant may be helpful, but “TORCH” screening alone is usually insufficient in newborns in whom a congenital infection is suspected because not all transplacental infections are included in this test and its sensitivity and specificity are limited. Specialized serological studies, culture of the organism, PCR for the organism’s DNA, or other more specific approaches are usually necessary to diagnose a congenital infection with certainty in an affected infant (47).

#### 36.3.1.1 Viruses.

**36.3.1.1.1 Rubella.** Abnormalities associated with prenatal infection with rubella vary substantially in frequency, severity, and type according to the month of gestation in which the infection occurred (48,49). From 40–85% of infants born to women with serologically proven rubella infection during the first trimester of pregnancy exhibit associated clinical abnormalities in early infancy. The birth of severely affected children drops off rapidly with maternal rubella infection after the first trimester of pregnancy, but later-appearing manifestations, such as hearing loss, delayed intellectual development, and diabetes, may be encountered in offspring of women infected later in pregnancy.

Rubella infection of the embryo may lead to miscarriage. Infants who are born after first-trimester rubella virus infection may display a wide variety of birth defects and health problems, including intrauterine growth retardation, subsequent failure to thrive, and congenital anomalies (Figure 36-1). Ocular defects such as cataracts, pigmentary retinopathy, microphthalmia, and glaucoma are often present. Various cardiovascular anomalies, including patent ductus arteriosus, valvular and peripheral pulmonary arterial stenoses, atrial and ventricular septal defects, and possibly other vascular stenotic lesions and tetralogy of Fallot, may occur. Myocardial damage, presumably stemming from myocarditis, has also been observed. Central nervous system abnormalities may include microcephaly, intellectual disability, hypotonia, and convulsions. Signs of acute meningoencephalitis or progressive panencephalitis may occur, and sensorineural deafness or other sensory or functional disturbances are often present. Signs and symptoms of widespread systemic infection are common and may include hepatosplenomegaly, jaundice, thrombocytopenia, anemia, irregularities of ossification of the long bones, and delayed ossification of the calvarium. Affected children may also exhibit pneumonitis,



**FIGURE 36-1** A child with rubella embryopathy. Note the “blueberry muffin” petechial rash. (Photograph courtesy of Susan Reef, MD, National Congenital Rubella Syndrome Registry, Centers for Disease Control and Prevention.)

a chronic rubelliform rash, generalized adenopathy, chronic diarrhea, diabetes mellitus, or thyroid disease. A variety of immune defects, such as thymic hypoplasia and hypogammaglobulinemia, may occur, often in association with recurrent or persistent infections. It is particularly important to note that hearing and other neurologic deficits and endocrine disturbances that are not apparent in the neonatal period may develop after several months or years of age (48).

Intrauterine diagnosis of fetal rubella infection can be accomplished by immunologic or molecular methods in the second trimester of pregnancy, but these methods cannot distinguish fetuses that will have rubella embryopathy from those that will have asymptomatic infections at birth (50,51). Manifestations of rubella embryopathy such as cardiac defects or fetal growth retardation can sometimes be identified prenatally by detailed ultrasound examination.

Prevention of congenital rubella syndrome is possible through routine immunization of children with rubella vaccine (48). Although use of attenuated live rubella vaccines in pregnant women is contraindicated, inadvertent immunization of women with such vaccines early in pregnancy has not been associated with an increased risk to the fetus (52).

**36.3.1.1.2 Cytomegalovirus.** Congenital cytomegalovirus (CMV) infection has assumed a position of increasing importance in recent years as a recognized cause of perinatal morbidity and mortality. Indeed, in periods when rubella is not epidemic, cytomegalovirus may represent the most common congenital infectious cause of intellectual disability and other central nervous system disorders, including deafness (53).



Congenital CMV infection is common, but most infected infants are asymptomatic. Between 0.2% and 2.2% of newborn infants are congenitally infected with CMV, but only about 10% of these infants exhibit serious manifestations at birth (53,54). The risk of symptomatic involvement is highest in the children of women who had a primary CMV infection during the first 6 months of gestation. Previous maternal infection or the presence of antibodies in the mother's blood does not prevent fetal infection, although such antibodies appear to reduce the risk of symptomatic disease.

The classical picture of severe congenital CMV infection includes central nervous system manifestations such as microcephaly, typically associated with diffuse periventricular calcifications reflecting extensive encephalitis (53,55). Less frequently, hydrocephalus may develop secondary to obstruction of the flow of cerebrospinal fluid. These central nervous system abnormalities are associated with functional disturbances, including intellectual disability, spasticity, hypotonia, seizures, and strabismus. Ocular involvement is common with chorioretinitis, optic atrophy, microphthalmia, cataracts, retinal necrosis, calcifications, and anomalies of the anterior chamber and optic disk, all of which may produce severe visual impairment. Hepatitis with resultant hepatosplenomegaly and jaundice may occur, and bone marrow disturbances may result in thrombocytopenia, with a generalized petechial rash or hemolytic anemia. Non-CNS malformations do not appear to be unusually frequent among infants with congenital CMV infections.

Although most infants infected in utero with CMV do not display clinical symptoms in the neonatal period, 10–15% of these children develop neurodevelopmental handicaps later in life as a result of their infection. Sensorineural hearing loss is most common. Other manifestations may include intellectual disability, movement and coordination disorders, behavioral disturbances, and chorioretinitis (53,56).

Detailed ultrasound examination and fetal magnetic resonance imaging (MRI) can be used for prenatal diagnosis of fetal ventriculomegaly, cerebral calcification, and some other serious manifestations of fetal CMV infection in the second or third trimester of pregnancy (57–59), although neurological dysfunction may occur in infants with congenital CMV infection even if the prenatal imaging studies are normal. Invasive testing can be used to demonstrate fetal infection but does not distinguish symptomatic from asymptomatic involvement of the fetus. Invasive testing is most informative when there is evidence of fetal disease on ultrasound examination.

Vaccination of seronegative women may be useful in reducing the risk of fetal CMV infection associated with primary maternal infection during subsequent pregnancies (60). Treatment of pregnant women with primary CMV infections and documented amniotic fluid involvement with specific hyperimmune globulin may

reduce the frequency of symptomatic CMV disease in the children (61,62).

**36.3.1.1.3 Varicella-Zoster Virus.** Although varicella infections and zosteriform eruptions during pregnancy are relatively infrequent, a congenital varicella syndrome has been recognized since 1947 (63). The risk for the fetus to display significant teratogenic effects when the mother contracts varicella during pregnancy is small—2% or less, depending on the gestational age (64,65). The most susceptible period of pregnancy for such effects is between 13 and 20 weeks' gestation. The risk of fetal damage related to maternal zoster during pregnancy appears to be much lower than with maternal varicella (65–67).

Children infected with varicella-zoster virus in utero may display growth deficiency and microcephaly with cortical atrophy, often resulting in convulsions and intellectual disability. Other neurologic sequelae may include peripheral nerve palsies, muscle weakness, and paralysis or muscular atrophy. Secondary positional limb deformities may occur, and ocular involvement—microphthalmia, cataracts, and chorioretinitis—may be seen. Limb anomalies may include distal phalangeal hypoplasia or hypoplasia of an entire limb, possibly secondary to denervation. Cutaneous abnormalities—scars, vesicles, epidermal hypoplasia, and other bizarre craniofacial or limb anomalies, possibly secondary to in utero ulceration—may be present. Respiratory distress, pneumonitis, repeated infections, and hearing deficiency have occasionally been reported (66,68).

Some manifestations of varicella embryopathy can be detected prenatally by ultrasound or fetal MRI examination (68), but affected fetuses cannot always be identified. No reliable means has been established for prenatal diagnosis of varicella embryopathy by amniocentesis or chorionic villus sampling (CVS) (66,68).

Varicella infection of children or adults usually confers long-term immunity to chicken pox, although reactivation of a latent varicella-zoster virus infection may occur, producing zoster. Passive immunization with varicella-zoster immune globulin (VZIG) within 96 h of exposure is recommended for pregnant women who have been in close contact with someone with varicella or zoster and who are seronegative or have no history of having had chicken pox themselves (66–68). Passive immunization of a pregnant woman who has already developed clinical manifestations of chicken pox seems unlikely to prevent transmission of varicella-zoster virus to the fetus. There is no evidence that acyclovir or vancyclovir treatment of a pregnant woman who develops chicken pox affects the likelihood that she will transmit varicella virus to her fetus (67,68).

Administration of varicella vaccine after exposure to chicken pox does not prevent development of varicella and is contraindicated during pregnancy. Outcome data from a registry of pregnancies in which the mother unintentionally received live attenuated varicella virus vaccine

after conceiving show no indication of an adverse effect on the fetus (69), but the data are insufficient to rule out an effect similar in magnitude to that seen with natural maternal chicken pox during pregnancy. Secondary transmission of vaccine virus may occur.

**36.3.1.1.4 Human Immunodeficiency Virus (HIV; AIDS).** Rates of human immunodeficiency virus (HIV) transmission from mother to fetus range from 15–30% in studies from the United States and Europe in which maternal antiretroviral treatment was not given during pregnancy (70). Maternal antiretroviral treatment late in pregnancy substantially decreases the rate of vertical transmission of HIV (71–74). No evidence of a substantial teratogenic risk has been found in association with commonly prescribed antiretroviral treatments in pregnancy in a voluntary Antiviral Pregnancy Registry sponsored by pharmaceutical companies that manufacture these medications ([http://www.apregistry.com/forms/interim\\_report.pdf](http://www.apregistry.com/forms/interim_report.pdf)).

Congenital HIV infections usually follow one of two courses in untreated children (71,75,76). Most have a slow progression that resembles AIDS in adults, but 10–25% of congenitally infected infants become symptomatic in the first year of life, suffer rapid progression, and die very early. Affected infants may exhibit failure to thrive, interstitial pneumonia, recurrent bacterial and other infections, chronic diarrhea, and generalized lymphadenopathy. Growth retardation is common, and microcephaly, developmental delay, progressive encephalopathy, and other neurological abnormalities are often seen. Malformations do not appear to be unusually frequent among the children of HIV-infected women (71,77).

**36.3.1.1.5 Parvovirus B19.** Parvovirus infection causes fifth disease (erythema infectiosum) in children. Infections in adults may produce a rash or arthropathy but are often asymptomatic. Fetal infection with parvovirus B19 can cause severe anemia, hydrops, and death (78–81). Many infected fetuses that develop hydrops die, but the hydrops may spontaneously resolve. Less severely affected fetuses may have meconium peritonitis, isolated ascites, increased nuchal thickness, or pleural or pericardial effusions.

Transplacental parvovirus B19 infection occurs in 25–50% of cases of maternal primary infection during pregnancy, but in most instances, there is no apparent adverse effect on the fetus (78–80). The excess fetal loss attributable to parvovirus B19 infection during the first 20 weeks of gestation is estimated to be between 2% and 9% in various studies. The risk of fetal death is lower with maternal infection later in pregnancy.

Persistent congenital anemia has been observed in infants born after intrauterine parvovirus B19 infection (80). Myocarditis, myositis, arthrogryposis, and ocular and central nervous system anomalies have also been reported after documented intrauterine parvovirus B19 infection (78,80), but such observations are rare. In most studies, no measurable increase in the frequency of

malformations or neurological abnormalities was found among the infants of women who had parvovirus B19 infections during pregnancy (82,83).

Testing for specific IgM antibody in serum is the standard means of diagnosing parvovirus B19 infection and distinguishing primary from secondary infection in a pregnant woman (78,84,85). Prenatal diagnosis of affected fetuses is often possible by ultrasonography and detection of parvovirus B19 DNA in amniotic fluid (78,84,85). Intrauterine transfusion may be beneficial in some cases of fetal hydrops associated with parvovirus B19 infection (84,85).

**36.3.1.1.6 Other Possible Teratogenic Viral Infections.** Herpes simplex virus infections are very common, and once they occur, they are persistent and recurrent. Transplacental fetal infection with herpes simplex virus is, fortunately, rare, but the effects on the fetus can be devastating (86). They may include chorioretinitis, microcephaly, various disruptive structural lesions of the brain, growth retardation, and typical skin lesions. Disseminated infection with death in the neonatal period may occur; surviving children may be intellectually disabled. This severe herpes simplex embryopathy must be distinguished from neonatal herpes simplex infection acquired during or immediately after delivery, a much more frequent and better recognized entity (86).

Transplacental infection with lymphocytic choriomeningitis virus is an uncommon occurrence that is often asymptomatic in the mother. Fetal infection has been associated with abortion and with hydrocephalus and chorioretinitis in surviving infants (87).

Infections with West Nile virus, a mosquito-borne virus that can produce encephalitis in adults, have become common in North America. Transplacental transmission of the West Nile virus has been reported after maternal infection during pregnancy and may be associated with central nervous system disruption in the fetus (88,89).

Concerns have been raised about the possible teratogenic effects of several other viruses, including influenza virus, Epstein–Barr virus, measles virus, mumps virus, and various enteroviruses (66,87,90). Most of these viruses have been associated with miscarriage in epidemiological studies, but evidence that these agents infect the embryo or fetus and cause serious disruptive lesions is lacking or very limited and controversial. It can be anticipated that future studies will clarify these questions and identify other teratogenic viral infections.

**36.3.1.2 Bacteria.** Although a host of bacterial agents may infect the fetus antenatally, only syphilis is thought to have a significant teratogenic potential. Nevertheless, many intra-amniotic bacterial infections can have devastating fetal consequences. Furthermore, as discussed later, high sustained fever associated with such infections may itself cause damage. The clinical signs and symptoms, which could be used as indicators of a possible prenatal bacterial infection, are very similar to those for the various viral agents described previously.

Transplacental transmission of *Borrelia burgdorferi*, the spirochete that causes Lyme disease, has been documented in humans, but whether this causes miscarriage or teratogenic damage to the embryo or fetus is controversial (91–93). Maternal Lyme disease during pregnancy appears to be associated with very little, if any, increased risk of congenital anomalies in the infant, especially if the maternal illness is treated promptly with appropriate antibiotics.

**36.3.1.2.1 Syphilis.** Congenital syphilis is certainly the oldest if not the most venerable of the known prenatal teratogenic infections, having been recognized for more than 500 years. The clinical manifestations of prenatal syphilis are related to both the time of gestation in which the fetal infection occurs and the duration of the untreated infection in the mother before pregnancy (94). Although little direct information is available to confirm such a conclusion, it has been generally believed that infection of the fetus usually does not occur before the fourth month of pregnancy. The frequency of congenital syphilis in the child of a woman who has untreated primary or secondary syphilis during pregnancy is 40–50%. Many such infants are delivered prematurely, and many of the infants are stillborn or die in the perinatal period. Syphilitic infections later in pregnancy result in lower risks to the fetus, with approximately 70% of late syphilitic infections resulting in the birth of normal healthy infants and only 10% showing signs of congenital syphilis. Fetal growth retardation may occur, and a wide variety of congenital problems may result.

The manifestations in the fetus may be overlooked in the newborn infant, and structural congenital anomalies are not frequent. Signs of syphilis in the child have been grouped into those that present within the first 2 years of life (early congenital syphilis) and those that appear later. Infants with early congenital syphilis are often hydropic, have a relatively large placenta, and may display widespread evidence of hematogenous infection such as hepatosplenomegaly, jaundice, generalized lymphadenitis, anemia, thrombocytopenia, and leukemoid reactions. Rhinitis and various other cutaneous and mucosal rashes and lesions—including vesicular or bullous eruptions, maculopapular rashes that may desquamate, mucous patches, petechial lesions, edema, and condylomata lata—may occur in up to 60% of patients. Osteitis is frequent and may mimic an Erb's palsy or present as an irritable infant. Syphilitic nephrosis may appear during the second or third month of life, and other evidence of generalized infection such as bronchopneumonia, failure to thrive, or malabsorption may be encountered. Nervous system manifestations such as meningitis, cranial nerve palsies, intracerebral vascular lesions, and progressive hydrocephalus are frequent. Convulsions and hemiplegia may also occur. There may be a generalized chorioretinitis and uveitis, and optic atrophy, glaucoma, and chancres of the eyelid may be seen (94).

Later manifestations of congenital syphilis, commonly occurring beyond the age of 2 years, are also widespread. The most characteristic features are Hutchinson teeth (peg-shaped and notched permanent upper central incisors), mulberry molars, interstitial keratitis, and sensorineural deafness. Frontal bossing is also a frequent, but not specific, finding. Poor maxillary growth and saddle nose deformity are frequent craniofacial manifestations, and deep linear facial scars, particularly around body orifices (rhagades), are typical late cutaneous manifestations. Late neurological features include intellectual disability, hydrocephalus, convulsions, cranial nerve palsies, and optic atrophy (94).

Prompt treatment of mother and infant is extremely important in congenital syphilis, particularly if the infant is symptomatic. Penicillin is the drug of choice (95).

**36.3.1.3 Parasites.** Although several parasitic agents are known to cross the placenta and infect the fetus, only toxoplasmosis has been clearly shown to produce congenital anomalies. Maternal malarial infection during pregnancy has been associated with fetal growth retardation and premature delivery (96,97). Fetal infection is usually asymptomatic, although intrauterine or neonatal death may occur (96).

**36.3.1.3.1 Toxoplasmosis.** Congenital toxoplasmosis has been recognized for several decades. The major risk to the fetus arises from primary toxoplasmosis infections during pregnancy. The frequency and severity of fetal effects are strongly related to the stage of gestation in which the exposure occurs. The risk of transmission with primary maternal infection increases from about 1% around the time of conception to more than 90% near term. Most fetal infections do not cause permanent damage, but clinical manifestations of the disease are more likely and more severe with infections earlier in pregnancy. The highest risk for having a baby with severe manifestations is estimated to be 10–40% with maternal infection between 10 and 24 weeks of gestation (98–101). Many more infants, especially those whose mothers acquire *Toxoplasma* infection in the second or third trimester of pregnancy, have mild or subclinical involvement, but these infants may develop chorioretinitis or neurological abnormalities later in childhood if not treated after birth (98,99,101).

As with other prenatal infections, congenital toxoplasmosis includes a wide range of clinical manifestations (98,99,101). It is often overlooked in the newborn period because of the subtlety of its signs. Clinically apparent disease is commonly associated with central nervous system abnormalities. These may include microcephaly, hydrocephalus, intracranial calcification, intellectual disability, and seizures. In addition, chorioretinitis, microphthalmia, glaucoma, and cataracts are frequent. Other signs of generalized sepsis may occur with hepatitis, resulting in hepatomegaly and jaundice, and thrombocytopenia, resulting in anemia. Pneumonitis, diarrhea, skin rash, lymphadenopathy, and other

signs of generalized infection may be present. Even if the features are more subtle in the neonatal period, long-term follow-up has revealed a high percentage of intellectual disability, convulsions, spasticity, visual impairment, and deafness in children with serious toxoplasmosis infections. *Toxoplasma* infection during pregnancy can also cause miscarriage.

Diagnosis of primary maternal infection by *Toxoplasma* is usually based on serological studies. Prenatal diagnosis by amniocentesis can be used to demonstrate most, but not all, fetal infections in pregnant women with primary *Toxoplasma* infections (98,99,102). Ultrasound examination is useful in identifying hydrocephalus, hydrops, and other severe manifestations of fetal infection but is not a reliable means of determining whether transmission of *Toxoplasma* to the fetus has occurred. Prompt treatment of the infant is important when congenital toxoplasmosis is diagnosed at birth (98,99). Treatment of the mother is often recommended when maternal *Toxoplasma* infection is documented early in pregnancy (98–100,102). Screening of pregnant women remains controversial and is not generally recommended in North America (98,100,102). The only satisfactory preventive measure is avoidance of *Toxoplasma* infection during pregnancy by avoiding ingestion of infected foods and contact with the oocysts. Generally, this means avoiding handling or eating raw meat (especially red meat) and eggs and avoiding exposure to materials such as cat feces, which may contain the oocysts.

### 36.3.2 Physical Agents

A wide variety of physical agents are potentially teratogenic for the fetus. Among these, the most important are ionizing radiation, mechanical factors, and possibly heat. Although public concern continues, present evidence does not support any causal relationship between birth defects in humans and commonly encountered low-energy exposures to sound waves, ultrasound examination, MRI, microwaves, or computer terminals (103–106).

**36.3.2.1 Ionizing Radiation.** A considerable amount of attention has been directed to the possibility of adverse effects from high-energy radiation on fetal growth and development. In particular, episodes such as the Fukushima nuclear reactor accident have unrealistically heightened the concern over the role that high-energy radiation plays in human malformations. A careful examination of available information must address three related concerns: teratogenesis, mutagenesis, and carcinogenesis. Although most exposures to high-energy radiation during pregnancy are avoidable, some exposures in both medical facilities and in the workplace continue to occur. In some instances, valid medical indications exist for the deliberate exposure of a pregnant woman to ionizing radiation. In such cases, as with the use of any potentially teratogenic agent during pregnancy, risk/benefit comparisons must be carefully made and a decision

reached by the patient and her physician after careful discussion and consideration. When exposure of a pregnant woman to ionizing radiation is necessary, the dosage to the fetus should be minimized.

**36.3.2.1.1 Teratogenesis.** Concern over possible teratogenic effects of high-energy radiation comes both from studies with experimental animals and epidemiologic studies of the offspring of survivors of nuclear explosions at the end of World War II. These experiences suggest that very high doses of high-energy radiation (>200cGy) can produce prenatal-onset growth retardation, central nervous system damage, including microcephaly, and ocular defects (107,108). Other abnormalities produced by large doses of high-energy radiation appear to be exceedingly rare in humans, if they exist at all. Furthermore, both the rate and fractionation of the radiation dosage are extremely important considerations, as slow dosage rates or division of the dose over a period of days markedly reduces the effects of the total dosage. Thus, it would appear that under ordinary circumstances, only therapeutic levels of radiation delivered to the region of the developing embryo during the first months of pregnancy have a demonstrable risk of significant anomalies for the fetus (107,108).

Much lower doses of radiation, as might occur from gastrointestinal tract radiography, CT scanning, or other diagnostic studies are, of course, far more frequent in the population. Exposure of a pregnant woman to such low-radiation doses at any time during gestation produces an extremely low or negligible risk of malformations in the offspring (107,108). This is not to imply that radiographic exposures can be conducted with total impunity during pregnancy. Even an extremely low risk should be avoided unless there is an important benefit expected.

**36.3.2.1.2 Mutagenesis.** The mutagenic effect of high-energy radiation is well known. Present evidence suggests that there may be no threshold for this effect. Thus, even low-dose natural exposures to radiation contribute to the total population load of mutations. By the same token, radiation exposures even at diagnostic levels may have potential mutational implications for an exposed fetus (107,108). It is estimated that 1cGy of radiation to a particular gene locus in a particular cell produces a risk of mutation on the order of  $10^{-7}$ . This would suggest that a germ cell exposed to 1cGy of radiation may have on the order of 1 chance in 1000 to have undergone a mutation of some type. However, most such mutations are probably either without phenotypic effect or lethal, so the chance for fertilization of that cell to result in an individual with a genetic disorder is probably substantially less.

Ordinarily, the radiation-exposed fetus would itself not suffer from a genetic disorder produced in this manner. Rather, potential offspring of that fetus in subsequent generations might manifest the disorder. By implication then, gonadal irradiation at any time before conception may have some small risk of producing a



pathogenic mutation in the offspring. The load of such mutations presumably increases throughout a person's lifetime but in the aggregate is still very small in absolute terms under ordinary circumstances.

**36.3.2.1.3 Carcinogenesis.** Concern over the carcinogenic potential of prenatal radiation may be a slightly more realistic concern (109). However, the magnitude of this risk is again substantially lower than is ordinarily understood by the public and appears to be less than the risk associated with similar exposures in early childhood (108). Studies of leukemogenesis stemming from prenatal exposures to X-irradiation suggest that the relative risk from common low-dose exposures is no greater than 1.5 and may be substantially less. In other words, the absolute risk, at worst, may rise from 1 chance in 3000 to 1 chance in 2000 for such an exposed individual to develop leukemia.

**36.3.2.1.4 Summary.** Commonly occurring exposures of a pregnant woman to high-energy radiation through diagnostic X-ray examination or other similar low-dose exposures produce very little or no measurable risk to the child (107,108). Although such exposures should be avoided whenever possible as a matter of prudence, inadvertent exposures or exposures for valid medical indications should not be a major source of concern.

**36.3.2.2 Heat.** Possible sources of fetal hyperthermia include high fever of any cause and other factors that produce substantial elevation in the maternal body temperature, such as excessive use of a steam bath or hot tub. Studies in laboratory animals strongly suggest that hyperthermia may be teratogenic at critical stages of neural tube development (110,111). The situation is less clear for humans, but most studies are limited by poor documentation of the degree and duration of fever or other form of hyperthermia that occurred during pregnancy. In addition, fever usually accompanies serious infections, and such infections or their treatment may also have teratogenic potential in some instances. Nevertheless, an increased frequency of neural tube defects has been found in association with high fever during the period of neural tube closure in some studies (110,112). A number of anecdotal cases of neuromigrational errors, Moebius syndrome, and neurogenic arthrogryposis have also been reported in the offspring of women who had hyperthermic events during the first or second trimester of pregnancy (110). The risk associated with substantial hyperthermia during pregnancy has not been clearly defined and probably depends on the severity, duration, and gestational timing of the fever or other exposure. It would seem prudent to avoid extreme prolonged hyperthermic exposures whenever possible during pregnancy.

Serial detailed ultrasound examination is useful for prenatal diagnosis of some kinds of severe central nervous system damage following such episodes, and maternal serum  $\alpha$ -fetoprotein screening for neural tube defects is indicated if the exposure occurred between 4 and 6 weeks after the last normal menstrual period.

### 36.3.2.3 Mechanical Factors.

**36.3.2.3.1 Constraint.** Although the effects of fetal constraint on fetal growth have been recognized for many years, relatively little attention has been paid to the effects of in utero mechanical factors on fetal morphogenesis. Fetal growth or movement may be constrained by a variety of mechanical factors, including uterine malformations, large uterine myomas, oligohydramnios (whether of maternal or fetal origin), intra-amniotic fibrous bands, or multifetal gestation.

It is easy to understand how fetal constraint could result in *deformations*. Such anomalies are the result of mechanical forces that entrap and physically compress structures of the developing fetus (113). Included in this category are plagiocephaly and such positional limb deformities as clubfoot, dislocated hips, and perhaps arthrogryposis, particularly in predisposed fetuses. Such anomalies could theoretically be produced at any time during prenatal life and would seem particularly likely to occur if the degree of constraint is severe or if the fetus is predisposed because of neurologic impairment. All these fetal anomalies are etiologically heterogeneous, and similar defects may result from mechanisms other than constraint.

Recognition of factors that predispose to fetal constraint, such as uterine malformation, oligohydramnios, or multifetal gestation, should alert the physician to the possibility of adverse consequences to the fetus or the newborn. These anomalies are often effectively treated by taking advantage of the relatively normal growth potential of the "deformed" tissues through casting or other essentially mechanical forms of therapy.

By the same token, recognition of deformations in the newborn infant should alert the physician to the possibility of fetal constraint in utero. For instance, features of severe in utero compression are a common part of the so-called Potter sequence as a consequence of oligohydramnios, whether of fetal (renal hypoplasia) or non-fetal (premature rupture of the fetal membranes with amniotic fluid leakage) origin. The presence of deformations should alert the practitioner to the possibility of other disturbances of fetal growth that may commonly be associated, e.g. clubfoot and pulmonary hypoplasia with consequent respiratory distress resulting from oligohydramnios.

**36.3.2.3.2 Early Invasive Prenatal Diagnosis.** Evidence that the mechanical trauma associated with early CVS or amniocentesis may occasionally cause fetal anomalies is of particular concern to medical geneticists. CVS before 10–11 weeks' gestation, i.e. during or shortly after formation of the limbs in the embryo, has been associated with an increased risk of limb reduction defects and oromandibular-limb hypogenesis syndrome (114,115). The risk appears to be greater and the defects more severe with earlier procedures. It is unclear whether or not an increased risk for fetal limb defects exists with later CVS procedures; if so, the risk is very small

(115,116). There is also some evidence that cavernous hemangiomas, intestinal atresia, gastroschisis, and club-foot are more common than expected among children born to women who had CVS (117), but further study is needed to define these risks more clearly and to determine whether they also depend on the gestational age at which the procedure is done.

Two randomized controlled trials of early amniocentesis found a small but significantly increased frequency of talipes equinovarus among infants born to women who had undergone the procedure between 11 weeks and 12 weeks 6 days of gestation (118–120). The risk was much greater when amniotic fluid leakage occurred following early amniocentesis. Talipes equinovarus does not appear to be more frequent than expected among the children of women who undergo amniocentesis after 15 weeks' gestation.

### 36.3.3 Drug and Chemical Agents

Since the thalidomide disaster, increasing attention has been focused on the role of drug and chemical agents in the environment to which pregnant women are exposed. Unfortunately, there has been a proliferation of both therapeutic agents and environmental chemicals during the past five decades, and it has also become increasingly clear that self-medication by pregnant women using a variety of over-the-counter, herbal, and traditional medicines is a common practice. In addition, many pregnancies are unplanned, and inadvertent chemical or drug exposures are frequent. Finally, women may have medical conditions for which treatment is necessary regardless of pregnancy.

For all these reasons, health professionals who care for pregnant women often must deal with their concerns about possible teratogenic effects of drug and other chemical exposures. Far too little is known about the teratogenic potential of most drug and chemical exposures. Several major categories of agents are considered in this section: environmental chemicals, nonprescription drugs, and prescription drugs.

**36.3.3.1 Environmental Agents.** Recognition of the pollution of our environment by an ever-proliferating group of compounds has caused serious concern regarding the potential impact on the developing fetus. Relatively little is known about the teratogenic potential of many of these exposures in humans. Fortunately, however, efforts to limit exposures for the public at large and for occupationally exposed adults provide a measure of protection for the fetus as well. Nevertheless, organic mercurial compounds demonstrate the teratogenic potential that environmental exposures can have.

**36.3.3.1.1 Organic Mercury Compounds.** Ingestion by a pregnant woman of food that is heavily contaminated with methylmercury compounds can cause severe damage to the developing central nervous system of her fetus (121–123). Clusters of affected infants

have been observed after maternal consumption of methylmercury-contaminated fish in Minamata, Japan, and of grain treated with methylmercury fungicides in Iraq. The frequent outcome of such pregnancies is children with severe central nervous system damage and microcephaly, resulting in static encephalopathy that presents as “cerebral palsy.” Maternal neurotoxicity often occurs in these cases as well, but the fetus appears to be more sensitive than the adult to this effect. Malformations do not appear to be unusually frequent in affected infants.

Subtle effects on fetal central nervous system development of much lower maternal exposures to organic mercury compounds, as may occur when the mother eats large amounts of swordfish or shark, have been reported in some studies but not others (123). These studies remain controversial, but the United States Food and Drug Administration has advised pregnant women to eat fish with lower rather than relatively high mercury contents (<http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/FoodbornePathogensContaminants/Methylmercury/ucm115662.htm>).

**36.3.3.1.2 Other Environmental Chemicals.** Women who used cooking oil that was heavily contaminated with PCBs during pregnancy had infants with intra-uterine growth retardation and a transient dark-brown staining of the skin (“cola-colored babies”) (124–126). Developmental delay was also observed in these children. Subsequent studies of infants whose mothers consumed PCBs in much smaller amounts during pregnancy have produced inconsistent findings (127,128).

Many natural and man-made chemicals have been shown to function as “endocrine disrupters” in experimental systems, and some adverse reproductive effects in wildlife have been linked to the presence of these chemicals in the environment (129–133). Although similar adverse effects, including teratogenesis, have been suggested in humans, a measurable impact at the levels of exposure usually encountered seems unlikely (134).

There has been considerable concern about maternal exposures to other environmental contaminants, but there is no compelling evidence of teratogenic effects of such exposures in humans. Adequate studies are often difficult to perform because of uncertainty about the magnitude of individual exposures and the causal heterogeneity of birth defects. Avoiding toxic exposures during pregnancy as much as possible is certainly a prudent precaution.

**36.3.3.2 Recreational Drugs.** Among the host of drugs that individuals in our society habitually use and abuse, alcohol and tobacco clearly stand out as the most important public health problems for both adults and the developing embryo or fetus. Adverse effects on the offspring have also been associated with maternal abuse of cocaine and some other “recreational” drugs during pregnancy.

**36.3.3.2.1 Ethyl Alcohol.** Ethanol leads the list of abused drugs in the United States and many other parts of the world. Thus, it is perhaps surprising that recognition

of the adverse effects of this agent on fetal growth and development did not occur sooner. Historical reviews suggest that such a possibility was indeed recognized by at least some individuals for hundreds if not thousands of years (135,136). However, the studies of Smith and Jones (40,137) and others (138–140) during the 1970s led to general acceptance of the teratogenic potential of maternal alcohol abuse during pregnancy. Pathogenesis, treatment, and prevention of alcohol-related birth defects are now subjects of intensive investigation, and recognition of the fetal alcohol syndrome has resulted in important changes in public policy and social norms regarding drinking by pregnant women (136,141,142).

Prenatal exposure to ethanol can result in a wide spectrum of effects on the embryo and fetus. The frequency and severity of these anomalies appear to be dose related. They range from apparently unaffected children to severely affected individuals with the fetal alcohol syndrome. Severe fetal alcohol syndrome occurs among infants born to chronically alcoholic women, but there is considerable disagreement about the amount of ethanol necessary to cause milder degrees of fetal damage (143–147). Some evidence suggests that consumption of as little as two drinks per day or periodic binge drinking (e.g. five or more drinks on a single occasion) in early pregnancy may be associated with recognizable (though milder) abnormalities in a significant percentage of exposed newborns. Full clinically recognizable fetal alcohol syndrome occurs in about 6% of children of women who drink heavily during pregnancy, although the risk is much higher for alcoholic women who have already had an affected child. Less-severe alcohol-related birth defects and neurocognitive deficits occur in a much larger proportion of these children, but the estimated frequency varies widely in different studies. The effects are less severe among the children of alcoholic women who stop drinking early in pregnancy.

The fetal alcohol syndrome is characterized by a distinctive facial appearance, prenatal-onset growth deficiency, developmental delay and intellectual disability, and an increased frequency of major congenital anomalies (147–150). Children with this syndrome display a flat midface with narrow palpebral fissures, a low nasal bridge, short upturned nose, and long smooth philtrum with a narrow vermilion border of the upper lip. Presenting as small-for-gestational-age babies in the neonatal period, they continue to grow poorly and often are admitted to the hospital for evaluation of “failure to thrive.” They may be described as jittery or tremulous babies, a feature that often results in confusion with drug-withdrawal symptoms. However, these neurologic abnormalities persist, and, in addition to having developmental delay and intellectual disability, such children are often poorly coordinated, tremulous, and sometimes hyperactive in later life. A wide variety of congenital anomalies has been associated with this syndrome, including cleft palate, cardiac malformations (especially

atrial and ventricular septal defects), microphthalmia, joint anomalies, and a variety of dermal and skeletal abnormalities. Cranial MRI often shows abnormalities of neuronal migration, occasionally associated with microcephaly, hydrocephalus, absence of the corpus callosum, other midline anomalies, or cerebellar abnormalities (151,152). Maternal alcohol use during pregnancy has been associated with an increased risk of stillbirth in some studies (153,154).

Other studies have reported associations between acute myeloid leukemia and other malignant neoplasms in the offspring and maternal drinking during pregnancy, although these associations have not always been found (155,156). If an association between maternal drinking and development of malignancy in the children does exist, the risk is probably substantially less than 1%.

Epidemiologic studies suggest that prenatal damage from maternal alcohol abuse may be one of the most frequent recognizable causes of intellectual disability in the United States (157). Disabilities due to prenatal alcohol abuse are an important public health problem (157)—an observation that is particularly distressing because virtually all such exposures are avoidable. Pregnant women, or women who might become pregnant, should avoid drinking alcohol as much as possible—the safest amount being none at all.

**36.3.3.2 Tobacco Smoking.** Maternal smoking during pregnancy interferes with fetal growth (158,159). Birth weight, length, and head circumference tend to be decreased in exposed babies. This growth deficiency is dose related and reversible in early childhood, although obesity appears to be more frequent than expected among the offspring of women who smoked during pregnancy (160). Women who smoke heavily during pregnancy also have higher-than-expected rates of spontaneous abortion, late fetal death, neonatal death, and prematurity (161).

Slightly lower measured intelligence levels and increased frequencies of hyperactivity have been reported in these children (162,163). Maternal smoking during pregnancy has also been associated with a small increase in some birth defects, especially cleft lip and palate and cleft palate alone (164). The risk appears to be greater among a subset of infants who have genetic variants that predispose to orofacial clefting (165,166).

The common association between smoking and concomitant alcohol and other drug abuse raises concern about potential interactive effects between tobacco and other potentially teratogenic exposures. Pregnant women should be advised to avoid smoking. Smokers who are unable to stop should be advised to reduce their smoking as much as possible, as this appears to improve fetal outcome.

**36.3.3.2.3 Cocaine.** The extensive medical literature on the effects of maternal cocaine use in pregnancy is difficult to interpret (167,168). Most studies are bedeviled by confounding factors, deficiencies in design, and poor documentation of the frequency, timing, and

dosage of the mothers' use of cocaine, other illicit drugs, and alcohol. Moreover, there appears to be a systematic publication bias in favor of studies that show an association and against those that do not find an association between maternal cocaine use and untoward pregnancy outcomes (46,169).

Vascular disruptions occur with increased frequency in infants whose mothers abused cocaine during pregnancy, especially in the second or third trimester. Involvement of the central nervous system has been reported most often (170). Other congenital anomalies thought to result from vascular disruption—segmental intestinal atresia, gastroschisis, sirenomelia, limb-body wall complex, and limb reduction defects—have also been associated with maternal abuse of cocaine during pregnancy in some studies (170). Associations with other kinds of congenital anomalies have been reported as well, but the findings are inconsistent. Cocaine's vasoconstrictive and hypertensive actions probably account for the increased frequency of placental abruption observed among pregnant women who abuse this drug (171).

Prenatal growth retardation has consistently been noted among infants whose mothers abused cocaine during pregnancy, but the effect appears to be due at least in part to concomitant exposure to alcohol or cigarette smoking (144). By school age, the growth of these children is indistinguishable from controls in most studies (172).

Many studies have demonstrated a subtle pattern of neonatal behavioral abnormalities among infants born to women who abused cocaine during pregnancy (170). This pattern has been characterized as affecting “the four A's of infancy”: attention, arousal, affect, and action. Studies in older children have been inconsistent, and interpretation is confounded by other differences that distinguish mothers who abuse cocaine during pregnancy from other women (170,172,173).

**36.3.3.2.4 Toluene Abuse.** Maternal abuse of toluene by inhalation during pregnancy can produce a characteristic toluene embryopathy in the offspring (174–176). Affected children exhibit central nervous system dysfunction, developmental delay, attention deficit disorder, microcephaly, growth deficiency, short palpebral fissures, deep-set eyes, micrognathia, abnormal auricles, and small fingernails. The features resemble fetal alcohol syndrome. Toluene embryopathy is associated with maternal inhalation of toluene in acute doses that may be 10–100 times greater than the occupational limit, which is averaged over a workday. Adverse fetal effects are unlikely with maternal exposure to less than the occupational limit of toluene during pregnancy, but it is prudent to avoid such exposure if possible.

**36.3.3.2.5 Other Drugs of Abuse.** Although many conflicting claims have been made regarding the “recreational” use of drugs such as amphetamines, LSD, and other hallucinogens, there is little objective evidence that implicates maternal abuse of such drugs as a cause of

birth defects (177,178). Maternal drug abuse during pregnancy is associated with fetal growth retardation, increased perinatal mortality, and neonatal behavioral abnormalities, including drug withdrawal (177–179). Children of women who abuse drugs during pregnancy are clearly at increased risk for adverse outcomes, but it is difficult to determine whether these are effects of the drugs themselves or of social problems, infectious diseases, poor nutrition, or abuse of alcohol or cocaine, all of which often accompany other drug abuse. For many reasons, including potential hazards to the fetus associated with all these factors, pregnant women should not abuse drugs.

**36.3.3.2.6 Caffeine.** While caffeine is teratogenic in high doses in some species, no convincing evidence linking this substance to human congenital anomalies has emerged (180,181). Associations between maternal coffee drinking during pregnancy and miscarriage or poor fetal growth have been repeatedly observed in epidemiological studies, but these studies are often confounded by cigarette smoking and other factors (181,182). It is unlikely that moderate coffee drinking by pregnant women adversely influences fetal growth or the rate of miscarriage, but it is sensible for pregnant women to avoid excessive caffeine intake.

**36.3.3.3 Nonprescription Drugs.** Many over-the-counter preparations are widely used for the treatment of viral illnesses, allergies, headache, aches and pains, sleep disturbances, anxiety, and gastrointestinal discomfort. Little formal evaluation has been conducted of the potential reproductive toxicity of most over-the-counter drugs. In the absence of specific information, it seems prudent to avoid these medications whenever possible, particularly in the earliest part of pregnancy.

**36.3.3.3.1 Salicylates and Other Anti-inflammatories.** Although aspirin and other salicylate compounds are believed to be among the safest and most effective drugs in the marketplace, excessive use during pregnancy may be associated with an increased risk of fetal hemorrhages, and limited evidence suggests that chronic aspirin consumption may interfere with fetal growth (183,184). Malformations, in general, do not appear to be associated with the prenatal use of salicylates (184,185), but there is evidence that gastroschisis may be about three times more common among the infants of mothers who take aspirin early in pregnancy (184). No consistent evidence of an effect on intelligence has been identified (186,187).

Epidemiological studies of children whose mothers took acetaminophen during the first trimester of pregnancy have found no consistent association with birth defects (188,189).

The available data regarding maternal use of ibuprofen during pregnancy are more limited, but treatment during the first trimester seems unlikely to pose a major teratogenic risk (190). A small increase in certain kinds of cardiac malformations has been found in some studies



but not others. However, neonatal renal failure, oligohydramnios, and in utero closure of the ductus arteriosus have been associated with maternal ibuprofen treatment to arrest premature labor later in pregnancy (191).

**36.3.3.4 Prescription Drugs.** Half of all pregnant women take at least one prescription medication during the first trimester (192,193). Treatment with some prescription medications is potentially teratogenic when used at conventional therapeutic doses. Other drugs can be used safely during pregnancy, and in some instances, such treatment is highly beneficial to both the mother and the fetus. Unfortunately, however, available data are insufficient to determine whether maternal treatment with most prescription drugs during pregnancy poses a substantial teratogenic risk. Postmarketing studies of prescription drugs for potential teratogenicity need to be greatly expanded to permit more adequate counseling of pregnant women. In the absence of specific information strongly supporting the safety of treatment with a particular agent during pregnancy, prudence dictates that such treatment be avoided whenever possible. All women of reproductive age who are given prescription drugs should be counseled regarding the potential teratogenic effects, because many exposures occur when women become pregnant unintentionally or before they recognize that they are pregnant.

**36.3.3.4.1 Thalidomide.** The most dramatic epidemic of drug-induced birth defects ever recognized occurred in the early 1960s when thalidomide was sold in several countries, but not the United States, as an over-the-counter sedative. The dramatic events that followed are now a matter of historical record: over 10,000 babies were damaged by this drug between 1958 and 1963. The association between maternal thalidomide treatment and birth defects was independently noted by Widukind Lenz (1962) and William McBride (1961). Subsequent studies indicated that the susceptible period for the embryo was between 20 and 35 days after conception (17).

The thalidomide embryopathy includes a very unusual and characteristic pattern of congenital anomalies (42). The manifestations depend primarily on the stage of embryonic development at which the exposure occurred. Typical anomalies are phocomelia and other limb reduction malformations, anomalies of the external ear, ocular anomalies, and cardiovascular malformations ranging from septal defects to complex conotruncal defects. Involvement of the central nervous system and other organ systems may occur but is less common.

For many years after the recognition of thalidomide embryopathy, the drug was not marketed in most countries. However, the discovery of thalidomide's immunomodulatory action has led to its use in a variety of neoplasms and immunopathic disorders, including AIDS. Currently, it is being prescribed in the United States and other countries under strict controls designed to avoid use by pregnant women.

#### **36.3.3.4.2 Folic Acid Antagonists.**

**36.3.3.4.2.1 Trimethoprim and Other Weak Folic Acid Antagonists.** A 60–80% increase in the frequency of birth defects has been observed among the infants of women who were treated with trimethoprim, an antibiotic that acts as a weak dihydrofolate reductase inhibitor, during the first trimester of pregnancy (194). The risk among these infants appears to be greatest for neural tube defects, cardiovascular malformations, oral clefts, and urinary tract defects, which occur two to five times more often than expected (194–196). Similar associations have been found among infants whose mothers were treated early in pregnancy with other weak folic acid antagonists such as triamterene, sulfasalazine, and anticonvulsant agents (195,197). These risks are reduced if the mother also takes a folic acid supplement early in pregnancy (194,195,197).

**36.3.3.4.2.2 Aminopterin and Methotrexate.** An unusual and characteristic pattern of congenital anomalies has been reported in more than two dozen children whose mothers took aminopterin or methotrexate during pregnancy. In addition to its use as an antineoplastic agent, methotrexate is employed in the therapy of rheumatoid arthritis and other immunopathic diseases and as part of a medical regimen to induce abortion.

Children with aminopterin or methotrexate embryopathy have distinctive craniofacial anomalies with abnormal head shape and ocular hypertelorism, shallow orbits, mild midfacial hypoplasia, micrognathia, cleft palate, and facial asymmetry (198–200). Malformations of the auricles, skin tags, and numerous skeletal anomalies, especially vertebral segmentation abnormalities with anomalous ribs, abnormalities of ossification of sacral structures, ectrodactyly, syndactyly, longitudinal limb reduction malformations, and positional limb deformities are common. Central nervous system abnormalities, may occur, and developmental delay is the rule in childhood. However, affected adults may have normal or only mildly reduced intelligence. Women who are treated with methotrexate or aminopterin in pregnancy have increased rates of miscarriage, early fetal growth retardation, stillbirth, and neonatal death.

The risk of a teratogenic effect after first-trimester maternal treatment with methotrexate is probably dose related, and much lower doses of the drug are used to treat immunopathic diseases than neoplasia. Most babies born to women treated with low-dose methotrexate during pregnancy appear normal at birth (201), but typical folic acid embryopathy has been reported even with low-dose treatment (202,203). This may reflect a particular genetic susceptibility.

**36.3.3.4.3 Anticancer Agents.** As the therapeutic efficacy of antineoplastic agents is dependent on their ability to kill cells, particularly undifferentiated cells, treatment with any of these agents should be regarded as potentially teratogenic. The strong folic acid antagonists and several of the alkylating agents, in particular, have

caused concern (199,204). Maternal treatment early in pregnancy with various cancer chemotherapeutic agents has been anecdotally associated with a variety of fetal anomalies, including severe intrauterine growth retardation, microphthalmia, cleft palate, genitourinary anomalies, and limb reduction defects (199,204). The risk for such exposure to produce malformations has not been clearly defined, but the early stage of pregnancy appears to be a particularly hazardous time.

Treatment with high doses of most anticancer agents is teratogenic in laboratory animals (199). Unlike most kinds of drug therapy, cancer chemotherapy is often given at the maximum dose tolerated by the mother, i.e. the doses are near or within the range of maternal toxicity. Under such conditions, treatment may carry a substantial risk of teratogenic effects, especially when it occurs during the first trimester and with several drugs at once. However, apparently normal children have been born to women who underwent cancer chemotherapy during the first trimester or later in pregnancy (204–206), and the risk of teratogenic effects in a particular case depends on the agent or combination of agents, dose, route, and gestational timing in a complex way that is unique for almost every woman.

In general, the risk of miscarriage appears to be increased with many kinds of cancer chemotherapy during the first trimester of pregnancy, and the risks of premature delivery and fetal death, growth retardation, and myelosuppression may be increased with treatment later in pregnancy (205,206). Women who are undergoing cancer chemotherapy should be advised to avoid pregnancy during the period of treatment. Patients who require such treatment during pregnancy should receive appropriate counseling regarding the risk of congenital anomalies and other adverse effects in exposed offspring.

**36.3.3.4.3.1 Trastuzumab (Herceptin®).** Anhydramnios and oligohydramnios are frequent in pregnancies in which the mother was treated with trastuzumab during the second trimester (207,208). Oligohydramnios may resolve if the trastuzumab treatment is discontinued, but in some instances, renal failure may occur in the newborn. Little information is available on the effect of first-trimester maternal trastuzumab treatment on embryonic development.

**36.3.3.4.4 Warfarin Anticoagulants (Coumadin®).** A striking pattern of congenital anomalies, which is otherwise quite rare, has been reported repeatedly among children whose mothers were treated with coumarin derivatives during pregnancy (Figure 36-2) (209,210). Affected children typically have abnormal facial features with severe nasal hypoplasia, which may also affect the ethmoid complex and result in choanal atresia. Microcephaly is common, and optic atrophy has been repeatedly observed. Radiographic studies often reveal a lag in skeletal maturation with stippling of epiphyseal growth centers. Prenatal-onset growth deficiency with subsequent failure to thrive, developmental delay, intellectual



**FIGURE 36-2** Fetal warfarin syndrome, note severe nasal hypoplasia. (From Pauli et al. (360), with permission.)

disability, and other neurologic abnormalities are frequent features. Other serious congenital anomalies are less frequent.

The period of greatest concern for the production of fetal facial and skeletal anomalies is from 6 to 9 weeks after conception. Beyond 9 weeks of age, ocular defects and central nervous system malformations may be produced. Children with these abnormalities often have a poor outcome, although survivors who do not have intellectual disability usually do relatively well except for their craniofacial abnormalities. Children with similar defects whose mothers did not take coumarin derivatives during pregnancy have been described who have a genetic disorder of vitamin-K-dependent coagulation factors (211,212), suggesting that bleeding into developing tissues may be pathogenic in coumarin embryopathy.

The frequency of coumarin embryopathy among infants of women who are treated with warfarin throughout pregnancy has been estimated to be 3–6% (213,214). Miscarriage appears to occur more often than expected when the mother is treated with coumarin derivatives early in pregnancy. Stillbirth and fetal, placental, and neonatal hemorrhage are substantially more frequent when the mother is treated with coumarin anticoagulants late in pregnancy (213).

**36.3.3.4.5 Antibiotics and Other Anti-infective Agents.** Available studies have not revealed an increased risk to the fetus associated with prenatal treatment with most anti-infective agents, including

penicillins, sulfonamides, cephalosporins, or related agents (215,216). Exceptions to this generalization are treatment with trimethoprim, a folic acid antagonist discussed above, or with tetracyclines, aminoglycosides, high-dose quinine, or high-dose fluconazole. Most newer antibacterial and antiviral agents have not been adequately studied with regard to their teratogenic potential in human pregnancy.

**36.3.3.4.5.1 Tetracyclines.** Maternal treatment with tetracyclines during the second and third trimesters of pregnancy produces staining of the infant's primary teeth (217). The risk for malformations does not appear to be increased among the children of women who were treated with tetracyclines during pregnancy.

**36.3.3.4.5.2 Aminoglycosides.** Several cases of sensorineural deafness, sometimes with accompanying vestibular dysfunction, have been reported in children whose mothers were treated during pregnancy with streptomycin (218). Asymptomatic abnormalities of auditory or vestibular function have been observed in up to 10% of such children, but symptomatic disturbances of eighth cranial nerve function are much less common (219).

**36.3.3.4.5.3 Quinine and Related Antimalarials.** Maternal use of very high doses of quinine in an attempt to induce abortion have been associated with deafness and optic nerve abnormalities in the offspring in case reports and clinical series (220). A causal association seems possible because quinine treatment, especially in large doses, may cause auditory and visual damage in adults. Such abnormalities do not appear to be unusually common among the children of pregnant women who are given much lower doses of quinine to treat malaria or who take the usual prophylactic or therapeutic doses of chloroquine (221).

**36.3.3.4.5.4 Fluconazole.** A few children have been described with a very unusual pattern of congenital anomalies whose mothers were treated for coccidioidomycosis meningitis during the first trimester of pregnancy with daily high-dose fluconazole (222). The features in these infants include brachycephaly, abnormal calvarial development, cleft palate, arthrogryposis, and congenital heart disease. The pattern of anomalies resembles the Antley-Bixler syndrome, an autosomal recessive condition, but the occurrence of a similar rare pattern of congenital anomalies in children whose mothers received the same, very unusual, treatment during pregnancy suggests a causal relationship. A teratogenic effect is unlikely with the much lower doses of fluconazole used to treat vaginal candidiasis (223).

**36.3.3.4.6 Anticonvulsants.** Because seizures affect 0.5–1.0% of pregnant women, the potential teratogenicity of anticonvulsants has been a source of substantial concern for several decades. Although there was confusion in the past about the relative contribution of the underlying convulsive disorder and that of the medications used to treat it, a consensus has now emerged that treatment of pregnant women with a number of different

anticonvulsant medications increases the risk for fetal abnormalities two to three times that of the normal population. The risk is higher in women who require treatment with multiple anticonvulsants, especially combinations that include valproic acid, than in those who are adequately treated with a single medicine (224–226).

Various anticonvulsant agents are now being used for the treatment of bipolar disorder, neurogenic pain, and other conditions, but it is not known if the teratogenic potential of these drugs is different when they are used during pregnancy for indications other than control of seizures.

It seems prudent to suggest that all epileptic women be cautioned before beginning pregnancy about the potential adverse outcomes for their offspring. In some instances, a trial off drug therapy before conceiving may be warranted. In other cases, where it is deemed unsafe to take a woman off anticonvulsant therapy, she should be placed on the smallest number of agents and the lowest dosages compatible with adequate seizure control, and the potential hazards of anticonvulsant therapy to her offspring should be discussed in detail.

Maternal serum  $\alpha$ -fetoprotein measurement and detailed ultrasound examination are useful for prenatal diagnosis of fetal neural tube defects associated with maternal valproic acid or carbamazepine treatment during pregnancy (227). Some other serious malformations have also been diagnosed prenatally in fetuses of women treated with anticonvulsant medications during pregnancy, but the subtle dysmorphic syndromes and functional deficits that occur more frequently in these children cannot usually be identified before birth.

Many of the same major malformations and a similar pattern of minor anomalies, sometimes called the “fetal anticonvulsant syndrome,” have been reported among children of epileptic women treated with a variety of anticonvulsant medications (228). The discussion below emphasizes these similarities as well as some serious malformations that are only associated with maternal use of particular drugs during pregnancy.

**36.3.3.4.6.1 Trimethadione.** Trimethadione is the only oxazolidine anticonvulsant that is still used clinically. It is prescribed infrequently, usually for the treatment of petit mal epilepsy—an uncommon seizure disorder among women of reproductive age. There is little justification for use of this particularly toxic drug in pregnant women. Nevertheless, trimethadione is important because it was the first maternal anticonvulsant treatment found to produce a unique pattern of abnormalities of growth and development in infants exposed prenatally (229,230). Moreover, maternal trimethadione treatment during pregnancy, especially when used in combination with other drugs, appears to cause serious birth defects in an unusually large proportion of exposed pregnancies (231,232).

**36.3.3.4.6.2 Hydantoin Anticonvulsants.** The fetal hydantoin syndrome, a recurrent pattern of minor



anomalies that occurs in about 10% of infants born to epileptic women treated with phenytoin during pregnancy, has been recognized for more than 35 years (233). An additional 30% of children who are exposed during embryonic development show lesser degrees of alteration. The abnormalities found in children with fetal hydantoin syndrome include a characteristic facial appearance with midface hypoplasia, low nasal bridge, ocular hypertelorism, and an accentuated cupid's bow of the upper lip (Figure 36-3) (234,235). Prenatal-onset growth deficiency, including poor growth for weight, length, and head circumference, is occasionally seen. Increased frequencies of distal digital hypoplasia and of major malformations, particularly clefts of the lip and palate and cardiovascular anomalies, have been noted (235,236). In addition, small but significant reductions in cognitive function have been observed among the children of epileptic women treated with phenytoin during pregnancy (237). Genetic differences in maternal or fetal metabolism appear to be important risk factors for congenital anomalies among the children of epileptic women treated with phenytoin during pregnancy (11,238).

Children whose mothers were treated with phenytoin during pregnancy also have a higher-than-expected risk of developing neuroblastoma (239). Fortunately, neoplasia is rare in childhood, and such tumors are uncommon even in children whose mothers took phenytoin during pregnancy.

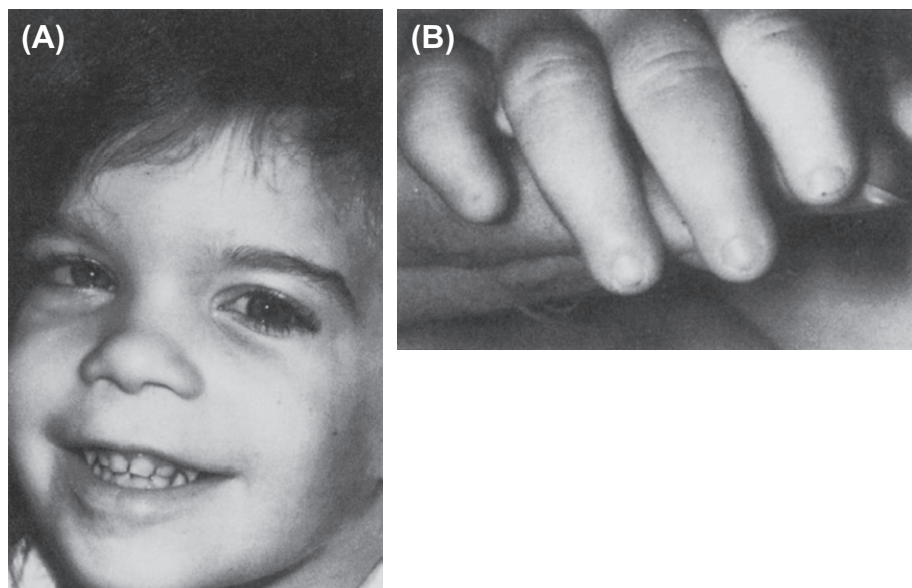
**36.3.3.4.6.3 Valproic Acid.** A characteristic pattern of craniofacial and other anomalies has been observed in up to half of children born to epileptic women treated with valproic acid during pregnancy (228,240,241). Features of this "fetal valproate syndrome" include

abnormalities of the calvaria with metopic ridging, trigonocephaly, narrow bifrontal diameter, relative deficiency of the outer orbital region, midfacial hypoplasia, short upturned nose with a broad flat bridge, and long flat philtrum with a thin vermilion border of the upper lip.

Major malformations occur in about 10% of infants whose mothers are treated with valproic acid for epilepsy during the first trimester of pregnancy; the rate is higher than that associated with most other anticonvulsants (225,240,242,243). The risk of spina bifida is about 2% among these children. The risk for anencephaly does not appear to be increased, suggesting that the pathogenetic mechanism may not be related to neural tube closure but rather to factors that affect canalization caudal to the posterior neuropore. Inherited variations in maternal drug metabolism may increase the susceptibility of certain pregnancies to these adverse effects (240,244).

**36.3.3.4.6.4 Carbamazepine.** A fetal anticonvulsant syndrome has also been observed among the children of epileptic women who were treated with carbamazepine during pregnancy (228). The features include minor craniofacial anomalies, fingernail hypoplasia, and delayed growth and development. The frequency of major malformations among the children of epileptic women treated with carbamazepine during pregnancy appears to be similar to that observed in children of women treated with other anticonvulsants (225,242,243). Spina bifida occurs in about 1% of children whose mothers took carbamazepine while they were pregnant.

**36.3.3.4.6.5 Other Anticonvulsant Agents.** Phenobarbital is not often used as an anticonvulsant in adults, but primidone, which is partially metabolized to phenobarbital, is sometimes used. In most studies, the frequency of congenital anomalies among children of



**FIGURE 36-3** Features of the fetal hydantoin syndrome. (A) Facial and (B) digital. Note the ocular hypertelorism, ptosis and strabismus, short nose with low bridge, and accentuated "cupid's bow" of lip. Distal phalangeal and nail hypoplasia are common digital anomalies. (From Hanson (234), with permission.)



epileptic women treated with these drugs during pregnancy is similar to that observed with other anticonvulsants and greater than that expected without such treatment (225,242,243,245). A distinctive pattern of minor dysmorphic features and poor growth, i.e. a “fetal anticonvulsant syndrome” occurs in some children whose mothers were treated for epilepsy with either phenobarbital or primidone during pregnancy (228,235).

Insufficient data are available to determine the risk of congenital anomalies in the children of pregnant women treated with the succinimide anticonvulsants, ethosuximide or methsuximide. Treatment of maternal epilepsy with lamotrigine during pregnancy appears to be associated with similar rates of congenital anomalies among the infants as treatment with older anticonvulsants (225,242,243). Information available on the teratogenic potential of other more recently developed anticonvulsants, including vigabatrin, topiramate, felbamate, gabapentin, clobazam, and clonazepam, is limited. It is not yet known if the risk of birth defects among the children of women who are treated with these newer drugs during pregnancy is higher, lower, or similar to that of children whose mothers took conventional anticonvulsants. Registries have been established in the United States (<http://www.mgh.harvard.edu/aed/>) and other countries to collect and disseminate information on the teratogenic potential of maternal treatment with anticonvulsant agents during pregnancy.

**36.3.3.4.7 Endocrine Agents.** A number of reports have suggested possible teratogenic effects of agents used for treatment of endocrine disorders. These drugs are often taken by women of reproductive age.

**36.3.3.4.7.1 Female Sex Hormones.** Agents with female sex-hormone-like activity, especially various synthetic progestins and estrogens, have received considerable attention because of their widespread use as contraceptive agents and for other medical purposes that could result in fetal exposures.

**36.3.3.4.7.1.1 Diethylstilbestrol.** Diethylstilbestrol (DES) was widely used in the 1950s as a treatment for threatened abortion and for estrogen replacement during pregnancy. The daughters of women who received such treatment during pregnancy have a greatly increased risk of gross structural anomalies of the uterus and vagina and of developing vaginal adenosis and clear cell adenocarcinoma of the vagina or cervix (246,247). At least one-third to one-half of women who were exposed in utero to diethylstilbestrol have gross or histological abnormalities of the genital tract, but the absolute risk of developing vaginal malignancy is fortunately quite small. Ectopic pregnancy, miscarriage, and premature delivery also occur with increased frequency among “DES daughters.” The sons of women who were treated with DES during pregnancy have higher-than-expected frequencies of epididymal cysts, hypoplastic testes, and cryptorchidism, but fertility and sexual function are usually not impaired (246).

**36.3.3.4.7.1.2 Other Estrogens and Progestins.** Other estrogens have not been clearly associated with similar risks. Early reports of reduced masculinization of male fetuses after maternal treatment with other estrogens during pregnancy have not been substantiated.

Maternal treatment during pregnancy with high doses of androgenic progestins is associated with an increased risk of masculinization of the external genitalia in female fetuses. The degree of masculinization depends on the time of treatment and is unlikely to occur after the 12th week of gestation. The magnitude of this risk is no more than 1% with high doses and is less with lower doses. Maternal treatment with high doses of norethindrone is particularly likely to produce such abnormalities (248). Despite reports associating maternal progestin use during pregnancy with various other malformations in the offspring, no consistent pattern of abnormalities has emerged, and most epidemiologic studies have failed to confirm such associations.

Combinations of estrogens and lower-dose progestins in the form of contraceptive agents have also been alleged to produce fetal anomalies when taken after conception. Studies claiming a relationship between maternal use of these agents and various congenital anomalies have been reported, but the results have generally not been reproducible (248,249). A woman who has inadvertently become pregnant while taking an oral contraceptive can be reassured that her use of birth control pills is very unlikely to have harmed the fetus.

#### **36.3.3.4.7.2 Other Endocrine-Active Agents.**

**36.3.3.4.7.2.1 Clomiphene.** Induction of ovulation with clomiphene has been associated with an increased risk of neural tube defects in some studies, but most investigations do not show such an association (250,251). The effects of treatment with this agent are often confounded with the effects of underlying maternal disorders that led to the need for therapy.

About 5–10% of pregnancies that result from ovulation induced by clomiphene treatment are multifetal (252,253), and these twin, triplet, or higher multiple pregnancies are at increased risk for premature delivery and positional limb deformations.

**36.3.3.4.7.2.2 Male Sex Hormone Agents.** Maternal treatment with androgens during pregnancy can cause masculinization of the external genitalia of female fetuses (248). The effect appears to be dose related. Similar masculinization of female fetuses may occur after maternal treatment with large doses of anabolic steroids such as danazol, which have androgenic and antiestrogenic activity (254). No clear evidence of other teratogenic effects of androgens has been reported.

**36.3.3.4.7.2.3 Corticosteroids.** Some epidemiological studies have found an association of maternal treatment with systemic corticosteroids early in pregnancy with orofacial clefts in the offspring (255–257). Even if this association is real and causal, the absolute risk for orofacial clefts among children of women who are

treated with systemic corticosteroids during the first trimester would be less than 1% (258). The risk for malformations of other kinds does not appear to be substantially increased in these children (259).

**36.3.3.4.7.2.4 Antithyroid Agents.** Maternal treatment during pregnancy with antithyroid agents such as radioactive iodine ( $^{131}\text{I}$ ), propylthiouracil, or methimazole is associated with an increased risk of hypothyroidism and consequent intellectual disability in the offspring (260). In addition, it appears that maternal treatment with methimazole or the related thioamide, carbimazole, during pregnancy can occasionally cause cutis aplasia of the scalp or, rarely, a methimazole embryopathy in the fetus (261,262). The features of this unusual pattern of anomalies include developmental delay, choanal atresia, esophageal atresia, absent or hypoplastic nipples, and scalp defects. Congenital goiter, hypothyroidism, or both may also occur among the children of women treated with methimazole or carbimazole during pregnancy (263).

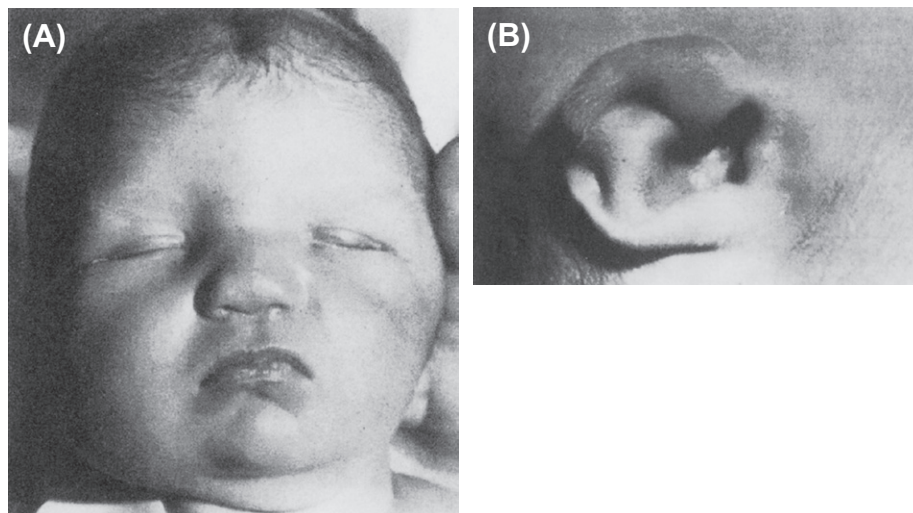
Iodides have the potential of producing neonatal goiter and hypothyroidism, particularly when taken by a pregnant woman after the first trimester (260). Fetal goiter can also be produced by maternal treatment with other medications, such as amiodarone, that contain large amounts of iodine (264).

**36.3.3.4.8 Retinoids and Vitamin A.** Preformed retinoids, including retinol, retinaldehyde, and retinoic acid, possess vitamin A activity directly. They are contained in substantial amounts in some foods, especially liver. Isotretinoin, etretinate, and acitretin are synthetic retinoid congeners that are used orally and topically to treat a variety of skin disorders. Absorption of the topical forms is usually quite limited.  $\beta$ -Carotene and related carotenoids, which are contained in many orange or dark green leafy vegetables, can be metabolized by the body to vitamin A.

Although information linking the ingestion of large amounts of vitamin A to birth defects has been available in laboratory animals since the 1950s, the relevance of these observations to human beings remained uncertain until a characteristic pattern of birth defects was recognized in children whose mothers had been treated with isotretinoin during pregnancy (43). A large body of studies subsequently demonstrated the teratogenic potential of oral therapy with isotretinoin or etretinate, and anecdotal evidence suggests that acitretin and preformed vitamin A itself in very high doses may have similar potential (265–267).

The characteristic retinoid embryopathy has been most clearly delineated in children of women who were treated with isotretinoin during the first trimester of pregnancy (43,267). Typical craniofacial anomalies include microcephaly, facial asymmetry with midfacial hypoplasia and facial nerve palsy (Figure 36-4a), microphthalmia, cleft palate, micrognathia, and microtia (Figure 36-4b) or anotia. Central nervous system abnormalities, such as hydrocephalus or posterior fossa cysts, and cardiovascular malformations, especially conotruncal defects, are common. Thymic hypoplasia and genitourinary anomalies, including hypoplastic kidneys and hydroureter, are also frequent. Children with isotretinoin embryopathy who survive often have intellectual deficiency (268).

Dosage and timing are major factors, the highest risk being with maternal treatment between 2 and 5 weeks after conception. However, it should be noted that both etretinate and retinol have half-lives of weeks to months, leading both to longer risk periods after discontinuation of use and the possibility of substantial bioaccumulation (269). The half-life of isotretinoin is much shorter—less than 1 day. Despite a program designed by the manufacturer to prevent use of isotretinoin in pregnancy, substantial numbers of fetal exposures continue to occur (270–272). Women who are pregnant or may become



**FIGURE 36-4** Fetal retinoid syndrome. (A) Dysmorphic facial features and (B) Microtia. ((A) From Lammer (361), with permission; (B) From Fernhoff and Lammer (362), with permission.)

pregnant should not be treated systemically with isotretinoin or related drugs.

Concern about the teratogenic potential of preformed vitamin A in doses as low as 10,000 IU/day has been raised (273) but appears to be unfounded (266,274,275). An exact threshold dose for the teratogenic risk associated with use of preformed vitamin A in pregnancy has not been determined but is likely to be greater than 30,000 IU/day. Nevertheless, pregnant women should not take more than the recommended dietary allowance of preformed vitamin A (266,276). Liver contains large amounts of retinol, and it has been recommended that pregnant women avoid eating excessive amounts of liver during pregnancy (277).

Ingestion of  $\beta$ -carotene, even in high doses, has not been associated with a teratogenic risk (266,278). Vitamin supplements containing  $\beta$ -carotene are, therefore, preferable to those containing preformed vitamin A for women of reproductive age.

**36.3.3.4.9 Lithium.** Children of women who are treated with lithium during pregnancy appear to have an increased risk of Ebstein's anomaly of the heart, although epidemiological studies indicate that this risk is likely to be small (27–29). Fetal echocardiography should be offered to women who are treated with lithium during the first trimester of pregnancy for prenatal diagnosis of fetal cardiac disease.

Treatment of severe depression in pregnant women and those of reproductive age presents a clinical dilemma. The small but serious teratogenic risk associated with lithium use may need to be balanced against a substantial risk to the mother's health if treatment is stopped and against possible maternal or fetal risks associated with alternative treatments (279,280).

**36.3.3.4.10 Selective Serotonin Reuptake Inhibitors.** Selective serotonin reuptake inhibitors (SSRIs) are very widely used as antidepressants. These drugs are also given to treat obsessive compulsive disorder, panic disorders, and other psychiatric illnesses. Several large epidemiological studies have found associations between maternal treatment with SSRIs, especially paroxetine, during early pregnancy and cardiac malformations or other birth defects in the infant, but other studies have not shown such associations (281–286). Although interpretation of these data remains controversial, it seems likely that the frequency of congenital anomalies among children of women who take an SSRI during the first trimester of pregnancy is increased by only a small amount overall. Transient behavioral alterations and other abnormalities of neonatal adaptation may occur in infants whose mothers were treated with an SSRI close to the time of delivery.

**36.3.3.4.11 Codeine and Other Opioid Analgesics.** A small increase in the frequency of congenital heart defects has been observed repeatedly in case-control studies of children whose mothers had been treated with codeine or another opioid analgesic early in pregnancy

(287–290). An increased frequency of spina bifida or cleft lip and palate has also been observed in some studies but not others (290–293). Even if all observed associations are causal, the overall increase in the risk of congenital anomalies among infants of women who took opioid analgesics early in pregnancy is probably less than 1%.

Narcotic withdrawal symptoms may occur in newborn infants whose mothers were treated chronically with opioid analgesics late in pregnancy (294).

**36.3.3.4.12 Misoprostol.** Misoprostol is a prostaglandin analog that is used in the prevention and treatment of peptic ulcer disease. The drug is also used in combination with mifepristone (RU-486), a progesterone blocker, to induce abortion. Associations have been observed between unsuccessful maternal use of misoprostol to induce abortion in the first trimester of pregnancy and the occurrence of Moebius syndrome, terminal transverse limb reduction defects, arthrogryposis, and brain defects such as holoprosencephaly and hydrocephalus in the offspring (295–297). Vascular disruption is a biologically plausible explanation for these associations (298). The risks of severe anomalies among fetuses surviving attempted induced abortion with mifepristone and misoprostol should be explained to women contemplating such treatment. These risks contribute to the recommendation that pregnancies that continue in spite of attempted medical abortion be surgically terminated.

Oral doses of misoprostol similar to those used for pregnancy termination are taken chronically for prophylaxis or treatment of peptic ulcer disease. Pharmacological induction of abortion and the teratogenic risks would be expected to be at least as great when the drug is taken early in pregnancy for peptic ulcer disease as when it is taken to induce abortion, but little information is available on the outcome of such pregnancies.

**36.3.3.4.13 Mycophenolate Mofetil.** An unusual pattern of malformations has been repeatedly observed among children whose mothers were treated with mycophenolate mofetil early in pregnancy (38,299–301). Frequent features include microtia or anotia, auditory canal atresia, conductive deafness, cleft lip, cleft palate, dysmorphic facial features of the face, congenital heart defects, and short fifth finger. The natural history of this embryopathy has not yet been delineated.

**36.3.3.4.14 Penicillamine.** Several infants whose mothers were treated with penicillamine during pregnancy have been reported to have an unusual syndrome resembling cutis laxa (see Chapter 155) (302,303). Although such connective tissue abnormalities are uncommon among the children of women who take penicillamine during pregnancy, a causal relationship seems likely. Similar skin abnormalities occur as a rare complication of chronic penicillamine therapy in adults, and such treatment is known to affect cross-linking of elastin and collagen.



### 36.3.3.4.15 *Inhibitors of the Renin-Angiotensin System.*

**36.3.3.4.15.1 Angiotensin-Converting Enzyme (ACE) Inhibitors.** Neonatal renal failure and hypotension as well as fetal anuria resulting in oligohydramnios, joint contractures, pulmonary hypoplasia, and death have been observed repeatedly after maternal treatment with captopril, enalapril, or lisinopril during pregnancy (304). Several cases of neonatal hypocalvaria and other skeletal anomalies have also been noted after maternal treatment during pregnancy with one of these ACE inhibitors. Accurate risk estimates are not available, but the effects appear to result from hypersensitivity of the fetus to the pharmacologic action of ACE inhibitors during the second half of pregnancy (304). There is some evidence suggesting that maternal ACE inhibitor therapy early in pregnancy increases the risk of cardiovascular and central nervous system malformations among infants (305), but further study is necessary to determine whether the observed associations are causal. Women who conceive while taking an ACE inhibitor should be switched during the first trimester to an antihypertensive agent of a different class if treatment continues to be necessary.

**36.3.3.4.15.2 Angiotensin II Receptor Inhibitors.** Losartan, candesartan, valsartan, and other antihypertensive drugs of the “sartan” class block the activity of the renin-angiotensin system by a mechanism different from ACE inhibitors. Several case reports describe oligohydramnios, fetal growth retardation, pulmonary hypoplasia, limb contractures, and calvarial hypoplasia in various combinations in association with maternal sartan treatment during the second or third trimester of pregnancy (304,306). Stillbirth or neonatal death is frequent in these reports, and surviving infants may exhibit renal damage. The fetal abnormalities, which are strikingly similar to those produced by maternal treatment with ACE inhibitors during pregnancy, are probably related to inhibition of the fetal renin-angiotensin system.

**36.3.3.4.16 Indomethacin and Other Prostaglandin Synthesis Inhibitors.** Maternal treatment with indomethacin late in pregnancy has been associated with decreased fetal urinary output and oligohydramnios, as well as with premature closure of the ductus arteriosus in the fetus and consequent persistent pulmonary hypertension in the infant (307). These effects appear to result from transplacental pharmacological activity of this prostaglandin synthesis inhibitor. Maternal treatment late in pregnancy with other inhibitors of prostaglandin synthesis, such as ibuprofen and ketoprofen, may have similar action on the fetal ductus arteriosus and kidneys (308). There is no indication that maternal treatment with these drugs early in pregnancy poses a substantial teratogenic risk.

**36.3.3.4.17 Methylene Blue.** Up to 20% of twins born after genetic amniocentesis in which methylene blue was used as a marker develop small bowel atresia (21). The affected twin was the one whose amniotic sac

was injected with the dye, and the risk appears to be greater with higher doses. The risk of fetal death was also increased after injection of methylene blue during genetic amniocentesis (309). It is important to note that these risks are only associated with intra-amniotic instillation of methylene blue, not with oral or topical use by the mother during pregnancy.

**36.3.3.4.18 Bendectin® (Diclectin®).** A fixed combination of pyridoxine (vitamin B<sub>6</sub>) and doxylamine (an antihistamine), marketed in the past as Bendectin® and more recently as Diclectin®, is used in the treatment of nausea and vomiting that are present during pregnancy. This medication was removed from the market by its manufacturer and has been unavailable for many years in the United States because of excessive litigation alleging that Bendectin® caused a variety of serious birth defects in children whose mothers took it early in pregnancy. However, extensive epidemiological studies provide no indication that maternal use of this medication during pregnancy increases the risk of congenital anomalies above the rate expected in the general population (310).

**36.3.3.5 Maternal Metabolic Factors.** Although not environmental in the strictest sense, factors that affect maternal metabolism may alter the intrauterine environment. Thus, an additional category of potential teratogenic effects is maternal metabolic factors. Of particular importance in this category is maternal diabetes mellitus, but maternal phenylketonuria (PKU) and inadequate folic acid intake are also of concern.

**36.3.3.5.1 Folic Acid Intake.** The recognition that the risk of many malformations, including neural tube defects, can be substantially reduced by maternal folic acid supplementation has provided an unparalleled opportunity for the prevention of birth defects. Dietary supplementation with folic acid before and after conception may reduce the overall risk of birth defects by 20% or more (311). Not all malformations are affected equally—neural tube defects may be reduced by as much as 85% (311–313), but the reductions in orofacial clefts, cardiac malformations, and renal anomalies are smaller (311,314). The mechanism of prevention is not understood (315,316), but certain genetic variants (e.g. in folate metabolism) may put some patients at greater risk than others for birth defects when folic acid supplementation is not provided (316,317).

All women of childbearing age should take 0.4 mg of supplemental folic acid daily (318). Larger doses of folic acid are sometimes recommended for women who are at increased risk of having a child with a neural tube defect. As compliance with regimens requiring daily vitamin supplements is problematical, grain products are fortified with folic acid in some countries (319).

**36.3.3.5.2 Diabetes Mellitus.** The principal maternal metabolic disorder that raises concern for the developing fetus is type 1 diabetes mellitus. The risk of congenital anomalies in infants of women who have insulin-dependent diabetes is two to three times greater



than that in the general population (320,321). A variety of congenital anomalies is associated with maternal diabetes, but congenital heart defects and neural tube defects are most common. Preconceptional and early postconceptional folic acid supplementation is, therefore, especially important for women with type 1 diabetes, and pregnant diabetic women should be offered prenatal diagnosis by detailed ultrasound examination, fetal echocardiography, and serum  $\alpha$ -fetoprotein measurement.

Caudal regression, focal femoral hypoplasia, and holoprosencephaly are also more frequent than expected among children of women with type 1 diabetes mellitus, but these malformations are, fortunately, uncommon. Diabetic pregnancies are at increased risk for spontaneous abortion, abnormal fetal growth, neonatal hypoglycemia, and various obstetrical complications (322,323). Risks for congenital anomalies and adverse neonatal outcomes can be minimized by very good control of maternal diabetes from the time of conception and throughout pregnancy (321–324).

Type 2 diabetes is occurring with increasing frequency in women of reproductive age, often in association with obesity. Although less well studied, infants of mothers with type 2 diabetes appear to have increased risks of similar kinds of malformations and other complications of pregnancy as infants of mothers with type 1 diabetes (324–327).

There is controversy over whether women with gestational diabetes also are at increased risk to have children with malformations. When maternal diabetes does not develop until after the first trimester of pregnancy, an effect on embryogenesis would seem unlikely. However, some women with gestational diabetes have unusually high blood glucose levels at the time of diagnosis and may actually have preexisting abnormalities of glucose metabolism. The infants of such women may be more likely to have malformations of the types seen in children of mothers with preexisting type 1 diabetes (328–330).

**36.3.3.5.3 Phenylketonuria.** About 75–90% of children of women with PKU who are not adequately treated during pregnancy are intellectually disabled and microcephalic (331,332). These children may also have prenatal growth retardation, congenital heart disease, and an appearance that is reminiscent of fetal alcohol syndrome. Children of women with PKU are usually heterozygous carriers and do not have PKU themselves. Children are damaged during gestation by exposure to very high levels of phenylalanine, phenylpyruvic acid, and other potentially toxic metabolites in the maternal blood (332).

Effective treatment of the mother beginning before conception and continuing throughout pregnancy reduces the risk substantially (332,333). It is extremely important to ensure that all females with PKU receive this information before and during their reproductive years. Unfortunately, adult women with undiagnosed PKU have occasionally been identified after they have become pregnant (334,335). A few of these women have

near-normal intelligence; others present with major psychotic disorders or have milder degrees of intellectual deficit than is commonly associated with classical PKU. Such women may come to attention only through the birth of an abnormal child. Thus, maternal screening for PKU subsequent to the birth of a child with the above-described abnormalities is one way of preventing the birth of additional affected children.

**36.3.3.5.4 Obesity.** Obesity is usually defined in relationship to height as the body mass index ( $\text{BMI} = \text{weight in kilograms}/(\text{height in meters})^2$ ). A BMI in the range of 25.0–29.9 is conventionally considered to indicate overweight; a BMI greater than 30.0, to indicate obesity. The overall frequency of major congenital anomalies was significantly correlated with the mother's prepregnancy BMI in the Collaborative Perinatal Project (336,337). An association of maternal obesity with congenital anomalies among the infants was also found in a record linkage study of 41,013 singleton pregnancies (338).

Several epidemiological studies have found that the risk of neural tube defects is almost doubled among infants of obese mothers (339). The results of studies of congenital heart defects, cleft palate, or cleft lip and palate among the infants of obese women are more heterogeneous, but most studies show a weak association (339). Associations of maternal obesity with hydrocephalus, anorectal atresia, diaphragmatic hernia, omphalocele, and hypospadias have also been reported, although these conditions are less well studied.

**36.3.3.6 Autoimmune and Isoimmune Disease.** Rh hemolytic disease, which results from transplacental transfer of maternal antibodies reactive against fetal red blood cells, has been recognized as a cause of hydrops fetalis for more than 70 years. Isoimmunization of an Rh<sup>-</sup> woman usually occurs during pregnancy by fetomaternal hemorrhage; amniocentesis or chorionic CVS may be a predisposing factor. Sensitization of the mother, and thus Rh hemolytic disease in the fetus in subsequent pregnancies, can largely be prevented by appropriate administration of Rh immunoglobulin (340,341) (see Chapter 74).

Fetal and neonatal disease may also be caused by transplacental transmission of various other maternal antibodies. Examples include Graves disease (342), idiopathic thrombocytopenia purpura (343,344), and systemic lupus erythematosus (345). Although exposure to these maternal antibodies stops at the time of delivery, permanent damage to relevant organs or tissues may occur in the fetus. For example, maternal anti-Ro/La antibodies can cause permanent heart block in an infant (346,347), fetal isoimmune thrombocytopenia can produce cerebral infarction and consequent porencephaly (348), and maternal antiacetylcholine receptor antibodies associated with myasthenia gravis can cause arthrogryposis in a child (349). A particularly interesting but poorly understood association exists between maternal systemic lupus erythematosus and the occurrence

of chondrodysplasia punctata in the infant (350). Fortunately, all these occurrences are rare.

### 36.4 PATERNAL EXPOSURES AND MATERNAL EXPOSURES BEFORE OR SHORTLY AFTER CONCEPTION

Concern is often voiced about the potential role of paternal exposures to toxic agents in the pathogenesis of birth defects (351). However, it seems unlikely that such paternal exposures could produce birth defects in a subsequent child except by germ cell mutation or alteration in the pattern of imprinting (352–354). Although these possibilities certainly exist for many agents to which fathers might potentially be exposed, the overall risk would presumably apply to many different genetic loci, and one would not expect to see any consistent pattern of abnormalities among the offspring of an exposed male. Rather, a low-frequency increase in a variety of disorders resulting from new autosomal dominant or chromosomal mutations or from altered imprinting would be anticipated.

No paternal exposure to any chemical or physical agent, including a number of known mutagens, has been convincingly shown to increase the risk of birth defects in subsequently conceived children (355). Germ cell mutations do occur, but their contribution to the burden of malformations appears to be too small to measure in comparison to the background risk. Paternal mutagenic exposures may contribute to infertility or early miscarriage and may be of importance from a population perspective, especially when considered over many generations. However, an individual couple concerned about exposure of the father to a potentially teratogenic or mutagenic agent can be reassured that such exposures probably present minimal risk to the fetus if they occur before conception and none at all if they occur postconceptionally.

Similar advice can be provided to women who were exposed to radiation or mutagenic chemicals, such as many cancer therapeutic agents, before conception. Available evidence indicates that the risk of congenital anomalies is not measurably increased among the children of women who have had preconceptional exposures in comparison to unexposed women (103,356).

The first two weeks after conception—the time between creation of the zygote and formation of the third germ layer, which marks the beginning of the embryonic period—is sometimes characterized as the “all-or-none period” with respect to teratogenic risk. Adverse environmental exposures that occur during this time are usually thought to either kill the conceptus, with loss of the pregnancy before it is recognized, or produce no permanent adverse effect because the multipotent cells that are present at this stage replace other cells that have been damaged or lost. The concept of the all-or-none period was developed on the basis of animal experiments that

showed lack of sensitivity to the teratogenic effects of ionizing radiation during this earliest phase of pregnancy (108).

Although many subsequent studies support the idea that embryogenesis proper is a time of greater susceptibility to teratogenic effects, it is now clear that some congenital anomalies may be induced during the cleavage, blastocyst, or bilaminar disk phases of development. The most compelling evidence for this comes from studies of mice exposed very early in pregnancy to several mutagenic chemicals (357) and studies of human infants who were conceived by in vitro fertilization. Alterations of imprinting are thought to be responsible for the increased risks of birth defects such as Prader–Willi syndrome and Silver–Russell syndrome that have been associated with in vitro fertilization in humans (358,359).

### 36.5 CONCLUSION

The need for reliable information on potentially teratogenic agents is likely to increase as a result of new technologies. One outcome of the Human Genome Project is a rapidly expanding capability to identify molecular targets for new therapeutic agents. However, in addition to mediating disease processes in adults, these targets may also be involved in morphogenesis or other critical functions in the embryo or fetus. Thus, in some cases the use of these agents in women who are pregnant or who become pregnant during therapy may pose specific and substantial risks for fetal development. The teratogenic potential of such agents will, therefore, be an especially important consideration.

Nanotechnologies (molecular level devices or interventions) are being developed for both diagnostic and therapeutic purposes. Very little is known about the risks of human exposures to such agents at present, and nothing is known about their possible effects on embryonic and fetal development.

Our recognition of epigenetic mechanisms (e.g. imprinting) that may modify gene expression and may also create transgenerational consequences opens new possibilities that will need to be considered in terms of risk assessment. These concerns may be especially important in the context of in vitro fertilization, intracytoplasmic sperm injection, and other assisted reproductive technologies that may bypass normal epigenetic control mechanisms that operate around the time of conception.

These new concerns are superimposed on our limited knowledge of the teratogenic effects of existing therapeutic, environmental, and dietary agents. There is an urgent need to develop approaches to screening for risks related to such mechanisms and for the appropriate evaluation of human exposures.

In conclusion, a wide variety of potentially teratogenic exposures may be encountered during pregnancy. Insufficient evidence is available for the complete characterization of most such exposures, and further information in this area is badly needed. At present, emphasis should be placed on

the avoidance of potentially hazardous agents unless benefits to the mother or infant from the proposed exposure clearly outweigh the hazards to the fetus. Well-informed women, supported by knowledgeable and sensitive health care providers, form one of the strongest lines of defense for the fetus against potentially hazardous exposures.

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## Biographies



**Jan M. Friedman, MD, PhD, FAAP, FABMG, FCCMG, FRCPC**, received his MD and MS degrees from the Tulane University in New Orleans and his PhD in Genetics from the University of Washington in Seattle. He completed a residency in Pediatrics at Chicago Children's Memorial Hospital and a clinical fellowship in Medical Genetics at the University of Washington. He is certified by the American Board of Pediatrics, the American Board of Medical Genetics, the Canadian College of Medical Geneticists, and the Royal College of Physicians and Surgeons of Canada.

Dr Friedman is a professor of Medical Genetics at the University of British Columbia. From 1989–1999, he served as the head of this department. He is currently Acting Executive Director of the Child & Family Research Institute and Acting Associate Dean (Research) in the UBC Faculty of Medicine.

Dr Friedman is an author of more than 225 articles and 8 books. He has served as president of the Teratology Society, Founding President of the Association of Professors of Human Genetics, President of the Canadian College of Medical Geneticists, Treasurer of the American Society of Human Genetics, and member of the Advisory Board of the CIHR Institute of Genetics.

Dr Friedman's honors include the receipt of awards for excellence in both clinical and basic science teaching from the UBC Department of Medical Genetics, the UBC Killam Teaching Award, the Thomas Shepard Lectureship of the Organization of Teratogen Information Specialists, The Robert L Brent Lectureship of the Teratology Society, The Bock Prize and Lectureship in Developmental Biology and Genetics of the Alfred I Dupont Hospital for Children, the Irene Uchida Lectureship at the University of Manitoba, the Joseph Warkany Lectureship of the Teratology Society, and the Terry Klassen Lectureship of the Women's & Children's Health Research Institute in Edmonton, Alberta.

Dr Friedman's current research focuses on the use of genomic technologies to identify causes of intellectual disability. He also studies the effects of various medications on human embryonic and fetal development and has made important contributions to the clinical and epidemiological understanding of neurofibromatosis.



**Dr James W. Hanson, MD** is the Director of the Center for Developmental Biology and Perinatal Medicine (CDBPM), at the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), NIH. He attended the Johns Hopkins University and subsequently received his Doctor of Medicine degree from the University of Iowa, College of Medicine. His residency in pediatrics was at the Johns Hopkins Hospital. He spent 2 years as a medical epidemiologist in the Birth Defects Section at the US Centers for Disease Control and Prevention in Atlanta, Georgia. From 1974 through 1976, he was a postdoctoral fellow in dysmorphology with Dr David Smith at the University of Washington. In 1976, he joined the Department of Pediatrics at the University of Iowa, and he became Director of the Division of Medical Genetics in 1977.

In 1991, Dr Hanson was a Joseph P Kennedy, Jr. Foundation Fellow in Public Policy, assigned to the US Senate Subcommittee on Disability Policy. Other subsequent federal roles include Senior Advisor for Provider Liaison to the National Vaccine Program Office, Office of the Assistant Secretary for Health; Special Assistant to the Director, Center for Research for Mothers and Children, NICHD; and Senior Advisor, Office of Policy Analysis, Office of the Administrator, Agency for Health Care Policy and Research (now AHRQ). He was detailed to the Office of Public Health and Science, DHHS, on policy issues related to the future of academic health centers. He was appointed to the position of Senior Advisor for Medical Genetics and Acting Chief of the Clinical and Genetic Epidemiology Research Branch, Genetics and Epidemiology Program, Division of Cancer Control and Population Sciences, NCI, in 1998, to develop a National Cancer Genetics Research Network. In 2002, he became Chief of the Mental Retardation and Developmental Disabilities Branch at NICHD. In 2003, he was appointed to his current position.

Dr Hanson's research interests include the effects of environmental agents on fetal growth and development, patterns of malformation and abnormal fetal development, newborn screening, prenatal screening, birth defects epidemiology, cancer genetics, information/communications technology, nanotechnology and point of care technologies, especially as they relate to public health and public policy aspects of genetics, preventive health care, and both domestic and international children's health policy issues. He has written numerous scientific articles and has been a member of the American Medical Association, the American Academy of Pediatrics, the American Pediatric Society, the Society for Pediatric Research, the American Society of Human Genetics, the American Public Health Association, the Great Plains Genetics Services Network, and the Iowa Medical Society. He is a Past-President of the Teratology Society. He is a founding fellow and was a founding member of the Board of Directors of the American College of Medical Genetics.

# CHAPTER

# 37

## Neurodevelopmental Disabilities: Global Developmental Delay, Intellectual Disability, and Autism

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### 37.1 INTELLECTUAL DISABILITY AND GLOBAL DEVELOPMENTAL DELAY

Intellectual disability (ID) is a developmental disability presenting in infancy or the early childhood years though cannot be diagnosed until the child is older than approximately 5 years of age when standardized measures of developmental skills become reliable and valid. The American Association on Intellectual and Developmental Disability defines ID using measures of three domains: intelligence, adaptive behavior, and systems of supports afforded the individual (1). Thus, one cannot rely solely on the measure of IQ to define ID. The term “intellectual disability” largely has replaced “mental retardation,” at least in the United States (1). Often, it is solely intelligence (typically measured by an intelligence quotient (IQ)) that is used to characterize intellectual function in the medical literature; however, standardized measures of “adaptive behavior” and of “social supports” should be considered to provide a more comprehensive and reliable data set for describing a person. Such measures may affect the medical genetic evaluation process and outcomes. The psychology profession requires completion of all measures before one can establish the diagnosis of ID in an individual. The gradations of disability are not different with this change in language: borderline, mild, moderate, severe and profound.

For the purpose of this chapter, we define intellectual disability as a disability characterized by significant limitations both in intellectual functioning and in adaptive behavior as expressed in conceptual, social and practical adaptive skills. The disability originates before 18 years of age (1). The prevalence of ID is estimated to be between 1% and 3% (2). Costs (direct and indirect) to support individuals with ID are large, estimated to be approximately \$1M average lifetime costs per person (3).

It has been estimated that the lifetime costs for medical care of U.S. children born with an ID in 2000 will be \$50 billion (4).

### 37.2 GLOBAL DEVELOPMENTAL DELAY

The type of disability identified is an important preliminary step because the type influences somewhat the diagnostic investigation pathway. Global developmental delay (GDD) is defined as a significant delay in two or more developmental domains including gross or fine motor, speech/language, cognitive, social/personal, and activities of daily living and is thought to predict a future diagnosis of ID (5). Such delays require accurate documentation using norm-referenced and age-appropriate standardized measures of development administered by experienced developmental specialists. The term global developmental delay is usually reserved for younger children (typically <5 years of age). Children with GDDs are those who present with delays in the attainment of developmental milestones at the expected age; this implies deficits in learning and adaptation, which suggests that the delays are significant and predict later ID. However, delays in development, especially those that are mild, may be transient and lack predictive reliability for ID or other developmental disabilities. This requires the clinician to judge the significance of the presenting signs and symptoms and the likelihood that they imply a pathological problem and not a normal variation in development in the infant or very young child. This, in turn, will determine the optimal diagnostic evaluation pathway. The prevalence of GDD is estimated to be 1–3% (5–7).

The purpose of identifying the cause is to address treatment options, discuss the expected clinical course and offer information regarding risk of recurrences for the

family. For parents or family members, the motivations to understand the cause of the disability vary. For example, Lenhard et al. (8) assessed the psychological benefit of a diagnosis by comparing the level of anxiety, guilt, and emotional burden among three groups: mothers of nondisabled children, mothers of children with Down syndrome, and mothers of children with idiopathic ID. They reported that mothers of children with Down syndrome and nondisabled children had similar scores for these measures, whereas mothers of children with idiopathic ID had “broad psychoemotional disadvantages.” The authors offered the opinion that Down syndrome is well known such that mothers of affected children may have different experiences from mothers of children with ID of other causes.

Graungaard and Skov (9) interviewed parents of eight children aged 1–27 months who had recently been diagnosed with significant physical and mental disabilities and concluded that a diagnosis “could enable parents to regain predictability and control in their life” and provide them with active coping strategies.

Because “we do not know if the parents’ ability to adapt to and cope with their child’s ID is actually improved by knowing a specific genetic cause,” Makela et al. (10) set out to address this question and interviewed families in depth in an effort to address the “value” of a specific etiological diagnosis in the child with an ID. Their qualitative research involving interviews of families of children with ID with and without an etiologic diagnosis and found these 20 families had specific stated needs and feelings about what a diagnosis offers:

1. Validation. A diagnosis established that the problem (ID) was credible, which empowered them to advocate for their child.
2. Information. A diagnosis was felt to help guide expectations and management immediately, and provide hope for treatment or cure in future.
3. Procuring services. The diagnosis assisted families in obtaining desired services, particularly in schools.
4. Support. Families expressed the need for emotional companionship that a specific diagnosis (or “similar challenges”) assisted in accessing.
5. Need to know. Families widely differed in their “need to know” a specific diagnosis, ranging from strong to indifferent.
6. Prenatal testing. Families varied in their emotions, thoughts and actions regarding prenatal genetic diagnosis.

Families differ in the importance of the specific etiology of their family member’s disability and this may change with time (lessening as the child ages to adult (11)). The medical geneticist might be advised to explore the motivations with families as the evaluation process and treatment outcome are valued differently by families.

### 37.3 DEFINITION OF A DIAGNOSIS

There is no unanimity in the definition of a “genetic diagnosis.” For many families and physicians, the diagnosis of the neurodevelopmental disability (e.g. autism, mild ID, etc.) is a sufficient clinical diagnosis and it does not always lead to counseling the patient or family about the potential value of the etiological evaluation (7,10). Furthermore, there is neither shared definitions of “treatment” and “improved outcomes” resulting from a genetics evaluation, nor a body of literature demonstrating the value of a diagnosis in reference to the long-term outcome for those with neurodevelopmental disabilities. Nevertheless, expert opinion with support of many case series indicate that a comprehensive evaluation is required to plan health care for individuals with neurodevelopmental disorders (5,7,12–16).

Schaefer and Bodensteiner (13) wrote that a specific diagnosis is that which “can be translated into useful clinical information for the family, including providing information about prognosis, recurrence risks, and preferred modes of available therapy.” Van Karnebeek et al. (17) defined etiologic diagnosis as “sufficient literature evidence ... to make a causal relationship of the disorder with mental retardation likely, *and* if it met the Schaefer–Bodensteiner definition.”

This chapter defines the “genetic diagnosis” as that in which there is sufficient literature to make a causal inference between the clinical and laboratory diagnostic information and the patient’s phenotype and thus informing treatment options, recurrence risks and patient outcomes.

For some patients, the clinical diagnosis cannot be confirmed by genetic diagnostic testing—the clinical recognition of a well-known genetic syndrome (e.g. Cornelia de Lange syndrome) for which comprehensive genetic diagnostic testing is negative for the known mutations (18).

Furthermore, at this time in the history of the practice of medical genetics and given the advances in exome and genome sequencing, the approach in the clinic to an individual patient is about to experience a sea-change from “phenotyping” the patient to determine which test should be ordered toward interpreting genomes and applying focused phenotyping based on that sequence result (19–21). Nevertheless, for the time being, the clinical approach to the patient with neurodevelopmental disability will continue to be in the tradition of the profession to include these elements: the child medical history (including prenatal and birth histories); the family history, which includes construction and analysis of a pedigree of three generations or more; the physical and neurologic examinations emphasizing the examination for minor anomalies (the “dysmorphology exam”); and, the examination for neurologic or behavioral signs that might suggest a specific recognizable syndrome or diagnosis. After the clinical genetic evaluation, judicious use of the laboratory tests, imaging, and other consultants based on best evidence are important in establishing the diagnosis (Table 37-1).

**TABLE 37-1** The Medical Genetics Evaluation for Global Developmental Delays or ID

## Medical Genetics Evaluation Process:

1. Clinical history
2. Family history
3. Physical examination (especially for minor anomalies)
4. Neurological examination
5. Specific confirmatory genetic tests for suspected syndromes
6. Microarray CGH
7. Fragile X molecular genetic testing
8. Metabolic screening in all<sup>a</sup>
9. Targeted MRI brain imaging

<sup>a</sup>See Table 37-5.

Adapted from Moeschler et al. (7), Moeschler (82), Michelson et al. (77), and Van Karnebeek and Stockler (80).

The medical geneticist experienced in the diagnostic evaluation and treatment of patients with neurodevelopmental remains the essential element in the evaluation process. This expert must be facile with the dysmorphology examination (and morphological pattern recognition) as well as the standard neurological examination. Recently, an international group of clinicians expert in the field of dysmorphology has published a proposed nomenclature to be used to describe human morphology (22). This effort is designed to improve the description of patient phenotype and facilitate comparison of findings among patients. It also serves to bring consistency and precision needed for communicating with colleagues in the research community. Pattern recognition of minor morphologic abnormalities (i.e. “dysmorphology” (23)) remains a pivotal skill of the medical geneticist in the evaluation of the patient with neurodevelopmental disability. Currently, the recognition of the pattern of dysmorphology, when present in the patient with neurodevelopmental disability, facilitates the efficient diagnostic evaluation process.

### 37.4 GENETIC MECHANISMS OF ID

The number of genes identified that cause ID suggests that an ID phenotype can emerge as the final common pathway of many different types of abnormal cellular processing and that no one overriding mechanism is likely to be the cause of intellectual (24). There are over 2500 genes or phenotypes listed in OMIM with “mental retardation” as the search term, and it is estimated that one-third of the entire genome is expressed in the human brain. Mutations in more than 450 genes have been identified to cause intellectual disabilities and related cognitive disorders (25). There are more than 200 X-linked genes for ID cloned or mapped (15). There are many inferred mechanisms by which pathogenic mutations affect CNS function, i.e. cause ID. It is emerging that ID can result from a wide range of protein abnormalities.

The genes identified at the earliest were frequently signaling molecules in the RhoGTPase pathway (*GDI*, *PAK3*, *ARHGEF6*) or associated with chromatin remodeling (*RPS6KA3*, *ATRX*). Most recently, components of the synaptic vesicle or components necessary for its formation have been identified as defective (*SYN1*, *SLC6A8*, *NLGN4* and *DLG3*) and a number of novel transcription factors (*ZNF41*, 81 and 674) have been found. Inlow and Restifo (26) identified the following categories of mechanism or dysfunction by an in silico review: enzymatic, mediators of signal transduction, binding proteins, transporter proteins, cell adhesion molecules, structural molecules, motor proteins, tRNAs, apoptosis regulators, chaperones, and enzyme regulators. There are at least two curated online resources for X-linked genes causing ID: <http://xlmr.interfree.it/home.htm> and [http://www.ggc.org/xlmr\\_update.htm](http://www.ggc.org/xlmr_update.htm).

### 37.5 DIAGNOSTIC TESTING OF PATIENTS WITH ID OF UNKNOWN CAUSE

Often, the expert medical geneticist or other specialist will recognize the likely or certain cause of the ID upon complete of the history and examination (27). More often, the cause of the disability is not certain and calls for a stepwise diagnostic evaluation with laboratory, imaging and consultations with other expert clinicians.

#### 37.5.1 Cytogenomic Copy Number Abnormalities

Advances in molecular genetic technology have quickened the pace of the discovery of the genetic underpinnings of disorders. DNA and RNA arrays now enable the detection of deletions, duplications and reduced expression of candidate genes. This applies to those with ID and/or autism spectrum disorders (ASDs). The clinical application of the chromosomal microarray has replaced the standard karyotype, with certain clinical exceptions (such as in use of the standard karyotype for confirming common syndromes such as Down syndrome) (28). There are also recognizably syndromes associated with chromosomal abnormalities that are detectable by conventional karyotyping or by targeted fluorescent in situ hybridization (FISH) analysis based on suspicion of the specific syndrome, such as Prader–Willi and Angelman syndrome (15q11.2–q13), Williams–Beuren syndrome (7q11.23), Smith–Magenis syndrome (17p11.2), DiGeorge syndrome (22q11.2), and monosomy of 1p36.1. Some balanced chromosomal translocations, not detectable by microarray, may be causal of the ID by mechanisms such as position effects (29).

Nevertheless, the cytogenomic microarray is more sensitive than the standard karyotype at identifying genomic imbalance as the cause of ID or ASDs. The



types of clinical laboratory microarrays vary somewhat (28) and there is little published data with head-to-head comparison of test performance by array type (30). The published diagnostic rates among study populations with ID range from 5 to 20%, about twice the rate by conventional karyotype (28,31–33). Most of these cases are microdeletion/duplications with many recurrent patients with similar recognizable phenotypes (Table 37-2 taken from Vissers et al. (33)). The pathogenic mechanisms for these deletions or duplications are challenging to predict, i.e. whether a syndrome is due to a mutation in a single gene or the result of deletion, duplication or dysregulation of multiple genes. For example, application of microarray to known syndromes, similar to CHARGE syndrome, has allowed for identification of haploinsufficiency of a single gene as cause for this known syndrome (see Table 37-3 for a list of others). Many of the microdeletions/duplications are rare or unique. As a consequence, there are several efforts to catalog patient phenotypes associated with copy number abnormalities (28,34,35). Table 37-3 provides a partial listing of prevalent genes by proposed mechanism.

Cytogenomic microarray studies may identify candidate genes for autosomal-dominant ID. Autosomal-recessive forms of ID are not well studied; much more is understood about X-linked genes causing disability. Only three different genes for autosomal-recessive ID have been identified thus far (36–38) including forms that are associated with microcephaly.

### 37.5.2 X-Linked ID

A number of factors have contributed to the progress in understanding ID due to X-linked genes, particularly when the pace of discovery is compared to that of autosomal genes (15). Some of these factors include longstanding awareness of the excess of males in many population studies dating to the nineteenth century; a large number of published clinical reports of large X-linked pedigrees dating back 70 years (39); the intense interest in fragile X syndrome (FXS) for some 40 years (40); and, the relative ease of mapping and identifying X-linked genes as compared to autosomal genes. More recently, advances in molecular genetic technology have quickened the pace of X-linked gene discovery, particularly the utilization of genes and genomic DNA in arrays has allowed for detection of deletions, duplications and reduced expression of candidate genes (41). Finally, advances in genome sequencing technology has made it possible for laboratories to sequence all X-linked genes in a single individual to identify pathogenic mutations (42).

Approximately 200 X-linked genes cause ID; about half have been identified and another 20% are regionally mapped. All genes responsible for X-linked intellectual disability (XLID) families reported before the rediscovery of FXS have now been identified; and, most, if not all, are the high-prevalence genes, that is, those

of a prevalence of 1% or greater. Nevertheless, there remain many genes unmapped and mapped yet unidentified and together they comprise a substantial proportion of XLID.

XLID often is subdivided into syndromic (S-XLID) and nonsyndromic (NS-XLID) forms, depending on whether further abnormalities are found on physical examination, laboratory investigation and brain imaging (15,43,44). Roughly two-thirds of XLMR cases are thought to be nonsyndromic (45); however, as the possibilities for classifying families through molecular studies improve, and as patients are examined in more detail, it is likely that the proportion of syndromic cases that are diagnosed will increase, with a concomitant decrease in the nonsyndromic cases. This is illustrated by the FXS, which was initially described as nonsyndromic and is now considered to be the most frequent example of S-XLMR. Moreover, mutations in several XLID genes can give rise to both nonsyndromic and syndromic forms, which demonstrates the difficulties in such classification schema.

### 37.5.3 Fragile X Syndrome

In 1969, Lubs (46) identified a family with four male members diagnosed with ID, each of whom had an unusual chromosomal gap on his X chromosome long arm, a “marker X chromosome” later confirmed by Sutherland et al. (47,48) to be related to certain cell culture conditions that render the chromosome “fragile.” The *FMR1* gene was cloned in 1991 (49) and in almost all cases, the causative mutation is the expansion of a CGG repeat located in the 5′ untranslated region (UTR) of *FMR1*. The length of the CGG repeat in the healthy population repeat is from 6 to 54 repeats (50). When the number of repeats exceeds 200, the expansion is referred to as a full mutation allele and results in FXS. At the molecular level, the large number of CGG repeats in the full mutation leads to marked methylation of both the CGG repeats and the *FMR1* promoter, hypoacetylation of associated histones, and chromatin condensation; these epigenetic changes result in transcriptional silencing of *FMR1* and subsequent loss of its protein product, fragile X mental retardation protein (FMRP) (51). Alleles with an intermediate number (55–200) of repeats are referred to as premutation alleles. Premutation alleles do not cause an FXS phenotype but are prone to large increases in repeat length during meiosis, especially female meiosis (50).

There are well-characterized clinical features in males with FXS that females, and rare males with a large expansion, may lack. Thus, it is recommended that any child who presents with developmental delay, borderline intellectual abilities, or ID, or has a diagnosis of autism without a specific etiology should undergo molecular testing for FXS (52). It is estimated that 2–6% of such patients will be found to have an *FMR1* mutation.

**TABLE 37-2 Recurrent Interstitial CMA Deletions and Duplications in ID**

Name	Size (Mb) <sup>a</sup>	LCR	MIM	Clinical Features
1q21.1 microdeletion	1.1	+	612474	Mild-to-moderate MR, MC, cardiac abnormalities, cataracts, clear incomplete penetrance
1q21.1 microduplication	1.1	+	612475	Autism or autistic behaviors, mild-to-moderate MR, microcephaly, mild FD
1q41q42 microdeletion	1.2	—	—	MR, seizures, various dysmorphisms, cleft palate, diaphragmatic hernia
2p15q16.1 microdeletion	3.9	—	—	MR, MC, receding forehead, ptosis, telecanthus, short palpebral fissures, downslanting palpebral fissures, broad/high nasal bridge, long/straight eyelashes, smooth and long philtrum, smooth upper vermilion border, everted lower lip, high narrow palate, hydronephrosis, optic nerve hypoplasia
3q29 microdeletion	1.6	+	609425	MR, mild FD, including high nasal bridge and short philtrum
3q29 microduplication	1.6	+	611936	Mild/moderate MR, MC, obesity
7q11.23 microduplication	1.5	+	609757	MR, speech and language delay, autism spectrum disorders, mild FD
9q22.3 microdeletion	6.5	—	—	MR, hyperactivity, overgrowth, trigonocephaly, macrocephaly, FD
12q14 microdeletion	3.4	—	—	Mild MR, failure to thrive, proportionate short stature and osteopoikilosis
14q11.2 microdeletion	0.4	—	—	MR, widely spaced eyes, short nose with flat nasal bridge, long philtrum, Cupid's bow of the upper lip, full lower lip, auricular anomalies
15q13.3 microdeletion	1.5	+	612001	MR, epilepsy, hypotonia, short stature, microcephaly and cardiac defects
15q24 microdeletion	1.7	+	—	MR, growth retardation, MC, digital abnormalities, genital abnormalities, hypospadias, loose connective tissue, high frontal hairline, broad medial eyebrows, downslanted palpebral fissures, long philtrum
16p11.2 microdeletion/duplication	0.6	+	611913	Association with MR, autism, schizophrenia
16p11.2p12.2 microdeletion	7.1	+	—	MR, flat facies, downslanting palpebral fissures, low-set and malformed ears, eye anomalies, orofacial clefting, heart defects, frequent ear infections, short stature, minor hand and foot anomalies, feeding difficulties, hypotonia
16p13.1 microduplication	1.6	+	—	Association with autism, significance uncertain
16p13.1 microdeletion	1.6	+	—	MR, MC, epilepsy, short stature, phenotypic variability
17p11.2 microduplication <sup>b</sup>	3.7	+	610883	MR, infantile hypotonia, failure to thrive, autistic features, sleep apnea, and structural cardiovascular anomalies
17q21.31 microdeletion	0.5	+	610443	MR, hypotonia, long hypotonic face with ptosis, large and low set ears, tubular or pear shaped nose with bulbous nasal tip, long columella with hypoplastic alae nasi, broad chin
19q13.11 microdeletion	0.7	—	—	MR, pre- and postnatal growth retardation, primary microcephaly, hypospadias, ectodermal dysplasia including scalp aplasia, dysplastic nails and dry skin
22q11.2 microduplication	3.7	+	608363	Highly variable. MR, FD, for example widely spaced eyes and downslanting palpebral fissures, velopharyngeal insufficiency, conotruncal heart disease
22q11.2 distal microdeletion	1.4–2.1	+	611867	MR, prematurity, prenatal/postnatal growth delay, mild skeletal abnormalities, arched eyebrows, deep set eyes, smooth philtrum, thin upper lip, hypoplastic alae nasi, small pointed chin
Xq28 microduplication	0.4–0.8	—	—	MR, severe hypotonia, progressive lower limb spasticity, absent or very limited speech

FD, facial dysmorphisms; LCR, low copy repeat; MC, microcephaly; MR, mental retardation.

<sup>a</sup>Common region.

<sup>b</sup>Potocki–Lupski syndrome.

From Vissers et al. (33).

### 37.5.4 Autism Spectrum Disorders

Autism is a pervasive developmental disorder involving deficits in three domains: social skills, communication, and repetitive behaviors or restricted interests, all before the age of 36 months (53). Typically, the behavioral and developmental symptoms are noted shortly

after age of 1 year and before the second birthday. The ASDs (54) include those with autism, pervasive developmental disorders—not otherwise specified, and Asperger syndrome.

There are several reports of a recent rise in prevalence of ASDs worldwide. Current estimates of prevalence for

**TABLE 37-3 Dosage-Sensitive Genes Causing ID and Identified by Deletion and/or Duplication Strategies**

Syndrome	Chromosome	Size	Gene(s) Involved	MIM	Reference
Cystinuria with mitochondrial disease	del(2)(p16)	179 kb	<i>SLC3A1, PPM1B, KIAA0436</i>	606407	Parvari et al.
Adrenal hyperplasia with hypermobility	del(6)(p21)	33 kb	<i>TNFX, CYP21A</i>	—	Koppens et al.
CHARGE syndrome	del(8)(q12)	2300 kb	<i>CHD7</i>	214800	Vissers et al.
Oto-facial-cervical syndrome	del(8)(q13.3)	316 kb	<i>EYA1</i>	166780	Rickard et al.
9q subtelomeric deletion syndrome	del(9)(q34)	Diverse	<i>EHMT1</i>	610253	Kleefstra et al.
Potocki-Shaffer syndrome	del(11)(p11.2)	2100 kb	<i>EXT2, ALX4</i>	601224	Potocki et al.
Infantile hyperinsulinism enteropathy and deafness	del(11)(p15p14)	122 kb	<i>USH1C, ABCC8, KCNJ11</i>	606528	Bitner-Glindzicz et al.
12q14 microdeletion syndrome	del(12)(q14)	3440 kb	<i>LEMD3, HMGA2, GRIP1</i>	—	Menten et al.
Peters Plus syndrome	del(13)q12.3q13.1)	1500 kb	<i>B3GALT1</i>	261540	Lesnik Oberstein et al.
Tuberous sclerosis polycystic kidney disease	del(16)(p13)	87 kb	<i>TSC2, PKD1</i>	173900	Brook-Carter et al.
Potocki-Lupski syndrome	dup(17)(p11.2p11.2)	3700 kb	<i>RAI1</i>	610883	Potcoki et al.
Alport leiomyomatosis	del(X)(q22.3)	133 kb	<i>COL4A5, COL4A6</i>	301050	Zhou et al.
MECP2 duplication syndrome	dup(X)(q28)	Variable	<i>MECP2</i>	—	Van Esch et al.

From Vissers et al. (33).

all pervasive developmental disorders are in the range of 20–80 per 10,000 individuals (55–58). A review of studies completed between 1998 and 2001 from several countries concluded that the prevalence of autism is about 13 per 10,000 individuals and for pervasive developmental disorders more generally, 37 per 10,000 individuals (56). Examination of possible factors behind the rising reported incidence has led to one group to report that the incidence of autism rose seven to eightfold in California from the early 1990s through 2007. Quantitative analysis of the changes in diagnostic criteria, the inclusion of milder cases, and an earlier age at diagnosis during this period suggests that these factors probably contributed 2.2-, 1.56-, and 1.24-fold increases in autism, respectively, but cannot fully explain the magnitude of the rise in autism in California. Differential migration also likely played a minor role. Wider awareness, greater motivation of parents to seek services as a result of expanding treatment options, and increased funding may each have contributed, but documentation or quantification of these effects is lacking. Having demonstrated the challenges of documenting an incidence trends in ASDs, these authors concluded that “with no evidence of a leveling off, the possibility of a true increase in incidence deserves serious consideration” (59). If there is a biological or environmental explanation for the observed increased incidence of the diagnosis of ASDs, it is not yet known (60).

ASDs are four times more common in males than females (61). There is a strong genetic basis to ASDs as indicated by the recurrence risk in families, twin studies, and the cooccurrence with chromosomal disorders and rare genetic syndromes. More than 100 genes or genomic disorders are known to cause ASDs (62).

The clinical presentation of the child with an ASD overlaps that with GDD, particularly in the early

years. For example, in the Childhood Autism Risks from Genetics and the Environment Study (63), which recruits preschool children from the California administrative data system, 71% of those who meet criteria for autism on both the Autism Diagnostic Observation Schedule (ADOS) (64) and Autism Diagnostic Inventory-Revised (ADI-R) also met criteria for ID. Many with ASDs will “outgrow” a diagnosis of GDD or ID in large part because the ability of the professionals to measure abilities improves as a child ages (59). This overlap complicated the diagnostic decision-making by the medical specialist as well as the study of the genetic and metabolic causes of ASDs. For example, at least 103 specific genes and 44 genomic loci have been reported in patients with ASD or autistic behavior. These genes and loci have all been causally implicated in ID, indicating that these two neurodevelopmental disorders share common genetic bases (62).

The genetic architecture of ASDs is highly heterogeneous (65). About 10–20% of individuals with an ASD have an identified genetic etiology. Microscopically visible chromosomal alterations have been reported in about 5% of cases; the most frequent abnormalities are 15q11–q13 duplications, and 2q37, 22q11.2 and 22q13.3 deletions. ASDs can also be due to mutations of single genes involved in autosomal-dominant, autosomal-recessive and X-linked disorders. The most common single-gene mutation in ASDs is FXS (*FMR1*), present in about 2% of cases. Other monogenic disorders described in ASDs include tuberous sclerosis (*TSC1, TSC2*), neurofibromatosis (*NF1*), Angelman syndrome (*UBE3A*), Rett syndrome (*MECP2*) and *PTEN* mutations in patients with macrocephaly and autism (Table 37-4) (66). Rare mutations have been identified in synaptic genes,

**TABLE 37-4 Monogenic Causes of Intellectual Disability, by Mechanism**

Gene	Locus	Disorder/Phenotype	Function of Encoded Protein; Subcellular Localization <sup>a</sup>
<i>Genes Required for Neurogenesis</i>			
Microcephalin	MCPH1/8p22-pter	Microcephaly vera	Cell cycle control and DNA repair
CDK5RAP2	MCPH3/q34	Microcephaly vera	Mitotic spindle function in embryonic neuroblasts
ASPM	MCPH5/1q31	Microcephaly vera	Formation of mitotic spindle during mitosis and meiosis
CENPJ	13q12.2	Microcephaly vera	Localization to the spindle poles of mitotic cells
<i>Genes Required for Neuronal Migration</i>			
LIS1	17p13.3	Miller Dieker syndrome: type 1 lissencephaly, pachygyria, subcortical band heterotopia (double cortex)	Interacts with dynein and plays a role in several function, including nuclear migration and differentiation
DCX/Dcn	Xq22.3	Type 1 lissencephaly, pachygyria, subcortical band heterotopia (double cortex)	Microtubule-associated protein (MAP)
RELN	7q22	Lissencephaly with cerebellar hypoplasia	Extracellular matrix (ECM) molecule, reelin pathway
VLDLR	9p24	Lissencephaly with cerebellar hypoplasia	Low-density lipoprotein receptor, reelin pathway
POMT1	9q34	Walker–Warburg syndrome (also known as HARD syndrome <sup>b</sup> )	Protein <i>o</i> -mannosyltransferase 1 (glycosylation of alpha-dystroglycan)
POMT2	14q24.3	Walker–Warburg syndrome	Protein <i>o</i> -mannosyltransferase 2 (glycosylation of alpha-dystroglycan)
POMGnT1	1p34	Muscle–eye–brain disease (MEB)	Protein <i>o</i> -mannose beta-1,2-n-acetylglucosaminyltransferase
Fukutin	9q31	Fukuyama congenital muscular dystrophy (FCMD) with type 2 lissencephaly	Homology with glycoprotein-modifying enzymes (no biochemical activity has been reported)
FLNA	Xq28	Bilateral periventricular nodular heterotopia (BPNH)	Filamin-1 (actin cross-linking phosphoprotein)
<i>Genes Required for Cellular Processes Involved in Neuronal and Synaptic Functions</i>			
FMR1	Xq27	FXS (Facial anomalies with macro-orchidism)	mRNA-binding protein, role in mRNA translation; potential regulation by RhoGTPase pathways; postsynaptic localization
FGD1	Xp11.2	Aarskog–Scott syndrome (Facial, digital and genital anomalies)	RhoGEF protein (GTP exchange factor), activate Rac1 and Cdc42
PAK3	Xq21.3	Nonsyndromic XLMR	P21-activated kinase 3; effector of Rac1 and Cdc42
ARHGEF6	Xq26	Nonsyndromic XLMR	RhoGEF protein, integrin-mediated activation of Rac1 and Cdc42
OPHN1	Xq12	MR with cerebella and vermis hypoplasia	RhoGAP protein (negative control of RhoGTPases; stimulates GTPase activity of RhoA, Rac1 and Cdc42; pre- and post synaptic localization)
TM4SF2	Xq11	Nonsyndromic XLMR	Member of the tetraspanin family, integrin-mediated RhoGTPase pathway regulation
NLGN4	Xp22.3	Nonsyndromic XLMR, autism, Asperger syndrome	Member of the neuroligin family, role in synapse formation and activity; post synaptic localization
DLG3	Xq13.1	Nonsyndromic XLMR	Protein involved in postsynaptic density structures; postsynaptic localization
GDI <sub>1</sub>	Xq28	Nonsyndromic XLMR	Regulation of Rab4 and Rab5 activity, and of synaptic vesicle recycling; pre- and post synaptic localization
IL1RAPL	Xp22.1	Nonsyndromic XLMR	Potential involvement in exocytosis and ion channel activity
<i>Transcription Signaling Cascade, Remodeling and Transcription Factors</i>			
NF1	17q11	Neurofibromatosis type 1 (NF1); MR is present in 50% of NF1 cases	RasGAP function, involved in Ras/ERK/MAPK signaling transcription cascade; postsynaptic protein
RSK2	Xp22.2	Coffin–Lowry syndrome (facial and skeletal anomalies)	Serine–threonine protein kinase, phosphorylates CREB, involved in Ras/ERK/MAPK signaling cascade, present in the postsynaptic compartment
CDKL5	Xp22.2	Rett-like syndrome with infantile spasms	Serine–threonine kinase (STK9), interacts with MECP2, potential implication in chromatin remodeling
CBP	16p13.3	Rubinstein–Taybi syndrome (mental retardation, broad thumbs and toes, dysmorphic face)	CREB (cAMP response element-binding protein 1) binding protein; chromatin-remodeling factor involved in Ras/ERK/MAPK signaling cascade
EP300	22q13.1	Rubinstein–Taybi syndrome	Transcriptional coactivator similar to CBP, with potent histone acetyl transferase: chromatin-remodeling factor

Continued



**TABLE 37-4 Monogenic Causes of Intellectual Disability, by Mechanism—cont'd**

Gene	Locus	Disorder/Phenotype	Function of Encoded Protein; Subcellular Localization <sup>a</sup>
<i>XNP</i>	Xq13	Large spectrum of phenotypes including ATRX syndrome (microcephaly, facial dysmorphic face, skeletal anomalies and alpha-thalassemia)	Homology with DNA helicases of the SNF2/SWI2 family, chromatin-remodeling factor, regulation of gene expression
<i>MECP2</i>	Xq28	Rett syndrome (female-specific syndrome) and other phenotypes including nonsyndromic MR	Methy-CpG-binding protein 2; chromatin-remodeling factor, involved in a transcriptional silencer complex
<i>DNMT3B</i>	20q11.2	ICF syndrome: immune deficiency associated with centromeric instability, facial dysmorphism and MR	DNA methyltransferase 3B, involved in chromatin remodeling
<i>ARX</i>	Xp22.1	Large spectrum of MR phenotypes: XLAG (X-linked lissencephaly and abnormal genitalia); West syndrome, Partington syndrome; nonsyndromic MR	Transcription factor of the aristaless homeoprotein-related proteins family
<i>JARID1C</i>	Xp11.2	Spectrum of phenotypes: MR with microcephaly, short stature, epilepsy, facial anomalies and nonsyndromic MR	Transcription factor and chromatin remodeling
<i>FMR2</i>	Xq28	Nonsyndromic MR	Potential transcription factor
<i>SOX3</i>	Xq27	Isolated GH deficiency, short stature and MR	SRY-BOX 3: transcription factor
<i>PHF8</i>	Xp11.2	MR with cleft lip or palate	PHD zinc-finger protein, potential role in transcription
<i>ZNF41</i>	Xp11.2	Nonsyndromic MR	Potential transcription factor
<i>GTF2I</i>			
<i>GTF2RD1</i>	7q11.23	Williams syndrome	Transcription factors, potential regulator of c-Fos and immediate-early gene expression
<i>PHF6</i>	Xq26	Börjeson–Forssman–Lehmann syndrome (hypogonadism, obesity, facial anomalies, epilepsy)	Homeodomain-like transcription factor
<i>Other Genes Involved in MR</i>			
<i>RPSS12</i>	4q24	Nonsyndromic ARMR	Member of the trypsin-like serine protease family, enriched in the presynaptic compartment
<i>CRBN</i>	3p25	Nonsyndromic ARMR	ATP-dependent protease; regulation of mitochondrial energy metabolism
<i>CC2D1A</i>	19p13	Nonsyndromic ARMR	Unknown function, protein contains C2 and DM14 domains
<i>FTSJ1</i>	Xq11.2	Nonsyndromic XLMR	Role in tRNA modification and mRNA translation
<i>PQBP1</i>	Xq11.2	Large spectrum of MR phenotypes including nonsyndromic MR	Polyglutamine-binding protein, potentially involved in pre-mRNA splicing
<i>FACL4</i>	Xq22.3	Nonsyndromic XLMR	Fatty-acid synthase-CoA ligase 4; possible role in membrane synthesis and/or recycling
<i>SLC6A8</i>	Xq28	Creatine deficiency syndrome (MR with epilepsy and dysmorphic features) and nonsyndromic MR	Creatine transporter, role in homeostasis of creatine in the brain
<i>OCRL1</i>	Xq25	Lowe syndrome (MR, bilateral cataract and renal Fanconi syndrome)	Inositolpolyphosphate 5-phosphatase (central domain) and RHoGAP-like C-terminal domain
<i>AGTR2</i>	Xq24	Nonsyndromic XLMR	Angiotensin II receptor type 2, signaling pathway
<i>SLC16A2</i>	Xq13.2	Severe syndromic form MR with abnormal levels of thyroid hormones	Monocarbohydrate transporter, T3 transporter
<i>SMS</i>	Xp22.1	Snyder–Robinson syndrome (macrocephaly, scoliosis, dysmorphic features)	Spermin synthase, CNS development/function (neuron excitability)
<i>UBE3A</i>	15q11	Angelman syndrome	Ubiquitin–protein ligase E3A; protein degradation (proteasome): CNS development/function (neuron differentiation)

The table does not represent an exhaustive list of genes involved in MR disorders. For additional genes, see the review by Inlow and Restifo (26), and online resources: <http://xlmr.interfree.it/home.htm> and [http://www.ggc.org/xlmr\\_update.htm](http://www.ggc.org/xlmr_update.htm).

<sup>a</sup>Subcellular localization is indicated mainly for protein shown to be present in the pre- and/or postsynaptic compartments.

<sup>b</sup>HARD syndrome includes hydrocephalus (H), agyria (A), retinal dysplasia (RD), with or without encephalocele, often associated with congenital muscular dystrophies.

From Chelly et al. (83).

including *NLGN3*, *NLGN4X* (67), *SHANK3* (68), and *SHANK2* (69). Recent whole-genome microarray studies have revealed submicroscopic deletions and duplications, called copy number variation (CNV), affecting many loci and including de novo events in 5–10% of ASD cases (70–74).

### 37.5.5 Inborn Errors of Metabolism and ID

The inherited metabolic conditions that cause ID typically are associated with other neurological or systemic signs and symptoms. In several series of patients presenting with intellectual disabilities, the incidence of diagnosed metabolic disorders ranges from 1% or less to about 5% (75–79). While this incidence is lower than genomic or single-gene conditions, the potential for response to treatment is relatively better (80). In one systematic review of the literature (80), 81 inborn errors of metabolism (IEM) that were responsive to treatment and associated with ID were identified; of these, 50 were diagnosed with routine metabolic screening tests (Table 37-5): plasma amino acids, total serum homocysteine and acylcarnitine profile by tandem mass spectroscopy; urine organic acids, glycosaminoglycans, oligosaccharides, purines and pyrimidines, and creatine metabolites (Table 37-6). Several of the 81 identified to be associated with ID are detectable by routine newborn screening currently in place in the United States and around the world. Of those IEMs not identified by the metabolic tests listed in Table 37-5, the identification is based on clinical judgment and a “test-by-test strategy.” For 13 of these 81 IEMs that cause ID, whole-genome sequencing may be the best solution for diagnostic testing (80) (Table 37-7) because of difficulties with current diagnostic: a biomarker is not available or is unreliable and/or the test requires invasive testing, and/or the test is difficult to access (80).

### 37.5.6 CNS Malformations, ID and Brain Imaging

CNS malformations are a common finding in those with ID—abnormal findings on MRI are seen in approximately 30% of children with DD/ID. However, only in a fraction of these children does MRI lead to an etiologic or syndromic diagnosis.

Current recommendations range from performing brain images on all patients with DD/ID (14) to performing it only on those with indications on clinical examination, to being considered as a second-line investigation to be undertaken when features in addition to GDD are detected either on history or physical examination (81). The finding of a brain abnormality or anomaly on neuroimaging may lead to the recognition of a specific cause for an individual child’s DD/ID in the same way that a dysmorphic examination might lead to the inference of a particular clinical diagnosis. However, similar to other major or minor anomalies noted

**TABLE 37-5** X-Chromosome Genes Causing both ID and ASDs<sup>a</sup>

Gene	OMIM	Phenotype or Syndrome
<i>MID1</i>	300502	Opitz syndrome
<i>NLGN4X</i>	300427	
<i>AP1S2</i>	300629	Brain calcifications
<i>NHS</i>	300457	Nance–Horan s.
<i>CDKL5</i>	300203	Rett-like infantile spasms
<i>PTCHD1</i>	300828	
<i>ARX</i>	300382	XLAG
<i>DMD</i>	300377	Duchenne/Becker dystrophy
<i>OTC</i>	300461	Ornithine transcarbamylase deficiency
<i>CASK</i>	300172	Microcephaly, pontine, cerebellar hypoplasia
<i>IL1RAPL1</i>	300206	MRX 21
<i>ZNF674</i>	300573	
<i>SYN1</i>	313440	Epilepsy
<i>ZNF81</i>	314998	
<i>FTSJ1</i>	300499	
<i>PQBP1</i>	300463	Renpenning s.
<i>NDP</i>	300658	Norrie disease
<i>CACNA1F</i>	300110	Severe congenital stationary night blindness
<i>SMC1A</i>	300040	Cornelia de Lange syndrome
<i>PHF8</i>	300560	Siderius–Hamel s.
<i>JARID1C</i>	314690	Microcephaly, spasticity, epilepsy
<i>IQSEC2</i>	300522	
<i>FGD1</i>	300546	Aarskog s.
<i>OPHN1</i>	300127	Cerebellar Hypoplasia
<i>MED12</i>	300188	FG/Opitz-Kavaggia s.
<i>NLGN3</i>	300336	
<i>KIAA2022</i>	300524	Progressive quadriplegia
<i>ATRX</i>	300032	ATRX s.
<i>PCDH19</i>	300460	Female-limited epileptic encephalopathy
<i>DCX</i>	300121	Lissencephaly
<i>ACSL4</i>	300157	
<i>AGTR2</i>	300034	
<i>UPF3B</i>	300298	
<i>LAMP2</i>	309060	Danon disease
<i>GRIA3</i>	305915	
<i>OCRL</i>	300535	Lowe s.
<i>PHF6</i>	300414	Borjeson–Forssman–Lehmann s.
<i>ARHGEF6</i>	300267	
<i>SLC9A6</i>	300231	Christianson s.
<i>FMR1</i>	309550	Fragile X s.
<i>AFF2</i>	300806	
<i>SLC6A8</i>	300036	Creatine Transporter def.
<i>MECP2</i>	300005	Rett s.
<i>L1CAM</i>	308840	MASA s.
<i>RAB39B</i>	300774	Epilepsy, Macrocephaly

<sup>a</sup>If phenotype/syndrome not indicated, there are only “nonsyndromic” families noted to date.

Modified from Bentacur (62).

on physical examination, abnormalities on neuroimaging typically are not sufficient for determining the cause of the DD/ID; the underlying precise, and presumably frequently genetic in origin, cause of the brain anomaly is often left unknown. Thus, although a CNS anomaly (often also called a “CNS dysgenesis”) is a useful finding, and indeed may be considered a useful “diagnosis.”

**TABLE 37-6 Routine Metabolic Screening Tests in Patients with ID or ASDs****Bargraph depicting the yield of 'Metabolic Screening Tests'**

Summary of all treatable IEM (n=50/62%) which can be detected by 'Metabolic Screening Tests', each of which is affordable and accessible with the potential to identify at least 2 IEM (and up to 22). Each bar represents the yield of the specific screening test, and lists the number and types of treatable IEM it can identify.

**Urine Tests****Urine Organic Acids (n=22)**

- ▶ β-Ketothiolase Deficiency
- ▶ Cobalamin A Deficiency
- ▶ Cobalamin B Deficiency
- ▶ Cobalamin C Deficiency (& tHcy)
- ▶ Cobalamin D deficiency (& tHcy)
- ▶ Cobalamin F deficiency (& tHcy)
- ▶ Ethylmalonic Encephalopathy (&ACP)
- ▶ Glutaric Acidemia type I
- ▶ Glutaric Acidemia type II
- ▶ HMG-CoA Lyase Deficiency
- ▶ Holocarboxylase Synthetase Deficiency
- ▶ Homocystinuria
- ▶ L.o. Isovaleric Acidemia (&ACP)
- ▶ 3-Methylcrotonyl Glycinuria (&ACP)
- ▶ 3-Methylglutaconic Aciduria
- ▶ L.o. Methylmalonic Acidemia (&ACP)
- ▶ MHBD Deficiency
- ▶ mHMG-CoA Synthase Deficiency
- ▶ L.o. Propionic Acidemia (&ACP)
- ▶ SCOT Deficiency
- ▶ SSADH deficiency
- ▶ Tyrosinemia type II (&PAA)

**Urine Glycosaminoglycans (n=7)**

- ▶ Hunter syndrome (MPS II)
- ▶ Sanfilippo syndrome (type a, b, c, d)
- ▶ Hurler Syndrome (MPS I)
- ▶ Sly syndrome (MPS VI)

**Urine Creatine Metabolites (n=3)**

- ▶ AGAT deficiency
- ▶ Creatine Transporter Defect
- ▶ GAMT deficiency

**Urine oligosaccharides (n=2)**

- ▶ α-Mannosidosis
- ▶ Aspartylglucosaminuria

**Urine Purines & Pyrimidines (n=2)**

- ▶ Pyrimidine 5'nucleotidase superactivity
- ▶ Molybdenum Cofactor Type A deficiency

**Blood Tests****Plasma Amino-Acids (n=13)**

- ▶ L.o. Argininosuccinic Aciduria
- ▶ L.o. Citrullinemia
- ▶ L.o. Citrullinemia Type II
- ▶ L.o. CPS Deficiency
- ▶ L.o. Argininemia
- ▶ HHH syndrome
- ▶ Maple Syrup Urine Disease (Variant)
- ▶ L.o. MTHFR Deficiency (&tHcy)
- ▶ L.o. NAGS Deficiency
- ▶ L.o. OTC Deficiency
- ▶ Phenylketonuria
- ▶ PDH Complex Deficiency
- ▶ Tyrosinemia type II (&UOA)

**Plasma Total Homocysteine (n=9)**

- ▶ Homocystinuria (&UOA)
- ▶ L.o. MTHFR Deficiency (&PAA)
- ▶ Cobalamin C Deficiency (& UOA)
- ▶ Cobalamin D Deficiency (& UOA)
- ▶ Cobalamin E Deficiency
- ▶ Cobalamin F Deficiency (& UOA)
- ▶ Cobalamin G Deficiency

**Legend** Abbreviations: plasma amino acids (PAA), total homocysteine (tHcy), plasma acylcarnitine profile (ACP), urine organic acids (UOA).

For the mucopolysaccharidoses, enzyme activity should be measured as a next step: Hurler (Iduronidase); Hunter syndrome (Iduronate-2-sulphatase); Sanfilippo syndrome (IIa = Heparan-N-sulfatase, IIb = N-acetyl-glucosaminidase, IIc = Acetyl CoA glucosamine N-acetyl transferase, IIId = N-Acetyl-glucosamine-6-sulfatase); Sly syndrome = β-Glucuronidase

After Van Karnebeek and Stockler (80).

**TABLE 37-7 IEMs for which Molecular Genetic Testing is Required**

IEMs	Gene	OMIM Numbers
AGAT deficiency	AGAT	612718
Biotin responsive basal ganglia disease	SLC19A3	606152
Cerebral glucose transporter defect	SLC6A19	608893
Co enzyme Q10 deficiency	COQ2, APTX, PDSS1, PDSS2, CABC1, COQ9	609825, 606350, 607429, 610564, 612837
CPS deficiency	CPS	117700
Creatine transporter deficiency	SLC6A8	300306
Hyperinsulinism–hyperammonia syndrome	GDH	138090
MELAS	MTTL1, MTTQ, MTTT, MTTK, MTTT, MTTT1, MTND1, MTND5, MTND6, MTTT2	590050, 590030, 590040, 590060, 590020, 590080, 516000, 516005, 516006, 590085
NAGS deficiency	NAGS	608300
Niemann–Pick disease type C	NPC1 & NPC2	607623, 601015
Serine biosynthesis defects	PHGDH, PSAT, PSPH	606879, 610963, 172480
Sjögren–Larsen disease	FALDH	609523
Thiamine-responsive encephalopathy	SLC19A3	606152

**TABLE 37-8 Key Clinical Findings in ASDs**

Key Finding	Key Considerations
X-linked pedigree	<ul style="list-style-type: none"> <li>Fragile X testing</li> <li>Urine guanidinoacetate and creatine for disorders of creatine synthesis and/or transport</li> <li>XLID genetic testing; X-chromosome sequencing</li> </ul>
Affected siblings	None (see X-linked; nonspecific) Rare autosomal recessive gene: e.g. <i>HOXA1</i>
Female gender	Rett syndrome genetic testing ( <i>MECP2</i> , <i>CDKL5</i> )
Macrocephaly	<ul style="list-style-type: none"> <li>Sotos syndrome genetic testing</li> <li>PTEN-associated disorders (Riley–Ruvalcaba–Bannayan syndrome) particularly in males with genital freckling</li> <li>Brain MRI</li> </ul>
Microcephaly	<ul style="list-style-type: none"> <li>Brain MRI</li> <li>7-dehydrocholesterol for Smith–Lemli–Opitz syndrome</li> </ul>
Dysmorphology	<ul style="list-style-type: none"> <li>Testing for specific suspected syndrome</li> <li>Microarray CGH</li> </ul>
Short stature	<ul style="list-style-type: none"> <li>Testing for specific suspected syndrome</li> <li>Microarray CGH</li> <li>Other common metabolic tests</li> </ul>
Somatic overgrowth	<ul style="list-style-type: none"> <li>Fragile X testing</li> <li>Sotos syndrome</li> <li>PTEN-associated disorders testing</li> </ul>
Dermatologic findings	<ul style="list-style-type: none"> <li>Neurofibromatosis, type 1</li> <li>Tuberous Sclerosis</li> <li>PTEN-associated conditions</li> </ul>
Motor delays, tone and coordination disorders	<ul style="list-style-type: none"> <li>Metabolic screening</li> <li>Brain MRI, MRS</li> <li>Mitochondrial disorders</li> </ul>
Seizure disorder	<ul style="list-style-type: none"> <li>Neurology consultation</li> <li>Landau–Kleffner syndrome (overnight sleep EEG)</li> <li>Metabolic testing<sup>a</sup></li> </ul>
Coarse features, organomegaly	<ul style="list-style-type: none"> <li>Metabolic testing<sup>a</sup></li> </ul>

**TABLE 37-9 Primary Autosomal-Recessive Microcephaly Genes**

Gene	OMIM #	Percentage of Cases (%)	Citations
<i>MCPH1</i>	607117	<5	Jackson et al. (84)
<i>CDK5RAP2</i>	608201	<5	Bond et al. (85)
<i>ASPM</i>	605481	37–45	Bond et al. (37)
<i>CENPJ</i>	609279	<5	Bond et al. (85)
<i>STIL</i>	181590	<5	Kumar et al. (86)

It is, however, frequently not an etiologic or syndromic diagnosis in the usual sense of the term. This distinction is not always made in the literature on the utility of neuroimaging in the evaluation of children with DD/ID. The lack of a consistent use of this distinction has led to confusion regarding this particular issue.

Shevell et al. (5) reported a range of finding in their review. For example, in three studies totaling 329 children with developmental delay in which CT was used in almost all patients, and MRI was used in but a small sample, a specific cause was determined in 27–32% of

the children. In their systematic review of the literature, van Karnebeek et al. (17) reported nine studies on the use of MRI in children with ID. The mean rate of abnormalities found was 30%, with a range of 6.2% to 48.7%. These investigators noted that more abnormalities were found in children with moderate to profound ID versus those with borderline to mild ID (mean yield of 30% and 21.2%, respectively). These authors also noted that none of the studies reported on the value of the absence of any neurologic abnormality for a diagnostic work-up and concluded that “the value for finding abnormalities or the absence of abnormalities must be higher” than the 30% mean rate implied.

If neuroimaging is performed in only selected cases, such as those children with an abnormal head circumference or an abnormal focal neurologic finding, the rate of abnormalities detected is increased further than when utilized on a screening basis in children with a normal neurologic examination except for that the documentation of developmental delay.

Brain imaging is often useful in guiding the etiological investigations based on the specific CNS abnormalities noted on imaging. To illustrate just two examples,



**TABLE 37-10 Selected Syndromes with Agenesis of Corpus Callosum**

Syndrome	OMIM #	Gene(s)	Phenotype
Chondrodysplasia punctata 2, XL type	302960	<i>EBP</i>	Increased concentration of 8(9)-cholesterol, & 8-dehydrocholesterol
Donnai–Barrow s.	222448	<i>LRP2</i>	Hypertelorism, ocular coloboma, diaphragmatic hernia
Fumarate hydratase deficiency	136850	<i>FH</i>	Isolated increased concentration of fumaric acid on urine organic acid analysis
L1 syndrome	308840	<i>L1CAM</i>	Hydrocephaly, spasticity, adducted thumbs
Mowat–Wilson s.	235730	<i>ZEB2</i>	Distinctive facial features, Hirschsprung disease
Aicardi s.	304050	?	Chorioretinal lacunae, infantile spasms
Hereditary motor and sensory neuropathy with agenesis of corpus callosum	218000	<i>SLC12A6</i>	Mild to severe ID
Kallman s.	300836, 136350, 607123, 607002, 605806, 600483	<i>KAL1, FGFR1, PROKR2, PROK2, CHD7, and FGF8</i>	Anosmia, GnRH deficiency
X-linked Opitz/BBB syndrome	300000	<i>MID1</i>	Hypertelorism, hypospadias
Tetra-Amelia s.	273395	<i>WNT3</i>	Absence of all four limbs
Oro–facial–digital syndrome, type 3		<i>OFD1</i>	Cleft palate, lobed tongue, polydactyly, syndactyly

findings such as microcephaly (Tables 37-8 and 37-9) or agenesis of the corpus callosum (Table 37-10) can often lead to a specific genetic diagnosis.

### 37.6 SUMMARY

The developmental disabilities discussed in this chapter—ID and autism—are important public health problems. The accurate etiologic diagnosis provides the opportunity to improve health outcomes and quality of life as well as save health and social services costs. These are extremely heterogeneous conditions with varied responses to treatment. The specialist is able to provide the proper diagnostic approach for individual patients and families. In the near future, whole-genome sequencing may obviate the test-by-test approach of such expert clinicians and move the field of medical genetics to clarification of individual phenotypic variation and natural history as well as to personalize the treatment plans for such patients.

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### Biography



**John B Moeschler** is a professor of Pediatrics, Dartmouth Medical School and director of the Division of Medical Genetics at Dartmouth–Hitchcock Medical Center. His research interest is in the clinical description and delineation of genetic syndromes, birth defects and developmental disabilities; genotype–phenotype correlations; and the natural history of such conditions. His research has been in collaboration with those in many other disciplines including molecular genetics and genomics and genetic epidemiology. His research interest became focused on genetic aspects of neurodevelopmental disabilities in 1978 during a fellowship in developmental disabilities at the University of Washington. In 2005, he received his Master of Science degree from The Dartmouth Institute for Health Policy and Clinical Practice. This study stimulated his other research interest, health care services research, particularly in those factors that improve health and health care for those with genetic conditions.



# CHAPTER

# 38

## Abnormal Body Size and Proportion

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### 38.1 INTRODUCTION

Normal stature varies widely among ethnic groups, and also varies within each ethnic group, approximating a normal distribution. Short stature and tall stature are therefore relative terms and related to a person's ethnic, familial, and nutritional status. Before beginning a complex diagnostic evaluation or contemplating growth-promoting or growth-limiting therapy, one must be able to differentiate between pathologic short or tall stature and normal variants at each end of the normal range. Utilizing standardized growth curves allows for such differentiation. However, growth curves must be appropriate for the individual's genetic and ethnic background. In addition, allowances must be made for parental height, nutritional background, and the impact of chronic disease before an individual can be declared pathologically short or tall. One must also rule out constitutional delay or acceleration of maturation by correlating bone age and height. This normal variation in the timing of growth accounts for a large proportion of children referred for variations in growth. Adult height is a classic polygenic trait, influenced by genetic variants in at least 180 loci (1).

### 38.2 PATHOLOGIC SHORT STATURE

When it is established that a person is truly short for his or her genetic background, and does not simply have constitutional delay, the exact cause of the pathologic short stature must be delineated. If possible, it is essential that

a specific diagnosis be made because there are literally hundreds of causes of short stature that have differing prognoses, complications, and responses to treatments. A specific diagnosis is essential to provide appropriate treatment and accurate genetic counseling. The first step in the clinical evaluation of short stature is to determine whether the body habitus is proportionate or disproportionate (Figure 38-1). Children with disproportionate short stature usually have a skeletal dysplasia or metabolic bone disease (see Chapter 158), while those with proportionate short stature may have a more generalized disorder (i.e. malnutrition, chronic disease, psychosocial dwarfism, endocrine disorder, genetic syndrome, or chromosomal or teratogenic disorder, as discussed in Chapters 36, 43–45, and 83). Exceptions to this rule occur, and disproportionate dwarfism may occur in cases of severe cretinism, while proportionate shortening may occur in persons with osteogenesis imperfecta.

A mildly disproportionate body habitus may not be apparent on casual examination, and anthropometric measurements such as sitting height, upper/lower segment ratio, and/or arm span must be made before a relatively mild skeletal dysplasia (2) can be excluded. Once a person with short stature is found to be proportionate, it is helpful to determine whether the growth deficiency was of prenatal or postnatal-onset. Prenatal-onset growth deficiency usually implicates a fetal environmental insult or a generalized cellular genetic defect that may limit cellular mitosis.

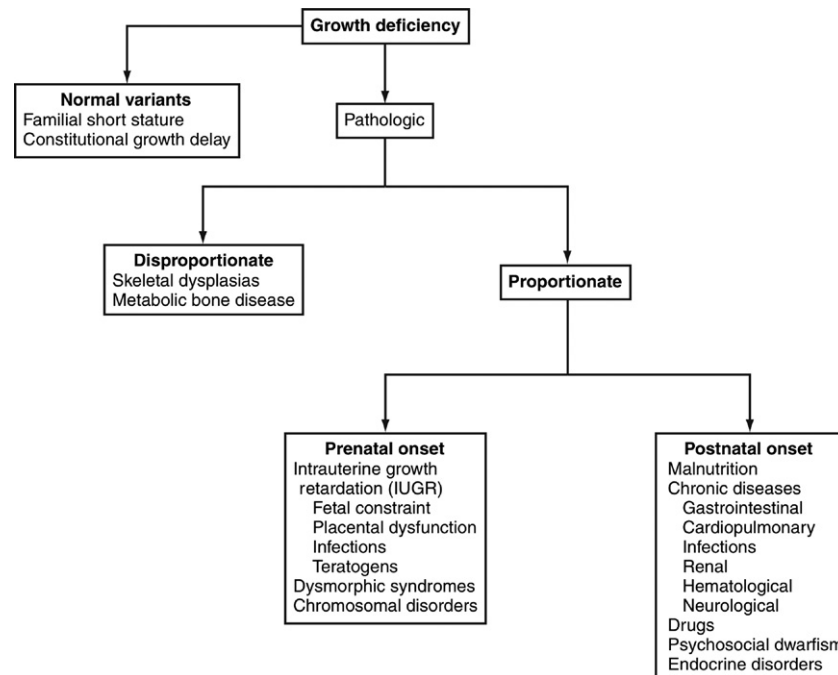


FIGURE 38-1 Classification of short stature.

Late fetal insults are more likely to demonstrate catch-up growth after birth when compared with environmental factors, which have an early impact in fetal life (3). On the other hand, postnatal-onset of proportionate growth deficiency usually implicates a postnatal environmental insult, such as infection, chronic disease, malnutrition, or an endocrine, psychological, or malabsorption disorder. These generalizations hold true whether or not the cause of the short stature is a single-system disorder, such as growth hormone deficiency or diabetes mellitus, or the short stature is associated with other abnormalities in a complex syndrome.

### 38.2.1 Proportionate Short Stature of Prenatal-Onset

Prenatal growth deficiency or intrauterine growth retardation (IUGR) can be due to numerous causes, both genetic and acquired. Mechanical late gestational constraint has been emphasized as the most common cause of prenatal growth deficiency (3). Most commonly, such small babies are born to small mothers with fathers of normal size, and they readily catch-up into the normal range within the first few months of postnatal life. Late fetal causes of growth deficiency, such as preeclampsia or placental insufficiency, usually show prompt resolution with postnatal catch-up growth. However, infants with disorders that adversely affect growth from early fetal life, such as chronic maternal hypertension, maternal diabetes mellitus with vascular changes, and heavy maternal smoking, may not show catch-up growth during the first six months of postnatal life. These conditions may result in diminished maternal blood flow to the placenta.

There are a wide variety of fetal malformation syndromes caused by single gene disorders, chromosomal

disorders, teratogens, or in utero infections in which fetal hypoplasia is part of the overall disorder (4,5) (Table 38-1). Infants with such malformation syndromes would not be expected to show catch-up growth, and some of these disorders can be associated with a high degree of perinatal lethality. Although etiologically heterogeneous, small size at birth may be associated with infant, childhood, and adult health implications (6–8). Long-term follow-up of over 1000 adults who were born small for gestational age (SGA) at term with no congenital abnormalities demonstrated small but significant deficits in academic achievement, with teachers less likely to rate them in the top 15th percentile of the class at age 16 years and more likely to recommend special education (9).

At age 26 years, those who had been born SGA were less likely to have professional or managerial jobs, with significantly lower levels of weekly income. Those born SGA with a small head size at age 5 years had substantially lower rates of professional attainment than those born SGA who had a normal head size at age 5 years. Also, adults born SGA had significant adult height deficits of approximately 5 cm (9). These findings have provided a rationale for treating infants with growth hormone, who were born SGA and did not catch-up in size by age 2 years (10). It is unknown if the lower rates of professional attainment in adults born SGA is due to their shorter stature or the underlying cause for their intrauterine growth deficiency, such as fetal alcohol syndrome, which would clearly impact both head size and professional attainment.

**38.2.1.1 Syndromic Disorders.** When the onset of growth deficiency is prenatal, one must differentiate between the numerous forms of intrauterine growth restriction (IUGR,

TABLE 38-1 Prenatal-Onset Growth Deficiency Disorders

Disorder	Key Clinical Findings		
Teratogenic disorders			
Fetal alcohol effects	Microcephaly, thin smooth philtrum, short palpebral fissures		
Fetal hydantoin effects	Ocular hypertelorism, hypoplastic nails, short nose		
Fetal trimethadione effects	Arched eyebrows, cupped helix, heart defects		
Fetal warfarin effects	Hypoplastic nose, stippled epiphyses, short limbs		
Fetal rubella effects	Deafness, cataracts, patent ductus arteriosus		
Fetal varicella effects	Cicatricial skin defects, limb hypoplasia, mental deficiency, seizures		
Maternal phenylketonuria effects	Microcephaly, cardiac defects, mental deficiency		
Chromosome Abnormalities			
Trisomy 18	Clenched hands, short sternum, low arch dermatoglyphic pattern		
Trisomy 13	Scalp defects, polydactyly, holoprosencephaly facies		
Triploidy	3-4 Syndactyly, dysplastic calvaria, cystic placenta		
4p syndrome	Ocular hypertelorism, hypospadias, preauricular pits		
5p syndrome	Cat-like cry, microcephaly, down-slanted palpebral fissures		
13q syndrome	Central nervous system defects, short thumbs, anal anomalies		
18p syndrome	Ptosis, protruding ears, mental deficiency		
18q syndrome	Prominent antihelix, midface hypoplasia, long palms		
45,X (Turner) syndrome	Lymphedema of hands and feet, webbed neck, broad chest with wide-set nipples		
Proportionate syndromes			
Disorder	Key Clinical Findings	Inheritance	Gene Testing
Brachmann–de Lange (122470)	Microcephaly, synophrys, thin downturned upper lip, small widely spaced teeth, micromelia, hirsutism, autistic tendencies, MR (IQ 30–102), occasionally with cardiac septal defects, hearing loss, myopia, gastrointestinal dysfunction, hypoplastic genitalia/cryptorchidism (99)	Sporadic, autosomal dominant X-linked (fewer than 1 percent pts have an affected parent)	3 genes encoding components of the Cohesin Complex: <i>NIPBL</i> (Nipped B-like) at 5p13.1 (100) <i>SMC1A</i> (segregation of mitotic chromosomes 1) gene at Xp11.22 (101) <i>SMC3</i> at 10q25
Rubinstein–Taybi (180849)	Normal prenatal growth with slowing of growth (height, weight and head circumference) in first few months of life, broad thumbs and halluces, down-slanted palpebral fissures, “Beaked nose” with prominent nasal septum, high arched palate, grimacing smile and talon cusp, moderate to severe mental retardation (IQ from 25 to 79), childhood/adolescent obesity. Variable features: cataracts, colobomas, congenital renal or cardiac defects, cryptorchidism (102)	Sporadic, autosomal dominant	<i>CREBBP</i> ( <i>Creb-binding protein</i> ) 16p13.3 (103) <i>EP300</i> at 22q13 (104)
Russell–Silver (180860)	IUGR with persistent postnatal growth deficiency, triangular face, normal-sized cranium, incurved (clinodactyly) 5th fingers, asymmetric limb length (may lead to decreased growth on the affected side with hemihypertrophy), risk of motor and cognitive developmental delay with learning disabilities (20)	Sporadic heterogeneous	10%—maternal disomy for 7p11.2 11p15 region: <i>H19</i> —maternal (105) <i>IGF2</i> gene (106)
Dubowitz (223370)	IUGR with postnatal growth deficiency, microcephaly with a high or sloping forehead and a broad and flat nasal bridge, flat or shallow supraorbital ridges, ptosis and blepharophimosis, with scarce lateral eyebrows, eczema, normal intelligence to mild to moderate MR, behavioral problems including hyperactivity (ADHD). Clinodactyly of the 5th fingers, and cutaneous syndactyly of the 2nd and 3rd toes. Genitourinary abnormalities may include hypospadias and cryptorchidism (107)	Autosomal recessive	<i>NSUN2</i> at 5p15.31 (107)

Continued

**TABLE 38-1 Prenatal-Onset Growth Deficiency Disorders—Cont'd**

Disorder	Key Clinical Findings	Inheritance	Gene Testing
Bloom (210900)	Microcephaly, malar erythema/sun sensitivity/telangiectasia, malar hypoplasia, Gastrointestinal Reflux (possibly contributing to infections of lung, middle ear and upper respiratory tract), sparse subcutaneous fat through infancy and early childhood, normal intelligence with poorly defined learning disability, early susceptibility to medical complications such as obstructive pulmonary disease, diabetes mellitus, and various cancers (12)	Autosomal recessive	<i>BLM</i> (DNA Helicase) at 15q26.1 (108) (quadriradial (Qr) in cultured blood lymphocytes and/or increased sister chromatid exchanges (SCEs) in any type of cultured cells)
Fanconi pancytopenia (227650)	Radial hypoplasia, hypoplastic thumbs, hyperpigmentation, pancytopenia (progressive bone marrow failure), increased risk of malignancy-acute myelogenous leukemia or myelodysplastic syndrome and solid tumors) (109). May have malformations of the kidneys/urinary tract, heart, GI system, oral cavity, CNS, ears (including hearing loss), developmental delay.	Autosomal recessive (110) Except <i>FANCB</i> mutations X-linked inheritance	Diagnostic test is detection of DNA aberrations when exposed to dipoxybutane (DEB) or mitomycin C (MMC) 15 Genes in the FA complementation group (111) ( <i>FANCA</i> -16q24.3, <i>FANCB</i> -Xp22.31, <i>FANCC</i> -9q22.3, <i>FANCD1</i> [ <i>BRCA2</i> ]-13q12.3, <i>FANCD</i> -3p25.3, <i>FANCE</i> -6p22-p21, <i>FANCF</i> -11p15, <i>FANCG</i> -9q13, <i>FANCI</i> -15q25q26, <i>FANCI</i> [ <i>BRIP1</i> ]-17q22, <i>FANCL</i> -2q16.1, <i>FANCM</i> -14q21.3, <i>FANCN</i> [ <i>PALB2</i> ]-16p12, <i>FANCO</i> [ <i>RAD51C</i> ]-17q22, and <i>FANCP</i> [ <i>SLX4</i> ]-16p13.3)
De Sanctis–Cacchione (278800)	Xeroderma pigmentosum, hypogonadism, microcephaly	Autosomal recessive	<i>ERCC6</i> (excision repair cross-complementing) gene at 10q11
Johanson–Blizzard (243800)	Hypoplastic alae nasi, microcephaly, midline scalp defects, sensorineural deafness, exocrine pancreatic insufficiency, nasolacrimal system malformations, absent permanent teeth, hypothyroidism (112)	Autosomal recessive	<i>UBR1</i> (ubiquitin protein ligase E3 component N-recognin 1) gene at 15q15-q21.1
Donohue leprechaunism (246200)	Severe IUGR adipose deficiency, thick lips, islet cell hyperplasia results in hyperinsulinemia	Autosomal recessive	<i>INSR</i> (insulin receptor) gene at 19p13.2
Seckel syndrome (210600, 606744, 608664, 61376)	IUGR with microcephaly, prominent nose, micrognathia	Autosomal recessive, genetically heterogeneous	<i>SCKL1-ATR</i> (Ataxia-telangiectasia and RAD3-related) at 3q22.1q24 <i>SCKL2</i> —18p11.31–q11.2 (113) <i>SCKL3</i> —14q21–q22 (114) <i>SCKL4</i> — <i>CENPJ</i> -13q12.2 (115)
Hallermann–Streiff (234100)	Microphthalmia, small pinched nose, hypotrichosis, dental anomalies, cutaneous atrophy	Sporadic	
Smith–Lemli–Opitz (270400)	Deficiency of enzyme 7-dehydrocholesterol reductase. Prenatal-onset growth deficiency with postnatal persistence secondary to abnormal cholesterol metabolism. Microcephaly, ptosis, cleft palate, syndactyly of 2nd and 3rd toes, postaxial polydactyly, hypospadias (116)	Autosomal recessive	<i>DHCR7</i> at 11q12-q13 (117) (diagnostic test is elevation of serum 7DHC)
Williams (130160)	Prominent lips, periorbital fullness, supraaortic stenosis (or other cardiovascular disease), mild MR, specific cognitive profile (overly friendly), endocrine abnormalities (118)	Sporadic, autosomal dominant	Genes at 7q11.2 <i>ELN</i> gene <i>LIMK1</i> <i>GTF2I</i> <i>MAGI2</i> (119) <i>HIP1</i> <i>YWHAQ</i>
Noonan (163950)	Webbed neck, pectus excavatum, pulmonary stenosis, hypertelorism, downward slanting palpebral fissures, motor delay	Autosomal dominant	<i>PTPN11</i> gene 12q24.1. Also <i>SOS1</i> , <i>KRAS</i> , <i>BRAF</i> , <i>SHOC</i>



**TABLE 38-1 Prenatal-Onset Growth Deficiency Disorders—Cont'd**

Disorder	Key Clinical Findings	Inheritance	Gene Testing
Aarskog (100050)	Ocular hypertelorism, optic nerve hypoplasia, retinal vessel tortuosity, deficient ocular elevation, hyperopia, and anisometropia (120) brachydactyly, shawl scrotum,	X-linked	<i>FGD1</i> gene at Xp11.2 (121)
Robinow (180700)	Broad forehead with flat facial profile, short forearms, hypoplastic genitalia	Autosomal dominant Autosomal recessive	<i>ROR2</i> on 9q22 (122)
Opitz (300000)	Ocular hypertelorism, hypospadias, swallowing difficulties secondary to abnormalities of the trachea/esophagus and larynx; also associated with cardiac anomalies, imperforate anus, and developmental delay; anteverted nares and posterior pharyngeal cleft were seen only in the X-linked form. (122)	Autosomal dominant & X-linked	<i>MID1</i> on Xp22
Coffin–Siris (135900)	Hypoplastic 5th digits and nails, coarse facies, hirsutism with sparse scalp hair, psychomotor delay/MR (123)	Autosomal dominant	<i>ARID1B</i> at 6q25 and other <i>SWI/SNF</i> subunit genes (123)

also known as fetal growth restriction) (3,5,11). This is a very heterogeneous group of disorders with multiple causes ranging from placental insufficiency to teratogenic disorders to specific genetic and chromosomal syndromes. When other causes of IUGR have been ruled out, and placental pathology supports the diagnosis, uteroplacental insufficiency is used to identify fetuses whose poor growth is due to an inadequate placental supply of nutrition. Declining body growth in response to inadequate intra-uterine supply with relative preservation of brain growth is viewed as an adaptive response to protect fetal brain development. There is no agreement whether a diagnosis of SGA should be based on racial, ethnic, or gender norms, but most SGA infants are identified on the basis of their birth weight for gestational age. Infants delivered preterm (as a group) are much smaller than fetuses that remained in utero and delivered closer to term, and these data suggest an overlap between preterm birth and IUGR. When preterm IUGR infants and infants of multiple gestations are born after 33–34 weeks gestation, they may have fewer complications of prematurity than expected for their gestational age, while infants born with IUGR before 34 weeks gestation have greater mortality and morbidity than preterm appropriate-for-gestational-age infants of the same gestational age. These factors must be taken into account while comparing growth parameters at birth with subsequent growth and development.

In many cases, a specific diagnosis of a recognizable syndrome can be made on the basis of associated characteristic clinical findings (e.g. Brachmann–de Lange syndrome (Figure 38-3A), Rubinstein–Taybi syndrome (Figure 38-3C), Russell–Silver syndrome (Figure 38-2), Johanson–Blizzard syndrome, Hallermann–Streiff syndrome, Williams syndrome (Figure 38-3B), Noonan syndrome (Figure 38-3D), or Aarskog syndrome (Figure 38-3E).) In some cases, limb malformations may represent distinctive features of the syndrome (e.g. Brachmann–de Lange syndrome

or Rubinstein–Taybi syndrome) (Table 38-1). Thus, the clinical evaluation and analysis of the dysmorphic features present in each case often permits reaching a conclusive diagnosis.

In certain syndromes, diagnosis can be supported by specific laboratory studies, such as an increased rate of sister chromatid exchanges in Bloom syndrome (now confirmed by mutation analysis of the DNA helicase gene *BLM*) (12); analysis of chromosome breakage in the Fanconi pancytopenia syndrome, which can be caused by mutations in at least 15 different genes (13); assessment of DNA repair genes in Cockayne syndrome, cerebro-oculofacial-skeletal syndrome (Figure 38-3F), and xeroderma pigmentosum (14,15); use of array comparative genomic hybridization (CGH) in Williams syndrome (Figure 38-3B) or velocardiofacial syndrome; and insulin receptor mutation analysis in leprechaunism (caused by mutations in the insulin receptor gene *INSR* at 19p13.2). As more genes for various syndromes become known, mutation analysis via clinical molecular testing will become more available and useful, but such testing seldom demonstrates mutations in 100% of cases, so clinical diagnosis is still the basis for diagnosing many recognizable syndromes. For families with a known mutation demonstrated in a research laboratory, this mutation can be confirmed in a clinical molecular laboratory and used for clinical purposes. Array CGH is rapidly replacing chromosome analysis and fluorescence in situ hybridization (FISH) analysis and can also be used to uncover the genomic basis for previously unknown causes of syndromic short stature, as well as to detect the deletion of a gene when mutation analysis of the causative gene demonstrates a normal sequence.

Some proportionate growth deficiency syndromes are inherited as autosomal recessive disorders, such as Bloom, Donohue, and microcephalic osteodysplastic primordial dwarfism type II (MOPDII), whereas others usually occur

(A)



(B)



**FIGURE 38-2** (A) This 3-year-old child with Russell–Silver syndrome had prenatal-onset growth deficiency that persisted after birth, triangular facial shape, fifth finger clinodactyly, mild skeletal asymmetry and reduced adipose tissue that placed this child at risk for fasting hypoglycemia. (B) This 4.5-year-old child with Russell–Silver phenotype did not respond to growth hormone and developed subtle skeletal signs of 3M syndrome.

sporadically, such as Hallermann–Streiff, Rubinstein–Taybi, Russell–Silver, Brachmann–de Lange, and Williams syndromes. Some disorders, such as Brachmann–de Lange syndrome and Noonan syndrome, are genetically heterogeneous and may be due to a new mutation. Others may result from either a gene mutation or a submicroscopic chromosomal deletion, as seen in Rubinstein–Taybi syndrome. In such cases, both mutation analysis and FISH or CGH analysis may be necessary, because if there is a whole-gene deletion and mutation analysis is undertaken, only the remaining normal allele will be sequenced and will be found to have a normal coding sequence.

**38.2.1.2 Chromosomal Disorders.** With the exception of sex chromosome aneuploidy, trisomy 21, and trisomy 8 mosaicism, most chromosomal disorders result in some degree of IUGR (see Chapters 43–45). In those syndromes compatible with survival beyond infancy, such as Turner syndrome and a variety of autosomal

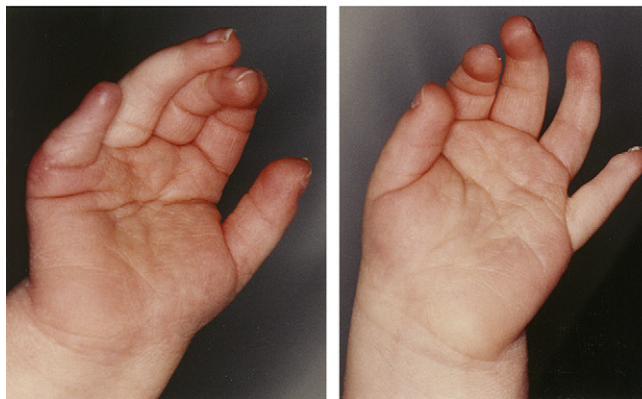
partial deletion or duplication syndromes, there is continued diminished growth throughout childhood and a blunted pubertal growth spurt as well. As the number of recognized chromosomal disorders increases because of new technologies, such as array CGH, it remains important to make an accurate clinical diagnosis to select the appropriate laboratory techniques. A patient with IUGR, developmental delay, and dysmorphic features who does not manifest a recognizable syndrome may have an underlying genomic disorder, which could be detected by array CGH. For example, deletion of 1q24q25 can result in primordial short stature with severe microcephaly due to deletion of the *CENPL* gene, a centrosomal gene (16), and deletion of 12q14 can result in short stature possibly secondary to the deletion of *HMGA2* (17).

In patients with a symptom complex suggestive of a specific chromosomal deletion syndrome (e.g. 4p- syndrome Figure 38-4 or 18q- syndrome Figure 38-5), it may be useful to begin the diagnostic evaluation with array CGH, particularly if previous chromosomal studies were normal. Some chromosomal syndromes may only be detectable in skin fibroblast samples (e.g. diploid–triploid mixoploidy or tetrasomy 12p). It is also important to remember that some girls with Turner syndrome, especially those with mosaicism, express few phenotypic features prepubertally, except for short stature. Thus chromosomal studies should be done on girls who appear to be short for their family background, with relatively normal body proportions, and for whom another diagnosis has not been made.

Several chromosomal deletion syndromes have also been found to be associated with growth hormone (GH) insufficiency, indicating the locations of genes or chromosomal regions that result in short stature. Since the *GH1* gene is on 17q22–q24, the growth hormone receptor *GHR* gene on 5p13–12, the *IGF1* gene on 12q22q24.1, and the *IGF1R* receptor gene on 15q25q26, it is not surprising that mutations or deletions of any one of these genes will result in growth deficiency, often on an autosomal recessive basis (18). Likewise, Donohue syndrome (leprechaunism) is due to mutations in the insulin receptor gene on 19p13.2, resulting in insulin resistance and IUGR, since insulin is a major fetal growth factor (Chapter 86). For some of the more common short stature and chromosome disorders, syndrome-specific growth curves are available through the Internet to aid in following these patients, and these curves are quite useful for detecting when secondary hormonal problems might be present. Growth deficiency in Turner syndrome has been attributed to haplo-insufficiency of the *SHOX* gene at Xpter-p22.32 (19), and altered imprinting on chromosome 7 or 11p15 has been identified in around half of patients with Russell–Silver syndrome (20). Autosomal recessive disorders such as 3M syndrome and Bloom syndrome also need to be considered in patients with the Russell–Silver phenotype.



(A)



(B)

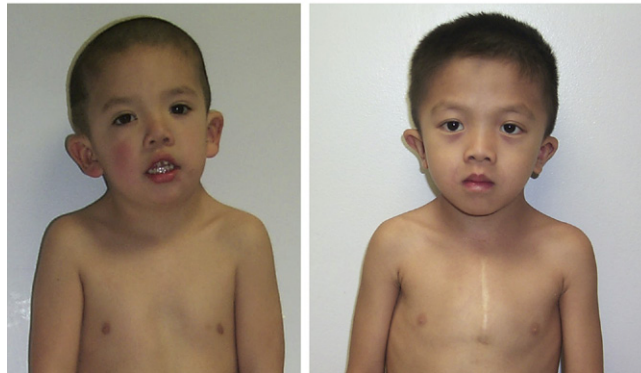


**FIGURE 38-3** (A) This child with classic Brachmann-de Lange syndrome has not had any testing to determine the cause for her disorder. (B) This child (left) with typical facial features for Williams syndrome and supravalvular aortic stenosis with peripheral pulmonic stenosis has an atypical 1.26–1.31 Mb deletion that includes *GTF2IRD1* but not *GTF2I*. She has typical visual-spatial construction defects without the usual increased eye contact and indiscriminant approach to strangers seen in children with the typical 1.55 MB deletion that includes *GTF2I*, illustrating the usefulness of chromosomal microarrays in understanding the clinical features of Williams syndrome. On the right are shown children with the typical deletion at different ages.

(C)



(D)



(E)



**FIGURE 38-3—cont'd** (C) This infant (top left) with Rubinstein-Taybi syndrome shows the characteristic facial features, with broad and often angulated thumbs and great toes, short stature, and moderate to severe mental retardation. The characteristic craniofacial features include downslanting palpebral fissures with nasal columella extending below the nares. The individual on the bottom left is shown at age 4 years and age 30 years with similar features due to a 169.92 kb deletion on 16p13.3 which included *CREBBP*. (D) These two children with Noonan syndrome show similar facial features with proportionate short stature, hypertrophic cardiomyopathy (left) and ASD with pulmonic stenosis (right). Both had feeding problems, with mildly delayed development later. (E) This child with Aarskog syndrome has proportionate short stature, ocular hypertelorism, anteverted nostrils, broad upper lip, with shawl scrotum, hyperextensible fingers, wide flat feet, ovoid periumbilical depression and ADHD.



(F)



**FIGURE 38-3—cont'd** (F) This 12-year-old boy with Cockayne syndrome (top) is the size of a two-year-old with typical features of Cockayne syndrome. These dizygotic twins with cerebro-oculofacial-skeletal (COFS) syndrome were from the Manitoba aboriginal family that was first reported with this syndrome. They were found to have a mutation in CSB, confirming that COFS syndrome and Cockayne syndrome are allelic (From Meira, L. B.; Graham, J. M., Jr, et al. *Am. J. Hum. Genet.* **2000**, 66, 1221–1228.)



**FIGURE 38-4** Two patients with 4p-syndrome (Wolf-Hirschhorn syndrome). Note ocular hypertelorism, with cleft lip in a patient on the left. Also present were hypospadias and preauricular pits with severe cognitive disability and intrauterine growth deficiency that persisted after birth.



**FIGURE 38-5** This patient with 18q-syndrome has typical mid-face deficiency, conductive hearing loss, deep-set eyes, prominent antitragus and long palms with proximally placed thumbs and long tapered fingers.

**38.2.1.3 Teratogens.** A variety of teratogenic exposures are associated with IUGR (Table 38-1) (see Chapter 36). Although maternal smoking may result in otherwise normal SGA babies, most other teratogens result

in well-characterized teratogenic syndromes. It must be stressed, however, that the clinical variability in these teratogenic syndromes is great, and it is the exceptional case that has all the features of a teratogenic syndrome. Therefore, a careful history of maternal drug and teratogen ingestion must be taken for all children who demonstrate IUGR. The most frequent teratogenic cause of growth deficiency is fetal alcohol syndrome (Figure 38-6). Maternal disease that can result in the accumulation of



**FIGURE 38-6** This 8-year-old girl with fetal alcohol syndrome has mild cognitive disability, ADHD, proportionate short stature, microcephaly, short palpebral fissures, short nose, thin smooth upper lip, and camptodactyly with mild fingertip and nail hypoplasia.

toxic substances that cross the placenta also can result in teratogenic effects on the fetus. This is clearly evident in the maternal phenylketonuria syndrome (5). Intra-uterine infections with rubella, syphilis, toxoplasmosis, and cytomegalic inclusion disease can produce prenatal growth retardation that results in postnatal proportionate short stature. Affected neonates may show systemic manifestations, including microcephaly, chorioretinitis, hepatosplenomegaly, petechiae, and seizures. The diagnosis should be suspected at birth, so that appropriate studies can be done to document the diagnosis and initiate therapy.

### 38.2.2 Proportionate Short Stature of Postnatal-Onset

Persons with postnatal-onset proportionate short stature usually have some kind of postnatal environmental insult, such as a chronic disease or an endocrine disorder, all of which may be associated with decreased insulin-like growth factor (IGF) or peripheral unresponsiveness to its action (Chapter 83). There may be direct suppression of hepatic IGF synthesis, deficient GH secretion with

secondarily reduced IGF production, inability to respond to GH stimulation of IGF, circulating IGF inhibitors, or peripheral unresponsiveness to the actions of IGF. These children usually have a bone age commensurate with, or even more severely retarded than, their height age, depending on the state of their gonadal and thyroid systems.

An accurate diagnosis is especially important for persons with this group of disorders because many of them will respond to specific therapeutic measures.

**38.2.2.1 Psychosocial Dwarfism.** In certain children, emotional disturbances may result in pronounced growth retardation (21). Although these children usually come from homes where marital discord, separation, alcoholism, and social upheaval are found, they can also be seen in well-to-do and seemingly well-adjusted families. There is usually a poor plasma GH response to GH stimulation tests, and levels of circulating IGF are low. Bone age maturation is usually greatly delayed. When removed from their adverse environment, these children usually show a striking catch-up in their bone age, and GH and IGF levels return to normal. Psychosocial dwarfism is thought to represent functional hypopituitarism in which psychological factors have produced a pituitary insufficiency through hypothalamic suppression. This condition appears to be relatively common, but it usually occurs in only one member of a family.

**38.2.2.2 Malnutrition.** Chronic malnutrition retards growth and is associated with reduced synthesis of IGF (22). The absence of an adequate substrate for growth may contribute to growth failure in malnutrition as well. Malnutrition resulting from malabsorption may also result in growth retardation (23). Asymptomatic celiac disease can result in proportionate growth retardation (24). Small-bowel biopsies are sometimes helpful in the workup of such children in whom other causes of growth retardation have been excluded.

**38.2.2.3 Chronic Disease.** Many chronic childhood diseases are associated with growth failure. Chronic liver disease, renal disease, celiac disease, regional enteritis, infectious disease, diabetes mellitus, hemoglobinopathies, asthma, congenital heart disease, and many others fit into this category. Often, reduced growth may be the dominant clinical feature. Low IGF levels are usually found, and, in addition, many patients are in a state of negative nitrogen balance. After cure or control of the basic disease, there is usually significant catch-up growth, and some children may ultimately reach a near-normal final adult stature, depending on the duration and severity of the disease, the age at the onset of the disorder, and its therapy. For example, children with celiac disease and persistent short stature may reach normal stature when treated with GH (24).

**38.2.2.4 Drug Administration.** In addition to various intrinsic causes of reduced human GH secretion, certain medications used in treating hyperactivity, such as methylphenidate hydrochloride or dextroamphetamine,



may alter the normal regulation of GH (25,26). Children often show reduced growth rates during the first few years after treatment commences, but tolerance to the growth suppression may develop later. Similarly, glucocorticoids used in treating certain diseases, such as asthma, nephrotic syndrome, juvenile rheumatoid arthritis, and leukemia, may significantly retard growth. The growth-suppressing effects of steroids may be seen with relatively small doses that are often thought to be harmless, such as prolonged use of topical ointments, nasal spray, or eye drops containing these compounds. The long-term effects of these and other drugs on final adult height have not been well established.

**38.2.2.5 Endocrine Disorders.** Many disturbances in the endocrine system can impair growth (18). Thyroid hormone deficiency can result in either proportionate or disproportionate short stature, depending on the degree of secondary epiphyseal dysplasia. Cushing disease can lead to proportionate short stature similar to that seen with the exogenous administration of steroids. Excess androgen secretion, as in the adrenogenital syndrome, can lead to accelerated growth with advanced bone age in early childhood but significant adult short stature resulting from premature closure of epiphyses (see Chapters 84 and 87). The prototype of endocrine short stature is GH deficiency. GH-related causes of short stature can result from many interruptions in the hypothalamic–pituitary–peripheral tissue axis. The various types of pituitary dwarfism can be classified on the basis of the level of the defect, whether genetic or acquired. If genetic, is it associated with an obvious developmental or degenerative disease of the hypothalamus? If pituitary, is the deficiency monotropic (isolated GH deficiency) or multitropic? Is it due to a defect in GH action; is IGF generation normal or defective (18)? The most common causes of acquired pituitary insufficiency are birth trauma, cranial irradiation for neoplasia, craniopharyngioma with the expanding tumor mass compromising pituitary function, and hemosiderosis following chronic transfusion therapy. In addition, trauma, surgical damage, infection, and sarcoidosis can also lead to pituitary insufficiency.

**38.2.2.6 Developmental Anomalies and Genetic Syndromes Associated with Hypopituitarism.** A number of developmental anomalies or genetic syndromes affecting the hypothalamus and pituitary may result in GH deficiency with or without other tropic hormone deficiencies (see Chapter 83). Many of these syndromes are associated with facial or optic anomalies. In certain disorders, such as congenital absence of the pituitary, empty sella syndrome, transsphenoidal encephalocele, and two rare syndromes associated with an unusual or large sella turcica, lateral skull radiographs may be sufficient to suspect the intracranial anomaly. In other disorders, however, such as holoprosencephaly and septo-optic dysplasia, magnetic resonance imaging of the brain and hypothalamus may be necessary to detect the malformations. Therefore, any

GH-deficient child with malformations of the face or eyes should have imaging of the brain and sella turcica to rule out a malformation affecting the hypothalamus or pituitary gland.

A number of genetic syndromes that are not associated with known developmental malformations of the hypothalamus or pituitary have pituitary insufficiency as a common component. Children with Pallister–Hall syndrome (hypothalamic hamartoma or hamartoblastoma, polydactyly, anal or renal anomalies, and facial changes) may present with hypopituitarism, and this disorder is caused by mutations of *GLI3* (27). In other disorders, such as histiocytosis X and hemochromatosis, the hormonal deficiencies are due to a degenerative disease of the hypothalamus and pituitary. In other syndromes, however, the pathogenesis of the pituitary insufficiency is unknown. Both sickle cell anemia and thalassemia are associated with delayed growth and sexual development that result in adult short stature. In thalassemia, this is apparently due to post-transfusion therapy, resulting in hemosiderosis that affects the pituitary. In persons with sickle-cell anemia, the exact cause of the short stature is unknown.

**38.2.2.7 GH Deficiency Unassociated with Developmental Anomalies or Multisystem Genetic Syndromes.** The most common form of GH deficiency is the “idiopathic type” (i.e. one in which no organic lesions exist). This heterogeneous group of disorders can be classified on the basis of whether only GH is deficient or there are multitropic hormone deficiencies, and if it is genetic or acquired. In addition, these disorders must be distinguished from the various forms of IGF or GH resistance. Prenatal problems, such as breech or forceps deliveries, early vaginal bleeding, or prolonged or unusually short labors, frequently occur in persons with acquired hypopituitarism, as do signs of intrapartum fetal stress or anoxia. It has been suggested that these perinatal insults lead to the hypopituitarism in the nongenetic forms of this disease. The many genetic forms of GH deficiency or resistance are discussed in Chapter 83.

### 38.2.3 Disproportionate Short Stature

If a disproportionate body habitus is found on physical examination, the patient is likely to have a form of skeletal dysplasia (Chapter 158). This is a heterogeneous group of heritable disorders affecting skeletal connective tissues in which the predominant clinical feature is dwarfism.

The osteochondrodysplasias have been divided into 37 groups of disorders and 3 groups of dysostoses based on clinical and radiographic features (2). Furthermore, each of these disorders is associated with a variety of skeletal and nonskeletal complications, so that an accurate diagnosis will help the clinician to develop a realistic treatment plan. These osteochondrodysplasias and dysostoses have also been classified

using molecular, pathogenetic, and developmental approaches based on the structure and function of the causative genes and proteins; these classification systems are discussed in detail by Warman et al. (2). As illustrated in Figure 38-1, the diagnostic workup for short stature should be approached rationally, before determining the mode of therapy. Disproportionate short stature may require a therapeutic approach entirely different from what is used for proportionate short stature.

Likewise, prenatal-onset disorders must be separated from postnatal-onset disorders, and each category of growth deficiency will manifest different responses to specific treatments. In general, prenatal-onset growth deficiency that persists after birth is generally assumed to be due to a genetic, syndromal, cytogenetic, or teratogenic disorder that results in generalized hypoplasia or disproportionate skeletal hypoplasia. Growth deficiency that is secondary to chronic disease, endocrine insufficiency, nutritional insufficiency, or psychosocial deprivation responds best to treatment of the primary disease state. Recurrence risk counseling must also be individualized based on the underlying diagnosis.

The use of more advanced molecular genetic and cytogenetic techniques has augmented the clinical diagnosis of known syndromes and has helped define clinically recognizable syndromes based on gene mutation analysis and copy number variants that were previously undetectable using standard cytogenetic techniques (16,17).

### 38.3 PATHOLOGIC OVERGROWTH

The array of pathologic conditions that can result in overgrowth is more restricted than the numerous conditions that have a negative impact on growth. There are a number of growth factors that function at various times during development and impact the growth of specific tissues in either a generalized or localized way. Similarly, other factors function to suppress growth. Thus, overgrowth disorders result in either localized or generalized effects on growth.

As with short stature, the pathologic determination of overgrowth should be made in relation to family and ethnic background. Once a careful assessment is made and pathologic overgrowth is apparent, every effort should be made to reach an accurate diagnosis in order to provide adequate medical management and genetic counseling. Most prenatal overgrowth disorders persist after birth, and, in some cases, mental deficiency, risk for tumor development, or both, are important associated features. The risk for tumor development usually involves embryonal neoplasms, perhaps related to overexpression of growth factors or lack of suppressing factors. A comprehensive review of overgrowth disorders has been presented in *Gorlin's Syndromes of the Head and Neck* (4).

#### 38.3.1 Generalized Overgrowth Disorders

The optimum birth weight for the lowest perinatal mortality and morbidity is 3500–4000g, with 5% of newborns exceeding 4000g and 0.5–1% exceeding 4500g (28). Factors related to large fetal weight include genetic predispositions; excessive maternal weight gain, obesity, and diabetes; postmaturity; and multiparity (29). Although many macrosomic infants demonstrate proportionate overgrowth, infants of diabetic mothers are often disproportionately overgrown, with increased weight-to-length ratios. Infants born to diabetic and/or obese mothers also have an increased risk for certain malformations (30,31). Some infants with anasarca are macrosomic primarily due to increased extracellular fluid volumes. True somatic overgrowth is often accompanied by increased placental size, as is commonly seen in Beckwith–Wiedemann syndrome (BWS) (32,33).

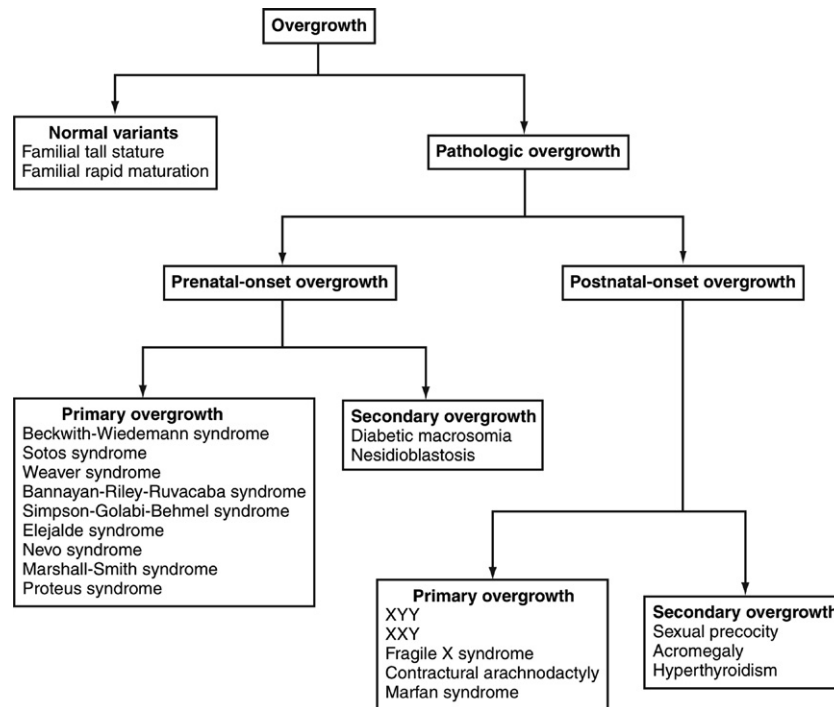
At the cellular level, the onset of most prenatal overgrowth results from (1) excessive cellular proliferation (hyperplasia), (2) excessive cellular size (hypertrophy), (3) an increase in the interstitium (e.g. anasarca), or (4) a combination of these factors. Overgrowth conditions can be subdivided between normal variants, such as familial tall stature or familial rapid maturation, prenatal-onset growth excess, or postnatal-onset growth excess (Figure 38-7). However, primary growth excess results from intrinsic cellular hyperplasia, while secondary growth excess is due to humorally mediated factors outside the skeletal system.

Among pathologic overgrowth disorders, most prenatal-onset growth excess is of the primary type, as is seen with BWS or Sotos syndrome. Secondary growth excess of prenatal-onset is usually not syndromic. The best examples include diabetic macrosomia and congenital nesidioblastosis (34).

In this disorder, a diffuse abnormality of the pancreas results in extensive and often disorganized formation of new islets of Langerhans, overproduction of insulin, and consequent severe neonatal hypoglycemia requiring pancreatectomy. Persistent hyperinsulinemic hypoglycemia of infancy is caused by mutations in the beta-cell high-affinity sulfonylurea receptor gene (*SUR1*) (35) or in the inwardly rectifying potassium channel gene (*KCNJ11*), and both these genes are located on 11p15, where the phenotype of persistent hyperinsulinemic hypoglycemia of infancy has been mapped (36). Autosomal dominant familial hyperinsulinism has been shown to be due in some instances to an activating mutation in the glucokinase gene (*GCK*) or, associated with hyperammonemia, in the *GLUT1* gene (37).

Postnatal-onset growth excess is usually secondary, involving overproduction of estrogens or androgens (precocious puberty), overproduction of GH (acromegaly), or overproduction of thyroid hormone. A few primary excess growth disorders manifest the majority of their overgrowth in the postnatal period (e.g. XYY syndrome,





**FIGURE 38-7** Classification of overgrowth disorders.

XXY syndrome, fragile X syndrome, and Marfan syndrome). The management of these syndromes is discussed in detailed in Chapters 44, 107, and 153.

**38.3.1.1 Prenatal Overgrowth Syndromes.** It is important to recognize the major diagnostic features for prenatal-onset overgrowth syndromes. Because of their importance and frequency, a few of these disorders are discussed in more detail in the following sections.

**38.3.1.2 Cerebral Gigantism (Sotos Syndrome).** Although the exact prevalence is not known, Sotos syndrome is estimated to be between 1:10,000 and 1:50,000; the diagnosis is largely based on clinical examination, and the features have been well described (38). Cerebral gigantism is associated with prenatal-onset overgrowth and affects length more than weight. Rapid linear growth, more pronounced in the first year of life, is accompanied by variable advanced osseous maturation and large hands and feet (39). The head is usually large and dolichocephalic with frontoparietal balding. The facial features are distinctive: the round face with a disproportionately prominent forehead seen in infancy elongates with age to a slim face with a pointed chin seen in adolescence (40). Other distinctive facial features include flushed cheeks and premature eruption of teeth (41) (Figure 38-8).

Complications commonly seen in the neonatal period include jaundice secondary to hyperbilirubinemia or hypoglycemia, often associated with hypotonia and difficulties with feedings secondary to trouble sucking or swallowing and gastroesophageal reflux (40). In addition, there are a number of neurological features to Sotos syndrome including developmental delay affecting

the development of both motor and speech functions, with associated clumsiness, and electroencephalographic abnormalities. There is a wide spectrum of psychosocial and behavioral problems with some degree of learning difficulties and risk of developing attention deficit disorder, hyperactivity disorder, and temper tantrums (42–44). On cranial imaging, there is delayed or underdevelopment of the brain, particularly of midline structures (45). Defects most commonly seen include dilation of cerebral ventricles (46) and hypoplastic or absent corpus callosum (47). Other defects seen in Sotos syndrome may include cardiac defects, usually atrial septal defect or patent ductus arteriosus (PDA) (46,48), and genitourinary anomalies (41). Although difficult to predict, final height tends to normalize after puberty with the most prominent feature seen in adults being macrocephaly (38).

Most cases are sporadic occurrences in otherwise normal families, with associated increased paternal age and de novo point mutations or whole-gene deletion of *NSD1* on 5q35. Roughly 93% of cases of Sotos syndrome result from a mutation of *NSD1*. In Japan, the frequency of mutations is marginally lower (83% *NSD1* mutations) and deletions are more frequent (10%) (49). Autosomal dominant inheritance has been demonstrated in some cases, with the affected parent showing mild mental retardation, long facies, and large hands and feet, but without tall stature (38). Based on a review of the literature, the tumor frequency of patients with Sotos syndrome is 3.9% (4).

**38.3.1.3 Weaver Syndrome.** In Weaver syndrome, overgrowth is usually evident at birth, with most affected

(A)



(B)



**FIGURE 38-8** (A) This 3-year-old girl with Sotos syndrome demonstrates typical facial features macrocephaly, dolichocephaly, frontoparietal balding, down-slanted palpebral fissures, flushed cheeks, prominently pointed chin, with premature eruption of teeth and accelerated osseous maturation. (B) Both these boys have Sotos syndrome with similar facial features, but the boy on the left has a NSD1 mutation with normal cognitive skills and the boy on the right is deleted for NSD1 with cognitive disability, a tethered cord and sacral malformations.

individuals at or above the 97th percentile for length and weight by age 1 year (4). Overgrowth continues throughout life, with adult height and weight above the 97th percentile, and markedly accelerated osseous maturation during childhood. Most affected individuals are mentally deficient. Their distinctive features include hypertonia, hoarse low-pitched cry, macrocephaly, round face, ocular hypertelorism, downslanting palpebral fissures, long philtrum, large ears, micrognathia, camptodactyly, clinodactyly, broad thumbs, prominent fingertip pads, and limited elbow and knee extension (Figure 38-9). Younger children with Weaver syndrome have been known to have difficult airways to intubate secondary to the relative micrognathia, anterior and cephalad positioning of the larynx, and a relatively short neck; this difficulty seems to decrease with the growth of the mandible over



**FIGURE 38-9** Both these unrelated boys have Weaver syndrome at ages 18 months and 11 months (From Weaver, D. D.; Graham, C. B.; Thomas, I. T.; Smith, D. W. A New Overgrowth Syndrome with Accelerated Skeletal Maturation, Unusual Facies, and Camptodactyly. *J. Pediatr.* **1974**, 84, 547–552.)



**FIGURE 38-10** This infant with Beckwith-Wiedemann syndrome was born macrosomic with macroglossia, omphalocele, glabella, nevus flammeus, infraorbital creases, and posterior auricular ear pits. These are typical features in this common overgrowth disorder.

time (50). Frequency of solid tumors and hematologic malignancies among reported literature cases was found to be 10.9% (51), but this may be an overestimate based on a failure to report cases of Weaver syndrome uncomplicated by malignancy. Weaver syndrome is caused by *de novo* mutations in *EZH2* (including one of the original two patients on Figure 38-9 (125).

**38.3.1.4 Beckwith-Wiedemann Syndrome.** BWS is the most common congenital overgrowth syndrome, with a frequency of 1 case per 13,700 births (33). It is associated with abdominal wall defects, macroglossia, prenatal-onset overgrowth, neonatal hypoglycemia, and visceromegaly. Other distinctive features include earlobe creases and pits, facial nevus flammeus, and infraorbital creases (Figure 38-10). Patients with BWS may have elevated serum alpha fetoprotein (AFP) levels postnatally, which tend to decrease over the first year of life at a slower rate than in normal children, (53) but this



elevated AFP is not yet attributed to an increased risk for hepatoma or other known causes of elevated AFP in the general adult population. Large size at birth is associated with placental overgrowth, long umbilical cord, and polyhydramnios, which may result in premature delivery, contributing to an increased perinatal mortality (54). The large size for age continues through early childhood and is associated with accelerated osseous maturation, but overgrowth is usually no longer evident by adulthood. Prompt recognition of this disorder is important because of the dangers associated with hypoglycemia and prematurity in overgrown neonates and associated risk for embryonal neoplasms affecting abdominal organs (primarily nephroblastoma, adrenocortical carcinoma, and hepatoblastoma). Intelligence is usually normal in the absence of complications related to prematurity and/or neonatal hypoglycemia.

BWS is caused by genetic alterations in chromosomal region 11p15, an area that is subject to imprinting (it is also known as the BWS critical region) and functionally separated into two regions: imprinting cluster 1 (IC1), regulating the expression of *IGF2* and *H19*, and IC2, regulating the expression of *KCNQ1*, *CDKN1C*, and *KCNQ10T1*. These regions are differentially methylated (IC1 is normally methylated on the paternal 11p15, and IC2 is normally methylated on the maternal 11p15). For example, *IGF2* is a paternally active growth-enhancing gene with imprinting of the maternal allele, while *H19* appears to be a maternally active growth suppressor gene with imprinting of the paternal allele. About 80% of cases of BWS can arise through one of the following molecular presentations (as detected by genetic testing):

- (1) Methylation at IC2 on maternal chromosome is abnormal/lost (~50 percent cases BWS) (54).
- (2) There is gain of methylation at IC1 on the maternal chromosome (~5 percent of BWS cases) (54).
- (3) There is mutation of the maternal *CDKN1C* allele (in 40% of patients with a positive family history of BWS) (55). Cleft palate can be associated with BWS when there are mutations in the gene (54).
- (4) Cases have paternal uniparental disomy of 11p15 (about 20% of cases) (56) (Figure 38-11) or maternal translocations or inversions with breakpoints in 11p15.

Familial cases (15% of all people diagnosed with BWS) are inherited in an autosomal dominant fashion with incomplete penetrance, and most are born to female carriers. Sporadic cases can arise through de novo germ line or somatic mutations. Among cases with normal chromosomes, paternal uniparental disomy is present in 10–20% of cases, and somatic mosaicism for partial paternal isodisomy occurs in some cases of hemihyperplasia (54). About half of the sporadic cases show biallelic expression of *IGF2*. Hemihyperplasia (Figure 38-12) rarely occurs in familial cases, and hemihyperplasia and neoplasms seem to occur more frequently in sporadic



**FIGURE 38-11** This fetus has a paternally derived duplication of 11p15 with deletion of 18q- due to inheriting an unbalanced 11p;18q chromosomal translocation. She shows features of both disorders with large size and macroglossia plus clubfeet and abnormal hands.

cases, possibly because of an imbalance in the biallelic expression of *IGF2* and *H19*. Paternal trisomy or uniparental disomy for 11p15 could lead to an excess of expressed paternal alleles, while relaxation of the maternal imprint for 11p15 might give rise to biallelic expression of *IGF2* and/or suppression of *H19*. Overexpression of *IGF2* appears to be the most common pathogenetic mechanism for prenatal overgrowth.

Because of the risk of tumor development in BWS and in some common types of congenital hemi-hypertrophy, Shuman et al. (54) recommends the following screening protocol: (1) baseline abdominal computed tomography scan at age 6 months (or at the time of initial diagnosis);



**FIGURE 38-12** This boy was born with hemihyperplasia of the right side of his body, including his tongue. These features are usually sporadic and noninheritable. They are typical of the features seen in paternal uniparental disomy for 11p15, which is with the highest risk for hepatoblastoma and Wilms tumor.

(2) abdominal ultrasound every 3 months until age 7 years, then every 6 months until completion of skeletal growth; (3) abdominal palpation and urinalysis every 3 months until age 7 years, then every 6 months until skeletal maturity; and (4) annual analysis of serum AFP because of the risk of hepatic tumor development. Patients with hemihyperplasia may need more frequent determinations because of their increased risk for hepatoblastoma (54).

**38.3.1.5 Simpson–Golabi–Behmel Syndrome.** This distinctive X-linked syndrome, with partial expression in female carriers, was confused with BWS in earlier reports because of striking prenatal-onset overgrowth, which persists into adulthood. Features of this syndrome, which can aid in prenatal diagnosis, include an increased amniotic fluid AFP and increased nuchal translucency accompanied by body wall edema. By 30 weeks of gestation, macroglossia, polyhydramnios, nephromegaly, and hepatosplenomegaly, in addition to macrosomia, may also aid with prenatal diagnosis (57). Distinctive features include congenital macrocephaly, normal intelligence to severe mental deficiency (language delay most characteristic), ocular hypertelorism with short broad nose, cleft palate, wide mouth, central groove of the lower lip, supernumerary nipples, pectus excavatum, rib and vertebral abnormalities, hypotonia, postaxial polydactyly of the hands, nail hypoplasia (especially involving the index fingers) (58), partial cutaneous syndactyly, cryptorchidism, heart defects, and large cystic kidneys (59). Malformations of the central nervous system (CNS) include agenesis of the corpus callosum, Chiari malformations and hydrocephalus (60), and aplasia of the cerebellar vermis (59). Features in common with BWS include prenatal overgrowth, macroglossia, earlobe creases and pits, umbilical hernia, intestinal malrotation, and hypoglycemia from excessive

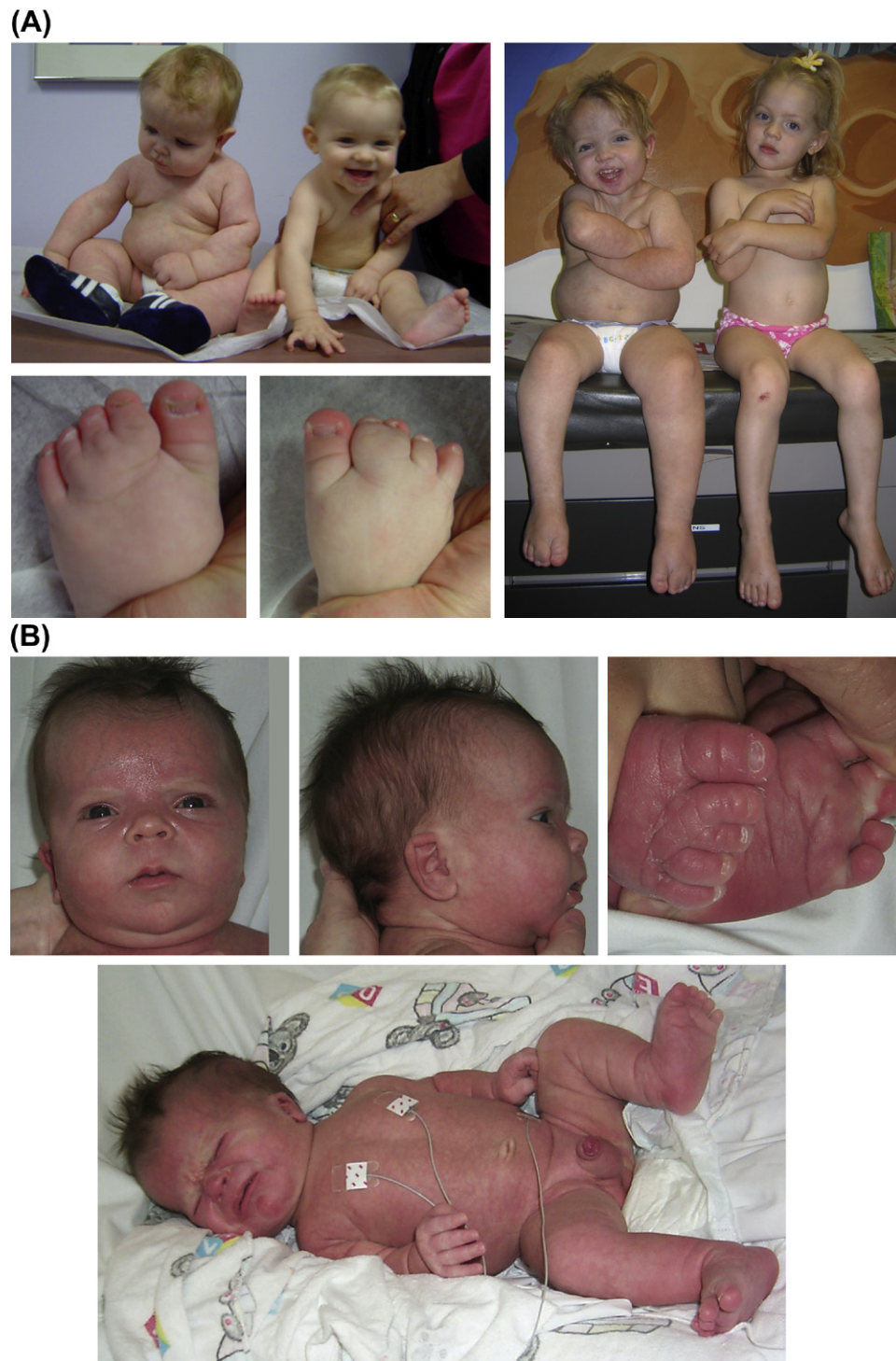
islets of Langerhans. Infant mortality is approximately 50%, occurring primarily from cardiorespiratory causes. This disorder results from mutations in the cell surface heparan sulfate proteoglycan glypican gene *GPL3*, which appears to play a role in controlling the growth of mesodermal tissues and binds to *IGF2*, whereby it helps to modulate its action (61). The reported frequency of tumors is 10% (59,62), including Wilms tumor, hepatoblastoma, adrenal neuroblastoma, gonadoblastoma, and hepatocellular carcinoma (63).

**38.3.1.6 PTEN Hamartoma Tumor Syndrome.** *PTEN* hamartoma tumor syndrome is an umbrella term used to cover the continuum of autosomal dominant overgrowth conditions including Cowden syndrome and Bannayan–Riley–Ruvalcaba syndrome associated with multiple hamartomas and detectable mutations of the *PTEN* gene (64,65). Cowden syndrome is a hamartomatous disorder with pathognomic features including cerebellar dysplastic gangliocytoma (adult Lhermitte–Duclos disease) and mucocutaneous lesions including facial trichilemmomas, acral keratoses, mucosal lesions, and papillomatous lesions. Other major features of this syndrome include macrocephaly, carcinoma of the thyroid (particularly follicular) and endometrium, and breast cancer. Occasional features include vascular malformations, lipomas, hamartomatous intestinal polyps, pigmented penile macules, Hashimoto thyroiditis (or other thyroid lesions), fibrocystic disease of the breast, uterine fibroids, and genitourinary malformations or tumors (especially renal cell carcinoma) (64).

Bannayan–Riley–Ruvalcaba syndrome is much rarer and has a classic triad of macrocephaly, intestinal polyposis, and genital lentiginosis (66). Birth length and weight are usually greater than the 97th percentile, although growth tends to normalize by midchildhood, with the final height within the normal adult range. Bone age is usually consistent with chronologic age, and macrocephaly persists into adulthood with normal ventricular size. Delayed gross motor development may be associated with a lipid storage myopathy, which appears to respond to treatment with carnitine. About 85% of patients with clinical features of Cowden syndrome (67) and 65% of patients with Bannayan–Riley–Ruvalcaba syndrome (67) have detectable *PTEN* gene mutations (68). *PTEN* is a tumor suppressor gene that functions as a dual-specificity phosphatase trafficking within and outside the nucleus (69,70). The nuclear functions of *PTEN* suggest it may be a genome stabilizer (71), autoregulating its activity through stabilization of p53, another tumor suppressor gene (72). Loss of the *PTEN* gene leads to the progression of brain tumors from benign to malignant secondary to the induction of angiogenesis (65,73).

**38.3.1.7 Megalencephaly Capillary Malformation Syndrome.** Moore et al. (74) initially proposed a sporadic distinct clinical syndrome of megalencephaly with capillary malformations (M-CAP) with features of cutis marmorata, nevus flammeus, CNS malformations with neurologic abnormalities, asymmetric growth,





**FIGURE 38-13** (A) One of dizygotic twins with megalecephaly capillary malformation syndrome at 12 and 40 months showing typical macrocephaly with capillary malformations fading and postnatally decreased growth in comparison with his twin sister. (B) Typical facial and limb features of macrocephaly capillary malformation syndrome at birth. The child experienced massive brain overgrowth with acquired tonsillar herniation requiring neurosurgical intervention and polymicrogyria resulting in seizures.

and macrocephaly (Figure 38-13). Thus macrocephaly-capillary malformation syndrome has come to be characterized clinically by prenatal-onset overgrowth with variable features of cerebral and somatic asymmetry, macrocephaly, neonatal hypotonia, cutaneous vascular malformations, and an abnormal neurocognitive profile

(75,76) and characteristic facial features. Conway et al. (77) analyzed the neurological features and underlying magnetic resonance imaging (MRI) findings in 17 cases of M-CAP syndrome and found that over half the patients have acquired cerebellar tonsillar herniation (versus congenital Chiari I malformation) with dilated

dural sinuses and ventriculomegaly secondary to rapid brain growth and progressive crowding of the posterior fossa often accompanied by (obstructive) hydrocephalus. This disorder is caused by post-zygotic mosaic mutations in *PIK3CA* (128).

### 38.3.1.8 Array CGH and Overgrowth Syndromes.

Array CGH has been found to be useful in the diagnosis of unknown overgrowth disorders (78). Among genetic causes of syndromic overgrowth, chromosomal deletions and duplications such as *dup(4)(p16.3)*, *dup(15)(q26qter)*, *del(9)(q22.32q22.33)*, *del(22)(q13)*, and *del(5)(q35)* have been identified, as well as a number of other rare copy number variations (CNVs), emphasizing the usefulness of this technique in unknown overgrowth disorders (78). Tatton-Brown et al. (79) reported a series of five patients with trisomy or tetrasomy of 15q, all of whom shared clinical features of overgrowth and learning difficulties with characteristic facial features including a long, thin face and prominent chin and nose. Three of these patients had renal anomalies including renal agenesis, horseshoe kidney, and hydronephrosis. Dolan et al. (80) reported a series of five patients with a Sotos-like syndrome and copy number variants within 19p13.13. The four patients with a deletion shared a phenotype of overgrowth with macrocephaly, ophthalmologic anomalies, gastrointestinal abnormalities, and learning disabilities. The patient with a duplication within 19p13.13 had microcephaly and short stature, with height affected more than weight. The smallest region of overlap a 311–340kb segment from base pair 12,793,474 to base pair 13,104,643 enriched with 16 genes including *MAST1*, *NFIX* and *CALR*. Malan et al. (81) demonstrated that mutations in *NFIX* resulted in the same phenotype, and that patients with Marshall–Smith syndrome also had mutations in *NFIX*. Marshall–Smith syndrome is an overgrowth syndrome characterized by accelerated osseous maturation; failure to thrive with limited life expectancy, usually secondary to respiratory dysfunction; hypotonia, moderate to severe developmental delay; and craniofacial anomalies including a prominent forehead, shallow orbits resulting in prominent eyes, blue sclere, depressed nasal bridge, and micrognathia (82,83). Diab et al. (84) underscored osseous fragility as an important aspect of this syndrome, and recent studies emphasize multiple nontraumatic fractures as significant clinical complication (82,85). Shaw et al. (83) found no copy number variants of clinical significance in a series of 12 patients with Marshall–Smith syndrome.

## 38.3.2 Segmental Overgrowth Disorders

Some disorders result in segmental or regional overgrowth (e.g. familial macrocephaly, congenital hemihyperplasia, Klippel–Trenaunay syndrome (KTS), Maffucci syndrome, Ollier disease, Patterson–David syndrome, and Proteus syndrome). Parameter-specific overgrowth disorders contrast with generalized overgrowth disorders

in that usually only a single growth parameter (e.g. weight) is affected. Examples of such disorders include those associated with obesity (e.g. Prader–Willi syndrome, Cohen syndrome, Börjeson–Forssman–Lehmann syndrome, Bardet–Biedle syndrome, and familial idiopathic obesity).

**38.3.2.1 Klippel–Trenaunay Syndrome.** KTS is a rare sporadic triad of congenital anomalies including cutaneous capillary malformations of a limb, venous malformations, and accompanying segmental hypertrophy of soft tissue and/or bone (86) or hypotrophy of a limb (usually lower limb) (87). Parkes–Weber syndrome (PWS) includes a clinically significant arteriovenous fistula (88). Ultrasound (Doppler) is used to distinguish KTS from PWS, as the prognosis and treatment are different, with magnetic resonance (MR) angiography as the gold standard for diagnosis to confirm involvement of superficial or deep venous involvement (87,89). Enoxaparin maintenance and recombinant coagulation factor VIIa have been successfully used to control potentially fatal consumptive coagulopathies resultant from the arteriovenous malformations (AVMs) of PWS (90).

**38.3.2.2 Proteus Syndrome.** Proteus syndrome consists of disproportionate or segmental overgrowth of limbs and macrocephaly associated with cranial hyperostoses, plantar hyperplasia; hemangiomas, lipomas or lymphangiomas, verrucous epidermal nevi, and variable psychomotor deficiency. Overgrowth and asymmetry are occasionally present at birth in 17.5% of cases (91). Among 205 reported cases of Proteus syndrome, which were reviewed to reassess the application of published diagnostic criteria, Turner et al. (91) concluded that there was overlap with other overgrowth syndromes. These authors emphasized that Proteus syndrome had asymmetric overgrowth with some asymmetry present from birth. Postnatal overgrowth of bone and soft tissue increases at a faster rate than the rest of the body (91), and this overgrowth tends to plateau at adolescence (92). Proteus seems to affect males more than females (1.9: 1) and has serious complications including premature death; scoliosis with megaspondyly; abnormalities of the CNS; ophthalmologic complications (especially strabismus); pulmonary, otolaryngologic, and dental complications; and elongation of the long bones with thinning (91). Most reports of Proteus suggest a sporadic disorder, and Happle (93) hypothesized that the cause of the syndrome was mosaicism for heterozygosity of an allele that would be lethal if present in all cells. Reported cases of monozygotic twins that are discordant for Proteus syndrome support the idea of postzygotic mosaicism (94). This disorder is caused by a post-zygotic activating mutation in *AKT1* (129).

**38.3.2.3 Hemihyperplasia-Lipomatosis and CLOVES Syndrome.** Hemihyperplasia-lipomatosis (HHML) syndrome and congenital lipomatous overgrowth, vascular malformations, and epidermal nevi (CLOVE) syndrome are segmental overgrowth syndromes that should

be considered in the differential diagnosis of Proteus syndrome. HHML syndrome was first delineated and distinguished from Proteus syndrome by Biesecker et al. (95) and is characterized by subcutaneous lipomatosis, asymmetric overgrowth (hemihyperplasia), and occasional vascular malformations. The hemihyperplasia is not as severe and progressive as is seen in PWS (95). HHML has been since reviewed by Dalal et al. (96) and Schulte et al. (97). Schulte et al. clarified the stark differences in the two syndromes as follows: unlike Proteus syndrome, lipomatosis is a main feature of HHML; likewise, the vascular malformations of HHML are capillary malformations without the involvement of the deep venous or lymphatic systems as seen in Proteus syndrome or KTS, and these are less susceptible to thrombosis (97). Some authors suggest that patients with HHML be screened for Wilms' tumor and hepatoblastoma (96,97).

CLOVE syndrome is an overgrowth syndrome characterized by congenital lipomatous overgrowth,

vascular malformations, and epidermal nevi. Sapp et al. (98) presented a series of seven patients previously diagnosed with proteus syndrome but with clinical features that deviated from the classic natural history and recognized diagnostic criteria. In addition to the features mentioned, these patients also shared varying degrees of scoliosis and bony structures that were enlarged but without the progressive enlargement seen in proteus syndrome (98). In this series of seven patients, several were noted to have plantar or palmar overgrowth with wrinkling of the skin. Both HHML and CLOVE syndrome are caused by post-zygotic activating mutations in *PIK3CA* (like M-CAP and fibroadipose hyperplasia (130,131)).

A few other important and distinctive prenatal-onset overgrowth syndromes are described in Table 38-2. Further analysis and study of these interesting syndromes should help shed light on the normal processes of human growth and morphogenesis.

**TABLE 38-2 Prenatal-Onset Overgrowth Syndromes**

Syndrome	Key Clinical Findings	Inheritance	Gene Testing
<b>Proportionate Syndromes</b>			
Beckwith–Wiedemann (130650)	Macroglossia, infraorbital creases, earlobe creases and pits, abdominal wall defects, neonatal hypoglycemia, visceromegaly, risk for abdominal neoplasms, hemi-hypertrophy, polyhydramnios, large placenta (54)	Sporadic, autosomal dominant	<i>H19</i> <i>LIT1</i> <i>CDKN1C/p57(KIP2)</i>
Perlman (267000)	Hypotonia, mental retardation, serration of upper alveolar ridge, nephromegaly, bilateral cortical hamartomas, nephroblastomatosis (124)	Autosomal recessive	
Sotos (117550)	Macrocephaly, dolichocephaly, down-slanted palpebral fissures, hypertelorism, prognathism, high narrow palate, premature eruption of teeth, large hands and feet, kyphoscoliosis, mental deficiency (38)	Sporadic, autosomal dominant	<i>NSD1</i> at 5q35
Weaver (277590)	Mental retardation, hypertonia, hoarse voice, macrocephaly, round face, ocular hypertelorism, down-slanted palpebral fissures, long philtrum, large ears, micrognathia, camptodactyly, thin deep-set nails, prominent fingertip pads (4)	Sporadic, autosomal dominant	<i>EZH2</i> at 7q35
Marshall–Smith (602535)	Accelerated linear growth and skeletal maturation, postnatal failure to thrive, hypotonia, developmental delay, structural brain anomalies, respiratory tract anomalies, recurrent pneumonia, pulmonary hypertension, dolichocephaly, coarse eyebrows, shallow orbits, blue sclere, upturned nose, low nasal bridge, small mandibular ramus, hypertrichosis, umbilical hernia, choanal atresia, omphalocele (82,83,85)	Sporadic, autosomal dominant	<i>NFIX</i> at 19p13.13
Bannayan–Riley–Ruv-alcaba (153480)	Delayed gross motor development, hypotonia, speech delay, mental deficiency, macrocephaly, prominent Schwalbe rings, prominent corneal nerves, pseudopapilledema, mesodermal hamartomas, pigmented penile macules, lipid storage myopathy (67)	Autosomal dominant	<i>PTEN</i> 10q23.31
Simpson–Golabi–Behmel (300209)/(312870)	Macrocephaly, ocular hypertelorism, short broad nose, large mouth, macroglossia, variable mental retardation, hypotonia, postaxial polydactyly of hands, nail hypoplasia, partial cutaneous syndactyly, cryptorchidism, supernumerary nipples, cardiac defects, gastrointestinal defects, large cystic kidneys (57)	X-linked recessive	Type 1 <i>CXORF5</i> Xp22.3-p21.2 Type 2 <i>GPC3</i> Xq26
Elejalde (256710)	Craniosynostosis, gross edema, short limbs, postaxial polydactyly, redundant neck skin, cystic renal dysplasia, congenital heart defect, spleen anomaly, micromelia (126)	Autosomal recessive	

*Continued*



**TABLE 38-2 Prenatal-Onset Overgrowth Syndromes—Cont'd**

Syndrome	Key Clinical Findings	Inheritance	Gene Testing
Cantu (239850)	Congenital hypertrichosis, neonatal macrosomia, macrocephaly, coarse facial features, broad nasal bridge, epicanthal folds, wide mouth, thick lips, and distinctive osteochondrodysplasia	Autosomal dominant	<i>ABCC9</i> at 12p12.1 (127)
19q13.13 Deletion (613638)	Proportional overgrowth, macrocephaly, frontal bossing, downslanting palpebral fissures with optic/ophthalmologic abnormalities like strabismus and/or optic nerve hypoplasia or atrophy, absent corpus callosum, autism (81)	Sporadic	<i>NFIX</i> deletion
<b>Disproportionate syndromes</b>			
Proteus (176920)	Regional overgrowth of hands and/or feet, asymmetry of limbs, plantar hyperplasia, hemangiomas, lipomas, lymphangiomas, varicosities, verrucous epidermal nevi, macrocephaly, cranial hyperostoses, long bone overgrowth, variable moderate mental deficiency (91,128)	Sporadic	Mosaic <i>AKT1</i>
Hemihyperplasia, lipomatosis, CLOVE		Sporadic	Mosaic <i>PIK3CA</i>
Megalencephaly capillary malformation syndrome (602501)	Megalencephaly, prenatal overgrowth, thickened subcutaneous tissue; over half of patients have acquired cerebellar tonsillar herniation (versus congenital Chiari I malformation) with dilated dural sinuses and ventriculomegaly secondary to rapid brain growth and progressive crowding of the posterior fossa (75–77)	Sporadic	Mosaic <i>PIK3CA</i>
Klippel–Trenaunay syndrome	Cutaneous capillary malformations of a limb, venous malformations, and accompanying segmental hypertrophy of soft tissue and/or bone (86)	Sporadic	

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### Biographies



**David L Rimoin MD, PhD** During the final preparation of this book, we lost a major pioneer in the field of Medical Genetics: David L Rimoin, MD, PhD Dr. He died May 27th, 2012 following a diagnosis of pancreatic cancer only a few days earlier, having seen patients even on the Tuesday before he died. Dr Rimoin's achievements as Steven Spielberg Chair and Director of the Medical Genetics Institute at Cedars-Sinai, as the former Chairman of the Department of Pediatrics and Professor of Medicine and of Pediatrics at UCLA and founding director of the multi-campus UCLA Genetics Training Program, were profound. Children, adults and families from across the country and the world who turned to him for his medical expertise have lost a uniquely skilled and caring physician who was singularly devoted to their health and wellbeing. His medical contributions will continue to bring healing for generations—through the books he authored, his leadership and mentoring, the papers he presented, his lectures and speeches, and through the research that gave birth to lasting medical progress. We honor David Rimoin's passion for excellence, scholarship, innovation, his contributions to transformational medicine and his ceaseless kindness and integrity.



**John M Graham, Jr, MD, ScD** is a board-certified pediatrician and medical geneticist, with over 40 years of training and experience in clinical genetics, dysmorphology, clinical teratology, developmental disabilities, communicative disorders, and public health aspects of birth defects. He completed a pediatric internship and residency, as well as fellowships in developmental disabilities and dysmorphology and did original research on the teratogenic effects of alcohol, fetal constraint, and maternal hyperthermia. He is a steering committee member and co-founder of the David W Smith Workshop on Malformations and Morphogenesis and a Professor of Pediatrics (Step VIII) at UCLA School of Medicine, he teaches medical school courses on teratology and clinical genetics. Since 1988, he has been Director of Clinical Genetics and Dysmorphology at Cedars Sinai Medical Center in Los Angeles, and between 1981 and 1988, he held similar positions at Dartmouth Medical School in New Hampshire. He has contributed to a number of research efforts that have discovered the genes for a number of common conditions, which affect growth. These activities have resulted in new diagnostic tests for these conditions and contributed toward understanding the natural history and best modes of treatment for these disorders.

**Deepika D Burkardt, DO** is a categorical resident in her first year of training through the Wright State University General Surgery Residency Program. She completed her baccalaureate education through the College of Arts and Sciences and the College of Engineering at the University of Dayton. Her research efforts contributed to the synthesis and characterization of the crystal structure of novel inorganic chemical molecules with temperature sensitive luminescence.

She completed her medical education at Michigan State University College of Osteopathic Medicine. During her medical education, she was awarded the grant through the American Pediatric Society/Society for Pediatric Research Student Research Program to work at the Medical Genetics Institute at Cedars Sinai Medical Center under the mentorship of Dr David L Rimoin and Dr John M Graham, Jr where her research involved characterization of clinical features of patients with 1q24-q25 deletion syndrome along with involvement in several other projects related to skeletal dysplasias, limb defects, and seizures.

# CHAPTER

# 39

## Susceptibility and Response to Infection

Michael F Murray

### 39.1 INTRODUCTION

In every instance of human infection three forces intersect: the individual patient's genome, the microbial genome(s), and environmental factors. These vectors meet, and each influences the clinical outcome, and a change in any one of these three forces can alter the outcome. In 2011, the United States Center for Disease Control and Prevention reported an unfortunate case that serves to demonstrate the genome vs. genome vs. environment nature of human infection (1). The case report gave details of a fatal laboratory-acquired infection with an "avirulent strain" of *Yersinia pestis*, the causative agent of plague. The attenuated *Yersinia* strain, known as KIM D27, has an iron acquisition defect associated with a 102 kb deletion of the *pgm* locus and is thus rendered nonvirulent to most human hosts (2). This strain has been used in laboratory research for many years, without high-level precautions because of its attenuated nature. However, in this case, the laboratory worker appears to have been rendered susceptible to this strain of *Y. pestis* because of his state of clinical iron overload. The worker's iron overload state and hemochromatosis (*HFE* C282Y homozygous) were diagnosed postmortem. It appears that the change in the human genome at *HFE* set in motion events that ultimately allowed a *Y. pestis* with a *pgm* deletion to become pathogenic in the setting of an iron-rich host following exposure in the unique environment of the research laboratory.

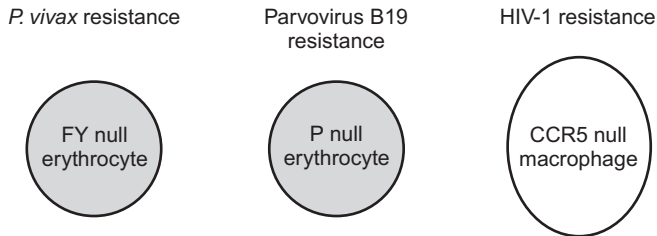
While this case report example demonstrates the concept in a unique instance, the genome vs. genome vs. environment interaction can play out over a time course of many human generations as common deadly infections, such as malaria, sculpt the human genome in chronically exposed populations (3). Thus, infection is a clear example in which nonhuman genomes exert influence on human health.

It has been suggested that microbial infection likely represents a primary driving force of natural selection in humans, particularly in the past five thousand years (4). In the spirit of his day, Haldane (5) suggested that

"the abolition of infectious diseases, which is now a possibility, and may be a reality in 50 years, should lead not only to an immediate improvement in human physique, but to a progressive improvement through many centuries." We are now more than 50 years hence, and it has become clear that despite the optimism associated with the antibiotic revolution of the mid-twentieth century, both new (e.g. human immunodeficiency virus) and old (e.g. malaria) infections are continuing to exert substantial selective pressure on human populations (6). Although infectious diseases will likely persist because of microbial and host evolution, a detailed understanding of host genetic susceptibility and response to infection will facilitate improvements in existing therapies and the development of new therapies.

As in other areas of clinical medicine, information regarding the impact of human genetics on infectious diseases is rapidly expanding beyond our classical appreciation of this interaction. A classical notion of genetic susceptibility to infection is typified by many of the primary immunodeficiency states, where monogenic mutations produce broad susceptibility to infection. Instead, this chapter will examine a wide range of human polymorphisms and mutations that alter susceptibility to infection, often by action outside of what is typically considered the immune system. In many cases, the altered susceptibility results from an altered dynamic phenotype, i.e. a phenotype appreciated in the context of an infection, although otherwise unrecognized.

The term "genetic susceptibility" as used in this chapter and in the referenced literature has several different connotations that are worth delineating. There are instances of alterations in "susceptibility" to (1) acute infection, (2) chronic infection, and (3) the morbidity and mortality associated with infection. There are only three known instances in which genetic resistance to acute infection is essentially complete (Figure 39-1). The special circumstances shared in these instances of "complete resistance" are (1) an infecting microbe whose life cycle requires an intracellular stage within an obligate human cell type, (2) a cell surface protein that



**FIGURE 39-1** Instances in which genetic resistance to acute infection is essentially complete.

acts as a cellular receptor for the microbe and whose lack of expression can be tolerated by the human host, (3) the existence of a genetic variant that leads to a null obligate cell (i.e. null for the specific cell surface protein in question). The details of the gene variants for the examples of “complete resistance” to specific infection (that are outlined in Figure 39-1) are included in the text of this chapter. Given a general lack of dispensability among human surface proteins, the mechanism for “complete resistance” exemplified here seems unlikely to have broad applicability. Instead, it is reasonable to expect that most instances of host resistance to the establishment of acute infection will ultimately prove to be cases of “relative resistance.”

The chapter is organized such that the key principle of heterozygous advantage is examined first, the contribution of recent genome-wide association studies (GWAS) to our current understanding of the topic next, and then finally a look at the knowledge base associated with individual genes, organized by location of the gene product in the context of the cell. The categories of cellular locations are cell surface proteins, intracellular proteins, and extracellular proteins.

### 39.2 HETEROZYGOUS ADVANTAGE AND HOMOZYGOUS DISADVANTAGE

The notion of heterozygous advantage with respect to infectious disease is typified by the example of sickle-cell disease and malaria (7). It is important to note that under the selective pressure of malaria there is the complementary phenomenon of homozygous disadvantage, such that both the person homozygous for the normal  $\beta$ -globin allele and the person homozygous for the mutant  $\beta$ -globin allele are at a disadvantage despite the fact that the  $\beta$ -globin<sup>S</sup>/ $\beta$ -globin<sup>S</sup> genotype offers additional antimalarial advantage compared to the heterozygote. With regard to human leukocyte antigen (HLA) heterozygosity there is evidence for advantages (see HLA discussion later). However, while this phenomenon has been suggested for other common recessive alleles, such as *CFTR*  $\Delta$ F508, a clear proof has been somewhat elusive (8).

In 2010, an important new example of heterozygous advantage with respect to selective pressure of infection in Africa was recognized. A link was made between an

unlikely infectious-disease-associated gene, apoprotein L1 (*APOL1*), and an increased risk of renal disease in African-Americans (9). Researchers went on to prove that two common variant alleles of this gene (i.e. *APOL1* G1 or *APOL1* G2), which are present in West African populations, are associated with protection against African trypanosomiasis, but when found in the homozygous state (G1/G1 or G2/G2) appear to be a risk for renal failure. Like the sickle hemoglobin–malaria situation, the scenario of *APOL1*-trypanosomiasis appears to be one where away from the selective pressure associated with infection (e.g. North America), there is a genetic disadvantage but no clear advantage to carrying the *APOL1* alleles. Unlike the sickle cell–malaria scenario, the homozygous disadvantage of the variants do not likely exert pressure on reproductive fitness in Africa since renal disease typically occurs later in life, beyond the life expectancy of most native Africans.

The work of Lyons and colleagues (10) suggests that more examples may exist, including examples of homozygous disadvantage for invasive bacterial infection. In their study of microsatellite markers in African children who died of invasive bacterial infection and controls they found that homozygosity at a relatively small sample of markers was associated with significant increases in the odds ratio for mortality due to invasive bacteria, these markers segregated differently in some cases based on Gram-positive vs. Gram negative bacteria. The most significant odds ratio (40.7; 95% confidence interval (CI), 4.28–387) was observed for Gram-negative infection in those children who were homozygous for both a site at 7q31 (D7S486) as well as a second site at 16p13.3 (D16S423). The mapping of the pathogenic genetic variants linked to this recessive disease risk has not yet been reported.

### 39.3 GENOME-WIDE ASSOCIATION STUDIES AND HUMAN INFECTION

A number of studies have been published looking for genome-wide associations between human infectious diseases and human single-nucleotide polymorphisms (SNPs). As with GWAS in other human diseases, these findings have the potential to lead to the discovery of new and important biological pathways for understanding disease pathogenesis. Of 910 GWAS publications listed on the NHGRI Catalog of Published Genome-Wide Association Studies (11), there are 21 that examine human infection and associated morbidity. The infections examined include human immunodeficiency virus 1 (HIV-1), hepatitis B virus (HBV), hepatitis C virus (HCV), *Neisseria meningitidis*, *Mycobacterium tuberculosis*, *Mycobacterium lepra*, and bovine spongiform encephalopathy (BSE) prions. Published work has also been carried out on other infections including *Plasmodium falciparum*, the causative agent of human malaria. In addition to the many new research leads,

these studies confirmed two long coding variants as associated with altered disease susceptibility; these are (1) the association of the HBB sickle variant (rs334) with malaria resistance (12) and (2) the association of the PRNP missense variant (rs1799990) with susceptibility to prion disease (13).

A GWAS on susceptibility to leprosy was carried out within a Chinese population by Zhang and colleagues (14). Associations with SNPs implicated four genes (*C13orf31*, *NOD2*, *TNFSF15*, *RIPK2*), which are also linked to inflammatory bowel disease. The strongest association was with an SNP that confers a missense change in *C13orf31*, a gene of unknown function; this same SNP has also been associated with Crohn disease (15). This variant of *C13orf31* encodes an amino acid substitution (Ile254Val) and has also been associated with leprosy susceptibility in Indian and West African populations (16). In this way, the “unbiased” GWAS approaches bring back into focus a long-speculated connection between mycobacterium susceptibility and Crohn disease susceptibility (17), although the fine details of both of these pathways remain to be worked out.

Of the dozens of additional associations in the GWAS that are listed in the NHGRI catalog (11), several SNPs are within genes that seem at this time to have a greater likelihood than others of being the causative variant in the association. First, there are two missense mutations in the *HCP5* (HLA complex P5) gene within the human major histocompatibility complex (MHC) at 6p21.33, in particular the SNP rs2395029, which has been associated with the control of HIV-1 viremia and the rate of progression to AIDS in three different studies (18–20). Second, there is an SNP conferring a nonsense stop to *ZNF229* (rs1434579), which is associated with tuberculosis susceptibility (21). Last, there are three separate associations between 3′ untranslated region (UTR) SNPs and infectious diseases; these of course raise the possibility for alterations in the modulation of the genes via microRNAs. The 3′ UTR SNPs associations include one with chronic HBV infection, *HLADPB1* rs9277535 (22), and two with HIV-1, *TRIM10* rs9468692 (18) and *ZNRD1* rs8321 (19).

## 39.4 CELL SURFACE PROTEINS

### 39.4.1 The ABO Blood Group Gene (ABO) and the Fucosyltransferase Genes (*FUT1*, *FUT2*, and *FUT3*)

Four genes coding for five different glycosyltransferases determine three polymorphic traits: classical “blood group” type, “secretor” status, and Lewis antigens. The monogenic basis of the ABO blood groups was predicted by Bernstein in the first half of the twentieth century and then demonstrated by Yamamoto and colleagues more than 50 years later to be encoded by the polymorphic single gene, known as *ABO*, located in the long arm of

chromosome 9 (9q34) (23,24). What were functionally characterized as separate transferases arising from this locus are in fact variants of the same protein. The alpha 1–3-N-actylgalactosaminyltransferase (transferase A) and the alpha 1–3-galactosyltransferase (transferase B) are differentiated by four amino acid residues. The transferases act upon the “H” antigen to form the oligosaccharide blood group antigens “A” and “B.” Frameshift mutations in the same gene result in the absence of any enzyme function in some individuals. In the absence of transferase activity, the unmodified “H” antigen defines the “O” phenotype. The enzymatic activity of fucosyltransferase 1 (*FUT1*) provides the oligosaccharide substrate for the ABO glycosyltransferases by creating the H antigen. Mutations in *FUT1*, which lead to the absence of the H antigen, exist but have no specific link to altered infectious diseases susceptibility. ABH antigen types are codominant in their expression (25).

ABH antigens have a wide distribution in addition to erythrocytes, including vascular endothelial cells and intestinal, cervical, urothelial, and mammary epithelial cells. These antigens can also be found in the body fluids of persons with inherited FUT 2 activity—i.e. positive “secretor” status (25). The nonerythrocytic distribution of antigens appears to account for many reported infectious disease associations.

The genes encoding FUT 1 and FUT 2 lie in the long arm of chromosome 19 (19q13.3). Individuals with the nonsecretor phenotype are homozygous for mutations associated with the nonfunctional FUT 2 enzyme, thereby giving the nonsecretor status an autosomal recessive inheritance pattern. In an American study, homozygous alleles for a nonsense mutation were found at codon 143 of *FUT2* in 20% of persons tested (26), while different mutations resulting in a nonfunctional enzyme have been found in other populations (27). Secretor status is associated with FUT 2 activity and results in secreted ABH antigens in saliva, plasma, urine, milk, and feces.

Since the first reported associations of ABO blood groups to disease states, numerous attempts have linked infectious diseases with specific ABH antigens and secretor status, and this extensive literature has been reviewed elsewhere (28,29). It appears that ABH antigens and secretor status influence susceptibility to infectious diseases in most cases by acting at mucosal sites of microbial exposure. The mucosal sites involved include the oropharyngeal, respiratory, gastrointestinal, and genitourinary tracts. Three common agents of bacterial meningitis gain access to the central nervous system via the upper respiratory tract mucosa and are linked to nonsecretor status (30,31). An association between the presence of the O blood group and cholera susceptibility has been demonstrated in multiple studies (32,33). In a Senegalese population, women who were exposed to HIV-1-positive heterosexual partners appeared to be at increased risk of infection if they were secretors, suggesting that the presence of ABH antigens in vaginal secretions increased



the risk of HIV-1 transmission (34). A reported association between resistance to infection by some strains of the Norwalk virus (a common cause of gastroenteritis) and blood group B is consistent with the concept of these antigens influencing infectious disease outcomes at mucosal surfaces (35). However, reported associations of blood group antigens with malaria have recently been described (36,37), and this suggests a link between an erythrocyte antigen and intraerythrocytic infection by *P. falciparum* separate from any mucosal surface.

A third fucosyltransferase gene at locus 19q13.3 (i.e. *FUT3*) determines the expression of Lewis antigen. Although the results have been debated, studies have linked Lewis antigen status to *Helicobacter pylori* infection in the gastric antrum (38) and gram-negative urinary tract infections. Expression of particular Lewis antigens by gastric epithelial cells may enhance colonization and infection by *H. pylori*. In addition, *H. pylori* expresses microbial “Lewis antigen,” although the precise role of this microbial lipopolysaccharide (LPS) is unclear (39)

### 39.4.2 Alpha 1,4-Galactosyltransferase (A4GALT)

Erythrocyte P antigens have been characterized by serologic studies to yield at least five surface antigenic variants. P antigens are present in multiple cell types, including lymphocytes, in which they comprise part of the Gb3/CD77 molecule (40). The P null (or p) phenotype is quite rare in the general population. However, in northern Sweden there is a carrier rate of up to 2% for the null phenotype in some population groups (41). In this group, persons who are homozygous for a point mutation in the *A4GALT* gene in chromosome 22q13.2 are phenotypically P null (42). These individuals lack active “P<sup>K</sup> synthase” activity, that is, the specific galactosyltransferase activity required to convert lactosylceramide to globotriaosylceramide (42). All the *A4GALT* alleles reported to date in P null individuals from Sweden and Japan have mutations within the coding sequence (42–44), in contrast to the regulatory sequence change leading to FY-null erythrocytes.

Parvovirus B19 is a ubiquitous human pathogen infecting most individuals around the world, with a seroprevalence of up to 90% in some adult populations (45). It causes a range of diseases including erythema infectiosum, hydrops fetalis, and transient aplastic anemia (46). Since P antigen is the erythrocyte receptor for human parvovirus B19, P null individuals are uniquely resistant to parvovirus B19 infection (47) (Figure 39-1). However, this rare phenotype does not appear to afford a significant selective advantage, and it is believed that the elevated P null frequencies in northern Sweden result from founder effect and genetic drift (48). This membrane protein also acts as a receptor molecule for Shiga-like toxins from *Escherichia coli* O157, the microbe that induces hemolytic uremic syndrome. However, there is

no research to date linking *A4GALT* genotypic variations and human clinical phenotypes in the setting of this bacteria (49).

### 39.4.3 Chemokine, CC Motif, Receptor 5 (CCR5) and Chemokine, CC Motif, Receptor 2 (CCR2)

Chemokine receptor 5 (CCR5) serves as a natural receptor for  $\beta$ -chemokines including CCL5, CCL3, and CCL3L1. CCR5 also acts as a viral coreceptor for macrophage tropic (M-tropic) strains of HIV-1 (50,51). This receptor is encoded in 3p21, and the gene product is expressed on the cell surface of macrophages.

A 32-base-pair deletion (nucleotides 794 to 825) in CCR5 (CCR5  $\Delta$ 32) (52) results in the loss of receptor expression on cell surfaces; this confers resistance to HIV-1 infection in the homozygous state (53) (Figure 39-1) and slower progression to AIDS in the heterozygous state (54). Meta-analysis revealed a consistently lower serum viral load associated with the presence of the CCR5  $\Delta$ 32 mutation (55). In one study of 334 men in Amsterdam, wild-type CCR5 was associated with an increased relative risk for HIV-1 disease progression (by a factor of 2.5) compared to heterozygous CCR5  $\Delta$ 32 (56).

A survey involving 4166 individuals suggested that CCR5  $\Delta$ 32 was absent in African, American Indian, and East Asian populations but present in 0–14% of Eurasian groups. Stephens and colleagues (57) estimated that the CCR5  $\Delta$ 32 mutation arose approximately 700 years ago (range, 275–1875). It has been suggested that a strong positive selective pressure, such as an infection via the CCR5 coreceptor, drove a recently increased gene frequency. Since HIV-1 is estimated to have infected human populations only after the 1940s (58), CCR5  $\Delta$ 32 is postulated to provide protection against at least one other as yet unidentified microbe. While the loss of a functional receptor associated with this allele appears to be inconsequential to the health of most heterozygotes, one study has suggested an associated increased risk of primary sclerosing cholangitis (59).

Two other protective polymorphisms and one deleterious polymorphism have been associated with CCR5 and HIV-1 infection. Homozygous individuals with a point mutation at position –502 relative to the transcription initiation site had delayed onset (3.8 years) of AIDS when compared to individuals with wild-type CCR5 (60). This variant was very common (43–68%) compared to either CCR5  $\Delta$ 32 or the third protective variant, known as the m303 mutation. While m303 occurred in less than 2% of individuals in the surveyed population, it was discovered in a multiply exposed HIV-1-negative individual. The compound heterozygous state with m303 and CCR5  $\Delta$ 32 in one patient conferred complete in vitro resistance to HIV-1 (61). In contrast, Martin and colleagues (62) attributed rapid HIV-1 disease progression (i.e.

development of AIDS in less than or equal to 3.5 years) to homozygosity for a polymorphism in the *CCR5* promoter region (*CCR5* P1). Homozygosity of *CCR5* P1 occurred in 12.7% of Caucasian Americans and 6.7% of African-Americans.

Chemokine receptor 2 (*CCR2*) is encoded at gene locus 3p21, in close proximity to *CCR5*. A point mutation causing the neutral substitution of isoleucine for valine (V641) has been studied as a potential AIDS susceptibility polymorphism. *CCR2* V641 was present in 10% of Caucasian Americans, 15% of African-Americans, 17% of Hispanic Americans, and 25% of Asian-Americans in one large study (63). Wild-type *CCR2* is in strong linkage disequilibrium with *CCR5*  $\Delta$ 32, which facilitates examination of distinct effects of each of the two polymorphisms (63). *CCR2* V641 is not associated with differences in HIV infectivity, but it is associated with a 2–4-year delay in the onset of AIDS in HIV-infected persons (63).

*CCR2* is unlikely to be a primary cellular coreceptor for HIV-1 when compared to *CCR5* and *CXCR4*. Evidence suggests that the *CCR2* V641 mutation could inhibit HIV-1 indirectly, through the desensitization of *CCR5* and *CXCR4* receptors to their normal ligand responses (64). However, the worldwide presence of exceptionally strong linkage disequilibrium between *CCR2* V641 and *CCR5* promoter polymorphism (*CCR5* 59635T), and the fact that V641 encodes a neutral amino acid change in a transmembrane domain, has led to speculation that *CCR2* V641 could be a marker for a different biologically significant mutation (65).

#### 39.4.4 CD14 Antigen (CD14)

The CD14 gene locus is at 5q31.1. CD14 is a surface protein on macrophages that has been implicated in bacterial LPS signaling (66). An SNP in the promoter (C-159T) has been associated with enhanced transcriptional activity for the gene (67). Homozygosity for CD14 159T was associated with Gram-negative infection and an increased prevalence of positive bacterial cultures in critically ill adults (68). The same promoter polymorphism has also been associated with chronic infection of peripheral blood monocytes with *Chlamydia pneumonia* (69). The notion that chronic *C. pneumonia* infection may be a risk factor for coronary artery disease, while controversial, is indirectly supported in this context by other studies that have linked this promoter polymorphism with myocardial infarction in a patient group that was considered “low risk” for atherosclerosis when assessed by a standard risk profile (70).

#### 39.4.5 Cystic Fibrosis Transmembrane Conductance Regulator

The cystic fibrosis transmembrane conductance regulator (CFTR) functions as a cytoplasmic membrane

chloride channel and ion transport regulatory protein, and its gene, *CFTR*, has been localized in chromosome 7q31. Cystic fibrosis (CF) is an autosomal recessive disease associated with the inheritance of mutant *CFTR* in both homozygous or compound heterozygote fashion (Chapter 58), and the increased susceptibility to infection associated with the CF phenotype is limited to the sinopulmonary tract.

*CFTR* mutations result in defects in the internalization and clearing of *Pseudomonas aeruginosa* in the respiratory tract. In the absence of normal clearing by the respiratory epithelial cells, *P. aeruginosa* infection occurs in 75–90% of patients with CF. Notably, *Pseudomonas* strains in the respiratory tract of CF patients adapt to this host alteration by transitioning from isolates with smooth LPS and no extracellular mucoid exopolysaccharide to isolates with rough LPS and mucoid hyperexpression. The microbial advantage gained by this adaptation has not been fully elucidated (71). Wild-type *CFTR* also serves as a receptor for *Salmonella typhi* on gastrointestinal epithelial cells (72). Mutant *CFTR* results in diminished translocation of *S. typhi* across the mucosal barrier; this diminished invasiveness of *Salmonella* is postulated to represent the heterozygous advantage of the carrier state, which allowed the carrier frequency of mutated *CFTR* to reach 4% in some Caucasian populations. A recent study also suggested that a disproportionate number of cases of chronic sinusitis might be associated with CF carrier status, i.e. a mutation in just one *CFTR* allele (73).

There is growing evidence that variations in other “modifying” genes act in concert with *CFTR* to influence infectious disease susceptibility. Genes encoding variants of MBL2 have been associated with increased liability for infection, decreased pulmonary function, and decreased survival time (74,75). DeRose and colleagues recently reported on a cohort of CF patients with a matched *CFTR* genotype ( $\Delta$ F508/ $\Delta$ F508) in whom allelic variants of *FCGR2A* were significantly associated with an altered risk of acquiring chronic *P. aeruginosa* infection (76).

The clinical use of the aminoglycoside antibiotic gentamicin to induce translational read through of stop mutations in *CFTR* resulting in expression of functional *CFTR* has been reported (77). A small molecular therapy based on this observation is in clinical trials (78); however, since only a minority of CF patients has *CFTR* alleles with stop mutations, the practical utility of this intervention may be quite limited.

#### 39.4.6 Chemokine, CX3C Motif, Receptor 1 (CX3CR1)

The chemokine receptor CX3CR1 is a leukocyte chemotactic and adhesion receptor for the chemokine CX3CL1 (formerly known as fractalkine) (79). The gene for this receptor lies in the short arm of chromosome 3 (3p21). In addition to being a receptor for CX3CL1, this cell

surface molecule also acts as a coreceptor for HIV-1. HIV-1-infected adult patients homozygous for a polymorphic allele including two point mutations (V249I and T280M) were statistically more likely to have rapid progression of disease relative to those with the wild-type gene (80). Although this allele had a frequency of 13.5% in the uninfected French population studied and ranged from 12.6 to 19.8 % in three infected cohorts examined, the polymorphism was not detected in the non-Caucasian populations tested (80). Homozygosity for the I249 allele has also been linked with HIV-1 progression in children (81). Interestingly, both M280 and I249 have now also been associated with atherosclerotic disease (82,83).

### 39.4.7 Chemokine, CXC Motif, Receptor 4 (CXCR4)

CXCR4 is a chemokine receptor for CXCL12 (which is also known as SDF1); *CXCR4* is at 2q21. The gene product is a seven transmembrane receptor protein that is highly expressed on many cell surfaces including breast cancer cells (84). Although CXCR4 is also a coreceptor for HIV-1, there have not been any associations to date between polymorphisms in this gene and this retrovirus. A recent report, however, has linked mutations in this gene with an autosomal dominant immunodeficiency syndrome known as WHIM syndrome (OMIM 193670); this syndrome is characterized by recurrent bacterial sinopulmonary infection and widespread chronic human papilloma virus (HPV) infection (85). The authors point out that their observations linking this gene and this syndrome suggest an association between CXCR4 and the normal control of HPV.

### 39.4.8 Duffy Blood Group (FY)

Chromosome 1q22–23 includes *FY*, which encodes the glycoprotein (GP) “duffy blood group” antigen or “duffy antigen receptor for chemokines” (DARC). This antigen has had many names in the literature including Duffy antigen, FY antigen, and glycoprotein D (GPD). DARC binds interleukin-8 (IL-8), CXCL1, CCL5, and CCL2 (86). Vascular endothelial cell expression of DARC may underlie its primary chemokine receptor function (87).

DARC was first described in a patient named Duffy with alloantibodies against this GP (88). Three common alleles exist in US populations, i.e. *FY*\*A, *FY*\*B, and *FY*\*O (also known as Duffy null or *FY*\*B<sup>ES</sup>). These codominant alleles lead to four serologic red blood cell phenotypes i.e. Fy (a+b–), Fy (a–b+), Fy (a+b+), and Fy (a–b–). The Fy (a–b–) phenotype, which is genotypically homozygous for the *FY*\*O allele, is common in African-Americans and West Africans (89). The coding sequence for *FY*\*B and *FY*\*O is identical (90). An SNP in the *FY*\*B 5'UTR leads to disruption of the GATA binding

site for erythroid transcription factor (91). Hence, *FY*\*O is the “erythroid silent” allele (*FY*\*B<sup>ES</sup>) but is present in a typical distribution on nonerythroid cells.

*Plasmodium vivax*, the second most common cause of human malaria, is incapable of establishing infection in individuals homozygous for *FY*\*O (92). Thus, the *FY*\*O allele is an example of a human polymorphism that prevents a specific microbial infection (Figure 39-1). In a remarkable demonstration of the bidirectional selective pressure in human–microbial populations, the region of West Africa where *FY*\*O predominates is free of *P. vivax* infection, thereby making this region unique within its latitude (93).

### 39.4.9 FC Fragment of IgG, Low Affinity IIA, Receptor (FCGR2A)

FCGR2A is a cell surface receptor that was formerly known as CD32. The gene locus is at 1q23; the gene product is expressed on a wide variety of cell types, and it mediates multiple cell-type specific functions including phagocytosis, release of inflammatory mediators, and clearance of immune complexes (76). A polymorphism of the gene has been reported, in which a single-nucleotide change results in either arginine (R) or histidine (H) at amino acid 131 of the mature protein. This polymorphism has been associated with both noninfectious and infectious risks, including an altered risk for nephritis in the setting of lupus (94). *P. aeruginosa* infection in the setting of CF (76), increased risk of respiratory tract infections (95,96), and susceptibility to perinatal infection in infants of HIV-1-infected mothers (97).

### 39.4.10 Human Leukocyte Antigen (HLA) Genes

The HLA genes display a high degree of polymorphism and serve as a model for human genes in an apparent state of coevolution with infecting microbes. They are encoded in the short arm of chromosome 6 (6p21.3) and have been implicated as a modulating influence in a large number of infections (Table 39-1). The HLA system, which is part of the human MHC, has been concisely reviewed elsewhere (98,99). MHC proteins, encoded by class Ia genes including *HLA-A*, *HLA-B*, and *HLA-C*, are expressed on most nucleated cells. The class II genes, also known as *HLA-D*, are designated with a three-letter code (e.g. *HLA-DRB*), which indicates class (D), family (M, O, P, Q, or R), and chain (A or B). Proteins encoded by class II genes are expressed on leukocytes (both B and T), macrophages, dendritic cells, and thymus cells. In both classes, numbers are assigned to each allele; this nomenclature is different for sequence-defined and for serologically defined variants. An asterisk followed by a number indicates a genotypic variant (e.g. *HLA-A*\*0101), while a number without an asterisk represents a serologic variant (e.g. *HLA-A*1) (100). An online

TABLE 39-1 HLA Class Ia and Class II Associated Diseases

Microbe	Type of Organism	Disease(s)	Class	References
Human T-lymphotropic virus-1 (HTLV-1)	RNA virus	Myelopathy	Ia	(269)
Human immunodeficiency virus-1 (HIV-1)	RNA virus	AIDS	Ia and II	(271) (65) (276)
Hepatitis C virus	RNA virus	Viremia	II	(114)
		Mixed cryoglobulinemia	Ia and II	(124)
Dengue virus	RNA virus	Hemorrhagic fever	Ia	(279)
		Shock		
Puumala Hantavirus	RNA virus	Renal disease	Ia and II	(278)
Ross River virus	RNA virus	Arthritis	II	(264)
Measles	RNA virus	Subacute sclerosing panencephalitis	Ia	(270)
Hepatitis B virus	DNA virus	Viremia	II	(108)
Herpes simplex virus 2 (HSV-2)	DNA virus	Genital ulcers	Ia	(125)
Epstein-Barr virus	DNA virus	Nasopharyngeal cancer	Ia and II	(117)
Cytomegalovirus	DNA virus	Retinitis	Ia and II	(283)
Human herpes virus 8 (HHV-8)	DNA virus	Kaposi sarcoma	Ia	(281)
Human papilloma virus	DNA virus	Cervical cancer	Ia and II	(259) (116)
<i>Mycobacterium tuberculosis</i>	Bacteria	Tuberculosis	II	(266) (127)
<i>Mycobacterium leprae</i>	Bacteria	Leprosy	II	(127)
<i>Mycobacterium avium</i>	Bacteria	Bacteremia	II	(274)
		Pneumonitis	Ia and II	(272)
<i>Chlamydia trachomatis</i>	Bacteria	Trachoma	Ia	(261)
		Fallopian tube scarring	II	(260)
<i>Haemophilus influenzae</i>	Bacteria	Meningitis and bacteremia	Ia	(284)
<i>Helicobacter pylori</i>	Bacteria	Atrophic gastritis	II	(282)
<i>Borrelia burgdorferi</i>	Bacteria	Lyme arthritis	II	(267)
<i>Streptococcus pyogenes</i>	Bacteria	Rheumatic fever	II	(287)
<i>Plasmodium falciparum</i>	Protozoa	Malaria	Ia and II	(7)
<i>Trypanosoma cruzi</i>	Protozoa	Chagas disease	Ia	(275)
<i>Toxoplasma gondi</i>	Protozoa	Congenital hydrocephalus	II	(277)
<i>Leishmania</i> spp.	Protozoa	Mucocutaneous ulceration	II	(280)
<i>Giardia lamblia</i>	Protozoa	Gastroenteritis	II	(263)
<i>Schistosomiasis</i> spp.	Helminth	Hepatic fibrosis	II	(268)
<i>Echinococcus</i> spp.	Helminth	Liver abscess	II	(262)
<i>Brugia</i> spp.	Helminth	Elephantiasis	Ia and II	(288)
<i>Onchocerca volvulus</i>	Helminth	River blindness	II	(126)
<i>Paracoccidioides</i> spp.	Fungus	Mycosis	Ia	(273)
<i>Fonsecaea pedrosoi</i>	Fungus	Chromoblastomycosis	Ia	(285)
<i>Trichosporon</i> spp.	Fungus	Hypersensitivity pneumonitis	II	(258)
<i>Cryptococcus neoformans</i>	Fungus	Meningitis	Ia	(286)

“Immunogenetics (IMGT) HLA Sequence Database” is available at <<http://www.ebi.ac.uk/imgt/hla>> (101).

The class II molecules are heterodimers, which allows for alpha/beta matching within and between families (e.g. DRA and DQB), and result in approximately 12 different class II antigens available on the cell surfaces of heterozygotes (102). Heterozygotes also express six different class Ia antigens. Owing to codominant expression, homozygotes likely express half as many different surface antigens as heterozygotes. Recombination within the MHC region allows for individuals who are

homozygous for one locus or for an entire haplotype. Thus, there is a range of expressed HLA types within human populations.

HLAs are pivotal in the processing and presentation of microbial antigens to the human immune system. Inefficient or failed peptide presentation can result in disadvantages for the host when combating infection. This was recently demonstrated in a cohort of HIV-infected patients, where a single amino acid change in a region critical to peptide presentation was associated with significant risk of rapid disease progression (103).



Twenty-one highly polymorphic HLA genes with more than 2000 recognized HLA alleles have been described (<http://www.ebi.ac.uk/imgt/hla>). This diversity is as yet unmatched elsewhere in the human genome. Infections may represent a driving force of HLA genetic diversity. Proposed mechanisms of infection-driven diversity include overdominant selection (or heterozygous advantage) and frequency-dependent selection (104). The overdominant selection hypothesis holds that the individual with the most available HLA types will be best suited to handle the largest variety of microbial antigens. The frequency-dependent selection hypothesis suggests that in a population, the selective force of the microbe on the host will favor uncommon HLAs to which the microbe has not yet adapted. Both mechanisms favor diversity, and they are not mutually exclusive processes.

The overdominant selection hypothesis has been supported both in principle (105) and with empirical data of specific infectious diseases. Heterozygous advantage has been demonstrated in three chronic viral infections: HPV, HBV, and HIV-1 (106–108). On the other hand, although not predicted by those seeking to explain HLA diversity, homozygous advantage has also been demonstrated (7,109). Homozygous advantage in the context of a specific microbial infection can be explained by increased numbers of a “preferred” HLA protein that is capable of superior presentation of specific “constant” microbial peptide antigens.

Frequency-dependent selection can be invoked to explain variations in the frequency of HLA-B53 in Africa. This HLA is associated with protection against severe malaria in studies performed in Gambia (7). While population frequencies in black South Africans are only about 2%, class Ia antigen reaches frequencies of up to 40% in malaria-prone regions of West Africa. HLA-B53 is extremely rare or altogether absent in most non-African populations. Malaria appears to have acted as a directional selective force on local populations for 10,000 years. Plasmodial infections may have increased the frequency of HLA-B53 to levels greater than expected by genetic drift, as observed currently in West Africa (110).

Coevolution of humans and microbes is an important concept in the study of HLA and infection. An African study of malaria peptides and HLA-B35 found evidence for bidirectional effects of human and parasitic alleles (111). Although observations such as this are still relatively rare, the opportunity for important observations in this area will grow with acquisition of human and microbial sequence data. For example, the use of a HIV-1 sequence database together with knowledge of HLA peptide constraints and epidemiological data on HLA influence on outcomes has allowed one group to correlate protective HLA types with their anticipated capacity to bind the observed range of motifs in the highly mutable HIV-1 (112).

Specific HLA alleles have been associated with cancers linked with chronic viral infections. HLA associations have been reported for several viral infections, including HBV, HCV, HPV, and Epstein–Barr virus (EBV). Hepatocellular carcinoma is associated with persistent viremia in both HBV and HCV, and while there is little direct evidence at this point for HLA associations with hepatocellular carcinoma, HLA associations have been reported with persistent viremia in chronic HBV or HCV infection (113,114). HPV is a primary etiologic factor in the development of cervical carcinoma (115), and HLA associations have been documented in cervical dysplasia and cervical carcinoma (116). Undifferentiated nasopharyngeal carcinoma is tightly linked with chronic EBV infection and has been associated with specific HLA antigens (117).

The exclusive application of serology-based (phenotypic) rather than sequence-defined (genotypic) HLAs represents a significant limitation with earlier HLA association studies. HLA genotyping emerged in the 1990s and yielded relatively greater specificity in HLA association studies. HLA antigens defined by serotyping represent phenotypes that have been useful with particular rheumatologic disease associations (e.g. HLA-B27 and ankylosing spondylitis; Chapter 78), but relatively nonspecific results obtained by such studies may be confounding. An example of the relative specificity of HLA genotyping and its ability to clarify otherwise contradictory data was demonstrated in the case of HLA-DR1 and HIV-1 infection. One group reported DR1 to be protective (118), while another group’s data suggested that DR1 increased risk for progression (119). In a third report, it was found that *HLA-DRB1\*0101* was associated with a risk for progression and *HLA-DRB1\*0102* appeared to be protective against progression; both these alleles are serologically grouped as HLA-DR1 (120). It can be expected that most studies will use genotypically defined alleles alone or in combination with serologically defined groups in the future.

Linkage disequilibrium between HLA and non-HLA genes within the MHC region may confound results obtained in the study of HLA and infection. HLA haplotypes, such as the “8.1 haplotype” (A1, B8, DR3) (121), have been associated with several infectious diseases; the association of the 8.1 haplotype with HIV-1 (122,123), HCV (124), or herpes simplex virus (HSV) (125) infection may be due to individual HLA loci, the combination of HLA loci, or the non-HLA genes carried with the MHC. Non-HLA effector genes closely linked with HLA loci include tumor necrosis factor (TNF), lymphotoxin A (LTA), transporters associated with antigen processing (TAP), and complement component genes.

The prevalence and cumulative effects of multiple infections, each with a different selective influence and occurring within the same human population must be considered when studying the role of HLA antigens in

infection. Most studies have attempted to evaluate the influence of a single microbe with the HLAs of a discrete human population. However, human populations are subject to numerous microbial infections that occur sequentially and concomitantly during a lifetime. This multipronged attack may favor HLA diversity, although it makes isolating the effects of one infection from another in a given population challenging. Insight may be gained into this situation by reexamining the interaction between HLA and two infections: one that exerts strong directional selection and one that does not exert obvious selective pressure. In the study referred to previously, HLA protection from malaria was associated with both class Ia and class II antigens (7). Howard (110) predicted that increases in specific HLA frequencies in response to malaria would ultimately result in either a counter-response from *P. falciparum* or the opportunity for another pathogen to “take advantage.” Three years later, the *DQB1*\*0501 allele, which together with HLA-B53 had been associated with protection against severe malaria, was linked with susceptibility to generalized *Onchocerca volvulus* infection (126). This chronic helminthic infection, which causes “river blindness” late in life, has no recognized effects on reproductive fitness, while malaria (fatal in 1% of local children prior to their fifth birthday) has a major effect on reproductive fitness. The price of 10,000 years of selection for HLAs protective against malaria appears to be susceptibility to *Onchocerca* infection.

Several HLA class and loci associations have been repeatedly reported with specific bacterial and viral infections. Tuberculosis studies performed in multiple countries since 1989 have consistently found class II, in particular DRB1, associations with infectious outcomes (127). Class I associations have consistently been significant in HIV-1 outcomes (106).

In summary, the study of HLA–microbe interactions continues to be a complex task of studying two moving targets, i.e. the highly polymorphic human MHC region and continuously evolving microbial pathogens. Published studies have been limited by numerous variables, but a greater understanding of the complex relationship between HLA and infection is emerging. Despite reports of HLA antigens functioning as coreceptors for viral entry (128), most available evidence suggests that the variability in infectious disease outcomes associated with specific HLA alleles is due to qualitative differences in the microbial peptide presentation to the immune system. Unfavorable outcomes are generally attributed to inefficient peptide presentation; however, it has been suggested that overly efficient HLA peptide presentation may lead to an excessive inflammatory reaction and poor outcomes (129). The role of HLA in host susceptibility to infection continues to be defined, and although some reported associations may ultimately prove to be due to genes linked to the MHC other than HLA, two principles have emerged: (1) HLA and microbial

populations coevolve, and consequently (2) multiple alleles at specific loci appear to be linked to outcomes in different populations.

### 39.4.11 Interferon Gamma Receptor Genes (*IFNGR1* AND *IFNGR2*)

The interferon gamma receptor (IFN $\gamma$ R) is expressed in all nucleated cells as a tetramer composed of two IFN $\gamma$ R1 and two IFN $\gamma$ R2 subunits (130). *IFNGR1* maps to 6q23–24, and *IFNGR2* maps to 21q22.1–22.2. Mutations in both *IFNGR1* and *IFNGR2* have been reported, although *IFNGR1* mutations appear to be more common. The increased infectious disease risks associated in both cases appear to be *Salmonella* and mycobacterial infections. Three cases attributable to *IFNGR2* were associated with three different homozygous mutations. IFNGR1 deficiency can be divided into two groups: patients with homozygous mutations leading to a complete loss of expressed gene product and patients with a single mutant allele arising at a common mutational “hot spot” and resulting in inheritable risk for recurrent infections with a dominant inheritance pattern (i). In a study of 22 patients with autosomal recessive “complete” IFNGR1 deficiency and 38 patients with autosomal dominant “partial” deficiency, a genotype–phenotype correlation was made linking “complete” deficiency to more severe risk of morbidity and mortality (132).

### 39.4.12 Interleukin-12 Receptor, Beta-1 (*IL12RB1*)

The cell surface receptor for interleukin-12 (IL-12) is a heterodimer composed of beta 1 ( $\beta$ 1) and beta 2 ( $\beta$ 2) chains. The  $\beta$ 1 receptor is encoded at chromosome 19p13.1, and the  $\beta$ 2 gene is in 1p31.2. Coexpression of both functional subunits is required for IL-12 binding.

Homozygous mutations *IL12RB1* have been associated with *Salmonella* and *Mycobacterium* infections in seven published cases (133,134). Six different mutations were demonstrated in *IL12RB1* of six different patients (130). One child who died at eight years of age without sequence analysis was presumed homozygous for the same mutation as his brother. The six patients included in the sequence analysis had intermittent infections but were apparently free of other adverse consequences (135).

### 39.4.13 Toll-Like Receptor 2

Toll-like receptor 2 (TLR2) is one of at least nine different human Toll-like receptors (TLRs) that have been described, and their name is based on homology with the *Drosophila* toll protein (136). In general, TLRs appear to function as bacterial sensors. The gene locus for *TLR2* is at 4q32, and it encodes a 784 amino acid protein. Experimental data suggests that a highly polymorphic (GT)

dinucleotide repeat, which is 100 nucleotides upstream from the *TLR2* translational start site in intron 2, is associated with variable expression of *TLR2* in response to cytokine stimulation (137). The numbers of GT repeats found in participants of this study varied from 12 to 28, and there were significant differences in allele distribution between African-Americans and Caucasians ( $P=0.008$ ) and between African-Americans and Koreans ( $P=0.0003$ ). Functionally, *TLR2* genes with shorter or longer GT repeats had higher promoter activity than mid-sized GT repeats when stimulated with interferon-gamma (IFN- $\gamma$ ) (137).

Association studies have examined several *TLR2* polymorphisms in relation to infectious diseases. A promoter polymorphism in *TLR2* (T-16933A) was used to define two groups in a study of the prevalence of infection in critically ill adults, and the A/A genotype was significantly associated with both sepsis and Gram-positive infection (68). In a small study, a *TLR2* missense mutation was found in 9% of patients with staphylococcal sepsis (Arg 753Gln), compared to less than 3% of normal controls (138). While the association of this polymorphism with Staphylococcal infection was not supported in the study by Moore and colleagues (139), the importance of *TLR2* in protection against staphylococcal infection has been supported in a murine knockout model (140). Another human missense mutation, the Arg677Trp polymorphism (C2029T nucleotide substitution), has been associated with tuberculosis in Tunisian patients ( $P<0.0001$ ) (141) and leprosy in Korean patients (142). However, a recent report suggesting that C2029T may only be found in a pseudogene that lies 23 kb upstream from *TLR2* (143) has called the associations with this SNP into question, and further study will be required to discern whether or not it is indeed associated with altered risk of mycobacterial infection.

#### 39.4.14 Toll-Like Receptor 4 (TLR4)

The locus for *TLR4* is at 9q32–q33, and the gene product is known to exist in at least four isoforms of 639 to 839 amino acid length. Of the human TLRs, it appears that the data to support the notion that they function as “endotoxin sensors” is best developed in relation to *TLR4*. The Asp299Gly amino acid substitution in the extracellular domain of *TLR4* (created by the A896G polymorphism) is associated with a blunted response to inhaled bacterial LPS. In vitro experiments support the notion that it is the Asp299Gly substitution rather than another common substitution (i.e. C1196T, which results in Thr399Ile) that interrupts LPS-mediated *TLR4* signaling (144). Several studies have now found that the 896G and the 1196T polymorphisms are in fact linked in the majority of patients. The 896G allele was associated with both a diminished inflammatory response to gram-negative pathogens and a decreased risk of

atherosclerosis in a study of patients in Bruneck, Italy (145). In this study, the 55 subjects who had the 896G allele were shown to have lower levels of proinflammatory cytokines, more susceptibility to severe bacterial infections, a lower risk of carotid atherosclerosis, and a smaller intima-media thickness in the common carotid artery. In these patients, the 896G allele was heterozygous in 53 and homozygous in 2 of the subjects, and 896G cosegregated with 1196T in 46 of the 55 patients (145). In another study of the same *TLR4* A896G polymorphism in relationship to a *Legionella pneumophila* outbreak in The Netherlands, the 896G allele showed a protective association (146). While further study of these links are clearly needed, associations between the 896G allele and both “protection from” *L. pneumophila* as well as “susceptibility to” other Gram-negative bacteria are not inherently contradictory. Since *L. pneumophila* and other bacteria differ greatly in both their LPS and their pathogenesis, the optimal protective *TLR4* response could also vary significantly.

#### 39.4.15 Toll-Like Receptor 5 (TLR5)

The gene encoding *TLR5* is on chromosome 1 (1q41). A common SNP (C1174T) has been described in Caucasians with the allele frequency of approximately 10%. This cytosine–thymidine transition changes the arginine at amino acid 392 to a stop codon (*TLR5* 392X) and is predicted to prematurely truncate *TLR5* in the extracellular domain and cause the loss of the transmembrane domain and the entire signaling cytoplasmic tail (147). In a separate analysis of the same *L. pneumophila* outbreak discussed earlier, Hawn and colleagues concluded that *TLR5* 392X exerted a dominant effect and that heterozygous *TLR5* 392X individuals are more susceptible to *L. pneumophila* because of impaired production of proinflammatory cytokines (147).

### 39.5 INTRACELLULAR PROTEINS

#### 39.5.1 Cytokine-Inducible SH2-Containing Protein

The cytokine-inducible SH2-containing protein (CISH) has been linked to risk for several different infectious diseases, and its gene, *CISH*, is located in chromosome 3 (3p21.3). Its pivotal role in different infectious diseases is explained by its intracellular role in interleukin-2 (IL-2) signaling. A recent report suggests that variant *CISH* alleles that are associated with decreased *CISH* expression are associated with an increase in the overall risk of tuberculosis, malaria, and bacteremia (148). In a study of over 8000 individuals in Africa and Asia, these authors found that having one of the common variants associated with decreased *CISH* expression was associated with an 18% increased risk of one of these major infections (148).

### 39.5.2 Galactose-1-Phosphate Uridyltransferase (GALT)

The gene encoding galactose-1-phosphate uridyltransferase (*GALT*) is located in chromosome 9 (9p13). Galactosemia, caused primarily by *GALT* deficiency, is an autosomal recessive inborn error of metabolism that has been associated with greater than 100 different mutant genotypes (149). This metabolic disorder is discussed in detail in Chapter 93, but warrants mention here because of the association of neonatal Gram-negative sepsis with this disorder (150). Although a comprehensive understanding of the pathogenesis of this bacteremic state is not available, impaired neutrophil function in neonates attributed to increased galactose is likely to be a significant contributing factor (151).

### 39.5.3 Glucose-6-Phosphate Dehydrogenase (G6PD)

The gene encoding glucose-6-phosphate dehydrogenase (*G6PD*) is located in chromosome Xq28. This enzyme is believed to be essential to cellular function, and quantitative variations in enzyme function appear to be clinically significant in mature erythrocytes. The phenomenon of X-linked inactivation in females was uncovered in the study of *G6PD*-deficiency; this results in heterozygous females having two cellular populations: *G6PD*-deficient and *G6PD*-nondeficient cells (152). At least 95 different mutations have been associated with *G6PD* deficiency (149), and these sequence changes account for the observed range of enzyme activities (from mild to severe deficiency). The known mutations are almost entirely point mutations, although four small in-frame deletions (of three or six base pairs (bp)) have been reported. It appears that a mutation that would result in a completely nonfunctional enzyme would be lethal (153).

In the 1960s, when the increased population prevalence of *G6PD*-deficiency in malarious zones was noted, it was suggested that, like sickle-cell disease and thalassemia, *G6PD* deficiency likely offers a survival advantage in malaria. However, published data to support this hypothesis was minimal until 1995 when data suggesting that both female heterozygotes and male hemizygotes have a 46–58% reduced risk for severe malaria was reported (154). While many mechanisms of selective advantage have been proposed, data suggests that early phagocytosis of *P. falciparum*-infected erythrocytes occurs. Phagocytosis is induced by parasite-associated erythrocyte membrane damage more readily in *G6PD*-deficient cells (155).

A selective disadvantage of *G6PD* deficiency has been proposed to explain the lack of allelic fixation in sub-Saharan African populations (154). The disadvantage is not clear, but an increased incidence of bacterial sepsis in neonates may be a contributing factor (156).

### 39.5.4 Alpha-Globin (HBA1 and HBA2)

Four functional  $\alpha$ -globin (*HBA*) alleles are present per diploid erythroblast, with two tandem *HBA* alleles at each 16p13.3 locus; these genes, *HBA1* and *HBA2*, have identical coding sequences with some variation in the noncoding portion. The protein  $\alpha$ -globin combines with  $\beta$ -globin to form the functional  $\alpha_2\beta_2$  tetramer of adult hemoglobin. “Thalassemia” is used to describe inherited defects in globin synthesis;  $\alpha$ -thalassemia is secondary to *HBA* mutations (Chapter 71). Greater than 30 different  $\alpha$ -thalassemia-inducing mutations exist (149). Persons with  $\alpha$ -thalassemia trait have three functional alleles and an essentially normal hematologic phenotype, while persons with a mild hypochromic anemia generally have two functional copies.

Before the mid-1980s, it was difficult to dissect the interactions of  $\alpha$ -thalassemia and infection from the effects of the  $\beta$ -hemoglobinopathies. Flint and colleagues studied human populations in the Southwest Pacific nations of Papua New Guinea and Melanesia, who have  $\alpha$ -thalassemia and no  $\beta$ -thalassemia or sickle-cell disease. This study suggested that the level of malaria endemicity correlated with the gene frequency of  $\alpha$ -thalassemia and argued that a selective pressure was being exerted by malaria in favor of  $\alpha$ -thalassemia (157). Further study has now demonstrated that the  $\alpha$ -thalassemia trait is associated with a reduced risk of severe malaria (0.66) with an even greater reduced risk associated with  $\alpha$ -thalassemia itself (0.40) (158). There was also a reduced risk for other serious infections associated with  $\alpha$ -thalassemia and trait in this study, although the reason for this association is not apparent (158).

### 39.5.5 Beta-Globin (HBB)

Two functional alleles encode wild-type  $\beta$ -globin, one at each 11p15 locus. This difference in gene copy number distinguishes the hemoglobinopathies associated with *HBB* from the  $\alpha$ -hemoglobinopathies that stem from mutations in any of the four functional *HBA* alleles. In addition to  $\beta$ -thalassemias associated with defects in  $\beta$ -globin synthesis, there are important structural  $\beta$ -hemoglobinopathies (159).

Sickle hemoglobin (HgbS) is encoded by a *HBB* allele with a point mutation in the sixth codon. In a series of scientific discoveries spanning from 1949 to 1977, this structural hemoglobinopathy was first described as “a molecular disease” based on hemoglobin electrophoresis differences (160), then as a single amino acid substitution linked disease (161), and finally as an SNP-linked disease (162). The A to T change that codes for a glutamic acid to valine substitution constituted the first host microbial resistance trait and the first human disease linked to a SNP.

In countries with endemic malaria, HgbS demonstrates the principle of balanced polymorphism since the



heterozygous state (i.e. sickle-cell trait) is associated with a reduced risk of severe malaria. Either homozygous state confers a disadvantage: HgbS/HgbS causing sickle-cell anemia and  $\beta$ Hgb/ $\beta$ Hgb risking severe morbidity or even mortality due to malaria. Heterozygous advantage may be responsible for the success of the five different HgbS alleles occurring as separate mutational events in five different human populations (163,164). In addition to its multiple clinical manifestations, sickle-cell anemia is associated with an increased susceptibility to a number of infectious complications including parvovirus-induced aplastic crisis and bacterial infections with encapsulated bacterial pathogens such as *Streptococcus*, *Haemophilus*, and *Salmonella* organisms (165).

Unlike HgbS,  $\beta$ -thalassemia is associated with a variety of genetic alterations including SNPs, substitutions, deletions, insertions, and rearrangements. The shared features of these various mutations are quantitative defects in  $\beta$ Hgb synthesis. The heterogeneous group of mutations that lead to  $\beta$ -thalassemia occurs in a manner that suggests that  $\beta$ -thalassemia affords protection against malaria (166).

### 39.5.6 Parkinson Disease (Autosomal Recessive, Juvenile) 2, Parkin (*PARK2*), and *PARK2* Coregulated (*PACRG*)

A bidirectional promoter at chromosome 6q26 is shared by *PARK2* and *PACRG*. The gene products of both of these genes are involved in intercellular ubiquitin-mediated proteolysis. The first disease association at this locus was juvenile-onset Parkinson disease, and there are now multiple different alleles of *PARK2* associated with this form of autosomal recessive Parkinson disease (i.e. onset at less than 20 years of age). In a Vietnamese cohort, Mira and colleagues (2004) found significant associations between leprosy and polymorphisms in the 5' regulatory region shared by *PARK2* and *PACRG*. They went on to confirm an association between this regulatory region and leprosy in a Brazilian cohort. Using reverse transcription polymerase chain reaction (RT-PCR) analysis they detected expression of *PARK2*, and to a lesser extent *PACRG*, in the primary target cells of the causative agent of leprosy (i.e. *Mycobacterium leprae*) (167).

### 39.5.7 Solute Carrier Family 11, Member 1 (*SLC11A1*)

*SLC11A1* was formerly known as natural resistance associated macrophage protein 1 (NRAMP). NRAMP 1 is a human macrophage membrane protein that is recruited to the phagosomal membrane after phagocytosis and apparently acts as a divalent cation transporter (168). The human gene and its protein product were sought following identification of the murine homolog in which a point mutation was associated with increased

susceptibility to intracellular parasites (169). *NRAMP1* is located in chromosome 2q35 (170) and is expressed exclusively in immune phagocytes (171).

Eleven *NRAMP1* polymorphisms have been identified (172), including four mutations that have been associated with increased susceptibility to active tuberculosis (172–174). Data suggests that NRAMP 1 plays a role in the development of lepromatous leprosy (175) and HIV-1 infection (176), as well as idiopathic conditions such as sarcoidosis (177) and autoimmune diseases (178). A recent study examined the association of four intragenic polymorphisms (274C/T, 469+14G/C, D543N, and 1729+55del4) with tuberculosis in a pediatric population (179). This group found the 274C polymorphism to be most significantly associated with rapid progression from infection to clinical disease.

### 39.5.8 Vitamin D Receptor (VDR)

The vitamin D receptor (VDR) is an intracellular protein, which is believed to act as a transcriptional regulatory factor (180). This receptor binds to 1,25-dihydroxyvitamin D3. VDR maps to 12q12–q14 and contains a total of 11 exons including eight protein coding exons. Along with their role in calcium metabolism, vitamin D and VDR are also involved in the immunoregulatory functions of monocytes and activated lymphocytes.

The VDR allele containing a T to C substitution at codon 352 may confer susceptibility to several important infectious diseases. Although this “silent” polymorphism does not alter the protein’s amino acid sequence, it is associated with increased mRNA quantities (181). Mutant alleles containing the C substitution are labeled as “t” genotype, while “T” genotype represents the wild-type allele. Homozygotes for “t” (i.e. tt) appear to have a decreased risk for chronic hepatitis B and decreased active tuberculosis (182,183). Heterozygotes for Tt appear less likely to develop leprosy (184). Whether this SNP is causally related or simply linked to the causative mutation is not yet fully elucidated (181).

## 39.6 EXTRACELLULAR PROTEINS

### 39.6.1 Chemokine, CC Motif, Ligand 3-Like 1 (*CCL3L1* and *CCL3L3*)

Segmental duplication within chromosome 17q21.1 has led to the inclusion of two copies of *CCL3L1* in the reference sequence of the human genome; *CCL3L3* is used to designate the centromeric copy (185). *CCL3L1* is among a group of chemokine receptor ligands that were formally designated as macrophage inflammatory proteins (MIP1 $\alpha$  and MIP1 $\beta$ ), and the gene product of *CCL3L1* has been shown to bind to several chemokine receptors including chemokine binding protein 2 (CCBP2) and chemokine (C-C motif) receptor 5 (CCR5). The gene copy numbers within individuals can

vary between zero and six, although it appears that only rare individuals have either zero copies or more than four copies (186).

The endogenous ligands of CCR5, namely, CCL3L1, as well as CCL3 and CCL5, can block the entry of HIV-1 into cells. This blockade is a consequence of inhibiting CCR5's coreceptor function for macrophage-tropic strains of HIV-1 (50) and (51). The increased affinity of CCL3L1 binding to CCR5, when compared to other CCR5 ligands such as CCL5 or CCL3, suggested that it might have a central *in vivo* role in inhibiting HIV-1 replication (187). The notion that CCL3L1 has a significant functional role in blocking HIV-1 pathogenesis is supported by the demonstration that the possession of a *CCL3L1* copy number lower than the population average is associated with a markedly enhanced risk for HIV-1-associated disease progression (188).

### 39.6.2 Chemokine, C-C Motif, Ligand 5 (CCL5)

CCL5 acts as a ligand for the receptor CCR5 and has previously been referred to as RANTES and SCYA5 in the literature. The gene locus is found at 17q11.2–q12, and the 91 amino acid gene product, which is secreted by CD8 T-cells, acts as a chemoattractant for monocytes and CD4 T-cells (189).

The binding of CCL5 to CCR5 competitively inhibits HIV-1 binding and entry into cells. There have been two noncoding polymorphisms identified associated with variable CCL5 expressions and thereby associated with altered clinical course of HIV-1 infection. The CCL5 promoter polymorphism -28G in a study of Japanese patients, while not associated with incidence of infection, was associated with increased gene expression in infected individuals and delayed HIV-1 disease progression (190). An intronic polymorphism "In1.1T/C" (T168923C) is associated with diminished gene product and accelerated progression to AIDS; in one study 168923C was present in 36% of African-Americans (191). In addition to its observed role in HIV progression, there are data emerging to suggest that 16923C is also associated with diminished response to antiviral therapy for chronic HCV infection (192).

### 39.6.3 Chemokine, CXC Motif, Ligand 12 (CXCL12)

The gene for CXCL12, formerly known as stromal-cell-derived growth factor 1 (SDF1), is found at 10q11.1. This gene yields two isoforms by alternate splicing (193): CXCL12 transcript variant 1 is associated with a 3560 bp mRNA derived from four exons, and CXCL12 transcript variant 2 is associated with a 1940 bp mRNA derived from three exons. CXCL12 binds to CXCR4 on the surface of lymphocytes and downregulates CXCR4 expression by inducing endocytosis (194,195).

A polymorphic variation in the 3'-UTR of transcript variant 2 (or SDF1-beta) has been associated with altered HIV-1 progression. "SDF1-3'A" was used by the authors to designate the allele with a G-to-A transition at position 801 (counting from the ATG start codon) in the 3' UTR. HIV-1 infected persons who were homozygous for 801A (SDF1-3'A/3'A) showed a delay in the onset of AIDS (196).

### 39.6.4 Chitinase 1 (CHIT1)

The human chitinase gene (*CHIT1*) has been assigned to chromosome 1q31–q32, and its gene product is a circulating enzyme also known as plasma chitotriosidase or methylumbelliferyl tetra-*N*-acetylchitotetraoside hydrolase. This enzyme is excreted by macrophages and is believed to play a role in degrading chitin, which is a cellulose-like structural polysaccharide that is generated by numerous microorganisms. Although the pathophysiological implications are unclear, the median activity of chitotriosidase in patients with Gaucher disease was more than 600 times the median value in plasma of healthy volunteers, and during enzyme supplementation therapy of Gaucher disease chitotriosidase activity declined dramatically (197).

A polymorphism consisting of a 24 bp duplication in exon 10, resulting in the activation of a cryptic 3' splice site and an in-frame deletion of 87 nucleotides has been described (198). This polymorphism leads to diminished chitinase activity in heterozygotes and an absence of chitinase activity in homozygotes (198). This polymorphism exists in diverse populations, with a reported allele frequency of 40% in South Asia, 20–25% in Europe, and 0–2% in Africa (198–201). A study in southern India found an association between chronic filariae infection with *Wuchereria bancrofti* and homozygosity for the allele carrying the 24 bp duplication (199).

### 39.6.5 Interleukin-1, Beta (IL-1B) and Interleukin-1 Receptor Antagonist (IL1RN)

Interleukin-1 activity, defined as its capacity to induce IL-2 synthesis in T-lymphocyte cell lines, was attributed to two distinct genes, which lie adjacent to each other on chromosome 2: *IL1A* and *IL1B* (202). *IL1B*, along with eight other interleukin-1 family genes, forms a cytokine gene cluster at 2q14. Proteolytic cleavage by CASP1 is required to convert the IL-1B protein precursor to functional IL-1 $\beta$  (203). Evidence suggests that some microbial pathogens (such as *Streptococcus pyogenes*) may directly alter production of active IL-1 $\beta$  through the expression of a bacterial cysteine protease that cleaves the human IL-1B precursor to yield active IL-1 $\beta$  (204). IL-1 $\beta$  helps drive T-helper lymphocyte 1 (Th1) responses and stimulate the synthesis of T cell proliferative cytokines such as IL-2 (205).

The interleukin-1 receptor antagonist gene (*IL1RN*) is located within the same cytokine gene cluster at 2q14 as *IL1B*. The *IL1RN* protein competitively inhibits IL-1A and IL-1B binding to the IL1R. As such, when *IL1RN* was described in 1990, it was the first specific cytokine receptor antagonist identified (206). *IL1RN*\*2 has been used to designate the polymorphic variant with two 86bp tandem repeats (VNTR) in intron 2 of *IL1RN* (207).

Wilkinson and colleagues studied potential associations between *IL1B* and *IL1RN* polymorphisms and clinical tuberculosis in a group of London patients who were of Indian origin (208). They concluded that *IL1RN*\*2 was associated with elevated *IL1RN* production and a reduced Mantoux response to purified protein derivative of *M. tuberculosis*. On the other hand, a non-*IL1RN*\*2 haplotype was associated with increased IL-1B production and linked to pleural tuberculosis.

An *IL1B* promoter polymorphism (C-31T) has been linked to expression of the gene; the T allele is associated with increased IL-1 $\beta$  concentrations and is therefore considered “proinflammatory.” *IL1B*-31T has been associated with hypochlorhydria induced by gastric infection with *H. pylori* and the associated development of gastric carcinoma (209). In this study, carriers of *IL1B*-31T had an autosomal dominant increased gastric cancer risk at an odds ratio of 1.9 (95% CI, 1.5–2.6).

### 39.6.6 Interleukin-4 (IL-4)

The IL-4 gene is adjacent to the IL-13 gene on chromosome 5q31.1, and these genes together with *IL5* and others form a cytokine cluster in the chromosome region. A long-range regulatory element, spread over 120kb, is believed to coordinate the expression of *IL4*, *IL5*, and *IL13* (210). IL-4 is a pleiotropic cytokine that regulates Th2 lymphocyte subset development and IgE production (205,211). IL-4 downregulates CCR5 expression and upregulates the expression of CXCR4 (212). A promoter polymorphism (C-589T) has been correlated with altered promoter activity, and the -589T allele, which is associated with increased activity, was found to have an allele frequency of 0.15 in a French population and 0.64 in a Japanese population (212).

HIV-1 viral load was lower in patients with IL-4 -589T and was associated with delayed progression to AIDS and death. These results are consistent with a protective effect due to a reduction in HIV replication, and it is in agreement with in vitro experiments that showed that IL-4 downregulates CCR5 expression and inhibits replication of R5 HIV-1 strains (212). Interestingly, IL-4 -589T enhances acquisition of X4 HIV-1 strains, and in the homozygous state this IL-4 polymorphism may negatively impact late-stage prognosis (213).

Choi and colleagues attempted to correlate the development of chronic disseminated candidiasis in patients

with acute leukemia with a more detailed polymorphic analysis of the IL-4 promoter. They defined three polymorphic haplotypes at positions -1098, -589, and -33; those haplotypes are TTT, TCC, and GCC (214). They associate greater risk for chronic disseminated candidiasis, a form of fungal infection observed primarily in patients with acute leukemia, with TCC, and apparent protection from CDC with TTT (214).

### 39.6.7 Interleukin-10 (IL-10)

The human IL-10 gene maps to chromosome 1q31–1q32 and multiple cytokine response elements are located in its 5' regulatory region including target sites for TNF, IFN- $\gamma$ , and IL-6 (215). Viral pathogens such as EBV (216) and the LPS of gram-negative bacteria (217) induce expression of human IL-10 from mononuclear cells. Elevated levels of serum IL-10 have been observed in patients with chronic HCV (218,219) and EBV (216) infections. Overall, much of the available data is consistent with a model in which diminished levels of IL-10 increase the risk for some autoimmune disease.

*IL10* promoter polymorphisms at positions -1082, -819, and -592 have been identified, and the GCC, ACC, and ATA haplotypes are linked, respectively, with high, intermediate, and low in vitro IL-10 production (220). The GCC haplotype as well as high in vitro IL-10 levels were associated with poor treatment response to interferon- $\alpha$  (IFN- $\alpha$ ) in patients with chronic HCV infection (221). Two studies of *IL10* promoter polymorphism and EBV were reported by Helminen and colleagues in a Finnish population; in this population the IL-10 ATA haplotype was associated with protection against primary EBV infection as well as increased in vivo IL-10 levels, and another report by this group associated the GCC haplotype with EBV seronegativity and ACC haplotype with severe EBV infection (222,223). The basis for the differences in the observed in vitro versus in vivo IL-10 production associated with the haplotypes has not been elucidated. Shin and colleagues (224) reported an increased susceptibility to HIV-1 infection and more rapid progression to AIDS in patients with a C-to-A polymorphism at position -592 of the *IL10* promoter. This -592A association appeared to be autosomal dominant in its effect.

### 39.6.8 Interleukin-28B (IL-28B)

The gene for interleukin-28B (*IL28B*) is found along with *IL28A* and *IL29* in a cytokine cluster at 19q13.13 and codes for IFN $\lambda$ 3 (225). This gene is associated with the spontaneous clearance of HCV infection and with the response to standard therapy with IFN- $\alpha$  and ribavirin treatment in individuals with chronic HCV infection (226–229). The fine mapping of the pathogenic variant is being pursued (230). The upstream haplotype associated with viral clearance at SNPs rs12979860 and

rs8099917 is CT, and the persistence haplotype at these SNPs is TG (225).

### 39.6.9 Mannose Binding Lectin 2 (MBL2)

*MLB2* at chromosome 10q11.2 encodes a serum protein that is secreted by hepatocytes and facilitates the opsonization and phagocytosis of extracellular microbial pathogens (231).

In 1991, Sumiya and colleagues linked recurrent infection, low serum concentration of this protein, and an amino acid change at codon 54 (G54D). Since that time, a body of literature has evolved that examines the relationship between serum MBL2 protein levels and six polymorphisms (three coding SNPs, one 5' UTR SNP, and two promoter SNPs). Within various populations studied, the coding SNPs are in linkage disequilibrium with the noncoding SNPs (232). Each of the three coding SNPs is associated with lower serum MBL2 protein levels, and each disrupts the tandem motif repeat (Gly-X-Y) in the collagenous region of the protein; the variants are frequently referred to as allele A (wild-type), allele B (G226A and gly54asp), allele C (G235A and gly57glu), and allele D (C219T and arg52cys) based on the chronological order in which they were identified (233–235). The three variants have a combined allele frequency of 20% in Caucasians, and the serum protein concentrations are diminished in heterozygotes and essentially undetectable in individuals with either homozygosity for a variant allele or compound heterozygosity for variant alleles (236). Along with infectious disease associations, the genotypes causing low levels of MBL2 have also been associated with autoimmune diseases; for example, there are studies linking low-concentration genotypes with erosive rheumatoid arthritis in both Chinese and Caucasian patient groups (237,238).

The most striking infectious disease association with *MBL2* to date is the association of the genotypes that cause low MBL2 protein concentration and invasive meningococcal infections (239). The “low concentration” variant alleles have been strongly associated with invasive pneumococcal infection, as well as febrile neutropenia and infection in the setting of chemotherapy and hematopoietic stem cell transplant (240). The clinical course and response to therapy of numerous other infections have been associated with low-concentration alleles, including mycobacterial infection, HIV-1, hepatitis B and C viruses, and aspergillosis (240).

The high frequencies of these variant alleles in many populations has led to the suggestion that MBL structural variants could represent a case of balanced polymorphisms. One theory suggests that heterozygosity for MBL variant proteins may confer protection against intracellular parasites that use opsonization via complement as a route of gaining cellular entrance, and another theory suggests that the MBL-mediated route of

complement activation may, in some circumstances, be detrimental for the host (232).

### 39.6.10 Surfactant, Pulmonary-Associated Protein B

Surfactant, Pulmonary-Associated Protein B (*SFTPB*) (2p12–p11.2) encodes a highly hydrophobic protein component of pulmonary surfactant. The surfactant lowers surface tension in the alveoli of the lung, and an autosomal recessive condition known as “congenital alveolar proteinosis” is associated with a deficiency of pulmonary surfactant protein B (241). A polymorphism in the *SFTPB* coding sequence (T1580C) leads to an amino acid substitution that results in the loss of a N-glycosylation site. In a study of 402 patients with community-acquired pneumonia, comparing the 1580C and the 1580T alleles, patients with C/C were at a significantly higher risk of respiratory failure compared to those with T/C and T/T (242).

### 39.6.11 Transforming Growth Factor Beta-1

Transforming growth factor  $\beta$ -1 (*TGFB1*) is encoded at 19q13.1; *TGFB1* shares significant sequence identity with *TGFB2* and *TGFB3* (243). A polymorphism at position +915 (codon 25) resulting in the homozygous arginine/arginine genotype has been associated with high levels of TGF  $\beta$ -1 production in stimulated lymphocytes (244). HCV-associated hepatic fibrosis (245) has been linked with the arginine/arginine sequence genotype.

### 39.6.12 Tumor Necrosis Factor (TNF) and Lymphotoxin A (LTA)

TNF is a proinflammatory cytokine mainly secreted by cells of monocyte lineage (246). The TNF (formerly known as  $\text{TNF}\alpha$ ) and the LTA (formerly known as  $\text{TNF}\beta$ ) genes are separated by only 1.2 kb at chromosome 6p21.3. These genes lie in the class III region of the human MHC complex of chromosome 6, approximately 250 kb centromeric of the HLA-B locus and 850 kb telomeric of *HLA-DR*. It may actually be polymorphisms within these genes that contribute to some of the associations that have been made with MHC alleles with specific autoimmune and infectious diseases. The recent introduction of therapeutic agents that block TNF signaling has led to an association between pharmacological TNF blockade and tuberculosis reactivation and thereby reinforced the notion that effective TNF signaling is necessary for control of infection (247).

*TNF* promoter polymorphisms have been associated with altered expression of TNF. Association studies, which have used the *TNF* promoter polymorphism G-308A to examine an extended MHC haplotype, often use the designations *TNF1* (for -308G)



and *TNF2* (for -308A). The A substitution at position -308 ("TNF2") results in a much stronger transcriptional activator and increased levels of TNF expression (248). This -308A allele is associated with worse outcomes in cerebral malaria (249) and leishmaniasis (250). Presumably, increased levels of TNF may contribute to disease severity and morbidity in systemic infections. Increased levels of plasma TNF were present in Gambian children with cerebral malaria, and the highest TNF levels were noted in fatal cases (251). Gambian children homozygous for the -308A allele were at significantly increased risk for death or severe neurologic sequelae due to cerebral malaria (249). The *TNF* -308A allele has also been associated with the development of HIV-1 dementia (252), Guillain-Barre syndrome following campylobacter infection (253), and lepromatous leprosy (254).

Cerebral malaria has also been associated with the *TNF* -376GA allele (255). In another study, severe malarial anemia was shown to be significantly associated with *TNF* -238A after stratification for the HLA

haplotype (256). Polymorphisms in both TNF and LTA are related to the development of ulcer disease in patients with *H. pylori* (257).

### 39.7 CONCLUSION

There is a growing list of human mutations and polymorphisms that are associated with variable susceptibilities to infection. For the most part, the polymorphism associations have been made in cases where both the infectious disease and the genetic variants are common, and a picture of host defenses extending beyond the classical immune system is emerging in conjunction with these findings. The susceptibility phenotypes fall into classic Mendelian inheritance patterns in many cases (Table 39-2). In addition to the examples of altered susceptibility to infections reviewed in this chapter, there is a growing database of information regarding variable responses to antibiotics and other therapeutic interventions in infection. As a host genetic basis of clinical infectious disease management, which includes both

**TABLE 39-2 Non-HLA Microbe–Gene Susceptibility Associations**

Microbe	Gene/Gene Product	Associated Susceptibility
HIV-1	FUT 2 CCR5 CCR2 CX <sub>3</sub> CR1 NRAMP1 SDF1-3'A	Heterosexually acquired infection Infection and progression to AIDS Progression to AIDS Progression to AIDS Progression to AIDS Progression to AIDS
Parvovirus B19	PK synthase	Infection
Hepatitis B virus	VDR	Persistent infection
Hepatitis C virus	IL-28B	Persistent infection Treatment response
<i>Haemophilus influenzae</i>	FUT 2	Invasive infection
<i>Streptococcus pneumoniae</i>	FUT 2	Invasive infection
<i>Neisseria meningitidis</i>	FUT 2	Invasive infection
<i>Pseudomonas aeruginosa</i>	CFTR	Pulmonary infection
<i>Salmonella</i> spp.	CFTR IFNGR	Gastrointestinal infection Systemic infection
<i>Vibrio cholerae</i>	Blood group O	Gastrointestinal infection
<i>Escherichia coli</i>	GALT	Bacteremia
<i>Helicobacter pylori</i>	Lewis Ag IL-1β	Gastric infection Gastric carcinoma
<i>Mycobacterium</i> spp.	VDR IFNGR IL12RB1 NRAMP 1 IL-1β TNF2 MBP	Symptomatic infection Systemic infection Systemic infection Symptomatic infection Pleural tuberculosis Symptomatic infection Pulmonary tuberculosis
<i>Plasmodium falciparum</i>	Blood group A α Hgb βHgb G6PD TNF2	Severe infection Severe infection Severe infection Severe infection Cerebral malaria
<i>Plasmodium vivax</i>	DARC	Infection
<i>Leishmania</i> spp.	TNF2	Mucocutaneous infection

susceptibility and therapeutic response, becomes better understood the opportunities for improving patient care will follow.

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### Biography



**Michael F Murray, MD**, a former primary care provider, is now the clinical chief of genetics at Brigham and Women's Hospital in Boston. He trained in internal medicine at the Cleveland Clinic and then went on to do fellowships in Infectious Diseases and Medical Genetics. Dr Murray directs the annual course in "The Genetic Basis of Adult Medicine: What the Primary Care Provider Needs to Know" and is the program director for a combined training program in Internal Medicine and Medical Genetics. He leads the Adult Genetics Clinic at Brigham and Women's Hospital where over 300 patients per year are evaluated, diagnosed, and managed. His research interests include the integration of electronic family health history tools into medical practice and the use of whole genome testing in medicine.

# CHAPTER

# 40

## Transplantation Genetics

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### ABBREVIATIONS

APC – Antigen-presenting cells  
ARMS – Amplification refractory mutation system  
BMT – Bone marrow transplantation  
C' – Complement  
CAPSeq – Clustering and alignment of polymorphic sequences  
CTL – Cytotoxic T-lymphocytes  
DKO – Double knockout  
dNTP – Deoxynucleotide triphosphate  
EB – Epstein–Barr  
ES – Embryonic stem  
GATK – Genome analysis toolkit  
GVHD – Graft-versus-host disease  
HAR – Hyperacute rejection  
HLA – Human leukocyte antigen  
HSC – Hematopoietic stem cells  
HTC – Homozygous typing cells  
iPS – Induced pluripotent stem  
KIR – Killer Ig-like receptors  
KO – Knockout  
mH – Minor histocompatibility  
MHC – Major histocompatibility gene complex  
MLC – Mixed lymphocyte culture  
MLR – Mixed lymphocyte reactivity  
NK – Natural killer  
NMDP – National Marrow Donor Program  
PCR – Polymerase chain reaction  
PE – R-phycoerythrin  
piPSC – protein-induced pluripotent stem cells  
PLT – Primed lymphocyte test  
PRA – Percentages of reactive antibodies  
PSBT – Pyrosequence-based typing  
RFLP – Restriction fragment length polymorphism  
RR – Relative response  
SI – Stimulation index  
SSOP – Sequence-specific oligonucleotide probe  
SSP – Sequence-specific primer

TCR – T-cell receptor

Th – T-helper cells

UNOS – United Network for Organ Sharing

### 40.1 INTRODUCTION

The idea of replacing a diseased or damaged part of the body of an individual—the recipient—with the same part provided by another healthy individual—the donor—has always been present in the human mind. Greek mythology is full of examples of chimeras—imaginary animals composed of parts coming from individuals of different species. If, on one hand, chimeras can represent an artistic version of a successful xenotransplantation intervention (i.e. a transplant between individuals of different species), on the other hand, picturesque examples of dreamed allotransplantation (i.e. a transplant between two genetically different individuals of the same species) are also available (1).

The most elegant representation of this mental concept is perhaps the famous painting of Fra Angelico (1387–1455), an altarpiece in the San Marco Church in Florence, Italy (Figure 40-1). The painter here represented the miracle performed by Saints Cosmas and Damian in Constantinople as narrated in a third-century legend. According to the legend, these twin brothers, one is a physician and the other a surgeon, replaced the cancerous leg of a white church sacristan with a healthy limb from a recently deceased Moor. The story is told that the sacristan walked around for the rest of his life with one white leg and one black leg (1–3).

There is, however, evidence that transplantation surgery was successfully performed more than 2000 years ago by ancient Hindu vaidya (1,4). The reported



**FIGURE 40-1** The healing of Justinian by Saint Cosmas and Saint Damian as portrayed by Fra Angelico in his painting conserved in the Museo di San Marco, Florence, Italy.

clinical interventions that involved the reconstruction of the nose using pedicle flap grafts from the patient's own forehead were similar in approach to the one described by the historian Calinzio who, in 1503, wrote in praise of the surgeon Branca who reconstructed the nose of a gentleman using skin removed from his slave (5). Calinzio added that: "for a time the new nose functioned well," then "suddenly grew cold," "turned blue," and "eventually fell off." Because the slave died on the same day, the slave's demise was blamed for the loss of the engrafted nose. Today, we know that the difference between these successful and unsuccessful transplants is that the first was an autologous transplant, and the other, an allotransplant. Branca's patient actually suffered a typical graft rejection, whereas the physician Gaspare Tagliacozzi was instead successful when, 100 years later (the year 1597), he tried to reproduce the vaidya's results by performing similar reconstructive rhinoplasty using skin flaps collected from the patient's arm (1,3).

We had to wait until the beginning of the twentieth century to find evidence of a fresh new look to the transplantation problem. It was, in fact, in 1912 when George Schöne clearly summarized conclusions that he gathered from different transplant attempts mostly performed in animals. The "laws of transplantation" were so defined: (1) autografts generally succeed; (2) allografts usually fail; and (3) xenografts invariably fail. "Blood relationship" was proposed as the biological barrier that separates donor from recipient: The closer the relationship, the more likely the graft will be accepted (1,3).

Twenty-five years later, Peter Gorer, an expert on tumor resistance in mice, defined the scientific basis

of this postulated blood relationship (6). Gorer was interested in explaining why tumors that could often be transplanted from one mouse to another failed to grow when transplanted in another strain of recipient mice. To determine what was different between the acceptor versus the rejecting strains, Gorer performed tumor transplants in  $F_1$ ,  $F_2$ , and backcross progeny of the three inbred strains of mice. The permissive trait was attributed to two independent genetic loci. On the basis of his knowledge of the blood groups, Gorer also attempted to distinguish the tumor-susceptible from the tumor-resistant animals on the basis of mouse erythrocyte agglutination driven by human serum. The successful experiments also proved that, similar to that for the ABO system, agglutination behaved as a simple Mendelian-dominant trait. Furthermore, rabbit anti-mouse erythrocyte sera were sufficient to define three mouse red blood cell antigens. The detection in the serum of the mice of anti-type-II-antigen antibodies coincided with rejection propensity (7). It was on this basis that Gorer joined forces with George Snell at the Jackson Laboratory in Bar Harbor, Maine, where they expanded the studies to Snell's "congenic" inbred mouse strains (8). Congenic strains are genetically identical except at a single chromosomal locus. Using congenic strains of mice, Gorer and Snell were able to determine that engrafted tumors were most likely able to "take" when the recipient mouse was of the same strain as the donor (9). More importantly, they also realized that survival of donor tissue was not limited to tumors but applied to any graft including skin. In addition, donor-specific antibodies first noted by Gorer in the graft-rejecting recipient were induced



not only by tumor grafting but also by any other tissue graft or blood transfusion (reviewed in Ref. (2)). By appropriate genetic crosses and backcrosses among their congenic inbred strains, Snell and Gorer identified a number of genetically independent loci controlling these immune responses, which they designated H for histocompatibility (i.e. compatibility between different tissues), followed by an arabic numeral in the order of discovery. The locus designated H-2 appeared to be more immunogenic than the others and corresponded to Gorer's original type-II antigen. This locus became known with time as the murine major histocompatibility gene complex (MHC). The MHC can be considered the molecular embodiment of Schöne's "blood relationship" (1).

Sir Peter Medawar tried to apply these concepts to help soldiers burned during World War II with skin grafts (10,11). He also came to the conclusion that, while autografts succeed, allografts actually fail after an initial period of survival. A second allograft from the same donor did not experience the latent period of the first; rather, it rejected in an accelerated manner. The survival of a second graft from a different donor was instead similar to that of the original graft, rejecting only after a latent period. Because the accelerated rejection could also be induced by injection into the recipient of donor leukocytes before engraftment, he hypothesized that "destruction of the foreign epidermis was brought about by a mechanism of active immunization." Specific antigens analogous to those of the ABO blood groups were supposed to be shared by skin and leukocytes and were responsible for the rejection response. Medawar's conclusions constituted the theoretical basis to postulate that immunosuppression might overcome the laws of transplantation.

In 1954, the first successful kidney transplant was performed between monozygotic twins, for which immunosuppression was not necessary (12). In 1959, the same team used an efficient immunosuppressive regimen to perform the first kidney graft between unrelated individuals (13). The graft survived for 20 years.

The observations that antibodies against transplantation antigens could be generated as the result of an immune response against transfused blood components suggested the possibility of identifying human histocompatibility antigens by serologic methods. In 1958, Jean Dausset produced the first human histocompatibility antiserum by deliberate immunization of one blood donor with leukocytes from another (14). This antiserum agglutinated the white blood cells of about 60% of his French donor panel. Concurrently, Payne and Rolfs showed that the sera of multiparous women often contained leukoagglutinins (15). This important finding promised readily available sources of reagent antisera with which to characterize human leukocyte antigens (HLA).

## 40.2 THE PHYSIOLOGIC FUNCTION OF MHC MOLECULES

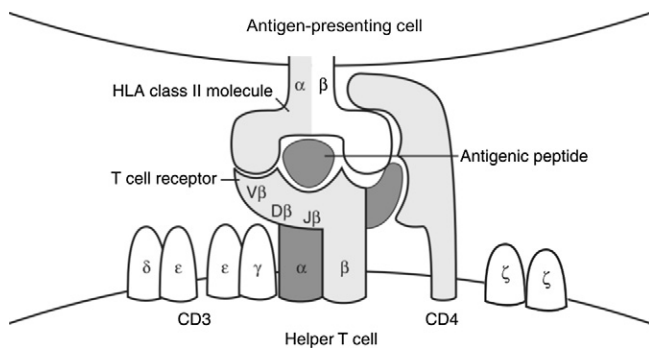
The interpretation of the genetics of histocompatibility initially was, as presented, based on experiments, in which the histocompatibility antigens were only seen as the factors limiting tissue transplantation by promoting graft rejection. Later on, Pamela Bjorkman et al. discerned the physiologic function of the MHC molecules when they solved the X-ray crystallography structure of HLA-A2 and recognized the presence of a heterogenic population of bound peptides to what is now referred to as *Bjorkman's groove* (16,17).

Although with different efficiency, macrophages, monocytes, dendritic cells, and mature B-cells are all able to phagocytose foreign molecules, thus capturing "nonself" proteins invading our body and enzymatically cleaving them into smaller segments called peptides. This function is called "processing" of the antigenic protein. The peptides generated as the result of antigen processing are then protected from further cleavage and brought to the cell surface by MHC molecules able to accommodate the peptides in their antigen-combining site (18). The function of this site, the so-called peptide-binding groove discovered by Bjorkman et al. (reviewed by Bjorkman in Ref. (19)), was clearly determined once the interpretation of the crystal structure of the HLA molecule was completed (17). The HLA and peptide complex is then exposed at the cell surface (i.e. "presented") to a T lymphocyte that recognizes and engages it by its T-cell receptor (TCR) molecule. The antigen-presenting cell (APC) in this way activates T lymphocytes, causing them to divide and secrete specific factors (20). The signal from the engaged TCR to the inside of the cell is transmitted by a chain of molecules, the CD3 complex consisting of a series of dimers such as the CD3  $\delta\epsilon$ ,  $\gamma\epsilon$ , and  $\zeta\epsilon$  chains, that ultimately activate the enzymes necessary to stimulate cell division, cytokine receptor upregulation, and cytokine production (20,21) (Figure 40-2). Cytokines are secreted cellular hormones that can inhibit or stimulate other cells to become activated.

T-cells producing "helper" factors are known as T-helper cells (Th). On the basis of preferential secretion of certain cytokines, Th cells are conventionally divided into Th<sub>1</sub>, Th<sub>2</sub>, and Th<sub>17</sub> subgroups (22). Th<sub>1</sub> cells, which preferentially secrete interleukin 2, interferon- $\gamma$ , and tumor necrosis factor (TNF)- $\alpha$ , help other T cells, already committed to the cytotoxic T-cell lineage, to proliferate. These cytotoxic T lymphocytes (CTLs) destroy cells in our bodies that are infected or altered by the same foreign antigen to which the Th initially reacted. Th<sub>2</sub> cells primarily promote activation of B lymphocytes and subsequent antibody production via secretion of interleukins 4, 5, 6, 10, and 13 (22). Th<sub>17</sub> cells secrete interleukins 17A, 17F, 21, 22, and 26, and acting primarily at mucosal sites contribute to immune-mediated protection against bacteria and fungi (22).



T-helper cells can be recognized and distinguished from CTL cells by differences in coreceptor expression and MHC restriction. For example, CD4 and CD8 are T-cell coreceptor molecules that stabilize the TCR/antigenic peptide/MHC molecule complex while recognition is taking place (Figure 40-2). In general, CD4<sup>+</sup> T-cells preferentially recognize MHC class II molecules (see later) that have bound peptides derived from internalized, exogenous proteins and give rise to T-helper cell lineages, whereas CD8<sup>+</sup> T-cells react with MHC class I molecules (see later) complexed with cell-derived endogenous peptides and are activated to generate CTLs.

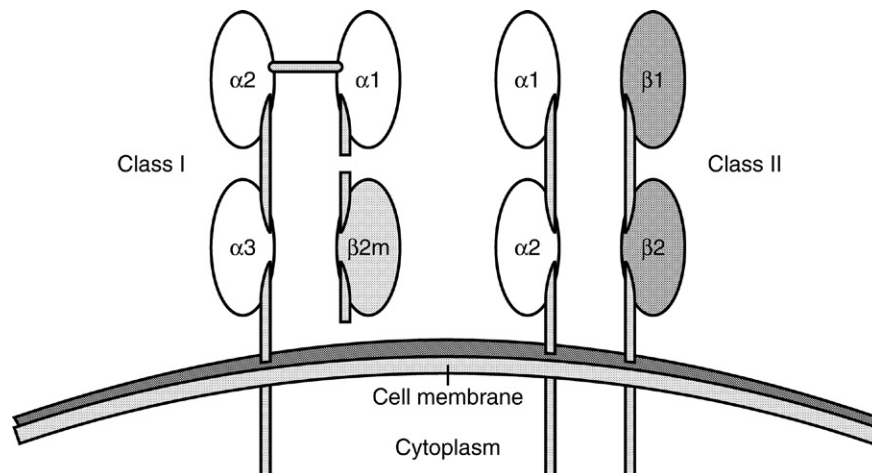


**FIGURE 40-2** The HLA class II molecule, expressed at the surface of an APC, interacts with the processed antigenic peptide, lodged in the Bjorkman groove of the molecule. The helper T cell recognizes the complex HLA molecule/antigenic peptide with its  $\alpha/\beta$  TCR. The accessory molecule CD4 stabilizes the binding of the TCR with the HLA molecule. CD4 provides antigen-mediated specific T-cell activation functions. The CD3 complex, composed of gamma ( $\gamma$ ), delta ( $\delta$ ), epsilon ( $\epsilon$ ), and zeta ( $\zeta$ ) molecules, transfers the signal of the successfully engaged TCR into the cytoplasm of the T cell. (From Conrad, B.; Trucco, M. *Superantigens as Etiopathogenetic Factors in the Development of Insulin Dependent Diabetes Mellitus*. Diabetes Metab. Rev. 1994, 10, 309–338.)

### 40.3 THE STRUCTURE OF HUMAN HISTOCOMPATIBILITY MOLECULES

HLA molecules are grouped into two classes based on their structure, function, and cellular expression. Class I (HLA-A, -B, and -C molecules) are characterized by a heavy (43 kDa)  $\alpha$  chain with three domains ( $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$ ) (Figure 40-3) that dimerizes with the  $\beta_2$  microglobulin, a short (12 kDa) protein, which provides the fourth domain necessary to stabilize the HLA molecule (23). Class I molecules are coexpressed on nearly all nucleated cells and platelets and are anchored via the transmembrane portion and the short cytoplasmic tail of the  $\alpha$ -chain only. Class II molecules (HLA-DR, -DQ, and -DP) consist of  $\alpha$  (34 kDa) and  $\beta$  (29 kDa) chains, each with two domains. Class II molecules are anchored by both chains, each of which can communicate across the membrane with cytoplasmic structures (Figure 40-3). These molecules are expressed primarily on B lymphocytes, macrophages, dendritic cells, activated T cells, and endothelial cells.

The two outermost domains of each molecule ( $\alpha_1$  and  $\alpha_2$  in class I;  $\alpha_1$  and  $\beta_1$  in class II) fold together to form the cleft in which the antigenic peptide can find appropriate lodging (Figure 40-4). These first external domains are approximately 90 amino acids, or 270 base pairs in length. The antigen-combining site (also known as the Bjorkman's groove) is composed of a  $\beta$ -pleated sheet with eight antiparallel strands forming the floor of the groove, and sides assuming the shape of  $\alpha$  helices (Figure 40-5). The amino acid residues forming this cleft are very polymorphic and are particularly immunologically relevant in the  $\alpha$ -helical regions. As the protein folds into the tertiary structure, some of the hypervariable regions assume a location pointing into the cleft (i.e. an optimal position



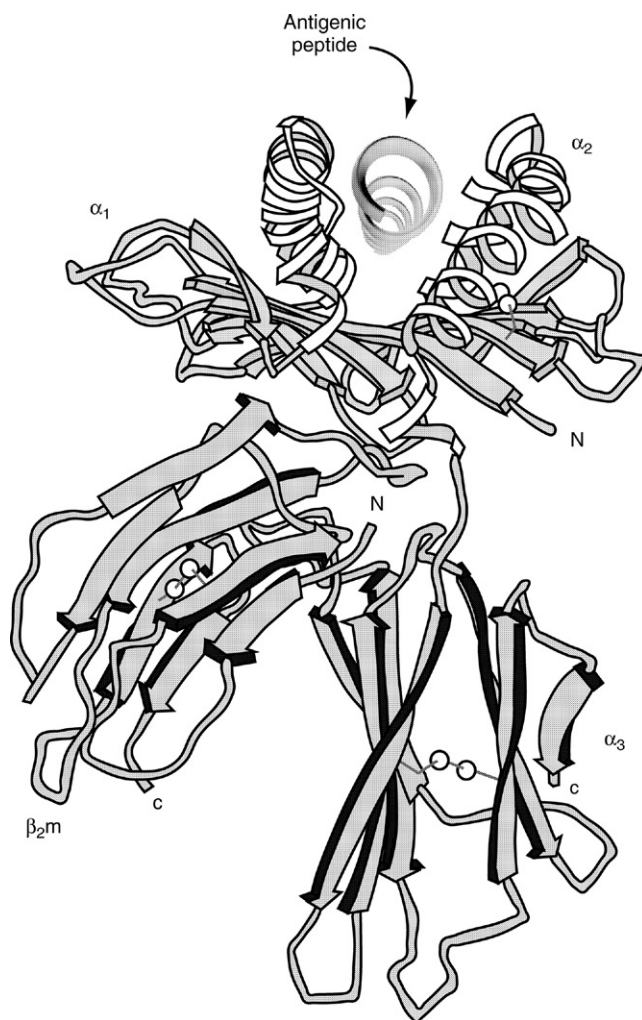
**FIGURE 40-3** The secondary structure of the HLA class I molecule with the  $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$  domains (i.e. amino acid sequences that show similarities and are present more than once in the same polypeptide) of the chain completed by the  $\beta_2$ -microglobulin is here compared with the structure of the class II molecule with its  $\alpha_1$  and  $\alpha_2$  domains of the  $\alpha$  chain. Domains  $\beta_1$  and  $\beta_2$  are characteristic of the class II  $\beta$ -chain only. Both class I and class II heterodimers form, at their most external end, a peptide-combining site composed of the  $\alpha_1$  and  $\alpha_2$  domains for class I, and  $\alpha_1$  and  $\beta_1$  domains for class II molecules. (From Rosner, G.; Martell, J.; Trucco, M. *Histocompatibility*. In Hematopoietic Stem Cell Therapy; Lister, J., Ball, E. D., Eds.; Churchill Livingstone: Philadelphia, 2000, pp 233–251.)

for interaction with the processed antigen). Other hyper-variable segments are instead located on the external part of the molecule and constitute the determinants potentially involved with TCR recognition. These same hyper-variable regions form the molecular basis of alloreactivity in tissue transplantation (i.e. donor differences in these amino acids are recognized as “foreign” by the recipient who immunologically reacts against them). These same, antigenically relevant differences are the HLA originally recognized by Dausset and Payne.

The HLA class I and class II molecules constitute the most highly polymorphic genetic system in humans (24,25). A stable, inherited polymorphism gives rise to alternative forms of the protein, the alleles. Nearly, all

the HLA molecules have various alleles, with HLA-B being the most polymorphic, having on average 86 SNPs per kb (26) and more than 1800 reported alleles, with the numbers consistently rising (27).

The molecular basis for the HLA polymorphism resides in nucleotide sequence differences present in the coding regions of the HLA genes. The polymorphism is clustered into three or four discrete hypervariable regions (Figure 40-6, panel A), which become more evident when the amino acid sequences of these alleles are aligned (Figure 40-6, panel B). These nucleic acid sequence differences account for amino acid differences among the various HLA molecules. Although most HLA alleles occur in all ethnic groups, they may vary in frequency from one group to another (28,29).

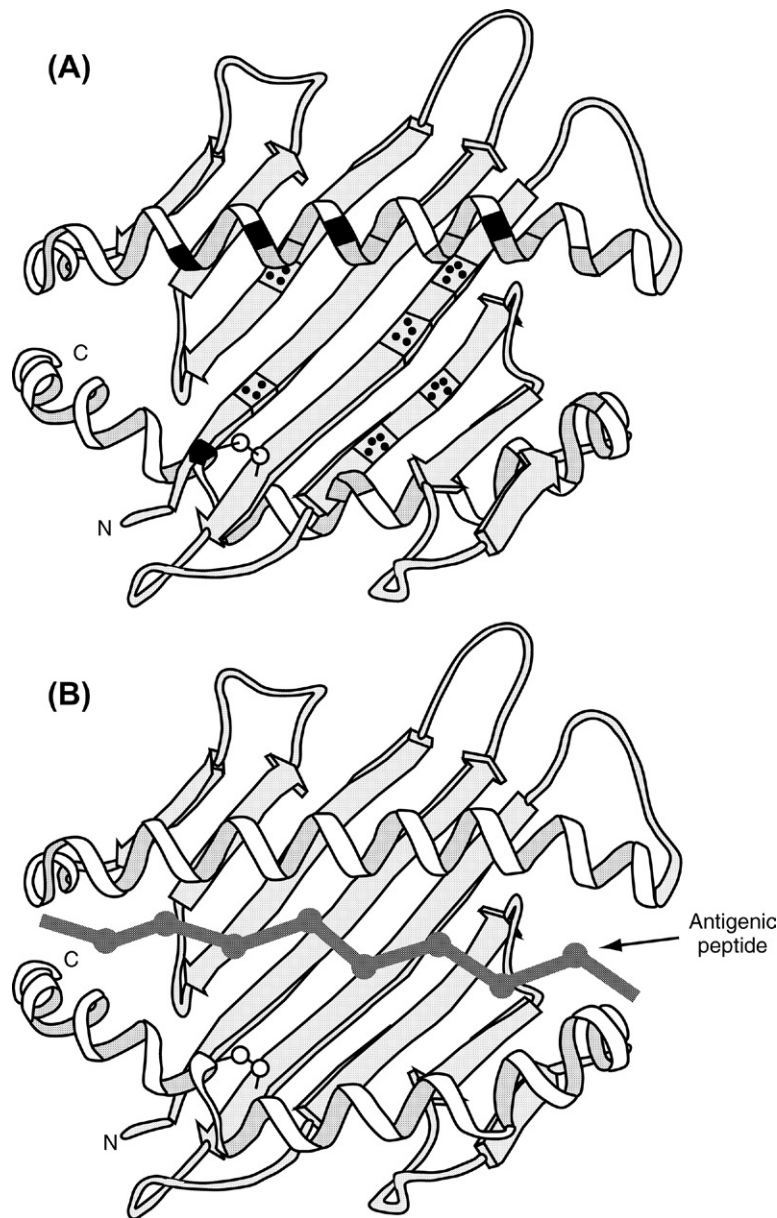


**FIGURE 40-4** The resolution of the structure of crystallized HLA-A2 class I molecule. The molecule, shown here from the side, has its most external domains ( $\alpha_1$  and  $\alpha_2$ ) able to form the antigen-combining site in which the processed antigenic peptide is lodged. In this position, the complex HLA molecule/antigenic peptide is recognized by the T cell via its receptor molecule. (From Faas, S.; Trucco, M. *Major Histocompatibility Locus and Other Genes that Determine Risk of Development of Insulin-Dependent Diabetes Mellitus*. In *Diabetes Mellitus: A Fundamental and Clinical Text*; LeRoith, D., Taylor, S., Olefsky, J. M., Eds.; J. B. Lippincott Co: Philadelphia, 1996, pp 326–333.)

#### 40.4 THE CHROMOSOMAL ORGANIZATION OF THE HLA COMPLEX

The genes encoding HLA class I and class II molecules are located in >4000-kilobase (kb)-long DNA segment present on the short arm of chromosome 6 (26,30,31). This DNA segment can be subdivided into three major regions that encode class I molecules (2000 kb), class III molecules (1000 kb), and class II molecules (1000 kb) (30). The organization of the genes included in the HLA complex is shown in Figure 40-7. The physical location, the locus, of each HLA gene determines its order on the chromosome, while the nomenclature instead recapitulates the chronological characterization of the genes at the various loci. Thus, the A locus is most distant from the centromere and was the first to be characterized. The characterization of the B locus immediately followed that of the A locus, however, A and B are geographically separated by the C locus, which was the last to be characterized. The first class II locus was characterized by cellular tests after C and was then called D. Subsequently, by absorbing out anti-class-I antibodies, sera able to recognize the same alleles defined by mixed lymphocyte reaction (MLR) were also obtained. The alleles recognized by these anti-class-II antibodies were then defined as D related and eventually became known as DR alleles. Class II loci -DQ and -DP were characterized after -DR in this chronological order and actually are in the same physical order on chromosome 6, closer to the centromere than class I loci with -DP as the most centromeric (Figure 40-7). The class I and class II regions are physically separated on the chromosome by genes encoding MHC class III molecules. Class III genes encode components of the complement cascade, C2, Bf, C4A, C4B, the functional steroid hydroxylase gene CYP21, a CYP21 pseudogene, and the genes for TNF- $\alpha$  and TNF- $\beta$  that are not relevant in the context of histocompatibility.

The class I genes present at the A-, B-, and C-loci encode molecules that can efficiently present antigenic peptides once paired with the  $\beta_2$  microglobulin, which is encoded on chromosome 15. The class I region also



**FIGURE 40-5** The antigen-combining site in which the antigenic peptide can find appropriate lodging. In (A), the HLA molecule is seen from the top. The polymorphic regions are indicated in different nuances of color from gray to black. In (B), a schematic, a 9-amino-acid-long peptide is shown present in the groove of the molecule. The antigenic peptides found in HLA class I molecule grooves are normally of this same size, while the antigenic peptides most frequently found associated with HLA class II molecules are longer than nine amino acids and can vary considerably in size. (Modified from Trucco, M. *To Be, or Not To Be Asp 57, that is the Question*. *Diabetes Care* 1992, 15, 705–715).

contains nonfunctional genes (i.e. pseudogenes HLA-H, -J, -K, and -L) and genes that are potentially functional, but whose gene products are not yet fully elucidated such as HLA-E and -F. HLA-G, a protein preferentially expressed at the surface of cytotrophoblasts at the maternal–fetal interface of the placenta, has been shown by Pazmany et al. (32) to sufficiently protect otherwise susceptible target cells from lysis promoted by activated natural killer (NK) cells. In addition to the ones at the -DR, -DQ, and -DP loci, the class II region includes genes encoding proteins involved in antigen processing and in peptide transport from the cytoplasm to the cell surface (33). Besides the large multifunctional proteases and

transporter associated with antigen processing encoding genes, others (i.e. the alpha chain encoding genes—DMA and DOA—or the beta chain encoding genes—DOB and DMB) are present in this region. These nonclassical class II loci likely function by regulating peptide loading onto HLA loci -DR, -DQ, and -DP (34,35).

The class II molecules are heterodimers, that is, molecules consisting of two MHC-encoded polypeptide chains: the  $\alpha$ , (encoded by “A” genes), noncovalently bound to the  $\beta$  (encoded by “B” genes) (Figure 40-3). For -DQ molecules, only the -DQA1 and -DQB1 gene products are expressed as functional -DQ  $\alpha/\beta$  heterodimers. The -DQA2 and -DQB2 genes are pseudogenes



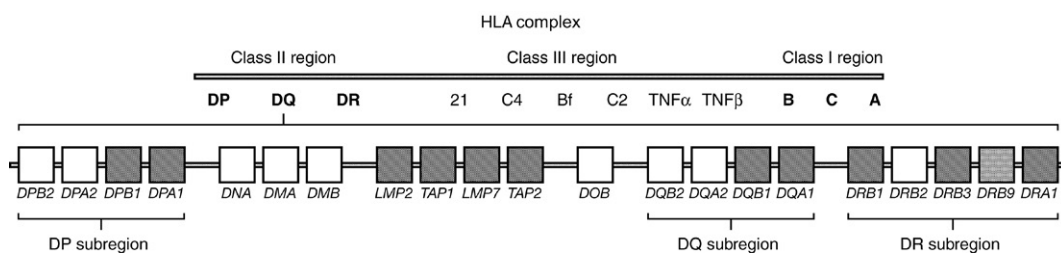
## (A)

AA Codon	10										15										20										25										30																			
DQB1*05:01:01	AG	GAT	TTC	GTG	TAC	CAG	TTT	AAG	GGC	CTG	TGC	TAC	TTC	ACC	AAC	GGG	ACG	GAG	CGC	GTG	CGG	GGT	GTG	ACC	AGA	CAC	ATC	TAT	AAC	CGA																														
DQB1*02:01:01									A									A				T	CT		G	AG																																		
DQB1*03:01:01									C	A												T	TA				T																																	
DQB1*03:02:01										A												T	CT				T																																	
DQB1*04:02:01					T					A										C							T																																	
DQB1*06:02:01					T					A												T	CT				T																																	
DQB1*06:03:01										A													T	CT	A																																			
DQB1*06:04:01										A														T	CT	A																																		
DQB1*06:09										A														T	CT	A			T																															
AA Codon	35										40										45										50										55										60									
DQB1*05:01:01	GAG	GAG	TAC	GTG	CGC	TTC	GAC	AGC	GAC	GTG	GGG	GTG	TAC	CGG	GCA	GTG	ACG	CGG	CAG	GGG	CGG	OCT	GTT	GCC	GAG	TAC	TGG	AAC	AGC	CAG																														
DQB1*02:01:01	A		AT										A	T			G		T	T			CC																																					
DQB1*03:01:01				CA								A					G			T		C		AC																																				
DQB1*03:02:01				CA									T				G			T		C		CC																																				
DQB1*04:02:01				C									T				G			T			AC					T																																
DQB1*06:02:01				C												C	G						A																																					
DQB1*06:03:01				C												C	G						A																																					
DQB1*06:04:01				C													G																																											
DQB1*06:09				C													G																																											
AA Codon	65										70										75										80										85										90									
DQB1*05:01:01	AAG	GAA	GTC	CTG	GAG	GGG	GCC	CGG	GCG	TCG	GTG	GAC	AGG	GTG	TGC	AGA	CAC	AAC	TAC	GAG	GTG	GCG	TAC	CGC	GGG	ATC	CTG	CAG	AGG	AGA	G																													
DQB1*02:01:01		C	A				A	AAA			G									C	T	A	CT		AC	C	T		C	C																														
DQB1*03:01:01							A	A			GA	T		C						C	T	A	CT		AC	C	T		C	C																														
DQB1*03:02:01							A	A			GA	T		C						C	T	A	CT		AC	C	T		C	C																														
DQB1*04:02:01		C	A				A	A						CC	A					C	T	A	CT		AC	C	T		C	C																														
DQB1*06:02:01							A				GA	T		C													T																																	
DQB1*06:03:01							A				GA	T		C													T																																	
DQB1*06:04:01							A	A			GA	T		C																																														
DQB1*06:09							A	A			GA	T		C																																														

## (B)

AA Pos.	10	20	30	40	50	60	70	80	90
DQB1*05:01:01	DFVYQ FKGLCYFING TERVGVTRH IYNREYVRF DSDVGVIYRAV TPQGRFVAEY WNSQKEVLEG ARASVDKVC R HNYEVAYRGI LQRR								
DQB1*02:01:01	---	---	---	---	---	---	---	---	---
DQB1*03:01:01	---	---	---	---	---	---	---	---	---
DQB1*03:02:01	---	---	---	---	---	---	---	---	---
DQB1*04:02:01	---	---	---	---	---	---	---	---	---
DQB1*06:02:01	---	---	---	---	---	---	---	---	---
DQB1*06:03:01	---	---	---	---	---	---	---	---	---
DQB1*06:04:01	---	---	---	---	---	---	---	---	---
DQB1*06:09	---	---	---	---	---	---	---	---	---

**FIGURE 40-6** In the case of HLA class II molecules, the most polymorphic (i.e. hypervariable) regions between allelic chains are encoded by nucleotides contained in the second exon of the gene (Panel A) corresponding to amino acids 6 through 94 (Panel B). The nucleotides in the sequences are here grouped three-by-three to reflect the amino acid composition of the most external domain of the molecule (i.e. amino acids 6 through 94). In this example, commonly occurring allelic nucleotide sequences of the HLA-DQB1 gene are shown.



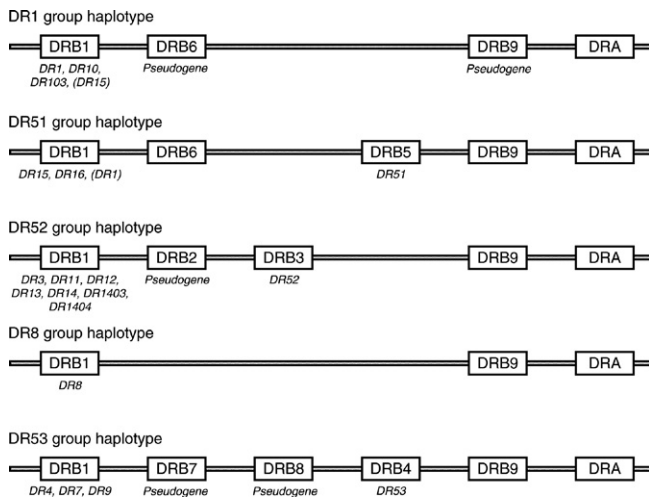
**FIGURE 40-7** The HLA complex present on human chromosome 6, can be subdivided into three regions: class I, class III, and class II molecules. The class II region is closest to the centromere of the chromosome and is conventionally subdivided into three additional subregions, -DR, -DQ, and -DP. In black, are the genes that encode functional molecules; in white are genes that are not functioning (i.e. pseudogenes) or that might encode proteins not yet characterized. The -DRA1 gene encodes a nonpolymorphic  $\alpha$  chain able to pair with the products of each of the functional -DRB genes. In this example, a -DR3 haplotype is shown. -DRB1 gene encodes for the -DR3 allele, -DRB2 and -DRB9 are pseudogenes, and -DRB3 encodes for the so-called -DR52 molecule, which is always found associated with -DR3. (Modified from Luppi, P.; Rossiello, M. R.; Faas, S.; Trucco, M. *Genetic Background and Environment Synergistically Contribute to the Onset of Autoimmune Diseases*. J. Mol. Med. 1995, 73, 381–393.)

(see Figure 40-7). Similarly, only the -DPA1 and -DPB1 genes encode the chains of functional -DP molecules. In contrast, more than one type of -DR molecule can be encoded on the same chromosome, and all are coexpressed at the surface of the same cell. Different -DRB genes are present in different number and in different

combinations on chromosome 6 in different individuals (Figures 40-7 and 40-8). Each type of -DR $\beta$ -chain pairs with the single nonpolymorphic  $\alpha$  chain encoded by the -DRA1 gene (Figure 40-7) (36).

Each individual has one maternal and one paternal chromosome 6, and consequently, one maternal and one





**FIGURE 40-8** DRB genes are present in different numbers and in different combinations on the chromosome 6 of different individuals. While -DRB1 gene encodes the serologically best defined -DR alleles, -DRB3, 4, and 5 encode for three molecules (-DR52, 53, and 51, respectively) always found associated with certain -DRB1 alleles such as -DR3, -DR4, and -DR1, respectively. Thus, -DR51, 52, and 53 are not allelic forms at a certain locus, such as the -DRB1 alleles, rather are different protein products, each encoded by their own genes at different loci, each only present in certain haplotypes. (From Rosner, G.; Martell, J.; Trucco, M. *Histocompatibility*. In Hematopoietic Stem Cell Therapy; Lister, J., Ball, E. D., Eds; Churchill Livingstone: Philadelphia, 2000, pp 233–251.)

paternal allele at any HLA locus. If the maternal allele and the paternal allele differ, as they often do, the person is said to be heterozygous at that HLA locus. If the inherited maternal and paternal alleles happen to be the same, the person is homozygous at that locus. As the maternal and paternal alleles are expressed codominantly, each cell of an individual will express two sets of each HLA molecule (e.g. two HLA-A molecules, two HLA-B molecules, etc.). The combined expression of both sets of molecules constitutes the HLA phenotype of the cells of an individual. The genotype of an individual is instead constituted by two (i.e. one paternal and one maternal) sets of arrays of closely linked genes, many with multiple alleles, inherited as a unit.

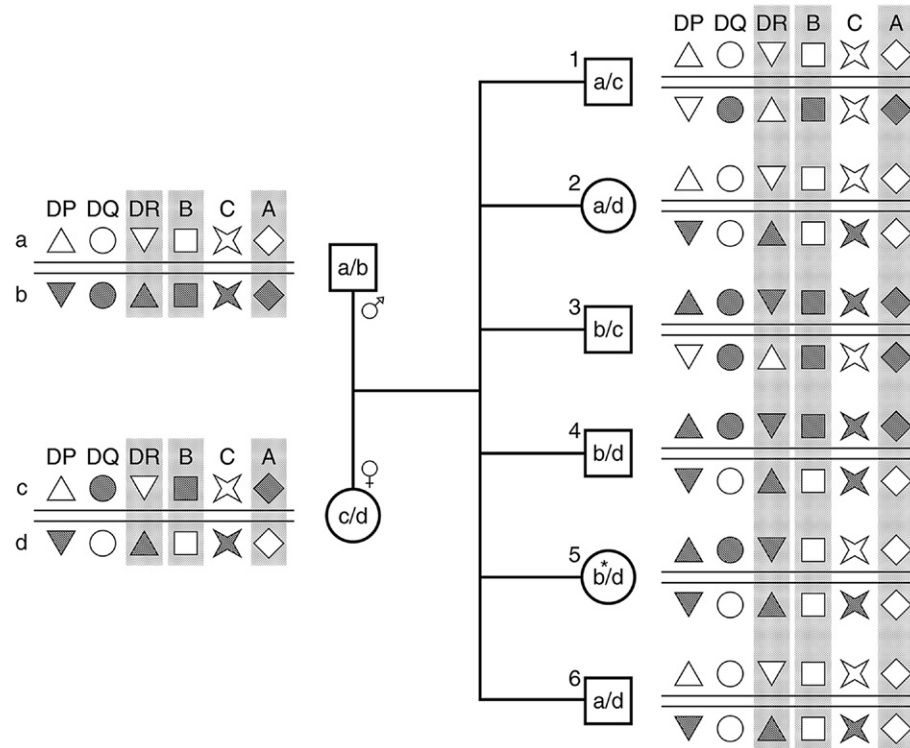
In 1967, Ceppellini coined the term “haplotype” to define each genetic unit inherited from one or the other parent (37). As originally defined, an HLA haplotype includes the class I, III, and II genes present on one chromosome 6 of an individual. The HLA genotype of an individual is composed of one paternal and one maternal HLA haplotype. Each HLA haplotype is transmitted from the parent to the child in Mendelian manner, so that each child shares one haplotype with each parent (Figure 40-9). Within a family, one can find siblings that share two haplotypes (i.e. they are HLA identical), one haplotype (i.e. they are HLA haploidentical), or no haplotypes (i.e. they are HLA different) with the probability of 25%, 50%, and 25%, respectively. Recombination may occur during parental gametogenesis by crossing over during meiosis. However, because of the relative proximity

of the HLA genes, recombination events between loci within a region, or even between regions, though possible, are quite rare. In addition, the selective pressure seems to maintain the genes of each established HLA haplotype together, that is, in linkage disequilibrium (38). With linkage disequilibrium, we define the nonrandom association of the alleles of linked loci present in one haplotype. For example, among Europeans, the most frequent HLA-A and HLA-B haplotype, if we disregard all other loci in the complex, encodes HLA-A\*101g; HLA-B\*801g molecules. This combination occurs in about 9.5% of the population. However, the allele frequencies are 0.172 and 0.125, respectively. If the population were in Hardy–Weinberg equilibrium, that is, noninfluenced by external forces and then in a situation occurring when the genes considered are independently segregating through successive generations, the HLA-A1; B8 haplotype frequency would be  $0.172 \times 0.125 = 0.022$ , or about one-fourth of the observed frequency. In its oldest and simplest measure, linkage disequilibrium, D, can be determined by calculating the difference between the observed haplotype frequency and the expected frequency (39). At equilibrium,  $D = 0$ . Whether or not D is significantly different from 0 is assessed using Fisher’s exact test. If it is significant then LD is said to exist. As a logical consequence, an HLA haplotype composed in part of the paternal and in part by the maternal genes (i.e. a recombinant haplotype) is only rarely observed.

On one hand, recombination is a very rare event across a few generations. On the other hand, it is considered the most efficient mechanism of HLA gene evolution. During the course of evolution, a primitive HLA gene duplicated and mutated; yet, by chance or because of selective advantage, the ancestral complex remained linked. Once gene duplication had occurred, reciprocal recombination was available to generate new haplotype arrangements. Sequence analyses suggest that both intraallelic and intergenic recombinational events were major contributors to HLA diversity (40,41). Some of these events have been likened to the one-way “gene conversion” process, first described in yeast, in which a segment of nucleotides from a “donor” gene is translocated or inserted into another gene (42).

One proposed example of an HLA intraallelic conversion is the specificity -DRB1\*1304 reported by Lee et al. (43). The nucleotide sequence of this allele is identical to that of -DRB1\*1102 except for codons 57, 58, and an intervening sequence that -DRB1\*1304 shares with -DRB1\*0801, \*0803, and \*0405. These authors suggested that one of the three latter alleles donated codons 57, 58, and the intervening sequence to \*1102, which led to serologic reactivity with antisera to -DR13.

A possible example of intergenic conversion is HLA-B46. This allele, which is relatively common in Asian Populations, appears to have derived from the donation of a cluster of nucleotides from HLA-Cw1 into HLA-B62. The donated -Cw1 nucleotides resulted in a



**FIGURE 40-9** With HLA typing we determine the HLA phenotype of an individual. The determination of the HLA phenotypes on at least the two parents and a child or on three HLA-different members of the same family permits segregation analysis, useful to determine the four haplotypes (normally called a, b, c, and d) present in the family, or the genotype of each individual. Consequently, the definition of individuals as heterozygous or homozygous at certain loci (e.g. sibling 1 is homozygous at -DP, -DR, and -C, both alleles are white; but heterozygous at -DQ, -B, and -A loci, 1 white and 1 black allele), together with the recognition of individuals who share one haplotype only (e.g. siblings 1 and 2 share the “a” haplotype, and are HLA haploidentical) or two haplotypes (e.g. siblings 2 and 6 share the “a” and the “d” haplotypes and are HLA identical), or none (e.g. siblings 2 and 3 and are HLA different) becomes possible, as well. A bone marrow transplant always offers the best chance of success if it is performed between HLA identical siblings. “Six-antigen matching” refers to the situation in which two individuals share both alleles at the locus -A, -B, and -DR (gray background) and to the unique situation in which national sharing of kidneys is considered mandatory. In this simplistic example, father and mother are phenotypically the same because they are sharing the alleles at the -A, -B, and -DR loci and constitute a six antigen-matching pair, although they are not HLA “identical,” similar to siblings 2 and 6, because their haplotypes are actually different. Although it is considered a very rare event, it is possible to find individuals, represented here by sibling 5, in which a crossing over between class I and class II gene regions, involving the paternal and maternal haplotypes of the father, cause an a–b recombination flagged here with an asterisk: b\*. (From Rosner, G.; Martell, J.; Trucco, M. *Histocompatibility*. In *Hematopoietic Stem Cell Therapy*; Lister, J., Ball, E. D., Eds.; Churchill Livingstone: Philadelphia, 2000, pp 233–251.)

lack of serologic reactivity with anti-B62 or any other B locus antisera, but an acquisition of reactivity to anti-Cw3 (44). HLA-B46 haplotypes typically carry the -Cw1 gene. If the B46<sup>+</sup> cell is heterozygous at the HLA-C locus, it will type for three HLA-C alleles, -Cw1, -Cw3, and the allele carried on the alternate haplotype.

By convention, the paternal haplotypes are designated as a and b and the maternal haplotypes as c and d. Children in a family are designated as genotypes ac, bc, ad, or bd. If a = HLA-A1;B8;C7;DR3;DQ2;DP4 while b = A2;B7;C7;DR2;DQ6;DP1, and c = A3;B65;C3;DR7;DQ2;DP2, or d = A11;B35;C4;DR1;DQ1;DP4, the phenotype of a child b/d will be A2,11; B7,35; C4,7; DR1,2; DQ1,6; DP1,4, and the recombinant haplotype resulting from a crossing over that took place somewhere in between class I and class II regions of the father’s paternal (a) and maternal (b) haplotypes, will be b\* A1;B8;C7;DR2;DQ6;DP1 as described in Figure 40-9 in simplified terms. Family studies are therefore

recommended, both to confirm the alleles present in each of the patient’s haplotypes and to rule out the possibility of recombination. Only a family study and the analysis of allele segregation can positively elucidate the both haplotypes of an individual.

## 40.5 MINOR HISTOCOMPATIBILITY SYSTEMS

Determining the HLA phenotype of an individual is the goal of histocompatibility testing (i.e. tissue typing) to determine who is a suitable donor for a particular recipient. It can be achieved using serologic and/or molecular techniques (see later and Ref. (45)). Generally, a sibling HLA-identical to the recipient constitutes the first choice as a donor although transplants from haploidentical donors are more frequently being performed today. In fact, although bone marrow transplantation (BMT) between genetically HLA identical siblings has the

highest chance of success, with the declining birthrate in the United States and Western Europe, the likelihood of finding a genotypically identical sibling has dwindled. An estimated 65–75% of individuals requiring BMT will not have a suitable donor in their family (46).

To overcome this problem, alternative sources of hematopoietic stem cells (HSCs) from phenotypically HLA-matched unrelated donors have been sought and used with much success. The National Marrow Donor Program (NMDP) has facilitated unrelated marrow transplants throughout the world, using HLA-matched marrow from donors that were chosen among the volunteers listed in the NMDP registry (47,48). Places such as the Placental Blood Center in New York are also having success with transplants using umbilical cord blood instead of bone marrow cells from HLA-matched and partially mismatched unrelated donors (49–51). Primarily used in pediatric patients, allogeneic cord blood sources accounted for >20% of blood and marrow transplants performed during the years 2004 through 2008. During the years 2006 through 2008, >700 cord blood transplantations were performed annually. Thus far, comparative studies of cord blood and BMT in both children and adults with leukemia exhibit statistically identical outcomes in regard to differences in relapse rates, despite the slower hematopoietic recovery associated with recipients of cord blood stem cells (52).

Unlike related donors who match at all loci within the haplotypes they share, unrelated donors can match at some, but not all loci. Therefore, in order to reduce the risk of graft-versus-host disease (GVHD), precise HLA typing at the various loci becomes essential.

Although to date, clinical experience in transplanting bone marrow from unrelated donors has proved that relatively long-term survival is achievable, the high risks of graft failure and serious, acute GVHD remain major obstacles when compared with transplants between related donors. One plausible explanation for the increased incidence of severe GVHD among allogeneic combinations of donor and recipient is that, unlike genotypically identical HLA-matched siblings who are “identical by descent” for HLA and other poorly characterized MHC or non-MHC (i.e. minor) histocompatibility antigens (53,54), the phenotypically HLA-matched unrelated donors may not be matched for a subset of antigens that can still contribute to alloreactivity (55). Furthermore, recent advances in HLA molecular typing have revealed additional polymorphisms only evident at the genetic level. These differences may have so far still undetermined functional and clinical relevance. The failure to identify these differences using older techniques suggests that unrelated donor/recipient pairs thought to be phenotypically matched, actually were somewhat mismatched (56).

All instances of graft rejection and most instances of GVHD in MHC-identical transplants can be attributed to non-MHC systems. The preponderance of evidence of

their impact on human transplantation comes primarily from BMT (54,57–59). Approximately 60% of patients who receive bone marrow from an HLA-identical sibling will have some evidence of GVHD, and in about 20%, it will be severe. In the mouse, the number of minor histocompatibility (mH) loci has been estimated to be more than 100, about 40 of which have been identified by skin graft experiments and genetic segregation analysis. It appears that the alleles of a particular locus vary in antigenic strength (as determined by mean survival time of skin grafts), and that the effects of different loci are additive (reviewed in Ref. (60)). This is probably true as well in humans because, in general, GVHD increases in severity with decreasing degree of relatedness even with HLA identity.

CTLs have been observed from patients with graft rejection after HLA-identical BMT for aplastic anemia, in multiply transfused patients, and in multiparous women (61–63). In some human and mouse CTL studies, the reactions have shown MHC class I restriction, including H-Y, a minor antigen encoded by a gene on the Y chromosome (53,64–66). Class II restriction of proliferating T cells reactive to mH antigens after HLA-identical BMT have been reported by Reinsmoen et al. (67) and van Els et al. (68). The MHC restriction of T-cell responses to these antigens suggests that they are naturally processed peptides of intracellular proteins that, presumably, associate with MHC antigen-binding sites for presentation to TCRs (59,69). However, not all responses to mH antigens appear to be MHC restricted. For example, as early as 1957, Snell showed that a B10.LP (H-2b) mouse preimmunized with tissue from C57BR (H-2k) rejected a C57BL/10 (H-2b) tumor. All B10.LP mice that were not preimmunized succumbed to the tumor. The tumor donor strain differed from the host only at the H-3 locus, whereas the C57BR strain differs from B10LP at the H-2 complex as well as H-3 and other minor loci. Confirmation that responses to mH are not always restricted was later provided by Murasko (70) who showed that BALB/c (H-2d) mice preimmunized against B10.D2 (H-2d) rejected C57BL/10 (H-2b) skin grafts in an accelerated manner. The interpretation was that the accelerated rejection was against mH antigens, because the preimmunizing strain was H-2 identical to the recipient, whereas the skin donor was not. Inasmuch as the mH loci probably encode a very heterogeneous group of molecules whose structures and primary functions are unknown, it is not surprising that both restriction and nonrestriction have been observed.

In humans, there is indirect evidence of the presence of an HLA-linked mH gene. The first report of this minor locus came from Eisvoogel et al. (71), who observed weak mixed lymphocyte reactivity between siblings, one of whom was a recombinant between HLA-A and HLA-B. This locus, which appeared to map near HLA-A, was designated MLR weak (MLRw). Later, Hsu et al. (72) reported a similar location for genes controlling *in vitro*



cellular responses to the synthetic polypeptides poly(L-tyrosine, L-glutamic acid)-poly(DL-alanine)-poly(L-lysine) and poly(L-histidine, L-glutamic acid)-poly(DL-alanine)-poly(L-lysine). Family studies suggested that the gene, or genes, lie distal to HLA-A. Then, in 1989, in an analysis of 142 HLA-identical sibling donor bone marrow transplants, Hopkins et al. (73) implicated this or a similar locus in the etiology of GVHD. These patients were selected from a total of 323 because they were informative for polymorphisms of clotting factor 13 (F13A), which maps distal to HLA-A, and the red cell enzyme glyoxalase-1 (GLO), which maps proximal to the HLA-D region. No double recombinants between these two loci were observed. Of 68 GLO-informative donor-recipient pairs, 22 were GLO mismatched, that is, one of each pair was a DR/GLO recombinant. There was no significant difference in acute GVHD between the DQ/GLO recombinant and nonrecombinant groups (32% vs 48%). Of 33 pairs informative for F13A, 18 had a donor or recipient recombinant. Among this HLA-A/F13A recombinant group, 67% of the patients had acute GVHD in contrast to 33% of patients in the nonrecombinant group, providing statistical significance to the difference. A relative risk of acute GVHD attributable to this putative immune response gene, that maps near but distal to HLA-A, is 4.0. This, or another, HLA-linked mH locus was described by Vinci et al. (74) who obtained a cytotoxic T-cell line from a patient who rejected bone marrow from an HLA-identical sibling. The cells lysed EBV-transformed B-cells of the donor and some, but not all, unrelated subjects who shared HLA-B44 with the patient. An EBV-transformed line of the patient's pretransplant B-cells were unaffected by the cytotoxic cells.

Neither transplantation nor transfusions elicit much demonstrable antibody production to mH antigens. For this reason, *in vitro* cellular assays, especially the generation of CTLs, has been the method of choice for studying mH antigens in both mouse and man. However, Bias et al. (75) found 31 unique non-MHC lymphocytotoxic antibodies in the sera of multitransfused kidney transplant patients who rejected HLA-identical organs, multitransfused bone marrow transplant candidates whose sera reacted with cells of HLA-identical siblings, and in multiparous women. These sera were tested against cells from 60 families with an average of seven children each. Analysis of reaction patterns revealed that the reacting antigens segregated independently of the MHC.

It is important to note that GVHD, unrelated to histoincompatibility, often occurs in syngeneic or autologous BMT (reviewed in Ref. (76)). It appears that the development of the immune system after BMT is similar to normal ontogeny. It has been shown in animal studies that syngeneic GVHD can be induced by a number of clinical treatments, including neonatal thymectomy, thymic irradiation before engraftment, and cyclosporin treatment for 6 weeks after engraftment followed by withdrawal of the drug. It appears that these treatments cause a

failure of the developing immune system to discriminate self from nonself, resulting in the generation of autoreactive cells, indicating that tolerance to self-antigens is controlled by regulatory T cells. Syngeneic or autologous GVHD is similar to chronic GVHD, a complex of reactions resulting from an imbalance of immunoregulatory cells. In acute GVHD, immunologic recovery occurs when the nonspecific regulatory T cells are able to control autoreactivity. Chronic GVHD appears to be an extension of acute GVHD in which the immune dysregulation continues, resulting in clinical manifestations characteristic of certain autoimmune diseases such as scleroderma.

## 40.6 SEROLOGIC METHODS FOR HLA TYPING

Tissue typing for transplantation refers to determination of the class I and class II specificities (i.e. the HLA phenotype) of both the potential donor(s) and the recipient. Finding the best donor for a kidney transplant generally means finding a "six-antigen match" by looking at each of the two alleles at HLA-A, -B, and -DR loci (Figure 40-9). For bone marrow transplant, however, HLA-C, -DQ and -DP are also typed and often considered (47).

Until recent years, the most largely used serological HLA typing method has been the microlymphocytotoxicity assay (77). This technique uses, as target cells, a suspension of pure and viable lymphocytes that have been separated from whole peripheral blood using density-gradient separation. The lymphocytes are incubated with a panel of alloreactive antisera and a source of complement (C'), usually rabbit serum. The anti-HLA antibodies present in the various sera are quite specific for the individual structural determinants that characterize the polymorphism of the HLA antigens. When a specific antigen-antibody complex is formed, C' activation results in cell lysis. Following addition of a vital dye, the percentage of dead versus live cells can be visualized by phase contrast microscopy. A positive reaction is scored when at least half of the cells are killed. The HLA type is assigned by interpreting the patterns of reactivity of dozens of sera whose specificities are known. Most antisera are obtained from donors who have been exposed by transfusion or multiple pregnancies to cells bearing foreign HLA molecules. As first proved by Rose Payne, multiparous women become immunized against the alleles encoded by the paternal HLA haplotype when blood from the fetus enters the mother's bloodstream at delivery (78,79).

Originally, it was the responsibility of the individual tissue typing laboratory to procure, test, and maintain quality control of the antisera used in testing. The pioneering HLA investigators soon realized, however, the need for standardizing their own reagents. This was achieved by organizing international workshops that have been held every 3-4 years since 1963. These international workshops, originally aimed at sharing and



consequently standardizing alloantisera, target the cells or cell lines, and technical procedures, soon became the forum for recognition of new loci, the definition of new alleles, the adoption of the standard methods, the acceptance and then the use of a common nomenclature.

Today, sera can be obtained locally or through national or international serum exchange programs. Commercial trays with well-characterized sera are most widely used. All accredited histocompatibility laboratories are required to adhere to rigorous standards regarding reagents, protocols, and quality assurance set forth by the American Society for Histocompatibility and Immunogenetics. In addition, most laboratories participate in several proficiency testing programs administered by such agencies as the College of American Pathologists.

However, even with all the refinement and standardization promoted through the years, many problems still confound the results obtainable by serologic methods. As mentioned, serology is encumbered by the requirement for relatively pure populations of viable lymphocytes. This has the greatest impact on class II typing, as HLA-DR, -DQ and -DP molecules are expressed most abundantly on B lymphocytes and less significantly on activated T cells and macrophages, which, all together, comprise at best 20% of the peripheral blood lymphocyte population. Therefore, a relatively large quantity of blood (30 mL) from each individual must be processed in order to obtain enough class II positive cells to allow a complete HLA typing. This may constitute a problem when the individual to be HLA typed is a child. In addition, if the cells are not essentially 100% viable at the beginning of the test procedure as a consequence of disease, chemotherapy, or the consumption of other drugs, the subsequent isolation procedures (e.g. spinning the blood through a Ficoll-Hypaque gradient, collecting cells at the serum/Ficoll interface, and separating B from T cell subpopulations to facilitate the typing of class II and class I alleles, respectively) may further damage them. This cell damage can generate a background of dead cells that, when the cytotoxic reaction is read, will make the results of the specific killing mediated by the antisera and C' indistinguishable from the negative controls. In addition, serology cannot be used to HLA type a patient recently treated with anti-CD3 antibodies, such as OKT3, because these antibodies are cytotoxic in the presence of C' and per se cause cell death, limiting the specificity of the assay. Finally, the C' itself can be a source of variability in these tests as it can, by itself, be slightly cytotoxic.

In leukemias (e.g. chronic myelogenous leukemia) and other disorders, insufficient numbers of B-cells and/or reduced antigen expression can completely preclude class II testing. Although Epstein-Barr (EB) virus transformation (80) and a variety of other procedures have been developed in attempts to procure sufficient numbers of B-cells for testing, they can be labor-intensive, time-consuming, and expensive, besides often being

quite unproductive (81). A major drawback to EB virus transformation is that the blastoid nature of the transformed B-cells makes them overly sensitive to C' activity. Although complement preadsorption with each cell line to be tested can avoid this problem, such treatment is impractical for a general clinical use.

In addition, cross-reactivity (i.e. lack of specificity) of alloantisera still remains an unsolved problem for serological typing. The HLA types defined by serologic methods are often not directly correlated to single alleles. Certain groups of class I antigens belong to cross-reactive groups, which share distinct immunogenic epitopes referred to as public, or broad specificities (82). All the cells sharing a public epitope react with the same antiserum. Further, the antisera used for class II typing are not naturally class II specific. These sera are frequently "contaminated" with class I antibodies, which may cause false-positive reactions. The donor's immune system is exposed simultaneously to the two classes of molecules both present on the immunizing cells. Although extensive absorption with platelets (which express class I, but not class II proteins) can provide a relatively pure preparation of the anti-class-II antibodies, these antisera have generally lower titers and are in shorter supply than anti-class-I antisera.

The need for ongoing screening programs to replenish sera is further complicated, in which two donors will never have exactly the same specificity. In addition, only 200 mL of blood can legally be collected postpartum and even serum obtained from the same donor after subsequent pregnancies can differ with respect to titer or range of reactivity. Accurate serologic HLA typing largely depends on experience and extensive knowledge of protocols and reagents of specialized HLA laboratory personnel.

In 1977, the development of mouse anti-human monoclonal antibodies was seen as the best alternative source to increase specificity and have an unlimited supply of standardized reagents (83). Their use, however, is not yet widespread because of the limited number of "private" specificities recognized by the mouse, which reacts preferentially against "public" specificities. Trays containing only monoclonal antibodies that allow a relatively informative typing are commercially available.

Despite its limitations, the microlymphocytotoxicity test remains the most popular test for clinical use to provide class I and class II typing at a moderate level of resolution in one working day. A drastic improvement in the analysis of percentages of reactive antibodies (PRA) has, however, improved the results of transplant (kidney, in particular) interventions, eliminating the negative conditions frequently found associated with fulminant or hyperacute rejections (HARs). The use of the flow cytometer to determine the presence in the recipient serum of anti-T-cell and anti-B-cell antibodies that are not reactive against the major histocompatibility antigens drastically improved the 4-year graft survival (84).

## 40.7 CELLULAR METHODS FOR HLA TYPING

Other alleles, including those at HLA-C, -DQ, -DP, and perhaps at other loci, are not strongly immunogenic and therefore do not elicit antibodies in large quantity or with high affinity. Reasons could be that these molecules are shed quite rapidly from the cell surface, are in a very limited quantity per single cell, or their critical amino acid differences point into the Bjorkman's groove instead of outside. They may, however, be clinically relevant and detectable by alloreactive T-cells.

As previously mentioned, the first cellular approach successfully used for recognition of HLA class II alleles was the so-called mixed lymphocyte culture (MLC) or MLR. To perform this test, lymphocytes from two individuals are coincubated for several days in tissue culture. If the two individuals are disparate for a class II allele or a non-MHC mH antigen undetectable by serology, both cell types undergo lymphoblastogenesis, synthesize DNA, and proliferate. In the presence of  $^3\text{H}$  thymidine, the degree of newly synthesized DNA can be estimated. Cells that are HLA-identical are nonreactive in MLC, and the incorporation of  $^3\text{H}$  thymidine equals the negative controls.

In 1969, in order to separately monitor the response of the recipient (host-versus-graft) and the donor (graft-versus-host), Fritz Bach proposed to perform two separate unidirectional MLRs (85). The cells chosen as stimulators are irradiated to inhibit DNA synthesis and  $^3\text{H}$  thymidine incorporation. The nonirradiated responders are instead still able to incorporate the thymidine. Every MLC typing test is set up as a complicated matrix of many one-way reactions. In general, three unrelated HLA-mismatched individuals are used as positive controls of the maximum proliferative ability of the responder cells. Every cell should be tested as both stimulator and responder. In addition, autologous controls (i.e. each individual's cells incubated with its own irradiated cells) are used to normalize the response of each cell type to its stimulators. The results are expressed as a relative response (RR) or as a stimulation index (SI). Ideally, when two individuals are HLA-identical, the proliferative responses are <20% of the positive controls (86).

The MLC test has been used most widely in BMT and was developed primarily to help select the most compatible (i.e. least stimulatory) donor when several compatible family members are available (85). It is best used for confirmation of genotypic identity in siblings, especially in cases in which a complete haplotype could not be determined, such as when one parent is unavailable for testing or one appears to be serologically homozygous for -DR or -DQ.

Although the MLC is a cellular cross-match predominantly triggered by class II alleles, it does not define an individual HLA type. One reason could be that

-DR is thought to have dominance over -DQ, perhaps because the expression at the cell surface of the former is 10 times higher than the one of the latter. The role of -DP, with 100 times fewer molecules than -DR expressed on a single cell, remains controversial although recent analysis of HLA-DPB1 mismatches and BMT outcomes suggest an important role (87). This is the reason cellular specificities recognized by MLC testing were originally designated as HLA-Dw ("D" because these specificities were considered to be encoded by a different locus than A, B, and C; "w" because they were still to be officially recognized in the context of an international workshop). HLA-Dw antigens were in reality immunogenic combinations of HLA-DR, -DQ, and/or -DP determinants recognized as such by alloreactive T cells, but not necessarily by alloantisera. By absorbing the antisera with platelets to remove anti-class-I antibodies and using purified B-cells as cytotoxicity test targets, the HLA-Dw determinants were also recognized by certain antisera. They were then defined as D-related serological specificities and eventually -DR alleles.

A variation of this test can be performed in which the lymphocytes to be tested are used as responders in an MLC test, in which a panel of HLA homozygous cells are used as irradiated stimulators. These homozygous typing cells (HTC) are able to define the HLA-Dw specificities but are quite difficult and costly to procure. Inasmuch as the majority of HTC are EB virus transformed cell lines, the results of the testing is frequently difficult to interpret. Other available cellular tests are the primed lymphocyte test, frequently used to better identify -DP alleles, and the cell-mediated lympholysis test, which primarily reflects the stimulatory and effector functions of CTLs in culture (88,89). Although proved extremely useful for research purposes, these tests are too complex and the results are too difficult to standardize to be included in routine typing procedures.

Until recent years, the convention was to use HLA-A, -B, and -DR serology (i.e. six antigens matching) to select a donor, with the MLC as the final arbiter of donor-recipient compatibility. In addition, a pretransplant cross-match of patient's serum against donor lymphocytes (i.e. PRA determination) is generally done to ensure that there is no recipient presensitization to donor HLA antigens, which could prevent engraftment. However, the MLC lacks sensitivity and specificity, in particular, when used for patients with hematologic disorders. Spontaneous proliferation of malignant blast cells in culture and/or poor HLA antigen expression can contribute to false positives or false negatives, respectively. The MLC is technically difficult and as many as 40% of the tests are uninterpretable. Furthermore, it does not have good predictive value for either rejection or GVHD. Therefore, despite being the only functional test for assessing class II histocompatibility, the NMDP no longer requires it before performing a bone marrow transplant (90).

## 40.8 MOLECULAR METHODS FOR HLA TYPING

The practical limitations of both serologic and cellular assays, as well as their inability to resolve clinically relevant subtypes, prompted the rapid development of molecular biologic techniques for HLA typing. Molecular techniques can precisely define differences missed by serologic typing and/or those suggested by an incompatible MLC.

In the 1980s, molecular biology technologies, such as gene cloning and sequencing, became simple to perform. This facilitated the isolation and characterization of the HLA genes at different loci from hundreds of individuals. By comparing sequence variations from person to person, the nature and locations of the polymorphism of the HLA complex were revealed (Figure 40-6).

The initial attempts to perform molecular HLA typing were based on restriction fragment length polymorphism (RFLP) analysis (91). RFLP testing uses a specific DNA probe to recognize the presence of a certain HLA gene on a set of DNA fragments. These fragments are generated by digestion of genomic DNA with commercially prepared bacterial restriction endonucleases (i.e. restriction enzymes) that recognize short, specific nucleotide sequences. The resulting array of restriction fragments are separated on agarose gels by electrophoresis, transferred by capillary action to a nitrocellulose or nylon membrane (Southern blotting), and sequentially hybridized with radiolabeled DNA probes specific for each HLA locus. After repetitive washings, the radioactive probes specifically hybridized to their DNA fragment are revealed by exposing the membranes to X-ray film. The positive fragments differ in size and number from an individual to another, thus creating a polymorphism useful for typing. Although by RFLP it was possible to generate results more precisely and completely than serological typing (92,93), in some laboratories, the use of radioactivity was problematic in that high-quality Southern blots could not be achieved. Also, the turnaround time for even a small number of tests was very long, thus precluding the use of these DNA tests in a clinical setting.

The Xth International Histocompatibility Workshop was organized with the specific aim of transferring RFLP technology to laboratories specialized in HLA serology. All participant laboratories received a common set of HTC lines and a panel of DNA probes. Although the proceeds of this workshop, published in 1987, were useful in establishing standards for RFLP analysis of class II loci, and the collective data obtained revealed strong associations between certain -DR and -DQ alleles and the RFLPs (94), the results were not definitive, largely because of strong homology and cross-reactivity between different alleles and even different loci. These obvious limiting aspects did not preclude, however, their application to fulfill specific aims, such as the characterization of HLA-DQ alleles that confer susceptibility to

type I diabetes (95), or the comparison between serologic and molecular typing in promoting kidney graft survival (96,97). As other techniques that directly analyze HLA polymorphism were being developed, RFLP analysis was, however, quickly deemed unsuitable for general clinical use.

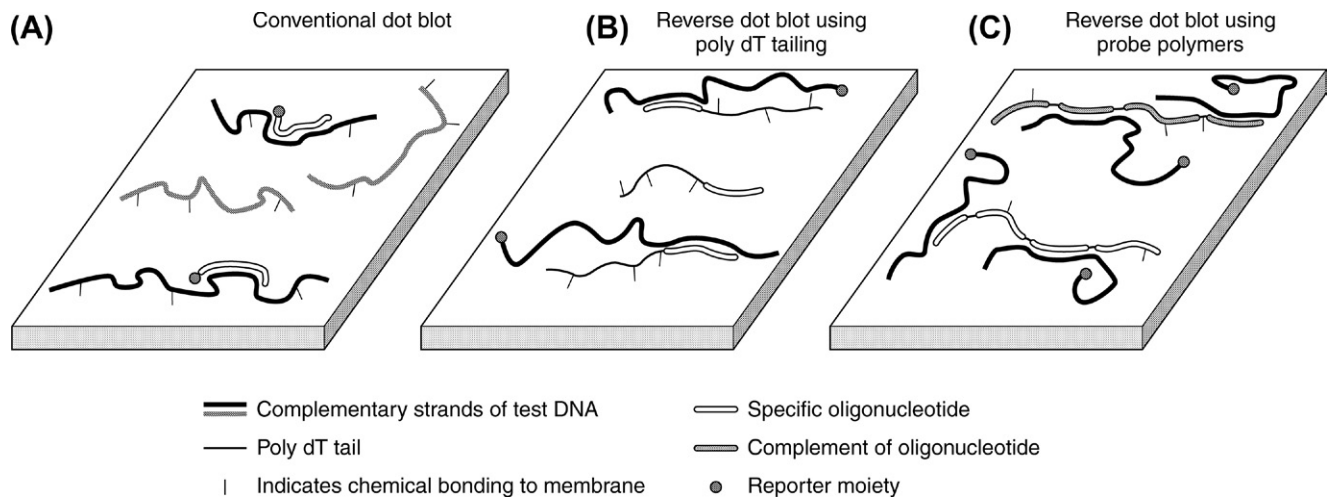
The introduction of polymerase chain reaction (PCR) (98,99) during the late 1980s revolutionized HLA molecular typing. PCR is a powerful and versatile technique for generating millions of copies of specific genomic DNA fragments from minute amounts of starting material once the nucleotide sequence of a certain gene is known. Amplification is accomplished automatically in a DNA thermal cycler by incubating nanogram quantities of DNA, isolated from any possible source in solution with buffer, free deoxynucleotide triphosphates (dNTPs), specific oligonucleotide primers, and thermostable DNA polymerase. The most common of analyses are performed by dot blotting a few microliters of the amplified DNA onto replicate nylon membranes. Each membrane is then hybridized with a different sequence-specific oligonucleotide probe (SSOP) labeled with a reporter moiety. After washing away the unbound excess, the retention of specific probe can be visualized by a variety of methods, the most common of which is chemiluminescence (Figure 40-10A). This approach allowed typing of hundreds of samples with a relatively small number of probes to determine, for example, that variants of sequences involving codon 57 of the second exon of the HLA-DQB1 gene were reliable markers for insulin-dependent diabetes mellitus susceptibility (100,101).

As the extremely large sequence diversity of the class II gene second exon was revealed, the possibility of using this technique for complete HLA class II typing seemed difficult, yet feasible. This daunting task was promoted and organized by the XIth International Histocompatibility Workshop, which provided standardized conditions and a battery of probes to each of its participant laboratories (102).

The advantages of this technique, often referred to as “oligotyping,” are many (Figure 40-10A). The requirement for viable, purified lymphocytes expressing HLA antigens is eliminated. Reagents can be standardized and synthesized in unlimited quantities. Functionally relevant subtypes and polymorphisms as small as one nucleotide substitution can be precisely defined as stringency conditions for hybridization can be perfected for each probe. However, to provide a high-resolution HLA typing, up to seven separate PCR amplifications may need to be done for -DRB and -DQB1 typing alone. A complete set of results, no matter how large or small the set, required 4–5 days to be finalized.

Therefore, this technique is not well suited for routine clinical applications, in which a relatively small number of samples should be tested as quickly as possible.

A different format referred to as the “reverse dot blot” has proved advantageous in reducing the amount



**FIGURE 40-10** “Conventional” dot blot used in SSOP typing (A) is compared with reverse dot blot after poly(d)T tailing of the various probes (B), and with reverse dot-blot of polymeric probes (C). (From Rudert, W. A.; Trucco, M. *A Novel Approach for a Rapid HLA Class II Molecular Typing*. In *Histocompatibility Testing*; Tsuji, K., Aizawa, M., Sasazuki, T., Eds.; Oxford University Press: New York, 1992, pp 352–356.)

of technical manipulation and in improving turnaround time (103,104). Reverse dot blots feature a premade membrane to which the entire battery of probes has been fixed. The DNA to be tested incorporates the reporter moiety during amplification and is then hybridized against the probes present on the membrane. Advantages include labeling one or few DNA samples per donor instead of many probes, performing an entire test with only one membrane, and obtaining results in one working day.

The canonical size of specific oligonucleotides normally used as probes is about 20 nucleotides, a size that has been determined as best for discriminating even 1 bp change. For the reverse dot blot, the direct attachment of probes of this size is inefficient and involves formations of chemical bonds to portions of the probe, resulting in reduced hybridization capability. These difficulties have been solved by enzymatically adding a large number of thymidine nucleotides to the end of the probe (poly dT tailing) (104). The increased length facilitates binding efficiency and favors formation of chemical bonds in the poly dT tails, thus leaving the sequence-specific parts of the probe available for hybridization (Figure 40-10B). This approach is being used in some commercial kits for HLA typing.

An alternative method (103) uses a different approach to bind nucleotide probes to the membrane. Long polymers, usually >25 repeats of the specific nucleotide sequence, are synthesized and bound to the membrane with high efficiency (105). Although chemical bonds between the membrane and the DNA will prevent the hybridization to some sequences, the result is that many more specific sequences remain available for hybridization. Another advantage of this method is that both complementary strands of probe sequence are bound to the membrane (i.e. the probes are double-stranded). This allows both strands of amplified DNA to hybridize to

the probe, further increasing the sensitivity of this assay (Figure 40-10C). Finally, a large quantity of polymers of the same size can be generated from those cloned into plasmid vectors. This level of reproducibility is not obtainable with poly dT tailing and would permit standardization of this technique for intralaboratory use.

Other “reverse” techniques involve fixing probes to 96-well plastic trays with either colorimetric or fluorescent detection (106,107).

Another technique based on the use of sequence-specific primer (SSP) pairs and PCR has been proved more suitable for clinical use. SSP consists of a battery of primer pairs, each capable of only amplifying a specific gene segment. The numerous PCR products obtained are then loaded onto an agarose gel. After electrophoresis and staining, the presence of the specifically amplified DNA segments can be visualized on a UV lightbox, so as to allow correct allele definition. The presence of determined band combinations allowed the attribution of each allele. SSP offers the possibility of obtaining moderate- to high-resolution HLA typing in a few hours. Techniques for -DRB molecular typing using 32 sequence-specific pairs of primers (108) and -DQB1 typing using 14 primer pairs (109) have been published. Several commercial companies offer SSP testing kits.

Both SSP and SSOP methodologies have also been applied to class I typing, which is proving to be a much more formidable task because of the high degree of widespread polymorphism compared with the class II genes and the complications presented by the presence of pseudogenes. One method for SSP typing (110) uses nine coding-strand and seven noncoding-strand primers in various combinations to generate HLA-A specific amplification products. The specificity of PCR priming is increased by taking advantage of the amplification refractory mutation system (ARMS), in which nonspecific reactions are inhibited by introducing a nucleotide



mismatch near the 3' end of the primer (111). Fifteen HLA-A group specificities can be successfully characterized using this method, which appears to be comparable to routine serology with respect to identification and resolution of HLA-A specificities. Subtypes of each group can be more specifically resolved using additional primer pairs (112). Low-resolution typing of the HLA-B alleles has also been described (113) using a similar approach. Twenty-four PCRs using 34 primers are sufficient to determine the majority of, but not all, serologically defined specificities at the HLA-B locus. ARMS-PCR has also been used to detect alleles at the HLA-C locus (114).

The SSP approach is especially amenable for use in clinical laboratories that do not have a great deal of molecular biology experience as the equipment and special techniques required are minimal. Because from DNA extraction to allele identification, reliable and easily interpretable results can be generated in <4 h, SSP typing is an especially attractive alternative to serology for clinical laboratories. However, for laboratories performing large-scale molecular typing, the SSP method may not be amenable.

Substantial progress has been made in converting the methods for molecular HLA typing to microchip technology (115–118). For example, working with HLA-B as a model system, an oligonucleotide array has been used to identify alleles occurring within a set of 100 samples obtained from unrelated individuals (116). Probes were optimized that represented all known polymorphisms encoded in HLA-B exons 2 and 3. Hybridization of PCR products allowed unambiguous detection of complex heterozygous SNP combinations, including some (e.g. HLA-B\*1502+\*2702 and HLA-B\*1513+\*2708) in which combinations of nearby SNPs cause ambiguous genotyping results when analyzed by conventional sequencing-based methods. More recently, the International MHC and Autoimmunity Genetics Network has exploited parallel genotyping of 1472 SNPs using microarrays to determine complete HLA class I and class II genotypes from more than 10,000 subjects (119). The accuracy of the method for determining 2- and 4-digit resolution at HLA class I and II loci has been estimated to be as great as 95% (118). Among its advantages, microarray technology can potentially accommodate thousands of perfectly distributed DNA spots on microarray platforms where hybridization can take place, saving reagent quantities, avoiding time-consuming manipulations, and providing automatically read results. Moreover, oligonucleotides-array-based strategies can provide high-throughput genotyping by virtue of parallel analysis of multiple genetic regions (115,119). A limitation of the microarray-based method, however, is the need for sophisticated, robust data handling protocols that are likely out of reach of the typical clinical typing laboratory. Thus, current use of this approach is limited to genome sequencing centers and a handful of specialized laboratories with interest in the HLA region.

In contrast to the microarray approach, microsphere technology has been widely adopted by the HLA typing community. The method exploits the use of color-coded beads developed in order to advance DNA hybridization-based typing methods (120). The major components of the system are benchtop flow cytometer, two-color microspheres, and biotinylated PCR-amplified product for probing specific oligonucleotides attached to individually color-coded beads. Flow cytometry analyzes individual microspheres by size and fluorescence, distinguishing three fluorescent colors (i.e. green at 530 nm, orange at 585 nm, and red at wavelengths above 650 nm). The ratio of two of the fluorescent signals is used for microsphere classification so that different two-color-coded beads, pre-labeled with probe DNAs, can be identified along with a third fluorescent signal to be used for quantification of hybridization signal. In this methodology, first applied toward HLA for genotyping of HLA-DQA1 alleles but later applied to class I as well as HLA-DRB1, -DQB1, and -DPB1 alleles, targeted DNA is PCR amplified using group-specific biotinylated primers (120–122). The biotin-labeled PCR product allows detection of hybridization between probe and amplified sequence by using R-phycoerythrin (PE)-conjugated streptavidin. Flow cytometry analysis enables quantification of the fluorescent signals on each microsphere. Assignment of HLA genotype is based on the hybridization reaction pattern compared with the pattern associated with published HLA gene sequences. In this manner, it is conceptually similar to traditional hybridization-based HLA typing approaches. At present, up to 100 individually color-coded microspheres, each containing a different allelic oligonucleotide probe, can be analyzed in a single mixture of oligonucleotide-coupled microspheres. The HLA loci are amplified from genomic DNA using sequence-specific oligonucleotide primer mixes that are optimized for PCR amplification of particular HLA alleles and have been used with dried blood spots as well as DNA isolated from blood draws and buccal swabs.

Advances to sequence-based typing methods have been applied to HLA genotyping. Pyrosequence-based typing (PSBT) has been developed to allow accurate sequencing of short stretches of DNA, initially for the analysis of expressed sequence tags (123,124), and has been applied to HLA genotyping (125–131). Sequencing of lengths between 50 and 150 nucleotides has been reported during analysis of HLA genotypes of loci HLA-DQB1 and HLA-DR. The method is suited for use during SNP analysis of HLA because of its ability to accurately resolve heterozygous nucleotides by enabling out-of-phase sequencing (130,132). Pyrosequencing is performed by addition of dNTPs individually in a predefined dispensation order, so that the nascent nucleotide chain is extended one nucleotide residue per dispensation event. Detection of nucleotide sequence is performed by way of a chain of enzymatic reactions involving the activities of

DNA polymerase, apyrase, ATP sulfurylase, as well as luciferase, respectively, allowing for the incorporation of complementary nucleotide, degradation of unused dNTP, generation of luciferase substrate from pyrophosphate and adenosine 5'-phosphosulfate, and emission of light from the ATP-driven conversion of luciferin to oxyluciferin (123,133). Incorporation of a particular nucleotide is displayed graphically in the form of a chart recording of nucleotide dispensation event versus the intensity of emitted light. This cascade of enzyme reactions is quantitative, in that increased light intensity is produced upon incorporation of multiple nucleotides (134).

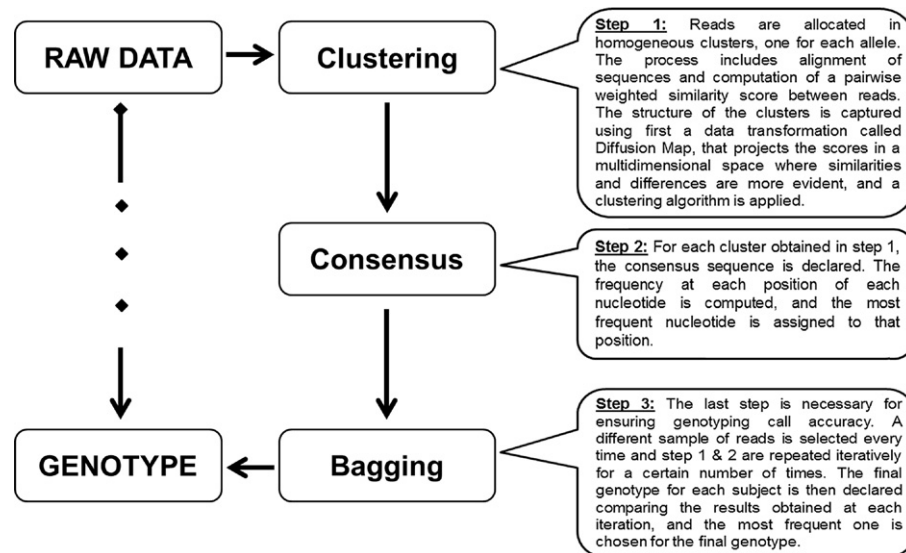
PSBT provides distinct advantages for genetic typing in that samples can be assayed in 96-well trays making it compatible with common laboratory automation instruments, thus increasing the rate of sample analysis, and the sensitivity of the technology requires little PCR-amplified material for each sequencing reaction (123). PSBT strategies can be designed using a minimal number of nucleotide dispensation events, so that an entire 96-well tray can be assayed at a rate of roughly 1 min per base and approximately 20 min for a 20-nucleotide sequence, sufficient for analysis of most HLA alleles when primers are placed adjacent to polymorphic regions. Moreover, out-of-phase pyrosequencing of alleles (130,132) allows unequivocal genotyping of allelic combinations that are ambiguous by conventional sequence-based typing techniques as well as those spaced too far apart to allow resolution by hybridization approaches (Figure 40-11). This results from the pyrosequencing system accepting nucleotides individually at each dispensation event, which directs the phase of primer extension of individual alleles. Out-of-phase pyrosequencing enables the detection of sequencing signal from each haplotype individually. This, in turn, enhances the ability to distinguish between genotypes for allelic combinations involving polymorphisms associated with crossing-over events (125,129,130). These sequences can be determined with greater accuracy because they can be sampled at multiple independent dispensation events tailored to provide allele discrimination from each haplotype.

In the past few years, pyrosequencing technology has advanced substantially allowing it to be used as an important method within the stable of next-generation sequencing technologies (135). The method was reengineered by the 454 Life Sciences in ways that enable efficient preparation of thousands of individually prepared clones for analysis within a single sequencing run, scaling of the reaction to picoliter volumes, and increasing read lengths to as great as 400 nucleotides. As the length of the polymorphic exons of HLA class I (exons 2 and 3) and class II loci (exon 2) are between 264 bp for HLA-DPB1 exon 2 and 276 bp for exon 3 of HLA-A, -B, and -C, the reengineered pyrosequencing method is capable of sequence-based typing of HLA alleles. In fact, a number of research laboratories have already reported achieving high-resolution typing of HLA class I and class

II loci using genomic DNA isolated from human cell lines as well as blood samples (136–138) and from cDNA created from isolations of mRNA (139). The main focus of research is now concerned with preparing high-quality template from DNA obtained using typical patient samples, including venous blood draws, dried blood spots, and buccal swabs, with the latter two examples being especially challenging to prepare because of the small amounts of DNA that are typically obtained. Along with translating the methodology from the research-based setting to the clinical HLA-typing laboratory, there is great need to develop computational methods for analyzing the data (138). For example, using only half of the 454 sequencer's capacity, Galan et al. (140) reported obtaining complete HLA-DRB exon 2 sequencing from 1407 samples. This resulted from analysis of >220,000 sequence reads and required substantial computational support in order to organize the data by subject, align the sequences, and identify the alleles involved. It seems likely that some of the alignment and assembly methods developed for the human genome project (141) along with advanced statistical methodology for sorting data obtained from heterozygous subjects will be required in order to fully achieve the goal of using next-generation sequencing as a paradigm for routine HLA typing (142,143). Given the proven ability of the methodology to generate high-resolution HLA typing from thousands of samples in a single-sequencing run and at low cost, these final hurdles will no doubt be overcome in the near future.

For example, a review of the literature revealed the availability of software for genotyping of HLA alleles using data generated by the 454 next-generation sequencer. The first is the CONEXIO ATF, which was used in a study conducted by Holcomb et al. (143), where the same set of genomic DNA samples were analyzed by eight different laboratory sites, and the results compared, showing the consistency of so-called the genotype. Although the methodology underlying the CONEXIO was not fully explained, it seems likely that the main step is the identification of the most abundant sequences generated, followed by assignment of the HLA genotype. The second HLA-typing algorithm was developed by Elrich et al. (142) and integrated the Genome Analysis Toolkit (GATK), a data processing tool for next-generation sequencing data (144). Given samples from heterozygous subjects, this methodology focuses on computing the posterior probability for all possible pairs of four-digit HLA alleles and assigns, as the genotype, the most likely pair. In particular, the posterior probability is computed as the product of the genotype, the phase and the frequency probability. The genotype probability is defined as the product of the probabilities of observing a base at a particular position given the data, as determined by GATK. The phase probability is computed as the product of the binomial probabilities that the phase orientation for each allele pair is consistent





**FIGURE 40-12** Clustering and Alignment of Polymorphic Sequences (CAPSeq) is a software application designed to genotype MHC alleles. Using next-generation data as input, the application enables clustering of similar sequences (i.e. those reflecting different alleles present in a heterozygous individual) and generation of a consensus sequence for each cluster. The consensus sequences are then compared with a list of known HLA allele sequences in order to identify the best match. The process is repeated in an iterative fashion using statistical method “Bagging” in order to increase the accuracy of the genotype call.

originating from pseudogenes). Diffusion maps are used to measure the “connectivity” of a dataset. The data are projected into diffusion space, in which the Euclidean distance between two points is small if the points are highly connected in the original feature space and large otherwise. Diffusion distance is a robust, non-linear measure that preserves the intrinsic structure of the data; which makes it an ideal choice for clustering problems involving related but highly polymorphic DNA sequences. Then the Kmeans algorithm is applied and the reads clustered in homogeneous groups. In the second step, the consensus sequence for each cluster is established by majority rule at each position. The genotype is called by comparing the consensus sequence with that of previously known HLA allele sequences. The third step is performed for ensuring classification accuracy. To this end, bagging (147), bootstrap aggregating, is used. Bagging is a machine learning technique that generates multiple versions of the same predictor, taking an “average” as final result. In the present example, it consists in sampling a fraction of reads from the total number of reads available, while performing steps one and two in an iterative manner. The process is repeated  $N$  times, and the final genotype declared by majority rule.

## 40.9 CLINICAL SIGNIFICANCE OF HLA MOLECULAR TYPING

A possible criticism to DNA typing is that it gives “too much” information. Although it has been hypothesized that even one nucleotide difference, and hence one amino acid disparity, can result in increased risk of alloreactivity in vivo (148), many nucleotide substitutions do not cause amino acid changes. Given the huge number of

detectable alleles at the various loci, it would be important to determine which mismatches are allowable and which ones are instead nonpermissive for a positive clinical outcome.

A number of studies have been done to determine the clinical impact of HLA matching in unrelated bone marrow transplants. Many reports have shown that the incidence of both acute GVHD and rejection are significantly higher among patients transplanted with phenotypically matched bone marrow cells from unrelated donors compared with cells from genotypically identical siblings (149,150), in particular when serologic techniques were used for HLA typing (151). Further, the incidence and severity tend to increase with increasing HLA mismatch, except in very young patients, in whom one HLA locus mismatch can be tolerated (152).

As described in a recent review (153), patients with acute myeloid leukemia can be favorably treated by BMT when paired with mismatched killer Ig-like receptors (KIR) and one HLA class I allele (154,155). Occurring in haploidentical BMT, the phenomena is believed to result from a failure to suppress NK cell killing when KIR-HLA class I mismatches occur (155). Reduced incidence of leukemia relapse results from NK cell cytokine-induced killing of recipient patient tumor cells and is regulated by the reduced KIR-HLA class I interaction between NK cells and mismatched target cells. In these cases, only one of the donor HLA-carrying chromosomes is matched with recipient, resulting from situations in which either parents or siblings matched at only one chromosome provided donor marrow. Under these conditions, a KIR-HLA class I mismatch will present and a fraction of donor NK cells will express KIRs not able to engage the HLA class I alleles of the recipient, thus



having the potential to kill the patient's leukemic cells (154–156). Mismatched NK cells do not cause GVHD, possibly because nonhematopoietic tissues may not express NK receptor activating ligands.

Bone marrow transplant is a case apart in general tissue transplantation because drastic immunosuppressive regimens cannot be applied as they are for any other type of organ to be transplanted. Immunosuppressive therapy is necessary to prevent graft rejection even when donor and recipient are HLA-identical siblings. It is the effectiveness of the immunosuppressive therapy that permits the survival, often for many years, of allogeneic grafts, many of which are poorly HLA matched. There is an abundance of data that show little or no difference between well-matched and poorly matched grafts as measured by 1-year survival (157). These considerations, added to the need for transplantation in a relatively short amount of time for the most severely diseased patients and to the limited number of donor organs available, have lead many to propose that organ allocation should be governed primarily by criteria other than HLA matching (158). This discussion was particularly contentious in those days (159–161). However, if we consider a longer time of transplanted organ survival, we can see how HLA matching of donors and recipients may actually have a relevant effect. In 1992, Terasaki et al. (162) developed logarithmic plots predicting 10-year rates of graft survival and rates of loss in organ half-life. They compared these predictions with actual graft survival of transplants done from 1975 to 1980. Their half-life actuarial estimate of 16,320 first cadaveric kidney grafts from the United Network for Organ Sharing (UNOS) registry was 10.3 years. From the 1975–1980 actual 10-year survival data, they calculated a half-life of 24.0 years when the donor was an HLA-identical sibling, 11.8 with a parental donor, and 7.9 with a cadaveric donor. When the cadaveric donor grafts were evaluated by HLA matching, the half-life was 19.7 for no -A, -B, -DR mismatch (or 6 antigens match) and 7.5 years for complete mismatches. The estimated half-life was similar though for one, two, three, four, and five mismatches. All other variables analyzed did not make any difference, including race, sex, original disease, and other nonhistocompatibility factors. On the basis of their conclusion that “HLA matching is the single most important factor that can improve nationwide 10-year graft survival rates,” the equitable system of organ allocation approved by UNOS was the following: the priority system adopted for nonrenal patients included the length of the time a patient has been on the waiting list, the severity of his or her illness, and the proximity of the available organ to the patient. For kidney allocation, the degree of HLA matching and the degree of HLA presensitization (i.e. PRA values or presence of antibodies against donor HLA specificities)

were added to the above criteria (163). UNOS has also mandated national sharing of perfect HLA matches, irrespective of other criteria. The probability for a kidney patient to find a perfect match anywhere is, however, no more than 5%.

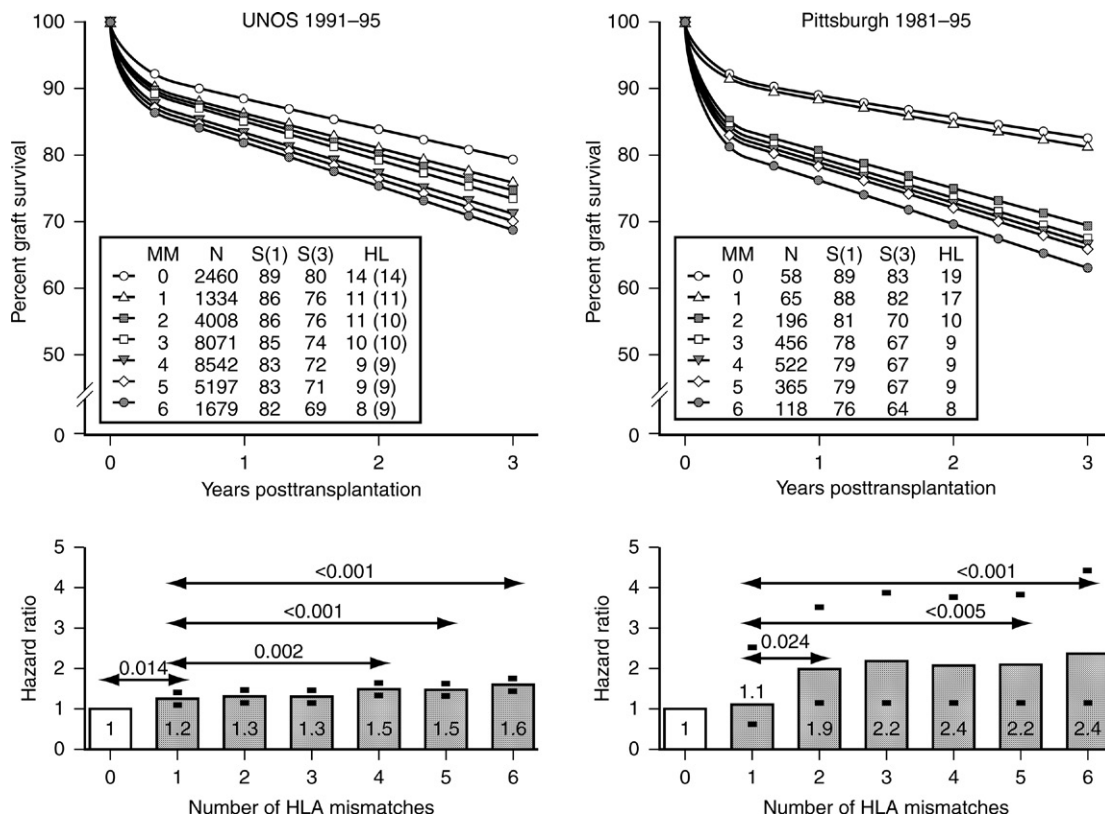
The results presented by Opelz et al. (164,165) relative to the cumulative results from various European transplant centers seem to confirm the appropriateness of these criteria. Starzl et al. (166), using updated results obtained from UNOS registry, have also proved the importance of HLA perfect matching for kidney transplants although the negative effect of mismatches seems to be the same for more than one or all the six alleles and limited to a small percentage even in a 10-year survival analysis (Figure 40-13). This is the reason why all kidney patients are still typed for HLA and ABO blood groups before entry in to the waiting list and their sera screened for anti-HLA antibodies.

With the advent of DNA methodologies for HLA typing, it has been proved that unrelated donors who appeared HLA-identical by serology were indeed not matched at the DNA level and that really matched pairs survived much longer. Studies using DNA methods for both class I and class II (167) typing have shown that molecular matching may reduce the risks of acute GVHD and patient mortality.

The likelihood of finding a matched donor in the registries also depends on whether the patient has a common or rare HLA genotype. Not only are some alleles more common or rare within a particular ethnic or racial group, but because of linkage disequilibrium, certain alleles can give rise to different haplotypes within different populations. NMDP is making an extraordinary effort to enroll in its registry HLA molecularly type different minority groups so to increase the likelihood that minority patients requiring any type of transplant, particularly kidney or bone marrow transplants, can find a more suitable HLA-matched donor.

Even considering all the possible genotypically identical siblings as donors and the large number of potential donors enrolled in the NMDP registry, still a substantial number of patients will not be able to find an HLA-matched donor. In addition, as the discovery of new loci and alleles that potentially must be matched increases, the odds of finding a complete match will decrease even further. Therefore, the most important charge for those involved in BMT is a more precise definition of HLA mismatches that are clinically permissible versus those that are functionally significant with respect to rejection, GVHD, and overall patient mortality.

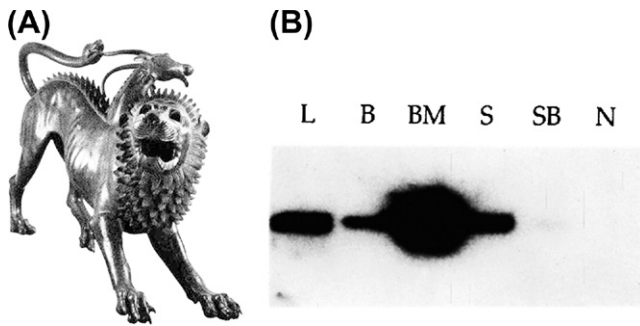
The transplantation of organs other than bone marrow or kidney offers, however, a completely different scenario inasmuch as the patients are routinely transplanted on the basis of their serious clinical conditions that impose this type of intervention to be



**FIGURE 40-13** Survival percentages of kidney transplants subdivided by 0 to 6 HLA antigen mismatches (MM). (From Starzl, T. E.; Eliasziw, M.; Gjertson, D., et al. HLA and Cross Reactive Antigen Group (CREG) Matching for Cadaver Kidney Allocation. *Transplantation* 1997, 64, 983-987.)

performed as soon as an organ becomes available. The HLA typing is generally performed a posteriori and the presence of reacting antibodies in the recipient only used to modify the immunosuppressive regimen. The possibility to successfully transplant organs such as the liver across the histocompatibility barriers seems to be due to the intrinsic characteristics of such a large human gland (168). First, the liver is able to absorb a large quantity of antibodies without having its function impaired; second, the quantity of immunocompetent cells present in the liver at the moment of the transplant is considered, by some, the possible reason for the liver's tolerogenic power. Under the protective umbrella of immunosuppression, immunocompetent stem cells present in the liver are able to migrate into the recipient body where they are not strongly confronted by a drug-impaired immune system of the recipient. In this peculiar context, the immune system of the recipient may become "accustomed" to the presence of immunocompetent cells of the donor, so that both systems harmoniously survive in the transplanted individual. This exceptional situation known as immunologic chimerism (Figure 40-14) can offer the invaluable advantage of generating a donor-specific type of tolerance in the recipient that *ipso facto* becomes able to host the foreign tissue without the need of a massive and,

in general, particularly dangerous immunosuppressive regimen. These types of considerations were actually supported by the finding of immunocompetent cells of donor origin in the tissues (e.g. skin) or in the blood of patients who received a liver transplant before 30 years (157,158,169-171). In different proportions, a certain degree of immunologic chimerism, or a "microchimerism," was also found in kidney and heart transplant patients years after transplantation (171). The enthusiasm produced by these incontrovertible findings promoted the implementation of therapeutic protocols aimed at favoring the establishment of immunologic chimerism. One protocol suggested the augmentation of immunocompetent cells obtainable by cotransplanting with a solid organ not generally rich in these cells (similar to the heart or the kidney), the bone marrow cells from the same donor (172). Although this therapeutic intervention did not cause any appreciable harm to the recipient, the benefits associated with the procedure were not easy to define in terms of graft survival. Quality of cells, quantity of cells, time of administration, and prolongation of chimeric status were all considered variables that supported the "feeling" of improved results, but did not offer any unquestionable and tangible parameters to quantify this sense of improvement (173-177).



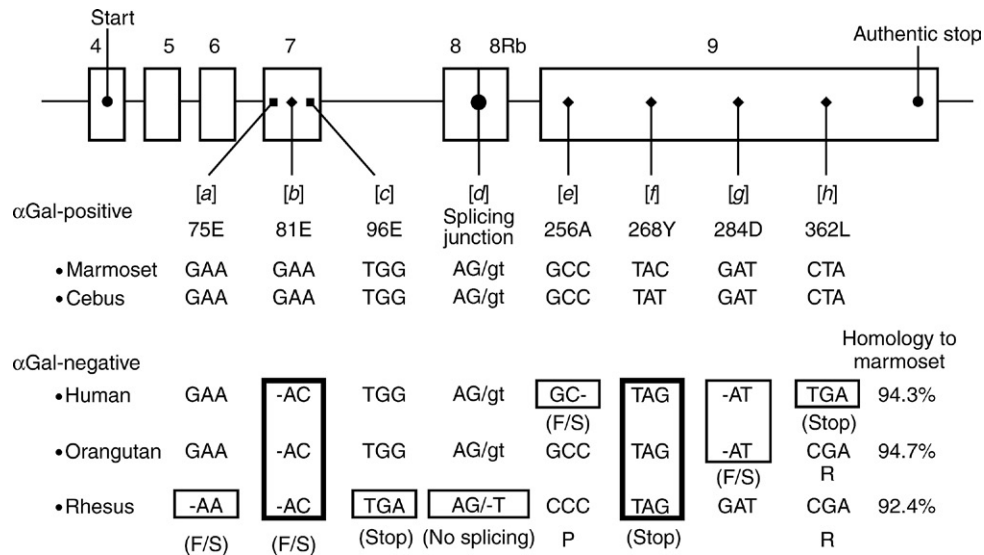
**FIGURE 40-14** Artistic representation of a chimera from Greek mythology and evidence of an immunologic chimera in a patient with Gaucher disease. In (A), Khimaira, also spelled in the Latin “Chimera,” was a powerful beast with the head of a male lion, the body of a goat, and, as a tail, a serpent. The legend states that she was slain by Bellerophon with a golden arrow, while he rode on the back of Pegasus, a horse with wings. The Chimera of Arezzo is conserved at the Archaeologic Museum in Florence, Italy. In (B), genomic DNA was extracted from the recipient’s tissues and amplified with HLA-DR beta-chain “generic” oligonucleotide primers to determine the subgroup of the donor’s alleles. “Specific” primers were then used to amplify the alleles selectively. The alleles were identified by hybridizing the amplified DNA to radiolabeled allele-specific probes. HLA-DR1 was only present in the donor. After HLA-DR1–specific amplification of DNA from the liver (L), blood (B), bone marrow (BM), skin (S), and small bowel (SB) of the recipient, the DNA was separated by electrophoresis on an agarose gel and then analyzed by Southern blotting. The denatured DNA present on the nylon membrane was hybridized to a labeled HLA-DR1 (donor)-specific oligonucleotide probe. For liver DNA, the quantity analyzed was reduced to 1% of the other samples. Although lower in intensity, the signal from donor DNA in the small bowel was clearly positive in the original film, although this can be seen here only faintly. The negative control was a reaction run without DNA (last lane; N = negative control). (From Starzl, T. E.; Demetris, A. J.; Trucco, M., et al. *Chimerism After Liver Transplantation for Type IV Glycogen Storage Disease and Type I Gaucher’s Disease*. N. Engl. J. Med. 1993; 328, 745–749.)

#### 40.10 GENETICS OF XENOTRANSPLANTATION

A more drastic but also theoretically valid alternative is the one originally proposed by David Sachs et al. (178,179) that considered the radiologic ablation of the recipient bone marrow followed by the transplant of mature-T-cell-free mixture of recipient and donor bone marrow cells. In the mouse, the simultaneous presence of the two immunologic systems, even from individuals with different histocompatibility phenotypes, allows the immunologic reconstitution of the recipient by both types of cells together. The established chimerism is, in this case, quite stronger in percentage of donor versus recipient cells than the one obtainable by bone marrow augmentation. Once proved durable, this chimerism is able to allow the acceptance of any organ coming from the same donor. More recently, autoimmune diabetes in the NOD mouse model can be treated with bone marrow from autoimmunity-free allogeneic donors (180) and that restoration of endogenous  $\beta$ -cell function to physiologically sufficient levels is achievable even if the

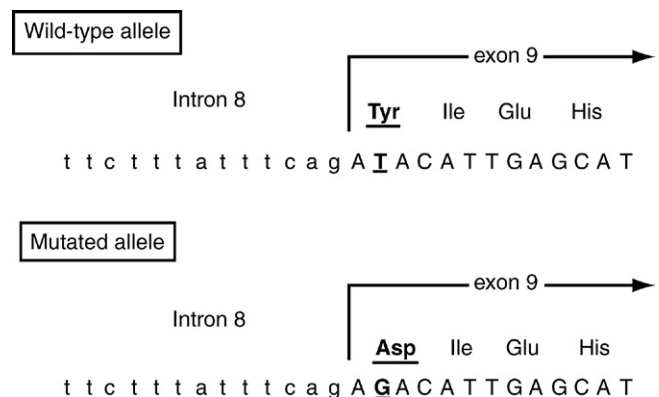
allogeneic BMT is performed after the clinical onset of diabetes (181).

The establishment of an immunologic chimerism has even been proposed to set the basis for possible xenotransplantation (178). An immediately available, unlimited source of tissues may be found in animals, and the pig seems to be the animal of choice because of its size and its already established, regulated, massive production for alimentary use (182). This possibility could not be further explored when it became clear that the  $\alpha$ 1,3 galactose ( $\alpha$ 1,3-Gal) epitopes on pig tissues were the targets of antibodies, normally present in the human serum, able to quickly reject the xenotransplant (183). Humans do not express a functional  $\alpha$ 1,3-galactosyltransferase ( $\alpha$ 1,3-GT) gene and, as a consequence, their tissues are  $\alpha$ 1,3-Gal-negative. However, it is likely that  $\alpha$ 1,3-Gal-positive microbial colonies within the human gastrointestinal tract result in immunological exposure to the antigen. As a result, humans develop antibodies against the antigen before xenograft exposure and promptly reject antigen-positive tissue. The only notable exception is Old World monkeys, which, similar to humans, are  $\alpha$ 1,3-Gal-negative. These antibodies are able to mount a rapid, deleterious reaction that is the major cause of xenograft loss in the first few hours after transplantation. This is now known as “hyperacute rejection.” To avoid the HAR of the tissues from  $\alpha$ 1,3-Gal-positive pigs, we considered the possibility of generating pigs genetically deprived of the activity of the enzyme  $\alpha$ 1,3-GT and, consequently, free of  $\alpha$ 1,3-Gal epitopes at their cell surface. Toward this end, we began cloning the  $\alpha$ 1,3-GT gene of the pig, together with its regulatory 5′ sequence. The cloning and sequencing of a stretch of genomic DNA ~30,000 nucleotides long, encompassing a regulatory sequence containing the gene promoter, 9 exons, and relative introns, was successfully completed at the end of the year 2000 (184). Then, it was our view that the best way to inactivate, in the pig, both alleles of the  $\alpha$ 1,3-GT gene, without creating lethal conditions for the pig embryo, could be determined by properly defining and evaluating the molecular reasons why the same gene is not functional in humans. Our comparison between human, orangutan, and rhesus (all  $\alpha$ 1,3-Gal-negative species) alternatively spliced  $\alpha$ 1,3-GT mRNAs, and marmoset and cebus ( $\alpha$ 1,3-Gal-positive species) normally translated  $\alpha$ 1,3-GT sequences, revealed a site, at the beginning of exon 9 of the gene, as possibly the most critical point for the enzyme proper function. A stop codon, TAG, substituted the amino acid at position 268 of the functioning enzyme (Figure 40-15) causing the production of a truncated protein, and the complete abrogation of the enzyme function (185). We began a close collaboration with the Virginia branch of Scottish PPL Therapeutics Inc., which had experts in the genetic modification of livestock and the application of homologous



**FIGURE 40-15** Comparison of crucial point mutations of the  $\alpha 1,3$  GT gene in primates. (From Koike, C.; Fung, J. J.; Geller, D. A., et al. *Molecular Basis of Evolutionary Loss of the  $\alpha 1,3$ -Galactosyltransferase Gene in Higher Primates*. J. Biol. Chem. 2002, 277, 10114–10120.)

recombination, gene targeting, and animal cloning (i.e. somatic cell nuclear transfer). As such, PPL Therapeutics was responsible for cloning the first mammal, “Dolly” the sheep. In addition, in March 2000, PPL Therapeutics generated the world’s first cloned pigs (186). Thus, genomic sequences encompassing the beginning of exon 9 were used to construct knockout (KO) vectors aimed at the targeted insertion of a selectable marker gene (e.g. neo), and at the inactivation of the  $\alpha 1,3$ -GT gene in pig fetal fibroblast cells. Cells carrying the  $\alpha 1,3$ -GT gene KO were then isolated and used as donors for somatic cell nuclear transfer (cloning) to produce a single  $\alpha 1,3$ -GT gene KO pigs (187). To produce animals useful for transplantation, it was necessary, however, to knockout also the second, yet noncompromised allele of the gene that was responsible for the still present expression of  $\alpha 1,3$ -Gal epitopes on the single KO pig cells. To knockout the second allele, then, an effective selection technique for the isolation of  $\alpha 1,3$ -Gal-negative fibroblasts was developed: the toxin A of *Clostridium difficile* uses the  $\alpha 1,3$ -Gal epitope as the receptor to get into the target cells, thus selectively killing  $\alpha 1,3$ -Gal-positive cells. After toxin A treatment, the surviving fibroblasts still carried the first KO allele of the  $\alpha 1,3$ -GT gene but also had the second allele of the  $\alpha 1,3$ -GT gene inactivated. When the second  $\alpha 1,3$ -GT gene allele was sequenced, we found a “T–G” transversion in the second base pair of exon 9 in its coding region, which caused a single amino acid change, from tyrosine (Tyr) to aspartic acid (Asp), in the  $\alpha 1,3$ -GT protein (Figure 40-16). This change is precisely located in the active site of the enzyme and totally impairs its function (188). These cells deprived of  $\alpha 1,3$ -GT activity were then used for nuclear transfer to produce the first  $\alpha 1,3$ -GT double knockout (DKO)

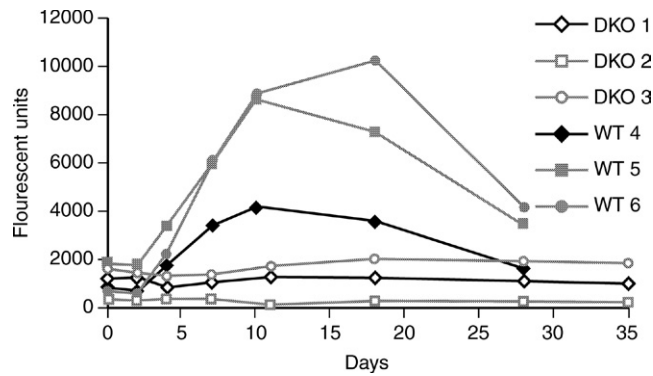


**FIGURE 40-16** Sequencing analysis of  $\alpha 1,3$  Gal-positive and  $\alpha 1,3$  Gal-negative alleles in the cloned pigs. (From Phelps, C. J.; Koike, C.; Vaught, T. D., et al. *Production of  $\alpha 1,3$ -Galactosyltransferase Deficient Pigs*. Science 2003, 299, 411–414.)

cloned pigs. “DKO” was a preferred definition because our pigs were not actually “homozygous” for the same KO allele. On the basis of immunohistochemistry, histologic staining of pig tissues, and immunoreactivity tests (Figure 40-17), it was shown that the cells of the newly generated DKO pigs did not express any  $\alpha 1,3$ -Gal epitopes at their surface (188).

Even without the  $\alpha 1,3$ -Gal epitopes, the tissues from the DKO pigs needed a strong immunosuppressive regimen for acceptance into a nonhuman primate recipient. The generation of an immunologic chimerism between these two species might be the only way to allow this type of xenotransplantation. Xenotransplantation can become a reliable treatment when new strategies are developed that overcome the immunologic hurdles and avoid the use of intensive immunosuppression. However, the risks involved in lethally treating the recipient have





**FIGURE 40-17** Anti- $\alpha$ 1,3-Gal IgM production in  $\alpha$ 1,3-Gal-negative mice (208) injected intraperitoneally (i.p.) with DKO or wild-type (WT) tissues. The tissue from the DKO piglet failed to generate any increase in anti- $\alpha$ 1,3-Gal IgM in the recipient  $\alpha$ 1,3-Gal-negative mice, whereas tissue from neonate wild-type piglets, used as a control, generated significant increases in the IgM titer, demonstrating, in vivo, that tissues from DKO pigs lack any immunogenic  $\alpha$ 1,3-Gal residue at their surface. (From Phelps, C. J.; Koike, C.; Vaught, T. D. *et al. Production of  $\alpha$ 1,3-Galactosyltransferase Deficient Pigs. Science* 2003, 299, 411–414.)

so far precluded any application of this type of protocol in humans but may constitute a very valuable tool for future improved applications. Along this line of reasoning, van der Windt *et al.* (189) reported long-term control of blood sugar in streptozotocin treated diabetic non-human primates, showing that expression of the human complement regulatory protein (hCD46) on pig islets improved the length of time in which recipient animals were normoglycemic. For example, four of five recipient animals exhibited graft survival and insulin independent normoglycemia for 3 months (end of experiment) and one recipient, selected at random, was normoglycemic for greater than 1 year post-transplantation (189).

## 40.11 STEM CELLS AND TRANSPLANTATION

Although all these approaches are designed to bypass the limitations imposed by different genetic profiles, a rather new and somewhat revolutionary alternative has recently been offered: Stem cells can be derived from the embryo or found in different tissues of an individual and are able to replace differentiated cells of different organs.

A stem cell is, by definition, the cell capable of duplicating itself and resuming its undifferentiated status, while also originating progeny that can differentiate into one or more final products that are physiologically defined by their specific functions (190). Proceeding through the differentiation pathway, stem cells can be categorized as totipotent, pluripotent, multipotent, oligopotent, and unipotent, depending on all their possibly reversible, progressively acquired characteristics (191). Embryonic stem (ES) cells are pluripotent cell lines derived from the inner cell mass of blastocyst-stage embryos. ES cells differentiating in culture may reproduce features characteristic of

early embryonic development. The ability to clone human embryos and to derive from them human ES cell lines is already a reality (192–195). Even imagining that tomorrow’s scientists could derive, by cloning, cell lines able to be differentiated toward cells with a desired phenotype and in sufficient number to satisfy the needs of the transplant recipient, to use them for clinical purposes, they should be carrying the diploid genome of somatic cell nuclei derived from individuals unrelated to the oocyte donor (i.e. the recipients). Even so, we would still need to isolate the broadest diversity of HLA polymorphisms, to more easily find a match between donor and recipient so to avoid the serious problem of allojection. In the case in which we opt to avoid all this demanding preparative effort for matching donor and recipient, it would be necessary to reduce the likelihood of allojection by other means (190). However, in the case of ES cells, it may be easier to achieve this goal because the already studied human ES cell lines seem to be able to down-regulate the expression of MHC antigens at their surface (196,197). Rapidly dividing cells with this unique phenotype have to spontaneously stop growing once a specific, predetermined, total population cell mass has been reached as they seem not even normally controlled by NK cells (196). In the case in which the ES cells do not stop proliferating, we will transplant cancer precursors into our patients. Furthermore, even assuming that we could overcome all these challenges, the use of human ES cell lines tailored ad personam would constitute an extremely demanding and expensive proposition (198). If ethical considerations will not block this type of experimentation, the stem cell avenue seems to be the most promising and exciting avenue for autologous, as well as allogeneic, transplantations.

More ethically acceptable certainly is the use of so-called “adult” stem cells, that is, precursor cells present in the tissue of already completely formed individuals. Years ago, Mulligan *et al.* (199) were able to show how stem cells isolated from the bone marrow were able to replace malfunctioning myocytes in a mouse model of Duchenne muscular dystrophy. This approach envisions isolating in vitro adult pluripotent stem cells from any manageable source (e.g. the bone marrow) and physically introducing these cells into the already existing, appropriate environment of living recipients. This assumes that the signals sent via host-secreted factors or by cell-to-cell contacts are powerful enough to guide the transplanted precursors to differentiate into the same type of cells surrounding them, even across different lineages. By receiving spatially and temporally restricted signals from the environment, the precursors may differentiate into the cells constitutive of that specific, target tissue. Using a cocktail of specific antibodies able to recognize a combination of distinct cell surface markers (c-kit<sup>high</sup>Thy<sup>low</sup>Lin<sup>+</sup>Sca-1<sup>+</sup>) on a single cell, Lagasse *et al.* (200) physically isolated HSCs from bone marrow and transplanted them into the fumarylacetoacetate-hydrolase-deficient mouse, an

animal model of fatal hereditary tyrosinemia type I. Four out of nine mutant mice had near-normal liver function (transaminase and tyrosine levels were slightly increased) when, three weeks after transplantation, the standard treatment with 2-(2-nitro-4-trifluoro-methylbenzyl)-1,3-cyclohexane-dione was discontinued. These mice survived for an additional 6 months without signs of progressive liver failure or renal tubular damage. When the experiment, at 7 months after transplantation, was interrupted, 30–50% of the liver mass showed cells expressing donor-derived markers. Similarly, by transferring precursors into the brain of a recipient mouse, Weimann et al. (201) were able to see purified HSCs differentiate into Purkinje neurons. Similar to HSCs, once transduced with a retrovirus to express bone morphogenetic protein 4 (BMP4), muscle-derived cell precursors were able to dramatically improve the healing of a spontaneously irreparable bone fracture (202). Taken together, these research reports suggest an abundance of promising opportunities for BMT in the treatment of chronic diseases. However, for the moment, the only tested clinical exploitation of adult stem cells is the one taking advantage of CD34<sup>+</sup> cells of the bone marrow.

In the most recent years, different groups established that 1–3% of cells in the bone marrow are capable of long-term multilineage hematopoietic reconstitution after myeloablation (203). These cells can be mobilized from the marrow to the peripheral circulation in clinically usable numbers, by the use of recombinant hematopoietic cytokines, similar to the granulocyte colony-stimulating factor. Once mobilized, these cells can be isolated from the peripheral blood by cell sorting using anti-CD34 antibodies. CD34<sup>+</sup> cells isolated from the peripheral blood have a number of advantages when compared with whole bone marrow for transplantation: they are potentially less tumor-contaminated than autologous marrow when used for autologous transplantation; their time for engraftment is shorter; the isolated CD34<sup>+</sup> cells are more amenable to *ex vivo* manipulations such as gene therapy; and even when used in the allogeneic setting, they eliminate acute GVHD and significantly reduced the incidence and severity of chronic GVHD. Unfortunately, these advantages are counterbalanced by the missing positive roles played by graft T-cells able to facilitate engraftment, eliminate residual leukemic cells, and transfer of donor immune memory (204).

In 2006, the creation of induced pluripotent stem (iPS) cells was described (205). Derived from mouse adult somatic cells by the ectopic expression of the transcription factors Oct3/4 (Pou5f1), Sox2, Klf4, and c-Myc, using viral as well as plasmid-based vectors has been shown to be sufficient to direct the reprogramming of adult somatic cells. During the following year, iPS cells were generated from human somatic cells (206) as a result of the combined expression of Oct3/4, Sox2, Klf4, c-Myc and independently with the expression of Oct3/4, Soc2, Nanog, and Lin28. The importance of iPS cells to

transplantation medicine occurs as a result of the cells being derived entirely from the recipient. Thus, these cells bypass transplantation complications such as GVHD and immune rejection. A recognized danger associated with the method for preparing iPS cells is the risk of inducing the expression of oncogenes, such as c-Myc, as well as the possible incorporation of transcription factors into unfavorable sites within the genomic DNA with the potential to induce coexpression of neighboring oncogenes. In 2009, Jaenisch et al. (207) reported the development of protocols for removing oncogenes after the induction of pluripotency, providing a possible solution to this complication. Moreover, it was reported in 2009 that iPS cells could be induced without alteration of genomic DNA (208). This was accomplished by repeated treatment with proteins Oct3/4, Sox2, Klf4, and c-Myc modified with polyarginine anchors to generate what has been termed protein-induced pluripotent stem cells (piPSCs). In the not-too-distant future, the use of iPS cells may circumvent the problem of stem-derived cell allotransplants (e.g. limiting material and immune-mediated rejection) making them equivalent to autotransplants, while also minimizing the risk of exposing the recipient to an uncontrolled neoplastic transformation of the cells.

## 40.12 CONCLUSION

The data presented clearly show that HLA matching does not and cannot influence solid organ distribution significantly. However, these analyses support the wisdom of the UNOS Board of Directors to mandate national sharing of the zero serologically mismatched HLA cadaveric donor kidneys in the rare cases where they occur. HLA matching for other solid organ grafts is even more problematic because the patients needing livers or thoracic organs are generally critically ill and cannot tolerate extensive waiting periods. The recent success in transplanting a single lobe of the organ from alive or cadaveric donors into one or more recipients, respectively, may expand these limited chances. On average, only about 30% of candidates for BMT will have an HLA-identical sibling or other suitable related donor. Leukemia is the most common diagnosis of BMT potential recipients. Survival and cure rates for acute nonlymphocytic leukemias are almost as great after autologous BMT as for HLA-identical sibling BMT. This is not the case for acute lymphocytic leukemia patients, however. Unfortunately, those patients with uncommon HLA phenotypes will not survive a lengthy search for an unrelated donor. At the present time, few chronic lymphocytic leukemias are considered candidates for bone marrow transplant; therefore, the NMDP and other registries of unrelated donors benefit primarily patients with chronic myelogenous leukemia. Thus far, the outcome of unrelated transplants for chronic myelogenous leukemia has been poor because of HLA mismatching not detected by serologic and cellular assays. It is anticipated that improvement will be seen

now that typing by DNA-based methods for class II are in place. Once this is accomplished, the next major effort for the future will be to identify which minor differences between variants can be tolerated with little or no risk of rejection or GVHD, or which tolerogenic protocol can offer safe but effective means to allow transplants across HLA boundaries or even across different species without the need for massive immunosuppression.

## ACKNOWLEDGMENTS

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Allele Frequencies in Worldwide Populations: <http://www.allele-frequencies.net>.

A centralized source for the storage of allele frequencies from different polymorphic areas in the human genome.

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## Biographies



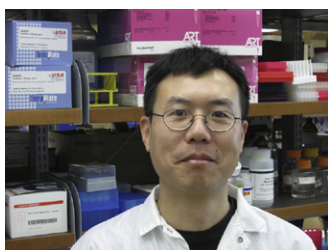
**Gaia Bellone** is a graduate student in the Department of Statistics at Carnegie Mellon University. She graduated in Economics from Bocconi University, Italy (BA 2005) and obtained her master degree in Statistics from Carnegie Mellon University (2008). She worked as Financial Analyst at a privately held financial institution. Her research focuses on the development of advanced statistical methods for analyzing sequencing data.



**Massimo Trucco** graduated summa cum laude in Medicine at the University of Turin, Italy, in 1974. In 1977, as assistant professor of Medical Genetics, was visiting the MRC laboratories in Cambridge, UK, working with Cesar Milstein; then moved to the Basel Institute of Immunology, Switzerland, directed by Niels Jerne. Here, Trucco published the characterization of the first anti-HLA monoclonal antibodies (*Nature* 273:666, 1978). In 1981, Trucco was at the Wistar Institute, University of Pennsylvania, where studied the molecular basis of HLA-DQB1 histocompatibility alleles (*Nature* 309:166, 1984). In 1986, Trucco moved to the University of Pittsburgh, appointed as associate professor in Pediatrics. With Dr McDevitt, Stanford University, the HLA-DQB1 alleles were characterized as the most-sensitive markers for type 1 diabetes susceptibility (*PNAS* 85:8111, 1988; *PNAS* 87:7370, 1990). In 1990, Trucco, as the Head of the Division of Immunogenetics, cloned the NK1-1 encoding gene (*Science* 249:1298:1990). In 1991, received the Henry Hillman endowed chair as Professor of Pediatric Immunology. In 1996, Trucco received the University of Pittsburgh Chancellor's Distinguished Award for his work on diabetes (*Nature* 371:351, 1994; *Nature* 380:284, 1996). This constituted the basis for opening the new Diabetes Institute, of which he became, and still is, the first Scientific Director.



**Steven Ringquist** is a research assistant professor at the Children's Hospital of Pittsburgh. He received bachelor degrees in Chemistry and Biology from California State University (BA 1982) and a doctorate degree in Biological Chemistry from the University of Illinois (PhD 1987). He went on to study within the field of genomics at Columbia University (1988–1989) where he mapped epigenetic changes occurring in genomic DNA. Later he worked at the University of Colorado (1990–1993) on developing experimental and computational models for measuring the influence of RNA elements that control gene expression. Beginning in 1994 his work at a pharmaceutical company developing modulators of protein activity resulted in the awarding of nine U.S. Patents. He returned to academia by obtaining a fellowship at the Sidney Kimmel Cancer Center where he investigated changes in expression of low copy number mRNA from tumor cells (1998–2000). In 2001, he moved to his current position at the Children's Hospital of Pittsburgh. Ringquist's research combines genetic, proteomic, and computational tools for the identification of molecular markers that predict transplantation outcome as well as susceptibility to autoimmune disease.



**Ying Lu** is a researcher at the Children's Hospital of Pittsburgh. He graduated from NanKai University, China (BS 1995) and pursued postgraduate studies in the Department of Computer Science at Rensselaer Polytechnic Institute (MS 2002) and in the Department of Chemistry at the University of Pittsburgh (MS 2005). He worked as adjunct staff at Proteomics Core Lab at Pitt (2003–2004). He then joined Dr Trucco's laboratory in 2005. His research focuses on the use of genomics and proteomics to elucidate the molecular mechanisms underlying diseases of the immune system.

# CHAPTER

# 41

## The Genetics of Disorders Affecting the Premature Newborn

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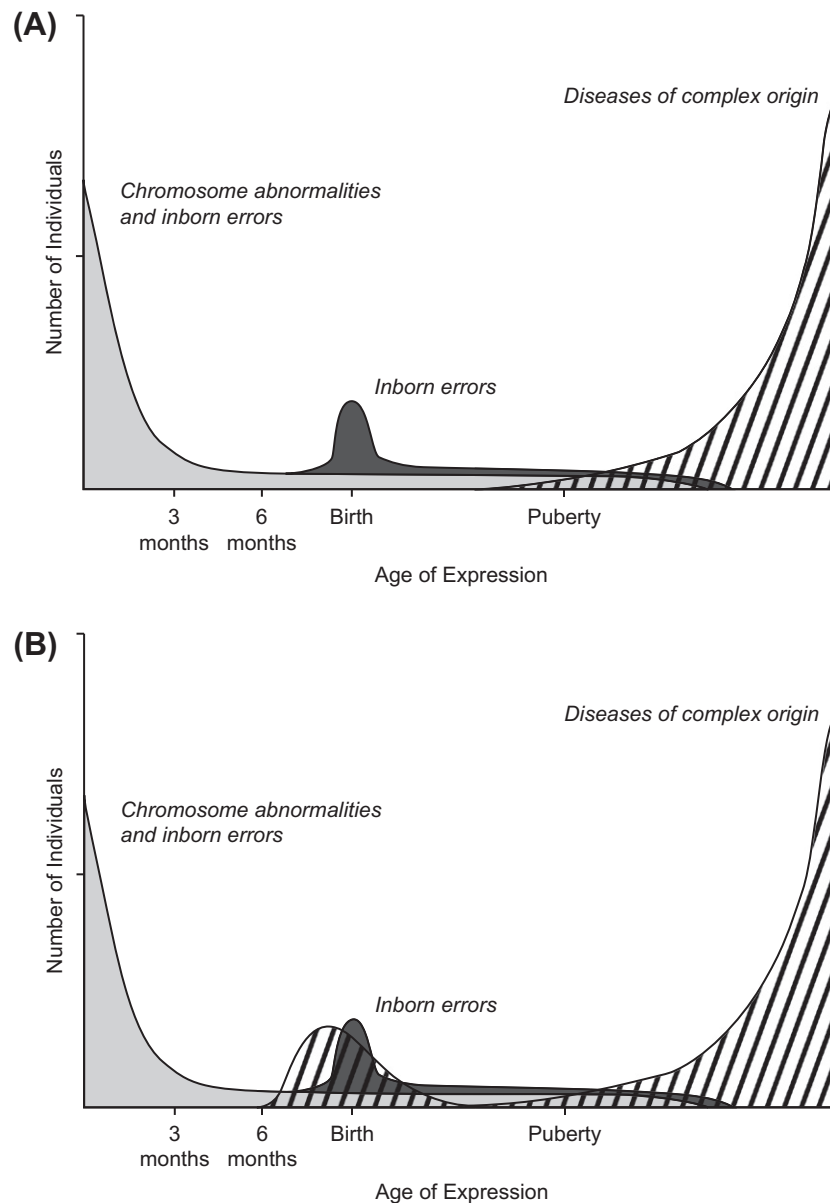
### 41.1 INTRODUCTION

Premature birth, occurring prior to 37 weeks of gestation, affects more than 12% of births in the USA. The rate of premature birth has risen 35% since 1981 (1) and is the leading cause of infant morbidity and mortality, with an estimated annual economic cost of \$26 billion. Despite improvements in perinatal and neonatal care over the past four decades, outcomes have remained stagnant over the last 15 years (2).

Immaturity of the immune system and increased oxidative stresses (3) in the setting of an immature antioxidant system are common pathways that contribute to the morbidity and mortality of prematurely born infants. There is a delicate balance that exists between the creation of reactive oxygen species (ROS) and cellular antioxidant defenses, and that balance is particularly perturbed in the preterm infant, placing the child at increased risk for oxidative damage (4). The preterm infant is exposed to increased ROS through the processes of hyperoxia, reperfusion, and inflammation. In addition, the antioxidant defenses are immature in preterm infants. Both upregulation of endogenous production of antioxidant enzymes (AOE) and maternal–fetal transfer of AOE occur in the final 12% of gestation, in preparation for the transition to the relative hyperoxia of extra-uterine life. A lack of mature ROS defenses may increase the risk for developing bronchopulmonary dysplasia

(BPD), retinopathy of prematurity, and periventricular leukomalacia, which have been linked to oxygen radical species (4).

In addition to the contributions that the premature physiologic state makes to the pathogenesis of the disorders commonly seen in the premature infant, genetics also plays a strong role (5). Historically, prenatal and perinatal disorders were considered to be more purely genetic, since chromosomal abnormalities and single-gene recessive errors of metabolism are responsible for many spontaneous abortions, miscarriages, and perinatal deaths (see Figure 41-1A). However, contemporary studies in the neonatal period permit a new paradigm in which disorders of the premature infant can be considered as complex, multifactorial, and polygenic, and the result of gene–environment interactions. Between 21 and 38 weeks of gestation, genetic factors can be thought of as influencing the newborn's tolerance to the inherent stressors of prematurity and to the toxicities of life-saving interventions such as positive pressure ventilation, oxygen therapy, and enteral feedings (see Figure 41-1B). Genetic approaches to the diseases of the neonatal period that address the pathophysiology of prematurity as tolerance to gene–environment interactions, beyond just global immaturity, will allow investigators to take advantage of new genetic resources and technologies that have been developed over the past two decades.



**FIGURE 41-1** Continuity of disease across the lifetime. (A) Conventional depiction of genetic disease from conception to adulthood. (B) Recognition of complex diseases in the premature very-low-birth weight neonate born between 24 weeks and term. White peaks are chromosomal aberrations. Grey peaks are Mendelian single-gene disorders. Hatched peaks are complex disorders. (Reprinted with permission from Bhandari, V.; Gruen, J. R. *The Genetics of Bronchopulmonary Dysplasia*. Semin. Perinatol. 2006, 30, 185–191.)

In the sections below, we present the evidence for heritability and consider candidate gene studies for the six most common causes of neonatal morbidity in infants born less than or equal to 28 weeks and 1500 g (28% overall morbidity): respiratory distress syndrome, BPD, patent ductus arteriosus (PDA), intraventricular hemorrhage (IVH), retinopathy of prematurity, and necrotizing enterocolitis (NEC) (6).

## 41.2 RESPIRATORY DISTRESS SYNDROME

Respiratory distress syndrome (RDS) is a condition that predominantly affects preterm infants. Formerly known

as hyaline membrane disease, RDS is caused by a lack of mature surfactant in the alveoli, resulting in higher than normal surface tension and thus decreased alveolar ventilation and atelectasis. The atelectasis causes ventilation-perfusion mismatching, hypoxia, and eventual respiratory failure. Infants are most at risk before 32 weeks of gestation, prior to the development of secondary septation/alveolarization that would result in a more mature lung (7).

RDS affects approximately 10% of all premature infants. Gestational age (GA) and birth weight (BW) are inversely proportional to the risk. It occurs in fewer than 5% of babies born after 34 weeks' gestation, in 30% of babies born between 28 and 34 weeks' gestation, and 60% of preterm infants born prior to 28 weeks' gestation (8).



Additional risk factors include male sex, white race, maternal gestational diabetes, perinatal asphyxia, hypothermia, and multiple gestations (9). Current treatment modalities have improved RDS mortality from nearly 100% to less than 10% (10), yet RDS is a leading cause of morbidity and mortality in preterm infants with an estimated annual economic cost of \$2.3 billion (8).

### 41.2.1 Twin Studies

Twin studies have suggested that genetic factors play an important role in determining susceptibility and severity. In 1996, Nagourney et al. (11) determined that second twin siblings had a significantly increased risk of RDS compared with the first twin, after controlling for confounding variables. In 1971, Myrianthopoulous et al. studied 31 twin pairs and showed a higher concordance between monozygotic (MZ) pairs compared to dizygotic (DZ) pairs (85% versus 44%, respectively) (12). Similarly, a retrospective study of 194 preterm (GA 30–34 weeks) twin pairs in the Netherlands found that RDS occurred more frequently in both twins when the twins were MZ (67% versus 29%,  $p < 0.05$ ) (13). While the study from the Netherlands suggested a strong genetic influence, it only included 80 pairs with a GA of 30–34 weeks, which is a GA of relatively low risk for developing RDS.

In contrast, Marttila et al. investigated 100 same-sex twins with RDS and found a concordance difference of only 10% (95% confidence interval (CI)  $-0.1$  to  $+0.3$ ,  $p = 0.32$ ) (14). In a follow-up study, the same group evaluated the concordance between same-sex and opposite-sex RDS rates within a collection of 23,278 twins from Europe (15). The authors found no difference in concordance between the two groups and concluded that environmental factors predominated over genetic factors. Both of these studies, however, lacked a formal determination of zygosity. In addition, several studies have shown the importance of sex in frequency and severity of RDS, calling into question the validity of these studies that were based on sex.

### 41.2.2 Heritability of RDS

Levit et al. performed a multicenter retrospective study of 332 twin pairs less than 32 weeks GA, and computed the heritability of RDS. Using placental pathology and sex concordance for determining zygosity, and after correcting for major known environmental risk factors, they found the heritability to be 49.7% ( $p = 0.04$ ) (16). Taken together, the twin and heritability studies suggest that genetic factors account for a significant proportion of the variance for RDS.

### 41.2.3 Candidate Genes

Candidate gene studies of surfactant proteins have shown evidence for genetic associations (see Table 41-1).

Surfactant is composed of a mixture of phospholipids (mainly phosphatidylcholine) and surfactant proteins (SPs) A, B, C, and D (64). SP-A regulates secretion and uptake of surfactant and plays a role in innate host defense, while SP-B and SP-C assist in adsorption and spreading of the phospholipid monolayer along the surface of the alveolus (65). SP-D is thought to participate in surfactant reuptake and recycling (66). Several studies have found associations between SP polymorphisms and the risk for developing RDS, particularly involving *SP-A* and *SP-B* alleles, reviewed by Hallman and Haataja (67).

### 41.2.4 *SP-A* and *SP-B* Studies from Finland

There have been five studies of RDS in prematurely born infants from Finland. Marttila et al. (50), in their study of 198 premature twin pairs, noted the *SP-A1* allele 6A2 ( $p = 0.030$ ), genotype 6A2/6A2 ( $p = 0.0042$ ), and haplotype 6A2-1A0 ( $p = 0.016$ ) were overrepresented in healthy premature twins when compared with twins with RDS. In healthy twin pairs, the genotype 6A2/6A2 was overrepresented, compared with concordant RDS twins (odds ratio (OR) 0.18, 95% CI 0.06–0.60,  $p = 0.0016$ ). A larger study by the same group collected data on 441 singletons and 480 multiples from Finland (51). Contrary to the prior study of twins, both the *SP-A1* allele 6A2 and the homozygous genotype 6A2/6A2 were overrepresented in small RDS singletons when the *SP-B* exon 4 genotype was Thr131/Thr131 ( $p = 0.009$  and  $p = 0.003$ , respectively). This combination of 6A2 and Thr131 was associated with a fourfold increase in risk. However, 6A2 alleles were underrepresented in RDS of near-term multiples when the *SP-B* genotype was either Ile/Thr ( $p = 0.012$  and  $p = 0.003$ , respectively) or Thr/Thr ( $p = 0.12$  and  $p = 0.018$ , respectively). In this study, the *SP-A* 6A2 allele with the *SP-B* Thr131 allele was detrimental to singleton infants but protective to multiples.

Ramet et al. showed that within a cohort of 176 Finnish preterm infants, the *SP-A1* allele 6A2 was more prevalent in infants with RDS (allelic frequency 0.65 versus 0.52,  $p = 0.017$ ), while the 6A3 allele frequency was less frequent in the RDS group (0.24 versus 0.34,  $p = 0.034$ ). The authors did not find any significant association between *SP-A2* alleles and RDS (52). A companion study by the same group investigated 684 premature infants, 184 with RDS, showing that the *SP-B* Ile131Thr genotype modulated the effect of the various *SP-A* alleles on RDS risk (increased with 6A2 and 1A0, decreased with 6A3 and 1A2) (53). In a later transmission disequilibrium study of 107 white parent-offspring trios from Finland, Haataja et al. showed that the *SP-A1-A2* haplotype 6A2-1A0 increased, and *SP-A1* 6A3-1A1 6A4-1A5 genotypes decreased the risk for developing RDS (56).

TABLE 41-1 Selected SNPs Evaluated by Multiple Investigators

Gene	Polymorphism	Potential Effect	Results	Ref #
<b>Vascular Genes</b>				
ACE	Intron 16 deletion	ACE activity ↑	BPD ↔	(17)
ACE	Intron 16 deletion	ACE activity ↑	BPD ↑	(18)
ACE	Intron 16 deletion	ACE activity ↑	BPD ↔	(19)
ACE	Ins/ins	Unknown	ROP ↑ (incidence)	(20)
ACE	Del/del	ACE concentration ↑	ROP ↑ (treatment)	(20)
ACE	Ins/del	ACE concentration ↑	ROP ↔	(21)
Factor V	Leiden	↑ Coagulation	IVH ↔	(22)
Factor V	Leiden	↑ Coagulation	IVH ↔	(23)
Factor V	Leiden	↑ Coagulation	IVH ↑	(24)
Factor V	Leiden	↑ Coagulation	IVH ↑	(25)
Factor V	Leiden	↑ Coagulation	NEC ↔	(26)
Factor XIII	Val34Leu	↓ Coagulation, ↑ hemorrhage	BPD ↓, IVH ↔	(22)
Factor XIII	Val34Leu	↓ Coagulation, ↑ hemorrhage	IVH ↑, white matter disease ↓	(27)
Prothrombin	G20210A	↑ Coagulation	IVH ↔	(22)
Prothrombin	G20210A	↑ Coagulation	IVH ↔	(23)
Prothrombin	G20210A	↑ Coagulation	IVH ↑	(28)
VEGF	-460TT/-634CC		ROP ↑	(29)
VEGF	(-2578)A		ROP ↓ (male)	(30)
VEGF	(-2578)A		NEC ↑	(31)
VEGF	-460C	↔	BPD ↓	(32)
VEGF	-460T	↔	BPD ↑	(32)
VEGF	-460T	↔	ROP ↑	(33)
VEGF	C(-460)T	↔	ROP ↔	(34)
VEGF	-634C	↓ VEGF expression	ROP ↑	(29)
VEGF	G(-634)C	↑ VEGF expression	ROP ↔	(34)
VEGF	G(-634)C	↑ VEGF expression	ROP ↔	(33)
VEGF	G(-634)C	↑ VEGF expression	ROP ↑	(35)
<b>Genes Related to Inflammation</b>				
IL-1 beta	C(3954)T	↑ IL-1 beta	BPD ↔	(36)
IL-1 beta	C(-511)T	↑ IL-1 beta	IVH ↑	(37)
IL-1 beta	T(-31)C	↓ IL-1 beta	NEC ↔	(38)
IL-1 beta	C(-511)T	↑ IL-1 beta	NEC ↔	(38)
IL-4 receptor alpha	A(+1902)G	↑ Signaling	NEC ↓	(39)
IL-4 receptor alpha	G(+1902)A	↑ Signaling	NEC ↔	(38)
IL-6	(-174)CC	↑ Production	IVH ↑	(40)
IL-6	(-174)CC	↑ Production	IVH ↔	(41)
IL-6	G(-174)C	↑ Production	BPD ↔	(36)
IL-6	G(-174)C	↑ Production	NEC ↔	(38)
IL-6	(-572)C	↑ Production	IVH ↔	(42)
IL-10	(-1082) GG/GA	↑ IL-10	RDS ↓	(43)
IL-10	G(-1082)A	↓ Transcription	BPD ↔	(44)
IL-10	G(-1082)A	↓ Transcription	BPD ↔	(36)
IL-10	G(-1082)A	↓ Transcription	NEC ↔	(38)
TGF beta	G(-509)T	↑ Production	ROP ↔	(35)
TGF beta	G(+915)C	↑ In vitro production	BPD ↔	(45)
TGF beta	G(+915)T	↓ Levels	BPD ↔	(46)
TNF alpha	G(-238)A	↑ Transcription	NEC ↔	(38)
TNF alpha	G(-238)A	↓ Function	BPD ↓	(47)
TNF alpha	G(-238)A	↓ Function	BPD ↔	(48)
TNF alpha	G(-308)A	↑ Transcription	ROP ↔	(35)
TNF alpha	G(-308)A	↑ Transcription	NEC ↔	(38)
TNF alpha	G(-308)A	↑ Transcription	BPD ↔	(45)
TNF alpha	G(-308)A	↑ Transcription	BPD ↔	(47)
TNF alpha	G(-308)A	↓ Function	BPD ↔	(36)
TNF alpha	G(-308)A	↑ Transcription	BPD ↔	(48)
TNF alpha	G(-308)A	↑ Transcription	IVH ↑	(45)
TNF alpha	G(-308)A	↑ Transcription	IVH ↔	(49)
TNF beta	A(250)G	↑ TNF alpha expression	BPD ↔	(47)

**TABLE 41-1 Selected SNPs Evaluated by Multiple Investigators—cont'd**

Gene	Polymorphism	Potential Effect	Results	Ref #
TNF beta	NcoI	↑ TNF alpha expression	IVH ↑ (male)	(49)
<b>Surfactant Protein Genes</b>				
SP-A1	6A2		RDS ↓	(50)
SP-A1	6A2		RDS ↔	(51)
SP-A1	6A2		RDS ↑	(52)
SP-A1	6A2		RDS ↑	(53)
SP-A1	6A2		RDS ↑ (white)	(54)
SP-A1	6A2-1A0	↓ Expression	RDS ↓	(50)
SP-A1	6A2/6A2		RDS ↑ (white)	(55)
SP-A1	6A3		RDS ↓	(52)
SP-A1	6A3		RDS ↓	(53)
SP-A1	6A3		RDS ↑ (black)	(54)
SP-A1	6A3-1A1/6A4-1A5		RDS ↓	(56)
SP-A1	6A4		RDS ↑ (white)	(54)
SP-A1	6A6		BPD ↑	(57)
SP-A1-A2	6A2-1A0		RDS ↑	(56)
SP-A2	1A0		RDS ↑	(53)
SP-A2	1A0		RDS ↑ (white)	(54)
SP-A2	1A0		RDS ↑ (white)	(55)
SP-A2	1A1		RDS ↑ (black)	(54)
SP-A2	1A2		RDS ↓	(53)
SP-A2	1A2		RDS ↑ (black)	(54)
SP-A2	6A3		RDS ↓ (black)	(55)
SP-B	Exon 4 Thr131		RDS ↔	(51)
SP-B	Exon 4 Thr131		RDS ↔	(53)
SP-B	G(8714)C		RDS ↑	(58)
SP-B	Intron 4 deletion	↑ SP-B expression	RDS ↑ (white male)	(55)
SP-B	Intron 4 deletion	↑ SP-B expression	BPD ↑	(59)
SP-B	Intron 4 deletion	↑ SP-B expression	BPD ↑	(60)
SP-B	Intron 4 deletion	↑ SP-B expression	BPD ↑	(61)
SP-B	Intron 4 insertion		RDS ↑ (black female)	(55)
SP-B	Intron 4 polymorphisms		RDS ↑	(59)
<b>Miscellaneous Genes</b>				
IGF-1R	G(+3174)A	IGF-1 ↓	ROP ↔	(62)
IGF-1R	G(+3174)A	IGF-1 ↓	BPD ↔	(63)

↑ = increased; ↓ = decreased; ↔ = no change; ACE = angiotensin-converting enzyme; BPD = bronchopulmonary dysplasia; del = deletion; IGF = insulin-like growth factor; IL = interleukin; ins = insertion; IVH = intraventricular hemorrhage; NEC = necrotizing enterocolitis; RDS = respiratory distress syndrome; ROP = retinopathy of prematurity; SP = surfactant protein; TGF = transforming growth factor; TNF = tumor necrosis factor; VEGF = vascular endothelial growth factor.

### 41.2.5 *SP-A* and *SP-B* in Preterm Infants from Germany

In an unrelated study of *SP-B* polymorphisms, Makri et al. looked at the rates of RDS among 140 white preterm infants from Germany (59). Among other findings, those infants carrying non-wild-type polymorphisms in *SP-B* intron 4 (either deletions or insertions) were found to have a significantly increased risk of developing RDS (93.1% versus 75.7%,  $p < 0.05$ ). Lyra et al. (58) also evaluated polymorphisms of *SP-B* in a cohort of 50 preterm infants with RDS and 100 healthy term infants. When only the white children were compared, there was a statistically significant difference in the RDS and control groups in the G(8714)C polymorphism rate ( $p = 0.028$ ).

### 41.2.6 Racial Differences

Studies by Kala and Floros showed a difference in risk factors conferred by *SP* genotypes between black and white subjects. In a study of 241 infants greater than 28 weeks GA, Kala et al. (54) found an overrepresentation of 1A0, 6A2, and 6A4 alleles in the RDS white subjects, whereas 1A1, 1A2, and 6A3 were significantly more frequent in the RDS black subjects. In addition, *SP-A2* 1A0/1A0 was found more frequently ( $p = 0.035$ ), and *SP-A2* 1A1 was found less frequently ( $p = 0.005$ ) in a subgroup of 82 white infants greater than 28 weeks GA with RDS compared with 83 controls. Synergistic positive association with RDS was also observed in white infants greater than 28 weeks GA when the 1A0 allele was present concurrently with a previously described *SP-B* intron

4 polymorphic variant. Floros et al. (55) investigated a group of 511 white and 72 black preterm infants and the interactions between *SP-A* and *SP-B* alleles in conferring RDS risks, in order to further understand the increased risks seen among white infants. They found that the *SP-A* 16A2/6A2 genotype and the *SP-A* 1A0/1A0 or 1A0/\* genotype were associated with an increased risk in the white population when the *SP-B* genotype was A(9306) G or (del/\*). The *SP-A* 16A3/6A3 and 6A3/\* genotypes, in the setting of *SP-B* genotype 1580(T/T), conferred reduced risk in the black population. In a similar fashion, an *SP-B* intron 4 insertion variant conferred increased risk to black females, while the *SP-B* intron 4 deletion variant conferred an increased risk in white males.

The efficacy of single nucleotide polymorphism (SNP)-based studies for identifying clinically significant polymorphisms for *SP-B* has been called into question. Hamvas et al. (68) evaluated the genomic stability of the *SP-B* gene, and showed excess low-frequency variation, intragenic recombination, and lack of common disruptive exonic variants. They concluded that SNP-based and linkage disequilibrium studies of the *SP-B* gene in diverse populations should involve complete resequencing.

#### 41.2.7 *SP-C* and RDS

Dominant mutations of the *SP-C* gene are known to be associated with interstitial lung disease. Lahti et al. (69) tested whether non-dominant *SP-C* alleles 138Asn (exon 4) or 186Asn (exon 5) could also affect the risk for RDS, after adjusting for sex. In a cohort of 245 preterm infants from Finland, both alleles were found to be independent risk factors for RDS (138Asn: OR 2.01, 95% CI 1.10–3.66,  $p=0.022$ ; 186Asn: OR 1.87, 95% CI 1.04–3.38,  $p=0.036$ ). They showed similar results for the haplotype 138Asn-18Asn (OR 2.05, 95% CI 1.12–3.74,  $p=0.020$ ). Of note, the associations for both alleles were observed to be stronger among male infants ( $p=0.018$  and  $p=0.045$ , respectively). Wambach et al. (70) investigated the complete *SP-C* gene sequences of 269 case-control white infants less than 34 weeks of gestation and identified 80 SNPs. They identified three SNPs within the promoter region as statistically associated with RDS: genomic position 1167 (OR 4.14, 95% CI 1.26–13.57,  $p=0.019$ ), genomic position 1647 (OR 12.75, 95% CI 1.58–103.15,  $p=0.017$ ), and genomic position 2385 (OR 13.0, 95% CI 1.60–105.09,  $p=0.016$ ).

#### 41.2.8 *SP-D* and RDS

There have been few studies of *SP-D* gene polymorphisms in the setting of RDS. Hilgendorff et al. (71) tested *SP-D* gene SNPs previously associated with adult pulmonary inflammatory disease. In a cohort of 284 premature infants, homozygote minor allele for SNP rs1923537 was associated with a lower prevalence of RDS (OR 1.733, 95% CI 1.139–2.636, adjusted  $p=0.0408$ ), lower number of total surfactant doses, less use of diuretics,

and a lower oxygen requirement at day 28 of life (71). In a haplotype transmission disequilibrium study of 132 families, Thomas et al. discovered that the two-marker *SP-D/SP-A* haplotype DA160 A/*SP-A* 1A1 reduced the risk of RDS ( $X^2=4.45$ ,  $p=0.035$ ) (72).

#### 41.2.9 Genes Related to Inflammation and RDS

The pathogenesis of RDS includes a significant inflammatory component. Capasso et al. (43) investigated known polymorphisms of inflammatory cytokines IL-8, IL-10, and TNF- $\alpha$  in 342 white newborns from Italy, 66 were prematurely born and had RDS. They found that the risk of RDS was significantly lower in preterm infants with the IL-10 genotype 1082 GG/GA than in those with the AA genotype (OR 0.48, 95% CI 0.24–0.95,  $p=0.03$ ). Further quantitative real-time polymerase chain reaction (RT-PCR) analysis demonstrated that IL-10 levels were higher in the GG/GA carriers ( $p=0.03$ ). A study by Bokodi et al. found that carriers of the cytokine allele IFN- $\gamma$  T(+874) A were at fourfold higher risk for developing RDS (OR 4.03, 95% CI 1.30–12.50,  $p=0.016$ ) (73). Additional work by this group found that a genotype associated with impaired heat shock protein cellular defense *HSP-72*(1267)GG genotype was associated with RDS (74).

#### 41.2.10 Meta-analysis

Treszl et al. (75) used a random forest technique to reanalyze previously published data of 24 SNPs among 135 preterm infants. Using relative importance scores (IS), they demonstrated that when including SNP information for E-selectin(128)Arg, TLR-4(399)Ile, IL-12 p40 CG, IL-18(-137)C, TNF- $\alpha$ (-238)A, IL-18(-607)A, *VEGF*(+405)G, and P-selectin(715)Pro (all IS > 1.2,  $p>0.1$ ), the accuracy of predicting the occurrence of RDS rose incrementally when compared to a baseline prediction utilizing only birth history data (0.72–0.77).

#### 41.2.11 Miscellaneous Genes and RDS

Other genes, in addition to *SP* genes, have been found to contribute to the development of RDS. *ABCA3*, an ATP-binding protein localized to the lamellar bodies of type II cells, is known to play a role in surfactant metabolism. *ABCA3* deficiency has been associated with cases of fatal RDS in full term infants (76). Karjalainen et al. found an association of the F353F allele in the *ABCA3* gene with a prolonged course of RDS in very premature infants (77). In an effort to study whether G-protein-coupled receptor for asthma susceptibility (GPRA) played a role in the pathogenesis of RDS, Pulkkinen et al. (78) performed a case-control study of 521 infants from Finland. They found that in preterm infants with RDS, the *GPRA* haplotype H1 was underrepresented in RDS, while the haplotype H4/H5 was associated with increased risk.



### 41.2.12 Summary of RDS

The genetic basis of RDS has been investigated by numerous twin studies, although concordance between twins was variable and could not uniformly determine the difference between familial and genetic factors. However, a multicenter study of 332 twin pairs with zygosity data showed that the heritability of RDS was 49.7%. To identify candidate genes associated with RDS, multiple investigators have studied the common polymorphisms of the four major surfactant protein genes (*SP-A*, *-B*, *-C* and *-D*) in cohorts of different sizes and demographics. The results from these studies were variable and often contradictory. Further studies of alternative candidate genes have been limited to six genes involved in either surfactant metabolism (*ABCA3*, *GPRA*) or the inflammatory cascade (*IL-8*, *IL-10*, *TNF- $\alpha$*  and *IFN- $\gamma$* ), without any independent validation. Meta-analysis of previously published polymorphism data suggests that genotypes may be incrementally helpful in determining the risk for developing RDS, but more systematic work on larger cohorts will be necessary to develop more robust and reproducible data.

## 41.3 BRONCHOPULMONARY DYSPLASIA

BPD is the most common chronic lung disease in infancy. It results from injury to the immature lung due to baro/volutrauma, exposure to hyperoxia, and in utero respiratory or systemic infections (79,80). Pathologically, BPD is defined as uniform inflation of the lung with impaired alveolarization, dysregulated vasculature, minimal airway metaplasia, smooth muscle hypertrophy, and fibrosis (81,82).

The diagnostic criterion for BPD is the requirement for supplemental oxygen at 36 weeks post-menstrual age (PMA) (79,82). It remains the most severe complication of preterm birth, affecting up to 23% of all infants with BWs between 500 and 1500 g (83). Demographic factors associated with BPD include GA, BW, male sex, white race, family history of asthma, and impaired growth for GA. During the perinatal period, low Apgar scores, asphyxia, PDA, pulmonary edema, duration of oxygen therapy, and various nutritional and mechanical ventilatory measures can also be associated with a higher risk (84). Prenatal maternal treatment with glucocorticoids decreases the risk. Long-term sequelae include general neurodevelopmental and quality of life impairments, increased risk of postnatal mortality, asthma, emphysema, pulmonary hypertension, systemic hypertension, and growth failure (84).

### 41.3.1 Heritability of BPD

There have been several studies that used multiple births to assess heritability. Parker et al. (85) were the first to

compare concordance among 108 very low birth weight (VLBW) twins. BPD status in one twin significantly predicted the risk in the other twin (adjusted OR 12.3,  $p < 0.001$ ) when adjusted for BW, GA, sex, RDS, pneumothorax, PDA, birth order, and Apgar scores. Bhandari et al. compared concordance in 450 twin pairs (86) and showed that 65.2% (95% CI 52.6–78.9%,  $p < 0.001$ ) of the variance could be accounted for by a combination of shared genetic plus environmental factors, after controlling for male sex, BW, and RDS. Zygosity data in a subgroup of subjects allowed for quantification of heritability through a comparison of MZ and DZ twins. After controlling for covariates, the heritability of BPD was 53% (95% CI 16–89%,  $p = 0.004$ ). A later study by Lavoie et al. (87) showed a higher heritability (>79%) after differentiating mild from more severe forms. In this study, mild BPD (oxygen dependence at 28 days of life), which usually resolves over time, was primarily attributable to shared environmental effects. Moderate to severe BPD (oxygen dependence at 36 weeks PMA), however, was attributable to primarily genetic factors. This suggests that genetic association studies should be stratified for severity or should only include subjects with moderate to severe disease.

### 41.3.2 Candidate Genes

BPD can be viewed as a disease of gene–environment interactions, rather than the traditional view of interrupted development. More recent studies have begun to test the large number of candidate genes that participate in the process of lung development, inflammatory regulation, oxygen toxicity, cell injury, tissue repair, surfactant production, and sepsis. These studies remain small and poorly replicated, but they highlight the shift from developmental to genetic factors in the investigation of this critical disease process.

### 41.3.3 Surfactant Genes and BPD

While surfactant proteins are frequently studied in relation to the pathogenesis of RDS (as described above), studies have shown an association with BPD as well. In a small study of 23 GA-matched preterm infants with BPD, Weber et al. (57) found that the *SP-A1* 6A6 allele was significantly overrepresented in the BPD group, suggesting that *SP-A* polymorphisms are an independent risk factor. Makri et al. (59) investigated the association between *SP-B* and BPD in a study of 140 preterm infants. The rate of BPD (using the older definition of requirement for supplemental oxygen on postnatal day 28) among infants with *SP-B* intron 4 deletion (*i4del*) was higher (21.6% versus 48.3%,  $p < 0.01$ ); however, the significance dropped out when the definition of BPD was extended to requirement for supplemental oxygen at 36 weeks PMA. A study of 140 preterm infants from Germany found similar associations between *SP-B* *i4del*

and BPD (60), particularly for the presenting twin. The intron 4 region of *SP-B* contains transcription factor binding sites that may allow for increased gene expression in response to infection or injury (88). A Finnish data mining study by Rova et al. (61) reported that the frequency of *SP-B* i4del, which may affect mRNA splicing and protein expression (89), was increased in 67 infants with BPD compared with 178 controls (OR 2.0, 95% CI 1.2–3.4,  $p=0.008$ ) (61). Pavlovic et al. found several associations with microsatellite markers from *SP-A*, *-B*, *-C*, and *-D* in 71 infants with mild or moderate BPD (90).

As described above, *ABCA3* is a protein involved in the storage and secretion of surfactant. Karjalainen et al. showed association of the *ABCA3* F353F allele with a prolonged course of RDS (77). In further studies they also showed that the F353F allele was more common in preterm infants with BPD than controls (OR 3.6, 95% CI 1.5–8.8,  $p=0.002$ ).

#### 41.3.4 Vascular Genes and BPD

Angiotensin converting enzyme (ACE), located in pulmonary endothelial cells, converts physiologic inactive angiotensin I to active angiotensin II (AGII). AGII functions as a direct vasoconstrictor as well as a stimulator of aldosterone production, which promotes extracellular fluid retention. In addition, recent studies suggest a role for AGII in the inflammatory cascade (91). Increased activity of ACE has been hypothesized to increase the risk of BPD. Yanamandra et al. (17) investigated whether a specific insertion/deletion polymorphism in intron 16 of *ACE*, previously associated with increased enzyme activity, contributed to the risk for BPD. They studied 245 mechanically ventilated infants weighing less than 1250 g at birth, and found no association between the *ACE* insertion/deletion polymorphism on mortality or development of BPD. In a similar, but smaller, study of 51 VLBW (BW less than 1000 g and GA equal to 28 weeks) infants with BPD compared with 60 controls, Kazzi and Quasney (18) found that homozygotes and heterozygotes for the high activity deletion polymorphism were more likely to have BPD than the group without the deletion (47% versus 22%,  $p=0.025$ ). The authors also suggested that the number of activity alleles correlated with severity, but only mild and moderate BPD was analyzed. In contrast, Bodoki et al. found no effect from the DD genotype on BPD in a study of 114 infants of greater GA (30 weeks) (19), perhaps highlighting the effect of GA on the impact of deleterious genetic factors on diseases of prematurity.

Several studies have demonstrated a role for vascular endothelial growth factor (VEGF) in lung angiogenesis and the protection of alveolar damage from hyperoxia, suggesting important roles in the pathogenesis of BPD (92). The *VEGF* 460C allele was associated with a dose-dependent reduction (OR 0.147, 95% CI 0.03–0.86,

$p=0.048$ ) in BPD risk in a cohort of 181 preterm infants, while the T allele had an increased risk of 9% (95% CI 2–14%) above baseline risk, after accounting for sex, GA, and length of supplemental oxygen therapy (32).

Hartel et al. (22) studied several coagulation factors, including factor V Leiden, prothrombin G20210A, factor VII-323 del/ins polymorphism, and factor XIII Val-34Leu polymorphisms. They found that among 1008 preterm infants, BPD was less prevalent in carriers than noncarriers (8.3% versus 14.6%,  $p=0.011$ ) of the factor XIII Val34Leu polymorphism. This polymorphism has been shown to be associated with alterations in the fibrin clot that protects against infarction and thrombus propagation. The authors suggested that the allele may be protective against BPD.

#### 41.3.5 Genes Related to Inflammation and BPD

Interferon gamma (IFN- $\gamma$ ) is a cytokine critical to both innate and adaptive immunity, and functions as the primary activator of macrophages, in addition to stimulating natural killer cells and neutrophils. Bokodi et al. hypothesized that functional variants of IFN- $\gamma$  influenced the risk for various perinatal complications involving immature immune responses (73). The authors found that carriers of the IFN- $\gamma$  T(+874)A allele required less oxygen and ventilatory support than those infants that did not, and that the allele protected against the development of BPD (OR 0.35, 95% CI 0.12–0.99,  $p=0.049$ ).

Bronchoalveolar lavage (BAL) samples from infants with BPD contain many inflammatory mediators (82). Interleukin 4 (IL-4) is a potent anti-inflammatory cytokine that inhibits the production of pro-inflammatory cytokines and chemokines by monocytes and macrophages that are found in BAL samples, suggesting that IL-4 may have a protective effect on the pathogenesis and progression of BPD. Lin et al. (93) investigated activity-altering IL-4 polymorphisms among 224 infants with RDS, but did not find any significant difference between those who developed BPD and those that did not. Interleukin 10 (IL-10) is another inhibitor of pro-inflammatory cytokines that plays a role in the inflammation and apoptosis associated with BPD. Yanamandra et al. (44) could not find any association with the presence of a low-production-related *IL-10* SNP in 294 VLBW infants and the later development of BPD. In addition, Bokodi et al. were unable to correlate any polymorphisms of multiple inflammatory cytokines including IL-1 $\beta$ , IL-6, IL-10, and IL-12, all known to be associated with lung pathology, or BPD or duration of ventilation (36).

Monocyte chemoattractant protein-1 (MCP-1) has been shown to be elevated in the BAL fluid of infants who developed BPD (94), but polymorphisms in the gene investigated by both Adcock et al. and Bokodi et al. (36,45) were associated with neither MCP-1 concentrations nor BPD. Within the innate immune system,

mannose-binding lectin (MBL2) facilitates the activation of the complement system. As infection is a risk factor for the development of BPD, variants of *MLB2* that decrease protein serum levels and function have been shown in two independent studies to influence the risk of BPD (95,96). Macrophage migration inhibitory factor (MIF) is a cytokine that has been shown to promote murine fetal lung development. Prencipe et al. (97) evaluated the *MIF* promoter polymorphism G(-173)C in 103 preterm infants for association with the risk of BPD. This polymorphism, which leads to increased MIF production, was associated with a lower incidence of BPD (OR 0.2, 95% CI 0.04–0.93,  $p=0.03$ ).

Transforming growth factor beta (TGF- $\beta$ ) is an anti-inflammatory molecule that stimulates the proliferation of fibroblasts and smooth muscle cells. While increased expression of TGF- $\beta$ 1 can lead to pathophysiology that mimics BPD in a neonatal mouse model (98), a noncoding polymorphism associated with decreased in vitro production was found to have no effect on the development of BPD in a study of 178 VLBW infants (45). Additionally, Atac et al. (46) prospectively studied the prevalence of the G(915)T variation, known to be associated with decreased TGF- $\beta$  protein levels, in a group of 192 preterm infants, 98 of whom developed BPD. The variation was found to be in Hardy–Weinberg equilibrium in both subjects who developed BPD and those who did not.

Tumor necrosis factor (TNF) plays a principle role in the inflammatory cascade and has been found to be elevated in the BAL fluid of infants who subsequently develop BPD (99). However, when Adcock et al. (45) investigated an SNP related to increased in vivo levels of TNF- $\alpha$  in 178 VLBW infants, they found no difference in the allele frequencies among those infants who developed BPD and those who did not. Likewise, a study by Kazzi et al. (47) showed similar allelic frequencies of TNF- $\alpha$  and TNF- $\beta$  SNPs among infants with and without BPD. The study also suggested that the number of hypofunctional adenine alleles in the TNF $\alpha$ -238 position was inversely correlated with the severity of BPD ( $r=-0.341$ ,  $p=0.003$ ). However, further studies of independent cohorts did not replicate these findings (36,48). Ultimately, a meta-analysis of 804 preterm infants by Chauhan et al. (100) showed that the hypofunctional 308A SNP of TNF- $\alpha$  was not a significant susceptibility factor for BPD (OR 1.04; 95% CI 0.85–1.25).

### 41.3.6 Antioxidant Genes and BPD

Dystroglycan is an extracellular matrix receptor involved in adhesion. This protein plays an important role in wound repair of epithelium, and thus may play a role in the progressive impairment of wound repair capacity that occurs in BPD. Concolino et al. (101) investigated the genotypes of 33 preterm infants and showed that the N494H homozygous genotype was associated with BPD ( $p=0.033$ ).

Glutathione S transferase (GST) is an important enzyme for cellular defense against ROS. SNPs in GST genes have been shown to alter the efficiency of reducing oxidative toxins. A pilot study of 35 BPD cases with 98 controls by Manar et al. (102) showed that BPD patients were less likely to have the more efficient valine/valine isoform and more likely to have the less efficient isoleucine isoform (OR 4.5, 95% CI 1.0–20.7,  $p=0.05$ ). The study was limited in size, resulting in marginal  $p$  values and wide confidence intervals.

### 41.3.7 Growth Factors and BPD

Insulin-like growth factor-I (IGF-I) and its receptor (IGF-IR) mediate both prenatal and postnatal lung growth. Hellstrom et al. (103) reported that low serum IGF-I was associated with BPD in 22 VLBW subjects. Chetty et al. (104) showed that while IGF-I and IGF-IR levels are low in fetal lung tissue, they are upregulated in RDS and BPD. Balogh et al. (63) found no association between a noncoding SNP in *IGF-1R* and BPD in 132 LBW infants, though the methods relating serum IGF-1 levels to the SNP have been called into question by further studies (5).

### 41.3.8 Miscellaneous Genes and BPD

Exposure to hyperoxia has been found to increase the levels of alternatively spliced isoforms of matrix metalloproteinase 16 (MMP16), a protein that plays a role in both lung embryogenesis and repair. After adjusting for BW and ethnic origin, the TT genotype MMP16 C/T (rs2664352) and the GG genotype of MMP16 A/6 (rs2664349) were protective for BPD and associated with decreased levels of the protein in tracheal aspirates of 284 infants born less than 28 weeks GA (105). Urokinase is an enzyme that participates in the stimulation of fibrinolysis and degradation of basement membrane glycoproteins. A 3'-UTR polymorphism was not found to be associated with BPD in a study by Lin et al. (106) of 204 ventilated infants born at less than 30 weeks GA.

### 41.3.9 Summary of BPD

Support for the genetic basis of BPD derives from two studies of twin pairs, showing heritability of 53–82%, depending on severity and definition of disease. A total of 25 candidate genes have been studied to date in variable numbers of subjects, but only 3 have been independently validated (*ACE* (17–19), *MBL2* (95,96), and *SP-B* (57,59–61,90)). Given that the ORs reported for any candidate gene was less than 2.0, it is likely that any combination of the known candidates accounts for less than 5% of the total variance. Of the 25 candidate genes, 18 were in the inflammatory cascade (mostly interleukins or interleukin modulators), and 7 were related to apoptosis. The choice of these genes was guided by what has been observed and described in pathologic specimens.

More expansive genome-wide studies beyond inflammation and apoptosis will likely identify key genetic factors not currently appreciated by conventional candidate approaches.

## 41.4 PATENT DUCTUS ARTERIOSUS

In fetal life, the ductus arteriosus is a patent vessel that diverts blood from the pulmonary artery to the aorta to reduce pulmonary blood flow. Failure to promptly close this muscular artery after birth and establish a mature circulatory pattern is termed persistent or PDA. A PDA frequently complicates the course of preterm infants. In preterm newborns, the PDA remains patent due to excess prostaglandin and nitric oxide sensitivity, the response to inflammatory mediators, and relative resistance to local hypoxia-ischemia (107). A review by Bokenkamp et al. (108) details further animal models of PDA that utilize targeted gene deletions to derive deeper insight into the complex physiology of the closing process.

With the increasing rates of preterm delivery, PDA is now the most common form of congenital heart disease in the newborn period (109), accounting for up to 13.7% of all cases of congenital heart disease (110). The persistence of the ductus in preterm infants, while physiologic in the setting of young GA, is not physiologic to the pulmonary-dependent circulation of the delivered neonate, and thus contributes to significant neonatal morbidity and mortality (111,112). The incidence of PDA in infants with a GA of 24, 25, and 26 weeks was 76.9%, 69.5%, and 61.5%, respectively (113). Risk factors for PDA include prematurity and presence of RDS (109).

### 41.4.1 Heritability of PDA

The contribution of genetic factors to the incidence of PDA in premature infants is increasingly being recognized. In a heritability study on premature twins, Bhandari et al. (114) modeled the genetic and non-genetic factors that contributed to the concordance of PDA among 333 DZ twin pairs and 99 MZ twin pairs from two different institutions. They showed that 76.1% of the variance was attributable to familial factors (genetic plus shared environmental components; 95% CI 62.5–89.8%,  $p=0.001$ ) and that heritability was 12.3%, though not statistically significant (95% CI 0–98%,  $p=0.779$ ).

### 41.4.2 Candidate Genes

There are few studies that investigate solely the effect of single-gene polymorphisms on a preterm child's risk of developing PDA. Derzbach et al. (115) studied the risks for various perinatal conditions related to the estrogen receptor- $\alpha$  gene *ESR1* PvuII marker locus, since premature withdrawal of maternal estrogens has been shown to play a role in preterm morbidity. They reported that

in a group of 141 low birth weight (LBW) neonates, male infants with the p-allele were less likely to have PDA (OR 0.24, 95% CI 0.05–0.97,  $p<0.05$ ). There was no effect of the p-allele on female infants. Altered immune regulation of both the adaptive and innate systems has also been implicated in a host of perinatal morbidities. Bokodi et al. (73) investigated the role of IFN- $\gamma$  and IL-12, which are regulators of macrophages and natural killer cells. In a study of 153 LBW newborns, they showed that the INF- $\gamma$  T(+847)A allele had a protective effect on the development of PDA (OR 0.43, 95% CI 0.19–0.97,  $p=0.043$ ).

In a study more tailored to the specific processes that contribute to PDA, Dagle et al. (116) studied 130 genes linked to the regulation of smooth muscle contraction, xenobiotic detoxification, and inflammation. They tested 377 SNPs in a cohort of 240 premature infants less than 32 weeks GA, and initially identified seven SNPs significantly associated with PDA. The seven SNPs were then tested in an additional 162 subjects, along with the angiotensin II type I receptor variant previously studied by Treszl et al. (117) in relation to indomethacin treatment of full-term infants with PDA. In the second analysis, only two genes remained significantly associated with PDA: the neural crest gene responsible for Char syndrome, called transcription factor AP-2 $\beta$  ( $p=0.003$ ), and a gene that controls NF- $\kappa$ B-mediated inflammation and apoptosis called *TNF-receptor-associated factor 1* ( $p=0.005$ ). Haplotype analysis showed that genetic variations in prostacyclin synthase, which produces the potent vasodilator prostaglandin I<sub>2</sub>, were also associated with PDA ( $p=0.01$ ). Notably in the second analysis, the angiotensin II type I receptor variant was not found to be significantly associated with PDA.

### 41.4.3 Meta-analysis

Treszl et al. (75) used a random forest technique to reanalyze previously published data on 24 SNPs studied in 135 preterm infants. Using relative importance scores, they showed that when including SNP information for *VEGF*(+405)G, *ACE* D, *Ang*(-35)C, and IL-12 p40 GC (all  $IS>1.5$ ,  $p>0.06$ ), the accuracy of predicting the occurrence of PDA improved when compared with a baseline prediction utilizing only GA and BW (0.57–0.64).

### 41.4.4 Genes and Response to Intervention for PDA

Further investigations into the pathogenesis and morbidity of preterm PDA have focused on the effects of single-gene variations on the efficacy of medical management. Treszl et al. (117) studied response to medical management with prophylactic indomethacin—a nonselective cyclooxygenase (COX) inhibitor commonly used to treat persistent PDA in preterm neonates—in



159 preterm infants with PDA. They showed that the CC(1166) genotype of the angiotensin II type I receptor gene (rs5186) conferred a lower risk of PDA than the AA or CA genotypes after early indomethacin administration (OR 0.067, 95% CI 0.005–0.821,  $p=0.035$ ). Durmeyer et al. (118) also used genotype to assess response to medical treatment. They studied whether variants of cytochrome P450 (CYP), known to influence the metabolism of the COX inhibitor ibuprofen, influenced how preterm infants responded to ibuprofen therapy. In their cohort of 111 preterm neonates less than 28 weeks GA with hemodynamically significant PDA, neonates with wild-type CYP2C8 or 2C9 alleles were more likely to respond to ibuprofen therapy compared with neonates with variant CYP2C8 and/or 2C9 (73% versus 52%,  $p=0.04$ ). However, when they compared responders (ductus closure;  $n=75$ ) to non-responders (surgical ligation;  $n=36$ ), the only two factors significantly associated with response to ibuprofen using multivariate analysis were greater GA and non-white race; CYP2C variants were not associated with PDA response to ibuprofen.

#### 41.4.5 Summary of PDA

The influence of genetic factors on the risk for PDA has not yet been definitively shown. A heritability study of 432 twin pairs showed that familial factors, which include both environmental and genetic factors, account for up to 76% of the variance in liability for PDA, while a sub-analysis of heritability was not significant likely due to lack of power. Variations of metabolic genes have been studied for their role in determining efficacy of medical management, though these SNPs were not uniformly shown to significantly affect the outcome of intervention. Studies of genes related to estrogen signaling and immune regulation identified variations that effect the risk for PDA, while a directed query of 377 polymorphisms uniquely selected for their possible role in the pathogenesis identified only three variations (AP-2 $\beta$ , TNF-receptor-associated factor 1, and prostacyclin synthase) that were significantly associated with the presence of PDA. Finally, random forest analysis suggested that, at the very least, certain polymorphisms may be useful in predicting the occurrence of PDA.

### 41.5 INTRAVENTRICULAR HEMORRHAGE

IVH is a major cause of acute brain injury in preterm infants, and can contribute to significant developmental disability and adverse outcomes. The subependymal germinal matrix is a temporary structure, yet critical to the production and maturation of nerve cells in the developing brain. The vasculature that supplies these tissues lacks the mature structure and stability of more permanent vessels, and thus is prone to rupture. IVH occurs during the first 1–3 days after birth, when these vessels

are most delicate (119). The severity of IVH is graded from I to IV, depending on characteristics such as acute distension of the cerebral ventricular system with blood and parenchymal venous infarction. The inflammation, blood pressure instability, and hypoxia associated with preterm birth are factors that are believed to be contributing factors (120).

IVH occurs almost exclusively in infants less than 32 weeks GA or below 1500 g, and is inversely associated with GA. Approximately 25% of infants born below 1500 g develop some form of IVH (120), though as the severity of prematurity increases, the proportion of higher grades of IVH increases (121). A recent large survey of preterm infants demonstrated that overall incidence of IVH was 11, 4, 7, and 5% for grades I, II, III, and IV, successively (83). Risk factors for IVH include young GA, male sex, unstable blood pressure with reperfusion injury, NEC, maternal chorioamnionitis, as well as respiratory complications such as RDS, hypercapnia, and pneumothorax. Antenatal steroids have been shown to decrease the risk (120). The long-term effects of IVH are related to the severity of the hemorrhage; up to three-quarters of children with a history of grade III or IV IVH develop cerebral palsy and/or major intellectual disabilities (120).

#### 41.5.1 Heritability of IVH

In addition to the strong association of GA and environmental factors with the development of IVH, studies of twins have quantified the familial (genetic plus shared environmental) components. An early study of 70 preterm twins by Viscardi et al. (122) showed significant effects of twinship and birth order on the development of IVH. A larger study by Bhandari et al. (86) of 450 preterm twin pairs showed that shared environmental and genetic factors accounted for 41.3% (95% CI 7.0–75.6%,  $p=0.02$ ) of the variance after adjusting for confounders. However, subset analysis of 252 twin pairs for which zygosity data was known did not show significantly different rates of concordance between MZ and DZ pairs, likely due to lack of power. The Bhandari study also confirmed that RDS (OR 3.59, 95% CI 1.85–6.97,  $p=0.001$ ) and GA (OR 0.85, 95% CI 0.73–0.98,  $p=0.02$ ) were significant independent variables for IVH (86). In a small study of 41 twin pairs, Perlman et al. (123) also did not find a difference in concordance for IVH between MZ and DZ twins.

#### 41.5.2 Candidate Genes

There are multiple overlapping factors and genetic pathways that likely contribute to the pathogenesis of IVH. These include angiogenesis and vascular pathology, neonatal cerebral blood flow, inflammation/infection, defense from reactive oxide species, and coagulation/thrombophilia mutations (120). Several studies have looked into genetic variations in these pathways that could predispose a preterm infant to IVH.

### 41.5.3 Hemostasis Genes and IVH

Consensus has not yet been reached on whether hemostasis gene mutations predispose preterm infants to IVH. Hartel et al. (22) investigated factor V Leiden, prothrombin G20210A, factor VII 323 del/ins polymorphism, and factor XIII Val34Leu polymorphisms among 1008 preterm infants, and found that, contrary to other reports, the frequencies of these polymorphisms were not higher in IVH compared to preterm controls. Another large study of 305 preterm neonates by Göpel et al. (23) found that the overall incidence of IVH was equivalent between those infants with factor V Leiden or the prothrombin G20210A mutation compared with controls (18.6% versus 16.4%). However, two factor V Leiden case-control studies have demonstrated increased risk of IVH. The first study by Petäjä et al. (24) of 51 preterm infants found that newborns with IVH were more likely to be carriers of the factor V Leiden Gln506-FV polymorphism than the general population, with an OR of 5.9 for being a carrier (95% CI 1.7–20.3,  $p=0.013$ ). They estimated that the absolute risk of IVH in an infant with factor V Leiden and less than 30 weeks GA was 80%, versus 14% among all infants born before 30 weeks GA. In the second case-control study, Komlosi et al. (25) found that within their group of 130 preterm infants, 10% of infants with grade I IVH had factor V Leiden (G1691A) mutation, versus 4.8% of controls. A separate case report by Ramenghi et al. (124) linked thrombophilic mutations such as in factor V Leiden with germinal matrix IVH (grade I). This report described a 24-week fetus with both factor V Leiden and methylenetetrahydrofolate reductase mutations, with severe ventriculomegaly, and IVH detected via intrauterine magnetic resonance imaging. This rare finding of prenatal IVH suggested a possible role of these mutations in the pathogenesis of preterm IVH.

Aronis et al. (28) studied the prothrombin G20210A mutation individually, and determined that it was more prevalent in a cohort of 17 newborns with IVH than in 38 similarly aged infants without IVH (12% versus 2%), though the difference was not statistically significant.

Göpel et al. (27) studied the factor XIII Val34Leu polymorphism in 531 preterm infants and full-term controls. They found that this polymorphism, known to be associated with decreased risk of adult brain infarct and increased risk of adult intracerebral hemorrhage, was associated with an increased rate of IVH (14.3% in carriers versus 10.1% in infants without the mutation,  $p=0.17$ ) but a decreased risk for isolated IVH and white matter disease in neonates (OR 0.3, 95% CI 0.1–0.7,  $p=0.005$ ). This study suggested that while a weaker clot can increase the rate of IVH (not statistically significant), the pathogenesis of white matter disease subsequent to IVH may rely, at least partially, on the post-hemorrhagic ischemia caused by the clots.

Collagen IV A1 (*COL4A1*) contributes one of the six alpha chains that together form collagen IV, a

critical component of basement membranes. Mutations in *COL4A1* have been shown to result in vascular damage and IVH in neonatal mice and porencephaly (a sequela of white matter damage in grade IV IVH) in human infants (125). Bilguvar et al. described a heterozygous *COL4A1* insertion mutation in 24-week DZ twins who developed grade III and grade IV IVH by day 2 of life (126). In addition, de Vries et al. (127) described a pair of siblings born at 33 and 31 weeks GA, both with antenatal IVH and the same deleterious *COL4A1* mutation inherited from their asymptomatic mother.

### 41.5.4 Genes Related to Inflammation and IVH

The preterm neonate is exposed to environmental insults that stimulate inflammatory factors that may increase the risk of neuronal damage. Variations known to enhance the inflammatory response have been associated with increased risk for developing IVH. A previously discussed study by Adcock et al. (45) investigated the role of variations in inflammatory cytokines in the development of BPD in a group of 178 ventilated preterm infants. While they did not find any significant associations with BPD, they showed that infants with the TNF- $\alpha$  promoter-308A allele (known to increase TNF- $\alpha$  production) had increased risk of IVH (relative risk (RR) 2.07, 95% CI 1.02–4.18,  $p=0.041$ ). Heep et al. (49) studied variations of TNF- $\alpha$  and TNF- $\beta$  in 27 preterm infants less than 32 weeks GA with severe IVH, compared to 102 healthy newborns greater than 32 weeks GA. They found that the male subjects had a significantly higher prevalence of the homozygous genotype (2/2) for the NcoI TNF- $\beta$ 2 allele ( $p<0.001$ ), which has been shown to be a marker for increased TNF- $\alpha$  expression. Notably, there were no significant findings to associate the TNF- $\alpha$  promoter-308 polymorphism with IVH. Another study of the inflammatory cytokine IL-1 $\beta$  by Baier et al. (37) in 215 ventilated preterm infants, found that the *IL-1 $\beta$* -511 T allele (known to increase IL-1 $\beta$  production) was associated with an increased risk for IVH (OR 3.0, 95% CI 1.4–6.4,  $p=0.003$ ).

Further investigations into the role of variations of the pro-inflammatory, neurocytopathogenic cytokine IL-6 have produced contradictory reports. Increased IL-6 production has been associated in neonates with the genotype *IL-6*-174CC. In a study of 148 preterm white infants from Britain, Harding et al. (40) showed that the CC genotype was associated with increased odds of IVH (OR 3.5, 95% CI 1.0–12.2,  $p=0.038$ ), as well as white matter disease and disability. Göpel et al. (41) refute prior studies on the connection between IL-6 variants and risk for IVH. They found that among a larger sample size of 1206 preterm white infants from Germany, the frequency of IVH was statistically equivalent between the three major genotypes at the 174 position. Harding et al. investigated the role of variants at the 572 position of the

IL-6 gene (42) as well. As with position 174, the C allele was associated with higher levels of IL-6. In this study of 113 preterm neonates, they found that although there was decreased long-term cognitive ability in children with the rare *IL-6-572C* allele, there was no increased rate of IVH or white matter disease.

IL-4 has been shown to modulate cytokine production in astrocytes, and increased concentrations can be seen in adult cerebral infarctions. The *IL-4 C(-590)T* variant is associated with increased IL-4 production. Yanamandra et al. (128) showed that the -590 T allele was associated with an increase in severe IVH in 235 preterm black infants from the USA.

Nitric oxide, a product of the endothelial NO synthase (*eNOS*) gene associated with anti-inflammatory and vasodilatory effects, has been found in decreased levels among infants with respiratory conditions and IVH. In a study of 124 preterm black infants from the USA, Vannemreddy et al. (129) showed that infants with IVH and either BPD or RDS had a twofold increase in the -786C mutation (OR 2.3,  $p=0.04$ ), though they did not present a subgroup analysis.

Derzbach et al. (115) tested a polymorphism of the estrogen receptor- $\alpha$  gene *ESR1* PvuII marker locus, due to the connection between premature withdrawal of maternal estrogens and preterm morbidity. In a study of 141 preterm neonates, male infants with the pp genotype had greater risk for IVH (OR 4.39, 95% CI 1.15–16.82,  $p<0.05$ ). There was no effect of the pp genotype on female infants.

### 41.5.5 Summary of IVH

The pathogenesis of IVH has both environmental and genetic elements. Twin studies have generally supported the familial role in determining the risk for IVH, although zygoty data has not yet shown a significant difference in concordance between MZ and DZ pairs, likely due to low power. At least 12 candidate genes have been proposed to play a role in IVH risk. Variants associated with altered coagulation states have been studied with variable results, perhaps reinforcing the nuanced view of IVH as a balance of both infarction and hemorrhage. Studies of inflammatory pathway variants showed contradictory results, but likely play a role. Studies of larger numbers of subjects with sufficient power to stratify for sex, demographics, and GA, will help to delineate the heritability of IVH and identify associated alleles.

## 41.6 RETINOPATHY OF PREMATURITY

Retinopathy of prematurity (ROP), a complication of preterm birth, is a leading cause of impaired vision and blindness in children. Retinal development depends on physiologic hypoxia to stimulate the production of angiogenic factors, such as VEGF and IGF-1, that promote maturation of the retinal vessels. After preterm birth, the hyperoxic environment halts angiogenesis so

that portions of the retina remain relatively avascular. A sharply demarcated boundary between the vascular and avascular retina is the definition of stage 1 ROP, and this becomes an elevated ridge in stage 2. However, as the preterm infant matures, IGF-1 levels rise and permit VEGF to stimulate a new wave of rapid and excessive angiogenesis. This neovascularization creates extra-retinal vessels, which can invade the vitreous (stage 3) or become dilated and tortuous (plus disease). Untreated, the condition can progress to partial (stage 4) or total (stage 5) retinal detachment and severe visual impairment (130).

In the last two decades, many centers have reported a decline in the incidence of ROP, despite an increase in the total numbers of surviving preterm infants. However, ROP has become an epidemic in some countries that provide quality care to preterm infants but lack sophisticated ophthalmologic resources (130). The risk for developing ROP is closely associated with BW and GA. Most infants born prior to 28 weeks GA develop some form of ROP, which can be seen on ophthalmologic examination by 34 weeks GA. Infants born greater than 32 weeks GA are not generally at risk for ROP, while infants born greater than 28 weeks GA will generally develop ROP but not require treatment (131). A recent population-based cohort study showed that the incidence of severe ROP was 10% in preterm infants with less than 32 weeks GA, though only 36% of cases were stage 3 or greater (132). Another multicenter study estimated the incidence of ROP at 68% in infants born less than 1251 g, and 93% in infants born less than 750 g (133). As GA decreases, the incidence and severity of ROP increases. Postnatal risk factors for ROP include IGF-1 levels, postnatal weight gain, sepsis, bradycardia, RDS, BPD, anemia, and IVH (131), but these measures of a “sick baby” apply most significantly to larger infants with older GA (134). Increased arterial oxygen tension also contributes to the development of ROP, due to the adverse effects of relative hyperoxia on the stimulation of normal retinal vessel development (131).

### 41.6.1 Twin Studies

While ROP is a multifactorial disease, there have been several studies suggesting that the risk for ROP is based in part on genetic factors. Variability in the incidence of ROP among different races, sex, and countries is well described and summarized in a review by Holmstrom (135). In addition, genetically modified animal models of ROP (135) emphasize the role of single-gene malfunctions in the pathogenesis. Twin studies of ROP have suggested a strong genetic component to the disease. In 56 twin pairs, Azad et al. (136) showed that 80% of the twin pairs had identical severity in the most severely affected retina, though these findings in twins did not delineate shared environment factors or shared alleles.



### 41.6.2 Heritability of ROP

Bizzarro et al. (137) compared concordance for ROP in 63 MZ and 137 DZ twin pairs in a multicenter study. They showed that GA (OR 0.65, 95% CI 0.45–0.94,  $p=0.024$ ) and duration of oxygen use (OR 1.03, 95% CI 1.01–1.05,  $p=0.003$ ) were significant risk factors for ROP, independent of BPD status. After controlling for both GA and environmental factors, they showed that the heritability was 70.1% (95% CI 9–100%,  $p=0.026$ ).

### 41.6.3 Candidate Genes

Candidate gene studies have focused on known mediators in the pathophysiology, particularly those involving angiogenesis in the developing retina and those linked to genetic syndromes with altered retinal vascularization.

### 41.6.4 Genetic Heterogeneity

Norrie disease is a rare X-linked disorder, due to a deficiency in the Norrie protein, that causes retarded retinal vascularization and a blinding retinopathy (135). There are conflicting results regarding the association of the Norrie disease gene and ROP, possibly due to ethnic differences between study populations. Small studies have identified Norrie mutations in 3–25% of preterm infants with ROP (138–140), although the largest study of 143 subjects by Hutcheson et al. reported that the prevalence of Norrie polymorphisms in infants with severe ROP was not significantly higher than the general population (141), concluding that the polymorphisms did not appear to be a major causative factor in the pathogenesis. Kim et al. (142) did not identify a single Norrie polymorphism among 18 Korean preterm infants with ROP. Additionally, Haider et al. (143,144) found no association between Norrie mutations and ROP in groups of 102 and 210 premature Kuwaiti infants, though a later study (145) of the same cohort of 210 infants showed a 5'-UTR polymorphism in the Norrie gene (C(597)A) that was associated with severe ROP. The AA genotype was found in 83.3% of severe ROP cases, 0% of spontaneously regressing ROP, and 10.4% of control cases ( $p<0.0001$ ). Dickinson et al. (146) identified deletions within the CT repeat region of the Norrie gene in three subjects with regressed ROP in a cohort of 31 Australian preterm infants with ROP and 91 preterm controls. The authors concluded, however, that these deletions did not contribute to severe ROP in their cohort.

Familial exudative vitreoretinopathy (FEVR) is another disorder with a pathogenesis similar to ROP. FEVR is a genetically heterogeneous bilateral eye disease that arises from deficient retinal vascularization in full-term infants. Mutations in the Norrie disease genes cause an X-linked form of FEVR. Four genes along the beta-catenin-mediated Wnt signaling pathway (*NDP*, *FZD4*, *LRP5*, and *TSPAN12*) have been implicated in retinal

vasculogenesis and in the pathogenesis of non-X-linked FEVR. Several studies of preterm infants have shown that a small percentage with severe ROP (3–11%) have variants in the same genes that can lead to FEVR, suggesting that alterations in the Wnt pathway may play a role in the abnormal retinal angiogenesis observed in both conditions (147).

### 41.6.5 Vascular Genes and ROP

Variations of the *VEGF* gene that affect protein production have been described in ROP. Cooke et al. (35) studied *VEGF* variants in 91 preterm infants with treatment-threshold ROP, and found that a significant number of ROP infants had the *VEGF* G(-634)C polymorphism (position also referred to as +405) compared with 97 controls without disease ( $p=0.03$ ). In addition, they noted that the G allele was a significant independent predictor of progression to threshold ROP ( $p=0.008$ ) and that infants with the GG genotype, associated with increased *VEGF* expression, were twice as likely to have threshold ROP (OR 2.0, 95% CI 1.11–3.69). Vannay et al. (29) studied 86 preterm infants that had been treated for ROP, and compared them with 115 preterm infants with ROP that did not require therapy. They found that the -634 C allele was significantly more common among infants with severe treatment-threshold ROP, doubling the risk for heterozygotes (-634 C OR 2.00, 95% CI 1.02–3.92,  $p=0.04$ ) and tripling the risk for homozygotes respectively (-634 C/C OR 3.37, 95% CI 1.17–9.65,  $p=0.007$ ). In addition, the 460TT/634CC haplotype was more prevalent in the treated patients (15% versus 1%,  $p<0.001$ ), and the association remained significant when risk factors such as GA, supplemental oxygen use, and sex were incorporated into the model. Kwinta et al. (33) also studied *VEGF* genotypes in 161 preterm infants, divided into three groups based on ROP severity: none, mild without treatment, and severe requiring treatment. The -460 T allele was overrepresented in the ROP treatment group compared to the group without ROP (54.2% versus 42.6%, OR 1.63, 95% CI 1.03–2.55).

However, these *VEGF*-ROP associations were not replicated in other studies. Shastry et al. (34) found no significant difference in the frequencies of *VEGF* G(-634) C and C(-460)T alleles in 61 preterm infants with advanced ROP compared to 61 full-term adult controls. The Kwinta study (33), discussed above, showed no association between the *VEGF* G(-634)C allele and ROP. In a study of 200 preterm infants, Banyasz et al. (30) did not find an association between the *VEGF*-2578 A allele and ROP, but their data suggested that -2578 A allele may affect severity in preterm males ( $p=0.044$ ) (30).

In another example of conflicting results, an *ACE* gene insertion/deletion (I/D) polymorphism was shown to play a role in ROP in a study of 181 preterm Kuwaiti infants by Haider et al. (20). They found that the I/I



genotype was more prevalent in ROP cases compared to non-ROP controls (24% versus 7%,  $p < 0.001$ ). However, the prevalence of the D/D genotype, associated with higher serum ACE concentration, was higher in ROP cases needing therapy (63%) compared with ROP cases that spontaneously regressed (63% versus 36%,  $p < 0.04$ ). The investigators postulated that the I allele increased risk for ROP, while the D allele and the higher levels of ACE increased risk for retina damage and neovascularization. In contrast, Spiegler et al. (21) studied the ACE I/D variant as well as the AGTR1 1166 A/C polymorphism in a cohort of 1100 preterm infants and found no significant associations with ROP.

In a study of 455 SNPs from 153 genes in 347 infants less than 32 weeks GA, Mohamed et al. (148) identified five genes associated with ROP with  $p$ -values less than 0.01, including the T(+867)G (rs427832) allele of AGTR1 ( $p = 0.005$ ), in support of Haider et al. The other four genes were: Indian hedgehog (IHH), related to vasculogenesis ( $p = 0.003$ ); T-box transcription factor of mesoderm (TBX5;  $p = 0.003$ ); cholesterol ester transfer protein (CETP;  $p = 0.004$ ); and glycoprotein 1b alpha polypeptide (GP1BA), a platelet surface protein ( $p = 0.005$ ). None of these five associations, however, withstood correction for multiple testing. In a multiple logistic regression analysis controlled for significant clinical risk factors, the Mohamed (148) study also showed that two variants of complement factor H (CFH) conferred protection against ROP (T allele for both variants,  $p = 0.01$ ). Both CFH variants were previously associated with neovascularization in macular degeneration. In addition, a polymorphism of the EPAS1 gene, a vascular endothelial cell transcription factor that activates VEGF function in response to hypoxia, was associated with ROP in the logistic regression analysis ( $p = 0.001$ ), while another EPAS1 polymorphism was identified through a family-based analysis strategy ( $p = 0.007$ ).

eNOS plays a central role in retinal angiogenesis and vasculogenesis. Rusai et al. (149) investigated the role of known functional polymorphisms of eNOS in the pathogenesis of ROP. In a study of 105 premature infants with treated ROP and 127 preterm infants with stage I/II ROP, they found that the genotype distribution of the eNOS 27-bp repeat polymorphism (b/a) was significantly different ( $p = 0.015$ ), and a multiple logistic regression analysis showed that male sex ( $p = 0.046$ ) and eNOS aa genotype ( $p = 0.047$  versus ab and  $p = 0.022$  versus bb) were significantly associated with severe ROP. Other studies have not shown any association between ROP and polymorphisms of TNF- $\alpha$ , TGF- $\beta$  (35), IGF-IR (62,150), or ANG2 (30,151).

#### 41.6.6 Meta-analysis

In a study by Dunai et al. (152), random forest analysis was used to evaluate whether previously investigated polymorphisms of genes that are thought to contribute to

the pathogenesis of ROP could be predictive of ROP at birth. A cohort of 103 preterm infants with ROP requiring treatment and 134 preterm controls was genotyped for seven polymorphisms (VEGF T(-460)C, VEGF G(-634)C, IGF-IR G(+3174)A, ANGII G(-35)C, ESR1 PvuII Pp, and eNOS 27-bp b/a). The genomic data was combined with birth data, and the resulting analysis suggested that the most important predictors of ROP were prematurity, LBW, intrauterine growth retardation, and Apgar scores. The polymorphisms were significantly less important than GA, LBW, intrauterine growth retardation, and Apgar scores for predicting the risk of treatment-threshold ROP.

#### 41.6.7 Summary of ROP

The genetic basis of ROP is supported by severity and heritability studies of 250 twin pairs. One study of 63 MZ and 137 DZ twins showed a heritability of 70%. Candidate gene studies have further investigated the role of genetic polymorphisms on the pathogenesis and risk. Genetic disorders with pathology similar to ROP have directed studies that have described associations between Norrie and FEVR mutations with ROP, although these findings were not reproduced in other studies. Similarly, some studies of genes related to vasculogenesis, including VEGF and angiotensin, identified associations with ROP but were not confirmed in other studies. A larger study of 455 SNPs in 153 candidate genes identified five polymorphisms associated with ROP, but they did not withstand correction for multiple testing. A meta-analysis of seven variants from vascular (VEGF, ANGII, eNOS), inflammation (IGF-IR), and estrogen receptor (ESR1) genes showed that polymorphisms of these genes were significantly less important than GA, LBW, intrauterine growth retardation, and Apgar scores for predicting the risk of treatment-threshold ROP.

### 41.7 NECROTIZING ENTEROCOLITIS

NEC is a common complication of prematurity and a cause of significant morbidity and mortality. The gastrointestinal tract of the preterm infant is immature in many ways: intestinal motility and absorption, barrier defenses, immune function, and mesenteric circulatory regulation. While the mechanism remains unknown, it is thought that these factors combine to result in a constellation of symptoms called NEC. Early signs of NEC include abdominal distention, feeding intolerance, and bloody stools, usually within the first 8–10 days of life. Radiographic signs progress from dilated loops of stagnant bowel to pneumatosis intestinalis and portal venous gas. Late-stage disease is characterized by peritonitis, bowel ischemia, and systemic hypotension. There are several unproven hypotheses that seek to explain the pathogenesis, including hypoxia-ischemia, altered microbial colonization, and intestinal immune dysregulation resulting in excessive inflammatory responses to bacteria (153).

Approximately 7% of infants born between 500 and 1500 g develop NEC, based on studies of large, multicenter neonatal network databases (154,155). This incidence is somewhat increased from prior decades, most likely due to advancements in perinatal care that allow for longer survival of very preterm infants. Many studies have attempted to define determinants of NEC risk; the only consistently identified risk factors are BW and GA. Feeding with human milk has been suggested to be protective, with up to a 10-fold reduction in risk (156). Age of onset varies inversely with GA, and the risk remains high in preterm infants until a GA of 35 weeks (157). The rate of death associated with NEC ranges between 20 and 30%, with higher rates found among infants who underwent surgical treatment (158).

### 41.7.1 Heritability of NEC

In a multicenter retrospective study of 450 preterm twin pairs, Bhandari et al. (86) confirmed that GA was a major determinant of NEC risk (OR 0.82, 95% CI 0.68–0.99,  $p=0.03$ ). After controlling for GA, the shared familial (genetic and environmental) contribution to the risk of NEC was 51.9% (95% CI 33.2–70.6%,  $p<0.001$ ), however the study lacked power to determine heritability.

### 41.7.2 Candidate Genes

Associations with candidate genes have been identified for NEC. Derzbach et al. (115) studied the association of the PvuII pP polymorphism of the estrogen receptor- $\alpha$  gene in a cohort of 141 preterm infants and 167 full-term controls. Male infants with the p allele were found to be at lower risk for NEC than males carrying the P allele (OR 0.24, 95% CI 0.07–0.83,  $p<0.05$ ). Bokodi et al. (73) studied a functional polymorphism of IL-12, which is an IFN- $\gamma$ -stimulating cytokine, in 153 premature infants with NEC compared with a control group of 172 healthy full-term infants. They showed that carriers of the IL-12 p40 CTCTAA allele were at increased risk for NEC (OR 2.37, 95% CI 1.01–5.53,  $p=0.046$ ). Heterozygote carriers of the IL-12 CTCTAA/GC polymorphism were also at increased risk (OR 2.91, 95% CI 1.41–6.02,  $p=0.004$ ). Treszl et al. (39) evaluated the frequency of a functional polymorphism of another cytokine, IL-4 receptor  $\alpha$  (IL-4R $\alpha$ ), in 46 preterm infants with NEC. They found that infants with NEC carried the variant less frequently than 90 preterm controls (12.5% versus 22.4%,  $p<0.05$ ), after adjusting for risk factors, and postulated that infants carrying this allele might have a predominance of Th2 cells that may protect against NEC.

Studies have noted decreased levels of L-arginine in children with NEC. Moonen et al. (159) studied the effect of a carbamoyl-phosphate synthetase 1 (*CPS1*) polymorphism that has been associated with low concentrations of L-arginine in neonates. They analyzed

the *CPS1* T(1405)N polymorphism in a cohort of 17 preterm infants with NEC, 34 preterm infants without NEC, and 25 healthy full-term infants. The incidence of NEC was found to correlate with the number of C alleles ( $p=0.037$ ), and the CC genotype was associated with an increased risk (OR 3.43, 95% CI 1.01–11.49,  $p=0.048$ ).

### 41.7.3 Vascular Genes and NEC

Banyasz et al. (31) studied functional polymorphisms of *VEGF*, which is a potent stimulator of vasculogenesis. In their study of 128 premature neonates and 200 healthy full-term infants, the *VEGF*-2578A allele was a risk factor (OR 2.77, 95% CI 1.00–7.65). Thrombosis of large mesenteric vessels is occasionally noted in some surgical cases of NEC. To investigate if pro-thrombotic genetic conditions play a role in the pathogenesis, Göpel et al. (26) sequenced the factor V Leiden mutation in a retrospective case-control study of 39 preterm infants with NEC. While one case of factor V Leiden was found in an infant noted to have thrombus in the mesenteric vein intraoperatively, the control group also had a single case of asymptomatic factor V Leiden, which coincides with the expected mutation frequency of approximately 5% in the general population.

### 41.7.4 Genes Related to Inflammation and NEC

Henderson et al. (38) studied the association of cytokine polymorphisms in the pathogenesis of NEC. In a multicenter case-control study of 100 preterm infants, they genotyped 10 candidate cytokine SNPs previously associated with infectious and inflammatory disease processes (TNF, IL-1 $\beta$ , IL-4R $\alpha$ , IL-6, IL-8, IL-10, IL-18), but found no significant association with NEC.

### 41.7.5 Meta-analysis

Findings from the Henderson study (38) described above were included in a random effects meta-analysis of prior genetic association studies, which increased the precision of the effect size, but did not identify any further associations (38). Treszl et al. (75) used a random forest technique to reanalyze previously published data of 24 SNPs in 135 preterm infants. Using relative importance scores, they showed that when including SNP information for IL-12 p40 GC, *IL-10*(-1082)C, *VEGF*(-460)C, and *ACE* D (all  $IS>1.7$ ,  $p>0.04$ ), the accuracy of predicting the occurrence of NEC increased when compared to a baseline prediction utilizing GA and BW (0.60–0.70).

### 41.7.6 Summary of NEC

While the familial susceptibility to NEC is close to 50%, studies to date have lacked power to determine heritability. However, polymorphisms of estrogen receptors,

interleukins, and metabolic enzymes have been found to be significantly associated with risk in single unreplicated studies. Investigations seeking to link NEC with the processes of vasculogenesis and coagulation through functional polymorphisms have found significant associations as well. An analysis of previously published SNPs associated with prematurity found that four polymorphisms (IL-12 p40 GC, IL-10(-1082)C, VEGF(-460) C, and ACE D) involving inflammation, vasculogenesis, and angiotensin could be used to improve the accuracy of predicting the development of NEC. An alternative meta-analysis found no statistically significant association between NEC and polymorphisms associated with infectious and inflammatory diseases.

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### Biographies



**Dr Aaron Prosnitz** graduated Magna Cum Laude with Honors in Biology from Brown University in Providence, RI, and then went on to complete a year of genetics research on autism at Mount Sinai School of Medicine in New York City. Afterward, he underwent medical school education at the University of Pennsylvania School of Medicine in Philadelphia, during which he participated in a NASPGHAN Medical Student Summer Research Fellowship. While finishing his first year of a residency in pediatrics at the Yale–New Haven Children’s Hospital in New Haven, CT, he presented original clinical research on pediatric Crohn disease at the PAS/SPR 2011 meeting. Upon completion of his residency training in 2013, he looks forward to entering fellowship to continue his career in academic medicine and research.



**Jeffrey R Gruen, MD**, is Professor of Pediatrics, Genetics, and Investigative Medicine at the Yale University School of Medicine and the Yale Child Health Research Center. He is a graduate of the Tulane University School of Medicine, and completed residency in pediatrics at Yale–New Haven Hospital. He completed fellowship training in neonatal medicine at Yale Medical School, while undertaking scientific training in molecular genetics. During fellowship, Dr Gruen mapped the short arm of human chromosome 6, including the major histocompatibility complex and hemochromatosis locus on 6p21.3. Later he shifted his mapping studies further up chromosome 6 to the *DYX2* locus for dyslexia, where he identified *DCDC2* as an important gene for dyslexia. Currently, the main focus of his lab is the genetics of language acquisition, reading, and dyslexia. In addition to mentoring graduate students and post-docs in genetics, Dr Gruen continues to attend as a neonatologist in the Newborn Intensive Care Unit of the Children’s Hospital at Yale–New Haven.



**Dr Vineet Bhandari** attended medical school at the Armed Forces Medical College, Pune, India, and then went on to do his pediatric and neonatology training at the Post Graduate Institute of Medical Education and Research, Chandigarh, India. He completed residency in pediatrics and fellowship training in neonatal–perinatal medicine at the University of Connecticut. He is board-certified in both pediatrics and neonatology by the American Board of Pediatrics. He is currently Associate Professor of Pediatrics, Obstetrics, Gynecology, and Reproductive Sciences at the Yale University School of Medicine and Director of the Program in Perinatal Research. He is also an attending neonatologist at the Yale–New Haven Children’s Hospital.



# CHAPTER

# 42

## Disorders of DNA Repair and Metabolism

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### 42.1 MENDELIAN PATTERN OF INHERITANCE

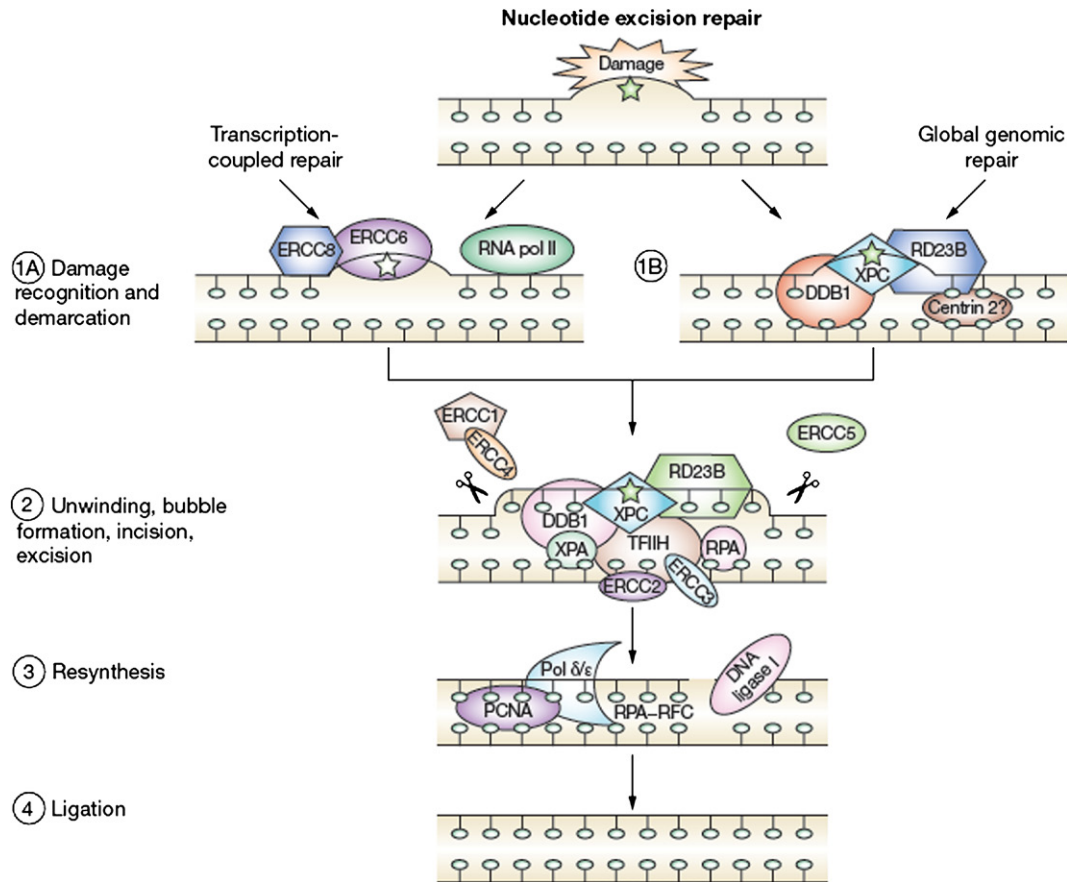
Somatic cells from patients with DNA repair deficiency syndromes often demonstrate an absence of repair activity and thus these syndromes result from autosomal or X-linked recessive mutations. Typically, individuals who inherit a single heterozygous inactivating mutation in a DNA repair gene are healthy and referred to as carriers. Overall, these autosomal recessive disorders that result in susceptibility to cancer are much rarer than the autosomal dominant cancer susceptibility disorders. Patients carrying founder mutations or families with consanguinity are seen frequently.

For some disorders, there can be multiple mechanisms of inheritance leading to a similar clinical phenotype. For example, disorders of telomere maintenance are often lumped together under the term dyskeratosis congenita, but can result from both recessive (autosomal and X-linked) and dominant mutation mechanisms (1). There is not enough data to know if the dominant disorders are secondary to dosage sensitivity for the heterozygous genes, e.g. *TINF2*, or secondary to a dominant negative impact of the heterozygous mutation. Work reported since approximately 2000 has defined a number of cases where distinct dominant and recessive cancer susceptibility syndromes are associated with mutations in the same gene. In these conditions, heterozygous parents have an adult-onset cancer phenotype with incomplete penetrance, whereas the children who inherit homozygous or compound heterozygous mutations (referred to here as biallelic mutations) have a significant cancer risk in childhood and may demonstrate congenital anomalies, developmental delay, and/or dermatologic features. Thus, there are an increasing number of disorders with a mixed model of inheritance where the cancer-susceptibility phenotype depends on whether the patient carries mutations in one or two alleles of the same DNA repair or metabolism gene.

### 42.2 DISORDERS OF NUCLEOTIDE EXCISION REPAIR: XERODERMA PIGMENTOSUM AND COCKAYNE SYNDROME

Xeroderma pigmentosum (XP) was one of the first genetic disorders to be recognized for its underlying DNA excision repair defect (2). Investigators at the US National Institutes of Health have carried out detailed characterization of the clinical phenotype and molecular abnormalities (3). Most striking are features related to cutaneous sensitivity to ultraviolet (UV) light, including very early onset of telangiectasia and freckling (4). The UV-light-exposed areas of the eye, including the cornea, lids, and conjunctivae, are affected with both corneal clouding and ocular malignancies. The clearest demonstration of the increased mutagenesis by UV light is the over three logs of increased risk of basal and squamous-cell skin carcinomas in sun-exposed areas. The age of onset, beginning in very early childhood, is nearly 40 years earlier than the general population (3). Melanoma skin cancers are also increased (approximately 5% lifetime risk). The specificity for increased cancer risk in sun-exposed areas is highlighted by the relatively modest increase in cancer risk for internal malignancies.

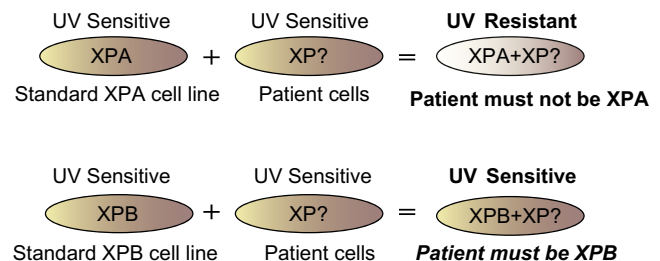
Nucleotide excision repair (NER) is one of the most important mechanisms for correcting the DNA adducts, including thymine dimers, that form after exposure to ultraviolet light (5). Detailed biochemical assays and analysis of XP patients from different complementation groups has allowed discernment of the protein complexes required to complete nucleotide excision repair (Figure 42-1) (6). As is true for a variety of DNA repair disorders, patients who carry mutations in genes that encode proteins that function together as a protein complex often demonstrate a similar clinical phenotype. For a newly diagnosed patient, complementation



**FIGURE 42-1** An outline of the nucleotide excision repair pathway, which includes global genomic repair (1B) and transcription-coupled repair (1A). The damaged base in the DNA is indicated by a green star. In global genomic repair, the damage is recognized by the heterotrimeric complex of XPC, RD23B, and centrin 2, whereas when the damage is in a gene that is being actively transcribed by RNA pol II, the Cockayne syndrome factors ERCC8 and ERCC6 have a crucial role in stalling the transcription process so that repair of the transcribed gene can be initiated. From this point onwards, the repair pathway is common to both mechanisms, and it proceeds by recruiting several other factors, as shown in (2), to effect unwinding, bubble formation of the strand harboring the damage, incision of the strand at discrete points on the 5' and 3' sides, and excision of the fragment containing the damage. The size of the bubble will depend on the nature of damage, which in turn is likely to determine the incision points for the removal of the damaged portion. In step (3), the gap created by the excision of the damaged strand is resynthesized by DNA pol  $\delta/\epsilon$ , with the help of auxiliary factors such as PCNA and RPA-RFC. Finally, DNA ligase I ligates the newly synthesized fragment to the downstream strand to complete the repair process and yield the repaired product (4). DDB1 = xeroderma pigmentosum E; ERCC1 = DNA excision repair protein ERCC-1; ERCC2–5 = excision repair complementing factors (formerly known as XPD/XPB/XPF/XPG); ERCC6/8 = Cockayne syndrome factor B/A; PCNA = proliferating cell nuclear antigen; pol  $\delta/\epsilon$  = polymerase  $\delta/\epsilon$ ; RD23B = RAD23 homolog B (*Saccharomyces cerevisiae*); RFC = replication factor C; RNA pol II = RNA polymerase II; RPA = replication protein A; TFIIH = active transcription complex; XPA = xeroderma pigmentosum A; XPC = xeroderma pigmentosum C. (From Rao, K. S. *Mechanisms of Disease: DNA Repair Defects and Neurological Disease*. Nat. Clin. Pract. Neurol. 2007, 3, 162–172.)

analysis is still used to determine which gene may be defective. Complementation analysis is carried out by fusing fibroblasts from a patient to standardized control cell lines from patients with known defects, and then assaying the fusion cells for restoration of NER (Figure 42-2). Although time-consuming and technically difficult, complementation analysis remains the primary tool used to differentiate the underlying mechanism in many DNA repair disorders because of the ability to test cells from patients for the specific repair defect.

Approximately 20% of XP patients have neurologic problems (3,6). This includes a group of XP patients (often in complementation group A) with neurologic abnormalities described as DeSanctis–Cacchione



**FIGURE 42-2** Complementation assay using UV sensitivity for cells from patients with xeroderma pigmentosum. The patient sample is fused to standard cell lines from each XP subtype. If the fusion cells demonstrate restored UV resistance, then the patient must carry a mutation in another subtype. If fusion cells do not restore UV resistance, then the patient carries mutations in the same subtype. (Modified from Plon, S. E. *Cancer Genetics and Molecular Oncology*. In *Principles of Molecular Medicine*, 2nd ed.; Humana Press, 2006.)

syndrome. In addition to the dermatologic features, patients have early onset progressive neurologic degeneration and immature sexual development. Some complementation group D patients with XP and later onset of neurologic difficulties also manifest brittle hair and ichthyosis, in a condition termed XPD/trichothiodystrophy (TTD). Cockayne syndrome shares some of the UV hypersensitivity of XP, but is also characterized by neurologic deficits, including developmental delay without skin cancer risk. There are at least three complementation groups for Cockayne syndrome. Utilizing knowledge of the specific biochemical defects in cells from patients with either XP or Cockayne syndrome, the genes responsible for this condition (*ERCC2*, 6, and 8) have been cloned (7). In a normal cell, DNA damage in actively transcribed genes is preferentially repaired before DNA from inactive parts of the genome, a process termed transcription-coupled repair (TCR). Cells from Cockayne patients are deficient in TCR, most commonly due to a mutation in the *ERCC2*-encoded DNA helicase, which is also a component of the active transcription complex TFIIH. The neurologic deficits in Cockayne syndrome may be attributed to the high transcriptional load of cells in the nervous system, and thus the central nervous system (CNS) may be more dependent on efficient TCR activity (6); although TCR is deficient the global repair rates are normal in cells from patients with Cockayne syndrome. This may explain the lack of cancer predisposition in this group of patients, which is in stark contrast to the high cancer rates described above for other forms of XP.

Another group of patients classified as XP variant, or XP<sup>v</sup>, demonstrate UV sensitivity but have normal nucleotide excision repair activity when tested in vitro. This disorder is due to mutation of a specialized DNA polymerase,  $\eta$ , belonging to the Y family of DNA polymerases, which function to place two adenine residues opposite a thymine dimer photoproduct, thus restoring the normal base sequence and reducing UV-induced mutagenesis (8).

### 42.3 DISORDERS OF BASE EXCISION REPAIR: *MUTYH* AND COLON CANCER RISK

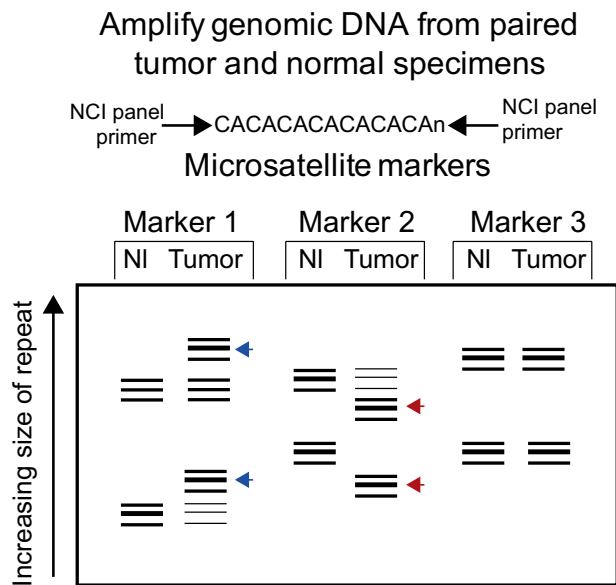
Inheriting mutations in the *MUTYH* base excision repair gene results in a limited range of tissue specificity with regard to cancer risk in the absence of other clinical features. This disorder was discovered through analysis of rare individuals and families in which affected members demonstrate an apparently autosomal recessive form of adenomatous polyposis coli, associated with the development of hundreds of colonic polyps and a high risk of colon cancer (9,10). Elegant work analyzing the tumors from these individuals revealed a somatic mutation pattern consistent with deficiency in base excision

repair, and eventually led to the identification of constitutional biallelic mutations in the *MUTYH* repair gene (also referred to as *MYH*). Specific mutations (Y165C and G382D) in the *MUTYH* gene are quite frequently detected in affected individuals (11). Analysis of cancer rates in individuals who carry one or two copies of these specific *MUTYH* mutations has been performed by a number of investigators. Homozygous or compound heterozygous mutation carriers develop hundreds of adenomatous polyps and have over 100-fold relative risk of colon cancer compared with the general population. Meta-analyses of multiple studies reveal that heterozygous carriers of a single *MUTYH* founder mutation may have at most a small increase in colon cancer rate compared with the general population (relative risk of 1.1–1.4) (12,13). In a population-based study of colon cancer in Canada, Croitoru et al. went further and sequenced the complete *MUTYH* coding region in heterozygous carriers of Y165C or G382D mutations (14). Their analysis yielded an odds ratio (OR) of 1.4 for colon cancer in this smaller group of validated heterozygous Y165C or G382D mutation carriers, but did not reach statistical significance; however, analysis of colon cancers from the heterozygous carriers did reveal somatic inactivation of the wild-type copy of *MUTYH* in 47% of samples tested (14). Thus, *MUTYH*-associated polyposis is an autosomal recessive colon cancer disorder with at most a minor cancer phenotype in heterozygous carriers.

In patients with XP, the predominant sites of cancer are explained by those tissues that are exposed to UV radiation and thus have a higher mutational load. In patients with *MUTYH* deficiency, there is also a very limited tissue spectrum, predominantly of benign and malignant tumors of the colonic epithelia without evidence for a significantly increased risk of other tumor types. This limited spectrum is despite the universal expression of *MUTYH* protein in different tissue types. Possible explanations for this tissue specificity include (1) that exposure of colonic epithelia to mutagens in food may increase the need for efficient base excision repair; or (2) in most tissues, other DNA repair pathways can substitute for *MUTYH*-dependent base excision repair.

### 42.4 DISORDERS OF MISMATCH REPAIR: LYNCH SYNDROME AND TURCOT SYNDROME

Lynch syndrome, with susceptibility to adult-onset cancers, including colorectal, endometrial, and ovarian, results from inheritance of a heterozygous mutation in one of four different mismatch repair genes: *MSH2*, *MLH1*, *MSH6*, and *PMS2* (15). These proteins function in heterodimers to recognize and repair mismatches caused by replication errors. Due to the striking colon cancer phenotype, Lynch syndrome is often referred to as hereditary non-polyposis colon cancer (HNPCC). The lifetime incidence of colon and endometrial cancers is



**FIGURE 42-3** Schematic diagram of a microsatellite instability assay used to evaluate tumor specimens in comparison to normal tissue from patients suspected of having Lynch syndrome or hereditary non-polyposis colon cancer. Microsatellite markers from the National Cancer Institute approved panel are analyzed from paired sets of normal and tumor tissue. Marker 1 and 2 show gain (blue arrowheads) or loss (red arrowheads) of repeat size in the tumor compared with the normal tissue, and are said to demonstrate microsatellite instability.

estimated to be 67–70%, with age of onset beginning in young adulthood and clustering in the 5th to 6th decades. There is also increased relative risk of ovarian, brain, biliary tract, and urinary tract cancers compared with the general population.

HNPCC results from inheriting a single deleterious mutation followed by second somatic inactivating event in the same gene through mutation, loss, or epigenetic silencing. Tumors from patients with HNPCC display an unusual DNA pattern, termed microsatellite instability (MSI) or replication errors positive (RER+), characterized by changes (both increases and decreases) in the length of repetitive sequences spread throughout the genome, due to mismatch repair deficiency in the tumor cell (Figure 42-3) (16). The presence of MSI or expression of mismatch repair (MMR) proteins in the tumor tissue is used to make decisions about germline testing in colon cancer patients. This represents one of the few examples where the molecular diagnostic test relies on a direct assessment of the localized repair deficiency in the tumor environment, as assessed by the number of microsatellite markers in the National Cancer Institute panel that demonstrate instability. Colon cancer incidence in heterozygous carriers of *MSH6* and *PMS2* mutations is lower than in carriers of *MSH2* and *MLH1* mutations (17–20). Thus, *MSH6* protein may play a subtly different biochemical role in mismatch repair than *MLH1* or *MSH2* proteins, as evidenced by the difference in cancer risk and the more frequent instability of mononucleotide repeats compared with dinucleotide repeats in tumors from *MSH6* mutation carriers (18).

The eponym “Turcot syndrome” has been associated with an overlapping set of conditions that presents with colonic polyposis, colon cancer, and brain tumors in childhood, and includes conditions transmitted in both autosomal dominant and recessive forms (21). The autosomal recessive form of Turcot syndrome is also designated “mismatch repair deficiency syndrome” (MMR-D), to clarify its distinct clinical phenotype from the dominant forms of Turcot syndrome (22,23). MMR-D syndrome describes children who inherit homozygous or compound heterozygous mutations in the same MMR genes (*MSH2*, *MLH1*, *MSH6*, and *PMS2*) as those associated with HNPCC. These children have a profound clinical phenotype resulting from mismatch repair deficiency in all cells of the body, including café au lait spots and axillary freckling usually associated with neurofibromatosis type 1 (24).

Tragically, children with MMR deficiency syndrome have a strikingly higher cancer incidence and tissue distribution compared with that of adults with HNPCC. Table 42-2 summarizes the cancer that developed in children documented to inherit two mutations in MMR genes (23). Among this group, the most common cancers were leukemias or lymphomas, brain tumors, and colorectal cancers. Mutations in *MSH2* and *MLH1* resulted in an earlier age of onset and somewhat different pattern of cancers compared with *MSH6* and *PMS2* carriers. All four types of MMR-D are associated with café au lait spots and childhood onset of one or more malignancies (22). Thus, complete deficiency of any one of the MMR proteins in somatic cells has a profound impact on genomic stability and cancer incidence.

The prevalence of childhood leukemias and lymphomas in MMR deficiency syndrome patients clearly demonstrates that the hematopoietic tissues are susceptible to malignant transformation when mismatch repair activity is absent. Mouse models have also demonstrated that *Msh2* homozygous null mice develop lymphomas, but not heterozygous *Msh2* mice (25). Similarly, careful analyses of HNPCC kindreds heterozygous for MMR gene mutations have not revealed an increased risk of hematopoietic cancers during childhood or adulthood (26). The differences in tumor types between individuals who inherit two or one mismatch repair gene mutation may reflect differential rates of second hits in different tumor types. For example, hematopoietic tissues may have a low spontaneous rate of second hits in the general population. Evidence to support this hypothesis that exogenous damage to hematopoietic tissues is needed to inactivate the MMR genes includes the finding that primary leukemias rarely demonstrate MSI, whereas samples from patients with therapy-related secondary leukemias (leukemia that develops after exposure of the patient to chemotherapy treatment for another cancer) demonstrate an MSI-high genotype (27).

The use of microsatellite assays focuses attention on alteration in short repeat sequences due to errors or



**TABLE 42-1 Representative DNA Repair Disorders**

Pathway	Representative Condition	Major Clinical Features	Diagnostic Test(s)
Nucleotide excision repair	Xeroderma pigmentosum	Extreme sunburns, early onset of skin cancers and photo-aging of skin	Sensitivity to UV light in fibroblasts
Base excision repair	MUTYH-associated polyposis	Development of hundreds of adenomatous polyps and early onset colon cancer	Directed mutation analysis of <i>MUTYH</i> sequence
Mismatch repair (See Table 42-2 for description of biallelic form)	Lynch syndrome (hereditary non-polyposis colon cancer)	Increased risk and earlier onset of colon, endometrial, ovarian, and biliary tract cancers	Microsatellite instability in tumor tissue
Double strand break recognition and repair	Ataxia-telangiectasia	Cerebella ataxia, telangiectasia, immunodeficiency, leukemias, lymphomas, and solid tumors	Increased alpha fetoprotein; sensitivity to ionizing radiation
Cross-link repair and homologous recombination	Fanconi anemia	Bone marrow failure, congenital anomalies, abnormal pigmentation, and increased risk of leukemias and head and neck and genitourinary malignancies	Chromosome breakage assay after exposure to diepoxybutane and/or mitomycin C
RECQ helicase disorders	Bloom syndrome	Short stature, butterfly rash on face, immunodeficiency, and increased risk of multiple cancer types	Increased sister chromatid exchange

**TABLE 42-2 Cancer Phenotype of Patients with Mismatch Repair Deficiency Syndrome**

Genes	Age of Diagnosis of 1st Tumor	Leukemia/Lymphoma—T Cell	Brain Tumors—Gliomas	HNPPC/Lynch Syndrome Tumors
<i>MSH2 MLH1</i> (n = 20)	3.5 years (0.4–35)	11	4	6
<i>MSH6 PMS2</i> (n = 5)	9 years (1–31)	16	32	35

HNPPC = hereditary non-polyposis colon cancer

Data obtained from Wimmer, K.; Kratz, C.P. Constitutional Mismatch Repair-Deficiency Syndrome. *Haematologica* 2010, 95, 699–701.

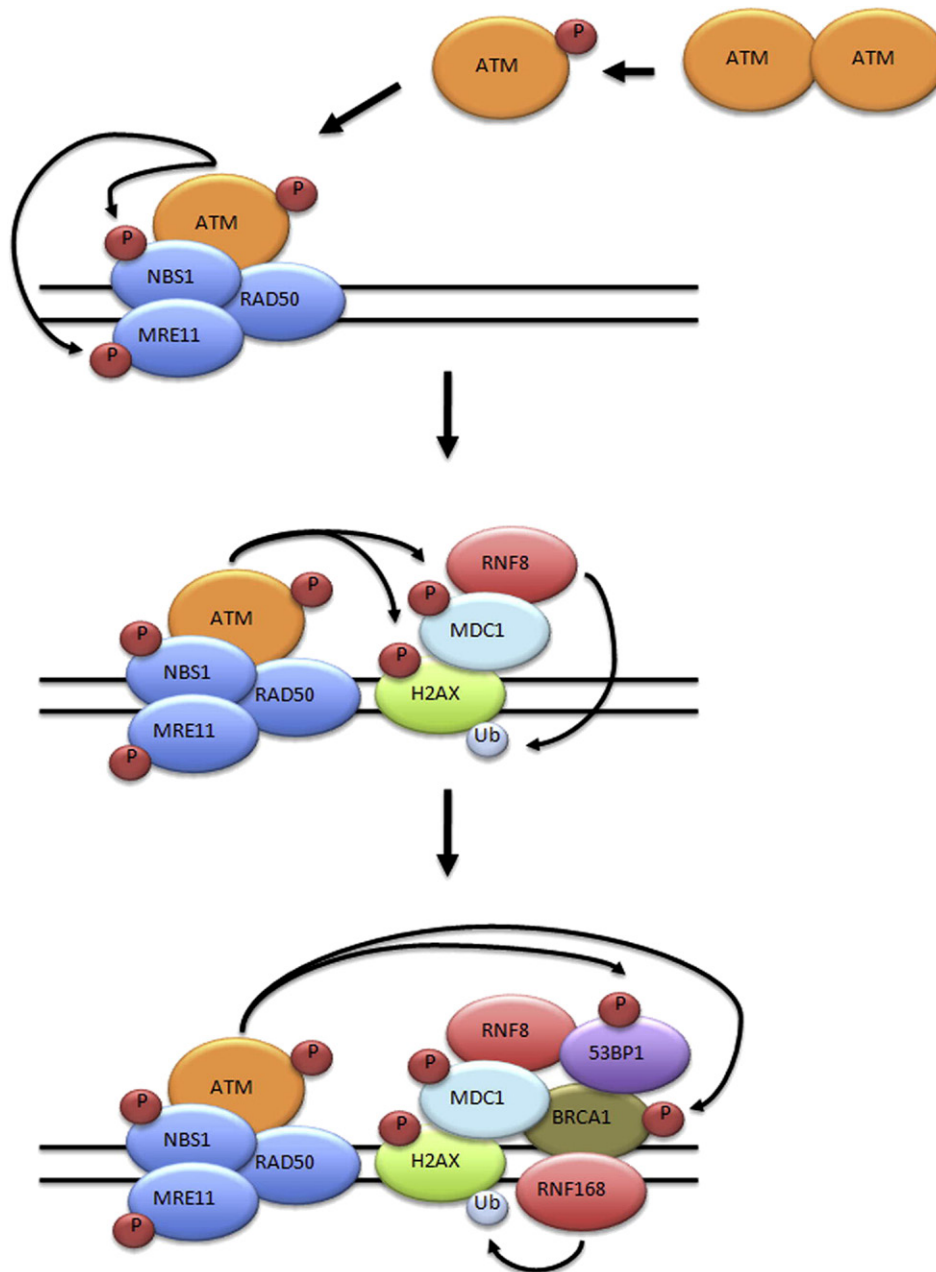
slippage in replication, but the role of MMR proteins in maintaining genomic stability is much greater. The ability of these protein complexes to recognize mismatches also allows them to inhibit recombination between heterologous sequences that share homology. For example, it was noted over 10 years ago that MMR-deficient cells show increased maintenance of telomere length by accelerated recombination events involving related telomere repeat sequences (28). Similarly, data from model organisms show that MMR-deficient cells demonstrate increased deletion and duplication events; again, secondary to the loss of this mechanism to inhibit recombination between heterologous repeats (29). Thus, the cancer phenotype seen in either the dominant or recessive condition may result from increase in several different types of genomic instability when mismatch repair function is lost.

## 42.5 DISORDERS ASSOCIATED WITH DOUBLE STRAND BREAK RECOGNITION AND REPAIR: ATAXIA-TELANGIECTASIA AND RELATED CONDITIONS

Children with ataxia-telangiectasia (AT) first develop truncal ataxia during early years of childhood, which

is progressive, eventually becoming wheelchair dependent (30). The disease name derives from the neurologic problems as well as oculocutaneous telangiectasia that is typically first apparent in the conjunctivae between the ages of 3 and 5 years. Individuals with AT have elevated alpha fetoprotein (AFP) levels, which can be helpful in making a diagnosis. AT is an autosomal recessive disorder due to inheritance of biallelic inactivating mutations of the *ATM* DNA damage response gene. Patients with AT have a significantly increased risk of leukemia and lymphoma as well as some increased risk for other tumor types (30). Women heterozygous for *ATM* mutations have an approximately twofold increase in risk of developing breast cancer during adulthood with no other clinical features of AT (31,32).

The cellular phenotype associated with AT is increased killing of lymphocytes (or immortalized lymphoblastoid cells) in response to ionizing radiation exposure. This quantitative assay is used as a clinical diagnostic test for patients suspected of having AT or related disorders (33). Radiation sensitivity exhibited by AT patients highlighted the important role that ATM plays in mediating the DNA damage response to double strand breaks (DSB), including DNA repair processes and cell



**FIGURE 42-4** Recruitment of DNA damage response proteins to a DNA double strand break. Prior to DNA damage, ATM exists as an inactive dimer. Following the induction of a DNA double strand break, ATM undergoes autophosphorylation, producing active ATM monomers. ATM and MRN (comprising MRE11, RAD50, and NBS1) are rapidly recruited to the site of the DNA double strand break. Upon recruitment, ATM phosphorylates MRE11, NBS1, and H2AX. The phosphorylation of H2AX leads to the recruitment of MDC1. MDC1 is phosphorylated by ATM and phosphorylated MDC1 serves as a docking site, recruiting the RING-finger ubiquitin ligase RNF8. RNF8 monoubiquitinates  $\gamma$ H2AX, resulting in the recruitment of 53BP1, BRCA1, and RNF168. The RING-finger ubiquitin ligase RNF168 maintains the ubiquitinated status of  $\gamma$ H2AX, aiding in the stabilization 53BP1 and BRCA1 at the break site. (From Derheimer, F. A.; Kastan, M. B. Multiple Roles of ATM in Monitoring and Maintaining DNA Integrity. *FEBS Lett.* 2010, 584, 3675–3681.)

cycle checkpoints (34). ATM protein resides in cells as a homodimer that is inactive (Figure 42-4) (35). After DNA damage or alteration in chromatin structure, the homodimer is disrupted and kinase function is activated. ATM phosphorylates a very large network of regulatory proteins that mediate recognition and repair of DNA lesions, including p53 and BRCA1 (36). Disruption of this network is thought to result in genomic instability and cancer development. Patients with AT who develop

cancer require substantially modified treatment regimens due to deficient DNA repair activity, which is manifested by clinically significant cytotoxicity in response to chemotherapy and radiation treatments (33). The link between ATM deficits and neurologic disability is much more complex and thought to potentially result from excess apoptosis in neurons that accumulate DNA damage, or perhaps accumulation of reactive-oxygen-species-mediated damage in neurons (6).

Defective double strand break repair (DSBR) also impacts the metabolism of DSB, seen as intermediates of normal VDJ recombination in lymphocytes. For example, repair of coding ends, where ATM normally accumulates, is thought to be delayed or defective in lymphocytes of AT patients (37). Abnormalities in VDJ recombination is thought to explain the lymphoid abnormalities seen in AT patients including: immunodeficiency with sinopulmonary infections, low levels of IgG, and prevalence of t(7;14) translocations in stimulated peripheral blood karyotypes.

In addition to ATM itself, there are a number of proteins required for DSBR, and the deficiency of these results in a series of related disorders (Table 42-3). The recombination-activating genes *RAG1* and *RAG2* play an important role in initiation of VDJ recombination. There are several autosomal recessive disorders that result from inheriting mutations in either of these two genes. Patients with two truncating mutations, e.g. nonsense or frameshift mutations, demonstrate classic severe combined immunodeficiency (SCID) impacting both T and B lymphocytes. Patients who inherit at least one missense allele, encoding a hypomorphic form of the RAG protein that generates greater than 1% enzymatic activity, have a more complex phenotype referred to as Omenn syndrome (38,39). Omenn syndrome is associated with immunodeficiency characterized by oligoclonal activated T cells and absent B cells. Classic Omenn syndrome includes erythroderma, lymphadenopathy, splenomegaly, granuloma formation in multiple organs, and infectious complications (38). Thus, genetic testing and identification of the specific mutation in the RAG genes is helpful in predicting the clinical course. The dysfunction of the immune system in the child (with either SCID or Omenn syndrome) can lead to engraftment of maternal T cells in the fetus, referred to as maternofetal transfusion, that may persist after birth (39). These maternal T cells perpetuate clinical problems in the child that are similar to graft versus host disease in patients undergoing bone marrow transplant.

Radiation-sensitive SCID (RS-SCID) results from inheritance of mutations in the *DCLRE1C* gene which encodes the Artemis protein (40). Artemis contains both endonuclease and exonuclease biochemical activities and is required for cleavage of the hairpin junctions specifically formed during VDJ recombination (41). As exemplified by the name of the disorder, patients with RS-SCID show a combined phenotype of significant radiation sensitivity and immunodeficiency, associated with accumulation of VDJ hairpin structures. Consistent with the concept that Artemis protein has a unique biochemical function in VDJ recombination, the gene is not essential and patients with RS-SCID demonstrate a variety of null and hypomorphic alleles (40). Patients with two null alleles do not demonstrate growth or developmental defects. A founder nonsense mutation in exon 8 of *DCLRE1C*, which results in nonsense-mediated decay of the *DCLRE1C* mRNA, is found in patients with RS-SCID from the Athabaskan Native American population (42).

Children with mutations in the gene that encodes DNA ligase IV (utilized during non-homologous end joining (NHEJ)) demonstrate microcephaly, immunodeficiency, and increased risk of lymphoma/leukemia (40). The *LIGIV* gene is essential, and patients with *LIGIV* deficiency carry a variety of hypomorphic alleles. *LIGIV* protein contains a BRCT motif, which is shared by other DSBR proteins including *BRCA1* (43). The clinical phenotype is also variable, with some patients demonstrating few of the features noted above and others having dysmorphic features accompanied by significant enough bone marrow failure to justify bone marrow transplantation (37,40). Cells from these patients demonstrate normal DNA damage checkpoint signaling and lack the ionizing radiation sensitivity seen in patients with AT.

AT-like disorder (ATLD) and Nijmegen breakage syndrome (NBS) are two disorders that result from mutations in genes encoding subunits of the MRN (*MRE11*, *RAD50*, *NBN*) heterotrimeric complex central to NHEJ repair (44,45). ATLD is a very rare disorder that results from biallelic mutations in *MRE11*. NBS results from biallelic mutations in the *NBN* gene, which encodes nibrin. The MRN complex comes to DSB independent of ATM activity. ATM then phosphorylates components of MRN as part of a complex DSBR signaling pathway (36,46). Despite the fact that *MRE11* and *NBN* function in the same complex, and cell lines derived from these patients demonstrate similar cellular defects, there are very distinct differences in their clinical phenotypes (44). Patients with ATLD demonstrate a similar neurodegeneration phenotype to patients with AT although with somewhat later age of onset. They lack the telangiectasia, elevated AFP, or reduction in immunoglobulins seen in patients with AT. To date, no patient with ATLD has been reported to develop leukemia or lymphoma; however, this is based on descriptions of less than 10 patients, so the cancer susceptibility is not well documented. NBS is quite distinct clinically from either AT or ATLD. Patients demonstrate microcephaly, developmental delay, dysmorphic features, reduced levels of immunoglobulins, radiation sensitivity, and increased risk of lymphoma (47).

The *ATR* (ataxia-telangiectasia and Rad3-related protein) gene encodes a protein highly related to *ATM*, which also encodes a member of the PI3 kinase family and targets many of the same proteins for phosphorylation when *ATR* is activated (34). Although it does not directly function in the double strand break repair pathway, the biochemical similarities justify inclusion in this section of the chapter. There are significant differences in the physiological impact of mutations in these two related genes. First, *ATM* is a non-essential gene, patients often inherit two truncating or null alleles, and there are no obvious growth defects or cognitive delays at birth. *ATR* encodes an essential function and thus patients with the *ATR*-related disorder Seckel syndrome are found to carry hypomorphic alleles of the *ATR* gene (40). *ATR* protein plays a crucial role in the response of

**TABLE 42-3 Disorders of Double Strand Break Response and Repair**

Condition	Gene/Protein	Clinical Features
Ataxia-telangiectasia (AT)	<i>ATM/ATM</i>	Cerebella ataxia, telangiectasia, immunodeficiency, leukemias, lymphomas and solid tumors
Ataxia-telangiectasia-like disorder (ATLD)	<i>MRE11/MRE11</i>	Neurologic features similar to AT with later age of onset, no telangiectasia, unclear cancer risk
Nijmegen breakage syndrome	<i>NBN/Nibrin</i>	Microcephaly, dysmorphic features, immunodeficiency, developmental delay and lymphoma risk
Radiation-sensitive–severe combined immunodeficiency disorder (RS-SCID)	<i>DCLRE1C/Artemis</i>	Sensitivity to ionizing radiation with severe immunodeficiency
Ligase IV deficiency	<i>LIGIV/ligase IV</i>	Microcephaly, immunodeficiency, anemia, developmental delay, lymphomas
SCID and Omenn Syndrome	<i>RAG1</i> and <i>RAG2/RAG1</i> and <i>RAG2</i>	Varying phenotype from isolated SCID to erythroderma, granulomas, lymphadenopathy and splenomegaly depending on mutation type. Can be complicated by persistent maternal T cells

cells to the single stranded DNA that is generated upon replication fork stalling, which occurs frequently even in the absence of exogenous damage. ATR also plays a role in repairing templates that contain bulky adducts. Thus, children with inherited deficiency in ATR function show severe growth defects with intrauterine growth retardation, small stature, and marked microcephaly (40). Patients also have very significant cognitive deficits and characteristic facial features. Consistent with the lack of a defect in response to double strand breaks, patients with Seckel syndrome and *ATR* mutations do not demonstrate significant ionizing radiation sensitivity or cancer predisposition. A mouse model of Seckel syndrome has been engineered by creating a murine line that has portions of the human *ATR* gene with an exon-skipping mutation swapped in the murine *Atr* locus. This mouse model recapitulates many of the features of Seckel syndrome, including microcephaly, and also demonstrates accelerated aging (48).

More recently, the use of homozygosity mapping and exome sequencing revealed mutations in two new genes, *CEP152* and *PCNT*, underlying some cases of Seckel syndrome. *PCNT* encodes pericentrin and is not essential. Patients with Seckel syndrome are often homozygous for a truncating allele (49). The proteins encoded by these two genes, CEP152 and pericentrin, respectively, bind each other and function in regulation of the centrosome. Absence of CEP152 function results in fragmented centrosomes, aneuploidy, and abnormal mitoses (50). The replicative stress evident in both ATR and CEP152/pericentrin deficient cells is thought to explain the similar clinical Seckel syndrome phenotype (49).

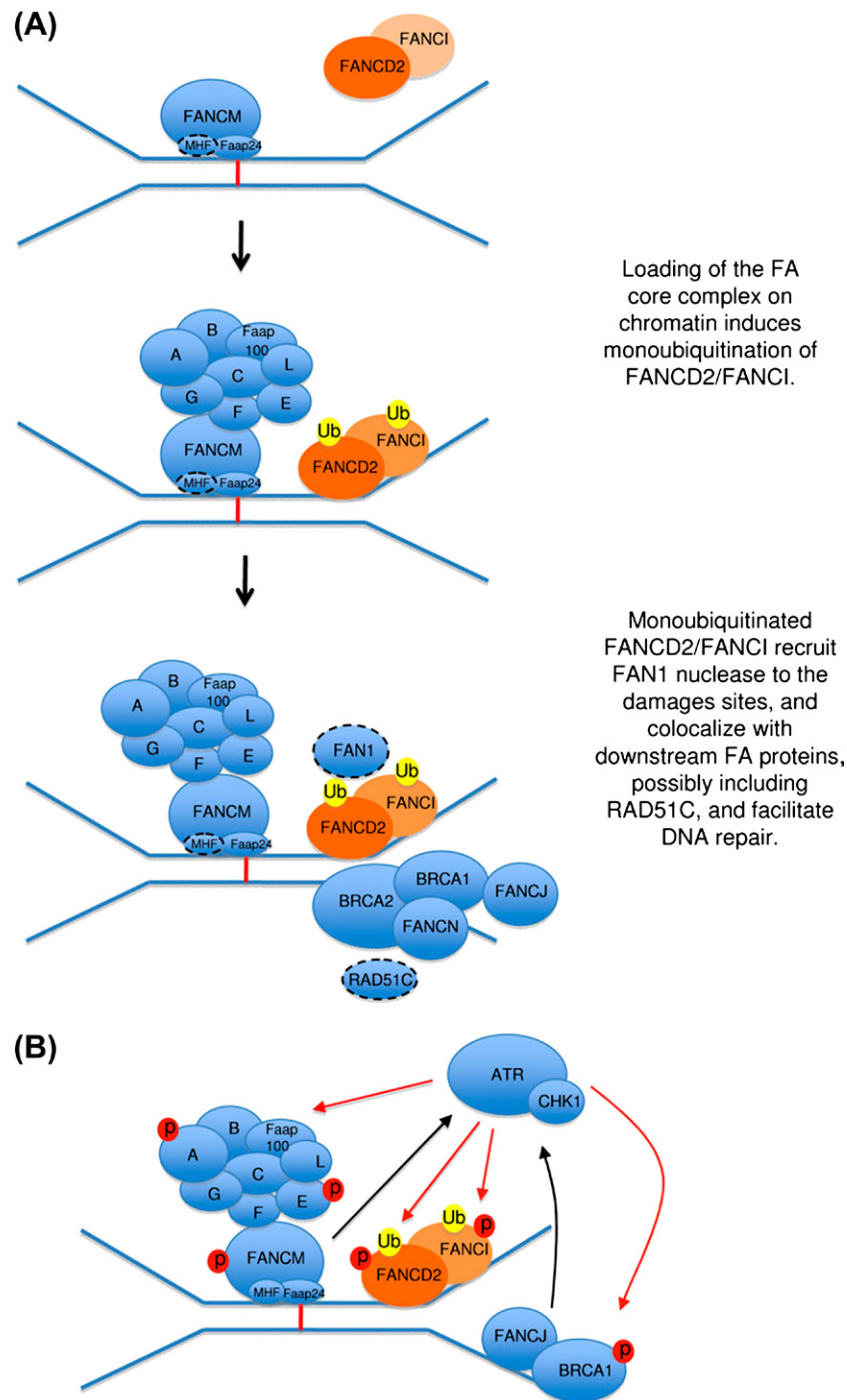
## 42.6 CROSSLINK REPAIR AND HOMOLOGOUS RECOMBINATION DEFECTS: BREAST-OVARIAN CANCER AND FANCONI ANEMIA

Extensive research on the genetic basis of hereditary breast and ovarian cancer was performed in the 1980s

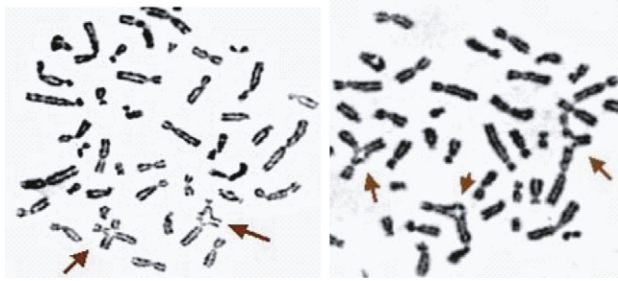
and 1990s. This work led to the identification of the *BRCA1* and *BRCA2* cancer-susceptibility genes as discussed in Chapter 89 and reviewed by Narod and Foulkes (51). Analysis of hundreds of individuals heterozygous for *BRCA2* mutations has revealed that, in addition to breast and ovarian cancer risk, there is a lower but still statistically significant increased risk of prostate, pancreatic, stomach, and gallbladder and bile duct cancers, and malignant melanoma compared with the general population (51,52). The relationship with Fanconi anemia was not expected, as there is no evidence for a statistically significant increase in hematopoietic cancers in individuals heterozygous for *BRCA2* mutations (52). Both *BRCA1* and *BRCA2* encode proteins which act through large protein complexes to respond to double strand DNA breaks and mediate homologous recombination (Figure 42-5). As for other tumor suppressor genes that play a role in DNA repair, the tumor tissue from *BRCA1* and *BRCA2* heterozygous mutation carriers demonstrates inactivation of the second allele, typically associated with loss-of-heterozygosity events, resulting in localized DNA repair deficiency, very significant genomic instability, and aneuploidy (51,52).

The *Brca2* gene is essential for viability, based on studies of mouse models containing null mutations (53). Starting with the observation that cells from *Brca2* mutant mice demonstrated increased sensitivity to cross-linking agents, Howlett et al. reported the startling finding that rare forms of Fanconi anemia (FA subtype D1) resulted from biallelic inheritance of *BRCA2* mutations, often with one mutation being a null allele and the second a hypomorphic allele (54,55). This finding has led to an explosion of research documenting the complex overlap between interstrand cross-link repair (hallmark of Fanconi phenotype) and homologous-recombination-mediated repair (*BRCA1*, *BRCA2*, and associated proteins) including the discovery of many new Fanconi genes for the rarer complementation groups, which encode proteins that provide functions required in the





**FIGURE 42-5** A schematic model for the FA pathway. (A) Activation of the FA pathway. DNA interstrand crosslinks (ICL) is directly recognized by FANCM–FAAP24–MHF protein complex. This complex recruits the FA core complex by direct interaction between FANCM and FANCF. The recruited FA core complex, containing a PHD E3 ubiquitin ligase domain in the FANCL subunit, subsequently monoubiquitinates its two substrates, FANCD2 and FANCI, on chromatin. The monoubiquitinated FANCD2–FANCI becomes an active form, recruits newly identified nuclease FAN1 or interacts with a series of DNA repair proteins (including BRCA1, PALB2 ½FANCL, BRCA2, and FANCI, BACH1/BRIP1) at the damaged sites, and facilitates downstream repair pathways. RAD51C, a newly identified FA-like protein, may have a functional interaction with FANCD2 at this step. FANCD2–FANCI probably also recruits other nucleases and TLS polymerases to process the ICL (not shown). The new players in the FA pathway described in the text are highlighted in dashed lines. (B) The FA pathway cross-talks with ATR–CHK1 checkpoint proteins. ATR and its effector kinase, CHK1, are required for damage-inducible activation of the FA pathway. ATR–CHK1 phosphorylates (red arrows) multiple FA and FA-associated proteins, including FANCA, FANCE, FANCD2, FANCI, and BRCA1. FANCM is also phosphorylated upon DNA damage by an unknown kinase. In turn, the stability and activity of ATR–CHK1 are promoted (black arrows) by the FANCM–FAAP24 heterodimer and FANCI by independent mechanisms. FANCM also mediates the formation of the BRAF complex, which contains the FA core complex members and the BLM complex containing Topo IIIa and RMI1/2 (not shown). (With permission from Kee, Y.; D’Andrea, A. D. *Expanded Roles of the Fanconi Anemia Pathway in Preserving Genomic Stability*, Genes Dev. 2010, 24, 1680–1694.)



**FIGURE 42-6** Karyotypes of diepoxybutane-treated peripheral blood samples from a patient with Fanconi anemia. Abnormal structures that are increased in FA patients include quadri-radial (arrows—left panel) and tri-radial (arrows—right panel) formations. (Photograph courtesy of Matthew Folsom and Rizwan Naeem, PhD—Texas Children's Hospital.)

overlap between the cross-link repair and homologous-recombination-mediated repair (56).

Fanconi anemia is an autosomal recessive condition (except for the X-linked FA-B group) associated with bone marrow failure, congenital anomalies, and a substantially increased risk of malignancy in children and young adults (57–60). The most common cancers include acute myelogenous leukemia in children and head and neck cancers and genitourinary cancers in young adults. The cellular phenotype of Fanconi cells includes a profound sensitivity to cross-linking agents such as diepoxybutane (DEB) and mitomycin C (59). Treatment of the bone marrow failure requires bone marrow transplantation, and this sensitivity to DNA-damaging agents requires the use of specialized conditioning regimens to prepare the patient for transplant.

In addition to increased cell death, there are a variety of cytogenetic abnormalities, which are the basis of the diepoxybutane breakage assay (Figure 42-6). Increased breakage in response to these agents is used as the diagnostic test for FA (61). Patients with FA may demonstrate normal DEB breakage analysis from a blood specimen secondary to *in vivo* complementation where recombination between the two mutant alleles results in lymphoid cells that have corrected their repair defect; this circumstance may necessitate use of fibroblast analysis for a clear diagnosis (62). The DEB assay is also used for complementation analysis, which, as described for XP, allows us to distinguish FA patients from among the 15 different complementation groups (corresponding to 15 different FANC genes). Complementation for FA can be performed by fusion of patient cells with cell lines of specified complementation group or by transduction of retroviruses that express wild-type FANC proteins (63). For example, if DEB breakage is normalized by the transduction with a FANCA-encoding retrovirus, then the patient is considered to have FA-A.

Dozens of biochemical and molecular studies combined with complementation analysis have demonstrated that the FANC proteins function in DNA repair through participation in a number of different complexes (see Figure

42-5) (60,64). FANCA, C, B, E, F, G, and L proteins make up the FA core complex, which is thought to function in the first portion of the FA pathway. Over 90% of FA patients carry mutations in one of the core genes, with FANCA, FANCC, and FANCG being the most common. Individuals who fall within the FA core complex complementation groups have the typical combined phenotype of congenital anomalies, short stature, bone marrow failure (onset at average age of 6–8 years), and increased risk of leukemias and genitourinary and head and neck cancers. When activated by DNA damage or replication fork stalling, the FA complex proteins result in monoubiquitination of two proteins, FANCD2 and FANCI, which together form a heterodimer. There is little to no increased cancer risk seen among heterozygous carriers of mutations in genes that encode FA complex proteins (58).

An additional upstream component is FANCM, which is found complexed with FAAP24. The identification of FANCM as a verified FA protein is unclear, as the original FA-M patient cell line was subsequently reported to also contain a mutation in the FANCA gene and no other biallelic FANCM mutant line has been identified (65). At a biochemical level, FANCM also acts differently from other FA complex proteins as it binds directly to DSBs and appears to be required for bringing the FA core complex to the site of the break (66). FANCM:FAAP24 binds directly to the ATR checkpoint kinase. FANCM-deficient cells have a broader spectrum of sensitivity to exogenous agents, including hydroxyurea and camptothecin, compared with other FA cell lines, and the complex plays a bridging role between the FA pathway and a number of other DNA repair and checkpoint pathways.

The presence of FANCD2 monoubiquitinated and non-ubiquitinated species can be detected by Western blot analysis, and this assay is used by some laboratories as a diagnostic test (59). In particular, if protein lysates from a patient with FA demonstrate loss of the normal FANCD2 monoubiquitination, when further accentuated by exposure to cross-linking agents, this provides strong evidence that the patient carries mutations in one of the genes encoding the core complex. In normal cells, once FANCD2 and FANCI are monoubiquitinated, this sends a signal which is transduced to the DNA repair complexes that include FANCD1/BRCA2, FANCI/BRIP1, FANCA-N/PALB2, and FANCP/RAD51C (60,67). These latter proteins play a direct role in repair pathways that utilize homologous recombination (68).

Patients in the FA-D1 complementation group, due to biallelic deleterious mutations in BRCA2, have a particularly severe cancer phenotype with the vast majority being diagnosed with malignancies by the age of 5 years (69). Overall, patients with two deleterious BRCA2 alleles appear to have an increased incidence of congenital anomalies as well as multiple malignancies, compared with patients with one null allele and one missense mutation (55). Childhood onset of solid tumors is also more frequent in the FA-D1 patients

TABLE 42-4 RECQ Helicase Disorders

Disease	Gene/Location	Cellular Phenotype and Proposed RECQ Function	Cancer Predisposition
Bloom syndrome	<i>BLM</i> /15q26.1	Increased sister chromatid exchange Repair of stalled replication forks	Leukemia/lymphoma and solid tumors
Werner syndrome	<i>WRN</i> /8p11	Premature senescence Maintenance of telomeres	Soft tissue sarcomas and skin cancers
Rothmund–Thomson syndrome	<i>RECQL4</i> /8q24.3 <sup>a</sup>	Minor excess cytotoxicity to S phase agents Replication initiation	Osteosarcoma and skin cancers

<sup>a</sup>Only approximately 60% of patients with Rothmund–Thomson syndrome carry mutations in the *RECQL4* gene. The risk of osteosarcoma is associated with positive *RECQL4* mutation status (95).

compared with patients with mutations in the core complex genes, with almost half of this cohort having solid or brain tumor diagnoses including medulloblastoma, glioblastoma, and Wilms tumor (55). Thus, many different tissue types are susceptible to cancer when there is constitutional *BRCA2* deficiency. This same paradigm has been found regarding mutations in the gene encoding the *BRCA2*-associated protein *PALB2* (70). Children carrying biallelic *PALB2* mutations, or FA-N complementation group, also demonstrate a severe FA phenotype similar to FA-D1 (71,72). In seven FA-N families, there were five cases of medulloblastoma, three of Wilms tumor, two additional cases of AML, and one case of neuroblastoma, all diagnosed by 4 years of age (72). In contrast, analyses of families with heterozygous carriers of *PALB2* mutations demonstrated a two- to fourfold relative risk of breast cancer and increased risk of pancreatic cancer compared with the general population (73,74). Thus, the relative risk of breast cancer in *PALB2* mutation carriers is less than that described for heterozygous carriers of *BRCA2* mutations.

The Fanconi subtypes FA-J and FA-I are due to mutations in the repair genes *BRIP1/FANCI* and *KIAA1794/FANCI* (75,76). Carriers of heterozygous *BRIP1/FANCI* mutations have a twofold increase in breast cancer risk (77). However, the cancer phenotype in biallelic cases has not been well described due to the very small number of FA-J patients, but includes at least one child with chronic myelomonocytic leukemia (78,79). The *BRIP1/FANCI* protein is a structure-specific helicase which appears to be important for unwinding G tracts that can form guanine quadruplex DNA structures (80). An example of overlap in functions between proteins encoded by distinct disease genes is the recent finding that *FANCI* helicase and *BLM* helicase (described in section 42.7) interact physically in foci after DNA damage (68). Degradation of *BLM* helicase occurs when *FANCI* protein is absent.

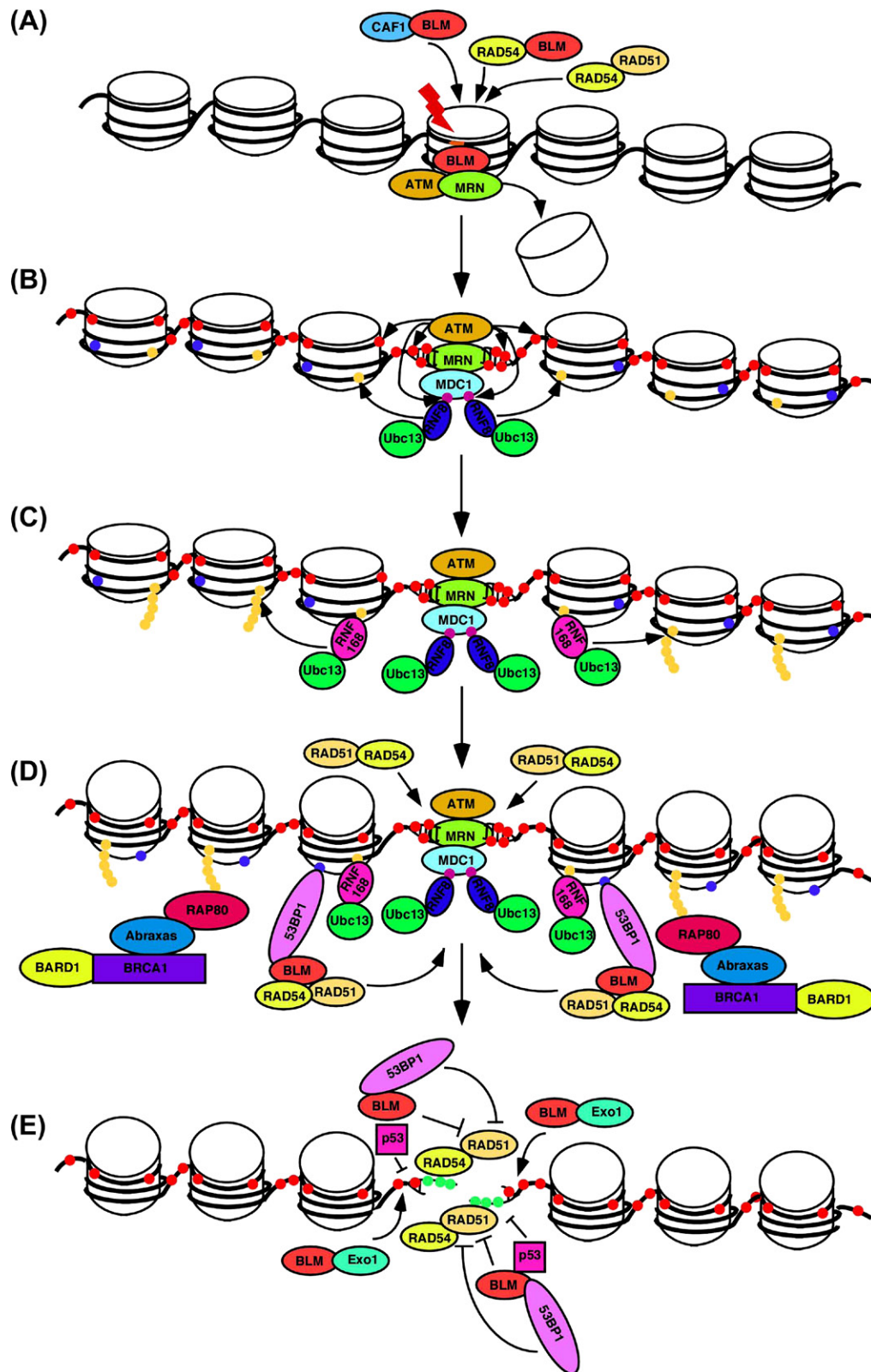
The most recently discovered Fanconi gene, *FANCP*, encodes the *RAD51C* homologous recombination protein (81). Again, biallelic carriers have Fanconi anemia FA-P, with significant childhood and young-adult cancer risk (82). In comparison to *PALB2* and *BRIP1*, heterozygous *RAD51C/FANCP* carriers have a breast and ovarian cancer risk close to that of *BRCA2* heterozygous

carriers (83). Thus, these distal members of the FA pathway play an important role in DNA repair that is mediated through homologous recombination. Mutations in these genes result in increased cancer risk in adult heterozygous carriers and a higher risk of solid tumors in children who are biallelic mutation carriers.

## 42.7 DISORDERS ASSOCIATED WITH RECQ HELICASE DEFICIENCY: BLOOM, WERNER, AND ROTHMUND–THOMSON SYNDROMES

There are three autosomal recessive disorders that demonstrate distinct clinical features but share predisposition to malignancy and are the result of mutations in the same family of helicases (Table 42-4). The three disorders, Bloom, Werner, and Rothmund–Thomson syndromes, are grouped together because they all result from biallelic mutations in members of the RECQ helicase family: *BLM*, *WRN*, and *RECQL4*, respectively (84). Extensive molecular biology and biochemical analyses demonstrate that these RECQ helicases play distinct roles in DNA metabolism.

Children with Bloom syndrome are very small at birth and have very significant lifelong short stature; a photosensitive rash, particularly prominent on the face; immunodeficiency; and predisposition to a variety of malignancies including leukemias/lymphomas and solid tumors (85,86). Similar to FA, the primary diagnostic test is a cytogenetic assay that monitors an increased number of sister chromatid exchange (SCE) events as the result of the hyper-recombination phenotype that results from loss of *BLM* activity. The cellular functions of the *BLM* helicase have been extensively studied and demonstrate an important role in resolving stalled replication forks and modulating recombination between sister chromatids (87). *BLM* helicase is a crucial component of homologous recombination and interacts directly with many of the other proteins described in the homologous recombination discussion (Section 42.6), such as *BRCA1* (Figure 42-7) (88). This high recombination rate is clinically evident in patients who are compound heterozygous for *BLM* mutations. In their lymphocytes, recombination between the two disease alleles can result in a corrected allele that expresses functional *BLM* protein (89). These





**FIGURE 42-7** Proposed model for the functions of BLM helicase during DNA damage response. (A) DNA double strand breaks (DSBs; red line) are recognized after BLM and/or RAD51-stimulated RAD54-dependent chromatin remodeling. BLM affects chromatin organization by interacting with and regulating the function of CAF-1. On remodeled chromatin, BLM accumulates and helps in the optimal ATM activation and MRN complex accumulation. (B) MRN complex promotes H2AX phosphorylation (γH2AX, red dots) which recruits MDC1. MRN complex, stabilized on the DNA lesion by MDC1, promotes further accumulation of activated ATM. ATM phosphorylates MDC1 (purple dots), promoting the binding and recruitment of RNF8/Ubc13 complex, which catalyzes the Lys63-linked ubiquitination of H2A and H2AX (yellow dots), causing a more accessible conformation of the chromatin. (C) RNF8/Ubc13-ubiquitinated histones recruit RNF168. RNF168/Ubc13 attaches K63-linked polyubiquitin moieties to RNF8-ubiquitylated histones (yellow dots). (D) Polyubiquitinated histones recruit RAP80, which helps in the accumulation of Abraxas/BRCA1/BARD1 at DSBs. Constitutive methylation of histones H3 and H4 (blue dots) are probably exposed due to RNF168/Ubc13-dependent ubiquitination. This results in the efficient recruitment of 53BP1 to the site of DNA damage. BLM again accumulates on the lesion in a 53BP1-dependent manner. Pro-recombinogenic proteins RAD51 and RAD54 interact with BLM, and accumulate at DSBs. (E) BLM functionally interacts with its partners, like RAD51, RAD54, 53BP1, and p53, during homologous recombination. RAD51 binds to the single stranded DNA by displacing replication protein A (green dots). While BLM, 53BP1, and p53 have anti-recombinogenic property, BLM also has a pro-recombinogenic resection function in coordination with Exo1. (From Tikoo, S.; Sengupta, S. *Time to Bloom*. Genome Integrity 2010, 1, 14.)



**FIGURE 42-8** Clinical features of Rothmund-Thomson syndrome (RTS). Shown are the hands of an adult with RTS who carries two deleterious mutations in the *RECQL4* gene, demonstrating the typical poikilodermatous rash, which often begins on the cheeks and spreads to the extremities, remaining lifelong. The absent thumb of the right hand is an example of the radial ray abnormalities seen in RTS patients with *RECQL4* mutations. (Photograph courtesy of L. L. Wang, MD—Baylor College of Medicine/Texas Children's Hospital.)

lymphocytes are visible due to the paucity of SCE events compared with the uncorrected cells.

Werner syndrome is characterized by premature aging (including early-onset atherosclerosis, diabetes, and cataracts beginning in the second decade) with increased incidence of soft tissue sarcomas (90). The premature aging is manifested at a cellular level as early senescence in fibroblasts from these patients. The WRN helicase encodes both a RECQ helicase module and 3'–5' nuclease activity (91). The aging phenotype demonstrated by Werner syndrome patients has resulted in extensive research into the role of the WRN helicase in maintenance of telomere integrity, despite the fact that WRN is not a subunit of telomerase itself (84). Current data suggests that WRN plays a role in the replication of the telomeric lagging strand, specifically in preventing telomere sister chromatid exchanges, which are associated with cellular senescence (92,93). Thus, BLM and WRN proteins decrease the number of exchanges, but in different chromosomal locations.

The third disorder in this group, Rothmund-Thomson syndrome (RTS), is characterized by a rash termed poikiloderma, which begins in infancy; skeletal dysplasias, including radial ray abnormalities; and cataracts (Figure 42-8). Children with RTS have a distinct predisposition to the development of osteosarcoma, and less frequently, skin cancers (94,95). Unlike other helicase disorders, there is not a clear cellular phenotype that can be used as a diagnostic test. RTS patients with osteosarcoma show little evidence for excess toxicity after chemotherapy treatment (96). Similarly, cells from RTS patients demonstrate only minor in vitro toxicity response to chemotherapy (97). In comparison, studies of the *RECQL4* helicase across species demonstrate that these helicases appear to have two fundamental roles in the initiation of DNA replication: (1) an essential function carried out by an N-terminal region of *RECQL4* that is homologous to the SLD2 family of proteins, and (2) functions associated with the intrinsic helicase domain (98). RAPADILLINO syndrome is a related autosomal recessive disorder, associated with individuals who carry at least one copy of a specific *RECQL4* founder mutation, which generates an abnormally spliced message with skipping of exon 7 (99). This abnormal *RECQL4* protein is mislocalized. Individuals with RAPADILLINO syndrome have an overlapping set of congenital anomalies with RTS, as well as an increased risk of lymphoma (100).

## 42.8 GENE-ENVIRONMENT INTERACTIONS: GORLIN-GOLTZ SYNDROME

Gorlin-Goltz syndrome, or nevus basal cell carcinoma syndrome (NBCCS), is an autosomal dominant disorder with many different features, including odontogenic jaw cysts, bifid ribs, palmar and plantar pits, frontal and biparietal bossing, calcification of the falx cerebri, and short fourth metacarpal bones (reviewed by Gorlin (101)). Heterozygous mutations in the *PTCH* gene, which encodes a homolog of the *Drosophila melanogaster* segment polarity gene, is the molecular basis for Gorlin-Goltz

syndrome (102,103). For this reason, NBCCS is not normally described in a chapter on DNA repair disorders but it highlights the gene–environment interaction that impacts cancer risk in a number of inherited disorders. The basal cell carcinomas (BCCs) are often first diagnosed in puberty. Adults with NBCCS may have hundreds of individual lesions. Exposure to both ultraviolet light and ionizing radiation can impact the number of BCCs that develop. For example, individuals of African-American descent have a later age of first diagnosis of NBCCS and an overall decreased number of BCC lesions compared with Caucasian populations, presumably due to the protective effect with regard to ultraviolet light exposure of increased melanin in the skin (104). In addition to BCC, there is a significant risk of medulloblastoma in childhood. Standard of care for treatment of medulloblastoma is radiation therapy to the corticospinal axis. Unfortunately, exposure of the skin to ionizing radiation (IR) can result in the development of hundreds of BCCs in the skin overlying the treatment field (104). These tumors develop with a latency of 5 years after radiation treatment, and therefore may appear at a much younger age than in patients with NBCCS who have not undergone radiation treatment. There is no evidence that the heterozygous constitutional cells have excess cytotoxicity in response to UV or IR. It is more likely that the exogenous exposure to radiation results in multiple forms of DNA damage, thus increasing the likelihood of a second hit in the wild-type *PTCH* locus facultative for tumorigenesis.

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# CHAPTER

# 43

## Autosomal Trisomies

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### 43.1 INTRODUCTION

It is now over 50 years since the recognition of the chromosomal basis for trisomy 21 (T21), Down syndrome (DS), first described clinically by the British physician John Langdon Down in the nineteenth century. Recognition of the other two major trisomies, trisomy 13 (T13) and trisomy 18 (T18), followed quickly after the discovery of T21 (1). Calculated live-birth rates of these conditions are 1 in 629 for T21, 1 in 6666 for T18, and 1 in 12,500 for T13 (2,3). The rates of all three autosomal trisomies increase markedly with advancing maternal age (4,5,6) and are lower than the rates observed in prenatal life since only about 25% of T21 conceptions, 5% of T18 conceptions, and 2.5% of T13 conceptions survive to birth, with most but not all intrauterine deaths occurring in early pregnancy.

Postnatal mortality associated with T18 and T13 is also very high, and the median survival time of infants with these trisomies is about 10 days. About 5–10% of T18 and T13 infants survive to 1 year of age (7,8,9). These survival figures have not changed in recent years, whereas T21 survival data indicate greatly improved life expectancy. This is due primarily to the longer survival of infants with heart disease—over 80% survival in many centers (10,11). Survival has also increased because of treatment of infections with antibiotics and generally enhanced surveillance for the medical complications of DS. This improved life expectancy has implications for health care provision for older individuals with DS (12,13) as well as informing decisions regarding health maintenance and intervention in the infant and child (14,15). In developed societies, increasing life expectancy has been accompanied by a decreasing birth prevalence of DS because of prenatal screening programs that offer invasive and noninvasive prenatal diagnosis (3,16,17,18).

### 43.2 GENETIC COUNSELING IN THE TRISOMIES

The diagnosis of an autosomal trisomy carries with it general issues, applicable in many cases, and specific or unique

issues in each individual case. From the geneticist and genetic counselor's perspective, there is no single approach that is suitable for all who are affected by a trisomy. The diagnosis of the affected fetus or infant is a fact that is usually conveyed under difficult psychological circumstances. Parents need to be treated with respect and with cultural sensitivity. Certainly the approach is different in the prenatal setting versus the neonatal setting (19,20).

Parents should be intimately involved in all aspects of this communication process and have consistently reported that the words used when they are first informed have a permanent effect on their later adjustments to their new situation, their feelings about their child, and their relationships with health professionals (21). In general, since we can now anticipate a long lifespan for the child with DS, optimism and encouragement seem warranted and should be conveyed, especially when counseling the family of a newborn (21,22).

Very early involvement of the clinical geneticist and counselor are essential in the prenatal and postnatal management of trisomies 13 and 18. It is critical to assess the family unit in making management decisions and to involve the parents fully. Variables such as family and financial resources, impact on siblings, and likely burdens of care need to be compassionately explored in making these decisions (23). Prenatal diagnosis by amniocentesis or chorionic villus biopsy (and occasionally ultrasound only) can help a family reach appropriate decisions about continuation of the pregnancy and management of labor and delivery, as well as decisions with respect to the health care of their affected child. In trisomies 18 and 13, counseling should emphasize a realistic appraisal of their child's prognosis, with an emphasis on support and comfort care. In these two trisomies, heroic measures such as intubation and cardiac surgery are usually not indicated because of the early lethality of these conditions (8). Nonetheless, some families will request intensive support of their newborn, and these decisions need to be respectfully discussed by the health care team and the family. Ongoing grief support is valuable and

should be offered to these families who may suffer for months or years after the loss of an affected child.

The Internet offers increasing accessibility of information and support for families and many of these sites offer easy connections to locally available services. Families with access to the Internet should be supported in their efforts to seek outside information and geneticists and counselors should act as facilitators in directing families to reputable sites. Sites such as those recommended by the Medical Library Association or organizations such as the National Institutes of Health are reputable. A consumer and patient health information section of the Medical Library Association has a link that lists “General Health Websites You Can Trust.” A list of the top 100 can be found at <http://caphis.mlanet.org/consumer>.

Many communities have excellent local resources and support groups for parents of children with DS and the National Down Syndrome Society also offers extensive information for families (<http://www.ndss.org>). The “Beyond the Basics” patient information library is open to all on the web and can be accessed through a patient information Website ([www.uptodate.com/patients](http://www.uptodate.com/patients)).

Families should be cautioned against sites offering unorthodox treatment protocols or hopes for “cures.” Families of affected children are uniquely vulnerable to claims of treatment success and often may spend substantial amounts of money in the pursuit of an improved developmental outcome. Counseling should address these alternative therapies and help direct families to legitimate sources of help.

The need for accurate information and authoritative and consistent support for parents of children with trisomies 18 and 13 has led to several organizations such as SOFT US (Support Organisation for Trisomies 18 and 13) and SOFT UK ([www.soft.org](http://www.soft.org)). These groups most often offer support and solace for those parents of affected babies surviving the immediate neonatal period; they also offer support for families whose children have died.

In advising families, general principles of counseling are always relevant. Many parents know of DS from personal experience and seek to understand the range of associations and the plans for specific investigation and management at an early stage. An initial offer of basic information leaflets with telephone or Internet contact details, as well as details of a local parent support service, is appropriate.

Genetic counseling may be offered to the family at one or several different stages: at the identification of risk, at confirmation of diagnosis, or at the time of another pregnancy for the parents or family planning for the unaffected siblings. Each family will have different expectations of counseling and vastly different reactions to the information it brings (21,24). The counselor who helps parents adjust to the diagnosis of an autosomal trisomy needs insight, intuition and compassion.

## 43.3 DOWN SYNDROME (TRISOMY 21)

### 43.3.1 Cytogenetic Diagnosis

In DS, no single clinical feature is pathognomonic, although the combination of facial dysmorphic features is highly specific (25). Chromosome analysis is necessary in each case to confirm the diagnosis and to assess genetic implications for the family (26).

In 95% of DS cases there is a complete extra chromosome 21. This is most often due to a nondisjunction event at the first meiotic division, and the chance of this occurring rises in older mothers. In 4% of DS cases, the extra chromosome 21 is translocated to or fused with another large or small acrocentric chromosome (#13, 14, and 15 are D-group acrocentrics; 21 and 22 are G-group acrocentrics). This fusion, sometimes described as a whole-arm exchange, is also called a Robertsonian translocation in recognition of the American cytogeneticist Robertson's contribution from his studies of chromosome fusion in insect cytogenetics early in the twentieth century. Usually, the Robertsonian chromosome is dicentric, although it may appear monocentric because of suppression of one centromere.

Nonhomologous (sometimes called heterologous) Robertsonian translocations comprise fusion of two different acrocentrics and are more frequent than homologous Robertsonian translocations. The prevalence of the balanced rob(14q21q) translocation is about 1 in 10,000, and the rob(21q21q) is about one-third as frequent. About a third of translocation DS cases are inherited from one parent, and usually it is the mother who is the balanced carrier. De novo rob(14q21q) DS also originates most often in maternal germ cells (27) and is due to a meiosis II nondisjunction event with an unusual pattern of genetic recombination in meiosis I preceding nondisjunction in meiosis II (28). Homologous Robertsonian translocations such as rob(21q21q) are mostly isochromosomes but can be true translocations if they are formed by fusion of maternal and paternal homologs postconception (29). Only about 7% of DS individuals with rob(21q21q) or rob(22q21q) have a carrier parent, and the mother is usually the carrier.

### 43.3.2 Trisomy 21 Mosaicism

In 1% of cases, T21 mosaicism is present (30). The first case reported in 1961 by Clarke and colleagues (31) was a 2-year-old girl who was selected for detailed cytogenetic study because she had physical features of DS but superior intellectual development. This girl had T21 in 13% of cells from blood leukocyte cultures and 34% of cells derived from fibroblast cultures; the remaining cells were 46,XX. There is a general tendency for the proportion of trisomic cells to be higher in fibroblasts and in early life, so the proportion of trisomic cells in one individual is not necessarily constant over time (32). In

general, in mosaic T21 the clinical signs of DS are less prominent (32,33). Patients with low-level mosaicism will likely be increasingly recognized with the utilization of microarray (34). A low level of mosaicism in an unaffected individual may not have immediate clinical significance, but the mosaic trisomy parent may be at high risk of offspring with full trisomy (35).

### 43.3.3 Prenatal Diagnosis

The incidence of DS increases in a nonlinear fashion and ranges from approximately 1/1500 in women from 15 to 25 years to 1/10 for a 48-year-old woman. The incidence remains constant between 15 and 25 and then rises slowly between 25 and 35, increasing by a factor of 4 from 35 to 40 and by a factor of 10 between 40 and 45. Initially all screening efforts in DS were directed at women at age 35 and older. This age was picked by consensus as the risk at that time was thought to approximately equal the procedure-related risks and to be cost effective (36,37).

In practice, the actual procedure-related risks are lower than originally thought. Some families place a high value on the definitive knowledge from a diagnostic test, even if there is risk, as they want the option of ending an affected pregnancy. Other families, especially those who have had prior unsuccessful or high-risk pregnancies, will choose serum screening tests and ultrasound to avoid the potential use of an invasive test (38). Based on these considerations, in 2007, the American College of Obstetrics and Gynecology recommended that all women be offered serum screening prior to 20 weeks gestation and that all women should have the option of definitive diagnostic testing regardless of maternal age (39).

### 43.3.4 First and Second Trimester Screening

Serum screening in the first trimester (11–13 weeks gestation) looks at two serum analytes, pregnancy-associated plasma protein A (PAPP-A) and human chorionic gonadotropin (hCG). The abnormal values in DS are observed

empirically and are incompletely understood. Altered placental function is an attractive hypothesis (40). Beta hCG performs best between 9 and 13 weeks of pregnancy and performance increases with increasing gestational age (41). In contrast to hCG, PAPP-A performance declines with increasing gestational age between 9 and 13 weeks (42,43).

At the same time or shortly before or after first-trimester serum screening there should be ultrasound determination of the nuchal translucency (NT). A small space in the posterior neck is a normal finding in all first-trimester fetuses, but enlargement of this space is associated with an increased risk of DS as well as other abnormalities such as Turner syndrome, Noonan syndrome, or congenital heart disease (44). An enlarged NT space is usually associated with distended jugular lymphatics, which are commonly found in a number of disorders. Even in an abnormal fetus the enlarged NT often resolves later in pregnancy. Risks associated with an increased NT should not be revised downwards even if the measurements revert to normal later in pregnancy (42). The optimal timing for the determination of NT is 11 weeks, but this can be performed between 10 and 14 weeks gestation. Early studies of NT revealed significant intraoperator variability and variability based on the quality of the ultrasound equipment. NT techniques have been clearly delineated and involve measuring the nuchal fluid space from inner to inner borders in a midsagittal plane (Figure 43-1). Distinguishing the amnion, which may not separate from the chorion until 16 weeks, is important, as the amnion can be confused with the posterior aspect of the fetal skin. Proper training and quality verification are now required elements in screening programs utilizing NT (44,45). NT in combination with serum screening can be used to determine those at very high risk for DS and early invasive testing by chorionic villus biopsy can be offered. In the State of California the prenatal screening program has determined that NT measurements equal to or over 3.5 mm constitute a risk for aneuploidy of greater than 1/5 and that all such mothers should be offered invasive diagnostic testing regardless of serum screening results. Several studies have examined



**FIGURE 43-1** Features of Down syndrome. (a) Brushfield spots. (b) Typical hand configuration with transverse crease and fifth finger clinodactyly. (c) “Sandal” gap—increased space between toes one and two (d) Typical ear, which is small (<3%) with overfolded helix.



the risks associated with enlarged NT. In general, the greater the enlargement the higher the risk for aneuploidy. Most fetuses with DS had NTs less than 4.5 mm, whereas fetuses with Turner syndrome and trisomies 18 and 13 had NTs in excess of 4.5 mm (46). The distinction between an enlarged NT and cystic hygroma is not entirely clear (47). In general, cystic hygromas tend to be larger and extend along the length of the fetus and are more often septated. Size, however, is the major determinant of outcome, probably not the distinction between cystic hygroma and isolated increased NT (48).

The optimal detection of DS involves testing in both the first and second trimester plus NT measurements. As discussed earlier, screening in the first trimester consists of two analytes: PAPP-A and beta hCG, plus NT. In the second trimester there are four analytes, which include alpha fetoprotein (AFP), unconjugated estriol (uE3), hCG and inhibin A, and these should be measured between 15 and 20 weeks gestation. When there is no local availability of NT measurement, a serum integrated test result can provide risk information, which has the highest detection rate without an NT measurement.

The FaSTER (First and Second Trimester Evaluation of Risk) trial looked at outcomes of pregnancy in 38,000 women followed in 15 centers in the United States who underwent first- and second-trimester serum screening plus or minus NT. At a detection rate of 85% there was a 4.8% false-positive rate for first trimester plus NT; a 4.4% false-positive rate for serum integrated, a 0.8% false-positive rate with fully integrated (plus NT) and a 7.3% false-positive rate for quadruple screening (43,49).

Many mothers do not enter prenatal care until the second trimester, and for such women the quadruple screening test (AFP, uE3, hCG and inhibin A) offers the best screening strategy. Preferably such testing is done in conjunction with an ultrasound scan for fetal anatomy. This can help refine risks, although ultrasound alone should generally not be used as a primary screening tool.

The pattern of analytes differs in fetuses affected with DS, T18, and Smith–Lemli–Opitz syndrome. In DS, the AFP and uE3 are depressed and hCG and inhibin are increased. In T18, AFP, uE3 and hCG are all low, whereas inhibin is unchanged. No risk information is obtained for T13. Risk calculations include adjustment for maternal weight, smoking, diabetes and multiple gestation.

### 43.3.5 Ultrasound Markers in Trisomies 21, 18 and 13

The prenatal detection of aneuploidy is a major goal of prenatal detection programs. Fetuses with T21 and T18 frequently have ultrasound evidence of congenital anomalies and, in addition, have what have been termed “soft markers,” which increase the risk for aneuploidy, but are also frequently seen in normal fetuses. These soft markers have no clinical sequelae by themselves and are usually fleeting, resolving by late gestation. The ultrasound

finding of an enlarged NT has been discussed. Other markers seen more frequently in aneuploidy include echogenic bowel, absent nasal bone, pyelectasis, mild shortening of the long bones, echogenic intracardiac focus and choroid plexus cysts. These findings can be seen in 11–17% of normal fetuses, but the presence of more than one marker increases the risk for aneuploidy (50). There has been significant controversy over the reporting of echogenic foci and choroid plexus cysts, as they are most often associated with a normal outcome and these findings provoke parental anxiety and may lead to invasive testing. Review of the evidence suggests that the finding of an isolated echogenic focus in the heart in an otherwise low-risk woman does not increase the risk for aneuploidy (51–53). Choroid plexus cysts are present in 30–50% of fetuses with T18 compared to 1–3% of all second-trimester fetuses; however, most large studies suggest that an isolated choroid plexus cyst in the presence of an otherwise normal fetal ultrasound is associated with a normal karyotype (54,55). Combining multiple markers, including serum screening, fetal NT and the presence or absence of the nasal bone increases DS detection. In one study, looking at these variables between 11 and 13 6/7 weeks in 20,000 pregnancies, 90% of DS was detected with a 2.5% false-positive rate (56).

In the second trimester, ultrasound may help refine the diagnosis of chromosomal aneuploidy. Over 1/3 of fetuses with T21 have structural malformations, including cardiac defects, central nervous system (CNS) anomalies (especially ventriculomegaly), anomalies of the gastrointestinal tract (especially duodenal atresia), and facial findings including brachycephaly, small ears, cystic hygroma and a thickened nuchal fold. A thickened nuchal fold is the most sensitive (40–50%) and specific (99%) single marker for DS detection in the second trimester (57). Soft markers such as mild long bone shortening, fifth finger clinodactyly, and sandal gap great toe should not be used as in isolation to detect DS in an otherwise low-risk pregnancy. Studies have suggested that one should not use second-trimester ultrasound alone to recommend an amniocentesis in women who have had normal serum screening. A study of 9000 women with abnormal serum screening and a normal ultrasound revealed a reduced risk of DS; however, in one study, about 45% of DS would have been missed in the presence of abnormal biochemical screening and normal ultrasounds in women declining amniocentesis. In addition, with normal serum screening results and an ultrasound with positive soft markers, 1200 amniocenteses would need to be performed to identify one fetus affected with T21 or T18 (58).

In T18, several second-trimester findings help identify affected fetuses. These include overlapping fingers, clubfeet, components of the Dandy Walker malformation, oral clefts, cardiac defects, micrognathia, omphalocele, diaphragmatic hernia, renal abnormalities and single umbilical artery. Intrauterine growth restriction and polyhydramnios are also common ultrasound findings in



T18. The detection rate for T18 is higher than 90% by combining ultrasound findings with abnormal first and/or second-trimester serum screening. Conversely, if the ultrasound is normal the risk of T18, even with abnormal serum screening, is low (59,60).

T13, the rarest of the autosomal trisomies, is associated with more severe structural abnormalities. Seventy five percent of T13 fetuses die in utero. Useful ultrasound findings include holoprosencephaly, midline facial abnormalities, polycystic kidneys, cardiac defects, neural tube defects, postaxial polydactyly, ventriculomegaly and posterior fossa abnormalities. Since some of these abnormalities can be visualized at 11–14 weeks gestation, first-trimester diagnostic rates are high (61).

An exciting development in the prenatal diagnosis of the major trisomies has been the analysis of cell-free nucleic acids in maternal blood. The presence of fetal material in the maternal circulation has been known for some time. Initially in the 1990s, the isolation of nucleated red blood cells along with fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR) allowed for detection of T21. The comparable effectiveness of ultrasound and serum screening and their lower costs kept this technology from acceptance in clinical practice, however. The knowledge that 3–6% of the cell-free nucleic acids in maternal plasma are fetal in origin along with technical advances, including digital PCR and massively parallel genomic sequencing, has led to some optimism that first-trimester noninvasive screening of maternal blood will soon allow for prenatal diagnosis of the trisomies as well as single gene disorders (62–64).

The postnatal confirmation of the diagnosis of T21 is critical to rule out a translocation or mosaicism and usually involves routine karyotyping. Recent efforts to reduce costs using new molecular techniques such as short tandem repeats for chromosome 21 are promising (65).

### 43.3.6 Clinical Diagnosis

DS is characterized by a recognizable pattern of dysmorphic features, congenital malformations and other

health conditions. In newborns, eight cardinal signs are redundant: neck skin, down turned mouth corners, hypotonia, flat face, small typical ears, epicanthal folds, “sandal gap” between first and second toes, and protruding tongue (66) (Figure 43-1). Clinical experience is an excellent predictor of DS, but the occasional infant will be atypical and cause even the most experienced clinician difficulty in diagnosis (25). Severe prematurity may also lead to a late diagnosis, as the findings may be subtle in this group. Ethnicity affects ease of diagnosis: In South Africa, delayed diagnosis was common in black neonates in whom facial features of DS were less readily recognized by the attending medical staff and by infants’ mothers (67,68).

In children under 2 years of age, 10 discriminating clinical signs of DS are brachycephaly, oblique palpebral fissures, nystagmus, flat nasal bridge or root, narrow palate, folded ear, short broad neck, incurved fifth finger, sandal gap between great toe and second toe, and hypotonia (Figure 43-2) (69). The adult with T21 has facial features that have changed with age (70) (Figure 43-2). The nasal root becomes more prominent, epicanthal folds become less prominent, and increased growth of the lower face occurs, just as it does in chromosomally normal individuals (Figures 43-3 and 43-4). Older literature photographs of adult individuals with T21 tend to reinforce old stereotypes, emphasizing features such as obesity, abnormal tongue fissuring and an open mouth.

### 43.3.7 Differential Diagnosis

In practice, one-third or more of requests for karyotyping to rule out DS give a normal result (25,71), but published clinical follow-up studies on the development of the infants who test negative are lacking. More rarely, features of the DS phenotype are present in cases with cryptic chromosome 21q imbalance (72) or undetected mosaicism for T21 (30). In newborns, Zellweger syndrome or tetrasomy 12p syndrome may be initially mistaken for T21. In older individuals, the facial appearance of Smith–Magenis syndrome can resemble DS. A “new”



**FIGURE 43-2** The changing face of Down syndrome with age. (a) infant, (b) young child, and (c) 47-year-old adult (note hearing aid, gray hair, edentulous).



**FIGURE 43-3** Transient myeloproliferative disease in a hydroptic newborn with Down syndrome.

chromosomal syndrome that has a close facial resemblance is the microdeletion of chromosome 9q34 (73).

### 43.3.8 Development, Personality, and Intellectual Disability

The stereotype of DS is a floppy, sociable, undemanding infant who turns into a placid, affectionate, good-natured, music-loving, moderately intellectually disabled adult. In fact, individuals with DS are just as different from each other as their chromosomally normal peers: some are happy and others are sad, some are flighty and impulsive and others are careful and steady. Just as in the general population, a minority of individuals exhibit deviant behaviors or suffer serious conditions such as autistic spectrum disorders (74). Overall, few individuals with DS have serious behavioral disturbances (75,76). There does appear to be a distinctive neurocognitive phenotype (77).

Many changes in the brain and behavior in DS are described (78,79), and correlation of structural CNS findings with cognitive abnormalities has been studied (80). Delayed myelination, reduction in growth of frontal lobes, narrowing of the superior temporal gyrus, and reduction in size of the brainstem and cerebellum are commonly reported neuropathologic changes in infancy. In older children, there is reduced brain volume, especially affecting the hippocampus, and, from mid-adult life onward, the predominant neuropathologies are changes similar to Alzheimer disease (see later). In one study, when followed over time there was a decrease in IQ, correlating somewhat with the age-related findings of early dementia and depression in DS adults (80).

Developmental delay becomes apparent during the first year of life, with motor skills being the most obviously affected milestones. The average age of sitting is 11 months; crawling 17 months; walking 26 months. Children with DS develop speech in the same order as

typical children, but the rate is slow, with the average time of first words at about 18 months (81–83). Parents should be encouraged that almost all DS individuals will learn to talk, although language is probably the most impaired area of functioning, hampering attempts to integrate individuals with DS into the community and their efforts to live independently. Abbeduto and colleagues (84) present an extensive review of language development in DS.

Virtually all children with DS have intellectual disability. Most are mildly to moderately impaired, with IQs in the 50–70 or 35–50 range. Some are severely impaired, with IQs of 20–35. The most common pattern of impairment is for receptive language to equal the mental age of the child, with expressive language being more delayed. Vocabulary continues to increase and surpass mental age in adolescence (85). Selective learning problems such as difficulty understanding sequences or rules of grammar have been described (86).

The histories of DS and Alzheimer dementia (AD) have been linked for a very long time. The characteristic brain findings of AD, including neuritic plaques, which are extracellular deposits of amyloid beta protein in the cerebral cortex and neurofibrillary tangles, are seen in DS as early as age 8 and precede the development of clinical dementia. It has previously been thought that nearly all individuals with DS have characteristic brain changes by 35–40 years, accompanied by clinical signs of AD, particularly with frontal lobe involvement. More recently, studies have suggested that the risks are slightly lower, with 50–70% of DS individuals having AD by age 60–70 (87). Along with the AD phenotype there is evidence of generalized accelerated aging, documented by age-related skin changes, osteoporosis, osteoarthritis, hypogonadism and cataracts (88).

### 43.3.9 Growth

At birth, infants with DS show mild growth retardation, with mean birth weight, length, and head circumference lying between the 10th and 15th percentiles calculated for chromosomally normal infants (89). In a study of 105 children with DS, length, weight and head circumference were below those of typical children and remained lower until puberty, with the growth spurt occurring earlier than in the normal population (11 in boys and 9.5 in girls) (90). Growth charts for children with DS are available in the United States and in several other countries (91–94). Successful efforts have also been reported to produce specific growth data for use in an electronic medical record (95).

Obesity is common in DS (96), and it is suggested that all those over 5 years with weight over 75th percentile should have the body mass index (BMI) charted. BMI above 98th percentile is an indication for further assessment. The majority of children with DS are obese by age three to four (82). The prevalence of obesity in DS

(BMI > 27.8/kg/m<sup>2</sup> in males and >27.3/kg/m<sup>2</sup> in adult females) is greater than in the general population (45 and 56%). Obese women with DS may have better verbal memory, with a suggestion that higher endogenous estrogen levels are contributory (97). Obesity in DS is not due simply to increased food intake with decreased energy expenditure. A systematic review of published literature failed to provide convincing evidence that increased aerobic exercise will improve physical or psychosocial outcomes (98). Intuitively, however, scheduled training sessions in the company of peers encourage friendship and social opportunities. Exercise also helps to combat low bone mass and osteoporosis, which are more prevalent in adults with DS (99,100).

Short stature is common in DS, especially in infancy and adolescence. Stature is most reduced in children with severe congenital heart disease (101). In addition, several medical conditions causing poor growth, such as celiac disease, are more common in DS. Medical and dietary assessments are indicated when growth measurements lie below the second percentile (102). The cause of most short stature in DS remains unclear. Low levels of IGF1 and decreased levels of growth hormone have been reported in some DS children (103).

Children with DS who are not growth hormone deficient do show a height response to growth hormone therapy (104,105), but such treatment is largely undertaken on a research basis and is still being assessed in the longer term. Blanket prescription of growth hormone is not recommended in the absence of proven growth hormone deficiency (105). Results from a study in Sweden did not demonstrate behavioral and cognitive improvements in treated young children (104). A study in 2010 (106) on early growth hormone treated children with DS revealed a greater head circumference in DS as well as improved performance on the Leiter and WISC III as compared to control DS children. Further studies are needed.

### 43.3.10 Congenital Heart Disease

Congenital heart defects (CHDs) are present in approximately 44% of individuals with DS and all newborn infants should undergo echocardiographic examination in the neonatal period. In the Atlanta Down Syndrome project, the breakdown of defects in the children with DS was as follows. Atrioventricular canal defect, comprising atrial septal defect with abnormality of the atrioventricular valves and ventricular septum, with or without other lesions, was seen in 45% of all CHD cases (107). In fetal life about 50% of fetuses with isolated canal defects have T21. Isolated ventricular septal defect with or without other lesions was seen in 35%, isolated secundum atrial septal defect in 8% and patent ductus arteriosus in 7%. Tetralogy of Fallot and other complex lesions made up the remaining 5%. In two studies of adults with DS, mitral valve prolapse was found in 46%, mitral regurgitation in 17% and aortic

regurgitation in about 7% of patients (108,109). This has implications, as such individuals should probably have prophylaxis for dental work to reduce the risk of bacterial endocarditis (110,111).

Medical and surgical treatments of heart defects in children with DS are similar to the treatments used in the care of chromosomally normal children, with modern postoperative morbidity and mortality rates similar in both groups (112,113); however, pulmonary hypertension is more common in DS (114,115). Postulated precipitating factors include anatomic variations such as maxillary hypoplasia and macroglossia, hypertrophy of the tonsils and adenoids, and sleep apnea with or without upper airway obstruction. Coronary artery disease, typically said to be rare in DS, may become more frequent as the population of older DS individuals increases (116).

### 43.3.11 Oral Health

Dental problems are common in DS. Malocclusion, tooth grinding, and tongue thrusting can impair chewing, while periodontal disease, akin to juvenile periodontitis, is especially common and may reflect an underlying immunologic predisposition. The tendency to poor oral health in DS children can be improved by regular dental care, at least as indicated by some studies (110,111,117). Despite the prevalence of the periodontal problems, caries may actually be less prevalent in DS individuals.

### 43.3.12 Gastrointestinal Complications

Gastrointestinal tract anomalies occur in about 5% of children with DS (118). Two major gastrointestinal anomalies associated with DS are duodenal atresia and Hirschsprung disease. About 2.5% of infants with DS have duodenal atresia or stenosis, sometimes in association with annular pancreas (86). About 20–30% of children with duodenal atresia have T21. Slightly less than 1% of patients with DS have Hirschsprung disease, which is higher than that in the general population (119). Two percent of patients with Hirschsprung disease have T21. A small number of T21 infants have both duodenal atresia and Hirschsprung disease, and in such cases dual pathology causes diagnostic problems because the presenting features of duodenal atresia overshadow signs of Hirschsprung disease. For these two major gastrointestinal anomalies, surgical outcome in the short term can be just as good as with chromosomally normal children, but presence of additional cardiovascular and respiratory malformations may lead to increase in delayed mortality (120–122).

Other congenital intestinal abnormalities that occur more frequently in T21 infants include tracheoesophageal (TE) fistula, imperforate anus, omphalocele, duodenal bands, annular pancreas, ileal and jejunal atresias, and microcolon (123). Esophageal motor disorders,



especially achalasia, are underdiagnosed complications of DS (124,125).

Even in the absence of Hirschprung disease, constipation is a common complaint in children with DS, and contributing factors are certainly hypotonia and relative inactivity. In the adult with DS chronic unexplained diarrhea may be seen in up to 20% of individuals. In addition, *Helicobacter pylori* infection has been found in as many as 66% of DS individuals (126).

Celiac disease has a biopsy-proven prevalence of between 5 and 16% in DS individuals. In order to avoid harmful consequences of undiagnosed and untreated celiac disease, serologic screening of children and adults using antiendomysial antibodies or tissue transglutaminase has been recommended, with biopsies for screen-positive cases (127,128). Positive cases should undergo small bowel biopsy. Negative cases should be rescreened at intervals or have human leukocyte antigen (HLA) typing that shows that they are not HLA DQ2 or DQ8 (129).

### 43.3.13 Otolaryngologic Problems

Ear, nose, and throat (ENT) complications cause much morbidity in DS, and upper airway problems can be life-threatening (130). Anatomic abnormalities affecting the external and internal ear contribute to the high prevalence of middle ear infection and hearing impairment. Hearing deficits occur in 38–78% of individuals with DS (131). The majority of DS children have a conductive hearing loss, 90% of which is caused by otitis media with effusion. Stenotic ear canals, impacted wax, ossicular chain, and cochlear malformations are also common. Ideally, all infants with DS should undergo newborn hearing screening and be followed by clinical examination, pure tone or behavioral audiometry, and tympanometry. It has been proposed that audiologic assessments with maximum frequency between 6 months and 1 year be performed, lessening in frequency thereafter and continuing into adulthood (132).

For infants with hearing impairment, there is a trend toward more aggressive treatment of conductive hearing loss and early amplification (131), but early insertion of polyethylene tubes and their regular replacement is not without complication and does not guarantee improvement in expressive language.

One upper airway abnormality that merits special mention is obstructive sleep apnea, a much underdiagnosed abnormality with multiple causes (133,134). Poor growth, noisy breathing, apnea–hypopnea, disturbed sleep, daytime sleepiness, and deterioration in behavior can all be consequences of nocturnal oxygen desaturation and chronic hypoxia. Treatments are not always successful but should be individually tailored in each case, with consideration given to weight control, relief of nasal allergies, and continuous positive airway pressure during sleep (135).

### 43.3.14 Ophthalmologic Disorders

Minor ocular findings in children and adults with DS include epicanthal folds, up-slanting palpebral fissures, and Brushfield spots on the iris (Figure 43-1). Asian children with DS seldom have Brushfield spots but do have epiblepharon and exotropia (136). Brushfield spots are also difficult to see in children with brown irides.

More important ocular complications, such as major refractive errors, cataract, glaucoma, nystagmus (137), nasolacrimal outflow drainage anomalies (138), and keratoconus (139), are important to detect. Therefore, all individuals with DS should have frequent visual screenings, starting in the neonatal period. One protocol suggests first examination at 1 month of age, then at 1 year of age, at 2–3 years of age, at 5–6 years of age (school start), and every 5 years thereafter (140). This should detect the accommodation deficit that is present in a majority of individuals and permit prescription of bifocal or progressive lenses.

### 43.3.15 Hematologic Disorders

Abnormalities affecting red cells, white cells and platelets are common in DS. At birth, 65% of DS infants have polycythemia (141). Interestingly, the mouse model of T21 demonstrates many of the same hematologic features of human DS including thrombocytosis, macrocytosis and a myeloproliferative disorder (142,143). Neutropenia and macrocytosis are also common in DS and the reasons are not known. Transient myeloproliferative disease (TMD) or transient leukemia almost exclusively affects infants with DS. Recent work in another mouse model for DS suggests that trisomy for the gene *ERG* may underlie this phenotype, as reduction to disomy for *ERG* corrected the myeloproliferative phenotype (144). The rate of TMD diagnosed prenatally or postnatally is 20%.

A reported prenatal presentation of TMD is that of generalized hydrops and is generally fatal (145) (Figure 43-3). Most cases of TMD are asymptomatic with resolution by 2–3 months of age, but some have severe disease (146,147). Presenting symptoms can include hepatosplenomegaly, obstructive jaundice, liver failure and ascites. Laboratory findings include leukocytosis; reduced, raised, or even normal platelet counts; and low, high, or normal hemoglobin. TMD is characterized by the presence of blasts in peripheral blood from a few to >200,000  $\mu$ L. With time the blasts decrease spontaneously. Unlike other forms of leukemia, the percentage of blasts in bone marrow is lower than in peripheral blood. Vesiculopapular skin findings are common, which may be a clue to the presence of TMD, and these findings resolve with hematological improvement (148,149).

Although spontaneous resolution in the first 3 months is usual, about 25% of affected children will, within 4 years, develop myelodysplastic syndrome and acute



megakaryoblastic leukemia (AMKL) (150). Rarely, life-threatening complications occur, including hepatic fibrosis and cardiopulmonary disease (151–154). White cell counts >100,000, direct hyperbilirubinemia, prematurity and hydrops with ascites are associated with increased risk of early death. Most patients do not require chemotherapy for TMD, but cases that do not resolve spontaneously or the high blast counts or liver dysfunction occasionally improve with low-dose cytosine arabinoside. Usually there is rapid response, with clearing of blast cells after about 7 days of treatment (155). When there is liver fibrosis the prognosis is poor, even with chemotherapy. The overall mortality in TMD is about 20% (154).

T21 specifically predisposes to leukemia, with a risk of 1–1.5% (156). The type of leukemia that has the highest relative risk of occurrence in T21 is AMKL. This leukemia arises in cells carrying somatic mutations in *GATA1*, an X-linked gene encoding a hematopoietic transcription factor seen invariably in AMKL, occurring prior to age four. In contrast, acute myelogenous leukemia diagnosed after age four is usually negative for *GATA1* mutations.

The leukemic cells in AMKL that carry somatic mutations in *GATA1* have been identified in transient leukemia blasts at birth, indicating that the somatic mutation occurs in utero (157). In one study of *GATA1* mutations, three of four children with a *GATA1* mutation detected in newborn screening blood spots had AMKL diagnosed at 12–26 months of age (158). Identification of *GATA1* mutations at birth could serve as a marker for the development of TMD and later AMKL. In a 2011 study, 88% of 134 DS patients with TMD had *GATA1* mutations and 85% of those DS patients with AMKL and DS had *GATA1* mutations (159). Thus, it is hypothesized that the leukemia that arises in DS with somatic *GATA1* mutations is a model for the stepwise process of leukemic transformation.

Another theory proposed by Da Vita and colleagues (160) is that disturbed early hematopoietic differentiation could be the cause of increased leukemia risk. They postulate that if a common mechanism is behind the risk of both major leukemia types, it would have to arise before the bifurcation to myeloid and lymphoid lineages. Using mouse embryonic stem cells bearing an extra human chromosome 21 they analyzed the early stages of hematopoietic commitment in vitro. They concluded that overdose of more than one extra 21 gene contributes to the disturbance of early hematopoiesis in DS and that one of the contributors is the gene *RUNX1*. As the observed hyperproduction of multipotential immature precursors in T21 precedes the bifurcation to lymphoid and myeloid lineages, they speculate that this could create conditions increasing the chance for acquisition of preleukemogenic rearrangements/mutations in both lymphoid and myeloid lineages during fetal hematopoiesis, thus contributing to the increased risk of both leukemia types in DS.

The risk of developing acute lymphoblastic leukemia (ALL) in DS is 10–20 times higher than in control children, accounting for 1–3% of children with ALL (161). Clinical symptomatology is similar to that seen in patients without DS. Mediastinal mass and CNS leukemia, unfavorable signs, are less likely to occur in patients with DS, as are T-cell leukemia and translocations 9;22 and 4;11. DS children, usually less than 10 years of age, respond to chemotherapy as well as control children with ALL. Children with DS are more likely to experience severe toxicity with standard chemotherapy regimens, particularly those requiring methotrexate, and often require reduced doses of chemotherapy (162,163).

Children with DS and ALL have an increased number of deaths caused by infection, a decreased five-year survival and more treatment-related complications as compared to children with ALL who do not have DS (150). These findings emphasize the need for providing aggressive supportive care for patients with DS and ALL.

### 43.3.16 Immune System Abnormalities

Abnormalities in virtually all components of the immune response have been reported in persons with DS. Commonly, immune system disturbances in DS manifest as a predisposition to infection, malignancies and autoimmune disorders, including celiac disease, diabetes mellitus, and autoimmune thyroiditis. Infections in DS are likely to be more severe and last longer than in typical children, and despite advances in treatment of infections, hospitalization due to infection is significantly more common in DS children. Immune factors are thought to play a role, but certainly the presence of tiny ear canals, gastroesophageal reflux and a small midface also contribute to infection risk.

The abnormalities of the immune system associated with DS include mild to moderate T- and B-cell lymphopenia, with marked decrease in naive lymphocytes, impaired mitogen-induced T-cell proliferation, reduced specific antibody responses to immunizations and defects of neutrophil chemotaxis. Limited evidence of genetic abnormalities secondary to trisomy of chromosome 21 affecting the immune system is available, such as the potential consequences of gene overexpression, most significantly superoxide dismutase (*SOD1*). Decreased intracellular killing in neutrophils is the most likely manifestation of a dosage effect from increased *SOD1* (164,165).

In a 2010 study, B-lymphocyte subpopulations in 95 children with DS were compared with 33 age-matched control children. DS serum immunoglobulin levels were compared with those of 962 non-DS children with recurrent infections. Transitional and naive B lymphocytes were profoundly decreased in the children with DS. This could be caused by an intrinsic B-lymphocyte defect resulting in (partial) failure of B-lymphocyte generation, decreased antigen-induced proliferation and/or increased

apoptosis, decreased proliferation due to deficient T-lymphocyte help, or a combination of these. The decreased number of CD27, CD21, and CD23 cells was reminiscent of common variable immunodeficiency and suggestive of disturbed peripheral B-lymphocyte maturation. Immunoglobulin levels in DS are subtly abnormal and different from those in non-DS children with recurrent infections. These authors concluded that the humoral immune system is abnormal in DS, but they could neither find a relation between B-lymphocyte subsets, immunoglobulins and clinical features of the children with DS nor answer the question whether DS lymphocytes are truly intrinsically deficient or are all findings explained by deficient T-lymphocyte helper cells (166). A 2011 study confirms the subtle abnormalities in the immune system and reports a normal antibody response to influenza vaccine but an impaired response to pneumococcal vaccine (167).

Immunologic alterations are often age related and may be one feature of a general, early-onset senescence in DS (168). The thymus in DS has histologic abnormalities that include reduced cortical area, thymocyte depletion, loss of corticomedullary demarcation, enlarged cystic Hassall corpuscles, and evidence of defective expansion of T-cell precursors. Reduced expansion of T-cell precursors may lead to an incomplete cell repertoire and abnormality of cell-mediated immune response. Evidence suggests that reduced thymic output and not peripheral output or T-cell dysfunction contributes the immunologic features of DS (169).

Most DS individuals do not have critical immunodeficiencies, but further study is needed to understand the immunologic role in their repeated infections. Immunologic evaluation should probably be reserved for those DS children with unusual and/or repeated infection, autoimmune disease, or other evidence of immunodeficiency.

Children with DS should receive the full childhood immunization schedule, including yearly influenza immunizations and pneumococcal vaccine. It is especially important for these children to receive hepatitis B immunization, as there is a significant risk for chronic carrier status (126).

### 43.3.17 Endocrine Disorders

Newborn infants with DS have levels of thyroid-stimulating hormone (TSH) and thyroxine slightly shifted to the right and left, respectively, compared with the general newborn population (170). A recommendation that all DS neonates should be treated for “mild congenital hypothyroidism” on the basis of evidence that mental and motor development is improved in thyroxine-supplemented infants (171) requires further assessment. Treatment is definitely required for severe congenital hypothyroidism due to thyroid gland dysgenesis. There has been the general conviction that hypothyroidism is frequent in DS, with a frequency varying between 3 and

54% in DS adults (172). In view of the high frequency of hypothyroidism and the nonspecificity of symptoms and clinical signs such as lethargy, increased weight, constipation, depression, and dementia, regular surveillance seems indicated. Health care guidelines published by the American Academy of Pediatrics (15) suggest regular checks of thyroid antibodies and thyroxine and TSH measurements at 1–2 year intervals. A 15-year follow-up study of hypothyroidism in 200 DS adults suggests that clinical hypothyroidism is relatively uncommon and that screening guidelines could be revised to suggest testing only every 5 years (173).

The risk of type 1 diabetes appears to be increased in DS (174). Interestingly, in one study, age of onset of diabetes was significantly earlier than in controls but diabetic control was better than in controls and less insulin was required. Accompanying additional autoimmune disorders were more frequent than in controls with type 1 diabetes (175). Diabetes was more likely to occur in females and in those with obesity (176).

Fertility in DS differs by sex, with males being generally infertile. This is probably due to impaired spermatogenesis. A 2002 study of young adult males with DS revealed normal levels of FSH, testosterone and dehydroepiandrosterone but showed elevated levels of luteinizing hormone (LH) and 17-OH progesterone (100). Rarely there have been reports of fertility in males. Females with DS appear to have normal menarche (12.6 years). There may be subtle abnormalities of the pituitary–adrenal axis, with increased levels of prolactin, LH, testosterone and 17-OH progesterone (100). DS women appear to have reduced fertility, but this may be due to lack of opportunity. Pregnancy has been reported on multiple occasions. Appropriate counseling and contraception are indicated for DS women. A higher rate of early menopause has been reported in women with DS (177).

### 43.3.18 Craniovertebral Junction Abnormalities, Including Atlantoaxial Subluxation

Atlantoaxial subluxation is a serious but infrequent complication of DS that causes neurologic symptoms and signs of spinal cord compression, including sudden or gradual onset of neck pain or postauricular pain, head tilt, ataxia, limb weakness, loss of bowel or bladder control, increased tendon reflexes, and spasticity. Such signs may occur at any age and are an indication for immediate referral to a specialist for investigation and, in some cases, surgical treatment (178).

In contrast, atlantoaxial instability is defined radiologically as an atlantoaxial gap of greater than 4 mm that may be observed, without any symptoms or clinical signs, in lateral-view radiographs of the cervical region in full flexion and extension. Instability probably does not predict increased risk of cord compression and seems to

have little or no predictive value for subsequent acute dislocation or subluxation at the atlantoaxial joint, such as might occur with a sports trauma or neck manipulation in the course of unguarded anesthesia (179,180). The American Academy of Pediatrics committee on genetics advises that a single radiograph should be obtained and such a radiograph may be required by organizations such as the Special Olympics prior to the child's or adult's active participation (15).

In older DS adults, the degree of ligamentous laxity lessens but degenerative cervical spine abnormalities may occur.

### 43.3.19 Neurologic Problems

Epileptic seizures are more common in DS individuals than in the general population. The prevalence of seizures peaks in infancy and in late adult life. Diagnosis remains clinical and is crucially dependent on a reliable history and accurate observation (181). The electroencephalogram may clarify the type of epilepsy and help manage treatments of certain epilepsies. Infantile spasms, myoclonic epilepsy, clonic seizures, reflex atonic seizures, and febrile seizures have all been reported.

In adults, onset of epileptic seizures, especially myoclonic epilepsy, is associated with the onset and progression of dementia (182,183). Other signs of dementia are personality change, cognitive decline, gait deterioration, loss of daily living skills, and incontinence. Functional decline in adults with DS is also a feature of treatable conditions such as depression and hypothyroidism.

The prevalence of dementia in DS rises from 10% at 50 years to more than 75% at over 60 years of age. Males with DS are more likely to become demented. Genotyping within 43 single-nucleotide polymorphism (SNPs) within 28 genes revealed significant associations with *APOE*, *SORL1*, *RUNX1*, *BACE1* and *ALDH18A1*, with the strongest associations with *APOE* (184).

Alzheimer's in DS is characterized by a specific type of slow and progressive neurodegeneration, which involves the abnormal hyperphosphorylation of the microtubule associated protein (MAP) tau. This hallmark, called neurofibrillary degeneration, is seen as neurofibrillary tangles, neuropil threads, and dystrophic neuritis. Apparently required for the clinical expression of AD, and related tauopathies, it leads to dementia in the absence of amyloid plaques. While normal tau promotes assembly and stabilizes microtubules, the nonfibrillized, abnormally hyperphosphorylated tau sequesters normal tau, MAP1 and MAP2 and disrupts microtubules. The abnormal hyperphosphorylation of tau, which can be generated by the catalysis of several different combinations of protein kinases, also promotes its misfolding, decrease in turnover, and self-assembly into tangles of paired helical and or straight filaments. Some of the abnormally hyperphosphorylated tau ends up both amino and C-terminally truncated. Disruption of microtubules by

the nonfibrillized abnormally hyperphosphorylated tau, as well as its aggregation as neurofibrillary tangles, probably impair axoplasmic flow and leads to slow progressive retrograde degeneration and loss of connectivity of the affected neurons (185).

Neuropathologic studies of adults with DS reveal cerebral amyloid (amyloid) plaques and neurofibrillary tangles. In young DS patients, there is evidence of intraneuronal amyloid in the hippocampus and cerebral cortex, and it is proposed that intracellular deposition precedes extracellular amyloid, followed by diffuse and neuritic plaques in the third decade and neurofibrillary tangles after age 40 years (186,187).

Patients with Alzheimer dementia have reduced cerebral production of choline acetyl transferase, which leads to a decrease in acetylcholine synthesis and impaired cortical cholinergic function. Adults with T21 might benefit from acetylcholinesterase inhibitors, which in clinical trials have conferred benefits to mildly and moderately affected Alzheimer disease patients. Results from treating small-sample-size populations of DS individuals have been favorable, but larger randomized studies are required (188).

### 43.3.20 Trisomy 21: Factors Influencing Chromosome Nondisjunction

Hassold and Sherman (189) reviewed their own studies and those of others who examined chromosome 21 DNA polymorphisms to demonstrate that approximately 90% of T21 cases result from a maternal meiosis nondisjunction error, 75% of which occur at maternal meiosis I. In cases in which the extra chromosome is paternal in origin, meiosis I and meiosis II nondisjunctions are equally frequent. The low frequency of a paternal origin may be due to a very low percentage of testicular mosaicism as compared to that seen in the ovary (190). Mosaic T21 may have postzygotic origin or be caused by a maternal meiotic error leading to trisomy in the zygote followed by loss of the extra chromosome during a mitotic cell division (191).

Chromosome recombination in oocytes occurs before birth, and the pattern of chromosome 21 genetic recombination affects susceptibility to nondisjunction (192,193,194). In one study, younger mothers of T21 cases had more pericentromeric and telomeric exchanges that increase susceptibility to nondisjunction, whereas older mothers of T21 cases had exchange patterns that mimicked the patterns observed in normally disjoining chromosome 21s. This, it was argued, suggested that the greatest risk factor for nondisjunction in younger women is a susceptible exchange pattern (195). The "two-hit" model suggests that ovaries of older women may be less efficient at "rescuing" the susceptible-to-nondisjunction meiosis I exchange pattern because of age-related perturbations in meiotic machinery (194). The spindle checkpoint delays the cell cycle when meiotic or mitotic

chromosomes are not properly attached to the spindle; spindle checkpoint proteins are being investigated to determine if gradual decline in the efficiency of the checkpoint explains maternal age-related probability of aneuploidy (196,197).

As maternal age-adjusted DS rates vary little across human populations (198), these are unlikely to be greatly influenced by environmental factors such as periconceptional multivitamin use (199), but putative associations such as between poor socioeconomic status and maternal meiosis II error, and between parity and DS, have been examined (200,201). Other factors put forward that might influence nondisjunction rates include changes in follicular development unrelated to the size of the oocyte pool (202,203); reduced ovarian complement, whether due to congenital absence or surgical removal (204); and accumulation of spontaneous mitochondrial DNA deletion mutations that diminish the supply of energy to cells surrounding the oocyte (205). Inheritance of methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms has been linked to chromosome 21 nondisjunction. Examination of transmission frequencies of the MTHFR 677T and 677C alleles from heterozygous parents to children with DS revealed that the 677T allele was transmitted to children with DS at a significantly higher rate and the 677C allele was transmitted at a significantly lower rate (206). Most conceptions with T21 end in pregnancy loss, and it was proposed that preferential transmission of the 677T allele in this population of live-born infants with DS could reflect a survival advantage. A putative association between birth of a child with folate-associated neural tube defect or hydrocephalus and increased risk of T21 is controversial (207,208). A 2010 study looking at predisposition to congenital heart disease in DS studied a group of 121 case families (mother, father, and proband with DS and CHD) and 122 control families (mother, father, and proband with DS and no CHD); tag SNPs were genotyped in and around five folate pathway genes: 5,10-MTHFR, methionine synthase (MTR), methionine synthase reductase (MTRR), cystathionine beta-synthase (CBS), and the reduced folate carrier (SLC19A1, RFC1). SLC19A1 was found to be associated with CHD using a multilocus allele-sharing test. In addition, the known functional polymorphism MTHFR C1298A was overtransmitted to cases with CHD and undertransmitted to controls. The authors concluded that the disruption of the folate pathway contributes to the incidence of CHD among individuals with DS (209). In summary, altered patterns of genetic recombination appear to be a common risk factor for T21 and for other maternal meiosis I trisomies, but the size and direction of the alteration varies with the chromosome involved (210). In addition, alterations in the folate pathway genes may impact the occurrence of congenital heart disease and possibly other defects in DS. A chromosome-nonspecific tendency to nondisjunction may explain why there is a slight excess of

other autosomal aneuploidies in women who have had a DS pregnancy at a young age (see “Down Syndrome Recurrence Risks” later) (211,212).

### 43.3.21 Chromosome 21 Down Syndrome Critical Region

Identification of cases with clinical features of DS in association with duplication of specific regions of chromosome 21 (Figure 43-5) led to the designation of a “Down syndrome critical region” at 21q22qter, hypothesized to harbor the most influential genes, extra copies of which determined most if not all DS features (213). At the same time, the “developmental instability” hypothesis proposed that DS features arose from specific disruptions of genetic homeostasis rather than from direct gene dosage effects (214). Fitzpatrick (215) incorporated both ideas in a testable hypothesis, proposing primary gene dosage abnormalities with sequential and complex transacting effects on disomic genes. Experimental evidence followed publication of the chromosome 21 DNA sequence with detailed annotation revealing over 200 genes, which is only half the number of genes on chromosome 22 (216,217).

Mouse models of DS have been generated because of the synteny existing between human genes on chromosome 21 and mouse genes on chromosome 16 (26.5 Mb), chromosome 10 (2.3 Mb) and chromosome 17 (1.1 Mb). Two kinds of murine models have been developed to investigate the molecular genetics of DS: segmental trisomic and transgenic models (218). The trisomic mouse mimics much of the clinical phenotype of DS, but single gene effects are difficult to analyze. In the transgenic mouse the effects of one or few genes can be studied in detail. Detailed statistical analysis of cranial shape in different trisomic mouse models (Ts1Cje, Ts65Dn, and Ms1Rhr/Ts65Dn) have shown that DS critical region genes alone are neither sufficient nor (largely) necessary to produce characteristic mouse craniofacial dysmorphology (219). This important study proposed that, in DS, triplicated genes have inconspicuous solitary effects but do contribute to the overall phenotype in combination with other genes, through as yet unidentified specific effects and interactions. Gene expression studies in Ts65Dn mice also point to complex regulation of expression of the aneuploid genes, since only a third are expressed at the theoretical 1.5-fold level, with significant proportions being expressed at levels above and below 1.5-fold (220). In yet another mouse model, there is evidence that triplication of the genes in the DS critical region does confer a characteristic neurological and behavioral phenotype (221). In several transgenic mouse models the important genes in the critical region, DYRK1A and RCAN1, have been shown to contribute to the learning and memory deficit, altered synaptic plasticity, impaired cell cycle regulation, and AD-like neuropathology in DS (222). Increasing evidence suggests that there is not



a single DS critical region but a number of susceptibility regions on chromosome 21, which are modified by other genes on chromosome 21 and genes elsewhere in the individual's genome. A review by Ruparelia and colleagues (223) summarizes how overexpression of genes on chromosome 21 contributes to the pathogenesis of DS. Whether or not the mouse phenotypes are directly applicable to high-level behavior and learning necessary for normal human development remains unclear. Nevertheless, better understanding of the complex genetic origins of the abnormal behaviors in the mouse model as well as illustrative human case reports, aid human DS genotype-phenotype analysis (224,225).

### 43.3.22 Down Syndrome Recurrence Risks

**43.3.22.1 Trisomy 21 and Mosaic Trisomy 21 Recurrence Risks.** Hook (226) summarized data on the chance of recurrence of T21 in children of mothers who had one live-born affected infant: when the mother was of age under 30, the recurrence risk was 1.4%, and this figure was clearly increased above the background rate of about 0.7%. But when the mother was older, the T21 recurrence risk was not clearly increased above the background maternal age risk. Hook also reviewed evidence from amniocentesis data that older women who had their first affected pregnancy under age 30 were more likely to have greater underlying predisposition to recurrence compared with older mothers who had their first affected pregnancy after age 35 years.

Hook's conclusions were confirmed in three more recent studies. Employing North American, United Kingdom and Australian data, Warburton and colleagues (227), De Souza and colleagues (212) and Morris and coworkers (5) compared observed numbers of trisomies at prenatal diagnosis with expected numbers. Comparisons of recurrence rates after diagnosis of the same trisomy (homotrisomy) and of a different trisomy (heterotrisomy) showed that younger women had the highest recurrence risk for homotrisomy, with both the index T21 and subsequent prenatal diagnosis at age less than 30 years. The risk was less for women with both pregnancies at age greater than or equal to 30 years. The study from the United Kingdom (4) showed women who had had a previous DS pregnancy have a constant absolute excess risk above their maternal age-related risk of having a subsequent affected pregnancy. The excess was determined by the age at which the affected pregnancy occurred and was considerably higher for women less than 30 years and negligible in women greater than 40 years.

When counseling families, these various recurrence risks may be perplexing. One approach is to emphasize that the overall chance of recurrence of T21 is still low: at about 1% at the time of prenatal diagnosis for mothers less than 30 years of age, this figure represents a six- to eightfold increase in the age-related risk; for mothers aged 31–40 years who had the index case in their 30s, the

age-related risk may be doubled; and for mothers over 40 years, there is negligible change in the maternal age-related risk. The chance of recurrence for any other viable trisomy after an index case with T21 may be double the age-related risk.

#### 43.3.22.2 Two Previous Trisomy 21 Conceptions.

About 3% of couples have a greater risk of recurrence of DS because of the presence of somatic or gonadal chromosome mosaicism in one parent (228). Such couples might have three or four pregnancies affected by trisomy 21. De novo recurrence of the same trisomy is another possibility, but studies suggest that the origin of recurrence is much more likely to be due to low-level oocyte mosaicism (228,229). Kovaleva (228) and Delhanty (229) evaluated records or literature reports on 80 families with recurrent T21. Postzygotic rescue of T21 due to error in meiosis I was proposed as a mechanism of parental gonadal mosaicism formation in 78% of the families with known origin of the T21. For the other 22%, rescue of errors in meiosis II or postzygotic mitotic nondisjunction was assumed. Mosaicism for T21 in successive generations has been reported in at least 12 families. Kovaleva and Delhanty (229) hypothesize that in many cases the mother was a trisomic conception with postzygotic trisomy rescue. It is interesting that in proven maternal mosaicism the maternal grandmother was over 35 at the time of the mother's conception. Parental gonadal mosaicism (mostly maternal) may contribute significantly to the occurrence and recurrence of DS in young women. In counseling of families with a recurrence, a suggested 10–20% represents an educated guess for the risk of a third pregnancy with T21. Preimplantation genetic diagnosis may be an option for some families with an apparently high recurrence risk (230).

**43.3.22.3 Parent with Trisomy 21.** Women with T21 may become pregnant, and in this situation the risk of T21 in the offspring is close to 50%. Fertility in males with T21 is exceptional, but there are at least three well-documented reports of an affected male fathering an unaffected child (231).

**43.3.22.4 Translocation and Isochromosome 21 Down Syndrome.** Following the diagnosis of translocation 21 or isochromosome 21 DS, if both parents have normal blood chromosomes, the translocation or isochromosome is usually presumed to have arisen as a result of a new mutation (de novo), and the observed recurrence risk in a series of translocation cases was less than 1% (232). Gonadal mosaicism for isochromosome 21 has been reported and recurrences of DS are recorded (233), so a rounded-up 1% risk of recurrence is appropriate, and prenatal cytogenetic diagnosis should be offered to cytogenetically normal parents who had a fetus or child with DS due to a de novo structural abnormality.

Where one parent is shown to carry a balanced Robertsonian translocation that has given rise to a child affected by DS, the risk of recurrence mainly depends on the sex of the carrier parent: if the mother is a

rob(14q21q) carrier, the risk of recurrence at amniocentesis is 15% (closer to 10% at term because of fetal demise after 16 weeks' gestation), compared with a much lower risk of less than 5%, even less than 1%, if the father is the translocation carrier. In parental translocation carriers of both the 14/21 translocation and the 13/14 translocation recurrence risk data was confirmed in a study of embryos from preimplantation genetic diagnosis (234). Although data are scanty, the same risks and sex difference most probably apply to other heterologous Robertsonian translocations involving chromosome 21 (rob(13q21q), rob(15q21q), and rob(21q22q)).

Translocation interchange T21 is a rarer cytogenetic variant that has been reported in more than 20 families with reciprocal translocations involving chromosome 21q. Usually, a large chromosome (numbers 1 through 12) is involved, and there is underlying 3:1 segregation, most often in the carrier mother, with a high risk of recurrence (235).

**43.3.22.5 Family History of Down Syndrome.** When the proband has confirmed T21, relatives other than the proband's parents may be advised that their positive family history does not confer an appreciably increased risk of T21, except, of course, when the family history absolutely suggests otherwise. Gair and colleagues (236) reported one such family with four cases of T21 in three generations.

In the case of an affected individual with unknown karyotype, based on the mother's age at the birth of the proband, the probability of translocation DS is very low when the mother is over 35 years and no greater than 10% at the youngest maternal age. Therefore, chromosome analysis carried out on a parent with a positive family history of DS rarely discloses a balanced Robertsonian translocation. Chromosome analysis may be offered to an expectant relative with explicit understanding that a normal result does not abolish risk of an abnormal pregnancy outcome. Aside from other chromosomal or inherited syndromes being mistaken for DS, cryptic or submicroscopic translocation involving chromosome 21q may cause recurrent DS with a normal karyotype (72).

## 43.4 TRISOMY 18

T18, or Edwards syndrome, was first diagnosed cytogenetically 1 year after T21 (237). It is the second most common trisomy. Neonatologists and pediatricians are usually familiar with the characteristic presentation and medical course of this serious trisomy.

### 43.4.1 Clinical Diagnosis

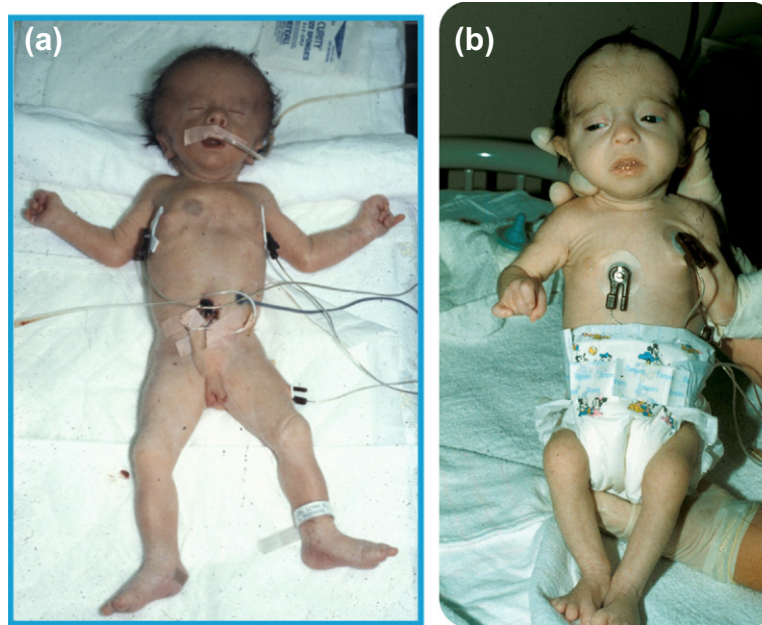
T18 is usually not a difficult clinical diagnosis, but may present difficulties to less experienced clinicians and in the setting of a neonatal resuscitation when the phenotype may be more challenging to appreciate. The

craniofacial features include dolichocephaly and a small triangular face that is seen by parents as "cute" but to the professional as distinct. The forehead is high, occupying more than one-third of the face. The nasal bridge is high for age and palpebral fissures often slant down and are short. The mouth is characteristically small, making intubation difficult. There is accompanying micrognathia. The ears have been called "windswept" because of posterior rotation and the helices are often poorly delineated. Preauricular tags and pits are not infrequent. The sternum is subjectively and objectively short. The hands reveal overlapping fingers, second and fifth over third and fourth (Figure 43-4). Nail hypoplasia is a consistent finding and is very helpful diagnostically. Phalangeal flexion creases are reduced to absent, especially distally. Prominence of the heels with convexity of the soles (rocker-bottom feet) and short, dorsiflexed great toes with a slight degree of second and third toe syndactyly are useful signs (Figure 43-5). The genitalia are often underdeveloped, with a characteristic decrease in fat stores of the mons veneris area in females. The neurological exam can reveal either hypotonia or hypertonia. General responsiveness is decreased and apnea/bradycardia are frequent findings. Structural birth defects are common, including congenital heart disease, esophageal atresia, omphalocele and the Dandy Walker malformation and its variants.

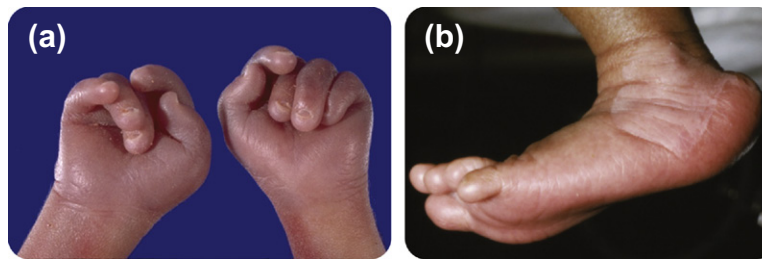
### 43.4.2 Prenatal Diagnosis

The prenatal diagnosis of T18 is accomplished by a variety of means depending on when the mother presents in pregnancy. There is increasing diagnosis in the first trimester because of early serum screening, NT determination and characteristic sonographic findings. In one study of 53 cases of T18 in the first trimester, all but one fetus had one or more sonographic abnormalities, the most common of which were an increased NT (91%), hypoplastic nasal bone (53%), generalized subcutaneous edema (49%), omphalocele (21%) and early growth restriction and bradycardia (26%) (238). This study confirms reports by Breathnach et al. (50) and Wapner et al. (239). First-trimester serum screening showing decreased levels of PAPP-A and decreased beta hCG are also helpful and may direct patients to early definitive testing via chorionic villus biopsy.

In mothers presenting for the first time in the second trimester there are a number of helpful diagnostic modalities. Serum quadruple screening provides useful information, which in combination with ultrasound can lead to accurate detection via invasive testing or a high suspicion of this diagnosis in women declining amniocentesis. The most common second-trimester findings in T18 include congenital heart disease, posterior fossa abnormalities in the Dandy Walker spectrum, choroid plexus cysts, and ventriculomegaly (240,241). Craniofacial abnormalities are frequently diagnosed by ultrasound with some specificity as to the chromosome involved (242). An isolated



**FIGURE 43-4** Two infants with trisomy 18. Note triangular face, small mouth, downsloping palpebral fissures, overlapping fingers, and short sternum.



**FIGURE 43-5** Trisomy 18. (a) Overlapping fingers in fetus. Note severe nail hypoplasia. (b) Rocker bottom foot.

choroid plexus cyst is a frequent normal developmental finding, which resolves in the third trimester, and in the absence of other findings is unlikely to be due to T18 (55). On the other hand, large and/or multiple choroid plexus cysts in the presence of other consistent anomalies are strongly suggestive of T18. A study by Lai and colleagues (243) reviewed 10 years' experience with 69 diagnoses of T18. The detection rate of fetal anomalies by ultrasound was 93% at <14 weeks and 100% at 18–21 weeks. A normal detailed ultrasound with normal fetal growth and normal amniotic fluid in the midtrimester essentially rules out T18.

A definitive diagnosis of T18 by chorionic villus biopsy or amniocentesis should prompt careful counseling of the family, which should take into account the family's cultural and religious beliefs. Most families receiving this diagnosis will elect termination after considering fully this serious handicapping condition. Some families will want to continue the pregnancy and should be supported in this decision (244). There should be a dialog between the obstetric/neonatal team and the family to achieve decisions that will be "in the best interests of the child." The practice of prenatal consultation with the family and

the neonatal team should be encouraged so that a birth and neonatal plan can be agreed upon prior to delivery. Merritt and colleagues (245) present an excellent comprehensive review of these complicated issues. Avoidance of cesarean section when fetal distress occurs in labor should be proposed to the family. Optimally the family will decide on vaginal delivery and supportive comfort care for the infant. Some families insist on "doing everything" for the baby even when all the facts are presented. This clearly presents a conflict between the physician's understanding of the medical facts and families' desire for autonomous decision making for their infant. In general, obviating the infant's pain and suffering and avoiding escalation of care, including intubation, should be goals for both families and treating physicians. These situations may present serious ethical challenges to the health care team (245–247). Ethical issues more commonly arise when there is no knowledge of a definitive diagnosis prenatally because of late prenatal care, refusal of diagnostic testing, or parental denial of the findings on ultrasound. In these situations, urgent consultation after birth by an experienced geneticist can lead to confirmation of the suspected clinical diagnosis and fact-based



supportive counseling. Utilization of a neonatal palliative care team, if available, and use of local infant hospice resources should be encouraged and can help families and health care providers achieve mutual goals for the infant and family. Even with full intervention including surgery and ventilator support the outlook for long-term life seems not to be improved, and these are the facts that families should understand in their decision-making.

A clinical diagnosis should always be confirmed by a postnatal karyotype or FISH. Array comparative genomic hybridization (array) will also confirm this diagnosis, but is an expensive option when the clinical diagnosis is unambiguous. FISH and array studies can be particularly useful on formalin fixed tissue when the infant is still-born and/or macerated and routine karyotyping is not successful.

### 43.4.3 Natural History

Case reports, epidemiologic studies, and interviews with parents have provided data on the natural history of T18 (7,248–252). After cytogenetic confirmation or a firm clinical diagnosis, parents may be told that median survival is 1–2 weeks, 90% of babies die by 6 months, and only 5–10% are still alive at age 1 year. Congenital heart disease or cardiopulmonary arrest are frequently stated to be the cause of death in T18, but this may be an oversimplification, with ill-defined CNS abnormalities and respiratory system problems contributing to “central apnea” (246).

Cardiac anomalies are common in surviving infants, with cyanosis present in the majority of newborns. The most common lesions are large septal defects and patent ductus arteriosus. Valvular dysplasias are present in most cases but are not associated with significant regurgitation or stenosis. Doppler evidence suggestive of elevated pulmonary artery pressure (low-velocity bidirectional flow across the ventricular septal defect and patent ductus arteriosus) is accompanied by greater than normal mean right ventricular cavity and free wall dimensions. The combined findings of frequent cyanosis and increased right ventricular dimensions suggest that factors such as pulmonary hypertension may contribute to early death. Cardiac surgery is not usually performed in the United Kingdom and Europe, but is more frequently undertaken in the United States and Japan (253). A recent survey of clinicians caring for these infants suggested that care takers often do not agree on their approach to recommending or offering surgery to these infants, with cardiologists more likely to offer intervention than neonatologists or geneticists (254). The literature on infants who have undergone full therapeutic interventions is not encouraging with respect to improving long-term survival. One study examined the outcomes of cardiac surgery in 34 patients with T18. Survival over the short term was statistically slightly better in the group of nine undergoing surgery, but the rate

of being discharged home alive was no different in the two groups (255).

In older survivors, profound physical and intellectual disability is inevitable and overall development usually does not progress beyond that of a 6-month-old, chromosomally normal infant. Vision and hearing impairments may be severe (256). Somatic growth is poor (257), and skeletal abnormalities such as severe kyphoscoliosis are frequent (258). Infection causes morbidity and mortality (259). Various malignant tumors are infrequently reported, including Wilms tumor (260) and hepatoblastoma (261).

### 43.4.4 Differential Diagnosis

“Pseudo-T18 syndrome” was formerly diagnosed in infants with some signs of T18, such as prominent occiput, abnormal ear helices, short palpebral fissures, distal limb contractures, and profound developmental retardation, who had a normal karyotype. Pseudo-T18 is practically never diagnosed today, but syndrome delineation in such challenging infants remains difficult. A rare condition that may resemble T18 includes Bowen-Conradi syndrome caused by mutations in the ribosome biogenesis gene *EMG1*. Infants with this autosomal recessive condition first described in the Hutterites, have many of the same features seen in T18 and have an equally poor prognosis for life and mental development (262). The heterogeneous Marden-Walker syndrome may have similar hand findings along with an immobile face and growth restriction. The complex and heterogeneous syndromes termed Pena Shokeir or cerebral ocular facial syndrome (COFS) are encompassed by the term fetal akinesia deformation sequence (FADS). This phenotype can be seen when a mother has myasthenia gravis with high titers of anti-acetylcholine receptors (263). In addition, FADS can be caused by homozygous mutations in *DOK7* or *RAPSN* (264,265). Other autosomal recessive causes of FADS await gene discovery. Autosomal recessive variegate aneuploidy with multiple mosaicism including T18 cells has also been reported. Using exome sequencing, this was recently found to be due to homozygous mutations in *CEP57*, a centrosomal protein involved in nucleating and stabilizing microtubules. The study indicates the crucial role of *CEP57* in maintaining correct chromosome number in cell division (266). Distal arthrogryposis may present in the newborn period with a T18-like hand, but lack of other features such as growth restriction and major anomalies reflects its much better prognosis.

### 43.4.5 Mosaic Trisomy 18

The phenotypes displayed by cases of T18 mosaicism range from full T18 syndrome through a milder, non-specific, dysmorphic phenotype often but not always



associated with growth deficiency (266) to a normal phenotype in cases ascertained serendipitously. If blood chromosomes are normal, clinical signs such as asymmetry of body proportions or cutaneous pigmentary abnormalities (267) are indications to undertake cytogenetic analysis of skin fibroblast cultures. Conversely, full T18 in blood cells with an unexpectedly mild clinical course should suggest chromosome mosaicism with a euploid cell line in other tissues (268).

#### 43.4.6 Cytogenetics of Trisomy 18

T18 syndrome is almost always due to three copies of chromosome 18, and, like T21, T18 is a maternal age-related autosomal trisomy with the rate at prenatal diagnosis or birth rising until 43 years and then leveling off (269). Studies employing polymorphic DNA markers indicated that the extra chromosome in T18 is usually of maternal origin; however, in contrast to T21, wherein maternal meiosis I errors are most frequent, maternal meiosis II errors predominate in T18 (193). Chromosome-specific factors complicate the simple model of susceptible chiasma distributions interacting with age-dependent deterioration of the meiotic mechanism. For chromosome 18, 30% of tetrads are nullichiasmate in maternal meiosis I nondisjunction, but nullichiasmata are not observed in maternal meiosis II nondisjunction. Maternal meiosis I errors, paternal meiosis errors, and post-zygotic errors do occur in a minority of T18 cases (270).

Very few cases are associated with translocations or any other chromosome 18 structural abnormalities such as the true 18q isochromosome (271). Detailed phenotype–karyotype correlations have thus been possible in a few cases only and led to the conclusion that no single region on 18q is sufficient to produce the T18 phenotype. Proximal and distal regions of 18q are both important, and severe mental retardation is associated with 18q isochromosome (272,273).

#### 43.4.7 Trisomy 18 Recurrence Risk

Recurrence of T18 is exceptionally rare, but over 40 years ago Hecht and colleagues (274) suggested increased risk of DS in families in which the index case had T18. Since then, there have been many recorded instances of occurrence in a sibship of T18 followed by or following a different trisomy (heterotrisomy). Recurrence of heterotrisomy cannot be accounted for by gonadal mosaicism in one parent. The risk of recurrence of heterotrisomy after a T18 index case is low, but in view of the evidence for predisposition to aneuploidy in younger women (227), highest risk estimates might be, first, for young mothers (<30 years) who have had an index case with T18, and a rounded-up 1% at the time of prenatal diagnosis may be offered as the chance of recurrence of any viable autosomal trisomy. In the case of mothers aged

30–40 years, twice the maternal age-related risk for DS represents the highest risk; for mothers over 40 years, the maternal-age specific risk of occurrence of trisomy is the best estimate. Prenatal diagnosis utilizing serum markers and ultrasound is customarily offered and some families will desire amniocentesis or chorionic villus sampling (CVS) for maximum reassurance.

If the T18 phenotype results from a chromosome 18 balanced structural rearrangement present in one parent, the risk of recurrence will almost always be significantly higher, but a more precise risk estimate will depend on the type of rearrangement and the pattern of its segregation in the extended family tree.

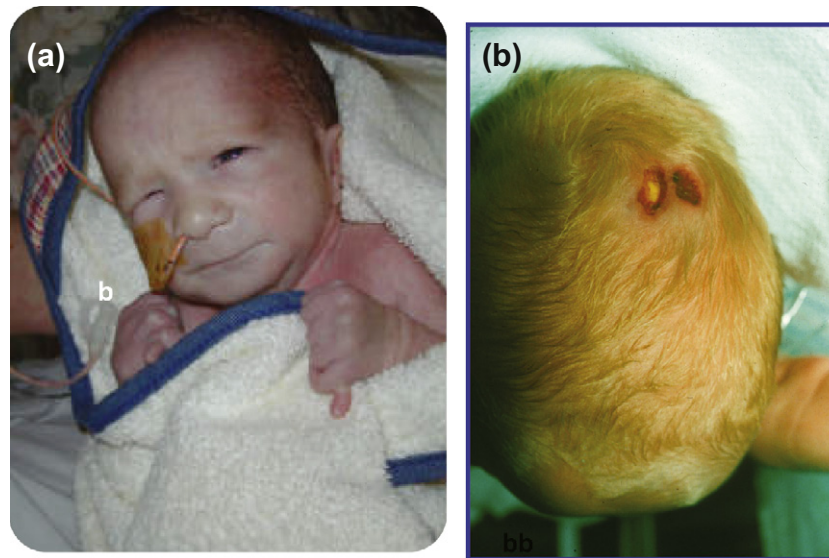
### 43.5 TRISOMY 13

In 1960, Patau and coworkers (275) reported T13 syndrome in the same issue of *The Lancet* that contained Edwards and colleagues' description of T18. That T13 infant had an extra D-group acrocentric chromosome, microcephaly, anophthalmia/microphthalmia, bilateral cleft lip and palate, and polydactyly. Notably, she was still alive at 13 months. The classic T13 syndrome phenotype was highly distinctive and had almost certainly been the subject of detailed case reports in earlier centuries (276).

#### 43.5.1 Clinical Diagnosis

A maternal history of severe and/or early-onset pre-eclampsia may be a prenatal clue to this diagnosis (277). Pre- or postnatally, the placenta may be recognized as abnormal or show changes similar to those present in a partial or mole (278). The mean birth weight is reduced at 2.6 kg. Postaxial polydactyly of the hands and feet plus any combination of microcephaly, ocular malformation (anophthalmia/microphthalmia), cleft lip and palate, heart defect, or renal abnormality is typical (Figure 43-6). Scalp defects in the region of the posterior fontanelle and present in nearly 50% of cases, are diagnostically helpful. Cardiac abnormalities, usually septal defects, occur in over 80% of cases. These defects are rarely the cause of infant death, that is more likely to be on a central basis.

Holoprosencephaly is present in about 66% of cases, with the face malformation (synophthalmia or hypotelorism and premaxillary agenesis) frequently, but not always, “predicting” the brain malformation. The cerebral malformation usually comprises a monoventricular cerebrum without corpus callosum, septum pellucidum, or fornices. Posterior fossa intracranial abnormalities with cerebellar malformation and heterotopias, microscopic abnormalities with pyramidal tract hypoplasia, neural tube defects, and cortical dysplasia are included among neuropathologic features of T13 that are neither constant nor obligatory (279). The diagnosis of T13 should always be suspected in an infant with holoprosencephaly and other anomalies. Occasionally the diagnosis



**FIGURE 43-6** Trisomy 13. (a) Typical face with bulbous nose and microphthalmia. Note postaxial polydactyly. (b) Typical scalp defects in trisomy 13.

of T13 can be unexpected, as when features of the Potter sequence obscure the craniofacial findings (personal experience).

Other pathologic findings in affected fetuses and infants include atrial and ventricular septal defects with patent ductus arteriosus, omphalocele, incomplete intestinal rotation or malrotation with unattached mesentery, enlarged lobulated kidneys with cystic change in the cortex and medulla, accessory spleen, abnormal liver lobation, microscopic pancreatic dysplasia, and changes in the morphology of the axial skeleton (280).

### 43.5.2 Prenatal Diagnosis

Prenatal diagnosis of T13 is common and increasingly cases are diagnosed in utero at 10–14 weeks on the basis of ultrasound abnormalities. The most common finding in the first trimester is increased nuchal edema (281), with about one-third of fetuses with this presentation having chromosome abnormalities, chiefly trisomies 21, 18, and 13 and Turner syndrome. Even more predictive of a chromosome abnormality is cystic hygroma in the first trimester as about half these fetuses will have chromosome abnormalities. Later in pregnancy hydrops fetalis is relatively common occurring in about 5–10 of T13 fetuses (282).

Congenital heart disease is seen in over 90% of T13 fetuses and infants, with the most common defects being ventricular septal defects, atrial septal defects, patent ductus, hypoplastic aorta, mitral and aortic valve abnormalities, pulmonic stenosis and total anomalous pulmonary venous return. Prenatal detection of these defects by ultrasound is high, averaging about 50% in multiple series (282).

Other major birth defects are common in T13 and can be detected as early as the first trimester. Reviewing

the published series, Chen noted omphalocele in 15% of fetal T13 and diaphragmatic hernia in 6–13% (282). Severe urinary tract malformations including most commonly hydronephrosis and rarely renal agenesis are reported in as many as 37% of T13 fetuses. Postaxial polydactyly seen in about 50% of T13 infants and about 25–60% of these anomalies are detected prenatally on ultrasound. Brain abnormalities, most commonly those in the holoprosencephaly spectrum, may also be suspected in the first trimester (283,284) and is the most common abnormality associated with T13. Its presence in a prenatal sonogram, especially when other abnormalities are documented, is highly suggestive of a diagnosis of T13.

### 43.5.3 Management

Given the evident seriousness of the malformations present in most affected infants, the immediate management of newborn infants with T13 and subsequent management of children with T13 raises ethical problems similar to those encountered in relation to management of T18 or other seriously malformed infants and children (discussed earlier). Median survival in T13 is less than 1 week (7), and more than 80% of affected infants die during the first month, but about 3% are alive at 6 months. If major congenital heart and renal defects are present, surgery is usually not undertaken. The issue of cardiac surgery may become important if the child survives to 2–3 months and is developing pulmonary hypertension. One series of nine patients who underwent surgery with trisomy 18 or 13 found that four of the nine remained alive at age 2 years (285). Individuals surviving to childhood and adulthood with T13 who do not have life-threatening malformations have profound intellectual impairment, severe sensory impairments, epilepsy, and

feeding difficulties, as well as difficult behaviors such as self-mutilation (246).

Decisions regarding management need to be made jointly by care providers and families. These decisions include whether or not to use oxygen and monitors or whether or not to consider intervention for cardiac disease. Usually the decision not to treat is considered “in the best interests of the child,” but decisions need to be individualized and accompanied by careful counseling (246).

#### 43.5.4 Differential Diagnosis

The overall pattern of anomalies in T13 usually allows for a rapid clinical diagnosis. There is significant overlap of the major features with other multiple congenital anomaly syndromes. The presence of postaxial polydactyly may suggest the diagnoses of Meckel–Gruber syndrome or Hydrolethalus. Usually the characteristic internal renal malformations and the presence of encephalocele allow the diagnosis of Meckel–Gruber and the brain findings differentiate Hydrolethalus. Another syndrome termed Holoprosencephaly Polydactyly syndrome is incompletely understood. It is not caused by known Holoprosencephaly gene mutations (286), but may be an autosomal recessive condition. A severe presentation of Smith-Lemli-Opitz syndrome also overlaps significantly with T13.

#### 43.5.5 Trisomy 13 Mosaicism

Like other mosaic autosomal trisomy phenotypes, mosaic T13 is variable, from normality to full T13 syndrome (287,288). As in other trisomies, there is little correlation between the percentage of mosaicism and the clinical phenotype (289). Skin pigment abnormalities such as hypomelanosis of Ito or the relatively specific phylloid hypomelanosis (defined as pigmentary mosaicism characterized by congenital hypochromic macules resembling a floral ornament with various elements such as round or oval patches, macules resembling the asymmetrical leaves of a begonia, or oblong lesions) are good clues (290). Mosaicism for T13 is most often a result of a meiosis I error followed by trisomy rescue (291). Overall, mosaicism for T13 is quite rare, with only 49 cases being reported in the most recent published review (292).

#### 43.5.6 Cytogenetics of Trisomy 13 Syndrome

Like trisomies 21 and 18, T13 is a maternal age-related autosomal trisomy, with the rate at prenatal diagnosis or birth rising until 43 years and then leveling off. About 75% of spontaneous abortuses and the same proportion of live-born infants with T13 syndrome have an extra, unattached chromosome 13. SNP arrays and DNA marker studies have revealed that the extra chromosome

is maternally derived in about 90% of these cases (292). Most of the time the stage of nondisjunction is maternal meiosis I but in the recent study of Bugge and colleagues (293) just under half the trisomies were meiosis II errors, a much higher rate than that seen in the other acrocentric chromosomes.

In about 20% of cases with T13 syndrome, the extra chromosome 13 is attached to another chromosome, usually a rob(13q14q), with the greater proportion arising from new mutations. This translocation carries a significant risk of unbalanced gametes and the segregation in males reveals more abnormal spermatozoa than previously predicted (294). In addition, in paternal carriers there is a risk for paternal uniparental disomy (UPD) 14 with a distinct clinical phenotype consisting of characteristic “coat hanger” rib findings, intellectual disability and obesity (295,296).

The majority of (13q13q) cases arise *de novo* and are isochromosomes arising from prezygotic fusion of long-arm sister chromatids. Barring effects of isozygosity for a single gene mutation, UPD 13 is expected to be harmless.

#### 43.5.7 Recurrence Risks

As with T18, recurrence of T13 is rare. In a questionnaire and telephone survey of families with trisomies 18 and 13 children, the sibling recurrence risk was less than 1% (248,249). As in DS there appears to be a risk for recurrence that is higher in younger mothers (212,227). The recurrence risk can be for T13 as well as other trisomies. Traditionally the highest risk estimates might be, first, for young mothers less than 30 years who have had an index case with T13; a rounded-up 1% at the time of prenatal diagnosis may be offered as the chance of recurrence of any viable autosomal trisomy. In the case of mothers aged 30–40 years, twice the maternal age-related risk for DS represents the highest risk. For mothers over 40 years, the maternal-age specific risk of occurrence of trisomy is the best estimate. Cytogenetic prenatal diagnosis is customarily offered. A recent study by Engels and colleagues (297) suggests that in translocation carriers the risk is probably somewhat higher than the 1% risk previously estimated. They looked at 101 carriers of a rob 13;14 translocation ascertained in a variety of ways. They found a miscarriage rate of 27%. Three of 42 amniocentesis in this group revealed T13 (7%). Although not addressed in this study the risk for paternal UPD 14 is increased in male carriers.

For male and female carriers of the rob(13q21q) and rob(13q22q), the risk of T13 syndrome is less than 1%, but the risk of DS in pregnancies of the female carrier of rob(13q21q) is 10–15%. For the rob(13q15q) carrier, there is a less than 1% risk for T13, but the significant risk for UPD 15 is notable.



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### Biography



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## Sex-Chromosome Abnormalities

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## 44.1 INTRODUCTION

The sex chromosomes X and Y are structurally distinct and subject to different forms of genetic regulation. Despite differences, they are thought to have descended from a common progenitor and during this process, the Y chromosome has become depleted of most genes. A number of genes retain copies on both the X and Y chromosomes, where of many are present in the two pseudoautosomal regions at the termini of the short and long arms of the sex chromosomes. Pairing at meiosis facilitates exchange of information at both pseudoautosomal regions. One X chromosome is inactivated in somatic cells of normal females. The Barr body, a chromatin mass visible in interphase, represents this inactive and late-replicating X. Therefore, the number of Barr bodies is always one less than the total number of X chromosomes per somatic cell. Inactivation is believed to occur early in embryonic life, completing by the end of the first week of development. The maternally and paternally derived X chromosomes are randomly inactivated in female somatic cells. Inactivation is permanent and clonal although new perspectives are constantly being added to our understanding challenging this view (1). These features are the basis of the Lyon hypothesis (2).

Three consequences of X inactivation are dosage compensation, variability of expression in heterozygotes, and mosaicism. X inactivation transcriptionally silences most, but not all, of the genes on one of the two Xs in females. In women, with two X chromosomes, random X inactivation takes place (3). This is a process that goes on in every single cell in the body. The process is governed by the X inactivation center (XIC) and initiated by *XIST*, a gene encoding a long intervening noncoding RNA (lincRNA). The *XIST* gene is located close to the centromere on the long arm of the X chromosome (4), and it orchestrates repressive histone modifications (recruiting *PRC2*) along the X chromosome leading to inactivation. In the remaining active X chromosome, *PRC2* is titrated away by the product of *TSIX*, which effectively leaves all females as

mosaics for the X chromosome with one of maternal and one of paternal origin. However, a great number of genes that are spread out on the X chromosome escape this X inactivation by unknown mechanisms (3), and dosage compensation takes place, so that expression between males and females are comparable for many genes (5,6). Approximately 65% of genes are fully silenced, while 15% completely escape X inactivation and 20% show variable expression, depending on tissue cell origin (7). LincRNAs are pervasively transcribed in the genome although their role in health and disease is poorly understood (8). Studies of dosage compensation, imprinting and homeotic gene expression suggest that lincRNAs function at the interface between DNA and chromatin remodeling (9–11) with further involvement in reprogramming of chromatin to promote cancer metastasis (12). To date, a range of different interactions has been hypothesized for lincRNAs in transcriptional regulation (9), and they may function both as intact interacting molecules as well as Dicer processed molecules that are chopped into small interfering RNAs that degrade other RNAs. The expression of a given gene may be regulated by another gene or genes, and thus, a gene only expressed on the one X chromosome because of X inactivation may still be reduced in expression in Turner syndrome (TS) if a regulatory gene avoiding X inactivation is missing, as seems to be the case with the biglycan gene (13). Biglycan is a glycosaminoglycan partaking in normal connective tissue function, and a reduced amount of this protein may explain some of the characteristics of TS.

The number and distribution of genes escaping inactivation are the subjects of considerable research. The clinical importance of genes that escape inactivation is uncertain; however, these genes may explain clinical features in sex-chromosome aneuploidy as gene products may be either under- or overexpressed in relation to normal females and males.

Because inactivation is random and occurs early in embryonic development when there are few cells, females



with one X carrying an altered gene, such as Duchenne muscular dystrophy or hemophilia A, have a varying proportion of cells, in which the altered allele is active. As a consequence, they exhibit variable phenotypes. The range can be extreme, from a normal female to one in whom there are significant and potentially severe clinical manifestations of the disorder. In addition, females have two populations of cells, in which one or the other X chromosome is active; in other words, they are mosaic with respect to their X-linked gene expression.

There are exceptions to random inactivation. In almost all individuals with structural abnormalities of the X chromosome, the structurally abnormal X is inactivated, mediating against unbalanced cells and their effect on phenotype. This preferential inactivation means that structural anomalies of the X chromosome are better tolerated than autosomal equivalents and are thus more frequently observed. Nonrandom inactivation is also the rule in X-autosome translocations. If the translocation is balanced, the normal X is preferentially inactivated, and the two parts of the translocated chromosome remain active, reflecting selection against a situation that would silence autosomal genes. In an unbalanced translocation, only the product carrying the XIC is present and this chromosome is invariably inactivated, the normal X being always active. These nonrandom events minimize the likelihood that a particular chromosomal defect will have clinical consequences.

Sex-chromosome abnormalities include monosomy X, polysomy of X and/or Y, structural changes in the X and Y chromosomes, and mosaicism. Early data on the health and developmental prognosis of these individuals were extremely biased by the method of ascertainment. Long-term prospective studies of unselected newborns and recent epidemiological studies have provided a more accurate natural history emphasizing not only how many aspects of these phenotypes fall within the range of “normal,” but also that morbidity and mortality are increased for all sex-chromosome abnormalities.

The parental origin of nondisjunction of the sex chromosomes has been studied. In XXY males, the origin of the extra X is equally likely to be maternal or paternal. In XXX females, the extra X is maternal in more than 90% of cases, reflecting a preponderance of maternal meiotic errors (14). In monosomy X, the paternal X is lost in approximately 80% of individuals. Research continues to explore a parent of origin effect (imprinting), particularly in monosomy X. One study found evidence of superior verbal and higher order executive function skills, which mediate social interactions, when the retained X was paternal (15,16), while others have not been able to replicate this finding (17).

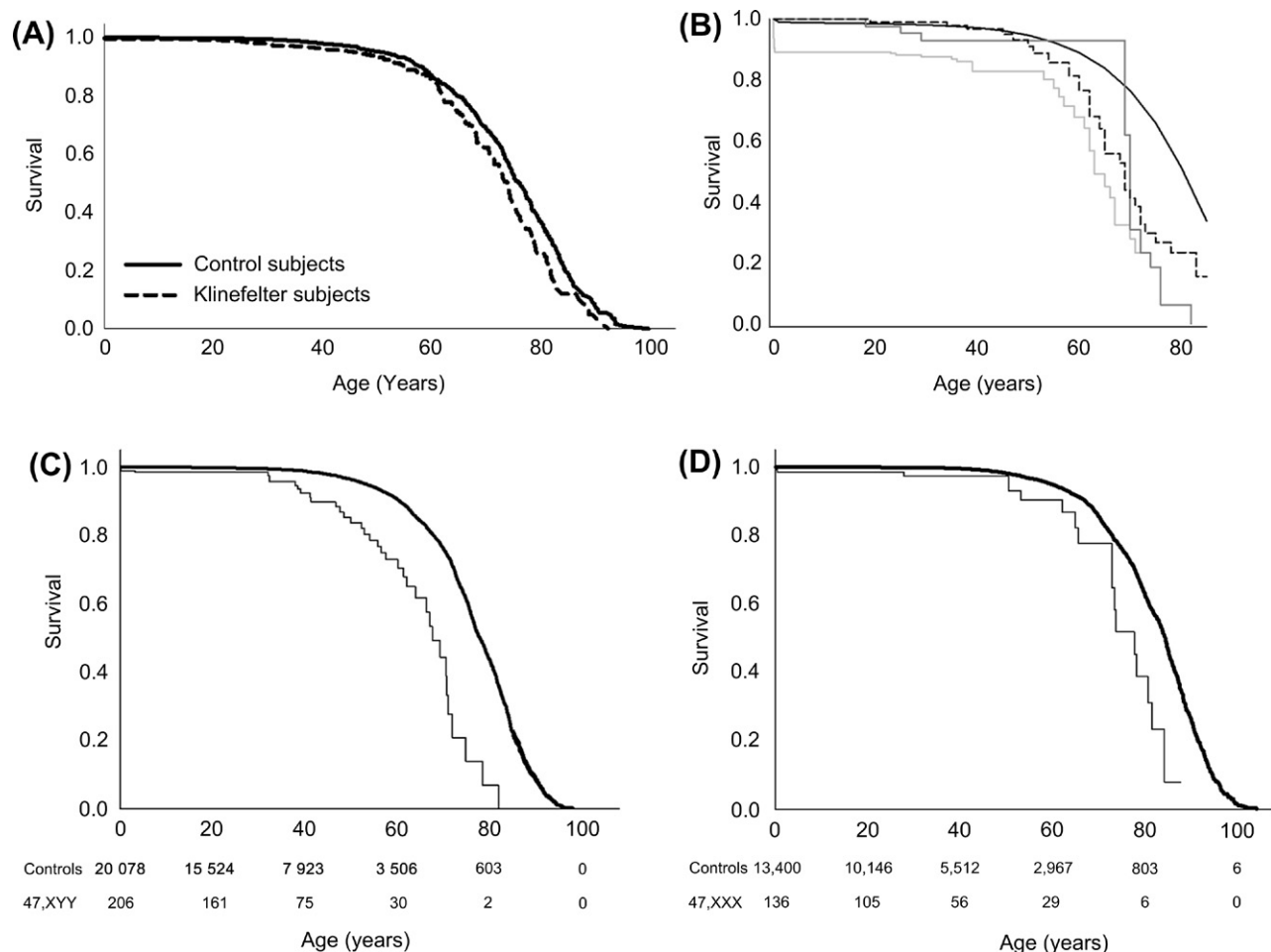
The risk of offspring with polysomy X rises with increasing maternal age, whereas mothers of offspring with monosomy X are predominantly younger than average (18). A sex-chromosome abnormality was diagnosed in about 1 in 250 amniocenteses performed on women

35 years of age and older. These abnormalities may be more common in chorionic villous sampling (CVS). This rate is higher than that found at term, suggesting that there is significant loss of these fetuses in the latter half of pregnancy (19). Since the advent of widespread maternal serum screening with  $\alpha$ -fetoprotein, estriol, and human chorionic gonadotropin (HCG), abnormal analytes may be detected in the presence of sex-chromosome aneuploidy, particularly 45,X (20).

## 44.2 THE EPIDEMIOLOGY OF SEX-CHROMOSOME ABNORMALITIES

Our estimates of the prevalence and incidence of sex-chromosome abnormalities are based on rather old studies dating back to the 1960s–1980s (21–28). These studies provided estimates for the prevalence of TS, Klinefelter syndrome (KS), 47,XXX syndrome and 47,XYY syndrome. Accordingly, we expect about 50 per 100,000 females with TS, 150 per 100,000 males with KS, 85 per 100,000 females with 47,XXX and 100 per 100,000 males with 47,XYY. It has even been suggested that the prevalence of KS is increasing (29). However, studies show significant delay in diagnosis or even nondiagnosis for all these four syndromes. It is estimated that only about 65% of TS, 25% of KS, 12% of 47,XXX and 14% of 47,XYY patients are diagnosed (30–34). The prevalence of prenatally detected cases with sex-chromosome trisomies is also low and a European study shows a termination of pregnancy rate of 36% (35). Thus, current clinical literature is, of course, based on diagnosed individuals and may therefore be biased by selection, especially, for example, if nondiagnosed individuals are less stigmatized or perhaps even more stigmatized suffering an early demise and thus remain undiagnosed. Furthermore, such figures highlight problems related to diagnosis and emphasize that the current diagnostic approach, which is most often by clinical indication, is not efficient in identifying individuals with sex-chromosome abnormalities (36). Work is underway to advance the use of high throughput methods for the diagnosis of sex-chromosome abnormalities (37).

Mortality is increased in all major sex-chromosome abnormalities (31,33,34,38–43), with hazard rates (or standardized mortality rates) ranging from 1.4 to 1.6 for KS, 2.1 to 2.5 for 47,XXX, 1.9 to 3.6 for 47,XYY and 2.9 to 4.2 for TS (Figure 44-1). The specific causes for the increased mortality rates are described below. Rates of morbidity have been studied in TS and KS and have been found to be increased (44,45). In TS, this increase is closely linked to a range of conditions known from clinical studies to be more prevalent (see Section 44.3.1). In KS, there is an increased risk of being admitted to hospital for virtually any disease, including psychiatric disorders and congenital malformations (45), which is difficult to attribute specifically to the chromosome aberrations or the known consequences of the condition—i.e.



**FIGURE 44-1** (A) Survival of KS subjects vs control subjects (hazard ratio, 1.40; 95% CI, 1.13–1.74;  $P < 0.002$ ). KS subjects lost 2.1 years (95% CI, 0.3–3.9 year; median survival) compared with control subjects (179). (B) Kaplan–Meier plots of cumulated mortality in the general population (black line), females with 45,X (light-gray line), females with an isochromosome Xq (dark-gray line), and females with all other karyotypes associated with TS (dashed-black line) (31). (C) Kaplan–Meier survival graphs in 47,XYY compared to an age-matched male background population. Solid line is for controls, and thin line is for persons. Survival is significantly lower in 47,XYY persons, log-rank  $P < 0.0001$ . Number of persons and controls are indicated below the figure (34). (D) Survival graph of all women with a 47,XXX karyotype (thin line) and their age- and gender-matched controls (solid line). Log-rank:  $P < 0.0001$ . Numbers of 47,XXX persons and controls are indicated below the figure (33).

hypogonadism, infertility and cognitive problems. The gene-dose effect may be a plausible cause of the congenital malformations and testicular failure and may also account for the increased risk of delayed speech, learning difficulties, and psychiatric diseases. Disturbed lateralization of the brain hemispheres has been associated with aneuploid number of X chromosomes and increased risk of dyslexia, disturbed verbal execution, and schizophrenia (46). There are no studies describing morbidity in 47,XXX and 47,XYY syndromes.

### 44.3 TURNER SYNDROME

A plethora of information has been added and our current understanding of the syndrome is continuously being broadened since the initial description of TS by Ullrich (47) in 1930 and later Turner in 1938 (48).

Others, such as the Russian physician Seresevskij, had described the syndrome before this time (49). The syndrome only affects females and medical care must include close collaboration between specialties such as genetics, embryology, pediatrics, gynecology and obstetrics, endocrinology, cardiology, gastroenterology, otorhinology and ophthalmology.

#### 44.3.1 Diagnosis, Genetics and Epidemiology

There are no evidence-based diagnostic criteria for TS. However, certain phenotypic traits coexisting with a spectrum of karyotypes comprise the diagnosis. The generally accepted cardinal physical stigmata include growth retardation with reduced adult height, gonadal insufficiency, and infertility (50). The genetic background of TS

is highly variable but includes complete or partial absence of a sex chromosome (the X or Y chromosomes). Mosaicism of two or more cell lines can be present with cutoff levels for low-grade mosaicism yet to be defined because some report 5% while others report 20% diagnostic levels (51,52). The spectrum of karyotypes results from sex-chromosome nondisjunction, events which may occur in the first or second division of meiosis during spermatogenesis or oogenesis or during later mitotic division in early embryonic development.

The classical karyotype 45,X accounts for 50% of cases with the remainder composed of mosaic karyotypes, karyotypes with an isochromosome of X—for example, i(Xq) or i(Xp)—or karyotypes with an entire or a part of Y chromosome (Table 44-1) (44). The genetic basis for the phenotype of TS is being unraveled. This includes improved knowledge of the functions of the *SHOX* gene, which is located on the X chromosome. Haploinsufficiency of *SHOX* explains growth retardation, changes in bone morphology, sensorineural deafness and other features. Additional genes are more than likely to be involved in the pathogenesis of TS but await discovery.

Prenatal prevalence of the syndrome is much higher than the postnatal prevalence, because of increased intrauterine mortality and induced abortions (53,54). Generally, the initial prenatal diagnosis of TS relies on an ultrasound scan focused on congenital malformations around week 19, which is neither sensitive nor specific enough to accurately establish the diagnosis of TS, and thus prenatal diagnosis of TS may not always be correct. Therefore, precise prenatal diagnosis must include high-resolution ultrasound scan, fetal echocardiography and other advanced imaging modalities, such as magnetic resonance imaging (MRI), augment fetal karyotyping. Following confirmation of diagnosis, most fetuses with TS are legally aborted. An European multicentre study found an induced abortion rate of 66%,

which confirms previous studies of legal abortion rates of 60–80% (44). However, this is only a fraction of fetuses with TS since <10% of any pregnant population are subjected to invasive methods of prenatal diagnosis and prenatal ultrasound scan only diagnoses fetuses at the most abnormal end of the spectrum of TS phenotypes, such as hydrops or increased nuchal fold (44). Maternal age and low height are risk factors for giving birth to a girl with TS and newborn girls with TS are smaller and shorter for gestational age than the background population (55).

Postnatal diagnoses are made at birth (15%), during teenage years (26%) or in adulthood (38%) with the remainder being diagnosed during childhood (36). The key to diagnosis is lymphedema in 97% during infancy and short stature in 82% in childhood and adolescence (36). Unfortunately, delay in diagnosing girls and adolescents is substantial (31).

Morbidity is considerably increased in TS. In a study of all females diagnosed with TS and compared with the general population of women, the relative risk (RR) of an endocrine diagnosis in TS patients is increased to 4.9 when compared with the female background population, with specific increases in hypothyroidism (RR 5.8), type 1 diabetes mellitus (T1DM) (RR 11.6) and type 2 diabetes mellitus (T2DM) (RR 4.4). The risk of ischemic heart disease and arteriosclerosis (RR 2.1), hypertension (RR 2.9), and vascular disease of the brain (RR 2.7) was also increased. Furthermore, the risk of other conditions such as cirrhosis of the liver (RR 5.7), osteoporosis (RR 10.1) and fractures (RR 2.16) was also increased, as were the risks for congenital malformations of the heart, the urinary system and the face, ears and neck. The risk for all cancers was comparable to other women (44). In addition to morbidity, mortality is increased in TS. In a British cohort study, the RR of premature death was increased to 4.2 (41) because of disease in the nervous, digestive, cardiovascular (CV), respiratory and genitourinary systems. Death due to cancer was lower than expected, corroborating morbidity studies. In Danish patients, the patterns of mortality were comparable, and there were important differences between patients with 45,X and those girls and women with an isochromosome, who had a fourfold increased mortality, whereas patients with other karyotypes only had a twofold increase in mortality (31).

### 44.3.2 TS and the CV System

Diseases of the heart and vasculature account for 50% of excess mortality in TS (40). A number of left-sided CV malformations occur with increased frequency. Elongated transverse aortic arch and aortic coarctation are seen in 50% and 4–14% of TS women, respectively (56,57). Bicuspid aortic valve occurs in 13–43% of TS patients versus 1–2% of general population (58) (Figure 44-2). Less commonly right-sided malformations, such as persistent left vena cava superior and partial anomalous venous

**TABLE 44-1** Distribution of Prenatal and Postnatal Turner Syndrome Karyotypes

Karyotype	Prenatal	Postnatal
45,X	134 (64%)	162 (47%)
45,X/46,XX	45 (22%)	59 (17%)
45,X/46,X,i(Xq) <sup>a</sup> ; 46,X,i(Xq); 45,X/46,X,i(Xq)/47,X, i(Xq),i(Xq) etc.	9 (4%)	41 (12%)
45,X/46,X,del(X); 46,X,del(X)	15 (7%)	27 (8%)
45,X/46,XX/47,XXX; 45,X/47,XXX; 45,X/46,XX/47,XXX/48,XXXX	5 (2%)	16 (5%)
45,X/46,X,r(X)	1 (<1%)	20 (6%)
45,X/46,XY	—	10 (3%)
Others with Y material	—	11 (3%)
	209 (100%)	346 (100%)

<sup>a</sup>i(Xq) = isochromosome X(q) (106).

return, are encountered (56). These congenital heart defects may cosegregate, and in a small proportion, the cardiac phenotype is more severe in its affection of the left ventricle and the thoracic aorta—the hypoplastic left heart syndrome.

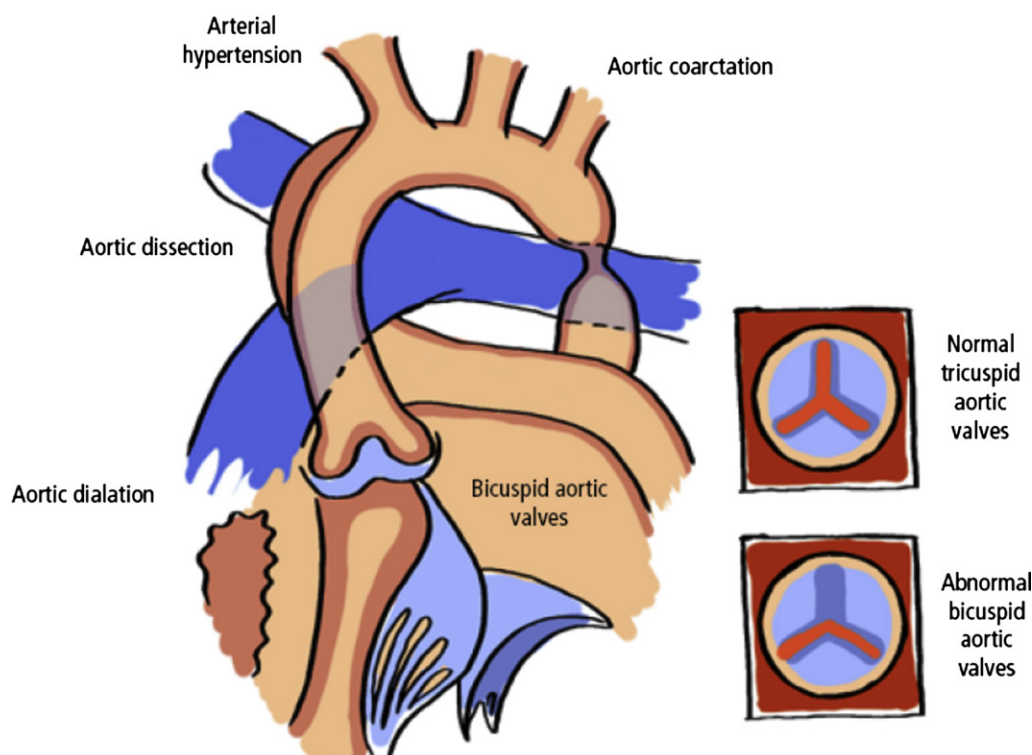
Some of the congenital CV defects call for early and potentially life-saving high-risk CV intervention, thereby contributing to both increased mortality in infancy and later on in life from complications of the congenital abnormalities disease and the necessary interventions (31). Other defects present less acutely, with symptoms associated with late life morbidity. Then again, some remain subclinical throughout the entire lifetime with an unresolved role to the pattern of morbidity and mortality in TS.

More than 30% of young girls and adolescents and 50% of adults with TS are mildly hypertensive on 24-h ambulatory measurements (59–63). Additionally, 50% have abnormal circadian blood pressure profiles (60,61,64). Though the pathological nature underlying hypertension in TS remains unknown, it is uniformly accepted that hypertension and sympathovagal dysfunction with nocturnal “nondipping” confers a high risk of future CV events, as also epidemiologically documented in TS (31). The presence of these risk factors adds to the other adverse indices of CV risk in TS: increased carotid intima thickness (63), propensity toward glucose intolerance and type 2 diabetes (61,65), nonalcoholic steatohepatitis (66–68), discordant lipid profile (69,70), estrogen deficiency and unfavorable body composition (71).

Beyond congenital cardiac malformations such as the bicuspid aortic valve and aortic coarctation, hypertension is thought to be a major contributing factor (62) to an extremely high incidence of aortic dissection of 40 per 100,000 TS syndrome years versus 6 per 100,000 general population years (72). Aortic dissection strikes TS patients at a median age of 35 years as opposed to 71 years in the general population (72). Normally, dilatation of the ascending thoracic aorta precedes dissection and rupture, and an abnormal aortic caliber is seen in up to 42% of nonselected adult patients with TS (73,74), increasing with age (75). Aortic caliber correlates with systolic blood pressure, but surprisingly, not with vascular atherosclerotic indices such as aortic stiffness or plasma lipids (62). An intrinsic arterial wall defect is highly likely as part of the generalized vasculopathy in TS, similar to the bicuspid aortic valve syndrome (63,76) (see Chapter 46).

Subclinical impairment of diastolic function associated with increased left-atrial dimensions is seen even in normotensive and strictly metabolically controlled TS (38). Additionally, the adverse prognostic marker N-terminal pro-brain natriuretic peptide (BNP) is elevated in the absence of symptoms of cardiac failure and without systolic dysfunction (64). Adding further prognostic burden to the CV phenotype, prolongation of the QTc interval is seen in 30% of girls, adolescents and adults with TS (77,78), a condition accepted as an independent predictor of sudden CV death.

Premature ovarian failure with estrogen deficiency is the most prevalent CV risk factor in TS. How premature



**FIGURE 44-2** Illustration of the occurrence of bicuspid aortic valves, aortic coarctation and the place in the ascending aorta where dissection often occurs. Furthermore, illustration of the frequent occurrence of hypertension.



estrogen deficiency impacts CV prognosis is unclear although young age at menopause in other populations confers a more adverse risk profile. Evidence confirming earlier observational findings of estrogen-derived cardioprotection is supported by animal and human studies showing anti-inflammatory, antioxidant and lipid-lowering effects with modification of disruptive vascular processes. Conversely, opposing such protective effects is the potential for the induction of myocardial hypertrophy, venous thromboembolic events, and dysrhythmia (79). In interventional studies, estrogen replacement therapy failed to reduce the risk of CV disease in primary and secondary prophylaxis of atherosclerosis in postmenopausal women. The potential for alleviation of adverse risk in premature ovarian failure has not been investigated. However, evidence is mounting to a beneficial effect of estrogen substitution on CV risk with the early introduction states of deficiency of female sex steroids—the so-called “timing hypothesis” (79).

Collectively, congenital and acquired CV disease occurs on a backdrop of endocrine deficits, resulting in a highly variable cardiac phenotype in TS where risk assessment and reduction is difficult. Therefore, all girls and women with TS should be regarded as having increased risk of CV events (80), and TS girls and women require a thorough baseline delineation of cardiac phenotype and regular follow-up by cardiologists trained in congenital heart disease (81). Depending on the presentations, examination should include an electrocardiogram, 24-h ambulatory blood pressure, echocardiography and MRI (81).

### 44.3.3 Ovarian Insufficiency and Hormone Replacement Therapy

Premature ovarian failure is the common denominator in most patients with TS (82). Early ovarian demise presents and estrogen insufficiency ensues. Ovarian germ-cell count is normal until week 18 of gestation, after which accelerated degeneration takes place. High levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are present in early childhood (2–5 years) and after the time of normal puberty onset (11–12 years) (83,84). During the time of normal puberty, the levels of FSH and LH increase to menopausal levels; however, many untreated girls show signs of puberty and/or have regular periods for a varying length of time (85). Ovarian follicles may be present in 45,X patients even in 12–19-year-olds (86), probably because of unrecognized mosaicism for a 46,XX cell line. Anti-Müllerian hormone (AMH) and inhibin B are emerging as possible markers of ovarian function (87,88). Understanding the processes in early follicular apoptosis in TS may, in the future, lead to a treatment sparing the follicles and maintaining fertility.

Timing of sex hormone replacement therapy (HRT) should allow onset of puberty at the same time as peers of the patient to avoid social problems secondary to delayed physical and psychological development. This also allows

optimal bone mineralization to take place. In most normal girls, puberty starts around 12 years of age. Since 30% of girls with TS undergo some spontaneous pubertal development and 2–5% have spontaneous menses with a small potential to achieve pregnancy without medical intervention (85,89), signs of puberty should be looked for before starting estrogen therapy. When FSH and LH are clearly elevated, AMH and inhibin B are low, and clinical signs of puberty lacking, pubertal induction should be started, although always considering individual circumstances. To induce pubertal development, the dosing and timing of estrogen therapy should aim at mimicking normal pubertal development. Doses should be individualized starting with very low doses of estrogen as monotherapy, either orally or percutaneously (90,91). The treatment should be monitored by the development of secondary sex characteristics (Tanner staging), serum LH and FSH, bone maturation or uterine volume. A gestagen is added when breakthrough bleeding occurs. Currently, it is not clear which gestagen is the most advantageous.

Estrogen therapy should always be coordinated with the use of growth hormone (GH). These initiatives should be individualized in order to optimize and coordinate growth and pubertal development. When growth is a priority, delaying estrogen therapy is an option to avoid compromising adult height. However, recent growth-promoting trials have documented that physiological timing of estrogen therapy does not compromise final height, when GH therapy is started early and dose is increased stepwise (92). HRT is a cornerstone of therapy of TS to optimize feminization and bone mineralization, and to enhance motor speed and verbal and nonverbal memory and processing. Females with TS present with a particular neurocognitive profile, with impaired performance on motor tasks, impaired visual-spatial ability, but normal verbal skills (93). The deficits in cognition are likely to be caused by haploinsufficiency of X-linked genes that normally escape X-inactivation, but these putative genes await further elucidation.

Infertility is rated as the most prominent problem in adult women with TS (94). Oocyte donation is possible in many countries and fertility preservation is an emerging option. The most recent studies show good results, or at least comparable to oocyte donation in other infertile patients. However, better preparation of the uterus for implantation (assessed by uterine size and endometrial thickness) with prolonged treatment with high daily doses of estradiol (4–6 mg or up to 8 mg of 17 $\beta$ -estradiol) may improve results in TS. Importantly, it is a high-risk endeavor for a TS woman to go through pregnancy, especially in relation with the CV system but also secondary to endocrine features. A high rate of maternal complications has been reported in some studies (95,96) although a recent study reported a much lower rate of complications after sufficient CV screening before pregnancy (89).

Androgen insufficiency is present (97), and one needs to evaluate the possible benefits of androgen substitution in TS.

During adulthood, it is important to continue HRT, even though a number of issues such as dose during the different ages, administration route, type of estrogen and type of gestagen are unresolved. Female hypogonadism is related to a number of other conditions, and HRT may reduce or even completely alleviate the risk of these. Currently, new studies indicate that the traditional dose of 2 mg of estradiol used in TS and other conditions of premature ovarian failure may be too low for reducing occlusive vascular risks and for normal growth of the uterus (98,99). The presently available evidence on different routes of administration in TS has not clearly demonstrated superiority of any particular route (61,66,100); newer studies point toward advantages with transdermal or subcutaneous application (101–103) but additional studies are needed to fully resolve this issue. A few studies have compared the contraceptive pill with physiological 17 $\beta$ -estradiol and a gestagen and they suggest that the contraceptive pill should not be used in the long-term treatment of TS (104,105).

#### 44.3.4 Decreased Stature in TS

Short stature is the cardinal finding in girls with TS affecting 95–99% (106). Growth retardation is already present in utero with birth weight approximately 1 SD below expected. Growth remains retarded in infancy and childhood, where height is 2 SD below normal and, because of absent pubertal growth spurt, the heights encountered at age 14 are 4 SD below expected if GH treatment has not been initiated (107). Overall, the growth phase is prolonged (if puberty is not induced) achieving a spontaneous final height of almost 3 SD or about 20 cm below normal height.

Part of the explanation of the small final height relates to the action of the *SHOX* gene located to the PAR1 region of the X and Y chromosomes; haploinsufficiency of *SHOX* leads to reduced final height as documented in Leri–Weill dyschondrosteosis. However, the height difference is only about 50–75% of what is seen in TS. Therefore, *SHOX* deficiency can only explain part of the height deficit in TS (108). BNP and fibroblast growth factor receptor 3 (FGFR3) are transcriptional targets of *SHOX* and present alongside at the growth plate in proliferative chondrocytes (109). Whether the lack of *SHOX*-induced BNP and FGFR3 is involved in CV malformations and diseases in TS remains to be elucidated.

Even though GH concentrations in TS have been found normal by some and reduced by others, it is generally concluded that girls with TS have a growth deficiency with reduced sensitivity to GH rather than a GH deficiency. GH treatment can increase growth velocity and final height. The effect is dose-dependent, and normalization of final height has been shown to be obtainable with high doses (92,110). In the only randomized controlled study, using GH treatment (0.3 mg/kg/week) or placebo, a significant increase in final height of 7.2 cm (CI: 6.0–8.0) was seen, with HRT started at age 13 (110).

If TS diagnosis is made early (before 1–2 years of age), treatment with GH should commence at an early stage. In a randomized controlled open-label study that administered GH to 9-months–4-year-old girls, growth retardation was corrected with growth catch up, bringing 93% of the toddlers back in the normal height range within 2 years. The placebo group had progressive growth failure (111).

Besides effects on growth and final height, GH treatment also has beneficial effects on body composition, with reduced fat mass and an increase in lean body mass. Concerns about the effect of GH on the heart has arisen from evidence that left ventricular hypertrophy is found in acromegalic patients with high levels of GH. A Dutch study showed no signs of left ventricular hypertrophy and no increase in blood pressure in TS girls undergoing 7 years of GH treatment (112).

#### 44.3.5 Endocrine and Metabolic Disorders

**44.3.5.1 Glucose Metabolism and Diabetes.** Both T1DM and T2DM occur more frequently in women with TS. Impaired glucose tolerance (IGT) has been reported in both girls and women with TS. An epidemiological study including 594 TS women found an increased RR of both T1DM and T2DM (44).

Generally, fasting glucose levels are not significantly different from controls, but fasting hyperinsulinemia has been documented and during an oral glucose tolerance test (OGTT), IGT has been found in 25–78% (61,65) of adult TS. In addition to higher glucose levels, the insulin response is increased and some have found a delayed insulin peak during an OGTT. The impaired glucose homeostasis seems to be explained by a decreased insulin sensitivity as well as a reduced “first phase insulin response,” which could be viewed as an inappropriately low  $\beta$ -cell response (61,65). Body composition is distinctly altered in TS with increased BMI, as well as decreased muscle mass, increased total fat mass and visceral fat mass. A more sedentary lifestyle and decreased VO<sub>2</sub>max is also found in this population. All factors contribute to the risk of developing reduced insulin sensitivity and diabetes (71,113).

Appropriate HRT in TS also seems to be important for glucose homeostasis even though the findings in TS are not uniform. HRT reduced fasting glucose and fasting insulin (114), and while not improving insulin sensitivity, fat-free mass and physical fitness did increase—both latter factors improve glucose homeostasis. In contrast to this, more subjects were found to have IGT during an OGTT while receiving HRT (61). On balance, HRT may slightly improve glycemic control in TS.

Insulin levels, both fasting and as an OGTT response, increase during GH treatment (115). The insulin levels decrease after termination of GH but they remain higher than pretreatment levels (92). GH therapy reduces insulin sensitivity during the first 6–12 months of treatment where after it stabilizes, but this effect subsides with

cessation of treatment (92). This stabilization could be due to changes in body composition with an increase in lean body mass and decrease in fat mass. The proportion of TS patients with IGT does not seem to increase significantly during treatment, and HbA<sub>1c</sub> remains unchanged or even decreases during GH therapy. While most of the effects on the glucose metabolism seem to reverse after cessation of GH treatment, the long-term effects of the GH-induced hyperinsulinism and insulin resistance are not known.

With widespread abnormalities in the glucose homeostasis and an increased risk of T1DM and T2DM, there is a need for attention to the increased risk of impaired glucose homeostasis and diabetes in TS. Recommendations for diagnosis and treatment of diabetes should adhere to general population guidelines. However, yearly screening of fasting glucose should be performed.

**44.3.5.2 Gastroenterology and Hepatology.** Increased concentrations of liver enzymes, especially alkaline phosphatase, alanine/aspartate aminotransferase and  $\gamma$ -glutamyl transferase (markers of hepatic cell lesion or turnover), are frequent in TS. Bilirubin (excretion marker) and coagulation parameters (production markers) are, in most cases, within the normal range. In a liver biopsy study in 27 women with TS who were biopsied because of persistently elevated liver serology (116), multiple abnormalities were found. These included marked nodular regenerative hyperplasia, multiple focal nodular hyperplasia, and cirrhosis, associated, in some, with obliterative portal venopathy. Other patients showed more moderate changes, including portal fibrosis, inflammatory infiltrates and nonalcoholic fatty liver disease. The authors conclude that the main causes of liver abnormalities in TS are vascular disorders thought to be congenital in origin, and nonalcoholic fatty liver disease, without signs of liver toxicity from concomitant estrogen therapy. This study is cornerstone for several reasons: it is the largest, it includes liver biopsies as well as thorough evaluation of other causes of liver disease (excluding viral, autoimmune and alcoholic causes), and it excludes HRT as causative to the liver abnormalities. In keeping with this, HRT normalizes measures of liver function and dynamic liver tests are normal and not affected by HRT in TS (68).

Inflammatory bowel disease also appears to be more frequent in TS (2–3%) and, especially, should be suspected in girls not responding to GH therapy. Celiac disease is present in 8% of patients (117,118) and because this may cause additional growth stunting, it should always and repeatedly be excluded. The general guidelines should be followed for these enteric diseases.

**44.3.5.3 Diseases of the Bone.** Peak bone mass depends on a number of factors, such as genetic background, nutrition, physical activity, local growth factors and a spectrum of hormones. Estradiol secretion is clearly deficient in childhood and adolescence. Children and younger and middle-aged adult patients with

TS have low bone mineral density (BMD), and studies show that fracture risk is increased (44,119,120). HRT is crucial to induce maximal peak bone mass in adolescents and young adults and to avoid a rapid decrease in BMD in adulthood (121). This is supported by longitudinal studies of estrogen-deficient and estrogen-replete adolescents with TS. Furthermore, patients with spontaneous menstruation have normal BMD, whereas absent menarche associates with a reduced BMD. A 3-year longitudinal study of 21 women with TS (age 20–40), with iliac crest biopsies before and 3 years after treatment with HRT, showed marked effects of estrogen on bone. Treatment consisted of estradiol implants (and an oral gestagen cyclically) (102) resulting in estradiol levels comparable to levels in premenopausal women, and considerably higher than levels achieved with regimens used hitherto (estradiol 2 mg orally or equivalent transdermal doses). Bone biopsies pointed toward an anabolic effect on the skeleton of estradiol in young TS (102). Furthermore, GH may improve BMD. In a recent 7-year study with GH treatment given at three different doses, BMD increased in a dose-dependent manner. However, estrogen was added after 4 years of GH treatment, and it is difficult to ascertain the individual effects of GH and estrogen in this study (122).

No very long-term studies (both follow-up and intervention studies) of the effect of estradiol have been published but 5 years of appropriate HRT maintains BMD in adulthood (123). There is a definite need for such studies to determine the ideal treatment regimen during adolescence in order to achieve two goals: attaining maximal peak bone mass and maintaining BMD without compromising adult height; and, with appropriate timing of pubertal induction. The optimal dosage of estrogen during adult life is also yet to be determined.

**44.3.5.4 Thyroid Disorders.** Thyroid disease is common in TS. Hypothyroidism is frequent and thyroid antibody formation even more so, with as many as 30% TS patients eventually developing manifest hypothyroidism. Correspondingly, a recent study showed a considerable increase in new cases with hypothyroidism during a 5-year follow-up period (124). It remains an enigma why so many TS patients suffer from diseases related to autoimmunity, and the basis for this grossly increased risk in TS (also including celiac disease, and diabetes (see Section 44.3.5.1)) is unaccounted for. A genetic basis seems probable although undocumented. GH treatment does not increase the frequency of autoantibodies. The treatment of hypothyroidism follows normal guidelines.

**44.3.5.5 Renal and Auditory System.** Structural anomalies of the urinary tract are frequent, but rarely give rise to problems (125) and are apparent on ultrasound.

Middle-ear infection is extremely frequent, especially in the first years of life and often amount to considerable morbidity (126). Some girls with TS already experience social hearing problems due to sensorineural hearing loss during teenage years, which very often leads to the use

of hearing aids (126,127). There are estrogen receptors in the inner ear and estrogen deficiency during childhood and adolescence may be involved in pathogenesis of the accelerated early presbycusis often seen in TS (127,128).

#### 44.3.6 Intelligence, Personality, and Behavior

Most females with TS have normal intelligence, and usually good verbal abilities. In one series of females with TS, including all karyotypes, mental retardation was diagnosed in approximately 10% (129), although this figure may well be too high. Intellectual handicap and karyotype and marker chromosomes derived from an X chromosome correlate. The group with the next highest prevalence of mental retardation (30%) had a ring (X) in their karyotype.

Specific learning disabilities are common, and a TS neurocognitive and psychosocial phenotype has been described. This profile includes impaired visual-spatial processing, social cognition, nonverbal problem solving, and psychomotor deficits (130). When school difficulties are appreciated, a full developmental assessment should be carried out and an individualized education program prepared. Difficulties may arise with attention, maturity, and social skills. Although overall psychosocial adjustment is good, there may be a risk for some social or adjustment problems, particularly with respect to social maturity, relationships, and self-esteem (130). Many girls with TS display immature behavior in comparison with their peers and experience particular isolation in adolescence.

They are at an older age than their peers when they begin dating, leave home, and marry (131,132). Adults also have fewer social contacts, but most do not perceive this as a problem and express satisfaction with employment and social life (131). Adult females with TS achieve similar educational levels as females in general (133) and rate their quality of life similar to matched controls (133–135).

The risk of psychiatric disease, including major reactive depression, affective disorder, psychosis, eating disorders, substance abuse, and obsessive-compulsive disorder, is as high as 10%, which is likely increased above the risk in the general population (136,137).

#### 44.3.7 Conclusions

Patients with TS need comprehensive care preferably from a multidisciplinary team, which can best be practiced from an outpatient clinic with centralized care enabling special emphasis on TS. Glucose metabolism, weight, thyroid function, bone metabolism, blood pressure, liver function, and CV status should be regularly assessed (Box 44-1). Estrogen deficiency should be treated, preferably with natural estrogens and a gestagen, and GH should be commenced early in life.

Importantly, knowledge concerning TS is still limited. There is a large deficit in our understanding of the syndrome with a hope to improve patient outcome through not only

#### BOX 44-1

#### Suggested Clinical Outpatient Program for Patients with Turner Syndrome

##### Baseline

Karyotype  
Renal and pelvic ultrasound  
Cardiac and aortic magnetic resonance imaging  
Echocardiography  
Thyroid status and antibodies  
Celiac screen  
Gonadotropins  
Renal and liver function  
Bone densitometry (DEXA scan)

##### Annually

Physical examination, including blood pressure and heart auscultation  
Thyroid function  
Body composition status (goal of BMI < 25), including physical exercise and diet instruction  
Fasting lipids  
Fasting blood glucose  
Renal and liver function

##### Every 3–5 years

Echocardiography, cardiac and aortic magnetic resonance imaging  
Bone densitometry (DEXA scan)  
Audiogram  
Celiac screen  
Thyroid antibodies (thyroid peroxidase)

a specialized multidisciplinary clinical approach but also a continuous effort to span disciplines in future research.

#### 44.3.8 45,X/46,XY Mosaicism

Substantial variation in the phenotype associated with 45,X/46,XY mosaicism exists, and much depends on whether the diagnosis is made postnatally, or, fortuitously, prenatally. In the latter circumstance, 90–95% of babies have a normal male phenotype at delivery (138,139). These boys have an increased risk of abnormal gonadal histology. Few prospective data are available on pubertal development, fertility, risk of testicular failure, and likelihood of gonadoblastomas, which is increased in comparison of TS patients with Y chromosome cell lines (140–142). When this diagnosis is made prenatally, detailed ultrasound evaluation will help to identify the minority with ambiguous genitalia and thus facilitate counseling regarding the natural history and prognosis (143). There does not appear to be any correlation between the phenotype and the proportion of cells with 45,X in lymphocyte or fibroblast studies.

When diagnosis of 45,X/46,XY is made postnatally, it is generally because of an abnormal phenotype; however, rare normal males have been identified. The most common presentation is TS, and both females and males with a 45,X/46,XY karyotype show poor growth and seem to benefit from GH treatment to achieve a normal growth



pattern (144). Less frequently, this karyotype is found in the investigation of male pseudohermaphroditism or mixed gonadal dysgenesis, with hypospadias, cryptorchidism, rudimentary phallus, urogenital sinus, or, rarely, true hermaphroditism (139). Associated mental retardation has been described but is unusual. Some apparently normal males, with normal genitalia, have been ascertained because of infertility, with abnormal sperm number or morphology. Some have found an increased risk of sex chromosomal and autosomal aneuploidy in sperm (145), while others have rates of aneuploidy similar to other patients with oligospermia (146).

#### 44.3.9 X;Y Translocations

X;Y translocations in humans are the result of abnormal male meiotic recombination. Translocations involving the X and Y chromosomes generally involve break points in Xp22 and Yq11, producing partial monosomy of Xp with the addition of Yq material. The normal two-copy complement of Xcen-Xq11, a putative TS critical interval, is present. The sex-determining region Y (SRY) is most often absent, so the individual with this translocation is usually a woman with normal intelligence and fertility but short stature. The latter feature likely reflects the absence of SHOX in the pseudoautosomal regions of Xp and Yp, but this can rarely be offset by the presence of GCY (a Y chromosome growth control gene), growth-promoting locus on Yq. Women with t(X;Y) may have demonstrable GH deficiency and may benefit from GH administration. A smaller percentage of women with break points proximal to Xp22 have gonadal dysgenesis, infertility, and variable Turner stigmata in addition to short stature.

Less often, the translocation break point is in Yq, and the product retains SRY, but important coding regions of the Y long arm may be deleted. The phenotype is highly variable. These males may have hypogonadism, infertility, dysmorphism, and a degree of cognitive impairment. When the Y chromosome translocation break point is more distal and only heterochromatic long arm material appears to be lost, there may still be submicroscopic loss of euchromatin that can have important clinical consequences. This possibility should be addressed by molecular methods.

### 44.4 KLINEFELTER SYNDROME

KS is the most common sex-chromosome aneuploidy and was first described by Harry F. Klinefelter, Edward C. Reifenstein and Fuller Albright in 1942, when they published nine cases of a syndrome characterized by gynecomastia, azoospermia, hyalinized small testes, elevated levels of FSH and hypogonadism (147). The cause was unknown until 1959 when Jacobs and Strong demonstrated the extra X-chromosome in the karyotype of KS (148).

#### BOX 44-2 Abnormalities Associated with KS and their Tentative Frequencies

Feature	Frequency (%)
Infertility (adults) (152)	>99
Small testes (<4 ml) (152)	>95
Increased gonadotropin levels (152)	>95
Azoospermia (adults) (152)	>95
Learning disabilities (children) (151)	>75
Decreased testosterone levels (152)	63–85
Decreased facial hair (adults) (152)	60–80
Decreased pubic hair (adults) (152)	30–60
Gynecomastia (children) (151,168)	38–75
Delay of speech development (children) (151)	40–?
Increased height (prepubertal) (151)	30–?
Abdominal adiposity (adults) (175)	~50
Metabolic syndrome (adults) (175)	46
Osteopenia (adults) (194)	~40
Type 2 diabetes (adults) (175)	10–39
Cryptorchism (151,162)	27–37
Decreased penile size (children) (151)	10–25
Psychiatric disturbances (children) (151)	25
Congenital malformations cleft palate, inguinal hernia (204)	~18
Osteoporosis (adults) (194)	10
Mitral valve prolapse (adults) (188)	?–55
Breast cancer (adults) (198,199)	Increased risk (~50-fold)
Mediastinal cancers (children) (197)	Increased risk (~500-fold)
Fractures (43,179)	Increased risk (2–40-fold)

Since then, a number of studies have increased knowledge about the syndrome, especially the chromosome surveys on large number of newborns, with follow-up through childhood and adolescence performed in the 1970s and 1980s (149–151). These studies on unselected newborn individuals with sex-chromosome aberrations focused mainly on development in infancy, childhood and adolescence, leaving the natural history of the adult with KS less well described. The typical man suffering from KS is described as tall, with narrow shoulders, broad hips, sparse body hair, gynecomastia, small testes, androgen deficiency and reduced intelligence (152), but a less well-defined phenotype is present in many patients (153). Typical features are listed in Box 44-2.

#### 44.4.1 Genetic Background

KS is characterized by the presence of one Y chromosome and two or more X chromosomes in a phenotypic male. The most abundant karyotype is 47,XXY but supranumerous X chromosomes and mosaics with mixture of normal cells are not uncommon. In the diagnosed Danish population of KS patients, 90% had the 47,XXY karyotype, while the remaining had rarer karyotypes (Table 44-2), with similar figures found in a British study (43).

**TABLE 44-2 Karyotype Distribution in a Large Series of KS Males**

Karyotype	Prenatal	Postnatal	Total
47,XXY	147 (90.2)	714 (89.7)	861 (89.8)
46,XY/47,XXY	15 (9.2)	48 (6.0)	63 (6.6)
48,XXXY	0	11 (1.4)	11 (1.1)
49,XXXXY	1 (0.6)	16 (2.0)	17 (1.8)
47,XXY/48,XXXY	0	2 (0.3)	2 (0.2)
48,XXY,+18	0	5 (0.6)	5 (0.5)
Total	163 (100.0)	796 (100.0)	959 (100.0)

Bojesen et al. (32).

Nonmosaic 47,XXY most often occurs because of nondisjunction of the sex chromosomes during the first or second meiotic division of gametogenesis in either parent. There is evidence that failure of the paternal XY or maternal XX chromosomes to pair and recombine in the usual way is a causative factor, as is also the case for autosomal trisomies (153,154). Paternal meiosis I errors account for approximately 50% of 47,XXY karyotypes (154). Of the remaining maternally derived karyotypes, 22–48% are due to meiosis I errors, 9–29% to meiosis II errors, 7% to unknown meiotic errors, and 3–16% to postzygotic mitotic nondisjunction (153–155). In prenatally ascertained cases, 54% are paternally derived and 46% are maternally derived (155). The maternal meiosis I errors contribute to a small overall association with advanced maternal age (156). Consistent with the situation for autosomal trisomies, advanced paternal age does not play a role. The phenotype does not appear to be influenced by the parental origin of the extra X chromosome, though few studies have specifically addressed this issue (157). The molecular basis of KS remains poorly understood. The phenotype is assumed to be caused by the expression of X-linked genes in one or more of the four pseudoautosomal regions of the X chromosome that escape inactivation. Preliminary data suggest that there are many more genes in this category on Xp than on Xq (153), but the identity and function of the critical genes have not been determined. Presence of an extra copy of the SHOX gene is probably the basis for the increased height in KS (158).

Although there is no consensus regarding a clinical definition of KS, a KS diagnosis should always be based on clinical findings combined with a confirmatory cytogenetic evaluation. On the other hand, a phenotypically normal man with very low-grade mosaicism should probably not have the diagnosis of KS.

Underdiagnosis and delayed diagnosis of KS is a major problem (32). Importantly, early diagnosis permits identification of speech problems and scholastic difficulties that require special measures to improve. Moreover, early diagnosis facilitates prevention or remediation of the long-term consequences of gonadal insufficiency.

From the earliest study, the syndrome was described as “not uncommon” (159) but the prevalence was unknown

until several large-scale sex-chromosome surveys were performed on newborns (22,27,160). Summarizing data from these studies across countries and ethnicities yields a prevalence estimate of 152 per 100,000 (32). Newer studies confirm that the true prevalence is in fact around 150 per 100,000 males or one in every 660 males (32), but with a major discrepancy between the prenatal and postnatal prevalence, indicating a severe lack of diagnostic activity, with only 25% of the expected number of KS being diagnosed postnatally and <10% of these being diagnosed before puberty. British data similarly find that only 26% of the expected number of adults with KS were diagnosed and only 4% of 10-year-olds (161). PCR-based screening methods to detect sex-chromosome aneuploidy are available but have not yet been validated on recent newborn population samples. If molecular screening for KS is offered, cytogenomic confirmation will be needed as well as infrastructure for follow-up and treatment of the patients with sex-chromosome abnormalities, and support services to help parents and caregivers deal with the uncertainties inherent in this type of diagnosis. Prenatal diagnosis of a fetus with 47,XXY karyotype should lead to professional genetic counseling, in order to inform the pregnant couple about the relatively good prognosis, but at the moment, 75% of couples expecting a child with KS choose termination (32). Owing to the fact that only approximately 25% of the expected number of men with KS is diagnosed, ascertainment bias is a major problem when interpreting data from different studies on different populations of KS patients. The phenotype of a KS patient diagnosed in childhood or at puberty may be different from what is seen in an adult KS patient. Most published data so far are hampered by such ascertainment bias. The most unbiased data available are the data from the prospective studies on boys diagnosed at birth, but the numbers of patients in these studies are small and hence the variability of estimates of certain clinical findings will be considerable.

As a consequence, one has to interpret prevalence data on clinical findings with great caution, and not readily extrapolate prevalence rates found in one population of KS patients to all KS patients.

#### 44.4.2 Congenital Malformations

There are no specific stigmata at birth, but an increased incidence of congenital malformations has been found (162). Minor congenital abnormalities were found in 26% with clinodactyly of the fifth finger as the most frequent (163) and major congenital abnormalities were found in 18% of KS boys, with cleft palate, inguinal hernia and cryptorchidism being the most frequent (163). Further evidence of highly increased prevalence of cryptorchidism comes from a large cross-sectional study from an andrological clinic, where 27% of KS patients had a history of maldescent of testes compared to 8% of the total number of patients attending the same clinic (162).

### 44.4.3 Testicular Development

The typical testicular histology includes hyalinization of seminiferous tubules, loss of germ cells and Leydig cell hyperplasia. In some KS patients, focal spermiogenesis may be found with the possibility of surgically extraction of sperm for in vitro fertilization (IVF) (164). The cause of the hyalinization of the testes with subsequent hypogonadism and infertility is unknown. There is a loss of spermatogonia from infancy (165) while hyalinization of the seminiferous tubules does probably not occur until midpuberty (166). At the beginning of puberty (167), testes grow to approximately 4 ml and thereafter shrink to the pathological adult size of <4 ml (168,169). Testes may be malfunctioning already during intrauterine life since micropenis seen in some newborn males with KS may be a result of decreased testosterone production in utero (170). Some find the normal surge in testosterone seen in the first 1–6 months of life attenuated in KS boys (170,171), while others find it similar to controls (172). Longitudinal studies on boys with KS before and during puberty have shown that even before puberty, testes are smaller than in normal boys (173). The levels of FSH, LH and testosterone are normal during the prepubertal period, but after the onset of puberty, a rise in FSH and LH and a decline in testosterone occur, compared to normal boys (168). In adult KS patients, not only decreased levels of testosterone but also decreased levels of insulin-like factor 3, a marker of Leydig cell function, are present (174). There is normal function of Sertoli cells in infancy since normal values of both inhibin B and AMH has been reported (171). At the end of puberty, inhibin B level diminishes, reflecting a loss of Sertoli cells (167). The hypogonadism, being regarded as a hallmark of KS, is relative rather than absolute as most of the KS patients have testosterone levels just below the normal range (162,175). Even though the levels of testosterone for many patients are within the normal range, levels of gonadotropins are usually elevated (152,162,175), indicating a relative hypogonadism that is reflected by the increased pituitary drive, and hence may be one of the causes of Leydig cell hyperplasia. The level of 17 $\beta$ -estradiol is relatively elevated compared to the low level of testosterone, being reflected by an elevated ratio between 17 $\beta$ -estradiol and testosterone (175).

### 44.4.4 Fertility

KS patients are usually considered infertile, but with the development of the testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) methodologies, it is now possible to extract viable sperm from the testes by surgical biopsy and inject it directly into an extracted ovum. Worldwide more than 60 children have been born after successful ICSI treatment in KS couples (164,176). There have been concerns about an increased risk of chromosome aberrations in the offspring because an increased

number of both autosomal and sex-chromosome aneuploidy has been found in spermatozoas extracted or ejaculated from KS men (177). As a consequence, professional genetic counseling and options of prenatal diagnosis or even preimplantation genetic diagnosis should always be offered to couples seeking infertility treatment where the male is suffering from KS.

### 44.4.5 Hypogonadism

The frequent hypogonadism present may delay or reduce the development of normal male secondary sexual characteristics with less growth of beard, muscle bulk, and secondary body hair (152). The sexual function in KS patients has not been investigated in detail, but decreased libido has been reported in 70% after the age of 25 years (162). Hypogonadism may also be involved in the neurocognitive phenotype seen in KS. The long-term consequences of hypogonadism in KS are difficult to separate from the gene-dose effects of having an extra X-chromosome, because studies on comparable hypogonadal diseases (e.g. hypogonadotropic hypogonadism) are lacking because of their infrequency.

**44.4.5.1 Gynecomastia.** Gynecomastia is relatively frequent in puberty in normal boys and may be very troublesome. In KS, the prevalence is markedly increased, up to 50% (152) in some series, although the true prevalence probably is much lower (178). The decreased testosterone level in concert with an elevation in estradiol may cause gynecomastia (168). Testosterone treatment may lead to a regression of gynecomastia, but some KS patients choose to have the breast tissue removed surgically.

**44.4.5.2 Diabetes and the Metabolic Syndrome.** Epidemiological studies on both morbidity (45) and mortality (179) show an increased risk of diabetes, and clinical studies describe a strikingly high incidence of the metabolic syndrome and insulin resistance in KS (175,180), with increased cholesterol. KS subjects accumulate excessive amounts of body fat and especially truncal fat, and typically have a low level of physical fitness. Prospective studies in other populations have shown that low levels of testosterone (and sex hormone-binding globulin) can predict abdominal adiposity (181), the metabolic syndrome (182) and type 2 diabetes (183). Apart from the association between low testosterone, body composition, the metabolic syndrome and insulin sensitivity, low testosterone has been associated with an adverse CV risk profile, with increased C-reactive protein and triglycerides and decreased HDL-cholesterol (184). Although speculative, it seems possible that, on one hand, the hypogonadism contributes to the development of metabolic syndrome (and increase cardiac risk factors) but, on the other hand, protects against ischemic heart disease by increasing the amount of adiponectin (185). Corroborating this theory are data from a study on castrated men, showing a decreased risk of dying from acute myocardial infarction, in spite of a generally



increased mortality risk (186). It seems plausible that a vicious circle may exist where hypogonadism leads to abdominal obesity that leads to insulin resistance that may further aggravate the hypogonadism (187).

**44.4.5.3 CV Disease.** Mortality from CV diseases is increased in KS (43,179), even though the mortality from ischemic heart disease is reduced (43). One reason for the increased mortality may be mitral valve prolapse, which is described in a large proportion in one study (188), a condition that is related to increased risk of sudden death. The risk of hypostatic leg ulcers may be significantly increased (189) and may cause more serious morbidity and mortality from pulmonary embolism (43). Dysfunction of the fibrinolytic system has been proposed as a reason for this and increased activity of plasminogen activator inhibitor 1 in KS patients was described in one study (190), but further studies are needed to elucidate this.

**44.4.5.4 Osteoporosis.** Hypogonadism is a known cause of secondary osteoporosis in both females and males (191). A majority of studies shows a significant reduction in BMD compared to normal men although frank osteoporosis is less frequent (192–195). Epidemiological studies on morbidity and mortality did show an increased risk of being admitted to hospital with an osteoporotic fracture (forearm, hip and spinal fractures) (45) and an increased risk of dying from hip fractures (43), indicating that the reported reduction in BMD may mirror an important clinical problem. In a mouse model of KS (41,XXY), testosterone deficiency did not explain the reduced BMD, raising the possibility that the chromosome abnormality *per se* leads to reduced BMD (196).

**44.4.5.5 Cancer.** It has been debated whether gynecomastia may lead to increased risk of breast cancer. A register-based study on cancer in KS did not show an increased risk of breast cancer in KS (197), but a Swedish study (198) using karyotyping on nonmetastatic lymph nodes from men with breast cancer showed 47,XXY karyotype in 7.5% of the examined patients, equivalent to a 50-fold increase in risk of breast cancer. A study on mortality and incidence of cancer in a large cohort of KS subjects ( $n=3518$ ) showed that the risk of breast cancer was significantly elevated (199). In addition, a significantly increased risk of mediastinal germ cell tumors (197), an increased mortality risk from lung cancer, and non-Hodgkin lymphoma, and a significantly decreased risk of dying from prostate cancer are present.

**44.4.5.6 Cognitive Disturbances.** KS was found at increased prevalence among inmates of penalty institutions and institutions for the mentally retarded in studies in the early 1960s (200–202). This leads to the conclusion that KS was associated with criminal behavior and low intelligence, stigmata that were challenged by prospective studies of newborns screened for sex-chromosome disorders during the 1970s. In these later studies with long-term follow-up (150,151,203), the global intelligence was found to be near normal, but with decreased verbal

intelligence, delayed development of speech and high proportion of educational problems (204–207). A study on prenatally diagnosed KS patients showed a remarkably good outcome regarding intellectual performance (208), but this may reflect a higher socioeconomic status and better motivation in parents choosing to continue the pregnancy in spite of the KS diagnosis. One study on adults with sex-chromosome abnormalities detected at birth showed that the decreased verbal IQ and reading impairment persisted in adulthood (209), and also that a large variation in educational and vocational achievement was present. The reason for the delay in speech and decreased verbal intelligence is unclear, but an MRI study of the whole brain found diminished left temporal gray matter volume in five untreated KS patients, compared to five testosterone-treated KS patients and to normal controls (210). Another study on brain perfusion in KS patients, using single photon emission computed tomography, described a diminished lateralization of perfusion to the left, compared to normal controls and a negative correlation between lower perfusion in regions involved in language processing and scores in verbal tests (211). This may, in part, explain the decreased verbal performance seen in KS. Although the results are based on small sample sizes, they point toward a possible positive effect of testosterone on verbal performance in KS. This is in accordance with a study on verbal intelligence, pubertal development and testosterone levels, showing a positive association between testosterone levels and verbal IQ (212). Interestingly, a recent study showed impaired learning and memory function as well as testicular failure in XXY mouse model (41,XXY) (213,214) although there were no impairments in sociability (215). In another recent mouse model (41,XXY\*), there were no differences between wild type and the 41,XXY\* in locomotion, exploration and anxiety-related behavior, but memory recognition was clearly impaired (216).

Sexuality may be affected—fewer 47,XXY men are married, experience greater sexual dissatisfaction in general, and acknowledge unconventional sexual experiences compared to a control group and demonstrate a less-masculine-gender role (217).

**44.4.5.7 Psychiatric Diseases.** Increased incidence of psychiatric illness was reported in KS primarily based on screening for sex-chromosome abnormalities in penalty institutions, psychiatric hospitals and institutions for the mentally retarded (202). In a long-term follow-up study, an increased frequency of referral to psychiatric treatment was seen (151) and a survey for sex-chromosome aberrations among patients with schizophrenia found a four- to fivefold excess of KS (218). An epidemiological study on hospital admissions in KS showed a significantly increased risk of discharge with a psychiatric diagnosis (45), and patients may show an increase in schizophrenia-spectrum pathology (219). Overexpression of X-linked genes escaping X inactivation is thought to be involved in some of the psychiatric disturbances seen in KS (220).



**44.4.5.8 Criminality.** One follow-up study found no increased frequency of criminal behavior in KS judged by self-reported penalties (221). Another study following 34 KS patients for 10 and 20 years showed increased criminal behavior at 10 years of follow-up, compared with both hypogonadal men (from other causes than KS) and a control group (203) but after 20 years of follow-up, there was no significant difference in criminality (222). Recently published data on 32 KS patients diagnosed prepubertally or during puberty showed a large proportion of patients with severe psychosocial problems, with 69% having problems with controlling aggression, 28% offending the law and 18% having convictions (223).

**44.4.5.9 Treatment.** Treatment and care of patients with KS is a multidisciplinary task that ideally should involve speech therapists, psychologists, general practitioners, pediatricians, endocrinologists, urologists and infertility specialists. Infants with KS are rarely diagnosed because they lack KS-specific stigmata. However, some KS boys have micropenis, which in some have been treated successfully with topic testosterone cream or single injections with intramuscular testosterone. The most serious problem in early childhood is the delay of speech development affecting perhaps half of the boys with KS (151). Careful observation is needed in order to refer these boys to speech therapists if delay of speech is observed. The same holds true for learning disabilities, which were observed in 77% of boys with KS followed from birth to adulthood (151).

At puberty, when gonadotropins are rising, testosterone treatment should be initiated in order to secure a proper masculine development of sexual characteristics and also to secure a sufficient increase in muscle bulk and BMD (224). Testosterone treatment in pubertal KS boys has also been reported to increase energy, endurance, better mood and concentration and relations to others (225). Data on a group of KS diagnosed before and during puberty showed increased psychosocial problems in periods without testosterone treatment (223).

Treatment is recommended to be continued life-long in order to prevent osteoporosis, obesity, metabolic syndrome, and diabetes and to ensure optimal cognitive development. Although most clinicians taking care of KS patients believe that testosterone treatment has a positive impact, both physically and psychologically, there is no evidence-based confirmation of such an effect. Treatment in young hypogonadal men has been shown to have a positive impact on fat mass, muscle mass, and muscle strength, as well as sexual activity and related areas, and it improves positive aspects of mood (226). In older hypogonadal males, data suggest positive effects of treatment on visiospatial cognition and verbal memory (227).

Even though some KS patients have normal testosterone values, virtually all have increased gonadotropin levels, and all KS patients should receive testosterone treatment if their gonadotropins are elevated, even though their testosterone levels may be within the lower end of

the normal range. Certainly, patients should be treated if they are suffering from hypogonadal symptoms (lack of energy, decreased libido, and also including abdominal adiposity).

The aim of treatment should include normalization in LH and testosterone levels in the midnormal range, rather than low-normal nadir values of testosterone. LH can be normalized in virtually all KS patients if the testosterone dose is carefully titrated. It follows that many KS patients are not sufficiently treated because LH is often high. One should also focus on the subjective symptoms reported by the patient, especially to avoid high levels of testosterone, which can occur with injection therapy and may cause discomfort.

A possible negative effect of exogenous testosterone on fertility (or outcome of TESE and ICSI) has been debated, as well as treatment with aromatase inhibitors and HCG to increase intratesticular testosterone in order to increase the chance of sperm recovery by TESE (176). These interesting data are based on less recovery of sperm in five KS patients on long-term testosterone treatment and should be confirmed, preferentially in a randomized setting, before withholding testosterone treatment to young men with KS.

An outpatient management program for patients with KS is suggested in Box 44-3. Initially, testosterone

#### BOX 44-3 Proposed Assessment and Follow-Up Program for Patients with Klinefelter Syndrome

##### At baseline

Confirmation of karyotype, if necessary  
Sex hormones: testosterone, estrogen, SHBG, FSH and LH  
Fasting glucose and lipids  
Thyroid status, hemoglobin, hematocrit, Prostate specific antigen (PSA)  
Physical examination including BP, height, weight, waist, testes, breasts and varicose veins  
Bone desitometry (DEXA scan) and vitamin D status, p- calcium  
Information about the syndrome  
Initiation of androgen treatment (injections, transdermally or oral)  
Questions about well-being, physical activity, energy, sexual activity, libido  
Echocardiography  
Fertility issues

##### Annual (every three months initially)

Physical examination including BP, height, weight, waist, testes, breasts and varices  
Sex hormones: testosterone, estrogen, SHBG, FSH and LH (nadir values)  
Fasting glucose and lipids  
Thyroid status, hemoglobin  
Questions about well-being, physical activity, energy, sexual activity, libido

##### Every 2–5–10 years

Bone densitometry (DXA scan) and vitamin D status, plasma calcium, PSA

treatment should be followed with visits every 3 months until testosterone dose is adjusted, thereafter annually.

**44.4.5.10 Perspectives.** Not a single randomized, placebo-controlled study addressing the effects of testosterone in KS patients has been published. Of course, there is a major ethical problem in comparing, what we think of as a potent medication with placebo, especially in puberty where lack of treatment may lead to long-term negative consequences for the patients. But at least randomized, placebo-controlled studies on adults with KS could and should be performed, with testosterone preparations that will restore testosterone to normal values, in a population large enough to detect small changes in e.g. BMD, body composition, insulin sensitivity and also modalities of quality of life. Future studies will hopefully provide the evidence that is essential for optimizing the treatment of KS patients.

**44.4.5.11 Conclusions.** KS is a frequent cause of hypogonadism and infertility. It is the most common sex-chromosome disorder in man, with 47,XXY being the most common karyotype. The syndrome affects one in every 660 males but is severely underdiagnosed; only 25% of the expected number is diagnosed and only a minority of them before puberty.

In children, the main clinical findings are delayed speech development and learning disabilities as well as increased height and chryptorchism. In adults, the main reasons for diagnosing KS are infertility and hypogonadism.

KS is associated with a significant increase in risk of death and morbidity from a number of diseases. Testosterone treatment is recommended to all KS patients with elevated gonadotropins, in order to counteract the many harmful effects of hypogonadism on body composition, glucose and lipid metabolism, well-being, sexual function and other functions, both mentally and physically.

## 44.5 47,XXX SYNDROME

The 47,XXX karyotype occurs in approximately 84 per 100,000 female newborns when pooling data from large cytogenetic surveys (21,25–28,228–233) and was first described in 1959 by Jacobs et al. Unlike TS, this is essentially the same incidence found at amniocentesis (19), suggesting that prenatal loss after midgestation is rare. Prospective studies (209,234–239) have documented that females with 47,XXX have no distinguishing physical features, generally normal pubertal development and fertility, and intelligence that is within the normal range although as a group, full-scale IQ is lower than in control females (240). They are at risk for developmental delays, learning disabilities, and difficulties with psychosocial adjustment, while demonstrating significant individual variability (240). Importantly, only a minority of the expected number of females with 47,XXX are ever diagnosed (13%) (33). There is a substantial delay to diagnosis of a median of 18 years with wide variations. In other words, half of all diagnosed females with 47,XXX are

not diagnosed before adulthood. Mortality is increased in 47,XXX with a hazard ratio of 2.5 (95% confidence interval: 1.6–3.9), due to CV, urological and chromosomal diseases and congenital defects (33).

### 44.5.1 Genetic Background

In a study of 50 females with 47,XXX, 90% of the extra X chromosome was maternally derived (14). Molecular studies indicated that 64% of these were the result of meiosis I nondisjunction, 18% were the result of meiosis II nondisjunction, and 16% were the result of postzygotic nondisjunction. In another 31 conceptuses with 47,XXX karyotypes, 93.5% resulted from maternal nondisjunction with increasing maternal age being present in meiosis I errors but not in the meiosis II errors (241). There is no excess intrauterine mortality after amniocentesis (208).

### 44.5.2 Growth and Physical Development

Females with X chromosome trisomy cannot be identified by the presence of distinct physical differences at any age (240). The mean birth weights are somewhat lower than those of 46,XX girls. Many girls with 47,XXX will reach the 80th percentile for height during adolescence. The greater height seems to be attributable to increased growth of the lower segment relative to the trunk. Variability is great and stature is also influenced by parental heights in the usual multifactorial manner (158). Relative microcephaly is present in a minority, but average head circumference is significantly smaller than in controls (242). Those who have been followed prospectively from birth have a higher incidence of minor anomalies such as epicanthic folds, ear anomalies, and clinodactyly than females with an euploid karyotype.

### 44.5.3 Gonadal Function and Fertility

The vast majority of adult women with an extra X chromosome, probably go through puberty in the usual manner, is no different with regard to their sexual orientation, and lead a normal sexual life. Most also have normal reproductive capacities and bear children without sex-chromosome aneuploidy. Precocious puberty, early-onset menarche, and primary or secondary amenorrhea, including premature ovarian failure, have also been reported (240), and premature ovarian failure seems to be more prevalent than in controls (242). The incidence of sex-chromosome aneuploidy in the offspring of women with 47,XXX does not appear to be substantially increased, but has been reported.

### 44.5.4 Intelligence, Personality, and Behavior

Neuromotor deficits with an increased tendency for delays in the acquisition of gross motor skills are frequently

present and usually mild but may result in poor coordination and reduced athleticism. To somewhat greater extent than other individuals with sex chromosome abnormalities, females with 47,XXX karyotypes are often delayed in the acquisition of language and academic skills. IQ is usually 10–15 points below that of siblings, but mental retardation (IQ < 70) is rare (242). The average IQ in a prospectively followed group of 46 females was between 85 and 90 with a broad range (53–112) (238). Verbal IQ was significantly less than performance IQ, with deficits in both expressive and receptive language present in approximately 50%. A total of 70% had learning disabilities that required some form of educational intervention. A study of 11 adolescent girls with 47,XXX found, in addition to a reduced mean full-scale IQ, impaired scores on individual tests of attention, concept formation, mental flexibility, spatial thinking, verbal fluency, and basic academic skills compared with controls (234). Retention and retrieval of previously learned information did not differ from controls. These 11 adolescent girls with 47,XXX found the academic demands of high school particularly problematic. Nine of them required remedial placements but less than half completed their high-school degrees (243). From a social point of view, these girls were immature, passive, and prone to aberrant behavior and depression. Two of the 11, however, attended college and graduated. Employment tended to be unskilled but did range to include a self-owned small business (235).

A study examining the transition from adolescence to adulthood in these 11 young women with 47,XXX and nine female sibling controls found the former group to be less well-adapted, to experience more stress, to have more problems with work, leisure, and relationships, and to demonstrate more psychopathology (236). Psychological dysfunction was not wholly attributable to lowered intelligence though the latter did appear to exert an influence. Nevertheless, most of the group experienced mild disturbances and functioned reasonably well. The authors concluded that previously reported outcomes of antisocial behavior and severe psychopathology, including schizophrenia, are rare in women with 47,XXX and that the variability in behavioral phenotype is much larger than was originally appreciated. In a follow-up study of the same cohort, the 47,XXX group had the greatest impairment in IQ in comparison with patients with 45,X and 47,XXY (209). Behavioral characteristics are also present (149). There also seems to be an increased frequency of attention deficit/hyperactivity disorder, anxiety and mood disorders (209,234), and studies have found an increased risk of seizures and frequent EEG abnormalities (244,245).

#### 44.5.5 Renal System

Urogenital malformations, including cloacal exstrophy, Müllerian anomalies, renal anomalies, and lower

mesodermal defects (single umbilical artery, sacral vertebral fusions, and anal agenesis) have been reported in a small number of individuals (246), but an increased association with 47,XXX has not been substantiated by prospective studies. Routine investigation of the urogenital tract in females who are incidentally found to carry an extra X chromosome is probably unwarranted.

**44.5.5.1 Management.** Clinical problems should be dealt with in a similar manner as in other groups of patients. Many patients will probably benefit from speech therapy, occupational therapy and educational support during childhood and adolescence. The diagnosis of specific cognitive disorders is important and should preferably be made early in order to allow supportive measures in school (240,242). In cases of premature ovarian failure, HRT should be offered just as in TS (106).

### 44.6 X CHROMOSOME MOSAICISM

45,X/47,XXX, 45,X/46,XX/47,XXX and 46,XX/47,XXX mosaics are the product of nondisjunction in the mitotic division of a 46,XX zygote. The increased frequency with which these mosaic diagnoses are made prenatally indicates that many mosaic individuals are not identified postnatally.

The majority of these women have been described in the obstetrical literature, indicating that they do not, for the most part, have physical stigmata of TS, but some have and this often lead to early diagnosis. A study examining growth in approximately 50 women with triple X mosaicism found a direct correlation between height SD scores and the proportion of 46,XX cells in peripheral lymphocytes and an indirect correlation between these scores and the proportion of 45,X cells (247). The 47,XXX cell line did not seem to have a significant influence on stature. The interpretation of studies relying on one tissue in mosaic patients must be cautious, however, because the frequency of the cell lines in other tissues remains unknown. In vivo selection for the euploid cell line may occur over time.

Spontaneous pubertal development and menarche occur in many girls with these mosaic karyotypes. Premature ovarian failure or recurrent miscarriages have been observed in some women, but the majority have chromosomally and phenotypically normal pregnancy outcomes.

Adolescent women with this karyotype generally have neuropsychological profiles that are indistinguishable from controls, and developmental or learning problems are the exceptions rather than the rule (234–238,243).

### 44.7 SEX CHROMOSOME TETRASOMY AND PENTASOMY (POLYSOMY)

Although 45,X, 47,XXX, 47,XXY, and 47,YYY collectively occur with an incidence of approximately 1 in 400 newborns, sex-chromosome polysomies are much

less-frequent events. Unfortunately, their rarity precludes prospective studies that would present an unbiased picture. These individuals all have been identified clinically. The additional sex chromosomes seem to produce a lowering of IQ and an increase in somatic malformations. Similar to the trisomic sex-chromosome abnormalities (47,XXX, 47,XXY, 47,XYY) where adult height is increased in comparison with the background population, height is higher than normal in persons with four sex chromosomes, but seems to be progressively lower, the more sex chromosomes, and thus SHOX genes, are present (158). Whether this effect on height is explained by the increased expression of other genes on the sex chromosomes remains to be determined.

#### 44.7.1 Male—48,XXXY, 49,XXXXY, 48,YYYY, 48,XYYY and 49,XYYYY

The most significant effects of additional X chromosomes on phenotype are a reduction in intellectual functioning and an increase in malformations. Approximately 1 in 85,000 males is born with a 49,XXXXY karyotype (248), whereas 48,XXXY is somewhat more common with a phenotype that is intermediate between 47,XXY and 49,XXXXY. The latter diagnosis is usually made postnatally because of variable growth deficiency, facial coarseness and dysmorphism, hypogenitalism, and malformations of the heart and skeleton such as radioulnar synostosis or congenital hip dislocation. These men have significant cognitive and behavioral problems with a variable IQ between 20 and 60. There are no consistent prenatal findings, but prenatal diagnosis has been described in fetuses with cystic hygroma, abnormal posturing of the lower extremities, and small genitalia (248). Testicular histology resembles the situation in KS and most will experience hypergonadotropic hypogonadism and should probably receive testosterone supplementation in the same manner as patients with KS (249).

Males with poly-Y karyotypes containing three Y chromosomes (48,XYYY) are usually identified by tall stature and aberrant behavior (impulsivity and low frustration tolerance), with low-normal or subnormal intelligence, developmental delay, abnormal dentition, radioulnar synostosis, and hypergonadotropic hypogonadism (250). Normal-appearing external genitalia seem to be the rule, but testicular biopsies have demonstrated variable abnormalities, and azoospermia is common. Recurrent respiratory infections, inguinal hernias, and additional abnormalities of the humerus, radius, and ulna have also been reported. Minor anomalies include brachydactyly, clinodactyly, and single transverse palmar creases. The phenotype is, however, inconsistent.

Males with four Y chromosomes are even fewer in number. In addition to the features outlined above, case reports suggest that these males are ascertained at birth because of the presence of craniofacial dysmorphism, including trigonocephaly, hypertelorism, upslanting palpebral fissures,

epicanthic folds, low-set ears, and micrognathia or prognathia. These boys are not uniformly tall and appear to have more severe intellectual deficits, hypotonia, motor delay, and speech delay than their counterparts with three Y chromosomes. Little is known about the extent of phenotypic variability (250).

#### 44.7.2 Female—48,XXXX and 49,XXXXX

More than 40 women with a 48,XXXX karyotype have been described. These individuals are often tall with an average height of 169cm (250). Otherwise there are no consistent clinical features. Epicanthic folds, hypertelorism, nystagmus, radioulnar synostosis, and clinodactyly are often reported. Genitalia are generally normal, but secondary sex characteristics may be incompletely developed. In one series, half of the adult women with this karyotype had normal menarche and menopause (250). With one exception, mental retardation has been consistently reported, with an average IQ of 60, but a range from 30 to 75. There is no characteristic behavioral phenotype.

The penta-X karyotype is less common than 48,XXXX, and there is a paucity of information on adult women with this karyotype, but primary ovarian failure has been noted in one case (251). All these individuals demonstrate a more markedly abnormal physical and developmental phenotype than is seen in girls with quadruple-X karyotypes. Unlike other sex-chromosome polysomies, girls with 49,XXXXX often demonstrate intrauterine and postnatal growth retardation, so they are more likely to be short than tall. The phenotype consists of microcephaly and coarse facial features, including hypertelorism, epicanthic folds, up-slanting palpebral fissures, a depressed and/or broad nasal bridge, and a short broad neck. Congenital heart defects, particularly ventricular septal defect and patent ductus arteriosus, radioulnar synostosis, generalized joint laxity with multiple dislocations, and talipes equinovarus have also been reported. Minor anomalies include single transverse palmar creases and clinodactyly. Puberty is delayed, secondary sex characteristics are incompletely developed, and several girls have been noted to have small uteri. Fertility remains unknown. Mental retardation has been universal with an average IQ of 50 and no distinct behavioral profile. The mechanism of origin seems to be successive maternal meiosis I and II nondisjunction in most instances (252–254).

### 44.8 47,XYY KARYOTYPE

47,XYY syndrome is present in approximately 1 in 1000 newborn boys, however, only a minority of about 15% are diagnosed postnatally, leaving the entire literature on 47,XYY heavily biased (34). The median age at diagnosis is 17.1 years, thus, half of all cases are diagnosed as adults. This sex-chromosome aneuploidy



is not characterized by distinct physical features and, because there does not appear to be a recognizable pattern of neurodevelopmental or behavioral characteristics, the use of the term *syndrome* may be inappropriate. Males with an extra Y chromosome are phenotypically normal and most never come to medical attention. This seems to be reflected in the paucity of recent clinical literature on individuals with this karyotype. There are relatively small numbers of controlled prospective studies, so knowledge of the medical and psychological profiles of boys with this chromosome constitution remains subjected to both ascertainment and reporting biases. However, Ratcliffe (151) reported the clinical features of 19 males ascertained at birth by consecutive newborn cytogenetic screening and followed to the ages of 16–27.

Mortality is clearly increased in 47,XYY syndrome with a hazard ratio of 3.6 (95% CI 2.6–5.1) corresponding to an average loss in lifespan of 12.4 years (34). The increased mortality is due to a range of causes such as cancer, pulmonary, neurological and urological diseases (34).

### 44.8.1 Etiology

The additional Y chromosome is derived from paternal meiosis II nondisjunction or postzygotic mitotic nondisjunction (255). 47,XYY syndrome is therefore not associated with increased maternal or paternal age. Not surprisingly, the incidence of 47,XYY syndrome at amniocentesis is similar to that at birth, indicating that few of these fetuses are lost in the second and third trimesters of pregnancy.

### 44.8.2 Growth and Physical Development

The length and weight of newborns with an extra Y chromosome are indistinguishable from others and there are few, if any, clinical differences to suggest the karyotype (256,257). There have been reports that the presence of the extra Y chromosome is more often associated with renal malformations and Potter sequence (258). There are also reports describing CNS anomalies in fetuses with this karyotype, including one with isolated agenesis of the corpus callosum and another with Dandy Walker malformation (259). Coincidental association is possible though it is intriguing that a fetus with 49,XYYYYY was found to have hypoplasia of the corpus callosum and cerebellum and a large posterior fossa cyst causing secondary hydrocephalus (260). No increase in any major anomaly has been seen in prospective studies of this karyotype with unbiased ascertainment at birth. There are rare reports of radioulnar synostosis in boys with 47,XYY (261) though this finding is more consistently associated with the presence of more than two Y chromosomes (48,XYYY and 49,XYYYYY) or with mosaic karyotypes that include multiple Y chromosomes.

Although an increased frequency of minor anomalies such as clinodactyly, inguinal hernia, and pectus carinatum has also been suggested, no consistent clinical picture has emerged. Similarly, no distinction in growth or appearance can be made between infants with 47,XYY and those with 46,XY karyotypes. For this reason, the karyotype is often found coincidentally.

Accelerated linear growth is present as early as age 2 and boys with 47,XYY are an average of 7.6 cm taller than their peers by the onset of puberty (151). The onset of puberty is on average 6 months later than in controls and the pubertal growth spurt is of longer duration, producing an average final height of 188 cm (151). Gron et al. documented larger craniofacial dimensions in these males than were present in controls (262), and Geerts et al. found increased height, weight, and head circumference in this population (257), and the increased height is thought to be due to the expression of additional genes involved in linear growth (158).

### 44.8.3 Gonadal Function and Fertility

Pubertal development, testicular histology, and spermatogenesis are most often normal. In a number of instances, smaller-than-normal testes, decreased spermatogenesis, spermatogenic arrest, subfertility, and sterility have been reported. Persistence of the extra Y chromosome in germ cells can impair testicular tubular development and result in low sperm counts. However, it appears that XY pairing and recombination usually occur normally in 47,XYY, the extra Y chromosome being lost during spermatogenesis (263,264), so that many XYY men have fathered chromosomally normal children. It has generally been observed that reproductive risks for males with 47,XYY are no higher than for euploid males, despite the fact that in situ hybridization studies demonstrate a lower frequency of single Y-bearing sperm than expected and a variably higher rate of disomic XX, XY, and YY spermatozoa in males with 47,XYY (263–265). However, results vary substantially between individuals (264). These observations, based on the collective analysis of several hundred sperm in 47,XYY and control men, reach a very high degree of statistical significance. However, the absolute frequencies of XX-, XY-, and YY-bearing sperm from 47,XYY men remain low at <1% (264). It appears that the increased rate of sperm aneuploidy is no greater than in males with oligoasthenoteratozoospermia and a normal karyotype (146). With occasional exceptions (266), the sperm of men with 47,XYY do not appear to have a higher rate of autosomal disomy than those of controls. In half of the case reports describing autosomal aneuploidy in offspring born to men with 47,XYY, the aneuploidy has been of maternal origin (267). There is no indication that the sexual orientation of boys and men with 47,XYY is any different than it is in those with a 46,XY karyotype.

#### 44.8.4 Intelligence, Personality, and Behavior

The commonest indications for karyotyping in boys with 47,XYY are developmental delay and/or behavioral difficulties. Population-based studies have demonstrated that intellectual abilities tend to be slightly lower on average than those of siblings and matched controls and boys with an extra Y chromosome are more likely to require educational help. However, intelligence is usually well within the normal range. Ratcliffe (151) reported average verbal and performance IQs of 99 and 104 among 19 boys and men identified at birth and followed prospectively, compared with 114 and 115 in controls. Interestingly, boys with KS, as well as females with 47,XXX, have significantly smaller whole-brain volumes on MRI than age- and sex-matched controls, whereas this difference is not seen in males with 47,XYY (268). During school age, learning disabilities requiring educational intervention are present in approximately 50% and are as responsive to therapy as they are in children with normal chromosomes (151,221). Expressive and receptive language delays and reading disorders are common. Hyperactivity, distractibility, and temper tantrums have been present in some boys and may affect their school performance (269). Aggression is not frequently observed.

Older psychological studies suggested that certain personality traits, such as infantilism, lack of emotional control, increased impulsiveness after emotional stimulation, and a weak concept of self were so characteristic that men with 47,XYY could be recognized by psychological tests alone (270). Such findings are largely contradicted by prospective studies, in which males with 47,XYY are behaviorally indistinguishable from controls.

There may be a weak predisposition to behavioral problems in the setting of a stressful environment as adolescents and young adults with 47,XYY from dysfunctional families seem to be less well adjusted than their siblings. This is not true in those whose environments have been more supportive. An important bias is that any psychosocial difficulties experienced by an individual with 47,XYY are likely to be attributed to his karyotype when they could well be caused or influenced by a myriad of environmental factors.

There are case reports describing pervasive developmental delay in a few boys with 47,XYY but these may be the result of ascertainment bias. Because the vast majority of boys with an extra Y chromosome do not have pervasive developmental delay, Nicolson et al. (271) speculate that the extra Y chromosome might set the stage for aberrant brain development. Pervasive developmental delay could then develop in a small number of these boys when a sufficient number of additional predisposing factors, such as a family history of learning or social difficulties, are present.

The karyotype 47,XYY has probably aroused more public interest than any other chromosome abnormality,

because the extra Y chromosome was demonstrated in tall, mentally retarded criminals. Prospective studies of XYY males detected at birth are now starting to yield data on adults. Gotz et al. (221) identified 16 newborns with 47,XYY and 13 newborns with 47,XXY by population screening over a 12-year period ending in 1979. Twenty years later, these men were compared with 45 controls with normal karyotypes and normal intelligence for rates of antisocial behavior and criminal convictions. The males with 47,XYY demonstrated a significantly higher frequency of criminal convictions and antisocial behavior in adolescence and adulthood than did controls, but multiple regression analysis suggested that these differences were associated with lowered intelligence. The same observations were not seen in the Klinefelter group, but the small number of subjects precluded any firm conclusions.

There is some suggestion, therefore, that the extra Y chromosome leads to a slightly increased risk of social maladjustment. There is little information on adaptation in an unselected group of 47,XYY adults. However, it would seem that the above-noted cognitive and psychological characteristics combined with environmental stressors might increase their risk for problems with the law.

#### 44.8.5 Health

Severe facial acne has occasionally been reported. There is poor evidence for a purported increase in varicose veins, electroencephalographic, or electrocardiographic abnormalities. Several reports of hematologic malignancies in boys and men with 47,XYY appear to be coincidental and due to the routine inclusion of a karyotype in the diagnostic workup (272,273). The increased mortality in 47,XYY syndrome warrants future studies investigating the specific background.

### 44.9 STRUCTURAL ABNORMALITIES OF THE Y CHROMOSOME

The human Y chromosome shows considerable inter-individual variation in structure, particularly length. This trait is stable and inherited, father and son generally having identical Y chromosomes. When a structural abnormality is diagnosed, especially in utero, comparison with the paternal Y chromosomes is essential for interpretation, and the possibility of nonpaternity must always be considered. Structural abnormalities of the Y chromosome are not usually well characterized by routine karyotyping, so molecular and in situ hybridization studies are generally recommended.

Clinical consequences of structurally abnormal Y chromosomes depend on the loci deleted, any associated mosaicism, and the tissue distribution of various cell lines. Derivative Y chromosomes are unstable and often associated with mosaicism. Broad categories include

those with loss of SRY on the Y chromosome short arm leading to a female phenotype in the TS spectrum, loss of azoospermia factors (AZF) on the long arm leading to phenotypically normal males with infertility, and mosaics typically with 45,X whose phenotypes range from males to mixed gonadal dysgenesis to females with TS.

### 44.9.1 Yp Deletions

Individuals who have neither molecular nor cytogenetic evidence of the testis-determining factor (SRY) on Yp11, just proximal to the pseudoautosomal region, are not usually masculinized. Persons with 46,X,del(Yp), therefore, have a Turner phenotype that often includes lymphedema, streak gonads, infertility, and a significant risk for gonadoblastoma. They may differ from TS by the absence of short stature (274).

### 44.9.2 Yq Deletions

Deletions of the long arm are thought to be the result of intrachromosomal recombination involving highly repetitive, palindromic DNA sequences unique to the Y chromosome. These deletions do not involve the SRY locus and do not affect testicular determination; hence, they produce phenotypic males. Some such patients have normal- or near-normal-sized testes with histologic evidence of defective spermatogenesis, whereas a few have had cryptorchidism or small testes. Each of the currently recognized common microdeletions of AZF regions contains several genes thought to be relevant to spermatogenesis, and each gene is often present in multiple copies. The complexity of Y chromosome sequences makes investigations challenging (275). Although some Yq deletions are visible on karyotype, molecular techniques are commonly required.

The palindromes characterized thus far include the proviral sequences HERV15yq1 and HERV15yq2; recombination between these results in a proximal deletion of Yq11 (276). The region lost in this deletion is identified as AZFa. Several candidate genes for spermatogenesis are found in this region including DEAD-box protein 3 and ubiquitin-specific protease 9 (USP9Y) (277). Point mutations and deletion of USP9Y have been discovered in a few males with milder deficits in spermatogenesis (277). Typically, deletions of AZFa result in severe azoospermia with Sertoli-cell-only syndrome, in which there is a complete absence of germ cells on testicular biopsy (278).

Initially thought to be nonoverlapping, the other commonly deleted regions were termed AZFb and AZFc in order from proximal to more distal Yq. Although overlapping deletions have now been identified, the terminology remains widespread. Within AZFb, deletions occur between palindromes P5 and proximal-P1, while recombinations between palindromes P5 and distal-P1 and P4 with distal-P1 result in deletions that overlap AZFb and

AZFc (277). The most common deletion involves loss of the AZFc region of Yq11 between palindromes b2 and b4 (279). Spermatogenesis candidate genes in AZFb include RBMY1A1, an RNA-binding motif protein, and in AZFc, deleted in azoospermia. Located proximal to AZFa is a putative growth control locus (GCY) as deletions involving this region are associated with short stature (280). The incidence of Yq deletion among azoospermic males varies widely, with figures as high as 55.5% in a group with narrowly defined idiopathic Sertoli-cell-only syndrome (281). Foresta et al. (282) reviewed more than 4800 published patients and found that the overall prevalence of these microdeletions ranged from 2.9% in unselected oligospermic men to 11% if other causes of oligospermia were ruled out (“idiopathic”), whereas in azoospermic men, the prevalence was higher, ranging from 7.3% in unselected patients to 18% in the “idiopathic” group.

Interestingly, a small number of families have been reported, in which Yq microdeletions of AZFc have been transmitted in natural conceptions to male offspring. Most cases involve a father having a single infertile son. Two men have been described who passed on large AZFc deletions to infertile sons; three in one case (283) and four in another (284). These families demonstrate that Y-chromosome microdeletions are subjected to variable expressivity and do not necessarily imply a life-long history of infertility.

A couple in which the male has a Yq microdeletion can be offered assisted reproduction. If sperms are retrievable, either from ejaculate in the case of oligospermia or by testicular biopsy, IVF with ICSI into a harvested egg can be performed. Fertilization and pregnancy rates do not appear to be affected by the presence or absence of Yq deletions (285). Male offspring in these situations would necessarily inherit their father's Y chromosome anomaly and attendant infertility. One son reportedly inherited a larger deletion than his father's (286). Concern has also been raised about a potential association with 45,X/46,XY mosaicism and Yq microdeletions, suggesting that transmission of Y chromosomes with microdeletions could give rise to mosaic offspring (287). Approximately 5% of oligospermic males participating in ICSI programs have nonmosaic Y-chromosome microdeletions (288). It has therefore been recommended that all males with reduced or absent sperm counts seeking assisted reproductive technologies be screened for Y-chromosome deletions.

### 44.9.3 Dicentric Yp

Dicentric Yp is among the most common Y-chromosome rearrangements and is often associated with the mosaic karyotype 45,X/46,X,dic(Yp). The dicentric Y chromosome may be created during male gametogenesis, the observed mosaicism presumably reflecting postzygotic instability. It may also result from a very early postconceptional mitotic event. Not surprisingly, the phenotype is highly variable and includes females with varying degrees

of masculinization and TS characteristics, to males with or without genital anomalies. One review found 27% of individuals with idic(Yp) had a male phenotype (274).

The determinants of phenotype within this wide spectrum are not clearly understood. Though the presence or absence of SRY and other Y chromosome genes for spermatogenesis and stature, the presence of a 45,X cell line, and the proportion and tissue distribution of these various cell lines would all reasonably be expected to contribute to phenotype, only limited genotype–phenotype correlations can be made (289,290). Cytogenetic studies to estimate the proportion of cells containing the dicentric Y chromosome may be misleading because of inherent dicentric instability. It appears that the majority of dicentrics contain two copies of most of the functional euchromatin with break points close to or within the heterochromatic region, with SRY usually present. Occasionally, males with this karyotype are fertile, presumably because of the presence of one or more Y-linked genes controlling spermatogenesis. More frequently, however, phenotypic males have significant deletions of Yq resulting in short stature and azoospermia.

The presence of Y material is known to predispose to gonadoblastoma, so gonadectomy is recommended for these individuals.

#### 44.9.4 Dicentric Yq

Dicentric Yq is less common than dicentric Yp, but they share many characteristics. It is generally associated with 45,X mosaicism, and the phenotypic spectrum is comparable. The recombinant chromosome loses a variable amount of Yp, so that some of these individuals are males and others are females. Once again, the presence of SRY in a proportion of cells does not guarantee a male phenotype (290). Isochromosome Yq is rare and, when present, usually results in females with little or no masculinization. Gonadoblastoma has been reported (291).

#### 44.9.5 Ring Y

Rings and very small Y-derived marker chromosomes can best be characterized with the use of molecular and in situ hybridization techniques. Many of the rings represent complex Y-chromosome rearrangements, some have multiple centromeres, and all are generally unstable. The phenotype varies widely depending to some degree on the extent of Y-chromosome rearrangement and deletion, but generally a 45,X cell line is also present and there is an overlap with the dicentric spectrum described above (292).

#### 44.9.6 Pericentric Inversions of the Y

Pericentric inversions of the Y are heritable and have no phenotypic effects. One form leading to an almost metacentric Y chromosome is common in India and probably

represents an ancient mutation. The paternal Y chromosome should be checked when the condition is diagnosed prenatally.

### 44.10 PRENATAL DIAGNOSIS OF SEX CHROMOSOME ABNORMALITIES

As more couples are availing themselves of CVS or amniocentesis for both fetal and maternal indications, sex-chromosome aneuploidy is an increasingly common prenatal finding. Most geneticists and obstetricians would agree that these counseling sessions are more complex and challenging than those for autosomal trisomies, in which the phenotypes are generally less subtle from a physical, developmental and prognostic point of view. The goal of counseling is to present the couple with accurate information that empowers them to make an informed decision about the pregnancy (see also Chapter 21).

The counselor can be faced with at least two different scenarios. When there has been a sonographic identification of major fetal anomalies such as hydrops, the couple will have received some information before the prenatal diagnostic procedure. In this scenario, they generally arrive for counseling with a highly variable degree of understanding that there are physical problems with the fetus that may be the result of a chromosome abnormality. On the contrary, when prenatal diagnosis has been performed because of advanced maternal age, the presence of “soft” markers for autosomal trisomies, or a positive prenatal screen, the finding of sex-chromosome aneuploidy is often incidental and completely unanticipated.

Irrespective of the particular situation, the general principles of prenatal counseling apply. The couple is often aware of “an abnormality” on arrival at the counselor’s door. For many couples, the term *chromosome abnormality* is synonymous with Down syndrome because this is the most common type of aneuploidy in live-born children and the only one with which they may have personal experience. They are usually in a state of extreme distress that is exacerbated by fear of what they are about to be told. A compassionate acknowledgment of this at the outset is often helpful and should be followed by a brief attempt to elicit what details the couple already knows. The counselor should then proceed as quickly as possible to a discussion of the particular chromosome finding and its implications, allowing the couple to dictate the agenda with their questions. Management options, which generally include continuing or ending the pregnancy, should be approached in a nonjudgmental and nondirective manner. As in any counseling situation, empathic verbal and body language are extremely important. The counselor must be prepared to respond compassionately to expressions of grief and to repeat the information as often as necessary.

Of the more common sex-chromosome aneuploidies (45,X, 47,XXX, 47,XXY, and 47,XYY), only TS and related mosaic karyotypes are often associated with



major congenital anomalies and an increased risk of pregnancy loss. TS counseling will depend to a great extent on the mode of ascertainment. If the indication for prenatal diagnosis is fetal hydrops on a midtrimester ultrasound examination, there is a high probability of intrauterine death. Because the majority of 45,X losses occur in the first trimester, this is not true of a fetus with 45,X and normal sonographic findings that are incidentally ascertained by second-trimester amniocentesis for advanced maternal age. The content and emphasis of the counseling session will be dramatically different in these two situations.

In general, however, counseling for common sex-chromosome aneuploidies should include a thorough discussion of the highly variable spectrum of physical findings, which may be cosmetically significant in 45,X and to a lesser extent 47,XXY, but is essentially restricted to taller than average stature in 47,XXX and 47,XYY, but with increased mortality rates in all four syndromes. The involvement of sex chromosomes suggests to many couples a fundamental difference in the gender of their child. The counselor should volunteer the fact that the extra or missing chromosome usually precludes fertility in KS and TS but has no known impact on sexual orientation and the capacity for normal sexual function. The mention of hypogonadism in TS and KS must be accompanied by a review of available treatments, including cosmetic surgery for gynecomastia and testicular enlargement in 47,XXY. HRT is a mainstay of treatment in both conditions, and GH therapy is an option in the former. Under some circumstances, a referral to a pediatric endocrinologist during the pregnancy is appropriate and helpful with regard to decision-making. The couple should also be made aware of the availability of alternative parenting options (IVF with donor egg or sperm, ICSI, adoption) for the adult individual with TS or KS.

Perhaps most importantly, the counselor must thoroughly review the intellectual, psychological, and behavioral observations summarized above and make absolutely clear the difference between distinct learning disabilities (which are often present and amenable to educational support) and mental retardation (which is almost always absent and implies a permanent degree of disability). For 47,XXX and 47,XYY, emphasis should be placed on the fact that most of these children would have gone through life undetected, particularly in a stable environment. The importance of a supportive home environment cannot be overemphasized as children with sex chromosome aneuploidy from stable and nurturing families have no more psychological disorders than do their siblings (237). Abramsky and Chapple (161) recognized that the label of a “chromosome abnormality” may lead to a worse perception of the phenotype (and an increased likelihood of pregnancy termination) than would otherwise be the case. How many couples, they wondered, would consider termination of a euploid pregnancy if they were told the child’s IQ would be normal but 10–15

points lower than that of his or her siblings? They also draw attention to the fact that background rates of learning disability, developmental delay, and psychological difficulties in chromosomally normal children should be quoted to enable the couple to put the implications for children with sex-chromosome aneuploidy in context. There is no such thing as a “perfect” child.

At least 15 studies published between 1982 and 2011 examined pregnancy termination rates after the prenatal diagnosis of sex-chromosome abnormalities in individual centers in North America, Europe, Scandinavia, Israel, and Australia (35,293). Termination rates were highly discrepant among studies and across regions, some of which excluded pregnancies ascertained because of ultrasound anomalies and many of which were complicated by a small number of patients and/or the involvement of a number of different counselors. Termination rates were highest for 45,X (54–100%), reflecting the fact that more pregnancies with monosomy X were ascertained because of ultrasound findings. The rates varied markedly for the other sex-chromosome aneuploidies. Between 17% and 92% of pregnancies diagnosed with 47,XXY were ended, while the equivalent figures were 17–67% for pregnancies with 47,XXX and 10–75% for pregnancies with 47,XYY. The average rate of termination for all sex-chromosome abnormalities was 50%.

A number of factors may influence the decision about whether to continue a pregnancy in the face of these karyotype results. At least two studies suggest that older parents with a higher number of previous pregnancies are more likely to continue their pregnancies (35,294), whereas another found couples with a higher number of previous children were less likely to continue their pregnancies (295). Others suggest that black, Hispanic, and Arab parents are more likely to continue their pregnancies than Caucasian and Jewish parents (296), but many have not found any racial predilection for one choice over the other. Not surprisingly, pregnancies with normal ultrasound findings and mosaic sex-chromosome abnormalities are continued more often than those without (297). Higher termination rates have been found when counseling is performed by an obstetrician as opposed to a medical geneticist, genetic counselor, or pediatrician (294,298,299). There is speculation that a greater awareness of medicolegal risk and less familiarity with the natural history of sex-chromosome aneuploidies influences the counseling of obstetricians. Many studies have demonstrated a significant decrease in the percentage of patients choosing termination of pregnancy over time (293,300), hopefully reflecting an increasing understanding and more accurately optimistic portrayal of sex-chromosome abnormalities resulting from long-term prospective studies.

In one of the few large reviews, of 169 pregnancies in which 45,X, 47,XXX, 47,XXY, and 47,XYY were diagnosed between 1971 and 1997, 32% of couples chose to continue their pregnancies (300).

Of those who had normal ultrasound examinations, 40% continued their pregnancies; the equivalent figure in pregnancies with abnormal ultrasound examinations was 18%. Half of the remaining pregnancies were ended on the basis of ultrasound findings alone as many of them consisted of 45,X-associated fetal hydrops or cystic hygroma, both of which predict a poor prognosis. Among pregnancies with a normal ultrasound examination, those with a diagnosis of 47,XXX and 47,XYY were continued significantly more often than those with a diagnosis of 45,X and 47,XXY. Fifty percent of sonographically normal pregnancies with mosaic findings were continued, compared with 35% of pregnancies with nonmosaic karyotypes. The method of prenatal diagnosis as well as the marital status, education, and profession of parents did not exert a significant influence on decision-making. There was a significant difference in parental decision-making over the course of the study, likely related to the lack of adult outcome data before 1990. None of the couples continued their pregnancies between 1976 and 1979, in contrast with a 46% continuation rate between 1995 and 1997. Recent studies have reported continuation rates as high as 60–70%. Interestingly, follow-up of a small number of these children suggests that they are developmentally ahead of children with sex-chromosome aneuploidies that were diagnosed by newborn screening. The fact that these parents made a conscious decision to continue their pregnancies, possibly combined with the advantage of early anticipatory intervention, appears to have had a positive effect on outcome.

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## FURTHER READING

Turner—Know Your Body. An Information Book on Turner Syndrome. <http://www.medical-research.dk/turner-know-your-body/> Turner Syndrome Society USA. <http://www.turnersyndrome.org>

## CROSS REFERENCES

Chapters 21, 27, 30, 31, 33, 35, and 46.

## Biography



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Gravholt is currently working as a consultant and senior researcher at Aarhus University Hospital, Denmark, Department of Endocrinology and Internal Medicine, and the Department of Molecular Medicine. He has worked clinically and scientifically with sex-chromosome abnormalities for the last 18 years. He has performed clinical, genetic, epidemiological and experimental studies. He has published more than 100 original publications and review papers. He is an active participant in the international Turner and Klinefelter syndromes research community, as well as national and international research societies.

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# CHAPTER

# 45

## Deletions and Other Structural Abnormalities of the Autosomes

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### 45.1 INTRODUCTION

Over the past two decades, the knowledge generated by the completion of the Human Genome Project (1990–2001) has refined and revolutionized our understanding of the role of genomic deletion, duplication and rearrangement in human disease. Utilization of new laboratory techniques such as chromosome microarray analysis (CMA) and application of these new tools to genomic analysis of patients with a wide variety of disorders has been highly informative. It is now the standard of care to utilize CMA testing as a first-tier test for many diagnostic categories, resulting in improved diagnostic accuracy, more precise delineation of deletion and duplication breakpoints, and the discovery of new genomic syndromes (1). In addition, the fruits of the Human Genome Project have produced extensive genomic databases, and it is now possible to correlate the genomic coordinates of deletions or duplications with the genome sequence and identify the genes whose dosage is altered. Our knowledge about deletions and other structural abnormalities of the autosomes has therefore changed dramatically over the past 5 years. This is manifest in more precise delineation of “classic” microdeletion syndromes, description of new microdeletion syndromes, and increased knowledge of the underlying genomic architecture that predisposes to the recurrent rearrangements.

The human genome is composed of  $3 \times 10^9$  base pairs of DNA, which are estimated to code for about 22,000–23,000 structural genes (2). Approximately 10–15% of the DNA codes for genes (transcripts and their regulatory sequences), while the remainder consists of repeated

sequences and sequences of unknown function. When examined at metaphase at the 550-band stage (standard cytogenetic analysis), an “average” chromosomal band contains approximately  $5 \times 10^6$  DNA base pairs that could code for as many as 100–200 genes. Therefore, patients with cytogenetically visible chromosomal abnormalities involving gains or losses of a single band or part of a band have abnormal dosage for numerous genes. Given this, it is not surprising that almost all individuals with a cytogenetically detectable chromosomal imbalance demonstrate some phenotypic abnormality. However, CMA analysis can identify deletions and duplications that are more than an order of magnitude smaller than karyotyping, and recent work demonstrates that phenotypically normal individuals also have deletions and duplications of genomic material, including regions that contain genes (3). In fact, the total number of genes in a healthy individual can vary by more than 100 genes, indicating that loss of one allele of some genes is not deleterious. These deletions and duplications found in healthy individuals have been termed copy number variants (CNVs), and a large part of the work involved in interpreting the results of a CMA analysis is focused on determining which CNVs are pathogenic and which are benign. In general, pathogenic alterations are copy number changes that are not seen in the normal population and contain genes that cause disease when deleted or duplicated; however, this is currently an incomplete science, as only a subset of dosage-sensitive genes are known, and analysis of copy number variation in controls has not been done systematically.



The curated data on controls is available through public databases that collect information from a wide variety of studies using different array platforms, different bioinformatics algorithms and different analytical standards (e.g. Database of Genomic Variants; <http://projects.tcag.ca/variation/>). Nevertheless, many new syndromes caused by deletions and duplications have been identified, and work on both patients and controls is ongoing.

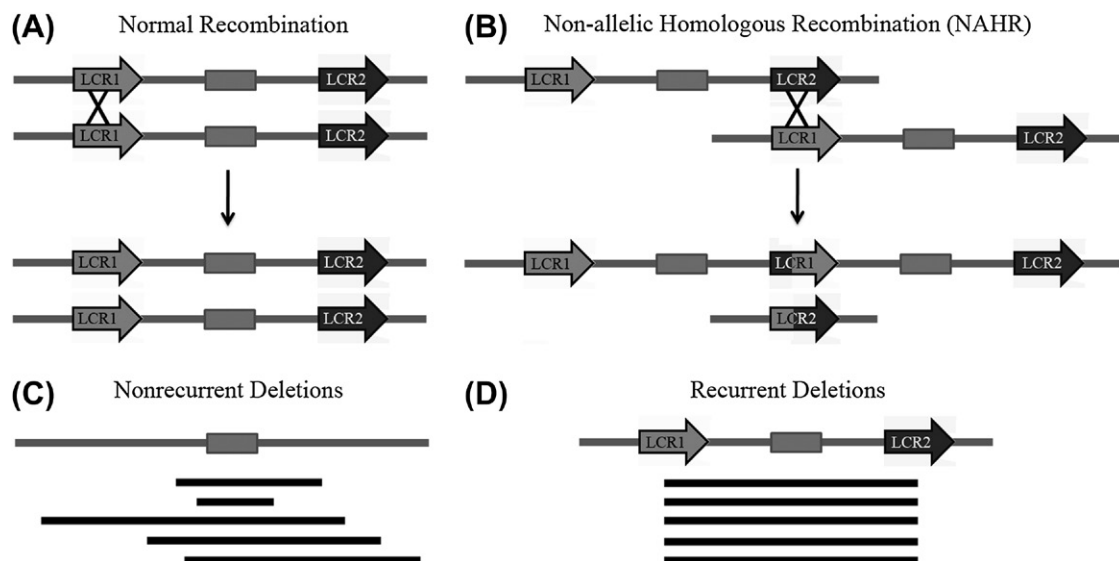
The diagnostic yield of CMA testing reported in the literature ranges from 5–20% of patients tested, depending on the clinical indications for testing and the characteristics of the microarray used (1,4–6). Deletions are the most common type of genomic alteration associated with human constitutional disease. Clinically relevant deletions account for approximately 56% of the abnormalities identified by CMA analysis, with clinically relevant duplications accounting for about 27%, and whole chromosome aneuploidy accounting for about 17% (7).

Genomic architecture has been shown to be a predisposing factor to DNA rearrangements that underlie disease, and it is estimated that 23% of the clinically relevant deletions and duplications identified arise because of flanking low copy repeats (LCRs) (Figure 45-1B and D) (8). Therefore, the majority of clinically relevant deletions and duplications are unique with variable breakpoints (Figure 45-1C). For diagnostic purposes, each gene within the identified deletions or duplications must be evaluated to determine if it is likely to be dosage sensitive, with an estimated 5–10% of genes causing a phenotype when deleted or duplicated (9). Determination of dosage sensitivity relies on review of published data for each gene, or chromosomal region. Nonrecurrent, but clinically recognizable deletion syndromes include

Wolf-Hirschhorn syndrome (WHS) (4p16), Cri du Chat syndrome (5p15), and the 18p and 18q deletions syndromes. These classic syndromes were all initially defined by clinical recognition of the phenotype, with subsequent identification of the genomic alteration based on cytogenetics, and narrowing of a critical region associated with specific phenotypes.

LCRs or segmental duplications (SDs) predispose to mispairing in meiosis, with subsequent deletion or duplication following recombination (10,11) (Figure 45-1A–D; Table 45-1). Classic microdeletion syndromes that are recurrent and LCR mediated include Williams syndrome (7q11.23 deletion), Prader-Willi/Angelman syndromes (15q11.2 deletions), Smith-Magenis syndrome (SMS) (17p11.2 deletions) and DiGeorge/Velocardio-facial syndromes (22q11.2 deletions). There are also several recurrent microdeletion syndromes that have been identified following the increased usage of CMA and these include deletions of 1q21.1, 8p23.1, 15q13.3, 16p11.2, 17q12, and 17q21.3. The 17q21.3 microdeletion syndrome was the first new disorder identified using CMA (12,13), and the features of this syndrome are consistent, such that the phenotype can now be clinically recognized. However, many of these recently identified microdeletion disorders have proven challenging, in that the deletions are often inherited from a mildly affected or phenotypically normal parent, suggesting that these deletions are characterized by marked variable expressivity. Included in this group with unclear phenotypes are the 1q21.1 and 15q13.3 recurrent microdeletions (Table 45-1).

There are several recurrent microduplications that have known phenotypic consequences including duplications of



**FIGURE 45-1** (A) Diagram demonstrates a genomic section with a gene (rectangle) flanked by low copy repeats (LCR1,2) undergoing normal recombination, which maintains the content of the gene with no deletions or duplications. (B) Demonstration of mispairing of the homologous repeats (LCR1,2) resulting in a deletion and a duplication. Following a recombination event within the mispaired repeats, one chromatid has a duplication of the gene, while the other chromatid is deleted for the gene. (C) Demonstration of random breakpoints associated with deletions that are not mediated by low copy repeats. (D) Demonstration of recurrent breakpoints resulting from nonallelic homologous recombination (NAHR) between repeats LCR1 and LCR2, which flank a gene.

TABLE 45-1 Recurrent Reciprocal Copy Number Changes (CNC) and Their Clinical Significance								
Chromosome Bands	Deletions		Duplications		Critical/ Candidate Gene	Approximate Size (Mb)	Approximate Breakpoint (Mb, hg19)	
	Alternate Name	Phenotype	Alternate Name	Phenotype			Start	End
1q21.1	TAR	Uncertain		Likely benign		0.2	145.3	145.9
1q21.1		Uncertain		Uncertain	<i>HYDIN2</i>	1.4	146.5	147.5
3q29		Uncertain		Uncertain		1.6	195.4	197.4
5q35.2q35.3		Pathogenic		Likely pathogenic	<i>NSD1</i>	2.1	175.4	177.2
7q11.23	Williams	Pathogenic		Pathogenic	<i>ELN, LIMK1, BAZ1B, GTF2I</i>	1.5	72.6	74.3
8p23.1		Pathogenic		Pathogenic	<i>GATA4</i>	5.0	6.6–7.8	11.8–
15q11.2	Angelman	Likely benign		Likely benign	<i>NIPA1</i>	0.5	22.7	23.3
15q11.2q13		Pathogenic		Pathogenic	<i>UBE3A</i>	5.1	23.4	28.7
15q11.2q13		Pathogenic		Pathogenic	<i>snoRNA, SNRPN</i>	5.1	23.4	28.7
15q13.3		Likely pathogenic		Uncertain	<i>CHRNA7</i>	1.5	30.9	32.5
16p13.11		Likely pathogenic		Likely benign	<i>NDE1, NTAN1</i>	1.0	15.4	16.5
16p12.2 <sup>a</sup>		Uncertain		Likely benign		0.6	21.9	22.5
16p11.2		Likely pathogenic		Likely pathogenic		0.6	29.5	30.4
17p12	HNPP	Pathogenic	CMT1a	Pathogenic	<i>PMP22</i>	1.4	14.1	15.5
17p11.2	Smith-Magenis	Pathogenic	Potocki-Lupski	Pathogenic	<i>RAI1</i>	3.7	16.6	20.4
17q11.2	Neurofibromatosis 1	Pathogenic		Uncertain	<i>NF1</i>	1.5	28.9	30.4
17q12	RCAD	Pathogenic		Uncertain	<i>HNF1B</i>	1.5	34.5	36.5
17q21.31		Pathogenic		Likely pathogenic	<i>MAPT</i>	0.7	43.6	44.4
22q11.2	22q11.2 Deletion syndrome	Pathogenic		Likely pathogenic	<i>TBX1</i>	2.6	18.9	21.5
Xp22.31	Ichthyosis	Pathogenic		Likely benign	<i>STS</i>	1.5	6.5	8.5
Yq11.223q11.23	Azospemia	Pathogenic		Likely benign	<i>DAZ</i>	3.6	24.5	28.4

Pathogenic = CNC known to result in clinically significant phenotype, rarely seen in unaffected individuals; Likely pathogenic = newly described CNC, rarely seen in unaffected individuals; Uncertain = associated with a range of phenotypes, also seen in unaffected individuals; Likely benign = few reports being associated with mild phenotype, but a low penetrance, seen at equal frequency in affected and control populations.

TAR, thrombocytopenia-absent radius; HNPP, hereditary neuropathy with liability to pressure palsies; CMT1a, Charcot-Marie-Tooth type 1a; RCAD, renal cysts and diabetes syndrome.

<sup>a</sup>Band changed between hg18 and hg19 build; 16p12.1 in hg18 build.

7q11.23 (reciprocal to the Williams syndrome deletions), 8p23.1, 16p11.2, 17p11.2 (Potocki-Lupski syndrome (PTLS)), 17p12 (Charcot-Marie-Tooth (CMT) associated duplication), 17q12 (*HNF1B* associated duplication), and 22q11.2 (reciprocal to the DiGeorge syndrome (DGS) associated deletion), all of which are associated with LCRs (14) (Table 45-1). Other microduplication syndromes have been reported, but the duplications are often inherited from a normal parent, and there is limited phenotypic data. Further studies are needed to determine if these microduplications are benign variants or pathogenic; these duplications include 1q21.1, 3q29, 15q11.2 (*NIPAI* associated duplication), 16p13.1, 22q11.2 and the Xp22.31 (steroid sulfatase (STS)-associated duplication).

Besides deletions and duplications, there are several other types of chromosome rearrangements that lead to human disease including translocations, inversions, ring chromosomes and isochromosomes (a specific type of deletion/duplication). In this chapter, we also include a discussion of uniparental disomy (UPD), as it occurs via abnormal chromosome segregation. In addition, the increasingly frequent use of single-nucleotide polymorphism (SNP) arrays in the diagnostic laboratory makes identification of UPD increasingly common in clinical practice.

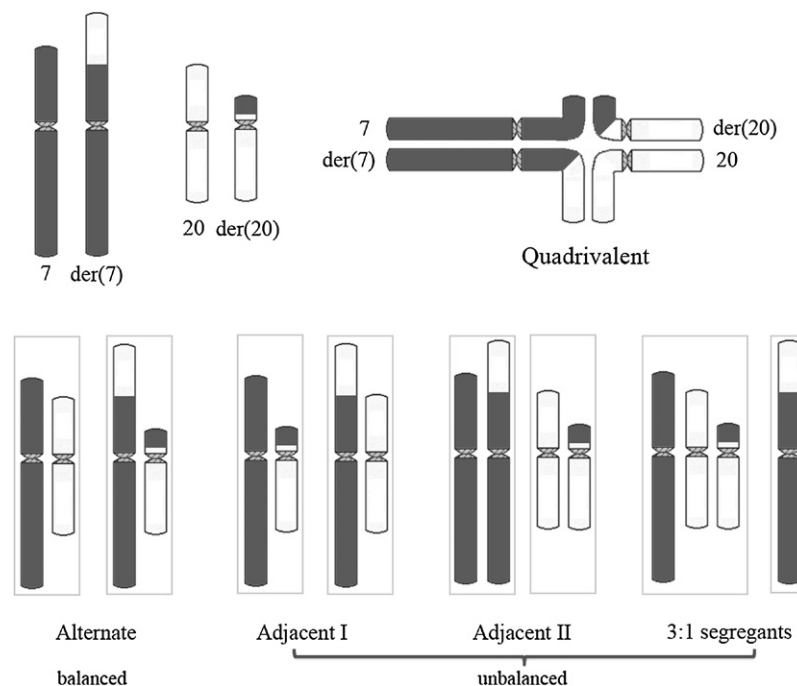
We review the most frequently observed deletions, duplications and translocations to provide access to information on the most common of the myriad of cytogenetic abnormalities. In addition, we present several rare disorders, which provide insights into the mechanisms of cytogenetic disease.

## 45.2 TRANSLOCATIONS

### 45.2.1 Cytogenetically Balanced Translocations

A translocation results from interchange of chromosomal segments between at least two chromosomes. Often, translocations occur between nonhomologous chromosomes (Figure 45-2). Translocations are considered balanced if the transfer of genetic information between the chromosomes is reciprocal, and no genetic material is lost or gained. Robertsonian translocations (discussed later) are associated with a loss of genetic material, but generally are not associated with phenotypic abnormalities. Translocations can be inherited (familial) or *de novo* (newly occurring).

Balanced translocations occur in approximately 1/500–1/600 individuals (15,16). The majority of



**FIGURE 45-2** Pachytene diagram of a hypothetical translocation between chromosomes 7p and 20p is shown. The normal 7 and 20 and derivative 7 and 20 all line up together, with several possible segregation outcomes. If alternate centromeres move into the same gamete, then the resulting fetus would either be normal with respect to chromosomes 7 and 20, or have a balanced rearrangement, similar to the parent. However, if adjacent centromeres move into the same gamete, several types of unbalanced gametes will form. The normal 7 paired with the der(20) will result in monosomy for part of 20p, and trisomy for part of 7p; or if the normal 20 segregates with the der(7), partial trisomy 20p and monosomy 7p results. These latter alternatives, with proper segregation of the different centromeres, but partial monosomy and trisomy for distal segments, are considered Adjacent I segregants. Adjacent 2 segregation occurs when the chromosome 7 and der(7) or 20 and der(20) segregate together, resulting in more profound partial trisomy or monosomy, including the centromeric area. Three to one segregation occurs when three centromeres move into one gamete, with only one chromosome in another. This is a common observation in the recurrent translocation 11;22.

balanced translocation carriers do not have associated clinical problems except decreased fertility and the risk of abnormalities to their offspring as a result of malsegregation of the translocation (Figure 45-2). However, the frequency of apparently balanced translocations, particularly *de novo* rearrangements, is higher in the mentally retarded population than in the newborn population (17–19). The frequency of clinical abnormalities among carriers of *de novo* reciprocal translocations is about 6% (20). In rare cases, balanced translocation carriers manifest features of a recognizable, dominant genetic disease due to interruption of a specific disease gene at the breakpoint of the translocation. Several autosomal dominant and X-linked diseases have been caused by a reciprocal translocation including Duchenne muscular dystrophy (21), neurofibromatosis 1 (22), supravulvar aortic stenosis (23), aniridia (24) and Alagille syndrome (25). CMA has shown that reciprocal translocations in phenotypically abnormal individuals are more likely to be associated with imbalances at the translocation breakpoints that cannot be seen by karyotype analysis (26). Another explanation for the association of congenital anomalies and apparently balanced reciprocal translocations is that the presence of a balanced rearrangement in a parent may be a predisposing factor for UPD in their offspring. This is discussed in some detail in a later section of this chapter.

### 45.2.2 Consequences of and Counseling for Reciprocal Translocations

Balanced translocation carriers are at risk for abnormal chromosomal segregation during meiosis. The consequences include infertility, multiple pregnancy losses, and live-born offspring with multiple congenital malformations as a result of chromosomal imbalance.

During meiosis, homologous chromosomes pair to form a synaptonemal complex. When there is a balanced translocation, all four chromosomes (the two chromosomes involved in the translocation and the two noninvolved) pair to produce a quadrivalent configuration (Figure 45-2). Normal gamete formation relies upon the segregation of alternating centromeres into two daughter cells, producing either a normal gamete or a gamete containing both partners of the balanced translocation. However, if adjacent centromeres segregate together, chromosomally unbalanced gametes are produced (Figure 45-2). For couples with a balanced translocation, abnormal segregations lead to recurrent miscarriages, stillborn offspring, or live-born offspring with multiple congenital abnormalities. Fertilization of a gamete containing adjacent segregants by a normal gamete results in a zygote with partial trisomy (duplication) and partial monosomy (deletion) for the relevant portions of the involved chromosomes. Pregnancy outcome is related to the size and composition of the rearranged chromosomal segments. In some

cases the imbalance is so severe that it results in miscarriage before a pregnancy is recognized. Such early pregnancy losses present clinically as infertility. Most balanced rearrangements are unique (i.e. they are only observed within a given pedigree), making it difficult to provide precise risk figures for each of the meiotic outcomes. If an individual with a balanced translocation was ascertained because of the birth of a chromosomally abnormal child, then it is known that at least one of the unbalanced karyotypes is compatible with life, and the risk for producing a subsequent affected child is high (5–30% by empirical data) with a significant risk for miscarriage or stillbirth (27–29). In cases where the balanced carrier was ascertained in an unbiased fashion (e.g. newborn screening studies) the risk for a chromosomally unbalanced child is lower (1–5%), with a significant risk for miscarriage or stillbirth (30). In this latter group there are many reciprocal translocations whose unbalanced segregants result in a phenotype that is severe and incompatible with life.

### 45.2.3 Recurrent Translocations

Most reciprocal translocations occur uniquely, at apparently random positions throughout the genome, with a few exceptions. To date, at least five recurrent translocations have been described. All these are mediated by repeat sequences within the genome, including both LCRs and palindromic, AT-rich regions that are prone to instability. These recurrent translocations include t(4;8)(p16;p23), t(4;11)(p16.2;p15.4), t(8;12)(p23.1;p13.31), t(8;22)(q24.13;q11.2), and t(11;22)(q23;q11.2).

### 45.2.4 t(11;22) Syndrome

The t(11;22)(q23;q11.2) is the most common, non-Robertsonian, constitutional translocation seen in humans (31–33). Several hundred families with this rearrangement have been described. The translocation appears to be independent of environmental factors and is estimated to have a low rate of mutation based on the paucity of *de novo* cases. In multiple unrelated families the breakpoints on both chromosomes 11 and 22 are tightly clustered (34,35). Further, similar breakpoints in numerous families occur within AT-rich repeat regions on chromosomes 11q23 and 22q11, suggesting genomic instability as the mechanism mediating the rearrangement (36,37). An unexpectedly high rate of *de novo* constitutional t(11;22) translocations in sperm from normal males has been reported, indicating that this translocation originates during meiosis (38).

Carriers of the constitutional t(11;22) are phenotypically normal, but come to attention subsequent to the birth of an abnormal offspring with the derivative chromosome 22 as a supernumerary chromosome, or upon being treated for infertility. Only one type of unbalanced karyotype is seen in live-born offspring in association



with this translocation, 47,XX(Y), +der(22)t(11;22)(q11.2;q23). This unbalanced karyotype is a result of missegregation during meiosis, referred to as 3:1 segregation, to produce a gamete with an extra chromosome (Figure 45-2). Presumably, unbalanced karyotypes with 46 chromosomes and the der(11) or the der(22) are non-viable. The minimal overall recurrence risk of balanced carriers for producing unbalanced offspring is estimated to be 2%. The risk could be as high as 5.6%, if one includes the nonkaryotyped stillborn children who were reported as malformed (32). The spontaneous abortion frequency is 27%. There is apparently no difference in the risk to male versus female carriers but there appears to be an excess of female balanced carriers among the phenotypically normal offspring of females with the t(11;22).

The syndrome associated with the presence of a supernumerary chromosome in the proband is designated the supernumerary der(22)t(11;22) syndrome, +der(22) syndrome, or Emanuel syndrome (OMIM# 609029). The phenotype of individuals with the +der(22) is discussed later in this chapter.

### 45.2.5 Other Recurrent Translocations

The t(4;8)(p16;p23) rearrangement, in the unbalanced form, has been reported multiple times in association with congenital anomalies. It has been shown to result from recombination between the olfactory receptor gene cluster LCRs (39,40). However, for the recurrent t(4;8), the precise breakpoint location has not been determined at nucleotide sequence resolution. Individuals with an unbalanced karyotype containing the der(4) show typical features of the Wolf-Hirschhorn deletion syndrome (41,42), while those with the der(8) show a milder dysmorphic syndrome (39).

The reported cases of the t(4;11)(p16.2;p15.4) have the same breakpoint intervals by CMA, suggesting this is a novel recurrent translocation. The formation of the t(4;11) appears to involve interchromosomal paralogous LCRs of 130 kb in length and 94.7% DNA sequence identity located in olfactory receptor gene clusters (43). The t(4;11) recurrent rearrangements occur within SDs with sequence homology at the breakpoints.

To date, two patients with a der(8)t(8;12)(p23.1;p13.31) have been identified (43). LCR clusters on both 8p23.1 and 12p13.31 that contain a 285 kb region of >94% homology, providing evidence that the translocations likely arose via interchromosomal nonallelic homologous recombination (NAHR). These patients had deletion of 8p23.1 to pter, with triplication of 12p13.31 to pter.

Another recurrent palindrome-mediated rearrangement is the t(8;22)(q24.13;q11.21) (44). Nearly identical palindromic AT-rich repeats on chromosomes 8 and 22 at the translocation breakpoints have been validated in several of the new and previously reported cases.

A syndrome characterized by extremity anomalies, mild dysmorphism and intellectual impairment is caused by 3:1 meiotic segregation of this rearrangement. The affected individuals have 47 chromosomes with a supernumerary der(22)t(8;22). Similar to the t(11;22), polymerase chain reaction (PCR) analysis of sperm DNA from healthy males indicates that the t(8;22) arises *de novo* during gametogenesis in some, but not all, individuals.

### 45.2.6 Robertsonian Translocations

Robertsonian translocations are a specific class of translocations in which two acrocentric chromosomes fuse at their centric ends (45). In humans, chromosomes 13, 14, 15, 21, and 22 are acrocentric, and all of these chromosomes are associated with Robertsonian translocations. The short arms of all of the acrocentric chromosomes contain numerous copies of the genes coding for ribosomal RNA. Because counting the number of centromeres in a metaphase spread assesses chromosome number, individuals with a Robertsonian translocation have 45 chromosomes. The small reciprocal product, which contains the remnants of the short arms of the two fused chromosomes, is usually lost. Loss of the short arms of two acrocentric chromosomes is not deleterious and individuals with this type of rearrangement are generally phenotypically normal. Robertsonian translocation carriers may be at increased risk for having children with abnormalities either because of malsegregation of the translocation chromosome or as a consequence of their increased risk for having offspring with UPD.

### 45.2.7 Counseling for Robertsonian Translocations

As with reciprocal translocation carriers, there is a risk to carriers of balanced Robertsonian translocations for producing unbalanced offspring. In general, Robertsonian translocation carriers are at increased risk for spontaneous abortions (29) and an association has been observed between Robertsonian translocations and male infertility (46). Clinically, the most significant is for carriers of a Robertsonian translocation that involves chromosome 21. These individuals are at risk for producing a child with Down syndrome. Female carriers have approximately a 15% risk for a live-born child with Down syndrome and male carriers have a much lower risk (about 1–2%) (30). In patients with Down syndrome, 4% have a Robertsonian translocation involving chromosome 21. The translocation is familial (inherited) in 25% of these patients and *de novo* in 75% (47). Theoretically, carriers of Robertsonian translocations involving chromosome 13 could produce offspring with trisomy 13 (Patau syndrome), but this is rarely observed. Robertsonian translocations between homologous chromosomes (e.g. 21;21 or 13;13) pose unique problems, since carriers are unable to produce normal gametes.

### 45.3 UNIPARENTAL DISOMY

UPD is the inheritance of a pair of chromosomes (or a portion of a pair) from only one parent (48). UPD is most easily identified by the study of polymorphic loci, which reveals a pattern consistent with the presence of two alleles from the one parent, and the absence of inherited alleles from the other parent. UPD is sometimes associated with clinical abnormalities, and this can occur by two mechanisms: having two copies of a recessive disease gene or absence of an imprinted gene.

Uniparental *isodisomy* (the inheritance of two copies of one homolog from a parent) can be associated with disease when there is a mutant allele for a recessive disorder present on the affected chromosome. In these cases, the patient would present with a homozygous mutation even though only one parent is a disease carrier. The identification of UPD is usually subsequent to the unexpected identification of a homozygous mutation in the patient. This has been observed for several recessively inherited diseases, including cystic fibrosis (49,50) and Bloom syndrome (51).

UPD is also associated with disease through the uniparental inheritance of a region of the genome containing an imprinted gene (see Chapter 10). Imprinted genes are differentially expressed depending on whether they are inherited from the male or female parent (52). In the case of imprinting-associated disease, the UPD can be *isodisomy* or *heterodisomy* (the inheritance of both parental homologs from one parent). The best-studied examples of this phenomenon in humans are Prader-Willi and Angelman syndromes, which are discussed later in this chapter. Both these disorders can be associated with loss of expression of imprinted genes located on chromosome 15q11q13. The loss of gene expression can be caused by deletion of one parental chromosome, or UPD. The absence of a paternal chromosome, due to either maternal UPD (two copies of a maternally inherited chromosome 15 and no copies of the paternally inherited chromosome 15) or a deletion of 15q11q13 on the paternal chromosome leads to the Prader-Willi syndrome (PWS). The absence of a maternal chromosome, due to either paternal UPD or a deletion of 15q11q13 on the maternal chromosome, leads to Angelman syndrome (AS), a clinically distinct syndrome (53,54). Another imprinting disorder is the Russell-Silver syndrome, which is characterized by pre- and postnatal growth retardation, triangular facies, and facial, limb or truncal asymmetry. Imprinted genes on chromosome 7 and chromosome 11 have been associated with this disorder. Maternal UPD for chromosome 7 is seen in about 10% of cases (55). Three imprinted genes within chromosome 7q31 have been identified, and patients with segmental maternal isodisomy of this region have been reported (56). Duplication of maternal genes within 7p11.2-p13 is also associated with this disorder. Therefore, Russell-Silver syndrome appears to be caused by extra copies of maternal genes rather than by

loss of a paternally imprinted gene (57). Russell-Silver syndrome is also associated with imprinted genes located within chromosome 11p15.5. As with the imprinted locus at 15q11q13, the parent of origin determines the clinical presentation, with maternal UPD of 11p15 associated with Russell-Silver syndrome, and paternal UPD associated with Beckwith-Wiedemann syndrome, an overgrowth disorder.

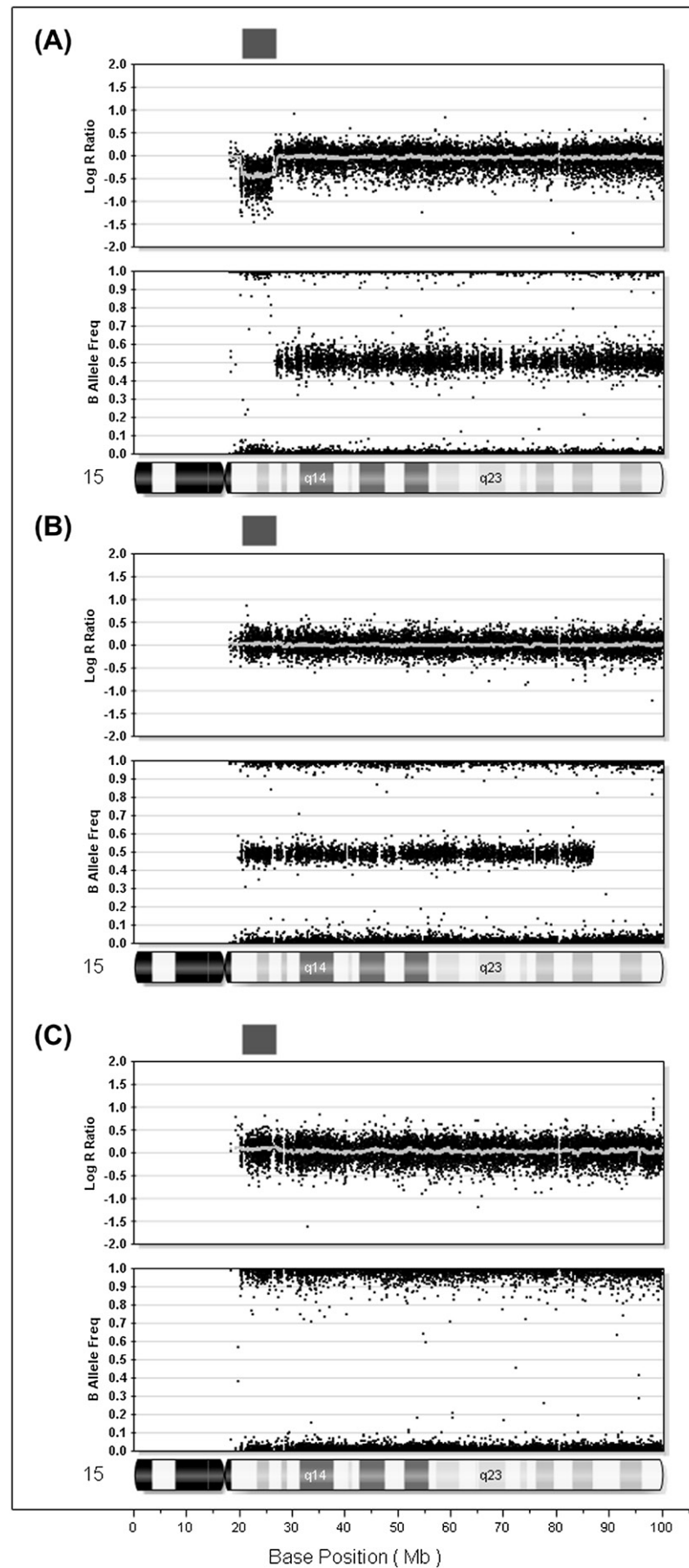
UPD often involves an entire chromosome, and may have originated through a trisomy rescue or monosomy rescue (55). Utilization of SNP arrays has increased our ability to detect UPD, and in some cases, examination of the genotyping patterns on the SNP arrays allows detection of the mechanism by which the UPD has occurred (Figure 45-3B and C) (58).

An association has been observed between UPD and inherited or *de novo* Robertsonian translocations. Malsegregation of familial Robertsonian translocations can result in UPD for one of the chromosomes involved in the translocation in multiple cases (59–62). UPD has also been observed in association with *de novo* Robertsonian translocations (63–67). Among the acrocentric chromosomes, UPD for chromosomes 13, 21 or 22 does not appear to be associated with phenotypic abnormalities, while UPD for chromosome 15 is associated with Prader-Willi or Angelman syndromes and UPD for chromosome 14 has been associated with multiple abnormalities (68).

### 45.4 DELETION

Loss or deletion of a chromosomal segment potentially results in an individual having only one copy of a portion of the genome, leading to hemizyosity for one or more loci. A deletion may be terminal (at the end of the chromosome) or it may be interstitial (within the chromosome). The clinical consequences of deletions depend on the size of the deleted segment and the number and function of the genes involved in the deletion. Deletions can either occur *de novo* or be inherited from a parent. Deletions may also result from malsegregation of a parental balanced translocation, or other chromosomal rearrangements. Translocation-mediated deletions are generally accompanied by partial trisomy of another chromosomal region, resulting in partial duplication accompanying the deletion. There have been isolated reports of deletions of many regions of the genome. As described earlier, some deletions occur sporadically, with only rare cases of similar breakpoints, while other deletions are recurrent. As discussed in the introduction, some of these deletions were originally identified based on clinical phenotypes and karyotypic analysis, while others were identified via CMA, with subsequent comparison of the phenotypes of patients with the deletion or duplication.

Genomic deletion disorders are generally characterized clinically by abnormalities in multiple systems. In



some cases, disorders that were originally associated with deletions were subsequently found to be caused by a single gene mapping within the deletion (e.g. Alagille (69) and Rubenstein-Taybi syndromes (70)). Even for the disorders caused by loss of multiple genes, it is unlikely that all the genes that map within the deleted segment play an important role in the causation of the major features of the phenotype. The fine mapping that is possible using chromosomal microarrays has brought us a long way toward defining the genes that cause an abnormal phenotype when deleted (haploinsufficient genes), while genes that are commonly found deleted in control individuals do not appear to be associated with clinical abnormalities (haplosufficient genes). Deletions that have been associated with clinically recognizable syndromes are discussed below. A list of recurrent chromosomal deletions is given in Table 45-1.

### 45.4.1 Chromosome 1p36 Deletion Syndrome

Early case reports described patients with deletions of 1p36 (94,95); however, it was not described as a clinically recognizable syndrome until sufficient numbers of patients with deletions were identified (MIM# 607872) (96). The 1p36 deletion is thought to be the most common terminal deletion, with a frequency of 1/5000–1/10,000 live births (97). There is a wide range of deletion sizes and no common breakpoint.

**45.4.1.1 Clinical Presentation.** Affected individuals have a characteristic facies consisting of midface hypoplasia, frontal bossing, deep set eyes, long philtrum, and pointed chin. Late closure of anterior fontanel, structural brain malformations including ventriculomegaly, cardiac defects, seizures, hypotonia, hearing loss, and vision problems are noted in most individuals with this deletion. Skeletal and genitourinary abnormalities are reported frequently as well. Developmental delay, poor to absent speech and intellectual disability is present in every individual with variable severity (97–99). Recently, a few cases with PWS-like features including hypotonia, obesity, and cognitive delays

have been associated with the 1p36 deletion (100,101). More than 10 cases of 1p36 deletion syndrome have been diagnosed prenatally. The most common prenatal findings are ventriculomegaly, midface hypoplasia and heart defects (102,103).

#### 45.4.1.2 Cytogenetics and Molecular Cytogenetics.

The 1p36 deletion syndrome is associated with pure terminal deletions, interstitial deletions and complex rearrangements. More than half the cases are *de novo* terminal deletions, one-quarter are interstitial deletions, and about 3% of cases are due to malsegregation of a balanced parental translocation. The size of the deletion is variable, ranging from 0.5 Mb to greater than 10 Mb, with no clear correlation between the extent of the deletion and severity of the features (99).

### 45.4.2 1q21.1 Microdeletion

The use of CMA analysis in individuals with intellectual disability, multiple congenital anomalies and developmental delay has resulted in the diagnosis of several new genomic disorders including the 1q21.1 deletion syndrome (MIM# 612474) (104). The 1q21.1 deletion is a recurrent microdeletion, which is not visible by standard chromosome karyotype analysis (104) (Table 45-1). Since its discovery, the 1q21.1 deletion has been associated with a variety of features (see later); however, the deletion is also seen in clinically unaffected individuals. This recurrent microdeletion therefore presents variable expressivity and incomplete penetrance.

**45.4.2.1 Clinical Presentation.** The 1q21.1 deletion syndrome (OMIM# 612474) does not present as a clinically recognizable syndrome; however, approximately half the individuals with this deletion have microcephaly, mild dysmorphic features and intellectual disability. This deletion has been associated with a variety of phenotypes including microcephaly, schizophrenia, epilepsy, cardiac disease, genitourinary abnormalities, cataract, and hypotonia (72,73,105). The 1q21.1 deletion has been found to be *de novo*, or inherited from a clinically unaffected or mildly affected parent. The variable expressivity, wide range of clinical findings, and inheritance from

**FIGURE 45-3** Data from the utilization of a genome-wide SNP array demonstrating diagnosis of genomic abnormalities. In each panel, the data are presented using two transformations, the  $\log_2 R$  ratio (top) and B allele frequency (bottom). For each panel, each SNP is represented by a dot, and the data are displayed along the length of the chromosome, with the ideogram underneath the B allele frequency plot. The  $\log_2 R$  ratio indicates the intensity of the signal at each SNP, compared to a set of “control” individuals, who presumably have two copies at that locus. Therefore if the subject has two copies, the SNPs should plot out at 0 [ $\log_2(2/2) = \log_2(1) = 0$ ]. Deletions are seen as negative values, while duplications are seen as positive values. The genotype of each SNP is also determined, and transformed into a B allele frequency. Each SNP is bi-allelic, with each allele being either an “A” or “B”. The B allele frequencies are then determined by the number of B alleles present. A genotype of AA has a B allele frequency of 0%, AB has a B allele frequency of 50%, and BB has a B allele frequency of 100%. Panel A shows a deletion of chromosome 15q11q13. Note the decreased  $\log_2 R$  ratio where the intensity drops, as well as the lack of heterozygotes shown in the B allele frequency track. This is a deletion associated with Prader-Willi or Angelman syndrome. Panel B demonstrates a normal  $\log_2 R$  ratio across all of chromosome 15, with a region of homozygosity toward the q terminus. This patient has uniparental disomy for chromosome 15, with heterodisomy near the centromere (presence of AB genotypes), and isodisomy near the telomere (absence of AB genotypes). This transition of hetero/isodisomy marks a meiotic crossover (372). Panel C demonstrates a patient with uniparental isodisomy for chromosome 15. Note the normal  $\log_2 R$  ratio, but complete lack of heterozygosity across the entire chromosome. The critical region for Prader-Willi or Angelman syndrome is noted by a dark gray rectangle above the array plots.



apparently unaffected parents poses a significant counseling dilemma for this syndrome.

#### 45.4.2.2 Cytogenetics and Molecular Cytogenetics.

The 1q21.1 microdeletion is a recurrent deletion associated with LCRs. The minimum deletion size is 1.35 Mb, which includes at least nine genes, including PRKAB2, FMO5, CHD1L, BCL9, ACP6, GJA5, GJA8, GPR8 9B and a paralog of the HYDIN (HYDIN2) gene. Mutations in HYDIN are known to be involved in hydrocephalus, and HYDIN2 has been suggested as a candidate for the microcephaly associated with 1q21 deletions (72). Note that there is a second, distinct, microdeletion syndrome within 1q21.1 associated with the thrombocytopenia, absent radius (TAR) syndrome, which is distinct from and is located proximal to the 1q21.1 syndrome described here (71).

### 45.4.3 Wolf-Hirschhorn Syndrome (WHS; 4p-)

Partial monosomy of the short arm of chromosome 4 is associated with a clinically distinct syndrome characterized by recognizable facial dysmorphism in combination with marked growth retardation of prenatal onset and severe mental deficiency (106–108). The true incidence of this syndrome is not known, with estimated rates of 1/50,000–1/95,896 (109–111). WHS was originally defined by the clinical phenotype, and subsequently found to be caused by deletions of chromosome 4p. In the majority of cases, the breakpoints of the deletions are nonrecurrent; however, recurrent unbalanced translocations involving chromosome 4p16 have been identified (39,43).

**45.4.3.1 Clinical Presentation.** The distinctive face has been called the “Greek helmet” facies, characterized by microcephaly and frontal bossing with a high frontal hairline; prominent glabella, ocular hypertelorism; proptosis due to hypoplasia of the orbital ridges; slanted palpebral fissures and epicanthal folds; a broad and beaked nose with a large, prominent bridge and shallow septum. Strabismus is a common finding. The ears are low-set, large, and simple, and are commonly seen with preauricular pits or tags. The mouth is downturned, with a short upper lip and philtrum, and micrognathia is present. Closure defects are common including cleft lip and/or palate in about 15% of patients, coloboma of the iris, cardiac septal defects (present in more than 50% of patients and a leading cause of mortality); and genital anomalies such as hypospadias, scrotal hypoplasia, cryptorchidism or testicular hypoplasia. Low birth weight, growth retardation and failure to thrive are commonly reported.

Intellectual disability is usually severe, with IQs below 20. Seizures are frequent and usually begin within the first two years of life. Heart disease and a high susceptibility to infection are frequently the cause of death.

Postnatal mortality has been reported as high, with one-third of patients dying during the first year of life; however, more recent analyses have noted that risk of death is significantly skewed toward patients with larger deletions (111).

#### 45.4.3.2 Cytogenetics and Molecular Cytogenetics.

WHS is caused by partial deletion of the short arm of chromosome 4 (4p-). The critical region for this disorder (Wolf-Hirschhorn critical region, WHCR) is located approximately 2 Mb from the telomere, within band 4p16.3 (112). Approximately 45–50% of deletions are associated with an unbalanced translocation, and the remaining patients have *de novo* pure terminal or interstitial deletions (113). The size of the deleted region can vary greatly among patients, although such variations do not apparently result in important differences in phenotype, suggesting that there are relatively few genes responsible for the major characteristics of this syndrome (108); however, the high frequency of unbalanced translocations associated with this disorder complicates the ability to determine the genotype/phenotype relationship. Two recurrent translocations have been associated with WHS (der(4)t(4;8)(4p16.3;8p23.1) and der(4)(4;11)(p16.2;p15.4)).

A number of patients have been described with very subtle translocations and deletions. In many of these cases, cytogenetic studies were normal, but a small deletion within 4p16 was detectable by molecular analysis or CMA (114). The presence of phenotypic features of WHS and a normal karyotype warrant further molecular investigation of the patient, as many of the translocations associated with Wolf-Hirschhorn may be cryptic. If a submicroscopic deletion is detected by microarray testing, analysis of parental chromosomes by molecular cytogenetic techniques (fluorescence in situ hybridization (FISH)) is of primary importance, since one of the parents could carry a cryptic translocation. If this is the case, prenatal diagnosis for future pregnancies could be offered.

Analysis of the deletions seen in patients with WHS has led to the identification of a 165 kb critical region within 4p16.3 (115). At least nine independent putative genes have been identified within this region. A novel developmental gene, *WHSC1*, maps in the distal part of the WHS critical region (116,117). A second critical region (WHSCR2), located 300–600 kb distal to *WHSC1*, has been described. Deletions of WHSCR2 have been associated with milder clinical features. WHSCR2 contains the *LETM1* gene, which is suggested to be associated with the seizure phenotype (41).

Submicroscopic deletions of 4p16.3 have also been found in patients with the Pitt-Rogers-Danks syndrome. The critical regions for the deletions in WHS and the Pitt-Rogers-Danks syndromes have been shown to be overlapping and may represent a clinical spectrum of the same disorder (115,117).

#### 45.4.4 Cri du Chat Syndrome (5p-)

Partial deletion of the short arm of chromosome 5 results in the Cri du Chat syndrome, which was first described in 1963 (118). The syndrome is named because it is associated with a laryngeal defect, which causes a distinctive cry resembling the mewing of a cat. The incidence has been reported to range from 1/15,000 to 1/50,000 newborns (110,119). This syndrome was originally defined by the clinical phenotype, and subsequently shown to be caused by deletions of chromosome 5p. The breakpoints of the deletions are nonrecurrent and not associated with LCRs.

**45.4.4.1 Clinical Presentation.** Patients present in the newborn period with low birth weight, the characteristic high-pitched, catlike cry and craniofacial dysmorphism, consisting of microcephaly, a round (moonlike) face, hypertelorism, epicanthal folds and broad nasal bridge (119,120). Most patients demonstrate hypotonia, and strabismus is common. About 1/3 of patients demonstrate variable types of heart disease.

The catlike cry disappears over the first year and development is severely delayed. Head control is attained at about 1 year, sitting occurs at 2 years and walking begins at 4 years. Speech development is particularly slow. Mortality is about 10% in the first year of life, but it improves for patients beyond this age, with reports of individuals living into their fifties (121). There is a report of an intellectually disabled woman with the Cri du Chat syndrome and an interstitial deletion of 5p who delivered a similarly affected daughter (122). Intellectual disability is usually severe with IQs below 50 in infants and below 20 in adults (123). Early intensive programs of special education have been beneficial, resulting in social and psychomotor development at the level of a 5- to 6-year-old child (121,124–127).

**45.4.4.2 Cytogenetics and Molecular Cytogenetics.** The Cri du Chat syndrome results from *de novo* deletions of 5p in 90% of patients, *de novo* unbalanced translocations in 5% of patients, and missegregation of familial translocations in 5% of patients (121). The extent of the deletion in patients with features of the Cri du Chat syndrome varies widely, but critical chromosomal regions for specific features of the syndrome have been identified. CMA has been used to map DNA copy number differences in 94 patients with Cri du Chat (128), allowing for genotype–phenotype correlations and localization of several clinical features to specific genomic regions. The characteristic cry localizes to a 1.5 Mb region in distal 5p15.31, while speech delay localizes to an approximately 3 Mb region of 5p15.33–5p15.32. Dysmorphic facial features localized to a 2.4 Mb region of 5p15.31–p15.2. The severity of developmental delay was correlated to the size of the deletion and its location in all cases with an isolated 5p deletion. A few genes associated with clinical phenotypes have been identified within the critical region

for Cri du Chat. One such gene is the telomerase reverse transcriptase or *TERT* gene on 5p15.33. Inherited haploinsufficiency for *TERT* results in progressive telomere shortening and autosomal dominant dyskeratosis congenita (DC), characterized by progressive bone marrow failure, nail dystrophy, abnormal skin pigmentation and mucosal leukoplakia. DC is not generally diagnosed in the Cri du Chat syndrome, and there is evidence that the DC phenotype requires several generations of telomere shortening, as could be seen with an inherited, dominant mutation (129). The key genes associated with the phenotypes seen in Cri du Chat syndrome have not yet been identified; however, analysis of transcripts within critical regions is leading to identification of candidate genes for specific features, such as a possible role for Delta-catenin in intellectual disability (130).

#### 45.4.5 Williams-Beuren Syndrome

Williams-Beuren syndrome (WBS) is also referred to as Williams syndrome (MIM# 194050). The clinical features of this syndrome were described in the 1960s (131,132) and in 1993 the genetic basis of this disorder was identified to be a microdeletion on chromosome 7q11.23 involving the elastin gene (133). The estimated frequency of this disorder is 1/7500–1/20,000 live births (23,134,135). This is a recurrent microdeletion, with a common deletion seen in most patients.

**45.4.5.1 Clinical Presentation.** WBS is characterized by cardiovascular defects, recognizable facial features, idiopathic infantile hypercalcemia, connective tissue anomalies, intellectual disability and an outgoing, highly social personality (reviewed in (136)). Patients may present at birth with cardiovascular disease (supravalvular aortic stenosis in 70% of patients and peripheral pulmonary stenosis in 27%), and the syndromic nature of the disorder may go unrecognized in the newborn period (135). Failure to thrive is a common feature during infancy. The characteristic facial features include a broad forehead, medial eyebrow flare, periorbital fullness, strabismus, stellate iris pattern, flat nasal bridge, malar flattening, full cheeks and lips, a long smooth philtrum, pointed chin and wide mouth. Endocrine features of hypercalcemia, hypercalciuria, hypothyroidism and early puberty are frequent. Connective tissue abnormalities are common and include umbilical hernia, rectal prolapse, lax joints or contractures. Individuals with WBS have some degree of intellectual disability, with severe limitations in visuospatial construction skills (deficit in the ability to visualize objects as a set of parts); however, patients usually show exceptional language abilities. Hearing and vision abnormalities are common and early intervention for these is crucial to optimize learning. Many adults tend to experience notable anxiety and neuropsychiatric conditions (reviewed in (136)), and hypertension is common among older individuals (137). The American

Academy of Pediatrics has published management guidelines for individuals with WBS (138).

#### 45.4.5.2 Cytogenetics and Molecular Cytogenetics.

WBS is a true contiguous gene deletion syndrome (139). The majority of the deletions associated with WBS are recurrent deletions mediated by LCRs. An inversion polymorphism on chromosome 7 is seen in only 6% of the general population, while up to 30% of the parents of individuals with WBS are found to carry an inversion polymorphism on chromosome 7, which suggests the inversion predisposes to the deletion (140,141). The commonly deleted region in patients with Williams syndrome is about 1.5 Mb in size and contains 25 genes including ELN (associated with cardiovascular and connective tissue phenotypes), LIMK1 (associated with impaired visual motor integration), and BAZ1B (associated with hypercalcemia) (142–144). A slightly larger, overlapping deletion of 1.8 Mb is seen in 5% of patients (141). A few individuals have atypical, nonrecurrent, deletions. In these individuals, the phenotype depends on the size and genomic content of the deletions. For example, individuals with deletions that do not include the GTF2I gene do not have intellectual disability (76,144).

### 45.4.6 8p23.1 Microdeletion

Terminal deletions, recurrent interstitial deletions, and complex rearrangements involving chromosome band 8p23.1 have been reported with congenital heart defect, diaphragmatic hernia, microcephaly, and intellectual disability (reviewed in (77)). The genomic architecture of chromosome band 8p23.1 includes clusters of olfactory receptor genes, which predispose this region to various genomic alterations including deletions, duplications, and translocations (145). Only the recurrent deletions of 8p23.1 are discussed here.

**45.4.6.1 Clinical Presentation.** Structural cardiovascular defects with or without conduction defects are observed in more than 60% of the individuals with the recurrent interstitial 8p23.1 deletion. These include atrial and ventricular septal defects, double outlet right ventricle, dextrocardia, pulmonary stenosis, and hypoplastic left heart (146,147). Congenital diaphragmatic hernia is seen in ~20% individuals with this deletion (148). Intellectual disability, seizures and aggressive behavior are observed frequently (149). Prenatal diagnosis of the 8p23.1 deletion has been made in at least four cases referred for complex heart defects (150).

#### 45.4.6.2 Cytogenetics and Molecular Cytogenetics.

The size of the 8p23.1 deletion ranges from 2.95 to 6 Mb and this deletion is mediated by LCRs. All the deletions identified to date have been reported to be *de novo*. Haploinsufficiency of GATA4 is thought to result in the heart defect; however, the severity of the condition is greater in patients with this contiguous

deletion as compared to patients with point mutations in GATA4 (151,152). Dosage effects of other genes in the region (e.g. SOX7) are thought to play a role in the severity of the cardiac phenotypes. Of note, there are multiple benign CNVs with the cytogenetic band 8p23.1, which are distinct from this GATA4-containing deletion (see Table 45-1 for breakpoints); therefore, deletions within chromosome band 8p23.1 should be interpreted with caution.

### 45.4.7 Langer-Giedion Syndrome (Trichorhinophalangeal Syndrome Type II)

The Langer-Giedion syndrome, or trichorhinophalangeal syndrome (TRPS) type II was first described clinically and subsequently found to be associated with interstitial deletion of the long arm of chromosome 8 (153). The deletion breakpoints in the Langer-Giedion syndrome are of variable sizes and are nonrecurrent.

**45.4.7.1 Clinical Presentation.** The Langer-Giedion syndrome is one of three forms of TRPS. TRPS II is characterized by multiple cartilaginous exostoses in combination with the clinical features of TRPS type I. TRPS II is characterized by sparse scalp hair, large and laterally protruding ears, bushy eyebrows that are sometimes thinning laterally, broad nasal bridge and bulbous nose, an elongated upper lip, and thin upper vermilion border. Malocclusion, mandibular retrognathia and dental abnormalities are common. There is frequently marked laxity of the skin in infancy and early childhood, which diminishes with age. Brittle nails and multiple nevi are seen frequently. Patients have winged scapulae and short stature of postnatal onset. Multiple cartilaginous exostoses are a defining feature of the disorder. The exostoses may appear in the first year, but may not develop until the fifth year, and they usually increase in size and number until skeletal maturation. There is considerable variability of intelligence and while mild to moderate intellectual disability has been seen in many patients, it does not appear to be a constant feature of the disorder (154). The third form of TRPS (TRPS III) is the most severe and shows the same dysmorphic features as TRPS I, with more severe shortening of all phalanges and metacarpals, short stature (<2 SD) and other skeletal alterations that have been described (155).

#### 45.4.7.2 Cytogenetics and Molecular Cytogenetics.

In 1980, Buhler et al. reported a girl with a terminal deletion of chromosome 8q (del(8)(q24)) with features of Langer-Giedion syndrome. Since that time, deletions or other rearrangements involving 8q24 have been identified in a high percentage of patients (153,156). The deletions are of variable sizes, and most patients with intellectual disability have larger deletions, suggesting that there is a correlation between the size of the deletions and the variable clinical features (157). All deletions associated with TRPS II include both the

TRPS1 gene and the exostosin 1 (EXT1) gene. TRPS1 is a zinc finger transcription factor gene that represses transcription (158,159) and EXT1 catalyzes an essential factor in a signal transduction cascade for regulation of chondrocyte differentiation, ossification, and apoptosis (160).

#### 45.4.8 9q Subtelomeric Deletion (9q-, Kleeftstra Syndrome)

The 9q- syndrome was first described in 2004 following clinical utilization of a FISH-based subtelomere assay as a screen for chromosomal differences in patients with intellectual disability (161–164). A translocation involving interruption of the *EHMT1* gene was instructive in identifying a critical gene responsible for the 9q subtelomere phenotype (165,166). The breakpoints of this terminal deletion syndrome are variable (166).

**45.4.8.1 Clinical Presentation.** Kleeftstra syndrome (MIM# 610253) is characterized by intellectual disability, childhood hypotonia, and a characteristic facial appearance, with some features such as congenital heart disease, renal defects, microcephaly, epilepsy, obesity and behavioral problems occurring in a smaller percentage (165–169). Patients present in the newborn period with hypotonia and delayed early milestones including head control (170). Patients have a characteristic facial dysmorphism with microcephaly or relative microcephaly. Stature is generally short and congenital heart disease is seen in about 50% of patients studied, with conotruncal malformations being the most common. Oculomotor abnormalities included amblyopia, esotropia, divergent strabismus, exotropia, bilateral hypermetropia and convergent squint. There are also abnormalities of the ears, hands and feet, and abnormalities of connective tissue were noted in 50% of the patients (168). All patients demonstrate motor and developmental delay, with seizures described in 40% of patients and behavior problems in about 50%. Behavior problems can include food-seeking behavior leading to obesity, autistic features, hyperactivity, and sleep disturbances.

**45.4.8.2 Cytogenetics and Molecular Cytogenetics.** Cytogenetically visible deletions of the long arm of chromosome 9 are very rare, suggesting that these larger deletions are likely lethal during development (171,172). The 9q deletions have been found to be isolated deletions (70%) or derived from rearrangement with another chromosome (30%) (167–169). Molecular mapping of the deletion breakpoints in patients has demonstrated deletions from 380kb to more than 3Mb (167,173). The identification of a patient with a balanced translocation (t(X:9)(p11.23;q34.3)) and the clinical features of the 9q- deletion syndrome lead to the recognition that haploinsufficiency of the Euchromatic histone-lysine N-methyltransferase 1 (*EHMT1*) gene is the likely cause for the phenotype of this disorder (165). There are repeated regions within 9q, but there is not a common deletion size seen in patients.

#### 45.4.9 15q11.2q13.1 Microdeletion Syndromes

There are two distinct disorders associated with interstitial deletions of proximal chromosome 15q. These are the Prader-Willi and Angelman syndromes. The deleted regions in these two clinically distinct conditions are often overlapping, although it has been determined that PWS results when the deletion is on the paternally inherited chromosome, while AS results from deletion on the maternally derived chromosome. These disorders highlight the importance of imprinting in the etiology of human disease. The deletions in this disorder occur between LCRs, and this is a recurrent deletion (Figure 45-3A) (79). The repeats in this region also mediate the formation of the supernumerary inverted duplication of 15q, discussed later in this chapter.

##### 45.4.9.1 Prader-Willi Syndrome.

**45.4.9.1.1 Clinical Presentation.** PWS is characterized by severe muscular hypotonia, poor suck, and hypogonadism in the neonatal period. Decreased fetal activity has been documented and serves as a prenatal indication to pursue cytogenetic and molecular studies in the neonatal period. During the second year poor feeding is followed by hyperphagia leading to obesity. PWS patients gain weight even when subjected to restricted diets. Patients have mild to moderate intellectual disability, behavioral disorders and characteristic facial features including almond shaped eyes, narrow bitemporal diameter, upslanting palpebral fissures and strabismus. Additional clinical features include short stature, small hands and feet and in about 75% of patients, fair hair and skin color as compared to other family members (174,175). Adults frequently suffer from diabetes and cardiac disease, secondary to weight gain. PWS occurs with an estimated incidence of 1/10,000–1/20,000 live births (176).

**45.4.9.1.2 Cytogenetics and Molecular Cytogenetics.** PWS is caused by the lack of expression of paternal genes from chromosome 15q11.2q13.1 and by three main mechanisms: (1) 15q microdeletions (70–75%), (2) maternal UPD for chromosome 15 (20–25%), and (3) abnormalities of imprinting (2–4%). Deletions of 15q11.2q13 were first described in four patients with PWS in 1981 (177). The majority of deletions are sporadic, *de novo* deletions, mediated by the repeat structure in the 15q11.2q13 region. In all PWS patients studied, the deletion of chromosome 15 occurs on the paternally inherited chromosome (178–180). This unusual finding led to studies of the parental origin of chromosome 15q11.2q13 in chromosomally normal PWS patients. These studies demonstrated that a number of patients had UPD for chromosome 15 (181). In a small percentage of cases, abnormalities in the imprinting process occur, such that all patterns of methylation are altered on the paternal chromosome and hence imprinting balance is abnormal (182). Finally, a few patients have been identified who have apparently balanced translocations with no evidence for deletion (183,184).



The genetic findings in PWS patients suggest that loss of the paternal 15q11.2q13 region, whether by deletion or maternal UPD, causes PWS syndrome. Most cases result from a deletion of 5–7 Mb, which contains a number of imprinted and nonimprinted genes. There are five paternal-only protein-coding genes within the PWS critical region (*MKRN3*, *MAGEL2*, *NECDIN*, *C15ORF2*, and *SNURF-SNRPN*), and a family of paternal-only-expressed snoRNA genes (*HBII-346*, *HBII-13*, *HBII-438*, *HBII-85* and *HBII-52*). While no single genetic alteration has been found that can cause all of the features of PWS, there is evidence for a critical region that includes only the *HBII-85* snoRNA gene (185). *SNRPN* (small nuclear ribonucleoprotein polypeptide N) has been a leading candidate for the PWS disease gene; however, it is not sufficient to cause the disease based on patients with interruption of *SNRPN* who do not demonstrate the full phenotype (183). It is most likely that PWS is caused by loss of more than one gene.

Diagnosis of PWS can be carried out by a combination of molecular studies, CMA, or both. Parent-specific imprinting differences can be detected at the DNA level by examination of the methylation pattern of specific regions of DNA, which is diagnostic in the majority of PWS patients (i.e. those due to deletion, UPD or imprinting center mutations). If a maternal-only pattern of methylation is revealed, the diagnosis of PWS can be confirmed, but further studies are needed to determine the molecular class of the mutation (deletion, UPD or imprinting center mutation).

Recurrence risks for PWS vary with the molecular mechanism identified. Recurrence is low in the case of *de novo* deletions and UPD. However, multiple familial cases have been identified in which there is an imprinting center defect (182) or balanced chromosomal rearrangement (186,187).

#### 45.4.9.2 Angelman Syndrome.

**45.4.9.2.1 Clinical Presentation.** Features of AS include severe motor and intellectual disability, ataxic gait, hypotonia, epilepsy, absence of speech, microcephaly, and a large prominent mandible with open-mouthed expression. Patients are reported to have a happy disposition and demonstrate bursts of excessive, inappropriate laughter. Seizures are a pronounced feature of AS, and have a characteristic EEG pattern. This rhythmic 2–3 Hz high-amplitude delta wave activity is predominantly over the frontal regions, and 3–4 Hz spikes tend to be found more posteriorly. These patterns are not encountered in PWS, in which seizures are not typical (188). The frequency of AS is estimated at 1/15,000.

**45.4.9.2.2 Cytogenetics and Molecular Cytogenetics.** Like PWS, the genetic etiology of AS is complex and is dependent on the parent of origin of the genetic defect. AS is caused by the lack of expression of maternal genes from chromosome 15q11.2q13.1 and is caused by four main mechanisms: (1) 15q microdeletions (70–75%), (2) paternal UPD for chromosome 15 (3–5%), (3) abnormalities of imprinting (4–6%), and

(4) mutation of the *UBE3A* gene (4–6%). Approximately 60% of patients with AS have a cytogenetically detectable deletion of 15q11.2q13 that is identical to that seen in association with PWS. Another 10–15% have a normal karyotype, but a submicroscopic deletion is detectable by FISH (189). In 1994, the *UBE3A* gene (E6-AP ubiquitin-protein ligase) was mapped to 15q11.2q13 within the deletion critical region for AS. Studies demonstrated that *UBE3A* is imprinted in some parts of the brain and, therefore, maternally inherited mutations would be expected to result in phenotypic abnormalities, consistent with findings in AS. *UBE3A* is not imprinted in lymphoblastoid and fibroblast cells (190). Four to six percent of Angelman patients have mutations in *UBE3A*, and these mutations have been found to account for a significant proportion of familial cases (191). The *UBE3A* gene functions to catalyze the transfer of ubiquitin to substrates, which are then targeted for degradation by the proteasome. This is the first member of the ubiquitination pathway to be implicated in a genetic disease; however, the mechanism by which loss of function of *UBE3A* leads to AS is not understood. As in the PWS, there is also a class of Angelman patients (4–6%) who have imprinting mutations. That is, there is a defect in the imprinting center such that the affected chromosome is incapable of becoming appropriately imprinted, resulting in failure to express maternal-only genes. In some cases, these mutations are caused by small deletions and, in others, the precise defect has not been identified (191). In addition to all the mechanisms of mutations leading to AS discussed earlier, there are still 10–14% of patients in whom no molecular defect has yet been found.

The most straightforward diagnostic test for AS is analysis of methylation status, which can detect imprinting differences. If methylation analysis is shown to be abnormal, the molecular class of the abnormality can be determined in order to provide accurate counseling information (low recurrence for large deletions and UPD, higher for small deletions that affect the imprinting center). If methylation studies are normal, point mutations in the *UBE3A* gene can be investigated by mutation analysis.

#### 45.4.10 15q13.3 Microdeletion

The 15q13.3 deletion is a newly characterized recurrent deletion syndrome (192) (MIM# 612001). The deletion was identified by using CMA in a cohort of individuals with intellectual disability, which revealed a 1.5 Mb deletion that occurs between two repeat clusters (BP4 and BP5) within chromosome band 15q13.3. Variable expressivity and incomplete penetrance is observed in this syndrome.

**45.4.10.1 Clinical Presentation.** The features associated with this deletion are highly variable, and include intellectual disability, seizures, autism spectrum disorder, developmental delay, mildly dysmorphic features and schizophrenia (105,192–196). More than 120 cases of

15q13.3 deletion have been reported, and when parental testing was available, most of these deletions were seen to be inherited from an unaffected or mildly affected parent. Three individuals with more severe phenotypes were found to have homozygous deletions within 15q13.3, and presented with profound intellectual disability, hypotonia, eye abnormalities and refractile seizures (197,198).

**45.4.10.2 Cytogenetics and Molecular Cytogenetics.** The 15q13.3 deletion is an LCR-mediated recurrent deletion (192). The deletion size ranges from 1.5 to 2 Mb and the critical region contains 7 genes including CHRNA7, ARHGAP11B, MTMR15, MTMR10, TRPM1, KLF13, and OTUD7A. The 15q13.3 deletion is usually inherited (57–90%) from a mildly affected or unaffected parent (195,199). The critical region for this deletion syndrome is flanked by multiple SDs, and several distinct deletions within chromosome band 15q13.3 that do not involve the critical genes have been shown to be present in normal controls (benign CNVs).

#### 45.4.11 16p13.1 Microdeletion

Deletions of 16p13.1 were first identified using CMA analysis in 2007 (200). The 16p13.1 microdeletion is a recurrent deletion.

**45.4.11.1 Clinical Presentation.** The 16p13.1 microdeletion is characterized by extreme variability of phenotypes. Deletion of 16p13.1 has been reported in individuals with autism and intellectual disability (200). Deletions of 16p13.1 have also been associated with developmental delay, schizophrenia and epilepsy (201–204). Other reported features include microcephaly, mild dysmorphic features, and short stature. This deletion was identified prenatally in three cases, one case with a midline defect, one case with increased nuchal translucency, and the third case with structural brain malformation (203,205). When parental testing was performed, roughly half were found to be *de novo* and half were inherited from unaffected or variably affected parents. Variable expressivity and incomplete penetrance has been observed in each family studied.

**45.4.11.2 Cytogenetics and Molecular Cytogenetics.** The 16p13.1 deletion is recurrent, with a common size of about 1.13 Mb. Fourteen annotated genes are within the common deletion, with reduced dosage of the NDE1 and NTAN1 genes thought to be the cause of the neurocognitive phenotype seen with this deletion (81). NDE1, also known as NUDE, is expressed in the brain and the protein product interacts with Lis1, which is important for normal brain formation. NTAN1 deficiency is related to memory dysfunction and social behavior in the mouse (206).

#### 45.4.12 16p11.2 Microdeletion

The 16p11.2 deletion is a newly characterized syndrome without a consistent phenotypic pattern but with almost

complete penetrance. It is one of the most commonly detected deletions in individuals tested by CMA in the clinical setting. This deletion was first described in 2007 by two independent groups, in a report of *de novo* copy number change in a cohort of individuals with autism spectrum disorder (207) and in a set of monozygotic twins with cardiac defect, intellectual disability and seizures (208). Since the initial reports, more than 100 individuals with 16p11.2 deletion have been reported in the literature (83,209).

**45.4.12.1 Clinical Presentation.** The 16p11.2 deletion has been associated with cardiac defects, developmental delay, skeletal abnormalities, genitourinary tract anomalies, obesity, neurobehavioral abnormalities (including autism), and dysmorphic features. Despite the variability of the phenotypes, some features are more common including developmental delay, intellectual disability, and/or autism spectrum disorder, and obesity (210). Delays are observed more in language and cognition than in motor skills. Recently, some individuals with normal intellect have been reported (211). Carriers of the 16p11.2 deletion are thought to have a higher risk of seizures as compared to noncarriers; however, long-term follow-up data is not available. Most of the deletions are *de novo* with several examples of inheritance from an affected parent; however, affected parents have presented with clinical features that are different from those seen in the probands (212).

**45.4.12.2 Cytogenetics and Molecular Cytogenetics.** The 16p11.2 deletion is a recurrent deletion mediated by LCRs. The deletion is commonly about 580 kb and contains 25 annotated genes including ALDOA, DOC2A, SEZ6L2, TAOK2, HIRIP3, MAPK3, MAZ, and PPP4C. Of note, there is another recurrent 200 kb deletion on 16p11.2 distal to the one described here. This distal 200 kb deletion includes the SH2B1 gene and has been associated with developmental delay and obesity (213).

#### 45.4.13 Miller-Dieker Syndrome

Deletion of the distal end of the short arm of chromosome 17 is associated with a distinct syndrome of facial dysmorphism in combination with lissencephaly (214). Some patients with isolated lissencephaly (ILS), in the absence of other dysmorphic features, have also been found to have deletions of this region of the genome (215). The deletions in this disorder do not occur between LCRs; the breakpoints are variable and nonrecurrent.

**45.4.13.1 Clinical Presentation.** Miller-Dieker syndrome (MDS) is characterized by type I lissencephaly (smooth brain, absent gyri and smooth cortical surface) and distinctive facial features including a prominent forehead, bitemporal hollowing, midface hypoplasia, a small jaw, short nose with upturned nares, and a protuberant upper lip with thin vermilion border. At the clinical level, MDS and ILS can be differentiated by the presence of

significant dysmorphic facial features and a more severe grade of lissencephaly in MDS.

Patients also demonstrate severe postnatal growth retardation and seizures (216–218). Seizures often set in early after birth and are reported to be of various types (219). Polyhydramnios is common during pregnancy, probably because of poor fetal swallowing movements. All patients have profound intellectual disability, diminished spontaneous movements, prominent opisthotonus and limb spasticity. In one study of 26 patients, 6 had died by the age of 4 years (214).

#### 45.4.13.2 Cytogenetics and Molecular Cytogenetics.

The occurrence of several families in which there were multiple affected siblings initially suggested that MDS was a recessive condition. It has now been shown that most of the known familial cases are due to a parental balanced translocation or pericentric inversion (220,221). MDS is associated with a deletion of chromosome 17p13.3, which is visible by high-resolution cytogenetic methods in about 50% of cases (214). The size of the deletion in these patients is variable, and there is no apparent difference in clinical manifestation among patients with different size deletions, suggesting that only one or a small number of genes within 17p13.3 are responsible for the phenotype.

Microdeletions of 17p13.3 have been detected in many patients with apparently normal chromosomes and features of MDS. Using cytogenetic and molecular techniques, deletions of 17p13 are detectable in about 90% of MDS patients and in about 15% of patients with ILS (215,222). Studies comparing deletion breakpoints in patients identified a critical 350kb region for MDS, and identified *LIS1* (lissencephaly-1, also known as *PAFAH1B1*) as a critical gene in lissencephaly. Point mutations have been identified in the *LIS1* gene in patients with the ILS sequence. Deletions of 17p13.3 and the *LIS1* gene result in a phenotype that can vary from the ILS sequence to MDS. Deletion of the *CRK* and 14-3-3 epsilon (*YWHAE*) genes occurs in patients with the most severe lissencephaly grade. *YWHAE* plays a role in cortical development and may contribute to the more severe form of lissencephaly seen only in patients with MDS (223). Facial dysmorphism and other anomalies in MDS patients appear to be the consequence of deletion of genes distal to *LIS1*.

#### 45.4.14 Hereditary Neuropathy with Liability to Pressure Palsies (HNPP)

Approximately 80% of individuals with hereditary neuropathy with liability to pressure palsies (HNPP) (MIM:162500) have a recurrent deletion on 17p12, while the reciprocal duplication is associated with CMT type 1A, and this pair of disorders has been shown to occur via NAHR.

**45.4.14.1 Clinical Presentation.** HNPP is a focal compression peripheral neuropathy that is characterized by episodic numbness, tingling or weakness in response to

nerve injury from pressure. Absent ankle reflexes are seen in about half of the individuals with this condition. Neuropathy is seen of both motor and sensory nerves. The common presenting symptom is neuropathy of a single nerve without any pain (224). The peroneal nerve is the most commonly affected nerve resulting in a foot drop. The ulnar nerve, brachial plexus, and the radial nerve are also frequently seen affected. Nerve conduction delays are noted on electrophysiologic studies (225). Subclinical central nervous system lesions (in white matter) are observed occasionally (226). The nerve injury causes demyelination with gradual recovery over time, although recovery may not be complete for several months. Onset is usually seen within the first and second decades of life. In the pediatric population, the 17p11.2 deletion can be detected as an incidental finding by CMA (227). Variability in the severity is often noted and some individuals are reported to be asymptomatic. Carpal tunnel is often seen in individuals with HNPP; however, HNPP is not a cause of most cases of isolated carpal tunnel syndrome (228).

#### 45.4.14.2 Cytogenetics and Molecular Cytogenetics.

A 1.4 Mb deletion involving the *PMP22* gene is the cause of HNPP in 80% of patients, with the remainder of cases resulting from intragenic mutations of this gene. The deletion is recurrent and lies between LCRs (CMT1A-REP). The CMT1A and HNPP reciprocal duplication/deletion syndromes have been used as a model to work out the mechanisms for recurrent deletion/duplication syndromes (229).

#### 45.4.15 Smith-Magenis Syndrome

SMS was first described in 1986, and patients have a constellation of features including facial dysmorphism, short stature, cleft palate, behavioral problems and intellectual disability. The syndrome is associated with a cytogenetically detectable 3.7-Mb interstitial deletion within chromosome band 17p11.2 (230,231). The incidence of birth is estimated at 1/25,000. The deletion is recurrent, with greater than 90% of patients presenting with a common deletion.

**45.4.15.1 Clinical Presentation.** The phenotype includes brachycephaly, midfacial hypoplasia with a broad flat midface, broad nasal bridge, prognathism, hoarse voice, speech delay, cognitive delay, psychomotor and growth retardation, and behavioral problems (232). The brows are heavy with excess lateral extension of the eyebrows. The nose has a depressed root and, in the young child, a scooped nasal bridge. Hearing loss is present in ~50% of cases and hyperactivity and behavioral problems are present in most. Two-thirds of the patients demonstrate self-injurious behavior including pulling out fingernails and toenails, insertion of foreign bodies into body orifices, and head banging. Ocular abnormalities are frequent and include high myopia, retinal detachments, iris anomalies, microcornea, strabismus and cataracts (230,231,233–235).



Patients demonstrate normal nerve conduction velocities, despite the fact that signs suggestive of peripheral neuropathy are found in 75% of patients (e.g. decreased or absent deep tendon reflexes, pes planus or pes cavus, decreased sensitivity to pain, and decreased leg muscle mass) (232). Notable symptoms of sleep disturbance (difficulty falling asleep, difficulty staying asleep, and frequent awakening during the night) have been observed in a significant number of patients (235). In 57% of patients, abnormalities in rapid eye movement (REM) sleep have been demonstrated by polysomnography (232,235). Patients with SMS manifest unusual hand- or arm-clasping behavior (self-hugging), which appears to be part of a complex upper body tic, exacerbated by happiness, excitement or over stimulation (236).

#### 45.4.15.2 Cytogenetics and Molecular Cytogenetics.

In the majority of patients, the syndrome is related to a cytogenetically visible, *de novo*, interstitial deletion of a portion of 17p11.2 (230,231). One patient with complete deletion of band 17p11.2 was more severely affected with facial malformations, cleft palate, and major anomalies of the cardiac, skeletal and genitourinary systems. Parent-of-origin studies on 15 patients demonstrated 9 paternal and 6 maternal deletions. The apparent random parental origin suggested that genomic imprinting does not play a role in the expression of the SMS clinical phenotype (233).

It has been demonstrated that NAHR between copies of a repeat gene cluster (SMS-REP) that flank the deletion is responsible for this microdeletion disorder (237). Several patients with SMS with atypical interstitial deletions at 17p11.2 demonstrated deletions of the retinoic acid induced 1 (*RAI1*) gene (238). Further, point mutations in the *RAI1* gene, which lies within the SMS critical interval, have been identified in patients with many SMS features in whom no deletion was detected (239,240).

### 45.4.16 17q12 Microdeletion

The 17q12 microdeletion is a recurrent 1.5 Mb deletion that encompasses the *HNF1B* (*TCF2*) gene. The first deletions involving the *HNF1B* gene were identified in patients with MODY 5 (241), and this is the first microdeletion to be associated with diabetes.

**45.4.16.1 Clinical Presentation.** The phenotypic spectrum of this disorder includes diabetes, pancreatic abnormalities as well as autism spectrum disorder, mood disorders, intellectual disability, speech delay, seizures and structural brain malformations (87,242,243). Neurocognitive features demonstrate reduced penetrance. Mefford et al. reported a *de novo* deletion in a fetal autopsy sample with dysplastic kidneys (244). Deletions also result in kidney cysts, hypo/dysplastic kidneys, and single kidneys (245,246).

**45.4.16.2 Cytogenetics and Molecular Cytogenetics.** Deletions of 17q12 are recurrent and mediated by SD. The common size of the deletion is between 1.06 and

2 Mb. The minimum deleted region (~1 Mb) includes 13 annotated genes including *HNF1B* also known as *TCF2*, *LHX1* and *ACACA*. Mutations of *HNF1B* result in renal cysts and diabetes syndrome (MIM# 137920), features that are seen in the 17q12 deletion. *LHX1* haploinsufficiency is hypothesized to take part in the neurocognitive phenotype of the syndrome (87).

### 45.4.17 17q21.31 Microdeletion

The 17q21.31 microdeletion syndrome was one of the first syndromes identified with the use of CMA technology (12,13). The 17q21.31 microdeletion has a clinically recognizable phenotype that includes characteristic facial features, hypotonia, developmental delay, abnormal hair structure and a characteristic, friendly disposition (88,247). This is a recurrent microdeletion.

**45.4.17.1 Clinical Presentation.** To date 40 patients with this deletion have been reported (12,13,88,247–249). The common dysmorphic features of these individuals include pear-shaped nose with bulbous tip, abnormal hair structure, ptosis, blepharophimosis, long face with broad forehead, and anteverted ears. Global developmental delay is evident in early childhood; language and speech is significantly affected. The degree of cognitive impairment is variable. Hypotonia and a friendly personality are seen in ~80% of the deletion carriers. Seizures, lax joints, cryptorchidism, and structural brain abnormalities are noted in roughly half of all the reported individuals. Scoliosis/kyphosis, ocular and cardiac abnormalities are noted frequently as well.

**45.4.17.2 Cytogenetics and Molecular Cytogenetics.** The commonly deleted size of this recurrent deletion is 500–700 kb, and a 424 kb critical region within the commonly deleted region has been identified (88). Six genes (*C17orf69*, *IMP5*, *STH*, *KIAA1267*, *CRHR1*, and *MAPT*) reside within the critical region. Haploinsufficiency of *MAPT* is thought to be the cause of this syndrome. Of note, gain-of-function mutations of *MAPT* result in progressive dementias (MIM# 601104; 260540; and 600274). In all the reported patients this deletion was found to be *de novo* when parental testing was available. An inversion of chromosome band 17q21.31 is detected in all originating parental chromosomes (250). This 900 kb inversion is present in 20% of individuals with European ancestry, and may predispose carriers for the 17q21.31 deletion in offspring (251).

### 45.4.18 18p Monosomy

The 18p- syndrome was first described in 1963 (252). The incidence is estimated to be about 1:50,000 live-born infants. Deletions of 18p have variable breakpoints and this is not a recurrent disorder.

**45.4.18.1 Clinical Presentation.** The main clinical manifestations include intellectual disability, growth retardation, round face, dysplastic ears, wide mouth with



dental anomalies, and abnormalities of the limbs, genitalia, brain, eyes, and heart. The pattern of abnormalities seen in patients with deletion of 18p may not be striking at birth, but becomes more evident by 3 years of age. Newborns have mild to moderate growth retardation (mean birth weight at the 5–10 percentile). Affected neonates demonstrate brachycephaly, a broad face with ptosis of the upper lids, strabismus, hypertelorism, a broad nose, downturned corners of the mouth, micrognathia with a wide mouth and large protruding ears. Teeth are irregularly set and are particularly prone to caries (253). Holoprosencephaly is present in at least 10% of patients. Heart defects, primarily septal defects are seen in less than 10% of patients. IQs range from 25 to 75, with an average of 45–50. Speech is particularly delayed and many patients do not speak before 7 years. Prognosis is poor for patients with holoprosencephaly, but patients without this defect do not have a shortened life expectancy (254). There are at least four reports of patients with partial deletion of 18p (with breakpoints in 18p11.2 or 18p11.3) who have reproduced (255–258). Consistent features in these cases included mild intellectual disability with short stature, ocular abnormalities (cataracts, extropia, myopia), and microcephaly reported in some cases.

Multiple cases have been identified on prenatal diagnosis, following abnormalities seen on ultrasound. In one case, holoprosencephaly was identified at ultrasound, with 18p deletion confirmed on follow-up chromosomes (259). In another case, a chromosome abnormality was picked up after amniocentesis for advanced maternal age, but an ultrasound at 20 weeks revealed increased nuchal translucency as the only abnormality (260).

#### 45.4.18.2 Cytogenetics and Molecular Cytogenetics.

Most 18p- patients have a cytogenetically detectable deletion of almost the entire short arm of chromosome 18. This is not a recurrent abnormality, and is not mediated by LCRs. This deletion occurs *de novo* in about 85% of patients, with the remainder of 18p- individuals resulting from the malsegregation of familial rearrangements. There have also been a few instances of *de novo* unbalanced rearrangements involving translocation of the 18 centromere and long arm onto the short arm of an acrocentric chromosome, resulting in monosomy 18p (259,260). Some deletions have been the result of cryptic translocations involving other subtelomeric regions (261).

### 45.4.19 18q Monosomy

Partial deletion of the long arm of chromosome 18 leads to a clinically recognizable, although variable syndrome. The deletions of 18q that have been studied have highly variable breakpoints and there are good correlations between genotypes and phenotypes (262,263). These highly variable deletions are nonrecurrent and are not associated with specific genomic architecture. There are SDs within chromosome 18q, but there does not seem

to be a preference for breakpoints that are mediated by these, with a couple of exceptions (264).

**45.4.19.1 Clinical Presentation.** Clinical features of the 18q- syndrome are variable, consistent with the variable sizes of the deletions. Consistent features include hypotonia, dysmyelination of the central nervous system, decreased or absent deep tendon reflexes, short stature, and hearing impairment. Facial dysmorphism consists of midface hypoplasia, deeply set eyes, short nose, and a “carp” mouth (large, open mouth with corners slanting downward, short upper lip, absent Cupid’s bow). Ears are characteristic with prominent antitragus and anti-helix. Atresia of the external auditory canal has been identified in about 50–65% of patients, and hearing loss has been noted. Fingers are long, thin and tapered with protuberant fingertips and toes are irregularly implanted and overriding. Both sexes demonstrate abnormal genitalia, with cryptorchidism and hypospadias in males and hypoplasia of the labia minora in females. Growth hormone insufficiency is a common cause of growth failure in 18q- individuals. Neurologic findings include hypotonia, poor coordination, choreoathetotic movements and spinal muscular atrophy. Intellectual disability is generally present, but varies from mild to severe. Patients have been reported with only learning disabilities, although half the patients have an IQ between 30 and 50 (265). Life expectancy is apparently normal and many affected adults have been described. There are multiple reports of this chromosomal abnormality being transmitted from parent to child (266,267).

#### 45.4.19.2 Cytogenetics and Molecular Cytogenetics.

The breakpoint of the deletions on the long arm of chromosome 18 have been found to vary between 18q21 and 18q23 with widely ranging deletion sizes from 0.5 Mb to more than 30 Mb. The highly variable breakpoints in this syndrome do not appear to be mediated by LCRs. Several different types of cytogenetic abnormalities have been found to be associated with deletions of 18q, and of 189 patients who were studied, 130 (69%) had simple terminal deletions, 11 (6%) had deletion/duplication events, 29 (15%) had interstitial deletions, 4 (2%) had deletions resulting from a translocation, and 15 (8%) had deletions resulting from capture of another chromosome’s telomere. About 94% of the alterations were *de novo*, and 87% of *de novo* deletions occurred on the paternal chromosome (264). Critical regions have been identified for several features including dysmyelination, kidney malformations, and aural atresia; however, key genes have not been identified to date (263).

### 45.4.20 Chromosome 22q11.2 Deletion Syndrome

Deletions of the proximal long arm of chromosome 22 have been associated with several well-studied, clinically identified syndromes. They include DiGeorge Syndrome (DGS), the velocardiofacial syndrome (VCFS) (268,269)

and the conotruncal anomaly face syndrome (CAFS) (270). All three syndromes share the same molecular lesion, a deletion within band 22q11.2. Conotruncal cardiac defects are common to all three described syndromes and studies have shown that there is a subgroup of patients with conotruncal defects who demonstrate deletions of 22q that can be identified at birth (271). It is now known that the majority of patients with DGS, VCFS, and CAFS have deletions of 22q11 (269). Thus, these syndromes are presumed to be slightly different manifestations of the same disorder. The 22q11.2 deletions occur between LCRs in the 22q11.2 region and are recurrent deletions (272,273). The overall frequency of the 22q11.2 deletion syndrome (22q11.2 DS) is estimated to be approximately 1/2000–1/6000 live births (274–276).

**45.4.20.1 Clinical Presentation.** The 22q11.2 deletion syndrome is characterized by cognitive, behavioral and physical anomalies (277). Patients demonstrate extreme phenotypic variability, and intrafamilial variability is common. Conotruncal cardiac malformations occur with an increased frequency in patients. Interrupted aortic arch type B (IAA), truncus arteriosus (TA), and tetralogy of Fallot (TOF) are the predominant cardiac lesions observed in DGS patients while TOF and ventricular septal defects (VSDs) are common in VCFS. Goldmuntz and colleagues (271) showed that 20–30% of newborns and infants with apparently nonsyndromic conotruncal cardiac defects have microdeletions of 22q11. Deletions of 22q11 have been described in cases of familial CHD as well (see Chapter 46). Careful examination of these individuals has identified subtle dysmorphic facial features or learning disabilities that are more suggestive of the diagnosis of DGS/VCFS; however, some of these features may not be apparent until a child reaches school age.

DGS was originally characterized by the absence or hypoplasia of the thymus and the parathyroid glands, cardiac malformations and dysmorphic facial features (278). Affected organs are derived from the embryonic third and fourth pharyngeal pouches and the syndrome is presumably a developmental defect of these structures. Absence or hypoplasia of the parathyroid glands may lead to hypocalcemia that presents as neonatal tetany or seizures. Hypoplasia of the thymus causes susceptibility to infection because of a functional deficit of T cells. The cardiac malformations include TOF, IAA, TA, conoventricular septal defect, right-sided aortic arch and aberrant right subclavian artery. Craniofacial dysmorphism can include micrognathia, cleft palate, bifid uvula, low-set ears and hypertelorism with short palpebral fissures. Defects in other organ systems have been reported (278–280). VCFS was initially characterized in patients with cleft palate, cardiac defects, speech and learning disabilities and a typical facial appearance (281–283). CAFS has been characterized by the presence of conotruncal cardiac defects in association with a characteristic facial appearance. The facial features include ocular hypertelorism,

lateral displacement of the inner canthi, flat nasal bridge, small mouth, narrow palpebral fissures, bloated eyelids, and malformed ears.

Since the disorder and the microdeletion segregate in an autosomal dominant mode, one should pay particular attention to the presence of minor features such as facial dysmorphism and mild cognitive impairment in the parents of deletion-positive probands. Parents of deletion-positive children should be examined by a clinical geneticist and tested to ascertain their carrier status with regard to a 22q11 deletion. Individuals with a 22q11 deletion have a 50% risk of transmitting the deletion-bearing chromosome to his or her offspring. Antenatal detection of the deletion has been successfully performed on cultured amniocytes and chorionic villi obtained from at-risk pregnancies. However, as there is a wide range of phenotypic features seen in association with the 22q11 deletion, one cannot predict the phenotypic outcome based on the results of molecular cytogenetic studies.

**45.4.20.2 Cytogenetics and Molecular Cytogenetics.** Approximately 15–20% of patients with DGS have abnormalities on karyotype (280). The majority of these cytogenetically abnormal cases involve chromosome 22. These are either unbalanced translocations with monosomy 22pter-->q11 (280,284–288) or interstitial deletions, del(22)(q11.21q11.23) (268,280,289–291). Even with high-resolution banding techniques, translocation-mediated or interstitial deletions are observed only in less than 25% of cases. Using molecular techniques (FISH, multiplex ligation-dependent probe amplification (MLPA), quantitative PCR (qPCR) or CMA), deletions of 22q11 can be demonstrated in the majority of patients diagnosed with either DGS or VCFS (268,291,292). Mutations of TBX1, located within the chromosome band 22q11.21, have been identified in several patients without the 22q11.2 deletion (293,294). These results suggest that the TBX1 gene is a major genetic determinant of the features of the 22q11.2 deletion syndrome.

Although the majority of cases of DGS are now attributable to a 22q11 deletion, other chromosome defects have been seen in association with DGS, including 10p13 monosomy, and deletion of 18q21.33 (280,295). In addition, there is evidence that teratogens and maternal diabetes are potential causes of the DGS phenotype, especially in individuals without a 22q11 deletion. The diagnostic management of a patient with DGS should include a molecular test to determine whether there is a 22q11.2 deletion. In the case of a deletion-positive proband, parents should be screened to determine their carrier status.

#### 45.4.21 22q13 Subtelomere Deletion (Phelan-McDermid Syndrome)

The Phelan-McDermid syndrome results from the loss of 22q13 by simple deletion, unbalanced translocation, ring

chromosome formation, or other unbalanced structural change. It is not mediated by LCRs.

**45.4.21.1 Clinical Presentation.** Numerous patients with deletions of 22q13 have now been reported in the literature (296–298). The clinical phenotype is characterized by hypotonia, developmental delay, severe language delay, and mild facial dysmorphism (epicanthal folds, large dysplastic ears, supraorbital fullness, upturned/blunted nasal tip and smooth philtrum). Features seen in a subset of patients include dolichocephaly, two to three toe syndactyly, joint hyperextensibility, seizures and abnormal nails. Most patients demonstrate normal to advanced growth parameters. In a study of 37 individuals with deletions of 22q13 ascertained through a 22q13 family support group, the age range was from 12 months to 26 years, and the number of males and females was similar (17 males, 20 females) (296). There have also been reports of individuals with deletion of 22q13 on one chromosome 22 homolog in whom a recessive allele in the *ARSA* gene (located at 22q13) was unmasked, resulting in metachromatic leukodystrophy (299). This should be considered particularly in patients with metachromatic leukodystrophy who manifest more severe neurologic involvement.

**45.4.21.2 Cytogenetics and Molecular Cytogenetics.** Deletions involving 22q13 have been detected both cytogenetically (deletions, ring chromosomes) and by FISH in individuals with an apparently normal karyotype. In some cases, these deletions have been identified after referral to rule out DGS/VCFS, and FISH using the DiGeorge/VCF probe set revealed incidental deletion of the control probe that maps within the *ARSA* (arylsulfatase A) gene (300). Chromosome 22q13 deletions have also been identified after subtelomere analysis (162,301).

Abnormalities of 22q13 can be pure deletions, or unbalanced rearrangements with involvement of another chromosome. In the study reported by Phelan et al. (296) there were 29 terminal deletions and 8 unbalanced translocations. There was one patient identified with an apparently reciprocal translocation, 46,XY,t(12;22)(q24.1;q13.3), who demonstrated features consistent with those seen in the 22q13.3 deletion syndrome (302). Clinical features in this patient included developmental delay, severely compromised expressive language, mild intellectual disability (IQ 54), hypotonia and minor dysmorphic features (dolichocephaly, epicanthal folds, and saddle nose with bulbous tip). Molecular analysis of this translocation revealed that it bisected the proline-rich synapse-associated protein 2 (*ProSAP2*) gene. *ProSAP2*, also known as *SHANK3*, encodes a scaffold protein that is involved in the postsynaptic density of excitatory synapses, and is preferentially expressed in the cerebral cortex and cerebellum. Haploinsufficiency of this gene is the cause of the major neurological features associated with deletion 22q13 (303,304).

## 45.5 DUPLICATION

### 45.5.1 Supernumerary Chromosomes

Small supernumerary chromosomes are seen in approximately 0.06% of the population (305). These small chromosomes are sometimes referred to as “marker” chromosomes. Marker chromosomes are defined as abnormal chromosomes that cannot be fully characterized based on standard cytogenetic analyses because of their paucity of banding landmarks. FISH and CMA analyses have shown that these markers are heterogeneous with respect to size and composition and some are associated with intellectual disability and other abnormalities, while others seem to have no recognizable phenotypic effects.

Correlation between the phenotype and the chromosomal origin may permit a distinction between those markers that carry a high risk for phenotypic abnormality and those markers with a low risk. The risk for phenotypic abnormality is correlated with whether the marker is *de novo* or familial (with the risk being higher for *de novo* markers), and with the chromosomal origin of the marker. In some cases, the marker chromosomes are large enough and have some banding characteristics, which allow the cytogeneticist to make educated guesses about their origin. Other markers are very small and have no cytogenetic landmarks. In a study of 50 marker chromosomes, Blennow et al. identified 37 markers that were of acrocentric origin (25 from chromosome 15), 5 isochromosomes for 18p, one isochromosome for 12p (Pallister Killian), and one each derived from chromosomes 4, 7, 8, 9, 19, and 20. In this section, we describe five syndromes associated with relatively well-studied supernumerary chromosomes.

### 45.5.2 Pallister-Killian Syndrome

The Pallister-Killian syndrome is a relatively rare disorder characterized by multiple dysmorphic features and severe intellectual disability and tissue-specific mosaicism. The cytogenetic abnormality is not generally observed in peripheral blood lymphocytes analyzed by G-banding and therefore the diagnosis could be missed if the proper diagnostic test is not carried out.

**45.5.2.1 Clinical Presentation.** Patients with Pallister-Killian syndrome have a very coarse face with pigmentary skin anomalies, localized alopecia, profound intellectual disability and seizures. In infancy the hair is sparse, particularly in the frontal area. Patients have a prominent, high forehead, hypertelorism, epicanthal folds, flat nasal bridge, large mouth with downturned corners and abnormal ears. The neck is often short and webbed, with excess nuchal skin. There is a relatively high incidence of diaphragmatic defects and supernumerary nipples. Affected newborns are profoundly hypotonic. Adults with the disorder demonstrate severe intellectual disability, epilepsy,

coarse facies and macroglossia (306–309). There have been a few exceptional patients who have been reported to have a mild phenotype, including mild intellectual disability (310).

The consistently mosaic nature of this chromosome abnormality can make prenatal diagnosis difficult. Prenatal findings on ultrasound that indicate a risk of the Pallister-Killian syndrome include broad neck, short limbs, abnormal hands or feet, diaphragmatic hernia and hydramnios. The diagnosis has been made after an abnormal screen for nuchal translucency (311).

#### 45.5.2.2 Cytogenetics and Molecular Cytogenetics.

Historically, in most Pallister-Killian patients studied by cytogenetic (G-banding) analysis, lymphocyte chromosomes are found to be normal but fibroblasts show an extra, small metacentric chromosome. The supernumerary chromosome has been demonstrated to be an isochromosome for the short arm of chromosome 12 (i(12p)) (312). The i(12p) has been demonstrated in a high percentage of cells on direct bone marrow analysis and in a lower, but significant percentage of cells from cultured bone marrow, and it has been seen in fibroblasts from skin, lung, and testes. Recent work demonstrates that the isochromosome can be detected by CMA of DNA purified from peripheral blood. It has been hypothesized that failure to detect the isochromosome by standard cytogenetics is a result of selection against it in the cells that respond to phytohemagglutinin (PHA) stimulation. When culturing is not carried out the isochromosome may be detected, either by microarray or by interphase FISH (313). Nevertheless, it is still important to consider the possibility of tissue-limited mosaicism when a negative result is obtained in one tissue (314,315). The percentage of cells containing the supernumerary i(12p) has varied widely among patients studied. The frequency of the i(12p) has been found to be higher in amniotic fluid than in chorionic villi or cord blood. The percentage of fibroblasts containing the marker has been observed to decrease over time (316–318). The marker can be inherited from either parent, although of six cases reported, it was maternal in five (319).

### 45.5.3 Isodicentric 15 Syndrome (Tetrasomy 15q)

The isodicentric 15 syndrome (tetrasomy 15q) accounts for approximately 40% of marker chromosomes, with an incidence of about 1/30,000. The rearrangement is mediated by the genomic architecture within 15q11q13, which is highly repetitive as described for Prader-Willi and Angelman syndromes. The marker associated with the tetrasomy 15q syndrome is genetically distinct from smaller, benign marker chromosomes derived from chromosome 15. Markers that do not extend into the Prader-Willi/Angelman syndrome critical region are often benign and may be familial, while markers that include this region are pathogenic (320–324). Thus, the clinical

presentation is dependent on the size of the marker, with smaller markers found in normal individuals (325,326), while the larger markers are associated with the tetrasomy 15q syndrome.

**45.5.3.1 Clinical Presentation.** Patients with tetrasomy 15q syndrome present with clinical abnormalities including central hypotonia, developmental delay, intellectual disability, epilepsy and autistic behavior (327,328). Patients with tetrasomy 15q can present during the first year with hypotonia and acquisition of milestones is delayed. Expressive language is particularly poor with echolalia. Affected children are often categorized as autistic (321,322,329–331). Many reported cases of tetrasomy 15q have been identified in patients studied because of intellectual disability. These patients are generally nondysmorphic, although facial features have been described as coarse and various minor dysmorphic features have been described, including flattened nasal bridge and anteverted nares (330). Affected individuals demonstrate developmental delay from an early age and other associated features include psychomotor retardation, seizures, hyperactivity and behavioral disturbances, including violent behavior (328,329,332,333). There have been a few cases of hyperpigmentation, which may be associated with duplication of the P gene, which is located within 15q11q13, and is known to be involved in pigmentation (334). Lifespan does not appear to be reduced, although there have been a few reports of progressive loss of function (335).

#### 45.5.3.2 Cytogenetics and Molecular Cytogenetics.

Isodicentric chromosomes consist of two copies of the p arm (satellites), centromere, and proximal long arm of chromosome 15, which are fused at the proximal long arm. The presence of SDs in this region is consistent with the high frequency of this marker chromosome. These regions are known to contain a large number of LCR sequences, and it has been proposed that illegitimate recombination between these repeats may be involved as a mechanism in the formation of structural anomalies involving 15q11q13 (336). Historically, this chromosome marker was described as an inverted duplication 15 (inv dup(15)); however, the nomenclature has been updated to reflect the origin of the marker, which is known to arise from both chromosome 15 homologs (dic(15;15)). While it may be difficult to differentiate the benign smaller dic(15;15) markers from the larger pathogenic ones by cytogenetics alone, the determination of genetic composition of the dic(15;15) is now straightforward with the use of array-based techniques. Pathogenic dic(15;15) markers include the Prader-Willi/Angelman syndrome critical region (337).

### 45.5.4 Isochromosome 18p (Tetrasomy 18p Syndrome)

The isochromosome 18p occurs as a supernumerary marker chromosome, with two copies of the short arm



of chromosome 18, resulting in tetrasomy 18p. This isochromosome is presumably compatible with life, because chromosome 18p is relatively gene poor.

**45.5.4.1 Clinical Presentation.** Patients with i(18p) demonstrate a recognizable phenotype characterized by low birth weight, microcephaly, hypotonia, camptodactyly or adducted thumbs and a typical facial appearance. There is hypotonia and feeding difficulties in the first year and spasticity develops by the second year. Intellectual disability in the moderate to severe range is present in all individuals studied. Some patients (about 50%) suffer from seizures. Facial dysmorphism includes dolichocephaly, a round face with small, low-set ears, short palpebral fissures and small mouth, although with increasing age, prognathism develops and the mouth and nose become more normal in size (338–340). Skeletal abnormalities including scoliosis, kyphosis, hypoplasia of the iliac wings, long narrow fingers and toes and rib abnormalities are prevalent. Orthopedic problems are a common issue among adult patients.

**45.5.4.2 Cytogenetics and Molecular Cytogenetics.** The marker chromosome is a small, metacentric marker, which can usually be identified as an i(18p) on the basis of its size and banding pattern. Callen et al. (340) studied nine patients with marker chromosomes thought to be an i(18p) based on banding and confirmed their origin from chromosome 18 using a chromosome 18 alpha satellite probe in combination with a probe mapping to 18p11.3. This marker chromosome usually occurs *de novo*; however, a few familial cases have been reported (341–343). In most, but not all, cases studied, the marker was maternally derived (344,345).

## 45.5.5 Cat Eye Syndrome

The name for this syndrome is derived from the “cat-eye-like” appearance of the pupil, which results from coloboma of the iris, one of the features of the syndrome. There is a high variability of clinical features noted. Cat eye syndrome (CES; MIM# 115470) is most often associated with a supernumerary, bisatellited, dicentric marker, although some patients with interstitial duplications of the 22q11.2 region have also been identified (346–348). The marker chromosome and interstitial duplications are mediated by LCRs within chromosome 22q and there is overlap between the genes that are involved in CES and the der(22) syndrome (Emanuel syndrome) (349). This is a recurrent disorder, and the marker often appears in mosaic form, presumably due to somatic loss.

**45.5.5.1 Clinical Presentation.** CES demonstrates significant variable expressivity (350). In addition to the characteristic ocular anomaly, other clinical features of CES include renal malformations, urinary tract anomalies, congenital heart defects, anal atresia with fistula, imperforate anus, preauricular tags or fistulas, heart malformations, and mild to moderate intellectual disability

Some patients have normal intelligence, but display psychiatric symptoms. Inferior iris coloboma occurs in less than half the cases. Preauricular skin tags and/or pits constitute the most consistent feature. There have been numerous examples of transmission of the marker from parent to child with variability in the phenotype (351,352).

**45.5.5.2 Cytogenetics and Molecular Cytogenetics.** The characteristic chromosomal change is the presence of an additional acrocentric marker chromosome, usually bisatellited, derived from chromosome 22. The marker chromosomes are variable in size and can be asymmetric with regard to the region duplicated (352). Three sizes of supernumerary CES chromosomes have been identified but the phenotype associated with the larger duplicated chromosome is no more severe than that of the smaller cat eye chromosome, and sizing of the duplicated segment has little prognostic value (353). A 2–2.5 Mb critical region contains 14 genes, with CECR1 (cat eye critical region 1) and CECR2 as possible candidates based on function and expression pattern (349). In addition to a supernumerary marker, there has been a case of CES exhibiting all the major symptoms including coloboma, preauricular anomalies, heart defect, kidney malformation, and anal atresia caused by interstitial duplication of the CES critical region on 22q11.2, resulting in partial trisomy 22q11.2 (348).

## 45.5.6 Supernumerary der(22)t(11;22) Syndrome (Emanuel Syndrome)

**45.5.6.1 Clinical Presentation.** Patients with the +der(22) t(11;22) syndrome (Emanuel syndrome) have a distinctive phenotype, which consists of severe intellectual disability, preauricular tag or sinus, ear anomaly, cleft or high-arched palate, micrognathia, heart defects (354) and genital abnormalities in the male. The cytogenetic findings associated with this syndrome have been discussed previously in this chapter (see Recurrent Translocations).

## 45.5.7 Recurrent Microduplications

These duplications are mediated by LCRs, and are reciprocal to the deletions of these regions that have been described earlier. The critical regions and mechanisms were covered in the earlier section on deletions.

**45.5.7.1 7q11.23 Microduplication.** The 7q11.23 microduplication is a recurrent duplication, and involves the same critical region that is deleted in patients with Williams syndrome. The duplication was identified fortuitously, when a sequence from within the duplication was used as a control in a real-time qPCR test to detect 22q11 deletions (355). The associated phenotype has a consistent clinical presentation. The duplicated genetic material is generally found in a tandem orientation (355).

The most consistent clinical feature seen in patients with the 7q11.23 duplication is speech delay, which is seen in all patients. Developmental delay is seen in close to 90% of patients, with a wide range in severity. Normal intelligence has been reported in about 10% of cases. Other features include hypotonia (70% of cases), epilepsy (20%), nonspecific magnetic resonance imaging (MRI) abnormalities (70%), autism (40%), congenital heart defects (<20%) and joint laxity (>20%). Age at diagnosis varies from the first year of life if hypotonia or seizures are present up to ages of 10 or 11 (355,356). The duplication is inherited in about 60% of cases and parents with the duplication are often found to have some consistent features ranging from intellectual disability to a history of expressive speech delay. There are characteristic facial features among the affected individuals, which can be subtle (356).

**45.5.7.2 8p23.1 Microduplication.** In the late 1990s several individuals were identified with cytogenetically visible duplication of 8p23.1 that was described as clinically inconsequential (357,358). Subsequently, 8p23.1 duplications were also associated with heart defects and Coffin Lowry syndrome-like features (359). These cytogenetically indistinguishable duplications have been characterized as two separate entities with the use of molecular cytogenetic techniques. The duplications associated with normal phenotypes were found to be copy number polymorphisms of the *defensin* gene clusters, while the clinically significant duplications were found to be the reciprocal duplication of the critical region of the 8p23.1 microdeletion syndrome. The recurrent 8p23.1 microduplication syndrome is characterized by variable features of developmental delay, heart defect, and mild dysmorphic features (360,361). These duplications are sometimes *de novo*, and sometimes they are inherited from an affected parent (362). These duplications manifest variable expressivity, but all carriers of the duplication have been found to present with some associated phenotypes.

**45.5.7.3 16p11.2 Microduplication.** The 16p11.2 microduplication has been associated with variable phenotypes including developmental delay, especially in motor skills (as opposed to the severe language delays seen in the reciprocal deletion), autism spectrum disorder, dysmorphic features, and microcephaly (83,363). The duplication occurs *de novo* as well as inherited from affected parents. Similar to the 16p11.2 deletion, these duplications are characterized by variable expressivity but complete penetrance.

**45.5.7.4 Charcot-Marie-Tooth Disease Type 1A (CMT1A).** CMT disease type 1A (MIM# 118220) is a peripheral neuropathy associated with a 1.4Mb recurrent duplication within 17p12 that includes the dosage-sensitive *PMP22* gene (364). The involved region is the reciprocal of the region that is deleted in patients with HNPP, discussed previously. CMT syndrome is a demyelinating peripheral neuropathy that is characterized by

progressive weakness of the peripheral muscles. Symptom onset may occur between 5 and 25 years of age, with the most common onset in mid-adolescence or early adulthood. Muscles in the lower legs are usually the most affected, but affected individuals rarely require a wheelchair. Sensory deficits such as decreased sensitivity to heat, cold or touch may also occur. CMT is genetically heterogeneous, with multiple loci.

**45.5.7.5 Potocki-Lupski (17p11.2 duplication) Syndrome.** PTLs was first described in 2000, when targeted analysis for duplication of the repeat rich 17p11.2 region was undertaken (365). This duplication is reciprocal to the region deleted in SMS. PTLs is characterized by hypotonia and failure to thrive in infancy and early childhood, which is hypothesized to result from oropharyngeal dysphagia and hypotonia. Other features include developmental delay, intellectual disability including a severe communication disorder, and features of autistic spectrum disorder (365–367). There are characteristic sleep disturbances that include sleep-disordered breathing that can be diagnosed on sleep studies, although parents have not reported obvious sleep pattern difficulties (366). Electroencephalography (EEG) abnormalities can be detected, although patients do not experience overt seizures. More than half the patients have cardiovascular anomalies including secundum-type atrial septal defect, bicommissural aortic valve and patent foramen ovale, and mild dilated pulmonary annulus. Hypermetropia is also seen (366). While the failure to thrive is evident during the first year, this disorder has more often been diagnosed after 2 years of age in children who present with developmental delay, intellectual disability or autistic-like features (367).

While some individuals with clinical features of PTLs have been found to have duplications that are larger than the 3.7Mb common duplication, no specific additional clinical features have been delineated in these patients (368). The duplication can be difficult to identify cytogenetically, but is readily identified by chromosomal microarray studies. In a 2007 study, 35 subjects with dup(17)(p11.2p11.2) were studied, and 22 had the common, repeat flanked duplication, while the remaining 13 had duplications ranging in size from 1.3 to 15.2Mb. The common duplication occurs on both maternal and paternal chromosomes, and the precipitating crossing over event can occur via both interchromosomal and intrachromosomal mechanisms (366). In addition to the common recurrent duplication, there is another set of LCRs, and an uncommon, NAHR-mediated duplication of 5 Mb has been detected in two PTLs patients (368). Molecular analysis of a total of 74 subjects with PTLs revealed a shortest region of overlap of all the duplications (recurrent and nonrecurrent) of 125kb, which only has one gene, *RAI1*, the critical region gene for SMS.

**45.5.7.6 22q11.2 Microduplication.** The 22q11.2 microduplication is reciprocal to the 22q11.2 deletion syndromes. These duplications were identified by FISH in individuals suspected to have DGS/VCFs

(272,369). It is the most frequently identified microduplication in individuals tested clinically. Phenotypes associated with this duplication are highly variable and may be clinically unrecognizable. The phenotypic spectrum ranges from developmental delay/intellectual disability, hypotonia, hearing loss, growth retardation, and cardiac defects to a completely normal phenotype. This syndrome shares some features of its reciprocal deletion including heart defect, cleft palate, and genitourinary abnormalities; however, the phenotypes associated with the duplications are usually milder than those of the deletions (90,370,371). Although some of the duplications occur *de novo*, most are inherited from a normal or mildly affected parent. Variable expressivity and incomplete penetrance make interpretation of the clinical significance of this duplication challenging.

## CROSS REFERENCES

10. Chromosomal Basis of Inheritance, 22. Cytogenetic Analysis, 46. Congenital Heart Disease, 110. Autism and Autism Spectrum.

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### Biographies



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**Laura K Conlin, PhD**, is the Assistant Director of the Clinical Cytogenomics Laboratory at The Children's Hospital of Philadelphia since 2010. Laura completed her PhD training at the University of Pennsylvania in 2008, and completed her Fellowship training in Cytogenetics and Molecular Biology at The Children's Hospital of Philadelphia. Her research interests are focused on understanding the contribution of mosaicism to human genomic disease, and she is working on several disorders including Pallister-Killian syndrome, Ring Chromosome 20, as well as developing models for analysis of complex conditions such as chimerism.



**Surabhi Mulchandani, MS**, is a Genetic Counselor in the Dept of Pathology at The Children's Hospital of Philadelphia. Surabhi Mulchandani is a genetic counselor in the Cytogenomics lab at the Children's Hospital of Philadelphia. She received a Masters degree in Molecular and Human Genetics from Benaras Hindu University, Varanasi, India, and a Masters degree in genetic counseling from California State University, Northridge. Surabhi interprets the complex data generated by genome-wide array technology in context of patient's clinical information, provides genetic education, and serves as a bridge between clinicians and the laboratory. She has developed genetic education tools for the patients and providers. She plans to expand genetic education and counseling to a diverse cross-section of people.



**Beverly S Emanuel, PhD**, holds the Charles E.H. Upham Professor of Pediatrics at the Perelman School of Medicine of the University of Pennsylvania and is the Division Chief of Human Genetics at The Children's Hospital of Philadelphia. Dr Emanuel is a human geneticist investigating several diseases caused by abnormalities of human chromosomes, particularly chromosome 22. Her laboratory developed the standard diagnostic test, used by laboratories worldwide, to assess the presence of a 22q11.21 deletion and the likelihood of recurrence of deletion-based DGS/VCFS. She also investigated the duplication of genes on this chromosome that give rise to other chromosomal disorders such as supernumerary der(22) syndrome. Dr Emanuel serves on multiple editorial boards and has received several awards including the Benjamin Franklin Founders Award and the Herbert and Esther Bennet Brandwein Award in Genetic Research at The University of Connecticut.



# CHAPTER

# 46

## Congenital Heart Defects

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### 46.1 INTRODUCTION

Congenital heart defects are both common and serious. With an estimated prevalence of approximately 8 in 1000, they are the most common birth defect worldwide, and they are the most common cause of birth defect-related death (1). As a result of advances in echocardiography, fetal echocardiography, cardiac catheterization, electrophysiology, and cardiovascular surgery, many individuals with even the most severe heart lesions now survive to adulthood and lead productive lives (2). This has raised awareness of the need to identify genes involved in all forms of congenital heart disease, isolated as well as syndromic, as patients and their families ask for information regarding their risks of having a child with a congenital heart defect (3).

Although the majority of heart defects are isolated, about one-third occurs as one component of a genetic syndrome (4). Often, it is the congenital heart lesion, the presenting finding, which alerts the healthcare team to the possibility of a genetic syndrome. As with any patient with a birth defect, a primary role of the medical geneticist is to assess whether the finding is an isolated finding, or represents one manifestation of an underlying syndrome. A syndrome is typically suspected when the heart lesion is associated with findings identified on the medical, prenatal or family history, or specific dysmorphic findings (major and minor anomalies) on physical examination. In these cases, confirmatory testing may be ordered, be it chromosome analysis, fluorescence in situ hybridization (FISH), or single-gene analysis. For other cases with additional findings that are not in a recognizable pattern, array comparative genome hybridization (aCGH) is a useful comprehensive screen in the evaluation of the patient with a congenital heart defect.

Making an accurate genetic diagnosis for a patient with a congenital heart defect has several clear benefits

including the ability to provide accurate counseling on prognosis, precise causality, and recurrence risk for both the parents and the child (5). Although the same benefits exist for an isolated heart lesion, unfortunately there is simply less information. For example, counseling a couple, who have just had a child with an isolated congenital heart defect, about the risk of having another affected child remains largely unchanged by molecular analyses, and is based on empiric recurrence data (3).

Despite advances in basic science and technology, our understanding of the genetic basis of congenital heart defects remains limited. That is because congenital heart defects are not variable manifestations of a single developmental aberration. Rather, they are an etiologically heterogeneous collection of malformations, with overlapping genetic and environmental factors. Further, hindering genetic discovery is the fact that it is rare for isolated heart defects to show Mendelian inheritance, so most of the progress in this area has utilized animal models (6). However, advances in understanding the underlying genetic etiology highlight the importance of follow-up of undiagnosed patients.

This chapter provides an overview of congenital heart defects from a perspective most relevant to the clinical geneticist. Cardiac embryology is reviewed and correlated with specific cardiac lesions. We review the common genetic syndromes that have a cardiac defect as a major manifestation, categorized by etiology: chromosomal/aneuploidy syndromes, segmental chromosomal deletion/duplication syndromes, single-gene mutations, and teratogenic. Lastly, recurrence risk counseling is reviewed.

### 46.2 THE EVALUATION OF THE PATIENT WITH CONGENITAL HEART DEFECT

For the physician caring for the patient with a congenital heart defect, an important first step is to determine

whether there is an underlying syndrome or whether the congenital heart defect occurred as an isolated birth defect. Appropriate identification of a syndrome, if present, can direct the team to investigate for other organ system involvement, provide the team with important prognostic information, particularly with regard to natural history, as well as providing reproductive risk counseling for the patient or family (7). This first step can be challenging as patients can present anytime from prenatal referrals based on abnormal imaging, to the adult patient seeking recurrence risk information. Although some genetic syndromes can be readily recognized through history and physical examination, other syndromes often have variable manifestations and phenotypes that can be less apparent at birth or become less obvious with age. Therefore, the diagnostic evaluation must be approached with this continuum in mind.

The patient's medical history can be rich with diagnostic clues and it is from this foundation that the differential diagnosis can be established to guide testing strategies. Regardless of age, a careful review of the patient's medical history, prenatal history, family history, previous evaluations if obtained, can often provide the first clues in determining whether the heart defect is syndromic or isolated.

The physical examination of the patient should seek out patterns of major and minor anomalies. The best clues to an underlying diagnosis are not always the most obvious anomalies nor those with the most important impact on a patient's health, but the rarest or most atypical (8).

After review of the history, and performing a physical examination, additional investigations may be necessary to identify other anomalies. Additional evaluations can include, but are not limited to, head ultrasound or brain magnetic resonance imaging, renal ultrasound, hearing evaluation, dilated eye examination, or skeletal imaging. Identification of additional anomalies can be applied to the differential diagnosis and further refine the genetic testing strategy. In those with obvious patterns of malformations suggestive of aneuploidy, such as Trisomy 21 or Turner syndrome, a standard chromosome analysis or cytogenomic array should be obtained. Those individuals with recognized microdeletion syndromes that could be missed from standard chromosome analysis might benefit from FISH of the region of interest. Lastly, individuals presenting with patterns of malformation due to single-gene disorders would need analysis of the particular gene(s) in question. For those cases with multiple anomalies but without a recognizable syndrome, cytogenomic array analysis (aCGH) should be performed as a first-line screening test.

After a thorough clinical evaluation, it may be determined that a particular heart defect is isolated. Although the genetics of some isolated heart defects are known (see Table 46-2), genetic testing for a particular gene may not be clinically available for confirmation, or the underlying

genetic etiology remains unknown. For the infant, this may be the result of an unrecognized syndrome whose manifestations have not been fully expressed at such a young age. For older individuals, syndromes with clinical variability should be considered before concluding that a specific cardiac defect is isolated.

In the event where a specific genetic diagnosis is not made, follow-up of the patient should be arranged. Our knowledge and understanding of the genetics underlying birth defects is advancing at a rapid pace with new and affordable molecular technologies to accurately identify the underlying genetic abnormalities. Re-evaluation of undiagnosed patients is an important part of the evaluation strategy. This is particularly relevant for the adult patient who may not have had access to new testing technologies.

### 46.2.2 Embryology

A discussion of embryology is important to the understanding of isolated and syndromic congenital heart defects. The cardiovascular system is the first major system to function in the embryo as a result of the nutritional and oxygen demands of the growing embryo, which cannot be sustained by diffusion alone. Two distinct heart fields that share a common origin appear to contribute to the developing heart identified as the first and second heart fields (9). By week 3 in the humans, cells from the first heart field coalesce along the ventral midline to form the primitive heart tube. As the heart tube develops, cells from the second heart field migrate in and, with rightward looping of the heart tube, populate much of the outflow tract, future right ventricle, and atria (10). Precursors of the left ventricle are largely derived from the first heart field. Both first and second heart fields are regulated by bone morphogenic proteins, fibroblast growth factors, and Wnt and Notch proteins, from signals that arise from the adjacent endoderm (6). Once formed, the primitive heart tube must break the pre-existing L–R symmetry and undergo a series of septation events that culminates in the formation of a four-chambered heart.

L–R symmetry is first broken at 3 weeks with the rightward looping of the heart tube that occurs due to the rapid growth of the bulbus cordis and the outer curvature of the right ventricle. The mechanisms of this looping are largely unknown in humans (Figure 46-1a–e). More than 40 genes have been associated with L–R patterning in mammals and there are approximately 10 genes currently implicated in humans (11) (see Table 46-1).

During the fourth week, endocardial cushions form to begin the partitioning of the atrioventricular canal. The endocardial cushions form from the cardiac jelly, fuse with the developing atrial septum and muscular interventricular septum, remodel, and form the atrioventricular valves and septa. Transforming growth factor B, bone morphogenic proteins (BMP2A and BMP4),

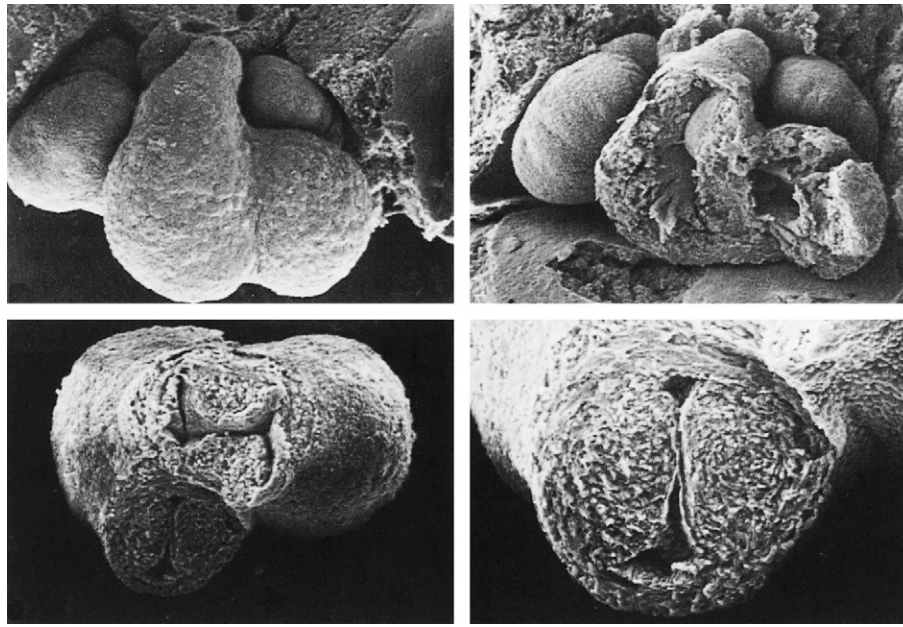


FIGURE 46-1

and *ChALK2* have been implicated in this process (12). *PitX2*, *GATA4*, and *FOG-2* through its interaction with *GATA4* are also involved in the formation of the atrioventricular septum (13).

Atrial septation occurs through the growth of two septa; the septum primum that grows from the ventral and posterior walls of the atrium, and the septum secundum. As the atria enlarge, the septum primum grows toward the developing atrioventricular canal, later divided by the superior and inferior endocardial cushions. Eventually, the septum primum fuses with the atrioventricular cushions narrowing the opening between the two atria, which is then defined as the ostium primum. The septum secundum grows adjacent to the septum primum as a thick muscular fold. The septum secundum partly overlaps the septum primum and the flap-like opening is the foreamen ovale (14) (Figure 46-2 E1–H1 only). In the mouse, *Nkx2.5* is required for normal chamber formation. In humans, mutations in *NKX2-5* are associated with septal and conduction defects (15). *TBX5*, the gene implicated in Holt–Oram syndrome, is also essential for normal atrial formation and may modulate *NKX2-5* transcriptional activity (16). Additional genes implicated in abnormal atrial septal formation are listed in Table 46-2.

The atria develop on the left and right sides of the heart and are thus truly lateralized structures. The two ventricles, on the other hand, develop from the single ventricle and bulbus cordis. Ventricular septation begins at the floor of the primitive ventricle through the proliferation of the interventricular septum. A foramen exists until the end of week 7 with the formation of the membranous part of the interventricular septum with tissue contributed from the right and left bulbar ridges, and the

endocardial cushion (17) (Figure 46-3A–E). This septum eventually fuses with the aorticpulmonary septum resulting in the alignment and communication of the right ventricle with the pulmonary trunk and the aorta with the left ventricle. Failure of this process results in the common and relatively minor membranous ventricular septal defect (VSD). Although VSDs are the most common congenital heart lesion, familial clustering has been described only in rare instances and single-gene disorders have yet to be identified. Additional genes implicated in septal defects are listed in Table 46-2.

Most of the wall between the ventricles comprises the myocardium, which becomes part of the pump chamber. Much of the surface is trabeculated, giving it a web-like appearance. The right ventricle is distinguished from the left by the coarser structure of the trabecule. The programmed cell death involved in this process is a likely factor in the appearance of holes through the wall, allowing blood to cross from left to right after birth. These muscular VSDs often close spontaneously as hypertrophy of the surrounding muscle obstructs the flow. Membranous VSDs are sometimes closed secondarily by valve tissue from the tricuspid valve. In genetic counseling terms, these resolving heart murmurs are important. Failure of equal and appropriate growth of the two ventricles may result from abnormal heart looping, abnormal inlet orifices, or obstruction of the outlet vessels. The most serious is failure of growth of the left ventricle, known as hypoplastic left heart (HLHS). Mutations in *HAND1* and *GJA1* have both been implicated in the development of HLHS (18). A more specific defect of development of the right ventricular muscular wall results in a distended saclike right ventricle with various names, including right ventricular dysplasia and Uhl anomaly.

TABLE 46-1

	Gene	Gene Function	Cardiac Anomalies	Other Reported Anomalies	Inheritance	
Laterality defects	ACVR2B	Member of transforming growth factor-beta (TGF-beta) superfamily of structurally related signaling proteins	Transposition of the great arteries, double-outlet right ventricle, ventricular inversion, aberrant systemic venous return	Polysplenia, asplenia, midline liver, malroation	AD	6
	CFC1	Member of the epidermal growth factor (EGF)- Cripto, Frl-1, and Cryptic (CFC) family. Plays key role in intercellular signaling pathways during vertebrate embryogenesis including left-right asymmetry in the heart.	Dextrocardia, right atrial isomerism, left atrial isomerism, transposition of the great arteries, double-outlet right ventricle, aberrant systemic venous return	Holoprosencephaly, agenesis of the corpus callosum, microcephaly, malrotation, polysplenia, asplenia, neural tube defects, pulmonary isomerism	AD, reduced penetrance	6 6
	CRELD1	Member of a subfamily of epidermal growth factor-related protein.	Atrioventricular septal defect, dextrocardia, double-outlet right ventricle	None	AD	6 6
	LEFTY2	Member of the TGF-beta family of proteins. Plays a role in left-right asymmetry determination of organ systems during development.	Hypoplastic left heart, atrioventricular septal defect, dextrocardia, aberrant systemic venous return	Malrotation, midline liver, polysplenia		6
	NODAL	Member of TGF-b superfamily. Plays a key role in specification and patterning in mammalian embryogenesis	DORV, transposition of the great arteries, total anomalous pulmonary venous return, partial anomalous pulmonary venous return, ASD, VSD, aortic coarctation, PDA	Holoprosencephaly, malrotation, asplenia, hydronephrosis	AR	2 6
	THRAP2	Putative member of the TRAP family involved in early embryonic patterning expressed in brain, heart, skeletal muscle, kidney, placenta, and peripheral blood leukocytes	Transposition of the great arteries, VSD, aortic coarctation	Mental retardation, microcephaly	AD, reduced penetrance	6 6
	ZIC3	Member of the ZIC family of C2H2-type zinc finger proteins. Functions as a transcription factor in early stages of left-right asymmetry.	Transposition of the great arteries, DORV, right atrial isomerism, total anomalous pulmonary venous return, PDA, aberrant systemic venous return	Asplenia, biliary atresia, vertebral anomalies, anal anomalies, malrotation,	X-linked	3 3 3



**TABLE 46-2 Nonsyndromic CHD**

Heart Defect	Gene	Gene Function	OMIM
Atrioventricular canal	CRELD1	Member of a subfamily of epidermal growth factor-related protein.	606217
	GDF1	Member of the bone morphogenetic protein (BMP) family and the TGF-beta superfamily. The members of this family are regulators of cell growth and differentiation in both embryonic and adult tissues. Studies suggest that this protein is involved in the establishment of left-right asymmetry in early embryogenesis.	600309
	GJA1	Member of the connexin gene family. Major protein of gap junctions in the heart that are thought to have a crucial role in the synchronized contraction of the heart and in embryonic development.	121014
Hypoplastic left heart	GJA1	Member of the connexin gene family. Major protein of gap junctions in the heart that are thought to have a crucial role in the synchronized contraction of the heart and in embryonic development.	121014
	HAND1	Part of a family of basic helix-loop-helix transcription factors. One of two HAND proteins asymmetrically expressed in the developing ventricular chambers. Working in a complementary fashion, they function in the formation of the right ventricle and aortic arch arteries.	241550
	HAND1	Part of a family of basic helix-loop-helix transcription factors. One of two HAND proteins asymmetrically expressed in the developing ventricular chambers. Working in a complementary fashion, they function in the formation of the right ventricle and aortic arch arteries.	241550
Septal defects	NKX2.5	Functions in early determination of the heart field and may play a role in formation of the cardiac conduction system	602406
	CITED2	Inhibits transactivation of HIF1A-induced genes by competing with binding of HIF1a to p300-CH1	108900
	GATA4	Family of zinc-finger transcription factors. Regulates genes involved in embryogenesis and in myocardial differentiation and function.	600584
	HAND1	Part of a family of basic helix-loop-helix transcription factors. One of two HAND proteins asymmetrically expressed in the developing ventricular chambers. Working in a complementary fashion, they function in the formation of the right ventricle and aortic arch arteries.	602937
	HAND2	Part of a family of basic helix-loop-helix transcription factors. One of two HAND proteins asymmetrically expressed in the developing ventricular chambers. Working in a complementary fashion, they function in the formation of the right ventricle and aortic arch arteries.	607941
	TBX20	Interacts physically, functionally, and genetically with other cardiac transcription factors, including NKX2-5, GATA4, and TBX5	241550
	ACTC1	Actins are highly conserved proteins that are involved in various types of cell motility and is major constituent of the contractile apparatus of the heart.	602406
	MYH6 THRAP2	Encodes the alpha heavy chain subunit of cardiac myosin. Putative member of the TRAP family involved in early embryonic patterning expressed in brain, heart, skeletal muscle, kidney, placenta, and peripheral blood leukocytes	602407
Double-outlet right ventricle	CFC1	Member of the epidermal growth factor (EGF)- Cripto, Frl-1, and Cryptic (CFC) family. Plays key role in inter-cellular signaling pathways during vertebrate embryogenesis including left-right patterning in the heart.	611363
	GDF1	Member of the bone morphogenetic protein (BMP) family and the TGF-beta superfamily. The members of this family are regulators of cell growth and differentiation in both embryonic and adult tissues. Studies suggest that this protein is involved in the establishment of left-right asymmetry in early embryogenesis.	612794
	NKX2.5	Functions in early determination of the heart field and may play a role in formation of the cardiac conduction system	160710

TABLE 46-2 Nonsyndromic CHD—Cont'd			
Heart Defect	Gene	Gene Function	OMIM
Tetralogy of Fallot	GATA4	Member of the GATA family of zinc-finger transcription factors. Thought to regulate genes involved in embryogenesis and in myocardial differentiation and function.	187500
	JAG1	Ligand for the receptor notch 1	600576
	NKX2.5	Functions in early determination of the heart field and may play a role in formation of the cardiac conduction system	187500
	ZFPM2/FOG2	Member of the FOG family of transcription factors. Modulates the activity of GATA family proteins.	601920
	GDF1	Member of the bone morphogenetic protein (BMP) family and the TGF-beta superfamily. The members of this family are regulators of cell growth and differentiation in both embryonic and adult tissues. Studies suggest that this protein is involved in the establishment of left-right asymmetry in early embryogenesis.	600584
Bicuspid aortic valve	NOTCH1	Member of the Notch family of proteins which function as a receptor for membrane bound ligands, and may play multiple roles during development	187500
Supravalvular aortic stenosis	ELN	Protein product is one of the two components of elastic fibers	603693
Transposition of the great arteries	CFC1	Member of the epidermal growth factor (EGF)- Cripto, Frl-1, and Cryptic (CFC) family. Plays key role in intercellular signaling pathways during vertebrate embryogenesis including left–right patterning in the heart.	109730
	GDF1	Member of the bone morphogenetic protein (BMP) family and the TGF-beta superfamily. The members of this family are regulators of cell growth and differentiation in both embryonic and adult tissues. Studies suggest that this protein is involved in the establishment of left–right asymmetry in early embryogenesis.	130160
	THRAP2	Putative member of the TRAP family involved in early embryonic patterning expressed in brain, heart, skeletal muscle, kidney, placenta, and peripheral blood leukocytes	185500
Aortic coarctation	THRAP2	Putative member of the TRAP family involved in early embryonic patterning expressed in brain, heart, skeletal muscle, kidney, placenta, and peripheral blood leukocytes	217095

Partitioning of the outflow tract begins during the fifth week. There are two pairs of ridges (bulbar and truncal) that fuse to form a spiral septum that separates the aortic and pulmonary outflow tracts. These ridges are derived from cardiac neural crest cells. Abnormal contributions of cells from the cardiac neural crest have been implicated in the 22q11 phenotype and ablation of the cardiac neural crest cells in chick embryos results in outflow tract and right ventricular hypoplasia (19). Complete failure of septation results in a common arterial trunk or, as it is more commonly known, persistent truncus arteriosus (Figure 46-4). This spiraling separation ultimately results

in placing the aorta on the left and the pulmonary artery on the right (Figure 46-5A–H). If this process is incomplete or distorted, the aorta overrides the upper margin of the ventricular septum; the membranous septum remains incomplete, and the outlet of the right ventricle is narrowed. These malformations combine to cause hypertrophy of the right ventricle. The combination of an aorta overriding a VSD with right ventricular outflow tract obstruction and right ventricular hypertrophy constitutes the tetralogy of Fallot (Figure 46-6). If the process is further distorted, the aorta lies predominantly over the right ventricle and the term double-outlet right

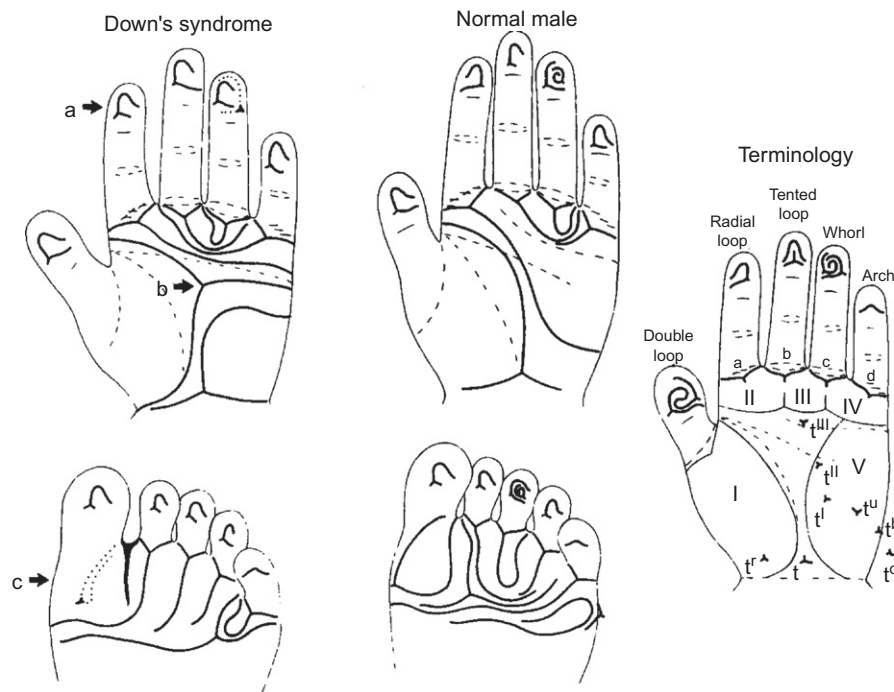


FIGURE 46-2



FIGURE 46-3



FIGURE 46-4



FIGURE 46-5



FIGURE 46-6

ventricle is applied (Figure 46-7). This anomaly may also form part of the spectrum that results in an abnormal plane of the outflow septum such that the anterior vessel becomes connected to the left ventricle and the morphologic aorta to the right ventricle. Transposition of the great arteries (TGA) is thus distinct from most outflow defects in that it results from a malformation of the septum rather than a neural crest migration anomaly. This distinction is reflected in the syndrome associations and recurrence risks (Figure 46-8).

Malformations involving the major vessels connected to the heart are generally included with heart defects in discussions of cause and recurrence risk. Systemic venous drainage is rarely of major genetic importance, although absence of the last segment of the inferior vena cava and its replacement by an azygous connection to the superior vena cava is a valuable diagnostic sign of lack of a morphologic right atrium in a left isomerism sequence. Abnormality of pulmonary venous drainage is of much greater significance. Four pulmonary veins rendezvous with an outgrowth from the back of the left atrium. The coalescence incorporates into the posterior wall, producing four separate orifices. If one or more orifices are displaced, the term anomalous pulmonary venous drainage is applied, which may be partial, involving up to three



FIGURE 46-7



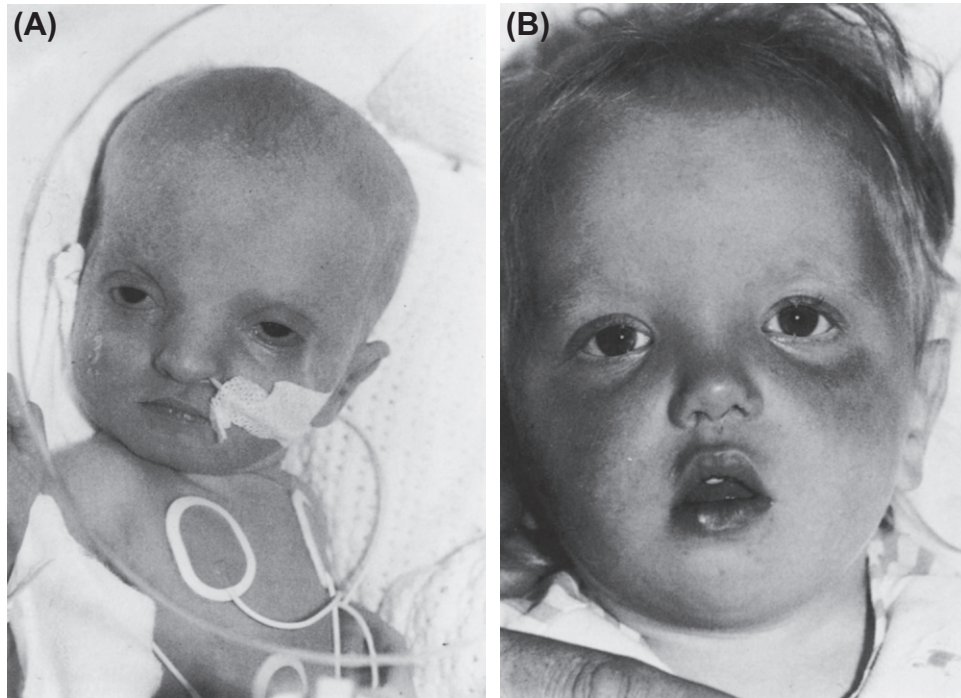


FIGURE 46-8

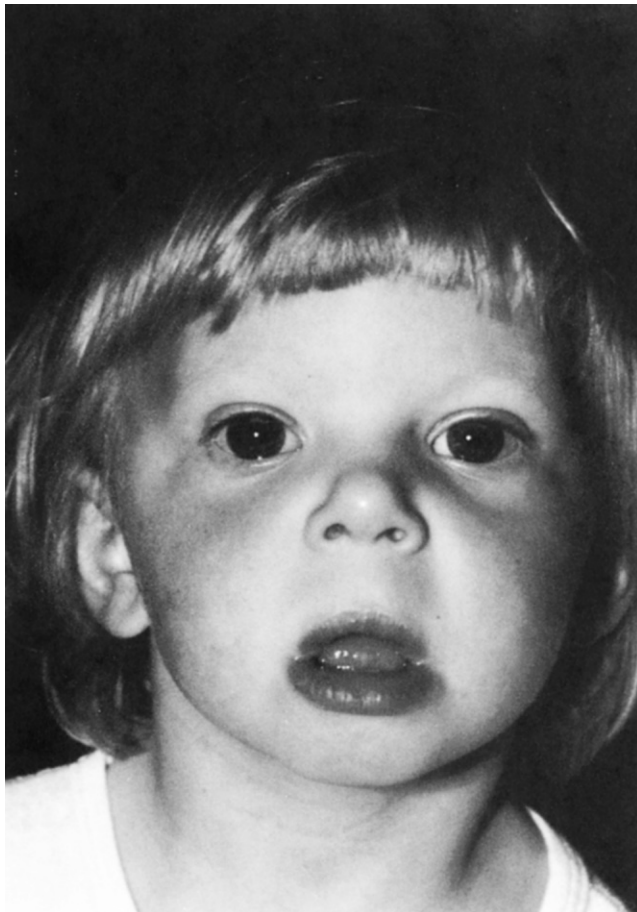


FIGURE 46-9

vessels, or complete, when it is known as total anomalous pulmonary venous return (Figure 46-9).

The ductus arteriosus (arterial duct) connects the right ventricular outflow to the descending aorta (Figure 46-10C only). It is usually called the sixth aortic arch, but it is morphologically distinct and has evolved to have an oxygen-sensitive lining, which allows it to constrict and occlude after birth, allowing the pulmonary circulation to open. It often stays open after birth, particularly in preterm infants, in whom the duct is not mature. The persistent ductus arteriosus (PDA) is, therefore, one of the most common anomalies of the cardiovascular system in postnatal life, but an essential physiologic structure *in utero* (Figure 46-11).

This brief overview has focused on anomalies relevant to a group of major defects that are serious after birth but compatible with intrauterine survival. The dependence of the embryo and fetus on a functional circulation means that any systemic defect that compromises more generalized intrauterine structure, function or both causes loss at an early stage.

### 46.3 SPECIFIC SYNDROMES WITH CONGENITAL HEART DEFECT

It is not surprising given the complexity of cardiovascular embryology and the large number of genes involved in normal heart development that the list of syndromes associated with congenital heart defects is not short. Appendix 1-11 contains a list of well-researched syndromes as grouped by their cardiac malformation. This appendix draws heavily on the previous edition and is

recommended as a reference source for the evaluation of syndromic causes of congenital heart defects. Common syndromes are discussed in more detail in the following sections.

## 46.4 CHROMOSOMAL DISORDERS

Although the application of aCGH has quickly become the diagnostic screening test of choice, chromosome analysis continues to be clinically useful particularly as a prenatal screening test and in the patient with findings suggestive of an aneuploidy syndrome. We have highlighted in the next section those aneuploidy syndromes commonly encountered by the medical geneticist.

### 46.4.1 Trisomy 21 (Down Syndrome)

Down syndrome is one of the most common genetic syndromes, with an estimated birth prevalence of 1 per 700 live births (20). Clinical features can include midface hypoplasia, epicanthal folds, up-slanting palpebral fissures, Brushfield spots on the irides, single palmar crease, brachydactyly, and increased sandal gap (Figure 46-2). Approximately 40% of patients with Down syndrome will have a congenital heart defect and of these, almost half will have the otherwise rare atrioventricular septal defect (AVSD) (21). Tetralogy of Fallot occurs in 10–15%. To date, no single gene or set of genes on chromosome 21 has been shown to cause heart defects in Down syndrome. Recently, mutations in *CRELD1* have been identified in patients with Down syndrome and AVSD, implicating *CRELD1* mutations, together with Trisomy 21 and possible environmental factors, as a risk susceptibility gene (22). For additional information on Trisomy 21, see Chapter 43.

### 46.4.2 Trisomy 18 (Edwards Syndrome)

Trisomy 18 is the second most common autosomal aneuploidy after Down syndrome. This is an important

bedside diagnosis to confirm due to the very poor prognosis and markedly diminished life expectancy that may influence medical management. Interestingly, recent studies have suggested that aggressive management is becoming more common, despite this well-recognized poor prognosis. The craniofacial manifestations of an infant with Trisomy 18 may be subtle, but typically include: prominent occiput, low-set ears, micrognathia, small palpebral fissures, short sternum and typical finger clenching (Figure 46-7). Heart defects are a recognized association and are a cause of early demise. Heart defects are often more complex in boys than in girls with Trisomy 18 and associated with increased mortality (23). For additional information on Trisomy 18 see Chapter 43.

### 46.4.3 Trisomy 13 (Patau Syndrome)

The birth incidence of Trisomy 13 is about one in 7000. Survival beyond the first year of life is rare (24). The characteristic clinical features are postaxial polydactyly, cleft lip and palate (often bilateral and severe), and hypotelorism associated with holoprosencephaly (Figure 46-9–46-11). There is a high incidence of cardiac defects, in particular atrial septal defects and VSDs. Disturbance of cardiac position, including dextrocardia, is common, suggesting a role for a gene or genes on chromosome 13 in laterality development (25). Appropriate identification of Trisomy 13 is important for recurrence risk counseling as well as directing clinical management, as aggressive medical interventions are not recommended given the high mortality rate and poor prognosis in long-term survivors. For additional information on Trisomy 13, see Chapter 43.

### 46.4.4 Turner Syndrome (Ulrich Turner Syndrome)

Turner syndrome has a birth prevalence of about 1 in 1850 live female births. In addition, a large proportion of affected fetuses are lost as early miscarriages, many of which can be attributed to the presence of a severe congenital heart defects, particularly HLHS (12). For live births, about 10% of Turner syndrome females have a clinically evident heart defect, with an additional 10% having an anomaly on echocardiogram, such as a bicuspid aortic valve. The most common abnormality is bicuspid aortic valve (16%) and coarctation of the aorta (11%); however, structural defects such as PAPVR and atrial and VSDs are also seen (26). And while the congenital heart defect may be detected and repaired in childhood, all patients with Turner syndrome require life-long cardiac follow-up because of the potential for progressive dilatation of the ascending aorta and an increased risk of thoracic aortic dissection (27). Additional findings are variable and include short stature, gonadal dysgenesis, and a variable dysmorphic appearance with neck webbing (pterygium



FIGURE 46-10

colli), down-slanting palpebral fissures and low-set ears (Figure 46-1–46-2). Patients with ring X chromosomes are at risk for mental retardation although classic Turner patients can struggle with learning disabilities. For additional information, see Chapter 44.

### 46.5 MICRODELETIONS/ MICRODUPLICATION SYNDROMES

Advances in molecular cytogenetic technology have significantly increased the ability to detect smaller and smaller chromosomal imbalances. This includes both

FISH and, more recently, aCGH, which has quickly become the most important diagnostic screening tool in patients with congenital heart disease who do not have a genetic syndrome readily identified by clinical examination (28). Although the identification of copy number variants of unknown clinical significance has been a significant limitation of CGH, this technology has been instrumental in defining the loss or gain of chromosomal material with such detail as to permit knowing which gene/s are involved. This has led to further identification of genes that may have critical roles in both syndromic and nonsyndromic congenital heart defects.

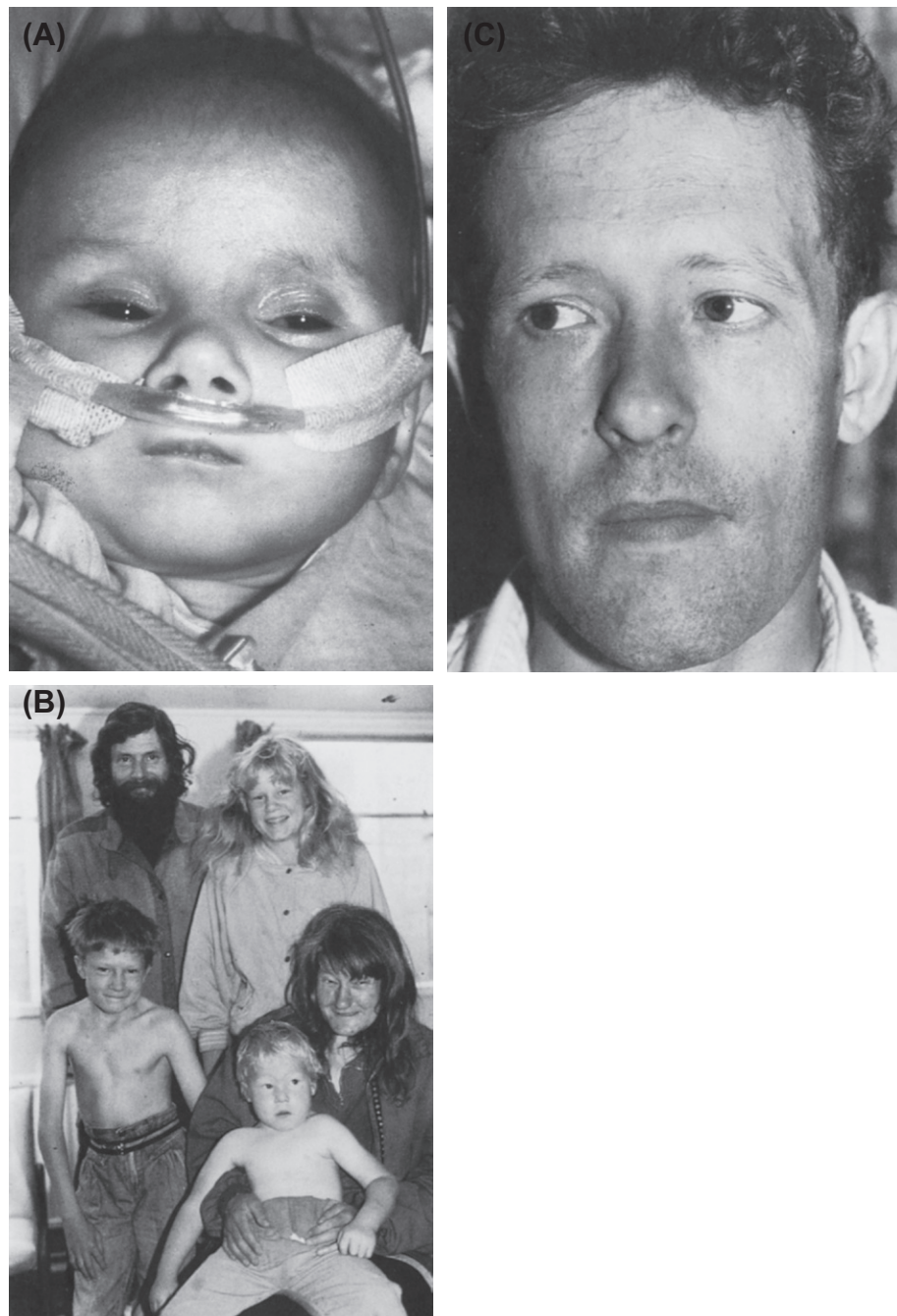


FIGURE 46-11



### 46.5.1 22q11 Deletion Syndrome

22q11 deletion syndrome is the most common human chromosomal deletion syndrome occurring in approximately one per 4000–6000 live births (29). Clinical features include learning impairments, palate anomalies (including velopharyngeal insufficiency), characteristic facial appearance (Figure 46-12 but 52-11 from previous version), neonatal hypocalcemia, thymic hypoplasia, and immune deficiencies. Approximately 15% of cases are familial segregating as an autosomal dominant trait with marked variability. Although the deletion is visible by routine G-banded cytogenetic testing in some, about two-thirds of cases require FISH testing to confirm the diagnosis. Therefore, a clinical suspicion is required. The most commonly reported heart defects include tetralogy of Fallot, tetralogy of Fallot with pulmonary atresia, VSD, interrupted aortic arch (type B), and truncus arteriosus (30). Individuals with a cardiac defect and an anomaly of the aortic arch are more likely to harbor a 22q11 deletion than those with other heart defects such as double-outlet right ventricle or TGA. 22q11 deletions are rarely identified in such non-conotruncal defects (7). Most individuals with 22q11 deletion syndrome harbor either a 3- or 1.5-Mb deletion in 22q11.22. This region includes *TBX1*, which has emerged as a major genetic determinant of the 22q11 deletion phenotype (31). Point mutations in *TBX1* occur in patients with findings suggestive of the 22q11 deletion syndrome phenotype but with normal FISH studies. Appropriate identification of the cardiac patient with a 22q11 deletion is important to facilitate identification of associated anomalies, renal defects, and possible calcium abnormalities. In

addition, a higher operative mortality may exist in those individuals with a 22q11 deletion (32).

### 46.5.2 Williams Syndrome

Williams syndrome, or Williams–Beuren syndrome, is characterized by learning disability with a unique personality profile: a “cocktail party” personality with a readiness to converse in a friendly outgoing fashion but with little content. The dysmorphic features are easily recognized and include: malar flattening, periorbital fullness, heavy sagging cheeks, short nose, poorly developed Cupid’s bow on the upper lip, and everted lower lip (Figure 46-13, Figure 52-9 from previous edition). The cardinal cardiovascular malformation in this syndrome is supravalvular aortic stenosis (SVAS), which affects about one-third of cases and tends to be progressive with age. Less well-recognized and more difficult to detect are peripheral pulmonary artery stenoses, which produce murmurs over the lung fields. The striking association with SVAS, which had been described as an isolated autosomal dominant trait, prompted the speculation that Williams syndrome would turn out to be a deletion involving the gene responsible for SVAS (33). The identification of a balanced translocation associated with SVAS provided an additional clue. Curran and associates first showed that dominant SVAS mapped to chromosome 7 at a translocation breakpoint and, subsequently, that most Williams syndrome cases could be shown to have a deletion of this region of chromosome 7 (34). The gene encoding tropoelastin (ELN) was shown to be causative in isolated SVAS by the demonstration of loss-of-function mutations (35,36).

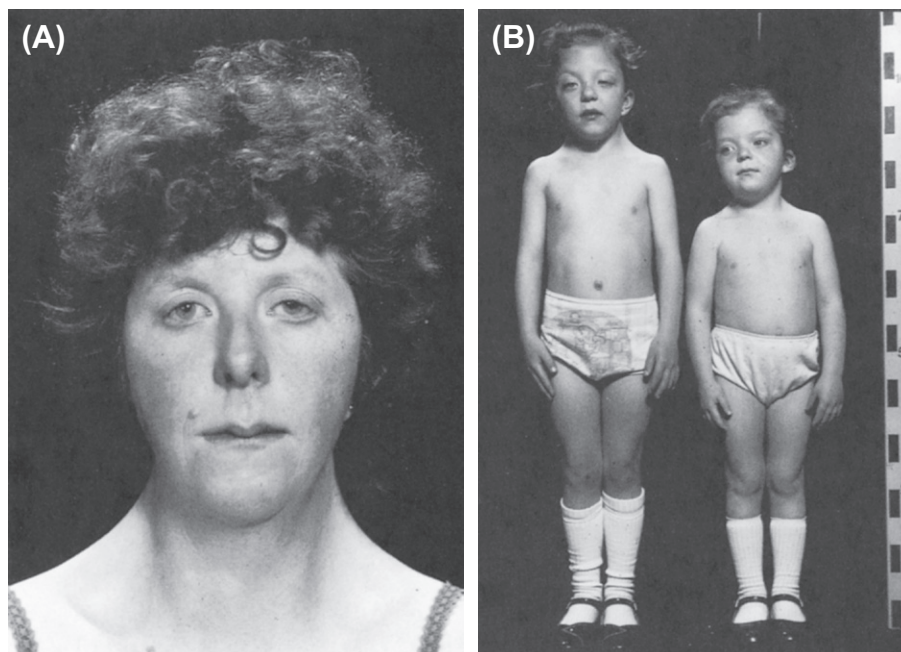


FIGURE 46-12



### 46.5.3 Alagille Syndrome

Alagille syndrome is characterized by hypoplasia of the intrahepatic bile ducts, leading to a variable degree of cholestasis, which may present with neonatal jaundice or become apparent later in life (37). Up to 90% of affected probands have single or multiple areas of peripheral pulmonary artery stenosis. In about one-third, a variety of other intra- and extracardiac malformations are seen. The face is mildly dysmorphic, with a prominent forehead, deep-set eyes, and thin nose (Figure 46-14, 46-10 from previous edition). Other typical clinical features include skeletal defects (particularly butterfly vertebrae) and anterior chamber eye defects, confusingly called posterior embryotoxon. Externally, the striking feature is a pale ring around the iris known as arcus juvenilis (Figure 46-15) (38). A microdeletion of 20p12, which includes JAG1, is demonstrable in 7% of cases. JAG1 is part of the Notch signaling pathway and point mutations are responsible for the phenotype in approximately 89% of cases; mutations in NOTCH2 account for approximately 1% of cases (39).

## 46.6 SINGLE-GENE DISORDERS

Chromosome analysis, FISH, and CGH are useful diagnostic tools in the evaluation of syndromic causes of congenital heart defects. However, these technologies are not able to detect abnormalities at the single-gene level. So, if they are used without recognition of the single-gene causes of many syndromic congenital heart defects, important diagnoses will be missed. A few of these syndromes are highlighted in the following section.

### 46.6.1 Noonan Syndrome

One of the best-recognized syndromes in the pediatric cardiology clinic is Noonan syndrome. Earliest

descriptions have been traced back over a century, but the syndrome derives its name from the report by Noonan and Ehmke in 1963 of nine children with valvar pulmonary stenosis, short stature, mild learning difficulties, and dysmorphic appearance. The mother and two daughters in Figure 46-16 (Figure 46-12 from previous edition) illustrate the variable phenotype. Collectively they have the full picture, yet none has the complete pattern.

In infancy, the striking features are the hypertelorism, down-slanting eyes with low-set, posteriorly rotated ears, and low posterior hairline. Later, the face becomes more triangular and rather coarse in appearance. In adulthood, the eyes are less prominent and the nose has a thinner bridge and pinched root with wide base; the neck becomes longer, accentuating the prominent trapezius



FIGURE 46-14

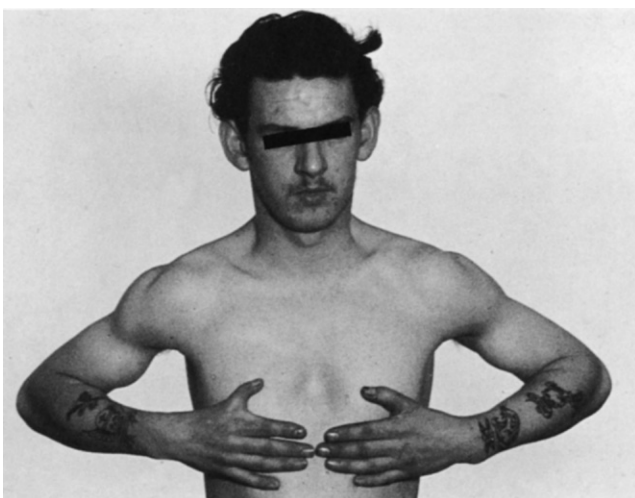


FIGURE 46-13

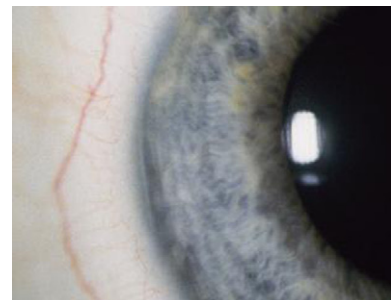


FIGURE 46-15 Posterior embryotoxon is an anteriorly displaced Schwalbe's line (the most peripheral edge of Descemet's membrane).

and/or neck webbing, which caused original confusion with Turner syndrome in the literature. Other important features are the feeding difficulties of infancy, a characteristic anterior chest deformity, wide spaced nipples, cryptorchidism, a predisposition to lymphatic dysplasia and bleeding diathesis, and a high frequency of nevi and café-au-lait macules.

Two-third of children with Noonan syndrome have a heart defect, with valvar pulmonary stenosis in 50%. Often the valve is dysplastic, making balloon dilation more difficult. Among a variety of other defects reported, the most frequent are ASD, asymmetrical septal hypertrophy, and PDA; VSD occurs in about 5% (40,41). The electrocardiogram typically shows left axis deviation with a wide QRS complex, giant Q waves, and a negative pattern in the left precordial leads (42). There is a phenotypic overlap with a number of syndromes, particularly LEOPARD syndrome (an allelic disorder with mutations in *PTPN11*), neurofibromatosis type 1, Costello syndrome, and cardiofaciocutaneous syndrome. Noonan syndrome is genetically heterogeneous disorder due to dysregulation of the Ras/MAPK (mitogen-activated protein kinase) signal transduction pathway. The Ras/MAPK pathway is implicated in growth factor-mediated cell proliferation, differentiation, and cell death. Over nine genes have been identified in association with altered Ras/MAPK signaling with resulting overlapping phenotypes. Mutations identified in *PTPN11*, *SOS1*, *RAF1*, and *KRAS* are responsible for the Noonan syndrome phenotype. Some genotype/phenotype correlations exist; for example, hypertrophic cardiomyopathy is a complication in about 20% of patients with Noonan syndrome, and more frequent in patients with *RAF1* mutations. However, there is insufficient evidence to correlate genotype with occurrence of a specific type of congenital heart defect (43).

Life-long cardiac follow-up is important for adults with Noonan syndrome as left-sided obstructive

lesions may develop in adulthood. Pulmonary valve insufficiency and right ventricular dysfunction are also potential problems after early pulmonary valve surgery (44).

## 46.7 HOLT-ORAM SYNDROME

Holt-Oram syndrome is the best-recognized of the heart-hand syndromes and is caused by mutations in the gene encoding T-box transcription factor *TBX5*. The characteristic anomalies are underdevelopment of the shoulder girdle with triphalangeal thumb and ASD (45) (Figure 46-17, Figure 46-13 from previous edition). The limb defects can vary from phocomelia to minor anomalies of joint movement at the thumb, elbow, or shoulder. About half of gene carriers have a secundum ASD, with occasional reports including VSD, AVSD, and truncus arteriosus. Patients may also present with mild to severe cardiac arrhythmias, commonly atrioventricular block (46).

## 46.8 CHARGE SYNDROME

CHARGE is a mnemonic that stands for coloboma, heart defects, choanal atresia, retarded growth and development, genital abnormalities, and ear anomalies. Formerly an association, the specific developmental anomalies of the optic vesicle, otic capsule, midline CNS structures, and upper pharynx have been attributed, in the majority of cases, to mutations in *CHD7*. Updated diagnostic criteria for CHARGE have been proposed and emphasize the importance of additional findings, for example, anomalies of the semi-circular canals, not included in the mnemonic (47). Congenital heart defects are seen in approximately 75% of children diagnosed with CHARGE syndrome, with tetralogy of Fallot or Fallot spectrum being the most common (48,49) (Figure 46-18).



FIGURE 46-16



FIGURE 46-17

### 46.8.1 Heart Malformation and Metabolic Disorders

The inborn errors of metabolism, such as the mucopolysaccharidoses (discussed in Chapter 102), develop cardiac involvement as a postnatal event, particularly involving valve dysfunction due to deposition of storage material and, most dramatically in type 2 glycogenosis or Pompe disease, with accumulation of material in the cardiac muscle. Some metabolic defects are not corrected by the maternal influence and can produce congenital malformations that may include the heart. Two are worthy of mention, since simple diagnostic biochemical tests exist, which facilitates prenatal diagnosis.

### 46.9 ZELLWEGER SYNDROME

Zellweger (cerebrohepatorenal) syndrome was originally described as a lethal multiple malformation syndrome of infancy characterized by profound hypotonia, a marked paucity of facial movement, open anterior fontanelle, hypoplastic supraorbital ridges, and a broad nasal bridge (Figure 46-19, Figure 46-15 from previous edition). Hepatomegaly, renal cortical cysts, deafness, cataracts, and calcific stippling of the patellae complete the typical phenotype. The discovery of a peroxisomal defect in a child with this syndrome emphasized that this pattern represents one end of a clinical spectrum that may result from at least 10 genetic defects in peroxisomal

assembly or the function of the single matrix enzymes, including PEX1, on chromosome 7q (50). Peroxisomes are single-membrane-bound organelles present in virtually all eukaryotic cells. They are involved in numerous metabolic processes, both anabolic and catabolic, including  $\beta$ -oxidation of very-long-chain fatty acids, the basis of the standard diagnostic test (51). Severe cerebral dysfunction results from premature arrest of migrating neuroblasts. Thymic hypoplasia and outflow tract cardiac malformations occur (52).

### 46.10 SMITH-LEMLI-OPITZ SYNDROME

Smith-Lemli-Opitz (SLO) syndrome is characterized by severe learning disability, failure to thrive, cleft palate, hypospadias in males, and 2/3 syndactyly. The facies are dysmorphic, with bi-temporal narrowing and anteverted nares (Figure 46-20, Figure 46-16 from previous edition). In one series, 16% of patients with SLO were found to have an AVSD, 7% with TAPVR (53).

The discovery that affected individuals have hypocholesterolemia and markedly elevated levels of 7-dehydrocholesterol due to a defect in the 7-dehydrocholesterol reductase gene showed that another multiple malformation syndrome had a metabolic basis (54). By 2003, 91 different pathologic mutations had been reported in the gene (55). How and why a defect in the cholesterol biosynthesis pathway should have such an adverse effect on, for example, the septation of the ventricular inlet

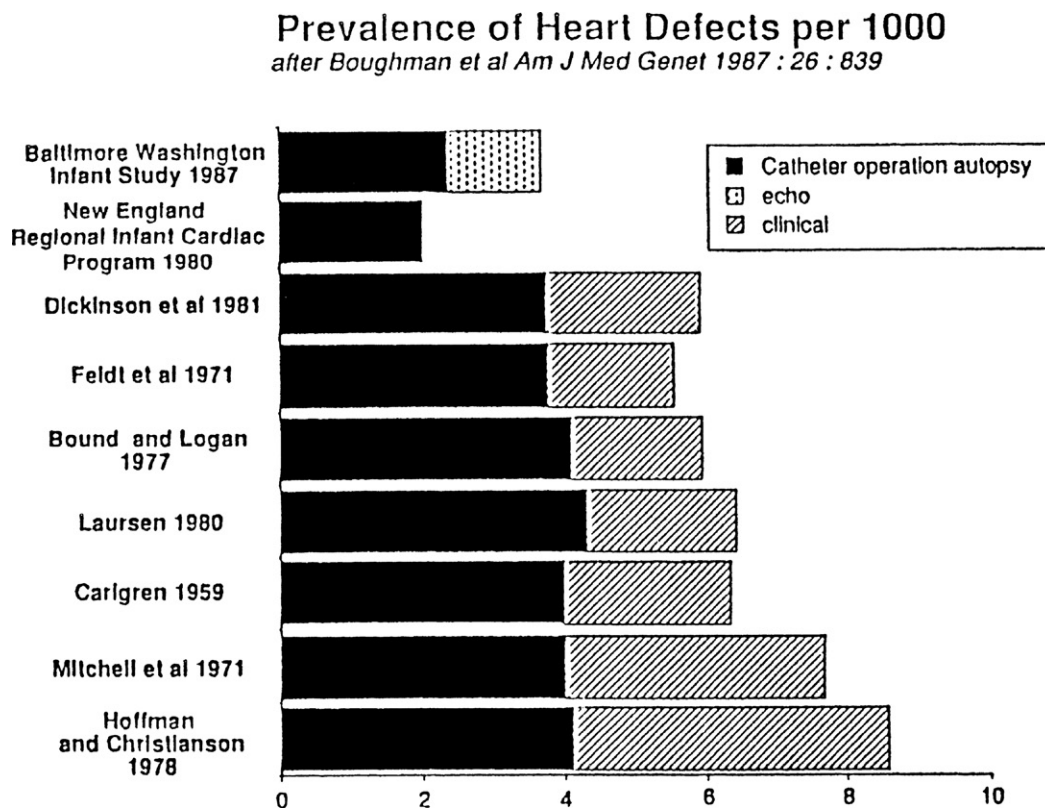


FIGURE 46-18



remains to be established, but from the practical perspective, the possibility of SLO syndrome must be considered in a dysmorphic infant with a heart defect in view of the recessive mode of inheritance and the availability of pre- and postnatal diagnostic tests.

### 46.10.1 Genes Responsible for Congenital Heart Malformations as Mongogenic Traits

Although congenital heart defects rarely occur in the context of large families, there are some case reports of heart malformations segregating as a monogenic trait. Coarctation of the aorta, interrupted aortic arch, and hypoplastic left heart have been identified in multigenerations with clinical variability and reduced penetrance (56,57). Overall, only a small number of single-gene causes of isolated heart defects have been identified, suggesting that congenital heart defects in general are extremely heterogeneous and genetically complex (Table 46-2). The investigation of genomic disorders due to the deletion of one or more genes and traditional mapping have identified the location, and in some cases the identity, of genes that, when defective, can produce isolated heart defects. The classic examples are *ELN* as

a cause of SVAS and *JAG1* leading to peripheral pulmonary artery stenosis. In addition, mapping studies and candidate gene analysis led to the discovery of *ZIC3* as a cause of X-linked laterality syndrome (Table 46-1), and the same approach yielded *TFAP2B* as the cause of autosomal dominant PDA (58). Mutations in *GATA4* and *CRELD1* have been identified in patients with isolated AVSD (59,60).

### 46.10.2 Environmental Causes and the Teratogen Syndromes

The search for specific environmental associations of congenital heart defects has scored notable successes. This began with congenital rubella, once a major cause of congenital heart defects, has now receded in most developed countries in which maternal vaccination is widespread. Table 46-3 lists several teratogens that are well-recognized as causes of congenital heart disease. In general terms, the evidence for a major contribution from specific environmental exposures is not strong (61). In practical terms, maternal diabetes and alcohol abuse remain the most important external threats to normal heart development, but it is important to take a history of drug ingestion before offering recurrence risks. This is important when the consultation is with an affected individual, since recognition of a teratogenic cause enables the counselor to give a very small recurrence risk. This assumes, of course, that it is possible to remove the cause in the next pregnancy.

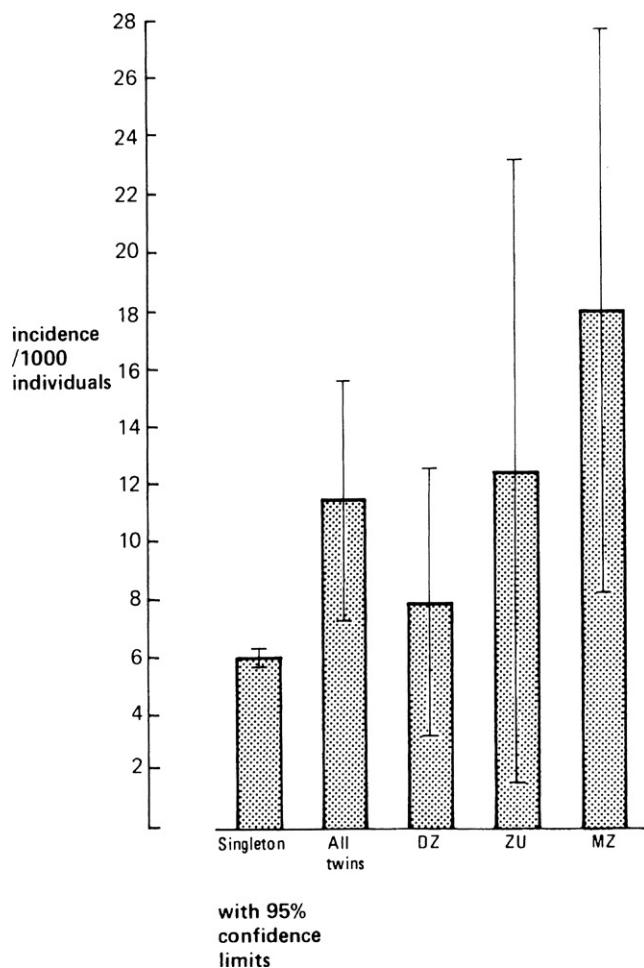


FIGURE 46-19

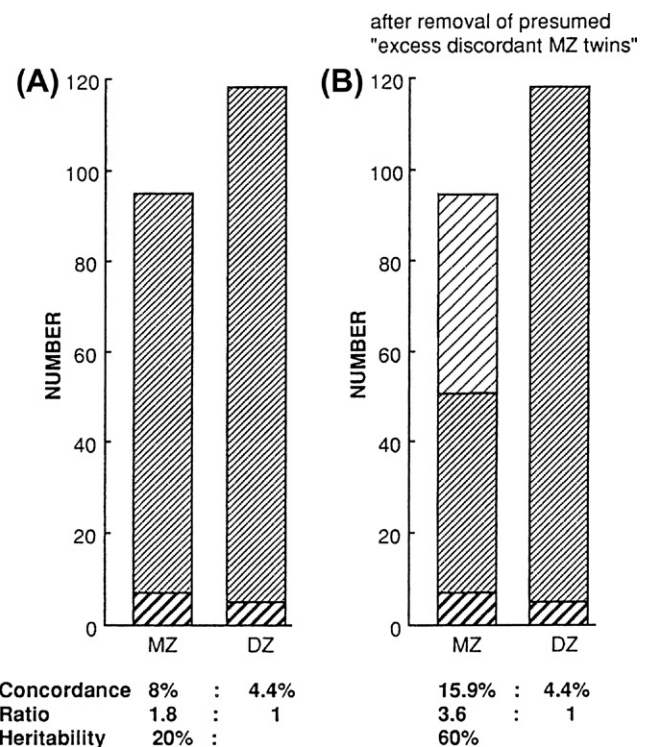


FIGURE 46-20



### 46.11 MATERNAL DIABETES

Fetuses born to mothers with diabetes mellitus are at a five-fold increased risk of birth defects, of which congenital heart defects are the most common. Defects in laterality, looping, and conotruncal septation predominate, suggesting that exposure in utero may affect cardiogenesis as early as 7-week gestation (62). The risk of fetal congenital abnormalities in pregnant women with diabetes mellitus is related to the level of glycemic control in early pregnancy; thus, strict metabolic targets as close to normal glycosylated hemoglobin (HbA1c) (ie, 4.0%-6.0%) are recommended (63). The mechanism of this

TABLE 46-3 Some Common Teratogens		
Teratogenic Influence	Risk of Heart Defect (%)	Most Common Type
Maternal rubella	35	PDA, peripheral pulmonary artery stenosis, septal defects
Maternal diabetes	3–5	VSD, coarctation, TGA
Maternal phenylketonuria	25–50	Tetralogy of Fallot
Systemic lupus erythematosus	20–40	Complete heart block
Maternal alcohol abuse	25–30	Septal defects
Hydantoin	2–3	Pulmonary and aortic stenosis, coarctation of aorta, PDA
Trimethadione	15–30	Tetralogy of Fallot, TGA, hypoplastic left heart
Thalidomide	<5	Tetralogy of Fallot, septal defects, truncus arteriosus
Lithium		Ebstein anomaly, tricuspid atresia, ASD
Retinoic acid	10–20	Conotruncal heart defects
Cocaine	5	Excess situs disturbance

relationship is poorly understood, however emerging evidence suggests that mutations in *RasGRP3* contribute to the developmental defects in mouse embryos born to diabetic mothers by affecting endothelial cell migration (64).

### 46.12 MATERNAL CIGARETTE SMOKING

There is growing evidence to suggest an increased risk of congenital heart defects due to fetuses exposed to toxins from tobacco. Maternal smoking during pregnancy has been associated with septal and right-sided obstructive defects as well as intrauterine growth retardation (65). However, other studies show conflicting risks (66). In the United States, an estimated 28% of reproductive-aged women smoke cigarettes, and a significant portion of those women continue to smoke during pregnancy (67). Therefore, an investigation of maternal tobacco use before and during pregnancy may provide insight to the pathogenesis of certain congenital heart defects and counseling opportunity for future risk prevention.

### 46.13 MATERNAL DRUG INGESTION

#### 46.13.1 Alcohol

Alcohol is the non-therapeutic drug most frequently ingested by pregnant women. Alcohol induces heart defects in some exposed fetuses; VSD is most common, but ASD, tetralogy of Fallot, and other defects can also occur (68). Fetal alcohol syndrome produces a recognizable phenotype, but the facial characteristics may not be present in all affected children (Figure 46-21, Figure 46-17 from previous edition). The overall incidence of fetal alcohol syndrome in the general population may be 0.1 to 3.3 per 1000, with cardiovascular defects present in 28% of affected infants (68). TGA may be more common in infants exposed to alcohol (66). Heavy alcohol drinkers, defined as women consuming a

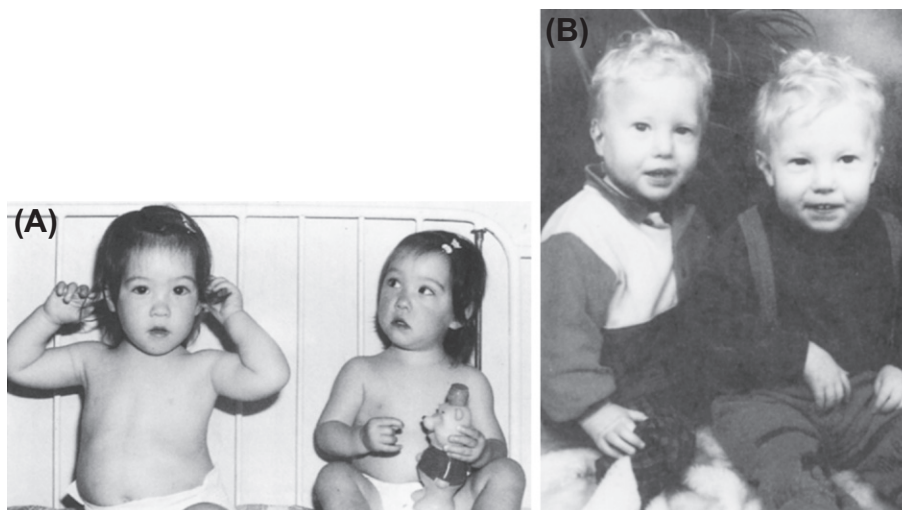


FIGURE 46-21

daily average equivalent of 45 mL (1.5 oz) or more absolute alcohol, had offspring with a higher incidence of major anomalies compared to more moderate drinkers. A “safe” level of alcohol intake during pregnancy has not been established and alcohol exposure prenatally remains an important cardiac teratogen.

### 46.13.2 Cocaine

Prenatal cocaine exposure affects the developing heart including an increased rate of arrhythmias and cardiac defects associated with heterotaxy. Fetal cocaine exposure in animal models suggests epigenetic alterations with long-term consequences in the offspring, even in the absence of gross malformations (69). More studies are needed to understand better both the short- and long-term consequences of prenatal cocaine exposure.

### 46.13.3 Lithium

Lithium, used in the treatment of depression, was first recognized as a teratogen because of the increased number of infants born with Ebstein anomaly, a relatively rare defect of the tricuspid valve. One study of infants exposed to lithium in utero found high rates of malformations with congenital heart defects accounting for the vast majority. Of these infants nearly half had Ebstein anomaly plus other cardiac malformations (70). However, more recent studies have failed to confirm this high risk.

### 46.13.4 Maternal Phenylketonuria

Successful treatment of phenylketonuria through newborn screening and dietary modification means homozygous females of normal intellect reach child-bearing age (71). A woman who has abandoned the dietary restrictions may enter pregnancy with high blood levels of phenylalanine, which are associated with microcephaly and mental retardation in around

90% of offspring. In addition, congenital heart defects are present in 12% pregnancies in untreated mothers, commonly tetralogy of Fallot, aortic coarctation, and HLHS (72).

## 46.14 FOLIC ACID SUPPLEMENTATION

Folic acid supplementation reduces by over one-half the number of neural tube defects in offspring. A less well-recognized effect is a probable reduction in conotruncal heart malformations. One study reported a 52% reduction in heart defects (73). Additional studies are needed to prove efficacy. The decision of the United States to supplement flour supplies will provide further evidence on this issue in coming years.

### 46.14.1 The Adult with Congenital Heart Defect

Advances in cardiac imaging, cardiac catheterization, electrophysiology, and improving surgical techniques have resulted in the exponential growth of the population of adult patients with congenital heart defects, both syndromic and isolated ones. As this population transitions from the care of the pediatric provider to the adult provider, it is important for members of the medical team to appreciate the specific needs unique to this population of patients. Long-term follow-up has identified patients with previously undiagnosed syndromes that are presenting later in life. For example, mouse models of *NKX2.5* mutations develop arrhythmias and abnormal contractility as adults (74). An accurate genetic diagnosis can provide anticipatory rather than reactive guidance that allows the medical team to be proactive in the care of the adult with congenital heart defects rather than reactive.

Studies on adult patients with more common genetic syndromes is emerging and personalization of their care to their unique medical needs, depending on specific diagnosis, is necessary to prevent significant morbidity and mortality (75). Table 46-4 is an excerpt

**TABLE 46-4** Recurrence Rates in Offspring of Males and Females with Heart Defects

Authors	Defect	% Offspring Affected		
		Male	Female	Ratio F:M
Williamson (532)	ASD	0	11.1	
Taussig et al. (476)	Tetralogy of Fallot	0	3.1	
Zetterqvist (552)	ASD, PDA, coarctation	1.6	3.1	1.94
Dennis and Warren (115)	Pulmonary valve stenosis, pulmonary	3.0	2.9	0.97
Czeizel et al. (109)	Mixed	1.4	5.1	3.64
Emanuel et al. (138)	AVSD	0	13.9	
Rose et al. (417)	Aortic stenosis, ASD,	7.3	13.0	1.78
Nora and Nora (351),	Mixed	2.4	5.5	2.29
				Romano-Zelekha et al. (414)
	2.7	3.3	3.1	

from a recent review exploring the history and management of the adult patient with a genetic syndrome diagnosis.

Pregnancy represents a major challenge for the woman with coexisting congenital heart defects. Discussions about the risks of pregnancy, including the impact of heart disease on fertility, recurrence risks and potential teratogenic effects from medications should be considered around reproductive age. For syndromes such as Turner and Marfan, guidelines exist to help direct clinical management of the pregnant patient (76,77). For those with other congenital heart defects, less information is available. Pregnancy related risks could include increased risks for thromboembolism, heart failure, arrhythmias and dissection depending on the specific heart lesion (78). Studies on pregnant patients with tetralogy of Fallot have reported higher rates of mortality, prematurity and intrauterine growth restriction in offspring (79).

#### 46.15 EMPIRICAL RISKS FOR SIBS OF CHILDREN WITH ISOLATED HEART DEFECTS

Table 46-5 (Table 46-3 from previous edition) reviews the major traditional studies of recurrence risk in sibs of children with heart defects. The general principle that sib risks fall into the range of 2–5%, so often used by genetic counselors, is apparent. Most of the studies did not distinguish between sibs born before and after the proband, which may have distorted figures, since families who have had a child with a heart defect may be less likely to have additional children. Nevertheless, the counseling figures shown are sufficient in practical terms, since the advances in fetal echocardiography and postnatal care of affected children have improved to the point where it is reasonable to counsel in terms of the risk of recurrence being small.

#### 46.16 EMPIRICAL RISKS FOR OFFSPRING

In early studies of recurrence risks in the offspring of adults born with major heart defects, the limited available literature suggested a recurrence risk in offspring of 3–5%, comparable to sibs and compatible with a multi-factorial model of inheritance. However, studies have shown a much higher risk of congenital heart defects in offspring of parents with various congenital heart defects, in the range of 10–16% (80,81,82).

Although these higher offspring risks are now widely quoted, the possibility of selection bias cannot be excluded. Additional confounded factors include studies evaluating recurrence risks of different heart defects with some studies identifying an overall risk of 4% (83). In all, multiple studies report varying results and Table 46-6 (Table 46-4 from previous edition) summarizes this literature.

##### 46.16.1 Future Developments

As technology continues to drive discoveries in clinical genetics, the ability to identify genetic changes and their associated risk for birth defects will become routine in the medical genetics clinic. Completion of the Human Genome Project and the increasing affordability of whole genome sequencing and its interpretation will drive our understanding of key developmental pathways to identify genetic determinants of congenital heart defects. The future will involve a large-scale collaborative effort to bring together the necessary clinical resources to permit evaluation of the contribution of these genes and ultimately drive the personalization of medicine. The coming decade will bring greater understanding of the genetic contribution to congenital heart disease creating better treatment options and better outcomes; prevention outside the prenatal clinics, will have to wait much longer.

**TABLE 46-5 The British Offspring Study: Affected and Total Offspring According to Proband Sex and Malformations**

	Proband No.	Offspring			
		Of Males		Of Females	
		Affected/Total	%	Affected/Total	%
TAPVD	38	0/14	—	1/20	5.0
TGA	97	0/10	—	0/3	—
Abnormal situs and other abnormal connections	90	1/14	7.1	2/13	15.4
AVSD	85	1/12	8.3	4/38	10.5
Tetralogy of Fallot	384	2/129	1.6	6/127	4.7
<b>Totals</b>		<b>Males</b>	<b>%</b>	<b>Females</b>	<b>%</b>
Heart defects		4/179	2.2	13/201	6.5 <sup>a</sup>
All major malformations		4/179	2.2	16/201	

Section IV Approaches to Specific Disorders

<sup>a</sup>M:F contrast: P = 0.01.

**TABLE 46-6 Common management issues for adults with genetics syndromes and cardiovascular. Abnormalities: Genetic**  
**primary care providers<sup>a</sup>**

Syndrome	Heart Defect	Complications (Postoperative Unless Specified)	Management
Chromosome Abnormality			
22q11.2 deletion	Interrupted aortic arch type B	Left ventricular outflow tract obstruction. Residual coarctation.	Depending on degree of obstruction, surgical correction may be required.
	22q11.2 deletion	Right ventricular outflow tract obstruction or insufficiency. Truncal right ventricular, or truncal valve regurgitation and stenosis.	Depending on the degree of obstruction, surgery may be required, e.g., aortic valve replacement.
	Tetralogy of Fallot	Right ventricular outflow tract obstruction, including pulmonary regurgitation, right ventricular dilation.	Depending on degree of obstruction, surgery may be required.
Down syndrome	Complete AVC <sup>b</sup>	Common atrioventricular valve regurgitation or stenosis.	Depending on degree of regurgitation, surgery may be required.
		Residual shunt at the site of the septal defect closure.	Depending on size of shunt or regurgitation, surgery may be required.
	Arrhythmia	Postoperative bradycardia, syncope	Monitor all cases. May need pacemaker.
	Mitral valve prolapse, less frequently aortic and tricuspid prolapse. Coronary artery disease	Usually asymptomatic. Mitral, aortic and tricuspid regurgitation may develop Not known to have increased risk of angina or myocardial infarction.	Auscultation in all individuals; echocardiography if abnormal. Routine population screening.
Turner Syndrome	Bicuspid aortic valve	Can progress to aortic stenosis. As in general population, there is associated risk of aortic dilatation, aneurysm and rupture.	Monitor all cases with meticulous echocardiography or MRI.
	Coarctation	Residual obstruction at the site of coarctation repair may lead to hypertension. Some mild coarctation may go undetected until adulthood. Repaired or unrepaired, associated with risk of aortic dilatation, dissection, and rupture.	Depending on severity of obstruction, anti-hypertensive medication, catheterization or surgical repair may be required. Meticulous imaging using echocardiography or MRI.
	Hypertension	Can be mild to severe.	Seek underlying cause, e.g., an aortic aneurysm, and treat aggressively when hypertension is present. Hypertension is a risk factor for aortic dissection.
	Aortic root dilation	Aortic dissection and rupture area associated with dilatation, although the natural history from dilatation to dissection has not been completely delineated. Beta-blockade can be useful.	Baseline imaging of aorta should be performed; at least one MRI examination should be provided to provide optimal imaging. In some cases, surgery may be indicated.
	ECG abnormalities	Aside from predisposition to resting tachycardia, not known to have pathologic arrhythmias.	Because of observed cardiac conduction abnormalities, avoid drugs that prolong QT interval.
Williams–Beuren Syndrome	Supravalvar aortic stenosis, focal or diffuse	Residual left ventricular outflow tract obstruction	Depending on degree of obstruction, surgery may be needed.
	Long-segment stenosis/hypoplasia Of thoracic or descending aorta ("coarctation")	Re-stenosis interventional catheterization	Depending on severity of obstruction, anti-hypertensive medication, catheterization or surgery may be required.
	Coronary artery stenosis (due to medial hypertrophy or dysplastic Aortic valve leaflet)	Coronary insufficiency	Screen coronary patency by echocardiography (if indicated), CTA, MRA or catheterization (if indicated).
	Hypertension Pulmonary artery stenoses (PPS, main branch stenoses)	Can occur at any age. Treat with antihypertensive medication. Balloon dilatation or stenting for significant obstruction. Complications such as tears or aneurysms reported.	Exercise stress testing is of limited value. Screen for renovascular cause.



Gene Abnormality			
Holt–Oram Syndrome	ASD, secundum-type, VSD, membranous, muscular	Residual shunt, rarely hemodynamic risk. Potential for stroke.	Depending on size of shunt, management
	ASD, secundum-type, VSD, Membranous, muscular	Eisenmenger syndrome	Medication for pulmonary hypertension
	Conduction block	Can be progressive; when complete heart block is present, may be associated with atrial fibrillation.	Need regular monitoring with Holter monitoring in those with known conduction system disease for development and/or progression; may require permanent pacemaker; anticoagulants (for atrial fibrillation)
Marfan Syndrome	Marfan Syndrome	Aortic dissection, rupture aortic regurgitation	Prophylactic aortic root repair (if >50 mm); valve sparing techniques
	Aortic dilatation	Progressive dilatation, rupture	Prophylactic grafting of any portion at risk; risks of surgery are less than medical, or in the case of end-stage disease, pain.
	Mitral valve prolapse	Mitral regurgitation, heart failure	Mitral valve repair or replacement
TGF- <i>B</i> receptor syndromes	Aortic dilatation	Aortic dissection, rupture	Prophylactic aortic root repair (if >40 mm using aortic diameter criteria for Marfan syndrome (>40 mm in adult))
	Aneurysm of other major arteries	Arterial dissection, rupture	Prophylactic repair
Hereditary Hemorrhagic Telangiectasia	Pulmonary arterio-venous malformations	Cyanosis; paradoxical embolization leading to stroke or brain abscess	Therapeutic or prophylactic embolization of coils when the diameter of the lesion is >10 mm or greater
	Hepatic arteriovenous malformations	Cirrhosis; hepatic encephalopathy	Medical management of liver disease
Noonan Syndrome	Pulmonic stenosis	Can present in adulthood for the first time; those with previous surgery or balloon angioplasty in childhood can have residual PS and/or pulmonary insufficiency	Options for severe pulmonary stenosis include valvuloplasty or valve replacement; pulmonary insufficiency often requires medical management
	ASD, secundum type	Residual shunt after repair in childhood; some adults have undetected large defect and fatigue symptoms; in combination with pulmonary stenosis may have risk for stroke	Depending on size of shunt may require closure by a septal occluder
	Hypertrophic cardiomyopathy	Right ventricular outflow obstruction; can coexist with a CHD such as pulmonary stenosis; risk of arrhythmias	Treatment considerations include beta blockers; careful monitoring; may also include treatment such as defibrillator

ASD, atrial septal defect; AVC, atrioventricular canal defect; CAVC, complete atrioventricular canal; CHD, congenital heart defects; CTA, CT angiography; ECG, electrocardiography

<sup>a</sup>Guidelines provide general information for primary care providers, but individuals require specific management by cardiologists. In the interest of brevity, each cardiac abnormality is not discussed in detail.

<sup>b</sup>ASD, VSD and tetralogy of Fallot were not discussed. Antibiotic prophylaxis for subacute bacterial endocarditis should be offered in appropriate doses per standard practice.

Lin, A. E., C. T. Basson, et al. (2008). "Adults with genetic syndromes and cardiovascular abnormalities: clinical history and management." *Genet Med* 10(7): 469–494.

APPENDIX

The following tables list syndromes in which a heart malformation may occur as grouped by the major heart defect. Space does not allow for every case report or occasional observation. Omim, GeneReviews and the Oxford Dysmorphology Database are excellent resources. In this table, GeneReviews are included where app. Several syndromes appear in more than one table.

Cardiac Defect <sup>a,b</sup>	Syndrome Name	Cardinal Features	Etiology	Inheritance	OMIM	Gene Reviews
AVSD	CHARGE	Colobomata (iris and/or retina, heart defects, atresia choanae, retardation of growth and development and/or central nervous system anomalies, genital hypoplasia in males, ears cup shaped and hearing loss (pulmonary valve stenosis)	CHD7 gene	AD	214800	Click link
	Down	Hypotonia, mental retardation, up-slanting palpebral fissures, epicanthal folds, brachycephaly, single palmar crease	Trisomy 21	Nondysjunction, unbalanced robertsonian translocations	190685	
	Ellis–Creveld	Short stature, postaxial polydactyly, miso/ acromelic shortening of limbs, short ribs,	EVC1 and EVC2	AR	225500	
	FG	Macrocephaly (absolute and relative), congenital hypotonia, small ears, agenesis of the corpus callosum, constipation, mental retardation	MED12	X-linked	305450	Click link
	Ivemark?	Postaxial polydactyly, hydrometrocolpos	MKKS	AR	236700	Click link
	McKusick-Kaufman Noonan	Short stature, pectus excavatum/carinatum, hypertelorism, low-set ears, mild mental retardation	PTPN11, SOS1, KRAS, RAF1	AD	163950	Click link
	Smith–Lemli–Opitz	Mental retardation, failure to thrive, feeding, difficulties, premature death, microcephaly, micrognathia, ptosis, anteverted nostrils, 2/3 syndactyly of toes, broad alveolar ridges, short stature, genital anomalies; occasional, polydactyly, cleft palate, various other, visceral malformations, cataract	DHCR7	AR	270400	Click link
Bicuspid aortic valve	1q21.1 microdeletion	Microcephaly, mild facial dysmorphism, autism, various heart defects	1q21.1 deletion	AD, reduced penetrance	612474	Click link
	17q21.31 deletion	Mental retardation, hypotonia, and characteristic face	Deletion 17q21.31	AD	610443	Click link
	9q subtelomeric deletion (Kleefstra)	Moderate to severe mental retardation, hypotonia, facial dysmorphisms, urogenital defects, seizures, behavior problems	9q34.3 deletion resulting in haploinsufficiency of EHMT1	AD, sporadic	610253	

	Beals	Arachnodactyly, large joint contractures, camptodactyly, "crumpled" ears, kyphoscoliosis,	FBN2	AD	121050	Click link
	Cranioectodermal dysplasia-1	Sagittal craniosynostosis facial dysmorphism, ectodermal and skeletal anomalies	IFT122	AR	218330	
	Familial thoracic aortic aneurysm and dissection	Aortic aneurysms and dissections, PDA	MYH11 ACTA2	AD	132900 611788	Click link
	Loeys–Dietz	Arterial tortuosity and aneurysms, hypertelorism, bifid uvula or cleft palate	TGFBR1 TGFBR2	AD	609192 610168	Click link
	Noonan	Short stature, pectus excavatum/carinatum, hypertelorism, low-set ears, mild mental retardation	PTPN11, SOS1, KRAS, RAF1	AD	163950	Click link
	Periventricular nodular heterotopia	Majority female with in utero male lethality, PDA, aortic dilation, periventricular heterotopic nodules, seizures, mild mental retardation, coagulopathy, strokes	FLNA	X-linked	300049	Click link
	Rubenstein–Taybi	Mental retardation, postnatal growth deficiency, microcephaly, broad thumbs and halluces, and dysmorphic facial features	CREBBP EP300	AD, de novo	180849	Click link
	Turner	Short stature, gonadal dysgenesis, short webbed neck, wide-spaced nipples, renal anomalies	Monosomy X	Sporadic		
	Williams	Distinctive facial characteristics, mental retardation unique personality, growth abnormalities, and endocrine abnormalities (hypercalcemia, hypercalciuria, hypothyroidism)	Deletion chromosome 7q11.23	AD	194050	Click link
Aortic valve stenosis	Alkaptonuria	Homogentisic acid in the urine, ochronosis (blue-black pigmentation in connective tissue), and arthritis of the spine and large joints.	HGD	AR	203500	Click link
	Morquio B (mucopolysaccharidosis type IVB)	Skeletal dysplasia, corneal clouding	GLB1	AR	253010	
	Pallister–Killian	Severe mental retardation, seizures, short stature, coarse face, frontal bossing, temporal balding, upturned nose, full cheeks, droopy mouth, patchy hypopigmented skin, diaphragmatic hernia, supernumerary nipples	Mosaic 12 p tetrasomy	sporadic	601803	
	Scheie (mucopolysaccharidosis type V)	Coarse facial features, cloudy cornea, stiff joints	IDUA	AR	607016	
	Weil–Marchesani	Microspherophakia, ectopia lentis, short stature, brachydactyly, joint stiffness	FBN1 ADAMTS10	AD AR	608328	Click link

Cardiac Defect <sup>a,b</sup>	Syndrome Name	Cardinal Features	Etiology	Inheritance	OMIM	Gene Reviews
Coarctation of the aorta	1q21.1 microdeletion	Microcephaly, mild facial dysmorphism, autism, various heart defects	1q21.1 deletion	AD, reduced penetrance	612474	<a href="#">Click link</a>
	22q11 deletion	Immune deficiency, palate abnormalities, hypocalcemia, psychiatric illness	22q11 deletion	AD	188400 192430	<a href="#">Click link</a>
	9q subtelomeric deletion (Kleefstra)	Moderate to severe mental retardation, hypotonia, facial dysmorphisms, urogenital defects, seizures, behavior problems	9q34.3 deletion resulting in haploinsufficiency of EHMT1	AD, sporadic	610253	
	Adams–Oliver Alagille	Scalp defects, terminal transverse defects, Intrahepatic cholestasis (95%); defects of the anterior chamber of the eye (80%), mainly posterior embryotoxon; abnormal facies (90%); vertebral anomalies (70%)	Unknown JAG1, NOTCH1	AD, AR AD	100300 118450 610205	<a href="#">Click link</a>
	Alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV)	Failure of formation and in-growth of alveolar with medial muscular thickening of small pulmonary arterioles presenting as persistent pulmonary hypertension of the newborn, multiple congenital anomalies: gastrointestinal, genitourinary, musculoskeletal, and/ or disruption of the normal right–left asymmetry of intra-thoracic or intra-abdominal organs	FOX transcription factor gene cluster on chromosome 16q24.1q24.2	AD	265380	
	Apert	Severe craniosynostosis, flat face, hypertelorism syndactyly (mitten hands), occasional cleft palate, deafness, mental retardation, renal and genitourinary anomalies	FGFR2	AD	101200	<a href="#">Click link</a>
	Cornelia de Lange	Mental retardation, growth failure, microbrachycephaly, hirsutism, synophrys, variable upper limb reduction defects	NIPBL SMC1A	AD X-linked	122470 300590	<a href="#">Click link</a>
	Cutis laxa	Lax skin, prematurely aged appearance, beaked nose, hoarse voice, joint dislocations, hernias	ELN FBLN5	AD	123700 604580	<a href="#">Click link</a>
	Diamond–Blackfan Anemia	Growth retardation, normochromic, macrocytic, anemia, craniofacial, upper limb, or genitourinary malformations	RPS19, RPL5, RPL11, RPL35A, RPS24, RPS17, RPS7, RPS10, RPS26	AD	105650	<a href="#">Click link</a>
	Goldenhar	Hemifacial microsomia, epibulbar dermoid, preauricular ear tags, deafness, microtia, eyelid coloboma, cleft palate, vertebral anomalies	Unknown	Sporadic, AD, AR	164210	<a href="#">Click link</a>



	Kabuki	Mental retardation, short stature, characteristic face with tented eyebrows and everted lower palpebral fissures, prominent ears, occasional skeletal and visceral malformations	MLL2 gene	AD	147920	
	Microphthalmia-9	Bilateral microphthalmia, IUGR, short stature, bilateral pulmonary hypoplasia/ agensis, diaphragmatic hernia, mental retardation, hypotonia, early death	STRA6	AR	601186	
	Mowat–Wilson	Distinctive facial appearance, Hirschsprung disease or chronic constipation, fleshy upturned ear lobules	ZEB2 gene	AD, de novo	235730	<a href="#">Click link</a>
	Neurofibromatosis type I	Cafe-au-lait macules, Lisch nodules in the eye, fibromatous tumors of the skin	NF1	AD	162200	<a href="#">Click link</a>
	Noonan	Short stature, pectus excavatum/carinatum, hypertelorism, low-set ears, mild mental retardation	PTPN11, SOS1, KRAS, RAF1	AD	163950	<a href="#">Click link</a>
	Opitz G/BBB	Hypertelorism, hypospadias, cleft lip/palate, laryngotracheoesophageal abnormalities, imperforate anus, developmental delay	MID1 gene	X-linked	300000	<a href="#">Click link</a>
	Pallister–Killian	Severe mental retardation, seizures, short stature, coarse face, frontal bossing, temporal balding, upturned nose, full cheeks, droopy mouth, patchy hypopigmented skin, diaphragmatic hernia, supernumerary nipples	Mosaic 12p tetrasomy	sporadic	601803	
	Rubenstein–Taybi	Mental retardation, postnatal growth deficiency, microcephaly, broad thumbs and halluces, and dysmorphic facial features	CREBBP EP300	AD, de novo	180849	<a href="#">Click link</a>
	Thrombocytopenia–absent radius (TAR)	Bilateral absent radius, (thumbs are present) ulnar hypoplasia, thumbs present, occasionally mentally retarded, squint,	200 kb deletion, chromosome 1q21.1	Unknown	274000	<a href="#">Click link</a>
	Turner	Short stature, gonadal dysgenesis, short webbed neck, wide-spaced nipples, renal anomalies	Monosomy X	Sporadic		
	Williams	Distinctive facial characteristics, mental retardation unique personality, growth abnormalities, and endocrine abnormalities (hypercalcemia, hypercalciuria, hypothyroidism)	Deletion chromosome 7q11.23	AD	194050	<a href="#">Click link</a>
Double-outlet right ventricle	CHARGE	Colobomata (iris and/or retina, heart defects, atresia choanae, retardation of growth and development and/or central nervous system anomalies, genital hypoplasia in males, ears cup shaped and hearing loss (pulmonary valve stenosis)	CHD7 gene	AD-variable expressivity	214800	<a href="#">Click link</a>

Cardiac Defect <sup>a,b</sup>	Syndrome Name	Cardinal Features	Etiology	Inheritance	OMIM	Gene Reviews
	Congenital disorders of glycosylation Ia	Developmental delay, hypotonia, failure to thrive, hepatic dysfunction, coagulopathy, abnormal subcutaneous fat , seizures, cerebellar hypoplasia/atrophy and small brain stem	PMM2	AR	212065	Click link
	Edwards	Growth failure, hypertonia, small palpebral Fissures, clenched hands with overlapping digits, omphalocele, neural tube defect,	Trisomy 18	Sporadic		
	Frank–ter Haar	Brachycephaly, wide fontanels, prominent forehead, hypertelorism, prominent eyes, macrocornea, glaucoma, full cheeks, small chin, bowing of the long bones, and flexion deformity of the fingers	TKS4	AR	249420	
	Goldenhar	Hemifacial microsomia, epibulbar dermoid, preauricular ear tags, deafness, microtia, eyelid coloboma, cleft palate, vertebral anomalies	Unknown	Sporadic, AD, AR	164210	Click link
	Opitz G/BBB	Hypertelorism, hypospadias, cleft lip/palate, laryngotracheoesophageal abnormalities, imperforate anus, developmental delay	MID1	X-linked	300000	Click link
	Pentalogy of Cantrell	Anterior body wall defect, short or bifid sternum, diaphragmatic pericardium defect,	Unknown with some cases linked to Xq26.1	Sporadic	313850	
Hypoplastic left heart	16p12.1 deletion	Developmental delay, craniofacial Dysmorphology, bipolar disorder, seizures, growth abnormalities	Deletion 16p12.1	AD, variable expressivity	136570	
	Alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV)	Failure of formation and in-growth of alveolar with medial muscular thickening of small pulmonary arterioles presenting as persistent pulmonary hypertension of the newborn, multiple congenital anomalies: gastrointestinal, genitourinary, musculoskeletal, and/ or disruption of the normal right–left asymmetry of intra-thoracic or intra-abdominal organs	FOX transcription factor gene cluster on chromosome 16q24.1q24.2	AD	265380	
	CHARGE	Colobomata (iris and/or retina, heart defects, atresia choanae, retardation of growth and development and/or central nervous system anomalies, genital hypoplasia in males, ears cup shaped and hearing loss (pulmonary valve stenosis)	CHD7 gene	AD-variable expressivity	214800	Click link
	Ellis–van Creveld	Short stature, postaxial polydactyly, miso/ acromelic shortening of limbs, short ribs,	EVC1 and EVC2	AR	225500	

	Holt–Oram	Triphalangeal or hypoplastic to absent thumb bifid thumb, variable hypoplasia of first metacarpal, radius or whole limb, narrow shoulders.	TBX5	AD	142900	<a href="#">Click link</a>
	Jacobsen	Growth retardation, mental retardation, dysmorphic features, strabismus, thrombocytopenia	terminal deletion 11q	AD	147791	
	Smith–Lemli–Opitz	Mental retardation, failure to thrive, feeding, difficulties, premature death, microcephaly, micrognathia, ptosis, anteverted nostrils, 2/3 syndactyly of toes, broad alveolar ridges, short stature, genital anomalies; occasional, polydactyly, cleft palate, vari- ous other, visceral malformations, cataract	DHCR7	AR	270400	<a href="#">Click link</a>
Patent Ductus Arteriosus (PDA)	1q21.1 microdeletion	Microcephaly, mild facial dysmorphism, autism, various heart defects	1q21.1 deletion	AD, reduced penetrance	612474	<a href="#">Click link</a>
	1p36 deletion	IUGR, failure to thrive, microcephaly, dysmorphic facial features, hearing loss, CL/P, seizures	Deletion 1p36	AD, sporadic	607872	<a href="#">Click link</a>
	22q11 deletion	Immune deficiency, palate abnormalities, hypocalcemia, psychiatric illness	22q11 deletion	AD	188400 192430	<a href="#">Click link</a>
	Adams–Oliver	Scalp defects, terminal transverse defects, CL/P	Unknown	AD, AR	100300	
	Agenesis of the corpus callosum with mental retardation, ocular coloboma, and micrognathia	Agenesis of the corpus callosum, mental retardation, coloboma, micrognathia	IGBP1	X-linked	300472	
	Axenfeld–Rieger syndrome type 3	Axenfeld–Rieger anomaly, sensorineural hearing loss	FOXC1	AD	602482	
	C (Opitz trigonocephaly)	Facial dysmorphism, mental retardation, redundant skin, omphalocele, hepatomegaly	CD96	AR	211750	
	CHAR	Facial dysmorphism, abnormal fifth digit	TFAP2B	AD	169100	<a href="#">Click link</a>
	CHARGE	Colobomata (iris and/or retina, heart defects, atresia choanae, retardation of growth and development and/or central nervous system anomalies, genital hypoplasia in males, ears cup shaped and hearing loss (pulmonary valve stenosis)	CHD7 gene	AD	214800	<a href="#">Click link</a>
	Chronic idiopathic intestinal pseudoobstruction	Gastrointestinal dysmotility, mild facial dysmorphism, thrombocytopenia, large platelets,	FLNA duplication	X-linked	300048	
	Coffin–Siris	Growth deficiency, mental retardation, microcephaly	Unknown	?AR	135900	

Cardiac Defect <sup>a,b</sup>	Syndrome Name	Cardinal Features	Etiology	Inheritance	OMIM	Gene Reviews
	Combined oxidative phosphorylation deficiency-3 (COXPD3)	Cardiomyopathy, respiratory failure, neonatal hypotonia, seizures, lactic acidosis, facial dysmorphism	TSFM	AR	610505	
	Combined oxidative phosphorylation deficiency-2 (COXPD2)	Agenesis of the corpus callosum, dysmorphism, fatal neonatal lactic acidosis	MRPS16	AR	610498	
	Congenital disorder of glycosylation type Ie	Acquired microcephaly, failure to thrive, hepatomegaly, splenomegaly, hypotonia, seizures, mental retardation	DPM1	AR	608799	Click link
	Congenital alveolar capillary dysplasia with misalignment of pulmonary veins	Respiratory distress at birth due to congenital alveolar dysplasia,	FOXF1	AR	265380	
	Desmosterolosis	Microcephaly, facial dysmorphic features, osteoclerosis,	DHCR24	AR	602398	
	Familial thoracic aortic aneurysm and dissection	Aortic aneurysms and dissections, BAV	MYH11 ACTA2	AD	132900 611788	Click link
	Feingold	Abnormalities of the hands and feet, short palpebral fissures, microcephaly, learning disability, esophageal/duodenal atresia	MYCN	AD	164280	Click link
	Goldenhar	Hemifacial microsomia, epibulbar dermoid, preauricular ear tags, deafness, microtia, eyelid coloboma, cleft palate, vertebral anomalies	Unknown	Sporadic, AD, AR	164210	Click link
	Fryns	Diaphragmatic defects, cloudy cornea, hypoplastic distal digits	Unknown	presumed AR	229850	Click link
	Goltz (Focal dermal hypoplasia)	Focal dermal hypoplasia, oligodactyly, hypoplastic teeth, microphthalmos, coloboma, alopecia, mental retardation	PORCN gene	X-linked	305600	Click link
	Kabuki	Mental retardation, short stature, characteristic face with tented eyebrows and everted lower palpebral fissures, prominent ears, occasional skeletal and visceral malformations	MLL2 gene	AD	147920	
	Loeys Dietz	Arterial tortuosity and aneurysms, hypertelorism, bifid uvula or cleft palate	TGFBR1 TGFBR2	AD	609192 610168	Click link
	Meckel–Gruber	Renal cysts, CNS anomalies (typically encephalocele), hepatic ductal dysplasia and cysts, and polydactyly	Genetic heterogeneity— MKS1, CC2D2A, CEP290, RPGRIP1L, TMEM67/MKS3	AR	249000	



Microphthalmia-9	Bilateral microphthalmia, IUGR, short stature, bilateral pulmonary hypoplasia/ agensis, diaphragmatic hernia, mental retardation, hypotonia, early death	STRA6	AR	601186	
Mowat–Wilson	Distinctive facial appearance, Hirschsprung disease or chronic constipation, fleshy upturned ear lobules	ZEB2 gene	AD, de novo	235730	<a href="#">Click link</a>
Noonan	Short stature, pectus excavatum/carinatum, hypertelorism, low-set ears, mild mental retardation	PTPN11, SOS1, KRAS, RAF1	AD	163950	<a href="#">Click link</a>
Opitz G/BBB	Hypertelorism, hypospadias, cleft lip/palate, laryngotracheoesophageal abnormalities, imperforate anus, developmental delay	MID1 gene	X-linked	300000	<a href="#">Click link</a>
Pallister–Killian	Severe mental retardation, seizures, short stature, coarse face, frontal bossing, temporal balding, upturned nose, full cheeks, droopy mouth, patchy hypopigmented skin, diaphragmatic hernia, supernumerary nipples	Mosaic 12 p tetrasomy	Sporadic	601803	
Pallister–Hall	Hypothalamic hamartoma, pituitary dysfunction, central polydactyly, and visceral malformations	GLI3	AD	146510	<a href="#">Click link</a>
Periventricular nodular heterotopia	Majority female with in utero male lethality, BAV, aortic dilation, periventricular heterotopic nodules, seizures, mild mental retardation, coagulopathy, strokes	FLNA	X-linked	300049	<a href="#">Click link</a>
Primary hypertrophic osteoarthropathy	Digital clubbing and osteoarthropathy, variable features of pachydermia, delayed closure of the fontanel	HPGD	AR	259100	
Renal–hepatic–pancreatic dysplasia (RHPD)	Potter facies, oligohydramnios, cystic malformations of the kidneys, liver, and pancreas	NPHP3	AR	208540	
Rubenstein–Taybi	Mental retardation, postnatal growth deficiency, microcephaly, broad thumbs and halluces, and dysmorphic facial features	CREBBP EP300	AD, de novo	180849	<a href="#">Click link</a>
Severe congenital neutropenia-4 (SCN4)	Congenital neutropenia, prominent superficial venous patterning	G6PC3	AR	612541	
Sotos	Pre- and postnatal overgrowth, macrocephaly, prominent forehead, down-slanting palpebral, fissures, pointed chin, mental retardation	<i>FGD1</i>	AD, de novo	117550	<a href="#">Click link</a>
Townes–Brocks	Imperforate anus, dysplastic ears, hearing loss, thumb malformations, renal impairment/ anomalies	SALL1	AD	107480	<a href="#">Click link</a>

Cardiac Defect <sup>a,b</sup>	Syndrome Name	Cardinal Features	Etiology	Inheritance	OMIM	Gene Reviews
	VATER/ VACTERL	Vertebral defects (V), anal atresia (A), tracheoesophageal fistula with esophageal atresia (TE), and radial or renal dysplasia (R), cardiac malformations (C) and limb anomalies (L) Three or more of the above is suggestive of the diagnosis	Unknown	Unknown, sporadic	192350	
	Weil–Marchesani	Microspherophakia, ectopia lentis, short stature, brachydactyly, joint stiffness	FBN1 ADAMTS10	AD AR	608328	<a href="#">Click link</a>
	Zellweger	Failure to thrive, facial dysmorphism, hearing loss, cataracts, glaucoma, liver dysfunction	<i>PEX1, 2, 3, 5, 6, 10, 12, 13, 14, 16, 19, 26</i>	AR	214100	<a href="#">Click link</a>
Pulmonary valve	17q21.31 deletion	Mental retardation, hypotonia, and characteristic face (pulmonary stenosis)	Deletion 17q21.31	AD	610443	<a href="#">Click link</a>
	9q subtelomeric deletion (Kleefstra)	Moderate to severe mental retardation, hypotonia, facial dysmorphisms, urogenital defects, seizures, behavior problems	9q34.3 deletion resulting in haploinsufficiency of EHMT1	AD, sporadic	610253	
	Adams–Oliver	Scalp defects, terminal transverse defects, CL/P	Unknown	AD, AR	100300	
	Cardiofaciocutaneous	Noonan-like, sparse curly hair, short stature, mental retardation	BRAF, MAP2K1, MAP2K2, and KRAS genes	AD, de novo mutation	115150	<a href="#">Click link</a>
	Carpenter	Craniosynostosis, preaxial polydactyly, brachysyndactyly, ptosis, obesity	RAB23	AR	201000	
	CHARGE	Colobomata (iris and/or retina, <i>heart</i> defects, <i>atresia</i> choanae, <i>retardation</i> of growth and development and/or central nervous system anomalies, <i>genital</i> hypoplasia in males, ears cup shaped and hearing loss (pulmonary valve stenosis)	CHD7 gene	AD-variable expressivity	214800	<a href="#">Click link</a>
	Costello	Polyhydramnios, mental retardation, coarse face, thick lips, deep plamer/ plantar creases	HRAS gene	AD, de novo mutation	218040	<a href="#">Click link</a>
	Keutel	Brachytelephalangism, calcification of	MGP	AR	245150	<a href="#">Click link</a>
	LEOPARD	<i>Lentigenes</i> , ECG abnormalities, ocular hypertelorism, <i>pulmonic</i> stenosis, <i>abnormal</i> genitalia, <i>retardation</i> of growth, and sensorineural <i>deafness</i>	PTPN11 RAF1 BRAF	AD	151100 611554 613707	
	Microphthalmia-9	Bilateral microphthalmia, IUGR, short stature, bilateral pulmonary hypoplasia/ agensis, diaphragmatic hernia, mental retardation, hypotonia, early death	STRA6	AR	601186	
	Neurofibromatosis type I	Café-au-lait macules, Lisch nodules in the eye, fibromatous tumors of the skin	NF1	AD	162200	<a href="#">Click link</a>

	Noonan	Short stature, pectus excavatum/carinatum, hypertelorism, low-set ears, mild mental retardation	PTPN11, SOS1, KRAS, RAF1	AD	163950	<a href="#">Click link</a>
	Simpson–Golabi–Behmel	Pre- and postnatal overgrowth, coarse facies, hypertelorism, broad nose, wide mouth, macroglossia, prominent jaw, broad hands, cleft/high palate, extra nipples, occasional, hypoplastic fingernails, polydactyly, hernias, renal tract abnormalities, mild mental retardation, embryonal tumors	GPC3 CXORF5	X-linked	312870	<a href="#">Click link</a>
	Townes–Brocks	Imperforate anus, dysplastic ears, hearing loss, thumb malformations, renal impairment/ anomalies	SALL1	AD	107480	<a href="#">Click link</a>
	Weil–Marchesani	Microspherophakia, ectopia lentis, short stature, brachydactyly, joint stiffness (pulmonary valve stenosis)	FBN1 ADAMTS10	AD AR	608328	<a href="#">Click link</a>
	Williams	Distinctive facial characteristics, mental retardation unique personality, growth abnormalities, and endocrine abnormalities (hypercalcemia, hypercalciuria, hypothyroidism)	Deletion chromosome 7q11.23	AD	194050	<a href="#">Click link</a>
Mitral valve anomalies	Adams–Oliver	Scalp defects, terminal transverse defects,	Unknown	AD, AR	100300	
	Axenfeld–Rieger syndrome type 3	Axenfeld–Rieger anomaly, sensorineural hearing loss	FOXC1	AD	602482	
	Beals	Arachnodactyly, large joint contractures, camptodactyly, “crumpled” ears, kyphoscoliosis,	FBN2	AD	121050	<a href="#">Click link</a>
	Brittle cornea	Blue sclere, corneal rupture, keratoconus or keratoglobus, hyperelastic skin, hypermobile joints	ZNF469	AR	229200	
	Cohen	Truncal obesity, mental retardation, hypotonia, microcephaly, delayed puberty, distinctive face, chorioretinal dystrophy, neutropenia	COH1	AR	216550	<a href="#">Click link</a>
	Costello	Polyhydramnios, mental retardation, coarse face, thick lips, deep plamer/ plantar creases	HRAS gene	AD-de novo mutation	218040	<a href="#">Click link</a>
	Ehlers–Danlos classic type	Joint hypermobility, increased skin elasticity, abnormal wound healing	COL5A1 COL5A2	AD	130000	<a href="#">Click link</a>
	Ehlers–Danlos vascular type	Connective tissue fragility with spontaneous rupture of arteries, bowel, poor wound healing	COL3A1	AD	130050	<a href="#">Click link</a>
	Fabry disease	Acroparesthesia, abdominal pain, hypertrophic cardiomyopathy, angiokeratomas, corneal opacities, renal failure, myocardial ischemia	GLA	X-linked	301500	<a href="#">Click link</a>

Cardiac Defect <sup>a,b</sup>	Syndrome Name	Cardinal Features	Etiology	Inheritance	OMIM	Gene Reviews
	Fragile X	Mental retardation, long face, large ears, enlarged testes	FMR1	X-linked	300624	Click link
	Frank–ter Haar	Brachycephaly, wide fontanel, prominent forehead, hypertelorism, prominent eyes, macrocornea, glaucoma, full cheeks, small chin, bowing of the long bones, and flexion deformity of the fingers	TKS4	AR	249420	
	Humeroapical dysostosis (spondyloepiphyseal dysplasia with congenital joint dislocations)	Congenital dislocations of knees and/or hips, clubfoot, elbow joint dysplasia with subluxation, limited extension, short stature	CHST3	AD	143095	
	Hurler (MPS IH;) Scheie (MPS IS;) Hurler–Scheie (MPS IH/S)	Coarse facial features, cloudy cornea, stiff joints	IDUA	AR	607014 607016	
	Kabuki	Mental retardation, short stature, characteristic face with tented eyebrows and everted lower palpebral fissures, prominent ears, occasional skeletal and visceral malformations	MLL2 gene	AD	147920	
	Loeys–Dietz	Arterial tortuosity and aneurysms, hypertelorism, bifid uvula or cleft palate	TGFBR1 TGFBR2	AD	609192 610168	Click link
	Marfan	Ectopia lentis, dilated aorta, skeletal features including pectus, scoliosis	FBN1	Autosomal dominant;	154700	Click link
	Morquio (mucopolysaccharidosis type IVA, IVB)	Coarse face, short stature, severe kyphoscoliosis, genu valgum, odontoid corneal clouding, hearing loss	GALNS (IVA) GLB1 (IVB)	AR	253000 253010	
	Myotonic dystrophy type 1	Myotonia, cardiac arrhythmia, cataracts, frontal balding	DMPK	AD	160900	Click link
	Oculofaciocardiodental (OFCD)	Congenital cataracts, microphthalmia, palate abnormalities	BCOR	X-linked	300166	Click link
	Osteogenesis imperfecta (type 1)	Multiple fractures, osteopenia, wormian bones, blue sclera, abnormal tooth enamel, progressive hearing loss	COL1A1 COL1A2	AD	166200	Click link
	Otopalatodigital (Melnick–Needles)	Skeletal dysplasia, facial dysmorphism, cleft palate	FLNA	X-linked	309350	Click link
	Progressive external ophthalmoplegia	Adult-onset weakness of the external eye muscles, exercise intolerance	POLG	AR	258450	Click link
	Pseudoxanthoma elasticum	Flexural cutaneous yellowish papular lesions, mild skin redundancy, retinal angioid streaks, chorioretinopathy	ABCC6	AR with mild manifestations in carriers	264800	Click link
	Shprintzen–Goldberg	Craniosynostosis, exophthalmos, maxillary and mandibular hypoplasia, arachnodactyly, camptodactyly	FBN1	AD	182212	Click link



	Stickler	High myopia, hearing loss, Pierre Robin sequence, cleft palate, vitreous anomaly	COL2A1 COL11A1 COL11A2 COL9A1	AD AR	108300	<a href="#">Click link</a>
	Weil–Marchesani	Microspherophakia, ectopia lentis, short stature, brachydactyly, joint stiffness (pulmonary valve stenosis)	FBN1 ADAMTS10	AD AR	608328	<a href="#">Click link</a>
	Williams	Distinctive facial characteristics, mental retardation unique personality, growth abnormalities, and endocrine abnormalities (hypercalcemia, hypercalciuria, hypothyroidism)	Deletion chromosome 7q11.23	AD	194050	<a href="#">Click link</a>
SEPTAL DEFECTS	1p36 deletion	IUGR, failure to thrive, microcephaly, dysmorphic facial features, hearing loss, CL/P, seizures	Deletion 1p36	AD, sporadic	607872	<a href="#">Click link</a>
	17q21.31 deletion	Mental retardation, hypotonia, and characteristic face	Deletion 17q21.31	AD	610443	<a href="#">Click link</a>
	18q deletion	Mental retardation, microcephaly, short stature, hypotonia, hearing loss	deletion 18q	AD, sporadic	601808	
	2q37 deletion	Short stature, brachydactyly, mental retardation	Contiguous gene deletion	AD, sporadic	600430	
	22q11 deletion	Immune deficiency, palate abnormalities, hypocalcemia, psychiatric illness	22q11 deletion	AD	188400 192430	<a href="#">Click link</a>
	9q subtelomeric deletion	Moderate to severe mental retardation, hypotonia, facial dysmorphisms, urogenital defects, seizures, behavior problems	9q34.3 deletion resulting in haploinsufficiency of EHMT1	AD, sporadic	610253	
	Adams–Oliver	Scalp defects, terminal transverse defects, CL/P	Unknown	AD, AR	100300	
	Agenesis of the corpus callosum with mental retardation, ocular coloboma, and micrognathia	Agenesis of the corpus callosum, mental retardation, coloboma, micrognathia	IGBP1	X-linked	300472	
	Alagille	Intrahepatic cholestasis (95%); defects of the anterior chamber of the eye (80%), mainly posterior embryotoxon; abnormal facies (90%); vertebral anomalies (70%)	JAG1, NOTCH1	AD	118450 610205	<a href="#">Click link</a>
	Alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV)	Failure of formation and in-growth of alveolar with medial muscular thickening of small pulmonary arterioles presenting as persistent pulmonary hypertension of the newborn, multiple congenital anomalies: gastrointestinal, genitourinary, musculoskeletal, and/ or disruption of the normal right–left asymmetry of intra-thoracic or intra-abdominal organs	FOX transcription factor gene cluster on chromosome 16q24.1q24.2	AD	265380	

Cardiac Defect <sup>a,b</sup>	Syndrome Name	Cardinal Features	Etiology	Inheritance	OMIM	Gene Reviews
	Antley–Bixler	Coronal and lambdoid suture synostosis, frontal bossing, severe midface hypoplasia, proptosis, choanal stenosis, bowed femora and ulnae, vaginal atresia, renal anomalies	POR	AR	207410	<a href="#">Click link</a>
	Apert	Severe craniosynostosis, flat face, hypertelorism syndactyly (mitten hands), occasional cleft palate, deafness, mental retardation, renal and genitourinary anomalies	FGFR2	AD	101200	<a href="#">Click link</a>
	Arthrogryposis, renal dysfunction, and cholestasis-1 (ARCS1)	arthrogryposis multiplex congenita with jaundice and renal dysfunction, dysmorphic features	VPS33B	AR	208085	
	Athabaskan brainstem dysgenesis	Horizontal gaze palsy, sensorineural deafness, central hypoventilation, developmental delay	HOXA1	AR	601536	
	Axenfeld–Rieger syndrome type 3	Axenfeld–Rieger anomaly, sensorineural hearing loss	FOXC1	AD	602482	
	Baller–Gerold	Craniosynostosis, radial aplasia, missing thumbs, anal anomalies, skeletal defects, central nervous system defects, urogenital defects	RECQL4	AR	218600	<a href="#">Click link</a>
	Bardet–Biedl	Mental retardation, ulnar polydactyly, obesity, retinal dystrophy, hypogenitalism, kidney disease, occasional short stature, hearing loss, vaginal atresia	Fourteen genes: <i>BBS1</i> , <i>BBS2</i> , <i>ARL6/BBS3</i> , <i>BBS4</i> , <i>BBS5</i> , <i>MKKS</i> / <i>BBS6</i> , <i>BBS7</i> , <i>TTC8</i> / <i>BBS8</i> , <i>B1/BBS9</i> , <i>BBS10</i> , <i>TRIM32</i> / <i>BBS11</i> , <i>BBS12</i> , <i>MKS1/BBS13</i> , <i>CEP290/BBS14</i>	AR	209900	<a href="#">Click link</a>
	Beals	Arachnodactyly, large joint contractures, camptodactyly, “crumpled” ears, kyphoscoliosis,	FBN2	AD	121050	<a href="#">Click link</a>
	C (Opitz trigonocephaly)	Facial dysmorphism, mental retardation, redundant skin, omphalocele, hepatomegaly	CD96	AR	211750	
	Camptomelic dysplasia	Pierre Robin sequence with cleft palate, shortening and bowing of long bones, and club feet, sex reversal	SOX9	AD	114290	<a href="#">Click link</a>
	Cardiofaciocutaneous	Noonan-like, sparse curly hair, short stature, mental retardation	BRAF, MAP2K1, MAP2K2, KRAS	AD, de novo mutation	115150	<a href="#">Click link</a>
	Carpenter	Craniosynostosis, preaxial polydactyly, brachysyndactyly, ptosis, obesity	RAB23	AR	201000	

Cat eye	Iris coloboma, anal atresia, preauricular tags renal malformations	Chromosome 22 partial tetrasomy	Sporadic	115470	
CHARGE	Colobomata (iris and/or retina, heart defects, atresia choanae, retardation of growth and development and/or central nervous system anomalies, genital hypoplasia in males, ears cup shaped and hearing loss (pulmonary valve stenosis)	CHD7 gene	AD- variable expressivity	214800	<a href="#">Click link</a>
CHILD syndrome	Congenital hemidysplasia, ichthyosiform nevus, limb defects; occasional visceral hypoplasia, CNS lesions and mental retardation	NSDHL	X-linked	308050	<a href="#">Click link</a>
Coffin–Siris	Growth deficiency, mental retardation, microcephaly	Unknown	?AR	135900	
Congenital disorders of glycosylation type IIa	Facial dysmorphism, stereotypic hand movements, seizures, mental retardation variable	ALG2	AR	212066	<a href="#">Click link</a>
Chondrodysplasia Punctata 2	Asymmetrical shortening of limbs, stippled epiphysis, contractures, patchy skin changes, epiphysis, contractures, patchy skin changes, sparse hair, flat facies	EBP	X-linked	302960	
Cohen	Truncal obesity, mental retardation, hypotonia, microcephaly, delayed puberty, distinctive face, chorioretinal dystrophy, neutropenia	COH1	AR	216550	<a href="#">Click link</a>
Cornelia de Lange	Mental retardation, growth failure, microbrachycephaly, hirsutism, synophrys, variable upper limb reduction defects	NIPBL SMC1A	AD X-linked	122470 300590	<a href="#">Click link</a>
Costello	Polyhydramnios, mental retardation, coarse face, thick lips, deep plamer/ plantar creases	HRAS gene	AD, de novo mutation	218040	<a href="#">Click link</a>
Cri Du Chat (5 p-)	Growth deficiency, catlike cry, developmental delay, microcephaly, round face, hypertelorism	deletion 5 p	Sporadic; 5 p translocation in 10-15%	123450	
Diamond–Blackfan Anemia	Growth retardation, normochromic, macrocytic, anemia, craniofacial, upper limb, or genitourinary malformations	RPS19, RPL5, RPL11, RPL35A, RPS24, RPS17, RPS7, RPS10, RPS26	AD	105650	<a href="#">Click link</a>
Down	Hypotonia, mental retardation, up-slanting palpebral fissures, epicanthal folds, brachycephaly, single palmar crease	Trisomy 21	Nondysjunction, unbalanced robertsonian translocations	190685	
Duane–Radial–Ray	Upper limb anomalies, ocular anomalies, renal anomalies	SALL4	AD	607323	<a href="#">Click link</a>

Cardiac Defect <sup>a,b</sup>	Syndrome Name	Cardinal Features	Etiology	Inheritance	OMIM	Gene Reviews
	Edwards	Growth failure, hypertonia, small palpebral fissures, clenched hands with overlapping digits, omphalocele, neural tube defect,	Trisomy 18	Sporadic		
	Ellis–van Creveld	Short stature, postaxial polydactyly, miso/ acromelic shortening of limbs, short ribs,	EVC1 and EVC2	AR	225500	
	Goldenhar	Hemifacial microsomia, epibulbar dermoid, preauricular ear tags, deafness, microtia, eyelid coloboma, cleft palate, vertebral anomalies	Unknown	Sporadic, AD, AR	164210	<a href="#">Click link</a>
	Fanconi anemia	Pancytopenia, leukemia, short stature, café-au-lait macules absent/hypoplastic thumb, small genitalia, microcephaly, renal abnormalities, mental retardation, small eyes, hearing loss, no physical manifestations in ~25-40%	<i>FANCA, FANCB, FANCC, BRCA2 (FANCD1), FANCD2, FANCE, FANCF, FANCG (XRCC9), FANCI, BRIP1 (FANCI or BACH1), FANCL, FANCM, PALB2 (FANCN), RAD51C (FANCO), SLX4 (FANCP)</i>	AR	227650	<a href="#">Click link</a>
	FG	Macrocephaly (absolute and relative), congenital hypotonia, small ears, agenesis of the corpus callosum, constipation, mental retardation	MED12	X-linked	305450	<a href="#">Click link</a>
	Fryns	Diaphragmatic defects, corneal clouding, hypoplastic distal digits	Unknown	Presumed AR	229850	<a href="#">Click link</a>
	Goltz (Focal dermal hypoplasia)	Focal dermal hypoplasia, oligodactyly, hypoplastic teeth, microphthalmos, coloboma, alopecia, mental retardation	PORCN gene	X-linked	305600	<a href="#">Click link</a>
	H syndrome	Cutaneous hyperpigmentation, hypertrichosis, hepatosplenomegaly, hypogonadism hearing loss, short stature	SLC29A3	AR	612391	
	Hallerman–Streiff	Microphthalmia, cataract, small jaw, frontal prominence, thin pointed nose, dental anomalies, hypotrichosis, skin atrophy, short stature	Unknown	Sporadic	234100	



Hennekam lymphangiectasia-lymphedema	Early-onset lymphedema, intestinal, lymphangiectasia, growth retardation, mental retardation, seizures, flat midface, epicanthus, hypertelorism, ear anomalies, tooth anomalies, renourinary malformations, deafness, skeletal anomalies	CCBE1	AR	235510	
Holt–Oram	Triphalangeal or hypoplastic to absent thumb bifid thumb, variable hypoplasia of first metacarpal, radius or whole limy, narrow shoulders.	TBX5	AD	142900	<a href="#">Click link</a>
Jacobsen	Growth retardation, mental retardation, dysmorphic features, strabismus, thrombocytopenia	Terminal deletion 11q	AD	147791	
Johannson–Blizzard	Failure to thrive, pancreatic insufficiency, hypothyroidism, aplasia cutis congenital, abnormal hair, hypoplastic alae nasi, mental retardation, sensorineural deafness	UBR1 gene	AR	243800	
Kabuki	Mental retardation, short stature, characteristic face with tented eyebrows and everted lower palpebral fissures, prominent ears, occasional skeletal and visceral malformations	MLL2 gene	AD	147920	
Keutel Lujan Fryns	Brachytelephalangism, calcification of Marfanoid habitus, sharp nose, hypernasal voice, behavior problems	MGP MED12	AR	245150 309520	<a href="#">Click link</a>
Lymphedema-Distichiasis	Extra row of eyelashes, late-onset lymphedema; occasional cleft palate, micrognathia, pterygium colli, ptosis, Arnold-Chiari malformation, urinary tract malformations, spinal extradural cysts, vertebral anomalies, double uterus	FOXC2	AD	153400	<a href="#">Click link</a>
Meckel–Gruber	Renal cysts, CNS anomalies (typically encephalocele), hepatic ductal dysplasia and cysts, and polydactyly	Genetic heterogeneity- MKS1, CC2D2A, CEP290, RPGRIP1L, TMEM67/MKS3	AR	249000	
Marshall–Smith	Accelerated linear growth, failure to thrive, mental retardation, and characteristic facial appearance (prominent forehead, shallow orbits, blue sclere, depressed nasal bridge, and micrognathia)	Unknown	Sporadic	602535	
McKusick–Kaufman Microcephalic osteodysplastic primordial dwarfism (MOPD) I	Postaxial polydactyly, hydrometrocolpos Primordial dwarfism	MKKS Unknown	AR AR	236700 210710	<a href="#">Click link</a>

Cardiac Defect <sup>a,b</sup>	Syndrome Name	Cardinal Features	Etiology	Inheritance	OMIM	Gene Reviews
	Microphthalmia-7 (Microphthalmia with linear skin defects)	Unilateral or bilateral microphthalmia and linear skin defects	HCCS	X-linked	309801	<a href="#">Click link</a>
	Microphthalmia-9	Bilateral microphthalmia, IUGR, short stature, bilateral pulmonary hypoplasia/ agensis, diaphragmatic hernia, mental retardation, hypotonia, early death	STRA6	AR	601186	
	Miller	Severe micrognathia, cleft lip and/or palate, hypoplasia or aplasia of the postaxial elements of the limbs, coloboma of the eyelids, and supernumerary nipples	DHODH gene	AR	263750	
	Mowat–Wilson	Distinctive facial appearance, Hirschsprung disease or chronic constipation, fleshy upturned ear lobules	ZEB2 gene	AD, de novo mutation	235730	<a href="#">Click link</a>
	Myhre	Short stature, joint limitations, thick skin, muscular hypertrophy, autism spectrum disorder	Unknown	?AR	139210	
	Noonan	Short stature, pectus excavatum/carinatum, hypertelorism, low-set ears, mild mental retardation	PTPN11, SOS1, KRAS, RAF1	AD	163950	<a href="#">Click link</a>
	Oculofaciocardiodental (OFCD)	Congenital cataracts, microphthalmia, palate abnormalities	BCOR	X-linked	300166	<a href="#">Click link</a>
	Odho	Mental retardation, blepharophimosis, ptosis, hypoplastic teeth, dysplastic ears, characteristic nose, small mouth, hearing loss	Unknown	AD	249620	
	Opitz G/BBB	Hypertelorism, hypospadias, cleft lip/palate, laryngotracheoesophageal abnormalities, imperforate anus, developmental delay	MID1 gene	X-linked	300000	<a href="#">Click link</a>
	Pallister–Hall	Hypothalamic hamartoma, pituitary dysfunction, central polydactyly, and visceral malformations	GLI3	AD	146510	<a href="#">Click link</a>
	Pallister–Killian	Severe mental retardation, seizures, short stature, coarse face, frontal bossing, temporal balding, upturned nose, full cheeks, droopy mouth, patchy hypopigmented skin, diaphragmatic hernia, supernumerary nipples	Mosaic 12 p tetrasomy	Sporadic	601803	
	Patau (Trisomy 13)	Mental retardation, incomplete development of forebrain, polydactyly, microcephaly, microphthalmia, cleft lip, cleft palate, deafness, cryptorchidism, scalp defects	Trisomy 13	Sporadic		
	Pentalogy of Cantrell	Anterior body wall defect, short or bifid sternum, diaphragmatic pericardium defect,	Unknown with some cases linked to Xq26.1	Sporadic	313850	

Peters-plus	Peters anomaly, short stature, short limbs, brachydactyly, round face, thin lips, long philtrum, mental retardation, occasional cleft lip/palate, renal abnormalities	B3GALT1	AR	261540	<a href="#">Click link</a>
Renal–hepatic–pancreatic dysplasia (RHPD)	Potter facies, oligohydramnios, cystic malformations of the kidneys, liver, and pancreas	NPHP3	AR	208540	
Renpenning (Golabi–Ito–Hall) Roberts	Mental retardation, microcephaly, short stature, and small testes Variable-reduction limb defects, growth delay, cleft lip and palate, hypertelorism, delay, renal, defects, neonatal death, premature centromere separation	PQBP1 gene ESCO2	X-linked AR	309500 269000	<a href="#">Click link</a>
Rubenstein–Taybi	Mental retardation, postnatal growth deficiency, microcephaly, broad thumbs and halluces, and dysmorphic facial features	CREBBP EP300	AD, de novo	180849	<a href="#">Click link</a>
Schinzel–Giedion	Mental retardation, severe midface hypoplasia, hirsutism, hydronephrosis, genital, anomalies, skeletal anomalies, choanal stenosis, brain malformations, sacrococcygeal tumors	SETBP1	AD, de novo	269150	
Simpson–Golabi–Behmel	Pre- and postnatal overgrowth, coarse facies, hypertelorism, broad nose, wide mouth, macroglossia, prominent jaw, broad hands, cleft/high palate, extra nipples, occasional, hypoplastic fingernails, polydactyly, hernias, renal tract abnormalities, mild mental retardation, embryonal tumors	GPC3	X-linked	312870	<a href="#">Click link</a>
Smith–Lemli–Opitz	Mental retardation, failure to thrive, feeding, difficulties, premature death, microcephaly, micrognathia, ptosis, anteverted nostrils, 2/3 syndactyly of toes, broad alveolar ridges, short stature, genital anomalies; occasional, polydactyly, cleft palate, various other, visceral malformations, cataract	DHCR7	AR	270400	<a href="#">Click link</a>
Sotos	Pre- and postnatal overgrowth, macrocephaly, prominent forehead, down-slanting palpebral, fissures, pointed chin, mental retardation	FGD1	AD, de novo	117550	<a href="#">Click link</a>
Spinal muscular atrophy type I	Muscle weakness, symmetric, proximal (lower limbs more affected than upper limbs), muscle atrophy, EMG shows neurogenic abnormalities, areflexia	SMN1, SMN2	AR	253300	<a href="#">Click link</a>

Cardiac Defect <sup>a,b</sup>	Syndrome Name	Cardinal Features	Etiology	Inheritance	OMIM	Gene Reviews
	Thanatophoric dysplasia	Severe short-limbed dwarfism, narrowed chest, neonatal death, macrocephaly, depressed nasal bridge, curved short femurs, metaphyseal flaring, H-shaped flattened vertebrae, brain anomalies; occasional cloverleaf skull	FGFR3	AD de novo	187600	<a href="#">Click link</a>
	Thrombocytopenia—absent radius (TAR)	Bilateral absent radius, (thumbs are present) ulnar hypoplasia, thumbs present, occasionally mentally retarded, squint,	200 kb deletion, chromosome 1q21.1	Unknown	274000	<a href="#">Click link</a>
	Timothy	Lethal arrhythmias, syndactyly, facial dysmorphism, immune deficiency, intermittent hypoglycemia, mental retardation, autism	CACNA1C	AD, de novo	601005	<a href="#">Click link</a>
	Townes–Brocks	Imperforate anus, dysplastic ears, hearing loss, thumb malformations, renal impairment/ anomalies	SALL1	AD	107480	<a href="#">Click link</a>
	Uniparental disomy chromosome 16 (maternal)	Asymmetry, hypertelorism, small hands and feet, 5th finger clinodactyly, microcephaly, hypospadias, scoliosis	Maternal uniparental disomy chromosome 16	Sporadic		
	Neonatal progeria	Low birth weight, aged appearance from birth, pinched features, development delay, sparse hair, lack of subcutaneous fat apart from pads over buttocks	Unknown	AR	264090	
	VATER/ VACTERL	Vertebral defects (V), anal atresia (A), tracheoesophageal fistula with esophageal atresia (TE), and radial or renal dysplasia (R), cardiac malformations (C) and limb anomalies (L) Three or more of the above is suggestive of the diagnosis	Unknown	Sporadic	192350	
	Weil–Marchesani	Microspherophakia, ectopia lentis, short stature, brachydactyly, joint stiffness	FBN1 ADAMTS10	AD AR	608328	<a href="#">Click link</a>
	Williams	Distinctive facial characteristics, mental retardation unique personality, growth abnormalities, and endocrine abnormalities (hypercalcemia, hypercalciuria, hypothyroidism)	Deletion chromosome 7q11.23	AD	194050	<a href="#">Click link</a>
	Wolff–Hirschhorn	Growth deficiency, severe mental retardation, microcephaly, seizures, hypertelorism, prominent glabella, highly arched eyebrows, proptosis, strabismus, cleft lip/palate, agenesis of corpus of cases callosum, talipes, hypospadias, cryptorchidism	Deletion in 165-kb critical region chromosome 4p, translocations in 10%–15%	Sporadic; de novo	194190	<a href="#">Click link</a>



	Yunis–Varon	Dolichocephaly, wide anterior fontanelles, sparse hair, dysmorphic, thin lips, short philtrum, micrognathia, absent thumbs, hypoplastic clavicles		AR	216340	
	Zellweger	Failure to thrive, facial dysmorphism, hearing loss, cataracts, glaucoma, liver dysfunction	<i>PEX1, 2, 3, 5, 6, 10, 12, 13, 14, 16, 19, 26</i>	AR	214100	<a href="#">Click link</a>
Transposition of the great arteries	1q21.1 microdeletion	Microcephaly, mild facial dysmorphism, autism, various heart defects	1q21.1 deletion	AD, reduced penetrance	612474	<a href="#">Click link</a>
	22q11 deletion	Immune deficiency, palate abnormalities, hypocalcemia, psychiatric illness	22q11 deletion	AD	188400	<a href="#">Click link</a>
	Antley–Bixler	Coronal and lambdoid suture synostosis, frontal bossing, severe midface hypoplasia, proptosis, choanal stenosis, bowed femora and ulnae, vaginal atresia, renal anomalies	POR	AR	192430 207410	<a href="#">Click link</a>
	Congenital disorders of glycosylation Ia	Developmental delay, hypotonia, failure to thrive, hepatic dysfunction, coagulopathy, abnormal subcutaneous fat, seizures, cerebellar hypoplasia/atrophy and small brain stem	PMM2	AR	212065	<a href="#">Click link</a>
	Ellis–van Creveld	Short stature, postaxial polydactyly, miso/acromelic shortening of limbs, short ribs,	EVC1 and EVC2	AR	225500	
Truncus arteriosus	1q21.1 microdeletion	Microcephaly, mild facial dysmorphism, autism, various heart defects	1q21.1 deletion	AD, reduced penetrance	612474	<a href="#">Click link</a>
	22q11 deletion	Immune deficiency, palate abnormalities, hypocalcemia, psychiatric illness	22q11 deletion	AD	188400 192430	<a href="#">Click link</a>
	Congenital disorders of glycosylation Ia	Developmental delay, hypotonia, failure to thrive, hepatic dysfunction, coagulopathy, abnormal subcutaneous fat, seizures, cerebellar hypoplasia/atrophy and small brain stem	PMM2	AR	212065	<a href="#">Click link</a>
	Goltz (Focal dermal hypoplasia)	Focal dermal hypoplasia, oligodactyly, hypoplastic teeth, microphthalmos, coloboma, alopecia, mental retardation	PORCN gene	X-linked	305600	<a href="#">Click link</a>
	Holt–Oram	Triphalangeal or hypoplastic to absent thumb bifid thumb, variable hypoplasia of first metacarpal, radius or whole limy, narrow shoulders.	TBX5	AD	142900	<a href="#">Click link</a>
	Microphthalmia-9	Bilateral microphthalmia, IUGR, short stature, bilateral pulmonary hypoplasia/ agensis, diaphragmatic hernia, mental retardation, hypotonia, early death	STRA6	AR	601186	

Cardiac Defect <sup>a,b</sup>	Syndrome Name	Cardinal Features	Etiology	Inheritance	OMIM	Gene Reviews
	Thrombocytopenia—absent radius (TAR)	Bilateral absent radius, (thumbs are present) ulnar hypoplasia, thumbs present, occasionally mentally retarded, squint,	200 kb deletion, chromosome 1q21.1	Unknown	274000	Click link
	Townes–Brocks	Imperforate anus, dysplastic ears, hearing loss, thumb malformations, renal impairment/ anomalies	SALL1	AD	107480	Click link
Total	22q11.2 microduplication	Highly variable phenotype from normal to mental retardation, growth retardation, and/or hypotonia	3-Mb or 1.5-Mb 22q11 duplication	AD	608363	Click link
anomalous pulmonary venous return	Athabaskan brainstem dysgenesis	Horizontal gaze palsy, sensorineural deafness, central hypoventilation, developmental delay	HOXA1	AR	601536	
	Cat eye	Iris coloboma, anal atresia, preauricular tags renal malformations	Chromosome 22 partial tetrasomy	Sporadic	115470	
	Desmosterolosis	Microcephaly, microcephaly, cleft palate, facial dysmorphic features, osteoclerosis,	DHCR24	AR	602398	
	Holt–Oram	Triphalangeal or hypoplastic to absent thumb bifid thumb, variable hypoplasia of first metacarpal, radius or whole limy, narrow shoulders.	TBX5	AD	142900	Click link
	Smith–Lemli–Opitz	Mental retardation, failure to thrive, feeding, difficulties, premature death, microcephaly, micrognathia, ptosis, anteverted nostrils, 2/3 syndactyly of toes, broad alveolar ridges, short stature, genital anomalies; occasional, polydactyly, cleft palate, various other, visceral malformations, cataract	DHCR7	AR	270400	Click link
Syndromes Tetralogy						
Tetralogy of Fallot	1p36 deletion	Short stature, facial dysmorphism, microcephaly, hearing loss, hypotonia, developmental delay, facial clefting	1p36 deletion	AD	607872	Click link
	15q13.3 deletion	Mental retardation, seizures, autism, schizophrenia	15q13.3 deletion	AD	612001	Click link
	16p11.2 deletion	Mental retardation, autism	16p11.2 deletion	AD	611913	Click link
	22q11 deletion	Immune deficiency, palate abnormalities, hypocalcemia, psychiatric illness	22q11 deletion	AD	188400	Click link
	9q subtelomeric deletion	Moderate to severe mental retardation, hypotonia, facial dysmorphisms, urogenital defects, seizures, behavior problems	TBX1 mutation		192430	
			9q34.3 deletion resulting in haploinsufficiency of EHMT1	AD, sporadic	610253	
	Adams–Oliver	Scalp defects, terminal transverse defects, CL/P	Unknown	AD, AR	100300	
	Alagille	Intrahepatic cholestasis (95%); defects of the arterior chamber of the eye (80%), mainly posterior embryotoxon; abnormal facies (90%); vertebral anomalies (70%)	JAG1, NOTCH1	AD	118450	Click link
					610205	

Athabaskan brainstem dysgenesis	Horizontal gaze palsy, sensorineural deafness, central hypoventilation, developmental delay	HOXA1	AR	601536	
Baller–Gerold	Craniosynostosis, radial aplasia, missing thumbs, anal anomalies, skeletal defects, central nervous system defects, urogenital defects	RECQL4	AR	218600	<a href="#">Click link</a>
C syndrome	Mental retardation, metopic ridge, broad alveolar ridges, up-slanting eyes, epicanthus, forehead hemangioma, upturned nose; joint, kidney, genital, and brain anomalies	CD96	AR	605039	
Camptomelic dysplasia	Pierre Robin sequence with cleft palate, shortening and bowing of long bones, and club feet, sex reversal	SOX9	AD	114290	<a href="#">Click link</a>
Carpenter	Craniosynostosis, preaxial polydactyly, brachysyndactyly, ptosis, obesity	RAB23 gene	AR	201000	
CHARGE	Colobomata (iris and/or retina, heart defects, atresia choanae, retardation of growth and development and/or central nervous system anomalies, genital hypoplasia in males, ears cup shaped and hearing loss	CHD7 gene	AD, variable expressivity	214800	<a href="#">Click link</a>
Coffin–Siris	Growth deficiency, mental retardation, microcephaly	Unknown	?AR	135900	
Congenital disorders of glycosylation Ia	Developmental delay, hypotonia, failure to thrive, hepatic dysfunction, coagulopathy, abnormal subcutaneous fat, seizures, cerebellar hypoplasia/atrophy and small brain stem	PMM2	AR	212065	<a href="#">Click link</a>
Cornelia de Lange	Mental retardation, growth failure, microbrachycephaly, hirsutism, synophrys, variable upper limb reduction defects	NIPBL SMC1A	AD X-linked	122470 300590	<a href="#">Click link</a>
Diamond–Blackfan Anemia	Growth retardation, normochromic, macrocytic, anemia, craniofacial, upper limb, or genitourinary malformations	RPS19, RPL5, RPL11, RPL35A, RPS24, RPS17, RPS7, RPS10, RPS26	AD	105650	<a href="#">Click link</a>
Down	Hypotonia, mental retardation, up-slanting palpebral fissures, epicanthal folds, brachycephaly, single palmar crease	Trisomy 21	Nondysjunction, unbalanced Robertsonian translocations	190685	
Edwards	Growth failure, hypertonia, small palpebral fissures, clenched hands with overlapping digits, omphalocele, neural tube defect,	Trisomy 18	Sporadic		
Frontonasal dysplasia	Hypertelorism, broad nasal root, median nasal cleft, short stature, microcephaly	ALX3	Sporadic	136760	

Cardiac Defect <sup>a,b</sup>	Syndrome Name	Cardinal Features	Etiology	Inheritance	OMIM	Gene Reviews
	Goldenhar	Hemifacial microsomia, epibulbar dermoid, preauricular ear tags, deafness, microtia, eyelid coloboma, cleft palate, vertebral anomalies	Unknown	Sporadic, AD, AR	164210	<a href="#">Click link</a>
	Hallerman–Streiff	Microphthalmia, cataract, small jaw, frontal prominence, thin pointed nose, dental anomalies, hypotrichosis, skin atrophy, short stature	Unknown	Sporadic	234100	
	Holt–Oram	Triphalangeal or hypoplastic to absent thumb bifid thumb, variable hypoplasia of first metacarpal, radius or whole limy, narrow shoulders. <i>HOS excluded with malformations involving:</i> ulnar ray only, kidney, vertebra, craniofacies, hearing loss or ear malformations, lower limb, anus, or eye.	TBX5	AD	142900	<a href="#">Click link</a>
	Hypoparathyroidism, sensorineural deafness, and renal disease (HDR)	Hypoparathyroidism, sensorineural deafness, renal disease	Microdeletion of 10p including GATA3	AD	146255	
	Kabuki	Mental retardation, short stature, characteristic face with tented eyebrows and everted lower palpebral fissures, prominent ears, occasional skeletal and visceral malformations	MLL2 gene	AD	147920	
	Lymphedema-Distichiasis	Distichiasis, late-onset lymphedema; occasional cleft palate, micrognathia, pterygium colli, ptosis, Arnold–Chiari malformation, urinary tract malformations, spinal extradural cysts, vertebral anomalies, double uterus	FOXC2	AD	153400	<a href="#">Click link</a>
	McKusick–Kaufman	Postaxial polydactyly, hydrometrocolpos	MKKS	AR	236700	<a href="#">Click link</a>
	Microcephalic osteodysplastic primordial dwarfism (MOPD) I	Primordial dwarfism	Unknown	AR	210710	
	Microphthalmia-9	Bilateral microphthalmia, IUGR, short stature, bilateral pulmonary hypoplasia/ agensis, diaphragmatic hernia, mental retardation, hypotonia, early death	STRA6	AR	601186	
	Miller–Dieker	Lissencephaly type I, severe retardation, seizures, growth failure, tall furrowed forehead, bi-temporal narrowing, short upturned nose,	Deletions at 17p13.3 including LIS1, sequence variations LIS1	AD	247200	<a href="#">Click link</a>
	Nager acrofacial dysostosis	Acrofacial dysostosis resembling Treacher–Collins syndrome combined with predominantly radial limb defects	Unknown	AD	154400	



Opitz G/BBB	Hypertelorism, hypospadias, cleft lip/palate, laryngotracheoesophageal abnormalities, imperforate anus, developmental delay	MID1 gene	X-linked	300000	<a href="#">Click link</a>
Pentalogy of Cantrell	Anterior body wall defect, short or bifid sternum, diaphragmatic pericardium defect,	Unknown with some cases linked to Xq26.1	Sporadic	313850	
Renpenning (Golabi–Ito–Hall)	Mental retardation, microcephaly, short stature, and small testes	PQBP1 gene	X-linked	309500	
Smith–Lemli–Opitz	Mental retardation, failure to thrive, feeding, difficulties, premature death, microcephaly, micrognathia, ptosis, anteverted nostrils, 2/3 syndactyly of toes, broad alveolar ridges, short stature, genital anomalies; occasional, polydactyly, cleft palate, various other, visceral malformations, cataract	<i>DHCR7</i>	AR	270400	<a href="#">Click link</a>
Thrombocytopenia—absent radius (TAR)	Bilateral absent radius, (thumbs are present) ulnar hypoplasia, thumbs present, occasionally mentally retarded, squint,	200 kb deletion, chromosome 1q21.1	Unknown	274000	<a href="#">Click link</a>
Timothy	Lethal arrhythmias, syndactyly, immune deficiency, intermittent hypoglycemia, mental retardation, autism	CACNA1C	AD, de novo	601005	<a href="#">Click link</a>
Townes–Brocks	Imperforate anus, dysplastic ears, hearing loss, thumb malformations, renal impairment/ anomalies	SALL1	AD	107480	<a href="#">Click link</a>
VATER/ VACTERL	Vertebral defects (V), anal atresia (A), tracheoesophageal fistula with esophageal atresia (TE), and radial or renal dysplasia (R), cardiac malformations (C) and limb anomalies (L) Three or more of the above is suggestive of the diagnosis	Unknown	Sporadic	192350	
Yunis–Varon	Dolichocephaly, wide anterior fontanelles, sparse hair, dysmorphic, thin lips, short philtrum, micrognathia, absent thumbs, hypoplastic clavicles		AR	216340	

<sup>a</sup>Syndromes may be associated with multiple types of cardiac defects.

<sup>b</sup>Space does not permit the inclusion of every case report or occasional observation.

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Online Mendelian Inheritance in Man: <http://www.ncbi.nlm.nih.gov/omim>.  
 GeneReviews: <http://www.ncbi.nlm.nih.gov/books/NBK1116/>.

## Biographies

**Dr Rocio Moran, MD, FACMG**  
Assistant Professor of Pediatrics  
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Rocio Moran is the Medical Director and section head of Pediatric Genetics at the Center for Personalized Genetic Healthcare, in the Genomic Medicine Institute of the Cleveland Clinic Foundation in Cleveland, Ohio. Dr Moran earned her BS from The Ohio State University in Molecular Genetics and her MD from Case Western Reserve University School of Medicine. She did her post-graduate training at the University Hospitals of Cleveland/ Rainbow Babies and Children's Hospital in Cleveland, Ohio and is boarded in Pediatrics and Medical Genetics. She joined the Cleveland Clinic Foundation in 2006. Her clinical interests include the natural history, diagnosis and management of connective tissue disorders, the evaluation of patients with birth defects, and the integration of genetics and genetic counseling into primary care and sub-specialty clinical practice.

For more information on Dr Moran, please see: [http://my.clevelandclinic.org/staff\\_directory/physician\\_name\\_search.aspx#7447](http://my.clevelandclinic.org/staff_directory/physician_name_search.aspx#7447).

**Dr Nathaniel H Robin, MD (FACMG)**  
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Dr Robin attended Albert Einstein College of Medicine in the Bronx, New York, where he also completed a residency in pediatrics. He then proceeded to do a fellowship in Genetics at The Children's Hospital Philadelphia/University of Pennsylvania. His first faculty position was in the Department of Genetics at Case Western Reserve University School of Medicine in 1995. In 2003, Dr Robin joined the newly created Department of Genetics at the University of Alabama at Birmingham. Dr Robin is board certified in Clinical Genetics by the American Board of Medical Genetics. His clinical practice is primarily as a general medical geneticist, with expertise in pediatric genetics, syndrome identification and genetic counseling. His areas of focused interest include craniofacial disorders, and the genetics of deafness. While maintaining an active clinical practice, Dr Robin has authored one book (*Medical Genetics: Its Application to Speech, Hearing, and Craniofacial Disorders*), 15 book chapters, over a dozen invited editorials and over one hundred peer-reviewed publications. His writings cover a wide range of topics in genetics and include descriptions and studies on a variety of genetic syndromes. He has published studies that have looked at genetics testing for deafness, and on ethical issues in genetic testing, including issues of confidentiality and duty to warn at risk relatives. Dr Robin is the Medical Genetics residency program director at The University of Alabama at Birmingham. For more information on Dr Robin, please see [http://135.25.56.115/depts/MEB/SOMResearchFaculty/currentfacultydata.asp?s\\_lname=robin&s\\_keyword=&s\\_fname=nathaniel&s\\_Department\\_Name=genetics&s\\_ResearchTitle=&ID=robin](http://135.25.56.115/depts/MEB/SOMResearchFaculty/currentfacultydata.asp?s_lname=robin&s_keyword=&s_fname=nathaniel&s_Department_Name=genetics&s_ResearchTitle=&ID=robin).

# CHAPTER

# 47

## Inherited Cardiomyopathies

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### 47.1 INTRODUCTION

Cardiomyopathies are disorders of the heart muscle (myocardium) that produce characteristic changes in cardiac morphology and function, typically described as hypertrophic or dilated remodeling. Although a variety of etiologies account for compensatory forms of cardiac remodeling are recognized, inherited and de novo gene mutations are the most prevalent causes for primary cardiomyopathies. Discovery of the gene mutations that cause cardiomyopathies has advanced knowledge of the complex cellular and molecular events that propel cardiac remodeling. Despite similar clinical presentations, not all cases of cardiomyopathies that are classified as dilated or hypertrophic reflect a common underlying pathophysiology. Elucidating the different molecular mechanisms leading from genetic mutation to the clinical expression of these disorders has had profound impact on our understanding of basic myocyte biology. This knowledge is increasingly impacting opportunities for personalized approaches to disease management for cardiomyopathy patients.

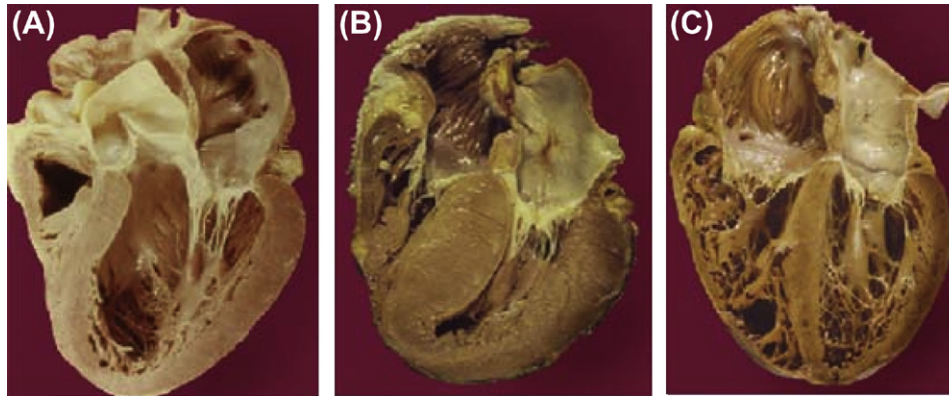
### 47.2 HYPERTROPHIC CARDIOMYOPATHY

Hypertrophic cardiomyopathy (HCM) was first described centuries ago, but modern clinical descriptions appeared in the mid 1950s and over the past 20 years, the molecular genetic basis of HCM has been elucidated. HCM is defined by the presence of unexplained cardiac

hypertrophy in a nondilated ventricle (Figure 47-1). “Unexplained” indicates that left ventricular hypertrophy (LVH) develops in the absence of any systemic or cardiac condition (such as hypertension or valvular heart disease) that may impose an increased load on the heart. HCM is usually familial and inherited as an autosomal dominant trait. Genetic studies have defined HCM to be a disease of the sarcomere, caused by mutations in eight major genes that encode different components of the contractile apparatus.

Many historical descriptions of HCM reported severe morphologic manifestations of the condition, including profound hypertrophic remodeling, marked left ventricular outflow tract obstruction, and adverse clinical outcomes, most notably sudden death. However, with the contemporary widespread use of robust noninvasive imaging techniques, HCM is now recognized to be a prevalent cardiovascular disorder with highly variable manifestations. Clinical presentation can occur throughout life, including late in adulthood. Hypertrophy is usually modest and typically involves the interventricular septum. Significant outflow tract obstruction occurs in ~25% of patients, although a provokable gradient may be detected in larger proportion of patients by administering medications or through maneuvers that impact preload or cardiac contractility. Importantly, for of the most patients, life expectancy is normal and symptoms do not necessitate major interventions.





**FIGURE 47-1** A comparison of normal cardiac anatomy and cardiac remodeling. A. Normal myocardium with left ventricular (LV) wall thickness  $\leq 11$  mm and normal LV volume. B. Hypertrophic cardiomyopathy cause increased LV wall thickness without cavity dilation. C. DCM causes enlargement of LV and occasionally right, ventricular cavity size. (Reproduced with permission from Ahmad, F.; Seidman, J. G.; Seidman, C. E. *The Genetic Basis for Cardiac Remodeling*. Annu. Rev. Genomics Hum. Genet. **2005**, 6,185–216.)

### 47.2.1 Prevalence

In a general population of young adults, the prevalence of unexplained LVH, the prototypic clinical feature of HCM, is 1 in 500 (1). Among a community-based cohort, the Framingham Heart Study, ~3% of participants have unexplained LVH. While the prevalence of unexplained LVH that is familial is unknown, these epidemiologic data support the claim that HCM is the most common genetic cardiovascular disorder (2,3). The prevalence of HCM in the general population may explain why this diagnosis accounts for most non-violent sudden deaths in individuals under the age of 35 and is the most common cause of sudden death in athletes, occurring in one-third of these events (4).

A small number of genetic epidemiologic studies have been performed to investigate the prevalence of sarcomere mutations in different populations. Direct DNA sequencing of six sarcomere genes (MYH7, TNNT2, TNNI3, TPM1, ACTC1, and MYBPC3) was performed on a cohort of 389 unrelated probands referred for management of HCM to a single center. Of these, 147/389 (38%) were found to have sarcomere mutations, most commonly involving MYH7 and MYBPC3 (87% of mutation positive individuals) (5–8). In contrast, a separate study that analyzed eight sarcomere protein genes (including MYL3 and MYL2) in subjects with familial HCM, identified sarcomere gene mutations in 75% of cases (9), with the majority of mutations occurring in MYH7 and MYBPC3. Together these studies indicate that a positive family history is a strong predictor of positive genetic testing.

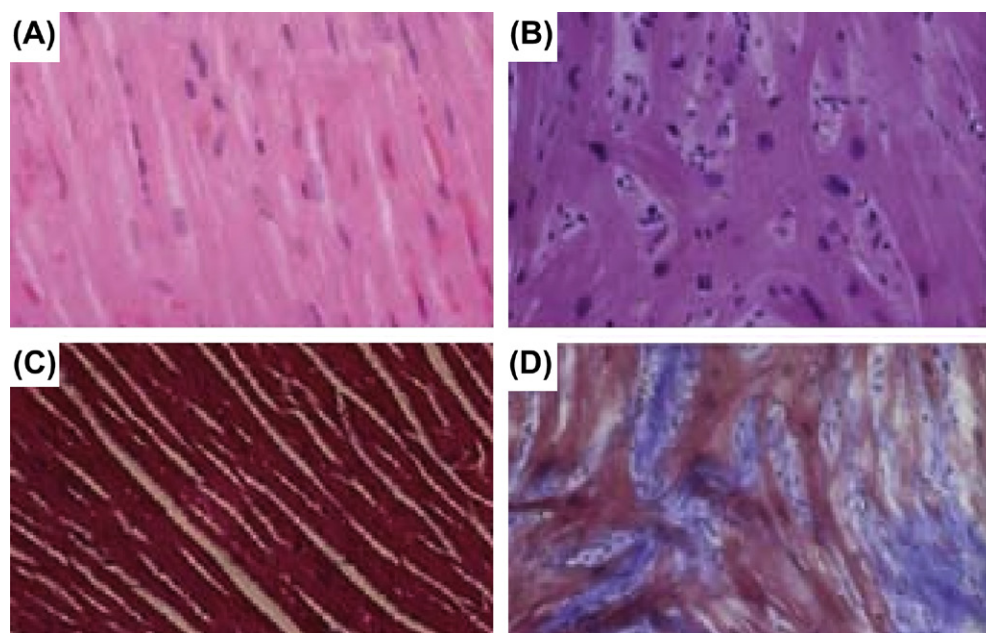
Genetic analyses have begun to address whether sarcomere protein genes also contribute to LVH that occurs in the context of other common cardiovascular disorders. Analyses of 50 participants in the Framingham Heart Study with LVH that occurred in the context of hypertension and atherosclerosis, a rare sarcomere protein gene mutation was identified 16%, while only normal gene sequences were observed in Framingham Heart Study participants with no evidence of LVH (10).

### 47.2.2 Pathology

HCM causes several patterns of hypertrophy, including asymmetric involvement of the interventricular septum (Figure 47-1), concentric hypertrophy and apical hypertrophy (11). There is significant variation in both the location and extent of LVH in affected individuals and no correlation between genetic etiology and cardiac morphology (12). The histopathologic hallmarks of HCM are myocyte enlargement, myocyte disarray and increased amounts of myocardial fibrosis (Figure 47-2). Although small amounts of myocyte disarray may be seen in other forms of cardiac disease, the higher degree of disarray present in HCM is distinctive. The distribution of disarray may be patchy and typically affects the deeper myocardial layers; therefore, catheter-based endomyocardial biopsy is often nondiagnostic. Small vessel disease with thickening of the walls of intramural arteries is also seen in HCM and may be correlated with clinical ischemia.

### 47.2.3 Phenotype and Natural History

**47.2.3.1 Phenotype.** The clinical spectrum of HCM is extremely diverse. Whereas some individuals experience only minor symptoms and are diagnosed incidentally or in the course of family screening, others may develop refractory symptoms of pulmonary congestion or end-stage heart failure requiring cardiac transplantation. In a small subset of patients, sudden cardiac death is the presenting event (13,14–16). Shortness of breath or dyspnea, particularly on exertion, is the most common symptom of HCM, occurring in ~90% of patients. In case that diastolic function is severely impaired, shortness of breath can happen with the absence of left ventricular outflow tract obstruction or valvular dysfunction. Other common clinical manifestations include exertional chest pain (~30% of patients), palpitations, atrial fibrillation (~25%) with associated risk of stroke, orthostatic lightheadedness, presyncope (>50%) and syncope (15–25%), orthopnea/paroxysmal nocturnal dyspnea, and fatigue (16,17).



**FIGURE 47-2** Normal and pathologic myocardial histology. A and C. Normal cardiac histology is characterized by orderly myocyte structure and minimal interstitial fibrosis. B. Hypertrophic cardiomyopathy is characterized by marked myocyte disarray, hypertrophy and interstitial fibrosis. D. Compared with normal tissue, increased interstitial fibrosis (blue) is evident by the Masson trichrome stain in both HCM. (*Reproduced with permission from Ahmad, F.; Seidman, J. G.; Seidman, C. E. The Genetic Basis for Cardiac Remodeling. Annu. Rev. Genomics Hum. Genet. 2005, 6, 185–216.*)

Symptoms and the incidence of atrial fibrillation typically increase with disease duration. In patients with outflow tract obstruction, cold weather leading to reflex vasoconstriction can lessen symptoms due to increased arterial pressure and subsequently decreased outflow obstruction. This finding is opposite in patients with coronary artery disease (11). Although there is limited correlation between morphologic findings and clinical manifestations, more recent studies suggest that the presence of significant outflow tract obstruction  $>50$  mmHg should be considered as an important determinant of adverse outcomes in HCM (18).

Typical findings on physical examination include a prominent left ventricular apical impulse or lift, a fourth heart sound (S<sub>4</sub>), and a brisk, occasionally bifid carotid upstroke. If obstruction is present, there may be a harsh crescendo–decrescendo systolic murmur that is typically the best heard at the lower left sternal border and apex, radiating to the axilla and base, but usually not to the neck as classically audible in valvular aortic stenosis. A separate murmur of mitral regurgitation may also be present, related either to concomitant intrinsic structural abnormalities of the mitral valve or as a consequence of systolic anterior motion (SAM) of the mitral apparatus if obstructive physiology is present. Maneuvers that alter preload and afterload are recommended for discriminating of HCM and valvular aortic stenosis. Preload or afterload reduction from these maneuvers enhances the dynamic gradient and accentuates the intensity of systolic murmur. Conversely, increased preload and afterload in response to maneuvers decrease murmur intensity. Although Valsava maneuver, leg-raising,

amyl nitrite inhalation can change murmur intensity, the classic maneuver that has been recommended is standing–squatting–standing. From standing to squatting, the murmur decreases in intensity due to increased preload and afterload. While the patient is standing from squatting position, the loudness of murmur is then accentuated as a result of rapidly decreased afterload.

The traditional description of HCM relies on the finding of unexplained LVH, usually via electrocardiography (EKG) or echocardiography. Magnetic resonance imaging (MRI) can complement echocardiography when findings are inconclusive. LV wall thickness in excess of two standard deviations above normal or  $>13$  mm in the adult population are the widely accepted echocardiographic criteria for establishing the diagnosis of HCM. Although considered diagnostic of the condition, LVH is not uniformly present in all individuals with sarcomere gene mutations, particularly early in life due to age-dependent penetrance (19,20). Furthermore, the severity of symptoms associated with HCM is not directly related to the magnitude of hypertrophy (13,15). Although a pattern of asymmetric septal hypertrophy is most common, any pattern of LVH may be seen, including isolated apical hypertrophy, concentric hypertrophy or asymmetric hypertrophy involving other myocardial segments (21).

Left ventricular systolic function is typically preserved in HCM and ejection fraction is often increased (22). However, abnormal diastolic function has been well documented and may largely account for symptoms of pulmonary congestion and exercise intolerance (22–24).

Electrocardiographic changes are present in ~90% of affected individuals and may be detected prior to the development of echocardiographic manifestations. However, given the high incidence of nonspecific EKG changes in the general population, EKG findings do not have a higher sensitivity or specificity for diagnosing HCM than echocardiography. Prototypical EKG findings include pathologic Q waves in at least two leads, T wave inversion in at least two leads, and voltage criteria for LVH (25,26). The occurrence of other EKG abnormalities such as pre-excitation and AV block may indicate the presence of other gene mutations that clinically mimic HCM (see below).

HCM patients may have an abnormal fall in blood pressure in response to exercise, possibly related to abnormal vasomotor tone or inability to augment stroke volume in response to increased demand. This finding may indicate a worse prognosis with a higher risk for sudden death (18,27,28).

**47.2.3.2 Natural History.** The natural history of HCM varies, even between family members who have inherited the same causal mutation. Although it is not uncommon for HCM to present in infancy or early childhood; development of LVH typically occurs in adolescence in conjunction with the pubertal growth spurt. The age of onset of hypertrophy may be determined to some extent by the specific nature of the underlying gene defect (20,29). Disease caused by mutations in the  $\beta$ -myosin heavy chain (MYH7) gene is typically associated with clinically obvious disease and nearly uniform development of LVH by the second decade of life. In contrast, clinically evident hypertrophy may not be present until the fourth or fifth decade of life in disease caused by mutations that introduce a premature truncation in the cardiac myosin binding protein C (MYBPC3) gene. MYBPC mutations have also been associated with elderly onset HCM (20). Recent studies indicate that mutation-positive HCM patients have a higher degree of phenotypic severity than HCM patients with a negative genetic test, suggesting that positive genetic result is associated with adverse outcome in HCM patients (8,30).

Estimates of annual mortality in HCM are imprecisely defined. Evaluation of populations drawn from specialized referral centers suggests a significant annual mortality rate of 4–6%. In contrast, community-based studies, which may be less subject to selection bias, demonstrate a more benign course with a projected annual mortality rate of 1 to 2% (17). HCM causes premature morbidity and mortality by sudden cardiac death (accounting for approximately half of HCM-related deaths), progressive heart failure, atrial fibrillation associated with an increased risk of thromboembolism and stroke, and heart failure. Sudden death is the most feared complication of HCM, and accurate estimation of an individual's risk for sudden death is a considerable clinical challenge. The annual risk for sudden death in the overall HCM population varies from 1 to 5% with ~10–20%

of patients at the highest risk (13,14,31). Heart failure may develop in patients with HCM and is associated with an annual mortality of 0.5%. Less than 10–20% of the patients progress to the “burnt-out” phase of HCM, marked by worsening symptomatic heart failure, left ventricular systolic dysfunction, progressive LV wall thinning, and chamber dilatation (13–15). These patients may ultimately require cardiac transplantation for end-stage heart failure.

## 47.2.4 Genetics

HCM is inherited as a Mendelian autosomal dominant trait. Linkage analysis of large kindreds with HCM initially identified several disease loci on chromosomes 1 (32), 11 (33), 14 (34), and 15 (35). Positional cloning and candidate gene analysis identified definitive pathogenic mutations in genes that encode different elements of the contractile apparatus, including cardiac  $\beta$ -myosin heavy chain (MYH7), cardiac troponin T (TNNT2) and I (TNNI3), cardiac myosin binding protein C (MYBPC3),  $\alpha$ -tropomyosin (TPM1), actin (ACTC1), and the essential (MYL3) and regulatory (MYL2) myosin light chains (Table 47-1; reviewed in (36,37). Also see *Cardiogenomics* website. Thus, genetic studies established the paradigm of HCM as a disease of the sarcomere (Figure 47-3)).

The sarcomere is the functional unit of contraction of the myocyte. Proteins are organized into thick (myosin heavy and light chains) and thin (actin, the troponin complex, and  $\alpha$ -tropomyosin) filaments that interdigitate during muscle fiber shortening and lengthening. The detachment and attachment of actin and the myosin head drive contraction and relaxation at the molecular level. The hydrolysis of adenosine triphosphate (ATP) provides fuel for this motor and carefully orchestrated fluxes in intracellular  $\text{Ca}^{2+}$  concentration coordinate thick- and thin-filament interaction (reviewed in References (38,39)). Membrane depolarization by the action potential elicits calcium influx through cell membrane L-type calcium channels. Ryanodine receptors (RyR2) on the sarcoplasmic reticulum (SR) are then activated to trigger calcium-induced calcium release. The resultant rise in intracellular  $\text{Ca}^{2+}$  concentration leads to calcium binding of troponin C and causes conformational changes in the troponin complex, releasing troponin I inhibition of actin and allowing actin-myosin cross-bridge formation. Myosin then hydrolyzes ATP and undergoes conformational changes that allow the myosin head to be propelled against the thin filament. Activation of the sarcoplasmic/endoplasmic  $\text{Ca}^{2+}$ -ATPase membrane pump, SERCA, causes sequestration of cytosolic  $\text{Ca}^{2+}$  back into the SR. The myosin head detaches from actin, troponin I inhibition of actomyosin interaction is reestablished, and myocyte relaxation ensues (Figure 47-4).

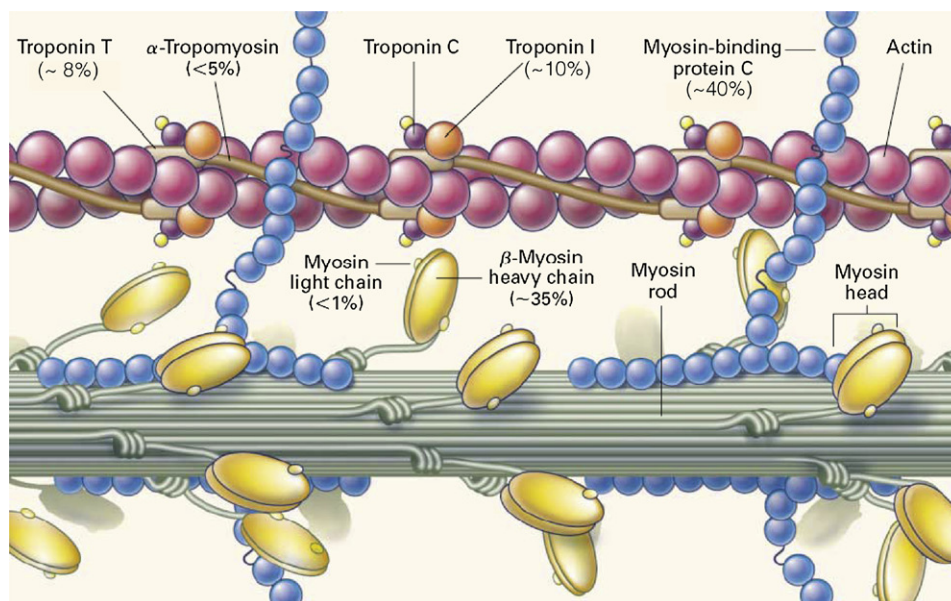
To date, more than 900 individual mutations have been identified in genes that encode different components of the contractile apparatus (40,41), summarized in



**TABLE 47-1 Gene Mutations That Cause Unexplained Left Ventricular Hypertrophy**

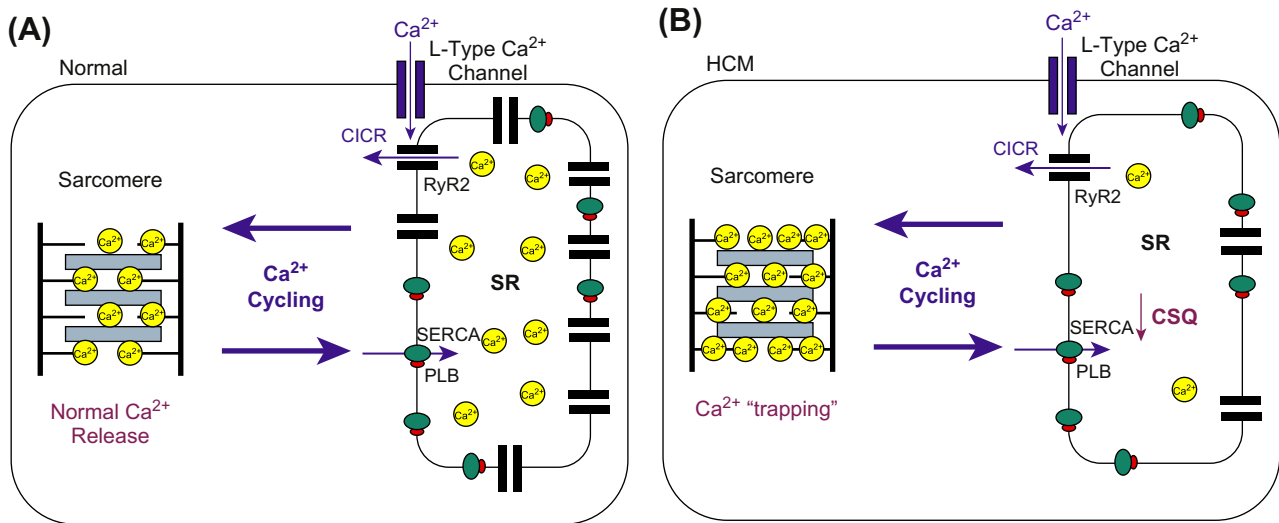
	Gene	Designation	Chromosome	Frequency	Number of Mutations	Phenotypic Correlation
HCM-sarcomere proteins	$\beta$ -Myosin heavy chain	MYH7	14q1	~35%	>200	Typically obvious disease with significant LVH; several severe phenotypes (end-stage heart failure and sudden death)
	Cardiac myosin binding protein C	MYBPC3	11q1	~40%	>150	Typically more mild disease but severe phenotypes have been described; early-onset HCM
	Cardiac troponin T	cTnT	1q3	~8%	>30	Typically mild LVH but increased associated with sudden death
	Cardiac troponin I	cTnI	19p1	~10%	27	
	$\alpha$ -Tropomyosin	TPM1	15q2	~5%	11	
	Myosin essential light chain	MYL3	3p	Rare	5	Skeletal myopathy
	Myosin regulatory light chain	MYL2	12q	~2%	10	Skeletal myopathy
Inherited LVH	Actin	ACTC1	11q	Rare	7	
	$\gamma$ 2-Subunit AMP	PRKAG2	7q3	?	4	Glucose metabolism; pre-excitation and conduction disease; CPMVT
	Linked lysosome associated membrane protein	LAMP2	X	?	6	
	Muscle LIM protein	CRP3	11p	?	3	

Data from Marian, A. J.; Roberts, R. The Molecular Genetic Basis for Hypertrophic Cardiomyopathy. *J. Mol. Cell Cardiol.* **2001**, *33*, 655–670; Roberts, R.; Sigwart, U. New Concepts in Hypertrophic Cardiomyopathies, Part I. *Circulation* **2001**, *104*, 2113–2116; Seidman, J. G.; Seidman, C. The Genetic Basis for Cardiomyopathy: From Mutation Identification to Mechanistic Paradigms. *Cell* **2001**, *104*, 557–567; and see Cardiogenomics website.



**FIGURE 47-3** Mutations in sarcomere protein genes are the genetic etiology of hypertrophic cardiomyopathy as well as some forms of DCM. (Modified and reproduced with permission from Spirito, P.; Seidman, C. E.; McKenna, W. J., et al. *The Management of Hypertrophic Cardiomyopathy*. N. Engl. J. Med. **1997**, *336*, 775–785.)





**FIGURE 47-4** Normal intracellular  $\text{Ca}^{2+}$  cycling (top) is altered in HCM (bottom). A. Electrical impulses generated by the cardiac action potential trigger the influx of  $\text{Ca}^{2+}$  via L-type calcium channels, causing a larger calcium-induced calcium release (CICR) in which  $\text{Ca}^{2+}$  from SR stores is released into the cytoplasm via ryanodine receptors (RyR2) on the SR membrane.  $\text{Ca}^{2+}$  binds to the troponin complex of the sarcomere, releasing inhibition of troponin I, allowing actomyosin interaction and the generation of the power stroke.  $\text{Ca}^{2+}$  returns to the SR via reuptake by the sarcoplasmic/endoplasmic  $\text{Ca}^{2+}$ -ATPase pump (SERCA), which is regulated by phospholamban (PLB). (Adapted with permission from Semsarian, C.; Ahmad, I.; Giewat, M., et al. 2002) B. Sarcomere mutations (asterisk) alter  $\text{Ca}^{2+}$  cycling, so that SR is diminished, resulting in depletion of SR calsequestrin (CSQ), and RyR2. (Adapted with permission from Semsarian, C.; Ahmad, I.; Giewat, M., et al. *The L-type Calcium Channel Inhibitor Diltiazem Prevents Cardiomyopathy in a Mouse Model*. J. Clin. Invest. **2002**, 109, 1013, with permission of The American Society for Clinical Investigation.)

**Table 47-1 (36,42–44).** A variety of types of mutations have been described, including missense, nonsense, short insertions and deletions, and alteration of splice donor or acceptor sites. De novo or sporadic mutations are also well described, although the relative prevalence of sporadic mutations versus familial disease remains unclear. The vast majority of mutations tend to be “private” or unique from family to family with rare reappearances in unrelated kindreds and haplotype analyses of unrelated patients who carry a shared HCM mutation often demonstrate that these genetic defects arose independently (45).

In contrast, several founding HCM mutations have been identified in some populations, as evidenced by shared haplotype architecture amongst mutation carriers. Some of these have been identified in homogenous populations with geographic or societal isolation, including subsets of populations in India (46), Finland (47), The Netherlands (48), Japan (49), South Africa (50), and in the United States (45). The maintenance of founding mutations in these populations is likely to reflect neutral evolutionary pressure due to benign or late-onset HCM manifestations and adverse outcomes that are delayed until after the reproductive age (37). Given a markedly increased human longevity, the effect of these genetic defects has also become even more apparent, as these contribute to an increased risk for heart failure in mutation carriers (46).

Mutations in cardiac  $\beta$ -myosin heavy chain, cardiac myosin binding protein C, and cardiac troponin T and I account for ~80–90% of described cases of HCM (43). Clinical correlates associated with mutations in different HCM genes have emerged (51) and are broadly outlined

in **Table 47-1**; however, numerous exceptions to these themes have been documented (52). However, a discrete number of mutations identified in some families are associated with higher incidence of adverse events, including sudden death and/or heart failure, in mutations carriers. With the exception of these, genotype in isolation is often insufficiently predictive of outcome, and integration of existing information associated with a particular mutation with clinical risk assessment is appropriate. Further identification of causal mutations and more accurate definition of genotype–phenotype correlations remain a work in progress, but the wide genetic and clinical spectrum of HCM make this a challenging task. As our ability to more precisely and sensitively assess the full spectrum of the HCM phenotype improves, more accurate associations will emerge. It remains unclear why some sarcomere mutations cause more severe disease than others and why individuals with the same mutation have a wide range of clinical features. Description of wider genetic and environmental factors that shape the expression of the underlying mutation is an active area of investigation.

#### 47.2.4.1 Cardiac $\beta$ -Myosin Heavy Chain (MYH7).

Myosin heavy chains account for ~1% of total myocyte protein. They are large molecules of >200,000 kDa, organized into two functional domains: an amino terminal globular head that interacts with actin and a carboxyl terminal rod (53,54). Force is transduced via a hinge region between these two domains. There are two cardiac-specific myosin heavy chain isoforms:  $\alpha$ -cardiac MHC (MYH6) and  $\beta$ -cardiac MHC (MYH7). Both genes are encoded in tandem on chromosome 14. The  $\alpha$ -isoform predominates

in fetal life and in the adult atria; the  $\beta$ -isoform predominates in the adult, accounting for more than 70% of total ventricular myosin (55). The majority of HCM-causing mutations is of the missense variety and clustered within the globular head. The genetic basis of HCM was first described as a missense mutation in this gene resulting in the substitution of glutamine for arginine at residue 403 (Arg403Gln) (56).

Hundreds of different MYH7 missense mutations have been reported in both familial and sporadic disease. Overall MYH7 mutations account for ~35% of cases of HCM (43,44,57). The phenotypic expression of these mutations is usually obvious with significant degrees of LVH apparent by late adolescence. However, recent studies indicate that 33.3% of familial childhood-onset HCM and 17.6% of sporadic childhood-onset HCM carry MYH7 mutations (9). Another independent study shows that a total of 16.7% of HCM patients with sarcomeric mutations were diagnosed even before 1 year of age. More than half of these pediatric patients also have family history of HCM (58). These findings could be attributable to background genotypes, lifestyle and environmental factors, presumably contributing to the clinical onset of HCM with MYH7 mutations or other gene mutations. Although heterogeneous, the clinical course of certain MYH7 mutations has been quite severe, associated with an increased risk of sudden death or development of end-stage heart failure. The precise determinants of the relationship between prognosis and underlying gene mutation are likely multifactorial and currently incompletely understood. Mutations that result in a change in the charge of the substituted amino acid may result in more severe disease, presumably due to more dramatic effects on protein structure and function.

**47.2.4.2 Cardiac Myosin Binding Protein C (MYBPC3).** The cardiac myosin binding protein C gene (MYBPC3) contains 37 exons spanning 24kb of chromosome 11 and encoding a 1274-amino-acid (137kDa) protein. Cardiac myosin binding protein C is arrayed into strips that provide structural support to the sarcomere by binding myosin heavy chain and titin (59,60). Functionally, cardiac myosin binding protein C protein may provide structural integrity to the sarcomere as well as modulate myosin ATPase activity and cardiac contractility in response to adrenergic stimulation. HCM mutations in MYBPC3 include missense, splice site, and deletion/insertion mutations and account for ~45% of cases of HCM (9,43,44). In a significant subset of individuals with MYBPC3 mutations, the development of clinically apparent LVH is delayed until age 50 years or above (20,29). MYBPC3 mutations have also been identified in 20% of a cohort of individuals with elderly-onset HCM, which is distinguished by late-onset hypertrophy and absence of family history (20). Similar to MYH7 mutations, MYBPC3 mutations have been identified in 21.2% of familial childhood-onset HCM and 25.5% of sporadic childhood-onset HCM (9). Most of childhood-onset

mutations are missense (76%) while 60% of previously reported mutations in adults encode truncation. This observation implies higher functional consequences of missense mutations than those of truncation mutations.

Several founding mutations have been identified in MYBPC3 gene, most of which cause truncated amino acid sequences. Amongst south Asians, a 25-bp deletion in intron 32 in MYBPC3 accounts for HCM in 4% of Indian subcontinental population (46). This common variant is associated with disorganized cardiac myocyte architecture in vitro and a sevenfold increased risk of chronic heart failure. This data suggests that analysis of HCM-causing genes would emerge as a promising strategy for identifying persons at high risk for cardiac remodeling and heart failure (61).

**47.2.4.3 Cardiac Troponin T (TNNT2).** Cardiac troponin T links the troponin complex to  $\alpha$ -tropomyosin and thus plays a central role in the regulation of contractile function. The cardiac troponin T gene (TNNT2) spans 17kb of DNA on chromosome 1 and encodes a 288-amino-acid peptide (36–39kDa). Several distinct isoforms are expressed in cardiac tissue via alternative splicing of 16 exons (62). Approximately 10% of HCM is thought to be attributable to TNNT2 mutations. Historically, the clinical phenotype resulting from TNNT2 mutations has been characterized by modest or clinically unapparent amounts of LVH but increased risk of sudden death. However, this classic description is based on information from a highly selected, referral-based population, and “benign” TNNT2 mutations have also been reported (63,64).

**47.2.4.4  $\alpha$ -Cardiac Actin (ACTC1).** This 375-amino-acid protein (41kDa) is encoded by the cardiac actin gene (ACTC1) and organized into six exons on chromosome 15 (65). Mutations in actin are typically located in proximity to the putative myosin binding site and are a rare cause of HCM (57,66). Actin mutations have also been identified as a rare cause of familial dilated cardiomyopathy (DCM) (~1%) (67).

**47.2.4.5  $\alpha$ -Tropomyosin (TPM1).** The  $\alpha$ -tropomyosin gene (TPM1) is organized into 15 exons on chromosome 15 (68) and encodes a 284-amino-acid protein expressed in both fast skeletal and cardiac muscle (69).  $\alpha$ -tropomyosin forms a complex with troponin T that regulates actin-myosin interaction in response to intracellular  $\text{Ca}^{2+}$  concentration. Calcium binding to troponin C causes conformational changes in troponins I and T that ultimately allow actin-myosin cross-bridge formation. Mutations in TPM1 are thought to account for a small proportion (~5%) of familial and sporadic HCM (70,71), including a potential founding mutation that arose in the Finnish population (72).

Although  $\alpha$ -tropomyosin is expressed in both cardiac and skeletal muscle, the clinical expression of TPM1 mutations is dominated by HCM, rather than skeletal myopathy. The cardiac specificity of phenotype may be due to the fact that the identified mutations alter the

portions of the  $\alpha$ -tropomyosin molecule that interact with the cardiac-specific isoform of troponin T. Alterations in  $\text{Ca}^{2+}$  sensitivity may also play a role in the tissue-specificity of TPM1 mutations (73).

**47.2.4.6 Cardiac Troponin I (TNNI3).** Cardiac troponin I is the inhibitory subunit of the troponin complex, acting to inhibit actin-myosin interaction. When  $\text{Ca}^{2+}$  binds to troponin C, conformational changes occur in troponin I that release the inhibition of troponin T and  $\alpha$ -tropomyosin, allowing actomyosin cross-bridge formation to occur. The cardiac-specific isoform of troponin I (TNNI3) spans eight exons on chromosome 19 and encodes a 210-amino-acid (27–31 kDa) protein (74). Direct DNA sequence analysis of TNNI3 in patients with HCM suggests that mutations in this gene account for ~10% of disease. Detailed survival analyses have not been performed.

**47.2.4.7 Myosin Light Chains.** The regulatory (MYL2, chromosome 12) and essential (MYL3, chromosome 19) myosin light chains belong to the EF-hand superfamily of proteins with a helix–loop–helix motif (75). They may be involved in determining the speed and force of actomyosin sliding by interacting with the head-rod junction of myosin molecules (76,77). Mutations in myosin light chains are rarely reported genetic etiologies of HCM, accounting for 1–3% of disease (78).

**47.2.4.8 Putative HCM Genes.** Despite substantially rare occurrences in only a small number of isolated probands, mutations in other genes, including  $\alpha$ -actinin (79),  $\alpha$ -MHC (20), cardiac ankyrin repeat protein (ANKRD1; Ref. (80)), cardiac troponin C (TNNC1; Ref. (81)), metavinculin (VCL) and MLP (CSRP3; Ref. (82)), myozenin 2 (MYOZ2; Ref. (83)), phospholamban (PLN; Ref. (84)), telethonin (TCAP Ref. (85)), titin (TTN; Ref. (86)), and vinculin (VCL; Ref. (87)) have been reported in association with HCM. Clinical significances of these genes have not yet been definitely determined due to a paucity of strong evidence that these are definitive HCM genes. Most of these genes are not included in clinical genetic testing for HCM.

## 47.2.5 Animal Models of HCM

Mutations in sarcomere proteins may alter actin-myosin interaction, intracellular calcium cycling, force generation or the transmission of force. However, the heterogeneity of HCM in the human population has challenged the dissection of the precise molecular mechanisms that lead from inherited gene defect to clinical phenotype.

To evaluate the consequences of specific mutations experimental cell and in vivo models of HCM have been developed. Genetically modified animals have been developed incorporating different human mutations in MYH7, MYBPC3, and TNNT, and TNNI3. Rodents carrying these gene mutations develop a phenotype that recapitulates human HCM, including age-dependent myocardial hypertrophy, fibrosis, and myocyte disarray. Interrogation of the biophysical properties of sarcomeres

with particular mutations with myocardial contractility, myocyte biochemistry and transcriptional responses has helped to clarify the molecular events associated with specific HCM mutations.

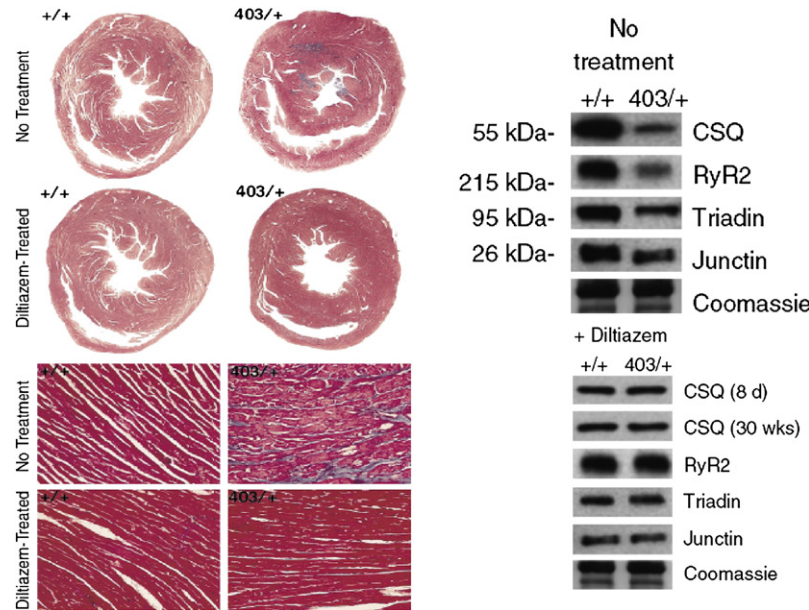
An HCM model (denoted as  $\alpha$ -MHC<sup>403/+</sup>) that expresses the human myosin heavy chain mutation, Arg403Gln (88) has been extensively studied. The cardiac phenotype of  $\alpha$ -MHC<sup>403/+</sup> mice closely mirrors human HCM histopathology. Although general life expectancy is not significantly altered, heterozygous mice demonstrate increased arrhythmogenicity and risk for exercise-induced sudden death as compared with their wild-type littermates (89). Enhanced systolic contractile performance (dominant-activating effect) was identified by examining single mutant myosin molecules from  $\alpha$ -MHC<sup>403/+</sup> myocytes as well as intact heart preparations (90–92). Diastolic function has been shown to be significantly impaired even prior to the development of hypertrophy (93).

Why sarcomere protein mutations in cardiac myocytes that enhance biophysical properties lead to cardiac hypertrophy became an important mechanistic question. Biochemical studies highlighted the crucial role of intracellular  $\text{Ca}^{2+}$  handling plays in linking alterations of muscle contraction to myocyte hypertrophy.  $\alpha$ -MHC<sup>403/+</sup> myocytes have diminished SR release of calcium in response to caffeine and decreased levels of calcium binding proteins calsequestrin, ryanidine receptor, triadin, and junctin (94). These biochemical alterations may worsen cardiac diastolic dysfunction in HCM. Abnormal calcium signaling has been demonstrated in other mouse models harboring a human mutation in troponin T (95) and regulatory myosin light chains (96) and has been proposed to be a remarkable characteristic of other cardiomyopathies (97,98).

The biochemical defects identified in HCM models has raised the possibility that diminishing calcium signals might be beneficial in HCM. To test this, the L-type calcium channel blocker, diltiazem was administered to young  $\alpha$ -MHC<sup>403/+</sup> mice (99) prior to the development of histologic or morphologic abnormalities (prehypertrophic phase: age 6–8 weeks). Diltiazem treatment was associated with a decreased degree of fibrosis, and hypertrophy, both at the gross and histologic levels. Aberrant myocyte biochemistry was also improved with normalization of levels of calcium binding proteins (Figure 47-5) (94,100). One consequence of the Arg-403Gln mutation may be abnormal sequestration of calcium by the mutant sarcomere with ultimate depletion of SR  $\text{Ca}^{2+}$  levels. Diltiazem may serve to blunt this effect by restoring more appropriate calcium cycling between the SR and the cytoplasm and thereby interrupting the signals leading to hypertrophic remodeling. Fundamental dysregulation of calcium handling caused by myosin mutations may in part mediate the hypertrophic response of the myocyte (Figure 47-4).

Calcium cycling abnormalities may enhance the probability for after depolarization in cardiac myocytes, a



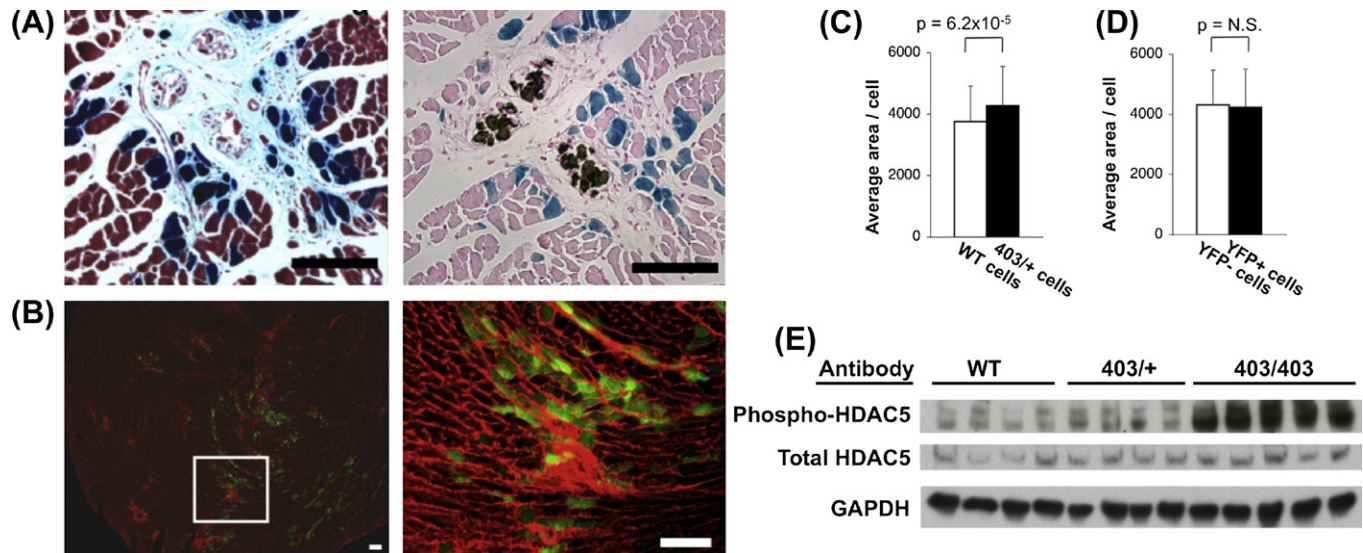


**FIGURE 47-5** Normalization of abnormal  $\text{Ca}^{2+}$  improves histopathology in a mouse model of HCM. Left panel. Histopathology of diltiazem-treated wild-type and  $\alpha\text{-MHC}^{403/+}$  mice as compared with untreated mice. Whole heart sections (top; magnification =  $2.5\times$ ) stained with Masson trichrome reveals fibrosis (blue staining) in untreated HCM mice. Histology (bottom; magnification =  $100\times$ ) shows a reduction in myocyte hypertrophy, disarray, and fibrosis in diltiazem-treated animals compared with untreated animals. Right panel. Cardiac myocyte  $\text{Ca}^{2+}$ -related protein expression is normalized by diltiazem treatment. Western blot analysis of calsequestrin (CSQ), ryanodine receptor (RyR2), triadin, and junctin shows a reduction in the amount of protein expression in untreated  $\alpha\text{-MHC}^{403/+}$  mice. Diltiazem treatment for 8 days (CSQ) or 30 weeks (triadin, junctin, RyR2, CSQ) leads to normalization of the expression levels of these proteins. Coomassie staining is shown to indicate sample loading in each lane. (Adapted from Semsarian, C., Ahmad, I., Giewat, M., et al. *The L-type Calcium Channel Inhibitor Diltiazem Prevents Cardiomyopathy in a Mouse Model*. *J. Clin. Invest.* **2002**, 109, 1013, with permission of The American Society for Clinical Investigation.)

cellular substrate for cardiac arrhythmias, a rampant complication in HCM (101). Electrophysiologic studies in  $\text{MHC}^{403/+}$  mice showed more ventricular arrhythmias than in mice with low-risk mutations in the myosin binding protein C (102,103).  $\text{MHC}^{403/+}$  mice have also been used to assess the correlation between HCM histopathology and arrhythmic events. In human HCM, myocardial fibrosis has been recognized in post-mortem hearts from HCM patients who succumbed to SCD (104) and myocardial fibrosis has been proposed to be a significant contributor to life-threatening arrhythmias (105,106). However, analyses of arrhythmia susceptibility in  $\alpha\text{-MHC}^{403/+}$  mice in the context of myocardial fibrosis, hypertrophy and myocyte disarray demonstrated a significant correlation only between inducible ventricular arrhythmias and the degree of hypertrophy (107). Consistent with these experimental data, substantial LVH (LV wall thickness  $\geq 30$  mm) is established risk factor for sudden death in human patients (108). Given these findings, the strategies that prevent hypertrophy in HCM would potentially reduce the risk for arrhythmia development and sudden cardiac death. Alteration of biophysical properties and intracellular  $\text{Ca}^{2+}$  in HCM myocytes as well as higher energy demands impose great stress on cardiac myocytes. Molecular studies in the  $\alpha\text{-MHC}^{403/+}$  mouse model also indicate that fetal cardiac genes typically repressed following embryonic development

re-expressed with myocyte stress (109), a finding similar to the studies in human HCM hearts (110). Recent investigations have also uncovered a relationship between the early onset of calcium cycling defect in stressed myocytes and the premature myocyte demise and myocardial scarring (interstitial matrix expansion and focal replacement fibrosis) in HCM mouse models (111). The myocyte enhancer factor-2 (Mef2) family of transcription factors is activated in several myocardial pathologies that are marked by cardiac hypertrophy and myocardial fibrosis (112) and Mef2 expression identifies stressed myocytes. Mef2 activity has been assessed in  $\alpha\text{-MHC}^{403/+}$  mice, using a Mef2-LacZ ( $\beta$ -galactosidase) transgene. Mef2 activity in HCM hearts was strikingly inhomogeneous and closely associated with foci of myocyte necrosis and scarring (Figure 47-6). In homozygous mutant ( $\alpha\text{-MHC}^{403/403}$ ) mice that have marked and relentless pathologic remodeling until death at day 10, Mef2 activation was substantially accentuated and evident shortly before rampant myocyte demise, implicating Mef2 activation in HCM as a molecular marker of stressed myocytes that are destined to die (111). Abnormal calcium signaling can lead to Mef2 activation via calcium/calmodulin dependent protein kinase II (CaMKII) phosphorylation (113,114), a pathway that may link aberrant calcium signaling to premature myocyte death and focal myocardial scarring in HCM hearts.





**FIGURE 47-6** Mef2 activation in HCM myocytes. A. Colocalization of Mef2-dependent reporter activity with fibrosis (left, Gomori trichrome staining) and necrosis (right, von Kossa staining) in 30-week-old  $\alpha\text{MHC}403/+/\text{Mef2-LacZ}$  hearts. B.  $\beta\text{MHC}$  re-expression (green, identified by YFP) occurred in regions of myocardial scars (red, identified by WGA) in 30-week-old  $\alpha\text{MHC}403/+/\text{Mef2-LacZ}/\beta\text{MHC-YFP}$  hearts. Right shows magnified view of inset. C. Cross-sectional areas of myocytes from 30-week-old WT/ $\text{Mef2-LacZ}/\beta\text{MHC-YFP}$  hearts (WT cells; open bar) and  $\alpha\text{MHC}403/+/\text{Mef2-LacZ}/\beta\text{MHC-YFP}$  hearts (403/+ cells; black bars) irrespective of their fluorescence. D. Cross-sectional areas of the 403/+ cells grouped according to their YFP fluorescence (YFP- and YFP+, respectively). E. Increased phosphorylation of HDAC5 and Mef2-dependent reporter activity in  $\alpha\text{MHC}403/403$  hearts. Western blot analyses of protein lysates from 6-day-old WT,  $\alpha\text{MHC}403/+ (403/+)$ , and  $\alpha\text{MHC}403/403 (403/403)$  hearts, reacted with antibodies specific Phospho-HDAC5, total HDAC5, and GAPDH. (Adapted from Konno, T.; Chen, D.; Wang, L., et al. *Heterogeneous Myocyte Enhancer Factor-2 (Mef2) Activation in Myocytes Predicts Focal Scarring in Hypertrophic Cardiomyopathy*. PNAS **2010**, 107, 18097–18102.)

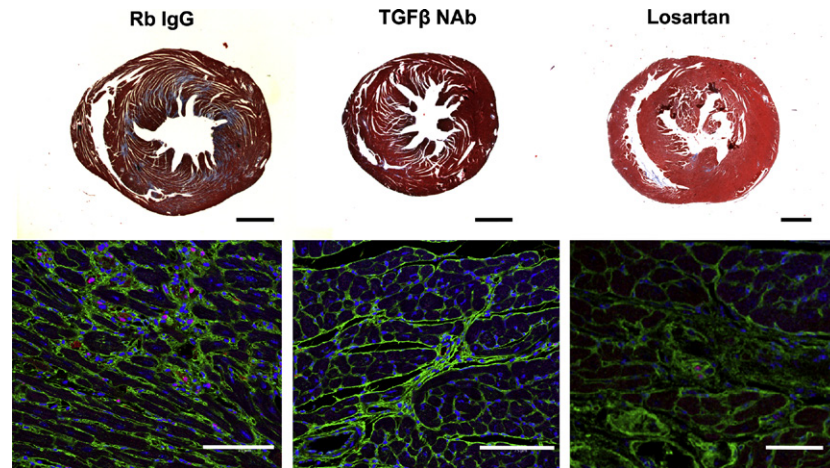
HCM mice have also been studied to define the early transcriptional changes that occur in response to a sarcomere gene mutation (109,115). All expressed RNAs in mutant myocyte and non-myocyte cells from the hearts of young prehypertrophic mice were compared with RNAs found in myocytes and non-myocytes from wild-type mice. These experiments revealed a striking increase in the expression of pro-fibrotic molecules within the transforming growth factor (Tgf) signaling pathway (including Tgf- $\beta$ 1, Tgf- $\beta$ 2, periostin and connective tissue growth factor) and cell-cycle proteins in non-myocyte cells of prehypertrophic hearts. BrdU labeling and Ki617 immunostaining confirmed transcriptional profiling data and revealed increased proliferation of non-myocytes.

To assess whether silencing of these profibrotic molecules could prevent the emergence of HCM pathology, anti-Tgf- $\beta$  neutralizing antibody was administered to young prehypertrophic mutant mice, a treatment that markedly diminished the emergence of myocardial fibrosis and hypertrophy (Figure 47-7) (115). To extend these studies, losartan, an angiotensin II (type 1) receptor antagonist was also used to inhibit Tgf- $\beta$  pathway signals. Similar to the neutral antibodies, chronic administration of losartan to prehypertrophic mutant mice prevented the emergence of LV hypertrophy and HCM pathologic features (Figure 47-7). Taken together, these data define Tgf- $\beta$  signaling as a pivotal mechanism for myocardial fibrosis development and a potentially important contributor to diastolic dysfunction and heart failure (Figure 47-8). Preemptive pharmacologic antagonism of Tgf- $\beta$  signals

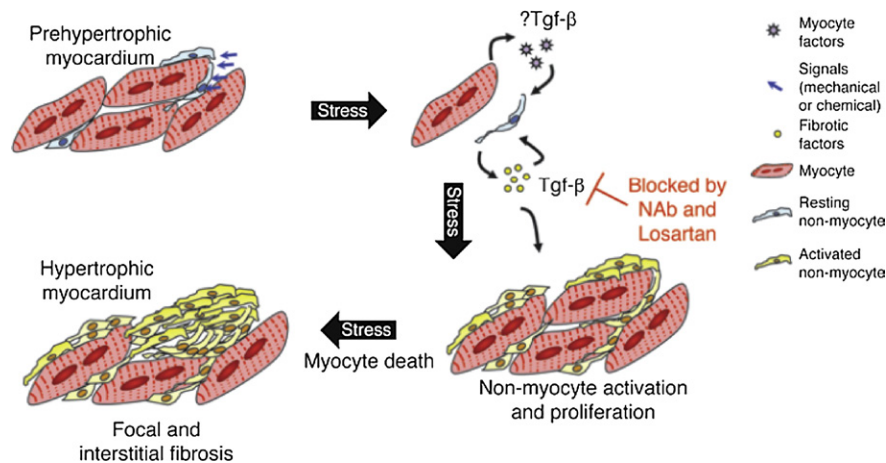
warrants clinical study in asymptomatic patients with sarcomere gene mutations.

In addition to the  $\alpha\text{-MHC}$  mouse model, a variety of other genetically modified mice, rats, and rabbits have been developed, incorporating mutations in  $\beta\text{-myosin}$  heavy chain, cMyBPC,  $\alpha\text{-tropomyosin}$ , and cardiac troponin T (93,116–120). These models typically show myocyte disarray, variable degrees of hypertrophy, systolic and diastolic dysfunction. The  $\beta\text{-MHC}^{403/+}$  transgenic rabbit model (117) is an attractive model, as the  $\beta$ -isoform of MHC is predominant in rabbit and adult human hearts, whereas the  $\alpha$ -isoform predominates in mice and rats. These rabbits show significant hypertrophy, fibrosis, diastolic dysfunction and an increased risk of sudden death (117). As with the  $\alpha\text{-MHC}^{403/+}$  mouse model, impaired myocardial relaxation develops in advance of hypertrophy (121). This finding has recently been replicated and extended to human patients with HCM (122).

Pharmacologic studies have also been performed to target fibrosis pathways rather than intracellular calcium handling and to reverse myocardial fibrosis in some other animal models of HCM. For examples, angiotensin II receptor type 1 blockade was associated with the reversal of interstitial fibrosis and decreased expression of collagen Ia and Tgf- $\beta$ 1 in mice with a cardiac troponin T mutation (123) who received losartan treatment (124).  $\beta\text{-MHC}^{403/+}$  transgenic rabbits were administered the HMG-CoA reductase inhibitor, simvastatin, for 12 weeks. As compared with placebo-treated transgenic



**FIGURE 47-7** Treatment with anti-TGF- $\beta$  neutralizing antibody (TGF- $\beta$  NAb) or losartan prevents the development of cardiac hypertrophy and fibrosis. Upper panel. Whole heart sections from mice treated with control IgG (Rb IgG) shows fibrosis (blue, Mason trichrome stain), unlike mice treated with TGF- $\beta$  NAb or losartan. Lower panel. Histologic sections stained with wheat-germ agglutinin (green) to demonstrate fibrosis and DAPI (blue) from BrdU-treated mice show nonmyocyte cell proliferation (magenta) in Rb IgG-treated mice that is reduced by TGF- $\beta$  NAb or losartan treatment. (Adapted with permission from Teekakirikul, P.; Eminaga, S.; Toka, O., et al. *Cardiac Fibrosis in Mice with Hypertrophic Cardiomyopathy Is Mediated by Non-myocyte Proliferation and Requires Tgf- $\beta$* . J. Clin. Invest. **2010**, 120, 3520–3529, with permission of The American Society for Clinical Investigation.)



**FIGURE 47-8** A model for fibrosis and diastolic dysfunction from HCM sarcomere gene mutations. Mutant myocytes have increased biophysical properties and abnormal  $\text{Ca}^{2+}$  homeostasis, factors that trigger mechanical and/or biochemical signals that activate gene transcription, e.g. Tgf- $\beta$ . Tgf- $\beta$  then stimulates nonmyocyte proliferation and expression of profibrotic molecules. Activated nonmyocyte cells secrete profibrotic factors that expand the interstitium, generate more stresses imposed on mutant myocytes, and promote myocyte death with resultant focal scarring. Tgf- $\beta$ -mediated increased interstitial and focal fibrosis may contribute to diastolic dysfunction in HCM hearts. Antagonism of Tgf- $\beta$  signaling by a neutralizing antibody (NAb) or losartan attenuates nonmyocyte proliferation and profibrotic gene expression, thereby preventing cardiac fibrosis. (Adapted with permission from Teekakirikul, P.; Eminaga, S.; Toka, O., et al. *Cardiac Fibrosis in Mice with Hypertrophic Cardiomyopathy Is Mediated by Non-myocyte Proliferation and Requires Tgf- $\beta$* . J. Clin. Invest. **2010**, 120, 3520–3529, with permission of The American Society for Clinical Investigation.)

animals, simvastatin therapy was associated with regression of hypertrophy and fibrosis as well as improved cardiac function and decreased intracardiac filling pressures. In addition, levels of activated stress-responsive signaling kinases (ERK 1/2) were reduced, suggesting that these pathways that trigger myocardial fibrosis and dysfunction may be modulated to improve disease expression (123).

Elevated myocardial aldosterone and aldosterone synthase mRNA levels have been demonstrated in both

humans with HCM and in cTnT-Q (123) mice. Treatment with the mineralocorticoid receptor antagonist, spironolactone, reversed interstitial fibrosis, attenuated myocyte disarray, and improved diastolic function (125). These promising results in animal studies highlight the importance of ongoing collaborative basic science and human clinical investigation to further our understanding of the pathophysiology of HCM and to allow for the development of novel treatment strategies for human disease.



## 47.2.6 New Paradigms of Inherited Cardiac Hypertrophy

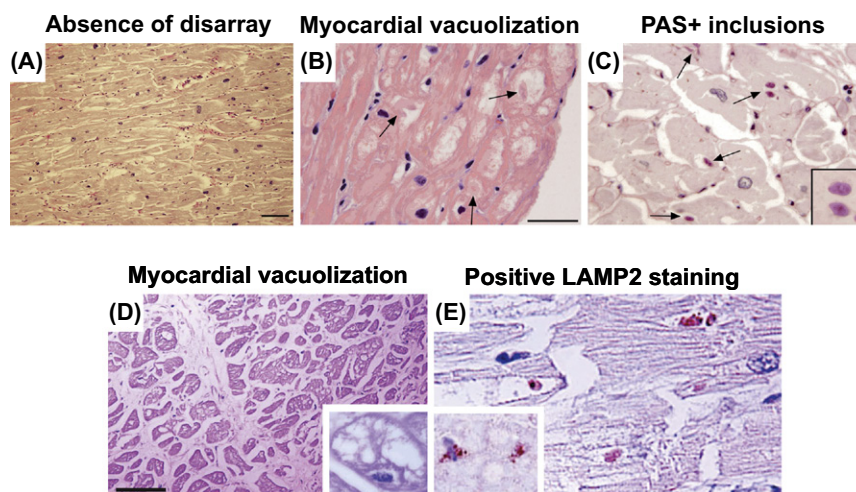
**47.2.6.1 Deficits of Energy Production and Regulation.** Gene mutations have been described in non-sarcomere proteins that produce clinical manifestations that mimic HCM phenotypes. Unexplained LVH that occurs with conduction abnormalities (progressive atrioventricular block, atrial fibrillation, and ventricular pre-excitation/Wolff–Parkinson–White syndrome) can be due to mutations in the  $\gamma$ -2 regulatory subunit (PRKAG2) of adenosine monophosphate (AMP)-activated protein kinase (AMPK), an enzyme involved with glucose metabolism, as well as mutations in the X-linked lysosome associated membrane protein (LAMP2) gene (12,126,127). In these patients, ventricular pre-excitation typically occurs early in life and is often symptomatic. These pathologies are not genetically related to the pre-excitation that occurs in isolated WPW (128), an arrhythmia that occurs in structurally normal hearts. Despite an increased prevalence of WPW in first-degree relatives of affected individuals, the genetic cause(s) of isolated WPW has not been elucidated (129).

Several important differences help to discriminate between HCM and PRKAG2 cardiomyopathies. The myocyte histopathology produced by PRKAG2 mutations lacks both the myocyte disarray and markedly increased fibrosis found in HCM and instead shows glycogen and amylopectin accumulation (Figure 47-9). Progressive cardiac conduction system disease occurs with increasing age in patients with PRKAG2 mutations, such that permanent pacemaker implantation is required in 30% of affected individuals (130). Severe

clinical outcomes were noted in a subset of patients with PRKAG2 mutations, including progression to end-stage heart failure or transplantation and sudden cardiac death.

LAMP2 mutations produce profound cardiac remodeling. Encoded on chromosome X, LAMP2 mutations are distinguished from HCM (Figure 47-10; Table 47-2) caused by sarcomere mutations due to the presence of ventricular pre-excitation, male-predominance, earlier age of presentation, striking LVH that can be massive (LV wall thickness >50mm) and is typically concentric, prominent voltage on EKGs and prevalent arrhythmias and progression to heart failure (131,132). LAMP2 cardiomyopathy causes early death in affected males, typically before age 30. Despite male preponderance, LAMP2 cardiomyopathy can also occur in female patients, presumably due to X-inactivation of the normal chromosome. LAMP2 mutations produce the multisystemic disorder, Danon disease, in which neurologic, skeletal muscle, and hepatic involvement occur with cardiomyopathy.

Despite the common finding of cardiac hypertrophy, LVH caused by PRKAG2 or LAMP2 mutations define a new paradigm for hypertrophic remodeling. This is a disease entity distinct from HCM caused by sarcomere protein mutations. Despite sharing a phenotype of cardiac hypertrophy with HCM, the different molecular signaling pathways triggered by PRKAG2 and LAMP2 mutations are likely to be different from those produced by sarcomere gene mutations. These differences suggest that the clinical approach to individuals with PRKAG2 and LAMP2 mutations should not be predicated on

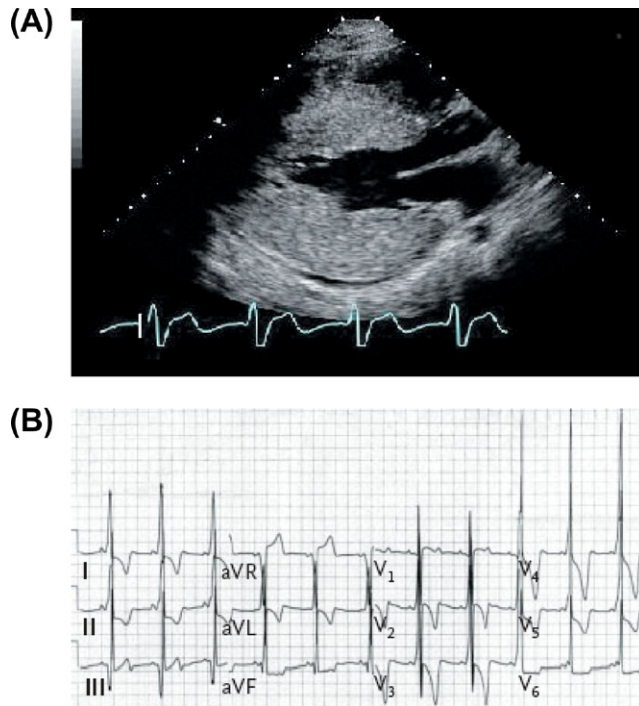


**FIGURE 47-9** Histopathology of glycogen storage cardiomyopathies. A and B. PRKAG2 mutations cause nonmembrane bound vacuoles (arrows) that stain for glycogen and amylopectin in myocytes. There is less fibrosis than in HCM and no myocyte disarray. C. Homogenous inclusions within vacuoles (arrows and inset) stained positive with PAS are mostly diastase-resistant. (Reproduced from Arad, M.; Benson, D. W.; Perez-Atayde, A. R., et al. *Constitutively Active AMP Kinase Mutations Cause Glycogen Storage Disease Mimicking Hypertrophic Cardiomyopathy*. *J. Clin. Invest.* **2002**, 109, 357–362, with permission of The American Society for Clinical Investigation.) D. LAMP2 mutations cause myocyte enlargement with prominent pleomorphic nuclei, and numerous cytoplasmic vacuoles. A vacuolated myocyte with a “spider cell” (inset) resembles rhabdomyoma cells. E. Immunohistochemical staining with LAMP2-specific antibodies exhibits positive (red) staining within vacuoles. (Adapted with permission from Arad, M.; Maron, B. J.; Gorham, J. M., et al. *Glycogen Storage Diseases Presenting as Hypertrophic Cardiomyopathy*. *N. Engl. J. Med.* **2005**, 352, 362–372.)

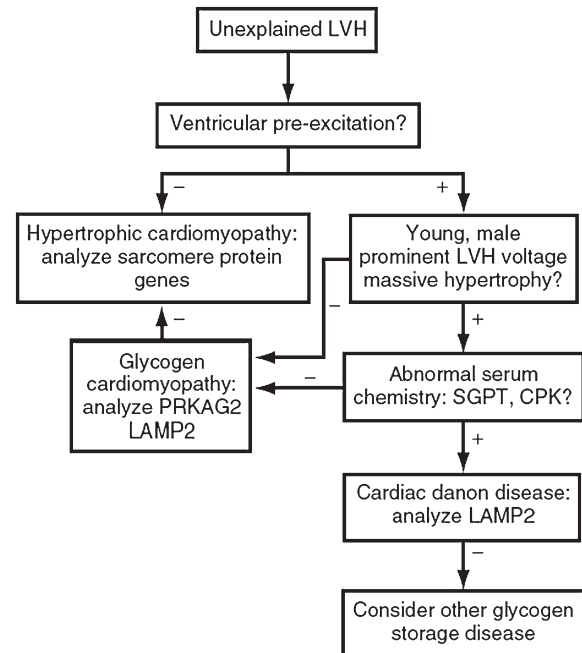
management tenets of HCM. An algorithm to differentiate HCM from glycogen storage cardiomyopathies is illustrated in Figure 47-11.

**47.2.6.2 Animal Models for PRKAG2 Cardiomyopathy.** A transgenic mouse model harboring the PRKAG2 Asn488Ile mutation under the cardiac  $\alpha$ -MHC promoter was constructed and recapitulated hallmarks of human

disease (133). Transgenic mice exhibited elevated AMPK activity, increased glycogen accumulation, massive LVH, progressive impairment of contractility, and ventricular pre-excitation and sinus node dysfunction. Ventricular pre-excitation occurred postnatally within the first



**FIGURE 47-10** LAMP2 mutations are associated with striking evidence of LVH. A. An electrocardiogram demonstrates a short PR interval, delta waves, and prominent voltage (R waves >50mm in precordial leads V4 and V5). B. The parasternal, long-axis view of an echocardiogram shows marked hypertrophy (maximal left ventricular wall thickness, >35mm) involving the interventricular septum and posterior left ventricular wall. (Adapted with permission from Arad, M.; Maron, B. J.; Gorham, J. M., et al. *Glycogen Storage Diseases Presenting as Hypertrophic Cardiomyopathy*. *N. Engl. J. Med.* **2005**, 352, 362–372.)



**FIGURE 47-11** An algorithm for the diagnosis of unexplained left ventricular hypertrophy (LVH). HCM is suggested by an autosomal dominant pattern of inheritance of LVH that occurs without systemic manifestations or conduction system abnormalities. Glycogen storage cardiomyopathy is suggested by the presence of ventricular pre-excitation in conjunction with unexplained LVH. PRKAG2 mutations are suggested by autosomal dominant inheritance and the absence of systemic manifestations. X-linked LAMP2 mutations are suggested by early-onset of cardiac-manifestations (Figure 47-10), male gender and progressive abnormalities in liver, musculoskeletal or neurologic function. (Adapted with permission from Arad, M.; Maron, B. J.; Gorham, J. M., et al. *Glycogen Storage Diseases Presenting as Hypertrophic Cardiomyopathy*. *N. Engl. J. Med.* **2005**, 352, 362–372.)

**TABLE 47-2** Cardiac Findings Associated with Mutations in Sarcomere-Protein Genes, PRKAG2, and LAMP2

	Sarcomere Protein Genes (N = 40) <sup>a,b</sup>	PRKAG2 (N = 32) <sup>b</sup>	LAMP2 (N = 7) <sup>b</sup>
Age at diagnosis (year)	33 ± 17 <sup>c</sup>	31 ± 15 <sup>c</sup>	15 ± 4
No. of distinct mutations	35	4	6
Preexcitation (%)	0 <sup>c</sup>	9 (28) <sup>d</sup>	6 (86)
Maximal left ventricular wall thickness (mm)	24 ± 10 <sup>c</sup>	17 ± 8 <sup>c</sup>	35 ± 15
S <sub>V1</sub> or S <sub>V2</sub> + R <sub>V5</sub> or R <sub>V6</sub>	48 ± 21 <sup>c</sup>	40 ± 21 <sup>d</sup>	92 ± 46
Maximal R or S (mV)	34 ± 13 <sup>c</sup>	32 ± 11 <sup>c</sup>	67 ± 17

Statistical analyses are comparisons to LAMP2 data using the chi-square test to compare pre-excitation and the Wilcoxon rank-sum test for all other parameters.

<sup>a</sup>Subjects with HCM with a defined sarcomere gene mutation.

<sup>b</sup>Numbers of subjects include probands and clinically affected family members.

<sup>c</sup>P < 0.002.

<sup>d</sup>P < 0.01.

Data from Arad, M.; Maron, B. J.; Gorham, J. M., et al. *Glycogen Storage Diseases Presenting as Hypertrophic Cardiomyopathy*. *N. Engl. J. Med.* **2005**, 352, 362–372.



month of life. A continuous thick fibrous layer (annulus fibrosis), which typically electrically insulates the ventricles from the atria was rendered discontinuous by vacuolated myocytes in transgenic mice (133,134). Phenotypic manifestations are mediated through enhanced activity of  $\alpha 2$ -subunit complexes (135). To assess whether cardiomyopathy, ventricular pre-excitation, and conduction system disease in PRKAG mutant hearts are prevented or reversed by glycogen modulation, the tetracycline-regulated transcriptional activator system was used in this transgenic mouse (PRKAG2<sup>Asn488Ile</sup>). It appeared that PRKAG2 cardiomyopathy, cardiac dysfunction, and conduction deficits were reversible mechanisms. Transgene suppression during early postnatal development completely prevented the emergence of accessory electrical pathways but not cardiomyopathy or progressive conduction system degeneration (136). These data suggest that developmental timing of glycogen depletion is critical to prevent accessory pathway, and imply that myocardial dysfunction and conduction system disease in PRKAG2 cardiomyopathy could potentially be treated by modulation of glycogen content, a viable alternative treatment option to device implantation and cardiac transplantation for patients with glycogen storage cardiomyopathy and arrhythmias.

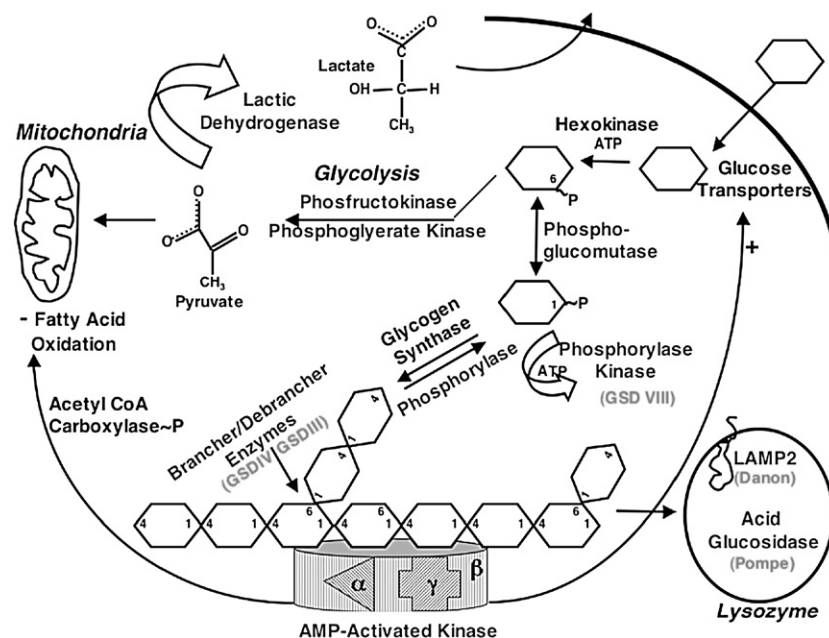
These non-sarcomere cardiomyopathies now become part of a subgroup of glycogen storage diseases (Figure

47-12), such as Pompe disease, a glycogen storage disease with acid maltase deficiency in infants and Fabry disease, an X-linked recessive glycosphingolipid disorder caused by the lysosomal enzyme  $\alpha$ -galactosidase A deficiency (137). Many other mutations leading to LV hypertrophy by defects in sarcomere, metabolic, and other genes remain to be discovered.

### 47.2.7 Diagnosis

The identification of unexplained LVH, typically via echocardiographic imaging, has traditionally formed the basis for the diagnosis of HCM. However, the presence of LVH is not an infallible marker for disease and is not universally present throughout life or in all individuals with sarcomere gene mutations. Thus, unexplained LVH is not the most specific or sensitive manifestation of HCM and further definition of the full spectrum of the HCM phenotype is required. Animal and human studies have indicated that diastolic abnormalities are present prior to the development of LVH (88,121,122,138). Biochemical abnormalities, namely alterations in intracellular calcium handling, may represent an even more fundamental manifestation of sarcomere gene mutations (94,99).

Myocardial fibrosis, a pathological hallmark of HCM, contributing to sudden cardiac death, cardiac

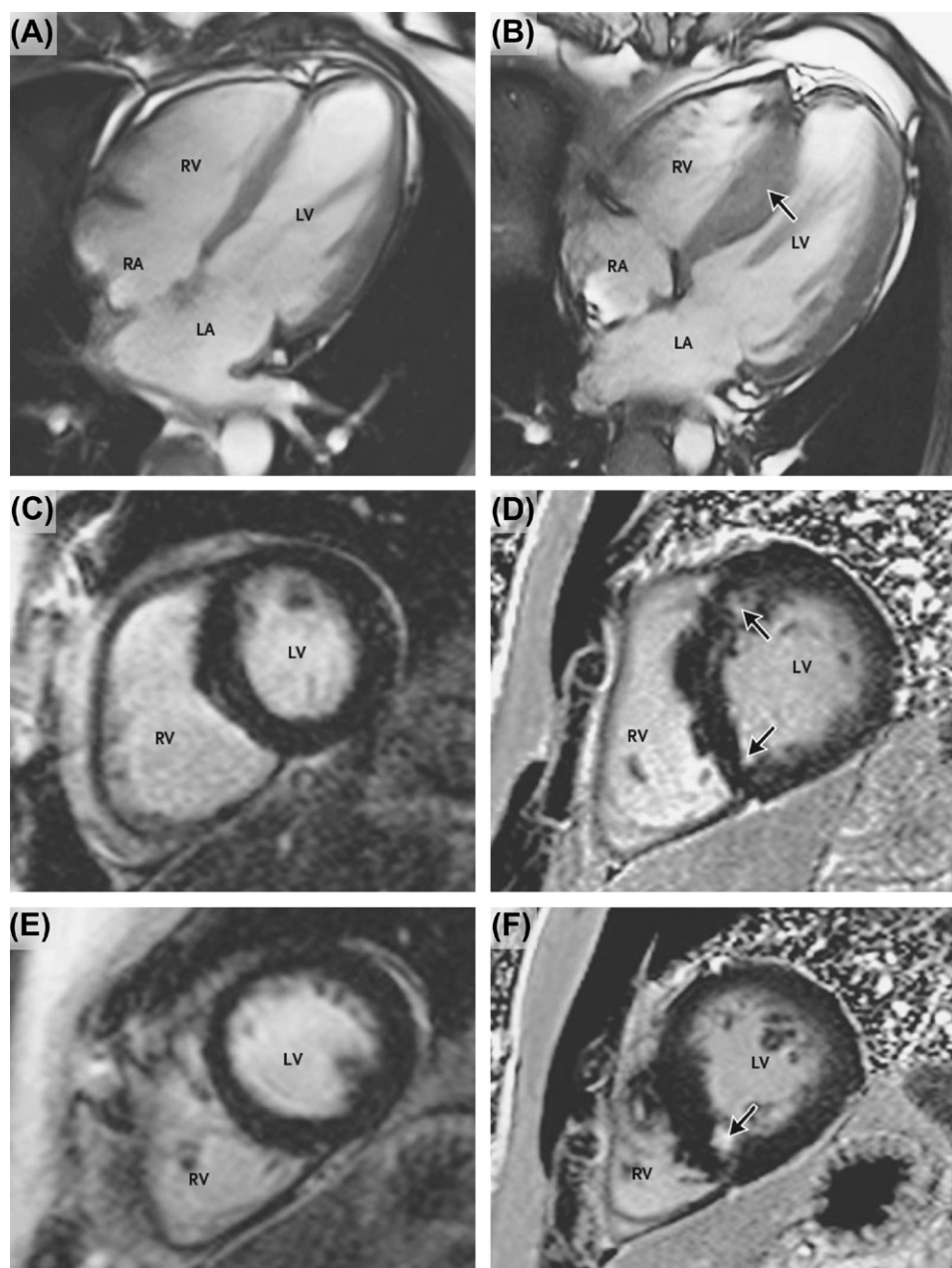


**FIGURE 47-12** Muscle glycogen metabolism pathways associated with cardiac diseases. Glucose enters the myocyte through transmembrane transport proteins and undergoes phosphorylation by hexokinase. It then enters pathways for glycolysis or glycogen synthesis by glycogen synthase. Glycogen, a branched glucose polymer, is a dynamic energy reservoir for muscles, as dictated by enzyme activity to change phosphorylation state. Glycogen metabolism is also influenced by AMP-activated protein kinase (regulates glucose uptake and fatty-acid oxidation via acetyl CoA carboxylase) and by lysosome activity. Defects in pathways of glycogen degradation (phosphorylase, phosphoglucomutase, phosphofructokinase, phosphoglycerate kinase, lactic dehydrogenase, and brancher/debrancher enzymes) result in glycogen accumulation. Mutations in PRKAG2 (the regulatory  $\gamma$  subunit of AMP kinase) or LAMP2 may cause glycogen accumulation resulting in cardiac hypertrophy and electrophysiological abnormalities. (Adapted with permission from Arad, M.; Maron, B. J.; Gorham, J. M., et al. *Glycogen Storage Diseases Presenting as Hypertrophic Cardiomyopathy*. N. Engl. J. Med. 2005, 352, 362–372.)

arrhythmias, LV dysfunction, and progressive heart failure (139–141) can be visualized with histologic evaluation and noninvasively with gadolinium-enhanced cardiac MRI (Figure 47-13) (105,142). Recent studies of serologic biomarkers of collagen metabolism in asymptomatic carriers of sarcomere mutations indicate that profibrotic milieu is significantly enhanced, preceding the development of LVH and myocardial fibrosis detectable on MRI (142). These findings suggest that sarcomere mutations have a prominent effect on the heart prior to

the onset of LVH. Their clinical and diagnostic implications may include identifying and monitoring persons at risk for cardiac complications as well as developing novel therapeutic strategies to attenuate fibrosis in HCM.

Genetic testing allows for precise and age-independent identification of individuals at risk for developing HCM and should be incorporated into the contemporary diagnosis of this disorder. Currently genetic diagnosis is accomplished by direct DNA sequence analysis of the exons and intron/exon boundaries of sarcomere

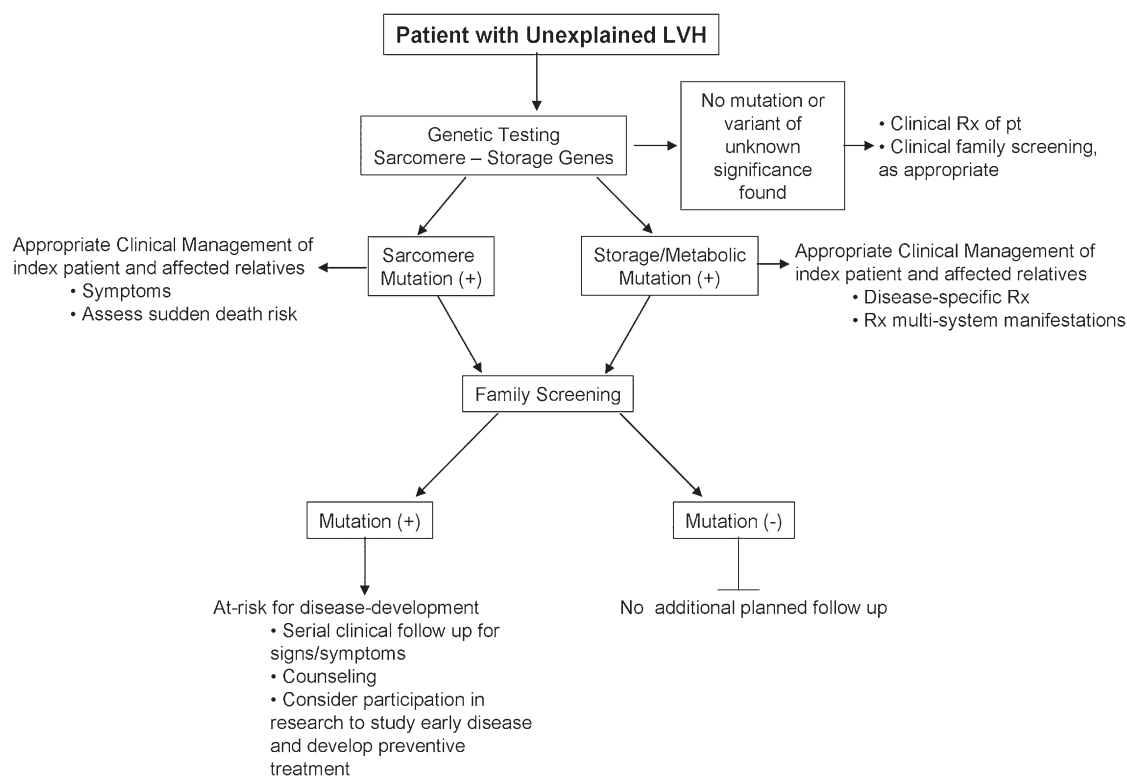


**FIGURE 47-13** Gadolinium-enhanced cardiac MRI in patients with overt HCM and in preclinical mutation carriers. A, C, and E. Young individuals with a sarcomere gene mutation have neither LVH nor late gadolinium enhancement of the myocardium. B, D, and F. Overt HCM shows a markedly thickened septum (B, arrow) associated with areas of heterogeneous delayed enhancement, which involves the anteroseptum (D, arrows) and the inferoseptum (D and F, arrows). LA, left atrium; LV, left ventricle; RA, right atrium; and RV, right ventricle. (Reproduced with permission from Ho, C. Y.; Lopez, B.; Coelho-Filho, O. R., et al. *Myocardial Fibrosis as an Early Manifestation of Hypertrophic Cardiomyopathy*. N. Engl. J. Med. **2010**, 363, 552–563.)

genes in order to identify potential disease-associated sequence variants. Once a sequence variant is identified, pathogenicity is typically determined by verifying that the sequence or structure of the encoded protein is altered, that the sequence variant absent from at least 2000 ethnically matched chromosomes from unaffected subjects, and that appropriate cosegregation with disease occurs in familial cases, or that the variant arises de novo. This is a daunting task that underscores the need for more efficient yet reliable techniques for screening large stretches of DNA. Application of emerging next generation sequencing technologies at subgenomic (up to ~200 genes) and whole exome/genome levels are increasingly allowing high-throughput clinical genetic screening and further discovery of novel causative and disease-modifying genes in patients with HCM. The identification of a sarcomere gene mutation in the appropriate clinical setting allows the definitive diagnosis of HCM and establishes the exact genetic etiology. Mutation confirmation can then be performed in family members in a straightforward manner. Individuals found to carry the family-specific mutation despite the absence of clinical manifestations are at risk for developing HCM and require longitudinal clinical follow-up as described later in this chapter. Such individuals should also be counseled of the 50% chance of transmission of the mutation to offspring. Family members who do not carry the mutation have no risk for developing HCM or

transmitting the condition. Longitudinal clinical follow-up is not necessary. An algorithm to incorporate clinical molecular genetic testing in the approach to patients and families with unexplained LVH is illustrated in Figure 47-14 (51).

There are significant limitations to this strategy of genetic diagnosis. Mutations in sarcomere genes, PRKAG2 and LAMP2 are thought to account for 60–75% of cases of inherited LVH. As such mutations are not detected in all individuals with unexplained LVH. A negative result from molecular screens can not exclude a genetic etiology, but simply eliminates previously identified genes from the list of suspects. Furthermore, in the absence of specific clinical correlates that are associated with genotype, this information remains most useful in establishing certain diagnosis of HCM or future risk for developing the disease. Although management of mutation carriers without clinical disease remains an emerging clinical field, longitudinal follow-up of this subset population is essential and limitations on competitive athletic participation is probably prudent. Continued efforts to elucidate how gene mutations lead to HCM may ultimately allow development of new strategies of disease management that will aim to alter the phenotype rather than merely palliating symptoms. Genetic diagnosis will play a crucial role in allowing us to identify and target therapy to individuals with gene mutations but with clinically undetectable disease.



**FIGURE 47-14** An algorithm for incorporating clinical molecular genetic testing in the approach to patients and families with unexplained left ventricular hypertrophy (LVH). (Reproduced with permission from Ho, C. Y. *Is Genotype Clinically Useful in Predicting Prognosis in Hypertrophic Cardiomyopathy?* *Circulation* **2010**, 122, 2430–2440.)

### 47.2.8 Management

There are two major aspects to the management of HCM: alleviation of symptoms and assessment of the risk for sudden cardiac death. Medical therapy is the cornerstone of treatment for symptomatic HCM and typically incorporates agents such as  $\beta$ -adrenergic and calcium channel antagonists to increase diastolic filling time, slow the heart rate, decrease contractility, and help to normalize intracardiac filling pressures. However, dihydropyridine calcium channel antagonists that have vasodilating effect should not be administered in patients with LVOT because they can enhance the severity of LVOT by decreasing afterload. Diuretics may be used judiciously for symptoms of congestion; however, due to altered LV compliance characteristics, overly aggressive diuresis may be associated with an abrupt fall in blood pressure and worsening of LV outflow tract obstruction. Disopyramide may also be used to attempt to alleviate outflow tract obstruction due to its negative inotropic effects. Afterload reduction should be avoided in patients with outflow tract obstruction or dynamic intracavitary gradients as obstructive physiology may be worsened.

Whether early pharmacological intervention of the molecular signals triggered by sarcomeric mutations might attenuate ventricular remodeling and symptoms has become an important question. Based upon experimental studies (94,99,115), clinical studies of sarcomere-mutation carriers without overt HCM have been characterized. Serum biomarker profiles revealed elevated levels of C-terminal propeptide of type I procollagen (PICP), a finding that suggests that the profibrotic state identified in the hearts of mouse HCM models also occurs in prehypertrophic humans with sarcomere mutations (142). Pharmacologic interventions (that parallel losartan treatment in HCM mice) are planned to assess whether attenuation of profibrotic signals in prehypertrophic patients impacts the emergence of overt disease.

An HCM prevention trial (*Clinical Trials* website) is underway, which uses diltiazem to modify calcium cycling based on studies of HCM mouse models (94). A pilot cohort of 40 prehypertrophic patients with any molecularly identified sarcomere mutation treated with either diltiazem or placebo will be assessed for signs of the development of HCM, including diastolic dysfunction, electrocardiographic abnormalities, and LVH. Estimated study completion date is December 2013.

Mechanical relief of obstructive physiology is warranted in HCM patients with persistent symptoms despite good medical regimens. Interventions to relieve outflow tract obstruction include surgical septal myectomy or catheterization-based alcohol septal ablation (16,143). Careful consideration of the mechanism of obstruction is warranted to assess the potential benefit from invasive procedures and to choose the appropriate strategy.

A number of clinical indicators of increased risk for sudden death have been identified including a prior

history of cardiac arrest or sustained ventricular tachycardia; unexplained syncope; significant nonsustained ventricular tachycardia on ambulatory monitoring; an abnormal blood pressure response to exercise in patients younger than 50 years; massive LVH (>30–35 mm); and a family history of recurrent SCD or identification of a malignant genotype. However estimation of an individual's risk is imprecise. If two or more risk factors are present, an increased risk of SCD is present with an estimated annual mortality of 4–6% (14,31,144). The positive predictive value of each risk factor individually is relatively low. In contrast, the negative predictive value of these risk factors is more useful (18)—if none of these risk predictors is present, the individual is likely at low annual risk for sudden death (<1%). Sudden death may be the initial presenting symptom of HCM or occur in only mildly symptomatic patients. Although sudden death has been associated with vigorous physical activity, many episodes have occurred in the setting of only mild or no exercise. Implantation of an implantable cardioverter-defibrillator (ICD) is effective in decreasing the risk of sudden death from lethal arrhythmias in appropriate individuals (145).

Given the familial nature of HCM, clinical screening of first-degree relatives of affected individuals is recommended. This consists of history, physical examination, 12-lead EKG, and echocardiography. As the penetrance of LVH is age-dependent, the lack of clinical findings on initial assessment does not exclude the possibility of disease or the inheritance of an underlying sarcomere mutation. Serial screening and longitudinal follow-up is required to evaluate for the development of phenotypic manifestations with aging. The age at which screening should begin is not clearly defined, and the following strategy has been proposed for familial HCM (146). Because overt manifestations are unusual in early childhood, screening of children under the age of 12 years is optional unless there are symptoms, a malignant family history of premature HCM-related death, early onset hypertrophy or other serious complications, or if the child is a competitive athlete. Serial screening every 12–18 months is recommended between the ages of 12 and 21 years and then follow-up should be continued at 3- to 5-year intervals thereafter until clinical manifestations develop or until a more definitive genetic diagnosis is established. Recent availability of clinical HCM genetic testing at many molecular diagnostic laboratories (*Gene Tests* website), should promote longitudinal clinical evaluation only to those at risk individuals who have inherited a pathogenic mutation.

## 47.3 DILATED CARDIOMYOPATHY

Pathologic remodeling of the heart resulting in increased chamber volume and diminished contractile function is termed DCM (Figure 47-1). Cardiac mass is increased due to enlargement of the cardiac chambers with only



modest increase in ventricular wall thickness. Histopathologic changes may be relatively subtle with only minor myocyte hypertrophy, degeneration, and interstitial fibrosis. The less distinctive clinical manifestation of DCM compared with hypertrophic remodeling has challenged precise characterization.

DCM that occurs in the absence of disorders that may cause cardiac dilation and dysfunction (coronary artery disease, alcohol abuse, untreated tachycardia, thyroid disease, viral myocarditis, and infiltrative disorders such as hemochromatosis) leads to the diagnosis of “idiopathic” DCM, a disorder that often is recognized in families. Increasingly, the application of molecular genetic studies to “idiopathic” DCM has resulted in the identification of underlying gene mutations. There is considerably more genetic diversity in DCM than in gene mutations that trigger hypertrophic remodeling.

### 47.3.1 Prevalence

The prevalence of genetic DCM is unclear, largely because most estimates assess primarily symptomatic disease, even though DCM, like HCM, can be clinically silent for many years. DCM occurs as a sporadic or familial trait. Familial cardiomegaly was first reported in 1948 and with systematic screening of family members in the 1990s more accurate estimates of its prevalence emerged. Clinical studies in which first-degree relatives of DCM probands were assessed with physical examination, 12-lead EKG, and echocardiography suggest a familial component in 30–50% of cases (147–150), implicating a genetic etiology. However, pathogenic mutations have been identified in only 20–30% of all DCM cases (151). Amongst 235 consecutive unrelated probands with DCM presenting to a single center between 1995 and 2002, clinical evaluations determined that 102/235 (43%) cases were familial based on a family history of premature sudden death or clinical findings of cardiovascular involvement in at least one other relative. Of these, over 85% demonstrated autosomal dominant inheritance (150,152).

### 47.3.2 Pathology

Typical anatomic findings of DCM are four-chamber cardiac enlargement without proportional hypertrophy of the ventricular walls (Figure 47-1). The histopathology is generally nonspecific with degenerating myocytes with mild to moderate hypertrophy and variable amounts of interstitial fibrosis. Although small foci of myocyte disarray may be found, the proportion is much below that required to establish the diagnosis of HCM. This lack of disarray is an important discriminating feature to differentiate true DCM from the end stage, “burnt-out” phase of HCM in which a small proportion of patients exhibit regression of LVH, cavity enlargement, and diminished left ventricular systolic dysfunction, although the myocyte disarray characteristic of HCM remains present.

## 47.3.3 Phenotype and Natural History

**47.3.3.1 Phenotype.** Clinical manifestations may be somewhat protean, particularly in the early stages of disease. Characteristic symptoms include exertional dyspnea, fatigue, orthopnea, and lower extremity edema. The age of onset can range from early childhood to late adulthood, although most patients present during the fourth or fifth decades of life. Familial DCM may be associated with additional phenotypes that cosegregate with disease (153), including additional cardiac involvement (mitral valve prolapse and conduction system disease) and extra-cardiac conditions (sensorineural hearing loss and muscular dystrophies). These additional manifestations may be expressed in advance of cardiac dilation or dysfunction and therefore serve as surrogate parameters to assign phenotypic status.

### 47.3.4 Natural History

The substantial genetic and clinical heterogeneity of DCM accounts for a natural history that is highly variable. Specific genotype–phenotype correlations are in general not well delineated. The spectrum of manifestations may range from asymptomatic left ventricular enlargement or dysfunction to progressive heart failure requiring cardiac transplantation. DCM is the most common underlying diagnosis in patients requiring cardiac transplantation in the United States and a common cause of heart failure with a world-wide prevalence of ~36.5 per 100,000 (154). Life expectancy in DCM is reduced due to severe heart failure or sudden death.

### 47.3.5 Genetics

The genetics of DCM are much more diverse than in HCM and remained incompletely defined. The most common mode of inheritance of familial DCM is autosomal dominant, but infrequent autosomal recessive, X-linked, and mitochondrial transmissions have been described (Table 47-3). A variety of molecular pathways have been identified that ultimately result in dilated remodeling of the heart. Mutations in a broad group of diverse genes have been implicated. These genes may be divided broadly into alterations of force generation and transmission, alterations of energy production and regulation, and alterations of intracellular calcium handling.

#### 47.3.5.1 Deficits in Force Generation and Transmission

The foundation of cardiac function is the generation of contractile force by the sarcomere and the transmission of force to the extracellular matrix. DCM can occur from mutations in genes that encode components of the force-generating sarcomere or in genes that encode proteins involved in forces transmission through the extracellular matrix (Figure 47-15). Notably, sarcomere protein gene mutations produce either DCM or HCM, depending on

**TABLE 47-3 Gene Mutations Associated with Dilated Cardiomyopathy<sup>a</sup>**

Gene	Protein	OMIM	% of DCM Caused by Mutations	Associated Phenotypes <sup>b</sup>
<b>Autosomal Dominant</b>				
ACTC1	$\alpha$ -cardiac actin	102540	<1%	
DES	Desmin	125660	<1%	Desminopathy, Myofibrillar myopathy
LMNA	Lamin-A/C	150330	7–8%	Partial lipodystrophy, CMT2B1, Emery–Dreifuss muscular dystrophy, Hutchinson–Gilford progeria syndrome, LGMD1B
SGCD	$\delta$ -sarcoglycan	601411	Unknown	Delta sarcoglycanopathy (LGMD2F)
MYH7	Myosin-7	160760	5–8%	Laing distal myopathy
TNNT2	Troponin T	191045	2–4%	
TPM1	$\alpha$ -tropomyosin	191010	Unknown	
TTN	Titin	188840	Unknown	Congenital myopathy, hereditary myopathy with early respiratory failure, LGMD, tibial muscular dystrophy
VCL	Vinculin	193065	Unknown	
MYBPC3	Cardiac myosin-binding protein C	600958	Unknown	
PLN	Cardiac phospholamban	172405	Unknown	
LDB3	LIM domain-binding protein 3	605906	Unknown	
ACTN2	$\alpha$ -actinin-2	102573	Unknown	
CSRP3	Cysteine and glycine-rich protein 3	600824	Unknown	
MYH6	Myosin-6	160710	Unknown	
ABCC9	ATP-binding cassette transporter sub-family C member 9	601439	Unknown	
TNNC1	Troponin C	191040	Unknown	
TCAP	Telethonin	604488	Unknown	LGMD2G
SCN5A	Sodium channel protein type 5 subunit alpha	600163	2–4%	Long QT syndrome type 3, Brugada syndrome, idiopathic ventricular fibrillation, sick sinus syndrome, cardiac conduction system disease
PSEN1	Presenilin-1	104311	<1%	Early-onset Alzheimer disease
PSEN2	Presenilin-2	600759	<1%	Early- and late-onset Alzheimer disease
FCMD	Fukutin	607440	Unknown	Fukuyama congenital muscular dystrophy
<b>X-Linked</b>				
DMD	Dystrophin	300377	Unknown	Dystrophinopathies (Duchenne muscular dystrophy, Becker muscular dystrophy)
TAZ (G4.5)	Tafazzin	30094	Unknown	Barth syndrome, endocardial fibroelastosis type 2, familial isolated noncompaction of the left ventricular myocardium
<b>Autosomal Recessive</b>				
TNNI3	Cardiac troponin I	191044	Unknown	Restrictive cardiomyopathy

LGMD, limb-girdle muscular dystrophy.

<sup>a</sup>Table adapted from Hershberger RE, Kushner JD, and Parks SB. Dilated cardiomyopathy overview. GeneReviews [Internet]. PMID: 20301486.

<sup>b</sup>Other phenotypes caused by mutation in the same gene.

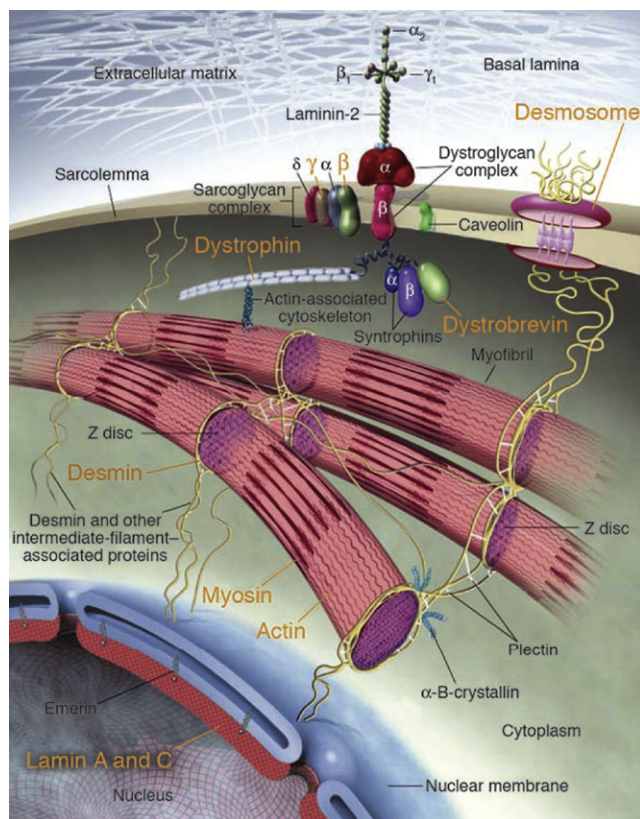
the precise residue that is altered by mutation and the resultant impact on contractile functions.

#### 47.3.5.2 Sarcomere Protein Mutations.

**47.3.5.2.1 Cardiac Actin (ACTC1).** Mutations in ACTC1 associated with DCM were first identified by candidate gene screening approaches. ACTC1 mutations are associated with early onset autosomal dominant DCM that occurs without additional cardiac or extracardiac manifestations (57,67). As described earlier in this chapter, actin plays a central role in both the production and transmission of the contractile force of the sarcomere. DCM-causing mutations in actin are hypothesized to disrupt force transmission, as they involve amino acid residues that dictate actin-cytoskeleton interactions (at

the Z line of the sarcomere). Rare actin mutations that alter actin-myosin interactions and presumably force generation may cause HCM (38,153,155).

**47.3.5.2.2  $\beta$ -Myosin Heavy Chain (MYH7).** This sarcomere protein was first implicated in DCM via genetic linkage studies of a large family with DCM that mapped to chromosome 14q (156). A  $\beta$ -myosin heavy chain missense mutation was identified in that family and subsequently in other DCM families that altered residues that have not been implicated in HCM. Biophysical analyses of DCM mutations in  $\beta$ -myosin heavy chain (e.g. Ser532Pro) indicate that these can diminish force generation by disruption of actomyosin binding. In contrast, another DCM mutations (e.g. Phe764Leu)



**FIGURE 47-15** Mutations in components of the myocyte cytoskeleton and extracellular matrix are associated with DCM and heart failure. Contractile force generated by the sarcomere is propagated through the actin cytoskeleton and dystrophin into the dystrophin-associated glycoprotein complex ( $\alpha$ - and  $\beta$ -dystroglycans,  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycans, caveolin-3, syntrophin, and dystrobrevin). Protein elements of the desmosome, plakoglobin, desmoplakin, and plakophilin-2 provide functional and structural contacts among neighboring cells. Intermediate filament proteins, including desmin, link this network to the nuclear membrane, where lamin A/C is located. (Reproduced from Morita, H.; Seidman, J.; Seidman, C. E. *Genetic Causes of Human Heart Failure*. J. Clin. Invest. **2005**, 115, 518–526, with permission of The American Society for Clinical Investigation.)

located in the head-neck hinge region of myosin alters the mechanics of contractile efficiency or force transmission (157).

**47.3.5.2.3 Cardiac Troponin T (TNNT).** DCM mutations in cardiac troponin T were discovered via candidate gene screening approaches. An in-frame deletion of a lysine residue located in the putative  $\text{Ca}^{2+}$ -sensitive troponin C binding region ( $\Delta\text{Lys210}$ ) originally identified in two unrelated DCM kindreds (156) has been subsequently identified in several other DCM kindreds. Missense mutations in cardiac troponin T missense mutations have been described. Clinical phenotypes associated with cardiac troponin T mutations are variable. While some mutation carriers have only modest clinical symptoms throughout life, others develop severe manifestations of DCM early in life, increased risk of sudden cardiac death, and rapid progression of heart failure (152).

**47.3.5.2.4 Cardiac Troponin I (TNNI3).** Mutations in cardiac troponin I are an uncommon cause of familial DCM. cTnI mutations Lys36Gln and Asn185Lys accounted for severe and early-onset DCM in two families (158). Functional studies demonstrate that both mutations diminished  $\text{Ca}^{2+}$  sensitivity and maximum ATPase activities, findings that are comparable with deficits produced by other DCM mutations in thin filament mutant proteins.

**47.3.5.2.5  $\alpha$ -Tropomyosin (TPM1).** Mutations in  $\alpha$ -tropomyosin were initially associated with DCM via a candidate gene screening approach of 350 probands with familial and sporadic DCM (159). Subsequent TPM1 mutations were identified to segregate with disease in DCM families. Clinical manifestations of DCM caused by some TPM1 mutations can vary considerably with patient age (160). Childhood presentation includes severe heart failure and high risk for sudden death that can necessitate transplantation. But with medical therapies remarkable recovery has been documented followed by long term survival with minimal symptoms and modest LV dysfunction.

In contrast to HCM-associated  $\alpha$ -tropomyosin mutations, which are typically localized to force generation regions involved in troponin T binding and  $\text{Ca}^{2+}$  sensitivity, some DCM mutations in TPM1 alter amino residues involved in surface polarity, therein disrupting actin interactions and compromising force transmission. Recent biochemical analyses of human DCM TPM1 mutations (160) confirm this model and also demonstrated marked reductions in  $\text{Ca}^{2+}$  sensitivity and affinity as well as lower maximum activation, changes that are predicted to reduce force generation.

**47.3.5.2.6 Titin (TTN).** The largest protein and one of the most abundant striated muscle proteins in humans, is the 3-kDa molecule (161) encoded by the TTN gene on chromosome 2. One titin protein spans an entire half of the sarcomere from Z-disc to M-line and functions in assembling contractile filaments, providing elasticity through serial spring-like elements and the majority of passive force, regulating active contractile force (162–165).

Due to the very large size of TTN (>100 kb of coding sequence), this gene has been studied in few DCM patients and to date fewer than 10 mutations (nonsense and missense) have been reported (166,167). Recessive mutations in TTN gene have been also reported in DCM that occurs in the context of congenital myopathies, including hereditary myopathy with early respiratory failure, tibial muscular dystrophy, and limb-girdle muscular dystrophy (168–170). Mechanisms by which different titin mutations produce isolated DCM or disease affecting multiple striated muscles are poorly understood.

**47.3.5.3 Intermediate Filament Mutations.** Intermediate filament proteins (Figure 47-15) connect actin to the dystrophin-sarcoglycan complex beneath the plasma



membrane of all muscle cells. Components of the sarco-glycan complex also extend outside of myocytes to the extracellular matrix (153). As such, mutations in intermediate filament elements are recognized causes of skeletal muscular dystrophies. Varying degrees of cardiac involvement are also seen.

**47.3.5.3.1 Desmin (DES).** The type III intermediate filament protein desmin is a 470-amino-acid muscle-specific protein that is expressed in cardiac and skeletal muscle. It is encoded by the DES gene, an 8.4-kb gene with nine exons; the resultant protein has a structure typical of intermediate filaments with a central  $\alpha$ -helical coiled-coil rod domain and flanking non-helical amino and carboxy terminals (38). Missense mutations in DES have been associated with familial desmin-related myopathy characterized by skeletal myopathy, cardiac conduction system abnormalities, restrictive cardiomyopathy, and intracytoplasmic accumulation of desmin-reactive deposits. Recent studies suggest that the ubiquitin-proteasome system plays a causative role in desmin-related cardiomyopathy, marked by an accumulation of desmin in cardiac muscle (171).

**47.3.5.3.2 Dystrophin (DMD).** Dystrophin is a large cytoskeletal protein that is expressed primarily in muscle. Its function is incompletely understood but thought to involve force transduction and membrane stabilization. Dystrophin is encoded by the DMD gene, the largest known gene at a length of 2.4 Mb on chromosome X (38). DMD mutations were first described as the genetic etiology of Duchenne muscular dystrophy and less severe phenotype called Becker muscular dystrophy. Duchenne muscular dystrophy is an X-linked recessive disorder with a prevalence of one in 3500 male births (172). Disease onset occurs in childhood with development of altered gait, progressive weakness of pelvic, femoral, shoulder girdle, neck flexor, and trunk muscles such that affected children may be wheelchair-dependent by adolescence. Cardiac involvement may consist of cardiomyopathy and conduction disease and typically develops later in the course of disease.

An X-linked primary DCM may also be caused by dystrophin mutations. The prevalence of DMD mutations in DCM is unclear. A study of adult onset DCM revealed dystrophin mutations in 13/201 probands. There was evidence of inherited disease in four of these cases (173). Affected males typically present in late adolescence with heart failure, which is often rapidly progressive and associated with premature death. Clinically evident skeletal myopathy may not be present; however, elevation of serum creatine kinase levels is often present. Female carriers have a predictably less severe phenotype with slower progression and later onset of DCM. A variety of mutation types have been described, most clustered at the amino terminal, including the promoter region, as well as the mid-rod domain (174). Quantitative and qualitative defects in protein expression have

been demonstrated (174). The basis for the cardiac selective phenotype is unclear.

**47.3.5.3.3 Sarcoglycans.** The dystrophin-associated glycoprotein complex is composed of  $\alpha$ - and  $\beta$ -dystroglycans,  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -sarcoglycans, caveolin-3, syntrophin, and dystrobrevin. Via dystrophin and actin interactions, this complex serves to stabilize the sarcomere and transmit force to the extracellular matrix.

$\delta$ -Sarcoglycan is a 290-amino-acid protein encoded by the SGCD gene (>100 kb; eight exons). The protein has a small intracellular domain, a single membrane spanning domain, and a large extracellular carboxyl terminal (38). SGCD mutations were first associated with autosomal recessive limb girdle muscular dystrophy (175). Candidate gene approaches revealed the deletion mutation,  $\Delta$ Lys28 in a family with autosomal dominant DCM, sudden death, and heart failure, but no evidence of clinically obvious skeletal myopathy (176). Fukutin-related protein (FKRP) is necessary for the dystrophin glycoprotein complex to remain intact. Mutations in the FKRP gene produce a clinical spectrum ranging from limb girdle muscular dystrophy with DCM to DCM with slight muscle weakness (177).

**47.3.5.3.4 Z-Disc Associated and Intercalated Disc Proteins.** For contractile force to be generated, one end of the thin filament must be tethered. The Z-disc defines the sarcomere's lateral boundary where actin, titin, and nebulin filaments are anchored. Metavinculin assists in the attachment of titin to the plasma membrane and therefore plays a key role in the transmission of force.

**47.3.5.3.5 Metavinculin and Vinculin (VCL).** Splice variants of the 75-kb VCL gene give rise to metavinculin and vinculin. Vinculin is a ubiquitously expressed component of the subsarcolemmal costameres and intercalated discs; metavinculin is coexpressed exclusively in cardiac, skeletal, and smooth muscle (38). Altered expression levels are associated with heart failure (178,179) suggesting a role in disease pathogenesis. VCL mutations have been recently identified by systematic genetic screening of a cohort of 350 individuals with sporadic and familial DCM (180).

**47.3.5.3.6 LIM protein (MLP) and Cypher/ZASP (LDB3).** Several Z-disc proteins have been implicated in DCM, including  $\alpha$ -actinin which functions to align actin and titin from adjacent sarcomeres, muscle LIM protein (MLP, interacts with  $\alpha$ -actinin), and telethonin which interacts with titin. Collectively these molecules may serve a role as mechano stretch receptors (181). Mutations in these proteins have been implicated in DCM (181,182). Cypher/ZASP is another Z-disc protein that is encoded by LDB3. At least six different isoforms have been characterized that have variable expression in cardiac and skeletal muscle. Human LDB3 mutations can cause isolated DCM as well as in left ventricular non-compaction (LVNC), a related cardiomyopathy characterized by poor systolic function of a dilated left ventricle in which deep trabeculations are present (177,182).



#### 47.3.5.4 Nuclear Intermediate Filament Proteins.

**47.3.5.4.1 Lamin A/C (LMNA).** The inner aspect of the nuclear membrane contains a complex containing composed of emerin and lamin A/C protein. Lamin A and C are widely expressed intermediate-filament proteins that arise from alternate splicing of the human LMNA gene, a 24-kb gene spanning 12 exons, and directly bind emerin. The function of lamins is incompletely understood, but these molecules participate in maintaining the structural integrity of the nuclear membrane, reassembly of the nuclear membrane during mitosis, and interacting with other proteins, including tissue-specific transcription factors (183).

Dominant mutations in the lamin A and C proteins (isoforms encoded by the LMNA gene) cause 16 different diseases with phenotypic variability, including isolated DCM, cardiac and skeletal myopathies, lipodystrophy, peripheral neuropathy, and progeria (184). Mutations that disrupt the terminal amino acids of lamin A cause progeria, while other LMNA mutations can cause tissue-specific disorders via unknown mechanisms. Mutations in emerin produce X-linked and autosomal recessive Emery–Dreifuss muscular dystrophy, which is characterized by joint contractures, cardiac conduction abnormalities, and DCM.

Cardiac phenotypes are particularly prevalent in LMNA disorders, including dominant Emery–Dreifuss muscular dystrophy (185), limb girdle muscular dystrophy (186), and DCM with prominent conduction system involvement (progressive atrioventricular (AV) block and atrial arrhythmias) (187,188). Electrophysiologic deficits usually precede DCM and may be the only cardiac manifestation (189). Electrophysiologic defects include sinus node dysfunction, paroxysmal atrial fibrillation, progressive AV block and ventricular arrhythmias (173,186,188). Postmortem findings of myocardium and conduction system with LMNA mutations exhibit fibro-fatty infiltration of the atrioventricular node (188). Several mutations which create null alleles cause Emery–Dreifuss muscular dystrophy whereas most missense mutations associated with isolated cardiac disease are predicted to function through a dominant-negative mechanism (188,190).

#### 47.3.5.5 Alterations in Energy Production and Regulation.

**47.3.5.5.1 Mitochondrial Mutations.** Five major multiprotein complexes within the mitochondria generate ATP by oxidative phosphorylation. Thirteen components of the machinery involved in oxidative phosphorylation are encoded by 37 genes in the mitochondrial genome, rather than the nuclear genome. The interplay between cardiac energetics and metabolism and pathways of cardiac remodeling are just beginning to be deciphered. Mitochondrial gene mutations demonstrate matrilineal inheritance as the mitochondria are derived exclusively from the maternal cell lineage. Additionally, heteroplasmy may be present with mutations

affecting some but not all copies of the mitochondrial genome in any given cell. As a result, there is marked clinical diversity in the expression of mitochondrial gene mutations. Syndromic disorders are most common with dilated or hypertrophic cardiac remodeling occurring in conjunction with Kearns–Sayre syndrome (ptosis, ophthalmoplegia, and pigmentary retinopathy, proximal muscle myopathy, diabetes and additional hormonal deficits and cardiac conduction abnormalities), MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes), and MERRF (myoclonic epilepsy with ragged-red fibers) (191). Less common predominant or exclusive cardiac disease has been described, particularly DCM (192–194).

Nuclear encoded metabolic mutations have been described in association with hypertrophic remodeling via glycogen accumulation in protein or lipid-associated complexes. These glycogen storage cardiomyopathies are described earlier in this chapter.

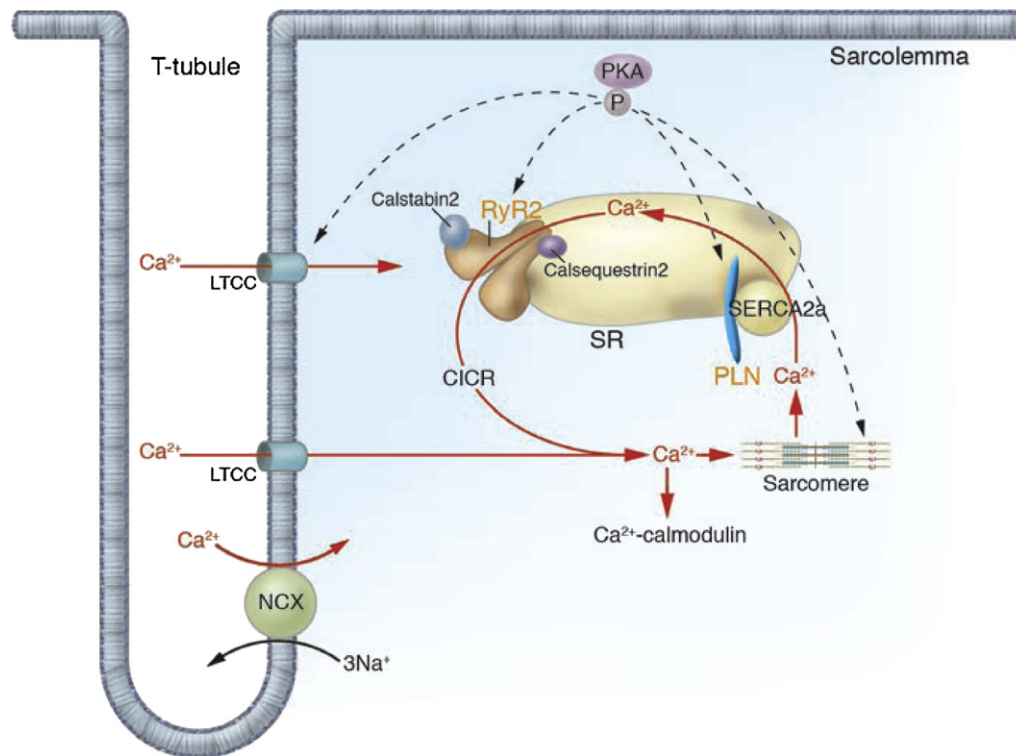
**47.3.5.6 Alterations in Intracellular Calcium Cycling.** Increasing evidence points to the importance of appropriate myocyte calcium homeostasis, which, if altered, may be a common and basic mechanism for heart failure. Abnormalities in the transcription and translation of important modulators of  $\text{Ca}^{2+}$  have been associated with both acquired and inherited cardiomyopathies and models of heart failure (Figure 47-16). Excitation–contraction coupling is the fundamental mechanism linking electrical signaling to the mechanical work of myocytes and is reviewed earlier in this chapter. Key mediators of this process are troponin–actin interactions, ryanodine receptors (RyR2) channels triggering calcium-induced calcium release from the SR), and the SR  $\text{Ca}^{2+}$ -ATPase pump (SERCA2a, which triggers relaxation and  $\text{Ca}^{2+}$  reuptake into the SR as modulated by phospholamban).

**47.3.5.6.1 Phospholamban.** Mutations in phospholamban are rare causes of familial DCM and heart failure (195,196). Biochemical studies on human specimens suggest that both chronically enhanced and decreased phospholamban activity have detrimental effects on cardiac function (195,197).

**47.3.5.7 Other DCM Genes.** Additional gene mutations have been demonstrated to cause DCM through unknown mechanisms. Mutations in ABCC9 encoding SUR2A, a regulatory subunit of the ATP-sensitive potassium channel ( $\text{K}_{\text{ATP}}$ ), have been identified in idiopathic DCM. Recent study reports missense and frameshift mutations in ABCC9, leading to defective catalysis-mediated pore regulation and resulting in DCM (198).

Dominant mutations in heart shock protein co-chaperone BCL2-associated athanogene 3 (BAG3) cause familial DCM (199). In addition, a nonsynonymous SNP located within the coding sequencing of BAG3 has been associated with sporadic cases of DCM (200).

Mutations in G4.5 (tafazzin), an X chromosome-encoded acyltransferase protein of unknown function,



**FIGURE 47-16** Mutations in proteins involved in intracellular calcium cycling cause cardiac remodeling (DCM, HCM, ARVD) and heart failure. Intracellular  $\text{Ca}^{2+}$  trafficking coordinates excitation–contraction coupling and myocyte contraction and relaxation. Calcium entry through transmembrane L-type calcium channels triggers a larger amount of calcium-induced calcium release (CICR) from the SR reservoir through the ryanodine receptor (RyR2), as regulated by calstabin2 and calsequestrin2. Sarcomere contraction then occurs. Relaxation occurs with  $\text{Ca}^{2+}$  reuptake into the SR via sarcoplasmic/endoplasmic calcium ATPase (SERCA2a), modulated by phospholamban. These molecules are regulated in a dynamic fashion by PKA-mediated phosphorylation.  $\text{Ca}^{2+}$  is also involved in other intracellular cascades, including calcium-calmodulin. (Reproduced from Morita, H.; Seidman, J.; Seidman, C. E. *Genetic Causes of Human Heart Failure*. J. Clin. Invest. **2005**, 115, 518–526, with permission of The American Society for Clinical Investigation.)

give rise to Barth syndrome, an X-linked cardioskeletal myopathy and triad of DCM, neutropenia, and 3-methylglutaconicaciduria in infants (201). Mutations in cardiac sodium channel (SCN5A), a known genetic defect of Brugada syndrome and long QT syndrome type III, can cause DCM with conduction system disease (177,202,203).

### 47.3.6 Animal Models of DCM

Mouse models of mutations represented in human DCM assist in the investigation of the consequences of these mutations as well as in the identification of important downstream pathways. Mice that carry myosin mutations that cause human DCM develop morphological and functional characteristics of DCM. Isolated myocytes from these mice exhibit depressed contractile function and reduced capacity to translocate actin, biophysical deficits that are the opposite of those that occur with HCM mutations (157,204). Remarkably, mice that carry two copies of dominant human HCM mutations (in myosin heavy chain or in MyBPC) develop DCM, heart failure and a predisposition for premature death (118,205–207). That mutant gene dosage can convert HCM to DCM may indicate a critical threshold effect of

sarcomere dysfunction, beyond which decompensation and myocyte death occurs.

Mouse gene mutations in the muscle LIM protein (208), desmin (209), and  $\delta$ -sarcoglycan (210) indicate that dysfunction of different elements of the cytoskeleton plays a causal role in the development of the DCM phenotype. Signals that ensue from these deficits that remodel the heart remain unknown.

There are inherent limitations in extrapolating results derived from mouse models to human pathophysiology. Mice null for  $\delta$ -sarcoglycan develop coronary artery spasm with eventual development of DCM related to multiple prior myocardial infarctions and myocyte necrosis (211). Administration of the L-type calcium channel blocker, verapamil, diminishes spasm and improves cardiac function. In contrast, human DCM associated with  $\delta$ -sarcoglycan mutations are thought to result in a deleterious dominant-negative effect on the dystrophin–sarcoglycan complex. Coronary artery abnormalities have not been described (176).

The molecular mechanisms of one pathogenic phospholamban mutation have been investigated via development of a transgenic mouse model of the Arg9Cys mutation (195). Biochemical studies suggested the presence of abnormal protein kinase A interactions that

inhibited the phosphorylation of mutant and wild-type phospholamban. This is predicted to result in the constitutive inhibition of SERCA2a due to excessive phospholamban activity. Indeed, mutant mice showed prolonged calcium transients, delayed myocyte relaxation, and decreased responsiveness to  $\beta$ -adrenergic stimulation. At the organ level, transgenic mice developed marked biventricular cardiac dilation and heart failure.

In addition to studying the effects of introducing mutations into the mouse genome, functional insights have been gained by studying the ability of other genetic manipulations to complement or rescue phenotype. For example, knocking out the muscle LIM protein (MLP) in mice causes neonatal DCM (208). If these MLP mice are crossed with mice lacking phospholamban, normal cardiac function results (197). Phospholamban functions to inhibit SERCA2a, an ATPase responsible for reuptake of calcium into the SR during myocyte relaxation. The observation that phospholamban inhibition leads to functional improvement of hearts genetically predisposed to the development of heart failure highlights the fundamental importance of intracellular calcium handling in pathways of heart failure and suggests potential novel treatment strategies.

Studies of lamin A/C mutations in mice show markedly elongated and dysmorphic nuclear architecture, fragmentation of heterochromatin, and defects in mechanotransduction (190,212,213). Myocytes carrying lamin A/C mutations have poor myocyte viability and are histologically similar to myocytes with mutations in desmosome proteins. This has led to speculation that lamins may serve as myocyte mechanosensors (155). Heterozygous lamin A/C mutations (LMNA<sup>+/-</sup>) in mice reduce cardiac lamin levels and predominantly induce early-onset apoptosis in electrically active myocytes and progressive electrophysiologic disease. Ultimately, nonconducting ventricular myocytes of LMNA<sup>+/-</sup> hearts succumb to diminished lamin levels resulting in late-onset DCM and heart failure, which presumably arise independently from electrophysiologic deficits (190).

Using genome-wide expression profiling in hearts from mice carrying a LMNA missense mutation led to the identification of activation of mitogen-activated protein kinase (MAPK) pathways that preceded clinically detectable DCM (186). Pharmacological treatment of LMNA mutant mice with ERK or JNK inhibitors after the onset of cardiac phenotypes prevents LV end-systolic dilation, increases cardiac ejection fraction, and reduces cardiac fibrosis. These findings suggest that inhibitors of ERK and JNK inhibitors may potentially benefit human patients with LMNA cardiomyopathy.

### 47.3.7 Diagnosis

DCM is a heterogeneous entity characterized by considerable genetic and clinical heterogeneity, a multitude of different molecular pathways, and a relatively low

prevalence of specific mutation types. The diagnosis of DCM is based on clinical evaluation and the finding of dilated cardiac chambers with only modest hypertrophy. There is great variability both in the degree of systolic dysfunction and the presence of associated phenotypes. As described earlier, these associated phenotypes may be cardiac (conduction system disease, arrhythmias, valvular abnormalities, congenital defects) or noncardiac (affecting skeletal muscle, metabolic pathways, and neurologic function). A careful and detailed family history should be obtained in all presenting individuals with a low threshold to perform systematic family clinical screening to assess for the presence of genetic disease. Individuals who are identified to have asymptomatic left ventricular dysfunction and/or enlargement should be followed closely and treated aggressively to attempt to prolong the asymptomatic phase and abrogate the development of a more profound phenotype. Recent availability of clinical genetic testing at many molecular diagnostic laboratories is listed at the National Center for Biotechnology Information Gene Tests Website (*Gene Tests* website). Incorporation of next-generation sequencing technology into clinical molecular genetic screening will facilitate the identification of variants in DCM and substantially improve the detection rate of pathogenic mutations.

### 47.3.8 Management

The management of genetic DCM follows management strategies of all-cause heart failure. Over the past decade, there have been significant strides in the management of systolic dysfunction heart failure. Standard medical regimens have been developed that have been shown to improve the morbidity and mortality of heart failure patients (reviewed in Reference (214)). Strategies incorporate neurohormonal inhibition [angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers combined with beta-adrenergic blockade and, when appropriate, mineralocorticoid antagonism], diuresis to reduce systemic and pulmonary venous congestion, and optimization of heart rate, rhythm, and blood pressure. Medically refractory heart failure may be addressed with novel surgical and mechanical therapies, including cardiac resynchronization therapy (biventricular pacing), valve repair/replacement, ventricular reconstruction surgery, and mechanical cardiac assist. Patients with asymptomatic left ventricular dysfunction have also been shown to benefit from early medical intervention, particularly early initiation of ACE inhibitors (215). This highlights the need for early detection of disease by routine and systematic evaluation of family members.

## 47.4 ATYPICAL CARDIOMYOPATHIES

A genetic etiology has also been described in less common forms of cardiomyopathy, including arrhythmogenic

right ventricular dysplasia (ARVD), restrictive cardiomyopathy, and ventricular noncompaction. Knowledge of molecular mechanisms in these rare types of cardiac disease is considerably less sophisticated than in more commonly encountered forms of hypertrophic and DCMs.

## 47.4.1 Arrhythmogenic Right Ventricular Dysplasia

**47.4.1.1 Prevalence.** Originally described in 1977, ARVD is an inherited form of cardiomyopathy, also known as arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) (216). The prevalence is unclear, but estimated at 1/5000 (217–219). ARVD has been cited as the leading cause of sudden death in competitive athletes in Italy (220). Thirty percent of cases are thought to be familial, typically with autosomal dominant inheritance.

**47.4.1.2 Phenotype and Natural History.** In addition to the characteristic morphologic changes to the right ventricle, ARVD is associated with cardiac arrhythmias and an increased risk of sudden cardiac death. The risk of sudden death is notable, occurring at a rate of ~2.5% per year and accounting for ~20% of sudden deaths in athletes and individuals <35 years of age (220). Sick sinus syndrome and complete atrioventricular conduction block are late manifestations. Heart failure may also occur in ~20% of cases (38).

## 47.4.2 Pathology

ARVD is characterized by necrotic and/or apoptotic myocyte loss and fibrofatty replacement of the myocardium. The right ventricle is typically predominantly affected, although there may also be involvement of the left ventricle (220).

## 47.4.3 Genetics

**47.4.3.1 Cardiac Desmosomes.** Dominant mutations have been identified in genes encoding protein components of the desmosomes: plakoglobin (JUP), desmoplakin (DSP), desmocollin-2 (DSC2), desmoglein-2 (DSG2) and plakophilin-2 (PKP2) (221–225). Approximately 50% of ARVD patients are found to carry a mutation in one of these genes (226), with ~20–30% occurring in PKP2 (reviewed in Refs (227,228)). However, some symptomatic individuals of ARVD harbor homozygous or compound heterozygous mutations. A rare autosomal recessive form of ARVD is present in Naxos syndrome with associated clinical features of nonepidermolytic palmoplantar keratoderma and woolly hair. Distinct mutations in the plakoglobin and desmoplakin genes have been associated with this syndrome (229,230). Proteins implicated in ARVD are components of desmosomes and adherens junctions, therefore likely playing a role in cell-cell communication and transmission of force.

**47.4.3.2 Atypical ARVD Genes.** A mutation in the RYR2, which encodes the cardiac ryanodine receptor was initially identified as a cause of ARVD (231). More commonly, RYR2 mutations produce catecholeminergic polymorphic ventricular tachycardia (CPMVT), a rare genetic disorder characterized by sudden cardiac death during physical or emotional stress despite the presence of apparently normal cardiac morphology (232,233). The cardiac ryanodine receptor is located with the SR and is involved with calcium-induced calcium-release in response to the initial action potential triggered influx of calcium via L-type channels.

Mutations in the untranslated regions of TGF- $\beta$ 3 have been reported to cause ARVD in one family and unrelated ARVD patients (234).

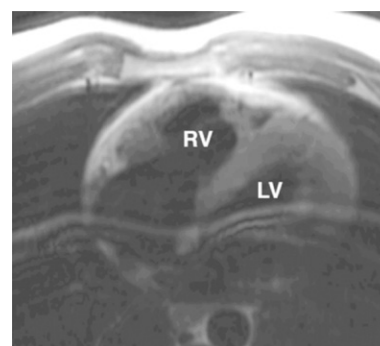
A founding mutation in the gene encoding transmembrane protein 43 (TMEM43) caused ARVD that exhibited gender-related lethality. The median life expectancy in affected men was 41 years compared with 71 years in affected women. Heart failure was a late-onset consequence in survivors (235).

Four other chromosomal loci have been linked to familial ARVD, including chromosome 14q12–22 (219), 2q32 (219), 10p12–14 (236) and 10q22 (237,238).

## 47.4.4 Diagnosis and Management

Making the clinical diagnosis of ARVD is often difficult due to the variability of morphologic findings and technical difficulties in imaging the right ventricular myocardium. Echocardiography and cardiac MRI studies are typically used to assess right ventricular size and function (Figure 47-17). ARVD is suggested by the presence of an enlarged, oftentimes dysfunctional right ventricle. MRI may be better able to assess for the presence of fatty infiltration into the RV wall.

Standard medical therapy with ACE inhibition,  $\beta$ -adrenergic blockade, and diuresis is indicated for



**FIGURE 47-17** Cardiac MRI images of a patient with ARVD. Four-chamber black-blood fast-spin echo sequence demonstrates high signal intensity in the epicardial aspects of the right ventricular free wall suggestive of fatty infiltration of the right ventricle and matching the regional RV dysfunction seen on cine imaging. (Courtesy of Raymond Kwong, MD, Cardiovascular Division, Brigham and Women's Hospital, Boston, MA.)



symptomatic heart failure. Because of the increased risk of ventricular tachyarrhythmias and sudden death, ambulatory EKG monitoring should be performed at regular intervals. Patients at higher risk for sudden death on the basis of symptoms (syncope, ventricular ectopic activity) or family history should be considered for prophylactic implantable cardioverter defibrillator (ICD) placement. Clinical screening of first-degree relatives is also recommended. Comprehensive DNA sequence analysis of the known ARVD-associated genes currently identifies a causative mutation in ~50% of ARVD probands (227). Clinical molecular genetic testing for ARVD is presently provided at several molecular diagnostic centers in the US (*Gene Tests* website). Genetic counseling is also recommended for individuals with ARVD and their relatives.

### 47.4.5 Ventricular Noncompaction

Noncompaction of the ventricular myocardium is a poorly understood cardiomyopathy thought to be caused by failure of the spongiform myocardium to fully compact during embryonic development. This results in a highly trabeculated appearance of the ventricular myocardium, particularly at the apex. It is also known as LVNC, spongy myocardium or left ventricular hypertrabeculation.

**47.4.5.1 Prevalence.** The largest case series of ventricular noncompaction suggests a prevalence of 0.014% of patients referred for echocardiography (239). A male predominance of 56–82% has been reported (239–241). Patients typically present in childhood or early adulthood, but the age at presentation has been quite variable.

**47.4.5.2 Phenotype and Natural History.** In the early stages of embryogenesis, the myocardium is organized in a loose network of fibers with deep crevices separating the myocardium from the left ventricular cavity. A process of compaction of this spongy tissue occurs between 5 and 8 weeks of development resulting in a densely trabeculated myocardium. This is coincident with the development of the coronary circulation (242). Isolated ventricular noncompaction was first described in 1990 and characterized as persistence of embryonic myocardial morphology in the absence of coexisting cardiac anomalies (243). The left ventricle is universally affected; right ventricular involvement is present in <50% of cases (241,242). The clinical course may be marked by heart failure, dysrhythmias, and an increased risk of thromboembolic events. There is also an association with neuromuscular disorders and facial dysmorphism in children (239,241).

Although LV systolic function may be preserved, the majority of patients in reported series have had symptomatic heart failure and impaired systolic and diastolic function. Cardiac arrhythmias also occur commonly, including atrial fibrillation, ventricular tachyarrhythmias, supraventricular tachycardia, and heart block. Ventricular tachyarrhythmias may be present in >40% of patients and sudden cardiac death may account for half of patient deaths (239,242,243).

An increased risk of thromboembolic events to the cerebrovascular, pulmonary, and mesenteric circulations has been associated with noncompaction with estimates of incidence ranging from 21% to 38% (242). The mechanism is unclear, but may be related to an increased predisposition to left ventricular thrombus formation due to extensive trabeculations and decreased systolic function as well as superimposed atrial fibrillation in a subset of patients. One series of pediatric patients with noncompaction reported no systemic embolic events, suggesting that the risk increases with increasing age (244).

Information on prognosis is variable and remains a work in progress. Initial studies suggested a severe prognosis with a 60% incidence of death or cardiac transplantation within 6 years of diagnosis (241). The prognosis appears less severe in the pediatric population with a lower incidence of thromboembolic events, ventricular tachyarrhythmias, and premature death (244). There is likely a spectrum of findings and earlier observations may be confounded by referral bias. Higher risk features have been suggested, including larger presenting left ventricular end-diastolic diameter, NYHA class III to IV heart failure symptoms, atrial fibrillation, and bundle branch block. These features were more frequently documented in nonsurvivors than survivors, and such patients should be considered for more pro-active and pre-emptive treatment, including ICD implantation and evaluation for cardiac transplantation (239).

### 47.4.6 Pathology

Histologically, isolated ventricular noncompaction differs from noncompaction seen in association with congenital heart disease in that there is communication between the intertrabecular recesses and the LV cavity in the former and both the LV cavity and the coronary circulation in the latter (239,242). There are no pathognomonic histologic findings, although increased interstitial fibrosis has been described. Necrotic myocytes has been observed within the prominent trabeculations of individuals with noncompaction.

### 47.4.7 Genetics

Both sporadic and familial types of isolated ventricular noncompaction have been reported. The initial study of isolated ventricular noncompaction, which mainly involved children, reports familial recurrence in ~50% of patients (243). However, the largest report of adult cohort with isolated noncompaction describes both sporadic and familial forms with a familial incidence roughly approximated at 18% (244). As compared with the initial report, the lower percentage of familial recurrence could be contributed to incomplete screening of siblings (242).

The search for genes responsible for isolated ventricular noncompaction remains a work in progress. Gene mutations have not yet been identified for sporadic disease. To date, ventricular noncompaction has been reported in several patients with mitochondrial disorders. However, only a few causative mutations, e.g. mtDNA 3243A>G and 8381A>G, have been reported in such patients (245).

**47.4.7.1 G4.5.** This gene encodes a novel protein family, tafazzins. Family with X-linked ventricular noncompaction was found to have a mutation in this gene in a region of the X chromosome (Xq28) where linkage has been identified in other cardiomyopathic syndromes, including Barth syndrome, myotubular myopathy, and muscular dystrophies (246,247). No mutations in G4.5 were identified in noncompaction individuals associated with congenital heart disease. To date, mutations in G4.5 have been predominantly reported in young male patients rather than adult patients.

**47.4.7.2 LDB3.** Mutations in the cypher/ZASP/LDB3 gene on chromosome 10q23.2 encoding for an LIM-binding protein, a component of the Z-line in both cardiac and skeletal muscles (182,248), cause myofibrillar myopathy or isolated cardiomyopathy. Also, it has been reported as a pathogenesis for both DCM with or without isolated ventricular noncompaction, linking this gene defect to left ventricular dysfunction and dilation (182).

**47.4.7.3 LMNA.** Recent study reported one LMNA mutation (Arg190Trp) in a DCM patient with features of ventricular noncompaction, suggesting that noncompaction may be part of the clinical spectrum of laminopathies (249).

**47.4.7.4  $\alpha$ -dystrobrevin.** This gene is alternatively spliced and only expressed in the heart. Mutations in  $\alpha$ -dystrobrevin (DTNA) gene have been identified in noncompaction individuals associated with congenital heart disease (240). Furthermore, mutations in DTNA have been demonstrated to result in muscular dystrophy in humans as well as skeletal myopathy and cardiomyopathy in mouse models (250).

**47.4.7.5 NKX2.5.** One cardiac-specific homeobox gene, NKX2.5, has also been suggested as a candidate gene

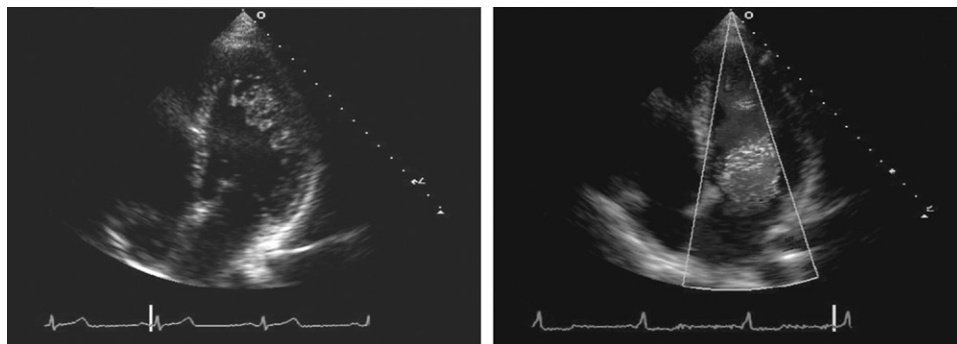
due to the finding of noncompaction in a child with a deletion of chromosome 5q where the gene is encoded (251).

**47.4.7.6 Sarcomere Proteins.** Mutations in some cardiac sarcomere genes (ACTC1, MYH7, MYBPC3, and TNNT2) have been associated with ventricular noncompaction with either traditionally described DCM or HCM. To date, ACTC1 (20%) and MYH7 (22%) appear to be sarcomere genes frequently found in association with ventricular compaction (252).

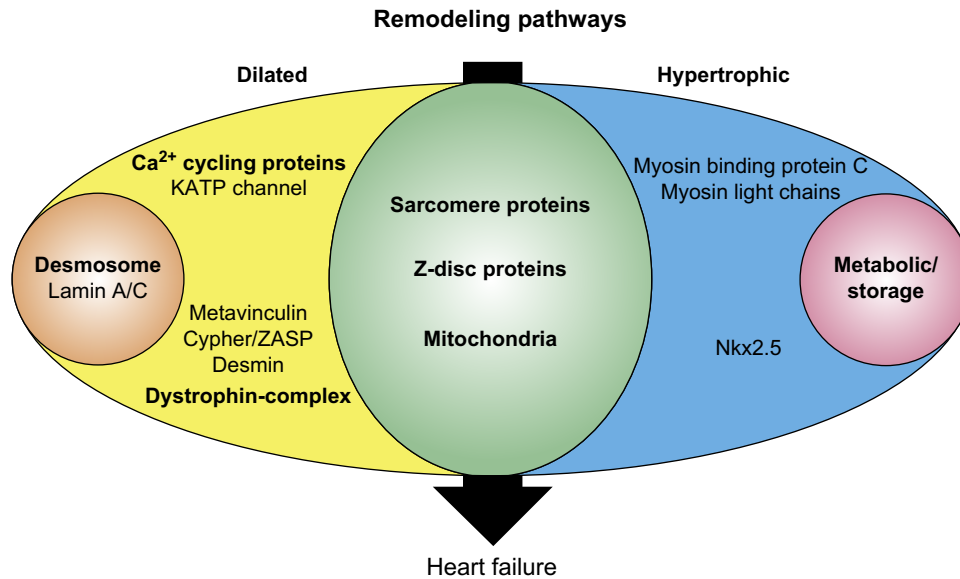
## 47.4.8 Diagnosis and Management

Ventricular noncompaction is typically diagnosed based on echocardiographic findings of multiple prominent ventricular trabeculations with multiple deep intertrabecular recesses. Color flow Doppler imaging can help to demonstrate communication of blood flow into these intertrabecular recesses (Figure 47-18). A two-layered endomyocardium with an increased ratio of noncompacted to compacted thickness at end systole is suggestive. (>1.4 in children or infants and >2.0 in adults; Reference (252)). The left ventricular apex is most commonly affected. LV systolic function is variable. Earlier reports suggested that decreased ejection fraction was ubiquitous, but there is clearly a spectrum of functional defects ranging from normal to severely impaired. Alternate imaging modalities including left ventriculography, computed tomography, and cardiac MRI may also assist in resolving ventricular morphology.

Management consists of standard medical therapy for functional abnormalities (e.g. symptomatic heart failure or asymptomatic LV systolic dysfunction). Because of the increased risk of associated comorbidities including atrial fibrillation, ventricular tachyarrhythmias, and sudden death, ambulatory EKG monitoring should be performed at regular intervals. ICD placement should be considered for patients at higher risk for sudden death based on findings on ambulatory monitoring, symptoms, syncopal episodes or a family history of sudden death. Systemic anticoagulation should also be considered and is mandatory if atrial fibrillation is identified. Aspirin



**FIGURE 47-18** Echocardiographic images from a patient with isolated ventricular noncompaction. Note the spongy appearance of the myocardium with prominent apical trabeculations interspersed with deep recesses (left). Color flow Doppler imaging (right) can highlight the blood flow interdigitating between the trabeculations.



**FIGURE 47-19** Human gene mutations can cause cardiac hypertrophy (blue), dilation (yellow), or both (green). Pathways that lead to pathologic remodeling of the heart involve force generation and transmission (sarcomere proteins and cytoskeletal elements), energy production and regulation [metabolic/glycogen storage proteins (pink) and mitochondrial proteins], and intracellular calcium cycling (sarcomere proteins, Ca<sup>2+</sup>-cycling proteins). (Reproduced with permission from Morita, H.; Seidman, J.; Seidman, C. E. *Genetic Causes of Human Heart Failure*. J. Clin. Invest. **2005**, 115, 518–526.)

may be administered for reducing the risk of systemic embolism in patients with isolated ventricular noncompaction. Clinical screening of first-degree relatives is also recommended. Clinical molecular genetic testing for isolated ventricular noncompaction-associated genes is currently available (*Gene Tests* website). Genetic counseling is also recommended for affected individuals and their family members.

## 47.5 CONCLUSION

Unraveling the molecular and genetic basis of inherited cardiomyopathies will provide further insight into their fundamental pathophysiology. The study of gene mutations associated with structural heart disease has been instrumental in the identification of key pathways involved in the development of abnormal cardiac morphology, including deficits in force transmission and generation (elements of the sarcomere apparatus and cytoskeleton), energy production and regulation (mitochondrial, metabolic, and glucose storage genes), and intracellular calcium handling (sarcomere and Ca<sup>2+</sup>-cycling proteins) (Figure 47-19). This information will lead to better understanding of the mechanisms underlying cardiac development and the more common forms of acquired dilated and hypertrophic heart disease. Knowledge gleaned from progress in the basic science arena will ultimately be translated into clinical practice. For example, screening of family members in DCM kindreds may identify individuals with asymptomatic left ventricular dysfunction or dilatation. Early initiation of medical therapy at this stage improves symptoms, morbidity, and prognosis. Genetic identification and biomarker

detection of individuals carrying gene mutations associated with HCM prior to the development of overt clinical disease provides a critical opportunity to intervene to alter phenotypic development rather than relying on the contemporary and suboptimal strategy of symptom palliation. Whole genome next-generation sequencing technologies would enable high-throughput investigation of the roles of genetic modifiers in HCM with atypical clinical expression. More definitive treatment to interrupt the natural history of dilated and hypertrophic cardiomyopathies will be drawn from better mechanistic understanding of how inherited gene defects remodel the heart.

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Chapter 125. The muscular dystrophies; Chapter 127. Congenital (structural) myopathies.

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## RELEVANT WEBSITES

- “Clinical Trials” <http://clinicaltrials.gov/ct2/show/NCT00319982>
- “Gene Tests” [www.genetests.org](http://www.genetests.org)
- “Cardiogenomics” <http://cardiogenomics.med.harvard.edu>



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# CHAPTER

# 48

## Heritable and Idiopathic Forms of Pulmonary Arterial Hypertension

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### 48.1 HISTORICAL PERSPECTIVES AND INTRODUCTION

Heritable and idiopathic pulmonary arterial hypertension (PAH), together formerly known as primary pulmonary hypertension (PPH; OMIM 178600), are clinically identical progressive disorders characterized by elevated pulmonary artery pressure with pathologic changes originating in the precapillary pulmonary arteries. Characteristic pathologic changes were described in autopsy specimens as early as 1891 by Romberg but the disease was not reliably diagnosed clinically until the development of cardiac catheterization. The term *primary pulmonary hypertension* was coined in 1951 to describe what we now recognize as PAH, with the first clinical description of the disease by Dresdale et al. (1). The term PPH was used exclusively for fifty years and typically referred to those with either familial or sporadic precapillary pulmonary hypertension. The 2003 World Congress on PAH changed the nomenclature from PPH to familial PAH (FPAH) for patients with the disease and either a positive family history or a PAH-associated gene mutation, and idiopathic PAH (IPAH) for those patients who have neither. *BMPR2* is the gene encoding bone morphogenetic protein receptor type II (*BMPR2*; OMIM 600799), which is mutated in over 75% of families with PAH. The

discovery of the *BMPR2* gene's association with PAH, as well as the rare occurrence of mutations in other members of the transforming growth factor  $\beta$  (TGF $\beta$ ) superfamily of receptors with PAH (*ACVRL1* and *ENG*), prompted a revision of nomenclature at the 2008 World Congress on PAH: patients with either a family history of PAH or a detectable mutation in an associated gene are now considered to have heritable PAH (HPAH), whereas patients with neither have sporadic PAH known as IPAH (2).

PAH is defined clinically by the presence of pulmonary hypertension in the absence of known secondary causes such as lung disease, heart disease, and thromboembolism. As with its initial epidemiologic descriptions, PAH affects females more than twice as often as males and occurs at all ages although whether severity is gender-specific remains in debate. Before disease-specific therapy was identified in the mid-1990s starting with intravenous epoprostenol, the disease characteristically progressed to right heart failure and death in just a few years from the time of diagnosis. Now, several therapies specifically approved to treat PAH patients exist although whether any of these agents only improve functional status or also specifically increase survival is unclear. However, with vital new understanding about the basic pathogenesis and novel treatment regimen trials now in progress, the outlook for PAH patients is more hopeful than ever before.

## 48.2 NOMENCLATURE

PAH was previously known as PPH although this label no longer exists. Several classification schemes have been used to characterize PAH since its initial descriptions in the 1950s as PPH by Dresdale et al. (1). The currently accepted system was established at the most recent World Symposium on Pulmonary Hypertension in 2008 (Dana Point, CA, USA). Group 1 pulmonary hypertension, known as PAH, includes PAH that occurs both in families and as a sporadic disease (Table 48-1). In recognition of genetic advances in understanding, including the discovery that mutations in selected genes occur among patients without family history of PAH previously felt to have sporadic disease, patients with PAH are labeled HPAH if they possess a family history of PAH and/or a detectable mutation in a PAH-associated gene. Those subjects who would otherwise meet criteria for what is classically considered PPH but lack family history or detectable mutation are considered to have the sporadic disease IPAH. It is likely that as our genetic understanding evolves and detection methods improve, the distinction between HPAH and IPAH will further narrow, but that remains to be seen.

## 48.3 INCIDENCE AND PREVALENCE OF HPAH AND IPAH

Current prevalence estimates of PAH due to HPAH and IPAH causes probably underrepresent the burden of

disease because of a number of factors, including recognition and diagnosis of disease as well as disease classification (3). However, current conservative estimates suggest approximately 6–15 cases per million in North America and Europe. It has generally been accepted that owing to the intervention of new pharmacologic agents, the prevalence of disease is increasing although this has not been proven. The number of new cases of IPAH or HPAH (incident cases) is estimated to be 1–2 per million population per year in North America and Europe. Insufficient information is available to determine whether specific populations have different frequencies of PAH although reports from groups of different ancestries are beginning to emerge. For example, it is clear that among Chinese and Japanese patients, as with those of European descent, *BMPT2* mutations are detectable in both HPAH families and IPAH patients. However, the exact prevalence is unknown as full sequencing augmented by multiplex ligation-dependent probe amplification or other assays to detect large deletions and duplications have not been performed in large patient cohorts (4,5).

## 48.4 PHENOTYPE AND NATURAL HISTORY OF HPAH AND IPAH

PAH is characterized by widespread obstruction or obliteration of the smallest pulmonary arteries (6). These arteries are obstructed by changes in all the layers of the vascular wall, including smooth muscle hypertrophy in the vascular media, obstruction of the lumen by concentric intimal fibrosis and microthrombi, and changes in the surrounding adventitia as well (7,8). When a sufficient number of vessels are occluded, the resistance to blood flow through the lungs increases. In order to maintain adequate pulmonary blood flow, the right ventricle compensates by generating higher pressure. The early phase of disease results in few or no symptoms until the right ventricle can no longer compensate for the increased resistance, and then progressive heart failure ensues. Initial symptoms include dyspnea, fatigue, chest pain, palpitation, syncope, or edema (9). Individuals who have HPAH have identical symptoms, signs, and clinical course as those with IPAH (9). The discovery of mutations in *BMPT2* as the major genetic association provided the first real understanding of the central pathogenesis and provided a basis for genetic testing (10,11), as well as the potential for new and innovative future therapies to address the basic pathogenesis at the molecular level. However, to date, the exact mechanism by which *BMPT2* gene mutations increase the susceptibility to PAH has not been elucidated and is a focus of intense investigation.

PAH affects individuals of all ages, including the very young and the elderly. While females are more than twice as likely to be affected as males, historically, the severity and outcome of disease have not appeared to vary according to gender. However, recent epidemiologic data

**TABLE 48-1 Pulmonary Hypertension (PH), Clinical Classification**

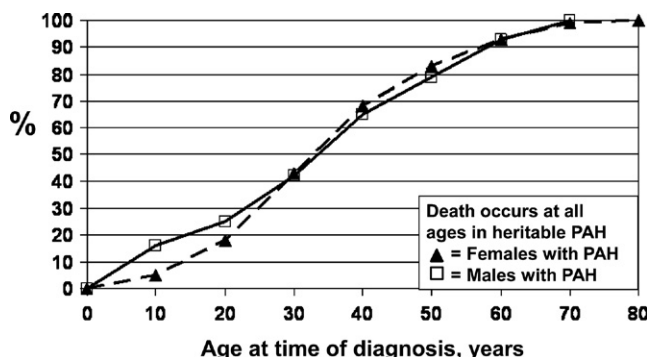
Group 1 PH: Pulmonary Arterial Hypertension (PAH):
<b>Idiopathic PAH</b>
No known associated gene mutation and no family history of PAH
<b>Heritable PAH</b>
Possess a genetic mutation known to associate with PAH (e.g. <i>BMPT2</i> , <i>ALK1</i> , <i>ENG</i> )
Subject in a family with documented PAH, but no detectable gene mutation of relevance
<b>PAH associated with drug or toxin use (e.g. fenfluramine exposure)</b>
<b>PAH associated with other disease states</b>
Connective tissue diseases
HIV
Portal hypertension
Congenital heart disease
Schistosomiasis
Chronic hemolytic anemia
<b>Persistent pulmonary hypertension of the newborn</b>
Group 1' PH: pulmonary veno-occlusive disease (PVOD); pulmonary capillary hemangiomatosis (PCH)
Group 2 PH: presence of left heart disease
Group 3 PH: presence of lung disease and/or hypoxia
Group 4 PH: chronic thromboembolic PH (CTEPH)
Group 5 PH: less-distinct and likely multifactorial mechanisms

Adapted from Simonneau et al., JACC 2009 Jun 30, 54 (Suppl. 1), S43–S54.

does suggest that while more likely to develop disease, among subjects with PAH, females may have improved survival, especially when compared to older males. In HPAH, mortality occurs at all ages regardless of gender, and we find that 20% of patients died before age 20, and 20% died after age 50 (Figure 48-1) (12). The clinical characteristics and natural history of untreated PAH (then known as PPH) were reported in a multicenter study (9), which was conducted before the identification of the first widely effective therapies. This study included 194 affected individuals from 32 US centers, where other causes of pulmonary hypertension were rigorously excluded. The mean age at diagnosis was 36 years, and while the clinical course varied, most untreated individuals gradually deteriorated with an overall mean survival of only 2.8 years. Preceding the availability of effective therapy, the clinical functional capacity correlated very closely with survival. Patients who were New York Heart Association (NYHA) class IV had a mean survival of 6 months, whereas those who were class III had a mean survival of 2.5 years, and those who were class I or II had a mean survival of 5 years (13). Family history was positive for only 6% of cases in that initial report although the method of acquisition of family history was not uniform across the cases reported.

## 48.5 INHERITANCE AND GENETICS OF PAH IN FAMILIES

PAH can occur as a sporadic (idiopathic, IPAH) disease or present among subjects within families (heritable, HPAH) known to possess PAH risk. Regardless, these two disorders are clinically and histologically indistinguishable. PAH in families (now classified as HPAH) represents at least 6% of all cases (9) and displays an autosomal dominant mode of transmission with reduced penetrance and genetic anticipation (Figure 48-2) (14–16). Significantly reduced penetrance in most families likely confounds the recognition that the disease is familial, and not uncommonly, a subject initially assigned the diagnosis of sporadic IPAH will possess a detectable *BMPR2* mutation—the diagnosis then changes to HPAH (40).



**FIGURE 48-1** Cumulative mortality of patients with HPAH in families. Circles represent females; squares represent males.

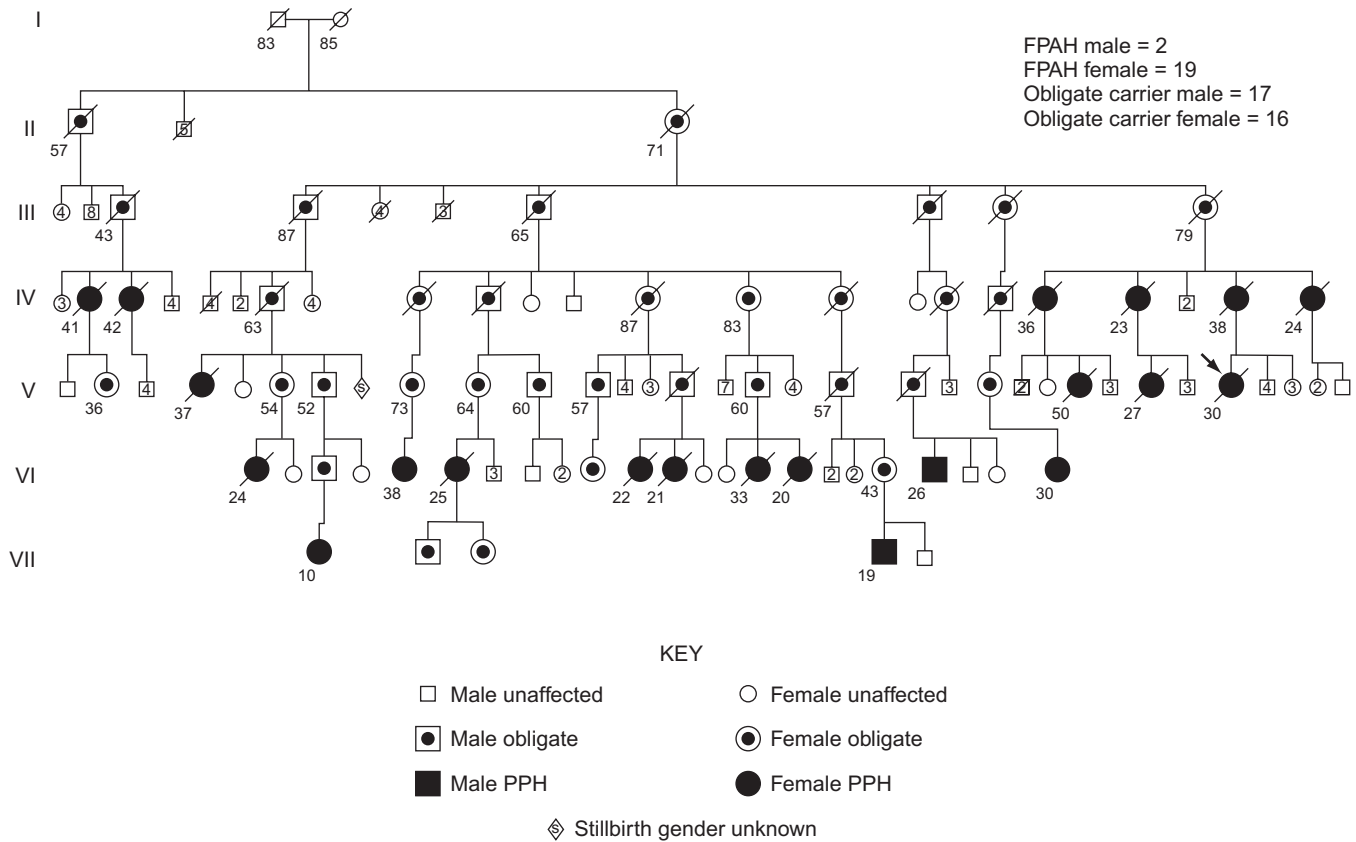
In fact, heterogenous germline mutations in *BMPR2* have now been found in the majority of familial (>75% tested) (17) as well as about 15% of cases previously labeled IPAH (18–21). Irrespective of the family history, subjects with an identifiable germline mutation in *BMPR2* have heritable disease (HPAH) by definition (2).

*BMPR2* is a receptor within the TGF $\beta$  superfamily of receptors critical to cell signaling. The gene encoding *BMPR2* resides on chromosome 2q33 and produces multiple isoforms—the most common comprises all 13 exons (Figure 48-3). It is a large gene, spanning 182,965 bp. The receptor has four major domains (Figure 48-3), including an extracellular (exons 1 through 3), a transmembrane (exon 4), and an intracellular serine/threonine kinase (exons 5 through 11) domain and a large C-terminus tail (exons 12 and 13); the latter is not shared by other TGF $\beta$  receptors. Patients within families with PAH related to *BMPR2* mutations share the same mutation although most mutations differ across families. To date, over 300 mutations have been reported throughout the gene from patients with PAH (17). The reported *BMPR2* mutations are heterogenous, including frameshift, partial deletion, splice site, nonsense, and missense mutations (17). A large proportion of mutations presumably result in a haploinsufficiency of *BMPR2* although some mutations promote a dominant negative effect (22).

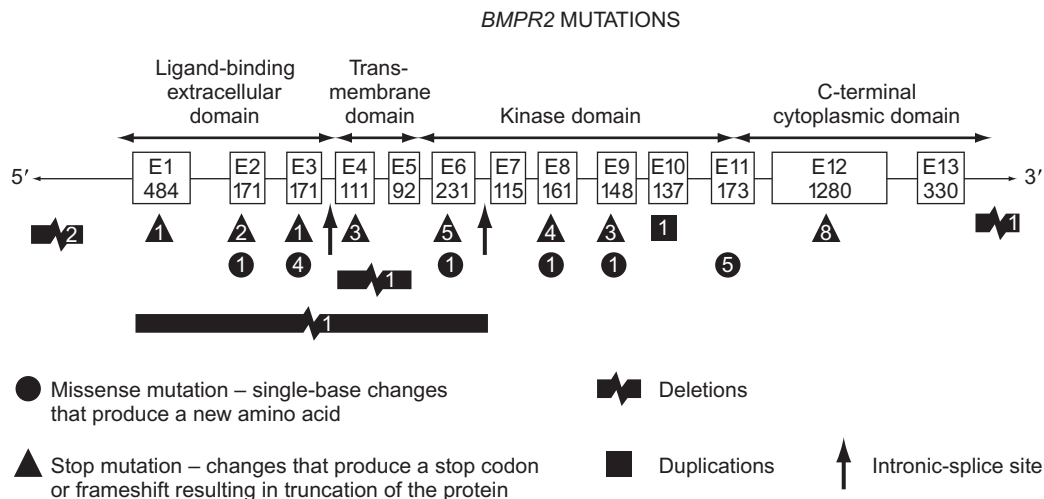
*BMPR2* mutations remain the most commonly identified genetic susceptibility for PAH (17). However, rare variations in three additional loci are associated with PAH. As with *BMPR2*, each of these is directly related to TGF $\beta$  receptor superfamily signaling. Located on chromosome 13, *SMAD9* (encodes Smad8) was found to be mutated in one patient with sporadic PAH (23) and its discovery makes sense given the importance of Smad signaling to PAH, although additional cases have yet to be reported. In a small percentage of cases, patients with hereditary hemorrhagic telangiectasia (HHT) develop PAH. HHT is a vascular dysplasia characterized by mucocutaneous telangiectasias, recurrent epistaxis, gastrointestinal bleeding, and arteriovenous malformations (AVMs) of multiple organs including the lungs. When PAH is present in HHT, it is clinically and histologically identical to HPAH and IPAH (24). However, in HHT, right heart failure is most often due to shunting through pulmonary AVMs and not PAH. Mutations in activin receptor-like kinase 1 (*ACVRL1*) located on chromosome 12 and endoglin (*ENG*) on chromosome 9 both associate with PAH and HHT although the great majority of patients have mutation in *ACVRL1* (25,26). Interestingly, *ACVRL1* and *ENG* encode proteins of the TGF $\beta$  superfamily of receptors. *BMPR2*, *ALK1* and *ENG* all signal intracellularly via the Smad family of coactivators (27,28). Similar to the *BMPR2*-associated HPAH, reduced penetrance and variable expressivity of PAH is seen in HHT (20,21).

All PAH-associated genes described to date, *BMPR2* being the most common, are mutated in the germline and





**FIGURE 48-2** Pedigree of a family with PAH and a detectable *BMPR2* gene mutation.



**FIGURE 48-3** Schematic diagram of *BMPR2* gene domains and mutations. Mutations have been described throughout this large gene.

are not specific to the lungs. These germline mutations have either been passed down through a family over time or occurred *de novo*, presumably in an oocyte or a spermatocyte. Regardless, the reduced penetrance of disease suggests the possibility that expression among susceptible subjects requires one or more additional “second

hits,” of which somatic changes in the lung are a possibility (29). In fact, small vessel obliteration due to exuberant cellular proliferation, evidence of endothelial cell monoclonality and somatic endothelial cell gene mutations, all are features that suggest PAH may be similar to an angioproliferative disorder with neoplastic features

(30–33). This prompted Aldred et al. to test the hypothesis that the acquisition of somatic mutations within the lung promotes the development of complex vascular lesions with neoplastic features. Interestingly, they demonstrated somatic chromosome abnormalities in the lung pulmonary artery endothelial cells of PAH patients, including one with a *BMPR2* mutation, as well as evidence of X chromosome loss and/or inactivation (34). While they documented somatic mutations in the endothelial cells of end-stage human PAH lungs, their findings require replication and further exploration before somatic changes are a widely accepted component of PAH pathogenesis (29).

The higher incidence and prevalence of HPAH among females compared to males is one of the many complicated and poorly understood factors involved in the inheritance and genetics of HPAH. HPAH also displays reduced penetrance within families and variable penetrance across families as well as genetic anticipation and variable expressivity.

### 48.5.1 Reduced Penetrance

*Penetrance* refers to the frequency with which a specific trait (phenotype) is expressed by individuals with a genotype known to be able to cause that trait. Owing to reduced penetrance, only about 20% of individuals with an HPAH-causing genetic mutation in *BMPR2* will develop detectable PAH during their lifetime. This characteristic of mutant *BMPR2* alleles suggests that possession of one mutant *BMPR2* allele is required but not sufficient to precipitate clinical expression of disease in most cases (32). To date, the mechanisms of reduced penetrance are unknown, and suggest a role for additional genetic and/or environmental modifiers of disease expression (33). For example, recent data suggest that the level of *BMPR2* gene expression by the wild-type allele may influence penetrance, with lower wild-type expression associated with higher penetrance (35). Epigenetic modification is an additional possible modifier of penetrance although it has been incompletely studied to date. No human is reported to have two *BMPR2* gene mutations, and animals genetically modified to lack the *BMPR2* gene do not survive embryonic development.

### 48.5.2 Gender Dimorphism

Aside from the presence of an identifiable mutation in a PAH-associated gene, gender is the most consistent determinant of HPAH expression. Females outnumber males  $\geq 2:1$  among both incident and prevalent cases of HPAH and IPAH. While not every family displays a strong gender bias, the vast majority are female predominant. The reasons for this disparity remain elusive. An increased incidence of HPAH among females suggests that either fewer males are successfully born to families with HPAH or females are more susceptible to having

detectable disease. On the basis of meticulous evaluation of our family pedigrees, we previously speculated that increased male fetal losses contributed to the gender discrepancy although this has not been independently validated (14). Alternatively, unclear genetic or hormonal factors may promote higher susceptibility for females relative to males. Reports from the French registry that the female/male ratio was identical in healthy *BMPR2* mutation carriers and noncarriers within PAH families suggested that the gender disequilibrium among PAH patients may result from specific gender-dependent factors such as hormones acting later in life rather than embryonic lethality or meiotic drive (16).

Interestingly, recent reports from the two large epidemiologic registries of PAH in France and in the United States found improved survival among females although it was unclear if this was due to effects on the pulmonary vasculature, heart or both (36,37). In addition, the survival disadvantage found in the United States study (REVEAL) was specific to men over age 60 years compared with younger men and compared with women at any age. Detailed prospective studies with validation will be necessary to fully appreciate the degree to which gender participates in survival if at all.

### 48.5.3 Variable Expressivity

The degree (mild to severe) to which rare genetic variation causes disease is not uniform among *BMPR2* mutation carriers, indicating variable expression of disease. For example, the age of onset and diagnosis among *BMPR2* mutants with PAH is highly variable, ranging from infancy to over 65 years of age (38). This variation is evident both within families (all affected subjects share the same *BMPR2* mutation) and across families (in most cases, different families have different *BMPR2* mutations). This again suggests that factors beyond the possession of a *BMPR2* mutation modify disease expression among susceptible subjects.

### 48.5.4 Genetic Anticipation

This refers to the increased severity or an earlier age of onset of disease for patients in subsequent generations in a pedigree. Initially perceived as predominantly a function of ascertainment bias, some evidence suggests that this truly occurs among families susceptible to PAH. While not uniform, the age at death in successive generations decreases consistently by about a decade in each generation among some families (14). However, this remains a finding in need of independent validation, and the concept is not without controversy since it lacks biological evidence of causation. However, progressively earlier ages of death continue to be a feature among our families with PAH, and recently were demonstrated by French researchers as well (39). This phenomenon is well described in several neurologic diseases, including fragile

X syndrome and Huntington disease. These neurodegenerative disorders associate with trinucleotide repeat expansions, which have not been identified in *BMPR2* or other PAH genes. While not reported in HPAH, progressive telomere shortening causes anticipation in autosomal dominant dyskeratosis congenita, which also associates with pulmonary fibrosis (40).

## 48.6 CONNECTING *BMPR2* TO PAH

Sufficient numbers of families with PAH (then known as PPH) were identified and DNAs were banked so that adequate power was available to conduct linkage analysis for a chromosomal location of a highly associated gene by the mid-1990s. In 1997, a microsatellite marker search established linkage to a 30-Mbp region on chromosome 2q33 (41,42).

### 48.6.1 Identification of Mutations in *BMPR2* in PAH That Occurs in Families

Because the sequence of the human genome had not been completed when the locus associated with PAH in families was mapped in 1997 and the critical interval for the familial locus was 30 Mbp (41), the subsequent search employed a candidate gene approach. Several genes with relevant functions, including those for an insulin-like growth factor and for neuropilin, a vascular endothelial growth factor receptor, were identified in the linkage peaks, but variations related to disease were not detected when DNAs of affected family members were analyzed. *BMPR2* was the third candidate studied, and it was selected on a functional basis because of its membership in the TGF $\beta$  family of receptors, which participate in the regulation of cell proliferation among many other roles.

We amplified exonic segments of *BMPR2* alleles from genomic DNAs of individuals with PPH and screened the amplicons for alterations by dideoxy fingerprinting, and found an altered pattern in a patient and healthy parent in one family (10). By DNA sequencing, we found that the change was a T deletion (2579–80 delT) that deleted a restriction site for *AseI* from this amplicon. We amplified the segment containing this mutation from DNAs of all available subjects in this family. The amplified segments were subjected to restriction endonuclease digestion, and the result was analyzed by agarose gel electrophoresis. These studies showed that, in this family, the 2579–80 delT mutation in *BMPR2* cosegregated faithfully and completely with the FPAH phenotype, greatly strengthening the evidence that the mutation was the real cause of the disease (10). In nearly the same time frame, another group independently reported FPAH linkage and the identification of *BMPR2* as the associated gene of interest (11).

Since the association of mutations in *BMPR2* with PAH, the majority of all families analyzed have been shown to possess a *BMPR2* mutation. In addition,

between 5% and 26% of cases of PAH thought to occur sporadically have also been found to associate with germline *BMPR2* mutations (4,19,43,44), with some documented cases of *de novo* mutations. The true frequency of mutations may yet be underestimated as the techniques employed in most of these studies may have failed to search for large gene deletions or rearrangements. Such mutations are well known to occur in PAH and may be missed by traditional sequencing techniques; in fact, advanced techniques that detect these mutations are now recommended for clinical testing for mutations among PAH patients (17,45,46).

### 48.6.2 Range of *BMPR2* Mutations

Within each FPAH kindred, every affected member has the same mutation, and only rarely do clearly unrelated kindreds share the same mutation. For example, our Vanderbilt HPAH cohort contains only a few truly unrelated families who share the same *BMPR2* mutation. The most common mutation among our families, and one of the few shared among families, is a nonsense mutation in the tail domain (exon 12; arginine to termination mutation at amino acid 899) (Figure 48-3). *BMPR2* is highly conserved in nature, and each amino acid substitution that results from the reported point mutations is predicted to alter receptor function. Mutations are equally divided among frameshift, nonsense, and missense, all predicted to alter the encoded transcript and result in a dysfunctional receptor. None of the mutations that are associated with PAH results in a receptor structure that would appear to be compatible with normal or increased function. Except for possible differences in penetrance across families, there are no other apparent differences, including gender, age of onset, and severity of disease, based on the location of mutations. However, owing to the rarity of mutations and heterogeneity, comprehensive studies of subjects lack sufficient power to explore effectively this question, and there are certainly families in whom the gender disequilibrium is more obvious than others.

### 48.6.3 *BMPR2* Mutations in PAH That Occurs Sporadically

Whereas HPAH is a complex genetic disease and there are no definitive influences on variable expression other than gender, in sporadic PAH, there are recognized environmental and biological triggers for disease, including associations with appetite suppressants (47), HIV infection (48), portal hypertension (49), and rheumatologic diseases (38,50). The best-documented environmental associations are aminorex, fenfluramine, and stimulants such as amphetamines (51). There is currently active study in the contribution of obesity, glucose intolerance, insulin resistance, and metabolic syndrome to sporadic PAH, and some data to suggest that many patients

struggle with these comorbidities—whether they are causative or simply associative is not yet clear (52–54). Studies are needed to examine the role of other factors, including hormone therapy, number of pregnancies, cigarette smoke, depression, systemic hypertension, altitude dwelling, diet, medications, lifestyles, and other potential modifying environmental influences on development of disease. These areas are now of great interest as presumably PAH develops because of the confluence of multiple factors that converge to promote disease among subjects with some form of underlying disease risk (12,34,54,55).

One such risk is the presence of a *BMPR2* gene mutation; however, among subjects with no prior family history, this is impossible to know before symptom onset. This was initially demonstrated by Thomson et al., who conducted a search for *BMPR2* mutations among 50 patients with “sporadic” PAH, in which the investigators sequenced the entire coding region and intron–exon boundaries of *BMPR2* (19). Germline mutations were found in 13 patients, each predicted to alter cell signaling response to ligands. While the precise prevalence of *BMPR2* mutations will require a comprehensive study of several hundred sporadic patients of varying ethnicities for whom family histories are well documented to be negative, it appears true that a proportion of sporadic PAH patients of multiple ethnic backgrounds possess a *BMPR2* mutation. These patients thus have heritable disease (HPAH), irrespective of family history. The several series published to date suggest that approximately 5–26% of patients with “sporadic” disease in fact have a *BMPR2* mutation (4,5,18–21,56). Our cohort at Vanderbilt represents a biased sample as we deeply probe family histories with our patients, and thus in our cohort, the percentage of “sporadic” patients with a *BMPR2* mutation is low (~6%) (22). As noted above, “sporadic” PAH patients with a *BMPR2* mutation are considered HPAH patients, while those without are classified as IPAH patients.

To determine whether *BMPR2* mutations occurred *de novo* in sporadic patients, or whether they were inherited, will require analysis of parental samples. There are many possible reasons why most “sporadic” PAH patients and a minority of HPAH kindreds do not yet have recognized *BMPR2* mutations. For example, the promoter, the intronic regions, and the large 3′ untranslated region of *BMPR2* in most sporadic studies were not examined. In fact, intronic mutations in *BMPR2* may be responsible for another 25% of familial cases that link to chromosome 2q33 (46). While it remains possible that rare variants in other primary genes associate with sporadic PAH, to date, no genes outside of the TGFβ receptor superfamily have been identified. Whole exome and whole genome sequencing, RNA sequencing, and other next generation genetic techniques provide novel platforms to evaluate more thoroughly this issue.

#### 48.6.4 *BMPR2* Mutations in Other Disorders

Other types of PAH have been studied for the presence of *BMPR2* mutations. One family with pulmonary veno-occlusive disease due to *BMPR2* mutation in exon 1 was described (58). In a cohort of 33 patients with PAH associated with fenfluramine ingestion, three had *BMPR2* mutations predicted to alter receptor function (21). Six individuals with congenital heart disease (complete type C atrioventricular canals, atrial septal defect, patent ductus arteriosus, partial anomalous pulmonary venous return, and aortopulmonary window with a ventricular septal defect) had responsible *BMPR2* variations (50).

*BMPR2* mutations were not found in 24 patients with PAH related to the scleroderma spectrum of disease (37). In addition, *BMPR2* mutations were not found in three studies of a total of 55 patients with HIV-related PAH (42). Importantly, *BMPR2* mutations that would be predicted to alter receptor function have not been found in any of 350 normal control subjects (not kin of subjects with PAH) reported to date.

#### 48.6.5 Additional Genetic Modifiers of IPAH and HPAH

While the search for rare variants associated with IPAH and HPAH has been challenging, the search for modifying exposures and genes has proven even more elusive. Analyses of a number of genes chosen for their potential to affect vascular function have been performed with variable success. For example, candidate gene approaches that select those genes or pathways that have a role in vascular structure and function and that have known variants that confer differences in function have been used most commonly. The list of explored candidate modifier loci is long, and includes serotonin transporter (5-HTT) and receptors, nitric oxide (NO) synthases, TGFβ1, endothelin and endothelin (ETA) receptors, vasoactive intestinal polypeptide, urea cycle enzymes (including carbamyl phosphate synthetase 1), ETA receptors, ion channels, monoamine oxidases, and sex hormone pathway members.

Given the challenge of correctly hypothesizing a biologically relevant candidate or candidates, discovery-based methods to determine contributing common variants such as the use of genome-wide association studies are an appealing approach. Rodriguez-Murillo et al. employed this approach to study HPAH in 15 families that segregated *BMPR2* mutations. They combined dense single-nucleotide polymorphism array data with novel biostatistical analyses to perform a multiscan linkage procedure aimed at improving power and precision for localization of linked loci. Their findings suggest several additional loci that may contain modifiers of disease penetrance among *BMPR2* mutation carriers, the strongest of which was a region on 3q22 (median log



of the odds score of 3.43). While a lack of power due to relatively small sample size made it impossible to identify precisely the associated gene or genes in this region, this locus is now a region of intense interest (57).

While IPAH and HPAH are diseases specifically of the pulmonary vasculature, few studies to date have explored the genetic variations specific to the lungs themselves. Recently, Aldred et al. advanced the hypothesis that the acquisition of somatic mutations within the lung participates in disease pathogenesis among patients with IPAH and HPAH with and without *BMPR2* mutations (34). Using a genome-wide copy number microarray approach, confirmed by fluorescent in situ hybridization as appropriate, they found evidence of several somatic alterations throughout the lung vasculature of patients with end-stage IPAH and HPAH. Specifically, they detected acquired chromosomal abnormalities (including chromosome 13 loss as well as female-specific X chromosome inactivation) in a small number of patients with PAH. These changes were fairly specific to the endothelial cells, and present throughout the lungs including plexiform lesions (34). This work is provocative as the first study to document somatic mutations in the endothelial cells of end-stage PAH lungs, and suggests additional loci of interest. Furthermore, the finding of differential changes between endothelial and smooth muscle cells would be consistent with the perceived central role of endothelial cells in the pathogenesis of disease (58). Additional studies are needed to determine that somatic mutations are causative in PAH, including evidence that such mutations occur before any detectable disease (29).

## 48.7 MOLECULAR AND CELLULAR PATHOGENESIS

Regardless of cause, PAH is a devastating pulmonary vascular disease that results in a progressive increase in pulmonary vascular resistance. This ultimately leads to right ventricular failure and death (59). While persistent vasoconstriction is certainly a component of disease pathogenesis, the predominant histopathologic finding in PAH is a profound remodeling of the smallest pulmonary arteries, characterized by intimal hyperplasia, medial hypertrophy, proliferation and fibrotic changes of the adventitial layer as well as infiltration by circulating cells (60). The molecular and cellular processes involved remain unclear. The prevailing hypothesis involves proliferation and dysfunction of both endothelial and smooth muscle cells, as well as fibroblast activation and the recruitment of circulating cells (58). Intense cellular proliferation, as well as prior evidence of endothelial cell monoclonality and somatic endothelial cell gene mutations, highlights the features that suggest PAH may be a variant of an angioproliferative disorder with neoplastic features as noted above (30–34). Naturally, molecular and cellular pathogenesis is an area of investigation

in the field, with a considerable interest in the role of *BMPR2*, but not limited to this or other TGF $\beta$  receptors and their signaling pathways.

### 48.7.1 *BMPR2*-Mediated Pathogenesis

The TGF $\beta$  superfamily is composed of receptors and their small ligands that, in concert, regulate many sequences of cell and tissue growth, wound healing and inflammatory responses; they are even involved in neoplastic transformation of cells (61). This “superfamily” is made up of serine–threonine cell surface receptors that signal through intracellular pathways by phosphorylating Smad proteins, which translocate to the nucleus to regulate gene expression via various transcription factors. The receptors’ stimulants to signal are ligand cytokines (most typically various TGFs or BMPs). Evidence is growing that imbalanced activation of TGF $\beta$  receptor superfamily signaling contributes to the pathogenesis of PAH, and complicates our understanding of the origins of disease. Specifically, enhanced activation of the traditional TGF $\beta$  receptors, coupled with reduced activity of mutated *BMPR2*, may promote the pathogenetic events that cause PAH (62).

While many hypotheses exist to explain the contribution of *BMPR2* to pathogenesis, the prevailing view centers on TGF $\beta$  “imbalance.” That is, TGF $\beta$  family signaling promotes proliferation of endothelial and smooth muscle cells in the smallest pulmonary arteries without sufficient counter regulatory control by *BMPR2*. Signaling through *BMPR2* is unable to serve as a molecular “brake” on exuberant cell proliferation (63,64). Consistent with this hypothesis, *BMPR2* is reduced in the lungs of patients with mutations, as well as patients with other forms of PAH including but not limited to IPAH (63).

*BMPR2* has been shown to influence intracellular signaling via at least two groups of molecules, Smads and mitogen-activated protein kinases (MAPKs) (Figure 48-4) (55). *BMPR2* mutations alter Smad signaling in a mutation-specific manner but predictably result in potentiation of p38 MAPK signaling and cell proliferation in vitro (58,65).

**48.7.1.1 Smad-Dependent BMP Signaling.** In the presence of ligand, activated receptors phosphorylate cytoplasmic signaling proteins known as Smads, which are responsible for TGF $\beta$  superfamily canonical signaling transduction. BMPs signal via a specific set of receptor-mediated Smads (R-Smads), Smad 1, 5, and 8, which form a complex with the common partner Smad (Co-Smad), Smad-4, and translocate to the nucleus (4). Target gene transcription is regulated by a variety of mechanisms, including direct binding of the Smad complex to DNA, interaction with other DNA proteins, such as AP-1 and TFE-3, and recruitment of transcriptional coactivators or corepressors (66).

**48.7.1.2 MAPK-Dependent BMP Signaling.** Although Smad signaling is recognized as the canonical BMP

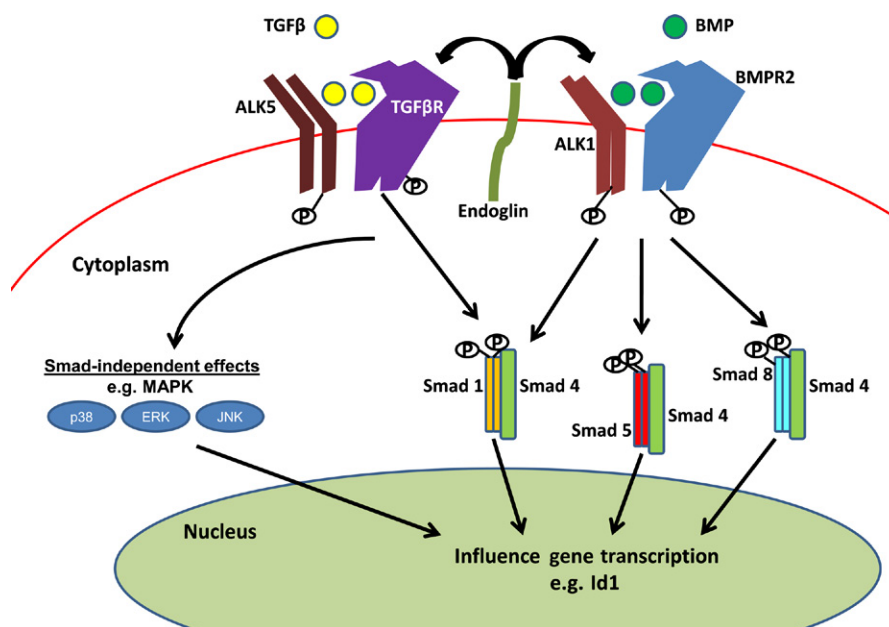


FIGURE 48-4 Schematic diagram of BMP signaling.

signaling pathway, there is an evidence that MAPKs, including  $p38^{\text{MAPK}}$  (MAPK14; OMIM 600289),  $p42^{\text{MAPK}}$  (MAPK1 or ERK2; OMIM 176948),  $p44^{\text{MAPK}}$  (MAPK3 or ERK1; OMIM 601795), and JNK/SAPK are modulated by BMPs and TGFβs in certain cell types (65). Disruption of Smad-dependent signaling may not be the most important pathway downstream of BMPR2 in the pathogenesis of PPH. The specific pathway activated by BMPR2 may depend on whether preformed type I/type II heterodimers are stimulated by ligand (Smad-dependent pathway), or whether ligand leads to recruitment of type I and II receptors to the signaling complex (MAPK-dependent pathway). It appears that preformed complexes are heterodimers containing one type I and one type II receptor, but ligand-recruited complexes consist of homo-oligomerized type I receptors and one type II receptor. Structural differences in the receptor complex may account for the selection of distinct signaling pathways.

**48.7.1.3 ACVRL1 and Endoglin.** Mutations in the TGFβ type I receptor, *ACVRL1* (OMIM 601284), *ENG* (OMIM 131195) (a TGFβ receptor accessory protein), and *SMAD4* associate with HHT. HHT is a condition frequently complicated by complex vascular lesions as well as variable expressivity; in some situations, PAH clinically identical to IPAH and HPAH will occur (67,68). The presence of a similar phenotype to IPAH and HPAH in HHT highlights the notion that the effects of TGFβ on vascular cell differentiation and proliferation are complex, and are likely influenced by additional genetic and environmental factors.

**48.7.1.4 Serotonin.** Serotonin (5-hydroxytryptamine; 5-HT) is implicated in the pathogenesis of PAH although human genetic and functional studies have shown mixed results. Pulmonary artery smooth muscle cells (PASMCs)

from patients with PAH display a greater proliferative response to 5-HT when compared to PASMCs derived from normal controls (69). Patients with PAH have a sustained increase in circulating plasma levels of 5-HT (70). Ingestion of anorexigens of the fenfluramine group is reported to increase the risk of developing PAH, and these drugs are known to inhibit 5-HT uptake by transporters and to augment circulating 5-HT levels. Lung tissue taken from PAH patients displays increased expression of 5-HTT (69). Homozygosity for a 5-HTT (SLC6A4; OMIM 182138) promoter polymorphism (L-allele) is found to be significantly increased in a PAH patient population as compared to a control population (65% vs 27%); this polymorphism is associated with increased gene transcription and 5-HT-induced PASMC proliferation (69). However, efforts to validate the L-allele polymorphism's association with PAH have yielded mixed results due to either small effect or insufficient sample size, or both (71,72).

**48.7.1.5 Transforming Growth Factor β1.** Given the interest in a potential signaling imbalance between canonical TGFβ signaling and BMP signaling, enhanced TGFβ signaling has been postulated to promote PAH among susceptible subjects. For example, among *BMPR2* mutation carriers, there may be common variants that promote disease expression such as penetrance or younger age at diagnosis. While not yet independently replicated, among our cohort of HPAH families, TGFβ1 common polymorphisms appear to modify disease expression. Specifically, *BMPR2* mutation carriers with TGFβ1 common variants that enhance TGFβ signaling had a younger mean age at diagnosis (about 7–10 years) of HPAH compared to those patients with variants associated with reduced TGFβ activity. Furthermore, those with the “less-active” TGFβ1 common variants had later age at diagnosis, and

lung Smad2 protein expression (a downstream marker of TGF $\beta$  activity) was higher among HPAH patients compared to controls (73).

**48.7.1.6 Other Contributors to Pathogenesis.** PSMCs derived from PAH patients have low messenger RNA (mRNA) levels for a voltage-gated potassium channel ( $K_{\text{vee}}$  channel) and decreased channel current, together with a corresponding increase in intracellular calcium levels as compared to controls (74,75). Intracellular calcium is a widely recognized stimulant for PSMC contraction and hypertrophy. The  $K_{\text{vee}}$  channel hypothesis is strengthened by the finding that some anorexigens block these channels in PSMCs.

Patients with PAH produce increased levels of thromboxane and endothelin-1, both vasoconstrictors, and decreased levels of both prostacyclin and NO synthase, which would inhibit formation of these vasodilators (76,77). It is possible that an imbalance between vasodilators and vasoconstrictors contributes to the pathogenesis of PAH.

Several studies have identified a possible role for angiopoietins (ANGPT1; OMIM 601667) in PAH, but whether these growth factors cause or prevent the disease remains unclear (78–80). One study demonstrated that administration of angiopoietin-1 (Ang-1) had protective effects in the rat model of monocrotaline-induced pulmonary hypertension, and hypothesized that this was due to the rescue of endothelial cells from apoptosis (80). Another study reported elevated levels of Ang-1 in lung tissue from PPH patients and demonstrated an associated decrease in BMPR-1A levels (79). The authors suggested that Ang-1 promotes smooth muscle cell hyperplasia through down-regulation of BMP signaling pathways. Subsequent work suggested overexpression of Ang-1 in rats caused pulmonary hypertension, while alternative studies have shown Ang-1 signaling is protective (81,82). While not indicative of causation, plasma levels of the angiogenic factors Ang-1, angiopoietin-2 (Ang-2), vascular endothelial growth factor, and soluble Tie2 were recently demonstrated to be elevated in IPAH patients. Of those factors, only Ang-2 improved with therapy and associated with improvement in hemodynamics. Ang-2 mRNA and protein were elevated in the plexiform lesions of IPAH patients, as well (83).

## 48.8 DIAGNOSIS

A normal resting mean pulmonary artery pressure is 8–20 mmHg. The diagnosis of any type of pulmonary hypertension requires the presence of a resting mean pulmonary artery pressure of  $\geq 25$  mmHg measured by cardiac catheterization. IPAH and HPAH are established clinically by confirming the presence of precapillary pulmonary hypertension (PAH), which is defined as pulmonary hypertension (mean pulmonary artery pressure  $> 25$  mmHg at rest) with a pulmonary capillary wedge pressure measurement  $\leq 15$  mmHg on cardiac

catheterization, in conjunction with the exclusion of other secondary causes of pulmonary hypertension (84). Secondary causes of PAH are more common and should be carefully excluded before the diagnosis of IPAH or HPAH is established. Some secondary causes of PAH include lung disease, heart disease, pulmonary embolism, and various other conditions including rheumatologic disease, cirrhosis, and HIV infection (see Table 48-1).

PAH may be suspected in individuals with the following symptoms when other known causes are absent. Initial symptoms include dyspnea (60%), fatigue (19%), chest pain (7%), palpitation (5%), syncope (8%), and edema (3%) (9). The clinical diagnosis of PAH was often delayed in the past, perhaps in part related to the fact that the typical symptoms are nonspecific, and also that the index of suspicion by general physicians was relatively low. General awareness has improved and widespread availability of echocardiography has greatly facilitated timely diagnosis.

Physical examination may reveal right ventricular heave, accentuation of the pulmonic component of the second heart sound, or cardiac murmur, such as tricuspid regurgitation occurring as a result of right ventricular dilation (9). Later in the course of disease, signs of right ventricular failure such as increased venous pressure, edema, or hepatomegaly occur in most patients. Electrocardiography (ECG) may reveal changes suggestive of right atrial or right ventricular hypertrophy. In individuals with pulmonary hypertension associated with cardiac causes, ECG may also reveal additional changes (left atrial hypertrophy) that suggest causes other than PPH. Pulmonary function testing may show mild restriction or may be normal in patients with PAH, but in patients with parenchymal lung diseases will usually reveal evidence of either obstructive or restrictive disorders or both (38).

In patients with IPAH and HPAH, chest radiography may show enlargement of pulmonary arteries and cardiomegaly from right ventricular or atrial enlargement, but the lung parenchyma appears normal. In individuals with pulmonary hypertension associated with parenchymal lung disease, chest radiography may reveal changes typical of interstitial or obstructive lung disease. Perfusion lung scanning is normal or mottled, or alternatively may reveal segmental or larger perfusion defects suggestive of pulmonary embolism. Chest computed tomography (CT) scanning shows normal lung parenchyma in individuals with IPAH and HPAH, while high-resolution imaging may show changes of interstitial lung diseases or emphysema in pulmonary hypertension associated with parenchymal lung disease. CT angiography has improved greatly and is noninvasive, so it may be helpful in the evaluation for suspected intravascular thrombi in the central pulmonary vasculature in some patients.

While not the definitive diagnostic study, echocardiography can provide an estimate of systolic pulmonary artery pressure and reveal changes of the right ventricle due to pulmonary hypertension (85). Echocardiography

is also valuable to screen for valvular or left ventricular diseases as alternative causes of pulmonary hypertension. Clinical confirmation of the diagnosis of PAH requires cardiac catheterization to measure pulmonary artery pressure and to exclude other cardiac abnormalities (84). When noninvasive testing suggests PAH, the catheterization should be performed in a laboratory that is experienced and prepared to perform an acute pulmonary vasodilator trial, which is often done using inhaled NO. Alternatively, provocative testing for other cardiac defects should be routinely employed when appropriate, such as observing the hemodynamic response of filling pressures to fluid challenge when there is clinical suspicion of left ventricular diastolic dysfunction.

Cardiac catheterization is necessary for confirmation of the disease in patients with suspected pulmonary hypertension (38,84), but in the current era, it rarely reveals unsuspected new findings. Recent advances of noninvasive thoracic and cardiac imaging often provide a specific diagnosis as the cause of pulmonary hypertension even before confirmation by cardiac catheterization. Many conditions that are associated with pulmonary hypertension, such as pulmonary embolism or parenchymal lung disease, are accurately and safely diagnosed by noninvasive imaging in the current era. Modern thoracic and vascular imaging by CT or magnetic resonance imaging provide high-resolution images that are comparable to invasive angiography.

Lung biopsy is not indicated to confirm a clinical diagnosis of PAH, but when histopathology is available in individuals with IPAH and HPAH (e.g. a lung explanted at the time of transplant), it usually confirms occlusion of small pulmonary arteries and in many cases, plexiform lesions with other lung structure being normal. Several pathophysiologic features contribute to small pulmonary artery occlusion, including proliferation of the intima and media of the vessel wall, vasospasm, and microthrombosis (7). Because surgical lung biopsy requires general anesthesia, the accompanying risk is rarely appropriate in patients in whom the clinical testing indicates IPAH or HPAH, but rarely lung biopsy does reveal other conditions (86). Extremely rarely, some patients demonstrate the typical clinical features of IPAH or HPAH but have a rapidly fatal course, and postmortem studies discover microembolic tumor occluding pulmonary microvessels.

Improvements in diagnosis are needed and will depend on the development of a method to identify and validate biomarkers that reflect the underlying vascular disease process itself, rather than the pulmonary hypertension per se, which is not the root problem itself, but is instead the consequence of the underlying pulmonary vascular disease. Pulmonary hypertension does not even begin until the pulmonary vascular disease is already in advanced stages, so any increase in pulmonary artery pressure reflects an advanced degree of vascular damage. Changes in right ventricular function and anatomy are even further distant in the sequence of events. Improved

molecular and cellular understanding about the central pathogenesis of PAH should lead to the development of early biomarkers of the vascular disease itself.

## 48.9 MANAGEMENT

Optimal therapy for PAH continues to improve, and many new and promising agents are still in investigational phases, so all patients should be offered consultation in specialized centers to access the best current treatment. Specialty centers for diagnosis and therapy of PPH are available all across the United States (see the Pulmonary Hypertension Association website at <http://www.phassociation.org>). New medications for PAH are costly and their availability differs by country, in part due to approval by regulatory agencies, so optimal therapy may differ geographically around the world (87). Most patients seek medical care only after symptoms develop, which occurs late in the course after extensive numbers of small pulmonary arteries have been lost. For the future, as further knowledge develops about the earliest pathogenesis of PAH, biomarkers of the vascular disease itself will be identified to diagnose the disease before extensive vascular loss, at a phase during which prevention of progression should have a greater possibility of success.

### 48.9.1 Approved Therapies in the United States

Despite considerable advances in the genetic, molecular, and cellular understanding of PAH, disease-specific medicinal options remain limited for patients but are discussed briefly below.

**48.9.1.1 Adjunctive Therapies.** Diagnosis of PAH does not necessarily preclude an active lifestyle. Patients should engage in activities appropriate to their functional status in order to prevent deconditioning. Regular exercise also provides a benchmark for patients to ascertain any change in their condition. Appropriate caution concerning physical activity is advised for patients with advanced pulmonary hypertension with symptoms of dizziness, chest pain, or severe dyspnea because there is increased risk for life-threatening syncope. In general, patients are cautioned to maintain physical activity at a level that is tolerated but not stressful to them physically or mentally. The role for cardiopulmonary rehabilitation and maintenance is an area of active investigation, with evidence that it is safe and efficacious in a monitored setting (88).

Chronic anticoagulation therapy, diuretics, and supplemental oxygen are used routinely in the care of individuals with pulmonary hypertension, but have not been rigorously studied (87). The rationale for anticoagulant therapy is based on the presence of risk factors for venous thromboembolism, such as heart failure and a sedentary lifestyle. Warfarin has been evaluated in only



two small uncontrolled studies, but is routinely recommended with a target International Normalized Ratio between 1.5 and 2.5.

Chronic hypoxemia occurs in advanced stages of disease and is often the result of impaired cardiac output, which results in desaturation of mixed venous blood. It may also be caused by right-to-left shunting through a patent foramen ovale. When chronic hypoxemia develops, oxygen administration is indicated to maintain arterial oxygen saturation at a level above 93%.

Substantial clinical improvement in functional status as well as in fluid balance can be achieved by diuresis alone in many patients with right heart failure (87). The value of cardiac glycosides in treating isolated right heart dysfunction is controversial. Because neurohormonal sympathetic activation has been demonstrated in pulmonary hypertension, some authors propose treatment with digoxin for its sympatholytic properties, but no data are available from prospective, randomized, placebo-controlled trials to provide clear guidelines.

Pregnancy and labor increase the demand on the cardiorespiratory system, so they are generally contraindicated in patients with pulmonary hypertension, and contraception is generally recommended for women of childbearing age who have PAH. However, recent interest in the potential role of sex hormones in the pathogenesis of PAH, as well as concerns about sex hormone exposures in cardiovascular disease in general, some clinicians recommend alternative forms of birth control to medical hormone therapy. Finally, for all patients vaccination, including annual influenza vaccination, is encouraged unless contraindicated.

**48.9.1.2 Calcium Channel Blockers.** Because these oral agents are widely used for other disorders, they are familiar to most physicians. It is widely accepted that a small minority (~10%) of PAH patients respond favorably to calcium channel blockers (CCBs), sometimes for many years or indefinitely, but randomized controlled trials have not been performed (87). As noted above, all patients should undergo acute vasodilator testing with a short-acting vasodilator (inhaled NO, adenosine, epoprostenol) during diagnostic heart catheterization (84). Patients who may benefit from long-term therapy with CCBs can be identified by a favorable response to an acute vasodilator challenge. The safest approach is with the use of short-acting agents, such as intravenous prostacyclin, adenosine, or inhaled NO, during right heart catheterization. In a retrospective analysis of 557 consecutive patients with PAH, Humbert et al. found that fewer than 7% had a sustained benefit from therapy with a calcium channel blocker (87). During acute vasodilator challenge, most patients who had a long-term response to CCBs had a marked improvement in their pulmonary hemodynamics (i.e. mean pulmonary artery pressure decreased by >10 mmHg, to a value >40 mmHg, with a normal or high cardiac output). The occurrence of severe, life-threatening hemodynamic compromise

during an acute vasodilator challenge with CCBs is well documented, and these agents should not be used as initial agents for acute pulmonary vasodilator testing.

**48.9.1.3 Prostaglandin Analogs.** Prostacyclin is a prostaglandin produced by both vascular smooth muscle and endothelium that stimulates vasodilation, inhibits smooth muscle growth (antiproliferative effects), and inhibits platelet aggregation. Prostaglandins target the prostaglandin I receptor, which is found in blood vessels, leukocytes, and thrombocytes. This receptor is coupled with Gs proteins and when stimulated, activates adenylate cyclase, leading to an increase in intracellular cyclic adenosine monophosphate (89).

Epoprostenol (FLOLAN) is a prostacyclin analog that is administered by continuous intravenous infusion because its half-life is only 3 min in the circulation. For chronic home administration, epoprostenol is infused by a portable infusion pump connected to a permanent tunneled catheter in the subclavian vein. Approval of epoprostenol in the United States followed a randomized controlled trial in 81 patients with PAH in NYHA functional class III or IV (90). Participants were randomly assigned to receive either conventional therapy alone (including warfarin, diuretics, oxygen, and oral vasodilators) or conventional therapy plus continuous intravenous epoprostenol. After 12 weeks, patients in the epoprostenol group had functional improvement as demonstrated by improved 6-min walk-test results. In the epoprostenol group, the mean pulmonary artery pressure decreased 8% in the epoprostenol group, while it increased 3% in the control group. Mean pulmonary vascular resistance decreased 21% in the epoprostenol group and increased 9% in controls. Eight patients in the conventional therapy group died during the study, whereas no deaths occurred in the epoprostenol group.

Common epoprostenol side effects include jaw pain, nausea, headache, flushing, and diarrhea. Complications from the delivery system include exit-site infections and bleeding, bacteremia/sepsis, catheter fracture, delivery malfunction that may result in sudden severe decompensation, and paradoxical embolization. In the past, patients began continuous intravenous epoprostenol with the understanding that they would continue it lifelong or until lung transplantation. As other effective therapies have become available in the past few years, data are emerging to suggest that selected patients may successfully transition off parenteral therapy to alternative prostanoids or other PAH-specific therapies.

Complications related to the central venous catheter required for intravenous infusion of prostacyclin led to the development of treprostinil, a stable prostacyclin analog approved in the United States to be administered by continuous intravenous or subcutaneous infusion (91,92). It otherwise displays the same pharmacologic and hemodynamic effects as seen with epoprostenol. Treprostinil has been an approved therapy for PAH in the United States since 2002 and some patients with

PAH in whom life-threatening complications developed with intravenous epoprostenol have been safely switched to subcutaneous treprostinil (93). Inhaled treprostinil appears efficacious although placebo-controlled trials using this agent are limited (94). The inhaled delivery system facilitates deposition in alveoli, thereby improving selective effect on pulmonary versus systemic vessels, preferentially on those pulmonary vessels located in well-ventilated regions. The relatively short duration of action requires dosing six or more times daily, which may hinder adherence to therapy.

**48.9.1.4 ET Receptor Antagonists.** There are two ET receptor antagonists currently approved for use in the United States: bosentan and ambrisentan. While similar, these drugs have different affinities for endothelin type A (ET<sub>A</sub>) and endothelin type B (ET<sub>B</sub>) receptor antagonism, which may theoretically lead to differing pharmacologic and therapeutic effects.

Bosentan (Tracleer) is an orally active dual ET receptor (ENDRA and ENDRB; OMIM 131243 and 131244) antagonist. Endothelin-1 has many different biological actions, including vasoconstriction and stimulation of proliferation of vascular smooth muscle cells. The effects of endothelin-1 are mediated through the ET<sub>A</sub> and ET<sub>B</sub> ET receptors. Activation of ET<sub>A</sub> receptors causes vasoconstriction and proliferation of vascular smooth muscle cells, whereas ET<sub>B</sub> receptors mediate pulmonary endothelin clearance and induce the production of NO and prostacyclin by endothelial cells. Two randomized, double-blind, placebo-controlled trials have evaluated the efficacy of oral bosentan in patients with PAH. In a study of 33 patients in NYHA in functional class III (95) who were randomly assigned to receive placebo or bosentan, patients receiving bosentan had an increase of 76 m in the 6-min walk test, as well as significant improvements in pulmonary artery pressure, cardiac output, and pulmonary vascular resistance. In a subsequent study (96), 213 patients in NYHA functional class III or IV were randomly assigned to receive placebo or bosentan; the treatment was associated with increase in the 6-min walk of 52 m in patients with PAH. Patients receiving bosentan also had improvement in the time to clinical worsening. Although there is some preliminary evidence of sustained efficacy with 12 months of therapy, the long-term effects of bosentan require further evaluation. Bosentan is metabolized by the liver and may induce an increase in hepatic aminotransferase levels. Development of abnormal hepatic function appeared to be dose dependent, in support of the approved dose of 125 mg twice daily. Elevations in aminotransferase levels to more than eight times the upper limit of normal occurred in 3% of patients receiving 125 mg twice daily and 7% of patients receiving 250 mg twice daily. Monthly monitoring of liver function tests is necessary, but there are no reports of permanent liver dysfunction or failure with bosentan, even though thousands have received the drug.

Ambrisentan is an oral selective ET<sub>A</sub> receptor antagonist, approved in the United States since 2007. This specific antagonism presumably blocks the vasoconstrictor effects of ET receptors while maintaining the vasodilator and clearance effects of ET<sub>B</sub> receptors. It was shown to improve exercise capacity and delay clinical worsening in patients with PAH in two concurrently run, 12-week multicenter randomized controlled trials (i.e. Ambrisentan in PAH, Randomized, Double-Blind, Placebo-Controlled, Multicenter, Efficacy Studies 1 and 2 (ARIES-1 and ARIES-2)) (97). This drug may provide sustained improvements in pulmonary hemodynamics in patients with PAH who receive long-term treatment and these changes correlate with improvements in exercise capacity, but further studies are needed (98). Cases of acute hepatitis have been described in patients taking selective ET<sub>A</sub> blockers, including one fatality, which emphasizes the importance of serial monitoring of liver function.

**48.9.1.5 Phosphodiesterase-5 Inhibitors.** Phosphodiesterase type 5 (PDE-5) inhibitors, such as sildenafil (Viagra), have an acute pulmonary vasodilator effect. NO, released by vascular endothelial cells, causes vasodilation of the lung vessels by increasing production of cyclic guanosine monophosphate (cGMP) via activation of soluble guanylyl cyclase, ultimately leading to smooth muscle cell relaxation (99). In the lung, cGMP is hydrolyzed rapidly by PDE-5 to inactive compounds. Inhibition of cGMP results in vasodilation (100). Two PDE-5 inhibitors, both oral delivery, are approved for PAH therapy in the United States: sildenafil and tadalafil. For each, the most common side effects are headache, flushing, back pain, dyspepsia, and insomnia. Patients may experience transient mismatching of ventilation and perfusion upon initiation, so caution should be taken in particular when starting these or any other PAH-specific medications.

## 48.9.2 Surgical Therapy: Lung Transplantation

Lung transplantation is an effective treatment for some individuals with PAH (26,27). Transplantation of both lungs is currently recommended although single-lung and heart-lung (heart and both lungs en bloc) transplantation has been used successfully. Typical upper age limits for recipients are 50 years for heart-lung transplantation, 60 years for bilateral lung transplantation, and 65 years for single-lung transplantation. Insufficient availability of donor lungs is a major limitation, but even for those fortunate enough to be recipients, long-term survival is limited by chronic rejection for most, so that the mean survival after lung transplantation is only 4 years. Although some patients have done very well for long periods, even sometimes for decades after lung transplantation, they represent only a small minority of all the recipients.

Ironically, the availability of effective medical therapy has complicated the decision about timing for transplantation. Before the availability of effective medical therapies, functional status correlated closely with survival in individual patients, so that lung transplantation was appropriate for patients with severely limited functional capacity (i.e. NYHA class III or IV). In the current era of effective medical therapy, it is much more difficult to estimate survival for individual PAH patients, and it is thereby problematic to decide when the high risk of lung transplantation is warranted. Medical therapy may appear effective in many patients who have good functional status, but their ability to survive even a modest intercurrent illness may be severely compromised by limited cardiorespiratory reserve.

## 48.10 COUNSELING

### 48.10.1 Clinical Screening and Surveillance for PAH

Clinical surveillance of asymptomatic family members at risk has been recommended since the 1998 World Health Organization Symposium on PAH. Most recently, guidelines from the 2008 meeting suggested echocardiographic screening of family members who are at risk, at intervals of 3–5 years, to enable early detection and treatment (17). There are no reports of the frequency of compliance with this recommendation, and its performance is influenced by the fact that many health insurers do not provide coverage for screening tests for individuals unless symptoms or signs are present. At Vanderbilt, we have on several occasions detected pulmonary hypertension in asymptomatic carriers of *BMPR2* gene mutations, the most recent of whom was an adolescent male who subsequently had PAH confirmed by cardiac catheterization.

### 48.10.2 Agents and Circumstances for At-Risk Subjects to Avoid

Appetite suppressant medications, such as fenfluramine/phentermine, dexfenfluramine, and amfepramone (diethylpropion), have all been associated with PAH (2,48). Amphetamines, cocaine, and related compounds causing vasoconstriction have anecdotal association with PAH and could be risk factors (22). Methamphetamine has recently been associated with cases of PAH, of great concern given the surge in the use of this drug of abuse (101).

Other medications that have anecdotal suggestion of risk include estrogen compounds used as oral contraceptive or replacement therapy (22). Anecdotal reports of association of pregnancy with onset of PAH give concern about the possible risk of pregnancy as a trigger; however, there is no consensus about the safest effective birth control in women who have PAH or who are at risk for it in PAH families. The physiologic stress of pregnancy in an affected individual is significant, and maternal

mortality is believed to be substantial, but newer effective therapies may decrease this risk (102).

Hypoxia that accompanies high altitude is associated with pulmonary vasoconstriction and PAH in susceptible individuals. Therefore, individuals with PAH should avoid hypoxia.

### 48.10.3 Genetic Counseling

PAH in families is inherited in an autosomal dominant manner. However, the average penetrance of *BMPR2* mutations is ~20%, and this reduced penetrance makes predictions of who will develop PAH in their lifetime unreliable. If a parent of a proband has a *BMPR2* mutation, the risk to the sibs of inheriting the gene mutation is ~50%; however, because of reduced penetrance, the risk to a sib of developing HPAH is ~10% (50% risk of inheriting the disease allele  $\times$  20% risk of penetrance). Similarly, the children of an individual with a *BMPR2* mutation are at 50% risk of inheriting the mutant allele; however, because of reduced penetrance, the risk to offspring of developing HPAH is ~10% (50% risk of inheriting the disease allele  $\times$  20% risk of penetrance). Prenatal testing is available through laboratories offering such testing although decisions on the use of prenatal testing should depend on the family at risk. There is no consensus on the appropriateness of prenatal testing at this time.

### 48.10.4 Mutation Testing of Relatives at Risk

Direct molecular genetic testing of *BMPR2* alleles for mutations is available in at least four clinical laboratories in the United States. Genetic testing for *BMPR2* gene mutations should occur within the framework of a comprehensive approach to provide not only medical care as needed but also genetic counseling irrespective of the result of mutation testing. The Genetic Information Non-discrimination Act of 2008 (103) guarantees the protection of subjects tested for genetic variants although the ramifications of testing may extend far beyond that of health insurance and employment, and thus appropriate counseling is critical.

The use of *BMPR2* molecular genetic testing to clarify the genetic status of at-risk relatives may be helpful, especially in families in whom a specific mutation is known. A negative test result in which the subject does not have a mutation can be very helpful because it indicates that the subject and his/her progeny possess the same risk as the general population; thus, they can safely forego clinical screening. As noted above, because of reduced penetrance, the approximate pretest probability for the first-degree relative of an affected member of an HPAH family is ~10% (50% risk of inheriting the disease allele  $\times$  20% risk of penetrance =  $0.50 \times 0.20 = 0.10$ ) of developing HPAH during the subject's lifetime. If there is



a known mutation in the family for which there is a reliable test, then if the first-degree relative tests negative for the mutation, his or her risk for developing HPAH falls from ~10% to that of the population (~1–15 per million subjects), so it changes 100,000-fold. Conversely, if he or she tests positive for the mutation, the risk changes from ~10% to ~20%, or a twofold change. Thus a positive-test result changes the pretest probability only slightly, whereas a negative-test result changes risk dramatically.

Detection of a responsible germline mutation in a sporadic PAH patient (those typically considered to have IPAH) may have the greatest overall impact, because it converts the concept of the disease from sporadic to familial, thereby revealing the possibility of a heritable disease for which one's bloodline kin are now at increased risk. If the patient has siblings or children, they may be surprised and alarmed by the new knowledge and the unanticipated personal risk. Of course, the risk already existed before testing, and was simply unrecognized, but the emotional impact of revealing the true risk has many possible complications—this highlights the critical role of extensive pretest counseling.

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## ABBREVIATIONS

PAH – pulmonary arterial hypertension

HPAH – heritable pulmonary arterial hypertension

IPAH – idiopathic pulmonary arterial hypertension

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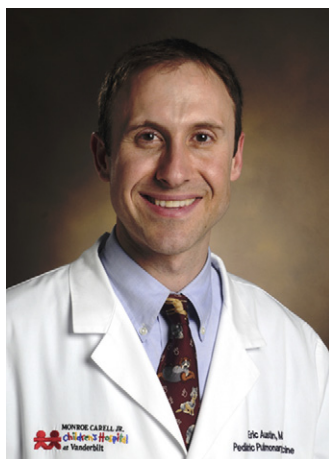
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## RELEVANT WEBSITES

Gene <http://www.ncbi.nlm.nih.gov/sites/GeneTests/review>.  
Phassociation <http://www.phassociation.org/>.

## Biographies



**Dr Eric D Austin, MD, MSCI**, is a pediatric pulmonologist and physician–scientist with a combined laboratory-based and patient-oriented translational research program that focuses on pulmonary hypertension and other cardiopulmonary morbidities in children and adults with and without pre-existing known genetic risks. Following pediatric training at the University of Colorado, he performed subspecialty training in pulmonary medicine at Vanderbilt University. Dr Austin joined the faculty at Vanderbilt in 2008, and remains an Assistant Professor of Pediatrics in the Division of Allergy, Immunology, and Pulmonary Medicine. In addition to the care of children with simple and complex pulmonary diseases, his specialty training was also in translational research techniques, including the design and maintenance of longitudinal cohorts of patients and family members with cardiopulmonary disease or genetic risk of disease. His research work focuses on evaluating the manner in which genetic, biochemical, environmental and other abnormalities promote phenotypic expression of pulmonary hypertension and other cardiopulmonary diseases.



**John A Phillips III, MD, PhD**, is the David T. Karzon Professor of Pediatrics at Vanderbilt University School of Medicine. He is also the Director, Division of Medical Genetics and Genomic Medicine, Professor of Pathology, Microbiology, Immunology and Medicine. Dr Phillips' has successfully cared for children and adults with genetic disorders for many years while maintaining a thriving research program as well. His research interests are in determining the molecular basis, pathogenesis, and treatment of Mendelian disorders. Current studies include the following: 1) genetic defects in the growth hormone (GH) synthetic pathway and regulation of alternative splicing of GH1 transcripts; 2) genetic basis of pulmonary arterial hypertension (PAH)—this work includes his discovery along with Drs. Loyd, Newman and colleagues of BMPR2 gene defects in familial pulmonary arterial hypertension; 3) genetic basis of autonomic disorders; 4) genetic basis of idiopathic pulmonary fibrosis (IPF) and, 5) determining the therapeutic effects of BH4 on blood Phenylalanine reduction, development and global function of subjects with Phenylketonuria (PKU).





**John H Newman, MD**, is Professor of Medicine and the Elsa S Hanigan Chair in Pulmonary Medicine at Vanderbilt University School of Medicine. For nearly 30 years, Dr Newman has successfully studied the complex nature of the pulmonary circulation, including the genetic basis of pulmonary hypertension—an interest that started in 1980 when he and Dr Loyd encountered their first cases of familial pulmonary hypertension. Mentor, physician-scientist, and clinician, Dr Newman has authored over a hundred articles in scientific publications and numerous books, book chapters, and review papers. Dr Newman continues to investigate a wide variety of questions to examine such issues as gene-to-gene interactions in pulmonary hypertension, the role of phosphodiesterase type 5 (PDE5) as a modulator in right ventricular (RV) failure, and gender and endothelin determinants of RV function in pulmonary hypertension.



**James E Loyd, MD**, Rudy W Jacobson Professor of Medicine Dr Loyd is a physician-scientist with a successful record of translational research and clinical care of patients with complex cardiopulmonary diseases. His primary areas of research focus have been pulmonary vascular disease, pulmonary fibrosis, and fibrosing mediastinitis. For example, Dr Loyd, along with Dr Newman and colleagues, has investigated pulmonary hypertension continuously since 1980. Their work with Dr Phillips and other investigators using a cohort of families with pulmonary hypertension resulted in their identification of BMPR2 as the primary gene associated with familial pulmonary arterial hypertension in 2000. Dr Loyd continues to explore genetic and other modifiers of pulmonary arterial hypertension, using translational research approaches incorporating cellular and animal models as well as human studies of disease expression.

# CHAPTER

# 49

## Hereditary Hemorrhagic Telangiectasia (Osler–Weber–Rendu Syndrome)

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### 49.1 INTRODUCTION

#### 49.1.1 Historic Perspective

The evolution of our recognition and understanding of hereditary hemorrhagic telangiectasia (HHT; OMIM 187300) begins with the descriptive science of the nineteenth century and continues through the current era of molecular medicine. Only in the past two decades have modern genetic approaches made clear that the previous view of HHT as a single entity was overly simplistic, and that a more accurate concept is of a family of closely related conditions that share similar, but not identical, molecular bases and clinical courses.

The history of HHT might be said to start in 1864, when Sutton described a family with apparent autosomal dominant epistaxis, including at least two affected individuals with a history of hemoptysis, one of whom died at the age of 60 of what was said to be “ruptured blood vessel of the lungs” (1). Even with no mention of such other symptoms and signs as telangiectasias, this likely represents what we now regard as HHT.

Although the following 30 years included a number of reports of families with various signs and symptoms of what may well have been HHT, it was Rendu who, in 1896, first reported the combination of telangiectases and hereditary epistaxis as a discrete entity, and one that he distinguished from the more widely known hemophilia (2). In the following decade, a number of reports of this condition appeared, most importantly by Osler (3) and Weber (4), whose names soon joined that of Rendu to form the triple eponymic appellation, that in varying order, has been used by many to label the disease ever since. In 1909, Haynes coined the term hereditary

hemorrhagic telangiectasia for the condition, in recognition of the three features—its hereditary nature, frequent epistaxis, and multiple telangiectases—that by then were thought to characterize it (5).

The remainder of the first half of the twentieth century witnessed numerous case reports, which both reflected and caused somewhat wider medical recognition of HHT; however, the diagnosis was apparently still often not entertained even in the face of “textbook” presentations, and there was relatively little progress in better understanding the condition’s basic underlying biology or in treating it more effectively. In the second half of the twentieth century, several developments began to change this picture—first in terms of therapy, and later in terms of biological understanding. Among major therapeutic advances were the development, starting in the 1950s, of septal dermoplasty for persistent epistaxis by William Saunders and others (6) and the development of transcatheter embolotherapy for pulmonary arteriovenous malformations (PAVMs), starting in the late 1970s, by Robert I. White, Jr, Peter Terry, and others (7). The finding in the 1980s by Bruce Jacobson and others of a patient registry that soon grew into the Hereditary Hemorrhagic Telangiectasia Foundation International provided an impetus for increased patient and provider education, as well for both basic and clinical research.

At the end of the twentieth century, new genetic approaches led to dramatic breakthroughs in understanding of the basic biology of HHT. Over 1000 primary research publications have appeared since 1995. In rapid succession in the mid-1990s, Marchuk, LeTarte and others identified two different causative genes, endoglin (*ENG*) on chromosome 9 and activin-like kinase 1 (*ACVRL1*) on chromosome 12 (see Section 49.2.3) (8,9).

That both genes were involved in the transforming growth factor beta (TGF $\beta$ ) and bone morphogenetic protein (BMP) signaling pathways suggested the biology that lay at the core of the HHT phenotype. That two different genes could cause what had previously appeared to be a single clinical condition raised the question of whether HHT was, in fact, a single clinical entity, or a family of related, phenotypically overlapping conditions.

The 1990s also saw the establishment of forums for communication among the growing international HHT research community, including regular international meetings that advanced both research and clinical collaborations (10,11). The last years of the twentieth century and the first decade of the twenty-first century witnessed other advances, such as the establishment of the first animal (mouse) models for HHT (12–15) and the identification of families segregating features of both juvenile polyposis and HHT due to mutations in SMAD4 (16).

### 49.1.2 Prevalence

Several population-based surveys suggest the prevalence is at least one per 5000–8000 (17). In the County of Fyn, Denmark, the prevalence is 15.6 per 100,000 (18). In the Netherlands Antilles, the prevalence is much higher, 1 per 1331, at least in part due to founder effect in an island population (19). As with many other dominant conditions, age dependency of features contributes to incomplete ascertainment in any family or population-based investigation. No prevalence survey has yet employed molecular diagnosis.

## 49.2 PHENOTYPE AND NATURAL HISTORY

### 49.2.1 Overview

The fundamental problem is in the development of blood vessels, especially the connections between arteries and veins. Problems start in the embryo, but they often do not become clinically important until adulthood. If the tiniest connections, the capillaries, are involved, then the problems involve telangiectases, or 1- to 2 mm enlargements that appear dark red on the skin, the lips, the tongue, or the nasal or the bowel mucosa. Because the telangiectases are close to the surface and have very thin walls, they bleed easily. This accounts for nosebleeds being an early sign of HHT, and gastrointestinal (GI) hemorrhage being a problem in adulthood. When the abnormal connections are larger, they are termed AVMs. The brain, the lung, and the liver are the primary sites of AVMs, but other organs can be involved. The absence of a capillary connection is established early in life, and the individual lesion may or may not expand slowly over many years. An AVM causes problems because capillaries are bypassed. An AVM anywhere results in increased cardiac output, which places strain on the heart. In the lungs, an

AVM provides a direct connection through which bacteria and blood clots in veins can travel directly to the arterial circulation without being filtered by lung capillaries. The result can be a stroke or cerebral abscess if the final resting place is the brain, or ischemic damage to an end organ such as the kidney. Also, pulmonary AVMs provide a shunt that prevents venous blood from being oxygenated, so the partial pressure of oxygen in arterial blood is reduced proportional to the number, size, and location of the lung lesions.

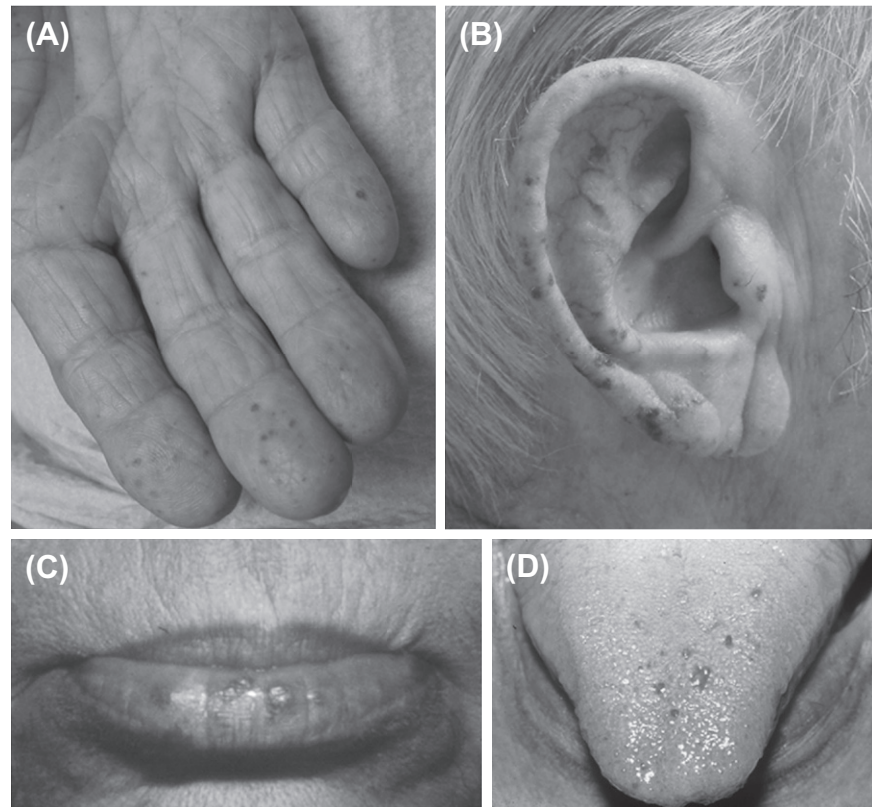
The condition is often misdiagnosed, both because the features vary considerably among those affected (even within a single family), and features accumulate over the lifespan of patients. Formal studies of quality of life show that the clinical complications of HHT, especially epistaxis, have a significant negative impact (20). Both marked inter- and intrafamilial variabilities characterize HHT. Interfamilial variability is due, in part, to different mutations in different genes. Intrafamilial variability is typical of many dominant disorders (21); the precise biology by which it occurs in HHT is still unknown.

**49.2.1.1 Mucocutaneous Telangiectases.** A telangiectasia can appear as a central core with dilated capillaries extending out from it (spider telangiectases) or as a single macular or slightly elevated, more-or-less circular lesion. Spider telangiectases typically occur because of sun exposure or chronic liver disease. Single, circular lesions are typical of HHT. When slight pressure is applied, telangiectases blanch, then quickly refill when the pressure is released. This distinguishes telangiectases from a petechia and an angiokeratoma, which do not blanch, and a cherry angioma, which at best may blanch minimally. In HHT, punctuate telangiectases can appear anywhere on the skin or mucus membranes, but are most common on the fingers, palms, face, lips, buccal cheek, and tongue (Figure 49-1). Microscopy of the nail folds often shows dilated loops between capillaries and can be an early diagnostic sign (22).

Telangiectases may be present in children, but they typically appear later and increase with age. Numerous telangiectases in young children, in the absence of brain or lung AVMs or a family history of HHT, should prompt consideration of other diagnoses, such as ataxia-telangiectasia or benign familial telangiectasia. In adults, cutaneous and oral telangiectases occasionally bleed when traumatized, but they generally are of cosmetic concern only.

Two areas where telangiectases of the mucus membrane pose substantial clinical risk are the nose and the GI tract.

**49.2.1.2 Epistaxis.** Often the first sign of HHT is recurrent bleeding from the nose (epistaxis). Because epistaxis occurs so frequently in the general population, until the episodes become frequent or severe, the patient or parent may not seek medical attention. Even then, the primary care or emergency physician usually treats or refers to the rhinologist but does not delve into the cause. The family



**FIGURE 49-1** Dermal and mucocutaneous features of HHT. A. Digits. B. Ear. C. Lips. D. Tongue.

history of a child that reveals severe epistaxis in close relatives can be the most important piece of evidence that a familial disorder should be entertained, and HHT is at the top of a very short list. Bleeding occurs from mucosal telangiectases and eventually affects about 90% of people with HHT. The average age of onset is 12 years and the average frequency is 18 episodes per month. Nocturnal epistaxis is common. Factors that predispose to bleeding include low atmospheric humidity and digital trauma.

**49.2.1.3 Gastrointestinal.** Bleeding from mucosal telangiectases in any portion of the GI track can lead to chronic anemia. At the most proximal end, lesions on the lips, tongue, and oral mucosa are of cosmetic concern primarily, but bleeding can be annoying and embarrassing. Bleeding from the stomach, small intestine, and colon becomes more common with age; rarely, this is a problem in children or adolescents. Upper and lower endoscopy can reveal the site of blood loss but often shows a myriad of lesions that appear capable of bleeding at any time. Examination of stool for occult blood is of limited use in HHT because swallowed blood from epistaxis yields a positive result.

One uncommon form of HHT is associated with juvenile polyposis and is due to mutations in *SMAD4* (16). Juvenile polyps can bleed or obstruct but are most worrisome because of their susceptibility to malignancy. Mutations in *ENG* can also predispose to juvenile polyps, apparently in the absence of signs of HHT (23). Involvement of both *SMAD4* and *ENG* in both HHT

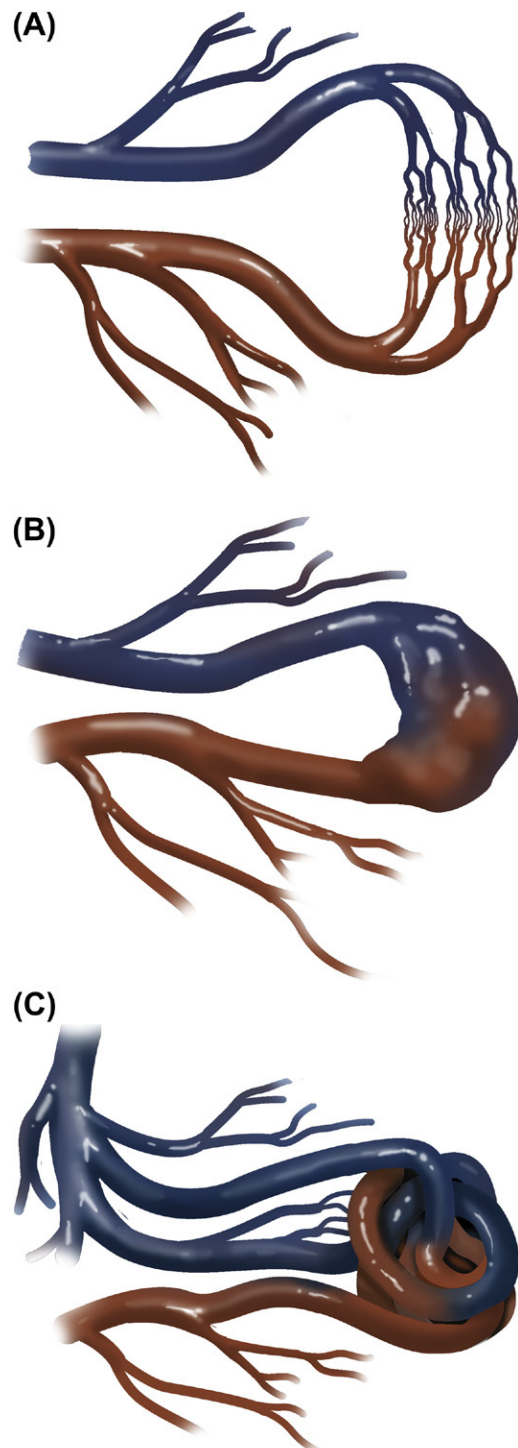
and polyposis emphasizes the importance of the TGF $\beta$ –BMP signaling pathway in the control of a variety of developmental systems.

**49.2.1.4 Central Nervous System.** Three forms of developmental lesions and one acquired form are common in the brain and spinal column in HHT (24). Telangiectases, venous malformations, and cerebral arteriovenous malformations (CAVMs) are often clinically silent. Their frequency and natural history are unclear because most patients have not been screened adequately or repetitively. Screening by magnetic resonance imaging (MRI) found a 23% prevalence of some cerebral vascular malformation in patients with HHT (25); however, this study also showed that angiography is more sensitive for both detecting and characterizing cerebral lesions. The risk of bleeding from any of these lesions is also unclear; one study suggested a risk of 0.5% per year (26), whereas another found a risk of 1.4–2.0% per year (27). In terms of cerebral hemorrhage of any cause, the risk for a man younger than 45 years of age was 20 times greater in HHT (27). If a malformation is large enough or bleeds, the complications include headache, altered mental status, seizure, and stroke. Migraine-like headaches are of increased frequency especially in those who have an intrapulmonary right-to-left shunt (28,29).

The major acquired lesion is brain abscess, which is thought because of paradoxical embolization of bacteria through a PAVM (30).



**49.2.1.5 Lung.** The single clinical finding that is most likely to prompt consideration of the diagnosis of HHT is the PAVM (Figure 49-2A). It remains uncertain as to what fraction of people with an apparently isolated PAVM actually has HHT. In part, many patients do



**FIGURE 49-2** Filtration function of pulmonary capillary bed and how a pulmonary arteriovenous malformation (PAVM) bypasses that function to produce paradoxical emboli can be envisioned from these renderings. A. Normal capillary bed. (Reprinted with permission from Ref. 139.) B. A simple PAVM. C. A complex, large PAVM. (Reprinted with permission from Ref. 139.)

not undergo detailed screening of the rest of their lungs to detect small lesions. In addition, molecular genetic screening has been available since 2003. PAVMs should be looked for carefully in any person with, or at risk for, HHT, including infants (31), because considerable morbidity results from these lesions. A PAVM of any size can serve as a conduit for bacteria, air bubbles, or clots from the venous circulation to be transmitted directly to the systemic arterial circulation. The risk of cerebral infarction due to embolization is greater in patients with multiple PAVMs (32,33). The PAVM also serves as a right-to-left shunt in terms of oxygenation, and the decrease in arterial  $PO_2$  is generally determined by the size, number, and location of PAVMs. The patient may present with progressively worsening dyspnea on exertion, cyanosis, and clubbing. Polycythemia leading to plethora is uncommon, because most patients with HHT have chronic blood loss from one source or another. Hemoptysis from rupture of a PAVM or bleeding from bronchial telangiectases is not uncommon (~10% over a lifetime), and can be life-threatening when massive (34). Chronic right-to-left shunting can also lead to pulmonary hypertension and failure of the right side of the heart.

Infants and children should be screened for PAVMs since they may have one or more of clinical importance. Typically, however, PAVMs emerge in adolescence and young adulthood. A lesion has a tendency to expand over time, and new ones emerge, which emphasizes the need for life-long assessment. Some young adults will have no evidence of an intrapulmonary shunt, and are unlikely to develop any.

A small fraction of patients with HHT have pulmonary hypertension unrelated to their degree of shunting. These patients typically have a mutation in *ALK1*. Some families with primary pulmonary hypertension but without signs of HHT have mutations in *ALK1* (OMIM 178600; see Chapter 48).

**49.2.1.6 Liver.** Four types of hepatic vascular lesions occur in HHT: (1) telangiectases; (2) direct communication of hepatic arteries to hepatic veins; (3) direct communication of hepatic arteries to portal veins; and (4) portal vein-to-hepatic vein connections (35,36). Several recent surveys using ultrasound, computed tomography (CT), or MRI have shown the frequency of hepatic vascular involvement to be much higher than previously thought, in the range of 40–75% (37,38). Discrepancies in prevalence relate to methods of diagnosis, with CT and MRI being more sensitive than ultrasound and auscultation for a bruit. Angiography is not necessary for routine assessment or screening (36). Hepatic artery-to-hepatic vein shunts place a strain on the circulation and can lead to congestive heart failure. Direct connections between the systemic arterial and portal circulations can cause portal hypertension, splenomegaly, spider-like telangiectases (which are distinct from the telangiectases typical of HHT), and esophageal and hemorrhoidal varices.

Communication between the portal circulation and the systemic venous circulation leads to hepatic encephalopathy, ascites, and an increase in GI hemorrhage. Despite hyperperfusion through the liver, areas of the organ can be relatively ischemic, and areas of fibrosis in a nodular pattern suggest classic cirrhosis (39) or even malignancy. Standard liver function tests are often normal because areas of the liver are relatively spared. Other patients have a pattern suggestive of ischemia in peribiliary regions leading to strictures, bile cysts, increased alkaline phosphatase and, eventually, hyperbilirubinemia (35). Neither percutaneous liver biopsy nor retrograde cholangiopancreatography is usually necessary, and either is of increased risk in HHT.

An HHT referral center in Italy assessed 502 patients for hepatic involvement and found vascular malformations in 154 (40). Followed for a mean of 44 months, 5.2% died of hepatic complications and 25.3% suffered complications. Therapies of various types (discussed in Section 6) successfully treated two-thirds.

**49.2.1.7 Other Manifestations.** Vascular malformations in the kidneys, bladder, retina, and other organs have been reported (41) as has aneurysm of the aorta and coronary arteries (42).

## 49.2.2 Pathology

**49.2.2.1 Gross Pathology.** The fundamental problem is in the development of blood vessels, especially the connections between arteries and veins. Problems start in the embryo, but they often do not become clinically important until adulthood. If the tiniest connections, the capillaries, are involved, then the problems involve telangiectases. Because the cutaneous and mucosal telangiectases are close to the surface and have very thin walls, they bleed easily. This accounts for epistaxis being an early sign of HHT, and GI hemorrhage being a problem in adulthood. A variety of other dysplastic vascular lesions occur, including AVMs. The brain, the lung, and the liver are the primary sites of AVMs, but other organs can be involved. The vascular precursor of an AVM is established early in life, and the individual lesion expands slowly over many years. Other lesions include arterial aneurysms, dilated veins, and complex AVMs with multiple feeding arteries and a mass of channels connecting to multiple veins (Figure 49-2B).

**49.2.2.2 Histology and Ultrastructure.** The absence of capillaries causes dilatation of the postcapillary venule, which is the essence of the punctuate telangiectases.

## 49.2.3 Genetics

The familial nature of HHT was recognized from the earliest descriptions of the phenotype. Inheritance is autosomal dominant with high penetrance if careful phenotypic assessment is performed (41,43). However, considerable intrafamilial variability occurs.

**49.2.3.1 HHT1 and Endoglin (ENG).** Genetic linkage for HHT families was first established to markers on chromosome 9q33–q34 (44,45). Mutations in the gene encoding endoglin were subsequently identified in HHT1 kindreds (9). Endoglin is a homodimeric integral membrane protein expressed at high levels on human vascular endothelial cells of all blood vessels (46). On endothelial cells, endoglin is the most abundant TGF $\beta$ -binding protein (47). The 90 kDa endoglin protein is encoded by a gene comprising 15 exons (48,49). In addition to the originally identified endoglin cDNA, a splice variant was detected called S-endoglin (for short endoglin), coding for an 85 kDa protein (50). The extracellular and transmembrane domains of S-endoglin and the longer endoglin version (L-endoglin) are identical, whereas the alternative splicing creates a novel, 14-amino acid residue cytoplasmic domain for S-endoglin. These two isoforms are coexpressed in different cell types although the majority of the transcripts correspond to L-endoglin. Although the physiologic distinctions between L- and S-endoglin are not yet completely known, in at least two assays of signaling, they differ in their response to ligand (51).

With the advent of large-scale diagnostic mutation analysis for HHT in a number of countries, hundreds of distinct mutations have been identified in the endoglin gene. At the outset, these mutations appeared to be primarily family specific. This led to difficulty in interpreting novel sequence variants, especially putative missense mutations, because each new family harbored a novel mutation that could not be cross-referenced with a previously described mutation. With the great number of mutations now identified and cataloged in databases, recurrence of the same mutation in apparently unrelated families is slightly more common. Nonetheless, novel sequence variants continue to predominate. In this context, cross-reference to normal sequence polymorphisms identified in the appropriate population helps to identify those alterations that merely represent normal sequence polymorphisms.

Mutations thus far identified in the endoglin gene include missense mutations, nonsense mutations, splice-site changes, and small nucleotide insertions and deletions leading to frameshifts and premature stop codons, all found throughout most of the exons of the gene. Expression data from a number of frameshift and nonsense mutations show that many of these create unstable messages (48,52), and therefore, little to no mutant proteins would be produced. Thus, these mutations would create null alleles. Other clear examples of null alleles include large deletions, duplications, and other genomic rearrangements involving multiple exons of the gene. All genetic testing centers for HHT now routinely search for such genomic rearrangements, and one center recommends that the search for DNA sequence variants and genomic rearrangements should occur simultaneously, rather than sequentially, to ensure identification of all possible mutations in the genes (53). Larger genomic mutations contribute to at least some of the

approximately 20% of HHT cases, in which no mutation can be identified in *ENG*, *ACVRL1* or *SMAD4*.

**49.2.3.2 HHT2 and ALK1 (*ACVRL1*).** A second HHT locus (HHT2; OMIM 600376) was identified in the pericentromeric region of chromosome 12 (8). A potential candidate gene, *ACVRL1*, encoding the ALK1 protein (activin receptor-like kinase 1), was shown to map within this interval, and mutations were identified within this gene in HHT2 families (8).

ALK1 protein is expressed primarily on endothelial cells and in highly vascularized tissues. Studies using a reporter gene trap within the mouse *alk1* gene suggest that its transcript is expressed most highly, and possibly exclusively, in the arterial endothelium (49,54). By sequence homology, ALK1 had been considered a type I cell surface receptor for the TGF $\beta$  superfamily of ligands. Until recently, however, the authentic ligand that activated ALK1 was unknown. Two groups independently demonstrated that BMP9 and the related BMP10 could specifically activate ALK1 (55,56). Subsequent studies have confirmed the critical role of BMP9/ALK1 signaling in angiogenesis. Most data support a role for ALK1/BMP9 signaling in inhibition of angiogenesis (55–58), whereas one study concluded that this pair stimulates angiogenesis (59). These discordant results may be due to the use of different endothelial cell subtypes in each study (59) since the specific response to the signal might be modulated by the differential expression of any one of several different type II coreceptors in an endothelial cell subtype-specific manner (58). Regardless, the discovery of BMP9 as a ligand for ALK1 has spawned a new avenue of research to determine whether HHT is caused by a defect in TGF $\beta$  signaling, BMP9 signaling, or an imbalance between the two.

Human *ACVRL1* contains 10 exons, nine of which encode the protein sequence (60). With large-scale mutation screening being performed worldwide in HHT families, hundreds of mutations have been identified in *ACVRL1*. Mutations in *ACVRL1*, similar to those in *ENG*, are for the most part family specific, again leading to difficulties of confirmation by cross-referencing with previously described mutations. Careful cataloging of normal sequence variants will help in sorting out mutations from population polymorphisms.

Mutations are found throughout the gene and fall into classes of nonsense, frameshift, splice-site, and missense mutations. Genomic rearrangements of *ACVRL1* are also found, and tests for such genomic mutations are now routinely included in genetic testing for HHT. Overall, missense mutations in *ACVRL1* appear to be more common than in endoglin. RNA expression data for some of the *ACVRL1* nonsense and frameshift mutations show that little or no message can be detected from the mutant allele (60). These data, in combination with the sum of the mutation data, demonstrate that most mutations create null alleles, which should lead to reduced signaling through the ALK1 receptor. Reduced signaling capacity

has been validated now for representative *ACVRL1* missense mutations (61). These authors developed the first functional assay for ALK1 signaling based on the discovery of BMP9 as the specific ligand for the receptor (61). Previously identified missense mutations in *ACVRL1* all showed defective BMP9 signaling, whereas known coding polymorphisms did not affect receptor function. Significantly, missense variants of uncertain significance could now be functionally categorized as either mutations or benign polymorphisms, demonstrating the utility of their signaling assay as a tool to inform DNA-based diagnostics.

**49.2.3.3 HHT3.** Evidence for a third locus and gene for HHT (HHT3; OMIM 601101) was provided by a single HHT family (62) that was unlinked to both the HHT1 and HHT2 loci. The locus in this family and in an additional smaller family was mapped to chromosome 5q31–q32 (63). Higher resolution mapping has further narrowed the candidate interval to a 5.7 Mb interval (64). The gene has yet to be identified, but no previously known receptor or effector for TGF $\beta$ /BMP signaling maps within the revised interval. The eventual identification of this gene is likely to shed new light on HHT pathogenesis, by either disclosing the identity of a heretofore unknown factor in TGF $\beta$ /BMP signaling, or by uncovering an unrelated, but parallel signaling pathway leading to the same clinical phenotype. The prevalence of HHT3 also remains uncertain, because to date, only two HHT families have shown statistically significant linkage to this region. Nonetheless, HHT in some of the families negative for mutations in *ENG*, *ACVRL1*, and *SMAD4* that have confounded diagnostic DNA testing laboratories will likely be shown to be due to mutations in this novel HHT3 gene.

**49.2.3.4 HHT4.** Evidence for a fourth locus and gene for HHT (HHT4; OMIM 61055) was provided by a single, large HHT family that maps to chromosome 7p14 (65). An obvious candidate gene mapping within the interval, bone morphogenetic protein endothelial receptor (BMPER), was ruled out early on by DNA sequence analysis. Loss-of-function BMPER mutations were subsequently identified in an autosomal recessive, perinatal lethal skeletal disorder diaphanospondylodysostosis (66). The HHT4 gene has yet to be identified but since there are no other obvious biological candidate genes mapping within the interval, its discovery should also prove invaluable to our understanding of HHT pathogenesis. As with HHT3, the prevalence of HHT4 remains unknown, but at least some of the families negative for mutations in *ENG*, *ACVRL1* and *SMAD4* will likely be shown to be due to mutations in this novel HHT4 gene.

**49.2.3.5 Juvenile Polyposis–HHT: A Syndromic HHT Phenotype (OMIM 175050).** A number of case reports suggested an association of juvenile polyposis (JP) and HHT, or with one or more features of HHT, especially, PAVMs (67–71). One of the two genes mutated in JP is *SMAD4*, encoding the Smad4 protein, a downstream effector of TGF $\beta$  signaling. The other gene mutated in JP is *BMPRIA*, encoding a type I receptor for another



ligand in the TGF $\beta$  superfamily (72). It is now known that most patients with JP harboring an *SMAD4* mutation display a combined syndrome of juvenile polyps and HHT (16,73). Careful clinical screening for both GI polyps and the various vascular malformations associated with HHT has shown that affected individuals meet diagnostic criterion for both JP and HHT (16). The combined syndromic phenotype is estimated to occur in 15–22% of individuals with an *SMAD4* mutation (74), but this may be an underestimate because of a lack of recognition of the HHT phenotype in individuals with clinically silent vascular phenotypes.

Thus far, only mutations in the *SMAD4* gene have been identified in JP–HHT syndrome. The mutations tend to cluster in the carboxyl terminal of the *SMAD4* protein, particularly in the MH2 domain. However, the majority of *SMAD4* mutations previously described in JP also cluster in the same region, ruling out a clear genotype:phenotype correlation. The molecular analysis of a larger number of JP–HHT cases shows that mutations throughout the *SMAD4* gene can cause the syndrome (75). Therefore, any patient who tests positive for an *SMAD4* mutation is at risk for the combined syndrome of JP–HHT, and should be monitored accordingly. Although JP–HHT cases are usually first identified as JP patients, some individuals may first present with the HHT phenotype. Of 30 patients with HHT and no clinical evidence of JP, who were negative for mutations in *ENG* and *ALK1*, screening for mutations in *SMAD4* found three positive (76). These *SMAD4*-positive patients should be screened intensively for polyps. The determination of the true penetrance of JP–HHT syndrome in *SMAD4* mutation carriers requires clinical screening of all JP patients (harboring any *SMAD4* germline mutation) for HHT-associated phenotypes.

Because patients with these two disorders are generally ascertained through distinct medical specialties, genetic testing is recommended for patients presenting with either phenotype to identify those at risk of this syndrome. In particular, patients with juvenile polyps who harbor an *SMAD4* mutation should be screened for the presence of vascular lesions associated with HHT, especially occult AVMs in visceral organs that may otherwise present suddenly with serious medical consequences.

## 49.3 ETIOLOGY

### 49.3.1 Molecular Pathology

**49.3.1.1 Genotype–phenotype Correlations in HHT.** Large-scale DNA sequencing for diagnostics in multiple countries and populations has provided a few consensus genotype–phenotype correlations in HHT. These correlations can be valuable in genetic counseling and in establishment of guidelines for routine medical care and screening for HHT patients. Although one study found that truncating mutations in the endoglin gene are

associated with a more severe outcome than missense mutations (77), and another suggested that truncating mutations in *ALK1* were associated with a higher frequency of epistaxis and telangiectasia (78), in general, mutation-specific genotype–phenotype correlations have not been uniform in all populations. There seems to be limited utility in using the nature of the germline mutation to predict clinical outcomes. This is perhaps not surprising, because the variability in clinical presentation among family members harboring the same mutation can be as broad as the variability among different families each with a distinct mutation.

The variability among family members with the same germline mutation suggests that yet undiscovered biological (such as hormonal), environmental, genetic and epigenetic factors influence the clinical presentation of HHT. Evidence for the role of other genes in modulating the phenotype comes from genetically engineered mutant mouse strains. The phenotype of mice with an engineered mutation in the murine endoglin gene is heavily influenced by the genetic background (that is, the particular inbred lineage) of the mice (79). Similarly, mice lacking the TGF $\beta$ 1 ligand show profound differences in the resulting phenotype depending on inbred strain background carrying the deleted allele. The strain-specific differences for the loss of TGF $\beta$ 1 have been exploited to map multiple chromosomal loci that modify the resulting phenotype (80–82). However, the genes underlying these modifier loci have yet to be identified in the mouse, and thus, the human orthologs cannot yet be investigated as potential modifiers of the human HHT phenotype.

There is, however, clear evidence for a correlation between the incidence of various clinical manifestations of HHT and the particular gene mutated. An initial subjective observation was made that the families that were linked to HHT1 (endoglin) seemed to have a much higher incidence of PAVMs reported than families for whom this locus was excluded (21,43). Further analysis confirmed that this difference was genuine (83–85). However, these initial results were interpreted with caution because they relied on incomplete screening for the pulmonary AVMs, such that only patients with symptomatic pulmonary lesions were scored positive for these lesions. Multiple large studies have now confirmed that patients with endoglin mutations exhibit anywhere from a twofold to up to a 10-fold higher incidence of PAVMs than patients with *ALK1* mutations (78,86–88).

Some of the variabilities observed among studies are likely due to differing ascertainment biases for the pulmonary phenotype. Similarly, cerebral AVMs are also more common in patients with endoglin mutations than those with *ALK1* mutations, and in some studies, appear to be almost exclusively found in HHT1 patients (86,88). One study found neurological complications secondary to cAVMs and pAVMs only in HHT1 patients (88).

In comparison to HHT1, HHT2 patients (*ALK1* mutation) more commonly exhibit gastrointestinal bleeding



(78) and show a higher frequency of hepatic AVMs (78,83,86,88,89), with two studies observing severe or symptomatic hepatic disease only in HHT2 (78,88). HHT2 is also associated with an increased risk to develop pulmonary hypertension (89–93). Pulmonary hypertension has also been observed in HHT1 cases (94–97).

Despite the wealth of genotype:phenotype data that has emerged since 2007, the establishment of correlations such as these continue to be fraught with difficulties due to ascertainment bias. Unless each family member is carefully and consistently screened using the same modality and for the entire spectrum of clinical manifestations regardless of current clinical presentation, these correlations will continue to be incomplete. The best data have come from the more recently published studies from large HHT centers of excellence, where comprehensive clinical screening was coordinated with molecular diagnostics. But even these more robust estimates of the incidence of clinical phenotypes should be used cautiously because these different incidence rates merely represent averages over large numbers of patients. Numerous individual cases are known that would violate the averages, and negligence in screening for any of the visceral manifestations associated with HHT could result later in serious medical consequences for the patient.

## 49.4 PATHOGENESIS

### 49.4.1 Animal Models

The roles of the genes encoding endoglin and ALK1 in vascular development have also been probed by disruption of these genes in the mouse. Homozygous disruption of either genes in the mouse results in embryonic lethality because of arrested endothelial remodeling. Three different groups have disrupted the endoglin gene (12–14). The primary defect is the maturation arrest of the primitive vascular plexus of the yolk sac into defined vessels, leading to channel dilation and rupture. Embryos show distended blood yolk sac vessels by E9.5, a lack of vascular organization by E10.5, and embryos are resorbed by E11.5. Smooth muscle cell differentiation and recruitment to the vessels is also defective. Various heart defects have been reported, including abnormal cardiac looping, and enlarged cardiac ventricles and pericardial sac. Heart valve formation is also disrupted, with reduction in the size of the atrioventricular endocardial cushions and disorganization of the endothelial surface of the cushions. Thus, endoglin plays a crucial role in the heart development. Careful analysis of endoglin null mice shows that they can develop AVMs during the embryonic stage (98). These data suggest a very early defect in the maintenance of arteriovenous vascular beds.

Embryos homozygous for null *alk1* mutations die *in utero* because of the defects in vascular development (99,100). By E9.5, they show absence of mature blood vessels in the yolk sac, and the embryos are resorbed

by E10.5. Histological analysis of the mutant embryos shows excessive fusion of capillary plexus into cavernous vessels. Hyperdilation of large vessels and deficient differentiation and recruitment of smooth muscle cells are also evident. The endocardium and myocardium are also immature, suggesting a role for ALK1 in heart development.

ALK1 function *in vivo* has also been probed using the powerful zebrafish (*Danio rerio*) experimental system. A germline mutation in the zebrafish *ALK1* orthologous gene (*acvrl1*) or morpholino knockdown of its transcript results in dilated cranial vessels because of an increased number of endothelial cells (101). The increase in endothelial cell number in the *acvrl1*-mutant fish is consistent with the majority of cell signaling studies that find that ALK1/BMP9 signaling inhibits angiogenesis. The zebrafish system has also been exploited to investigate the *in vivo* effects of *ALK1* mutations found in HHT2 patients (102), and to explore the phenotypic consequence of loss of TAK1, a downstream kinase that is activated by ALK1 (103). Loss of TAK1 in the zebrafish mirrors the ALK1-mutant phenotype, and TAK1 overexpression can rescue the animals from the phenotypic consequences of loss of ALK1. Morpholino knockdown of both ALK1 and TAK1 shows a synergistic effect on the vascular phenotype. These combined data suggest that *TAK1* might be a novel HHT gene, or possibly a genetic modifier of the clinical phenotype.

In mice heterozygous for both *ENG* and *ALK1* knockouts, the proper genetic model for the HHT phenotype shows that an HHT-related phenotype will develop over time. Lesions such as cutaneous or mucocutaneous telangiectasias as well as visceral AVMs are observed and these resemble the lesions seen in human patients (13,79,104–106). Detailed immunohistochemical characterization of the vessels of mice heterozygous for a mutation in endoglin has led to the hypothesis that HHT involves a generalized vascular abnormality that includes dilated postcapillary venules, vascular walls lacking smooth muscle cells, and irregular collagen and elastin staining (106). Bleeding was associated with regions of inflammation, which may provide a clue to therapy for the bleeding associated with the human patients.

The endoglin mouse model has been used to investigate the earliest events in the pathogenesis of HHT-associated vascular dysplasia. In addition to its role in TGFβ signaling, the mouse models have uncovered a role for endoglin in the regulation of vascular tone. Resistance arteries from *Eng*<sup>+/-</sup> mice display an endothelial nitric oxide synthase (eNOS)-dependent dilation and impairment of the myogenic response (107). Endoglin physically associates with eNOS (107) and also regulates eNOS expression at the transcriptional level via Smad2-dependent TGFβ signaling (108). Endoglin-deficient endothelial cells exhibit uncoupled eNOS activity, producing less NO, and instead generating more eNOS-derived superoxide (107). Intriguingly, the uncoupled activity was not observed in the lungs of newborn *Eng*<sup>+/-</sup> mice, but increased as the

mice aged to adulthood (109). This age-dependent eNOS uncoupling correlated with vasorelaxation in the adult mice, and may explain the development of the pulmonary lesions in adolescents with HHT.

The data from the *Eng*<sup>+/−</sup> mice also suggest a molecular explanation for the association of HHT with pulmonary hypertension. The HHT1 mouse model exhibits increased right ventricular systolic pressure, degeneration of the distal pulmonary vasculature, and muscularization of the small arteries of the lung, each of which can be attributed to uncoupled eNOS activity (110).

These physiologic analyses of the blood vessels in HHT mouse models have helped to elucidate the underlying pathogenesis of HHT. These models suggest that even the “normal” (nonlesional) vessels in HHT harbor an intrinsic structural or physiological defect. The connection with vasodilation, the myogenic response, and nitric oxide levels has provided novel and testable hypotheses concerning the nature of the initiation events leading to vascular lesion development. Increased superoxide may create endothelial cell damage, and this may be an important clue to the initiation of vascular lesion development.

Although the mechanisms whereby endoglin and ALK1 affect TGF $\beta$  signaling pathways are rapidly coming into focus, their specific roles in modulating vascular endothelial and smooth muscle cell properties are just beginning to be elucidated. Despite these positive signs, the specific factor or factors responsible for vascular lesion formation remain unknown. A reduction to 50% of functional endoglin or ALK1 levels is compatible with development of a normal vascular system *in utero* since there is no evidence of increased miscarriage in HHT families. Yet mutation carriers are at nearly 100% risk of developing the vascular lesions observed in HHT. The vascular lesions in HHT are localized to discrete regions within specific organs in the affected tissue, with little evidence of pathology outside the lesions themselves. This suggests that some genetic, physiological, or mechanical event initiates the formation of each vascular lesion. The pathobiology of the disease may be related to remodeling of the vascular endothelium following an unknown initiating event. TGF $\beta$ 1 mediates vascular remodeling through effects on extracellular matrix production by endothelial cells, stromal interstitial cells, smooth muscle cells, and pericytes. Perturbations in the TGF $\beta$  signaling pathway in HHT may lead to altered repair of vascular endothelium and remodeling of the vascular tissue via changes in expression profiles of extracellular matrix proteins. Continued clinical research on this disorder, in combination with studies of animal models and basic biochemical characterization of the signaling pathways involved, will play an important role in elucidating the pathogenesis of HHT.

## 49.5 DIAGNOSIS

Until very recently, the diagnosis of HHT could only be made on clinical grounds. However, with the

clinical availability of DNA-based genetic testing, this has changed. In some situations, when signs, symptoms, and/or family history raise the possibility of HHT, genetic testing can now provide a definitive answer. However, because of technical challenges, including that HHT can be due to a mutation in any one of several genes, and that most families tested to date have had unique mutations, genetic testing is not always currently simple or definitive. Genetic testing is particularly helpful when the “familial” mutation has been identified; in such cases, presence or absence of the mutation, and thus of affected status, in other family members can usually be readily determined. In other situations, clinical grounds remain the mainstay for making the diagnosis. Investigators in Canada compared the use of molecular diagnosis for identifying which relatives in an HHT family were affected, and then performing clinical screening only in those who test positive, with the current protocol of performing clinical screening of every relative at risk (111). From a cost perspective, based on expenses incurred for both molecular and clinical testing in Canada, traditional clinical screening was 50% more expensive. A similar study was conducted in the USA, where many health economic issues are quite different from Canada (112). However, the conclusion that molecular genetic screening of at-risk relatives is highly cost effective was affirmed. These results may not be generalized to countries with substantially different cost structures for the various tests.

The classic findings in HHT are the quadrad of recurrent epistaxis, multiple telangiectasias, visceral AVMs, and a positive family history. However, clinical diagnosis can be challenging, especially because many patients with HHT do not show evidence of all features and recurrent epistaxis (as an isolated finding or an indication of an underlining bleeding diathesis, such as von Willebrand disease) and, to a lesser extent, multiple telangiectasias (as an isolated finding, or due to CREST syndrome, ataxia–telangiectasia, hereditary benign telangiectasia, chronic liver disease, or pregnancy) are each common findings in the general population. However, when the full triad of recurrent epistaxis, multiple telangiectasias, and family history is present, the diagnosis of HHT is both straightforward and rarely incorrect.

The presence of such characteristic visceral lesions as AVMs can also be a key to clinical diagnosis, particularly PAVMs, because a majority of those with a pulmonary AVM prove to have HHT (this is especially true if there are multiple PAVMs). Similarly, GI telangiectasias are often, but not always, a sign of HHT. Indeed, any individual with pulmonary, cerebral, hepatic, or spinal AVMs or GI telangiectases should be evaluated for the possibility of having HHT.

Perhaps the most useful framework for making the diagnosis of HHT on clinical grounds is the so-called Curacao criteria, devised by a number of clinicians from

around the world experienced in the disorder (11). The four criteria in this construct are as follows:

- Epistaxis
- Multiple telangiectasias at characteristic sites (lips, oral cavity, fingers, nose)
- Visceral lesions (such as GI telangiectasias or pulmonary, cerebral, hepatic, or spinal AVMs)
- Family history of a first-degree relative with HHT according to these criteria.

The diagnosis of HHT is believed to be definite if three or four of these criteria are present, possible if two are present, and unlikely if fewer than two criteria are present. The authors of the Curacao criteria also noted that “Within HHT families, a firm diagnosis can be made on the basis of two separate visceral manifestations, though this is not applicable to the general population” (11).

## 49.6 MANAGEMENT

### 49.6.1 Mucocutaneous Telangiectasias

Lesions on the tongue, lips, and fingers may bleed when traumatized. Photocoagulation with a laser can be effective in stopping acute bleeding. Smaller lesions can be eliminated, and this is of some cosmetic utility around the face.

### 49.6.2 Epistaxis

Often the most frustrating and debilitating feature of HHT is recurrent bleeding from the nose. Bleeding often starts spontaneously, even during sleep, and can be profuse. The unpredictability of epistaxis and the difficulty in stemming the acute hemorrhage may severely affect a patient’s occupation, social interactions, and activities. Local pressure by pinching the bridge of the nose is obviously the first maneuver, but the actual telangiectasias that is bleeding may be so proximal as to not be compressible externally. Various devices have been used for internal compression of nares. Some have been designed by patients, whereas others (such as Foley catheters, with the balloon providing tamponade) have gained currency in emergency departments. None is routinely efficacious, and some carry risks. Typically, the clot will be dislodged when the gauze or device is removed. Prolonged pressure on the mucosal surface can cause ischemia or infarction. Cauterizing agents, such as silver nitrate, may be useful for an initial, small bleed, but are useless for large ones. Angiographic procedures to localize the feeding artery and embolize it are fraught with hazards, especially necrosis of nasal tissue or ischemia of the vascular supply to the eye. The combined septenopalatine and ethmoid arteries supply about two-thirds of the blood to the septum; occluding one or both leads to collateral formation within a few months.

Once the acute event ends, endoscopic examination of the nares typically reveals many telangiectasias, and the ones most likely to bleed can be treated with laser phototherapy. A variety of wavelengths have been studied, and rhinologists often have their favorite instruments. Some patients achieve long-term improvement from periodic photocoagulation. However, those who continue to bleed enough to require transfusion of packed erythrocytes on a regular basis need to be considered for septal dermoplasty. In this major procedure, the inferior turbinate is resected and a skin autograft is placed by suturing to the septum superiorly and then packing the nose (113). After several weeks, the graft adheres to the nasal mucosa and often provides relief for several years. Unfortunately, telangiectases eventually can appear on the surface of the graft and lead to renewed epistaxis. Additionally, the grafted skin must be kept moist and can be a site of bacterial overgrowth.

Prevention of epistaxis, although imperfect, is important to practice. The goals in severe cases should be to reduce or eliminate the need for visits to emergency rooms, reduce or eliminate the need for transfusions, and improve the quality of life. The ambient humidity should be kept as high as feasible. Allergies should be controlled to the extent possible and airborne irritants, such as cigarette smoke, should be excluded. Spraying the nares with dilute saline or carefully applying an emollient such as petroleum jelly reduces crusting. Various medications, including hormones and antifibrinolytics, have also been useful in selected patients, but all have important risks and contraindications (114–116). Local or systemic agents that produce vasoconstriction can be beneficial in the short term, but they cause a rebound inflammation in the nasal mucosa that is counterproductive. Several agents have been reported to be effective in reducing severe epistaxis in individual patients or small, nonrandomized series. The anti-VEGF drug, bevacizumab, given topically or systemically, holds promise (117,118) but can be associated with severe adverse effects. Thalidomide and tranexamic acid also being studied more systematically, now that case reports and small series demonstrating encouraging results have appeared (119).

Drugs that alter platelet function, such as aspirin and nonsteroidal anti-inflammatory agents, should be avoided. However, if a patient with HHT develops a clinical problem that requires systemic anticoagulation, such as atrial fibrillation or deep venous thrombosis, then warfarin can be used with attention to keeping the INR in the low end of the therapeutic range. There is as yet no experience in HHT with the newer oral anticoagulants such as inhibitors of thrombin or Factor Xa.

### 49.6.3 Central Nervous System

Vascular malformations present at birth are usually asymptomatic, but large venous malformations and CAVMs, in the absence of bleeding, may cause problems

because of their size or location. Whether any particular lesion needs to be treated by radiation, surgery, or vascular occlusion needs to be considered on the basis of the patient's symptoms and age (26,120).

Brain abscess, due to paradoxical embolization of bacteria through a PAVM, needs to be treated in several ways. First, based on the neurologic and general health status, a decision about drainage and antibiotics must be made. Second, further central nervous system (CNS) embolization must be minimized by treating all sources of right-to-left shunts. Just because PAVMs are the most likely culprit in HHT, the presence of a patent foramen ovale or atrial septal defect should not be overlooked.

Because of the risk of CNS embolization in HHT, particular care must be paid to any instance when bacteria or air might enter the blood stream. All people with HHT should adhere to routine prophylaxis against endocarditis. Additionally, all intravenous lines, except for the infusion of blood or radiographic contrast, should have special filters to prevent air bubbles from entering the vein.

#### 49.6.4 Lung

Plain chest radiography, pulse oximetry, arterial blood gases, or a combination thereof are insensitive for detecting all but major right-to-left shunts in the lung. The contrast echocardiogram, in which agitated saline is injected into an antecubital vein as the four-chambered view of the heart is observed, is a useful screening tool for PAVMs (121). In the normal situation, the microbubbles are absorbed in the lung capillaries and do not appear in the left atrium. Appearance of contrast ("bubbles" in common parlance, but not of a size to endanger the CNS) in the left atrium immediately (i.e. one to three heart beats) after it enters the right side of the heart strongly suggests a septal defect, typically a patent foramen ovale. If this occurs, then a pulmonary shunt cannot be diagnosed, and the patient must have a CT scan. If contrast does not appear for four to eight heart beats, then there is likely a shunt in the pulmonary circulation. However, a few percent of "normal" individuals will have delayed passage of a few bubbles (122,123). Whether this represents a "false positive" or the presence of small and clinically irrelevant intrapulmonary shunts is unclear. Quantity of the contrast in the left atrium is a rough guide to the number and size of the PAVMs. Delayed appearance of more than a few bubbles requires follow-up imaging.

The standard protocol to address the size, number, and location of PAVMs is a high-resolution CT scan of the lung before and after injection of radio-opaque contrast. The ability to reformat the images is especially useful to distinguish potential PAVMs from lung nodules of various types and to define the number and size of the feeding arteries and the draining veins.

Until the early 1980s, patients with a PAVM of any cause were not treated unless they had recurrent

hemoptysis, severe arterial desaturation, or high-output heart failure. Then, the segment or segments of lung with the offending or largest PAVMs were resected. Unfortunately, because PAVMs in HHT can progress over time, this surgical approach could be used only a limited number of times. Robert White, a radiologist, and Peter Terry, a pulmonologist, at Johns Hopkins Hospital developed the ability to occlude PAVMs by means of balloons inserted into the feeding artery and then filled with saline containing radio-opaque dye (124,125). This approach, since modified to involve wire coils that invoke a thrombus in the feeding artery, has revolutionized the management of the lung in HHT. Now, under fluoroscopic guidance, four to six or more PAVMs can be treated in one session (Figure 49-3). Complications include pleuritic chest pain that typically resolves in a few days, failure to occlude feeder vessels completely (more common in complex AVMs or when a feeding artery is large), transient ischemic episode, rare paradoxical embolization of a device, and recanalization (126–128).

Until recently, PAVMs with feeding arteries of <3 mm diameter were left untreated. However, some episodes of cerebral embolization occurred (129), and we and others now recommend occluding any PAVM that can be reached by a catheter. This aggressive approach requires an expert interventional radiologist who has considerable experience with HHT. After all PAVMs that can be occluded are, the patient should have a repeat CT scan in 6 months to document that all remain occluded (i.e. that no recanalization has occurred). Subsequently, a CT scan should be performed every five years to monitor emergence or growth of any additional lesions (Figure 49-4).

Major complications related to PAVMs can occur during pregnancy and in the postpartum period due to pulmonary hemorrhage, increased right-to-left shunting, or cerebrovascular events (130). Any patient diagnosed with HHT during pregnancy should undergo high-resolution CT of the lung with contrast. Embolotherapy is indicated during pregnancy for PAVMs that can be reached by an intervention catheter (131).

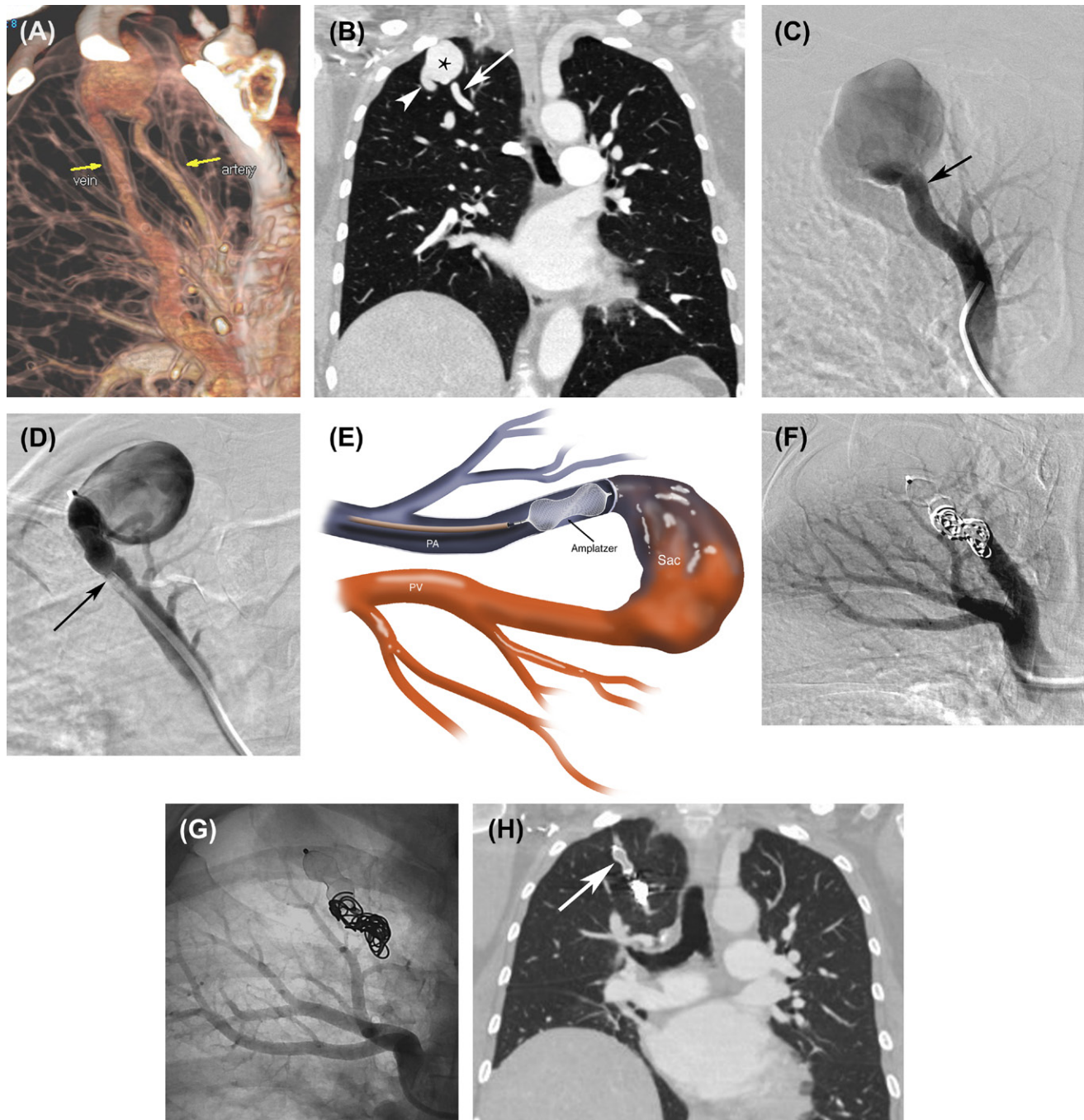
#### 49.6.5 Liver

Vascular lesions in the liver are much less amenable to treatment than are PAVMs. Occlusion of the feeding vessel or vessels, although technically feasible, carries an unacceptable risk of infarction of substantial regions of liver parenchyma. There also is an increased risk of infection associated with endoscopic retrograde cannulation of the pancreas, so ERCP is contraindicated in HHT.

Patients who have hepatic encephalopathy can be treated with nitrogen restriction and lactulose. Those with high-output cardiac failure respond initially to diuretics.

The therapy of last resort is liver transplantation. Unfortunately, because tests of liver function often





**FIGURE 49-3** Fifty-two-year-old woman with known right upper lobe PAVM. Patient was followed for 14 years at another institution and told treatment was not needed. She has obvious HHT with a strong family history, epistaxis, and telangiectases, and was known by her primary care physician to have HHT. She was asymptomatic. A. Color-enhanced coronal reconstruction from CT shows simple PAVM in the right upper lobe; feeding artery and draining vein are labeled. Feeding artery measured 12 mm in diameter. B. Representative image showing feeding artery (arrow), sac (asterisk), and draining vein (arrowhead). C. Image from selective right upper lobe pulmonary angiography confirms simple angio-architecture; the feeding artery is indicated. D. Image obtained during contrast injection with 16 mm Amplatzer vascular occluder deployed at the mouth of PAVM sac. Arrow shows occluder still attached to delivery cable. E. Diagrammatic representation of panel D. F and G. Subtracted (F) and unsubtracted (G) postembolization pulmonary arteriograms after placement of coils in addition to Amplatzer vascular occluder show occlusion of PAVM. H. Representative image from coronal CT reconstruction 6 months after embolization. Sac has almost completely disappeared. Characteristic appearance of Amplatzer device (arrow). Patient reported marked improvement in her exercise tolerance. (*Reprinted with permission from Ref. 139.*)

remain relatively normal despite severe vascular disease in the liver, patients have difficulty gaining access to the transplant list. One report found vascular dilatation in the allografts of two patients of 8 and 10 years

following transplant; whether these findings were in some way related to the underlying HHT, and if so, how common and important this issue will remain to be studied (116).



**FIGURE 49-4** Multiple, treated and untreated PAVMs in one lung. (Reprinted with permission from Ref. 139.)

### 49.6.6 Gastrointestinal

Hemorrhage from the GI tract can be one of the most debilitating and frustrating problems of HHT, especially in older people. There is no good way to monitor chronic bleeding. Testing the stool for occult blood is useless because most patients swallow blood from bouts of epistaxis, which turns the test positive. Patients should monitor their bowel movements for signs of moderate GI bleeding (e.g. tarry stools). However, most patients who become anemic have mild, chronic bleeding that escapes their attention. Upper and lower endoscopy typically shows multiple mucosal telangiectasias from the oral cavity to the duodenum and from the rectum to the ileocecal valve. The number of telangiectasias roughly correlates with the anemia and transfusion requirement (132). Any lesion that appears to be bleeding can be cauterized. However, there is no point in attempting to treat every telangiectasia, especially if repeated endoscopies are contemplated. Capsule endoscopy confirms that telangiectasias exist throughout the jejunum and ileum; but push enterostomy or major surgery are rarely required (133). As with refractory epistaxis, hormonal (estrogen/progesterone in women without contraindications, and danazol in men) and antifibrinolytic therapy may have a role in chronic GI bleeding (132,134). Similarly, experimental agents such as thalidomide and bevacizumab are worth studying (118,119). Selective mesenteric arteriography can reveal intestinal AVMs that can be embolized

(135) but the site or sites of chronic blood loss usually remain obscure.

### 49.6.7 Anemia

Unless a contraindication exists, virtually all adults with HHT should take supplemental iron. The amount and form of iron depend on the severity of the anemia (136). Oral iron can produce adverse effects including constipation and nausea. Patients who do not tolerate oral iron supplements or do not absorb iron from the intestine (e.g. celiac disease) need intravenous iron. All preparations can produce allergic reactions. Those with lower iron content can be infused faster with less risk of adverse effects; however, multiple infusions are necessary. Those with higher iron content must be infused slower, but if successful, may replete stores with one dose. Additional cofactors, folate, and B12 should be considered. Inability to replete iron stores due to severe blood loss despite iron supplementation requires episodic or even regular transfusions of packed erythrocytes. Any person being treated for anemia should have periodic assessment of blood counts and iron stores.

### 49.6.8 Counseling

The same issues that arise in most serious autosomal dominant disorders occur in HHT. The recent availability of molecular diagnosis offers promise of presymptomatic and prenatal diagnosis. However, complexities often arise because of intergenic heterogeneity, “negative” results, and DNA sequence variants of uncertain pathogenic importance (137).

The Hereditary Hemorrhagic Telangiectasia Foundation International ([www.hht.org](http://www.hht.org)) plays an important role in several areas. The organization facilitates the establishment of centers at academic hospitals that provide comprehensive medical and counseling services for patients and families with HHT. There are now 33 centers worldwide, including 15 in North America. The Foundation also sponsors national conferences for patient education and support, and biennial international research symposia.

### 49.6.9 Life Expectancy

Infants and children with HHT and unusually severe vascular malformations in the CNS or lung can die. Many adults with HHT live into their eighth and ninth decade despite severe anemia from epistaxis and GI bleeding. In 1999, a mortality analysis of patients in one county in Denmark showed that patients younger than age 60 died at twice the rate of the population average (18). A more recent study from an HHT center in Italy confirmed an increased peak in mortality rate in patients under the age of 50 years, and somewhat higher mortality in those ages 60–79 (138). Life expectancy was not related to gender or to genotype.



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### Biography



**Reed Pyeritz**, completed his PhD and MD at Harvard and trained in internal medicine at the Peter Bent Brigham Hospital in Boston and medical genetics at the Johns Hopkins Hospital in Baltimore. He is a professor of Medicine and Genetics at the Raymond and Ruth Perelman School of Medicine of the University of Pennsylvania in Philadelphia, where he directs the Center for the Integration of Genetic Healthcare Technologies and serves as vice-chair for academic affairs of the Department of Medicine. He is a senior fellow in both the Center for Bioethics and the Leonard Davis Institute for Health Economics.

His research has focused on several areas, including clinical investigations of heritable disorders of connective tissue and cardiovascular diseases, especially those affecting the thoracic aorta. Currently he is studying how more refined methods of investigating genetic variation, such as cytogenomic arrays and whole genome sequencing, actually increase the level of uncertainty in applying the results clinically.

# CHAPTER

# 50

## Hereditary Disorders of the Lymphatic System and Varicose Veins

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### 50.1 DEVELOPMENT OF THE LYMPHATIC SYSTEM

The lymphatic vessels arise from the cardinal vein after the cardiovascular system is established and functional. Lymphatic vessel development commences about embryonic weeks six to seven in humans, when a distinct subpopulation of endothelial cells in the lateral part of the anterior cardinal veins become committed to a lymphatic lineage and sprout laterally to form the vascular sacs. The peripheral lymphatic vasculature is generated by the sprouting of lymphatic vessels from the lymph sacs, followed by merging of the separate lymphatic capillary networks and remodeling and maturation of the lymphatic capillary plexus. LYVE1, a specific lymphatic endothelial marker, is expressed in a subset of endothelial cells in the central veins and is the first indicator of lymphatic commitment. In adults, LYVE1 expression decreases in the collecting lymphatics but remains high in the lymphatic capillaries. PROX1 expression is specific for lymphatic vessels and, in the mouse, Prox1 knockout embryos do not form lymph sacs or lymphatic vessels (1,2). Overexpression of Prox1 in human blood vascular endothelial cells (VECs) downregulates genes specific for VECs and upregulates lymphatic endothelial cell (LEC) gene expression (3,4).

Signals leading to the polarized expression of PROX1 are poorly understood, but Francois and colleagues (5) showed that the expression of homeobox transcription factor SOX18, mutations in which lead to human hypotrichosis-lymphedema-telangiectasia syndrome (OMIM 206823), precedes PROX1 expression and that there are SOX18 binding sites in the promoter of PROX1, indicating that SOX18 acts upstream of

PROX1 in initiating the LEC differentiation program. The protein kinase receptor VEGFR3 is expressed on all endothelial cells early in development but becomes increasingly specific for endothelial cells committed to a lymphatic lineage (3,6). VEGFR3 is activated by VEGF-C and VEGF-D, but during development VEGF-C expression is predominantly in regions where the lymphatic vessels develop (6,7). Mice null for VEGF-C have complete absence of lymphatics, and the heterozygote for the null allele shows severe lymphatic hypoplasia (7). Overexpression of VEGF-C leads to lymphatic hyperplasia in the mouse, although VEGFC mutation has not been reported in humans.

Direct communication between the lymphatic network and the vascular system occurs around 40 days postconception in humans, whereby the lymphatic drainage of the right and left sides of the upper body, which had collected in bilateral jugular lymph sacs, empty into the ipsilateral jugular veins. Failure or delay in completing these communications leads to widespread distortion of lymphatic development termed the jugular lymphatic obstruction sequence (8). The clinical consequences of edema distal to the obstruction will vary depending on whether communication is established and when it occurs. Complete absence of communication is lethal. Distention of the jugular sac produces, among other findings, a cystic hygroma, which causes excess skin growth over the neck, the posterior hairline and hair pattern to be disturbed, and the ears to protrude and rotate. If the jugular sac does not decompress until late in fetal development, the infant will have pterygium coli, or webbing of the neck. Edema of the hands and feet may not be fully resolved at birth, and permanent effects on distal



development will be noted, such as predominance of digital whorls and narrow, hyperconvex nails. If drainage of lymph from the lower quadrants of the fetus is primarily affected, the ileac lymph sacs become distended and excess skin grows over the lower abdomen. Prenatal resolution can result in the prune-belly birth defect.

Defective lymphatic development has consequences for the cardiovascular system (3). Peripheral veins tend to be of large caliber, presumably because of increased venous return from edematous tissues. The frequency of left-sided flow defects (e.g. aortic coarctation, bicuspid aortic valve, hypoplastic left heart) is increased, in part because of the space occupied by the distended jugular sacs.

## 50.2 DISORDERS OF THE LYMPHATIC SYSTEM

Diffuse, acquired blockage of lymphatics, such as by fibrosis, tumor or infection (e.g. *Microfilaria bancrofti*), usually results in edema of the body parts distal to the blockage. However, if the thoracic duct is blocked, anastomosing channels develop between the lymphatic system and systemic veins, and edema does not persist.

A prenatal cystic hygroma is often seen in fetal aneuploidy. If the fetal karyotype is normal, a cystic hygroma detected in the first trimester carries a good prognosis; if it resolves by the mid-second trimester, little distortion of the infant is likely to be noted (70).

Both Turner syndrome (Chapter 50) and Noonan syndrome (Chapter 46) have prominent, multisystem malformations due to fetal lymphatic obstruction. Failure of the jugular lymphatic–venous communication to occur may be the major reason that 98% of embryos with a 45,X karyotype do not survive to term.

The National Lymphedema Network, Inc., maintains a list of various support groups; while many pertain to acquired lymphedema, issues pertinent to various congenital and hereditary conditions can be found ([www.lymphnet.org](http://www.lymphnet.org)).

### 50.2.1 Hereditary Lymphedema I

Hereditary lymphedema 1A (OMIM 153100) is a mainly congenital onset lymphedema, the swelling of the extremities due to failure in the development and/or function of the lymphatics, leading to the accumulation of lymph in the interstitial fluid space. It is highly variable in expression and penetrance, and unaffected obligate heterozygous individuals occur in almost all pedigrees of sufficient size. The age at onset varies from embryonic (as seen on prenatal ultrasound) to middle age. The classic presentation, termed Milroy disease after the physician who gave the first detailed description of the condition in 1892, manifests as congenital edema of the lower half of the body with no other obvious manifestations. The

major features of the disease, variable age at onset and expressivity and reduced penetrance, were described by Milroy (9) over the course of 35 years, in a single family. The complications of Milroy disease include hypoproteinemia due to intestinal loss of albumin, increased susceptibility to infection in the affected limb, and, in some cases, angiosarcoma.

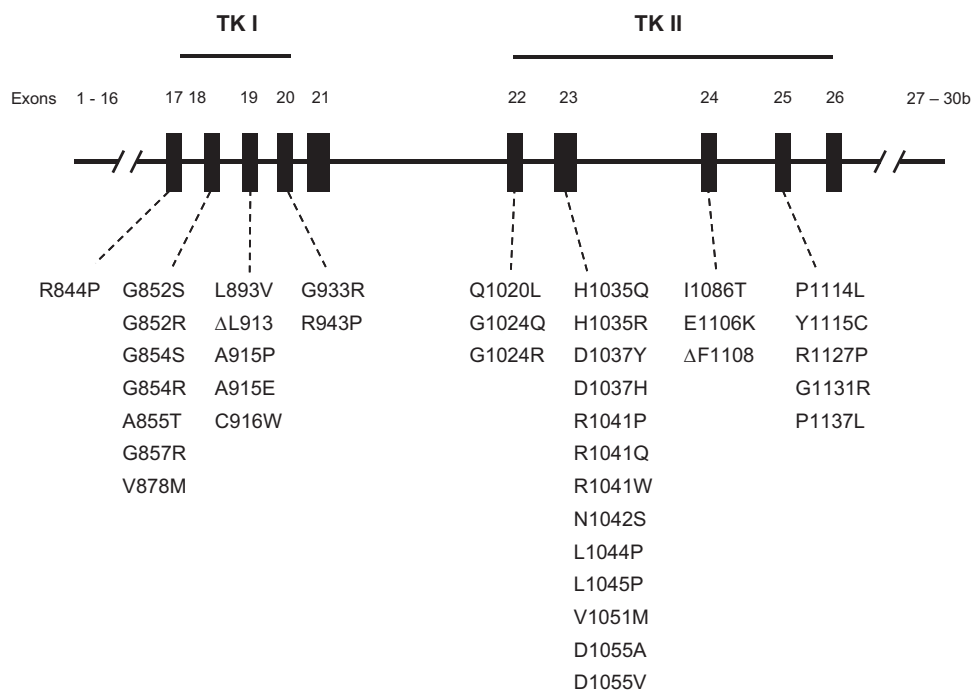
Management is symptomatic. Decongestive therapy consisting of manual lymphatic drainage, wearing of compression garments, skin care and remedial exercise results in improvement of the lymphedema, but is generally temporary. There is no proven medical therapy.

Milroy disease maps to chromosome 5q34-q35 (10–12) and is caused by mutations in the kinase domains of the vascular endothelial growth factor receptor-3 (*VEGF3*, *FLT4*) (13,14). The majority of the mutations are missense mutations occurring in one of the two kinase domains of *VEGFR3*, although a single case of Milroy disease due to a recessively inherited mutation in the ATP binding domain of *VEGFR3* has been reported (15). These mutations are summarized in Figure 50-1. The kinase domain mutations lack the autophosphorylation activity resulting in reduced signal transduction in response to VEGF-C/-D, an obligatory step in lymphoangiogenesis. The autosomal dominant inheritance is apparently due to the accumulation of the nonfunctional receptor on the cell surface, perhaps due to the failure in recycling of the receptor containing one or two copies of the kinase negative receptor subunit.

*VEGFR3* null mice die at around embryonic day E10.5 because of failure in maturation of the perineural vascular plexus into a dendriform hierarchy of large and small vessels (16), although the heterozygote is grossly phenotypically normal. *VEGFR3* is expressed in all endothelial cells during early embryogenesis, but in the course of development its expression in VECs declines, and in the adult, *VEGFR3* expression is largely restricted to the lymphatic endothelium (17). The *chy* mouse, with a *VEGFR3* (I1953F) kinase domain mutation, is characterized by the appearance of chylous fluid in the abdomen, which resolves, and sparse, large cutaneous lymphatic vessels, similar to humans with Milroy disease (18). For a detailed review of lymphangiogenesis see the article by Tammela and Alitalo (19).

Lymphoscintigraphy in Milroy disease shows little or no uptake by peripheral lymphatics of the foot and consequently little or no uptake in the ilioinguinal lymph nodes (20). Generalized in utero edema has been observed in individuals with *VEGFR3* mutations, but this edema resolved spontaneously, either in utero or after birth (21).

Hereditary lymphedema 1B (OMIM 611944) was described by Malik and Grzeschid (22) in a single Pakistani family and assigned to the interval 6q16.2-q22.1 by microsatellite linkage analysis. The lymphedema



**FIGURE 50-1** VEGFR-3 (FLT4) mutations causing hereditary lymphedema.

was congenital, progressive, and confined to the lower limbs. Full expression occurred by puberty and resolved by 40–45 years of age. Autosomal dominant inheritance with reduced penetrance was observed, as in Milroy disease. Region 5q34-35 was ruled out by linkage analysis, and pathological mutations in the candidate gene *FOXO3A* were not found by sequence analysis of the coding region of *FOXO3A*. The region of linkage contains approximately 70 genes, several of which are plausible candidate genes for lymphedema.

Hereditary lymphedema 1C (OMIM 613480) has an early onset of uncomplicated lymphedema of the legs and hands, and is inherited in an autosomal dominant pattern, with variation in age at onset, penetrance and expressivity. Phenotypically it is indistinguishable from Milroy disease, but is caused by missense mutations in *GJC2* coding for connexin 47. These mutations occur in both the extracellular and intracellular domains of connexin 47, and the only common feature of the six mutations that have been described is that they change an amino acid that is highly conserved in mammalian evolution (23). In the two extracellular mutations, expression of the mutant connexin 47 failed to form gap junction plaques when expressed in HeLa cells in culture, and thus failed to communicate electrically with adjacent cells. The four intracellular mutations formed plaques, but their gap junction activity was abnormal. This suggests that these *GJC2* mutations cause lymphedema through failure of intracellular communication. Whether this failure to communicate is between adjacent lymphatic endothelial

cells or endothelial cell and lymphatic smooth muscle cells is unknown. Inactivating mutations in *GJC2* cause Pelizaeus-Merzbacher-like disease (PMLD; OMIM 608804) and connexin 47 is usually considered a central nervous system (CNS) connexin. The relationship between the severe CNS dysmyelination seen in autosomal recessive PMLD and the phenotype of lymphedema seen in dominantly inherited lymphedema in individuals with missense mutations in the same gene is unexplored.

### 50.2.2 Hereditary Lymphedema II

Hereditary lymphedema II (OMIM 153200) is a dominantly inherited, pubertal or adult-onset lymphedema predominantly affecting the legs, but with involvement of the arms, face and larynx in some families. Meige (24) described eight affected individuals in a four-generation family, and the disorder often bears his eponymous designation. Some individuals are first diagnosed with myxedema, and pleural effusion may be present. The nosology of hereditary lymphedema II is unclear, and until the specific gene(s) involved are identified the existence of hereditary lymphedema II as a distinct genetic entity is uncertain.

### 50.2.3 Lymphedema-Distichiasis Syndrome

Lymphedema-distichiasis syndrome (OMIM 153400) is characterized by bilateral lymphedema, usually of the legs, and having a peripubertal age at onset and

distichiasis. These patients often come to medical attention because of corneal abrasions caused by the presence of a double row of eyelashes (distichiasis) (25). The disorder was localized to 16q24.3 by linkage in three families (26), and *FOXC2* was implicated in the study of a patient with a (Y;16) chromosomal translocation (27). Fang et al. (27) went on to identify truncation mutations in two families in the *FOXC2* gene, a member of the forkhead/winged-helix family of transcription factors. This was followed by numerous reports of truncation mutations, leading to haploinsufficiency for *FOXC2* causing lymphedema-distichiasis syndrome (28,29). Mellor and colleagues (30) reported abnormal isotope uptake on lymphoscintigrams, and significant lymphatic backflow, and that all patients with *FOXC2* mutations studied demonstrated backflow on Doppler ultrasound examination, consistent with valve failure.

Studies of the *FOXC2* null mice showed that they died at approximately E12.5, with severe abnormalities of the developing mesenchyme (31). The heterozygous null animals uniformly showed lymphedema due to defective valves and enhanced recruitment of vascular mural cells to cutaneous lymphatic capillaries, and aberrant hair follicles arising from the meibomian gland (32,33), recapitulating the phenotypes observed in humans with lymphedema-distichiasis syndrome. Cederberg and colleagues (34) reported reduced body weight, relatively greater insulin sensitivity, and hypertriglyceridemia in heterozygous null mice compared to normal mice, consistent with the diabetes mellitus reported in members of a family with *FOXC2* mutation.

Multiple cases of lymphedema, occurring in the presence and absence of distichiasis, have occurred in patients with missense substitutions in *FOXC2* (30,35). These missense mutations alter the transactivational activity of *FOXC2*, indicating that the haploinsufficiency in lymphedema-distichiasis could arise by multiple molecular mechanisms (35).

#### 50.2.4 Hennekam Lymphangiectasia-Lymphedema Syndrome

Hennekam lymphangiectasia-lymphedema syndrome (OMIM 235510) is an autosomal recessive syndrome of intestinal lymphangiectasia with severe lymphedema of the face and genitalia and severe mental retardation first reported in a highly inbred Dutch kindred (36). The phenotype was localized by homozygosity mapping to chromosome 18q21, and analysis of the candidate gene *CCBE1* showed that mutations in the calcium-binding EGF domain of this gene caused generalized lymphatic dysplasia in a subset of Hennekam patients (37,38). *CCBE1* was identified as a candidate gene based on the finding that mutation in the same domain of the zebrafish *ccbe1* gene led to the failure of embryonic

lymphangiogenesis and venous sprouting (39). Alders and colleagues (37) found that non-disease causing changes in human *CCBE1* could rescue the zebrafish phenotype, but that the homologous Hennekam mutations could not. *CCBE1* mutations occurred in only a subset of patients as evidence for genetic heterogeneity in Hennekam syndrome (37).

#### 50.2.5 Hypotrichosis-Lymphedema-Telangiectasia Syndrome

Hypotrichosis-lymphedema-telangiectasia syndrome (OMIM 607823) was localized to chromosome 20q13 by homozygosity mapping in two consanguineous families, and the *SOX18* transcription factor was considered a plausible candidate gene for the human disease because of the combination of hair and cardiovascular anomalies in the mouse ragged phenotype (40). Both dominant and recessive inheritance occurred, with the alleles causing dominant inheritance having a mutation occurring in a position homologous to that of the four dominant ragged mutations in the transactivation domain of the mouse. The two recessive mutations occurred in the alpha-helix of the DNA-binding domain of *SOX18*. *SOX18* directly activates *Prox1* transcription by binding to its proximal promoter. *PROX1* is considered a “master regulator” of lymphatic development. The overexpression of *Sox18* in blood VECs induces the expression of *Prox1* and other lymphatic endothelial markers, and the *Prox1* null mouse is characterized by a complete blockage of lymphatic endothelial cell differentiation from the cardinal vein (5). The *Ra<sup>op</sup>* allele of the mouse leads to endothelial hyperplasia in the microvasculature and inadequate recruitment of pericytes to the maturing blood microvessels reminiscent of the pericyte maturation defect noted in the *Foxc2* null heterozygote (41). *Sox7* and *Sox17* are strain-specific modifiers of the lymphoangiogenic defects noted in *Sox18* null mice, suggesting that they may also be involved in the expression of lymphatic defects in humans (42).

#### 50.2.6 Cholestasis-Lymphedema Syndrome (Aagaes Syndrome)

Cholestasis-lymphedema syndrome (LCS; OMIM 124900) was first described in consanguineous families of Norwegian origin. It is characterized by lymphedema and early onset, recurrent cholestasis. Aagaes syndrome was mapped to a 6.6 cM interval of chromosome 15q by Bull et al. (43), and Fruwirth et al. (44) later mapped a second locus for LCS on chromosome 18 by a combination of linkage and haplotype mapping. Phenotypic similarity between the families suggested a second locus, LCS2, for cholestasis-lymphedema syndrome. The causative genes for LCS1 and LCS2 and the insight they would provide

to the pathogenesis of these disorders have eluded investigators.

### 50.2.7 Choanal Atresia and Lymphedema

Choanal atresia and lymphedema (OMIM 613611) was first reported in a consanguineous Yemenite family by Qazi et al. (45), and mapped to the region 1q32-q41 by homozygosity mapping (46). This interval contains the *PTPN14* protein tyrosine phosphatase gene, and reasoning that lymphedema is part of the phenotype of Noonan syndrome (OMIM 176876), which is caused by mutations in the homologous *PTPN11* gene, Au et al. (46) identified a patient homozygous for a frameshift mutation at residue 194, which led to a premature chain termination at residue 211. This mutation interrupted a FREM domain (erzin-radixin-meosin family of cell adhesion molecules) in *PTPN14*. They examined two mouse embryonic stem cell lines in which exon-trapping vectors were integrated within the *Ptpn14* gene. In three chimeric animals generated from the exon-trap located in intron 5, homozygous for the intron-5 trap, they observed lymphedema in a fraction of the offspring, and they confirmed high levels of expression of *PTPN14* in lymphatic endothelial cells, and concluded that this gene was responsible for the phenotype observed in the mouse and in humans.

Mutations in transcription factors *FOXC2*, *SOX18*, *CCBE1* and *NEMO* cause lymphedema-related syndromes OMIM153400, OMIM 607823, OMIM 235510 and OMIM 300248, respectively; mutation in *PTPN11* causes Noonan syndrome associated with lymphedema; and tyrosine kinase *VEGFR3* mutation causes Milroy disease (OMIM 153100), indicating both the genetic heterogeneity of lymphedema and the complexity of lymphoangiogenesis.

## 50.3 MENDELIAN DISORDERS AFFECTING BOTH THE LYMPHATIC AND VENOUS SYSTEM

### 50.3.1 Klippel-Trenaunay Syndrome

Klippel-Trenaunay syndrome (KTS; OMIM 14900) is defined by cutaneous hemangiomata and hypertrophy of bones and soft tissues (47). There is clear overlap with the Parkes Weber syndrome (OMIM 608355), which has hypertrophy due to arteriovenous malformations. Lymphatic abnormalities are restricted to KTS. Very few familial cases of KTS have been described (48). As with Milroy disease, the only effective treatment of lymphedema is physical therapy involving decongestive therapy, the wearing of pressure garments, remedial exercise and particular attention to hygiene in the affected area. Surgical and therapeutic interventions have not proved effective.

**TABLE 50-1 Mendelian Disorders Affecting the Lymphatics**

Disorder	Inheritance	OMIM No	Locus	Gene	Features
Lymphedema, hereditary, 1A, Milroy's	AD	153100	5q34q35	<i>FLT4 (VEGFR3)</i>	Congenital onset
Lymphedema, hereditary, 1B	AD	611944	6q16.2-q02.1		Congenital onset, fully expressive at puberty
Lymphedema, hereditary, 1C	AD	613480	1q41-q42	<i>GJC2</i>	Congenital (leg), variable onset (arm), pubertal to adult
Lymphedema, hereditary, II	AD	153200			Pubertal or adult onset
Lymphedema-distichiasis syndrome	AD	153400	16q24.3	<i>FOXC2</i>	Bilateral dilated lymphatics; double row of eyelashes
Lymphedema, microcephaly-chorioretinopathy syndrome	AD	152950			Microcephaly frequently with normal intelligence; chorioretinopathy
Hennekam lymphangiectasia-lymphedema syndrome	AR	235510	18q21.32	<i>CCBE1</i>	Generalized lymphatic dysplasia; intestinal lymphangiectasia
Hypotrichosis-lymphedema-telangiectasia syndrome	AR/AD	607823	20q13.33	<i>SOX18</i>	Autosomal dominant and autosomal recessive inheritance reported
Cholestasis-lymphedema syndrome	AR	124900	15q		Aagaes syndrome, jaundice and lymphatic hyperplasia
Yellow nail syndrome	AR	153300			With frequent respiratory tract involvement
Choanal atresia and lymphedema	AR	613611	1q32	<i>PTPN14</i>	Listed under OMIM 603155. May be the same disorder
Ectodermal dysplasia, anhidrotic, with immunodeficiency, osteopetrosis and lymphedema	X	300301	Xq28		Possible mutation in NEMO

AD, autosomal dominant; AR, autosomal recessive; X, X-linked.



## 50.4 VARICOSE VEINS

The common disorder, varicose veins, is considered in this chapter rather than in Chapter 56. This term most commonly refers to increased caliber and tortuosity associated with engorgement of the superficial veins of the leg, with the saphenous being the most prominent. Varicosities may be congenital, but develop in middle age in the vast majority of affected people. As much as 10% of the population of developed countries have varicosities of the legs, and perhaps 10% of them have symptomatic venous insufficiency. Anything that increases pressure and flow in the superficial veins, such as proximal obstruction (pregnant uterus, tumor) or thrombosis of the deep veins, can increase the luminal diameter. Little dilatation is necessary before the venous valves become incompetent, which in turn increases pressure upstream and causes further dilatation. Chronic dilatation results in vascular remodeling and persistent valvular failure. The clinical results are discomfort, mild edema, susceptibility to venous rupture with minor trauma, and venous stasis, which increases the susceptibility to thrombosis. About 20% of people with chronic, symptomatic venous varicosities develop skin breakdown (varicose ulcers). Conservative treatment of support stockings and elevation of the legs can provide temporary relief of discomfort but does not address the fundamental problem. Surgical treatment has involved ligation and stripping of larger vessels and sclerosis of smaller ones. In all cases, recurrence rates are high (40%). In 1999, the US Food and Drug Administration approved the use of radiofrequency heat to collapse distended veins. Although this procedure is much less invasive than other surgical approaches and produces little short-term disability, the long-term effectiveness is unclear.

*Varicose veins are the result of an improper selection of grandparents.*

—William Osler, *Aphorism 335*

Many factors predispose to varicose veins and venous insufficiency of the lower extremity, including age (more common the older the subject), gender (increased in females), number of pregnancies, weight (increased in obesity), lifestyle (increased with prolonged standing), and positive family history (49–51). The impact of family history is subject to the typical inadequacies (imperfect sensitivity and specificity) of this method (52,53). Certain heritable disorders of connective tissue, especially Ehlers-Danlos of the classical and vascular types and the Marfan syndrome, predispose to varicose veins. However, the vast majority of venous varicosities are not simply inherited. Multifactorial inheritance was suggested by studies of large families (54). Another study of 134 relatively small families found that when neither parent was affected, the risk that a child had varicosities was 20%. When one parent was affected, the risk to a

son was 25% and to a daughter was 62%. When both parents were affected, the risk to any child was 90%. Having an affected mother may confer greater risk than if the father is the one parent involved (55).

Whether a specific tendency to defective or absent venous valves is inherited remains uncertain (56). Venous capacity and compliance show modest heritability (0.3) when studied in twins (57). Attempts to identify predisposing genes began in the past decade (51).

In part because of the occurrence of venous varicosities in certain heritable disorders of connective tissue, levels of various signaling molecules and production of components of the extracellular matrix have been examined in tissue from patients. For example, transcription of vascular endothelial growth factor (VEGF) and some of its receptors is altered in segments of varicose veins (58). In addition, a variety of genes encoding proteins involved in the extracellular matrix and its remodeling are differentially expressed in smooth muscle cells isolated from varicose veins (59,60). However, abnormalities in the actual tissue are always subject to issues of cause and effect. Examination of cultured cells from varicose veins might surmount that obstacle. Synthesis of type III collagen was reduced in both cultured smooth muscle cells and dermal fibroblasts compared with controls (61,62).

Immunostaining of the intima of varicose veins found increased expression of HIF-1 $\alpha$ , suggesting a role for hypoxia (63). A suggestion of endothelial cell dysfunction in varicose veins (64,65) led Sverdlova and colleagues (66) to examine the prevalence of a thermolabile variant of methylene-tetrahydrofolate reductase (MTHFR), C677T, in patients with lower limb varicose veins. They observed a twofold increase in the risk of varicose veins among individuals homozygous or heterozygous for the thermolabile allele. The C677T variant of MTHFR is both thermolabile and less active than the normal allele and is associated with elevated levels of plasma homocysteine (67). Hyperhomocysteinemia due to polymorphic variation in *MTHFR*, mutations in cystathionine  $\beta$ -synthase, folate, and vitamin B<sub>12</sub> deficiency, and other factors is common and potentially predisposes to varicose veins (68).

Rosbotham and colleagues (69) reported venous abnormalities in affected members of a lymphedema-distichiasis family in which lymphedema-distichiasis was linked to chromosome 16q24 and presumed to carry a mutation in the forked head transcription factor, FOXC2. Brice and colleagues (20) reported that 49% of affected individuals, in a sample of lymphedema-distichiasis families with FOXC2 mutations or linkage to 16q24, had early-onset varicose veins. These results support an important role for FOXC2 in the development of both the venous and lymphatic systems (30). Studies of FOXC2 in patients or families ascertained through the presence of varicose veins without lymphedema-distichiasis are required to

evaluate the role of this gene in the development of varicose veins in the general population.

## 50.5 GENETIC COUNSELING

Genetic diseases that affect the lymphatics are rare and heterogeneous, making them problematic from a counseling perspective. Milroy disease, hereditary lymphedema 1C, lymphedema-distichiasis syndrome and Hennekam syndrome are amenable to molecular diagnosis, but there are no prevalent mutations that can be screened in the general population. In families with known mutations, screening while possible, is complicated by the extreme variation occurrence of any feature (because of lack of penetrance), of age of onset of features, and of severity of the disease in carriers of the mutation. Lymphedema can be detected by prenatal ultrasound, but the specificity of ultrasound for the detection of lymphedema is uncertain and the clinical outcome, in cases where an abnormal ultrasound is present, cannot be reliably predicted.

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## WEBPAGES

[www.lymphnet.org](http://www.lymphnet.org)

<http://www.nhs.uk/conditions/varicose-veins/pages/whatarevaricoseveins.aspx>

## CROSS REFERENCES

Disorders of the Veins; Disorders of the Capillaries.

### Biography



Reed Pyeritz completed his PhD and MD at Harvard and trained in internal medicine at the Peter Bent Brigham Hospital in Boston and medical genetics at the Johns Hopkins Hospital in Baltimore. He is a professor of Medicine and Genetics at the Raymond and Ruth Perelman School of Medicine of the University of Pennsylvania in Philadelphia, where he directs the Center for the Integration of Genetic Healthcare Technologies and serves as vice-chair for academic affairs of the Department of Medicine. He is a senior fellow in both the Center for Bioethics and the Leonard Davis Institute for Health Economics.

His research has focused on several areas, including clinical investigations of heritable disorders of connective tissue and cardiovascular diseases, especially those affecting the thoracic aorta. Currently he is studying how more refined methods of investigating genetic variation, such as cytogenomic arrays and whole genome sequencing, actually increase the level of uncertainty in applying the results clinically.



# CHAPTER

# 51

## The Genetics of Cardiac Electrophysiology in Humans

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### 51.1 INTRODUCTION

Dysrhythmias typically are thought to occur because of primary or secondary abnormalities in cardiac electrophysiology. These abnormalities can include primary alterations in myocardial conduction and repolarization or those occurring as a result of structural heart disease. A significant portion of the individuals found to have abnormalities of rhythm and conduction are now known to have a genetic basis, with familial inheritance notable. Familial inheritance of dysrhythmias and conduction disorders indicates that genetic factors play an integral role in development of these abnormalities. Understanding the underlying genetic defects responsible for these disorders has indeed provided insights into the mechanisms leading to the clinical picture and promises to impact the therapeutic strategies used in the care of these patients. Over the course of the past 15 years, our understanding of the genetic abnormalities in a variety of cardiomyopathies, long-QT syndrome (LQTS), Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia (CPVT), and newer disorders has led to general concepts of cardiovascular disease. In this chapter, the clinical features and management of familial dysrhythmias and conduction disorders are discussed and the current understanding of the genetic abnormalities associated with these disorders is reviewed.

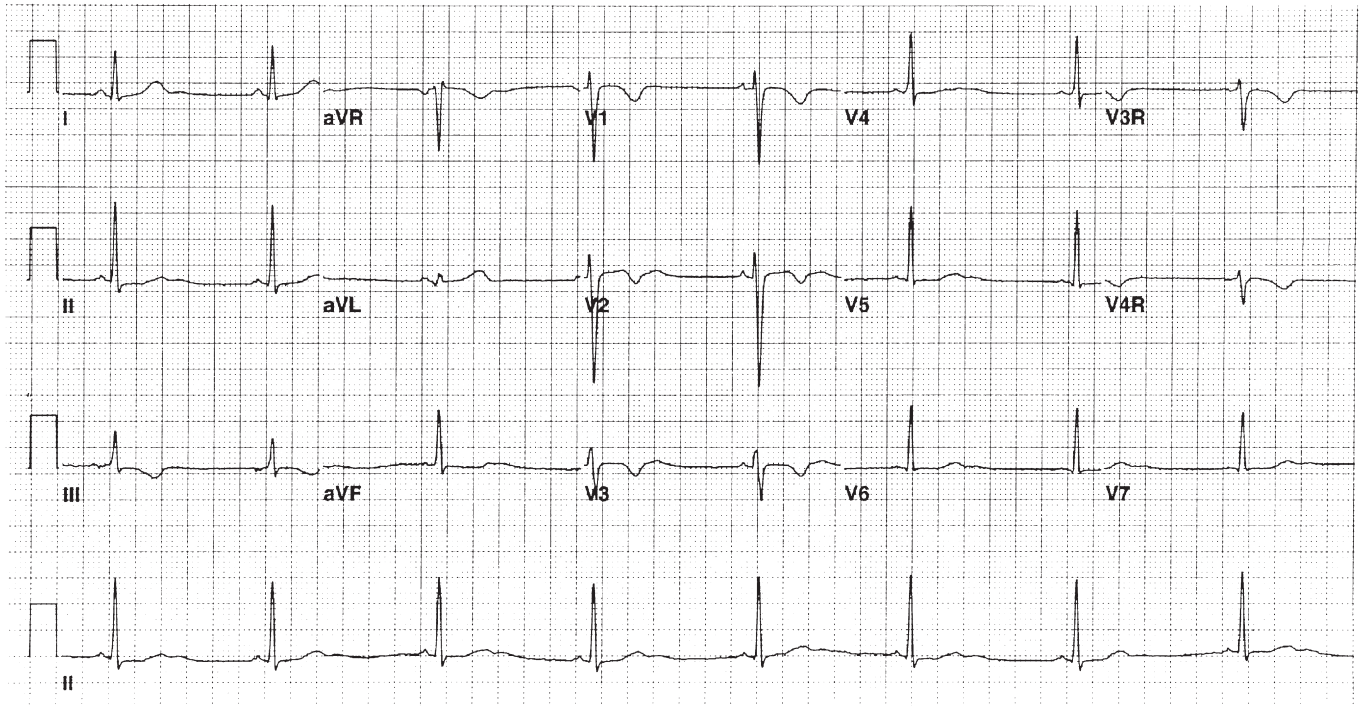
### 51.2 SPECIFIC CARDIAC DYSRHYTHMIAS

The entire cardiac electrical system can be affected by genetic abnormalities, leading to atrial and ventricular tachydysrhythmias, sinus node dysfunction, or atrioventricular (AV) block. Primary dysrhythmias and secondary dysrhythmias (i.e. those associated with structural heart disease) are discussed next.

### 51.3 PRIMARY ABNORMALITIES IN CARDIAC RHYTHM: VENTRICULAR TACHYDYSRHYTHMIAS

#### 51.3.1 Long-QT Syndromes

LQTSs are primary disorders of cardiac repolarization (1), in which prolongation of the QT interval corrected for heart rate (QTc) is seen on the surface electrocardiogram (ECG) along with abnormalities of T wave morphology and sinus bradycardia (Figure 51-1). Syncope, seizures, and sudden death are the clinical features that are commonly seen, occurring because of ventricular tachycardia (VT), especially polymorphic VT or torsade de pointes (Figure 51-2), which can degenerate into ventricular fibrillation (VF). Torsade de pointes, meaning “turning of the points,” is common in all forms of LQTS and describes the varying axis of the QRS complex during VT (1). This dysrhythmia is a subset of polymorphic VT, to be distinguished from monomorphic VT, which has a different morphology and mechanism of development (2,3). Monomorphic VT usually results from reentrant mechanisms and typically can be induced by programmed electrical stimulation. On the other hand, polymorphic VT cannot be induced using programmed electrical stimulation, suggesting that the mechanism is unlikely to be primary reentry. Torsade de pointes, in fact, is thought to be initiated by abnormal automaticity and then maintained by reentrant mechanisms (4). Development of early afterdepolarizations (EADs) appears to be an important mechanism whereby drug-induced action potential prolongation initiates torsade (5,6). Under normal conditions, the ventricle is activated from subendocardium to epicardium by impulses arising in the subendocardial Purkinje network. Mapping data in animal models support the idea that the initial beat in torsade arises in the subendocardium, consistent with a triggered beat arising from an EAD in the Purkinje



**FIGURE 51-1** Electrocardiogram of a 14-year-old female with long-QT syndrome. Note the sinus bradycardia (48 beats/min) and very prolonged QTc of 600ms, with low-amplitude, bifid T waves.



**FIGURE 51-2** Torsade de pointes polymorphic ventricular tachycardia.

system (7). The conditions that evoke EADs markedly prolong repolarization in the mid-myocardium (M-cell region) as well, resulting in a situation in which propagation of EAD-related triggered beats from the subendocardium may be blocked in regions where M-cell action potentials have become especially long, setting up intramural reentrant excitation with a circuit that varies from

beat to beat (8). This perhaps accounts for the distinctive morphology of torsade de pointes (2,9).

Recently, the concept has emerged that defects in currents important for repolarization prolong the action potential but are not directly arrhythmogenic. Rather, action potential duration creates a milieu in which genetically normal, drug-unmodified ion channels or other

electrogenic phenomena further prolong repolarization and precipitate arrhythmias.

The LQTSs have been classified into acquired and genetically inherited forms. Acquired long-QT syndrome (aLQTS) is the most common form of LQTS, with drug-induced LQTS particularly common. Drugs implicated in aLQTS include antiarrhythmic agents such as quinidine or sotalol, tricyclic antidepressants, antibiotics (especially macrolide antibiotics such as erythromycin), antihistamines such as terfenidate, and inhalational anesthetics. aLQTS has also been seen in association with metabolic derangements, including hypokalemia, hypomagnesemia, and hypercalcemia. Additionally, aLQTS has been identified in patients with other cardiac diseases such as cardiomyopathies and myocardial ischemia, as well as under circumstances of intracranial disease (i.e. intracranial surgery, subarachnoid hemorrhage, and increased intracranial pressure).

Four forms of inherited LQTS are known. Romano-Ward syndrome (10,11) and Jervell and Lange-Nielsen syndrome (JLNS; (12)) are the classically described forms of LQTS. More recently, two other disorders, Andersen syndrome (13) and Timothy syndrome (14,15), have been described and studied and the underlying causes were determined. Another disorder, sudden infant death syndrome (SIDS), has also been showed to be LQTS-like in some babies (16).

Romano-Ward syndrome is characterized by autosomal-dominant inheritance with reduced penetrance and is the most common inherited form of LQTS, estimated to have an incidence of one in 10,000 live births worldwide (17–19). JLNS is a seemingly rare condition, with an estimated incidence of one in 1.6 million live births (17–19). This disorder, which is defined as LQTS associated with sensorineural deafness, has been described as having autosomal recessive inheritance since its initial description in 1957 (12). In the past decade, molecular genetic studies have clarified its inheritance, suggesting it to be autosomal-dominant LQTS associated with autosomal-recessive deafness. Finally, SIDS has been shown to be another presentation of inherited LQTS (16). Supportive data were provided by the identification of mutations in the cardiac sodium-channel gene, *SCN5A*, by Ackerman et al. (20), followed by identification of *HERG/KCNH2* mutations in other infants with SIDS (21).

**51.3.1.1 Clinical Features of LQTS.** LQTS is typically identified in individuals presenting with syncope, seizures, or sudden cardiac death that results from episodic ventricular tachydysrhythmias, particularly torsade de pointes and VF (1,17–19,22). Cardiac dysrhythmias have been reported in up to 24% of cases, but the risk of sudden death has been estimated to be <1% per year. These estimates are almost certainly inaccurate, however, as a significant percentage of the 300,000–400,000 sudden deaths occurring in the United States yearly (23) are likely to be the result of this disorder (which goes

unrecognized), and many living patients are asymptomatic and can have normal QT intervals on screening ECGs because of reduced penetrance. Today, many asymptomatic family members are being identified via both screening ECGs and molecular genetic family screening, and therefore, better estimates are likely in the future.

As LQTS may be difficult to diagnose, a set of criteria have been developed by Schwartz et al. (1,1a,22,24). These criteria use a point system in the diagnostic scheme, relying heavily on classical features (Table 51-1). This approach is thought to improve diagnostic accuracy by including major criteria (prolonged QTc > 440ms, stress-induced syncope, family history of LQTS) and minor criteria (congenital deafness, T wave alternans, relative bradycardia, abnormal ventricular repolarization) (Figure 51-3).

The clinical features of LQTS, which occur because of dysrhythmias, are typically associated with triggering events. The most well-described triggers include exercise, anxiety or excitement, auditory events (i.e. telephone or alarm clock ringing), swimming or diving into a pool, and postpartum status. Some patients have events or die during sleep, which is thought to be associated with bradycardia.

In most cases of LQTS, no other abnormalities occur. However, sensorineural deafness is associated with the JLNS form of LQTS, distinguishing it from Romano-Ward syndrome. LQTS has been reported to occur in approximately 1% of children with congenital deafness, and this has led to the recommendation for screening ECGs in this group of children. A recent study from Thailand demonstrated a possible prevalence of 0.7% of JLNS in children with sensorineural deafness at a school for the deaf (25). These children also appear to have more severe ECG abnormalities than Romano-Ward syndrome patients and have a worse prognosis. Other associated abnormalities have been reported rarely in more complex patients with LQTS. Marks et al. (14) described LQTS patients with syndactyly; in this patient subset, LQTS appears to be quite severe, with a high percentage of patients dying in infancy. In most of these patients, the initial presentation included 2:1 AV block due to marked QT prolongation. These patients were initially found in sporadic cases, with both genders being represented, but more recently, families have been identified, and a new syndrome termed Timothy syndrome (15) has been described (Section 51.3.1.4.3). Other patients with LQTS have been described with associated diabetes mellitus or asthma (26,27), as well as those with potassium-sensitive periodic paralysis, dysmorphic features, and skeletal abnormalities, termed Andersen syndrome (13).

**51.3.1.2 Genetics of LQTS.** As previously noted, four forms of inherited LQTS have been described, including autosomal dominant (Romano-Ward syndrome), autosomal recessive (JLNS), and other complex forms (Andersen syndrome and Timothy syndrome), in addition to sporadic cases. Over the past 15 years, the genetic

TABLE 51-1 Diagnostic Criteria in LQTS

Clinical Finding	Points <sup>a</sup>
Electrocardiographic findings <sup>b</sup>	
QT <sub>c</sub> <sup>c</sup>	
>480 ms <sup>1/2</sup>	3
460–470 ms <sup>1/2</sup>	2
450 (male) ms <sup>1/2</sup>	1
Torsades de pointes <sup>d</sup>	2
T wave alternans	1
Notched T wave in three leads	1
Low heart rate for age <sup>e</sup>	0.5
Clinical history	
Syncope <sup>d</sup>	
With stress	2
Without stress	1
Congenital deafness	0.5
Family history <sup>f</sup>	
Family members with definite LQTS <sup>g</sup>	1
Unexplained sudden cardiac death below	0.5 age 30 among immediate family members

<sup>a</sup>Scoring: <1 point = low probability of LQTS, 2–3 points = intermediate probability of LQTS, >4 points = high probability of LQTS.

<sup>b</sup>In the absence of medications or disorders known to affect these ECG features.

<sup>c</sup>QT<sub>c</sub> calculated by Bazett's formula, where QT<sub>c</sub> = QT.

<sup>d</sup>Mutually exclusive.

<sup>e</sup>Resting heart rate below the second percentile for age.

<sup>f</sup>The same family member cannot be counted twice.

<sup>g</sup>Definite LQTS is defined by an LQTS score >4.

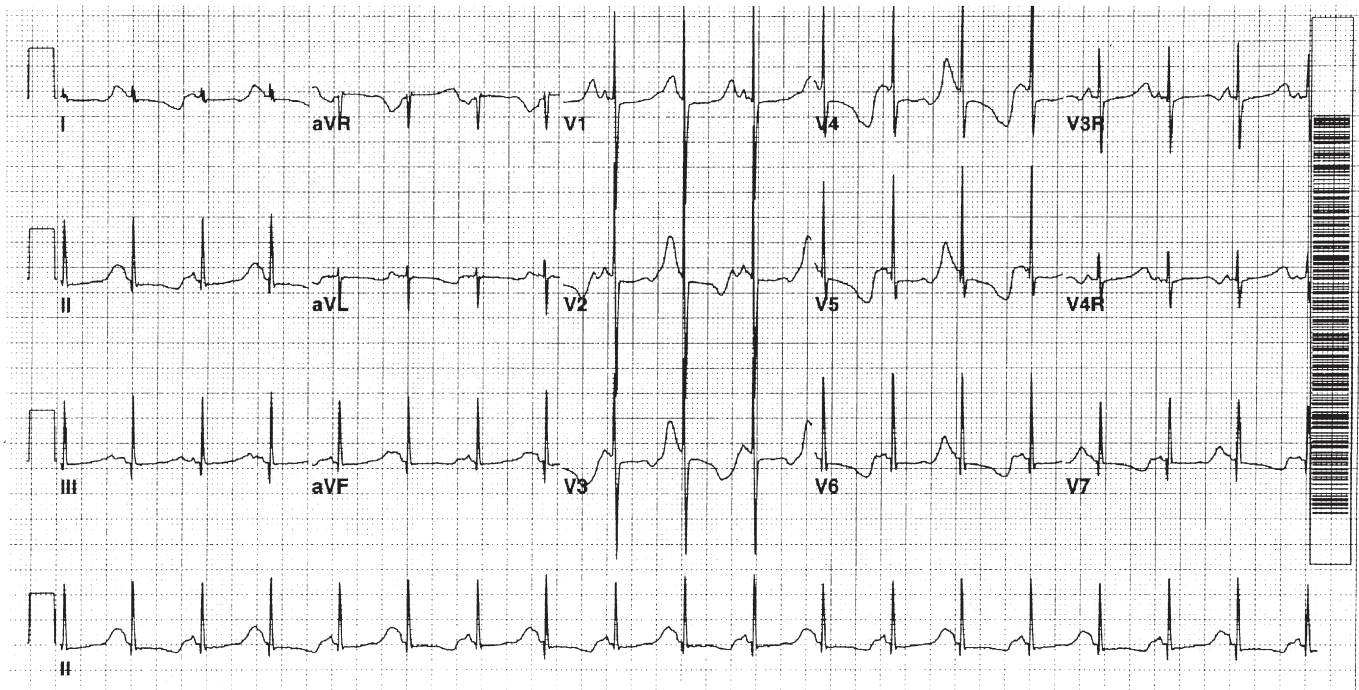


FIGURE 51-3 T wave alternans in a child with long-QT syndrome.

aspects of all four forms of LQTS have been unraveled. In the case of the most common inherited form, Romano-Ward syndrome, the key genes have been identified for all of the mapped subtypes. In 1991, Keating et al. identified genetic linkage to the short arm of chromosome 11 (11p15.5) in several families with Romano-Ward

syndrome (28,29). Shortly thereafter, we demonstrated genetic heterogeneity, several laboratories confirmed this subsequently (30–33). In a collaborative effort (34), linkage was shown for several families to two new loci, the long arm of chromosome 7 (7q35–36) and the short arm of chromosome 3 (3p21). The three loci were later termed



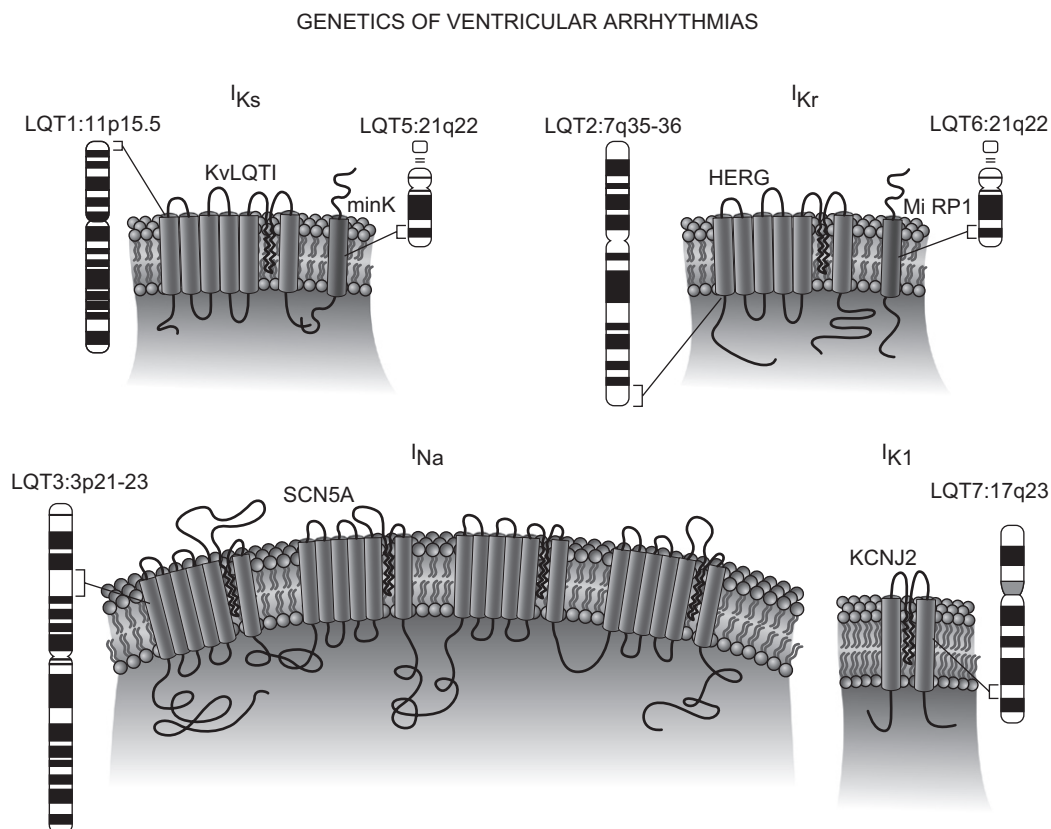
LQT1 (11p15.5), LQT2 (7q35–56), and LQT3 (3p21). A fourth locus (LQT4) on chromosome 4q (4q25–27) was later described as well (35). Most recently, two other genes, both located on chromosome 21q22 (loci LQT5 and LQT6), were identified (36–38) (Figure 51-4). Penetrance in Romano–Ward syndrome is reduced and, in some families, Romano–Ward syndrome appears to occur in a recessively inherited pattern (39).

### 51.3.1.3 Gene Identification in Romano–Ward Syndrome.

**51.3.1.3.1 KVLQT1 or KCNQ1: The LQT1 Gene.** The first of the genes mapped for LQTS, termed LQT1, required 5 years from the time that mapping to chromosome 11p15.5 was first reported to gene cloning (Table 51-2). This gene, originally named *KVLQT1* but more recently called *KCNQ1*, is a novel potassium-channel gene that consists of 16 exons, spans approximately 400 kb, and is widely expressed in human tissues including heart, inner ear, kidney, lung, placenta, and pancreas, but not in skeletal muscle, liver, or brain. In the original report, 11 different mutations (deletion and missense mutations) were identified in 16 LQTS families, establishing *KVLQT1* as LQT1. To date, more than 100 families with *KVLQT1* mutations have been described. Although most of the mutations are “private” (i.e. only seen in one family), there is at least one frequently mutated region (called a “hot spot”) of *KVLQT1* (40,41).

Analysis of the predicted amino acid sequence of KVLQT1 suggests that it encodes a potassium-channel  $\alpha$  subunit (37,38,42) with a conserved potassium-selective pore-signature sequence flanked by six membrane-spanning segments similar to shaker-type channels (Figure 51-4). A putative voltage sensor is found in the fourth membrane-spanning domains (S4) and the selective pore loop is between the fifth and sixth membrane-spanning domains (S5 and S6). Biophysical characterization of the KVLQT1 protein confirmed that KVLQT1 is a voltage-gated potassium-channel protein subunit that requires coassembly (37,38) with a  $\beta$  subunit called minK to function properly (Figure 51-4). Expression of either KVLQT1 or minK alone results in inefficient (or no) current development. When minK and KVLQT1 are coexpressed in either mammalian cell lines or *Xenopus* oocytes, however, the slowly activating delayed-rectifier potassium current ( $I_{Ks}$ ) is developed in cardiac myocytes. Combination of normal and mutant KVLQT1 subunits forms abnormal  $I_{Ks}$  channels and these mutations are believed to act through a dominant-negative mechanism (the mutant form of KVLQT1 interferes with the function of the normal wild-type form through a “poison pill”-type mechanism) or a loss-of-function mechanism (only the mutant form loses activity) (43–45).

Because KVLQT1 and minK form a unit, mutations in minK were also expected to cause LQTS (38), and this fact was subsequently demonstrated (Section 51.3.1.3.5).



**FIGURE 51-4** Chromosomal loci and genes responsible for long QT syndrome.

**TABLE 51-2 Familial Primary Dysrhythmias**

Disease	Rhythm Abnormality	Inheritance	Chromosome Location	Gene
Ventricular arrhythmias				
Romano–Ward syndrome	TdP, VT, VF	AD	3p21, 4q25, 7q35, 11p15.5, 21q22 <sup>b</sup> (3p21); ANK $\beta$ (4q25)	KVLQT1/KCNQ1 (11p15.5); HERG/KCNH2 (7q35); SCN5A minK/KCNE1 (21q22); MiRP1/KCNE2(21q2)
Jervell and Lange-Nielsen syndrome	TdP, VT, VF	AD/AR <sup>a</sup>	11p15.5, 21q22	KVLQT1 (11p15.5); minK (21q22)
Andersen syndrome	TdP, VT, VF, AF	AD	17q23	Kir2.1 (KCNJ2)
Timothy syndrome	VT, VF	AD	12p13.3	Ca(V) 1.2
Brugada syndrome	VT, VF	AD	3p21	SCN5A
Sudden unexpected death syndrome	VT/VF	AD	3p21	SCN5A
Short QT syndrome	TdP, VT, VF	AD	7q35, 11p15.5, 17q23	KCNQ1, KCNH2, KCNJ2
Familial VT	VT	AD	?	?
Familial bidirectional VT	VT	AD	1q42	RyR2
Catecholaminergic polymorphic VT	VT	AD	1q42b	RyR2, CASQ2
Supraventricular arrhythmias				
Familial atrial fibrillation	AF	AD	6q14–16, 10q22, 11p15.5, 21q22 (21q22)	KvLQT1/KCNQ1 (11p15.5); KCNE KCNE2(21q22)
Familial total atrial standstill	SND, AF	AD	?	?
Familial absence of sinus rhythm	SND, AF	AD	?	?
Wolff–Parkinson–White syndrome	AVRT, AF, VF	AD	7q3	AMP-kinase/PRKAG2
Familial PJRT	AVRT	AD	?	?
Conduction abnormalities				
Familial AV block	AVB, AF, SND, VT, SD	AD	19q13	?
Lev-Lengre syndrome	AVB, AF, SND, VT, SD	AD	3p21	SCN5A
Isolated conduction disease	AVB/SND	AD	3p21	SCN5A
Sick sinus syndrome	AVB, SND, VT	AR	3p21	SCN5A
Familial bundle-branch block	RBBB	?	?	?

AD, autosomal dominant; AF, atrial fibrillation; AR, autosomal recessive; AVB, atrioventricular block; AVRT, atrioventricular reciprocating tachycardia; PJRT, permanent form of junctional reciprocating tachycardia; RBBB, right bundle-branch block; SD, sudden death; SND, sinus node dysfunction; TdP, torsade de pointes; VF, ventricular fibrillation; VT, ventricular tachycardia.

<sup>a</sup>Jervell and Lange-Nielsen syndrome: autosomal dominant rhythm abnormality and autosomal recessive sensorineural deafness.

<sup>b</sup>At least one other unknown.

The vast majority of mutations in *KVLQT1* and *minK* are heterozygous mutations in Romano–Ward syndrome patients (38,46,47). *KVLQT1* appears to be the most commonly mutated gene in LQTS.

**51.3.1.3.2 HERG or KCNH2: The LQT2 Gene.** After the initial localization of LQT2 to chromosome 7q35–36 by Jiang et al. (34) (Table 51-2), candidate genes (i.e. genes encoding proteins that could cause repolarization abnormalities if mutated, such as ion channels, modulators of ion channels, members of the sympathetic nervous system) in this chromosomal region were analyzed. Human ether-a-go-go-related gene (*HERG*)—a cardiac potassium-channel gene originally cloned from a brain complementary DNA library (48) and that is expressed in neural crest-derived neurons (49), microglia (50), a wide variety of tumor cell lines (51), and the heart (52)—was found to be mutated in patients with clinical evidence of LQTS (52). Six LQTS-associated mutations were initially identified in *HERG*, including missense mutations, intragenic deletions, and a splicing mutation. Later, Schulze-Bahr et al. (53) confirmed *HERG*, which

is now known as *KCNH2*, as the *LQT2* gene, identifying mutations in other families. Currently, this gene is thought to be the second most common gene mutated in LQTS (second to *KVLQT1*). As with *KVLQT1*, “private” mutations that are scattered throughout the entire gene without preferential clustering are seen.

The *KCNH2* gene consists of 16 exons and spans 55kb of genomic sequence (52). The predicted topology of *HERG* is shown in Figure 51-4 and is similar to *KVLQT1*. Unlike *KVLQT1*, *HERG* has extensive intracellular amino and carboxyl termini, with a region in the carboxyl-terminal domain having sequence similarity to nucleotide-binding domains.

Electrophysiologic and biophysical characterization of *HERG* expressed in *Xenopus* oocytes established that *HERG* encodes the rapidly activating delayed-rectifier potassium current ( $I_{Kr}$ ; (54,55)), and electrophysiologic studies of LQTS-associated mutations demonstrated a loss-of-function or a dominant-negative (56) mechanism of action. In addition, protein trafficking abnormalities have been shown to occur (57,58). This channel has been

shown to coassemble with  $\beta$  subunits for normal function, similar to that seen in  $I_{Ks}$ . McDonald et al. (59) initially suggested that the complexing of *HERG* with *minK* is needed to regulate the  $I_{Kr}$  potassium current. Bianchi et al. (60) provided confirmatory evidence that *minK* is involved in regulation of both  $I_{Ks}$  and  $I_{Kr}$ . Most recently, Abbott et al. (36) identified MiRP1 as a  $\beta$  subunit for *HERG* (Section 51.3.1.3.6).

Recently, Zhang et al. (61) identified an intronic variant in *KCNH2* (T1945+6C) in which splicing assay analysis showing downstream intron retention and complementary DNA with the retained intron 7 failing to produce functional channels, consistent with potential disease-causing dysfunction. This finding potentially expands the disease-causing mechanisms in LQTS.

**51.3.1.3.3 SCN5A: The LQT3 Gene.** Utilization of the candidate gene approach established that the gene responsible for chromosome 3-linked LQTS (LQT3) (62) is the cardiac sodium-channel gene *SCN5A* (63,64) (Table 51-2). *SCN5A* is highly expressed in human myocardium and brain, but not in skeletal muscle, liver, or uterus (64,65). It consists of 28 exons that span 80kb and encodes a protein of 2016 amino acids with a putative structure that consists of four homologous domains (DI–DIV), each of which contains six membrane-spanning segments (S1–S6) similar to the structure of the potassium-channel  $\alpha$  subunits (Figure 51-4) (66). Mutation analysis identified three mutations—one 9 bp intragenic deletion ( $\Delta K_{1505}P_{1506}Q_{1507}$ ) and two missense mutations ( $R_{1644}H$  and  $N_{1525}S$ )—in six LQTS families (63,64); when expressed in *Xenopus* oocytes, all mutations generated a late phase of inactivation-resistant, mexiletine- and tetrodotoxin-sensitive whole-cell current via different mechanisms (67,68). Two of the three mutations showed dispersed reopening after the initial transient, but the other mutation showed both dispersed reopening and long-lasting bursts. These results suggested that *SCN5A* mutations act through a gain-of-function mechanism (the mutant channel functions normally, but with altered properties such as delayed inactivation) and that the mechanism of chromosome 3-linked LQTS is persistent nonactivated sodium current in the plateau phase of the action potential. An et al. (69) also showed that not all mutations in *SCN5A* are associated with persistent current and demonstrated that *SCN5A* interacted with  $\beta$  subunits. Furthermore, mutations in *SCN5A* have been shown to result in widely different clinical phenotypes and different responses to medications (70). Novel mutations in *SCN5A* were identified by our laboratory in patients with Brugada syndrome and idiopathic VF (71), disorders with normal QT interval but ST segment elevation in the right precordial leads (Table 51-2). Electrophysiologically, these mutations result in more rapid recovery from inactivation of the mutant channels or loss of function causing the Brugada syndrome-type phenotype (Section 51.3.2). In addition, mutations in this gene cause conduction system disease (72) and SIDS (1,20,22),

sudden unexplained nocturnal death syndrome (see later section), and, in cases with recessive inheritance and homozygous mutations, sick sinus syndrome (73) (Table 51-2).

**51.3.1.3.4 Ankyrinb or AnkK: The LQT4 Gene.** Initially mapped in 1995 to chromosome 4q25–25 by Schott et al. (35) in a large French family with autosomal-dominant LQTS associated with sinus bradycardia due to sinus node dysfunction and atrial fibrillation (AF), the gene remained elusive until 2003. Mohler et al. (74) identified ankyrinb or ankyrin2 (*AnkK*) as the disease-causing gene. This gene encodes a protein with three major isoforms with molecular weights of 440, 220, and 150kDa generated by alternative splicing. The major isoform in the heart is the 220kDa form. Ankyrins are adapter proteins that link integral membrane proteins to the spectrin-based cytoskeleton (75) and contain three functional domains that consist of the membrane-binding domain, the spectrin-binding domain, and the regulatory domain. These proteins bind to several ion-channel proteins, including the voltage-sensitive sodium channel ( $I_{Na}$ ), anion exchanger ( $Cl^-/HCO_3^-$  exchanger), sodium–potassium ATPase, sodium–calcium exchanger (NCX or  $I_{Na-Ca}$ ), and calcium-release channels, including those mediated by the ryanodine receptor (RyR2) and the inositol triphosphate receptor. Mutations in *ANKK* appear to act by a loss-of-function mechanism. It is likely that mutations in this gene cause a clinical phenotype by disrupting the cytoskeletal framework, which results in compromise of channel function or of the trafficking of channels to their proper locale. A murine model of mutant *ANKK* results in clinical features similar to those seen in humans, and findings indicative of calcium-handling abnormalities (74) and sodium-channel dysfunction (76) have been reported.

**51.3.1.3.5 minK or KCNE1: The LQT5 Gene.** The *minK* (*IsK* or *KCNE1*) gene was initially localized to chromosome 21 (21q22.1) and found to consist of three exons that span approximately 40 kb (77). It encodes a short protein consisting of 130 amino acids and has only one transmembrane-spanning segment with small extracellular and intercellular regions (Figure 51-4). When expressed in *Xenopus* oocytes, it produces potassium current that closely resembles  $I_{Ks}$  in cardiac cells (37,38). The fact that the *minK* clone was expressed only in *Xenopus* oocytes and not in mammalian cell lines raised the question whether *minK* is a human-channel protein. With the cloning of *KVLQT1* and coexpression of *KVLQT1* and *minK* in both mammalian cell lines and *Xenopus* oocytes, it became clear that *KVLQT1* interacts with *minK* to form the cardiac  $I_{Ks}$  (37,38); *minK* alone cannot form a functional channel but induces  $I_{Ks}$  by interacting with endogenous *KVLQT1* protein in *Xenopus* oocytes and mammalian cells. Bianchi et al. (60) also showed that mutant *minK* results in abnormalities of  $I_{Ks}$  and  $I_{Kr}$  and in protein-trafficking abnormalities. McDonald et al. (59) showed that *minK* also interacts with *HERG*,

regulating  $I_{K_r}$ . Splawski et al. (78) demonstrated that *minK* mutations cause LQT5 when they identified mutations in two families with LQTS (Table 51-2). In both cases, missense mutations (S74I; D76N) were identified that reduced  $I_{K_s}$  by shifting the voltage dependence of activation and accelerating channel deactivation. This was later confirmed by others (46,47) and further supported by the fact that a mouse model with mutant *minK* (79) developed a phenotype (which included deafness). The functional consequences of these mutations include delayed cardiac repolarization and, hence, an increased risk of dysrhythmias (46,47,80).

**51.3.1.3.6 MiRP1 or KCNE2: The LQT6 Gene.** The *MiRP1* (minK-related peptide 1 or *KCNE2*) gene is a novel potassium-channel gene cloned and characterized by Abbott et al. (36). This small integral membrane subunit protein assembles with *HERG* (LQT2) to alter its function and enable full development of  $I_{K_r}$  (Figure 51-4). *MiRP1* is a 123-amino-acid-channel protein with a single predicted transmembrane segment similar to that described for *minK*. Chromosomal localization studies mapped this *KCNE2* gene to chromosome 21q22.1, within 79kb of *KCNE1* (*minK*) and arrayed in opposite orientation. The open reading frames of these two genes share 34% identity, and both are contained in a single exon, suggesting that they are related through gene duplication and divergent evolution.

Three missense mutations associated with dysrhythmias were identified in *KCNE2* by Abbott et al. (36), and biophysical analysis demonstrated that these mutants form channels that open slowly and close rapidly, thus diminishing potassium currents. In one case, the missense mutation, a C-to-G transversion at nucleotide 25 that produced a glutamine (Q)-to-glutamic acid (E) substitution at codon 9 (Q9E) in the putative extracellular domain of *MiRP1*, led to the development of torsade de pointes and VF after intravenous clarithromycin infusion (i.e. drug induced). Therefore, similar to *minK*, this channel protein acts as a  $\beta$  subunit but, by itself, leads to risk of ventricular arrhythmia when mutated (Table 51-2). These similar channel proteins (i.e. *minK* and *MiRP1*) suggest that a family of channels exist that regulate ion-channel  $\alpha$  subunits. The specific role of this subunit and its stoichiometry remain unclear and are currently hotly debated.

#### 51.3.1.4 Complex Forms of LQTS.

##### 51.3.1.4.1 Jervell and Lange-Nielsen Syndrome.

**51.3.1.4.1.1 Clinical Features.** As noted previously, patients with JLNS have severe QT interval prolongation; episodic tachydysrhythmias, including torsade de pointes, syncope, and/or sudden death; and sensorineural deafness (1,12). The deafness is autosomal recessive, whereas the LQTS is autosomal dominant but severe (19).

**51.3.1.4.1.2 Genetics.** Neyroud et al. (81) reported the first molecular abnormality in patients with JLNS when they reported on two families in which three children were affected by JLNS, finding a novel homozygous

deletion–insertion mutation of *KVLQT1* (Table 51-2). A deletion of 7bp and an insertion of 8bp at the same location led to premature termination at the C-terminal end of the *KVLQT1* channel. At the same time, Splawski et al. (15a) identified a homozygous insertion of a single nucleotide that caused a frameshift in the coding sequence after the second putative transmembrane domain (S2) of *KVLQT1*. Together, these data strongly suggested that at least one form of JLNS is caused by homozygous mutations in *KVLQT1*. This has been confirmed by others (40,45,82–84).

As a general rule, heterozygous mutations in *KVLQT1* cause Romano–Ward syndrome (LQTS only), whereas homozygous (or compound heterozygous) mutations in *KVLQT1* cause JLNS (LQTS and deafness). The hypothetical explanation suggests that although heterozygous *KVLQT1* mutations act by a dominant-negative mechanism (85), some functional *KVLQT1* potassium channels still exist in the stria vascularis of the inner ear. Therefore, congenital deafness is averted in patients with heterozygous *KVLQT1* mutations. For patients with homozygous *KVLQT1* mutations, no functional *KVLQT1* potassium channels can be formed. It has been shown by in situ hybridization that *KVLQT1* is expressed in the inner ear (81), suggesting that homozygous *KVLQT1* mutations can cause the dysfunction of potassium secretion in the inner ear and lead to deafness (79). However, it should be noted that incomplete penetrance exists and not all heterozygous or homozygous mutations follow this rule (86).

As with Romano–Ward syndrome, if *KVLQT1* mutations can cause the phenotype, it could be expected that *minK* mutations could also be causative of the phenotype (JLNS). Schulze-Bahr et al. (83), in fact, showed that mutations in *minK* result in JLNS syndrome as well (Table 51-2), and this was confirmed subsequently (47,84). Hence, abnormal  $I_{K_s}$ , whether it occurs because of homozygous or compound heterozygous mutations in *KVLQT1* or *minK*, results in LQTS and deafness.

##### 51.3.1.4.2 Andersen Syndrome (LQT7).

**51.3.1.4.2.1 Clinical Aspects.** Andersen et al. (13) identified a complex phenotype including ventricular arrhythmias, potassium-sensitive periodic paralysis, and dysmorphic features. The dysmorphisms included hypertelorism, broad nasal root, defects of the soft and hard palate, and short stature. More recently, skeletal abnormalities have broadened the phenotype (87). These skeletal features include micrognathia, clinodactyly, syndactyly, and scoliosis. The associated cardiac abnormalities include QTc prolongation, VT, VF, and atrial arrhythmias. Torsades de pointes and bidirectional VT have been seen. In addition, repolarization abnormalities affecting late repolarization and resembling giant U waves are common. Sudden death has not been reported as a major risk in this disorder. Andelfinger et al. (87) also reported sex-specific variable expression, as well as other clinical features



including unilateral dysplastic kidney and congenital heart disease (bicuspid aortic valve, coarctation of the aorta, valvular pulmonic stenosis).

**51.3.1.4.2.2 Genetics: Kir2.1 or KCNJ2: The LQT7 Gene.** Andersen syndrome was originally mapped to chromosome 17q23–q24.2 by Plaster et al. (88) using genome-wide linkage analysis. The critical region within this locus was narrowed and candidate gene mutation screening identified mutations in *KCNJ2*, which encodes an inward-rectifier potassium channel called Kir2.1 ( $I_{K1}$ ; 588). This channel is highly expressed in the heart and plays a role in phase 4 repolarization and in the resting membrane potential. Multiple gene mutations have been identified to date, with relatively high penetrance noted. Functional studies have demonstrated reduction or suppression of  $I_{K1}$  by a haploinsufficiency or dominant-negative effect (89,90). Lange et al. (89) generated known *KCNJ2* mutants that did not yield any measurable potassium currents in CHO cells, consistent with failure to form functional homomultimeric complexes and non-functional channels. In addition, Bendahhou et al. (91) demonstrated that defective Kir2.1 channels may not traffic to the membrane properly. This gene may play a role in developmental signaling pathways as well, which is believed to be the cause of the dysmorphisms (87).

Analysis of a variety of *KCNJ2* mutations demonstrated that many of these mutations included residues implicated in binding membrane-associated phosphatidylinositol 4,5-bisphosphate (92). It should be noted that nearly 40% of cases do not segregate with this gene, suggesting that genetic heterogeneity exists (93).

#### **51.3.1.4.3 Timothy Syndrome.**

**51.3.1.4.3.1 Clinical Aspects.** This complex disorder is characterized by multisystem dysfunction, including webbing of fingers and toes (syndactyly), immune deficiency, intermittent hypoglycemia, a cognitive immune deficiency, cognitive abnormalities, autism, dysmorphic features, various forms of congenital heart disease, and lethal arrhythmias associated with LQTS (14,15a).

**51.3.1.4.3.2 Genetics.** This autosomal-dominant disorder was described initially by Marks et al. (14), and more recently, the genetic basis of this syndrome was described by Splawski et al. (15a). Mutations in *Ca(V)1.2*, the L-type calcium channel, which is expressed in all affected tissues, was shown to produce maintained inward  $Ca^{2+}$  overload in multiple cell types and, in the heart, causes prolonged  $Ca^{2+}$  current, delaying cardiomyocyte repolarization and increasing arrhythmia risk.

**51.3.1.5 Genotype–Phenotype Correlations in LQTS.** Moss et al. (94) showed that the ECG manifestations of LQTS were in great part determined by the channel mutated. Different T wave patterns were clearly evident when comparing tracings from patients with mutations in LQT1, LQT2, and LQT3. More recently, Zareba et al. (95) showed that the mutated gene may result in a specific clinical phenotype with different triggers and may predict outcome. For instance, these

authors suggested that mutations in LQT1 and LQT2 resulted in early symptoms (i.e. syncope), but the risk of sudden death was relatively low for any event. In contrast, mutations in LQT3 resulted in a paucity of symptoms but, when symptoms occurred, they were associated with a high likelihood of sudden death. Zareba et al. (96) showed that the location of mutations in *KCNQ1* plays no role in clinical course. However, Shimizu et al. (97) showed mutation site-specific differences in arrhythmic risk and sensitivity to sympathetic stimulation in LQT1 patients, with those having transmembrane mutations having a greater risk of early-onset cardiac events and greater sensitivity to sympathetic stimulation compared with patients having C-terminal mutations. Moss et al. showed that pore mutations in LQT2 have higher risk, and effects of age and gender based on genotype were also reported (98). In the latter case, Zareba et al. showed that, during childhood, the risk of cardiac events was significantly higher in LQT1 and LQT3 males than females, with no gender-related differences in the risk of cardiac events among LQT2 and LQT3 carriers. In adulthood, LQT2 females and LQT1 females had a significantly higher risk of cardiac events than respective males. The lethality of cardiac events was higher in LQT3 males and females, and higher in LQT1 and LQT2 males than females. Compound mutation, which reportedly occurs in approximately 8% of subjects, appears to cause longer QTc intervals, worse and more frequent cardiac events, and, in general, a more severe phenotype than other mutations, and worse disease than expected (99).

Neonates appear to have gene-specific clinical features as well. Lupoglazoff et al. (100) studied 23 neonate probands and demonstrated that 2:1 AV block is common and usually associated with LQT2 mutations, with a poor prognosis during the first month of life. In contrast, LQT1 mutations in this patient subgroup appeared to be correlated with sinus bradycardia and good short-term prognosis with  $\beta$ -blocker therapy. Ackerman et al. (20) and Moss et al. (101) showed that swimming is a common trigger for symptoms in LQT1 patients, whereas Wilde et al. (102) have found auditory triggers to be common in LQT2. LQT3, on the other hand, appears to be associated with sleep-associated symptoms. More recently, Choi et al. (103) evaluated 388 subjects with LQTS for LQT gene mutations and identified 43 individuals with swimming-related events (11%). Among this group, 65% had mutations in LQT1, with 21% having RyR2 mutations and 5% LQT2 mutations. Coupled with the findings by Moss et al. (101), it could be suggested that understanding the underlying cause of LQTS in any individual could be used to improve survival by prevention and gene-specific therapy.

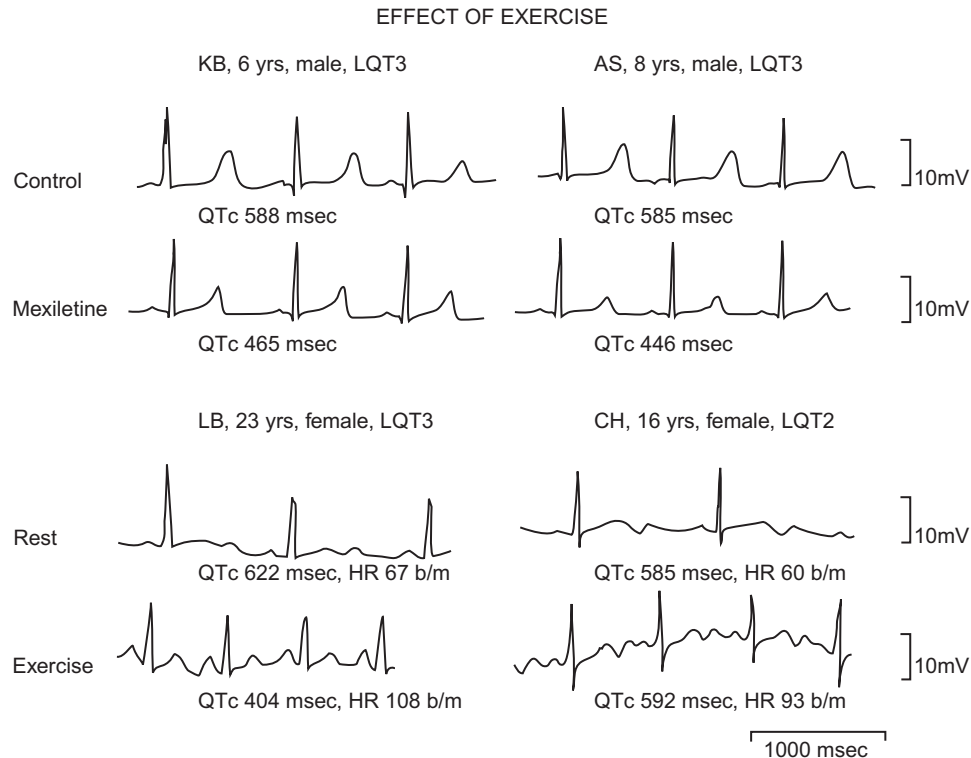
**51.3.1.6 Management of LQTS.** At present, there are four classical modalities for treatment of LQTS that have withstood the test of time: (i)  $\beta$ -blockers (104–107), (ii) pacemakers (108–110), (iii) left cervicothoracic sympathetic ganglionectomy (24,111,112), and,

most recently, (iv) internal cardioverter–defibrillator (ICD) (113,114). The mortality of untreated symptomatic patients with LQTS exceeds 20% in the year after their first syncopal episode and approaches 50% within 10 years of initial presentation (115). With institution of the classical therapy, this can be reduced to 2–4% in 5 years after initial presentation (105,115). Despite the lack of a placebo-controlled, randomized clinical trial, strong evidence supports the use of antiadrenergic interventions as the mainstay of therapy for most patients. The trigger for many life-threatening events appears to involve sudden increases in sympathetic activity (i.e. emotional or physical stress), and therefore, antiadrenergic therapy makes physiologic sense. The  $\beta$ -adrenergic blocking agents prevent new syncopal episodes in approximately 65–75% of patients (105). Suppression of complex ventricular arrhythmias (i.e. couplets and VT) seems desirable. Villain et al. (107) showed that a low incidence of cardiac events occurs in children with LQTS treated with  $\beta$ -blockers. In the 122 children studied, only four deaths occurred. Of the remainder, 111 children (92%) were treated with  $\beta$ -blockers alone, with no deaths and only five nonfatal cardiac events (4.5%) noted. None of these children had LQT1 mutations. Priori et al. (106) studied 335 patients treated with  $\beta$ -blockers for an average of 5 years and found 10% of LQT1 patients, 23% of LQT2 patients, and 32% of LQT3 patients had events while being treated, with the highest risk of events in LQT2 and LQT3 patients. Chatrath et al. (116) found that 25% of LQTS probands had cardiac events, with LQT1 most prevalent. In addition, they found that the highest rate of events occurred during treatment with atenolol, while propranolol appeared more protective. The addition of an IB agent (mexilitine) to  $\beta$ -blocker therapy may be helpful, particularly in patients with the LQT3 genotype (117). High-risk patients with drug-resistant, symptomatic VT are referred for left cardiac sympathetic denervation, which apparently provides additional protection (24,111,112). In addition, the use of cardiac pacing as an adjunct to  $\beta$ -blockers appears to be most rational in patients with evidence of pause-dependent or bradycardia-dependent arrhythmias (108–110). Symptomatic bradycardia due to LQTS or induced by  $\beta$ -blocker therapy should also be considered an indication for elective pacing.  $\beta$ -Blocker therapy is monitored with treadmill exercise testing, with the desired result a blunting of the heart rate response to exercise. However, Kaltman et al. (118) suggested that little difference occurs between pretreated and treated patients regarding QTc at any phase, QTc dispersion, or other QTc measures. Unfortunately, in some patients, this comes at the expense of excessive sinus bradycardia at rest and with minimal levels of exertion. Excessive fatigue, inattentiveness, and irritability may result in discontinuance of therapy by the patient. Compliance, especially

in the adolescent population, may be enhanced by returning the patient to a relatively normal lifestyle by the elimination of chronotropic incompetence with a pacemaker.

Other less time-tested therapies are also available. In some rare cases, torsade de pointes persists despite therapy with the classical modalities. The ICD has been used successfully in this setting (119,120). Until recently, it had not been considered to be the first-line therapy because shocks from the device can precipitate further emotional stress and set off a circuitous response of persistent malignant arrhythmias. However, the Multicenter Automatic Defibrillator Implantation Trial (MADIT), which demonstrated dramatic superiority of therapy with automatic implantable defibrillators over “best conventional therapy” in patients with coronary disease at high risk for ventricular arrhythmias (121), has made this therapeutic approach somewhat appealing. Automatic implantable defibrillators have been used more commonly in LQTS patients because of the results of the MADIT trial. However, whether this is the best approach is still not clear, and long-term data are needed to determine the answer to this question. Zareba et al. (114) have reported a 3-year follow-up analysis with 1.3% death in ICD patients versus 16% in non-ICD patients. Reports of efficacy in neonates have been published as well (113).

Another new approach to treating patients with LQTS is the so-called gene-specific approach. With the identification of the precise molecular defect in some patients with LQTS, specific mechanism-based therapies have been devised and small therapeutic trials were performed. Schwartz et al. (117) were the first to use this approach when they used the sodium-channel blocker mexilitine in patients with mutations in the sodium-channel gene *SCN5A* (LQT3). In these patients, the QTc was dramatically shortened in a statistically significant manner (Figure 51-5). Patients with potassium-channel mutations (HERG, LQT2) treated with mexilitine had no change in the QTc. However, no data currently exist that demonstrate clinical efficacy of this approach in either decreasing the number of syncopal events or improving the survival. Other sodium-channel blockers have had similar effects (27). Recently, Benhorin et al. (70) showed that flecainide shortened the QTc in patients with a D1790G *SCN5A* mutation while lidocaine was ineffective, suggesting that allele-specific therapies may be needed. Although the data are intriguing, use of sodium-channel blockers alone for patients with *SCN5A* mutations should still be considered experimental. Other gene-specific trials have also been performed with similar results. Compton et al. (122) used intravenous potassium to elevate the serum potassium to  $>4.8$  mEq/L in patients with LQT2 and found significant shortening of the QTc. Again, no data on survival or symptom improvement exist for this therapy, but this result has been confirmed (123). Etheridge et al. (124) showed that QTc reduction



**FIGURE 51-5** Mexiletine, a sodium channel blocker, shortens the QTc in two children with extreme QTc prolongation (>580 ms) and an *SCN5A* mutation.

may be large, and QT dispersion and T wave morphology improved, but outcome data were not available. Potassium-channel openers may have the same effect (125). Other gene-specific therapeutic approaches are currently being developed.

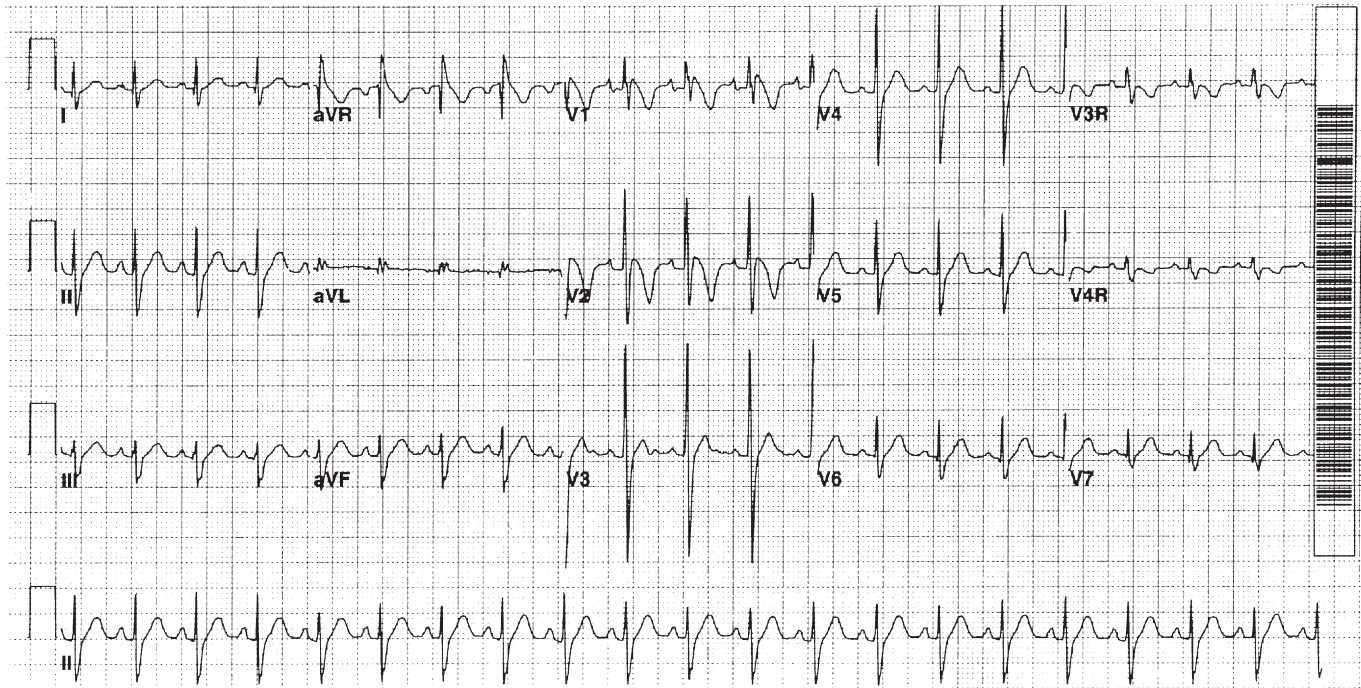
### 51.3.2 Brugada Syndrome

**51.3.2.1 Clinical Features.** This disorder is characterized by ST segment elevation in the right precordial leads ( $V_1$ – $V_3$ ) with or without right bundle-branch block (RBBB) (Figure 51-6) and episodic VF (Figure 51-7) (126). The first identification of the ECG pattern of RBBB with ST elevation in leads  $V_1$ – $V_3$  was reported by Osher and Wolff (127). Shortly thereafter, Edeiken (128) identified persistent ST elevation without RBBB in 10 asymptomatic males, and Levine et al. (129) described ST elevation in the right chest leads and conduction block in the right ventricle in patients with severe hyperkalemia. The first association of this ECG pattern with sudden death was described by Martini et al. (130) and later by Aihara et al. (131), and further confirmed in 1991 by Pedro and Josep Brugada (132), who described four patients with sudden death and aborted sudden death with ECGs demonstrating RBBB and persistent ST elevation in leads  $V_1$ – $V_3$ . In 1991, these authors characterized what they believed to be a distinct clinical and ECG syndrome (132,133). In some patients, the surface ECG appears normal, even in

familial cases. Provocation studies in the catheterization laboratory using ajmaline, flecainide, or procainamide will result in ST segment elevation in the right precordial leads in affected patients (134–137).

The finding of ST elevation in the right chest leads has been observed in a variety of clinical and experimental settings and is not unique or diagnostic of Brugada syndrome by itself. Situations in which these ECG findings occur include electrolyte or metabolic disorders, pulmonary or inflammatory diseases, and abnormalities of the central or peripheral nervous system. In the absence of these abnormalities, the term *idiopathic ST elevation* is often used and may identify Brugada syndrome patients (4). Two consensus conferences have been held to define the clinical signs, diagnostic approaches, therapies, and diagnostic studies (138–140).

The ECG findings and associated sudden and unexpected death have been reported as a common problem in Southeast Asia, where it most commonly affects men during sleep (141). This disorder, known as sudden unexplained death syndrome (SUDS) or sudden unexplained nocturnal death syndrome (SUNDS), has many names in Southeast Asia, including bangungut (to rise and moan in sleep) in the Philippines; non-laitai (sleep-death) in Laos; lai-tai (died during sleep) in Thailand; and pokkuri (sudden and unexpectedly deceased phenomena) in Japan (141,142). General characteristics of SUDS include young, healthy males in whom death occurs suddenly with a groan, usually during sleep late



**FIGURE 51-6** Brugada syndrome in a 4-year-old. Note the right ventricular conduction delay and ST segment elevation in leads  $V_1$  and  $V_2$ . Procainamide infusion worsened the ST elevation.

at night. No precipitating factors are identified, and autopsy findings are generally negative (143). Life-threatening ventricular tachydysrhythmias as a primary cause of SUDS have been demonstrated, with VF occurring in most cases (144).

**51.3.2.2 Brugada Syndrome and Arrhythmogenic Right Ventricular Dysplasia.** Controversy exists concerning the possible association of Brugada syndrome and arrhythmogenic right ventricular dysplasia (ARVD), with some investigators arguing that these are the same disorders or that at least one is a forme fruste of the other (145–150). However, the classical echocardiographic, angiographic, and magnetic resonance image (MRI) findings of ARVD are not seen in Brugada syndrome patients. In addition, Brugada syndrome patients typically are without the histopathologic findings of ARVD. Further, the morphology of VT/VF differs as noted in Table 51-2.

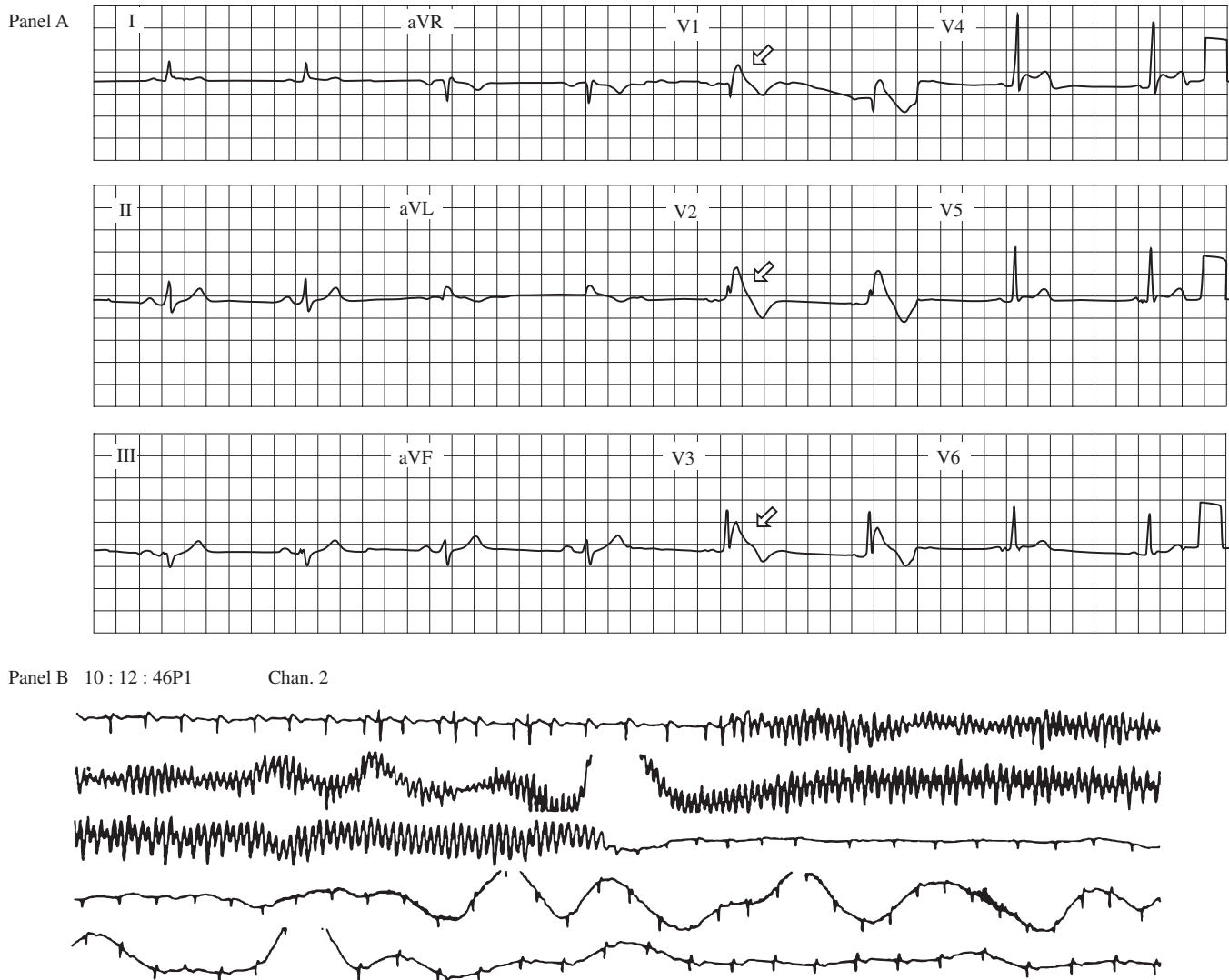
**51.3.2.3 Clinical Genetics.** Most of the families identified to date with Brugada syndrome have autosomal-dominant inheritance (151–154). It appears as if penetrance is reduced, as many patients have a normal ECG and no symptoms until provocation studies are performed or fever unmasks the ECG abnormalities (155,156). Although the number of families reported has been small, it is likely that this is due to underrecognition as well as premature and unexpected death.

**51.3.2.4 Molecular Genetics.** In animal studies, blockade of the calcium-independent 4-aminopyridine-sensitive transient outward potassium current ( $I_{to}$ ) results in surface ECG findings similar to that seen in Brugada

syndrome and includes elevated, downsloping ST segments (4), and occurs because of greater abbreviation in the epicardial action potential compared with the endocardium (which lacks a plateau phase). Loss of the action potential plateau (or dome) in the epicardium but not the endocardium would be expected to cause ST segment elevation and, because loss of the dome is caused by an outward shift in the balance of currents active at the end of phase I of the action potential (principally  $I_{to}$  and  $I_{Ca}$ ), autonomic neurotransmitters such as acetylcholine facilitate loss of the action potential dome by suppressing calcium current and augmenting potassium current, whereas  $\beta$ -adrenergic agonists (i.e. isoproterenol, dobutamine) restore the dome by augmenting  $I_{Ca}$ . Sodium-channel blockers also facilitate loss of the canine right ventricular action potential dome as a result of a negative shift in the voltage at which phase I begins (4,157). On the basis of this information, candidate genes ( $I_{to}$ ,  $I_{Ca}$ , and  $I_{Na}$ ) were selected for the study.

In 1998, our laboratory reported the findings on six families and several sporadic cases of Brugada syndrome (71) (Table 51-2). The families were initially studied by linkage analysis using markers to the known ARVD loci, and linkage was excluded. Candidate gene screening was performed and *SCN5A* was chosen for study, based on physiologic speculation (4). In three families, mutations in *SCN5A* (Figure 51-8) were identified (71). Since then, many groups have confirmed these findings and mutations have been identified throughout the channel topography. A second locus on chromosome 3 at 3p22–25 was





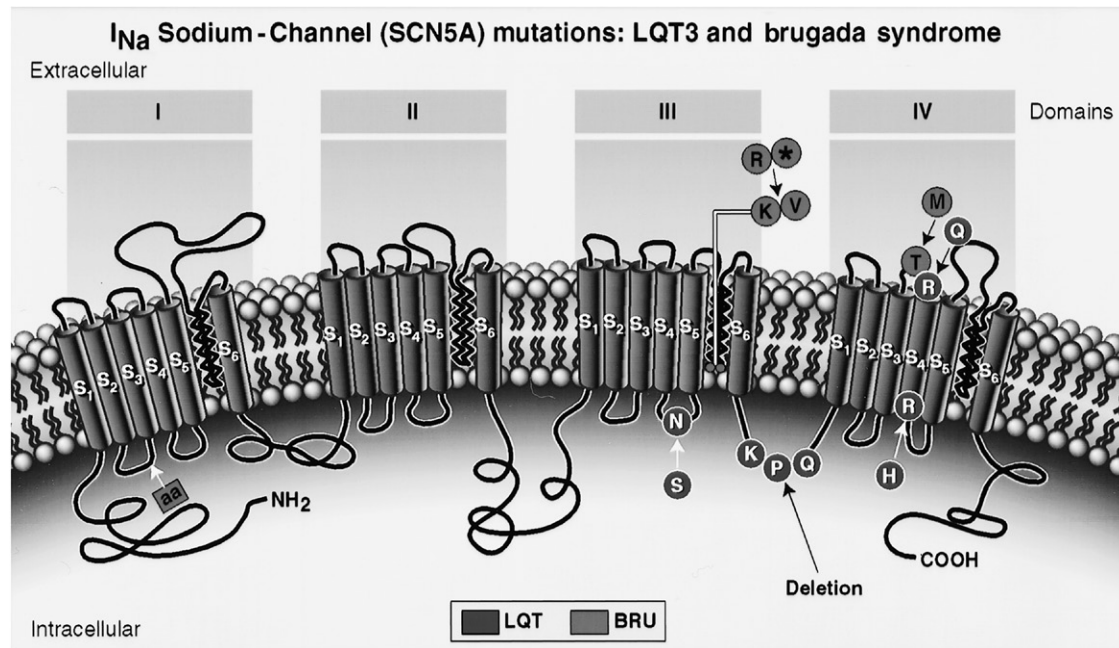
**FIGURE 51-7** Ventricular fibrillation in a patient with Brugada syndrome.

subsequently identified (158), but the gene has remained elusive.

In our original study, biophysical analysis of the mutants in *Xenopus* oocytes demonstrated a reduction in the number of functional sodium channels in some mutations, which promoted development of reentrant dysrhythmias, while in other mutations, sodium channels recovered from inactivation more rapidly than normal and were temperature dependent (155,156). In this case, the presence of both normal and mutant channels in the same tissue would be expected to promote heterogeneity of the refractory period, a well-established mechanism causing dysrhythmias. Inhibition of  $I_{Na}$  causes heterogeneous loss of the action potential dome in the right ventricular epicardium, leading to a marked dispersion of depolarization and refractoriness, an ideal substrate for development of reentrant dysrhythmias. Phase 2 reentry produced by the same substrate is believed to provide the premature beat necessary for initiation of the VT and VF responsible for symptoms

in these patients. Interestingly, however, Kambouris et al. (159) identified a mutation (R1623H) in essentially the same region of *SCN5A* as one of the original (T1620M) mutations, but the clinical and biophysical features of this mutation were found to be consistent with LQT3 and not Brugada syndrome. The same authors also demonstrated these mutant channels to have a propensity to inactivate without ever opening (i.e. closed-state inactivation), suggesting novel physiologic mechanisms can occur in a heterogeneous manner in similar regions of this ion channel (160). In some cases, LQT3 and Brugada syndrome occur in the same family (161,162).

**51.3.2.5 Therapy.** No good medical therapy has been clearly identified for these patients thus far. Belhassen et al. (163,164) have argued that quinidine has a role in chronic therapy for these patients, but this is based on a small number of clinical vignettes. In addition, Glatter et al. (165) suggested that sotalol may be effective. Currently, implantation of an ICD is the therapy of choice,



**FIGURE 51-8** Mutations in *SCN5A* in patients with Brugada syndrome versus those with long-QT syndrome.

but this remains controversial. The prognosis of these patients appears to be good (166). Ajiro et al. have suggested that late potentials on signal-averaged ECG irrespective of mutation (167). This is not confirmed by others (168,169).

### 51.3.3 Sudden Unexpected Nocturnal Death Syndrome

**51.3.3.1 Clinical Features.** SUNDS is a disorder that occurs in Southeast Asia, particularly Thailand, Cambodia, and the Philippines, as well as in Japan. The clinical features of this disorder are highlighted by the flamboyant names given this disease in the various Asian countries: lai-tai (died during sleep) in Thailand, pokkuri (sudden unexpected death at night) in Japan, and bangungut (moaning and dying during sleep) in the Philippines. SUNDS is the most common cause of “natural” death in these countries, typically occurring during sleep in males (141,142). The clinical features include an abnormal surface ECG with ST segment elevation in the right precordial leads (V<sub>1</sub>–V<sub>3</sub>), with or without RBBB. VF leads to the clinical symptoms of syncope, sudden death, and resuscitated sudden death. SUNDS is an allelic form of Brugada syndrome as the gene responsible for SUNDS is the same as the Brugada syndrome gene (Table 51-2).

**51.3.3.2 Genetics and Management.** SUNDS has not been considered an inherited disorder, but we have identified some families in which SUNDS is inherited as an autosomal dominant trait with mutations in *SCN5A*, the cardiac sodium-channel gene (170). The genetic findings have been confirmed by others (142,171). It is not currently clear why SUNDS afflicts men almost exclusively,

but it is certain that this is not transmitted in an X-linked manner, as male-to-male transmission occurs.

The management of SUNDS depends on implantation of an ICD in order to rapidly treat any episodes of VF. No medical therapy has proven effective for this disorder, but if quinidine therapy “pans out” for Brugada syndrome, it is likely that it would be used for SUNDS as well. Another important step necessary for optimal therapy of SUNDS is the screening of all family members by surface ECG testing. It is possible that provocation tests, similar to those described in Brugada syndrome, may be useful, but this has not been proven thus far.

### 51.3.4 SQT Interval Syndrome

**51.3.4.1 Clinical Features.** In 2000, Gussak et al. (172) identified a new familial clinical syndrome characterized by an abbreviated QTc interval (<300 ms), predisposition to life-threatening arrhythmias, and a high rate of sudden death. AF may occur and, on electrophysiologic evaluation, short refractory periods may be identified. Age of onset of symptoms may be young (<1 year of age) and in some cases, the syndrome may be responsible for SIDS.

**51.3.4.2 Genetics of Short-QT Syndrome.** Short-QT (SQT) syndrome was initially shown by Gussak et al. (172) to have autosomal-dominant inheritance. Multiple families and sporadic cases have been reported (172–176). Mutations in three ion-channel genes (SQT1, *HERG/KCNH2*; SQT2, *KvLQT1/KCNQ1*; SQT3, *Kir2.1/KCNJ2*) have been reported to date (173,174,176). In the case of SQT1, mutations dramatically increased I<sub>Kr</sub>, leading to heterogenous abbreviation of action potential duration and refractoriness

and reduced the affinity of the channels to  $I_K$  blockers. Bellocq et al. (173) performed functional studies on the *KCNQ1* mutations that revealed a pronounced shift of the half-activation kinetics, which led to a gain of function of  $I_{Ks}$  and repolarization shortening. In the case of the SQT3-causing mutations in *KCNJ2*, whole-cell patch-clamp studies of this *Kir2.1* ( $I_{K1}$ ) demonstrated a larger outward  $I_{K1}$  than wild type and shifting of peak current, as well as acceleration of the final phase of repolarization leading to shortened action potential duration. Clinically, this leads to tall and asymmetrical T waves, a finding that appears to be distinct for SQT3 (176).

**51.3.4.3 Therapy in SQT Syndrome.** As sudden death occurs commonly in this disorder, defibrillator implantation is indicated. Gaita et al. (177) evaluated the role of medical therapy, testing QTc interval response to flecainide, sotalol, ibutilide, and quinidine, with only quinidine producing QTc prolongation (normalization). However, no definitive treatment approaches have been described to date.

### 51.3.5 Right Ventricular Outflow Tract Tachycardia

**51.3.5.1 Clinical Features.** This rare form of VT occurs in the setting of an apparently normal, healthy individual who develops repetitive monomorphic VT arising from the right ventricular outflow tract (RVOT) (178–180). These VTs are characterized by left bundle-branch block (LBBB) morphology with an inferior frontal plane axis, which may be either leftward or rightward directed. Unlike ARVD and arrhythmogenic right ventricular cardiomyopathy (ARVC), which are also associated with LBBB/inferior axis VT, the ECG in normal sinus rhythm is typically normal. VT episodes tend to be characterized by frequent salvos of nonsustained VT or are separated by short periods of normal sinus rhythm and may be entirely asymptomatic. In some cases, however, catecholamines or exercise may provoke sustained episodes of VT. O'Donnell et al. (181) studied 33 patients with RVOT and compared them to 17 patients with ARVD/ARVC. Structural abnormalities were uniform by MRI in the ARVD/ARVC group, whereas many RVOT patients also had abnormalities in MRI. Electrophysiologic study showed a triggered automatic basis in 97% of RVOT cases, whereas the ARVD/ARVC patients displayed reentry in more than 80% of cases. Tada et al. showed that 9% of RVOT originated from the free wall, with 91% from the RVOT septum (182).

**51.3.5.2 Genetics.** No genetic data exist regarding RVOT VT. It is not known how often genetic inheritance occurs or what type of inheritance pattern occurs. At the molecular level, no gene has been identified.

**51.3.5.3 Management.** In some cases, these tachycardias appear to be adenosine sensitive, terminating with an intravenous bolus of adenosine. In other cases, verapamil has been reported to terminate the VT. Implantation of

an ICD may be considered in symptomatic patients with recurrent VT refractory to medical therapies.

### 51.3.6 Familial VT/CPVT

**51.3.6.1 Clinical Features.** This inherited form of VT was first described in detail by Rubin et al. (183) and is another form of VT that occurs in the absence of structural heart disease. Clinically, these patients present with frequent runs of nonsustained VT at rest (Figure 51-9), which may decrease or extinguish with exercise (183,184). In some cases, salvos of nonsustained VT may be incessant, causing palpitations, dizziness, or syncope. The ECG is usually normal during sinus rhythm, with a normal QT interval. Electrophysiologic evaluation may not consistently show inducible VT in these patients, suggesting that the mechanism in this dysrhythmia is not reentry as with more common forms of VT. Instead, it is believed that this form of inherited dysrhythmia is due to enhanced automaticity (i.e. an increased rate of electrical firing of a ventricular myocyte) or triggering (i.e. resulting from secondary depolarizations that occur during or immediately after repolarization).

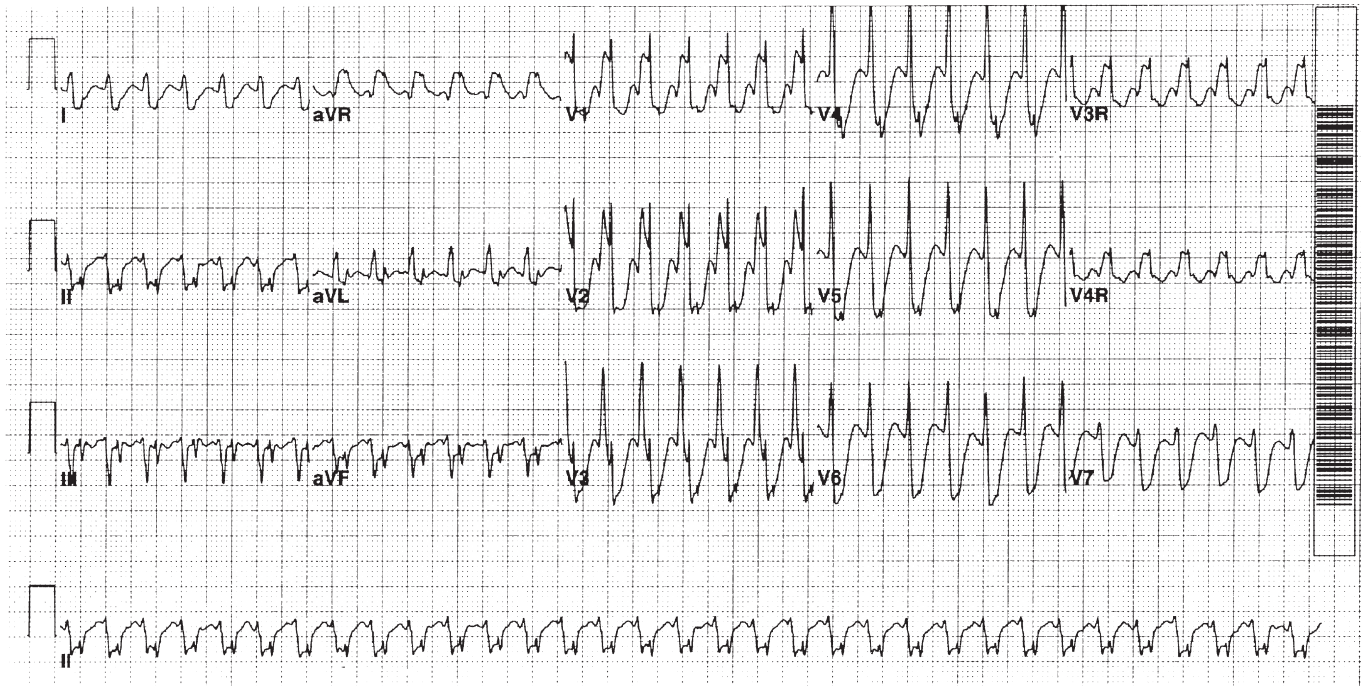
Sumitomo et al. (185) described the ECG characteristics of CPVT and the clinical features in detail. The initial manifestations included young age at presentation (mean age of onset, 10.3 years), syncope (79%), cardiac arrest (7%), and family history (14%). Electrocardiograms demonstrated sinus bradycardia and normal QTc. The CPVT morphology included polymorphic (62%), polymorphic and bidirectional (21%), bidirectional (10%), and polymorphic with VF (7%).

**51.3.6.2 Genetics and Management.** This inherited tachydysrhythmia is transmitted as an autosomal-dominant trait (Table 51-2) (186). Mutations in *RyR2* have been identified as causative in CPVT (41,187–189). In addition, mutations of the calsequestrin gene have been found in CPVT (162a,190,191); they appear to be relatively uncommon, but the incidence and prevalence are not known. The therapy of this disorder appears to rely on the use of  $\beta$ -adrenergic blocking agents, which effectively suppress this rhythm disturbance. However, Sumitomo et al. showed complete  $\beta$ -blocker control in only 31% of cases (185). In addition, the use of class I and class III antiarrhythmics have reportedly been successful in treating this disorder. Sumitomo et al. also showed that calcium-channel antagonists partially suppressed CPVT in some familial autosomal-dominant cases. The use of ICDs has not been reported in this disorder, but this is certainly a likely option in familial or difficult cases.

### 51.3.7 Bidirectional VT

**51.3.7.1 Clinical Features.** Bidirectional VT occurs as an acquired disorder as well as an inherited abnormality. The acquired form occurs secondary to digitalis intoxication





**FIGURE 51-9** Monomorphic ventricular tachycardia (200 beats/min).

(192), whereas the inherited form occurs in families (193–195). This latter form of VT occurs in the absence of drugs or structural heart disease. Clinically, this disease appears to be catecholamine sensitive (196–199), with increased adrenergic stimulation inducing polymorphic ventricular premature complexes followed by directional and polymorphic VT (196). Owing to this characteristic feature, the disorder has also been termed *catecholaminergic polymorphic VT*. Many patients present during the first decade of life with syncope or sudden cardiac death although in many cases, the individual may be asymptomatic. Bidirectional VT has a characteristic ECG appearance that includes VT with an RBBB morphology, alternation in the frontal plane QRS axis from  $-60^\circ$  to  $-80^\circ$  to  $+120^\circ$ , and a rate of 140–200 beats/min (200). This form of VT may be confused with a supraventricular rhythm arising from above the AV node, but several differentiating features are known. These include dissociation of the atria from the ventricles during tachycardia and the ability to identify the His bundle during tachycardia (195), which both occur with bidirectional VT.

**51.3.7.2 Genetics and Management.** This disorder appears to be transmitted as an autosomal-dominant trait with variable penetrance (Table 51-2). A family history of syncope or sudden death is reported in 30% of cases. The therapy for this disorder is not completely clarified, but it appears as if exercise or increase in atrial rate by atrial overdrive pacing (194,195) suppresses the VT. Recurrence of the dysrhythmia has been prevented by  $\beta$ -blockers (193). Fisher et al. (197) have reported 25 years of follow-up in patients with this form of VT in which medical therapy, predominantly  $\beta$ -blockers, was successfully based on serial exercise-pharmacologic

testing. In these patients, early identification of exercise- or catecholamine-induced dysrhythmias, as demonstrated on exercise stress tests predominantly, led to successful therapy.

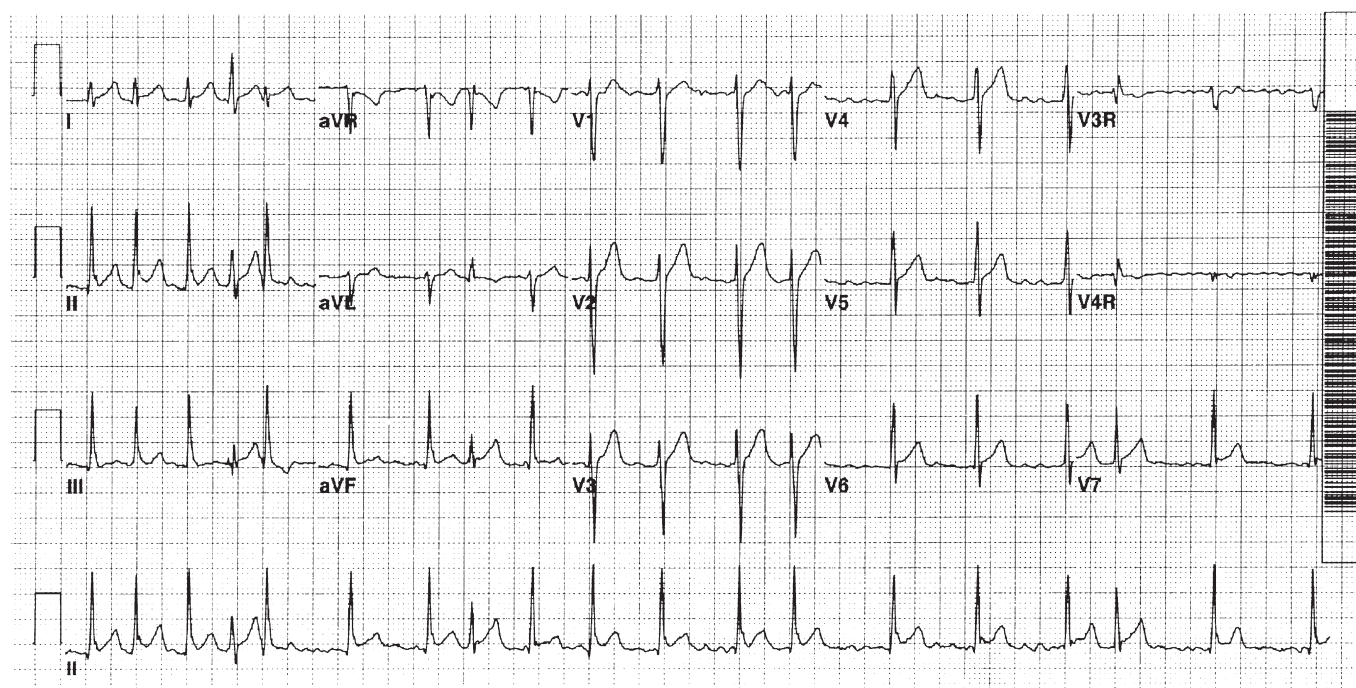
Swan et al. (201) initially mapped a gene for malignant polymorphic VT in two families with structurally normal hearts to chromosome 1q42–q43 (Table 51-2). Previously, based on our final common pathway hypothesis, we speculated that all ventricular tachyarrhythmia disorders would be due to ion-channel abnormalities (i.e. ion channelopathy) and suggested that identification of this gene would help to clarify this mystery. As noted, mutations in RyR2 were identified, and subsequently mutations in calsequestrin were also found.

## 51.4 PRIMARY ABNORMALITIES IN CARDIAC RHYTHM: SUPRAVENTRICULAR DYSRHYTHMIAS

### 51.4.1 Familial AF

**51.4.1.1 Clinical Features.** AF is considered the most common sustained dysrhythmia worldwide (Figure 51-10) and is believed to be the most common cause of embolic stroke, accounting for 75,000 strokes per year in the United States (202). Currently, there are more than 3 million cases of AF in the United States, with an overall prevalence of 0.5–1%, increasing to approximately 10% in individuals older than the age of 70 years. AF accounts for about one-third of strokes in people over the age of 65 years and leads to more hospital admissions than any other dysrhythmia (203). However, AF can also occur in children and in fetal life (204).





**FIGURE 51-10** Atrial fibrillation with its irregularly irregular rhythm and fibrillation pattern, which is well seen in the right chest leads (V3R, V4R) and rhythm strip.

**51.4.1.2 Genetics.** Familial AF was initially considered to be rare, but Brugada and Brugada (205) identified multiple families with familial AF from Spain and noted the penetrance to be high. Generally, the disease was found to be chronic, but some individuals presented with paroxysmal AF, some having palpitations. Left atrial dilation has developed in select individuals; syncope and dyspnea also occur in these patients. This disorder appears to be inherited as an autosomal-dominant trait, and many of these patients develop AF very early in childhood (204,206–208). Brugada et al. (152) identified the first locus for familial AF to chromosome 10q22–q24 (Table 51-2), and genetic heterogeneity was subsequently identified. The first gene for familial AF was found to be *KCNQ1*, the same gene causing LQT1 (209). This gene, located in chromosome 11p15.5, causes AF via gain-of-function mutations, in contrast to the dominant-negative or loss-of-function mutation that causes LQT1. In addition, *minK*, the other subunit associated with  $I_{Ks}$ , was shown by Lai et al. (210) to have a polymorphism (38G) that is associated with disease and possibly plays a role in control of AF pathogenesis. More recently, Yang et al. (211) identified mutations in *KCNE2/MiRP1* causing AF via a gain of function. Finally, a locus on chromosome 6q14–16 has been shown to be another AF locus (212).

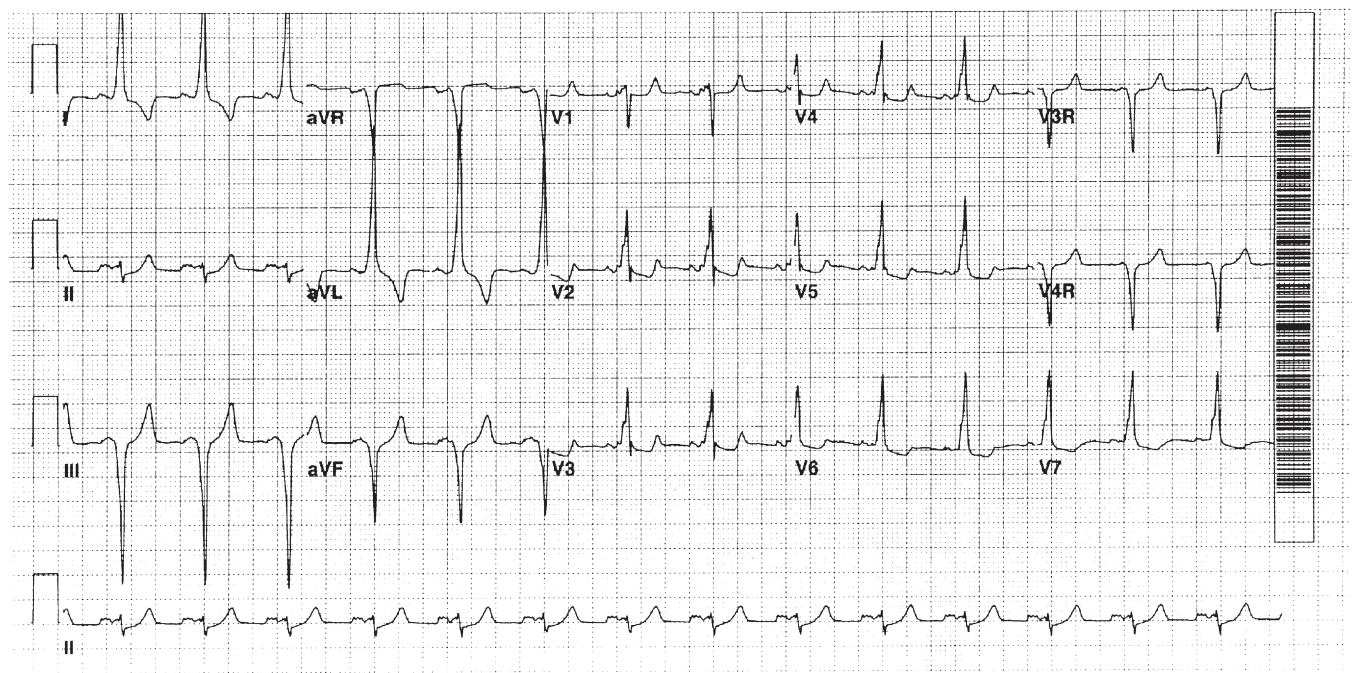
## 51.4.2 Wolff–Parkinson–White Syndrome

**51.4.2.1 Clinical Features.** In the normal heart, the atria and ventricles are electrically connected via a single pathway through the AV node. In Wolff–Parkinson–White (WPW) syndrome (213), an accessory AV

bypass pathway is present, creating the substrate for tachydysrhythmias (214). The electrical conduction properties of accessory pathways in WPW syndrome are more like those seen in ventricular myocardium than in AV nodal tissue and may conduct very rapidly with a short refractory period (215). During sinus rhythm, ventricular excitation occurs via the accessory pathway before excitation through the AV node, a process referred to as *preexcitation*. It is this preexcitation that is responsible for the characteristic ECG findings of a short PR interval and the so-called delta wave, a slurring of the upstroke of the QRS complex (Figure 51-11).

The most common dysrhythmia seen in patients with WPW syndrome is orthodromic AV reciprocating tachycardia (AVRT), which uses the AV node as the antegrade limb and the accessory pathway (i.e. bypass tract) as the retrograde limb (216). This tachydysrhythmia commonly causes palpitations, chest pain, dizziness and lightheadness, shortness of breath, and, in some cases, syncope. When sustained, AVRT may precipitate onset of AF, which under certain circumstances may be life-threatening because of its ability to rapidly conduct via the accessory pathway, inducing VF. These patients are, therefore, at increased risk of sudden death.

**51.4.2.2 Genetics and Management.** Although most cases of WPW syndrome are believed to be sporadic, autosomal-dominant inherited WPW syndrome is well described (217–219). The incidence of preexcitation in first-degree relatives has been reported to be <1%, with the prevalence in the general population thought to be approximately 0.15% (215,219). Notably, patients with multiple pathways have an increased prevalence



**FIGURE 51-11** Electrocardiogram of a 15-year-old female with Wolff–Parkinson–White syndrome. The electrocardiographic features include a short P–R interval and delta wave.

of preexcitation in first-degree relatives, approximately 1.7%. WPW syndrome may also occur in the setting of congenital heart disease, particularly Ebstein anomaly, or myocardial disease (i.e. hypertrophic cardiomyopathy [HCM]). It is not clear whether WPW in this circumstance has increased familial incidence. The permanent form of junctional reciprocating tachycardia (PJRT), in which a septal accessory pathway is present and has decremental properties similar to the AV node, appears to have familial inheritance as well but is probably uncommon. Neither pure WPW syndrome nor familial PJRT has been deciphered from a genetic standpoint. However, a form of WPW has been genetically identified.

In WPW syndrome families with WPW associated with left ventricular hypertrophy (LVH)/HCM, a gene was mapped to chromosome 7q3 (220), but the gene remained elusive until Gollob et al. (221) and Blair et al. (222) identified the AMP-kinase gene *PRKAG2* as causative (Table 51-2). This gene was cloned in 2000 (223) and was shown to act as a major regulator of cellular ATP levels and to protect cells against stresses that cause ATP depletion. Since its initial description, multiple mutations have been identified in patients with WPW with or without LVH (224–228). Murphy et al. (226) showed that 78% of patients with mutations had LVH, there was 91% survival at 12 years of follow-up, and progressive conduction disease occurred in 40%. Some patients also have skeletal myopathy. Murine models have been made that demonstrate this disease to be equivalent to a glycogen storage disease (224,229). Loss of function of the gene was also noted.

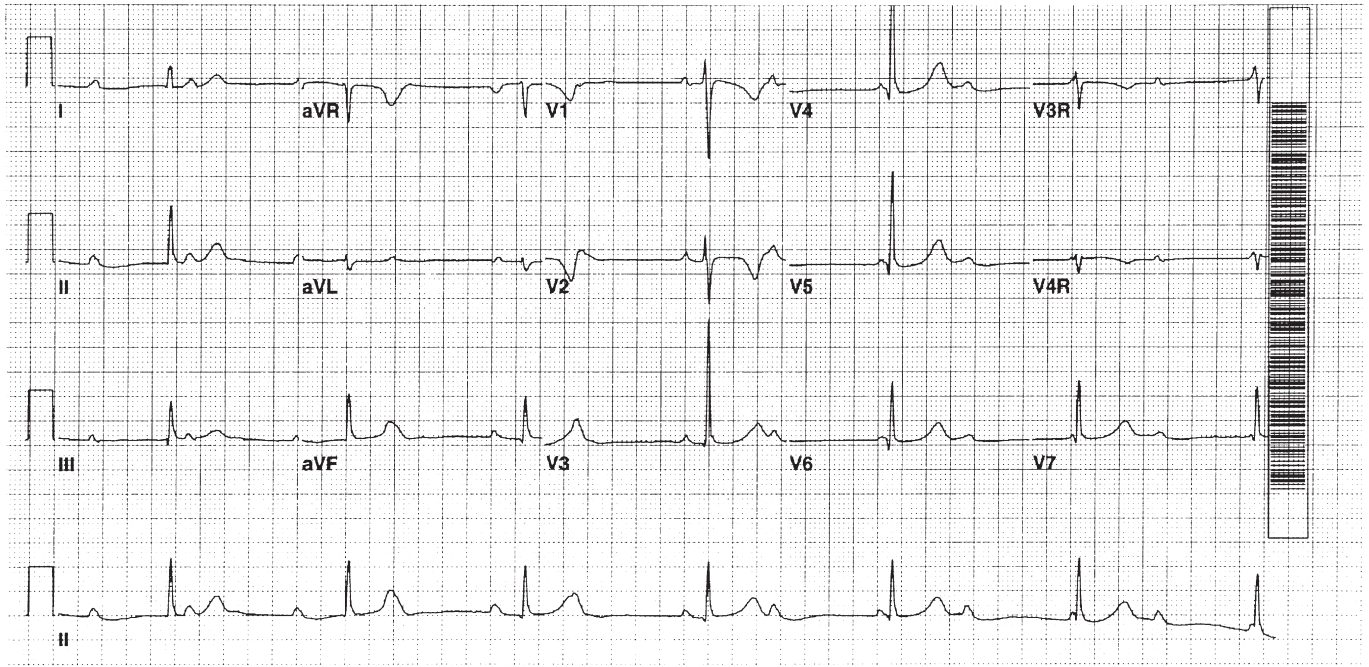
The current first-line therapy for WPW is radiofrequency ablation, which, in most cases, permanently disrupts the accessory pathway (230). In the small percentage of patients not undergoing radiofrequency ablation, medical therapy may be used.

## 51.5 PRIMARY CONDUCTION ABNORMALITIES

### 51.5.1 Lev-Lenegre Progressive Cardiac Conduction Disease

**51.5.1.1 Clinical Features.** This syndrome, also known as progressive cardiac conduction defect (PCCD), is among the most common cardiac conduction disorders in the world and represents the major cause for pacemaker implantation worldwide, at 0.15 pacemaker implantations per 1000 inhabitants per year in developed countries (72). PCCD is characterized by progressive worsening of cardiac conduction through the His–Purkinje system, resulting in RBBB or LBBB and widening of the QRS complex (231–233). Patients with this disorder ultimately develop complete AV block (Figure 51-14) and, in many instances, present with syncope or sudden death. Etiologically, PCCD has been considered to be a primary degenerative disease or an exaggerated age-related process, in which sclerosis of the conduction system occurs.

**51.5.1.2 Genetics.** The first gene for PCCD was mapped to chromosome 19q13.3 (Table 51-2) in families with autosomal dominantly transmitted disease (234,235). Genetic heterogeneity was identified when



**FIGURE 51-12** Complete atrioventricular block in an individual with bradycardia.

Schott et al. demonstrated linkage to chromosome 3p21 and identified mutations in the cardiac sodium-channel gene *SCN5A* (72) (Table 51-2). This gene had previously been shown to cause a form of LQTS (LQT3) as well as Brugada syndrome (63,64,71). Other supportive evidence of *SCN5A* mutations causing AV block have been reported. Miura et al. (236) identified *SCN5A* mutations in congenital LQT and 2:1 AV block, while Shirai et al. (237) showed that mutations in this gene may cause overlapping features of Brugada syndrome and cardiac conduction disease. Tan et al. (238) showed that mutations in *SCN5A* result in isolated conduction system disease as well, and this was confirmed by Wang et al. (239). In addition, Viswanathan et al. (240) showed that a common *SCN5A* polymorphism (H558R) mitigates the effects of mutations on channel function and the clinical response.

**51.5.1.3 Management.** Patients with PCCD require pacemaker therapy once bradycardia occurs. In cases in which syncope precedes diagnosis, immediate implantation is appropriate. Before pacemaker implantation, serial ECGs and Holter monitor studies are necessary for close monitoring.

### 51.5.2 Familial Complete Atrioventricular Heart Block

This disorder, when familial, presents with adult onset (age 20–50 years) and has an autosomal-dominant inheritance pattern (241–243). Approximately 50 families have been identified with this disorder and, in all cases, transmission is consistent with autosomal-dominant inheritance with full penetrance and variable

expression. Whether all these conditions represent a single disorder is not known. The common presentation of this disease includes one of the following: (i) RBBB alone, (ii) left axis deviation (LAD) alone, (iii) RBBB plus LAD, or (iv) complete heart block (Figure 51-12). In addition, AV block has been associated with dilated cardiomyopathy (DCM) and skeletal myopathy, and several genetic loci have been identified for those cases (Sections 51.6.5 and 51.7.3). As previously noted, another gene has been mapped to chromosome 19q13 in a family with AV block (Table 51-2) but without DCM (234), while the gene for Lev-Lenegr PCCD and for isolated conduction disease has been found to be the cardiac sodium-channel gene *SCN5A* (72,238,239) (Table 51-2).

### 51.5.3 Familial Total Atrial Standstill

**51.5.3.1 Clinical Features.** Atrial standstill is characterized by the complete absence of spontaneous atrial electrical activity and inability to pace the atria. In addition to loss of electrical activity, there is a loss of mechanical function of the atria documented by absence of A waves on jugular venous pulse and right atrial pressure tracing, and absence of atrial contraction as detected by echocardiography. Primary atrial standstill can occur as sporadic cases or in families. Familial cases have been reported in an isolated community having a high rate of inbreeding (244–246). Familial atrial standstill can be associated with other cardiac abnormalities. Ebstein anomaly (ventricular displacement of the tricuspid valve) has been reported in one family with persistent atrial standstill (247). Certain types of muscular dystrophy



(e.g. Emery–Dreifuss muscular dystrophy (EDMD)) and Kugelberg–Wielander syndrome can present with atrial standstill. These disorders are discussed in further detail below.

Atrial standstill and abnormal sinus node function are common dysrhythmias in familial cases of amyloidosis. Often other systemic effects of amyloidosis, such as peripheral neuropathy, cardiomyopathy, or renal dysfunction, are not seen in these cases. Histologic evidence of amyloid deposits may be identified in atrial or ventricular myocardium (248).

The clinical symptoms of atrial standstill, whether primary or secondary to a systemic disease, may be due to absence of atrial activity or to intermittent atrial tachydysrhythmias. Patients may present with bradycardia due to loss of atrial activity or with palpitations, presyncope, dyspnea, or chest pain as a result of atrial tachydysrhythmias. A high rate of unexplained sudden death has been reported (244,246). Loss of atrial mechanical function may predispose to the development of atrial thrombi, and cerebral and peripheral emboli are reported in up to 30% of cases (244,247).

**51.5.3.2 Genetics and Management.** The mode of inheritance is not known with certainty as there are few reports of large affected families, but it is most likely autosomal dominant (244). No gene has been identified thus far (Table 51-2). Treatment consists of permanent ventricular pacing for symptomatic bradycardia and chronic anticoagulation to prevent formation of atrial thrombi.

### 51.5.4 Sinus Node Dysfunction/Sick Sinus Syndrome

**51.5.4.1 Clinical Features.** Congenital absence of sinus rhythm (249) or sinus node dysfunction (250,251) occurs in familial forms. The clinical presentation is generally due to symptomatic bradycardia, but it also may be associated with paroxysmal AF and other atrial tachydysrhythmias. Histologic data are limited, but one report of a single member of a family who suffered sudden death showed mononuclear cell infiltration of the sinus node, fibrosis of the sinus and AV nodes and atrial tissue, and atrophy of the right and left bundle branches (252).

**51.5.4.2 Genetics and Management.** The inheritance pattern of familial congenital absence of sinus node function is autosomal dominant with a high degree of penetrance (Table 51-2). The gene for this disorder remains unknown. However, Benson et al. (73) identified homozygous mutations in the cardiac sodium-channel gene *SCN5A* in individuals with sick sinus syndrome.

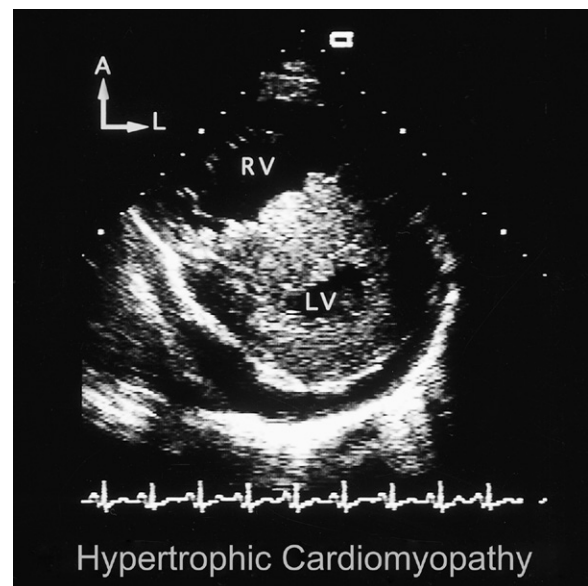
Treatment consists of permanent pacing for symptomatic bradycardia, and, unlike persistent atrial standstill, in which the atria are unable to be paced because of inability to produce electrical excitation, atrial or dual-chamber pacing may be used. If AF is a prominent

feature, antiarrhythmic medications and/or chronic anticoagulation may be indicated.

## 51.6 FAMILIAL DYSRHYTHMIAS ASSOCIATED WITH MYOCARDIAL DISEASE

### 51.6.1 Hypertrophic Cardiomyopathy

HCM is characterized by myocardial hypertrophy with a wide spectrum of symptoms, including dyspnea, chest pain, and syncope (253). The annual mortality rate was initially reported to be 2–4%, primarily due to sudden death and often occurring in asymptomatic individuals, but is now believed to be 0.1% per year. This disorder is considered to be the leading cause of sudden death in the young and in athletes (254). The annual incidence of sudden death is higher in younger patients with familial HCM than in the elderly. The diagnosis is based on typical clinical features and the demonstration of unexplained left ventricular, right ventricular, or biventricular hypertrophy on two-dimensional echocardiography. The LVH is commonly asymmetrical, localized to the septum, but may involve the entire ventricle in a concentric pattern (Figure 51-13). Isolated right ventricular hypertrophy occurs in fewer than 5% of cases. Isolated apical hypertrophy is rare except in Japan, where it is claimed to account for 20–30% of the cases, or in elderly patients. Dynamic outflow tract obstruction occurs in about 30% of patients. Histologically, the myocardial hypertrophy consists of myocyte hypertrophy, cellular and myofibrillar disarray, and myocardial fibrosis, and one of the hallmarks of familial HCM is myocyte and myofibrillar disarray (255).



**FIGURE 51-13** Echocardiogram demonstrating hypertrophic cardiomyopathy with left ventricular posterior wall and interventricular septal hypertrophy. LV, left ventricle; RV, right ventricle.



**51.6.1.1 Mapping of *FHC* Genes.** The first gene for familial hypertrophic cardiomyopathy (FHC) was mapped to chromosome 14q11.2–q12 using genome-wide linkage analysis in a large Canadian family (256). Soon after, FHC locus heterogeneity was reported (257–259) and subsequently confirmed by Watkins et al. when they mapped the second FHC locus to chromosome 1q3 (260), and by Thierfelder et al. (261), who mapped the third locus to chromosome 15q2 (261) (Table 51-3). Carrier et al. (262) mapped the fourth FHC locus to chromosome 11p11.2. Five other loci were subsequently reported, located on chromosomes 7q3 (220), 3p21.2–3p21.3, (263), 12q23–q24.3 (264), 15q14 (265), and 2q31 (266) (Figure 51-14). Several other families were not linked to any known FHC loci, indicating the existence of additional FHC-causing genes. These genes have been identified in patients presenting late in childhood or adulthood and not in neonates, infants, and very young children.

**51.6.1.2 Gene Identification in FHC.** Most of the disease genes encode proteins that are part of the sarcomere (i.e. a sarcomyopathy), which is a complex structure with an exact stoichiometry and multiple sites of protein–protein interactions (267,268). The encoded proteins (Figure 51-16) include three myofilament proteins,  $\beta$ -myosin heavy chain (269,270) and ventricular myosin essential light chain 1 and ventricular myosin regulatory light chain 2 (263); four thin filament proteins, cardiac actin (265), cardiac troponin T (267), cardiac troponin I (264), and  $\alpha$ -tropomyosin (267); and finally one myosin-binding protein, the cardiac myosin-binding protein C (271,272). Titin and muscle LIM proteins, both Z-disk proteins, were described as well (266) (Table 51-3). Each of these proteins is encoded by multigene families that exhibit tissue-specific, developmental, and physiologically regulated patterns of expression. More recently, mutations in the *PRKAG2* gene encoding AMP-kinase on chromosome 7q3 (222,273), and the *LAMP-2* gene on Xq28 have been shown to be metabolic causes of LVH (224).

### 51.6.1.3 Thick-Filament Proteins (Figure 51-14).

**51.6.1.3.1 Myosin Subunits.** Myosin is the molecular motor that transduces energy from the hydrolysis of ATP into directed movement and that, by doing so, drives sarcomere shortening and muscle contraction. Cardiac myosin consists of two heavy chains (MyHC) and two pairs of ventricular light chains (MLC), referred to as essential (or alkali) light chains (MLC-1) and regulatory (or phosphorylatable) light chains (MLC-2) (274). The myosin molecule is highly asymmetrical, consisting of two globular heads joined to a long rod-like tail. The light chains are arranged in tandem in the head–tail junction. Their function is not fully understood. Neither myosin light chain type is required for the adenosine triphosphatase (ATPase) activity of the myosin head, but they probably modulate it in the presence of actin and contribute to the rigidity of the neck, which is hypothesized to function as a lever arm for

generating an effective power stroke. Mutations have been found in the heavy chains and in the two types of ventricular light chains.

Concerning the heavy chains, the  $\beta$  isoform ( $\beta$ -MyHC) is the major isoform of the human ventricle and of slow-twitch skeletal fibers. It is encoded by *MYH7*. At least 50 mutations have been found in unrelated families with FHC, and three hot spots for mutations have been identified, including codons 403, 719 and 741. All but three of these mutations are missense mutations located in the head or in the head–rod junction of the molecule (275). The three exceptions are two 3 bp deletions that do not disrupt the reading frame (codon 10, codon 930) and a 2.4 kb deletion in the 3' region. In the kindred with the latter mutation, only the proband developed clinical evidence of HCM, and in this case, it occurred at a very late age of onset (59 years).

Animal models were initially reported by Vikstrom et al. (276) and Geisterfer-Lowrance et al. (277). The mice produced by these groups developed HCM-like disease. Heterozygotes have been produced with the Arg403Gln mutation and have been shown to have normal survival, whereas homozygotes had premature death, mimicking the human mutations. Fatkin et al. (278) demonstrated neonatal cardiomyopathy in homozygotes. In addition, Bevilacqua et al. (279) demonstrated QT dispersion in these animals, whereas Berul et al. (280) showed gender differences in the electrophysiologic abnormalities seen in these animals.

As for the light chains, the isoforms expressed in the ventricular myocardium and in the slow-twitch muscles are the so-called ventricular myosin regulatory light chain (MLC-2s/v), encoded by *MYL2*, and the ventricular myosin essential light chain (MLC-1s/v), encoded by *MYL3*. They both belong to the superfamily of EF-hand proteins. Two missense mutations have been reported in *MYL3* and five in *MYL2*.

**51.6.1.3.2 Myosin-Binding Protein C.** Myosin-binding protein C (MyBP-C) is a part of the thick filaments of the sarcomere, being located at the level of the transverse stripes, 43 nm apart, seen by electron microscopy in the sarcomere A band. Its function is uncertain, but it is believed to play both structural and regulatory roles. Partial extraction of the cardiac isoform (cMyBP-C) from rat-skinned cardiac myocytes and rabbit skeletal muscle fibers has been shown to alter  $\text{Ca}^{2+}$ -sensitive tension (281). In addition, the phosphorylation of cMyBP-C was shown to alter myosin cross-bridges in native thick filaments, suggesting that cMyBP-C can modify force production in activated cardiac muscles.

cMyBP-C is encoded by the *MYBPC3* gene (272,282), which has three distinct regions that are specific to the cardiac isoform: the  $\text{NH}_2$ -terminal domain C0 immunoglobulin I (IgI), containing 101 residues; the MyBP-C motif, a 105-residue stretch linking the C1 and C2 IgI domains; and a 28-residue loop inserted in the C5 IgI domain (283). It was later shown that cMyBP-C is

**TABLE 51-3 Familial Disorders of Cardiac Structure Associated with Dysrhythmias**

Disease	Rhythm Abnormality	Inheritance	Chromosome Location	Gene
<b>Ventricular arrhythmias</b>				
Hypertrophic cardiomyopathy	VT, VF	AD	1q3, 2q31, 3p21.2, 7q3, 11p11.2, 11p15.1, 12q23, 14q11, 15q14, 15q2, 19p12	Cardiac troponin T (1q3), titin, myosin essential light chain, myosin regulatory light chain, $\beta$ -myosin heavy chain, $\alpha$ -actin, MLP, $\alpha$ -tropomyosin, cardiac troponin I
ARVD/cardiomyopathy	VT, VF	AD	1q42–43, 2q32, 3p23, 6p24, 10p12–14, 10p22, 12p11, 14q12–22, 14q24.3	RyR2, desmoplakin, plakophilin-2
ARVD/cardiomyopathy with myofibrillar myopathy	VT, VF	AD	10q22.3	?
Familial palmoplantar keratosis	VT, IVCD	AR	17q21	Plakoglobin with ARVD (Naxos syndrome)
Familial palmoplantar keratosis	VT, IVCD	AR	6p24	Desmoplakin with ALVD (Carvajal syndrome)
Dilated cardiomyopathy	VT	AD	1q32, 1q42, 2q31, 2q35, 5q33, 6q12, 6q22.1, 9q13, 9q22, 10q21, 10q22.3, 11p11, 11p15.1, 14q12, 15q14, 15q22 titin (2q31)	Desmin, $\delta$ -sarcoglycan, $\alpha$ -actin, titin, $\beta$ -myosin heavy chain, $\alpha$ -tropomyosin, MLP, $\alpha$ -actinin-2, ZASP, Phospholamban
Dilated cardiomyopathy	VT	X-linked	Xp21	Dystrophin
Mitral valve prolapse	AF, ?SD	AD	16p11.2–12.1	?
Barth syndrome	VT	X-linked	Xq28	G4.5 (tafazzin)
Left ventricular noncompaction	VT	X-linked	Xq28	G4.5 (tafazzin)
Left ventricular noncompaction	VT	AD	10q22.3, 11p15, 18q12	ZASP; $\alpha$ -dystrobrevin
<b>Supraventricular arrhythmias</b>				
Familial amyloidosis	Atrial standstill, AF	AD	TTR locus	Prealbumin (transthyretin)
<b>Conduction abnormalities</b>				
Idiopathic restrictive	AVB, VT	AD,AR	2q31, <sup>a</sup> TTR locus; 19p12.2	Desmin (2q31), prealbumin cardiomyopathy (transthyretin), troponin I
Familial amyloidosis	AVB	AD	TTR locus	Prealbumin (transthyretin)
Dilated cardiomyopathy with	AVB	AD	1p1–1q1, 3p22, 6q23	Lamin A/C, SCN5A conduction disease
Holt–Oram syndrome	AVB, AT	AD, X-linked	12q24.1	TBX-5
Familial atrial septal defect	AVB, AF, SD	AD, ?	5q35, 5 p	Nkx2-5
Noonan syndrome	AVB, BBB, IVCD	AD	12q24	PTPN11
LEOPARD syndrome	AVB, BBB, IVCD	AD	17, 12q24	Neurofibromin; PTPN11
Heterotaxy	AVB, SVT, AF	X-linked	Xp26	Zic3
Heterotaxy	AVB, SVT, AF	AD	—	NODAL, LEFTY A, LEFTY B, activin type IIB
<b>Neurologic disorders</b>				
<b>Muscular dystrophies</b>				
Duchenne	ST, AT, IVCD, VT	X-linked	Xp21	Dystrophin
Becker	AVB SVT, AF	X-linked	Xp21	Dystrophin
Limb-girdle	AVB	AR	2p13, 4q12, 5q33–34, 13q12, 15q15, 17q11–12, 17q21 <sup>a</sup>	Dysferlin, $\beta$ -sarcoglycan, $\delta$ -sarcoglycan, $\gamma$ -sarcoglycan, calpain-3, telethonin, $\alpha$ -sarcoglycan
Limb-girdle	AVB, VT	AD	1q11–21, 3p25, 5q31, 6q23	Lamin A/C, caveolin-3 (3p25), myotilin
Facioscapulohumeral	Atrial standstill, AF	AD	4q35, 10q	D4Z4
Emery–Dreifuss	Atrial standstill, AF	X-linked	Xq28	Emerin
Emery–Dreifuss	Atrial standstill, AF	AD	1p1–1q1	Lamin A/C
Myotonic dystrophy	AVB, VT	AD	19q13.3	Myotonin protein kinase

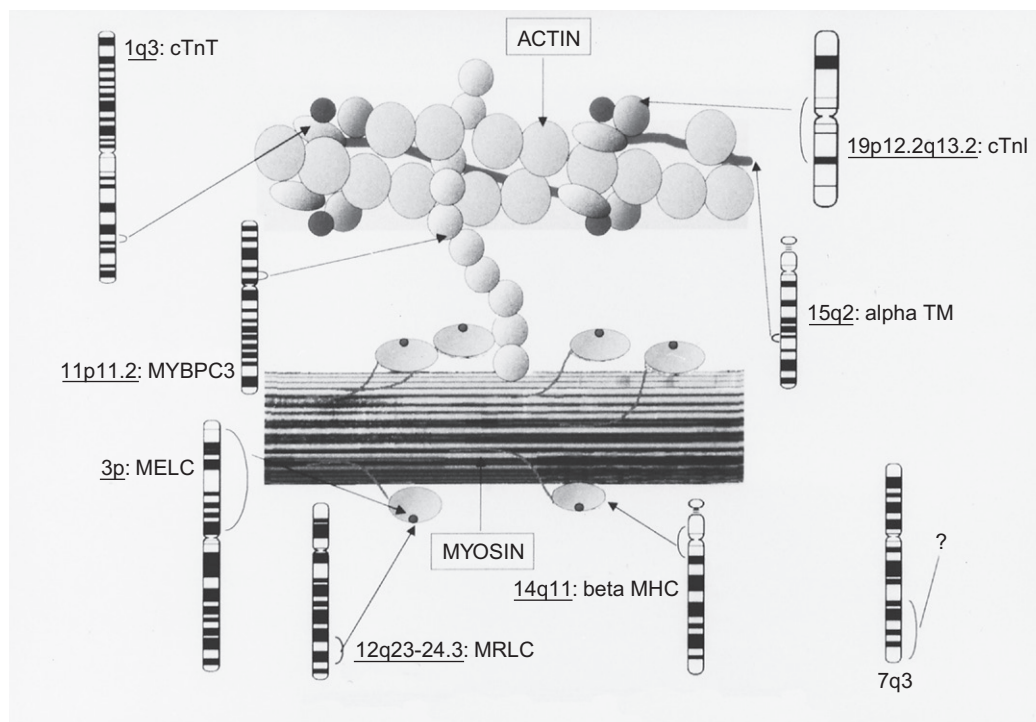
**TABLE 51-3 Familial Disorders of Cardiac Structure Associated with Dysrhythmias—cont'd**

Disease	Rhythm Abnormality	Inheritance	Chromosome Location	Gene
Other neurologic disorders				
Freiderich ataxia	Variable	AR	9q13–31.3	Frataxin
Kearns–Sayre syndrome	AVB	Mitochondrial	mtDNA	tRNA <sup>Leu</sup> (UUR)-3243
MELAS syndrome	AVB, VT	Mitochondrial	mtDNA	
MERRF syndrome	AVB, VT	Mitochondrial	mtDNA	
McArdle syndrome	SD, AVB	AR, AD	?	?
Kugelberg–Wielander syndrome	Atrial standstill, AF, AVB	AR	?	?

AD autosomal dominant; AF, atrial fibrillation; ALVD, arrhythmogenic left ventricular dysplasia; AR, autosomal recessive; ARVD, arrhythmogenic right ventricular dysplasia; AT, atrial tachycardia; AVB, atrioventricular block; BBB, bundle branch block; IVCD, intraventricular conduction delay; mtDNA, mitochondrial genome; SD, sudden death; ST, sinus tachycardia; SVT, supraventricular tachycardia; VF, ventricular fibrillation; VT, ventricular tachycardia.

<sup>a</sup>At least one other unknown.

## MOLECULAR GENETICS OF HCM



**FIGURE 51-14** Loci, genes, and proteins responsible for familial hypertrophic cardiomyopathy. All genes causing HCM encode proteins of the sarcomere.

specifically expressed in the heart during human and murine development (284,285).

Approximately 30 MYBPC3 mutations have been identified in unrelated families with FHC. In the majority of cases, the mutations result in aberrant transcripts that are predicted to encode COOH-terminal truncated cardiac MyBP-C polypeptides lacking at least the myosin-binding domain. Of the remaining reported mutations, mutated or deleted proteins occur without disruption of the reading frame; most of these are missense mutations (exons 6, 17, 21, and 23), but a splice donor site mutation (intron 27) and an 18-residue duplication (exon 33)

have also been reported (271,272,286–289). In addition, three mutations are predicted to produce either a mutated protein or a truncated one: two are missense mutations in exon 15 and 17 and the other is a branch-point mutation in intron 23. It has been suggested that most mutations result in a dominant-negative action of the mutant protein. Another possibility is defective processing and rapid degradation of partially glycosylated protein. This rapid degradation of a truncated protein may be a critical step preventing myofibrillogenesis, with filament formation (rather than the function of assembled filaments) being disturbed (286,290,291).

A mouse model was reported by McConnell et al. (292), in which mice expressing altered forms of *MYBPC3* were produced. The engineered mutations encoded truncated forms of MyBP-C in which the cardiac MyHC-binding and titin-binding domain were replaced with novel amino acid residues. Homozygous mice exhibited neonatal onset of a progressive DCM with myocyte hypertrophy, myofibrillar disarray, fibrosis, and dystrophic calcification on histopathology. Left ventricular dilation with reduced contractility was noted on echocardiography in the neonatal period, but myocardial hypertrophy and diastolic dysfunction developed later in the life of these animals. Previously, Yang et al. (293) reported a mouse model of HCM due to mutant MyBP-C, and this clinical phenotype was more similar to the human disorder. Here, however, the transgenic animals had very high levels of mutant gene (driven by the  $\alpha$ -MHC promoter) and diminished wild-type gene expression to very low levels.

**51.6.1.4 Thin-Filament Proteins (Figure 51-14).** The thin filament contains actin, the troponin complex, and tropomyosin. The troponin complex and tropomyosin constitute the  $\text{Ca}^{2+}$ -sensitive switch that regulates the contraction of cardiac muscle fibers. Mutations were found in  $\alpha$ -tropomyosin ( $\alpha$ -TM) (267) and in two of the subunits of the troponin complex: cardiac troponin I (cTnI), the inhibitory subunit, and cardiac troponin T (cTnT), the tropomyosin-binding subunit (264,267). Recently, actin mutations have also been reported (265).

$\alpha$ -TM is encoded by *TPM1*. The cardiac isoform is expressed both in the ventricular myocardium and in fast-twitch skeletal muscles (294). It shares the overall structure of other tropomyosins that are rod-like proteins that possess a simple dimeric  $\alpha$ -coiled-coil structure in parallel orientation along their entire length. Four missense mutations were initially described in unrelated FHC families. Two of them, A63V and K70T, are located in exon 2b within the consensus pattern of sequence repeats of  $\alpha$ -TM and could alter tropomyosin binding to actin. Mutations D175N and E180G are both located within constitutive exon 5, in a region near the C190 and near the calcium-dependent troponin T-binding domain. This appears to be a relatively rare cause of HCM. Michelle et al. (295) later demonstrated that the known  $\alpha$ -TM mutations rely on the calcium-sensitizing effects of these mutations to define the severity of disease.

cTnT is encoded by *TNNT2*. In human cardiac muscle, multiple isoforms of cTnT have been described that are expressed in the fetal, adult, and diseased heart, and that result from alternative splicing of the single gene *TNNT2* (296,297). The precise physiologic relevance of these isoforms is currently poorly understood, but the organization of the human gene has been partially established (298,299), thus allowing precise identification of the position of the mutations within exons, including those alternatively spliced during development.

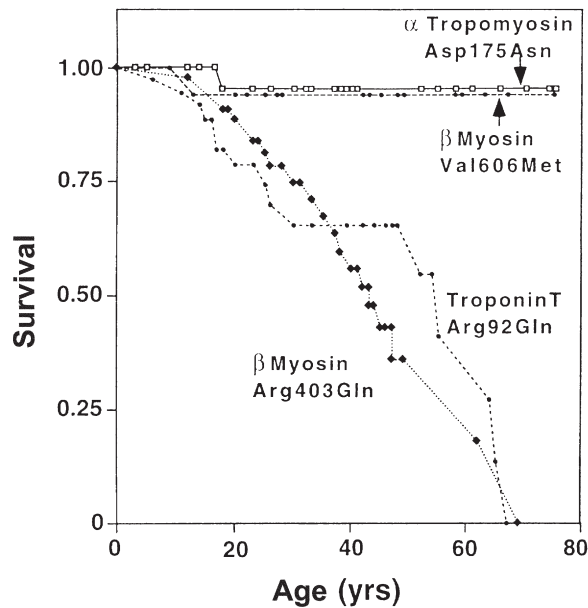
cTnI is encoded by *TNNI3*. The cTnI isoform is expressed only in the cardiac muscles (300). Cooperative binding of cTnI-actin-tropomyosin is a unique property of the cardiac variant (301). A small number of troponin I mutations have been identified in FHC, with others found in patients with restrictive physiology mimicking restrictive cardiomyopathy (265a).

$\alpha$ -Cardiac actin was also identified as a cause of FHC. Mogensen et al. (265) studied a family with heterogeneous phenotypes, ranging from asymptomatic with mild hypertrophy to pronounced septal hypertrophy and left ventricular outflow tract obstruction. Using linkage analysis and mutation screening, the gene was mapped to chromosome 15q14 with a LOD score of 3.6, and a missense mutation (G $\rightarrow$ T in position 253, exon 5) resulted in an Ala295Ser amino acid substitution. The mutation is localized at the surface of actin in proximity to a putative myosin-binding site. This mutation, which causes FHC, differs from the two mutations reported to cause DCM, which were localized in the immobilized end of actin that cross-binds to the anchor polypeptides in the Z-bands (302). It appears that mutations near the dystrophin-binding region result in DCM, whereas mutations affecting the sarcomeric end result in an HCM phenotype.

**51.6.1.5 Genotype-Phenotype Relations in FHC.** The pattern and extent of LVH in patients with HCM vary greatly even in first-degree relatives, and a high incidence of sudden deaths is reported in selected families. An important issue, therefore, is to determine whether the genotype heterogeneity observed in FHC accounts for the phenotypic diversity of the disease. However, the results must be seen as preliminary, because the available data relate to only a few hundred individuals. Several concepts nevertheless begin to emerge, at least for mutations in the *MYH7*, *TNNT2*, and *MYBPC3* genes. For *MYH7*, it is clear that prognosis for patients with different mutations varies considerably (303). For example, the R403Q mutation appears to be associated with markedly reduced survival, whereas some others, such as V606M, appear more benign (Figure 51-15). The disease caused by *TNNT2* mutations is usually associated with a 20% incidence of nonpenetrance, a relatively mild (and sometimes subclinical) hypertrophy, but a high incidence of sudden death that can occur even in the absence of significant clinical LVH (304,305). However, one family with a *TNNT2* mutation reportedly had complete penetrance, and echocardiographic data showed a wide range of hypertrophy and there was no sudden cardiac death (298). Thus, more data are needed before final conclusions are drawn. Mutations in *MYBPC3* seem to be characterized by specific clinical features, with a mild phenotype in young subjects, a delayed age at onset of symptoms, and a favorable prognosis before the age of 40 years (271,304–307).

Genetic studies have also revealed the presence of clinically healthy individuals carrying a mutant allele (no matter which gene is affected), which is associated





**FIGURE 51-15** Kaplan-Meier survival curve in hypertrophic cardiomyopathy comparing mutations of  $\beta$ -myosin heavy chain, cardiac troponin T, and  $\alpha$ -tropomyosin.

in first-degree relatives with a typical phenotype of the disease. Several mechanisms could account for the large variability of the phenotypic expression of the mutations: the role of environmental differences and acquired traits (e.g. differences in lifestyle, risk factors, and exercise), and finally, the existence of modifier genes and/or polymorphisms that could modulate the phenotypic expression of the disease. The only significant results obtained so far concern the influence of the angiotensin I-converting enzyme insertion/deletion polymorphism. Association studies showed that, compared to a control population, the D allele is more common in patients with HCM and in patients with a high incidence of sudden cardiac death (308,309). It was recently shown that the association between the D allele and hypertrophy is observed in the case of MYH7 R403 codon mutations, but not with MYBPC3 mutation carriers (310), raising the concept of multiple genetic modifiers in FHC.

**51.6.1.5.1 Elucidation of the Pathogenesis from Genetic Models.** On the basis of in vitro and in vivo studies in genetic animal models of HCM, it now appears that the primary defect is impaired contractility. Analysis of a human heart from a patient with the Arg403Gln mutation showed that the ratio of myosin to actin was normal (311), indicating there is no deficiency of the  $\beta$ -MHC protein. All the responsible mutant genes encode for a sarcomeric protein and appear, in some way, to impair systolic contraction or diastolic relaxation. In feline adult cardiac myocytes, in which  $\beta$ -MHC is the predominant myosin form, expression of human mutant  $\beta$ -MHC gene, Arg403Gln, was associated with sarcomere disassembly (312). Expression of the human mutant troponin T in this model also induced sarcomere disassembly and was associated with impaired rate of

cell shortening as detected by laser (313). Furthermore, it was shown that the expressed mutant protein was incorporated into the sarcomere. In a transgenic mouse, expression of troponin T gene (cTnT-Gln92) (314) exhibited sarcomere disarray, increased fibrous tissue, and sudden death, but only minimal hypertrophy. In this model, it was also shown that increased expression of the mutant protein was associated with a more severe phenotype, confirming the mutant protein has a dominant-negative effect as expected. These results have been confirmed by several investigators (276,277,312,315). Expression of the mutant MyHC gene or the troponin gene in the mouse has, in general, been associated with less hypertrophy than expected (314). Recently, a transgenic rabbit model (rabbit has  $\beta$ -MHC as its cardiac myosin) has been developed expressing the  $\beta$ -MHC (Arg403Gln) mutation, which exhibits sarcomere disarray, hypertrophy, and increased fibrous tissue virtually identical to that observed in humans (316).  $\alpha$ -TM mutations in an animal model caused cardiac dysfunction as well (317). Thus, the overall postulated pathogenesis of FHC may be summarized briefly as follows: the mutant protein is incorporated into the sarcomere and acts as a “poison peptide” that impairs contractility of that particular cell, which, in turn, provides the stimulus for the mitogenic response (probably several growth factors) of compensatory hypertrophy. The growth stimulus, as in acquired disorders, appears highly localized and mediated by autocrine or intracrine factors, given that the hypertrophy is localized in many patients, primarily to the interventricular septum. The growth factors also stimulate fibroblast proliferation and increased matrix formation. The relationship between the hypertrophy and increased fibrous tissue response to sudden death and arrhythmias remains to be determined. It is postulated that the fibrous tissue leads to delayed electrical conduction and predisposes to arrhythmias and sudden death. The future elucidation of the molecular basis for the pathogenesis of this disease, however, must provide a rationale for three puzzling, consistent features of the pathology of FHC: (i) predominance of hypertrophy in the septum, (ii) sarcomere and myocyte disarray, and (iii) the supernormal systolic function. The diastolic stiffness or decreased compliance is expected with hypertrophy whether it is primary or compensatory, but these other features are not seen in compensatory hypertrophy associated with myocardial infarction or pressure overload.

**51.6.1.6 Management.** Medical therapy is typically indicated (318). The most commonly used medications include  $\beta$ -blockers and calcium-channel blockers. Good hydration status is also important: antiarrhythmic agents are used when necessary; and poorly controlled ventricular dysrhythmias or syncope may lead to ICD implantation. In cases in which severe obstruction occurs, myomectomy or pacemaker implantation (DDD mode) has been utilized. More recently, alcohol ablation has

been employed in adults. Cardiac transplantation may be necessary in cases with dilation and systolic dysfunction.

### 51.6.2 Neonatal HCM

Clinically, this appears to be the same disorder as that seen in older children and adults (319). Echocardiograms and ECGs tend to mimic the findings described for adults with HCM. However, the underlying etiologies appear to differ from the sarcomeric gene-related disorders of adults. In the neonate and infant with HCM, abnormalities of mitochondrial function and metabolic disorders tend to predominate. In addition, genetic syndromes are common in this age group as well. Mott et al. (320) have demonstrated that Noonan syndrome, mitochondrial myopathies, and metabolic disorders such as glycogen storage diseases predominate, along with abnormalities of fatty acid oxidation. Although sarcomeric gene mutations could be involved as well, few patients have been described to date in the literature. Instead, it is likely that the sarcomere is secondarily affected by the mitochondrial and metabolic abnormalities, which probably create an energy production–utilization mismatch (224,321–323).

Finally, neonates with a dilated form of HCM with combined systolic and diastolic dysfunction are well described. In some cases, deep endomyocardial trabeculations are noted, consistent with left ventricular non-compaction (324,325) (Figure 51-16). Males with this abnormality have been shown, in some cases, to carry mutations in G4.5, an X-linked gene (*Xq28*) that encodes a novel protein called tafazzin (326–328) (Table 51-3), which is thought to function as an acyltransferase. Clinically, these children have abnormal mitochondria on electron microscopy and abnormal energy production. This gene also causes a more complex phenotype called

*Barth syndrome*, an X-linked cardioskeletal myopathy with cyclic neutropenia, 3-methylglutaconic aciduria, cardiolipin deficiency, short stature in youth, and cholesterol depletion (274a,329–331). Other genes have also been identified, including  $\alpha$ -dystrobrevin in left ventricular noncompaction associated with congenital heart disease (325a) and Z-disk-associated PDZ domain protein (ZASP; (332)). These genes encode cytoskeletal proteins and Z-disk-localized proteins. Other children with early presentation of dilated HCM have been found to have mitochondrial abnormalities as well, and this occurs in both sexes (266a).

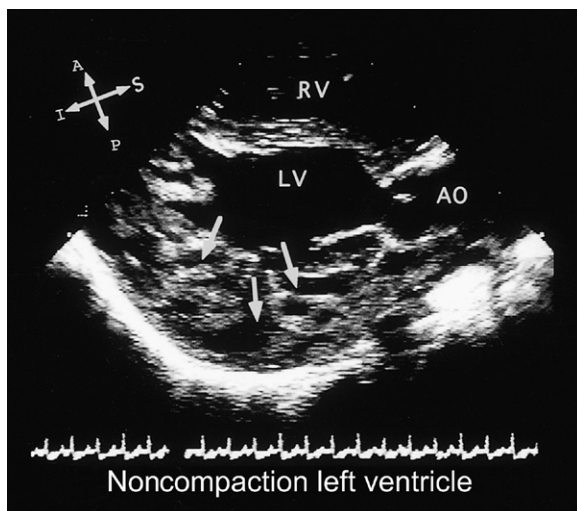
Evaluation of these children should include urinalysis for organic acids, serum amino acids, acylcarnitine profile, and skeletal muscle biopsy for microscopy, electron microscopy, and electron transport chain analysis (319).

**51.6.2.1 Management.** Therapy includes  $\beta$ -blockers in patients with HCM and hypercontractile function, with or without obstruction. In metabolic mitochondrial disorders, the metabolic derangement should be treated. Carnitine, coenzyme Q10, riboflavin, thiamine, and other vitamins are useful in some cases. In cases in which ventricular dysfunction occurs, anticongestive measures are necessary. Dysrhythmias are treated with medical therapy.

### 51.6.3 Dilated Cardiomyopathy

**51.6.3.1 Clinical Features.** DCM is characterized by increased ventricular chamber size, decreased wall thickness, and impaired systolic ventricular function (333). Secondary diastolic dysfunction may also occur. Owing to the left ventricular dilation, the mitral ring stretches and the development of mitral regurgitation is common. When mitral regurgitation is significant, left atrial enlargement occurs and the development of pulmonary hypertension may occur. The most common clinical presentation is that of congestive heart failure (CHF), with resting tachycardia (probably due to increased sympathetic tone), dyspnea due to pulmonary edema, hepatomegaly, and poor perfusion due to low cardiac output (334–336). Syncope and sudden death may occur, and in many of these cases, VT/VF is the cause (238a,337–341). Many patients may have pericardial and/or pleural effusion as well. In addition to the ventricular tachydysrhythmias, supraventricular tachycardia (SVT) can occur. In some patients, conduction abnormalities are prominent.

**51.6.3.2 Genetics.** The prevalence of idiopathic DCM has been estimated to be approximately 40 cases per 100,000 (342–344). Familial DCM (FDCM) is estimated to account for 30–40% of patients with idiopathic DCM. Inheritance of FDCM is usually autosomal dominant, but X-linked, mitochondrial, and autosomal-recessive DCM also occurs. Genetic heterogeneity has been found for the autosomal-dominant form of FDCM. The first autosomal-dominant gene was identified by our



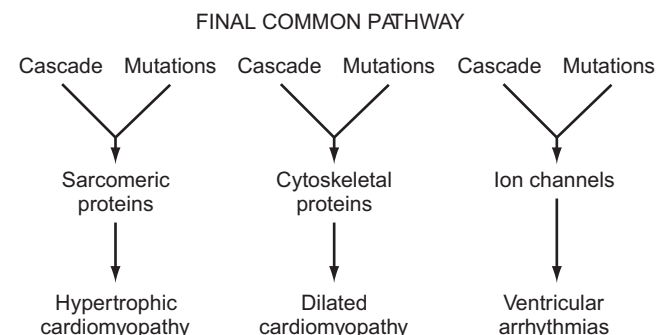
**FIGURE 51-16** Echocardiogram of left ventricular noncompaction. Note the deep endomyocardial trabeculations in the left ventricle (LV). AO, aorta; RV, right ventricle.

group on chromosome 1q32 (345). Five other chromosomal loci have been identified by us and by others, on chromosomes 10q21–23 (346), 5q33 (347), 9q13 (348), 15q14 (302), and 2q31 (349) (Table 51-3). Four additional chromosomal loci have been mapped to 1p1–1q1 (350), 1q11–1q21 (351), 3p22–25 (352), and 6q23 (353), in families having DCM in association with conduction defects; also notable is that one family linked to chromosome 6q23 has associated limb-girdle muscular dystrophy (LGMD) as well (353), while two other families had sensorineural deafness (354). In the families with conduction disease, transient dysrhythmias, which present in the second or third decade, become sustained and common place by the third or fourth decade (355). The abnormal rhythms included second- or third-degree AV block, AF, and marked bradycardia, commonly requiring a pacemaker. DCM usually developed in the fourth or fifth decade, generally out of proportion to the severity of the rhythm disturbance. Sudden death commonly occurred in the late stages of the disease. On autopsy, marked right and left ventricular dilation, interstitial fibrosis, myocyte degeneration characterized by cytoplasmic vacuolization, and AV nodal cell replacement by fibrous tissue were noted. The gene for the chromosome 1p–1q-linked disorder was shown to be lamin A/C (356), the same gene that causes autosomal-dominant EDMD (357). The other genes have remained elusive (Table 51-3). In the pure forms of FDCM, the genes identified generally encode proteins of the cytoskeleton, sarcomere, or sarcomere–sarcolemma link. The first three genes identified included  $\alpha$ -actin (15q14; (302)), desmin (2q31; (358)), and  $\delta$ -sarcoglycan (5q33; (347)) (Table 51-3). In addition to forming portions of the thin filament of the sarcomere, which is essential to the generation of force,  $\alpha$ -actin is also an important cytoskeletal protein involved in structural integrity and the transmission of force. Mutations in actin responsible for FDCM are located in the domain that is immobilized and attached to the Z-band or intercalated disk and involved with transmitting force. In contrast, it has also been shown that mutations in the actin domain affecting the myosin cross-bridges and the generation of force (sarcomere) give rise to familial HCM (265). Desmin is the specific intermediate filament for muscle and an essential cytoskeletal protein for maintaining cardiac structure and for the transmission of force and other signals to the cytoplasm and the nucleus of the cell. Desmin stretches from its attachment to the sarcomere Z-band to the nuclear membrane and other organelles (359). Mutations in desmin have been shown to be associated with cardiac and skeletal abnormalities (360). A missense mutation (Ile451Met) was found to be responsible for DCM in a family without any skeletal or smooth muscle abnormalities. Mutations leading to combined skeletal and cardiac abnormalities have all been in the rod region of desmin. In contrast, the Ile451Met mutation responsible for the restricted cardiac phenotype of

DCM encodes for the tail domain of human desmin, located in codon 451 with cytosine substituting for guanine. This would suggest a possible unique cardiac function for the domain in this region. Elimination of the desmin gene in a knockout mouse was associated with a phenotype of DCM exhibiting impaired cardiac function and myocyte necrosis.

Several new genes have been identified since 2000. Other cytoskeletal genes, such as metavinculin (352a) and  $\beta$ -sarcoglycan (361), were identified, along with those of the Z-disk, including  $\alpha$ -actinin-2 (85a), muscle LIM protein (MLP) (85a,362), the ZASP gene (332), and titin (352a). In addition, other sarcomeric proteins known to cause FHCM, such as  $\beta$ -MyHC (160a), cTnT (160a,363),  $\alpha$ -TM (352a), and MyBP-C (328a), have also been shown to cause DCM. A calcium homeostasis gene, phospholamban, was also shown to cause DCM when mutated. In all the cases described, irrespective of the gene, ventricular arrhythmias were prominent clinical features of the affected subject.

It is of note that the genes identified to cause DCM have also been shown to be associated with musculo-skeletal disorders. For instance, Duchenne and Becker muscular dystrophies (DMD and BMD) are due to dystrophin mutations,  $\beta$ - and  $\delta$ -sarcoglycan cause LGMD, lamin A/C causes EDMD, and desmin causes myofibrillar myopathy (364–367). Therefore, these cytoskeletal proteins appear to cause cardiac and skeletal muscle disease. There is, thus, a strong suggestion that FDCM may be a disease of the cytoskeletal proteins and the link with the sarcomere (368,369), analogous to HCM being a disease of the sarcomere (267) and LQTS and Brugada syndrome being an ion channelopathy (19,370). This has been termed the final common pathway hypothesis by Towbin (368,369) (Figure 51-17), and the concept has been reiterated by Katz (371).



**FIGURE 51-17** Final common pathway hypothesis. The concept developed here is that phenotypes result from mutations in genes encoding specific subtypes of proteins or cascade pathways that affect the function of these targets. For instance, hypertrophic heart disease results from mutations in sarcomeric proteins or in cascade pathways affecting these target proteins. Similarly, ventricular arrhythmias and dilated, dysfunctional hearts are caused by abnormalities of ion channels and cytoskeletal/sarcolemmal proteins, respectively, or cascade pathways involved in the function of these target proteins.

### 51.6.4 X-Linked DCM

**51.6.4.1 Clinical Features.** Berko and Swift (372) reported a five-generation kindred with DCM, X-linked inheritance, and no clinical evidence of skeletal myopathy. Males presented in their teens or early 1920s with clinical evidence of mitral regurgitation and an echocardiographic diagnosis of DCM. Episodes of VT were noted in several patients. The males progressed rapidly (within 1 or 2 years) to death or cardiac transplantation. Manifesting female carriers developed mild cardiomyopathy in the fourth or fifth decade and progressed slowly. Right ventricular endomyocardial biopsy revealed minimal interstitial fibrosis, while postmortem evaluation showed marked dilation, widespread patchy fibrosis (worst in the posterior wall), and normal mitochondria on electron microscopy. There were no pathognomonic findings differentiating this cardiomyopathy from other dilated forms, except for the apparent X-linked inheritance and elevation of the muscle isoform of creatine kinase (CK-MM) in the serum of affected males and female carriers.

**51.6.4.2 Genetics.** Towbin et al. (373) demonstrated linkage of X-linked dilated cardiomyopathy (XLCM) to the dystrophin (Table 51-4) locus at Xp21 (i.e. the gene responsible for DMD and BMD) in the family described previously, as well as in a second family. Evaluation of the protein defect in XLCM showed absence (or low abundance) of the N-terminal and rod portion of the dystrophin protein, while skeletal muscle total protein was normal (373). The 156 kDa dystrophin-associated glycoprotein (known as  $\alpha$ -dystroglycan) (373,374), a membrane-bound constituent of the dystrophin-associated glycoprotein complex, was decreased in abundance in cardiac tissue as well, and it was suggested that the entire dystrophin-associated glycoprotein complex

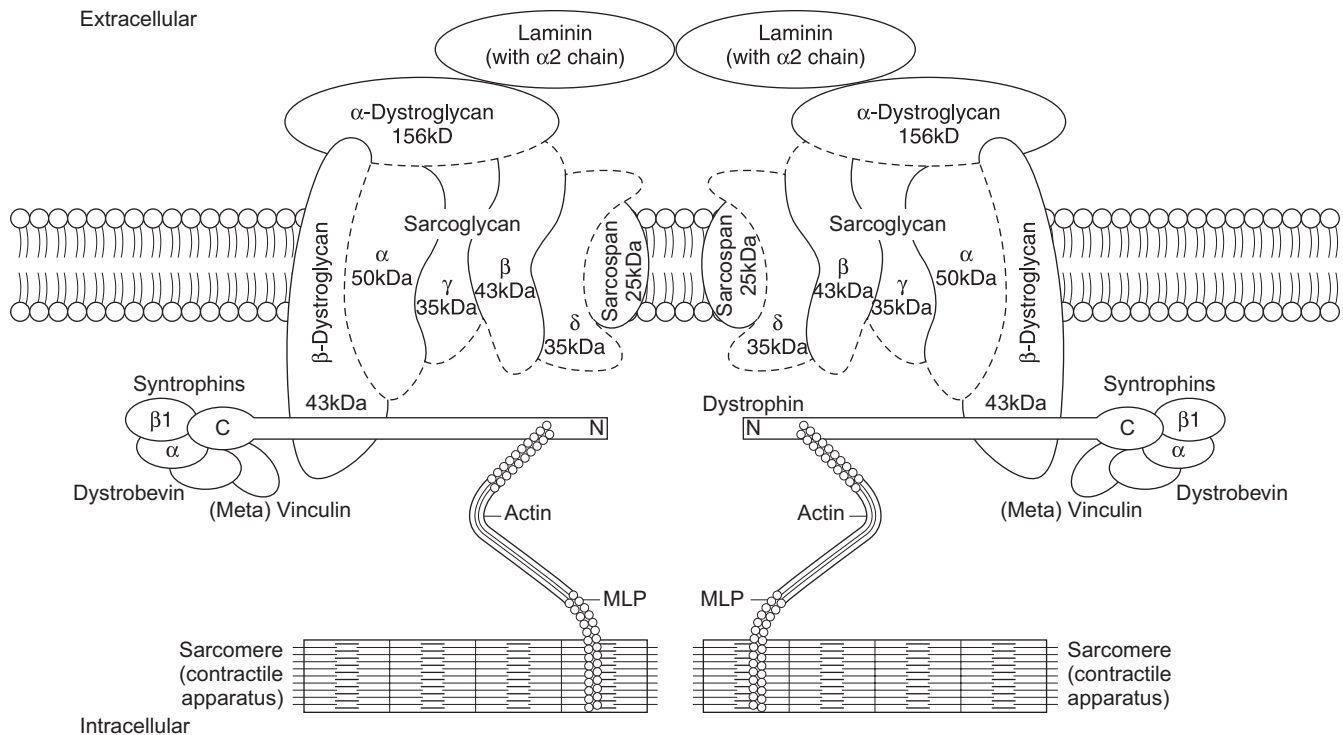
was involved (374) (Figure 51-18). This was later confirmed by others (202a,375–379). Diverse mutations leading to XLCM have been shown by Towbin and Ortiz-Lopez (380), Yoshida et al. (381), Muntoni et al. (382), Milasin et al. (383), and Ortiz-Lopez et al. (384), with most mutations residing in the 5' portion of the gene. Franz et al. (377) reported on a mutation in exon 29, however. In all cases, it appears that the DCM occurs because of mechanical destabilization of the muscle membrane. Other novel mutations in the 5' end of dystrophin, including a transposon (385) insertion and an Alu-rearrangement (376), were found to result in XLCM. Further, mutations in dystrophin have been shown to cause sporadic DCM (202a,202b). In addition, our group has shown that the N-terminal portion of dystrophin near the actin-binding domain is a “fragile site” in all forms of DCM, whether or not the dystrophin gene is mutated. Using immunohistochemistry and Western blots, all forms of DCM were shown by Vatta et al. (170a,170b,332) to have loss of N-terminal dystrophin, which may normalize during the process of reverse remodeling.

**51.6.4.3 Management.** Treatment of CHF and ventricular arrhythmias is necessary, and transplantation is common. Medical therapy includes an oral anticongestive regimen of digoxin, diuretic, and an angiotensin-converting enzyme inhibitor such as captopril or enalapril. Recently, the addition of a  $\beta$ -blocker such as metoprolol or carvedilol has become standard practice. Owing to the poor systolic function, the potential for the development of thrombi and embolic phenomena has led to the institution of therapy with aspirin or other medications. In some patients, inotropic support intravenously is needed; the usual approach includes use of renal-dose dopamine, milrinone, and possibly

**TABLE 51-4 Limb-Girdle Muscular Dystrophies**

Genetic Designation	Locus	Gene	Cardiac Disease
Autosomal dominant (LGMD1)			
A	5q31	Myotilin	None
B	1q11–21	?	Dysrhythmias, AVB
C	3p25	Caveolin-3	Dysrhythmias, AVB
D	6q23	?	Dysrhythmias, AVB
E	?	?	?
F	7q32.1	?	?
G	4p21	?	Dysrhythmias, AVB
Autosomal recessive (LGMD2)			
A	15q15	Calpain-3	None
B	2p13	Dysferlin	None
C	13q12	$\gamma$ -Sarcoglycan	Cardiomyopathy, AVB
D	17q21	$\alpha$ -Sarcoglycan	Cardiomyopathy, AVB
E	4q12	$\beta$ -Sarcoglycan	Cardiomyopathy, AVB
F	5q33–34	$\delta$ -Sarcoglycan	Cardiomyopathy, AVB
G	17q11–12	Telethonin	Cardiomyopathy, AVB
H	9q32	TRIM32	Cardiomyopathy, AVB
I	19q13.3	FKRP	Cardiomyopathy, AVB
J	2q31	Titin	Cardiomyopathy, AVB





**FIGURE 51-18** Dystrophin-associated glycoprotein complex. Abnormalities in proteins comprising this complex result in myocardial disease with or without skeletal muscle disease.

other medications such as dobutamine, epinephrine, and norepinephrine. Ventricular assist devices or intra-aortic balloon pumps may be indicated in severe cases as a bridge to transplantation. Ventricular assist devices have recently been used as a bridge to recovery. We have shown that this approach normalizes dystrophin, fibrosis, and myocyte hypertrophy, enabling reverse remodeling (*170a,170b,332a*). Antiarrhythmic agents may be needed in some patients; ICD implantation is reserved for patients with life-threatening dysrhythmias.

### 51.6.5 Conduction Disease with DCM

**51.6.5.1 Clinical Features.** Families are described with variable conduction disease ranging from first-degree to second-degree AV block that occurs in the second or third decade of life and progresses to complete AV block over one or two decades (*355*). In these families, DCM occurs late in the course of the disease and is out of proportion to the conduction disease severity. Typically, these are autosomal-dominant transmitted disorders, and in some cases, skeletal myopathy is notable. A variety of genes have been identified thus far.

**51.6.5.2 Genetics.** In 2001–2010, several families have been studied using linkage analysis. The first gene for conduction disease with dilated cardiomyopathy (CDDC) was identified by Kass et al. (*350*) to be a chromosome 1p1–1q1-linked gene (*Table 51-3*). Subsequently, Olson and Keating mapped a second locus to 3p25–3p22 (*352*). In these families, no skeletal

myopathy was demonstrated. However, Messina et al. localized a gene to 6q23 in a family with skeletal myopathy (*353*), and Van der Kooi et al. (*351*) described another family, which they suggested as having LGMD (LGMD1B) with CDDC, and which they mapped to chromosome 1q11–1q21 (*271a,386*). The gene for the 1p1–1q1-linked CDDC was identified as lamin A/C (000a, (*66a,270a,356,387–389*)) (*Table 51-3*). This gene encodes two proteins that are members of the intermediate filament class of cytoskeletal proteins. The families identified had variable skeletal myopathy as well. The gene also causes autosomal-dominant EDMD, which has a similar phenotype although skeletal myopathy is central to the diagnosis in these patients (*357*). In CDDC, tachydysrhythmias including nonsustained VT, SVT, and AF occur in some of the affected patients, including young individuals (*390,391*).

Although the 3p25–p22-linked gene and 6q25-linked gene have not been identified, another group of similar patients have been shown to have mutations in the chromosome 2q-linked gene desmin, a myofibrillar protein that accumulates in the muscle fibers of affected patients (*358*). In skeletal and cardiac muscles, normal desmin encircles the Z-bands that hold together the actin filaments and help transmit tension along the myofibrils, protecting their structural integrity during repeated muscle contractions over time. Mutant desmin appears to cause fragility of the myofibrils and impair contraction. In mice lacking desmin, the same phenotype occurs but only later in life.

Most recently, Olson et al. (392) identified mutations in *SCN5A* in a family with DCM and AF as well as conduction abnormalities.

**51.6.5.3 Management.** The management of CDDC requires pacemaker implantation for symptomatic bradycardia. Signs and symptoms of CHF are treated with standard CHF therapy.

### 51.6.6 Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy

ARVD/ARVC is characterized by fatty infiltration of the right ventricle, fibrosis, and ultimately thinning of the right ventricular wall (Figure 51-19) with chamber dilation (393–395). The residual myocardial fibers are often embedded in fibrous tissue, and this substrate is conducive to right ventricular reentrant arrhythmias. This is the most common cause of sudden cardiac death in the young in Italy (395) and is said to account for about 17% of sudden death in the young in the United States (393–396).

**51.6.6.1 Clinical Features.** The clinical presentation of ARVD/ARVC is highly variable and includes palpitations, syncope, right heart (or biventricular) failure, and sudden death (397). Some patients may be asymptomatic. Clinical manifestations are rare before puberty, with typical onset between 12 and 45 years of age, and sudden death is a common first symptom. It has been estimated that 3–4% of sudden deaths during sports (and 17% of all sudden deaths in young persons) occur because of this disorder, and it is well known that the dysrhythmias in this disease are most likely to occur during exercise.

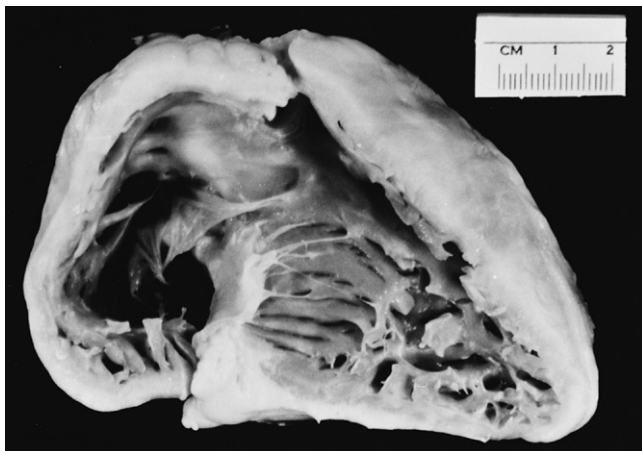
ARVD/ARVC is extremely difficult to diagnose and, since there is no diagnostic standard, consensus criteria were developed (398) on the basis of structural, functional, and electrical manifestations. The structural changes most commonly noted include increased right ventricular chamber size, deep fissuring and trabeculation of the RVOT, localized akinetic or dyskinetic bulges

or outpouchings (particularly the infundibulum at the apex and posterior sub-tricuspid areas), and localized thinning of the right ventricular wall (399).

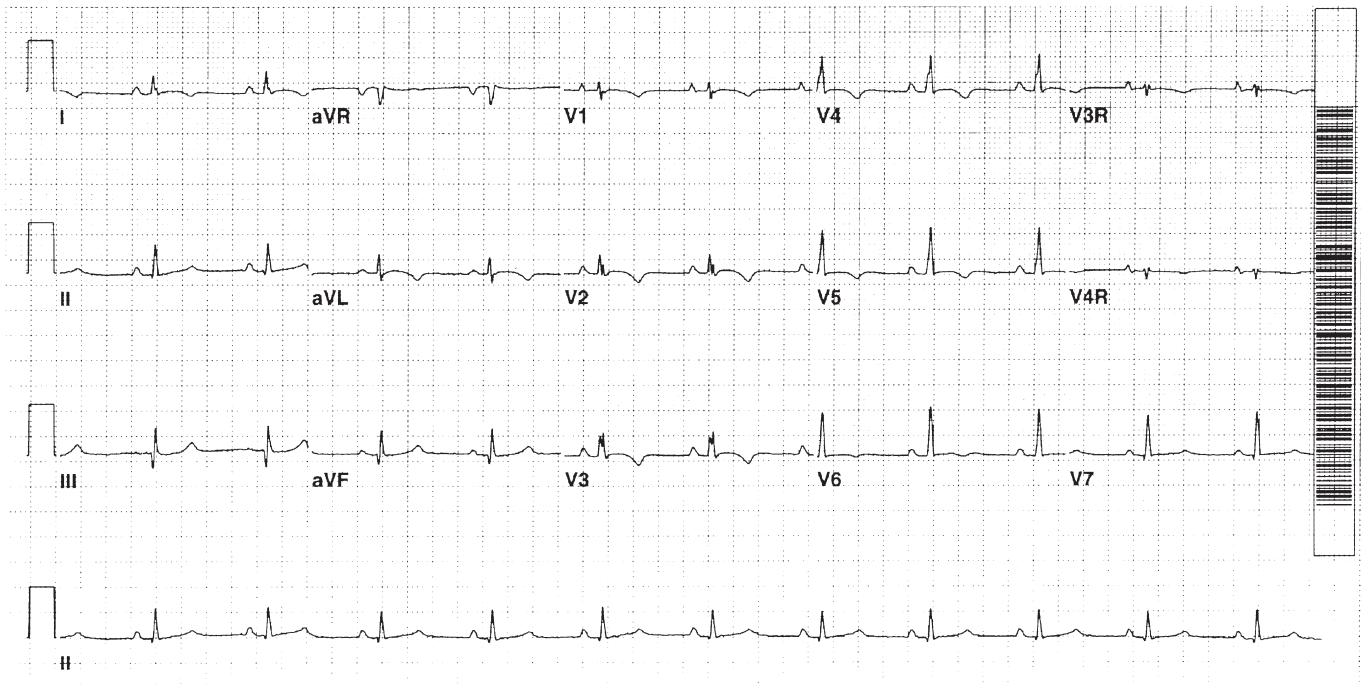
Morphologically, the most striking feature of ARVD/ARVC is fatty replacement of the epicardium and mid-myocardium of the right ventricular free wall (394,395). The endocardium is usually spared, as is the septum. As the disease progresses, the left ventricle may become involved, particularly the epicardial surface. Focal right ventricular thinning, especially in the infundibular region, in the diaphragmatic inferior wall, or at the apex, is typical. Histology demonstrates severe myocardial atrophy with residual myocytes interspersed with fibrofatty tissue. Evidence of patchy and acute inflammation with myocyte death and focal round cell infiltrate (lymphocytes) is present in approximately 70% of cases (400).

Clinically, the key feature of this disease is its propensity to tachydysrhythmias (397). During sinus rhythm, there are abnormalities of the QRS configuration that are due to slow conduction of electrical activity in the Purkinje system of the right ventricular wall, known as parietal block. In addition, there may be abnormalities of repolarization secondary to the underlying abnormal anatomy of the right ventricular free wall and chamber enlargement. These abnormalities include (i) prolongation of the QRS duration in the right precordial leads ( $V_1$ – $V_3$ ), representing alterations of conduction delay predominantly affecting the right ventricular free wall, the last portion of the heart to be depolarized; (ii) epsilon waves, small electrical potentials occurring immediately after the QRS complex and representing delayed right ventricular depolarization; (iii) presence of late potentials by signal-averaged ECG, also suggestive of delayed depolarization and typically associated with reduced right ventricular ejection fraction; (iv) T wave inversion beyond lead  $V_1$  in a patient older than 12 years of age, the extent of which correlates with the extent of right ventricular involvement; and (v) ventricular dysrhythmias (Figure 51-20). These are almost always of LBBB morphology as they arise from the right ventricular free wall (Figure 51-21). The propensity for dysrhythmias has been suggested to occur because of abnormalities of cardiac sympathetic innervation, with significant reduction of myocardial  $\beta$ -adrenergic receptor density (401). Owing to the high-risk features of ARVD/ARVC, it has been suggested that a registry be created (402).

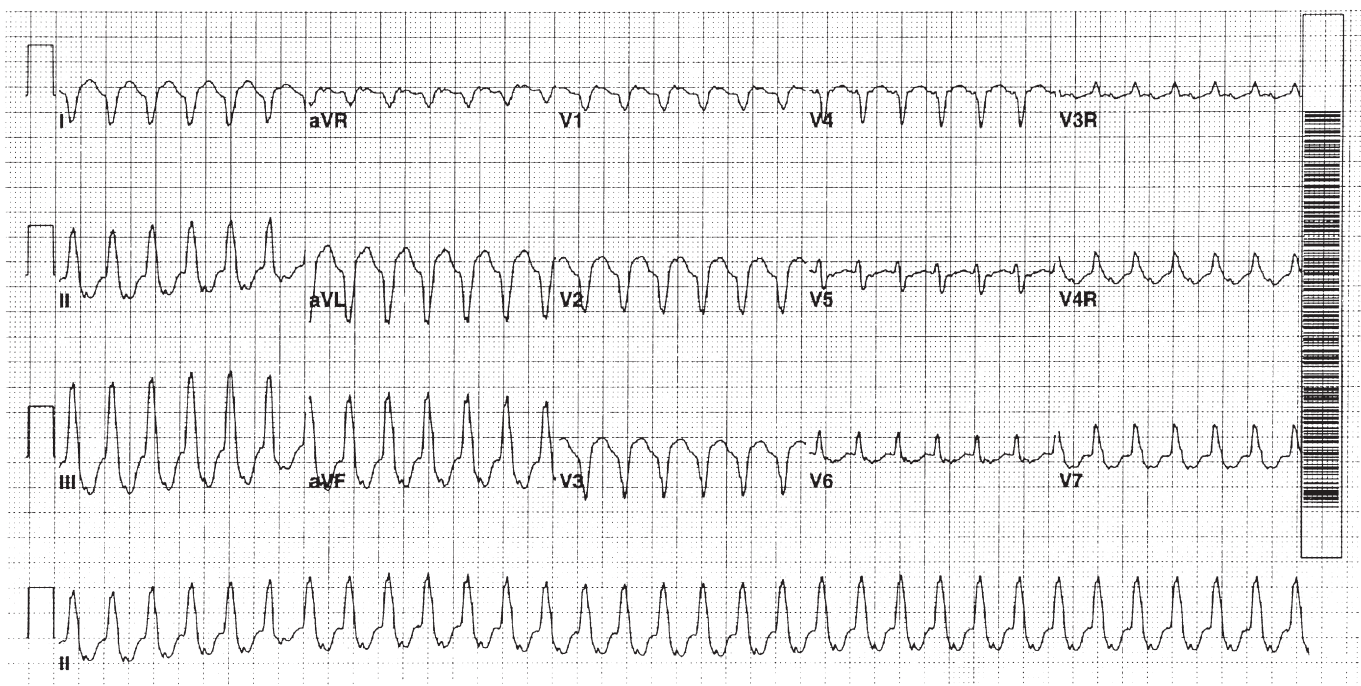
**51.6.6.2 Genetics.** ARVD/ARVC is commonly inherited with a family history of disease being present in 30–50% of cases (397). With the exception of complex variants of the disease found in Naxos, Greece, and in Ecuador with a recessive mode of transmission (39a,403,404), the disease is transmitted in an autosomal-dominant pattern with various degrees of clinical expression. The autosomal-dominant form is genetically heterogeneous, with six genes for a pure form of ARVD/ARVC mapped to chromosomes 14q24.3 (ARVD1; (405)), 1q42–q43 (ARVD2; (406)), 14q12–q22 (ARVD3; (407)), 2q32.1–q32.3



**FIGURE 51-19** Gross anatomic findings in ARVD. Note the right ventricle free wall thinning with fatty replacement.



**FIGURE 51-20** Features of ARVD are seen in this electrocardiogram. Epsilon waves and T wave inversion in the lateral leads are seen.



**FIGURE 51-21** Left bundle-branch ventricular tachycardia in ARVD.

(ARVD4; (408)), 3p23 (ARVD5; 5), and 10p12–p14 (ARVD6; (363a)) (Table 51-3). To date, three genes for this disorder have been identified. The first gene, RyR2, is involved in calcium homeostasis. It has been identified as the gene for CPVT. The other genes, plakophilin-2 and desmoplakin, encode desmosomal proteins. A locus has also been identified for ARVD/ARVC associated with myofibrillar myopathy, a skeletal myopathy with axial and distal weakness, myopathic electromyographic

findings, and abnormal biopsies with myopathic changes; rimmed vacuoles; accumulation of desmin, dystrophin, and sarcoglycan; and myofibril disorganization. This gene was mapped to chromosome 10q22.3 (Table 51-3), but the gene has not yet been identified (409).

Two autosomal-recessive forms of ARVD/ARVC, Naxos disease and Carvajal syndrome, have been identified. The first of these, Naxos disease, was mapped to chromosome 17q21 (403). This complex disorder consists



of ARVD/ARVC associated with diffuse nonepidermolytic palmoplantar keratoderma and wooly hair, and has been identified on the Greek island of Naxos. The gene for this disorder was identified as plakoglobin (Table 51-3), a desmosomal protein (410). The second disease, Carvajal syndrome, has the same clinical phenotype as Naxos disease except left ventricular involvement occurs. This gene, localized to chromosome 6p21, has been identified as desmoplakin, another desmosomal protein. It is interesting that the autosomal-dominant myofibrillar myopathy/ARVC has desmin-like inclusions, suggesting that a similar gene product will be found to cause this disorder. As noted, the genes involved in the pure form of ARVD/ARVC also are involved in desmosomal function.

**51.6.6.3 Management.** Medical therapy consists of antiarrhythmic drugs to suppress ventricular arrhythmias, with sotalol (most common), amiodarone, flecainide, and propafenone reportedly used (399,411). Although sotalol appears to be effective in preventing recurrence of sustained VT, whether it is effective in preventing sudden death remains unknown. Catheter ablation of VT has been achieved in selected patients, but recurrences are not uncommon, probably because of the progressive nature of the disease. ICDs are currently recommended for patients who have had cardiac arrest or are deemed to be at high risk of syncope/sudden death or who have polymorphic VT (399). Owing to the fatty infiltrate, implantation (and function) of the ICD may be problematic.

## 51.6.7 Restrictive Cardiomyopathy

**51.6.7.1 Clinical Features.** Restrictive cardiomyopathy (RCM) is the least common of the four major categories of cardiomyopathy (412,413). It is a primary abnormality of diastolic function caused by derangement in the dynamics of ventricular filling, resulting in an increase in ventricular end-diastolic pressures and dilated atria (Figure 51-22). Systolic

function is usually preserved. Pulmonary hypertension commonly occurs (414). The diagnosis may be made by an ECG in which extreme P wave (atrial) voltage and width is notable and an echocardiogram with severe atrial dilation with normal ventricular size and function (Figure 51-23). Ischemia and ventricular tachydysrhythmias may occur as do atrial tachydysrhythmias and conduction abnormalities. Sudden death is common (371a,412,415–417). Secondary RCM can develop in the late stages of HCM (418); DCM; valvular, hypertensive, and ischemic heart disease; or specific heart muscle disease, such as amyloidosis (419,420). The most common cause of secondary restrictive cardiomyopathy in adults is myocardial amyloid. Patients manifest exercise intolerance due to their inability to increase cardiac output by tachycardia without further compromising ventricular filling. Weakness and dyspnea are often prominent, and chest pain may also occur. At the end stage, the findings are those of cardiac failure with anasarca (414,421).

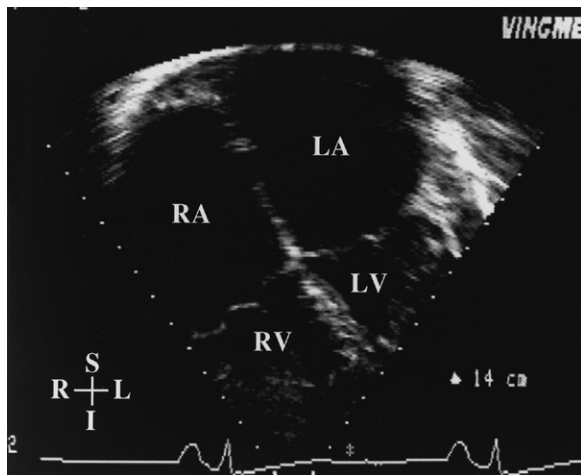
**51.6.7.2 Genetics.** Mutations in the transthyretin (TTR) gene (389a,422,423), which codes for the TTR serum protein, have been found to be associated with RCM (Table 51-3). This protein contains four subunits, each with 127 amino acids, encoded by four exons within a 7kb gene. Many TTR point mutations cause TTR to form amyloid, which occurs primarily in the heart, leading to heart failure.

Familial forms of RCM have also been seen. In these cases, autosomal-dominant and autosomal-recessive inheritance have been described. One such family was found to have mutations in the desmin gene (424) (Table 51-3), and mice have been created with desmin mutations that have a phenotype similar to the clinical condition (425,426). Another gene, troponin I, was identified in cases of RCM with and without associated LVH.

**51.6.7.3 Management.** In patients with CHF, anticongestive therapy is appropriate (412,414). However, most patients do not improve with any medical therapy and, because of the high risk of sudden death, transplantation may be the first-line therapy. The use of ICDs is controversial.

## 51.7 NEUROLOGIC DISORDERS ASSOCIATED WITH DYSRHYTHMIAS AND CONDUCTION DISEASE

Numerous atrial and ventricular dysrhythmias can be seen in certain familial neurologic diseases as can myocardial diseases (DCM, HCM, RCM). Generally, the most common rhythm disturbances associated with neurologic diseases are due to abnormalities of the cardiac conduction system. The following neurologic disorders are discussed: DMD, BMD, EDMD, LGMDs, congenital muscular dystrophies, myotonic dystrophy (DM), Friedreich ataxia, and mitochondrial myopathies.



**FIGURE 51-22** Restrictive cardiomyopathy. This echocardiogram identifies the diagnostic features: dilated atria with normal ventricular size, thickness, and systolic function. LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle.



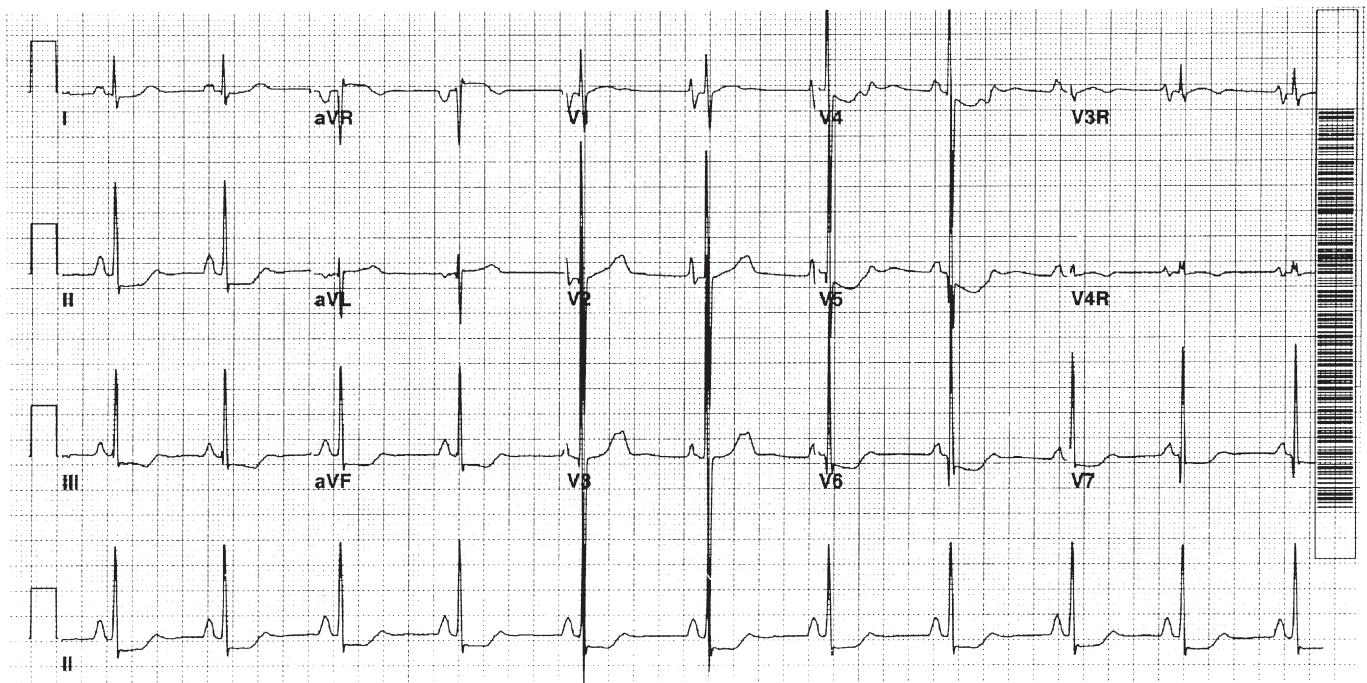
### 51.7.1 Duchenne Muscular Dystrophy

**51.7.1.1 Clinical Features.** DMD is an X-linked disorder characterized by the early onset of progressive, generalized muscle weakness and “pseudohypertrophy” of certain muscle groups (427,428). The incidence of DMD is estimated to be one in 3300 live male births, with little ethnic variation, and the calculated mutation rate of  $10^4$  is an order of magnitude higher than for most other genetic diseases. Approximately 35% of cases arise by spontaneous mutation, with the remaining two-thirds occurring by inheritance of the disease-causing gene from the carrier mother. Female carriers of DMD are usually asymptomatic but occasionally have a slowly progressive myopathy of moderate severity (429,430). This “manifesting female carrier” state occurs in approximately 8% of carriers and is thought to occur because of random X-inactivation. Cardiomyopathy may be the first clinical manifestation of female carriers (431). The disease may also be expressed in females with Turner syndrome having a single X chromosome and in females with X-autosome translocations that disrupt the *DMD* gene. In the latter case, the translocation not only disrupts the *DMD* gene but also causes the nonrandom inactivation of the normal allele on the other X chromosome, resulting in the expression of the disease phenotype.

Although evidence of skeletal muscle disease in boys with DMD is evident in the neonatal period, as seen by high serum muscle enzymes (particularly CK-MM), clinical disease is not. There may be a mild developmental delay, with walking later than expected, but weakness

is usually not appreciated until at least 2 or 3 years of age (428). Early symptoms reported by parents include difficulty in running or climbing stairs, frequent falling, and enlargement of calf muscles. Pelvic girdle weakness is more obvious than shoulder girdle weakness in the early stages. The gait becomes lordotic and waddling, and the child usually walks with the heels raised slightly off the ground (i.e. toe walking). As pelvic girdle weakness increases, the child has increasing difficulty rising from a seated position. In order to rise from the floor to a standing position, the child must brace the arms against the front of the thigh and climb up the legs, the so-called *Gower sign*. Muscle pseudohypertrophy usually appears by 5–6 years of age, with muscle enlargement most commonly occurring in the calf muscles; the quadriceps, infraspinatus, deltoid, and gluteal muscles may also be involved, however. The upper and lower extremities become progressively weaker with age, and joint contractures may appear because of uneven weakness of agonist and antagonist muscles. Contractures of the hip flexors, iliotibial bands, and heel cords develop in 70% of patients between 6 and 10 years of age. Most patients are wheelchair-bound by the end of the first decade of life. After ambulation is lost, fixed contractures occur and paraspinal muscle weakness leads to progressive kyphoscoliosis. Significant weakness of the respiratory muscles occurs early in the second decade and is a common cause of demise.

While most cases of DMD can be recognized on the basis of the patient’s history and clinical signs alone, laboratory evaluation is important to confirm the diagnosis (427). As previously noted, extremely high levels of



**FIGURE 51-23** Restrictive cardiomyopathy. Batrial enlargement (tall, wide P waves) and left ventricular hypertrophy (large-voltage QRS in  $V_6$ ) are noted along with ST segment depression in the inferior lateral leads, which is particularly noticeable in the rhythm strip.

CK-MM are found in the early stages of disease, as early as birth, and precede evidence of clinical involvement. Other muscle enzymes, including aldolase, aspartate aminotransferase, lactate dehydrogenase, and pyruvate kinase, are also grossly elevated. In the end stages of the disease, enzyme levels fall but do not reach normal values. Electromyographic examination may also be useful, demonstrating the characteristic features of a myopathy (432). Insertional activity is normal or increased initially, but decreases in the advanced stages of the disease, when fibrosis replaces muscle fibers. Fibrillation potentials and positive sharp waves occur in the early stages of the disease due to the splitting of muscle fibers. The motor unit potentials are small and polyphasic, and an early recruitment pattern with minimal effort is present. Mild intellectual impairment is common in patients with DMD. The retardation is present at an early age, is nonprogressive, and does not correlate well with the stage of the disease. Approximately one-third of patients have IQs less than 75, characterized primarily by impaired verbal ability.

The heart is commonly involved in DMD, with ECG abnormalities and DCM being most typical (427,433,434). Cardiac symptoms, however, are reportedly unusual before the terminal stages of the disease. CHF tends to occur (435). A midsystolic click and late systolic murmur associated with mitral valve prolapse (MVP) are also common. In addition, an  $S_3$  or  $S_4$  gallop, sinus tachycardia, and a mitral regurgitation murmur are usually heard along with cardiomegaly and increased pulmonary vascular markings on chest radiograph; at this stage, bilateral diaphragmatic elevations may be seen because of diaphragmatic dystrophy. Unlike the late-onset findings of DCM, the ECG is abnormal early in the course of DMD, with a tall R wave and an abnormally increased R/S ratio in the right precordial chest leads and a deep, narrow Q wave in leads I,  $aV_L$ ,  $V_5$ , and  $V_6$  (434). These abnormalities progress over time and are attributed to the finding that the greatest dystrophic myocardial changes are in the posterobasal and contiguous lateral left ventricular myocardium. In addition, P waves with negative terminal deflections in  $V_1$  exceeding 20 ms and 0.1 mV appear in 20–45% of patients and, in the absence of left atrial enlargement on echocardiogram, are attributed to an intrinsic disorder of left atrial or intra-atrial conduction. A short P–R interval may be seen in up to 50% of patients but is not thought to be due to a bypass tract as seen in WPW syndrome. Infranodal conduction abnormalities, however, may be seen in patients with DMD; these include complete or incomplete bundle-branch block and left anterior or posterior fascicular block. Atrial and ventricular premature beats and atrial flutter are seen in some patients.

Echocardiography reveals left ventricular dilation and dysfunction, with significantly reduced shortening fraction or ejection fraction, and left ventricular

hypokinesis of the posterobasal ventricular wall is identified (365,373,436). Doppler and color Doppler commonly demonstrate mitral regurgitation, secondary either to the DCM or to the associated MVP, which occurs secondary to papillary muscle dysfunction (437). In some patients, systolic function appears normal but diastolic dysfunction is present.

Histopathologic abnormalities of the heart and skeletal muscle are universal in patients with DMD, and those of skeletal muscle are widespread even in the early stages of disease (427,438). Typical findings are rounding of the muscle fibers, increased variability in fiber size, increased central nucleation, and fiber splitting. Necrotic and regenerating fibers are present along with large, round hyaline fibers. In the late stages, muscle may be virtually replaced by fat and fibrous tissue. In the heart, degenerative changes in muscle fibers and areas of fibrosis in the ventricles, atria, and conduction system occur, with most pronounced changes in the posterobasal region and adjacent lateral wall of the left ventricle. The underlying cause of cardiac disease is not currently known, but it is speculated that the gene defect in DMD leads to instability of the translated cytoskeletal protein, leading to weakening of the myocyte membrane and subsequent myocyte death due to mechanical stress (368,369,436).

**51.7.1.2 Genetics.** The dystrophin gene (Table 51-3), on the short arm of the X chromosome at Xp21 (427,439), may cause either low-level production of a nonfunctional protein or complete absence of dystrophin in the heart and skeletal muscle of affected patients when mutated. It is among the largest genes discovered thus far, comprising approximately 2.5 Mb and transcribing a 14 kb messenger RNA (mRNA) molecule (440–442). This cytoskeletal protein-encoding gene is normally expressed in striated and smooth muscle, as well as in brain. In muscle tissue, the dystrophin protein has been localized to the cytoplasmic surface of the sarcolemma and is associated with several integral membrane glycoproteins (443). This glycoprotein–dystrophin complex, which involves the sarcoglycans, dystroglycan, syntrophins, and dystrobrevins, connects dystrophin to the sarcolemma and links to the extracellular matrix (Figure 51-18); it may be involved in the regulation of intracellular calcium, which in dystrophin-deficient muscle is increased along with increased calcium channel transport (443–446).

The diagnostic approaches to DMD have changed dramatically in 2001–2010. Previously, serum CK-MM level and muscle biopsy were the standard approaches. Today, DMD is diagnosed primarily by molecular analysis (447), which is rapid and accurate and may predict clinical course. Most commonly, dystrophin mutations that cause a frameshift (441,448,449) of the nucleotide sequence result in the severe form of muscular dystrophy, DMD.

**51.7.1.3 Management.** Management of the CHF associated with the DCM seen in DMD is identical to that

used for patients with other causes of heart failure and dysrhythmias. Pacing is not usually necessary.

### 51.7.2 Becker Muscular Dystrophy

**51.7.2.1 Clinical Features.** BMD is an X-linked disorder that differs in both severity and time of onset from DMD (427,432), despite being due to allelic mutations in dystrophin, the gene responsible for DMD. BMD appears later and progresses more slowly than DMD, so that survival to middle age is seen. The pattern of muscle weakness, however, is identical to that in DMD, with early involvement of the pelvic girdle and proximal lower extremities (427). The initial signs of weakness usually appear during the second decade, but may occur as late as the third decade. The weakness gradually progresses, with the upper extremities becoming involved after 5–10 years. Patients generally remain ambulatory until their mid-30s. Similar to DMD, muscle hypertrophy is common; intellectual impairment, however, is less common and less severe. As in DMD, life expectancy is also reduced in BMD, with only 50% of patients surviving to 40 years of age.

Cardiac involvement may be seen in adolescence and ultimately affects 80% of patients (450). As in DMD, DCM and cardiac failure are the usual abnormalities encountered and are often the ultimate cause of death. Conduction abnormalities manifesting as fascicular block or complete heart block are also seen. As in DMD, muscle enzyme activity is markedly elevated in BMD, and preclinical cases may be detected by elevated CK-MM. Electromyographic examination shows a “myopathic” pattern with small, polyphasic motor units and early recruitment of motor units. The histology of BMD is similar to that of other forms of muscular dystrophy. In contrast to DMD, hyaline fibers are rarely seen. Electrocardiographic changes are similar to those seen in DMD. Other ECG abnormalities encountered include LAD, RBBB, LBBB, and complete heart block. The echocardiogram may demonstrate the features of DCM.

**51.7.2.2 Genetics and Management.** BMD is also due to mutations within the dystrophin gene; that is, it is allelic with DMD (Table 51-3). As is the case with DMD, more than 30% of patients with BMD have no family history of the disease, an indication that they represent spontaneous mutations. The phenotypic difference between DMD and BMD patients has been speculated to be due to frameshift mutations leading to more severe disease (DMD) while out-of-frame mutations cause less severe (BMD) disease (448,449,451,452). The frameshift hypothesis explains more than 90% of the cases of DMD versus BMD. The cardiac abnormalities in BMD, as with those described for DMD, require further study (381). However, cardiomyopathy is quite common (381,430,435,436,450,453,454). The treatment of CHF

and arrhythmias is similar to other patients with these signs and symptoms.

Animal models have been created during the past several years that help to characterize the roles of dystrophin and the associated complexes. Loss-of-function mutations of dystrophin lead to a DMD or BMD phenotype, while utrophin-deficient mice have defects in the postsynaptic membrane folds at the neuromuscular junction. Mice lacking both dystrophin and utrophin display a severe muscular dystrophy with premature death (455–458). Sarcoglycan-deficient mice also demonstrate severe muscular dystrophy but, in addition, severe HCM and/or DCM have/has been seen (459–461). This has recently been supported by the identification of  $\delta$ -sarcoglycan mutations in humans with FDCM by our laboratory (347).

Various methods evaluating the possibility of gene therapy for dystrophinopathies have been reported over the past several years in mice with varying degrees of success. Mini-gene and stem-cell transplantation have both been considered promising using dystrophin and utrophin (386a,462–467).

### 51.7.3 Limb-Girdle Muscular Dystrophy

**51.7.3.1 Clinical Features.** LGMDs are a heterogeneous group of muscle disorders first described in 1954 and characterized by predominant weakness and wasting of muscles of the pelvic and shoulder girdle (468). Facial and extraocular muscles are usually spared. There is a broad clinical heterogeneity, and this is paralleled by genetic heterogeneity (469,470). The heart is affected in several forms of LGMD, including cardiomyopathy (dilated and hypertrophic) and conduction system abnormalities (365,427,443). Onset can occur at any age, in childhood or adulthood, but usually has its onset after walking has started. Autosomal-dominant and autosomal-recessive forms of LGMD have been described, with the age of onset generally earlier in the autosomal-recessive forms (469).

**51.7.3.2 Genetics.** A classification scheme for the LGMDs has been devised that depends on the pattern of inheritance (469). The autosomal-dominant forms of LGMD are designated LGMD1 (LGMD1A–1D), whereas the autosomal-recessive forms are designated LGMD2 (LGMD2A–2G). This classification scheme is based on the inheritance and the specific gene causing the disorder (Table 51-4). The majority of recessive LGMDs are due to mutations in the dystrophin-associated protein subcomplex sarcoglycan (471–479), with LGMD2C, 2D, 2E, and 2F being caused by  $\alpha$ - (480),  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan, respectively (i.e. sarcoglycanopathies) (Table 51-3). Although quite variable in severity, these LGMDs tend to have a course that is often reminiscent of DMD in its severity. In all cases, cardiac disease can occur (365) and includes conduction system disease and cardiomyopathy (HCM and/or DCM). The other recessive



forms of LGMD are caused by mutations in calpain-3 (481,482), a muscle-specific calcium-dependent protease (LGMD2A), resulting in a somewhat milder course than the sarcoglycanopathies. Recently, LGMD2B was characterized as being caused by mutations in dysferlin (483,484), a novel muscle protein of uncertain function, and LGMD2G was found to be caused by mutations in the sarcomeric protein telethonin, a protein localized to the Z-disk of muscle (485). In this form, cardiomyopathy occurs (Table 51-4).

The autosomal-dominant forms of LGMD have been more difficult to identify thus far (486,487). The only gene identified to date is caveolin-3 (488), which causes LGMD1D. In LGMD1B, and 1C, and 1D, cardiac dysrhythmias can become evident concomitant with the initial stages of muscular weakness (Table 51-3). Cardiomyopathy is found as well (351,353) (Table 51-4).

Multiple animal models of the LGMDs have been reported, with most having cardiac disease. Cardiomyopathy and premature death consistently occur. In addition to the murine models, a naturally occurring hamster model with associated cardiomyopathy has been well described and occurs due to  $\delta$ -sarcoglycan mutation (366,460,461,489–491).

## 51.7.4 Emery–Dreifuss Muscular Dystrophy

**51.7.4.1 Clinical Features.** EDMD is a relatively rare disorder characterized by weakness in the humeroperoneal distribution, early joint contractures, and DCM (311a,492–495), with X-linked (493) inheritance. Occasionally, autosomal-dominant (496,497) or autosomal-recessive (498,499) inheritance is seen. The onset of disease in these patients occurs between 2 and 10 years of age, with weakness initially noted in the shoulder girdles and upper extremities. Contractures of the elbows and posterior cervical muscles appear early. The disease is slowly progressive, with involvement of the distal leg musculature following that of the upper extremities; contractures of the knees and ankles follow contractures of the elbows. Unlike DMD and BMD, muscle pseudohypertrophy does not occur (492). The disease evolves slowly and usually stabilizes in the third decade, with most patients remaining ambulatory. DCM is a common occurrence, but the severity of disease varies from family to family (492,493). Varying degrees of AV block are common, and atrial standstill may occur. These electrical abnormalities may lead to episodes of syncope, transient ischemic attacks, stroke, and sudden death. A pacemaker is commonly required. AF has also been observed. As in DMD and BMD, muscle enzyme activity is elevated, albeit to a lesser extent. Skeletal muscle biopsy histopathologic findings are similar to those associated with other forms of muscular dystrophy. Type I fiber atrophy has been described in some cases.

**51.7.4.2 Genetics.** The gene responsible for X-linked EDMD was localized to Xq28 (500) before being cloned

(501) (Table 51-3). The gene, called emerin (or STA), was shown to have an open reading frame of 762 nucleotides that encodes a serine-rich 254-amino-acid protein with probable mechanical/structural function (501). Emerin mRNA shows ubiquitous tissue distribution, with the highest expression in skeletal and cardiac muscles. The complementary DNA sequence of emerin predicts a tail-anchor membrane protein with amino acid sequence similar to thymopoietins, a group of nuclear lamina-associated proteins (502). Nagano et al. (331a) and Manilal et al. (503) both showed that emerin is a 34kDa nuclear membrane protein in skeletal and cardiac muscle that is absent in EDMD. Emerin belongs to a family of type II integral membrane proteins that are anchored to the inner nuclear membrane via hydrophilic tails, with the remainder of the molecule projecting into the nucleoplasm (503,504). This family includes lamins, lamin receptor, and thymopoietin  $\beta$ . Emerin is also found at intercalated disks and is thought to result in conduction defects due to this localization (505) and its interactions with desmosomes and fascial adherans.

The autosomal-dominant form of EDMD was initially mapped to chromosome 1 (1q11–q23) and the gene was identified as lamin A/C (357) (Table 51-3). The encoded protein is also a nuclear lamina-associated protein and is expressed in myocytes and adipocytes. In the nuclear inner membrane, this protein has a distribution that mirrors that of emerin (506). The phenotypic spectrum of this gene appears to be broad when mutated. In some cases, only a DCM phenotype with conduction disease (356,387) occurs (in the absence of clinical skeletal muscle disease), while in other cases, familial Dunnigan-type lipodystrophy (507) occurs. It is believed that the nuclear membrane either plays a critical communication role or a structural role through interactions with cytoskeletal proteins (506,508).

**51.7.4.3 Management.** The DCM associated with EDMD is treated with the usual anticongestive medical regimen, and conduction disease typically requires a pacemaker.

## 51.7.5 Myotonic Dystrophy

The most common adult-onset muscular dystrophy in humans, DM, is a progressive, multisystem disorder that manifests as a highly pathologic phenotype of the skeletal muscle, smooth muscle, brain, lens, testicular function, glucose metabolism, and heart (509,510). The variable phenotype in DM is further expanded by clinical “anticipation,” a phenomenon in which the severity of symptoms increases while the age of disease onset decreases with each successive generation within a family (511). Thus, the spectrum of DM symptoms can vary from mild, where only cataracts develops late in life, to a severe congenital form associated with skeletal muscle hypotonia, often resulting in respiratory arrest and death. An intermediate adult-onset form also occurs,



which manifests with one or more characteristic feature of DM, including myotonia (an abnormal muscle membrane depolarization resulting in abnormally delayed muscle relaxation time after strong muscle contraction), skeletal muscle weakness and wasting, cardiac conduction system disease with or without DCM, cataracts, endocrine dysfunction, frontal balding, epitheliomas, and mental retardation (Table 51-5).

**51.7.5.1 Clinical Features.** Although a range of skeletal muscles can be affected in DM, fast-twitch muscles are usually more severely affected than slow-twitch muscles. During the course of the disease, it is common to find early involvement of facial and neck muscles, with later involvement of extensor forearm and anterior tibial muscles, and muscles of the hands and feet. The heart is affected in approximately 80% of patients (512,513), and sudden death occurs with a high incidence (15–30%). Cardiac abnormalities are primarily conduction disorders, which occur in 45–80% of DM patients, and the increased incidence of sudden cardiac death occurs from either complete AV block or ventricular dysrhythmias (514–516). Disorders of impulse formation in the sinus node also occur, and a significant number of patients exhibit sinus bradycardia (Table 51-5). The cardiac

conduction abnormalities typically have a gradual but predictable increase in severity, often progressing from intermittent to stable first-degree AV block followed by more serious intraventricular block, including bundle-branch blocks and ultimately progressive AV block to complete heart block (514,517,518). Ventricular dysrhythmias, including VT, may also occur.

A rough correlation exists between the severity of the neuromuscular disease and progression of the cardiac features. DM is occasionally associated with overt cardiac failure, with 28% of patients having systolic dysfunction (519). MVP is seen in 17–32% of patients as well (520,521). Similar to conduction abnormalities, echocardiographic abnormalities usually are more prevalent in older patients with severe neuromuscular symptoms.

**51.7.5.2 Genetics.** Myotonic dystrophy, because of its variable presentation, may be underdiagnosed, and therefore, only conservative estimates of incidence can be made. Currently, it is believed that the incidence is 4.5–5.5 per 100,000 live births (522). DM is inherited as an autosomal-dominant disorder but does demonstrate two unique non-Mendelian features. Both the sex and the position of the parent within the pedigree affect the severity of disease in the subsequent generation due to genetic anticipation (511). In addition, the adult-onset form is inherited more frequently through the father, while congenital DM is almost exclusively maternally inherited.

The gene responsible for DM was initially mapped (523) to the long arm of chromosome 19 (19q13.3), and subsequently, the *DM* gene was identified as myotonin protein kinase (524–526), a serine/threonine kinase (DMPK) (527) (Table 51-3). The causative mutations are CTG repeat sequence expansions in the 3'-untranslated region of DMPK, which cause alterations in the expansion of neighboring genes when large enough (528,529). The probability and amplitude of the expansion increases as a function of repeat tract length, and the severity of the disease correlates with length of the repeat sequence (530). Two distinct patterns of CTG expansion have been described. Small changes in repeat size appear to predominate at repeat tract lengths <150 bp (35 repeats) in length. Such changes in tract size are reflected in the range of repeat tracts observed in the normal population (5–35 repeats). Once a threshold of >50 repeats is reached, however, the pattern of instability appears to change dramatically, causing the frequency of large repeats (>200 repeats) to greatly increase. In congenital DM, enormous expansions occur, reaching values >10 kb.

It is currently believed that nuclear retention of mutant DMPK transcripts occur in the nuclei of DM fibroblasts and muscle, causing a decrease in the pool of translatable DMPK mRNA in the cytoplasm (531). Titration of CUG-BP (a novel CUG-binding protein believed to play a role in processing and/or transport of

**TABLE 51-5 Systemic Involvement in DM**

<b>Muscle</b>	
Skeletal muscle	Myotonia, weakness and dystrophy predominately of facial, neck, and distal limb muscles
Heart	Conduction disorders more prominent than overt cardiomyopathy (dilated). Conduction abnormalities include disorders of impulse formation (sinus bradycardia, prolonged SA node recovery time, SA node dysfunction); disorders of impulse conduction (AV block including primary through complete AV block; intraventricular conduction defects including fascicular block, bundle branch block); and dysrhythmias, including premature atrial beats, atrial fibrillation and flutter, premature ventricular beats, and VT.
Smooth muscle	Widespread involvement of pharynx, esophagus, colon, and uterus. Delayed relaxation and abnormal peristalsis.
<b>Central nervous system</b>	
Brain	Mental retardation, hypersomnolence
Eye	Cataracts, decreased vision independent of cataracts
<b>Endocrine</b>	
Testis	Gonadal atrophy
Metabolism	Abnormal carbohydrate metabolism
<b>Skeletal/Skin</b>	
Skeletal	Cranial and facial abnormalities, talipes
Skin	Premature balding, calcifying epithelioma
<b>Lungs</b>	
Respiration	Hypoventilation

AV, atrioventricular; SA, sinoatrial.

CUG-encoding mRNAs) by the mutant DMPK message could allow DMPK levels to drop below 50% of normal (532–534). Reddy et al. (535) developed a mouse model, in which DMPK is functionally inactivated (DMPK<sup>-/-</sup>) and showed that loss of DMPK results in late-onset skeletal myopathy characterized by 30–50% loss in muscle twitch and tetanic force development by 11 months of age. Ultrastructural changes included Z-line loss, mitochondrial and sarcoplasmic reticulum abnormalities, and loss of sarcomere organization, suggesting that impaired excitation–contraction coupling or contractile apparatus abnormalities underlie the progressive muscle weakness seen in DM. Although DMPK is clearly an important component of muscle, loss of its expression cannot explain the complete DM phenotype. Timchenko (534) has suggested that RNA CUG repeats play a significant role in the development of the full phenotype. Wang et al. (536) initially demonstrated that DMPK poly(A)<sup>+</sup> RNA is significantly reduced in muscle biopsies from DM patients although total RNA had normal DMPK transcript levels, and suggested that the repeat expansion caused abnormalities in RNA processing. Timchenko et al. (537) further suggested that CUG repeat-containing DMPK RNA could affect processing of other mRNAs, perhaps by sequestering specific RNA-binding proteins, and thereby causing tissue-specific abnormalities.

Genetic heterogeneity exists for DM as well. Ranum et al. (538) identified a second locus for DM, called DM2, on chromosome 3q. Although the gene at this locus is not yet known, it is likely that DM2 could be associated with CTG repeats in a gene different from (but similar to) DMPK or with another type of triplet repeat expansion, and that RNA processing could modify the clinical features of this gene as well.

Finally, animal models have been created that mimic the clinical findings in humans (535,539–541). These animals continue to be studied in order to further unravel the molecular physiology responsible for this disorder.

**51.7.5.3 Management.** Management of DM involves the treatment of the skeletal myopathy with occupational and physical therapy and, when needed, use of a wheelchair. Cardiac abnormalities require pacemaker implantation for the conduction disorder and anticongestive therapy for patients with heart failure.

## 51.7.6 Friedreich Ataxia

**51.7.6.1 Clinical Features.** Friedreich ataxia is the most common of the hereditary spinal cerebellar degenerations, with an incidence of 1 in 50,000 and a carrier frequency of 1 in 110 (542). This autosomal-recessive form of spinocerebellar degeneration is characterized by progressive limb ataxia, loss of deep tendon reflexes, sensory abnormalities, and musculoskeletal deformities. The symptoms of Friedreich ataxia usually appear insidiously during childhood or early adolescence. Progressive weakness of the upper

and lower extremities gradually becomes obvious. Gait difficulties are often the first symptom, which progress slowly followed by unsteadiness in the arms and hands. Difficulty in writing and handling eating utensils subsequently becomes apparent.

Cardiac involvement (542) occurs in 50–90% of patients, and the most common abnormality is HCM; DCM occurs rarely. Thus, the most common cardiac symptoms relate to cardiac failure and dysrhythmias (543–545). Left ventricular outflow tract obstruction due to asymmetrical septal hypertrophy may be evident, and approximately 50% of patients die of cardiac disease. Patients are followed for development of dysrhythmias and the signs and symptoms of cardiac failure.

Involvement of the heart is readily detected by ECG and echocardiography (544). The ECG abnormalities are found in 90% of patients and include repolarization abnormalities manifesting as inverted or biphasic T waves in the inferior limb leads and left precordial leads, a short P–R interval, left and right ventricular hypertrophy, and left and right axis deviation. Premature atrial contractions, atrial flutter/fibrillation, and premature ventricular contractions are common (543,544). Echocardiography detects cardiac involvement in 60–100% of patients, with the most common finding being concentric hypertrophy, but asymmetrical septal hypertrophy accompanied by systolic anterior motion of the mitral valve is also common. Left ventricular chamber diameter may be normal or decreased, and ejection fraction or shortening fraction is usually normal or depressed, although DCM (left ventricular dilation and reduced contractility) is seen occasionally. There is no specific treatment for the cardiac manifestations except symptomatic treatment if cardiac failure ensues.

**51.7.6.2 Genetics.** Friedreich ataxia is inherited as an autosomal-recessive disorder, and parental consanguinity has been noted in some cases. The gene was initially mapped to chromosome 9q13–31.1 (546) and, in 1996, the gene was identified (547). This gene is 40 kb, contains five exons, has a 1.3 kb transcript, and encodes a 210-amino-acid protein called frataxin (Table 51-3). The highest level of expression is within the heart, while intermediate levels are seen in liver, skeletal muscle, and pancreas; minimal levels are identified in other tissues, including the brain. Although a few affected patients were found to have a point mutation of frataxin, the majority (~95%) are homozygous for an unstable GAA trinucleotide expansion in the first intron (547). The remaining patients are compound heterozygotes for the expansion. In patients homozygous for the expansion, there is a correlation between the number of GAA repeats on the smaller allele, age of onset, disease progression, and cardiomyopathy (223a,542,548–550), confirming that the expansion is the primary cause of disease. The expansion results in severely reduced levels of mature frataxin mRNA (547). Using immunocytofluorescence and immunocytoelectron microscopic evaluation,

Campuzano et al. (551) demonstrated that frataxin is localized to the mitochondria, associated with the mitochondrial membranes and crests. They suggested that reduction in frataxin results in oxidative damage. Subsequently, Rotig et al. (552) suggested that frataxin regulates mitochondrial iron transport and that deficiency of iron–sulfur cluster-containing subunits of mitochondrial respiratory complexes I and II and the iron–sulfur protein aconitase occurs. Hence, it appears that Friedreich ataxia is a mitochondrial disorder (362a,553). As these patients have HCM, diabetes, ataxia, and apparent free radical toxicity, the mitochondrial basis of this disorder clarifies the clinical features.

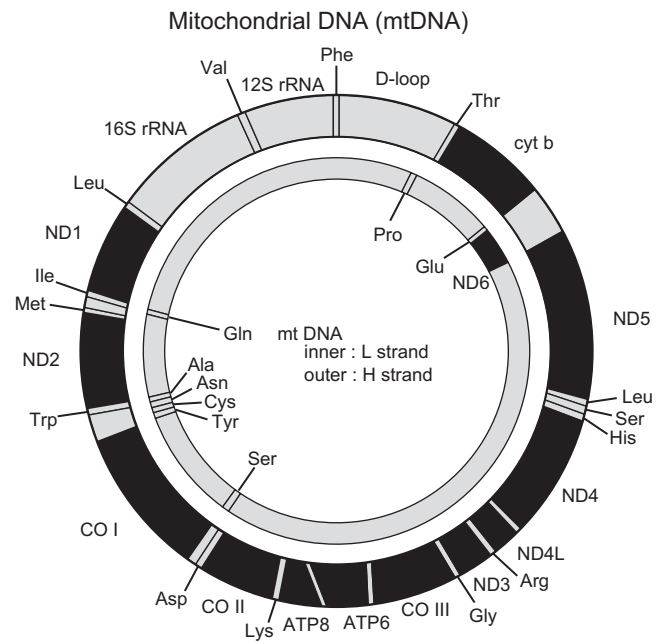
**51.7.6.3 Management.** The management of this disorder is symptomatic. The HCM is typically treated with a  $\beta$ -blocker or calcium-channel blocker, while the other symptoms are increasingly treated with mitochondria-based therapy, such as vitamins and free-radical-reducing agents (i.e. coenzyme Q10, ibedenone, riboflavin, thiamine, vitamin K).

## 51.7.7 Mitochondrial Disorders

Mitochondria are intracellular organelles that participate in several metabolic pathways, including ATP synthesis by oxidative phosphorylation (333,554,555). Mitochondria are unique organelles because they contain a genome that encodes some of the polypeptides in the oxidative phosphorylation pathway and all the transfer RNA (tRNA) and ribosomal RNA (rRNA). Nuclear genes encode the remaining mitochondrial proteins. Mitochondria self-replicate and are derived from oocytes. Thus, mutations in the mitochondrial genome are maternally inherited (554).

**51.7.7.1 Clinical Features.** Cardiac abnormalities are common in mitochondrial disorders and include conduction defects, HCM, DCM, and combined HCM/DCM, which may in part represent progression from the hypertrophic form (556,557). Leber hereditary optic neuropathy, a disorder in which there is a loss of central vision due to degeneration of the retinal ganglion cells and the optic nerve axons, commonly has associated WPW syndrome (404a,558–561). Other mitochondrial mutations are also associated with WPW syndrome; these include MELAS syndrome (mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes) (562,563) and MERRF (myoclonic epilepsy with ragged red fibers) syndrome (564,565). Another mitochondrial myopathy associated with cardiac disease is Kearns–Sayre syndrome, in which DCM and conduction disease are common findings (566).

**51.7.7.2 Genetics.** The human mitochondrial genome (567) is a small circular DNA molecule (Figure 51-24) that is maternally inherited. Mitochondrial DNA (mtDNA) encodes 13 of the 69 proteins required for oxidative metabolism, 22 tRNAs, and two rRNAs required for their translation. As mtDNA has much less redundancy than



**FIGURE 51-24** Mitochondrial DNA, a small circular molecule that encodes 13 of the 69 proteins required for oxidative metabolism, 22 tRNAs, and 2 rRNAs.

the nuclear genome (in which essentially identical information is received from both parents), and tRNAs and rRNAs are present in multiple copies, the mitochondrial genome is an excellent target for mutations giving rise to human disease (568,569). Mitochondria are dependent on nucleocytoplasmic mechanisms for most structural components, but do contribute vital peptides that are central to cellular respiration. The electron transport chain, which generates cellular ATP, is organized into complexes I–IV and the ATP synthase (complex V) (Figure 51-25). The 13 mtDNA genes that encode enzymes in the respiratory chain include seven complex I (569,570) subunits (ND1, 2, 3, 4L, 4, 5, and 6); one complex III subunit (cytochrome b); three complex IV subunits (COI, II, and III); and two complex V subunits (ATPase 6 and 8) (571–573). Each cell contains numerous mitochondria, and each mitochondrion contains multiple copies of mtDNA. In most mitochondrial disorders, patients carry a mix of mutant and normal mitochondria—a condition known as *heteroplasmy*, with the proportions varying from tissue to tissue and individual to individual within a pedigree, in a manner correlating with severity of phenotype.

Mitochondrial diseases often produce disturbances of brain and muscle function, and are usually evident during infancy or early childhood (555). Cardiac disease is most commonly seen with respiratory chain defects (556). Ragged red fibers are present in muscle biopsy specimens (574) almost invariably when the molecular defect involves mtDNA (564,575) (Table 51-3). These defects represent the genetics of ATP production. The diverse clinical syndromes associated with various respiratory chain complexes are thought to result from involvement of tissue-specific isoforms in some cases,



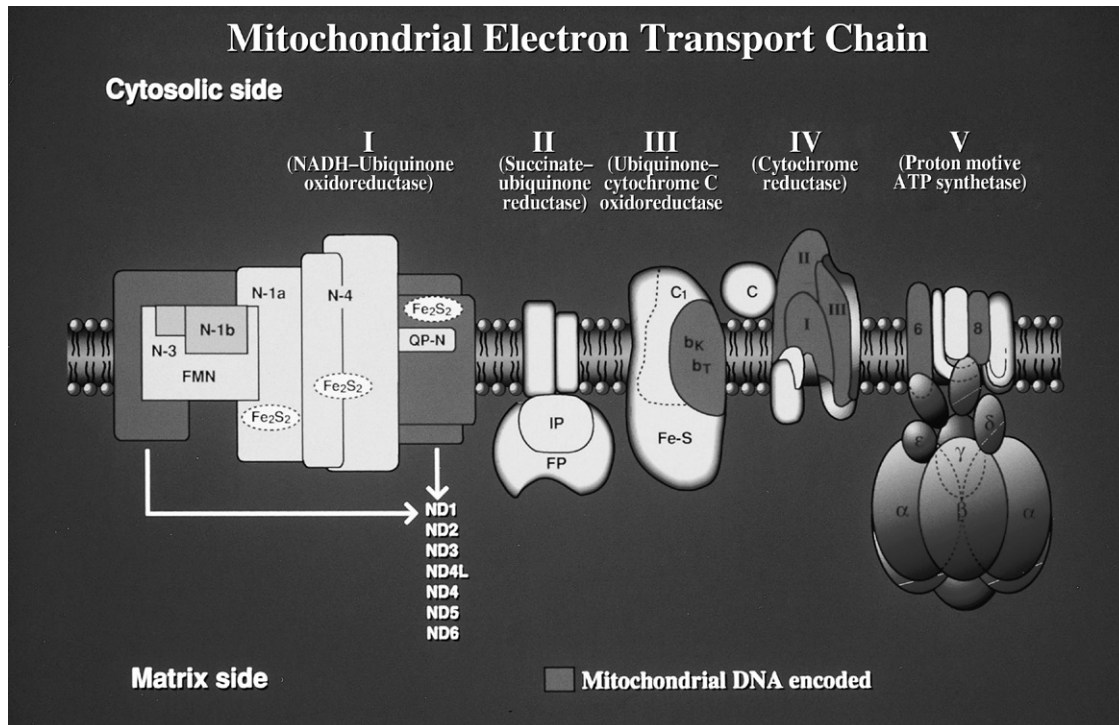


FIGURE 51-25 Oxidative phosphorylation pathway.

involvement of tissue-nonspecific (generalized) subunits in other cases, and the residual enzyme activity in affected tissues (575). The cardiac diseases seen associated with mitochondrial defects include both HCM and DCM (266a,556,569,576).

Mitochondrial gene mapping, in contrast to mapping of the nuclear genome, does not require genetic linkage. One simply has to show that the disease exhibits transmission through all mothers and no fathers in a sufficiently large family. Once this is established, the mitochondrial genome can be sequenced to identify the mutation, which must be shown to segregate with the disease, as there are many apparently harmless polymorphisms.

**51.7.7.3 Management.** Therapy for these disorders is generally symptom based. Conduction disturbance generally requires placement of a permanent pacemaker, and heart failure is treated with the usual therapy. In some patients,  $\beta$ -blockers may be useful. Hypertrophic heart disease is usually treated in a manner similar to that for other forms of HCM. Mitochondrial-based therapy may include coenzyme Q10, carnitine, or vitamins, but these therapeutic approaches typically do not alter the clinical course.

**51.7.7.4 Kearns-Sayre Syndrome.** This mitochondrial myopathy is characterized by ptosis, chronic progressive external ophthalmoplegia, abnormal retinal pigmentation, and cardiac conduction defects, as well as DCM (566). Hearing loss and limb weakness are frequently associated, as are endocrinopathies such as diabetes mellitus, hypoparathyroidism, and growth hormone deficiency. Approximately 20% of Kearns-Sayre syndrome patients have cardiac involvement and,

of these, the majority usually has conduction defects causing progressive heart block (388,577,578). These patients generally have large heterogenous deletions in the mitochondrial genome, of which tRNA<sup>leu(UUR)</sup>-3243 is most common.

Clinically, conduction abnormalities, bifascicular block, or progressive high-grade block may define the requirement for permanent pacemaker implantation. Symptomatic improvement using mitochondrial therapies may occasionally be seen with coenzyme Q10 therapy. The major function of coenzyme Q10 in mitochondria is to shuttle electrons from complexes I and II to complex III, while stabilizing the respiratory chain complexes. Vitamins such as phyloquinone (vitamin K<sub>1</sub>), menadione (vitamin K<sub>3</sub>), and ascorbic acid (vitamin C) have been used to donate electrons directly to cytochrome c. In addition, the endocrine abnormalities and heart failure should be treated in the usual way.

**51.7.7.5 LEOPARD Syndrome.** LEOPARD syndrome is an inherited disorder involving multiple organ systems. The acronym represents the abnormalities characteristic of this syndrome: multiple lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness. The conduction abnormalities include first-degree AV block, intraventricular conduction delay, and bundle-branch block (579,580). Progression to complete heart block is generally not seen. LEOPARD syndrome has been shown to be genetically heterogenous. A report of mapping to chromosome 17 with identification of the neurofibromin gene is documented (Table 51-3). In addition, mutations



in the *PTPN11* gene, which causes Noonan syndrome, have been shown to cause LEOPARD syndrome in a substantial percentage of patients (581). Therapy is supportive. HCM occurs in some patients with this disorder. A gene for LEOPARD syndrome was initially reported to map to chromosome 17, with subsequent identification of the neurofibromin gene. More recently, *PTPN11*, the gene identified for Noonan syndrome, has been shown to cause LEOPARD syndrome (157a,297a,582). Clearly, however, other currently unidentified genes for this disorder exist (583).

## 51.8 CONGENITAL HEART DISEASE AND DYSRHYTHMIAS OR CONDUCTION DISEASE

### 51.8.1 Atrial Septal Defects

**51.8.1.1 Clinical Features.** Atrial septal defects (ASDs) are common congenital heart malformations that account for more than 10% of isolated congenital heart defects (584). Ostium secundum ASD located in the region of the fossa ovalis is the most prevalent subtype and one thought to arise by malformation of the septum primum. This results in incomplete coverage of the fossa ovalis and shunting across the defect. Blood flow through the ASD depends on a variety of physiologic factors, including the ratio of left-sided (systemic) to right-sided (pulmonary) resistance, the ratio of left ventricular to right ventricular compliance, and defect size. Uncorrected ASDs can lead to increased pulmonary blood flow with secondary development of pulmonary artery hypertension, right ventricular hypertrophy, right-sided volume overload, heart failure, and death. In addition, ECG abnormalities are common and include variable degrees of AV block, atrial tachydysrhythmias (such as AF or atrial flutter), and abnormal ECG findings, including RBBB, right axis deviation, and right ventricular hypertrophy with strain (585,586).

**51.8.1.2 Genetics and Management.** Although most cases of ASD are sporadic, autosomal-dominant familial transmission has been well reported, particularly ASD associated with conduction system defects (584,587–589). Age-related penetrance has also been noted. Linkage analysis initially identified a locus on chromosome 5q35 (Table 51-3), and subsequently mutations in the transcription factor *Nkx2-5* were identified (590–592). Mutations in this gene were thought to destabilize the protein or disrupt the ability of *Nkx2-5* to target DNA binding. *Nkx2-5* defects appear to cause haploinsufficiency of a transcription factor meant to play a central role in cardiac morphogenesis, including the atrioventricular node. A second locus (5p) has been (was later) identified (Table 51-3), but the gene remains elusive (590). (Most recently, Robinson et al. (593) identified a locus on chromosome 3p25 in isolated cases and found mutations in the cell adhesion molecule *CRELD1*. These

authors also noted mutations in *CRELD1* in subjects with heterotaxy and ASD.)

Therapy of ASDs includes diuretic therapy when significant left-to-right shunting occurs. In patients with conduction disease, pacemakers may play a role.

### 51.8.2 Holt–Oram Syndrome

Da Vinci was the first to recognize that communication between right- and left-sided cardiac chambers resulted from defects in the atrial and ventricular septa. Approximately 100 years later, an association between congenital cardiac malformations, particularly septal defects, and upper limb deformities was first recognized (594), but it was not until 1960 that familial cases of a heart–hand syndrome were described by Holt and Oram (595). Holt–Oram syndrome (HOS) is characterized by malformed thumbs associated with ASDs, normal intelligence, and normal (i.e. nondysmorphic) features. Essentially all individuals with HOS exhibit some upper limb skeletal abnormality in the developmental distribution of the preaxial radial ray (596,597). These skeletal deformities may be bilateral and asymmetrical or unilateral and, in either limb, may be mild and subclinical or severe and overt. The most common is malformation of one or more carpal bones. The wrist bones may be absent, misshapen, or fused. Another feature, which has been considered to be a typical and expected finding, is a triphalangial or hypoplastic/absent thumb. Some children present with severe foreshortening of the radius that extends proximally in a preaxial distribution to include the humerus and shoulder girdle and may present as phocomelia.

Approximately 75% of individuals with HOS have cardiac manifestations (596) that vary in magnitude from severe to clinically insignificant and are not dependent on the severity of skeletal disease. The cardiac abnormalities may manifest as congenital cardiac structural disease or conduction disease. Most commonly, individuals present with ASDs (usually ostium secundum ASD); many individuals, however, have ventricular septal defects (VSDs), typically in the membranous portion of the ventricular septum. Rarely, more complex congenital heart disease (i.e. tetralogy of Fallot, truncus arteriosus, hypoplastic left heart syndrome, heterotaxia, total anomalous pulmonary venous return) occurs (598–600).

Cardiac conduction abnormality may occur in the presence or absence of structural heart disease, and is commonly progressive. It may manifest initially as sinus tachycardia with mild first-degree AV block, but, over time, the function of the sinus node can deteriorate and, in addition, AV node block can occur. High-grade heart block and AF may occur in patients with HOS (600).

**51.8.2.1 Genetics and Management.** HOS has autosomal-dominant inheritance with complete penetrance (601). The expression of the syndrome, as noted previously, is quite variable, however. The prevalence is believed to be approximately 1 per 100,000 live births.

As 85% of these are new mutations, the mutation rate is calculated to be 1 per million (602).

No consistent familial cytogenetic abnormalities have been associated with HOS although karyotypic abnormalities have been reported in sporadic cases (603,604). Linkage analysis initially identified a locus on chromosome 12q2 in families with HOS (Table 51-3), and subsequent refining studies localized an *HOS* gene to chromosome 12q24.1 (605,606). Cloning of this region identified the human *TBX5* gene (Table 51-3) within this region of the genome, and mutation analysis identified disease-causing mutations (596,607–610). The gene is expressed in skeletal and cardiac tissue, with *TBX5* protein found in the atria, and, similar to the *Nkx2-5* mutations in familial ASD, ventricles and haploinsufficiency appear to be the mechanism of disease in many patients (608,611). In others, it is speculated that dominant-negative effects occur by stoichiometric inhibition of *TBX5* binding to DNA. Genetic heterogeneity exists (609), but the remaining genes are currently unknown.

The management of HOS focuses on treatment of the left-to-right shunt in patients with ASDs or VSDs (potentially requiring diuretics) and ultimately closure of the defect. When more severe congenital heart disease occurs, surgical palliation is required. The conduction disorder must also be monitored closely, and in some cases, a pacemaker is needed.

### 51.8.3 Laterality Defects

The clinical presentation of children with laterality defects is varied (332a,612) and includes abnormal visceral and atrial situs resulting in situs inversus or heterotaxy, abnormal looping of the cardiac tube resulting in ventricular inversion, and abnormal positioning of the heart in the chest (i.e. dextrocardia) (613,614). In some cases, complex cardiac anomalies occur and may be associated with conduction disease (615,616). Conduction defects occur most often in patients with heterotaxy or with “corrected” (left) transposition of the great arteries (L-TGA).

**51.8.3.1 Heterotaxy Syndrome.** The fundamental characteristics of heterotaxy syndrome include abnormal symmetry of the viscera and veins (such as lungs, liver, and vena cava) and situs discordance between various organ systems, as well as between the various segments of the heart (332a,612,617,618). Patients with heterotaxy have a random orientation of various organs, including asplenia or polysplenia syndrome (619) and loss of pulmonary and cardiac asymmetry (isomersion) at some levels, resulting in apparent bilaterally right- or left-sidedness, respectively. Patients with heterotaxy syndromes tend to have significant noncardiac midline defects as well (620,621).

In asplenia syndrome, both lungs are frequently trilobed, both bronchi are eparterial, the spleen is hypoplastic or absent, and the liver commonly overrides

the midline. The cardiac abnormalities found in these patients include right atrial appendage isomerism, common atrium, complete AV canal, double-outlet right ventricle, pulmonic stenosis, TGA, and anomalous pulmonary venous connections (332a,612,622). Atrioventricular block rarely occurs. Several reports of bilateral sinus nodes have been published, and dual AV nodes appear to predispose these patients to SVT, particularly in patients with two ventricles (623). The mechanism for SVT has been postulated to be “AV nodal to AV nodal” reentry, with the retrograde accessory pathway for SVT mapped to an anterior AV node, left lateral pathway, or tricuspid valve.

Polysplenia syndrome includes bilateral left-sidedness with mirror-image lungs (i.e. both lungs have the appearance of the left lung, including two lobes and hyparterial bronchi), anomalous pulmonary venous return, and renal-to-hepatic inferior vena cava segment absence with return of blood from the lower body occurring via the azygos or hemiazygos system (624). Bilateral left atrial appendages may occur, and typical cardiac defects include ASD, VSD, pulmonic stenosis, AV canal, and others. Unlike asplenia, conduction disturbances are common in polysplenia (625), including congenital complete AV block in 20–30% of cases (626,627). The mechanism for AV block has been postulated to be discontinuity between the AV node and the ventricular conduction pathway, usually at the level of the penetrating bundle (628).

**51.8.3.2 Ventricular Inversion.** Isolated ventricular inversion is relatively rare, with the vast majority of patients having associated congenital heart disease. In 99% of cases of ventricular inversion, TGA, resulting in physiologically corrected transposition (L-TGA), is associated (332a,629,630). In addition to L-TGA, other lesions include abnormal left-sided systemic tricuspid valves, malformations of the left-sided systemic right ventricle, VSD, pulmonic stenosis, and dextrocardia. Rhythm disturbances are frequently seen, including preexcitation and atrial tachydysrhythmias, including AF or atrial flutter or SVT (629). In some cases, the dysrhythmias occur because of AV valve regurgitation or ventricular dysfunction, while other cases are due to accessory pathways. Heart block is also common, even in patients with L-TGA only (i.e. no other associated cardiac lesions) (631,632). The site of block may be above, within, or below the His bundle. The AV node is usually located anteriorly in the right atrium of patients with L-TGA that connects right atrium to the His bundle, which encircles the anterior lateral region of the pulmonary valve. In most cases, the posterior AV node is hypoplastic.

#### 51.8.3.1 Genetics.

**51.8.3.1.1 Mouse Models.** Mouse models of aberrant left–right asymmetry have been useful in dissecting the embryologic and genetic basis of laterality. The *inversus viscerum* (iv) mouse, in which heterotaxy occurs

in 50% of homozygous progeny (with the remainder developing normal visceral situs), is due to mutations in the left–right dynein (*lrd*) gene, an axonemal dynein heavy chain gene that is expressed in the embryonic midline along the node and rostral floorplate (633). Another model, the *inv* mouse, has situs inversus in 85% of cases and {of} heterotaxy in 15% of cases of homozygous offspring; in this mouse, the defective gene is an ankyrin-repeat protein called *inversin* (634).

Other mice thus far developed include a model in which *lefty*, a ligand in the transforming growth factor- $\beta$  (TGF- $\beta$ ) pathway that is important for left–right determination, is selectively disrupted (635). In this model, thoracic left isomerism with bilateral left lungs and left atria occurs and appears similar to human polysplenia syndrome. Mice with mutations in the activin receptor type IIB gene develop abnormal spleens and other signs of heterotaxia, as well as bilateral right-sidedness of the pulmonary and atrial anatomy consistent with human asplenia syndrome (636).

**51.8.3.1.2 Human Genetics.** To date, a number of genes have been identified for laterality defects (637). In X-linked heterotaxy, Gebbia et al. (638) identified mutations in *Zic3*, a zinc-finger transcription factor that functions in the early stages of left–right body axis formation (Table 51-3). In autosomal-dominant forms of disease, mutations in the human activin type IIB gene (639), *NODAL*, *LEFTY* (640), and *HNF-3 $\beta$* , all transcription factors (Table 51-3) that are members of the TGF- $\beta$  pathway, have been identified in patients with laterality defects (641,642). (Most recently, Robinson et al. (593) identified mutations in *CRELD1*, a cell adhesion molecule mapped to chromosome 3p25, as causative of heterotaxy associated with ASDs.)

## 51.8.4 Familial MVP

**51.8.4.1 Clinical Features.** MVP is a very common clinical diagnosis, particularly in young, healthy women (329a). The diagnosis is usually made on the basis of auscultatory findings of a midsystolic click with or without a mitral regurgitation murmur heard at the cardiac apex (329a,361a). Echocardiography may identify the valve cusps prolapsing into the left atrium during systole and the redundant valve leaflets; in cases of mitral regurgitation, Doppler and color Doppler may clearly demonstrate the regurgitant jet. Typical symptoms include chest pain, palpitations, and anxiety. Dysrhythmias are common. The dysrhythmias occurring in the MVP syndrome include atrial premature complexes, nonsustained atrial tachycardia, and nonsustained ventricular ectopy. It has been speculated that MVP has an associated increased risk of sudden death, possibly due to dysrhythmias; severe mitral regurgitation and ruptured mitral chordae tendineae have also been cited as causes of sudden death. In addition, these patients have an increased risk of endocarditis.

**51.8.4.2 Genetics and Management.** MVP is commonly inherited, with autosomal-dominant transmission seen (643). Expression may be variable, depending on age and gender. Full expression is found in adolescent and young adult women (11–50 years of age), affecting approximately 50% of this population in families with MVP. On the other hand, only 10–30% of older women, older men, and children in affected families demonstrate MVP, consistent with incomplete penetrance.

Linkage to chromosome 16 at 16p11.2–p12.1 (Table 51-3) was first identified in families with MVP in 1999 (644). Interestingly, males and females were equally affected clinically. This gene for this form, *MVP1*, has not yet been identified, but we speculated that a connective tissue protein-encoding gene would be at the root of the abnormality (645). MVP commonly accompanies connective tissue disorders, such as Marfan syndrome and Ehlers–Danlos syndrome, which have been shown to occur because of mutations in fibrillin and various collagens. It is likely that MVP syndrome is a forme fruste of a connective tissue disorder (645). More recently, linkage has been reported in familial MVP (MVP2) on chromosome 11p15.4 (646), while a third locus on Xq28 was reported in a familial form of inherited myxomatous valvular dystrophy (647) in which degeneration of the aortic valve was also noted. The genes for MVP2 and the X-linked disorder have not yet been identified.

## 51.9 SUMMARY

The genetics of cardiac diseases, including those in which dysrhythmias are primary or secondary findings, has come of age as the twenty-first century begins. As the Human Genome Project comes to completion, it is expected that further understanding of the genes responsible for the disorders of the heart will also be better understood. In the next decade, the relationships between mutated genes, modifier genes, and environmental factors are likely to be important areas of study that will enable better therapies to be developed.

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### Biography



Reed Pyeritz completed his PhD and MD at Harvard and trained in internal medicine at the Peter Bent Brigham Hospital in Boston and medical genetics at the Johns Hopkins Hospital in Baltimore. He is a professor of Medicine and Genetics at the Raymond and Ruth Perelman School of Medicine of the University of Pennsylvania in Philadelphia, where he directs the Center for the Integration of Genetic Healthcare Technologies and serves as vice-chair for academic affairs of the Department of Medicine. He is a senior fellow in both the Center for Bioethics and the Leonard Davis Institute for Health Economics.

His research has focused on several areas, including clinical investigations of heritable disorders of connective tissue and cardiovascular diseases, especially those affecting the thoracic aorta. Currently he is studying how more refined methods of investigating genetic variation, such as cytogenomic arrays and whole genome sequencing, actually increase the level of uncertainty in applying the results clinically.

# CHAPTER

# 52

## Genetics of Blood Pressure Regulation

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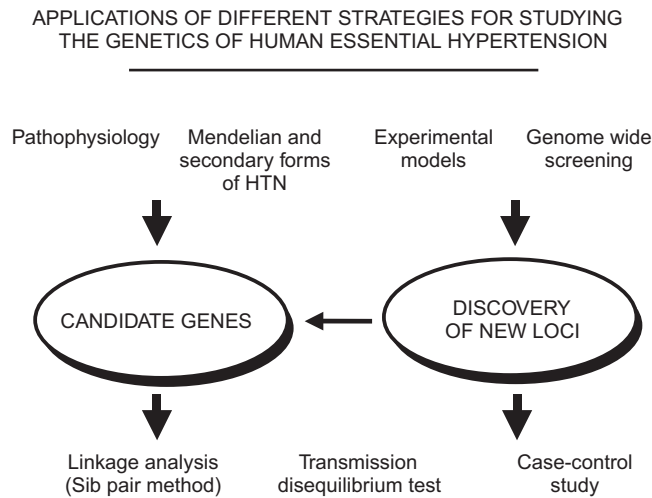
### 52.1 INTRODUCTION

Hypertension, or high blood pressure, is the leading cause of cardiovascular disease worldwide and is highly prevalent in the United States and around the world. The overall prevalence of hypertension in the United States is approximately 29% (1,2). As expected, the majority of hypertension is accounted for by people older than 60 years of age; however, the prevalence of hypertension among younger people is far from negligible—7.3% prevalence in ages 18–39 and 32.6% prevalence in ages 40–59 (3). Even though there has been some improvement in blood pressure control in both sexes, non-Hispanic blacks, and Mexican-Americans in recent years, the prevalence of hypertension has not declined (3). Hypertension is defined as blood pressure >140/90 mmHg and prehypertension as blood pressure between 120 and 139/80 and 89 mmHg (4). About 90–95% of hypertension cases have no obvious underlying medical cause and are referred to as primary “essential” hypertension, whereas the remaining 5–10% are caused by other conditions that affect the kidney, arteries, heart or endocrine system (5).

A number of epidemiologic studies have shown that individual blood pressure levels result from both genetic predisposition and environmental factors. Aside from the well-known environmental factors such as obesity, lack of exercise, smoking, stress, excessive sodium consumption, and excessive alcohol consumption, other attributes such as family history also play a part in causing essential hypertension. The heritable component of blood pressure has been documented in familial and in twin studies. However, the total number of genetic loci involved in hypertension, the frequency of deleterious alleles, their mode of transmission, and the quantitative effect of any single allele on blood pressure remain unclear. The unimodal distribution of blood pressure

within each age group and in each sex strongly suggests that multiple loci are involved. Several strategies based on the biological tools available at the genetic level and on different approaches for comparing hypertensive and normotensive subjects (case-control studies, family-based association studies, sibling pairs studies) have been used to identify major genes or suggest susceptibility genes. The discovery of a positive linkage between a given locus and the high blood pressure promotes new studies for finding a functional gene variant and for identifying new intermediate phenotypes associated with the locus. Multiple genes have been identified as risk factors for hypertension, but even so, only a fraction of the heritability is sufficiently explained (6).

During the past twenty years, numerous investigators have attempted to identify the genes involved in blood pressure regulation, their respective importance in determining blood pressure level, and sometimes their interaction with other genes and environmental components. Different strategies have been undertaken (Figure 52-1). The first and most successful approach, mainly taken by Lifton et al. (7–9), was to identify genes causing rare Mendelian forms of hypertension. This has led to the confirmation of the importance of some enzymes, channels, and receptors implicated in sodium handling in the regulation of blood pressure. The second approach was to study genes that may contribute to the variance of blood pressure because of their well-known effect on the cardiovascular system. The genes of the renin–angiotensin–aldosterone system are a good illustration of such a “candidate gene” approach since this system is involved in the control of blood pressure and in the pathogenesis of several forms of experimental and human hypertension. The third approach has been the genome-wide association studies (GWAS), with genotyping of genetic



**FIGURE 52-1** Main strategies used for studying the genetics of arterial hypertension. (Revised by Reed E. Pyeritz, April 2006.)

markers and analyzing their cosegregation with blood pressure. With the advent and ever-decreasing cost of next-generation sequencing, recent developments in the genetics of hypertension involve exome sequencing and will eventually incorporate whole-genome sequencing. There have also been numerous fruitful research performed based on experimental rodent models on the genetics of blood pressure regulation (10–14).

There are several risk factors and sequelae of high blood pressure. A major factor is an imbalance of the renin–angiotensin–aldosterone system. Renin converts angiotensinogen to angiotensin I, which is further converted by the angiotensin-converting enzyme (ACE) to angiotensin II. Angiotensin II is responsible for the constriction of blood vessels as well as the production of aldosterone, which promotes fluid retention. Both angiotensin II and aldosterone increase blood pressure, and therefore, promote hypertension. There are cases, though, where renin is not overproduced, and patients with this sort of hypertension are said to have “low-renin” hypertension. Vascular remodeling, a result of the increased resistance in blood vessels required to push the blood through due to increased pressure, is precarious as even a small amount of blockage greatly reduces blood flow rate. Such blood vessel blockage was found in 34% of hypertensive patients in a study comparing arteries of hypertensives versus normotensives (15). Vascular inflammation also plays a great part in the pathophysiology of hypertension, increasing blood pressure through restricting blood flow by constricting blood vessels. High blood pressure promotes inflammation through the release of vasoactive peptides such as angiotensin-II and endothelin-1, resulting in a detrimental feedback loop (16,17). Carotid intima-media thickness (cIMT), commonly used as a surrogate for atherosclerosis, is also correlated with hypertension and microalbuminuria and GWAS has contributed in identifying substantial genetic contribution to systolic blood pressure (SBP), renal function, and cIMT (18).

Salt-sensitive patients have a greater risk of developing renal failure and other cardiovascular issues. The main reason for this renal failure due to high salt intake is a decrease in blood flow inside the kidneys. Since the blood flow has decreased, the kidneys must increase their filtration fraction so as to continue blood regulation (19). In salt-resistant subjects, changes in plasma volume and cardiac output had no significant effect on blood pressure as the blood vessel dilates to compensate, whereas in salt-sensitive subjects, the rise in sodium resulted in a steady increase in blood pressure with no vasodilation (20). Sodium intake itself is found to be correlated to increased arterial pressure. High salt intake, however, does not increase the volume of blood in blood vessels in patients with essential hypertension. In fact, the blood volume is decreased in such patients, and the blood pressure increase is a consequence of increased intracellular sodium, and consequently, intracellular calcium. This results in increased flow resistance inside blood vessels and increased blood pressure (21). Furthermore, chronic kidney disease (CKD) itself is a significant risk factor for hypertension. For every 100mEq/g increase in urinary sodium, there is a 1.61-fold increase in risk for progression of patients to end-stage renal disease (22). In a study of non-CKD and CKD patients, patients with cardiovascular disease were found to have a significantly greater arterial stiffness (23). This stiffness, in turn, increases vascular resistance, and therefore, increases blood pressure. Recently, variants in the *LSD1* gene were found to be associated with salt-sensitive hypertension in African-American hypertensive cohort and functional studies were also carried out in *LSD1*-knockout mice (24).

The dangers of dyslipidemia are widespread, including increased risk of atherosclerosis and consequently, increased blood pressure, heart disease, and stroke (25). Atherosclerotic plaque blocks blood flow and this blockage results in oxygen starvation and increased blood pressure. In severe cases, starved tissues may undergo necrosis, or blood vessels may rupture. Furthermore, cholesterol levels were found to be a principle risk factor for cardiovascular disease in a study measuring blood pressure response during exercise for over 2100 subjects (26). In a study of 3110 men without hypertension, men with dyslipidemia had an increase of 23% in their risk of developing hypertension (27). One of the first genome-wide scan for genes contributing to blood pressure was conducted in a set of 18 Dutch families exhibiting familial combined hyperlipidemia with the revelation of a locus on chromosome 4 that exhibited a significant logarithm of the odds (LOD) score of 3.9 (and 4.6 when adjusted for age and gender) with SBP (28). In the same study, there was also a suggestive evidence of linkage of diastolic blood pressure to the lipoprotein lipase gene locus on chromosome 8p with a LOD score of 1.8 (28). Prehypertension is also associated with high triglycerides and low-density lipoprotein (LDL) in both females and males (29).



In nondiabetic individuals with hypertension, insulin resistance is a common finding. In a study of untreated patients with essential hypertension, glucose uptake was found to be inversely proportional to mean blood pressure (30). Since glucose uptake is hindered by insulin resistance, the higher the insulin resistance, the lower the glucose uptake, and therefore, the higher the blood pressure (30). Correlation and pathway analysis examining relationships among traits within and between generations found that genetic effects unrelated to body mass index (BMI) accounted for 60.8% of the variation in SBP and 36.8% of the variation in insulin sensitivity, whereas heritable effects related to BMI accounted for an additional 14% of variation in SBP and 26.8% of variation in insulin sensitivity (31). One of the first studies to successfully identify the genetic loci responsible for variations in blood pressure was a quantitative sibpair linkage analysis with candidate loci for blood pressure control, insulin resistance and lipid metabolism. A genetic region at the lipoprotein lipase (LPL) locus on the short arm of chromosome 8 was found to be linked to SBP (32). Further studies demonstrated that LPL haplotypes showed linkage to glucose infusion rate, a direct physiologic measurement of insulin sensitivity, in a family-based population of Mexican-Americans (33). Later, another study independently replicated this finding that LPL haplotypes 1 and 4 are associated with insulin sensitivity and resistance (34). In addition, it was suggested that variation in the 3' untranslated region of LPL affects LPL expression and activity, consequently influencing risk of insulin resistance (35).

A blood pressure and insulin resistance relationship was further elucidated with additional genetic linkage studies, including the finding of a major gene in chromosome 7q linked to blood pressure, leptin levels, and homeostasis assessment (HOMA) (36). Later, it was found that genes in the blood pressure pathway, namely *ADD1*, *ADRB2*, *AGT*, *NOS3*, *NPPA*, and *SCNN1A*, may be the genetic markers for insulin resistance in the Mexican-American Coronary Artery Disease cohort (37). In the Insulin Resistance Atherosclerosis family study, it was further determined that both albuminuria and blood pressure exhibit familial aggregation in nondiabetic Hispanic- and African-Americans (38). In the same cohort, it was also concluded that a significant relationship exists between visceral adipose tissue and hypertension in men with insulin resistance (39). A good example of gene discovery without genome-wide screening, but with careful multivariate subanalyses is the finding that variations in the *CAV1* gene are associated with insulin resistance and hypertension (40).

## 52.2 MENDELIAN FORMS OF HYPERTENSION

Rare Mendelian forms of hypertension are usually characterized by early-onset hypertension, frequent familial history of stroke, and strong penetrance. Most forms

are also characterized by extracellular volume expansion leading to a suppression of plasma renin activity, confirming the importance of salt and water homeostasis in blood pressure regulation (41).

### 52.2.1 Mutations Affecting Circulating Mineralocorticoid and Its Receptor

**52.2.1.1 Glucocorticoid-Remediable Aldosteronism (OMIM #103900).** In glucocorticoid-remediable aldosteronism (GRA), also referred to as glucocorticoid-suppressible hyperaldosteronism (GSH) or familial hyperaldosteronism type I, there is a variable degree of hyperaldosteronism and an increased urinary excretion of 18-hydroxylated and 18-oxocortisol metabolites, 18-hydroxycortisol and 18-oxocortisol. These abnormalities, including hypertension, can be corrected by the suppression of adrenocorticotrophic hormone (ACTH) by dexamethasone. It had been proposed that the disease was due to an abnormality of aldosterone synthase—CYP11B2, the enzyme responsible for the conversion of corticosterone into aldosterone—in the zona fasciculata. Lifton et al. showed that this disorder was linked to an abnormal aldosterone synthase gene (8). They studied a large kindred and found a gene duplication arising from unequal crossing-over, resulting in fusion of the 11 $\beta$ -hydroxylase (CYP11B1) promoter with the coding sequence of aldosterone synthase. The chimeric gene encodes a protein that can hydroxylate cortisol (the steroid substrate present in the zona fasciculata) in the 18 position. This gene is under the control of 11 $\beta$ -hydroxylase gene regulatory region, whose expression is under ACTH control and can be downregulated by exogenous glucocorticoid administration. All the phenotypic abnormalities of GSH could be explained by this mutation.

Since this original description, a few other families affected with this syndrome have been identified (42). In a case of GSH with an adrenal tumor, expression of the chimeric gene was found in the tumoral tissue and in the zona fasciculata of the surrounding adrenal tissue, and sensitivity of the corresponding adrenal cells to ACTH was observed (43). In all families reported thus far, the chimeric gene derives from unequal homologous recombination between exons 2 and 4 of the 11 $\beta$ -hydroxylase and aldosterone synthase genes, respectively. It has been shown by site-directed mutagenesis that such hybrid genes expressed in heterologous eukaryotic cells are able to encode chimeric proteins that hydroxylate cortisol in the 18 position (44). More recently, the transfection into COS-7 cells of a series of CYP11B1 complementary DNAs (cDNAs) containing various regions of CYP11B2 sequence showed that two amino acid changes, Ser-288Gly and Val320Ala, are sufficient to convert CYP11B1 into an efficient aldosterone-producing enzyme (45). Although these results suggest that a gene conversion involving exons 5 and 6—in which these residues

are encoded—could cause a novel form of GSH, no such molecular abnormalities have yet been found in a series of patients with essential hypertension and primary aldosteronism (46,47). GSH is very rare; however, one of the major interests of this diagnosis is its therapeutic implication, with a clinical and biological improvement with small doses of dexamethasone that can be associated with an antialdosterone treatment and/or a more usual antihypertensive therapy (48,49).

Gordon et al. described another form of familial primary aldosteronism, called familial hyperaldosteronism type II (FH-II), that corresponds to few families, with about 30% of the affected patients presenting an aldosterone-producing tumor and with probably an autosomal-dominant inheritance (50,51). The screening of the entire genome in a large family with FH-II showed linkage with chromosome 7p22; a gene has not been identified (52).

**52.2.1.2 Hypertension, Early-Onset, Autosomal-Dominant, with Exacerbation in Pregnancy (OMIM #605115).** In the family described by Geller et al., all women bearing the mutation had severe pregnancy-induced hypertension with hypoaldosteronism, which was probably caused by the massive increased production of progesterone during pregnancy (53). This condition is characterized by an early-onset and severe form of hypertension associated with low plasma levels of renin and aldosterone (53). It is caused by an activating missense gain-of-function mutation (Ser810Leu) of the human mineralocorticoid receptor. This mutation is located within the hormone-binding domain and results in constitutive mineralocorticoid receptor activity and an alteration of the receptor specificity. The receptor becomes abnormally activated by progesterone and other steroids lacking 21-hydroxyl groups, which act normally as antagonists. These original findings open the possibility of such mutations underlying some cases of pregnancy-induced hypertension.

**52.2.1.3 Apparent Mineralocorticoid Excess (OMIM #218030).** The syndrome of apparent mineralocorticoid excess (AME) is a rare autosomal recessive form of hypertension diagnosed in children and characterized by very early and severe hypertension associated with hyporeninism and hypoaldosteronism (54). It corresponds to a defect of 11 $\beta$ -hydroxysteroid dehydrogenase type 2, which is expressed in the kidney and converts cortisol into cortisone and corticosterone into 11-dehydrocorticosterone. Classical deleterious mutations have been found in affected patients who are either homozygotes for the mutation, especially in consanguineous families, or composite heterozygotes (55–57). The enzymatic defect leads to increased renal concentrations of cortisol and corticosterone, which are themselves powerful agonists of the human mineralocorticoid receptor, contrary to cortisone and 11-dehydrocorticosterone. Thus, affected patients are characterized by high values of the cortisol:cortisone ratio in plasma and urine, and by arterial hypertension, mimicking primary aldosteronism (58). The genetic diagnosis

can be obtained rapidly by direct sequencing of the five exons of the gene.

**52.2.1.4 Pseudohypoaldosteronism Type IIA (OMIM #145260).** Pseudohypoaldosteronism type II (PHA-II), also known as *Gordon syndrome*, is an autosomal-dominant form of volume-dependent hypertension characterized by hyperkalemia and hyperchloremic acidosis without any glomerular insufficiency (59). The low renin levels are thought to be the consequence of volume repletion, whereas plasma aldosterone seems to vary according to the opposite influences of low renin and high potassium levels. At least two arguments suggest a primary renal tubular defect along the distal convoluted tubule: affected patients are highly sensitive to small doses of thiazide diuretics; and the features of PHA-II are the mirror image of the Bartter–Gitelman syndrome, in which inactivating mutations in the thiazide-sensitive sodium chloride cotransporter gene (*SLC12A3*) have been demonstrated (60). However, the pathophysiology of PHA-II is certainly more complex, and other authors have proposed an associated reduced proximal reabsorption, a chloride reabsorption defect, and an altered sensitivity to mineralocorticoids (61). Lifton et al. discovered mutations in *WNK4*, which maps to 17q21–q22, in several families with this form of pseudohypoaldosteronism, including a family originally reported to show linkage to chromosome 12 (62,63).

**52.2.1.5 Hypertension with Brachydactyly (OMIM #112410).** A rare form of an autosomal-dominant syndrome associating arterial hypertension and brachydactyly has been reported in a few families, especially one extended Turkish pedigree (64,65). In 100% of the affected patients, hypertension is accompanied by short stature and brachydactyly but with no other special biological or hormonal phenotype (66). A particular defect of cerebral vascularization—loops in the posterior/inferior cerebellar artery—has been discussed as the possible origin of neurovascular compression and high blood pressure (67). The gene responsible for the disease was mapped to chromosome 12p but has not yet been identified (68,69).

## 52.2.2 Mutations Altering Renal Ion Channels and Transporters

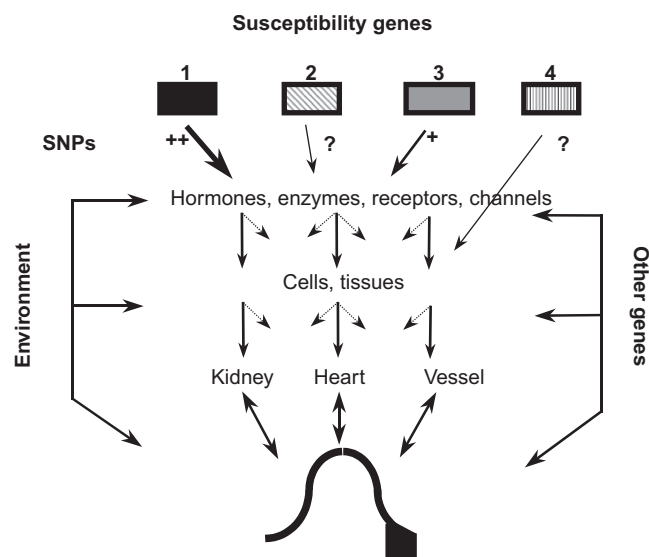
**52.2.2.1 Liddle Syndrome (OMIM #177200).** Liddle and associates described a family with hypertension and an abnormality of Na<sup>+</sup> reabsorption at the level of the renal distal tubule, which simulated primary aldosteronism but had negligible basal and stimulated aldosterone secretion (70,71). Blood pressure and hypokalemia were not influenced by spironolactone treatment, however, triamterene, a specific inhibitor of the distal renal epithelial sodium channel, corrected these abnormalities. The authors proposed that the primary abnormality was a constitutive activation of the epithelial sodium channel. Some 30 years later, this hypothesis was reinvestigated in the proband and originally described pedigree. The index

case developed renal failure and renal transplantation corrected the aldosterone and renin response to salt restriction, showing the involvement of the kidneys in the disease, and making the gene for the epithelial amiloride-sensitive sodium channel (ENaC) located in the collecting cortical tubule a logical candidate gene for this disease (72). This channel consists of at least three homologous subunits,  $\alpha$ ,  $\beta$ , and  $\gamma$ , which act together to confer its low sodium conductance and its high selectivity for sodium and amiloride (73).

Loss-of-function mutations of the gene for either the  $\beta$  or the  $\gamma$  subunits of the ENaC result in the recessive form of pseudohypoaldosteronism type I, characterized by excessive salt wasting accompanied by hyperkalemic acidosis, increased plasma and urinary aldosterone, and increased plasma renin activity (74). Analyzing the original Liddle pedigree, which demonstrated autosomal dominant inheritance, Shimkets et al. showed complete linkage of the gene encoding the  $\beta$  subunit of this channel, located at chromosome 16p13–12 (75). In this pedigree and in other unrelated kindreds, a premature stop codon, a frameshift mutation, and other deleterious mutations were found, all located in the last exon of the gene encoding for the intracellular carboxy-terminal domain of the  $\beta$  subunit (75). These mutations were showed to be gain-of-function mutations, with an increased amiloride-sensitive  $\text{Na}^+$  current after transfection of the corresponding mutant subunits together with  $\alpha$  and  $\gamma$  wild-type subunits. In a Portuguese family affected with this syndrome, a 32-bp deletion leading to a premature termination of the carboxyl end of the same subunit was found (76,77). A point mutation affecting the same region of the  $\gamma$  subunit has also been found to cause Liddle syndrome, but no mutation of  $\alpha$ -ENaC has been discovered (68). Comprehensive studies have shown that the mechanism by which truncation of the C-terminus of the  $\beta$  and  $\gamma$  subunits alters the function of the epithelial sodium channel corresponded to a much conserved motif (PPXXY) in the C-terminus of all three subunits of the sodium channel (78–80). Normally, a specific interaction between the PY motif and cytosolic proteins (Nedd4 and other related WW proteins) leads to degradation of part of the newly synthesized subunits (81). Both deletion and missense mutations of this PY motif increased surface expression of the mutant proteins and thus increased the number of sodium channels in the apical membrane, favoring renal sodium absorption and hypertension (82).

## 52.3 CANDIDATE GENES

Although it is likely that genes interact with each other and with environmental factors (Figure 52-2), most of the studies performed so far have been conducted using a very simple design, only testing the possible role of each candidate gene—that is, each gene coding for a protein (hormone, enzyme, receptor, channel, etc.) known to be involved in blood pressure regulation.



**FIGURE 52-2** Interaction between genes and environment in the regulation of blood pressure.

### 52.3.1 Renin–Angiotensin System

The importance of the renin–angiotensin system in the salt and water homeostasis and in the regulation of vascular tone, as well as the effectiveness of inhibitors of this system in the treatment of hypertension and of its morbid consequences, has justified the genetic analysis of this system for many years. All the genes of the human renin–angiotensin system have been mapped and cloned, and genetic markers were identified. Since very complete reviews have already been published on this subject, we only summarize the main findings obtained that concern essential hypertension, blood pressure regulation, or both (83–88).

**52.3.1.1 The Renin Gene (OMIM \*179820).** The renin gene (*REN*; 1q32) is important because the renin–angiotensinogen reaction is the first and rate-limiting step leading to angiotensin II (Ang II) production. Numerous studies have involved renin to some degree in experimental forms of hypertension and in human hypertension. This was especially the case in the fulminant hypertension that develops in transgenic rats harboring the mouse *ren2* gene and of the pioneer observation made by Rapp et al. of cosegregation between a renin gene polymorphism and blood pressure level in Dahl rats (89,90). In humans, about 30% of subjects with essential hypertension have higher renin levels compared to normotensive subjects of the same age when examined under the same metabolic conditions (91). Interestingly, renin levels seem at least partly heritable as observed by Grim et al. in twins submitted to very standardized conditions of posture and sodium diet, and confirmed by Rossi et al. (92,93). The analysis of 175 sibling pairs and trios who participated in a multicenter trial showed very significant correlations of plasma renin levels either on a high-salt or a low-salt diet (94).

Association studies (most of which being of limited power) performed with several polymorphisms of *REN*

largely have been negative (85,95–97). To explore the potential role of the renin gene as a genetic determinant of hypertension, Jeunemaitre et al. analyzed the same renin gene haplotypes in 98 sibling pairs with severe essential hypertension (98). Allelic concordance for all sibpairs according to sibship size was not significantly different from that expected under the hypothesis of no linkage, reflecting only a small excess of renin alleles shared by the hypertensive sibs. These results suggest that REN does not have a frequent and/or important role in the pathogenesis of essential hypertension. However, the definitive exclusion of a contribution of the renin gene in the heritability of essential hypertension will require more powerful linkage studies, such as the use of a reliable renin intermediate phenotype, and a more informative marker of the renin locus. Some of the recent genome scans have found the chromosome 1q32 region to be a possible locus for hypertension.

**52.3.1.2 Angiotensin I-Converting Enzyme Gene (OMIM +106180).** Angiotensin I-converting enzyme (ACE) is a zinc metalloprotease whose main functions are to convert angiotensin I into angiotensin II and to inactivate bradykinin. It is assumed that this step of the renin–angiotensin system is not rate limiting in plasma, and indeed there is no indication that plasma ACE levels are directly related to blood pressure levels. However, the local generation of angiotensin I and the degradation of a bradykinin may depend on the level of ACE expressed in tissues. Molecular cloning of the human endothelial ACE cDNA revealed that the enzyme consists of two highly homologous and functionally active domains resulting from gene duplication (96). The organization of the human ACE gene (17q23) provides further support for the duplication of an ancestral gene (99). Ong et al. have made the recent discoveries that mice lacking the N-domain of ACE have increased response to angiotensin-II induced hypertension, increased inflammatory cytokines, and protection against bleomycin-induced pulmonary fibrosis (100,101). There are two ACE promoters, a somatic promoter localized 5' to the first exon of the gene and a germinal intragenic promoter located 5' to the specific testicular ACE messenger RNA (99,102,103). The two alternate promoters of ACE exhibit highly contrasting cell specificities as the somatic promoter is active in endothelial, epithelial, and neuronal cell types, whereas the testis promoter is only active in a stage-specific manner in male germinal cells (102). It is thought that plasma ACE concentration reflects the level of the synthesis of the enzyme at the somatic level. Bernstein et al. have made many discoveries in this area, elucidating both the traditional and nontraditional roles of ACE (14,104–107).

For a geneticist, one of the big advantages of plasma ACE concentration as a genetic marker is its large inter-individual variation (from 1 to 8 at the extremes of the distribution) and its reproducibility when measured repeatedly in a given subject (108). This important

interindividual variability is due, in large part, to a major genetic effect, as shown by Cambien et al. in a family population study, in which there was an intrafamilial resemblance between plasma ACE levels accounting for approximately 30% and 75% of the ACE variance in parents and offspring, respectively (109). The role of ACE in the genetic control of plasma ACE was assessed using an ACE polymorphism consisting of the presence or the absence of a 287-base pair DNA fragment (insertion [I]/deletion [D]) (110). In this seminal observation of 80 healthy subjects, Rigat et al. showed that DD subjects had an immunoreactive ACE level almost twice as high as patients homozygous for the I allele, whereas heterozygous patients had an intermediate ACE level. This I/D polymorphism accounted for 47% of the total variance of serum ACE, showing that the ACE locus plays an important role in determining serum ACE levels (110). Similar to serum, T-lymphocyte ACE levels are significantly higher in patients homozygous for the D allele than in the other subjects (111). The ACE I/D polymorphism is not directly involved in the genetic regulation of serum and tissue ACE, and the causative variant responsible for the increase in ACE has yet to be found; indeed, another study combining segregation and linkage analysis in 98 healthy nuclear families showed that the ACE I/D polymorphism is only a neutral marker in strong linkage disequilibrium with the putative functional variant (112). Soubrier's group performed an extensive study of eight new polymorphisms in 95 healthy nuclear families (113). After adjustment for the I/D polymorphism, all polymorphisms of the 5' group remained significantly associated with ACE levels, which suggested the existence of two quantitative trait loci acting additively on ACE levels and which would explain 38% and 49% of the ACE variance in parents and offspring, respectively. More recently, Rieder et al. analyzed the complete genomic sequence of ACE from 11 individuals, representing the longest contiguous scan (24kb) for sequence variation in human DNA. They identified 78 variable sites in 22 chromosomes that resolved into 13 distinct haplotypes; 17 of these chromosomes were in absolute linkage disequilibrium with the ACE I/D polymorphism, producing two distinct and distantly related clades and suggesting that the causal variant should be located within the 3' part of the gene (114).

The observation that plasma ACE levels are under the direct control of genetic variation in ACE, coupled with the role of the ACE enzyme in two main enzymatic cascades (renin–angiotensin system and kallikrein–kinin system) in cardiovascular physiology, rapidly made the ACE I/D polymorphism one of the most popular markers tested in cardiovascular diseases. The first genome scans, performed in an F2 rat population generated from stroke-prone spontaneously hypertensive rats and normotensive genetically hypertensive rats, made this hypothesis even more attractive (115,116). Both groups of investigators found a significant linkage between NaCl-loaded



hypertension and a gene locus on rat chromosome 10 that contained the ACE locus and contributed as much as 20% of blood pressure variance under high salt intake. Several hundred reported and unreported studies have been performed since then (85,87,88). We summarize here only some of the main findings using ACE as a candidate gene for human essential hypertension.

One association study comparing a normotensive and a hypertensive Australian population with two hypertensive parents showed an association of hypertension with an ACE polymorphism (117). In fact, the significant difference between the I/D genotype originated only from a subgroup of patients of age 50 years or older, in which the D genotype was less frequent than in normotensives. This finding was interpreted as owing to an overrisk of cardiovascular events in hypertensive patients carrying the D allele (118). Harrap et al. investigated the distribution of the ACE I/D gene polymorphism described previously in young adults with contrasting genetic predisposition to high blood pressure (the “four-corners approach”) (119,120). Young adults with high blood pressure and two parents with high blood pressure did not show any significant difference in the I/D allele frequencies of ACE when compared with adults of the same age but with low blood pressure and no genetic predisposition to high blood pressure. Other association studies were also negative (121,122). In a large series of hypertensive sibpairs from Utah, no evidence of linkage between hypertension and a growth hormone gene polymorphic marker in complete linkage disequilibrium with ACE emerged (123).

However, some studies suggested that ACE might influence blood pressure variability in a sex-specific manner. In a logistic regression analysis of 3095 participants in the Framingham Heart Study, the adjusted odds ratios for hypertension among men for the DD and DI genotypes were 1.59 and 1.18, respectively, versus II, where no effect was observed in women (124). Positive results were also observed by Fornage and associates in the analysis of a large population-based sample of 1488 siblings having a mean age of 15 years and belonging to the youngest generation of 583 randomly ascertained three-generation pedigrees from Rochester, Minnesota (125). In sex-specific analyses, genetic variation in and around ACE explained as much as 35% of the interindividual blood pressure variation in males but not in females. Finally, Julier et al. conducted an affected sibpair analysis in French and U.K. families, based on the fact that one of the principal blood pressure loci in rat hypertensive strains lies on chromosome 10, a region homologous to human chromosome 17 (126). Significant evidence of linkage was found near two closely linked microsatellite markers, D17S183 and D17S934, that reside 18 cM proximal to ACE in the homology region (126). Taken together, these results suggest that genetic variation in ACE does not play a major role in blood pressure variance in the overall population, but that it could influence

variation either in a sex-specific manner or indirectly by its role in different tissues (kidney, heart, vessels) (85,87,88).

**52.3.1.3 The Angiotensinogen Gene (OMIM +106150).** Angiotensinogen (angiotensin I; AGT), the renin substrate, is mainly synthesized by the liver and is the unique substrate for renin. Plasma angiotensinogen concentration is within a range, in which variations in its concentration directly affect the angiotensin I production rate. Indeed, angiotensinogen levels are around the K range of renin, and therefore, it is logical to suspect that a chronic state of increased plasma angiotensinogen might increase angiotensin I and facilitate hypertension and/or cardiovascular diseases. The role of angiotensinogen in human hypertension was suspected in an epidemiologic study in which a strong correlation was found between plasma angiotensinogen concentration and blood pressure, and in another study in which offspring of hypertensive patients had elevated plasma angiotensinogen levels (127,128).

The human angiotensinogen gene (AGT) belongs to the superfamily of serpins and is localized to chromosome 1q42.3, in the same region as human renin (129,130). Jeunemaitre et al. suggested a role of AGT in human essential hypertension (131). An extensive study was performed in two large series of hypertensive sibships yielding a total of 379 sibpairs (Salt Lake City, Utah, USA and Paris, France) and using a highly polymorphic microsatellite marker at AGT. An excess of AGT allele sharing was found mainly in severely hypertensive sibpairs and in men. Other linkage studies have since been reported with discrepant results. Caulfield et al. showed a strong linkage and an association of AGT to essential hypertension in a set of British families (132,133). To the contrary, no evidence for linkage was found in a large European study involving 630 affected sibling pairs, either in the whole panel or in family subsets selected for severity or early onset of disease (134). Linkage of AGT to essential hypertension was also found in 63 affected African–Caribbean sibling pairs (135).

Similarly, positive albeit modest significant excess of AGT allele sharing was found in 46 extended Mexican–American families (136). No linkage was found between AGT and hypertension in 310 hypertensive Chinese sibling pairs (137,138). Together, these results probably highlight the modest effect of AGT in the overall population and the difficulty of identifying susceptibility genes by linkage analysis in complex diseases.

Among the 15 polymorphisms initially identified, two of them leading to amino acid changes, 174M and M235T, were found to be associated with hypertension and with plasma angiotensinogen concentration (98,123,131). This association between plasma angiotensinogen level and the M235T genotype was further confirmed in white children (139). In this study, a strong and independent relationship of serum angiotensinogen with BMI and race was also observed.

Confirmation of the impact of an increased effect of angiotensinogen on blood pressure was obtained by Smithies et al., who performed genetic manipulations in mice leading to animals with several functional copies of a given gene (13). Mice bearing one, two, three, or four functional *AGT* copies displayed a gene-dosage effect on plasma angiotensinogen concentration and blood pressure level, paralleling the M235T variant effect observed in humans (140,141). The threonine residue at position 235 is not conserved across species and is in complete linkage disequilibrium with another nucleotide substitution (G > A) at position 6 in the promoter of the gene, both alleles being indistinguishable (77). Lalouel and his group were able to show that this variant is associated in vitro with increased expression of *AGT* and probably explains the association with increased plasma angiotensinogen (142). However, the true biologic effect may be more complex since other polymorphisms (C-532T, C-18T, A-20C, T+31C) also in linkage disequilibrium with G-6A and M235T might play a role in the variation in transcription of the gene (143–146).

There is a strong effect of race on the allele frequency of most of these polymorphisms. The 235T allele frequency varies from 40% in whites to 80% in the Asian and African-American populations and as much as 93% in Nigerians (43). This high prevalence of the 235T allele may explain why no relation between 235T or 174M allele frequencies and hypertension was observed in a study of African-Americans (147). In the Japanese population, a significant association between this allele and high blood pressure was found in several separate case-control studies (148–151). These differences in allele frequencies might also facilitate false-positive results in case of admixture in the case-control studies (152). The association between the M235T polymorphism and essential hypertension has been tested in a large number of case-control studies (153). A meta-analysis of case-control studies representing 5493 white patients showed that the *AGT* 235T allele was significantly but mildly associated with hypertension and this association increased in studies with positive family history (154). This polymorphism represents a good example of susceptibility alleles, which are quite common in the population, but explains only a small part of the blood pressure variance in the overall population.

One possibility is that molecular variants of *AGT* might represent also a susceptible allele for pregnancy-induced hypertension, for which a familial tendency has been documented (155,156). Ward et al. found a significant association between the *AGT* 235T variant and preeclampsia in both white and Japanese samples (157). Using another strategy, analysis of the allelic inheritance of *AGT* repeat in 52 sibling pairs of preeclamptic sisters, Arnggrimsson et al. showed a significant linkage between *AGT* and preeclampsia in Icelandic and Scottish families (158). The association in Japanese women was confirmed in a larger case-control study involving 139

women with pregnancy-induced hypertension and 278 matched controls (159). However, other studies limited in their sample size found no indication of association or linkage between preeclampsia and *AGT* in white women (160–162). More recently, a significant increase of the T235 allele frequency in a large series of preeclamptic women of African origin identified on the island of Mauritius was found (163). Interestingly, nulligravid women homozygous for T235 have a reduced plasma volume during the follicular phase of the menstrual cycle than those bearing the MT or MM genotype (164).

The pathogenesis of preeclampsia is probably influenced by a variety of genetic (maternal and paternal) and nongenetic factors. Recent GWS studies have reported possible loci on chromosomes 4q and 2p (162,165,166). The gene coding for nitric oxide synthase (NOS) has also been linked to the pathology (160). The possible pathophysiologic relationship between *AGT* and pregnancy-induced hypertension may be due to both an increased expression of angiotensinogen in decidual arteries and decreased formation of high-molecular-mass angiotensinogen, an aggregate form of the protein that is particularly abundant during pregnancy (144,167). The M235T polymorphism was also found to be associated with increased response of plasma angiotensinogen to a 1-week exposure to ethinyl estradiol in normal volunteers (168). From all these studies, *AGT* appears to be involved in the determination of human familial hypertension and some forms of pregnancy-induced hypertension. However, its exact quantitative effect on blood pressure is unknown but might be weak and modulated by a variety of interacting loci and environmental factors, accounting for the negative results in some underpowered studies (169).

**52.3.1.4 The Angiotensin II Type 1 Receptor (OMIM \*106165).** Angiotensin II receptors, which mediate all the biologic and physiologic effects of the renin-angiotensin system, are also candidate genes for essential hypertension. The angiotensin II type 1 receptor (AT1R) subtype is a G-coupled receptor inserted into the plasma membrane of angiotensin II target cells, vascular smooth muscle cells, renal vasculature and mesangial cells, adrenal glands, and brain. The human gene (*AT1R*) has been cloned and is located on chromosome 3q21–3q25 (170,171).

In the first series of experiments, Jeunemaitre et al. looked for putative molecular variants in the coding region of the gene that would result in overactivation of the receptor. Such variants have been reported for other G-coupled seven transmembrane-domain receptors, such as the human thyrotropin and luteinizing hormone receptors, resulting in a constitutive activation or hyperresponsiveness of the receptors (172,173). In the case of hypertension, similar functional variants of angiotensin II receptors could theoretically lead to some forms of essential hypertension or to tumoral sporadic primary aldosteronism. However, no evidence of such mutations

in the coding region of AT1R was found in 60 probands of hypertensive families and in 20 cases of tumoral primary aldosteronism (174,175). There was no evidence of linkage between a microsatellite marker of AT1R and hypertension in a hypertensive sibpair study. However, an informative diallelic marker (A1166C) present in the 3' untranslated region of AT1R was found significantly more frequently in 206 hypertensive subjects than in 298 normotensive controls, suggesting that a variant of the AT1 receptor exerts a small effect on blood pressure (174). This specific polymorphism was significantly associated with hypertension in a Japanese woman during her first pregnancy (176).

Other polymorphisms have been described, especially at the promoter region of AT1R. However, none of these polymorphisms has shown evidence for association with hypertension in a large white population-based sample (177). Studies performed in Finland suggest linkage at the AT1R locus and association with the A1166C polymorphism (178,179). It is interesting to note that the A1166C polymorphism of AT1R has also been associated with aortic stiffness in essential hypertension, left ventricular mass, coronary vasoconstriction, different responses to Ang II in isolated human arteries, and myocardial infarction in interaction with the ACE I/D polymorphism (180–184). However, these associations have been disputed in other studies, and no relation was found between AT1R density in platelets and the A1166C genotype (146).

## 52.3.2 Sodium Channels

**52.3.2.1 Subunits of the Epithelial Sodium Channel.** Epithelial sodium channel (ENaC) is the rate-limiting step of Na<sup>+</sup> reabsorption by the kidney distal tubule. As discussed in Section 52.2.2.1, the presence of gain-of-function mutations in the genes coding for the  $\beta$ - and  $\gamma$ -ENaC subunits suggests the possibility of more subtle polymorphisms at those genes that might modify ENaC activity, especially in salt-sensitive patients or those with a low-renin profile. Several groups, therefore, have extended the screening for mutations in essential hypertension to  $\beta$ - and  $\gamma$ -ENaC. In a series of more than 400 hypertensive subjects, seven missense mutations were found at the gene coding for the  $\beta$ -ENaC subunit (SCNEB; 16p13–p12), almost all of them in patients of African descent (185). Whereas these variants led to no significant increase in Na<sup>+</sup> current after expression in *Xenopus* oocytes, data obtained in human B lymphocytes suggest that at least one of them (T594M) could have an effect on Na<sup>+</sup> reabsorption (186). In a case-control study involving black residents of London, Baker and associates found a significant increase of the 594M frequency in the 206 hypertensive patients (8.3%) compared to the normotensive subjects (2.1%), the statistical significance persisting after adjustment for sex and BMI (187). In the subset of patients in whom plasma

renin activity was measured, the T594M polymorphism was also associated with a low-renin profile, suggesting that it could raise blood pressure in affected people by increasing renal tubular sodium reabsorption. However, the impact of this polymorphism is still debated.

Several polymorphisms have also been detected in  $\gamma$ -ENaC (SCNNIG; 16p13–p12) including four neutral polymorphisms—three in the third exon of the gene (T387C, T474C, C549T) and one in the last exon (C1990G) (188). These four variants had similar frequencies in 453 hypertensive and 245 normotensive white subjects as well as in patients with a low-renin profile. Two rare mutations were also found in three hypertensive subjects, 594insP and R631H, that produced no significant increase in Na<sup>+</sup> current in *Xenopus* oocytes. Results obtained by other groups confirm that this subunit is infrequently involved in essential hypertension (189). The analysis of  $\gamma$ -ENaC showed an interesting missense polymorphism (W493R) located in the extracellular loop of the subunit and in a rather well-conserved sequence not far from the amino acids responsible for the sensitivity to amiloride. However, in whites, this polymorphism was found at a similar allele frequency in hypertensive and normotensive individuals and did not change the amiloride-sensitive current when expressed in *Xenopus* oocytes (190). Other polymorphisms (G442V at  $\beta$ -ENaC and T663A at  $\alpha$ -ENaC) have been shown to be associated with changes in the aldosterone:potassium ratio in urine and with essential hypertension (191). It is also interesting to note that a sibpair linkage study performed on 286 white Australian families showed significant linkage between SBP and microsatellites at chromosome 16p12, located in the vicinity of the genes encoding the  $\beta$  and  $\gamma$  subunits of ENaC (192).

**52.3.2.2 Guanine Nucleotide-Binding Protein (OMIM \*139130).** Several studies have suggested increased activity of the sodium-proton exchanger in about 50% of subjects with essential hypertension. Increased ion transport activity has also been found in immortalized lymphoblasts and in fibroblasts from hypertensive subjects that might be due to altered intercellular signal transduction and enhanced activation of pertussis toxin-sensitive G proteins (193). The heterotrimeric G proteins are formed by the assembly of several subunits ( $\alpha$ ,  $\beta$ , and  $\gamma$ ). After having excluded mutations of several of these subunits, Siffert et al. found a very interesting genetic polymorphism (C825T) in exon 10 of the gene coding for the  $\beta_3$  subunit (GNB3; 12p13) (194). This polymorphism is not a missense mutation but favors the use of an alternative splicing of exon 9 of the gene and thus the expression of a protein with a deletion of 41 amino acids. The expression of the deleted protein in insect cells showed that it was functional but that it presents an enhanced reactivity to pharmacologic activators. Finally, in this pioneering study, the 825T allele was more frequent in 426 hypertensives (0.31) than in 427 controls (0.25), suggesting its role as a susceptibility factor for

essential hypertension. However, this association with essential hypertension has been controversial.

In a population-based study at Augsburg (Germany) selected from the MONICA survey, the C825T polymorphism showed a weak, albeit significant, association with diastolic blood pressure (195). Interestingly, plasma renin levels were lower in patients bearing the 825T allele, suggesting that it might be associated with a low-renin profile. An association study performed in 110 Australian white hypertensives, each of whom was the offspring of two hypertensive parents, and 189 controls whose parents were both normotensive beyond the age of 50 years, found a significant difference in the 825T allele frequency, supporting a role for this variant (196). In contrast, Brand et al. analyzed the GNB3 variant in two large case-control studies selected for essential hypertension (PEGASE study) and myocardial infarction (ECTIM study) (197). The odds ratios for hypertension and myocardial infarction associated with T-allele carrying were 1.23 (95% CI, 0.94–1.62;  $P=0.13$ ) and 1.11 (95% CI, 0.88–1.39;  $P=0.37$ ), respectively. There was no association of the GNB3 polymorphism with early onset of hypertension, familial history of hypertension, or blood pressure. In a German case-control study involving 479 hypertensives and 1000 gender- and age-matched controls, the 825T allele was associated with an OR of 1.5 (95% CI, 1.1–2.2) versus non-T carriers for the presence of hypertension (198). However, in both the whole group and the untreated group, blood pressure levels were virtually identical between the genotypic groups. In a study performed in 447 Indians of the Oji-Cree tribe in Canada, the frequency of the 825T allele was 0.50, much greater than those usually observed in whites (199). Surprisingly, the 825T allele was associated with a lower mean SBP (CC, 126.7 mmHg; CT, 123.5 mmHg; TT, 119.6 mmHg;  $P<0.05$ ) and was not associated with significant difference in diastolic blood pressure. A Japanese study seems to confirm these negative results (200). The frequency of the 825T allele was very similar (0.49) in 718 subjects with confirmed essential hypertension, 191 with borderline hypertension and 515 controls, all from the Tokyo area.

The hypothesis of a genetic polymorphism of the GNB3 subunit favoring high blood pressure is attractive because of its possible ubiquitous effect, especially on the vascular tone on the renal sodium handling. In this regard, the measurement of this polymorphism in 197 blacks and 190 non-Hispanic whites who underwent monotherapy with hydrochlorothiazide for 4 weeks showed that 825TT homozygotes had greater response of both diastolic and SBP than the CC homozygotes (201).

### 52.3.3 Other Candidate Genes

Many other genes can be considered as excellent candidates for essential hypertension. A list of more than

100 genes that may act directly or indirectly on blood pressure is easily established (Table 52-1). Among them are genes coding for proteins of the adrenergic systems (dopamine receptors,  $\alpha$ - and  $\beta$ -adrenergic receptors in particular), for proteins involved in salt–water homeostasis (natriuretic peptides and their receptors, subunits of the epithelial sodium channel, renal transporters, kallikrein–kinin system, etc.), for proteins involved in the hormonal regulation of blood pressure (enzymes and receptors of the mineralo- and glucocorticoid pathways), and for proteins involved in the structure and/or the regulation of vascular tone (endothelins and their receptors in particular).

It is, of course, not possible to summarize here the studies that have been performed for all these candidate genes, except to note that none of them has been firmly established as bearing polymorphisms strongly favoring changes in blood pressure. For example, the gene coding for the endothelial cell NOS was very attractive since nitric oxide plays an important role in regulating blood flow and pressure. Several reports have suggested that hypertensive subjects have a blunted endothelium-dependent vasodilation that might be secondary to decreased nitric oxide production. However, linkage and case-control studies performed in French hypertensive and normotensive subjects failed to show association or linkage with essential hypertension, suggesting that common variants of this isoform of NOS are not involved in common hypertension (202). Similar negative results were obtained in humans for the acyl-CoA synthetase medium chain family (ACSM3 or SA; OMIM \*145505) locus, which was linked to blood pressure levels in several crosses involving different strains of genetically hypertensive rats (203,204).

The gene coding for aldosterone synthase (CYP11B2; OMIM \*124080) is also an attractive candidate (205). It has been tested for its possible association with essential hypertension and primary aldosteronism (206–209). A polymorphism in the promoter region of the gene (C-344T) is located at a steroidogenic transcription factor (SF-1 binding site) and could play a role in the expression of the gene. Despite the fact that the Ang II,  $K^+$ , and cyclic AMP signaling pathways utilize in vitro a CRE-like *cis*-element and another SF-1 binding site that is located closer to the transcription start to regulate human CYP11B2 expression, the C-344T polymorphism has been associated with variations in plasma aldosterone, suggesting that it could favor sodium retention and high blood pressure (206,210,211). Contradictory results have been obtained according to the populations and the biochemical and clinical parameters studied (212–214).

The group headed by G. Bianchi in Milan has extensively studied the possible role of  $\alpha$ - and  $\beta$ -adducin genes in rat and human hypertension (215). Adducin is a cytoskeletal protein that favors the association between spectrin and actin, but it can also directly interact with actin.



**TABLE 52-1** Some Loci Associated with Abnormal Regulation of Blood Pressure or Hypertension Determined by Large-Scale GWAS

Ethnicity	Phenotypes	Locus	P value	Nearest gene
Asian	SBP	12-88,584,717	$9.1 \times 10^{-7}$	ATPB1
Asian	DBP	12-88,584,719	$1.2 \times 10^{-6}$	
Americans of African descent	SBP	2-190,446,083	$2.1 \times 10^{-11}$	<i>PMS1</i>
		6-16,031,005	$3.4 \times 10^{-9}$	MYLIP
		8-102,026,053	$1.6 \times 10^{-8}$	<i>YWHAZ</i>
		11-9,388,666	$4.8 \times 10^{-8}$	<i>IPO7</i>
		14-91,877,083	$1.5 \times 10^{-8}$	<i>SLC24A4</i>
European	SBP	5-162,604,350	$3.5 \times 10^{-7}$	CCNG1
European	HTN	12-24,872,878	$7.4 \times 10^{-6}$	<i>BCAT1</i>
European	SBP	1-11,785,365	$1 \times 10^{-5}$	<i>MTHFR</i>
Mixed	SBP	10-104,836,168	$3 \times 10^{-7}$	<i>NT5C2</i>
	SBP	17-40,563,647	$4 \times 10^{-6}$	<i>PLCD3</i>
	DBP	4-81,403,365	$7 \times 10^{-9}$	<i>FGF5</i>
	DBP	10-63,194,597	$3 \times 10^{-6}$	C10orf107
	DBP	12-110,492,139	$1 \times 10^{-7}$	ATXN2
	DBP	15-72,865,396	$6 \times 10^{-8}$	<i>CSK</i>
	DBP	17-44,795,465	$5 \times 10^{-6}$	<i>ZNF652</i>
	SBP	10-104,584,497	$2 \times 10^{-6}$	<i>CYP17A1</i>
European	SBP	11-16,858,844	$5.8 \times 10^{-7}$	<i>PLEKH7</i>
	SBP	12-88,537,220	$3 \times 10^{-11}$	<i>ATP2B1</i>
	DBP, HTN	12-88,533,090	$3.7 \times 10^{-7}$	<i>SH2B3</i>
	SBP, DBP	12-110,368,991	$5.7 \times 10^{-7}$	<i>ULK4</i>
	DBP	10-18,748,804	$8.7 \times 10^{-7}$	<i>CACNB2</i>
	DBP	15-72,912,698	$8.1 \times 10^{-7}$	<i>CPLX3</i>

Phenotypes: DBP=diastolic blood pressure; SBP=systolic blood pressure; HTN = hypertension.

Locus: chromosome position.

Adapted from Ehret, G. B. Genome-Wide Association Studies: Contribution of Genomics to Understanding Blood Pressure and Essential Hypertension. *Curr. Hypertens. Rep.* 2010, 12 (1), 17–25.

Thus, it might be involved in cellular signal transduction and interact with membrane cytoskeletal proteins that affect ion transport across the cell membrane. Adducin is a heterodimeric protein that is composed of three subunits,  $\alpha$ ,  $\beta$ , and  $\gamma$ . Each subunit is composed of two distinct domains: an N-terminal globular hydrophobic domain and a C-terminal tail responsible for its interaction with actin and spectrin. The  $\alpha$ -adducin gene (*ADD1*) is located at human chromosome 4p16.3 and was identified because of its proximity to the gene responsible for Huntington disease. The  $\alpha$ -adducin gene is widely expressed, but is predominantly expressed in brain and kidney tissue. The  $\beta$ -adducin gene is 50% identical, and is expressed as different tissue-specific isoforms. Thus, there are physiologic quantitative and qualitative differences in assembly of these subunits according to the tissue in which they are expressed.

Bianchi et al. hypothesized that variations in adducin could lead to variations in the activity of ion transporters or channels, especially the  $\text{Na}^+\text{-K}^+\text{-Cl}^-$  cotransporter in the thick ascending limb and  $\text{Na}^+\text{-K}^+\text{-ATPase}$  all along the renal tubule, thus leading to increased salt reabsorption and hypertension. The Milan hypertensive strain (MHS) of rat has characteristics that suggest a renal abnormality of sodium handling: increased glomerular filtration rate during the first weeks of life, low-renin

status, and increased sensitivity to furosemide compared to the Milan normotensive strain (MNS) (215). Differences in the amino acid sequence of the adducin genes between the MHS and MNS strains could explain a significant part of the blood pressure difference between these two strains. Indeed, two amino acid changes were found in the  $\alpha$ -(F316Y) and  $\beta$ -(Q529R) adducin genes. The transfection of wild or mutant subunits in renal epithelial cells showed that these mutations induce an increased activity of the  $\text{Na}^+\text{-K}^+$  pump, thus establishing a pathophysiologic link with increased renal sodium handling and high blood pressure.

In humans, several recently concordant studies establish a relation between the  $\alpha$ -adducin gene and salt sensitivity and blood pressure, even though its effect is probably mild at the population level. In a study involving French and Italian hypertensive sibpairs, significant linkage was found between several markers surrounding the  $\alpha$ -adducin locus and essential hypertension (216). A positive association was also found when the authors compared the genotype frequencies of the G460W polymorphism of the  $\alpha$ -subunit of the adducin gene in 190 hypertensive patients and 126 controls. The blood pressure response to the chronic administration of hydrochlorothiazide was also significantly more important in subjects bearing the 460W allele, suggesting that this

variant could predispose to salt sensitivity and to hypertension. Since then, other studies have been performed with conflicting results. The association with low-renin status was recently confirmed in a multicenter international study investigating intermediate phenotypes in hypertension (217). Allayee et al. found no association between ADD1 polymorphisms and SBP in Dutch subjects (28). Determination of the exact importance of the adducin locus in human hypertension will probably require large-scale studies as well as a search for interaction with other genes and sodium content of the diet.

## 52.4 GENOME-WIDE ASSOCIATION STUDIES

GWAS were undertaken with the primary aim of identifying novel genetic loci associated with variation in risk of clinical diseases, subclinical disease, and levels of risk factors. Taking into account the major advances accomplished in recent years on the genetic and physical map of the human genome, this approach has become a major strategy to identify major loci implicated in complex traits. Once the regions of interest have been identified, a systematic analysis of their genes is undertaken based on their possible function (i.e. the positional candidate gene approach) (218). Because it does not make any *a priori* assumption as to the genes to identify, this strategy is designed for discovering new pathways involved in blood pressure regulation.

However, there are strong statistical limits to this approach that have been well delineated (219–222). Among them are the problem of power to identify loci that have only a small influence on blood pressure level; the requirement of nonparametric methods, necessitating the use of hundreds of sibpairs and/or particular study designs such as discordant sibpairs; the necessity of adjustment of the statistical significance for multiple testing, which might prevent the identification of genes with mild effects on the trait; the requirement of replication of any positive result in another sample with the same characteristics using either the same design or a complementary approach such as family-based association studies or case-control studies; the low power of replication for loci with only a small blood pressure effect; the possible heterogeneity of genes causing essential hypertension according to the ethnic background; and the confounding factors such as obesity or associated diseases such as dyslipidemia and diabetes. Finally, one must be aware that GWAS, at least in the initial stage, does not take into account the interaction among genes and between genes and environment, which are the mechanisms at work at the phenotypic level. Thus, negative results can be obtained for a locus with a true biologic and clinical effect that would be detected only if analyzed in combination with other loci or with environment. Despite these limits, several GWAS have produced interesting results. A nonexhaustive summary of these studies is presented in this section and in Table 52-2.

**TABLE 52-2** Some Mendelian and Mitochondrial Conditions Associated with Hypertension

Phenotype	OMIM No.	Locus	Gene
Apparent mineralocorticoid excess	218030	16q22.1	HSD11B2
Early-onset hypertension	605115	4q31.23	NR3C2
Glucocorticoid remediable aldosteronism	103900	8q21	CYP11B1
Hypomagnesemia, hypertension, hypercholesterolemia	500005	Mitochondrial	tRNA Ile
IgA nephropathy	106150	1q42.2	AGT
Liddle syndrome	177200	16p12.2	SCNN1G
		16p12.2	SCNN1B
Pregnancy-induced hypertension	189800	7q36.1	NOS3
		2p13	
Progressive renal failure	161900	1q21	
Pseudohypoaldosteronism, type 2A	145260	1q31-q42	
Pseudohypoaldosteronism, type 2B	614491	17q21.31	WNK4
Pseudohypoaldosteronism, type 2C	614492	12p13.33	WNK1
Pseudohypoaldosteronism, type 2D	614495	5q31.2	KLHL3
Susceptibility to hypertension	145500	1p36.12	ECE1
		1q42.2	AGT
		3q14	AGTR1
		7q36.1	NOS3
		12p13.31	GNB3
		12p12.2-p12.1	HYT4
		15q	HYT2
		17q	HYT1
		20q13.13	PTGIS
Williams–Beuren syndrome	194050	7q11.23	

One of the first GWAS to be published was that performed by the group of Boerwinkle based on the Family Heart Program in Rochester, Minnesota, that included 3974 subjects from 583 families. The first study was performed based on the selection of 69 sibling pairs highly discordant for their blood pressure level (above the eightieth percentile or below the twentieth percentile) (223). Four genomic regions (2p22, 5q33, 6q23, 15q25) were suggested to be linked with SBP. In a second study, the authors analyzed the candidate genes contained in the most promising region (5q33); among them, the dopaminergic receptor type 1A, the  $\alpha$ 1B-adrenergic receptor, and the  $\beta$ 2-adrenergic receptor (224). For each of those, an extensive search for polymorphisms was performed followed by genotyping the study population. Most results

**TABLE 52-3** Some Mendelian Conditions Associated with Hypotension

Phenotype	OMIM No.	Locus	Gene
Achasia-Addisonianism-Alacrima syndrome	231550	12q13.13	
Aromatic L-amino acid decarboxylase def.	608643	7p12.1	<i>DDC</i>
Corticosteroid binding globulin deficiency	611489	14q32.13	<i>SERPINA6</i>
Corticosterone methyloxidase def., type II	610600	8q24.3	<i>CYP11B2</i>
Demyelinating leukodystrophy	169500	5q23.2	<i>LMNB1</i>
Fabry disease	301500	Xq22	<i>GLA</i>
Hereditary amyloidosis	105210	18q12.1	<i>TTR</i>
Hereditary sensory and autonomic neuropathy	223900	9q13.3	<i>IKBKAP</i>
Multiple system atrophy	223360	4q22.1	<i>SNCA</i>
	223360	9q34.2	<i>DBH</i>
Orthostatic hypotensive disorder	143850	18q	
Pseudohypoaldosteronism, type 1	264350	12p13.31	<i>SCNN1A</i>
		16p12.2	<i>SCNN1G</i>
		16p12.2	<i>SCNN1B</i>
	177735	4q31.23	<i>NR3C2</i>

did not show any consistent association between these polymorphisms and blood pressure. The polymorphism (Arg16Gly) of the  $\beta$ 2-adrenergic receptor appeared the most interesting because of its association with significant, although minor, changes (2–3 mmHg) of SBP. Another study was performed in England on 119 white families selected based on the presence of at least three hypertensive subjects in the same sibship (147). The genome scan used 262 markers and identified a suggestive region on chromosome 11q. However, the analysis of 124 additional families did not confirm that location. A statistical simulation confirmed the genetic complexity of essential hypertension, with a multipoint exclusion-linkage analysis showing that at least three genetic loci were necessary to explain the familial aggregation of the trait.

The Amish population seems ideal for genetic studies looking at blood pressure because of both the large size of the families and the homogeneous environment compared with outbred populations. The study reported by Hsueh and colleagues involved 694 subjects from 28 families, each containing 3–69 individuals (225). A total of 357 microsatellite markers have been genotyped in 436 parent–siblings couples, 1326 sibling pairs, 1342 relations between first-degree relatives, and 1311 relations between first cousins. In these families, heritability was 0.23 for systolic pressure and 0.29 for diastolic pressure, estimations similar to previous observations. The only

region that was linked without ambiguity to the trait was located in 2q31–34, with LOD scores of 1.64 for systolic and of 3.36 for diastolic blood pressure. The GWAS was performed on 1702 subjects from 332 families selected from the Framingham survey (226). For each individual, blood pressure was measured at least three times over a period of at least 10 years. The quantitative trait analyzed was the blood pressure averaged on this duration and adjusted for age and BMI. A 10-cM genome scan was performed that allowed the analysis of the following relative pairs: 1545 sibling pairs, 933 parent–offspring pairs, 742 cousin pairs, and 468 pairs of other first-degree relatives. The statistical analysis showed only one region of interest—chromosome 9—for diastolic blood pressure. For SBP, a suggestion of linkage was found for several regions on chromosomes 5, 10, and 17. On chromosome 17, two regions were suggested, with the one located on 17q12–21 giving the most promising multipoint LOD score (4.7).

This chromosome 17 region corresponds with what was also found by Julier et al. (126). In this study involving French hypertensive sibpairs, French diabetic pairs with hypertension, and U.K. families, significant linkage and association were found using markers of the same region that contains the ACE locus at its telomeric end. However, no particular gene has yet been proven as the one responsible for this statistical association. Other arguments make this a region of interest. First, it corresponds to the chromosome 10 region in the rat genome, a region that has been shown by two groups to contain a major locus, explaining up to 20% of the blood pressure variance in the stroke-prone spontaneously hypertensive rat compared to its normotensive counterpart, the WKY rat (115,116). Second, it is also a region containing one of the genes responsible for pseudohypoaldosteronism (PHA2B), an autosomal dominant hypertensive condition associated with hyperkalemia despite normal glomerular function (227). Finally, a missense polymorphism (Gly40Ser) at the glucagon receptor gene located at 17q25 has been associated with hypertension with an allele frequency of 0.08 in a limited set of white hypertensives who have two hypertensive parents compared to only 0.01 in normotensive controls (228). This polymorphism was not significantly associated with hypertension in the overall sample of 741 French hypertensive subjects compared to 412 controls (229) (Table 52-3).

The Finnish Twin Cohort Study has also been used for a GWAS of blood pressure (179). The strategy was to study a limited number of pairs with an early-onset phenotype (mean age at discovery, 38.3 years) and to diminish the effect of possible confounding factors (at least one sib with BMI < 27 kg/m<sup>2</sup>, exclusion of diabetes). Only 47 sibpairs were studied, comprising 36 dizygotic twins and 11 nontwin siblings, in the first stage of the analysis. In stage 2, genotyping was focused on the regions of suggestive linkage, with the addition of all available family members of the 47 probands (total of 138 subjects).

Despite these relatively small numbers, several regions gave a maximum likelihood score  $>1.5$  (1q, 2q, 22q, and Xp). The most significant result was obtained on chromosome 3q21–25 (maximum likelihood score = 3.38 on the entire sample). This region contains AT1R, and the stronger result was obtained with the intragenic CA repeat at this locus. Analysis of candidate genes in a German population, using blood pressure as a quantitative trait, also suggested linkage with AT1R (230). GWAS was also performed in 125 random and 81 obese families participating in the Québec Family Study (231). Multiple linkage regions were suggested for SBP, especially on chromosomes 1p, 2p, 5p, 7q, 8q, and 19p. One of the main interests of this study was to show the importance of gene–gene or gene–environment interactions, suggested by the fact that most of the regions that were suggested in lean families did not show any linkage when analyzed in families in which one or more members had a BMI  $>32$  kg/m<sup>2</sup>.

In order to increase the sample size and to replicate findings, multiple large population-based cohort studies collaborated. One of these endeavors, the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium gathered genome-wide data for about 38,000 individuals, with large numbers of detailed health-related phenotypes measured in similar ways (232). The CHARGE blood pressure group with a joint meta-analysis with the Global BPgen Consortium identified four SNPs associated with SBP in ATP2B1, CYP17A1, PLEKHA7, SH2B3, six SNPs associated with diastolic blood pressure in ATP2B1, CACNB2, CSK-ULK3, SH2B3, TBX3-TBX5, ULK4, and one SNP associated with hypertension in ATP2B1 (233).

In the largest GWAS to date with over 200,000 individuals of European descent, the International Consortium for Blood Pressure Genome-Wide Association Studies identified 16 novel loci, including six loci containing genes previously known to regulate blood pressure (GUCY1A3-GUCY1B3, NPR3-C5orf23, ADM, FURIN-FES, GOSR2, GNAS-EDN3) (234). It was concluded that multiple common genetic variants contribute to interindividual BP variation in people of European and non-European ancestry. Even though the effect sizes of risk alleles were small, 1/0.5 mmHg, the aggregate effect on BP appears clinically relevant (234). It is interesting to note that one of the loci identified contains a gene, CYP17A1, known to be responsible for the Mendelian form of hypertension—congenital adrenal hyperplasia. Another locus contains ADM, which encodes adrenomedullin, which has natriuretic, vasodilatory and BP-lowering properties. Other loci identified include GNAS, which encodes a subunit of G-protein, mediating signal transduction at adrenergic receptors, and SLC4A7, which encodes a sodium/bicarbonate cotransporter.

With the goal of discovery of new variations associated with traits using a candidate gene approach and the discovery of new variants using the genome-wide

association mapping approach, the Candidate Gene Association Resource (CARE) was developed to perform cross-cohort analysis of genetic variation in cardiovascular, pulmonary, and other traits, comprising over 40,000 participants in nine community-based cohorts (235). In 8090 African-Americans the CARE project identified and replicated 17 loci previously associated with coronary heart disease or its risk factors including high blood pressure in Caucasians, including CDKN2A/CDKN2B and FADS1–3, PLTP, LPL and ABCA1 (236). Admixture mapping analysis for SBP and diastolic blood pressure was also employed to detect disease variants with substantial allele frequency differences followed by trait-marker association analysis in ancestral populations in the CARE consortium. It was found that in 6303 unrelated African-Americans and subsequently replicated in 11,882 independent of African-American subjects, that a novel variant on chromosome 5 between the SUB1 and NPR3 genes was associated with both SBP and DBP (237). Using candidate gene SNP data on a 50K cardiovascular gene-centric array, the CARE consortium found an association for SBP near SLC25A42 and for DBP near HLA-B (238). Previously reported European-American blood pressure SNPs were also replicated in this African-American samples, including SH2B3, TBX3-TBX5 and CSK-ULK3 (238). It is also of note that genome-wide studies undertaken to study metabolic syndrome has identified SNPs that contribute to variation in SBP, further demonstrating the complicated polygenic nature of this common disease (239).

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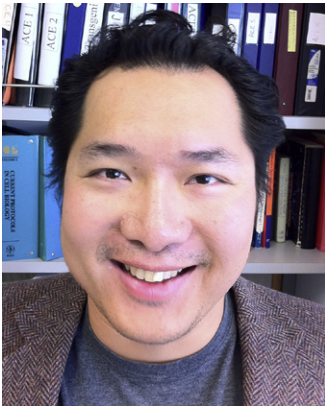


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### Biographies



**Frank S Ong MD**, Dr Ong graduated with high honors from the University of Southern California in Gerontology (BS), Biological Sciences (BA) and Medicine (MD). He then served as resident physician in the Department of Family Medicine at Geffen School of Medicine, UCLA. He completed a research fellowship in genetics of hypertension at Cedars-Sinai Medical Center and a clinical fellowship in molecular genetics at UCLA. Dr Ong is now the faculty of Biomedical Sciences at Cedars-Sinai Medical Center. He has served as a peer-reviewer for *Hypertension*, *Journal of the American College of Cardiology*, *The Pharmacogenomics Journal*, *Pharmacogenetics and Genomics*, and *PLoS ONE*. He is also a Western Regional Councilor and Institutional Representative for the American Federation of Medical Research. He has received numerous awards, including American Heart Association Kidney Council New Investigator Award for High Blood Pressure Research, Western Society for Clinical Investigation Award, Cedars-Sinai Medical Center Sports Spectacular Endowed Fellowship Award and the American Federation of Medical Research Henry Christian Award. He has been funded by the NIH for work on the angiotensin-converting enzyme and injury. He is also active in several population genetics consortia focusing on the genetics of blood pressure regulation and pharmacogenetics. He enjoys playing the violin and hiking.



**Kenneth E Bernstein MD**, Dr Bernstein is currently a Professor of Biomedical Sciences and Pathology and Laboratory Medicine at Cedars-Sinai Medical Center. He began his research training as a postdoctoral Fellow in the Laboratory of Immunology, NIH, Bethesda, MD. In 1987, he relocated to Emory University where he remained until 2008, when he came to Cedars-Sinai. Bernstein has studied ACE, angiotensin II, and the renin-angiotensin system for more than 25 years and published over 145 papers describing original findings. He has made many important contributions including cloning the gene encoding the angiotensin converting enzyme and angiotensin II AT1 receptor, identifying the testis ACE promoter, elucidating novel kinase signaling by the AT1 receptor (Jak/STAT and Src signaling), and most recently, using targeted homologous recombination to create several novel mouse models of ACE expression. Dr Bernstein received the AHA's Novartis Prize for Hypertension Research in 2005 and the AHA's Basic Science Prize in 2007, only the fourth person to win both these international awards.



# CHAPTER

# 53

## Preeclampsia

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### 53.1 INTRODUCTION

Abnormalities of blood pressure regulation can precede, arise during, or appear for the first time in the puerperium of pregnancy. The terminology used to describe these conditions has varied across time and the definitions applied to many of these terms have also changed. These fluctuations confound the interpretation of aggregate data from the literature. This includes the data that address the epidemiology, etiology and management of these conditions. Preeclampsia is a hypertensive disorder of pregnancy that is associated with proteinuria. The prevalence varies across geographic regions (115) and racial/ethnic groups (97). In one prospective cohort study, the relative risk for preeclampsia in black women was 2.4 (95% confidence interval 1.1–5.6) compared to white women (81). Socioeconomic factors also appear to influence the general demographics for this condition. The adjusted odds ratio for preeclampsia was 4.0 (95% confidence interval 1.1–14.0) for persons who receive no prenatal care (60). Data extrapolated from renal biopsies suggests that parity is important, with a greater risk of preeclampsia among nulliparous women. Furthermore, underlying renal disease is an important covariable among multiparous women who display the condition (105). In a review that compared the onset of preeclampsia in mothers carrying twin vs singleton pregnancies, the relative risk of developing preeclampsia was increased among those carrying twins (2.8–4.4) (85). In addition, preexisting medical conditions, wherein hypertension and renal disease may be observed simultaneously (e.g. diabetes, chronic hypertension, autoimmune disorders), increase the risk of developing preeclampsia (111). Generally speaking, preeclampsia occurs in 1–6% of all pregnancies with much higher numbers (10–20%) reported for nulliparous women (115,125). Of course, accepting any statement on prevalence is tempered by varied nomenclature used to describe diseases characterized by hypertension during pregnancy and the specific definitions used to define preeclampsia. In the United States, preeclampsia ranks among the top three causes of maternal death (15.7%, 1.8/100,000 live births) compared to embolism (19.6%,

2.3/100,000) and hemorrhage (17.2%, 2.0/100,000) (29). Preeclampsia also takes a toll on fetal/neonatal well-being. Mothers with preeclampsia are more likely to deliver preterm neonates that are small for gestational age (i.e. fetuses have intrauterine growth restricted). This leads to higher perinatal morbidity and mortality rates.

Preeclampsia is a quantitative trait disorder that is likely to be polygenic and result from multiple factors, some genetic and some environmental. Proteins and the genes that encode them are emerging as are the interactions between environmental factors and these proteins. Whether the aggregate of these has their greatest impact at the maternal–placental interface, within the maternal or fetal compartments, or are in fact playing their roles in various compartments depending on the gestational age of the pregnancy remains to be clarified. Independent of our ability to understand the proteins and interactions that give rise to preeclampsia, one thing seems certain: preeclampsia has a heritable basis.

### 53.2 CLASSIFICATION

Before 2000, consensus regarding the nomenclature for the disorders characterized by hypertension during pregnancy had been lacking. More recently, several national (e.g. American College of Obstetricians and Gynecologists) and international entities (e.g. International Society for the Study of Hypertension in Pregnancy) have adopted the nomenclature put forth by the National Institutes of Health/Working Group Report on High Blood Pressure in Pregnancy 2000 (Table 53-1) (125). More devastating than the problem of delay in adopting an agreed-upon nomenclature for disorders of hypertension during pregnancy, the criteria used to define preeclampsia have been changed on multiple occasions over time. Currently, there is not a single agreed-upon definition (125). The unifying characteristics among those proposed are hypertension and proteinuria; after this, there is a great deal of divergence—some subtle and some glaring.

**TABLE 53-1 Classification Scheme for Conditions Characterized by Hypertension during Pregnancy**

	Pre-eclampsia	Chronic Hypertension with Superimposed Preeclampsia	Chronic Hypertension	Gestational Hypertension	Transient Hypertension	Pregnancy Induced Hypertension	Unclassified
NIH/NICHD (126)	X	X	X	X			
NIH/NICHD (115)	X	X	X		X		
ACOG (2)			X			X	
ACOG (67)	X	X	X	X			X
Canadian Hypertension Society (108)		X	X	X			X
WHO (152)	X		X				

### 53.2.1 Hypertension

Blood pressure is a quantitative trait (see Chapters 76 and 77). The threshold values for systolic (140 mmHg) and diastolic (90 mmHg) blood pressure used to discriminate between normotensive and hypertensive measures were first adopted in 1952 (146). These values apply to pregnant women independent of their age. In conflict with these values are published norms for teenage girls. In this subgroup of patients, blood pressure values that exceed the mid-130 mmHg systolic or the mid-80 mmHg diastolic are above the ninety-fifth percentile value (126). Yet pregnant teens do not meet current blood pressure criteria for preeclampsia until either the systolic or diastolic values meet or exceed 140 mmHg or 90 mmHg, respectively. This represents the most obvious example that for quantitative disorders (e.g. hypertension) that are defined by threshold values, age-specific normative data are required. There are also racial differences with respect to blood pressure changes across pregnancy (97). In a recent report, fifth and ninety-fifth percentile confidence intervals were generated for blood pressure across pregnancy (83). Importantly, this was completed for a defined population, Mexican-Hispanics. An additional consideration includes variation in techniques used to measure blood pressure. The National Institutes of Health/Working Group Report on High Blood Pressure in Pregnancy 2000 has adopted Korotkoff sound 5 for use in determining diastolic blood pressure. This report requires that blood pressure meet or exceed 140 mmHg systolic or 90 mmHg diastolic after 20 weeks of gestation. Furthermore, this threshold must have been reached on at least two occasions (125).

### 53.2.2 Proteinuria

Proteinuria in pregnancy is most often determined using a 24 h collection, and protein values are expressed per unit time (24 h). Values exceeding 300 mg are considered pathologic if they appear for the first time after 20 weeks gestation. This defining feature varies little among groups that have demonstrated an interest in

setting disease defining thresholds for this quantitative trait. More recently, attention has been paid to indentifying patients who might not require a 24 h urine collection. The suggestion has been made that a specific protein/creatinine (P/C) ratio threshold might establish those that are more likely to have a negative 24 h urine collection, thus sparing the health care system and patients cost and inconvenience, respectively. In a review of 10 publications (1994–2004) reporting on the use of a random urine calculation of the P/C ratio in pregnancy, there was a strong correlation between this and a formal 24 h urine calculation of total protein (122). These authors recommend obtaining a formal 24 h urine collection when the P/C ratio exceeds a predetermined cutoff. The International Society for the Study of Hypertension in Pregnancy (ISSHP) has endorsed a P/C ratio cutoff  $\geq 30$  mg/mmol as equivalent to a 24 h specimen (22).

In conclusion, preeclampsia is a quantitative trait disorder that is identified only in females during a specific lifetime event—pregnancy. Furthermore, this condition requires defined thresholds for elevated blood pressure and renal dysfunction, in the form of proteinuria, to establish the disease. Unfortunately, these criteria may not be generalizable to all pregnant patients (e.g. teenagers, patients with underlying renal disease).

### 53.2.3 Other Features

Once the diagnosis of preeclampsia is established, management decisions are based upon clinical features that include the gestational age of the fetus, degree of blood pressure elevation at the time of diagnosis, and the response of blood pressure to therapeutic interventions (e.g. bed rest, medication). Severe disease often results in the need for preterm delivery. Severe disease is defined by blood pressure, quantitative measure of urine protein, platelet count, and serum chemistries. Severe disease is also considered to be present when tests of fetal well-being demonstrate compromise and there are specific maternal signs and symptoms (Table 53-2) (58). Although protein in a 24 h urine collection is used to define severe disease,

**TABLE 53-2** Criteria Used to Establish Severe Preeclampsia

Severe blood pressure elevation
Elevated liver function studies
Low platelet count (100,000)
Eclampsia
Persistent maternal headache
Scotomata/blurred vision
Proteinuria ( $\geq 5$ g/24 h) and at least 34 weeks' gestation
Nonreassuring tests of fetal well-being
Oligohydramnios
Fetal growth restriction
Placental abruption

recent data suggests that if this is the only criterion of severe disease, the pregnancy can be allowed to continue until 32–34 weeks' gestation (35,132). Patients with preeclampsia do not follow a defined disease course. Severe disease is associated with a greater prevalence of maternal and fetal morbidity and mortality (98,137,138). For this reason, severe disease often results in preterm delivery and its sequelae.

### 53.2.4 Future Reproduction and Health Implications

Genetic counseling may be desired by patients who experienced preeclampsia in a previous pregnancy. Generally speaking, severity of disease in the index case influences recurrence in a subsequent pregnancy. Numerous clinical studies describe the recurrence risk of preeclampsia in a subsequent pregnancy. Recurrence estimates for preeclampsia range from 17.9% up to 55% if the index pregnancy was complicated by early severe preeclampsia with hemolysis, elevated liver enzymes and thrombocytopenia, or eclampsia (Table 53-3) (28,66,136). Since the risk of recurrent preterm delivery is a primary concern voiced by patients presenting for counseling on recurrence risk, a perspective on this risk can be helpful. A recent report of 334 patients delivered before 34 weeks because of a hypertensive disorder of pregnancy identified that 64% of patients refrained from

pregnancy. Of the remaining, 211 had a subsequent pregnancy, 17% had a recurrent preterm (<34 weeks) delivery due to a complication of hypertension and 14% delivered between 34 and 37 weeks. Of those delivering after 37 weeks, a minority had an uneventful pregnancy course (89).

The estimated incidence of chronic hypertension following a pregnancy complicated by preeclampsia has ranged from 0% to 78% with a mean of 23.8% (30,46,136). Long-term follow-up studies show that a higher proportion of eclamptic and preeclamptic women have developed chronic hypertension within 10 years of the index pregnancy (4.4%) compared with normotensive women (1.5%). The cumulative risk increased more with age in the eclamptic and preeclamptic group. In women observed for more than 10 years, the proportion was 51% compared with 14% in the normotensive group (136). Gestational age at first detection of high blood pressure seemed to be an important risk factor. Women with onset of high blood pressure before 31 weeks had a 2.3 times higher risk of developing chronic hypertension than those with later onset disease. Therefore, women who develop preeclampsia early in pregnancy are more likely to have the complications of hypertension and proteinuria in more than one pregnancy (recurrent preeclampsia), and those who develop preeclampsia for the first time in their second or later pregnancy are at risk of developing chronic hypertension. Although some still debate whether women with preeclampsia or transient hypertension have an increase risk of future hypertension compared to women without prior preeclampsia, the literature leaves little room for disagreement (4,93,133).

One way to measure whether the preeclampsia syndrome is a risk factor for future cardiovascular disorders is to determine the proportion of women who develop hypertensive complications or die from such complications. A significant increase in mortality rate has been observed in women (black and white) classified as parous at the time of the index pregnancy. The death rate was also significantly higher in black primigravid women but not in primigravid white women. The majority of the deaths (82%) were due to hypertensive cardiovascular complications, which was over three times the rate in

**TABLE 53-3** The Recurrence Risk of Severe Preeclampsia or the Risk of Chronic Hypertension When the Index Pregnancy was Complicated by Severe Preeclampsia

Index Pregnancy	Complicated by Early-Onset, Severe Preeclampsia (HELLP) Syndrome, or Eclampsia (28,66,136)	Early-Onset Preeclampsia (Delivery <34 Weeks) (89)	Eclampsia or Preeclampsia (136)
<b>Future Pregnancy</b>			
Overall recurrence	17.9–55%		
Complication requiring delivery <34 weeks		17%	
Chronic Hypertension with 10 years			4.4%
Chronic Hypertension greater than 10 years later			51%

women without pregnancy-related hypertension. Nearly half of this mortality was secondary to cerebral hemorrhage. Women with recurrent preeclampsia syndrome were also at high risk of early death from cardiac disease (31). This has been the case in the Icelandic population as well, where the death rate among women with previous hypertensive complications in pregnancy was significantly higher than expected. About 55% of the excess deaths were attributed to ischemic heart disease. An association with the severity of the pregnancy disease and the risk of developing ischemic heart disease later in life was also noted (72). The clinical symptoms at the time of pregnancy do not allow differentiation between those who are destined to develop chronic hypertension or heart disease in later life and those who will not do so. The same general advice on preventive measures to promote a healthy lifestyle must be targeted to all those at potential risk, at least until it becomes possible to separate subgroups at definite risk for subsequent cardiovascular disease.

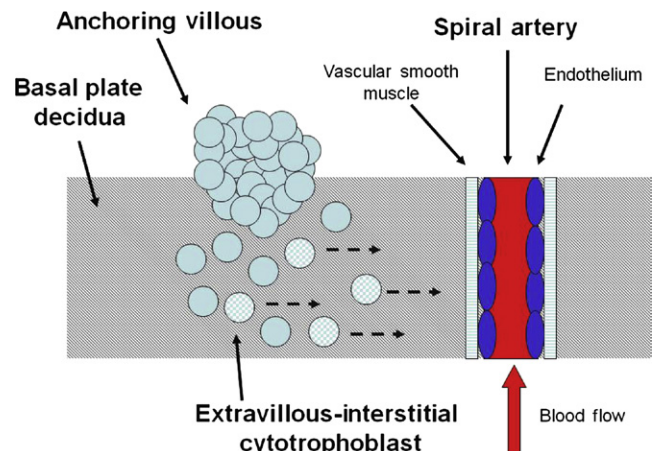
### 53.3 PATHOLOGIC AND MOLECULAR BASIS

The pathogenesis of preeclampsia is not well understood. However, trophoblast invasion may hold the key. In normal pregnancy, there are two primary trophoblast invasion processes. One takes place as the trophoblast of the anchoring villi invades the decidua and the other centers on trophoblast invasion of the spiral arteries. If either of these processes become dysregulated poor placentation and a successful pregnancy outcome has the potential to be compromised. Research suggests that cytokine production by immune-regulatory cells, a hypoxic microenvironment within the decidua, abnormal immune recognition at the maternal-fetal interface, circulating “toxins” as a cause of endothelial dysfunction and altered vascular smooth muscle responses during pregnancy are probably the contributors to the hypertension-associated conditions of pregnancy, and more specifically, preeclampsia. It is unlikely that one of these alone determines the pathophysiologic events leading to preeclampsia. Since preeclampsia is a condition defined by quantitative traits, it is likely that perturbations in several genes, biologic pathways, and tissues lead to the aberrant blood pressure regulation and proteinuria that define this disease. Placental implantation represents a logical starting point to explore the pathogenesis of preeclampsia, because it is widely appreciated that once the placenta is removed (i.e. the pregnancy is discontinued), preeclampsia resolves. It is precisely this dictum that is responsibility for the preterm birth burden of this condition.

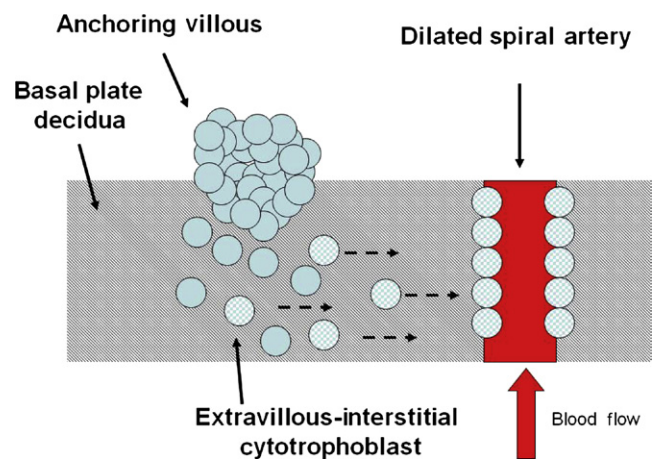
#### 53.3.1 Normal Placentation

As mentioned, normal placentation is characterized by two distinct processes of trophoblast invasion: invasion of the decidua and invasion of the spiral arteries.

In normal placentation anchoring villi, consisting of villous cytotrophoblast, must first invade and thereby attach to the decidua. The cells of the anchoring villous proliferate and some develop into progenitors of extravillous cytotrophoblast cells. The latter detach from the anchoring villi in the first weeks of pregnancy and migrate through the decidua as interstitial cytotrophoblast (Figure 53-1). A subset of these cells invades the spiral arteries. The endovascular invasion of the spiral arteries by interstitial cytotrophoblast cells is thought to occur at two distinct times in pregnancy; the first occurs during the first trimester probably around 12 weeks gestation, and the second wave of invasion peaks in the second trimester (16–20 weeks) (18). The invasion of spiral arteries by cytotrophoblast cells results in plugging followed by recanalization (pseudovascuogenesis) of the spiral arteries and then replacement of endothelium and vascular smooth muscle cells (Figure 53-2). During normal pregnancy, this process takes place along the length



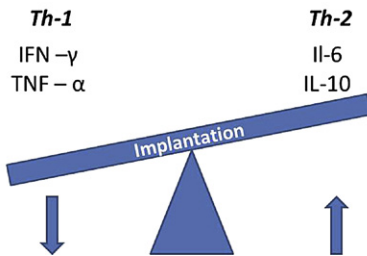
**FIGURE 53-1** The relationship of anchoring villi, decidua, and spiral artery at implantation. A population of extravillous cytotrophoblast migrates toward the spiral artery.



**FIGURE 53-2** Migration of extravillous cytotrophoblasts results in endovascular invasion of the spiral artery. Trophoblasts replace endothelial cells and vascular smooth muscle cells. The result is a low resistance and dilated spiral artery.



### Implantation of anchoring villi



**FIGURE 53-3** A shift in the balance of Th-1 proinflammatory to Th-2 anti-inflammatory cytokines is implicated in trophoblast invasion of the decidua.

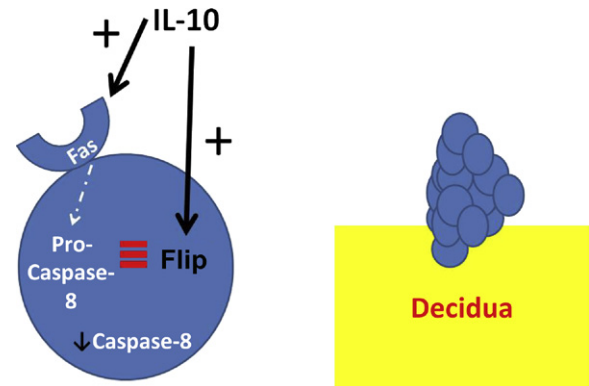
of the spiral artery endothelium. The process of endovascular invasion is believed to transform spiral arteries into dilated low-resistance conduits of blood flow (19,20,120).

### 53.3.2 Abnormal Invasion of Decidua

A great deal of attention has been paid to the process of early trophoblast invasion into the decidua. Anchoring villi must invade the decidua before extravillous cytotrophoblast can migrate through the decidua to invade the spiral arteries. Furthermore, premature trophoblast cell death could result in the release of toxins and cellular debris into the maternal circulation with downstream systemic effects that characterize the clinical features of this condition (e.g. hypertension, renal dysfunction).

Investigations of the Fas/FasL pathway have provided possible insights surrounding trophoblast invasion of the decidua by anchoring villi structures. It seems clear that this represents a dynamic process requiring a shift in cytokine production from a Th-1-dominated (proinflammatory) to a Th-2 dominated (anti-inflammatory) milieu (Figure 53-3). The critical cytokines in the former are IFN- $\gamma$  and TNF- $\alpha$  and those in the latter group are IL-6 and IL-10. Regulation of the Fas/FasL pathway by cytokines appears to be a well-established process in normal invasion of the decidua (Figure 53-4) (12). The exact origin of the cytokines responsible for the Th-1 to Th-2 shift—whether from macrophages or natural killer cells—is less certain. When first-trimester trophoblast cells derived from a cell line (H8) are exposed *in vitro* to serum from patients with preeclampsia, cell viability was reduced and the Fas/FasL pathway was implicated (116).

Originally, the Fas/FasL pathway was described as a regulator of the life span of peripheral blood activated mature T-cells (113,140,141). Fas (CD95) is a marker of activated T-lymphocytes and is also found as a soluble molecule in serum. Fas is a membrane protein (TNF-R superfamily) and in the immune system, is a marker of activated T-cells where it is the initiator of apoptosis.



**FIGURE 53-4** IL-10 upregulates the expression of Fas, however, apoptosis is not increased because IL-10 also upregulates FLIP. FLIP binds (red bars) to pro-caspase-8, which prevents cleavage to the active enzyme caspase-8. Trophoblast cells remain viable and capable of implantation.

Soluble Fas is present in serum too and in this form is capable of binding to FasL and thus prevent apoptosis. Many investigators have implicated cell-mediated immunity in the pathogenesis of preeclampsia (100,124,130). Peripheral blood CD8<sup>+</sup> T cells obtained from patients with preeclampsia demonstrated greater expression of Fas compared to controls, suggesting enhanced cell-mediated immunity. In addition, soluble Fas in serum was greater suggesting an accumulation of these cells and slower turnover due to the decreased availability of FasL (82).

### 53.3.3 Abnormal Invasion of Spiral Arteries

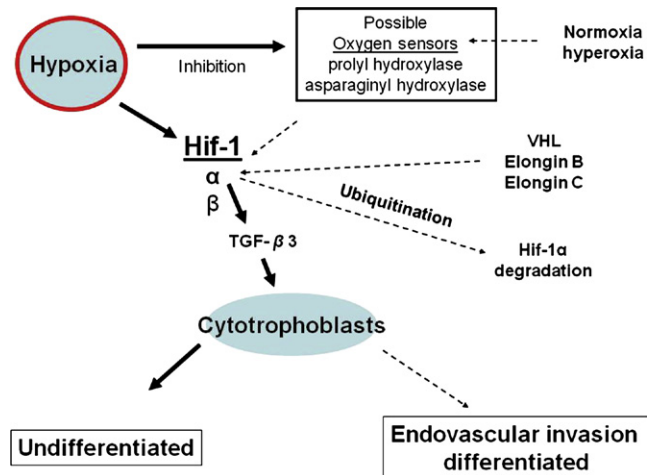
**53.3.3.1 Histologic Features.** Supporting the clinical observations that implicate the placenta in the pathogenesis of preeclampsia are abnormal histologic features of trophoblast cells (Table 53-4) and the spiral arteries. Cytotrophoblast cells from placentas of patients with preeclampsia have distinctly different cell adhesion markers and invasive properties compared to cells from control placentas (Table 53-3). There is less transformation of spiral arteries by cytotrophoblast and they appear to be occluded by fibrin, IgM, complement (C<sub>3</sub>), apolipoprotein-a, platelets, lipid-laden macrophages, and leukocytes (80,86,106,128). In aggregate, these make up the histologic lesion called “acute atherosclerosis,” which is considered to be the pathognomonic lesion of the placenta in preeclampsia (157). There is, however, some overlap between “acute atherosclerosis” of the placenta described in preeclampsia and the lesion seen in cases of idiopathic fetal growth slowing (IUGR), chronic hypertension, and miscarriage also conditions with a placental origin (18,53,106). In the latter conditions, the defining features of preeclampsia, hypertension and proteinuria are absent. The observation of a similar lesion has led to speculation that acute atherosclerosis occurs secondary to more “upstream” etiologic events (120).

**TABLE 53-4 Histologic Change in Trophoblast from Patients with Preeclampsia vs Normal Pregnancies (6,8,11,17,70)**

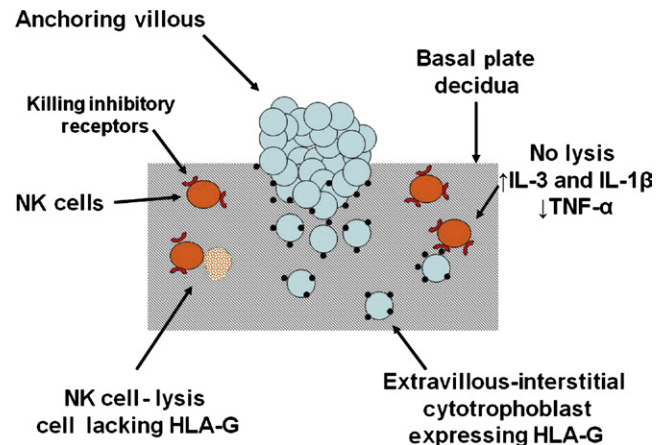
Syncytiotrophoblast	Smaller Mass Higher Glycogen Content
Interstitial Cytotrophoblast	Shallow Invasion Greater Number
Endovascular Trophoblast	Fewer Numbers Fewer Vessels Transformed Patchy and Discontinuous

**53.3.3.2 Oxygen and the Microenvironment.** The triggers for abnormal cytotrophoblast differentiation and migration toward the spiral arteries are not completely understood. Recent work implicates altered oxygen concentrations at the maternal–fetal interface (Figure 53-5). When villous explants are cultured under hypoxic conditions, they are induced to proliferate (47,79); however, assays of differentiation that rely on cell surface protein expression suggests a failure to differentiate (51,52). The proliferative response to hypoxic conditions may be mediated by the transcription factor Hif-1. Hif-1 has  $\alpha$  and  $\beta$  subunits. It is the  $\alpha$  subunit that is upregulated during hypoxic conditions, whereas, under either normoxic or hyperoxic conditions, the degradation of this subunit occurs (102). Within placental tissue, Hif-1 was shown to be upregulated during conditions of hypoxia within the placental microenvironment in vivo and in vitro experiments (123,150). Hif-1 also appears to regulate transcription of TGF- $\beta$  3 (131). Strong evidence suggests TGF- $\beta$  3 inhibits differentiation and endovascular invasion by cytotrophoblasts (24,25). Thus, Hif-1 may be a key regulator of gene expression important in trophoblast differentiation and migration.

**53.3.3.3 Immune Regulation of Trophoblast Migration.** It is remarkable that placental implantation proceeds uninterrupted despite having the potential, as a semi-allogenic graft, to initiate rejection by the maternal immune system (107). Tissue is rejected by the maternal immune system through the interaction of several cell types and antigens. Unchallenged observations, related to the interaction of fetal antigens and maternal capacity to reject fetal tissues, include the lack of classical HLA Ia gene expression by trophoblast cells (45,55,69), the expression of nonclassical HLA-G by extravillous trophoblast, and trophoblast cells that make up distal anchoring villi (42,84). Key cell types at the maternal–fetal interface include maternal immunoreactive cells (natural killer (NK) cells, and CD8<sup>+</sup> and CD4<sup>+</sup> T-Lymphocytes) and fetoplacental cytotrophoblast (villous and extravillous). NK cells are the primary macrophage subtype in the decidua. NK cells have the ability to destroy the cells that lack classical HLA (class Ia) antigens (A, B, and C), such as tumor cells and cytotrophoblast. They can also initiate a viral immune



**FIGURE 53-5** Hypoxia within the microenvironment of implantation may be responsible for abnormal placentation observed in preeclampsia. The  $\alpha$ -subunit of the transcription factor Hif-1 is under control of oxygen sensors and ubiquitinated under normoxic or hyperoxic condition. Solid line, hypoxia; dashed line, normoxia.



**FIGURE 53-6** Migration of extravillous-interstitial cytotrophoblast is permissive through interactions of HLA-G and KIR receptors on NK cells. Lysis of foreign cells is prevented subsequent to these interactions.

response and they can secrete cytokines in response to various stimuli. In addition to receptors that promote lysis or production of cytokines, NK cells have killing inhibitory receptors (KIR). Protein interactions include those between membrane-bound and soluble HLA-G, and the receptors on NK cells, CD8<sup>+</sup>, and CD4<sup>+</sup> cells. At least two noncompeting theories of immune tolerance at the maternal–fetal interface have been put forth. One holds that cytotrophoblasts that express HLA-G can migrate safely through the decidua to invade the maternal spiral artery without being lysed by NK cells (Figure 53-6). The protective effect of HLA-G is likely mediated through KIR on NK cells. There are at least three NK KIR (LIR-1/ILT2, p49, and BY55) that interact with HLA-G (5,26,36). However, trophoblast cells are resistant to NK cell lysis (15,78). Another mechanism (Figure 53-6) holds that through the interaction of HLA-G with

alternative subsets of KIR on NK cells, cytokine production (IL-3 and IL-1 $\beta$ ) is decreased while tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interferon- $\gamma$  are increased (73,99). Evidence suggests that cytokines are important in the process of vascular invasion by extravillous trophoblast cells (13). Thus, HLA-G offers “immune privilege” to trophoblast cells and its presence may be important in guarding against the development of preeclampsia (90).

### 53.3.4 Serum Toxins and Endothelium

The multiorgan nature of preeclampsia is thought to be due to widespread endothelial dysfunction and decreased organ perfusion (127). The factors that lead to this are not clear. The longest surviving theory held that the placenta produced toxins that were released into the maternal blood stream. This gave rise to the terms “toxemia of pregnancy,” which were the earliest used to describe the clinical entity—preeclampsia (40). Observations that serum from patients with preeclampsia has mitogenic and cytotoxic effects on endothelial cells and fibroblasts, respectively, lend support to the theory of circulating toxins (129,144). Mediators of apoptosis (e.g. soluble Fas) and angiogenesis have been proposed and are found to circulate at varied levels among patients with preeclampsia compared with controls. These could function as the reputed circulating factors proposed by others.

Apoptosis is one explanation for the derivation of serum toxins originating from placental tissue. The relationship between the Fas/FasL pathway has been studied with respect to trophoblast invasion of the spiral arteries too. It seems the expression of FasL by migrating trophoblast cells induces apoptosis of activated lymphocytes (75). Thus, the Fas/FasL pathway may be important in both processes of trophoblast invasion—decidua for attachment and spiral artery invasion for appropriate perfusion. This apoptosis pathway may also play a role in recanalization of spiral arteries (pseudovasculogenesis) plugged by trophoblast after vascular invasion, and the subsequent death of endothelial cells that line the spiral arteries (14). When pseudovasculogenesis fails to occur, there is a poor placental perfusion and a milieu of low oxygen tension potentially important antecedents of preeclampsia.

Neutrophil activation results in the release of elastase, reactive oxygen species, and leukotrienes that can damage endothelial cells (57). These may play a role in causing widespread endothelial dysfunction. Patients with preeclampsia have higher serum concentrations of cytokines and cell adhesion molecules (93) capable of attracting neutrophils to the endothelium and inciting neutrophil activation, prostaglandin production and the release of superoxide anions and hydrogen peroxide. These free radicals can cause damage when the defense system (e.g. vitamin E, glutathione peroxidase, superoxide dismutase, and catalase) is not maintained or is overwhelmed. Observations of increased oxygen-free

radicals and lipid peroxides along with lowered serum concentrations of vitamin E in cases of more severe disease adds credibility to this theory of hematogenous toxins (71,147,149). Animal models support observations in humans (76,139). Hyperlipidemia normally seen in pregnancy is exacerbated in pregnancies complicated by preeclampsia (121). It is conceivable that abnormal lipid metabolism could contribute to the observations of endothelial cell dysfunction (7). Observations in human pregnancy support these concepts (101,117,151).

Reduced organ perfusion as a cause of organ dysfunction originates from several converging mechanisms. Most simplistically, the increased systemic vascular resistance leads to greater capillary oncotic pressure and proteinuria leads to decreased osmotic pressure (44). The result is a decreased intravascular volume and increased resistance to blood flow, both of which result in the potential for compromised end-organ function. Additionally, peptides such as natriuretic peptides (e.g. atrial natriuretic peptide) function to compensate for hypertension by increasing sodium and water excretion (62,108). Preeclampsia is characterized by decreased intravascular volume (48,63).

If adequate blood supply for the delivery of critical oxygen concentrations, nutrients that maintain homeostasis, and a channel for disposal of metabolic fuel is a determinant of placental development and vascular tone and organ function, then endothelial-dependent relaxation is likely a key process. Arteries from specific tissues of patients with preeclampsia demonstrate alterations in endothelial-dependent relaxation (104) and enhanced pressure response to angiotensin II (1). These observations appear to be secondary to endothelial dysfunction rather than smooth muscle dysfunction. Local regulation of vasoactive substances in tissue beds—especially the uterus—may play an important role in this relaxation (21,54). Altered renal proximal tubular function, activation of the coagulation cascade, and increased sensitivity to vasopressors antedate the overt rise in blood pressure seen in preeclamptic patients. The cause of increased sensitivity to circulating pressor agents such as angiotensin II (50) still requires elucidation. An imbalance of endothelial-derived prostaglandins in favor of the endogenous vasoconstrictor thromboxane as compared to the vasodilator prostacyclin could explain this unusual sensitivity to angiotensin II (22,155). Alternatively, there could be altered regulation of angiotensin II receptor concentrations (16) or nitric oxide production (114).

### 53.3.5 Angiogenic Factors

Angiogenic factors are thought to play an important role in the process of pseudovasculogenesis. The logic is as follows. The process of pseudovasculogenesis is characterized by transformation of trophoblast cells that have plugged the spiral arteries. These cells express a unique set of cell-surface markers (Table 53-4) and assume the role

played by endothelial cells. One well-described angiogenic factor vascular endothelial growth factor (VEGF) is capable of transforming stem cells to endothelial cells (118). When pseudovasculogenesis within the placenta fails, the histologic finding “acute atherosclerosis” is seen.

Recently, attention has been directed to circulating angiogenic proteins, VEGF and placental growth factor (PlGF). Evidence suggests that circulating levels of these proteins may be reduced in patients with preeclampsia. This reduction likely results from the upregulation of the soluble fms-like tyrosine kinase-1, sFlt1, which is similar to VEGF receptor (77,91,103,134). It works to “soak up” circulating VEGF and PlGF; by doing so, the function of these angiogenic factors is lost and pseudovasculogenesis is compromised. In addition to inducing angiogenesis, VEGF is known to stimulate the release of endogenous vasodilatory compounds (e.g. nitric oxide) (64,103,109). Capable of binding VEGF and PlGF, sFlt1 may be an important mediator of the clinical manifestations seen in preeclampsia (103). Furthermore, these molecules may hold promise as biomarkers for disease since changes in serum concentration are seen before the clinical phenotype (91,92).

In summary, that the exact pathogenesis of preeclampsia has remained elusive should not be surprising. This disorder is defined by quantitative traits and as such, the genetic basis is likely to involve multiple genes in multiple pathways. In addition, there are almost certain to be environmental factors that impact gene expression. A common theme in the pathogenesis of this condition is abnormal trophoblast invasion that predisposes the placenta to poor perfusion and apoptosis. The increase in vasomotor tone, a feature of this condition, establishes a cycle of events with poor perfusion a central theme. The cycle is not broken without removal of the offending organ—the placenta (Figure 53-7). Whether a hypoxic milieu, perturbations in the immune system at the maternal–fetal interface, or aberrations in the process of pseudovasculogenesis are the inciting factors on a case-by-case basis seems relevant to the future approaches aimed at prevention and/or treatment of this condition.

## 53.4 GENETIC BASIS OF PREECLAMPSIA

Three lines of evidence support the heritability of preeclampsia. The least robust of those currently available are simple descriptive studies. These were followed by epidemiologic studies that led to views of a Mendelian inheritance pattern. Finally, advancing technologies in genetics enabled the search for genetic associations.

### 53.4.1 Family Studies

The earliest reports of multiple affected family members date to the late 1800s (Table 53-5). These were followed by the first prospective study to document

**TABLE 53-5 Cytotrophoblast Surface Molecules during Control and Preeclamptic<sup>a</sup> Pregnancy (158)**

Protein	Proximal Anchoring Villi	Distal Anchoring Villi	Interstitial	Endo-vascular
E-Cadherin	+	+/-	+/-	-
VE-Cadherin	+	+	+	+
$\alpha$ V $\beta$ <sub>6</sub> Integrin	+/-	-	-	-
$\alpha$ V $\beta$ <sub>3</sub> Integrin	-	+	+	+
VCAM-1	-	+	+	+
PECAM-1	+	+	+	+

<sup>a</sup>Results for preeclamptic pregnancies are in shaded rows for each protein.

heritability (34). Among 296 eclamptic women observed between 1931 and 1960, data from 98 of 110 daughters born showed 32% developed preeclampsia in their first pregnancy and 39% developed preeclampsia in any pregnancy. Furthermore, there were 147 sisters of the 296 eclamptic women; 37% developed preeclampsia and 4% became eclamptic. Although a control group was not included in this descriptive study, the data presented support a higher than expected frequency of preeclampsia in some families. This and similar studies (3,9,32,33,37,38,68,94,142) were used to promote the concept that preeclampsia was a Mendelian disorder with either a recessive or a dominant mode of transmission. However, there were flaws in the rationale for supporting a simple Mendelian approach to inheritance. Today, we recognize that preeclampsia is a complex disorder characterized by quantitative traits (hypertension and proteinuria). Thus, multiple genes and possibly environmental factors are implicated in the disease phenotype.

### 53.4.2 Association Studies

With the background from epidemiologic studies that support a single-gene theory for preeclampsia and the ready availability of polymerase chain reaction as a laboratory technique to test for genetic alterations, the natural progression of a search for single genes that might explain this condition was embarked upon. A plethora of genetic association studies followed, and these studies continue to be published today. Some of those that demonstrated positive association (Table 53-6) were often refuted by other investigators within months. In nearly every case, the genes chosen had a biologic basis; however, one could critique patient selection, criteria used to define disease, control groups, and sample sizes



**TABLE 53-6 Familial Predisposition (34)**

Elliot	1873	Mother and four daughters with eclampsia
Lohlein	1879	Three sisters with eclampsia
Favra	1885	Mother and two daughters with eclampsia
Stewart	1893	Eclampsia in two sisters
Haneman	1896	Eclampsia in twin sisters
Morawick	1898s	Eclampsia in mother and daughter
Harig	1901	Fatal eclampsia in twin sisters
Newman	1912	Eclampsia in mother and daughter
Schroderus	1931	Three generations of toxemia

**TABLE 53-7 Genetic Association and Preeclampsia**

Author	Gene	Polymorphism
Ward (148)	AGT	M235 T
Dizon-Towns (41)	Factor V	Leiden (on)
Grandone (54)	MTHFR	C677 T
Yamada (154)	PAI-1	4 G/5 G
Yoshimura (156)	eNOS	Glu298Asp
Tempfer (145)	eNOS	Intron-4 (27 bp)

**TABLE 53-8 Genome Mapping**

Author	Region	LOD Score
Oudejans (119)	10q22	3.6
Laivuori (88)	2p25	3.77
Laivuori (88)	9p13	3.74
Laivuori (88)	4q32	3.13
Lachmeijer (87)	12q	2.1
Arngrimsson (10)	2p13	4.70
Moses (110)	2	2.58
Moses (110)	11q23–24	2.02
Harrison (61)	4q	2.9

used to draw conclusions. Thus far, genetic association studies have not been transformative in guiding clinical management, disease prediction or prognosis.

### 53.4.3 Genome-Wide Scans

Technologic advances recently led to the application of genome-wide scanning to identifying genomic areas of interest for quantitative traits (Table 53-7). These studies have varied in their general approach and the criteria used to establish linkage. Of note, the literature reflects a general trend toward higher LOD scores over time. Overall, at least six chromosomal regions have been suggested to contribute genes important in preeclampsia, while genes located on chromosomes 2 and 4 have been implicated in more than one study (Table 53-8).

### 53.4.4 Parent of Origin

The suggestion that preeclampsia was rooted in a Mendelian inheritance pattern raised the question of whether any gene effects were due solely to parental genes present in the fetus, the maternal environment owing to mother's genes, or a function of maternal and paternal genes combined. Clinical observations that mothers are less likely to develop preeclampsia in subsequent pregnancies with the same father, and that they establish an increased recurrence risk in subsequent pregnancies with a different father support the importance of a paternal contribution. Animal models lend credibility to theories that favor a combination of maternal and paternal gene effects (74,143). Using a Utah database of children born after pregnancies complicated by preeclampsia, a paternal gene effect was demonstrated (43). When the male children from these pregnancies conceived their own children, pregnancies were complicated by preeclampsia nearly two times greater than among control patients (2.7% vs 1.3%, respectively). Thus, it is likely that maternal and paternal genes contribute to the maternal phenotype we define as preeclampsia.

Making sense of these lines of evidence requires an integrated approach, one that recognizes the limitations of the single-gene theory yet accepts that specific genes are important. Preeclampsia fits a quantitative trait model of disease. Therefore, one is left trying to explain its genetics under one of two models: (i) multiple genes with small but equal effects, and (ii) a major gene effect with multiple modifying genes (96). However, neither model is mutually exclusive. The first model would argue a normal distribution of the phenotype (e.g. hypertension), while the second would argue a skewed distribution. The possibility of time-specific expression of a major gene (skewing of the phenotype) over the course of pregnancy cannot be excluded after considering available evidence (83).

## 53.5 ANIMAL MODELS

There has been a paucity of data derived from nonhuman primates that can be used to explain the pathogenesis or redefine therapies for preeclampsia. Despite early reports that showed that nonhuman primates with placental ischemia had clinical features of preeclampsia (27), other species have received greater attention. Exploiting a candidate pathway recognized for its importance in physiologic studies (49) and for which genetic association was suggested, investigators perturbed the renin/angiotensin system in mice. Pregnant female mice that overexpressed human angiotensinogen and were exposed to excess human renin from placental tissue demonstrated features remarkably similar to preeclampsia, including late-pregnancy hypertension and proteinuria (143). Nitric oxide, a potent endogenous vasorelaxing autoid, was investigated. Rats exposed to an inhibitor of nitric oxide synthase showed progressive hypertension with reduced growth of pups (153). Contrary to these

observations, a mouse model with a targeted disruption of endothelial nitric oxide synthase demonstrated higher blood pressure throughout pregnancy, but no evidence of proteinuria (59,65). Two additional rodent models of preeclampsia have been described. A mouse line (BPH/5) with spontaneous hypertension shows late-pregnancy hypertension with normalization of blood pressure postpartum in addition to proteinuria and renal lesions seen in human disease. The genetic perturbations in this line are unknown (39). Recently, the paternally imprinted cyclin-dependent kinase inhibitor gene, *p57Kip2*, has been implicated and the phenotype is strikingly similar to preeclampsia. A decrease or loss of expression of this gene in mice results in placental development that is similar to that observed in some cases of human disease (74). The precise utility of the animal models described, which demonstrate features of preeclampsia, is unclear. Except for the latter model (i.e. *p57Kip2*) and perhaps the as yet unknown perturbation in the BPH/5 line, models have focused primarily on genes or pathways that likely play a role in the human condition. Although the approach to animal modeling was to substantiate evidence derived from human studies, provide new direction for pathogenesis, and to enable a therapeutic alternative to human disease, each model appears to have fallen short of these goals. However, examinations of all previously described models are incomplete and hold the promise of offering new insights into the genetic basis of preeclampsia.

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- NICHD Working Group Report on High Blood Pressure in Pregnancy (July 2000)  
[http://www.nhlbi.nih.gov/guidelines/archives/hbp\\_preg/index.htm](http://www.nhlbi.nih.gov/guidelines/archives/hbp_preg/index.htm).

# Common Genetic Determinants of Coagulation and Fibrinolysis

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## 54.1 INTRODUCTION

Thrombotic disorders are a major cause of morbidity and mortality and can be classified into venous thrombosis, characterized by the deposition of fibrin-rich thrombi, and arterial thrombus, characterized by platelet-rich thrombi superimposed upon atheromatous plaque. Elucidating the mechanisms involved in the development and progression of these disorders has been a major health care initiative for many years. It has become clear that for both venous and arterial thrombotic disorders, abnormalities of hemostasis play a key role.

The hemostatic system maintains a delicate balance between the processes of coagulation and anticoagulation, platelet activation and platelet inhibition, and activation/inhibition of fibrinolysis to achieve hemostasis while conserving vascular patency. Activation of the coagulation cascade and platelets occurs in rapid response to endothelial cell injury, leading to thrombin generation, platelet deposition and fibrin formation (Figure 54-1). Activation of the anticoagulant pathway by interaction of thrombin with membrane-bound thrombomodulin and activation of protein C inhibit further thrombin generation and serve to localize coagulation at the site of vascular injury and prevent widespread systemic coagulation. Finally, the fibrinolytic system helps to maintain vascular patency by lysis of the fibrin matrix.

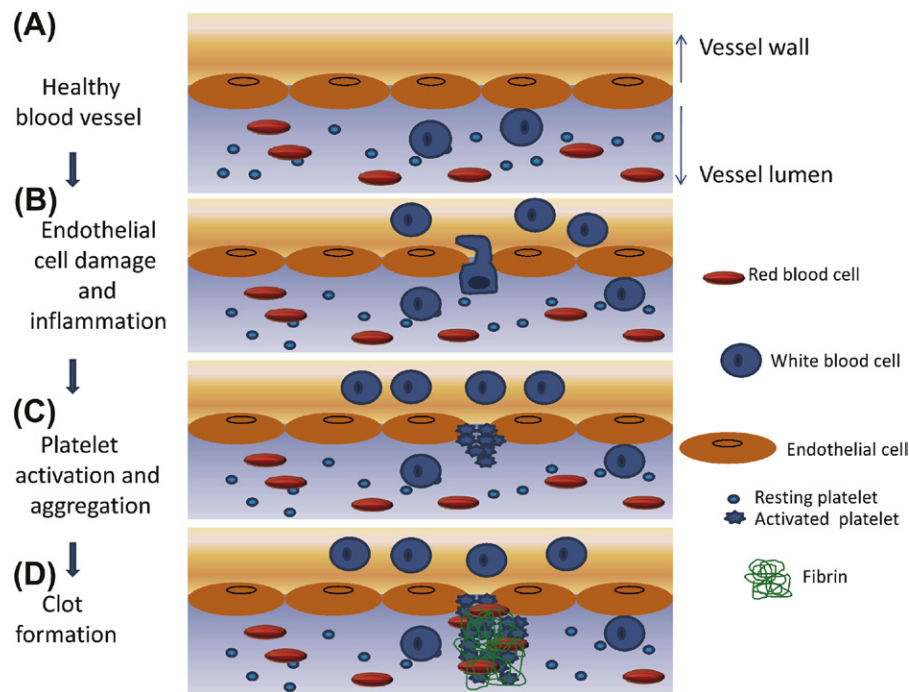
Perturbation of these processes may give rise to bleeding disorders (these tend to be rare familial defects and are not within the scope of this chapter), or to thrombosis. Perturbation of hemostasis may arise through a variety of environmental and genetic influences and ultimately the duration of exposure and the accumulation of risk factors will determine risk of thrombosis.

The venous system is a low-pressure, low-flow environment where a tendency to hypercoagulability has clear links to pathogenesis of disease, particularly where blood flow is compromised, for instance, by periods of immobility. The contribution of genetic factors to

the pathogenesis of venous thrombosis is indicated by a strong family history in many individuals presenting with premature thrombosis. Commonly, deficiencies in the anticoagulant proteins, protein C, protein S and anti-thrombin are identified in these subjects, which highlight the importance of abnormalities of hemostasis, and anticoagulant pathways in particular, in the pathogenesis of venous thrombosis. The contribution of genetic factors to the pathogenesis of venous thrombosis is most apparent in young subjects in whom spontaneous thrombosis may occur without obvious environmental stimuli such as surgery, pregnancy or malignancy.

The development of arterial thrombosis involves complex interactions between environmental and genetic factors. In comparison to the venous circulation, the arterial system is a high-flow, high-pressure system in which the development of atherosclerotic plaques in the vessel wall is followed by plaque instability and rupture, to precipitate the acute thrombotic event. A number of large prospective and case-control studies have addressed whether changes in plasma levels of hemostatic components are related to the pathogenesis of cardiovascular disorders. These studies, which have been reviewed extensively (1–4), have demonstrated associations between several coagulation and fibrinolytic factors (fibrinogen, factor VII (FVII), tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1)) and atherothrombotic events. It is apparent that whatever the predisposing factors, activation of coagulation is intimately involved in both the progression of atherosclerosis and the manifestation of acute thrombosis. As with venous thrombosis, the contribution of genetic factors has been indicated by the clustering of cardiovascular disease (CVD) within families; however, due to the complex pathogenesis of coronary artery disease (CAD), elucidating the genetic factors contributing to acute thrombotic events has proved to be more complicated.

Additive genetic factors contribute to variance in hemostasis components, as evidenced by studies in families and



**FIGURE 54-1** Dynamic process of clot formation. Clotting is initiated upon endothelial cell injury. The normal vessel endothelium is characterized by an anti-inflammatory anti-thrombotic phenotype that maintains vascular patency (A). Proinflammatory cells such as monocytes migrate from the vessel lumen into the vessel wall and release cytokines, which further promote inflammatory cell transmigration (B). Exposure of subendothelial collagen and von Willebrand factor lead to platelet adhesion activation and aggregation forming a platelet plug at the site of injury (C). Exposure of tissue factor at the site of injury initiates activation of the coagulation cascade (Figure 54-2), which results in thrombin generation and the formation of a fibrin mesh that traps circulatory blood cells such as platelets and erythrocytes to enable consolidation of the thrombus (D).

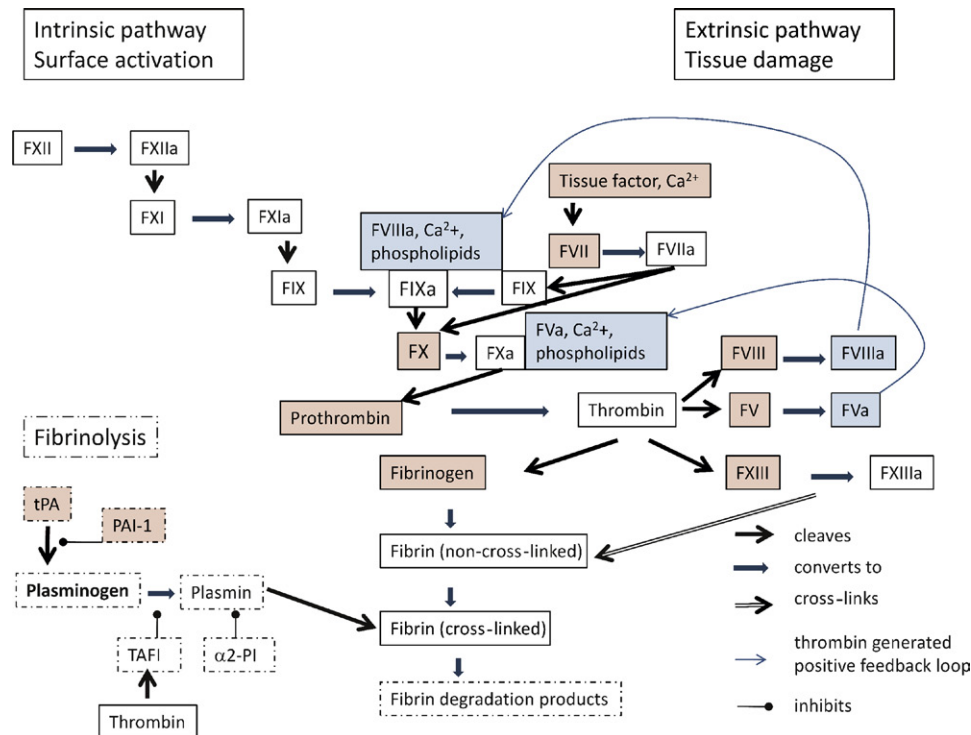
twins, which have identified a significant heritable component for many factors. Potentially, all the genes coding for proteins involved in coagulation, anticoagulation, fibrinolysis and platelet function (Figures 54-1–54-3) are candidate genes for thrombosis. In the Leeds Family study, the heritability estimates for FVII, fibrinogen, factor XIII (FXIII) complex, PAI-1 and tPA were 0.40, 0.37, 0.52, 0.28 and 0.27, respectively (5). Similar heritability estimates have been reported in Spanish families (6), with higher estimates observed in twins (7). A major contribution of additive genetic factors in determining the risk for thrombosis was indicated by the Genetic Analysis of Idiopathic Thrombophilia (GAIT) study, which showed that genetic factors accounted for ~60% of variation in risk (8). Importantly, significant genetic correlations between various hemostatic factors and thrombosis were identified in the GAIT study (8), supporting the concept that genetic variants influencing individual hemostatic factors also influence susceptibility to thrombosis.

Genetic variants contribute to thrombosis in a variety of ways. Nonsynonymous polymorphisms in the genes encoding individual hemostatic components can influence the structure and/or the function of the encoded protein to influence protein/protein interactions or protein concentration through influences on protein secretion or degradation. Polymorphisms in the key regulatory regions of the genes encoding individual hemostatic components can influence mRNA expression or stability to

affect the concentration of the encoded protein. Finally, genetic variants in key regulatory proteins may modulate the posttranslational modification and/or synthesis and secretion of hemostatic components.

Historically, a hypothesis-driven candidate gene approach has been favored in which the genes encoding individual hemostatic components were screened for novel polymorphisms, which were tested for association with intermediate and disease phenotypes, and functional studies carried out to elucidate mechanisms of action. Recent technological advances, including development of systems for high-throughput genotyping, improved bioinformatics methods for data mining and improved statistical methods for data analysis, have enabled identification of genetic variants in putative regulatory regions. In particular, genome-wide analysis of genetic variants associated with intermediate hemostatic phenotypes and thrombotic disorders through linkage analysis in twin- and family-based studies and genome-wide association studies (GWAS) has identified novel factors associated with hemostasis and thrombosis. While genome-wide analyses for disease end points may be criticized for being entirely hypothesis free and frequently difficult to characterize, genome-wide analysis of intermediate phenotypes has the advantage of a more hypothesis-driven approach, with more accurate measurement providing greater opportunities for identifying key regulatory proteins and understanding potential functional effects.





**FIGURE 54-2** The coagulation cascade. Exposure of tissue factor and contact activation lead to the initiation of the clotting cascade, which culminates in the conversion of prothrombin to thrombin. A positive feedback loop results in further activation of cofactors V and VIII, leading to a burst in thrombin activity sufficient to ensure effective fibrinogen conversion. Fibrinogen is cleaved by thrombin to give rise to polymerized fibrin, which in turn becomes cross-linked by FXIII, leading to the formation of a stable fibrin clot. Fibrin formation is counteracted by the activation of the fibrinolytic cascade, in which plasminogen is converted by tissue plasminogen activator (tPA) to plasmin, which digests the fibrin clot, giving rise to fibrin degradation products. The process of fibrinolysis is halted by the incorporation of plasmin inhibitor ( $\alpha$ 2-antiplasmin) and TAFI into the clot (the latter being activated by thrombin). Areas shaded in pink are components of the coagulation/fibrinolysis system, which are discussed in more detail in this chapter. Areas shaded in blue denote cofactor activity.

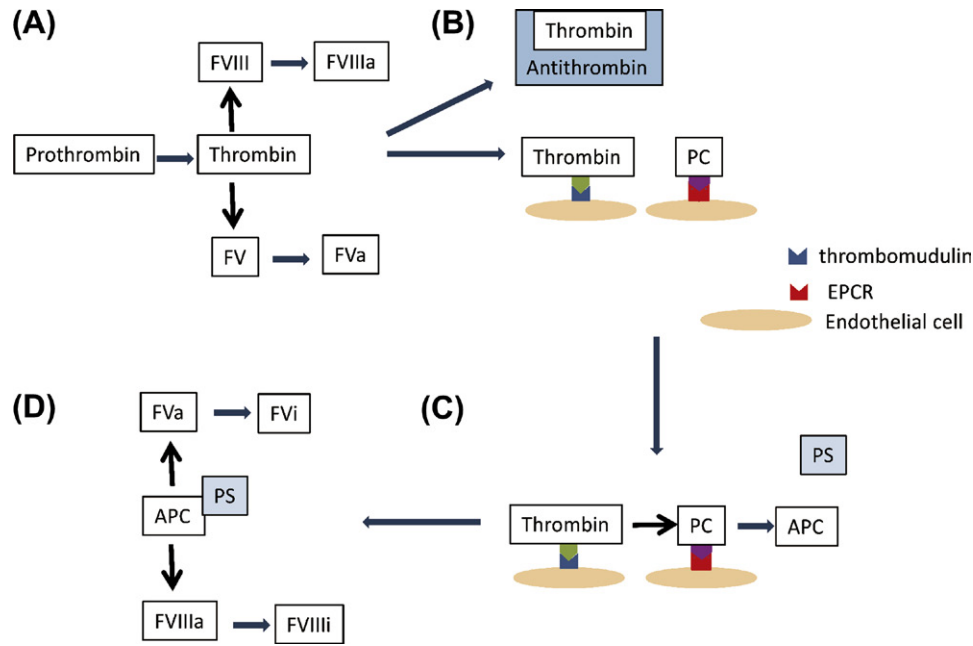
In the following sections of this chapter we describe the common genetic variants influencing coagulation, anticoagulation, fibrinolysis and platelet function that have been associated with venous and/or arterial thrombosis. Particular emphasis is placed on discussing functional polymorphisms where there is evidence of an association of the genetic variants with alterations in the levels or function of the particular protein and where there is evidence of an association of the genetic variants with thrombosis. We also provide relevant information from recent GWAS of hemostatic intermediate phenotypes and thrombotic disease. We conclude this chapter by considering the impact of genetic risk factors on the clinical management of thrombosis.

## 54.2 GENETIC VARIANTS INFLUENCING COMPONENTS OF THE COAGULATION CASCADE

### 54.2.1 Factor VIII and Von Willebrand Factor

Factor VIII (FVIII) circulates in the blood bound to von Willebrand factor (vWF), which protects FVIII from proteolytic cleavage. As vWF concentration is an important

determinant of FVIII concentration, we have considered these proteins together, although genetic variants influencing vWF concentration may also affect platelet function. Elevated plasma FVIII and vWF have been associated with increased risk for vascular disease (9–11). Evidence of a genetic contribution to variance in plasma FVIII and vWF levels originated from observation of associations between FVIII, vWF and ABO blood group, with the lowest levels observed in those of blood group O, and clustering of FVIII and vWF levels in families (12). Viel et al. (13) carried out resequencing of the exons and splice junctions of the gene encoding FVIII (*F8*) in individuals without hemophilia to identify genetic variants associated with variance in FVIII concentration. They identified 47 polymorphisms across the gene, which showed considerable racial differences in frequency. Two variants, D1241E and 56010G>A (in intron 7), were associated with plasma FVIII concentration, although these single-nucleotide polymorphisms (SNPs) were in strong linkage disequilibrium. Further analysis of D1241E indicated that this SNP accounted for ~10% of variance in FVIII independent of other covariates including ABO genotypes, indicating that it may be functional or in linkage disequilibrium with a functional polymorphism in *F8* (13).



**FIGURE 54-3** Natural anticoagulants. Thrombin generated by the coagulation cascade (A, see Figure 54-2) also exhibits an anticoagulant effect by binding to thrombomodulin situated on endothelial cells (B). Free thrombin is inhibited by binding to antithrombin (B). Thrombin then undergoes a functional switch and cleaves protein C bound to endothelial protein C receptors (C). This leads to activation of protein C (APC), which together with cofactor protein S inhibits the coagulation process by cleaving FVa and FVIIIa to their inactive cleavage products FVi and FVIIIi, thus inhibiting the positive feedback loop of thrombin generation (D).

Common polymorphisms in the vWF gene (*VWF*) have been identified, including four within the 5' gene regulatory region: -1793 C/G, -1234 C/T, -1185 A/G, and -1051 G/A (14,15). Differences in nuclear protein binding have been reported for -1234 C/T and -1051 G/A, and -1234 C/T, -1185 A/G and -1051 G/A polymorphisms are in strong linkage disequilibrium forming two major haplotypes (haplotype 1: -1234C/-1185A/-1051G; haplotype 2: -1234T/-1185G/-1051A) (15). These haplotypes associate with plasma vWF levels in subjects of blood group O, with highest levels in subjects homozygous for haplotype 1 and lowest in those homozygous for haplotype 2 (15). In the population-based Rotterdam Study, the -1793 C/G polymorphism was associated with risk for CVD, but only in individuals with advanced atherosclerosis at baseline (16).

A recent GWAS analysis of FVIII and vWF in >20,000 individuals identified 191 SNPs associated with FVIII concentration and 400 SNPs associated with vWF concentration with genome-wide significance ( $p < 0.5 \times 10^{-8}$ ) (17). SNPs associated with FVIII were localized to five chromosomal regions (6q24, 8p21, 9q34, 12p13, and 12q23) and SNPs associated with vWF were localized to eight chromosomal regions, five of which coincided with those for FVIII with an additional three unique loci (12q24.3, 14q32, and 19p13.2). The complete concordance of genetic loci for FVIII with those for vWF supports vWF as a major determinant of plasma FVIII concentration. For the 9q34 locus, SNPs spanned 11 genes including *ABO* and after accounting for SNPs tagging *ABO* blood groups, no other SNPs were

significantly associated with FVIII or vWF in this region, suggesting that *ABO* is the functional gene, supporting previous studies. SNPs in the 12p13 locus were localized to *VWF*, with rs1063857, a synonymous SNP at amino acid 795, giving the highest p value. Interestingly, SNPs in the *F8* gene were not identified as a significant determinant of FVIII or vWF in this study. The most informative SNPs at the 6q24, 8p21, 12q23, 12q24.3, 14q32, and 19p13.2 loci were in the vicinity of the genes encoding syntaxin-binding protein 5 (*STXBP5*, rs9390459); scavenger receptor class A, member 5 (*SCARA5*, rs2726953); stabilin 2 (*STAB2*, rs4981022); syntaxin 2 (*STX2*, rs7978987); tandem C2 domains, nuclear (*TC2N*, rs10133762); and C-type lectin domain family 4, member M (*CLEC4M*, rs868875), respectively. The SNPs in this study accounted for ~10% of variance in FVIII and ~13% of variance in vWF and support *ABO* as an important determinant of both FVIII and vWF, through influences on vWF (17). The novel candidate genes for FVIII/vWF regulation included genes with putative roles in Weibel–Palade exocytosis and protein uptake/clearance (17). The influence of these genetic variants on venous and cardiovascular thrombosis is currently unknown.

### 54.2.2 Factor VII

FVII is a vitamin K-dependent coagulation factor circulating as an inactive zymogen; FVII is essential for tissue-factor-dependent activation of coagulation. In the Northwick Park Heart Study, elevated levels

of FVII coagulant activity were associated with fatal (but not nonfatal) coronary events (18). However, this association has not been substantially confirmed by other large studies (19,20). Six common polymorphisms have been identified within the FVII gene (-122 T/C, -401 G/T, -402 G/A, a 10bp insertion at -323, Arg353Gln, and an intron 7 polymorphism), giving rise to three different alleles H5, H6 and H7 (21–23). The 10bp insertion and Arg353Gln are in strong linkage disequilibrium and account for approximately 20% of variance in plasma FVII. Gln353 is less efficiently secreted than Arg353 in vitro and associated with lower plasma FVII levels (24). Alleles -401 G/T and -402 G/A together account for ~30% of variation in plasma FVII levels, with -401G and -402A alleles associated with increased transcriptional activity in vitro and elevated plasma FVII levels (22). In relation to disease, the association of these polymorphisms with CAD remains controversial, with most studies showing no association; this issue has been discussed in detail elsewhere (25).

Smith et al. (17) carried out GWAS analysis of FVII and identified 305 SNPs associated with FVII at genome-wide significance. SNPs were clustered in five distinct chromosomal regions, 2p23, 4q25, 11q12, 13q34, and 20q11.2. SNPs in 13q34 localized to the FVII structural gene (*F7*) and nearby genes including the gene encoding factor X (FX) (*F10*). After adjusting for Arg353Gln, 22 SNPs localized to *F10* (rs 3211727) and *MCF2L* (*MCF.2* cell-line-derived transforming sequence-like, rs1755693) retained GWAS significance. SNPs in 2p23 were localized to the glucokinase regulatory protein gene (*GCKR*; rs1260326: P446L) and those in 4q25 to the alcohol dehydrogenase genes (rs1126670 and rs896992, respectively). SNPs in 11q12 localized to genes encoding members 2 and 6A of the membrane spanning four domains, subfamily A (*MS4A2* and *MS4A6A*; rs11230180 and rs2847666, respectively). The functional relationship between these proteins and FVII is unclear at present. SNPs in 20q11.2 were in strong linkage disequilibrium with a nonsynonymous SNP in the gene encoding the protein C receptor (*PROCR*; rs867186: S219G). This finding is consistent with evidence indicating that the protein C receptor binds FVIIa leading to endocytosis (26) and that FVII and FVIIa are higher in individuals possessing the G219 allele (27).

### 54.2.3 Factor V

Factor V (FV) plays central roles in both coagulation and anticoagulation processes. Activated FV (FVa) is a cofactor in the conversion of prothrombin to thrombin, whereas zymogen factor V acts as a cofactor with protein S for activated protein C-mediated inhibition of FVIIIa (28). Resistance to activated protein C (APCR) is strongly associated with venous thromboembolism (VTE) (29) and a missense polymorphism of the

factor V gene (Arg506Gln, Factor V Leiden) accounts for the majority of the observed APCR (29). Gln506 is the most commonly occurring genetic risk factor for venous thrombosis identified to date, occurring in ~5% of Caucasians, although there are considerable regional differences in incidence. The Gln506 variant of FVa is less readily inactivated by APC and is less effective as a cofactor for the inactivation of FVIIIa, leading to a hypercoagulable state (reviewed by Lane and Grant (25)). The risk for venous thrombosis associated with Gln506 is estimated to be between 3- and 8-fold in heterozygotes and as high as 50- to 80-fold in homozygotes. A number of other polymorphisms of the FV gene have been identified, one of which (A4070G) gives rise to His1299Arg. A haplotype including nine FV polymorphisms always associates with Arg1299 and is referred to as HR2. This haplotype has been associated with reduced plasma FV levels (30), APCR (even in the presence of Gln506) and risk for VTE in some studies (31,32). A recent meta-analysis supported a statistically significant relationship between both Gln506 and HR2 and venous thrombosis (33).

### 54.2.4 Prothrombin

The conversion of prothrombin to thrombin is the final step in the common coagulation pathway, thrombin acting to convert fibrinogen to fibrin and activate FXIII and platelets. A polymorphism in the 3'-untranslated region of the gene, G20210A, occurs at the last base of the prothrombin mRNA before the poly(A) addition site (34) and is associated with plasma levels of prothrombin, with highest levels in those homozygous for the 20210A allele (34,35). A number of mechanisms have been proposed to explain this relationship, including effects on mRNA processing and stability (36–39). Allele 20210A has been related to VTE, which in the Leiden Thrombophilia Study was accounted for entirely by its association with plasma prothrombin level (34). An additional polymorphism of the prothrombin gene, A19911G in intron M, also relates to plasma prothrombin levels (40) and influences prothrombin mRNA splicing efficiency (41). An interaction between the G20210A and A19911G polymorphisms in relation to venous thrombosis has been reported in a number of studies (40,42,43).

### 54.2.5 Fibrinogen

Fibrinogen (or Factor I) is a soluble glycoprotein (GP) with a central role in blood clotting both as the substrate for fibrin formation and as the ligand for the platelet  $\alpha_{IIb}\beta_{III}$  receptor, which promotes platelet aggregation. Thrombin cleaves fibrinogen to fibrin monomers, which spontaneously polymerize to form half-staggered protofibrils. These aggregate to fibers and result in a three-dimensional fibrin network stabilized by activated FXIII, which serves as a scaffold for the emerging blood clot. Fibrinogen is a

large and complex protein comprising pairs of three non-identical polypeptides linked by a central domain. The subunits are referred to as  $\alpha$ ,  $\beta$  and  $\gamma$  chains (roman capital letters denoting intact chains prior to proteolytic cleavage by thrombin), which are encoded by separate genes (*FGA*, *FGB*, *FGG*) located in the fibrinogen module on the short arm of chromosome 4 (44,45). Individual chains are produced and assembled in hepatocytes. In addition to its function as a coagulation factor, fibrinogen is also an acute phase protein, and raised levels have been consistently associated with CVD (18,46,47). Both environmental and genetic factors influence fibrinogen levels, and in heritability studies, the contribution of genetic factors to plasma levels of fibrinogen have been estimated to be in the region of 50% (5,6,48). There are a number of polymorphisms in all three chains, but in vitro studies suggest that  $\beta$ -chain synthesis is rate limiting in the synthesis of the fibrinogen molecule and research has focused on this area. The substitution of G to A in fibrinogen  $\beta$ -455 is consistently associated with increased fibrinogen levels, in addition to promoter polymorphisms of the fibrinogen  $\beta$  gene (-1420G/A, -993C/T and -148 C/T), which are in complete linkage disequilibrium with  $\beta$ -455 G/A. All three fibrinogen genes have interleukin (IL)-6 responsive sequences, but only SNPs in the  $\beta$ -chain have been shown to modulate fibrinogen synthesis in response to IL-6 (49). This may explain why some environmental factors, such as smoking, result in higher plasma fibrinogen levels only in carriers of these SNPs, and underlines the confounding effect of genetic and environmental factors on plasma fibrinogen. More recent work identified common SNPs and haplotypes of the *FGA* and *FGB* genes as a major determinant for the variability of fibrinogen levels, concluding that genetic background represents a major determinant of fibrinogen, which modulates the response to proinflammatory stimuli (50). Two coding polymorphisms have been identified to date (Arg448Lys in the  $\beta$ -chain, and Thr312Ala in the  $\alpha$  chain). Arg448Lys is in strong linkage disequilibrium with  $\beta$ -chain promoter polymorphisms and is associated with variation in plasma fibrinogen levels, functional properties of the fibrinogen molecule (51) and fibrin phenotype. Clots formed from recombinant as well as plasma-purified Lys448 fibrin have a more compact structure with thinner fibers and smaller pores and increased stiffness, even before cross-linking by FXIII (51). In addition, clots made from fibrinogen-depleted plasma substituted with recombinant Lys448 clots are more difficult to lyse (51). Such phenotypic properties have been associated with increased risk for CVD (52–55). In clinical studies, the Lys448 allele was associated with severity of CAD (56) and with stroke in female patients (57). In Caucasians, the polymorphism is estimated to occur at a frequency of 15–20%.

Thr312Ala occurs in a region of  $\alpha$ -fibrinogen important in a number of FXIII-dependent processes, including  $\alpha$ -fibrin/ $\alpha$ -fibrin and  $\alpha$ -fibrin/ $\alpha$ 2-antiplasmin cross-linking (amino acid residues A $\alpha$ 328 and 303, respectively

(58,59)). The region of  $\alpha$ -fibrinogen encompassing this polymorphism (residues A $\alpha$  242–424) is important in promoting the dissociation of the FXIII A and B subunit dimers thereby enhancing activation of FXIII itself (60,61). We found that fibrin clots formed from plasma-purified Ala312 fibrinogen have increased  $\alpha$ -chain cross-linking, fibrin fiber diameter and clot stiffness compared with clots from Thr312 fibrinogen (62). In clinical studies we reported associations of the Ala312 allele with poststroke mortality in subjects with atrial fibrillation (63) and with pulmonary embolism (64) supporting a role for Ala312 in clot stability. Ala312 is in linkage disequilibrium with a haplotype on *FGG* that is associated with decreased plasma levels of the gamma chain variant  $\gamma'$ . This modified version of the gamma chain arises from alternative processing of  $\gamma$ -fibrinogen mRNA resulting in the inclusion of intron 9 and polyadenylation of the transcript at an alternative polyadenylation site in intron 9 (65). This leads to replacement of the last 4 amino acids by 20 alternative amino acids, which significantly alters the properties of the  $\gamma$  chain (see also review by Uitte de Willige (66)). Between 7 and 15% of plasma fibrinogen molecules contain  $\gamma$ A/ $\gamma'$ , which has been shown to bind FXIII zymogen, and it may represent a mechanism whereby the local concentration of FXIII is increased during clot formation (67). Fibrinogen  $\gamma'$  has a decreased platelet aggregation potential. Interaction of fibrinogen with the platelet  $\alpha_{IIb}\beta_3$  receptor is decreased in recombinant, homodimeric (A $\alpha$ B $\beta$  $\gamma'$ )<sub>2</sub>, indicating that the binding of platelets to the fibrinogen  $\gamma$  chain is mediated by the four C-terminal residues of the  $\gamma$ A chain (68). Clots made from  $\gamma$ A/ $\gamma'$  fibrinogen in the presence of FXIII are more highly cross-linked and lyse more slowly than clots from  $\gamma$ A/ $\gamma$ A fibrinogen (69), and  $\gamma$ A/ $\gamma'$  fibrinogen has also been shown to enhance the activation of FXIII (70). Furthermore, clots formed with  $\gamma$ A/ $\gamma$  fibrinogen have a thinner average fiber diameter and a higher proportion of branch points than clots formed from  $\gamma$ A/ $\gamma$ A fibrinogen (71). A functional role for  $\gamma$ A/ $\gamma$  fibrinogen in thrombosis is supported by findings by Lovely et al. (72) who found that  $\gamma$ A/ $\gamma'$  fibrinogen levels are increased in patients with CAD compared with healthy controls. Similar findings were reported by Mannila et al., (73) who found significantly increased  $\gamma$ A/ $\gamma'$  levels in patients with myocardial infarction (MI) compared to age- and sex-matched controls. In contrast, Uitte de Willige et al. (74) report decreased  $\gamma$ A/ $\gamma'$  levels in patients suffering from deep venous thrombosis (DVT) compared to healthy controls in the Leiden Thrombophilia Study. This could suggest that the relationship between the  $\gamma'$  splice variant and thrombosis is dependent on the type of CVD, and exerts a pro-thrombotic effect in arterial disease, but is associated with protection against thrombotic events in venous disease. The putative mechanisms behind these observations—potentially mediated by differences in fibrin structure, platelet binding and FXIII activation—require further investigation.



Owing to the consistent association of increased plasma fibrinogen with CVD and heritability of ~50%, a number of studies have searched for loci associated with variance in plasma fibrinogen and two recent studies have reported GWAS analysis for plasma fibrinogen (75,76). In the study by Dehghan et al., (75) which involved a meta-analysis of >20,000 individuals from six independent studies, 73 SNPs localized to four chromosomal regions were identified with GWAS significant associations with fibrinogen. The most significant SNP was rs1800789 at 4q31.3 within the FGB gene; this SNP is in linkage disequilibrium with the -148 C/T and -455 G/A polymorphisms, which have been consistently associated with plasma fibrinogen (see earlier). Rs2522056 and rs1539019 were the most significant SNPs at 5q23.3 (3' to the gene encoding interferon regulatory factor 1 [*IRF1*]) and 1q44 (NLR family, pyrin domain containing 3 isoforms [*NLRD3*], respectively, both of which are involved in the regulation of inflammatory processes (75)). Rs511154 at 3q22.3 occurs in intron 1 of the gene encoding propionyl coenzyme A carboxylase  $\beta$ -polypeptide [*PCCB*]. Together these polymorphisms accounted for <2% of the variance in fibrinogen. In an independent study of >17,000 healthy participants in the Women's Genome Health Study, Danick et al. (76) identified 19 SNPs meeting GWAS significance, localized to five distinct chromosomal regions. The strongest associations were with SNPs in 4q32.1 in the vicinity of the fibrinogen structural genes (rs6056 and rs1800788 were the most informative SNPs in this region), supporting the findings of Dehghan et al. The most significant SNP at 1q21.3 was rs8192784 (76), a nonsynonymous Asp358Ala polymorphism in the gene encoding the IL-6 receptor (*IL6R*), which has been shown to influence plasma levels of soluble IL6R and IL-6 (77). The other significant SNPs were rs7422339, a nonsynonymous Asp1405Thr polymorphism at 2q34 (carbamoyl phosphate synthetase 1 [*CPS1*]), rs1016988 and rs10479002 at 5q31.1 (solute carrier family 22, members 4 and 5 [*SLC22A4*, *SLC22A5*]) and rs10512597 at 17q25.1 (in the vicinity of a cluster of immunoglobulin superfamily members including *CD300LF*). Together these SNPs accounted for <2% of the variance in fibrinogen. Interestingly, rs8192284 and rs10512597 were also associated with plasma C-reactive protein (76), highlighting the role of inflammatory factors in the regulation of fibrinogen.

### 54.2.6 Factor XIII

Zymogen FXIII is a transglutaminase that, when activated, plays a crucial role in fibrin clot formation by cross-linking adjacent  $\gamma$  and  $\alpha$  chains of polymerized fibrin, to ensure the structural integrity of the clot (78). To convert FXIII to activated FXIII (FXIIIa), thrombin cleaves a 37 amino activation peptide from the N-terminus of the A-subunit. In addition to clot stabilization, FXIIIa incorporates plasmin inhibitor ( $\alpha$ 2-antiplasmin)

into the growing clot, which significantly increases resistance to lysis by plasmin. FXIII deficiency is associated with severe bleeding but there is little information regarding elevated plasma FXIII and vascular disease. Results from animal studies indicate that FXIII is a major determinant of endogenous and exogenous fibrinolysis and suggest associations with MI and pulmonary embolism (79,80). Common polymorphisms of the FXIII gene have been identified, including four missense polymorphisms, Val34Leu, Pro564Leu, Val650Ile and Glu651Gln (81,82). While none of these polymorphisms are linked to plasma levels of FXIII A subunit antigen or FXIII B subunit antigen, Val34Leu and Pro564Leu are significantly associated with FXIII cross-linking activity, with enhanced activity associated with possession of Leu34 and Pro564. Joint linkage and association analysis indicated that Val34Leu is the predominant genetic determinant of FXIII activity (83). The substitution in codon 34 is three amino acids away from the thrombin cleavage site between Arg37 and Gly38 and results in major changes in functionality of the protein. Activation of Leu34 FXIII by thrombin occurs more rapidly than Val34, with a 2.5 increase in catalytic efficiency (84). Work from our group has shown that these differences in cleavage rates lead to cross-linking at different times during the fibrin polymerization process, which influence fibrin clot properties, with Val34 clots characterized by thin fibers and small pores and Leu34 clots by thicker fibers and a more porous gel. Furthermore, the Leu34 allele is activated at a lower concentration of thrombin than the Val34 allele (85). Val34Leu is the only FXIII polymorphism that has been consistently associated with vascular disease in a number of case-control studies of venous and arterial thrombosis; weak but significant inverse associations of Leu34 with MI and VTE are supported by several meta-analyses (33,86–88).

## 54.3 GENETIC VARIANTS INFLUENCING NATURAL ANTICOAGULANTS

### 54.3.1 Thrombomodulin

Thrombomodulin is an endothelial cell thrombin receptor that converts thrombin into an anticoagulant capable of activating protein C, suggesting a protective role in thrombosis. An inverse association between soluble thrombomodulin and risk of coronary heart disease was reported in the Atherosclerosis Risk in Communities (ARIC) Study (89). Thrombomodulin plays additional functional roles in the downregulation of fibrinolysis and inflammation through activation of thrombin-activatable fibrinolysis inhibitor (TAFI) (see the review by Weiler and Isermann (90) for further information). Polymorphisms of the thrombomodulin gene (*THBD*) have been identified, including several promoter polymorphisms, many of which are of very low frequency, and a more common missense polymorphism Ala455Val (91,92). Information

regarding relationships between polymorphisms and levels of soluble thrombomodulin are inconsistent, although the rare -33 G/A polymorphism confers a weak decrease in promoter activity in a luciferase reporter gene assay (93). Association studies of thrombomodulin gene variants with thrombosis have been contradictory (91,92). However, in a murine model involving targeted endothelial cell deletion of thrombomodulin, deficiency resulted in spontaneous, and ultimately lethal, arterial and venous thrombosis, which could be prevented by treatment with warfarin, suggesting a direct effect on coagulation rather than inflammation (94).

### 54.3.2 Protein C

Protein C is a major anticoagulant and deficiency is associated with VTE. Protein C is activated by the thrombin/thrombomodulin complex and exerts its anticoagulant action via inactivation of activated factors V (FVa) and VIII (FVIIIa), a process that requires the cofactor protein S (see later). Many mutations have been identified in the gene encoding protein C (*PROC*), which are causal in the development of familial venous thrombosis but are rare in the general population. These rare mutations are beyond the scope of this chapter (see the review by Spek and Reitsma (95) for further information). Three common SNPs in the 5' regulatory region of *PROC* have been identified: -1654 C/T, -1641 A/G and -1476 A/T, which are in close linkage disequilibrium (96), with the -1654 C/T and -1641 A/G and which define the most common haplotypes (CC/GG, CT/AG, CC/AG, CT/AA, TT/AA). These haplotypes associate with differences in plasma protein C, with lowest levels in individuals with the CC/GG haplotype, highest with the TT/AA haplotype and intermediate in the other haplotype groups (97–99). Consistent with this, the CC/GG haplotype associated with increased risk for venous thrombosis in a number of studies (97–99).

A GWAS of variants influencing protein C concentration in the ARIC Study identified 504 SNPs associated with protein C with genome-wide significance ( $p < 5 \times 10^{-8}$ ) (100), with the SNPs most strongly associated with protein C localized to four chromosomal regions, 2p23, 2q13–q14, 7q11.23 and 20q11. The SNP most strongly associated with protein C was rs867186 in 20q11, a nonsynonymous SNP (S219L, also associated with FVII) in the gene encoding the endothelial protein C receptor (*PROCR*, also known as *EPCR*), which accounted for ~10% of variance in protein C. The other most informative SNPs were rs1158867 (2q13–q14), an intronic SNP in *PROC*; rs1260326 (2p23), a nonsynonymous (P446L) SNP in the glucokinase regulatory protein gene (*GCKR*), the same SNP that was associated with variance in FVII (described earlier); rs1714573 and rs1178977 (7q11.23), both intronic SNPs in the gene encoding bromodomain adjacent to zinc finger domain 1B (*BAZ1B*); and rs6120849 (20q11), an intronic SNP in the gene encoding mannosidase alpha-like 2 gene (*EDEM2*). *EDEM2*,

*GCKR* and *BAZ1B* represent novel regulatory genes for protein C, with putative roles in protein degradation, posttranslational modification and transcriptional regulation, respectively; however, each of the SNPs in these genes accounted for <2% of total variance in protein C. Their influence on venous thrombosis is not known.

### 54.3.3 Protein S

Protein S is a cofactor to activated protein C in the inactivation of factors Va and VIIIa. As with protein C, deficiency of protein S is associated with venous thrombosis and mutations have been identified that give rise to rare forms of familial venous thrombosis (see de Frutos et al. (101) for further details (101)). Common polymorphisms of the protein S gene (*PROS1*) have been described, including 2148 A/G (Pro626), 2698 C/A (located 520bp from the termination codon in the 3'-untranslated region) and a T/A polymorphism in intron K (102,103). The Pro626 and the 2698 C/A polymorphisms significantly associate with plasma levels of total protein S antigen, the highest levels in those of AA/AA genotype and the lowest in those of GG/CC genotype (103). It has also been shown that Pro626 is associated with free protein S, a more specific risk factor for venous thrombosis (104). The mechanism(s) by which the Pro626 and 2698 C/A polymorphisms influence plasma levels is unknown; however, the Pro626 polymorphism was not found to be associated with venous thrombosis in relatives of patients with protein S deficiency (105).

### 54.3.4 Antithrombin

Antithrombin is an important anticoagulant factor that inhibits several activated components of the coagulation cascade including thrombin and factor Xa. Deficiency of antithrombin is a risk factor for venous thrombosis and mutations have been identified that account for familial cases of venous thrombosis (106). A number of polymorphisms have also been identified in the antithrombin gene (*SERPINC1*) including a trinucleotide short tandem repeat, (ATT)<sub>n</sub>, in the fifth intron, a -159 T/C polymorphism and silent coding polymorphisms including Asn450, Gln377 and V327 (107,108). The relationships of these polymorphisms to plasma antithrombin concentration or venous thrombosis have not been reported; however, an SNP in intron 1 (rs2227589) has recently been found to be associated with venous thrombosis in the Leiden Thrombophilia Study (109).

### 54.3.5 Tissue Factor Pathway Inhibitor

Tissue factor pathway inhibitor (TFPI) is a heparin-releasable inhibitor of tissue-factor-dependent coagulation and functions by formation of a quaternary FXa/TFPI/FVIIIa/TF complex (110). Decreased plasma TFPI has been associated with both arterial and venous

thromboses (111,112). A number of polymorphisms have been identified that are associated with plasma TFPI, including Val264Met, -287 T/C and -33 T/C (113–115), and in genome-wide linkage analysis the chromosomal region with the strongest linkage to plasma TFPI was in the vicinity of the TFPI structural gene (116). The -287 T/C and -33 T/C polymorphisms have also been shown to influence reporter gene activity and binding of nuclear proteins to suggest they are functional polymorphisms influencing plasma TFPI through effects on gene transcription (117). The -33 T/C polymorphism has been associated with risk of venous thrombosis (115,118).

## 54.4 GENETIC VARIANTS INFLUENCING COMPONENTS OF THE FIBRINOLYTIC CASCADE

### 54.4.1 Tissue-Type Plasminogen Activator

tPA is an endothelial cell-derived activator of the fibrinolytic system that is considered to be protective against thrombotic disorders, a view supported by the clinical use of recombinant tPA following acute MI. In contradiction, however, elevated plasma levels of tPA have been associated with an increased risk of MI in a number of prospective and case-control studies (119,120). The reason for this paradoxical observation is unclear but may reflect the association of tPA with plasma PAI-1 or other risk factors for vascular disease. However, the role of elevated levels of tPA in the pathogenesis of CVD remains unclear at present.

Polymorphisms in the tPA gene include a common Alu insertion/deletion (Alu I/D) in intron 8, and -7351 C/T, -125 T/C, Arg129Trp, 20099 T/C and 27445 T/A (121). There is no association of these polymorphisms with basal tPA levels, although an association with vascular tPA release in a perfused forearm model was found for the Alu insertion/deletion, -7351 C/T, 20099 T/C and 27445 T/A polymorphisms (121–123). Higher release rates were observed in subjects homozygous for the I allele, the -7351 C allele, the 20099 C allele and the 27445 T, relative to those possessing the alternative allele at these sites. The I allele has also been associated with MI in the Rotterdam Study (124), although this has not been confirmed in other studies (25). The T allele of the -7351 polymorphism associated with MI and lacunar stroke (125,126), suggesting that elevated vascular release of tPA in those homozygous for the C allele may be protective against acute thrombosis. In a GWAS analysis from the Framingham Heart Study no SNPs associated with plasma tPA at genome-wide significance (127).

### 54.4.2 Plasminogen Activator Inhibitor-1

PAI-1 is a serine protease inhibitor that is the major circulating inhibitor of tPA. Elevated PAI-1 associates with CVD (128–130) and PAI-1 is found in atherosclerotic

plaques (131). PAI-1 clusters with features of the insulin resistance syndrome (132), which is itself associated with CVD. Common polymorphisms of the PAI-1 gene have been identified, including a 4G/5G insertion/deletion at -675, -844 A/G, Ala15Thr, a CA dinucleotide repeat within intron 3 and a 3' *Hind* III restriction fragment length polymorphism (RFLP) (133). The only consistent associations with plasma PAI-1 have been observed for the -675 4G/5G polymorphism, with approximately 25% higher levels in those homozygous for the 4G compared to the 5G allele (134–136). In vitro studies have confirmed higher rates of transcription associated with the 4G allele and a difference in binding of nuclear factors, with the 4G allele binding an enhancer, whereas the 5G allele binds both an enhancer and a repressor resulting in decreased transcription of the 5G allele relative to the 4G allele (134). As with many gene–disease association studies, a number have reported associations with disease, while others fail to confirm these associations. However, meta-analyses support significant associations of the 4G allele with MI (137) and venous thrombosis (33). In a GWAS in the Framingham Heart Study no SNPs associated with plasma PAI-1 at genome-wide significance (127).

## 54.5 GENETIC VARIANTS INFLUENCING PLATELET FUNCTION

### 54.5.1 Platelet Glycoprotein Receptor Variants

Polymorphisms in the platelet receptors  $\alpha_{IIb}\beta_3$  (GPIIb-IIIa),  $\alpha_2\beta_1$  (GPIa-IIa), GPIb-V-IX and GPVI have been extensively studied in relation to thrombosis. The GPIb-V-IX receptor plays an essential role in primary hemostasis, mediating initial adhesion of platelets under high shear stress to vWF bound to collagen exposed at sites of vascular injury (138), and deficiency of GPIb-V-IX is associated with a bleeding tendency (Bernard-Soulier syndrome). The  $\alpha_2\beta_1$  and GPVI receptors mediate platelet interactions with collagen exposed at sites of vascular injury, with  $\alpha_2\beta_1$  primarily mediating platelet adhesion to collagen and GPVI primarily mediating collagen-induced signaling pathways (139). The  $\alpha_{IIb}\beta_3$  receptor is the final common mediator of platelet aggregation and predominantly interacts with fibrinogen and vWF. Deficiency of  $\alpha_{IIb}\beta_3$  is associated with a bleeding tendency (Glanzmann thrombasthenia) and the importance of  $\alpha_{IIb}\beta_3$  in thrombosis is highlighted by the effective clinical use of anti- $\alpha_{IIb}\beta_3$  agents in the management of acute coronary syndromes (140,141). Polymorphisms in the genes encoding these platelet receptors have been identified and recently reviewed by Williams et al. (142) and are summarized briefly in the following.

$\alpha_{IIb}\beta_3$  polymorphisms include the  $\beta_3$  -468 A/T, -425 A/C, -400 A/C, Leu33Pro and Arg489Gln polymorphisms and the  $\alpha_{IIb}$  Ile843Ser and 10480 C/G



polymorphisms (143). The Leu33Pro polymorphism has been studied extensively, with functional analyses indicating that Leu33Pro does not influence platelet  $\alpha_{IIb}\beta_3$  receptor expression (144). However, Pro33 is associated with a lower threshold for  $\alpha_{IIb}\beta_3$  activation, enhanced  $\alpha$ -granule release, clot retraction and adhesion and binding to fibrinogen (145–147). Haplotypes comprising the -468 A/T, -425 A/C, -400 A/C polymorphisms were shown to influence reporter gene activity but were not associated with  $\alpha_{IIb}\beta_3$  receptor density, although haplotypes comprising these three promoter polymorphisms and Leu33Pro were associated with increased plasma vWF activity, suggesting a potential effect on platelet  $\alpha$ -granule and/or endothelial cell Weibel–Palade body secretion (144). Associations between Leu33Pro and CVD have been described, although results have been conflicting. Meta-analyses have indicated a modest association of Pro33 with an ~10% increased risk for MI (148,149), although this is not consistent in all meta-analyses, as reviewed by Williams et al. (142).

The most extensively studied polymorphisms of GPIb-IX-V in relation to vascular disease are located in the GPIb $\alpha$  gene and include -5 T/C, a 39 base pair variable number tandem repeat (VNTR) polymorphism and Thr145Met (HPA-2) (142). The VNTR polymorphism results in one to four copies of a 13 amino acid repeat sequence within the macroglycopeptide region of GPIb and influences platelet plug formation under high shear rates (150). The Thr145Met polymorphism influences the conformation of the N-terminal region of GPIb $\alpha$  (amino acids 1–59) and binding of vWF (151). The -5 C/T polymorphism lies within the Kozak sequence of GPIb $\alpha$  mRNA and the C allele has been associated with increased receptor density as a result of more efficient translation of mRNA (152). The most extensively studied polymorphisms in  $\alpha_2\beta_1$  are the  $\alpha_2$  Lys505Glu (HPA-5) and the synonymous 807 C/T (Phe224) and 873 G/A (Thr246) polymorphisms (153). The 807 C/T and 873 G/A polymorphisms are in strong linkage disequilibrium and the 807 C and 873 G alleles are associated with lower  $\alpha_2\beta_1$  receptor density (153), with decreased receptor density associated with decreased adhesion to type I collagen (154). There are few reports relating these polymorphisms to vascular disease and the results are conflicting (142); however, an increased incidence of the  $\alpha_2$  807 C allele in patients with Type I von Willebrand disease suggests an association with bleeding (155).

**54.5.1.1 Genome-Wide Association Analysis of Platelet Function.** Moderate to high heritabilities have been reported for measures of platelet aggregation in response to different agonists (156,157), suggesting that additive genetic factors are important determinants of interindividual variance in platelet function. Recent GWAS of platelet function identified a number of novel loci associated with platelet responses (158,159). Johnson et al. (158) identified SNPs localized to three

regions associated with ADP-induced platelet aggregation (1q23.1: rs12566888 in *PEAR1* encoding platelet endothelial aggregation receptor-1; 11p15.4: rs7940646 in *MRV11* encoding murine retrovirus integration site 1 homolog; 7q36.3: rs2363910 in *SHH* encoding sonic hedgehog), three regions significantly associated with epinephrine-induced aggregation (10q25.2: rs4311994 in *ADRA2A* encoding adrenergic  $\alpha_2A$  receptor; 1q23.1: rs12566888 in *PEAR1*; 10q21.2: rs10761741 in *JMJD1C* encoding jumonji domain containing 1C) and one region associated with collagen-induced aggregation (19q13.42: rs1671152 [T323K] in *GP6* encoding platelet GPVI). The genes identified in this study were biologically plausible, encoding either known platelet receptors (*PEAR1*, *ADRA2A*, *GP6*) or intracellular signaling pathways (*MRV11*, *SHH*, *JMJD1C*). Mathias et al. (159) used combined linkage and association to identify genetic loci influencing platelet function at baseline and following 14 days of aspirin treatment in GenSTAR participants of African–American and European American families with a history of premature CVD (159). In linkage analysis, several chromosomal regions showed suggestive linkage with platelet function phenotypes in both ethnic groups, although there was little consistency in loci or phenotypes between the families of European and African descent. The only region of significant linkage was region 5q11.2, which was linked with post-aspirin ADP-induced whole blood aggregation in African–Americans (159). Combined linkage and association analysis identified 31 SNPs significantly associated with one or more platelet function phenotypes; although the majority did not lie in regions with likely candidate genes, SNPs in the vicinity of *MME* (membrane metalloendopeptidase rs1436634), *GLIS3* (GLIS family zinc finger 3 rs10116901) and *IPCEF1* (interaction protein for cytohesin exchange factors 1, also known as *PIP3-E*, rs1534446) were associated with collagen responses, and SNPs in the vicinity of *LDHAL6A* (lactate dehydrogenase A-like 6A rs11024665) and *ANKS1B* (ankyrin repeat and sterile alpha motif domain containing 1B, rs17029861 and rs2373201) were associated with ADP responses (159). There is limited information relating these SNPs and thrombosis. Most convincing evidence supports relationships of the GPVI T323K polymorphism and the strongly linked S219P polymorphism with GPVI intracellular signaling (160) and venous thrombosis (109,161).

## 54.6 GENOME-WIDE ASSOCIATION ANALYSIS FOR THROMBOSIS

### 54.6.1 Venous Thromboembolism

In a hypothesis-driven analysis of associations between ~20,000 SNPs with putative functional roles and DVT, Bezemer et al. (109) identified 7 SNPs that consistently associated with DVT. These SNPs were localized to



*CYP4V2* (ch4, gene encoding cytochrome P450 family 4, 2), *KLKB1* (ch4, encoding prekallikrein), *F11* (ch4, encoding factor XI (FXI)), *SERPINC1* (ch1, encoding antithrombin) and *GP6* (ch19, encoding platelet GPVI). The five SNPs on ch4 were also associated with plasma FXI, with the risk allele for DVT associated with increased FXI in each case (109); adjusting for FXI did not completely abolish associations between each of the SNPs and DVT suggesting potential mechanisms independent of FXI. In a meta-analysis of 28 SNPs in 21 genes previously associated with VTE comprising >120,000 cases and controls Gohil et al. (33) confirmed significant associations of the minor alleles of the following SNPs in hemostatic genes with VTE: factor V Arg506Gln, factor V H2, prothrombin G20210A, prothrombin G11991A, PAI-1 4G/5G, and  $\alpha$  fibrinogen Thr312Ala; in addition, the minor alleles of FXIII V34L and  $\beta$  fibrinogen -455 G/A were associated with protection from VTE. In a GWAS to identify genetic variants associated with VTE, Tregouet et al. (161) identified only five SNPs (rs2420371, rs1208134, rs505922, rs657152, and rs630014) potentially associated with VTE. The rs2420371 and rs1208134 SNPs in the FV gene were in strong linkage disequilibrium with FV Arg506Gln and were not associated with VTE after adjusting for Arg506Gln. The rs505922, rs657152 and rs630014 polymorphisms occur in the *ABO* gene and after adjusting for *ABO* blood group were not significantly associated with VTE (161). The authors additionally applied a candidate gene approach to their analysis by specifically testing associations between SNPs in genes encoding hemostatic factors and VTE and identified rs6825454 in the fibrinogen gene locus (in strong linkage disequilibrium with the  $\alpha$  fibrinogen Thr312Ala and  $\gamma$  fibrinogen rs2066865 polymorphisms) and rs867186 in *PROCR* associated with VTE at  $p < 1 \times 10^{-3}$ . In a recent update to this initial study Morange et al. (162) described an additional association between rs169713, an SNP ~90 kb 5' to the *HIVEP1* gene (encoding a DNA-binding protein involved in transcription regulation), and VTE. The sample size of these studies was relatively small in the context of GWAS and larger studies will be required to identify polymorphic variants associated with more modest increased risks for VTE in the order of 10–20%. In addition, further GWAS analyses of intermediate hemostatic phenotypes are likely to reveal additional polymorphic variants that influence risk for VTE; these analyses are currently underway as part of the Euro-CLOT study (163).

### 54.6.2 Arterial Thrombosis

A number of GWAS have been carried out for CVD, including coronary atherosclerosis, MI and stroke (reviewed by Malarstig and Hamsten (164) and briefly summarized later). Initial GWAS analyses for CVD provided clear replication for a susceptibility locus at

9p21.3, a relatively gene-sparse region of the genome with no obvious candidate genes (165–167). Interestingly, the most informative SNPs in this region are not associated with conventional cardiovascular risk factors (168). However, significant associations between SNPs across the 9p21.3 susceptibility locus and measures of platelet aggregation in response to collagen and epinephrine and also with coronary artery calcification have been identified; however, no clear mechanism has been identified to explain the observed associations (169). A 2011 study compared patients with angiographic evidence of CAD with and without MI to delineate the genetic factors predisposing to the development of atherosclerosis and those leading to plaque rupture and thrombosis (170). The locus most strongly associated with MI in this study was *ABO*, with SNPs tagging non-O blood groups associated with CAD with MI but not CAD alone (170). *ABO* blood groups influence carbohydrate structures on vWF, which influence vWF clearance and plasma levels of both vWF and FVIII, which are lowest in individuals of blood group O (171). Therefore, the associations between *ABO*, venous thrombosis and MI may result from decreased plasma vWF and FVIII, conferring protection from thrombosis.

## 54.7 HOW DO WE ACCOUNT FOR THE MISSING HERITABILITY?

Perhaps the most striking observations from GWAS analyses of intermediate phenotypes for arterial and venous thrombosis is the minor proportion of variance (or risk) explained by the individual SNPs despite the strong genetic component estimated for many phenotypes in twin and family studies. This is an area of much recent debate (172) and in the remainder of this chapter we will consider potential explanations for the discrepancy between heritability estimates and the total genetic contribution suggested by GWAS studies. Evidence from GWAS studies supports a contribution of many genetic variants, with minor individual effect sizes, to the pathogenesis of venous and arterial thrombosis. Studies to date have focused on the identification of novel genetic variants with the strongest associations above a predefined genome-wide significance  $p$  value. For the most part studies have excluded SNPs with  $p$  values below this threshold even in the face of supporting biological evidence indicating the potential for a functional role for a variety of SNPs. Several methods have been recently proposed that enable greater interrogation of GWAS data to evaluate the potential additive contributions of multiple genetic variants to variance in intermediate phenotypes (173) and disease end points (174,175); through these approaches a greater proportion of the heritability for various traits could be accounted for by genotyped SNPs, although a significant proportion of heritability remains unexplained. These methods have yet to be applied to GWAS data

for intermediate hemostatic risk factors for venous and arterial thrombotic disease.

For the most part GWAS studies have not analyzed haplotypes, gene/gene interactions or the combined effects of genes and environment despite a wealth of evidence that migrants attain the cardiovascular risk of the country to which they migrate rather than retaining that of their country of origin, emphasizing the importance of environmental factors. Although genetic variants identified by GWAS have yet to be evaluated, there are numerous examples in the literature that highlight the importance of both gene/gene and gene/environment interactions in modulating hemostatic intermediate phenotypes; a few examples are outlined below to illustrate the concepts more clearly.

The genetic architecture of venous thrombosis is less complex than that of arterial thrombosis and gene/gene and gene/environment interactions have been described in relation to risk for venous thrombosis conferred by the FV Arg506Gln polymorphism. Interactions between Arg506Gln and prothrombin 20210 G/A, oral contraceptive use, pregnancy, air travel and rare inherited disorders of protein C, protein S and antithrombin have all been described (176–180). For example, in the study by Legnani et al. (178) the relative risk (RR) for thrombosis in carriers of Gln506 was 10.3, for carriers of the 20210A allele was 4.7, whereas the RR for those that carried both the Gln506 and 20210A alleles was 45.6. Similarly, the RR for use of oral contraceptives was 4.1 but the risk associated with oral contraceptive use for those that carried both the Gln506 and 20210A alleles was 86.5.

Work from our group has shown that the influence of the FXIII Val34Leu polymorphism on clot structure (determined by analysis of clot permeability) is modulated by fibrinogen concentration and this may influence susceptibility to venous and arterial thrombosis. In subjects homozygous for Val34, clot permeability was found to decrease with increasing fibrinogen concentration, indicating the formation of a denser clot structure with increasing fibrinogen. In subjects homozygous for Leu34, there was no difference in clot permeability with increasing fibrinogen concentration, and intermediate results were obtained for subjects heterozygous for Val34Leu (181). These results therefore indicate that possession of the Leu34 allele would be expected to be protective for thrombosis (relative to the Val34 allele) only in the presence of high fibrinogen. In addition, a significant interaction between Val34Leu and the  $\alpha$ -fibrinogen Thr312Ala polymorphism has been identified in relation to pulmonary embolism, with the apparent protective effect of Leu34 being lost in the presence of the Ala312 allele (64). This finding was supported by in vitro data indicating that the influence of Ala312 is also modulated by fibrinogen level, with a greater decrease in clot permeability with increasing fibrinogen being observed for subjects possessing Ala312, which may counteract the beneficial

effect of Leu34 at high fibrinogen concentrations (181). These studies highlight the importance of considering the accumulated risk conferred by possession of multiple risk alleles and environmental risk factors in evaluating individual risk for VTE.

Gene/gene and gene/environment interactions have also been reported for a number of polymorphic variants that contribute to arterial thrombosis. Plasma PAI-1 levels significantly correlate with plasma triglyceride level and a 4G/5G genotype-specific response to triglyceride has been reported, with the highest levels of PAI-1 being observed in subjects with 4G/4G genotype and high plasma triglyceride levels (182,183). A triglyceride responsive region has since been identified within the PAI-1 5' region adjacent to the 4G/5G site (184). Furthermore, plasma PAI-1 demonstrates diurnal variation and this appears to be related to the 4G/5G genotype, with the most pronounced diurnal variation observed in subjects with the 4G/4G genotype (185,186), linking impaired fibrinolysis with diurnal variation in risk for arterial thrombosis. We have also shown that patients with MI who possessed the Leu34 allele had higher plasma levels of PAI-1 and an increased prevalence of the PAI-1 4G/4G genotype compared with those homozygous for the Val34 allele (187). These findings suggest that the FXIII Leu34 allele is protective for MI unless there is coexistent suppression of fibrinolysis occurring in association with genetic and environmental factors associated with elevated PAI-1.

For a comprehensive review of the impact of gene/gene and gene/environment interactions in studying complex diseases the reader is directed to a review by Wade (188). In this review the issue is succinctly summarized by the statement “although the human genome consists of ‘only’ 30–40,000 genes, this number of genes permits 450–800 million two-gene interactions to say nothing of the myriad of possible multi-locus interactions.” The majority of GWAS studies described earlier have focused on considering one or two SNPs with the highest *p* value rather than evaluating the haplotype structure of the genes in the vicinity of the most strongly associated SNPs. However, many genes have several polymorphic variants that are associated with the phenotype of interest at genome-wide significance and analysis of the haplotype distributions may be more informative where this is the case. A haplotype is defined as the combination of alleles for different polymorphisms that occur on the same chromosome (189), and for any given stretch of chromosomal DNA an individual will have two haplotypes, although at a population level there may be numerous haplotypes for any given stretch of chromosomal DNA. Since haplotypes can extend over many kilobases of a chromosome, haplotype analyses may also reveal informative gene/gene interactions. For the GWAS studies, analysis of haplotype structures and interactions between environmental factors and the genetic factors that predict variance in hemostatic factors and thrombosis (both single

variant, single gene and gene/gene interactions) are yet to be evaluated. Analysis of the cumulative effect of gene/gene and gene/environment interactions will most likely be a major focus in the GWAS community in the next few years and may help to reveal the true extent to which individual genetic factors contribute to estimated heritabilities.

It is also important to understand the methodological issues that may impact identifying genetic variants that influence hemostatic intermediate phenotypes and thrombosis and perhaps more importantly in identifying gene/gene and gene/environment interactions. These include consideration of sample size, population stratification and poorly defined phenotypes that can have a profound influence on the outcome of genetic analyses. While recent extensive international collaborations have resulted in sample sizes sufficient to detect genetic variants conferring an increased risk in the region of 10% or greater, the impact of poorly or inconsistently defined phenotypes (both intermediate and disease end points) is perhaps of greater issue in these large consortia. In this context, it remains unclear how large a sample size would be required to evaluate adequately potential gene/gene and gene/environment interactions.

### 54.8 IMPACT OF GENETIC RISK FACTORS ON THE CLINICAL MANAGEMENT OF THROMBOSIS

In concluding this chapter, it is important to consider how knowledge of the role of common polymorphisms in coagulant and anticoagulant genes has influenced (or will influence) clinical management of thrombosis. In relation to arterial thrombus, it is clear that knowledge of an individual's genotype has had no influence on management, perhaps in part because of the complexity of the disease. Although FV Arg506Gln strongly associates with VTE, a similar situation exists in relation to management of venous thrombosis. What seems most important is to evaluate the total risk profile of each individual, whether a venous thromboembolic event was sporadic or precipitated by a known event such as surgery, whether the event was a first or recurrent event and whether there is a strong family history. Even in the face of such knowledge it is still not clear whether knowledge of an individual's genetic makeup would influence their subsequent behavior or their clinical management. For instance, should knowledge of a woman's genetic makeup be used to influence the decision to prescribe oral contraceptives, particularly when reporting a personal or family history of venous thrombosis? The increased risk of unwanted pregnancies associated with alternative forms of contraception and the increased risk of VTE in pregnancy highlights that this is not a simple decision. At present, despite compelling evidence for a role of common genetic variants in influencing susceptibility to VTE, there seems to be no consensus as to how information regarding

genetic risk factors should be utilized clinically (190). The current absence of evidence that treatment strategies are influenced by knowledge of thrombophilia genotypes or evidence that such knowledge influences lifestyle choices to modulate risk for VTE suggests it is likely that this debate will continue.

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History of Medical Genetics, Medicine in a Genetic Context, Epigenetics, Human Gene Mutation in Inherited Disease: Molecular Mechanisms and Clinical Consequences, Analysis of Genetic Linkage, Chromosomal Basis of Inheritance, Multifactorial Inheritance and Complex Diseases, Population Genetics, Twins and Twinning, Genetic Evaluation for Common Diseases of Adulthood, Genetics of Atherosclerotic Cardiovascular Disease, Genetics of Stroke, Disorders of the Venous System.

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### Biographies



**Peter Grant** is a Consultant Diabetologist in the Leeds Acute Teaching Hospitals Trust, with a particular interest in management of vascular risk in patients with diabetes. He is also professor of Medicine and Head of the Division of Cardiovascular and Diabetes Research within the Faculty of Medicine and Health at the University of Leeds. The Division consists of approximately 50 researchers including clinical academic cardiologists and diabetologists, postdoctoral staff and PhD students. He has a current grant income of ~£3.3m, including a British Heart Foundation Programme Grant and funding for two senior clinical fellowships.

The mission of the Division is to understand the molecular basis of the strong biological link between diabetes and cardiovascular disease and professor Grant's major research theme in this strategy is the genetic and environmental determinants of thrombotic risk in relation to insulin resistance and cardiovascular disease. This has included case-control family and twin studies and he has published over 200 original articles in international peer-reviewed journals including those in *Diabetes*, *Blood*, *Circulation* and *The Lancet*.

Professor Grant is editor-in-chief of the international journal, *Diabetes and Vascular Disease Research*, which is a new peer-reviewed journal referenced on medline that publishes research that links these two common conditions.



**Angela Carter** is a principal research fellow in the Division of Cardiovascular and Diabetes Research at the University of Leeds. Her research is focused on the identification and functional analysis of genetic and environmental risk factors for cardiovascular disease with a particular emphasis on the interactions between haemostatic and inflammatory cardiovascular risk factors and their influence on thrombosis.



**Kristina Standeven** is a research fellow in the Division of Cardiovascular and Diabetes Research at the University of Leeds. Her research focuses on the regulation of the end stage in hemostasis, including genetic and environmental factors leading to posttranscriptional and posttranslational modifications in fibrinogen and their impact on structure and function of blood clots.

# CHAPTER

# 55

## Genetics of Atherosclerotic Cardiovascular Disease

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### ABBREVIATIONS

ALOX5AP – Arachidonate 5-Lipoxygenase-Activating Protein  
 ANGPTL3 – Angiopoietin-like 3 protein  
 BP – Blood pressure  
 CAC – Coronary artery calcium  
 CAD – Coronary artery disease  
 CHARGE – Cohorts for Heart and Aging Research in Genetic Epidemiology  
 CHD – Coronary heart disease  
 cIMT – Carotid intima-media thickness  
 CNV – Copy number variation  
 CRP – C reactive protein (CRP)  
 CVD – Cardiovascular disease  
 dKO – Double knockout  
 eQTLs – Expression quantitative trait loci  
 FH – Familial hypercholesterolemia  
 FLAP – 5-lipoxygenase (5-LO) activating protein  
 GerMI – German MI  
 GLGC – Global Lipids Genetics Consortium  
 GWAS – Genome Wide Association Studies  
 ICAM-1 – Intercellular adhesion molecule-1  
 ICBP – International Consortium for Blood Pressure  
 IL6 – Interleukin-6  
 LpPLA2 – Lipoprotein-associated phospholipase A2  
 LRP6 – low-density lipoprotein receptor-related protein 6  
 MI – Myocardial infarction  
 OR – Odds ratio  
 PDE-4D – Phosphodiesterase 4D  
 QTL – Quantitative trait locus  
 vWF – von Willebrand Factor  
 WTCCC – Wellcome Trust Case-Control Consortium

### 55.1 INTRODUCTION

Atherosclerotic cardiovascular disease (CVD) and its major clinical manifestation, coronary heart disease (CHD), are common conditions responsible for one in three deaths in the United States claiming more than

2200 lives every day (1). While there are many established risk factors for CHD, both acquired and inherited, it is thought that a sizeable proportion of the causality of CHD is heritable. Estimates of the heritability for CVD range from 30% to 60% (2,3) with some studies suggesting that a number closer to 60% may be more accurate, including one large ( $n > 20,000$ ) twin study that used over 30 years of follow-up data (4).

Such studies make a strong case for family history as a robust risk factor for premature CHD and have been reaffirmed in numerous population-based studies including INTERHEART (5) and the Framingham Offspring Study (6). In the latter, those who had at least one parent with premature CHD had a 1.7–2.0-fold increased risk of CHD compared to those without a family history (6). Nevertheless, we are still only beginning to understand how to translate the heritability of CHD into novel prevention strategies and personalized therapeutics.

Much has changed since the last edition of this chapter, with the emergence and maturation of genome wide association studies (GWAS) and the initiation of exome and whole genome sequencing in cardio-metabolic disease. Indeed, the past five years have been paradigm shifting with discovery, through GWAS, of more loci for CHD than identified in the previous 50 years. This, however, may only represent the tip of the iceberg. Further discoveries as well as functional and clinical translation are expected to continue to accelerate and ultimately lead to substantial prognostic and therapeutic advances in clinic. As with other diseases (type 1 and type 2 diabetes mellitus, macular degeneration, and so forth), recent genomic findings are starting to redefine the pathogenetics of human atherosclerosis (see Chapter 86). There is a current intense focus on identification of causal genes at novel CHD loci identified through GWAS, definition of directional effects of loss of function and gain of function in human and animal model systems for genes within CHD loci, exome sequencing

for rare coding variants, development of therapeutics based on these novel genomic discoveries, and nascent advances in risk prediction and personalized genomic medicine for CHD.

This chapter will review recent advances and discoveries, discuss continued challenges to studying the genetics of CVD, and highlight ongoing efforts that will drive continued rapid evolution of the genomics of atherosclerosis and CHD.

## 55.2 CHALLENGES TO GENETIC STUDIES OF CHD

### 55.2.1 Heterogeneity of the Disease Traits

Despite being a common disease overall, there are numerous challenges researchers must overcome to unlock the genetic determinants of CHD. The first is that the CHD phenotype is quite heterogeneous and encompasses a broad variety of conditions. In general, it can encompass any disease related to the heart vessels, of which there are many. These include acute coronary syndromes due to a ruptured plaque within an epicardial coronary vessel, chronic progressive atherosclerosis causing vessel lumen narrowing and/or outward remodeling of coronary vessels, coronary artery dissection, and microvascular coronary disease, to just name a few. For the purposes of this chapter, we focus on genetics or heritability relating to the process of atherosclerosis of the coronary arteries, resulting in chronic ischemic heart disease or acute coronary syndromes. Even within this more limited definition, differences in heritability may exist by location of disease within the coronary tree. For example, some studies suggest that proximal coronary artery disease, including left main disease, may be more heritable compared to distal disease (3,7).

**55.2.1.1 Late Onset Disease.** Because atherosclerosis is not usually evident until clinical events occur in middle or older age, use of classical family studies along generations is difficult. Youngest members of generations may not express detectable atherosclerosis for many years. Relatives in the oldest generations may have died without clear cause or without sufficient documentation to be certain that atherosclerosis was involved. While this means that CHD-related genes are more likely to become passed on to offspring, it may be difficult to discover cases for family-based studies, except in the most extreme cases with early onset CHD. For population-based studies, identifying true controls (those who will not develop the disease at a later date) may also be difficult for a late onset disease unless older individuals are chosen as a control population. Thus, population-based association studies—whether genome wide or candidate gene—have typically used large number of cases who are relatively young and controls who are older.

### 55.2.2 Non-Mendelian Inheritance and Gene–Environment Interactions

Only a small proportion of CHD is monogenic with Mendelian inheritance patterns. In the overwhelming majority of cases, CHD is considered to be a complex trait with multiple genes and gene–environment interactions that contribute to the phenotype. Furthermore, the risk factors for CHD are also polygenic. Even apparent environmental risk factors, such as tobacco use (8,9), also have complex gene and gene–environment basis further impacting the overall complexity of downstream atherosclerosis and making the genetics of atherosclerotic phenotype more difficult to study. Multiple old and recent studies have reviewed the genetics of common CVD risk factors such as dyslipidemia (10–14), the presence of type 2 diabetes (15), and elevated blood pressure (BP) (16,17) showing their heritability in many cases to be complex. These risk factors are the subject of additional chapters in this text (Chapters 52, 86, and 96).

**55.2.2.1 Use of Surrogate Phenotypes.** The study of CHD and atherosclerosis is further complicated by the widespread use of surrogate phenotypes that are commonly used to represent CHD such as follows: atherosclerotic coronary artery disease (CAD) burden seen on coronary angiogram, amount of coronary artery calcium (CAC) seen on cardiac CT, or the degree of carotid intima-media thickness (cIMT) as seen with carotid ultrasound. These phenotypes may act as markers for clinical events such as myocardial infarction (MI) or cardiovascular death; they also may be indicative of the different pathophysiologic consequences of atherosclerosis and each has different strengths and limitations compared to using clinical events alone when studying CHD. For clinical events, it is important to consider the complexity of the underlying athero-thrombotic processes even when focused on an apparently homogenous phenotype such as early onset MI, for example, our group has examined angiographic CAD sub-phenotypes and found that almost all recently identified GWAS loci that associate with MI do so through a relationship to coronary atherosclerosis, while few associate with MI *per se* in the presence of established CAD (18). Such heterogeneity in phenotypic associations has implications for how discovered loci might be used in risk prediction and in therapeutic targeting (e.g., for prevention of atherosclerosis or in reducing plaque rupture or thrombosis).

It is crucial, therefore, to define the CHD phenotype under study, strive to reduce clinical heterogeneity, and to appreciate the potential clinical relevance and translation. At the same time, it is important to recognize that recent GWAS discoveries were successful largely because they used the largest sample sizes possible and did not focus excessively on narrow phenotyping. Simply stated, sample size and power overcame phenotype heterogeneity in these initial GWAS discoveries. Moving forward, particularly with sequencing technologies, a focus on



extreme phenotypes and more homogenous phenotypes may provide powerful insights into the genetics of specific CHD traits.

### 55.2.3 Translation of Animal Model Discoveries into Humans

Human translation of discoveries in animal models of atherosclerosis has been challenging. Mouse models have become the predominant animal model for CHD over the last several decades using both endogenous genetic manipulation and environmental manipulation through diet-induced hypercholesterolemia. This has facilitated testing pharmacologic and genetic hypotheses; however, there also have been many inconsistencies regarding the role of specific pathways (19). These are discussed in more detail later in this chapter.

### 55.2.4 Overcoming the Challenges

Ultimately, it is likely that many different combinations of genetic risks interact with the environment, with chance, and with each other in different ways among individuals, families, and populations giving rise to the heterogeneity of CHD phenotypes. As such, a combination of different techniques including animal models, GWAS, family studies, and whole-exome and whole-genome studies will be needed to help further clarify individual rare and common genetic contributions and their interactions with each other and environmental factors. Finding the genetic contributions that overlap between risk factors (e.g., lipid traits) and CHD phenotypes (e.g., acute coronary syndrome) has only recently become possible with the application of large GWAS, and these should be further elucidated through sequencing-based discoveries.

## 55.3 MOUSE MODELS OF ATHEROSCLEROSIS

The genetic and molecular mechanisms of atherosclerosis have been elucidated, in part, by mouse models although significant caveats will be outlined below. In rodent models, over 100 different genes have been shown to affect atherosclerosis. There has been a significant disconnect, however, between the large phenotypic differences for individual genes in mouse models and their weak or absent associations with atherosclerosis and CHD in humans. With recent gains in human genetics, it is now possible to start to reconcile some of these discrepancies, and it is hoped to move toward more informed and sophisticated application of mouse modeling of this human disease.

Because wild-type mice on a chow diet are not susceptible to atherosclerosis, three main mouse models have been used for examining atherosclerosis: (1) cholesterol-cholate diet or the “Paigen Diet,” which induces enough hypercholesterolemia in wild-type mice to induce

atherosclerosis (20–22); (2) the low-density lipoprotein receptor knockout (KO) mice (*Ldlr*<sup>−/−</sup>), which lacks the primary receptor for LDL cholesterol leading to hypercholesterolemia and atherosclerosis (23) on a Western-type diet; and (3) apolipoprotein-E KO mice (*Apoe*<sup>−/−</sup>), which lack a key lipoprotein for uptake via hepatic lipoprotein receptors leading to marked hypercholesterolemia and atherosclerosis on a chow diet (23–25). In addition, transgenic models (especially for genes not present in mice, e.g., CETP), bone marrow transplantation, and approaches utilizing antibodies or small molecule inhibitors have been used in the wild-type and KO models above to model-specific genes and proteins (26,27).

### 55.3.1 Candidate Gene Approach Using KO Mice

This has been the most popular approach with candidates chosen based on basic biology and human biomarker association as well as on often questionable human genetic associations. Using mouse models, almost always backcrossed onto either the *Apoe*<sup>−/−</sup> or *Ldlr*<sup>−/−</sup> background to generate double knockout (dKO) mice, there are at least 100 genes in published literature with reported significant differences in atherosclerosis in mice. Of these, around one-third also significantly affected total plasma cholesterol levels. Thus, the mechanism of affecting atherosclerosis is likely through lipid-induced pathways, while approximately two-thirds affect atherosclerosis but do not affect plasma cholesterol levels (Table 55-1). These latter genes are mostly candidates chosen for their hypothesized functions in endothelial cells, vascular smooth muscle cells, and specific leukocytes, particularly mechanisms that modulate directly vascular signaling and inflammation. Overall, these candidate gene studies have provided remarkable mechanistic insight into the role of specific lipoproteins and lipid genes and pathways as well as endothelial cell, monocyte-macrophages and T-cell (28) signaling, and inflammatory pathways in experimental atherosclerosis. Although biased in selection, the ratio of lipid to non-lipid candidate genes is somewhat surprising because the traditional thinking has been that atherosclerosis is primarily a lipid disease.

### 55.3.2 Unbiased Mapping in Mouse Models

Mice have also been used to try to identify atherosclerosis genes through unbiased mapping, for example, quantitative trait locus (QTL) analysis (see Chapter 5) (29,30). So far, over 40 mouse atherosclerosis QTLs have been mapped (31), typically analyzing second (F2) generation progeny of two different progenitor strains, one prone to and the other protected against the disease, applying one of the three atherosclerosis models

**TABLE 55-1 Genes Associated with Atherosclerosis in Mice in Double KO Models**

Background Knock-out <sup>a</sup>	Chr	Candidate Gene Knockout	Association with Atherosclerosis <sup>b</sup>	Effects on Plasma Cholesterol <sup>b</sup>	Reference
Apoe <sup>-/-</sup>	X	Ace2	↑	No	(333)
Apoe <sup>-/-</sup>	1	Fn1	↓	Yes	(334)
Apoe <sup>-/-</sup>	1	Selp	↓	No	(335)
Apoe <sup>-/-</sup>	2	Aplnr	↓	Yes	(336)
Apoe <sup>-/-</sup>	2	Cst3	↑	Yes	(337)
Apoe <sup>-/-</sup>	2	Fut7	↓	No	(338)
Apoe <sup>-/-</sup>	2	Nfe2l2	↓	No	(339)
Apoe <sup>-/-</sup>	2	Mmp9	↑	No	(310)
Apoe <sup>-/-</sup>	3	Fabp5	↓	Yes	(311)
Apoe <sup>-/-</sup>	3	Fabp4	↓	Yes	(312)
Apoe <sup>-/-</sup>	3	Pdzk1	↑	Yes	(313)
Apoe <sup>-/-</sup>	3	Csf1	↓	Yes	(314)
Apoe <sup>-/-</sup>	3	Nceh1	↑	No	(315)
Apoe <sup>-/-</sup>	3	Cd1d1	↓	No	(316)
Apoe <sup>-/-</sup>	3	Gja5	↑	No	(317)
Apoe <sup>-/-</sup>	4	Angptl3	↓	Yes	(318)
Apoe <sup>-/-</sup>	4	Ttpa	↑	No	(319)
Apoe <sup>-/-</sup>	4	Tlr4	↓	No	(320)
Apoe <sup>-/-</sup>	5	Cd36	↓	Yes	(321)
Apoe <sup>-/-</sup>	5	Il6	↑	Yes	(322)
Apoe <sup>-/-</sup>	5	Scarb1	↑	Yes	(323)
Apoe <sup>-/-</sup>	5	Nos3	↑	No	(324)
Apoe <sup>-/-</sup>	5	Cxcl10	↓	No	(325)
Apoe <sup>-/-</sup>	5	Spp1	↓	No	(326)
Apoe <sup>-/-</sup>	5	Ncf1	↓	No	(327)
Apoe <sup>-/-</sup>	6	Cav1	↓	Yes	(328)
Apoe <sup>-/-</sup>	8	Msr1	↓	Yes	(329)
Apoe <sup>-/-</sup>	8	Tlr3	↑	No	(330)
Apoe <sup>-/-</sup>	8	Cx3cl1	↓	No	(331)
Apoe <sup>-/-</sup>	8	Hsd11b2	↑	No	(332)
Apoe <sup>-/-</sup>	8	Pla2g15	↑	No	(333)
Apoe <sup>-/-</sup>	9	Lipc	↓	Yes	(334)
Apoe <sup>-/-</sup>	9	Mmp12	↓	No	(310)
Apoe <sup>-/-</sup>	9	Mmp3	↑	No	(310)
Apoe <sup>-/-</sup>	9	Fut4	↓	No	(338)
Apoe <sup>-/-</sup>	9	Icam1	↓	No	(335)
Apoe <sup>-/-</sup>	9	Gpx1	↑	No	(336)
Apoe <sup>-/-</sup>	9	Cx3cr1	↓	No	(337)
Apoe <sup>-/-</sup>	9	Ccr2	↓	No	(338)
Apoe <sup>-/-</sup>	10	Ifngr1	↓	Yes	(339)
Apoe <sup>-/-</sup>	10	Nr1h4	↑	Yes	(340)
Apoe <sup>-/-</sup>	11	Mapk9	↓	No	(341)
Apoe <sup>-/-</sup>	11	Alox15	↓	No	(342)
Apoe <sup>-/-</sup>	11	Nos2	↓	No	(343)
Apoe <sup>-/-</sup>	11	Ace (+/-)	↓	No	(344)
Apoe <sup>-/-</sup>	12	Pik3cg	↓	No	(345)
Apoe <sup>-/-</sup>	14	Lgals3	↓	No	(346)
Apoe <sup>-/-</sup>	14	Ltb4r1	↓	No	(347)
Apoe <sup>-/-</sup>	14	Clu	↓	No	(348)
Apoe <sup>-/-</sup>	15	Soat2	↓	Yes	(349)
Apoe <sup>-/-</sup>	15	C6	↓	No	(350)
Apoe <sup>-/-</sup>	15	Tnfrsf11b	↑	No	(351)
Apoe <sup>-/-</sup>	16	Serpind1	↑	No	(352)
Apoe <sup>-/-</sup>	17	Angptl4	↓	Yes	(353)
Apoe <sup>-/-</sup>	17	Plg	↑	No	(354)
Apoe <sup>-/-</sup>	17	Cdkn1a	↓	No	(355)
Apoe <sup>-/-</sup>	18	Lipg	↓	Yes	(356)
Apoe <sup>-/-</sup>	18	Egr1	↓	No	(357)

TABLE 55-1 Genes Associated with Atherosclerosis in Mice in Double KO Models—cont'd

Background Knock-out <sup>a</sup>	Chr	Candidate Gene Knockout	Association with Atherosclerosis <sup>b</sup>	Effects on Plasma Cholesterol <sup>b</sup>	Reference
Apoe <sup>-/-</sup>	19	Fas	↑	Yes	(358)
Apoe <sup>-/-</sup> and Ldlr <sup>-/-</sup>	1	Soat1	↓	Yes	(359)
Apoe <sup>-/-</sup> and Ldlr <sup>-/-</sup>	4	Abca1	↑	Yes	(360)
Apoe <sup>-/-</sup> and Ldlr <sup>-/-</sup>	8	Lcat	↓	Yes	(361)
Apoe <sup>-/-</sup> and Ldlr <sup>-/-</sup>	11	Itgb3	↓	No	(362)
Apoe <sup>-/-</sup> with Paigen diet	1	Il1r1	↓	No	(363)
Apoe <sup>-/-</sup> with Paigen diet	2	Hdc	↓	Yes	(364)
Apoe <sup>-/-</sup> with Paigen diet	10	Bax	↑	No	(365)
Ldlr <sup>-/-</sup>	1	Mapkapk2	↓	Yes	(366)
Ldlr <sup>-/-</sup>	1	Tnfsf4	↓	Yes	(367)
Ldlr <sup>-/-</sup>	1	Fcgr3	↓	Yes	(368)
Ldlr <sup>-/-</sup>	1	Il10	↑	No	(369)
Ldlr <sup>-/-</sup>	3	Cd5l	↓	No	(370)
Ldlr <sup>-/-</sup>	3	Ctss	↓	No	(371)
Ldlr <sup>-/-</sup>	3	Vcam1	↓	No	(372)
Ldlr <sup>-/-</sup>	3	Ptgfr	↓	No	(373)
Ldlr <sup>-/-</sup>	5	Cxcl1	↓	No	(374)
Ldlr <sup>-/-</sup>	6	Lep	↑	Yes	(375)
Ldlr <sup>-/-</sup>	6	Apobec1	↑	Yes	(376)
Ldlr <sup>-/-</sup>	6	Olr1	↓	Yes	(377)
Ldlr <sup>-/-</sup>	6	Vwf	↓	No	(378)
Ldlr <sup>-/-</sup>	8	Ikbbk	↑	No	(379)
Ldlr <sup>-/-</sup>	9	Apoa1	↓	Yes	(380)
Ldlr <sup>-/-</sup>	10	Ifng	↓	No	(381)
Ldlr <sup>-/-</sup>	10	Lrp1	↑	No	(382)
Ldlr <sup>-/-</sup>	11	Cxcl16	↑	No	(383)
Ldlr <sup>-/-</sup>	11	Pfn1 (+/-)	↓	No	(384)
Ldlr <sup>-/-</sup>	11	Ccl2	↓	No	(385)
Ldlr <sup>-/-</sup>	11	Mpo	↑	No	(386)
Ldlr <sup>-/-</sup>	11	Tbx21	↓	No	(387)
Ldlr <sup>-/-</sup>	11	Pecam1	↑	No	(388)
Ldlr <sup>-/-</sup>	12	Gpr132	↓	No	(389)
Ldlr <sup>-/-</sup>	17	Ddr1	↓	No	(390)
Ldlr <sup>-/-</sup> and Paigen diet	6	Alox5	↓	No	(391)
Paigen diet	5	Hps4	↓	No	(392)
Paigen diet	6	Pon2	↑	Yes	(393)
Paigen diet	6	Pon1	↑	No	(394)
Paigen diet	7	Hps5	↓	No	(392)
Paigen diet	9	Rora	↑	Yes	(395)
Paigen diet	12	Lipin1	↑	No	(396)
Paigen diet	19	Hps1	↑	No	(392)
Paigen diet	19	Hps6	↓	No	(392)

Supplemental Table 55-1 References.

<sup>a</sup>The knockout is homozygous unless otherwise indicated as heterozygous by '(+/-)'.<sup>b</sup>Positive studies are those with  $p < 0.01$  for cases compared to controls for atherosclerosis effects and  $p < 0.05$  for effects on plasma cholesterol.Adapted from Stylianou, I. M.; Bauer, R. C.; Reilly, M. P.; Rader, D. J. Genetic Basis of Atherosclerosis: Insights from Mice and Humans. *Circ. Res.* 2012, 110 (2), 337–355.

described above. Because the linkage regions implicated in these QTLs are wide and contain hundreds of genes, there has been limited success in identifying the culprit genes. In fact, only three genes have been identified. The *Ath1* locus on mouse Chr 1 (32) was identified and confirmed in a KO model as tumor necrosis factor (ligand) superfamily member 4 (*Tnfsf4*, also known as *Ox40l*) (33). The *Artles1* locus on mouse Chr 6 was positionally cloned as arachidonate 5-lipoxygenase (*Alox5*) (34) and confirmed in *Alox5*<sup>-/-</sup> mice (35). *Adam17* has been

implicated as possible gene at *Ascla5* locus on mouse Chr 12 (36). Although these are plausible candidate genes in humans, there remains uncertainty. In KO mice, the region could carry from the donor strain not just the KO gene but also polymorphisms in regional genes (so-called “passenger gene” effect (37)) that affect the phenotype leading to a false conclusion that the KO gene is solely responsible for the effect (37,38). For example, although human genetic data do implicate *ALOX5* in atherosclerosis, this has not been convincingly proven

particularly in current GWAS data. *CXCL12*, which is associated with human CAD in GWAS, maps to mouse Chr 6 less than 1Mb away from *Alox5* within the so-called “passenger gene” region of the *Alox5*<sup>-/-</sup> strain; thus, polymorphisms in *Cxcl12* might be a significant contributor to the *Artles1* QTL.

### 55.3.3 Challenges with Mouse Models

Mice have proven extremely powerful in studying genes and loci that affect atherosclerosis. However, translation of these findings to human CHD has been slow and unconvincing, partly due to lack of human genetic data. On the other hand, the limitations of mouse models may be much more substantial and pernicious than previously recognized. It is apparent from recent human GWAS that there is almost no overlap with rodent KO or QTL models even now that over 30 loci for human CHD have been identified. Although, some loci in mouse models may indeed contain genes for human atherosclerosis and may emerge in future work (e.g. larger GWAS, pathway analysis, exome sequencing), many are likely to lack relevance to human CHD. It is therefore important to enumerate the potential limitations of prior mouse modeling so that they can be avoided or minimized in future functional translation.

The key issues include (1) the need to create models with extreme hypercholesterolemia in order to induce atherosclerosis—this may be a particularly non-physiological model for the study of non-lipid candidate genes, which appear to be more abundant than lipid genes based on recent human GWAS; (2) lack of homology between mouse and human for genes of interest (e.g. CETP is not expressed in mouse and the 9p21 locus, a major human GWAS discovery, is markedly different between human and mouse); (3) biased candidate gene selection for KO studies in mice due to lack of human data (now no longer the case); and (4) the failure of linkage and mapping strategies in mice. Moving forward, a more human-translational strategy might be worthwhile with mouse studies focused largely on validated human loci and genes, the requirement that there be reasonable genetic homology between mouse and human, and a renewed effort to apply new and improved models when studying human genes, particularly with less extreme lipids.

## 55.4 CANDIDATE GENE STUDIES IN HUMANS

Using the candidate gene approach, polymorphisms in specific genes with plausible biological relevance (based on rodent studies and basic biology, genes from promising family studies, genes in pathways related to proven CHD genes (e.g. lipid pathways), and more recently genes for CHD risk factors arising from GWAS) have been genotyped in populations to determine association with atherosclerosis and CHD and traits. The rationale is

that there should be numerous genes underlying the susceptibility to atherosclerosis in the general population, each with predicted small effects (39).

Indeed, prior to the GWAS era, there were hundreds of specific candidates purported to be associated with premature CAD and atherosclerotic traits, but only a few genes showed a reproducible association with either surrogate outcomes or increased CVD risk. The limitations of prior work included sample sizes and power that were inadequate to detect small but real effects, false positives due to a failure to control for the multiple testing or to replicate findings in independent samples, and, in retrospect, poor selection of candidate genes with many likely not involved or having redundant actions in the human disease. In many cases, candidate gene findings from family-based studies could not be replicated in population association studies (e.g. *MEF2A*), or genes that modulated experimental atherosclerosis in mice did not appear to have relevance in humans (see above), or for some that appeared promising from the mechanistic and causative perspectives (e.g. *MTHFR*), conflicting, and ultimately negative data regarding clinical outcomes during treatment makes such candidates less relevant. The realization of marked false positives in prior publication eventually led to more rigorous design, more cautious interpretation, and ultimately the current relatively conservative approach in the GWAS and post-GWAS era.

The candidate gene approach, however, has revealed a small number of true CAD genes, both lipoprotein and non-lipoprotein related. Based on recent large studies as well as current standards for multiple testing and replication, the list of definite genes, however, is even shorter than that which we had suggested at the last update of this chapter (Table 55-2). For example *LPA* and *PCSK9*, genes for circulating Lp(a) and LDL-C respectively, have reproducible associations with CAD and MI (40–46), subclinical atherosclerosis traits (47–49), as well as their intermediate lipoprotein traits (40,50–53). Indeed, recent Mendelian randomization (see below) studies provide strong support for causal roles of Lp(a) and LDL-C (and related genes) in atherosclerosis and CHD (40–43,50,54–56).

For a substantial set of biologically plausible candidate genes with suggestive evidence in human studies, large studies showing a lack of association have also been reported (57), and thus confidence in those genes remains tenuous. The ITMAT Broad CARE (IBC) 50K CAD Consortium recently completed the most comprehensive study of candidate genes for CAD and MI (57). As a complement to recent GWAS, they conducted a large and systematic candidate gene case-control study of CAD susceptibility, including analysis of many uncommon and functional variants in candidate genes (57). Over 49,000 SNPs, mostly in ~2100 genes of cardiovascular relevance, were genotyped on a custom array in 15,596 CAD cases and 34,992 controls (including almost a quarter of South Asian origin) and replication was attempted in an



TABLE 55-2 Putative Candidate Genes Associated with Cardiovascular Disease

Category	Gene/Gene Product	Intermediate Biochemical Trait	Intermediate Clinical Phenotypes	Moderate Prior Association with CHD	Strong Contemporary Evidence for Association with CHD <sup>a</sup>
<b>Lipid</b>					
	LDL-R	LDL-C levels	Familial hypercholesterolemia	(228,229)	(85,230,110)
	ApoB-100	LDL-C levels	Familial hypercholesterolemia	(231,232)	(55,210)
	PCSK9	LDL-C levels	Familial hypercholesterolemia	(233)	(44,85,110,210,234)
	Lp(a)	Lp(a) levels		(235)	(42,43,50)
	ApoE	TC, LDL-C, TG, VLDL-C levels	Familial dysbetalipoproteinemia	(236,237)	(57)
	USF-1	TC, LDL-C, TG, VLDL-C levels	Familial combined hyperlipidemia	(238)	
	LPL	TG, CM levels	Familial chylomicronemia syndrome, Familial combined hyperlipidemia	(239)	
	HL	HDL-C levels	—	(240)	
	EL(LIPG)	Phospholipase activity	—	(241)	
		HDL-C levels	—		
		TC <sup>106</sup>	—		
		Phospholipase activity	—		
		EL mass levels	—		
	Lp-PLA <sub>2</sub>	Lp-PLA <sub>2</sub> activity and mass levels	—	(242)	
	PON 1	PON activity	—	(243,244)	
	ABCA1	HDL-C levels	Tangiers Disease	(245,246)	(55,106,121,210,234,247)
	Apo A-I	HDL-C levels	—	(248,249)	(85,210,106,55,247)
	LCAT	HDL-C levels	Familial LCAT deficiency	(250)	(121,234,210)
	CETP	HDL-C levels, CETP mass levels or activity	—	(251)	(106,121,252)
	ACAT	—	—		
	SR-BI	—	—	(253)	
	CD-36	Fatty acid levels	Insulin resistance	(254)	
	SR-A	—	—		
	LOX-1	LOX-1 levels	—	(255,256)	
	ApoA5	—	—		
	ABCG8	LDL-C phytosterol levels	—	(165)	(57,86)
	TRIB1	TG levels	—	(144)	(57,86)
	LIPA	Lipase A levels	Wolman's disease	(257)	(57,84,130)
<b>Inflammatory</b>					
<b>Acute Phase</b>					
	CRP	CRP levels		(258)	
	SAA	SAA levels		(259)	
<b>Adhesion-Chemokine</b>					
	P-selectin	Sol P-selectin levels		(260,261)	
	E-selectin	E-selectin levels		(262)	
	ICAM-1	Sol ICAM-1 levels		(263)	
	VCAM-1	Sol VCAM-1 levels			
	CXC3L1	—		(264)	
	CCR2	—		(265)	
	MCP-1	MCP-1 levels		(266,267)	
	RANTES	RANTES levels		(268,269)	

Continued

TABLE 55-2 Putative Candidate Genes Associated with Cardiovascular Disease—cont'd

Category	Gene/Gene Product	Intermediate Biochemical Trait	Intermediate Clinical Phenotypes	Moderate Prior Association with CHD	Strong Contemporary Evidence for Association with CHD <sup>a</sup>
Leukocyte/ Cytokine/ Macrophage related	TLR4	—		(270,271)	
	TNF $\alpha$	TNF $\alpha$ levels			
	TNF-Rs	TNF-R levels			
	A20				
	LTA (TNF $\beta$ )	—		(75,272)	
	IL-1 gene cluster	IL-1/IL-RA levels		(273,274)	
	IL-6	IL-6 levels		(275,276)	
	IL-10	IL-10 levels			
	5-LO	—		(277)	
	FLAP	—		(70,278)	
	COX-2	—		(279)	
	MPO	MPO levels		(280)	
	MMP-3	MMP-3 levels		(281,282)	
	MMP-9	MMP-9 levels		(283,281)	
Lymphocyte related	IL-4	IL-4 levels		(261)	
	IL-12	IL-12 levels		(284)	
	IL-18	IL-18		(285)	
	IFN $\gamma$	IFN $\gamma$ levels			
	CD40-40L	SoI CD40L levels			
	IL-5	IL-5 levels		(286)	(57)
Metabolic	Adiponectin	Adiponectin levels	Insulin resistance, type 2 diabetes	(287,288)	
	Leptin	Leptin levels	Insulin resistance, type 2 diabetes		
	Resistin	Resistin levels			
	PPAR $\gamma$	—	Insulin resistance, type 2 diabetes	(289,290)	
	PPAR $\delta$	—	Insulin resistance, type 2 diabetes	(290)	
	UCP2	—		(291,292,293)	
	IRS-1		Insulin resistance, type 2 diabetes	(294,295,296)	
	Lamin A/C	—	Hutchinson–Gilford progeria Lipodystrophy	(74,297)	
	11 $\beta$ HSD2	Cortisol/cortisone levels	Hypertension	(298,299)	
	Mineralo- corticoid receptor		Hypertension	(300,301)	
Vascular/ Endothelial/ Matrix	MGP	MGP levels		(302)	
	OPG	OPG levels		(303)	
	OPN	OPN levels		(304)	
	ACE	ACE levels	Hypertension, diabetic nephropathy	(305,306,307)	
	PDE4-D			(73)	
	eNOS	Nitrite levels		(308,309)	
	MEF2A	—		(64)	

**TABLE 55-2 Putative Candidate Genes Associated with Cardiovascular Disease—cont'd**

Category	Gene/Gene Product	Intermediate Biochemical Trait	Intermediate Clinical Phenotypes	Moderate Prior Association with CHD	Strong Contemporary Evidence for Association with CHD <sup>a</sup>
Thrombosis	Connexin 37	—		(310)	
	ATM	—		(311)	
	MTHFR	Homocysteine levels		(312,313,314)	
	Fibrinogen	Fibrinogen		(315)	
	PAI-1	PAI-1 mass levels or activity		(315)	
	Prothrombin			(316)	
	Factor VII	Factor VII levels		(317,318)	
	Factor V	Factor V levels		(305,314,316)	
	GP IIIa	—		(313,319)	
	TM	TM levels		(318,320)	
	vWf	vWF levels		(321)	

LDL-R = low-density lipoprotein receptor; apo = apolipoprotein; PCSK-9 = Proprotein convertase subtilisin kexin 9; USF-1 = Upstream stimulatory factor-1 (USF-1); LPL = lipoprotein lipase; HL = hepatic lipase; EL = endothelial lipase; LpPLA<sub>2</sub> = lipoprotein associated phospholipase A<sub>2</sub>; PON-1 = paraoxanase 1; ABCA1 = ATP binding cassette-1; LCAT = lecithin:cholesterol acyl transferase; CETP = cholesterol ester transfer protein; ACAT = Acyl-CoA:cholesterol acyltransferase; SR-BI = scavenger receptor BI; SR-A = scavenger receptor A; LOX-1 = lectin-like oxidized low-density lipoprotein receptor; CRP = C reactive protein; SAA = serum amyloid A; ICAM-1 = intracellular adhesion molecule-1; VCAM-1 = vascular cell adhesion molecule; CXCL1 = Fractalkine; CCR2 = CC-chemokine receptor 2; MCP-1 = monocyte chemoattractant protein-1; RANTES = Regulated on activation normal T cell expressed and secreted; TLR4 = toll like receptor 4; TNF $\alpha$  = tumor necrosis factor alpha; TNF-Rs = TNF receptors; LTA = lymphotoxin A; IL = interleukin; 5-LO = 5-lipoxygenase; FLAP = 5-LO activating protein; COX-2 = cyclooxygenase-2; MPO = myeloperoxidase; MMP = matrix metalloproteinase; IFN $\gamma$  = interferon gamma; CD40L = CD40 ligand; PPAR = peroxisome proliferator-activated receptor; UCP-2 = uncoupling protein 2; IRS-1 = insulin receptor substrate-1; 11 $\beta$ HSD2 = 11 beta-hydroxysteroid dehydrogenase type 2; MGP = matrix gla protein; OPG = osteoprotegerin; OPN = osteopontin; ACE = angiotensin converting enzyme; PDE4-D = phosphodiesterase 4D; eNOS = endothelial nitric oxide synthase; MEF2A = Myocyte enhancer factor 2A; ATM = ataxia-telangiectasia mutated gene; MTHFR = Methylenetetrahydrofolate reductase; PAI-1 = plasminogen activator inhibitor-1; GP IIIa = glycoprotein IIIa; TM = thrombomodulin; vWf = von Willebrand factor.

additional 17,121 CAD cases and 40,473 controls. Several prior CAD susceptibility loci were confirmed (e.g., LPA, and from GWAS, 9p21.3 and SORT1 at 1p13.3).

Notably, results were essentially negative for most previously suggested CAD candidate genes. Further, apart from the previously reported variants in LPA, none of the other ~4500 low frequency and functional variants showed a strong effect. However, in the replication phase, novel associations were identified for common variants in or near *LIPA*, *IL5*, *TRIB1*, and *ABCG5/ABCG8* with per-allele odds ratios (ORs) ranging from 1.06 to 1.09. Thus, this contemporary large-scale gene-centric analysis identified several novel candidate genes for CAD that relate to lipid and inflammatory pathways, clarified the literature with regard to many previously suggested genes (largely negative), and confirmed some recent GWAS findings (see below).

In an ongoing effort, the National Human Genome Research Institute and several partners have devised the “MetaboChip,” which fine maps all top loci from GWAS of CAD and CVD risk factors (lipids, hypertension, diabetes, obesity, and biomarkers) as well as genotypes all suggestive SNP signals ( $p < 10^{-3}$ ) from those same GWAS (58). The MetaboChip has just completed genotyping in very large case-control samples of CAD and MI. This work will (1) clarify the literature with regard to causal genes and variants at the leading CVD loci suggested by GWAS,

(2) which validated risk factor loci actually do relate to CAD, and (3) by incorporating SNPs from GWAS reaching a lesser  $p$ -value threshold in large focused replication, also overcome GWAS false negatives, an acknowledged limitation of stringent genome-wide Bonferroni correction (59). These latter SNPs will be examined with greater power in the MetaboChip CHD project.

### 55.4.1 Family-Based Studies in Humans

**55.4.1.1 Family-Based Linkage Studies.** In rare instances, atherosclerosis can appear as a Mendelian disorder, such as in familial hypercholesterolemia (FH), where mutations in the LDL-R receptor greatly increase plasma cholesterol and consequently atherosclerosis susceptibility (60). Although largely overtaken by GWAS, family-based linkage studies were deployed quite extensively in the 1990s. Such linkage studies can be considered in three broad strategies: (1) by examining a single very large family under a Mendelian inheritance hypothesis; (2) using several large families in a QTL type approach; or (3) by examining many sibships or trios in families with premature CAD. As with the mouse QTL mapping, the linkage regions using these approaches is typically too large to easily identify plausible causal genes (61). Further, the paucity of strong CAD loci and genes in population samples underscores the weakness of linkage

for common diseases that is to identify rare high-risk alleles, which in reality are infrequent in the population for complex traits. This reality is highlighted by negative results of the largest linkage studies of premature CHD that included 4175 subjects in 1933 affected UK families (62). In at least one large family, however, the Mendelian inheritance approach identified a strong candidate gene in the low-density lipoprotein receptor-related protein 6 (LRP6) (63). This locus as well as additional important examples are described below.

**55.4.1.1.1 Low-Density Lipoprotein Receptor-Related Protein 6 (LRP6).** *LRP6* was identified as the likely causal gene at 12p13 from a linkage study of 28 individuals across four generations of a family with autosomal dominant inheritance pattern of early onset CHD and osteoporosis (63). A missense variant in *LRP6* that segregated with disease was associated with LDL-C, metabolic syndrome features, and other CHD risk factors. The variant was not found in controls.

**55.4.1.1.2 Myocyte-Specific Enhancer Factor 2A (MEF2A).** *MEF2A* is a gene selected from 93 candidate genes in the locus Ch15q26 where a signal was found within a family that had three generations of CHD among 13 members, 9 of whom had an MI (64). *MEF2A* is a gene within this locus that has biological plausibility for a relationship with CHD because of its expression in coronary vasculature in the embryo (64). A seven amino acid deletion was found through re-sequencing of family members with the phenotype and initial functional studies showed this deletion impaired nuclear localization of the *MEF2A* product. However, impaired nuclear localization could not be confirmed in follow-up studies (65) and additional population-based studies showed that while the mutation existed at low frequency, it had no association with sporadic cases of premature MI (66). More recent papers continue to contest the importance of genetic variation in *MEF2A* without a clear consensus, suggesting that potentially other gene(s) in the 15q26 locus may be causative for CVD (67–69).

**55.4.1.1.3 Arachidonate 5-Lipoxygenase-Activating Protein (ALOX5AP).** In work that was transformative for the field, the DeCODE group took advantage of the nationalized medical databases and the detailed genealogical records of family relatedness to perform large population-wide linkage analyses and follow-up association studies of CHD traits in Iceland. Using about 300 Icelandic families, they identified the *ALOX5AP* gene on chromosome 13 encoding 5-lipoxygenase (5-LO) activating protein (FLAP), a leukotriene inflammatory mediator, as the gene underlying approximate 2-fold increase in MI and stroke risk (70). FLAP is important in leukotriene production (70), lipid mediators of inflammation. Such findings have been replicated in other samples, although with weaker effects (71,72) and haplotypes in leukotriene A4 (*LTA4*) hydrolase, a related pathway gene, were also associated with increased MI risk. Using similar approaches, DeCODE also identified phosphodiesterase 4D (*PDE-4D*)

on chromosome 5 as a candidate gene for ischemic stroke (73). However, findings for *ALOX5AP*, *LTA4*, and *PDE4D* have not yet been replicated in MI GWAS studies.

**55.4.1.1.4 A-Type Lamin (LMNA).** *LMNA* has been implicated in multiple Mendelian disorders including cardiomyopathy and lipodystrophy as well as progeria and atherosclerosis. In families with the Hutchinson–Gilford progeria syndrome, both autosomal dominant and sporadic mutations in the A-type lamin (*LMNA*) gene have been identified as causal in premature atherosclerosis and aging (74).

## 55.4.2 Family-Based Association Studies

Family-based association studies provide an elegant approach to studying candidate genes, which overcomes the problem of racial admixture that is a significant concern for association studies of unrelated individuals. In fact, the PROCARDIS consortium demonstrated the feasibility of recruiting a large multicenter sample of parent offspring trios (447 families across four European countries) as well as sib-pairs with premature acute coronary syndrome to perform family-based transmission disequilibrium-based association studies of candidate genes (75). However, such family studies are difficult to recruit and do not overcome the inherent lack of power to detect small effects. Because GWAS of unrelated individuals can more easily recruit large samples and provide methods based on genome wide SNP data to control for population stratification, family-based association studies are less popular and are now mostly used in conjunction with population-based associations studies through meta-analysis strategies designed to increase overall sample size and power. The CARDIoGRAM consortium GWAS of CAD, described in detail below, provides a contemporary example with Framingham and DeCODE Icelandic family data being combined with population-based data for GWAS discovery of heart disease genes.

## 55.4.3 Future Applications of Family-Based Studies

As new techniques such as whole exome and whole genome sequencing emerge, there is renewed interest in family-based studies, independent of the methods of linkage, through combining unbiased sequence data in families with extreme traits with informatic approaches to identify novel genes for CHD traits. In one noteworthy example, Musunuru and colleagues found a novel genetic cause of an inherited lipid disorder by performing whole exome sequencing of family members with extreme familial hypolipidemia—low LDL, TGs, and HDL (76). They found two distinct nonsense mutations in a single gene that co-segregated in affected family members (77). This gene, angiopoietin-like 3 protein (*ANGPTL3*), has been shown in rodents to increase TG and HDL levels



via inhibition of lipoprotein lipase and endothelial lipase (78–81) and was recently identified through GWAS as a locus for plasma lipoproteins and CAD.

## 55.5 GENOME WIDE ASSOCIATION STUDIES

A GWAS is a study where genetic information is obtained for individuals with known phenotypes of interest using a large array of genetic markers. These markers represent common variation in the human genome. The goal of GWAS is to map susceptibility genes by way of detection of associations between the genetic markers and the phenotype of interest (82). GWAS has driven the discovery of multiple novel loci and genes for many complex diseases including CHD. Since identification in 2007 of the Chr 9p21 locus as the first GWAS discovery for CAD, there are now approximately 30 loci for atherosclerotic traits revealed by the GWAS approach (Table 55-3). We provide a brief introduction to GWAS and then discuss findings for CAD and MI.

GWAS are typically designed as case control studies. Because GWAS are focused on common variants, it is not expected that any individual variant will have a very strong association with disease; typically the OR for complex traits is between 1.05 and 1.5. Given the large number of tests and the need to correct for multiple comparisons (typically Bonferroni correction for up to 1 million independent SNPs), large sample sizes (thousands of cases and controls) are required to detect the expected small-to-moderate effects. In addition, a staged strategy has usually been employed whereby a small number of SNPs that reach a pre-specified *p*-value threshold are selected for testing in an additional independent sample. This strategy enhances power (83) and facilitates independent replication while also taking advantage of targeted “look-ups” of top SNPs in existing independent GWAS datasets, thus increasing efficiency and reducing costs. In fact, the most recent wave of secondary GWAS for complex cardio-metabolic traits have typically involved meta-analysis of tens of thousands of individuals (84–88) many of whom were already the subject of smaller GWAS that lacked power to detect anything except the strongest loci. Different populations, may have different linkage disequilibrium structures (89), thus most GWAS are done among homogenous populations. There is continued need to pursue additional GWAS in distinct ethnic groups and to perform replication across ethnicities.

The GWAS approach is not hypothesis driven; rather it is a discovery-based method for detecting novel genetic markers for specific phenotypes. The technique is designed to detect modest association of common variants with complex traits and is relatively unbiased in that a relatively large number of common SNPs are typed with broad coverage across the genome. However, these arrays do not include rare variants and therefore

cannot detect directly effects of stronger disease causing mutations. When a variant is identified that has a reproducible association with CAD, there are several possible explanations: (1) the common causal allele (non-synonymous or regulatory) has a small effect via gene at that locus (e.g. SORT1 locus); (2) the common causal allele has a regulatory effect on gene some distance from the locus (long distance regulatory sites) (e.g., Chr 10q locus downstream of CXCL12); (3) the SNP is in linkage disequilibrium (correlation via common inheritance) with a rarer causal variant nearby at that locus (e.g., LPA SNP); or (4) the SNP is in long-distance linkage disequilibrium with causal variant elsewhere on the chromosome (e.g. likely the case for the Chr 12 pleiotropic CHD locus (90,91)). For a given SNP association, therefore, it is often unclear what is the causal gene at that locus or even if the causal gene resides at that locus.

For many loci, fine mapping and re-sequencing are essential to identify the causal gene even before attempting to establish the direction and mechanism of causal gene relationships. Knowledge of the linkage disequilibrium structure of the region and bioinformatic tools can accelerate such efforts by narrowing the focus on functional elements (e.g. transcription factor binding site, microRNAs, or epigenetic marks) within the locus. Interrogation of expression quantitative trait loci (eQTLs)—that is, SNP associations with mRNA expression (92,93) in disease relevant tissue (e.g. macrophages)—can often be performed *in silico* in existing datasets (e.g. CARDIoGRAM (85)) and point toward causal genes or at least genes in regulated pathways (see Chapter 5).

There are many limitations and precautions when performing GWAS some of which have particular relevance to atherosclerotic CHD and its risk factors. These include (1) the need for very large samples given empiric evidence of small effects; (2) adequate control for population admixture, a cause of confounding especially when using controls that come from different geographic regions than CAD cases; (3) stringent correction for multiple testing (typical *p*-value thresholds of  $<5 \times 10^{-8}$ ); and (4) the need for multiple independent replications, particularly given phenotypic heterogeneity when combining atherosclerotic traits (e.g. CAD and MI). The historical problem of widespread false positives in small candidate gene association studies rightly led to stringent thresholds for genome-wide significance. However, this has led to a significant rate of false negatives where some SNPs with *p*-values between  $1 \times 10^{-3}$  and  $5 \times 10^{-8}$  mark loci that are causal. For example, in GWAS meta-analysis of height, using a maximum likelihood method which makes use of the complete distribution of *p*-values, up to 29% of the phenotypic variance of height could be explained by genetic variants, which was 3-fold higher than using only significant GWAS SNPs (94,95). Strategies for mining these GWAS data in

<b>Loci</b>	<b>Probable Causal Gene(s)</b>	<b>Lead SNP</b>	<b>Risk Allele</b>	<b>CVD-Related Phenotypes</b>	<b>OR</b>	<b>p-value</b>	<b>Primary Reference<sup>a</sup></b>
1p13.3	SORT1	rs599839	A	LDL, Lp-PLA2 activity and mass	1.11 [1.08–1.15]	$2.89 \times 10^{-10}$	(85)
1p32.3	PPAP2B	rs17114036	A		1.17 [1.13–1.22]	$3.81 \times 10^{-19}$	(85)
1p32.3	PCSK9	rs11206510	T	LDL-C	1.08 [1.05–1.11]	$9.10 \times 10^{-8}$	(85)
1q41	MIA3, AIDA	rs17465637	C		1.14 [1.09–1.20]	$1.36 \times 10^{-8}$	(85)
2q33.2	WDR12	rs6725887	C		1.14 [1.09–1.19]	$1.12 \times 10^{-9}$	(85)
3q22.3	MRAS, ESYT3	rs2306374	C		1.12 [1.07–1.16]	$3.34 \times 10^{-8}$	(85)
6p21.31	ANKS1A	rs17609940	G		1.07 [1.05–1.10]	$1.36 \times 10^{-8}$	(85)
6p24.1	PHACTR1	rs12526453	C	CAC	1.1 [1.06–1.13]	$1.15 \times 10^{-9}$	(85)
6q23.2	TCF21	rs12190287	C		1.08 [1.06–1.10]	$1.07 \times 10^{-12}$	(85)
6q25.3	LPA	rs3798220	C		1.51 [1.33–1.70]	$3.00 \times 10^{-11}$	(85)
7q22.3	BCAP29, DUS4L	rs10953541	C		1.08 [1.05–1.11]	$3 \times 10^{-8}$	(84)
7q32.2	ZC3HC1	rs11556924	C		1.09 [1.07–1.12]	$9.18 \times 10^{-18}$	(85)
9p21.3	CDKN2A, CDKN2B, ANRIL	rs4977574	G	Intracranial aneurysm, CAC	1.29 [1.23–1.36]	$1.35 \times 10^{-22}$	(85)
9q34.2	ABO	rs579459	C	TC, LDL, VTE	1.1 [1.07–1.13]	$4.08 \times 10^{-14}$	(85)
10q11.21	CXCL12	rs1746048	C		1.09 [1.07–1.13]	$2.93 \times 10^{-10}$	(85)
10q11.23	KIAA1462	rs2505083	C		1.15 [1.11–1.20]	$1 \times 10^{-11}$	(129)
10q23.31	LIPA	rs1412444	T		1.09 [1.07–1.12]	$3 \times 10^{-13}$	(84)
10q24.32	CYP17A1, CNNM2, NT5C2	rs12413409	G	HTN, Intracranial aneurysm	1.12 [1.08–1.16]	$1.03 \times 10^{-9}$	(85)
11q22.3	PDGFD	rs974819	T		1.07 [1.04–1.09]	$2 \times 10^{-9}$	(84)
11q23.3	APOA5-A4-C3-A1, ZNF259	rs964184	G	TC, HDL, LDL, Lp-PLA2 activity and mass	1.13 [1.10–1.16]	$1.02 \times 10^{-17}$	(85)
12q24.12	SH2B3, ATXN2	rs3184504	T	HTN, DM, CKD	1.07 [1.04–1.10]	$6.35 \times 10^{-6}$	(85)
13q34	COL4A1-A2	rs4773144	G		1.07 [1.05–1.09]	$3.84 \times 10^{-9}$	(85)
14q32.2	HHIPL1, CYP46A1	rs2895811	C		1.07 [1.05–1.10]	$1.14 \times 10^{-10}$	(85)
15q25.1	ADAMTS7	rs3825807	A		1.08 [1.06–1.10]	$1.07 \times 10^{-12}$	(85)
17p11.2	PEMT	rs12936587	G		1.07 [1.05–1.09]	$4.45 \times 10^{-10}$	(85)
17p13.3	SMG6, SRR	rs216172	C	Aortic root size	1.07 [1.05–1.09]	$1.15 \times 10^{-9}$	(85)
17q21.32	UBE2Z, GIP, ATP5G1, SNF8	rs46522	T		1.06 [1.04–1.08]	$1.81 \times 10^{-8}$	(85)
19p13.2	LDLR	rs1122608	G	LDL	1.14 [1.09–1.18]	$9.73 \times 10^{-10}$	(85)
21q22.11	MRPS6, SLC5A3, KCNE2	rs9982601	T		1.18 [1.12–1.24]	$4.22 \times 10^{-10}$	(85)

VTE = venous thromboembolism, TC = total cholesterol, LDL = low-density lipoprotein cholesterol, HDL = high density lipoprotein cholesterol, DM = diabetes mellitus, HTN = hypertension, CAC = coronary artery calcium.

<sup>a</sup>Primary reference denotes most recent primary study or meta-analysis from which OR and p-value are reported.

order to reduce false negatives have yet to become standardized but promise to increase the yield from existing GWAS over time.

### 55.5.1 GWAS Findings for Atherosclerotic Traits

The CVD phenotypes most commonly studied by GWAS are MI, the presence of angiographic CAD, surrogate measures such as CAC and IMT, (96,97) and risk factors for CVD including plasma lipids, hypertension (HTN), diabetes, smoking, and circulating biomarkers. Here we focus on published and emerging data for CAD and MI, for surrogate measures of atherosclerosis and selectively for CHD risk factors and biomarkers. Using GWAS, multiple novel CHD loci have been discovered and replicated (Table 55-2), many of which lack a defined mechanism for their association with CHD. In other cases, loci which were well-known to relate to CHD and its risk factors have been identified from these studies, confirming the utility of GWAS approach. In addition, there has been some overlap between GWAS loci for clinical CHD and those for surrogate measures (e.g. coronary calcium) and for risk factors, particularly plasma LDL cholesterol (86).

**55.5.1.1 The 9p21 Locus.** To date, the Chr 9p21.3 locus is the most well known and reproduced for CHD (both MI and angiographic CAD) in Caucasians although prior to GWAS it was completely unknown in CHD. This locus was first identified almost simultaneously in three studies from the Wellcome Trust Case-Control Consortium (WTCCC), the Ottawa Heart Study, and DeCODE Genetics (98–101) followed by corroboration in almost all subsequent reasonably powered analyses in European ancestry samples (41,54,102). A 2008 meta-analysis, including seven case control studies, found a highly associated SNP within the 9p21 locus (rs1333049), with an OR of 1.29 (95% CI 1.22–1.37) per copy of the risk allele (103). This risk allele is relatively common with ~50% and 25% of individuals of European descent carrying one or two alleles respectively. Thus, this discovery is typical of many in GWAS of complex traits in that it appears to confer a small to moderate effect but, because it is common, it may contribute significantly to the risk of disease in the population (sometimes called the population attributable risk) (89).

Follow-up studies of CHD across a wide spectrum of European ancestry samples have been largely confirmatory (104–106). However, a few studies have been negative (107,108). In particular, those that attempt to discriminate between association with angiographic CAD and risk of MI suggest that the 9p21 locus relates to MI and CHD via coronary atherosclerosis. Indeed, our group found that this locus was associated with CAD defined by presence or absence of angiographic CAD but did not relate to MI among patients with evidence of angiographic CAD, supporting the notion that the 9p21 alleles confer risk of atherosclerosis rather than MI *per*

*se* (18). This concept is indirectly supported by recent work that found significant association of the 9p21 locus with CAC, a surrogate measure of coronary atherosclerosis. In fact, the Cohorts for Heart and Ageing Research in Genetic Epidemiology (CHARGE) consortium, the 9p21 CAD risk allele was the most significant (top SNP rs1333049, meta-analysis  $\beta$  0.269, SE 0.03,  $p = 7.58 \times 10^{-19}$ ) for CAC in a meta-analysis of ~10,000 European ancestry individuals (109). Remarkably, other non-atherosclerotic vascular wall traits, including aortic (110) and intracranial aneurysms (111) have been associated with 9p21 CHD SNPs, suggesting a fundamental role for this locus in several pathophysiologies involving medial arterial remodeling, not just atherosclerosis. Also noteworthy is the lack of association with any traditional risk factors including plasma lipoproteins and hypertension (112–114). To date, studies of cIMT (103,115) as well as aortic IMT (116) and aortic plaques measured at ultrasound (117) have been negative including analysis of carotid ultrasound traits in over 40,000 individuals in the CHARGE consortium GWAS (97). Overall, these data may point toward medial arterial pathophysiologies in its relationship to vascular diseases.

The 9p21 locus association with CAD has been extended to non-European ethnicities including east Asian (Koreans (105), Japanese (115,118,119)), south Asian (120,84), Hispanic, and African-American samples (121). However, the top CAD SNP at the 9p21 locus did vary in some ethnicities, suggesting either distinct causal variants or shared ethnic signals marked by smaller chromosomal regions. For example, the NHLBI CARE Project found modest evidence of replication for the 9p21 locus with CHD in African-Americans but the lead SNP (rs6475606; African-Americans OR 2.0,  $P = 6.4 \times 10^{-4}$ ) was not correlated with the index CAD European ancestry SNP (rs4977574; African-Americans OR 1.18,  $P = 0.17$ ) in HapMap samples of African descent (YRI) ( $r^2 = 0.001$ ) whereas it was in perfect LD with rs4977574 in HapMap CEU ( $r^2 = 1.0$ ). This pattern of fine mapping suggests shared ethnic causal signals marked by a smaller chromosomal region in African ancestry that will facilitate more focused re-sequencing to identify such causal variant(s).

To date, the biology of the association of CVD with the 50–60 kb 9p21 region, containing the CAD risk alleles, has been difficult to elucidate. The closest protein-encoding genes are 100 kb away from these SNPs. In European ancestry samples, there is a large LD block at this locus. At the 3' end there is CDKN2B, encoding a cyclin-dependent kinase inhibitor tumor suppressor, p15<sup>INK4B</sup>, as well as CDKN2A that encodes another tumor suppressor, p16<sup>INK4B</sup>. These kinases are cell cycle regulators of TGF- $\beta$ , which in smooth muscle cells is implicated in aortic aneurysms (122,123) and plays a role in atherosclerosis in experimental models (124). CDKN2A/B may therefore be directly involved in the association with CVD. Notably at the GWAS region, there is a predicted non-coding RNA designated ANRIL,

which encodes multiple microRNA isoforms expressed in vascular cells that regulate target genes, including CDKN2A/B, relevant to vascular homeostasis and disease (125,126). Visel and colleagues have knocked out the orthologous 70-kb non-coding interval on mouse chromosome 4, creating mice that are viable, but which have increased mortality and decreased expression of CDKN2A/B, suggesting loss of a regulatory element for these genes. Furthermore, they showed that cultures of aortic smooth muscle cells from these mice had excessive proliferation and diminished senescence (127).

Others have identified an enhancer hot-spot in this GWAS region that can impact inflammatory pathways involved in CVD. Harismendy and colleagues found marked enrichment of enhancers within the 9p21 region and that two CHD risk allele SNPs disrupt binding of STAT1 and related INF gamma signaling in endothelial cells (128). Overall, the 9p21 locus is a major reproducible GWAS discovery for vascular diseases that appears to act via completely novel molecular and pathophysiological actions. Remarkably, four years on since its discovery, we still do not understand the precise genetic or molecular mechanism of its association, have not yet defined loss of function or gain of function alleles and their impact, and have enabled little work on therapeutic translation.

**55.5.1.2 Additional GWAS Loci for CHD.** Since 2007 and the identification of the 9p21 locus, there have been significant advances particularly in 2009 and 2011 (18,84,85,99,100,110,129–132) (Table 55-3). In 2007, Samani and colleagues combined data from the WTCCC and German MI (GerMI) studies to replicate the 9p21 locus and to identify an additional five putative loci for CAD and MI (100). Kathiresan and colleagues extended these findings in a 2009 report of 2967 early MI cases and 3075 controls replicating six prior findings and identifying three novel loci (110). Simultaneously, additional loci were reported increasing to 13 the total number of replicated CAD and MI loci (43,132). A recent wave of larger studies, most involving meta-analysis of several independent GWAS (18,85,98,130), has increased to over 30 the number of independent genes and loci associated with human CAD and MI. We performed two parallel but complementary multistage GWAS of angiographically defined CAD in order to discover loci related to coronary atherosclerosis distinctly from those associated with MI in the presence of atherosclerosis (18). Using this approach, we identified variants in *ADAMTS7* on Chr 15 as a locus for coronary atherosclerosis whereas *ABO* genotypes, which were surrogates for the loss-of-function O blood group, conferred protection from MI in patients with established CAD.

The CARDIoGRAM consortium, which combined through meta-analysis 14 published and unpublished European ancestry GWAS of CAD and MI including 22,223 cases and 64,762 controls with replication in 56,682 additional individuals, replicated almost all prior loci while identifying and replicating 13 new regions including *ADAMTS7* and *ABO* (85). Work by the

CD4 Consortium, published simultaneously, combined GWAS data across European and South Asian ancestry studies ( $n = 8424$  Europeans and 6996 South Asians with 15,062 controls, replication in 21,408 cases and 19,185 controls). Like CARDIoGRAM, C4D identified the 9p21 locus, *ADAMTS7* at 15q25, *SORT1* at 1p13 but also reported consistent findings across ethnicity and identified four additional loci including SNPs in the lysosomal acid lipase A (*LIPA*) gene at 10q23, *PDGFD* on 11q22, *BCAP29* on 7q22, and *KIAA1462* on 10p11 (84).

## 55.5.2 GWAS of Subclinical Atherosclerosis Traits

CAC and cIMT are two widely studied non-invasive measures of subclinical atherosclerosis. Recent guidelines support use of both measures in the clinic for risk prediction and prevention of CHD (133,134). CIMT is an ultrasound measurement of thickening of the intima and media layers of the carotid artery at specific locations along the common carotid artery (common cIMT) or internal carotid artery (internal cIMT). Both abnormal thickness and the presence of non-obstructive carotid plaque have been shown in multiple independent studies to predict CHD events and stroke (135,136). CAC, measured by cardiac CT is also strongly associated with CHD events (137–139).

The CHARGE consortium recently completed the first comprehensive GWAS of three carotid artery phenotypes, carotid plaque, and both internal and common cIMT among 31,211 subjects across nine population-based studies (97). They found three independent loci for common cIMT that met genome-wide significance: rs11781551 at 8q24 near the *ZHX2* gene, a member of nuclear homodimeric transcriptional repressors; rs445925 near *APOC1* on 19q13, a locus which also includes *APOE*, *APOC2*, and *APOC4*; and rs6601530 at 8q23.1, within *PINX1*, a telomerase inhibitor (Table 55-4). Two loci met genome-wide significance for plaque: rs17398575 at 7q22 near the *PIK3CG* gene, which encodes the PI3/PI4-kinase family of genes and rs1878406 on 4q31 near *EDNRA* or endothelin receptor type A, which has been associated nominally with BP (140), atherosclerosis (141), and CHD (142) in candidate gene studies. The latter SNP region also had quite a strong association ( $p = 2 \times 10^{-6}$ ) with CAD in CARDIoGRAM data. No SNPs achieved genome-wide significance for internal cIMT and none of the genome-wide significant SNPs for MI and CAD in CARDIoGRAM had a significant association with any of these carotid phenotypes (85).

The CHARGE consortium also conducted a meta-analysis across 9961 subjects from five cohorts with significant findings for CAC. As noted, the 9p21 region was rich in SNPs with genome-wide significance for CAC. A second notable finding was on 6p24 locus within the *PHACTR1* gene (top SNP  $p = 2.65 \times 10^{-11}$ ), which also reached genome-wide significance in several GWAS of



**TABLE 55-4 Genome-Wide Significant Loci<sup>a</sup> Associated with CAC and cIMT**

Phenotype	Locus	Probable Causal Gene(s)	Top SNP	P-value <sup>b</sup>
CAC	9p21	CDKN2A CDKN2B, ANRIL	rs1333049	$3.33 \times 10^{-24}$
	6p24	PHACTR1	rs9349379	$3.90 \times 10^{-22}$
Common cIMT	8q24	ZHX2	rs11781551	$2.4 \times 10^{-11}$
	19q13	APOC1 APOE, APOC2, and APOC4	rs445925	$1.7 \times 10^{-8}$
Carotid plaque	8q23.1	PINX1	rs6601530	$1.7 \times 10^{-8}$
	7q22	PIK3CG	rs17398575	$2.3 \times 10^{-12}$
	4q31	EDNRA	rs1878406	$6.9 \times 10^{-12}$

<sup>a</sup>Only genome-wide significant loci are shown ( $p < 5 \times 10^{-8}$ ). Data is taken from studies by the CHARGE consortium (97) and O'Donnell and colleagues (109).

<sup>b</sup>P-values represent those for discovery and replication combined but were genome-wide significant in discovery phase as well.

MI and CAD (85,110). Of all, CAD-related SNPs in CARDIoGRAM, seven had associations with CAC after Bonferroni correction for a significant  $p$ -value being less than  $1 \times 10^{-3}$  (109).

### 55.5.3 Summary of Atherosclerosis-Related Loci

To date, 29 loci for CAD, MI, or subclinical atherosclerosis have met genome-wide significance in large-scale GWAS (Table 55-3). It is worthwhile to briefly review the current knowledge for these loci and related genes. Only a minority of GWAS loci are significantly associated with established CHD risk factors such as plasma lipids, diabetes, or HTN. Several appear to have more pleiotropic effects on diverse disease traits (e.g. ABO, Chr 12). Some loci contain genes, such as *LDL-R* and *LPA* that previously were implicated in atherosclerosis through specific causal pathways and candidate gene studies. Recently, data emerged implicating genes and specific mechanisms for novel loci in atherosclerosis; for example, *SORT1* (143,144).

Some markers, however, are in gene deserts and most have no relation to known risk factors for CHD, contain genes with unknown function, or have described functions that lack obvious mechanisms to account for the association with CAD. One theme that may be emerging is that some loci encode transcription factors, regulatory domains, and signaling proteins that are expressed in vascular cells suggesting direct effect on atherosclerosis. Regardless, these GWAS discoveries point to an emerging paradigm shift in our understanding of the causes of atherosclerosis and have prompted an unprecedented focus on as yet unidentified mechanisms and pathways for atherosclerosis and MI independent of traditional risk factors. Several novel loci have been studied to varying extents and here we will review briefly a few examples that have emerging mechanistic data in atherosclerosis.

**55.5.3.1 Chr 1p13 Locus (SORT1).** The 1p13 locus has genetic and functional data that support its association with CAD and MI. It is both a LDL-C and CHD-associated locus. Homozygotes for the major alleles have a

40% increased risk of MI and increased LDL levels. A non-coding SNP, rs12740374, is likely causal. This SNP creates a C/EBP transcription factor binding site and modulates liver expression of *SORT1* which encodes the sortilin-1 protein. Levels of sortilin are higher in heterozygotes than homozygotes for the major allele. *SORT1* modulates hepatic VLDL secretion and may also regulate hepatic LDL clearance (143). However, the relative role of *SORT1* on production vs. clearance in regulating LDL remains to be defined; this pathway appears to have clear potential as a drug target.

**55.5.3.2 Chr 1p32.2 (PPAP2B).** The rs17114036 SNP is located in the *PPAP2B* (*LPP3*) gene, which encodes lipid phosphate phosphohydrolase 3 (*LPP3*). LPPs are membrane proteins that dephosphorylate lipid phosphates (145). Different LPPs perform distinct functions depending on cell localization and substrates (146), suggesting that novel lipid metabolites may exert an important role in atherosclerosis. Targeted disruption of *PPAP2B* in the mouse results in embryonic lethality due to developmental vascular defects (147).

**55.5.3.3 Chr 6q23.2 (TCF21).** *TCF21* (also named *POD-1* or *Capsulin*) encodes a basic helix-loop-helix transcription factor. Two transcript variants encoding the same protein have been found for this gene (148). *TCF21* is a tumor suppressor gene whose product regulates mesenchymal cell transition into epithelial cells. It is frequently lost in human malignancies (149,150). Expression of *TCF21* marks the spiral septum of the heart and progenitor cells that give rise to the pericardium and coronary arteries (151). It is also expressed in vascular smooth muscle cells (152,153).

**55.5.3.4 Chr 9q34.2 (ABO).** The ABO (transferase A, alpha 1-3-N-acetylgalactosaminyltransferase; transferase B, alpha 1-3-galactosyltransferase) gene encodes proteins for the first discovered blood group system, ABO (154). ABO blood groups reflect the presence of three main carbohydrate antigens and distinct glycotransferase activities: A, B, and H. Individuals with the A, B, and AB alleles express glycosyltransferase activities that convert the H antigen to the A antigen or to the B antigen. The

O blood group, with unmodified H antigens, is caused by a deletion of guanine-258 near the N-terminus, which results in a frameshift and a protein lacking glycosyl-transferase activity (154,155).

Associations of the ABO blood groups with plasma lipoproteins (156) and CHD were reported several decades ago (157–159). In a recent meta-analysis, Wu et al. found a pooled ORs of 1.25 (1.14–1.36) for MI in non-O relative to blood group O carriers (160). ABO-related thrombosis is believed to be mediated by A and B glycosyltransferase-modification of von Willebrand Factor (vWF) resulting in impaired proteolysis and higher circulating vWF and Factor VIII (161). However, ABO also modifies multiple glycoproteins on endothelium and platelets. Remarkably, multiple GWAS have now identified ABO as a locus associated with VTE (162), plasma vWF and Factor VIII (163), plasma LDL-C (164), and sitosterol (165), inflammatory risk markers E-selectin, P-selectin and sol-ICAM1 (166–168), and angiotensin-converting enzyme (169).

The mechanisms underlying how different ABO groups affect CHD and other CVD risk traits remain unknown probably because studying glycobiology is more challenging than studying nucleic acids or proteins (170). Furthermore, there is a lack of appropriate mouse models for the human ABO locus (171). Overall, ABO is notable because of its diverse genetic disease associations and because it highlights a potentially important role of glycobiology in human CVD.

**55.5.3.5 Chr 10q11 (CXCL12).** The only novel CAD GWAS loci related to a known inflammatory gene is the 10q11 locus which is downstream of the chemokine (C-X-C motif) ligand 12 (CXCL12) gene, previously called stromal cell derived factor 1 (SDF-1). The CAD-associated SNPs at this locus are in a gene desert with CXCL12 located ~100,000 bases away. CXCL12 has two common splice variants and up to six additional coding variants. Preliminary data suggest that the 10q11 CAD-associated SNP, rs1746048, is associated with circulating CXCL12 levels and higher transcript levels of CXCL12  $\alpha$ -isoform in human natural killer cells and the liver (172).

Human data remain conflicting as to whether CXCL12 is pro- or anti-atherogenic (173). It is likely, however, that differential tissue-specific roles may be involved, that mice may differ from humans, and that acute vascular injury models may differ from chronic atherosclerosis models. CXCL12 plays a role in angiogenesis and has anti-inflammatory properties, preventing the accumulation of leukocytes in inflamed tissue (174). KO mouse data (Cxcl12<sup>-/-</sup>) suggest that deficiency is detrimental to health as it is required for B-cell lymphopoiesis, myelopoiesis, correct formation of the ventricular septum (175,176), and T-cell development (177). Tissue-specific deletion and over-expression of CXCL12 and the GWAS region in mice, as well as sequencing and functional genomics in humans, will be needed to clarify the biological effect of LOF and GOF alleles in atherosclerosis and MI.

**55.5.3.6 Chr11q23.3.** This locus includes several apolipoprotein genes, APOA5, APOA4, APOC3, and APOA1, as well as ZNF259, the zinc finger protein 259; ZPR1. The lipoprotein genes have greatest mechanistic support for being candidates in atherosclerosis, however, which specific gene or genes remain uncertain. ApoA5 decreases plasma triglycerides by enhancing lipoprotein lipase-mediated triglyceride hydrolysis (178). ApoC3 has opposite effects on plasma triglycerides due to inhibition of triglyceride hydrolysis (179,180). ZNF259 is a mitogenic protein that interacts with tyrosine kinase receptors, including the epidermal growth factor receptor (181) raising the possibility of a functional role in atherosclerosis.

**55.5.3.7 Chr 15q25.1 (ADAMTS7).** The protein encoded by the ADAMTS7 gene is a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family. Members of this family have a similar domain organization, including a prepro-region, a reprolysin-type catalytic domain, a disintegrin-like domain, a thrombospondin type-1 (TS) module, a cysteine-rich domain, a spacer domain without cysteine residues, and a COOH-terminal TS module (182,183). ADAMTS are important targets of PTHrP signaling and participate in the degradation of cartilage extracellular matrix proteins (184,185). ADAMTS13 is the protease that cleaves vWF, and mutations in ADAMTS13 cause thrombotic thrombocytopenic purpura (TTP, OMIM: 274150). Many ADAMTS proteins are known to bind and cleave proteoglycans found in the arterial wall including aggrecan, versican, and brevican (186). Indeed, several lines of evidence link the ADAMTS family with atherosclerosis (187–189) and human CVD (189,190).

ADAMTS7 binds and cleaves cartilage oligomeric matrix protein (COMP), an interaction thought to play a role in the progression of arthritis (183). More recently, ADAMTS7 over-expression was shown to increase post-injury neointimal formation in a rat carotid artery balloon-injury model (191). Subsequent knock down of ADAMTS7 expression by topical application of siRNAs decreased post-injury neointimal formation. Over- and under-expression of ADAMTS7 also increased and decreased primary VSMC migration, respectively (191). Overall, human genetic and rodent experimental data suggest that ADAMTS7 is likely to be atherogenic, perhaps through enhanced VSMC infiltration and migration in plaques. These results suggest that ADAMTS7 may be a potential target for therapeutic intervention.

## 55.6 GWAS FINDINGS FOR CVD RISK FACTORS

A complementary strategy for discovery of novel CVD genes is through an indirect GWAS discovery focus on CHD risk factors (86). For loci with reproducible GWAS associations with a specific risk factor, a candidate gene approach (initially with less stringent multiple testing criteria than applied in unfocused GWAS) can be used to

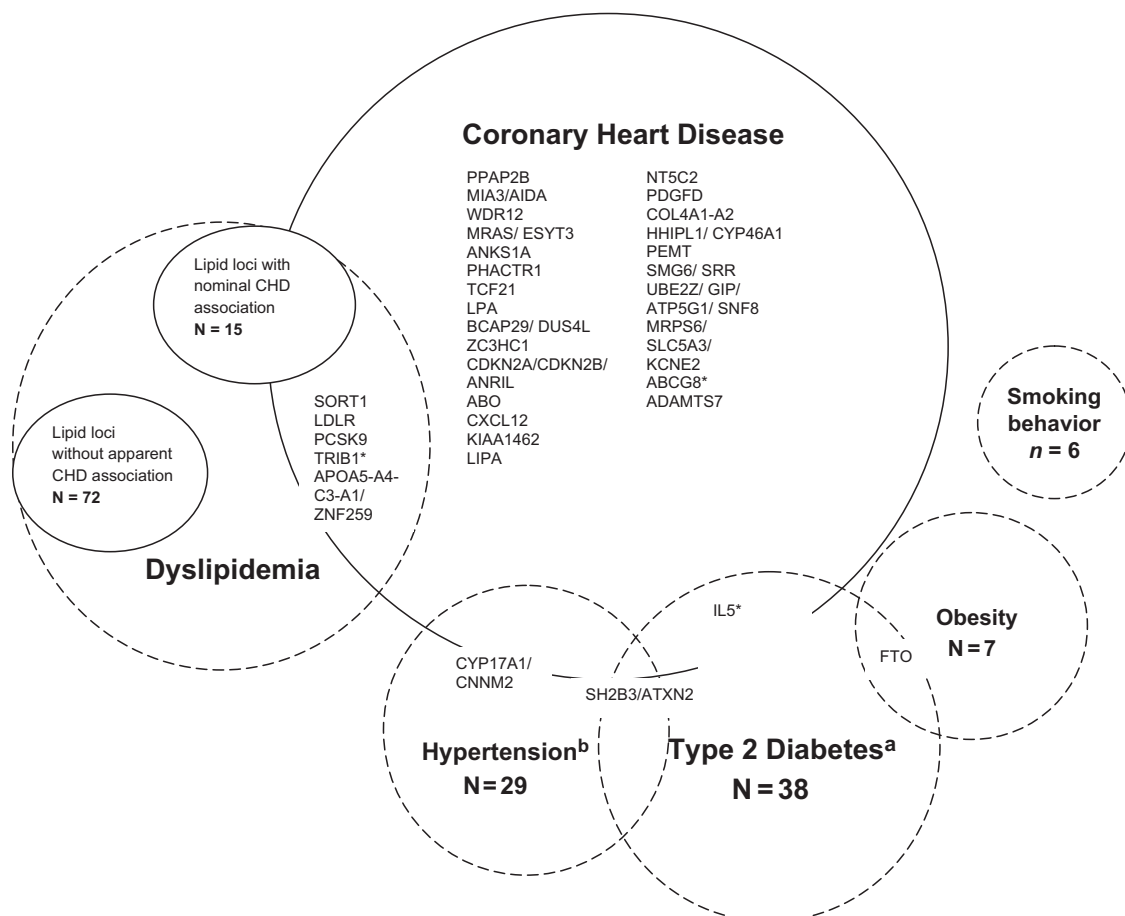
assess whether that locus is also related to CAD and MI. Based on risk factor association, the prior probability is higher and focus is narrower and therefore the chance of discovery of true CHD loci is increased. A related human genomic strategy, Mendelian randomization (see below), also provides a powerful tool under certain circumstances to prove or disprove if a specific risk factor/biomarker for CHD is actually causal. Below, we provide a brief discussion of GWAS for specific CHD loci. **Figure 55-1** presents an overview of which specific loci for specific CHD risk factors, discovered through GWAS, actually also have evidence for association with CHD.

### 55.6.1 GWAS of Plasma Lipids

The genetics of plasma lipoproteins is covered elsewhere (Chapter 96), but we will summarize the most relevant findings for GWAS of plasma lipids as these have proven to be remarkably informative in advancing our understanding of lipid metabolism pathways in CHD. The largest study today by the Global Lipids Genetics Consortium (GLGC) has assembled data on plasma lipid

concentrations for more than 100,000 individuals of European descent and identified 95 significantly associated loci ( $p < 5 \times 10^{-8}$ ), with 59 of these representing completely novel discoveries (86).

Prior lipid loci discovered by smaller GWAS were replicated (41,44,55,106,192,193) and all loci harboring rare mutations that cause Mendelian lipid disorders (e.g., ABCA1, LDLR, and PCSK9) were identified as containing common variation that also associated plasma lipids in the general population. This marked overlap of GWAS findings with Mendelian lipid disorders has provided a form of validation more broadly for the GWAS strategy in complex cardio-metabolic traits and supports the concept that many novel GWAS loci will harbor biologically important genes and rare mutations that cause rare Mendelian lipid disorders. In fact, this has already proven to be the case for ANGPTL3, where rare loss of function alleles are associated with familial cases of extremely low LDL-C, TG, and HDL-C (86). Of the 59 novel loci, 39 were associated with total cholesterol, 22 with LDL-C, 21 with HDL-C, and 16 with TGs.



**FIGURE 55-1** Overlapping loci of CHD and related risk factors from GWAS: <sup>a</sup>These loci were obtained from a large-scale study using the ITMAT/Broad/CARE (IBC) 50k SNP Array (57). <sup>b</sup>Hypertension includes systolic and diastolic blood pressure as well as a history of hypertension. Note: Loci had to meet genome-wide specific threshold criteria for a particular study to be listed in this figure. References include the following: Hypertension (56,205,323), Dyslipidemia (86), Type 2 Diabetes (87), Obesity (324–329), and Smoking Behavior (330–332). Studies for Obesity and Smoking Behavior are less well replicated and validated among large sample size populations and *via* meta-analysis.

The most highly associated locus in the genome for LDL-C was at Chr 1p13, containing SORT1 the causal gene, independently related through GWAS to MI and CAD (41,100) (discussed above). Several loci (e.g. SORT1 (143), MYLIP/IDOL (194), and TRIB1 (144)) have undergone genetic and functional interrogation with definition of causal genes and mechanism of lipid effects as well as initiation of therapeutic programs for targeting these novel genes in lipid disorders and CHD.

A controversial unanswered question for the field is whether genes that relate to HDL-C and TG have equal impact on CHD as loci for LDL-C or whether loci for HDL-C and TG lack systematic effects but rather have gene-specific mechanism-based impact that are only causal for CHD for a limited number of these genes. This would be consistent with some clinical trials and epidemiological literature as well as emerging Mendelian randomization studies (see below). The GLGC data provides an intriguing insight into this question. In 24,607 CHD cases and 66,197 controls, they assessed the relation of the top SNP at each of the 95 lipid loci for association with CAD and MI. Their findings can be summarized as follows: (1) many LDL-C loci had significant associations (corrected for the number of candidate SNPs tested) with CHD and all effects on CHD were in the direction expected based on their relation with LDL-C; (2) most HDL-C and TG loci did not have significant associations with CHD and the observed number with CHD association was barely different from the number expected by chance; and (3) HDL-C and TG loci (e.g., TRIB1) that did have significant association with CHD tended to be those with association with more than one lipid trait. Overall, these data provided strong support for the LDL hypothesis in atherosclerosis, have raised substantial questions regarding the causal association of HDL-C and TG in atherosclerosis (vs. confounding or reverse causation), and suggest strongly that the specific function and mechanism of HDL and TG loci will determine whether they also relate to atherosclerosis and CHD. Thus, therapeutic targeting to lower CHD of genes for HDL-C and TG will remain tentative and restricted to those loci and genes with multiple lipid effects and reproducible human genetic evidence for CHD association.

### 55.6.2 GWAS of Diabetes and Hypertension

Over 30 loci for type 2 DM (Chapter 86) (195–200) and over 25 loci for HTN (Chapter 52) (56,115,195,201,205) have been identified through GWAS, some related to genes that are known drug targets (202). Most loci though are novel and have led to new paradigms in disease pathophysiology, especially for type-2 diabetes where many new loci appear to regulate pancreatic beta cell function. Unlike lipids, there appears to be almost no relationship of type-2 diabetic loci to CHD (Figure 55-1). These diabetic data further support clinical trial

literature in suggesting that most genes for type-diabetes as well as glycemia-diabetes *per se* are not causally related to CHD.

GWAS discoveries for BP and HTN have been challenging due to the dynamic nature of BP and the limitations (e.g., misclassification) of applying the case-control study design to this continuous trait. More recently, particularly through larger studies (203–205) the use of quantitative phenotypes novel loci for BP have been identified. The largest and most recent of these studies completed by the International Consortium for Blood Pressure (ICBP) found, across 200,000 subjects of European descent, 16 novel BP-related loci. Six of these contained genes already known to be related to BP. The ICBP study also confirmed 12 of 13 previously identified loci at the level of genome-wide significance ( $p < 5 \times 10^{-8}$ ) (205). Based upon all significant SNPs from both novel and prior findings, the authors created a genetic risk score, weighted by the average systolic and diastolic BP effect of each SNP. The top decile of risk had a 29% prevalence of HTN and the bottom decile a 16% prevalence. The overall score was quite strongly associated with CHD and stroke (205), but it is not clear yet which HTN loci confer the most risk of atherosclerotic disease. Thus, some BP/HTN loci do relate to CHD and stroke, providing genetic evidence for a causal relationship between BP and atherosclerotic CVD, a finding which is well supported by clinical trials of HTN. As noted already, a few loci (e.g. SH2B3/ATXN2 and CYP17A1/CNNM2) discovered for CAD and MI are also related to BP/HTN providing reciprocal indirect support for causation. In addition to larger GWAS and exome or genome sequencing of extreme HTN phenotypes, an evaluation of each BP/HTN locus for CHD risk is required to determine if there is a systematic association or specific and individual and specific mechanisms of BP gene relationships with CHD. This knowledge combined with functional work may greatly accelerate the translation to clinic for CHD risk prediction and novel therapeutics targeting BP and HTN-related CHD.

### 55.6.3 GWAS of Novel Biomarkers

Many large GWAS of novel CVD biomarkers, particularly inflammatory and thrombotic proteins, have been reported or are ongoing. Some of these biomarkers are not themselves causally related to CHD (e.g. C reactive protein (CRP)), while others may be (e.g. lipoprotein-associated phospholipase A2 (LpPLA2), interleukin-6 (IL6), intercellular adhesion molecule-1 (ICAM-1), and von Willebrand Factor (vWF)). One unanswered question is whether very large GWAS of these markers can reveal novel genes and pathways that are causal for CHD regardless of whether the biomarker itself is or not. Early indications suggest that this may be the case. For example, IL6 and IL6R genes are highly significant in GWAS of plasma CRP levels (206,207) and this cytokine pathway has emerging evidence for causation in CHD. The top GWAS hit for plasma vWF and



Factor VIII is the ABO locus (163) and, as noted, ABO has also been identified in GWAS of MI (18,85).

In addition to a systematic assessment of all biomarker GWAS loci for their CHD relationship in large datasets, specific interrogation in cohort studies using the Mendelian randomization strategy, when appropriate, will provide human genetic evidence for which inflammatory and thrombotic biomarkers are causal in CHD and warrant further focus for therapeutic targeting in CHD. Below, we provide a brief review of Mendelian randomization with some examples related to CHD.

#### 55.6.4 Next Steps for Novel CVD Risk Loci

Each GWAS locus is at a different stage of follow-up investigation, but the majority lack even certainty regarding the specific gene or genetic mechanism and all require genetic mapping and functional work. In general terms, the next steps are to (Figure 55-2) (1) identify the causal gene or genetic mechanism (e.g., non-coding regulatory region); (2) map mutations/causal variation through sequencing and fine mapping; (3) define through functional studies whether the discovered mutations are loss of function (LOF) or gain of function (GOF) for the candidate gene/genetic effect; (4) assess in large human samples whether low frequency LOF or GOF variation increases disease risk in order to understand causal direction of locus on CHD; (5) develop therapeutic translation based on biochemical, functional, and directional characteristics of the gene/locus; and (6) in parallel integrate human genomic data across these loci and future discoveries in order to advance personalized risk assessment in clinical practice (see below).

#### 55.6.5 Mendelian Randomization

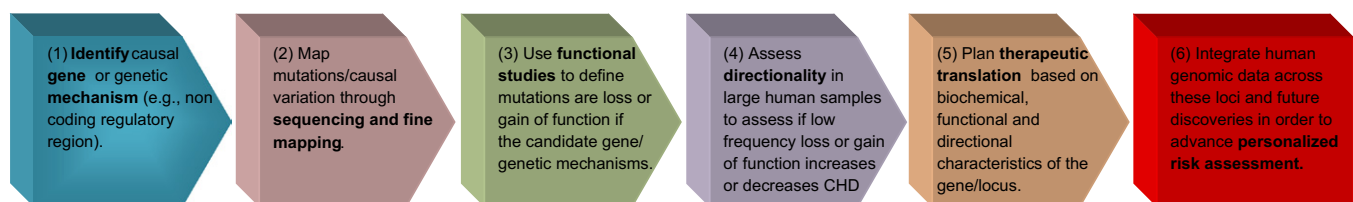
This concept has been mentioned above in relation to GWAS of CHD risk factors and discriminating between causal risk factors and non-causal biomarkers of CHD. In this technique, genetic variants are used to help provide evidence that a risk factor/intermediate phenotype is causative for a disease. The intermediate phenotype can be any risk factor under study, such as a biomarker. The genetic variant must directly affect the intermediate phenotype. For example, genetic variants can change the stability of the transcript for the biomarker of interest. If the intermediate phenotype truly causes the disease, then

biomarker-related genetic variants should also be associated with the disease after taking into account the size of the effect of the variant on the intermediate phenotype and the size of the effect of the intermediate phenotype on the disease. This concept is similar to a randomized clinical trial, except here randomization to the exposure (a genetic variant) occurs at meiosis, when genotypes are randomly assigned to gametes. This assignment, being otherwise random, is also free of confounding as each allele as a 50% chance of being transmitted to the zygote. Problems with Mendelian randomization include that the technique is only as good as the estimates of the effect sizes of the variant on the intermediate phenotype and this phenotype on the disease. There is also an assumption that the DNA variant does not impact the disease by other means than the intermediate phenotype of interest (i.e. assumed to be non-pleiotropic). Certain biomarkers, therefore, are unsuited to the Mendelian randomization strategy. Several recent examples for CHD biomarkers are illustrative for this methodology.

**55.6.5.1 PCSK9.** Nonsense variants in PCSK9 that reduce LDL (intermediate phenotype) are observed to be associated with less CHD (disease) in African-American cohorts (44–46). Cohen and colleagues showed that in European populations a common missense variant is associated with 15% lower LDL levels and predicted a 47% lower risk of incident MI. This and other genetic data supports the role of LDL as a causative factor for CHD events.

**55.6.5.2 Lp(a).** The lipoprotein(a) (LPA) story is perhaps one of the better examples of this methodology. For years LPA had been a putative biomarker for CHD and it was unclear whether it was causative. Four major genetic studies have now shown that LPA is causally related to CHD. SNPs at the LPA locus in Europeans are clearly associated with increased LPA levels and higher LPA levels are associated with increased risk for CHD events (40–43,50). These studies showed that individuals with LPA variants causing higher Lp(a) levels are at higher risk for CHD events; a risk that was abolished with adjustment for Lp(a) levels.

**55.6.5.3 CRP.** For CRP, however, Mendelian randomization suggests that there is no causal relationship to CHD and that CRP is more likely a marker for disease *via* reverse causation or confounding. Three large Mendelian randomization studies have not been able to show an association between CRP variants and CHD or



**FIGURE 55-2** Suggested steps for novel GWAS loci. (Adapted from Stylianou, I. M.; Bauer, R. C.; Reilly, M. P.; Rader, D. J.; *Genetic Basis of Atherosclerosis: Insights from Mice and Humans*. Circ. Res. 2012, 110 (2), 337–355).

ischemic vascular disease (106,200,208). Despite this, the use of CRP as a marker for disease may continue to find clinical relevance as it appears to help stratify some patients and direct treatment (209). Nevertheless, finding the etiology or causative component among the inflammatory and risk biomarker pathways is a critical step in determining which specific biomarkers warrant trials of therapeutic interventions to determine efficacy in treating CHD. Mendelian randomization type-studies for other intermediate CHD biomarkers have been completed (e.g. apo E (200,210) and apoA5 (211)) or are ongoing (e.g. homocysteine and IL6/IL6R).

### 55.6.6 Copy Number Variation

Copy number variation (CNV) describes large polymorphic DNA deletions or duplications from 1 kb up to several MB in size, accounting for about 12% of human genomic DNA. CNVs have been associated with specific complex diseases including autism and schizophrenia, but not CAD/MI to date. Indeed, one large GWAS case-control study of early MI by the MI Gen Consortium found no relation of CNVs, defined by about 2 million SNP and CNV probes, despite positive associations of common SNPs with MI in the same sample (44). However, CNVs have been implicated in a vascular wall disorder, sporadic thoracic aortic aneurysm, and dissections (TAAD) that may have some overlap with coronary atherosclerosis pathophysiology (212,213). CNVs in cases with TAAD were enriched for genes regulating cell adhesion *via* interaction with the smooth muscle actin and myosin. Interestingly, mutations in pathway-related genes are reported in those with familial TAAD (212).

**55.6.6.1 Missing Heritability.** Ever since the genome was sequenced, we assumed that we would easily find and explain all of the heritability for CHD traits, which is estimated at 30–50%. In order to provide a similar discrimination as current risk prediction models, it is estimated that at least 20% of the heritability needs to be explained with additive genetic information (214). However, with the totality of information from current approaches and studies only perhaps 10% of the heritability of CAD/MI has been explained (85).

There are several possible reasons for this. First, rare mutations are not covered in GWAS, which focus on common variants. It is these rare variants at known and to-be-discovered risk loci that may impart more severe phenotypes and contribute collectively to a more significant proportion of heritability. Usually these alleles will impart much higher ORs, of disease in those that carry them than the ORs of 1.1–1.3 observed with GWAS-identified common variants. Ongoing and future studies using exome and whole genome sequencing in CHD will identify rare functional variation and with that information we can determine the true contribution to heritability of rare vs. common alleles at risk loci. Second, gene–gene and gene–environment interactions are not accounted for in most

current approaches and likely make up a significant component of CHD risk. Some individuals may be more genetically predisposed to develop CHD if they smoke compared to others. Third, there may be additional unmeasured complex heritability based on tissue-specific epigenetic and transcriptomic regulation, known to have a heritable basis (215). Indeed, the contribution of post-translational proteomic and proteoglycomic modification to heritability of disease traits remains to be studied. Finally, the degree of heritability of CHD may have been overestimated based on older less-powerful datasets and less-sophisticated methodologies. However, it remains likely that at least 30% of CHD traits have a heritable basis.

## 55.7 GENETIC RISK SCORES AND PREDICTION ALGORITHMS FOR PERSONALIZED MEDICINE

A primary goal of determining causative genetic variants associated with CHD is to be able to generate a risk prediction score or algorithm to help personalize CHD risk and inform decisions on treatment. Although often touted as the inevitable product of recent GWAS efforts, this aspiration is arguably the most challenging and will follow the more immediate successes, that is the transformation of our understanding of the causes and pathophysiology of complex CHD and the development of therapeutic programs targeting specific genes.

To date, several nascent attempts have been made to augment traditional risk scores like the Framingham risk score (which does not include family history), or Reynolds risk score (which includes family history and CRP data), as these do not always accurately predict CHD events. For a prediction scheme to be truly successful, clinicians need also be able to act upon the increased risk. For example, moving a high-risk individual to an even higher risk category may not change current treatment regimens, while moving someone from low to intermediate or intermediate to high risk may change treatment and outcome.

Attempts to incorporate the most striking finding from GWAS, the 9p21 locus, into risk predication models have been disappointing. As judged by the C statistic, the addition of 9p21 data to traditional risk factors in one study by Paynter and colleagues did not improve risk discrimination (216). A separate study within the ARIC cohort did show a small increase in the ROC with addition of the 9p21 allele which reclassified 12% of individuals in the low to moderate risk score categories (217). However, one common allele with small effects on a complex trait may add little in predictive modeling. As noted above, some estimates suggest that genetic data accounting for at least 20% of the heritability will have to be used in order to provide substantial discrimination in risk prediction models (214). Thus, use of combinations of independent genetic risk data through additive scores may be

required to improve risk prediction and discrimination at the population level.

Kathiresan and colleagues looked at genotype score combining data for nine (mostly common) SNPs associated with LDL-C and HDL-C and found that this score was an independent predictor of incident CHD in a prospective cohort study after adjustment for traditional RF including plasma lipids themselves (44). However, the score did not improve risk discrimination and only modestly improved risk reclassification beyond standard risk factors. This same research group examined a genetic risk score of 13 common variants, identified by case-control GWAS of MI and CAD, in prospective studies of MI and found small improvements in ROC, discrimination, and reclassification (218). It should not be surprising that we are unable to improve substantially on CHD risk prediction based on current knowledge because, as noted above, only a small portion of the heritable component of CHD has been identified.

In the coming decade, however, there should be exponential advances in risk prediction and personalized preventive medicine. Additional common risk alleles undoubtedly will be identified through even larger and more sophisticated GWAS and candidate genes studies and we have barely started the process of identifying rare, high-impact causal alleles in the existing genomic regions as well as novel CHD loci. In particular, knowledge of rare functional alleles is likely to greatly enhance complex disease prediction and discrimination. Lack of this information is problematic for two reasons. First, inclusion of such data should improve risk prediction and discrimination. More perniciously, use of common alleles without rare allele data introduces substantial potential for measurement error whereby many people with common “risk” alleles that are markers for less frequent disease causing variants are not at any increased risk for CHD if they do not carry the actual rare causal variant.

Current approaches will continue to be limited until such time as we have a more complete picture of all rare and common causal variants across the genome. Such data will emerge through large-scale exome and whole-genome sequencing projects but the task of proving which of the rare alleles are causal remains daunting without a clear, scalable solution. Further, it is possible that the addition of individualized tissue-specific (of greatest relevance to the disease) epigenetic, transcriptomic, proteomic, and other -omic dynamic profiles may add greatly to the assessment of individualized risk over the lifespan of an individual. These developments may evolve rapidly. More likely, however, it will take decades to unlock the potential for genomics to improve personalized medicine.

## 55.8 SUMMARY AND FUTURE DIRECTIONS

Much of the last decade has seen a fundamental shift in the genetic approaches to the study of complex human

diseases. At the start of the last decade, DNA chips and arrays were just beginning to emerge based on the HAPMAP project and nascent knowledge of the human genome. At that time, high-throughput sequencing technologies were still conceptual. Thus, technology and genomic knowledge limited progress. Over the last few years, we have seen the widespread application of GWAS, directly genotyping up to a million SNPs in each individual, in increasingly larger CVD studies. This has led to discoveries of many new CHD genes and the current transformation in how we understand and study atherosclerosis and its clinical complications. However, these are still the early stages in this scientific and clinical journey. Up to now, the GWAS approach has focused on less than 1% of the human genome, which has been biased toward common less-impactful variation and applications have been relatively crude with focus on main effects in case-control studies of end-stage clinical outcomes.

In order to advance clinical practice in terms of therapeutic advances and personalized risk determination, huge genomic efforts as well as mechanistic and translational science needs to be performed. The focus of the next few years will be whole exome sequencing (to identify casual variation in the “lower hanging fruit” of coding sequence), whole genome sequencing (to identify causal variation in the non-coding regulatory genome), and perhaps larger-scale incorporation of whole-transcriptome and epigenome data (to layer on top the tissue/cell specific manifestation of genomic regulation). Moderately sized whole-exome projects in MI and CHD traits are already a reality through NIH-NHLBI-funded efforts such as the Exome Sequence Project of Early Onset MI (ESP-EOMI) and related traits, which should report data in 2013.

Given the large amount of existing and emerging data on CHD loci and genes, there is great need for higher throughput functional modeling in human and animal systems. Scaling rodent and other humanized models of atherosclerosis to provide rapid screening of genes at a given locus and to define the direction of impact of LOF on disease is essential. Yet, animal models may fail to recapitulate the human reality or prove challenging to model the human state as appears to be the case for several emerging loci (9p21, CXCL12, ABO, and others). Thus, there is great need for tools to interrogate tissue-specific function in cells that are directly relevant to the human disease process including endothelial cells, vascular smooth muscle cells, and macrophages as well as hepatocytes and adipocytes. The emergence of human induced pluripotent stem cells (hiPSC) reprogramming technology (219–222) and the ability to differentiate hiPSC to specific somatic cells (223–225) provides the potential to address cell specific function and mechanisms in human for proven common and rare CHD genotypes. It remains to be seen if hiPSC will serve as reliable models, if epigenomic and transcriptomic programs can be fully manipulated to execute full reprogramming and



subsequent complete somatic differentiation, and if full reprogramming is required to recapitulate true primary cell functions (226,227).

The next few years have the potential to fundamentally change current model of human atherosclerosis. The use of high-throughput sequencing, scalable functional studies as well as creative clinical studies, and use of families will define the full spectrum of causal genes and variants. Understanding the effect of LOF and GOF for specific CHD risk alleles will be critical to comprehensive clinical translation in personalized CHD risk and therapeutics. The coming years of human CHD genomics promise to be even more exciting and transformative than the last several when this chapter was previously revised.

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### Biographies



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Dr Qasim is the author of several peer-reviewed manuscripts in this area. Along with Dr Muredach Reilly, he co-authored the previous edition of the chapter on the Genetics of Atherosclerosis in this textbook. He has written a handbook of medicine for medical students and residents, *The Hitchhiker's Guide to Internal Medicine*, and is the author of several chapters in cardiology texts relating to non-invasive imaging and general cardiology. He is also the founder of CardioQnA, a company which centers on medical education, as well as the website [www.echocardiographer.org](http://www.echocardiographer.org), a learning resource and image library for echocardiography.



**Muredach P Reilly** is an associate professor of Medicine and Pharmacology in the Perelman School of Medicine at the University of Pennsylvania (Penn). He earned his medical degree at University College Dublin, Ireland and completed training in medicine and cardiology as well as epidemiology at Penn. He leads a translational cardio-metabolic research program at Penn with specific focus on unbiased genomic discovery approaches, including GWAS, to CHD traits and functional studies of newly identified loci for CHD. His clinical specialty is in Preventive Cardiology and oversight of acute coronary care. He maintains multiple NIH awards for his cardio-metabolic research program and has published over 100 peer-reviewed research articles as well as many articles relating to genomics of CHD and its metabolic risk factors.



# CHAPTER

# 56

## Disorders of the Venous System

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### 56.1 INTRODUCTION

The vasculature is the first organ system to develop during embryogenesis, delivering nutrients, growth factors, and oxygen to tissues and removing waste products. It is therefore essential for development. The vascular system comprises four major compartments (arterial, capillary, and venous blood vessels, and lymphatic vessels), which have in common the presence of endothelial cells (ECs) forming the innermost layer. In blood vessels, the endothelial tubes are supported by a layer of vascular smooth muscle cells (vSMCs) and/or pericytes (together called mural cells) of variable thickness, whereas only a few SMCs are present in certain areas of large lymphatic vessels.

The mechanisms that give rise to the mature vascular network are complex. These developmental processes, termed vasculogenesis, angiogenesis, and lymphangiogenesis, involve a plethora of signaling molecules and their receptors at various time-points. The best known are the angiopoietins (ANGPTs), ephrins, fibroblast growth factors, platelet-derived growth factor, transforming growth factor beta (TGF $\beta$ ), and vascular endothelial growth factors. As suggested by numerous murine models showing abnormal vascular development, many other factors, including those participating in cell–cell and cell–matrix interactions, are probably also involved.

Vascular anomalies result from localized defects that occur during vascular development (1,2). They are subdivided into vascular tumors (mainly hemangiomas) and vascular malformations, which in turn are subcategorized according to the type(s) of vessel(s) altered (3,4).

Thus, capillary, venous, arteriovenous, lymphatic, and combined malformations can be distinguished.

### 56.2 THE VENOUS SYSTEM

Veins collect blood from the capillary network and contain about 75–80 % of the total volume of blood in the body. These vessels are larger than arteries, but their walls consist of a thinner layer of vSMCs and a thicker adventitia. The presence of valves allows for proper orientation of blood flow in veins. Venous flow is passive, essentially mediated by physical movements of the body and the aspirating effect exerted by the heart. Some veins, especially in the legs, are exposed to high pressure due to gravitational forces and subject to permanent distortions (i.e. varicose veins).

During vascular development, a primary capillary plexus is formed early on by in situ differentiation of hemangioblasts, originating from the mesoderm, into ECs that assemble to form endothelial tubes (vasculogenesis). In response to the hemodynamic forces of local blood flow, this plexus is progressively remodeled during angiogenesis to form the three types of blood vessels. At the cellular level, the “venous” phenotype of the ECs is determined very early, even before the vessel is committed to a vein (5). In the trunk of the embryo, the venous system is first present as symmetrical bilateral vessels. Then, the left part regresses progressively while the right-sided veins evolve into the superior and inferior vena cava. Defective remodeling may lead to retention of embryonic veins, such as persistence of the left superior vena cava (6,7), or the right umbilical vein (8).



Among abnormalities of the veins, a persistent saphenous vein is sometimes observed in Klippel–Trenaunay patients (9).

## 56.3 DISORDERS OF THE VENOUS SYSTEM

Venous malformations (VM), the main focus of this chapter, are the most frequent malformations seen in interdisciplinary vascular anomaly centers. Varicose veins and varicosities are discussed with the disorders affecting lymphatic vessels (Chapter 50). Malformations with capillary or arteriovenous components are described in Chapter 57. Neither will we discuss the diagnostics and management of acute and chronic venous disorders, which have been extensively reviewed elsewhere (10).

### 56.3.1 Glomuvenous Malformation

The most frequent inherited VM, glomuvenous malformation (GVM; Online Mendelian Inheritance in Man (OMIM) 138000) accounts for roughly 5% of venous malformations (VMs) (11), and is often improperly called “glomangioma” or “(multiple) glomus tumor” although it is not a tumor and never becomes malignant. GVM is characterized by the presence of a variable number of “glomus cells” around distended venous channels (12,13). Similar rounded cells are present in paraganglioma (OMIM 115310, 168000, and 605373) and (subungual) solitary glomus tumor. All are sometimes referred to as “glomus tumor.” However, paragangliomas are tumors of the parasympathetic ganglia, most commonly located in the head and neck area, caused by mutations in three different subunits of the succinate dehydrogenase enzyme complex, namely SDH-B, SDH-C, and SDH-D, and more rarely in SDHAF2 (14–19). Solitary glomus tumors are usually subungual painful lesions histologically characterized by the presence of glomus cells, but without important vascular component (20).

GVM segregates as an autosomal dominant disease, with incomplete penetrance and variable expressivity (13,21–24). Frequently, a single individual in a family is more severely affected and is brought to the medical attention, whereas most of the other affected individuals have small lesions and never consider treatment (25). As penetrance is below 100% and many individuals have tiny asymptomatic lesions, it is likely that the inherited nature of patients’ lesions is overlooked.

Clinical distinction of GVM from VMs and multiple cutaneous and mucosal venous malformations (VMCM) can be difficult in patients with few small lesions and without familial history of the disease (25). Yet, a series of clinical criteria and a few biomarkers have been defined (26). A GVM is usually raised, nodular, present at birth, and slowly expands during childhood (Figure 56-1). It is often multifocal and hyperkeratotic. Its color varies from pink to purplish-dark blue (25). Sometimes, it is flat and purple



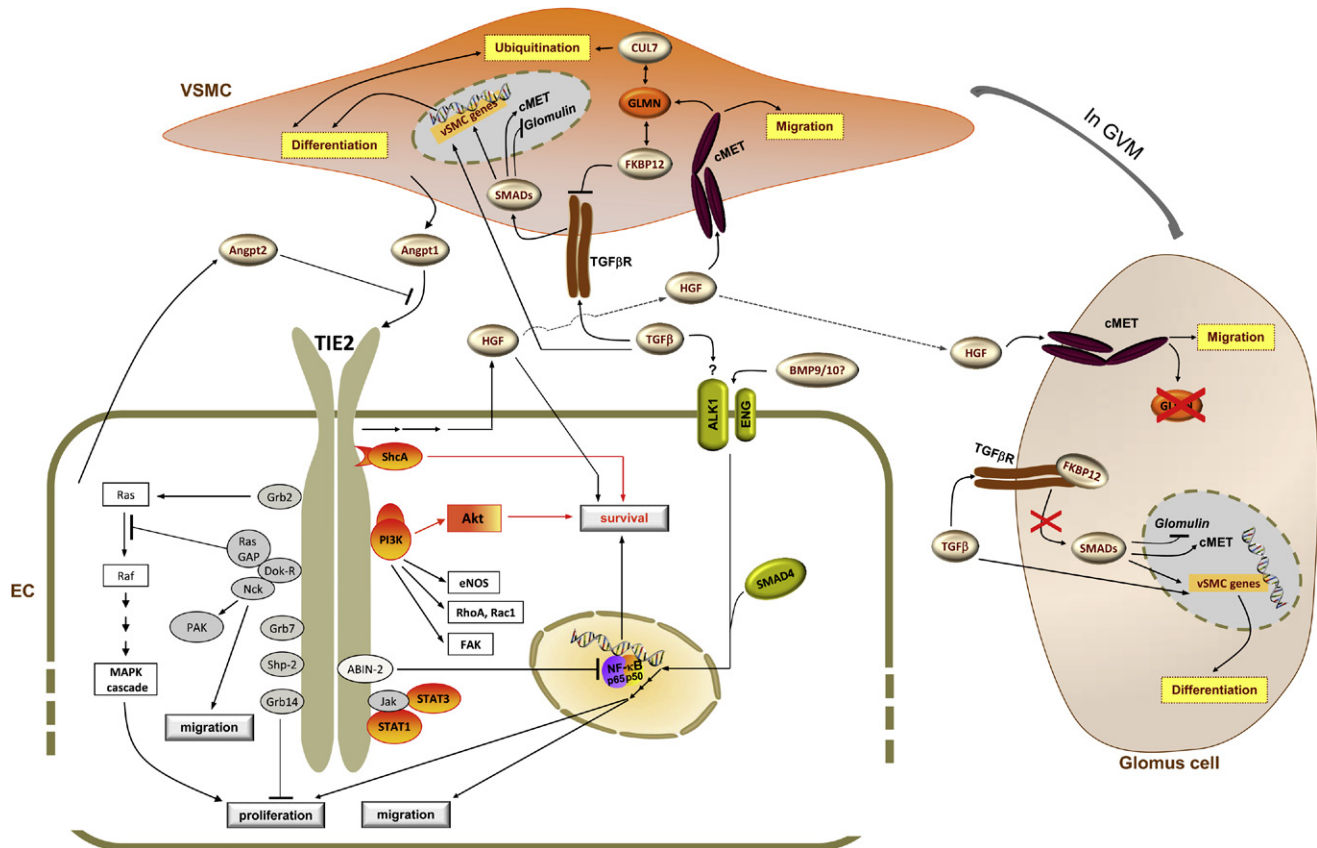
**FIGURE 56-1** Venous anomalies: GVM on leg; cutaneous and mucosal venous malformations (VMCMs) on lips; sporadic VM on foot; HCCVM on arm; multiple spindle cell hemangioendotheliomas and enchondromas on hand of patient with Maffucci syndrome; intestinal and cutaneous VMs of patient with BRBN syndrome; CLVM on legs of patient with KTS (left side more affected).

in color, especially in the newborn. This plaque-like GVM usually darkens with time (27). GVMs are mainly located on the extremities, involve skin and subcutis, and are often painful on palpation. They cannot be completely emptied by compression. Contrary to a VM, a GVM is rarely encountered in the mucosa (25). Patients with GVM have normal mental and physical development, normal blood cell counts, coagulation, liver and renal function, electrolytes, plasma proteins, and urinalysis. They do not have intestinal hemorrhage, which distinguishes them from patients suffering from blue rubber bleb nevus (BRBN) syndrome (OMIM 112200) (28). D-dimer level is normal in GVM (29). In two patients, thoracic-plaque-type GVM was associated with pleural effusion (30).

The alterations in the mutated gene seem to cause specifically abnormal cutaneous vascular development. Currently, the best therapy is surgical resection of the entire GVM, which is curative, and is facilitated by the non-infiltration of GVM in underlying tissues (25). Alternatively, sclerotherapy can be used for a GVM that is soft on palpation.

GVMs are caused by loss-of-function mutations in the gene encoding glomulin (*GLMN*); the function of the protein is unknown (31). Glomulin has 594 amino acids. So far, 40 distinct mutations have been reported in 162 families (22,31–34). Sixteen of the mutations were detected in several families, accounting for almost 85% of the pedigrees with a *GLMN* mutation. The most frequent mutation is *c.157\_161del* (a.k.a. *157delAAGAA*), present in 72 kindreds (44.4%). Screening for these shared mutations first accelerates genetic diagnosis (22).

There is no correlation between the position of a mutation in the *GLMN* gene and characteristics of the disorder, such as the number of glomus cells in the lesion, the extent of the lesion, or the number of lesions. Yet, for the same mutation, the expressivity is variable



**FIGURE 56-2** Signaling pathways involving glomulin (GLMN) and TIE-2 in normal and pathological endothelial and vSMCs. Lack of GLMN signaling results in aberrant differentiation towards glomus cells. Pathways chronically activated by *TIE-2* mutations highlighted in orange; with the exception of STAT1, all are also activated by wild-type receptor, upon stimulation.

from patient to patient (22). Several observations suggest a paradigmatic mode of inheritance for GVM, instead of a simple autosomal dominant transmission. GVM has an age-dependant variation in penetrance, which reaches its maximum (92.7%) by 20 years of age (31). Unaffected mutation carriers have been identified in GVM families and affected individuals develop new lesions by time, albeit they stay small (25). This multifocality can be explained by the occurrence of double-hit mutations in glomulin (31,35).

In GVMs, the mural glomus cells are round or polygonal, instead of elongated like the normal vSMCs (12,36). Glomus cells stain positively for smooth muscle  $\alpha$ -actin and vimentin, whereas they are negative for desmin, von Willebrand factor, and S-100 neuronal marker (36). During murine development, glomulin RNA was first detected at E10.5 dpc in cardiac outflow tracts, and later, strong expression was seen in vSMCs (37). In contrast, glomus cells do not express the late markers of vSMC differentiation glomulin and smoothelin-b, whereas two earlier markers, smooth muscle myosin heavy chain and h-caldesmon, were detected (35). Thus, it seems that glomus cells have deviated in their differentiation process due to lack of glomulin expression.

Glomulin has no known motif or conserved domain. It may act synergistically to control both TGF $\beta$  and

hepatocyte growth factor (HGF) pathways (Figure 56-2), which are crucial for vSMC differentiation (38,39). In vitro, glomulin interacts with FKBP12 and might impede its binding and inhibition of TGF $\beta$  receptor signaling (40,41). FKBP12 also inhibits downstream signaling of mTOR (42,43). Complete loss of glomulin in GVMs could thus result in inhibition of TGF $\beta$  and mTOR signaling. Glomulin also interacts with the intracellular part of the HGF receptor c-Met. Upon HGF binding, glomulin is tyrosine-phosphorylated, released, and induces phosphorylation of p70S6-kinase (41), thereby controlling protein synthesis (42,43). As HGF triggers downstream signaling mediating vSMC migration (39), lack of glomulin in GVMs likely alters this signaling. Glomulin was also reported to interact with Cul7, with which it forms an SCF-like complex (44). These complexes are E3-ubiquitin-ligases, determining the specificity for the substrate to ubiquitinate. Thus, glomulin–Cul7-containing complex may regulate protein degradation in vSMCs, and affect their differentiation.

### 56.3.2 Inherited Venous Malformation

A second, less common inherited VM is the autosomal dominant multiple cutaneous and mucosal venous malformation (VMCM; OMIM 600195), which accounts

for another 1–2% of all VMs (11). It is characterized by multiple small, compressible blue lesions on the skin and mucosa, commonly located in the cervicofacial region and the limbs, and less often on the trunk (Figure 56-1). Histologically, distended venous channels are lined by a single endothelial cell layer surrounded by sparse, irregularly distributed vSMCs (45). VMCM is caused by mutations in *TEK*, located on chromosome 9p21 (45–47). *TEK* (also called TIE-2) is an endothelial cell tyrosine kinase receptor that binds the angiopoietins ANGPT 1, 2, and 4 (Angpt3 in mice) (48–50). The resulting modulation of downstream signaling molecules, including PI3K/Akt and mitogen-activated protein kinase (MAPK) (51,52), is critical to endothelial cell survival and function (Figure 56-2) (49,53,54). Eight intracellular mutations have been identified, which cause widely variable ligand-independent hyperphosphorylation of the receptor in vitro (45–47). The most commonly identified change, R849W (10/17 families reported), was shown to be accompanied by a somatic loss-of-function of the wild-type receptor in one lesion, locally abolishing some putative protective/competitive effect of the latter (55). As in the case of GVM, this likely explains why the germ line mutations do not cause generalized vascular abnormalities despite being ubiquitous, instead resulting in highly focal malformations only where their effects are compounded by an additional somatic hit.

### 56.3.3 Sporadic Venous Malformation

By far the most frequent (95%) of the VMs are those that occur sporadically, and therefore, unpredictably. Histologically similar to VMCMs, sporadic VMs (OMIM 600195) are usually much larger, single lesions that affect the skin and mucosa (Figure 56-1), but can also infiltrate underlying tissues in various organ systems (11). They can cause significant morbidity due to their size, localization, or expansion, making them the malformations most frequently treated at centers specializing in vascular anomalies (11). In about 42% of patients, localized intravascular coagulopathy, characterized by elevated levels of D-dimers, is observed, and correlates with the size and depth of lesions and the presence of phleboliths (29,56). While compression, sclerotherapy, and surgical removal are currently employed, these approaches can be problematic and ineffective, depending on the nature and location of the lesion, and regrowth is common (26). More targeted treatments, guided by insights into the pathogenic mechanisms involved, are therefore sought.

An appreciation of the widespread role of somatic events in inherited vascular malformations prompted researchers to focus on surgically resected lesions. This led to the discovery that somatic intracellular mutations in *TEK* cause at least 50% of sporadic VMs (55). As with VMCM, these are characterized by ligand-independent hyperphosphorylation of the receptor in vitro. Amongst the changes is the frequent L914F (69%

of mutation-positive samples), which has never been observed germ line among VMCM families, suggesting it is lethal when ubiquitous (55). The somatic changes also include a series of double mutations that occur in *cis* (on the same allele) (55,57). The constituent single mutations include three that cause premature truncation in the C-terminal tail domain, yet cause receptor phosphorylation (57), likely by abolishing its customary inhibitory fold. Double *cis*-mutations seem to predispose to multifocal sporadic VMs. Patients with this rare form have the same mutations in their multiple lesions, although they are undetectable in the blood (55) (Soblet et al., unpublished). This would suggest the multiple VMs were all seeded by a common cellular progenitor in which the somatic mutation had occurred.

The mechanism by which chronic TIE-2 activation causes VMs is unknown. R849W, being the first mutation identified, is the most studied, and has been shown to have a Shc- and pAkt-dependent pro-survival effect on ECs (Figure 56-2) (58–60). While this could account for why they survive despite the lack of support and cross-talk with smooth muscle cells, it does not explain the defective recruitment of the latter. R849W does not seem to influence proliferation or migration in vitro, but does lead to the formation of more unstable tubes as compared with cells bearing the wild-type receptor (58). It causes increased basal as well as lipopolysaccharide-induced phosphorylation and activation of STAT1, an inflammatory mediator (58,61). What this implies for VM-pathology is unknown. Inherited R849W and somatic L914F both affect the subcellular localization of the receptor in overexpressing human umbilical vein endothelial cells (HUVECs) (55). While R849W is evenly distributed on the cell surface, similarly to the wild-type receptor, L914F seems to be sequestered in the Golgi and ER, where it is strongly hyperphosphorylated. It is not exclusively perinuclear in distribution, however, as evidenced by the fact that a proportion translocates to the retracting edge of cells in response to ANGPT 1 (55). This movement is much more pronounced in the case of R849W, which is also more highly concentrated and phosphorylated in long retracting fibers than the wild-type receptor (55). The two common changes, therefore, seem to have distinct if overlapping functional effects. The lack of any clear correlation between patient phenotype and the strength of receptor phosphorylation (55,57) suggests that pathogenesis results, not merely from a quantitative increase, but also qualitative abnormalities in signaling downstream of TIE-2, potentially due to its localization in compartments with inappropriate interacting molecules.

### 56.3.4 Hyperkeratotic Cutaneous Capillary–Venous Malformation

Multiple cutaneous- and capillary-VMs sometimes occur in association with cerebral cavernous malformation



(CCM; OMIM 116860), described in detail in Chapter 57. Characterized by distended capillary-like channels without any intervening brain parenchyma, CCM can be asymptomatic, but often causes headaches, focal neurological defects, seizures, or hemorrhage (62). A systematic analysis of the cutaneous lesions associated with familial forms of the anomaly in a large cohort showed that hyperkeratotic cutaneous capillary–venous malformations (HCCVMs; OMIM 166860) are exclusively associated with mutations in *CCM1* (*KRIT1*) (63). Nodular VMs are associated with mutations in *CCM3* (*PDCD10*), and sometimes *CCM1*, albeit less frequently (63). Mutations in the third gene known to cause CCM, *CCM2* (malcavernin/*MGC4607*), are rarely, if ever, associated with VMs. HCCVMs are plaque-like, irregular, black or crimson lesions, with the ECs of the venous channels surrounded by two or more layers of smooth muscle cells (Figure 56-1). Nodular VMs appear more similar to multifocal sporadic VMs (64). Histologically, they are similar to the cerebral lesions of CCM, and consist of several thin-walled channels, packed together and surrounded by a fibrous layer containing smooth muscle cells (63).

### 56.3.5 Other VM-Associated Syndromes

VMs also arise in the context of sporadic syndromes such as BRBN (OMIM 112200) and Klippel–Trenaunay (OMIM 149000), the etiologies of which are unknown. BRBN is characterized by multiple small, rubbery cutaneous VMs, often on the plantar surfaces (Figure 56-1). Their association with gastrointestinal VMs, which can cause bleeding and anemia, forms the basis of the

diagnostic criteria (11,26). Klippel–Trenaunay syndrome (KTS), a detailed description of which is included in Chapter 50 (Hereditary Disorders of the Lymphatic System), also involves the venous component of the vascular system. It is characterized by capillary–lymphatic–venous malformations (CLVMs) on a hypertrophic extremity, most often a lower limb (Figure 56-1) (11,65). Abnormal capillary–venous channels in the form of spindle cell hemangioendotheliomas are seen in Maffucci syndrome (OMIM 166000), which also involves multiple enchondromas, benign tumors of the cartilage (Figure 56-1). There is a high rate of malignant transformation, most often to chondrosarcoma (11). *PTHR1*, the gene that can be mutated in enchondromatosis in Olliers disease (66), and *PTPN11*, which can be mutated in metachondromatosis (67), have not been found to be mutated in Maffucci syndrome. Genome-wide single nucleotide polymorphism mapping arrays, performed on the blood and tissue from patients, have revealed several somatic deletions and losses of heterozygosity in the tumors, although none was common to all or most samples (68).

## 56.4 CONCLUSION

The discovery that loss of glomulin function or aberrant TIE-2 activation causes more than 50% of all VMs makes known or novel inhibitors of the related pathways potentially useful therapeutic candidates. Further insights into the pathways dysregulated by TIE-2, *GLMN*, and other molecules that cause venous disorders could reveal additional suitable targets for these anomalies (see Table 56-1 for a summary). The availability of good animal models

**TABLE 56-1 Disorders Affecting the Venous System**

Malformation/ Syndrome	Inheritance <sup>a</sup>	OMIM	Locus	Mutated Gene	Features
GVM	AD	138000	1p21–22	<i>GLMN</i>	Raised hyperkeratotic bluish nodules on skin, often painful; enlarged venous channels with rounded vSMCs (i.e. glomus cells)
Multiple cutaneous and mucosal venous malformation (VMCM)	AD	600195	9p21	<i>TIE-2 (TEK)</i>	Soft bluish masses on skin and mucosa, often multiple; enlarged venous channels with patchy vSMCs
Sporadic VM	SP	600195	1p21–22	<i>TIE-2 (TEK)</i> somatic	Same as VMCM, usually a single large lesion; no family history
BRBN syndrome	SP	112200	?	?	Resembles VMCM, associated with gastrointestinal lesions that can bleed
KTS	SP	149000	?	?	CLVMs on hypertrophic extremity (usually lower limb)
Maffucci syndrome	SP	166000	?	?	Abnormal capillary–venous channels associated with multiple enchondromas and frequently chondrosarcomas
Cerebral cavernous malformation (CCM) with nodular VM	AD	116860	7q11–22	<i>KRIT1</i>	Nodular VM associated with brain, spinal cord, retinal, and cutaneous dilated channels with loose ECs, causing headaches, seizures, and hemorrhages
HCCVM	AD	116860	7q11–22	<i>KRIT1</i>	Same as CCM, with plaque-like hyperkeratotic cutaneous lesions

<sup>a</sup>AD, autosomal dominant; SP, sporadic; ?, unknown.



of (glomus) VMs, currently lacking, would allow for the testing of potential therapies for their safety and efficacy.

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## RELEVANT WEBSITES

Human Molecular Genetics Laboratory: [http://www.deduveinstitute.be/genetics\\_of\\_human\\_diseases.php](http://www.deduveinstitute.be/genetics_of_human_diseases.php).  
 Center for Vascular Anomalies: <http://www.saintluc.be/en/services/vascular-anomalies/index.php>.  
 Vascular Anomaly Database: <http://www.icp.ucl.ac.be/vikkula/VAdb/>.

## Biographies



Dr Miikka Vikkula, MD, PhD, obtained his MD at the University of Helsinki in 1992 and his PhD in molecular genetics, with a thesis on collagens and cartilage disorders, in 1993 (Prof. Leena Peltonen). He was a research associate at the Department of Cell Biology at Harvard Medical School (Prof. Bjorn R Olsen) between 1993 and 1997, during which time he became interested in vascular anomalies. With his wife, Prof. Laurence Boon, MD, PhD, plastic surgeon, the couple discovered the gene for familial venous malformation in 1996. They settled in Brussels in 1997, where Dr Vikkula started to develop his own laboratory as a guest investigator at the de Duve Institute between 1997 and 2000. He obtained a “docentship PhD” in human molecular genetics at the Université catholique de Louvain (UCL), Brussels, in 2000, and was thereafter nominated assistant professor at the Faculty of Medicine in UCL. He has been a full member of the de Duve Institute, and a member of the directorat since 2004. He was nominated Professor of Human Genetics at the Université catholique de Louvain on 1 October 2008. Prof. Vikkula is well known internationally as a major contributor to the understanding of the molecular basis of lymphatic and vascular anomalies and lymphedema, with many high impact publications. He has been an invited speaker at numerous major scientific meetings. He has also received numerous honors and awards from a number of prestigious organizations for his academic achievements; most recently, the Lacroix Prize on cardiovascular disorders, 2010; the Pfizer Scientific Award, 2006; and the Eugène de Sommer Scientific Award, UCL, Belgium, 2006.



Dr Nisha Limaye obtained her PhD at the University of Texas Southwestern Medical Center in Dallas, Texas, in the laboratory of Dr Edward K Wakeland. Her work focused on the genetic basis of pathogenesis in a murine model of the complex autoimmune disease systemic lupus erythematosus. She moved to Brussels, Belgium, in 2005, and became interested in the genetic underpinnings of vascular anomalies in the course of her postdoctoral work in the laboratory of Prof. Miikka Vikkula, where she remains as an associate member of the de Duve Institute in Brussels, Belgium. In 2010, she was nominated a “Chercheur Qualifié”, becoming a permanent member of the National Research Funding Agency of Belgium.





**Pascal Brouillard**, PhD, graduated in biochemistry at the University of Liège, Belgium, in 1997. The same year, he started as a PhD student in the Laboratory of Human Molecular Genetics (Prof. Vikkula), de Duve Institute, Université catholique de Louvain (UCL), Brussels, Belgium, where he studied vascular anomalies. More precisely, he discovered the glomulin gene, mutations in which cause glomuvenous malformations. Moreover, he found that the lesions need a second-hit mutation for development. It was the first time such a mechanism was reported in vascular anomalies. He obtained his PhD in 2003. Since then, he has pursued his postdoctoral studies in the same laboratory, mainly focusing on the generation of various glomulin mouse models. Between 2003 and 2007, he was a research associate of the Fonds National de la Recherche Scientifique (FNRS, Belgium), and is now a research associate of UCL. Pascal was laureate of the FBBF Belgian Biotechnology Fund Research Award in 2005, and is a member of the Belgian Society of Human Genetics and of NAVBO.



**Professor Laurence Boon**, MD, PhD, obtained her MD at the Université catholique de Louvain, Brussels, Belgium, in 1989, and became a plastic surgeon in 1996. During her training in plastic surgery, she spent two years at the Children's Hospital, Boston, and the Harvard Medical School, under the supervision of Professor John B. Mulliken, to specialize in vascular anomalies. At the same time, she was a research associate at the Department of Cell Biology, and with her husband, discovered in 1996 the gene for familial venous malformation. Since 1996, she has been in charge of the Center for Vascular Anomalies at the University Hospital of St Luc, Brussels, Belgium. She obtained a PhD in plastic surgery in 2003, with a thesis on "New Clinical and Molecular Genetic Criteria for Hemangiomas and Venous Malformations."

Prof. Boon is well known internationally for the management of vascular anomalies and as a major contributor to the understanding of the molecular basis of vascular anomalies, with several high impact publications, reviews, and chapters on vascular anomalies. In 2003, she was invited to give the Sidney Hurwitz Memorial Lecture at the American Pediatric Dermatology Meeting in Seattle, USA. Since 2008, she has been the chair of the scientific committee of the ISSVA (International Society for the Study of Vascular Anomalies). She has been an invited speaker at numerous major scientific meetings. She continues to develop the multidisciplinary Center for Vascular Anomalies in Cliniques universitaires St Luc in Brussels. The Center will celebrate its 20th anniversary in 2011. Prof. Boon is involved in several research projects aimed at elucidating the pathophysiology of these anomalies as well as in clinical trials of new medications for vascular anomalies.



# CHAPTER

# 57

## Capillary Malformation/ Arteriovenous Malformation

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### 57.1 INTRODUCTION

Vascular anomalies are a heterogeneous group of disorders divided into tumors and malformations based on clinical, radiologic, and immunohistochemical studies. Vascular malformations are considered to be localized defects of vascular morphogenesis. They are present at birth, even if not always visible, and based on the type of the affected vessel, they are classified into capillary, venous, arterial, and lymphatic malformations. Sometimes, more than one vessel type is involved (combined vascular malformations), such as arteriovenous malformation, capillary–venous malformation, etc. Vascular malformations are in general sporadic, but familial forms exist, enabling genetic studies and the identification of genes involved in vascular morphogenesis, which can lead to proper genetic counseling and development of targeted therapies. This chapter reviews sporadic and familial capillary malformation with a special emphasis on cerebral cavernous malformation and capillary malformation–arteriovenous malformation. Hereditary hemorrhagic telangiectasia is discussed in Chapter 49.

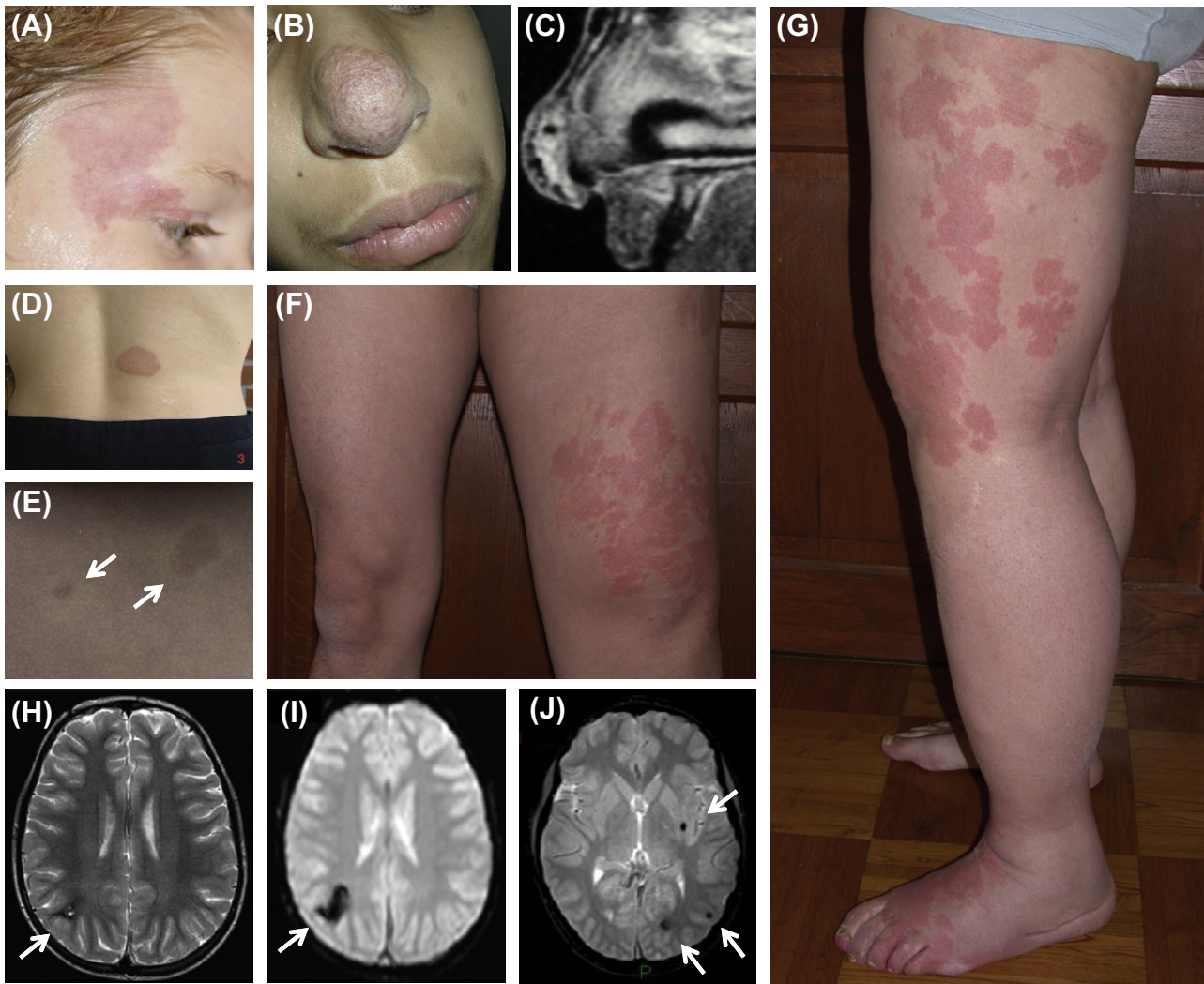
### 57.2 CAPILLARY MALFORMATION

Capillary malformation (CM) (Figure 57-1A) or “port-wine stain,” with an incidence of 3/1000 is the most frequent vascular malformation (1). Histologically, CM is formed of dilated capillary-like channels,

the number of which increases with age. It is usually an isolated, sporadic, solitary flat lesion, present at birth, and grows proportionately with the child and persists throughout life. CM is a slow-flow, homogenous lesion of variable size, with geographic borders, located mainly on the skin and sometimes on the mucosa, appearing as a red macula that darkens and often thickens with age. It is located on the head, neck, trunk, or limbs, and the underlying tissues (skin, fat, muscle, and bone) can be hypertrophic.

CMs are asymptomatic, but can generate important psychosocial distress. Often they do not require any treatment. When necessary for unsightly reasons, laser, mainly pulsed-dye, is the first line treatment. Laser treatment reduces the coloration in 75% of patients without modifying skin texture. It requires many sessions and necessitates general anesthesia in children and in adults with large CMs. When laser is ineffective, surgery is an option and it can also be used to reduce tissue hyperplasia.

CMs have to be differentiated from common nevus flammeus, present in up to 50% of newborns and located on the back of the neck, forehead, eyelids, or upper lip. Hypertrophic CM located on a limb has to be differentiated from Klippel–Trenaunay syndrome (capillary–lymphatic–venous malformation; Online Mendelian Inheritance in Man (OMIM) 149000; see Chapter 50) and Parkes Weber syndrome (capillary malformation, arteriovenous micro fistulas, and hypertrophy; OMIM 608355; see Section 57.4).



**FIGURE 57-1** Disorders of the capillaries. (A) Sporadic capillary malformation (CM). (B–G) Phenotypic variation in capillary malformation–arteriovenous malformation (CM-AVM): (B) Nose AVM and small CM on the left cheek; (C) T1-weighted MRI enhanced sequence of the AVM shown in B; (D and E) CM of CM-AVM—small multifocal CM with halo (arrows); (F and G) Parkes Weber syndrome in the lower extremity: patchy CM and hypertrophy. (H and I) sporadic cerebral cavernous malformation (CCM) lesion (arrow) in the parietal lobe: axial T2-weighted MRI sequence (H) and axial T2-weighted gradient-echo sequence (I). (J) Multiple supratentorial CCMs (arrows)—axial T2-weighted gradient-echo sequence.

Sometimes, a capillary blush can be observed on top of an arteriovenous malformation and can be misinterpreted as a CM.

### 57.3 STURGE-WEBER SYNDROME

Particular attention has to be paid to CM located on the territory of the first branch of the trigeminal nerve (V1), which in 10% of cases is part of Sturge–Weber syndrome (OMIM 185300). This is a sporadic, severe neurocutaneous disorder, with an estimated incidence at 1/50,000 and unknown etiology. Males and females are equally affected. It manifests as a unilateral capillary malformation present at birth and located on the forehead and the upper eyelid (V1), ipsilateral leptomeningeal capillary-venous anomaly, and ocular involvement. The capillary malformation can be bilateral and/or more

extensive, covering the territory of the maxillary (V2) and mandibular (V3) branches of the trigeminal nerve, and sometimes the trunk and the limbs. In rare cases, the CM can be absent.

Seventy-five percent of children with intracranial vascular anomaly develop seizures, most often before age 2 years, with a risk of contralateral neurologic deficit and learning difficulties. Gyral calcifications can be observed. The major ocular complication is glaucoma, occurring in more than 50% of patients, requiring regular ophthalmologic follow-up throughout life, but especially in infants. Management of epilepsy and glaucoma is an emergency. It is still questioned if prophylactic anticonvulsant treatment is recommended or not. Pulsed-dye laser can be used to treat the facial CM, once the seizures are controlled. In patients with intractable seizures, surgery (lobectomy) may be required.

Even if most CMs are sporadic, some are familial, as those seen in CM-AVM (capillary malformation–arteriovenous malformation), CCM (cerebral cavernous malformation) or HHT (hereditary hemorrhagic telangiectasia). These three conditions have an autosomal dominant transmission. The first two conditions are discussed here, whereas HHT is discussed in Chapter 49.

## 57.4 CAPILLARY MALFORMATION–ARTERIOVENOUS MALFORMATION

Linkage studies in families with inherited capillary malformations allowed the identification of a susceptibility locus on 5q (2,3) and subsequently the recognition of *RASA1* on 5q14.1 (OMIM 139150) as the mutated gene (4). About 130 families with heterozygous *RASA1* mutations have been identified until now (4,5). This autosomal dominant condition is characterized by multifocal CMs and fast-flow vascular malformations: arteriovenous malformations (AVM), arteriovenous fistulas (AVF) and Parkes Weber syndrome (Figure 57-1B–G). The condition has been named capillary malformation–arteriovenous malformation (CM-AVM; OMIM 608354).

The penetrance is high, at more than 95%, and de novo occurrence is around 30%. The expressivity is variable even within the same family and the prevalence at least 1/100,000. All the affected people have CMs, but in contrast to classical CM, CMs of CM-AVM are often multifocal and small, less than 1 cm, round or oval, randomly distributed, and frequently surrounded by a pale halo. New lesions can appear during childhood.

About 30% of individuals with *RASA1* mutations have, in addition to multifocal CMs, fast-flow vascular malformations located in or outside of the central nervous system (CNS). In contrast with hereditary hemorrhagic telangiectasia, no lung or liver AVMs/AVFs have been observed. Lesions in the CNS include intracranial pial AVM/AVF, vein-of-Galen aneurysmal malformation, and spinal lesions (4–8). Around 10–12% of patients have symptomatic CNS fast-flow lesions; they present with cardiac failure or neurologic signs usually early in life, at birth or, before the age of 6 years. Neurologic symptoms include migraine, hemorrhage, epilepsy, and focal neurologic deficit. In about 20% of patients with *RASA1* mutation, the fast-flow vascular malformation is located outside the CNS. Half are AVM/AVF on the head and neck region or extremities, involving cutis and subcutis and sometimes muscles and bones, and half have Parkes–Weber syndrome (PKWS; OMIM 608,355), which is characterized by a capillary blush on an extremity, multiple arteriovenous microfistulas, and bony and soft tissue hypertrophy.

CM-AVM was described only in 2003. Thus, understanding of the natural and clinical history is insufficient for developing guidelines for clinical management. Meanwhile, patients should be examined once a year by

a clinician aware of the phenotype and its complications (6). CMs are harmless lesions. In contrast to sporadic CM, CMs of CM-AVM do not respond well to pulsed-dye laser, although most of them are small and well tolerated. Fast-flow lesions in the CNS in CM-AVM patients seem to manifest early in life. Nevertheless, regular brain magnetic resonance imaging (MRI) is recommended until natural history is better understood. These lesions, as well as extracranial fast-flow lesions, require a multidisciplinary approach, as in isolated AVM and AVF. Patients with Parkes Weber syndrome should be treated conservatively; epiphysiodesis for leg length discrepancy should be avoided when possible as it can aggravate the fast-flow vascular malformation (9).

Most of the *RASA1* mutations are nonsense, splicing, or out-of-frame deletion/duplication mutations, leading to premature termination and suggesting loss of function. This is further supported by the recent observation of large genomic *RASA1* rearrangements in the context of a new microdeletion syndrome in 5q14.3 (10). These patients present with severe mental retardation and absent speech, hypotonia, stereotypic movements, epilepsy, cerebral malformations, and mild facial dysmorphism due to *MEF2C* haploinsufficiency (10–12). When *RASA1* is included in the microdeletion, multifocal CMs are an important clue for the diagnosis.

The pathophysiology of CM-AVM is unknown, but the number of lesions and their localized nature could indicate that a somatic second hit is required for the lesions to develop, as previously reported in other multifocal vascular malformations (13–18). *RASA1* encodes p120RASGAP, a multi-domain cytoplasmic protein that acts as a negative regulator of the RAS-signaling pathway. P120RASGAP is a GTPase-activating protein (GAP) of RAS p21. It accelerates the intrinsic GTPase activity of RAS p21, switching it from active GTP-bound form to inactive GDP-bound. In addition, independent from its GAP activity, p120RASGAP plays a role in proliferation, differentiation, and pro- and anti-apoptosis (19). In mice, the complete loss of *Rasa1* is lethal at mid-gestation with abnormal angiogenesis; chimeric murine embryos constituted from wild-type and *Rasa1*-deficient cells exhibit severe edema and grossly abnormal vasculature (20).

There are several other disorders caused by mutations in genes encoding proteins that regulate the RAS/MAPK (mitogen-activated protein kinase) pathway. Germline mutations in the *NF1* gene, homolog to *RASA1*, cause neurofibromatosis type 1 (OMIM 162200), and other disorders, such as Noonan syndrome, LEOPARD syndrome, cardio–facio–cutaneous syndrome, Costello syndrome and Legius syndrome, are caused by germline mutations in genes that encode components of the RAS/MAPK pathway. With a certain overlap, they are characterized by craniofacial features, and cardiac, cutaneous, and neurologic abnormalities, and some of them exhibit cancer predisposition. These



entities are grouped under the term of RASopathies (21,22).

### 57.5 CEREBRAL CAVERNOUS MALFORMATION

Cerebral cavernous malformation (CCM; OMIM 116860) is a vascular anomaly located in the CNS, either intracranial (Figure 57-1H–J) or intraspinal. It can be associated with retinal or cutaneous lesions. CCM consists of clusters of dilated capillary-like vessels in a dense collagenous matrix, without normal vascular supporting cells and with abnormal blood–brain barrier. Based on cerebral magnetic resonance and autopsy studies, the prevalence is considered to be 1/200–1/1000 (23,24).

Sporadic and familial forms have been described; usually one lesion is observed in sporadic patients and multifocal lesions in familial patients by gradient-echo MRI. The familial form represents around 50% in Hispanic American patients of Mexican descent, due to a founder effect, and 10–40% in other populations. Linkage studies followed by sequencing of positional candidate genes or loss-of-heterozygosity mapping identified three CCM genes: *CCM1* (7q; *KRIT1*), *CCM2* (7p; *MGC4607*) and *CCM3* (3q; *PDCD10*) (reviewed by (25,26)). A heterozygous mutation (72% in *CCM1*, 18% in *CCM2*, and 10% in *CCM3*) is identified in about 95% of patients with multifocal CCMs and positive family history, but in only about half of the patients with multifocal CCMs and unaffected parents (27,28). No CCM germline mutation is expected in sporadic cases with one cerebral lesion on gradient-echo MRI.

About 60% of the mutation carriers are symptomatic; associated symptoms are mainly seizures and cerebral hemorrhages, but also headaches and focal neurologic deficit, irrespective of the gene involved. The radiologic penetrance is much higher but not complete, even with the very sensitive gradient-echo MRI sequences, and this observation is important for genetic counseling. Even if symptoms can be observed at any age, most often the onset is around 30 years in familial CCM. Importantly, new lesions appear with age and the size of the lesions tends to increase. Symptoms do not seem to correlate with the number of lesions, but rather with their location. The most severe outcome is associated with those in brainstem and basal ganglia (25). Cutaneous lesions, including capillary malformation, venous malformation, and more specific hyperkeratotic cutaneous capillary–venous malformation (HCCVM), are seen in about 9% of patients, mainly associated with *CCM1* mutations (29). Current clinical guidelines for symptomatic patients recommend medical treatment for seizures and surgical removal in case of hemorrhage, focal neurologic deficit, or lesions associated with intractable epilepsy. Gradient-echo MRI is the gold standard technique for diagnosis and it is recommended in patients with neurologic symptoms. Some

clinicians also recommend brain MRI for surveillance of asymptomatic patients, but this is controversial, as asymptomatic lesions are usually not treated.

More than 150 distinct mutations in the three CCM genes have been reported (30–35). Most of them are nonsense, frame-shift or splice site mutations, suggesting loss of function as the most plausible mechanism. To explain the localized nature of the malformation and the number of lesions (one in sporadic versus multifocal in familial CCM), a double-hit mechanism with biallelic loss was proposed. The efforts to confirm this hypothesis have recently been successful with the identification of a second hit in each of the three CCM genes (13,16) in one lesion from a few patients. Using laser capture microdissection, the mutations were shown to occur in a subset of endothelial cells, but not in non-endothelial cells (13) and this was consistent with immunohistochemistry studies (36).

In parallel with the genetic studies, many biochemical and in vivo studies have been conducted in an effort to unravel the disease mechanism. Yet, many pieces from the puzzle are still missing. The three CCM genes are conserved across species. *CCM1* encodes KRIT1 (Krev interaction trapped 1; OMIM 604214), a scaffold protein, which contains several domains involved in protein–protein interaction. *CCM2* (*MGC4607*; OMIM 607929) encodes malcavernin, a scaffold protein containing a phosphotyrosine-binding domain (PTB), similar to that of ICAP1 $\alpha$ , one of the KRIT1 partners. *CCM3* (*PDCD10*; OMIM 603285) encodes PDCD10, a protein with no known functional domain. Studies in zebra fish showed that loss of *krit1*, *ccm2*, or *ccm3* orthologs causes cardiovascular phenotypes with dilated and thin-walled heart and vessels (37–40). Mice lacking *Krit1* or *Ccm2* die in mid-gestation with vascular defects and heterozygous mice, unlike human beings, do not develop vascular lesions (reviewed by (41)). Loss of *Pdcd10* in murine embryos leads to growth arrest at E8.0 prior to the onset of circulation (42).

The CCM proteins are expressed in a variety of tissues, yet the disorder involves mainly CNS small vessels. Although it is not completely proven, it appears that CCMs are due to a defect in endothelial cells. Loss of *Ccm2* in murine endothelial cells, but not in neuroglial precursor cells, leads to vascular defects (43–45). Yet, loss of *Ccm3* in murine neuroglia also leads to vascular defects (44). It has been shown that the three CCM proteins interact (*CCM2* is the linker between *CCM1* and *CCM3*), but they also have specific partners (28,40,46–48). These proteins seem to play a role in cell–cell junctions, cell shape and polarity, and possibly cell–extracellular matrix adhesion (reviewed by (49)).

Besides the disorders described in this chapter, CMs are observed as part of many other rare entities/syndromes of unknown etiology, such as macrocephaly–capillary



malformation (OMIM 602501; previously described as M-CMTC (macrocephaly–cutis marmorata telangiectatica congenita)) and diffuse capillary malformation with overgrowth, or Wyburn–Mason syndrome, etc.

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## CROSS REFERENCES

Hereditary disorders of the lymphatic system; Hereditary hemorrhagic telangiectasia; Neurofibromatosis type 1; the disorders associated with RAS/MAPK pathway (RASopathies); M-CM, and other Overgrowth syndromes.

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## RELEVANT WEBSITES

Capillary malformation-arteriovenous malformation, <http://www.ncbi.nlm.nih.gov/books/NBK52764/>.  
 LOVD—Leiden Open Variation Database, [http://www.icp.ucl.ac.be/vikkula/VAdb/home.php?select\\_db=RASA1](http://www.icp.ucl.ac.be/vikkula/VAdb/home.php?select_db=RASA1).  
 Cerebral cavernous malformation, [http://www.orpha.net/consor/cgi-bin/Disease\\_Search.php?lng=EN&data\\_id=1072&Disease\\_Disease\\_Search\\_diseaseGroup=cerebral-cavernous-&Disease\\_Disease\\_Search\\_diseaseType=Pat&Disease\(s\)/group%20of%20diseases=Cerebral-cavernous-malformations&title=Cerebral-cavernous-malformations&search=Disease\\_Search\\_Simple](http://www.orpha.net/consor/cgi-bin/Disease_Search.php?lng=EN&data_id=1072&Disease_Disease_Search_diseaseGroup=cerebral-cavernous-&Disease_Disease_Search_diseaseType=Pat&Disease(http://www.orpha.net/consor/cgi-bin/Disease_Search.php?lng=EN&data_id=1072&Disease_Disease_Search_diseaseGroup=cerebral-cavernous-&Disease_Disease_Search_diseaseType=Pat&Disease(s)/group%20of%20diseases=Cerebral-cavernous-malformations&title=Cerebral-cavernous-malformations&search=Disease_Search_Simple)  
<http://www.ncbi.nlm.nih.gov/books/NBK1293/>.

### Biographies



**Dr Nicole Revencu, MD, PhD**, has been a consultant in clinical genetics since 2008. She qualified from the Université catholique de Louvain, Brussels, Belgium, in 1996. She then undertook clinical training in pediatrics at Saint-Luc Hospital/Université catholique de Louvain, and in 2001 she moved into clinical genetic training. From 2004 to 2008 she undertook a research project on genetics of vascular malformations in Professor Miikka Vikkula's lab. Her main clinical interests are dysmorphology, cleft lip and palate, and vascular anomalies. She participates to the teaching of clinical genetics to medical undergraduates and is a supervisor for residents in pediatrics in the clinical genetics module.



**Dr Miikka Vikkula, MD, PhD**, obtained his MD at the University of Helsinki in 1992 and his PhD in molecular genetics, with a thesis on collagens and cartilage disorders, in 1993 (Prof. Leena Peltonen). He was a research associate at the Department of Cell Biology at Harvard Medical School (Prof. Bjorn R Olsen) between 1993 and 1997, during which time he became interested in vascular anomalies. With his wife, Prof. Laurence Boon, MD, PhD, plastic surgeon, the couple discovered the gene for familial venous malformation in 1996. They settled in Brussels in 1997, where Dr Vikkula started to develop his own laboratory as a guest investigator at the de Duve Institute between 1997 and 2000. He obtained a "doctentship PhD" in human molecular genetics at the Université catholique de Louvain (UCL), Brussels, in 2000, and was thereafter nominated assistant professor at the Faculty of Medicine in UCL. He has been a full member of the de Duve Institute, and a member of the directorat since 2004. He was nominated Professor of Human Genetics at the Université catholique de Louvain on 1 October 2008. Prof. Vikkula is well known internationally as a major contributor to the understanding of the molecular basis of lymphatic and vascular anomalies and lymphedema, with many high impact publications. He has been an invited speaker at numerous major scientific meetings. He has also received numerous honors and awards from a number of prestigious organizations for his academic achievements; most recently, the Lacroix Prize on cardiovascular disorders, 2010; the Pfizer Scientific Award, 2006; and the Eugène de Sommer Scientific Award, UCL, Belgium, 2006.



**Professor Laurence Boon, MD, PhD**, obtained her MD at the Université catholique de Louvain, Brussels, Belgium, in 1989, and became a plastic surgeon in 1996. During her training in plastic surgery, she spent two years at the Children's Hospital, Boston, and the Harvard Medical School, under the supervision of Professor John B. Mulliken, to specialize in vascular anomalies. At the same time, she was a research associate at the Department of Cell Biology, and with her husband, discovered in 1996 the gene for familial venous malformation. Since 1996, she has been in charge of the Center for Vascular Anomalies at the University Hospital of St Luc, Brussels, Belgium. She obtained a PhD in plastic surgery in 2003, with a thesis on "New Clinical and Molecular Genetic Criteria for Hemangiomas and Venous Malformations."

Prof. Boon is well known internationally for the management of vascular anomalies and as a major contributor to the understanding of the molecular basis of vascular anomalies, with several high impact publications, reviews, and chapters on vascular anomalies. In 2003, she was invited to give the Sidney Hurwitz Memorial Lecture at the American Pediatric Dermatology Meeting in Seattle, USA. Since 2008, she has been the chair of the scientific committee of the ISSVA (International Society for the Study of Vascular Anomalies). She has been an invited speaker at numerous major scientific meetings. She continues to develop the multidisciplinary Center for Vascular Anomalies in Cliniques universitaires St Luc in Brussels. The Center will celebrate its 20th anniversary in 2011. Prof. Boon is involved in several research projects aimed at elucidating the pathophysiology of these anomalies as well as in clinical trials of new medications for vascular anomalies.



# CHAPTER

# 58

## Cystic Fibrosis

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### ABBREVIATION LIST

AAV – Adeno-associated virus  
 ABC – ATP-binding cassette  
 ASL – Airway surface liquid  
 CBAVD – Congenital bilateral absence of the vas deferens  
 CF – Cystic fibrosis  
 CFRD – CF-related diabetes  
 CFTR – Cystic fibrosis transmembrane conductance  
   regulator  
 DZ – Dizygous  
 ENaC – Epithelial Na<sup>+</sup> channel  
 FEV1 – Forced expiratory volume in 1 second  
 GI – Gastrointestinal  
 IRT – Immunoreactive test  
 IRT – Immunoreactive trypsinogen test  
 MZ – Monozygous  
 NPD – Nasal potential difference  
 ORCC – Outwardly rectifying chloride channel  
 PD – Potential differences  
 PI – Pancreatic insufficient  
 PS – Pancreatic sufficient  
 PTC – Premature termination codon  
 SES – Socioeconomic status  
 SNP – Single nucleotide polymorphism  
 TMD – Transmembrane domain

One of the earliest clinical descriptions of cystic fibrosis (CF) appeared in 1936 (1). It was termed CF of the pancreas in recognition of the feature that distinguished this illness from other causes of chronic gastrointestinal (GI) malabsorption (1). Recurrence of CF in families indicated a genetic etiology. Extensive pedigree analyses published in 1946 suggested an autosomal recessive pattern of inheritance (2). This implied that CF is caused by defects at a single locus. Monogenic inheritance was supported by review of CF pedigrees in Italian church records. Using consanguineous matings to expose recessive traits, Romeo and coworkers concluded that their results were most consistent with CF being a single-gene disorder (3). Cloning of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene on chromosome 7 and the

subsequent demonstration that CF patients have disease-producing mutations in *CFTR* confirmed the monogenic hypothesis for the classic form of the disease (4–6). However, it appears that genetic heterogeneity may account for a minor fraction of non-classic CF cases (7,8).

### 58.1 INCIDENCE OF CYSTIC FIBROSIS

Retrospective population-wide studies estimate that 1 in 3000 Caucasian newborns is affected with CF. This figure varies, however, by geographic location and population. A significantly higher frequency of CF, presumably as a result of genetic drift and/or founder effect, has been reported in several ethnic groups. Notable examples are the Hutterites in Alberta, Canada (incidence of 1 in 313), Afrikaaners in Southwestern Africa (1 in 622), and French-Canadians in the Saguenay-Lac St. Jean region (1 in 895) (9–11). Epidemiologic studies of more diverse populations have produced incidence estimates ranging from 1 newborn with CF per 1857 in Ireland (12) to an estimated 1 in 8000 to 1 in 9000 for the Hispanic population in Mexico (13). Overall, the majority of European, US, and Australian studies report an incidence of CF between 1 in 2000 and 1 in 4000 in Caucasians (14,15).

Newborn screening studies provide a different approach to estimating the incidence of CF. These studies utilized the serum immunoreactive test (IRT) either alone or in combination with DNA analysis (16–18). The incidence of CF in US newborn screening programs ranges from 1 in 3073 Caucasians in Colorado, 1 in 3938 Caucasians in Wisconsin to 1 in 5287 newborns in Western Pennsylvania (17,19,20). Adjustment of the Colorado data for testing compliance increased the estimated incidence to 1 in 2521, consistent with the generally accepted figure from retrospective studies. The lower estimate from Pennsylvania is likely the result of racial mixture (19) but CF may be less common among Wisconsin Caucasians (20). Two-tiered screening (IRT and DNA analysis) in South Australia revealed an incidence of 1 in 2821 newborns which compares reasonably



with the established incidence for that region (1 in 2200 newborns) (18,21). Studies from the United Kingdom, France, and Italy estimated similar newborn incidence of 1 in 2575, 1 in 2913, and 1 in 2650, respectively (22–24).

A third estimate for the incidence of CF in a mixed Caucasian population can be derived from the US CF Foundation Patient Registry. Clinical and epidemiologic data from all patients under care at CF Foundation Care Centers in the United States are collected by this registry. Over a 3-year period from 1990 to 1992, an average of 824 newly diagnosed Caucasian patients were reported to the CFF Registry. Adjusting by a coefficient of 1.21 for patients not followed in CFF Care Centers suggests an incidence of 1 in 3200 based upon 3,201,678 live Caucasian births (25). A fourth approach to estimate incidence utilizes heterozygote frequencies determined from population-based mutation screening. This method is particularly useful for determining incidence in a discrete population where the CF mutation frequencies are firmly established. For example, screening of Israeli Ashkenazi Jews for 5 CF mutations indicates a carrier frequency of 1 in 29 and estimates a population incidence of 1 in 3364 (26). Similar types of studies estimate an incidence of 1 in 1984 in Scotland (27) and 1 in 2916 in US Caucasians (28). Finally, comprehensive analysis of published CF incidence and corresponding frequencies of the common CF mutation  $\Delta F508$  from the same region

show significant correlation. This indicates that estimates of incidence based on newborn registries are a reasonably accurate reflection of the true regional incidence of this disease (29). Taken together, these studies indicate that an incidence of 1 in 2000 to 1 in 4000 newborns is most appropriate for Caucasians of European extraction. Epidemiologic and screening approaches both show variation in CF incidence by population, indicating that population-specific data should be used whenever possible.

The incidence of CF varies dramatically among different races. Case reports of the disease in native Africans and native Asians have been published but CF is rare in both populations (14,30,31). Reliable incidence estimates are not available for either race with the exception of one study of Asian Pacific Islanders living in Hawaii (1 in 90,000) (32). The incidence in black South Africans (1 in 12,000) and in African-Americans (1 in 15,000) is higher than native Africans probably due to Caucasian admixture (25,33). The same appears to be the case for individuals of Asian ancestry living in Canada or the United Kingdom (34,35). Indeed, CF incidence worldwide appears to be primarily determined by the degree of Caucasian admixture (Figure 58-1). However, screening of South African blacks for “African” CF mutations suggests that the incidence of CF may be considerably higher in native Africans (36). Case reports have also described CF in patients of Native American, Lebanese, Indian,



FIGURE 58-1 Worldwide Incidence of CF.

and Arabian background (14). Therefore, the diagnosis of CF should not be discounted on the basis of racial background, especially in multiracial countries such as the United States.

## 58.2 CLINICAL FEATURES

CF is a highly variable disorder. Classic CF usually manifests as recurrent pulmonary and/or GI disease in early childhood. Failure to thrive due to malabsorption and increased energy consumption is a common feature. These clinical features accompanied by an elevated concentration of chloride in sweat ( $>60$  mM) confirm the diagnosis of classic CF (see Section 6.4). About 60% of all CF patients are diagnosed before 12 months of age and 90% by 10 years old (37). Although an increasing number of patients are being diagnosed with CF as adults and almost half of the patients in the 2009 CF Foundation Patient Registry were 18 years or older, the median age of diagnosis has not changed substantially from prior decades (5 months of age in 2009) (38). Predicted survival age of patients in the United States in 2009 was 35.9 years (38). Pulmonary disease is the primary cause of mortality, accounting for nearly 90% of deaths (39,40). Liver disease and suicide account for another 2–3% of deaths (40). Not all features have to be present to make a diagnosis of CF. Patients with less obvious signs and symptoms of CF are frequently diagnosed in adulthood with non-classic forms of CF (Table 58-1) (41). Features of the CF phenotype in males are also observed in a separate autosomal recessive disorder called, congenital bilateral absence of the vas deferens (CBAVD) (Table 58-1) (42,43). Cloning of *CFTR*, the gene responsible for CF and identification of disease-associated mutations in patients with non-classic CF and CBAVD, confirmed that these variant phenotypes have the same molecular etiology as classic CF (44,45). However, there is evidence of genetic heterogeneity in CBAVD, and in non-classic CF (8,46).

### 58.2.1 Classic Cystic Fibrosis

Chronic cough, pulmonary infiltrates, reactive airway disease, and watery rhinorrhea are frequent symptoms.

TABLE 58-1 Phenotypes Associated with Mutations in the <i>CFTR</i> Gene			
	Classic CF	Non-classic	CBAVD
Chronic pulmonary disease	+	+	±
Pancreatic exocrine disease	+	±	–
Elevated sweat chloride ( $>60$ mM)	+	±	±
Male infertility	+	±	+

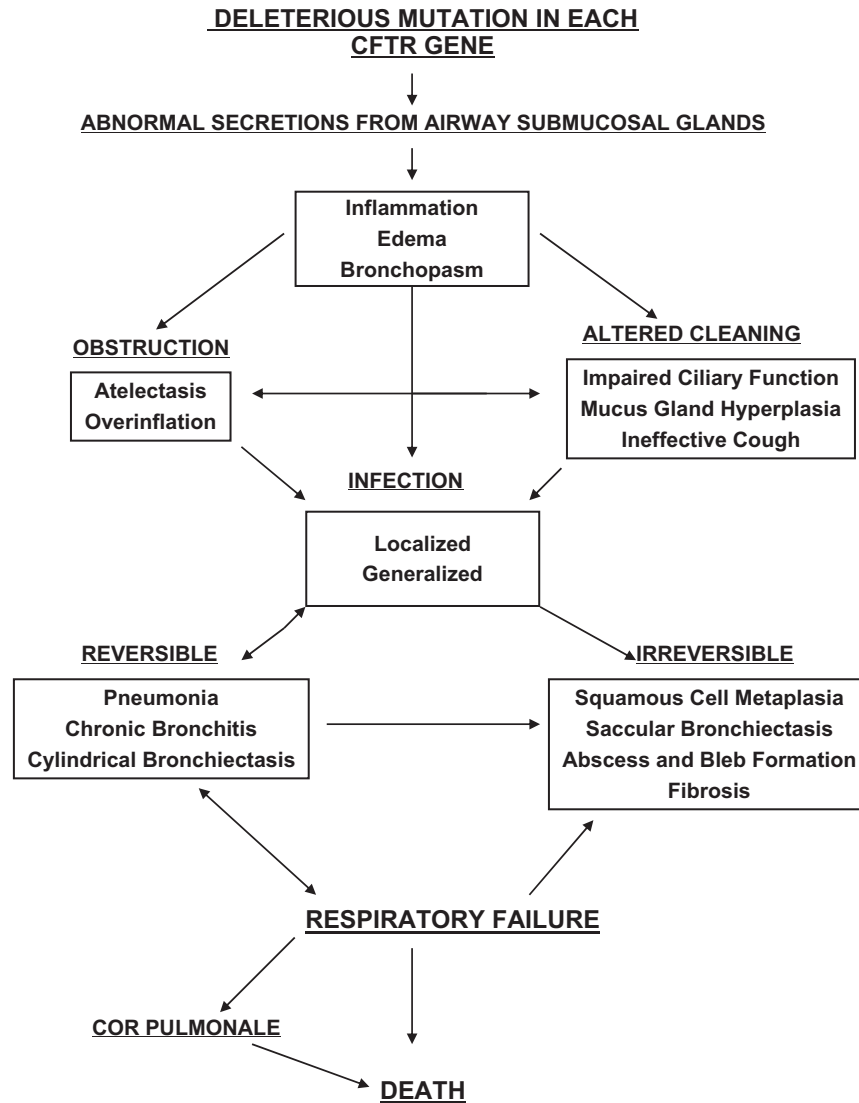
Neonatal intestinal obstruction due to meconium ileus (MI) and nasal polyposis in early childhood are particularly suggestive of this disorder (47–49). Pancreatic exocrine deficiency is present in 85–95% of patients and usually manifests as failure to thrive (50,51). Patients present with signs and symptoms of chronic protein and fat malabsorption including abdominal distension, and foul-smelling fatty stools (steatorrhea). Less common signs and symptoms at the time of diagnosis include electrolyte imbalance, rectal prolapse, and sinus disease (Table 58-2). Newborn screening accounted for 49.8% of the CF patients diagnosed in the United States in 2009 (38). It is likely that diagnosis of CF in the newborn period will become the most common form of presentation due to widespread use of screening in North America, Europe, and Australasia.

**58.2.1.1 Respiratory Disease.** Lung disease in CF manifests as recurrent episodes of infection and inflammation that leads to tissue destruction, fibrosis, and respiratory failure (Figure 58-2). Abnormally viscous mucus secretions initiate this downward spiral in lung function. At birth, the lungs of CF patients appear normal (52,53). Submucous glands in the large airways of CF newborns are similar in number and distribution to their non-CF counterparts (52,53). However, autopsy examination of infants dying from MI or other causes reveal dilation of acinar and duct lumens of submucosal glands due to obstruction with abnormal mucus secretions (53,54). Secretions from individual submucosal glands in nasal biopsies confirm that CF patients have high viscosity compared to normal subjects, leading to lower gland secretion rates in CF vs. normal subjects (55). Mucus impaction in the small airways (bronchioles) is also a consistent early finding (54,56). However, bronchioles have neither submucous glands nor evidence of prior infection (57). Identification of young infants

TABLE 58-2 Signs and Symptoms Suggesting a CF Diagnosis<sup>a</sup>

	Percent
Respiratory symptoms	51.6
Failure to thrive/malnutrition	43.2
Steatorrhea/abnormal stools	34.8
Meconium ileus/intestinal obstruction	20.6
Family history	17.1
Electrolyte imbalance	5.3
Rectal prolapsed	3.8
Neonatal screening	3.3
Nasal polyps/sinus disease	2.7
Genotype	2.3
Prenatal diagnosis	1.3
Liver problems	1.1
Edema/hypoproteinemia/hypoalbuminemia	0.4
Other	2.0
Unknown	2.4

<sup>a</sup>Patients may present with one or more sign or symptom. Data from Ref. (40).



**FIGURE 58-2** Pathogenesis of Cystic Fibrosis in the Lungs.

with CF by newborn screening has enabled detailed in vivo study of the early manifestations of the disease. Significant increases in segmented neutrophils, elastase, and interleukin 8 levels in bronchoalveolar lavage fluid have been observed early in life and in the absence of infection (58). Inhibition of CFTR in normal airways increases submucous gland secretions (59). Both autopsy and in vivo studies indicate that submucous gland dysfunction may be the earliest manifestation of CF in the lungs (56,58). Gland dysfunction may lead to inflammatory changes in the lung and abnormal secretions that compromise lung defenses leading to airway infections that are a hallmark of CF (60) (Figure 58-2).

The next phase is local and generalized chronic bacterial infection confined to the lungs. CF lung disease is distinctive due to the unusual organisms involved. The initial infection is most frequently with *Staphylococcus aureus*, with *Hemophilus influenza* and *Pseudomonas aeruginosa* being less common (56,61). Over time, the airways become chronically infected, with *P. aeruginosa*

becoming the predominant organism (62). Colonization with this bacterium, especially mucoid strains, is highly unusual for normal lungs but almost pathognomonic for CF (63). Altered adherence properties of respiratory epithelium in CF patients may explain the predilection for specific organisms (64,65). Once established, eradication of these resident microbes is virtually impossible. Chronic *P. aeruginosa* lung infection with altered local defenses sets the stage for recurrent cycles of infection, inflammation, and tissue destruction (56,62,66,67). Over a period of years, this process moves from region to region decreasing the amount of functional lung tissue (56). Submucosal glands become hypertrophied, increase in numbers, and appear in the small airways (68). Dilation of the small and large airways (bronchiolectasis and bronchiectasis) is a characteristic finding on chest X-ray (69). The latter can lead to the development of cysts, usually affecting the upper lobes. Episodes of bacterial respiratory infection occur throughout the lifetime of the patient; often this process is peribronchial but interstitial

pneumonia also occurs in later stages. Damaged tissue is replaced by fibrosis adding restrictive lung disease to the underlying obstructive process. Ventilation/perfusion mismatches occur resulting in hypoxemia and cor pulmonale (70). At this final stage, death may occur due to severe hypoxemia, acute bronchial artery hemorrhage, tension pneumothorax, or cardiac complications (71).

Clinically, lung disease in CF presents as an intermittent but persistent cough. In many children, this may be accompanied by persistent wheezing viral infection and infiltrates (56,72). Over time, the cough becomes daily and productive. This situation usually remains stable for a number of years, occasionally interrupted by acute worsening of symptoms called pulmonary exacerbations. The episodes are treated with intensive antibiotic therapy and mucus clearance techniques to recover lung function. Tissue destruction accompanies these exacerbations, which become more frequent over time, causing an increased rate of progression of disease. Pulmonary function measures are used to document progression of diseases, among which, the forced expiratory volume in 1 second (FEV1) has proved most useful. FEV1 has been shown to be highly predictive of survival (73,74). It has been estimated that lung function declines exponentially by about 3% per year for the average patient, slightly more rapid for females than males (74,75).

The age of onset and the rate and pattern of lung disease progression are highly variable (56). The role of *Pseudomonas* infection in disease onset and progression is a matter of debate (61,67,76–78). However, conversion to mucoid strains of this bacterium or infection with relatively drug-resistant organisms (*Burkholderia cepacia*) has been associated with rapid disease progression (63,78–81). Other studies indicate that *B. cepacia* colonization is associated with a variable clinical course (82,83). Patients with residual pancreatic function may have less severe lung disease (74,84,85). Furthermore, certain CF mutations appear to be associated with milder lung disease than that observed in patients homozygous for the common CF mutation  $\Delta F508$  (74,86–88). However, in general, genotype is not predictive of pulmonary phenotype (85,89). Therefore, predicting the course of lung disease for a newly diagnosed patient is not possible due to the lack of accurate prognostic factors.

Epithelia similar to that found in the lung are also present in the nasal sinuses. Consequently, the mucus produced by the sinus epithelium is altered leading to obstruction of the sinus ostia (90). Thus, almost all patients with CF have radiographic evidence of sinus disease (91). A smaller fraction of patients have clinically significant sinus disease. The reported incidence of a complication of sinus disease, nasal polyposis, varies from 6 to 48% of CF patients (92). In general, about one in four patients has this complication (92). Polyps from CF patients have different histologic features than those found in patients with allergic symptoms (93). Chronic sinusitis associated with *Pseudomonas* infection

is a characteristic finding although the infection rarely spreads beyond the paranasal spaces (94). Fungus infection may also be present in the sinuses of some CF patients (95).

**58.2.1.2 Pancreatic Disease.** Dysfunction of the exocrine pancreas occurs to some degree in almost all patients (51). Malabsorption caused by pancreatic insufficiency was the prominent life-limiting feature of CF. Fortunately, replacement of digestive enzymes and vitamin supplementation has greatly reduced the complications associated with this manifestation. The pathologic process in the pancreas is similar to that observed in the lung (96). Abnormal mucus secretions cause obstruction and dilation of the pancreatic ducts. Pancreatic enzymes continue to be produced by the acini causing tissue destruction, cyst formation, and fibrosis. This process begins in utero and progressive destruction of the entire organ is ongoing (96). The degree of destruction usually correlates with the age of the patient (51).

Deficiency of pancreatic enzymes leads to malabsorption of protein, fat, and fat-soluble vitamins. Carbohydrate absorption is not significantly affected (97). Untreated, this situation can lead to a multitude of problems: steatorrhea and absence of adipose tissue, hypoproteinemia and edema, central nervous system, and visual disturbances due to deficiency of vitamins A and E, and bone demineralization and coagulopathy due to deficiency of vitamins D and K (51). These symptoms are rarely encountered in CF patients since most are treated with pancreatic enzyme supplementation. In 2009, 89.1% of patients in the US CF Foundation Patient Registry were taking enzyme supplements (38).

The severity of pancreatic disease is variable. About 5–15% of patients retain some degree of pancreatic function (51,96). These patients are termed pancreatic sufficient (PS), a condition associated with CFTR genotype (6,98,99). Serum levels of the pancreatic precursor enzyme, immunoreactive cationic trypsinogen, can distinguish between pancreatic insufficient (PI) and pancreatic sufficient (PS) patients over 7 years of age (100,101). Patients with residual pancreatic function may manifest recurrent episodes of pancreatitis (102).

Loss of function of the endocrine pancreas also occurs in CF. As the median age of CF patients has increased, CF-related diabetes (CFRD) has become as significant contributor to morbidity and mortality. About a quarter to half of CF patients have an abnormal glucose tolerance test by the third decade and over 25% of CF patients 35–44 years of age had a diagnosis of diabetes (38,51,103,104). Diabetes in CF patients displays a number of pathologic similarities with type 2 diabetes seen in the general population; incidence increases steadily with age (105), pancreatic islets have evidence of amyloid polypeptide accumulation (106), and insulin secretion decreases over time (107). Family-based studies demonstrated that a history of type 2 diabetes in one first-degree or at least two second-degree relatives was associated



with a substantial increase in risk of diabetes in a CF patient ( $OR = 3.1$ ) (108). Thus, pathways involved in the development of diabetes in CF appear to overlap with type 2 diabetes in the general population. Diabetes in CF patients has been associated with reduced pulmonary function, especially in females, poorer nutritional status, and reduced survival (109–112).

**58.2.1.3 Gastrointestinal Disease.** Disease in the GI tract of CF patients is usually less prominent than lung and pancreatic manifestations. However, several conditions are characteristic of CF. In neonates, obstruction of the distal small bowel by abnormally viscous meconium is virtually diagnostic of CF (47,48). This condition, termed meconium ileus, is believed to be the consequence of inadequate enzymatic digestion and/or hydration of the intraluminal contents of the small intestine (48). Clinically, the patient manifests symptoms of small bowel obstruction that can proceed to perforation and peritonitis (113). Presence of the abnormal meconium can interfere with fetal intestinal development producing atresia or volvulus (113).

MI with obstruction occurs in approximately estimate that 13–20% of CF patients present with this feature (47,48,89). Obstruction can occur in the large intestine of neonates (“meconium plug syndrome”) causing delay in the passage of the first stool and evidence of colonic obstruction (114). In uncomplicated cases of MI, radiologic studies using contrast enemas are not only diagnostic, but may also be therapeutic (115). Surgery is usually required for more difficult cases but survival rates are excellent and long-term prognosis is similar to CF patients without MI (113).

In children and adults, recurrent episodes of bowel obstruction due to distal intestinal obstruction syndrome (DIOS) are also characteristics of CF (71). The prevalence of DIOS is highly variable in CF patients ranging from 2.4% to 41.3% in different reports (116–118). Patients with MI have a significantly increased risk of DIOS supporting a common etiology. However, analysis of affected twins and siblings reveals that surgical treatment for MI appears to be the risk factor for developing DIOS in later life (119). Other forms of surgery, such as lung transplantation, also appear to be associated with higher rates of DIOS (120,121). Rectal prolapse in childhood is a very unusual situation but is not uncommon in children with CF (about 20%) (122). Other intestinal complications include intussusception and mucoid impaction of the appendix (123).

**58.2.1.4 Hepatobiliary Disease.** Liver disease occurs in a significant fraction of CF patients. Twenty-five percent of patients have focal biliary cirrhosis present at autopsy (124). However, clinical evidence of hepatic cirrhosis and portal hypertension occurs in only 2–3% of children and only 5% of adults (124). Radiographic studies reveal gall bladder abnormalities in about half of CF patients (125) but only 12% develop gallstones (cholelithiasis) (126). Jaundice and hepatocellular failure

are generally absent until end-stage disease develops. Abnormal mucus secretions in the bile ductules cause obstruction, dilation, inflammation, and focal biliary cirrhosis that progress to multilobular cirrhosis over time (127). Cholestasis in the neonatal period may present as prolonged jaundice (124). On exam, this cirrhotic process manifests as hepatosplenomegaly. Hemorrhage from esophageal varices can be life-threatening. Risk factors for the development of CF-related liver disease include male sex, a history of MI, and poor nutritional status (128–130). Whether CFTR genotype contributes to the development of liver disease is not clear (129–133).

**58.2.1.5 Sweat Glands.** Demonstration of elevated concentrations of chloride and sodium in eccrine sweat has been the diagnostic standard for CF since 1953 (134). The vast majority of patients with clinical features consistent with CF have chloride concentrations greater than 60 mM. Ninety-six percent of patients in the US Registry have sweat chlorides between 60 and 165 mM (104). About 3% are above 166 mM while 1.4% are less than 60 mM (discussed below as Atypical CF). Since values above 160 mM are not physiologically possible, it is likely that they represent testing error (135). At first glance, the high chloride concentration in sweat seems inconsistent with the underlying functional defect in CF; deficient chloride secretion. However, the sweat gland is composed of two regions: a secretory coil and a resorptive duct and both components are abnormal in CF patients. The coil secretes an ultrafiltrate in response to cholinergic and  $\beta$ -adrenergic agonists that is nearly isotonic with plasma. The  $\beta$ -adrenergic response of the secretory coil is consistently absent in CF patients (136). In healthy individuals, chloride and sodium are absorbed as the ultrafiltrate passes through the water-impermeable duct producing hypotonic sweat. In CF, chloride and sodium absorption of the duct is defective, leading to elevation of both electrolytes in sweat collected at the skin surface (137).

The major clinical manifestation of abnormal sweat gland function is salt depletion. This condition can present in young children as hypochloremic, hyponatremic alkalosis, and dehydration (103). Heat waves may precipitate acute volume depletion, whereas chronic salt-loss can be a common presenting sign in arid climates (138,139).

**58.2.1.6 Reproductive Tract Abnormalities.** A consistent abnormality caused by mutations in each CFTR gene is absence of atrophy or fibrosis of the vas deferens in men. This condition manifests as male infertility due to azoospermia in 96–97% of male patients with CF (140). Absence of secretions from the seminal vesicles causes chemical abnormalities in semen and a reduction in ejaculate volume (141). The pathogenesis of the vas deferens abnormality is unknown but is believed to be a consequence of mucus obstruction of the developing genitourinary tract. A separate genetic condition, CBAVD, is also associated with CFTR dysfunction (see below).

About 2–3% of males with CF are fertile and compound heterozygotes for two CF mutations have had offspring (45). Fertility in women with CF is reduced but not to the same degree as their male counterparts. Thick cervical secretions and menstrual irregularity due to chronic disease contribute to difficulties conceiving (103). Pregnancy was reported in 130 (4%) of 3,249 US female patients of reproductive age in 1993 (104). Just over half of these pregnancies resulted in a live birth. In 2009, 226 pregnancies were reported in 12,762 US females with CF.

### 58.2.2 Non-Classic Cystic Fibrosis and CFTR-Related Disorders

In the decade following the introduction of sweat test as the diagnostic standard for CF, it became apparent that adults could present with mild or incomplete phenotypes (142). Increased vigilance for “milder” forms of CF uncovered patients without clinical, chemical, or histological evidence of pancreatic disease (50,143–145). Diagnosis in these situations relied upon pulmonary illness consistent with CF and elevated sweat chloride concentrations. In males, azoospermia was an additional useful criterion for diagnosis (146). However, patients with suggestive features but sweat electrolyte concentrations below the range typical for CF (i.e., <60 mM) presented a diagnostic challenge, particularly if pancreatic function is preserved (147,148). Over the years, these patients have been variably referred to as non-classic, PS, intermediate, borderline, or atypical forms of CF (149,150). In this chapter, the term non-classic will be used to denote patients who have CF (based on clinical grounds) but manifest disease in only one or two of the classic triad of organ systems: lung, pancreas, and skin (i.e., sweat gland).

These observations indicated the existence of non-classic forms of CF where pulmonary history and exam suggested the diagnosis but sweat chloride concentrations were normal, or slightly above the normal range, but not diagnostic of CF (142,147). In 1978, Stern and colleagues presented seven patients fitting this profile (144). Sweat chloride concentrations were described as “intermediate” (40–60 mM). Since a significant fraction of normal adults have values in this range, additional criteria were used to suggest that these patients represented a variant of CF (144,151). Case reports of patients felt to have CF but with normal sweat chloride values (<40 mM) have also been published (148). The discovery of the CF gene has facilitated a molecular approach to the problem. Mutations in each CFTR gene have been identified in patients with non-classic disease confirming their inclusion in the CF spectrum (44,45,152). However, dysfunction of CFTR does not underlie all cases of non-classic CF (7,8).

More recently, non-classic CF has been differentiated from CFTR-related disorders such as male infertility due to CBAVD and pancreatitis where CFTR mutations are

present. The operating definition has been “a clinical entity associated with CFTR dysfunction that does not fulfill diagnostic criteria for CF” (150). An underlying reason to distinguish these two main categories is that classic and non-classic forms of CF are highly likely to result in reduced longevity while CFTR-related disorders are not. Thus, counseling and treatment decisions will differ considerably for the two categories.

## 58.3 GENETICS

CF is the most common lethal autosomal recessive disorder in Caucasians affecting about 1 in 3000–4000 newborns. This high incidence implies that approximately 1 in 30 individuals of European descent is a carrier of the disease. Explanations for the high frequency of CF alleles in Caucasians have included the coexistence of multiple CF loci (14), high mutation rate (153), genetic drift (32), founder effect (154), sex ratio and segregation distortion in favor of mutant alleles (155–160), and heterozygote advantage (161). Linkage analyses eliminated the hypothesis of multiple CF loci for classic CF (162). The identification of the CFTR gene and discovery of the major CF mutation ( $\Delta F508$ ) that is common in Caucasians, but rare in populations with a comparatively low incidence of CF (e.g., native Africans and Asians), provided a molecular basis for the high frequency of CF in Caucasians (4,6,29,163). Demonstration of a single origin of all  $\Delta F508$  mutations excluded high mutation rate (164). The high frequency of  $\Delta F508$  in large and heterogeneous European-derived populations argued strongly against genetic drift and founder effect, with the exception of ethnic isolates (154). Studies of sex ratio, segregation distortion, and a “hitchhiker” effect have been either inconclusive (155–159) or lack confirmatory evidence (160,165,166).

Heterozygote advantage of CF carriers remains as an attractive hypothesis. However, it is difficult to prove since it has been estimated that an advantage of as little as 1.6% per generation could account for the high incidence of CF (14,161). Various forms of heterozygote advantage have been proposed, including higher fertility of CF carriers (14,167,168), protection against bronchial asthma (169), and increased resistance to common infectious diseases (170–176). Fertility differences were found unlikely to have played an important role (177). Mouse models of CF have suggested heterozygote resistance to chloride-secreting bacterial diarrheas (176). However, this issue has been controversial since CF mutation distribution does not correlate with the geographic distribution of cholera (178). Furthermore, a second murine study and a human study did not find reduced intestinal chloride secretion in CF heterozygotes (179,180). These observations suggest that other factors may contribute to the high frequency of CF in Caucasians (181).

At least three cases of CF have been reported due to inheritance of both copies of chromosome 7 from one

parent (uniparental isodisomy) (182–184). The parent in each case was a heterozygous carrier of a CF mutation, thereby giving rise to a homozygous affected child. Although an interesting genetic phenomenon, this form of inheritance is extremely rare and should not be used to alter the risk estimates for healthy carriers of CF alleles (185).

### 58.3.1 Identification of the Gene Responsible for Cystic Fibrosis

**58.3.1.1 Mapping the Gene.** The first breakthrough in the mapping of the CF gene was a report of linkage between paraoxanase, a polymorphic enzyme in many populations and the CF locus (186). Soon thereafter, a DNA marker (DOCRI-917) was linked to the CF locus at a distance of approximately 15 cM (187). Mapping of this marker to the middle of the long arm of chromosome 7 located the gene responsible for CF, and facilitated the identification of additional markers (MET and D7S8) closer to the CF locus (188,189). Two collaborative studies demonstrated that these markers flanked the CF gene (190,191). The availability of DNA markers closely linked to the CF locus enabled accurate prenatal diagnosis and carrier detection to be performed in families with an affected individual (192).

**58.3.1.2 Approaches to Identify the Gene on Chromosome 7 Responsible for CF.** Scambler and colleagues took advantage of the close physical relationship between the MET oncogene and the CF locus (193). Using chromosome-mediated gene transfer, a short region of unmethylated CG-rich DNA was identified in a region encompassing the CF locus (194). A candidate for the gene responsible for CF was discovered close to the CG-rich region (194). The predicted amino acid sequence of the candidate gene resembled secreted “growth factors,” making this gene (termed IRP for *int*-related protein) an unlikely candidate for causing CF (195). Furthermore, the discovery of five families, each with a recombination event between IRP and the CF locus, eliminated IRP as the gene responsible for CF (195). However, two new DNA markers, XV2c in the IRP gene and KM19 from the 5′ region of IRP, had very few recombinations with CF in family studies (194). This indicated that XV2c and KM19 were considerably closer to the CF locus than either of the previously identified markers, MET and D7S8 (196,197). Furthermore, one particular pattern (haplotype) of the XV2c/KM19 markers was found on 85% of the CF-bearing chromosomes but was present on only 15% of normal chromosomes in North American and European Caucasian families (30,196,197). The high degree of linkage disequilibrium between XV2c/KM19-derived haplotypes and the CF locus confirmed the closeness of these markers to the gene, and suggested that a single mutation of this gene predominates in the Caucasian population.

**58.3.1.3 Cloning and Characterization of the *CFTR* Gene.** The gene causing CF, the *CF Transmembrane Conductance Regulator* or *CFTR*, was identified by a combination of chromosome jumping, physical mapping, isolation of exon sequences, and genetic analysis (4–6). *CFTR* is composed of 27 exons spanning 189 kb of DNA (198,199). The gene is transcribed into a mature mRNA of approximately 6500 basepairs (5). Alternative splicing of several exons has been described (200) and some of the resulting transcripts encode a functional isoform of CFTR (201–203). Several lines of evidence confirmed that *CFTR* is the gene responsible for CF (6). First, a common mutation, the deletion of three nucleotides ( $\Delta F508$ ), was found only on chromosomes from CF patients. Second, there were no recombinations between  $\Delta F508$  and the CF phenotype. Third, the  $\Delta F508$  mutation was almost exclusively associated with haplotype in strong disequilibrium with the CF locus. Finally, normal chromosomes with the haplotype common to CF chromosomes did not carry  $\Delta F508$ , indicating that this deletion was not a polymorphism linked to the “CF” haplotype (6).

*CFTR* mRNA is expressed in epithelial tissues classically affected in CF, such as the lung, pancreas, sweat gland, and liver (5). It has also been found in tissues of unclear or unknown involvement in CF, such as the large intestine and testis (204,205). RNA studies indicate a low level of *CFTR* transcripts (~1 per cell) in adult respiratory epithelium but much higher levels in pancreas and intestine (204,206). *CFTR* RNA can be detected in many regions of the developing lung but appears confined to airway epithelium of large and small airways by the perinatal period (207–209). Extensive *in situ* studies of *CFTR* expression in the rat reveals developmentally regulated expression in the male reproductive structures (204). Studies of *CFTR* protein expression have been generally consistent with RNA data. *CFTR* is barely detectable in surface epithelia of the lungs, although it is abundant in the pancreas, sweat gland, kidney, and intestines (205,210–214). *CFTR* expression in the lung is prominent in the submucosal glands, particularly cells in serous tubules and in occasional cells of the ducts (212,215). More detailed histochemical studies reveal that *CFTR* expression is confined to apical membranes of ciliated cells in the surface epithelium and gland ducts (216).

Although *CFTR* expression displays exquisite temporal and spatial regulation, sequences that control *CFTR* transcription are incompletely understood. The consensus of a number of studies indicates that the major transcription initiation site is approximately 70 nucleotides 5′ of the start site for translation (217–220). Elements for basal levels of expression reside within 360 nucleotides of the translation initiation codon (219). This region contains consensus sequences for an inverted CCAAT box and a cAMP response element (221–224). DNase I hypersensitive sites have been found within and flanking

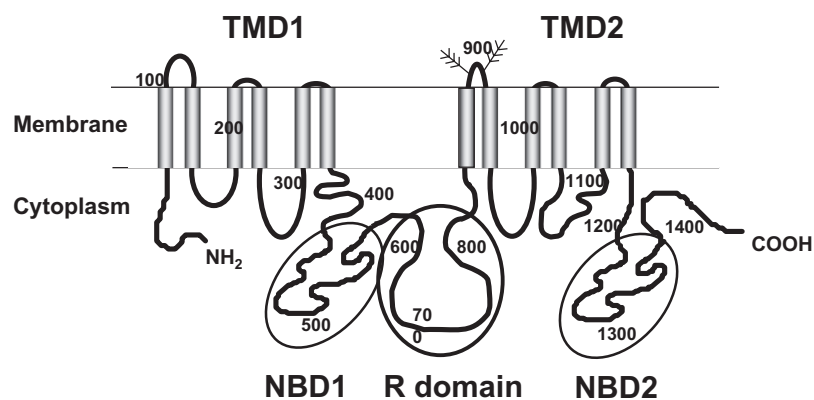
the CFTR gene. These elements may facilitate tissue regulation of CFTR expression (225). Employment of newer techniques that map regulatory elements (e.g., enhancers) has identified DNA elements distant from the *CFTR* promoter (226,227) and within the *CFTR* gene (228,229). Other approaches to this problem include the identification of genomic regions that confer strain-specific differences in the expression of *Cftr* in mice and cross-species analysis to identify conserved non-coding regions that might harbor binding sites for regulators (230,231). The basal CFTR promoter does not seem to be regulated by methylation (203,232) although microRNAs that target the 3' region may play influence *CFTR* expression (233).

### 58.3.2 Properties of CFTR

**58.3.2.1 CFTR Is an Integral Membrane Protein.** The CFTR gene is predicted to encode a protein of 1480 amino acids that has two repeated structures each containing a transmembrane domain (TMD) composed of six hydrophobic segments and a putative nucleotide binding domain (NBD). These structures are separated by a 241 amino acid region with many charged residues and multiple potential sites for phosphorylation by protein kinases A and C (5) (Figure 58-3). The latter region was postulated to be involved in controlling protein function and was named the Regulatory or R domain. The overall structure of CFTR is most similar to members of the multi-drug resistance family and STE6, a yeast protein (5). These proteins belong to the superfamily of ATP-binding cassette (ABC) proteins which import or export small molecules (drugs, proteins, sugars, and ions) across cell membranes (234,235). Members of this group of proteins are most similar in the hydrophilic region containing an ATP-binding site (5,236). Therefore, by comparison with this family of proteins, it was originally proposed that CFTR may function as an ATP-dependent transporter, possibly exporting molecules across cell membranes (5).

Expression of CFTR, or portions of CFTR, demonstrates that most of the initial predictions of the topology and functional domains of CFTR were quite accurate. Localization of CFTR in vivo with antibodies is consistent with its presence in the apical cell membrane (205,210,216,237). Insertion of novel N-glycosylation sites indicates that the 12 putative hydrophobic segments traverse the cell membrane (238) (Figure 58-3). The putative NBDs both bind ATP, and the first domain (NBD1) hydrolyzes ATP (239–245). Finally, phosphorylation of several residues in the R domain by protein kinase A (PKA) plays an important role in regulating CFTR function (246,247).

**58.3.2.2 CFTR Is a cAMP-Regulated Chloride Channel.** Although the overall structure of CFTR is similar to ABC transporters, experimental evidence convincingly demonstrates that it functions as a chloride channel (248–250). Expression of CFTR resulted in the appearance of novel cAMP-stimulated chloride secretion in a variety of cells derived from non-epithelial tissue that do not have cAMP-activated chloride channels (251,252). Furthermore, the magnitude of this secretion was correlated in a linear fashion with the amount of CFTR protein present (251,252). Similar results were obtained by heterologous expression of CFTR in mouse cells and *Xenopus* oocytes (253–256). Second, reconstitution of CFTR into planar lipid bilayers was associated with cAMP-regulated chloride channel activity. The protein was initially purified from Sf9 insect cells and its identity confirmed by immunoblotting, isoelectric point analysis, amino-terminal sequencing, and amino acid composition (257). Following reconstitution into phospholipid vesicles, cAMP-activated channels were identified with properties virtually identical to chloride channels in Sf9 and hamster (CHO) cells transfected with CFTR and human colonic epithelial (T84) cells that normally express CFTR (257). A third line of evidence is derived from functional studies of mutated CFTR. Based on the hypothesis that charged residues in membrane-spanning regions play



**FIGURE 58-3** Structural and Functional Domains of CFTR. CFTR is composed of two TMDs (TMD1 and TMD2) each containing six hydrophobic segments inserted into the cell membrane, two intracellular NBDs (NBD1 and NBD2) that interact with ATP and an intracellular regulatory (R) domain that is phosphorylated by protein kinase A. The fourth extracellular loop has two sites for asparagine-linked glycosylation. Residue numbers are shown.



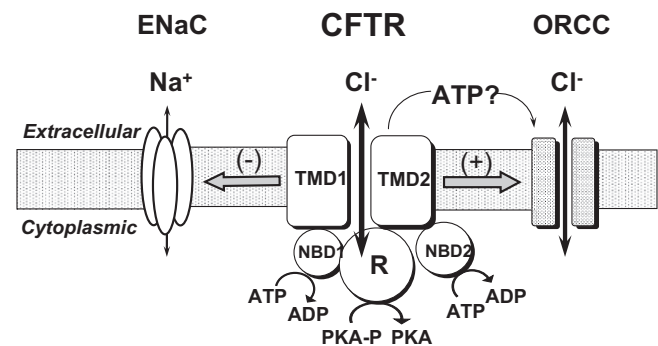
an important role in ion conductivity of the channel, Anderson and colleagues changed four of six evolutionarily conserved basic amino acids in putative transmembrane segments of CFTR (258). One mutation, lysine to glutamic acid at codon 335 (K335E), caused changes in the anionic permeability and ion conductivity of CFTR. The preference of wild-type CFTR for chloride over iodide was reversed in the mutant channel (258). Since numerous other properties of CFTR were unaltered, it was suggested that lysine at position 335 was critical for determining anion selectivity of CFTR and infers that CFTR itself forms a chloride channel. The demonstration that disease-producing mutations affect the chloride conductance properties associated with CFTR probably provided the most convincing argument that CFTR is a channel. Alterations found in patients with milder pancreatic disease (R117H, R334W and R347P) reduce but do not abolish the conductance of CFTR bearing these mutations (259,260).

Which regions of CFTR form the channel pore? Several different but complementary approaches have progressively narrowed the critical region of CFTR involved in ion conduction. Deletion of a portion of the R domain produced a functional CFTR mutant with altered regulation (261). Channel function was lost upon removal of the entire R domain and upon deletion of NBD1, NBD2, or both NBDs (262). Truncation of the carboxy-terminus up to, but not including, NBD2 was associated with chloride channel properties similar to wild-type CFTR (262). These results suggested that several domains of CFTR had to be intact to create a functional channel. Expression of the amino-terminal half of CFTR containing TMD1, NBD1, and the R domain generated cAMP-regulated chloride conductances (263). The anion selectivity and the current-voltage relationship of the channel formed by this CFTR mutant were similar to wild-type. However, the magnitude of current was significantly lower for the half-molecule and its regulation was also altered (263). The last transmembrane segment in the second half of CFTR (TM12) may also play a role in channel formation (264). Amino acids in the first part of CFTR have been removed and shown not to be involved in channel formation. Mutant CFTR with the first four transmembrane segments of TMD1 deleted produces a functional channel with ion selectivity that is identical to wild-type (265). These truncation studies indicate that the fifth and sixth transmembrane segments (TM5 and TM6) in TMD1, NBD1, and the R domain are required to form a chloride-selective channel. Mutation of individual amino acids in TM6, particularly the arginine residue at codon 347, altered the conductance properties of the CFTR channel (264,266), suggesting that TM6 is intimately associated with the channel pore. The importance of TM6 in channel formation has been confirmed by the scanning-cysteine-accessibility method (267). Regulation of channel opening is mediated by ATP binding to each NBD and dimerization of these domains.

Hydrolysis of ATP bound to the second NBD appears to facilitate channel closing (268–270).

**58.3.2.3 CFTR Is a Regulator of Other Proteins Involved in Ion Transport.** It has been shown that CFTR is capable of regulating the function a number of proteins, an outwardly rectifying chloride channel (ORCC), an amiloride-sensitive epithelial  $\text{Na}^+$  channel (ENaC), an inwardly rectifying ATP-sensitive renal potassium channel (ROMK2), and members of the SLC26 family of anion transporters (271–276). There is an interesting history behind the discovery that CFTR regulates a separate chloride channel. In 1983, Quinton made the seminal discovery that sweat duct cells of CF patients had low permeability to chloride (277). Following this observation, electrophysiologic studies implicated abnormal regulation of an outwardly rectifying, epithelial chloride channel as the molecular defect in CF (278–281). The channel characterized in these studies was present in membranes of CF cells but failed to activate upon the addition of either protein kinases A or C (282,283). Following the cloning and characterization of CFTR, it was realized that the abnormally functioning ORCC in CF tissues was not CFTR (284,285). An intimate relationship between CFTR and ORCC was illustrated by expression of CFTR in an epithelial cell line derived from a CF patient. PKA-activation of ORCC was recovered, and regulation of ORCC in complemented cells was similar to normal epithelial cell lines (271,286). This discovery implied that either CFTR formed more than one type of channel or that the ORCC was a separate entity (Figure 58-4).

The molecular identity of ORCCs and the mechanism by which CFTR regulates the ORCC have not been resolved. Several independent observations implicate ATP as the mediator between CFTR and ORCC.



**FIGURE 58-4** CFTR Regulation of the Outwardly Rectifying Chloride Channel (ORCC) and the Epithelial Sodium Channel (ENaC) in Secretory Epithelia. CFTR is activated by phosphorylation of the R domain and ATP interaction with the NBDs. Activation of CFTR has a positive regulatory effect upon the ORCC and a negative regulatory effect upon ENaC. The former is postulated to occur by an autocrine mechanism involving CFTR-mediated export of ATP. The mechanism underlying the interaction between CFTR and ENaC is unknown. The direction of chloride and sodium movement through these channels depends upon the intracellular and extracellular concentrations of each ion and the membrane potential (see Figure 62-4).

Stutts and colleagues demonstrated that extracellular ATP increased the amount of time that ORCCs in epithelial cells were open. It was hypothesized that this interaction occurred via a purinergic receptor (P<sub>2U</sub>) (287,288). Concurrently, it was shown that CFTR is permeable to ATP in addition to chloride (289). These observations have been unified into a single mechanism by which CFTR regulates the ORCC via an autocrine mechanism involving ATP (290) (Figure 58-4). This process may be mediated by sequences in the first NBD and/or the R domain (291,292). It had been proposed that an isoform of the voltage-gated channel 3 (CIC-3B) generates ORCC-like currents (293). However, studies of colocalization and interaction with CFTR have been inconsistent (294). Thus, the identity of ORCC remains a mystery. Other proteins, such as SLC26A9, that contribute to chloride conductance in secretory epithelia also appear to be regulated by CFTR (295).

A regulatory relationship between CFTR and the amiloride-sensitive ENaC has also been demonstrated (Figure 58-4). Increased absorption of sodium by respiratory epithelia compounds the difficulty of hydrating mucus secretions in CF patients (296,297). This electrophysiologic abnormality has been attributed to CFTR dysfunction; nasal epithelial cells from “CF mice” demonstrate increased Na<sup>+</sup> absorption (298) and heterologous expression of CFTR in primary CF airway cells normalizes Na<sup>+</sup> absorption (299). Cloning of the three subunits that form the amiloride-sensitive ENaC enabled coexpression studies confirming the interaction between CFTR and this separate channel (272,300). Activated CFTR exerts a negative regulatory effect upon the ENaC, decreasing sodium absorption (Figure 58-4). On the other hand, studies of sweat duct cells indicate that CFTR has a positive regulatory effect upon ENaC (301). The mechanism underlying the interaction between CFTR and ENaC is not certain although there is evidence that the first NBD of CFTR (302) and proteolytic mechanisms (303) are involved. Interestingly, the first nucleotide binding domain of CFTR appears to be required for regulation of the potassium channel ROMK2 (304). Finally, it has been suggested that CFTR modulates bicarbonate secretion by regulation of chloride–bicarbonate exchangers (305–307). This hypothesis is attractive since bicarbonate secretion is impaired in CF cells (308,309). Members of the SLC26 family of chloride–bicarbonate exchangers have been shown to interact and regulate CFTR (276,276).

**58.3.2.4 Other Proposed Functions of CFTR.** In addition to functioning as a chloride channel and as a regulator of separate ion channels and transporters, several other functions have been proposed for CFTR. CFTR appears to play a role in the regulation of volume-regulated anion channels and gap junction channels (310,311). Other proposed functions of CFTR include sensor for intracellular ATP levels (312), conductor of a variety of small solutes and water across cell membranes

(313), and facilitator of acidification of intracellular organelles (314,315). Loss of these properties could explain a number of metabolic derangements in CF cells. However, insufficient corroborating evidence is available to assign these functions to CFTR.

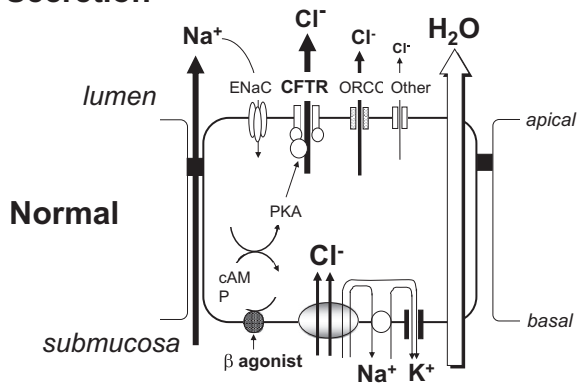
**58.3.2.5 CFTR Interacts with Cytosolic Proteins.** The amino and carboxy terminal regions of CFTR interact with a number of proteins (316). Interaction of the amino terminus of CFTR with syntaxin 1A mediates chloride channel activity and regulates trafficking of CFTR (317–319). The carboxy terminus of CFTR contains sequences that influence stability (320,321) and facilitate entry into clathrin-coated vesicles, recycling from the plasma membrane, and localization in polarized epithelium (322–324). Several lines of evidence suggest that CFTR physically interacts with a macromolecular complex at the apical membrane via a PDZ domain that augments CFTR function, and links CFTR to the cytoskeleton and metabolic state of the cell (see Ref. (325) for a comprehensive review). Components in the macromolecular complex may include several of the aforementioned proteins regulated by CFTR (e.g., SLKC26 and RomK) (276,326) as well as upstream mediators of CFTR activation such as the  $\beta$  adrenergic receptor and phosphodiesterase 4D (327,328).

**58.3.2.6 Abnormal CFTR Function Causes the Epithelial Ion Transport Defect in Patients with CF.** Abnormal transport of sodium and chloride in epithelial cells lining the airways of the lungs and in the sweat glands of CF patients is the biochemical defect in CF (277,329). A number of studies have shown that provision of normal CFTR to epithelial cells derived from CF patients revert cellular phenotypes to normal. Using a retroviral vector, Drumm and colleagues transferred the normal CFTR cDNA to a pancreatic adenocarcinoma cell line from a CF patient (CFPAC-1) (330). Stimulation of transfected cells with cAMP or forskolin (which increases intracellular cAMP) produced chloride secretion that was absent in cells transfected with the virus alone. Similarly, expression of wild-type CFTR in CF airway cells also corrected the ion transport defect (331). This correction did not occur when mutant CFTR containing the common CF mutation  $\Delta F508$  (described below) was used. Furthermore, reduction in endogenous CFTR levels is associated with loss of cAMP-activated chloride transport in epithelial cells. This has been achieved by decreasing CFTR mRNA transcription using a phorbol ester or interfering with translation with antisense oligonucleotides (332–334). These studies confirmed that CFTR plays a central role in the chloride transport that is defective in cells of CF patients. More recently, the development of a porcine model of CF has led to a re-evaluation of the role of aberrant sodium transport at the earliest stages of CF (335). Studies of primary airway cells from humans with CF suggest that the alteration in currents caused by the sodium channel inhibitor amiloride reflects changes in chloride rather than sodium transport (336).

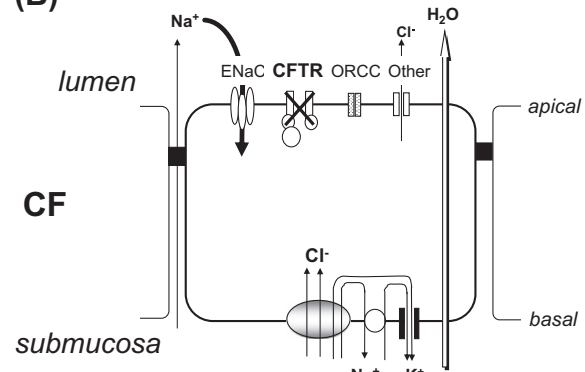
Although a great deal is known at the molecular and cellular level, the mechanism by which CFTR dysfunction leads to disease manifestation at the organ level is poorly understood. This is particularly true for pulmonary disease, the primary cause of morbidity and mortality in CF. Indeed, a major debate in CF research is whether the underlying pathophysiology in CF is due to a loss of electrolyte and fluid secretion or absorption, or a combination of both (Figure 58-5) (337). Attention was focused upon this issue after publication of a provocative study by Smith, Welsh, and colleagues. These investigators suggest that high salt concentrations in the airway surface liquid (ASL) of CF patients inhibited bacterial killing mediated by antimicrobial peptides (338).

Shortly thereafter,  $\beta$ -defensin-1, a human antimicrobial peptide present in respiratory epithelia, was shown to have reduced anti-pseudomonas activity in high salt solutions (339). However, the “high salt” concept ran counter to the concept that epithelial cells in the airways are highly permeant to water and that sodium absorption is increased in CF patients (297,329,340). The latter “low volume” theory implied that the ionic composition of ASL should not differ between normals and CF, but the volume of ASL would be reduced leading to altered viscosity of ASL and impaired clearance of pulmonary secretions. Measurements of ion concentrations in ASL have been difficult, and results comparing CF and normal have been conflicting (341–343). However, there

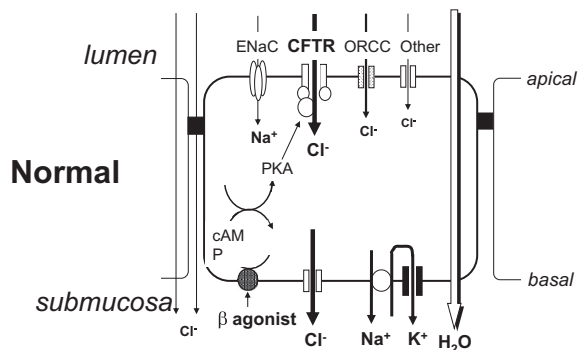
### (A) Secretion



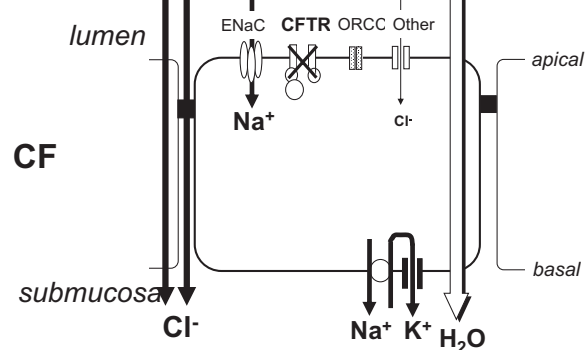
### (B)



### (C) Absorption



### (D)



**FIGURE 58-5** Simplified Model of Electrolyte and Water Transport across Normal and Cystic Fibrosis Airway Epithelia. A. Secretion. The top panels demonstrate the process of secretion in normal and CF airway cells. The  $\text{NaK}_2\text{Cl}$  co-transporter (shaded oval) pumps chloride from the submucosa into the cell. Sodium and potassium that are transported with chloride exit via the  $\text{NaK}$  ATPase (open circle) and by the basolateral  $\text{K}^+$  channel (filled rectangles). The net result is an increase in the intracellular concentration of chloride. Binding of a  $\beta$  agonist to receptors located on basolateral membranes increases the intracellular levels of cAMP and activated PKA. Phosphorylation of CFTR by PKA causes activation of CFTR and ORCC, and chloride, driven by its concentration gradient, flows out the cell across the apical membrane. Other apical membrane chloride channels also participate in chloride transport out of the cell. Sodium follows the electrochemical gradient created by chloride ion secretion and flows from the submucosa to the lumen via a paracellular pathway. A minor fraction of sodium is reabsorbed via ENaC (downregulated by activated CFTR). The movement of sodium and chloride ions from submucosa to the lumen drives water secretion across the epithelial cell layer. In CF cells, CFTR function is absent leading to severely reduced chloride transport into the lumen, hyperabsorption of sodium due to increased activity of ENaC, and impaired water secretion. B. Absorption. The two bottom panels illustrate the process of absorption in normal and CF airway cells. Activation of CFTR permits chloride movement from the lumen, across the cell and into the submucosa. The “low volume” hypothesis of CF pathogenesis proposes that in the absence of functional CFTR, sodium is hyperabsorbed due to loss of ENaC regulation. Chloride is also hyperabsorbed through transcellular and paracellular pathways. Due to the presence of water channels in airway epithelial cells, the increased absorption of sodium and chloride into the submucosa leads to excessive water absorption. The movement of isotonic fluid into the cell layer reduces the volume of the airway surface liquid.

is substantial evidence that the salt concentration of ASL from CF patients and normals is similar and that altered ASL properties affect clearance of airway secretions in CF patients (344–346). Continued investigation of the pathophysiology of CF airway disease is likely to reap therapeutic dividends (347–349).

### 58.3.3 Mutations in the *CFTR* Gene

**58.3.3.1 Nature of Mutations.** Over 1800 putative disease-causing mutations have been reported to the CF Mutation Database (<http://www.genet.sickkids.on.ca/PicturePage.html>). The majority of these mutations involve one or a few single nucleotides; about 40% of the reported mutations cause a change in the amino acid and are termed missense, 8.5% change the amino acid to a termination codon and are termed nonsense, and approximately 12% alter nucleotides known to be crucial to the proper splicing of RNA into messenger RNA. Most of the remaining point mutations involve an insertion or deletion of nucleotides that changes the reading frame of the mRNA and usually introducing a premature termination signal. About 2% of mutations are exonic deletions that leave the reading frame intact. Insertions or deletions involving many nucleotides from one or more *CFTR* exons account for nearly 3% of CF alleles. Several recur in select populations (350–353).

Numerous examples of multiple mutations in the same *CFTR* gene have been published (354–361). In at least three cases, the two alterations appear to work in concert to alter the phenotype produced (354,357,361). Putative disease-associated mutations in the start-site for translation have also been identified (362,363). Several deleterious mutations of the *CFTR* promoter have been reported and correlated with altered *CFTR* RNA expression (364,365).

Over 200 of the mutations reported in *CFTR* appear to be “benign” polymorphisms that do not cause CF ([www.genet.sickkids.on.ca/](http://www.genet.sickkids.on.ca/)). Most notable is the observation that the phenylalanine residue at codon 508 can be changed to cysteine and the isoleucines at codon 506 or 507 can be changed to valine without apparent effect (366). Furthermore, these three missense changes do not cause CF when found *in trans* with a known CF-causing mutation such as F508del. However, there is evidence that mutations that do not change an amino acid can have deleterious effects upon RNA processing. These “synonymous” mutations alter regions that interact with splicing factors that enhance or suppress inclusion of exons in the mature RNA (367,368). Furthermore, population analysis indicates that it is difficult to distinguish rare exonic variants of deleterious effect from those that are benign (369). Thus, variations in *CFTR* exons occurring in a clinical context consistent with CF have to be interpreted with caution, even if there is no predicted change in the encoded amino acid. Numerous DNA sequence variants in the non-coding region of the

*CFTR* gene have also been reported to the Consortium. The majority are in introns within 100 bp of the splice sites. While most of these non-coding variants are likely to have no effect, some may affect splicing by activating cryptic splice sites (370) or by affecting binding of splicing factors as noted above. Two intronic point mutations distant from the splice acceptor and donor sites have been well characterized. One occurs in intron 19, 10 kb from the 3' end of exon 19 while the other is 1.6 kb from the end of exon 11 (43,45). Both activate cryptic splice donor sites and cause the inclusion of novel sequence into the *CFTR* mRNA transcripts.

The advent of nationwide newborn screening has led to a reassessment of the utility of DNA testing to diagnose CF (38). To assess the disease-causing liability of CF mutations, the Clinical and Functional Translation of *CFTR* (CFTR2) project was initiated with support from the CF Foundation in the United States (371). The ambitious goal of the project is to annotate the disease implications of all *CFTR* mutations and to provide this information on a public website. Release of the first version of CFTR2 is imminent.

**58.3.3.2 Frequency and Distribution of Mutations.** One mutation has been found to occur in about 70% of *CFTR* genes from CF patients (29). The mutation is a deletion of three nucleotides which causes the omission of a phenylalanine residue at position 508 in the protein. The mutation has been named F508del, F is the single-letter code for a phenylalanine residue, 508 denotes the codon affected, and del is shorthand for deletion. Although this is the most common CF mutation, its frequency varies considerably among human populations. The highest frequency has been found in Northern European populations where it accounts for 75–88% of CF alleles. The frequency drops to 50–60% in Southern European populations and even lower in the Ashkenazi and African-Americans (29). In Europe, the frequency of  $\Delta$ F508 increases along a southeast to northwest gradient (29,372).

Apart from F508del, a subset of approximately 20 mutations occur with a frequency greater than 0.1% in mixed European Caucasian populations (Table 58-3). In ethnically discrete populations, such as Ashkenazi Jews, Hutterites in Alberta Canada, French-Canadians in the Saguenay-Lac St. Jean region of Quebec, and Native Americans, a few mutations account for nearly 100% of CF alleles (Table 58-4). It has been surmised that carriers of these mutations must have been among the founders of these ethnic groups. Marriages within these groups then maintained these mutations at higher frequencies than observed in the general populations. Screening with the appropriate panel of CF alleles can achieve detection rates over 90% in geographically defined populations (Table 58-4). However, a very large number of mutations (>300) have to be screened to achieve detection above 90% in diverse populations such as France (373). Over 30 mutations occur at high frequency only in select



**TABLE 58-3** CF-Causing Mutations That Occur at a Frequency of 0.001 (0.1%) or Higher in Three Samples of Caucasian CF Patients

Mutation	Consequence	% of CF Alleles		
		CFGAC <sup>a</sup> (n = 43,849)	U.S. CFF <sup>b</sup> (n = 25,030)	Biomed <sup>c</sup> (n = 27,177)
G85E	Gly → Glu at 85	0.15	0.17	0.21
R117H*	Arg → His at 117	0.30	0.67	0.29
621 + 1G → T	mRNA splicing defect	0.72	0.90	0.54
711 + 1G → T	mRNA splicing defect	0.11	<0.1	0.11
1078delT	Frameshift with premature termination	0.13	<0.1	0.13
R334W	Arg → Trp at 334	0.12	0.22	0.16
R347P	Arg → Pro at 347	0.24	0.24	0.20
A455E*	Ala → Glu at 455	0.14	0.12	0.20
ΔI507	Deletion of Ile at 507	0.21	0.31	0.23
ΔF508	Deletion of Phe at 508	66.0	68.8	66.8
1717-1G → T	mRNA splicing defect	0.65	0.28	0.83
G542X	Nonsense-mediated mRNA decay	2.42	2.43	2.64
G551D	Gly → Asp at 551	1.64	2.12	1.50
R533X	Nonsense-mediated mRNA decay	0.73	0.90	0.75
R560T	Arg → Thr at 560	0.15	0.19	0.18
1898 + 1G → A	mRNA splicing defect	0.12	<0.11	0.22
2789 + 5G → A*	mRNA splicing defect	0.12	0.31	0.11
R1162X	Premature termination	0.29	0.22	0.51
3569delC	Frameshift with premature termination	0.12	0.17	0.14
3849 + 10 kbC → T*	mRNA splicing defect	0.24	0.67	0.15
W1282X	Nonsense-mediated mRNA decay	1.22	1.41	1.0
N1303K	Asn → Lys at 1303	1.34	1.25	1.64

Some patients were included in more than one sample.

Asterisk indicates a mutation that may be associated with normal or borderline sweat Cl<sup>-</sup> values.

<sup>a</sup>Data from Cystic Fibrosis Genetic Analysis Consortium (374).

<sup>b</sup>Data from CFF Patient Registry, 1997.

<sup>c</sup>Data from Estivill et al. (760).

**TABLE 58-4** Populations Where Greater Than 90% of CF Alleles Have Been Identified

	# Mutations	% CF Alleles	%ΔF508	Reference
Alberta Hutterites	2	100	31	(761)
Saguenay-Lac St. Jean	11	100	60	(762)
Tyrol, Austria	16	100	74.6	(763)
Native American	4	100	0	(379)
Welsh	29	99.5	71.6	(764)
Brittany Celts	19	98	81.16	(765)
Ashkenazi Jews	5	97	43	(26)
Germany	54	95.4	72.0	(766)
Belgian	17	94.3	78.9	(767)
Northern Ireland	30	93.9	68	(768)
France	310	93.56	67.18	(373)
Northwest England	11	91.5	81.15	(769)
Southern France	40	91.2	64	(770)
Spain	75	90	53.2	(771)

populations (374). Examples include the Y122X mutation, which accounts for 48% of CF alleles in Reunion Island but is rare elsewhere (375). The remainder of the mutations reported to the CF Mutation Database is rare, occurring on only one or a few chromosomes.

Mutations in the CFTR genes of non-Caucasian CF patients have also been reported. Individuals of African descent living in the United States have been extensively studied. Similar to Caucasians, ΔF508 is the most common mutation (48%) in this population probably due to

admixture (376). Several of the African-American mutations appear to be of native African origin (36,376,377). A study of 82 African-Americans identified putative deleterious CF mutations in 96% of patients and discovered a subset of eight common “African-American” mutations accounted for 23% of CF alleles (376). For example, the second most common mutation in African-Americans, 3120 + 1G → A was found in four of six CF chromosomes from native Africans (377). Three mutations have been found to account for all CF alleles in the Zunis tribe of native Americans (378,379). Interestingly, one of these mutations was originally reported in Northern Italian Caucasians. A small study of Asian CF chromosomes from Japan, Korea, Thailand, and Vietnam revealed five different mutations, one in homozygosity (380). A different mutation, 1898 + 5G → T, was found on 3 of 10 chromosomes in Chinese patients living in Taiwan (381). None of these Asian mutations was previously reported in Caucasian patients. Together, these studies demonstrate that non-Caucasian populations have CF mutations that are unique as well as those acquired by admixture with Caucasians.

To determine the possible origin of disease-causing mutations, DNA polymorphism haplotypes created by CF-linked markers have been analyzed on chromosomes bearing some of the more common CF gene mutations. Several studies demonstrate that the predominant mutation, F508del, is almost exclusively associated with one marker haplotype (6,382,383). This strongly suggests that this common mutation is of a single origin. The timing of this mutation in human evolution is a matter of some debate with estimates ranging from 3000 to 53,000 years ago (164,384). Haplotype analysis indicates that mutations G551D and G542X also have single origins (385). The frequency of these less common mutations also varies among populations. The G551D mutation is found on 5% of CF chromosomes from Scottish patients, 3% of CF chromosomes in North American Caucasians, but rarely on CF chromosomes from patients in Southern Europe (386,387). The nonsense mutation, G542X, appears to occur in most populations and varies between 1% of CF chromosomes from African-American patients to 12% of chromosomes from Ashkenazi Jewish patients (26,355). These differences in population distribution may reflect differences in the timing of each mutation during human evolution, differences in selective pressures exerted upon each mutation, or both. One mutation, R553X, is associated with a significantly different haplotype in African-Americans than that observed in Northern Europeans. Since this mutation occurs at a region of DNA vulnerable to mutation (a CG dinucleotide), most likely this mutation has arisen several times during human evolution on chromosomes with different haplotypes (385). Several other CF mutations have recurred during human evolution including R117H, R334W, R347P, R1162X, and 3849 + 10 kb C → T (357,388).

### 58.3.4 Effects of CF Mutations on CFTR Function

Understanding the consequences of mutations upon function provides a starting point for dissecting the complicated pathway from the gene defect to pathology of the organism, and opens important avenues for novel therapeutic approaches to CF. The effects of only a small fraction of the over 1800 mutations upon CFTR function have been assessed. In most cases, the effect of a mutation has been inferred from studies of mutant CFTR overexpressed in immortalized cells. A few studies have analyzed mutated CFTR in primary cells or native tissue. Therefore, our understanding of the consequences of mutations upon CFTR function is still evolving. However, a clear picture has been drawn for the common mutation F508del and several of the less common mutations. The effects of mutations upon CFTR function have been grouped into classes (389). The number of mutation classes differs somewhat among publications (149,390) and the originally proposed five classes of mutations has high utility since it correlates well with disease severity (133). Mutations in Class I, II, and III generally have severe effects upon CFTR synthesis or function and are associated with more severe forms of the disease while those in Class IV and above can have moderate effects resulting in milder forms of the disease.

**58.3.4.1 Mutations that Alter RNA Processing that Severely Reduce or Cause Loss of Protein Synthesis (Class I).** About one half of the mutations reported to the CF Mutation Database are predicted to affect RNA processing. Several of these mutations are less common than F508del but not rare in Caucasians (Table 58-3). Some introduce a premature signal for termination of translation. This group includes those that substitute an amino acid with a termination signal (nonsense mutation), those that delete or insert one or more nucleotides that are not multiples of 3 (frameshift mutation), and those that cause aberrant exon splicing (splice-site mutation). The common effect of these mutations is severe reduction in mRNA transcript level from the gene containing the alteration due to a phenomenon known as nonsense-mediated mRNA decay (391–396). The mechanism underlying this process is conserved from yeast to mammals and it plays a key role in regulating gene expression (397). The effect of the W1282X nonsense mutation (most common mutation in Ashkenazi CF patients) is not clear since two studies report severe reduction and one reports no significant reduction in mRNA levels (392,396,398). A nonsense mutation that is not associated with RNA decay is R1162X, an allele, common in Northern Italy (396,399). Severe reduction in transcript levels would be expected to cause a corresponding reduction or more likely absence of the CFTR protein. This has been demonstrated for patients carrying two nonsense mutations (400,401).

One nonsense mutation in *CFTR* (E831X) was found to be unexpectedly associated with mild PS CF (402). Further investigation revealed that the mutation occurred near a 5' splice site and that alternative splicing enabled the production of a RNA transcript free of the premature termination codon (PTC). Loss of the three nucleotides encoding the PTC caused omission of the glutamic acid at codon 831. However, CFTR missing this residue was properly folded, trafficked to the cell membrane, and partially functional. Retention of some CFTR function appears to explain the mild phenotype noted in individuals with two copies of E831X (402).

Premature termination mutations can also cause exon skipping and aberrant RNA splicing. Three nonsense mutations (E60X, R75X, and R553X) have been reported to cause skipping of the exon in which they reside (396,403). In each case, loss of the exon causes a frameshift and introduction of additional PTCs. The nonsense mutation E92X, caused by an alteration in the first nucleotide of exon 4, is associated with exon 4 skipping and tissue-specific aberrant splicing (404). In nasal epithelial cells, a novel exon is added to the full-length *CFTR* mRNA. The novel exon, however, contains in-frame termination codons that would be expected to lead to loss of the mRNA with the novel exon.

Splice-site mutations affect RNA processing by altering the composition of the *CFTR* messenger RNA. Two examples are 621 + 1G → T and 711 + 1G → T that account for 0.7% and 0.1% of mutant CF alleles, respectively (Table 58-3). The first mutation alters the splice-site immediately following exon 4 of *CFTR* and produces two aberrantly spliced RNAs, both of which retain an open reading frame (405,406). The 711 + 1G → T mutation changes the splice-site following exon 5 causes skipping of the entire exon. Since the number of nucleotides forming exon 5 is a multiple of 3, the loss of this exon also leaves the reading frame intact (405). These aberrant transcripts may be translated into protein products that may be unstable, non-functional, or partially functional. Determining which of these possibilities occur usually requires protein expression and analysis.

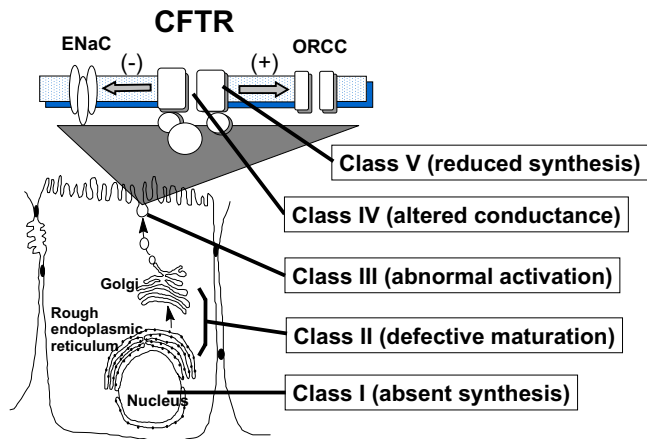
**58.3.4.2 Mutations that Cause Defective CFTR Maturation (Class II).** In a seminal paper, Smith and colleagues suggested that the majority of CF mutations that change a single amino acid caused abnormal processing of the mutant protein (407). Indeed, the study of CFTR processing has emphasized the critical role that cellular quality control mechanisms play in distinguishing misfolded mutant protein (408,409). For example, two mutations, F508del and the most common missense mutation G551D (~1.6% worldwide) had very different effects when transiently expressed in heterologous cells. CFTR bearing  $\Delta$ F508 was incompletely glycosylated suggesting that it was being improperly processed (407). On the other hand, CFTR bearing G551D was fully glycosylated and predicted to be properly processed. Localization of mutant CFTR by immunocytochemical stains of

sweat glands from CF patients showed that  $\Delta$ F508-CFTR was contained within the cytoplasm in a perinuclear distribution consistent with abnormal processing, while G551D-CFTR localized to the cell membrane similar to properly processed wild-type CFTR (210,401). Whether F508del-CFTR is misprocessed in a tissue-specific manner has been a matter of debate (410).

Further studies have shown that  $\Delta$ F508 is a temperature-sensitive mutation (411). CFTR bearing  $\Delta$ F508 can be found in the cell membrane when expressed in cells growing at lower temperatures (25 °C instead of 37 °C) such as *Xenopus* oocytes and cells from an insect (fall armyworm) (251,253). In fact, F508del-CFTR isolated from the insect cells can function as a cAMP-dependent chloride channel although its stability appears to be reduced (412,413). A variety of other CF missense mutations have been shown to interfere with CFTR processing (414–416). The missense mutation G480C has been found in three African-American patients with pancreatic insufficiency. CFTR bearing this mutation functioned similar to wild-type CFTR in *Xenopus* oocytes at 19 °C but was improperly processed in mammalian cells at 37 °C (417). Two mutations A455E and P574H associated with a milder form of pancreatic disease were shown to alter CFTR processing. However, some mature protein was synthesized that functioned similar to wild-type CFTR (418).

**58.3.4.3 Mutations Associated with Partially Functional CFTR (Classes III and IV).** If mutant CFTR protein is trafficked to the cell membrane, the possibility exists that the protein may retain some function. Determination of the amount of residual function can provide considerable insight into the role of CFTR dysfunction in CF pathophysiology. Structure/function studies of select CF missense mutations indicate that one or more aspects of CFTR function can be affected. Two mutations of the same amino acid in the first ATP-binding domain (G551S and G551D) alter the activation of CFTR (Figure 58-6; Class III) (255). High levels of stimulation achieved by using non-hydrolyzable forms of cAMP (CPT-cAMP) and a potent phosphodiesterase inhibitor (IBMX) can activate these mutant molecules (255,256). Newer CFTR potentiators selected to activate CFTR bearing gating mutations such as G551D have shown considerable therapeutic promise (419,420).

Missense mutations in cytoplasmic loops of the TMDs appear to affect CFTR channel regulation (414,415,421,422). Other mutations affect conductance through the CFTR chloride channel (Figure 58-6; Class IV). Five mutations (R117H, R334W, R347P, R347H and P574H) associated with non-classic forms of CF have been extensively evaluated. In each case, the mutant CFTR was shown to be functional although the degree of retained function varied considerably (259,260,266,423). The effects of the R347P and R347H mutations were primarily confined to changes in ion flow through the CFTR chloride channel (260,266) while the



**FIGURE 58-6** The Cellular Consequences of Five Classes of Mutations.

P574H mutation selectively altered the duration of channel opening (418,423). However, the R117H mutation altered the rate of ion flow through the CFTR channel and reduced the amount of time the channel remained open (259,260). The observation that two properties of the channel were altered by a single mutation suggests that the region of CFTR that forms the channel and the region that regulates the chloride channel may overlap. Conversely, this mutation may have a pervasive allosteric effect on the two functional domains of CFTR.

**58.3.4.4 Mutations Associated with Reduced Levels of Wild-Type CFTR (Class V).** Some splice-site mutations have an intermediate effect upon splicing that permits the production of a low level of properly spliced mRNA. An excellent example is the 3849 + 10kbC → T mutation. This mutation activates a cryptic splice donor site in intron 19 causing the inclusion of a novel 84 basepair exon into CFTR mRNA transcripts (45). This insertion does not alter the reading frame but would be predicted to encode a truncated protein. However, the mutation does not alter all transcripts produced by a CFTR gene carrying the 3849 + 10kbC → T mutation. The 3849 + 10kbC → T mutation is found on 0.2% of CF chromosomes worldwide and is relatively common in the Ashkenazi Jewish population (4% of CF alleles) (26,374). About 8% of nasal epithelial CFTR mRNA transcripts are properly spliced in a patient homozygous for this mutation (45) and would be expected to produce a small amount of wild-type CFTR protein. Therefore, some splice-site mutations are predicted to reduce the amount of wild-type CFTR protein produced.

**58.3.4.5 Mutations with Other Effects.** Some mutations have been shown to affect the ability of CFTR to regulate other proteins. For example, CFTR bearing G551D, a mutation that causes pancreatic insufficiency and severe pulmonary disease, retains some chloride channel function but cannot activate the ORCC (424). However, A455E, a mutation associated with mild lung disease, permits CFTR to function as a chloride channel and as a regulator of the ORCC. These results indicate

that the two functions of CFTR are separately mutable and suggest that alteration of the regulatory function of CFTR may have some consequence upon phenotype (424). Interestingly, CFTR regulation of the ENaC and of the inwardly rectifying potassium channel ROMK2 is also altered by the G551D mutation (304,425,426). Analysis of select disease-associated missense mutations in other regions of CFTR did not show altered regulation of the ORCC (292). Thus, one region of CFTR may be essential for its regulatory function. Mutations have also been shown to affect CFTR localization in polarized epithelia. Nonsense mutations in the last CFTR exon such as S1455X produce transcripts that escape nonsense-mediated mRNA decay (427). These transcripts are predicted to encode truncated forms of CFTR that are missing regions that mediate efficient apical retention of CFTR (428–431). Other mutations have been reported that create a novel internalization signal that reduces cell surface CFTR (432).

**58.3.4.6 Correlating CFTR Function and CF Phenotype.** Our understanding of this relationship falls into three groups: CFTR that is absent or degraded (Class I and II), abnormally functioning (Class III and IV), or reduced (Class V). Complete loss of functional CFTR, whether due to absence of the mRNA, defective protein processing, or inactivation by a mutation, results in classic CF: pancreatic exocrine dysfunction, elevated sweat electrolytes, absence of the vas deferens, and chronic life-limiting pulmonary disease. Mutations that alter but do not abolish the function of CFTR can give rise to mild CF. For example, residual CFTR-mediated chloride secretion in rectal epithelia correlates with CFTR genotype and with later onset of disease, higher frequency of pancreatic sufficiency, and milder lung disease (433). Changes in the conduction properties of the channel are generally associated with less severe phenotypes (e.g., R117H, R334W, R347P) (260). In some cases, the amount of partially functional protein is correlated with the development and severity of lung disease (e.g., R117H and P574H) (357,418,423). Reduction in the level of normally functioning CFTR due to mRNA splicing defects generally produces non-classic forms of CF (45,434). Thus, the degree of residual CFTR function appears to correlate with severity of phenotype.

### 58.3.5 Relationship Between CFTR Genotype and Phenotype

The section above described our current understanding of the effect of disease-associated mutations upon CFTR function, a fundamental aspect of pathogenesis. Extensive studies have also been performed to determine the degree to which CFTR genotype correlates with the features that compose the CF phenotype. Two general approaches have been taken to assess the association between CFTR genotype and phenotype. One method is to determine severity of disease in patients selected by



genotype, while the second involves determining genotypes in patients with similar degrees of illness. From these studies it is apparent that some features of the disease are correlated with genotype. CFTR genotype influences severity of pancreatic disease, degree of sweat chloride abnormality, and age of onset of disease (Table 58-5). For pulmonary disease, an association exists but is confounded by other variables including genetic background and environment. Complications such as MI and pancreatitis are influenced by the nature of the CF mutation (435–437).

**58.3.5.1 CFTR Genotypes Associated with Classic CF.** The commonness of the F508del mutation has facilitated a thorough analysis of the phenotype associated with this mutation. As expected, homozygosity for F508del is associated with the classic form of CF including pancreatic insufficiency, significantly elevated sweat chloride levels, and chronic lung disease (89,438) Pulmonary disease in F508del homozygotes can vary from mild to very severe (85,89,438,439). Collaborative reports

have been published comparing the phenotypes of compound heterozygotes carrying one of the less common CF mutation and F508del with patients homozygous for F508del. Patients heterozygous for G551D/ΔF508 were clinically indistinguishable from ΔF508 homozygotes, apart from a decreased risk of MI (435,440). Interestingly, a higher rate of MI was observed in a small group of patients with the G542X/ΔF508 genotype (99).

Shortly after the cloning of the CFTR gene, two patients with mild pulmonary disease were discovered carrying nonsense mutations in each CFTR gene (441). This observation indicated that the CFTR protein was not essential for life and suggested that the absence of the CFTR protein may be less harmful than the presence of mutant protein. Subsequent studies on larger groups of patients analyzing a nonsense mutation common in the Ashkenazi population (W1282X) indicated that individuals homozygous for this mutation had disease that was very similar to that observed in individuals carrying this mutation paired with ΔF508 or with individuals

**TABLE 58-5 Association Between CFTR Mutations and Specific Features of the CF Phenotype**

Mutation <sup>a</sup>	FEV1% Predicted	% Pancreatic Sufficient (n)	Sweat Chloride Concentration in mM (n)	Age at Diagnosis in Years (n)	Reference
<i>Classic CF</i>					
621 + 1G → T	73 ± 26 (41)	2.0 (51)	100 ± 20 (22)	0.8 ± 1.1 (51)*	(89)
R347P <sup>b</sup>	Not reported	8 (12)	114 (9)	Not reported	(772)
ΔF508	70 ± 27 (269)	2.5 (396)	106 ± 22 (328)	1.7 ± 3.0 (392)	(89)
1717 – 1G → A	68 ± 26 (20)	3.3 (30)	107 ± 36 (26)	2.0 ± 4.4 (28)	(89)
G542X	67 ± 27 (81)	0 (147)	109 ± 23 (128)	1.6 ± 3.1 (147)	(89)
S549R(T → G) <sup>c</sup>	Not reported	9 (16)	117 ± 25 (14)	0.9 ± 1.0 (16)	(361)
G551D	65 ± 24 (61)	1.4 (73)	101 ± 16 (58)	1.9 ± 2.8 (79)	(435)
R553X	64 ± 25 (36)	2.0 (51)	105 ± 18 (46)	1.7 ± 2.7 (52)	(89)
1811 + 1.6 kbA → G <sup>d</sup>	65 ± 25 (17)	0 (17)	98 ± 12 (17)	2.8 ± 4 (17)	(43)
R1066C <sup>e</sup>	64 ± 23 (15)	0 (21)	112 ± 29 (18)	26 ± 4.0 (21)	(773)
W1282X	75 ± 26 (12)	0 (16)	110 ± 18 (13)	4.0 ± 9.9 (17)	(89)
N1303K	69 ± 24 (42)	0 (59)	104 ± 24 (56)	1.5 ± 2.7 (58)	(89)
<i>Non-classic CF</i>					
102T → A + S549R(T → G) <sup>f</sup>	Not reported	50 (6)*	79 ± 27 (5)	9.0 ± 13 (6)	(361)
P67L <sup>b</sup>	Not reported	77 (12)	57 ± 9 (12)	22.5 ± 11.3 (12)	(774)
G85E <sup>g</sup>	80 ± 8 (6)	31 (13)**	107 ± 4 (5)	4.2 ± 4.7 (13)*	(775)
R117H	73 ± 22 (22)	87 (23)***	82 ± 19 (20)***	10.2 ± 10.5 (23)**	(89)
R334W	61 ± 29 (6)	40 (10)***	108 ± 19 (10)	6.3 ± 7.0 (10)	(446)
A455E <sup>h</sup>	74 ± 26 (29)**	79 (33)***	Not reported	15.0 ± 10.6 (33)***	(86)
A455E	85 ± 19 (9)**	78 (9)*	79 ± 19 (9)**	5.7 ± 4.8 (9)**	(87)
3272 – 26 A → G <sup>b</sup>	63 (5)	80 (5)	105 (5)	14.7 (5)	(370)
3849 + 10 kb C → T <sup>b,i</sup>	63 (10)	73 (11)	40 (11)	Not reported	(45)
3849 + 10 kb C → T <sup>b,j</sup>	51 ± 20 (14)	67 (15)	62 ± 17 (14)	12.5 ± 8.8 (15)	(455)

<sup>a</sup>Patients carried ΔF508 on their other CF chromosome unless indicated and phenotype features were compared to age- and sex-matched ΔF508 homozygotes from the same treatment center. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

<sup>b</sup>Statistical comparison with ΔF508 homozygotes was not performed.

<sup>c</sup>Patients carried S549R (T → G) on their other CF chromosome.

<sup>d</sup>Patients carried mutations ΔF508, ΔI507, 1609 del CA, G542X, K710X, and N1303K on their other CF chromosome.

<sup>e</sup>Patients carried ΔF508 (14), R1066 (2), R334W (1), G542X(1), 712 – 1G → T (1) and 3905insT(1) on their other CF chromosome.

<sup>f</sup>Patients carried –102T → A + S549R(1), R334W(1), ΔF508(2), G542X(1), or S945L on their other CF chromosome.

<sup>g</sup>Patients carried ΔF508(9), ΔI507(2), 712 – 1G → T(1), or G85E(1) on their other CF chromosome.

<sup>h</sup>Patients carried ΔF508(25), E60X(4), G542X(2), R553X(1), or 1717 – 1G → A(1) on their other CF chromosome.

<sup>i</sup>Patients carried ΔF508(9) or W1282X(2) on the other CF chromosome.

<sup>j</sup>Patients carried ΔF508(5), W1282X(5), G85E(1), or unknown(4) on their other CF chromosome.

homozygous for  $\Delta F508$  (442). Studies of other nonsense mutations such as R1162X and R553X have revealed subtle phenotypic differences from  $\Delta F508$  homozygotes (354,443,444). A collaborative study involving 32 laboratories worldwide studied this issue in a large group of patients carrying  $\Delta F508$  compounded with the nonsense mutations G542X, R553X, or W1282X and 18 patients carrying nonsense mutations in each gene (Table 58-5). No significant differences were seen in the compound heterozygotes when compared to individuals homozygous for  $\Delta F508$  (89). These data indicate that nonsense mutations are generally associated with the same phenotype as observed in  $\Delta F508$  homozygotes, although at least one exception exists (see above).

**58.3.5.2 CFTR Genotypes and Severity of CF Lung Disease.** Pulmonary disease is the primary cause of morbidity and mortality in CF patients. Thus, the relationship between CFTR mutations and lung disease severity has been of high importance in CF research. Studies of small numbers of monozygotic and dizygotic twins and siblings with CF indicated that genetic factors exist that influence pulmonary disease severity (439,445). Furthermore, pancreatic sufficiency appeared to be associated with improved respiratory function (84). Whether this was due to improved nutritional status or a direct consequence of the mutations carried by PS patients was unclear. However, initial genotype/phenotype studies did not show a correlation between mutations associated with a high likelihood of pancreatic sufficiency, such as R117H and R334W and severity of pulmonary disease (89,446). However, a mutation associated with pancreatic sufficiency, A455E, was shown to confer a milder pulmonary phenotype (Table 58-5). The commonness of this mutation in the Netherlands and in French-Canada facilitated the comparison of A455E heterozygotes with age and sex matched  $\Delta F508$  homozygotes from the same population (86,87). As with other studies of mutations associated with non-classic CF, patients with A455E were diagnosed later and most but not all were PS. Pulmonary function was improved in patients bearing A455E with FEV1 and forced vital capacity being significantly higher than in  $\Delta F508$  homozygotes. A455E patients also had lower rates of colonization with *P. aeruginosa* than their  $\Delta F508$  homozygote counterparts (86). A study of 267 German patients suggested an association between the type and location of CFTR mutations and predilection to chronic *P. aeruginosa* colonization (447). These studies raise the possibility that CFTR plays a role in bacterial colonization, an important aspect of lung pathophysiology in CF patients. Whether CFTR binds bacteria directly or is involved in the bacterial adherence properties of the respiratory epithelia is not clear (65,338,448–452).

**58.3.5.3 CFTR Genotypes Associated with Non-Classical CF.** Selection of patients with non-classic phenotypes and subsequent determination of mutations has provided considerable insight into the role of CFTR in CF pathogenesis. Prior to the identification of the

CFTR gene, pedigree analysis and DNA studies suggested that pancreatic status was determined by genetic factors (98,453). An extensive analysis of over 500 CF patients attending a single clinic in Toronto, Canada, provided evidence that genotype strongly influences pancreatic phenotype. Five mutations (R117H, R334W, R347P, A455E, and P574H) were found exclusively in PS patients (99). The influence of CFTR genotype upon severity of pancreatic disease has been confirmed by multicenter collaborative studies (Table 58-5). Analysis of 23 patients with the genotype R117H/ $\Delta F508$  revealed a high association with pancreatic sufficiency and provided evidence that R117H also influences the degree of sweat chloride abnormality and age of onset of disease (89). The association between mutation and pancreatic status is not absolute; however, since 3 of the 23 R117H/ $\Delta F508$  compound heterozygotes in the latter study were PI and 10 of the 396  $\Delta F508$  homozygotes were PS (89). Incomplete association with pancreatic sufficiency has been demonstrated for several other mutations (Table 58-5). For example, 9 of 15 patients carrying R334W combined with either  $\Delta F508$ ,  $\Delta I507$ , N1303K, G542X, or R1162X were PS (379). Therefore, these mutations can be associated with a milder pancreatic phenotype but other factors such as environment and genetic background must play a role in severity of pancreatic disease.

Family and DNA marker studies also suggested that patients with non-classic CF had different CFTR mutations than those with classic CF (454). Not unexpectedly, these patients carry splice mutations 3849 + 10 kb C  $\rightarrow$  T and 2789 + 5G  $\rightarrow$  T (45,434,455). A third mutation, a substitution of serine for glycine at codon 551 (G551S) was found in two older sisters with atypical CF (44). This mutation is interesting since a different substitution in the same amino acid (aspartic acid for glycine (G551D) causes classic PI CF. Other mutations associated with non-classic CF phenotypes in addition to those listed in Table 58-5 include P205S and L206W (456–458).

**58.3.5.4 Contribution of Modifier Genes to the CF Phenotype.** CF is a variable disorder, even among patients with identical CFTR genotypes (438). Case reports of patients who deviate from the clinical profile associated with a particular CFTR genotype emphasize the importance of other factors at the level of the individual patient (441,459–461). Several lines of evidence suggest that differences in genetic background accounts for a considerable fraction of disease variability, especially for the lungs (450,462,463). One of the most effective methods to determine the degree to which genes influence the occurrence of disease manifestations is to study related individuals. Siblings affected with CF demonstrate substantial similarity for a number of CF traits including MI, pancreatic status, and lung disease severity (%FEV1) (49,113,439,464,465). Although siblings share, on average, 50% of the variation in each autosomal gene, they also share similar environments (e.g., household, schooling, medical care). Dissection

of the genetic and environmental contribution to traits that are similar in siblings is facilitated by twin study. However, collection of data from sufficient numbers of affected twins requires large multicenter collaborative projects. The European CF Twin and Sibling Study demonstrated that a combined measure of body mass (weight for height percentage) and pulmonary disease showed greater similarity among 29 monozygous (MZ) twins than 12 dizygous (DZ) and 277 siblings pairs affected with CF. This difference was interpreted as evidence of genetic effect since MZ twins share 100% of their genes and DZ twins and sibling share 50% of their genes (466). A subsequent study of a larger number of twins and siblings was able to demonstrate that modifier genes play a substantial role in the variation in lung function as exhibited by robust estimates of heritability (0.54–1.0) (467). Affected MZ twins were also shown to have more similar measurements of chloride secretion across rectal and airway epithelia than DZ twins and siblings, indicating that modifier genes independent of CFTR influence this trait (468,469).

Identification of the genes and specific variant therein responsible for variation in traits under genetic control has been approached by candidate gene and genome-wide analysis (470,471). CF patients grouped according to disease status have been analyzed for significant differences in genotype frequencies in a number of biologically plausible candidate genes. Of the two dozen or so genes reported to show association with some aspect of CF disease severity, only a few have shown association in two or more independent samplings of CF patients. The latter is a key issue since population stratification can lead to false positive associations (472). Two genes, *transforming growth factor  $\beta$ 1* and *mannose binding lectin 2*, have stood the test of replication, although not all studies come to the same conclusions (473–478). Meta-analysis of published studies supports a role of *MBL2* in earlier acquisition of *P. aeruginosa*, lower lung function, and higher rate of lung transplantation (479). However, as more studies are performed in different CF populations, it is likely that true modifier genes will consistently demonstrate association, as noted for other complex traits (480).

Diabetes is a complication that increases in prevalence with age such that 40–50% of adults with CF are affected (481). Twin and sibling analysis revealed that risk for diabetes in CF is highly heritable with estimates approaching 1.0 (482). Evidence that a family history of type 2 diabetes increases risk for diabetes in a family member with CF (OR 3.1) suggested that the two forms of diabetes might have common genetic origins (108). Indeed, a single nucleotide polymorphism (SNP) near the *TCF7L2* gene that has been consistently associated with risk for type 2 diabetes demonstrates significant association with diabetes in CF (108). As over 40 genes have been associated with diabetes in the general population, it is likely that other risk genes will occur in common between CFRD and type 2 diabetes.

Liver disease provides a second example where a risk gene in the general population acts as a modifier of the same trait in CF. Hepatocellular disease is common in CF patients (see above). The *SERPINA1* Z variant that has been associated with risk for a variety of liver diseases in the general population was shown to dramatically modify risk for CF-related portal hypertension (483). Although the Z alleles are rare (~2%), the high risk of severe hepatic disease associated with this variant (OR ~ 5) provides a potentially useful clinical marker for this important complication.

While association studies provide a method to test candidate genes for influence upon phenotype, a different approach, akin to positional cloning, is required to find novel modifier genes. Using this approach in mice, Rozmahel and coworkers identified a locus in CF mice responsible for prolonged survival from fatal intestinal obstruction (484). Strain-specific differences in survival were used to map a locus on mouse chromosome 7 that was associated with survival. Additional loci for intestinal obstruction, body weight, and lung disease manifestations in CF mice have been reported (485–488). Studies of surviving mice suggest that a compensatory mechanism exists in their epithelial cells that facilitates sodium and chloride transport in the absence of CFTR (484). Since 15% of newborn CF patients exhibit a similar trait (neonatal intestinal obstruction or MI), a region of conserved synteny on human chromosome 19 was investigated for a modifying effect upon the MI phenotype. DNA markers from the human locus showed a high degree of allele sharing in siblings concordant for and a significantly lower than expected degree of allele sharing in sibs discordant for MI (489). These data suggested the existence of genetic variant (CFM1) independent of CFTR that contributes to the development of MI in humans. As of this time, a modifier gene has not been identified. Studies of twins in the U.S. CF Twin and Sibling Study have shown that the MI trait is primarily determined by genetic factors (119). Variable DNA markers from the CFM-1 region on chromosome 19 fail to demonstrate linkage of this region to MI in a larger number of CF siblings. However, suggestive linkage was observed to several chromosomal regions (119). Regional analysis of the chromosome 8p23.1 locus revealed significant association between MI and a haplotype composed of three SNPs located in intro 3 of the methionine sulfoxide reductase (*MSRA*) gene (490).

Genome-wide association and linkage methods have been applied to the search for genetic modifiers of lung function variation in CF. The *interferon-regulated developmental regulator-1* (*IFRD1*) was identified as a potential modifier of CF lung disease by performing genome-wide analysis on pooled CF patient with severe or mild lung disease. Promising candidates such as *IFRD1* were selected using hierarchical analysis. Replication of association of *IFRD1* variation and lung function in CF siblings and knock-out studies in mice supported



a modifier role for *IFRD1*. *IFRD1* modulates transcription during neutrophil differentiation, and loss of *IFRD1* leads to decreased effector function and reduced inflammation. Wright and colleagues identified two loci (chr 11p and chr 20q) that contained genetic modifiers of the pulmonary function measure FEV1 scaled as a percentile of severity among CF patients (491,492). The chr11 locus was identified by association and it is flanked by two genes (*APiP* and *EHF*) that have roles in apoptosis and lung development. The *IFRD1* and *APiP/EHF* loci likely contain common variants that account for a small fraction of the variation in CF lung function (<2%). The chr20 locus was identified by linkage in affected siblings and region-wide SNP association was noted within the linkage region although a specific gene or mechanism was not implicated. The responsible modifiers in the chr 20 region are likely to be rare (or less frequent) DNA variants as the regional association accounted for only a small fraction of the linkage signal in the CF siblings (492). Further, genome-wide analyses are expected and are likely to confirm novel loci and genes, most of which are unlikely to have been chosen in a candidate gene approach.

**58.3.5.5 Contribution of Environment to the CF Phenotype.** Analysis of lung function measures in twins and siblings when living together and when subsequently living apart demonstrated that genetic factors independent of *CFTR* and environmental factors each accounted for half of the variation in lung function (493). The same appears to be true for nutritional factors (494). Assessing environmental contribution to phenotype is challenging due to the variety of components involved and the lack of objective measurements for many components. Despite these limitations, evidence has been generated linking passive cigarette smoking with reduced pulmonary function (495–497). Interaction between secondhand smoke and variation in the modifier *TGFβ1* causes considerable reduction in lung function measurements (498). Air quality also influences lung function measurements and frequency of pulmonary exacerbations in CF patients (499). Pulmonary colonization with *P. aeruginosa* and *B. cepacia* is an environmentally mediated event that is associated with reduced longevity (78,500,501). The variable effect of both organisms upon pulmonary function indicates that other factors contribute to outcome for the individual patient. These factors are likely to include genetic variability in the bacteria and the host (65,83,502,503). Aggressive nutritional intervention has also been shown to affect outcome (75,504–506). Interestingly, patients vary in their response to nutritional treatment, suggesting that other environmental factors, genetic factors, or both may play a role (507). Socioeconomic status (SES) has a significant influence upon outcome. Not surprisingly, low SES has been associated with poorer growth, higher frequency of pulmonary exacerbations, and reduced survival (508,509).

**58.3.5.6 CFTR Genotypes Associated with Non-CF Phenotypes.** A series of conditions with one or more phenotypic features in common with CF have been shown to be associated with mutations in *CFTR*. In most cases, disease in one organ system (vas deferens, pancreas, sweat gland, sinuses) is similar to that observed in CF patients. Infertility due to CBAVD is an almost invariable feature in males with CF (510). This finding has also been reported in otherwise healthy males and was defined as a separate disorder inherited in an autosomal recessive fashion (511). Screening of these patients for mutations observed in CF patients has revealed that a significant portion carry the ΔF508 mutation (40%) while another 20% are heterozygous for a less common CF mutation and about 10% carry two CF mutations (42,43,512). More extensive mutation analyses of men with CBAVD uncovered a putative disease-associated mutation in both *CFTR* genes in approximately 70% (373).

The distribution of *CFTR* mutations clearly differs between CF and CBAVD patients with partially functional alleles at much higher frequency in CBAVD patients (43,373,513). Furthermore, the 5T splice variant of the intron 8 splice acceptor site is commonly found in men with CBAVD (43). However, the 5T variant is also found in about 10% of individuals in the general population and in fathers of CF patients who also carry ΔF508 on their other chromosome. Thus, 5T is variably penetrant with estimates of penetrance ranging from 0.56 to 0.60 (43,514). The partial penetrance appears to be explained by variability in the length of a polymorphic TG tract immediately adjacent to the 5T variant (515,516). Males with a CF mutation paired with a *CFTR* gene bearing the 5T variant that has 12 or 13 adjacent TG repeats are much more likely to have CBAVD than those with the same *CFTR* genotype but 11 TG repeats (517). Variation in TG repeats affects the efficiency of exon 9 splicing, although the mechanism underlying this process is a matter of debate (518–520).

Males with CBAVD can have normal or elevated sweat chloride values and can display defective chloride conductance in airway epithelia that is qualitatively different from patients with CF (521,522). Although extensive longitudinal data are lacking, it appears that some men with isolated CBAVD and mutations in each *CFTR* gene do not develop chronic, life-limiting pulmonary disease (523). However, detailed study of bronchoalveolar lavage fluid from CBAVD males with two *CFTR* mutations revealed subtle evidence of infection and inflammation (524). Thus, subclinical lung disease may exist in this group of individuals, suggesting that follow-up for signs of progressive lung disease characteristic of CF may be prudent.

Investigation of males with CBAVD has provided important insights into the influence of genotype upon the development of lung disease. This is illustrated by the R117H mutation that permits partial *CFTR* function and



occurs in approximately 10% of CBAVD patients (usually paired with  $\Delta F508$ ) and about 0.3% of CF patients (374,512). How this mutation was able to give rise to phenotypes with and without pulmonary disease was unclear until the genetic context in which the mutation occurs was analyzed. The R117H mutation occurred at least twice but in CFTR genes that have subtle but important differences (357). One of the R117H mutations occurs exclusively in CF patients while the other was seen less frequently in CF patients but exclusively in CBAVD patients. Sequencing of CFTR genes containing each version of R117H revealed that the R117H mutation associated with CF has the inefficient splice variant 5T while CBAVD males carry R117H mutations that have an efficient variant of this splice acceptor site (357). Therefore, the splice variant is predicted to alter the amount of mutant CFTR produced. Higher levels of mutant CFTR bearing R117H appear to cause the CBAVD phenotype while reduced levels produce chronic lung disease characteristic of CF (357). More recent studies have confirmed that the status of the splice variant is correlated with phenotype in patients carrying the R117H mutation (28,373,513). CFTR mutations have been found at higher than expected frequencies in several other conditions with phenotypic similarity to CF. These include idiopathic pancreatitis (525–528), allergic bronchopulmonary aspergillosis (529), asthma (530), disseminated bronchiectasis (531–535), isolated sweat gland dysfunction (427), and chronic rhinosinusitis (536–538).

## 58.4 DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

CF has been recognized as a distinct clinical entity for over 50 years. Initially, multisystem involvement and inheritance pattern differentiated this disorder from other illnesses of infancy and early childhood (2,539). The development of the sweat chloride test in the 1950s provided an objective diagnostic tool. Subsequently, milder forms of the illness associated with diagnostic elevations in sweat chloride concentration were recognized in older children and adults (142). In the late 1960s, the discovery that almost all male patients have congenital reproductive system abnormalities resulted in the diagnosis of older male patients with “borderline” abnormal sweat electrolytes (141,510). In the past decade, the description of patients carrying mutations in each CFTR gene who do not meet clinical criteria for the diagnosis of classic CF further broadened the phenotypic spectrum (44,45). These observations prompted a revision of diagnostic criteria for CF to include advances in our understanding of CF at the molecular level as shown in Table 58-6. The diagnosis of CF requires a characteristic clinical finding and evidence of CFTR dysfunction (540). The advent of nationwide newborn screening for CF prompted adaptation of the diagnostic criteria to account for the lower

sweat chloride concentrations that occur in newborns with CF (541,542).

Each of the clinical features listed on Table 58-7 are suggestive of CF and should prompt testing of CFTR function, especially neonatal MI, steatorrhea, *P. aeruginosa* respiratory tract infection, or the coexistence of multiple features in one patient (37). Many other illnesses can have one or more of these features. Therefore, the diagnosis of CF relies upon accurate testing of CFTR function. Determination of sweat chloride concentrations has been and continues to be the “gold standard” for evaluating CFTR function. Delineation of a panel of common disease-causing mutations in CFTR and the development of assays of CFTR function in respiratory epithelia (nasal potential difference or NPD) provide additional objective methods for diagnosis. DNA and NPD testing can

**TABLE 58-6 Criteria for the Diagnosis of CF**

One or more characteristic phenotypic features (see Table 62-7)
or a history of CF in a sibling
or a positive newborn screening test result
And an increased sweat chloride concentration by pilocarpine iontophoresis or two or more occasions
or identification of two CF mutations
or demonstration of abnormal nasal epithelial ion transport

Data from Rosenstein and Cutting (540).

**TABLE 58-7 Phenotypic Features Consistent with a Diagnosis of CF**

1. Chronic sinopulmonary disease manifested by
a. Persistent colonization/infection with typical CF pathogens including <i>Staphylococcus aureus</i> , nontypeable <i>Haemophilus influenzae</i> , mucoid and nonmucoid <i>Pseudomonas aeruginosa</i> , and <i>Burkholderia cepacia</i>
b. Chronic cough and sputum production
c. Persistent chest radiograph abnormalities (e.g., bronchiectasis, atelectasis, infiltrates, hyperinflation)
d. Airway obstruction manifested by wheezing and air trapping
e. Nasal polyps; radiographic or computed tomographic abnormalities of the paranasal sinuses
f. Digital clubbing
2. Gastrointestinal and nutritional abnormalities including
a. Intestinal: meconium ileus, distal intestinal obstruction syndrome, rectal prolapse
b. Pancreatic: pancreatic insufficiency, recurrent pancreatitis
c. Hepatic: chronic hepatic disease manifested by clinical or histologic evidence of focal biliary cirrhosis or multilobular cirrhosis
d. Nutritional: failure to thrive (protein-calorie malnutrition), hypoproteinemia and edema, complications secondary to fat-soluble vitamin deficiency
3. Salt loss syndromes: acute salt depletion, chronic metabolic alkalosis
4. Male urogenital abnormalities resulting in obstructive azoospermia (CBAVD)

be especially useful in patients presenting with non-classic forms of CF (see below).

The standard method for sweat test is quantitative pilocarpine iontophoresis, in which sweat is collected, weighed, and then subjected to electrolyte determination (543). Specific guidelines for the proper performance and interpretation of this test have been published (135). Collection of adequate volumes of sweat (at least 75 mg, preferably 100 mg or more) is critical to test reliability and accuracy (135). Elevated sweat chloride concentrations have been reported in patients with endocrine, metabolic, and other conditions that are clinically distinct from CF (135). The situation is more complicated for the small fraction of patients (1–2%) who have suggestive clinical features, but sweat chloride concentrations that persist in the borderline or normal range (144,148,151,544). Additional testing in these cases is required to establish the diagnosis. Demonstration of azoospermia in males (510), pansinusitis by CT scan (545), or abnormal pancreatic function by fecal fat determination or elevated serum trypsinogen levels can be helpful (101). Measurement of  $\beta$ -adrenergic stimulated sweat rate may become another diagnostic tool for CF diagnosis (546). This test can distinguish between normals and heterozygotes, and therefore could be particularly useful for the evaluation of symptomatic patients with normal sweat chloride concentrations (136,547,548).

Assessment of ion conductivity in epithelia tissue by measuring NPDs provides an in vivo assessment of CFTR function (296,329,549,550). This procedure is technically challenging and prone to variability depending upon the anatomic site of measurement, test protocol, and equipment used (296,551). However, in centers with extensive experience, CF patients can be reliably distinguished on the basis of higher mean basal potential differences (PD) and minimal change in PD in response to perfusion with a chloride-free solution in the presence of isoproterenol ( $\beta$  agonist) and amiloride (sodium channel blocker) (552). Furthermore, NPD measurement may be a more sensitive assay of CFTR function than determination of sweat chloride concentration. Thus, NPD testing can help establish a CF diagnosis in some patients with borderline abnormal sweat tests and clinical features of CF (553–555).

According to the currently accepted criteria, the identification of deleterious mutations in each CFTR gene of a patient with suggestive clinical features can confirm the diagnosis of CF, even in the absence of an abnormal sweat test (540). An important caveat to the criteria listed in Table 58-6 is how a CF mutation is defined. Many rare mutations have been reported to the CF Mutation Database. The consequences of many of these mutations are unknown. Thus, DNA diagnosis of CF should be based upon mutations shown to alter CFTR function, mutations predicted with a high degree of confidence to have a deleterious effect (e.g., nonsense and frame-shift), or mutations that have been reported in numerous

patients such as those listed in Table 58-3. Preferably, a CF mutation would meet several of these conditions. As noted above, the CFTR2 project has addressed the disease-liability of approximately 160 mutations that each occur at a frequency of 0.01% or greater in approximately 40,000 CF patients. Testing for these mutations identifies 97% of CF-causing alleles which is a considerable improvement over the sensitivity of the current panel of mutations approved by the American College of Medical Genetics (75–87% sensitivity; see below).

An important consideration is the accuracy of the genetic testing. A survey of laboratories offering CF mutation analysis in Europe revealed that only 48% made no mistakes on a standard panel (556). Laboratories that processed more samples made considerably fewer errors. To obtain the highest level of accuracy it is recommended that testing be performed only in certified laboratories that participate in proficiency testing (557–559). A number of academic and commercial DNA diagnosis laboratories in North America and Europe offer testing for the F508del mutation and 5–100 other less frequent CF alleles (560,561). The sensitivity of the test depends upon the population being tested. For heterogeneous European-derived Caucasian populations like those in North America, detection ranges from 75% to 87% of CF alleles (Table 58-3). DNA testing is of greater utility in populations where a limited number of mutations account for 90% or more of CF alleles (Table 58-4). In non-Caucasian races such as native Africans, the test has very low sensitivity. Detection rates approach 75% in African-Americans presumably due to admixture with Caucasians in the United States (376). Ethnicity also contributes to the distribution of CFTR mutations. Individuals self-identified as Hispanic from the southwest United States carry a subset of mutations that are rarely seen in other groups, which may be the result of genetic drift or founder effect (562). Customization of CF mutation panels for mutations observed in Hispanic CF individuals increases sensitivity to levels similar to those obtained in Caucasian CF patients using the ACMG mutation panel (563).

Buccal mucosal cells, peripheral blood samples, or dried blood samples on Guthrie cards can be sent to distant laboratories for DNA testing (564,565). Therefore, DNA diagnosis might substitute for the sweat test in situations where sweat testing is unavailable or unreliable. DNA analysis can also be useful when sweat chloride test results are ambiguous. For example, the mutation 3849 + 10kbC  $\rightarrow$  T has been associated with normal to borderline sweat chloride levels but pulmonary and/or GI manifestations characteristic of CF (45,455,566,567). Testing for CF mutations can assist with diagnosis since the 3849 + 10kbC  $\rightarrow$  T mutation is included in panels used by many diagnostic labs. DNA testing of fetuses with an echogenic bowel can be useful. There is an association between this finding and CF, but the precise risk that a fetus with echogenic bowel will be affected

with CF is not known (568,569). Estimates of risk range from 1.3% to 13.3% (570). Since an accurate prior probability is unavailable, Bayesian analysis for fetuses found to have one CF mutation is unreliable. However, if two CF mutations are discovered in the fetus, or both parents are shown to be carriers, then clinical management can be adjusted accordingly. Furthermore, the nature of the mutation in *CFTR* may influence the risk for fetal echogenic bowel. Deletions of *CFTR* have been found in a higher than expected fraction of CF-fetuses with echogenic bowel, loop dilation, or gall bladder anomalies (571).

### 58.4.1 Prenatal Diagnosis

Highly accurate DNA-based prenatal diagnosis is available for families with a previously affected child and for couples where both members are known carriers of a CF mutation (572–574). Healthy siblings can also be tested to determine carrier status. However, about 25% of Caucasian families will carry a rare mutation not included on CF mutation panels. Although a variety of techniques have been devised to screen an entire gene for mutations, none is currently practical for routine diagnostic testing (574,575). Linkage analysis with polymorphic markers can be used in families where one or neither mutation can be identified (573,576,577). Use of polymorphic microsatellite markers flanking and within the *CFTR* gene virtually eliminates mistyping due to recombination, providing an accuracy of 99% or greater for prenatal and carrier diagnosis (382,578–580). More recently, preimplantation genetic diagnosis for CF has been successfully performed upon single cells derived from in vitro fertilized embryos (581). This technique provides an alternative to some couples who would not undergo fetal testing.

### 58.4.2 Diagnosis by Newborn Screening

The issue of screening newborns for CF has been controversial. Central to this proposal is whether early diagnosis alters outcome for patients and, if so, does the degree of improvement outweigh the costs. These points were moot until the development of an inexpensive and reliable test with acceptable levels of specificity and sensitivity. In the early 1980s, the immunoreactive trypsinogen test (IRT) was shown to be a promising method for CF neonatal screening (16,582). The sensitivity and specificity of this test depends upon the concentration that is used to determine an abnormal result. In a study of 220,865 newborns from Wisconsin, the test had a sensitivity of 87% using a high “cutoff” value of 180 ng/ml (583). Sweat testing confirmed the diagnosis in 12.5% of neonates with elevated IRT concentrations in that study. Use of a lower “cutoff” value (140 ng/ml) followed by a second IRT test resulted in a sensitivity of 95.2% and positive predictive value of 32% (17).

In 1983, an Ad Hoc Committee Task Force on newborn screening sponsored by the U.S. CF Foundation raised several important concerns and recommended that screening not be implemented (584). In addition to acknowledging that the benefits of early intervention were unclear, the committee questioned the possible effects of screening upon parent–child bonding and reproductive decision making, and the reliability of the IRT. Pilot screening studies performed in Europe, Australia, and the United States have addressed most of the concerns of the Task Force (17,23,585,586). Furthermore, coupling of IRT with DNA testing considerably improves the positive predictive value of newborn screening (18,22,583,587). Two tier (IRT and DNA) screening also reduces false positives, and eliminates the need for second IRT measurements (21,75). Unwanted carrier detection does occur as a consequence of the two tier test (21). However, it appears that a substantial fraction of infants with a positive IRT who carry one CF mutation have a mild form of CF (588–590).

Neonatal screening for CF is now widespread in North America, Europe, and Australasia as optimization of molecular-based therapies may require early intervention. For example, patients identified by newborn screening in Colorado demonstrated airway inflammation before symptoms of lung disease evolved (58). These data suggest that therapy directed at the basic defect may have to be initiated in infancy. Neonatal screening may be the only reliable method to identify patients before considerable morbidity is present. Furthermore, evidence is accumulating that CF patients identified by newborn screening have better outcomes than those diagnosed by traditional methods. Improvements in nutrition (20,591,592) and lung function (592,593) have been observed as benefits of earlier diagnosis. These benefits have translated into fewer complications, reduced need for therapy and improved survival (594–596). Thus, the risk to benefit ratio may be tipping in favor of implementing newborn screening for most populations (597).

### 58.4.3 Screening for CF Carriers in the General Population

The discovery that one mutation is common in CF raised the possibility of population screening for CF carriers. Opinions vary considerably regarding the feasibility of large-scale screening programs (598–603). Comprehensive reviews on this topic are available (604–607). Since it is not possible to detect all mutations easily and inexpensively, concern has arisen regarding the use of a test that cannot detect all carriers (608). A negative test at an 85% level of mutation detection reduces the chance that a Caucasian individual is a CF carrier from about 1 in 30 to 1 in 170. This may appear to be a valuable reduction in risk. However, in a population-based screening program, a large number of couples will be identified

with one member being a CF carrier. For the majority of couples, the other member will have a negative test. Since a negative test does not eliminate the chance that an individual is a carrier, many couples will be given an increased risk of having an affected child (about twice the population risk) without the possibility of further testing.

In the United States, a consensus report was issued by an NIH Advisory Council recommending that screening not take place until 95% of CF mutations can be detected (609). Similar recommendations for CF carrier screening have been issued by the American Society of Human Genetics and World Health Organization (606,608). At a 95% level of mutation detection, couples in which one partner is a CF carrier and the other has a negative mutation test will have the same risk as if they did not have any testing. Studies have been performed to assess the impact of carrier screening upon the general population with inconclusive results (586,610–613). Small-scale population studies (<5000 individuals) involving pregnant patients enrolled in HMOs or attending an academic medical center clinic indicated that screening for CF was desired and the necessary pre- and post-test phases could be accomplished (28,614). In 1997, a 14-member organization by the NIH in the United States concluded that testing for CF mutations should be offered to couples seeking prenatal care even though mutation detection rates in the general population had not exceeded 90% and large-scale screening projects utilizing a wide variety of clinical settings had not been performed. Guidelines for the implementation of CF mutation testing in the general population have been issues considered by the American College of Medical Genetics (615). A pan-ethnic panel consisting of mutations that exceed 0.1% frequency in the general population was recommended and has been amended based upon screening results (616,617). Important issues remain regarding the utility of screening in non-Caucasian couples and implementation in clinical practices (618,619). There have been reports that implementation of population-based carrier screening has resulted in a decrease in the incidence of CF (620,621).

#### 58.4.4 Differentiating Between CF and Congenital Bilateral Absence of the Vas Deferens

Obstructive azoospermia due to absence (or malformation) of the vas deferens, an almost invariable feature in males with CF, has been reported as a separate autosomal recessive disorder (141,146,511,622). The first clinical report of CBAVD appears to be that of Michelson in 1949 (623). The disorder is rare in healthy men (0.04%) but is the cause of infertility in about 2% men with obstructive azoospermia (624). The finding of chronic sinus and respiratory disease in some men with CBAVD lead to the suggestion that it may be a variant of CF (510,625). This perception has been strengthened

by the detection of CF mutations in a large fraction of men with CBAVD (see above). Presence of persistent pulmonary or pancreatic disease in males with CBAVD and elevated sweat chloride concentrations would indicate a CF diagnosis (Table 58-5). DNA and NDP testing can be useful in less obvious cases. A diagnosis of CF should be entertained in men with CBAVD carrying two *CFTR* mutations (Table 58-3) and/or having chloride conductance abnormalities in nasal epithelia similar to that observed in CF patients (626). Identification of mutations in *CFTR* primarily associated with isolated CBAVD (42,43,373,513) or NPD measurements that are not consistent with CF (521) or normal (626) would suggest retention of the CBAVD diagnosis. Considering the implications of a diagnosis of CF, labeling these otherwise healthy males as a variant of CF is not appropriate. Furthermore, evidence of genetic heterogeneity indicates that *CFTR* may not be abnormal in all men with this condition (46). Finally, some men with CBAVD have lung disease and some do not, so the long-term prognosis of this group is unknown (523,524,627). Until further data becomes available, monitoring of men with CBAVD for clinical signs of lung or pancreatic disease characteristic of CF appears prudent.

#### 58.4.5 Non-Classic CF in the Absence of *CFTR* Mutations

As noted previously, patients with abnormal *CFTR* function may present with disease in a subset of organ systems affected in classic CF. These patients can present as diagnostic dilemmas since the reduction in *CFTR* function may be moderate, thereby creating subtle phenotypes with borderline abnormalities in the sweat test or NPD. Etiologic heterogeneity can further complicate diagnosis of patients presenting with non-classic CF (7,8). Some patients with non-classic presentations have been found with mutations in the *SBDS* gene responsible for Shwachman–Diamond syndrome or in the  $\beta$  subunit of the ENaC (628,629). More recently, patients presenting with hyponatremic dehydration and elevated sweat chloride concentration have been found with a mutation in *carbonic anhydrase 12*. The same mutation (G143K) occurred in homozygosity in two different consanguineous Bedouin pedigrees (630,631). These patients did not manifest other features of CF. Although these cases of genetic heterogeneity for CF traits such as elevated sweat chloride concentration are likely to be rare, distinguishing their disease from CF is important for the new insight into organ system disease in CF, accurate prognosis, and appropriate therapy.

### 58.5 MANAGEMENT

Therapy of CF has the following aims: preservation of lung function, optimization of nutritional status, minimization of complications, and maintenance of psychosocial



well-being. Achievement of these goals requires a multifaceted management plan delivered by a team of health care professionals. For this reason, many CF patients receive care at specialized “CF Centers.” Implementation of multidisciplinary care and improvement in traditional forms of therapy have led to progressive increases in survival with most patients now living well into their third decade (38).

### 58.5.1 Pulmonary Therapy

Lung disease is the life-limiting factor for most CF patients. For this reason, a great deal of effort is expended to retard progression of pulmonary damage. At the present time, therapy is targeted at mucus clearance and control of infection (Table 58-8). Coughing is a potent method to expel mucus from the airways and is the predominant large airway clearance mechanism for CF patients (632). Chest percussion and/or postural drainage may assist this process in the small airways for some patients (633-635). Exercise has been advocated on the basis of patient well-being but may also promote more efficient sputum expectoration (636,637). A significant fraction of patients have a reactive airway component to their disease that can be improved by  $\beta$ -adrenergic bronchodilators (638). Control of bacterial infection, especially *P. aeruginosa*, is the mainstay of pulmonary treatment (56). Reduction of the inflammatory response using steroids appeared promising but this benefit may

be outweighed by significant complications (639,640). Improved methods of mucus removal can aid in the clearance of secretions from the airways. Mucus secretions from CF airways contain large amounts of DNA that significantly increases its viscosity. Cloning of human DNase I provided a means to digest DNA in mucus (641). Clinical trials demonstrated improvement in lung function and a reduction in the frequency of respiratory tract exacerbations in patients receiving aerosolized rhDNase I (Pulmozyme) (642-644). Actin is also abundant in secretions from CF patients. Low-dose long-term macrolide antibiotics are efficacious in the treatment of diffuse panbronchiolitis, a chronic lung disease with several features in common with CF. On this basis, macrolide treatment has been tried in CF patients with positive results (645-647). However, the mechanism by which macrolides improve outcome for CF patients is unclear (648). Lung transplantation is the last resort in selected patients with extensive irreversible damage (649).

### 58.5.2 Novel Therapies for CF Lung Disease

An increased understanding of the etiology and pathogenesis of CF at the molecular level has opened a whole new array of therapeutic possibilities (650,651). These range from provision of normal CFTR to airway cells to modulators of the immune system (Table 58-8). Of all the treatment regimens being considered for CF, gene

**TABLE 58-8 Treatment of CF Lung Disease**

Pathology	Treatment Goal	Current Options	Future Possibilities
Defective CFTR gene	Replace with normal gene	None	Gene therapy
	Translation of RNA transcripts containing nonsense mutations	None	Premature termination suppressors
Abnormal protein	Replace with normal protein	None	Protein replacement
	Enhance mutant protein folding	None	Chemical chaperones, bisaminomethylbithiazoles
	Augment mutant protein function	None	Potentiators of channel gating, phosphodiesterase inhibitors, protein phosphatases
Altered ion transport	Utilize alternative pathway of chloride secretion	None	ATP/UTP
	Block sodium hyperabsorption	None	Amiloride
Abnormal airway mucus	Reduce viscosity	Pulmozyme Hypertonic saline	Gelsolin
Obstruction	Facilitate clearance	Airway clearance techniques	?
Airway reactivity	Reduce reactivity	$\beta_2$ -agonists Cromolyn Aerosolized steroids	?
Infection	Control bacterial and viral replication	Antibiotics Influenza vaccine	<i>Pseudomonas</i> vaccines Immunoglobulin therapy Antimicrobial peptides
Inflammation	Decrease immune response	Corticosteroids <sup>a</sup> Ibuprofen	Protease inhibitors Anti-oxidants
Irreversible airway destruction	Replace damaged tissue	Lung transplantation	?

<sup>a</sup>Limited to cases complicated by bronchiolitis, ABPA, or severe airway reactivity.

therapy has received the greatest attention, although small chemical correctors and potentiators are showing considerable promise.

Providing affected epithelial tissues, especially those in the lungs, with a long-lasting source of normally functioning CFTR is an attractive and desirable goal. Consequently, a considerable amount of effort has been directed toward this goal and, although efficacy has remained elusive, the problems limiting successful gene replacement have been defined (652,653). Recombinant adenovirus is an efficient vector for transient expression that can be produced in titers sufficiently high enough for human trials. Expression of CFTR transcripts and protein from this vector has been detected for as long as 6 weeks in tracheal cells from cotton rats and rhesus monkeys (654,655). However, adenovirus vectors produce an inflammatory response in animals and have caused adverse events in human subjects (656–660). Since efficacy has not been demonstrated and safety concerns remain, adenovirus trials are no longer being conducted for CF. Adeno-associated virus (AAV) is a non-pathogenic parvovirus that can integrate into the genome of the host. Correction of the chloride conduction deficiency in CF cells and RNA expression for up to 6 months has been achieved using AAV-CFTR vectors (661,662). AAV vectors appear to have low toxicity and can be administered repeatedly although efficacy for CF has not yet been convincingly demonstrated (663–665). Furthermore, recombinant AAV vectors are difficult to produce in high titers and their ability to integrate appears to be altered from wild-type AAV (666,667). Other vectors that have been considered include sendai virus and feline immunodeficiency virus (668,669). Delivery of naked plasmid DNA mixed with cationic liposomes has shown some promise in animal models (670,671), although results have been mixed in human trials (672). Synthetic vectors that utilize a receptor-mediated endocytosis pathway for DNA delivery may be more efficient and might avoid some of the problems of biologic vectors (673).

The difficult issues presented by gene therapy have lead to renewed efforts to target the molecular defect (674,675). The discovery that CFTR containing the common mutation,  $\Delta F508$ , is partially functional suggested that the majority of CF patients might be treated by increasing the amount of mutant CFTR at the cell surface (254,412,676). Altering the fate of misfolded mutant CFTR is an interesting but difficult task. It may require manipulation of the normal process of intracellular trafficking which could have detrimental side effects. Several strategies have been suggested: use of chaperones, alteration of trafficking regulated by G-proteins, and increased CFTR expression (676–681). A second phase of this theoretical treatment would involve maximization of mutant CFTR function. Application of high levels of the phosphodiesterase inhibitor IBMX appears to stimulate the chloride currents exhibited by mutant CFTR expressed in *Xenopus* oocytes (255). However,

IBMX did not affect chloride secretion in nasal epithelial cells of CF patients (682). Mixed results have been obtained with Milronone, another phosphodiesterase inhibitor (683,684). The protein phosphatase genistein shows promise as a direct activator of wild-type and mutant forms of CFTR (685,686). High throughput small molecule screens have identified new candidates for CFTR activation and gating (419). One compound termed VX-770 that augments the function of CFTR bearing the gating mutation G551D has shown dramatic effect in human trials (420). Pronounced reduction in sweat chloride concentration and improvement in lung function (FEV1%pred) were maintained over a 4-week period. The success of this approach will probably drive a search for other compounds that augment CFTR bearing mutations that affect other functional properties. Indeed, several compounds show promise for correcting the folding defect of CFTR with the F508del mutation (687,688).

Therapies selective for other CFTR genotypes have been developed. Clinical trials are underway based on the observation in vitro that high doses of aminoglycoside antibiotics cause read through of premature termination codons (689,690). Administration of aminoglycoside-derived compounds to nasal epithelia corrects the ion transport defect in patients carrying a nonsense mutation (691). Although this approach appears promising, only a small fraction of CF patients carry mutations that would respond to this therapy.

Epithelial cells contain chloride channels that are distinct from the channel encoded by CFTR (284,692,693). These channels can be activated by different mechanisms than those employed by CFTR. It may, therefore, be possible to exploit other pathways of epithelial chloride secretion and compensate for electrolyte abnormalities caused by mutations in CFTR (694). The observation that ATP and UTP applied to airway epithelial cells elicit chloride secretion in normal and CF patients demonstrates the clinical feasibility of this approach (340,695). Alternatively, it may be possible to improve the hydration of epithelial secretions by affecting the movement of ions other than chloride (340). For example, blockage of sodium absorption in airway epithelia of CF patients by aerosolized amiloride resulted in reduced sputum viscosity and elasticity and subtle signs of improved pulmonary status (696,697). Alternatively, rehydration of ASL by administration of aerosolized hypertonic saline appears to improve mucus clearance and reduce inflammation (698–700).

Novel approaches have also been devised to control viral and bacterial infection and the inflammatory response they elicit. Vaccination of CF patients against *P. aeruginosa* has been attempted using a variety of antigens derived from the bacteria (701,702). Although well-tolerated and eliciting significant rises in titers of anti-*P. aeruginosa* antibody, clinical status has not been affected by immunization (701). Administration

of immunoglobulin enriched for anti-*P. aeruginosa* antibodies is an alternative approach to the control of chronic *Pseudomonas* lung infection (703). An increased understanding of the molecular adaptations of *Pseudomonas* that permit colonization of CF patients should identify new therapeutic targets (502,704–706). Bacterial genes that regulate formation of biofilms are particularly attractive (707,708). By the same token, greater insight into role of CFTR dysfunction in the pathogenesis of CF lung disease has suggested novel treatments such as antimicrobial peptides and agents that alter electrolyte concentration in ASL (347,348,709–711).

Inflammation and subsequent tissue destruction is a major component of CF lung damage (712). It appears that long-term alternate-day with anti-inflammatory steroids such as prednisone may improve lung function but treatment side effects remain problematic for this medication (713,714). Non-steroidal anti-inflammatory agents appear more promising. Ibuprofen given over a 4-year period reduced the rate of pulmonary function decline with few adverse reactions (714). Other clinical strategies to control inflammation include modulators of the NFκB pathway, anti-oxidants, and anti-proteases (715–717). Aerosol delivery of α1-antitrypsin or secretory leukoprotease inhibitor may be useful to counteract neutrophil elastase in epithelial airway secretions (718–720).

### 58.5.3 Nutritional and Other Therapies

Normal growth and development in children and maintenance of nutritional status in adults are important goals in the management of CF patients. These are achieved by providing exogenous pancreatic enzymes, supplementing fat-soluble vitamins (A, D, E, and K) and providing sufficient calories to compensate for less efficient absorption and increased energy requirements. Enzyme replacement remains central to the treatment of malabsorption. Pancreatic extract has been used for CF since the 1930s (51). Dosage is empirically based; sufficient amounts are taken at each meal-time to control steatorrhea and allow appropriate weight gain (51). Considerable patient-to-patient variation exists in enzyme requirements. Normal fat absorption, even at very high enzyme doses, is rarely achieved (721,722). Efficacy of pancreatic enzyme replacement can be limited by gastric acid inactivation; therefore, current preparations of pancreatic enzymes have an enteric coating that dissolves at pH levels that exist in the small intestine. Reduction of gastric activity with H<sub>2</sub>-blockers, sodium bicarbonate, and prostaglandin analog can improve enzyme effectiveness in some patients (723–725). Although relatively safe, some patients taking high-dose pancreatic supplements have had colonic strictures (726,727).

Dietary manipulation to reduce fat intake had been in vogue in the past, but a normal diet with moderate amounts of fat is currently recommended (51). Reduction

in fat intake may be contraindicated since CF patients have deficiencies in essential fatty acids that cannot be attributed to malnutrition (728). Impaired uptake and/or metabolism of fatty acids appears to be associated with CFTR dysfunction, although the precise mechanism is unknown (729–731). Correlation between the degree of fatty acid deficiency and severity of lung disease has been documented (732,733). Correlation between *CFTR* genotype, pancreatic status, and level of the fatty acid docosahexaenoic acid (derived from n-3 synthetic pathway) has been reported, but correlation with levels of the fatty acid arachidonic acid (derived from n-6 pathway) has been inconsistent (734,735). More recent studies of CF mice have also questioned the connection between essential fatty acid deficiency and CFTR dysfunction (736). The lipid imbalance in CF mice can be corrected by oral administration of lipid emulsions (730). These observations have prompted clinical trials to evaluate the potential therapeutic efficacy of correcting lipid imbalances in CF patients. Calorie supplementation of the diet is advocated for many CF patients due to increased energy consumption for respiration and infection control. The most important factor may be attention to the dietary preferences of the patient to ensure that adequate calories are consumed (737). For some patients, enteral supplementation is being used to provide adequate caloric intake (506).

Liver disease is the second most common cause of death for CF patients (40). As outlined for lung disease, treatment is aimed at several phases of pathogenesis. The goals are prevention of mucus plugging of bile ducts and reduction of hepatotoxicity caused by bile acids. Choleric agents can be used to reduce bile viscosity and prevent mucus plugging. Bile acid therapy is the predominant treatment for chronic cholestasis (738). Ursodeoxycholic acid is used to alter biliary bile and composition to minimize cell damage. Portal hypertension can be treated by sclerosis of varices. For patients with advanced disease, liver transplantation is the only therapeutic option.

### 58.5.4 Animal Models for CF and Their Role in Therapy Development

Generation of mice with defective *CFTR* genes (CF mice) have led to advances in both the understanding of CF pathogenesis and treatment (739). A number of CF mice lines have been created by “knockout” techniques (740,741). Although epithelial chloride transport is abnormal in the lungs and GI tract of these animals, the clinical phenotype differs from their human counterparts (740–742). Intestinal obstruction and perforation are a frequent life-limiting complication in the mice but significant lung disease is not readily apparent (740). However, the lungs of CF mice are not normal. CF mice appear to have more difficulty than their normal littermates in clearing pulmonary infections after

repeated exposure to bacteria that colonize human patients (*S. aureus* and *B. cepacia*) (743–745). Furthermore, CF mice are significantly more susceptible to chronic infection with *P. aeruginosa* that express alginate, a polysaccharide found on the surface of *Pseudomonas* that colonize CF patients (746,747). Finally, it appears that pathology in the lungs, liver, and pancreas of CF mice that mimics those seen in CF patients develops over time, and may be substantially influenced by differences in genetic background (i.e., strain differences) (485,748). These strain differences may be a consequence of variation in alternative pathways of chloride transport, which are more active in mice than in humans (749). Analysis of different CF mice strains suggests that the magnitude of conductance via the alternative pathways is genetically determined (750). Furthermore, studies of inbred mouse strains reveal differences in susceptibility to colonization with *Pseudomonas* (751). By combining these observations, it may be possible to create a murine model of CF lung disease that closely approximates the human situation.

Mice bearing CF mutations observed in humans such as  $\Delta F508$  and G551D have also been generated (752–754). Similar to their human counterparts,  $\Delta F508$  mice manifest intracellular processing defects of CFTR that are relieved by temperature reduction. The G551D CF mice had reduced rates of fatal intestinal blockage, a phenotype reminiscent of the lower frequency of MI in CF patients with the G551D mutation (435,754). Even if CF mice do not have a phenotype that precisely matches with those observed in humans, imitation of the biochemical phenotype of CF at the cellular level will be invaluable for the design and preliminary testing of the various therapies currently under consideration (683,684,755). Careful study of phenotype variation in CF mice will facilitate the identification of genetic modifiers of disease severity (484–487). Murine models can also be used to test the contribution of genetic modifiers to disease variation. For example, dysregulation of the ENaC in the absence of CFTR leads to hyperabsorption of sodium in the lungs of CF patients (see above). Transgenic mice that overexpress the  $\beta$  subunit of ENaC (but not  $\alpha$  or  $\gamma$ ) in their lungs develop pulmonary disease that closely resembles the pathology observed in CF patients even though functional CFTR is present (756). Intriguingly, two patients with CF-like lung disease in the absence of CFTR mutations had mutations in the  $\beta$  subunit of ENaC (629). Modifiers such as the  $\beta$  subunit of ENaC could provide targets that are more amenable to manipulation than CFTR.

Two new animal models of CF hold considerable promise for advancing our understanding of CF pathophysiology, especially for early stage disease. A large mammal model for CF has been developed using the pig. The porcine model for CF shares a number of features with CF in humans including neonatal intestinal obstruction, pancreatic endocrine and exocrine

dysfunction, hepatocellular disease (757), and polymicrobial lung infection (758). Of note, the CF pig develops intestinal obstruction that is morphologically and temporally similar to MI in CF neonates except that nearly 100% of CF piglets are affected. A high rate of intestinal obstruction (~75%) is also observed in a ferret model of CF (759) suggesting that the lower rate of this complication in humans with CF may be due to the presence of modifying factors. Treating obstruction in the pig and ferret model is challenging and currently limiting full utilization of these models. Once intestinal obstruction has been addressed, these animals will aid the search and evaluation of therapies for CF as they have pulmonary systems that are quite similar to humans.

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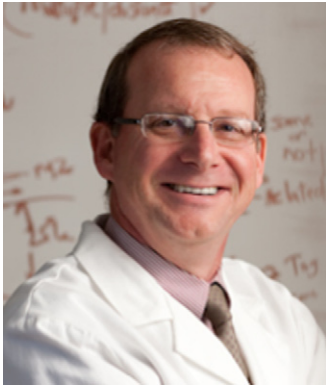
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### Biography



**Dr. Garry Cutting** is Professor of Pediatrics and Medicine and the Aetna/U.S. Healthcare Professor of Medical Genetics at Johns Hopkins University School of Medicine. Dr. Cutting received his undergraduate and medical degree at the University of Connecticut. In 1983, he went to Johns Hopkins University where he completed his residency in pediatrics and a fellowship in medical genetics before joining the faculty in 1989. Dr. Cutting's primary studies have been in the molecular genetics of cystic fibrosis and more recently elucidating the factors underlying variation in the severity of cystic fibrosis. His previous research achievements include the cloning and characterization of a new class of GABA receptor subunits (GABAc) and structure/function studies of voltage gated chloride channels. He also directs the Postdoctoral Clinical Laboratory Training Program in Medical Genetics and he is the Director of the DNA Diagnostic Laboratory at Johns Hopkins University School of Medicine. Dr. Cutting is the co-editor of the journal *Human Mutation* and Board Member of the Federation of American Societies for Experimental Biology. He is the recipient of numerous grants including a MERIT award from the NIH and has published over 150 peer-reviewed articles.

# CHAPTER

# 59

## Genetic Underpinnings of Asthma and Related Traits

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### GLOSSARY

**Bronchial hyperresponsiveness** – A condition of lung airways characterized by easily induced and/or exaggerated constriction of airways following some triggering event.

**Candidate gene study** – A hypothesis-driven form of genetic study in which genes suspected in disease pathology are examined for variations between cases and control individuals. Candidate gene studies require a previous understanding of disease biology, as candidate genes are selected largely on the basis of biological plausibility.

**Forced expiratory volume in 1 second (FEV<sub>1</sub>)** – A measure of lung function in which the amount of air a patient can expel in 1 s following a deep inhalation is measured by spirometry.

**Forced vital capacity (FVC)** – A measure of lung function in which the full volume of air a patient can expel following a deep inhalation is measured by spirometry.

**Gene–environment interaction** – Phenotypic effects resulting from effects of the environment on given genotypes. In the context of asthma genetics, this would refer to the influence of environmental factors on both susceptibility to asthma and the severity of disease.

**Gene–gene interaction** – Also called epistasis. Refers to phenotypes that arise only when specific alleles are present at two or more different loci.

**Genome-wide association study (GWAS)** – A hypothesis-free (and often hypothesis-generating) form of genetic study in which hundreds of thousands of single nucleotide polymorphisms are genotyped in large cohorts of cases and controls. The large panel of polymorphisms is analyzed for variations in frequency between cases and controls, allowing for identification of disease susceptibility loci.

**Genome-wide linkage study** – A hypothesis-free (and often hypothesis-generating) form of genetic study in which the genomes of disease-affected and unaffected family members are screened with a panel of genetic markers. Owing to the smaller amount of recombination expected between family members, regions containing susceptibility loci can be identified with a comparatively small number of genetic markers.

**Peak expiratory flow (PEF)** – A measure of lung function in which the speed of a patient's exhalation is measured, through the use of a peak flow meter.

**Pharmacogenetics** – The study of genetic variation that affects the responses of individuals to medication.

### NOMENCLATURE

FEV<sub>1</sub> – Forced expiratory volume in one second

FVC – Forced vital capacity

GWAS – Genome-wide association screen

PEF – Peak expiratory flow

SNP – Single-nucleotide polymorphism

### 59.1 INTRODUCTION

#### 59.1.1 Definition

Asthma is a common, chronic respiratory disorder featuring episodic shortness of breath, often at night and usually accompanied by a nonproductive cough. Wheezing is usually observed on clinical examination during exacerbations, with normal breath sounds when asthma is well controlled. Asthma is characterized by episodic airway obstruction accompanied by airway inflammation and, in some cases, irreversible structural alterations to

airways. There are many different definitions of asthma. The most recent Global Initiative for Asthma's Global Strategy for Asthma Management and Prevention report (updated in 2009) provides the following definition, which integrates symptoms and addresses the underlying cellular mechanisms of asthma:

*Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment (1).*

Acute asthma attacks are episodes in which patients experience difficulty breathing, usually as a result of constriction in the airways of the lung. Symptoms of acute attacks can usually be alleviated through the use of inhaled bronchodilators. These medications relax the smooth muscle of the airways, opening the airways and alleviating the increased work of breathing. Over the long term, structural changes can occur in the lungs that impact lung function, with excess mucus production, infiltration of cells of the immune system mediating chronic inflammation, and thickening of the walls of the airway that irreversibly limits air space. Some of these changes, such as immune-mediated inflammation, can be controlled through medication.

Although asthma is usually recognized through the acute episodes of asthma attacks, including wheezing and sometimes irreversible declines in lung function, asthma has an important immune system component as well. Roles for many immune cells and mediators have been described. There is a clear connection between asthma and atopy, although this connection is not absolute. Atopic individuals are more prone to developing asthma, and much of the prevalence of asthma can be linked to specific allergies (2). However, not all atopic individuals develop asthma, and not all asthmatics have detectably elevated allergic responses. Nonetheless, dysregulated immunity appears important in the development of asthma, with elevated serum IgE levels, excess release of allergic mediators from mast cells, infiltration of eosinophils into the lungs and inflammation in the airways, and skewed  $T_H1$  and  $T_H2$  responses frequently observed in asthmatics. Reduction of chronic inflammation in the lungs is part of the strategy for long-term control of asthma, through the use of anti-inflammatory agents like inhaled glucocorticoids.

### 59.1.2 Asthma Diagnosis

Asthma sufferers can present with a variety of symptoms, and the transient nature of the acute attacks (the most obvious symptom) can make actual diagnosis difficult.

Most asthmatics will report recurrent episodes of difficulty breathing, and wheezing will often (although not always) be observed on examination. A series of secondary characteristics can be evaluated to aid in diagnosis. Bronchial hyperresponsiveness (BHR), the tendency of asthmatic airways to overreact to irritants or stimuli, can be measured by either direct or indirect means (3). Direct challenges are the most commonly performed tests, and they specifically target the response of airway smooth muscle. These involve exposure of the airways to a substance, like methacholine, that binds to receptors on smooth muscle cells and promotes muscle contraction, constricting the airways. The reduction in lung function can be assayed, as can the response to treatment with bronchodilators. Most asthmatics will display larger-than-expected reactions in direct challenge tests (4). Indirect challenge tests involve stimuli that will cause activation of the immune cells that are resident in the asthmatic lung. These immune cells will release mediators that act on smooth muscle in the airways causing constriction (5). Common indirect stimuli include exercise, dry or cold air, and adenosine monophosphate. Indirect tests mimic the triggers of most asthma attacks in daily life, and therefore may be more clinically relevant (3). Overreactions to indirect challenges indicate the presence of immune cells like mast cells and eosinophils in the lung, indicative of asthma.

Several measures of lung function are commonly used (1). Peak expiratory flow (PEF) is a measurement of the patient's maximum speed of expiration. It is measured with a peak flow meter; modern meters are inexpensive, portable plastic devices, which enable home measurement and recording of PEF that aids in the monitoring of early deterioration and response to therapy. Forced expiratory volume in 1 s ( $FEV_1$ ) is the amount of air exhaled in 1 s following a deep inhalation, while forced vital capacity (FVC) is the maximal amount of air that can be expelled following a full inhalation. Both  $FEV_1$  and FVC are measured by spirometry. The reversibility of  $FEV_1$  by treatment indicates a diagnosis of asthma (6).

As mentioned previously, there is a clear but imperfect connection between asthma and allergy. As such, it is often useful to determine a patient's allergy status, either through skin tests or measurement of allergen-specific or total IgE levels in serum. Allergies and allergic disease (especially allergic rhinitis) increase the probability of an asthma diagnosis. In addition, identifying allergens to which a patient reacts can aid in identifying environmental exposures that trigger asthma attacks.

In the description of studies of the genetics of asthma that follows, many different phenotypes are considered end points for studies. Some studies consider actual asthma diagnoses, but others rely on different criteria. Measurements of lung function, allergic reactions, levels of serum IgE, and eosinophilia are cited as end points. In this chapter, these other criteria that serve somewhat as surrogates for asthma will be described as "asthma-related phenotypes."

### 59.1.3 Asthma Prevalence and Severity

Owing to the variability of the disease and a lack of generally agreed upon standards for diagnosis, it is difficult to estimate the prevalence of asthma, and variations in practice from country to country complicate worldwide estimates. In the United States, it is estimated that at least 22.9 million Americans suffer from asthma. Asthma is the leading chronic illness in US children, with 6.8 million affected in 2006 (2). Global rates of asthma range from 1 to 18% of the population from different countries (1). Prevalence is rising in locations where rates were previously low, and variation in rates from country to country appears to be diminishing (7).

Asthma was previously categorized based on the severity of symptoms in untreated individuals. Four groups were recognized: intermittent, mild persistent, moderate persistent, and severe persistent (8). However, these classifications did not account for the difficulty of controlling symptoms with treatment. Mild persistent asthma in one individual might be resistant to treatment while severe persistent asthma in another person might respond well and be thoroughly controlled with proper medication. Current consensus is to classify asthma on the basis of both severity of symptoms and the level of control of those symptoms (8,9). Mild asthma is therefore well controlled with low-intensity treatment, while severe asthma requires high-intensity treatment for full, and sometimes only partial, control. The advent of new treatments, allowing better control in certain situations, will hopefully allow currently severe forms of asthma to be reclassified as mild asthma in the future. Further characterization of severe asthma continues, with lower lung function, more frequent sinopulmonary infections, more persistent symptoms and higher health care requirements in severe asthmatics (10). Additional work has shown the existence of multiple distinct groups among severe asthma sufferers; differences between groups are apparent in age, age of onset of disease, presence of atopy, requirements for medication and health care, and impairment and reversibility of lung function (11). Appreciation of these groups will likely aid in care, as the underlying causes and therefore the most appropriate treatments for each type of severe asthma are likely to be different.

## 59.2 THE GENETICS OF ASTHMA AND ALLERGIC DISEASES

Twin studies have shown that there is a genetic element to asthma susceptibility, with heritability of the condition estimated at between 0.36 and 0.77 (12–15). The first study to link a genetic locus (chromosome 11q13) to asthma was published in 1989 (16). Since then, more than 600 candidate genes described in more than 1000 publications have been found in connection to asthma or an associated phenotype, like elevated IgE levels, BHR, or eosinophilia.

### 59.2.1 An Overview of the Analysis of the Genetic Contributions in Asthma

Researchers have been successful in identifying the genetic underpinning of over 3000 single-gene disorders. However, it has been comparatively difficult to identify the genetic basis of complex genetic disorders, such as asthma, allergic disorders, and autoimmune diseases, with multifactorial inheritance and significant environmental contributors. Three study designs are routinely employed to investigate genetic contributions in complex diseases: candidate gene association studies, genome-wide linkage studies and genome-wide association studies.

**59.2.1.1 Candidate Gene Studies.** In a candidate gene association study, a particular gene (or set of genes) is selected for study based on its biological plausibility or suspected role in the phenotype of interest. The incidence of variants in this gene is compared between a group of individuals affected with the phenotype (cases) and a group of controls. The strength of such a design lies in the statistical power and relative ease of recruiting large cohorts, compared to family-based studies. The main limitations of such a design are its inability to identify novel or unsuspected genes and pathways contributing to the pathogenesis of a disorder, and its susceptibility to unknown population structures in cases or controls. Candidate gene association studies are best suited to identifying common genetic variants of modest effect (17).

More than 1000 papers have been published with candidate gene studies examining asthma and related phenotypes, identifying more than 600 candidate genes. However, surprisingly few of these candidate gene discoveries have been rigorously replicated, and many have been examined and failed replication in subsequent studies (18–20). Among genes identified in candidate studies are receptors for detection of microbial products (TLRs, *CD14*, *CARD15*, among others); various cytokines and cytokine signaling proteins involved in T-cell survival, proliferation, and differentiation; genes involved in lung function, development, and response to stimuli (*ADRB2*, *CFTR*, *SPINK5*, etc.); genes involved in epithelial barrier function and innate immunity (*FLG* and *DEFB1*) (21,22); and genes believed to be involved in the responses to environmental exposures (*GSTM1*, *GSTP1*, and *GSTT1*) (19,23–25). Genes that have been extensively replicated include the beta2 adrenergic receptor gene (26–28); the cytokines, receptors, signaling proteins, and transcription factors involved in  $T_H1$  and  $T_H2$  differentiation of T cells, such as *IL4*, *IL4RA*, *IFNG*, *IFNGR1*, *STAT6*, *GATA3*, and *TBX21* (29–36); and genes involved in the cellular responses that characterize atopic disease, such as *IL13* and its receptor and the *FCER1B* gene (37–41). Genes that have been identified in five or more candidate gene studies as having a positive association with asthma, or asthma-related phenotypes, are listed in Table 59-1.



**TABLE 59-1 Summary of Well-Replicated Loci Identified Through Candidate Gene Studies**

Gene	Chromosomal Locus	Function
Immune function		
<i>IL10</i>	1q31-q32	Cytokine—immune regulation
<i>CTLA4</i>	2q33	Control/inhibition of T-cell responses/immune regulation
<i>IL13</i>	5q31	Induces T <sub>H</sub> 2 effector functions
<i>IL4</i>	5q31.1	T <sub>H</sub> 2 differentiation
<i>CD14</i>	5q31.1	Microbe detection—recognizes pathogen-associated molecular patterns
<i>HAVCR1</i>	5q33.2	T-cell responses—hepatitis A virus receptor
<i>LTC4S</i>	5q35	Leukotriene synthase—inflammatory mediator
<i>LTA</i>	6p21.3	Inflammatory mediator
<i>TNF</i>	6p21.3	Inflammatory mediator
<i>HLA-DRB1</i>	6p21	Major histocompatibility complex class II—antigen presentation
<i>HLA-DQB1</i>	6p21	
<i>HLA-DPB1</i>	6p21	
<i>FCER1B</i>	11q13	Receptor for IgE—atopy
<i>IL18</i>	11q22.2-q22.3	Inflammation
<i>STAT6</i>	12q13	IL-4 and IL-13 signaling
<i>CMA1</i>	14q11.2	Chymase—mast cell expressed serine protease
<i>IL4R</i>	16p12.1-p12.2	Alpha chain of receptors for IL-4 and IL-13
Barrier function/innate immunity		
<i>FLG</i>	1q21.3	Epithelial integrity and barrier function
<i>SPINK5</i>	5q32	Epithelial serine protease inhibitor
<i>CC16</i>	11q12.3-q13.1	Potential immunoregulatory function—epithelial expression
<i>NOS1</i>	12q24.2-q24.31	Nitric oxide synthase—cellular communication
<i>CCL11</i>	17q21.1-q21.2	Eotaxin-1: eosinophil chemoattractant
<i>CCL5</i>	17q11.2-q12	RANTES: chemoattractant for T cells, eosinophils, basophils
Tissue response		
<i>GSTM1</i>	1p13.3	Detoxification, removal of products of oxidative stress
<i>ADRB2</i>	5q31-q32	Smooth muscle relaxation
<i>GPRA</i>	7p14.3	Regulation of metalloprotease expression, neuronal effects
<i>NAT2</i>	8p22	Detoxification
<i>GSTP1</i>	11q13	Detoxification, removal of products of oxidative stress
<i>ACE</i>	17q23.3	Regulation of inflammation
<i>TBXA2R</i>	19p13.3	Platelet aggregation
<i>TGFB1</i>	19q13.1	Influences cell growth, differentiation, proliferation, apoptosis
<i>ADAM33</i>	20p13	Cell–cell and cell–matrix interactions
<i>GSTT1</i>	22q11.23	Detoxification, removal of products of oxidative stress

Genes identified as asthma susceptibility loci in candidate gene studies. Genes are grouped loosely based on their functions in immunity, epithelial barrier function, or tissue response and remodeling.

**59.2.1.2 Linkage Studies.** Genome-wide linkage study design focuses on families affected by the disease of interest. With less genetic recombination occurring between closely related individuals, it is possible to screen the entire genome with a panel of relatively few, evenly spaced markers, searching for variants that are either unique to or over-represented in affected individuals. If such a region is found, it is said to be linked with the disease trait, and the genes within this region can become candidates for further analysis, including association study followed by positional cloning of the gene. Unlike the candidate gene association study, this study design allows for the identification of genes and pathways previously not suspected of contributing to the disease in question. However, because large families of affected individuals are needed, these studies are expensive and difficult to conduct. Moreover, while they are effective at

identifying genes with low-frequency variants with high penetrance and large effects, they often lack the statistical power to identify genes of modest effect that are attributed to common alleles. This is in contrast to genome-wide association studies (discussed later), which are best suited to the identification of common variants with lower penetrance and smaller effects. In this way, linkage studies and association studies are used to address different questions, and are, in fact, complementary.

Approximately 20 genome-wide linkage screens have been reported in different populations investigating chromosomal regions that are linked to asthma and atopy, or related phenotypes such as elevated IgE levels, wheezing, and BHR. A number of chromosomal regions have been repeatedly identified across multiple studies that contain genes of biological relevance to asthma and allergic disease, including the cytokine cluster on chromosome

**TABLE 59-2** Table of Loci Identified through Linkage Studies and Positional Cloning

Gene	Chromosomal Locus	Reference
<i>CYFIP2</i>	5q33.3	Noguchi et al., 2005 (42)
<i>DPP10</i>	2q14.1	Allen et al., 2003 (43)
<i>HLA</i>	6p21.33	Nicolae et al., 2003 (44)
<i>PHF11</i>	13q14.3	Zhang et al., 2003 (45)
<i>GPRA</i>	7p14.3	Laitinen et al., 2003 (46)
<i>ADAM33</i>	20p13	Van Eerdewegh et al., 2003 (47)

5q (containing interleukin-3 (*IL3*), *IL5*, and granulocyte/macrophage colony-stimulating factor (*GMCSF*)), *FCER1B* on 11q, *IFNG* (interferon  $\gamma$ ) and *STAT6* on 12q, and *IL4R* (the IL-4R $\alpha$  chain, also part of the IL-13R) on 16p. Linkage studies followed by positional cloning approaches have resulted in the identification of a handful of novel asthma susceptibility genes, including *CYFIP2* (42), *DPP10* (43), *HLA* (44), *PHF11* (45), *GPRA* (46), and *ADAM33* (47). *GPRA* (G protein-coupled receptor for asthma) and *ADAM33* (a disintegrin and metalloproteinase domain-containing protein 33) have generated considerable interest, as their expression in bronchial smooth muscle cells suggests roles in the pathobiology of asthma and pulmonary allergic disease (46).

**59.2.1.3 Genome-Wide Association Studies.** Rapid advances in microarray technology that now permit the high-throughput genotyping of hundreds of thousands of single nucleotide polymorphisms (SNPs) have allowed for the development of a third type of study, the genome-wide association study (GWAS). In this design many SNPs (hundreds of thousands) are compared across the entire genome between cases and controls. Like the candidate gene association study, this design requires the collection of a large number of cases and controls for analysis, to achieve statistical power. In contrast to the candidate gene approach, however, it permits a hypothesis-free search for gene variants associated with disease, revealing new targets for researchers. As mentioned earlier, GWAS is well suited for discovery of common alleles with relatively small effects. The results of GWAS targeting asthma or related phenotypes are summarized in Table 59-2.

In 2007, the first GWAS that focused on bronchial asthma as an end point was reported (48), and identified multiple markers on chromosome 17q21 reproducibly associated with childhood-onset asthma. The findings were replicated in German and British cohorts. Independent replication of the 17q21 association has been reported in multiple populations of diverse ethnic backgrounds (49–53).

Variable expression of two genes within this region, *ORMDL3* and *GSDML*, was linked to asthma susceptibility (48). *ORMDL3* is a member of a gene family that

encodes transmembrane proteins anchored in the endoplasmic reticulum (54). *GSDML* encodes a member of the gasdermin proteins that are expressed in epithelial cells and regulate apoptosis. Both sequencing and functional data will be required to identify the causal gene; however, this finding does represent the first step in unraveling the complex genetics underlying asthma susceptibility in a hypothesis-independent manner.

A case-control GWAS of North American asthmatics of European ancestry from the Childhood Asthma Management Program (CAMP) cohort has also been reported. No loci reached genome-wide significance in their discovery cohort, whereas the strongest association was to variants of the *PDE4D* gene on chromosome 5q12. In seven Caucasian replication cohorts, two of seven *PDE4D* SNPs were marginally associated. No significant associations were observed at the *PDE4D* locus in populations of African ancestry (55). *PDE4D* is a lung-expressed phosphodiesterase that has been implicated in airway contractility; as such it is a plausible asthma candidate gene that warrants further investigation. In a separate study, genome-wide association data from the CAMP cohort was investigated for replication of previously reported candidate gene associations. Approximately 30 genes were investigated with five SNP-based associations replicating to a nominal significance in the *IRAK-3*, *PHF11*, *IL10*, *ITGB3*, and *IL4R* genes (56).

Another GWAS on allergic asthma was recently reported, with children 6 years of age, whose mothers had participated in an earlier study of asthma in pregnancy (Perinatal Risk of Asthma in Infants with Asthmatic Mothers—PRAM). An initial genome-wide association screen was performed on small numbers of cases and controls of mixed ethnic descent, and the most significant SNPs were further analyzed in a larger collection of samples. Although no single SNP achieved genome-wide significance, one SNP in an intron of *PDE11A* was cited as potentially interesting. *PDE11A* encodes a phosphodiesterase related to *PDE4D*, suggesting that this family of proteins may play a broader role in asthma pathogenesis (57).

A genome-wide association was also reported on two independent populations of African descent ascertained through the Genomic Research on Asthma in the African Diaspora (GRAAD) consortium, which included African-American asthmatics and controls from the Baltimore-Washington, DC, area and African Caribbean asthmatics and their family members from Barbados (58). A meta-analysis of the two populations did not yield any genome-wide significant associations, illustrating the complexity of identifying associations for a complex disease in admixed and heterogeneous populations.

An association was reported between several SNPs in the transducin-like enhancer of split 4 (*TLE4*) gene on chromosome 9q with asthma in a population of 492 Mexican children with asthma, but these associations also did not reach genome-wide significance (59).

However, the investigators replicated these findings in an independent cohort of 177 Mexican case-parent trios. *TLE4* had not previously been linked to the pathogenesis of asthma, but does play a role in early B-cell development (60).

Association of asthma with SNPs in multiple genes was reported in a recent study containing more than 10,000 individuals with physician-diagnosed asthma and 16,000 unaffected persons matched for ancestry (61). SNPs in several loci achieved genome-wide significance, including a block on chromosome 2 that includes *IL1RL1* and *IL18R*, *HLA-DQ* on chromosome 6, *IL33* on chromosome 9, *SMAD3* on chromosome 15, and *IL2RB* on chromosome 22. The authors observed association with the previously reported *ORMDL3/GSDMB* locus on chromosome 17 only in childhood-onset asthma. Many of these genes have direct or indirect roles in T-cell responses (*IL2RB*, *HLA-DQ*) and the development of T<sub>H</sub>1 (*IL18R1*) or T<sub>H</sub>2 (*IL33*) responses. *SMAD3* is involved in the response to transforming growth factor  $\beta$ , which controls proliferation, differentiation, and apoptosis depending on the cellular context.

A GWAS from our group was recently reported on a series of pediatric asthma patients consisting of North American cases of European ancestry with persistent asthma requiring daily inhaled glucocorticoids for symptom control, and matched controls without asthma. In addition to the previously reported 17q21 locus, we uncovered association to a novel asthma locus on chromosome 1q31 in the discovery cohort and replicated the finding in an independent cohort of Northern European ancestry. The locus contains *DENND1B*, a gene that is expressed by natural killer (NK) cells and dendritic cells (62). Homologs of the *DENND1B* protein have been shown to interact with the TNF $\alpha$  receptor (63). To determine whether the 1q31 locus also contributes to asthma in children of African ancestry, we also tested for association of the chromosome 1q31 locus and asthma in African-American cases and ancestrally matched controls. A total of 17 of 20 SNPs were significantly associated with asthma, although the associated allele at each SNP was the alternative allele to that associated with asthma in the discovery set. Allele reversal at shared risk loci can be attributed to differences in the underlying genomic architecture at the loci between populations of different ancestry and as a result is being tagged differently. The *DENND1B* gene has since been replicated in inflammatory bowel disease, in separate studies of both Crohn disease and ulcerative colitis, and in primary biliary cirrhosis.

Six GWAS have been reported using intermediate phenotypes and quantitative traits, rather than asthma itself, as study end points. The first report used genome-wide associations to identify variants that modulate serum protein levels (64). A promoter SNP in the *CHI3L1* gene that encodes the chitinase-like protein YKL-40 was shown to influence serum YKL-40 levels and was also shown to be weakly associated with asthma, bronchial responsiveness,

and pulmonary function in the Hutterite population. A GWAS showed significant association of the *FCER1A* and *RAD50* genes with expression of CHI3L1, and evidence for association of the *STAT6* gene with IgE levels. IgE levels are closely correlated with the clinical expression and severity of both asthma and allergy. The *RAD50* variants were further shown to be associated with increased risk of asthma and atopic eczema (65).

Eosinophils are leukocytes that play an important role in the initiation and propagation of inflammatory signals. This makes them likely mediators of inflammatory disease and a GWAS was performed examining blood eosinophil counts. Five loci reached genome-wide association significance, one of which, *IL1RL1*, was also shown to be associated with asthma in a collection of 10 different populations (66).

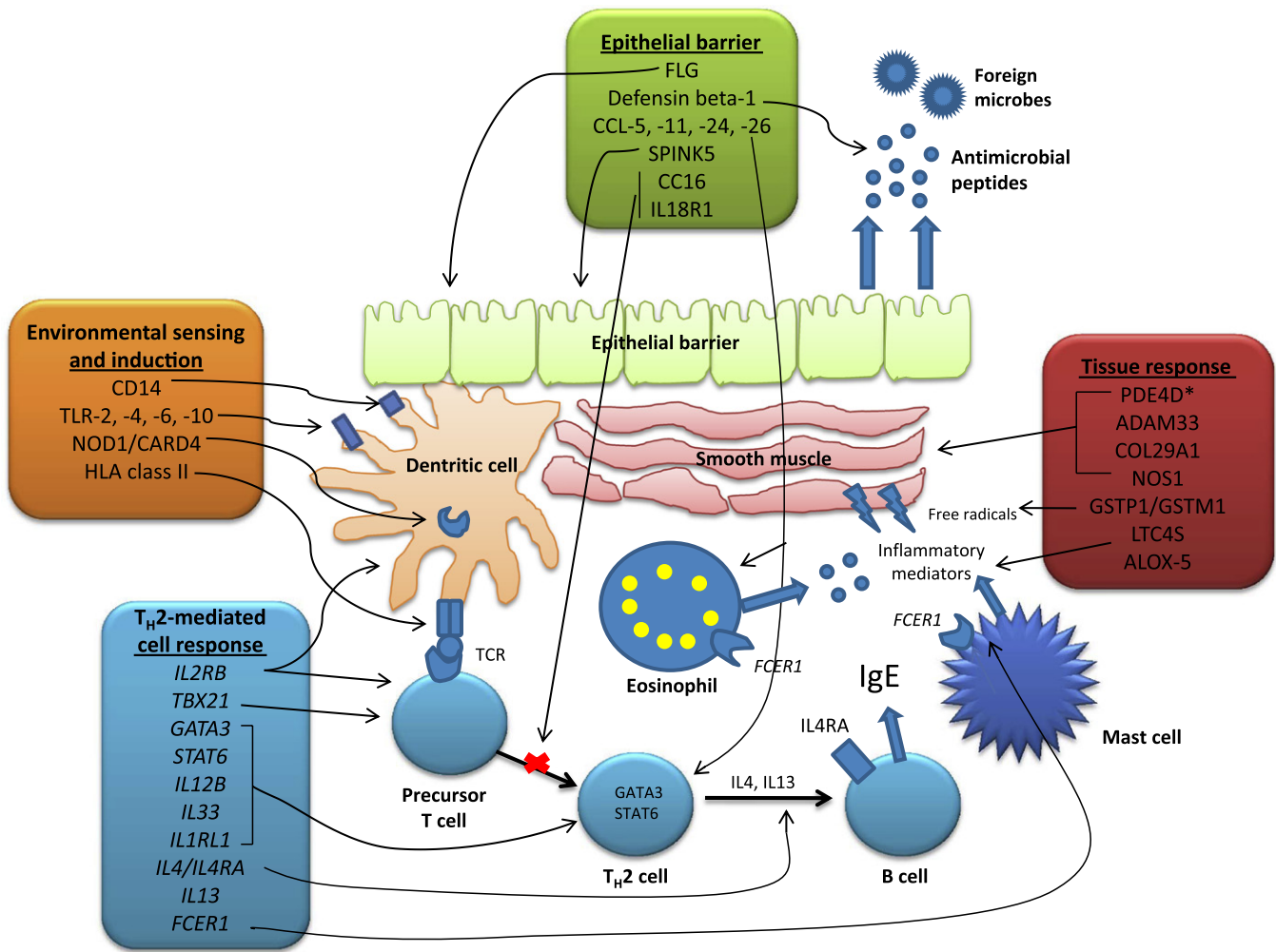
One GWAS focused on chronic obstructive pulmonary disease (COPD), and three other studies performed GWAS on lung function using a quantitative metric of lung function as a measure of airflow obstruction. Altered lung function, and airflow obstruction in particular, is associated with both asthma and COPD. Two SNPs at the  $\alpha$ -nicotinic acetylcholine receptor (*CHRNA 3/5*) surpassed genome-wide significance in the study and replicated in two of three independent cohorts. The *CHRNA 3/5* locus had previously been associated with lung cancer and nicotine dependence (67,68). The authors also reported that SNPs at the *HHIP* locus on chromosome 4 showed association and were consistently replicated across the study cohorts but did not reach genome-wide significance (69). In the first of the three lung-function GWAS that included 7691 Framingham Heart Study participants, the only locus to surpass genome-wide significance for association with FEV<sub>1</sub>/FVC ratio and replicate in an independent cohort of 835 Family Heart Study participants was *HHIP* (152).

The final two studies resulted in the identification of eleven novel loci associated with measures of lung function; both studies also replicated the previously reported association of the *HHIP* locus (70,71). These novel loci will not only shed further light on the pathways associated with pulmonary function but may also provide potential targets for respiratory disease such as asthma and COPD.

## 59.2.2 Themes Revealed by Genetic Analysis of Asthma Susceptibility

The numerous genome-wide linkage, candidate gene, and genome-wide association studies performed on asthma and asthma-related phenotypes have resulted in an increasingly large list of genes implicated in asthma susceptibility and pathogenesis. This list can be categorized into four broad functional groups (19,72–74), from which several themes have emerged, as summarized in Figure 59-1.

**59.2.2.1 T<sub>H</sub>2-Mediated Cell Response.** T<sub>H</sub>2-cell-mediated adaptive immune responses have been widely recognized as a crucial component of allergic disease.



**FIGURE 59-1** Genes implicated in asthma susceptibility and pathogenesis.

Genes involved in T<sub>H</sub>2-cell differentiation and function have been extensively studied in asthma candidate gene association studies, and as one might expect, SNPs in many of these genes have been associated with asthma and other allergic phenotypes. Genes important for T<sub>H</sub>1 versus T<sub>H</sub>2 T-cell polarization, such as *GATA3*, *TBX21*, *IL4*, *IL4RA*, *STAT6*, and *IL12B*, have been implicated in asthma and allergy (29–38,75,76). The genes encoding IL-13 and the beta-chain of the IgE receptor FcεR1 are well-replicated contributors to asthma susceptibility (37,39–41,75,77). These two molecules play critical roles in allergic disease.

**59.2.2.2 Environmental Sensing and Immune Detection.** A second class of associated genes is involved in detection of pathogens and allergens. These genes include pattern recognition receptors and extracellular receptors, such as *CD14*, toll-like receptor 2 (*TLR2*), *TLR4*, *TLR6*, *TLR10*, and intracellular receptors, such as nucleotide-binding oligomerization domain containing 1 (*NOD1/CARD4*) (78–83). Additional studies identified variations in the human leukocyte antigen (HLA) class II genes associated with asthma and allergen-specific IgE responses (77). These molecules are important in the immune response and shaping of the T-cell repertoire;

their involvement in an immune-mediated inflammatory disorder like asthma is unsurprising.

**59.2.2.3 Tissue Response.** A variety of genes involved in mediating the response to allergic inflammation and oxidant stress on the tissue level appear to be important contributors to asthma susceptibility. Examples include *ADAM33*, a disintegrin and metalloprotease expressed in lung fibroblasts and smooth muscle cells; the alpha-1 chain of type 29 collagen (*COL29A1*); leukotriene C4 synthase (*LTC4S*); glutathione S-transferase (*GSTP1*, *GSTM1*); arachidonate 5-lipoxygenase (*ALOX-5*); and nitric oxide synthase 1 (*NOS1*) (47,84–90).

**59.2.2.4 Epithelial Barrier Function.** Studies of asthma genetics have raised new interest in the body's first line of immune defense, the epithelial barrier, in the pathogenesis of asthma. Mutations in the filaggrin gene (*FLG*) were initially identified in the rare single-gene disorder ichthyosis vulgaris (91), but subsequently loss-of-function variants were reported to be strongly associated with atopic dermatitis, eczema, and asthma, both dependent and independent of atopic dermatitis (22,92–94). Filaggrin, a protein involved in keratin aggregation, is not expressed in the bronchial mucosa (95), which has lead others to



suggest that asthma susceptibility in patients with loss-of-function *FLG* variants may be due to allergic sensitization that occurs after breakdown of the epithelial barrier (96).

Several epithelial genes with important roles in innate and adaptive immune function have also been implicated in asthma. These genes include defensin-beta1 (an antimicrobial peptide), uteroglobin/Clara cell 16-kD protein (*CC16*) (an inhibitor of dendritic cell-mediated  $T_H2$ -cell differentiation), and several chemokines (*CCL-5*, -11, -24, and -26) involved in the recruitment of T cells and eosinophils (21,97–102). Variations in *SPINK5*, a serine protease inhibitor limited to the epithelium and the causative factor in Netherton syndrome (103), have been associated with asthma, but with conflicting results (75,81,104,105).

### 59.2.3 The Future of Asthma Genetics

Many studies that have been aimed at asthma and allergies have revealed considerable new information about the genetic variants that underlie susceptibility to the condition as well as its severity. It is through this work that we have come to appreciate the importance of the barrier function of epithelium and molecules involved in the sensing and effector arms of innate immunity. In addition, we have a much better picture of the critical roles played by both  $T_H2$  skewing and the molecules involved in the development and remodeling of the lungs. The application of GWAS to asthma, with the possibility of discovering new genes that are currently unsuspected in asthma pathobiology, has the potential to greatly and rapidly expand our knowledge of the genetic and biological factors contributing to this complex genetic disease. However, challenges remain in the understanding of the genetic contribution to asthma and only a relatively small proportion of its heritability is explained despite recent advances. Although the other contributing factors are not necessarily specific to asthma genetics, they are worth illuminating here.

**59.2.3.1 Gene–Environment Interactions.** Asthma, as an immune-mediated disease, involves the response of the body to the environment, in the form of pollutants, allergens, viruses, and other pathogens and irritants. These environmental factors interact with genetic variation to influence the development or severity of disease. Researchers are finding that specific genetic variants affect susceptibility to, and the severity of, asthma in different ways depending on the environments of the individuals carrying those variants, a phenomenon known as gene–environment interaction. Several examples of gene–environment interaction exist in asthma, with perhaps the best characterized being *CD14*. Interest in *CD14* as an asthma susceptibility locus originates with linkage studies identifying an association between asthma (and related phenotypes) and chromosome 5q, where *CD14* is located (106–110). The protein product of *CD14* acts to optimize the immune response to pathogen-associated molecular pathogens, such as lipopolysaccharide (LPS)/endotoxin (111,112). This function made *CD14* an interesting candidate gene within the chromosome 5q region linked to asthma. A functional SNP

(denoted *CD14*-260C>T) in the promoter of *CD14* was associated with increased *CD14* protein levels in serum and reduced serum IgE levels (113,114). This polymorphism has been examined in candidate gene association studies of asthma, with conflicting results. Studies have demonstrated a protective role for the T allele at this SNP, including reduced serum IgE levels and allergic symptoms in individuals bearing this allele (115,116). Conversely, other studies showed that the T allele does not protect from asthma or allergy, with association of this allele with higher IgE levels in laboratory workers (117), increased positive skin tests (118), and food allergies (119) in different study populations. In two German cohorts, *CD14*-260T was not associated with asthma, atopy, or IgE levels (120,121). The reasons for such conflicting results were unclear, until studies of potential environmental influences were performed. The *CD14*-260C allele was associated with higher IgE levels in children with pets like cats and dogs, while the opposite allele was associated with the same phenotype in children exposed to stable animals such as horses (122). Homozygotes for *CD14*-260T were found to be at lower risk for asthma if exposed to comparatively low levels of house dust endotoxin, but at higher risk at higher endotoxin exposures (123). These alleles have opposite effects on allergic reactions in individuals of Karelian ethnicity that live in either Finland or Russia (124), suggesting key environmental differences between these locales. This SNP has also been associated with effects on IgE by alcohol consumption (125) and *Helicobacter pylori* (126). Other polymorphisms at the *CD14* locus have been associated with different outcomes in specific populations, depending on environmental exposure (117). Given the large number of identified asthma susceptibility loci, and the daunting amount of environmental variables that may influence complex diseases, much work remains before we have a reasonable understanding of the roles of gene–environment interactions in asthma.

**59.2.3.2 Gene–Gene Interactions.** A comparatively small number of studies have been published to date examining the role in asthma of gene–gene interactions, where variation at one locus alters the effects of variations at a second locus, reflecting epistasis between two or more genes. The existing literature consists mainly of studies in which researchers have chosen two or more specific genes (and occasionally specific variants of those genes) to examine in the context of asthma, looking for evidence of interactions between the two loci. Examples of gene–gene interactions that have been observed in association with asthma include *IL9* and *IL9R* polymorphisms in Koreans (127), *TGFBR2* and *FOXP3* in specific IgE production (128), *IL13* and *IL4* in Dutch cohorts (129), and *LTA4H* and *ALOX5AP* in Latinos (90). Larger scale analysis examining 169 SNPs in 29 genes identified a number of gene–gene interactions affecting both total and antigen-specific IgE levels (130). Methods are actively being developed to enable large-scale and unbiased analysis of gene–gene interactions (131) and visualization of the resulting networks (132), but these

efforts are in their relative infancy. Given the number of previously identified relevant genes, and the possibilities for discovery of new loci, the combinatorial potential for interactions between gene effects is daunting. Much work remains in the development of methods and tools before we will truly grasp these vast possibilities.

**59.2.3.3 Replication.** It has been a long-term concern for the field of asthma genetics (and for the study of many other common, complex diseases) that many discoveries fail to be replicated in subsequent studies (133,134). While concerns about poorly constructed or statistically underpowered studies are constant, an appreciation has developed for other issues that may affect study results (135). Heterogeneity among cases and controls is the most basic confounder of genetic analyses. While tools have been developed to address the most obvious source of heterogeneity, racial and ethnic diversity (136–138), other less apparent sources may remain. As described earlier, asthma is influenced by gene–environment interactions, with genetic and environmental variability interacting in unexpected ways. The search for statistical power has led to studies with increasingly large sample sizes; large populations are required to detect the common variants with small effect sizes that are hypothesized to underlie complex diseases like asthma. However, these growing study populations carry risks as well as benefits, as it becomes more difficult to account for diversity among large populations. While computational tools exist to deal with racial stratification in study groups, less attention has historically been paid to geographic and environmental diversity. And while genetic analyses like GWAS often inherently include the information required to identify racial stratification, accounting for environmental variations requires additional effort in the form of questionnaires or surveys answered by study participants, or observation and sampling of the participants' environments. These concerns may directly affect the issue of replication, as the assembly of ever larger cohorts may result in the mixing of environmental influences, diluting the effects of gene–environment interactions and making the detection of the connected loci difficult if not impossible. In fact, the example of *CD14* described earlier demonstrates that studies focused on smaller groups can yield informative results, if the smaller cohorts are accompanied by reports on environmental influences. However, in the case of asthma the list of environmental influences may be extraordinarily long. Endotoxin, various allergens, cigarette smoke, airborne pollutants, and indoor pets or stable animals have all been considered environmental factors for asthma, as have less obvious connections, such as whether and for how long an infant was breast-fed (139,140). Controlling for these impacts will be increasingly laborious, and lack of accounting for some of these factors may account for failures of genes to be replicated.

Replication in asthma studies faces an additional complication in the varying phenotypes that can be considered asthma-associated, and the degree to which a specific locus needs to be identified to be considered replicated. Both phenotypes and genotypes can be subjected to varying

stringent criteria, generally referred to as strict or loose replication (141). For phenotypes, strict replication would involve studies that consider only the same end point (i.e. diagnosis of asthma, BHR, serum IgE levels, allergic skin test responses), while loose replication would consider studies that identified the same locus using varying but related end points. For genotypes, strict replication would require identification of precisely the same variant of a gene (SNP, copy number variation, insertion or deletion) in multiple studies, while loose replication considers the gene itself to be the unit of replication, including all variants identified at a locus. Use of the gene as the unit of replication has been advocated (18,142), and is becoming more common, but worries persist that this is too permissive a standard and may lead to inappropriate conclusions of replication (141). Obviously, convincing demonstration of replicated results will be difficult if researchers cannot agree on even the standard of replication required.

**59.2.3.4 Pharmacogenetics.** Pharmacogenetics, in which variations in genotype are examined for their effects on the response to treatments, is of growing interest with asthma, with the hope that it will increase efficacy and reduce toxic side effects of medications. It is a nascent field, and as such there are still few studies that involve more than a few hundred subjects, and most are limited to only one or two candidate genes selected for their known or suspected roles in response to specific medication. The best example at this time is provided by beta-adrenergic receptor agonists (or simply beta-agonists), which are prescribed to treat bronchoconstriction and provide long-term symptom control for asthmatics. The *ARDB2* locus encodes the beta<sub>2</sub>-adrenergic receptor, which binds to and is activated by beta-agonists. This activation leads to several downstream effects, including relaxation of airway smooth muscle, thereby alleviating the acute symptoms of an asthma attack. Two studies have implicated variations in *ARDB2* as modulators of response to inhaled bronchodilators (143,144). However, a recent randomized, double-blind study was performed in which subjects were genotyped before being enrolled, so that they could be stratified by genotype before receiving prescriptions (145). This study showed no association of genotype with the response to beta-agonists. One study showed that an arginine/glycine polymorphism at position 16 of the *ARDB2* protein influences the response to regularly administered albuterol, with the Arg/Arg genotype receiving less relief from regular, long-term use of short-acting beta-agonists (146). Yet another group has shown that genotype *ARDB2* does not affect the response to combined beta-agonist and inhaled corticosteroid treatment (147).

While several other genes that are targeted by current asthma treatments have been identified as asthma susceptibility loci, very few have actually been analyzed for the effects of variations in genotype on treatment. Genes involved in the biosynthesis of leukotrienes, such as *ALOX-5*, *ALOX5AP*, and *LTC4S*, have been identified as asthma susceptibility loci; drugs targeting the leukotriene pathway, such as 5-lipoxygenase inhibitors

and cysteinyl leukotriene receptor 1 antagonists, have been approved for treatment of asthma. One group has shown that variations at *ALOX5* correlate with the response to a 5-lipoxygenase inhibitor (148), while another demonstrated that an SNP in the gene *LTA4H*, which encodes a leukotriene cleaving enzyme (LTA<sub>4</sub> hydrolase), associates with variability in the response to a cysteinyl leukotriene receptor 1 antagonist (149). Two studies have shown that variants of genes involved in the synthesis of or the response to glucocorticoids impact the response to inhaled glucocorticoids in asthmatics. Polymorphisms in corticotrophin-releasing hormone receptor (*CRHR1*) (150) and the *STIP1* gene (involved in the signaling initiated by glucocorticoids) (151) associate with variable FEV<sub>1</sub> response after

inhaled glucocorticoid treatment, as do polymorphisms in *TBX21*, encoding a transcription factor important in the generation of T<sub>H</sub>1 cells (35). This last study demonstrates that variations in genes not directly involved in the metabolism or signaling cascades of a drug can be important modulators of the response to that drug. This point will be critical in the future of pharmacogenetics, as researchers design the studies and tools necessary to examine drug–gene interactions in an unbiased way. Just as GWAS and linkage studies allow identification of susceptibility loci that would not have been suspected based on known function, unbiased pharmacogenetic studies could allow identification of variants that affect the response to treatment that cannot be predicted based on current information (Table 59-3).

**TABLE 59-3 Summary of GWAS Loci Referenced in this Review, Including Chromosome Location, the Most Significant SNP Identified, and the End Point of the Study**

Reported Gene	Locus	Top SNP	End Point Analyzed	Reference
<i>RAD50</i>	5q31.1	rs2244012	Asthma	Li et al., 2010 (77)
<i>HLA-DR/DQ</i>	6p21.32	rs3998159		
<i>DENND1B</i>	1q31.3	rs2786098	Asthma	Sleiman et al., 2010 (62)
<i>TLE4</i>	9q21.31	rs2378383	Asthma	Hancock et al., 2009 (59)
<i>PDE4D</i>	5q12.1	rs2548659	Asthma	Himes et al., 2009 (55)
<i>ORMDL3</i>	17q12	rs7216389	Asthma	Moffatt et al., 2007 (48)
<i>PDE11A</i>	2q31.2	rs11684634	Asthma	DeWan et al., 2010 (57)
<i>CHI3L1</i>	1q32.1	rs4950928	Asthma/YKL-40 serum levels	Ober et al., 2008 (64)
<i>FCER1A</i>	1q23.2	rs2251746	Serum IgE levels	Weidinger et al., 2008 (65)
<i>STAT6</i>	12q13	rs12368672		
<i>RAD50</i>	5q31.1	rs2040704		
<i>IL1RL1</i>	2q12.1	rs1420101	Blood eosinophil count/asthma	Gudbjartsson et al., 2009 (66)
<i>IKZF2</i>	5q31.1	rs12619285		
<i>GATA2</i>	3q21.3	rs4857855		
<i>IL5</i>	2q12.1	rs4143832		
<i>SH2B3</i>	12q24.12	rs3184504		
<i>CHRNA 3/5</i>	15q24	rs8034191	COPD	Pillai et al., 2009 (69)
<i>HHIP</i>	4q31.22	rs13147785	FEV <sub>1</sub> /FVC	Wilk et al. 2009 (152)
<i>TNS1</i>	2q35	rs2571445	FEV <sub>1</sub>	Repapi et al., 2010 (71)
<i>GSTCD</i>	4q24	rs10516526	FEV <sub>1</sub>	
<i>HTR4</i>	5q33.1	rs3995090	FEV <sub>1</sub>	
<i>AGER</i>	6p21.32	rs2070600	FEV <sub>1</sub> /FVC	
<i>THSD4</i>	15q23	rs12899618	FEV <sub>1</sub> /FVC	
<i>GPR126</i>	6q24.1	rs3817928	FEV <sub>1</sub> /FVC	Hancock et al., 2010 (70)
<i>ADAM19</i>	5q33	rs2277027	FEV <sub>1</sub> /FVC	
<i>AGER-PPT2</i>	6p21.3	rs2070600	FEV <sub>1</sub> /FVC	
<i>FAM13A</i>	4q22.1	rs2869967	FEV <sub>1</sub> /FVC	
<i>PTCH1</i>	9q22.32	rs16909898	FEV <sub>1</sub> /FVC	
<i>PID1</i>	2q36.3	rs1435867	FEV <sub>1</sub> /FVC	
<i>HTR4</i>	5q33.1	rs7735184	FEV <sub>1</sub> /FVC	
<i>INTS12-GSTCD-NPNT</i>	4q24	rs17331332	FEV <sub>1</sub>	
<i>IL1RL1/IL18R1</i>	2q12.1	rs3771166	Asthma	Moffatt et al., 2010 (61)
<i>HLA-DQ</i>	6p21.32	rs9273349		
<i>IL33</i>	9p24.1	rs1342326		
<i>SMAD3</i>	15q22.33	rs744910		
<i>IL2RB</i>	22q12.3	rs2284033		
<i>ORMDL3/GSDMB</i>	17q12	rs2305480	Childhood onset asthma	

### 59.3 CONCLUSION

Despite the progress that has been made in understanding the genetic contribution to asthma and allergic disease in recent years, much work remains to be done before this information can be used to improve the diagnosis and management of individuals suffering from these conditions. Today's discoveries unfortunately only explain a fraction of the heritability of asthma. Referred to as "missing heritability," this difficult-to-fill void in our understanding has become a common theme in the analysis of complex genetic traits. As more GWAS of increasingly greater size are performed, it is likely that additional loci will be identified that will close a portion of this gap. However, GWAS are not well suited to the detection of rare variants with potentially large effects. More importantly, the development of techniques to describe gene–gene and gene–environment interactions, which likely play a key role in the susceptibility to allergic disease, is only in its infancy. Very little is known how epigenetic phenomena, heritable but reversible changes to DNA (such as methylation) that are not detected by standard genotyping, may contribute to asthma and allergy pathogenesis. Advancement in sequencing technologies is likely to enable strategies capable of thoroughly examining the entire genome, including all genetic and epigenetic variations and their complex interaction. Together with transcriptome sequencing and in silico, in vitro, and in vivo models of human-derived tissues through induced pluripotent stem (IPS) cell cultures, eventually we will be able to complete the journey from genetic discovery to improvements in asthma patient care.

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### CROSS REFERENCES

Genomics and Proteomics; Epigenetics; Analysis of Genetic Linkage; Multifactorial Inheritance and Complex Traits; Population Genetics; Pharmacogenetics and Pharmacogenomics; Bioinformatics; Genetic Risk Assessment for Common Disease.

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## RELEVANT WEB PAGES

The Global Initiative for Asthma: <http://www.ginasthma.org>.  
 The American Lung Association: <http://www.lungusa.org/>.

## Biographies



**Hakon Hakonarson, MD, PhD**, is an associate professor of Pediatrics at The University of Pennsylvania School of Medicine. He is a physician-scientist and pediatric pulmonologist by training and director of The Children's Hospital of Philadelphia's Center for Applied Genomics (CAG), a high-throughput highly automated genotyping facility founded to identify the genetic causes of complex medical disorders in children, such as autism and cancer, with the objective of developing new therapies. The Center represents a \$40 million commitment from CHOP to genotype approximately 100,000 children, a research undertaking that has gained nationwide attention, including news features in the *Wall Street Journal*, *New York Times*, *Time Magazine*, *Nature* and *Science*. Dr Hakonarson has an extensive track record in human genetics and has developed an international reputation among his peers. He has served previously in several senior posts in the biopharmaceutical industry, including as the Director of Inflammatory and Pharmacogenomics Research and the Vice President of Clinical Sciences and Development and CSO. Dr Hakonarson has also been the principal and co-principal investigator on several NIH-sponsored grants, and he has published numerous high-impact papers on genomic discoveries and their translations in some of the most prestigious scientific medical journals, including *Nature*, *Nature Genetics* and *The New England Journal of Medicine*. *Time Magazine* listed Dr Hakonarson's autism gene discovery reported in *Nature*, 2009, among the top 10 medical breakthroughs of that year. With over 10 years of experience in pioneering genomics research and genome-wide mapping and association studies in asthma and other complex diseases, Dr Hakonarson has intimate knowledge of the complexities of large-scale genomics projects and has put together the necessary infrastructure and workflow processes to unravel these complexities.



**Michael E March, PhD**, is a research scientist at The Children's Hospital of Philadelphia's Center for Applied Genomics (CAG). He earned his PhD in microbiology from the University of Virginia in 2003 for research into the molecular mechanisms of Fc-receptor-mediated inhibition of antigen stimulation in B cells, in the laboratory of Kodimangalam S. Ravichandran, PhD. He served as a postdoctoral fellow in the laboratory of Eric O. Long, at the National Institute for Allergy and Infectious Diseases, where he engagement. Since joining CAG in 2010, Dr March has undertaken the molecular characterization of *DENND1B*, a molecule identified by Dr Patrick Sleiman, colleagues in CAG, and a host of collaborators as a novel asthma susceptibility gene with unknown mechanisms for influencing asthma or immune function.



**Patrick M A Sleiman, PhD**, is currently Assistant Professor of Pediatrics at the Children's Hospital of Philadelphia and the University of Pennsylvania Medical School. He obtained a PhD in genetics from University College London and was subsequently a postdoctoral research fellow at the Institute of Neurology in London and then at the Center for Applied Genomics in Philadelphia. His research interests are in identifying the genetic basis of Mendelian and complex human disease, specifically within the domains of asthma and allergies, neurodevelopment and neurodegeneration. Dr Sleiman has been involved in the discovery of several novel disease genes in asthma, Parkinson's disease and dementia and has authored over 70 peer-reviewed publications.

# CHAPTER

# 60

## Hereditary Pulmonary Emphysema

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### ABBREVIATIONS

AAT – alpha 1-antitrypsin  
 CEPH – Centre d'Etude du Polymorphisme Humain  
 COPD – chronic obstructive pulmonary disease  
 COPDGene – Genetic Epidemiology of COPD study  
 CT – computed tomography  
 DLCO – diffusing capacity for carbon monoxide  
 ECLIPSE – Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points study  
 EXACTLE – exacerbations and CT scan as lung endpoints study  
 FEF25–75 – forced expiratory flow rate between 25% and 75% of forced vital capacity  
 FEV1 – forced expiratory volume in 1s  
 FVC – forced vital capacity  
 GOLD – global initiative for chronic obstructive lung disease  
 HPLC – high-performance liquid chromatography  
 HRCT – high-resolution computed tomography  
 MMP – matrix metalloproteinase  
 NAS – normative aging study  
 NETT – National Emphysema Treatment Trial  
 PI – protease inhibitor  
 SHARe – SNP Health Association Resource  
 SNP – single nucleotide polymorphisms  
 SPEP – serum protein electrophoresis  
 SPIROMICS – SubPopulations and InteRmediate Outcome Measures In COPD Study  
 STR – short tandem repeat  
 SVC – slow vital capacity

### 60.1 INTRODUCTION

Pulmonary emphysema has been a recognized clinical problem since Bonet described the condition of “voluminous lungs” in 1679. The first description of enlarged airspaces in emphysema was by Ruysh in 1721, and Baillie described the destructive character of the condition in 1789, which was followed by the comprehensive description by Laennec in 1819 (1). In the 1893 edition of his *Principles and Practice of Medicine*, Osler described several forms of

emphysema, including hypertrophic emphysema, which involved “distention of the air-cells and atrophy of their walls, and clinically by the imperfect aeration of the blood and more or less marked dyspnoea” (2). In addition to this remarkably modern description of emphysema, Osler noted “the markedly hereditary character of the disease.”

Chronic obstructive pulmonary disease (COPD) includes emphysema and chronic bronchitis. Because emphysema is encompassed within the diagnostic rubric of COPD, we focus our discussion on COPD in general and only refer to emphysema when specifically warranted. Since the mass production of cigarettes began in the early 1900s, the development of COPD has become increasingly common. COPD is the fourth leading cause of morbidity and mortality in the United States and is expected to rank third as the cause of death worldwide by 2020 (3). There are estimated to be 10 million individuals in the United States with physician-diagnosed COPD and many more affected individuals who are undiagnosed (4).

Although case reports of familial COPD were published in the 1950s (5), interest in the role of genetic factors in COPD largely began with the discovery of severe alpha 1-antitrypsin (AAT) deficiency by Laurell and Eriksson in 1963 (6). AAT deficiency is a proven genetic determinant of COPD; therefore, we discuss the molecular and population genetics of AAT deficiency in detail. In addition, we review the evidence for genetic factors in non-AAT deficiency COPD, including assessment of risk to relatives, segregation analysis, linkage analysis, and association studies. We also discuss the utility of animal models in identifying the genetic determinants of COPD.

### 60.2 DISEASES WITH AIRFLOW LIMITATION: DEFINITIONS

Pulmonary emphysema is included with chronic bronchitis and small airway disease in the syndrome of COPD. COPD is defined as a “disease state

characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases” (7). Thus, airflow limitation, also known as airflow obstruction, is central to the definition of COPD. In addition to chronic bronchitis, emphysema, and small airway disease, COPD has also been used to describe the subset of asthmatic subjects who have chronic airflow obstruction as well as subjects with less common conditions such as bronchiectasis. For this discussion, we restrict COPD to include individuals with chronic bronchitis, small airway disease, and/or pulmonary emphysema.

### 60.2.1 Pulmonary Emphysema

Pulmonary emphysema is an anatomically defined condition characterized by abnormal airspace enlargement and destruction of airspaces beyond the terminal bronchioles but without obvious fibrosis (8). This destructive process reduces elastic recoil of the lung, accounting for airflow obstruction. In addition, the surface available for gas exchange is reduced, which, in severe cases, limits the capacity for gas exchange to occur and results in hypoxemia. A cross-section of an emphysematous lung is shown in Figure 60-1.

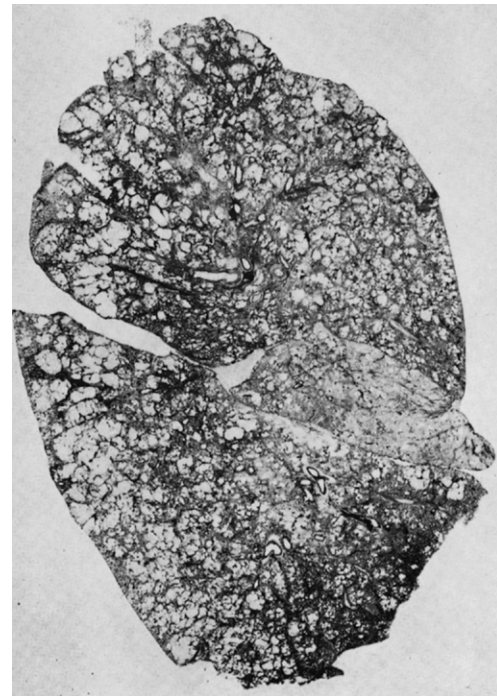
### 60.2.2 Chronic Bronchitis

Chronic bronchitis is a condition defined on a clinical basis by the presence of cough productive of phlegm for at least 3 months per year for at least two consecutive years (8). This excess mucus production should not be caused by other conditions, such as bronchiectasis or tuberculosis. Subjects with chronic bronchitis frequently do not have chronic airflow obstruction; for this discussion, we consider individuals with chronic bronchitis to have COPD only if they also have chronic airflow obstruction.

### 60.2.3 Small Airway Disease

COPD also includes small airway disease—a poorly understood entity that includes inflammation of the terminal and respiratory bronchioles as well as fibrosis of airway walls with narrowing (8). Some inflammation of the terminal and respiratory bronchioles is likely present in all cigarette smokers. However, only a subset of smokers develops fibrosis and narrowing of the small airways, with associated airflow obstruction.

The classification of disease processes encompassed within COPD emphasizes the heterogeneity of this disorder. Some investigators have attempted to define subsets of COPD based on clinical and/or physiological criteria. For example, Burrows suggested that chronic airflow obstruction included a group of patients with



**FIGURE 60-1** Paper mounted whole lung section from a subject who died with COPD. This subject had severe emphysema, which was primarily the centrilobular form. Destruction of respiratory bronchioles produced increased airspaces throughout the lung. (From Thurlbeck et al., 1970 (246).)

emphysema, who were largely male smokers with progressive airflow limitation, and a group of patients with chronic asthmatic bronchitis, who were largely female with a more benign clinical course (9). However, dissection of the syndrome of COPD will likely require improved understanding of the pathophysiological basis of disease, and, potentially, the genetic determinants of this condition (10).

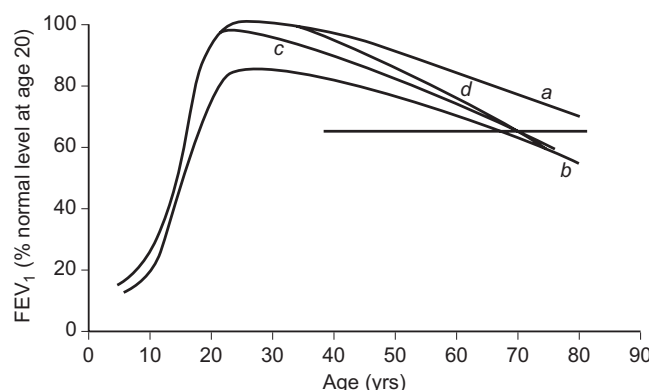
## 60.3 PHENOTYPIC EVALUATION IN COPD

A variety of phenotypes are useful in genetic and epidemiologic studies of COPD, including pulmonary function test data, chest computed tomography (CT) scan measurements, and questionnaire-derived assessments of respiratory symptoms. Development of biochemical markers that relate to the pathophysiology of COPD has been less successful.

### 60.3.1 Pulmonary Function Tests

As noted above, fixed airflow obstruction is essential for the definition of COPD. Airflow obstruction is typically determined by spirometry, which includes forced expiratory maneuvers after the subject has inhaled to total lung capacity. Key phenotypes obtained from spirometry include the volume of air exhaled within the first second of the forced expiratory volume in 1 s (FEV<sub>1</sub>) and





**FIGURE 60-2** Hypothetical tracking curves of FEV<sub>1</sub> which can lead to COPD. The normal pattern of growth and decline in FEV<sub>1</sub> is shown by curve a. Significant reductions in FEV<sub>1</sub> (below a threshold of 65% predicted) can occur by: (1) normal rate of decline after a reduced maximally attained FEV<sub>1</sub> (curve b); (2) early initiation of FEV<sub>1</sub> decline following normal growth (curve c); and (3) accelerated decline in FEV<sub>1</sub> following normal growth (curve d). (From Rijcken, 1991 (17).) B. Bronchial responsiveness and COPD risk: An epidemiological study (postdoctoral dissertation). (Adapted from Fletcher and Peto, 1977 (16).)

the total volume of air exhaled during the entire maneuver (forced vital capacity [FVC]). Subjects with airflow obstruction have a reduced ratio of FEV<sub>1</sub>/FVC; however, in addition to COPD, airflow obstruction can result from other diseases, such as asthma. Spirometric values are typically expressed as a percentage of predicted values, to adjust for known effects of age, gender, height, and ethnicity on these parameters.

In normal subjects, maximally rapid vital capacity (i.e. FVC) is equal to the relaxed or slow vital capacity (SVC). In subjects with significant airflow limitation, SVC is often larger than FVC because airways in such subjects tend to close before the lung region that they serve has fully emptied. Therefore, airflow obstruction may be more sensitively determined by the use of the FEV<sub>1</sub>/SVC rather than FEV<sub>1</sub>/FVC. Other spirometric measures, such as the forced expiratory flow rate between 25% and 75% of the FVC (FEF<sub>25-75</sub>), may also be used as phenotypes in the study of COPD. Some investigators contend that FEF<sub>25-75</sub> reflects obstruction in small airways, but the large coefficient of variation associated with this measurement may limit its utility.

Reduced FEV<sub>1</sub> has been repeatedly demonstrated to be a major risk factor for mortality from chronic lung disease (11,12) and from all causes (13,14). Since reduced FEV<sub>1</sub> can result from restrictive lung diseases (e.g. interstitial lung diseases, neuromuscular diseases) as well as obstructive lung diseases, analysis of the FEV<sub>1</sub>/FVC ratio selects for conditions associated with airflow obstruction. The relationship between emphysema and airflow obstruction is controversial; some investigators suggest that emphysema is not the major cause of airflow obstruction in COPD (15).

FEV<sub>1</sub> is particularly valuable in the assessment of COPD because it tends to track consistently throughout the life of an individual. FEV<sub>1</sub> follows a pattern of growth, plateau, and then decline with increasing age. There are at least three mutually independent ways that one can reach a low level of FEV<sub>1</sub> in later adult life.

Specifically, one can have reduced growth, premature decline, or accelerated decline in lung function. A simplified graphic depiction of the natural history of COPD is shown as a function of the influences on tracking curves of FEV<sub>1</sub> in Figure 60-2 (16,17). Death or disability can result from normal rate of decline after a reduced growth phase (curve b), early initiation of pulmonary function decline after normal growth (curve c), or accelerated decline after normal growth (curve d). The rate of decline can be modified by changing environmental exposures (i.e. quitting smoking), with smoking cessation at an earlier age leading to a more beneficial effect than smoking cessation after disability has developed.

Although rare individuals may experience precipitous declines in pulmonary function values, most individuals follow a steady trajectory of increased pulmonary function with growth during childhood and adolescence, followed by gradual decline with aging (18,19). Children appear to track in their quantile of pulmonary function based on environmental, developmental, and familial factors that put them on different tracks at an early age.

In addition to spirometry, other pulmonary function tests demonstrate abnormalities in COPD. Patients with emphysema tend to have increased total lung capacity and residual volume, related to the hyperinflation that accompanies alveolar destruction. Reduced diffusing capacity for carbon monoxide (DLCO) is also frequently noted in emphysema. However, DLCO is not reduced in some subjects with emphysema, and DLCO may be reduced in subjects with small airway disease rather than emphysema (20,21). A variety of pulmonary function tests were developed to assess early small airway disease, in an effort to identify a susceptible subpopulation of cigarette smokers who would develop severe COPD; such measurements, including the closing volume, tended to be abnormal in all smokers and were not useful in the identification of susceptible smokers (22).

### 60.3.2 Chest CT

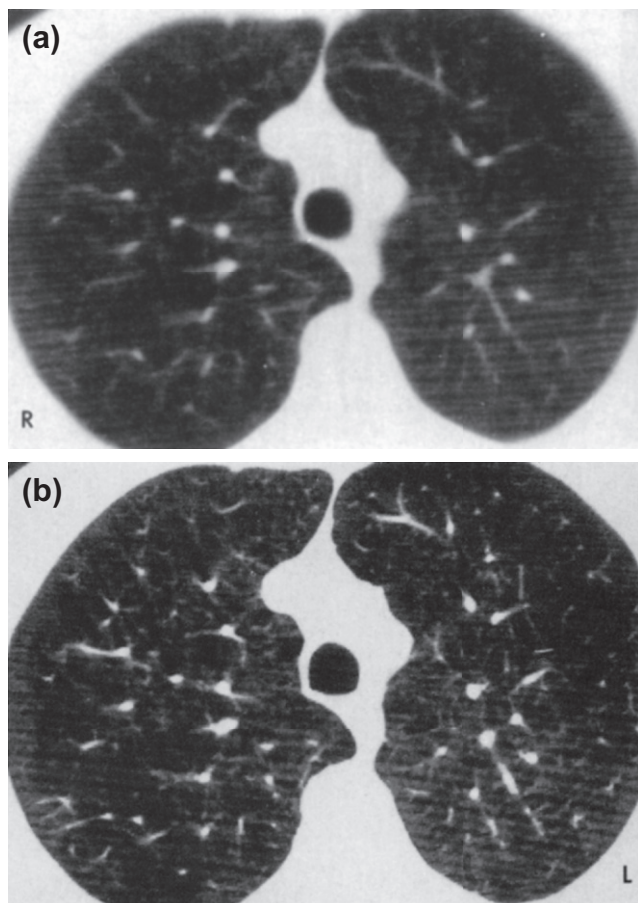
The development of high-resolution CT (HRCT) chest scans has provided a powerful, noninvasive tool for assessment of the anatomic presence of emphysema. HRCT scans are much more sensitive than conventional chest X-rays for the detection of emphysema. High-resolution (thin cut) images allow identification and quantification of the extent of emphysema, which was not possible with conventional (thick cut) chest CT scan images (23). An example of the improved detection of emphysema by HRCT compared with conventional chest CT is shown in Figure 60-3.

Both qualitative (presence/absence or severity scales determined by radiologist scoring) and quantitative (computerized image analysis) approaches have been used to analyze the severity and distribution of emphysema on HRCT. Radiologist scoring typically involves grading the severity of emphysema in different lung regions and obtaining an overall emphysema score; independent scoring by multiple radiologists probably improves the quality of the emphysema scoring data. Using computerized

image analysis of the frequency distribution of X-ray attenuation values within the lung, thresholds can be defined corresponding to the presence of emphysema, and the fraction of lung below those thresholds can be used to assess the percentage of emphysema within the lung (24,25). Furthermore, Gupta et al. demonstrated that various quantitative and qualitative HRCT features correlate with patients' characteristics, spirometric indices, and health-related quality-of-life score, suggesting that HRCT is useful not only in detecting emphysema and its various subtypes but also in predicting the extent and severity of COPD (26).

Assessment of small airway disease using chest CT scanning has been quite challenging because the small airways responsible for airflow obstruction in COPD (<2 mm in internal diameter) are below the resolution of current chest CT scanners (27,28). However, Nakano et al. have shown that measurements of larger airways reflect the degree of remodeling in the small airways (29).

Kitaguchi et al. reported three COPD phenotypes based on HRCT evaluation: absence of emphysema, with little emphysema with or without bronchial wall thickening (A phenotype); emphysema without bronchial wall thickening (E phenotype); and emphysema with bronchial wall thickening phenotype (M-phenotype) (30). The presence of bronchial wall thickening in A- and M-phenotype groups was significantly associated with reversibility responsive to treatment with inhaled corticosteroid and sputum eosinophilia. These findings suggest that morphological phenotypes assessed by HRCT may help to evaluate the severity of COPD and identify patients who will respond to selective therapy. Although chest CT scanning holds great promise for COPD genetic studies, the optimal chest CT phenotypes for studies of COPD in general, and COPD genetic studies in particular, remain to be defined.



**FIGURE 60-3** Comparison of standard chest CT (image a) and high-resolution chest CT (image b) of a subject with severe AAT deficiency. Emphysema is not detected by standard chest CT, but regions of low-density and pulmonary vascular attenuation caused by emphysema are apparent on the high-resolution CT image. (From Guest and Hansell, 1992 (23).)

### 60.3.3 Questionnaire Phenotypes

A standardized questionnaire for evaluation of key epidemiological variables in respiratory studies was developed by the National Heart, Lung, and Blood Institute's (NHLBI) Epidemiology Standardization Project in 1978 (31). With minor modifications, the adult and children's versions of this questionnaire have been widely used in epidemiological and genetic studies of COPD. Questionnaire responses to items related to chronic cough and chronic phlegm production are essential for the clinical definition of chronic bronchitis; use of questions related to physician-diagnosed chronic bronchitis and physician-diagnosed emphysema are less accurate for the diagnosis of these conditions.

It is important to recognize that the physiological definition of COPD can be contrasted with the clinical criteria for the definition of chronic bronchitis (determined by questionnaire) and the anatomic criteria for the definition of emphysema (determined by radiologic or

pathologic studies). Airflow obstruction is more likely with greater severity of anatomic emphysema, but a substantial fraction of subjects with airflow obstruction do not have significant emphysema, and some subjects with significant emphysema do not have airflow obstruction (32,33). To date, it remains unclear what genetic, environmental, and pathobiological factors contribute to this less-than-perfect correlation.

### 60.3.4 Biochemical Markers

A variety of biochemical markers have been developed in an effort to reflect the inflammation and tissue destruction of COPD. The decline in pulmonary function tests, such as FEV<sub>1</sub>, in COPD is a gradual process. Therefore, detection of a change in the rate of decline in FEV<sub>1</sub> in response to treatment may require a long period of observation in a large number of subjects. If biochemical assays in readily obtainable samples (e.g. blood, urine) could be used as surrogate markers for the pathophysiological process leading to loss of pulmonary function in COPD, the efficacy of novel treatment approaches could be assessed more rapidly.

As lung elastin degradation appears to be critical for the development of emphysema, serum levels of elastin-derived peptides and urine levels of desmosine (a specific breakdown product of elastin) have been developed (34,35). As desmosine is exclusively excreted in the urine, urine measurements of desmosine levels have typically been performed (36). A radioimmunoassay for desmosine measurement was reported by King et al. in 1980 (36). Pelham et al. compared the desmosine levels in urine samples that had been hydrolyzed with hydrochloric acid to release desmosine from elastin in 17 severely AAT-deficient subjects (PI Z) with COPD and 27 healthy control subjects; no differences in urine desmosine levels were detected between these groups (37). Subsequently, Starcher and Scott demonstrated that urine contained several substances that interfered with the accurate measurement of desmosine levels by immunoassay; they developed purification procedures, including hydrolysis with acetone and extraction with chloroform/ethanol, to remove these contaminants (38). Stone et al. developed an alternative approach to remove urine contaminants; they developed a high-performance liquid chromatography (HPLC) assay for desmosine measurement that also measures isodesmosine (another specific elastin breakdown product) and hydroxylsypyrindoline and lysypyrindoline (specific collagen breakdown products) (39). Stone et al. subsequently compared urine desmosine levels using this HPLC assay in 22 lifelong nonsmokers, 13 current smokers without airflow obstruction, and 21 COPD patients (34). In univariate analysis, desmosine and isodesmosine levels in smokers with and without COPD were similar; both groups had elevated levels compared to nonsmokers. In multivariate analysis, COPD appeared to be associated with

increased desmosine and isodesmosine levels. Of interest, Stone et al. found elevated levels of the specific collagen breakdown products in COPD patients compared to either smokers without airflow obstruction or lifelong nonsmokers. More recently, Ma et al. have developed a more sensitive method for measuring desmosine and isodesmosine using HPLC followed by electrospray ionization mass spectroscopy; this method is able to detect the low amounts of free desmosine and isodesmosine in urine, and total desmosine and isodesmosine in sputum from COPD subjects (40).

Additional efforts to develop novel biochemical markers in COPD have focused on the assessment of inflammation and oxidative stress. For example, Montuschi et al. found elevated levels of 8-isoprostane, a potential biomarker of oxidative stress, in exhaled breath of COPD subjects compared to both smoking and nonsmoking control subjects (41). Subsequently, Biernacki et al. showed that 8-isoprostane levels in exhaled breath increase during COPD exacerbations (42).

However, despite the potential utility of a biomarker for elastin degradation or COPD-related inflammation, none of these various biochemical phenotypes has been consistently validated in large numbers of subjects. Moreover, they have not been applied as phenotypes in genetic studies of COPD.

Recently, several large-scale clinical studies have been conducted to identify novel biomarkers. One such study is Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points (ECLIPSE), a 3-year longitudinal study to define the parameters that characterize subgroups and predict disease progression in more than 2000 individuals with COPD (43). Lomas et al. evaluated 2083 individuals aged 40–75 years with COPD and a smoking history of  $\geq 10$  pack-years, 332 controls with a smoking history of  $\geq 10$  pack-years and normal lung function, and 237 nonsmoking controls in the ECLIPSE cohort. They demonstrated that serum clara cell (CC) protein-16 levels were reduced in individuals with COPD and that there is a weak correlation with disease severity in former smokers (44). Lomas et al. also demonstrated that serum surfactant protein D was significantly elevated in 1888 individuals with COPD compared to 296 current and former smokers without airflow obstruction and 201 nonsmokers although there was no correlation with the severity of COPD (45). In 488 COPD patients from the ECLIPSE cohort, Singh et al. demonstrated that sputum neutrophil levels are associated weakly with FEV<sub>1</sub> % predicted and health status (46). The value of sputum counts in COPD appears to be principally as a tool for measuring the burden of neutrophils in the airways although sputum counts do not appear to be a major surrogate biomarker in COPD.

Another study is SubPopulations and Intermediate Outcome Measures In COPD Study (SPIROMICS), which will follow several thousand patients with COPD



across six US sites and is sponsored by the National Institutes of Health ([www.csc.unc.edu/spir/](http://www.csc.unc.edu/spir/)).

More recently, the Genetic Epidemiology of COPD (COPDGene) study, which is a multicenter observational study, was designed to identify genetic factors associated with COPD and characterize chest CT phenotypes in subjects with COPD, including assessment of emphysema, gas trapping, and airway wall thickening. Subtypes of COPD based on these phenotypes will be used in a comprehensive genome-wide study to identify COPD-susceptibility genes. The COPDGene study will enroll 10,000 smokers with and without COPD across the Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages. Two groups are studied: non-Hispanic whites and non-Hispanic African-Americans (47).

Budoff et al. demonstrated that correlations between gated and ungated coronary artery calcification and thoracic aortic calcification were excellent ( $r = 0.96$ ) in a study of 50 patients enrolled in the COPDGene study (48).

## 60.4 CIGARETTE SMOKING AND COPD

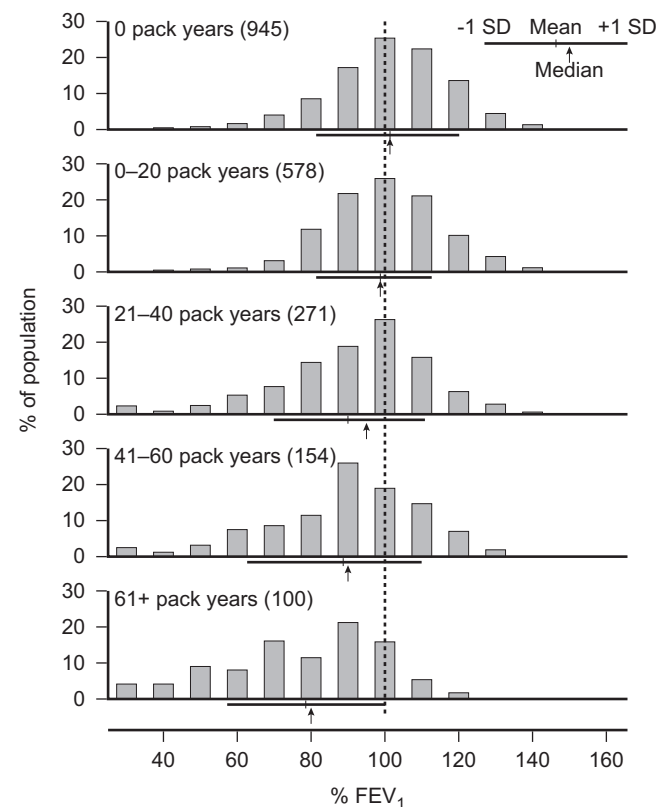
### 60.4.1 Causal but Variable Relationship of Smoking and COPD

In the 1950s, the most widely advanced hypothesis for the etiology of COPD was that progressive lung damage was caused by air pollution or recurrent respiratory infections. By 1964, adequate epidemiological evidence had accumulated for the Advisory Committee to the Surgeon General to conclude that “Cigarette smoking is the most important of the causes of chronic bronchitis in the United States, and increases the risk of dying from chronic bronchitis and emphysema” (page 31) (49). Ezzati et al. reported that the estimated fraction of COPD mortality attributable to smoking was 54% for men aged 30–69 years and 52% for men aged  $\geq 70$  years (50). Nearly all of the studies on which the 1964 Surgeon General recommendations were based were cross-sectional in design. Subsequent longitudinal studies have shown accelerated decline in FEV<sub>1</sub> in a dose-response relationship to cigarette smoking, related to both the duration of smoking and the amount smoked (51,52). This dose-response relationship of pulmonary function decline with cigarette smoking accounts for the observed higher prevalence rates for COPD with increasing age. The historically higher rate of smoking among males is the likely explanation for higher prevalence of COPD among males; however, the prevalence of COPD among females is increasing, in accordance with more recent increases in the rate of smoking among women (53).

It is well known that cigarette smoking is a major risk factor for the development of COPD. In 1977, Burrows et al. demonstrated a dose-response relationship between FEV<sub>1</sub> (percentage predicted) and pack-years of cigarette smoking in a study of 2369 individuals (51). The variable

relationship between FEV<sub>1</sub> and cigarette smoking intensity in the Burrows study is demonstrated in Figure 60-4. Heavier smokers were more likely to develop airflow obstruction, indicated by reduced FEV<sub>1</sub>. However, many smokers had pulmonary function within the normal range. In the Burrows study, pack-years, defined as the average number of packs of cigarettes smoked per day multiplied by the number of years of smoking, was the smoking-related variable that correlated most closely with FEV<sub>1</sub>. However, pack-years of smoking only accounted for about 15% of the variability in FEV<sub>1</sub>.

Pathologic studies of emphysema have demonstrated variability in the development of emphysema as well. Auerbach et al. performed an autopsy study of smokers; many smokers had pathologic evidence for emphysema, but the extent of emphysema varied widely (54). In another early autopsy study, Petty et al. also demonstrated marked variability in the development of emphysema among smokers (55). More recently, a study of lung pathology in smokers demonstrated that microscopic emphysema, assessed by quantitative measurements of airspace wall surface area per unit volume of lung tissue, was present in only 26% of smokers (56). On the basis of these pathologic studies, variability in the development



**FIGURE 60-4** Distributions of FEV<sub>1</sub> (% predicted) values with varying pack-year histories of smoking among adult subjects in the study by Burrows et al. Mean, median, and  $\pm 1$  standard deviation for FEV<sub>1</sub> are shown for each group. A dose-response relationship between increased smoking intensity and reduced FEV<sub>1</sub> is present, but many heavy cigarette smokers have FEV<sub>1</sub> values within the normal range. (From Burrows et al., 1977 (51).)



of emphysema in response to cigarette smoking is clearly present. However, it remains unclear if only a minority of smokers develops COPD (the susceptible smokers) or if all smokers develop some pathologic changes of COPD, but only a minority develops severe disease.

In part, the variable relationship between cigarette smoking and COPD relates to competing risks; cigarette smokers may die from other smoking-related illnesses such as coronary artery disease or lung cancer before the development of COPD. However, as discussed, genetic factors likely also influence the variable susceptibility to develop COPD. Because cigarette smoking is a proven major environmental risk factor for COPD, which can be readily assessed with questionnaires, COPD offers unique opportunities and challenges to incorporate environmental influences in the study of genetics of complex diseases. Genetic determinants of COPD may influence the development of COPD through a genotype-by-environment interaction between cigarette smoking and susceptibility genes. According to an official American Thoracic Society public policy statement on novel risk factors and the global burden of COPD in 2010, population-attributable fraction for smoking as a cause of COPD ranged from 9.7% to 97.9% but was <80% in most studies, indicating a substantial burden of disease attributable to nonsmoking risk factors (57).

### 60.4.2 Effects of Smoking at Different Life Stages

The effects of cigarette smoking, and potentially other environmental influences, on pulmonary function likely depend on the intensity of the environmental exposure, the timing of the exposure during growth, and the baseline lung function of the individual. Cunningham et al. estimated that maternal smoking during pregnancy resulted in a 1.3% reduction in FEV<sub>1</sub> when exposed children reach the ages of 8–12 years (58). Tager et al. examined the effect of actively smoking cigarettes during adolescence; individuals who smoked cigarettes from age 15 through age 20 were estimated to have an 8% reduction in FEV<sub>1</sub> (59). Xu et al., with data from the Vlagtwedde/Vlaardingen Study in The Netherlands, demonstrated that there was a large effect of cigarette smoking to decrease maximal level of lung function in individuals age <20 that far exceeded the effect of cigarette smoking on decline in lung function in older age groups (60). Therefore, dose and timing of exposure to cigarette smoke can have a profound impact on disease expression at different stages of the life cycle. Indeed, exposure to cigarette smoking in utero or active smoking in adolescence may have far greater effects on pulmonary function many years later than active smoking after the age of 35.

Cigarette smoking is the major environmental risk factor for the development of COPD. A variety of other potential environmental risk factors also have been

suggested although the supporting data are much less compelling; these potential risk factors include respiratory infections, occupational exposures, ambient air pollution, passive smoke exposure, and diet (61).

## 60.5 SEVERE AAT DEFICIENCY

A small percentage of COPD patients (estimated at 1–2%) inherit severe AAT deficiency (62). We discuss AAT deficiency in some detail because it is a proven genetic risk factor for COPD, and because it can serve as a model of the manner, in which genetic and environmental factors can interact to lead to COPD.

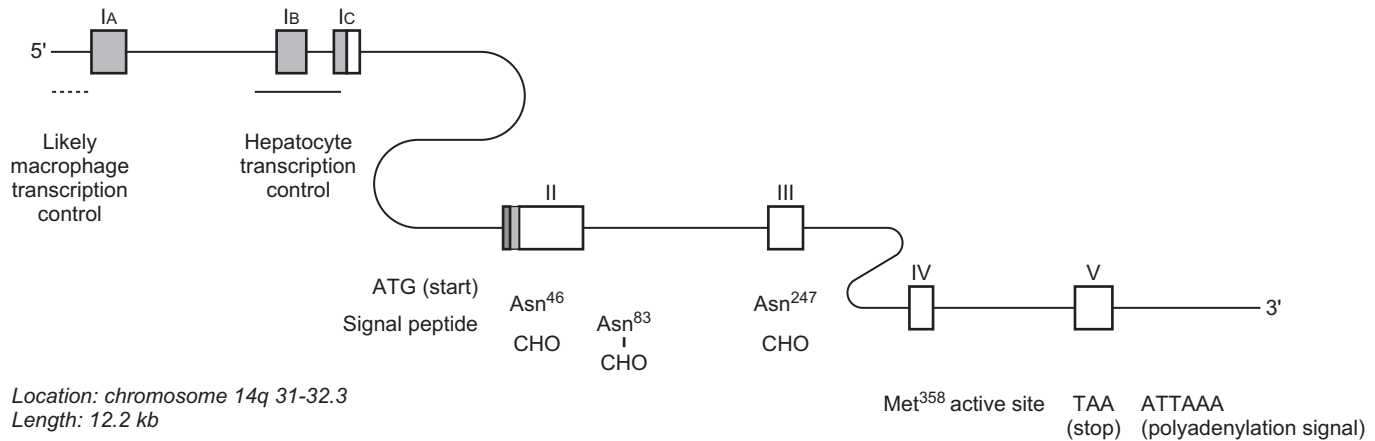
### 60.5.1 Description of the Protease Inhibitor Locus and Protease Inhibitor Alleles

AAT is encoded by the protease inhibitor (PI) locus (Human Genome Organisation name SERPINA1) on chromosome 14q32.1 (63,64). The PI gene, which is 12.2kb in length, has seven exons and six introns; the structure of the PI locus is shown in Figure 60-5. The first two exons and a short sequence of the third exon are encoded in the transcript produced by macrophages, but not by hepatocytes. Most of the fourth exon, and all of the remaining three exons, encode the protein sequence. The active inhibitor site P1 amino acid residue, methionine 358, is encoded by exon V. A “sequence-related gene,” which is likely a pseudogene, is found 12kb downstream from the PI locus (65).

The dominant site of synthesis of AAT is the liver but AAT is also synthesized in monocytes, macrophages, and neutrophils (66,67). AAT is a 394-amino acid protein (52 kDa); salt bridges within the molecule include glutamic acid 342 to lysine 298 and glutamic acid 264 to lysine 387.

The discovery of AAT deficiency was made by observing the absence of an alpha 1-globulin band in serum protein electrophoresis (6). Subsequently, differences in electrophoretic mobility of various AAT forms, related to differences in molecular surface charge, were demonstrated (68). The most common variant was designated M for medium rate of migration. Variants that migrated faster and slower than M were called F and S, for faster and slower migration, respectively. The Z form of the protein migrated much slower than the S form.

Approximately 100 different PI alleles have been identified, a few of which result in decreased serum levels of AAT (69). A partial list of PI variants is provided in Table 60-1. The common M allele, with an allele frequency >95% in US populations, is associated with normal AAT levels. The S allele, which is associated with slightly reduced AAT levels, and the Z allele, which is associated with markedly reduced AAT levels, also occur in US populations. A small percentage of subjects inherit null alleles, which lead to the absence of any AAT



**FIGURE 60-5** Structure of PI locus. The gene includes seven exons (IA, IB, IC, II, III, IV, and V); the coding sequence is contained in exons II to V. A signal peptide is encoded by exon II, following the translation start site (ATG) for the AAT mRNA. Hepatocyte transcription begins within exon IC and is controlled by the region denoted by the solid line. Transcription in monocytes and macrophages begins with exon IA. The three carbohydrate attachment sites are noted at amino acid residues 46, 83, and 247. The site of the critical active site methionine (amino acid residue 358) is also indicated. (From Brantly et al., 1988 (70).)

**TABLE 60-1** Structure of Representative Allelic Variants at the PI Locus

		Amino Acid Residue Change			
PI Allele	Type of Mutation	AA#	From	To	Cellular Defect
Normal alleles					
M1 (Ala213 or Val213)	Substitution (1 bp)	213	ala	val	None
M2	Substitution (1 bp)	101	arg	his	None
M2	Substitution (1 bp)	376	glu	asp	None
M3	Substitution (1 bp)	376	glu	asp	None
F	Substitution (1 bp)	223	arg	cys	None
Xchristchurch	Substitution (1 bp)	363	glu	lys	None
Deficient alleles					
S	Substitution (1 bp)	264	glu	val	IC degradation
Z	Substitution (1 bp)	342	glu	lys	IC accumulation
Mmalton	Deletion (3 bp)	52	phe	none	IC accumulation
Siiyama	Substitution (1 bp)	53	ser	phe	IC accumulation
Mheerlen	Substitution (1 bp)	369	pro	leu	IC degradation
Mprocida	Substitution (1 bp)	41	leu	pro	IC degradation
Null (QO) alleles					
QOgranite falls	Deletion (1 bp)	160	tyr	stop	Stop at 160—no mRNA
QOludwigshafen	Substitution (1 bp)	92	ile	asn	No protein
QOclayton	Insertion (1 bp)	363	glu	arg	Stop at 376; IC degradation
QOhongkong	Deletion (2 bp)	318	leu	leu	Stop at 334; IC accumulation
QOisola di procida	Deletion (17 kbp)				No mRNA
Dysfunctional Alleles					
Pittsburgh	Substitution (1 bp)	358	met	arg	Antithrombin 3 activity

IC corresponds to intracellular.

Only a partial list of PI alleles is presented; see Huber and Carrell (Huber and Carrell 1989) and Brantly et al. (Brantly et al. 1988a) for more details.

production through a heterogeneous collection of mutations (see below) (70). Individuals with two Z alleles or one Z and one null allele are referred to as PI Z because they cannot be distinguished by the isoelectric focusing technique commonly used to assess PI type. PI Z individuals have approximately 15% of normal plasma antitrypsin levels.

Additional improvements of isoelectric focusing techniques led to the discovery of variants within the class

of PI\*M alleles (71,72). The M subtypes are designated numerically as M1, M2, M3, and so on. The M subtypes, which are all associated with normal AAT serum levels, follow the codominant inheritance pattern for protein expression of other PI alleles.

The PI locus was initially designated as Pi; however, the International System for Human Gene Nomenclature proposed using only capital letters for locus designation, with the symbol PI for the PI locus (73). Although both

Pi and PI are still used to designate the PI locus, we have chosen to use PI in this chapter.

An international committee's recommendations for nomenclature at the PI locus, developed at a meeting in 1978, included designation of alleles with capital letters based on their relative positions by isoelectric focusing of serum (74). Alleles may be designated as superscripts or following an asterisk; for example, the S allele was PI<sup>S</sup> or PI\*S. If no unused letter is available, a number is added to the nearest lettered allele (e.g. M2, M3) or the place of origin of the first person identified with the allele is included adjacent to the letter (e.g. Mheerlen). Phenotypes from PI typing are designated as PI M, PI Z, and so on. Phenotypes from PI typing are designated with a single letter to represent data from electrophoresis results, such as PI M; however, if family data or M allele subtypes (e.g. M1 and M2) support the presence of, for example, 2 PI\*M alleles in an individual, the designation PI MM is appropriate. In practice, the applied nomenclature has varied widely, and superscripts, asterisks, and other terms are used inconsistently to designate specific alleles.

Null alleles (also referred to as QO alleles) lead to the absence of AAT protein production through a variety of mechanisms. Single base substitutions, insertions, and deletions resulting in a premature stop codon have been reported; the consequences of premature termination of mRNA synthesis include the absence of mRNA production (as seen in QOgranite falls), intracellular accumulation of a truncated protein (as seen in QOhongkong), and intracellular degradation of a truncated protein (as seen in QOclayton) (75–77). Mechanisms other than premature stop codon formation that have resulted in null alleles include gene deletion (PI\*QOisola di procida) and critical amino acid substitutions (PI\*QOludwigshafen) (78,79). Brantly et al. noted that several different null alleles have been described that result from mutations in a series of seven cytosine nucleotides from codons 360 to 362 (77).

Differences in nomenclature of null alleles persist; some investigators have used PI\*QO followed by the birthplace of the subject; others have used the term “null” followed by the birthplace of the individual. A few null–null individuals have been identified, who have no detectable serum AAT (80); for example, Garver et al. presented a 35-year-old nonsmoker with severe COPD who was null–null, and Cox and Levison described three null–null siblings with COPD (81,82). Null–null individuals seem to have an extremely high predisposition to develop COPD but the number of subjects that have been identified is too small to quantitate the risk relative to PI Z individuals with certainty (82).

In addition to null alleles, a variety of very rare PI alleles with electrophoretic mobility similar to the M protein have been described, which are associated with very low serum levels of AAT. Some of these rare variants, such as Mheerlen and Mprocida, are associated with reduced AAT levels because of intracellular degradation

of AAT; other rare variants, such as Mmalton, Mni-chinan, and Siiyama, are associated with reduced AAT levels because of intracellular accumulation of protein (Table 60-1). An extremely rare PI variant, antitrypsin Pittsburgh, involved a substitution of arginine for methionine at the critical P1 active site residue (83). This variant inhibited thrombin instead of leukocyte elastase, and acute-phase responses resulted in elevated levels of antithrombin activity, which led to severe hemorrhagic complications.

### 60.5.2 Diagnosis of AAT Deficiency

The clinical laboratory test used most frequently to screen for AAT deficiency is measurement of the immunological level of AAT in serum. The definitive diagnosis of AAT deficiency requires PI type determination, but measurement of serum immunological or functional levels can be used for screening, as long as appropriate methods to quantitate AAT levels are used. Available assays for measurement of immunological AAT levels include immunoelectrophoresis (rocket test), turbidometric assays, enzyme-linked immunoassays, and radial immunodiffusion plates. Methods that are based on quantitation of the alpha 1-globulin peak (which is largely AAT) from serum protein electrophoresis (SPEP) are unreliable, because the large albumin peak in SPEP analysis could provide falsely elevated estimates of alpha 1 globulin levels.

Problems with commercial standards used to measure serum levels of AAT have led to overestimation of measured levels by up to 40% (84). To avoid confusion with commercial laboratory measurements, which are typically reported in units of milligrams per deciliter, investigators using purified laboratory standards have typically reported AAT concentrations in units of micromolar values.

Isoelectric focusing of serum in polyacrylamide gels in the pH range of 4.0–5.0 remains the most common method for the determination of PI type. AAT is a negatively charged glycoprotein that resolves into eight bands when subjected to electrophoresis near its isoelectric point. Isoelectric focusing of serum can accurately determine PI type, which reflects the genotype at the PI locus for the common alleles. Molecular genotyping can distinguish the common PI alleles (M, S, and Z) using restriction fragment length polymorphism-based assays, oligonucleotide hybridization, or a variety of other molecular genotyping approaches, using blood, dried blood spots, or buccal brushings (85,86). However, high-throughput molecular genetic tests for rare deficiency alleles are not widely available, so rare severe deficiency alleles (e.g. null alleles) can be misclassified as normal if only these molecular genetic approaches are used. Thus, additional testing, such as measurement of serum AAT level, is required in addition to molecular genotyping of the common alleles in order to exclude rare deficiency states.

The PI locus demonstrates autosomal codominant expression of protein variants because individuals with, for example, PI MZ will produce both M and Z proteins. However, the development of COPD and liver disease are expressed in an autosomal-recessive mode because two deficiency alleles are required to cause a significant increase in risk for these diseases (see discussion of controversy regarding the risk for Z allele heterozygotes below).

The genotype at the PI locus is the primary genetic determinant of the serum AAT level. AAT is an acute-phase reactant protein that increases in the bloodstream with systemic infection or other stresses; therefore, environmental factors can influence AAT serum levels. Martin et al. studied immunological and functional levels of serum AAT in 583 individuals from 114 pedigrees (87). They noted that the PI locus appeared to be the only important genetic determinant of serum AAT level because adding polygenic determinants to quantitative genetic models that included PI type effects did not improve the fit of their models. Using measured genotype analysis, Silverman et al. estimated that depending on adjustment for covariates, PI type explained between 72% and 92% of variation in immunological serum AAT levels within families of PI Z subjects (88). Thus, most, if not all, of the genetic variation in serum AAT level appears to be controlled by the PI locus.

### 60.5.3 Pathogenesis of COPD in Severe AAT Deficiency

AAT is a member of the serpin family of PIs, which are involved in the modulation of serine proteases—a group of enzymes that trigger such important inflammatory cascades as the activation of complement, coagulation, kinin release, and fibrinolysis. The critical P1 amino acid residue at the active inhibitor site differs between members of the serpin family and tends to dictate specificity of the inhibitor. Typically, enzyme inhibition with the serpins involves proteolytic cleavage during formation of the complex with enzyme and permanent inactivation of the enzyme.

AAT is the major plasma PI of leukocyte elastase (encoded by the *ELA2* gene)—one of the enzymes that has been hypothesized to play a role in the development of emphysema (89). AAT also demonstrates protease inhibitor activity against proteinase 3, cathepsin G, chymotrypsin, trypsin, plasmin, and thrombin, but the highest rate constant of association is for leukocyte elastase (90). Functionally, the most important residue of AAT is methionine at position 358, the P1 residue at the active inhibitory site. This active site has been localized to a tensely strained loop of 16 amino acid residues that protrudes from one end of the molecule in a pattern that provides an attractive bait for the active site of leukocyte elastase (91). Inhibition of leukocyte elastase involves hydrolysis of the 358–359 bond between methionine and

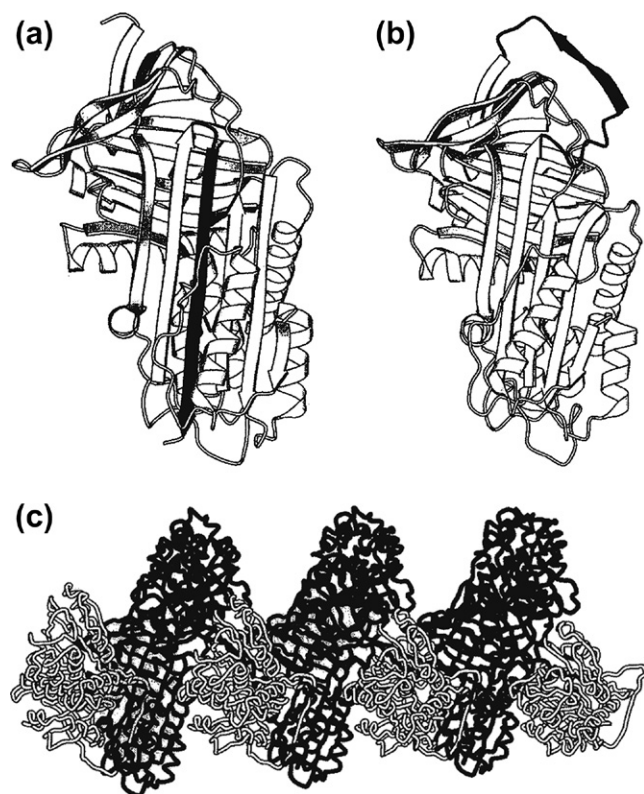
serine; after hydrolysis, elastase remains bound to the antitrypsin molecule, preventing further enzyme activity.

The Z allele encodes a single base substitution that replaces glutamic acid at amino acid position 342 with lysine; this substitution eliminates a salt bridge that is usually present between amino acid residue 342 and the lysine at amino acid residue 290. PI Z individuals have approximately 15% of normal plasma antitrypsin levels because the Z protein polymerizes within the endoplasmic reticulum of hepatocytes (92). Although the Z form of the AAT protein is somewhat less functional as a PI than the M form on a molecule-by-molecule basis (93), the primary mechanism for reduced AAT activity in PI Z individuals is the reduced serum level of the Z protein in PI Z individuals.

The molecular mechanism for reduced secretion of Z AAT has been deduced by Mahadeva et al. (92,94). The Z protein accumulates in hepatocytes in globules within the endoplasmic reticulum that are strongly stained by periodic acid-Schiff stain after diastase treatment. The formation of these aggregates of Z protein results directly from the point mutation at amino acid residue 342, which is located at the base of the reactive center loop of the AAT molecule. The reactive center loop of one M antitrypsin molecule can move in and out of the A beta-sheet in an adjacent M antitrypsin molecule; with elevated temperatures (41°), the M protein forms polymers of AAT molecules. However, the mutation in the Z allele enhances the tendency toward this loop-sheet polymerization, which occurs spontaneously at 37° with the Z protein (94). The structure of the AAT protein and a model of loop-sheet polymerization are shown in Figure 60-6. Lomas et al. also demonstrated that loop-sheet polymerization was found with the two other PI variants that result in intracellular accumulation of AAT, Siyama and Mmalton (95,96). Extracellular polymerization of AAT Z molecules has been demonstrated, which represents an additional mechanism that reduces the antiprotease activity in PI Z subjects (97). Of interest, these extracellular antitrypsin polymers are chemotactic for neutrophils, which may contribute to increased inflammation and lung parenchymal destruction in PI Z subjects (98).

About 90% of the elastase inhibitor activity in the lower respiratory tract is provided by AAT; the remainder is due to secretory leukoprotease inhibitor, elafin, and alpha 2-macroglobulin (99,100). Lung epithelial lining fluid from cigarette smokers contains normal levels of AAT measured immunologically but sharply decreased levels of elastase inhibitory activity (101). The methionine 358 P1 active site residue of AAT may be oxidized and inactivated by exposure to cigarette smoke or to oxidative bursts from inflammatory cells (102,103). Oxidative inactivation has been proposed as a potential mechanism for the pathogenesis of emphysema in cigarette smokers with normal PI types, by creating a functionally antitrypsin deficient state (104). AAT





**FIGURE 60-6** Structure of the AAT molecule and model for loop-sheet polymerization. (A) Ribbon diagram of AAT molecule with a cleaved reactive center loop (in black), which is inserted into the A beta-sheet of the AAT molecule. Beta-pleated sheets and alpha-helices are shown in gray. (B) The active inhibitory molecule of AAT includes a reactive center loop which is exposed for binding with proteinase. (C) Adjacent molecules of active AAT can polymerize by insertion of the reactive center loop of one molecule into the A beta-sheet of an adjacent molecule. Extended polymer formation results in hepatocyte globule formation in PI Z subjects. (From Elliott et al., 1996 (247).)

in the lung lining fluid can be inactivated by oxidation, complex formation with elastase, or fragmentation; the relative importance of these mechanisms of inactivation remains uncertain.

The recognition of an association between heritable AAT deficiency and pulmonary emphysema was a central event in the development of the protease–antiprotease hypothesis for the pathogenesis of emphysema. This hypothesis generally states that when proteolytic enzymes (especially leukocyte elastase) are released into the lungs, PIs normally prevent lung tissue destruction. The PI in highest concentration and with the highest association constant for leukocyte elastase is AAT (89). When PI levels are too low relative to protease levels, the uninhibited proteases cause lung destruction—elastin degradation is apparently critical—which eventually leads to emphysema (105,106).

The requirement for contact between inflammatory cell and extracellular matrix substrate raises important issues regarding the biochemical microenvironment in the lung interstitium. Campbell pointed out that proteolytic

events may be spatially controlled in the extracellular matrix at or near the cell membrane (107). This has been demonstrated by addition of neutrophils to a fibronectin-coated plate in the presence of excess AAT. Degradation of fibronectin occurs only where the cells contact the plate in such a manner as to exclude the PI. Thus, selected areas of the microenvironment have enzyme-inhibitor balance that differs from the whole lung. Campbell et al. have also demonstrated that proteolytic damage related to individual azurophil granules of neutrophils could be detected. The diameter of the region of extracellular matrix destruction from individual azurophil granules was substantially larger when serum from PI Z subjects was used than when serum from PI M subjects was used to provide the antiproteases (108).

#### 60.5.4 Prevalence of Severe AAT Deficiency

The prevalence of AAT deficiency is especially elevated in populations of Scandinavian descent. Analysis of the haplotypes of polymorphic loci adjacent to the AAT Z allele suggests that there was likely a common mutational origin for the majority of Z alleles, which may have occurred in northern Europe (109).

A variety of screening studies for AAT deficiency have been performed. Because of the rarity of severe AAT deficiency (e.g. PI ZZ), the prevalence of severe AAT deficiency is often estimated from the Z allele frequency, using Hardy–Weinberg equilibrium assumptions. Hutchison reviewed the European studies that estimated Z allele frequency and noted that the highest Z allele frequencies were found in the north-western section of Europe (including southern Sweden, Norway, Denmark, and the United Kingdom) (110). Prevalence surveys have typically found low Z allele frequencies in populations of African and Asian descent (111). de Serres performed a comprehensive search of S and Z allele frequency estimates in previously published control cohorts from around the world (112). He noted that there may be substantial numbers of PI ZZ subjects in almost every region of the world, including Asia and Africa. de Serres et al. later reported striking differences in the prevalences of both the PIS and PIZ alleles among these 69 countries and the numbers at risk for AAT deficiency in a given country in specific geographic regions (113). Data on the prevalence of the PIS and PIZ alleles as well as the numbers in those phenotypic classes known to be at risk for AAT deficiency are considered critical for the identification of individuals at risk for adverse health effects associated with AAT deficiency, as well as the treatment and management of those individuals identified in a given country. In the United States, de Serres et al. reported that the highest risk for AAT deficiency is found in whites, followed by Hispanics and blacks, with the lowest prevalence among Mexican Americans and no risk among Asians (114).

An alternative approach to estimate the prevalence of severe AAT deficiency involves direct screening for severely deficient individuals. The largest screening study was a survey of 200,000 newborns in Sweden from 1972 to 1974; a prevalence estimate of 1 in 1639 for PI Z individuals was determined from this screening effort (115). A large newborn screening program in Oregon identified 21 PI Z infants in a population of 107,038, for a prevalence estimate of 1 in 5097 (116). However, confirmation of PI Z in the Oregon study required a follow-up test, so some PI Z infants may have been missed, resulting in an underestimate of PI Z prevalence. In a population of 20,000 blood donors in the United States, Silverman et al. found seven PI Z subjects, for a prevalence of approximately 1 in 2667 (117). Pooled data from 26 other screening studies of populations, which included Z alleles (excluding Sweden and the United States) revealed a prevalence of eight PI Z subjects in 19,768 individuals screened (1 in 2471) (117). Thus, although the highest prevalence rates of PI Z subjects has been noted in Sweden, the differences between the prevalence of PI Z subjects in Sweden compared to other Caucasian populations may not be as great as previously assumed.

### 60.5.5 Natural History of PI Z

PI Z individuals often have early-onset COPD. Among PI Z subjects with COPD, panacinar emphysema with predominant involvement of the lung bases has been classically described. However, recent data based on chest CT scans indicate that PI Z subjects with COPD frequently have diffuse emphysema, without a basilar predominance, or even upper lobe predominant emphysema (118).

Only three of the first five PI Z patients had clinical manifestations of airflow limitation (6); thus, variability in the development of pulmonary complications was evident from the initial description of severe AAT deficiency. Published series of PI Z individuals have usually included many PI Z subjects with COPD; however, these studies have largely included PI Z individuals who were tested for AAT deficiency because they already had COPD (119–122). Thus, the fraction of PI Z individuals who will develop COPD and the age-of-onset distribution for the development of COPD in PI Z subjects remain unknown. PI Z subjects are also at increased risk for liver diseases, including hepatic cirrhosis; however, discussion of liver disease is beyond the scope of this chapter.

Several large series of AAT-deficient individuals reported in the 1970s and 1980s clearly demonstrated that PI Z subjects who smoke cigarettes tend to develop more severe pulmonary impairment at an earlier age than nonsmoking PI Z individuals (119–121). Larsson estimated survival probabilities in PI Z subjects using a life table approach (120). He estimated that the median

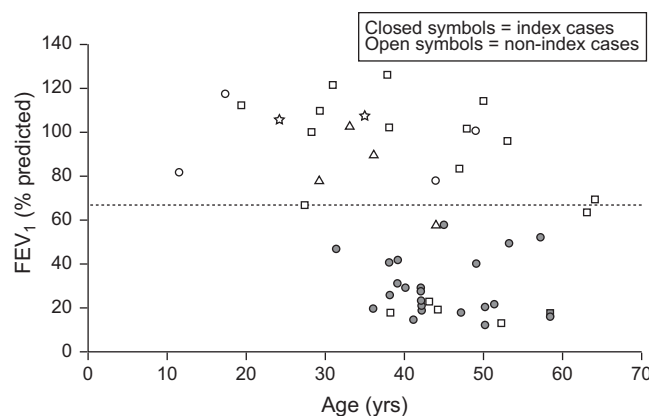
survival of PI Z smokers would be 23 years less than PI Z nonsmokers. Subsequently, Seersholm et al. confirmed the markedly different mortality rates in PI Z smokers and nonsmokers using the Danish Alpha 1-antitrypsin Register; lifelong nonsmokers had similar mortality rates to the general Danish population (123).

Few studies have considered whether factors other than smoking influence the development of lung disease in PI Z subjects. Black and Kueppers studied 18 nonsmoking PI Z individuals and 36 PI Z subjects who were current or exsmokers (124). They found significant variability in pulmonary function and clinical symptoms, especially among nonsmoking PI Z individuals. For instance, nine of 18 nonsmoking PI Z subjects had a chronic cough, while 33 of 36 smoking PI Z subjects reported a chronic cough. Pulmonary function tests, which included FEF<sub>25–75</sub> and diffusing capacity, were widely variable among the nonsmokers and did not decrease systematically with increasing age. They speculated that unidentified host factors contribute to this variability.

Identification of many PI Z subjects, because they already have COPD, has influenced perceptions about the natural history of this disorder. In a study performed in St. Louis, Silverman et al. included 52 PI Z subjects, ascertained as noted below (125); significant variability in pulmonary function was found (Figure 60-7). In Figure 60-7, index PI Z subjects, who were tested for AAT deficiency because they had COPD and who were the first PI Z identified in their family, all had significantly reduced FEV<sub>1</sub> values. Non-index PI Z subjects, who were ascertained by a variety of other means, including family studies, population screening, and liver disease, suggest a much different natural history for severe AAT deficiency than index PI Z subjects. Many non-index PI Z subjects have preserved pulmonary function. With subjects identified from the Danish Alpha 1-antitrypsin Register, Seersholm et al. also found significantly higher FEV<sub>1</sub> values in non-index PI Z subjects (not identified because they had COPD) compared to index PI Z subjects (identified because they had COPD) despite similar ages and smoking histories (126).

Piitulainen et al. assessed environmental determinants of reduced lung function other than cigarette smoking in a group of 205 lifelong nonsmoking PI Z subjects in Sweden. They found no relationship between reported passive smoke exposure and reduced FEV<sub>1</sub>; however, use of a kerosene heater and working in an agricultural occupation were significantly associated with reduced lung function (127). In a series of 128 PI Z individuals, Mayer et al. found that high mineral dust exposure was associated with increased cough and reduced FEV<sub>1</sub> values (128).

The natural history of PI Z subjects in the general population remains uncertain. There are likely three groups of unidentified PI Z subjects in the general population: (1) PI Z subjects with diagnosed COPD who have



**FIGURE 60-7** Effect of ascertainment bias on percent predicted FEV<sub>1</sub> among 52 severely AAT deficient subjects (PI Z) in the study by Silverman et al. Closed circles represent index PI Z subjects (individuals diagnosed with severe AAT deficiency because they had COPD, and who were the first PI Z subject identified in their family); open symbols represent non-index subjects. Nonindex subjects were ascertained by liver disease (open circles), family studies (squares), population screening (triangles), and other pulmonary problems (stars). Marked variability in FEV<sub>1</sub> values is evident for the non-index PI Z subjects. (From Silverman et al., 1989 (125).)

not been tested for AAT deficiency (possibly because they were not diagnosed with early-onset COPD); (2) PI Z subjects with significant but unrecognized airflow obstruction who have not been diagnosed with COPD (and thus AAT deficiency was not considered); and (3) PI Z subjects with normal pulmonary function. However, the relative proportions of these groups are unknown.

### 60.5.6 Other Familial Factors Influencing the Expression of AAT Deficiency

The primary focus of the St. Louis AAT study was to determine if genetic factors other than PI type influenced the variable development of lung disease among PI Z subjects. Therefore, first-degree relatives of the 52 PI Z subjects were enrolled. Despite comparable smoking histories, parents of PI Z subjects with reduced FEV<sub>1</sub> tended to have lower FEV<sub>1</sub> values themselves compared to parents of PI Z subjects with preserved FEV<sub>1</sub> (129). Elevated total serum immunoglobulin E levels in relatives of COPD PI Z subjects compared to relatives of healthy PI Z subjects suggested a relationship between familial factors influencing reduced pulmonary function and atopy. These results, as well as the findings with segregation analysis from the St. Louis study discussed below, suggested that additional genetic factors influence the development of airflow obstruction in AAT deficiency. Moreover, Silverman et al. demonstrated that significant genotype-by-environment interaction between PI type and pack-years of smoking was present (88).

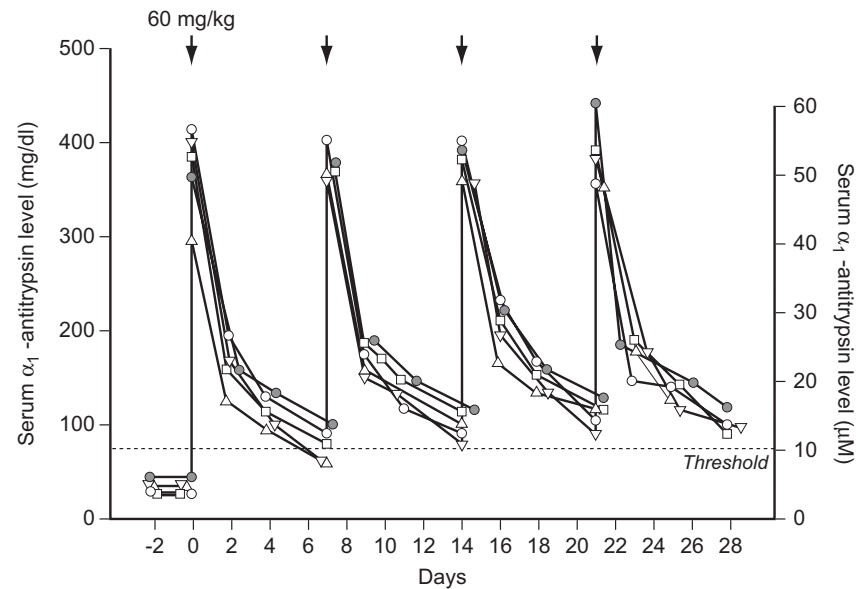
Novoradovsky et al. assessed whether variation in the endothelial nitric oxide synthase gene (NOS3) could be one of the genetic factors that influences the variable development of airflow obstruction in PI Z subjects (130). They identified polymorphisms in the NOS3 gene, and they tested for genetic association of these variants with airflow obstruction in 55 PI Z subjects with FEV<sub>1</sub> < 35% predicted, 122 PI Z subjects with FEV<sub>1</sub> > 35% predicted,

and 93 control subjects. Two polymorphisms in the coding region, which likely do not lead to functionally important changes in the NOS3 protein, were associated with severe airflow obstruction in PI Z subjects. Further work to replicate this finding and to identify a functionally important variant will be required, but NOS3 is an intriguing possible contributor to the variable development of airflow obstruction in PI Z subjects.

### 60.5.7 Treatment of Severe AAT Deficiency

The most important therapeutic measure for subjects with severe AAT deficiency who smoke cigarettes or other tobacco products is smoking cessation. In addition to conventional treatment for COPD (smoking cessation, influenza/pneumococcal vaccinations, bronchodilator treatment, pulmonary rehabilitation, etc.), specific treatment for AAT deficiency in the form of intravenous augmentation therapy has been developed.

Research at the Pulmonary Branch of the NHLBI demonstrated that commercial scale enrichment for AAT from human plasma (Cohn Fraction IV) could be performed, and that intravenous augmentation therapy with this partially purified preparation was feasible and safe. Heat treatment at 60° for 10 h was shown to reduce the likelihood of transmission of viral pathogens (131). Wewers et al. reported results from a feasibility study of AAT augmentation therapy in 21 PI Z patients with COPD (100). These PI Z patients received weekly intravenous infusions of 60 mg/kg of functionally active AAT for 6 months. The stated goal was to achieve AAT levels in the bloodstream and lung that were greater than levels in PI SZ subjects (approximately 11 μM), because those subjects appear to be near the threshold level for increased COPD risk. As shown in Figure 60-8, serum AAT levels were maintained above the 11 μM threshold with weekly infusions of partially purified antitrypsin.



**FIGURE 60-8** Serum levels of alpha 1-antitrypsin measured by radial immunodiffusion in five patients with severe alpha 1-antitrypsin deficiency who received weekly intravenous alpha 1-antitrypsin augmentation therapy of 60 mg/kg. Serum alpha 1-antitrypsin levels are expressed relative to a commercial standard (mg/dL on left vertical axis) and relative to a laboratory standard ( $\mu\text{M}$  on right vertical axis). Each patient is denoted by a different symbol (e.g. open circle, open triangle); values were measured 30 min, 2 days, 4 days, and 7 days after each infusion. The target threshold level is indicated by a dashed line. (From Wewers et al., 1987 (100).)

Bronchoalveolar lavage was performed periodically in the subjects who were receiving augmentation therapy in the trial by Wewers et al.; significant increases in AAT levels in the lung epithelial lining fluid were demonstrated (100). Although there is considerable experience with weekly AAT infusions, monthly infusions also provide AAT levels above  $11 \mu\text{M}$  ( $80 \text{ mg/dL}$ ) for the majority of the following months (132). The therapeutic impact of AAT levels that fall below the  $11 \mu\text{M}$  threshold before the subsequent monthly infusion is unknown.

Since 1988, purified AAT has been marketed in the United States for the intravenous treatment of severe AAT deficiency; four commercial products are currently available (Prolastin-C, Aralast NP, Zemaira, and Glassia). The only FDA-approved treatment regimen is a weekly infusion of 60 mg/kg. A once-monthly infusion of larger amounts has also been shown to be safe and to have nearly equivalent biochemical effectiveness (132). Despite efforts to inactivate viruses in these products and the absence of reported cases of viral infection as a result of augmentation therapy, hepatitis B vaccination is recommended before starting augmentation therapy. Intravenous augmentation therapy (initially, Prolastin-C) was released by the FDA after biochemical efficacy had been demonstrated; intravenous AAT treatment resulted in increased AAT levels in serum and bronchoalveolar lavage fluid with increased antielastase activity. However, a randomized controlled trial of AAT replacement therapy was not performed at that time. Instead, a registry of AAT-deficient subjects was created in order to follow the natural history of AAT-deficient subjects with and without augmentation therapy (133). By 1992, 1129 severely AAT-deficient subjects, defined as serum AAT

level below  $11 \mu\text{M}$ , were enrolled in the NHLBI Registry (134). During 4 years of observation, 67% of registry participants were receiving augmentation therapy for at least part of the study. Among all subjects in the registry, receiving augmentation therapy was not associated with a significant difference in the rate of decline in  $\text{FEV}_1$ ; however, among subjects with  $\text{FEV}_1$  values in the range of 35–49% of predicted, augmentation therapy was associated with a slower rate of  $\text{FEV}_1$  decline ( $P = 0.03$ ). Because it was not a randomized controlled trial, differences in mortality and  $\text{FEV}_1$  decline in subjects receiving augmentation therapy could be influenced by selection bias. However, the NHLBI Registry data suggested that a subset of severely AAT-deficient subjects may benefit from augmentation therapy. An observational study comparing 198 exsmoking German PI Z subjects receiving weekly AAT augmentation therapy with 97 exsmoking Danish PI Z subjects who did not receive this treatment found similar results; a slower rate of  $\text{FEV}_1$  decline was observed in subjects with baseline  $\text{FEV}_1$  values between 31% and 65% predicted (135).

Development of a randomized controlled trial of augmentation therapy was not performed before FDA approval of Prolastin-C in the United States, because the estimated sample size required to demonstrating a change in the rate of decline in  $\text{FEV}_1$  was judged to be impractical (136). Investigators in Denmark and The Netherlands reported a randomized controlled trial of augmentation therapy in 56 PI Z subjects with  $\text{FEV}_1$  values between 30% and 80% of predicted (137). Subjects received monthly infusions of AAT for 3–5 years; in addition to pulmonary function measurement every 3 months at the hospital, patients measured spirometry at



home on a daily basis. Annual chest CT scans also were performed. No significant differences in FEV<sub>1</sub> decline were noted in this relatively small study population; however, a trend toward reduction in longitudinal decline of chest CT density was noted in subjects receiving augmentation therapy ( $P = 0.07$ ). Therefore, chest CT scans may provide more sensitive phenotypes to detect beneficial effects of augmentation therapy in future studies.

More recently, the EXAcacerbations and CT scan as Lung Endpoints (EXACTLE) study (77 patients studied over 24–30 months), using a similar placebo-controlled trial design of intravenous AAT, explored CT densitometry as the primary outcome (NCT00263887) (138). CT densitometry showed a trend toward treatment benefit although the exacerbation frequency was not affected. This confirmed similar results from an earlier trial (137). Because of the similar study design and method of CT densitometry, Stockley et al. combined the raw data from these two studies (137,138) to increase the statistical power. The overall results of the combined analysis of two separate trials of comparable design, and the only two controlled clinical trials completed to date, have confirmed that intravenous AAT augmentation therapy significantly reduces the decline in lung density and may therefore reduce the future risk of mortality in patients with AAT deficiency-related emphysema (139).

A meta-analysis of randomized and nonrandomized studies included 1509 patients and found, however, that augmentation had a modest effect in slowing lung function decline in patients with AAT deficiency, and that patients with moderate obstruction are most likely to benefit (140).

Currently, eligibility for AAT augmentation therapy requires the demonstration of severe AAT deficiency, with a serum AAT level below 11  $\mu\text{M}$ . Augmentation therapy is not indicated for use in individuals with normal AAT levels or with deficient phenotypes associated with a level above 11  $\mu\text{M}$ . Typically, PI Z individuals will qualify although other rare types associated with severe deficiency (e.g. null-null) are also eligible. PI SZ individuals are technically eligible for augmentation therapy if their serum levels are below 11  $\mu\text{M}$ . However, it is unclear if PI SZ individuals with one or more measured serum values below 11  $\mu\text{M}$  have a different risk of COPD than PI SZ subjects with serum values above 11  $\mu\text{M}$ ; as noted above, the primary genetic determinant of AAT level is the PI type, and the significance of variation in serum level within a PI type has not been determined. AAT augmentation therapy is not indicated in PI MZ individuals.

At this time, AAT augmentation therapy is not recommended for severely deficient subjects with normal pulmonary function and without emphysema for the following reasons: (1) there is no compelling evidence that AAT augmentation therapy prevents the development of emphysema; (2) augmentation therapy is a very expensive treatment that requires weekly (or at least monthly) intravenous infusions; and (3) the subset of PI Z subjects

with preserved pulmonary function who will eventually develop COPD cannot be identified with certainty. PI Z subjects with normal pulmonary function but radiological evidence of emphysema represent a problematic group, in which the decision regarding augmentation therapy is controversial.

A variety of additional specific treatments have been considered for severe AAT deficiency. Recombinant AAT avoids the potential risks associated with a blood product, but the half-life of intravenous recombinant AAT, which lacks carbohydrate side chains, is considerably shorter than AAT prepared from plasma. To address this problem and to provide a more convenient mode of administration, aerosolized recombinant AAT was developed and shown to increase the antielastase capacity of the lung (141). Aerosolized treatment with Prolastin-C has also been shown to have biochemical efficacy (142); in a study of 30 normal volunteers who received 200 mg of aerosolized Prolastin-C, the medication was tolerated well and provided an increase in the antielastase activity of bronchoalveolar lavage fluid for at least 36 h after the dose was given (143). However, inhaled AAT has not been approved by the FDA.

Synthetic leukocyte elastase inhibitors have also been developed, but they have not yet been released for treatment purposes. Surgical treatments for COPD, which are not specific for AAT deficiency, such as lung volume reduction surgery and lung transplantation, have also been used in AAT-deficient patients with severe COPD.

### 60.5.8 Who should Be Tested for AAT Deficiency?

Selection of individuals for AAT deficiency testing involves a variety of complex issues. Severe AAT deficiency is associated with increased risks of COPD and liver cirrhosis, but the magnitude of these risks is uncertain. Specific treatment for AAT-related lung disease is available in the form of augmentation therapy but it is expensive and of uncertain therapeutic efficacy. Smoking cessation is beneficial for AAT-deficient subjects, but it is unknown if revealing the diagnosis of AAT deficiency will result in significantly increased rates of smoking cessation.

One study that suggested a beneficial effect of AAT screening for smoking avoidance in PI Z subjects resulted from the large newborn screening effort in Sweden (144). Follow-up of 50 PI Z children, who were diagnosed with AAT deficiency at birth, when they were 18–20 years old, found self-reported current smoking in 6% and exsmoking in 6% of these PI Z young adults. Among 48 matched control subjects, 17% were current smokers and 19% were exsmokers; these smoking rates were significantly higher than the rates for the PI Z subjects. Although smoking rates in PI Z individuals identified at birth appear to have been positively affected by

diagnosing AAT deficiency, there were concerns about the psychological impact on families of learning that their newborn had AAT deficiency (145). In light of these issues, general population screening for AAT deficiency has not yet been widely accepted.

In the American Thoracic Society's "Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease," a relatively restrictive set of indications for AAT testing was presented (146). AAT testing was recommended for individuals in each of the following categories: (1) nonsmokers with chronic bronchitis and airflow obstruction; (2) bronchiectasis; (3) moderate or severe COPD by age 50; (4) basilar predominance of emphysema; (5) unremitting asthma, especially if under age 50; (6) family history of AAT deficiency or early-onset COPD; and (7) cirrhosis without apparent risk factors.

More recently, a Task Force of the American Thoracic Society and European Respiratory Society prepared a new set of standards for the diagnosis and management of AAT deficiency (69). In recognition of the marked underdetection of AAT deficiency and the fact that there are no studies of the efficacy of genetic testing programs in AAT-deficient subjects, they weighed the available evidence and provided recommendations. One of the most interesting and potentially controversial recommendations is for AAT testing of all symptomatic adults with COPD, emphysema, and/or asthma with irreversible airflow obstruction. This recommendation for widespread targeted detection efforts among individuals with chronic airflow obstruction represents a substantial change in clinical practice. Additional strong recommendations for AAT testing were provided for individuals with unexplained liver disease, individuals with necrotizing panniculitis (a rare manifestation of severe AAT deficiency), and siblings of PI Z subjects, who typically have a 25% risk of also having the PI Z type.

Currently, in the United States, only a minority of COPD patients who are diagnosed at later ages are tested for AAT deficiency. Testing additional people with COPD does raise significant economic and social issues; however, acceptance of a more uniform approach to screening for AAT deficiency would certainly be desirable.

### 60.5.9 Lessons from Severe AAT Deficiency

Although most subjects with COPD are not AAT deficient, AAT deficiency serves as an example of the manner in which genetic factors can predispose to COPD and a model of how genetic and environmental factors can interact. The identification of genetic factors predisposing to a disease can provide important insights into disease pathophysiology. The recognition of an association between heritable AAT deficiency and pulmonary emphysema was a central event in the development of the protease–antiprotease hypothesis for the pathogenesis

of emphysema (105). In addition to contributing to the development of this pathogenetic model of emphysema, AAT deficiency is an important risk factor for the development of COPD in a small minority of COPD patients. Searching for additional genetic factors influencing COPD may provide novel insights into the pathogenesis of COPD and other obstructive airways diseases, which potentially could lead to important therapeutic or preventative strategies.

## 60.6 RISK OF COPD IN Z ALLELE HETEROZYGOTES

### 60.6.1 PI MZ

The risk of lung disease in heterozygous PI MZ individuals has been a subject of considerable controversy for many years. PI MZ individuals do have reduced serum AAT concentrations (approximately 60% of PI M levels). However, random population surveys (including a study by Bruce et al. that matched subjects for age, race, sex, and smoking history), have typically found no differences in pulmonary function between PI MZ and PI M individuals (147,148). A study by Larsson et al. showed that PI MZ individuals who smoke cigarettes show some deficits in sensitive pulmonary function tests compared to smoking PI M subjects, but there was no evidence for increased airflow obstruction in smoking PI MZ subjects (assessed by the ratio of FEV<sub>1</sub> to vital capacity) (149). Larsson et al. did not find any significant differences in pulmonary function between nonsmoking PI M and PI MZ individuals. On the other hand, case–control studies comparing the prevalence of the PI MZ type in patients with COPD and control subjects have usually discovered an excess of PI MZ individuals among COPD patients (62,150,151).

Many of the previous studies that have attempted to assess the potential risk to PI MZ subjects have been limited by small sample size. Seersholm et al. matched 1551 PI MZ subjects from the Danish Alpha 1-antitrypsin Deficiency Registry with control subjects from the Danish general population (with 10 controls for each PI MZ subject). Using the Danish Hospital Discharge Registry, they compared the rates of hospitalization for obstructive lung diseases among the PI MZ and control subjects (152). The PI MZ subjects were significantly more likely to be hospitalized for asthma, emphysema, and chronic bronchitis than the control subjects; however, this increased risk for hospitalization was limited to the 565 subjects who were first-degree relatives of PI Z subjects with severe COPD. This study is limited by the lack of data related to smoking history on the PI MZ and control subjects, but the results suggest that the subset of PI MZ subjects who are first-degree relatives of PI Z subjects with COPD may be at increased risk for obstructive lung disease. The relative importance of PI MZ versus other

familial factors in determining the risk for COPD in the study by Seersholm et al. is uncertain.

In a study of 9187 adults in the Copenhagen City Heart Study, Dahl et al. found a slightly faster rate of FEV<sub>1</sub> decline among PI MZ subjects (25 mL/year) than among PI M subjects (21 mL/year) ( $P=0.048$ ) (153). However, in a general population sample of 2016 adults in Arizona, Silva et al. found no evidence for accelerated decline in FEV<sub>1</sub> among PI MZ compared to PI M subjects (154).

In an effort to synthesize the results from many studies that have examined PI MZ risk for COPD, Hersh et al. recently performed a meta-analysis (155). Among case-control and cross-sectional studies that compared the presence or absence of COPD among PI MZ and PI M subjects, a significantly increased risk of COPD among PI MZ subjects was found (odds ratio, 2.3; 95% CI, 1.6–3.4). However, among population-based studies that compared FEV<sub>1</sub> values between PI MZ and PI M subjects, no differences in average FEV<sub>1</sub> were found. Since COPD is defined by reductions in FEV<sub>1</sub>, the discordance between the results for COPD and FEV<sub>1</sub> was surprising. It is possible that all PI MZ subjects have a slightly increased risk for COPD, or that there is a significant risk for a subset of PI MZ subjects because of gene–gene or gene–environment interactions. Sørheim et al. examined the associations between PI MZ heterozygosity and COPD-related phenotypes in two large populations: a case-control study from Norway and a multicenter family study from Europe and North America (156). In this study, they demonstrated that PI MZ heterozygotes had lower FEV<sub>1</sub>/FVC ratio than PI MM individuals, suggesting that PI MZ individuals may be slightly more susceptible to the development of airflow obstruction than PI MM individuals.

### 60.6.2 PI SZ

The risk of COPD associated with the PI SZ type is also uncertain. PI SZ subjects have serum AAT levels that are approximately 35% of PI MM levels (157). Turino et al. evaluated 50 PI SZ individuals from the NHLBI Alpha 1-antitrypsin Deficiency Registry who provided spirometric data (158). PI SZ subjects who were lifelong nonsmokers had airflow obstruction less frequently than lifelong nonsmoking PI Z subjects. Among smokers, PI SZ and PI Z subjects had similar levels of pulmonary function impairment. However, 50% of these PI SZ subjects were identified because they had lung disease. More recently, Holme et al. demonstrated that subjects with PiSZ showed less emphysema on CT scans and less abnormal respiratory physiology test results (159). However, both the PiSZ and the PiZZ groups had similar health status impairment. The reasons for this finding remain unclear.

Seersholm and Kok-Jensen assessed 94 PI SZ subjects ascertained from the Danish Alpha 1-antitrypsin

Deficiency Register (160); they included 28 index cases who were identified because they had respiratory problems and 66 nonindex cases who were identified through family studies. Nonindex PI SZ subjects had no increased mortality compared to population norms, and the mean FEV<sub>1</sub> of this group was 94.7% predicted. In a series of 25 PI SZ subjects, Hutchison et al. found FEV<sub>1</sub> below 80% predicted in 11 of 14 index PI SZ subjects who were identified through a chest clinic, but only in 1 of 11 non-index PI SZ subjects who were identified through family studies (157). In summary, PI SZ subjects ascertained with lung disease, not surprisingly, have reduced pulmonary function; PI SZ subjects who are not ascertained because of pulmonary problems often have preserved pulmonary function. Whether PI SZ subjects are at significantly increased risk for COPD, even if they smoke, has not been definitively established.

## 60.7 COPD AND COPD-RELATED PHENOTYPES IN OTHER GENETIC SYNDROMES

Several rare genetic syndromes are associated with COPD or have COPD-like features. Cutis laxa is a rare syndrome associated with loose, redundant, and inelastic skin. Several modes of inheritance of cutis laxa have been described. Autosomal-recessive cutis laxa is often associated with emphysema, even in childhood. At least some cases of autosomal-recessive cutis laxa are caused by mutations in the fibulin-5 (*FBLN5*) gene (161). Fibulin-5 is critical for normal elastic fiber development, and fibulin-5 knockout mice develop severe emphysema (162,163).

Autosomal-dominant cutis laxa can be caused by frameshift mutations in the distal part of the elastin (*ELN*) gene; although emphysema has been less consistently reported in autosomal-dominant than autosomal-recessive cutis laxa, several individuals with autosomal-dominant cutis laxa and emphysema have been reported (164,165). A novel mutation in the fibulin-4 gene (*FBLN4*; 11q13) was recently identified in autosomal-recessive cutis laxa with developmental emphysema (166). The mutation caused an amino acid substitution in an epidermal-growth-factor-like domain of fibulin-4, leading to very low levels of extracellular protein.

Another rare genetic syndrome potentially related to the development of emphysema is familial spontaneous pneumothorax, which has been described in multiple families. Recently, an extended pedigree from Finland was included in a genome-wide linkage study; suggestive linkage of familial spontaneous pneumothorax to chromosome 17p was found (167). The linkage peak was located near the folliculin gene (*FLCN*); mutations in the *FLCN* gene can lead to Birt–Hogg–Dube Syndrome—a rare syndrome associated with cystic lung changes. In the Finnish pedigree with familial spontaneous

pneumothorax, a 4-bp deletion in the *FLCN* gene was found to correlate perfectly with emphysema-like cystic lung changes on chest CT scan. The relationship of these cystic lung changes to airflow obstruction was not discussed.

Menkes disease is a condition characterized by abnormal hair and dysmorphic features and caused by mutations in an intracellular copper transporter (*ATP7A*; Xq13.3). The clinical features are due to defective connective tissue synthesis probably because of the result of dysfunction of lysyl oxidase. This copper-dependent enzyme is required for proper cross-linking of both collagen and elastin fibers. A recent case report described a child with Menkes disease and severe bilateral pan-lobular emphysema who died at the age of only 14 months (168). Gene sequencing revealed a splice-site mutation in *ATP7A*, suggesting that proper ECM cross-linking is vital for stability of the lung parenchyma.

Although rare genetic syndromes such as cutis laxa, familial spontaneous pneumothorax, and Menkes disease account for a tiny fraction of COPD cases, they may point to pathways relevant for the development of COPD in the general population.

## 60.8 RISK TO RELATIVES FOR NON-AAT COPD

### 60.8.1 Familial Aggregation of Spirometry and COPD

Several types of studies have suggested that genetic factors other than AAT type may be involved in the susceptibility to develop COPD. We review the evidence from general population studies and from COPD families; subsequently, we consider severe, early-onset COPD.

A variety of studies of pulmonary function measurements performed in the general population and in twins have suggested that genetic factors influence variation in pulmonary function. Redline et al. assessed spirometry in 256 monozygotic and 158 dizygotic twins who were not selected for respiratory problems (169). For FEV<sub>1</sub>, higher correlation in monozygotic twins (0.72) than dizygotic twins (0.27) suggested that genetic factors influence variation in pulmonary function. However, such general population studies do not address whether genetic factors influence the development of significant airflow obstruction.

Studies in relatives of COPD patients also have supported a role for genetic factors. Several studies in the 1970s reported higher rates of airflow obstruction in first-degree relatives of COPD patients than in control subjects. For example, Larson et al. compared spirometry in 156 first-degree relatives of COPD patients to 86 spouse controls with similar pack-year history of smoking (170). Airflow obstruction was found in 23% of first-degree relatives, but only 9% of control subjects.

Although this study did show familial aggregation for airflow obstruction, it had several weaknesses. AAT deficiency was not rigorously excluded as a potential contributor to airflow obstruction. In addition, a higher percentage of the first-degree relatives than controls were smokers, so at least some of the observed differences may relate to differences in smoking behavior. Nonetheless, genetic predisposition to COPD was suggested by this study.

Kueppers et al. studied 114 subjects with COPD and control subjects matched based on age, gender, occupation, and smoking history (171). Siblings of COPD and control subjects also were included; the mean FEV<sub>1</sub> among siblings of COPD subjects (90% of predicted) was significantly lower than the siblings of control subjects (103% of predicted). This difference in pulmonary function between siblings of COPD and control subjects remained significant after adjustment for smoking history.

A large study of COPD in families was performed at Johns Hopkins (172,173). Familial aggregation for airflow obstruction was demonstrated; after adjusting for age, sex, race, and smoking history, reduced FEV<sub>1</sub>/FVC was found in 28% of first-degree relatives of COPD probands compared with 19% of relatives of nonpulmonary patients (173). The high rate of airflow obstruction in the relatives of nonpulmonary patients is unexplained. Variance component analysis of their data suggested a significant genetic contribution to FEV<sub>1</sub> and the ratio of FEV<sub>1</sub>/FVC (174).

More recently, McCloskey et al. assessed familial aggregation of airflow obstruction in 173 siblings of probands with severe COPD compared to a population-based control cohort in the United Kingdom (175). None of the nonsmoking siblings had evidence for airflow obstruction on spirometry. However, smoking siblings of COPD probands had a significantly increased risk of airflow obstruction compared to matched population-based control subjects, with an odds ratio of 4.7 ( $P < 0.0001$ ).

Additional studies have demonstrated familial aggregation of chronic bronchitis. In a survey of 9226 residents of Tecumseh, Higgins and Keller found approximately threefold higher rates of chronic bronchitis in offspring when at least one parent had chronic bronchitis than if neither parent had chronic bronchitis; this analysis did not include adjustment for cigarette smoking (176). Speizer et al. analyzed National Health Interview Survey data from 1970 for chronic respiratory conditions and found higher rates of bronchitis or emphysema among offspring when at least one parent had bronchitis or emphysema; this effect appeared to be independent of cigarette smoking (177). Finally, in a study in East Boston, Tager et al. found approximately twofold higher rates of chronic bronchitis or airflow obstruction in first-degree relatives of probands with chronic bronchitis or airflow obstruction than in first-degree relatives of



control probands; this familial aggregation also appeared to be independent of cigarette smoking (178).

### 60.8.2 Familial Aggregation of Severe, Early-Onset COPD

In an effort to identify novel genetic risk factors for COPD, Silverman et al. have focused on subjects with severe, early-onset COPD unrelated to severe AAT deficiency (179). In multiple previous successful studies of complex trait genetics, focusing on early-onset cases led to the identification of susceptibility genes for breast cancer, Alzheimer's disease, glaucoma, and diabetes mellitus (maturity-onset diabetes of the young) (180–184). By studying severe, early-onset COPD probands and their relatives, the study population may be enriched for genetic influences.

Probands in the Boston early-onset COPD study had FEV<sub>1</sub> <40% predicted at age younger than 53 years, without severe AAT deficiency (179). All available first-degree relatives, spouses, and older second-degree relatives (aunts, uncles, and grandparents) were included. The initial phase of the Boston early-onset COPD study included 44 early-onset COPD probands, with 204 first-degree relatives, 54 second-degree relatives, and 20 spouses. Spirometric and demographic data for the first-degree relatives of early-onset COPD probands, stratified by smoking status, are shown in Table 60-2. Highly significant differences in FEV<sub>1</sub> and FEV<sub>1</sub>/FVC were found when current or exsmoking first-degree relatives of early-onset COPD probands were compared to control subjects. No significant differences in age or pack-years of smoking were noted.

No significant differences in FEV<sub>1</sub> or FEV<sub>1</sub>/FVC were found when lifelong nonsmoking first-degree relatives of early-onset COPD probands were compared to lifelong nonsmoking control subjects. In fact, the mean FEV<sub>1</sub> values were 93.4% of predicted in both groups of nonsmokers. This pattern would be consistent with genetic risk factors that interact with smoking to result in COPD. A similar pattern of smoking-related susceptibility was also seen for chronic bronchitis (179). To account for potential familial correlations and for the effects of age and pack-years of smoking, generalized estimating

equations were used to calculate odds ratios of developing chronic bronchitis and various levels of reduction in FEV<sub>1</sub>. When all first-degree relatives of early-onset COPD probands were compared to all controls, an increased risk of FEV<sub>1</sub> <80% was seen. However, stratification by smoking status revealed that this risk was exclusively found in smoking first-degree relatives; with significant odds ratios of 4.5 for FEV<sub>1</sub> <80% predicted and 3.6 for chronic bronchitis, and a nearly significant odds ratio of 3.5 for FEV<sub>1</sub> <60% predicted. Lifelong nonsmokers had no increased risk for reduced FEV<sub>1</sub> or chronic bronchitis. Smoking-related increased spirometric bronchodilator responses were also observed in smoking first-degree relatives of severe, early-onset COPD probands compared to smoking control subjects (185). Subsequent analyses of an increased number of first-degree relatives in the Boston early-onset COPD study have shown that nonsmoking first-degree relatives of severe, early-onset COPD probands do have significantly lower values of some more sensitive spirometric measures (e.g. FEF<sub>25–75</sub>) compared to nonsmoking control subjects, suggesting that even nonsmoking first-degree relatives of severe, early-onset COPD probands may have mild airflow abnormalities (186).

A remarkably high percentage of females (>70%) was noted among the early-onset COPD probands (187). This female predominance differed from previous studies of severe COPD, which had found a male predominance (188–191). The explanation for the female predominance found by Silverman et al. is uncertain but this study included a group of probands who were younger, more severely affected, and more recently collected than earlier series of severe COPD subjects. In a follow-up study of these severe, early-onset COPD probands, reduced survival of male subjects with severe COPD did not appear to account for the observed female predominance (192); thus, it is certainly possible that there is a biological basis for the increased female susceptibility, due to hormonal or other factors, that could mediate a genotype-by-gender interaction in early-onset COPD pedigrees.

In summary, the Boston early-onset COPD study has led to the identification of a variety of phenotypes that demonstrate smoking-related susceptibility in first-degree

**TABLE 60-2 Age, Smoking History and Spirometry in First-Degree Relatives of Boston Early-Onset COPD Study Probands Compared to Control Subjects<sup>a</sup>**

Group	n	FEV <sub>1</sub> /FVC (% Pred)	FEV <sub>1</sub> (% Pred)	Age	Pack-Years
Smoking first-degree relatives	112	83.5 ± 16.1 <sup>b</sup>	76.1 ± 20.9 <sup>b</sup>	45.9 ± 17.3	28.5 ± 26.6
Smoking control subjects	48	94.3 ± 10.3	89.2 ± 14.4	48.6 ± 13.9	22.1 ± 22.1
Nonsmoking first-degree relatives	91	92.7 ± 7.6	93.4 ± 12.9	34.5 ± 18.9	0.00
Nonsmoking control subjects	35	95.5 ± 7.2	93.4 ± 14.2	39.9 ± 18.2	0.00

<sup>a</sup>Values are mean ± SD. No other pairwise comparisons between first-degree relatives and control subjects with similar smoking histories were significant at  $P < 0.05$ .

<sup>b</sup>Indicates  $P < 0.01$  compared to control subjects with similar smoking history.

Adapted from Silverman et al., 1998 (179).

relatives of early COPD probands including FEV<sub>1</sub>, FEV<sub>1</sub>/FVC, chronic bronchitis, FEF<sub>25–75</sub>, and bronchodilator responsiveness. These phenotypes will probably be useful in further genetic studies of COPD.

## 60.9 SEGREGATION ANALYSIS

Segregation analysis is a statistical technique that attempts to determine if the pattern of phenotypes within families is consistent with the transmission of a major gene for that phenotype. Segregation analysis has been performed with spirometric phenotypes in several general population studies. Chen et al. performed segregation analysis with class D regressive models in a general population sample of 799 individuals from 214 nuclear families in Saskatchewan (193). They found no evidence for a major gene effect for FEV<sub>1</sub> or FEF<sub>25–75</sub>. Givelber et al. performed segregation analysis of FEV<sub>1</sub> with class D regressive models in 5003 individuals from 1408 pedigrees in the Framingham Study (194). They took advantage of the longitudinal follow-up of Framingham families by selecting spirometric values from parents in the 1960s and offspring in the 1990s; thus, spirometric values from parental and offspring generations were compared at similar ages. Although they found evidence for significant familial correlations for FEV<sub>1</sub>, they did not demonstrate evidence for a major gene influencing FEV<sub>1</sub>. Wilk et al. also used class D regressive models to perform segregation analysis in 1964 Caucasian individuals in 455 families (195). They found evidence for a dominant major gene influencing FEV<sub>1</sub>. In summary, segregation analysis of pulmonary function measurements in the general population has supported genetic influences, but they have provided inconsistent evidence for major gene effects.

In COPD families, the Johns Hopkins COPD study included segregation analysis of spirometric phenotypes. The investigators found support for the existence of a major gene influencing FEV<sub>1</sub> in families with COPD (196). However, they performed segregation analysis with class A regressive models, which subsequently have been shown to be susceptible to false-positive assignments of major genes when they are applied to quantitative traits such as FEV<sub>1</sub> (197).

Segregation analysis has also been performed in AAT-deficient families to determine if there is evidence for an additional major gene influencing pulmonary function in those families (198). To determine if the variability in pulmonary function among AAT-deficient families was determined by environmental factors or genetic factors other than PI type, segregation analysis was performed after adjustment for the effects of PI type on the mean and variance of spirometric measures. Segregation analysis, performed with the mixed model incorporated in the POINTER computer program, provided preliminary evidence for an additional major gene influencing FEV<sub>1</sub>. However, significant evidence for an

additional major gene could not be demonstrated after the effects of cigarette smoking and the interaction of cigarette smoking with PI type were included. Although the failure to demonstrate evidence for a major gene after adjustment for cigarette smoking may indicate that the effect of smoking provided false evidence for a major gene initially, these results could indicate the existence of a major gene that enhances susceptibility to the effects of cigarette smoking. Thus, the presence of additional genetic factors influencing the development of COPD in PI Z individuals, potentially mediated by variable susceptibility to smoking, remains intriguing but speculative.

## 60.10 LINKAGE ANALYSIS

### 60.10.1 Linkage Analysis in COPD Families

In the Boston early-onset COPD study, 585 members of 72 early-onset COPD pedigrees were included in genome-wide linkage analysis. Initially, qualitative COPD-related phenotypes of airflow obstruction and chronic bronchitis were analyzed with multipoint non-parametric linkage analysis (199). In the initial qualitative phenotype genome scan, no genomic regions met criteria for suggestive linkage to airflow obstruction or chronic bronchitis (200). However, limiting analysis to cigarette smokers substantially increased the evidence for linkage of chronic bronchitis to chromosome 22 (LOD = 2.08 at 36 cM).

Genome-wide linkage analysis of quantitative spirometric phenotypes provided more compelling evidence for linkage, especially using postbronchodilator spirometric values (201,202). Postbronchodilator FEV<sub>1</sub> showed at least suggestive evidence for linkage to chromosome 8p (LOD = 3.30 at 2 cM) and 1p (LOD = 2.24 at 136 cM). Postbronchodilator FEV<sub>1</sub>/FVC was also linked to multiple regions, most significantly to markers on chromosome 2q (LOD = 4.42 at 222 cM) and 1p (LOD = 2.52 at 118 cM).

Genotyping additional short tandem repeat (STR) markers to increase the genotypic information for linkage analysis provided stable to increased evidence for linkage of quantitative spirometric phenotypes on chromosomes 2q, 12p, and 19q (203,204). Stratified linkage analysis of smokers only also provided stable to increased evidence for linkage to these genomic regions, potentially related to genotype-by-smoking interactions.

### 60.10.2 Linkage Analysis of Pulmonary Function in the General Population

Genome-wide linkage studies of spirometric measures have also been performed in families from the general population (summarized in Table 60-3). In the Framingham Study, Joost et al. analyzed quantitative prebronchodilator spirometric measurements in 1578

**TABLE 60-3 Linkage Analysis of Quantitative Spirometric Phenotypes in the General Population<sup>a</sup>**

Author (Date)	Population (n)	FEV <sub>1</sub>		FEV <sub>1</sub> /FVC	
		Region	Maximum LOD	Region	Maximum LOD
Joost (2002) (205)	Framingham study (n = 1578)	4p	1.6	—	—
		6q	2.4	—	—
Wilk (2000) (195)	Family heart Study (n = 2178)	3q	2.0	1p	1.8 <sup>b</sup>
				4p	2.6 <sup>b</sup>
				8p	1.6 <sup>b</sup>
				11q	1.9 <sup>b</sup>
				15q	1.6 <sup>b</sup>
Malhotra (2003) (207)	CEPH Pedigrees (n = 264)			2q	2.0
				5q	2.2 <sup>c</sup>

<sup>a</sup>LOD scores above 1.5 are presented.<sup>b</sup>On the basis of normalized phenotypic values for FEV<sub>1</sub>/FVC.<sup>c</sup>Corresponds to parametric heterogeneity LOD score.

individuals from 330 pedigrees using variance component linkage analysis (205). They found suggestive linkage of FEV<sub>1</sub> to chromosome 6q (LOD = 2.4). After genotyping additional STR markers in a subset of their study population, increased evidence for linkage to FEV<sub>1</sub> to 6q was found (LOD = 5.0) (206).

With 2178 participants in the Family Heart Study, Wilk et al. performed genome-wide linkage analysis with prebronchodilator spirometric phenotypes including FEV<sub>1</sub> and FEV<sub>1</sub>/FVC (195). They found suggestive evidence for linkage of FEV<sub>1</sub> to chromosome 3q and FEV<sub>1</sub>/FVC to chromosome 4p. Of interest, they also demonstrated some evidence for linkage of FEV<sub>1</sub>/FVC to chromosome 1p (LOD = 1.8) near the region of linkage to FEV<sub>1</sub>/FVC in the Boston early-onset COPD study.

Using 26 extended Centre d'Etude du Polymorphisme Humain (CEPH) pedigrees, Malhotra et al. also performed genome-wide linkage analysis of prebronchodilator spirometric measurements (207). They found suggestive evidence for linkage of FEV<sub>1</sub>/FVC to chromosome 2q in a similar region to the significant FEV<sub>1</sub>/FVC linkage reported in the Boston early-onset COPD study, as well as suggestive linkage of FEV<sub>1</sub>/FVC to chromosome 5q.

The linkage results of spirometric measurements in the Boston early-onset COPD study pedigrees and general population pedigrees have been somewhat inconsistent, except for the FEV<sub>1</sub>/FVC linkage to chromosome 2q in the CEPH pedigrees and the FEV<sub>1</sub>/FVC linkage to 1p in the Family Heart Study. Inconsistent linkage results between COPD families and general population pedigrees could relate to different genetic determinants influencing normal variation in spirometry and COPD, false-positive evidence for linkage in some regions, and/or inadequate power to replicate all linkage signals in the different studies.

Silverman's group reported a genome-wide linkage analysis in the Boston early-onset COPD study, which found significant linkage to a region on chromosome 2q for COPD-related traits (201,203). DeMeo et al. identified *SERPINE2* as a COPD-susceptibility gene

on chromosome 2q (208). Single nucleotide polymorphisms (SNPs) in *SERPINE2* were demonstrated to explain some of the chromosome 2q linkage signal. In addition, the presumed functional variant(s) in *SERPINE2* have yet to be identified. Furthermore, chromosome 2q was also linked to lung function traits in a general population sample of 264 individuals in 26 families from Utah (207) and in a Dutch study of 1183 individuals in 200 families ascertained through a proband with asthma (209). This suggests that additional COPD-susceptibility genes are located on chromosome 2q and that these genes can be identified by merging SNP-based fine mapping data from multiple COPD study populations. Hersh et al. identified *XRCC5*, an ATP-dependent DNA helicase, as a potential COPD-susceptibility gene by genotyping SNPs in the chromosome 2q region in four study samples—two case-control and two family-based—and tested for association with COPD and related traits (210).

## 60.11 GENETIC ASSOCIATION STUDIES

A large number of association studies have compared the distribution of variants in candidate genes hypothesized to be involved in the development of COPD in COPD patients and control subjects. For example, Smith and Harrison demonstrated associations between two polymorphisms in the microsomal epoxide hydrolase gene (*EPHX1*) with COPD by comparing two groups of COPD patients to 203 blood donors in Scotland (211). They included 94 patients with pathologically demonstrated emphysema from lung tissue surgically resected during lung cancer surgery; they also included a group of 68 patients with COPD from a respiratory clinic. Previously, a variant in exon 3 had been associated with reduced *EPHX1* activity compared to wild-type, while a variant in exon 4 had been associated with increased *EPHX1* activity compared to wild-type. Because *EPHX1* is involved in the metabolism of reactive epoxide intermediates, which can be produced by cigarette smoke

exposure, these functional variants were hypothesized to be involved in the variable development of COPD. In comparisons across genotypes, the 68 COPD patients and the 94 pathologically proven emphysema patients were each more likely to include homozygotes for the slow allele in exon 3. However, these authors also found an association of the variant allele in exon 4, conferring fast enzyme activity, with COPD in the respiratory clinic patients. Thus, the functional significance of reduced *EPHX1* activity is unclear.

A variety of candidate genetic loci have been studied with the case-control association analysis approach. A nonexhaustive list of loci that have been associated with COPD is presented in Table 60-4. A representative study supporting the association is shown for polymorphic variants located beyond the 3' end of the AAT gene, the vitamin D-binding protein, the cystic fibrosis transmembrane regulator gene, ABO blood group, alpha 1-antichymotrypsin, and ABH secretor status (172,212–215). In some cases, more than one study exists to support an association. However, in each case, at least one study refutes the association (216–219).

Several factors could contribute to the inconsistent results of case-control genetic association studies in COPD. Genetic heterogeneity in different populations could contribute to difficulty in replicating associations between studies. In addition, false-positive or false-negative results could contribute to inconsistent replication. A potentially important factor is that case-control association studies are susceptible to supporting associations based purely on population stratification (220). Population stratification can result from incomplete matching between cases and controls, including differences in ethnicity and geographic origin. In addition, most published COPD genetic association studies have not focused on genomic regions linked to COPD-related phenotypes—regions in which association studies may be more fruitful.

Only one family-based genetic association study has been reported in COPD (204), a study design that is typically immune to population stratification effects. This study focused on genetic variants in *TGFB1*, a gene that is located within the region of linkage to FEV<sub>1</sub> on chromosome 19q in the Boston early-onset COPD study and that was associated with COPD in one previously

reported case-control genetic association study (221). Five *TGFB1* SNPs were genotyped in Boston early-onset COPD study families; using family-based association analysis, one SNP in the promoter region of *TGFB1* (rs2241712) and two SNPs in the 3' untranslated region of *TGFB1* (rs2241718 and rs6957) were significantly associated with FEV<sub>1</sub> ( $P < 0.05$ ). Among 304 severe COPD cases from the National Emphysema Treatment Trial (NETT) and 441 smoking control subjects from the Normative Aging Study (NAS), two SNPs in the promoter region of *TGFB1* (rs2241712 and rs1800469) and one SNP in exon 1 of *TGFB1* (rs1982073) were significantly associated with COPD ( $P \leq 0.02$ ) (204). Additional research to replicate the genetic associations in *TGFB1* and to identify the functional variants in or near *TGFB1* will be required. Genetic variation in *TGFB1* is also a modifier of the lung involvement in classic cystic fibrosis.

The Framingham Heart Study has collected spirometry and smoking history data on three generations of adults, and these research participants provided DNA samples that have recently been genotyped for 550,000 SNPs using microarray technology. These genotype and phenotype data, which have been made publicly available through the NHLBI's SNP Health Association Resource (SHARe) initiative, provide a powerful resource to conduct genome-wide association studies to identify novel genetic risk factors for airflow obstruction. Wilk et al. performed a genome-wide association study in 7691 Framingham Heart Study participants to identify SNPs associated with the FEV<sub>1</sub>/FVC ratio, analyzed as a percentage of the predicted value (222). Four SNPs in tight linkage disequilibrium on chromosome 4q31 were associated with the percentage predicted FEV<sub>1</sub>/FVC ratio with  $P$  values of genome-wide significance in the Framingham sample (best  $P$  value =  $3.6 \times 10^{-9}$ ). Quantitative spirometry traits were examined in a previous genome-wide association study using 70,987 SNPs in about 1220 related individuals in the Framingham Heart Study (223). Although no results met the strict criteria for genome-wide significance, *GSTO2* emerged as a candidate gene that warranted replication studies.

Matrix metalloproteinase (MMP)-12-mediated pathologic degradation of the extracellular matrix and the subsequent repair cycles influence the airway changes in

**TABLE 60-4** Conflicting Evidence from Case-Control Genetic Association Studies in COPD

	Support Association	Do Not Support Association
A1AT 3' flanking region	Kalsheker (1990) (214)	Sandford (1997) (217)
Vitamin D-binding protein	Schellenberg (1998) (215)	Kauffmann (1983) (248)
CFTR	Gervais (1993) (212)	Artlich (1995) (219)
ABO blood group	Cohen (1980) (172)	Vestbo (1993) (218)
$\alpha$ 1-Antichymotrypsin	Poller (1992) (213)	Sandford (1998) (216)
ABH secretor status	Cohen (1980) (172)	Vestbo (1993) (218)

Selected references that support or do not support an association to the specified locus are presented.



patients with COPD and asthma. Haplotypes containing the MMP12 Asn357Ser (rs652438) allele are associated with lung function decrease in smokers with COPD (224). Recently, Mukhopadhyay et al. demonstrated that the common serine variant at codon 357 of the MMP12 gene (rs652438) is associated with increased severity of in patients with COPD (225).

Thus far, a variety of candidate genes have been examined in COPD with genetic association studies, but no genetic loci other than SERPINA1 (AAT) have been definitively proven as significant risk factors for COPD.

## 60.12 ANIMAL MODELS OF COPD

Animal models have played an important role in the elucidation of emphysema pathophysiology. In 1964, Gross et al. were assessing the effects of intratracheal injection of a variety of substances, including papain, human serum, and tap water, on the development of silicosis in a rat model (226). Surprisingly, the animals treated with papain developed severe emphysema, which was not detected in the other experimental groups. The development of emphysema was related to the proteolytic activity of papain; subsequently, a variety of other proteolytic enzymes, including leukocyte elastase and proteinase 3, have been shown to cause experimental emphysema in rodents following intratracheal instillation (227). The proteolytic cleavage of elastin appears to be critical for the development of protease-induced experimental emphysema. This work has provided critical evidence for the protease–antiprotease hypothesis for the development of emphysema—particularly regarding the importance of elastase and antielastase activities to this hypothesis.

Advances in molecular biological techniques have led to a variety of creative applications of animal models in COPD research. Transgenic mice that overexpress interstitial collagenase (MMP1) develop histologic changes of emphysema (228). These interstitial collagenase transgenic mice do not have associated inflammation or a decrease in elastin fibers. Therefore, it is unclear if the MMP1 transgenic model represents a failure of alveolar septation in development or if it corresponds to adult-onset emphysema. However, transgenic mice that inducibly overexpress interleukin-13 develop emphysema and inflammation, with characteristics more similar to

human COPD (229). Transgenic mice that inducibly overexpress interferon gamma in the adult lung also develop emphysema and inflammation (230). Both the interleukin-13 and interferon gamma transgenic mouse models demonstrate increased activity of a variety of cathepsins and MMPs.

“Knockout” mouse models have also provided useful insights into COPD pathophysiology. Macrophages from a macrophage elastase knockout mouse had impaired ability to invade extracellular matrix and degrade elastin (231). Subsequently, Hautamaki et al. compared the effects of cigarette smoke exposure on these macrophage elastase knockout mice with their wild-type litter mates (232). In wild-type animals, macrophage elastase was only expressed in mice that had been exposed to cigarette smoke; these smoke-exposed wild-type mice developed emphysema after 6 months of cigarette smoke exposure. However, macrophage elastase knockout mice did not develop emphysema after 6 months of cigarette smoke exposure. The changes in alveolar duct area and mean linear intercept in wild-type and macrophage elastase knockout mice are demonstrated in Table 60-5.

To demonstrate that the development of emphysema was not simply a reflection of the increased number of lung macrophages in smoke-exposed mice, Hautamaki et al. instilled monocyte chemoattractant protein-1 intratracheally into the macrophage elastase knockout mice. Although the number of pulmonary macrophages increased substantially in response to monocyte chemoattractant protein-1 instillation, the macrophage elastase knockout mice still did not develop emphysema with cigarette smoke exposure. The importance of macrophage elastase in human emphysema remains to be determined, but it is an attractive candidate proteinase in COPD pathophysiology.

Other knockout mice develop emphysema even without cigarette smoke exposure. Mice that underwent targeted disruption of the platelet-derived growth factor A chain developed lung hyperinflation and air-filled cavities; alveolar septation was absent (233). This developmental defect resulted from a failure of alveolar development with a lack of elastin fiber production, which was caused by an absence of alveolar myofibroblast cells. Knockout mice for the surfactant protein D (SFTPD), integrin  $\beta 6$  (ITGB6), and tissue inhibitor of metalloproteinase-3

**TABLE 60-5 Effects of Cigarette Smoke Exposure on Control (MME<sup>+</sup>/MME<sup>+</sup>) and Macrophage Elastase Knockout (MME<sup>-</sup>/MME<sup>-</sup>) Mice**

Group	Alveolar Duct Area (mm <sup>2</sup> )	Mean Linear Intercept (μm)
Control mice: no smoke exposure	7.6 ± 1.2	50.4 ± 1.5
Control mice: with smoke exposure	12.2 ± 2.0 <sup>b</sup>	66.6 ± 4.8 <sup>b</sup>
MME <sup>-</sup> /MME <sup>-</sup> mice: no smoke exposure	6.7 ± 0.9	47.9 ± 2.2
MME <sup>-</sup> /MME <sup>-</sup> mice: with smoke exposure	7.8 ± 1.2	49.6 ± 3.5

<sup>a</sup> Indicates  $P < 0.05$  compared to mice without smoke exposure.

<sup>b</sup> Indicates  $P < 0.001$  compared to mice without smoke exposure.

Adapted from Hautamaki et al., 1997 (232); values are mean ± SD.

(TIMP3) genes spontaneously develop progressive emphysema as they get older (234–236). Because these knockout animals develop inflammation with increased MMP activity, they may provide useful models to study COPD pathophysiology.

The klothe and fibroblast-growth-factor-23-deficient mice models both develop spontaneous emphysema as part of a “rapid aging” phenotype that is potentially related to perturbations of vitamin D metabolism (237,238). Tight-skin mice develop emphysema and features resembling Marfan syndrome due to a mutation in the fibrillin 1 gene (*FBN1*) (239).

While these monogenetic mouse models have provided insights into COPD pathogenesis, susceptibility for developing complex syndromes such as COPD are more likely to be the result of a combination of allelic variants that leave an individual more susceptible to the effects of cigarette smoke exposure. There have been several groups that have started to compare the degree of cigarette-smoke-induced lung damage among different strains of mice to understand the genetic factors that may underlie COPD susceptibility. The earliest approaches compared the effects of smoke exposure in a few mouse strains with differences in specific molecular phenotypic traits, i.e. different levels of AAT (240), different antioxidant responses (241), and different major histocompatibility complex haplotypes (242).

In addition to transgenic and knockout mouse models, several naturally occurring mouse models have emphysema-like features (243). One such spontaneously occurring mutation, the tight-skin mouse, was shown to result from a mutation in *FBN1* (244). This *FBN1* mutation interferes with elastic fiber assembly, resulting in enlarged airspaces; thus, it likely represents a developmental defect resulting in airspace enlargement rather than a destructive process of normally developed, mature lung tissue.

Although rodent models have provided important insights into potential biochemical mechanisms of COPD, identification of susceptibility loci using experimental crosses of relatively susceptible and relatively nonsusceptible strains with quantitative trait locus analysis has not yet been reported. Significant differences in susceptibility between murine strains to the development of smoking-induced COPD have been demonstrated (242). Utilization of these strain-specific differences in COPD susceptibility to perform quantitative trait locus mapping may provide unique opportunities to uncover genetic determinants of COPD (245).

### 60.13 CONCLUSIONS

Severe AAT deficiency is a proven genetic risk factor for COPD. Although considerable insight into the pathogenesis of COPD has been provided by studies of AAT deficiency, fundamental questions about the natural history of AAT deficiency remain unanswered.

Only a small percentage of COPD patients inherit severe AAT deficiency, and additional genetic factors likely influence the development of COPD. Further efforts in linkage analysis, association studies, and animal models may lead to the identification of such factors. To achieve a complete understanding of COPD pathophysiology, characterization of the interactions between genetic determinants and cigarette smoking (and potentially other environmental factors) will be required. Identification of genetic factors influencing the development of COPD unrelated to AAT deficiency could clarify the biochemical mechanisms causing COPD, allow identification of highly susceptible individuals, and lead to new therapeutic interventions for COPD.

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## RELEVANT WEBSITES

[www.cdc.gov/tobacco/data\\_statistics/sgr/2004/index.htm](http://www.cdc.gov/tobacco/data_statistics/sgr/2004/index.htm).  
[www.csc.unc.edu/spir/](http://www.csc.unc.edu/spir/).  
<http://www.copdgene.org/>.

## Biographies



**Dr Chad K Oh, MD** received his medical education at Kyung-Hee University School of Medicine in Seoul, Korea, and completed his pediatric residency training at the Rush University School of Medicine in Chicago, IL, and allergy immunology fellowship training at the National Institutes of Health in Bethesda, MD. He is certified by the American Board of Pediatrics and the American Board of Allergy and Immunology. He joined the faculty at UCLA in 1995 and was promoted through the assistant and associate Professor levels. He served as the Program Director of the Harbor-UCLA Conjoint Adult/Pediatric Clinical Immunology and Allergy Fellowship Training Program for six years. Dr Oh was the Chief of Allergy and Immunology Division at Harbor-UCLA Medical Center in Torrance, CA. He has authored more than 40 publications in prestigious journals, including *The Journal of Immunology*, *The Journal of Allergy and Clinical Immunology*, *Biochemical Journal*, and *Proceedings of the National Academy of Sciences USA*. He authored the book, “How to live with a nut allergy” by McGraw-Hill, as well as six other book chapters and 70 abstracts. Dr Oh has served as a member or chairman on numerous national committees and as an officer for professional organizations, including the Executive Committee of the American Academy of Allergy, Asthma and Immunology and the American College of Allergy, Asthma and Immunology, and as President of the Los Angeles Society of Allergy Asthma Immunology. He has served on the editorial board of *Experimental Medicine* and has been a reviewer for numerous journals, including *The Journal of Allergy and Clinical Immunology*, *The Journal of Immunology*, *Pediatrics*, and *Allergy & Asthma Proceedings*. Dr Oh has received several honors and awards, including young investigator awards from the National American Lung Association and an Innovative Research Award from the American Academy of Allergy, Asthma & Immunology. He joined MedImmune, LLC, in 2008 as a Medical Director to participate in the development of novel therapeutic targets in asthma and other lung diseases.



**Dr Néstor Molfino, MD, MSc, FCCP** joined MedImmune, LLC, in June 2007 as senior Director and was named Vice President, Clinical Development, and Clinical Therapeutic Area Head, Pulmonary Disease in July 2008. In this role, he is responsible for all phases of global clinical development for pulmonary conditions. Dr Molfino has 14 years of biopharmaceutical industry experience. Before joining MedImmune, Dr Molfino was senior Director, Clinical Development at Otsuka Maryland Research Institute for four years. He also served as Director, Medical Affairs at Baxter Bioscience and Vice President, Research and Development and Scientific Affairs at Theratechnologies, Inc, in Canada. For the past 14 years, Dr Molfino has also served as a pulmonary and allergy consultant to Fermin Salaberry, a nonprofit public community hospital in Argentina. Dr Molfino received a master's degree in molecular and cell biology from the University of Toronto. He holds a medical degree from Universidad Nacional de Rosario in Argentina, completing an internship and residency in internal medicine and respiratory medicine, after which he went to Toronto, Canada, for a postdoctoral fellowship at the University of Toronto, where he was appointed assistant professor in 2004. In 2006, he was also appointed adjunct professor at McGill University in Montreal, Canada.

# CHAPTER

# 61

## Interstitial and Restrictive Pulmonary Disorders

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### 61.1 INTRODUCTION

The interstitial lung diseases (ILDs) represent a heterogeneous group of lung disorders that are characterized by inflammation and/or fibrosis in the interstitial space between alveoli as well as adjacent perialveolar tissue and other contiguous supporting structures. In most instances, ILDs lead to restrictive physiology on pulmonary function testing (PFT) and many lead to hypoxemia and respiratory failure. Over 150 diseases have been associated with ILD, some with known causes that include environmental exposures such as asbestos, autoimmune and rheumatologic disease, and underlying genetic syndromes that include manifestations of ILD, several of which are covered in this chapter. However, a significant component of individuals with ILD actually fall into the idiopathic interstitial pneumonia (IIP) category, a group of ILDs in which the cause remains unknown. Over the past decade, evidence has emerged suggesting that at least a component of these IIPs may have a genetic basis, holding promise that one day the cause of these diseases will be known and targeted therapies developed. We have divided this chapter into three main categories: (1) the role of genetics in the IIPs, (2) common diseases that can include ILD as a manifestation and that likely have a genetic contribution to disease, and (3) defined genetic syndromes that include ILD as one manifestation.

### 61.2 IDIOPATHIC INTERSTITIAL PNEUMONIA AND FAMILIAL INTERSTITIAL PNEUMONIA

#### 61.2.1 Idiopathic Interstitial Pneumonia (IIP)

When a patient presents with a clinical presentation consistent with ILD, the physician must try to determine if an

underlying disease process explains the presence of ILD. However, in many instances, the cause of ILD remains unknown and the patient's diagnosis falls into a group of disorders known as the IIPs. By the latest American Thoracic Society Consensus Statements (1,2), the IIPs break down into the following categories: idiopathic pulmonary fibrosis (IPF) and its pathologic correlate usual interstitial pneumonia (UIP), nonspecific interstitial pneumonia (NSIP), acute interstitial pneumonia (AIP), cryptogenic organizing pneumonia (COP), lymphocytic interstitial pneumonia (LIP), desquamative interstitial pneumonia (DIP) and respiratory bronchiolitis interstitial lung disease (RBILD). The IIPs can present in either childhood or adulthood. However, the IIPs are much more common in the adult population. Of these IIPs, IPF is the most common and most severe form and has thus received the most attention in pulmonary research.

Clinically, patients with IPF typically present with shortness of breath and decreased exercise tolerance (3). PFT reveals restriction and radiographic imaging reveals interstitial fibrosis on chest roentgenogram and high-resolution computed tomography (HRCT) of the chest. Patients typically progress to hypoxemia and respiratory failure, with most patients dying from the disease within 5 years of diagnosis. Unfortunately, no effective treatments are available short of lung transplantation, a therapy with its own complications and feasible for only a small percentage of patients. The incidence of disease is approximately 20 per 100,000 males and 13 per 100,000 females (1), but rising. Most individuals present between ages 50 and 75, but patients can present outside this interval in either direction. Many individuals can be diagnosed with disease based on clinical presentation and classic findings on HRCT of the chest. When an individual does require a lung biopsy for diagnosis, a pathologic pattern referred to as UIP is noted (2).



While the cause remains unknown in IPF, over the past decade genetic discoveries in familial forms of disease have shown promise in helping the medical and scientific community gain significant insights into the pathogenesis of this disease.

### 61.2.2 Familial Interstitial Pneumonia (FIP)

Familial interstitial pneumonia (FIP) is defined when two or more individuals have an IIP within a single family (4–7). FIP has been described in the literature for over a century, but it has been the past decade that has greatly increased our understanding of this category of ILD, including some cases in which genetic links have been revealed (8–11). Studies from the United Kingdom and Finland suggested that familial forms of the disease account for approximately 2–4% of all cases of IPF (12,13). However, it is possible that this percentage may actually be higher (5,7). Studies from the United Kingdom and the Mayo Clinic in Minnesota suggest that adult patients who present with FIP are essentially indistinguishable from sporadic IPF patients in terms of clinical presentation, radiographic examination, histopathologic findings, and survival except that patients with FIP tended to present at an earlier age (6,12). Most families analyzed to date reveal that FIP is inherited in an autosomal dominant pattern (4,5,12). While penetrance of disease is clearly not 100%, it must be noted that this determination is greatly limited by the fact that patients typically present late in adulthood when other variables may lead to less frequent diagnoses.

In a recent collaborative study from the National Jewish Medical Center/Colorado University, Duke University, and Vanderbilt University, investigators reported the largest FIP cohort analyzed to date (111 families, 309 patients, 360 unaffected controls) (4). The results from this study revealed that male gender (55.7 vs. 37.2%,  $p < 0.0001$ ), older age (68.3 vs. 53.1 years,  $p < 0.0001$ ), and history of cigarette smoking (67.3 vs. 34.1%,  $p < 0.0001$ ) were risk factors for the presence of disease in these families. In this cohort, among individuals who had a biopsy performed, 85% were classified as IPF/UIP. However, interestingly, pathologic heterogeneity was observed within many individual families. While UIP was the only IIP pathology observed in 55% of families, the remaining 45% of families had two or more pathologic forms represented, with many families having both UIP and NSIP represented. These observations of pathologic heterogeneity within single families supported previous observations (8,9). Together, these studies suggest that, although described as different diseases based on pathologic description, these IIP disease categories may in fact have similar origins. Furthermore, the finding that cigarette smoking was a risk factor for the development of FIP suggests that environmental factors can influence the presentation of genetic-based familial ILD.

Among avenues to pursue genetic relationships in the IIPs, analyzing FIP cohorts are the most promising. Over the past decade, FIP families have been analyzed with candidate gene and linkage approaches with limited success. To date, four genes have been clearly linked to cases of adult FIP (surfactant protein C (SPC), surfactant protein A2, telomerase reverse transcriptase, and telomerase RNA component) in a causative manner, but together only account for approximately 15% of FIP cases (9–11,14). Pediatric ILD has been linked to genetic mutations in SPC and ATP-binding cassette transporter A3 (8,15). Newer generation genetic approaches such as whole exome sequencing (WES) and genome-wide association studies (GWAS) are now being employed, but results as yet are unknown. Genetic studies in FIP have been greatly limited by the fact that highly genetically informative families are rare in FIP, possibly because of decreased disease penetrance in some families, and also more likely because patients present late in life and blood or tissue specimens may not be available for those who are already deceased. While autosomal dominant inheritance suggests a single causative genetic defect for any given family, it is now clear that there is likely considerable locus heterogeneity in FIP, with many different individual genes across different families contributing to disease. However, despite these limitations, over the past decade genetic mutations have been linked to several cases of FIP as described later, and ongoing studies hold promise for further discoveries, all of which will improve our understanding of the pathogenesis of both familial and sporadic forms of this disease.

### 61.2.3 Surfactant Protein C Mutations and Pediatric ILD and Adult FIP/UIP

SPC is one of four surfactant proteins expressed in the alveolar space, with its prominent role being to maintain alveolar surface tension such that alveoli do not collapse. It is expressed throughout the lung epithelium during lung development, but in the mature lung is localized specifically to type II alveolar epithelial cells (AECs) (16). Over the past decade, some cases of both pediatric and adult ILD have been linked to genetic mutations in the SPC (*SFTPC*) gene. In 2001, Nogee and colleagues (8) made the initial report of *SFTPC* mutation-mediated ILD when they reported an infant with NSIP whose mother had previously been diagnosed with DIP in her first year of life. Genetic sequencing revealed a heterozygous G to A transition in the first base of intron 4 (IVS4+1 G to A) on a single allele in both child and mother, consistent with inheritance in an autosomal dominant pattern. This *SFTPC* mutation caused deletion of exon 4 and its 37 amino acids and is referred to in the literature as the *SFTPC*Δ<sup>exon4</sup> mutation. Mature SPC was not detected in the lung of either subject, but a misprocessed form of the pro-protein, pro-SPC, was noted in type II AECs. Following this initial study, *SFTPC* mutations have been

described in many cases of pediatric ILD, many of which are found in the carboxy-terminal region of pro-SPC (17–21).

After these reports linking *SFTPC* mutations to childhood ILD, questions arose as to whether disease occurred because of altered SPC function in the alveolar space or because of dysfunction in the type II AEC population including possible impairments in regenerative capacity (16). Shortly after the report by Nogee et al. (8), Amin and colleagues (22) reported a mother and two children who had chronic ILD and SPC deficiency (lack of mature SPC in the alveolar space), but no mutations in *SFTPC* (or surfactant protein B—*SFTPB*) were noted. Subsequently, studies have shown that mice deficient in SPC have a greater propensity to develop experimental lung fibrosis (23,24), suggesting that SPC deficiency may have a role in pulmonary fibrosis.

While *SFTPC* mutations may lead to altered expression of SPC in the alveolar space, multiple lines of evidence implicate aberrant intracellular processing of pro-SPC with type II AEC toxicity as the most likely result of these mutations (16,25). Transcription of the *SFTPC* gene and subsequent mRNA translation yield a 197 amino acid precursor protein (pro-SPC). The pro-SPC product is then processed through the endoplasmic reticulum (ER), Golgi, and distal secretory pathway, resulting in the mature 35 amino acid SPC protein that is secreted into the alveolar space. Carboxy-terminal *SFTPC* mutations lead to the production of a pro-SPC product that cannot be folded normally in the ER, leading to accumulation of protein, ER stress, activation of the unfolded protein response (UPR), and type II AEC injury (26–28).

Following the identification of *SFTPC* mutations linked to childhood cases of ILD, investigators evaluated whether or not similar mutations might explain cases of adult FIP. In 2002, Thomas and colleagues (9) reported a family in which 11 adults had ILD (six with biopsy-confirmed UIP/IPF and five with IPF on clinical grounds) and three children had NSIP. In this large family, a heterozygous exon 5 +128 T to A transversion leads to the substitution of glutamine for leucine at amino acid position 188 (L188Q) of pro-SPC. Immunostaining for pro-SPC on lung tissue sections from affected individuals revealed an abnormal staining pattern, while accumulation of atypical intracytoplasmic vesicles was noted in type II AECs on electron microscopy. Mouse lung epithelial (MLE) cells expressing the mutant L188Q *SFTPC* were also noted to have similar intracytoplasmic vesicles, suggesting aberrancy in SPC processing. Subsequently, in vitro studies revealed that this L188Q *SFTPC* mutation results in a pro-SPC product that cannot be folded properly, leading to ER stress and caspase pathway activation (28,29). Subsequent to this initial study by Thomas and colleagues, other *SFTPC* mutations have been found in adult FIP cohorts, including a recent study by Van Moersel and colleagues (30) in which *SFTPC* mutations

represented approximately 25% of FIP cases in a Dutch cohort, a percentage higher than what has been observed in other cohorts.

While detected in FIP cases, mutations in *SFTPC* are rarely encountered in sporadic cases of IPF. In 2004, Lawson and colleagues (31) reported a study of 89 patients with UIP, 46 patients with NSIP, and 104 normal subjects and found only one mutation that resulted in an amino acid sequence change in pro-SPC. In this individual, a genetic mutation in exon 3 caused a substitution of threonine to isoleucine at amino acid 73 (I73T). This I73T mutation is likely the most common of the *SFTPC* mutations reported to date, occurring in both pediatric cases of ILD (17–21) and adult cases of FIP (30).

Although rarely found in IPF, the fact that mutations in *SFTPC* can lead to biopsy-proven UIP points to the type II AEC as the critical cell in the pathogenesis of this disease. One question that arose as a result of the genetic studies was whether or not pathways important in *SFTPC* mutation-mediated disease might also be important in sporadic cases of IPF. In 2008, two separate groups of investigators reported studies that suggested a role for AEC ER stress in non-*SFTPC* mutation-associated ILD (29,32). Lawson and colleagues (29) found that ER stress markers were prominent in AECs lining areas of fibrosis in lung biopsies from individuals with non-*SFTPC* mutation-associated FIP as well as from individuals with sporadic IPF. Soon thereafter, Korfei and colleagues (32) reported a cohort of sporadic IPF/UIP lung biopsies with similar findings of AEC ER stress, but also demonstrated that caspase pathway activation was present in these same AECs. Together, these two studies demonstrated that ER stress pathways may be important not only in the pathogenesis of *SFTPC* mutation-associated disease, but in sporadic cases of IPF as well.

#### 61.2.4 Surfactant Protein A2 Mutations and Adult FIP

Considering that *SFTPC* mutations were found in cases of FIP, suspicion arose on whether or not mutations in the genes for the other surfactant proteins might be found in other cases of FIP. In 2009, Wang and colleagues (14) reported such a finding related to *SFTPA2*, one of two genes that encode for surfactant protein A. In that study, they analyzed a highly genetically informative family in which 15 affected individuals had FIP and/or lung adenocarcinoma with bronchoalveolar cell carcinoma features. Starting with a whole genome linkage analysis, they found a 15.7Mb region on chromosome 10 that segregated with disease. This region contains both *SFTPA1* and *SFTPA2*, as well as *SFTPD*, the gene for surfactant protein D. Sequencing of all three of these candidates was performed, and from this a heterozygous mutation in *SFTPA2* was found. A G to T transversion in codon 231 was noted, which predicted a substitution of valine for glycine at the highly conserved amino acid

position 231 (G231V). Subsequently, 58 additional families were analyzed, with one family having a different *SFTPA2* mutation, a T to C transversion that resulted in the substitution of serine for phenylalanine at amino acid position 198 (F198S). Both these mutations (G231V and F198S) are in the protein's carbohydrate recognition domain. Subsequent in vitro analyses by these investigators demonstrated that these mutations result in a protein product that is retained in the ER, leading to ER stress (14,33), raising further questions on the roles that aberrant protein processing and ER stress in type AECs play in the pathogenesis of IPF.

### 61.2.5 *ABCA3* Gene Mutations in Pediatric ILD

ATP-binding cassette transporter A3 (*ABCA3*) is expressed in the lamellar bodies of type II AECs, suggesting an important role for this protein in surfactant processing. In 2004, Shulenin and colleagues (34) reported a study in which they analyzed 21 infants with severe neonatal surfactant deficiency in whom the etiology for their deficiency was unknown, observing that 16 of the 21 individuals (76%) had mutations in the *ABCA3* gene. These patients were either homozygous for the same mutation, or compound heterozygous for different mutations. Analysis of lung tissue sections, which were available from a subset of patients, by light and electron microscopy revealed markedly abnormal lamellar bodies. Taken together, these studies reveal that *ABCA3* is critical for surfactant processing and the proper formation of lamellar bodies.

After this report on *ABCA3* mutations and neonatal surfactant deficiency, Bullard and colleagues (15) evaluated a large cohort of children with pediatric ILD for mutations in *ABCA3*. From a cohort of 165 subjects who were known to not have mutations in *SFTPC* or *SFTPB*, four patients with a diagnosis of DIP who were over 10 years of age were selected for genetic sequencing of the 30 coding exons of *ABCA3*. Three of the four patients were compound heterozygotes for mutations in *ABCA3*, with each of these three individuals carrying one copy of a specific mutation, E292V, which had been described previously (34). Screening of an additional 153 subjects from the cohort was then performed for this specific mutation with an additional seven subjects identified as being E292V *ABCA3* mutation carriers. Of these seven additional patients, five had a second *ABCA3* mutation, while two did not. Thus, of the 165 subjects in the cohort, a total of 10 carried the E292V mutation, eight of whom were compound heterozygotes for *ABCA3* mutations (15). However, this analysis did not evaluate the possibility that other children in the cohort could have had different *ABCA3* mutations. Subsequently, Young and colleagues (35) reported a teenage boy who had ILD with a biopsy pattern of UIP and an *ABCA3* mutation. Otherwise, to date, studies in the adult population have

yet to clearly link *ABCA3* mutations to adult cases of FIP (30).

### 61.2.6 *ABCA3* Mutations May Modify Clinical Expression of *SFTPC* Mutations

To determine if *ABCA3* mutations might have a disease-modifying effect on *SFTPC* mutations, Bullard and Nogee (36) evaluated four infants with pediatric ILD who had the I73T *SFTPC* mutation and noted that three had concomitant heterozygous mutations in *ABCA3*. In all cases, all parents were asymptomatic from a respiratory standpoint. Pedigree analysis indicated that each child had inherited the *SFTPC* mutation from one parent and the *ABCA3* mutation from the other. Taken together, the fact that both parents were disease free, yet the child had prominent childhood ILD raised suspicion that heterozygous *ABCA3* mutations may modify the effect of the *SFTPC* mutation, leading to the development of clinical ILD. Following this pediatric study, Crossno and colleagues (37) reported a small adult FIP family in which the I73T *SFTPC* mutation was found in conjunction with a heterozygous *ABCA3* variation, although given the small pedigree it is not clear to what role this *ABCA3* variant played in the presentation of ILD.

### 61.2.7 Telomerase Mutations in FIP/UIP

Multiple genetic diseases can include manifestations of pulmonary fibrosis, including dyskeratosis congenita (DKC) (4,5,38). DKC is discussed later in this chapter, but warrants a brief introduction here because observations in this disease led to important discoveries related to telomerase mutations in FIP. DKC was originally described based on a triad of abnormal skin pigmentation, nail dystrophy, and oral leukoplakia, but actually involves many different organ systems (38). Among these, the lungs are frequently affected, with 20% of DKC individuals having pulmonary fibrosis. In fact, behind aplastic anemia, respiratory failure is the second most frequent cause of death in DKC subjects (39). Genetic mutations in *DKC1*, the gene encoding for dyskerin, are responsible for the X-linked form of DKC (40,41). Some autosomal dominant forms of DKC have been linked to genetic mutations in telomerase reverse transcriptase (*TERT*) and telomerase RNA component (*TERC*) (42–44). These components are essential for the normal operation of the telomerase complex to maintain telomere integrity, and multiple lines of evidence suggest that it is telomere shortening that leads to disease manifestations when these mutations are present.

In 2005, Armanios and colleagues (44) reported a *TERT* mutation in a DKC family in which pulmonary fibrosis was a predominant finding. This led the investigator to question whether or not FIP cases might also be linked to mutations in components of the telomerase complex, and indeed that proved to be the case. In 2007,

Armanios and colleagues analyzed 73 FIP families and found that five of these families had genetic mutations in *TERT* and one family had a mutation in *TERC* (10). Of the six probands from these families, five had lung biopsies consistent with UIP, while one had a biopsy that read as IIP. Across the six families, 19 individuals were affected by FIP. Classic mucocutaneous manifestations of DKC were not present in any affected subjects. Mutation carriers without lung disease were noted, but they tended to be younger than their affected relatives. In vitro studies of the identified mutations demonstrated decreased telomerase activity. Furthermore, telomere shortening in peripheral blood lymphocytes was prominently noted in the mutation carriers compared to nonmutation carriers. In fact, all mutation carriers had average telomere lengths that were at or below the 10th percentile when compared to age-based telomere length distribution curves.

Shortly after this initial report, Tsakiri and colleagues (11) reported similar findings in another FIP cohort. While the Armanios study relied on a candidate gene approach, the Tsakiri study utilized two highly genetically informative families to link to a region on chromosome 5. At that point, *TERT* was selected as a potential candidate and two separate mutations in the two families were discovered. Subsequently, an additional 44 FIP families were analyzed, with four having heterozygous *TERT* mutations and one a heterozygous *TERC* mutation. As in the Armanios study, in vitro evaluations of the mutations revealed decreased telomerase activity. Furthermore, on peripheral blood leukocytes, telomere lengths were shorter in mutation carriers compared to age-matched noncarriers. In addition, the investigators also analyzed an additional 44 cases of sporadic ILD and identified a single subject with a mutation in *TERT*. When considered together, these two studies suggest that telomerase mutations are the underlying cause of disease in approximately 10% of FIP.

A year following their genetic studies, both the aforementioned groups reported that telomere shortening was prominent in individuals with pulmonary fibrosis even without defined mutations in *TERC* or *TERT* (45,46). In the first report, Cronkhite and colleagues (45) analyzed a cohort of pulmonary fibrosis patients who did not have mutations in *TERT* and *TERC*, including probands from 59 different FIP families and 73 subjects with sporadic IPF. In this study, the investigators found that 24% of the FIP subjects and 23% of the sporadic IPF subjects had peripheral blood leukocyte telomere lengths that were below the 10th percentile compared to controls. Subsequently, Alder and colleagues (46) analyzed 100 cases of sporadic IPF and found one subject with a mutation in *TERC* (none found in *TERT*). Among these individuals, 62 had telomere length measurements on peripheral blood lymphocytes available, with 60 of the 62 (97%) having telomere lengths less than the median of healthy controls and six of 62 (10%) with telomere lengths less than the first percentile compared to healthy controls.

Furthermore, using fluorescent in situ hybridization techniques, these investigators also demonstrated that AECs in these individuals also had telomere shortening. Taken together, these two studies suggest a potentially critical role for telomere shortening in the pathogenesis of IPF, even when defined telomerase mutations are not present.

Building on the genetic observations by Armanios and colleagues and Tsakiri and colleagues mentioned earlier, in 2008, researchers from Japan analyzed a cohort of patients with IPF and controls with GWAS and identified an association of a common *TERT* variant with susceptibility to IPF (47). This A to C SNP was in intron 2 of *TERT* (rs2736100). Among 242 IPF cases and 1469 controls, the minor allele frequency in diseased individuals was 0.277 compared to 0.409 in controls ( $p = 2.9 \times 10^{-8}$ ), thus indicating that the more common A was the risk allele in this cohort.

### 61.2.8 *ELMOD2* Linkage in FIP Cases in Finland

In 2002, Hodgson and colleagues (13) reported 17 multiplex FIP families in Finland. In this evaluation, familial clustering was described, including the likelihood of a distant shared ancestor. Subsequently, they evaluated six of these families using a genomewide scan approach, identifying five potential loci on chromosomes 3, 4, 9, 12, and 13 (48). When hierarchical mapping was performed on 24 families, a shared haplotype on chromosome 4q31 emerged in eight of the 24 families. Compared to population controls, this haplotype was significantly more frequent in affected individuals (odds ratio (OR), 6.3; 95% confidence interval (CI) 2.5–15.9;  $p < 0.0001$ ). The shared haplotype region encompassed a gene known as *LOC152586* and the first three exons of a gene known as *ELMOD2*. The function of neither gene is known, but gene expression evaluation suggested expression of *LOC152586* in the testis and not in the lung. In contrast, *ELMOD2* was expressed in both alveolar macrophages and AECs in normal lung, but not detected in IPF/UIP lung biopsies. Real-time polymerase chain reaction (PCR) studies of mRNA expression from frozen lung tissue revealed that *ELMOD2* expression was markedly decreased in IPF/UIP lung compared to normal lung. However, sequencing of *ELMOD2*'s nine exons and exon–intron boundaries in affected individuals from the linked families did not reveal any genetic mutations. Despite this observation, however, given the strength of the shared haplotype, *ELMOD2* remains a candidate gene for further evaluation in FIP.

### 61.2.9 Genetic Polymorphism Associations in IIP

Common polymorphisms in multiple genes have been analyzed for potential associations with IPF. However, all these studies have been in small cohorts, and in many



instances the results have never been confirmed in other populations. A brief summary of some of these studies is described below.

**61.2.9.1 Interleukin-1 Receptor Antagonist and Tumor Necrosis Factor- $\alpha$ .** Interleukin-1 $\alpha$  (IL-1 $\alpha$ ) and IL-1 $\beta$  are key proinflammatory cytokines and bind to the IL-1 receptor to initiate a proinflammatory response. However, when bound to the IL-1 receptor antagonist (IL-1RN), no response is elicited, with this system acting as a downregulation mechanism. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is another prominent proinflammatory cytokine. IL-1RN+2018 and TNF- $\alpha$ -308 polymorphisms have both been implicated in inflammatory diseases. In 2000, Whyte and colleagues (49) observed an association with IPF with the IL-1RN+2018C>T polymorphism in a cohort of English and Italian patients. However, two subsequent studies analyzed an *IL-1RN* intron variable number tandem repeat that is in strong linkage disequilibrium with the +2018C>T polymorphism and found no association with IPF (50,51). The TNF- $\alpha$ -308G>A allele was found to be associated with IPF in the cohort of English and Italian patients (49). The study by Riha and colleagues (51) confirmed this finding in an Australian cohort, but a later study by Pantelidis and colleagues (52) did not find an association with the TNF- $\alpha$ -308G>A allele.

**61.2.9.2 Surfactant Proteins.** Because of their expression in the lung, the genes for surfactant proteins are attractive targets for evaluating for disease risk-associated polymorphisms. In 2003, Selman and colleagues (53) analyzed an IPF cohort for common polymorphisms in the four surfactant protein genes (*SFTPA1*, *SFTPA2*, *SFTPB*, *SFTPC*, *SFTPD*) but found no associations with IPF. However, in subgroup analysis, the *SFTPA1*\_6A4 allele was found to be associated with increased IPF risk in nonsmoking subjects, while the *SFTPB* B1580\_C allele was associated with increased IPF risk in smokers. This *SFTPB* allele has previously been reported as having a higher risk for chronic obstructive pulmonary disease (COPD), a smoking-related lung disease (54). *SFTPC* has two common polymorphisms (T138N in exon 4 of pro-SPC and S186N in exon 5), but there is no clear evidence that either affects SPC processing or function. In a study from Finland of infants with respiratory distress syndrome, the asparagine allele at position 186 was found in greater frequency in affected individuals compared to controls ( $p=0.040$ ). In the same study, the asparagine allele at position 138 approached statistical significance for increased frequency in affected individuals ( $p=0.071$ ) (55). In contrast, in a study from Japan of 41 subjects with FIP or sporadic IIP, the serine allele at position 186 was found in greater frequency in affected individuals compared to controls (56). However, other studies have failed to identify any association between either the T138N or S186N *SFTPC* polymorphisms, including the Selman study (53) and another study by Markart and colleagues (57).

**61.2.9.3 Angiotensin II Pathways.** Angiotensin converting enzyme (ACE) converts angiotensin I to II. Blocking the actions of angiotensin II, either through ACE inhibitors or angiotensin II type 1 receptor blockers, attenuates lung fibrosis in mouse models (58,59). Serum ACE levels are affected by an ACE insertion/deletion polymorphism in intron 16. In a 2001 study of 24 patients with either UIP or fibrotic NSIP, Morrison and colleagues (60) found that the D allele frequency was greater in affected subjects compared to controls (69 vs. 54%;  $p=0.04$ ). In 2008, Molina and colleagues (61) reported that the G-6A polymorphism in the promoter of the gene (*AGT*) encoding angiotensinogen, the precursor for angiotensin II, did not predispose individuals to develop IPF, but was associated with disease progression over time, with worse oxygenation in IPF subjects carrying the G genotype.

**61.2.9.4 Transforming Growth Factor- $\beta$ 1.** Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is one of the principal profibrotic cytokines, with multiple lines of evidence revealing a prominent role in the pathogenesis of lung fibrosis. Polymorphisms at codons 10 (L10P caused by a position 869T>C substitution) and 25 (R25P caused by a 915G>C substitution) appear to affect the level of TGF- $\beta$ 1 produced. The codon 915C>C polymorphism affecting codon 25 has been reported to be associated with post-lung transplant allograft fibrosis (62). The CC genotype of the codon 10 869T>C polymorphism is associated with worse lung disease in cystic fibrosis (63). Neither the codon 10 nor the codon 25 polymorphisms have been associated with IPF. However, in a study by Xaubet and colleagues (64), the L10P change was associated with progression of gas exchange abnormalities in those individuals with IPF.

**61.2.9.5 Complement Receptor 1.** A common polymorphism in exon 33 (5507C>G) of the gene for erythrocyte complement receptor 1 (*CR1*) affects the level of CR1 expressed per erythrocyte. In 2002, Zorzetto and colleagues (65) reported that the homozygous GG allele was associated with sarcoidosis. This group subsequently analyzed a group of IPF patients and noted that the GG genotype was more commonly represented in IPF subjects than in controls (66). However, a second study analyzing two separate cohorts of patients found no association with this *CR1* polymorphism and IPF (67). Furthermore, another study did not identify an association with this *CR1* polymorphism and sarcoidosis (68).

**61.2.9.6 Other Candidate Genes Evaluated.** Other polymorphism evaluations have been performed in IPF (69). Human leukocyte antigen (HLA) associations with IPF have been reported including HLA-Dw6, HLA-DR2, HLA-B8, HLA-B12, and HLA-B15 (70–73). The plasminogen activator inhibitor-1 (*PAI-1*) promoter polymorphism 4G/5G has been reported to be associated with increased risk of NSIP, but not UIP (74). In 2001, Pantelidis and colleagues (52) reported that while polymorphisms in IL-6 (intron 4G) and TNF receptor II (1690C) were not individually associated with IPF, the

combination of both alleles was associated with greater risk of disease. Two different matrix metalloproteinase promoter polymorphisms were analyzed by Checa and colleagues (75), with the -1607 polymorphism being associated with risk for IPF in general and the -755 polymorphism conferring IPF risk in individuals that smoke. In a study by Xaubet and colleagues (76), the carriage of double homozygous alleles with two separate cyclooxygenase-2 polymorphisms conferred a mild increased risk for IPF. In 2009, Aquino-Galvez and colleagues (77) reported an association with IPF and a polymorphism in the major histocompatibility complex (MHC) class 1 chain-related gene A. Two separate studies by Bournazos and colleagues (78) analyzed Fcγ receptor (FcγR) polymorphisms in IPF cohorts, noting that an FcγR IIIb polymorphism was associated with IPF risk, while an FcγRIIa polymorphism was associated with the severity of IPF, but not the presence of IPF (79). Specific polymorphisms in many other selected genes have been analyzed with no evidence of association with IPF (69). However, it is likely that many other gene candidates have been analyzed in IPF cohorts, but have never been reported because of the difficulties with publishing negative results.

### 61.3 ASSOCIATED POLYMORPHISMS IN OTHER COMMON FORMS OF ILD

#### 61.3.1 Systemic Sclerosis-Related ILD

In many of the rheumatologic disorders, ILD may develop and even be a prominent finding. Of these, systemic sclerosis, or scleroderma is best described in association with pulmonary fibrosis. Systemic sclerosis is characterized by excessive deposition of connective tissue components including collagen in the skin and other tissues, prominent alterations in the microvasculature, and abnormalities in cellular and humoral immunology. Among those tissues that can be affected, the lung is sometimes involved with significant pulmonary fibrosis, and affected patients typically present with respiratory symptoms similar to those of individuals with IIP. When lung biopsy is performed, NSIP is a commonly encountered histological pattern, although UIP is also observed (80). Over 90% of patients with systemic sclerosis have detectable antinuclear antibodies in the serum. Three predominant antibodies are observed: (1) anti-RNA polymerase antibodies, (2) anti-centromere antibodies, and (3) antitopoisomerase antibodies (ATAs), each consistent with clinical disease subsets with ATA (also known as anti-Scl-70) strongly associated with lung fibrosis (81,82).

Since it is thought that pathogenesis of systemic sclerosis involves a T-cell-mediated response to an antigen, early genetic studies evaluated the MHC on chromosome 6. In 2001, Gilchrist and colleagues (81) reported that 39 of 54 (72%) ATA-positive patients carried an HLA-DRB1\*11 allele in comparison to 18% of controls ( $p=0.00006$ ). Although not as strong, they also found

an association with another allele, HLA-DPB1\*1301. A separate study found similar associations with HLA-DRB1\*11, specifically finding that the allelic subtype \*1104 was associated with the presence of ATAs and lung fibrosis (83). Other genes have been analyzed as well using a candidate gene approach. In a study by Sato and colleagues (84), TNF alleles were not associated with lung fibrosis, but the TNF -863A allele was strong associated with anticentromere antibodies, which are seen less often in pulmonary fibrosis compared to ATAs. Weak associations with lung fibrosis have been found with both secreted protein acidic and rich in cysteine/osteonectin (SPARC) and fibronectin 1 (FN1) (85,86). Two haplotypes (Hap-5 and Hap-6) in the fibrillin 1 gene (FBN1) have been associated with systemic sclerosis, with Hap-5 specifically associated with pulmonary fibrosis (87). Two separate studies detailed an association between the TGF-β L10P codon and lung fibrosis in systemic sclerosis (88,89). In a study by Beretta and colleagues (90), 104 subjects with systemic sclerosis were followed over time, with 25 (12.3%) of these individuals developing severe lung fibrosis. In this cohort, the interleukin-1β (IL-1β) C+3962T polymorphism was found to be associated with the presence of severe restrictive lung disease ( $p=0.003$  for TT versus CC; hazard ratio, 6.61; 95% CI, 2.28–19.15).

#### 61.3.2 Sarcoidosis

Sarcoidosis is a multisystem disorder in which granulomas form and accumulate in the tissues. This process can occur in any organ, but over 90% of individuals present with pulmonary findings, ocular involvement, and/or skin manifestations (91). Pulmonary presentations range from incidental detection of hilar adenopathy on chest radiograph in asymptomatic subjects to chronic respiratory failure due to severe parenchymal lung fibrosis. Given the fact that the granuloma is the central pathologic finding in sarcoidosis, a significant aspect of research into the pathogenesis of this disease has targeted searches for an inciting antigen or pathogen (91).

Familial clustering of cases of sarcoidosis could potentially occur because of either genetic disposition or because most families share similar environmental exposures. A Case-Control Etiologic Sarcoidosis Study (ACCESS), the most comprehensive evaluation of familial sarcoidosis, analyzed 706 case-control pairs, with the study revealing that relatives of individuals with sarcoidosis had a much greater likelihood of having sarcoidosis compared to relatives of controls (OR, 5.8; 95% CI, 2.1–15.9 for siblings; OR, 3.8; 95% CI, 1.2–11.3 for parents) (92). Because of important immunologic features of sarcoidosis, HLA alleles have been analyzed with class I HLA-B8 (93) and class II HLA-DRB1 and DQB1 found in association with sarcoidosis (94,95).

Genome-wide linkage approaches have had some success in identifying genetic associations with sarcoidosis.

In the Sarcoidosis Genetic Analysis Consortium (SAGA) study, an analysis of African-American families with sarcoidosis, 15 potential associations (as based on  $p \leq 0.05$ ) were identified, with the most prominent of these being on chromosome 5q11 ( $p = 0.0005$ ) (96,97). Following these initial SAGA findings, fine mapping in African-American families not only further refined this disease susceptibility association to 5q11.2 but also noted a protective association with genes on chromosome 5p15.2 (98). In a separate and earlier genome-wide linkage study, an analysis of 63 German sarcoidosis families suggested linkage to chromosome 6p21 (and five other potential associations) (99). Following this observation, Valentonyte and colleagues (100) performed fine mapping of the 6p21 region and observed an association between sarcoidosis and a polymorphism in the butyrophilin-like2 gene (*BTNL2*). Subsequently, a disease-associated truncating splice site mutation in this gene, r22076530, was described. This association between sarcoidosis and *BTNL2* has since been confirmed in studies of other populations (96,98,101,102). A member of the B7 family (which includes CD80 (B7.1) and CD86 (B7.2)), *BTNL2* is thought to act as a negative costimulatory molecule (101), although its exact function remains largely unknown (91).

### 61.3.3 Hypersensitivity Pneumonitis and Chronic Beryllium Disease

Hypersensitivity pneumonitis encompasses a group of lung diseases characterized by both inflammation and fibrosis that results from a broad range of specific environmental exposures. Both acute and chronic forms exist, with lung fibrosis potentially progressing to end-stage lung disease with prolonged exposure to some of the offending agents. HLA associations have been reported with pigeon breeder's lung, one of the more commonly encountered forms of hypersensitivity pneumonitis, including HLA-DR7 (103), HLA-DRB1\*1305, and HLA-DQB1\*0501 (104). An association with the TNF allele TNF-2(-)(308) has also been described (104).

Exposure to beryllium can lead to prominent ILD. Among those individuals who have the appropriate level of exposure to beryllium, the HLA-DPB1 Glu69 polymorphism has been associated with a 10-fold greater risk of developing lung disease (105).

## 61.4 GENETIC SYNDROMES WITH ILD MANIFESTATIONS

Several Mendelian disorders can have prominent pulmonary involvement including manifestations of ILD. Some of these relevant disorders have been selected for discussion in this section of the chapter. Considering that many of these are covered in other sections of this text, these diseases with their associated ILD manifestations are presented in a brief format.

### 61.4.1 Dyskeratosis Congenita

DKC, mentioned previously in this chapter in the section related to telomerase mutations and FIP, is a rare hereditary disorder initially described based on a triad of mucocutaneous manifestations—oral leukoplakia, nail dystrophy, and abnormal pigmentation of the skin. However, it is actually a multisystem disorder with the potential to have manifestations in tissue sites that require ongoing cellular turnover. Among the classic mucocutaneous features, 78% of DKC patients have oral leukoplakia, 88% have nail dystrophy, and 89% have abnormal skin pigmentation, based on DKC registry estimates. Bone marrow failure occurs in approximately 86% of DKC patients and is the leading cause of death. Pulmonary fibrosis occurs in approximately 20% of DKC patients and is the second leading cause of death. In individuals with pulmonary fibrosis, clinical presentations are similar to those of individuals with IIP, and when lung biopsies are performed, the histologic pattern of UIP is commonly observed. Additional possible DKC manifestations include short stature, learning difficulties, dental disease, liver disease, epiphora, esophageal strictures, and increased malignancy risk (38).

On evaluation of the DKC registry in 2006, over half of the individuals had not yet had a disease causative genetic mutation described (38). However, mutations in genes that encode for proteins in the telomerase complex have been linked to DKC. The X-linked form of the disease was mapped to chromosome Xq28 (40,106), with the subsequent identification of mutations in *DKC1*, the gene encoding for the protein dyskerin (41). Dyskerin is expressed in all tissues, is known to be involved in ribosome production, and serves an important role in the telomerase complex. Ultimately, genetic mutations in *DKC1* are found in approximately 33% of DKC registry cases (38). DKC also can present in autosomal dominant forms, with some cases linked to heterozygous mutations in telomerase reverse transcriptase (*TERT*) (44) and the telomerase RNA component (*TERC*) (42,43). In addition, heterozygous mutations in *TINF2*, a gene that encodes one of the proteins in the shelterin complex that protects telomeres, have been reported (107,108). Patients with autosomal recessive DKC have mutations in *TERT* (109) as well as two other genes encoding proteins in the telomerase complex—*NOP10* (110) and *NHP2* (111). Telomere shortening occurs with mutations in *DKC*, *TERT*, *TERC*, *TINF2*, *NOP10*, and *NHP2*. Multiple lines of evidence, including mouse model work, suggest that DKC manifestations are related to the progressive telomere shortening that occurs because of decreased telomerase activity associated with the mutations (112–116). In contrast, a recent report linked cases of DKC to autosomal recessive mutations in the gene *C16orf57*, but telomere lengths in affected subjects were normal (117). Pulmonary fibrosis has been noted in DKC cases caused by *DKC*, *TERT*, and *TERC* mutations, but to



date information is limited on the prominence of pulmonary fibrosis in DKC cases caused by *TINF2*, *NOP10*, *NHP2*, or *C16orf57* mutations.

### 61.4.2 Hermansky-Pudlak Syndrome

Hermansky-Pudlak syndrome (HPS) is a group of heterogeneous autosomal recessive disorders. Eight different subtypes have been described, each with a different responsible gene. For each human form of *HPS*, a similar murine gene defect has been described; however, seven additional *HPS* genes in mice occur (118). All eight human subtypes have the clinical findings of oculocutaneous albinism and prolonged bleeding from defective platelet function. HPS is classically diagnosed when an individual with albinism and easy bruising is evaluated and an absence of platelet dense granules is noted on electron microscopy. Other manifestations of disease can be present and depend on the given subtype. Multiple lines of investigation suggest that *HPS* genetic mutations result in aberrancies in protein trafficking with dysfunction of lysosome-related organelles (118–120). *HPS* genes appear to be expressed ubiquitously, but gene defects appear to affect specific cell populations, including melanocytes and pigment epithelial cells, neutrophils, and platelets. In the case of those subtypes that result in ILD, the cell of interest appears to be the type II AEC.

For most of the *HPS* genes, the function of the encoded protein remains unknown, although most have regions that predict association with other proteins (121,122). HPS is the most commonly encountered genetic disease in Puerto Rico, with 1/1800 individuals in northwest Puerto Rico affected (123). HPS-1 is the most common subtype, accounting for approximately 50% of HPS cases outside the Puerto Rican population. Over 20 disease-causing mutations have been reported in *HPS1* (118), located on chromosome 10q23.1-23.3 (124). HPS-2 is caused by mutations in *AP3B* (125). *HPS3* is on chromosome 3q24 (126), *HPS4* on chromosome 22q11.2-q12.2 (127), *HPS5* on chromosome 11p14 (128), *HPS6* on chromosome 10q24.32 (128), *DTNBP1*, the gene for HPS-7 on chromosome 6p22.3 (129), and *BLOC1S3*, the gene for HPS-8, on chromosome 19q13 (130).

Among the eight different subtypes, pulmonary fibrosis occurs in HPS-1 and HPS-4. The proteins encoded by *HPS1* and *HPS4* function together in the BLOC-3 protein complex (131,132). In the lung of individuals with HPS-1 or HPS-4, the lamellar body, a lysosomal-related organelle, of type II AECs appears to be affected with subsequent aberrancies in trafficking of an as yet unidentified protein (118). In most individuals with HPS-1 and HPS-4 who survive to adulthood, ILD can be found, with many patients having severe pulmonary fibrosis that progresses to respiratory failure (133,134). Individuals who present with lung fibrosis have an average age of onset of symptoms of 35 and an average age of death related to respiratory failure of 37. Clinical

presentations for HPS-1- and HPS-4-related pulmonary fibrosis mirror that seen with the IIPs. In those individuals who have a lung biopsy performed, a pathologic pattern similar to UIP is often observed. Interestingly, hyperplastic AECs filled with phospholipid-rich droplets and enlarged lamellar bodies are frequently prominent on these biopsies, an observation that points to possible defects in the secretory pathway (135). Compared to HPS-1, HPS-4 is much less frequently encountered, but lung manifestations occur in the same pattern as noted in HPS-1 (127,136), a finding likely related to the fact that they are involved in the same BLOC-3 protein complex.

### 61.4.3 Lymphangioleiomyomatosis and Tuberous Sclerosis Complex

Lymphangioleiomyomatosis (LAM) is characterized by diffuse infiltration of benign smooth muscle-like cells in the lung parenchyma with resultant progressive cyst formation (137). In pulmonary clinics, the most commonly encountered form falls into the category of “sporadic”-LAM, or S-LAM. Patients typically present with chronic dyspnea on exertion and can have chest X-rays that suggest an interstitial pattern, but computed tomography reveals the burden of lung cysts. Patients can present with patterns of obstruction and/or restriction on PFT. Spontaneous pneumothoraces are also common. The sporadic form of LAM occurs exclusively in women. Pulmonary manifestations of LAM are also encountered in the genetic disease tuberous sclerosis complex (TSC), and in reality, TSC with LAM (TSC-LAM) is more common, although many of these TSC individuals never present with respiratory symptoms.

TSC is an autosomal dominant tumor suppressor syndrome, with individuals carrying a heterozygous mutation in one copy of an allele and disease manifestations occurring when the second allele is lost through somatic mutation (138). TSC is caused by mutations in one of two different genes that encode for proteins that function together. *TSC1* is on chromosome 9q34, encoding for hamartin, and *TSC2* is on chromosome 16p13.3, encoding for tuberin (139–141). Hamartin and tuberin interact in the cell and are involved in regulating cellular growth and proliferation (142). Over 600 mutations have been reported in these two genes (143–145). TSC is characterized by hamartomas and dysplasias in multiple organs, including abdominal angiomyolipomas (AMLs) of the kidney, liver, or spleen, retinal hamartomas, and skin angiofibromas. In addition, the central nervous system (CNS) is frequently affected by subependymal nodules, giant cell astrocytomas, and cortical tubers (138). LAM can frequently occur, but the degree of respiratory compromise is quite variable among patients. In addition, multifocal micronodular pneumocyte hyperplasia (MMPH), a nodular proliferation of type II AECs, can be found in some TSC cases (146). With S-LAM, affected individuals not only predominantly present with severe



respiratory symptoms but may also have AMLs of the kidneys, lymphangiomyomas, and axial thoracic and abdominal adenopathy. However, the other manifestations of TSC, such as CNS involvement, are not found.

Similarities between S-LAM and TSC-LAM suggested that aberrancies in *TSC1* or *TSC2* might underlie the sporadic form as well. In 1998, two groups of investigators analyzed tissue from AMLs and lymph nodes from S-LAM patients and found loss of heterozygosity for *TSC2* with no *TSC2* mutations noted in peripheral blood leukocytes (147,148). Following this breakthrough, Carsillo and colleagues (149) analyzed S-LAM lesions from lung and kidney and found *TSC2* missense and truncating mutations in the affected tissues, while normal tissue surrounding the lesions as well as peripheral blood leukocytes had no *TSC2* mutations. These results suggested that diseased tissues were affected by the occurrence of two somatic *TSC2* mutations, and in fact it is now thought that most cases of S-LAM are caused in this manner.

#### 61.4.4 Pulmonary Langerhan Cell Histiocytosis

Pulmonary Langerhan cell histiocytosis (PLCH) is a lung disorder, most frequently encountered in smokers, in which cells of dendritic lineage (referred to as Langerhan cells) proliferate in the lung parenchyma leading to prominent pulmonary manifestations (150,151). Most individuals present with complaints of dyspnea on exertion and cough, and as with LAM, spontaneous pneumothorax is a common occurrence. Radiographic findings depend on the extent of disease, with initial chest radiographics and computed tomography potentially revealing a micronodular or reticular appearance. However, with disease progression, cystic lung disease becomes prominent and can lead to severe end-stage lung destruction. Multiple lines of evidence point to PLCH being caused by cigarette smoke, and in fact, some patients will dramatically improve with smoking cessation. Familial cases have been described, but no clear genetic association is known. One study suggested an association with the TNF $\alpha$ -308 promoter allele (152), but such an association was not observed in a subsequent study (153). However, this second study did suggest an increased frequency of HLA Cw7 and DR4 in PLCH (153).

#### 61.4.5 Pulmonary Alveolar Proteinosis

Pulmonary alveolar proteinosis (PAP) is a rare lung disease in which surfactant-rich lipoproteinaceous material accumulates within the alveolar space (154). The acquired or idiopathic form of PAP is the most common, but secondary and congenital forms occur as well. Patients with idiopathic PAP typically have clinical symptoms of progressive dyspnea and cough with a chest radiograph

that suggests an alveolar filling process. Computed tomography reveals a pattern of ground glass opacification with intralobular and interlobular septal thickening. Bronchoalveolar lavage yields a milky opaque fluid rich in surfactant, while lung biopsy reveals eosinophilic material in the airspaces, but with relatively preserved alveolar architecture. For many years, whole-lung lavage remained the main therapeutic approach (154). Murine models provided significant insight into the pathogenesis of PAP, when it was noted that mice deficient in granulocyte-macrophage colony-stimulating factor (GM-CSF) developed pulmonary disease with the same histologic features noted in PAP (155,156). Similar results were observed in mice deficient in the  $\beta$ c chain of the GM-CSF receptor (157,158). Evaluations of these models delineated that macrophage function was impaired leading to the accumulation of foamy macrophages in the airspaces that were filled with surfactant-rich material, an observation identical to that seen in PAP. Individuals with idiopathic PAP revealed have antibodies against GM-CSF in the serum and bronchoalveolar lavage (159,160), leading to aberrant alveolar macrophage function and accumulation of the surfactant-rich material. Subsequently, multiple studies have suggested that exogenously administered GM-CSF has a therapeutic benefit in some subjects with idiopathic PAP (161–163).

Secondary forms of PAP are caused by impaired function or decreased numbers in the macrophage population, such as can be seen with immunosuppression, some types of hematologic malignancies, some infections, and inhalation of toxic dusts or fumes (154). Congenital forms of PAP are caused by mutations in *SFTPC*, *SFTPB*, and the  $\beta$ c chain of the GM-CSF receptor (8,21,164–167). Surfactant protein B (SPB) deficiency leads to respiratory distress on the first day of life, with death typically in the first six months, with the lungs of affected infants having a histopathologic pattern similar to that seen in idiopathic PAP (164). Multiple *SFTPB* mutations have been described (165,168) and deletion of *SFTPB* in a murine model essentially recapitulated the clinical findings. *SFTPC* mutations can not only cause ILD in childhood but have also been associated with pediatric cases of PAP (21). Finally, some cases of pediatric PAP have been linked to mutations in the  $\beta$ c chain of the GM-CSF receptor (166).

#### 61.4.6 Lysinuric Protein Intolerance

Lysinuric protein intolerance (LPI) is an autosomal recessive disorder of cationic amino acid transport in the small intestine and kidney. A rare disorder, it is usually diagnosed at 5–6 years of age (169). LPI is caused by mutation in the heterotrimeric amino acid transporter (HAT)  $\gamma$ -LAT-1 gene *SLC7A* (170,171) located on chromosome 14q11.2 (172). HATs are responsible for amino acid transport, and in LPI, dibasic amino acid transport is defective, predominantly in the small intestine and

kidney epithelium, but also likely in the lung and liver (169). LIP families have been described in Italy, Finland, and Japan. Affected individuals have increased urinary excretion of arginine, ornithine, and lysine, with the low plasma concentration of these dibasic amino acids leading to urea cycle dysfunction and hyperammonemia (169). Mental retardation, delayed physical development, intestinal malabsorption, vomiting, and failure to thrive are the prominent clinical manifestations. However, respiratory disease is frequently reported as well, with progressive alveolar proteinosis or alveolar hemorrhage both described (173,174). In a study from Finland, one-third (8 of 25) of asymptomatic LPI individuals had findings suggestive of lung fibrosis on chest radiography while two-thirds (8 of 14) had ILD on computed tomography of the chest (174). While the mechanism causing surfactant accumulation is not well known, like PAP, whole-lung lavage has been associated some therapeutic benefit (175).

### 61.4.7 Neurofibromatosis

Neurofibromatosis 1 (NF1) and neurofibromatosis 2 (NF2) are autosomal dominant tumor suppressor syndromes with genetic and clinical features that are distinct from each other (176). While both forms of NF can be inherited, sporadic mutations account for approximately half the cases. NF1 has a prevalence of about 1/5000, while NF2 is much rarer, with a prevalence of ~1/200,000. Patients with NF1 typically present with cutaneous neurofibromas, Lisch nodules of the iris, café-au-lait macules, and skin freckling in intertriginous areas of the axilla or groin. Mental retardation, scoliosis, pseudoarthrosis of the tibia, hypoglycemia, hypertension, optic pathway glial tumors, pheochromocytomas, and leukemias have also been described. The skin manifestations seen in NF1 are typically absent in NF2 (176). NF1, located on chromosome 17q11.2, encodes neurofibromin, a protein involved in controlling cellular proliferation (177–179). NF2, located on chromosome 22q12.1, encodes merlin, a cytoskeletal protein (180–182). Pulmonary manifestations can occur in NF1, but are not typically seen in NF2. The degree to which pulmonary involvement occurs is not well known, with different reports describing a wide range; however, the most recent analysis reported that only 1.9% (3 of 156) individuals with NF had ILD (183). Thoracic manifestations are not limited to ILD, as individuals can also present with bronchiectasis, pulmonary hypertension, emphysema, intrapulmonary neurofibromas, intrathoracic schwannomas, AEC hyperplasia, and phrenic nerve involvement with diaphragmatic paralysis (184,185). Severe restriction on pulmonary function can occur because of scoliosis or skeletal disease affecting the chest wall. Lung biopsies from individuals with NF associated ILD reveal a peripheral and subpleural pattern similar to that observed in UIP, but

accumulation of alveolar macrophages sometimes suggests a pathologic pattern of DIP (186).

### 61.4.8 Familial Hypocalciuric Hypercalcemia

Familial hypocalciuric hypercalcemia (FHH), a disorder characterized by elevated serum calcium and magnesium and low urinary calcium secretion is caused by inactivating mutations in *CaR*, a calcium-sensing receptor gene located on chromosome 3 (187,188). *CaR* encodes a seven-transmembrane G protein-coupled receptor that functions to set the extracellular level of calcium (189). Heterozygous inactivating mutations in *CaR* decrease the sensitivity of the receptor to calcium, requiring a higher serum calcium level for reduction of parathyroid hormone release from the parathyroid glands (190) and increased reabsorption of calcium and magnesium in the nephron (191,192). When homozygous, such mutations cause lethal neonatal hyperparathyroidism (LNH) (187). In contrast, activating mutations in *CaR* also occur, resulting in autosomal dominant hypocalcemic hypercalciuria (193,194). Further, some cases of apparent idiopathic hypoparathyroidism have been explained by activating *CaR* mutations (194). A second FHH locus known as HHC3 has been mapped to 19q13 (195).

Pulmonary fibrosis was first described in association with FHH by Auwerx and colleagues (196). Patients present with pulmonary symptoms similar to those seen with the IIPs, most prominently progressive dyspnea. Recurrent lung infections are sometimes encountered, thought to be due to granulocyte dysfunction and low myeloperoxidase levels (197,198). Chest radiographic imaging may reveal reticulonodular infiltrates, while PFT reveals restriction with a reduction in diffusion capacity. Most affected individuals present in the fourth decade, and some individuals can progress to severe end-stage pulmonary fibrosis. Evaluation of bronchoalveolar lavage fluid reveals elevated cell counts including neutrophils, macrophages, and multinucleated giant cells. Lung biopsies are characterized by interstitial inflammation with conchoid-body aggregates, multinucleated giant cells, and loosely formed granulomas (196,197). The role that the *CaR* encoded calcium sensing receptor plays in the pathogenesis of ILD in this disorder remains unclear, but it is thought that it may impact the effect of some key profibrotic mediators (199).

### 61.4.9 Pulmonary Alveolar Microlithiasis

Pulmonary alveolar microlithiasis (PAM) is a rare diffuse lung disease with a likely autosomal recessive mode of inheritance, which is characterized by calcium phosphate microlith accumulation within the alveolar spaces. Over 500 individuals with PAM have been reported across the globe, with patients ranging from

infancy to 80 years of age (200). Over 100 cases have been described from Japan alone (201). Approximately 50% of the cases appear to be inherited, with consanguinity playing a prominent role (202). Using a genome wide SNP analysis, investigators evaluated Japanese PAM patients and identified homozygous mutations in the gene *SLC34A2* (201), which encodes for a type IIb sodium phosphate cotransporter. In vitro studies revealed that mutant *SLC34A2* protein led to loss of phosphate transport function. Additional studies have revealed that *SLC34A2* is highly expressed in type II AECs in the lung (203,204).

Among patients with PAM, the most common presenting symptom is dyspnea on exertion, with non-productive cough often presenting as well. On rare occasions, hemoptysis may be encountered. With PAM, innumerable alveolar calcifications develop, leading to a sandlike micronodular appearance on chest radiograph (205). A calcified reticular pattern with interlobular septal thickening in a predominantly peripheral and basal distribution is observed on computed tomography of the chest (206). Radiographic findings do not necessarily correlate well with clinical symptoms, as striking radiographic images have been noted in asymptomatic individuals. However, in the later stages of the disease, patients can present with severe pulmonary fibrosis and associated pulmonary hypertension and respiratory failure. Calcospherites may be observed on bronchoalveolar lavage, and lung biopsies reveal prominent calcified foci (200). At the present time, no effective treatments are available for PAM except for lung transplantation (207), but it is hoped that, based on the genetic link mentioned, agents targeting phosphate metabolism might one day be therapeutic.

#### 61.4.10 Lipoid Proteinosis

Lipoid proteinosis, also known hyalinosis cutis et mucosae (Urbach–Wiethe disease), is characterized by the widespread deposition of a glycoprotein material in multiple tissues including the lung (208). A rare autosomal recessive disorder, over 250 cases have been described worldwide with many cases involving consanguinity (209). In a study by Hamada and colleagues (208) in 2002, evaluation of three affected siblings from a family in Saudi Arabia linked the disease to chromosome 1q21. Further evaluation of five more families with 28 affected individuals narrowed the region of interest to 6.5 Mb, from which a candidate gene approach yielded six different homozygous mutations in the gene encoding extracellular matrix protein 1 (*ECM1*). *ECM1* is a glycoprotein implicated in angiogenesis, keratinocyte differentiation, and endobronchial bone formation (210–212).

Since multiple tissues may be affected, clinical manifestations may also be diverse, including papules in the skin and along eyelid margins, thickening of the tongue

and frenulum, skin blistering and/or scarring, alopecia, nail dystrophy, dental abnormalities, hoarseness from laryngeal involvement, epilepsy, and neuropsychiatric abnormalities (213–215). Pulmonary manifestations can occur, but are rare. Chest radiograph may reveal reticulonodular changes suggesting diffuse interstitial fibrosis (214). In affected lungs, histologic evaluation reveals expansion of amorphous eosinophilic material in the alveolar septa (216).

#### 61.4.11 Gaucher Disease

The most prevalent of the lysosomal storage diseases, Gaucher disease, is caused by defective hydrolysis of glucosylceramides (217). The disease is autosomal recessive, with most cases caused by mutations in the gene encoding for  $\beta$ -glucosidase, the enzyme responsible for catalyzing glucosylceramide (218). Some cases are caused by mutations in the genes encoding for glucocerebrosidase or lysosomal hydrolase. With the resultant decreased enzymatic activity, inclusions of glucocerebroside accumulate in macrophages in the reticuloendothelial system. Infiltration of these Gaucher cells in multiple tissues leads to neurologic impairment in the most severe (infantile-onset) forms, skeletal disease, hepatosplenomegaly, anemia, and thrombocytopenia. Lung disease may also manifest with presentations including pulmonary hypertension and/or ILD (218–220). In fact, a prospective study of Gaucher patients indicated that PFT abnormalities were quite common, with evidence of both obstruction and restriction in the cohort. Diffusion capacity abnormalities were common as well (221). Reticulonodular or miliary patterns may be noted on chest radiograph with HRCT of the chest also revealing bilateral interstitial changes, sometimes with areas of ground glass attenuation. Gaucher cells may be observed in bronchoalveolar lavage fluid (222). With lung biopsies, two histopathologic lung patterns have been described: (1) isolated alveolar space accumulation of Gaucher cells and (2) infiltration of Gaucher cells into the alveolar, interstitial, and pleural components (223). Exogenous  $\beta$ -glucosidase therapy can be employed to treat Gaucher disease, with some reports suggesting that pulmonary abnormalities slowing improving with this enzyme replacement therapy (218,224).

#### 61.4.12 Niemann-Pick Disease

Niemann-Pick disease (NPD) is a lipid storage disease in which sphingomyelin and cholesterol accumulate in the cell (225,226). A rare autosomal recessive disorder, NPD has six variants (A–F). In NPD, lipid-laden Pick cells infiltrate the reticuloendothelial system, leading to hepatosplenomegaly and anemia because of bone marrow involvement. Patients with type A NPD have variable degrees of neurologic involvement, with

some cases of severe neurodegeneration and death in childhood. In contrast, patients with type B NPD survive into adulthood without manifesting neurologic disease. Biochemical defects vary across the six subtypes and range from deficiency of lysosomal sphingomyelinase to alterations in cholesterol processing in the cell (226). As examples, NPD types A and B result from mutations in the *SMPD1* locus (located on chromosome 11p15.4-15.1), types C1 and D from *NPC1* mutations (located on 18q11-18q12), and type C2 from *NPC2* (*HE1*) mutations (located on 14q24.3). Parenchymal lung tissue infiltration and fibrosis can occur, and is typically more common in the neuronotropic infantile forms than in the adult forms. PFTs may reveal restriction and a decrement in the diffusing capacity, and patients may have oxygen desaturation (227–230). Radiologic imaging reveals predominantly basilar reticulonodular infiltrates on chest radiographs (231) and upper lung zone nodular and ground-glass changes and lower lung zone interlobular septal thickening on HRCT (228). On lung biopsy specimens, NP cells accumulate in the interstitium and alveoli, histiocytes are present, and the alveolar septa are expanded with excess matrix.

### 61.4.13 Fabry Disease

Fabry disease is a lysosomal storage disorder of glycosphingolipid metabolism due to deficiency of  $\alpha$ -galactosidase A ( $\alpha$ -gal A). The  $\alpha$ -galactosidase gene (*GLA*) is located on chromosome Xq22, with multiple mutations reported that result in loss of function of the encoded enzyme (232,233). As might be expected, disease manifestations are more severe and occur earlier in men, but women can be affected by the disease as well. With deficiency of  $\alpha$ -galactosidase A, catabolism of glycolipids is impaired, leading to their accumulation in the vascular endothelium as well as visceral tissues. The disease commonly affects the skin, with the appearance of angiokeratoma, a characteristic early sign. Involvement of the renal and cardiovascular systems is common, with renal failure, myocardial infarction, heart failure, and stroke the major causes of death. The lungs can be affected as well by glycolipid accumulation, with pulmonary manifestations including dyspnea, oxygen desaturation, airway obstruction, wheezing, hemoptysis, pneumothorax, frequent respiratory infections, pulmonary vascular infiltration, and interstitial infiltrates. Enzyme replacement therapy with human  $\alpha$ -gal A is now used to treat Fabry disease; this treatment reduces painful crises, improves quality of life (234,235), reduces cardiac hypertrophy, stabilizes renal function (235), and improves pulmonary symptoms and oxygenation (236).

### 61.4.14 GM<sub>1</sub> Gangliosidosis

GM<sub>1</sub> gangliosidosis is a rare storage disorder resulting from  $\beta$ -galactosidase deficiency. Also known as

Morquio syndrome type B or mucopolysaccharidosis type IVB, the disease is secondary to mutations in the gene for  $\beta$ -galactosidase on chromosome 13p21.33 (237). Affected individuals have severe cerebral degeneration that usually leads to death before a year of age. GM<sub>1</sub> ganglioside accumulates in neurons, reticuloendothelial cells, and renal epithelial cells, and causes hepatomegaly and skeletal deformities. Pulmonary involvement with respiratory insufficiency has been reported in some individuals. Miliary and reticulonodular changes are observed on chest radiography while foamy macrophage infiltration of the alveoli is seen on lung histology (238,239).

### 61.4.15 Marfan Syndrome

Marfan syndrome is a hereditary disorder with prominent skeletal, cardiovascular, or ocular manifestations and is caused by mutations in the gene for fibrillin-1 (*FBN-1*) on chromosome 15q21.1 (240–242). Fibrillin is a large glycoprotein that not only is involved in the structural framework of the connective tissue, facilitating elasticity, but also regulates the activity of TGF- $\beta$ . Prominent skeletal manifestations include tall stature, long limbs, anterior chest deformity, scoliosis, high arched palate, and joint laxity. Subluxation of the lens is the most clinically important ocular finding. Cardiovascular complications can be severe and include aortic aneurysm, annuloaortic ectasia, and mitral and aortic valvular regurgitation, with acute aortic dissection the leading cause of early death. The most common pulmonary presentation is spontaneous pneumothorax. While interstitial fibrosis in the apices has been described, it is rare. In contrast, restriction on the pulmonary system caused by thoracic skeletal abnormalities such as pectus deformities and scoliosis are commonly encountered (243,244).

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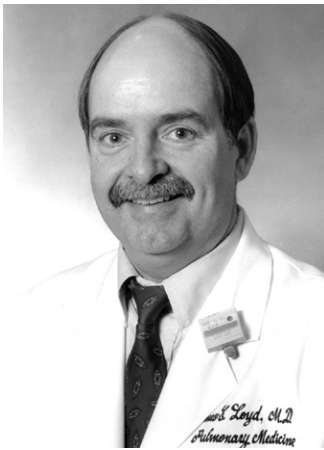
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# CHAPTER

# 62

## Congenital Anomalies of the Kidney and Urinary Tract

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### GLOSSARY

- Amnion nodosum** – a nodular condition of the fetal surface of the amnion, observed in oligohydramnios associated with absence of the kidneys of the fetus.
- Cloaca** – the structure at the far end of the hindgut that divides to form a rectum, a bladder, and genitalia.
- Gubernaculum** – the fetal ligament attached at one end to the lower end of the epididymis and testis and at the other end to the bottom of the scrotum; it is present during the descent of the testis into the scrotum and then atrophies.
- Mesonephric (Wolffian) duct** – an embryonic duct of the mesonephros, which in the male develops into the epididymis, ductus deferens and its ampulla, seminal vesicles, and ejaculatory duct and in the female is largely obliterated.
- Nephrogenic blastema (or metanephric mesenchyme)** – developmental precursor that gives rise to the nephrons in the kidney.
- Paramesonephric duct** – either of the paired embryonic ducts developing into the uterine tubes, uterus, and vagina in the female and becoming largely obliterated in the male.
- Pelvic ectasia** – dilation of the renal pelvis and calyces without any evidence of obstruction.
- Renal dysplasia** – kidney that contains poorly branched and abnormally differentiated nephrons and collecting ducts, increased stroma and occasionally, cysts and metaplastic tissues such as cartilage.
- Renal hypoplasia** – abnormally small kidney (two standard deviations smaller than the mean size for age) that contains intact nephrons that are reduced in number.
- Urachus** – a canal connecting the bladder of the fetus with the allantois, a structure that contributes to the formation of the umbilical cord. The lumen of the urachus is

normally obliterated during development, transforming the urachus into a solid cord, a functionless remnant. If still patent at birth, urine will leak from the umbilicus.

**Ureteric bud** – developmental precursor that branches in a highly reproducible manner; at each ureteric bud tip nephrons are induced. These branches will form the collecting system, including collecting ducts, renal pelvis, ureter, and bladder trigone.

**Verumontanum** – an elevation, or crest, in the wall of the urethra where the seminal ducts enter it.

### 62.1 INTRODUCTION

Congenital anomalies of the kidney and urinary tract (CAKUT) include anomalies of the kidneys, ureters, bladder, and urethra. CAKUT occurs in approximately 1 in 500 fetal ultrasound examinations (1) and represents 35–45% of all congenital abnormalities. Anomalies specific to the kidney are found in 3–6 per 1000 births. In necropsy studies of infants and children up to age 12 years, congenital anomalies of the kidney and urinary tract are present in 2–6.3% (2,3). As expected by the high incidence rate, many cases of CAKUT remain asymptomatic (3).

CAKUT occurs frequently with other structural anomalies (3) and associated syndromes. Accepted classifications of renal anomalies have avoided classifying multiple malformation disorders, placing them in one category: genetic causes (4). This chapter is divided into three main categories: (i) errors of organogenesis, (ii) errors of migration and position, and (iii) obstruction. Associated anomalies are common in disorders with errors of organogenesis. More discrete anomalies result

from disorders of histogenesis. Contiguous anomalies in the caudal part of the embryo provide evidence for developmental fields. Noncontiguous anomalies most likely result from expression of genes important for development expressed at different times during embryogenesis.

## 62.2 CLINICAL FEATURES

Structural anomalies of most organ systems have an increased risk for associated CAKUT (Table 62-1). CAKUT associated with other major anomalies, increased minor anomalies, or recurrence within a family is probably caused by an underlying syndrome or a monogenic or chromosomal disorder. In evaluating a family for inheritance of CAKUT, it is important to remember that many CAKUT cases are asymptomatic and that abdominal examination fails to detect most malformations. However, there is improved detection of clinically

significant CAKUT by routine ultrasonographic screening. A trial of early screening by renal ultrasonography in 5700 healthy 1-month-old infants in Japan was effective in detecting CAKUT in 3.5%, and surgical intervention occurred as a result of screening in 0.1% (5). In screening 6088 full-term newborns after 72 h of life with abdominal ultrasound, Hálek et al. discovered renal abnormalities in 250, some of whom required surgery (6). Because universal newborn screening for CAKUT in infants is not currently in practice, search for an underlying disease is recommended in individuals with structural anomalies that place them at increased risk for CAKUT (Table 62-1) or with the following clinical signs and symptoms:

Abnormal kidneys on prenatal ultrasound  
Anuria-oliguria  
Poor urinary stream  
Edema

**TABLE 62-1 Frequency of Urinary Tract Anomalies in Cases with Other Structural Defects**

Structural Anomaly/ Pattern	Percentage with Urinary Tract Anomalies	Most Common Urinary Tract Defects (in Relative Order of Frequency)
Absent gall bladder	32	Cystic dysplasia, renal agenesis, horseshoe kidneys
Agenesis of the corpus callosum	45–55	Reflux, ureterocele, unilateral renal agenesis, crossed fused renal ectopia, bladder diverticulae
Anencephaly	5–16	Hydronephrosis, horseshoe kidneys, polycystic kidneys, renal agenesis, renal hypoplasia, urethral atresia
Anorectal malformation	26–58 <sup>a</sup>	Hydronephrosis, unilateral renal agenesis, cystic dysplasia, reflux, cystic dysplasia, renal ectopia, cloacal exstrophy
Biliary atresia	3	Double ureter, hydronephrosis, renal cysts
Caudal dysplasia	40	Renal agenesis, hypoplasia, cystic dysplasia; horseshoe kidneys, crossed renal ectopia, urachal anomalies
CHARGE	42	Unilateral renal agenesis, hydronephrosis, renal hypoplasia
Diaphragmatic hernia	15–18	Renal agenesis, cystic dysplasia, hydronephrosis, ureteropelvic obstruction
Esophageal atresia and tracheoesophageal fistula	33	Unilateral renal agenesis, horseshoe kidneys, reflux
Gastroschisis	15	Unilateral renal agenesis, horseshoe kidneys
Heart defect	5–39 <sup>a</sup>	Duplex collecting system, unilateral renal agenesis, renal ectopia
Lateral body wall defect	50–65	Renal agenesis, urethral atresia, hydronephrosis
Limb reduction defects	9	Renal agenesis, hydronephrosis, cystic dysplasia, horseshoe kidney
MURCS association	28–80	Renal agenesis, renal ectopia
Myelomeningocele	9	Renal agenesis, horseshoe kidney
Omphalocele	11–47	Cloacal exstrophy, horseshoe kidneys, patent urachus
Oral clefts	4	Renal agenesis, horseshoe kidney
Penoscrotal transposition	90	Renal agenesis, cystic dysplasia, ectopia, horseshoe kidneys
Persistent cloaca	83	Cloacal and bladder anomalies
Pulmonary hypoplasia	18–21	Cystic dysplasia, renal agenesis, horseshoe kidney, polycystic kidney, urethral atresia
Single umbilical artery (isolated)	26	Dilated renal pelvis, duplicated renal pelvis, reflux, hydronephrosis, horseshoe kidneys, unilateral renal agenesis
Sirenomelia	100	Renal agenesis, cystic dysplasia, urethral atresia
Supernumerary nipples	4	No specific pattern
Tracheal agenesis	38	Renal agenesis, cystic dysplasia, horseshoe kidney
VACTERL association	82–87	Reflux, unilateral renal agenesis, ureteropelvic junction obstruction, crossed fused ectopia
Vertebral defects	27–46	Unilateral renal agenesis, duplicated ureter, renal ectopia, horseshoe kidney

<sup>a</sup>Higher numbers seen in autopsy series and/or those with  $\geq 3$  congenital anomalies.

Source: From Stevenson, R. E. Human Malformations and Related Anomalies, 2nd ed.; Oxford University Press: Oxford, New York, 2006; p 1167.

Persistent wetness  
 Polydipsia, polyuria  
 Recurrent urinary tract infections  
 Hypertension  
 Ambiguous genitalia, abnormal external genitalia  
 Abdominal mass  
 Oligohydramnios  
 High imperforate anus  
 Exstrophy of the bladder  
 Prune-belly abdomen  
 Aniridia, hemihypertrophy  
 Preauricular pits or ear tags accompanied by any of the following: dysmorphic features; facial asymmetry; coloboma; choanal atresia; jaw hypoplasia; branchial cysts or sinuses; cardiac murmurs; distal limb anomalies; imperforate or anteriorly placed anus; family history of deafness; or a maternal history of gestational diabetes (7).

## 62.3 GENETICS

Over 250 syndromes and monogenic disorders have been reported to have an increased risk for CAKUT (4,8–11). Many isolated structural anomalies of the urinary tract have multifactorial inheritance and an empiric recurrence risk that is greater than the background population risk. The type of anomaly is frequently helpful in revealing the nature of the underlying disorder and the presence of other medically significant structural anomalies. Disorders associated with specific renal anomalies are summarized in the tables of each section. CAKUT associated with chromosome abnormalities and teratogen exposures are summarized in Tables 62-2 and 62-3.

## 62.4 ERRORS OF ORGANOGENESIS

### 62.4.1 Renal Agenesis

Unilateral or bilateral renal agenesis refers to the complete absence of one or both kidneys. The presence of residual renal tissue with or without the ipsilateral ureter, by definition, excludes a diagnosis of renal agenesis. Estimates of the prevalence of unilateral renal agenesis from autopsy studies range from 1:52 to 1:1286; one study showed the incidence in newborns to be 0.15 per 1000 (12). Newer studies note an incidence of unilateral renal agenesis as 1 in 5000 newborns (13,14). The incidence of renal aplasia, defined as the presence of renal parenchyma without any function is 1 in 1300 (15). Interestingly, Hiraoka et al. (15) proposed that a significant proportion of renal agenesis diagnosed clinically thus far might more correctly be renal aplasia. In their study, ultrasounds in the neonatal period could identify the aplastic kidney, which had a reniform shape, not rudimentary, during the newborn period, and regressed rapidly thereafter.

The incidence of bilateral renal agenesis is variable, depending on the method of ascertainment and the age of the cohort. An incidence of 0.12 per 1000 newborns was reported in a population study in British Columbia (12). The sex ratio shows a male predominance of 2.45:1. In 12,000 autopsies of fetuses and infants less than 1 year old, 1 of 240 had bilateral renal agenesis (16,17), compared with 1 of 2653 and 1 of 2812 of autopsies of children under age 15 years and of all ages, respectively (18). More recent studies note an incidence of bilateral renal agenesis as 1 in 30,000 newborns (13,14).

**62.4.1.1 Clinical Features.** Bilateral renal agenesis presents in utero with severe oligohydramnios as early as 14 weeks gestation. At birth, bilateral renal agenesis presents with severe respiratory distress due to pulmonary hypoplasia secondary to inadequate renal function and inadequate amniotic fluid during pregnancy, nonpalpable kidneys, and morphologic features of Potter sequence (16). Unilateral renal agenesis may be detected on ultrasonographic screening, or secondary to investigation for symptoms of genitourinary tract disease. The majority of individuals with unilateral renal agenesis are asymptomatic and identified because of other medical problems or at autopsy.

Associated congenital anomalies appear frequently, most commonly involving (i) contiguous structures of the renal developmental field, (ii) noncontiguous structures, and (iii) deformations resulting from oligohydramnios. Anomalies of the contiguous structures in bilateral renal agenesis include complete and bilateral absence of the ureters and hypoplasia of the bladder. In 85% of females, the vagina and the uterus (paramesonephric duct derivatives) are absent or abnormal. In males, the testes are usually undescended, and the ductus deferens and the seminal vesicles (mesonephric duct derivatives) are abnormal or absent (17). With unilateral renal agenesis, the ipsilateral ureter and fallopian tube may be absent, and various uterine and vaginal abnormalities may occur. In 60% of patients, other anomalies of the contiguous developmental fields, including anal atresia, imperforate anus, malrotation, and Meckel diverticulum are present, and 33–99% have sacral and coccyx anomalies, sirenomelia, and talipes equinovarus (4).

Frequently, anomalies of noncontiguous structures are found (17), especially limb malformations, cardiac anomalies (19), tracheal agenesis (20), esophageal or duodenal atresia, cleft lip or palate, hydrocephalus (21), and other brain malformations.

Oligohydramnios results in anomalies due to deformation (Potter sequence), including breech presentation (60%), apparent hypertelorism, infraorbital creases, flattened nasal tip, hypoplastic mandible, enlarged and posteriorly rotated ears, redundancy of skin, dry skin, genu varum, talipes, and metatarsus adductus (4,8). Pulmonary hypoplasia, with arrest of alveolar development at 12–16 weeks gestational age, occurs because of either severe oligohydramnios or the underlying disorder.



**TABLE 62-2 Common Chromosomal Disorders Associated with Urinary Tract Anomalies**

Chromosome Disorder	Urinary Tract Anomaly	Frequency
2q terminal deletion	Wilms tumor, horseshoe kidney, dysplastic kidney, renal hypoplasia, ureteral stenosis	11%
4p	Renal agenesis or hypoplasia, vesicoureteral reflux, hydronephrosis	33%
4q partial duplication	Horseshoe kidney, renal hypoplasia, renal duplication, ectasia of distal tubule	Frequent
5p	Horseshoe kidney, renal agenesis, renal duplication, ectasia of distal tubules	Occasional
6q partial duplication	Unilateral renal agenesis, cystic dysplasia	Uncertain <sup>a</sup>
7 trisomy	Cystic dysplasia, enlarged kidneys	Frequent
7q partial deletion (Williams–Beuren)	Renal aplasia/hypoplasia, duplicated kidney, bladder diverticula	18%
8 trisomy, 8 trisomy mosaicism	Cystic dysplasia, enlarged kidneys, small cortical cysts, hydronephrosis, duplication of kidneys/ureters/pelvis	Frequent
9p partial duplication	Horseshoe kidney, hydronephrosis	Frequent
9 trisomy	Hydronephrosis, cystic dysplasia, duplication kidneys and ureters	Frequent
10p partial duplication	Unilateral agenesis, cystic kidney, renal dysplasia	Uncertain
10q partial duplication	Hypoplastic kidney, hydronephrosis	Frequent
11p13 deletion	Wilms tumor	Frequent
13q	Hydronephrosis, vesicoureteral junction obstruction	Uncommon
13 ring	Renal hypoplasia and ectopy, duplication of kidney and ureter, unilateral renal agenesis, polycystic kidney	Frequent
13 trisomy	Hydronephrosis, cystic dysplasia, micropolycystic dysplasia, hydroureter, horseshoe kidney, ureteral duplication, duplication of renal pelvis, small cortical cysts	60–70%
17p partial deletion (Smith–Magenis)	Variable; no characteristic structural abnormality	19%
17p13.3 deletion (Miller–Dieker)	Cystic or pelvic kidney	Uncertain
18q	Horseshoe kidney, unilateral agenesis, hydronephrosis	40%
18 ring	Hydronephrosis, tubular dilation	20%
18 trisomy	Horseshoe kidney, ectopia, ureteral duplication, cortical cysts, exstrophy of the cloaca, hydronephrosis	70%
21q	Unilateral renal agenesis, abnormal kidney shape, dilated calyces	Uncertain <sup>a</sup>
21 trisomy	Renal agenesis, hypoplasia, horseshoe kidney, posterior urethral valves, hydronephrosis, ureteropelvic junction obstruction, vesicoureteral reflux, fetal hydronephrosis	3–7%
22q11 deletion	Renal agenesis, dysplasia, multicystic kidneys	36%
22 partial trisomy	Renal agenesis, horseshoe kidney, hydronephrosis	Frequent
22 partial tetrasomy (cat eye)	Unilateral or bilateral renal agenesis, hydronephrosis, supernumerary kidneys	Occasional
45,X (plus other Turner karyotype abnormalities)	Horseshoe kidney, duplication collecting system, abnormal rotation, ureteropelvic junction obstruction, cystic malformation of collecting tubules; cystic, double, and ectopic kidneys, intrarenal vascular changes	60–80%
XXXXY	Hydronephrosis	10%
XXXXX	Renal hypoplasia, dysplasia	Uncertain <sup>a</sup>
XXY (Klinefelter)	Renal cysts, hydronephrosis	Uncertain <sup>a</sup>
Triploidy	Hydronephrosis, renal cysts, polycystic kidneys, cystic dysplasia	Frequent

<sup>a</sup>Reported abnormalities may not be in excess over background risk for all urinary tract anomalies.

Single umbilical artery was present in 12% of fetuses and stillbirths with bilateral renal agenesis in an autopsy series reported by Potter (16). Amnion nodosum usually accompanies oligohydramnios.

**62.4.1.2 Pathogenesis.** Renal agenesis results from an error in embryogenesis occurring before the end of week four of development (4). Complete absence of the kidney is hypothesized to result from failure of the ureteric bud derived from the wolffian (or mesonephric) duct to make contact with the nephrogenic blastema (or metanephric mesenchyme); this can result from the absence of the wolffian duct, failure of the ureteric bud to form, failure of the bud to reach the blastema, an inadequate inductive influence at the appropriate time in embryogenesis, or absence of the metanephrogenic mesenchyme. There are numerous transcription/growth factors and

intracellular signaling molecules that are involved in the induction networks that signal the kidney and ureteral precursors. Key to the induction of the outgrowth of the ureteric bud from the mesonephric duct appears to be the expression of the signaling molecule glial-derived neurotrophic factor (GDNF) secreted from the metanephric mesenchyme, which then binds to its receptors rearranged during transfection (RET) and GDNF-family receptor  $\alpha 1$  (GFRA1) in the mesonephric duct (22,23). A disruption of this GDNF-RET signaling pathway has been implicated in renal agenesis and hypodysplasia.

GDNF levels and its spatial expression are regulated by multiple transcription factors such as Eyes absent homolog 1 (EYA1) (24,25), the sine oculis homeobox homologs SIX1 and SIX4 (26,27), Sal-like 1 (SALL1) (28), members of the paired-box gene family (PAX2)

**TABLE 62-3 Urinary Tract Anomalies Associated with Teratogen Exposures**

Teratogen	Major Features	Renal Anomaly
Alcohol	Prenatal onset of growth retardation, microcephaly, hypotelorism, short palpebral fissures, smooth philtrum, variable other structural anomalies, including heart and skeletal anomalies	Small rotated kidneys, horseshoe kidney, renal dysplasia, micromulticystic dysplasia, hydronephrosis
Alkylating agents (busulphan, chlorambucil, cyclophosphamide, mechlorethamine)	Growth retardation; cleft palate; microphthalmia; digit anomalies; cardiac defects; anomalies of larynx, trachea, and esophagus	Agenesis of kidneys, hydronephrosis, hydroureter
Angiotensin-converting enzyme (ACE) inhibitors	Growth retardation, hypocalvaria, pulmonary hypoplasia oligohydramnios, patent ductus arteriosus, limb anomalies, fetal hypotension, increased fetal and neonatal mortality	Glomerulopathy, interstitial nephritis, nephrotic syndrome, progressive renal failure, renal artery stenosis, anuria, neonatal acute kidney injury, renal tubular dysgenesis
Cocaine	Structural anomalies resulting from vascular disruption	Renal and ureteral agenesis, hydronephrosis, prune-belly syndrome, hypospadias, ambiguous genitalia
Maternal diabetes	Caudal regression, neural tube defects, congenital heart, and other anomalies	Renal agenesis, ureter anomalies, urethral anomalies, cystic dysplasia
Rubella	Cataracts, microphthalmia, pigmentary retinopathy, growth retardation, heart anomalies (patent ductus arteriosus, peripheral pulmonary stenosis), skeletal anomalies, sensorineural deafness, neurologic impairment, microcephaly	Stenosis of renal artery, polycystic kidney, duplication of the ureters, unilateral renal agenesis
Thalidomide	Limb reduction anomalies, micrognathia, neural tube defects; vertebral, heart, and visceral anomalies	Renal agenesis, obstructive uropathy, abnormal kidney rotation, pelvic kidney, horseshoe kidney
Trimethadione	Distinctive facial features, developmental delay, prenatal and postnatal growth impairment, omphalocele; heart, skeletal, and limb anomalies	Absent kidney and ureter, fetal lobulation of kidneys
Vitamin A congeners	Micrognathia, cleft palate, microphthalmia, midfacial hypoplasia, hearing anomalies, anotia, microtia, neural tube defects	Hypoplastic kidneys, hydronephrosis

(29), members of the HOX11 paralogous family (30), and signaling molecule growth and differentiation factor 11 (GDF11) (31). Mutations in EYA1 (32), SIX1 (33) or SIX5 (34), all regulators of GDNF, result in brachio-oto-renal syndrome, which is characterized by defects in branchial arch, ear and renal development. Heterozygous mutations in SALL1 are implicated in Townes-Brocks syndrome, characterized by external ear anomalies with sensorineural hearing loss, limb anomalies, and renal and anorectal malformations (35). Recent papers have identified heterozygous mutations in *RET* and *UPK3A* genes in individuals with renal agenesis (36–38).

In addition to anomalies due to errors in embryogenesis of the mesonephric and paramesonephric ducts, abnormalities of the hindgut and cloacal membrane are common. A larger field of abnormal embryonic development can affect the entire caudal end of the embryo (e.g. caudal regression sequence and sirenomelia). Other developmental field defects that are frequently associated with renal agenesis are the acrorenal field defect (limb, renal, and other anomalies), the MURCS association (Mullerian duct aplasia, renal aplasia, cervicothoracic somitic (vertebral) defects, hypoplastic uterus, absent vagina, short stature)

(39), the VACTERL association (vertebral defects, anal atresia, cardiac defect, tracheoesophageal fistula, renal anomalies, limb anomalies), the CHARGE syndrome (coloboma, heart defect, choanal atresia, mental retardation, genital hypoplasia, ear anomalies, growth impairment, deafness), and the cerebro-renal-digital field defect (brain malformations and digital, renal, and other anomalies).

**62.4.1.3 Genetics.** There is etiologic heterogeneity for unilateral and bilateral renal agenesis (Tables 62-4–62-6). Reviews of newborns with the Potter sequence have determined that approximately 80% of cases are non-syndromic (40). Of newborns with Potter sequence due to all causes, 21% have bilateral renal agenesis; 48% cystic dysplasia; 25% obstructive uropathy; and 6% other renal pathology.

Hereditary factors are partly responsible for renal agenesis, as evidenced by familial aggregation of disease. For example, using renal ultrasound, Roodhooft et al. (40) found a 9% incidence rate of asymptomatic urogenital malformations and a 4.4% incidence rate of renal malformations in first-degree relatives of patients with congenital absence or severe dysgenesis of both kidneys. Familial recurrence with autosomal dominant and recessive patterns

**TABLE 62-4 Renal Agenesis: Disorders with Multiple Congenital Anomalies**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Acrorenal field defect syndromes	Limb anomalies, variable other anomalies (Table 62-5)	Renal agenesis, dysplasia, hypoplasia or ectopia	Heterogeneous	
Alagille (arteriohepatic dysplasia)	Peripheral pulmonary stenosis, biliary tract anomalies, bile duct atresia, posterior embryotoxon, skeletal anomalies	Renal dysplasia, renal cysts, renal artery stenosis, mesangiolipidosis, azotemia, nephrolithiasis, urinary concentrating defects, small kidneys, single kidney, microcystic tubular dilation with interstitial fibrosis	AD (118450)	<i>JAG1</i> <i>NOTCH2</i>
Branchio-oculo-facial syndrome	Branchial defects, lacrimal duct obstruction, pseudocleft of upper lip	Renal agenesis	AD (113620)	<i>TFAP2A</i>
Branchio-oto-renal	Mixed hearing loss, pinnae anomalies, branchial cleft fistulas, preauricular pits, Mondini cochlear malformations	Renal agenesis, cystic dysplastic kidneys ureteral anomalies, calyceal cyst or diverticulum	AD (113650)	<i>EYA1</i> <i>SIX1</i> <i>SIX5</i>
Campomelic dysplasia	Congenital bowing or angulation of long bones, cleft palate, sex reversal (XY female), respiratory tract and brain anomalies, other skeletal anomalies	Renal agenesis or dysplasia	AD (114290)	<i>SOX9</i>
Caudal dysplasia (caudal regression)	Sacral agenesis/hypoplasia, lower limb anomalies, anal atresia, genital anomalies	Renal agenesis or dysplasia; anomalies of ureters, urethra and bladder	Heterogeneous, maternal diabetes in some patients	
Cerebro-renal-digital syndromes	See Table 62-6	Renal agenesis, dysplasia, or ectopia; ureteral anomalies	Heterogeneous	
CHARGE syndrome	Coloboma, heart defect, choanal atresia, mental retardation, genital hypoplasia, ear anomalies, growth impairment, deafness	Renal agenesis, cystic renal dysplasia, ureteral abnormalities, fused/ectopic kidneys	AD (214800)	<i>CHD7</i>
CHILD syndrome	Congenital hemidysplasia, ichthyosiform erythroderma, and limb defects	Unilateral renal agenesis Hydronephrosis	X-linked (308050)	<i>NSDHL</i>
Chromosomal abnormalities	See Table 62-2			
Cornelia de Lange	Distinctive facies, microcephaly, short stature, micromelia, oligodactyly, mental retardation	Renal agenesis, dysplasia, or hypoplasia	AD (122470)	<i>NIPBL</i>
Diabetes, maternal	Prenatal overgrowth, caudal regression, neural tube defects, congenital heart defects	Renal agenesis, ureteral and urethral anomalies	Metabolic teratogens	
Digeorge syndrome	Sacral meningocele, hydrocephalus, conotruncal heart anomalies	Unilateral renal agenesis, dysplasia, hydronephrosis	AD (188400)	22q11.2 deletion
Dysgnathia complex (otocephaly)	Agnathia, synotia, microstomia, holoprosencephaly, cleft lip/palate	Renal agenesis, fusion or hypoplasia	Heterogeneous	
Early amnion rupture (ADAM, amniotic bands)	Digital and limb amputations, ring constrictions, facial clefts, body wall defects, brain anomalies	Renal agenesis, dysplasia, or ectopia; ureteric and urethral anomalies	Sporadic	
Fanconi pancytopenia	Anemia, pancytopenia, radial aplasia or hypoplasia, microcephaly, short stature, variable microphthalmia, ear anomalies, heart defects, chromosome breakage	Renal agenesis (39%) or dysplasia; duplication of pelvis and/or ureter; ectopic or horseshoe kidney, hydronephrosis	AR (227650)	<i>FANCA</i> (A, C-G, I, J, L-P)
Fraser (cryptophthalmos)	Cryptophthalmia, cleft lip/palate, genital anomalies, atresia of ear canal, anal atresia, syndactyly	Renal agenesis or dysplasia, ureteric anomalies	AR (219000)	<i>FRAS1</i>

**TABLE 62-4 Renal Agenesis: Disorders with Multiple Congenital Anomalies—cont'd**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Kallmann syndrome	Hypogonadotropic hypogonadism, anosmia, cryptorchidism	Unilateral renal agenesis	AD, AR, XLR (147950, 244200, 308700, 308750)	<i>FGFR1</i>
Lenz microphthalmia	Microphthalmia; coloboma; mental retardation; anomalies of the dental, cardiovascular, genital and skeletal systems	Renal agenesis or dysplasia, hydroureter	XLR (309800)	<i>BCOR</i>
LEOPARD syndrome	Short stature, sensorineural hearing loss, hypertelorism, hyposmia, cleft palate, pulmonic stenosis, hypertrophic cardiomyopathy, hypospadias, cryptorchidism, absent ovary	Unilateral renal agenesis	AD (151100)	<i>PTPN11</i> <i>RAF1</i> <i>BRAF</i>
Limb–body wall complex (lateral body wall defect)	Lateral body wall deficiency, limb reduction anomalies, neural tube defects, heart anomalies	Renal agenesis or dysplasia, ureter anomalies, bladder exstrophy, fused/ectopic kidneys	Sporadic	
Mayer–Rokitansky–Hauser syndrome	Absent uterus, cervix, and upper vagina; vertebral defects	Renal agenesis or hypoplasia, double anomalies	AD (277000)	
Microcephaly, congenital heart disease, unilateral renal agenesis, and hypossegmented lungs	Microcephaly, congenital heart disease, hyplobulated lungs, cleft palate, preauricular pits, hydranencephaly	Unilateral renal agenesis	AR (601355)	
MURCS association	Müllerian duct aplasia, renal agenesis, cervicothoracic somitic (vertebral) defects, hypoplastic uterus, absent vagina, short stature	Renal agenesis, dysplasia, or ectopia; ureteral anomalies	Unknown (601076)	
Neu-Laxova, type II (cerebroarthrodigital)	Microcephaly, severe growth retardation, exophthalmos, absent eyelids, micrognathia, lissencephaly, other brain anomalies, joint pterygium	Renal agenesis	AR (256520)	
Neural tube spectrum	Meningomyelocele, anencephaly, encephalocele, vertebral anomalies, midline anomalies	Renal agenesis, hypoplasia, dysplasia, or fusion; ureteral anomalies, hydronephrosis	Heterogeneous, multifactorial	
Papillorenal syndrome	Optic nerve coloboma (“morning glory” anomaly of the optic disc), renal anomalies	Oligomeganephronia, renal hypoplasia, multicystic dysplasia	AD (120330)	<i>PAX2</i>
Partial aniridia with unilateral renal agenesis with psychomotor retardation	Partial aniridia, glaucoma, mental retardation	Unilateral renal agenesis	AR (206750)	
Potter oligohydramnios sequence	Flattened facies, low-set ears, suborbital creases, wrinkled and redundant skin, pulmonary hypoplasia, skeletal deformations	Bilateral renal agenesis, aplasia, hypoplasia, or severe dysplasia	Heterogeneous (i.e. any disorder resulting in severely decreased urine production)	
Renal adysplasia	Anomalies of internal genital system, anus, heart, spine, hands, and feet	Renal agenesis, hypoplasia, or dysplasia; ureteral and urethral anomalies, 50–90% risk for any renal anomaly, 30–40% risk for bilateral renal agenesis	AD (191830)	<i>RET</i> <i>UPK3A</i>
Renal cysts and diabetes syndrome	Pancreas atrophy, diabetes, genital abnormalities	Unilateral kidney agenesis, hypoplasia, cortical atrophy, interstitial fibrosis, renal cysts	AD (137920)	<i>TCF2</i>
Renal–genital–ear	Anomalies of middle ear ossicles, kidneys, and vagina	Renal aplasia or hypoplasia	AR (267400)	

Continued



**TABLE 62-4 Renal Agenesis: Disorders with Multiple Congenital Anomalies—cont'd**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Roberts (SC phocomelia)	Prenatal growth retardation, limb malformations, craniofacial abnormalities, mental retardation	Renal agenesis, dysplasia, horseshoe kidney, polycystic kidney	AR (268300)	<i>ESCO2</i>
Sacral defect with anterior meningocele (sirenomelia)	Fusion of lower limbs, genital and cloacal anomalies, anal atresia, sacral and other vertebral anomalies	Renal agenesis or hypoplasia, dysplasia, ectopia, or fusion; ureteral and urethral anomalies	AD (600145)	<i>VANGL1</i>
Smith–Lemli–Opitz	Microcephaly, postaxial polydactyly, ambiguous genitalia, facial dysmorphism, 2–3 syndactyly, disorder of cholesterol metabolism	Unilateral renal agenesis, cystic dysplasia and fusion, hydronephrosis, ureteropelvic junction obstruction	AR (270400)	<i>DHCR7</i>
Teratogen exposures	See Table 62-3			
Thoracoabdominal syndrome	Diaphragmatic and ventral hernia, hypoplastic lung, cardiac anomalies, cleft lip/palate	Renal agenesis	X-linked (313850)	
Thymic–renal–anal–lung dysplasia	Unilobed or absent thymus, imperforate anus, unilobed lung	Renal agenesis/dysgenesis, ureteral agenesis/dysgenesis	AR (274265)	
Townes–Brocks	Anal atresia, anteriorly placed anus, radial ray duplication or reduction, ear anomalies, sensorineural hearing deficit, spectrum of hemifacial microsomia	Renal agenesis or hypoplasia, vesicoureteral reflux, posterior urethral valves, multicystic kidneys	AD (107480)	<i>SALL1</i>
Twin reversed arterial perfusion (TRAP sequence)	Co-twin with incomplete development of all organ systems, limbs, and body form; upper body more severely affected than lower body	Renal agenesis, hypoplasia or cystic dysplasia; ureteral and urethral anomalies	Sporadic, occurs in MZ twins, triplets	
Urorectal septum malformation	Pseudohermaphroditism, cloacal and Müllerian duct anomalies, ambiguous genitalia, imperforate anus	Renal agenesis, hypoplasia, and cystic dysplasia; ureteral and urethral anomalies	Sporadic, maternal diabetes in some patients	
Uterus bicornis bicollis with partial vaginal septum and unilateral hematocolpos with ipsilateral renal agenesis	Double uterus, two cervixes, partial vaginal septum	Unilateral renal agenesis	AD (192050)	
VACTERL association	Vertebral defects, anal atresia, cardiac malformations, tracheoesophageal fistula, radial ray aplasia or hypoplasia, genitourinary and limb anomalies	Renal agenesis, hypoplasia, or cystic dysplasia; ureteric and urethral anomalies	Sporadic (192350)	

AD, autosomal dominant; AR, autosomal recessive; XLR, X-linked recessive; MZ, monozygotic.

of inheritance has been reported, both with respect to rare multiple malformation syndromes (Table 62-4) and isolated renal aplasia (41–48). X-linked inheritance of renal agenesis has also been reported, as in Kallmann syndrome (49).

**62.4.1.4 Differential and Diagnosis.** Diagnosis of renal agenesis is made with abdominal ultrasound. If the ultrasound imaging is equivocal, computed tomography (CT) scan or magnetic resonance imaging (MRI) can be used for confirmation. However, CT and MRI contrast agents

should not be used in the presence of poor kidney function. Severe renal dysplasia and renal hypoplasia can present with a similar clinical presentation. Earlier investigation of the structural and functional abnormalities of the kidney with renal scan and intravenous pyelogram (IVP) was not reliable when the kidney was severely dysplastic or hypoplastic. Cystoscopy for identification of the ureteral orifice is also unreliable when there is unilateral renal agenesis because of the variable association with ipsilateral absence

**TABLE 62-5 Disorders of the Acrorenal Field Defect with Renal Agenesis**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Acrorenal mandibular	Split hand/foot; anomalies of vertebrae, ribs and uterus	Renal agenesis, polycystic kidneys	AR (200980)	
Acrorenal syndrome	Tetraectrodactyly, lobster-claw deformity, clinodactyly	Renal hypoplasia	AR (201310)	
AREDYLD	Acrorenal field defect, ectodermal dysplasia, and lipoatrophic diabetes; hypotrichosis, absent dentition, aplasia of the breast	Ureter dysfunction, secondary renal cystic disease, hypoplasia of the renal calyx	AR (207780)	
Cerebro–renal–digital syndromes	Digital and limb anomalies, brain malformations, mental retardation and other anomalies. See Table 62-6	Renal aplasia, ectopia, and cystic dysplasia; ureteral anomalies	Heterogeneous	
CHARGE syndrome	Coloboma, congenital heart anomalies, choanal atresia, mental retardation, genital hypoplasia, ear anomalies, deafness	Renal agenesis, cystic dysplasia, fusion, or ectopia; ureteric anomalies	AD (214800)	<i>CHD7</i>
Chromosomal abnormalities	See Table 62-2			
DK phocomelia	Phocomelia, encephalocele, absent corpus callosum, thrombocytopenia	Renal agenesis, fused-ectopic kidney, ureteric anomalies, horseshoe kidney	AR (223340)	
Duane-radial ray syndrome	Duane anomaly, coloboma, ear anomaly, radial ray aplasia or hypoplasia, thumb hypoplasia, preaxial polydactyly	Renal agenesis, hypoplasia, dystopia or ectopia, malrotation of the kidney, horseshoe kidney, vesicoureteral reflux, pelvicalyceal dilation	AD (607323)	<i>SALL4</i>
Ectrodactyly-ectodermal dysplasia-clefting (EEC)	Ectrodactyly, ectodermal dysplasia, cleft lip/palate, abnormal hearing, internal genital tract anomalies, anal atresia	Renal agenesis or cystic dysplasia, ureteral and bladder anomalies, duplicated collecting system	AD (129900)	<i>EEC1</i> <i>EEC2</i> <i>TP63</i>
Fanconi pancytopenia	Pancytopenia; anemia; radial aplasia/hypoplasia; microcephaly; short stature; variable eye, ear, and heart anomalies; increased chromosome breakage	Renal agenesis (39%) or dysplasia, duplication of pelvis and/or ureter, ectopic or horseshoe kidney, hydronephrosis	AR (227650)	<i>FANCA</i> (A, C–G, I, J, L–P)
Hirschsprung disease with polydactyly, renal agenesis and deafness	Hypertelorism, Hirschsprung disease, congenital sensorineural hearing loss, polydactyly	Unilateral renal agenesis	AR (235740)	
Lacrimoauriculodento-digital (Levy–Hollister, LADD)	Digital anomalies, bifid/triphalangeal thumb, aplasia or hypoplasia of lacrimal puncta and salivary glands, dacryocystitis, cup-shaped pinnae, conductive deafness, peg teeth, enamel dysplasia	Renal agenesis, nephrosclerosis	AD (149730)	<i>FGFR2</i> <i>FGFR3</i> <i>FGF10</i>
Partial lower limb duplication-renal agenesis	Partial duplication of lower leg, hyperelasticity of skin, ectopic anus	Unilateral renal agenesis, duplicated ureter, hydronephrosis	Sporadic	
Townes–Brocks	Anal anomaly, imperforate anus, bifid or triphalangeal thumbs, features of hemifacial microsomia	Renal agenesis, ureteral and urethral anomalies, renal cystic dysplasia	AD (107480)	
Ulnar-mammary (Schinzel–Pallister)	Ulnar ray defects, oligodactyly, aplasia or hypoplasia of mammary glands and nipples, hypoplasia of apocrine glands, genital anomalies	Unilateral renal agenesis	AD (181450)	<i>TBX3</i>

AD, autosomal dominant; AR, autosomal recessive.

**TABLE 62-6 Disorders of the Cerebro–Renal–Digital Field Defect Associated with Renal Agenesis and Dysplasia**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Acrocallosal	Agenesis of corpus callosum, macrocephaly, polymicrogyria, post- or preaxial polydactyly, cleft lip/palate, congenital heart anomalies, facial dysmorphism	Renal agenesis, cystic renal dysplasia	AR (200990)	<i>GLI3</i>
Acrocephalopolydactylous dysplasia	Swollen globular body, omphalocele, short limbs, overgrowth, anotia, craniosynostosis, polydactyly, lethal	Renal cystic dysplasia, hydronephrosis, megacystis, megaureter	AR (200995)	
Acrorenal–mandibular	Split hand/foot; anomalies of genitals, uterus, vertebrae, and ribs	Renal agenesis, ureteral anomalies, renal dysplasia, cystic kidneys	AR (200980)	
Cerebellar vermis aplasia with associated features suggesting Smith–Lemli–Opitz syndrome and Meckel syndrome	Agenesis of cerebellar vermis, occipital encephalocele, hepatic fibrosis, postaxial polydactyly, genital anomalies	Renal agenesis or cystic dysplasia	AR (213010)	
Cerebro–oculofacioskeletal (COFS, Pena–Shokier)	Microcephaly, hypotonia, microphthalmia, cataracts, blepharophimosis, facial dysmorphism, camptodactyly, skeletal anomalies	Renal agenesis, cystic dysplasia, and hypoplasia	AR (214150)	<i>ERCC6</i>
Cerebro–renal–digital, Kousseff type	Cerebral hypoplasia, lissencephaly, intrauterine growth retardation, talipes equinovarus	Renal dysplasia, unilateral renal agenesis	AR	
Duane–radial ray syndrome	Duane anomaly, coloboma, ear anomaly, radial ray aplasia or hypoplasia, thumb hypoplasia, preaxial polydactyly	Renal agenesis, hypoplasia or ectopia, malrotation of the kidney, vesicoureteral reflux, pelvicalyceal dilation horseshoe kidney	AD (607323)	<i>SALL4</i>
Fryns	Diaphragmatic defects, distal digital hypoplasia, distinct facial features, pulmonary hypoplasia, eye and brain anomalies, lethal	Renal dysplasia, cortical cysts	AR (229850)	
Meckel–Gruber	Occipital encephalocele, polydactyly, cleft lip/palate, microcephaly, microphthalmia, small or ambiguous genitalia, brain anomalies, biliary dysgenesis, pancreatic dysplasia	Renal cystic dysplasia or hypodysplasia, ureteral hypoplasia or aplasia, urethral agenesis, hypoplastic bladder	AR (249000)	<i>MKS1</i>
Neurofaciodigitorenal	Megaloencephaly, mental retardation, hypotonia, short stature, triphalangeal thumb, “bifid” nasal tip	Unilateral renal agenesis	AR (256690)	
Orofaciodigital, type IV (Baraitser–Burn)	Cleft palate, lobed tongue, tongue hamartoma, oral frenula, hypertelorism, polydactyly, syndactyly, porencephaly, cerebral atrophy and malformations, tibial dysplasia, mesomelic limb shortening	Renal agenesis, cystic dysplastic kidneys	AR (258860)	
Orofaciodigital, type VI (Varadi)	Lobed tongue, oral frenula, medial cleft lip, hypertelorism, central polydactyly, brachydactyly, cerebellar anomalies, Dandy–Walker anomaly, key-hole foramen magnum, brain hamartoma	Renal agenesis, dysplasia	AR (277170)	
Pallister–Hall	Hypothalamic hamartoblastoma, postaxial polydactyly, imperforate anus, prenatal growth retardation, multiple buccal frenula, lethal	Renal agenesis, renal dysplasia	AD (146510)	<i>GLI3</i>

**TABLE 62-6 Disorders of the Cerebro–Renal–Digital Field Defect Associated with Renal Agenesis and Dysplasia—cont'd**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Papillorenal syndrome	Optic nerve coloboma, “morning glory” optic nerve	Renal agenesis, hypoplasia, renal fibrosis, tubular atrophy, vesicoureteral reflux	AD (120330)	<i>PAX2</i>
Pfeiffer	Craniosynostosis, broad thumbs, broad great toes, partial syndactyly, occasional brain anomalies	Renal agenesis (unusual)	AD (101600)	<i>FGFR1</i> <i>FGFR2</i>
Renal dysplasia, retinal pigmentary dystrophy, cerebellar ataxia, and skeletal dysplasia, (Mainzer–Saldino syndrome)	Retinal dysplasia, cerebellar hypoplasia, ataxia, hepatic fibrosis, skeletal anomalies, cone epiphyses	Renal medullary cysts, renal agenesis	AR (266920)	
Rubinstein–Taybi	Broad thumbs and toes, distinctive facies, mental retardation, microcephaly, cryptorchidism, small phallus	Renal dysplasia, absent or extra kidneys, double renal pelvis, renal calculi, posterior urethral valves, abnormal bladder shape	AD (180849)	<i>CREBBP</i>
Short rib polydactyly, type II (Majewsky)	Median cleft lip; preaxial or postaxial polydactyly; short ribs and limbs; disproportionate shortening of tibia; anomalies of genitalia, larynx, epiglottis, and viscera; pachygyria; lethal	Renal dysplasia, polycystic kidneys, glomerular and renal tubular cysts	AR (263520)	<i>NEK1</i>
Short rib polydactyly, type I (Saldino–Noonan)	Extremely short and narrow thorax, marked micromelia, absent fibula, postaxial polydactyly, genital and heart anomalies, lethal	Renal agenesis and dysplasia, polycystic kidneys	AR (263530)	
Smith–Lemli–Opitz	Microcephaly, postaxial polydactyly, ambiguous genitalia, dysmorphism, 2–3 toe syndactyly, cerebral dysgenesis, hamartoma, anomalies of other organs, defect of cholesterol synthesis	Unilateral renal agenesis, horseshoe kidney, cystic dysplasia	AR (270400)	<i>DHCR7</i>
Zellweger	Hypotonia, seizures, cirrhosis, peroxisomal enzyme deficiency of dihydroxyacetone acyltransferase	Renal cortical microcysts, absent renal peroxisomes, hydronephrosis	AR (214100)	<i>PEX 1-3,5,6,10, 12-14,16, 19,26</i>

AD, autosomal dominant; AR, autosomal recessive.

of the ureter, ureteral orifice, and hemitrigone (50). However, more recently, isotopic  $^{99m}\text{Tc}$ -EC/dimercaptosuccinic acid (DMSA) scans are being used in children with renal agenesis or multicystic dysplastic kidney to detect other associated urinary abnormalities (51).

Other renal disorders that frequently appear with anuria and oligohydramnios include autosomal recessive polycystic kidney disease (ARPKD), infantile presentation of autosomal dominant polycystic kidney disease, renal tubular dysgenesis due to autosomal recessive inheritance, in utero exposure to angiotensin-converting enzyme (ACE) inhibitors, severe obstructive uropathy, chronic placental hypoperfusion, and severe perinatal asphyxia.

**62.4.1.5 Prenatal Diagnosis.** Fetal ultrasonographic examination can detect oligohydramnios as early as 14–16 weeks gestation (4). Absence of the kidneys can

be detected by ultrasound imaging as early as 15 weeks gestation; however, a confident diagnosis may not be made until after 18–19 weeks of pregnancy. Ultrasound imaging can also be used to detect unilateral renal agenesis with or without renal dysplasia. Identification of associated anomalies, including a two-vessel umbilical cord, can also be used for detection of disorders with multiple congenital anomalies. Severe oligohydramnios is a strong predictor of pulmonary hypoplasia and postnatal respiratory insufficiency.

**62.4.1.6 Management and Outcome.** Bilateral renal agenesis is associated with a grim prognosis (52). Prenatal loss is estimated to be as high as 38%. Neonatal death occurs within 24 h in a majority of affected infants, secondary to pulmonary insufficiency rather than uremia. If the pulmonary hypoplasia is not critical, chronic



peritoneal dialysis might be feasible depending on the size of the infant and presence of other comorbidities. Unilateral renal agenesis is associated with abnormalities of the remaining kidney in as high as 30–50% of individuals receiving medical attention (4). Clinical problems in the contralateral kidney include renal dysplasia and ectopy, pyelonephritis, ureteropelvic junction obstruction, ureterovesical junction obstruction, vesicoureteral reflux, and stone formation (18,51).

Doray and colleagues (53) reported a family with affected individuals in three generations. Individuals affected with, or who are obligate heterozygotes for, hereditary renal dysplasia have an empiric recurrence risk of 15–20% for having a child with unilateral or bilateral renal agenesis. Renal ultrasonographic examination of first-degree relatives and, when indicated, second-degree relatives is necessary to exclude variable expression of an autosomal dominant disorder with reduced penetrance.

In the late 1980s and early 1990s, Brenner and Chertow (54) hypothesized that low birth weight (LBW) constitutes a risk factor for diseases in adult life such as systemic arterial hypertension and chronic kidney disease (CKD); it built on the Barker hypothesis, which first established a framework for the intrauterine origin of diseases suffered in adulthood (55). They introduced concepts of nephron endowment, hyperfiltration and intraglomerular hypertension in the available nephrons for playing a potential role in the development of systemic hypertension in adulthood. A small study by Keller et al. (56) has also shown a strong correlation between the number of nephrons and the risk of developing primary hypertension, with the average white hypertensive patient having 46% fewer glomeruli than healthy individuals.

At 10-year follow-up of children with congenital solitary kidney without uropathy, there was no increased risk for systemic hypertension or proteinuria, and there was better compensation of renal function when compared to acquired solitary kidney (57,58). However, by age 30, a diagnosis of solitary kidney predicted worse outcome (hazard ratio, 2.43; 95% confidence interval (CI), 1.9, 3.7) for initiation of dialysis as compared to patients with unilateral or bilateral renal hypodysplasia (RHD), or multicystic or horseshoe kidney, independent of other prognostic factors (59).

Management is uncertain, but general recommendation is to avoid nephrotoxic medications such as nonsteroidal anti-inflammatory drugs (NSAIDs) and tubular toxins, to avoid high protein diets and to maintain a normal body weight. Ericksson et al. (60) demonstrated in a Helsinki cohort that hypertensive adults had LBW as children, and gained weight more rapidly in childhood. Hodgin et al. (61) reported cases of secondary focal segmental glomerulosclerosis (FSGS) in adults who were LBW, presumably who had lower nephron endowment. Obesity can also predispose to secondary FSGS; therefore, it is wise to maintain a normal body weight especially with unilateral renal agenesis. It is also recommended that individuals with unilateral renal agenesis

should not participate in contact sports or other activities that place the single kidney at risk of injury.

## 62.4.2 Renal Hypoplasia/Dysplasia

RHD, encompassing the diagnosis of renal aplasia, hypoplasia, and dysplasia, is the second leading cause of chronic renal insufficiency (creatinine clearance  $\leq 75$  mL/min/1.73 m<sup>2</sup>) in the pediatric population (62). In the North American Pediatric Renal Trials and Collaborative Studies (NAPRTCS) database, RHD was the primary diagnosis in 17.3% of the children with CKD, 14% of children on dialysis, and 15.9% of children with renal transplants (63).

Unilateral dysplastic kidneys occur in 1 in 1000, and bilateral dysplasia in 1 in 5000 of the general population (64). Strictly speaking, renal hypoplasia is defined as a small kidney, which contains intact nephrons that are reduced in number, whereas a dysplastic kidney contains disorganized elements and poorly differentiated tissue.

**62.4.2.1 Clinical Features.** Severe renal dysplasia and renal hypoplasia can have a similar clinical presentation. These two entities can be distinguished based on histological examination of renal tissue obtained from a kidney biopsy or surgical nephrectomy, which is rarely performed for diagnostic purposes. In practice, the diagnosis of primary renal hypoplasia is favored when the following criteria are met: (i) reduction of renal size by two standard deviations from the mean size for the age, (ii) exclusion of renal scarring by DMSA scan, and (iii) presence of compensatory hypertrophy of the contralateral kidney.

**62.4.2.2 Pathogenesis.** The cause of RHD is aberrant interactions between the ureteric bud and metanephric mesenchyme. Histologically, there is a reduced number of ureteral bud branches, due to high levels of apoptosis, and nephrons that are fully formed, resulting in a small kidney size (65,66). Kidney size is largely determined by the number of nephrons that are formed during development, which ends at 32–36 weeks gestation. The average number of nephrons varies between 300,000 and 1 million in each kidney (67).

While most cases of RHD are sporadic, nonsyndromic disease, studies have discovered mutations in PAX2 and HNF1B in up to 15% of European Caucasian (68–71) and 10% of Japanese children with RHD (72). Thomas et al. (73) studied a multiethnic cohort of 73 North American children with CKD due to RHD, and found mutations only in Caucasian children (14%), perhaps because of the small sample size of the African-American children and children of more than one race.

PAX2, a member of the “paired-box” transcription factor gene family, is one of the earliest genes expressed during fetal kidney development. There is an embryonic continuum of expression of many genes, especially PAX2, in which the metanephros can develop into a normal kidney and ureter, a hypoplastic kidney with malformed ureter (underexpression of PAX2), or a tumor or multicystic dysplastic kidney (overexpression of PAX2) (74–76). Mutations in PAX2 result in renal coloboma

syndrome, characterized by optic nerve colobomas, renal hypoplasia and vesicoureteral reflux (77). Alterations in *PAX2*, which normally activates the expression of *RET* and *GDNF*, may contribute to renal hypoplasia by reducing *GDNF*-*RET* signaling (29,78). A 2007 study has also shown that a common variant of the *PAX2* gene is associated with reduced newborn kidney size (79).

Hepatocyte nuclear factor 1-beta (*HNF1B*) is a critical regulator of a genetic cascade essential to controlling the proliferation and differentiation of renal tubular epithelial cells. It also controls the expression of the *PKHD1* gene (the gene mutated in recessive polycystic kidney disease), accounting for the cystic renal phenotype in mutation carriers (80,81). The 1.4Mb region of the human chromosome 17 containing *HNF1B* is highly susceptible to copy number variation as it is flanked by areas of segmental duplications, which are sites for recurrent rearrangements (82,83). Whole gene deletions of *HNF1B* are a common finding in RHD (68,84). Less commonly other renal developmental genes, such as *EYA1*, *SIX1*, *SALL1* are implicated in RHD (70).

**62.4.2.3 Genetics.** One of the more common autosomal dominant disorders, hereditary urogenital adysplasia (OMIM 191830), can present with unilateral or bilateral renal aplasia or dysplasia or both. Although the etiology of hereditary urogenital adysplasia is heterogeneous, a *PAX2* gene mutation was found in one family with isolated renal hypoplasia (85). There are several autosomal dominant syndromes associated with RHD: renal coloboma syndrome (*PAX2*), renal cysts and diabetes syndrome (*HNF1B*), branchio-oto-renal syndrome (*EYA1*, *SIX1*), and Townes-Brocks syndrome (*SALL1*).

There is increasing research being conducted investigating the role of epigenetics in gene expression and disease causality. Epigenetic mechanisms of gene regulation include DNA or chromatin protein methylation, acetylation and chromatin remodeling. Post-translational modification of histones (via acetylation or methylation) may modify chromatin to alter gene expression. To date Patel et al. (86) demonstrated a link between *PAX2* and chromatin methylation. Future studies will hopefully elucidate further roles for epigenetic modifications in the pathogenesis of CAKUT.

**62.4.2.4 Prenatal Diagnosis.** Ultrasound imaging can also be used to detect bilateral or unilateral hypoplasia. Identification of associated anomalies, including a two-vessel umbilical cord, can also be used for detection of disorders with multiple congenital anomalies. Severe oligohydramnios is a strong predictor of pulmonary hypoplasia and postnatal respiratory insufficiency.

**62.4.2.5 Management and Outcome.** There is a strong rationale for mutation screening of all children with RHD (68–73). Clinical genetic testing is available for *PAX2* or *HNF1B*. Mutation identification may aid in monitoring for extrarenal manifestations such as diabetes with *HNF1B* mutations and eye or ear abnormalities with *PAX2* mutations. Previous reports have indicated that as many as half the mutations in *HNF1B* and *PAX2*

occur de novo (70,71,84). Knowing whether the mutation was inherited or occurred de novo would implicate a role for screening siblings of the affected individual and estimating recurrence risk.

The likelihood of chronic renal failure developing in patients with bilateral dysplasia has been correlated with a calculated glomerular filtration rate (GFR) of <15 mL/min/1.73 m<sup>2</sup> at 6 months of age; children with calculated GFR >15 mL/min/1.73 m<sup>2</sup> at that age tend to show improvement in renal function at follow-up (87). A small study by Keller et al. (56) showed a strong correlation between the number of nephrons and the risk of developing primary hypertension, with the average white hypertensive patient having 46% fewer glomeruli than healthy individuals.

### 62.4.3 Renal Cystic Disorders

Renal cystic disorders are discussed in Chapter 63 with particular reference to autosomal dominant and recessive polycystic kidney diseases, the clinical spectrum, and molecular genetics of these disorders. This chapter emphasizes monogenic, multiple congenital anomaly (MCA) (43) and chromosomal and teratogenic disorders associated with cystic diseases of the kidneys. Over 250 congenital disorders are associated with renal cystic disease, the most common of which are summarized in the tables. A population-based study in Manitoba reports a birth incidence of renal cysts of 1 in 1824 total births (19); multicystic dysplasias occur in 1 in 3226 births; cysts due to lower urinary tract obstruction occur in 1 in 13,982 births; and infantile polycystic kidney disease occurs in 1 in 20,793 births (4).

### 62.4.4 Cystic Dysplastic Kidneys

Renal dysplasia refers specifically to renal anomalies resulting from poorly branched and abnormally differentiated nephrons and collecting ducts, increased stroma and, occasionally, cysts and metaplastic tissues, such as cartilage (88). Dysplastic changes can be diffuse, segmental, or focal. Cysts may or may not be present and may vary in size. Dysplastic kidneys can present on gross examination as (i) normal or enlarged in size with multiple cysts (Potter type IIA kidney, multicystic dysplasia, multicystic kidney, and multicystic dysplastic kidney), (ii) small or rudimentary in size with few or only small cysts (Potter type IIB kidney, dysgenetic kidney, aplastic kidney, solid cystic dysplasia, or a rudimentary kidney), (iii) rudimentary nubbins of disorganized tissue with dysplastic metanephric elements resulting from severe dysplasia (renal aplasia); or (iv) segmental dysplasia with only a part of the kidney—most commonly the upper pole—involved.

The incidence of multicystic dysplasia of the kidney is estimated to be 1 in 3226 births (19). The prevalence of Potter syndrome due to renal agenesis or dysplasia in one or both kidneys is estimated at 1 in 6369 (40). Over 80 syndromes and individual case reports of MCA disorders have been reported with renal dysplasia. The most common of these disorders are given in Tables 62-6–62-8.

**TABLE 62-7 Disorders Associated with Renal Dysplasia**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Asplenia with cardiovascular anomalies	Bilateral right or left embryonic primordia, asplenia, polysplenia, complex heart anomalies	Renal dysplasia	AR (208530)	
Bardet–Biedl	Mental retardation, pigmentary retinopathy, polydactyly, obesity, hypogenitalism	Renal dysplasia, cystic tubular disease, focal segmental glomerulosclerosis, bladder detrusor instability	AR (209900)	<i>BBS 1-15</i>
Beckwith–Wiedemann	Omphalocele, macroglossia, organomegaly, islet cell hyperplasia, adrenal cytomegaly, embryonic tumors, macrosomia	Nephromegaly, renal medullary dysplasia, Wilms tumor, nephrocalcinosis	AD (130650)	<i>CDKN1C</i> imprinting error, parental disomy, duplication, inversion or translocation of 11p15.5
Branchio–oto–renal	Mixed hearing loss, Mondini cochlear malformations, pinnae anomalies, branchial cleft fistulas, preauricular pits	Renal dysplasia, agenesis, and ectopy; ureteral anomalies	AD (113650)	<i>EYA1</i> <i>SIX1</i> <i>SIX5</i>
Caudal dysplasia	Sacral agenesis/hypoplasia, lower limb and skeletal anomalies, anal atresia, anomalies of the uterus	Renal dysplasia and agenesis; anomalies of urethra, and bladder	Heterogeneous, maternal diabetes in some patients	
Cerebro–renal–digital	Digital and limb anomalies, brain malformations, other anomalies. See <a href="#">Table 62-6</a>	Renal dysplasia, ectopy, agenesis, ureteral anomalies	Heterogeneous	
CHARGE syndrome	Coloboma, heart anomalies, choanal atresia, mental retardation, genital hypoplasia, ear anomalies, deafness	Cystic renal dysplasia, renal agenesis, ureteric anomalies, fused/ectopic kidneys	AD (214800)	<i>CHD7</i>
Chromosome abnormalities	See <a href="#">Table 62-2</a>			
Cloacal exstrophy	Persistent cloaca, exstrophy of cloaca, failure of fusion of genital tubercles, omphalocele, vertebral anomalies, spina bifida cystica, abnormal genital structures	Duplication of urethra; urethral and ureteral anomalies; exstrophy of the bladder; renal dysplasia, agenesis, and ectopy	Heterogeneous	
Cornelia de Lange	Microcephaly, in utero growth retardation, distinct facies, micromelia, oligodactyly, heart and other anomalies, mental retardation	Renal dysplasia, agenesis, hypoplasia	AD (122470)	<i>NIPBL</i>
Craniosynostosis–mental retardation–clefting syndrome	Craniosynostosis (coronal), hypertelorism, choroidal coloboma, mild mesomelic shortening of limbs, developmental delay, seizures	Segmental renal dysplasia, cystic dysplasia	AR (218650)	
Cystic hamartoma of lung and kidney	Hamartomatous pulmonary cysts	Medullary dysplasia, cellular mesoblastic nephroma	Unknown	
Denys–Drash syndrome	Male pseudohermaphroditism, ambiguous genitalia, gonadal dysgenesis, gonadoblastoma	Diffuse mesangial sclerosis glomerulopathy, nephroblastoma (Wilms tumor), nephrotic syndrome, focal segmental glomerulosclerosis	AD (194080)	<i>WT1</i>

**TABLE 62-7 Disorders Associated with Renal Dysplasia—cont'd**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Digeorge syndrome	Sacral meningocele, hydrocephalus, conotruncal heart anomalies, hypoplasia of the thymus and parathyroid glands, hypocalcemia	Unilateral renal agenesis, dysplasia, hydronephrosis	AD (188400)	22q11.2 deletion
Early amnion rupture	Digital and limb amputations, ring constrictions, facial clefts, body wall defects, brain anomalies	Renal dysplasia, agenesis, and ectopy; ureteric anomalies	Sporadic	
Ectrodactyly–ectodermal dysplasia–clefting (EEC)	Ectrodactyly, ectodermal dysplasia, cleft lip/palate, abnormal hearing, internal genital tract anomalies, anal atresia	Renal agenesis or cystic dysplasia, ureteral and bladder anomalies, duplicated collecting system	AD (129900)	<i>EEC1</i> <i>EEC2</i> <i>TP63</i>
Fanconi pancytopenia	Pancytopenia; anemia; radial aplasia/hypoplasia; microcephaly; short stature; variable eye, ear, and heart anomalies; increased chromosome breakage	Renal agenesis (39%) or dysplasia, duplication of pelvis and/or ureter, ectopic or horseshoe kidney, hydronephrosis	AR (227650)	<i>FANC</i> (A,C–G,I,J,L–P)
Fraser syndrome	Cryptophthalmia, cleft lip/palate, genital anomalies, atresia of ear canal, anal atresia, syndactyly	Renal dysplasia or agenesis; ureteric anomalies	AR (219000)	<i>FRAS1</i> <i>FREM2</i>
Genitopalatocardiac	Male pseudohermaphroditism, micrognathia, cleft palate, conotruncal cardiac anomalies, other anomalies	Renal cystic dysplasia	AR (231060)	
Harrod syndrome	Arachnodactyly, hypospadias, cryptorchidism, distinctive facial features, anomalous vasculature, gut malrotation	Cortical microcysts of the kidney, renal dysplasia, ureteral anomalies, vesicoureteral reflux	?AR (601095)	
Hemifacial microsomia (oculoauriculovertebral spectrum, Goldenhar)	Facial asymmetry, epibulbar dermoid, coloboma, anotia, preauricular tags, deafness, vertebral anomalies, heart anomalies, variable brain malformations	Renal cystic dysplasia, agenesis, or ectopia; hydronephrosis and hydroureter; abnormal blood supply to kidney	Heterogeneous AD (164210)	
Hypoparathyroidism, sensorineural deafness, and renal disease	Hypoparathyroidism, sensorineural hearing loss, and renal dysplasia	Renal dysplasia, nephrosis	AD (146255)	<i>GATA3</i>
Lenz microphthalmia	Microphthalmia, coloboma; mental retardation; skeletal, dental, genital, and cardiovascular anomalies	Renal agenesis or dysplasia, hydroureters	XLR (309800)	<i>BCOR</i>
Limb-body wall complex	Lateral body wall deficiency, limb reduction anomalies, neural tube defects, scoliosis, heart anomalies	Renal agenesis or dysplasia; ureteral and urethral anomalies; fused/ectopic kidneys; bladder exstrophy	Sporadic	
McKusick–Kaufman	Hydrometrocolpos, transverse vaginal membrane, vaginal septum, postaxial polydactyly, cardiac anomalies, hypospadias	Polycystic kidney, vesicovaginal fistula, hydronephrosis (secondary to ureteral compression from hydrometrocolpos)	AR (236700)	<i>BBS6</i>
Microcephaly, hiatus hernia and nephrotic syndrome (Galloway–Mowat)	Microcephaly, seizures, psychomotor retardation, eye anomalies	Nephrotic syndrome, microcystic dysplasia, focal glomerulosclerosis, diffuse mesangial sclerosis	AR (251300)	

Continued



**TABLE 62-7 Disorders Associated with Renal Dysplasia—cont'd**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
MURCS association	Müllerian duct aplasia, renal agenesis, absent vagina, uterus, cervicothoracic somitic vertebral anomalies, short stature	Renal agenesis, dysplasia, or ectopia; ureteral and urethral anomalies	Sporadic (601076)	
Multinodular goiter, cystic renal disease, and digital anomalies	Multinodular goiter, triphalangeal thumbs, preaxial polydactyly of feet	Renal dysplasia, polycystic kidney anomalies	AD (138790)	
Neural tube defects	Meningomyelocele, anencephaly, encephalocele, vertebral anomalies, anomalies of schisis association, midline anomalies	Renal agenesis, hypoplasia, dysplasia, or fusion; ureteral anomalies	Heterogeneous multifactorial	
Renal hamartomas, nephroblastomatosis, and fetal gigantism (Perlman)	Fetal macrosomia, hypotonia, psychomotor retardation, seration of upper alveolar ridge, can be perinatal lethal	Bilateral renal hamartomas, nephroblastomatosis, Wilms tumor	AR (267000)	
Nephronophthisis 2	Hypertension, pulmonary hypoplasia	Cortical microcysts, tubular atrophy, chronic tubulointerstitial nephritis, enlarged hyperechoic kidneys, absence of corticomedullary differentiation, renal failure by age 3	AR (602088)	<i>INVS</i>
Potter oligohydramnios sequence	Clinical features resulting from oligohydramnios	Bilateral renal agenesis, aplasia, hypoplasia, or dysplasia	Heterogeneous	
Renal adysplasia	Internal genital tract anomalies; occasional anomalies of anus, heart, spine, hands, and feet	Renal agenesis, hypoplasia, or dysplasia; ureteral and urethral anomalies	AD (191830)	<i>RET, UPK3A</i>
Renal–hepatic–pancreatic	See <a href="#">Table 62-9</a>			
Sacral defect with anterior meningocele (sirenomelia)	Fusion of lower limbs, sacral agenesis, anal atresia, uterine/vaginal anomalies, cardiac defects	Urethral atresia, ectopic urethra, posterior urethral valves, renal agenesis and dysplasia, ureteral and bladder anomalies	AD (600145)	<i>VANGL1</i>
Senior–Loken (renal-retinal)	Pigmentary retinal dysplasia, occasional hypotonia, seizures, hearing loss, psychomotor retardation	Renal dysplasia, juvenile nephronophthisis, medullary cystic disease	AR (266900)	<i>NPHP1</i>
Simpson–Golabi–Behmel	Prenatal and postnatal overgrowth, variable mental function, characteristic facial appearance, postaxial polydactyly, structural anomalies of organ systems	Large cystic dysplastic kidneys, hydronephrosis, duplication of renal pelvis, Wilms tumor	XLR (312870)	
Smith–Lemli–Opitz	Microcephaly, postaxial polydactyly, ambiguous genitalia, facial dysmorphism; 2–3 toe syndactyly, disorder of cholesterol metabolism	Unilateral renal agenesis or cystic dysplasia, fused kidneys	AR (27400)	<i>DHCR7</i>
Teratogen exposures	See <a href="#">Table 62-3</a>			
Tuberous sclerosis	Hypopigmented macules, adenoma sebaceum, retinal and brain tumors or phakomas, mental retardation, seizures	Renal angiomyolipomas (70%), renal epithelial cysts (20–30%), oncocytoma, renal cell carcinoma, polycystic kidney disease	AD (191100)	<i>TSC1 TSC2</i>

**TABLE 62-7 Disorders Associated with Renal Dysplasia—cont'd**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Urethral obstruction	Deficient abdominal muscle, urinary obstruction/distension, undescended testes, malrotation of the gut, clubfeet, limb reduction anomalies	Posterior urethral valves, urethral atresia, ureteral duplication, bladder distension, renal dysplasia, hydronephrosis	Heterogeneous (prune-belly syndrome)	
Urorectal septum malformation	Müllerian duct anomalies, persistent cloaca, ambiguous genitalia, imperforate anus, other anomalies	Renal agenesis, hypoplasia, and dysplasia; ureteral and urethral anomalies	Sporadic, maternal diabetes in some patients	
VACTERL association	Vertebral defects, anal atresia, cardiac malformation, tracheoesophageal fistula, radial ray hypoplasia/aplasia, genitourinary and limb anomalies	Renal agenesis, hypoplasia, and dysplasia; ureteric and urethral anomalies	Sporadic (192350)	
Zellweger	Hypotonia, seizures, cirrhosis, peroxisomal enzyme deficiency of dihydroxyacetone phosphate (DHAP) acyltransferase	Renal cortical microcysts, absent renal peroxisomes, hydronephrosis	AR (214100)	<i>PEX 1-3,5,6,10,12-14,16,19,26</i>

AD, autosomal dominant; AR, autosomal recessive; XLR, X-linked recessive.

Often associated with renal dysplasia is renal hypoplasia (earlier section), where there is a reduction in kidney size without abnormal development.

**62.4.4.1 Clinical Features.** Renal dysplasia is the most common cause of an abdominal mass in the newborn and the most common abnormality of the kidneys in children with CAKUT. The clinical signs, symptoms, and age of presentation of patients are usually determined by the severity of renal dysplasia and the associated congenital anomalies. Severe renal dysplasia can present in utero with oligohydramnios, followed by neonatal death from pulmonary insufficiency (Potter sequence). Less severe renal dysplasia includes single cysts and segmental and unilateral anomalies that may be asymptomatic and not identified on routine ultrasound imaging.

Clinical symptoms in infants and children include anuria, oliguria, impairment of urinary concentration and acidification secondary to malformed nephrons and tubules (polyuria with polydipsia, urinary salt-wasting, renal tubular acidosis), hematuria, hypertension, uremia, back pain, growth delay, chronic or progressive renal failure, renal osteodystrophy, and other symptoms of renal dysfunction. Unilateral or segmental involvement may be clinically silent, or associated with high blood pressure, and other clinical symptoms, including back pain. Single or multilocular cysts may increase the risk for renal tumors (89), but the risk for development of Wilms tumor in multicystic, dysplastic kidneys has been overemphasized (90).

Associated anomalies of other urinary tract structures, especially ureters, are present in a large majority of patients with renal dysplasia. Frequently there are anomalies of noncontiguous structures in patients with renal

dysplasia (19). Many of these anomalies are indicators of an underlying syndrome, a chromosomal, embryonic developmental field defect, or monogenic disorders.

Anomalies of noncontiguous structures often associated with bilateral renal dysplasia include cardiovascular anomalies (19), central nervous system (CNS) abnormalities (anencephaly, hydrocephalus, iniencephaly, spina bifida, and encephalocele), diaphragmatic hernia, cleft palate, microphthalmia, duodenal stenosis, imperforate anus, tracheoesophageal fistula, and bilateral absence of the radius and thumb. Cystic changes of the liver, pancreas, or other parenchymatous organs are not usually a feature of renal dysplasia, but are present in some disorders (Table 62-9).

Structural anomalies of other organ systems are frequently associated with unilateral renal dysplasia. Cardiovascular anomalies occur in 28–48% of patients (19); the contralateral urinary tract is frequently involved in the following anomalies: segmental renal dysplasia, renal hypoplasia, renal agenesis, hydronephrosis, ureterocele, ectopic ureters, duplication of the collecting system, and vesicoureteral reflux.

The most useful diagnostic investigations of the urinary tract are as follows:

1. Determination of renal function based on serum creatinine, urea, electrolytes, and creatinine clearance
2. Assessment of impaired urinary concentration and abnormal acidification in renal dysplasia
3. Renal ultrasonography for suspected structural renal anomalies and serial monitoring
4. Nuclear renography for evaluating size, morphology, and overall and segmental function of each kidney

**TABLE 62-8 Disorders with Renal Dysplasia and Anomalies in the Osteorenal Developmental Field**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Acrocephalopolydactylous dysplasia	Swollen globular body, omphalocele, short limbs, overgrowth, craniosynostosis, lethal	Renal cystic dysplasia, hydronephrosis, megabladder, megaureter	AR (200995)	
Alagille (arteriohepatic dysplasia)	Peripheral pulmonary stenosis, biliary tract anomalies, bile duct atresia, posterior embryotoxon, skeletal anomalies	Renal dysplasia, renal cysts, renal artery stenosis, mesangiolipidosis, azotemia, nephrolithiasis, urinary concentrating defects, small kidneys, single kidney, microcystic tubular dilation with interstitial fibrosis	AD (118450)	<i>JAG1</i> <i>NOTCH2</i>
Brachymesomelia-renal	Severe upper limb brachymesomelia, corneal opacities, heart anomalies, abnormalities of cranium and face	Glomerulocystic renal dysplasia, large kidneys	Isolated case (113470)	
Campomelia, Cumming type	Severe shortening and bowing of long bones, vertebral anomalies, cystic dysplasia of liver and pancreas, short gut, pulmonary hypoplasia, polysplenia	Renal cystic dysplasia	AR (211890)	
Campomelic dysplasia	Congenital bowing or angulation of long bones, other skeletal anomalies, cleft palate, sex reversal (XY female), respiratory tract and brain anomalies	Renal dysplasia, hypoplasia, and agenesis; hydronephrosis; calyceal dilation (33%)	AD (114290)	<i>SOX9</i>
Cerebrocostomandibular	Multiple posterior rib-gap defects, severe micrognathia, psychomotor retardation, anterior meningocele	Renal dysplasia, hydroureter	AD (117650)	<i>COG1</i> (2 cases)
Digitorenocerebral syndrome	Absent distal phalanges, dilated cerebral ventricle, seizures	Cystic renal dysplasia, double ureter	AR (222760)	
Ellis–Van Creveld (chondroectodermal dysplasia)	Short stature, short limbs, polydactyly, pelvic dysplasia, neonatal teeth, nail dysplasia, heart anomalies	Renal agenesis, renal dysplasia, multicystic kidneys, hypertension, nephronophthisis	AR (225500)	<i>EVC1</i> <i>EVC2</i>
Fryns	Short-limbed dysplasia, spondylocostal dysostosis, cleft palate, heart anomalies, duplication of uterus and vagina, Dandy–Walker cysts, hydrocephalus, absent corpus callosum, lethal	Renal dysplasia (hypodysplastic), hydroureter, cortical cysts	AR (229850)	
Hereditary osteolysis of carpal bones with nephropathy	Osteolysis of carpal and tarsal bones, hypotelorism, micrognathia, tall stature, proteinuria, end-stage renal disease	Arterial and arteriolar sclerosis, hematuria, azotemia, mesangial proliferation, glomerular basement membrane abnormalities, focal segmental glomerulosclerosis, nephrotic syndrome, end-stage renal disease	AD (166300)	
Hutterite cerebro-osteo-nephrodysplasia	Short stature with mild spondylorhizomelic dysplasia, failure to grow, severe mental retardation, postnatal microcephaly, seizures	Terminal nephrotic syndrome, nephrosis, proteinuria, end-stage renal disease	AR (236450)	
Jeune (asphyxiating thoracic dysplasia)	Small, bell-shaped thorax; pulmonary hypoplasia, polydactyly, variable rhizomelic limb-shortening, trident pelvis, hepatic fibrosis, pancreatic dysplasia	Renal dysplasia, glomerulonephritis, juvenile nephronophthisis, stenosis of ureterovesical junction, hydroureter	AR (208500)	

**TABLE 62-8 Disorders with Renal Dysplasia and Anomalies in the Osteorenal Developmental Field—cont'd**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Lethal short-limbed dysplasia, type Piepkorn	Lack of ossification of all bones except clavicle, extremely short limbs, cleft palate, vascular and urogenital anomalies	Renal dysplasia	Unknown	
Nail–patella syndrome	Short stature, sensorineural hearing loss, ocular anomalies, cleft lip/palate, hypoplastic or absent patella, other bony abnormalities, muscle aplasia, spina bifida	Collagenation of glomerular basement membrane, glomerulonephritis	AD (161200)	<i>LMX1B</i>
Renal cysts and diabetes syndrome	Pancreas atrophy, diabetes, genital abnormalities	Unilateral kidney agenesis, hypoplasia, cortical atrophy, interstitial fibrosis, renal cysts	AD (137920)	<i>TCF2</i>
Renal dysplasia, retinal pigmentary dystrophy, cerebellar ataxia, and skeletal dysplasia (Mainzer–Saldino syndrome)	Retinal dysplasia, cerebellar hypoplasia, ataxia, hepatic fibrosis, skeletal anomalies, cone-shaped epiphyses	Renal dysplasia, hydronephrosis, megabladder, megaloureter	AR (266920)	
Renal dysplasia–limb defects (Ulbright)	Mesomelic bony dysplasia, hypoplasia of radius and tibia, characteristic facial appearance	Renal dysplasia	AR (266910)	
Roberts (SC phocomelia)	Tetraphocomelia, cleft lip/palate, severe psychomotor retardation, lethal; chromosomes with premature centromere separation	Renal dysplasia or agenesis, horseshoe kidney, hydronephrosis, polycystic kidney	AR (268300)	<i>ESCO2</i>
Short rib–polydactyly, type II (Majewski)	Median cleft lip; preaxial and postaxial polydactyly; short ribs and limbs; disproportionate shortening of tibia; anomalies of the genitalia, larynx, epiglottis and viscera, pachygyria, lethal	Renal dysplasia, polycystic kidneys, glomerular and renal tubular cysts	AR (263520)	<i>NEK1</i>
Short rib–polydactyly, type I (Saldino–Noonan)	Short limbs; metaphyseal dysplasia; defective ossification of calvarium, vertebrae, pelvis, carpals, and tarsals; trident pelvis; cardiac anomalies; GI atresia; genital anomalies; lethal	Renal dysplasia, polycystic kidneys	AR (263530)	
Smith–Lemli–Opitz	Cerebellar hypoplasia, mesomelic dysplasia, heart and eye anomalies, polydactyly, male pseudohermaphroditism, lethal	Renal dysplasia, hypodysplasia	AR (270400)	<i>DHCR7</i>

AD, autosomal dominant; AR, autosomal recessive.

5. Consider CT and MRI to define the anatomy of the kidney and contiguous structures
6. Banded karyotype for anomalies, abnormal growth, microcephaly, or developmental delay associated with chromosomal disorders in renal dysplasia
7. Voiding cystourethrogram

**62.4.4.2 Pathogenesis.** There are two basic processes that are believed to give rise to renal dysplasia. First, the development of the kidney from a few cells to a mature organ of a million nephrons requires a significant degree

of cell proliferation in nephrogenesis with a controlled balance of apoptosis in cells that need to be deleted. In dysplasia, there is upregulation of proliferation in dysplastic epithelia (which has a role in cyst development), and an excess of apoptosis in adjacent mesenchyme (which may be responsible for involution of dysplastic kidneys before or after birth) (91). Second, there is a disorder in the process of differentiation of renal precursor cells, which is still being clarified. Many genes have been identified, but their interactions in developmental pathways are still being studied. For example, Wilms tumor-1



**TABLE 62-9 Disorders Associated with Polycystic and Multicystic Kidneys, Including Disorders with Renal-Hepatic-Pancreatic Dysplasia**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Acrorenal–mandibular	Split hand/foot; genital, vertebral, rib and uterine anomalies	Renal agenesis, ureteral anomalies, cystic kidney, renal dysplasia	AR (200980)	
Asplenia with cardiovascular anomalies	Bilateral right or left embryonic primordia, asplenia, polysplenia, complex heart anomalies, situs inversus	Renal dysplasia, cortical cysts	AR (208530)	
Branchio–oto–renal (Melnick–Fraser)	Mixed hearing loss, Mondini cochlear malformation, pinnae anomalies, branchial cleft fistula, preauricular pits	Cystic dysplastic kidneys, renal agenesis, ectopia, ureteral anomalies	AD (113650)	<i>EYA1</i> <i>SIX1</i> <i>SIX5</i>
Campomelia, Cumming type	Severe shortening and bowing of long bones, cervical lymphocele, vertebral anomalies, cystic dysplasia of liver and pancreas, shortgut, polysplenia, pulmonary hypoplasia, other anomalies	Renal dysplasia, polycystic kidneys	AR (211890)	
Carnitine palmitoyltransferase deficiency, lethal neonatal	Dysmorphic face, cardiomegaly, respiratory failure, hepatomegaly, long digits, hypotonia, hypoglycemia	Enlarged polycystic kidneys, dysplastic renal parenchyma, lipid accumulation in kidney, especially proximal convoluted tubules, double ureters	AR (608836)	<i>CPT2</i>
Fryns	Neonatal lethal; diaphragmatic defects; distal digital hypoplasia; distinct facial features; pulmonary hypoplasia; eye, brain, and other anomalies	Renal dysplasia, cortical cysts	AR (229850)	
Glutaric acidemia, type II	Multiple acyl-CoA dehydrogenase deficiency, cerebral dysplasia, fatty liver, biliary dysgenesis, pancreatic dysplasia, Potter facies	Renal dysplasia, multicystic kidneys	AR (231680)	<i>ETFA</i> <i>ETFB</i> <i>ETFDH</i>
Hutterite cerebro–osteonephrodysplasia	Short stature with mild spondylorhizomelic dysplasia, failure to grow, severe mental retardation, postnatal microcephaly, seizures	Terminal nephrotic syndrome, nephrosis, proteinuria, end-stage renal disease	AR (236450)	
Jeune (asphyxiating thoracic dystrophy)	Small, bell-shaped thorax; pulmonary hypoplasia, polydactyly, variable rhizomelic limb shortening, trident pelvis, biliary dysgenesis, pancreatic dysplasia, other anomalies	Renal dysplasia, glomerulonephritis, juvenile nephrophthisis, stenosis of ureterovesical junction, hydroureters, multicystic kidneys	AR (208500)	
Joubert syndrome	Cerebellar vermic aplasia, ataxia, colobomata, Dandy–Walker malformation	Multicystic kidneys	AR (213300, 243910)	<i>INPP5E</i>
Meckel–Gruber	Occipital encephalocele, polydactyly, cleft lip/palate, microphthalmia, small or ambiguous genitalia, brain anomalies, biliary dysgenesis and pancreatic dysplasia	Renal dysplasia or hypodysplasia, ureteral hypoplasia or aplasia, hypoplastic bladder, urethral agenesis, renal cysts, hydroureter	AR (249000)	<i>MKS1</i>
Multinodular goiter, cystic renal disease, and digital anomalies	Multinodular goiter, triphalangeal thumbs, preaxial polydactyly of feet	Renal dysplasia, polycystic kidney	AD (138790)	
Orofaciodigital, type I	Lobulated tongue, median pseudocleft of lip, cleft palate, hypoplastic alae nasi, digital anomalies, mental impairment	Adult-onset polycystic kidneys	XLR (311200)	<i>CXORF5</i>

**TABLE 62-9 Disorders Associated with Polycystic and Multicystic Kidneys, Including Disorders with Renal-Hepatic-Pancreatic Dysplasia—cont'd**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Polycystic kidney, autosomal dominant	Hepatic cysts (hepatic fibrosis is unusual), mitral valve prolapse (25%), berry aneurysms of cerebral (10–36%) and abdominal vessels, cysts in pancreas, ovaries, lungs and other, diverticulitis	Cystic disease of renal medulla and corticomedullary nephrons and collecting tubules	AD (173900)	<i>PKD1</i> <i>PKD2</i>
Polycystic kidney, autosomal recessive	Hepatic fibrosis, biliary dysgenesis, pancreatic dysplasia	Medullary ductal ectasia, cortical cysts	AR (263200)	<i>PKHD1</i>
Polycystic kidney disease, Potter type I, with microbrachycephaly, hypertelorism and brachymelia	Microbrachycephaly, hypertelorism, brachymelia and distinctive facial features	Polycystic kidney disease, with cortical cysts and medullary ductal ectasia (Potter type I)	AR (263210)	
Senior-Loken (renal dysplasia-retinal aplasia)	Pigmentary retinal dysplasia, hypotonia, seizures, hearing loss, mental retardation	Renal dysplasia, juvenile nephrophthisis, medullary cystic disease	AR (266900)	<i>NPHP1</i>
Short rib polydactyly, type II (Majewski)	Median cleft lip; pre/postaxial polydactyly; short ribs and limbs; genital, laryngeal, epiglottic and visceral anomalies, pachygyria, biliary dysgenesis; pancreatic dysplasia; lethal	Renal dysplasia, multicystic kidneys, glomerular and renal tubular cysts	AR (263520)	<i>NEK1</i>
Short rib polydactyly, type I (Saldino-Noonan)	Phocomelia; metaphyseal dysplasia; defective ossification of calvarium, vertebrae, pelvis, carpal and tarsal bones; cardiac, GI and GU anomalies; biliary dysgenesis; pancreatic dysplasia; lethal	Renal dysplasia, multicystic kidneys	AR (263530)	
Trisomy 9	Potter sequence, cleft palate, joint immobility, biliary dysgenesis, pancreatic dysplasia	Renal dysplasia, multicystic kidneys	Chromosomal	
Tuberous sclerosis	Hypopigmented macules, adenoma sebaceum, retinal and brain tumors, seizures, mental retardation	Renal angiomyolipomas (70%), renal epithelial cysts (20–30%), oncocytoma, renal cell carcinoma, polycystic kidney disease	AD (191100)	<i>TSC1</i> <i>TSC2</i>
Von Hippel-Lindau	Retinal angiomas, cerebellar hemangioblastomas	Renal cysts, renal cell carcinoma (40%), bladder papillomas	AD (193300)	<i>VHL</i>
Zellweger syndrome	Death in early infancy, hypotonia, seizures, biliary dysgenesis, peroxisomal enzyme deficiency of dihydroxyacetonephosphate (DHAP) acyltransferase	Kidney with subcortical cysts, hydronephrosis, multicystic kidney	AR (214100)	<i>PEX 1-3,5,6,10, 12-14,16, 19,26</i>

AD, autosomal dominant; AR, autosomal recessive; XLR, X-linked recessive.

(WT1) is involved in mesenchymal-epithelial transformation and podocyte differentiation, and has a negative interaction with PAX2 (92,93). Of note, mutations in WT1 lead to the development of nephrogenic rests, an abnormal structure in the kidney that is formed by failure of the mesenchymal tissue to differentiate into nephrons; they are associated with the development of Wilms tumor (94). Nephrogenic rests are rarely seen in normal kidneys after 1 year of age, but are found in nearly all patients with multifocal or bilateral Wilms tumor and in 30% of children with sporadic Wilms tumor (95).

Homozygous mutations in the Wilms tumor suppressor gene (*WT1*) are found in 18% of Wilms tumors (96–98).

**62.4.4.3 Genetics.** There is etiologic heterogeneity for renal dysplasia, as is evident in Tables 62-6–62-9. Familial recurrence due to all patterns of inheritance has been reported for isolated renal dysplasia and when associated with MCA syndromes (Tables 62-6–62-9). Teratogenic agents have been associated with renal dysplasia or cystic changes in human and experimental animal models after in utero exposure to alcohol, abnormal metabolites

**TABLE 62-10 Risk for Chromosomal Aneuploidy in Fetuses with Renal Abnormalities Identified by Sonograms (106)**

Renal Anomaly	Cases	Chromosomal Anomaly (%)
<i>Total</i>	682	85 (12)
<i>Isolated</i>	476	16 (3)
<i>Other anomalies</i>	206	69 (34)
<i>Bilateral</i>	510	66 (13)
<i>Isolated</i>	342	14 (4)
<i>Other anomalies</i>	168	52 (31)
<i>Mild hydronephrosis</i>	258	35 (14)
<i>Isolated</i>	163	5 (3)
<i>Other anomalies</i>	95	30 (32)
<i>Moderate hydronephrosis</i>	119	15 (13)
<i>Isolated</i>	81	5 (6)
<i>Other anomalies</i>	38	10 (26)
<i>Multicystic kidneys</i>	109	13 (12)
<i>Isolated</i>	79	3 (4)
<i>Other anomalies</i>	30	10 (33)
<i>Renal agenesis</i>	24	3 (13)
<i>Isolated</i>	19	1 (5)
<i>Other anomalies</i>	5	2 (40)
<i>Unilateral</i>	172	19 (11)
<i>Isolated</i>	134	2 (2)
<i>Other anomalies</i>	38	17 (45)
<i>Mild hydronephrosis</i>	18	2 (11)
<i>Isolated</i>	10	0 (0)
<i>Other anomalies</i>	8	2 (25)
<i>Severe hydronephrosis</i>	87	8 (9)
<i>Isolated</i>	76	1 (1)
<i>Other anomalies</i>	11	7 (44)
<i>Multicystic kidneys</i>	64	8 (13)
<i>Isolated</i>	48	1 (2)
<i>Other anomalies</i>	16	7 (44)
<i>Renal agenesis</i>	3	1 (33)
<i>Isolated</i>	0	0 (0)
<i>Other anomalies</i>	3	1 (33)

because of maternal diabetes, maternal rubella, thalidomide, hypoxia, and ACE inhibitors.

Prenatally diagnosed chromosomal abnormalities in renal dysplasia occur in 10.3–28.5% of fetuses (Table 62-10). Conversely, 35% of individuals with chromosomal disorders have renal anomalies, most commonly renal dysplasia and micropolycystic kidneys.

Renal ultrasound examinations of first-degree and, when indicated, second-degree relatives are necessary to exclude variable expression of autosomal dominant disorders, particularly hereditary renal adysplasia. Using renal ultrasound results from parents and sibs of 41 probands with bilateral renal agenesis or cystic dysplasia or both, Roodhooft and colleagues (40) determined a 9% risk for related urogenital anomalies and a 4.4% risk for renal anomalies. Perinatal lethal renal disease is associated with a 3.6% recurrence

risk for sibs and a 0.2% risk for first cousins. Empiric recurrence risks can be used for families with a proband affected with renal dysplasia after excluding monogenic, syndromic, and chromosomal disorders. Affected individuals or obligate heterozygotes for hereditary renal dysplasia have an empiric recurrence risk of 15–20% for having a child with unilateral or bilateral renal agenesis.

There are several autosomal dominant syndromes associated with dysplasia: renal coloboma syndrome (PAX2), renal cysts and diabetes syndrome (HNF1B), branchio-oto-renal syndrome (EYA1, SIX1), and Townes–Brocks syndrome (SALL1).

**62.4.4.4 Prenatal Diagnosis.** Prenatal diagnosis of renal dysplasia has been successful using ultrasound imaging and, in selected cases, maternal serum alpha-fetoprotein screening (99). The classical antenatal presentation of dysplastic kidneys is large bright kidneys, with or without cortical cysts on the 20 week ultrasound. The degree of abnormality depends on the severity of the following variables: abnormality of size, brightness, visibility and number of cysts, amniotic fluid and abnormalities in other organ systems. In the multicystic dysplastic kidney, there are multiple thin-walled cysts at 20 weeks, which do not connect, and are distributed randomly throughout the kidney; often a renal pelvis or ureter may not be seen (64). Exclusion of associated structural anomalies and karyotype determination is recommended when renal malformations are identified. Of the fetuses identified by ultrasound as having one kidney with multicystic dysplasia, 40% have contralateral renal anomalies, 20% have bilateral multicystic dysplasia, 10% have contralateral renal agenesis, and 10% have contralateral hydronephrosis, most commonly due to ureteropelvic junction obstruction.

Chromosome abnormalities in fetuses identified to have cystic dysplastic kidneys on ultrasound scans vary, depending on the presence of associated structural anomalies (Table 62-10). The fetal risk for chromosomal abnormalities with unilateral multicystic kidneys is 2% and bilateral involvement 4%, if this is an isolated finding. When associated structural anomalies are apparent on ultrasound imaging, there is a risk for chromosomal aneuploidy of 44% for unilateral renal findings and 33% for bilateral renal abnormalities.

**62.4.4.5 Management and Outcome.** Many dysplastic kidneys involute over time, including before birth (100,101). In this case, the prognostic indicators are the presence of abnormalities and compensatory hypertrophy of the contralateral kidney (102). Fetal prognosis is dependent on the extent of renal involvement, the amount of amniotic fluid, and the associated structural malformations, as well as the underlying diagnosis. Progressive deterioration in renal function is the usual clinical course when dysplastic changes are bilateral or when contralateral renal agenesis or hypodysplasia is present. These children exhibit urinary concentrating defects, metabolic

acidosis, and growth failure. However, affected infants may experience improvement in renal function through normal physiologic adaptation to postnatal life during infancy with later gradual deterioration in function. Prophylactic antibiotics should be started at birth to prevent urinary tract infections, until the presence of vesicoureteral reflux and other ureteral abnormalities have been ruled out by further imaging. Radionuclide scan can be considered to evaluate split excretory function from either kidney.

Unilateral renal dysplasia may remain asymptomatic. Operative removal of unilateral dysplastic kidney may not be necessary in the absence of clinical symptomatology (e.g. pain, mass effect, or hypertension) (89). Tumors have infrequently been reported to arise in the cysts of the dysplastic kidneys (89), but the risk for development of Wilms tumor in multicystic, dysplastic kidneys has been overemphasized (90).

### 62.4.5 Ureteral Abnormalities

Agenesis of the ureters is not usually found in the absence of other urinary tract malformations. Renal agenesis is frequently associated with partial or complete agenesis of the ureters. Disorders such as sirenomelia, associated with caudal dysplasia, frequently have ureteral agenesis or abnormalities.

Duplication of the ureters is common, with a prevalence of 1:50 to 1:300 (18). Duplication may be complete with two ureters extending from the renal pelvis to the bladder, or the ureters may unite before entering the bladder. Unilateral duplication is five to six times more common than bilateral duplication. Ureteral anomalies may remain asymptomatic, presenting at autopsy, or can present with symptoms resulting from obstruction: infection, hydronephrosis, or ureterocele.

**62.4.5.1 Pathogenesis.** The ureters are derivatives of the ureteric buds, originally part of the distal mesonephric ducts, which open into the anterior portion of the hindgut, forming the cloaca. The terminal portions of both mesonephric ducts are incorporated into the endodermal vesicoureteral primordium, forming the vesical trigonum. Abnormal division during ureteric bud formation is postulated as the pathogenetic mechanism for duplication of the ureters, which occurs during developmental weeks six through 11. The point of origin of the ureteric bud becomes the ureteral orifice in the urinary bladder. Abnormal placement of the point of origin will lead to ureteral ectopia.

**62.4.5.2 Genetics.** Ureteral anomalies clearly have a hereditary basis, especially in the presence of associated anomalies (Tables 62-11 and 62-12; see also Tables 62-2–62-9). There is suggestion in the medical literature that isolated ureteral anomalies have a hereditary basis; however, information on empiric recurrence risk for bifid (double) ureters is limited. Atwell et al. (103) report a high frequency of unilateral or bilateral bifid

ureters among sibs and parents of 30 probands presenting clinically with this finding. When a bifid pelvicalyceal system is included, 20 of the 30 families studied have affected first-degree relatives. Male-to-male transmission is observed, suggesting autosomal dominant inheritance with variable expression. Renal ultrasound imaging will simplify diagnosis of severe ureteral anomalies among first-degree relatives, but will not identify duplicated ureters that are not dilated.

Duplication of the ureter is found as a nonobligatory feature in a number of syndromes, chromosomal abnormalities, and inherited disorders. Because of the high prevalence of ureter duplication as an isolated malformation, this finding may not be helpful with reference to syndrome diagnosis. Genetic disorders commonly associated with ureteral abnormalities include ectrodactyly–ectodermal dysplasia–clefting (EEC) syndrome, Kaufman–McKusick syndrome, von Mayer–Rokitansky–Kuster (MRK) anomaly, hypertelorism–microtia–clefting syndrome, restrictive dermatopathy, hereditary renal adysplasia, infants of diabetic mothers, as well as other malformation sequences, chromosomal abnormalities, and monogenic disorders listed in Tables 62-2 and 62-3.

Ureterocele, a saccular out-pouching of the distal ureter into the urinary bladder, has been reported in monozygotic and dizygotic twins, in sibs, and in apparent parent–child inheritance. Ureteroceles are very frequently associated with duplications of the kidney and ureter.

### 62.4.6 Bladder

Major anomalies of the bladder are frequently associated with other malformations including exstrophy of the bladder, exstrophy of the cloaca, anomalies of the urachus, and prune-belly anomaly (discussed later).

Agenesis of the bladder is an almost constant feature in sirenomelia. Other anomalies of the urinary and genital system are usually present as well, including absence of the ureters and uterus, or abnormal insertion of the ureters into the urethra or uterus. A hypoplastic bladder is expected in association with bilateral renal agenesis or other renal anomalies that prevent urine production and delivery to the bladder.

Bladder exstrophy, where the bladder is open anteriorly, is due to the lack of abdominal wall closure. It is often associated with epispadias and separation of the pubic rami. Numerous familial cases have been described. Bladder exstrophy can be part of the omphalocele–exstrophy–imperforate anus–spinal defects (OEIS) complex.

Abnormal configuration of the bladder may be seen in bladder diverticulum, persistence of the urachus, and incomplete or complete duplication of the bladder. True diverticulae are evaginations of the bladder wall. They may arise as herniations of the bladder wall because of distal obstruction or as intrinsic defects in bladder musculature. A diverticulum of the dome of the bladder may



**TABLE 62-11 Disorders with Abnormalities of the Urethra**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Amastia, bilateral with ureteral triplication	Anastia, unusual facies, hemivertebrae, mitral valve prolapse, umbilical hernia, syndactyly	Ureteral triplication, hydronephrosis	AD (104350)	
Aphalangy with hemivertebrae	Aphalangy, hemivertebrae, urogenital–intestinal dysgenesis, anal atresia, persistent cloaca	Urethral atresia, absent ureters, bilateral renal agenesis	AR (207620)	
Bardet–Biedl syndrome	Obesity, pigmentary retinopathy, polydactyly, hypogenitalism	Ectopic urethra, cystic kidneys	AR (209900)	<i>BBS1-15</i>
Caudal dysplasia (caudal regression)	Sacral agenesis/hypoplasia, lower limb and other skeletal anomalies, anal atresia, genital anomalies	Urethral, bladder and ureteral anomalies, renal agenesis or dysplasia	Heterogeneous, maternal diabetes	
Chromosomal abnormalities	See <a href="#">Table 62-2</a>			
Cloacal exstrophy	Persistent cloaca, exstrophy of cloaca, failure of fusion of genital tubercles, omphalocele, vertebral defects, spina bifida cystica, abnormal internal and external genitalia	Duplication of urethra; urethral and ureteral anomalies, exstrophy of bladder, renal dysplasia, renal agenesis, renal ectopy		
Conjoined twins	Urinary tract anomalies are most common in iliothoracopagus, ischiopagus, diprosopus, dicephalus, dipygus conjoined twins	All urinary anomalies may occur	Unknown	
Diabetes, maternal	Caudal regression, neural tube defects, heart and other anomalies	Urethral, bladder, and ureteric anomalies; renal agenesis; urethral atresia	Metabolic teratogenicity due to maternal diabetes	
Distal obstructive uropathy polydactyly	Polydactyly, oligohydramnios, stillbirth	Posterior urethral valve with hydronephrosis, hydroureter, dilated bladder	Unknown	
Early amnion rupture (ADAM, amniotic bands)	Digital and limb amputations, ring constrictions, facial clefts, other anomalies	Urethral duplication; other urethral anomalies; renal agenesis, dysplasia, ectopy; ureteral anomalies	Sporadic	
Facial–skeletal–genital, Rippberger type	Facial dysmorphism, neurosensory deafness, normal intelligence	Recurrent urethral strictures, urinary tract infections	Unknown	
Hand–foot–uterus (hand–foot–genital)	Hypoplasia of the thumbs and great toes, duplication of female internal genitalia, chordee and hypospadias in males	Duplication of urethra, ectopic ureteral orifices, intravaginal urethra, hydronephrosis, epididymal cyst	AD (140000)	<i>HOXA13</i>
McKusick–Kaufman	Hydrometrocolpos, transverse vaginal membrane, vaginal septum, postaxial polydactyly, cardiac anomalies, hypospadias	Hydroureter, ureteral duplication, urogenital sinus, ectopic urethra	AR (236700)	<i>BBS6</i>
Limb–body wall disruption	Lateral body wall deficiency, neural tube defect, severe scoliosis, absent or abnormal limb(s)	Urethral duplication or atresia; bladder exstrophy, renal agenesis, dysplasia fusion or ectopy; ureteral anomalies	Sporadic	
Meckel–Gruber	Occipital encephalocele, polydactyly, cleft lip/palate, microphthalmia, small or ambiguous genitalia, brain anomalies, biliary dysgenesis, pancreatic dysplasia	Renal dysplasia or hypodysplasia, ureteral hypoplasia or aplasia, hypoplastic bladder, urethral agenesis	AR (249000)	<i>MKS1</i>
Michels (oculopalatoskeletal)	Cleft lip/palate, deafness, blepharophimosis, anterior chamber eye anomalies, skeletal anomalies	Urethral and ureteral	AR (257920)	
Neurofibromatosis 1	Café-au-lait spots, neurofibromas, Lisch nodules, axillary freckling, local bony overgrowth, macrocephaly	Neurofibromas of the urethra, bladder, or ureter; renal artery stenosis; hydronephrosis	AD (162200)	<i>NF1</i>

**TABLE 62-11 Disorders with Abnormalities of the Urethra—cont'd**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Potter sequence	Craniofacial and skeletal compression deformities from oligohydramnios	Bilateral renal agenesis, aplasia, hypoplasia or dysplasia; urethral agenesis; posterior urethral valves	Heterogeneous	
Prune-belly syndrome	Deficient abdominal muscle, urinary obstruction/distention, undescended testes malrotation of the gut, clubfoot, limb reduction anomalies	Posterior urethral valves, urethral atresia, ureteral duplication, bladder distension, hydronephrosis, renal dysplasia	Heterogeneous (100100)	
Renal adysplasia	Anomalies of internal genitalia; occasional anomalies of anus, heart, spine, hands, and feet	Renal agenesis, hypoplasia, or dysplasia; ureteral and urethral anomalies	AD (191830)	<i>RET</i> <i>UPK3A</i>
Sacral defect with anterior meningocele (sirenomelia)	Fusion of lower limbs, sacral agenesis, anal atresia, uterine/vaginal anomalies, cardiac defects	Urethral atresia, ectopic urethra, posterior urethral valves, renal agenesis, cystic dysplasia, horseshoe kidneys, ureteral and bladder anomalies	AD (600145)	<i>VANGL1</i>
Townes–Brocks	Anal anomaly, imperforate anus, bifid or triphalangeal thumbs, features of hemifacial microsomia	Renal agenesis or dysplasia, ureteral and urethral anomalies	AD (107480)	<i>SALL1</i>
Urofacial syndrome (Ochoa)	“Inversion” of facial expression when laughing	Neuropathic bladder, posterior urethral valves, hydroureter	AR (236730)	<i>HPSE2</i>
Urorectal septum malformation	Ambiguous genitalia, Müllerian anomalies, persistent cloaca, imperforate anus, vaginal atresia	Urethral agenesis, renal agenesis or dysplasia, bladder dilation	Sporadic, maternal diabetes in some patients	
VACTERL association	Vertebral defects, anal atresia, cardiac malformation, tracheoesophageal fistula, radial ray hypoplasia/aplasia, limb anomalies	Renal agenesis, hypoplasia, or cystic dysplasia; ureteral and urethral anomalies	Sporadic (192350)	
Williams syndrome	“Elfin” facies, stellate iris, short stature, heart defects, hypercalcemia	Urethral stenosis, solitary kidney, pelvic kidney	AD (194050)	7q11.23 deletion

AD, autosomal dominant; AR, autosomal recessive.

represent persistence of the urachus. Lateral diverticulae may represent incomplete partition of the bladder and may or may not be associated with duplication of the urethra, genitalia, and rectum. Clinical presentation of bladder diverticulae is most commonly recurrent urinary tract infections, due to incomplete emptying with voiding. Diverticulae of the bladder may occur in association with diverticulae of the intestines, and with obstruction of the urethra due to prostatic hyperplasia or tumor, both of which have a familial predisposition.

Bladder diverticulae occur as a clinical feature of heritable disorders, particularly involving abnormal copper metabolism (i.e. cutis laxa, occipital horn syndrome, and Menkes syndrome). They can also occur in Ochoa syndrome, an autosomal dominant condition associated with neuropathic bladder, posterior urethral valves (PUVs), and hydroureter.

Congenital dilatation of the bladder, presenting in utero, has a number of etiologies. The obstruction is usually due to PUVs, particularly in males (see later

discussion). Deficiency of the abdominal musculature (prune-belly anomaly), ascites, hydroureter, and hydronephrosis are frequently associated anomalies. Bladder dilatation can result from neurologic impairments, including neural tube defects, mitochondrial myopathies, and agangliosis. Megacystis in the absence of recognized obstruction occurs in the megacystis–microcolon–hypo-peristalsis syndrome.

### 62.4.7 Urethral Agenesis and Atresia

Absence of the urethra is rare and reported predominantly in males. Congenital segmental urethral agenesis or atresia is more common than complete atresia. The level of obstruction is usually at the membranous urethra. Failure of formation of all or part of the urethra is an anomaly that is frequently associated with prune-belly syndrome (i.e. Eagle–Barrett syndrome).

**62.4.7.1 Clinical Features.** Presenting signs and symptoms of urethral agenesis or segmental absence include

**TABLE 62-12 Disorders Associated with Posterior Urethral Valves**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Amyloidosis, familial cutaneous	Amyloidosis, incontinentia pigmenti, failure to thrive, seizures, developmental delay, blindness	Urethral stricture	XLR (301220)	
Aphalangy with hemivertebrae	Aphalangy, hemivertebrae, urogenital–intestinal dysgenesis, anal atresia	Urethral atresia, absent ureters, bilateral renal agenesis	AR (207620)	
Caudal dysplasia (caudal regression)	Sacral agenesis/hypoplasia, lower limb and skeletal anomalies, anal atresia, genital anomalies	Urethral, bladder, and ureteral anomalies; renal agenesis or dysplasia	Heterogeneous, maternal diabetes in some patients	
Chromosomal abnormalities	See <a href="#">Table 62-2</a>			
Cloacal exstrophy	Persistent cloaca, exstrophy of cloaca, failure of fusion of genital tubercles, omphalocele, vertebral defects, spina bifida cystica, abnormal internal and external genitalia	Duplication of urethra, urethral and ureteral anomalies, exstrophy of bladder, renal dysplasia, renal agenesis or ectopy	Sporadic	
Diabetes, maternal	Caudal regression, neural tube defects, heart and other anomalies	Urethral, bladder, and ureteric anomalies; renal agenesis with caudal regression	Metabolic teratogens	
Distal obstructive uropathy polydactyly	Polydactyly, oligohydramnios, stillbirth	Posterior urethral valves with hydronephrosis, hydroureter, dilated bladder	Unknown	
Facial–skeletal–genital, Rippberger type	Facial dysmorphism, neurosensory deafness, normal intelligence	Recurrent urethral stricture, urinary tract infections	Unknown	
Hand–foot–uterus	Hypoplasia of thumbs and great toes, duplication of female internal genitalia; chordae and hypospadias in males	Ectopic ureteral orifices, intravaginal urethra, hydronephrosis, epididymal cyst, posterior urethral valves	AD (140000)	<i>HOXA13</i>
Limb–body wall complex (lateral body wall defect)	Lateral body wall defects, limb reduction anomalies, neural tube defects, heart and other anomalies	Urethral atresia or duplication (with bladder exstrophy); renal agenesis, dysplasia, fusion, or ectopy; ureteral anomalies, posterior urethral valves	Sporadic	
McKusick–Kaufman	Hydrometrocolpos, transverse vaginal membrane, vaginal septum, postaxial polydactyly, cardiac anomalies, hypospadias	Hydroureter, ureteral duplication, ectopic urethra, urogenital sinus, posterior urethral valves	AR (236700)	
Michels (oculopalatoskeletal)	Cleft lip/palate, deafness, blepharophimosis, anterior chamber eye anomalies, skeletal anomalies	Urethral and ureteral anomalies	AR (257920)	
Neurofibromatosis 1	Café au lait spots, neurofibromas, Lisch nodules, axillary freckling, local bony overgrowth, macrocephaly	Neurofibromas of the urethra, bladder, or ureter; renal artery stenosis; hydronephrosis, posterior urethral valves	AD (162200)	<i>NF1</i>
Potter sequence	Facial and skeletal deformations resulting from oligohydramnios, pulmonary hypoplasia	Bilateral renal agenesis, hypoplasia, or dysplasia; urethral agenesis; posterior urethral valves	Heterogeneous	
Prune-belly syndrome	Deficient abdominal muscles, urinary obstruction/distention, undescended testes, malrotation of the gut, clubfeet, limb reduction anomalies	Posterior urethral valves, urethral atresia, ureteral duplication, hydronephrosis, bladder distension, renal dysplasia	Heterogeneous (100100)	
Renal adysplasia	Anomalies of internal genitalia; occasional anomalies of anus, heart, spine, hands, and feet	Renal agenesis, hypoplasia, or dysplasia; ureteral and urethral anomalies	AD (191830)	<i>RET</i> <i>UPK3A</i>

**TABLE 62-12 Disorders Associated with Posterior Urethral Valves—cont'd**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Rubinstein–Taybi	Broad thumbs and toes, distinctive facial features, mental retardation, microcephaly, cryptorchidism, small phallus	Posterior urethral valves, absent or extra kidneys, ureteral anomalies, double renal pelvis, renal calculi, abnormal bladder shape	AD (180849)	<i>CREBBP</i>
Sacral defect with anterior meningocele (sirenomelia)	Fusion of lower limbs, sacral agenesis, anal atresia, uterine/vaginal anomalies, cardiac defects	Urethral atresia, ectopic urethra, posterior urethral valves, renal agenesis, cystic dysplasia, ureteral and bladder anomalies	AD (600145)	<i>VANGL1</i>
Townes–Brocks	Anal anomaly, imperforate anus, bifid or triphalangeal thumbs, features of hemifacial microsomia	Renal agenesis, ureteral and urethral anomalies	AD (107480)	<i>SALL1</i>
Urofacial syndrome (Ochoa)	“Inversion” of facial expression when laughing	Neuropathic bladder, posterior urethral valves, hydroureter	AR (236730)	<i>HPSE2</i>
Urorectal septum malformation	Ambiguous genitalia, Müllerian anomalies, persistent cloaca, imperforate anus, vaginal atresia	Urethral agenesis, renal agenesis or dysplasia, bladder dilation, posterior urethral valves	Sporadic, infant of diabetic mother in some cases	
VACTERL association	Vertebral defects, anal atresia, cardiac malformation, tracheoesophageal fistula, radial ray hypoplasia/aplasia, limb anomalies	Renal agenesis, hypoplasia, or cystic dysplasia; ureteral and urethral anomalies	Sporadic (192350)	

AD, autosomal dominant; AR, autosomal recessive; XLR, X-linked recessive.

abdominal distension due to enlarged bladder or urinary ascites, laxity of abdominal musculature, bilateral cryptorchidism, oligohydramnios, respiratory insufficiency, failure to pass urine, or evidence of urinary fistulae such as a patent urachus.

Associated anomalies can include megacystis, hydroureter, hydronephrosis, dysplastic kidneys, undescended testes, and pulmonary hypoplasia. Urachal, cloacal, urethrorectal, urethropenile, urethroscrotal, or vesicovaginal fistulae are frequently present. Anal atresia or ectopic anus and abnormal development of the prostate, urinary tract musculature, and gubernaculum are common. Hypoplastic abdominal musculature is present in prune-belly syndrome. Loops of bowel protruding under the thin abdominal wall give the characteristic prunelike appearance. Bladder distension can lead to obstruction of the umbilical arteries with lower limb ischemia, hypoplasia, or transverse reduction defects. Affected females may also have bicornuate or duplicated uterus, vaginal atresia, clitoral hypertrophy, anal atresia, and upper urinary tract anomalies.

**62.4.7.2 Genetics.** Disorders associated with urethral agenesis and atresia are listed in Table 62-11. When a specific disorder is not identified, recurrence risk for urethral atresia appears to be low.

**62.4.7.3 Diagnosis and Differential Diagnosis.** Abdominal distension with abdominal muscular hypoplasia so that the loops of bowel can be readily seen is pathognomonic for the prune-belly syndrome. Other frequent findings include features of the Potter sequence due to oligohydramnios, unless a urethral-visceral fistula or a patent urachus is present. Marked posterior urethral dilation, secondary to the obstruction at the membranous urethra, results in the typical appearance on

ultrasound of a dumbbell-shaped bladder or “keyhole sign,” identifiable both in utero and postnatally.

Diagnosis is often possible by fetal ultrasound. The differential diagnosis includes other urethral abnormalities resulting in severe obstruction, including PUVs and urethral duplication.

**62.4.7.4 Prenatal Diagnosis.** Prenatal detection by ultrasound imaging of urinary tract obstruction associated with megacystis, dumbbell-shaped bladder, increased abdominal circumference, and oligohydramnios suggest the possibility of urethral agenesis or atresia.

Detection of recurrence of significant urethral anomalies with subsequent pregnancies is possible with diagnostic fetal ultrasonography. Fetal ultrasonographic evaluation for evidence of urinary tract obstruction is recommended at 16–18, 24 and 32 weeks of pregnancy, since obstruction can present later in pregnancy. Severe obstruction may be diagnosable with high-level ultrasonography as early as 12–14 weeks of pregnancy.

**62.4.7.5 Management.** In utero urinary decompression with a vesicoamniotic shunt can be performed in a fetus with urethral atresia, but should only be attempted if there is residual renal function. Exclusion of underlying syndromic, chromosomal, and teratogenic etiologies associated with a poor prognosis is recommended before aggressive treatment. In the newborn with oligohydramnios, pulmonary insufficiency is frequently present, and usually requires aggressive management. Diagnostic investigations are directed toward determination of the cause of the obstruction and evidence of residual renal function.

Urinary diversion or vesicostomy may be necessary before reconstruction of the urethra. In prune-belly



syndrome, surgical management ultimately includes abdominoplasty, bilateral orchiopexy, reduction cystoplasty, and selective ureteral reconstruction and reimplantation. Kidney transplantation and the use of the bowel for partial reconstruction of the ureters and/or urinary bladder have improved the outcome in severe obstructive uropathy.

### 62.4.8 Duplication of the Urethra

Duplication of the urethra may be complete, with two separate external meati, or partial, with a variable outlet of the accessory urethra. Urethral duplications are subclassified anatomically (e.g. epispadias, hypospadias, and Y-type accessory channel opening into the perineum or anal canal) (104). The etiology of these conditions is unknown. Urethral duplication has occurred in males with VACTERL association and in males with PUVs.

### 62.4.9 Posterior Urethral Valves

PUVs are found in males, masculinized females, and, rarely, in normal females. The incidence is estimated to be 1 in 5000–8000 male infants. PUVs are recognized to be the most common cause of obstructive uropathy that may lead to renal failure in childhood. The “valves” are actually tissue folds located in the posterior urethra that functionally obstruct urine outflow.

**62.4.9.1 Clinical Features.** The age of presentation, clinical symptoms, and ease of diagnosis are variable and depend on the severity of obstruction. In regions of the world where prenatal ultrasonography is routine, the majority of these infants are detected in utero. Prior to the widespread use of prenatal ultrasonography, it was estimated that one-third to half of individuals with PUVs presented within the first 3–6 months of life and the majority within the first year of life.

Commonly presenting symptoms in those not diagnosed prenatally are abdominal mass (38–53%), oligohydramnios (32%), failure to grow (18–40%), sepsis or urinary tract infections (20%), or a weak urinary stream (14%). Associated anomalies in the urinary tract system include a thickened, trabeculated bladder, dysfunctional voiding, ureterovesical junction obstruction, vesicoureteral reflux, hydronephrosis, and renal dysplasia. Severe oligohydramnios is associated with pulmonary hypoplasia and respiratory insufficiency postnatally.

Valvular obstruction results in incomplete emptying of the bladder. Back pressure leads to dilation of the posterior urethra. The bladder neck and detrusor musculature hypertrophy and thicken. The bladder may become trabeculated and sacculated with thickened walls. Urinary ascites can develop by transudation of urine through small bladder perforations, fistulae with surrounding organs, or a patent urachus. The ureters are dilated in 70% of cases with PUVs. Functional obstruction of the ureterovesical junction can occur secondary

to thickened detrusors. Vesicoureteral reflux is present in 45% of cases with PUVs and is unilateral in 17%. Reflux, especially when associated with urinary tract infections, contributes to more rapid loss of renal function. Dysplasia of the kidneys can occur secondary to obstruction and from associated anomalies of the metanephric buds (4).

**62.4.9.2 Genetics.** Disorders of multiple congenital anomalies associated with PUVs are listed in Table 62-12. The majority of individuals with PUVs are otherwise normal. Isolated PUVs appear to have a recurrence risk of 2–6% (4).

**62.4.9.3 Diagnosis and Differential Diagnosis.** Initial evaluation of an infant with bladder obstruction includes determination of renal function and electrolyte balance. Diagnosis of PUVs is made with a voiding cystourethrogram and by urethral endoscopy. There is an appearance of elongation and marked dilation of the posterior urethra with a sharp cutoff distal to the verumontanum and a narrowed distal urethral segment. Diagnostic evaluation of the upper urinary tract system is indicated with ultrasound and renography with radioisotopes. CT urography is less useful in the newborn and others with decreased concentrating ability and uremia.

**62.4.9.4 Prenatal Diagnosis.** Urinary tract obstruction is diagnosed with increasing frequency during the fetal period by ultrasound. Fetal PUVs appear on ultrasound scans as a dilated bladder sometimes with a dilated posterior urethra (i.e. keyhole sign), variable dilatation of the upper urinary tract, and oligohydramnios, the severity of which are dependent on the degree of obstruction. The risk for chromosomal aneuploidy after identification of bilateral hydronephrosis, which can be due to causes other than PUV, is 4% when it is an isolated finding, and 31% when there are associated structural anomalies (Table 62-10). Prenatal diagnosis with fetal sonograms at 18, 24, and 32 weeks of pregnancy can detect occurrence of bladder enlargement and incomplete emptying, hydroureter, pelvocaliectasis, and oligohydramnios. PUVs can also present postnatally; therefore, postnatal ultrasonographic evaluation and urethral endoscopy are indicated in suspicious cases.

**62.4.9.5 Management.** Management and treatment are dependent on the anomalies present and the degree of urinary tract obstruction. During pregnancy, in utero drainage of the obstructed fetal bladder may be performed at perinatal centers. Treatment is intended to prevent continued renal damage and complications of oligohydramnios. Before in utero intervention, it is imperative to determine if there is adequate residual renal function by measuring amniotic fluid electrolytes. Postnatally, primary ablation of the valves with transurethral fulguration is usually successful. When the anterior urethral lumen is too narrow for endoscopy, a temporary vesicostomy may be necessary. When renal impairment is present, upper tract drainage with cutaneous pyelostomy or high ureterostomy may be indicated,

with later reconstruction. Dialysis and renal transplantation may be necessary later in infancy, childhood, or adolescence.

Survival is dependent on the severity of the obstruction, the amount of residual renal function, and the presence of associated anomalies. Fetuses with severe oligohydramnios and renal dysplasia secondary to PUVs rarely survive the newborn period or are stillborn. There is increased mortality in infants presenting within the first 3 months of the newborn period because of renal dysplasia and insufficiency. In prenatally diagnosed cases with severe genitourinary abnormalities, 32% of fetuses die. Among those children presenting after 1 year of age, mortality is less than 10%. Mortality is higher in individuals with associated nonrenal congenital anomalies.

Studies of long-term outcome have identified three risk factors for late development of renal failure: (i) presentation before age 1 year, (ii) bilateral vesicoureteral reflux at the time of presentation, and (iii) daytime urinary incontinence at age 5 years. Long-term complications include recurrent urinary tract infection, persistent vesicoureteral reflux, and hydronephrosis; urinary incontinence after ablation of the PUVs; bladder dysfunction; polyuria; late onset of renal failure; infertility; and sexual dysfunction.

## 62.5 ERRORS OF MIGRATION AND POSITION

### 62.5.1 Horseshoe Kidney

Horseshoe kidneys are the most common type of fused kidneys, accounting for 90% (18). Horseshoe kidneys have been found by radiographs in 1 in 200 individuals and at postmortem examination in 1 in 450 individuals (18); the male to female ratio is 2–3:1. Fused kidneys are unable to ascend from the embryonic pelvic position; horseshoe kidneys have an equal amount of renal tissue on each side of the midline. Fusion is usually at the lower poles, and the ureters do not cross the midline before entering the renal sinuses.

**62.5.1.1 Clinical Features.** It is estimated that one-third of individuals with horseshoe kidneys remain asymptomatic (18). Horseshoe kidneys are found in asymptomatic individuals in the course of evaluations for associated structural anomalies, investigations for unrelated medical problems, or at abdominal operation.

The most common clinical symptoms are pain, hematuria, and urinary tract symptoms from obstruction of the ureters. Infants and children may present with a lower abdominal mass. In one study of individuals with horseshoe kidneys, 40% presented with ureteropelvic junction obstruction, 18% with renal stones, and 22% with urinary tract infection (105).

The calyces usually point posteriorly, with a vertical pelvic axis, because of failure of normal rotation. Hydronephrosis is the most commonly associated

genitourinary anomaly, being present in 45% of horseshoe kidneys. The hydronephrosis is associated with reflux in 59%, ureteral obstruction in 17%, and megaureter in 24%. Only 6.5% of the hydronephrosis is due to extrinsic ureteral compression. Other associated common genitourinary anomalies are hypospadias and cryptorchidism.

Nongenitourinary tract anomalies are present in one-third of individuals with horseshoe kidneys—79% of infants, 28% of children, and 4% of adults. Anomalies are found most frequently in the musculoskeletal, cardiac, and central nervous systems (89), and are associated with vertebral malformations, meningomyelocele, sacral agenesis, and anorectal atresia with fused kidneys.

**62.5.1.2 Genetics.** Horseshoe kidneys are usually sporadic or related to the underlying genetic, chromosomal, or syndromic disorder (Table 62-13). Familial recurrence has been reported, as has concordance in monozygotic twins.

Three embryologic explanations for renal fusion may account for horseshoe kidneys: (i) primary fusion at gestational weeks four to six may occur because of close proximity of the lower poles of the kidneys, leading to adhesion of parenchymal elements from the two nephrogenic cords, or development of a fused kidney from a single nephrogenic cord that interacts with separate ureteric buds arising from two wolffian ducts; (ii) secondary fusion may occur as late as gestational weeks seven to nine because of disruption of vascular blood supply or premature ablation of the polar arteries during upward migration, resulting in partial necrosis and subsequent fusion of the kidneys; (iii) usually the lower poles of the two kidneys fuse before crossing the iliac arteries at 7–9 weeks gestation. Normal renal ascent from the pelvic area is prevented when the fused kidneys reach the junction of the aorta and inferior mesenteric artery.

**62.5.1.3 Diagnosis and Differential Diagnosis.** Initial investigation with ultrasound can identify the basic renal anatomy, evidence of obstruction, and cystic dilation of the calyces (89). CT urography or MRI is especially helpful in defining the anatomy of the ureters and kidneys. Angiography to define the blood supply is important if surgery is anticipated. Radioisotope tests can be useful in the initial and follow-up evaluations of renal function. Retrograde pyelography may be necessary to determine the entire course of the ureters. Crossed fused renal ectopy, lump kidney, and pancake kidney can present as an abdominal mass with similar urinary tract anomalies. The structural difference is apparent on ultrasound scans.

**62.5.1.4 Prenatal Diagnosis.** Routine fetal sonogram does not consistently identify horseshoe kidneys in the fetus. Fetal horseshoe kidneys may be identified if there are renal anatomic anomalies (e.g. obstructed ureters, relatively low placement of the kidney in the pelvis, or cysts), other associated anomalies, or chromosomal disorders (e.g. Turner syndrome) present.

**62.5.1.5 Management.** Only rarely is death of individuals with horseshoe kidneys attributable to the renal

**TABLE 62-13 Disorders Associated with Horseshoe Kidneys, Renal Ectopy, and Fusion**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Acrorenal	Hypertelorism, micrognathia, minor skeletal anomalies, horseshoe kidney	Renal agenesis, crossed ectopia	?AD (102520)	
Asplenia with cardiovascular anomalies	Bilateral right or left embryonic primordia, asplenia, polysplenia, complex heart anomalies, situs inversus	Renal dysplasia, fused/ectopic kidneys, horseshoe kidneys	AR (208530)	
Beckwith–Wiedemann	Omphalocele, macroglossia, organomegaly, islet cell hyperplasia, adrenal cytomegaly, embryonic tumors, macrosomia	Nephromegaly, renal medullary dysplasia, Wilms tumor, nephrocalcinosis	AD (130650)	<i>CDKN1C</i> imprinting error, uniparental disomy, duplication, inversion or translocation of 11p15.5
Branchio–oto–renal	Mixed hearing loss, Mondini cochlear malformations, pinna anomalies, branchial cleft fistulas, preauricular pits	Renal dysplasia, agenesis, and ectopy; horseshoe kidneys, ureteral anomalies (but rarely duplications)	AD (113650)	<i>EYA1</i> <i>SIX1</i> , <i>SIX5</i>
Caudal dysplasia	Sacral agenesis/hypoplasia, lower limb and skeletal anomalies, anal atresia, genital anomalies	Renal dysplasia and agenesis; ureteral, urethral, and bladder anomalies; horseshoe and fused pelvic kidney	Heterogeneous, maternal diabetes in some patients	
Cerebro–renal–digital syndromes	Digital and limb anomalies, brain malformations, other anomalies (Table 62-6)	Renal dysplasia, ectopy, and agenesis, ureteral anomalies, fused kidneys	Heterogeneous	
CHARGE syndrome	Coloboma, heart defect, choanal atresia, mental retardation, genital hypoplasia, ear anomalies, deafness	Cystic renal dysplasia, renal agenesis, ureteral anomalies, fused or ectopic kidneys	AD (214800)	<i>CHD7</i>
Chromosome abnormalities	See Table 62-2			
Cloacal exstrophy	Persistent cloaca, exstrophy of the cloaca, failure of fusion of genital tubercles, omphalocele, vertebral defects, spina bifida cystica, abnormal internal and external genitalia	Duplication of urethra, urethral and ureteral anomalies, exstrophy of bladder, renal dysplasia, agenesis and ectopy	Heterogeneous	
DK-phocomelia	Phocomelia, encephalocele, absent corpus callosum, thrombocytopenia	Renal agenesis, fused/ectopic kidney, ureter anomalies	AR (223340)	<i>SALL4</i>
Duane–radial ray syndrome	Radial defects, coloboma, duane anomaly	Renal malrotation/ectopy, partial horseshoe kidney	AD (607323)	
Dysgnathia complex, (Agnathia holoprosencephaly)	Agnathia, synotia, microstomia, holoprosencephaly, cleft lip/palate	Renal agenesis, fusion, or hypoplasia	AD (202650)	
Early amnion rupture (ADAM, amniotic bands)	Digital and limb amputations, ring constrictions, facial clefts, body wall deficiency, brain and other anomalies	Renal dysplasia, agenesis, and ectopy; ureter anomalies, fused/ectopic kidneys, horseshoe kidneys	Sporadic	
Faciocardiorenal syndrome (Eastman–Bixler)	Severe mental retardation, characteristic facies, heart defect	Horseshoe kidneys, hydroureter	AR (227280)	

**TABLE 62-13 Disorders Associated with Horseshoe Kidneys, Renal Ectopy, and Fusion—cont'd**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Fanconi pancytopenia	Anemia, pancytopenia, hyperpigmentation, radial aplasia/hypoplasia, microcephaly, short stature, variable microphthalmia, other anomalies, chromosome breakage	Renal dysplasia, absent kidney (39%), duplication of pelvis and/or ureter, ectopic or horseshoe kidney, hydronephrosis, renal cysts	AR (227650)	<i>FANC</i> (A, C–G, I, J, L–P)
Hand–foot–uterus	Hypoplasia of thumbs and great toes, duplication of female internal genitalia, chordee and hypospadias in males	Ectopic ureteral orifices, hydronephrosis, epididymal cysts, ectopic/fused kidneys, intravaginal urethra	AD (140000)	<i>HOXA13</i>
Hemifacialmicrosomia (oculoauriculovertebral spectrum, Goldenhar)	Facial asymmetry, epibulbar dermoid, coloboma, anotia, preauricular tags, deafness, vertebral anomalies, heart anomalies, variable brain malformations	Renal dysplasia, agenesis, ectopy; hydroureter; abnormal blood supply to kidney; hydronephrosis	Heterogeneous AD (164210)	
Limb–body wall complex	Lateral body wall deficiency, limb reduction anomalies, neural tube defects, severe scoliosis, amniotic entanglements, heart and other anomalies	Renal agenesis or dysplasia, bladder extrophy, fused/ectopic kidneys, ureter anomalies	Sporadic	
McKusick–Kaufman	Hydrometrocolpos, transverse vaginal membrane, vaginal septum, postaxial polydactyly, cardiac anomalies, hypospadias	Hydroureter, ureteral duplication, urogenital sinus, ectopic/fused kidneys ectopic urethra, horseshoe kidneys	AR (236700)	<i>BBS6</i>
MURCS association	Müllerian duct aplasia, renal aplasia, cervicothoracic somite (vertebral) defects, vaginal absence, hypoplastic uterus, short stature	Renal agenesis or dysplasia, ectopia, ureteral anomalies	Sporadic (601076)	
Neural tube defect spectrum	Meningomyelocele, anencephaly, encephalocele, vertebral anomalies, midline anomalies	Renal agenesis, hypoplasia, dysplasia, and fusion; ureteral anomalies	Heterogeneous, commonly multifactorial	
Orocraniodigital syndrome (Juberg–Hayward)	Cleft lip–palate microcephaly, hypoplastic thumbs, elbow deformities	Horseshoe kidneys	?AR (216100)	
Radial–renal syndrome	Hand and limb anomalies, heart anomalies, tracheo-esophageal fistula, split hand/foot	Ureteric reflux, crossed, renal ectopia	AD (179280)	
Roberts (SC phocomelia)	Tetraphocomelia, cleft lip/palate, severe psychomotor retardation, neonatal lethal; chromosomes with premature centromere separation	Renal dysplasia and agenesis, hydronephrosis, horseshoe kidney	AR (268300)	<i>ESCO2</i>
Rubinstein–Taybi	Broad thumbs and toes, distinctive facial features, mental retardation, microcephaly, cryptorchidism, small phallus	Renal dysplasia, absent or extra kidneys, double renal pelvis, renal calculi, posterior urethral valves, abnormal bladder shape, fused/ectopic kidneys	AD (180849)	<i>CREBBP</i>

Continued



**TABLE 62-13 Disorders Associated with Horseshoe Kidneys, Renal Ectopy, and Fusion—cont'd**

Disorder	Major Features	Renal Anomaly	Etiology	Genes
Sacral defect with anterior meningocele (sirenomelia)	Single lower limb, sacral agenesis, anal atresia, uterine/vaginal anomalies, cardiac anomalies	Urethral atresia, ectopic urethra, posterior urethral valves, renal agenesis, cystic dysplasia, ureteral and bladder anomalies	AD (600145)	<i>VANGL1</i>
Smith–Lemli–Opitz	Microcephaly, postaxial polydactyly, ambiguous genitalia, facial dysmorphism, 2–3 toe syndactyly, other anomalies	Unilateral renal agenesis, renal dysplasia and fusion	AR (270400)	<i>DHCR7</i>
Twin reversed arterial perfusion (TRAP sequence)	Co-twin with incomplete development of all organs, limbs and body form; upper body more severely affected than lower body	Renal agenesis, hypoplasia, and cystic dysplasia; anomalies of renal fusion/ectopy, horseshoe kidneys; anomalies of ureter, bladder, and urethra	Sporadic, in MZ twins and triplets	

AD, autosomal dominant; AR, autosomal recessive; MZ, monozygotic.

anomaly. Treatment of hydronephrosis and vesicoureteral reflux medically or surgically, when indicated, can greatly reduce associated clinical symptomatology and renal damage. Long-term outcome and mortality are largely determined by the severity of associated anomalies and the presence of disorders of multiple congenital anomalies.

Compared with the general population the risk for renal tumors is increased. Wilms tumor is most frequently reported; others reported include adenocarcinoma, teratomas, and transitional cell tumors (18,89).

## 62.6 ERRORS RESULTING IN OBSTRUCTION

Cystic changes in the kidney can result from obstruction or dysfunction of urinary outflow. Obstruction during embryogenesis and the early fetal period can result in kidney changes that can be difficult to distinguish from renal dysplasia. Obstruction during the late fetal period, infancy, and childhood results in a more characteristic appearance of the “obstructive kidney” with caliectasis and parenchymal scarring. Spina bifida is a common cause of ureteral and bladder dysfunction leading to obstructive changes of the urinary tract. Renal cystic changes caused by obstruction of urinary outflow occurring during the embryonic and fetal period are known as Potter type IV or peripheral cortical cystic dysplasia.

### 62.6.1 Clinical Features

The clinical presentation and course are dependent on the degree of renal parenchymal loss and the underlying etiology of the renal obstruction. The most common anomalies resulting in obstructive kidney(s) are bladder

outlet obstruction (e.g. from PUVs), hydronephrosis due to reflux or vesicoureteral junction obstruction, pelvicaliectasis due to ureteropelvic junction obstruction, ureteral duplication, ureteral strictures, and ureterocele.

Hydronephrosis can result from abnormal innervation of the ureters and bladder (e.g. myelomeningocele), as well as delay in maturation of ureteral muscle function and autonomic muscle weakness (e.g. mitochondrial myopathy). Other renal malformations, such as horseshoe kidney, which are associated with ureteral anomalies, can result in a mixed appearance of renal dysplasia from an error in embryogenesis and secondary changes in the kidney due to obstruction and urinary reflux.

Clinical symptoms are typical of those caused by obstruction, such as urinary tract infections, hematuria, hypertension, oliguria, and, prenatally, oligohydramnios (i.e. Potter) sequence. Other structural anomalies, such as meningo-myelocele, resulting in obstruction, are usually evident on examination. Segmental hypoplasia or Ask-Upmark kidney is characterized by one or more atrophic lobes, which appear as depressions or grooves on the capsular surface of the kidney. The majority of patients with Ask-Upmark kidney will have vesicoureteral and intrarenal reflux, presenting with hypertension or urinary tract infection.

### 62.6.2 Prenatal Diagnosis

Hydronephrosis, pelvicaliectasis, and obstructive cystic changes of the kidneys frequently present during the fetal period. However, fetuses and infants with high-grade vesicoureteral reflux might have normal renal ultrasounds. Dilatation of the ureters is readily detectable by fetal sonogram. Compared with the overall maternal age-related risk, the risk for fetal chromosomal abnormalities is three

times higher when there is an isolated hydronephrosis, and 30 times higher when there are associated malformations of other organ systems (106) (Table 62-10). For families at risk for fetuses affected with autosomal dominant hydronephrosis, serial ultrasound scans are recommended, since obstruction increases as fetal renal function improves. Fetal ultrasound is very reliable for prenatal diagnosis of obstruction of the urinary tract. In utero treatment of eligible fetuses is offered only in a few centers and remains experimental. Fetuses with evidence of significant hydronephrosis and some degree of oligohydramnios with potentially reversible renal damage, who do not have underlying disorders with a poor prognosis, are the best candidates for in utero drainage with catheter placement or fistulae formation between the dilated ureter and the amniotic sac.

### 62.6.3 Genetics

Autosomal dominant inheritance of unilateral hydronephrosis has been reported in families. Simpson and German (107) described seven families with multiple cases of urinary tract anomalies, mostly with obstructive uropathy, and reviewed cases in sibs, twins, and other relatives. Familial occurrence has also been reported for bilateral megalo ureters with hydronephrosis and for hydronephrosis due to ureteropelvic junction stricture, aberrant vessels, duplication of the ureter, unilateral hydronephrosis, and ureterocele. Ureteral anomalies and secondary obstruction are common and do not usually have enough specificity to be helpful in making a diagnosis of a specific disorder. Disorders commonly associated with ureteral anomalies and secondary obstructive renal cystic disease are discussed in previous sections.

Izquierdo and colleagues (108) found linkage to human leukocyte antigen (HLA) by analyzing five families with hereditary pelviureteric junction obstruction. Maximal lod scores were 3.090 at a recombination fraction of 0.11 with full penetrance, and 2.486 at a recombination fraction of 0.1 with 90% penetrance. Use of the HOMOG program suggested genetic heterogeneity with one locus on 6p in four of the families and a different locus in one family. After exclusion of the unlinked family, two-point analysis gave a maximal lod score of 3.9 at a recombination fraction of 0.05 with full penetrance, and 4.2 at a recombination fraction of 0.0 with 90% penetrance. Further evidence for the 6p locus being important in hydronephrosis is the report by Fryns and colleagues (109) of a de novo chromosome translocation t(6;19)(p23.1;q13.4) in a fetus with oligohydramnios and bilateral massive hydronephrosis and multicystic renal dysplasia secondary to pelviureteric junction obstruction.

### 62.6.4 Primary Vesicoureteral Reflux and Reflux Nephropathy

Vesicoureteral reflux has been comprehensively reviewed by Williams et al. (110). Primary vesicoureteral reflux refers

to the retrograde flow of urine from the urinary bladder toward the kidney. It is estimated to occur in about 1% of children, and in the majority of cases, it clears over time. Vesicoureteral reflux is usually discovered during imaging investigations of children with urinary tract infections. A small proportion of these patients progress to end-stage renal disease. This progressive condition, known as reflux nephropathy, consists of calyceal clubbing or deformity with overlying corticomedullary scarring. Vesicoureteral reflux in utero has been associated with renal hypoplasia and dysplasia; lesions seen in the end-stage kidney include chronic tubulointestinal nephritis and focal glomerulosclerosis.

**62.6.4.1 Pathogenesis of Primary Vesicoureteral Reflux.** The anatomic lesion is due to incompetence of the vesicoureteral junction. Normally the retrograde flow of urine from the bladder is impeded by the length of a submucosal ureteric “tunnel,” the size of the ureteric opening and the crossing of the bladder wall trigone muscles during voiding at the point at which the ureter inserts into the urinary bladder. The severity of vesicoureteral reflux is graded on a scale of I to V on voiding cystourethrography with a separate, more worrisome variant of intrarenal reflux where urine flows back into the renal parenchyma through the collecting ducts.

Developmentally primary vesicoureteral reflux (VUR) arises from disruption in complex signaling pathways and cellular differentiation that may also be influenced by environmental factors in utero. Abnormal ureteric budding and disturbed interactions between the ureteric bud and metanephric mesenchyme has been implicated from animal studies with knockout mice. Lu et al. (111) found evidence for defective SLIT-ROBO signaling pathways in the pathogenesis of a subset of human VUR. ROBO2 plays a key role in the formation of the ureteric bud; its inactivation in mice leads to supernumerary ureteric buds, lack of ureter remodeling, and improper insertion of the ureter into the bladder. Bertoli-Avella et al. (112) found a relatively high frequency of ROBO2 variants in familial cases of primary VUR in their cohort of patients. Animal models have suggested that RET-mediated signaling pathways are required for the final formation of the vesicoureteral junction. Yang et al. (38) found that a RET GLY691Ser mutation is associated with primary vesicoureteral reflux in a French-Canadian population from Quebec.

**62.6.4.2 Genetics.** Autosomal dominant inheritance with variable expression or multifactorial inheritance has been implicated for primary vesicoureteral reflux and reflux nephropathy (110). In a study of first-degree relatives (51 parents and 43 siblings) of 26 affected children, an overall risk of 17% was found, with 23% of siblings and 12% of parents having evidence of reflux nephropathy or vesicoureteral reflux. The risk for other family members being affected appears to be higher if the proband presents with renal failure. Kaefer and colleagues (113) found 100% concordance among very young monozygotic twins and 50% concordance among dizygotic twins. Feather and

colleagues (114) found that VUR mapped to a locus on chromosome 1 in several European families whose pedigree was consistent with dominant inheritance. As adults, 45% of these affected individuals will have vesicoureteral reflux or reflux nephropathy or both, and in 15%, renal failure will develop. Many clinicians recommend radionuclide voiding cystography as a screening investigation of all first-degree relatives. Weng et al. (115) performed a genome-wide linkage scan in 12 large families with primary VUR and found a linkage signal on chromosome 12p11-q13 suggesting that recessive inheritance may be operative in certain cases.

The HLA haplotypes A2-B8 and A9-B12 are more frequent in patients with reflux nephropathy than in patients with other end-stage renal diseases. Kuroda et al. (116) found that individuals with the transforming growth factor-beta1-509CC and 869TT genotype may have an increased susceptibility to vesicoureteral reflux.

**62.6.4.3 Diagnosis and Differential Diagnosis.** Diagnostic investigations include a voiding cystourethrogram to demonstrate reflux, CT urography or nuclear scan for detection of renal scarring, and CT urography or renal ultrasonography for detection of structural anomalies. An international classification system is used for grading of vesicoureteral reflux (110).

**62.6.4.4 Management.** The clinical course is dependent on the degree of renal parenchymal loss and the underlying etiology for the renal obstruction. Patient management is directed toward reducing ongoing damage to the kidneys from reflux nephropathy, and preventing recurrent infection through the use of prophylactic antibiotics (117, 119). Children with high grades of vesicoureteral reflux may be treated with surgical interventions such as ureteral reimplantation. The long-term use of ACE inhibitors does not seem to reduce the progressive loss of renal function (118).

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Cystic Diseases of the Kidney; Nephrotic Disorders; Renal Tubular Disorders; Cancer of the Kidney and Urogenital Tract.

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# CHAPTER

# 63

## Cystic Diseases of the Kidney

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### 63.1 INTRODUCTION

Cystic diseases of the kidney are some of the most significant monogenic causes of renal morbidity and mortality in both pediatric and adult populations. There have been considerable advances in the past decade in identifying and characterizing the genes responsible for these disorders. The genetic aspects, molecular pathogenesis, clinical features, and management of these disorders will be highlighted in this chapter. Although cystic renal disease can be acquired, the primary aim of this chapter will be to focus on those forms that are inherited.

### 63.2 AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE (MIM 173900)

#### 63.2.1 Clinical Features and Natural History

The dominant form of polycystic kidney disease (PKD), once called “adult polycystic kidney disease,” is one of the most significant monogenic causes of morbidity and mortality in the adult population. It is estimated to affect 12.5 million people worldwide with a prevalence of 1/800 live births and accounts for 5–10% of all cases of adult end-stage renal disease (ESRD) (1). Two genes, *PKD1* and *PKD2*, have been identified to cause autosomal dominant polycystic kidney disease (ADPKD). Patients with a *PKD1* mutation have earlier onset of disease and more complications, reach ESRD faster, and die sooner than patients with *PKD2* mutations.

ADPKD is usually asymptomatic until adulthood, typically beginning in the fourth to fifth decade of life. Hematuria, renal colic, recurrent urinary tract infection (UTI),

lower back discomfort, and hypertension are common presenting symptoms. Progression to ESRD is an invariant feature of the disease. Factors that predict earlier onset of ESRD include hypertension, proteinuria, and mutations in *PKD1*. Women with *PKD2* mutations preserve renal function longer than men with the same mutations, but no gender difference is observed with *PKD1* mutations. Median age of onset of ESRD or death is 53 years in *PKD1* patients and 69 years in *PKD2* patients (2).

Although ADPKD is a completely penetrant disorder, a significant degree of clinical variability exists. Approximately 50% of patients develop hypertension with a mean age of onset of 31 years, long before the development of ESRD and sometimes before the discovery of renal cysts (1). Cyst enlargement and compression of normal parenchyma is hypothesized to decrease intrarenal perfusion and activate the renin–angiotensin–aldosterone (RAA) system, leading to hypertension (3). However, this theory does not adequately explain why the age- and gender-adjusted prevalence of hypertension is four times greater in those with *PKD1* compared to *PKD2* mutations. About 25–30% of patients develop cyst infection, and 18–25% of patients develop gross hematuria from cyst hemorrhage. Nephrolithiasis, seen in 10% of all patients, occurs with equal frequency in *PKD1* and *PKD2*, and is due mostly to either oxalate or urate stones (2). Expanding cysts compressing the collecting system producing urinary stasis, along with low urine pH and hypocitraturia, contribute to stone formation.

Hepatic cysts present approximately 10 years after the development of renal cysts. The number and size of these cysts increases with age. Approximately 80% of ADPKD patients have hepatic cysts by age 50 (4,5). Female gender and increased gravidity also correlate with increased



burden of liver involvement. Polycystic liver disease is rare in children and is typically asymptomatic. Symptoms are being seen more commonly as the life span of ADPKD patients has lengthened with dialysis and transplantation (6). The primary complication of polycystic liver disease is severe hepatomegaly with abdominal discomfort and shortness of breath; hepatic function is preserved. Cyst infection, hemorrhage, and post-traumatic rupture occur much less often. Portal hypertension with ascites, variceal bleeding, and encephalopathy are very rare but are perhaps the most significant hepatic sequela of ADPKD (7).

Cardiac disease, specifically mitral valve prolapse (MVP), hemodynamically significant mitral regurgitation (MR), or aortic insufficiency (AI), is also seen in patients with ADPKD. The estimated prevalence of MVP is 25–30%, MR 13%, and AI 8% (8–10). Valvular changes are due to myxomatous degeneration and loss of collagen. Patients with hypertension also develop compensatory left ventricular hypertrophy. Dilatation of the aortic root and thoracic aortic dissection have also been reported. Although abdominal aortic aneurysms and dissection were thought to occur with increased frequency in ADPKD patients, subsequent studies have shown that this is not the case (11).

Intracranial aneurysms, typically small saccular aneurysms of the circle of Willis, are found in approximately 10% of all ADPKD patients and are less common in those younger than 30 years (12,13). PKD1 and PKD2 patients are equally at risk (14). Aneurysms are found in 22–25% of those with a family history of aneurysms and ADPKD, and 6% of those without a family history (15,16). Most aneurysms are small, with 90% less than 10mm in diameter and 70% less than 6mm in diameter (17). Over 95% of aneurysms identified in asymptomatic screening are less than 6mm in diameter (15–19). Aneurysm surveillance studies indicate that aneurysms smaller than 10mm have a low rupture risk of 0.05% per year, but since the studies were done on the general population, it is uncertain whether this low risk can be applied to ADPKD patients alone (20).

Aneurysmal rupture results in subarachnoid hemorrhage (SAH), perhaps the most sudden and devastating complication of ADPKD. Symptoms of rupture are no different from those in non-ADPKD patients and include sudden-onset severe headache, acute focal neurologic deficit, altered mental status, nausea, vomiting, or photophobia. The estimated immediate mortality of such an event is 10%, with 38% long-term, permanent morbidity in survivors (21). The mean age of rupture is 40 years, with 65–75% of all ruptures occurring prior to age 50 (13). About 10% of aneurysm rupture occurs in ADPKD patients younger than 20 years (22). Recognition of symptoms of SAH and emergent neurosurgical intervention are crucial for survival and reduction of long-term morbidity.

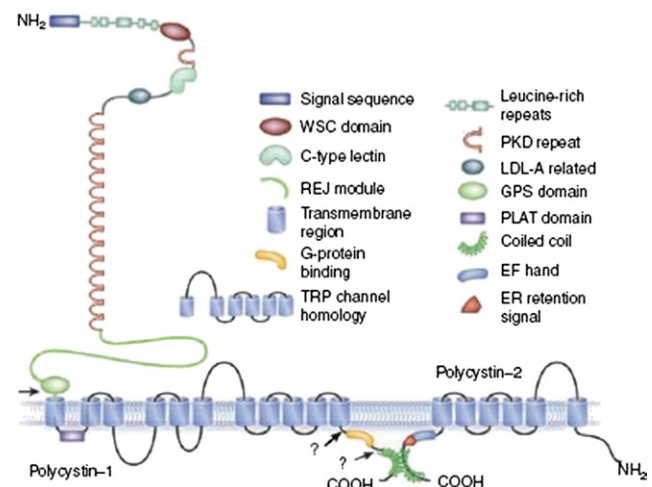
Colonic diverticular disease has been reported to occur with increased frequency in ADPKD patients with ESRD awaiting transplant, but not in patients without ESRD (23,24). Diverticulitis, if not recognized and

treated promptly, can lead to colonic perforation. Ventral and inguinal hernias have also been documented in ADPKD patients with ESRD (25–27).

In males, cysts can be found in the seminal vesicles, epididymis and prostate. Sperm abnormalities and defective motility can be also seen but rarely cause male infertility (28). Cysts of the pancreas are rare and usually asymptomatic. However, patients may develop recurrent pancreatitis and intraductal papillary mucinous tumor or carcinoma. Arachnoid cysts may occur and present with headache, diplopia, hearing loss and ataxia due to cerebrospinal fluid leak (29).

### 63.2.2 Gene Mapping, Structure, Function, and Genotype–Phenotype Correlations

Early studies demonstrated linkage between the *PKD* locus and the  $\alpha$ -globin locus on chromosome 16p. Subsequent linkage studies refined the locus to a 750 kilobase (kb) segment at 16p13 (30,31). The presence of several *PKD* pseudogenes made direct positional cloning difficult, but in 1995, sequencing of the *PKD* cDNA showed a 14,148 bp transcript with 46 exons spanning 52kb of genomic DNA (32). The *PKD* protein, initially termed polycystin and renamed polycystin-1 after the discovery of a second *PKD* locus, is a plasma membrane protein 4304 amino acids long weighing 463 kilodaltons (kDa) (Figure 63-1). The N-terminal extracellular region is composed of an aggregation of multiple domains involved in protein–protein and protein–carbohydrate interactions, while the C-terminal intracellular region ends in a series of protein–protein interaction motifs called coiled-coil regions. In addition to the extracellular and intracellular signaling regions, polycystin-1 contains a sea urchin receptor for egg jelly (suREJ) domain. This domain offers some insight into the function of the protein. The sea urchin homolog is involved in the activation of the sperm's acrosome reaction (33). Contact of



**FIGURE 63-1** Torress, V. E.; Harris, P. C. Autosomal Dominant Polycystic Kidney Disease: The Last 3 Years. *Nature* 2009, 76 (2).

the sperm with the egg's zona pellucida results in calcium ion influx triggered by the suREJ protein, leading to release of proteolytic enzymes and polymerization of globular actin.

Several years after linkage of PKD to the  $\alpha$ -globin locus, two families with PKD were described who did not link to chromosome 16p (34,35). Clinical characteristics of these families were indistinguishable from those linking to *PKD1*. Identification and study of more PKD families unlinked to the first PKD locus resulted in the discovery of a 680kb segment on chromosome 4q21, from which the *PKD2* gene, encoding the polycystin-2 protein, was cloned (36). The *PKD2* gene consists of 15 exons spanning 68kb and encodes a 5.4kb mRNA. The polycystin-2 translated product, a 110kDa protein, contains 968 amino acids and 6 transmembrane domains (Figure 63-1). Polycystin-2 belongs to a subfamily of transient receptor potential channels and functions as a calcium channel. The C-terminal intracellular region shares homology with the C-terminal region of *PKD1*, and co-immunoprecipitation studies indicate that *PKD1* and *PKD2* interact with one another via their C-terminal coiled-coil regions (37,38).

Polycystin-1 and polycystin-2 are ubiquitously expressed, at higher levels during embryonic development, then at lower levels during postnatal and adult life (39–41). mRNA expression is observed in the brain, cardiac and skeletal muscles, smooth muscle of blood vessels, vascular endothelium, breast, lung, liver, pancreas, kidney, and reproductive organs (42,43). Within the fetal kidney, the two proteins are expressed in maturing renal tubular epithelium and at lower levels at the distal ureteric buds. Both proteins are required for normal embryonic development; *PKD1* knockout mice die at birth because of pulmonary hypoplasia from massive cystic enlargement of the kidneys and pancreas while the *PKD2* knockout dies in the late embryonic period with abnormalities in cardiac atrioventricular septation and cysts in the kidneys and pancreas (44,45). Postnatally, the proteins are seen in the distal convoluted tubular and collecting duct epithelia.

Mutations in *PKD1* account for 85% of cases of ADPKD, while most of the remaining mutations are found in *PKD2* (46). To date, 864 germline *PKD1* mutations have been reported (47), most of which result in protein truncation. Of the 436 mutations that are classified as pathogenic, 134 (31%) are frameshift mutations, 110 (25%) nonsense mutations, 95 (22%) substitutions, 45 (10%) splice site mutations, and 36 (8%) insertions and deletions. The high mutation rate in the *PKD1* gene is thought to be related to the large polypyrimidine tract in intron 21, the longest such tract known in the human genome (48). The high cytosine and thymine content allows for triple-helix formation, which can result in mutagenesis.

Like mutations in *PKD1*, a large majority of germline *PKD2* mutations result in a truncated protein

product. A total of 139 mutations have been described, of which 115 have been classified as pathogenic (47). Of these, 50 (43%) are frameshift mutations, 30 (26%) nonsense mutations, 17 (15%) splice site mutations, 11 (10%) missense mutations, and 6 (5%) insertions or deletions. While the mutations are located throughout the *PKD2* gene, a slight clustering of mutations is seen in exons 4, 5, and 6, which make up 24.2% of the cDNA sequence but account for 38.6% of germline mutations. There are several unrelated families (by ethnicity and haplotype analysis) with identical mutations, indicating a few mutation-prone “hot spots,” but most *PKD2* germline mutations are unique to the affected family.

A very small percentage (<1%) of ADPKD families do not demonstrate linkage to either *PKD1* or *PKD2* (49). The existence of a third ADPKD gene has been suggested, but there has been no definitive evidence to support this hypothesis. Common confounders, such as genotyping errors, nonpaternity, and misdiagnosis (including phenocopies and nonpenetrance) may explain this apparent disparity (49).

Although all cells in an ADPKD kidney inherit the same germinal mutation, histopathologic studies indicate that only 1–5% of all nephrons have cysts (50). Experience gained from *Rb* mutations and hereditary retinoblastoma led to the hypothesis that a “second hit,” or somatic inactivation of the normal PKD gene, is necessary to trigger cystogenesis in both the kidney and the liver. The second hit hypothesis was confirmed in both *PKD1* and *PKD2* patients. Clonal loss of heterozygosity, specifically, loss of the normal *PKD1* haplotype, was demonstrated in cysts isolated from *PKD1* kidneys (51,52), and the same was demonstrated for *PKD2* kidney and liver (53). Truncating and missense mutations from the normal *PKD1* haplotype were discovered (54). Cysts from *PKD2* kidneys and livers were also found to have truncating mutations in the inherited wild-type *PKD2* gene (53,55). These studies indicate that, while inherited in a dominant fashion, ADPKD is recessive at the cellular level. In addition, a trans-heterozygous state can arise when compound heterozygous mutations occur in both *PKD1* and *PKD2* (54,56).

With regard to genotype–phenotype correlations, it is well known that mutations in *PKD1* are associated with more severe disease than those in *PKD2*. Individuals with *PKD1* mutations are diagnosed at an earlier age, have a higher incidence of hypertension and hematuria, and progress to ESRD on average 20 years earlier (2,57). *PKD1* kidneys are significantly larger than age-matched *PKD2* kidneys because of a greater number of cysts rather than the rate of cystic expansion (58,59). This finding is consistent with the two-hit model of cystogenesis as *PKD1* is more prone to mutation.

Mutations of any type at the 5' end of *PKD1* are correlated with decreased renal survival, earlier onset of ESRD, and increased risk of intracranial aneurysms

compared to mutations at the 3' end of the gene. There appears to be no significant difference in the severity of disease between families with truncating, in-frame, or missense deletions (14,60). With *PKD2*, splice site mutations appear to produce milder renal symptoms compared with other types of mutations (61). Excepting this association, no other genotype–phenotype correlations have been determined from mutational analysis (62,63). There is, however, a gender effect observed with *PKD2* disease, with females having milder renal involvement than males (61).

In addition to the phenotypic variation observed in families with different mutations, individuals within a family can manifest with differing degrees of clinical severity (63). Genetic modifiers have been purported to play a role in this intrafamilial variability, and their effect has been estimated to be between 18 and 59% in those with *PKD1* mutations (64). Thus far, association studies of the angiotensin-1-converting enzyme (*ACE*) gene and the endothelial nitric oxide synthase (*ENOS*) gene have shown equivocal results (65–68). One clear example of genetic background affecting phenotype expression is the contiguous gene deletion syndrome involving the tuberlin (*TSC2*) and *PKD1* genes, both located on chromosome 16p13.3 (Figure 63-2) (69). Affected individuals suffer from tuberous sclerosis and have severe polycystic kidney and liver disease typically arising in infancy to early childhood with rapid progression to ESRD. In the past, tuberlin was thought to function in localizing polycystin-1 to the plasma membrane (70). More recent studies have shown that the cytoplasmic tail of polycystin-1 directly interacts with tuberlin in the mammalian target of rapamycin (mTOR) pathway, and inhibition of this pathway reverses renal cystogenesis (71).

Nongenetic factors also contribute to phenotypic variation. Hormonal effects have been described, including the predominance of cystic liver disease in females due to the effects of estrogen (72). Smoking and other

promutagenic factors may confer higher risk by increasing the chance for somatic mutations, thereby providing the “second hit” (73).

### 63.2.3 Laboratory, Imaging, and Histopathologic Findings

Serum electrolyte studies are normal unless there is ESRD. Urinalysis reveals microscopic or gross hematuria in about 40–45%, especially if there is cyst hemorrhage (74). Dipstick proteinuria is present in 20% of patients and microalbuminuria in about 35% (75). Liver transaminases, alkaline phosphatase, and markers of hepatic synthetic function such as prothrombin time and partial thromboplastin time are usually normal even with severe cystic disease.  $\gamma$ -Glutamyltransferase levels correlate with hepatic cyst burden (7).

Abdominal ultrasound is the modality of choice for diagnosis of ADPKD. The Ravine criteria are highly specific (near 100%), but sensitivity is age dependent, i.e. the longer an individual remains negative, the lower the likelihood of disease (Table 63-1). Sensitivity is lower in *PKD2* patients less than 30 years of age (67%) because of later age of onset; therefore, an individual at risk for *PKD2* must undergo either molecular diagnostic testing or ultrasound surveillance continued beyond the age of 30 (76). Enlarged kidneys with multiple anechoic cysts

TABLE 63-1

Age in Years	PKD1 (%)	PKD2 (%)	Unknown Genotype (%)
15–30 <sup>1</sup>	PPV: 100 SEN: 94.3	PPV: 100 SEN: 69.5	PPV: 100 SEN: 81.7
30–39 <sup>1</sup>	PPV: 100 SEN: 96.6	PPV: 100 SEN: 94.3	PPV: 100 SEN: 95.5
40–59 <sup>2</sup>	PPV: 100 SEN: 92.6	PPV: 100 SEN: 88.8	PPV: 100 SEN: 90

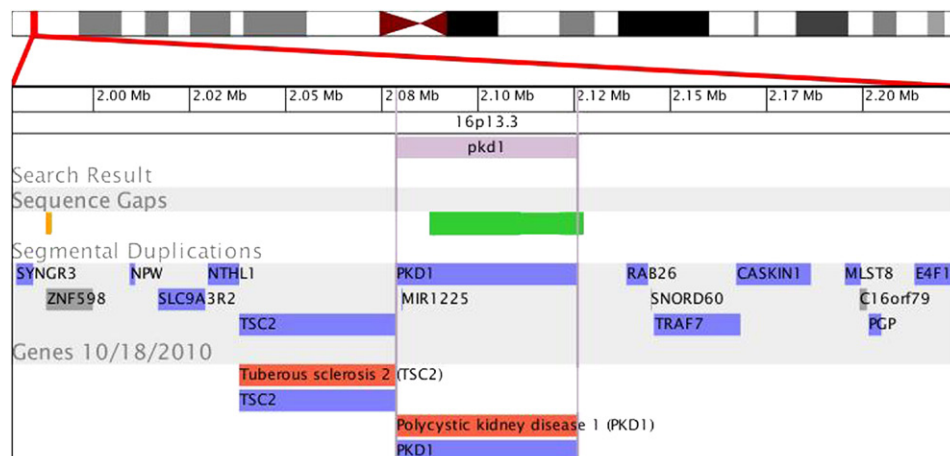


FIGURE 63-2 Figure of the genes *PKD1* *TSC2*. Signature genomic genome browser.

of varying sizes throughout the cortex distorting normal pelvicalyceal architecture are seen. Cyst hemorrhage or infection may be visualized as echogenic debris within the cyst. Hepatic, pancreatic, and splenic cysts can be visualized with ultrasound as well. Computed tomography (CT) with intravenous contrast or magnetic resonance imaging (MRI) can be used for diagnosis of ADPKD when higher resolution imaging is required.

Renal biopsy of patients with ADPKD is not recommended because of risk of bleeding. Gross renal specimens are enlarged with cysts present over the surface of the kidney. Cut specimens reveal cysts throughout the thickness of the kidney, filled with straw-colored, serosanguinous, or brown fluid. Infected cysts are filled with purulent material. Microscopically, interstitial fibrosis and intact nephrons can be seen in the parenchyma between cysts. Cysts are lined with renal epithelium originating primarily from the proximal convoluted tubule and collecting duct. Hepatic cysts are lined with biliary duct epithelium.

### 63.2.4 Molecular and Prenatal Diagnosis

As mentioned earlier, abdominal ultrasound is the diagnostic test of choice, especially for individuals at risk for PKD1. CT or MRI may be used as an adjunct if ultrasound is equivocal and higher resolution studies are required. Molecular diagnostic testing is commercially available for both *PKD1* and *PKD2*. Sequence analysis has a mutation detection rate of approximately 90% for *PKD1* (46). An additional 4% of patients may have gross rearrangements, which are detectable using multiplex ligation-dependent probe amplification (77). For *PKD2*, over 90% of mutations are identified with direct sequence analysis. There are limitations to molecular testing, however. Most *PKD1* and *PKD2* mutations are “private mutations,” with very few recurrent mutations. Consequently, sequencing results must be interpreted with caution, as some sequence variations may represent polymorphisms that are not pathogenic. In fact, each individual is known to carry more than 10 polymorphic variants in the *PKD1* gene (78). In addition, exon sequencing does not detect mutations in the noncoding regions of the genes.

Prenatal diagnosis can be performed via analysis of cells obtained from chorionic villus sampling or amniocentesis if linkage to a specific haplotype or disease-causing mutation has already been established. However, due to the availability of treatment, lack of cognitive impairment, and adult onset of disease, prenatal diagnosis is usually not performed unless there is a family history of a severely affected infant.

### 63.2.5 Management

Since there is no definitive treatment for the underlying genetic defect of ADPKD, management is limited to surveillance for and control of the disease complications.

However, currently there are a number of novel therapies in clinical trials that could be used in the management of these patients in the future.

**63.2.5.1 Hypertension.** Since hypertension and proteinuria are predictors of earlier onset of ESRD, and the RAA system is the key mechanism underlying hypertension in ADPKD, angiotensin-converting enzyme inhibitors (ACE-i), which target the RAA system, decrease blood pressure, and reduce proteinuria, would seem to be the treatment of choice. There have been epidemiologic and nonrandomized studies suggesting that ACE-i can slow the progression of renal insufficiency and reduce proteinuria (79–81). However, the few randomized, prospective studies on ACE-i have indicated otherwise. The Modification of Diet in Renal Disease (MDRD) Study Group was a randomized, prospective trial that included 200 patients with ADPKD and found that treatment with low-protein diet or ACE-i did not slow the loss of glomerular filtration rate (GFR) (81). The ACEi in Progressive Renal Insufficiency Study included 64 patients with ADPKD and found that treatment with benazepril did not decrease the number of patients who experienced doubling of serum creatinine or required initiation of dialysis (82,83). Finally, no difference in GFR decline was noted in hypertensive ADPKD patients treated with either atenolol (a  $\beta$ -blocker) or enalapril. No significant decrease in microalbuminuria was noted either. Normotensive ADPKD patients treated with enalapril did not have any significant decrease in GFR decline compared to those not treated (84). There are no data to suggest that antihypertensive therapy reduces the risk of intracranial hemorrhage or cardiac disease in ADPKD patients. However, a randomized study that compared standard versus strict control of hypertension (blood pressure less than 135–140/85–90 mmHg versus 120/80 mmHg) found significantly decreased left ventricular mass index, a cardiovascular disease risk factor, in patients with strict control (85). While this study indicates that antihypertensive therapy is probably beneficial in ADPKD, it did not examine cardiac end points such as myocardial infarction, cardiomyopathy, or congestive heart failure. Other therapies include angiotensin receptor blockers (ARBs), which act in a manner similar to that of ACE-i. Since hypertension is an independent factor that increases the morbidity of ESRD patients, therapy with ACE-i and/or ARBs is encouraged.

**63.2.5.2 Dialysis and Renal Transplantation.** At onset of ESRD, renal replacement therapy with dialysis should be initiated or, if living donors are available, patients should be evaluated for preemptive kidney transplantation. Assessment for ventral hernias, which interfere with peritoneal dialysis, should be performed prior to consideration for peritoneal catheter placement. Outcomes of renal transplantation are comparable with all non-diabetic renal transplant patients, with 73% 5-year and 67% 10-year graft survival and 84% 5-year and 73% 10-year patient survival rate (86,87). Malignancy due to



chronic immunosuppression is the most common cause of graft failure and patient death (88). Patients with ADPKD are excellent candidates for transplantation, but living related donor transplantation must be carefully assessed because of the high risk of disease in relatives and late onset of cysts in some of them. Owing to these concerns, potential living related donors should undergo genetic testing or be screened carefully for evidence of ADPKD before proceeding to living donation.

**63.2.5.3 Polycystic Liver Disease.** There are no effective medical therapies for polycystic liver disease, and since most patients have no symptoms, no treatment is recommended. The observation that postmenopausal women with ADPKD taking hormone replacement therapy have higher burden of hepatic cysts prompted a recommendation to avoid estrogen, but there are no studies that indicate that avoidance of estrogen or estrogen receptor blockade is effective (7). Patients with symptomatic liver disease and a few dominant cysts may be candidates for CT-guided aspiration/sclerosis or laparoscopic cyst fenestration, but most symptomatic patients have too many cysts for these techniques to improve symptoms. There is a risk of bleeding, bile leak, ascites, or recurrent symptoms with these techniques, and some patients may require conversion to open laparotomy. Partial liver resection has been attempted in patients with severe symptoms and hepatomegaly with relief of symptoms, but there is a high risk of perioperative morbidity (7). Orthotopic liver transplantation (OLT) or combined liver and kidney transplantation is reserved for those patients who have ESRD and severe refractory liver disease or for patients with symptomatic portal hypertension, Budd–Chiari syndrome, or intrahepatic biliary obstruction. Five-year survival is 69% for OLT alone and 76% for combined OLT and renal transplantation (7).

**63.2.5.4 Abdominal Pain.** Abdominal pain should not be disregarded in ADPKD patients, as nephrolithiasis, renal cyst bleeding, diverticulitis, lower UTI, and cyst (renal or hepatic) infection are common. Nephrolithiasis should be treated with analgesia, bed rest, and hydration. Renal cyst hemorrhage is usually self-limiting and treated in the same manner as nephrolithiasis, but caution must be exercised if pain worsens, as retroperitoneal hemorrhage with severe blood loss and hypovolemia has been reported. Diverticulitis in end-stage ADPKD patients must be recognized promptly and treated with antibiotics and bowel rest. Lower UTI presents in the usual manner with frequency, urgency, and dysuria. Oral antibiotics that cover gram-negative enterobacteriaceae should be administered for the standard duration.

Cyst infections, however, are more difficult to recognize and treat. Renal cyst infections are likely due to ascending infection, as suggested from the observation that 92% of cyst infections occur in females (89). Renal cyst infection classically presents with fever and focal tenderness overlying the affected kidney, hepatic cyst infection with fever and right upper quadrant

tenderness. However, some patients have more subtle clinical signs. Not all patients with renal cyst infection have pyuria or positive urine cultures. Leukocytosis and elevated erythrocyte sedimentation rate aid somewhat in diagnosis, but blood culture has been shown to be the most effective diagnostic test for both renal and hepatic cyst infection (89,90). 18F-fluorodeoxyglucose positron emission tomography has become a promising agent for detection of infected cysts, but can be expensive (91,92). Empiric therapy with an antibiotic that penetrates into the cyst, such as ciprofloxacin, trimethoprim-sulfisoxazole, or vancomycin, should be initiated in patients with suspected cyst infection. Enterobacteriaceae are the usual pathogens. If an organism is identified, the patient should receive an extended course of treatment lasting a minimum of four weeks to ensure adequate cyst penetration; CT-guided drainage may be necessary with cyst infections that do not respond.

**63.2.5.5 Cardiac Disease.** Periodic echocardiography should be performed to assess for valvular disease, left ventricular hypertrophy, and dilatation of the aortic root. Subacute bacterial endocarditis prophylaxis should be given prior to dental and gastrointestinal procedures if there is hemodynamically significant valvular insufficiency. Valve replacement is indicated for severe, symptomatic regurgitation.

Monthly to bimonthly monitoring of the aortic root should be performed if the aortic root reaches 4 cm, and aortic root replacement is indicated if the diameter of the aortic root exceeds 5 cm. This recommendation, however, is based on data from patients with Marfan syndrome. There are no ADPKD patient studies that follow the velocity of aortic root dilatation or assess the risk of aortic dissection in relation to aortic root diameter. It is also not known whether  $\beta$ -blockade in ADPKD patients with aortic root dilatation can successfully slow the rate of dilatation as in Marfan syndrome.

**63.2.5.6 Screening of Intracranial Aneurysm.** Prior to the development of MRI and magnetic resonance arteriography (MRA), routine screening of all ADPKD patients was not recommended because the screening test, intra-arterial angiography, held a significant risk of complications including permanent neurologic deficit (93,94). High-resolution time-of-flight MRA is now the modality of choice for aneurysm screening. While the technique is highly specific and sensitive for aneurysms 5 mm in diameter and above, the sensitivity drops considerably for smaller aneurysms (95). CT angiography has been shown to have better sensitivity, but the requirement of intravenous contrast may render this study unfeasible for patients with chronic renal insufficiency (CRI) or ESRD (96).

While screening relatives of patients with SAH successfully identified small, asymptomatic aneurysms and resulted in more surgical interventions performed, the resultant operative complications, postoperative morbidity, and long-term reduction in function outweighed any benefits gained from preventing SAH (20,97). Caution,

however, must be exercised in extrapolating the data to ADPKD patients, where only theoretical cost-benefit analyses have been performed (98,99).

The current recommendation is to screen only ADPKD patients with a past history of SAH or a family history of SAH or aneurysm, beginning at age 20. Since aneurysm rupture has been observed following renal transplantation, other groups have suggested screening of those planning to undergo major elective surgery. Individuals in “high-risk” professions such as airline pilots have also been identified as candidates for screening (22,100).

Very few individuals (2.4%) without aneurysm identified at initial screening developed an aneurysm in a 10-year longitudinal study (101). Studies of those with an aneurysm found at screening have shown that aneurysmal enlargement or development of new aneurysms occur slowly. In one study of 18 individuals with an aneurysm found at screening, one developed a new aneurysm and one experienced enlargement of the existing aneurysm (19). Both events occurred in the group of 10 patients imaged over an average period of 10 years and previously reported in a separate study (102). None of the patients followed for less than 10 years developed a new aneurysm. In another study, 25% (5 of 20) of individuals with previously identified aneurysms developed new aneurysms over an average 15-year follow-up period. Four of those five were initially discovered to have intracranial aneurysm when they experienced an intercranial bleed; none had recurrent intercranial bleeding (103). These findings suggest that those who have experienced SAH have a high incidence of developing new aneurysms, an observation born out by other studies that have also shown that they are at higher risk of aneurysm rupture (104). Although there are no specific recommendations for imaging intervals, these data indicate that those who screened negative should have the longest interval for reimaging (5–10 years), those with asymptomatic aneurysms an intermediate interval (3–5 years), and those with prior history of SAH the shortest (1–3 years). Clipping of an aneurysm or intra-arterial coiling should be performed in patients with aneurysms that are symptomatic or larger than 10 mm.

**63.2.5.7 Novel Therapies.** Better understanding of the pathophysiology and the availability of animal models have led to the identification of several promising drugs that are in clinical trials. Inhibition of cAMP has been shown to reduce the growth of cysts. Vasopressin V2 receptor antagonists (VPV2R) reduce renal levels of cAMP and therefore inhibit cyst development in animal models. Tolvaptan, a VPV2R, has completed phase II clinical trials, and a phase III trial is ongoing (NCT00428948). Somatostatin inhibits cAMP in the kidney and liver. Clinical trials with octreotide and lanreotide for PKD and cystic liver disease are ongoing (NCT00309283, NCT00426153, NCT00565097). Animal studies have also shown that the mTOR is involved in the coordination of cell growth and proliferation, the

uncontrolled activation of which has been implicated in the pathogenesis of ADPKD. In animal models, the mTOR inhibitors, sirolimus and/or everolimus, significantly retard cyst expansion and protect renal function. Prospective, randomized clinical trials with these drugs are in progress (NCT00346918, NCT00286156, NCT00414440) (6).

### 63.3 AUTOSOMAL RECESSIVE POLYCYSTIC KIDNEY DISEASE (MIM 263200)

#### 63.3.1 Clinical Features and Natural History

Autosomal recessive polycystic kidney disease (ARPKD) affects approximately 1:10,000–40,000 live births, without ethnic variation (105–108). The true incidence may be higher, as severely affected infants may die as newborns without a diagnosis, and patients with mild disease may not be diagnosed until later in life (109). The carrier frequency of the causative gene is reported to be 1:70 (110). ARPKD was considered to be a fatal condition of infancy, but more recent case series indicate a wider range of phenotypic severity and age of onset. Approximately 50% of cases are identified prenatally by ultrasound or at birth (108). Oligohydramnios, nephromegaly, echogenic kidney parenchyma, small cysts below 2 cm in diameter, and loss of corticomedullary differentiation are highly suggestive of ARPKD (111). However, ultrasound is unreliable for prenatal diagnosis, as not all affected fetuses, especially those with milder involvement, display these signs (112).

As a result of oligohydramnios, affected infants have Potter facies (hypertelorism; prominent infraorbital creases; flattened nasal bridge and tip; low-set, flattened, fleshy pinnae; and micrognathia), talipes equinovarus due to extrinsic compression, and respiratory insufficiency with subsequent air leaks (pneumothorax or pneumomediastinum) due to pulmonary hypoplasia. Neonatal mortality is estimated at 20–50% depending on the case series and is due to critical respiratory insufficiency, not from renal or liver disease (113–116).

Infants who survive the first month of life have a good chance of surviving the first year of life, with studies showing 78–87% 1-year and 50–87% 5-year survival rates (1,107,117). Ten-year survival is estimated at 87%, and 15-year survival ranges from 67 to 79% (113,114). Survivors face significant morbidities, and those who require neonatal mechanical ventilation have earlier onset of problems. About 20–25% of patients have growth retardation (108), 65% have systemic hypertension with median onset at 7 and 70 days of life in ventilated and nonventilated patients, respectively, and 40% have chronic renal failure with median onset at 1 day and 1 year of life in ventilated and nonventilated patients, respectively (107). This discrepancy is most likely the

effect of children who, because of milder symptomatology and lack of respiratory insufficiency, were not diagnosed with ARPKD until later in infancy or childhood. ESRD, found in 10–30% of patients, typically presents in the first decade of life (107,112,116). Unlike ADPKD, renal size does not increase with progression of renal insufficiency.

Clinical signs and imaging findings in children who present after the neonatal period are not as reliable. The more consistent feature in these children tends to be hepatic disease. Liver disease involves abnormal intrahepatic biliary duct hyperproliferation and dilatation, with subsequent hepatocellular damage, inflammation, and fibrosis, also known as Caroli's disease (Figure 63-3). Hepatic synthetic function is preserved. Portal hypertension, with associated esophageal varices and upper gastrointestinal bleeding, hepatosplenomegaly, and recurrent cholangitis are later complications of ARPKD. The North American case series showed 15–34% of patients had portal hypertension with median age of onset at 7 months and 4.6 years of life in ventilated and nonventilated patients, respectively. Severe portal hypertension required a small percentage (2.4–7%) of patients to have portocaval shunting or liver transplantation. Severity of renal disease and presence of systemic hypertension did not correlate with severity of hepatic disease (107).

### 63.3.2 Gene Mapping, Structure, Function, and Genotype–Phenotype Correlations

Initial studies excluded allelism of ARPKD to the *PKD1* locus (118,119). The first linkage studies identified the ARPKD locus, termed *PKHD1* (polycystic kidney and hepatic disease 1), at 6p21-cen (120), which was subsequently refined to a 3.8 centiMorgan (cM) segment from 6p21.1-12, without evidence of a second locus (121). Seven years later, two groups, one using a rat model system to backtrack to the human gene, the other searching within a cDNA transcription map of the *PKHD1* region, independently identified the gene within a month of each other (122,123).

The *PKHD1* gene, one of the largest disease genes in the human genome, contains a minimum of 86 exons with multiple splice forms, the longest of which spans 472 kb (Figure 63-4) (123). It encodes a 16.2 kb mRNA and is translated into a 447 kDa protein with 4074 amino acids.

Termed fibrocystin by one group and polyductin by the other, the gene has a single transmembrane domain, a large, highly glycosylated N-terminal segment containing multiple immunoglobulin-like and plexin domains, and a short C-terminal cytoplasmic tail. The function of fibrocystin is unclear, but domain analysis suggests that the protein may play a role in the regulation of cellular adhesion, repulsion, and proliferation, and/or the regulation and maintenance of renal collecting tubules and hepatic bile ducts (124). The *PKHD1* protein product localizes to primary cilia in renal tubular epithelial cells, with concentration in the basal body area (125,126).

To date, a total of 702 unique *PKHD1* mutations have been reported, and of these, 378 are classified as pathogenic (Figure 63-5) (127). Of these, 202 (53%) are missense mutations, 78 (21%) frameshift mutations, 56 (15%) nonsense mutations, and 36 (10%) splice site mutations. Large deletions of *PKHD1* have also been documented, although they make up a significantly smaller proportion of cases (128). Mutations are scattered throughout the gene without evidence of clustering. As with *PKD1* and *PKD2*, many mutations result in a truncated protein product and are unique to each family. One mutation, 107C>T (T36M), has been observed in unrelated patients of different ethnic origins and is likely a mutation “hot spot” (129). There are two founder mutations, R496X and V3471G, in the Finnish population (129).

While intrafamilial phenotypic heterogeneity and high rates of compound heterozygosity make genotype–phenotype correlation somewhat unreliable, there were some trends visible on mutation analysis. All patients carrying a truncating mutation on both parental alleles died shortly after birth regardless of the site of truncation (129). Analysis of families classified as “severe” (at least one child presenting with perinatal disease and neonatal demise) showed that 57% of the mutations detected were truncating. Families classified as “moderate” (survival or diagnosis beyond 1 month) had 70% missense mutations. However, seven missense mutations were correlated with perinatal demise (115). In some patients, only one mutant allele was identified, implying the existence of mutations within introns, regulatory elements, or larger genomic rearrangements undetectable by gene sequencing. Individuals with prominent hepatic involvement do not exhibit a specific pattern of mutation (63).

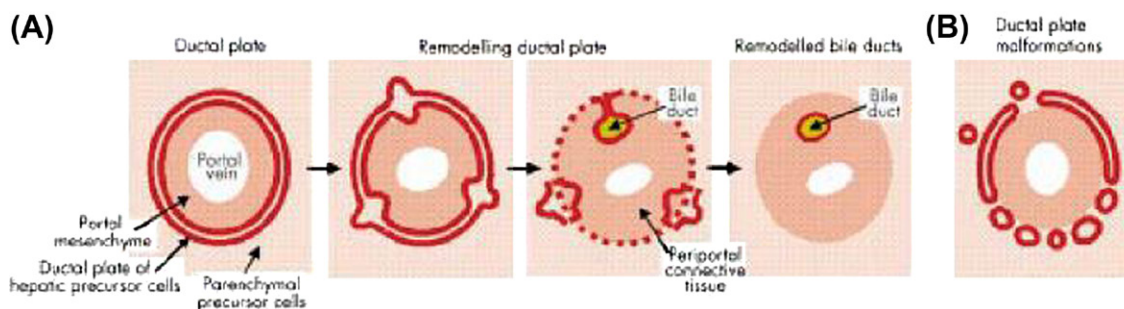


FIGURE 63-3

### 63.3.3 Laboratory, Imaging, and Histopathologic Findings

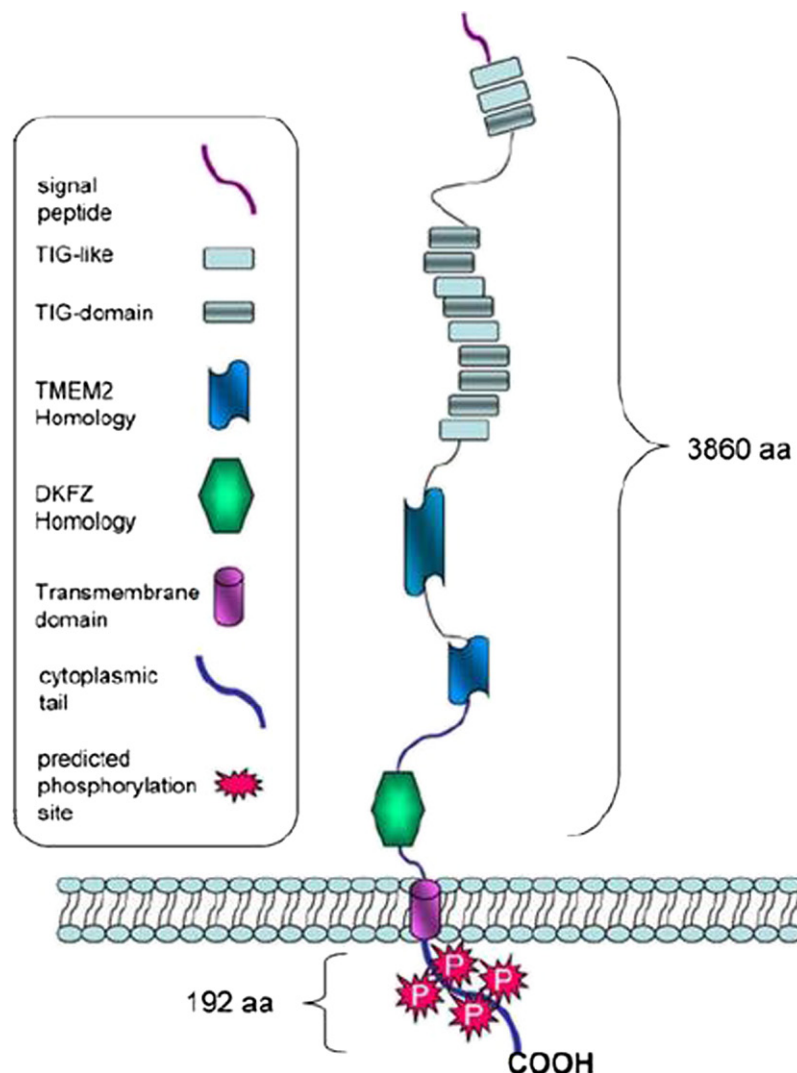
Decreased urinary concentrating ability is a universal finding in these patients. Hyponatremia (25%), elevated blood urea nitrogen (BUN) and serum creatinine (60–67%), and proteinuria (10–55%) are found on serum chemistries and urinalysis. Renal ultrasonography shows nephromegaly, increased echogenicity, loss of corticomedullary differentiation, and multiple small cysts. ARPKD can be diagnosed if the patient has characteristic renal ultrasonographic signs and is found to have hepatic fibrosis, parental consanguinity, absence of parental renal cysts, or a previous sibling diagnosed with ARPKD by renal/hepatic pathology.

Kidney specimens are spongelike in appearance, with dilated, elongated channels radiating from the medulla to the cortex at right angles to the cortical surface. Histopathologically, the cysts are derived from the distal convoluted tubules and collecting ducts, unlike ADPKD cysts, which can arise from any portion of the nephron

(107,108,130,131). Hepatic histopathology shows hepatic fibrosis and the typical ductal plate abnormality with biliary duct proliferation and dilatation.

### 63.3.4 Molecular and Prenatal Diagnosis

Molecular testing for ARPKD is clinically available and can be performed by direct sequencing of the *PKHD1* gene or by linkage analysis. As with *PKD1* and *PKD2*, interpretation of sequence results may be difficult because of the large number of private mutations and the uncertain nature of variants. With the increasing number of mutations cataloged in databases and new algorithms to determine pathogenicity of variants, the mutation detection rate now approaches 85% for the entire spectrum of ARPKD (132,133). Screening for large deletions or gross rearrangements in *PKHD1* may increase the diagnostic yield in some cases (128). *PKHD1* molecular testing has been suggested for children with isolated Caroli's disease, as one small series of 14 children with isolated



**FIGURE 63-4** Sweeney, W. E., Jr.; Avner, E. D. *Molecular and Cellular Pathophysiology of Autosomal Recessive Polycystic Kidney Disease (ARPKD)*. Cell and Tissue Research 2006, 326 (3), 675.





First described by Fanconi in 1950, nephronophthisis was previously classified together with medullary cystic kidney disease (MCKD) since both had similar presenting symptoms and renal histopathologic appearance. However, MCKD was later placed into its own category because of its autosomal dominant inheritance and later age of onset than familial nephronophthisis.

Nephronophthisis is an autosomal recessive disorder with variable presentation and considerable locus heterogeneity (140). Three different variants have been identified and are classified according to age of onset. Juvenile nephronophthisis usually presents by age 6 years, with development of ESRD by a median age of 13 years and generally between the first and second decades of life (141). Infantile nephronophthisis begins in infancy when mothers notice their babies producing an excessive number of wet diapers. The adolescent variant presents with ESRD at a median age of 19 years. In all types, progressive CRI is invariable, and anemia develops out of proportion to the degree of renal insufficiency. CRI progresses to ESRD, and if untreated, death occurs by uremia.

#### 63.4.1.1 Juvenile Nephronophthisis (MIM 256100).

Juvenile nephronophthisis is the most common of the three variants. Patients present with polyuria from tubular dysfunction and a decrease in urinary concentrating ability. Despite fluid deprivation, urine osmolality is low in these children and is not increased with the administration of vasopressin, mimicking nephrogenic diabetes insipidus (142). Hypertension is rare because of sodium wasting.

#### 63.4.1.2 Infantile Nephronophthisis (MIM 602088).

Infantile nephronophthisis was recognized as a distinct entity from other forms of nephronophthisis not only because of its extremely early presentation but also because of several phenotypic differences. Some infants present prenatally with an ARPKD-like phenotype of oligohydramnios, Potter facies, and severe respiratory insufficiency without hepatic fibrosis. Patients presenting in infancy and early childhood do not have the typical polyuria and polydipsia, but instead suffer from hypertension and hyperkalemia. *Situs inversus totalis* and cardiac defects may be present. The renal histological phenotype differs from that of juvenile nephronophthisis in that features of ARPKD (e.g. nephromegaly, microcystic cortical dilatation) are seen along with the characteristic tubular cell atrophy, interstitial cell infiltration, and fibrosis seen in nephronophthisis.

In most cases, mutations in the *NPHP2* gene cause infantile nephronophthisis. Recently, mutations in *NPHP3* and *NPHP9* have been reported in patients with very-early-onset nephronophthisis, thus establishing locus heterogeneity for infantile nephronophthisis (143,144).

#### 63.4.1.3 Adolescent Nephronophthisis (MIM 604387).

In adolescent nephronophthisis, ESRD is seen in late adolescence and young adulthood, with a reported median age of 19 years (range 4–37 years) (145). *NPHP3* was the first gene identified to cause adolescent nephronophthisis. In children with mutations in *NPHP1–4*, retinitis pigmentosa occurs in about 10% of families. However,

all patients with *NPHP5* mutations have retinitis pigmentosa (140,142).

### 63.4.2 Syndromic Forms of Nephronophthisis

An additional characteristic of nephronophthisis that distinguishes the condition from MCKD is its association with extrarenal malformations. Optic nerve colobomas, cone-shaped epiphyses, and liver fibrosis have been reported in separate kindreds. Other forms of nephronophthisis with specific extrarenal malformations have acquired syndromic nomenclature and are mentioned here.

**63.4.2.1 Joubert Syndrome (MIM 213300).** Joubert syndrome is an autosomal recessive condition characterized by congenital hypotonia, psychomotor retardation, an abnormal breathing pattern of tachypnea alternating with apnea, cerebellar ataxia, oculomotor apraxia, and most importantly, aplasia or hypoplasia of the cerebellar vermis giving the classic “molar tooth sign” on brain neuroimaging (Figure 63-6). Renal disease is present in 25% of patients with Joubert syndrome and most often manifests as nephronophthisis (146). Children typically present in the first decade of life with polyuria and polydipsia followed by CRI and ultimately renal failure by the second decade. Juvenile nephronophthisis is the most common form associated with Joubert syndrome, but infantile and adolescent onset nephronophthisis can occur.

At least 10 different genes are known to cause Joubert syndrome, and other loci are yet to be determined. Mutations in *AHI1*, *NPHP6*, *MKS3*, and *CC2D2A* each account for approximately 10% of cases (147–150). About 1–2% of patients have a homozygous *NPHP1* deletion (148).

#### 63.4.2.2 Senior-Løken Syndrome (MIM 266900).

Also known as Senior syndrome or renal-retinal syndrome, this disorder is a combination of nephronophthisis and degeneration of the retinal pigment epithelium and accounts for approximately 10–15% of all cases of nephronophthisis. Renal symptoms and histopathology are identical to that of isolated nephronophthisis, including the variability in age of onset. Similarly, onset of ocular pathology varies from birth to childhood. Newborns present with Leber’s congenital amaurosis, characterized by visual inattention, nystagmus, near-absent pupillary reaction, and blindness or severe visual impairment. Older children present with nyctalopia (night blindness), then develop progressive loss of bilateral peripheral vision. Color perception and central visual acuity are preserved until end-stage disease, which typically occurs in adolescence, although there is one report of an adult patient with some preservation of visual fields and acuity (151). Fundoscopic examination reveals attenuation of blood vessels and pallor of the optic disk without the “bone spicule” retinal pigmentary abnormalities typically seen in retinitis pigmentosa.

In the majority of cases, Senior-Løken syndrome is caused by mutations in *NPHP5*, but mutations in *NPHP1*,

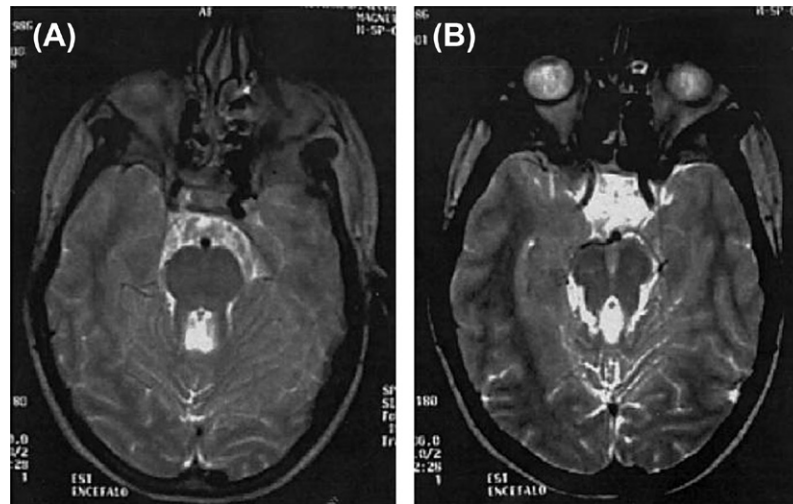


FIGURE 63-6

including the classic 290 kb deletion, *NPHP2*, *NPHP3*, and *NPHP4* have been reported (139,142,145,152–154). In addition, mutations in *NPHP6* cause 20% of cases of Leber congenital amaurosis, which is characterized by early onset of severe vision impairment from retinitis pigmentosa, without renal disease (155,156). The frequency of retinitis pigmentosa in Senior–Løken syndrome varies with the gene involved, from 6% with *NPHP1* to 100% with *NPHP5* and *NPHP6* (142).

#### 63.4.2.3 Meckel–Gruber Syndrome (MIM 249000).

Meckel–Gruber syndrome is a severe, perinatally lethal, multiple congenital anomaly syndrome with an estimated incidence between 1:140,000 to 1:13,250 births (157). Infants have bilaterally enlarged, cystic kidneys and hepatic ductal plate dysplasia, biliary proliferation, and fibrosis similar to those with ARPKD. Renal cysts involve both the cortex and the medulla. Cortical cysts tend to be small, thin walled, and surrounded by normal glomeruli, while medullary cysts are larger, up to several millimeters in diameter, and separated by thick fibromuscular walls (131). Central nervous system malformations are severe. Posterior encephalocele is the most common malformation, while aplasia or hypoplasia of various central nervous system (CNS) structures, such as the cerebellum, cerebrum, corpus callosum, and optic or olfactory tracts, are present in other patients. Preaxial or postaxial polydactyly is another cardinal feature of the syndrome. Prenatal diagnosis of Meckel–Gruber syndrome can be made with transvaginal ultrasound as early as 10–12 weeks gestational age.

The mode of inheritance is autosomal recessive, and locus heterogeneity has been well established. Mutations in *MKS1*, *TMEM216*, *MKS3/TMEM67*, *NPHP6/CEP290*, *NPHP8/RGRIP1L*, *NPHP3*, and *CC2D2A* all have been reported to cause Meckel–Gruber syndrome. Many of these genes overlap with those causing Joubert syndrome, suggesting that these disorders form a continuum of phenotypic variation rather than separate entities (158).

#### 63.4.2.4 Cogan-Type Oculomotor Apraxia (MIM 257550).

Patients with Cogan-type oculomotor apraxia suffer from an inability to make smooth voluntary horizontal eye movements and initiate horizontal saccades. In order to bring objects into view, they must make quick, jerking movements of their heads. Involuntary horizontal eye movements are not impaired, indicating that the oculomotor nerves are capable of firing normally. Nephronophthisis has been reported in association with this disorder, but unlike Joubert syndrome, these patients do not have the molar tooth sign or retinitis pigmentosa.

Mutations in *NPHP1* and *NPHP4* have been found in patients with Cogan-type oculomotor apraxia and nephronophthisis. Homozygosity of the classic *NPHP1* deletion has been reported in one patient, while another patient was found to have the classic deletion of one allele and an inactivating splice junction point mutation of the other (159). Homozygosity for a frameshifting 3272delT mutation in *NPHP4* has also been documented (160).

**63.4.2.5 Mainzer–Saldino Syndrome.** The association of nephronophthisis with cone-shaped epiphyses has been described. In addition, patients with this syndrome can also develop retinal degeneration and cerebral ataxia (161).

#### 63.4.3 Gene Mapping, Structure, Function, and Genotype–Phenotype Correlations

At least nine different genes are known to be associated with nephronophthisis in addition to those responsible for the syndromic forms of nephronophthisis (Table 63-2). The most common mutation in patients with nephronophthisis is a homozygous deletion of *NPHP1*, which accounts for 21% of cases (142). The remaining genes each comprise <3% of cases, and the mutation is unknown in 70%.

### TABLE 63-2

Gene (Protein)	Chromosome	Phenotype (Median Age at ESRD)	Extrarenal Symptoms	Mutation Frequency (%)	Intervention
<i>NPHP1</i> (nephrocystin-1)	2q13	NPHP (13 years)	RP (10%), OMA (2%), JS (rarely)	23.4, homozygous deletion 2.1, point mutation	Investigative
<i>NPHP2/INVS</i> (inversin)	9q31	Infantile NPHP (<5 years)	RP (10%), LF, <i>situs inversus</i> , VSD	1.4	Nephrectomy
<i>NPHP3</i> (nephrocystin-3)	3q22	Infantile and adolescent NPHP	LF, RP (10%), <i>situs inversus</i> , MKS	0.7, if truncating mutation infantile form	Nephrectomy
<i>NPHP4</i> (nephrocystin-4)	1p36	NPHP (21 years)	RP (10%), OMA, LF	2.6	Nephrectomy
<i>NPHP5/IQCB 1</i> (nephrocystin-5)	3q21	NPHP (13 years)	Early-onset RP	3.6	Gallbladder resection
<i>NPHP6/CEP290</i> (nephrocystin-6/CEP290)	12q21	NPHP	JS, MKS	1	ATF
<i>NPHP7/GLIS2</i> (nephrocystin-7/GLIS2)	16p	NPHP	–	0.1	–
<i>NPHP8/RPGRIP1L</i> (nephrocystin-8/RPGRIP1L)	16q	NPHP	JS, MKS	0.5	Nephrectomy
<i>NPHP9/NEK8</i> (nephrocystin-8/NEK8)	17q11	Infantile NPHP	–	0.1	–
<i>TMEM67/MKS3/NPHP11</i> (Mockelin/nephrocystin-11)	8q22.1	MKS, JS, NPHP + LF	JS, MKS	–	–
<i>NPHP1L/XPNPEP3</i> (nephrocystin-1 L/XPNPEP3)	22q13	NPHP	Cardiomyopathy, seizures	0.1	–

ATF4, Activating transcription factor 4; APC2, anaphase-promoting complex 2; BCAR 1, breast cancer antiestrogen resistance 1; CC2D2A, coiled-coil and C2 domain containing 2; JS, Joubert syndrome; LF, liver fibrosis; MKS, Meckel–Gruber syndrome; OMA, oculomotor apraxia; PTK2B, protein tyrosine kinase 2B; RP, retinitis pigmentosa; RPGR, retinitis pigmentosa GTPase regulator; VSD, ventricular septal defect. Matthias, T.; Wolg, F., et al. Nephronophthisis. *Pediatric Nephrology* 2010, 26 (2).



**63.4.3.1 NPHP1.** The first gene locus for nephronophthisis was mapped to 2p by linkage analysis of 22 multiplex families (162). The locus was refined first to 2q13, then with fine mapping techniques to a 2Mb critical region (163–167). An approximately 250kb deletion was discovered in 80% of familial cases of nephronophthisis mapping to the 2q13 locus (167). Two ORFs were identified within the deleted region, one encoding a small 148 amino acid protein involved in cellular trafficking and the other encoding a then-uncharacterized protein that was eventually shown to be mutated in patients with nephronophthisis (168,169).

The *NPHP1* protein, named nephrocystin-1, contains 20 exons spanning 83kb of the genome, and encodes a 4.5kb mRNA. The predicted protein is 732 amino acids long and weighs 83kDa. It contains coiled-coil domains and an *Src*-homology 3 domain (SH3), both of which are protein–protein interaction motifs (Figure 63-7). SH3 domains are typically found on “adapter” proteins that bring other proteins together. Through the yeast 2-hybrid system, nephrocystin-1 was shown to bind the focal adhesion complex protein p130cas. Immunofluorescence demonstrated that nephrocystin-1 and p130cas co-localize in vivo at focal adhesions of the basolateral cell membrane (170,171). p130cas is a cytosolic protein involved in the focal adhesion complex, which mediates signal transduction from the extracellular matrix to the nucleus (172). Nephrocystin-1 was also found to co-localize with E-cadherin at adherens junctions (173) and with  $\beta$ -tubulin in the primary cilia of renal cells, specifically, at the transition zone (174,175). In addition, it interacts with other nephrocystin proteins and Joubertin to form a multiprotein complex (145,160,174,176). These findings, along with similar results from PKD1, PKD2, and PKHD1 localization, indicate the important role of cilia, cell–cell, and cell–matrix interactions in inherited kidney disease (142,177,178).

As stated earlier, more than 80% of patients with juvenile nephronophthisis have a homozygous 290kb deletion of *NPHP1* (170,179). The gene is flanked by two 330kb inverted repeats, with two nearby 45kb repeats, making the segment prone to unequal homologous recombination and intrachromosomal loop excision, creating an interstitial deletion (180) (Figure 63-7).

Nonsense, frameshift, and splice site mutations in *NPHP1* have also been documented, and all likely result in loss-of-function of nephrocystin-1 (181). Only one missense mutation, 1024G>A, has been reported, leading to a nonconservative amino acid change in a residue that is conserved in mouse and dog *NPHP1* (182).

Why some patients with the classic *NPHP1* deletion have only renal manifestations while others have Senior–Løken syndrome, Joubert syndrome, or Cogan-type oculomotor apraxia is not clear. There may be other genes within the deleted region or around the deletion boundaries that are responsible for extrarenal disease. Another possible explanation is triallelic inheritance, in which a mutation in another gene, in addition to deletion of *NPHP*, is required for multisystemic disease. Other theories include subtle rearrangements of the break points undetectable by haplotype analysis, or disruption of enhancer sequences altering expression of genes outside the deletion region.

**63.4.3.2 NPHP2.** Linkage analysis in an inbred Bedouin family with infantile nephronophthisis localized the gene locus to a 12.9cM region at 9q22–q31 (183). The discovery of mutations of *inversin* in the *invlinv* mouse, which has enlarged, cystic kidneys, *situs inversus*, and histopathologic findings similar to nephronophthisis, and the location of human *inversin* within the NPHP2 critical region, strongly suggested that *inversin* was the candidate gene. Confirmation came when mutational analysis in seven families with infantile nephronophthisis showed recessive mutations in the gene (174), which was then named *NPHP2* or *INVS*.

*NPHP2* is a 17-exon gene spanning 100kb of genomic DNA, encoding a 1065 amino acid protein, nephrocystin-2/inversin, which is conserved from the zebrafish to the mouse. It has a highly conserved N-terminal domain containing 16 tandem ankyrin repeats, a split nuclear localization signal, two calmodulin-binding IQ domains, and two “destruction” or D boxes that bind a protein involved in the anaphase-promoting complex, a cell-cycle regulatory ubiquitin ligase that regulates cellular transition from metaphase to anaphase and mitosis to G<sub>1</sub>. Inversin co-localizes with nephrocystin-1 in the primary cilia of renal tubular epithelium, connecting it with other proteins that have a role in cilia formation, maintenance,

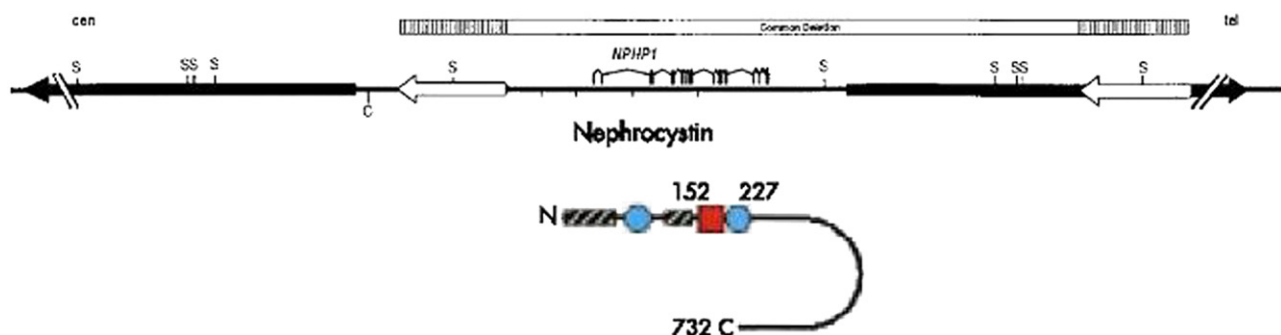


FIGURE 63-7

and intraflagellar transport (174) (Figure 63-8). In addition, cell-cycle-dependent expression of *NPHP2* and its role in the Wnt signaling pathway support the hypothesis that nephrocystin-2/inversin is involved in the maintenance of normal planar cell polarity (184,185).

Frameshift, missense, and splice site mutations have all been reported in *NPHP2*, and most mutations result in protein truncation. Five recurrent mutations have been found in unrelated families: R899X, R907X, Q485fsX509, Q671X, and E970fsX971 (144,181). No clear genotype–phenotype correlations have been established. In a cohort of seven families with *NPHP2* mutations, one patient with homozygous missense mutations did not have onset of ESRD until age 5 years, but neither did children of a family with compound heterozygous truncating mutations. The patient with *situs inversus totalis* had homozygous truncating R603X mutations and was the only patient with mutations in both alleles that removed all domains C-terminal to the ankyrin repeats, including the nuclear localization domains (174).

**63.4.3.3 NPHP3.** Examination of a large, inbred Venezuelan family with nephronophthisis indicated, at the time, that their disease was distinct from juvenile and infantile nephronophthisis on the basis of both the later age of onset and the gene locus. A total genome scan followed by higher resolution haplotype analysis mapped the family's locus to a 3.3 Mb interval at 3q22 (186,187). Seven genes with known function and eight expressed sequence tags (ESTs) were found within this interval.

The mapping of several families with Senior-Løken syndrome to the same region narrowed the candidate

genes to five ESTs, and sequencing revealed mutations in a 40.5 kb gene that was named *NPHP3*. *NPHP3* is composed of 27 exons and encodes a 3990 nucleotide mRNA, which is translated into a 1330 amino acid cytosolic protein product called nephrocystin-3. The protein has an N-terminal coiled-coil region and a tubulin-tyrosine ligase domain and interacts with nephrocystin-1 and nephrocystin-2/inversin (145,188) (Figure 63-9). In embryonic mice, the mRNA is expressed first in the node during gastrulation, then in the brain, retina, liver and biliary tract, renal tubules, and respiratory epithelium. Mutations in the murine ortholog *Nphp3* produce the *pcy* mouse mutant, which has cystic kidney disease due to a hypomorphic *Nphp3* allele (145). Interestingly, treating the *pcy* mouse with a vasopressin-2 receptor antagonist has been shown to ameliorate disease by lowering renal cAMP levels (136). This discovery may lead to future therapeutic interventions.

The phenotypic spectrum associated with *NPHP3* mutations has broadened as more cases are amassed and now includes infantile and adolescent nephronophthisis, nephronophthisis with liver fibrosis, nephronophthisis with retinitis pigmentosa, and embryonic patterning defects. With regard to genotype–phenotype correlations, truncating and splice site mutations in *NPHP3* produce a more severe phenotype similar to Meckel–Gruber syndrome, whereas missense and other nontruncating mutations are associated with milder and later-onset disease (139,144).

**63.4.3.4 NPHP4.** After the discovery of *NPHP1*, *NPHP2*, and *NPHP3*, there continued to be kindreds that did not map to any of these loci (189). Those

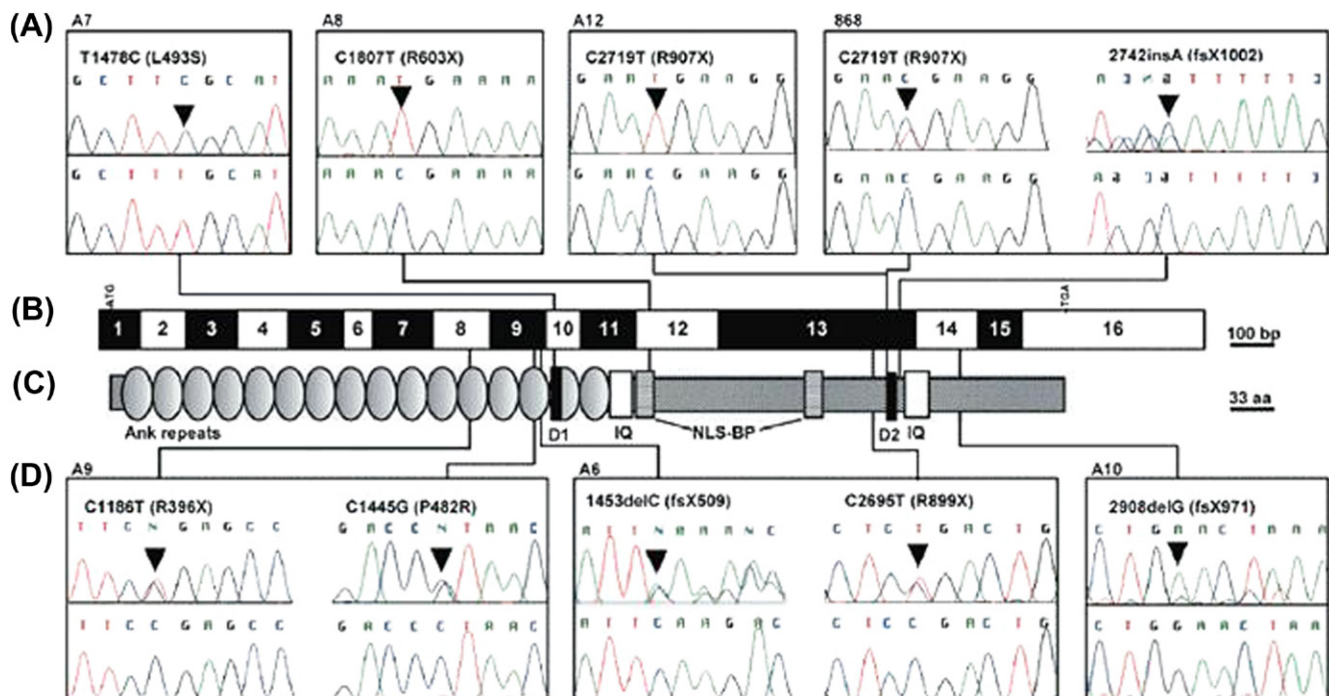


FIGURE 63-8

families were gathered, and linkage analysis mapped them to a 2Mb region within 1p36.31 (153). Higher resolution haplotype mapping narrowed the region to a 700kb interval containing six genes. Two groups identified mutations in the same gene and named it *NPHP4* (154,160).

*NPHP4* is a large, 30 exon gene that spans 130kb and encodes nephrocystin-4/nephroretinin, a 1426 amino acid protein product that has no known homologs in the human genome (Figure 63-10). It is a highly conserved protein, with orthologs found in the mouse, cow, pig, zebrafish, *Xenopus*, *Ascaris*, and *Caenorhabditis elegans*. The mouse ortholog shares 78% sequence identity, while the *C. elegans* ortholog has 24% identity with the human sequence. Domain analysis shows a putative nuclear localization signal, an acidic domain, and an SH3 domain, the latter two also being features of nephrocystin-1 (154). Nephrocystin-4 forms a complex with nephrocystin-1 and other proteins including p130Cas and Pyk2. It localizes to primary cilia, basal bodies, and centrosomes, consistent with the pathogenic mechanism of cystogenesis ascribed to other nephrocystin proteins (190). In addition, nephrocystin-4 localizes to the connecting cilium of photoreceptor cells where it interacts

with RPGRIPI (retinitis pigmentosa GTPase regulator interacting protein 1), deficiency of which is responsible for Leber congenital amaurosis (191).

A recent mutational analysis of *NPHP4* in 250 patients with nephronophthisis identified 23 novel sequence variants in 26 (10%) different patients (192). Of the six patients who had either homozygous or compound heterozygous mutations, 5/8 mutations (63%) were thought to be loss-of-function or truncating mutations. In the 20 patients with only one sequence variant detected, only 1 was a likely loss-of-function mutation. No genotype-phenotype correlations were recognized.

Previously reported mutations in *NPHP4* have included nonsense, missense, frameshift, and splice site mutations, most resulting in a truncated protein (154,160). Three kindreds with no extrarenal involvement were found to have homozygous missense mutations, G754R, R848W, and F991S, indicating a possible genotype-phenotype correlation between isolated nephronophthisis and missense mutations (154,160).

**63.4.3.5 NPHP5.** *NPHP5*, also called *IQCB1*, spans 65.7kb and contains 15 exons. The first two exons are not translated. *NPHP5* encodes nephrocystin-5, a 598 amino acid protein that is ubiquitously expressed in fetal

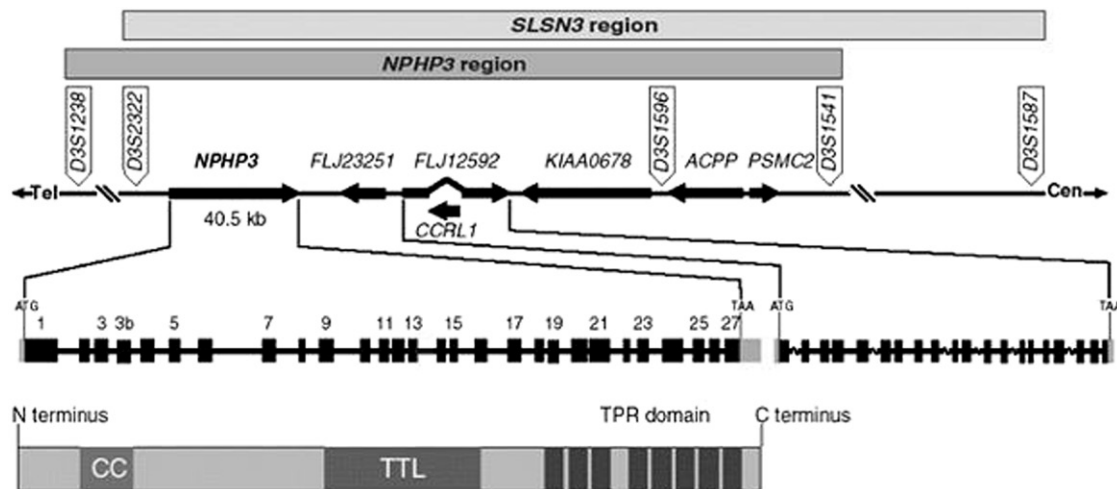


FIGURE 63-9

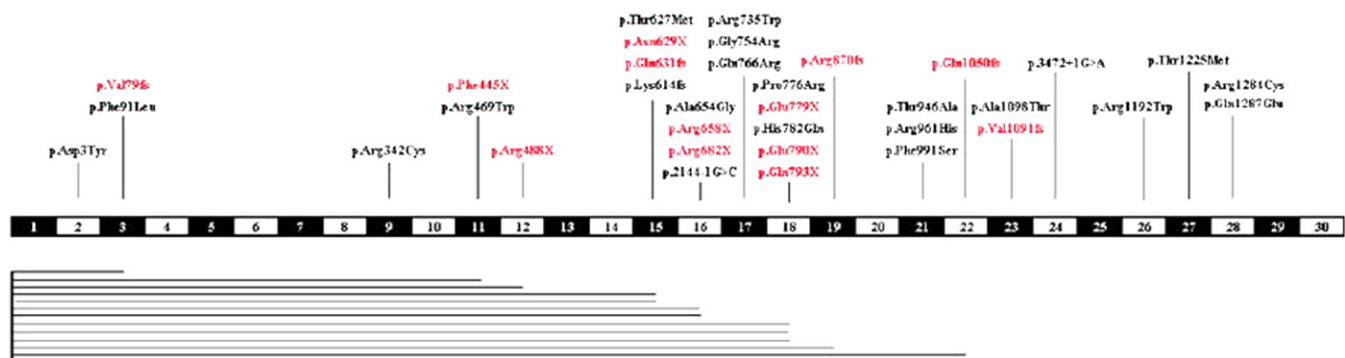


FIGURE 63-10 Reinhardt, Lehrack, S., et al. Mutational analysis of the *NPHP4* gene in 250 patients with nephronophthisis. Human Mutation 2005, 25 (4), 411.

and adult tissues (193). It has a coiled-coil domain and two IQ calmodulin-binding regions. Nephrocystin-5 directly interacts with calmodulin and forms a complex with retinitis pigmentosa GTPase regulator (RPGR) that localizes to primary cilia in renal epithelial cells and their homologous structures in the retina, photoreceptor connecting cilia (193). Mutations in *NPHP5* cause Senior-Løken syndrome, which is characterized by nephronophthisis and early-onset retinitis pigmentosa. All the eight mutations originally described resulted in protein truncation; no missense mutations were found (193). Most of the affected individuals had homozygous mutations.

**63.4.3.6 NPHP6.** *NPHP6*, also known as *CEP290*, spans 93.2kb and contains 55 exons. It encodes nephrocystin-6, a 290kDa protein of 2479 amino acids with 13 putative coiled-coil domains, a region with homology to structural maintenance of chromosomes (SMC) chromosome segregation ATPases<sup>14</sup>, a bipartite nuclear localization signal (NLS\_BP), six RepA/Rep<sup>+</sup> protein KID motifs (KID), three tropomyosin homology domains and an ATP/GTP-binding site motif A (P-loop) (149). Nephrocystin-6 localizes to centrosomes in renal epithelial cells during interphase and to connecting cilia of photoreceptors. It interacts with nephrocystin-5 and ATF4, a transcription factor involved in the regulation of the cell cycle and in cAMP-dependent renal cyst formation (136,149,158). Mutations in *NPHP6* can produce exclusive cystic renal disease, syndromic forms of nephronophthisis such as Joubert syndrome and Meckel-Gruber syndrome, as well as Leber congenital amaurosis. The nine initially reported mutations were all nonsense or frameshift mutations (149).

**63.4.3.7 NPHP7.** *NPHP7* is also known as *GLIS2* and contains six exons spanning more than 7.5kb. The protein nephrocystin-7 or “Gli-similar protein 2” is highly expressed in the kidney and localizes to primary cilia of renal epithelial cells and nuclei (194,195). The *GLIS2* protein is related to the Gli transcription factor, suggesting a possible link between cyst formation and the sonic hedgehog signaling network (142). Mutations in *NPHP7* were first reported in a consanguineous Oji-Cree kindred (195). Affected members had a homozygous transversion (IVS5 + 1G > T) affecting a splice donor site. Other than this index family, mutations in *NPHP7* appear to be a rare cause of nephronophthisis.

**63.4.3.8 NPHP8.** *NPHP8*, or *RPGRIP1L* as it is also known, is composed of 27 exons, the last of which is noncoding, and extends over 103.2kb. The encoded protein, nephrocystin-8/RPGRIP1L (retinitis pigmentosa GTPase regulator interacting protein 1-like), is ubiquitously expressed in human embryonic and fetal tissues. It contains five coiled-coil domains, a C-terminal region homologous to the RPGR-interacting domain of RPGRIP1, and a central region with two protein kinase C conserved region 2 (C2) motifs. Nephrocystin-8 co-localizes with nephrocystin-4 and nephrocystin-6 to

basal bodies, centrosomes, and primary cilia of renal tubular cells (196,197). The spectrum of phenotypic variability observed in individuals with *NPHP8* mutations can be explained in part by the type of mutation. Homozygous truncating mutations of *NPHP8* produce Meckel-Gruber syndrome or similar severe phenotypes, whereas the presence of at least one missense mutation causes milder and later-onset disease (139,196). Heterozygous and homozygous missense mutations as well as frameshift and splice site mutations have all been reported in individuals with Joubert syndrome (196–198). Other extrarenal features may include polydactyly, liver fibrosis, pituitary agenesis, and partial growth deficiency.

**63.4.3.9 NPHP9.** *NPHP9*, also known as *NEK8*, is an extremely rare cause of nephronophthisis. In a study of 588 patients with nephronophthisis, only three missense mutations were identified in *NPHP9*, and in one of these individuals, a homozygous mutation in *NPHP5* was also present, suggesting possible oligogenic inheritance (143). Like all known nephrocystin proteins, nephrocystin-9 localizes to primary cilia of renal tubular epithelial cells (143,199). It has been shown to interact with polycystin-2, and in mouse models, mutations in *Nek8* cause increased expression of polycystin-1 and polycystin-2 (199). Nephrocystin-9/NEK8 is also thought to play a role in cell cycle regulation (142).

In general, other than the specific genotype–phenotype correlations mentioned earlier, the phenotypic variation in nephronophthisis does not appear to be attributable solely to the gene involved or the type of mutation. Genetic modifiers have been shown to play a role. The R830W allele of the *AHI1* gene was found more frequently in patients with nephronophthisis and retinal degeneration than in those with nephronophthisis without retinal degeneration (25% compared to 1.8%,  $P = 5.36 \times 10^{-6}$ ), equaling a relative risk of 7.5 (200). This association was true regardless of the primary mutation. Similarly, the Thr229 variant of the *RPGRIP1L* gene was shown to be associated with retinitis pigmentosa in a small cohort with nephronophthisis (201). Other factors may become apparent as the mechanism of disease is further elucidated.

#### 63.4.4 Laboratory, Imaging, and Pathologic Findings

A complete blood count reveals severe microcytic, hypochromic, hypoproliferative anemia due to low erythropoietin secretion. Creatinine clearance is decreased, while serum creatinine and BUN are increased. Urine osmolality following water deprivation/dDAVP administration is inappropriately low compared to serum osmolality and reflects loss of urinary concentrating ability (also known as nephrogenic diabetes insipidus). Proteinuria and pyuria are rare. Renal ultrasonography shows normal to decreased renal size, increased



echogenicity, and diminished corticomedullary differentiation. Although corticomedullary cysts may be present, it is important to note that their presence is not necessary for diagnosis. Renal biopsy shows a characteristic set of histopathologic findings: disorganized tubular basement membranes, tubular atrophy with cyst formation, interstitial lymphohistiocytic infiltration, and periglomerular and interstitial fibrosis. Patients with nephronophthisis should also be screened for ocular involvement with fundoscopic examination and an electroretinogram if Senior-Løken syndrome is suspected. A brain MRI may show the molar tooth sign in patients with Joubert syndrome.

### 63.4.5 Molecular and Prenatal Diagnosis

Given the high degree of genetic heterogeneity and phenotypic variation, it may not be clear which gene to test first. Furthermore, molecular genetic testing is not available on a clinical basis for all genes associated with nephronophthisis. An algorithm has been suggested to aid in decision making (158). In children less than 5 years of age, testing for mutations in *NPHP2* should be sought first. In those older than 5 years, testing should begin with *NPHP1*, as the classic homozygous deletion accounts for a significant number of cases of nephronophthisis. If no mutations are detected, testing of other genes is dictated by the nature of the extrarenal complications. It has recently been shown that high throughput sequencing is an efficient method to detect rare mutations (202). Analysis of 18 genes associated with nephronophthisis and its variant forms in 120 patients led to a molecular diagnosis in 30 (25%). This and other types of advanced diagnostic techniques will become more widely available for clinical purposes in the future. Of note, no mutation is identified in over two-thirds of patients, implying that other genes are yet to be discovered. In all cases, a careful family history should be elicited, looking in particular for consanguinity or a history of early death or renal disease.

### 63.4.6 Management

There is currently no therapy for the underlying disease or the ophthalmologic and neurologic manifestations. The anemia responds to administration of erythropoietin. Supportive care should be given for complications of renal insufficiency such as acidosis, electrolyte disturbances, and hypovolemia. Periodic monitoring of renal function is necessary so that dialysis and enrollment in transplantation registries can be initiated at onset of ESRD. Analysis of the North American Pediatric Renal Trials and Collaborative Studies database demonstrates that nephronophthisis transplant recipients have excellent outcomes that are better than those of the general pediatric transplant population (203).

## 63.5 MEDULLARY CYSTIC KIDNEY DISEASE

### 63.5.1 Clinical Features and Natural History

The first family with MCKD was likely reported in 1966 (204). Patients can present with polyuria, polydipsia, or isosthenuria, which are the same symptoms of juvenile nephronophthisis. Unlike nephronophthisis however, MCKD is dominantly inherited and has adult onset of disease. In addition, most MCKD patients present with hypertension (40%), hyperuricemia (36%), gout (20%), or microalbuminuria (5%), which are not found in nephronophthisis (205,206). A bimodal pattern is observed in the age of onset of disease and ESRD, with one peak in the third to fourth decade of life and another in the fifth to sixth decade of life. This pattern reflects the difference in phenotype of the two loci identified for MCKD. MCKD1 generally presents later while MCKD2 presents earlier, although there is some overlap.

Regardless of the age of presentation, disease progresses rapidly: the average interval from diagnosis to ESRD is 5 years. Hyperuricemia tends to worsen as renal function worsens (207). Hypotension replaces hypertension in later stages of disease as patients lose renal concentrating capacity and become hyponatremic. If treatment via dialysis or transplant is not initiated, death occurs by uremia and renal failure.

### 63.5.2 Gene Mapping, Structure, Function, and Genotype–Phenotype Correlations

**63.5.2.1 MCKD1 (MIM 174000).** Initial studies excluded linkage to the *NPHP1* locus (208). Linkage analysis in a Cypriot family placed a locus for MCKD, later termed *MCKD1*, with the discovery of a second locus, at 1p21 (209). The critical region was narrowed to a 3.3 Mb region by fine mapping (210). Several candidate genes in the region, the natriuretic peptide receptor A (NPR-A) and the rhesus blood group B glycoprotein genes, were excluded when no mutations were found in either (205,211,212). The critical region was further narrowed to a 2.1 Mb interval (206). Mutational analysis of 37 genes within the critical region was performed, and three sequence variations were identified in three different genes, each segregating within a particular family (213). Thus, a single causative gene remains to be determined. Alternatively, multiple genes may be involved.

**63.5.2.2 MCKD2 (MIM 603860).** Several MCKD kindreds did not show linkage to the *MCKD1* critical region, and subsequent study of an Italian pedigree led to the identification of 16p12 as a second MCKD locus (214,215). The Italian MCKD2 family had earlier onset of disease, isosthenuria, and more severe hyperuricemia and gout, but another MCKD2 family did not have isosthenuria, hyperuricemia, or gout (216). Mapping of a

family with familial juvenile hyperuricemic nephropathy (FJHN) and medullary cysts to 16p12 suggested that MCKD2 and FJHN were allelic and narrowed the critical interval to a 1.3 Mb region (217).

Pathogenic mutations in the uromodulin gene, *UMOD*, were found in families with FJHN, and sequencing of *UMOD* in patients with MCKD2 revealed mutations as well (218). The uromodulin gene has 12 exons (10 coding) spanning 18.6 kb of the human genome. It is transcribed into a 2.4 kb mRNA, which is translated into a 640 amino acid protein that is also known as Tamm-Horsfall protein. Uromodulin/Tamm-Horsfall protein (UMOD/THP) is expressed in the epithelium of the thick ascending loop (TAL) of Henle and the early distal convoluted tubules (219). UMOD is anchored to the plasma membrane via a glycosyl phosphatidylinositol linkage, and is secreted via cleavage of the anchor. It contains two calcium-binding epidermal growth factor (cbEGF) domains and a zona pellucida (ZP) domain that facilitates its polymerization into fibers that forms a gel impermeable to water but allows ion flux (220) (Figure 63-11). Thus, uromodulin is thought to impart to the TAL its water impermeability, and therefore, enable the kidney to concentrate urine. It is also hypothesized to inhibit renal stone formation and protect against UTI and ischemia (221–224).

All the known *UMOD* mutations are missense or in-frame deletions/insertions in exon 4, which encodes the cbEGF domains. A family with homozygous mutations in *UMOD* was observed to survive to adulthood but have more severe hyperuricemia and faster progression to ESRD (225).

An interesting finding that may cast light on the pathogenesis of MCKD is that the majority of mutations affect conserved cysteine residues (219,226,227). Since

no free sulfhydryl groups are found on uromodulin, these mutations likely alter proper disulfide bonding and protein folding (228). Misfolded endoplasmic reticulum (ER) proteins tend to aggregate within the ER and are not secreted, a finding confirmed for mutated uromodulin (220,226). Decreased UMOD secretion in the TAL of the nephron may increase water permeability, and therefore, decrease renal concentrating ability, explaining the isosthenuria seen in patients with MCKD2. Increased salt and water excretion leads to compensatory distal convoluted tubule and collecting duct reabsorption and secondary urate reabsorption, leading to hyperuricemia. Tubular cells undergo apoptosis because of accumulation of undegradable, misfolded uromodulin (229). The body mounts an inflammatory response to the release of UMOD into the interstitium, eventually leading to fibrosis.

**63.5.2.3 Other Loci.** Linkage analysis of MCKD kindreds uncovered families that mapped neither to the *MCKD1* locus nor to *MCKD2*, suggesting the existence of more loci for MCKD (214,230).

### 63.5.3 Imaging and Histopathologic Findings

Renal ultrasonography shows normal to decreased renal size and reduced cortical thickness. Although bilateral corticomedullary or medullary cysts may be present (40%), they are not necessary for diagnosis, nor are they pathognomonic for MCKD (207). Histopathologic findings of renal biopsies are indistinguishable from biopsies of patients with nephronophthisis: disorganized tubular basement membranes, tubular atrophy with cyst formation, interstitial lymphohistiocytic infiltration, and periglomerular and interstitial fibrosis.

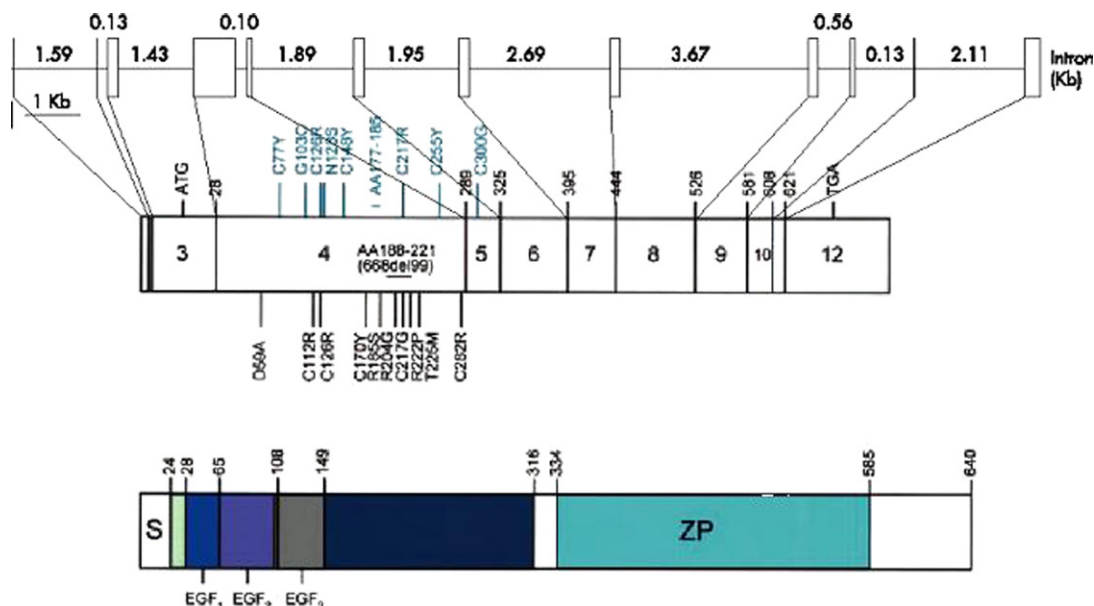


FIGURE 63-11

### 63.5.4 Molecular and Prenatal Diagnosis

Diagnosis is made primarily via a combination of suggestive clinical features, imaging, and (if available) renal histopathologic findings. Since no specific gene has been identified for MCKD1, testing of at-risk individuals requires a family with several affected members and sufficient size in order for linkage analysis to have sufficient power. Confirmatory molecular testing is available on a commercial basis for MCKD2. Prenatal testing or testing of presymptomatic children is not recommended for a disease that is both treatable and does not present until adulthood.

### 63.5.5 Management

Since there is no therapy for the underlying disorder, treatment is limited to supportive care. Gout and hyperuricemia are controlled with allopurinol. Interval assessment of renal function should be performed to detect onset of ESRD, when dialysis and renal transplantation should be initiated. MCKD must be excluded in family members who wish to be kidney donors. Outcomes of renal transplantation are excellent; the graft kidney does not develop cystic disease (231).

## 63.6 MULTICYSTIC DYSPLASTIC KIDNEY DISEASE (MKD)

By ultrasound, congenital MDK consists of cysts of various sizes with loss of lobar organization. Microscopically, there is loss of normal structure, with the presence of primitive ductules and cartilage, suggesting aberrations in renal differentiation. Dysplasia can result in cysts that are solid or cystic, large or small. Function is variable (232).

MDK usually presents as an abdominal mass in the newborn period. Other significant manifestations are uncommon and include flank pain, urinary frequency, dysuria, failure to thrive and UTI.

Most cases of MKD occur sporadically. However, a number of syndromes can have MKD as their renal manifestation. These include Meckel–Gruber syndrome, Jeune asphyxiating thoracic dystrophy, Zellweger syndrome, VACTERL association, brachio–oto–renal syndrome, Williams syndrome, Beckwith–Wiedemann syndrome and certain chromosomal trisomies.

## 63.7 GENETIC SYNDROMES WITH CYSTIC RENAL DISEASE AS A MAJOR COMPONENT

### 63.7.1 Tuberous Sclerosis Complex (MIM 191100, 613254)

Tuberous sclerosis complex is a neurocutaneous disorder with an incidence of 1 in 5000 to 10,000 live births (233) and is characterized by the involvement of many organ

systems including benign hamartomas of the brain, eye, heart, lung, liver, kidney, and skin (for more information, please refer to the chapter on Phakomatoses).

Renal manifestations include angiomyolipomas, which are the most common, followed by renal cysts and lymphangiomas. Angiomyolipomas are at increased risk of bleeding, especially with progressive enlargement. Renal cell carcinoma develops in 1–2% of patients. The Consensus Conference recommended the following surveillance imaging studies for patients with TSC (234). Renal ultrasonography should be performed at the time of diagnosis to ascertain the presence or absence of the polycystic variant of TSC or angiomyolipomas. All children with TSC should have screening with renal ultrasonography every 1–3 years (3 years if no prior lesions are seen on imaging). If malignant transformation or the development of large angiomyolipomas (greater than 3–4 cm) is detected, abdominal CT or MRI should be performed to evaluate the abnormalities. Interventional radiology can be consulted for treatment of the lesions greater than 3–4 cm to prevent hemorrhage.

Tuberous sclerosis complex is inherited in an autosomal dominant manner with variable expression. Two genes are known to cause the disorder: *TSC1*, located on chromosome 9q34, and *TSC2*, located on chromosome 16p13.3, which lies in close proximity to the *PKD1* gene. A contiguous gene deletion syndrome involving *TSC2* and *PKD1* results in a combined phenotype of tuberous sclerosis and ADPKD. *TSC1* encodes hamartin, and *TSC2* encodes tuberin. The two proteins interact to form a complex. In affected individuals meeting definite clinical criteria, 70–80% have an identifiable mutation in one of these two genes, with *TSC2* being more frequently implicated (235). Sequence variants account for the majority of mutations, but large deletions and rearrangements have also been reported in both genes (236). With regard to genotype–phenotype correlations, individuals with a *TSC1* mutation appear to have milder disease than those with a *TSC2* mutation. One study showed decreased frequency of seizures and mental retardation, fewer subependymal nodules and cortical tubers, milder kidney involvement, absence of retinal hamartomas, and less severe facial angiofibroma in patients with *TSC1* mutations (237). In addition, mutations in familial *TSC2* cases appear to be associated with less severe clinical manifestations than de novo *TSC2* mutations (238).

### 63.7.2 Bardet–Biedl Syndrome (MIM 209900)

Bardet–Biedl syndrome (BBS) is a genetically heterogeneous disorder with 14 gene loci identified to date (Table 63-3). It is usually inherited in an autosomal recessive manner, but some forms have a unique triallelic inheritance pattern requiring recessive mutations at one locus and an additional mutation at a second locus. Owing to considerable phenotypic heterogeneity,

TABLE 63-3

Gene	Locus
<i>BBS1</i>	11q13
<i>BBS2</i>	16q21
<i>BBS3/ARL6</i>	3p12–q13
<i>BBS4</i>	15q22.3–q23
<i>BBS5</i>	2q31
<i>BBS6/MKKS</i>	20p12
<i>BBS7</i>	4q27
<i>BBS8/TTC8</i>	14q32.1
<i>BBS9/B1</i>	7p14
<i>BBS10</i>	12q21.2
<i>BBS11/TRIM32</i>	9q31–34.1
<i>BBS12</i>	4q27
<i>BBS13/MKS1</i>	17q23
<i>BBS14/CEP290</i>	12q21.3

a classification system has been proposed in which a minimum of four major criteria are required for diagnosis. The major diagnostic criteria are learning disability (62–87%), rod–cone dystrophy (92–100%), postaxial polydactyly (58–74%), truncal obesity (72–96%), hypogonadism in males (74–96%), and renal anomalies (46–95%). Speech deficit (54%); motor and speech delay (50%); hypertension (50%); ataxia (40%); “difficult,” immature, volatile behavior with lack of inhibition and easy frustration (33%); dental crowding (27%); and asthma (25%) are other common features (239). Molecular diagnostic testing is commercially available.

BBS shares some features of nephronophthisis, with polyuria, polydipsia, nyctalopia progressing to total blindness in the second decade of life, hepatic fibrosis, and chronic renal failure reported in some patients. Renal pathology is sometimes indistinguishable from nephronophthisis and MCKD. Progression to ESRD, however, is rare. Structural renal anomalies include cortical cysts, calyceal blunting, hydronephrosis, vesicoureteral reflux with recurrent UTI, renal dysplasia, and fetal lobulation (240).

A possible link to inversin (*NPHP2*), cilia, and cell-cycle regulation was discovered when the *BBS8* protein was found to localize to centrosomes and co-localize with tubulin. Furthermore, patients with homozygous mutations of *BBS8* were found to have randomization of body situs (241). For the most part, BBS proteins localize to cilia, centrosomes, or basal bodies (241–246).

### 63.7.3 Jeune Asphyxiating Thoracic Dysplasia (MIM 208500) and the Short-Rib Polydactyly Syndromes

Patients with asphyxiating thoracic dysplasia (ATD) have extremely narrow, constricted thoracic cages caused by shortened, horizontal ribs. Death often occurs in infancy because of severe pulmonary hypoplasia as a consequence of the small chest cavity. Survivors face recurrent

pneumonia and the possibility of respiratory failure incurred by restrictive lung disease. Long bones and phalanges are shortened and some patients have postaxial polydactyly of the hands and/or feet. Radiographs show short ribs with irregular costochondral junctions, hypoplastic iliac wings with trident-shaped acetabulae, transient irregularity of epiphyses and metaphyses, and cone epiphyses of the phalanges. Short stature and relative size of the thoracic cage improve with age, but children also develop biliary ductal plate malformations and hepatic fibrosis, pancreatic cysts and fibrosis, and polycystic kidneys. Renal histopathology shows cystic renal tubular dysplasia and possibly disorganized tubular basement membranes, tubular atrophy, and interstitial infiltration and fibrosis seen in nephronophthisis. Interestingly, some patients with ATD are affected with tapetoretinal degeneration and situs inversus, which are also seen in patients with nephronophthisis (247,248). Currently, two causative genes, *IFT80* and *DYNC2H1*, are associated with ATD. Like the nephronophthisis and BBS genes, *IFT80* and *DYNC2H1* play a role in ciliary and/or microtubular function.

ATD belongs to the short-rib dysplasia family of skeletal dysplasias, which includes the four neonatal lethal short-rib polydactyly (SRP) syndromes, Ellis–van Creveld dysplasia, and Barnes thoracolumbar pelvic dysplasia. Infants with the SRP syndromes are hydropic and die in the perinatal period from respiratory insufficiency. Cystic renal disease is present in all four types of SRP. All have CNS malformations (Dandy–Walker malformation, pachygyria, holoprosencephaly, and anencephaly), gastrointestinal malformations (esophageal, intestinal, or anal atresia in SRP I, II, and III, malrotation in IV), narrow chest, micromelia, and postaxial polydactyly. SRP types II and IV have bifid epiglottis with median cleft lip and palate. SRP IV infants are also found to have hepatic and pancreatic cysts with fibrosis, much like ATD and ARPKD.

### 63.7.4 Hajdu–Cheney Syndrome (MIM 102500)

This autosomal dominant skeletal dysplasia is an acroosteolytic condition with progressive, slow, centrifugal resorption of the distal and middle phalanges of the hands and feet beginning in adolescence. The initial abnormality in affected infants is widening of the sagittal and/or lambdoid sutures. Other craniofacial abnormalities that develop with age are persistently open sutures, wormian bones, elongation of the sella turcica, coarse hair and facies, broad forehead, hypertelorism with downslanting palpebral fissures, broad nasal bridge, and premature loss of teeth. Short stature, osteoporosis, and joint laxity are also observed. Polycystic kidneys similar to ADPKD were noted in approximately 10–14% of affected children, often with concomitant hypertension and CRI (249–251). Other renal anomalies, such



as unilateral multicystic dysplastic kidney, vesicoureteral reflux, and glomerulonephritis have been noted.

### 63.7.5 Campomelia, Cumming Type (MIM 211890)

This autosomal recessive, skeletal dysplasia is characterized by premature stillbirth, cervical hygromas with hydrops fetalis, narrow chest, shortened limbs with bowing of all long bones, especially the tibiae and ulnae, and cloverleaf skull (252–254). Biliary ductal plate malformation and hepatic fibrosis, pancreatic fibrosis, and renal cortical and medullary cysts are seen (254,255). Abnormalities of situs, specifically dextrocardia, left-sided superior vena cava, right-sided aortic arch, total anomalous pulmonary venous return, and polysplenia have been reported in several fetuses with this disorder (252,255,256).

### 63.7.6 Glutaric Acidemia Type II (MIM 231680)

Glutaric acidemia type II, also known as multiple acyl-CoA dehydrogenase deficiency, is an autosomal recessive disorder caused by mutations in *ETFA*, *ETFB*, or *ETFDH*, genes that encode the electron transport flavoprotein (ETF) and ETF ubiquinone oxidoreductase (ETF:QO). ETF is the ultimate electron acceptor in branched-chain amino acid, long-chain fatty acid, and choline metabolism. ETF:QO transfers electrons from ETF to the electron transport chain. Deficiency in either enzyme causes a phenotype that is a combination of fatty acid oxidation disorders and isovaleric acidemia, with metabolic acidosis, hypoketotic hypoglycemia, hepatomegaly, hypotonia, and a “sweaty foot” odor. Malformations such as enlarged anterior fontanel, high forehead, flat nasal bridge, telecanthus, and ear anomalies may be present. Bilaterally enlarged, polycystic kidneys with medullary dysplasia can be seen, especially in patients with more severe disease. Presumptive diagnosis can be made from plasma acylcarnitine and urine organic acid analysis. Fibroblast assay of ETF and ETF:QO activity and molecular genetic testing of *ETFA*, *ETFB*, and *ETFDH* confirm the diagnosis.

### 63.7.7 Carnitine Palmitoyltransferase II Deficiency, Neonatal Lethal Form (MIM 608836)

The carnitine palmitoyltransferase II (CPT II) enzyme catalyzes the reconstitution of long and very-long-chain acylcarnitines to coenzyme A following translocation into the mitochondrion. Patients with CPT II deficiency are consequently unable to metabolize long-chain fatty acids and become energy deficient during periods of fasting. CPT II deficiency can present at any time, but severely affected infants who present in the first few

days of life with lethargy, poor feeding, tachypnea, metabolic acidosis, hepatomegaly, hypothermia, and hypoglycemia have been noted to have enlarged, polycystic kidneys with parenchymal dysplasia. Presumptive diagnosis can be made from free and total carnitine levels, plasma acylcarnitine profile, and urine organic acid analysis. The diagnosis is confirmed by measuring CPT II activity in fibroblasts or molecular testing of the *CPT2* gene.

### 63.7.8 Zellweger Syndrome (MIM 214100)

Also known as cerebrohepatorenal syndrome, Zellweger syndrome is a disorder of peroxisome biogenesis that lies at the severe end of a clinical spectrum that includes neonatal adrenoleukodystrophy and infantile Refsum disease. This group of disorders is genetically heterogeneous. Mutations have been found in 12 different genes, all encoding proteins necessary for normal peroxisome assembly. *PEX1* is the most commonly implicated, and the mode of inheritance for all types is autosomal recessive. Patients present in early infancy with severe hypotonia, hyporeflexia, and poor feeding. Many are initially suspected of having a type of myopathy. Dysmorphic examination shows macrocephaly with an extremely large anterior fontanelle, high forehead, flat facies, hypertelorism with upslanting palpebral fissures, and hepatomegaly. Plain-film X-rays reveal stippling of the patellae and long bone epiphyses. Most patients have brain malformations and suffer from myoclonic or generalized tonic-clonic seizures. A vast majority die before the first birthday; survivors are severely mentally retarded. Polycystic kidneys are present in most patients. Zellweger syndrome can be screened for by measuring plasma very-long-chain fatty acids, which are elevated in this condition. Additional testing may include analysis of plasmalogens in erythrocytes, bile acid intermediates, and measurement of phytanic acid and pristanic acids in plasma. Complementation studies can be performed on fibroblasts to identify which peroxisomal gene is deficient. Molecular diagnostic testing is commercially available for many of the *PEX* genes.

There are other genetic syndromes associated with renal cysts. These syndromes and their mode of inheritance are shown in Table 63-4.

## 63.8 MECHANISMS OF CYSTOGENESIS

How do mutations in the polycystins, fibrocystin, and nephrocystins result in cyst formation? While all the mechanisms are not clear, increasing knowledge of the primary cilia of renal tubular epithelial cells has shed light on some of the mechanisms.

Each epithelial cell of the distal convoluted tubule and collecting duct contains a single primary cilium that is nonmotile, with microtubules arranged in a 9+0 pattern. Once thought to be a vestigial organelle, the primary

cilium is now known to be necessary for sensing extracellular shear stress from perpendicular fluid flow and transducing an increase in intracellular calcium ion concentration (257). Studies have also shown that primary cilia are necessary for maintenance of planar cell polarity

through various cellular signaling pathways (e.g. Wnt signaling), and loss of cilia results in abnormal orientation of cell division and cystogenesis (258).

Polycystin-1, polycystin-2, fibrocystin, and the nephrocystins all localize to the primary cilia of renal epithelial cells, and some at basal bodies, centrosomes, focal adhesions or adherens junctions (Figure 63-12). Likewise, the genes associated with syndromic forms of cystic kidney disease (i.e. Joubert syndrome, BBS, Meckel–Gruber syndrome, etc.) demonstrate similar localization.

Polycystin-1 and polycystin-2 directly interact with one another through their C-terminal coiled-coil domains to form a complex. This complex mediates calcium influx in response to shear stress by sensing the bending of the primary cilium (259,260). The large extracellular domain of polycystin-1 functions as a mechanosensor of urine flow, which triggers calcium influx through polycystin-2 channels. The initial calcium current has been shown to “feed forward” and trigger release of intra-organellar calcium stores via the ryanodine receptor. The polycystin complex is also believed to play a role in regulation of the cell cycle (261). Expression of mutant *PKD1*, blockade with polycystin-1- and polycystin-2-specific antibodies, or other disruptions of this system results in impaired calcium influx, leading to increased cAMP levels, and ultimately, abnormal cell proliferation and cell dedifferentiation (Figure 63-13) (6,78,262). The flow-sensor function of the polycystins in the primary cilium may be a contributing pathway in the pathogenesis

TABLE 63-4
<b>Autosomal Recessive Inheritance</b>
Meckel syndrome
Kaufman–McKusick syndrome
Retina–renal dysplasia syndromes
Ivemark syndrome
Fryns syndrome
<b>Autosomal Dominant Inheritance</b>
Brachio–oto–renal syndrome
von Hippel Lindau syndrome
Townes–Brocks syndrome
<b>X-Linked</b>
Oro–facial–digital syndrome
<b>Chromosomal</b>
Trisomy 18
Trisomy 13
Trisomy 9
Triploidy
Deletion 3p
<b>Inheritance Variable</b>
VATER association
Proteus syndrome
Prune-belly syndrome

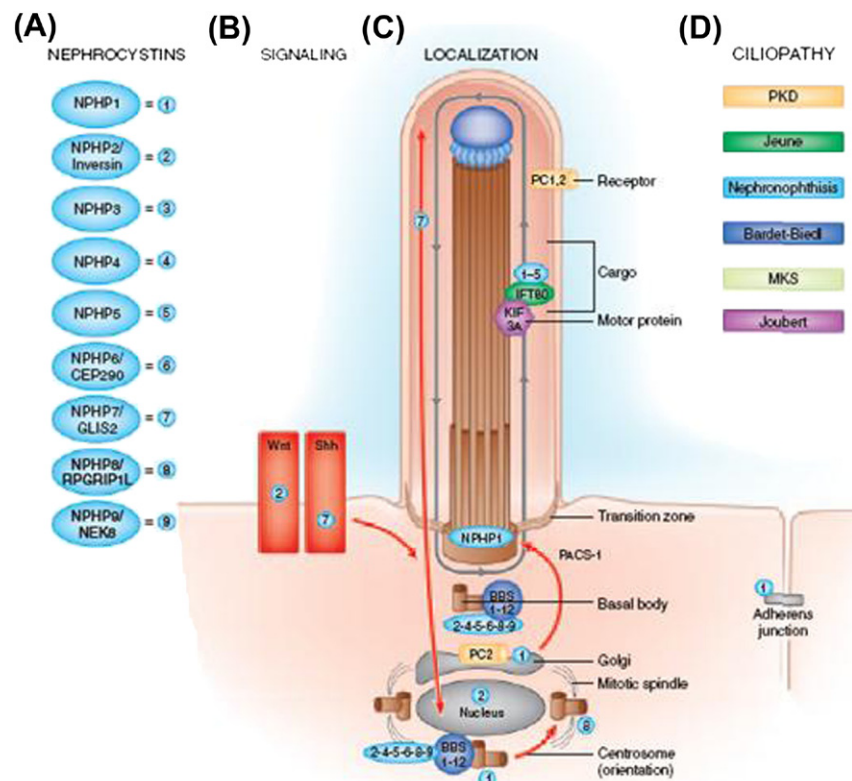


FIGURE 63-12 Hildebrand, F., et al. Nephronophthisis: Disease Mechanism of a Ciliopathy 2009, 20, 26, Fig 4.

## Polycystin and ADPKD

Autosomal dominant polycystic kidney disease (ADPKD) is due to mutations in one of two genes, PKD1 and PKD2, which code for the linked transmembrane proteins polycystin 1 and polycystin 2, found on the primary cilium present on almost all renal tubular cells (not on the intercalated cells) and collecting ducts. Polycystin 1 is expressed predominantly in the distal convoluted tubule and collecting ducts, while polycystin 2 is expressed predominantly in the distal convoluted tubule and loop of Henle.

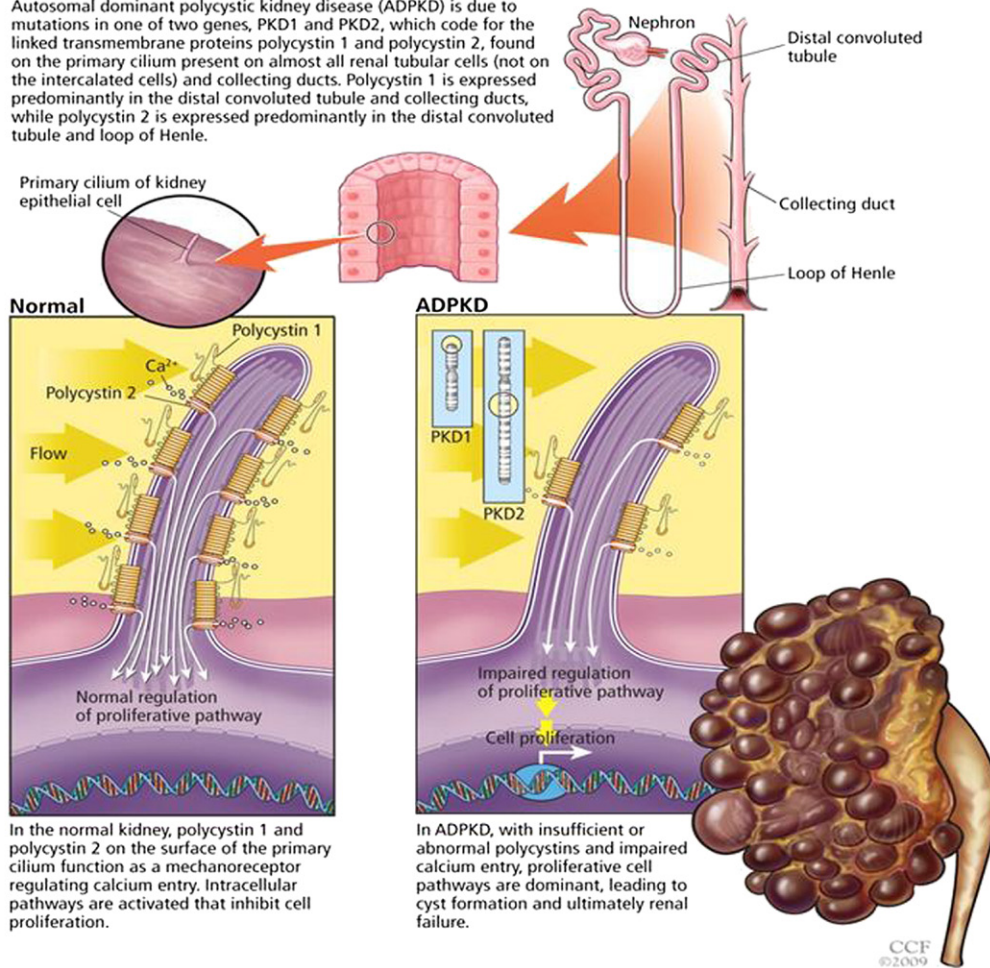


FIGURE 63-13

of cyst formation in kidneys with obstructed flow, such as ureteropelvic or ureterovesicular junction obstruction.

Nephrocystin-1 and fibrocystin have also been found at the primary cilium in association with the polycystin protein complex and are also thought to participate in the same mechanotransduction pathway (125,126,174,263,264).

The Wnt signaling pathway is an integral part of the pathogenic process of renal cyst formation (Figure 63-14). Under normal circumstances, cilia respond to fluid flow, and the calcium signaling cascade is initiated. As a result, several proteins, including nephrocystin-2/inversin and the BBS proteins, are activated and shift the Wnt pathway from its canonical to non-canonical mode (185,265,266). As a result of this shift,  $\beta$ -catenin, which normally acts as a transcriptional activator, is degraded by proteasomes, and normal planar cell polarity is maintained. When calcium signaling or ciliary proteins are defective, the canonical Wnt pathway predominates, and  $\beta$ -catenin is able to translocate into the nucleus where it forms an active transcription factor complex with the T-cell-specific transcription

factor/lymphoid enhancer binding factor (TCF/LEF) and promotes the expression of fetal genes to induce cell proliferation and differentiation, ultimately resulting in cyst formation.

Polycystin-1, polycystin-2, and nephrocystin-1 also aggregate into a complex that co-localizes in vivo with  $\alpha 2 \beta 1$  integrin and co-immunoprecipitate with multiple focal adhesion adaptor proteins, most notably focal adhesion kinase, c-src, and p130cas (170,171). In response to ligand binding, the polycystin complex activates c-Jun kinase and mitogen-associated protein kinase via focal adhesion adaptor proteins, leading to phosphorylation and activation of the c-Jun protein, a member of the AP-1 transcription factor family. Phosphorylated c-Jun dimerizes with ATF, another member of the AP-1 family, to create an active transcription factor that drives the expression of genes responsible for differentiation and reduction of proliferation in developing renal tubular epithelium (267) (Figure 63-15A). Inactivation of AP-1 transcription factor-mediated gene expression by abrogation of polycystin-1 and/or 2 signaling results in inability to downregulate proliferation and fetal gene



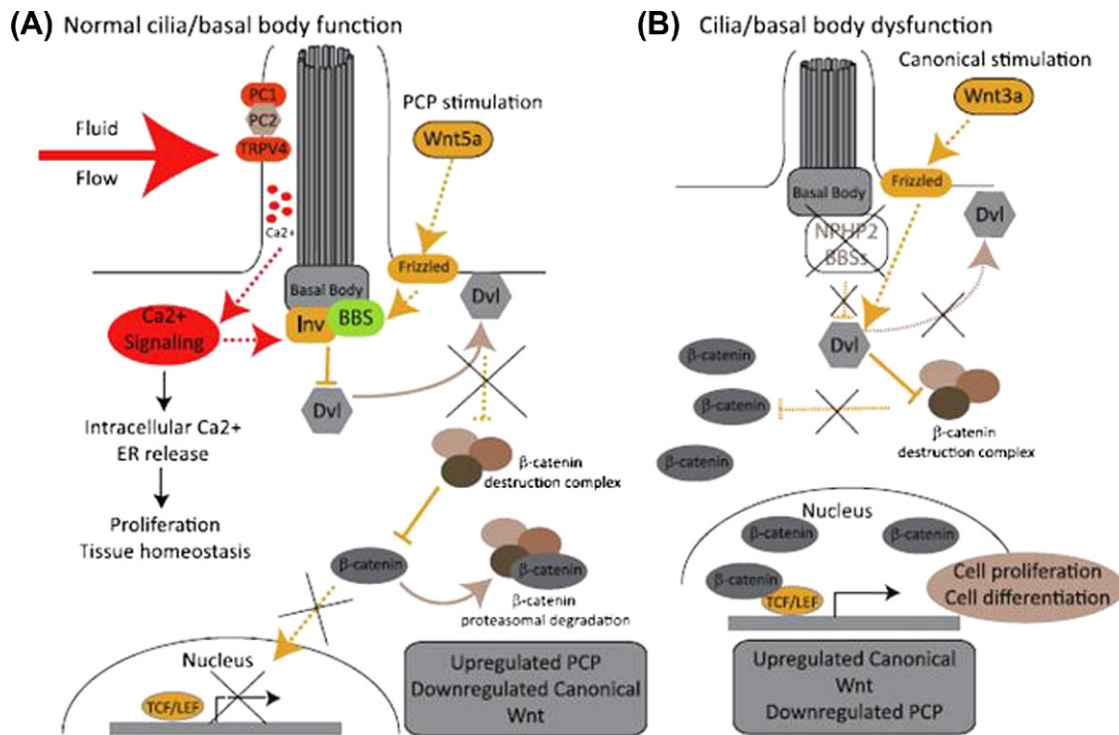
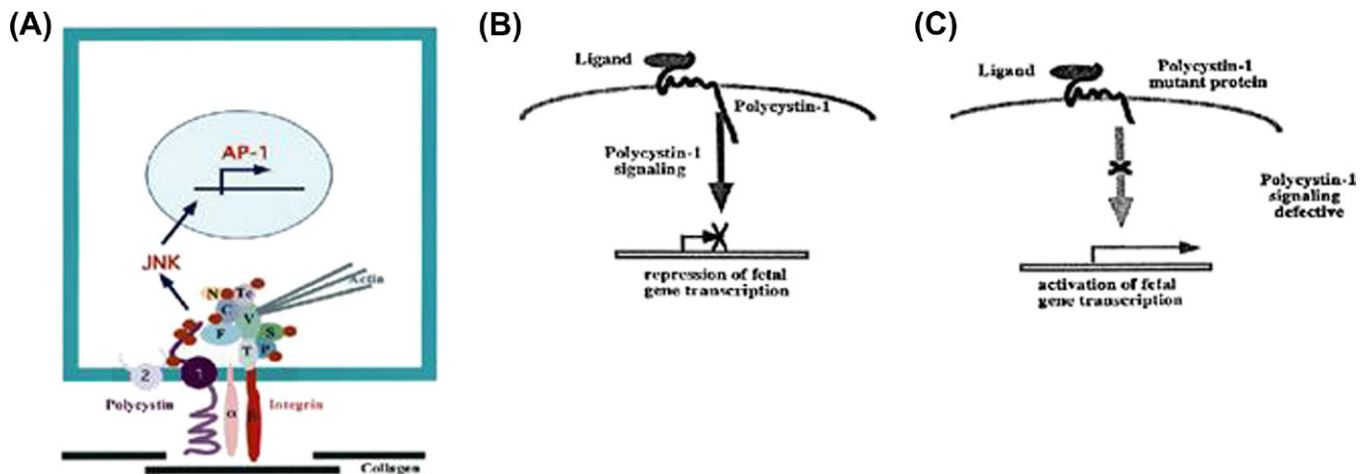
FIGURE 63-14 Katsanis, N., et al., *Pediatric Nephrology* 2010, 6, Fig 2.

FIGURE 63-15

expression, leading to conditions that foster cyst formation (268,269) (Figure 63-15B,C). The exact role of nephrocystin-1 at the focal adhesion complex is not well defined, although its SH3 domain may be responsible for recruiting c-src and p130cas to the polycystin protein complex.

The function of the other nephronophthisis genes, especially in relation to cerebral and ocular development, is less well defined. While nephrocystin-3 and nephrocystin-4 have been found to associate with nephrocystin-1, it is not yet known which signaling pathways they participate in and how. Inversin interacts with the anaphase-promoting complex via its two

“destruction-box” domains, possibly linking environmental stimuli sensed by primary cilia to the centrosome and regulation of cell division (154). Finally, inversin is involved in early embryogenesis as one of the determinants of left–right axis patterning, perhaps through its interaction with mechanosensory cilia (270,271).

Much progress has been made in our understanding of renal cystogenesis. Although questions remain to be answered, our increasing knowledge of the genetic and molecular basis of cystic kidney diseases will enhance insight into the pathogenesis, clinical consequences, and ultimately, the treatment of these disorders.



## CROSS REFERENCES

In the section on tuberous sclerosis complex, we would like to refer to the chapter on Phakomatoses.

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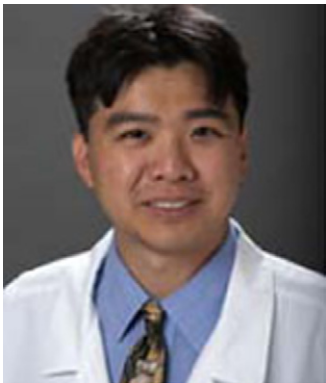
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### Biographies



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# CHAPTER

# 64

## Nephrotic Disorders

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### 64.1 INTRODUCTION

Nephrotic syndrome (NS) is a clinical diagnosis characterized by heavy proteinuria, hypoproteinemia, and edema. It occurs in various forms of acquired renal diseases, such as glomerulonephritides and membranous nephropathy, or may be part of systemic diseases, such as vasculitis, lupus erythematosus, and amyloidosis. NS may also be caused by infections, such as human immunodeficiency virus (HIV), maternal syphilis, toxoplasmosis, and malaria. Toxic agents such as mercury, insect stings, or maternal steroid–chlorpheniramine treatment are also associated with NS.

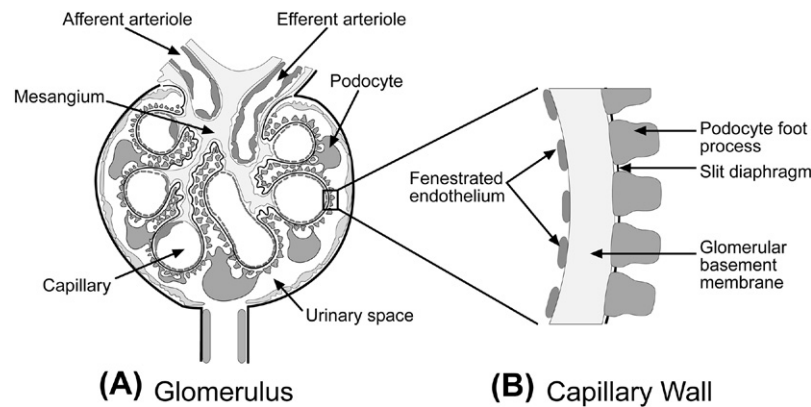
The most common variety of primary NS in children is steroid-sensitive nephrotic syndrome (SSNS). Approximately 80% of all children with NS respond to prednisone therapy and renal biopsy indicates minimal histological changes (MCNS) in 80% and focal segmental glomerulosclerosis (FSGS) in 20% (1). Twenty percent of NS in children show steroid resistance (SRNS). Seventy-five percent of these patients exhibit renal histological features of FSGS, and 20% demonstrate MCNS. Overall, MCNS and FSGS account for over 90% and 5% of NS in the pediatric age group, respectively. FSGS is especially common in African-Americans, and the incidence of FSGS in children has increased. In adults the situation is different, and FSGS is the most common type of NS. Typically FSGS manifests as NS but progresses to renal failure within months or years. FSGS is a significant cause of end-stage renal disease (ESRD), comprising up to 5% of adults and 20% of children with ESRD. The pathophysiology of SSNS and SRNS is mostly unknown, but T-lymphocyte dysfunction has been regarded as the basic event. In addition, circulating permeability factors have been suggested as the etiology for SSNS and SRNS, but their presence has not been verified (2).

During the past decade gene defects both in autosomal recessive and dominant forms of SRNS have been identified. To date, approximately 10–20% of sporadic and 30–40% of familial cases of SRNS are associated with a gene defect (3,4). These new genes include nephrin (*NPHS1*), podocin (*NPHS2*), Wilms' tumor factor 1 (*WT1*), phospholipase Ce1 (*PLCE1*), laminin  $\beta$ 2 (*LAMB2*), transient receptor potential C6 ion channel (*TRPC6*), CD2-associated protein (*CD2AP*), alpha-actinin-4 (*ACTN4*), and inverted formin 2 (*INF2*) genes. It is to be expected that more pathogenetic gene defects will be identified in the future. The known gene disorders cover about two-thirds of NS in early childhood and less than 20% of the cases with late-onset of NS.

Recent discoveries indicate that NS caused by a particular gene defect can manifest at various ages. Thus, the traditional classification of NS into congenital (CNS; onset <3 months of age), infantile (INS, onset at 4–12 months) and late-onset (>12 months) is not valid anymore. However, the terms are still useful in clinical practice. Similarly, the histological features of the kidney overlap in the different genetic and nongenetic entities, so that the microscopic findings do not provide the basis of the classification or tell the specific diagnosis. The clinical renal and extrarenal manifestations, kidney histology, and the age at onset, however, give a clue to the possible gene defect involved. This review focuses first on the major genetic entities and then deals with the diagnostics and management of NS patients.

### 64.2 GLOMERULAR FILTRATION BARRIER

In the kidney, filtration of plasma takes place in the glomerular capillary tuft surrounded by the urinary

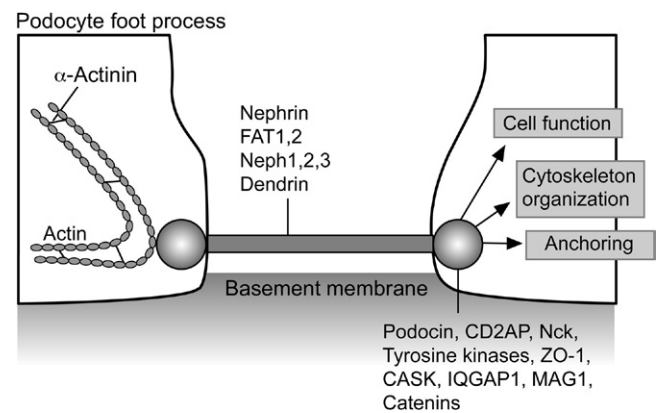


**FIGURE 64-1** The structure of kidney glomerulus (A) and glomerular capillary wall (B). Primary urine is formed in the glomerulus by passing of plasma water and small-molecular-weight molecules through the capillary wall (glomerular filtration barrier) into the urinary space. In nephrosis plasma proteins also leak through this barrier, which is composed of three layers: fenestrated endothelium, glomerular basement membrane, and podocyte foot processes. These are connected by the slit diaphragm, which is important for the proper function of the filtration barrier. (Figure 64-1A is modified from the original figure by Professor Wilhelm Kriz.)

space and Bowman's capsule (Figure 64-1). The ultrafiltration barrier in the capillary wall comprises three layers: fenestrated endothelium, glomerular basement membrane (GBM), and epithelial cell (podocyte) layer with distal foot processes and interposed slit diaphragms (SDs). The barrier is a highly sophisticated size- and charge-selective molecular sieve, and normally only water, electrolytes, and small plasma solutes pass through this barrier (5–7). The flow of glomerular filtrate follows the extracellular route, passing across the GBM and then across SDs, which bridge adjacent foot processes just above the GBM.

Studies performed during the past decade have clearly indicated that podocytes and the SD are essential in restricting the passage of plasma proteins into urine (8,9). The precise molecular structure of SD is still unresolved, but podocyte proteins nephrin, Nephrin, Nephrin, Fat1, Fat2, cadherins, and dendrin seem to be its essential components (10,11) (Figure 64-2). Nephrin and Nephrin are believed to associate with each other extracellularly and they form the backbone of the SD (12–14). The SD proteins interact with the adapter proteins podocin, CD2AP, ZO-1, CASK, IQGAP1, MAG I, catenins, and spectrins that are located in the cytosolic part of the podocyte foot process (Figure 64-2) (15–19). The adapter proteins connect the SD with the actin cytoskeleton of the podocyte foot process and take part in the signal transduction from the SD into podocyte (9,17).

The actin network and the interacting proteins, such as  $\alpha$ -actinin-4, are critical for the maintenance of the complex structure of the podocyte foot process (20). Practically all forms of NS lead to podocyte injury with foot process flattening (effacement) and distortion of normal SD structure (21). This is believed to reflect a defective interplay of the SD components, adapter proteins and the actin network itself. The discovery of NS caused by mutations in the GBM components (laminin- $\beta$ 2) highlights the importance of the GBM, podocyte foot



**FIGURE 64-2** A hypothetical model of the podocyte foot process and slit diaphragm. Nephrin and Nephrin possibly form the backbone of the extracellular part of the slit diaphragm. Other molecules, such as Fat 1 and dendrin have also been localized to this structure. Adapter proteins, such as podocin, CD2AP, ZO-1, CASK, IQGAP1, MAG1, Nck, catenins, and spectrins are intracellular molecules that interact with nephrin and Nephrin and connect the slit diaphragm to actin cytoskeleton of the podocyte foot process. The calcium-channel TRCP6 is also believed to interact with the slit diaphragm complex.

processes, and the SD in maintaining a properly functioning glomerular filtration barrier (22).

### 64.3 NEPHRIN GENE (*NPHS1*) MUTATIONS

Nephrin is a transmembrane adhesion protein of the immunoglobulin family. The extracellular part of nephrin contains eight immunoglobulin-like motifs and one type III fibronectin domain (8). The intracellular domain has nine tyrosine residues, some of which become phosphorylated during ligand binding. Nephrin is synthesized by glomerular podocytes and localized at the SD area between the podocyte foot processes (8). Nephrin is encoded by the *NPHS1* gene, located on chromosome 19q13.1. It consists of 29 exons and has a size of

26kb. Mutations in *NPHS1* causes congenital nephrotic syndrome of the Finnish type (CNF, *NPHS1*), which is a prototype of CNS.

### 64.3.1 Congenital Nephrotic Syndrome of the Finnish Type (CNF, *NPHS1*)

CNF (OMIM 602716) is an autosomal recessive disease first described in Finland. The incidence in Finland is 1 in 8000 live births. CNF has been reported from all over the world in different ethnic groups (23–25). A very high incidence of CNF has been observed among the Old Order Mennonites in Lancaster County, Pennsylvania. Exon sequencing analyses of Finnish patients revealed the presence of two truncating mutations in 94% of *NPHS1* chromosomes (Fin-major and Fin-minor) (8,26). The uniform mutation pattern seen in the Finnish population can be explained by the founder effect (27). Most non-Finns have individual mutations and to date roughly 150 different mutations have been described, spanning the whole gene. Mutations in the promoter area have also been found. Most missense mutations in *NPHS1* lead to the misfolding of nephrin molecule and a defective intracellular nephrin transport in the podocyte (28). In four recent reports of *NPHS1* mutations in the European and worldwide cohorts of CNS, nephrin gene mutations accounted from 39 to 80% of the cases. (29–32). Overall, about half of CNS seems to be caused by *NPHS1* mutations.

The majority of typical CNF children are born prematurely with a birth weight ranging between 1500 and 3500g (33). The index of placental weight/birth weight (ISP) is over 25% in practically all newborns. The reason for this is not known. Proteinuria begins in utero and is thus detectable in the first urine sample tested. Heavy protein losses result in hypogammaglobulinemia (34) as well as low antithrombin III levels and increased risk for thrombotic complications (26). Hyperlipidemia is also present, as in other nephroses. Infants with CNF do not have any major extrarenal malformations. Minor functional disorders in the central nervous system, cardiac hypertrophy, and muscular hypotonia, however, are common during the nephrotic stage.

No single histological finding is pathognomonic for CNF. In the early stage, proliferation of mesangial cells and increase of mesangial matrix are seen (35). Dilations of the proximal and distal tubules are the most characteristic findings. Their amount varies greatly, from an occasional dilation to a universal dispersion throughout the renal cortex. In the interstitium, fibrosis and inflammatory infiltrates increase with age. In electron microscopy, the principal finding is the fusion and effacement of podocyte foot processes, seen in many nephrotic kidney diseases (21). The Fin-major and Fin-minor mutations of the *NPHS1* lead to a complete absence of nephrin in the kidney glomerulus (33). These kidneys also lack the filamentous image of podocyte SDs as studied by electron

microscopy, indicating that the absence of nephrin leads to distortion of the SD and the leakage of plasma proteins into urine through the “empty” podocyte pores (11).

### 64.3.2 Childhood-Onset NS

Mutations in *NPHS1* are quite rare in cases manifesting after the first weeks of life (29,30). However, Philippe et al. (36) analyzed DNA from 160 patients in 142 families with NS presenting after 3 months of age. Putative disease-causing nephrin mutations were found in 10 families, with the age of onset ranging from 6 months to 8 years. Biopsy findings showed minimal changes in six of the patients and FSGS in three patients. ESRD had developed in five patients at the age of 6–25 years. Mutations were classified as severe or mild and the presence of at least one mild mutation in these patients likely explains the later onset and milder course of disease. Santin et al. (30) report a similar study of 97 FSGS patients from 89 unrelated families. Disease-causing *NPHS1* defects were observed in seven sporadic and five familial cases. Three of the individuals were 1 year of age or older, and one was 27 years old at the time of disease onset. The results broaden the spectrum of renal disease related to nephrin gene mutations.

## 64.4 PODOCIN GENE (*NPHS2*) MUTATIONS

Podocin is a hairpin-like protein of the stomatin-family and is exclusively expressed in podocytes, where it is an important component of the lipid rafts of the filtration slit (Figure 64-2) (37). Podocin plays a major role in the structural integrity and function of the SD (38). This protein is encoded by the *NPHS2* gene composed of eight exons and located on chromosome 1q25-q31. Genetic defects in *NPHS2* (OMIM 604766) can lead to NS starting at any age.

### 64.4.1 Congenital and Childhood SRNS

Koziell and colleagues (24) first reported that two of five CNS patients lacking *NPHS1* mutations had homozygous *NPHS2* mutations. Schultheiss and coworkers (39) reported *NPHS2* mutations in 11 CNS patients with FSGS histology in half the kidney biopsies. Half the patients developed ESRD at a median age of 6 years. In accordance with these two studies, 14 homozygous or compound heterozygous mutations were found in *NPHS2* in a cohort of 22 patients with CNS (40). Recently, Hinkes et al. (29) performed an analysis of *NPHS1*, *NPHS2*, *WT1* and *LAMB2* genes in a large European cohort of 89 children from 80 families with NS manifesting in the first year of life. They detected disease-causing mutations in 66% of the families. Interestingly, both *NPHS1* and *NPHS2* mutations accounted for an equal number (39.1%) of CNS cases. In patients with the infantile form

of NS, NPHS2 mutations were still responsible for 35% of cases, while NPHS1 mutations were not detected.

Besides CNS, NPHS2 mutations have been found in families with an autosomal-recessive form of SRNS manifesting later in life (41–44). In one study, NPHS2 mutation analysis was performed in 338 patients from 272 families with SRNS. The mutation detection rate in NPHS2 was 43% for the familial cases. NS in these patients manifested at the mean age of 3.4 years. In another study, mutation analysis of NPHS2 was performed on 190 patients with SRNS from 165 families (40). Homozygous or compound heterozygous mutations in NPHS2 were detected for 56 of 190 patients (29%). Twenty-seven of these 56 patients represented familial cases. The age of the patients with NPHS2 mutations varied from birth to 24 years, with a median of 3.5 years. In a 2008 study, a worldwide cohort of 430 patients from 404 different families with SRNS was screened for NPHS2 mutations (45). Recessive podocin mutations were present in 18.1% (73/404) of families. Patients with these mutations manifested symptoms at a significantly earlier age (mean onset < 1.75 years) than any other patient group.

To date, more than 100 different NPHS2 mutations have been identified in patients with SRNS. As a rule, they appear in both alleles (homozygous or compound heterozygous) in accordance with autosomal recessive inheritance. The mutations are distributed throughout the entire gene. Most mutations cause a severe disease with an early onset and development of ESRD within a few years. The molecular basis for this is that many of the disease-causing mutations in podocin seem to disrupt nephrin trafficking to the plasma membrane of podocytes, which severely interferes with the SD structure and function (46,47). The podocin gene mutations do not cause any extrarenal defects.

### 64.4.2 Late-Onset SRNS

NPHS2 mutations have also been described in familial cases of SRNS with adolescent or adult onset. Many of these late-onset cases are compound heterozygotes, with one allele harboring a R229Q mutation (44,48). The R229Q variant is present in the heterozygous state in approximately 4% of Western populations. It encodes a defective podocin protein with low affinity for binding to nephrin. Recently, Machuca et al. (49) sequenced the podocin gene in 546 patients from 455 families SRNS. Thirty-six patients from 27 families were compound heterozygotes for the p.R229Q variant and one pathogenic mutation. These patients had significantly later onset of NS than patients with two pathogenic mutations. Among 119 patients diagnosed after 18 years of age, 18 patients were found to have one pathogenic mutation and p.R229Q, but none had two pathogenic mutations. In the study by Santin et al. (50), mutation analysis was performed in 148 unrelated Spanish patients with SRNS.

Homozygous or compound heterozygous NPHS2 pathogenic mutations were identified in 7 of the 92 pediatric patients and in none of the adult FSGS patients. On the other hand, four of the 47 adult patients were compound heterozygotes for p.R229Q and one pathogenic mutation (mostly p.A284V).

Studies show that compound heterozygosity for p.R229Q is associated with adult-onset SRNS. Screening for the pR229Q variant is recommended in these patients, along with further NPHS2 mutation analysis in those carrying the variant. The role of p.R229Q homozygosity in SRNS is less well defined. It seems to increase the risk for SRNS although the magnitude of this effect remains unknown. NPHS2 mutations are the most common podocyte gene defects. Yet, mutations are found in a minority of SRNS patients. In a 2007 study, no homozygotes or compound heterozygotes were observed in 377 biopsy-confirmed late-onset FSGS (51). Similarly, no NPHS2 mutations were observed in 39 Brazilian patients with adult-onset FSGS (52) or in 18 African–American children with SRNS (53). He et al. (54) screened NPHS2 mutations in 87 adult patients with idiopathic FSGS (15 steroid-sensitive, 63 steroid-resistant, and nine familial cases) and found compound heterozygous mutations only in one patient and no homozygous mutations.

## 64.5 WILMS TUMOR SUPPRESSOR GENE (*WT1*) MUTATIONS

WT1 encodes for a nuclear WT1 protein, which is a transcription factor of the zinc finger family. WT1 plays a crucial role in the embryonic development of the kidney and genitalia. In the mature kidney, WT1 is expressed in podocytes and epithelial cells of the Bowman's capsule. WT1 contains 10 exons, the first six of which encode a proline/glutamine-rich transcriptional regulatory region. Exons 7–10 encode the four zinc fingers of the DNA-binding domain. Up to 24 different isoforms of WT1 may result from the combination of alternative translations sites, alternative RNA splicing, and RNA editing. The biological role of all these isoforms is not known (1,3).

A variety of WT1 mutations, which either affect development or induce tumor formation, have been identified (Chapter 59). Developmental defects include the WAGR syndrome, Denys–Drash syndrome (DDS), and Frasier syndrome (FS) (Chapter 66). Mutations in WT1 can also cause an isolated kidney disease with NS. The histopathological diagnosis in these cases may be diffuse mesangial sclerosis (DMS) or FSGS (55).

### 64.5.1 Denys–Drash Syndrome

DDS (OMIM 194080) classically meant a combination of NS showing a histopathologic picture of DMS, male pseudohermaphroditism, and Wilms tumor. Male pseudohermaphroditism in most published cases refers to an



XY karyotype, some testicular tissue elsewhere than in the scrotum, variable degrees of ambiguous genitalia, such as hypospadias and cryptorchidism in children appearing like boys, or hypertrophy of the clitoris in phenotypic girls. Some cases with an XX karyotype and female phenotype have been reported. Three clinical categories of DDS have been noted: genotypic males with all three abnormalities, genotypic males with nephropathy and ambiguous external and/or internal genitalia only, and genotypic females with nephropathy and Wilms' tumor only (1,3).

DDS is caused by heterozygous mutations in *WT1*. More than 60 germ line mutations (both familial and de novo) have been described in DDS patients. Most are missense mutations within exon 8 and 9, coding for zinc finger domains 2 and 3, which lead to alteration in the DNA-binding capacity of *WT1*. It is believed that the mutant protein actively suppresses the normal allele, which explains the more severe phenotype seen in DDS compared with children with complete deletion of one *WT1* allele. In most patients, the nuclear expression of *WT1* is absent or reduced in podocytes. The podocyte function is affected and, at the molecular level, upregulation of *PAX2* and downregulation of *nephrin* have been reported (56). In addition, the expression of growth factors that regulate glomerular capillary development is affected in DDS (57). Missense mutations can occur with or without *WT*. *WT1* analysis is important in young patients with NS for early detection and tumor prophylaxis. The nephropathy is usually discovered at the age of a few months, sometimes at birth. To avoid the development of Wilms' tumor, bilateral nephrectomy at the onset of terminal renal failure is recommended. Removal of native kidneys has been suggested for all patients with nephropathy caused by *WT1* mutations (58).

### 64.5.2 Frasier Syndrome

FS (OMIM 136680) is characterized by the association of male pseudohermaphroditism and glomerulopathy (1,3). There is complete male to female gender reversal in 46,XY patients. FS is associated with gonadoblastomas, but not with Wilms' tumor. Proteinuria is detected in childhood, usually between 2 and 6 years of age, and kidney biopsy reveals FSGS. The renal disease does not respond to medical therapy, but it has a slower progressive course to renal failure than DDS.

FS is caused by point mutations in intron 9 in *WT1*, which interferes with the recognition of the second splice donor site of *WT1*. This results in the loss of the lysine-threonine-serine (KTS)-containing isoforms of *WT1*. How this change in the relative expression of +KTS and -KTS isoforms results in the severe developmental defects in FS is not completely understood (1). In addition, mutations in exon 9 that do not alter the +KTS isoform expression have been reported in FS patients. On the other hand, typical FS mutations have

been observed in DDS patients. Based on this overlap, both diseases should be considered as part of a spectrum of *WT1* gene mutations, rather than as separate entities. Recently, Chernin et al. (59) followed 19 patients with mutations in intron 9 splice site (KTS mutations), 27 patients with missense mutations, and six patients with other mutations. Totally 24 different *WT1* mutations were detected. Sixteen of the 19 patients with KTS mutations were females. The results showed that KTS mutations cause isolated NS with the absence of Wilms tumor in 46,XX females. On the other hand, these mutations cause FS with gonadoblastoma risk in 46,XY phenotypic females.

### 64.5.3 Isolated Kidney Disease

Originally, three studies reported *WT1* mutations in patients with isolated DMS. Since then, the incidence of *WT1* mutations has been evaluated in sporadic cases of SRNS and SSNS (60). Mutations in exons 6–9 of *WT1* were identified in 8 of the 115 SRNS patients (7%). In two females, mutations in exon 9 consistent with the diagnosis of DDS were identified; one of them had Wilms tumor. In three male and three female patients, splice site mutations in exon 9 consistent with the diagnosis of FS were found. Two of the male patients presented with urinary or genital malformations, one of them with sexual reversal and bilateral gonadoblastoma. All three female patients presented with isolated FSGS. No mutations were found in 110 patients with sporadic SSNS. In a recent study, *WT1* mutations were observed in 5 of 110 patients (4.5%) with SRNS. All patients were less than 5 years of age at onset of NS (61). Interestingly, Mucha et al. (62) screened a worldwide cohort of 164 cases of sporadic SRNS, all of whom were 18 years or less at the onset of NS. All 10 exons of *WT1* were analyzed and 15 patients exhibited seven different mutations exclusively in exons 8 and 9 of *WT1*, suggesting that screening of *WT1* exons 8 and 9 in patients with sporadic SRNS is sufficient to detect pathogenic mutations.

## 64.6 PHOSPHOLIPASE C $\epsilon$ 1 GENE (*PLCE1*) MUTATIONS

PLC $\epsilon$ 1 belongs to the phospholipase family of proteins that catalyze hydrolysis of phosphoinositides, generating messengers such as IP<sub>3</sub> and DAG, which are involved in a wide spectrum of intracellular functions (63). Phospholipase C isoenzymes can be activated by G-protein-coupled receptors. The main action of IP<sub>3</sub> is the stimulation of calcium release from intracellular storage pools, which could be involved in cytoskeletal reorganization and modulate the function of other signal transduction cascades. PLC $\epsilon$ 1 was found to associate with IQGAP1, a protein that has been shown to interact with *nephrin*. A link between PLC $\epsilon$ 1 and RasMAP kinase signaling pathway has also been suggested (64).

The precise function of PLC $\epsilon$ 1 in podocytes, however, remains to be clarified.

In 2006, Hinkes et al. (65) identified mutations in *PLCE1* as causing early-onset NS (OMIM 610725). The age at onset in the 12 patients varied from 2 months to 4 years and the age at ESRD from 5 to over 13 years. No extrarenal manifestations were observed. Kidney histology showed DMS in most patients. Two siblings with a missense mutation had characteristics of FSGS. Two other affected individuals responded to immunosuppressive therapy, making this the first report of a molecular cause of NS that may resolve after therapy. The study showed that PLC $\epsilon$ 1 is expressed in developing and mature podocytes and the DMS histology possibly represents developmental arrest.

In a subsequent study, Gbadegesin et al. identified 40 children from 35 families with idiopathic DMS from a worldwide cohort of 1368 children with NS (66). The age of onset varied from 1 month to 6 years. Truncating mutations in *PLCE1* were detected in 28.6% (10/35) of the families and, interestingly, WT1 mutations in only 8.5% (3/35). Except in one family, all the mutations detected were homozygous loss-of-function mutations. One child had two compound heterozygous mutations. Fourteen children were placed on corticosteroid or cyclosporine therapy and no one responded. Age at ESRD varied from 8 months to 5 years.

So far, the results show that *PLCE1* is a major gene causing isolated DMS and subjects with this type of histological lesion should be screened for *PLCE1* mutations. The mutations may also lead to FSG with a relatively late-onset of proteinuria. *PLCE1* mutations, however, remain an infrequent cause of FSGS. A Dutch study did not find *PLCE1* mutations in 19 cases of childhood-onset FSGS (67) nor were mutations in this gene found in 69 families (median age of onset 26 years) with idiopathic or hereditary FSGS (68). Similarly, *PLCE1* mutations were not found in 125 Spanish patients with SRNS (ranging from congenital to adult onset) (61). Interestingly, some individuals with *PLCE1* mutations may also remain asymptomatic, implying that there may be modifier genes that interact with *PLCE1* to cause DMS/FSGS.

### 64.7 LAMININ- $\beta$ 2 GENE (*LAMB2*) MUTATIONS

Laminin  $\beta$ 2 is one of the laminin chains and component of laminin-521, which has an  $\alpha$ 5: $\beta$ 2: $\gamma$ 1 conformation. Laminin-521 is specifically expressed in the GBM and at some other sites such as intraocular muscles and neuromuscular synapses. Laminin is able to polymerize in a regular manner and has a critical role in maintaining the structural properties of the GBM. Laminin-521 also interacts with its receptor integrin  $\alpha$ 3 $\beta$ 1, which links the GBM to the actin cytoskeleton of the podocyte (22).

In 2004, Zenker et al. (69,70) reported that mutations in the *LAMB2* gene, encoding the laminin  $\beta$ 2 chain,

are associated with Pierson syndrome. This syndrome (OMIM 609049) is a rare autosomal recessive disorder characterized by early-onset NS with variable ocular and neurologic defects. The typical ophthalmic sign is a fixed narrowing of pupils (microcoria), which is due to a defect of the dilator pupillae.

After the original observation, milder variants of the syndrome have been reported with less prominent extrarenal abnormalities (71). Recently, Matejas et al. reviewed the findings in a total of 51 patients from 39 families with *LAMB2* mutations (72). The majority (71%) of the mutations was truncating and was evenly distributed along the *LAMB2* gene. The rest (29%) were missense mutations causing amino acid changes in the N-terminal region of laminin  $\beta$ 2, which is the critical area for interacting with  $\alpha$  and  $\gamma$  chains of neighboring laminin. The age of the patients at diagnosis was mostly less than 3 months, and was over 1 year in only four patients. The age at onset of ESRD was mainly under 1 year (from 1 week to 16 years). Renal biopsy showed DMS histology in 73%, FSGS in 14% and minimal changes in 8% of the patients. Neurodevelopmental deficits were observed in 82% of the patients and all but two patients had some ocular abnormalities. Among patients with isolated NS, *LAMB2* mutations are apparently rare. They were observed in only 2.5% (2/80) of the European patients with congenital or infantile NS (29). Similarly, no *LAMB2* mutations were observed in 33 patients with SRNS and FSGS histology, and there was ocular involvement in 8 patients (73).

### 64.8 TRANSIENT RECEPTOR POTENTIAL C6 ION CHANNEL GENE (*TRPC6*) MUTATIONS

TRPC6 is a receptor-operated cation channel that contributes to changes in the cytosolic free  $\text{Ca}^{2+}$  concentration. TRPC6 is expressed in the podocyte and clustered in the podocin–nephrin–lipid complex. It has been speculated that TRPC6 could be involved in monitoring the integrity of the SD (74). The alteration of TRPC6 function may impair the cytoskeletal adaptive response of podocytes to injury, eventually leading to progressive damage. Studies suggest that nephrin binds to phosphorylated TRPC6 via its cytoplasmic domain, competitively inhibiting TRPC6 expression and activation (75).

In 2005, two groups independently reported that the *TRPC6* gene is mutated in a subset of patients with the autosomal dominant form of FSGS (76,77). A missense mutation in *TRPC6* was reported to be a cause of familial FSGS in a large New Zealand family with FSGS. Subsequently, other families with different *TRPC6* mutations have been reported in both adults and children (78,79). The few published studies, to date, suggest that *TRPC6* mutations account for 3–7% of cases with familial adult-onset FSGS. The affected individuals typically present with high-grade proteinuria. The incomplete penetrance

of TRPC6 mutations to some extent limits its diagnostic value.

### 64.9 CD2-ASSOCIATED PROTEIN GENE (CD2AP) MUTATIONS

CD2-associated protein (CD2AP) is an adapter protein, which interacts with T-cell adhesion protein CD2 and is expressed in lymphoid and epithelial cells. In the kidney, CD2AP localizes to the SD of the podocyte where it links podocin and nephrin to the phosphoinositide 3-OH kinase to form a signaling complex. CD2AP-deficient mice develop severe proteinuria and renal dysfunction shortly after birth. Heterozygous mice develop an FSGS-like lesion around the age of 9 months (80).

The role of CD2AP in human FSGS is still being elucidated, but individuals with homozygous mutations in CD2AP have been reported to present with early-onset FSGS (OMIM 607832). A homozygous mutation (p.R612X) was identified in an infant who presented NS and severe glomerular sclerosis on renal biopsy. The truncated protein displayed a dramatic reduction in actin-binding efficiency in vitro (81). Homozygous mutations of CD2AP, however, are rare and in two studies no CD2AP mutations were detected in adult or pediatric patients with SRNS (61,82).

A heterozygous mutation in the *CD2AP* gene has been reported to be a predisposing factor toward developing a late-onset FSGS. Five different CD2AP heterozygous mutations have been identified in pediatric and adult FSGS patients (80–82), associated with reduced expression of CD2AP and downregulation of CD2AP, nephrin, and podocin expression on kidney biopsies. However, heterozygous CD2AP mutations have been found in unaffected individuals and the causal link between heterozygous CD2AP mutations and FSGS is not yet clear.

### 64.10 ALPHA-ACTININ-4 GENE (ACTN4) MUTATIONS

Alpha-actinin-4 is an actin filament cross-linking protein important for the integrity of the cytoskeleton in podocyte foot processes. While ACTN4 is highly expressed in several tissues, human disorders manifest only in the kidney. Transgenic ACTN4 mice develop a severe glomerular disease (83). Podocyte cell lines derived from these mice show less adherence to collagen IV and laminin, which are important components of the GBM (84).

Mutations in ACTN4 are associated with an autosomal dominant form of familial FSGS (OMIM 603278) (85). The mutated ACTN4 proteins showed higher binding affinity to F-actin, which may change the mechanical characteristics of the podocyte. Only a few ACTN4 missense mutations have been described (85,86). ACTN4 mutations were recently found in 4% of familial FSGS cases. On the other hand, no ACTN4 mutations were observed in 125 Spanish patients with SRNS (61) or in

42 pediatric patients with SRNS (82), indicating that ACTN4 mutations are rare in SRNS. Most reported patients show a mild to moderate degree of proteinuria during adolescence or later, and some patients gradually progress to ESRD. In contrast, Choi et al. (87) recently reported a familial case of FSGS in which two affected siblings showed rapidly progressing NS in early childhood. Renal pathological findings were of an FSGS collapsing variant.

### 64.11 NS CAUSED BY INVERTED FORMIN 2 GENE (INF2) MUTATIONS

INF2 is a member of formins that accelerate cytoplasmic filament nucleation and elongation (88). Formins are widely expressed proteins governing several dynamic events that require remodeling of the actin cytoskeleton such as cell polarity, morphogenesis and cytokinesis. INF2 has the unique ability to accelerate both actin polymerization and depolymerization (89). The regulatory mechanisms controlling INF2 and its cellular function are not well known. INF2 is a widely expressed protein with high expression in the kidney, including glomerular podocytes.

In 2010, Brown et al. (90) identified a locus for autosomal dominant FSGS on a region of chromosome 14q. By sequencing multiple genes in this region, they detected nine independent nonconservative missense *INF2* mutations in a total of 72 individuals from 11 families coming from United States, Canada and Mexico. Patients with INF2 mutations presented in adolescence or adulthood (age range 11–67 years), typically with moderate or nephrotic range proteinuria, but not with NS. The disease was progressive, often leading to ESRD (age range 13–67 years). All mutations altered highly conserved amino acid residues in the diaphanous inhibitory domain of the INF2 protein. These observations underscore the importance of fine regulation of actin polymerization in podocyte function.

A subsequent study by Boyer et al. (91) determined the prevalence of INF2 mutations in a cohort of 54 families (78 patients) with proteinuric disorder of apparent autosomal dominant inheritance. Seven missense mutations were found in nine families (28 patients), which translates to a detection rate of 16.7%. Median age at onset of proteinuria was 27 years and NS was noted in four patients. Median age at ESRD was 36 years (range 20–70 years). Significant intrafamilial phenotypic variability was evident with a wide range of age at presentation and ESRD. An additional familial case of FSGS associated with INF2 mutations was described (92). In this family, two siblings and their father had a heterozygous pE220K mutation. This mutation manifested in these three individuals as incidentally detected proteinuria without overt NS, but at different ages, 7, 9, and 30 years, respectively. Renal biopsy showed FSGS.

### 64.12 OTHER GENETIC FORMS OF NS

In the nail-patella syndrome, NS occurs in a minority of patients and is accompanied by changes in the expression of the GBM components and some podocyte proteins. This syndrome is caused by mutations in the *LMX1B* gene, which codes for a transcription factor strongly expressed in podocytes (93). The link between *LMX1B* mutations and the GBM alterations is not completely resolved (94).

The development of NS has been found in rare cases of mitochondrial disorders. Mutations in *COQ2*, located on chromosome 4q21, are associated with early onset of collapsing glomerulopathy-associated SRNS, with or without extrarenal symptoms (OMIM 607426) (95,96). In addition, mutations in decaprenyl diphosphate synthase subunit 2 (*PDSS2*), which is located on chromosome 6q21, led to both CoQ10 deficiency and NS in a patient with Leigh syndrome (OMIM 607426) (97). Mutations in mitochondrially encoded tRNA leucine 1 (*MT-TL1*) cause MELAS syndrome, which is sometimes associated with SRNS, characterized histologically by FSGS and extensive obliteration of podocyte foot processes (98). A single patient with NS and a genetic defect in mitochondrial respiratory chain activity has also been described (99).

Pathogenic mutations in the *MYH* gene, encoding the motor protein nonmuscle myosin heavy chain type II isoform A (*NMMHC-A*), are associated with the autosomal dominant giant-platelet disorders, which may include sensorineuronal deafness and glomerular disease. *NMMHC-A* acts as a component of the podocyte cytoskeleton contractile functions (100). Polymorphisms in *MYH9* were found to confer two to four times increased risk of ESRD in African-Americans compared with European Americans (101). Moreover, the presence of the same risk haplotype was associated with almost fivefold increased risk of FSGS (102).

Galloway-Mowat syndrome (GMS) is characterized by NS with central nervous system anomalies including microcephaly, psychomotor retardation, and macroscopic and microscopic brain anomalies (103). Other extrarenal disorders have also been reported, such as hiatus hernia, congenital spondylorhizomelic shortness, and diaphragmatic defects. NS appears usually at the age of a few months (0–34 months) and kidney biopsy may show MCNS, FSGS, DMS, or even mesangioproliferative glomerulonephritis. GMS is an autosomal recessive disorder, but the genetic defect is still unknown. In addition to GMS, there are solitary reports on other, unique combinations of NS and nonrenal defects.

### 64.13 DIAGNOSIS OF NS

NS is easily detected in newborns, infants, and children. Edema, hypoproteinemia, hyperlipidemia, and heavy proteinuria are the cardinal signs of NS. The typical

findings in full-blown NS include serum albumin level <10–20 g/L and urinary protein 2–100 g/L (after serum albumin has been corrected >15 g/L). The severity of proteinuria, however, shows variation and can be quite modest in the beginning. Serum creatinine can be normal during the first weeks or months. On the other hand, SRNS associated with DMS or FSGS often progresses to renal failure quite rapidly (6).

The exact etiologic diagnosis behind NS is much more difficult to unravel (104–106). The etiologic diagnosis, however, is helpful in assessing therapy and prognosis, in the follow-up of possible associated symptoms, and in genetic counseling of the family. Possible renal and nonrenal malformations, abnormalities of genitalia (karyotype), and neurological and ocular defects should be searched for, because they may give a clue to the etiology and help starting mutation search from the most likely gene. In newborns, placental weight >25% of birth weight is typically present in classic CNF, but may be seen in other entities with congenital NS. The indications for renal biopsy are not quite clear. Histological lesions, such as FSGS, MCNS, and DMS, are not specific for the gene defect involved. On the other hand, histology and clinical findings give a clue to the possible etiology. Severity of the histological lesions also helps to assess the treatment strategies.

In newborns with proteinuria during the first days of life, possible *NPHS1* mutations should be screened, followed by *NPHS2* analysis (104). If disease-causing mutations are found, kidney biopsy is perhaps not needed. If kidney biopsy has been performed and shows FSGS histology and/or signs of renal failure, search for *NPHS2* mutations is the first option in newborns as well as in older children. In these cases, if no mutation is detected, analysis of *NPHS1*, *WT1* and *PLCE1* can be performed (107). On the other hand, *WT1* and *PLCE1* analyses are indicated in all cases with DMS histology (108). In juvenile and adult patients with sporadic or autosomal recessive SRNS, screening for *NPHS2* mutations is the first step. A pR229Q change together with a disease-causing mutation is often found in adult-onset FSGS. In adults with autosomal dominant SRNS, screening for *INF2*, *TPRC6*, and *ACTN4* is indicated.

For the clinician, the situation is complicated: several genes can cause NS with overlapping phenotypes. Sequencing several genes one after the other takes time and is costly. However, if causative mutations are found the situation regarding treatment, prognosis as well as risk of recurrence and possibilities for prenatal diagnostics become much more clear. Careful clinical investigations to detect the possible syndromic cases as well as the suggested order of studying the genes in question hopefully helps in finding the etiologic diagnosis with reasonable effort.

Prenatal diagnosis of CNS by genetic analysis is feasible if the mutations in the family are already known.



It can be assumed that the combination of mutations in the family in question most probably leads to similar age of onset and severity as in the affected individuals in the same family. In case of no family history, CNF can still be detected prenatally based on elevated alpha-fetoprotein (AFP) in maternal serum and amniotic fluid. If the AFP concentration in amniotic fluid is very high and the ultrasound examination does not reveal fetal anencephaly or other malformations, CNF is a probable diagnosis. However, heterozygous fetal carriers of NPHS1 mutations may have temporarily elevated AFP levels in amniotic fluid and maternal serum and repeated measurement of amniotic fluid AFP before the 20th week of pregnancy is recommended in cases with high AFP levels (109). Prenatal diagnosis in families with a known risk for CNS should be based on mutation analysis whenever possible. If the mutations are known in advance, the results can be obtained rapidly. Sequencing of the possible genes is also possible, but it may not succeed in the short time frame during pregnancy.

## 64.14 MANAGEMENT OF NS PATIENTS

### 64.14.1 Supportive Therapy

The magnitude of the protein loss into urine is crucial for therapeutic decision making in patients with NS. Heavy and constant proteinuria (5–100 g/L) inevitably leads to life-threatening edema, and protein substitution by parenteral albumin infusions is mandatory (26). On the other hand, patients with moderate proteinuria (1–5 g/L) may manage without albumin substitution, especially if proteinuria is associated with renal failure and reduced urinary output. Patients with constant heavy proteinuria often have low levels of serum thyroid-binding globulin and thyroxine substitution is recommended. Imbalance of plasma coagulation factor levels contributes to hypercoagulability and risk for thrombosis, and the use of warfarin, aspirin and dipyridamole therapy has been recommended. Anti-thrombin III (50 U/kg) may be given before surgical procedures. Because of urinary losses of immunoglobulin, prophylactic use of antibiotics and immunoglobulin has been recommended, but in our experience, they are not helpful and may induce resistant bacterial strains. A high degree of suspicion for septic infections is warranted in NS patients. Infants with severe NS have traditionally been treated with a high-energy (130 kcal/kg/day) and high-protein (3–4 g/kg/day) diet. Glucose polymers are given to increase energy intake, and a mixture of rapeseed and sunflower oil is given to balance lipid levels. The children also receive vitamin D<sub>2</sub> (400 IU/day), multivitamin preparations, magnesium (50 mg/day) and calcium (500–1000 mg/day). Most infants need a nasogastric tube to guarantee their energy intake.

### 64.14.2 Medication and Kidney Transplantation

In nongenetic forms of NS (SSNS and SRNS) immunosuppressive medication with prednisone, calcineurin inhibitor (cyclosporine A (CsA), tacrolimus), antimetabolite (cyclophosphamide, chlorambucil, mycophenolic acid) or anti-CD20 antibodies (rituximab) often bring the disease into remission (110,111). Use of immunosuppressive drugs in hereditary forms of NS has traditionally been regarded unhelpful. However, few patients with proven hereditary SRNS and partial remission induced by Cs A have been reported (65,112). CsA and perhaps also glucocorticoids have a direct effect on podocyte cell signaling and maintenance of the actin network, which may explain the few cases with beneficial effects (113,114). In a recent report, the use of CsA, however, showed little effect in patients with genetic SRNS (115).

Reduction of protein losses by antiproteinuric drugs, ACE inhibitors, and indomethacin has been reported (26). These drugs lower the perfusion pressure in the glomerulus and have direct effects on the podocyte functions., e.g. nephrin expression (116), which lead to reduced protein leakage. In our experience, patients who have severe NPHS1 mutations (such as Fin-major and Fin-minor) do not respond to this medication. On the other hand, patients with mild (missense) mutations in podocyte genes may show a reduction in protein excretion and treatment with “antiproteinuric therapy” with ACE inhibitors is worth trying.

Renal transplantation is an established mode of therapy for children with NS not responding to medical therapy. Overall, the results of kidney transplantation in NS are quite similar to those obtained in other etiologies. Patient survival at 5 years is over 90% and graft survival over 80% in registry databases and in single centers (26). Recurrence of NS in the graft is rare but has occurred in 30% of the CNF children homozygous for Fin-major mutation. Antinephrin antibodies have been observed in these children after transplantation. Treatment of the recurrence with cyclophosphamide, plasmapheresis and anti-CD20 often leads to remission (117,118). The recurrence rate of NS in FSGS is in general about 30%. However, in hereditary FSGS this proportion is lower, about 8% (104).

## 64.15 CONCLUSIONS

Our knowledge on the genetic basis of NS has greatly increased during the past decade. Podocyte proteins play an important role in glomerular sieving and their genetic defects result in proteinuria and NS. Mutations in the NPHS1 gene lead especially to congenital NS, whereas NPHS2 mutations can cause NS at any age. The third important gene is WT1, which may cause syndromic and isolated NS. In addition, several other podocyte genes are responsible for rare cases of early- and late-onset NS.

Gene diagnostics is crucial in many cases with NS not responding to medical therapy.

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## LIST OF RELEVANT WEBSITES

1. [www.renalgene.org](http://www.renalgene.org)
2. [www.hcbl.nlm.nih.gov/omim](http://www.hcbl.nlm.nih.gov/omim)
3. [www.nephcure.org](http://www.nephcure.org)

## CROSS REFERENCE

Chapter 66.

### Biographies



**Hannu Jalanko, MD, PhD**, is a specialist in pediatric nephrology and transplantation. He has worked at the Children's Hospital, University of Helsinki, since 1984. During the past 10 years he has been the Head of Pediatric Nephrology and Transplantation Division at the Children's Hospital. He was appointed as professor in 2009. His research interests are nephrotic kidney disorders and management of pediatric transplant patients. He has published 160 original articles in these fields. He has acted as a section editor in journals of Pediatric Nephrology and Pediatric Transplantation and as an editorial board member of Transplantation; he is a member of the European Society of Pediatric Nephrology (ESPN) and American Society of Nephrology (ASN).



**Helena Kääriäinen, MD, PhD**, is a specialist in medical genetics. She started her career at the Family Federation of Finland, which is a nonprofit organization providing counseling, education and information in medical genetics. From 2003 to 2007 she was Professor of Medical Genetics at the University of Turku, Finland. Since then, she has been a Research Professor at the National Institute for Health and Welfare. She has been a board and executive board member of the European Society of Human Genetics (ESHG) since 2001, and Secretary General of the society during 2004–2010. In Finland, she has been a board member and President of the Finnish Society of Medical Genetics, and a member of several national and European committees related to genetics and research ethics. Her research interests are rare disorders, including rare renal diseases, genetic testing and counseling. She has written and edited books for medical doctors and lay people.

# CHAPTER

# 65

## Renal Tubular Disorders

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### 65.1 INTRODUCTION

Disorders of renal tubular function must be divided both by the segment of the tubule involved and by the substrate(s) whose transport is affected. Molecules are reabsorbed by mechanisms that are both specific and saturable. In addition, there is some suggestion that the entry step of substrates into the renal tubule from the brush border side (the lumen) is under different genetic control from the exit step through the basolateral membranes into the blood.

In the past 10 years most of the transport proteins involved in specific transport systems have been identified and mapped. Reviews of the genes involved in transport of amino acids have been published by Verrey et al. (1) and by Broer (2). (The genes are identified with the specific defects listed later.) However, the identification of the transport proteins has still left much to be explained. For example, it is unclear why a mutation in a transport protein may be expressed only in the kidney, only in the gut, or in both, with differing clinical manifestations. There is not yet a good correlation with specific mutations and clinical severity in most cases. A few defects have yet to be identified.

Transport in the proximal tubule is largely energy dependent and is driven by oxidative metabolism. Experimentally it can be supported by acetate, lactate, and a variety of other organic acids. The distal tubules, and particularly the collecting ducts, are more dependent on energy derived from glycolysis. Many substrates are cotransported with sodium, and the driving force for movement across membranes is provided by the electrochemical potential difference generated by  $\text{Na}^+$  and  $\text{H}^+$  movements. Thus, inherited defects in energy metabolism, such as those in cytochrome C oxidase, and compounds such as heavy metals, which interfere with energy metabolism, can have effects on many different transport systems in the tubule. Several of the specific defects in energy metabolism, particularly the mitochondrial defects, have also been mapped.

Most disorders of transport involve defects in the proximal tubule. Clinically significant systemic disease

is produced by generalized transport defects and defects in bicarbonate, phosphate, and L-carnitine reabsorption. Defects in cystine reabsorption cause stones. Defects in neutral amino acid transport, when also present in the intestine, can cause Hartnup disease. However, many of the defects in transport, although genetically well defined, were thought to be of little clinical significance. There is now some suggestion that they may be important to the nutrition of seniors.

### 65.2 GENERALIZED DISORDERS OF TUBULAR FUNCTION (FANCONI SYNDROME)

The Fanconi syndrome is a generalized tubular disorder that leads to multiple transport abnormalities, including increased urinary loss of several organic substrates—amino acids, glucose, bicarbonate, and organic acids—and the loss of inorganic ions essential for mineral homeostasis—calcium, magnesium, sodium, potassium and, perhaps most important clinically, phosphorus (3). Clinically, the syndrome is usually defined by the combination of glucosuria, phosphaturia, generalized aminoaciduria, and renal tubular acidosis (RTA). In addition there is usually increased renal excretion of small-molecular-weight proteins. These metabolic abnormalities can lead to metabolic bone disease (rickets or osteomalacia) and stunted growth. Water-soluble vitamins are also lost into urine.

The Fanconi syndrome can be caused by a variety of inherited diseases and environmental factors that are toxic to the transport mechanisms in the proximal tubule. Inherited causes are familial idiopathic cystinosis; disorders of phosphorylated sugar metabolism, including galactosemia, glycogen storage disease, and hereditary fructose intolerance; Lowe syndrome; Wilson disease; tyrosinemia; medullary cystic disease; and rickets with secondary hyperparathyroidism. In the absence of other known diseases, it can also exist in an isolated form that is inherited as an autosomal recessive or dominant trait and in an X-linked recessive form with nephrolithiasis and renal failure, which although discussed here, could

be considered a form of RTA. In the general population the most common causes of childhood Fanconi syndrome are probably cystinosis and galactosemia. In the inner cities, lead poisoning may be the leading noninherited form.

The clinical presentation of the Fanconi syndrome (4) depends on the age of the patient and the other manifestations of the primary disease. Idiopathic Fanconi syndrome may manifest itself primarily as bone disease or as a gastrointestinal problem with vomiting, anorexia, constipation probably due to volume depletion, and chronic acidosis or can be found incidentally on routine urine analysis. The diagnosis of idiopathic Fanconi syndrome can only be made after an exhaustive search for other primary causes.

Families with autosomal dominant inheritance have been reported by Luder and Sheldon (5), Ben-Ishay and colleagues (6), Hunt and colleagues (7), and in four successive generations by Smith and colleagues (8). All these families with dominant inheritance were reported to have mild disease and were termed the adult Fanconi syndrome. However, Patrick and associates (9) reported in a follow-up of Luder and Sheldon's family that three members had developed renal failure. Interfamilial and intrafamilial clinical variation has been great. Some patients have only had the urinary findings without overt clinical expression, whereas others have had severe rickets. It was stated in 1992 (10) that 11 families were described. The gene for adult Fanconi syndrome has been mapped to chromosome 15q15.3 by Lichter-Konecki and colleagues (11).

Two autosomal recessive forms have been differentiated. In the severe childhood form the picture is much like cystinosis, and some questions must be raised whether the early reports of this mode of inheritance might not represent undiagnosed secondary causes of this syndrome. The most convincing pedigree is that of Klajman and Arber (12) who described a consanguineous Iraqi Jewish family with six affected siblings. The existence of an autosomal recessive adult form of the disease has been called into question. Dent and Harris's (13) family was shown by Brenton and colleagues (14) to have a dominant pattern of inheritance.

A huge family, of Irish descent, with X-linked nephrolithiasis, nephrocalcinosis, renal tubular dysfunction, and renal insufficiency was described by Frymoyer and colleagues (15). Wong and colleagues (16) reported five additional families. Scheinman and colleagues (17) performed linkage analysis on 102 members from five generations in the family reported by Frymoyer's group. Linkage (LOD score 5.91) was established to Xp11.22 (probe DXS255) with 3.6% recombination. In five unrelated British families, Pook and colleagues (18) mapped the disease to the same position using four different markers. One of these families had a microdeletion using the DXS255. The gene has been definitely identified by Schurman and colleagues (19) as

the *CLCN5* gene, which is also the gene that causes the similar Dent disease.

### 65.3 DISORDERS OF AMINO ACID TRANSPORT

Except for tryptophan and perhaps homocysteine, which are tightly bound to protein, amino acids are filtered freely by the glomerulus. Most amino acids are normally reabsorbed in the proximal tubule with only very small amounts remaining in the urine. Glycine and histidine, which are less efficiently reabsorbed than the other amino acids, account for most of the  $\alpha$ -amino nitrogen found in the urine. Only very small infants, particularly premature babies, excrete significant quantities of the other amino acids. In these infants alanine, proline, hydroxyproline, serine, and threonine are commonly present. Cystine is also seen in premature and occasionally in other newborns. It is now a more common finding because cystine is added to infant formulas.

Amino acids are reabsorbed by energy-dependent mechanisms of high specificity (20). In most cases transport is coupled to the movement of sodium ions. Essentially, the transport systems can be considered group specific. The amino acid transport systems are as follows:

- Neutral amino acid system L (leucine preferring)
- Neutral amino acid system A (alanine preferring)
- Iminoglycine system
- Acidic amino acid system
- Dibasic amino acid system (includes cystine)

At least two transport systems are present for neutral amino acids with differing affinities. At least one transport system is present for amino acids containing two ammonium groups (the dibasic amino acids plus cystine). This transport system is the first in which a definite membrane-associated protein has been cloned and mapped. One system is present for acidic amino acids and another for the combination of glycine, proline, and hydroxyproline (the iminoglycine system). Studies in animals suggest that secondary systems with lower affinity are also present for the same substrates (21,22). This concept has been used to explain some of the variant amino acid disorders in humans, in which the urine pattern does not correspond to the listed systems. The transport systems described are all present in the brush border of the tubule and the gut and have been observed in a variety of cell types. A defect in basic amino acid transport in the basolateral membrane of the cell has been described for the dibasic amino acids, lysinuric protein intolerance (23). This system has a different inheritance and different affinities for amino acids than the dibasic system found in brush borders.

The genetics of amino acid transport are not always clear. In some families, defects are found only in kidney tubules, in some only in gut, and in others in both. It is hoped that the identification and mapping of proteins involved in transport will help to clarify these issues.

### 65.3.1 Glycine and the Imino Acids

Joseph and colleagues (24) described a child with marked increases in the urinary excretion of proline, hydroxyproline, and glycine. Since then numerous other reports have been published, and newborn screening studies (25) have reported an incidence as high as 1 in 20,000 in the general population. Initial association of iminoglycinuria with a variety of neurological disorders (e.g. deafness, blindness, mental retardation) was reported. However, family studies and population screening suggest that this is a benign condition inherited as an autosomal recessive trait (26). This transport system has a much higher affinity for the imino acids than for glycine (27). Heterozygotes may excrete glycine in the urine. This probably explains the dominant glycinuria reported in three generations by Greene and colleagues (28). However, Scriver (29) has suggested that there may be several different forms of hyperglycinuria. This transport system is quite late developing in humans (30), and these amino acids are commonly elevated in the urine of newborn infants, particularly premature ones. Like many of the other disorders of amino acid transport in the kidney, a defect in the intestine may or may not also be present (31). The primary importance of this hereditary condition is that it can be mistaken for a primary defect in glycine metabolism or a secondary defect due to organic aciduria. The molecular defect in this combination has not been described.

### 65.3.2 Dibasic Amino Acids and Cystine

**65.3.2.1 Classic Cystinuria.** Cystinuria is not only a clinically significant disease but also important historically because it was one of the original inborn errors reported by Garrod in his famous Croonian lectures (32). McKusick (33) quotes Marcet as suggesting in 1817 that the disease was familial. In classic cystinuria the urinary excretion of lysine, ornithine, arginine, and cystine is greatly increased compared to normal (34). Although the absolute increase in cystine is less than that of the other amino acids, because it is far less soluble, it forms stones. Serum amino acids are normal, and no nutritional deficiency in patients has been demonstrated. The mutation in this transport system was also the first that was found for amino acids.

Three hereditary patterns of excretion have been described in heterozygotes for classic cystinuria (35). Homozygotes, including compound heterozygotes between two types, cannot be distinguished. Heterozygotes can be separated based on their pattern of urinary amino acids and whether or not they also have an intestinal defect. Type I heterozygotes have normal excretion of cystine in their urine. Types II and III heterozygotes have increased excretion in their urine. They can only be distinguished by studying intestinal transport. Type II heterozygotes have a transport defect in the gut, but type III do not.

The incidence of clinical cystinuria is reported to vary widely in different populations. In Sweden the incidence is reported to be only 1 in 100,000 (36), whereas in Libyan Jews it is reported to be as high as 1 in 2500 (37). Neonatal screening suggests a high incidence in many populations (1 in 15,000 in Boston, 1 in 11,000 in Vienna, and 1 in 17,000 in Sydney), but these figures must be accepted very conditionally because of the increased secretion normally noted in newborns and particularly because of the observation by Scriver (personal communication, 1986) that newborn heterozygotes are difficult to distinguish from homozygotes. Our own observations suggest that the incidence is particularly high in the Old Order Mennonites, both in Pennsylvania and Missouri.

Cystinuria is the first amino acid transport disease in which the molecular defect was identified and mapped. Wells and Hediger (38) isolated a protein related to amino acid transport in the rat. Lee and colleagues (39) located a similar DNA out of a human library and localized it to chromosome 2p (SLC3A1 amino acid transferase gene). Pras and colleagues (40) demonstrated linkage of this gene to cystinuria. Calonge and colleagues (41) have now demonstrated six different missense mutations in eight different families with cystinuria (30% of all defects in the affected). Gasparini and coworkers (42) indicated that all mutations in SLC3A1 were in patients with type I disease. Cystinuria types II and III have been linked to the SLC7A9 amino acid transport gene by several authors (43).

#### 65.3.2.2 Other Forms of Dibasic Aminoaciduria.

Cystine excretion in the absence of other dibasic amino acids has been reported (44). The two siblings with this condition were without stones but were only 2 and 4 years old when reported. Dibasic aminoaciduria in the absence of cystine excretion was reported in 13 members of a family (45). These patients were also asymptomatic.

The description of lysinuric protein intolerance has led to the first documentation of a basolateral transport defect for dibasic amino acids (but not cystine) (46). This disorder leads to a marked protein intolerance because of hyperammonemia and suggests that the urea cycle is not perfect but requires the intake of the dibasic amino acids. The defect can be partially overcome by citrulline, which is not transported by the same system. The disease has been reported throughout the world but has a particularly high incidence in Finland (1 in 60,000).

### 65.3.3 Neutral Amino Acids

Hartnup disease is primarily a neutral amino acid transport defect. However, histidine, glutamine, and asparagine are also increased in the urine. It can be distinguished from a generalized aminoaciduria because the other amino acids are not increased. Often the disease is diagnosed from urine because of the presence of indoles and tryptamine produced by bacteria,



owing to malabsorption of tryptophan by the gut. Stool amino acids, when measured, are increased. The gene for Hartnup disease has been definitely identified with the SLC6A19 transporter gene on 5p15 in the original family with this disorder by Kleta and colleagues (47). The clinical manifestations of the disease are very variable (48). The patient may have any combination of cerebellar ataxia, emotional instability, delayed development, severe retardation, and a pellagra-like rash. However, recent evidence suggests that the renal defect in the absence of an intestinal transport defect is of less significance than the combined defect or an intestinal defect alone ((49); Scriver, personal communication, 1987). Many of the clinical manifestations have been believed to be due to tryptophan malabsorption, leading to niacin deficiency. However, in the author's experience patients who have clinical manifestations other than rash (primarily developmental delay and ataxia) continue to have slow development even when receiving large amounts of niacin and a good protein intake. Magnetic resonance imaging in these patients leads one to speculate that a transport defect may also be present in the brain.

The disease appears to have an autosomal recessive inheritance. Several reports of consanguineous cases have appeared. There seem to be three forms of the disease. The classic form has defects in both the renal tubular and intestinal systems (50). The form of the disease usually detected on newborn screening has only urinary manifestations (25) and may be benign. A disorder affecting only the gut transport systems was described, which was associated with growth and developmental delay (49). Subsequently, we have found another family with two affected siblings and lesser degrees of intestinal loss of amino acids in the mother and two siblings who were normal. This strongly supports autosomal recessive inheritance.

### 65.3.4 Acidic Amino Acids

Massive excretion of aspartic and glutamic acids has been reported twice in single patients (51,52). Presumably this condition has an autosomal recessive inheritance. The clinical manifestations in the two patients were quite different, and the significance of this condition is unknown.

## 65.4 RENAL TUBULAR ACIDOSIS

### 65.4.1 Proximal Renal Tubular Acidosis (Type II RTA)

Proximal RTA is caused by bicarbonate loss only in the proximal tubule. Because most bicarbonate is reabsorbed more distally, when the filtered load is decreased by a fall in serum bicarbonate, normal acidification mechanisms in the distal tubule can still lower the urine pH.

The serum electrolytes demonstrate a hyperchloremic acidosis.

RTA type II occurs as part of the Fanconi syndrome. Nearly all isolated cases have been in young boys, most before 2 years of age (3,53). The great predominance of males (4:1) suggests an X-linked recessive form of inheritance. This mode of inheritance has not been proven, however, because no family history can be demonstrated in a majority of cases (54). A mild RTA II with dominant inheritance has been studied (55).

### 65.4.2 Distal Renal Tubular Acidosis (Type I RTA)

In type I RTA bicarbonate is lost in the distal tubules. This disease presents with nephrocalcinosis, fixed urinary specific gravity, a low serum bicarbonate, and hypocalcemia. The bone manifestations are very variable but may be severe. Chaabani and colleagues (56) recently reported a large pedigree in which 28 members had RTA I, the clinical presentation being quite variable. Some had nephrocalcinosis and growth retardation, whereas others were clinically unaffected. An unstable form of carbonic anhydrase B in red cells was reported by Kondo and colleagues (57), but no correlation with clinical states is yet available.

RTA I has an autosomal dominant inheritance. Randall's (58) follow-up of the pedigree described with Targgart (59) clearly shows male to male transmission. Linkage is now established to 17q21-q22. This is the chloride-bicarbonate exchanger gene AE1 (now called SLC4A1 transporter gene) (60).

A distinct form of RTA I with perception deafness was reported by Konigsmark (personal communication, 1966), and Nance and Sweeney (61) provided evidence for autosomal recessive inheritance. Reports of carbonic anhydrase activity in this condition have varied widely, and the association is unclear. The condition is, however, clearly different from either classic RTA I or carbonic anhydrase II deficiency, described later.

### 65.4.3 Carbonic Anhydrase II Deficiency

RTA that is primarily distal but also proximal was described by Sly and colleagues (62-64) in three sisters with osteopetrosis and cerebral calcifications. Carbonic anhydrase II is found in both kidney and brain and was deficient in the patients and decreased in the obligate heterozygotes. About half of all the subsequently reported cases have occurred in families of Arab descent in the Middle East or North Africa. The gene has been localized to chromosome 8q22 (65,66). At least eight different mutations have now been described in patients with this deficiency. Hu and colleagues (67) found the same novel splicing mutation in six unrelated Arab families, and this should be useful for diagnosing patients in this ethnic group by polymerase chain reaction.

## 65.5 DISORDERS OF SUGAR TRANSPORT

At least two transport systems mediate the absorption in the gut or the reabsorption of sugars in the proximal tubule. The most important is the system for glucose and galactose. Also present in the proximal tubule are systems for the reabsorption of other sugars. Fructose is not actively transported by the tubule, and the condition known as fructosuria is due to overflow rather than a transport defect. As with the amino acid transport disorders, defects have been described that affect the intestine as well as the kidney. Because heterozygotes for these disorders may spill small amounts of sugar into the urine, these disorders have been described as having dominant modes of inheritance (68). However, heavy excretion is seen only in homozygotes and the conditions are considered autosomal recessive.

### 65.5.1 Renal Glycosuria

Clinically, renal glycosuria may be considered as two disorders. First, patients may have a low threshold for glucose but a normal total capacity to reabsorb it. Thus, these patients will spill glucose into their urine at normal serum concentrations, but their total daily loss is not great. This disorder is important mainly because it may be confused with diabetes mellitus and because a low renal threshold for glucose may make urine monitoring of a diabetic difficult. Second, patients may have a decreased capacity to reabsorb glucose with or without a decreased threshold. These patients may spill very large amounts of glucose in a day, sometimes as much as 100 g. These two defects appear to be allelic. Elsas and Rosenberg (69,70) showed that both types of defects can appear in the same family and that compound heterozygotes have clinical glycosuria. Oemar and colleagues (71) described a family in which glucose reabsorption appeared to be completely absent. Some of these patients have polydipsia and polyuria, and many of the women have yeast infections of the vagina. Most, however, have few clinical symptoms. Kanai and colleagues (72) recently provided evidence that the defect in these patients is in the low-affinity  $\text{Na}^+$  glucose cotransporter in the proximal tubule. This gene has not yet been studied in the clinically more important disease of the intestine and kidney, glucose–galactose malabsorption.

Linkage was initially related to the HLA site, but more recently it has been suggested that it is related to the low-affinity sodium/glucose cotransported SLC5A2 on chromosome 16 (72). A specific mutation in this gene (now labeled SLC5A2) was reported by van den Heuvel and colleagues (73).

### 65.5.2 Fructosuria

Fructosuria in the absence of fructose intolerance (fructose phosphate aldolase deficiency) is not a renal tubular

disease. Instead, this benign condition is the result of a defect in hepatic fructokinase. It is inherited as an autosomal recessive trait (74). The gene has been mapped to 2p23.3 by Bonthron and colleagues (75) who demonstrated the first mutations.

### 65.5.3 Pentosuria

Pentosuria was also one of the original inborn errors described in the Garrod lectures (32). Patients excrete 1–4 g of pentose, primarily 1-xylulose, daily (76). It is a benign condition that occurs primarily in Ashkenazi Jews of Polish origin and in Lebanese. This condition, like fructosuria, is usually a disorder of metabolism rather than transport. Wang and Van Eys (77) demonstrated that the defect is in the enzyme NADP-xylitol dehydrogenase. Heterozygotes have intermediate levels of the enzyme, confirming its autosomal recessive mode of inheritance.

## 65.6 HYPOPHOSPHATEMIC RICKETS

Hypophosphatemic rickets behaves as an X-linked dominant trait in most families. However, Scriver and coworkers (54) described an autosomal dominant pedigree. See Chapter 153 for a detailed discussion of this disorder.

## 65.7 CONCLUSION

The renal tubules reabsorb 80–98% of filtered small molecules by energy-dependent mechanisms. Disorders, primary or secondary, that interfere with these mechanisms can cause loss in the urine of sugars, amino acids, bicarbonate, phosphorus, and other cations, as well as a variety of organic acids. Specific transport systems are under genetic control. Thus, inherited disorders can affect the reabsorption of only one or a small group of compounds. Defects altering energy metabolism can produce a more generalized loss of renal tubular mechanisms. With the notable exceptions of bicarbonate and phosphorus, unless an intestinal defect is also present, these disorders produce little systemic disease and have not yet been implicated in nutritional deficiencies, although there is now some suggestion that they may be import in seniors. Cystine transport deficiency produces problems primarily because of the low solubility of this compound. Nonetheless, these disorders are important to recognize because they cause confusion with defects in metabolism, where increased serum levels of a compound cause the filtered load to exceed the tubules' capacity for reabsorption.

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# CHAPTER

# 66

## Cancer of the Kidney and Urogenital Tract

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### ABBREVIATIONS

BHD – Birt–Hogg–Dube  
BWS – Beckwith–Wiedemann syndrome  
DDS – Denys–Drash syndrome  
FCRC – Familial non-VHL clear cell renal cell carcinoma  
HPRC – Hereditary papillary renal carcinoma  
IC1 – Imprinting control region 1  
IC2 – Imprinting control region 2  
RCC – Renal cell carcinoma  
SGBS – Simpson–Golabi–Behmel syndrome  
UPD – Uniparental disomy  
VHL – von Hippel–Lindau disease  
WAGR – Wilms’ tumor, aniridia, genitourinary abnormalities, mental retardation

### 66.1 TUMORS OF THE KIDNEY

#### 66.1.1 Wilms’ Tumor

Wilms’ tumor (nephroblastoma) is the most common solid tumor in childhood and affects approximately 1 in 10,000 children. The median age at diagnosis is 3–4 years, with most children affected by age 6. Approximately 5% of patients have bilateral tumors, and these children are younger at diagnosis (mean 26 months) than those with unilateral tumors (mean 36 months) (1). Wilms’ tumor is believed to arise from remnants of immature kidney, and microscopic examination may show structures that resemble stages in normal renal development. Most tumors show a triphasic histology, with variable proportions of persistent blastemal cells, dysplastic tubules, and supporting stroma. The parallel between normal renal development and Wilms’ tumor is reinforced by the high incidence of developmental abnormalities in children with Wilms’ tumor, approximately 5% having genitourinary abnormalities. In addition, many Wilms’ tumors arise in kidneys containing foci of persistent renal stem cells known as nephrogenic rests. These are rare in normal kidneys after 1 year of

age but can be found in up to 40% of unilateral and in almost all bilateral Wilms’ tumor kidneys (2). Analysis of the age of onset of bilateral and unilateral Wilms’ tumor by Knudson and Strong (3) was compatible with a two-hit model of tumorigenesis, as previously reported for retinoblastoma. However, familial Wilms’ tumor is rare. A number of genetic disorders are associated with a predisposition to Wilms’ tumor including WAGR syndrome (see later discussion), Denys–Drash syndrome (DDS), Beckwith–Wiedemann syndrome (BWS), hemihypertrophy, familial Wilms’ tumor, Perlman syndrome, Simpson–Golabi–Behmel syndrome (SGBS) and mosaic variegated aneuploidy syndrome. Although only 2% of patients have an associated genetic disorder, the analysis of these patients, particularly those with the WAGR syndrome, has been critical to the isolation of Wilms’ tumor genes.

##### 66.1.1.1 WAGR Syndrome and the WT1 Gene.

Approximately 1% of children with Wilms’ tumor have aniridia, compared with 1 in 50,000 of the general population. These children may also have other characteristic abnormalities associated with the WAGR syndrome (Wilms’ tumor, aniridia, genitourinary abnormalities, and mental retardation). Most children with the WAGR syndrome have cytogenetically visible chromosome 11 deletions involving band 11p13, and this observation formed the basis for the assignment and subsequent isolation of the aniridia (PAX6) and Wilms’ tumor suppressor (WT1) genes from chromosome 11p13. Wilms’ tumor does not complicate familial aniridia caused by intragenic PAX6 mutations, but children with sporadic aniridia and a contiguous deletion of WT1 and PAX6 (which are approximately 700kb apart) are at risk. In such cases aniridia is fully penetrant, but Wilms’ tumor occurs in only 50% of patients with WAGR deletions (4). Similarly, the occurrence and severity of genitourinary abnormalities and mental retardation are variable. Inclusion of BDNF in WAGR syndrome deletion is associated with obesity (5).

For patients with sporadic aniridia, the risk of Wilms' tumor is about 15% (6). Although most patients with Wilms' tumor and aniridia have a cytogenetically visible chromosome 11p13 deletion, Wilms' tumor can also occur in those with submicroscopic deletions. Molecular cytogenetic and genetic analysis can determine the extent of a deletion and hence the risk of Wilms' tumor (7).

WT1 encodes a zinc finger-containing protein. WT1 has multiple functions, and it may appear to have differing effects on cell proliferation in different cellular contexts (8). Alternative splicing can result in a variety of proteins (e.g. with inclusion or exclusion of 17 amino acids encoded by exon 5 and three amino acids (KTS: lysine, threonine, and serine) between zinc fingers 3 and 4) and the precise nature, and relative levels of WT1 isoforms are important in the regulation of WT1 function. The WT1 gene product has four zinc finger motifs at the C terminus and a proline-glutamine-rich domain at the N terminus, suggesting a role in transcriptional regulation. In some cases WT1 may act as a transcriptional activator, but it is also proposed to repress the expression of important growth factors such as IGF2, IGF1R, and epidermal growth factor receptor (9). Although a role for WT1 in RNA processing has been suggested, transcriptional regulation is the best established function (8). About 10% of Wilms' tumors have WT1 mutations, and loss-of-heterozygosity studies have implicated a further gene (WT2) in chromosome 11p15 (which may be imprinted, as chromosome 11p allele loss in Wilms' tumor preferentially affects the maternal chromosome) in the pathogenesis of Wilms' tumor (10). The frequency of germline WT1 mutations in nonsyndromic Wilms' tumor patients is small (approximately 2%), but occasionally a WT1 mutation can present with a unilateral tumor and no genitourinary abnormality (11).

Genitourinary abnormalities in the WAGR syndrome result from deletion of WT1. The expression of WT1 is tightly controlled during normal development, with the strongest expression occurring in the fetal kidneys, gonads, and mesothelium (12,13). Within the kidney, WT1 mRNA expression is strongest in the renal vesicles and glomerular epithelium. A variety of investigations have suggested that WT1 is involved in normal genital and gonadal development, including the specific expression of WT1 mRNA in the developing gonad (12,13), the finding of WT1 mutations in DDS and Frasier syndrome (see below), and the failure of gonadal development in transgenic mice homozygous for WT1 mutations (14). Individuals with familial aniridia and sporadic aniridia without WAGR syndrome have inactivating mutations (usually frameshift or nonsense mutations) in the PAX6 gene (15–17).

**66.1.1.2 Denys–Drash Syndrome.** DDS is a rare disorder characterized by a progressive glomerulonephropathy causing renal failure, genital anomalies, and Wilms' tumor (18,19). Not all patients have the complete triad, and nephropathy plus one of the other features

is sufficient to make the diagnosis. The nephropathy is characterized by the presence of focal or diffuse glomerulosclerosis and usually presents with proteinuria progressing to nephrotic syndrome and hypertension, and then end-stage renal failure by 3 years of age (18). Males (XY karyotype) with DDS usually have ambiguous genitalia or phenotypically normal female external genitalia (male pseudohermaphroditism). In addition, the internal genitalia are frequently dysplastic or inappropriate (19). XX individuals usually have normal female external genitalia but the internal genitalia are frequently dysplastic. Wilms' tumor in DDS presents early (mean 18 months) and is frequently bilateral.

Renal failure is the most common cause of death in DDS, but recent advances in the management of renal failure and renal transplantation in childhood are improving the prognosis. Nevertheless, the management of DDS is difficult. Affected patients require surveillance for Wilms' tumor. Bilateral nephrectomy is indicated in patients with renal failure receiving renal replacement therapy (19,20), but patients with screen-detected Wilms' tumor without renal failure may be treated by nephron-sparing surgery (21).

Molecular genetic analysis of DDS patients has demonstrated de novo germline WT1 mutations in most cases (reviewed in (18,19,22)). Most mutations affect the second and third zinc finger domains and the most common mutation is a missense mutation at codon 394 in exon 9. DDS mutations are predicted to alter or impair the DNA-binding function of the WT1 gene product. The genital abnormalities in DDS are more severe than those in the WAGR syndrome, suggesting that DDS mutations produce their effect on genital development by a dominant negative mechanism (23). However, Wilms' tumor does not occur in all DDS patients, suggesting that DDS mutations do not have a dominant negative effect in the pathogenesis of Wilms' tumor (23,24). The phenotype of Meacham syndrome (male pseudohermaphroditism with abnormal internal female genitalia comprising a uterus and double or septate vagina, congenital heart defect and diaphragmatic abnormalities) overlaps with that of DDS and Suri et al. (25) demonstrated that the two disorders are allelic.

**66.1.1.3 Frasier Syndrome.** This rare disorder is defined by male pseudohermaphroditism, streak gonads, and renal failure from focal and segmental glomerulosclerosis. Gonadoblastoma is frequent, but Frasier syndrome is distinguished from the related DDS by the absence of Wilms' tumor and a more slowly progressive glomerulopathy (end-stage renal failure usually occurs in adolescence or early adulthood). Germline WT1 mutations in the donor splice site in intron 9 were described in Frasier syndrome by Barbaux et al. (26). WT1 mutations associated with Frasier syndrome lead to loss of the +KTS WT1 isoform (see previous discussion) (27,28). Identification of the molecular basis of Frasier syndrome has enabled this disorder to be

diagnosed in females with normal female genital development and focal segmental glomerulosclerosis (29). Thus the +KTS isoform appears to be critical for renal and testicular development (30), but not for ovarian development or function.

**66.1.1.4 Beckwith–Wiedemann Syndrome.** BWS, a congenital overgrowth syndrome with an estimated incidence of 1 in 14,000, is characterized by a triad of major features: macroglossia, pre- or postnatal gigantism (>90th centile), and anterior abdominal wall defects (exomphalos, umbilical hernia, or diastasis recti). Minor features include earlobe creases or posterior helical rim pits (75% of cases), facial naevus flammeus (62%), visceromegaly (liver, kidney, spleen), and renal abnormalities (including nephromegaly) (62%) (31). Neonatal hypoglycemia is frequent and occasionally severe or prolonged. Less-common features include hemihypertrophy (25%), cardiac defects (9%), and cryptorchidism. Mental retardation is uncommon and is usually associated with a history of prematurity or the presence of chromosome 11 duplication. Facial dysmorphisms (maxillary hypoplasia and macroglossia) become less apparent with age, and although surgical tongue reduction may be indicated, bone age is advanced and growth rate tends to slow down during late childhood. Most cases of BWS are sporadic, but approximately 15% are familial, with autosomal dominant inheritance with incomplete (and parent-of-origin effects) penetrance and variable expression (see (32) and references within). Penetrance is more complete when the mother is the transmitting parent (33). An excess of multiple births, particularly female monozygotic twins discordant for BWS, has been noted in BWS, and there is an increased incidence of BWS in children conceived with assisted reproductive technologies, such as in vitro fertilization and intracytoplasmic sperm injection (ICSI) (34,35). A small number of BWS patients have duplications or balanced translocations or inversions of chromosome 11p15, and duplications are invariably of paternal origin, whereas chromosome 11 inversions or balanced translocations are of maternal origin (see (32)). In addition, approximately 20% of sporadic patients with sporadic BWS have uniparental disomy (UPD) of chromosome 11p15 (36,37). UPD in BWS patients arises as a postzygotic mitotic event, so that affected individuals are mosaic for normal cells and cells with paternal isodisomy, including chromosome 11p15.5. There is a strong association between UPD and hemihypertrophy, such that most patients with hemihypertrophy have UPD, and the hemihypertrophy appears to reflect the variation in the proportion of disomic cells between the two sides of the body (37). A clinical association between hemihypertrophy and Wilms' tumor was reported (38); and there is a higher risk of Wilms' tumor in BWS patients with UPD.

The genetics of BWS are complex, but the key factors are that genetic or epigenetic alterations within

an imprinted gene cluster at 11p15.5 result in loss of activity of the maternally expressed candidate growth suppressors CDKN1C and H19 and/or increased expression of the paternally expressed growth promoter IGF2 (insulin-like growth factor 2). Thus in BWS patients with paternally derived 11p15.5 duplications and those with UPD there is increased IGF2 expression from the additional paternal IGF2 alleles. Sporadic BWS children without UPD have epigenetic alterations at two imprinting control regions within 11p15.5, resulting in loss of imprinting of IGF2 (biallelic expression) or silencing of CDKN1C (see later). Although elevated IGF2 expression provides a plausible explanation for many features of BWS (39), the maternally expressed cyclin-dependent kinase inhibitor CDKN1C (p57KIP2) is also implicated and germline CDKN1C mutations occur in approximately 40% of familial cases and 5% of sporadic cases (40,41).

Imprinting of IGF2 is closely linked to that of the reciprocally imprinted (maternally expressed RNA) H19 gene, and the imprinting of IGF2 and H19 is regulated by an intergenic imprinting control region (IC1) that contains CTCF binding sequences. IC1 is differentially methylated (paternal allele methylated and maternal unmethylated), but a subset of BWS children (approximately 5%) have biallelic ICR1 methylation and biallelic IGF2 expression and silencing of H19 expression due to IC1 deletions or, more commonly, epimutations (32,42).

Molecular mapping of rare balanced translocation and inversion breakpoints associated with maternally inherited BWS led to the recognition of a second more proximal, imprinting control center (IC2) within the 11p15.5 gene cluster (43). Within an intron of the KCNQ1 (KVLQT1) gene, which maps between CDKN1C and IGF2, there is a differentially methylated region (KvDMR1, paternal allele unmethylated and maternal allele methylated), and about 50% of sporadic BWS cases have loss of methylation (biallelic unmethylated alleles) at KvDMR1 (44–46). Loss of maternal KvDMR1 methylation is associated with loss of maternal CDKN1C expression (46,47). There is a very high frequency of KvDMR1 loss of methylation in BWS children born after assisted reproductive technologies, and although IC2 defects may be caused by genetic alterations (48), epimutations are the most common cause of IC2 defects. Genotype–phenotype correlations can explain some of the phenotypic variability seen in BWS. Germline CDKN1C mutations and IC2 defects are associated with a high frequency of exomphalos, suggesting that inactivation of CDKN1C is a critical factor in the pathogenesis of exomphalos in BWS (41). Approximately 5% of BWS patients develop an embryonal neoplasm, most commonly Wilms' tumor, but hepatoblastoma, adrenal cortical carcinoma, and neuroblastoma also occur. In contrast to children with WAGR syndrome and DDS, in whom Wilms' tumor occurs at a younger age than in



sporadic cases, the BWS patients with Wilms' tumor are not younger at diagnosis. Instruction of the parents in weekly abdominal palpation is routinely recommended, and many authorities suggest regular renal ultrasound scans (e.g. every 3 months upto the age of 7 years), and in some cases, serum  $\alpha$ -fetoprotein estimates for hepatoblastoma detection. Wilms' tumor risk is highest in BWS children with UPD or IC1 defects (i.e. those with biallelic IGF2 expression), but Wilms' tumor has not been associated with germline CDKN1C mutations or IC2 defects (though other tumors may occur). Hence knowledge of the molecular subtype of BWS can influence the tumor surveillance program for BWS children. Morrison et al. (49) described a small group of patients with overgrowth, Wilms' tumor and biallelic expression of IGF2 and H19 silencing, but no other evidence of BWS. Furthermore, Scott et al. (50) detected 11p15.5 constitutional abnormalities (e.g. uniparental disomy, IC1 deletions and epimutations) similar to those in BWS in 3% of patients with apparently nonsyndromic Wilms' tumor (12% of bilateral cases).

**66.1.1.5 Simpson–Golabi–Behmel Syndrome.** This X-linked disorder is characterized by pre- and postnatal overgrowth, macrocephaly, coarse facies, cleft palate, central cleft of the lower lip, midline tongue groove, polydactyly, accessory nipples, and a variety of congenital cardiac, gastrointestinal, and genitourinary malformations (51,52). Affected males are at risk of embryonal tumors, including Wilms' tumor and neuroblastoma (53).

SGBS should be differentiated from other overgrowth syndromes, in particular BWS. Although X-linked, carrier females may show some expression, so that the X-linked inheritance pattern may superficially resemble the parent-of-origin effects seen in familial BWS (53).

Mutations in GPC3, a glypican gene, cause SGBS, and it suggested that GPC3 mutations might modify IGF2 function (54). A second locus for SGBS has been mapped to Xp22 (55). Li and coworkers (56) suggested that hepatoblastoma and nephroblastomatosis are part of the Simpson–Golabi–Behmel syndrome phenotype and identified GPC3 deletion in two patients previously diagnosed as having Sotos syndrome and Perlman syndrome. Surveillance for Wilms' tumor should be performed by renal ultrasonography every 3–4 months until age 7 years (57).

**66.1.1.6 Perlman Syndrome.** Wilms' tumor occurs frequently (45% of patients) and has an early onset (in the first year of life) in this autosomal recessively inherited disorder. Other renal features include nephromegaly and nephroblastomatosis. Although there are similarities to BWS (polyhydramnios, macrosomia, nephromegaly, and hypoglycemia), the distinctive facial dysmorphism (deep-set eyes, broad/depressed nasal bridge, everted upper lip, macrocephaly) allows the two conditions to be distinguished (58). Cryptorchidism is common, and cardiac defects and diaphragmatic hernia have been reported (59). Perlman syndrome is

associated with a high neonatal death rate, and three of four patients who survived the neonatal period were mentally retarded.

**66.1.1.7 Fanconi Anemia Complementation Group D1.** Fanconi anemia is an autosomal recessively inherited disorder characterized by bone marrow failure and congenital anomalies. A subtype of Fanconi anemia, D1, is caused by homozygous mutations in BRCA2. Children with this form of Fanconi anemia are at risk of developing childhood tumors including Wilms' tumor (60).

**66.1.1.8 Mosaic Variegated Aneuploidy.** This rare autosomal recessive disorder is caused by mutations in BUB1B, which encodes BUBR1, a key protein in the mitotic spindle checkpoint (61). Mosaic variegated aneuploidy is characterized by mosaic aneuploidy (usually trisomy or monosomy), growth retardation, developmental delay and microcephaly, eye and other anomalies and a high risk of cancer including Wilms' tumor.

**66.1.1.9 Familial Nonsyndromic Wilms' Tumor and Other Wilms Tumor Genes.** Approximately 1% of Wilms' tumor patients have an affected relative. Familial predisposition is inherited as an autosomal dominant trait with incomplete penetrance (62). Germline mutations in WT1 are a rare cause of familial Wilms' tumor (unsurprisingly, in view of the importance of WT1 for normal genital and gonadal development), but Pelletier and colleagues (63) reported a father–son pair with Wilms' tumor (and hypospadias and cryptorchidism in the son) associated with a germline WT1 mutation. There is a paucity of data on the recurrence risk of Wilms' tumor, although Crawford (64) suggested that the risk for siblings of unilateral sporadic cases was approximately 0.1%, but that the risks were significantly higher in families with two affected siblings (33%) and for the children of survivors of Wilms' tumor: perhaps 2–10% for patients with unilateral tumors and approximately 33% for patients with bilateral or familial tumors. In addition to 11p15.5 abnormalities (see above), genetic linkage studies mapped Wilms' tumor susceptibility genes (FWT1 and FWT2) to chromosomes 17q12–q21 and 19q13.3–13.4, respectively (65,66) with evidence for further locus heterogeneity and a later age of onset than in sporadic cases in FWT1-linked families (67–69).

Germline DICER1 mutations in addition to predisposing to pleuropulmonary blastoma may also be associated with multilocular cystic nephroma and, rarely Wilms tumor (70,71).

The X chromosome gene WTX was identified through the detection of somatic mutations in sporadic Wilms' tumor and shown to be a negative regulator of wnt signaling (72,73). Germline mutations in WTX were found in osteopathia striata congenita with cranial sclerosis (74). Although germline WTX mutations have not been associated with Wilms' tumor (or other cancers), precursor lesions (nephrogenic rests) have been described in one mutation carrier (75).

### 66.1.2 Renal Cell Carcinoma

Renal cell carcinoma (RCC) is the most common adult renal neoplasm and accounts for almost 90% of malignant kidney tumors. Up to 3% of patients with RCC have an inherited predisposition. The most frequent cause of familial RCC is von Hippel–Lindau (VHL) disease, a multisystem familial cancer syndrome with variable expression. Tumor histopathology is useful in investigating the basis of familial RCC. VHL-related tumors are invariably clear cell, but familial non-VHL clear cell RCC (FCRC) also occurs (see later discussion) and rare cases of clear cell RCC may be associated with constitutional balanced rearrangements involving the proximal region of the short arm of chromosome 3 (76). Hereditary papillary RCC is associated with germline MET proto-oncogene mutations in many cases. Birt–Hogg–Dube (BHD) syndrome is associated with RCC susceptibility, and renal cancer may be associated with germline mutations in the Krebs cycle enzyme genes fumarate hydratase (FH) and succinate dehydrogenase (77). Although patients with tuberous sclerosis may rarely have an increased risk of RCC (78), angiomyolipoma is the most frequent renal tumor in this disorder.

**66.1.2.1 von Hippel–Lindau Disease.** VHL disease is autosomal dominantly inherited, with a minimal birth incidence of 3 per 100,000 persons and a prevalence of 2.5 per 100,000 (79). Expression of disease is variable, but the most frequent manifestations are retinal (60% of patients), cerebellar (60%), spinal (13–44%) and brain-stem hemangioblastoma (18%); RCC (28%); pheochromocytoma (10%); and renal, pancreatic, and epididymal cysts (77,80). Most patients with VHL disease present in the second or third decades with retinal or cerebellar hemangioblastomas, but the onset may rarely be in infancy and penetrance is almost complete by age 60 years. The risk of RCC is influenced by the underlying mutation (see later), but in many cases the risk of an affected patient developing RCC is approximately 70% by age 60 (80) (Figure 66-1). As with other familial cancer syndromes, patients with VHL disease have a greatly increased risk of RCC and tumors develop at an early age and are frequently multiple (81,82). Multiple renal cysts occur in up to 76% of patients, and histopathologic examination may show a continuum from simple benign cysts to frank RCC.

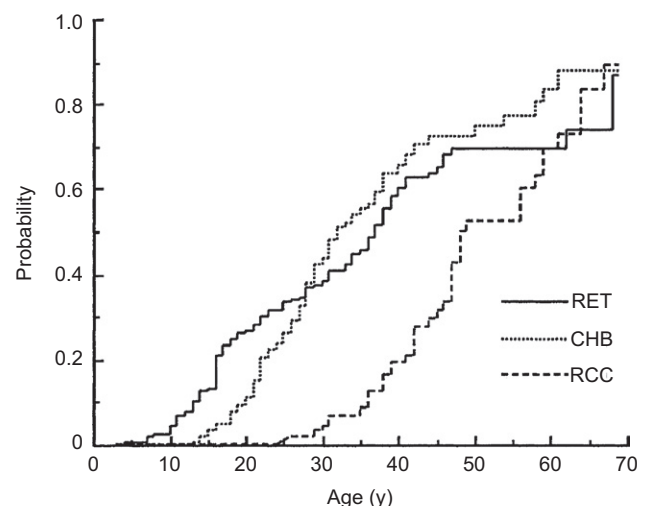
Interfamilial variation in predisposition to pheochromocytoma is well recognized, such that in some families pheochromocytoma is the most frequent complication but is rare in other families (80,83,84). Patients with familial or bilateral pheochromocytoma but no evidence of VHL disease may have germline VHL gene mutations, and in such cases careful investigation for subclinical evidence of VHL disease and for VHL gene mutations is indicated. Genotype–phenotype correlations have revealed that the incidence of pheochromocytoma is higher in families with missense mutations

than in those with large germline deletions or mutations predicted to produce a truncated protein product, and VHL disease families may be categorized according to the presence or absence of pheochromocytoma (types 2 and type 1, respectively). Most type 2 families are further subclassified as type 2B (pheochromocytoma plus hemangioblastomas and RCC), but type 2A (pheochromocytoma and hemangioblastomas but no RCC) and type 2C (pheochromocytoma only) kindreds are also distinguished.

Pancreatic cysts are frequent, and pancreatic tumors, usually nonfunctional islet cell, occur in approximately 5–10% of patients. Pancreatic tumors may become malignant, and detailed guidelines on the management of pancreatic tumors are available (85).

Endolymphatic sac tumors (ELST) are a recently recognized complication of VHL disease that can be detected (by MRI) in up to 11% of cases (86). Impaired hearing, tinnitus, or vertigo in a VHL patient should lead to investigations for an ELST.

When there is a family history of VHL disease a diagnosis can be made in the presence of a single hemangioblastoma or visceral tumor (e.g. pheochromocytoma or RCC). In isolated cases a clinical diagnosis requires two or more hemangioblastomas (retinal or central nervous system) or a single hemangioblastoma in association with a visceral tumor, but molecular genetic analysis provides an alternative method of diagnosis in patients who do not satisfy conventional diagnostic criteria. Germline VHL gene mutations have been reported in approximately 50% of patients with familial or bilateral pheochromocytoma and 4% of patients with isolated cerebellar haemangioblastoma and no clinical or radiological evidence of VHL disease (Jes et al., 2000,



**FIGURE 66-1** von Hippel–Lindau disease. Age-dependent probability of developing retinal hemangioblastoma (RET), cerebellar hemangioblastoma (CHB), and renal cell carcinoma (RCC). From Maher, E. R.; Yates, J. R. W.; Harries, R., et al. *Clinical Features and Natural History of von Hippel–Lindau Disease*. Q. J. Med. **1990**, 77, 1151–1163.

(87)). VHL gene mutation analysis is also indicated in isolated retinal hemangioblastoma and familial clear cell RCC.

Early detection and treatment of complications reduces morbidity and mortality from VHL disease, and all affected patients and at-risk relatives should be entered into a systematic screening protocol such as that described in Table 66-1. Increasingly the efficiency of screening protocols is enhanced by molecular genetic diagnosis, such that screening is confined to proven gene carriers. Relatives who have not inherited a VHL mutation are then spared unnecessary surveillance. Peripheral retinal hemangioblastoma can usually be treated by laser photocoagulation or cryosurgery though the treatment of optic disk lesions is more difficult because of the risk of laser-induced optic nerve damage. Cerebellar hemangioblastomas are benign tumors, and small asymptomatic tumors usually do not require treatment. Surgical removal of cerebellar hemisphere lesions usually produces a good result, but the treatment of spinal and brainstem hemangioblastomas is difficult, particularly in the presence of multiple tumors. Stereotactic radiotherapy may occasionally be used for small tumors when surgery is not possible. RCC is the leading cause of death in VHL disease, but early detection of renal tumors by ultrasound or MRI allows small localized renal tumors to be removed. Most centers employ a conservative renal-sparing approach to the management of RCC in VHL disease, with local excision of the tumor

or partial nephrectomy (88). Asymptomatic tumors are followed until they reach 3 cm, and then surgery is performed. At this time additional smaller tumors may also be removed (89). Although there is a high risk of developing further primary tumors, the aim is to preserve renal function. With repeater surgery, treatment for end-stage renal failure may become necessary, and although relatively few VHL patients have undergone renal transplantation, the morbidity and mortality of renal transplantation appear to be similar to those of non-VHL cases (90).

The VHL tumor suppressor gene was isolated from chromosome 3p25, and germline mutations may be detected in up to 100% of cases (91). Approximately 40% of patients have germline deletions, and the remainder are mainly truncating or missense mutations. Most germline VHL mutations occur rarely and recurrent mutations are relatively uncommon, but genotype–phenotype correlations have been established such that patients with germline deletions, truncating mutations or missense mutation predicted to compromise protein stability usually have a type 1 phenotype without pheochromocytoma. Conversely, surface missense mutations predominate in type 2 (pheochromocytoma present) kindreds (92–94). Specific missense mutations are associated with type 2 phenotype subtypes (2A, 2B and 2C) so that characterization of a germline VHL gene mutation not only allows accurate presymptomatic diagnosis but also can indicate the risk of pheochromocytoma and other tumors.

The mechanism of tumorigenesis in VHL disease is analogous to that in bilateral retinoblastoma, so that tumorigenesis requires inactivation of the wild-type allele (95). In addition, somatic most sporadic clear cell renal carcinomas demonstrate somatic VHL gene mutation, methylation or loss, resulting in homozygous VHL gene inactivation (96–98) consistent with the VHL gene having a critical RCC tumor suppressor function. The VHL tumor suppressor gene has multiple functions (99) but the best characterized is the regulation of hypoxia gene response pathways through its role in a ubiquitin ligase complex. Thus inactivation of VHL leads to upregulation of HIF-1 and HIF-2 (transcription factors that activate transcription of VEGF and other mRNAs in response to hypoxia) because of failure of VHL to target HIF-1 $\alpha$  and HIF-2 $\alpha$  subunits for proteolysis (100). Stabilization of HIF-1 and HIF-2 activates the hypoxic gene response that consists of a large repertoire of target genes implicated in diverse processes such as angiogenesis, proliferation, apoptosis and metabolism (e.g. VEGF, PDGF $\beta$ , TGF $\alpha$ , Cyclin D1 etc) (101), and HIF target genes are thought to have a key role in promoting the angiogenic phenotype characteristic of VHL disease tumors and HIF-2 targets directly promote cell proliferation—so providing a basis for the use of tyrosine kinase inhibitors in metastatic clear cell kidney cancers and, in some cases where surgery is not possible, VHL disease.

**TABLE 66-1** Example Surveillance Program for von Hippel–Lindau Disease

**Affected Patient**

1. Annual physical examination and urine testing
2. Annual direct and indirect ophthalmoscopy
3. MRI brain scan every 3 years up to the age of 50 years and every 5 years thereafter
4. Annual abdominal MRI scan (ultrasound if not available, but CT scan may be required if multiple cysts renal or pancreatic cysts are present)
5. Annual 24-h urine collection for catecholamines and VMAs

**At-Risk Relative**

1. Annual physical examination and urine testing
2. Annual direct and indirect ophthalmoscopy from age 3 to 60 years (fluorescein angiography or angiography may be used from age 10 to increase sensitivity)
3. MRI brain scan every 3 years from age 15 to 40 years and then every 5 years until age 60 years
4. Annual abdominal MRI scan (or ultrasound scan) from age 15 to 65 years<sup>a</sup>
5. Annual 24-h urine collection for catecholamines and VMAs from age 11<sup>a</sup>

MRI, magnetic resonance imaging; CT, computed tomography; VMA, vanillyl-mandelic acid.

<sup>a</sup>Screening for pheochromocytoma can be commenced earlier if there is a family history of pheochromocytoma or a mutation known to be associated with type 2 VHL disease.



**66.1.2.2 Familial Non-von Hippel–Lindau Renal Cell Carcinoma.** By 1991 the literature contained 23 reports of 105 patients with familial RCC and no clinical features of VHL disease (80). Among this group of patients there was an early age at onset (mean 48 years, similar to that in VHL disease and 15 years younger than in nonfamilial cases) (81,102) and evidence for autosomal dominant inheritance with age-dependent penetrance. Since then, the etiology of familial RCC has been clarified by the identification of RCC susceptibility genes and the realization that many genetic disorders are associated with specific RCC histopathological subtypes. Thus 80% of sporadic RCC have a clear cell appearance, and papillary (chromophilic) tumors are the most common cause of non-clear cell RCC. Familial clear cell RCC is associated with VHL disease, non-VHL familial clear cell RCC, and chromosome 3 translocations and a variety of disorders can cause inherited non-clear cell tumors.

FCRC is characterized by two or more relatives with clear cell RCC and no evidence of VHL disease. Convincing evidence for FCRC was provided by Teh and colleagues (103), who described two large kindreds with dominantly inherited clear cell RCC unlinked to the VHL locus. Although most cases of FCRC observed by Teh and colleagues (103) were diagnosed after age 50, Woodward and colleagues (104) reported FCRC kindreds with early-onset RCC and suggested that at-risk relatives should be screened by annual ultrasound from age 20. Extrarenal cancers do not appear to be a frequent feature of FCRC. The genetic basis for most cases of FCRC has not been elucidated, although some may harbor a constitutional translocation or germline VHL, FLCN or SDHB mutations (see later discussion) (105–107).

A constitutional translocation involving chromosome 3 has been reported in a number of families with inherited clear cell RCC. In a large kindred with familial RCC, originally reported by Cohen et al. (108), 10 patients in three generations developed RCC and two also developed papillary thyroid carcinoma (56). Within the family a balanced 3;8 translocation with breakpoints at 3p14.2 and 8q24.1 segregated with RCC susceptibility (translocation carriers were estimated to have an 87% risk of developing RCC by 60 years of age). Other chromosome 3 translocation kindreds associated with RCC have been described but the breakpoints in these families are heterogeneous (although mostly pericentromeric), and although in some cases putative tumor suppressor genes (e.g. FHIT, TRC8, NORE1, LSAMP, FBXW7) or fusion transcripts have been identified by breakpoint cloning, in others it has been suggested that RCC susceptibility results from instability of the derivative chromosome 3 (and loss of the derivative chromosome 3 through nondisjunction and subsequent somatic VHL gene mutation (three-hit model of tumorigenesis)) (76,109). Although familial RCC associated with chromosome

3 translocations is rare, cytogenetic analysis should be performed in FCRC and early-onset/multicentric RCC. However, in chromosome 3 translocation carriers without a personal or family history of RCC (and without disruption of known tumor suppressor genes) the risk of RCC appears to be small (110).

**66.1.2.2.1 Hereditary Papillary Renal Carcinoma.** Histopathologically, papillary cancer may be divided into two subtypes. Germline MET mutations are associated with type 1 tumors that are characterized by small basophilic cells with pale cytoplasm and inconspicuous nuclei (111,112) and type 2 papillary RCC susceptibility may be associated with germline FH mutations (113). Susceptibility to type 1 papillary RCC is rare (incidence approximately 0.1 per million) and is inherited as a dominant trait with incomplete penetrance (94,114). MET is a receptor tyrosine kinase and the mutations described in hereditary papillary renal carcinoma (HPRC) occur in the intracellular tyrosine kinase domain and activate MET activation (115) in a manner analogous to similar kinase domain RET mutations in MEN2B. Interestingly, RCC from HPRC cases with MET mutations demonstrate nonrandom duplication of the mutant chromosome 7 (116). Careful investigation of HPRC kindreds frequently identifies asymptomatic RCC in gene carriers and one MET mutation (H112R) had a penetrance of only 30% by age 50.

Homozygous recessive FH mutations cause FH deficiency, whereas heterozygous mutations predispose to cutaneous and uterine leiomyomatosis (fibroids). A subset of patients with hereditary leiomyomatosis develop type 2 papillary or collecting duct histology RCC (113,117,118) so this dominantly inherited condition is called hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome. Although only a minority of affected individuals develop RCC, the renal cancers are aggressive and should be removed when detected (in contrast to other familial RCC syndromes such as VHL disease and BHD syndrome in which renal tumors are usually managed conservatively until they reach 3 cm diameter). FH is a key component of the tricarboxylic acid cycle (Krebs cycle) and catalyzes the conversion of fumarate to malate. Tumors from HLRCC patients show activation of the hypoxic gene response pathways (as in VHL disease) secondary to accumulation of fumarate (119,120). In addition fumarate has been reported to promote succination and immunostaining for S-(2-succinyl) cysteine has been suggested as a biomarker for FH-deficient tumors (121).

Two inherited RCC syndromes associated with tumors of variable histopathology are the BHD syndrome and RCC associated with mutations in the succinate dehydrogenase subunit genes.

BHD syndrome was initially linked to familial oncocytoma by Toro and coworkers (122). This dominantly inherited disorder is characterized by cutaneous fibrofolliculomas, lipomas, and spontaneous pneumothoraces



and probably colonic polyps and cancers (123). A gene for BHD syndrome was mapped to 17p and a novel tumor suppressor gene (folliculin, FLCN) was characterized (124). A mutation hotspot within a mononucleotide tract in exon 11 accounts for about 50% of mutations. The function of the BHD gene product has not been defined, but there are two naturally occurring animal models: the Nihon rat and canine hereditary renal cystadenocarcinoma (125,126). BHD is associated with an approximately 30% lifetime risk of renal tumor and renal screening (probably from age 20) should be offered to affected patients and at-risk relatives (127). A variety of histopathological tumor types have been described in BHD syndrome, but the most common types are hybrid chromophore/oncocytoma and clear cell RCC.

Succinate dehydrogenase is a heterotetrameric protein located on the inner mitochondrial membrane, and consists of four subunits (A, B, C and D), and has a critical role in cellular energy metabolism through its dual role in the Krebs cycle and as part of the respiratory chain (mitochondrial complex 2). Heterozygous germline mutations in the gene encoding the D subunit (*SDHD*) were initially associated with familial head and neck paragangliomas (HNPGL) and then sporadic and familial pheochromocytoma (128,129). Thereafter germline mutations in the B subunit gene (*SDHB*) were also demonstrated to cause susceptibility to HNPGL and adrenal and extra-adrenal pheochromocytoma (130) and subsequent studies demonstrated that germline *SDHB* and *SDHD* mutations are a major cause of pheochromocytoma and HNPGL susceptibility. Furthermore, germline *SDHB* mutations are associated with a high risk of malignant pheochromocytoma and about 5% of patients with inherited RCC will have a *SDHB* mutation (105). A variety of histological subtypes of RCC may be associated with *SDHB* mutations (and less frequently *SDHD*) and the lifetime risk of RCC in *SDHB* mutation carriers was estimated to be about 15% (131).

### 66.1.3 Renal Angiomyolipoma

Renal angiomyolipoma is the classic renal lesion of tuberous sclerosis, found in approximately 50% of patients (132). Angiomyolipomas complicating tuberous sclerosis are frequently multiple and bilateral, but sporadic cases also occur and these are typically single, have a later age of onset, and have a female preponderance (133). Renal angiomyolipomas are usually asymptomatic, but severe hemorrhage can occur. Although RCC may complicate tuberous sclerosis, the absolute risk is considered to be low (approximately 2–4%) (134). However, patients with tuberous sclerosis who develop RCC are significantly younger than average and further data is required to define the lifetime risk of RCC (135). Renal imaging in tuberous sclerosis is usually performed for the detection and follow-up of angiomyolipomas, but CT is the most

helpful investigation for differentiating angiomyolipomas and renal cancers.

**66.1.3.1 Tumors of the Ureter and Renal Pelvis.** Carcinoma of the renal pelvis accounts for approximately 10% of all malignant renal tumors. Environmental causes include occupational exposure (as for bladder cancer) and prolonged excessive phenacetin ingestion.

Examples of familial ureteric and renal pelvis transitional cell carcinoma are uncommon but transitional carcinoma of the renal pelvis (together with cancer of the bladder and ureter) can be a feature of hereditary non-polyposis colorectal cancer syndrome (HNPCC, Lynch syndrome) (see discussion below). Sijmons and colleagues (136) estimated that the relative risk of developing transitional cell cancer of the ureter or renal pelvis in HNPCC was 14, although the cumulative risk was <3%. Renal medullary carcinoma arises in the renal parenchyma and is usually seen in young patients with sickle trait (137).

## 66.2 BLADDER NEOPLASMS

Carcinoma of the bladder has an incidence of approximately 30 and 10 per 100,000 in males and females, respectively. Most tumors are transitional cell carcinomas, and a notable feature is the propensity of the patients to develop further urothelial tumors. Environmental exposure to chemical carcinogens was first linked to bladder cancer 100 years ago. Occupational exposure to industrial chemicals, including arylamines, is now firmly established as a predisposing factor, but only a minority of patients will have a significant occupational history (138). Other agents associated with bladder cancer include tobacco smoking, chronic infection (bacterial, schistosomiasis), and phenacetin exposure. There has been much on the interaction of genetic and environmental factors in bladder cancer susceptibility. In particular detoxification of some arylamines is mediated by N-acetyltransferase and phenotypic variations in the activity of this enzyme reflect genetic polymorphisms such that “slow” and “fast” acetylator phenotypes can be distinguished. Cartwright and collaborators (139) found a strong association between slow acetylator phenotype in bladder cancer patients with a history of occupational exposure, although weaker associations have been found in patients with no occupational exposure. In a meta-analysis Johns and Houlston (140) detected a modest association (pooled odds ratio 1.31) with slow acetylator status. Similarly in a meta-analysis of polymorphisms in glutathione S-transferases and human diseases, Haddous et al. (141) observed significant associations between bladder cancer and the GSTM1\*0 null allele and GSTP1 polymorphism in smokers.

In epidemiologic studies, family history confers a twofold increase in bladder cancer risk, but it has been unclear whether this is related to genetic influences or shared environment. Evidence for familial transitional

cell carcinoma of the upper and lower urinary tract has been reviewed by Mueller and coworkers (2008). In addition to rare families suggestive of dominant inheritance with incomplete penetrance, patients with germline retinoblastoma mutations may have an increased risk of bladder cancer (142) and bladder cancer risk is increased in HNPCC (Lynch syndrome)—particularly in men with an MSH2 mutation (143). Nevertheless familial clustering of bladder cancer, although uncommon, has been reported (Mueller et al., 2008). Individuals who are at increased risk of bladder cancer can be screened by half-yearly urinalysis and urinary cytology, with cystourethroscopy when these tests are abnormal. Large genome-wide association studies have identified several low-penetrance risk alleles for bladder cancer and confirmed associations for the GSTM1 and NAT2 and interactions with smoking for both loci (144).

### 66.3 CARCINOMA OF THE PROSTATE

Prostate cancer is the most common cancer and the second cause of cancer death in North American men. Although the etiology is unknown, environmental factors such as a high-fat diet and androgen stimulation have been implicated, and in recent years the role of genetic factors has come under intense scrutiny. Genetic epidemiology studies in Mormon families by Cannon and colleagues (145) suggested that the inheritability of prostate cancer was greater than that of breast or colorectal cancers. Subsequent case-control studies have provided further evidence of familial clustering: Steinberg and colleagues (146) found that 15% of prostate cancer patients had an affected father or brother, compared with 8% of controls, and Spitz and colleagues (147) found a positive family history of prostate cancer in 13% of affected patients and 6% of controls. Overall the relative risk of prostate cancer in men with an affected first-degree relative is approximately twice that of men with no affected relatives and the relative risk increases with the number of affected relatives (146). Further evidence that the familial clustering of prostate cancer was caused by genetic factors rather than shared environment was the observation of Carter and associates (148) that there is a greater risk of familial prostate cancer in early-onset cases and complex segregation analysis suggested that prostate cancer clustering could be caused by a rare, highly penetrant dominantly inherited predisposition gene (frequency in the population 0.36%, penetrance 0.88 at age 85 years) (149). Under the most likely genetic model, 43% of early-onset prostate cancers (age <55) would occur in gene carriers, but only 9% of cases, in those under 85. Linkage studies of familial prostate cancer, although initially promising, proved to be more difficult than those for familial breast and colorectal cancer. A locus (HPC1) was mapped to chromosome 1q24-25 but confirmatory studies were inconsistent (150–152). Other susceptibility loci were linked (e.g. autosomal loci

including PCAP 1q32.2-43, CAPB 1p36, HPC2/ELA2 17p11, HPC20 20q13 and an X-linked locus (HPCX Xq27–q28)) (reviewed in (153)). Although mutations in candidate genes for some loci were reported (e.g. RNA-SEL and ELAC2 for HPC1 and HPC2 respectively), the role of variants within these genes in prostate cancer susceptibility appeared complex and the lack of reproducibility of linkage studies in prostate cancer has been considered as evidence of the fact that extensive locus heterogeneity with familial risk was most likely to be attributable to the inheritance of multiple moderate-risk genetic variants. With the advent of genome-wide association studies a large number of susceptibility alleles have been identified (154,155), although it is unclear how these findings might impact clinical practice (156).

Following reports that carriers of BRCA1 and BRCA2 mutations in familial breast/ovary cancer kindreds had an increased risk of prostate cancer, these genes were analyzed as candidate high-penetrance prostate cancer susceptibility genes and it was found that approximately 2% of men with early-onset prostate (<55 years) had a germline BRCA2 mutation (157). In many cases there was no family history of breast or ovarian cancer. A male BRCA2 mutation carrier was estimated to have a 23-fold increased risk of developing prostate cancer by age 55 years and it also appears that male BRCA2 carriers with prostate cancer have a poorer prognosis than similar noncarriers (158). An increased risk (16-fold) of prostate cancer has been reported in carriers of an Eastern European Nijmegen breakage syndrome (NBS1) mutation (159).

The recognition of genetic factors in determining prostate cancer susceptibility has led to suggestions that men with a high-risk family history should be offered screening from age 40 (148). Gronberg and colleagues (160) recommended that men with two or more close relatives with prostate cancer should be offered screening by PSA measurements and digital rectal examination from ages 50–70 years (screening could be commenced earlier in selected families with a young onset of prostate cancer). However, it is not yet known if such screening will be effective.

### 66.4 TESTICULAR NEOPLASMS

Testicular cancer is the most frequent malignancy in men between 20 and 40 years of age. Most tumors are of germ cell origin (seminoma, teratoma) but approximately 5–10% are sex cord/stromal tumors (e.g. Leydig and Sertoli cell), and gonadoblastoma contain germ cell and stromal elements. Epidemiologic studies have suggested that gonadal hormone drive is a major factor in the development of germ cell tumors. The most important risk factor is cryptorchidism, and this and other conditions (e.g. orchitis, infertility) are associated with reduced testicular function, which would produce increased gonadotropin drive to the testicles. Testicular

tumors are bilateral in about 4% of patients, which is suggestive of a genetic basis (161) and it is estimated that about 2% of all men with testicular cancer may have a genetic predisposition (161,162). In reviewing reports of familial testicular cancer, Patel and colleagues (161) noted that in 12 pairs of identical twins with testicular cancer the tumors were of the same histological type in 70% of cases but that in other affected relative pairs the histology was mostly different. In a Dutch single-center study, relative risk of testicular cancer was increased 9- to 13-fold in brothers (163) and in an analysis of the Swedish Family-Cancer Database, familial risks were increased to 3.8-fold for fathers, 8.3-fold for brothers, and 3.9-fold for sons (164). Large families with a high incidence of testicular cancer have been described but are rare (165,166) and the majority of families are relative pairs, usually brothers, suggesting that susceptibility may be caused by genes with small or moderate effects. An increased risk of testicular germ cell tumors has been reported in Down syndrome and Klinefelter syndrome (167).

Linkage studies in familial testicular cancer were hampered by the paucity of large multigenerational pedigrees and although candidate gene studies identified the Y chromosome *gr/gr* deletion as causing two- to three-fold increase in risk, this variant occurs in <3% of males (168). In contrast, genome-wide association studies have identified significant associations to a number of loci including KITLG, SPRY4, BAK1, DMRT1, TERT and ATF7IP (169,170). Interestingly these loci can be linked to three pathways: KIT, telomerase regulation and sex determination (167).

Genetic disorders predisposing to testicular tumors can be classified according to the presence or absence of abnormal sexual differentiation. Disorders of sex development (DSD) are defined as conditions of incompleter or disordered genital or gonadal development leading to discordances between genetic sex, gonadal sex and phenotypic sex and are subclassified into (1) sex chromosomal DSD, (2) 46XY DSD and (3) 46XX DSD (171). DSD patients with hypervirilization are not at risk of germ cell tumors and the risk is variable in patients with hypovirilization and gonadal dysgenesis.

Gonadoblastoma occurs in XY gonadal dysgenesis and in patients with the WAGR and Frasier syndromes (see previous discussion). The risk of germ cell tumors in gonadal dysgenesis is associated with the presence of Y chromosome material and, in particular, the presence of Yq material. Girls with Turner syndrome and 45X, 46XX, or 46X, del (X) are not at increased risk of germ cell tumors, but up to 20% of patients with gonadal dysgenesis and 45X/46XY or other karyotypes with Y chromosome material may develop gonadoblastoma (172). Gonadoblastoma usually develops in the second and third decades and may secrete estrogens or testosterone. Deletion mapping in patients with gonadoblastoma and Y chromosome deletions indicated that the genes

on the Y chromosome that induce gonadoblastoma are distinct from the sex-determining locus (173) and the TSPY (testis-specific protein Y-encoded) is thought to be the Y chromosome gonadoblastoma locus (GBY) (174). Although bilateral testicular germ cell tumors have been reported in Klinefelter syndrome (175,176), testicular tumors are not common and most germ cell tumors associated with Klinefelter syndrome have an extragonadal origin (177). The detection of low-level 45X/46XY mosaicism by sensitive molecular analysis in girls with Turner syndrome is helpful in identifying those who are at potential risks for gonadoblastoma (178). There is probably no increased risk of gonadoblastoma in Klinefelter syndrome ((51); XXY) or in the XYY syndrome. However, gonadoblastoma is associated with sex reversal caused by 9p deletion (179).

The association between the presence of a Y chromosome and gonadoblastoma is also a feature of single-gene disorder causes of DSD. XX forms of gonadal dysgenesis are probably not associated with an increased risk for gonadoblastomas, but XY pure gonadal dysgenesis (Swyer's syndrome, which may result from SRY mutations) is frequently complicated by this tumor. Affected individuals are of normal stature and do not have the features of Turner syndrome, but have streak gonads. The incidence of gonadal neoplasia in true hermaphrodites (individuals with both testicular and ovarian tissue) appears to be low, although both ovarian and testicular tumors have been reported. Complete androgen insensitivity syndrome (testicular feminization) is inherited as an X-linked recessive disorder and is associated with an increased risk of testicular malignancy (about 5%), most often seminoma. If malignancy develops it is usually after age 25 so that orchidectomy can be delayed until after the pubertal growth (172). An increased incidence of benign testicular tumors has been reported in congenital adrenal hyperplasia (180). Testicular germ cell tumor has also been reported in a man with familial male-limited precocious puberty secondary to a germline-activating mutation in the luteinizing hormone/chorionic gonadotropin receptor gene (181).

Mendelian disorders not associated with abnormal sexual development that predispose to testicular tumors include Peutz-Jegher syndrome, Carney complex and possibly X-linked ichthyosis. Males with Peutz-Jegher syndrome have an increased risk of testicular tumors, although the risk is less than that of ovarian tumors in affected females. Tumors have been classified as Sertoli cell or sex cord tumors and are usually present with childhood gynecomastia secondary to excess estrogen production (182,183). The Carney complex (synonyms NAME or LAMB syndrome) is a dominantly inherited disorder characterized by patchy skin pigmentation, cardiac myxomas, and endocrine abnormalities. Expression is variable, but Carney and colleagues (184) reported that 56% of affected males had testicular tumors, usually bilateral. Microscopic examination of the tumors



usually reveals a large-cell calcifying Sertoli cell appearance or a Leydig cell tumor. The average age at diagnosis is 17 years (range 5–83), and some patients present with sexual precocity (184). Carney complex is genetically heterogeneous PRKAR1A at 17q22–q24 (Type 1 Carney Complex (CNC1) or at a 2p16 locus (CNC2) (185)). Libé et al. (186) reported that PDE11A sequence variants were associated with the development of large-cell calcifying Sertoli cell tumors (in patients with Carney complex type 1).

Familial Leydig cell tumors with no associated abnormalities were reported in a father and son by Bokemeyer and associates (187). Patients with X-linked ichthyosis (steroid-sulfatase deficiency) have been suggested to be at increased risk of cryptorchidism and testicular tumors (188). Germ cell tumors have been reported in families with Li–Fraumeni syndrome (189), but there is no evidence to suggest that germline p53 mutations are a frequent cause of familial testicular tumors (190).

Individuals at high risk of testicular cancer should be offered appropriate preventive measures. Nonfunctioning testes, which present a significant risk for tumorigenesis (e.g. androgen insensitivity syndrome or XY gonadal dysgenesis), should be removed. Individuals thought to be at high risk of familial testicular tumors can be monitored by regular self-examination and ultrasonography.

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## CROSS REFERENCES

App to Spec Disorders: Congenital Anomalies of the Kidney and Urinary Tract (CAKUT); Cystic Diseases of the Kidney; Nephrotic Disorders; Genetic Disorders of the Adrenal Gland; The Phakomatoses; Primary Tumors of the Nervous System.

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# CHAPTER

# 67

## Gastrointestinal Tract and Hepatobiliary Duct System

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### ABBREVIATION

HSCR – Hirschsprung Critical Region. Refers to two basic types of this heterogeneous disorder, HSCR type 1 and HSCR type 2 (see text).

### 67.1 INTRODUCTION

The gastrointestinal (GI) tract is composed of the mouth and oral structures, salivary glands, pharynx, esophagus, stomach, small and large intestines, the biliary tract, pancreas, rectum, and anus, and other auxiliary organs that contribute to digestion and adsorption of food. In addition it functions as an organ of immunity. This chapter focuses on gross defects of the intestinal anatomical structures, genetic disorders of the small and the large intestines, and the biliary tract system with emphasis on two genetic disorders, congenital intestinal aganglionosis (Hirschsprung disease) and a complex multisystem disorder affecting the biliary duct system (Alagille syndrome (ALGS)).

GI polyposis, neoplasias involving the GI tract, and inflammatory bowel disease are presented in Chapters 67 and 68. Malformations of the GI tract are common in many chromosomal disorders (Chapter 43). Although the most important examples will be mentioned here, the reader is referred to the chapters on chromosomal disorders.

### 67.2 EMBRYOLOGICAL BACKGROUND

The different types of congenital malformations of the GI tract can best be understood on the basis of the underlying normal embryological development. In brief, the cells forming the gut derive from the endoderm for the inner lining, the mucosa, from the ectoderm for the wall, containing muscles and connective tissue, and from the neural crest for the intramural ganglion cells required for peristalsis (1–3). The early precursors of the gut originate from the

inward migration of cells from a small area on the surface of the blastula. In humans, the GI tract develops between the 5th and 12th week of fetal development from a simple ectodermal tube with a mucosal lining. During development the mucosa develops numerous folds and ridges and small fingerlike outgrowths, the villi, eventually to cover its entire surface (for reviews see References (1–3)).

Beginning at the 7-somite stage (Carnegie stage 10, day 22/23), different segments become apparent: the foregut, the midgut, and the hindgut (1). The rostral portion of the foregut becomes esophagus, stomach, and duodenum, with liver, biliary duct, and pancreas. The stomach rotates clockwise about 90°. The midgut becomes greatly elongated and forms a loop in the umbilicus outside the body cavity in the extraembryonic coelom. Because of the rapid growth of the midgut in relation to the abdominal cavity, the newly formed bowel appears to rotate in a counterclockwise direction (Carnegie stage 20, 18–22 mm crown-rump length, day 51–53). The gut is permanently positioned in the abdominal cavity after this 35-mm stage (10th week) (1).

The hindgut is formed by the secondary yolk sac and gives rise to the left third of the transverse colon, the descending and pelvic colons, the rectum and the upper part of the anal canal.

The liver develops during the early stages of duodenum formation (14-somite stage, Carnegie stage 11, day 24), followed by the bile duct system. The bile capillaries are produced by canalization of the minor intrahepatic divisions of the hepatic trabeculae. The pancreas develops from a structure that first reaches the mesenterium (ventral pancreas) and then follows the rotation of the stomach. The bile ducts and the pancreatic duct form a common duct that enters the duodenum.

The abdominal cavity and the pleural cavity become separated by development of the diaphragm. First a septum transversum forms above the liver during the 4th week of fetal development (Carnegie stage 13, 4- to

6-mm embryo, day 28–32). Then a pleuroperitoneal fold originating from the dorsal wall of the cavity extends to and fuses with the septum transversum to complete the diaphragm.

## 67.3 CLASSIFICATION OF GASTROINTESTINAL DISORDERS

Based on the embryological considerations GI disorders can be classified into (1) gross defects of the anatomical structures, (2) disorders resulting from failure of the intramural intestinal ganglion cells to migrate to the gut and establish function there (congenital intestinal aganglionosis, Hirschsprung disease), (3) disorders mainly due to malfunction or malformation of the hepatobiliary system, and (4) functional disorders. For additional information the reader is referred to other reviews (4,5), in particular to McKusick's classification of the Mendelian Inheritance of Man (MIM) (6) and its online version OMIM [www.ncbi.nlm.nih.gov/Omim]. For all such disorders mentioned in this chapter, the OMIM number is provided for easy access to additional information.

### 67.3.1 Gross Defects of the Intestinal Anatomical Structures

Various embryological malfunctions may result in failure of the gut to become fully patent (atresia), failure of the gut to rotate normally (malrotation), duplication of parts of the gut, disruption of the abdominal wall and umbilical structures (gastroschisis and omphalocele), defects of the diaphragm, and anorectal malformations (1–5).

**67.3.1.1 Atresias.** With normal development of the intestines in the human embryo, the proliferating mucosa closes the lumen of the gut during Carnegie stage 17 (11- to 14-mm stage, day 42–44 at the beginning of the 7th week). Subsequently the gut is recanalized and assumes its typical mucosal relief. Failure to recanalize will result in stenosis or atresia of the bowel for various extents at different locations.

*Atresia of the esophagus* (OMIM 189960) with or without a concomitant fistula to the trachea is a prominent cause of neonatal regurgitation and aspiration pneumonia. Its frequency can be estimated at about 1 in 3000 newborns. It may be nonsyndromic as an isolated anatomical defect or syndromic as part of a systemic malformation syndrome (see VATER complex, below). As a nonsyndromic malformation, esophageal atresia with or without tracheal fistula accounts for about one-third of patients with GI atresia. OMIM lists seven reports of familial occurrence, most of them in a child and a parent. However, in most cases atresia of the esophagus does not appear to result from genetic causes that establish an increased risk of recurrence in sibs or offspring of a patient. Most of these observations

occurred with systemic genetic disorders in which esophageal atresia was only one of several or many possible manifestations. It is unlikely that monogenic inheritance plays a role. A very low risk of recurrence can be given in genetic counseling. An interstitial deletion of chromosome 17 [del(17)(q22q23.3)] has been reported to be associated with esophageal atresia (7).

*Duodenal atresia* (OMIM 223400) accounts for about 10 % of atresias at a frequency of about 1 in 5000 newborns. OMIM lists five reports of familial occurrence, but in most cases a genetic origin is unlikely. Duodenal atresia is a characteristic concomitant malformation of trisomy 21 (8,9).

*Jejunal atresia* (OMIM 243600) occurs in about 1 in 4000 newborns, making the jejunum the most common site of bowel obstruction in the newborn. Although seven observations of familial occurrence have been reported in OMIM, a genetic cause appears to be uncommon. In the absence of a previously observed patient in the same family, a low risk of recurrence can be given in genetic counseling.

Multiple intestinal atresias (familial intestinal polyatresia syndrome or apple peel syndrome) may represent a distinct subtype. It has been reported in sibs and in an inbred kindred (see OMIM 243150). However, a monogenic cause is unlikely to account for most of these cases.

Five different anatomical types of jejunal obstruction have been distinguished (10,116): type I (mucosal web), type II (ends separated by a fibrous cord), type IIIa (ends separated by a V-shaped mesenteric defect), type IIIb (“apple peel” deformity), and type IV (multiple atresias). Type IIIb most commonly shows familial occurrence, although familial cases of types I, II, and IV have also been observed (see OMIM 243150).

*Anal atresia or imperforate anus* (OMIM 207500) occurs in about 1 per 5000 births. Although it has been observed in sibs (MIM 207500 and 301800), genetic factors probably contribute little to the etiology. For most isolated cases a low risk of recurrence can be assumed. However, in more than half of the patients, anal atresia occurs together with other malformations as one component of a Mendelian, chromosomal, or nongenetic syndrome. OMIM lists 20 entries under the heading *anal atresia*. Pinsky (11) listed 26 different disorders involving anorectal malformations.

Anal atresia is a frequent component of the VATER complex (MIM 192350). This is an acronym for vertebral, anal, tracheoesophageal, renal and radial limb malformations. Cardiovascular and nonradial limb malformations may also be present (VACTERL, OMIM 276950, 314390). This nongenetic malformation complex rarely includes all components in an individual patient. Usually, only a few of the malformations are manifest (8).

Anal atresia is a feature of the cat-eye syndrome (OMIM 115470), a chromosomal disorder resulting from an inverted duplication of 22q11, which leads



to tetrasomy of this region (the CES critical region, see chapter on chromosomal disorders). The cat-eye syndrome shows wide phenotypic variability, ranging from near normal to ones with severe malformations (12). Preauricular skin tags and/or pits constituted the most consistent features.

Anorectal malformations (imperforate anus and anal stenosis) are a hallmark of the autosomal dominant *Townes–Brock syndrome* (OMIM 107480) (10,13,14). Other variable manifestations are anomalies of the hands (preaxial polydactyly, triphalangeal thumbs, hypoplastic thumbs) and feet (syndactyly III/IV, short metatarsals, and overlapping toes), sensorineural deafness and malformed auricles, dysplastic kidneys, hypospadias, cardiac malformations (not only atrial and ventricular septal defects but also lethal truncus arteriosus in one case and pulmonary valve atresia in another), mental retardation, and other manifestations. This disorder results from mutations in the *SALL1* zinc finger transcription factor gene located on 16q12.1 (10,13,14). *SALL1* (OMIM 602218) is the human homolog of the *spalt* gene (*Sal*) in *Drosophila melanogaster*, a developmental regulator that is conserved throughout evolution. The expression patterns of *sal*-like genes in mouse, *Xenopus*, and the fish Medaka suggest that *sal* is regulated by sonic hedgehog (*SHH*; OMIM 600725).

Anal atresia or stenosis may be a feature of the CHARGE syndrome (Coloboma-Heart defect-Choanal atresia-Retardation-Genital and Ear Anomalies, OMIM 214800). Most cases are sporadic. However, rare familial occurrence, a high rate of concordance in monozygotic twins, and mutations in two genes (*CHD7* at 8q12.1 and *SEMA3E* at 7q21.11) suggest that genetic causes can occur.

**67.3.1.2 Defects of the Abdominal Wall.** *Gastroschisis* (OMIM 230750) is a disruption of the abdominal wall outside the umbilicus because of diverse causes. With a frequency of 1–2 per 10,000 births it represents a relatively common anatomical defect in the newborn (17). OMIM lists six entries for gastroschisis (230750), omphalocele (164750), constrictive bands (217100), and a mouse homolog called disorganization (223200). In most cases gastroschisis does not seem to result from a genetic cause, but exceptional occurrence in sibs has been reported (see OMIM 230750). Gastroschisis has been found to occur in 14.1% (469 of 3322) cases worldwide as part of manifestation of other disorders or multiple congenital anomalies (15). Other additional risk factors have been considered (16).

In a population-based registry, Torfs and Curry (15) found six other family members with this defect among 127 infants with gastroschisis, corresponding to a possible recurrence rate of 3.5%. Other observations, in particular of discordance in monozygous twins and absence of recurrence in 44 families (16–18), do not point to a genetic etiology. An abdominal wall defect may also be part of an amniotic band disruption

complex. An analysis of 274 cases of gastroschisis (79% isolated) in 21 regional registers in Europe (EUROCAT registers) during the period 1980–1990 indicated a prevalence rate of 0.94 per 10,000 (19,20). Gastroschisis can be recognized by ultrasonographic prenatal diagnosis. A gene–environmental model involving the VEG-NOS3 pathway has been invoked (21).

*Omphalocele* (OMIM 164750) is a defect of the abdominal wall at the base of an umbilical ring that has remained open and contains part of the intestines, occasionally of considerable extent. Although there have been three reports of isolated omphalocele in sibs (see OMIM 164750), a genetic etiology is unlikely in most cases. However, omphalocele is an important component of the Beckwith–Wiedemann (OMIM 130650) and the Shprintzen–Goldberg syndromes (OMIM 182210). OMIM lists 37 entries for omphalocele as a part of other systemic disorders.

Kanagawa et al. (22) reported a family with nine individuals in three generations affected with a presumptively autosomal dominant form of omphalocele that required surgical intervention within the first few days of life. Yatsenko et al. (23) observed a patient with omphalocele, dysmorphic features, and mild developmental delay associated with trisomy for region 3q27.3-qter and monosomy for 4q32.3-qter resulting from an unbalanced translocation of maternal origin. The association of a duplication for the long arm of chromosome 3 and an omphalocele has been reported previously in several instances (see OMIM 164750).

Katz et al. (24) suggested that mutations in the *PITX2* gene might contribute to the causes of omphalocele and VATER-like syndromes in some patients. They found a three base pair deletion in the 3′ untranslated region of *PITX2* in one patient with this combination, but neither in patients with isolated omphalocele nor in normal controls.

**67.3.1.3 Malrotation.** Malrotation (OMIM 193250) of variable extent is a common result of the normal embryonic origin of the GI tract. OMIM lists four familial reports, but a genetic basis for malrotation alone is unlikely. It is a frequent component of chromosomal and several monogenic disorders.

**67.3.1.4 Duplication.** Parts of the intestines may be duplicated as a result of unknown embryonic causes. No evidence for primary genetic causes exists.

**67.3.1.5 Meckel Diverticulum.** Meckel diverticulum (OMIM 155140) is a dilated proximal omphalomesenteric duct connected to the distal ileum. It occurs in 1–2 % of newborns, often without clinical consequences. Although familial occurrence has been reported, there is no evidence for genetic factors in most patients. It may also occur in the cat-eye syndrome (OMIM 115470), Simpson–Golabi–Behmel syndrome type 1 (OMIM 312870), Beckwith–Wiedemann syndrome (OMIM 130650), and Pena–Shoheir syndrome type 1 (OMIM 208150).

**67.3.1.6 Partial Agenesis of the Pancreas.** Agenesis of the dorsal pancreas (OMIM 167755) has been reported

in a mother and her two sons of different fathers (25). However, this appears to be a rare defect without a known genetic basis.

Yorifuji et al. (26) described hypoplasia of the pancreas in a mother and three of her offspring in a nonconsanguineous Japanese family (OMIM 600001). In addition, atrial septal defect (MIM 607411) and patent ductus arteriosus were present in the mother, whereas severe cardiac malformations (transposition of the great vessels, ventricular septal defects, pulmonic stenosis, atrial septal defect, and tetralogy of Fallot) were documented in her children.

Congenital agenesis of the pancreas OMIM (260370) has been associated with a gene at 13q12.1 (*PDX1/IPF1*) and the possibility that a mutation in the gene for insulin promoter factor-1 (*IPF1*; 600733) could be responsible. Pancreatic and cerebellar agenesis may be caused by mutation in the *PTF1A* gene (see OMIM 607194).

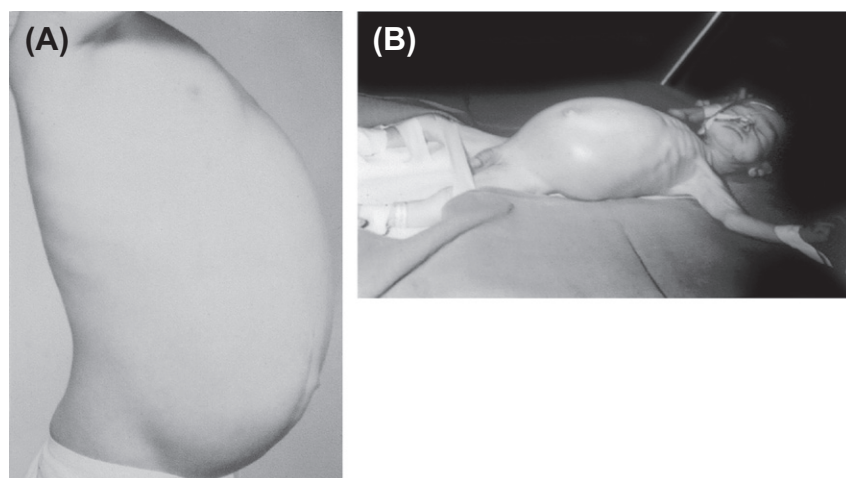
**67.3.1.7 Defects of the Diaphragm.** Congenital diaphragmatic hernia (CDH) is part of the heterogeneous group of defects in the development of the diaphragm with a prevalence among newborns of 1 in 2500–4000 with a mortality rate of 30–60% due to lethal pulmonary hypoplasia. Two types, DIH1 and DIH2 have been mapped to 15q26.1 and 8p23.1, respectively (OMIM 142340). A third form, DIH3, has been associated with a mutation in the zinc finger gene *ZFPM2* (OMIM 603693). A possible X-linked form exists (OMIM 306950).

For isolated CDH, including congenital absence of the diaphragm, an empirical risk of recurrence of about 3.5% might exist for sibs (26–31). CDH occurs as a syndromic part of the Fryns syndrome (OMIM 229850), Simpson–Golabi–Behmel syndrome (OMIM 312870), tetrasomy 12p (OMIM 601803), Brachmann–de Lange syndrome (OMIM 122470), and lethal multiple pterygium syndrome (OMIM 253250) (30).

## 67.3.2 Congenital Intestinal Aganglionosis (Hirschsprung Disease)

**67.3.2.1 Definition and Clinical Aspects.** Hirschsprung disease (OMIM 142623) is a genetically determined, multigenic, neonatal intestinal obstruction syndrome that is correctable by surgery. It is genetically heterogeneous and clinically variable, ranging from severe in neonates to mild in late infancy and childhood. The main clinical manifestations are chronic constipation, abdominal distension and a megacolon of variable length (Figure 67-1). A neonate may present with meconium ileus and sigmoid perforation; impaired defecation is a leading sign in all cases. The diagnosis is confirmed by rectal biopsy by demonstrating the absence of intramural intestinal ganglion cells. Surgical intervention by removing the aganglionic distended part of the colon is successful in most cases, but early recognition and therapy are important. With a population incidence of 1 in 5000 newborns, Hirschsprung disease is the most common cause of intestinal obstruction in neonates and during early childhood. The sex ratio is approximately four males to one female. Reliable diagnostic criteria, successful surgical intervention in most cases, follow-up management, and genetics have established the scientific basis for good patient care and genetic counseling. Congenital intestinal aganglionosis may be a component of numerous other genetic syndromes (see syndromic forms below).

Hirschsprung disease was first described in 1869 (32), but it became known as a GI pediatric disease entity following the description by Harald Hirschsprung in 1889 in Copenhagen (33). Its pathogenesis, absence of intramural ganglion cells in the myenteric (Auerbach) and submucosal (Meissner) plexuses of the intrinsic GI tract, was recognized in 1948 (34,35). Earlier observations had pointed in this direction, but did not draw the correct conclusions (36,37). The population incidence



**FIGURE 67-1** Hirschsprung disease. (A) Distended abdomen due to megacolon. (B) Infant with severe form of megacolon. (Photo courtesy Dr Lester Martin, Cincinnati.)

in European populations is about 1:5000, with a ratio of affected males to females of 4.5:1. Until the late 1950s, before the introduction of several successful surgical procedures, this disease was almost invariably fatal in infancy or early childhood. Observations of rare familial occurrence in sibs, and later in surviving affected parents, led to systematic genetic studies in the 1960s and 1970s. The era of molecular genetics of Hirschsprung disease began in 1973, when close linkage with the gene *RET* was first demonstrated (38,39). Hirschsprung disease may occur in syndromic form as a component of numerous other genetic disorders (see below). For comprehensive reviews see references (40–43).

**67.3.2.2 Types of Aganglionosis.** The terminal portion of the colon just above the rectum is always involved, but the upper (rostral) border is highly variable. Thus, Hirschsprung disease is classified into two basic types, according to the length of the aganglionic segment: short-segment Hirschsprung disease (also called type I or S-HSCR), occurring in approximately 60–85% of patients and long-segment Hirschsprung disease (type II or L-HSCR), occurring in 15–25%. The short-segment type is defined as aganglionosis below the upper sigmoid and the long-segment type as aganglionosis extending beyond the splenic flexure into the transverse colon, which may also involve part of the ascending colon. In a small proportion of patients (approximately 3–5%), the entire colon and various parts of the small intestine may be aganglionic (total colonic aganglionosis). Other rare variants are total intestinal aganglionosis, ultrashort-segment aganglionosis (from the distal rectum to the normal aganglionic zone 2 cm above the pectinate line), and segmental aganglionosis above a normal distal segment, which is reported in very few cases only (for reviews see References (40,43,44)). About 70% of patients with Hirschsprung disease have no other manifestations, approximately 18% have associated congenital malformations or a different genetic disorder, and approximately 12% have a chromosomal aberration, mostly trisomy 21 (see syndromic forms of Hirschsprung disease below).

#### 67.3.2.3 Formal Genetics.

**67.3.2.3.1 Inheritance Pattern.** Systematic genetic studies of the familial occurrence of nonsyndromic Hirschsprung disease preceded the identification of the genes involved (45–49), and these studies are still a reasonable basis for genetic counseling. The overall risk of occurrence in siblings is approximately 4%, approximately 200 times the population risk. In spite of the existence of pedigrees showing an autosomal dominant or an autosomal recessive mode of inheritance (50), a Mendelian inheritance pattern is absent in the families of most patients. For this reason and based on a detailed analysis, taking the gender of the index patient and affected siblings into account, a multigenic

(multifactorial) model has been invoked to explain the unusual inheritance pattern of Hirschsprung disease. This model assumes that the proportion of disease predisposing alleles is higher in females than in males. Furthermore, the length of the aganglionic segment greatly influences the genetic risk. The longer it is, the higher the risk of recurrence in a first-degree relative (Table 67-1).

Thus, the proportion of affected siblings is higher if the index patient is female (6–18%) than when the index patient is male (0.6–8%), which is the opposite of the sex ratio in the general population with four males to one female (Carter effect). The familial incidence is lower in S-HSCR than in L-HSCR (reviewed in References (40,43,49)).

**67.3.2.3.2 Genetic Counseling.** The risk of an affected sib is high if the index patient is female with a long aganglionic segment and the sib is male (18%), compared with 7% if the aganglionic segment is short. In contrast, female sibs of a male index patient have a low risk of 0.6% for type 1 (short) and 3% for type 2 (Table 67-1). Thus, the risk to sibs is higher if the index patient is female rather than male. This is the opposite of the population incidence, with a ratio of four males affected to one female. This seeming paradox, first described in the 1960s by C.O. Carter for pyloric stenosis (see below), is called the *Carter effect*. It is interpreted as being due to a multigenic population difference between males and females. They differ in a threshold effect: it takes more contributing genetic factors for disease manifestation in females than it does in males, the latter presumably being more liable to the disease than females. The gender of the parent and the length of the aganglionic segment also influences the average risk to

**TABLE 67-1 Empirical Risk for Nonsyndromic Hirschsprung Disease<sup>a</sup>**

Risk to Sibs (%)		
Sex of Index Patient	Male	Female
Male		
Type I (short) <sup>b</sup>	4	1
Type II (long)	11	8
Female		
Type I	6	2
Type II	23	18
Risk to Offspring of an Affected Parent (%)		
Affected Male		
Type I	0.5	0.5
Type II	28	22
Affected Female		
Type I	0.5	0.5
Type II	28	22

<sup>a</sup>Data from Bodian and Carter, (45); Madsen, (46); Passarge, (47), (49), and Chakravarti and Lyonnet, (40).

<sup>b</sup>Length of aganglionic segment defined as type I for absence of intestinal ganglion cells caudal to the splenic flexure, and as type II for absence anywhere further rostral to this point (see text).

offspring of a parent affected with Hirschsprung disease (Table 67-1).

Caution should be exercised when applying the empirically derived risk figures because the actual risk in a given family might come close to that of an autosomal dominant or recessive inheritance pattern. The risk of recurrence depends on the underlying disease if intestinal aganglionosis occurs as a component of a systemic disorder (syndromic Hirschsprung disease, see below).

**67.3.2.4 Diagnosis.** The current low mutation detection rate (up to about 60%) makes a molecular genetic confirmation of the diagnosis of Hirschsprung disease untenable in most cases. However, based on the history and clinical findings, and confirmed by rectal biopsy, a reliable diagnosis can be established without mutation analysis.

**67.3.2.4.1 Clinical Signs.** Failure to pass meconium within the first 48 h is the first and sometimes only manifestation in the neonatal period. Vomiting and abdominal distension occur within the first few weeks in about 20–70% and 30–90%, respectively. Beyond the neonatal period, constipation (in 68%) associated with abdominal distension (in 64%) with vomiting (in 37%) become leading signs. Hirschsprung disease should be considered in unexplained perforation of the caecum or appendix (44).

**67.3.2.4.2 X-Ray.** Regular abdominal X-rays show a distended proximal colon and small intestines above an empty rectum. Barium enema supports the diagnosis in most cases by demonstrating a small rectum with a transition zone between the narrow aganglionic and the dilated proximal colon. Anorectal manometry shows an absence of the normal relaxation of the internal sphincter in response to rectal distension. However, this normal rectoenteric reflex only develops within 2 weeks after birth and cannot be used in the newborn (40).

**67.3.2.4.3 Rectal Biopsy.** The absence of ganglion cells in the myenteric plexus (Auerbach) and the submucosal plexus (Meissner) of the colon over a variable length is the hallmark of Hirschsprung disease. The diagnosis

can be confirmed by full-thickness rectal biopsy or suction rectal biopsy. The former requires general anesthesia and experience in interpretation (40). Suction rectal biopsy can be taken without anesthesia but samples the superficial submucosal plexus only. In many cases this is sufficient to establish the diagnosis by showing either aganglionosis or absence of the regular neural network associated with aganglionosis.

The diagnosis rests on histological and histochemical demonstration of aganglionosis following a rectal biopsy. Since the intrinsic innervation of the anal canal is separated from that of the intestines by a zone above the pectinate line about 10–20 mm that normally does not contain ganglion cells, the rectal biopsy must be taken high enough to ensure diagnostic reliability. Since another zone 4–5 mm rostrally normally has relatively few ganglion cells, a rectal biopsy must be taken 20–30 mm above the pectinate line to reach both the myenteric and the submucosal layers and ensure diagnostic reliability.

#### 67.3.2.5 Molecular Genetics.

**67.3.2.5.1 Genes and Loci Involved.** As of April 2011, mutations at nine known partially interdependent genes are recognized to be involved in causing of Hirschsprung disease (Table 67.2). Autosomal dominant mutations are in the *RET* gene (Rearranged during Transfection protooncogene, OMIM 164761; HSCR1) and in its ligand, the *GDNF* (*glial-cell-derived neurotrophic factor*) gene on 5p13.1–p12 (OMIM 600837; HSCR3). Recessive mutations, observed in an inbred Mennonite population (51) are (1) in the *EDNRB* (*endothelin receptor type B*) gene on 13q22 (OMIM 131244; HSCR2) and its ligand, the *EDN3* (*endothelin-3*) gene on 20q13.2–q13.3 (OMIM 131242; HSCR4), (2) in the *SOX10* [*SRY (sex determining region Y)-box 10*] gene on 22q13 (OMIM 602229), and (3) in three further loci (Table 67-2).

Three different, presumably functionally unrelated signaling pathways are involved in the normal development of the neural-crest-derived intestinal ganglion cells. These are (1) the *RET* receptor tyrosine kinase pathway,

**TABLE 67-2 Genes Involved in the Causes of Hirschsprung Disease<sup>a</sup>**

Gene	Location	Main Effect	Penetrance	OMIM
<i>RET</i>	10q11.2	Dominant, loss of function	50–72%	164761
<i>GDNF</i>	5p13.1	Dominant/recessive	Unknown	600837
<i>EDNRB</i>	13q22	Recessive	8–85%	131244
<i>EDN3</i>	20q13	Recessive	Unknown	131242
<i>SOX10</i>	22q13	Dominant/recessive <sup>b</sup>	>80%	602229
<i>ECE1</i>	1p36	Dominant/recessive <sup>b</sup>	Unknown	600423
<i>NTN, NRTN</i>	19p13	Unknown	Unknown	601880
<i>ZEB2</i> (formerly <i>ZFHX1B, SIP1</i> )	2q22	Sporadic <sup>b</sup>	Unknown	605802
<i>PHOX2B</i>	4p12	Congenital hypoventilation <sup>c</sup>	Unknown	603851

<sup>a</sup>Most genes appear to be interdependent and mutations usually do not segregate in a Mendelian pattern. Data from Chakravarthi and Lyonnet 2001 (40); Passarge, 2011 (43). Predisposing chromosomal regions have been identified at 3p12, 9q31, 16q23, and 19q12 (see text).

<sup>b</sup>Syndromic forms.

<sup>c</sup>Sometimes associated with Hirschsprung disease.



consisting of the genes encoding the RET receptor and its ligand, glial-cell-line-derived neurotrophic factor (GDNF); (2) the endothelin type B receptor pathway, with the EDNRB receptor and its ligand, endothelin-3 (EDN3); and (3) the transcription factor SOX10 (41).

In addition to these genes, susceptibility loci have been identified at 3p12 (HSCR5), 4q31–q32 (HSCR9; OMIM 611644), 9p31 (HSCR6; OMIM 606874), 16q23 (HSCR8; OMIM 608462), and 19q12 (HSCR7; OMIM 606875).

All mutations have a reduced penetrance and variable expression with respect to the length of the aganglionic segment. Dominant mutations at the *RET* locus account for approximately 50% familial patients with Hirschsprung disease and approximately 35% of isolated patients, with a penetrance of 50–70%. Mutations at the *EDNRB* locus on 13q22 are recessive, each with reduced penetrance (30–85%). Mutations in *SOX10* have been found in a syndromic form, the Waardenburg–Shah syndrome (OMIM 277580), associated with Hirschsprung disease. In addition to the genes mentioned above, several modifier genes are thought to influence penetrance and expression of the different causative mutations. Mutations in the *endothelin converting enzyme 1 gene* (*ECE1*; OMIM 600423) seem to be very rare.

**67.3.2.5.2 Role of the RET Gene.** The *RET* gene (MIM 164761), located on the long arm of chromosome 10, region 1, band 1.2, plays a pivotal role in Hirschsprung disease. *RET* was originally described as a protooncogene (*RET* being a term used when this gene was discovered in a different context). Inactivating *RET* mutations are dominant and result in aganglionosis of different types with a penetrance of 50–70% (40,41,43,52–60).

They may occur as a new mutation or are transmitted from a parent. They account for about 50% of familial patients with Hirschsprung disease and about 35% of isolated patients. In short-segment type 1, mutations in the *RET* gene are found in 32%; in long-segment type 2, in 57% (40,41). In addition, changes in noncoding regions of *RET* contribute to the causes of Hirschsprung disease (see below).

The human *RET* gene, located on the long arm of chromosome 10, region 1, band 1.1 (10q11.1; OMIM 164761), encodes a cell surface molecule, receptor tyrosine kinase, expressed in derivatives of the neural crest and neuroectoderm, with functions in development and differentiation of neural crest cell lineages, from which the intramural intestinal ganglion cells also derive (40,41,61). The gene has 21 exons encompassing about 55kb of genomic DNA. Its transcript is alternatively spliced.

The RET receptor is a transmembrane protein of 1114 amino acids with different functionally distinct domains: a signal peptide; three extracellular domains, consisting of the putative ligand-binding domain, a cadherin-like domain, and a cysteine-rich domain; a transmembrane domain; and an intracellular catalytic

domain (Figure 67-2). The last domain is conserved in evolution and is similar to other growth factor receptors. Different mutations in the *RET* gene lead to five clinically different disorders: Hirschsprung disease, multiple endocrine neoplasia type IIA (MEN2A; OMIM 171400), multiple endocrine neoplasia type IIB (MEN2B; OMIM 162300), familial medullary thyroid carcinoma (MTC; OMIM 155240) and pheochromocytoma (OMIM 171300). In a syndromic form associated with congenital hypoventilation (OMIM 603851) mutations in the *PHOX2B* gene (paired-like homeobox 2B; OMIM 603851) have been observed (62).

Over 89 distinct *RET* mutations in Hirschsprung disease are distributed throughout the gene (40). In contrast, in MEN2A, mutations cluster in six cysteine residues in exon 10 (four residues) and exon 11 (two residues). In MEN2B, mutations are of one type only (M918T; (59)). Some mutations overlap in Hirschsprung disease and MEN2A. Hirschsprung disease and MEN2A have been observed in the same patient and can segregate in the same family (63). Loss-of-function mutations in *RET* are associated with Hirschsprung disease in a high proportion of patients, whereas the other conditions are associated with gain-of-function mutations. Presumably insufficient expression levels of RET on the surface of neural crest cells as a result of a mutation interfere with normal migration of ganglion cells to the colon or differentiation in situ. The types of mutations in the *RET* gene causing Hirschsprung disease include missense and nonsense mutations, including deletions. GDNF, one of the ligands of RET, is a transforming growth-factor- $\beta$ -related protein of 211 amino acids that can activate *RET* in cultured cells and is strongly expressed in developing murine gut and kidney mesenchyme (40,41). Mutations in the *GDNF* gene alone are rare. Usually other predisposing changes are present such as mutations in *RET* or trisomy 21. Mutations in *EDN3*,

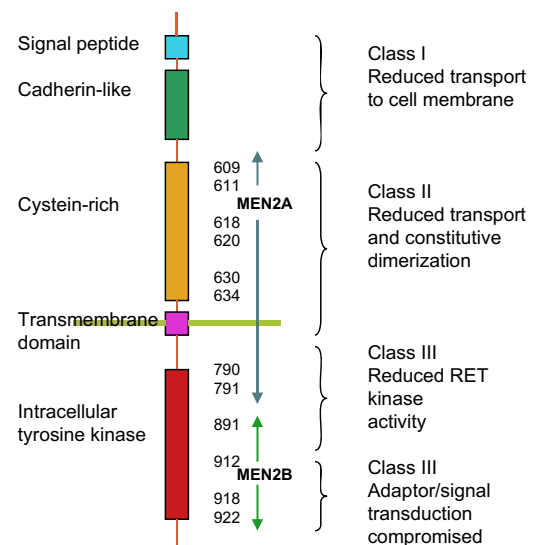


FIGURE 67-2 RET receptor.

*EDNRB* and *SOX10* are found in approximately 10% of patients with Hirschsprung disease.

Aside from *RET*, another gene expressed as a dosage effect is *EDNRB* on 13q22. Homozygotes for a missense mutation (G to T transversion) of a highly conserved tryptophan residue at position 276 (W276C) in the fifth transmembrane domain of this G-protein-coupled receptor have a 74% risk of developing Hirschsprung disease, whereas the risk is 21% in heterozygotes (64).

Some of the syndromic forms of Hirschsprung disease involve other cell types derived from the neural crest, such as precursors of melanocytes for pigment formation in the cranial region in Waardenburg–Shah syndrome or cells destined to function in the sensory components of the acoustic pathway in associated deafness.

In general, the genotypes and phenotypes, i.e. length of the aganglionic segment, in isolated Hirschsprung disease correlate poorly. Bolk et al. (65) and Bolk Gabriel et al. (66) established that *RET*, the major susceptibility gene, interacts in multiplicative model with one of three chromosomal regions at 3p12, 9q31, and 19q12. This is the first complete genetic dissection of a complex disorder (67).

The current, relatively low mutation detection rate (up to approximately 60%) makes a molecular genetic confirmation of the diagnosis of Hirschsprung disease untenable in most cases. However, based on the patient's history and the clinical findings, a reliable diagnosis (confirmed by rectal biopsy) can be established without mutation analysis. The latter, however, is useful for risk assessment in relatives of a patient.

**67.3.2.5.3 Genetic Contributions by Noncoding *RET* Sequences.** In the majority of families no coding-sequence mutations can be found. In isolated patients this proportion is even lower. However, several studies indicate that certain common polymorphic variants in the *RET* gene contribute to disease susceptibility (68–73). Chakravarti and coworkers (73) identified a common noncoding variant with enhancer function in intron 1 of the *RET* gene that was associated with a 10- to 20-fold increase in susceptibility to Hirschsprung disease. Although this common noncoding mutation (*RET*+3) explains only 2.63% and 1.14% in susceptibility in males and females, respectively, it contrasts with 0.1% of the total variance in susceptibility explained by all known coding mutations (73). Remarkably, a striking gender difference in parental transmission was observed: of 53 informative families, the mutant allele arose from mothers in 35 families and from fathers in 18. This is in keeping with the assumption that females with HSCR carry more susceptibility alleles than males. Interestingly, the worldwide allele frequencies of the mutant *RTE*+3 allele was virtually absent in Africa, but was present in 40% in China and Japan and in 25% in Europe. Emison et al. (73) consider strongly the possibility of a yet unidentified selective advantage similar to the high frequency of

hemoglobin disorders in regions with endemic malaria or chemokine receptors in HIV.

**67.3.2.6 Functional and Embryological Considerations.** Intestinal mobility is controlled by three distinct enteric nervous plexuses: the myenteric plexus of Auerbach, between the circular and longitudinal muscle layers of the muscularis propria, and the two plexuses in the submucosal region, the superficial submucosal plexus of Meissner, just beneath the muscularis mucosa, and the deep submucosal plexus of Henle. Intramural intestinal ganglion cells reach the alimentary tract by migrating from the cephalic neural crest between the 6th and 12th weeks of embryogenesis. This occurs in a defined time sequence with a cranial–caudal gradient (74–77). At 5 weeks' gestation, paired vagal fibers extend to the upper esophagus, but ganglion cells are still absent. At 6 weeks, neuroblasts are present in the esophagus outside the circular layer and the stomach. At 8 weeks (18-mm embryo), ganglion cells are present in the small intestine and the rectum, but not in the colon. At 12 weeks (70 mm) the entire plexus is innervated, presumably by further caudal ganglion cell migration. The most critical period seems to be between weeks 8 and 12, when most of the distal plexus develops.

The neuroblasts that first reach the alimentary tract form the myenteric plexus. The submucosal plexus is formed by neuroblasts migrating from the myenteric plexus across the circular muscle layer into the submucosa (76). The submucosal plexus is also formed in the caudal direction, but later, during the 3rd and 4th months of gestation. The outer longitudinal muscle layer develops from embryonic mesenchymal tissue after the myenteric plexus is formed during the 12th week (76). In contrast to the apparently direct role of vagal nerve fibers, sympathetic and pelvic parasympathetic nerves are not involved in the development of the intramural plexus.

The precursor cells of the intramural intestinal ganglion cells are derived from the neural crest. From here they migrate to the intestines and differentiate into the normal neuronal network, all phases under the control of different genes. For example, sufficient levels of *RET* expression on the surface of the cells involved are thought to be required for migration and differentiation. The failure of the intestinal ganglion cells to migrate from the neural crest or to proliferate, differentiate and survive in the intestinal wall results from several genetic causes, jointly called a neurocristopathy (78). Since other cell types also are derived from the neural crest, the syndromic co-occurrence of Hirschsprung disease with other neurocristopathy syndromes, becomes understandable. These other cells are sensory ganglia, sympathetic neurons, parasympathetic neurons of the autonomous nervous system, endocrine and paraendocrine cells (thyroid C-cells, adrenal medulla, carotid cells), pigment cells and craniofacial mesectoderm (for review, see References (40,117)).

**67.3.2.7 Syndromic Forms of Hirschsprung Disease.**

In approximately 12% of patients with Hirschsprung disease, an associated genetic syndrome is present (see Table 67-3), which often involves another cell lineage derived from the neural crest. In other syndromes, the origin of the association is less clear. The OMIM database lists 50 entries under the heading “Hirschsprung disease.” The most important monogenic disorders associated with Hirschsprung disease are Waardenburg syndrome type I (OMIM 193500) and type IIA (OMIM 193510), Waardenburg–Shah syndrome (OMIM 277580), cartilage–hair hypoplasia (OMIM 250250), Smith–Lemli–Opitz syndrome (OMIM 270400), congenital central hypoventilation syndrome (“congenital Ondine curse”; OMIM 209880), MEN2A (OMIM 171400) and MEN2B (OMIM 162300) (Table 67-3).

Best understood is the association of Hirschsprung disease with defects in other cells derived from the neural crest such as melanocytes in Waardenburg syndrome and sensory components of the acoustic pathway that lead to an association with deafness. The OMIM database lists 17 entries for deafness and Hirschsprung disease. A striking association between trisomy 21 and Hirschsprung disease has been noted in several studies, for example, up to 2.5% of patients with Hirschsprung disease showed this association, also having Down syndrome (45–49). A genetic modifier locus on 21q22 may be responsible for this frequent association (40).

Other chromosomal aberrations are detectable in approximately 8% of patients with Hirschsprung disease,

for example, interstitial deletions at 2p22, 13q22–q32 and 10q11.2 (40,66,67).

**67.3.2.8 Hirschsprung Disease in Mutant Mice.**

Neural-crest-derived associations of pigmentary anomalies and Hirschsprung disease occur in several different mutations in mice, including the recessive coat color mutations piebald, piebald-lethal and lethal-spotting (79–81). Mice homozygous for piebald-lethal and lethal-spotting also have aganglionosis. Approximately 10% of homozygous piebald and piebald–piebald-lethal compound heterozygotes have aganglionosis. These data indicate that mutations in different genes differ in penetrance. Perhaps the effect of mutations in the various genes involved in migration, differentiation and network formation of the intramural GI ganglion cells lies in a gene dosage gradient that eventually determines penetrance and expression. Overo-spotted horses, the lethal white foal syndrome with aganglionic megacolon, may be an equestrian model of human Hirschsprung disease (82).

Mice carrying mutations in the *Ret* gene pathway differ from humans in three aspects: (1) occurrence of aganglionosis only in homozygous null genotypes for *Ret* and *Gdnf*, (2) complete penetrance in most mice, and (3) absence of sex bias, in contrast to a two-fold higher penetrance in human males compared to females (41). On the other hand, penetrance of the phenotype of *Ret*<sup>+/−</sup>;*Ednrb*<sup>sls</sup> mice is greater in males than in females as it is in humans. This indicates that mutant alleles at the mouse genes *Ret* and *Ednrb* might interact as a cause of aganglionosis (41). If confirmed, this would be evidence of an interaction between two genes in two different signaling pathways.

**TABLE 67-3 Syndromic Forms of Hirschsprung disease<sup>a</sup>**

Disorder	OMIM
Multiple endocrine neoplasia type 2A	171400
Multiple endocrine neoplasia type 2B	162300
Familial medullary thyroid carcinoma	155240
Pheochromocytoma	171300
Waardenburg syndrome types 1 and 2	193500/10
Shah–Waardenburg syndrome	277580
Cartilage hair dysplasia	250250
Smith–Lemli–Opitz syndrome	270400
Hypoventilation syndrome	209880
Goldberg–Shprintzen syndrome	609360
Mowat–Wilson syndrome	235730
McKusick–Kaufman syndrome	236700
Polydactyly, renal dysgenesis, and deafness	235740
Brachydactyly type D	306980
Microcephaly and iris coloboma	235730
Dysmorphic facial features and nail hypoplasia	235760
Osteopetrosis, recessive type	259700
Bardet–Biedl syndrome	209900
Piebald trait	172800
Congenital deafness, isolated cases	277580

<sup>a</sup>Examples from OMIM (Online Mendelian Inheritance in Man: <http://www.ncbi.nlm.nih.gov/Omim/>).

For additional data see Chakravarti and Lyonnet (40), Amiel et al. (42).

**67.3.3 Genetic Defects of the Hepatobiliary Duct System**

The hepatobiliary duct system allows drainage of the bile from the biliary glands to the duodenum. Biliary atresia and genetically determined defects of the bile ducts are an important group of syndromic and nonsyndromic disorders.

**67.3.3.1 Biliary Atresia.** Biliary atresia (OMIM 210500) is defined as an infantile disorder characterized by obstructed bile flow due to lack of patent intra- or extrahepatic bile ducts. Often it is part of a systemic genetic disorder. This usually determines the clinical consequences. OMIM lists 17 disorders involving familial occurrence of biliary atresia: extrahepatic biliary atresia (210500), Lambert syndrome (245550, see below), biliary atresia with ichthyosis congenita (242400), multiple intestinal atresias (243150, see jejunal atresia), cat-eye syndrome (115470), biliary malformation with renal tubular insufficiency (210550), multiple GI abnormalities (601346, see below), ALGS (118450, see below), Kabuki syndrome (147920), Meckel syndrome type 1 (249000), Hardikar syndrome (612726), mental retardation type



Buenos Aires (Mutchinik syndrome, OMIM 249630), and others.

Mental retardation type Buenos Aires was originally described in 1972 in two children of consanguineous parents (83). They showed an apparently distinctive syndrome of mental and physical retardation, peculiar facies, and heart and renal malformations. Doerfler and colleagues (84) described two brothers with a similar phenotype. In addition, they determined that the two sib pairs, from Buenos Aires and the Ruhr area of Germany, originated from the same geographic region in Poland (former East Prussia). It could not be established whether they are in fact related. Tonoki and colleagues (85) described a Japanese girl with Mutchinick syndrome, indicating that this phenotype is not geographically or ethnically restricted.

Isolated biliary atresia (210500) is etiologically heterogeneous. A monogenic cause is unlikely in most cases in spite of some familial observations. Four sibs with branchial dysplasia, mental deficiency, club feet, inguinal hernia, and intrahepatic biliary atresia have been reported (MIM 245550). MIM 601346 lists three reports of familial occurrence (in sibs) of extrahepatic biliary ducts, duodenal atresia, hypoplastic pancreas and gallbladder, tracheoesophageal fistula, and hypoplastic intestines. The identity as an independent disorder has not been established.

### 67.3.3.2 Arteriohepatic Dysplasia Syndrome (Alagille Syndrome).

**67.3.3.2.1 Clinical Manifestations.** ALGS (OMIM 118450/601920) is a multisystem developmental disorder with important hepatic manifestations resulting from a paucity or atresia of intrahepatic bile ducts, which leads to cholestasis and liver insufficiency in early childhood (86–102). It progresses to cirrhosis and liver failure in a high proportion of patients. Neonatal jaundice is an early manifestation in many but not all patients (87,88). ALGS is the most common form of familial cholestatic liver disease in childhood with a frequency of about 1:70,000 live births. The hepatic manifestations are documented by liver biopsy showing a paucity of intrahepatic ducts in 80–100% of biopsies, to some extent depending on the age of the patient when the biopsy is taken. Elevated serum bile acids, conjugated bilirubin, alkaline phosphatase, cholesterol, and  $\gamma$ -glutamyl transferase are typical laboratory findings. Two forms, ALGS1 (OMIM 118450) and ALGS2 (OMIM 610205), are distinguished by mutation in different genes (see below).

Although originally described in 1969 and 1973, ALGS became established as a recognizable albeit variable disorder in 1975 (86). A particularly wide spectrum of manifestations has been recognized based on molecular genetic analysis (see below). Other organs that can be involved in a variable manner are the heart, skeleton, eyes, kidney, and the vascular system. In addition, growth retardation occurs in 50–90% of patients. Dysmorphic facial features include a broad forehead and prominent

mandible, giving the face a triangular appearance, bulbous nose, and deep-set eyes (Figure 67-3).

*Congenital cardiac malformations* have been reported in 80–95% of patients, most often as tetralogy of Fallot. Other malformations observed are ventricular and atrial septal defects, aortic stenosis, coarctation of the aorta, and pulmonic valvular and peripheral arterial stenoses. Isolated heart disease has been observed as the only manifestation (87–90). The *eye* is involved in 55–88% of patients. Most common are defects of the anterior chamber of the eye (posterior embryotoxon; Rieger and Axenfeld anomalies) visible by slit lamp examination. Since posterior embryotoxon occurs in 8–15% of the normal population, its presence might be difficult to assess. Pigmentary changes of the retina may be present. Other, rare ophthalmological manifestations include microcornea, keratokonus, cataracts, hypoplasia of the iris, and abnormal optic disks (88). Typical *skeletal manifestations* are vertebral malformations, “butterfly” vertebrae in 22–87%, and decreased interpediculate distances in the lumbar spine (90).

*Minor manifestations* are renal anomalies (solitary or ectopic kidney, cysts, dysplastic kidney, renal arterial stenosis, duplication of the renal pelvis or ureters). Intracranial bleeding may occur in 15% with a high rate of morbidity and mortality. The overall development is delayed and may include mental retardation in about 30%, decreased motor skills, and delayed puberty. Other manifestations are a high-pitched voice, tracheal and bronchial stenoses, jejunal and ileal atresia and stenosis, malrotation, macrocephaly, hypothyroidism, and insulin-dependent diabetes mellitus (88,89).

**67.3.3.2.2 Genetics.** The first descriptions of ALGS were based on familial occurrence consistent with autosomal dominant inheritance, but highly variable expressivity and reduced penetrance. The locus first identified was assigned to chromosome 20 at p12 based on microscopically visible deletions and translocations



FIGURE 67-3 Alagille syndrome.



involving this region (92–94). The gene carrying mutations in individuals with ALGS was also localized to this region in 1997 (95–97).

**67.3.3.2.3 Molecular Genetics.** In about 94% of patients, ALGS is caused by mutations in the gene, Jagged-1 (*JAG1*, OMIM 601920), encoding a cell surface membrane protein. It is a member of a family of ligands for transmembrane receptors that function in signal transduction in the Notch signaling pathway (98). This ligand is called Jagged-1 (*JAG1*) in humans. Its receptor, Notch, was originally identified in *D. melanogaster* and the nematode *Caenorhabditis elegans* in functions relating to cell fate decisions in many different cell types. Four receptors, Notch 1, 2, 3, and 4, occur in mammals. Notch is unique by functioning both in cell–cell interactions and as a highly conserved signal transducing system downstream of ligand binding (98). Thus, mutations in the ligand *JAG1* have pleiotropic effects, involving many different cell types and tissues, which is consistent with a central role of the Notch signaling pathway in cell specification, tissue patterning, and morphogenesis throughout development in vertebrates and invertebrates (94,98).

*JAG1* is a transmembrane protein with a single transmembrane domain, a small intracellular domain, a large extracellular domain consisting of 16 extracellular EGF-like (epidermal growth factor) repeats (40–50 amino acids each), three highly conserved regions named DLS (for ligands Delta and Serrate in *Drosophila* and Lag-2 in *C. elegans*) of 40 amino acids, and a 21-amino-acid signal peptide (Figure 67-4).

The *JAG1* gene has 26 exons spanning 36kb of genomic DNA with 88% homology with the rat gene but 96.7% homology for the coding regions (94,98). Its cDNA (GenBank accession number 4557678) is 6kb in size with a coding region of 3657 nucleotides (101). Mutations in *JAG1* can be detected in about 70% of patients. In about 56–70% they occur de novo. More

than 430 affected individuals have been studied to date (88). The types of mutation identified include 3–7% total gene deletions, 72% truncating nonsense mutations resulting in frameshift, 10% splice-site mutations, and 9% missense mutations (88).

Mutations are manifest in the heterozygous state, which is in agreement with the autosomal dominant mode of inheritance observed in families. However, carriers of a mutation with minimal or no manifestation have been observed. The clinical phenotype is not influenced by the type of mutation, since considerable clinical variability has been observed within families with the same mutation (94–100). Presumably a *JAG1* mutation causes haploinsufficiency either by relative lack of the gene product or because it has to be tightly regulated for this ligand to interact with its receptor (97). ALGS2 is due to mutations in the Notch-2 gene (*NOTCH2*, OMIM 600275).

**67.3.3.2.4 Diagnosis and Genetic Counseling.** In a typical constellation the diagnosis can be made easily on the basis of the main manifestations described above. However, a fair proportion of individuals carrying a mutation in the *JAG1* gene have been shown to manifest a few and mild signs only. Since this has been observed within the same family with the same mutation, a prognosis to be expected cannot readily be determined. This needs to be taken into account if prenatal diagnosis is considered. Cytogenetic fluorescence in situ hybridization using a probe containing *JAG1* will detect a deletion in 3–7% of patients. ALGS is transmitted as an autosomal dominant trait. Up to 70% of mutations are de novo. In the absence of a mutation in the parents of a child with proven mutation, a low risk of recurrence may be assumed. Germline mosaicism has not yet been described in a parent. However, it has been demonstrated in a few individuals without clinical manifestations (94,98,102).

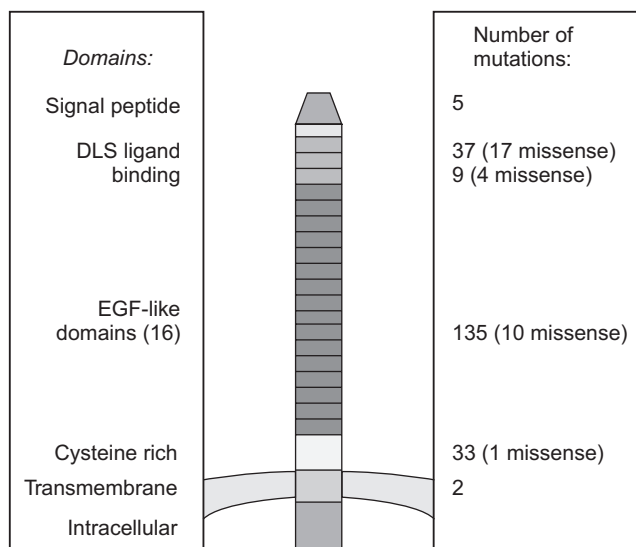
## 67.3.4 Functional Disorders

This is a loosely defined heterogeneous group of disorders. Hereditary pancreatitis, infantile hypertrophic pyloric stenosis (IHPS), achalasia, intestinal pseudo-obstruction, and intussusception are considered here.

**67.3.4.1 Hereditary Pancreatitis.** OMIM (167800) lists 10 reports of familial occurrence under this heading. Four chromosomal regions, 1p36.2, 5q32, 7q31.2 and 7q35, have been identified to be associated with hereditary pancreatitis.

Mutations in the cationic trypsinogen gene *PRSS1* (OMIM 276000) and the gene *SPINK1* (OMIM 167790). This gene encodes a pancreatic secretory trypsin inhibitor. In addition, idiopathic pancreatitis has been found to be associated with mutations in the cystic fibrosis gene (*CFTR*, OMIM 602421).

Audrezet and colleagues analyzed the entire coding sequence and exon/intron junctions of the *PRSS1* (276000), *SPINK1* (167790), and *CFTR* (602421) genes



**FIGURE 67-4** Schematic structure of the Jagged-1 protein.

in 39 white French patients with idiopathic chronic pancreatitis (103). They found a missense mutation (R122H; MIM 276000.0001) in the *PRSS1* gene; 4 patients had the same missense mutation in the *SPINK1* gene, 3 as heterozygotes and 1 as a homozygote (N34S; MIM 167790.0001); and 8 patients carried mutations in the *CFTR* gene.

#### 67.3.4.2 Infantile Hypertrophic Pyloric Stenosis.

OMIM (179010) lists seven entries under the heading IHPS. Five types of IHPS can be distinguished genetically by susceptibility loci on five chromosomal regions: chromosome 12q (IPHS1, OMIM 610260), 16q13-p12 (IPHS2, OMIM 610260), 11q11-q22 (IPHS3, OMIM 612017), Xq23 (IPHS4, OMIM 300711), and 16q24 (IPHS5, OMIM 612525).

IHPS usually manifests at the age of 2–6 weeks of age with projectile vomiting, a palpable pyloric tumor, and gastric peristalsis. It is one of the first and best analyzed examples of multigenic (multifactorial) inheritance in man. From these studies reliable risk figures have been derived, based on the data obtained by the late Cedric O. Carter in the 1960s (104) and summarized by Carter in 1983 in the first edition of this book (105). IHPS is a relatively common component of partial trisomy 9 involving bands q22–31 (106). Only the nonsyndromic type, not associated with other disorders, is considered here.

The incidence of pyloric stenosis in populations of European extraction is about 1.5–3 per 1000 live births (range 0.5–4.5) with an unusual sex ratio of four to five males to one female. Thus, the birth frequency is about 1 in 200–300 males and 1 in 1000–2000 females. The population incidence appears to be lower today than a few decades ago, when the studies by Carter were carried out. In populations of Asian and African origin the incidence is less than in Caucasians, about 1:10,000 and 1:20,000, respectively.

Pyloric stenosis occurs in 2.4–18.9% of first-degree relatives of patients and in 0.5–4.3% of second-degree relatives (Table 67-4), in stark contrast to the population incidence of 0.1–0.5 %. Contrary to what one

might expect, the frequency of affected relatives of female patients is higher than that of male patients. This inverse relationship is also called the Carter effect. It is assumed to result from a threshold for liability to the disease (104–106). The threshold model of a polygenic (or better multigenic) genetic disease assumes that beyond a certain threshold the disease will manifest, below it, it will not. It is the total aggregate of several individually unknown genetic and nongenetic factors that determine that an individual is positioned relative to the threshold. At the population level the distribution resembles a normal Gaussian curve. The patients less frequently affected in the population, in the case of pyloric hypertrophy the females, have a greater genetic contribution than the more frequently affected males. Presumably genetic factors contribute to a lesser degree in males than in females.

According to this threshold model of liability to disease, the highest frequency is in male relatives of female patients (18.9%) and the lowest is in sisters and daughters of male patients (2.4–2.8%), as shown in Table 67-4. As seen in the section on Hirschsprung disease, genetic dissection of multigenic complex disorders aims to identify the individual genes involved.

The gene locus for IHPS type 1 (IPHS1) has been mapped to 12q24.2–24.31. This is based on an altered expression of the neuronal nitric oxide synthase gene (*NOS1*, OMIM 179010) reported in patients with this type of IHPS (107–110). For these studies the investigators used the AAT motif repeat in the first intron of the *NOS1* gene. In a recent study of 16 infants with IHPS and 9 controls, Saur et al. (109) found a significantly decreased expression of *NOS1* mRNA by reverse transcription polymerase chain reaction (RT-PCR). Mutations were found in exon 1c in 3 of the 16 patients. Furthermore, carriers of a single nucleotide polymorphism at position 84 in the exon 1c promoter regions (G84A; OMIM 163731.0001) had an increased risk of developing IHPS. Thus, genetic alterations in the *NOS1* exon 1c regulatory region would appear to influence the expression of this gene and contribute to the pathogenesis of IHPS (109). On the other hand, no linkage between IHPS and *NOS1* was revealed in a study of three Swedish families with multiple affected members (110).

**67.3.4.3 Achalasia.** OMIM (200400) lists five entries under achalasia, which includes several reports of familial occurrence of esophageal achalasia. However, it is doubtful that genetic factors play a role in the etiology in most patients. In a study of 1012 first-degree relatives of 167 patients with achalasia, no other patient has been found (111,112).

**67.3.4.4 Intestinal Pseudo-Obstruction.** Intestinal pseudo-obstruction (OMIM 243180) usually occurs as part of a generalized disorder affecting smooth muscles, autosomal recessive visceral neuropathy. Reports of familial occurrence are listed in OMIM 243180. An autosomal dominant form has been described (OMIM

**TABLE 67-4 Risk of Pyloric Stenosis in Families**

Affected Relatives (%)	Index Patient	
	Male	Female
First-degree		
Brothers	6.5	10.8
Sisters	2.8	3.8
Sons	5.5	18.9
Daughters	2.4	7.0
Second-degree		
Males	2.2	0.5
Females	4.3	1.7
General population	0.5	0.1

Data from Carter (104,105).

609629). Recurrent intestinal pseudo-obstruction may occur in myotonic dystrophy (OMIM 160900).

*Neuronal intestinal dysplasia* (NID; MIM 601223) due to congenital hypoplasia or aplasia of the sympathetic innervation of the intestine may result in intestinal pseudo-obstruction. Two forms of NID are distinguished, NID A and B. NID A is very rare. In NID B a few familial clusters suggest autosomal dominant inheritance.

*Chronic idiopathic intestinal pseudo-obstruction* (CIIP) is caused by a severe abnormality of GI motility, leading to signs of intestinal obstruction without any mechanical lesion (113). It can occur as a manifestation of one of several disorders such as Chagas disease, myxedema, or Duchenne muscular dystrophy. For one form, resulting from qualitative abnormalities of the enteric ganglia and nerve fibers, autosomal recessive inheritance has been suggested. OMIM 243180 lists three familial observations. In general, however, the genetic risk can be considered low.

Auricchio and colleagues described a family with apparent X-linked inheritance (113). They mapped a locus (*CIIPX*) to Xq28. The authors raised the question of whether *CIIPX* may represent an additional susceptibility locus in Hirschsprung disease.

Bott and colleagues described congenital idiopathic intestinal pseudo-obstruction-associated X-linked hydrocephalus with stenosis of the aqueduct of Sylvius (OMIM 307000) in an infant who lacked Cajal cells (114). The patient carried a mutation in the *L1CAM* gene (OMIM 308840.0016) in exon 22, which encodes the fibronectin type III domain of the L1CAM protein. Other mutations of *L1CAM* are involved in Hirschsprung disease, contributing to a quantitative defect in the migration of neural crest cells to distal segments of the gut. Intestinal pseudo-obstruction may occur as part of mitochondrial neurogastrointestinal encephalopathy (OMIM 603041).

**67.3.4.5 Intussusception.** Isolated intussusception (OMIM 147710) is rare, there being only one report of five affected members in three generations of a family. One girl without intussusception had malignant hyperthermia. The authors considered this association fortuitous. Intussusception is an important complication of the Peutz-Jeghers syndrome (OMIM 175200) due to jejunal polyps and in juvenile intestinal polyposis (OMIM 174900).

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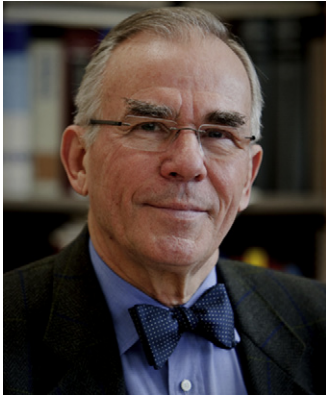
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## RELEVANT WEB PAGES

- EDN3 (endothelin 3); LocusID: 1908. LocusLink: <http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=1908>.
- EDN3 (endothelin 3); MIM number: 131242. OMIM: <http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispim?131242>.
- EDNRB (endothelin receptor type B); LocusID: 1910. LocusLink: <http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=1910>.
- EDNRB (endothelin receptor type B); MIM number: 131244. OMIM: <http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispim?131244>.
- GDNF (glial cell derived neurotrophic factor); LocusID: 2668. LocusLink: <http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=2668>.
- GDNF (glial cell derived neurotrophic factor); MIM number: 600837. OMIM: <http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispim?600837>.
- OMIM (Online Mendelian Inheritance in Man). <http://www.ncbi.nlm.nih.gov/Omim/>.
- RET (ret proto-oncogene (multiple endocrine neoplasia and medullary thyroid carcinoma 1, Hirschsprung disease)); LocusID: 5979. LocusLink: <http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=5979>.

### Biography



**Eberhard Passarge, MD**, was professor and Founding Chairman of the Department of Human Genetics, University of Essen, Germany, 1976 until mandatory retirement according to Federal University Law in 2001. He was Head of the Division of Cytogenetics and Clinical Genetics at the Department of Human Genetics, University of Hamburg, Germany 1968–1976. Following medical school 1955–1960 he graduated with an MD degree from the University of Freiburg, Germany.

He had postgraduate general medical training at the General Hospital Hamburg-Harburg, Germany, 1961–1962, and The Worcester Memorial Hospital, Worcester, Massachusetts, USA. He received further training in pediatrics and human genetics at the Children's Hospital Medical Center Cincinnati, Ohio, with Josef Warkany 1963–1966 and Cornell Medical Center New York with James German 1966–1968. Among his main interests are the scientific investigation of hereditary and congenital diseases and the application of this knowledge in genetic diagnosis and counseling. He is the author of about 240 articles in international peer-reviewed journals, author of chapters in several international textbooks and three books in human and medical genetics. His experience in teaching human genetics is reflected in his single-author book *Color Atlas of Genetics*, in an upcoming 4th edition. He was Secretary-General of the European Society of Human Genetics (1987–1990) and President of the German Society of Human Genetics (1990–1996), of which he became an honorary member in March 2011. He is a member of the American Society of Human Genetics, founding member of the European Society of Human Genetics, the Teratology Society, and the American Society for the Advancement of Science, corresponding member of the American College of Medical Genetics and other scientific societies. He is married to Mary Fetter Passarge, MD, a graduate of the University of Pennsylvania School of Medicine. Since February 2010 he is the Intermediary Chairman, Department of Human Genetics, University of Leipzig, Germany.

# CHAPTER

# 68

## Inflammatory Bowel Disease

Kent D Taylor, Huiying Yang and Jerome I Rotter

### ABBREVIATION

**ABCB1** – ATP-binding cassette, subfamily B (also known as MDR/TAP), member 1 (ABCB1, Gene Id #5243, OMIM 171050).  
**ANCA** – antineutrophil cytoplasmic antibody.  
**AS** – ankylosing spondylitis.  
**ASCA** – anti-*Saccharomyces cerevisiae* antibody.  
**CARD15** – caspase recruitment domain family, member 15 gene (Gene Id #64127)\*.  
**CD** – Crohn's disease.  
**cM** – centimorgan.  
**DLG5** – discs, large homolog 5 of *Drosophila* gene (Gene Id #9131, OMIM 604090)\*.  
**HLA** – human leukocyte antigen.  
**HLA-DRB1** – major histocompatibility complex, class II, DR beta 1 gene, (Gene Id #3123, OMIM 142857)\*.  
**HPS** – Hermansky–Pudlak syndrome.  
**IBD** – inflammatory bowel disease.  
**ICAM1** – intercellular adhesion molecule 1 (CD54), human rhinovirus receptor (Gene Id #3383, OMIM 147840).  
**IFNG** – interferon, gamma (Gene Id #3458, OMIM 147570)\*.  
**IL** – interleukin.  
**IL1RN** – interleukin 1 receptor antagonist (Gene Id #3557, OMIM 147679).  
**IPAA** – ileal pouch-anal anastomosis.  
**kB** – kilobase pairs (1000 base pairs).  
**LTA** – lymphotoxin alpha (TNF superfamily, member 1) (Gene Id #4049, OMIM 153440)\*.  
**LTB** – lymphotoxin beta (TNF superfamily, member 3) (Gene Id #4050, OMIM 600978)\*.  
**Mb** – megabase pairs (1 million base pairs).  
**MDR1** – see ABCB1.  
**MHC** – major histocompatibility complex, located at 6p21.3.  
**MLOD** – multipoint LOD score.  
**MTHFR** – 5,10-methylenetetrahydrofolate reductase (NADPH) (Gene Id #4524, OMIM 607093)\*.  
**NOD2** – same as CARD15. The mouse gene is named Nod2.  
**NPL** – nonparametric LOD score  
**OMIM** – online Mendelian Inheritance in Man. Online catalog of human genes and genetic disorders edited by Victor A. McKusick, available online from the National Center for Biotechnology Information, National

Institutes of Health, [www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=omim](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=omim).

**OR** – odds ratio.

**pANCA** – ANCA with perinuclear staining on indirect immunofluorescence.

**PSC** – primary sclerosing cholangitis.

**SCID** – severe combined immunodeficiency.

**SLC11A1** – solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1, also known as natural resistance-associated macrophage protein 1, (Gene Id #6556, OMIM 600266)\*.

**SLC22A4/A5** – solute carrier family 22 (organic cationic transporter), members 4 and 5, also known as OCTN1/2 (SLC22A4 Gene Id #6583, OMIM 604190; SLC22A5 Gene Id #6584, OMIM 603377).

**SNP** – single-nucleotide polymorphism.

**TCR** – T-cell receptor.

**TGF** – transforming growth factor.

**TNF** – tumor necrosis factor (TNF superfamily, member 2) (Gene Id #7124, OMIM 191160)\*.

**TNR** – trinucleotide repeat.

**UC** – ulcerative colitis.

**VNTR** – variable number of tandem repeats.

**95%CI** – 95% confidence interval of the odds ratio.

### 68.1 INTRODUCTION AND DISEASE DEFINITION

The inflammatory bowel diseases (IBDs) consist principally of ulcerative colitis (UC; OMIM#191390) and Crohn's disease (CD; OMIM#266600), two chronic idiopathic inflammatory diseases of the gastrointestinal tract. UC and CD are considered together because of their overlapping clinical, epidemiologic, and pathogenetic features, and shared complications and therapies. CD and UC also present distinct clinical, genetic, and pathologic characteristics as shown in [Table 68-1](#). These similarities and differences suggest that UC and CD may be part of a continuum of IBDs.

The common symptoms in UC and CD are diarrhea, abdominal pain, fever, and weight loss. UC is a chronic inflammation of the colonic and rectal mucosa, characterized by relapses and remissions of rectal bleeding. Inflammation in UC typically begins in the rectum and extends proximally in a continuous pattern without skipping



**TABLE 68-1 Diagnostic Criteria and Clinical Characteristics of Ulcerative Colitis (UC) and Crohn's Disease (CD)**

Method	Ulcerative Colitis	Crohn's Disease
Anatomic location (1)	Colon and rectum only	Any part of the alimentary tract with the most common form ileocolitis
Radiologic	Continuous distribution, fine granularity, fine superficial ulceration, fissures absent, strictures or fistulae rare fissures common, strictures or fistulae common	Discontinuous and segmented distribution, deep ulceration extending into submucosa (transmural), thickening of mucosal folds, luminal narrowing
Endoscopic (2)	Disease almost always involves the rectum and extends proximally for varying distances, inflammation diffuse and continuous, ulceration in inflamed mucosa, normal ileocecal valve, tracts, abscesses, strictures, "cobblestone" appearance	Rectum often spared, asymmetric and skip areas of inflammation, longitudinal ulceration, ileocecal involvement discrete ulcers in normal mucosa, fissures, sinus and fistulous
Histologic	Disease principally involves mucosa; mucosa irregularity, ulceration, increased chronic inflammatory cells in lamina propria. Goblet cell mucin depletion, and glandular dysplasia	Disease is transmural, granulomas, fissures, serositis, and fistulae, granulomas are key, but detectable in only 3% to 50% of CD cases
Extraintestinal (3–5)	Common extraintestinal manifestations include skin manifestations: lesions, arthropathies, ophthalmologic problems (6), disorders in hepatobiliary diseases occur in 21% of UC patients (7)	Frequency (24%) and type of common CD are similar as those in UC (8), thrombotic and thromboembolic vascular complications may occur in CD
Subgroupings	Proctitis, left-sided colitis, pancolitis	Based on anatomic location: ileocolonic, ileitis, jejunoileitis, colonic CD. Based on nature of disease: perforating, nonperforating

areas of the colon. Inflammation is superficial, involving the mucosal/submucosal layers (the inner lining of the gut). In contrast to UC, inflammation in CD may occur anywhere in the gastrointestinal tract, from the mouth to the anus, with both involved and normal segments (skipped) of the intestine observable histologically and endoscopically. The most common form of CD is ileocolitis, affecting both the small bowel and the colon (9). When only the small bowel is affected, CD is also known as *regional ileitis*. In contrast to the superficial inflammation in UC, inflammation in CD is transmural, involving all layers of the bowel wall, mucosa, submucosa, and muscular layer, and sometimes progresses through the bowel wall. The chronic transmural inflammatory nature of CD can result in granulomas, abscesses, fistulae, and perianal complications. There is no etiologic-specific treatment for either form of IBD although colectomy is a curative procedure for UC. Currently, therapy begins with supportive measures and anti-inflammatory agents (e.g. the sulfasalazines and corticosteroids), with the addition of immunosuppressive agents when severe disease does not respond. In our current era, IBD does not increase mortality by itself (10).

Traditionally, the diagnosis of CD and of UC has been based on clinical, radiologic, endoscopic, and histologic findings (reviewed in Table 68-1), but more recently, disease-specific antibodies have been explored for diagnosis (11). Serum antibodies to neutrophils staining with a perinuclear pattern (abbreviated pANCA) and serum anti-*Saccharomyces cerevisiae* antibodies (ASCA) are specific for the IBDs and symptomatic

patients with one of these serologic markers diagnoses UC or CD without further invasive testing although this is not yet general practice. However, because of their relatively low sensitivity, these markers cannot be used as screening tools. In the future, these and other serum antibodies may be useful for classifying individuals with indeterminate colitis. Other serum antibodies will be discussed later.

The main reason for lack of specific treatments for these diseases is our continued poor understanding of the etiology and pathogenesis of the various forms of IBD. On the basis of the current evidence, exogenous or infectious agents, including variation in gut microflora, damage mediated by the immune system, and underlying genetic factors combine to increase susceptibility to disease. Thus, genetic studies are essential for delineating etiologies underlying the continuum of the various forms of IBD. Understanding these etiologies promises to point to cellular and immune pathways that will allow the subsequent development of radically new and specific therapies for these disorders. This understanding will also eventually target those at high risk for disease prevention.

The remainder of this chapter reviews the supporting evidence for the genetic predisposition to various clinical groups of IBD and the progress in gene identification. This evidence includes ethnic differences in disease frequency, familial aggregation, an increased monozygotic (MZ) twin concordance rate compared with that in dizygotic (DZ) twins, the existence of genetic syndromes that feature IBD, loci for CD and UC identified by genome

scan and fine mapping, and finally, associations between various forms of IBD and specific genetic markers.

## 68.2 PHENOTYPIC HETEROGENEITY

Patients with UC or CD show great variability in their disease phenotypes for such characteristics as age of onset, rate of relapse, fibrostenosis, perforating/fistulizing, internal or perianal “penetrating disease” (fistulae, abscesses, or perforations), presence of diarrhea, bleeding or mucus discharge, duration of disease, location of disease (small bowel only, ileocolonic, colon only), number and types of surgery, development of chronic pouchitis after colectomy, and response to classes of medications such as corticosteroids, sulfasalazine or oral mesalamine, immunomodulatory agents (e.g. 6-mercapto-purine/azathioprine, methotrexate, cyclosporine), antibiotics, and topical therapies for distal colonic disease. Increasing evidence supports the clinical subgrouping of CD into perforating and nonperforating forms (12–14). Perforating CD is the more aggressive form, has a higher reoperation rate, and often results in abscesses and/or free perforation and fistulae. In contrast, the nonperforating form follows a more indolent clinical course and is associated with obstruction and bleeding (15). This clinical subgrouping has been further supported by cytokine mRNA profiles (16) and genetic marker studies (17–19). Some patients can be clearly classified as having CD based on histologic criteria yet have the UC-like features of left-sided colitis, more continuous and more shallow inflammation on endoscopy, and response to topical therapy (14).

These phenotypic differences may reflect underlying differences in immunologic processes. IL-1 beta and IL-1 receptor antagonist mRNAs are increased in resected intestinal tissue from patients with nonperforating CD (the more benign form of CD) when compared with perforating CD (16). CCR9-positive lymphocytes and CCR9 expressed in thymus distinguish small bowel from colonic CD (20). CD patients from families with multiple cases tend to have an early age at onset and more extensive disease as compared with those from families with no other cases (21). Within families with multiply affected cases of CD, common familial patterns have been observed in the disease aggressiveness, age of onset (22) and disease location (21,23). Such familial distribution of clinical features suggests that certain genetic or environmental factors shared by family members may determine the clinical course of the disease. Thus, it may be beneficial to study a clinically homogeneous group in order to understand the role of certain genetic or environmental factors.

Further observations support the concept that the serum expression of pANCA and ASCA reflect mucosal processes. For pANCA, B-cell clones taken from the mucosa of UC patients express pANCA (12), directly demonstrating pANCA in the intestinal mucosa. For ASCA, the specificity of the expression of antimannan

antibodies, rather than antigliadin or antiovalbumin in CD patients (24), of antibodies to *Saccharomyces* and not to *Candida* (25) and of the expression of ASCA in patients with CD, rather than with UC or other colitides, suggests that ASCA expression manifests a CD-related immune response rather than reflects a nonspecific intestinal insult resulting from the inflammation.

The expression of pANCA or ASCA has also been shown to extend the observed variability in IBD phenotype and provides further evidence for distinct subgroups within UC and CD. Within UC, the presence of pANCA has been associated with more severe forms of UC (treatment-resistant and left-sided UC (26), and the development of pouchitis following an ileal pouch-anal anastomosis (IPAA) procedure (27). Within CD, the presence of pANCA is associated with a more UC-like disease (more left-sided colitis, more distal, continuous, or shallow endoscopic appearance, more superficial histopathology) (14). Using pANCA as a phenotype, the authors have also provided evidence for heterogeneity within UC with family data (28) and this heterogeneity appears to have a genetic basis, as demonstrated by the use of HLA class II (29) and the intercellular adhesion molecule-1 molecular polymorphisms (see later, Candidate Genes) (30). High titers of ASCA in CD are associated with early age of disease onset as well as with both fibrostenosing and internal penetrating disease; in contrast, high ANCA levels are associated with a later age of onset and different disease location (13). These serum antibody observations are summarized along with other subclinical markers in Table 68-2.

Because increasing evidence points to the role of an aberrant innate immune response or T-cell response to enteric bacteria (see later), characterization of the serum expression of these antibodies may give clues to the specific microbial antigens involved in IBD pathogenesis. Western blots demonstrated that pANCA monoclonal antibody reacts to an unidentified *Bacteroides caccae* antigen and to an *Escherichia coli* outer membrane porin (OmpC) (54). Subtractive cloning, using tissue from CD patients taken from lesions and nearby uninvolved tissue, led to the identification of I2, a *Pseudomonas aeruginosa* sequence that acts as a potent superantigen for CD4<sup>+</sup> T-cell response (55,56). OmpC, I2, ASCA, and pANCA have all been shown to be expressed in the sera of CD patients with more severe disease, characterized by small bowel involvement, frequent disease progression, longer disease duration, and greater need for intestinal surgery, and expression of more than one antibody is associated with more severe disease (57,58). Serum expression screening has been used to identify specific bacterial antigens that drive IBD in the C3H/HeJBir mouse model of colitis. A lambda DNA expression library was first constructed from bacteria obtained from mouse ceca and then screened with sera from colitic mice. A large proportion of the positive clones from this study were sequences related to bacterial flagellin from *Butyrivibrio*,

**TABLE 68-2 Subclinical Markers for Inflammatory Bowel Disease**

Disease	Marker Studied	Type of Relatives	Observation	References
Combined IBD	Autoantibodies to epithelial-cell-associated component (ECAC) antigens	Family members	ECAC-C observed in 69.7% CD patients, 55.7% relatives, and only 8.0% control subjects	(31)
	Antinuclear autoantibodies (ANA)	Family members	ANA observed in 18% CD patients, 43% UC patients, 13% of relatives of CD patients, 24% of relatives of UC patients, 2% control subjects	(32)
	Goblet cell autoantibodies (GABs)	Family members	Positive GABs in 39% UC, 30% CD, 21% first-degree relatives of UC, 19% first-degree relatives of CD, 3% infectious enterocolitis, 2% healthy control subjects	(32)
	C-reactive protein (CRP)		High specificity to differentiate CD from other functional bowel disorders Higher levels associated with higher disease activity and need for colectomy	(33–35) (36)
Crohn's disease	C3 dysfunction	Family members	Greater C3 dysfunction in CD (38%) and relatives (18%) than in control subjects	(37)
	Intestinal permeability	Family members and twins	Increased in CD patients, conflicting results regarding relatives	(38–42)
	Obligate anaerobic fecal flora	Family members	The flora of CD patients contained more anaerobic gram-positive coccoid rods and more gram-negative rods than that of healthy subjects; during 5–7 years follow-up, three of nine children with CD with a CD floral pattern showed CD symptoms and one such child was diagnosed as CD. None of the 17 children with a normal flora showed CD symptoms	(43)
	Subclinical intestinal inflammation (fecal calprotectin)	Family members	Calprotectin in CD patients (47 mg/L) and relatives (11 mg/L) higher than controls (4 mg/L), but not spouses (4 mg/L)	(44)
	Anti- <i>Saccharomyces cerevisiae</i> antibodies (ASCA)	Family members and twins	ASCA increased in CD patients as compared with control subjects ASCA positive in 50% CD, increased in healthy relatives	(25,45,46). Also see text
	Pancreatic autoantibodies	Family members	Positive pancreatic autoantibodies in 27% CD (144% type I, 13% type II), subtype showed familial cluster, 0.5% in first-degree relatives	(47)
Ulcerative colitis	Antineutrophil cytoplasmic antibodies	Family members and twins	Specific for UC, increased in healthy relatives of UC patients, occur in a subset of CD patients	(48–51). Also see text
	Colonic mucins	Twins	HCM species IV (mucin subtype) reduced in both UC and healthy cotwins as compared with control subjects	(52)
	Mucosal production of IgG subclass	Twins	UC patients and their healthy MZ cotwins showed a raised proportion of IgG1 (78%) as compared with control subjects (56%)	(53)
	IgA against gliadin	Twins	High IgA titers against gliadin in both UC patients and their healthy cotwins	(24)

CD, Crohn's disease; HCM, human colonic mucin; IgA, immunoglobulin A; IgG, immunoglobulin G; MZ, monozygotic; UC, ulcerative colitis.

*Roseburia*, *Thermotoga*, and *Clostridium* species (27%). However, none of the sequences directly matched a flagellin already identified and in the GenBank database (59). The fact that bacterial flagellin is one of the ligands of the Toll-like receptor TLR5 supports the role of the innate immune system in the pathogenesis of IBD (see later) (60). The most reactive antigen, CBir1 flagellin, was also expressed in sera from human patients with CD independent of the expression of the others (59).

When taken together, these observations suggest that ASCA, pANCA, I2, OmpC, and CBir1 are serum markers for different mucosal inflammatory mechanisms that underlie distinct disease expression, and that genetic variation may underlie these mechanisms. Rather than a complete loss of tolerance to all microbial antigens, IBD is characterized by subsets of patients with different responses to specific microbial antigens (58,61). Use of clinical (fistulizing, perforating, fibrostenotic) and subclinical (ANCA,

**TABLE 68-3** Examples of Inflammatory Bowel Disease Incidence/Prevalence Rates (per 100,000) among Jewish and Non-Jewish Populations by Area and Time Period<sup>a</sup>

Area	Disease	Period	Jews	Non-Jews	Ratio <sup>b</sup>	Reference
<b>Incidence comparisons:</b>						
<i>North America</i>						
Baltimore, MD	IBD	1960–1963	16.7	5.4	3.1	(88)
	UC	1960–1963	13.3	3.4	3.9	
	CD	1960–1963	3.4	1.7	2.0	
New York	CD		12.6 <sup>c</sup>	5.4	2.3	(89)
<i>Scandinavia</i>						
Malmö, Sweden	CD	1965–1973	24.0 <sup>c</sup>	6.0	4.0	(90)
Stockholm, Sweden	CD	1960–1974	10.0	3.0	3.3	(91,92)
Western Cape, South Africa	CD	1970–1974	2.8	0.8	3.5	(93)
<b>Prevalence comparisons:</b>						
Edmonton, Canada	CD	1977–1981	309	143	2.2	(94)
	UC	1977–1981	143	76	1.9	
Southern Israel	CD	1990	30	3.2 <sup>d</sup>	9.4	(95)
	UC		89	9.8 <sup>d</sup>	9.1	

CD, Crohn's disease; IBD, inflammatory bowel disease; UC, ulcerative colitis.

<sup>a</sup>See also (Loftus, 2004a).

<sup>b</sup>Ratio of Jewish/Non-Jewish patients.

<sup>c</sup>As estimated in Reference (96).

<sup>d</sup>Israeli Arab.

ASCA) characteristics may be useful in subdividing patient populations into more etiologically homogeneous groups for genetic studies and thus be the means for unraveling the genetic heterogeneity of these diseases in the future. In addition to giving clues on the bacteria and the specific immune defects that lead to the pathogenesis of IBD, these antibodies hold future promise for diagnosis of subsets of IBD and thus for specific treatments.

### 68.3 RACIAL AND ETHNIC DIFFERENCES

Because the etiology of IBD is unknown, descriptive epidemiology plays a critical role in indicating the potential importance of genetic and environmental factors. However, owing to the difficulty of conducting population-based epidemiologic studies for these diseases, estimates of incidence and prevalence, especially in nonwhite populations, are imprecise. With the available data, the following can be generalized (62,63). Whites have a higher risk than nonwhite populations (64–66). There seems to be a north-to-south gradient in both North America and Europe (66–68), and IBD appears to be less common in developing countries. In North America and Europe, the most rapid increase of IBD occurred from 1960 to 1980 (63,66,69,70). For the white population in North America (Manitoba), the annual incidence rate of IBD (per 100,000) from 1989 to 1994 was 14.6 for CD and 14.3 for UC, and the prevalence (per 100,000) in 1994 was 198.5 for CD and 169.7 for UC (71). For a similar period (1983–1993), a somewhat lower incidence and prevalence was observed in Minnesota (incidence: 6.9 and 8.3 per 100,000 for CD and UC, respectively) (69,70,72). IBD risk in Europe is similar to that in North

America (67,73–77). A careful epidemiologic study of northern France has demonstrated that the incidence of CD has continued to increase in this region, rising from 5.2 per 100,000 from 1988 to 1990 to 6.4 per 100,000 from 1997 to 1999, and the incidence of UC has decreased from 4.2 to 3.5 per 100,000 in the same time periods (78).

In the United States, the white population has the highest risk among all ethnic groups, then blacks, followed by Mexican-Americans, and Asians (64,66,79,80). In the United States, blacks with CD have a higher incidence of associated arthritis and uveitis compared with whites, and Mexican-Americans with UC have a higher incidence of pANCA expression (100% for Mexican-Americans compared with 40% for blacks) (81).

Outside of North America and Europe, the incidence of CD has been increasing recently, for example, in Puerto Rico (from 0.49 to 2 per 100,000) (82), Taiwan (from 0.85 to 2.4 per 100,000) (83), and Hong Kong for both CD (from 0.3 to 1.0 per 100,000) (84). These increases suggest that an increase in Westernization may cause an increase in CD incidence (59).

Although the differences in IBD frequency among various ethnic groups can have both environmental and genetic explanations, important evidence for a genetic component to IBD susceptibility is the consistently increased incidence/prevalence in the Ashkenazi Jewish population compared with other ethnic groups in the same geographic location. The fact that the Jewish and non-Jewish differences occur across different time periods as well as different geographic areas (85–87) strongly suggests the existence of a genetic predisposition as the most parsimonious explanation (Table 68-3).



**TABLE 68-4 Positive Family History of Inflammatory Bowel Disease in Ulcerative Colitis (UC) and Crohn's Disease (CD) Probands<sup>a</sup>: An Example**

Disease in Probands	Number of Crohn's Probands <sup>b</sup>	Disease in Relatives			
		UC (%)	Disease (%)	Mixed <sup>c</sup> (%)	Total (%)
UC	269	37 (13.8)	6 (2.2)	4 (1.5)	47 (17.5)
Crohn's disease	258	19 (7.4)	32 (12.4)	9 (3.5)	60 (23.3)
Total	527	56 (10.6)	38 (7.2)	13 (2.5)	107 (20.3)

<sup>a</sup>See Reference (105).<sup>b</sup>Both Jews and non-Jews have the same trend.<sup>c</sup>With several affected relatives, some affected with UC and some with CD.

Further support for this hypothesis comes from studies of the historical origins of Jewish subgroups and their relation to IBD frequency (86,97–99).

## 68.4 FAMILIAL AGGREGATION

More than a dozen studies have demonstrated that familial aggregation is clearly increased in IBD although the data fit no simple Mendelian pattern of inheritance (30,76,87). Several studies have shown that there is an approximate 10- to 30-fold increase in disease prevalence among siblings compared with the communitywide prevalence (100–104). In addition to an increase in UC in relatives of UC patients and in CD among the relatives of CD patients, both UC and CD may exist in the same families, with an increased frequency higher than the co-occurrence by chance alone, suggesting an etiological relationship between UC and CD (Table 68-4) (105,106). These families may identify a specific subset of IBD (mixed IBD), which currently would be defined by family history (105). Positive family history is somewhat greater among CD patients than among UC patients (105–107), and the relatives of CD patients have a higher risk for IBD than those of UC patients (105,108). This suggests that CD is, to some degree, more often familial than UC and that CD has a greater genetic component (as will be seen later, this is supported by the twin data). The authors' family data represent the observations regarding familiarity well (Table 68-4; 105). In that study, the age of relatives was also taken into account to estimate the lifetime risks for disease. The lifetime risks for the relatives of non-Jewish patients were consistently lower than the corresponding risks for relatives of Jewish patients from the same geographic area (Table 68-5). This has implications for the mode of inheritance of genetic susceptibility (see later).

The serum expressions of ANCA and ASCA have also been observed to be familial traits. An increased frequency of ANCA expression has been observed in the clinically healthy relatives of UC patients compared with environmental controls (49,28). Second-degree relatives, who did not share the same household with the probands, had an increased prevalence of ANCAs, and

**TABLE 68-5 Empiric Risks for Inflammatory Bowel Disease (IBD) in First-Degree Relatives of Patients with Inflammatory Bowel Disease<sup>a</sup> (%)**

	Sibling	Parents	Offspring	Total
<b>Uncorrected empiric risks for relatives of IBD probands</b>				
<i>Jewish probands affected with</i>				
Crohn's disease	8.0	3.0	1.8	4.5
Ulcerative colitis	2.4	3.2	1.9	2.6
<i>Non-Jewish probands affected with</i>				
Crohn's disease	3.0	3.7	0	2.7
Ulcerative colitis	0.4	0.9	2.3	0.9
<b>Corrected empiric lifetime risks for relatives of IBD probands<sup>b</sup></b>				
<i>Jewish probands affected with</i>				
Crohn's disease	16.8	3.8	7.4	7.8
Ulcerative colitis	4.6	4.1	7.4	4.5
<i>Non-Jewish probands affected with</i>				
Crohn's disease	7.0	4.8	0	5.2
Ulcerative colitis	0.9	1.2	11.0	1.6

<sup>a</sup>See Reference (105).<sup>b</sup>Corrected for age of at-risk relatives, using age-specific incidence data.

the household controls were not at an increased risk for ANCA. These observations suggest that the familial aggregation of ANCAs is due to shared genetic factors among the family members, and not shared environmental factors. Furthermore, in these family studies, there was a significant difference in the frequency of ANCAs in the relatives of probands whose sera were ANCA(+) compared with the relatives of probands whose sera were ANCA(–). This concordant familial distribution indicates heterogeneity within UC. Thus, ANCAs may be used as a marker of an underlying immunologic disturbance that is genetically determined (50).

The authors have demonstrated that the level of serum ASCA expression is a quantitative trait that is also familial in both affected and unaffected relatives of CD patients using intraclass correlation analysis (109). A high percentage of CD patients (approximately half) and affected family members (also approximately half but with a lower level of expression) were seropositive for antimannan antibodies, compared with the normal control population (3.7%). Seropositivity or seronegativity was correlated

among all affected relatives and this association was stronger in affected first-degree relatives. Intraclass correlation revealed less variation in ASCA levels within, rather than between families, and a significant familial aggregation was observed. There was no significant correlation among marital pairs. These findings demonstrated that ASCA in family members affected and unaffected with CD is a familial trait for both affected and unaffected relatives. The lack of correlation in marital pairs suggested that this familial aggregation is due in part to a genetic factor or childhood environmental exposure.

## 68.5 TWIN AND SPOUSE STUDIES

Although it is possible for the familial aggregation described in the preceding section to be due to environmental factors alone, additional support for a major genetic component to IBD susceptibility is provided by increased MZ twin concordance rates, the rarity of IBD concordance in spouses, and the numerous instances of affected relatives completely separated geographically and temporally from other affected family members.

Even though twin data must be interpreted with caution, because concordant pairs are more likely to be reported than discordant pairs, several studies have shown an increased concordance rate of both CD and UC in MZ twins when compared with DZ twins (110,111). In order to circumvent this selection bias, population-based twin studies have also been conducted with the same higher concordance rate for MZ twins than that for DZ twins for both CD and UC (112–114). The concordance rate for CD is also higher than for UC (for example, the Swedish population-based study observed an MZ twin concordance of 58% for CD and 18% for UC). The higher concordance rate in MZ twins than in DZ twins supports the hypotheses that genetic factors are an important component in the development of IBD and that genetic factors account for much of the familial aggregation. The observation that <100% of MZ twins are concordant indicates that there is reduced penetrance for the IBD genotype, presumably due to nongenetic factors. These nongenetic factors may be environmental or they may be due to the random stochastic variation, for example, in the development of the B cells and T cells of the immune system (115,116). The observation that the risk to DZ twins is not higher than the risk to siblings argues that the environmental factors are population-wide in their effect.

From the limited number of family studies investigating the risk in spouses, it seems that the incidence of reported spouse concordance does not appear to be increased over population risks, and is dramatically less than the risk to siblings (30,102,111). This observation argues against any rapid-acting environmental agents; there are, however, case reports of husband–wife couples that both developed IBD after marriage (see also below, Multilocus Model, and Gene and Environmental Interactions) (117).

Therefore, these genetic epidemiologic studies of differences in IBD in various populations, of familial aggregation, and of twins and spouses of the affected have contributed to the basic concept that there is a major genetic contribution to susceptibility to IBD or diseases.

## 68.6 INFERENCES REGARDING MODE OF INHERITANCE

Although there is a strong familial aggregation of IBD and a major genetic component contributes to such familiarity, there is no one simple genetic model that can explain the mode of inheritance of this disorder. The following observations must be considered when exploring potential genetic models (118). From the MZ twin data, these are diseases with reduced penetrance, that is, genetic susceptibility does not appear to be the only determinant of disease (119). The fact that DZ twins have a risk similar to that of siblings suggest that it is macro- (over a wide area), not micro- (within an individual household) environmental factors that determine the risk to IBD (120). CD and UC are clearly genetically related, because they are (a) found together with increased frequency in families, (b) both increased in the Jewish population compared with the surrounding white population, and (c) distributed in the same countries of origin for US. Ashkenazi Jewish patients with IBD (62,121). On the basis of the stronger family history of IBD and greater MZ twin concordance for CD, genetic determinants appear to play a more deterministic role in CD than in UC. This does not mean that UC is any less genetic but may be more influenced by stochastic variation (122). There is now evidence for the involvement of specific loci and genes (Section 68.10) (123). Genetically modified animal models suggest that different genes can cause the same clinical phenotype (124). Animal models also indicate the importance of gene and environmental interactions (125). These observations and their implications for the mode of inheritance are summarized in Table 68-6.

### 68.6.1 Simple Mendelian Model

Segregation analysis of the aggregation of disease within families demonstrates that IBD is not inherited in any simple Mendelian mode of inheritance (105,126). Using a computerized genetic analytic technique termed *complex segregation analysis*, a recessive gene with incomplete penetrance was proposed for CD (127,128), and an additive major gene or a dominant major gene was proposed for UC (128,129). However, the conclusion of a major gene effect from complex segregation analysis is not equivalent to the conclusion of a major gene with simple Mendelian inheritance. Although these analyses argue strongly against the multifactorial/polygenic model (discussed later), they cannot distinguish

between simple Mendelian models and models with several major genes interacting (also known as multilocus or oligogenic model, see later), or models of genetic heterogeneity (i.e. several genetically distinct diseases with a similar phenotype). Nevertheless, keeping the genetic heterogeneity of IBD in mind (see later), the possibility of simple Mendelian susceptibility may be true for a subset of IBD. If so, even though these forms are rarer, the collection of families showing Mendelian segregation and subsequent locus and then gene identification within these families may be fruitful in showing biochemical or immunologic pathways relevant to the non-Mendelian and more frequent forms of IBD. This line of inquiry proved very fruitful for uncovering the roles of the low-density lipoprotein (LDL) receptor in cholesterol metabolism (130) and of ion transporters in hypertension (131).

**TABLE 68-6 Genetic Inferences Based on Evidence of Family Aggregation in Inflammatory Bowel Disease (IBD)**

1.	IBD exhibits familial aggregation	Increased risk factors within families
2.	(a) Monozygotic twin concordance > dizygotic twin concordance (b) No increase in spouses	Genetic factors responsible for familial aggregation
3.	(a) Positive family history in CD > UC  (b) Twin concordance of CD > UC	CD is more genetic or more deterministic; UC may be more stochastic and thus more immunologic
4.	Both CD and UC occur in the same families	Some genetic aspects of CD and UC are shared
5.	Very few "mixed" MZ twin pairs	Some genetic aspects of CD and UC are distinct
6.	Risks are much greater in families than in the population	Genetic susceptibility is not polygenic
7.	Familial risks in Jews > familial risks in non-Jews in neighboring area	Genetic susceptibility is not simple Mendelian
8.	Non-Jewish families have proportionally more "mixed" IBD (CD and UC) than Jewish families	"Mixed IBD" is not a simple overlap, but possibly a different disease, thus evidence for genetic heterogeneity
9.	Clinical features concordant in CD families	Genetic heterogeneity
10.	Increased risks to offspring when both parents have IBD	Limited number of disease genes
11.	Dizygotic twin concordance = sibling risk	Environmental factors are probably not micro-environmental but macroenvironmental

CD, Crohn's disease; MZ, monozygotic; UC, ulcerative colitis.

## 68.6.2 Multifactorial/Polygenic Model

In order to explain familial aggregation that does not follow a Mendelian pattern of inheritance, the polygenic model proposes that many genes, each with a small contribution to the phenotype, together provide the susceptibility to disease when the individual crosses a certain threshold. This model is termed multifactorial when these many genes must also interact with environmental factors. A polygenic model has been proposed to explain the association between CD and UC and to explain why the relatives of people with CD are much more likely to have IBD than the relatives of people with UC. The features of this model are the following: (1) a single genotype, comprising 10 or 15 genes, makes individuals susceptible to IBD (107); (2) if a person has only a few of these genes, they are more susceptible to UC, whereas if they have most of these genes (a more complete genotype), they are more susceptible to CD. Relatives of those patients with most of the genes (CD patients) would also be more likely to have a larger number of IBD susceptibility genes than the relatives of patients with only a moderate number of the risk genes (UC patients). Thus, CD patients would be more likely to have relatives with UC, whereas UC patients would be more likely to have relatives with too few of the high-risk genes to have any form of IBD.

However, the formal mathematical genetic analyses of large family data sets of both UC (128,129) and CD (127,128) have allowed the rejection of the polygenic model. Basically, the risk of relatives is too great to be explained by this model. In addition, genetically modified animal models (including knockouts) suggest that if one gene in the immunoregulatory pathway is sufficiently altered, it can lead to clinical disease; thus, multiple genes with equal but small effects may not be required.

## 68.6.3 Multilocus (Oligogenic) Model

There is increasing evidence that the genetic predisposition to a number of diseases is due to the interaction of two or more major genes, a form of inheritance termed two locus (if two major genes are involved), or multilocus or oligogenic (if more than two are involved) (132–138). This model is important for IBD for several reasons. First, it is etiologically attractive because it is able to explain the occurrence of more than one pathophysiologic defect in IBD patients. For example, in order to develop clinical CD, one may need both a permeability defect that leads to increased exposure of the body's immune system to antigenic substances that ordinarily do not cross the gut mucosal barrier and a particular genetically determined immune response. An analogous hypothesis might include abnormal mucins and certain autoantibodies as the etiologic factors in UC. Second, the multilocus model is able to explain the disparity between IBD recurrence risk estimates and typical

risk estimates for Mendelian disorders (134–136,138). Third, the multilocus model is able to explain the relationship of UC and CD in families. For example, one gene may be insufficient by itself to lead to clinical disease yet predisposes to both diseases, but clinical disease occurs only when a second more specific gene interacts with the first and leads to the specific disease, either UC or CD. Fourth and finally, the multilocus model makes genetic counseling and risk identification in relatives feasible once the first susceptibility locus is identified, as has been demonstrated for other autoimmune diseases (Section 68.10) (136).

In one intriguing study, the risk of IBD in offspring when both parents have IBD is (1) more than twice the empiric risk to offspring of couples when one parent has IBD, (2) similar to the empiric risk for identical twins, and (3) is higher for CD than for UC (139). The risk to offspring was similar whether both parents, one parent, or neither parent had developed symptoms of IBD at the time of the conception. Furthermore, the concordance rates for the type of IBD (UC or CD) in these couples were similar whether both experienced the onset of IBD before marriage, one before and one after, or both after marriage. These observations suggest that genetic and not acquired factors are essential in the development of IBD and in the determination of the type of IBD, whether UC or CD. Furthermore, although strong environmental determinants limited to certain families could be an explanation, these data support the concepts of a limited number of genetically determined forms of IBD, of a limited number of IBD-predisposing genes, and of the multilocus/oligogenic model for IBD susceptibility. If many different genes were required or if there were many different genetic forms of IBD, then genetic complementation would reduce the risk to offspring. However, if there are a limited number of such genes, then the offspring of two affected parents are more likely to be homozygous at the loci for such genes and consequently affected.

The number of genomic regions thus far identified using genome screen methods and the identification of several candidate genes further support the multilocus/oligogenic model (see later).

#### 68.6.4 Genetic Heterogeneity Model

The heterogeneity model proposes that IBD is not a single disease but rather several diseases with different etiologies but presenting the same clinical picture. Advantages of this model are that, first, the identification of clinical subgroups leads to understanding separate etiologies corresponding to each of the specific subtypes of IBD and, thus, unravels an otherwise bewildering array of phenotype/genotype relationships, and second, genetic heterogeneity leads to testable etiologic hypotheses. The following observations support the idea of the genetic heterogeneity of IBD. (1) IBD is phenotypically

heterogeneous with emerging evidence on clinical subtypes (see earlier). (2) IBD is a feature of several different genetic syndromes (see later). (3) Animal models engineered by targeted gene disruption (knockout mice) illustrate that there are many pathophysiologic pathways to the development of IBD (140–142,125); (4) The different relative frequencies of mixed disease (CD and UC) in the same family in Jewish versus non-Jewish families argue that the mixed form may be a distinct subgroup (105).

Genetic heterogeneity leads to the predictions that genetically determined physiologic abnormalities in the affected case are likely to be shared with their relatives and that these would be similar within the same clinical subgroup. In this way, further support for the genetic heterogeneity of IBD comes from (5) the genetic serum expression of pANCA or of ASCA define clinical subtypes of disease and are also present in the unaffected members of the family of affected (see later), and (6) the association of specific genetic markers with clinical subtypes within CD and UC associations also suggest heterogeneity within CD and within UC (see later).

Therefore, the available evidence support the concepts that IBD is a genetically heterogeneous group of disorders, with each subform an oligogenic disorder due primarily to the interaction of a limited number of genes, although there may be more minor contributions from modifying genes.

### 68.7 ASSOCIATION OF IBD WITH RARE GENETIC SYNDROMES

IBD is clearly associated with three well-defined genetic syndromes: Turner's syndrome (143), Hermansky–Pudlak syndrome (HPS) (OMIM #203300) (144–147), and glycogen storage disease type 1b (GSD1b) (OMIM #232220, Chr 11q23) (148,149). In addition, IBD has been less clearly associated with several immunodeficiency syndromes (Table 68-7).

**TABLE 68-7 Genetic Syndromes Associated with Inflammatory Bowel Disease**

Turner's syndrome
Hermansky–Pudlak syndrome
Glycogen storage disease type 1b
Sickle cell disease
Trisomy 9
Immunodeficiency disorders
Secretory immunoglobulin deficiency
Agammaglobulinemia/hypogammaglobulinemia
Selective IgA deficiency
Chediak–Higashi syndrome
Complement 2 (C2) deficiency
Hereditary angioedema
Pachydermoperiostosis

IgA, immunoglobulin A.



### 68.7.1 Turner's Syndrome

Several reports of patients with Turner's syndrome (Chapter 44), that is, individuals who lack all or part of one X chromosome, leave little doubt that the incidence of IBD is many times higher than that seen in the general population (85,150,151). This association may be due to the abnormal state of a single X chromosome; IBD has also been linked to the X chromosome in genome scans (152,153). Alternatively, patients with Turner's syndrome are known to be at increased risk of several diseases thought to involve autoimmunity (150). It may not be too surprising that IBD, also a potential autoimmune disease, is increased in these patients. CD and UC have been reported in about equal numbers in patients with Turner's syndrome.

### 68.7.2 Hermansky–Pudlak Syndrome

HPS (Chapter 145) is a tyrosinase-positive form of oculocutaneous albinism with a lysosomal storage defect leading to accumulation of ceroid lipofuscin and with a platelet aggregation defect leading to a bleeding diathesis (147). The pattern of inheritance is autosomal recessive; many genes have now been identified, showing genetic heterogeneity in this disorder and multiple mutations have been observed with different subphenotypes, showing locus heterogeneity (154,155). HPS1 is rare except in the Puerto Rican population (where it is the most common genetic disorder with an incidence of 1:1800) and an isolated village in the Swiss Alps. The *HPS1* gene (OMIM \*203300, Chr 10q23.1) was identified by linkage and positional cloning of families from these populations and is the homolog of the mouse "pale ear" gene (156–158). Different mutations at the HPS1 locus have been shown to be associated with IBD (159).

HPS2 (OMIM #608233, Chr 5q14.1) differs from the other forms of HPS in that patients are immune deficient and susceptible to infections. HPS2 mutations are located in the beta-3A subunit of the adaptor protein complex 3 (AP3); AP3 forms transport vesicles for sorting protein traffic to lysosomes, melanosomes, and platelet granules (AP3B1, OMIM \*603401) (160). The identification of the altered protein in HPS2 is, therefore, consistent with the pathophysiology of HPS and focuses further investigation on protein trafficking pathways for this disorder. Alterations in protein trafficking have also been observed to affect NK T-cell development (161) and antigen presentation because endocytosed microbes are delivered to lysosomes for binding to MHC and CD1 molecules (162).

Proteins HPS1 and HPS4 (OMIM \*606682, Chr 22q11.2-12.2) (163) form a complex that is involved in the biogenesis of lysosome-related organelles (BLOC-3, biogenesis of lysosome-related organelles complex 3) (164,165). The role of HPS2 in cellular trafficking suggests that BLOC-3 is involved in a perhaps earlier step

in sorting of proteins to organelles (166). HPS3 (OMIM \*606118, Chr 3q24; syntenic with murine *coa*), HPS5 (OMIM \*607521, Chr 11p15-p13), and HPS6 (OMIM \*607522, Chr 10q24.32) form complex BLOC-2 (167,168) and HPS3 is involved in the early stages of melanosome biogenesis and maturation (169,170).

Interstitial pulmonary fibrosis with pulmonary insufficiency has been reported as the most frequent and serious complication of HPS1 (147,166,171). In addition, a form of granulomatous colitis was reported in 5/9 HPS1 albinos in two Puerto Rican families living in New York and in 12/37 patients living in Puerto Rico (65,147). HPS and granulomatous colitis are rare in non-Puerto Ricans (144). The clinical diagnosis in these colitis patients was UC, but the resected colons showed long linear ulcerations and microscopically focal mucosal non-necrotizing granulomas similar to CD but with variable amounts of brown granular pigment (65). In general, colitis has been refractory to medical treatment and appears to require surgical resection (147). No defect in peripheral blood lymphocyte or neutrophil function was identified (146).

### 68.7.3 Glycogen Storage Disease Type Ib

GSDIb (OMIM #232220) is an inherited metabolic disorder caused by a defect in the glucose-6-phosphate transporter (G6PT1; OMIM #602671). GSDIb contrasts with GSDIa (OMIM +232200), caused by a deficiency in the glucose-6-phosphatase (G6Pase) activity (Chapter 93). GSDIb is distinguished clinically from classic GSDIa by a predisposition to recurrent infections due to neutropenia and neutrophil dysfunction (123,172,173). In a study of 36 GSDIb patients from North America, 75% of subjects had some gastrointestinal symptoms and 28% had documented CD, and an additional 22% had symptoms highly suggestive of CD but had not been diagnosed (174). CD is, therefore, associated with GSDIb, implicating neutrophil abnormalities in the pathogenesis of IBD (148,175–177). Neutrophils in GSDIa also show increased apoptosis (178). Long-term treatment with colony-stimulating factors (CSF2, OMIM \*1389600, alias granulocyte–macrophage colony-stimulating factor, GM-CSF, and CSF3, OMIM \*138970, alias granulocyte colony-stimulating factor, G-CSF) normalizes neutrophil count (179). Treatment with CSFs heals oral and intestinal mucosal lesions from CD in GSDIb (149,180). CSF3 is also in trial for treatment of non-GSDIb CD as an alternative to immunosuppression (181–183).

### 68.7.4 Immunodeficiency Syndromes

The role of neutrophils in CD pathogenesis is further supported by the observations of CD-like lesions in neutrophil or bone marrow stem cell disorders: chronic granulomatous disease (124,184,185), congenital neutropenia (186), autoimmune neutropenia (187), leukocyte adhesion deficiency (188), and

myelodysplastic syndromes (Table 68-7) (189–191,588). A very interesting observation is that gut inflammation disappeared after stem cell transplantation for myeloid leukemia and myelodysplastic syndrome; this alleviation of inflammation persisted after immunosuppression was discontinued (192).

## 68.8 ASSOCIATIONS WITH OTHER DISEASES

The association of IBD with many immune-related disorders with unknown etiology is well documented; these disorders include ankylosing spondylitis (AS), psoriasis, primary sclerosing cholangitis (PSC), and pouchitis. IBD may also be associated with autoimmune disease in general.

### 68.8.1 Ankylosing Spondylitis

In UC patients, the frequency of AS (OMIM #106300) ranges from 1% to 6%, and of arthritis from 2% to 15% (193). In CD patients, the frequency of all arthropathies ranges from 5% to 10% (193–195). The frequency of the MHC allele HLA-B27 is greatly increased in AS patients (90% compared with a frequency of 10% in the general population) (196–202). In the HLA-B27 transgenic rat model of IBD, gut bacteria have been associated with both gut inflammation and arthropathy (203). Thus, an increase in IBD risk is associated with factors that also increase the risk for AS, particularly HLA-B27, suggesting that IBD is a potent initiating or attenuating factor in the development of AS (204). It is still not certain whether AS should be considered a complication of IBD or an associated disease. Bowel permeability is altered in AS patients and their relatives (205), and bowel disease may be present with AS even when it is not manifested clinically (206–208). Patients with non-AS spondyloarthropathy and inflammatory gut lesions have a greater risk of developing AS than patients without gut inflammation (209–211). Furthermore, relatives of IBD patients have an increased risk for AS even when the IBD patients themselves have no evidence of AS (212).

### 68.8.2 Psoriasis

Psoriasis (OMIM #177900) is a chronic inflammatory dermatosis that affects 2% of the population in North America. Psoriasis is increased in CD patients (7–11% compared with 1.1–1.6% in the general population), as well as in the relatives of CD patients (213–215). The concurrence of psoriasis and CD at both the individual level and family level suggests the possibility of a genetic link between the two disorders. Genomewide scans for psoriasis have identified many susceptibility loci; 6p21.3 (HLA or major histocompatibility region, MHC) and 17q25 are well established, and there is some linkage

evidence for loci at 1p, 3q21, 4q31–q34, 17q, and 19p13 (216–222). These data are consistent with the hypothesis that some genetic determinants are common for both disorders (see later).

### 68.8.3 Primary Sclerosing Cholangitis

PSC (OMIM #260480) is familial (223), and the association between PSC and UC has been recognized for several decades (224–227). The prevalence of PSC among UC patients is approximately 5% overall and 10–15% among UC patients with hepatic abnormalities (225,228,229). The association is stronger in reverse, with approximately 50–70% of all PSC cases affected with UC. This may be an underestimate, because IBD onset may have a substantial subclinical phase of IBD far longer than previously appreciated (230). There is no difference in PSC-related survival among patients with or without IBD (231). Cigarette smoking and appendectomy increase the risk for all extraintestinal manifestations in UC, including PSC (232). The following studies have demonstrated similarities in the immune response between these two disorders. First, a higher proportion of patients with PSC and UC have antibodies to colon antigens and to portal tract antigens of the liver (47,224). Second, a colonic epithelial protein has been identified with unique epitopes that are shared by the skin and biliary tract epithelial cells (233). Third, both UC and PSC express ANCA (49,234–237). If there is a common antigenic target for immune-mediated attack on both colonic and biliary epithelial cells, identification of this antigen may facilitate the understanding of the basic immunoregulatory disorder underlying both diseases and may foster development of an improved assay for a marker of both diseases. And fourth, both PSC and UC share HLA class II genetic risk factors. PSC risk factors include HLA-DR2 serotype and the HLA haplotype A1-B8-DR3 (238–241), and UC risk factors include HLA-DR2 (see later) and HLA-DR3 in a population in which HLA DRB1\*1502 (serologically DR2) is rare (242).

### 68.8.4 Pouchitis

Restorative proctocolectomy with ileal reservoir is a widely accepted procedure in the surgical treatment of UC (243). However, the most frequent long-term complication after IPAA for UC is a nonspecific inflammation of the ileal reservoir known as *pouchitis* (244). This complication seems to be disease related and not operation related, because the occurrence of pouchitis in patients after IPAA for familial adenomatous polyposis is significantly lower than that in patients after IPAA for UC (245). The cumulative risk of developing pouchitis varies between 15% and 46%, depending on the duration of follow-up (246). The etiology and pathophysiology of pouchitis are not well understood, but many of the risk factors are similar to those for UC. Pouchitis has

also been associated with the presence of extraintestinal manifestations of the UC, including PSC (247,248), of pANCA (27,249), and of the protective effect of smoking (250). Therefore, it appears that pouchitis reflects the same underlying etiology and pathophysiology of UC, indicating the importance of host factors, such as genetic susceptibility and immunologic functions.

### 68.8.5 Multiple Sclerosis

The concurrence of multiple sclerosis (MS; OMIM #126200) with IBD has been observed both within families (251,252) and within individuals (253–256). Of interest, a study of intestinal permeability in MS patients found that a fourth of patients with MS also had increased intestinal permeability (257).

One explanation for the association of IBD with other diseases is that the genetic component is a generalized defect of the immune system, which then may predispose the patient to several different autoimmune diseases or conditions. This hypothesis is supported by (1) the increased frequency of classic organ-specific autoimmune disorders observed in a large series of UC patients (e.g. autoimmune thyroid disease, insulin-dependent diabetes, and systemic lupus erythematosus) but not in CD patients (258–261); (2) the concurrence of UC and celiac disease (262,263); and (3) genome scans for several autoimmune disorders has shown that susceptibility to many autoimmune diseases share genomic regions, suggesting common susceptibility genes (264).

The hypothesis further suggests that the etiology of the complications of IBD affecting only a portion of IBD patients may also share this generalized autoimmune disorder. This would be an example of genetic pleiotropy, one gene altering many phenotypes. These complications of IBD include uveitis, erythema nodosum, pyoderma gangrenosum, hepatitis, pancreatitis, and cirrhosis (107). In a large family with members affected with different autoimmune disorders, including rheumatoid arthritis, systemic lupus erythematosus, psoriasis, and IBD, it appears that family members with the same disease tend to share the same major histocompatibility complex (MHC) HLA haplotypes (265). Variation of the MHC class I polypeptide-related sequence A gene (*MICA*; GeneID 4276), also located within the MHC, has been associated with UC and with peripheral arthropathy (266). These observations suggest that one set of genes may determine the general autoimmunity of a patient, whereas other genes govern the specific constellation of phenotypes.

## 68.9 GENE AND ENVIRONMENTAL INTERACTIONS

The family and twin studies discussed earlier and the genetic linkage and association studies reviewed later are evidence that genetic factors play an essential role

in the development of IBD. However, the following observations argue for an additional environmental contribution to human IBD: (1) disease incidence has exhibited temporal trends and is correlated with industrialization or “Westernization”; (2) there is much less than a 100% concordance rate in MZ twins; (3) different disease risks have been observed for the same ethnic group residing in different geographic locations; and (4) mouse models of IBD have demonstrated that intestinal bacteria are necessary for disease development. Therefore, it is likely that both gene and environmental factors determine the risk of developing IBD, and IBD is, thus, a complex genetic trait.

Although the relationship of environment to IBD is complex, epidemiologic studies have identified several environmental factors that increase susceptibility to IBD.

### 68.9.1 Smoking

Smoking appears to have a protective effect for UC (267), with UC patients less likely to be smokers (OR=0.17) (52,241,268–271). Smoking may reduce overall symptoms (272,273), the need for surgery (274), and pouchitis (250). In contrast, smoking is associated with CD (275). A large meta-analysis across seven studies showed that smoking is associated with a greater susceptibility to CD (pooled odds ratio [OR]=2.0, 95% confidence interval [CI] 1.65–2.47, for current smokers; OR=1.8, 95% CI 1.3–2.5 for previous smokers) (269). CD smokers showed a higher recurrence of repeat surgery through a 10-year follow-up period, particularly for women (OR=4.2 for women; OR=1.5 in men) (276), and this risk of recurrence is reduced when CD patients stop smoking (277). Interestingly, one study demonstrated that CD patients do not know about the effect of smoking on their disease (278). Smoking may interact with the IBD4 locus (see linkage results later) (279). Randomized placebo-controlled trials of transdermal nicotine in UC patients have demonstrated some clinical improvement but not disease remission (246,280).

### 68.9.2 Westernization

As noted above, IBD appears to rise as a country becomes “Westernized” or industrialized (63). For example, a negative correlation between infant mortality or household hygiene and the incidence of CD has been demonstrated (73,281,282). Many crucial changes occur in food, housing, transport, leisure, and clothing during this process, so that identification of the important exposure may be impossible. One recent hypothesis is that cooling of food during distribution and home refrigerators accounts for the rise in CD and is based on the following observations: (1) increases in incidence parallel public acquisition of refrigerators in the United States, Sweden, United Kingdom, and Southern Europe; (2) pathogenic psychotropic bacteria exist that are capable of growth within

refrigerators, particularly *Yersinia enterocolitica*; (3) CD lesions occur in regions of high lymphoid follicles, suggesting the role of specific infection; (4) CARD15 mutations (see later) affect phagocytic cell lines, including monocytes, targets of *Yersinia* infection (283).

### 68.9.3 Appendectomy

A meta-analysis of 17 case control studies describing 3600 cases and 4600 controls showed that appendectomy was associated with a 69% reduction in subsequent risk of developing UC (284). A large Australian study observed that appendectomy was associated with more extensive UC but with a reduction in the requirement for immunosuppression or colectomy for the treatment of colitis (285).

### 68.9.4 Intestinal Bacteria

The study of animal models of IBD has given clear evidence that gut bacteria are necessary for intestinal inflammation and that inflammation results from an aberrant activation of the immune system driven by the gut lumen bacteria (141,142,279,286,287). Inflammation develops when these various animals are raised in normal and in pathogen-free (SPF) conditions and does not develop when the animals are raised in very strict germ-free (GF) conditions (288). For example, inflammation does not develop with the IL10 knockout (ko) and TCRalpha ko mouse models grown in GF conditions (289,290); recolonization with *Bacteroides vulgatus* induces inflammation in both models and *Helicobacter hepaticus* alone induces inflammation in the IL10-/- (IL10 knockout) model (these bacteria are murine pathogens) (291,292). An *H. hepaticus* flagellar hook protein has been identified as one of the specific antigens that induces colitis in the IL10-/- model (293). *H. hepaticus* alone induces inflammation in many other animal models and is thus under active investigation by many groups (294,295). These knockout models also demonstrate that variation in many different genes leads to intestinal inflammation and show the greatest promise for investigating the relationships between cytokine genes and intestinal bacteria as well as the steps that lead to an imbalance between TH1 and TH2 cells in the intestinal immune system (141,287). Another mouse model is adoptive transfer: mice homozygous for the SCID mutation develop IBD when isolated CD4<sup>+</sup> T cells from another donor strain expressing high levels of CD45RB<sup>high</sup> antigen on their surface are transferred. Intestinal bacteria in the recipient mouse are also required for intestinal inflammation. This adoptive transfer model has demonstrated that both intestinal inflammation promoting and inhibiting cells are present in the intestinal immune system, and shows the greatest promise in dissecting the events in T-cell differentiation that lead to inflammation, particularly the relationship between the intestinal immune system

and the thymus (296). In one rat transgenic model, the human HLA-B27 and beta-2 microglobulin genes have been introduced to replace the rat ortholog. Animals develop multiorgan disease that resembles the human spondyloarthropathies that accompany IBD in some individuals, colitis, arthritis, orchitis, and psoriasiform changes of the skin and nails (297,298); both colitis and arthritis do not occur when B27 transgenic rats are raised under GF conditions, implicating the normal enteric bacterial flora as the source of the antigens driving these diseases (299). *E. coli* and *Enterococcus* spp. are correlated with the amount of inflammation (300). This model has clearly demonstrated the role of the MHC class I antigen presentation system and shows the greatest promise to elucidate how presentation induces both intestinal and extraintestinal inflammation. New and unexpected ways to treat IBD should emerge from the intensive study of all of these types of models (287).

The studies examined thus far clearly indicate the role of both genetic and environmental factors in the etiology of IBD. Clearly identifying the specifics of these risk factors will depend on the relative distribution of the particular factor. For example, in western societies, a common environment may have brought the majority of people to an equal footing in terms of good hygiene, better nutrition, and improved maternal and pediatric care. In this case, when the environmental factors are common to all within the society, those individuals with the IBD susceptibility genes will be more likely to develop the disease as compared with those who do not have such genes. In this case, identifying the IBD susceptibility genes is possible, whereas identifying the IBD susceptibility environmental factors will be difficult. In contrast, the situation in which the environmental factors are not so common, as in developing countries that are experiencing a rising incidence of IBD, offers a window of opportunity for finding the specific environmental factors that contribute to IBD. Finding the environmental factors will be easier as the IBD susceptibility genes are identified. Therefore, well-designed genetic epidemiologic studies in developing countries are warranted as the incidence of IBD increases; these studies will have benefit for North America in addition to that particular developing country.

### 68.9.5 An Evolutionary Perspective

One question that should be raised in the context of this broad discussion of the genetic and the environmental risk factors for IBD is, "Why are IBD susceptibility alleles so frequent in the population?" There are three theoretical possibilities.

First, IBD susceptibility could be due to new mutations. However, IBD susceptibility alleles clearly cause clinical disease with major morbidity and even mortality. The mortality was certainly greater until modern supportive management became available. If new mutations were the explanation, the mutation rate would have to



equal both the mortality rate and the decreased reproduction due to the disease. For new mutations to explain the IBD disease frequency in the modern world, the mutation rate clearly would have to be much greater than the estimated range of 1 in 100,000 to 1 in 1 million/gene locus that appears to be the mutation rate in humans.

Second, IBD susceptibility alleles could be the result of the founder effect. The founder effect refers to the concept that a given gene appeared (presumably by mutation) in a small ancestral population (i.e. in a founder) and by random chance was transmitted to a large number of that founder's offspring. This would establish the gene in relatively high frequency in the original small population and its subsequent ancestors (301,302). Although this founder effect is a reasonable explanation for the high frequency of certain genes in certain ethnic groups, it is an inadequate explanation for a disease (or diseases) with as wide a distribution and as high a frequency as IBD.

Third, IBD susceptibility alleles may convey a selective advantage, either now or under conditions in the past; IBD susceptibility alleles are frequent, even though deleterious (disease causing), because they also provide some compensating advantage to those individuals who have them (303). This advantage would allow those humans with the allele (or alleles) to survive under a particular selective pressure, and thus, the IBD susceptibility alleles would increase in frequency in the population. A balance would be achieved when the advantageous effect of the alleles matches the frequency and severity of the disease for which they predispose. Understanding how IBD susceptibility alleles provide a selective advantage would be useful for two reasons. One reason is that this understanding would contribute to our general understanding of human evolution and history. The second reason is perhaps more important: understanding the selective advantage of IBD susceptibility alleles will provide knowledge of the underlying physiologic process at a very fundamental level that would promote gene identification, unraveling disease pathogenesis, and thus suggest entirely new therapeutic approaches.

Is there any information that would allow us to speculate on the selective advantage provided by the IBD susceptibility genes? Such speculation should be built on several basic observations:

IBD is clearly genetic, common, and occurs in many different ethnic groups throughout the world. Therefore, a selective advantage is likely to account for its high frequency.

IBD is clearly a complex genetic trait and is likely to encompass a spectrum of many diseases that present with similar characteristics. Therefore, there are many steps affecting many pathways in IBD pathogenesis that could contribute to any selective advantage, and the advantage could be related to other diseases or other pathways not directly related to aberrant intestinal inflammation.

IBD has clearly increased dramatically in the developed world over the twentieth century and is now appearing

(or being recognized) with increasing frequency in the developing world. This indicates that some environmental factor or factors still operating in recent historical times could provide the selection pressure in the developing world. This further suggests that the incidence of IBD rises because the selective advantage is no longer acting against the selection pressure because the environmental selection pressure is disappearing.

The DZ twin rate for IBD is no greater than the sibling recurrence risk, suggesting that the environmental factors for IBD susceptibility affect the entire population. That is, the environmental factors are probably ubiquitous, rather than varying dramatically in frequency between families within a population or over a short time period (the few years of childhood).

IBD is clearly an inflammatory and immunologic disease of the gut, suggesting that environmental factors that provide the selection pressure may have operated in the gut.

On the basis of these observations, the authors have proposed the following hypothesis: the genetic variation that predisposes to the various forms of IBD provided a selective advantage in the form of mucosal immunoprotection in an unsanitary world (282). Effective public sanitation is a development of modern civilization, and we propose that the development of such sanitation removes the selective advantage of IBD susceptibility alleles. However, the mucosal immunoprotection provided by IBD susceptibility alleles is still primed genetically in those with the *IBD* genes, armed, if you will, to defend the organism. Thus, we have proposed that when IBD predisposing alleles are not adequately used in mucosal defense, the situation of the developed world, either (1) later exposure to an infectious agent would result in hyperstimulation of the immune response and subsequent chronic inflammation (analogous to paralytic polio, which occurs when infection occurs after early infancy), or (2) failure of exposure to a potentially injurious agent would leave the gut immunologic system in a continuously primed state and thus lead to subsequent dysregulation, for example, an autoimmune reaction that results in the diseases we recognize as UC and CD (282,304).

This hypothesis could provide a possible explanation for the relatively higher frequency of IBD in the Jewish population (282). IBD appears to be highest in frequency in Ashkenazi Jews (i.e. Jew whose origin is middle and eastern Europe), suggesting that the selective factor or factors had their greatest influence after the Ashkenazi/Sephardic division, coinciding with the historical division of Europe and the Mediterranean between the Christian and Islamic kingdoms (86). However, the greatest frequency of IBD is distributed in those Ashkenazi Jews whose origin is in middle Europe (97,98) and is similar to the distribution of Tay-Sachs gene carriers (305). Historically, countries in middle Europe imposed the greatest ghetto urbanization on Jews, perhaps leading to the greatest overcrowding and to the consequent

greatest defects in sanitation. This situation eased when the Ashkenazi Jews were invited to settle in eastern Europe with less pressure from ghetto urbanization, that is, Poland, Ukraine, and Russia. More individuals that are Tay–Sachs heterozygotes or IBD patients come from middle as opposed to eastern Europe (98,305). The lower frequency of IBD in Israel when compared with that of western Europe or the United States could possibly result from the more developing nature of that society. Regardless, if the specific details of this hypothesis are correct, it will be important to determine, if indeed, a genetically hyper-responding mucosal immune system is responsible for the susceptibility to IBD.

## 68.10 GENE IDENTIFICATION

### 68.10.1 Genomewide Linkage Studies

The epidemiologic evidence demonstrating a major genetic contribution to IBD susceptibility has resulted in several genome scans for IBD susceptibility loci examining linkage to CD, UC, or IBD combined. Specific genomic regions identified by the whole genome approach have been further tested by yet more laboratories in more targeted linkage studies. This international effort is noteworthy within the study of the genetics of complex diseases in that several susceptibility loci for IBD have been identified by more than one laboratory.

Table 68-8 shows the major IBD susceptibility loci demonstrated by linkage studies. Consensus has been reached by an international consortium on IBD genetics (International IBD Genetics Consortium) that the genomewide approach provides evidence for susceptibility loci for IBD on chromosome 16q12 (IBD1) and on chromosome 12 (IBD2). Further agreement has been reached by some of the members of this consortium that there is evidence for loci at or near the MHC on 6p21.3 (IBD3), at 14q11–12 (IBD4), 5q31 (IBD5) and 19p13 (IBD6). More loci likely exist; some have

probably been detected by this effort already. If a cut-off is arbitrarily set at a logarithm at OR (LOD) = 2 or at  $p < 0.001$  or at replication in two studies, then additional tentative loci may also have been identified at 1p36 (IBD7) and 3p26 (IBD9), and on chromosomes 4, 5, and 7. Complete reviews of linkage results are available (125,337).

Even though some of these linkage results will represent false-positive findings incurred during the analysis of the genome screen data, clearly these results already support the following ideas: (1) there are multiple susceptibility loci for IBD, (2) CD and UC share some loci in common and do not share other loci, and (3) some of these loci interact, and (4) different loci play different roles in the IBD susceptibility of different populations.

### 68.10.2 Chromosome 16 (IBD1, OMIM #266600)

The chromosome 16 locus was the first locus to be identified by the whole genome approach (306), was quickly confirmed by the authors (314), and currently has the strongest experimental support worldwide. A two-point LOD score of 2.04 was observed for marker D16S409 in an initial family panel with 25 sibling pairs affected with CD with no known cases of UC (CD-only families) and in a second panel of 53 IBD families (306). This result was confirmed in an independent sample the same year with a peak multipoint LOD score (MLS) of 2.1 at D16S411 (314). After stratifying based on Jewish ethnicity, the MLS increased to 2.4 for the non-Jewish families and decreased to lack of evidence for linkage in the Jewish families. This suggested that the chromosome 16 region contained a susceptibility locus for CD that is important for the non-Jewish population. Fine-mapping to a 1-cM resolution has resulted in a MLS of  $Z = 2.81$  ( $p = 0.0003$ ) at D16S416–D16S3117 (312). Linkage of this region was also observed in families with UC only (NPL = 2.02,  $p = 0.02$  at D16S3120) but, surprisingly,

TABLE 68-8 Susceptibility Loci for Inflammatory Bowel Disease

Locus	Chromosomal Position	OMIM Reference	Susceptibility Gene(s) Identified	Associated Disease	References <sup>a</sup>
IBD1	16q14	#266600	CARD15	CD	(306) (152,307–315)
IBD2	12q14	%601458		UC	(316) (152,311,317–322)
IBD3	6p21.3 MHC	%604519	HLA-DRBI*0103 TNF	IBD	(323) (152,324–327)
IBD4	14q11	%606675		CD	(319) (279,328,329)
IBD5	5q31-33	%606348	SLC22A4/SLC22A5	CD	(327) (319,330,331)
IBD6	19p13	%606674	ICAM1 hypothesis	IBD	(327) (328,331–333)
IBD7	1p36	%605225		IBD	(310) (152,332)
IBD8	16p12	%606668		CD	(334) (331)
IBD9	3p26	%608448		IBD	(316) (331,335,336)

CD, Crohn's disease; IBD, inflammatory bowel disease; TNF, tumor necrosis factor; UC, ulcerative colitis.

<sup>a</sup>First reference is the initial report.

not in families with both CD and UC subjects (mixed families) (313).

In an effort to combine the observations of the various laboratories worldwide in a single nonparametric analysis, members of the International IBD Genetics Consortium combined genotype data for the same six markers broadly spanning the IBD1 region (308). A total of 581 families (382CD only, 91UC only, 108 mixed) had data available for both parents and at least two affected sibpairs. An MLS of 5.2 for this locus was observed in CD but not in UC. This is among the highest LOD scores observed in any complex trait and demonstrates the importance of the study of a large number of families in order to detect modest genetic effects by linkage. Approximately 10–15% of the susceptibility to CD was estimated to be accounted for by this locus. The interval containing multipoint evidence within 1 LOD of the peak spans less than a 10-cM region between D16S753 and the interval between the markers D16S411 and D16S419.

An extensive and elegant positional candidate approach by Hugot et al. (283), as well as a candidate gene approach by Cho et al. (338), has led to the identification of the *CARD15/NOD2* gene as the susceptibility gene for IBD at this locus (see below).

The existence of a second IBD gene within the initial linkage region has been proposed by Hampe et al. (334) based on their observation of other linkage signals on chromosome 16 after stratification on the presence or absence of one of the *CARD15* risk. One of these peaks contains the *CARD15* gene, but another region centered around D16S3068, 25 Mb from *CARD15*, appears in their analysis to confer IBD risk independently of the *CARD15* gene. The designation IBD8 (OMIM %604519) has been assigned to this locus. The authors of this chapter, as well as Van Heel et al., confirmed linkage of CD to chromosome 16 in the absence of the three major *CARD15* variants between D16S514 and D16S515 ( $p = 0.0007$ ) (331,339).

### 68.10.3 Chromosome 12 (IBD2, OMIM %601458)

The chromosome 12 locus (IBD2) was the second region to be identified by a whole genome approach (316). A two-point LOD score of 5.47 ( $p = 2.66 \times 10^{-7}$ ) was observed in a set of 186 sibpairs from CD only, UC only, and mixed families at the marker D12S83. An association between a “4-1-3” haplotype constructed with markers D12S83, D12S1662, and D12S1655 and UC was observed in further work using the transmission disequilibrium test (TDT) (340). Both the linkage and association findings have been replicated in North American studies: one observing an association to the same marker D12S83 in both CD and UC families using the TDT (318), and one to a marker 14cM away at D12S85 in CD families (322). The International IBD Genetics Consortium observed a greater linkage of UC to this region

rather than CD (308,320). Jewell et al. have proposed that this locus contributes more to UC than to CD (320); however, Lesage et al. disagree (321,341). A third possibility in this debate is that there may be two loci in this region, one for CD and one for UC.

No gene for IBD2 has been identified thus far; a few candidate genes have been tested for association to IBD, CD, or UC based on their location within the IBD2 region, and negative results have been reported for interferon gamma (IFNG) (342), keratin 8 (KRT8) (343), and the natural-resistance-associated macrophage protein (NRAMP2) (344).

### 68.10.4 Major Histocompatibility Complex (MHC; IBD3, 6P21.3)

On the basis of the hypothesis that IBD pathogenesis may involve immunoregulatory factors, candidate genes within the MHC on chromosome 6p21.3 have long been tested for IBD susceptibility with conflicting results (see later). However, linkage of the region to IBD has been established. In a study of the sharing of MHC haplotypes, which allowed the delineation of identity by descent of all markers (323), linkage to MHC was observed using several nonparametric methods: (1) an increased number of sibpairs sharing one or more haplotypes ( $p = 0.004$ ), (2) an increased mean proportion of sharing between concordant affected ( $p = 0.002$ ) and concordant unaffected ( $p = 0.03$ ) pairs, along with a decreased proportion in discordant pairs ( $p = 0.007$ ), (3) a significant linear relation between the similarity of phenotype between members of a sibpair and the proportion of their shared haplotypes by regression analysis ( $p = 0.00003$ ), and (4) an increased sharing between pairs more distantly related than sibpairs ( $p = 0.001$ ). Linkage was also observed in this region in a genome scan by the same investigators (319). Further support for linkage of the MHC region to IBD has been observed by additional laboratories following the systematic mapping approach: (1) an MLS of 4.2 for D6S461 in 284 German families (152); (2) a peak nonparametric LOD score of 2.3 ( $p = 0.0026$ ) for D6S1017, with other NPL scores higher than 2 for nearby markers in 158 Canadian families (327,345); (3) a peak nonparametric LOD score of 3.04 near D6S291 in 234 UK families (324); and (4) an MLS of 5.91 for male affected only in 428 European families (325).

Genes at this locus have been tested and several associations reported (see later). The fact that there are numerous candidate genes for IBD within the MHC will make finding-specific causal variants problematic.

### 68.10.5 Chromosome 14 (IBD4, OMIM %606675)

Evidence for linkage between CD and the marker D14S261 has been observed in a study of 46 families

from Los Angeles (MLOD 2.8,  $p = 0.0002$ ) (207) and of 62 families from Pittsburgh (MLOD 3.6) and to the general region of this marker in a third study of 89 Flemish families (D14S80, NPL 2.41,  $p = 0.008$ ) (329). An MLOD of 2.36 ( $p \leq 0.01$ ) was observed by the International IBD Genetics Consortium in 733 IBD families (279). This linkage was observed only in those families, in which at least one sibling smoked, suggesting that IBD4 interacts with this environmental factor. No gene for this locus has been identified to date.

### 68.10.6 Chromosome 5 (IBD5, OMIM %606348)

Evidence for linkage to the region D5S393–D5S673 has been observed in Jewish CD-only families (319) and in CD families with early-onset disease (MLOD 3.9) (327). This region is syntenic to a mouse region implicated in the dextran-sulfate-induced colitis rodent model (346) and maps to a cytokine gene cluster containing many candidate genes for IBD. Rioux et al. (347) applied a systematic linkage disequilibrium (LD) mapping approach in order to find the causal variation with this linkage region by constructing a dense genetic map composed of both microsatellite and single-nucleotide polymorphism (SNP) markers across the 18-cM region and identified a 250-kb-long haplotype block conferring susceptibility to CD. The LD across this block was so strong, however, that they were unable to identify causal variation for CD within the *SLC22A4*, *SLC22A5*, or *PDLIM3* genes that lie on this block. The association of this block to CD was subsequently confirmed in Germany (292:212 transmitted:nontransmitted in IBD trios,  $p = 0.0002$ ) (348) and in Britain (23% in CD compared with 14.7% in control,  $p = 0.0002$ , RR 2.0 95 CI 1.4–2.8 for homozygote; 163:114 transmitted:nontransmitted,  $p = 0.0066$ ) (349,350). Negoro et al. also tested for interactions between the IBD5 CD haplotype and CARD15 mutations and demonstrated that IBD5 and CARD15 independently contribute to CD.

### 68.10.7 Chromosome 19P13 (IB6, OMIM %606674)

Significant linkage of IBD to the marker D19S591 with an MLS of 4.6 was first observed in Canadian sibpair families (327). Some evidence for linkage to this same marker has also been reported by another study (328) (two-point LOD score 1.6,  $p = 0.0067$ ) and to a neighboring region at D19S1034–D19S586 by a third group (multipoint  $p = 0.0059$ ) (310). It is important that the linkage region in these studies spans almost the entire chromosome, so that gene finding for this locus will be difficult. Van Heel et al. (331) tested for interactions between this locus, CARD15 mutations, and IBD5 and reported greater linkage in subjects that did not have CARD15 mutations (D19S217, LOD 2.9,  $p = 0.0001$ , for

subjects without CARD15 mutations compared to LOD 0 for subjects with CARD15 mutations) and for subjects that did have one or two copies of the IBD5 CD susceptibility haplotype (D19S425, LOD 2.4,  $p = 0.0005$ ).

Because the linkage evidence to date covers most of chromosome 19, there are many possible candidate genes for this locus. The association of ICAM1 and CD and UC has been observed (51), and Low et al. (333) have proposed that ICAM1 is the susceptibility gene in this region (see later).

### 68.10.8 Chromosome 1P36 (IBD7, OMIM %605225)

Two studies have observed evidence for linkage to the D1S2670–D1S2682 region (MLOD 2.65,  $p = 0.0002$ ) (310) and (MLOD 2.08) (152). Families with linkage to this region also show greater linkage to IBD1, suggesting that this susceptibility locus interacts with IBD1 (310). Further narrowing of this region to 130 kb near D1S2697 and D1S3669 has been accomplished by homozygosity mapping in a collection of American Iraqi Chaldean families with IBD (332). Because both CD and UC are observed in these families, these authors have proposed that this locus determines a generic susceptibility to IBD, with the concomitant inheritance of IBD1 than determining CD.

### 68.10.9 Chromosome 3P26 (IBD9, OMIM %608448)

A suggestion of linkage to the region D3S1076–D3S1573 in CD-only families was also observed in the same study that reported the initial linkage to chromosome 12 (242,316). With further fine mapping, a peak single-point linkage to CD has been observed by this group at marker D3S3521 (LOD 3.5,  $p = 0.00003$ ), with the support region (LOD within 1 of the peak) spanning D3S11–D3S3559 (351). This result was confirmed by Hampe et al. (D3S1304, MLOD 1.65) (336) and by Duerr et al. (D3S1297, MLOD 3.39,  $p = 0.000052$ ) (335). A candidate gene has yet to be identified for this locus.

### 68.10.10 Meta-Analysis Across All Genome Scans

The field of the genetics of IBD is one of the leading fields in the genetics of complex traits in that there has been replication of several genomic regions. Nevertheless, many of the loci identified have not been reproduced across studies. This is somewhat expected because (1) there are differences in sampling between the various centers conducting these studies worldwide; (2) reliable detection of linkage requires a sample size that is larger than the usual linkage study performed in IBD genetics (~100 sibpairs) (122,352); and (3) even larger sample sizes are required for confirmation of a linkage result by a second study.



The effort of the International IBD Genetics Consortium to combine linkage studies and genotype large numbers of individuals with the same microsatellite markers has been one strategy to overcome these problems. Another strategy has been to combine data from many linkage studies. The genome search meta-analysis method (GMSA) divides the genome into 120 bins 30 cM in size and ranks the evidence of linkage for that bin in each genome scan (353,354). When applied to the genome scans for IBD susceptibility loci, IBD3 emerges as the most significant locus for IBD and CD (345,355,356), showing that a locus may attain greater significance in a combined data analysis than is apparent in a single-genome scan. The GMSA of four genome scans by Williams et al. ranked IBD1 next in importance for IBD, and IBD5 and IBD1 next for CD, and the GMSA of van Heel et al. ranked IBD1 and IBD6 for CD.

## 68.11 CANDIDATE GENE STUDIES

In the candidate gene approach, genetic variants are tested based on a prior hypothesis regarding the role of that gene or gene product in the pathophysiology of the disease. Such a hypothesis may be based on evidence from clinical observation or physiologic studies of affected individuals (in vivo studies), from studies of known disease-related processes (in vitro studies), from animal models of disease (transgenic construction or targeted disruption), and from the effects of drugs or chemicals on disease in either humans or animals (pharmacogenetic studies). For IBD, possible candidates could be selected from genes involved in the regulation of the balance between TH1 and TH2 cells; the response of humans to gut flora, particularly the innate immune system; and the release of cytokines during the immunologic destruction and healing of the intestinal mucosa.

### 68.11.1 The Major Histocompatibility Complex (MHC; IBD3)

The MHC on chromosome 6p21.3 is the most important genomic region for innate and adaptive immunity, containing genes involved in the regulation of the immune system and in antigen processing and presentation. Classically, the MHC contains 224 genes identified within a 3.6-Mb region (357); however, accumulating evidence supports the idea that the cluster of genes important for immune pathways spans 7.6 Mb and contains 421 genes (282 expressed, 139 pseudogenes) (358). Because the MHC is a candidate region for most, if not all, diseases with dysregulation of the immune system as the possible underlying pathophysiology, the MHC has long been studied in IBD genetics. However, assigning the specific role of a specific gene to the pathophysiology of IBD has been hampered by several factors: (1) the MHC includes the most polymorphic human proteins known, the HLA class I and class II molecules, and some of these proteins

have more than 100 allelic variants; (2) LD extends over great distances throughout this region such that associations can be observed with variants that are not located in the gene actually related to IBD pathophysiology; and (3) the size of many of the studies reported have not been large enough to demonstrate adequately the small effects of some of these genes and so have been difficult to replicate by other investigators.

**68.11.1.1 The MHC Class II Region.** In general, the class II molecules are dimeric, with an  $\alpha$ -chain and a  $\beta$ -chain that form a groove for presenting an extracellular antigenic peptide to CD4<sup>+</sup> T cells. The three class II molecules are HLA-DP, HLA-DQ, and HLA-DR. In each case, the  $\alpha$ - and  $\beta$ -chains are encoded by *A* and *B* genes, respectively. For HLA-DR, there is an invariant *HLA-DRA* gene and up to three distinct and highly polymorphic *HLA-DRB* genes. One of these *HLA-DRB* genes, *HLA-DRB1*, is present in all individuals and is the most polymorphic. The study of *HLA-DRB1* alleles has been an important tool in the study of the role of class II genes in IBD. Serologic methods identify several HLA-DR types and subtypes, and were used in the older studies, whereas molecular methods reveal even more subtypes at the nucleotide level and are the preferred techniques today. A meta-analysis of 29 studies reporting HLA-DR or HLA-DQ frequencies in CD and UC patients and in controls has been published (359). The results of this analysis and of recent association studies using molecular methods are summarized in Table 68-9.

The authors have identified an association between the combination of HLA-DR1/HLA-DQ w5 alleles and CD in whites in the United States and HLA-DR2 and UC (367). The association between HLA-DR1 and CD has also been observed in France (360) and in The Netherlands (373). Associations with HLA-DR3 and CD and DR13 and UC have been observed in Italy (374). Further molecular typing revealed that, of all the DR1 alleles, only the *HLA-DRB1\*0103* allele, and not other DR1 subtypes, was associated with CD as well as UC (see Table 68-9) (365). This finding pointed to the importance of molecular typing over serologic typing as the means to clarifying the associations between class II alleles and IBD; an example of the associations between IBD and HLA alleles typed using molecular methods in one of the largest studies to date is given in Table 68-10. An association between CD and *HLA-DRB1\*0405* ( $p = 0.001$ , OR 2.02) and *HLA-DRB1\*0410* ( $p = 0.002$ , OR 4.79) has been observed in Japan (375). An association between *HLA-DRB1\*0103* and UC has been reported in the Japanese population (242) and in Britain (119,363). In UC patients with colectomy, *HLA-DRB1\*0103* is associated with a shorter time to surgery, as determined by Kaplan–Meier survival analysis (9 months compared with 5.2 years,  $p = 0.002$ ), suggesting association with a more aggressive UC. Roussomoustakaki et al. observed that this allele is associated with extensive UC, extraintestinal manifestations of UC, or with some complications of UC (mouth ulcers,

**TABLE 68-9 Associations of Crohn's Disease (CD) and Ulcerative Colitis (UC) with Human Leukocyte Antigen (HLA) Class II Alleles**

Serologic DR	Genetic Observation	OR	p	Reference
DR1				
DRB1*01	15% CD, 9% controls	1.75	0.003 (corr)	(360)
DRB1*03	Not found in ANCA neg UC			(361)
DRB1*07	17% CD, 11% controls	1.58	0.008 (corr)	(360)
DRB1*07	18% UC, 10% controls	1.9 RR	0.0001	(362)
DRB1*0103	8.6% UC, 3.2% controls	2.9	0.0074	(242)
	14% UC (16% extensive UC), 23% extraintestinal manifestations		<0.0001	(363)
	6% UC, 0.2% control	27.6	0.0002	(364)
	Increased with extensive UC, colectomy	33, 84	<0.0001	(364)
	7.9% CD, 2.2 control	3.9	0.004	(365)
	8.9% UC, 2.2% control	4.4	0.001	(365)
	Meta-analysis, association with UC	3.42		(359)
DR2				
	70% UC, 31% controls	5.1 RR	<0.001	(366)
	41% UC, 21% control	2.6	0.008	(367)
	44% ANCA + UC, 21% ANCA – UC		0.01	(29)
	Meta-analysis, association with UC	2.0		(359)
DRB1*15	42% UC, 26% controls	2.1	0.006	(364)
	35% ANCA post-UC, 15% controls	2.9	0.004	(361)
	Meta-analysis, association with UC	1.65		(359)
DRB1*1501 & DRB1*1502	Increased in ANCA post UC			(361)
DRB1*1502	49% UC, 18% controls	2.8 RR	<0.0001	(368)
	12% UC, 5% controls	2.7	0.005	(365)
	Meta-analysis, association with UC	3.74		(359)
	Negative for DR2 alleles			(369)
	Negative for DRB1*1502			(370)
DR4				
DRB1*0410	13% CD, 3% controls	5.02 RR	0.001 (corr)	(371)
DRB1*0410/	13% CD, 2.7% controls			
DQA1*03/		5.6 RR	0.00011 (corr)	(371)
DQB1*0402				
haplotype				
DR13				
DRB1*1302/	21% CD, 5.4% controls			
DRB3*0301		4.6 RR	0.0066	(370,372)
haplotype				
DRB3*0301	Meta-analysis, association with CD	1.18		(359)
DP				
DPB1*0401	74% CD, 71% controls, stratified on Jew/non-Jew	1.6	0.015	(365)
DQ				
DQA1*03	88% CD, 68% control	3.36 RR	0.03 (corr)	(371)
DQA1*0201	19% CD, 11% control	1.9 RR	0.0001	(362)
DQB1*0402	19% CD, 6% controls	3.89 RR	0.001 (corr)	(371)
DQBI*0501	16% CD, 10% controls	10.6	0.01 (corr)	(360)

ANCA, anti-*Saccharomyces cerevisiae* antibodies; OR, odds ratio; RR, relative risk.

arthritis, or uveitis). In Britain, HLA-DRB1\*0103 has also been associated with the extraintestinal manifestation of peripheral arthropathy in IBD patients when compared with controls (376). The authors have further observed that the association of DR2 with UC was due to the HLA-DRB1\*1502 allele (365). In addition, a rare HLA-DRB1\*0103-DQA1\*0501-DQB1\*0301 haplotype was dramatically associated with IBD with an

OR of 6.6, and a more common DR1 haplotype, HLA-DRB1\*0103-DQA1\*0101-DQB1\*0510, was also associated with IBD. This result suggested that an interaction between HLA-DR and HLA-DQ may determine the extent of disease risk.

A new association at another MHC class II locus, HLA-DP, has been observed; the common HLA-DPB1\*0401 allele confers a modest risk for CD (365).

**TABLE 68-10 Summary of Class II Associations with Crohn's Disease (CD), Ulcerative Colitis (UC), or Both Inflammatory Bowel Disease (IBD)**

Class II Allele	Disease or Haplotype	% Affected	%Control	<i>p</i>	OR
IBD	DRB1*0103	8.3	2.2	0.001	4.6
–CD		7.9	¢¢	0.002	4.4
–UC		8.9	¢¢	0.001	4.9
IBD	DRB1*0103- DAQA1*0501- DQB1*0303	2.8	0.4	0.034	6.9
IBD	DRB1*0103- DAQA1*0101- DQB1*0501	6.4	2.2	0.007	3.5
UC	DRB1*1502	11.9	4.7	0.006	2.6
UC	DPB1*0401	74.1	64.7	0.014	1.6

Total subjects = 232 control, 304 for CD, 270 for UC.

OR, odds ratio.

From Trachtenberg, E. A.; Yang, H.; Hayes, E.; et al. HLA Class II Haplotype Associations with Inflammatory Bowel Disease in Jewish (Ashkenazi) and Non-Jewish Caucasian Population. *Hum. Immunol.* **2000**, 61, 326–333.

\*Genes numbers from Entrez Gene, National Center for Biotechnology Information, National Institutes of Health, [www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene). OMIM numbers from the On-line Mendelian Inheritance in Man database, [www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=omim](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=omim).

The HLA-DRB1 association has also been confirmed by a different method, genotyping DNA pooled from CD patients and controls and measuring differences in the allele frequencies (rs1729, major allele frequency 0.70 in CD patients and 0.90 in controls,  $p = 3.02 \times 10^{-9}$ ) (377).

The results summarized in Tables 68-9 and 68-10 clearly point to a role for the MHC class II genes in the pathogenesis of IBD. However, three lines of evidence suggest that the contribution of the MHC to IBD is likely to consist of more than that provided by the class II region: (1) There is some evidence of association to other MHC genes (see later). (2) Although some investigators have confirmed associations to the same class II alleles, others have observed associations to other class II alleles, particularly when studying other populations. This suggests that at least some MHC class II alleles are not the IBD susceptibility alleles but are in LD with susceptibility alleles. (3) The results of two genome scans (319,326) raise the question as to whether the peak linkage to IBD in this region may be several centimorgans telomeric from MHC and the result of the third (327) suggests that the peak may be centromeric. (4) The magnitude of the linkage effect is not explained by the relatively modest associations of MHC class II alleles heretofore observed. This evidence suggests that further fine LD mapping of the MHC and IBD is also warranted. Strategies to unravel the role of the potentially multiple MHC genes involved in the susceptibility to IBD are (1) to carry out this fine mapping using both case-control and family-based association tests and to follow leads that are positive with both methods, and (2) to examine the contribution of each locus in isolation from the others by methods that stratify the genotyping data at one locus with respect to another locus.

**68.11.1.2 NOTCH4 (Gene Id #4855).** A preliminary example of such a fine-mapping effort has revealed

further susceptibility loci in MHC class III region around NOTCH4. The NOTCH4 gene is a homolog of the *Drosophila* Notch gene, and in humans, is located in the MHC class III region close to the border with the class II region. The function of human NOTCH4 is not currently known, but the family of Notch proteins is involved in the control of cell fate decisions during development (378) including hematopoiesis (303) and gut epithelium (379). Notch receptors and ligands are also expressed in the thymus, and the overexpression of Notch1 in a mouse transgenic strain directs CD4<sup>+</sup> CD8<sup>+</sup> precursors to the CD8 lineage (380). Authors have observed that an allele of NOTCH4 is associated with CD in Ashkenazi Jews in both a case-control study and a family-based study by TDT (381). This genetic association is independent of the HLA-DRB1 associations discussed earlier and supports the idea that multiple genes in the MHC may be determining IBD. In a study of well-characterized CD patients, the same allele was also associated with a subset of CD patients characterized by an earlier age of onset of CD and no expression of pANCA combined with a high expression of ASCA (44). These observations suggest that either NOTCH4 itself or a gene close by may also determine one of the subtypes of CD.

**68.11.1.3 Tumor Necrosis Factor (TNF, Gene Id #7124).** Tumor necrosis factor (TNF) is involved in the regulation of inflammation at many levels; of particular interest for IBD is the role of TNF in the recruitment of circulating inflammatory cells to local tissue sites and in granuloma formation. TNF is an important proinflammatory cytokine in CD (382), and its pivotal role in CD is prominent in mouse models of intestinal inflammation: a transgenic that increases TNF expression has colitis (383) and a TNF knockout reduces the ability of chemicals to induce colitis (384). TNF is also of interest in CD because

anti-TNF antibody is an effective treatment of moderate to severe CD (385). The genes for three members of the TNF superfamily, TNF, lymphotoxin alpha (*LTA*, gene #4049) and lymphotoxin beta (*LTB*, gene #4050) are located adjacent to each other at the border between the class III and class I regions of MHC (358,386). Several studies have observed an association between polymorphisms in this region and (1) changes in TNF expression (387–389), (2) altered immune response to infectious diseases (162,390–393), and (3) increased joint damage in rheumatoid arthritis (394). The association between IBD and variants in the TNF gene has been difficult to demonstrate, probably because the magnitude of the effect is small. However, in a large British case-control study, the C allele of the TNF-857C/T promoter polymorphism has been associated with IBD (534/587 were CC with IBD compared with 231/278 controls,  $p = 0.0014$ ), UC (280/304 were CC,  $p = 0.001$ ), and with CD patients who did not have NOD2 mutations (see later) (222/241 were CC,  $p = 0.0022$ ) (389). This result has been confirmed in Australia by TDT of 170 multiplex families (395). The British study found that blood from individuals with the TNF-857 CC genotype produced higher levels of TNF when stimulated by lipopolysaccharide (LPS); these authors also demonstrated that OCT1 bound to the TNF promoter with the T allele, not the C allele, and so proposed that the T allele of this variant allowed OCT1 to interfere with normal TNF transcription (389). Two observations suggest that genetic variation in the TNF region may also determine the course of disease in subsets of IBD: (1) a haplotype in the *LTA* region may be associated with a lack of response to anti-TNF therapy in a group of patients with moderate to severe CD (396); and (2) TNF microsatellite a is associated with the level of expression of ASCA antibody (397).

### 68.11.2 CARD15/NOD2 (IBD1; GENE ID #64127; OMIM \*605956)

Hugot et al. (398) applied the positional cloning approach in order to identify the gene causing susceptibility to IBD within the IBD1 locus. These authors began with a fine-map of 26 microsatellite markers spaced ~1 cM apart spanning the IBD1 locus, obtained a borderline TDT result with one allele of marker D16S3136, and sequenced the region, identified SNPs, and genotyped CD patients for these SNPs. These SNPs in the surrounding region were in LD with each other, and several were associated with CD to varying degrees. At the time of their study, no genes had been identified in this region, but GRAIL analysis predicted a gene in the region, and the existence of this gene was then confirmed by clones sequenced from a human leukocyte cDNA library. Three SNPs altered amino acids in this predicted protein, R675W (SNP8), G1881R (SNP12), and 980-frameshift-981X (SNP13) were associated with CD; the pedigree disequilibrium test (PDT) was most significant for SNP13

( $p = 6 \times 10^{-6}$ ). The relative risk for CD for being a heterozygote with one of these mutations was 3; for being a homozygote, 38; and for being a compound heterozygote, 44. The frequency of one of these three mutations is 0.07 in controls, 0.05 in UC patients, and 0.29 in CD patients.

Simultaneously with the fine-mapping study by Hugot et al., Ogura et al. were following a candidate gene approach based on the discovery that the mouse *Nod2* gene, a member of a gene family that contains a caspase recruitment domain (CARD) and that activates NF-kappaB (399), mapped to the human IBD1 locus. *Nod2* is now known as *CARD15*. By sequencing this gene in CD patients, these authors identified the same frameshift mutation observed by Hugot et al. and demonstrated an association of the frameshift mutation to CD using both the TDT and case-control study designs (338). NF-kappaB signaling was defective in cells coinfecting with NF-kappaB reporter constructs containing human *CARD15* sequence with the frameshift mutation. This observation suggested that mutations in *CARD15* alter response to bacterial antigens.

The Hugot et al. and Ogura et al.'s papers were published side-by-side in the journal *Nature* and together gave convincing evidence that *CARD15* was the susceptibility gene for CD. Their result has been confirmed by many investigators (18,118,400–409). The identification of *CARD15* as a CD susceptibility gene represents the final argument that CD is a genetic disorder.

All the three predisposing mutations were found on the same background haplotype that included a fourth SNP, SNP5 identified by Hugot et al. This observation suggested the possibilities that CD susceptibility required (1) the combination of SNP5 with one of the other predisposing SNPs or (2) some other unidentified variant in LD with SNP5. The authors of this chapter observed, in contrast to Hugot et al., that this background haplotype alone, without one of the three predisposing mutations, was associated with CD in Ashkenazi Jewish CD subjects (OR = 3.13,  $p = 0.023$ ) (339). Sequencing of this haplotype revealed a new *CARD15* variant, JW1 (IV8+158), such that the *CARD15* SNP5-JW1 haplotype showed exhibited increased risk (OR = 5.75,  $p = 0.0005$ ) for CD and the highest population-attributable risk (15.1%) for CD among the reported CD susceptibility SNPs in Jews, as compared with non-Jews. Investigators in Israel have reported an association between the *CARD15* G908R SNP (SNP8) and a younger age of CD onset (25% pediatric CD patients compared with 9% of adult CD patients,  $p = 0.003$ ) (410). *CARD15* mutations are associated with CD in Israel (411). In New York, a higher frequency of *CARD15* mutations was found in Ashkenazi Jewish subjects from multiplex families with CD from Central (44%) compared with Eastern (24%) Europe origin and in Sephardic/Oriental Jews compared with controls (412).

None of the three major *CARD15* mutations was observed in 483 Japanese CD patients, and sequencing



portions of the exons 4, 8, and 11 from the *CARD15* gene in 96 patients did not demonstrate any variants of consequence (288); these SNPs were also absent in 126 Korean CD patients (413) and in 63 Chinese CD patients (414). These observations suggest that *CARD15* does not play a major role in CD susceptibility in Asia. Genotyping and sequencing also failed to demonstrate an association between *CARD15* and CD in a patient sample from Tunisia (415). Significantly lower frequencies of *CARD15* mutations have been observed in black and Hispanic children with CD (416).

Several reports have explored the relationship between *CARD15* mutations and CD clinical phenotypes. Work by the authors of this chapter using univariate analysis demonstrated that the three CD susceptibility variants were significantly associated with fibrostenosing disease (46% of patients with fibrostenosing disease carried at least one of the three variants compared with 24% of patients without fibrostenosing disease (OR 2.8, 95% CI 1.6–5.2) (118). Multivariate analysis showed that the primary association was with the fibrostenosing disease rather than small bowel disease. CD patients with two *CARD15* mutations are characterized by a younger age at onset of disease (16.9 years compared with 19.8 years,  $p = 0.01$ ), a stricturing phenotype (53% compared with 28%, OR 2.92,  $p = 0.00003$ ), and less-frequent colonic involvement (43% compared with 62%, OR 0.44,  $p = 0.003$ ) than CD patients with no *CARD15* mutations (407). Investigators in Italy observed that CD carriers of at least one of the three major *CARD15* variants were younger and more likely to have an ileal localization of disease, stenosing pattern, previous surgery, and ASCA expression (400). The association between *CARD15* mutations and ileal disease either with fibrostenotic complications or with younger age of onset was observed by others (18,417,418).

The identification of *CARD15* as a CD susceptibility gene immediately focused attention on the role of the innate immune system in developing colonic inflammation. The *CARD15* protein binds muramyl dipeptide that originates from bacterial peptidoglycan (419); peripheral blood mononuclear cells from individuals homozygous for the *CARD15* L1007fsinsC (SNP13) fail to respond to muramyl dipeptide, as measured by activation of NF- $\kappa$ B (420). Muramyl dipeptide induced secretion of IL8 in these cells and upregulates the TNF and IL1- $\beta$  secretion induced by other Toll-like receptor ligands (421), but these effects are abolished in cells from CD patients with *CARD15* L1007fsinsC (SNP13). Mice with targeted disruption of the *CARD15* gene (Nod2-/- knockout) showed no overt intestinal inflammation but were sensitive to bacterial infection through oral delivery, suggesting that *CARD15* is important for intestinal immunity (422). The observation that *CARD15* is expressed in Paneth cells at the base of the small intestinal crypts supports this role (423), because Paneth cells secrete antibacterial substances in response to bacteria (120,424). The role for *CARD15* acting as

an antibacterial factor is supported by the observation that intestinal epithelial cells (CaCo2 cell line) stably transfected with a wild-type *CARD15* construct were able to prevent invasion by *Salmonella typhimurium* but not with a construct carrying the *CARD15* L1007fsinsC insertion (425).

### 68.11.3 SLC22A4/SLC22A5 (IBD5; GENE IDs #6583 & 6584; OMIM 604190 & 603377)

As described earlier, high-resolution linkage LD mapping of the IBD5 locus led to the identification of a 250-kb haplotype conferring CD susceptibility, but that the LD across this region was too high to allow determination of the specific gene with this region that was involved in CD (347). Subsequently, 10 novel SNPs were identified by resequencing the five genes within this interval, and two of these were predicted to have functional effects, an L503F amino acid substitution in the solute carrier family 22 (organic cationic transporter) member 4 gene, *SLC22A4*, and a -207G->C transversion disrupting the heat shock element in the promoter of the *SLC22A5* gene (426). A T-C haplotype of these two SNPs were at a higher frequency in CD patients compared with controls (54% versus 42%,  $p = 0.0003$ ) and had a population attributable risk of 19% for heterozygotes and 27% for homozygotes. This haplotype does not confer risk to UC. These two SNPs were also associated with CD when they were located on other IBD5 haplotypes that were not the CD susceptibility haplotype (14.3% in CD compared with 9.8% in controls,  $p = 3.5 \times 10^{-9}$ ), arguing that these two SNPs, rather than nearby alleles, conferred CD susceptibility. Both proteins were expressed in human colon. The *SLC22A4* L503F SNP “F” allele decreased the sodium-dependent carnitine transport and carnitine affinity of this protein. The CD-associated variant in the promoter of *SLC22A5* was shown to disrupt formation of a protein complex by gel shift assay and to reduce transcription in a luciferase reporter assay. These authors have proposed that, because carnitine mediates fatty acid oxidation and inhibition of fatty acid evokes colitis, impaired carnitine transport may be the mechanism that *SLC22A4* confers CD susceptibility (427). Combination of the *SLC22A4/A5* T-C haplotype with one or more of the three common *CARD15* variants confers a 7.5-fold increased risk for CD ( $p = 9 \times 10^{-8}$ ) (49).

When taken together, this combination of genetic and cellular observations suggest that the *SLC22A4/A5* T-C haplotype confers CD susceptibility and comprises the functional variation of the IBD5 locus.

### 68.11.4 DLG5 (GENE ID #9231; OMIM 604090)

IBD was linked to the region D10S547–D10S192 (MLOD 2.07,  $p = 0.0033$  at D10S548) (152). With stages of fine-mapping, an association with CD was detected at

D10S201 ( $p < 0.01$ ), then with an SNP, rs1344966 (CD,  $p = 0.002$ , IBD,  $p = 0.0006$ ) (428). Of the two genes in the region, DLG5 and KCNMA1, the association signal was confined to DLG5, discs, large homolog 5 (so named because of its homology with a gene in *Drosophila*). SNP rs1248696, R30Q, is overtransmitted to CD patients by the TDT (82 transmitted:54 nontransmitted,  $p = 0.004$ ), suggesting that DLG5 is associated with CD. The R30Q SNP was associated in one experiment from a case-control study (11% or 73/591 in CD compared with 5.9% or 24/380 in controls,  $p = 0.003$ ) but not in a second, and was significantly associated in a TDT (355). These results support the hypothesis that the haplotype block containing R30Q is associated with CD. A study from Scotland (429) and another study from Germany (430) did not confirm this association. A weak association of CD to DLG5 was observed in Japan, but the R30Q SNP was not (431), suggesting this gene may be important for CD in Japan but that different variants have arisen in this ethnic group.

### 68.11.5 ICAM1 (IBD6?; GENE ID #3383; OMIM 147840)

Intercellular adhesion molecule 1 (ICAM1, CD54) is involved in one of the several steps of the normal capture and migration of leukocytes from the blood stream to the site of inflammation (432,433). Several observations suggest that ICAM1 plays a direct role in IBD: (1) In mucosa taken from IBD patients and controls, a massive infiltration of ICAM1-positive cells has been correlated with the amount of inflammation in IBD (434). The concentration of ICAM1 is significantly elevated in CD and UC patients and is also higher in active UC compared with inactive UC (435). (2) Plasma-soluble ICAM1 is also higher in patients with active UC, pouchitis, and CD (436). (3) Anti-ICAM1 treatment of rats reduces intestinal inflammation in the acetic acid-induced model of colitis (437). (4) Antisense ICAM1 oligonucleotide prevents colitis in dextran-sulfate-induced mice (438) and reduces inflammation and leukocyte adherence in indomethacin-treated rats (439). The authors observed that the frequency of the "R" allele of the ICAM1 G241R polymorphism was higher for the ANCA-negative UC and for ANCA-positive CD subsets of disease but not for CD or UC as a whole when compared with controls (440), suggesting that this polymorphism determines the disease course of subsets of IBD. An association of the ICAM1 G241R SNP and UC and the K499E and both UC and CD has been reported in Germany (e.g. R241, controls: 9%, UC 16%,  $p = 0.024$ , CD not significant) (441) and of the ICAM1 K469E in Italy (e.g. E/E genotype, controls, 11%, IBD 24.9%) (442) and Japan (controls: 59.5%, IBD: 63.0%,  $p = 0.0025$ ; CD: 65.2%,  $p = 0.0056$ ; UC, 61.7%,  $p = 0.017$ ) (443). British investigators have observed an association between the

ICAM1 K/K genotype and CD (control 29.4%, CD 39.9%,  $p = 0.0096$ ) and have proposed that the ICAM1 gene is the susceptibility gene for the IBD6 locus (333). A negative result was reported for ANCA-positive UC (360,441), but it should also be noted that the method of ANCA determination is not uniform on both sides of the Atlantic. Italian investigators have reported that the ICAM1 K469E polymorphism is associated with a greater extent of CD and with penetrating disease behavior (444), and British investigators have reported that the G241R is associated with fistulizing disease (21.8% versus 10.0%,  $p = 0.03$ ) (333).

These observations are very suggestive that the common ICAM1 G241R and K469E amino acid variants are associated with CD and UC; however, the linkage region on chromosome 19 is very broad and the conclusion that ICAM1 is the IBD6 susceptibility gene must remain provisional. Formal studies must address two issues: First, does the ICAM1 association account for the entire linkage signal? This question could be examined by stratifying the linkage signal on the presence and absence of ICAM1 polymorphisms. Second, are there other ICAM1 polymorphisms or an underlying haplotype structure that is associated with a greater IBD susceptibility than that observed with these two polymorphisms? This question could be examined by genotyping more variants in this gene and performing an analysis of the association of the haplotype structure with IBD.

### 68.11.6 Other Observed Associations

**68.11.6.1 Interleukin 1 Beta (IL1B, Gene Id #3553) and Interleukin 1 Receptor Antagonist (IL1RN, Gene Id #3557).** The interleukin 1 (IL1) family consists of three related proteins: interleukin-1-alpha (IL1A, Gene Id #6552), interleukin-1-beta (IL1B) and interleukin-1 receptor antagonist (IL1RN). The genes for all three proteins are located together on chromosome 2q14 but not within a region of linkage observed in a genome scan. IL1A and IL1B are cytokines with a wide spectrum of proinflammatory actions in many cell types, and IL1RA inhibits the action of IL1A and IL1B by blocking the interleukin 1 receptor (445). When these cytokines were measured in freshly isolated intestinal mucosal cells from IBD patients and controls, an imbalance in the ratio of IL1 to IL1RN was observed in both CD and UC patients, with the ratio correlating closely with the clinical severity of disease (446). This observation was confirmed using biopsies from the inflamed mucosa of IBD patients (447). Furthermore, removal of IL1RA by treating animals with anti-IL1RN or by gene knockout increases susceptibility to experimentally induced colitis (448). These observations support the hypothesis that an imbalance between IL1 and IL1 receptor antagonist is important in the etiology of IBD (445,449).

Several groups have observed an association between IL1RN and UC, with an increased frequency of allele 2 of an 86 bp variable number of tandem repeats (VNTR) polymorphism in intron 2 of IL1RN in UC (449,450). This increased frequency was observed in patients from the Los Angeles area, particularly in a subset of UC patients from the Ashkenazi Jewish population. Biopsies from IBD patients with this genotype have a slight reduction of IL1RN (447). These results suggest that genetic variation in the IL1RN may alter the ratio between interleukin 1 and its receptor antagonist and thus contribute to susceptibility to IBD. An association between polymorphisms in IL1RN has been observed in some but not all subsequent studies (19,451–454). Furthermore, some evidence supports the concept that IL1RN polymorphisms may also participate in determining the course and severity of IBD: (1) a significant association has been observed between two polymorphisms in the interleukin 1 beta gene (IL1B; variants at –511 in the promoter and in exon 5) and nonperforating CD but not with perforating-fistulizing disease (19), and (2) IL1RN allele 2 has a higher frequency in surgically treated UC patients compared with nonsurgically treated patients or controls (451). When taken together, the evidence supports an association between polymorphisms in IL1RN and both CD and UC; the differences in these reports may be due to sample size problems and differences between the ethnic groups studied in these reports.

**68.11.6.2 ATP-Binding Cassette, Subfamily B (MDR/TAP), Member 1 (ABCB1, Gene Id #5243, OMIM 171050).** This gene is also known as “multidrug resistance 1 (MDR1)” and “P-glycoprotein 170,” a transporter that pumps neutral and cationic hydrophobic compounds and acts to pump toxins back into the gut lumen (455). Early observations on mice with a targeted disruption of this gene suggested that loss of ABCB1 expression in intestinal epithelial cells leads to colitis (456). The MDR1<sup>–/–</sup> is currently studied as a mouse model for IBD (457,458). This gene was resequenced in IBD subjects, and the ABCB1 A893S/T SNP was associated with both CD and UC by case-control ( $p = 0.002$ ) and PDT ( $p = 0.0002$ ) in a large study from North America (459). The ABCB1 C3435T polymorphisms were also associated with UC in studies from Germany (460,461); however, this result was not confirmed by studies from Germany and Britain (462) and Greece (463). ABCB1 is located at suggestive linkage region on 7q21 and may account for this LS (464).

These results are very promising for the role of this gene in both CD and UC. Further work should address whether there are promoter or other SNPs that exert a greater susceptibility than or in addition to the MDR1 A893S/T and C3435T SNPs. In future, the combination of the human variability and the mouse model will provide a powerful tool for unraveling the pathophysiology of IBD represented by this gene.

**68.11.6.3 Interferon Gamma Receptor Subunit 1 (IFNGR1, Gene Id #3459).** Mutations in the IFNG receptor subunit 1 gene (IFNGR1) have been associated with impaired response to IFNG (121,465) and susceptibility to mycobacterial infection (466–468). The authors have observed an association between a polymorphism in this gene and the development of chronic pouchitis in UC patients who have IPAA surgery (469). This observation raises the intriguing possibility that this receptor plays a role in determining the course of severe forms of UC. This gene is located in a peak of linkage to IBD on 6q (319).

**68.11.6.4 Solute Carrier Family 11 (Proton-Coupled Divalent Metal Ion Transporters), Member 1 (SLC11A1, Gene Id #6556).** The *SLC11A1* gene is also known as the natural-resistance-associated macrophage protein 1 gene and may be an important gene controlling response to infection by pathogens, particularly to *Mycobacteria* spp. (364,470,471). An association has been observed between CD and a haplotype composed of two markers that flank *SLC11A1*, D2S434 and D2S1323 (3a) and CD and one allele of a *SLC11A1* promoter microsatellite (472). A negative result has also been reported (111).

Therefore, rapid progress is being made with gene identification for the IBDs. The linkage data clearly identify specific and several genetic loci as contributing to the etiology of IBD; nine loci have been identified, and more have been suggested from the current linkage data. Recently, the *CARD15* gene has been identified and has focused attention on the role of the innate immune system in the pathophysiology of IBD. Many more genes for innate immunity are currently being tested, for example, those in Toll-like Receptor pathways, along with genes based on other functional hypotheses and mouse models, and consensus will emerge on the role of these in the near future. Good evidence exists for the contribution of multiple genes in the MHC, in particular, HLA-DRB1 in the MHC class II region, as well as for *SLC22A5/A5*, *DLG5*, and *ICAM1*.

## 68.12 CLINICAL APPLICATION OF GENETIC INFORMATION

In general, the genetic information described here is not yet used in clinical practice. Typing for ANCA and ASCA is on the verge of utility in diagnosing CD versus UC and in clarifying the cases of “indeterminate colitis.” At the current time, genetic markers are not used diagnostically although the use of these is currently under discussion. As for the serum antibody markers pANCA and ASCA, they can be used in diagnosis. Even though they are found at increased risk in relatives, screening relatives clinically is not recommended because there is no recognized intervention. Counseling is based on empiric risks, which were reviewed in family epidemiology, and are, for the most part, modest.



In the long term, genotyping patients for CD- and UC-associated genes will enable the determination of risk to relatives of IBD patients and of the optimal therapy for each individual. Identifying the genes that predispose to each pathogenetic mechanism of IBD will point research into new directions to develop therapies and will enable the construction of mouse models for the testing of those therapies before starting clinical trials. The use of this genetic information in the nearest term is perhaps suggested by several preliminary IBD pharmacogenetic studies.

Because glucocorticoids are known substrates for a drug efflux pump protein (P-glycoprotein 170) expressed by the *MDR* gene (SLC11A1, *MDR1*), the expression of this pump on the surface of lymphocytes was compared between IBD patients and controls, and increased *MDR* expression was observed in CD and UC patients who required surgery because they failed medical therapy (473). This observation suggests that a genetic variation that affects the pharmacology of steroids may lead to the ineffectiveness of these drugs in some IBD patients, and so the genetic contribution of SLC11A1 to IBD may be of interest to choosing proper therapies for individual patients.

Clinical response to 6-mercaptopurine (6-MP) depends on its conversion to 6-thioguanine (6-TG) and reaching a TG level  $>235$  (pmol/ $8 \times 10^{-8}$  erythrocytes) (474). Patients heterozygous for mutations that reduce the level of the enzyme thiopurine methyltransferase (TPMT) are able to attain this therapeutic level more readily on a given dose of 6-MP because the drug is not converted into inactive nucleotides by this enzyme (e.g. 6-methylmercaptopurine or 6-MMP). These results suggest that TPMT genotyping may assist the clinician in optimizing the therapeutic response to 6-MP and in identifying individuals at increased risk for drug-induced toxicity.

Patients in the original successful clinical trial of the anti-TNF antibody (385) were also typed for ANCA status and genotyped in the TNF region of MHC (396). The response of pANCA patients was the lowest and not significantly different from placebo, and the response of sANCA patients (a different type of staining from pANCA) was the highest in this study. Homozygotes for the LTA Ncol-TNFC-aa13L-aa26 haplotype “1-1-1-1” did not respond to treatment. Although these results must be interpreted with a great deal of caution because of the many comparisons made in this small study, these results raise the intriguing possibility that sANCA may identify a CD subgroup with a better response to Infliximab and that pANCA and homozygosity for the LTA “1-1-1-1” haplotype may identify CD subgroups with a poorer response (396).

These studies each raise the possibility that the use of specific genetic markers, involved in either metabolism of the therapeutic agent or in the susceptibility to a form of IBD, may well guide therapy in the not-too-distant future.

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# CHAPTER

# 69

## Bile Pigment Metabolism and Its Disorders

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### 69.1 INTRODUCTION

Bilirubin, the major yellow pigment in plasma is the degradation product of heme, the iron-containing tetrapyrrole chromophore of hemoglobin and several important enzymes. Owing to its internal hydrogen bonding, bilirubin is very sparsely soluble in water and is toxic to neural and other tissues. It is rendered harmless by a series of physiologic processes, including binding to plasma proteins, rapid uptake by hepatocytes, conjugation with sugars to form polar derivatives, and efficient bile canalicular excretion. Perhaps because of its distinctive color, bilirubin has attracted the attention of physicians, chemists, and biologists since antiquity. Excessive accumulation of bilirubin in the serum is an indicator of liver dysfunction, and serum bilirubin analysis is used as a routine “liver function test.” Bilirubin throughput by the liver has also been studied as a model for hepatic disposal of other biologically important organic anions of limited aqueous solubility. Investigations into the molecular mechanisms of inherited disorders of bilirubin metabolism in humans and animals have provided important insights into bilirubin’s metabolic pathways. Newer aspects of bilirubin disposal and its relationship with biliary excretion of other substances are continuing to be unveiled by the efforts of numerous laboratories. Definitive treatment of some of these disorders remains a therapeutic challenge and continues to stimulate research. Although physicians have been mainly concerned with the toxic effect of bilirubin, the antioxidant property of the pigment may impart it a cytoprotective role.

This chapter provides a brief description of the chemistry, toxicity, and disposition of bilirubin, followed by a description of clinical situations in which various aspects of bilirubin throughput are disturbed.

### 69.2 FORMATION OF BILIRUBIN

Approximately 250–400 mg of bilirubin is produced daily in humans from heme catabolism. Normally, hemoglobin of senescent erythrocytes account for 80% of bilirubin production (1), and the remainder is derived from other heme-containing proteins and enzymes, and free heme. Intravenously administered radiolabeled heme precursors, glycine and  $\delta$ -aminolevulinic acid, are incorporated into bile pigments in two phases (1,2,4). The “early-labeled peak” of bilirubin (ELB) is excreted in bile during the first 3 days, and contains 20% of the radiolabel. The ELB consists of an initial “fast” component, comprising two-thirds of the peak in humans, and is largely derived from hepatic hemoproteins such as cytochromes, catalase, peroxidase, and tryptophan pyrrolase (4), and a rapidly turning over pool of free heme in the cytosol of hepatocytes (5), a fraction of which may be degraded without incorporation into heme proteins (6). Induction of hepatic cytochrome P-450 enhances the ELB (7). As  $\delta$ -aminolevulinic acid is preferentially incorporated into hepatic hemoproteins, when labeled  $\delta$ -aminolevulinic acid is used as a precursor, only the initial component of the ELB incorporates radioactivity (4). This is followed by the relatively “slower” phase of the ELB, which normally contains one-third of the peak and

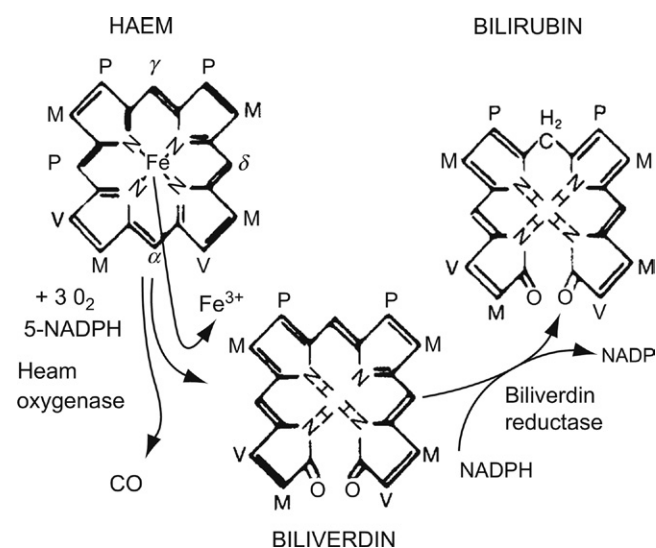
is derived from erythroid and nonerythroid sources. This phase is enhanced during “ineffective erythropoiesis,” as in congenital dyserythropoietic anemias, megaloblastic anemias, iron-deficiency anemia, and lead toxicity and erythropoietic porphyria (9). The slower component of the ELB is also enhanced during accelerated erythropoiesis from any cause because of intramedullary destruction of some normoblasts, destruction of reticulocytes in the peripheral circulation (10), and trimming of segments of reticulocytes during maturation (11). Normally, 80% of the radiolabel is excreted as the “late-labeled peak,” which is derived from the hemoglobin of senescent erythrocytes and appears approximately 50 days and 100 days after intravenous injection of the precursors in rats and humans, respectively (4). In hemolytic syndromes or acute intravascular or extravascular hemolysis, the life span of erythrocytes is shortened and the late-labeled peak appears earlier.

### 69.2.1 Enzyme-Mediated Opening of the Heme Ring

Heme (ferroprotoporphyrin IX) consists of four pyrrole rings connected by methane bridges and a single iron in the center (Figure 69-1). The ring is opened by oxidation of the  $\alpha$ -methene-bridge carbon, catalyzed by heme oxygenase, a group of enzymes located in the endoplasmic reticulum. This reaction requires NADPH and oxygen, and results in the elimination of the  $\alpha$ -methene-bridge carbon as CO (12); release of iron, which is reutilized; and formation of biliverdin, a green pigment (Figure 69-1). High levels of heme oxygenase activity are found in organs involved in hemoprotein breakdown, such as the spleen, where senescent erythrocytes are sequestered. In the liver, both hepatocytes and Kupffer cells have heme

oxygenase activity; the activity in the Kupffer cells is as high as in the spleen (13). Three forms of heme oxygenase have been identified (14). Heme oxygenase 1 (HO-1), a 32-kDa protein, is a stress-response protein. It is induced by stress-related agents, such as endotoxins (e.g. lipopolysaccharide), several cytokines, heavy metals, hypoxia, and reactive oxygen species. HO-1 is also induced by protoheme IX, oxidized and low-density lipoprotein, and probably also by the shear stress on endothelial cells in the cirrhotic liver (15–17). Nuclear factor- $\kappa$ B and p38 mitogen-activated protein kinase signaling pathways mediate the lipopolysaccharide-dependent induction of HO-1 via the proximal promoter region of the gene. The lipid peroxidation end product 4-hydroxy-nonenal (HNE) and phospholipids are natural components of oxidized LDL and mediate the stabilization and nuclear translocation of the anti-inflammatory nuclear factor E2-related factor 2 (Nrf2), thereby enhancing the transcription of HO-1 (18). By converting the pro-oxidant heme to antioxidant biliverdin and bilirubin, it serves as a cellular defense mechanism against oxidative injury. HO-1 expression in response to inflammatory mediators may contribute to resolving inflammatory responses, thereby protecting several organs against oxidative injury. Both CO and biliverdin/bilirubin, which are the products of HO-1, have been implicated in this response. In the gastrointestinal tract, HO-1 induction is associated with protection against injury caused by ischemia-reperfusion, indomethacin, lipopolysaccharide-associated sepsis, trinitrobenzene sulfonic acid, and dextran sulfate sodium (19). In the presence of hypercholesterolemia, HO-1-deficient mice develop early atherosclerosis. In humans, HO-1 deficiency is associated with growth retardation, hyperlipidemia, and endothelial damage, which may cause consumption coagulopathy and microangiopathic hemolytic anemia. Inducible HO-1 is an important cytoprotectant in the renal vascular endothelium and tubular epithelium. It is likely that HO-1 has additional effects that may not be mediated by CO or bilirubin. For example, human mesenchymal stem cells induce subsets of regulatory T-lymphocytes in an HO-1-dependent manner (20). In certain conditions, induction of HO-1 may result in increased iron deposition in tissues, with deleterious effects. In Alzheimer’s disease, HO-1 induction in astroglial cells by  $\beta$ -amyloid and hydrogen peroxide is thought to cause mitochondrial nontransferin iron sequestration, thereby contributing to bioenergy failure (21). Finally, the cytoprotective effect of HO-1 may have a deleterious effect as seen in the case of prostate cancer progression (22).

Heme oxygenase 2 is a constitutive protein, expressed primarily in the brain and the testis. The catalytic activity of heme oxygenase 3 is very low; this isoform may function mainly as a heme-binding protein. CO released by heme-oxygenase-mediated oxidation of the  $\alpha$ -carbon bridge is a potent vasodilator that regulates the vascular tone in the liver and in other organs, such as the heart, under conditions of stress.



**FIGURE 69-1** Mechanism of heme ring opening and subsequent reduction of biliverdin to bilirubin. Oxidation of the  $\alpha$ -carbon bridge catalyzed by microsomal heme oxygenase results in the elimination of the carbon as CO, resulting in opening of the heme ring.

**69.2.1.1 Heme Oxygenase Inhibitors.** Non iron metalloprotoporphyrins, such as tin- and zinc protoporphyrin, bind to heme oxygenase with greater affinity than heme (23), but are not degraded. These dead-end inhibitors of heme degradation (23,24) strongly suppress heme oxygenase activity, thereby suppressing bilirubin formation.

## 69.2.2 Reduction of Biliverdin to Bilirubin

In most mammals, biliverdin is converted to the orange pigment bilirubin by the action of biliverdin reductase, which utilizes NADH at pH 6.7 and NADPH at pH 8.5 as cofactors (25). Variants of cytosolic biliverdin reductase, isolated from rat liver and spleen (27), are post-translational derivatives of a single-gene product (25,28).

Because bilirubin is almost quantitatively excreted in bile, bilirubin production can be measured by determination of biliary excretion in experimental animals. In humans, it can be estimated by quantifying fecal and urinary urobilinogen and stercobilinogen, the bacterial degradation products of bilirubin (29). The rate of bilirubin formation can also be determined from the turnover of intravenously administered radioisotope-labeled bilirubin (30). More conveniently, bilirubin formation can be estimated by measuring CO production using a closed rebreathing system. Oxidation of the  $\alpha$ -carbon bridge of heme is the main source of endogenous CO production although a small fraction may be contributed by other sources. Therefore, CO production can be calculated from the CO concentration in the breathing chamber or from an increment in blood carboxyhemoglobin saturation (31).

## 69.3 STRUCTURE OF BILIRUBIN

The planar chemical structure of bilirubin (Figure 69-2) was determined by Fischer and Plieninger (32). Despite the presence of two propionic acid side chains and four amino groups, bilirubin IX $\alpha$  is nearly insoluble in water at physiologic pH. An explanation for this phenomenon was suggested by Fog and Jellum (34) and by Kuenzle et al. (35), who proposed that bilirubin IX $\alpha$  may be internally stabilized by hydrogen bonding between the propionic acid carboxyls and the two external pyrrolenone rings. Such intramolecular hydrogen bonding has been confirmed by X-ray diffraction studies (i). These hydrogen bonds constrain the molecule into a "ridge tile" conformation and engage all polar groups of the molecule, rendering it insoluble in water (Figure 69-2). For steric reasons, the hydrogen-bonded structure requires that the interpyrrolic bridges at the 4 and 15 positions of bilirubin should be in a trans- or Z configuration (37). Addition of methanol, ethanol or 6-M urea interferes with hydrogen-bonded structure and makes bilirubin more labile, water soluble, and rapidly reactive with diazo reagents. Conjugation of the propionic acid carboxyls

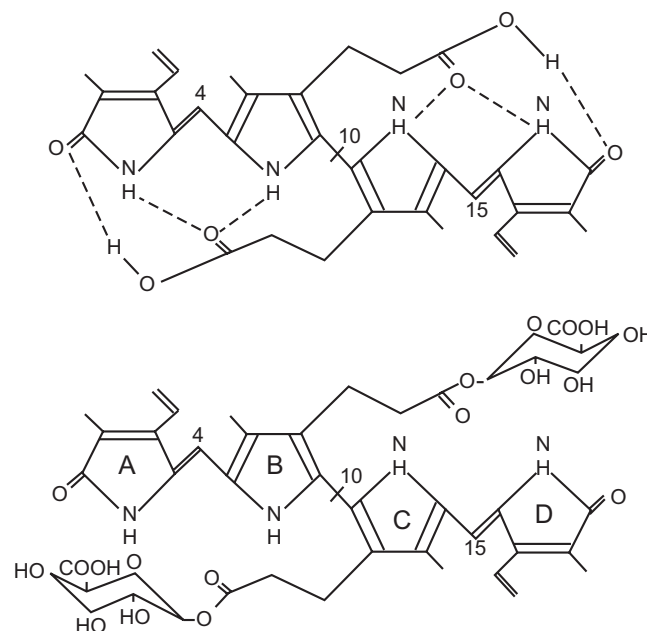
with sugar moieties disrupts the hydrogen bonds, resulting in the formation of water-soluble metabolites that are readily excreted in bile.

## 69.3.1 Absorption Spectra

Bilirubin IX $\alpha$  has a main absorption band at 450–474 nm in most organic solvents and an extinction coefficient of 48.0–63.4/mmole at its absorption maximum at 1-cm path length.

## 69.3.2 Photoisomerization of Bilirubin

Exposure of circulating bilirubin to light changes the configuration of one or both of the interpyrrolic bridges at the 5 and 15 positions to E or cis-configuration, which sterically hinders hydrogen bonding. The resulting 4Z-15E or 4E-15Z isomers lack hydrogen bonds in one half of the molecule, whereas bilirubin IX $\alpha$ -EE lacks hydrogen



**FIGURE 69-2 Upper Panel.** Hydrogen-bonded structure of bilirubin. The bilirubin molecule is contorted into a ridge-tile-like configuration caused by internal hydrogen bonding (interrupted line) of the propionic acid carboxyls to the amino groups and the lactam oxygen of the pyrrolenone rings of the opposite half of the molecule. The carbon bridges connecting pyrrolenone rings A and B (C-4) and C and D (C-15) are in the Z (trans-) configuration. By engaging the polar groups (propionic acid carboxyls and the amino and lactam groups), hydrogen bonding renders the bilirubin molecule very sparsely soluble in water. Because the central carbon bridge (C-10) is buried deep within the molecule, conjugated bilirubin reacts very slowly with diazo reagents, unless the hydrogen bonds are disrupted by adding "accelerator" reagents ("indirect diazo reaction"). **Lower Panel.** Glucuronidation of both propionic acid carboxyls results in the formation of bilirubin diglucuronide, the predominant pigment excreted in normal human bile. Glucuronidation disrupts the internal hydrogen bonds, making the molecule water soluble and exposing the central carbon bridge (C-10) to diazo reagents, resulting in "direct diazo reaction."



bonds on both halves of the molecule. Blue light is more efficient in mediating the conformational changes. Following absorption of two photons (38), the vinyl substituent at position C3 of bilirubin IX $\alpha$ -4E-15Z is cyclized with the methyl substituent on the internal pyrrole ring, forming the structural isomer E-cyclobilirubin or lumirubin (39,40). Although the rate of cyclization is slower than that of the formation of configurational isomers, because of the greater stability of lumirubin, this form may be quantitatively more important in phototherapy of neonatal jaundice (39). The photoisomers are more polar than is bilirubin IX $\alpha$ -ZZ and can be excreted in bile without conjugation (42).

## 69.4 POSSIBLE PHYSIOLOGIC BENEFITS OF BILIVERDIN AND BILIRUBIN

Generation of biliverdin and bilirubin, and the subsequent glucuronidation and canalicular transport of bilirubin, are all energetically expensive mechanisms. The evolutionary conservation of these pathways suggests a physiologic benefit of bilirubin. The antioxidant effect of bilirubin may be important during the neonatal period, when the body concentration of other natural antioxidants is low. In adults, a weak but statistically significant inverse relationship between serum bilirubin levels and the risk of coronary artery disease has been reported (43). Analysis of the Third National Health and Nutrition Examination Survey (NHANES III) database revealed that, in over 176 million individuals in the United States, the odds ratios of a history of colorectal cancer were reduced to 0.295 in men and 0.186 in women per 1-mg/dL increment of serum bilirubin levels (44). Similarly, an inverse relationship between serum bilirubin levels and cancer mortality was seen in a large study in Belgian population (45). These impressive statistical data, however, do not establish conclusively a cause-and-effect relationship, because of the possible existence of confounding variables.

## 69.5 TOXIC EFFECTS OF BILIRUBIN

The toxic effect of bilirubin on the neonatal brain has been known for many centuries. Bilirubin deposition at specific areas of the brain accompanied by structural damage is termed *kernicterus*. At elevated concentrations, unconjugated bilirubin, particularly its non-albumin-bound fraction, is toxic to astrocytes and neurons. Mitochondrial damage results in impaired energy metabolism and apoptosis, while injury to plasma membranes may disrupt the transport of neurotransmitters (46). The study of mutant Gunn rats that lack hepatic bilirubin glucuronidating activity (see later) has been pivotal in the understanding of the neurotoxicity of bilirubin. This is the only naturally mutant animal model, in which bilirubin-induced brain damage has been known to occur. Structural and functional damages of the cochlear nuclei, resulting in hearing deficiency, are

a common complication of several hyperbilirubinemia in human neonates. Cells of the auditory system that receive synaptic input from end-bulbs or calyces appear to be early targets (47). In Gunn rat pups, these morphologic changes correlate with brainstem auditory evoked potentials (48). Tight binding of unconjugated bilirubin to plasma albumin inhibits deposition of bilirubin in the brain. Displacement of bilirubin from albumin binding enhances the net transfer of bilirubin into neural tissues (50). Administration of sulfonamides in Gunn rats results in reversible brainstem auditory evoked potential abnormalities (51).

Purkinje cells of the cerebellum, hippocampus, and basal ganglia are also common targets of bilirubin deposition in Gunn rats and human infants with overt kernicterus. Purkinje cells of the cerebellum are affected in Gunn rats at the age of 7 days. Subsequent degeneration of these cells results in cerebellar hypoplasia (52). Purkinje cells that recover and persist into adult life may have abnormalities of synapse formation with other Purkinje cells or with neural cells of other types. There is enlargement and distortion of cerebellar mitochondria (54) and increased activities of the lysosomal enzymes in Gunn rats by the eighth day of life (55). Cerebellar cyclic GMP concentrations decrease progressively from day 15 to day 30, but cyclic AMP (cAMP) levels remain normal (56). Inhibition of cytochrome c oxidase activity and ascorbate-driven oxygen consumption in neonatal rat cortical neuronal cells by unconjugated bilirubin may lead to neuronal apoptosis (57).

### 69.5.1 Clinical Features of Bilirubin Neurotoxicity

In most cases, kernicterus occurs during the first few months of life. In patients with inherited absence of bilirubin glucuronidation, who may survive through infancy, kernicterus may occur during adolescence or early adulthood. Overt kernicterus usually presents between the third and sixth day of life with loss of the Moro reflex, hypotonia, athetoid movements, and reflex opisthotonus in response to startling stimuli. Left untreated, this progresses to atonia and death. In some cases, bilirubin encephalopathy may present late, with cerebellar symptoms as the presenting feature (58). Chronic effects of bilirubin encephalopathy may be observed in patients who survive acute kernicterus. These include chronic hearing abnormalities, athetoid movements, paralysis of upward gaze, and mental retardation.

Autopsy of infants dying during the acute phase of kernicterus shows bilirubin staining of the hippocampus, basal ganglia, and nuclei of the cerebellum and the brainstem (59). However, yellow staining is not found in children dying in the chronic stage of bilirubin encephalopathy. In these cases, focal necrosis of neurons and glia are observed. Gliosis of the affected area is seen in chronic cases (60).

Because histologic lesions are not present from the onset of clinical kernicterus (60), these may not be the initiating pathophysiologic events. Focal bilirubin staining of the brain may also occur in other forms of brain injury, such as cerebral hemorrhage (61), and does not, by itself, establish the diagnosis of kernicterus.

Serum unconjugated bilirubin levels above 20.0 mg/dL are usually considered dangerous (62). However, in some studies, serum bilirubin levels that are not high enough to cause kernicterus have been reported to result in an increased incidence of neurologic abnormalities or decreased intellectual performance later in life (63,64).

### 69.5.2 Pathophysiology of Kernicterus

Immaturity of the blood–brain barrier in neonates has been implicated in the high incidence of kernicterus in neonates. Tight junctions between capillary endothelial cells and foot processes of astroglial cells provide a structural barrier to equilibration of hydrophilic water-soluble substances and proteins in blood with those in the brain (65). The functional counterpart of this barrier consists of specific transport mechanisms that are involved in the translocation of ions, water, and nutrients from plasma to brain. Despite a long-standing interest in the blood–brain barrier in the newborn, it has been difficult to confirm an enhanced passage of labeled markers (66) into the immature brain, and the evidence for an immature blood–brain barrier in the neonate is not convincing.

Infusion of hypertonic urea (68) or arabinose in the carotid artery of newborn rats results in osmotic shrinkage of capillary endothelial cells, thereby opening the blood–brain barrier transiently. While the barrier remains open, intravenously administered albumin-bound bilirubin rapidly enters the brain. After discontinuation of the hyperosmolar infusion, the blood–brain barrier becomes reconstituted, and bilirubin is rapidly cleared from the brain (69). Accumulation of unconjugated bilirubin in the cerebrospinal fluid and the brain tissue is limited by its active export, probably mediated by multidrug-resistance-related protein-1 (MRP1/ABCB1), the ATP-dependent pump, present in choroid plexus epithelia, capillary endothelia, astrocytes, and neurons (46). Unconjugated bilirubin may downregulate MRP1/ABCB1 in cells constituting the blood–cerebrospinal fluid barrier, thereby potentiating bilirubin encephalopathy (70). Clearance of bilirubin may be inefficient in damaged and edematous brains, which may bind bilirubin (71). Therefore, bilirubin may be more toxic to brains that are already damaged from other causes.

### 69.5.3 Biochemical Mechanism of Bilirubin Toxicity

Bilirubin inhibits a vast array of metabolic, enzymatic, and synthetic functions of the brain. However, it is not

clear whether these toxic effects, observed in tissue culture or in vitro, are relevant in the clinical manifestation of bilirubin encephalopathy. Bilirubin may uncouple oxidative phosphorylation and inhibit ATPase activity of brain mitochondria (73). Bilirubin reduces local cerebral glucose utilization in immature rats, particularly in the auditory, visual, hypothalamic, and thalamic regions (74). Bilirubin inhibits non-cAMP-dependent protein kinase activity in vivo (75). Irreversible inhibition of  $\text{Ca}^{2+}$ -activated, phospholipid-dependent protein kinase C activity and cAMP-dependent protein kinase activity has been reported (76,77), suggesting that an abnormality of protein phosphorylation may play a role in bilirubin encephalopathy in the newborn.

### 69.5.4 Bilirubin Nephrotoxicity

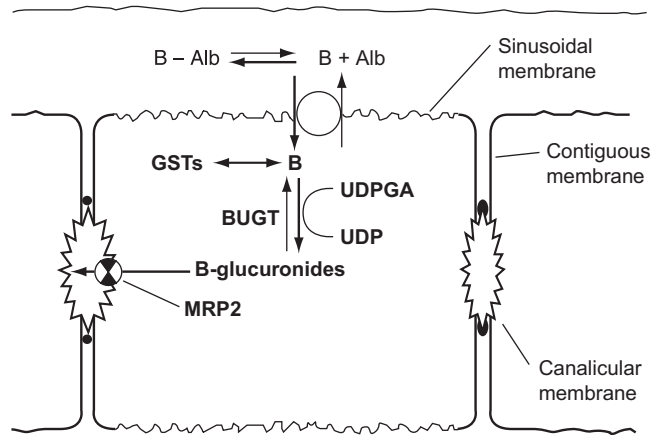
In Gunn rats (78) and hyperbilirubinemic infants (79), deposition of unconjugated bilirubin in the renal medulla results in medullary necrosis and formation of visible bilirubin crystals on the papillae. Impairment of urinary concentration resulting from an abnormality of the ascending loop of Henle occurs in Gunn rats (78,80), but not in mature neonates with hyperbilirubinemia (81) or adult patients with Crigler–Najjar syndrome type I (82).

## 69.6 DISPOSITION OF BILIRUBIN

Similar to many other water-insoluble substances, bilirubin remains tightly bound to plasma proteins, particularly albumin. In the liver, the albumin–bilirubin complex dissociates and bilirubin, but not albumin, is internalized. Within the cytosol, bilirubin is stored bound to cytosolic proteins, particularly to glutathione-S-transferases (GSTs). Bilirubin is rendered water soluble by conjugation with glucuronic acid, catalyzed by a microsomal bilirubin uridine diphosphoglucuronate glucuronosyltransferase (UGT1A1). Bilirubin glucuronides are transported across the bile canalicular membrane and are carried to the duodenum by the bile duct. Other substances that are sparingly water soluble, or are organic anions in nature, and are destined to be excreted in the bile, share some of these steps with bilirubin. The steps of bilirubin throughput are briefly described in this section and are schematically shown in Figure 69-3.

### 69.6.1 The Role of Albumin

Owing to its structural peculiarity (see earlier), bilirubin is only sparingly water soluble at physiologic pH. Binding to albumin keeps the pigment in solution and prevents its deposition into tissues, including the brain. Thus, albumin facilitates the transport of bilirubin to the liver and prevents its toxic effects. Although inherited analbuminemia is compatible with life, the biliary recovery of amphipathic compounds such as bilirubin



**FIGURE 69-3** A schematic diagram summarizing the metabolism of bilirubin by hepatocytes. In plasma, bilirubin is tightly but reversibly bound to albumin. In liver sinusoids, the albumin–bilirubin complex comes in direct contact with the basolateral domain of the hepatocyte plasma membrane (sinusoidal membrane) through fenestrae of the specialized hepatic endothelial cells, where the albumin–bilirubin complex dissociates. Bilirubin is taken up by the facilitated diffusion, the molecular basis of which is not fully understood. Within the hepatocyte, bilirubin is stored bound to glutathione-S-transferases (GSTs), which inhibit its efflux from the cell, thereby increasing the net uptake. Glucuronidation of bilirubin in the endoplasmic reticulum is catalyzed by uridine diphosphoglucuronate glucuronosyl-transferase-1 (UGT1A1), forming bilirubin monoglucuronide and diglucuronide. Conjugated bilirubin is secreted across the bile canaliculus by an energy-requiring transporter, MRP2, and to a lesser extent by electrogenic transport. Canalicular transport of bilirubin is shared by other many organic anions, including glucuronides and glutathione conjugates, but not by most bile salts.

and bromosulphophthalein (BSP) is reduced in genetically analbuminemic rats (83). In this situation, other plasma proteins take over some of the functions of albumin. The neural toxic effect of bilirubin is markedly enhanced in the analbuminemic rat.

There are a primary and a secondary binding site on albumin for bilirubin (84), and some weaker binding sites may exist (86,87). The lysine 240 in human albumin and lysine 238 in bovine serum albumin appear to be primary bilirubin-binding sites (88). Other ligands that bind at the same site as bilirubin, such as sulfonamides, anti-inflammatory drugs, and cholecystographic contrast media, may displace bilirubin competitively from albumin (86), thereby precipitating bilirubin encephalopathy in newborns (89), without changing serum bilirubin levels.

Normally, albumin is present in molar excess to bilirubin. The reserve bilirubin-binding capacity of albumin acts as a buffer for rapid fluctuations of serum bilirubin levels as in acute hemolysis. Owing to the influence of many metabolites and drugs on albumin binding of bilirubin, measurement of unbound plasma bilirubin and the reserve bilirubin-binding capacity provides a more accurate estimate of the risk of brain damage from unconjugated bilirubin than does the measurement of total bilirubin concentrations alone. Unbound bilirubin in serum can be quantified by the gel chromatography

(90), peroxidase treatment (52), electrophoretic analysis (91), and fluorometry (92). In conditions associated with the presence of conjugated bilirubin in plasma for a long duration, bilirubin becomes irreversibly bound to albumin (93). Owing to the long half-life of albumin, this fraction, which is not cleared by the liver or kidney, lingers for a long time in serum.

### 69.6.2 Bilirubin Uptake by Hepatocytes

Bilirubin is delivered to the liver bound to plasma albumin. Because the endothelial lining of hepatic sinusoids is fenestrated, the albumin–bilirubin complex comes in direct contact with the sinusoidal and basolateral plasma membrane domains of the hepatocyte. Bilirubin dissociates from albumin at or close to the hepatocyte surface before it is taken up into the hepatocyte. Whether this dissociation is facilitated by an albumin receptor at the hepatocyte surface remains speculative. In the presence of spontaneous portosystemic shunts, as in cirrhosis of the liver, or surgically induced diversion of portal blood from the liver, bilirubin produced in the spleen does not come in contact with hepatocytes in the first pass. This results in a mild unconjugated hyperbilirubinemia. An open ductus venosus in the newborn may exacerbate “physiologic” jaundice in infants by a similar mechanism.

Bilirubin is taken up by the hepatocyte by facilitated diffusion (94), which is bidirectional and does not consume energy, but needs the presence of inorganic anions, such as  $\text{Cl}^-$  (95,96). The mechanism of bilirubin uptake at the sinusoidal surface is not yet understood precisely (97,98). Unconjugated bilirubin is relatively nonpolar, and some investigators have suggested that it may diffuse through the sinusoidal membrane simply by dissociating from albumin, without requiring transporter proteins (99). However, concentrative bilirubin uptake is a hepatocyte-specific function (100). Therefore, hepatocyte-specific carrier proteins have been sought. OATP2 (also known as OATP-C or SLC21A6) in human liver and OATP4 (S1c21a10) in rat liver are high-affinity transporters of organic anions such as BSP, taurocholate, estradiol-17 $\beta$  glucuronide, LTC4, estrone-3-sulfate and estrone-1-sulfate, dehydroepiandrosterone sulfate, triiodothyronine, and thyroxine (101,102). Some studies have suggested that OATP-C also transports unconjugated bilirubin (101), but other studies did not support this conclusion (103). NTCP (SLC10A1) is a specialized carrier for the  $\text{Na}^+$ -dependent hepatic uptake of bile salts (104–107), which does not appear to be directly related to bilirubin uptake. Thus, the physiologically important bilirubin transporter remains to be identified at this time.

### 69.6.3 Storage of Bilirubin within the Hepatocyte

Within the hepatocyte, bilirubin binds to cytosolic proteins that keep it in solution predominantly. The

bilirubin-binding proteins were originally termed the Y (ligandins) and Z (fatty-acid-binding) proteins (108). Later, the Y proteins or ligandins were found to consist of the  $\alpha$  class of GSTs (109). Binding to GSTs does not affect the influx of bilirubin directly, but increases the net input by inhibiting its diffusion out of the hepatocytes (110).

### 69.6.4 Bilirubin Conjugation

**69.6.4.1 Conversion of Bilirubin to Polar Derivatives.** Conjugation of the propionic acid carboxyls with sugar moieties, particularly glucuronic acid (111), is the major mechanism by which the internal hydrogen bonds of bilirubin are disrupted and the molecule becomes water soluble. Depending on whether one or both propionic acid carboxyls are glucuronidated, bilirubin monoglucuronide or diglucuronide is formed (113), both of which are efficiently excreted in bile. Bilirubin diglucuronide is the major pigment in normal human and dog bile (111). In addition, smaller amounts of glucosyl and xylosyl conjugates have been described in human T-tube bile (113,115) and bile from other species (116).

**69.6.4.2 Enzyme-Catalyzed Glucuronidation of Bilirubin.** A family of enzymes termed uridine diphosphoglucuronate glucuronosyltransferases (UGTs; EC 2.4.1.17), located in the endoplasmic reticulum and nuclear envelope of a variety of cells (117), catalyzes the transfer of the glucuronosyl moiety of UDPglucuronate to aglycone substrates. Substrates of this group of enzymes comprise a wide spectrum of substances, including hormones (e.g. steroid hormones, thyroid hormones, and catecholamines), endogenous metabolites (e.g. bile salts and bilirubin), numerous drugs and their intermediate metabolites, toxins (e.g. carcinogens), and laboratory xenobiotics (118). The products of the transferase reaction are ether, ester, thiol, and N-glucuronides. Glucuronides are more polar than the aglycone substrates and usually less biologically active. Because of the wide array of substrates that are conjugated via UGTs, this group of enzymes constitutes a major detoxification system of the body.

The catalytic activity of UGTs is partly latent in microsomal vesicles. Full enzyme activity is obtained by perturbing the microsomal membrane by detergent (119) or enzymatic (120) treatment. UDP-N-acetylglucosamine, which activates hepatic microsomal UGT activity at low concentrations, may be a physiologic activator of the UGTs. According to one postulated model, the catalytic site of UGT is located inside the lumen of the endoplasmic reticulum, and the donor substrate, UDP-glucuronic acid, must be transported through the membrane barrier to the catalytic site (121). In this model, UDP-N-acetylglucosamine is thought to increase UDP-glucuronate transport by activating a putative “permease.” Detergents and other membrane-perturbing agents

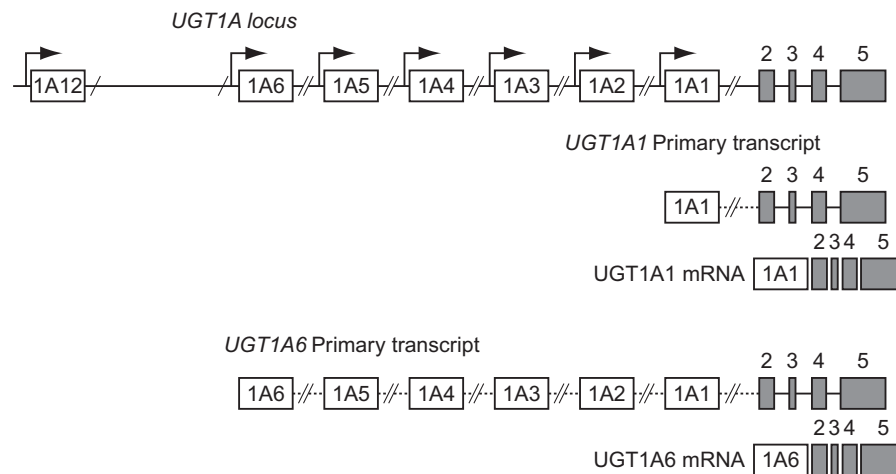
may enhance UGT activity in vitro by physically increasing the permeability of the membranes to UDP-glucuronic acid (122). An alternative model proposes that the physical or chemical activators of UGT act by releasing the membrane constraint of UGT (123), and UDP-N-acetylglucosamine acts as an allosteric activator of the enzyme. It should be noted that the two models may not be exclusive of each other.

The carboxy-terminal domain of all UGT isoforms has a high level of sequence homology, and is thought to bind the common substrate, UDPglucuronic acid (124). The amino-terminal domain is less homologous in structure and imparts aglycone substrate specificity to the enzymes (125).

**69.6.4.3 Families and Subfamilies of UGT.** The UGT system consists of a large number of structurally related enzymes, all of which accept UDPglucuronic acid as a donor substrate, but differ in aglycone substrate specificity (126–128,130,131). UGT isoforms differ in ontogenic development (132) and effect of enzyme inducing agents (133). On the basis of the extent of structural homology of the respective messenger RNAs (mRNAs), UGT isoforms have been classified into several families and subfamilies (134). The UGT1A locus expresses one group of UGT isoforms (135). The enzymes have identical carboxy-terminal domains (136–138), but variable amino-terminal regions. The UGT1A locus consists of four consecutive exons (exons 2–5) at the 3' end, which are used in the mRNAs of all isoforms expressed from this locus and encode the identical carboxy-terminal domain (Figure 69-4). Upstream to these are a series of 13 unique exons, each preceded by a promoter. Only one of these exons is used in a given UGT isoform. The process of transcription can start at any of the promoters, giving rise to transcripts of different lengths. The presence of a separate promoter upstream to each of these variable-region exons explains independent regulation of enzymes expressed from this gene and their different organ distribution and expression during ontogeny, enzyme induction, or carcinogenesis. Enzyme activity toward 4-nitrophenol and other simple phenolic substrates develops in late fetal life in rats, whereas activity toward bilirubin develops after birth (132). UGT1A6, a 3-methylcholanthrene-inducible isoform, is permanently overexpressed in carcinogen-induced preneoplastic nodules in rat liver (137). Triiodothyronine treatment results in a threefold increase in rat liver phenol-UGT activity, whereas bilirubin glucuronidation is reduced by 80% (139).

The unique exon that is located at the 5' end of the transcript is spliced to exon 2, and the intervening sequences are spliced out. In this manner, the UGT1A locus expresses nine UGT isoforms (the other four unique exons represent pseudogenes). Thus, the UGT1A locus is considered to comprise multiple overlapping genes, each of which is named according to the unique exon that is used in the processed mRNA. For example,





**FIGURE 69-4** A schematic representation of the human UGT1A locus. This locus is located in chromosome 2 at 2q37 (373). Exons 2–5 are shared by all UGT isoforms expressed from this locus. Upstream to these common-region exons, there is a series of variable-region exons, designated exons UGT1A1 through UGT1A12, only one of which is used in any given UGT isoform. Each of these exons encodes the variable amino-terminal region of one UGT isoform. Each variable-region exon has an upstream promoter element, and is differentially regulated. Depending on the promoter selection, primary transcripts of various lengths are produced. The variable exon 1 at the 5' end of the transcript is spliced to the common exons 2–5, resulting in the formation of a mature messenger RNA (mRNA) with a unique exon 1. Several species of UGT mRNAs are produced in this manner, all of which have identical 3' domains. Therefore, a genetic lesion in any of the common-region exons affects all mRNAs expressed from this locus.

if transcription starts at the 3'-most unique exon, the gene is termed *UGT1A1*. On the other hand, if the transcription starts at the sixth unique exon, the gene is termed *UGT1A6*. Initially, *UGT1A1* and *UGT1A4* were reported to have activity toward bilirubin in humans (140), but later, only *UGT1A1* was shown to contribute physiologically significant bilirubin glucuronidating activity (53). The *UGT1* gene in the rat has a similar exon organization (136), indicating that this gene is highly conserved during evolution.

### 69.6.5 Excretion of Conjugated Bilirubin across the Bile Canalculus

In contrast to the transport across the sinusoidal surface plasma membranes, canalicular secretion of conjugated bilirubin and other cholephilic compounds occurs against a high concentration gradient, whereby the liver:bile concentration ratio for organic anions, such as dibromosulfophthalein, may reach 1:1000 (143). Such large gradient cannot be accounted for by the  $-35$  mV potential difference across the canalicular membrane, and suggest the presence of active transport systems (144–147). MRP2 (also termed ABCC2), a member of the ATP-binding cassette (ABC) protein family, serves as the canalicular efflux pump for non-bile-acid organic anions, including bilirubin and other glucuronide and glutathione conjugates (148–150). Genetic deficiency of MRP2/ABCC2 in humans leads to the Dubin–Johnson syndrome, characterized by retention of conjugated bilirubin in plasma (152,153). The TR– rat, which lacks mrp2/ABCC2 function because of a single-nucleotide deletion of the *mrp2/abcc2* gene, is an animal model of Dubin–Johnson syndrome (154,155). Sequence analysis

of the human MRP2/ABCC2 promoter showed a number of putative consensus binding sites for both ubiquitous and liver-enriched transcription factors, including AP1, SP1, HNF1, and HNF3 $\beta$  (156,157), as well as the nuclear receptors CAR, PXR, and FXR.

Two other ABC family proteins, mrp1 and mrp3, are expressed at low levels in rat liver, but are upregulated during cholestasis (159,160). Both mrp1 and mrp3 are located in the basolateral membrane of the hepatocytes and pump accumulating bilirubin glucuronides out of the hepatocytes into the plasma (156,161). Mrp1 also pumps glutathione S-conjugates (162), as well as unconjugated bilirubin. Mrp3 expression is upregulated in the liver of mrp2-deficient rats and in UGT1A1-deficient Gunn rats (156). Thus, MRP3 (mrp3 in rodents) functions as a reverse transporter, which pumps substrates back to the blood when biliary excretion of glucuronides is reduced.

Several other ATP-dependent canalicular transporters have been identified and cloned. *FIC1* is a gene mapped to chromosome 18q21 (163), the product of which may couple the hydrolysis of ATP to the translocation of acidic phospholipids (e.g. phosphatidylserine and phosphatidylethanolamine) from the outer to the inner layer of the plasma membrane. The gene for another transport protein termed *bile salt export pump*, also known as sister of P-glycoprotein, is located on chromosome 2q24 (165). MDR3/ABCB4 in humans (*mdr2* in mice) mediates ATP-dependent translocation of phosphatidylcholine from the inner to the outer leaflet of the bile canalicular plasma membrane (166). Inherited defects of any of these genes result in disordered bilirubin excretion by direct or indirect mechanisms.

### 69.6.6 Nuclear Receptors

Nuclear receptors may orchestrate the various steps involved in bilirubin throughput: maximum capacities for bilirubin uptake, storage, conjugation, and canalicular excretion appear to be similar *in vivo*. Therefore, reduction of any of these steps can lead to hyperbilirubinemia. On the other hand, enhancement of bilirubin excretion, which may be needed when bilirubin production is increased, would require coordinated increase in the capacity of each. It has been proposed that the nuclear receptor, CAR, serves as the co-ordinating mechanism for physiologic modulation of each of these steps (167,168).

### 69.6.7 Degradation of Bilirubin in the Gastrointestinal Tract

The small amount of unconjugated bilirubin that reaches the intestine is partly reabsorbed. This reabsorption may be greater when the infant is fed maternal milk than when a baby formula is used. Therefore, breast-feeding may contribute to neonatal hyperbilirubinemia (169). Conjugated bilirubin is not substantially absorbed from the intestine (170). Intestinal bacteria deconjugate bilirubin (171) and degrade it to urobilinogens and related products (172). Urobilinogens absorbed from the intestine are re-excreted in the bile and, to a smaller extent, in the urine. In liver disease and increased bilirubin production, urinary urobilinogen excretion is increased. However, because the extent of reabsorption of urobilinogen by renal tubules varies, and the pigment is unstable in acid urine, quantification of urobilinogen excretion in urine is not of clinical benefit. However, complete absence of urobilinogen in stool and urine indicates complete bile duct obstruction. Urobilinogen is colorless. Its yellow oxidation product, urobilin, contributes to the characteristic color of urine and stool.

### 69.6.8 Extrahepatic Disposition of Bilirubin

During biliary obstruction, urinary excretion becomes the major excretory pathway for bilirubin (173). In children with biliary atresia, 50–90% of bilirubin excretion may occur through the kidney (174). Unconjugated bilirubin is tightly bound to albumin and is not filtered by normal renal glomeruli, and therefore does not appear in the urine. A fraction of conjugated bilirubin that is not bound to albumin is filtered by renal glomeruli (173) and is excreted in urine. Therefore, excretion of bilirubin in the urine, in the absence of albuminuria, indicates the presence of conjugated bilirubin in plasma. Unconjugated bilirubin entering the renal tubules is reabsorbed, but is not secreted by the tubules (175). Although UGT1A1 activity is present in the proximal small intestinal villi (117), the relative contribution of small intestine in bilirubin disposition is not known. Small amounts

of unconjugated bilirubin pass to the intestinal lumen across the intestinal epithelium or by exfoliation of the epithelial cells (176).

### 69.6.9 Alternative Pathways of Bilirubin Disposition

As discussed in the preceding sections of this chapter, exposure to light results in the formation of configurational (EZ, ZE, or EE forms) and cyclic (e.g. lumirubin) isomers of bilirubin formed in the presence of ambient light or during phototherapy. These isomers are more polar than bilirubin IX $\alpha$ -ZZ, and are excreted in bile in the unconjugated form (42). A significant fraction of bilirubin undergoes photodegradation to polar diazo-negative compounds that are excreted in bile and urine (38).

## 69.7 BILIRUBIN MEASUREMENT

Total serum bilirubin level and the conjugated bilirubin fraction are routinely determined as markers of liver function. In the newborn period, the fractional concentration of non-protein-bound bilirubin is a more reliable predictor of potential neurotoxicity, and helps in determining the need for institution of therapy to reduce serum bilirubin levels. Under some circumstances, the measurement of, covalently, albumin-bound bilirubin may add clinical insight to bilirubin disposition.

Serum bilirubin is usually measured after conversion to stable azo-derivatives, analysis of intact tetrapyrroles being mainly used for research on bilirubin metabolism (177).

### 69.7.1 Bilirubin Measurement Following Reaction with Diazo Reagents

Reaction with a diazonium ion cleaves the bilirubin molecule at the central carbon bridge, and derivatizes the two resulting dipyrroles at the C9 and C11 positions of bilirubin (178). Because conjugated bilirubin lacks internal hydrogen bonds, the central methane bridge is readily accessible to the diazo reagents. Therefore, conjugated bilirubin reacts rapidly with diazo reagents (“direct” fraction) (179). On addition of “accelerator” substances, such as methanol or caffeine, both conjugated and unconjugated bilirubin react rapidly (total bilirubin). The “indirect” fraction of bilirubin is calculated by subtracting the “direct” fraction from total bilirubin. In order to characterize the azodipyrroles, they can be separated by thin-layer chromatography (180) or high-performance liquid chromatography (HPLC) (181). Because up to 15% of unconjugated bilirubin in solution may exhibit direct diazo reaction, this method slightly overestimates conjugated bilirubin. Therefore, a direct-reacting bilirubin concentration of <15% of total bilirubin is considered normal although normally only up to 4% of serum bilirubin is conjugated. The fraction of

bilirubin that is covalently bound to albumin also gives a direct reaction (182). As the irreversibly protein-bound bilirubin is cleared slowly from serum after relief of biliary obstruction, the finding of direct-reacting bilirubin during this period may give a false impression of continued biliary obstruction.

### 69.7.2 Chromatographic Analysis of Bilirubin as Intact Tetrapyrrole

For accurate identification of bilirubin and its conjugates for research purposes, methods based on thin-layer chromatography (180) or HPLC (116,183–185) have been developed. When the identification of specific sugar conjugates is not required, bilirubin mono- and diconjugates can be converted to mono- and dimethyl esters, respectively, by alkaline methanolysis before analysis (186). To measure the covalently albumin-bound fraction of bilirubin ( $\delta$ -bilirubin), HPLC is performed on incompletely deproteinated serum (182). The use of chromatographic methods of bilirubin analysis is generally limited to research laboratories.

### 69.7.3 Slide Test

The Ektachem slide test measures conjugated, unconjugated, and irreversibly protein-bound bilirubin using a diazo technique. One slide measures total bilirubin, and another specially coated slide allows only the free and reversibly protein-bound bilirubins to react with the diazo reagent; irreversibly protein-bound bilirubin can be estimated from the difference (187).

### 69.7.4 Transcutaneous Bilirubinometry

To assess the risk of severe neonatal hyperbilirubinemia, it is useful to evaluate the rate of increase of serum bilirubin levels during the first 24–48 h of life. Repeated estimation of serum bilirubin levels can be accomplished in a noninvasive manner by measuring the yellow color of the skin in reflected light, using computer analysis to circumvent the interference by underlying skin color (188,189).

## 69.8 BILIRUBIN IN BODY FLUIDS

### 69.8.1 Bilirubin in plasma

In the plasma, 96% of total bilirubin is unconjugated although the direct-reacting fraction, as determined by using diazo reagents, may slightly overestimate the conjugated fraction. During bilirubin overproduction, both unconjugated and conjugated fractions increase and their proportion remains unchanged. In contrast, reduced levels of bilirubin glucuronidating activity result in a lower proportion of conjugated bilirubin. In cases of intrahepatic cholestasis, biliary obstruction, or hepatocellular diseases, as well as in Dubin–Johnson and Rotor

syndromes, both conjugated and unconjugated bilirubin accumulate in plasma, and the proportion of conjugated bilirubin increases. Under these conditions, MRP2 is downregulated, reducing biliary excretion of conjugated bilirubin. Bile pigments accumulating in hepatocytes may be pumped out into plasma by alternative pumps (e.g. MRP1 and MRP3), which are upregulated during cholestasis. Following prolonged accumulation of conjugated bilirubin, a fraction of the pigment binds to albumin irreversibly. This fraction, termed  $\delta$ -bilirubin, gives a direct diazo reaction and can be identified by chromatographic analysis. After resolution of biliary obstruction or intrahepatic cholestasis,  $\delta$ -bilirubin may linger in plasma for several weeks because it is not taken up by hepatocytes or excreted in the urine (190).

### 69.8.2 Bilirubin in Bile

In human bile, over 80% of bilirubin is diglucuronide, with only approximately 4% being unconjugated bilirubin. When UGT1A1 activity is absent, as in the case of Crigler–Najjar syndrome type I, little or no bilirubin glucuronides are excreted in bile. In Crigler–Najjar syndrome type II or Gilbert syndromes, in which hepatic UGT1A1 activity is lower than normal, proportions of bilirubin monoglucuronide and unconjugated bilirubin increase in bile. The presence of a significant amount of conjugated bilirubin in bile reliably differentiates Crigler–Najjar syndrome type I from Crigler–Najjar syndrome type II (see later).

## 69.9 DISORDERS OF BILIRUBIN METABOLISM

Hyperbilirubinemia may result from increased bilirubin production, reduced uptake from the circulation, abnormal intracellular storage, deficiency of UGT1A1 activity, or biliary excretion of bilirubin. In many acquired clinical disorders, such as hepatitis or cirrhosis, several steps of this process are affected. In contrast, in inherited disorders of bilirubin metabolism, a specific step of bilirubin throughput may be involved. From the standpoint of bilirubin metabolism, these disorders may be classified into those that cause unconjugated hyperbilirubinemia and those associated with significant retention of conjugated bilirubin in serum.

### 69.9.1 Metabolic Disorders Causing Unconjugated Hyperbilirubinemia

**69.9.1.1 Neonatal Hyperbilirubinemia.** Serum bilirubin levels are higher in newborns compared to those in normal adults, with obvious jaundice occurring in about 50% during the first 5 days of life. Normally, serum bilirubin concentrations increase from 1–2 mg/dL at birth to a peak of 5–6 mg/dL in about 72 h, and subsequently decline to <1 mg/dL in 7–10 days (129).

In normal newborns, serum bilirubin is predominantly unconjugated. Exaggeration of this physiologic jaundice raises the concern of bilirubin-induced neuropathy. Serum bilirubin concentrations reach 10 mg/dL in about 16% of newborns, and the level exceeds 15 mg/dL in 5% (192). Jaundice of the newborn results from an increased bilirubin load and a lower capacity of the liver to dispose of bilirubin. Exaggeration of these factors and/or superimposition of additional mechanisms may result in a pathologic level of hyperbilirubinemia. The mechanisms of neonatal jaundice were briefly considered here.

**69.9.1.1.1 Increased Bilirubin Load.** Bilirubin production, as measured by carbon monoxide production, is increased in the newborn period (193). The excess bilirubin is derived from erythroid and nonerythroid sources and from shortened erythrocyte half-life (194). Rh incompatibility between mother and fetus used to be a common cause of severe neonatal jaundice before treatment of the mother with anti-Rh immunoglobulins became available (72). ABO blood group incompatibility continues to be a common cause of exaggerated neonatal hyperbilirubinemia (196). Sick cell disease, hereditary spherocytosis, and toxic or allergic drug reactions are common causes of hemolytic jaundice in the newborn period. Excessive bilirubin production can result from ineffective erythropoiesis, as in thalassemia, vitamin B12 deficiency, and congenital dyserythropoietic anemias. In cases in which the rate of bilirubin production exceeds the bile canalicular excretory capacity, conjugated bilirubin may accumulate in the serum (197).

**69.9.1.1.2 Low Hepatic Bilirubin Uptake.** During the first few days of life, the rate of hepatic uptake of bilirubin is lower than that in adults. The low uptake rate may be related to low levels of cytosolic GSTs (199), which increase net bilirubin uptake by binding bilirubin and thereby reducing its efflux. Delayed closure of the ductus venosus may permit portal blood, which is enriched in conjugated bilirubin from the intestine, to bypass the liver.

**69.9.1.1.3 Reduced Bilirubin Glucuronidation.** Only 1% of the normal adult level of hepatic UGT1A1 activity is present at birth (200). Postnatal maturation of UGT1A1 is birth related, and increases rapidly to adult levels by 14 weeks, regardless of the gestational age at birth (201). In some disorders, UGT1A1 activity is inhibited by some inherited factor(s).

**69.9.1.1.4 Maternal-Milk Jaundice.** Serum bilirubin levels in breast-fed infants are, in general, higher than in formula-fed babies (202), and, in some cases, may increase to 15–24 mg/dL by the age of 10–19 days (203). The unconjugated hyperbilirubinemia may promptly disappear when breast-feeding is discontinued. If breast-feeding is continued, the hyperbilirubinemia may last for weeks. Maternal-milk jaundice is usually benign (203), but kernicterus has been reported in rare cases (204). The maternal milk contains an inhibitor of UGT1A1

activity in this syndrome (203). It has been postulated that polyunsaturated free fatty acids produced by the action of lipolytic enzymes present in some maternal milk samples may be responsible for the inhibition of the transferase (205). Consistent with this notion, the inhibitory effect of maternal milk on bilirubin glucuronidation increases on storage and is destroyed by heating at 56°C (205). Mild maternal-milk jaundice may resolve despite continuation of breast-feeding.

**69.9.1.1.5 Maternal-Serum Jaundice.** This syndrome, associated with moderate to severe unconjugated hyperbilirubinemia (8.9–65 mg/dL) within the first 4 days of life, was described by Lucey, Arias, and associates (207,208). The syndrome is thought to be caused by an unidentified inhibitor of UGT1A1 present in the serum of mothers of these infants. The jaundice may persist several weeks and is occasionally associated with kernicterus.

**69.9.1.1.6 Decreased Canalicular Bilirubin Excretory Capacity.** Maturation of canalicular excretion processes may take longer than does the maturation of uptake and conjugation. Therefore, in the late newborn period, canalicular excretion becomes rate limiting in hepatic bilirubin disposition. In cases in which the bilirubin load is higher than normal, conjugated bilirubin accumulates in serum (209).

**69.9.1.1.7 Increased Intestinal Reabsorption.** Intestinal  $\beta$ -glucuronidase-mediated deconjugation releases unconjugated bilirubin in the intestine (210). Because of the lack of an established intestinal flora in the newborn, there is reduced bacterial degradation of bilirubin, resulting in an increased absorption of unconjugated bilirubin (210). A greater fraction of unconjugated bilirubin is absorbed from the intestine in infants fed with maternal milk.

**69.9.1.2 Hyperbilirubinemia Due to Bilirubin Overproduction.** Where liver function is normal, overproduction of bilirubin rarely increases serum bilirubin levels to >3–4 mg/dL. Common causes of overproduction of bilirubin include hematologic conditions associated with hemolysis, such as hereditary spherocytosis, and toxic or idiosyncratic drug reactions. Ineffective erythropoiesis that occurs in thalassemia and vitamin B12 deficiency, and a rare group of disorders called *dyserythropoietic anemias*, result in unconjugated hyperbilirubinemia of various degrees (211). In patients with hemolytic jaundice but normal liver function, a small amount of conjugated bilirubin produced in the liver may appear in the circulation (212), but the unconjugated:conjugated bilirubin ratio remains normal. In sickle cell anemia, a combination of hemolysis and liver abnormalities may lead to the accumulation of both unconjugated and conjugated bilirubin.

**69.9.1.3 Inherited Disorders of Bilirubin Glucuronidation.** Three inherited syndromes associated with a deficiency of bilirubin glucuronidation have been described. A near-complete deficiency of UGT1A1



activity results in Crigler–Najjar syndrome type 1. Severe but incomplete deficiency of UGT1A1 activity results in Crigler–Najjar syndrome type 2 also known as *Arias syndrome*. A mild reduction of UGT1A1 activity results in a common benign disorder, *Gilbert syndrome*, in which a mild and fluctuating unconjugated hyperbilirubinemia is the only significant clinical feature. Clinical and biochemical features of these syndromes are summarized in Table 69-1 and are described in this section.

**69.9.1.3.1 Crigler–Najjar Syndrome Type 1.** Crigler and Najjar (213) described this rare, recessively inherited syndrome in 1952 in six infants from three unrelated families. After the discovery of UGT, this syndrome was found to result from an absence of UGT1A1 activity (214). All patients had lifelong severe nonhemolytic unconjugated hyperbilirubinemia. Five of the six infants in the initial report died of bilirubin-induced encephalopathy within 15 months. The sixth patient remained free of brain damage until the age of 15 years, when kernicterus developed and death followed in 6 months (214). Another related patient remained without brain damage until 18 years of age, but then developed bilirubin encephalopathy and died at the age of 24 (215). The original family described by Crigler and Najjar had a high degree of consanguinity. Several members of this family had other recessively inherited disorders, such as Morquio syndrome, homocystinuria, metachromatic leukodystrophy, and bird-headed dwarfism. In subsequently studied families, the other recessively inherited disorders were not observed. Since 1952, several hundred other patients with Crigler–Najjar syndrome type 1 have been described in all races, and an autosomal-recessive inheritance has been established (214). Icterus is often the only finding on physical examination. However, in many patients, residual neurologic abnormalities, a

sequel to a previous episode of bilirubin encephalopathy, may be detected. With routine use of phototherapy and plasmapheresis for reversing acute bilirubin encephalopathy, many patients with Crigler–Najjar syndrome type 1 now survive through childhood. However, many survivors develop kernicterus around puberty or in early adult life (215). Use of phototherapy usually prolongs survival without encephalopathy to adolescence, and liver transplantation results in long-term survival (see later). Because of a relatively high concentration of unconjugated bilirubin in bile, pigment stones are common.

**69.9.1.3.1.1 Laboratory Tests.** Serum bilirubin levels usually range from 20 to 25 mg/dL, but may reach 50 mg/dL (213–215). Serum bilirubin is unconjugated, and there is no bilirubinuria. The concentration of serum bilirubin increases during intercurrent illnesses, and decreases on exposure to the sun or bright artificial light (41). The bile is paler than normal (217) and contains only small amounts of unconjugated bilirubin (218). The stool color is normal despite reduced fecal urobilinogen excretion (213). There is no evidence of hemolysis (217), and bilirubin is produced at a normal rate (219). Because bile canalicular transport is normal, BSP (213) and indocyanin green (220) and cleared normally from serum, and the biliary tree is normally visualized by cholecystographic agents.

**69.9.1.3.1.2 Liver Histology.** Liver histology is normal, except that, in several patients, bilirubin plugs were found to be deposited in bile canaliculi and bile ducts (213,218), probably resulting from biliary excretion of unconjugated bilirubin or its photoisomers.

**69.9.1.3.1.3 Abnormalities of Hepatic UGTs.** Hepatic UGT activity toward bilirubin is virtually absent in all patients with Crigler–Najjar syndrome type 1. In addition, many of these patients have reduction of the

**TABLE 69-1 Inherited Disorders Causing Unconjugated Hyperbilirubinemia**

	<b>Crigler–Najjar Syndrome Type 1</b>	<b>Crigler–Najjar Syndrome Type 2</b>	<b>Gilbert Syndrome</b>
Serum bilirubin concentration	340–850 $\mu$ M	<340 $\mu$ M	Usually <50 $\mu$ M
Routine liver function tests	Normal	Normal	Normal
Serum bile acid levels	Normal	Normal	Normal
Oral cholecystography	Normal	Normal	Normal
Liver histology	Normal	Normal	Normal
Bile proportion of monoglucuronide	Usually pale; contains small amounts of unconjugated bilirubin	Increased proportion of bilirubin monoglucuronide	Increased bilirubin
Hepatic BUGT activity	None	10% of normal or less	25–40% of normal
Effect of phenobarbital	None	Reduction by 25% or more	Reduction on serum bilirubin
Mode of inheritance	Autosomal recessive	Autosomal recessive	Autosomal recessive
Prevalence	Rare	Uncommon	Common, ~5% in general population
Prognosis	Kernicterus is the rule	Usually benign; kernicterus is rare	Benign
Animal model monkey, Southdown sheep	Gunn rat	–	Bolivian squirrel mutant

BUGT, bilirubin uridine diphosphoglucuronate glucuronosyltransferase.

level of glucuronidation of phenolic substrates (221). Immunologic analysis using monoclonal and polyclonal antibodies revealed variability of expression of UGT proteins in the liver of different patients with Crigler–Najjar syndrome type 1 (221).

Cloning and characterization of the human UGT1A locus in 1992 (135,140) has permitted the elucidation of the genetic basis of Crigler–Najjar syndrome type 1 (48,49,223). Because UGT1A1 is the only UGT isoform that contributes significantly to bilirubin glucuronidation, genetic lesions within any of the five exons comprising the UGT1A1 gene may lead to complete or near-complete loss of hepatic bilirubin glucuronidation. Such genetic lesions may consist of point mutations, deletions, or insertions within the coding region. Intronic mutations at splice donor or acceptor sites may lead to inappropriate splicing, resulting in loss of bilirubin–UGT activity (224). The genetic lesions may result in mutation of a single critical amino acid or deletion of segments of the enzyme. The various genetic lesions described in the literature (Table 69-2) have been reviewed recently (226–230). In cases where the genetic lesions are present in exons 2–5, all UGT isoforms expressed from the UGT1A locus are affected. However, when the mutation is located in the unique first exon of UGT1A1, only bilirubin glucuronidation is affected.

A large number of mutations can result in Crigler–Najjar syndrome type 1, and, in most cases, no particular mutation is common in a given community. An exception to this is seen in the Amish–Mennonite community, in which Crigler–Najjar syndrome type I is relatively common and all patients carry the same mutation (N. Roy Chowdhury et al., unpublished observation), indicating transmission of this mutation from a common ancestor through intermarriage within the small community.

Molecular diagnosis of Crigler–Najjar syndrome is achieved by amplification of the five exons of UGT1A1 and their flanking sequences by polymerase chain reaction (PCR) and sequence determination of the amplicons. The same strategy can be applied to perform prenatal diagnosis using chorionic villus samples as the starting material (232). Although in a great majority of cases, inheritance of genetic lesions from both parents is required for the manifestation of Crigler–Najjar syndrome, recently an instance of uniparental isodisomy has been reported in which both mutant alleles were inherited from the father (233). The mother's UGT1A1 genotype was normal. This case highlights the desirability of analyzing the genotype of both parents to determine the mode of inheritance of Crigler–Najjar syndrome.

The Gunn rat, and animal model of Crigler–Najjar syndrome type 1. Gunn rats are a mutant strain of Wistar rats that exhibit lifelong nonhemolytic unconjugated hyperbilirubinemia inherited as an autosomal-recessive characteristic (234) because of the deficiency of UGT activity toward bilirubin (235). The Gunn rat is the only animal model that develops bilirubin encephalopathy

spontaneously (236). Studies performed in Gunn rats have provided important information on bilirubin toxicity and have helped in developing new therapeutic modalities for hyperbilirubinemia, including cell transplantation and gene therapies (224,237–239). Gunn rats have a single guanosine base deletion in the common-region exon 4, which results in a frameshift, leading to a premature termination codon. As a consequence, 150 amino acid residues at the carboxy-terminal end of all isoforms encoded by the UGT1A locus are deleted (240) and their activities affected (241). However, UGT isoforms expressed from other UGT genes are unaffected (242).

**69.9.1.3.1.4 Treatment of Crigler–Najjar Syndrome Type 1.** Temporizing measures are directed at reducing serum bilirubin levels. Definitive treatment consists of partial- or whole-liver transplantation. New experimental therapies based on liver cell transplantation and gene therapy are currently being developed. These treatment modalities are briefly discussed here.

**69.9.1.3.1.4.1 Phototherapy.** Phototherapy is the most commonly used medical therapy for severe unconjugated hyperbilirubinemia (218). Banks of fluorescent lamps with devices for shielding the eyes, or “light blankets,” lower serum bilirubin levels by converting bilirubin IX $\alpha$ -ZZ to its photoisomers (see the section on bilirubin chemistry). Thickening of skin, pigmentation, and decreased surface area compared to body mass result in a diminution of effect of phototherapy beyond the age of 3 or 4 years (37,218).

**69.9.1.3.1.4.2 Plasmapheresis.** During neurologic emergencies, serum bilirubin concentration can be acutely reduced by plasmapheresis (218). Following removal of bilirubin that is tightly bound to plasma albumin, bilirubin is mobilized from tissue stores to the plasma. Attempts to remove plasma bilirubin by affinity chromatography on albumin-conjugated agarose gel columns were hindered in human subjects because of the removal of formed elements of blood (243).

**69.9.1.3.1.4.3 Orthotopic Liver Transplantation.** Presently, the transplantation of whole liver or a segment of the liver is the only available definitive treatment for Crigler–Najjar syndrome type 1 (244,245). Although this procedure is associated with some risk in these patients, it has been curative in several cases and has dramatically improved the outlook for these patients.

**69.9.1.3.1.4.4 Experimental Methods for Reduction of Serum Bilirubin Levels.**

**69.9.1.3.1.4.4.1 Inhibition of Heme Oxygenase Activity.** Noniron metalloporphyrins are strong inhibitors of microsomal heme oxygenase activity (23). Administration of tin-protoporphyrin has been shown to suppress neonatal hyperbilirubinemia in rhesus monkeys (246,247). Injection of tin-mesoporphyrin at 0.5  $\mu$ mol/kg three times a week for 13–23 weeks in two 17-year-old boys with Crigler–Najjar syndrome type 1 resulted in a modest reduction in serum bilirubin concentrations

**TABLE 69-2 Structural Mutations in UGT1A1 Abolishing Enzyme Activity (Crigler–Najjar Syndrome Type I)**

Exon	Nucleic Acid Mutation <sup>a</sup>	Mutant Protein	Catalytic Activity	References
1	115>G	H39D	ND	(369)
1	120–121delC,T	Truncated protein	Inactive	(409)
1	222>A	Y74X	Inactive	(369)
1	295delT	Truncated protein	Inactive	(410)
1	474–475insT/508–510delTTC	Truncated protein/F170del	Inactive/Inactive	(66,333)
1	508–510delTTC	F170del	Inactive	(66,283,333,410)
1	513–515delCTT	Truncated protein	Inactive	(410)
1	517delC	Truncated protein	Inactive	(369)
1/2	529T>C/879–892del	C177R/truncated protein	Inactive/Inactive	(283)
1/4	576C>G/1184GC>T	Y192X/G395 V	Inactive	(94)
1	652–653insT/Promoter: (TA)6TAA/(TA)7TAA	Truncated protein	Inactive	(410)
1	715C>T	Q239X	Inactive	(75)
1	717–718delAG	Truncated protein	Inactive	(94)
1	722–723delAG	Truncated protein	Inactive	(369)
1	801delC	Truncated protein	Inactive	(94)
1	826G>T	G276R	Inactive	(283)
1/3	835A>T/1069C>T	B279Y/Q357X	ND/Inactive	(49,187,283)
1	840C>A	C280X	Inactive	(411)
Intron 1	IVS1, +1G>C	Truncated	Inactive	(28)
2	del Exon 2	Truncated	Inactive	(283)
2/4	872C>T/1282A>G	A291 V/K426E	Inactive	(187)
2	880T>A and 881–893del	Truncated protein	Inactive	(283,333)
2	877T>A and 878–890del	Truncated protein	Inactive	(184)
2/3	877T>A and 878–890del/1007G>A	Truncated protein/R336Q	Inactive	(184/94)
2	923G>A	G308E	Inactive	(187,405)
2	991C>T	Q331X	Inactive	(318)
3/4	1005G>A/1102G>A	W335X/A368 T	Inactive	(187)
3/4	1055G>A/1223–1224insT	W335X/truncated protein	Inactive/Inactive	(187)
3	1006C>T/N, Promoter: (TA)6TA/(TA)7TA	R336 W/N expression	Inactive/reduced	(214)
3	1007G>A/1124C>T	R336Q/S375F	Inactive	(94/405)
3	del13 blood pressure	361X (truncated protein)	Inactive	(410)
3/4	del13 bp/1100G>C	361X (truncated protein)/ R367P	Inactive	(410)
3	1021C>T	R341X	Inactive	(17)
3	1046delA	Truncated protein	Inactive	(369)
3	1069C>T	Q357X	Inactive	(49,187,283)
3/4	1069C>T/1201G>C	Q357X/A401P	Inactive	(49,187,283)
3	1070A>G	Q357R	Inactive	(187)
3/4	1081C>T/1159C>G, 1160C>T	Q361X/P387R	Inactive/Inactive	(409)
Intron 3	IVS3, –2A>G/145C>T	Truncated protein/Q49X	Inactive	(28) Exon 1
4	1124C>T	S376F	Inactive	(283,318,405)
4	1143C>G	S381R	Inactive	(187)
4	1159C>G, 1160C>T	P387R	Inactive	(409)
4	1159C>T/1184G>T	P387S/G395 V	Inactive	(94)
4/5	1176delC/1456T>G	Truncated protein/Y486D	Inactive	(410)
4	1184G>T	G395 V	Inactive	(94)
4	1201G>C	A401P	Inactive	(187)
4/5	1201G>C/1308A>T	A401P/K437X	Inactive	(187)
4	1205A>T	K402 T	Inactive	(410)
4	1205A>C	K402 T	Inactive	New 11
5	1223delA/N <sup>b</sup>	Truncated protein	Inactive	(369)
5	1381T>C	W461R	Inactive	(134)
5	1451G>A	W484X	Inactive	(134)
5	1452G>A	W484X	Inactive	(369)
5	1490T>A/N <sup>b</sup>	L497X/N	Inactive	(369)

N, normal; ND, not determined.

<sup>a</sup>Nomenclature of mutations is in accordance with den Dunnen and Antonarakis (85). A slash separating two mutations indicates that the patient carried two different mutations on the two alleles.<sup>b</sup>Mutations predicted to result in CN-1 in homozygous states.

(248). The place of this agent in the treatment of Crigler–Najjar syndrome type 1 is yet to be determined.

69.9.1.3.1.4.4.2 Oxidative Degradation of Bilirubin. Bilirubin oxidase from *Myrothecium verrucaria* (249) catalyzes the oxidation of bilirubin to a colorless product. Perfusion of human blood containing bilirubin through filters packed with immobilized bilirubin oxidase resulted in the degradation of 90% of the bilirubin per pass (249). When such columns were connected to the circulation of a Gunn rat, serum bilirubin levels are decreased by 50% in 30 min. There are some concerns, however, regarding the removal of formed elements of blood by these columns. Intravenous injection of bilirubin oxidase, linked to polyethylene glycol to increase its half-life in circulation, has resulted in significant reduction of serum bilirubin levels in Gunn rats, but only for a few hours (249).

69.9.1.3.1.4.4.3 Induction of P-450c. Induction of cytochrome P-450c activity results in increased oxidative degradation of bilirubin in Gunn rat liver, leading to the reduction of serum bilirubin levels. Several indoles present in cruciferous vegetables, such as cabbage, cauliflower, and Brussels sprouts, induce P4501A1 and P4501A2 in rat liver and intestine (250). Indole-3-carbinol, an inducer of P4501A2, is being studied for a potential therapeutic effect in Crigler–Najjar syndrome type 1 (250).

69.9.1.3.1.4.4.4 Replacement of UGT1A1 Activity. UGT1A1 activity is present in excess in normal liver. Therefore, partial replacement of UGT1A1 should reduce serum bilirubin levels in Crigler–Najjar syndrome type 1 to a nontoxic level. Transplantation of hepatocytes into the Gunn rat liver by portal venous infusion, injection into the splenic pulp (251), intraperitoneal injection of microcarrier-bound hepatocytes (85,252), or intraperitoneal implantation of alginate–polylysine-encapsulated hepatocytes (254) resulted in reduction of serum bilirubin levels in Gunn rats. After intrasplenic injection, a great majority of the hepatocytes rapidly translocate to the liver, where, in the absence of immune rejection, they survive and function throughout the life span of the recipient (255). On the basis of the experience gained from these preclinical studies, isolated allogenic human hepatocytes were transplanted into the liver of a patient with Crigler–Najjar syndrome type 1 through a catheter placed percutaneously into the portal vein (258,259). Transplantation of 7.5 billion hepatocytes resulted in the lowering of plasma bilirubin concentration by about 50% and permitted reduction of the duration of phototherapy. Two and half years later, bilirubin glucuronide excretion in bile continued, but serum bilirubin level gradually increased, probably because of increased bilirubin production or reduced effectiveness of phototherapy. The patient received an auxiliary liver transplantation, which has kept her serum bilirubin within normal limits (J. Roy Chowdhury, personal communication).

The clinical course of this case, as well as the experience with other patients who received hepatocyte transplantation (260), indicates that the number of adult hepatocytes that can be transplanted at a single procedure is not likely to be sufficient for curing inherited liver-based metabolic disorders (261). Moreover, there is an increasing shortage of good-quality donor livers for hepatocyte isolation (259,261). For these reasons, strategies are being explored to induce preferential proliferation of the transplanted normal hepatocytes over the mutant host cells. Since adult hepatocytes have a remarkable capacity to proliferate, massive repopulation of the liver by transplanted hepatocytes requires not only a proliferate stimulus to the engrafted cells, but also preparative manipulations of the host liver that prevent the replication of the host liver cells. Controlled hepatic irradiation in combination with a variety of mitotic stimuli is being evaluated for extensive repopulation of the liver by engrafted wild-type or genetically modified hepatocytes (141,264–266). Recent success in obtaining hepatocyte-like cells by differentiating human embryonic stem cells or induced pluripotent cells derived by reprogramming somatic cells, such as skin fibroblasts offer hopes for a renewable source of functional transplantable hepatocytes (24,268).

69.9.1.3.1.4.4.5 Gene Therapy. Supplementation with a normal *UGT1A1* gene is an attractive potential therapeutic modality. Methods for gene introduction into the liver using recombinant viruses or ligands that mediate receptor-directed endocytosis are being developed for this purpose. These approaches have been reviewed (224,269). In the ex vivo approach, liver cells isolated from a resected liver lobe of a mutant subject are established in primary culture and transduced with normal genes using recombinant retroviruses. The transduced cells are then transplanted into the subject, from whom the cells were obtained, thereby circumventing the need for immunosuppression. This approach resulted in modest reduction of serum low-density lipoprotein (LDL) cholesterol levels in LDL receptor–deficient rabbits (Watanabe Heritable Hyperlipidemic strain) (270) and in patients with familial hypercholesterolemia (271). However, advances in techniques of gene transfer (196) to the liver and ability to conditionally immortalize hepatocytes (272) should improve the possibility of success of ex vivo gene therapy. Recombinant adenoviruses are highly efficient in transferring genes to quiescent hepatocytes in vivo. Adenoviruses remain episomal and express transgenes very efficiently. Administration of these vectors to transfer human *UGT1A1* gene complementary DNA (cDNA) into Gunn rats resulted in rapid reduction in serum bilirubin levels.

However, these episomal vectors are eventually lost following cell division, and, because they are highly immunogenic, they cannot be administered repeatedly. The use of viral gene-deleted helper-dependent adenovectors can prolong the duration of transgene expression



and result in lifelong amelioration of jaundice in Gunn rats (273), but, for clinical gene therapy, the expression is not expected to be long enough to span the life of a human subject without repeated administration of the vectors. Repeated gene transfer using adenoviral vectors is possible by using generalized immunosuppression around the time of virus administration (274) or specific host tolerization to adenoviral proteins (275–278). However, this approach is difficult to translate into clinical application. In another approach, an immunomodulatory gene, such as adenoviral E3 or CTLA4-Ig, has been expressed along with the therapeutic transgene to prevent immune response against the cells infected with adenovirus (279,280). Coexpression of CTLA4-Ig, an inhibitor of costimulation of T lymphocytes by antigen-presenting cells, permits repeated administration of the adenovector, resulting in lifelong correction of jaundice in Gunn rats. However, the safety of abrogating host immunity toward adenoviruses, which are potential human pathogens, remains doubtful.

To avoid the need for readministration of gene-therapy vectors, we and others have explored the use of vectors that integrate into the host genome. Simian virus 40 (SV40) is a DNA virus of the papova family. Recombinant SV40 viruses have been developed by replacing the coding region for the T antigens with a target gene. These vectors can infect quiescent hepatocytes, are nonimmunogenic, and integrate into the host genome (281,282), permitting long-term expression of the transgene. Gunn rats treated with a recombinant SV40 virus expressing UGT1A1 showed significant long-term reduction in serum bilirubin levels (283). Because the recombinant virus does not evoke an immune response, the vector can be administered repeatedly.

Vectors based in nonrecombinant lentiviruses can integrate into the genome of both dividing and quiescent cells, such as hepatocytes. Administration of recombinant lentiviruses in utero on the nineteenth gestational day in Gunn rat embryos resulted in UGT1A1 expression in the liver and reduction of serum bilirubin levels in Gunn rats (284). Both recombinant SV40 and lentiviral vectors have a broad range of cellular targets and transduce many tissue types after systemic administration (283,284).

Nonviral, plasmid-based vectors are also being explored for gene therapy on the Gunn rat model. Both naked DNA and carriers containing ligands that are endocytosed via liver-specific receptors (e.g. the asialoglycoprotein receptor) are being evaluated (285–287). To enhance integration of the therapeutic transgene, some investigators are examining the feasibility of using the Sleeping Beauty transposon system, which results in the excision of the transgene at specific flanking sequences and splicing into the host chromosomal DNA (288). In a very different approach, oligonucleotides are used to correct single-base mutations or deletions utilizing the cell's intrinsic mismatch repair system (289). The nonviral

gene transfer or gene repair strategies hold great promise for safe gene therapy of inherited jaundice although, at their current state, these approaches are not efficient enough for immediate clinical application.

**69.9.1.3.2 Crigler–Najjar Syndrome Type 2.** Crigler–Najjar syndrome type 2 is also known as Arias Syndrome. Arias (8) reported a milder variant of the classical Crigler–Najjar syndrome, which is now termed Crigler–Najjar syndrome type 2, in which serum unconjugated bilirubin concentrations usually ranged from 8 to 18 mg/dL. During intercurrent illness, general anesthesia, or prolonged fasting, bilirubin levels could be as high as 40 mg/dL (291,292). Kernicterus is unusual in this group, but can be precipitated during episodes of exacerbated hyperbilirubinemia (8,217,291,292). As in Crigler–Najjar syndrome type 1, there is no evidence of hemolysis or other liver dysfunction. Crigler–Najjar syndrome type 2 is clinically differentiated from type 1 by >25% reduction of serum bilirubin after administration of enzyme-inducing agents (e.g. phenobarbital) and the presence of significant amounts of bilirubin glucuronides in bile although the proportion of bilirubin monoglucuronide exceeds 30% of total conjugated bilirubin (normal, ~10%) (292,293), reflecting a reduced hepatic UGT1A1 activity. The liver histology is normal, and UGT1A1 activity is usually reduced to approximately 10% of normal (292,293).

**69.9.1.3.2.1 Genetic Lesions.** Molecular genetic studies that have performed so far support an autosomal-recessive mode of inheritance (294). As in Crigler–Najjar syndrome type 1, the genetic lesions are found in the coding region of UGT1A1. However, in Crigler–Najjar syndrome type 2, these mutations always consists of a single amino acid transition that significantly reduces the UGT1A1 activity, without completely abolishing it. Table 69-3 lists the reported genetic lesions found in Crigler–Najjar syndrome type 2. In some cases, the mutation may increase the  $K_m$  for bilirubin (295). As the in vitro UGT1A1 assays are performed at high bilirubin concentrations, their measurements may overestimate the in vitro activity of the enzyme in these cases. The various lesions causing Crigler–Najjar syndrome type 2 have been reviewed (228).

**69.9.1.3.3 Gilbert Syndrome.** This disorder, associated with a mild, chronic, fluctuating unconjugated hyperbilirubinemia was described by Gilbert and Lereboullet (296). Although most patients present as isolated cases, familial incidence also has been noted (297).

**69.9.1.3.3.1 Clinical Features.** Gilbert syndrome is usually diagnosed in young adults who are incidentally found to have mild, predominantly unconjugated hyperbilirubinemia. Bilirubin levels may be elevated only intermittently and are usually <3 mg/dL, but may increase during intercurrent illness, stress, fasting, or menstrual periods (297). Mild icterus is the only positive finding on physical examination. Vague symptoms, such as fatigue and abdominal discomfort, reported by some patients

**TABLE 69-3 Structural Mutations in UGT1A1 Reducing Enzyme Activity (Crigler–Najjar Syndrome Type 2 and Gilbert Syndrome)**

Exon	Nucleic Acid Mutation <sup>a</sup>	Mutant Protein	Catalytic Activity	References
1	44T>G	L15R	Reduced activity	(212)
1	44T>G/572C>T	L15/R/S191F	Reduced activity/inactive	New #11
1/4	111C>A/1207C>T	P34Q/R403C	Reduced activity	(94)
1	211G>A <sup>b</sup>	G71R	Reduced activity	(9,371,412)
1	211G>A/N <sup>c</sup>	G71R/N	Reduced activity	(371,412,413)
1/5	211G>A and 1456T>G	G71R and Y486D	Reduced activity	
1	395T>C/N <sup>c</sup>	L132P/N	Reduced activity	(412)
1	524T>A	L175Q	Reduced activity	(283)
1/2	524T>A/973delG	L175Q/truncated protein	Reduced activity/truncated-inactive	(283)
1/4	576C>G/1130G>T/Promoter: (TA)6TAA/(TA)7TAA	Y192X/H377 V	Inactive/reduced activity	(94/369)
1	625T>C	R209 W	Reduced activity	(36,113,283)
1/4	625 T<C/1186delG	R209 W/truncated protein	Reduced activity/truncated-inactive	
1	671T>G/722-723delAG	V224 G/truncated protein	Residual activity/inactive	(369)
1	686C>A <sup>c</sup>	P229Q/N	Reduced activity	(371)
1	717–718delAG/671T>G	Truncated protein/V224 G	Inactive/reduced activity	(274)
Intron 1/3	865-1G>A/1007G>T	Splicing aberration/R336L	Inactive/reduced activity	(94)
2/3	877T>A&878–890del/1060T>C	Truncated protein/W354R	Inactive/reduced activity	(94,184)
2	881T>C/N, Promoter: (TA)6TAA1/(TA)7TAA	I294 T/N	Reduced activity	(214)
2	992A>G	Q331R	Reduced activity	(193)
2	991C>T/N <sup>c</sup>	Q331X/N	Reduced activity	(161)
3/intron 3	1006C>T/1304>1G>T	R336 W/splicing aberration	Reduced activity/inactive	214/94
3/5	1006C>T/1489delG	R336 W/A498PfsX3	Reduced activity/inactive	New #11
4	1099C>G/N <sup>c</sup>	R367 G/N	Reduced activity	(371,412)
4	1130A>G/N	H377R/N	ND/N	(369)
4	1133G>T	G377 V	ND	(369)
4	1160C>A	P387H	Reduced activity	New #11
5	1391A>C/N, Promoter: (TA)7TAA/(TA)7TAA	Z464A/N	Residual activity	(174)
5	1433C>A	A478D	Reduced activity	(94)
5	1456T>G <sup>b</sup>	Y486D	Reduced activity	(125,371)

A slash separating two mutations indicates that the patient carried two different mutations on the two alleles.

N, normal; ND, not determined.

<sup>a</sup>Nomenclature of mutations is in accordance with den Dunnen and Antonarakis (85).

<sup>b</sup>In these cases, the hyperbilirubinemia was mild and the patients were classified as Gilbert syndrome.

<sup>c</sup>In all these cases, a heterozygous mutation was found to be the cause of the phenotype and a dominant-negative mechanism has been proposed to explain the hyperbilirubinemia.

may be manifestations of anxiety. Except for the predominantly unconjugated hyperbilirubinemia, routine blood tests are negative. Because canalicular excretion processes are normal, oral cholecystography allows visualization of the gallbladder. Liver biopsy is not routinely indicated, but, when performed, shows normal histology, except for a nonspecific accumulation of lipofuscin pigment in the centrilobular zones (298).

**69.9.1.3.3.2 Incidence.** Gilbert syndrome is perhaps the most common inherited disorder in man, the reported incidence ranging from 3% to 7% of the population (299,300). Distribution of serum bilirubin levels in the general population follows a skewed (301) or bimodal (299,300) pattern rather than a Gaussian distribution, and it has been proposed that subjects with Gilbert syndrome should be considered to represent one end of this spectrum. Therefore, the definition of Gilbert syndrome is somewhat arbitrary, making the determination

of its precise incidence difficult. Although it is possible to distinguish patients with Gilbert syndrome from the general population by determination of biliary bilirubin diglucuronide:monoglucuronide ratio and UGT1A1 activity in liver biopsy specimens, these tests are impractical for population studies. Because of the higher average serum bilirubin levels in males (299), Gilbert syndrome is diagnosed with much more frequency in males than in females (300). Gilbert syndrome is often recognized around puberty, which may be related to the inhibition of bilirubin glucuronidation by endogenous steroid hormones (302).

**69.9.1.3.3.3 Bilirubin Glucuronidation.** Hepatic UGT1A1 activity, as determined by in vitro assays, is present at a consistently low level (around 30% of normal) in Gilbert syndrome (303,304).

**69.9.1.3.3.4 Organic Anion Uptake.** In addition to reduced UGT1A1 activity, some patients with Gilbert

syndrome exhibit abnormalities of organic anion transport (306). However, based on studies of handling to bilirubin loads and the pattern of bilirubin conjugates secreted in bile, other investigators have suggested that the initial uptake of bilirubin is normal for Gilbert syndrome and the reduced plasma clearance may be explained by a decreased rate of glucuronidation only (307). The plasma disappearance of organic anions other than bilirubin (e.g. BSP or indocyanin green) may be abnormal in a minority of cases of Gilbert syndrome (308,309). No mechanistic basis for the association of decreased UGT1A1 activity and reduced organic anion uptake in these cases is known, and the coexistence of the two abnormalities may be merely coincidental.

**69.9.1.3.3.5 Diagnosis.** Gilbert syndrome is diagnosed in individuals with mild unconjugated hyperbilirubinemia without evidence of hemolysis or structural liver disease. Although hemolysis is not a feature of Gilbert syndrome, coexistent compensated hemolysis is found in many patients because increased bilirubin load results in clinically obvious jaundice (310). A presumptive diagnosis of Gilbert syndrome is made when mild unconjugated hyperbilirubinemia is noted on several occasions, and serum levels of alanine and aspartate aminotransferases, alkaline phosphatase,  $\gamma$ -glutamyl transpeptidase, and fasting and postcibal bile acid are all normal. Sequence determination of the promoter region upstream to exon 1 of UGT1A1 provides a simple means of diagnosis. If confirmation of diagnosis is required, chromatographic determination of the relative content of bilirubin monoglucuronide and diglucuronide in bile is of potential use in the diagnosis of Gilbert syndrome. Fasting or nicotinic acid tests provide suggestive data, but are not specific for Gilbert syndrome. Needle biopsy of the liver is not necessary for the diagnosis.

**69.9.1.3.3.6 Effect of Fasting.** Reduction of daily caloric intake to 400 kcal for 48 h results in elevation of serum bilirubin levels in all individuals (26). However, this response may be exaggerated in Gilbert syndrome (312). Fasting may induce hyperbilirubinemia by multiple physiologic mechanisms. Both increased bilirubin production (313) and reduced hepatic bilirubin clearance (314) have been reported. Reduction of hepatic UDPglucuronic acid content has been proposed as a mechanism of the exaggerated hyperbilirubinemic response to fasting in rats (315). However, because fasting also exacerbates hyperbilirubinemia in homozygous Gunn rats that lack UGT1A1 activity (316), reduction of bilirubin glucuronidation cannot be a complete explanation for fasting hyperbilirubinemia. Because of the overlap with the normal response to caloric deprivation (312), the fasting test does not reliably distinguish Gilbert syndrome from other hyperbilirubinemic states.

**69.9.1.3.3.7 Nicotinic Acid Tests.** Intravenous administration of nicotinic acid increases unconjugated hyperbilirubinemia, probably by increasing erythrocyte fragility, splenic heme oxygenase activity, and splenic

bilirubin formation (317). Nicotinic acid administration has been proposed as a provocative test for Gilbert syndrome; however, similar to fasting, it does not clearly distinguish patients with Gilbert syndrome from normal subjects or those with hepatobiliary disease (317).

**69.9.1.3.3.8 Bilirubin Conjugates in Bile.** As in Crigler-Najjar syndrome type 2, bile in Gilbert syndrome contains an increased proportion of bilirubin monoglucuronide (293), reflecting reduced hepatic UGT1A1 activity in these syndromes.

**69.9.1.3.3.9 Genetic Basis of Gilbert Syndrome.** Gilbert syndrome is associated with a variant TATAA box in the promoter upstream to exon 1 of UGT1A1. The normal TATAA element has the sequence A[TA]6TAA, whereas subjects with Gilbert syndrome are homozygous for elongation of the dinucleotide repeat, so that the sequence of the TATAA element is T[TA]7TAA (318). All subjects with Gilbert syndrome of white, black, or Asian Indian origin who have had genetic analysis were found to be homozygous for this variant promoter. Promoter-reporter studies show that an increased TATAA box length reduces UGT1A1 expression (318). Patients with the Gilbert genotype have been shown to have lower hepatic microsomal UGT1A1 activity (319). UGT1A1 alleles carrying the Gilbert-type TATAA element have been designated as UGT1A1\*28 (320). However, all subjects who are homozygous for the variant promoter do not exhibit the clinical phenotype, indicating that additional variables, particularly the rate of bilirubin production, are important for the manifestation of the phenotype. Notably, although the inheritance is autosomal (chromosome 2q37), women with this genotype usually do not manifest jaundice, probably because of lower daily bilirubin production. The gene frequency of the Gilbert-type promoter in the Western hemisphere is approximately 0.3. Thus, 9% of the general population is homozygous for this genotype. The gene frequency may be lower in Japan. Some mutations of the coding region of UGT1A1 may be associated with mild hyperbilirubinemia, compatible with the clinical diagnosis of Gilbert syndrome (321,322) (Table 69-3). These mutations have not been observed in populations other than those of Far Eastern origin. Some of these mutations have been reported to be dominant negative, suggesting that they can partially inactivate a normal allele (323).

Because of the high frequency of the Gilbert-type TATAA element in the general population, some heterozygous carriers of structural mutations may carry the Gilbert-type promoter on the structurally normal allele. This reduces the expression of the only structurally normal allele. Therefore, a combination of these mutations may give rise to intermediate levels of hyperbilirubinemia (324,325). This explains the long-standing observation that intermediate levels of hyperbilirubinemia are common among relatives of patients with Crigler-Najjar syndrome. This type of combination is a more common

cause of intermediate levels of hyperbilirubinemia than is homozygous mutation of the coding region in UGT1A1.

**69.9.1.3.3.10 Health Implications of Gilbert Syndrome.** Gilbert syndrome is generally considered innocuous, and its recognition is considered important mainly for reassuring the patient and the physician that no underlying liver disease is responsible for the mild hyperbilirubinemia. However, the clinical and epidemiologic significance of Gilbert syndrome is continuing to be unveiled. The UGT1A1\*28 genotype has been reported to be associated with accelerated or prolonged neonatal hyperbilirubinemia (326,327). In children with a combination of glucose-6-phosphate dehydrogenase deficiency and the UGT1A1\*28 genotype, neonatal serum bilirubin concentrations can rise to dangerously high levels (328). Patients with hereditary spherocytosis who also have the UGT1A1\*28 genotype may develop pigment gallstones early in life (329).

Although the mild hyperbilirubinemia associated with Gilbert syndrome should not cause any significant toxicity in adults and may indeed be cytoprotective by virtue of the antioxidant effect of bilirubin, the reduced expression of UGT1A1 activity may have some significant consequences. The pharmacogenetics of UGT1A1 polymorphism is receiving increasing attention (320). Oxidative paracetamol metabolism is associated with drug toxicity. Conflicting evidence has been reported for increased oxidative metabolism of paracetamol and its decreased glucuronidation in subjects with the UGT1A1\*28 allele (330,331). Gilbert syndrome is associated with a high incidence of diarrhea in patients treated with the anticancer drug, irinotecan (332).

**69.9.1.3.3.11 Animal Model.** Bolivian squirrel monkeys have higher serum unconjugated bilirubin concentrations and a greater hyperbilirubinemic response to fasting than does a closely related Brazilian population

(333,334). Plasma clearance of intravenously administered bilirubin, hepatic UGT activity toward bilirubin, and bilirubin diglucuronide:monoglucuronide ratio in bile are lower in the Bolivian population. In these respects, Bolivian squirrel monkeys are a model of Gilbert syndrome. Fasting hyperbilirubinemia is rapidly reversed by oral or intravenous administration of carbohydrates, but not by lipid administration (333).

## 69.9.2 Disorders Associated with Predominantly Conjugated Hyperbilirubinemia

Accumulation of conjugated bilirubin in serum may result from leakage of bilirubin glucuronides from hepatocytes or regurgitation from bile in inflammatory or ischemic diseases of the hepatocyte, and intrahepatic cholestasis or biliary obstruction. However, in specific disorders of organic anion storage or transport (e.g. Rotor and Dubin–Johnson syndromes), conjugated bilirubin may be retained in plasma in the absence of other evidence of liver dysfunction. Table 69-4 provides a comparison of the clinical and pathophysiologic features of these inherited disorders. In addition, there are inherited abnormalities of bile canalicular transport proteins that cause a group of disorders collectively termed progressive familial intrahepatic cholestasis. In another genetic condition, paucity of bile ducts leads to conjugated hyperbilirubinemia. A brief description of these disorders follows.

**69.9.2.1 Dubin–Johnson Syndrome.** A syndrome characterized by chronic nonhemolytic jaundice due to accumulation of conjugated bilirubin in serum and grossly pigmented, but otherwise histologically normal, livers was described by Dubin and Johnson (335) and by Sprinz and Nelson (336). Mild icterus is the only significant physical finding. Rarely, hepatosplenomegaly has

**TABLE 69-4 Inherited Disorders Causing Retention of Conjugated Bilirubin**

	Dubin–Johnson Syndrome	Rotor Syndrome
Serum bilirubin	Predominantly conjugated, usually 50–85 $\mu$ M, can be as high as 340 $\mu$ M	Predominantly conjugated, usually 50–100 $\mu$ M, occasionally as high as 340 $\mu$ M
Routine liver function tests	Normal except for hyperbilirubinemia	Normal except for hyperbilirubinemia
Serum bile salt levels	Normal	Normal
Plasma BSP retention	Normal at 45 min; secondary rise at 90 min	Elevated; but no secondary rise at 90 min
BSP infusion studies	T <sub>m</sub> very low; storage normal	T <sub>m</sub> and storage both reduced
Oral cholecystogram	Usually does not visualize the gallbladder	Usually visualizes the gallbladder
Urinary coproporphyrin excretion	Total—normal; >80% as coproporphyrin I	Total—elevated; ~50–75%, as coproporphyrin I pattern
Appearance of liver	Grossly black	Normal
Histology of liver	Dark pigments, predominantly in centrilobular	Normal, no increase in pigmentation areas; otherwise normal
Mode of inheritance	Autosomal recessive	Autosomal recessive
Prevalence	Rare (1:1300 in Middle Eastern Jews)	Rare
Prognosis	Benign	Benign
Animal model	Mutant TR <sup>-</sup> rats/mutant Corriedale sheep/Golden lion tamarin monkey	None

BSP, bromosulphthalein.



been observed (337,338). Although patients are usually asymptomatic, an occasional patient complains of weakness and vague abdominal pain. Serum bile acid levels are normal (339), and pruritis is absent. Serum bilirubin levels are increased by intercurrent illness, oral contraceptives, and pregnancy (339). Although the syndrome is occasionally recognized during the neonatal period (340), the diagnosis is most often made after puberty. In some cases, jaundice is first noticed during pregnancy or contraceptive use (339).

**69.9.2.1.1 Laboratory Tests.** Liver function tests, including serum bile acid levels, are normal (339). Serum bilirubin levels fluctuate. Usual levels range from 2 to 5 mg/dL, but levels as high as 20–25 mg/dL have been observed. Over 50% of total serum bilirubin is direct-reacting, and bilirubin is excreted in urine. Because the canalicular excretion abnormality includes a wide variety of non-bile-acid organic anions, oral cholecystography, even using a “double dose” of contrast material, usually does not visualize the gallbladder. However, visualization may occur 4–6 h after intravenous administration of meglumine iodipamide (Biligradin) (341). Grossly, the liver is black, and light microscopy reveals a dense pigment (342). Following infusion of 3H-epinephrine into mutant Corriedale sheep (an animal model for Dubin–Johnson syndrome), the isotope is incorporated into the hepatic pigment (343). The pigment differs from authentic melanin (344), but could be composed of polymers of epinephrine metabolites (345). Following liver disease, such as acute viral hepatitis, the pigment is cleared from the liver and reaccumulates slowly after recovery (346).

**69.9.2.1.2 Organic Anion Transport.** The hepatic secretion of bilirubin glucuronides, the leukotriene LTC<sub>4</sub>, reduced and oxidized glutathione, and numerous glucuronide and glutathione conjugates are disturbed in these patients (347,348). In contrast, bile acid secretion is unaffected. Hepatic storage is normal, and the transport abnormality is limited to canalicular excretion (349). After intravenous injection of BSP, plasma BSP concentration decreases at near-normal rate for 45 min, indicating a normal sinusoidal uptake process. However, in 90% of patients, plasma BSP concentration increases after this time, so that the concentration at 90 min is greater than that at 45 min because of reflux of glutathione-conjugated BSP from hepatocytes into the circulation (67). A similar secondary rise occurs after intravenous administration of bilirubin (351). Because the secondary rise of plasma BSP occurs in other hepatobiliary disorders as well (352), this phenomenon is not pathognomonic of Dubin–Johnson syndrome.

**69.9.2.1.3 Urinary Coproporphyrin Excretion.** In normal subjects, approximately 75% of the urinary coproporphyrin is isomer III, which is the precursor of heme. The total urinary coproporphyrin excretion is normal in Dubin–Johnson syndrome, but over 80% of the coproporphyrin is isomer I (353). The proportion of urinary coproporphyrin I in the urine of neonates is higher than

in adults, but the levels are not as high as in Dubin–Johnson syndrome (354). In unaffected parents or children of subjects with Dubin–Johnson syndrome, total urinary coproporphyrin excretion was reduced by 40% as compared to normal, because of a 50% reduction in coproporphyrin III excretion (355). The mechanism of the abnormal pattern of urinary porphyrin excretion and its relationship to the organic anion transport defect is not known. When the history and physical examination are consistent, the urinary coproporphyrin excretion pattern is diagnostic of Dubin–Johnson syndrome.

Organic anions other than bile acids are transported into the bile canaliculus from the hepatocyte against a concentration gradient by an ATP-dependent energy-consuming process, mediated by a protein that had been originally termed the canalicular multispecific organic anion transporter (cMOAT), and is now known as MRP2 or ABC-C2 (3,149,150,357,358) (Figure 69-1). These anions include conjugated bilirubin and other glucuronide- or glutathione-conjugated substances. MRP2 is one of the ABC transporters (357). Direct evidence for its involvement in canalicular transport came from the discovery of a frameshift mutation in the gene encoding in the TR– rat (154).

Despite the absence of MRP2, the serum bilirubin levels are only mildly elevated in Dubin–Johnson syndrome, suggesting alternative pathways for the secretion of bilirubin conjugates. Currently it is not known whether other canalicular transport proteins mediate the residual canalicular transport of bilirubin glucuronides in Dubin–Johnson syndrome. As discussed earlier, absence of MRP2 leads to accumulation of organic anions within the hepatocytes and upregulation of the expression of MRP1 and MRP3 in the basolateral domain of the hepatocyte plasma membrane. These and possibly other ATP-consuming pumps may contribute to the active export of unconjugated bilirubin and bilirubin glucuronides from the hepatocyte to plasma via the space of Disse (159).

**69.9.2.1.4 Genetic Basis and Inheritance.** Dubin–Johnson syndrome has been reported in all races and both sexes. There is a high incidence (1:1300) in Persian Jews (338), in whom it is associated with clotting factor VII deficiency (359). On the basis of the urinary coproporphyrin excretion abnormality, the inheritance pattern is clearly recognized as autosomal recessive (355). Dubin–Johnson syndrome is caused by mutations of the MRP2 (ABCC2) gene, causing a deficiency of canalicular MRP2 expression or function (152,154,360).

The human cDNA has been isolated on the basis of homology with rat MRP2 (153). The human MRP2 gene has been localized to chromosome 10q23–q24 (361), and the exon–intron organization has been elucidated (362,363). Several different mutations (Table 69-5) have been identified in this gene in patients with Dubin–Johnson (153,362–365). Mutations most commonly involve the crucial ATP-binding region. Mutation at an intronic splice donor site has also been identified in a patient with

**TABLE 69-5 Mutations Identified in Patients with Dubin–Johnson Syndrome**

Exon	Mutation <sup>a</sup>	Mutation Protein	References
Nucleic Acid 3/28	298C>T/3928C>T	R100X/R1310X–	(374)
Truncated protein 13	1669–1815del	Truncated protein	(98)
Intron 15	IVS15, +2T>C	Truncated protein	(18)
16	2026G>C	G676R	(181)
17	2125T>C	W709R	(199)
18	2302C>T	R768 W	(98)
18	2272–2439del	Truncated protein	(98)
18 splice site/29	2439+2T>C/4145A>G	2272del168/Q1382R	(102)
23	3196C>T	R1066X–truncated protein	(150)
25	3449G>A	R1150H	(37)
25	3517A>T	I1173F	(37)
30	4175–4180del	Truncated protein	(266)

<sup>a</sup>Nomenclature of mutations is in accordance with den Dunnen and Antonarakis (85).

Dubin–Johnson syndrome (364). Some mutations may lead to impaired glycosylation of MRP2, leading to premature proteasome-dependent degradation (366). The genetic analyses have confirmed an autosomal-recessive pattern of inheritance.

#### 69.9.2.1.5 Animal Models

**69.9.2.1.5.1 Mutant Corriedale Sheep.** In this mutant strain, biliary excretion of conjugated bilirubin, glutathione-conjugated BSP, iopanoic acid, and indocyanine green is decreased, whereas taurocholate transport is normal (367). The secretion of the organic cation procaine amide ethobromide is unaffected (3) and, interestingly, the secretion of unconjugated BSP is unimpaired (368). There is a mild hyperbilirubinemia, with 60% of the bilirubin being conjugated. The liver is pigmented but otherwise of normal histology (343). Total urinary coproporphyrin excretion is normal, with increased excretion of coproporphyrin isomer I and decreased isomer III excretion. In all these respects, the mutant Corriedale sheep is a model of Dubin–Johnson syndrome.

**69.9.2.1.5.2 TR– Rats.** These rats have a metabolic defect strongly resembling that in people with Dubin–Johnson syndrome and the mutant Corriedale sheep. Biliary secretion of conjugated bilirubin and many other organic anions is impaired (369), and coproporphyrin I is the major porphyrin excreted in urine (370). The liver is not normally pigmented, but, when the rats are fed a diet enriched in tryptophan, tyrosine, and phenylalanine, intracellular pigment deposition can be demonstrated. Impaired excretion of anionic metabolites of tyrosine, phenylalanine, and tryptophan may result in their retention, oxidation, polymerization, and subsequent lysosomal accumulation in the liver (371). As in human Dubin–Johnson syndrome, TR– rats (372) and rats of the Eisai hyperbilirubinemic rats (EHBR) strain, which have a similar defect, absence of MRP2 (mrp2 in rats) in the canalicular membrane leads to impaired canalicular secretion of bilirubin and other glucuronides, the leukotriene LTC<sub>4</sub>, glutathione, and glutathione conjugates (347). Experiments with the rodent models showed that

bilirubin conjugates and bile acids are secreted into the bile canaliculus via different pathways. Bile acids with free 3-OH groups are transported by the bile salt export pump (BSEP), but 3-OH-conjugated bile acids are transported by mrp2, as evidenced by impaired secretion of bile acid conjugates in TR– and EHBR rats (373).

**69.9.2.1.5.3 A Non-Human Primate Model.** The Golden Lion tamarin monkey, which manifests conjugated hyperbilirubinemia, is another animal model for Dubin–Johnson syndrome (374).

**69.9.2.2 Rotor Syndrome.** Rotor, Manahan, and Florentin described several patients with chronic predominantly conjugated hyperbilirubinemia from two families (93). Hematologic tests and blood biochemistries were normal. Liver histology was normal and, in contrast to Dubin–Johnson syndrome, there was no pigmentation. The basic abnormality of this disorder is different from that of Dubin–Johnson syndrome (375,376). Rotor syndrome is harmless and is characterized by chronic predominantly conjugated hyperbilirubinemia without evidence of hemolysis (93,375,376). It is rare, but has been described in several races.

**69.9.2.2.1 Organic Anion Excretion.** After intravenous injection of 5 mg/kg BSP, over 25% of injected BSP is retained in serum at 45 min, but there is no secondary rise of plasma BSP level and conjugated BSP does not appear in the plasma (377). There is also marked plasma retention of intravenously administered unconjugated bilirubin (378) and ICG (379). Phenotypically normal heterozygotes have mild abnormal BSP retention at 45 min (377). In contrast to the findings in Dubin–Johnson syndrome, oral cholecystographic agents visualize the gallbladder in Rotor syndrome (380).

**69.9.2.2.2 Hepatic Storage.** Transport maximum and hepatic storage of BSP has been determined by a constant infusion technique (377). In Dubin–Johnson syndrome, the transport is markedly abnormal, but the hepatic storage capacity is normal. In contrast, in Rotor syndrome, the storage capacity was reduced by 75–90%, whereas the transport maximum was reduced by only

50% (377). The findings in Rotor syndrome are similar to those in “familial hepatic storage disease,” which is associated with predominantly conjugated hyperbilirubinemia and normal liver histology (381). These two disorders may represent the same pathophysiologic entity.

**69.9.2.2.3 Urinary Coproporphyrin Excretion.** In Rotor syndrome, total urinary coproporphyrin is increased two- to fivefold over normal. The proportion of coproporphyrin I in urine is approximately 65% of total (376). These findings are similar to those seen in many other hepatobiliary disorders (382), and distinguish Rotor syndrome from Dubin–Johnson syndrome. In a recent report, however, two brothers with clinical Rotor syndrome had over 80% of urinary coproporphyrins as isomer I (383). Obligate heterozygotes have a coproporphyrin excretory pattern that is intermediate between that of control subjects and patients with Rotor syndrome. By urinary coproporphyrin analysis, Rotor syndrome appears to be inherited as an autosomal-recessive characteristic (376). The urinary coproporphyrin abnormality may be due to reduced biliary excretion of coproporphyrins, with concomitant increase in renal excretion. The mechanism of the organic anion transport defect in Rotor syndrome is not known.

### 69.9.3 Hyperbilirubinemia Resulting from Inherited Cholestasis Syndromes

**69.9.3.1 Progressive Familial Intrahepatic Cholestasis.** These inherited disorders do not specifically affect bilirubin metabolism, but cause the retention of both unconjugated and conjugated bilirubin in plasma by affecting bile flow. Three life-threatening heterogeneous disorders, characterized by defective secretion of bile acids or other components of bile, may result in cholestasis of various degrees of severity and are collectively termed progressive familial intrahepatic cholestasis (PFIC) (384). Benign recurrent intrahepatic cholestasis (BRIC) is a disorder that is genetically related to PFIC I. These disorders usually present during infancy or childhood, and are often associated with growth failure and progressive liver disease. Several additional genetic cholestatic disorders have been described during the past few years. PFIC syndromes and the other inherited cholestatic diseases have been reviewed recently (385) and are discussed in brief here.

**69.9.3.1.1 Progressive Familial Intrahepatic Cholestasis Type I.** PFIC I was originally described in a family in the Amish-Mennonite community and was named Byler disease, after the family name of the first patient (386). The disease is associated with severe life-threatening cholestasis, and is caused by mutations in the P-type ATPase gene, FIC1 (also termed ATP8B1), which has been localized to chromosome 18q21 (163). FIC-1 couples the hydrolysis of ATP to the translocation of acidic phospholipids. How the mutation of FIC-1 causes cholestasis and abnormal bile acid

transport is understood only partially (387). Lack of FIC-1 may lead to diminished nuclear translocation of the nuclear receptor FXR (388). Cholestasis may result from consequent downregulation of the BSEP (Section 76.345.3.361.3).

**69.9.3.1.2 Benign Recurrent Intrahepatic Cholestasis.** This disorder was first described in 1959 (389). It presents in adolescence or early adulthood (390,391) with recurrent episodes of conjugated hyperbilirubinemia associated with malaise, anorexia, pruritis, weight loss, and malabsorption. Laboratory tests reveal biochemical evidence of cholestasis without severe hepatocellular injury (392,393). Such episodes last for weeks to months, followed by a complete return to normalcy clinically, biochemically, and histologically. In a given patient, recurrent attacks resemble each other in symptoms, signs, and duration. Liver histology reveals noninflammatory intrahepatic cholestasis without fibrosis regardless of the number and severity of attacks. During remission, liver histology returns to normal whether examined by light or electron microscopy (394).

Family studies suggest a recessive inheritance pattern. Surprisingly, this relatively benign disorder was also found to be associated with mutations in the *FIC-1* gene, other mutations of which cause the much more severe disorder PFIC I.

There is no specific treatment for BRIC. Liver transplantation has not been considered because of the episodic and nonprogressive nature of the disease.

**69.9.3.1.3 Progressive Familial Intrahepatic Cholestasis Type II.** This disorder resembles Byler disease clinically, but occurs in non-Byler families, mainly in the Middle East and Europe. It is caused by defects in the gene that codes for the sister P-glycoprotein (SPGP), also known as BSEP or ABCB11, which is a canalicular ATP-dependent transporter of bile acids from the hepatocytes into the bile (395). The BSEP gene has been localized to chromosome 2q24 (165). A large number of different point mutations in BSEP have been described in patients with PFIC II.

Although both PFIC I and PFIC II are associated with life-threatening cholestasis, serum  $\gamma$ -glutamyl transpeptidase levels are normal or nearly so in both disorders, which differentiate them from PFIC III (165,384). BSEP is expressed specifically in the liver and, as expected, liver transplantation ameliorates all manifestations of PFIC II.

**69.9.3.1.4 Progressive Familial Intrahepatic Cholestasis Type III.** PFIC III involves mutations in the multidrug resistance protein-3 P-glycoprotein (PGY3, also known as MDR3/ABCB4) (Figure 69-1). MDR3/ABCB4 is thought to transport phosphatidylcholine from the inner lipid leaflet of the bile canaliculus to the outer leaflet, thereby replenishing the phospholipid, which is continuously removed into the bile because of contact with bile acids. In the absence of phosphatidylcholine translocation, bile acids damage the canalicular



membrane, causing progressive destruction of small bile ducts (209,396). In contrast to the findings in PFIC I and II, serum  $\gamma$ -glutamyl transpeptidase activity is increased in PFIC III (384). MDR3/ABCB4 deficiency in humans has been associated with a wide spectrum of hepatobiliary disorders, including small-duct primary sclerosing cholangitis (397) and cholesterol gallstones (398). Interestingly, heterozygosity for nonsense or missense mutations of the MDR3/ABCB4 gene has been associated with the heretofore enigmatic familial intrahepatic cholestasis of pregnancy (399,400).

**69.9.3.1.5 Treatment.** The treatment options currently available for PFICs are limited. Experimental transplantation of normal hepatocytes in an *mdr2* knockout mouse model of PFIC III has resulted in massive repopulation of the liver with the transplanted cells, resulting in long-term amelioration of the phospholipid transport defect in that condition (401,402).

**69.9.3.1.6 Other Inherited Cholestatic Disorders.** The cholangiopathy of North American Indian childhood cirrhosis is caused by the genetic lesions of a gene (*CIRH1A*) that encodes a mitochondrial scaffold protein. This disorder resembles clinically extrahepatic biliary atresia, except that there is no biliary tract obstruction (403).

GRACILE syndrome, another form of potentially lethal intrahepatic cholestasis associated with fetal growth retardation, aminoaciduria, iron overload, and lactic acidosis (GRACILE), is caused by genetic lesions of another mitochondrial scaffold gene, *BCS1L* (404). Interestingly, although North American Indian childhood cirrhosis and GRACILE syndrome are both caused by mutations of genes expressing mitochondrial scaffold proteins, neither of these syndromes includes features of usual mitochondriopathy, such as central nervous system lesions or hepatocellular steatosis.

Alagille syndrome is a multisystem development anomaly affecting the hepatobiliary system, eyes, vertebrae (sagittal, cleft), heart, kidneys, and central nervous system (405). Most patients exhibit a characteristic facies. The central nervous system lesions can be secondary to malnutrition, but in patients with carefully corrected nutritional balance, intracranial bleeding is the most common central nervous system complication (406). The syndrome is inherited as an autosomal-dominant condition with markedly variable penetrance. Lesions of the Jagged1 (*JAG1*) gene, mapped to chromosome 20p12 (407), are responsible for this disease. Jagged-1 is a ligand of Notch, and plays a critical role in the Notch signaling pathway that is important in the normal development of various organs. In about 70% of the patients with Alagille syndrome, deletion or mutations of the *JAG1* gene can be found, the coding region being normal in the remaining patients. Interestingly, 50–70% of these mutations are de novo, and not found in the parents. Paucity of bile ducts, which was considered *sine qua non* for Alagille syndrome is found in only 89% of

cases (406). Liver transplantation is required eventually in 21–50% of patients who have hepatic symptoms in infancy.

Villin disease, related to lesions of the *VILLIN1* gene, was reported to be the basis of cholestatic disease in several children, who eventually developed cirrhosis, requiring liver transplantation (408).

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# CHAPTER 70

## Cancer of the Colon and Gastrointestinal Tract

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### 70.1 INTRODUCTION

#### 70.1.1 Familial Cancer and Cancer Genetics

All cancers are caused by abnormalities in genes. However, most cancers are not caused by *inherited* genetic disorders; most of the mutations in a cancer are somatic, acquired mutations. Familial clusters of cancer may be caused by shared environmental exposures, or by a germline mutation that creates an unusual sensitivity to ordinary environmental exposures. In some instances, the germline mutations facilitate the accelerated accumulation of somatic mutations, which helps to overcome statistical considerations that make it unlikely for any individual cell to accumulate the critical combination of mutations to overcome normal restraints on growth. In some instances, an inherited defect occurs in a tumor suppressor gene that is critical in regulating cell proliferation, such as the germline defect in the adenomatous polyposis coli (*APC*) gene. In other instances, a germline mutation occurs in a gene involved in maintaining the integrity of DNA. Inactivating germline mutations in the DNA mismatch repair (MMR) genes (*hMSH2*, *hMLH1*, *hMSH6* or *hPMS2*) cause Lynch syndrome, and biallelic mutations in the *MUTYH* gene cause a form of colonic polyposis.

In the cases of the *APC* gene or the DNA MMR genes, the inherited defect almost always occurs in one of the two alleles. This creates a situation in which the familial pattern of disease inheritance is autosomal dominant (i.e.

inheriting one mutant allele is sufficient for the predisposition to multiple colonic polyps), but mechanistically, the disease is actually recessive, and requires a second, “somatic” mutation of the other allele for the neoplasm to develop. This chapter reviews the general pathways by which certain gastrointestinal (GI) cancers develop, and focuses on germline mutations that predispose to the more dramatic, syndromic forms of familial GI cancer.

#### 70.1.2 Gastrointestinal Cancer

The GI tract is the site of more malignant tumors than any other organ system in the human body, which is one reason that much is known about genes that predispose to these cancers. The incidence of cancer in each organ of the GI tract varies enormously worldwide (1). The wide variations seen among different national groups appear to be explained almost entirely by environmental factors, principally dietary influences and the effects of chronic infections with different bacteria or viruses. For cancers of the esophagus, stomach, pancreas and hepatobiliary tree, environmental factors are overwhelmingly important, and familial factors play a minor role in carcinogenesis. However, in the colon and rectum, genetic considerations are somewhat more important, and are very important within the context of the developed world. In the extreme cases of familial adenomatous polyposis (FAP) and Lynch syndrome (or hereditary nonpolyposis colorectal cancer (HNPCC)), the germline mutations have powerful effects



that essentially overwhelm the influence of the environment. These two diseases have led to important insights into the formation of tumors in general (2).

## 70.2 THE GENETICS OF COLORECTAL CANCER

### 70.2.1 Somatic Genetics of Colorectal Cancer

Colorectal cancer was the first of the solid tumors to have undergone a sufficient degree of genetic analysis for the pathogenesis of the disease to be conceptualized. A step-wise series of events occurs whereby activating mutations in oncogenes and inactivating events in tumor suppressor genes accumulate, which account for the gradual evolution of colorectal neoplasia and the spectrum of premalignant tumors found in the colon (3). *K-RAS* was the first mutated oncogene found in colorectal cancer (4,5). Several different genetic lesions are involved in the inactivation of tumor suppressor genes, whose normal function is to inhibit cell proliferation or in some way prevent the emergence of the neoplastic phenotype. Tumor suppressor genes are functionally recessive inasmuch as the inactivation of both alleles is required for tumorigenesis. The recessive nature of tumor suppressor genes is the essential feature that permits them to be responsible for the familiarity of cancer. Inheritance occurs by a germline mutation in one allele, which affects every cell in the body. The second allele becomes inactivated in a somatic cell later in the life of the affected individual. Typically, the germline mutation is phenotypically silent, but the allele is inactivated in every somatic cell, greatly increasing the likelihood of tumor development, and decreasing the age at which this is likely to occur. The wild-type allele from the unaffected parent undergoes an acquired somatic alteration, referred to as the “second hit.” Each cell type has genes that specifically regulate its growth, which is why germline mutations in genes lead to tissue-specific cancers.

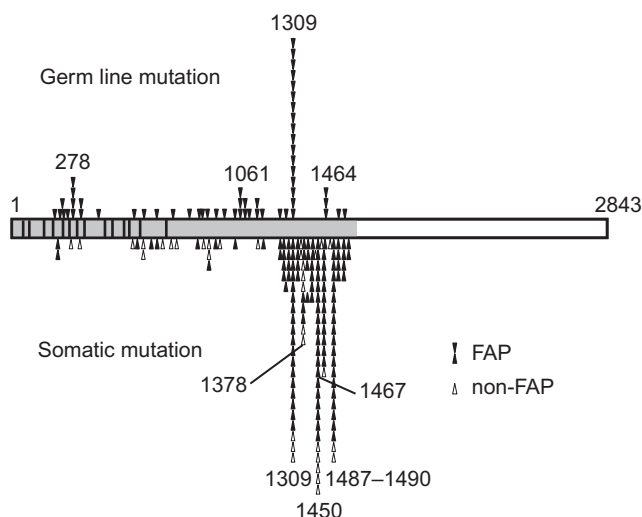
**70.2.1.1 The *APC* Gene.** An understanding of the somatic genetics of colorectal neoplasia began with an observation that occurred at the interface between sporadic and hereditary disease. A patient was identified who had multiple congenital abnormalities and features of FAP or Gardner’s syndrome (6). The patient’s colon was carpeted with adenomatous polyps, and he had two cancers in his colon. None of his relatives had FAP. It was assumed that he was an index case of FAP, and his karyotype revealed an interstitial deletion on 5q13–q22 that was not present in either parent. This clue led to the mapping of the gene for FAP at 5q21–q22 (7), and somatic deletions were found at this locus in 20% of sporadic colorectal cancers (8).

In 1991, two groups collaboratively reported cloning the adenomatous polyposis coli (*APC*) gene at the 5q locus, identified germline mutations in this gene in families with FAP, and reported mutations at this locus in sporadic colorectal cancers (9–12). The *APC* gene

encodes a 15-exon 8.5-kb transcript with a predicted protein structure that was unique at the time. The *APC* gene plays a major role in controlling epithelial cell proliferation. Mutations at the *APC* locus occur early in sporadic colorectal tumorigenesis; as it is one of the earliest genetic alterations in the multistep process, loss of function of *APC* has been termed the “gatekeeper” of neoplasia in the colon (13).

Neoplasia in the colon usually begins as a benign adenoma and, because of genomic instability that occurs along with progressive growth, additional mutations occur that permit the malignant phenotype to emerge. *APC* mutations are found in 63% of adenomas, including even the smallest of them (14). Most of the germline (and somatic) mutations create premature stop codons (nonsense mutations), or altered splice sites. Many of the mutations occur in the mutation cluster region in the fifteenth exon (Figure 70-1). This exon is a very long (>6000bp) open reading frame that accounts for about 75% of the *APC* transcript. Premature stop codons either lead to nonsense-mediated decay of the message, or result in the synthesis of a truncated *APC* protein. The multiple intestinal neoplasia (*min*) gene is the murine homolog of the human *APC* gene, and germline mutations at this locus cause multiple intestinal neoplasms in the mouse (15,16).

The *APC* gene product has multiple functions. One key role is to regulate intracellular concentrations of  $\beta$ -catenin (17), which can transactivate a cascade of genes involved in cellular proliferation, including *cyclin D*, *MYC*, *PPAR $\delta$*  and others. When the *APC* gene product is expressed in a cultured colon cancer cell line lacking wild-type *APC*, apoptosis ensues (18). The functions of  $\beta$ -catenin are complex, as this protein also participates in the intercellular adhesion junction (19,20). The earliest



**FIGURE 70-1** The *APC* gene germline and somatic mutations are depicted in FAP and non-FAP patients: 68 germline mutations in FAP patients and 241 somatic mutations in colorectal tumors from FAP and non-FAP patients are arranged on the *APC* sequence. Only the shaded regions were analyzed in this study. (From *Cancer Research* 1994, 54, 3011.)

colorectal neoplasms (adenomas) are characterized by excessive proliferation and the formation of a mass or polyp, which corresponds to at least some of the expected results of unregulated  $\beta$ -catenin expression. The APC protein is not expressed in the normally proliferating cells that are located at the base of the colonic crypt (21). APC protein is expressed as a function of colonic epithelial differentiation, and in mature epithelial cells it interacts with  $\beta$ -catenin, which leads to its phosphorylation and degradation. Loss of APC function usually occurs as a consequence of premature truncation, although more limited deletions of the APC-binding domain may be sufficient for loss of function (22). Missense mutations typically do not inactivate APC. In many of the colon cancers in which inactivating APC mutations cannot be found, stabilizing mutations in  $\beta$ -catenin that confer resistance to degradation by APC binding occur (23). Epigenetic silencing of the APC gene also occurs via hypermethylation of its promoter region (24,25). Hypermethylation-associated gene inactivation occurs in the promoters of numerous tumor suppressor genes, and is an important mechanism for tumor development (26).

**70.2.1.2 The K-RAS Gene.** The K-RAS-2 oncogene was among the first altered genes to be recognized in colorectal cancer (4,5,27). The *c-K-RAS-2* proto-oncogene is located on chromosome 12p; the gene product has GTPase activity and is a molecular switch for certain signal transduction pathways, some of which control growth and differentiation. Point mutations at codons 12, 13 and 61 frequently occur in colorectal cancer and, based on the three-dimensional structure of the *c-H-RAS* oncogene, these three amino acids interact with the phosphate groups of GTP. Critical mutations at these codons interfere with the regulation of the signal transduction, and deregulate cell growth (28). K-RAS-2 mutations occur in about half of benign adenomatous polyps in the colon (29), and it has been suggested that specific mutations at codon 12 may be associated with more aggressive tumor types (30,31). It appears that mutations in the APC gene are permissive of a neoplastic phenotype in the colon, whereas a RAS mutation in a cell might serve to facilitate additional clonal expansion. Although not an initiating event, RAS gene mutations appear to occur relatively early in the process of colon carcinogenesis, and copies of the mutated genes may be found in the stool or colonic effluents of patients with early disease, sometimes even in the absence of detectable colorectal neoplasia (32). Interestingly, RAS gene mutations in the absence of APC inactivation are found in hyperplastic polyps of the colon, which are not neoplastic, and not considered precursors of cancer, underscoring the importance of the sequence of the acquisition of mutations in carcinogenesis (33).

**70.2.1.3 Allelic Losses in Colorectal Cancer.** Vogelstein et al. (34) used restriction fragment length polymorphism (RFLP) analysis to search for allelic losses in colorectal carcinomas. In 56 paired specimens of normal colonic tissue and carcinoma studied with probes targeting most of

the chromosomal arms, 53 tumors demonstrated some degree of allelic loss or loss of heterozygosity (LOH). Some tumors lost a greater fraction of their allelic arms than others, and certain chromosomal arms—notably 17p and 18q—were lost in more than 75% of the tumors. Other allelic sites, namely, 1q, 4p, 5q, 6p, 6q, 8p, 9q, 18p and 22q, were lost in 25–50% of tumors. The recognition of frequent allelic loss in colorectal cancer led to a search for putative tumor suppressor genes on these chromosomes. The mechanism involved in this process remains incompletely understood and controversial. Several candidate mechanisms for chromosomal instability have been proposed (35). Importantly, the amount of allelic loss, expressed as “fractional allelic loss,” correlated with a more lethal outcome (36).

**70.2.1.4 The p53 Gene.** The *p53* gene on 17p was initially thought to be a possible oncogene (37). Colorectal cancers were found to have loss of one copy of *p53* in 75% of tumors, and the remaining allele was found to be mutant, fulfilling the prediction that biallelic losses would be required of tumor suppressor genes in cancer (38). Subsequently, *p53* mutations and deletions have been found in diverse types of tumors, including most of the common solid tumors in humans. It is currently thought that *p53* mutations may occur somewhat early in the tumor progression scheme, and that deletion of the wild-type allele occurs as a later event. A family of *p53* genes has been identified, and overlapping functions may complicate a simple interpretation of mutations in the *p53* gene (39). Using a combination of techniques, it has been observed that the allelic loss of markers linked to *p53* occurs either in focal carcinoma-in-adenoma (40), or precisely at the transition from adenoma to carcinoma (41). Families with germline mutations in *p53* (i.e. the Li-Fraumeni syndrome) are at increased risk for a variety of cancers, including sarcomas, breast cancer, and early-onset colorectal cancer (42).

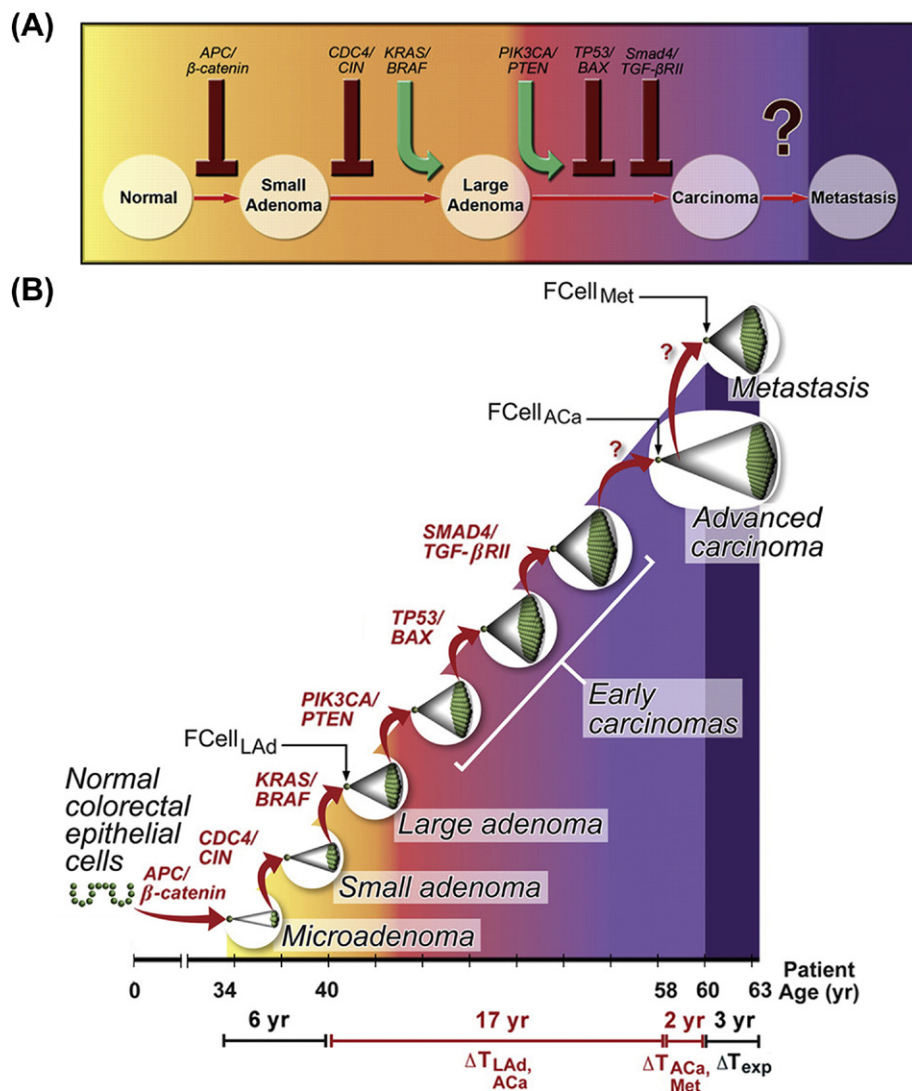
**70.2.1.5 Allelic Losses on 18q.** A high frequency of allelic loss in colorectal cancer was also noted on 18q (34). The first gene found at this locus was termed the “deleted in colorectal cancer” (*DCC*) gene (43), but the *SMAD4* gene also resides in this region (18q21.1) (44). Allelic loss at 18q appears to confer a worsening of five-year survival compared to patients who do not suffer this loss (45). Although this has been a somewhat controversial issue, there is evidence that the frequent chromosomal losses in this region and linkage with worse clinical outcomes are better explained by the loss of *SMAD* family genes, rather than *DCC* (46). Germ-line mutations in *DCC* have never been linked to familial cancer in humans or rodents. *DCC* knockout mice do not demonstrate abnormalities in intestinal growth, nor are they predisposed to cancer (47). Mice engineered to have mutations in both their *apc* and *smad4* genes (both of which are on mouse chromosome 18) have an enhanced neoplastic phenotype with larger and more malignant intestinal tumors (48). Moreover, mice with a

knockout of the *smad3* gene (which also corresponds to an 18q locus in humans) spontaneously develop aggressive, metastatic colon cancers (49).

**70.2.1.6 Multistep Carcinogenesis.** The concept of initiation and promotion, which was developed from animal models of skin tumorigenesis, has been replaced by the principle of multistep carcinogenesis (Figure 70-2A). An increase in the number of genetic lesions occurs during stepwise neoplastic progression. By analyzing the frequency of genetic alterations found at each stage of progressive neoplasia in the human colon, a model of multistep genetic damage has been proposed (3,50). The model proposes that a “genetic alteration” at the *APC* locus is an early and possibly initiating event in adenomatous tissue, which is the earliest identifiable colorectal neoplasm (14). *K-RAS* mutations are not found in tiny adenomatous polyps, but are

progressively more frequent in larger ones. *BRAF* mutations may substitute for *K-RAS* mutations, and appear to be involved in a separate “serrated pathway” that gives rise to neoplasms with somewhat different clinical features (33). Alterations on 18q are not common in small adenomas, but are more common in larger adenomas and those with more aggressive pathological appearances. As mentioned, biallelic losses of *p53* mediate the adenoma-to-carcinoma transition (41). By combining clinical and genetic data, it is possible to estimate the length of time required for multistep carcinogenesis to evolve in the colon (51). These estimates predict that it may take ~17 years for a large benign tumor to evolve into an advanced cancer, but less than 2 years for metastatic clones to arise within the cancer (Figure 70-2B).

**70.2.1.7 Genomic Instability.** Before 1993, attention had been focused on the identification of activated



**FIGURE 70-2** Sequential, multistep colorectal carcinogenesis. A. Fearon and Vogelstein proposed in 1990 that the sequential accumulation of genetic alterations was responsible for the morphological changes seen in tumor progression in the colon. Normal colonic tissue is represented on the left, and the sequential appearance of hyperproliferative epithelium, benign adenomatous polyps, and carcinoma are indicated with progression to the right. B. Colorectal cancers evolve by genetic alteration (mutation in this example), followed by clonal expansion, the generation of novel, diverse clones, and successive waves of expansion. However, the generation of a uniquely advantageous genetic alteration is a rare event, accounting for the long periods of latency that occur during the evolution of a lethal colorectal cancer. FCell indicates Founder Cells for advanced carcinoma or metastasis. (From PNAS 2008, 105, 4283.)

oncogenes and inactivated tumor suppressor genes in colorectal cancer. However, it had long been suspected that a phenotype of increased mutability might be required to account for the number of mutations found in many cancers (52). In 1993, three independent groups (53–55) reported that widespread mutations occurred at simple repeat genomic sequences in approximately 15% of colorectal cancers. These signature mutations are caused by loss of the DNA MMR function, and the result is referred to as microsatellite instability (MSI). MSI is defined by a critical number of mutations at microsatellite sequences in the DNA of a tumor (56). These observations ultimately led to the identification of mutations in DNA repair genes, and to the genetic basis of Lynch syndrome, previously called hereditary nonpolyposis colorectal cancer or HNPCC (57). Tumors with MSI are usually either diploid or near-diploid, and the mutational signature includes point mutations and insertion/deletion mutations at mono- and dinucleotide repeats, rather than as large cytogenetically detectable deletions, duplications or chromosomal rearrangements. Most colorectal cancers have intact DNA MMR activity, but are aneuploid. This latter process is not understood, and is referred to as chromosomal instability, or CIN (35,58).

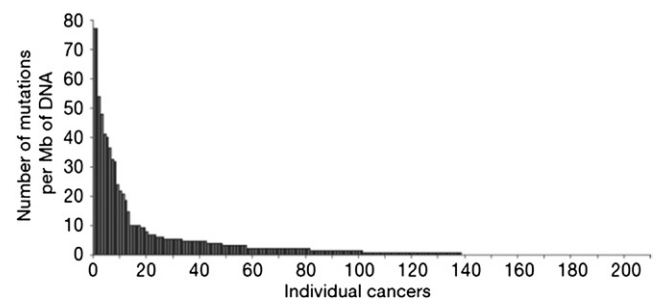
**70.2.1.8 CpG Island Methylation and Gene Silencing in Cancer.** Allelic losses, chromosomal rearrangements, and point mutations in the DNA sequence are frequent mechanisms for the loss of function of tumor suppressor genes. However, it has been found that methylation of cytosines at CpG islands, which are found around the 5' ends of about half of human genes, leads to transcriptional silencing of the gene. In part, this is a normal physiological process occurring with aging, but in some cancers, there is an excess of methylated promoters. Furthermore, certain tumor suppressor genes are methylated in a large fraction of cancers of certain organs (26). The phenomenon of excessive methylation in cancer has been termed the CpG island methylator phenotype, or CIMP (59), and is an important mechanism for tumor development. Some genes frequently methylated in CIMP cancers may be mutated in other tumors, or deleted in yet others. In fact, the *APC* gene can be inactivated by any of these three processes (24), and one can find both processes in the same tumor, since biallelic inactivation is required for loss of expression of tumor suppressor genes (25). Nearly one-third of colorectal cancers have little evidence of extensive CIN or MSI, and may be a result of CIMP (60). To make matters somewhat more complicated, essentially all sporadic colorectal cancers with MSI also have CIMP, and the MSI is a result of biallelic inactivation of the *hMLH1* gene due to promoter methylation (61).

**70.2.1.9 Somatic Mutations in Colorectal and Other GI Cancers.** One way to determine the spectrum of somatic mutations in tumors is to perform complete exomic or total genomic sequencing of the tumors, and this has been done for various types of cancer, including GI cancers. A number of important insights have been gained through this approach. First, the number of

point mutations in coding exons is surprising low: about 1 somatic mutation per megabase of DNA in colorectal cancers (62,63). Secondly, there is a relatively narrow range for the prevalence of somatic mutations among types of tumors (from about 0.12 to 4.21 per megabase), with a few outliers, such as DNA MMR-deficient tumors, which have 32.29 somatic mutations per megabase. In addition, among individual tumors, one finds occasional cancer genomes with >70 somatic mutations, and a large proportion of tumors with <10 (Figure 70-3) (62).

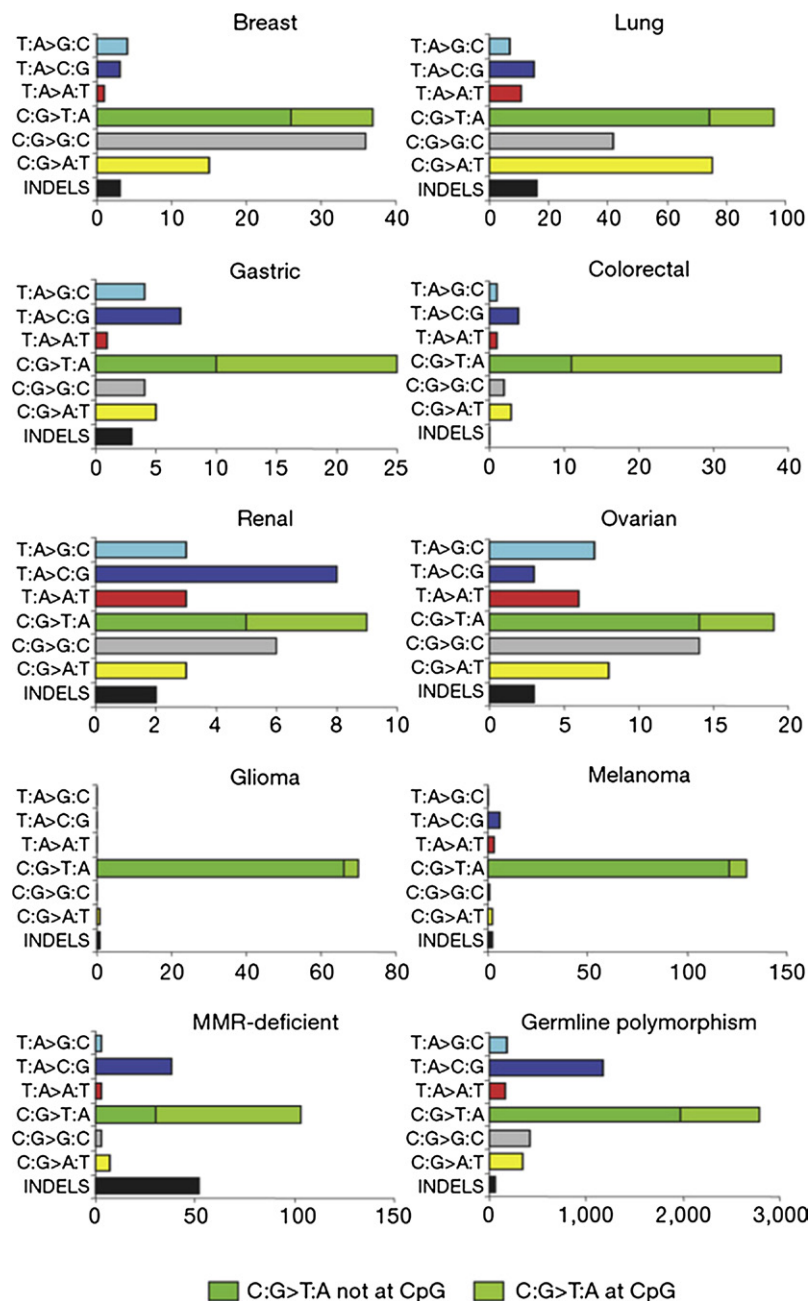
The mutational signatures for tumor groups are potentially enlightening. Colorectal and gastric cancers show a great propensity for C:G → T:A mutations, with the relative exclusion of other mutations, a fingerprint also shared with gliomas and melanomas. This spectrum stands in stark contrast to the broad spectrum of mutations found in lung, breast, renal and ovarian cancers (62). Although there is no experimental data to prove this, this mutational pattern is consistent with a failure of base excision repair to completely excise adducts created by oxidative stress, a relative failure of one part of the DNA MMR system, or perhaps an overwhelming of these repair systems by excessive proliferation of gut epithelium (see *MUTYH*-associated polyposis (MAP)). In any event, the signatures found in GI tract tumors are quite different from the patterns seen in other tumors (Figure 70-4).

Total exomic sequencing has also been used to compare individual colorectal or other cancers. A study of 11 colorectal cancers revealed that the mutational spectrum is so variable among tumors, that none are exactly alike, and only a very small number of mutations are common to all colorectal cancers (Figure 70-5). The same was true for breast cancers. Interestingly, the three most commonly mutated genes—*APC*, *KRAS* and *p53*—were the same genes initially identified with much less precise investigative techniques in the early 1990s (63). Studies of copy number variation reveal a very heterogeneous landscape, but the patterns of variation have yet to yield clinically relevant interpretations (64). This raises the vexing problem of how to find systemic therapies for widespread tumors when each is driven by a different set of mutated genes.



**FIGURE 70-3** Number of mutations in a human cancer. The number of somatic mutations (including base substitutions, insertion–deletion lesions, and complex mutations) per megabase of DNA in 210 human cancers. The number of mutations per megabase ranges from none to nearly 80, underscoring the diversity in human cancer. (From *Nature* 2007, 446, 154.)





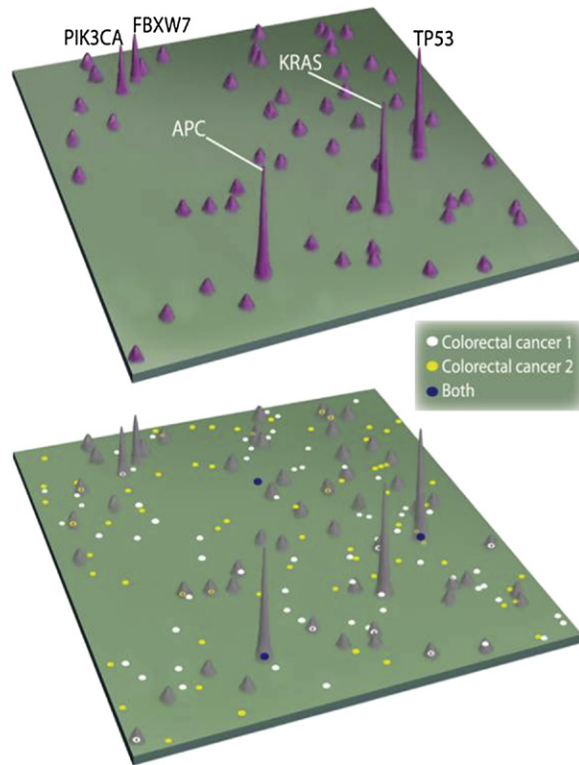
**FIGURE 70-4** Mutational spectrum in human cancers. The mutational spectrum in a series of 10 different types of human cancer suggests that cancers of different organs evolve through different mutagenic exposures. Colorectal and gastric cancers share some similarities but are distinct from lung and breast cancers, for example. The predominance of C:G → T:A mutations suggests a potential mutational mechanism (see text). INDELS indicates insertion or deletion mutations. (From *Nature* 2007, 446, 154.)

## 70.3 FAMILIAL COLORECTAL CANCER

### 70.3.1 Familial Adenomatous Polyposis (FAP), and Related Syndromes

FAP is an autosomal dominant disease in which affected individuals develop a large number of adenomatous polyps, predominantly in the colon. Adenomatous polyps may also occur in the small intestine and, rarely, in the stomach. Gastric polyps are common in FAP, but most of these are the result of fundic gland hyperplasia, and

actually, gastric cancer occurs in ~0.5% of FAP patients (65). Adenomatous polyps may bleed, or grow and cause obstructive symptoms, but the principal concern is the development of carcinoma. Cancer is nearly an inevitable consequence of FAP, as these benign neoplasms slowly grow and are subject to the same fate as sporadic adenomas. In FAP, however, there are hundreds or thousands of adenomas that begin in the teenage years, making malignant conversion a likely consequence because of the large number of adenomas, and their early appearance. The polyps are typical adenomas, which individually have a



**FIGURE 70-5** The genomic landscape of human colorectal cancer. Eleven colorectal cancers were subjected to total exomic sequencing, and the results plotted in two dimensions, representing all chromosomes, end to end, beginning with chromosome 1p in the left rear corner. Individual tumors had unique sets of mutations, as illustrated in the “landscape,” and only a handful of mutations were common to many tumors (creating the “mountains” in the landscape) or fewer (creating “hills”). The heterogeneity of the mutational landscape helps explain the variations in microscopic appearance, clinical behavior and therapeutic responses of individual colorectal cancers. (From *Science* 2007, 318, 1108.)

relatively low likelihood of malignant progression. It is the very large number that overwhelms the low risk for cancer in each individual polyp.

FAP begins with the asymptomatic appearance of a small number of polyps that progressively increase in number. Most of the polyps remain small, even without treatment. Over time, the colon may become studded with polyps. All histological varieties of neoplasia may be seen, including tubular adenomas, tubulovillous and villous adenomas, and carcinoma. Hyperplastic polyps and other types of pathological variants do not occur more commonly than in the general population. The finding of more than 100 adenomatous polyps in a young (<30 year old) individual is almost always FAP.

**70.3.1.1 Germline Mutations in the APC Gene and FAP.** As mentioned in the section on somatic genetics of colorectal cancer, FAP is caused by a germline mutation in the tumor suppressor gene called *APC*, located on chromosome 5q. A patient with FAP is asymptomatic at birth and phenotypically normal. Polyps of the colon begin to develop in the prepubertal and pubertal periods, and 50% have adenomatous polyps by age 16 (66). Early

reports of FAP series, which included a large number of patients who were not screened for the disease, indicated that the average age for the development of cancer was 39 years, and for death from cancer 42 years (67). This suggests that in the setting of FAP there is a lag of at least 23 years from the first adenomas to the occurrence of cancers. Work performed in humans with FAP, as well as the experimental rodent model of FAP, indicate that both *APC* alleles are inactivated in the adenomas in this disease (68,69). However, the mechanism of losing the second allele might be more complicated than a simple LOH event, and remains to be fully understood.

Most FAP is caused by inactivating germline mutations in one allele of *APC*, either premature stop codons, altered splice sites, or genetic deletions. A few well-characterized familial clusters of dominantly inherited adenomatous polyposis do not have a detectable germline lesion in *APC*, but these could be due to sequence alterations in a regulatory portion of the gene, or another cryptic mutation. It has been observed that 20–30% of FAP patients had new mutations, but there is phenotypic variation within families, and family members with identical *APC* mutations can show a variable number of polyps. Moreover, there is an autosomal recessive form of FAP involving a separate gene (*MUTYH*-associated polyposis). Identification of presymptomatic FAP patients can be achieved using a blood test to analyze the *APC* gene (70). Because the gene is large, an in vitro transcription–translation (IVTT) assay was initially used commercially, but this is not the most sensitive approach to diagnosis, and has been replaced by direct sequencing of the entire gene, and analyses for deletions or rearrangement. Some genetic testing performed in the 1990s may have used the IVTT approach, which may have given spuriously negative results to some patients. Genetic testing by DNA sequencing is commercially available, and information regarding laboratories that perform this can be found by consulting the Internet site <http://www.genetests.org>.

**70.3.1.2 Gardner Syndrome.** Gardner syndrome was initially thought to be a variant of FAP, with colonic polyposis and additional extraintestinal manifestations such as osteomas of the cranium and mandible, subcutaneous fibromas and lipomas, abnormal dentition, small intestinal polyps, etc. However, the identification of *APC* gene mutations as the basis for families with Gardner syndrome, and the recognition that all the extracolonic manifestations could be found in most FAP patients led to the realization that there was no specific difference between patients labeled as Gardner syndrome and FAP. The term Gardner syndrome remains as a historical reference to Dr Eldon Gardner, but it does not designate a unique disease.

**70.3.1.3 Variable Clinical Expression of FAP.** The phenotype in FAP may be either more or less virulent, based in part on the location of the *APC* mutation. The gene comprises 15 coding exons, the first 14 of which are of typical size. However, the very large open reading

frame of the 15th exon is a target for mutations. Particular hazard is associated with a mutation cluster region in exon 15 between codons 1250 and 1464. This is the most common site for mutations in sporadic adenomas, and germline mutations here are associated with a “profuse” phenotype with >5000 adenomas (Figure 70-6). Patients with germline mutations immediately upstream or downstream to this region typically develop fewer polyps (71,72). Furthermore, the average age to develop colorectal cancer in the profuse type of FAP is less than that observed in the sparse type (34 vs 42 years) (73). The mechanism underlying this remains uncertain, but this raises the possibility that some mutant alleles may encode a protein that exerts a dominant negative effect on the wild-type allele inherited from the unaffected parent.

The variable phenotypic expression in FAP is consistent with the influence of environmental factors, the presence of modifier genes, or both (74). In the mouse model of FAP, a major modifying locus called *mom-1* has been identified, which can affect the expression of the tumor phenotype (75). The situation may be more complicated, as a “modifier of min” has been located on the mouse chromosome 4, which may influence the mouse phenotype (76). Moreover, mutations at this locus, which encodes secretory phospholipase A2, are not involved in the clinical expression of FAP in humans.

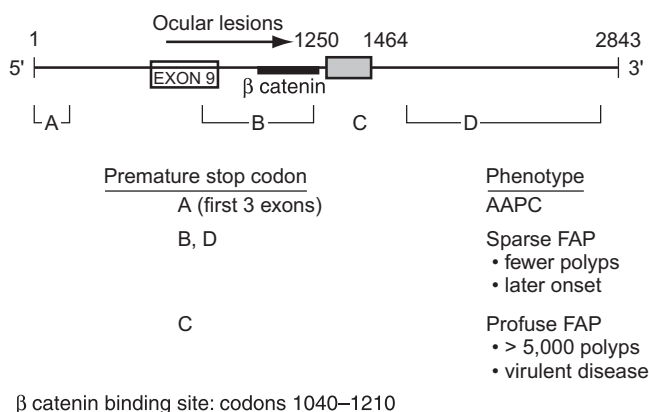
Congenital hypertrophy of retinal pigment epithelium (CHRPE) seen in FAP is rarely seen when the germline mutation of *APC* occurs in exons 1–7, but is frequently present when it occurs in exons 9–15 (77). A family has been reported with a germline mutation at the 3′ end

of the *APC* gene that results in a hereditary desmoid syndrome (78).

A genotypic variant of particular interest is when the premature truncation occurs in one of the first three exons of *APC*. The result is a form of the disease called attenuated adenomatous polyposis coli or attenuated FAP. In this condition, the number of polyps is smaller than in classic forms of the disease (79,80). In one large family with attenuated FAP, a considerable proportion of the members who carried the mutant gene had fewer than 50 polyps, and the average age for developing cancer was 54 years. However, several patients developed cancer in the absence of diffuse polyposis and, curiously, two members of the family had extensive polyposis. It has been demonstrated that the mechanism that accounts for the attenuation of the polyposis phenotype is the presence of an internal ribosomal entry site that permits transcription of the *APC* gene downstream of the mutation, skipping over the premature stop codon (81).

**70.3.1.4 Turcot Syndrome.** Turcot syndrome is a historical curiosity based on a few case reports of young patients with colon cancers or polyps and malignant tumors of the central nervous system. Most of these patients died at an early age, and often there was no apparent history of this disorder in the parents. Thus, inheritance was initially assumed to be autosomal recessive (82). Further attention was drawn to Turcot syndrome as a discrete entity when a family was found in which the manifestations were not linked to the *APC* gene (83). The issue was clarified by the characterization of 14 families with the manifestations of Turcot syndrome, of which 10 were found to have germline mutations in *APC* (84). The predominant brain tumor in those families was medulloblastoma. Furthermore, the relative risk (RR) of developing a cerebellar medulloblastoma in patients from FAP families was estimated to be 92 times that of the general population. Interestingly, in four other families, the brain tumors were glioblastoma multiforme, and three of these had germline mutations in one of the MMR genes, indicating that these patients actually had Lynch syndrome. Thus, the term Turcot syndrome does not actually indicate a unique disease, and these brain tumors in the presence of colorectal neoplasia in familial clusters or young patients should raise the possibility of either FAP or Lynch syndrome.

**70.3.1.5 The I1307K Variant of *APC* and Recurrent Adenomatous Polyps.** A curious wrinkle in the FAP phenotype is a predisposition to recurrent adenomatous polyps in the absence of diffuse polyposis, caused by a nontruncating sequence variation in *APC* termed I1307K (85). This might be considered a polymorphism in the gene, as no missense mutation has been identified, including this, which appears sufficient to cause FAP. The I1307K variation alters codon 1307 from isoleucine to lysine, but more importantly, converts the nucleotide sequence from AAATAAAA to A<sub>8</sub>, which is hypermutable and vulnerable to insertion or deletion mutations



**FIGURE 70-6** Germline mutations in the *APC* gene and the FAP phenotype. The *APC* gene is schematically depicted. Mutations occurring in segment A produce the attenuated FAP phenotype (AAPC). Mutations occurring from exon 9 to the 3′ end of the gene are associated with congenital hypertrophic retinal pigmented epithelium (CHRPE) lesions. Mutations occurring in segment C produce the profuse type of FAP with >5000 polyps, and earlier onset of polyps and cancer. Mutations occurring on either side of this (i.e. segments B and D) are associated with the sparse type of FAP, with a somewhat smaller number of polyps (~1000), and later onset of neoplasia. Segment C is also the most common site for acquired mutations in *APC*, and is situated immediately downstream of the β-catenin binding site.

during replication, which then creates a premature stop codon. This has been termed a premutation, which results in an increased risk of recurrent polyps and perhaps a two- to three-fold increase in the risk for colorectal cancer. This sequence variation occurs in 6% of Ashkenazi Jews and rarely presents outside of this population. This mild increase in predisposition to cancer can be managed by periodic colonoscopic screening, and does not ordinarily require prophylactic colectomy, as is required for severe forms of FAP. The clinical feature of I1307K is recurrent adenomatous polyps inherited in an autosomal dominant fashion. The I1307K allele requires a second, frameshift mutation within (or very close to) the  $A_8$  sequence for loss of function, and then a third mutation is required to inactivate the wild-type allele. The additional steps required are the likely explanation for the attenuated phenotype in carriers of this polymorphism, and the fragility of the  $A_8$  explains the increased risk for polyps. There have been contrary views on both the risk of cancer in individuals with this polymorphism, as well as the mechanism for inactivation of the APC alleles (86).

**70.3.1.6 MUTYH-Associated Polyposis (MAP): Autosomal Recessive FAP.** Some patients develop multiple colonic adenomas, but do not have a vertical family history of FAP; although there may be multiple polyps in more than one sibling, polyposis is not found in the parents. A systematic investigation of sibling clusters with multiple polyps in a British registry led to the discovery of an autosomal recessive genetic form of polyposis caused by germline mutations in the *MUTYH* gene (87), now called *MUTYH*-associated polyposis (MAP).

At least 1.3% of western European populations carry a missense mutation in one allele of the Mut Y homolog, *MUTYH*, initially called *MYH*, but changed as that name had been previously attached to another gene. *MUTYH* is a base excision repair (BER) gene, which participates in the process that repairs a mutagenic product of oxidative damage to DNA. The BER system can recognize 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG), which is a characteristic by-product of oxidative damage. During DNA replication, guanines with this adduct may mispair with adenines rather than cytosines, leading to G:C → T:A mutations. Three human BER genes cooperate to repair oxidative damage—*MTH1*, *OGG1*, and *MUTYH*. The *MTH1* gene (the human Mut T homolog) prevents incorporation of the oxidized guanine into newly synthesized DNA. The Mut M homolog, *OGG1*, selectively excises 8-oxo-dG adducts from the DNA strand. The Mut Y homolog, *MUTYH*, is an adenine-specific DNA glycosylase, which can recognize adenines that are inappropriately paired with 8-oxo-dG, and removes them (88).

Families were found with an autosomal recessive pattern of multiple polyp development, and had no germline mutations in *APC*. Each adenoma in these patients had unique *APC* mutations, and the mutational spectrum revealed a mutational signature (G:C → T:A) consistent

with defective BER activity. These patients were found to have specific germline mutations in the *MUTYH* gene: Y165C and G382D. Owing to renumbering of the codons, these are now termed Y179C and G396D. These mutations were in evolutionarily conserved regions, and were judged likely to be pathogenic (89). About 1.3% of the western European population carries one of these mutations (90); these are absent from Korean, Japanese and Jewish populations. Y179C is a nearly inactive allele, whereas G396D is hypomorphic. Thus, patients with biallelic Y179C mutations have cancers detected at a mean age of 46 years, versus 58 years for the G396D homozygotes, and 52 years for the compound heterozygotes (91). About 90% of MAP patients from western European populations will have at least one of these two mutations (92).

A US study of patients with MAP reported this to be a relatively mild disease with 3–100 adenomatous polyps, and later onset of polyps compared to classic FAP (93). However, a group of 170 Dutch patients with colonic polyposis who had no germline mutations in *APC* were screened for *MUTYH* mutations, and biallelic mutations were found in 24%. Colorectal cancer was present at the time of diagnosis in 65% of the affected individuals, at ages ranging from 21 to 67. Patients with this disease in whom the polyps were counted were as likely to have 10–99 polyps as 100–1000, but none had <10, or >1000, adenomas. Several patients had gastroduodenal polyps, one had duodenal carcinoma, and there was a significant increase in breast cancers among women (94). In an extended study of 276 Dutch, English and German cases, duodenal adenomas were found in 17%, and the estimated lifetime risk for duodenal cancer was 4% (95).

Interestingly, monoallelic *MUTYH* mutations are somewhat more common in US colorectal cancer patients who are older than 50 years of age, than in those who are younger than 50 (93). To sensitively screen for this disease, one must know the allele frequency of mutant genes in the population under study. The mutations Y179C and G396D accounted for 81% of the mutant alleles in one study from Great Britain (88). Among patients with 5–100 adenomas, 6.4% were found to have biallelic *MUTYH* mutations, so this does not account for all the *APC* mutation-negative individuals with polyposis. However, it should be considered among those with late-onset polyposis of a mild-moderate variety. In the British study, the median age for diagnosis of the biallelic mutation carrier state was 50 years, and the median number of adenomas was 40. There is a reported increase in the risk of colorectal cancer among heterozygous carriers of *MUTYH* mutations, with a two-fold increase in incidence (96). Interestingly, there is a reduced risk of colon cancer-associated mortality in this disease compared to sporadic colorectal cancer (96,97).

Clinical testing of the *MUTYH* gene is commercially available. A reasonable strategy to screen for this disease is to test for the two common point mutations in



suspected patients. Once the first mutation is found, then the entire gene should be sequenced to find the second mutation. The requirement for biallelic mutations in the disease improves the likelihood that one will be found by screening for the common mutations first.

### 70.3.2 Lynch Syndrome, and the Nonpolyposis Familial Colorectal Cancer Syndromes

Lynch syndrome is an autosomal dominant disease that predisposes to cancers of the colon and other organs, and is currently defined on the basis of documenting a germline mutation in a DNA MMR gene. Lynch syndrome was first recognized in a “cancerous fraternity” by the University of Michigan pathologist, AS Warthin (98). The index family was followed up 58 years later in a classic paper by Lynch, who described the continued risk for cancer in the offspring of affected family members, which illustrated the autosomal dominant mode of inheritance and high degree of penetrance (99). Subsequently, a number of large families have been described and the syndrome is recognized by the features listed in Table 70-1. The possible diagnosis of Lynch syndrome is supported when the family history meets the Amsterdam Criteria, which have been modified over the years to accommodate the fact that colorectal cancer is just one of the manifestations of the disease. Lynch syndrome is caused by a germline mutation in one of several DNA MMR genes, and its hallmark is the presence of MSI in the tumor (56,57). In this disease, the manifestations are determined, to some degree, by which of the four MMR genes is responsible (Table 70-2).

**TABLE 70-1 Clinical Features of Lynch Syndrome**

Autosomal Dominant Inheritance
Increased risk for colorectal cancer (70 and 40% penetrance for men and women, respectively)
Colon cancers have increased frequency of unique pathological features: 60–70% occur in the proximal colon, early age of onset (average age 40–45 years), mucinous or signet ring pathological appearance, Crohns-like lymphocytic infiltration of the tumor, poorly differentiated appearance
Multiple colonic malignancies (both simultaneous and metachronous)
Increased incidence of endometrial cancer (40–60%)
Increased risk for tumors of:
Stomach (observed/expected (o/e) = 4.1, $P < 0.001$ )
Small intestine (o/e = 25.0, $P < 0.001$ )
Ovary (o/e = 3.5, $P < 0.001$ )
Kidney (o/e = 3.2, $P < 0.01$ )
Ureter (o/e = 22.0, $P < 0.001$ )
Brain tumors: glioblastoma multiforme
Increased risk for pancreatic and prostate cancers
No increase in the frequency of lung, breast, or prostate cancers

o/e = observed/expected frequency of the tumor.

**70.3.2.1 The Genetic Basis of Lynch Syndrome.** In 1993, three groups reported that a subset of colon cancers was characterized by a large number of mutations at microsatellite loci (53–55). At the same time, Lynch syndrome was linked to a locus on chromosome 2p in two large kindreds (100), and it was immediately recognized that MSI was present in the Lynch syndrome colorectal cancers (Figure 70-7). In a remarkably short period, four human

**TABLE 70-2 Clinical Features of Lynch Syndrome: by Germline Mutation**

Germline Mutation	Unique Genetic and Clinical Features
hMSH2	Classic features of Lynch syndrome; common locus Nearly all colorectal cancers are MSI-H IHC shows absence of staining for hMSH2 and hMSH6 proteins Many of the germline mutations are large deletions (missed by DNA sequencing) May be associated with Muir–Torre syndrome (with skin lesions)
hMLH1	Classic features of Lynch syndrome; common locus Most colorectal cancers are MSI-H, but some mutations do not show this (i.e. D132H) IHC shows absence of staining for hMLH1 and hPMS2 proteins Some missense mutations in hMLH1 do not lead to loss of hMLH1 immunostaining Some mutations in hMLH1 will lead to isolated loss of hPMS2 immunostaining Must be distinguished from more common, acquired loss of hMLH1 (promoter methylation), which may be associated with mutations in BRAF
hMSH6	Less common locus for Lynch syndrome Produces an attenuated disease with delayed onset of colorectal cancers (average age 56 years) Penetrance same as with hMSH2 and hMLH1, but average age 10–20 years later Increased penetrance for endometrial cancer (71% by age 70) Tumors may be MSI-H (86%), but occasionally MSI-L (14%) MSI testing less sensitive in endometrial cancers IHC shows isolated loss of hMSH6 staining in 90%; hMSH2 staining intact Some mutations retain immunoreactivity, but lose function Families often fail to meet Amsterdam criteria because of later onset of cancers
hPMS2	Less common locus for Lynch syndrome Attenuated disease with later onset of cancer (>50 years old) and reduced penetrance Most colorectal cancers are MSI-H IHC shows isolated loss of hPMS2 protein, with intact hMLH1, hMSH2, hMSH6 Germline mutation detection problematic because of multiple (20) pseudogenes that mask mutations in the true gene

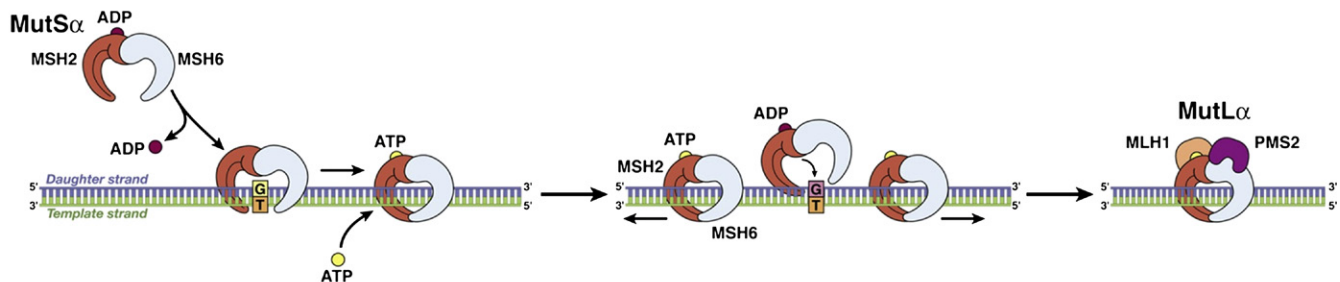
IHC, immunohistochemistry; MSI, microsatellite instability.

genes were cloned that account for most of the germline mutations in Lynch syndrome (101–105). These genes are all homologous to yeast MMR genes and were termed *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2*. However, linkage of *hPMS1* to familial cancer was spurious, and the gene is not considered to be a Lynch syndrome gene now. Subsequently, germline mutations in the *hMSH6* gene were linked to Lynch syndrome families as well (106–108). It has been proposed that both *hMLH3* (109,110) and *Exo1* (111) may be linked to familial colorectal cancer clusters, but convincing clinical evidence for these as Lynch syndrome genes is lacking (112–115). No Lynch syndrome families have ever been linked to germline mutations in the *hMSH3* gene. Thus, *hMSH2* and *hMLH1* are the “major” Lynch syndrome genes, and are the loci of mutation for most families with classic forms of the disease. *hMSH6* mutations less commonly cause Lynch syndrome,

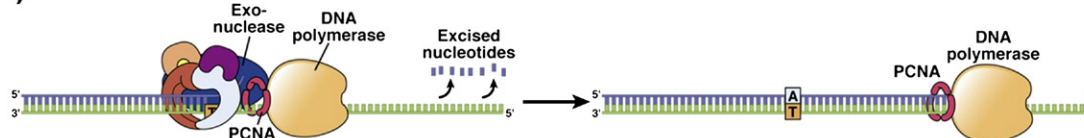
and one sees an attenuated form of the disease with these mutations. *hPMS2* also appears to produce an attenuated form of the disease, and is linked to a smaller, but as-yet undetermined, number of these cases (Table 70-3) (116).

The population prevalence of Lynch syndrome has not yet been established for the United States and western Europe, but a crude estimate would be at least one per 300 (117), based on the lifetime prevalence of colorectal cancer in the United States (~5/100), the proportion of colorectal cancers with MSI (~15%), and an estimate of what proportion of tumors with MSI are a result of Lynch syndrome (20–25% of those). This would provide an estimate of ~1.9/1000, but a higher estimate would account for the incomplete penetrance of colorectal cancer in this disease, which is in the range of 70% for men but may be about 40%, or even less, for women. Studies using population-based sequencing of the major DNA MMR genes

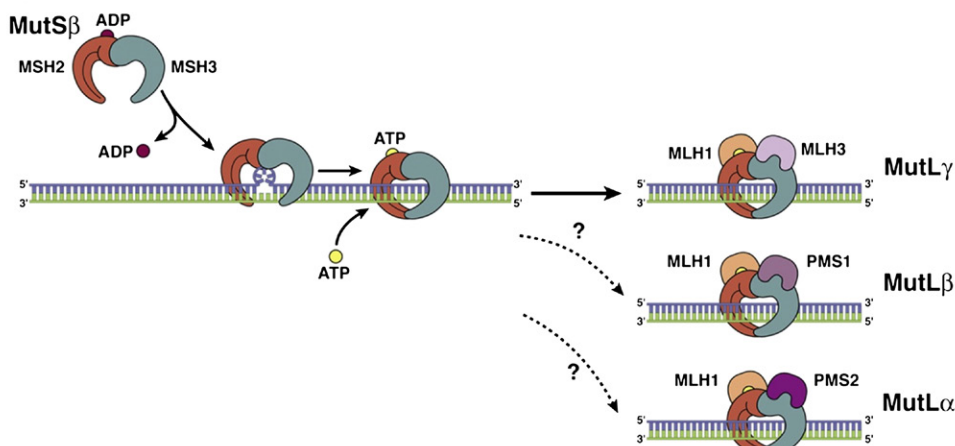
### (A) Single mismatch



### (B) Exonuclease complex and resynthesis



### (C) Insertion/deletion loop and variations in MutL complexes



**FIGURE 70-7** The DNA MMR system. This system requires one pair of heterodimerized proteins (either *hMSH2*+*hMSH6*—or *MutSα*—as shown in the top panel or *hMSH2*+*hMSH3*—called *MutSβ*—as shown in the bottom panel) to recognize a mismatch error in newly synthesized DNA. The mismatch triggers a conformational change in which the dimer forms a sliding clamp on the DNA, sliding away from the mispair toward the DNA polymerase complex. A second dimer (consisting of *hMLH1* + *hPMS2* (*MutLα*, as shown on the top), *hMLH1* + *hMLH3* (*MutLβ*) or *hMLH1* + *hPMS1* (*MutLγ*; all three shown in the bottom panel)), which interacts with PCNA and Exonuclease 1 to facilitate the excision of the mismatch, and ultimately, resynthesis to repair the error. (From *Gastroenterology* 2010, 138, 2073.)

TABLE 70-3 Lynch Syndrome Genes

Human Gene	Bacterial Homolog	Chromosomal Location	Base Pairs in cDNA	No. of Exons	Protein Size (aa-Amino Acids)
hMSH2	MutS	2p16	2727	16	934aa (105 kDa)
hMSH6	MutS	2p15–16	4245	10	1360aa (160 kDa)
hMLH1	MutL	3p21.3	2268	19	756aa (85 kDa)
hPMS2	MutL	7p22	2586	15	862aa (96 kDa)

*hMSH2* and *hMLH1* yield lower estimates for the prevalence of Lynch syndrome, largely because it has not been possible to detect all the disease-causing mutations using current technologies. Sequencing these two genes yields likely disease-causing mutations in 26% of familial clusters of colorectal cancer (118). Even accepting these limitations, documentable Lynch syndrome accounts for at least 2.2% of all colorectal cancers (119). Mutation databases are available at <http://www.nfdht.nl>, which assist in the attempts to distinguish innocuous sequence variations from disease-causing mutations in the DNA MMR genes.

**70.3.2.2 Mechanism of Tumor Development in Lynch Syndrome.** The basis of tumor development in Lynch syndrome is the result of a failure of the DNA MMR system. This DNA repair system monitors replication mismatches that occur as a result of misincorporation of bases by DNA polymerase, and “loop-outs,” which are errors that occur at repetitive sequences (i.e. mononucleotide or dinucleotide repeats) during DNA replication (Figure 70-7). A number of proteins are involved in this process. Loss of either of the two major MMR proteins (*hMSH2* or *hMLH1*) results in a total loss of DNA MMR. These major proteins are necessary for stabilization of the repair complex through heterodimerization with minor DNA MMR proteins, *hMSH6*, *hMSH3*, *hPMS2* and possibly others (120).

Individuals with Lynch syndrome have a germline mutation that inactivates one allele of a DNA MMR gene. The presence of the second, wild-type allele provides sufficient levels of gene expression and protein function that MSI is not found in normal tissues unless there are biallelic germline mutations, which are rare. Monoallelic inactivation of a DNA MMR gene is associated with a normal phenotype, but with an increased risk of developing cancer. When a second alteration occurs that inactivates the wild-type allele in a cell of a target tissue, there is loss of DNA MMR activity, resulting in MSI.

MSI can be found in about ~15% of all colorectal cancers (53–55). Not all of these are Lynch syndrome tumors. Criteria have been established for the performance and interpretation of testing for MSI (121,122). An international consensus conference led to the adoption of a panel of five microsatellite markers, which is now widely accepted for investigation or clinical diagnosis (56). However, other microsatellite panels have been found to perform at least as well for the detection of MSI

colorectal cancers (123–125). Moreover, the selection of appropriate marker obviates the need to use matched normal DNA to determine the presence of MSI (125).

Two types of MSI can be found in colorectal tumors, from which important implications can be drawn. When two or more of five markers are mutated, the tumor is designated MSI-high (MSI-H). These tumors share the phenotype described for Lynch syndrome, with a characteristic pathology, a proximal colonic predominance, and a better prognosis. About 20–25% of these tumors come from patients with Lynch syndrome, and the other 75–80% arise from acquired hypermethylation of the promoter of the *hMLH1* gene, which is unrelated to familiality (61,126,127). When only one of the five microsatellite markers is mutated, the tumor is designated MSI-low (MSI-L). These tumors behave clinically like tumors that show microsatellite stability (MSS), in which none of the five microsatellites tested is mutated. The mechanism of MSI-L may be multifactorial, but nongenetic downregulation of the *hMSH3* gene has been shown to produce MSI-L. Loss of *hMSH3* activity shown by immunohistochemistry correlates with the presence of somatic mutations in tri- and tetranucleotide repeats (which has been termed EMAST, for elevated microsatellite alterations at selected tetranucleotide repeats) (128). EMAST can be found in ~60% of colorectal cancers, and this is sometimes reflected as MSI-L, which may occur with no mutations at mononucleotide repeats, and occasional mutations at dinucleotide repeats.

MSI permits a very substantial increase in the accumulation of mutations at simple repetitive sequences called microsatellites. Some microsatellites occur in the coding regions of genes that control the growth of colorectal cells. When these genes are inactivated in the setting of MSI, the progeny will undergo clonal expansion, leading to the formation of a neoplasm. Repetitive sequences are under negative evolutionary selection pressure in the genome because they are intrinsically unstable. Nonetheless, a few genes are in our genome in which a coding exon has a mononucleotide repeat of six or more, and many are mutated in MSI-H cancers. The microsatellites in the diagnostic panel may be functionally unimportant, and simply passengers that are not directly involved in carcinogenesis.

Once MSI-H has developed, a cell becomes hypermutable (but not necessarily neoplastic). It is the mutational inactivation of target genes that leads to abnormal growth behaviors. The initial target genes found in

**TABLE 70-4 Target Genes in Colorectal Cancers with Microsatellite Instability**

Gene	Repeat	Frequency in Colorectal Cancers
TGF- $\beta$ R2	A10	85–90% (MSI)
IGF 2R	G8	9% of MSI tumors, 1/8 Lynch syndrome tumors
Bax	G8	51–54% (MSI); 52% in Lynch syndrome tumors
MSH6	C8	33% in Lynch syndrome cancers
MSH3	A8	52% in Lynch syndrome cancers
E2F4	(CAG) <sub>n</sub>	65% (in all MSI cancers)
Caspase-5	A10	62% in MSI cancers
ACVR	A8	50–75% in MSI cancers
TCF-4	A9	?
PTEN	A6 (2)	?

MSI = microsatellite instability.

Other genes frequently mutated at several sites in tumors with MSI include APC,  $\beta$ -catenin and  $\beta$ 2-microglobulin.

association with the mutator phenotype were the transforming growth factor  $\beta$ <sub>1</sub>-receptor type 2 gene, which contains an A<sub>10</sub> sequence (129); the insulin-like growth factor 2-receptor, which contains a G<sub>8</sub> tract (130); the BAX gene, which contains a G<sub>8</sub> tract (131); and several of the minor DNA MMR genes (132). A variety of other genes may be mutated in MSI-H tumors, including APC, PTEN, XTNNB1, CASP7, E2F4, and TCF-4 (56,133,134) (Table 70-4). Many of these are genes involved in the regulation of cell proliferation or apoptosis, and represent tumor suppressor genes that are mutated in this unique carcinogenic “pathway.”

The hypermutability caused by loss of DNA MMR activity leads to a great increase in frameshift mutations at repetitive sequences, and when the sequence is in an exon it typically creates a downstream stop codon, and either makes a nonfunctional protein, or is degraded through nonsense-mediated decay. If the inactivated gene is involved in growth regulation in colonic tissues, there may be clonal expansion, the accumulation of more mutations, and a multistep carcinogenesis pathway parallel to that initially described by Vogelstein in 1988, but involving a fundamentally different mechanism of genomic instability and a different set of target genes.

**70.3.2.3 Clinical Features of Lynch Syndrome and “Familial Colorectal Cancer Type X”.** Lynch syndrome is clinically heterogeneous, and the term “hereditary nonpolyposis colorectal cancer” (HNPCC) is both inadequate and misleading. It has been suggested that the term “Lynch syndrome” be used for the familial cancer syndromes in which a germline mutation in a DNA MMR gene has been identified (135). This is to avoid inclusion of the well-characterized familial clusters of early-onset colorectal cancer that are not classic Lynch syndrome, and are not caused by germline mutations in DNA MMR genes (136). By comparison, true Lynch

syndrome families have an earlier onset of colorectal cancer and extracolonic cancers, and there are elevated risks for cancers of the endometrium, ovaries, urinary tract (particularly renal pelvis and ureter), stomach, pancreas, brain, and small intestine, when compared with the familial clusters of colorectal cancer not associated with a germline mutation in a DNA MMR gene. It has been suggested that the latter families should perhaps be termed “familial colorectal cancer—type X” (136). They have no increase in relative incidences of cancer in organs other than the colorectum, a lower penetrance for colorectal cancer, and a later onset of the cancers. Compared to the general population, the relative risk (RR) for colorectal cancer in Lynch syndrome is 6.1, compared with 2.3 for “syndrome X.” The mean age of onset of colorectal cancer is 48.1 in the relatives of patients with Lynch syndrome vs 60.7 for the relatives of those with “syndrome X.” This concept has been confirmed in a Spanish cohort of patients (137).

The increased risk for cancer in Lynch syndrome is organ specific, most pronounced for the colon and endometrium, but increased risks for cancer are seen for other organs (138,139). Importantly, there are no significant increases in breast, lung or prostate cancers in Lynch syndrome families, indicating that this syndrome does not lead to an increase in cancer for every organ (136). A hallmark of Lynch syndrome is the early age at which tumors develop: colorectal and endometrial cancers typically occur two or more decades earlier than would be expected in the general population. The disease is inherited as a classic autosomal dominant condition, based on the inheritance of a germline mutation in a DNA MMR gene.

It is recognized that incomplete penetrance of the phenotype can occur in Lynch syndrome. There have been individuals who have developed as many as five or six tumors in several different organs (140), but most affected individuals tend to develop one or two in their lifetime. The risk of developing a cancer is linear and continuously increases by about 1.6% per year (141–143). Cancer risks for specific organs have been variably estimated by different registries, and may vary according to the DNA MMR gene affected, the presence of modifier genes in the population being studied, and confounding environmental influences. Polymorphisms in putative modifier genes, including *cyclin D* (144), codon 72 of the *p53* gene (145), and in several carcinogen-metabolizing enzymes (146,147), are associated with earlier tumor onset. Single nucleotide polymorphisms (SNPs) at 8q23.3 and 11q23.1, not associated with specific genes have been linked with two- to three-fold increased risks for colorectal cancer in Lynch syndrome (148). Lifetime risks for colorectal cancer have been estimated to be as high as 80% (141,149), but as the ability to diagnose these families more carefully at the genetic level improves, the estimates are likely to fall. For example, using known germline mutations (instead of simply family history), it has been reported that male gene carriers have a colorectal cancer risk of 74% by age 70, whereas female



gene carriers have a risk of 30%. Overall cancer risks were also modified by gender in this study, and men had a 91% risk for any cancer by age 70 vs 69% in women. Women in this study had a 42% risk of endometrial cancer, which is greater than their risk for colon cancer (150). Moreover, as families are characterized on the basis of germline mutational analysis, there may be a shift toward later onset of cancer, since many of the initial family clusters (identified on the basis of family history) may be over-represented with the most severely affected families.

#### 70.3.2.4 Pathological Features in Lynch Syndrome.

The pathologic features of the cancers are unique in Lynch syndrome (151). First, the majority of the colorectal cancers, 60–70%, occur in the proximal colon, and tend to be detected at an earlier stage. Approximately 37% have a poorly differentiated appearance, mucinous cancers are more common, excess mucin occurs in about one-third of cancers, and signet ring cells are more commonly seen (57,152). Flow cytometric studies indicate that 90% of these tumors are diploid or near-diploid (153), and there is less CIN in tumors that demonstrate the features seen in Lynch syndrome tumors (54,55). Lynch syndrome predisposes to multiple synchronous (18%) and metachronous (45% by 10 years if not treated with subtotal colectomy) cancers, indicating that these patients are at substantial risk for multicentric disease (154).

**70.3.2.5 Colonic Polyps in Lynch Syndrome.** Colonic polyposis, such as that seen in FAP, is not seen in Lynch syndrome. However, adenomatous polyps are more common in individuals at risk for Lynch syndrome. Adenomas can be found in 30% of the first-degree relatives of Lynch syndrome patients with at least one adenoma present at a mean age of 44 years, and 20% have multiple adenomas. Furthermore, like the cancers, the adenomas have a predilection for the proximal colon (155). Moreover, the adenomatous polyps tend to be larger and to be detected at more advanced pathologic stages, suggesting that they may grow faster and intrinsically be more aggressive than sporadic adenomas. Because of the shorter time in the evolution of an adenoma to carcinoma, seeing more than three adenomatous polyps before age 30, or more than six before age 50, suggests some diagnosis other than Lynch Syndrome (156).

**70.3.2.6 Prognosis in Lynch Syndrome.** Patients who develop colorectal cancers in the setting of Lynch syndrome have a significantly better prognosis than might be expected when compared with stage-matched controls. This is true for patients who meet the clinical criteria for Lynch syndrome (157), families with germline mutations in the *hMLH1* gene (158), and in young patients with MSI colon cancers, which would include many Lynch syndrome patients, and others who are not well characterized (159). Patients with Lynch syndrome-related colorectal cancers present with lower-stage disease and are less likely to present with distant metastases (160), and young patients whose tumors have MSI have a relative hazard ratio for death of 0.42 compared to tumors without MSI (159).

The penetrance for cancer of this disease is clearly less than 100%, and germline mutations in the DNA MMR genes are often found in families that do not meet the initial Amsterdam criteria (161). The tumor spectrum in Lynch syndrome was estimated from one study of 315 individuals at risk for the disease. In these families, 63% of the tumors were colorectal, 8% were endometrial, 6% were gastric, 4% were in the pancreaticobiliary tree, and other organs were occasionally affected (162).

**70.3.2.7 Clinical Variation in Lynch Syndrome.** It was suggested in 1984 that there may be two variants, initially termed Lynch syndromes I and II, in which type I was colon cancer specific, and type II had more extracolonic cancers (163). Although such familial phenotypes occur, disease variation is mainly linked to the DNA MMR genes that give rise to the disease. Germline mutations in the minor DNA MMR genes (*hMSH6* and *hPMS2*) give rise to an attenuated phenotype. Specifically, germline mutations in the *hMSH6* gene are associated with later onset of colon cancers (median age for cancer being in the sixties), with incomplete penetrance, and an increase in the relative impact of endometrial cancers in the women of these families. In addition, 86% of the tumors are MSI-H and 14% are MSI-L, and the latter may be missed, depending on the panel of microsatellites used to screen for this mutational signature (164). Lynch syndrome caused by *hPMS2* mutations is more complicated, but the penetrance appears to be less than that associated with the other Lynch Syndrome genes. When isolated loss of *hPMS2* expression was found in a large group of colorectal cancers, this was associated with early onset of disease, but not with a striking family history of cancer (165).

**70.3.2.8 Muir–Torre Syndrome.** Another variation of Lynch syndrome has been called the Muir–Torre syndrome, which is typical Lynch syndrome complicated by benign or malignant sebaceous tumors and keratoacanthomas of the skin. MSI has been found in the cutaneous and internal malignancies of Muir–Torre syndrome (166). Furthermore, several families have been linked to the *hMSH2* and *hMLH1* loci in this disease, which clearly identifies it as a member of the Lynch syndrome family of diseases (167,168). The presence of multiple sebaceous neoplasms (including sebaceous adenomas, sebaceous epitheliomas, and sebaceous carcinomas), or keratoacanthomas in any patient, strongly suggests the diagnosis of Muir–Torre syndrome.

**70.3.2.9 Diagnosis of Lynch Syndrome.** Traditionally, the diagnosis of Lynch syndrome is made on the basis of family history, with an emphasis on early onset of proximal colon cancers clustering in a family. In 1991, diagnostic criteria known as the Amsterdam criteria (169) were developed by an international collaborative group. They required (1) three or more family members with histologically verified colorectal cancer, one of whom is a first-degree relative of the other two; (2) colorectal cancer involving at least two successive generations; (3) one or more colorectal cancer cases diagnosed before age

50; and (4) FAP is excluded. The original Amsterdam criteria were useful to standardize collaborative studies, but were too restrictive for clinical use as they failed to acknowledge the contribution of endometrial and other extracolonic cancers, and made it relatively unlikely that a small family would fit the criteria. The modified Amsterdam criteria (170) and the revised Bethesda criteria (124) have been developed to improve our ability to diagnose this disease (Table 70-5), and these acknowledge the presence of tumors in nonintestinal organs in this disease (most prominently endometrial cancer), and the use of molecular diagnostics on tumor tissue.

The genetic diagnosis of Lynch syndrome has intrinsic problems depending on the gene involved. Mutations in *hMSH2* are frequently premature stop codons, which are almost always pathogenic. However, a substantial proportion of *hMSH2* mutations are caused by large deletions that are not detected by DNA sequencing, and require techniques that detect whether there are one or two alleles in the exon being sequenced (171). Deletions are a common problem with *hMSH2* because the 5' end of this gene is located in a cluster of *Alu* sequences, which are prone to recombination with excision of the DNA between the hybridized sequences. This genetic alteration is present in 20–25% of cases in which the germline mutation is suspected in *hMSH2*, but none is found (172). Curiously, *Alu*-mediated losses of the termination codon of the

*EPCAM* gene, which is immediately upstream of *hMSH2*, leads to methylation of the promoter of *hMSH2* in the tissues in which *EPCAM* is expressed, and produces a syndrome that mimics Lynch syndrome-*MSH2* type (173).

Many institutions will initiate their evaluation for Lynch syndrome using immunohistochemical staining of the tumor tissue for the two pairs of DNA MMR proteins, *hMSH2* plus *hMSH6*, and *hMLH1* plus *hPMS2*. The absence of staining of either *hMSH2* or *hMLH1* is accompanied by the loss of staining for *hMSH6* and *hPMS2*, respectively, as these are their heterodimeric partners.

The most common DNA MMR abnormality at immunohistochemistry in a group of unselected colorectal cancers will be the paired losses of *hMLH1* and *hPMS2*, which will most commonly reflect biallelic methylation of the promoters of *hMLH1*; this can be confirmed by an assay for promoter methylation or *BRAF* mutation (59). In the absence of methylation-mediated silencing, one needs to determine whether there is a germline mutation in the *hMLH1* gene. However, the interpretation of mutations at the *hMLH1* locus can be hazardous, as many are missense mutations, and it is not always apparent a priori whether such a mutation will abrogate function (174). Functional studies are cumbersome, but may be the only way to determine whether some of these mutations are truly pathogenic (175). Moreover, it is possible to find instances in which the germline mutation in *hMLH1* leads to MSI and isolated loss of *hPMS2* staining at immunohistochemistry. This scenario can be caused by a missense mutation in the *hMLH1* gene that abrogates the ability of the *hMLH1* protein to interact with the *hPMS2* protein. There have been instances of soma-wide methylation of *hMLH1*, with methylation present in blood, skin and gut epithelium, and this will mimic Lynch syndrome-*MLH1* type (176). Inheritance of this epigenetic germline alteration has also been reported, although the mechanism responsible for this is not known (177).

Germline mutations in the *hMSH6* gene cause a modified form of the disease. Families with *hMSH6* mutations have a delayed-onset form of Lynch syndrome in which the median onset of disease is 10–20 years later, colorectal cancer may be less prominent, and endometrial cancers become a more important clinical feature (108). Of particular importance, when these individuals reach their 70s and 80s, they have accumulated the same total prevalence of cancer as occurs in families with germline mutations in *hMSH2* or *hMLH1*, but the tumors appear later, and there is the very high incidence of endometrial cancer (164). Perhaps more importantly, 8% of familial clusters of colorectal cancer in which the Amsterdam criteria were not met were associated with germline mutations in *hMSH6* (107). Between 16 and 21% of colorectal cancer patients under age 50 who have no more than one first-degree relative with colorectal cancer have Lynch syndrome, which is over-represented with alterations in *hMSH6* and *hPMS2* (178,179).

**TABLE 70-5 Increased Suspicion for Lynch Syndrome: Clinical Criteria**

<p>A. Modified Amsterdam Criteria (for Lynch Syndrome) (Vasen et al., 1999)</p> <ol style="list-style-type: none"> <li>(1) Three relatives with pathologically verified colorectal cancer, or other Lynch syndrome-associated cancer<sup>a</sup></li> <li>(2) One patient is a first-degree relative of the other two</li> <li>(3) At least two successive generations are affected</li> <li>(4) FAP is excluded</li> <li>(5) One cancer has occurred &lt;50 years</li> </ol>
<p>B. Revised Bethesda criteria (to prompt MSI testing in colorectal cancer) (Umar et al., 2004)</p> <ol style="list-style-type: none"> <li>(1) Colorectal cancer in a patient &lt;50 years old</li> <li>(2) Multiple synchronous or metachronous Lynch syndrome-associated cancers<sup>a</sup></li> <li>(3) Colorectal cancer with the "MSI-H" histology<sup>b</sup> in a patient &lt;60 years old</li> <li>(4) Colorectal cancer in ≥1 first-degree relatives with a Lynch syndrome-related tumor,<sup>a</sup> in which one of the cancers is diagnosed in a person &lt;50 years old</li> <li>(5) Colorectal cancer diagnosed in ≥2 first- or second-degree relatives with a Lynch syndrome-related tumor,<sup>a</sup> regardless of age</li> </ol>

<sup>a</sup>Adenocarcinoma of the colon, rectum, endometrium, stomach, ovary, pancreas, small bowel, ureter or renal pelvis, biliary tract; also, glioblastoma multiforme of the CNS or tumors of the Muir-Torre syndrome spectrum (sebaceous neoplasms and keratoacanthomas).

<sup>b</sup>The presence of tumor-infiltrating lymphocytes, Crohn-like lymphocytic reaction, mucinous/signet ring differentiation, or medullary growth pattern.

Finally, it has been estimated that some proportion of Lynch syndrome is due to germline mutations in the *hPMS2* gene (116,180). This conclusion can be reached by finding isolated loss of hPMS2 staining at immunohistochemistry, and excluding a germline mutation in the *hMLH1* gene (116). However, detection of the germline mutation in *hPMS2* is very difficult because of the presence of many pseudogenes of hPMS2 in the human genome, which provides extra wild-type copies of the *hPMS2* sequence, which obfuscate the DNA sequencing results (181). Lynch syndrome caused by germline mutations in *hPMS2* may be attenuated as it is in the case of *hMSH6*, but too few families have been reported to be certain of the phenotype in this circumstance, and it is likely that many families have been overlooked because of the diagnostic challenges.

Genetic testing for Lynch syndrome using DNA sequencing is still imperfect. First, there are multiple genes that contribute to the syndrome, and for each gene the mutations are scattered over multiple exons. Direct sequencing strategies will not detect some disease-causing mutations, such as genomic deletions, which are often not detectable using exon-by-exon sequencing, as mentioned above for *hMSH2* (171). Germline mutations can be found in half (or fewer) of familial colon cancer clusters, regardless of the means of genetic testing (161). It is reasonable to consider testing for MSI in any colon cancer patient under age 70 (117), and then to pursue specific DNA MMR genes when this is positive. Two studies of cohorts of colorectal cancer patients who do not have more than one first-degree relative with colorectal cancer have shown that 16–20% of these patients have cryptic, unsuspected Lynch syndrome (178,179).

Biallelic germline mutations have been reported for all four Lynch syndrome genes. This causes a virulent, early-onset tumor diathesis beginning in childhood including hematological malignancies, and colorectal cancers before age 20. The patients may have café-au-lait spots, and be misdiagnosed as neurofibromatosis (182).

**70.3.2.10 Management of Lynch Syndrome.** Once a diagnosis of Lynch syndrome is definite, the patient may opt for one of two management options. Colonoscopic surveillance every 2–3 years has been shown to detect early-stage tumors and significantly reduce mortality from colon cancer (183). A consensus developed by several groups has recommended surveillance every 1–2 years, beginning at age 25 (117,184). The ordinary surveillance interval of 5 years used for patients with sporadic neoplasms is not adequate because of the rapid progression of adenoma to carcinoma in this setting (156,185). Some patients may prefer to avoid a large number of colonoscopic procedures and choose a prophylactic colectomy. Such patients do not require a total proctocolectomy, as the distal colon is relatively spared in this disease and can be managed with sigmoidoscopy annually.

If surgery is performed in Lynch syndrome, a subtotal colectomy is recommended whether the tumor is

in the proximal or the distal colon. It is not possible to make recommendations regarding the use of chemotherapy, or postoperative radiation (for rectal cancers). In vitro data suggest that these tumors will be relatively resistant to a wide range of chemotherapeutic agents (186,187), but so far there are no prospective clinical trials on which to base therapeutic decisions. The prognosis is significantly better for MSI-H tumors than for non-MSI-H tumors, in aggregate, or when matched for stage (159). A large, multicenter trial in which colorectal cancer patients were randomized to receive 5-fluorouracil (5-FU)-based chemotherapy was retrospectively analyzed for outcome based on the MSI status. No benefit was seen for adjuvant chemotherapy in patients whose colorectal cancer was MSI-H (but was observed for MSS tumors), and there was a suggestion that this treatment doubled the risk of death in such patients (188). The absence of benefit for adjuvant 5-FU-based chemotherapy has been confirmed in multiple studies (reviewed in (189)). Pooled analyses of multiple trials suggest that this is not a simple issue. Adjuvant chemotherapy may reduce distant metastatic recurrences in stage III MMR-deficient colorectal cancer patients, but result in an increase in intra-abdominal recurrences, and show no benefit in stage II tumors (190). One should be cautious when considering the use of adjuvant chemotherapy in patients with Lynch syndrome until data demonstrate that benefit will be derived from this treatment in each of these clinical subsets of patients.

**70.3.2.11 Management of Patients with a Family History of Colon Cancer.** Approximately 5% of the North American population will develop colorectal cancer. Depending on family size, therefore, many patients will seek advice from a physician when a family member develops an intestinal tumor. To advise such patients properly, it is essential to obtain a careful family history, document the tumors where possible, and inquire about the age of tumor onset and the multiplicity of tumors in the relatives. More tumors and earlier age of onset in a relative increase an individual's risk for cancer.

As mentioned earlier, colorectal cancer is common, so both positive and negative information will be important. In a large family in which many members have lived into, say, their eighties, and no tumors have developed, the risk for colorectal cancer is probably less than normal. In a small family even one colon cancer may be important, especially if it occurs in a young first-degree relative. At one end of the spectrum, patients in Lynch syndrome families have a 50% risk of inheriting a susceptibility gene, and the penetrance for tumor development may be as high as 80–90%. The Amsterdam criteria for Lynch syndrome require that three family members have colorectal cancer, more than one generation be involved, and at least one individual be affected before 50 years of age. More ambiguous degrees of a positive family history result in an estimated risk that would fall between 5 and 50%, and the involvement of



a genetic factor might also decrease the age at which a tumor may develop.

It has been estimated that the age-adjusted RR of colorectal cancer for men and women with any affected first-degree relative is 1.72 (95% confidence interval, 1.34–2.19), and the RR when there are two or more affected first-degree relatives is 2.75 (95% confidence interval, 1.34–5.63) (191). Therefore, when a patient has one first-degree relative with colorectal cancer, it should alert the clinician to the need for some type of screening. The presence of two or three affected family members, particularly when one or more cancers occur in younger patients (i.e. individuals less than 50–55 years), increases the suspicion of Lynch syndrome. Positive family histories for cancer also confer an earlier age of onset of the disease among the relatives (192).

If one suspects Lynch syndrome on the basis of multiple first-degree relative involvement or early age of tumor onset, colonoscopy is the most appropriate screening approach, and it should be undertaken at least every other year. Screening intervals of 3 years or more, which are appropriate for sporadic colorectal cancer, are not appropriate for Lynch syndrome, as interval cancers can occur (156,193). In patients whose histories do not suggest Lynch syndrome, less rigorous screening programs are appropriate, but should be tailored to the degree of estimated risk. In the general population, colonoscopy is the optimal diagnostic modality for screening, and a negative colonoscopic examination can usually assure a relatively low likelihood for the development of a lethal malignancy within the subsequent 3–5 years (194). Moreover, when there is a history of a single first-degree relative with colorectal adenoma or cancer at an older age (i.e. >60 years), there may be no important increase in risk for colorectal cancer compared to the general population (192), and a targeted, intense screening program is unnecessary. Nonetheless, the risk of colorectal cancer in North America and western Europe is sufficiently high that annual fecal occult blood testing and periodic flexible sigmoidoscopy have been recommended for the general population, and an estimation of increased risk affects the age at which screening begins, the use of colonoscopy (rather than only sigmoidoscopy), and the use of periodic surveillance examinations. Several studies have demonstrated that colonoscopy is relatively less effective in preventing proximal vs distal colorectal cancers, for a variety of reasons (195,196). Therefore, particular care must be exercised by the colonoscopist to carefully examine the proximal colon in Lynch syndrome patients, to avoid the unfortunate occurrence of “interval cancers” (156).

When syndromic colon cancer is strongly suspected, a more aggressive approach is indicated. If FAP is suspected in a family, the optimal approach is to find the germline mutation in a family member who is definitely affected with the disease. Then, first-degree, at-risk relatives should be offered testing accordingly. If a mutation

is found in an affected family member, this information can be very helpful in making the diagnosis of FAP or excluding it definitively. It is essential to definitively identify the mutation in an affected individual in a family, since not all mutations are detectable with the current diagnostic strategies; thus, obtaining a negative genetic test in the relatives of an affected individual in whom the germline mutation has not been found is not definitive. Once identified, one can test siblings and offspring, and if the test is negative for a known mutation, the patient can be reassured, and no more endoscopic surveillance is necessary.

#### 70.3.2.12 Other Possible Forms of Non-Lynch Syndrome, Nonpolyposis Familial Colorectal Cancer.

There are still forms of familial colorectal cancer for which the germline mutations are not known. Familial clusters of colorectal cancer with variable types of MSI (MSI-V) have been identified, but the genetic basis of this has not been found. These families always meet the Bethesda criteria, often meet the Amsterdam criteria (55% of families), frequently have *BRAF* mutations (V599E), frequently have methylation of the promoter of the *MINT31* gene, and may progress through a unique carcinogenetic pathway (197).

There is a slight increase in colorectal cancer among individuals with a germline sequence variation in the transforming growth factor type 1 receptor. This sequence variation, called *TGF $\beta$ R1*\*6A, does not produce a phenotype of highly penetrant familial cancer by itself (198). However, this could be a modifier gene that interacts with other factors, either environmental or genetic, to increase the risk of cancer.

### 70.3.3 Genome-Wide Association Studies of GI Cancers

Several large collaborations have attempted to find additional genes for familial GI cancer using genome-wide association studies (GWAS), in which a large number of SNPs are tested for linkage to a specific phenotype (such as colorectal cancer) in a large number of patients. One locus at 8q24.21 has been found repeatedly by several groups (199,200). Although there are very high levels of significance attributed to this locus (up to  $P = 10^{-14}$ ), the odds ratios (ORs) for colorectal cancer are relatively modest (OR = 1.27 and ~1.14 for the two studies). What is perhaps most interesting about this locus is that there is no gene or obvious functional genetic unit at or near the locus. Other collaborations and meta-analyses have confirmed the association (201–203), so the linkage is clearly real, but the mechanism by which this locus modifies risk is still an open question. One group has suggested a long-range interaction with the *MYC* locus (204), but the biology of how this modifies risk remains to be discovered.

Similarly, additional SNPs have been linked to colorectal cancer risk, including 9p24 (200,201), 8q23.3 (203,205), 10p14 (205), and 11q23 (202), typically with



similar findings: a relatively low increment of risk (all with RRs less than 2.0, most often about 1.2, and some with incremental risks <10%), and most are not located within or even near a gene. There are some exceptions, such as the identification of a SNP associated with the *SMAD7* gene (206), but generally, the hunt for common low-risk genetic variants has pointed to areas of the genome that are not populated by genes. The accumulation of multiple risk SNPs is modestly additive, but the total risk is not yet sufficient to be clinically useful as a predictive tool (discussed in (207) and (208)). Two SNPs (at 8q23.3 and 11q23.1) modify colorectal cancer risk in patients with Lynch syndrome (148), and this may be a direction in which this information can be applied clinically in the future.

## 70.4 OTHER FAMILIAL GASTROINTESTINAL POLYPOSIS SYNDROMES

The gastrointestinal polyposis syndromes are listed in Table 70-6.

### 70.4.1 Peutz–Jeghers Syndrome

Peutz–Jeghers syndrome is an autosomal dominant inherited disorder characterized by distinctive intestinal hamartomatous polyps in association with mucocutaneous melanocytic macules. The syndrome was named after Peutz, who first noted a relationship between the intestinal polyps and the mucocutaneous macules in

1921, and Jeghers' definitive descriptions in 1944 and 1949. Peutz–Jeghers syndrome occurs with a frequency between 1 in 70,000 and 1 in 200,000 people, making it one-tenth as common as FAP based on registry data. The syndrome has a male to female ratio of 1:1, and the average age at the time of diagnosis is 23 years in men and 26 years in women.

**70.4.1.1 Mechanistic Basis of Peutz–Jeghers Syndrome.** Peutz–Jeghers syndrome is inherited as a pleiotropic autosomal dominant condition with variable and incomplete penetrance (209). The germline mutation responsible for this disease is in a serine-threonine kinase on chromosome 19p13.1 referred to as *LKB1* (210–212) or *STK11* (213). Normal expression of *STK11/LKB1*, a protein containing 436 amino acids, occurs in a wide variety of adult and fetal tissues and tumors, but has predominance in epithelial cells and seminiferous tubules of the testis (214). LOH at this locus has been found in 70% of tumors of Peutz–Jeghers patients who have an *STK11/LKB1* germline mutation (215,216). In some breast carcinomas and hamartomatous polyps from Peutz–Jeghers patients, hypermethylation of the promoter of *STK11/LKB1* occurs (217).

Introduction of *STK11/LKB1* into cell lines with severely reduced mRNA levels and impaired *STK11/LKB1* kinase activity suppresses cell growth by causing a G1 cell cycle block (218). The mechanism of the cell cycle block may be due, in part, to the association of *STK11/LKB1* with p53 and regulation of p53-dependent apoptosis pathways (219). In addition, the *STK11/LKB1* protein associates with and regulates the brahma-related gene 1,

**TABLE 70-6 Intestinal Polyposis Syndromes**

Adenomatous Syndrome	Chromosomal Location(s)	Mutated Gene(s)	Inheritance Pattern
Familial adenomatous polyposis	5q21	<i>APC</i>	Autosomal dominant
Gardner's variant			
Turcot's variant			
MUTYH-associated polyposis	1p32-34	<i>MUTYH</i>	Autosomal recessive
Lynch syndrome	2p16, 3p21, 7p22, 2p16	<i>hMSH2, hMLH1, hPMS2, hMSH6</i>	Autosomal dominant
Muir–Torre variant			
Turcot's variant			
Syndrome X	?	?	Autosomal dominant?
Hamartomatous Syndrome	Chromosomal Location	Mutated Gene	Frequency in Germline (%)
<i>PTEN</i> Hamartoma syndrome			
Cowden's disease	10q22-23	<i>PTEN/MMAC1/TEP1</i>	>80
Lhermitte–Duclos variant	10q22-23	<i>KILLIN (methylation)</i>	?
Bannayan–Riley–Ruvalcaba syndrome	10q22-23	<i>PTEN/MMAC1/TEP1</i>	~60
Juvenile polyposis syndrome (with HHT overlap)	18q21.1	<i>SMAD4</i>	~20
	10q22-23	<i>BMPR1A/ALK3</i>	~25
	9q34	<i>ENG</i>	?
Peutz–Jeghers syndrome	19p13.3	<i>STK11/LKB1</i>	70–90
Hereditary mixed polyposis syndrome	15q13-q14	? <i>CRAC1</i>	?
	15q21	? <i>THBS1</i>	?
	10q23	? <i>BMPR1A/ALK3</i>	?
Hyperplastic polyposis syndrome	?1p	?	?

*Brg1*, which is an essential component of the chromatin remodeling complex (220). Mutations in *STK11/LKB1* compromise its ability to stimulate AMP-activated protein kinase, resulting in impaired downstream signaling inhibition of the mammalian-target of rapamycin complex 1 (mTORC1) (221,222), a common protein shared with TSC2 signaling and mutated in the benign tumor syndrome, tuberous sclerosis complex (223). Polyps from *lkb1*<sup>+/-</sup> mice show dramatic growth suppression with rapamycin, coincident with countering the parallel upregulation of *HIF-1α* and its downstream transcriptional targets (224). Furthermore, *STK11/LKB1* itself appears to be regulated by phosphorylation through pathways that include protein kinase A and EGF/RAF/ERK (225,226).

Knockout of the *stk11/lkb1* gene in mice resulted in embryonic death in midgestation, with embryos that had neural tube defects, mesenchymal cell death, and vascular abnormalities. In extraembryonic tissues such as the placenta, *VEGF* mRNA expression was downregulated. However, overall *VEGF* mRNA was markedly upregulated in the embryos (227). These findings established a link between *stk11/lkb1* and the *VEGF* signaling pathway, and the vascular defects seen in the embryos are at least in part mediated by *VEGF*. *VEGF* is known to be regulated by *mTOR* (228).

**70.4.1.2 Clinical Features of Peutz–Jeghers Syndrome.** In childhood and early adult life, the principal causes of morbidity in Peutz–Jeghers syndrome stem from the intestinal location of the polyps (small intestine >> colon > stomach). These include small intestinal obstruction and intussusception, abdominal pain, hematochezia, and rectal prolapse, which typically occur in the second and third decades of life. Pathologically, the Peutz–Jeghers polyps are unique hamartomas with an arborizing network of smooth muscle tissue covered with non-neoplastic-appearing epithelium. The smooth muscle bands extend throughout the head of the polyp and are sufficiently characteristic pathologically to establish the diagnosis. Benign glands within the polyp may be surrounded by the arborizing smooth muscle, and may extend into the submucosa or muscularis propria. The glandular epithelium is often described as “pseudoinvasive” into the deeper layers of the polyp. The polyps are almost always multiple, and there are characteristic extraintestinal manifestations that make this syndrome easily identifiable. Early in infancy, hypermelanotic macules (1–5 mm) may be found around the mouth (often crossing the vermilion border of the lips), nose, lips, buccal mucosa, hands and feet, and may also be present in the perianal and genital regions. The lesions are flat, may be brown or greenish black and, with the exception of the buccal pigmentation, may fade at puberty. It is important to distinguish these melanin deposits from ordinary freckles, which are sparse near the nostrils and mouth and usually absent at birth.

**70.4.1.3 Cancer in Peutz–Jeghers Syndrome.** As the Peutz–Jeghers patient ages, the morbidity risk shifts to

that of malignancy, particularly cancers of the luminal GI tract, pancreas, female reproductive tract, lung and breast. There is a 15-fold elevated RR of developing cancer over the general population, and 93% of Peutz–Jeghers patients develop cancer by age 64 (229). Thus, patients may present with symptoms of cancer if the diagnosis was not previously made. GI cancers have been reported that appear to have developed from foci of adenomatous epithelium within the Peutz–Jeghers polyps. This may be due to the mutant *STK11/LKB1* in epithelial cells, which cannot activate GSK3β, preventing phosphorylation of β-catenin (230,231). In addition, acquired mutations in *CTNNB1* (but not *APC*) and *p53* are thought to be involved in progression to cancer, when this occurs (216).

The Johns Hopkins Polyposis Registry has reported the strongest evidence for an increased risk of cancer in the Peutz–Jeghers syndrome (229), and the same is true for *STK11/LKB1* mutation carriers (232). The major sites in order of RR over the general population are small intestine (RR 520), stomach (RR 213), pancreas (RR 132), colon (RR 84), esophagus (RR 57), ovary (RR 27), lung (RR 17), uterus (RR 16), and breast (RR 15.2). In addition, other reproductive site cancers have been associated with Peutz–Jeghers syndrome, including adenoma malignum of the cervix, Sertoli cell tumors, and sex cord tumors with annular tubules. With the last condition, menstrual irregularities in females (due to hyperestrogenism from sex cord tumors with annular tubules), or gynecomastia and growth acceleration (due to a Sertoli cell tumor) may be the presentation in an undiagnosed patient. Biliary adenocarcinoma risk is also elevated in patients with mutations in the *STK11/LKB1* gene (233).

## 70.4.2 Juvenile Polyposis Syndrome (JPS)

Juvenile polyps are distinctive pathological lesions that occur as solitary rectal polyps in 1–2% of children. They have a smooth surface covered by normal colonic epithelium, and the lamina propria is edematous and contains inflammatory cells. JPS is a rare autosomal dominant entity consisting of multiple juvenile polyps. Traditionally, the presence of more than 10 juvenile polyps indicates JPS, but more than five should raise suspicion. The polyps will almost always recur after endoscopic removal. JPS patients usually present by age 30, with the mean age of presentation being 9.5 years. In some families only the colon is involved, but there are families with generalized GI juvenile polyposis (234). This syndrome is diagnosed by the pathological appearance of the polyps and exclusion of extraintestinal lesions seen with the other hamartomatous syndromes (235). An estimate for prevalence in the population is 1 in 250,000 individuals. JPS patients often present with rectal bleeding, but with a large number of polyps, patients can present with GI protein loss, malnutrition, cachexia, and failure to thrive, typically in the first decade of life. Large polyps can cause obstruction or altered bowel habits. The average age for

colorectal cancer presentation is 34 years, so anemia and obstruction from tumor can be presenting symptoms later in life.

**70.4.2.1 The Genetic Basis of JPS.** The genetic basis for JPS centers on bone morphogenic protein (BMP) signaling. Initially, some patients with JPS features showed deletions of chromosome 10q23 (236–238) and mutations in *PTEN*, the affected gene identified for Cowden syndrome and Bannayan–Riley–Ruvalcaba syndrome (BRRS). However, the deletions on chromosome 10q23 also encompassed or surrounded the locus for the type 1 receptor for BMP, *BMPR1A*. Moreover, young patients who, because of their age, could not be classified phenotypically into any hamartomatous polyp syndrome other than JPS, later developed the extraintestinal manifestations of Cowden disease, removing *PTEN* mutations as a cause for true JPS (239–241). No *PTEN* mutations were detected in 14 families and 11 sporadic cases of JPS (242).

Linkage analysis in families without *PTEN* mutations revealed that the aforementioned gene on chromosome 10q23, *BMPR1A*, is mutated in the germline of some JPS families (243). The mutations result in premature truncation of this cellular transmembrane protein that mediates BMP signaling. Several BMP ligands bind to specific type II BMP receptors, which then activate *BMPR1A* through a glycine-serine rich domain. Subsequently, *BMPR1A* phosphorylates SMAD1, SMAD5, and SMAD8, which then associate with SMAD4 to form heteromeric complexes that translocate from the cytoplasm to the nucleus, and associate with DNA-binding proteins to regulate transcription of genes that control apoptosis and mesoderm formation. The demonstration of germline mutations of *BMPR1A* in JPS patients was the first evidence that BMPs play a role in controlling colonic epithelial growth and neoplasia. BMP signaling can regulate *PTEN* via a non-SMAD pathway, suggesting interconnectivity among the hamartomatous polyposis syndromes (244).

A separate large kindred with JPS demonstrated linkage to chromosome 18q21.1 (245). Subsequently, three familial and two sporadic JPS cases demonstrated germline mutations in the *SMAD4* gene at chromosome 18q21.1, whose gene product encodes a key intracellular signal transducer and transcriptional regulator for the TGF $\beta$  superfamily of ligands and receptors, which includes BMP (246). The most common *SMAD4* mutation was a 4-bp deletion from codons 414–416; however, a 2-bp deletion from codon 348 and a 1-bp insertion at codon 229 have also been reported. All these mutations are predicted to cause a truncated SMAD4 protein that would prevent the requisite homotrimerization at its carboxyl terminus. The prevalence of *SMAD4* and *BMPR1A* mutations in JPS appears to be 18.2 and 20.8%, respectively, suggesting that additional genes or alternative means of inactivating the known genes are involved in this syndrome (247).

In addition, germline mutations of *SMAD4* can phenotypically cause hereditary hemorrhagic telangiectasia

(HHT), and thus careful examination for findings associated with both syndromes should be undertaken in these patients (248,249) (see Chapter 49). Mutations in another gene, *ENG*, encoding endoglin, a chaperone protein for TGF $\beta$  superfamily signaling, had been found to be a cause of HHT, and were also identified in the germline of some JPS patients (241,250). The overlap between JPS and HHT was borne out because of insights brought by genetics, and led to the discovery of a key phenotypic feature that had not been appreciated previously.

Germline mutations can be identified in only about ~40% of JPS patients. Large deletions that encompass *SMAD4* and *BMPR1A* are often missed by routine DNA sequence analysis, but when sought, may raise the identification of germline mutations to ~60% of patients (251,252). These large deletions are often seen in JPS with other associated congenital abnormalities, which can occur in up to 20% of JPS patients.

Patients with JPS are predisposed to juvenile hamartomatous polyps and GI cancer, with a 15% incidence of colorectal carcinoma in young patients and a cumulative risk of 68% by 60 years of life (234,253). BMP and its downstream signaler SMAD4 appear to suppress Wnt signaling, involved as a “gatekeeper” for colonic neoplasia (254). Juvenile polyps commonly have nuclear localization of  $\beta$ -catenin (255). Thus, dysregulated BMP signaling may predispose juvenile polyps to adenomatous evolution and later malignant transformation through this mechanism. Other tumors associated with JPS include pancreatic, gastric, and duodenal cancers (256).

### 70.4.3 PTEN Hamartoma Tumor Syndrome

Many researchers now consider BRRS and Cowden syndrome to be a single entity, with a phenotypic spectrum caused by mutations in the *PTEN* tumor suppressor gene (257). BRRS, also previously known as Bannayan–Zonana or Ruvalcaba–Myhre–Smith syndrome, was described as a rare autosomal dominant disease that consisted phenotypically of multiple hamartomatous polyps of the intestinal tract (typically juvenile polyposis), macrocephaly, lipomas and hemangiomas, cognitive and developmental delays, lipid storage myopathy, Hashimoto thyroiditis, and speckled macules of the penis or genital area (237). Cowden syndrome, also known as multiple hamartoma syndrome, was described in 1963 in which multiple hamartomas develop in the skin and mucous membranes, as well as throughout the GI tract. The hallmark of Cowden syndrome is multiple facial trichilemmomas (hamartomas of the hair follicle). All the features of Cowden syndrome and BRRS are now considered part of the PTEN hamartoma tumor syndrome. The true incidence of PTEN hamartoma tumor syndrome is not known; estimates are that it occurs in 1 in 200,000 individuals, with males and females affected equally, but it may be underdiagnosed, and much higher. It often occurs without a prior family history.

A number of unusual phenotypic features may be present in PTEN hamartoma tumor syndrome patients. These include a high-arched palate, beaked nose, and mandibular, maxillary and soft palate hypoplasia. Café-au-lait spots may be present, and some patients develop retinal gliomas, odontitis, dental caries, adenoid facies, kyphoscoliosis, and pectus excavatum. A number of extraintestinal hamartomas can develop. These may include those of mucous membranes, such as flat or verrucous papules on the tongue, gingiva, and oral mucosa. Other soft tissue tumors can also develop, including lipomas, hemangiomas, lymphangiomas, neurofibromas, meningiomas, and uterine leiomyomas. The hamartomatous polyps in the PTEN hamartoma tumor syndrome demonstrate a broad range of histology, and the most common intestinal polyp appears to be a protuberance of cytologically normal epithelium indigenous to the region from which the polyps arose; hyperplastic polyps are also common (258). Overall, the polyposis in the PTEN hamartoma tumor syndrome should be considered a mixed polyposis. Lipomas, juvenile polyps, inflammatory polyps, ganglioneuromas, and lymphoid hyperplasia can occur. A variant of Cowden's syndrome, the Lhermitte-Duclos syndrome, shares the phenotype and genotype of Cowden's syndrome, but also features macrocephaly, ataxia, and dysplastic cerebellar gangliocytomatosis (cerebellar hamartomas). Clinically, confusion of PTEN hamartoma tumor syndrome with multiple endocrine neoplasia (MEN) type 2B may occur, because both syndromes share intestinal ganglioneuromas and thyroid disease in their phenotype. However, medullary thyroid cancer occurs in MEN2B patients, whereas follicular thyroid cancer is characteristic of PTEN hamartoma tumor syndrome. Genetically, MEN2B is associated with germline mutations in *RET*, while the PTEN hamartoma tumor syndrome is associated with mutations in *PTEN*. Another syndrome that can be confused with the PTEN hamartoma tumor syndrome is the pheochromocytoma–paraganglioma syndrome, comprising papillary thyroid carcinomas, carcinoma of the breast, and renal cell carcinomas in addition to head and neck paragangliomas, adrenal and extra-adrenal pheochromocytomas (259). These two syndromes can also be distinguished by germline testing, as the pheochromocytoma–paraganglioma syndrome is associated with germline mutations in the succinate dehydrogenase genes, subunits B, C, and D (259,260). Surveillance for the appropriate tumors based on the natural history of these syndromes can then be instituted.

**70.4.3.1 The Genetic Basis of PTEN Hamartoma Tumor Syndrome (BRRS and Cowden Syndrome).** The genetic cause for PTEN hamartoma tumor syndrome is a germline mutation in *PTEN* (261), which encodes a dual-function phosphatase (phosphoserine/phosphothreonine and phosphotyrosine residues) that can dephosphorylate the lipid phosphatidylinositol-3,4,5-triphosphate after activation by the mitogenic enzyme phosphatidylinositol 3 kinase. *PTEN* mutations are found in 81%

of Cowden syndrome kindreds (262). Nearly half the mutations identified are in exon 5 of *PTEN*, the location encoding the phosphatase core motif. The PTEN protein is a tumor suppressor that, by its dephosphorylating ability, contributes to programmed cell death, and inhibits the cell's ability to migrate and invade. About 60% of BRRS patients have germline mutations in *PTEN* (263). Indeed, the reports of BRRS and Cowden disease occurring in the same family with the same *PTEN* mutation originally suggested that the two diseases might be allelic (262). Analysis of BRRS and Cowden syndrome patients with germline mutations in *PTEN* indicate a similar mutational spectrum, and correlations between *PTEN* mutations and cancers (or breast fibroadenomas) in Cowden syndrome, BRRS, or BRRS/Cowden disease families demonstrate considerable overlap (262). The overall implication was that *PTEN*-mutation-positive Cowden syndrome and BRRS were pleiotropic presentations of a single syndrome and thus were consolidated into the PTEN hamartoma tumor syndrome. Splice variants of *PTEN* may drive some of the phenotypic variations between Cowden syndrome and BRRS (264). Both should receive equal attention with respect to cancer surveillance.

Germline mutations in *PTEN* have not been found for all BRRS or Cowden syndrome patients (265). Some patients have gross chromosome deletions or rearrangements involving the *PTEN* locus on chromosome 10, thus making the patient haploinsufficient. Other patients, despite an exhaustive search for chromosomal deletions and mutations of *PTEN*, do not demonstrate abnormalities at this locus. *KILLIN*, encoding a 20-kDa protein that mediates p53-induced apoptosis, lies in close proximity to *PTEN* on chromosome 10, but is transcribed in the opposite direction to *PTEN* (266). Germline hypermethylation upstream to *PTEN* showed no transcriptional repression of *PTEN* in patients with *PTEN*-negative Cowden syndrome, but methylation of this region transcriptionally downregulated *KILLIN* more than 250-fold compared with controls (267). Patients with *KILLIN* promoter hypermethylation had a three-fold increase in breast cancer and two-fold increase in kidney cancer over individuals with germline *PTEN* mutations. Thus, germline promoter methylation of *KILLIN* may prove to be either a susceptibility gene for PTEN hamartoma tumor syndrome or part of the spectrum of its current genotype.

**70.4.3.2 The PTEN Hamartoma Tumor Syndrome and Cancer.** Two-thirds of PTEN hamartoma tumor syndrome patients will develop a goiter, and there is a 10% incidence of thyroid cancer in this condition. More than 75% of patients will have breast lesions, including fibrocystic breast disease and fibroadenomas, and 50% will develop breast cancer. The breast cancer is often bilateral, and the median age of diagnosis is 41 years. Other cancers such as renal cell carcinoma, Merkel cell carcinoma, lymphomas, endometrial cancer and melanomas are increased in PTEN hamartoma tumor syndrome.



An analysis of 127 *PTEN* mutation carriers demonstrated an increased risk for colorectal adenocarcinoma in those with polyposis, 224-fold over the general population, and all patients in the cohort with colon cancer were younger than 50 years of age (268). Thus, *PTEN* hamartoma tumor syndrome patients should be considered at high risk for early-onset colorectal cancer.

A heterozygous *pten* knockout mouse model causes neoplasia in multiple organ systems, including multiple intestinal polyps in association with the gut lymphoid tissue (269). The demonstration of an acquired DNA MMR defect within familial hamartomatous polyp epithelium suggests a potential mechanism for transformation into adenocarcinoma (270).

#### 70.4.4 Hereditary Mixed Polyposis Syndrome (HMPS)

Hereditary mixed polyposis syndrome (HMPS) is a very rare autosomal dominant syndrome in which affected family members have atypical juvenile polyps with mixed adenoma and hyperplastic elements, hyperplastic polyps, colonic tubular, villous and serrated adenomas, and progression to colonic adenocarcinomas (271). There appear to be few or no extraintestinal manifestations. At least five HMPS families have been described in the literature (272). Although the original description of a large family with HMPS had been linked to chromosome 5q (273), a subsequent analysis has determined linkage to chromosome 15q13-q14, a region containing a susceptibility locus for colorectal adenomas and carcinomas (*CRAC1*), as well as chromosome 15q21 where the tumor suppressor gene *THBS1*, encoding thrombospondin, is located (274). It remains to be determined if the HMPS locus and either *CRAC1* or *THBS1* are one and the same (272). Another analysis found linkage to chromosome 10q23, and one family had an 11-bp deletion in *BMPR1A*, the same gene involved with JPS; subsequently two of four HMPS families were found to have mutations in *BMPR1A* (275,276). The implication of this analysis is that HMPS is genotypically related to JPS, but this needs to be verified. Based on the analysis of the five known families, it is probable that all contain a common ancestral Ashkenazi haplotype. Commonly, rectal bleeding is the presenting symptom, and screening with regular colonoscopies and polyp removal has been proposed to reduce the incidence of colorectal cancer in this setting (277).

#### 70.4.5 Hyperplastic (Serrated) Polyposis

Multiple hyperplastic (serrated) polyps (>20 polyps in the colon) rarely occur in individuals and are not usually familial (278); the majority of isolated hyperplastic polyps are located in the rectum. It is now recognized that colorectal polyps with a serrated appearance may be either hyperplastic polyps (which have no apparent neoplastic potential), or serrated adenomas (which have

a considerable neoplastic potential) (279). When at least five histologically confirmed serrated polyps occur proximal to the sigmoid colon with 2 polyps >1 cm in size, when any number of serrated polyps are proximal to the sigmoid colon in a patient with a first-degree relative with hyperplastic polyposis, or when more than 30 serrated polyps of any size are distributed throughout the colon, the diagnosis of hyperplastic (serrated) polyposis should be given. Patients with hyperplastic or serrated polyposis have an increased risk for colon cancer (280,281), with predilection for the right colon, as do their first-degree relatives (282). Hyperplastic polyposis can be associated with a family history of colorectal cancer, and with alterations at chromosome 1p (278), but the genetic cause for this syndrome has yet to be identified. A familial syndrome of giant hyperplastic polyposis has been described in a single family that also met the criteria for Lynch syndrome (283). Up to 18% of MAP patients meet the criteria for hyperplastic polyposis, and an analysis of 17 MAP patients showed that 47% had serrated polyps (282). Somatic mutations in *BRAF* occur commonly in the polyps (including hyperplastic polyps, serrated adenomas, admixed hyperplastic adenomas, tubular adenomas, and carcinomas) from patients with hyperplastic polyposis (284) with few *KRAS* mutations, and many of these polyps have hypermethylation of several loci, suggesting that these lesions evolve through the CIMP pathway (33). The DNA MMR gene *bMLH1* is one of the genes susceptible to hypermethylation, so, some of these polyps may develop MSI because of loss of MMR function. Sessile serrated adenomas may be flat and relatively unapparent visually, making them difficult to observe at routine colonoscopy, and their predilection for the right colon gives rise to the predominance of right-sided cancers observed in patients with hyperplastic polyposis.

#### 70.4.6 Noninherited Forms of Intestinal Polyposis

GI polyposis may occur in a small number of conditions that represent acquired, noninherited diseases. One of these, Cronkhite–Canada syndrome, consists of diffuse GI polyposis, loss of fingernails, alopecia, cutaneous hyperpigmentation, diarrhea, weight loss and malnutrition. This syndrome typically occurs in middle-aged or older patients, and the diagnosis is made when multiple polyps are found that most resemble inflammatory polyps (285). Colorectal cancer has been reported to occur in association with the Cronkhite–Canada syndrome, but there is no evidence to indicate whether there is an excess incidence of neoplasia in this condition. In any event, the disease is not inherited and has never been reported in familial clusters, and little is known about its pathogenesis.

Other conditions may cause multiple polyps in the GI tract in the absence of any known genetic basis. These include the inflammatory polyps or pseudopolyps that

are seen in ulcerative colitis or other types of inflammatory bowel disease, lymphoid polyposis (both benign and malignant varieties), lipomatous polyposis, and pneumatosis cystoides intestinalis. These are isolated curiosities with no known genetic basis.

## 70.5 GENETICS OF GASTRIC CANCER

Gastric carcinoma is among the three most common causes of cancer worldwide. What is most remarkable about the epidemiology of stomach cancer is the relative absence of strong evidence for important inherited factors in this disease. Gastric carcinoma seems to be principally related to environmental factors, largely infection by *Helicobacter pylori*, and increased familial risks are probably related to this or other environmental factors. One commonly finds an increase in gastric cancer in Lynch syndrome kindreds, and in the largest family reported, it was noted that a predominance of gastric cancer evolved into a predominance of colon cancers during the twentieth century (99). Therefore, the first consideration in a familial cluster of gastric cancers is to consider the possibility of Lynch syndrome, particularly if the background incidence of gastric cancer is high in that geographical region (286). Gastric cancer is quite uncommon as a complication of FAP, but may be seen in Peutz–Jeghers syndrome (287).

### 70.5.1 Hereditary Diffuse Gastric Cancer (HDGC) and the *E-Cadherin* Gene (*CDH1*)

There is a rare form of hereditary gastric cancer that can be distinguished by unique pathological features, termed hereditary diffuse gastric cancer (HDGC). Germline mutations in the *CDH1* gene predispose to early-onset, diffuse gastric cancers with a linitis plastica phenotype (288). Inactivating mutations in *CDH1*, located on 16q22.1 are of high penetrance, were originally described in a Maori kindred from New Zealand (289), but have been found in patients from Europe and North America (290,291). The tumors are characterized by signet ring cells, and

begin as clinically silent multifocal clusters of intramucosal cancer. HDGC is inherited as an autosomal dominant disease, the majority of the germline mutations result in truncation of the protein, and the mutations identified thus far are scattered throughout the 16 exons (292,293). However, only 30–50% of HDGC families have germline mutations identified, making other genetic abnormalities likely (including Lynch syndrome, as described earlier). The loss of the adhesion protein E-cadherin leads to defective intracellular adhesion, which manifests as a diffuse, spreading, isolated cellular phenotype, which impedes early diagnosis. The virulence of the linitis plastica tumors has led to the recommendation for prophylactic gastrectomy in gene carriers (294). The population prevalence is unknown, but it appears to be rare.

### 70.5.2 Genetics of Pancreatic Cancer and Familial Pancreatic Cancer

Pancreatic cancer is largely thought to be caused by environmental factors, but the etiology of this disease remains enigmatic. There have been recurring reports of familial clusters of pancreatic cancer, and there appear to be many potential genetic culprits for this phenomenon. There is an excess of pancreatic cancer in a variety of genetic diseases that predispose to other GI cancers, but pancreatic cancer is not highly penetrant in any of them. It has been projected that as many as 5% of pancreatic cancer may have a genetic basis and the number of genes that might participate in this is large (295). Thus, one must consider the possibilities of germline mutations in the familial breast cancer genes *BRCA1* and *BRCA2*, in atypical mole/melanoma families with germline mutations in *p16*, in Peutz–Jeghers syndrome families (*STK11*, *PRSS1*), in Lynch syndrome (both *hMSH2* and *hMLH1*), ataxia telangiectasia (*ATM*), and in FAP (Table 70-7) (296–299). In the absence of syndromic cancer, the presence of  $\geq 3$  first-degree relatives with pancreatic cancer is associated with a 40% lifetime risk for that disease, suggesting that there are other forms of this disease yet to be discovered.

**TABLE 70-7 Hereditary Syndromes Associated with Familial Pancreatic Cancer**

Syndrome	Germline Mutation	Lifetime Risk for Pancreatic Cancer (%)
Hereditary Pancreatitis	<i>PRSS1</i> (cationic trypsinogen)	25–40
Familial atypical multiple mole melanoma syndrome (FAMMM)	<i>p16/CDKN2A</i> (cell cycle inhibitor)	10–17
Hereditary breast and ovarian cancer	<i>BRCA2</i> (DNA repair)	5
Hereditary breast and ovarian cancer	<i>BRCA1</i> (DNA repair)	3.6
Peutz–Jeghers syndrome	<i>STK11/LKB1</i> (kinase)	11–36
Lynch syndrome	DNA MMR genes (DNA repair)	3.7
Familial adenomatous polyposis	<i>APC</i> (tumor suppressor gene)	~2
Three or more affected first-degree relatives	Undetermined	40

Adapted from reference (299).

Finding the germline mutations is a challenge, since relatively few cases of multigenerational transmission of familial pancreatic cancer have been documented, and complicating this further, there are no simple preventive measures one might use to reduce the mortality from this disease. Surveillance is also a challenge even in patients with a known elevated risk, as no approaches are known to be effective in reducing the high mortality in pancreatic cancer.

### 70.5.3 Genetics of Esophageal Carcinoma and Familial Esophageal Cancer

Esophageal cancer occurs in two major histological forms, squamous cell carcinoma and adenocarcinoma. Squamous cell carcinoma is thought to be due to environmental factors, and has a wide range of incidence on a geographical basis. Esophageal adenocarcinoma occurs principally in patients with Barrett esophagus, a metaplastic condition in which the normal squamous lining is replaced by columnar mucosa. This condition is caused by chronic gastroesophageal reflux disease, and although families with Barrett esophagus have been reported (300,301), most cases of Barrett esophagus are apparently sporadic (302). The familial Barrett esophagus cases typically present at a younger age and may contribute to esophageal adenocarcinoma not recognized to be familial. Multiple SNPs are associated with both Barrett esophagus and esophageal adenocarcinoma, but it appears that a multitude of genes interacting with the environment may be involved in familial aggregations (reviewed in (303)).

One rare entity, esophageal tylosis, or keratosis palmaris et plantaris, is characterized by diffuse hyperkeratosis of the squamous epithelium of the esophagus, as well as hyperkeratosis of the palms and soles. This disease, previously referred to as the Howel–Evans syndrome, is associated with squamous cell carcinoma of the esophagus at an average age of 45 years (304). The gene for this has been mapped to 17q23-ter in a single large family (305). A candidate gene called *downregulated in multiple cancers 1* (*DMC1*) has been found in this region of chromosome 17q, and its expression is reduced in a number of cancers (306).

### 70.5.4 Other GI Cancers

Gastrointestinal stromal tumors (GISTs) may occasionally be familial. In one large kindred, the risk for GISTs was linked to a deletion of codon 579 in exon 11 of the *KIT* gene. Somatic loss of the wild-type *KIT* allele was demonstrated in the tumor of one individual. Affected individuals responded to treatment with imatinib, which has inhibitory activity for the *KIT* receptor tyrosine kinase (307). In another kindred, three sisters had intestinal neurofibromatosis and developed five GISTs. A germline mutation in the *PDGFRA* gene (Y555C) was found. This mutation in the juxtamembrane domain leads to autophosphorylation and activation of the receptor, and is a putative cause of this familial tumor cluster (308).

Familial GI carcinoid tumors have been reported, are rare, and have yet to be linked convincingly to specific genetic loci, except for multiple endocrine neoplasia (MEN1) kindreds (309). MEN1 families have germline mutations in the *MENIN* gene, and some have increases in pancreatic islet cell tumors as a feature of the disease. Pancreatic islet cell tumors may also occur in the context of von Hippel–Lindau (*VHL*) germline mutations (310).

A patient has been described with homozygous mutations (c.2386-11A>G, creating a de novo splice site) in the spindle-assembly checkpoint gene, *BUB1B*; he developed multiple early-onset cancers of the papilla of Vater, followed by later-onset adenomas and invasive cancers of the colon and stomach. No other characteristic features were identified (311).

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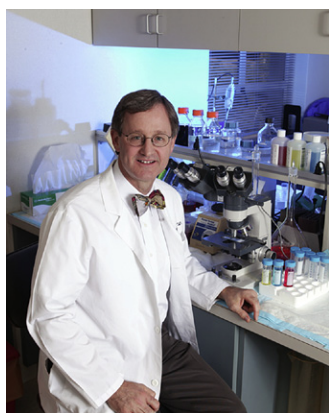


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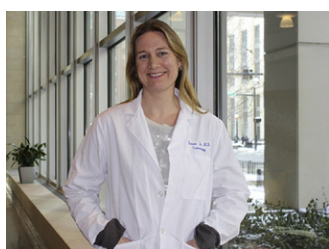


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## Biographies



**C Richard Boland, MD**, is Chief of Gastroenterology at Baylor University Medical Center in Dallas, Texas. He received degrees from The University of Notre Dame and Yale Medical School. He has a career-long research interest in colon cancer, specifically focusing on the causes of colon cancer and familial cancer syndromes. He was a Fellow and Assistant Professor at UCSF (1978–1984), moving to the University of Michigan as Associate and Professor from 1984 to 1995, and Professor of Medicine and Chief of the GI Division at UCSD from 1995 to 2003. He moved to Baylor, Dallas, in 2003 to focus on research with both laboratory-based and clinical programs designed to accelerate the translation of basic concepts into the diagnosis, prevention, and treatment of colorectal cancer. Dr Boland has been funded continuously by the NIH since 1979, served on multiple NIH Study Sections, has published >300 papers, has been elected to the AAP, and has trained numerous postdoctoral fellows who have followed him into the study of colorectal cancer. He served as President of the AGA from 2011 to 2012. His clinical activities are focused upon the familial forms of gastrointestinal neoplasia.



**Barbara Jung, MD**, is a Gastroenterologist at Northwestern Medical Faculty Foundation and an Associate Professor in the Department of Medicine at Northwestern. She received her MD and Doctorate in Medicine in 1996 from the Ludwig-Maximilians University, Munich, Germany. After a postdoctoral fellowship at the Sidney Kimmel Cancer Center in San Diego, she joined UCSD's physician-scientist training pathway in 1999 where she completed a residency in Internal Medicine followed by a fellowship in Gastroenterology. In 2004, she became faculty at UCSD as an Assistant Professor. She was awarded a K08 grant in 2005 and became Assistant Program Director for the GI Fellowship Program in 2006. In 2010, she relocated to Northwestern University in Chicago, where she heads a laboratory focused on CRC signaling, biomarker identification and validation and immune targets for CRC treatments. Further, she established and leads a multidisciplinary, personalized high-risk GI cancer clinic.



**John M Carethers, MD**, is currently Professor and Chair of the Department of Internal Medicine at the University of Michigan. Dr Carethers is a trained gastroenterologist and physician-scientist who focuses his research in the area of hereditary colon cancer genetics. Dr Carethers received his BS degree in Biological Sciences with a minor in Chemistry from Wayne State University, and his MD with high distinction from the same institution. Dr Carethers did his internship and residency in Internal Medicine at Massachusetts General Hospital, followed by a fellowship in gastroenterology at the University of Michigan. He was then recruited to the University of California San Diego where he grew his laboratory-based research in the area of DNA mismatch repair and colorectal cancer pathogenesis. Prior to being recruited to the University of Michigan, he served as the Gastroenterology Division Chief at UC San Diego.

# CHAPTER

# 71

## Hemoglobinopathies and Thalassemias

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### 71.1 INTRODUCTION

The hemoglobinopathies and the thalassemia syndromes are a diverse group of inherited disorders of hemoglobin synthesis that result from the qualitative defects (hemoglobinopathies) or quantitative defects (thalassemia syndromes) in globin synthesis. As a group they are the most common and clinically significant single gene disorder in the world and pose a serious health problem in many countries. The only definitive cure for the hemoglobinopathies is bone marrow transplantation, and thus only supportive management is available for the treatment of the vast majority of severely affected patients. The methods of clinical management have improved considerably during the past few years and the life expectancy of affected individuals has been significantly increased with patients now surviving to the third to fifth decade. However, the treatment required is very expensive and is not a realistic means of controlling the disorders for many developing countries. Therefore, many countries, especially those with a high incidence of  $\beta$ -thalassemia, are applying an alternative method of control that involves screening the population for carriers, identifying couples at risk, and providing prenatal diagnosis.

This chapter outlines the structure, function, and biosynthesis of normal hemoglobin, and then discusses the current knowledge of the clinical diseases associated with the defects in globin synthesis, their molecular pathology, diagnosis, and treatment. A more detailed description of the thalassemia syndromes may be found in the comprehensive book by Weatherall and Clegg (1), and for hemoglobin variants, in the book edited by Steinberg and Nagel (2).

### 71.2 NORMAL HUMAN HEMOGLOBIN

Hemoglobin (Hb) is a tetramer with a molecular weight of 64,500. It consists of two  $\alpha$  and two non- $\alpha$  globin polypeptide chains, each of which has a single covalently bound heme group. Each of the four heme groups is

made up of an iron atom bound within a protoporphyrin IX ring.

In humans, there are six known different globin polypeptide chains that form a number of hemoglobin tetramers. The polypeptide chains are designated  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$ . The  $\alpha$ - and  $\zeta$ -chains consist of 141 amino acids, while the  $\beta$ -,  $\gamma$ -,  $\delta$ -, and  $\epsilon$ -chains have 146 residues. The  $\epsilon$ -,  $\gamma$ -, and  $\delta$ -chains are more similar to  $\beta$ -chains than to  $\alpha$ -chains, differing from  $\beta$  at 36, 39, and 10 positions, respectively, and are designated  $\beta$ -like chains. The  $\zeta$  chain is an  $\alpha$ -like globin and is found in embryonic erythrocytes together with  $\epsilon$ -globin.

The hemoglobin composition of erythrocyte lysates can be quantified by cellulose acetate and starch gel electrophoresis. These procedures have been replaced in many laboratories by isoelectric focusing (IEF), which gives a much better resolution of the different hemoglobins, or by high performance liquid chromatography (HPLC), which provides a quick chromatographic method with a degree of automation. Different hemoglobin tetramers, their structure, percentage in normal adult lysate, and conditions in which levels are increased, are seen in Table 71-1

Hb A ( $\alpha_2\beta_2$ ) is the major component of hemoglobin in normal adults, usually comprising about 97% of the total hemoglobin. The remainder is Hb A<sub>2</sub> ( $\alpha_2\delta_2$ ), which usually constitutes about 2–3% in normal individuals. Hb A<sub>2</sub> is increased in the majority of the  $\beta$ -thalassemias and megaloblastic anemias and decreased in iron deficiency and sideroblastic anemias.

Hb A<sub>1c</sub> differs from Hb A by the posttranslational addition of a glucose at the NH<sub>2</sub> terminus of the  $\beta$ -chain; hence the tetramer's structure is  $\alpha_2(\beta\text{-N-glucose})_2$ . The percentage of Hb A<sub>1c</sub> (5% in normals) is related to the intracellular concentration of glucose and the red cell life span. In diabetic patients, the concentration of Hb A<sub>1c</sub> is increased about twofold because of the elevated glucose concentration in their red cells.

**TABLE 71-1 Human Hemoglobins**

Hb	Stage of Development	Structure	Percentage in Adults	Conditions in which Increased
A	Adult	$\alpha_2\beta_2$	92	
A <sub>1c</sub>		$\alpha_2(\beta\text{-N-glucose})_2$	5	Diabetes mellitus
A <sub>2</sub>		$\alpha_2\delta_2$	2–3	$\beta$ -Thalassemia
H		$\beta_4$	0	Some $\alpha$ -thalassemias
F	Fetal	$\alpha_2\gamma_2$	<1	Newborn, $\delta\beta$ -, $\beta$ -thalassemia, HPFH and marrow stress
Bart's		$\gamma_4$	0	Some $\alpha$ -thalassemias
Gower I	Embryonic	$\zeta_2\varepsilon_2$	0	Early embryos (<8 weeks)
Gower II	Embryonic	$\alpha_2\varepsilon_2$	0	Early embryos (>8 weeks)
Portland	Embryonic	$\zeta_2\gamma_2$	0	(<8 weeks) and $\alpha^0$ -thalassemia (hydrops fetalis)

Hb F ( $\alpha_2\gamma_2$ ), fetal hemoglobin, comprises the bulk of hemoglobin (50–85%) in human newborns, but declines rapidly after birth, reaching concentrations of 10–15% by 4 months of age. Subsequently, the decline is slower and adult levels of less than 1% are reached by 3–4 years of age. Fetal hemoglobin may be increased in  $\beta$ - and  $\delta\beta$ -thalassemia, hereditary persistence of fetal hemoglobin (HPFH), D1 trisomy, some cases of thyrotoxicosis, megaloblastic and aplastic anemias, leukemia and various malignancies involving marrow, sickle-cell anemia, and during pregnancy. Hb F is quantified by alkali resistance, electrophoresis, column chromatography, or HPLC. Hb F is heterogeneous because of the presence of two types of  $\gamma$ -globin chains, only differing in their composition at position 136. Those that contain glycine at this position are called  $G\gamma$ -chains and those with alanine at this position are called  $A\gamma$ -chains.

Hbs Gower I ( $\zeta_2\varepsilon_2$ ), Gower II ( $\alpha_2\varepsilon_2$ ), and Portland ( $\zeta_2\gamma_2$ ) are embryonic hemoglobins synthesized in the yolk sac before 8 weeks of gestation. At 4–5 weeks of gestation, a decrease in  $\zeta$ - and  $\varepsilon$ -chain production occurs, together with a compensating increase in  $\alpha$ - and  $\gamma$ -chain production. At this stage of gestation  $\beta$ -chain synthesis in reticulocytes accounts for 4% of non- $\alpha$ -chain synthesis and gradually increases thereafter. Then at around 8 weeks of gestation, the site of hemoglobin synthesis changes to the fetal liver, which produces Hb F plus a small amount of Hb A. At 18 weeks gestation, the liver is progressively replaced by bone marrow as the major site of red cell production and there is a gradual switch from Hb F to Hb A synthesis. The mechanism for this switch from Hb F to Hb A has been the subject of intense research for a number of years because of the therapeutic implications of increasing the amount of Hb F in affected patients with  $\beta$ -thalassemia and sickle-cell disease.

Hb H and Hb Bart's are tetramers of  $\beta$ - and  $\gamma$ -chains, respectively, and both function very poorly in transporting oxygen. These two hemoglobins may be increased in some types of  $\alpha$ -thalassemia in which the deficiency of  $\alpha$ -globin chains is sufficient to allow the production of unpaired  $\beta$ -like globin chains into soluble tetramers.

### 71.2.1 Primary and Secondary Structures

The primary structure of each globin chain is its amino acid sequence; it is composed of 141 amino acids in  $\alpha$ - and  $\zeta$ -chains and 146 amino acids in  $\beta$ -,  $\gamma$ -,  $\delta$ -, and  $\varepsilon$ -chains. The primary structure of  $\alpha$ - and  $\beta$ -chains is shown in [Figure 71-1](#).

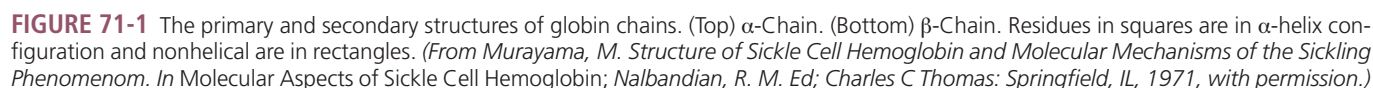
The relationship between adjacent amino acids along the chain enables interactions that can result in one of two basic configurations of secondary structure: the  $\alpha$ -helix or  $\beta$ -pleated sheet. The  $\alpha$ -helix, stabilized by hydrogen bonding between carbonyl and amino groups, has 3.6 amino acid residues per turn. About 75% of hemoglobin in its native state is in the  $\alpha$ -helix form, as shown in [Figure 71-1](#). The  $\beta$ -pleated sheet configuration predominates in other molecules, such as immunoglobulins and chymotrypsin.

At specific locations in the hemoglobin subunits, the rodlike  $\alpha$ -helix is interrupted by nonhelical segments that allow folding of the amino acid chain. On X-ray crystallography, the confirmations of  $\alpha$ - and  $\beta$ -hemoglobin subunits are seen to be similar. The  $\beta$ -globin chain has eight helical segments, A through H, and the secondary structure of the  $\alpha$ -globin corresponds to that of the  $\beta$ -globin except for the absence of residues forming the D helical region see ([Figure 71-1](#)). The histidine residue at position 8 of the F helical segment (F8) is linked covalently to the heme iron molecule. This histidine residue is located at position 87 in the  $\alpha$ -chain and 92 in the  $\beta$ -chain, and mutations altering it have important pathologic consequences. Amino acids with charged side groups, for example, lysine, arginine, and glutamic acid, lie on the external surface, while uncharged residues tend to be oriented toward the interior of the molecule.

### 71.2.2 Tertiary and Quaternary Structures

Tertiary structure refers to the configuration of a protein subunit in three-dimensional space, while quaternary structure refers to the relationships of the four subunits of hemoglobin to each other. The hemoglobin tetramer has been shown by X-ray crystallography to be an oblate spheroid with a diameter of 5.5 nm and a





single axis of symmetry. The globin chains are folded so that the four heme groups are in surface clefts equidistant from each other. The four subunits forming the tetramers are labeled  $\alpha_1$ ,  $\alpha_2$ ,  $\beta_1$ , and  $\beta_2$ . While there is no contact between the two  $\beta$ -chains, each  $\alpha$ -chain touches both  $\beta$ -chains. Bonds across the  $\alpha_1\beta_1$  interface are firmer than those at the  $\alpha_1\beta_2$  interface, and changes from oxy- to deoxyhemoglobin involve more extensive movement at the  $\alpha_1\beta_2$  interface. The quaternary structure changes markedly in going from oxy- to deoxyhemoglobin, and this accounts for many of the observed changes in physical properties. Hemoglobin mutations resulting in amino acid substitutions at these points can markedly alter specific functional properties.

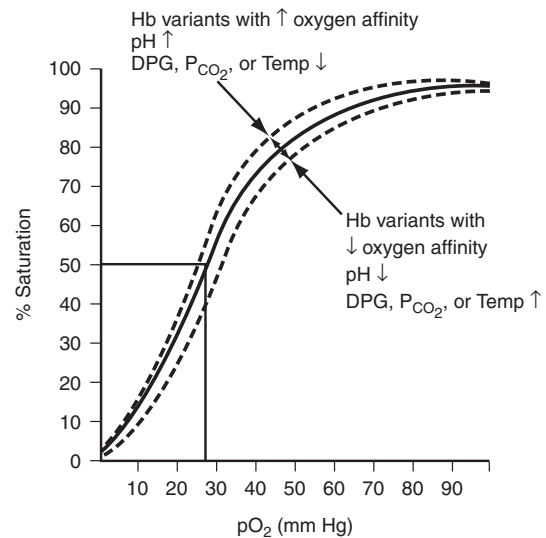
### 71.2.3 Functional Properties

For hemoglobin to fulfill its physiologic role, it must bind oxygen with a certain affinity. One measure of oxygen affinity is  $P_{50}$ , or the partial pressure of oxygen in millimeters of Hg that is required for 50% saturation of hemoglobin: a hemoglobin with increased  $P_{50}$  has decreased oxygen affinity (Figure 71-2). Oxygen affinity is also affected by a number of environmental factors including temperature, pH, organic phosphate concentration, and  $p\text{CO}_2$  see (Figure 71-2).

The sigmoidal shape of the oxyhemoglobin dissociation curve reflects heme–heme interaction; that is, successive oxygenation of each heme group in the tetramer increases the oxygen affinity of the remaining unoxygenated heme groups. The basis of heme–heme interaction is the decrease in the atomic radius of the heme iron that occurs with oxygenation, allowing the iron atom to fit into the plane of the porphyrin ring. This alteration is amplified by a series of conformational changes that affect the other heme groups. The resulting sigmoidal oxyhemoglobin dissociation curve has great physiologic importance because it enables large amounts of oxygen to be bound or released with a small increase or decrease in oxygen tension. In contrast to Hb A, Hb H ( $\beta_4$ ) and Hb Bart's ( $\gamma_4$ ) lack subunit interaction and have a hyperbolic rather than a sigmoidal oxyhemoglobin dissociation curve, which prevents oxygen release at physiologic oxygen tensions.

The Bohr effect is a change in oxygen affinity of hemoglobin with a change in pH. This effect is beneficial at the tissue level where the lower pH decreases oxygen affinity and promotes oxygen release see (Figure 71-2). Oxygen uptake in the lungs is enhanced by the opposite changes in pH and  $p\text{CO}_2$ .

Red cells have unusually high concentrations of 2,3-diphosphoglycerate (2,3-DPG). One molecule of 2,3-DPG sits in a pocket in deoxyhemoglobin bound to specific  $\beta$ -chain residues (1, 2, 82, and 143 of both  $\beta$ -chains). The importance of the binding is that 2,3-DPG stabilizes the deoxy form of hemoglobin in preference to the oxy form, thereby lowering the oxygen



**FIGURE 71-2** The oxyhemoglobin dissociation curve and effect of different factors on oxygen affinity.

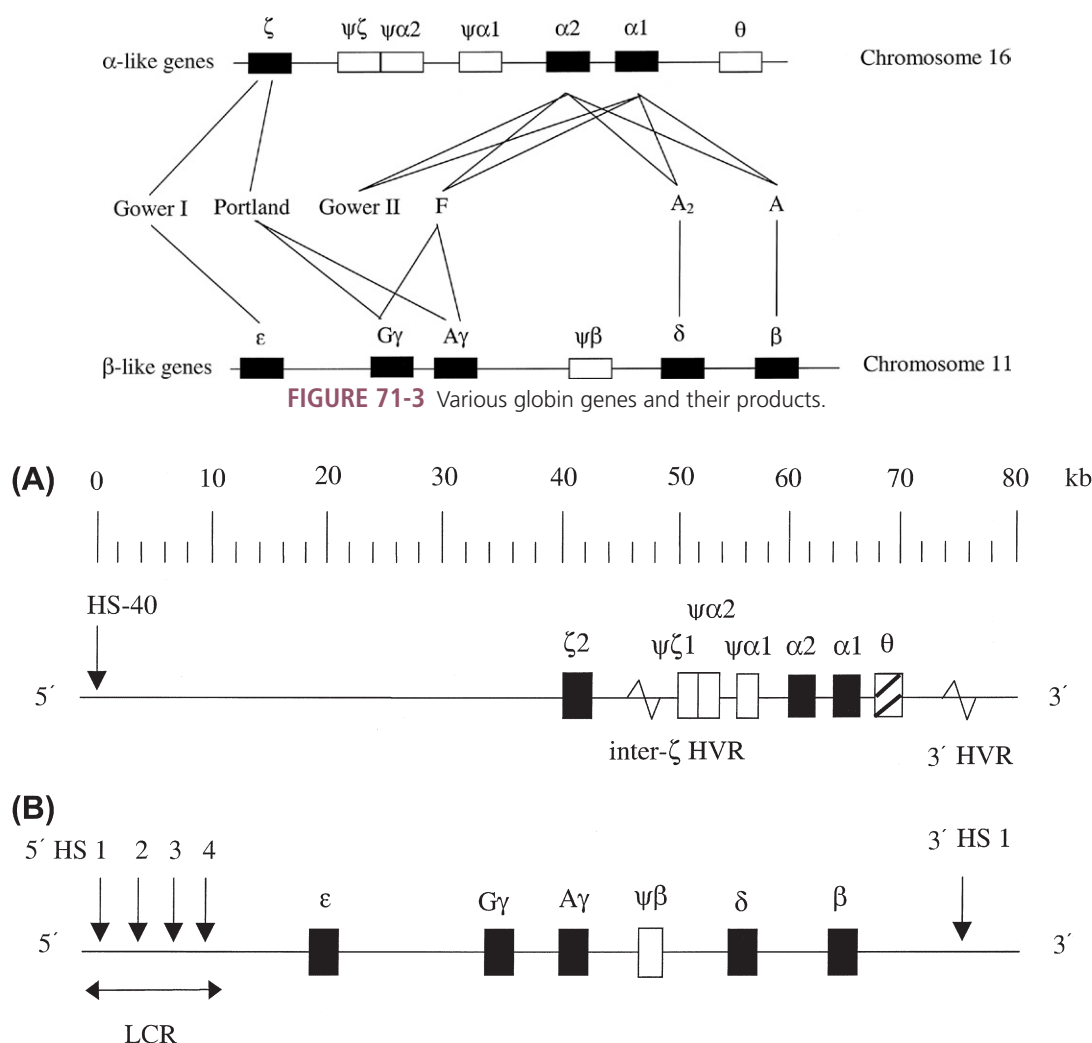
affinity of the molecule. The  $\gamma$ -chain of Hb F lacks the  $\beta^{143}$  histidine residue, and the resultant decrease in binding of 2,3-DPG to Hb F accounts for the increased oxygen affinity of fetal red cells compared with that of adult red cells.

## 71.3 HEMOGLOBIN BIOSYNTHESIS

### 71.3.1 Genetics

In humans there are eight different genetic loci that code for the six types of globin chains. In addition, there are at least four pseudogenes that have sequences similar to other globin genes but differ in that they are not expressed into globin proteins. A schematic representation of the interaction of the products of these genes is shown in Figure 71-3. Normally, globin tetramers are formed of two  $\alpha$  or  $\alpha$ -like chains and two  $\beta$  or  $\beta$ -like chains; since humans are diploid, that is, have a pair of each nonsex chromosome or autosome, they have two genes for each autosomal locus. Thus, as there are two loci encoding the structure of the  $\alpha$ -globin chain, there are four  $\alpha$ -chain genes. In contrast, there is only a single  $\beta$ -globin locus and, therefore, two  $\beta$ -genes see (Figure 71-3). The relative numbers of  $\alpha$ - and  $\beta$ -loci are important in understanding the different inheritance patterns of  $\alpha$ - and  $\beta$ -thalassemias, as well as the different relative amounts of variant hemoglobins in individuals carrying a variant  $\alpha$ - or  $\beta$ -globin gene. These quantitative differences correlate directly with the clinical severity of the various disorders.

The  $\beta$ -like globin genes form a linked cluster on the short arm of chromosome 11 (11p15.5), spread over a region of approximately 60 kilobases (kb), as depicted in Figure 71-4. There are five functional genes, arranged in the order of their developmental expression: 5'- $\epsilon$ - $\gamma$ - $\gamma$ - $\psi\beta$ - $\delta$ - $\beta$ -3'. The pseudogene ( $\psi\beta$ ) has sequences similar to



**FIGURE 71-4** Globin genes complexes. (A) β-Gene complex on chromosome 11. (B) α-Gene complex on 16. Distances along the chromosome are measured in kilobases (kb) at the top.

the β-gene, but differs in having altered sequences that prevent its expression and the production of functional globin chains. Each of the genes contains two intervening sequences (IVSs) that interrupt the coding sequence at the junctions of the codons for amino acids 30–31 and 104–105. The first IVS is 122–130 base pairs (bp), while the second is 850–904 bp in length. The globin gene sequences comprise only about 7 kb of the 60 kb of DNA in the β-gene region, while the remaining 53 kb are flanking sequences that contain sequences with specific regulatory roles. These include the locus control region, enhancer sequences, and the promoter regions of the globin genes.

The β-locus control region or LCR-β is found in a cluster of DNase I hypersensitive sites 6–18 kb 5' to the ε-globin gene. The LCR-β establishes a transcriptionally active domain spanning the entire β-globin gene cluster, allowing a high level of erythroid-specific expression of the genes. Transcription is dependent on the promoter regions of these genes becoming opposed to the LCR-β in association with the binding of a variety of regulatory

proteins consisting of both tissue-specific and ubiquitous transcription factors. The precise mechanism of the developmental switches of gene expression from ε to γ and from γ to β remains to be fully understood, but is known to be complex with respect to both gene silencing and gene competition for the LCR sequences.

Erythroid cells have been shown to contain a variety of erythroid-specific transcription factors, the most important being GATA-1 and NF-E2. GATA-1 belongs to a family of proteins recognizing the consensus sequence (T/A)GATA(A/G), and acts as a tissue-specific activator of transcription with binding sites both in the locus control regions and in the promoters and 32' flanking regions of the globin genes. NF-E2 binding sites have been identified in both the β- and α-locus control regions. Aberrant binding of GATA-1 to a mutant site has been implicated as the cause of one type of HPFH.

The α-like genes form a cluster on the tip of chromosome 16 at position 16p13.3 and are arranged in the order 5'-ε-ζ-ψζ-ψα2-ψα1-α2-α1-θ-3'. The cluster contains three functional genes (two α-genes that are 3.6 kb apart

and one  $\zeta$ -gene), three pseudogenes ( $\psi\zeta$ ,  $\psi\alpha 1$  and  $\psi\alpha 2$ ), and one gene of undetermined function ( $\theta$ ) as depicted in Figure 71-4. Unlike the pseudogenes, the  $\theta$ -gene does not contain any apparent defects in its sequence that would prevent its expression. In each case about 4kb separate the  $\zeta 1$ ,  $\psi\alpha 1$ ,  $\alpha 2$ , and  $\alpha 1$  loci, suggesting the existence of discrete duplication units in the DNA. The two  $\alpha$ -genes are the result of a duplication that took place about 60 million years ago. The  $\alpha$ -genes have smaller IVSs than are found in the  $\beta$ -like genes; IVS-I contains 114bp, while IVS-II contains 132bp. There is an  $\alpha$ -globin control region called HS-40 for the  $\alpha$ -globin gene cluster that is a nuclease-hypersensitive site analogous to LCR- $\beta$ , although its mechanism of action is different. Deletion of the LCR- $\alpha$  causes  $\alpha$ -thalassemia by inactivation of the adjacent intact  $\alpha$ -globin genes. The  $\alpha$ -gene cluster also contains several hypervariable repetitive minisatellite sequences (HVRs). These are the 5'-HVR, located 70kb upstream of the  $\zeta 2$ -gene; the inter- $\zeta$  HVR, between the  $\zeta 2$ - and  $\psi\zeta 1$ -genes; the intra- $\zeta$  HVR, within the introns of the  $\zeta 2$ - and  $\psi\zeta 1$ -genes; and the 3'-HVR, positioned at the 3' end of the cluster.

### 71.3.2 Ontogeny

The globin genes are expressed at different times and in different relative amounts during human development (Figure 71-5). The sequence of appearance of the various globin chains is helpful in understanding the timing of onset of clinical manifestations of the hemoglobinopathies and thalassemias. For example, a deficiency of  $\alpha$ - or  $\gamma$ -chain synthesis and  $\alpha$ - or  $\gamma$ -chain variants with abnormal functions should be observed at birth, while a deficiency of  $\beta$ -chains may not cause symptoms until several months of age. Finally, levels of  $\beta$ -chain variants, such as Hb S, progressively increase over the first months, so that

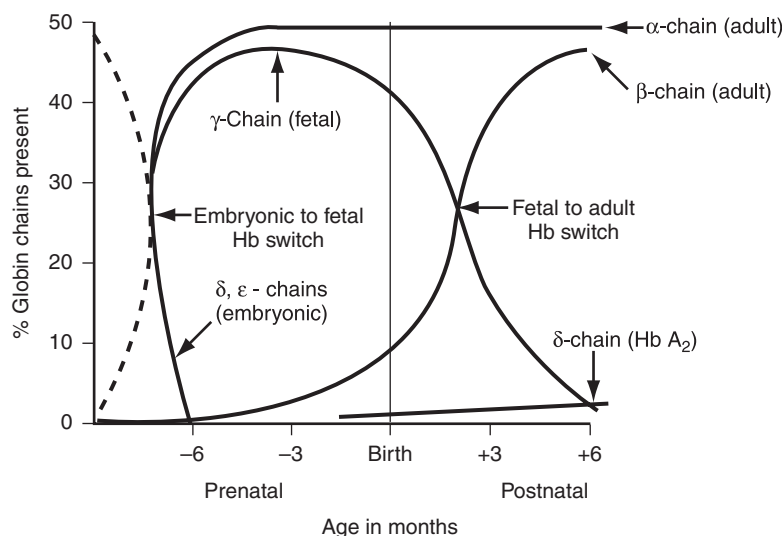
the onset of clinical manifestations may be delayed until the latter half of the first year of life.

### 71.3.3 Globin Biosynthesis

Each globin gene consists of a 5' promoter region, cap site, a 5' untranslated region (5' UTR), initiation codon, exon 1, intron 1 or IVS-I, exon 2, intron 2 or IVS-II, exon 3, termination codon, a 3' UTR, and a poly A signal (AATAAA). The gene is transcribed to produce messenger RNA precursor (pre-mRNA) (Figure 71-6), which starts at the cap site (3) nucleotides from the initiation codon and ends past the poly A site where the poly A tail is attached. The pre-mRNA molecule is then processed, undergoing excision of the IVSs and splicing of the three exon sequences.

Further processing occurs at each end of this RNA molecule. At the 5' end, guanosine is added in a special triphosphate linkage, and this guanosine and the next two nucleotides are then methylated. These 5' end modifications are called capping and methylation and while their function is not completely known, they have been shown to be vital for the initiation of translation of many mRNAs, including globin mRNAs. The 3' end modification involves the addition of about 150 adenylic acid nucleotides [poly(A)]. Poly(A) addition may also be important for the transport of mRNA to the cytoplasm and its subsequent stability. With aging of the mRNA, the poly(A) "tail" shortens.

Once the processed mRNA has been transported to the cytoplasm, it binds to ribosomes. The first step in translation (initiation) requires the binding of mRNA to the two ribosome subunits, amino acyl-tRNA, guanosine triphosphate, and protein initiation factors. Initiation occurs at the 5', or capped, end of mRNA that corresponds to the  $\text{NH}_2$ -terminal end of the globin chain.



**FIGURE 71-5** Qualitative and quantitative changes in globin chains during human development. Note that the percentage of  $\beta$ -chains accumulated in early fetal development is much less than the percentage of  $\beta$ -chains synthesized during fetal development. (Modified from Bunn, H. F.; Forget, B. G.; Ranney, H. M. *Hemoglobin Structure*. In *Human Hemoglobins*; WB Saunders: Philadelphia, 1977; p 4, with permission.)



Protein synthesis then proceeds toward the COOH-terminal end. Four to six chains of varying length (nascent chains) undergo translation on the same mRNA simultaneously. When these nascent chains have attained full length, a termination codon is reached. Since no tRNA is available for decoding this codon, polypeptide synthesis stops and, with the assistance of protein termination factors, the polypeptide chain is released from the ribosome and its mRNA. About one-third of the mature mRNA sequence is not used for translation, but these untranslated nucleotides, which are located at both ends of the molecule, may have other regulatory functions.

The protein chain assumes its secondary and tertiary structures because of interactions resulting from its amino acid sequence. Next, heme is bound and, in combination with other polypeptide subunits, the quaternary hemoglobin molecule is formed. These steps are shown in Figure 71-6.

## 71.4 HUMAN HEMOGLOBIN VARIANTS

### 71.4.1 Molecular Etiology

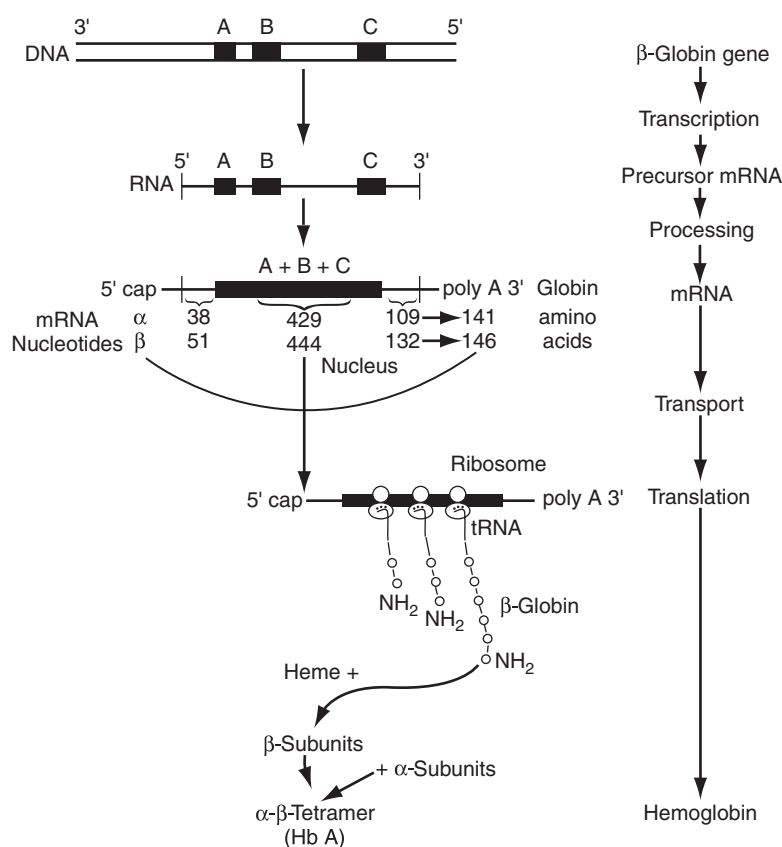
Abnormal hemoglobins result from mutations that change the sequence or number of nucleotides within the globin gene involved, or more rarely, from mispairing and crossover between two like genes during meiosis, creating a fusion protein of both gene sequences.

Mutation can cause substitution, addition, or deletion of one or more amino acids in the polypeptide sequence of the affected globin (Table 71-2).

Single base changes can result in single amino acid substitutions (e.g. Hb S ( $\beta 6$  Glu→Val)), shortened chains due to premature termination of translation (e.g. Hb McKees Rocks ( $\beta 145$  Tyr→Termination)), or elongated chains. Elongated chains result when the termination codon undergoes a mutation to a codon for an amino acid, such as UAA→CAA ( $\alpha 142$ ) in Hb Constant Spring. Two other elongated chains are Hb Icaria and Hb Koya Dora, both of which also have 31 additional residues and differ from Hb Constant Spring only at residue 142.

Single base deletions or additions can cause a frame-shift in the normal reading process. For example, in the variant Hb Wayne, a single base deletion (-A) at codon  $\alpha 139$  causes the subsequent sequence of two codons, the termination codon TAA and the 3' UTR to be read out of phase for seven triplets until a new terminating codon (TAG) is encountered.

Deletions of three, or multiples of three, nucleotides in the DNA cause deletions of one or more amino acids. It is interesting that of 13 examples of this type, all are  $\beta$ -chain variants, including Hb Leiden ( $\beta 6$  or 7 Glu→0) and Hb Gun Hill ( $\beta 91-95$  [Leu-His-Cys-Asp-Lys]→0). Deletions of segments of genes may be due to nonhomologous crossing over after mispairing in meiosis. This mechanism accounts for the Hb Lepore globins ( $\delta\beta$  fusion



**FIGURE 71-6** Steps involved in hemoglobin biosynthesis. Understanding of the order and function of various steps is incomplete.

**TABLE 71-2 Molecular Basis of the Hb Variants**

Mutation	Example Hb		Clinical Manifestation	Molecular Basis (Presumed)
Nucleotide base substitutions for				
One amino acid	Hb S	$\beta 6\text{Glu} \rightarrow \text{Val}$	Sickling	$\beta\text{:Cd 6 GAG} \rightarrow \text{GTG}$
Two amino acids	Hb C-Harlem	$\beta 6\text{Glu} \rightarrow \text{Val} + \beta 73\text{Asp} \rightarrow \text{Asn}$	Sickling	$\beta\text{:Cd 6 GAG} \rightarrow \text{GTG}$ & $\beta\text{:Cd 73 GAT} \rightarrow \text{AAT}$
Termination	Hb McKees Rocks	$\beta 145 \text{ Tyr} \rightarrow \text{Termination}$	Increased oxygen affinity and polycythemia	$\beta\text{:Cd 145 TAT} \rightarrow \text{TAA}$
Amino acid instead of termination	Hb Constant Spring	$\alpha 2\text{:142 Termination} \rightarrow \text{Gln}$	Decreased synthesis (thalassemia-like)	$\alpha 2\text{:Cd 142 TAA} \rightarrow \text{AAA}$
Nucleotide base deletions				
Single base deletion $\rightarrow$ frameshift	Hb Wayne	$\alpha 2\text{:139-146 Lys-Tyr-Arg} \rightarrow \text{Asn} + 7 \text{ residues}$	Normal	$\alpha 2\text{:Cd 139 (-A)}$
Triplet deletion $\rightarrow$ single amino acid	Hb Leiden	$\beta 6 \text{ or } 7 \text{ Glu} \rightarrow 0$	Unstable	$\beta\text{:Cd 6 or 7 (-GAG)}$
Multiple codon	Hb Gun Hill	$\beta 91-95 \text{ Leu-His-Cys-Asp-Lys} \rightarrow 0$	Unstable	$\beta\text{:Cd 91-95 (-15 bp)}$
Crossover	Hb Lepore	$\delta\beta$ -fusion with segments of $\delta$ and $\beta$ lost	Decreased synthesis (thalassemia-like)	$\delta\beta\text{:7.4 kb deletion}$
Nucleotide base additions				
Two bases added $\rightarrow$ frameshift	Hb Cranston	$\beta 144 \text{ Tyr-His} \rightarrow \text{Ser-Ile-Thr}$	Unstable	$\beta\text{:Cd 144/145 +CT}$
Multiple codon	Hb Grady	$\alpha 118 (+\text{Glu-Phe-Thr})$	Normal	$\alpha 2 \text{ or } \alpha 1\text{:Cd 118/119 (+9 bp)}$

**TABLE 71-3 Numbers of Known Hemoglobin Variants**

Type	Number
$\alpha$ Chain variants (total)	378
$\beta$ Chain variants (total)	529
$\gamma$ Chain variants (total)	87
$\delta$ Chain variants (total)	50
Variants with two amino acid replacements	19
Variants with hybrid chains	12
Variants with elongated chains	13
Variants with deletions, insertions and deletions/insertions	27
$\alpha$ Chain variants with the same mutation on both the $\alpha 1$ and $\alpha 2$ gene	14
$\alpha$ Chain variants with a different mutation on the $\alpha 1$ and $\alpha 2$ gene	2

chains), the anti-Lepore globins ( $\beta\delta$  fusion chains), and Hb Kenya globin ( $\gamma\beta$  fusion chain).

### 71.4.2 Known Variants

The numbers of known hemoglobin variants resulting from changes in the nucleotide base number or sequence in DNA are shown in Table 71-3. An updated listing is available on the Internet through the globin gene server Web site (HbVar): <http://globin.cse.psu.edu> (4). A total of 1044 variants are listed, most of these detected by gel electrophoresis or HPLC, which separate hemoglobins on the basis of charge differences resulting from the

amino acid substitutions. Since many mutations that do not change the protein's charge are not detected by this method, many undetected hemoglobin variants must still exist in the population. Hb variants were originally characterized by the identification of the substituted amino acid by peptide analysis, but now as new variants are added to the list, the substituted amino acid is identified from the changed gene sequence or by mass spectrometry. The number of  $\beta$ -chain variants (529) is more than that of  $\alpha$ -variants (378), even though there are two  $\alpha$ -loci compared to a single  $\beta$ -locus. The majority of the  $\alpha$ -chain variants have arisen from one mutation on either the  $\alpha 1$ -gene or the  $\alpha 2$ -gene. For  $\alpha$ -chain variants characterized by protein analysis techniques, the particular  $\alpha$ -gene carrying the variant is not known. However, gene-specific polymerase chain reaction (PCR)-based sequencing allows the identification of the variant  $\alpha$ -globin gene, and such studies have revealed that fourteen  $\alpha$ -chain variants result from the identical mutation on either of the two  $\alpha$ -genes, with the percentage Hb variant expressed by the  $\alpha 2$ -gene mutation being higher than that of the  $\alpha 1$ -gene mutation (5). Interestingly, DNA sequencing has also revealed that at least three  $\alpha$ -chain variants have arisen by different mutations, for example, Hb J-Broussais has arisen by the mutation G  $\rightarrow$  T on codon 90 of the  $\alpha 2$ -gene and G  $\rightarrow$  C on codon 90 of the  $\alpha 1$ -gene.

Table 71-4 compares the properties of the hemoglobin variants according to the different types of abnormal globin chains. The vast majority of these variants arise from a single base substitution, which results in a single amino acid substitution. Many of these substitutions are

**TABLE 71-4 Comparison of the Properties of Hb Variants**

Globin Chain	Total Variants	Clinically Silent (%)	Unstable (%)	Increased Oxygen Affinity (%)	Decreased Oxygen Affinity (%)	Ferric Hb (%)	Sickling (%)	Thalassemic (%)
$\alpha$	378	()	93 (26)	63 (17)	14 (4)	1 (<1)	0 (0)	38 (10)
$\beta$	529	187 (35)	207 (39)	141 (27)	62 (12)	7 (1)	12 (2)	38 (7)
$\gamma$	87	81 (93)	3 (3)	2 (2)	0 (0)	1 (1)	0 (0)	0 (0)
$\delta$	50	45 (10)	2 (4)	1 (2)	0 (0)	0 (0)	0 (0)	4 (8)
Hybrid	12	7 (58)	0 (0)	2 (17)	0 (0)	0 (0)	0 (0)	5 (42)

**TABLE 71-5 Clinical Manifestations of Hemoglobin Mutants**

Type	Example	Clinical Manifestation
Sickling	Hb S	Sickling due to decreased solubility
Unstable	Hb Bristol	Anemia with Heinz body formation
Abnormal oxygen affinity		
Decreased	Hb Kansas	Mild anemia possible
Increased	Hb Chesapeake	Polycythemia due to decreased oxygen transport
M hemoglobin	Hb M-Boston	Cyanosis due to ferric hemoglobin
Decreased synthesis	Hb Lepore	Thalassemia

clinically silent, including some of those that produce abnormal physical properties in the variant hemoglobin, and have been detected only through population screening. The amino acid substitutions can cause a number of abnormal physical properties. These are (i) instability of the tetramer; (ii) deformity of the three-dimensional structure; (iii) inhibition of ferric iron reduction; (iv) alteration of the residues that interact with heme, with 2,3-DPG, or at the  $\alpha$ - $\beta$  subunit contact site; or (v) abnormality of other properties of the molecule. The varieties of clinical phenotypes that arise from these abnormal physical properties are listed in Table 71-5.

The location of the amino acid changed by the mutation can often be correlated with the resultant phenotype. Unstable hemoglobin variants arise from several types of changes in the primary sequence that affects the secondary, tertiary, or quaternary structure. These substitutions tend to be at residues in the interior of the molecule, at contact points between chains, at residues that interact with the heme groups, or when a proline residue replaces another amino acid within an  $\alpha$ -helical region (Hb Genova [ $\beta$ 28(B10)Leu→Pro], Hb Abraham Lincoln [ $\beta$ 32(B14)Leu→Pro]), resulting in disruption of the helix. Hb Philly [ $\beta$ 35(C1)Tyr→Phe] is also unstable, secondary to a missing hydrogen bond normally found between the  $\alpha_1$  and  $\beta_1$  subunits. Many other unstable hemoglobins are the result of mutations affecting residues that bind

heme or are in the hydrophobic heme cleft, for example, Hb Gun Hill [ $\beta$ 91–95 (Leu-His-Cys-Asp-Lys)→0] and Hb Hammersmith ( $\beta$ 42 Phe→Ser).

Substitutions on the surface of the molecule usually do not affect tertiary structure or heme–heme interaction, but they may permit molecular interactions that decrease solubility under certain conditions [Hb S  $\beta$ 6 Glu→Val]. Substitution of tyrosine for either of the histidines that bind the iron molecule (E7 or F8) results in increased stability of the ferric (oxidized) iron state seen in M hemoglobins, M-Boston [ $\alpha$ 58(E7)His→Tyr], and M-Iwate [ $\beta$ 87(F8)His→Tyr]. Substitution at an  $\alpha_1\beta_1$  subunit contact point, such as  $\beta$ 99, can disturb heme–heme interactions causing increased oxygen affinity and polycythemia, as with Hb Kempsey [ $\beta$ 99(G1)Asp→Asn].

Although most hemoglobin variants are synthesized at a normal rate, several are associated with quantitative as well as qualitative abnormalities. One example, Hb E ( $\beta$ 26 Glu→Lys), is the second most common hemoglobinopathy worldwide and is associated predominantly with Southeast Asian populations. Both the heterozygous and homozygous states are associated with red cell microcytosis and hypochromia, and the thalassemic phenotype of the  $\beta^E$  gene is linked to activation of a cryptic donor splice site by the codon 26 mutation. Competition between the normal and abnormal alternative splice sites reduces  $\beta^E$ -mRNA production, resulting in a very mild  $\beta$ -thalassemia phenotype. Individuals homozygous for Hb E have a very mild disorder, being only slightly anemic with red cell indices similar to those of  $\beta$ -thalassemia trait. However, for reasons still not fully understood, Hb E trait combines with  $\beta$ -thalassemia trait to produce a serious thalassemic disorder, compound heterozygotes having a variable clinical picture ranging from thalassemia intermedia to homozygous  $\beta$ -thalassemia.

### 71.4.3 Inheritance of Hemoglobinopathies

Heterozygotes for a hemoglobin containing an abnormal  $\beta$ -globin chain have an abnormal as well as a normal  $\beta$ -gene at that locus, and their status is often described by the term “trait.” Since most variants are rare, they usually occur in the heterozygous state and, if they cause clinical symptoms, are examples of autosomal

dominant conditions. When both alleles code for the same common  $\beta$ -variant, the individual is then homozygous and is said to have the “disease” state. The most commonly encountered variants in the homozygous state are Hb S, C, D, and E and their phenotypes are listed in Table 71-6.

It should be noted that the term “sickle-cell disease” is often used to describe a similar phenotype that is seen when any of several genotypes (SS, SC, S/ $\beta$ -thalassemia, S/D-Punjab, or S/O-Arab) are exposed to a

certain environment (hypoxia), as listed in Table 71-6. Furthermore, under conditions of severe hypoxia a person with the AS genotype or “trait” can also manifest symptoms of the sickle-cell “disease” phenotype. This distinction between the genotype (homozygous and heterozygous) and the phenotype (trait and disease) is an important one. For these reasons, the patterns of inheritance of hemoglobin variants are more precisely expressed in terms of genotypes than in terms of phenotypes.

**TABLE 71-6** Phenotypes of Thalassemias, Sickle-cell Disorders and Various Hb Variant/Thalassemia Interactions

Type	Phenotype	DNA Diagnosis
<b>1. Homozygous states</b>		
$\alpha^0$ -Thalassemia (—/—)	Hb Bart's hydrops fetalis	Gap-PCR, MLPA
$\alpha^+$ -Thalassemia ( $-\alpha/-\alpha$ )	No clinical problems	Gap-PCR, MLPA
$\alpha^+$ -Thalassemia ( $\alpha^T\alpha/\alpha^T\alpha$ )	Hb H disease	ASO, sequencing
<b><math>\beta</math>-Thalassemia</b>		
$\beta^0$ or severe $\beta^+$ mutation	Thalassemia major	ASO, ARMS, sequencing
Mild $\beta^{++}$ mutation	Thalassemia intermedia	ASO, ARMS, sequencing
$\delta\beta^0$ -Thalassemia	Thalassemia intermedia	Gap-PCR, MLPA
HPFH	No clinical problems	Gap-PCR, MLPA
Hb Lepore	Variable: intermedia to major	Gap-PCR
Hb S	Sickle-cell disease	RE-PCR, ASO, ARMS
Hb C	Moderate anemia and splenomegaly	ASO, ARMS
Hb D-Punjab	No clinical problems	RE-PCR, ASO, ARMS
Hb E	No clinical problems	ASO, ARMS
<b>2. Compound heterozygous states</b>		
$\alpha^0$ -thal/ $\alpha^+$ -thal (—/— $\alpha$ )	Hb H disease	Gap-PCR, MLPA
$\alpha^0$ -thal/ $\alpha^+$ -thal (—/— $\alpha^T\alpha$ )	Severe Hb H disease or H hydrops	Gap-PCR, MLPA
$\beta^0$ -thal/severe $\beta^+$ -thal	Thalassemia major	ASO, ARMS, sequencing
Mild $\beta^+/\beta^0$ or severe $\beta^+$ -thal	Variable: intermedia to major	ASO, ARMS, sequencing
$\delta\beta^0$ -thal/ $\beta^0$ or severe $\beta^+$ -thal	Variable: intermedia to major	Gap-PCR, MLPA, as for $\beta^+/\beta^0$
$\delta\beta^0$ -thal/mild $\beta^+$ -thal	Mild thalassemia intermedia	Gap-PCR, MLPA, as for $\beta^+/\beta^0$
$\delta\beta^0$ -thal/Hb Lepore	Thalassemia intermedia	Gap-PCR, MLPA,
$\alpha\alpha/\beta^0$ or severe $\beta^+$ -thal	Mild thalassemia intermedia	Gap-PCR, as for $\beta^+/\beta^0$
Hb Lepore/ $\beta^0$ or severe $\beta^+$ -thal	Thalassemia major	Gap-PCR, MLPA, as for $\beta^+/\beta^0$
Hb C/ $\beta^0$ or severe $\beta^+$ -thal	Variable: $\beta$ -thal trait to intermedia	ASO, ARMS, sequencing
Hb C/mild $\beta^{++}$ -thal	No clinical problems	ASO, ARMS, sequencing
Hb D/ $\beta^0$ or severe $\beta^+$ -thal	No clinical problems	RE-PCR, ARMS, sequencing
Hb E/ $\beta^0$ or severe $\beta^+$ -thal	Variable: intermedia to major	ASO, ARMS, sequencing
Hb O-Arab/ $\beta^0$ -thal	Severe thalassemia intermedia	RE-PCR, ARMS, sequencing
Hb S/ $\beta^0$ or severe $\beta^+$ -thal	Sickle-cell disease	RE-PCR, ASO, ARMS
Hb S/mild $\beta^{++}$ -thal	Usually mild sickle-cell disease	RE-PCR, ASO, ARMS
Hb S/Hb C	Sickle-cell disease, variable severity	RE-PCR, ASO, ARMS
Hb S/Hb D-Punjab	Sickle-cell disease	RE-PCR, ASO, ARMS
Hb S/Hb O-Arab	Sickle-cell disease	RE-PCR, ASO, ARMS
Hb S/HPFH	Sickle-cell trait	RE-PCR, Gap-PCR, MLPA
<b>3. Hb E disorders</b>		
Hb E + $\alpha^0$ -thal/ $\alpha^+$ -thal (—/— $\alpha$ )	Hb AE Bart's disease see (Table 71-9)	Gap-PCR, ARMS,
Hb E + $\alpha^0$ -thal/ $\alpha^+$ -thal (—/— $\alpha^T\alpha$ )	Hb AE Bart's disease	Gap-PCR, ARMS, sequencing
Hb EE + $\alpha^+$ -thal/ $\alpha^+$ -thal ( $\alpha^T\alpha/\alpha^T\alpha$ )	Mild thalassemia intermedia	Gap-PCR, ARMS, sequencing
Hb EE + $\alpha^0$ -thal/ $\alpha^+$ -thal (—/— $\alpha$ )	Hb EF Bart's disease see (Table 71-9)	Gap-PCR, ARMS,
Hb EE + $\alpha^0$ -thal/ $\alpha^+$ -thal (—/— $\alpha^T\alpha$ )	Hb EF Bart's disease	Gap-PCR, ARMS, sequencing
Hb E/ $\beta^0$ + $\alpha^0$ -thal/ $\alpha^+$ -thal (—/— $\alpha$ )	Hb EF Bart's disease	Gap-PCR, ARMS, sequencing
Hb E/ $\beta^0$ + $\alpha^0$ -thal/ $\alpha^+$ -thal (—/— $\alpha^T\alpha$ )	Hb EF Bart's disease	Gap-PCR, ARMS, sequencing

Hb, hemoglobin; thal, thalassemia; HPFH, hereditary persistence of fetal hemoglobin; PCR, polymerase chain reaction; MLPA, multiplex ligation-dependent probe amplification; RE, restriction enzyme; ASO, allele specific oligonucleotide; ARMS, amplification refraction mutation system.



## 71.5 SICKLE-CELL ANEMIA AND RELATED DISORDERS

### 71.5.1 Molecular Basis and Geographical Distribution

The sickle-cell gene results from a point mutation at the second nucleotide of codon 6 of the  $\beta$ -globin gene. The base change of A to T causes the amino acid substitution of valine for glutamic acid (i.e. GAG to GTG); therefore, Hb S is  $\alpha_2\beta_2^{6 \text{ Glu} \rightarrow \text{Val}}$  ( $\beta^S$ ). The frequency of sickle trait (Hb AS) among United States blacks at birth is about 8%, and the incidence of sickle-cell anemia at birth should be around 0.16%, or 1 in 625 births. This contrasts with the higher carrier frequencies seen in some areas of equatorial Africa because of the protective advantage conferred by the carrier state against falciparum malaria (AS children have a relative resistance to malaria during early childhood, and are more likely to survive, breed, and pass on their genes). Hb S is known to occur at frequencies up to 20% in the Cameroons, Guinea, Zaire, Uganda, and Kenya. It also occurs at similar gene frequencies in the Quatif oases of eastern Saudi Arabia and parts of India. At lower gene frequencies of up to 5%, the sickle-cell gene has been reported in Nepal, in regions around the Mediterranean (e.g. Turkey, Lebanon, Syria, Greece, Portugal, and the coast of North Africa), in the Middle East and in Iran. The malaria hypothesis explains the distribution of Hb S in all these regions except those of the New World (United States, Caribbean islands, Brazil, etc.) and Europe. Hb S in the New World is found only in immigrants from malarious regions of the Old World and thus arrived in these regions by gene flow. Similarly, gene flow was responsible for the introduction of Hb S in the Mediterranean countries, by the slave trade in ancient times, and in European countries such as the United Kingdom by the voluntary population migration in the twentieth century.

### 71.5.2 Pathophysiology of Sickling

Substitution of valine for glutamic acid at the  $\beta 6$  residue causes a change on the surface of the deoxygenated  $\beta^S$  chain, which allows it to interact in a special way with other  $\beta$ -chains. This interaction results in the formation by  $\alpha_2\beta_2^S$  tetramers of a 14-stranded helical polymer of diameter 15–17 nm. The parallel alignment of these rod-like polymers, in turn, causes the sickle-shaped deformation of the erythrocytes. In sickle-cell anemia the sickling process may begin when the oxygen saturation of Hb S is decreased to 85%, but it does not occur in heterozygotes (Hb AS) until the oxygen saturation of hemoglobin is decreased to 40%. In addition to a decrease in oxygen tension, a reduction in pH or an increase in 2,3-DPG also promotes sickling. These factors probably interact in patients with sickle-cell anemia, since their blood normally has an increased 2,3-DPG concentration.

The viscosity of oxygenated sickle-cell blood is increased, primarily because of irreversibly sickled cells, and also because of increased gamma globulin levels. When the blood becomes deoxygenated, viscosity increases further because of the cellular rigidity that occurs with sickling. This, in turn, increases the exposure time of erythrocytes to a hypoxic environment, and the lower tissue pH decreases oxygen affinity, which further promotes sickling. The end result is occlusion of capillaries and small arteries and infarction of surrounding tissues. Deformed sickle cells also have a shorter survival time because of their increased mechanical fragility and damaged membranes, resulting in a more rapid red cell turnover and consequent anemia.

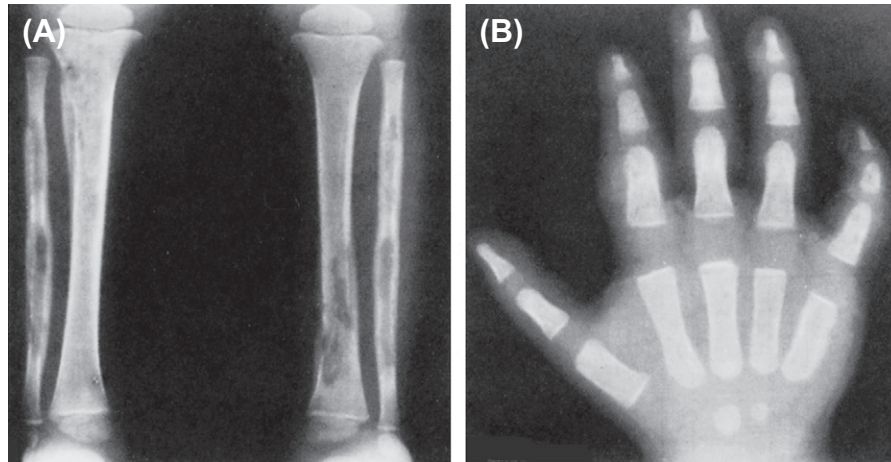
### 71.5.3 Clinical Aspects of Sickle-Cell Disease

As can be seen from Figure 71-6,  $\beta$ -chain production does not usually reach sufficient levels to cause symptoms until the second half of the first year of life. As higher concentrations of Hb S are reached in erythrocytes, the cells become susceptible to hemolysis and a progressive hemolytic anemia with splenomegaly is seen. The increased rate of erythropoiesis leads to erythroid marrow expansion and increased folic acid requirements. However, the two major problems for young children with SS disease are infections and vaso-occlusive crises.

Children with sickle-cell anemia have increased susceptibility to potentially life-threatening bacterial infections including sepsis and meningitis caused by *Streptococcus pneumoniae* and *Haemophilus influenzae*. The relative risk of sickle-cell anemia patients compared with that of normals for pneumococcal, *H. influenzae* and all bacterial meningitis is 579:1, 116:1, and 309:1, respectively. These patients are also susceptible to bacterial pneumonia (often *Pneumococcus*), osteomyelitis (*Salmonella* and *Staphylococcus*) (Figure 71-7), and urinary tract infections (*Escherichia coli* and *Klebsiella*). Increased susceptibility is also seen for *Shigella* and *Mycoplasma pneumoniae*. Several factors that contribute to this susceptibility are functional hyposplenism, impaired antibody response, decreased opsonization, impaired complement activation in the properdin pathway, and abnormal chemotaxis.

Bacterial infection is the most common reason for hospitalization of pediatric sickle-cell anemia patients and often leads to the diagnosis. Serious bacterial infections are seen in approximately one-third of children with sickle-cell anemia before 4 years of age. Infection, not crisis, is the most common cause of death in these children, although infections often precipitate crises.

Vaso-occlusive crises begin in infancy with dactylitis, or hand-and-foot syndrome see (Figure 71-7). Later crises may involve the periosteum, bones, or joints, resulting in infarction that must be differentiated from osteomyelitis and septic arthritis. Vaso-occlusive crises



**FIGURE 71-7** Radiographic changes in sickle-cell anemia. (A) Changes in the tibiae and fibulae secondary to *Salmonella osteomyelitis*. (B) Hand-and-foot syndrome with soft-tissue swelling and focal areas of cortical destruction and periosteal new bone formation. (Courtesy of Dr John Dorst.)

and sepsis are difficult to differentiate and often coexist in younger children.

Pulmonary crises with pleural pain and fever may be due to infection, in situ thrombosis, or embolism. Other clinical manifestations include splenic sequestration, abdominal and aplastic crises, cholelithiasis, hepatic infarcts, occlusion of cerebral vessels, ocular changes, hematuria, hyposthenuria, hyponatremia, priapism, and skin ulcers.

Sickle-cell disease has a broad spectrum of clinical severity. Because the  $\beta^S$ -gene has a stable single point mutation, the variability of clinical expression of sickle-cell disease is explained by the interaction of a number of factors, which can be genetic, cellular, or physiological. There are genetic factors that can be co-inherited with sickle-cell disease, such as  $\alpha$ -thalassemia and the determinants that increase Hb F (such as  $\beta$ -globin haplotypes), and those that interact with the sickle-cell trait (e.g. double heterozygosity for  $\beta$ -thalassemia, Hb C, Hb D-Punjab, or Hb O-Arab). The cellular factors include those that affect erythrocyte cell volume such as Hb concentration, pH and ion balance, and physiological factors such as vascular tone.

The effect of  $\alpha$ -thalassemia on the clinical picture of sickle-cell disease is complex.  $\alpha$ -Thalassemia lowers the mean cell volume (MCV) and the mean corpuscular hemoglobin concentration (MCHC). As the latter is one of the factors that determine the extent of polymerization of Hb S, the result of  $\alpha$ -thalassemia is a reduction in polymerization and a milder anemia. There is a reduction in hemolysis and fewer sickling-related manifestations such as leg ulceration and acute chest syndrome. However, the advantages of the inhibition of sickling are balanced by disadvantages of  $\alpha$ -thalassemia in causing a reduction in hemolysis and an increase in total hemoglobin. These effects result in an increase in blood viscosity, making patients more prone to painful crises and vaso-occlusive episodes such as a vascular necrosis.

Analysis of  $\beta^S$ -gene haplotypes has demonstrated four common patterns within African ethnic groups: the Benin, CAR (Central African Republic) or Bantu, Senegal, and Cameroon haplotypes. Although at least 17 different  $\beta^S$ -gene haplotypes have been identified, only these four haplotypes appear not to be related to each other by simple recombination events. Epidemiologic studies in Africa and the United States show that in SS patients, the presence of a CAR haplotype on one  $\beta^S$ -chromosome is associated with low Hb F levels and a higher risk of soft-tissue organ failure and early death. On the other hand, patients with the Senegal haplotype have higher levels of Hb F and less severe disease. The highest Hb F levels are exhibited in SS patients from eastern Saudi Arabia and India, resulting in a clinically benign disease. These patients are usually homozygous for a fifth  $\beta^S$ -gene haplotype, the Indian-Arab haplotype, indicating the Hb S mutation has occurred independently to the mutations in Africa. It is not clear in which direction the mutation traveled, originating in India and migrating to Arabia or vice versa.

The persistence of Hb F in adults inhibits the polymerization of Hb S as well as reduces the Hb S concentration. Indeed, patients doubly heterozygous for Hb S and the deletion form of HPFH have no clinically significant hematologic abnormalities despite having only Hb S and Hb F. Such compound heterozygotes have nearly normal red cells, each with 20–30% Hb F in a pancellular distribution.

### 71.5.4 Diagnosis

The peripheral blood smear of sickle-cell anemia patients may have normal-appearing, irreversibly sickled, target, and nucleated red cells. Howell-Jolly bodies and red cell fragments are also present, especially after functional asplenia develops (Table 71-8A). The clinical history of crises or severe infections with anemia, abnormal

red cell morphology on peripheral smear with a normal or elevated mean corpuscular volume (MCV), positive sickling test, and Hb S (greater than 80%) and Hb F on electrophoresis or HPLC, makes the diagnosis of sickle-cell anemia probable. Family studies indicating that both parents have the sickle-cell trait are helpful to exclude S/ $\beta^0$ -thalassemia and S/HPFH, although a definitive diagnosis is best made by DNA analysis for the Hb S mutation and the other possible interacting genotypes. In addition, sibs should also be tested to identify and treat previously undiagnosed cases.

### 71.5.5 Treatment

A number of antisickling agents have been tried to ameliorate sickle-cell disease and a number of new therapies have evolved from our improved understanding of the pathophysiology of sickle-cell disease (6). The three main approaches to therapy are (i) the chemical inhibition of Hb S polymerization to increase red cell hydration, (ii) reducing the MCHC by inhibiting potassium ion and water loss (e.g. with the antifungal agent clotrimazole), and (iii) the pharmacological induction of Hb F. Hb F concentrations are inversely related to morbidity in sickle-cell disease and the use of drugs that diminish Hb S polymerization by increasing the level of Hb F has been the most promising approach (7). Initially, cytotoxic agents such as 5-azacytadine were demonstrated to increase Hb F in patients, but then more benign drugs including hydroxyurea, erythropoietin, and short chain fatty acids such as butyrate were studied in sickle-cell anemia patients (7). The most successful of these agents has been hydroxyurea, which increases fetal hemoglobin production presumably through its effect in speeding up erythroid precursors through their maturation steps, the Hb level and the MCV, and reduces the frequency of painful crises. Hydroxyurea was approved 10 years ago specifically for the management of severe sickle-cell disease in adults and has now an established role in ameliorating the disease and improving life expectancy for most adult patients (8). The drug has also been shown to be effective in treating children with SS disease and adults with Hb S/ $\beta$ -thalassemia with beneficial effects (9).

The clinical management of sickle-cell anemia is basically supportive and prophylactic, involving prompt and effective analgesia, rehydration, antibiotics, and hospital admission as appropriate. Infections should be treated promptly with antibiotics. Newborn screening and close follow-up, especially early in life, has significantly improved survival. The clinical benefit of daily prophylactic penicillin in reducing the incidence of serious pneumococcal infections was demonstrated conclusively by the PROPS I study, and now represents standard practice. The associated anemia is usually tolerated well, but if folate deficiency occurs, the anemia becomes more severe and is associated with macrocytosis, hypersegmented granulocytes, and a decrease in the percentage

of reticulocytes. Folate deficiency is prevented easily by daily folic acid supplement. Exchange blood transfusions are seldom indicated for uncomplicated anemia because of the association with side effects such as alloimmunization, but can be effective for life-threatening vaso-occlusive crises (cerebral) or in preparation for surgery. As with  $\beta$ -thalassemia, sickle-cell patients on long-term transfusion programmes require iron chelation therapy to prevent iron overload. Crises should be managed with vigorous hydration because of the patient's inability to concentrate urine and the increased blood viscosity. Acidosis and hypoxia should be treated, and analgesics should be given for the accompanying severe pain.

Although hypertransfusion of packed red cells and drugs such as hydroxyurea may ameliorate the symptoms of sickle-cell disease, only bone marrow transplantation is truly curative at the present time. Bone marrow transplantation has been successfully applied to treat sickle-cell disease in children and is increasingly being used for patients with less severe, but symptomatic, diseases. Cord blood transplantation has been performed successfully when an HLA-matched sibling without sickle-cell anemia has been born. However, bone marrow transplantation is less suitable for adults who already have irreversible organ damage, and important barriers to more widespread use of bone marrow transplantation remain. These include limited availability of HLA-matched related donors, a short-term mortality of about 10% from the transplant procedure, and uncertainty as to which individuals will have severe symptomatic disease warranting such an intervention.

The concept of gene therapy in the sickle-cell syndromes holds promise for a curative approach in the future, although progress has been slow and frustrating, because of inadequate gene transfer and efficacy, and low gene expression (10). The development of a transgenic knock-out mouse model of sickle-cell disease, which undergoes sickling with deoxygenation, has provided an important animal model to study both therapeutic interventions and gene therapy manipulations to transfer globin genes or activate the endogenous  $\gamma$ -globin genes. The mouse sickle model has been corrected using a lentiviral vector to insert LCR and  $\gamma$ -globin gene sequences into the murine hematopoietic cells (11), but there remains some concerns about insertional mutagenesis using this approach. Interest has now switched to the technique of gene replacement therapy, using a protocol to replace the sickle globin gene with a normal copy of the  $\beta$ -globin gene by homologous recombination (12).

### 71.5.6 Prevention

During genetic counseling, AS $\times$ AS couples are advised of their 25% risk for having children with sickle-cell disease and certain couples may request prenatal diagnosis. However, the utilization of prenatal diagnosis for sickle-cell disease appears to be very low compared with that



for  $\beta$ -thalassemia because of the variable clinical course of the disease. An audit of more than 20 years experience of prenatal diagnosis for the hemoglobin disorders in the United Kingdom showed that only 13% of all couples at risk of having a child with sickle-cell disease underwent prenatal diagnosis.

The first method of prenatal diagnosis to be developed used fetal blood obtained by fetoscopy or placental aspiration, and the types of  $\beta$ -chains produced in fetal red cells were detected by biosynthetic studies. The significant risk of fetoscopy (6% fetal mortality), its limited availability, and the variable clinical course of the disease combined to limit the widespread use of these methods until methods involving mutation analysis of fetal DNA from amniotic fluid cells were developed in 1978. The sickle-cell gene was first detected by linkage analysis of a Hpa I restriction enzyme site, but this was quickly replaced by the direct method of restriction enzyme analysis using Mst II or Dde I, which cut the normal  $\beta^A$ -globin gene at codons 5–7 but fail to cut the  $\beta^S$ -globin gene. In 1982, the switch to using DNA from chorionic villi for prenatal diagnosis permitted prenatal diagnosis to be accomplished in the first trimester of pregnancy, although the Southern blotting technique took 5–10 days for a diagnosis to be made; the discovery and application of the PCR technique shortened the time for a diagnosis to potentially just a few hours. The application of PCR has permitted a wide variety of different methods of mutation analysis to be developed for diagnosing the sickle-cell gene, including allele-specific oligonucleotide (ASO) hybridization (known as dot blotting), reverse dot blotting, amplification specific refractory mutation system (ARMS), allele-specific priming, and oligonucleotide ligation (13). However, the simplest PCR-based method of detection, the use of Dde I to cut amplified DNA is still the most widely used approach.

### 71.5.7 Interactions with Sickle Hemoglobin

Sickle-cell disease comprises a number of compound heterozygous genotypes. Genotypes that are frequently encountered are Hb S/ $\beta$ -thalassemia or Hb S/ $\delta\beta$ -thalassemia, Hb S/C, Hb S/D-Punjab, and Hb S/O-Arab. There are also some rare interactions that have been reported in just a few families: Hb S/C-Harlem, Hb S/S-Antilles, Hb S/S-Oman, and the compound heterozygosity for Hb S and some unstable  $\beta$ -chain variants.

**71.5.7.1 Hb S/ $\beta$ -Thalassemia.** The interactions of the different types of  $\beta$ -thalassemia alleles ( $\beta^+$ ,  $\beta^0$ , and  $\delta\beta^0$ ) are described later in this chapter in the thalassemia section.

**71.5.7.2 Hb S/C Disease.** Hb C is found at frequencies of up to 0.15% in parts of West Africa, where it coexists with Hb S. Accordingly, Hb C trait occurs in about 3% of American blacks at birth, Hb SC disease in 1 in 833, and Hb C disease in about 1 in 1250. The Hb C mutation,  $\beta_6$  Glu→Lys, GAG→AAG, causes a decrease in solubility of

both the oxygenated and the deoxygenated forms of the hemoglobin, resulting in the formation of crystals instead of long polymers. In individuals homozygous for Hb C, the red cells become dehydrated and rigid, causing hemolytic anemia but such patients do not develop any sickling symptoms. The clinical importance of Hb C lies in its interaction with Hb S. The inheritance of the Hb C trait with the Hb S trait results in Hb SC disease, a milder version of sickle-cell disease. Patients with SC disease tend to have a variable course, with most complications occurring less frequently than in SS disease.

**71.5.7.3 Hb S/D Disease.** Hb S/Hb D-Punjab ( $\beta_{121}$ , Glu→Gln) results in a moderately severe form of sickle-cell disease. This compound heterozygous state has been observed in patients of African origin and from Central and South America and India, and in individuals with only Mediterranean or northern European ancestry. Patients have a mild to moderate hemolytic anemia (Hb of 5–10 g/dl) with sickling crises.

**71.5.7.4 Hb S/Hb O-Arab Disease.** Hb S/Hb O-Arab ( $\beta_{121}$ , Glu→Lys) results in a severe type of sickle-cell disorder. Hb S/Hb O-Arab patients have been observed in Arabs, Africans, Afro-Caribbeans, and Afro-Americans. The Hb concentration varies between 6 and 10 g/dl and the blood film is similar to sickle-cell anemia.

**71.5.7.5 Other Rare Sickle-Cell Disease Genotypes.** Hb S/C-Harlem ( $\beta_6$  Glu→Val and  $\beta_{73}$  Asp→Asn) is a severe sickle-cell disorder. Hb C-Harlem has two amino acid substitutions, the sickle cell substitution at codon 6, and one at codon 73 that makes the hemoglobin move like Hb C in electrophoresis at alkaline pH (7). In combination with Hb S it causes severe sickle-cell disease.

Hb S-Antilles ( $\beta_6$  Glu→Val and  $\beta_{23}$  Val→Ile) has two amino acid substitutions, similar to Hb C Harlem. It is more prone to sickling than Hb S itself and in the heterozygous state it results in a mild anemia and a moderate sickling disorder. In combination with Hb S, it is reported to produce a very severe form of sickle-cell disease with a severe chronic hemolytic anemia. Compound heterozygosity for Hb C and Hb S-Antilles also produces a severe sickle-cell disorder.

Hb S-Oman ( $\beta_6$  Glu→Val and  $\beta_{121}$  Glu→Lys) has two different phenotypes in the heterozygous state, depending on whether the patients have co-inherited heterozygous or homozygous for  $\alpha$ -thalassemia (all patients described with Hb S-Oman have  $\alpha$ -thalassemia). Patients with  $\alpha^+$ -thalassemia trait have about 20% Hb S and a moderate sickling disorder. The blood film shows a unique form of an irreversibly sickled cell called a “Napoleon Hat cell” or “yarn and knitting needle cell.” In contrast, patients with Hb S-Oman trait and homozygous  $\alpha^+$ -thalassemia have about 14% Hb S-Oman and are asymptomatic. The compound heterozygous state for Hb S and Hb S-Oman has been described in a few Omani patients. Patients have 25% Hb S and 11% Hb S-Oman, and the blood film shows Napoleon Hat cells. Patients have very severe disease, with a Hb level of 7 g/dl.



The interaction of Hb S with unstable  $\beta$ -variants may result in a mild form of sickle-cell disease. Three such variants have been described, namely, Hb Quebec-Chori, Hb Hofu, and Hb I-Toulouse. Hb S in combination with mildly unstable  $\beta$ -variants such as Hb Hope and Hb Siiraj can cause mild hemolysis.

## 71.6 UNSTABLE HEMOGLOBIN VARIANTS

### 71.6.1 Molecular Basis

At least 300 unstable hemoglobin variants are known see (Table 71-4). Among these,  $\beta$ -variants are two times more frequent than  $\alpha$ -variants, a discrepancy that may be due to the smaller percentage of unstable hemoglobin and hence milder clinical symptoms associated with the  $\alpha$ -chain variants. An individual with a single variant  $\alpha$ -gene has three normal  $\alpha$ -genes, so that the percentage of unstable hemoglobin in the red cells is very small (5–20%). In contrast, an individual with a variant  $\beta$ -gene has only a single normal  $\beta$ -gene; so the unstable hemoglobin containing the variant  $\beta$ -chain makes up a greater proportion of the total cellular hemoglobin synthesized (20–40%). Because the gene frequencies for these variants are extremely low, almost all affected individuals seen are heterozygotes.

The increased propensity of unstable hemoglobins to denature can result from several types of mutations. As mentioned previously, the  $\alpha$ -helix of  $\alpha$  or  $\beta$ -globin can be disrupted by proline replacing another amino acid within the helix. There are at least 10 examples of this type of disruption of primary and secondary structures, including Hb Bibba ( $\alpha 136$  Leu→Pro) and Hb Genova ( $\beta 28$  Leu→Pro). Deletions of amino acid residues alter primary and secondary structures as well as the conformation of the hemoglobin molecules, and 8 of the 10 variants of this type are unstable, for example, Hb Leiden ( $\beta 6$  or  $7$  Glu→0) and Hb Gun Hill ( $\beta 91$ – $95$  (Leu-His-Cys-Asp-Lys)→0). Interference with interchain contacts permits the dimers to dissociate into monomers, for example, Hb Philly ( $\beta 35$  Tyr→Phe) and Hb Tacoma ( $\beta 30$  Arg→Ser) lack hydrogen bonds normally linking the  $\alpha$  and  $\beta$  subunits. Substitutions that affect heme binding or disturb the hydrophobic heme pocket (certain nonpolar residues in the CD, E, F, and FG regions) decrease the molecule's stability see (Figure 71-1). There are over 30 such mutations, and most result in unstable hemoglobins, such as Hb Bristol ( $\beta 67$  Val→Asp) and Hb Köln ( $\beta 98$  Val→Mer). Finally, globin chain elongation can result in instability due to hydrophobic properties of the extended chain, for example, Hb Cranston ( $\beta 144$ – $151$ ).

These variant hemoglobins tend to denature spontaneously; the globin subunits precipitate in the red cell, forming aggregates or Heinz bodies. The Heinz bodies adhere to the red cell membrane and result in decreased pliability of the cell. Inflexible erythrocytes are then selectively trapped by the reticuloendothelial system.

### 71.6.2 Clinical Aspects of Unstable Hemoglobins

Patients often present in infancy or early childhood with a hemolytic anemia, jaundice, and splenomegaly, or later with cholelithiasis. Some variants also cause cyanosis because of their abnormal properties, that is, propensity to form methemoglobin or decreased oxygen affinity. Clinical severity varies with different unstable variants; for  $\beta$ -variants, symptoms appear after the  $\gamma$  to  $\beta$  transition in hemoglobin synthesis see (Figure 71-5). A number of Hb variants are associated with a thalassemic condition rather than a severe hemolytic anemia. These variants, such as Hb Agrinio,  $\alpha 29$  Leu→Pro, and Hb Quon Sze,  $\alpha 125$  Leu→Pro, are so unstable that their presence is difficult to detect except by DNA sequence analysis of the appropriate gene.

### 71.6.3 Diagnosis

The peripheral smear may be normal or hypochromic. Staining with a supravital stain, such as 1% methyl violet, demonstrates preformed Heinz bodies (Figure 71-8B). Heat instability of the variant hemoglobin is demonstrated by the formation of a hemoglobin precipitate when a hemolysate is incubated at 50 °C or higher, or at 37 °C in 17% isopropanol. Hemoglobin electrophoresis by usual methods may detect only about half of unstable variants since the charge of these variants is often unaltered by the substitutions. Oxygen saturation curves of whole blood may indicate normal (20% of the unstable variants), decreased (30%), or increased (50%) oxygen affinity.

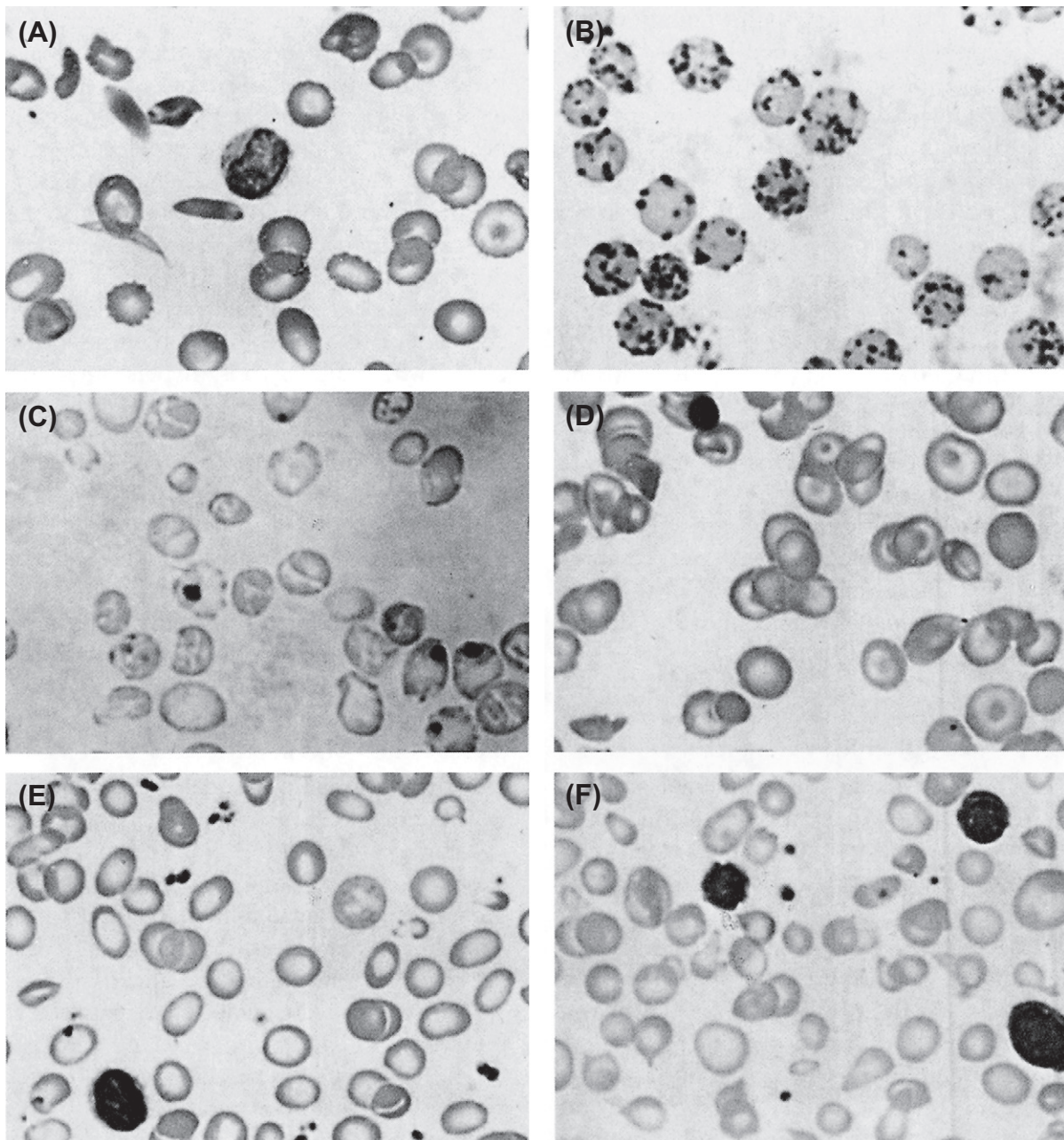
### 71.6.4 Treatment

Treatment is generally supportive. If hemolysis is severe, prophylactic folate may be indicated. Oxidant drugs, such as sulfonamides, increase hemolysis in some patients and should be avoided. Transfusions are indicated only in the treatment of aplastic crises. While splenectomy may result in improvement of the anemia, it also increases the risk of septicemia, especially in young patients. Because of the mortality associated with septicemia in splenectomized patients, the physician should reserve splenectomy for selected patients. Splenectomy should be postponed until the patient is at least 6 years of age, and the administration of pneumococcal vaccine and prophylactic antibiotics should be considered.

## 71.7 HEMOGLOBIN VARIANTS WITH ALTERED OXYGEN AFFINITY

### 71.7.1 Molecular Basis

The oxygen dissociation curve shown in Figure 71-2 is sigmoid shaped because of heme–heme interactions. Mutations that affect the heme–heme interaction, the Bohr effect, or the deoxyhemoglobin-2,3-DPG interaction can



**FIGURE 71-8** Peripheral blood smears from patients with various disorders of globin synthesis. (A) Homozygous sickle cell anemia. (B) Unstable Hb Zurich with Heinz bodies. (C) Hb H disease. (D) Sickle/ $\beta$ -thalassemia. (E)  $\beta$ -thalassemia trait. (F) Homozygous  $\beta$ -thalassemia. Figures B and C were prepared as follows: whole blood with EDTA was incubated at 41 °C for 3–6 hours, then a 1:1 mixture of blood and 0.5% rhodanile blue in 0.9% saline was made and immediately smeared. Hemoglobin precipitates formed secondary to heating are seen. (Courtesy of Dr William Zinkham.)

change the shape or position of the oxygen dissociation curve. Mutations affecting the  $\alpha_1\beta_2$  subunit contact point can alter heme–heme interaction by causing the deoxyhemoglobin conformation to be less stable. These mutations result in increased stability of the oxyhemoglobin conformation and increased oxygen affinity (e.g. Hb Kempsey ( $\beta 99 \text{ Asp} \rightarrow \text{Asn}$ )). Alternatively, the oxyhemoglobin conformation can be destabilized by mutations affecting the  $\alpha 94\beta 102$  contact point, resulting in decreased oxygen affinity (e.g. Hb Kansas ( $\beta 102 \text{ Asn} \rightarrow \text{Thr}$ )). Substitutions at the COOH-terminal ends of globin chains can lead to instability of deoxyhemoglobin conformations

and increased oxygen affinity (e.g. Hb Bethesda ( $\beta 145 \text{ Tyr} \rightarrow \text{His}$ )) as well as a reduction in the Bohr effect. 2,3-DPG binds to residues  $\beta 1$ , 2, 82, and 143 in the deoxygenated form. Substitutions altering these residues tend to have increased oxygen affinity (e.g. Hb F ( $\gamma$ -globin has a serine for histidine substitution at position 143)).

Variants with increased oxygen affinity cause a shift to the left of the oxygen dissociation curve see (Figure 71-2), resulting in less oxygen delivery per gram of hemoglobin. To compensate, hemoglobin concentration and/or blood flow increases to partially restore oxygen delivery to the

tissues. Some variants with increased oxygen affinity do not cause polycythemia because of the small fraction of the total hemoglobin they comprise, or the compensatory changes in the shape of the oxygen dissociation curve. Variants with decreased oxygen affinity have a shift to the right and increased oxygen delivery per gram of hemoglobin. As a result, the hemoglobin concentration is normal or decreased (e.g. Hb Beth Israel ( $\beta 102 \text{ Asn} \rightarrow \text{Ser}$ )).

### 71.7.2 Clinical Aspects and Diagnosis

Because the gene frequencies for nearly all variants of hemoglobins are very low, patients are nearly always heterozygotes. The great majority of patients are asymptomatic, and when oxygen affinity is increased the major finding is polycythemia with erythrocytosis, normal white blood cell and platelet counts, and absence of splenomegaly. Since about half of these variants cannot be detected on routine electrophoresis, whole blood oxygen affinity studies are required for diagnosis. Some concern has been raised regarding the risk to fetuses of mothers who have variants with increased oxygen affinity. The few data available regarding the outcome of such pregnancies do not in general seem to indicate increased fetal mortality.

### 71.7.3 Treatment

The condition is generally considered benign. It is important to avoid chemical treatment of the compensatory polycythemia unless hematocrit levels are high enough to cause increased viscosity.

## 71.8 M HEMOGLOBIN VARIANTS

### 71.8.1 Molecular Basis

There are five known variants (with 22 different names) of M hemoglobin involving  $\alpha$ - or  $\beta$ -chains. Four of these variants result from the substitution of tyrosine for histidine at positions  $\alpha 58$ ,  $\alpha 87$ ,  $\beta 63$ , and  $\beta 92$ : Hb M-Boston ( $\alpha 58 \text{ His} \rightarrow \text{Tyr}$ ), M-Iwate ( $\alpha 87 \text{ His} \rightarrow \text{Tyr}$ ), M-Saskatoon ( $\beta 63 \text{ His} \rightarrow \text{Tyr}$ ), and M-Hyde Park ( $\beta 92 \text{ His} \rightarrow \text{Tyr}$ ). The substituted tyrosine may form a stable bond with the ferric form of the heme iron. This bond prevents interaction of the ferric iron of the affected  $\alpha$ - or  $\beta$ -chain with oxygen, but it does not render the globin-heme unit unstable. Both the  $\alpha$ -variants (M-Boston and M-Iwate) have decreased oxygen affinity, two of the  $\beta$ -variants (M-Hyde Park and M-Saskatoon) have normal affinity, and the fifth Hb M variant, M-Milwaukee-I, ( $\beta 67 \text{ Val} \rightarrow \text{Glu}$ ) has decreased oxygen affinity. Two  $\gamma$ -chain Hb M variants have been described, Hb FM-Osaka ( $\gamma 63 \text{ His} \rightarrow \text{Tyr}$ ) and Hb FM-Fort Ripley ( $\gamma 92 \text{ His} \rightarrow \text{Tyr}$ ).

### 71.8.2 Clinical Aspects

M hemoglobin variants, like other rare hemoglobin disorders, are inherited in an autosomal dominant pattern.

The age of onset of cyanosis differs depending on whether the  $\alpha$ - or  $\beta$ -chain is affected. With  $\alpha$ -chain variants, cyanosis is seen at birth;  $\beta$ -globin variants develop cyanosis when  $\gamma$  to  $\beta$  switching is nearly complete, at about 6 months of age see (Figure 71-5).

Hb FM-Osaka and Hb FM-Fort Ripley were discovered in cyanotic newborns. The babies became normal as Hb F disappeared after birth.

### 71.8.3 Diagnosis

The blood is chocolate brown and does not change color on exposure to oxygen. Usually there is no anemia, and routine electrophoresis may be normal. Spectral analysis allows differentiation of M hemoglobins from methemoglobin secondary to diaphorase I deficiency. The latter is a red cell enzyme deficiency, which is inherited as an autosomal recessive. Because the modes of inheritance for M hemoglobins and diaphorase deficiency differ, usually one parent of a patient with the former is affected, while both parents of a patient with the latter disorder are unaffected.

### 71.8.4 Treatment

No treatment is indicated; however, the diagnosis should be made so that extensive cardiac and pulmonary evaluations can be avoided.

## 71.9 THALASSEMIAS

### 71.9.1 Quantitative Disorders of Globin Synthesis

The thalassemia syndromes are defined as a group of inherited disorders characterized by the absence or reduced synthesis of one or more of the normal globin chains of hemoglobin. They are subclassified according to the particular globin genes affected and also according to the effect on gene expression. For example, the  $\beta$ -thalassemias are disorders of  $\beta$ -globin gene expression, whereas the  $\delta\beta$ -thalassemias are disorders of both  $\delta$ - and  $\beta$ -gene expression. Absent globin synthesis is designated with an “o” superscript, for example,  $\beta^0$ -thalassemia, while the presence of some normal but insufficient gene product is noted by a “+” superscript, for example,  $\beta^+$ -thalassemia. The lack of synthesis of the affected globin chain results in an unbalanced  $\alpha/\beta$  globin chain ratio and therefore the defect is a quantitative one. This is in contrast with the hemoglobinopathies in which the variant hemoglobins are qualitatively or structurally abnormal.

### 71.9.2 Geographical Basis

Thalassemia is distributed primarily among people of Mediterranean, African, Middle Eastern, Asian Indian, Chinese, and Southeast Asian descent, but sporadic cases



have been reported in many ethnic groups. The thalassemias are most common in the Mediterranean region, Africa, and Asia, but have now become a global problem through migration of populations throughout Europe, the Americas, and Australia. The high carrier rates observed in many populations are thought to have resulted from a positive selection pressure due to *Falciparum* malaria. The mechanism by which the thalassemia trait provides protection against malaria is less clear than that with the sickle-cell trait, but there is a strong geographic correlation of gene frequencies with the incidence of malaria.

The thalassemias and the Hb variants are regionally specific, with each local population having its own characteristic spectrum of mutations. For most of these populations the range of mutations has been identified by molecular analysis and the frequency of each mutation relative to the others has been determined. This information is the first step required for the control of the thalassemias through an integrated program of carrier screening, genetic counseling, and prenatal diagnosis.

### 71.9.3 Molecular Basis of $\alpha$ -Thalassemia

Both the  $\alpha$ - and the  $\beta$ -thalassemia syndromes provide examples of defects at essentially all different steps of globin chain biosynthesis.  $\alpha$ -Thalassemia is characterized by a deficiency of  $\alpha$ -globin chain synthesis and defective gene expression may occur in either one globin gene (called  $\alpha$ -2 or  $\alpha^+$ -thalassemia) or in both (called  $\alpha$ -1 or  $\alpha^0$ -thalassemia). Most of the common  $\alpha$ -thalassemia alleles result from the deletion of gene sequences in the  $\alpha$ -globin gene cluster, as depicted in Figure 71-9. An updated listing of all  $\alpha$ -thalassemia alleles is available on the Internet through the HbVar globin gene server Web site (4) (<http://globin.cse.psu.edu>).

**71.9.3.1  $\alpha^+$ -Thalassemia.**  $\alpha^+$ -Thalassemia is most commonly caused by the deletion of one of the two  $\alpha$ -globin genes. Although five different deletions have been identified, only two are commonly encountered in practice. These are the 3.7 kb deletion ( $-\alpha^{3.7}$ ), which has reached high frequencies in the populations of Africa, the Mediterranean area, the Middle East, the Indian subcontinent and Melanesia, and the 4.2 kb deletion ( $-\alpha^{4.2}$ ), which is commonly found in Southeast Asian and Pacific populations. These deletions were created by unequal crossing over between homologous sequences in the  $\alpha$ -globin gene cluster, resulting in one chromosome with only one  $\alpha$ -gene ( $-\alpha$ ) and the other chromosome with three  $\alpha$ -genes ( $\alpha\alpha\alpha$ ). Further recombination events between the resulting chromosomes have given rise to a very rare quadruplicated  $\alpha$ -gene allele ( $\alpha\alpha\alpha\alpha$ ). Individuals with five or six  $\alpha$ -globin genes are hematologically normal. However, the excess  $\alpha$ -globin chains may be sufficient to cause thalassemia intermedia when these alleles are co-inherited with the  $\beta$ -thalassemia trait.

Various nondeletion defects have also been found to cause  $\alpha^+$ -thalassemia and a total of 93 mutations have been described to date (Table 71-7), mostly in populations from the Mediterranean area, Africa, and Southeast Asia. Although nondeletion mutations are generally much less frequent than deletion mutations, among Southeast Asians, the nondeletion  $\alpha^+$ -thalassemia allele for Hb Constant Spring gene has reached a reasonably high gene frequency. This nondeletion  $\alpha^+$ -thalassemia gene encodes an abnormal  $\alpha$ -chain that has 31 additional amino acids at the COOH-terminal end and is synthesized at about 3% of the rate of normal  $\alpha$ -chains.

The different types of nondeletion mutations are similar to those described for  $\beta$ -thalassemia. There are ones that affect RNA splicing, the poly A signal and the

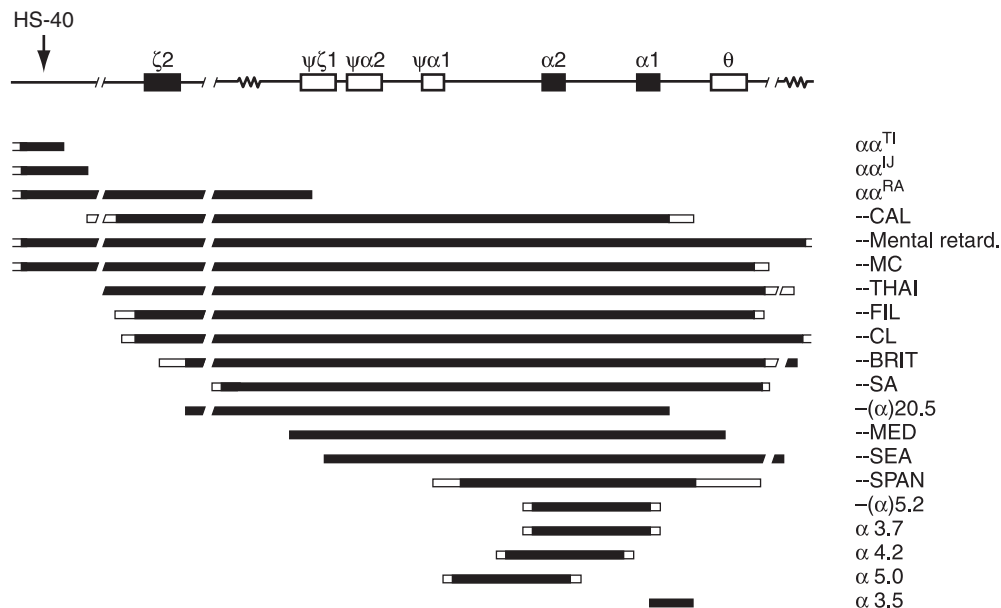


FIGURE 71-9 Deletions in the  $\alpha$ -globin gene cluster.



TABLE 71-7 Point Mutations Causing  $\alpha$ -Thalassemia

Mutant Class	HGVS	Hb Name	Phenotype	Origin
1. Transcriptional mutations				
<i>Promoter regulatory elements</i>				
$\alpha 1$ CAP+22 T→C	HBA1:c.-16T>C		$\alpha^+$	Italian
2. RNA processing mutations				
<i>Splice site mutations: donor</i>				
$\alpha 2$ IVS I (−5 bp) GAGGT-GAGG→GAGG-----	HBA2:c.95+2_95+6delTGAGG		$\alpha^+$	Mediterranean, Middle East
$\alpha 1$ IVS I-1 G→A	HBA1:c.95+1G>A		$\alpha^+$	Thai
$\alpha 2$ IVS II-2T→A	HBA2:c.300+2T>A		$\alpha^+$	North European
<i>Splice site mutations: acceptor</i>				
$\alpha 2$ IVS I-116 A→G	HBA2:c.96-2A>G		$\alpha^+$	North European
$\alpha 1$ IVS I-117 G→A	HBA1:c.96-1G>A		$\alpha^+$	Indian
$\alpha 1$ IVS II-148 A→G	HBA2:c.301-2A>G		$\alpha^+$	Iranian
$\alpha 2$ IVS II-149 G→A	HBA2:c.301-1G>A		$\alpha^+$	Argentinian
<i>Splice site mutations: consensus sequence</i>				
$\alpha 2$ IVS I-5 G→A	HBA2:c.95+5G>A		$\alpha^+$	Ashkanazi Jews
$\alpha 1$ IVS I-5 G→A	HBA1:c.95+5G>A		$\alpha^+$	African
<i>Creation of alternative splice site in exon</i>				
$\alpha 2$ Cd 22 C→T	HBA2:c.69C>T		$\alpha^+$	Surinamese
<i>Polyadenylation signal mutations</i>				
$\alpha 2$ AATAAA→AATAAG	HBA2:c.*+94A>G		$\alpha^+-\alpha^0$	Middle East, Mediterranean
$\alpha 2$ AATAAA→AATGAA	HBA2:c.*+92A>G		$\alpha^+-\alpha^0$	Mediterranean
$\alpha 2$ AATAAA→AATAAC	HBA2:c.*+94A>C		$\alpha^+-\alpha^0$	Surinam
$\alpha 2$ AATAAA→AATA--	HBA2:c.*+93_*+94delAA		$\alpha^+-\alpha^0$	Indian
$\alpha 2$ Poly A (−16 bp) (giving rise to CATAAA)	HBA2:c.*+74_*+89delCCCTTCCTGGTCTTTGA		$\alpha^+-\alpha^0$	Arabian
3. RNA translation mutations				
<i>Initiation codon mutations</i>				
$\alpha 1$ ATG→GTG	HBA1:c.1A>G		$\alpha^+$	Mediterranean
$\alpha 2$ ATG→ACG	HBA2:c.2T>C		$\alpha^+$	Mediterranean
$\alpha 2$ ATG→-TG	HBA2:c.1delA		$\alpha^+$	Southeast Asian
$\alpha 2$ ATG→A-G	HBA2:c.2delT		$\alpha^+$	Vietnamese
$\alpha 1$ ATG→GTG	HBA1:c.1A>G		$\alpha^+$	Mediterranean
$-\alpha^{3.7}$ ATG→GTG	HBA2:c.1A>G		$\alpha^0$	African-American
$-\alpha^{3.7}$ ACCATG→--CATG	HBA2:c.-2_-3delAC		$\alpha^+-\alpha^0$	North African, Mediterranean
<i>Termination codon mutations</i>				
$\alpha 2$ Cd 142 TAA→CAA	HBA2:c.427T>C	Hb Constant Spring	$\alpha^+$	Southeast Asian
$\alpha 2$ Cd 142 TAA→AAA	HBA2:c.427T>A	Hb Icaria	$\alpha^+$	Mediterranean
$\alpha 2$ Cd 142 TAA→TCA	HBA2:c.428A>C	Hb Koya Dora	$\alpha^+$	Indian
$\alpha 2$ Cd 142 TAA→GAA	HBA2:c.427T>G	Hb Seal Rock	$\alpha^+$	African-American
$\alpha 2$ Cd 142 TAA→TAT	HBA2:c.429A>T	Hb Paksé	$\alpha^+$	Laotian
<i>Frameshift mutations</i>				
$\alpha 2$ Cd 19 (−G)	HBA2:c.56delG		$\alpha^+$	Iranian
$\alpha 2$ Cd 22 (−C)	HBA2:c.69delC		$\alpha^+$	Portuguese
$-\alpha^{3.7}$ Cd 30–31 GAGAGG→GAG--G	HBA1:c.92_93delAG		$\alpha^0$	African-American
$\alpha 2$ Cd 39-41 (−9 bp, +8 bp) duplication	HBA1:c.108_146del9;ins8		$\alpha^+$	Yemenite-Jewish
$\alpha 2$ Cd 49 (−GC)	HBA2:c.149_150delGC		$\alpha^+$	Unknown
$\alpha 1$ Cd 51-55 (−13 bp)	HBA1:c.155_167delGCTCTGCCAGGT		$\alpha^+$	Spanish
$\alpha 1$ Cd 62 GTG→(−TG)	HBA1:c.187delG		$\alpha^+$	African-American
$\alpha 1$ Cd 78 (−C)			$\alpha^+$	African-American
$\alpha 1$ Cd 132 (+T)→75aa	HBA1:c.396_397insT	Hb Pak Num Po	$\alpha^+$	Thai
<i>Nonsense mutations</i>				
$\alpha 1$ Cd 14 TGG→TAG	HBA1:c.44A>G		$\alpha^+$	Iranian
$\alpha 2$ Cd 23 GAG→TAG	HBA2:c.70G>T		$\alpha^+$	Tunisian
$\alpha 2$ Cd 54 CAG→TAG	HBA2:c.163C>T		$\alpha^+$	Unknown

Continued

**TABLE 71-7 Point Mutations Causing  $\alpha$ -Thalassemia—cont'd**

Mutant Class	HGVS	Hb Name	Phenotype	Origin
$\alpha 2$ Cd 90 AAG $\rightarrow$ TAG	HBA2:c.271A>T		$\alpha^+$	Middle Eastern
$\alpha 2$ Cd 116 GAG $\rightarrow$ TAG	HBA2:c.349G>T		$\alpha^+$	African-American
4. Mutations causing posttranslational instability				
<i>Unstable <math>\alpha</math> chain variants due to point mutations</i>				
$\alpha 1$ Cd 14 TGG $\rightarrow$ CGG [Trp $\rightarrow$ Arg]	HBA1:c. 43T>C	Hb Evanston	$\alpha^+$	Indian
$-\alpha^{3.7}$ Cd 14 TGG $\rightarrow$ CGG [Trp $\rightarrow$ Arg]	HBA1:c. 43T>C	Hb Evanston	$\alpha^0$	Asian Indian, African-American
$-\alpha^{4.2}$ Cd 14 TGG $\rightarrow$ CGG [Trp $\rightarrow$ Arg]	HBA1:c. 43T>C	Hb Evanston	$\alpha^0$	Asian Indian
$\alpha 2$ Cd 21 GCT $\rightarrow$ TCT [Ala $\rightarrow$ Ser]	HBA2:c.64G>T	Hb Zoetermeer	$\alpha^+$	Dutch
$\alpha 2$ Cd 26 GCG $\rightarrow$ ACG [Ala $\rightarrow$ Thr]	HBA2:c.79G>A	Hb Caserta	$\alpha^+$	Italian
$\alpha 2$ Cd 29 CTG $\rightarrow$ CCG [Leu $\rightarrow$ Pro]	HBA2:c.89T>C	Hb Agrinio	$\alpha^+$	Greek
$\alpha 2$ Cd 32 ATG $\rightarrow$ ATA [Met $\rightarrow$ Ile]	HBA2:c.99G>A	Hb Amsterdam	$\alpha^+$	Surinamese
$\alpha 1$ or $\alpha 2$ Cd 35 (TCC $\rightarrow$ CCC) [Ser $\rightarrow$ Pro]	HBA1 or HBA2::c.106T>C	Hb Evora	$\alpha^+$	Filipino
$\alpha 2$ Cd 42 TAC $\rightarrow$ CAC [Tyr $\rightarrow$ His]	HBA2:c.127T>C	Hb Barika	$\alpha^+$	Unknown
$\alpha 2$ Cd 59 GGC $\rightarrow$ GAC [Gly $\rightarrow$ Asp]	HBA2:c.179G>A	Hb Adana	$\alpha^+$	Southeast Asian
$\alpha 1$ Cd 59 GGC $\rightarrow$ GAC [Gly $\rightarrow$ Asp]	HBA2:c.179G>A	Hb Adana	$\alpha^+$	Mediterranean
$\alpha 2$ Cd 59 (GGC $\rightarrow$ CGC) [Gly $\rightarrow$ Arg]	HBA2:c.178G>C	Hb Zurich Albisrieden	$\alpha^+$	Unknown
$\alpha 2$ Cd 66 CTG $\rightarrow$ CCG [Leu $\rightarrow$ Pro]	HBA2:c.190T>C	Hb Portsmouth	$\alpha^+$	Cambodian
$\alpha 2$ Cd 93 GTG $\rightarrow$ GGG [Val $\rightarrow$ Gly]	HBA2:c.281T>G	Hb Bronte	$\alpha^+$	Italian
$\alpha 2$ Cd 103 CAC $\rightarrow$ CTC [His $\rightarrow$ Leu]	HBA2:c.311A>T	Hb Bronovo	$\alpha^+$	Turkish
$\alpha 1$ Cd 104 TGC $\rightarrow$ AGC [Cys $\rightarrow$ Ser]	HBA1:c.313T>A	Hb Oegstgeest	$\alpha^+$	Surinamese
$\alpha 2$ Cd 104 TGC $\rightarrow$ TAC [Cys $\rightarrow$ Tyr]	HBA2:c.314G>A	Hb Sallanches	$\alpha^+$	Mediterranean
$\alpha 2$ Cd 108 ACC $\rightarrow$ AAC [Thr $\rightarrow$ Asn]	HBA2:c.327C>A	Hb Bleuland	$\alpha^+$	Surinamese
$\alpha 2$ Cd 109 CTG $\rightarrow$ CGG [Leu $\rightarrow$ Arg]	HBA2:c.329T>G	Hb Suan Dok	$\alpha^+$	Southeast Asian
$-\alpha^{3.7}$ Cd 109 CTG $\rightarrow$ CGG [Leu $\rightarrow$ Arg]	HBA2:c.329T>G	Hb Suan Dok	$\alpha^0$	Unknown
$\alpha 1$ or $\alpha 2$ Cd 110 GCC $\rightarrow$ GAC [Ala $\rightarrow$ Asp]	HBA1 or HBA2:c.332C>A	Hb Petah Tikva	$\alpha^+$	Iraqi-Jewish
$\alpha 2$ Cd 125 CTG $\rightarrow$ CCG [Leu $\rightarrow$ Pro]	HBA2:c.377T>C	Hb Quong Sze	$\alpha^+$	Southeast Asian
$\alpha 2$ Cd 117 TTC $\rightarrow$ TCC [Phe $\rightarrow$ Ser]	HBA2:c.353T>C	Hb Foggia	$\alpha^+$	Italian
$\alpha 1$ Cd 119 CCT $\rightarrow$ TCT [Pro $\rightarrow$ Ser]	HBA1:c.358C>T	Hb Groene Hart	$\alpha^+$	Moroccan
$\alpha 1$ Cd 123 GCC $\rightarrow$ CCC [Ala $\rightarrow$ Pro]	HBA1:c.370G>C	Hb Voreppe	$\alpha^+$	French
$\alpha 2$ Cd 125 CTG $\rightarrow$ CGG [Leu $\rightarrow$ Arg]	HBA2:c.377T>G	Hb Plasencia	$\alpha^+$	Spanish
$-\alpha^{3.7}$ Cd 125 CTG $\rightarrow$ CAG [Leu $\rightarrow$ Gln]	HBA2:c.377T>A	Hb Weste-Einde	$\alpha^0$	Kurdish-Jewish

TABLE 71-7 Point Mutations Causing  $\alpha$ -Thalassemia—cont'd

Mutant Class	HGVS	Hb Name	Phenotype	Origin
$\alpha 2$ Cd 129 CTG→CCG [Leu→Pro]	HBA2:c.389T>C	Hb Utrecht	$\alpha^+$	Unknown
$\alpha 1$ Cd 129 CTG→CCG [Leu→Pro]	HBA1:c.391T>C	Hb Tunis-Bizerte	$\alpha^+$	North African
$\alpha 2$ Cd 130 GCT→CCT [Ala→Pro]	HBA2:c.389G>C	Hb Sun Prairie	$\alpha^+$	Indian-Pakistani
$\alpha 2$ Cd 131 TCT→CCT [Ser→Pro]	HBA2:c.394T>C	Hb Questembert	$\alpha^+$	Yugoslavian
$\alpha 1$ or $\alpha 2$ Cd 132 GTG→GGG [Val→Gly]	HBA1 or HBA2:c.398T>G	Hb Caen	$\alpha^+$	French
$\alpha 2$ Cd 136 CTG→CCG [Leu→Pro]	HBA2:c.410T>C	Hb Bibba	$\alpha^+$	Caucasian
Unstable $\alpha$ chain variants due to small deletions or insertions				
$\alpha 2$ Cd 30 (–GAG) [–Glu]	HBA2:c.91_93delGAG		$\alpha^+$	Southeast Asian
$\alpha 1$ Cd 37 (–CCC) [–Pro]	HBA1:c.112_114delCCC	Hb Heraklion	$\alpha^+$	Greek
$\alpha 1$ Cd 38/39 (–ACC) [–Thr]	HBA1:c.118_120delACC	Hb Taybe	$\alpha^+$	Arabian
$\alpha 1$ Cd 60–61 (–AAG) [–Lys]	HBA1:c.184_186delAAG	Hb Clinic	$\alpha^+$	Spanish
$\alpha 1$ Cd 62 (–GTG) [–Val]	HBA1:c.187_189delGTG	Hb Aghia Sophia	$\alpha^+$	Greek
$\alpha 1$ Cd 64–74 (–33 bp)	HBA1:c.183_215del33		$\alpha^+$	Unknown
$\alpha 1$ Cd 74/75 (–GAC) [–Asp]	HBA1:c.212_214delGAC		$\alpha^+$	Mexican
$\alpha 1$ Cd 93–99 (+21 bp)	HBA1:c.280_300ins21		$\alpha^+$	Unknown
$\alpha 2$ Cd 113–116 (–12 bp)	HBA2:c.[339C>G;340_351delCTCCCCGCCGAG]	Hb Lleida	$\alpha^+$	Spanish

initiation or termination of mRNA translation; ones that cause frameshift and nonsense mutations; and ones that cause posttranslational instability. Most mutations are located in the dominant  $\alpha 2$ -globin gene and these are denoted as  $\alpha^T\alpha$  (signifying the affected gene). As there appears to be no associated compensatory change in the expression of the remaining functional  $\alpha 1$ -globin gene (as occurs with the  $-\alpha$  deletion alleles) they give rise to a slightly more severe reduction in  $\alpha$ -chain synthesis than the deletional forms of  $\alpha^+$ -thalassemia. Some have a more severe phenotype than expected, for example, the poly A (A→G) mutation and the  $-\alpha^{3.7}$  (–AC) mutation, which result in Hb H disease in the homozygous state (14), and some mutations that cause highly unstable variants that also have secondary effects on the red cell structure. The interaction of these severe nondeletional mutations with  $\alpha^0$ -thalassemia may result in a severe form of Hb H disease (15) or, very rarely, Hb Bart's hydrops fetalis syndrome in infants with a very low level of  $\alpha$ -globin chain synthesis (16).

**71.9.3.2  $\alpha^0$ -Thalassemia.**  $\alpha^0$ -Thalassemia results from deletions that involve both  $\alpha$ -globin genes in the  $\alpha$ -globin gene cluster. At least 34 different such deletions have been described. The deletions that have attained the highest gene frequencies are found in individuals from Southeast Asia and South China (–<sup>SEA</sup>), the Philippine Islands (–<sup>FIL</sup>), Thailand (–<sup>THAI</sup>), and a few Mediterranean countries such as Greece and Cyprus (–<sup>MED</sup> and  $-(\alpha)^{20.5}$ ). Although one  $\alpha^0$ -thalassemia mutation (–<sup>SA</sup>) has been described in Asian Indians it is extremely uncommon in this ethnic group. No  $\alpha^0$ -thalassemia deletions have

been reported in individuals from sub-Saharan Africa, although two very rare  $\alpha^0$ -thalassemia alleles involving the combination of the  $-\alpha^{3.7}$  deletion and a nondeletion mutation (e.g. the  $-\alpha^{3.7}$  CD 30–31 (–AG) allele) have been described. In Northern Europe  $\alpha$ -thalassemia only occurs sporadically because of the lack of natural selection, although one particular  $\alpha^0$ -thalassemia mutation (–<sup>BRIT</sup>) has been reported in a number of British families. Finally, in extremely rare instances,  $\alpha^0$ -thalassemia can also result from deletions that remove the  $\alpha$ -globin gene regulatory element about 62 kb upstream of the  $\alpha$ -globin gene complex but leave the  $\alpha$ -globin genes intact.

#### 71.9.4 Molecular Basis of $\beta$ -Thalassemia

$\beta$ -Thalassemia is caused by at least 200 different point mutations or small insertions/deletions of DNA sequence in and around the  $\beta$ -globin gene (Table 71-8), together with a much smaller number of gene deletions ranging from 25 bp to 67 kb. An updated listing of all  $\beta$ -thalassemia alleles is available on the Internet through the HbVar Web site (4). Although more than 230 different  $\beta$ -thalassemia alleles have been characterized, only approximately 30 mutations are found in at-risk groups at a frequency of 1% or greater and thus just a small number account for the majority of the mutations worldwide. All mutations are regionally specific and the spectrum of mutations has now been determined for most at-risk populations (17).

The mutations either reduce the expression of the  $\beta$ -globin gene ( $\beta^+$ -type) or result in the complete absence

TABLE 71-8 Point Mutations Causing  $\beta$ -Thalassemia

Mutations Class	HGVS	Hb Name	Phenotype	Origin
(1) Transcriptional mutations				
<i>Promoter regulatory elements</i>				
-102 C→A	c.-152C→A		$\beta^{++}$ (silent)	Unknown
-101 C→T	c.-151C→T		$\beta^{++}$ (silent)	Mediterranean
-101 C→G	c.-151C→G		$\beta^{++}$ (silent)	Italian
-93 C→G	c.-143C→G		$\beta^{+}$	Surinam
-92 C→T	c.-142C→T		$\beta^{++}$ (silent)	Mediterranean
-90 C→T	c.-140C→T		$\beta^{+}$	Portuguese
-88 C→T	c.-138C→T		$\beta^{++}$	African-American, Asian Indian
-88 C→A	c.-138C→A		$\beta^{+}$	Kurds
-87 C→G	c.-137C→G		$\beta^{++}$	Mediterranean
-87 C→T	c.-137C→T		$\beta^{++}$	German-Italian
-87 C→A	c.-137C→A		$\beta^{++}$	African-American, Yugoslavian
-86 C→G	c.-136C→G		$\beta^{+}$	Thai, Lebanese
-86 C→A	c.-136C→A		$\beta^{++}$	Italians
-73 C→A	c.-123C→A		$\beta^{++}$	Chinese
-56 G→C	c.-106G→C		?	Moroccan. Algerian
-50 G→A	c.-100G→A		?	Chinese
-32 C→A	c.-82C→A		$\beta^{+}$	Taiwanese
-32 C→T	c.-82C→T		$\beta^{+}$	Unknown
-31 A→G	c.-81A→G		$\beta^{+}$	Japanese
-31 A→C	c.-81A→C		$\beta^{+}$	Italian
-30 T→A	c.-80T→A		$\beta^{+}$	Mediterranean, Bulgarian
-30T→C	c.-80A→C		$\beta^{+}$	Chinese
-29 to -26 (-AA)	c.-79_-78delAA		$\beta^{+}$	African-American
-29 A→G	c.-79A→G		$\beta^{+}$	African-American, Chinese
-29 A→C	c.-79A→C		$\beta^{+}$	Jordanian
-29 G→A	c.-79 G→A		$\beta^{+}$	Turkish
-28 A→C	c.-78A→C		$\beta^{+}$	Kurds
-28 A→G	c.-78A→G		$\beta^{+}$	Blacks, Southeast Asians
-27 A→T	c.-77A→T		$\beta^{+}$	Corsican
-27 (-AA)	c.-77_-76delAA		?	Unknown
-25 G→C	c.-75G→C		$\beta^{+}$	Unknown
-23 to +23 (+45 bp duplication)	c.-73_-8del45		$\beta^{+}$	Maori
<i>5'-UTR mutations</i>				
CAP+1 A→C	c.-50A→C		$\beta^{++}$ (silent)	Asian Indian
CAP+8 C→T	c.-43G→A		$\beta^{++}$ (silent)	Chinese
CAP+10 (-T)	c.-41G→A		$\beta^{++}$ (silent)	Greeks
CAP+20 C→T	c.-31G→A		$\beta^{++}$	Bulgarian
CAP+22 G→A	c.-29G→A		$\beta^{++}$	Mediterranean, Bulgarian, Turkish
CAP+33 C→G	c.-18C→G		$\beta^{++}$ (silent)	Greek Cypriot
CAP+40 to +43 (-AAAC)	c.-11_-7delAAAC		$\beta^{+}$	Chinese
CAP+45 G→C	c.-3G→C		$\beta^{++}$ (silent)	Italian
(2) RNA Processing				
<i>Splice site mutations: donor</i>				
IVS I-1 G→A	c.92+1G→A		$\beta^0$	Mediterranean
IVS I-1 G→T	c.92+1G→T		$\beta^0$	Asian Indian, Southeast Asian, Chinese
IVS I-1 G→C	c.92+1G→C		$\beta^0$	UAE
IVS I-2 T→G	c.92+2T→G		$\beta^0$	Tunisian
IVS I-2 T→C	c.92+2T→C		$\beta^0$	African-American
IVS I-2 T→A	c.92+2T→A		$\beta^0$	Algerian, Italian
IVS II-1 G→A	c.315+1G→A		$\beta^0$	Mediterranean, African-American
IVS II-1 G→C	c.315+1G→C		$\beta^0$	Iranian



TABLE 71-8 Point Mutations Causing  $\beta$ -Thalassemia—cont'd

Mutations Class	HGVS	Hb Name	Phenotype	Origin
IVS II-2 T→A	c.315+2T→A		$\beta^+$	Turkish
IVS II-2 (–T)	c.315+2del		$\beta^0$	Chinese
IVS II-2,3 (+11, –2 bp)	c.315+2_315+delins ACGTTCTCTGA		$\beta^0$	Iranian
<i>Splice site mutations: acceptor</i>				
IVS I [3' end] (–17 bp)	c.93-17_93-1delTATTTT CCCACCCTTAG		$\beta^0$	Kuwaiti
IVS I [3' end] (–25 bp)	c.93-21_96del		$\beta^0$	Asian Indian, UAE
IVS I [3' end] (–44 bp)	c.76_92+27del		$\beta^0$	Mediterranean
IVS I [3' end] (+22 bp)			$\beta^0$	Thai
IVS I-129 A→G	c.93-2A→C		?	German
IVS I-129 A→G	c.93-2A→G		$\beta^0$	German
IVS I-130 G→C	c.93-1 G→C		$\beta^0$	Italian, Japanese
IVS I-130 G→A	c.93-1 G→A		$\beta^0$	Egyptian
IVS II-849 A→G	c.316-2A→G		$\beta^0$	African-American
IVS II-849 A→C	c.316-2A→C		$\beta^0$	African-American
IVS II-850 G→C	c.316-1 G→C		$\beta^0$	Yugoslavian
IVS II-850 G→A	c.316-1 G→A		$\beta^0$	English-Scottish
IVS II-850 G→T	c.316-1 G→T		$\beta^0$	Japanese
IVS II-850 (–G)	c.316-1delG		$\beta^0$	Italian
<i>Splice site consensus sequences</i>				
IVS I (–3) or CD 29, C→T; GGC→GGT	c.90C→T		$\beta^+$	Lebanese
IVS I (–2) or CD 30, A→G; AGG→GGG	c.91A→G		$\beta^0$	Sephardic Jews
IVS I (–2) or CD 30, A→C; AGG→CGG	c.91A→C		$\beta^0$	
IVS I (–1) or CD 30, G→C; AGG→ACG [Arg→Thr]	c.92 G→C	Hb Monroe	$\beta^0$	Mediterranean, African-American
IVS I (–1) or CD 30, G→A; AGG→AAG	c.92 G→A		$\beta^0$	Bulgarian
IVS I-5 G→C	c.92+5 G→C		$\beta^+$	Asian Indian, Southeast Asian, Melanesian
IVS I-5 G→T	c.92+5 G→T		$\beta^+$	Mediterranean, North European
IVS I-5 G→A	c.92+5 G→A		$\beta^+$	Mediterranean, Algerian
IVS I-6T→C	c.92+6T→C		$\beta^{++}$	Mediterranean
IVS I-7 A→T	c.92+7A→T		$\beta^+$	Italian
IVS I-128 T→G	c.93-3 T→G		$\beta^+$	Saudi Arabian
IVS I-130 (+1) or CD 30 G→C; AGG→AGC	c.93 G→C		$\beta^0$	Middle East
IVS II-4,5 (–AG)	c.315+4_315delAG		$\beta^0$	Portuguese
IVS II-5 G→C	c.315+5 G→C		$\beta^+$	Chinese
IVS II-843 T→G	c.316-8 T→G		$\beta^+$	Algerian
IVS II-844 C→G	c.316-7C→A		$\beta^{++}$ (silent)	Italian
IVS II-844 C→A	c.316-7C→A		$\beta^{++}$ (silent)	Ghanaian
IVS II-848 C→A	c.316-3C→A		$\beta^+$	African-American, Egyptian, Iranian
IVS II-848 C→G	c.316-3C→G		$\beta^+$	Japanese
<i>Creation of alternative splice site in intron</i>				
IVS I-110 G→A	c.316-3C→A		$\beta^+$	Mediterranean
IVS I-116T→G	c.316-3C→G		$\beta^0$	Mediterranean
IVS II-654 C→T	c.316-197C→T		$\beta^0/\beta^+$	Chinese, Southeast Asian, Japanese
IVS II-705 T→G	c.316-146 T→G		$\beta^+$	Mediterranean
IVS II-726 A→G	c.316-125A→G		?	Moroccan
IVS II-745 C→G	c.316-106C→G		$\beta^+$	Mediterranean

Continued

TABLE 71-8 Point Mutations Causing  $\beta$ -Thalassemia—cont'd

Mutations Class	HGVS	Hb Name	Phenotype	Origin
IVS II-815 C→T	c.316-36C→T		?	Thai
IVS II-837 T→G	c.316-14 T→G		$\beta^0/\beta^+$	Asian Indian
<i>Creation of alternative splice site in exon</i>				
CD 10 GCC→GCA	c.33C→A		$\beta^+$	Asian Indian
CD 19 AAC→AGC [Asn→Ser]	c.59A→G	Hb Malay	$\beta^{++}$	Southeast Asian
CD 24 GGT→GGA	c.75 T→A		$\beta^{++}$	African-American, Japanese
CD 26 GAG→AAG [Glu→Lys]	c.79 G→A	Hb E	$\beta^+$	Southeast Asian, European
CD 27 GCC→TCC [Ala→Ser]	c.82 G→T	Hb Knossos	$\beta^+$	Mediterranean
<i>3'-UTR: RNA cleavage &amp; polyadenylation</i>				
Poly A (A→C) AATAAA→CATAAA	c.*+108A→C		$\beta^{++}$	Chinese
Poly A (A→G) AATAAA→GATAAA	c.*+108A→G		$\beta^+$	Czechoslovakian
Poly A (T→C) AATAAA→AACAAA	c.*+110 T→C		$\beta^{++}$	African-American
Poly A (T→A) AATAAA→AAAAAA	c.*+110A→C		$\beta^{++}$	Tunisian
Poly A (T→G) AATAAA→AATGAA	c.*+111A→G		$\beta^{++}$	Mediterranean
Poly A (A→G) AATAAA→AATAGA	c.*+112A→G		$\beta^{++}$	Malay
Poly A (A→G) AATAAA→AATAAG	c.*+113A→G		$\beta^{++}$	Kurd
Poly A (–AT) AATAAA→A–AAA	c.[*+109_*+110delAT or *+110_*+111delTA]		$\beta^+$	French, African-American
Poly A (–AATAA) AATAAA→-----A	c.*+108_*+112delAATAA		$\beta^+$	Kurd, UAE
Poly A (–AATAAA) AATAAA→-----	c.*+108_*+112delAATAAA		$\beta^+$	Nigerian
Cap site+1480 (or term CD+6) C→G	c.*+6C→G		$\beta^{++}$ (silent)	Greek
Cap site+1522 (or term CD+47) C→G	c.*+47C→G		$\beta^{++}$	Armenian
Cap site+1565 (or term CD+90) (–13 bp)	c.*+91_*+103del GCATCTGGATTCT		$\beta^+$	Turkish
<i>(3) RNA translation</i>				
<i>Initiation codon</i>				
ATG→GTG	c.1A→G		$\beta^0$	Japanese
ATG→CTG	c.1A→C		$\beta^0$	British
ATG→ACG	c.2T→C		$\beta^0$	Yugoslavian
ATG→AGG	c.2T→G		$\beta^0$	Chinese
ATG→AAG	c.2T→A		$\beta^0$	North European
ATG→ATC	c.3G→C		$\beta^0$	Japanese
ATG→ATA	c.3G→A		$\beta^0$	Italian, Swedish
ATG→ATT	c.3G→T		$\beta^0$	Iranian
<i>Nonsense codon</i>				
CD 6 GAG→TAG, & CD 4 ACT→ACA, CD 5 CCA→TCA	c.15[A→T;c.16C→T;c.19 G→T]		$\beta^0$	Italian
CD 6 GAG→TAG	c.19 G→T		$\beta^0$	Brazilian
CD 7 GAG→TAG	c.22 G→T		$\beta^0$	English
CD 15 TGG→TAG	c.47 G→A		$\beta^0$	Asian Indian, Japanese
CD 15 TGG→TGA	c.48 G→A		$\beta^0$	Portuguese, Japanese
CD 17 AAG→TAG	c.52A→T		$\beta^0$	Chinese, Japanese
CD 22 GAA→TAA	c.67 G→T		$\beta^0$	Reunion Island
CD 26 GAG→TAG	c.79 G→T		$\beta^0$	Thai
CD 35 TAC→TGA	c.107 A→G		$\beta^0$	
CD 35 TAC→TAA	c.108C→A		$\beta^0$	Thai
CD 37 TGG→TAG	c.113 G→A		$\beta^0$	Afghani, Chinese
CD 37 TGG→TGA	c.114 G→A		$\beta^0$	Saudi Arabian
CD 39 CAG→TAG	c.118C→T		$\beta^0$	Mediterranean
CD 43 GAG→TAG	c.130 G→T		$\beta^0$	Chinese, Thai
CD 59 AAG→TAG	c.178A→T		$\beta^0$	
CD 61 AAG→TAG	c.184A→T		$\beta^0$	African-American
CD 66 AAA→TAA	c.199A→T		$\beta^0$	
CD 90 GAG→TAG	c.271 G→T		$\beta^0$	Japanese
CD 112 TGT→TGA	c.339 T→A		$\beta^0$	Slovenian

TABLE 71-8 Point Mutations Causing  $\beta$ -Thalassemia—cont'd

Mutations Class	HGVS	Hb Name	Phenotype	Origin
CD 121 GAA→TAA	c.364 G→T		$\beta^0$	Czechoslovakian
CD 127 CAG→TAG	c.382C→T		Dominant	English
CD 131 CAG→TAG	c.394C→T		$\beta^0$	Korean
CD 132 AAA→TAA	c.397A→T		$\beta^0$	African
<i>Frameshift</i>				
CD 1 (−G)	c.4delG		$\beta^0$	Mediterranean
CD 2-4 (−9 bp, +31 bp)	c.7_15delinsCCTGAGGTGAAGT CTGCCTGAGGAGAAGTCT		$\beta^0$	Algerian
CD 5 (−CT)	c.17_18delCT		$\beta^0$	Mediterranean
CD 6 (−A)	c.20delA		$\beta^0$	Mediterranean, African-American
CD 6 (−G)	c.20delG		$\beta^0$	German
CD 6-10 (−13 bp)	c.20_32del13		$\beta^0$	Sri Lankan
CD 8 (−AA)	c.25_26delAA		$\beta^0$	Mediterranean, UK
CD 8/9 (+G)	c.27_28insG		$\beta^0$	Asian Indian, Japanese
CD 9 (+TA)	c.28_29insTA		$\beta^0$	Tunisian
CD 9/10 (+T)	c.30_31insT		$\beta^0$	Arab
CD 11 (−T)	c.36delT		$\beta^0$	Mexican
CD 14 (+T)	c.44_45insT		$\beta^0$	Azerbaijan
CD 14/15 (+G)	c.45_46insG		$\beta^0$	Chinese
CD 15 (−T)	c.46delT		$\beta^0$	Asian Indian
CD 15/16 (+G)	c.48_49insG		$\beta^0$	Chinese
CD 15/16 (−G)	c.49delG		$\beta^0$	German
CD 16 (−C)	c.51delC		$\beta^0$	Asian Indian
CD 16 T→C	c.51 C→T		$\beta^0$	Asian Indian
CD 18 (+A)			$\beta^0$	Italian
CD 20/21 (−TGGA)	c.62_65delTGGA		?	Spanish
CD 20/21 (+G)	c.63_64insG		$\beta^0$	Ashkenazi Jewish
CD 22-24 (−8 bp) (−AAGTTGG)	c.68_74delAAGTTGG		$\beta^0$	Turkish
CD 24 (−G, +CAC)	c.74delinsCAC		$\beta^0$	Egyptian
CD 25/26 (+T)	c.78_79insT		$\beta^0$	Tunisian
CD 26 (+T)	c.79_80insT		$\beta^0$	Japanese
CD 27/28 (+C)	c.84_85insC		$\beta^0$	Chinese, Thai
CD 28 (−C)	c.85delC		$\beta^0$	Egyptian
CD 28/29 (−G)	c.88delG		$\beta^0$	Japanese, Egyptian
CD 31 (−C)	c.94delC		$\beta^0$	Chinese
CD 33-34 (−G)	c.102_103delG		$\beta^0$	Thai
CD 35 (−C)	c.108del		$\beta^0$	Malay
CD 36/37 (−T)	c.112delT		$\beta^0$	Kurdish, Iranian
CD 36-39 (−8 bp)	c.111_118delTTGGACCC		$\beta^0$	Indian
CD 37 (−G)	c.114delG		$\beta^0$	Kurdish
CD 37-39 (−7 bp): (−GACCCAG)	c.114_120delGACCCAG		$\beta^0$	Turkish
CD 38/39 (−C)	c.116delC		$\beta^0$	Czechoslovakian
CD 38/39 (−CC)	c.116_117delCC		$\beta^0$	Belgian
CD 39 (CAG→TAG)	c.118C→T		$\beta^0$	Mediterranean
CD 40 (−G)	c.123delG		$\beta^0$	Japanese
CD 40 (+86 bp)	c.121_122ins86		$\beta^0$	Portuguese
CD 40/41 (+T)	c.123_124insT		$\beta^0$	Chinese
CD 41 (−C)	c.126delC		$\beta^0$	Thai
CD 41/42 (−TTCT)	c.124_127delTTCT		$\beta^0$	Chinese, Southeast Asian, Indian
CD 42/43 (+T)	c.129_130insT		$\beta^0$	Japanese
CD 42/43 (+G)	c.129_130insG		$\beta^0$	Japanese
CD 44 (−C)	c.135delC		$\beta^0$	Kurdish
CD 45 (+T)	c.136_137insT		$\beta^0$	Turkish
CD 45/46 (+A)	c.38_139insA		$\beta^0$	
CD 45 (−T)	c.136delT		$\beta^0$	Pakistani
CD 47 (+A)	c.143_144insA		$\beta^0$	Surinamese
CD 47/48 (+ATCT)	c.146_147insATCT		$\beta^0$	Asian Indian

Continued

TABLE 71-8 Point Mutations Causing  $\beta$ -Thalassemia—cont'd

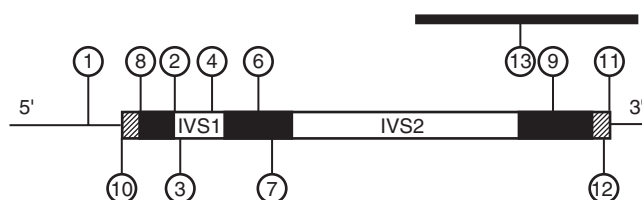
Mutations Class	HGVS	Hb Name	Phenotype	Origin
CD 49 (–C)	c.149delC		$\beta^0$	Jordanian
CD 50 (–T)	c.153delT		$\beta^0$	Unknown
CD 51 (–C)	c.154delC		$\beta^0$	Hungarian
CD 53 (–T)	c.161delT		$\beta^0$	Chinese
CD 53/54 (+G)	c.162_163insG		$\beta^0$	Japanese
CD 54 (–T)	c.165delT		$\beta^0$	Algerian
CD 54/55 (+A)	c.165_166insA		$\beta^0$	Asian Indian
CD 55 (–A)	c.166delA		$\beta^0$	Sri Lankan
CD 56–60 (+14bp)	c.168_181ins14		$\beta^0$	Iranian
CD 57/58 (+C)	c.174_175insC		$\beta^0$	Asian Indian
CD 59 (–A)	c.178delA		$\beta^0$	Italian
CD 61–63 (–7bp): (–GGCTCAT)	c.186_192delGGCTAT		$\beta^0$	Italian
CD 64 (–G)	c.193delG		$\beta^0$	Swiss
CD 67 (–TG)	c.202_203delGT		$\beta^0$	Filipino
CD 71/72 (+T)	c.216_217insT		$\beta^0$	Chinese
CD 71/72 (+A)	c.216_217insA		$\beta^0$	Chinese
CD 72–73 (–5, +1bp): (–AGTGA, +T)	c.217_221delinsT		$\beta^0$	British
CD 74/75 (–C)	c.225delC		$\beta^0$	Turkish
CD 76 (–GC)	c.229_230delGC		$\beta^0$	North African
CD 76 (–C)	c.230delC		$\beta^0$	Italian
CD 77/78 (–C)	c.232delC		$\beta^0$	Mexican
CD 80/81 (–C)	c.243delC		$\beta^0$	Iranian
CD 81–87 (–22bp)	c.244_265del22		$\beta^0$	Indian
CD 82/83 (–G)	c.250delG		$\beta^0$	Czech, Azerbaijan
CD 83–86 (–8bp): (–CACCTTTG)	c.252_259del		$\beta^0$	Japanese
CD 84/85 (+C)	c.255_256insC		$\beta^0$	Japanese
CD 84–86 (+T)	c.258_259insT		$\beta^0$	Japanese
CD 88 (+T)	c.266_267insT		$\beta^0$	Asian Indian
CD 88 (–TG)	c.266_267del		$\beta^0$	Japanese
CD 89/90 (–GT)	c.269_270delGT		$\beta^0$	Japanese
CD 91 (+T)	c.275_276insT		$\beta^0$	Belgian
CD 95 (+A)	c.287_288insA		$\beta^0$	Southeast Asian
CD 104 (–G)	c.314delG		$\beta^0$	German
CD 106/107 (+G)	c.321_322insG		$\beta^0$	African-American, Egyptian
CD 116 (+TGAT)	c.-61837_–61836insTGAT		$\beta^0$	Sri Lankan
CD 131/132 (+GCCT)	c.396_397insGCCT		?	German
CD 142 (–CC)	c.428_429delCC		$\beta^0$	French
Mutations causing posttranslational instability				
<i><math>\beta</math> Chain variants associated with <math>\beta</math>-thalassemia trait</i>				
CD 3 (+T), CD 5 (–C) [Leu-Thr-Pro→Ser-Asp-Ser]	c.[16delC; 9_10insT]	Hb Antalya	$\beta^+$	Turkish
CD 10 (GCC→GCA) [Ala→Ala]	c.33C→A		?	Asian Indian
CD 106 CTG→GTG [Leu→Val]	c.319C→G	Hb L'Aquila	$\beta^+$	Italian
CD 126 GTG→GGG [Val→Gly]	c.380 T→G	Hb Neapolis	$\beta^0$	Italian, German, Thai
<i>Hyperunstable <math>\beta</math> chain variants that result in dominant <math>\beta</math>-thalassemia</i>				
CD 28 CTG→CGG [Leu→Arg]	c.86T→G	Hb Chesterfield	$\beta^+$	English
CD 30/31 (+CGG)	c.93_94insCGG		$\beta^0$	Spanish
CD 32 CTG→CAG [Leu>Glu] in cis with CD 98 GTG→ATG	c.[295 G→A; 98 T→A]	Hb Medicine Lake	$\beta^0$	Caucasian
CD 33–34 (–GGT) [–Val]	c.102_104delGGT	Hb Korea	$\beta^0$	Korean
CD 33–35 (–6bp) [Val.Val.Tyr→Asp]	c.101_106delTGGTCT	Hb Dresden	$\beta^0$	German



TABLE 71-8 Point Mutations Causing  $\beta$ -Thalassemia—cont'd

Mutations Class	HGVS	Hb Name	Phenotype	Origin
CD 53 (–T)	c.161delT		$\beta^0$	Chinese
CD 60 GTG→GAG [Val→Glu]	c.182 T→A	Hb Cagliari	$\beta^+$	Italian
CD 91 (–T)→156aa	c.275del	Hb Morgantown	?	Irish
CD 94 (+TG)→156aa	c.283_284insTG	Hb Agnana	$\beta^0$	Italian
CD 98 GTG→ATG [Val→Met] (Hb Kohn) <i>in cis</i> with CD 32 CTG→CAG.	c.[295 G→A;98 T→A]	Hb Medicine Lake	$\beta^0$	Caucasian
CD 100 (–3, +8) (–CTT, +TCTGAGAACTT) →159aa	c.301_303delinsTCTGAGAACTT		$\beta^0$	South African
IVS II-535 to CD 108 (+23, –310, +28bp)→151aa	c.[316-300_327delinsCAGGTGCCA TCTGTCACCCCTTTTCTTTG;316- 316_316-315insAATATATTTTAA- ATATACTTTTT]	Hb Jambol	$\beta^0$	Bulgarian
CD 106 CTG→CGG [Leu→Arg]	c.320T→G	Hb Terre Haute	?	North European, French
CD 108–112 (–12bp) [Asn-Val-Leu-Val-Cys→Ser]	c.326_337delACGTGCTGGTCT		$\beta^0$	Swedish
CD 109 (–G) [GTG→TG]→156aa	c.328delG	Hb Manhattan	$\beta^0$	Lithuanian
CD 110 CTG→CCG [Leu→Pro]	c.332 T→C	Hb Showa-Yakushiji	$\beta^+$	Japanese
CD 113 (–G) [GTG→TG]→156aa	c.340delG		$\beta^0$	Canadian
CD 114 CTG→CCG [Leu→Pro]	c.344 T→C	Hb Durham-N.C.	$\beta^0$	Italian, Irish
CD 114 (–CT, +G)→156aa	c.343_344delinsG	Hb Geneva	$\beta^0$	Swiss-French
CD 115 GCC→GAC [Ala→Asp]	c.347C→A	Hb Hradec Kravlove	$\beta^+$	Czech
CD 118 (–T)→156aa	c.355delT	Hb Sainte Seve	?	French
CD 120 (–A)→156aa	c.361delA		$\beta^0$	
CD 120/121 (+A)→138aa	c.363_364insA		$\beta^0$	Filipino
CD 121 GAA→TAA	c.364 G→T		$\beta^0$	Caucasian
CD 123 (–A)→156aa	c.370delA	Hb Makabe	$\beta^0$	Japanese
CD 123-125 (–ACCCACCC) →135aa	c.370_378delACCCACCA	Hb Khon Kaen	$\beta^0$	Thai
CD 124 (–A)→156aa	c.375delA		$\beta^0$	Russian
CD 124–126 + CCA [+Pro]	c.378_379insCCA		?	Armenian
CD 125 (–A)→156aa	c.378delA		$\beta^0$	Japanese
CD 126 (–T)→156aa	c.380 T→G	Hb Neapolis	$\beta^0$	Italian
CD 126-131 (–17 bp) →132aa	c.380_396delTGACAG GCTGCTATCAG	Hb Westdale	$\beta^0$	Trinidad, Pakistani
CD 127 CAG→CCG [Glu→Pro]	c.383A→C	Hb Houston	?	English
CD 127 CAG→CTG [Glu→Arg]	c.383A→G	Hb Dieppe	?	French
CD 127 CAG→TAG	c.382C→T		$\beta^0$	English
CD 127/128 (–AGG) [Glu.Ala→Pro]	c.383_385delAGG	Hb Gunma	$\beta^0$	Japanese
CD 128/129 (–4, +5, –11bp) →153aa	c.[385_388delinsCCACA; 397_407delAAA GTGGTGCC]		$\beta^0$	Irish
CD 131/132 (–GA) →138aa	c.396_397delGA		$\beta^0$	Swiss
CD 131–134 (–11 bp) →134aa	c.394_404delCAGAAAGTGGT		?	Spanish
CD 134–137 –12, +6 bp	c.404_413delinsGCAG		$\beta^0$	Portuguese
CD 137–139 (–TGGCTA) Val-Ala-Asn→Asp	c.413_418delTGGCTA	Hb Stara Zagora	?	Bulgarian
CD 141 (–C)→156aa	c.424delC	Hb Florida	?	Argentine
CD 142 (–C)→156aa	c.428delC	Hb Montreal II	?	Unknown

of  $\beta$ -globin ( $\beta^0$ -type). A few of the  $\beta^+$ -types are associated with an unusually mild phenotype and are sometimes designated  $\beta^{++}$ -type. The mutations may affect globin gene transcription, RNA processing or translation, RNA cleavage and polyadenylation, or result in a highly unstable globin chain. Frameshift and nonsense codon mutations have been observed in all three exons and RNA processing mutations have been found in both introns and the four splice junctions. Examples of each type of mutation are shown in Figure 71-10.



**FIGURE 71-10** Diagram illustrating the  $\beta$ -globin gene and the position of thirteen examples of the different types of  $\beta$ -thalassemia mutations. Noncoding regions, hatched areas; exons, shaded areas; intervening sequences (IVS), blank areas. The types and numbers of mutations are listed below:

Type of Mutation	Total	Example	Position
Transcriptional mutations	32	-88 (C→T)	1
<b>RNA processing mutations</b>			
Splice junction changes	25	IVSI-1 (G→A)	2
Consensus site changes	19	IVSI-5 (G→C)	3
New internal splice site	8	IVSI-110 (G→A)	4
Activation of cryptic splice site in exons	5	CD 26 (G→A) (Hb E)	5
<b>RNA translation mutations</b>			
Nonsense mutations	23	CD 39 (C→T)	6
Frameshift mutations	88	CD 71/72 (+A)	7
Initiation codon mutations	8	ATG→GTG	8
Dominant $\beta$ -thalassemia and highly unstable $\beta$ -chain variants	46	CD 121 (G→T)	9
Cap site mutations	1	CAP+1 (A→C)	10
5' Untranslated region mutations	7	CAP+22 (G→A)	10
Polyadenylation site mutations	10	AATAAA→AACAAA	11
3' Untranslated region mutations	3	3' UTR+6 (C→G)	12
Gene deletions (25 bp–67 kb)	20	619 bp deletion	13

**71.9.4.1 Transcription Mutations.** Transcription mutations are mostly relatively mild and are commonly observed in  $\beta$ -thalassemia intermedia. They are single nucleotide substitutions in the TATA box at -30 from the transcription start site, in the CCAAT box at -70, in the ACACCC distal promoter region at -90, and in the 5' UTR. Some, such as the mutation -101 A→C, are extremely mild ( $\beta^{++}$ -type), with carriers having borderline-reduced/normal red cell indices and normal/borderline-raised HbA<sub>2</sub> values (silent thalassemia), with homozygotes having an extremely mild form of thalassemia intermedia. Compound heterozygotes for these transcriptional mutations and the more severe  $\beta$ -thalassemia mutations tend to have a milder clinical disorder, although there are some notable exceptions. The CAP+1 A→C mutation results in a very mild disorder in the homozygous state, yet when inherited with a  $\beta^0$ -type mutation may result in thalassemia major, and the -29 A→G mutation has a mild phenotype in homozygous African individuals but is reported to result in thalassemia major in Chinese individuals homozygous for the same mutation. This ethnic difference in phenotype is partly related to different chromosomal backgrounds for this mutation, as the African mutation is linked to the C→T polymorphism at position -158 5' to the  $\gamma$ -globin gene (XmnI restriction site), which is associated with increased Hb F production under conditions of erythropoietic stress.

**71.9.4.2 RNA Splicing Mutations.** RNA splicing mutations occur at splice junctions (the invariant donor 5' GT and acceptor 3' AG dinucleotides of the introns), in consensus sequences around splice junctions, in introns to produce new donor and acceptor cryptic splice sites, and in cryptic splice sites in exons. These latter mutations alter sequences that are similar to donor splice sites at the 5' ends of introns but are not normally used for splicing. By making these sequences resemble more closely the consensus sequence for a donor splice site, these mutations activate the cryptic site, and their use leads to the production of abnormal RNA and slowed normal splicing. The second most common hemoglobin variant, Hb E, is associated with a thalassemia phenotype because the  $\beta^E$  mutation activates a cryptic splice site in exon 1, creating abnormally spliced mRNA and decreasing the formation of functional  $\beta^E$ -mRNA. Mutations that affect either of the two invariant dinucleotides of the splice junctions completely abolish normal splicing and produce the phenotype of  $\beta^0$ -thalassemia.

**71.9.4.3 RNA Modification Defects.** RNA modification defects are found at both the 5' end or cap site and the 3' end in the RNA cleavage and polyadenylation signal. The cap site mutation, which changes the first A residue to a C, may work either by reducing transcription itself or by slowing the 5' capping process that may reduce mRNA stability. The mutations in the AATAAA polyadenylation signal at the 3' end of the transcript markedly reduce RNA cleavage and lead to elongated mRNA molecules that are probably unstable. The mutations have a moderately severe  $\beta^+$ -thalassemia phenotype as only approximately 10% of the mRNA is properly

cleaved. Several  $\beta^+$ -thalassemia mutations have also been discovered in the 3' UTR. Eight mutations have been discovered that affect the initiation codon (ATG) and all produce a  $\beta^0$ -thalassemia phenotype.

**71.9.4.4 Frameshift Mutations.** Frameshift mutations are deletions or additions of 1, 2, or 4 nucleotides that change the ribosome reading frame and cause premature termination of translation at a new nonsense or chain termination codon (TAA, TAG, and TGA). Likewise, insertions, deletions, and point mutations can all generate a nonsense codon mutation, directly stopping translation. Chain termination mutations result in the majority of cases in a shortened  $\beta$ -mRNA that is often unstable and is rapidly degraded. The majority of these mutations that occur within exons 1 and 2 results in the typical recessively inherited  $\beta^0$ -thalassemia phenotype. In contrast, frameshift and nonsense mutations that occur later in the  $\beta$ -globin sequence in exon 3 often produce a clinical phenotype more severe than typical  $\beta$ -thalassemia trait and are said to be dominantly inherited.

**71.9.4.5 Missense Mutations and Dominant  $\beta$ -Thalassemia.** Missense mutations in the  $\beta$ -globin gene may produce an unstable hemoglobin variant that is associated with a  $\beta$ -thalassemia phenotype more severe than that caused by a  $\beta^0$ -thalassemia allele. These mutations, usually in the second half of exon 3, lead to a highly unstable  $\beta$ -globin chain that is rapidly degraded, so that little or no mutant hemoglobin can be detected. The unstable  $\beta$ -globin chain interacts with  $\alpha$ -globin chains before it gets degraded, forming alpha beta dimers that are also unstable and get degraded, resulting in chronic hemolytic anemia. This phenomenon makes these mutations more severe than a recessive form of  $\beta$ -thalassemia, hence the designation “dominant  $\beta$ -thalassemia.” Examples are Hb Indianapolis and Hb Showa-Yakushiji. Heterozygotes for these mutations have a moderately severe anemia with jaundice, enlargement of the spleen, gross abnormalities of the erythrocytes, and well-formed inclusion bodies in the red cell precursors.

**71.9.4.6 Deletions.** Most deletions in the  $\beta$ -globin cluster affect more than the  $\beta$ -globin gene and produce  $\delta\beta$ -thalassemia,  $\gamma\delta\beta$ -thalassemia,  $\epsilon\gamma\delta\beta$ -thalassemia, or HPFH syndromes; the majority of these different types of deletions is depicted in Figure 71-12. However, 20 different deletions have been characterized that just delete or partially delete the  $\beta$ -globin gene and result in  $\beta^0$ -thalassemia. One, the 619 bp deletion, affects the 3' end of the  $\beta$ -globin gene and is one of the most common  $\beta$ -thalassemia allele in Asian Indians. The other small deletions of the  $\beta$ -globin gene have been seen rarely. The larger deletions that delete the promoter region of the  $\beta$ -globin gene are interesting because they are associated with unusually high Hb A<sub>2</sub> levels of 6–9% in heterozygotes. The increased expression of the  $\delta$ -globin gene may be due to the fact that these deletions remove the CACCC, CCAAT, and TATA elements of the  $\beta$ -globin gene, thus eliminating competition of the b and d promoter

sequences for transacting transcription factors. Four different Hb Lepore deletions produce fusion  $\delta\beta$  globins, due to unequal crossing over between mispaired  $\delta$  and  $\beta$ -globin genes at different points between the genes, and are all associated with a  $\beta$ -thalassemia phenotype. This is in contrast to the fusion  $\gamma\beta$ -globin Hb Kenya, which behaves like an HPFH allele.

## 71.9.5 Pathophysiology

In the thalassemia syndromes, there is reduced or absent synthesis of the affected globin chain; the unaffected chain continues to be synthesized at relatively normal levels. The result is an imbalance that causes aggregation and precipitation of excess unpaired chains. In  $\beta$ -thalassemia, free  $\alpha$ -chains aggregate; the aggregates are highly insoluble and form inclusions in nucleated erythroid precursors in the bone marrow. These inclusion bodies cause intramedullary hemolysis (ineffective erythropoiesis). In contrast, in  $\alpha$ -thalassemia the  $\gamma_4$  (Hb Bart's) and  $\beta_4$  (Hb H) tetramers that form are more soluble. Thus, in severe  $\alpha$ -thalassemias, inclusions are seen in mature erythrocytes and the ineffective erythropoiesis of  $\beta$ -thalassemia is absent. In any severe thalassemia, removal of these inclusions from erythrocytes by the reticuloendothelial system damages the cells and produces “teardrop” forms. Splenomegaly can be secondary to splenic congestion or hypersplenism. After the spleen is removed, cell destruction continues at a decreased rate in the liver, and the number of red cell inclusions may increase greatly. The large number of erythroid precursors expands the marrow cavities, and bone deformities, thinning, and occasional pathologic fractures result see (Figure 71-11).

Iron accumulation results from increased gastrointestinal absorption stimulated by the anemia, blood transfusions, and decreased utilization for hemoglobin synthesis. The deposition of excess iron causes damage to the heart, pancreas, and other tissues. Chelation therapy is essential to remove excess iron stores. Folic acid requirements are increased in thalassemia. If deficiencies develop, they may worsen the anemia.

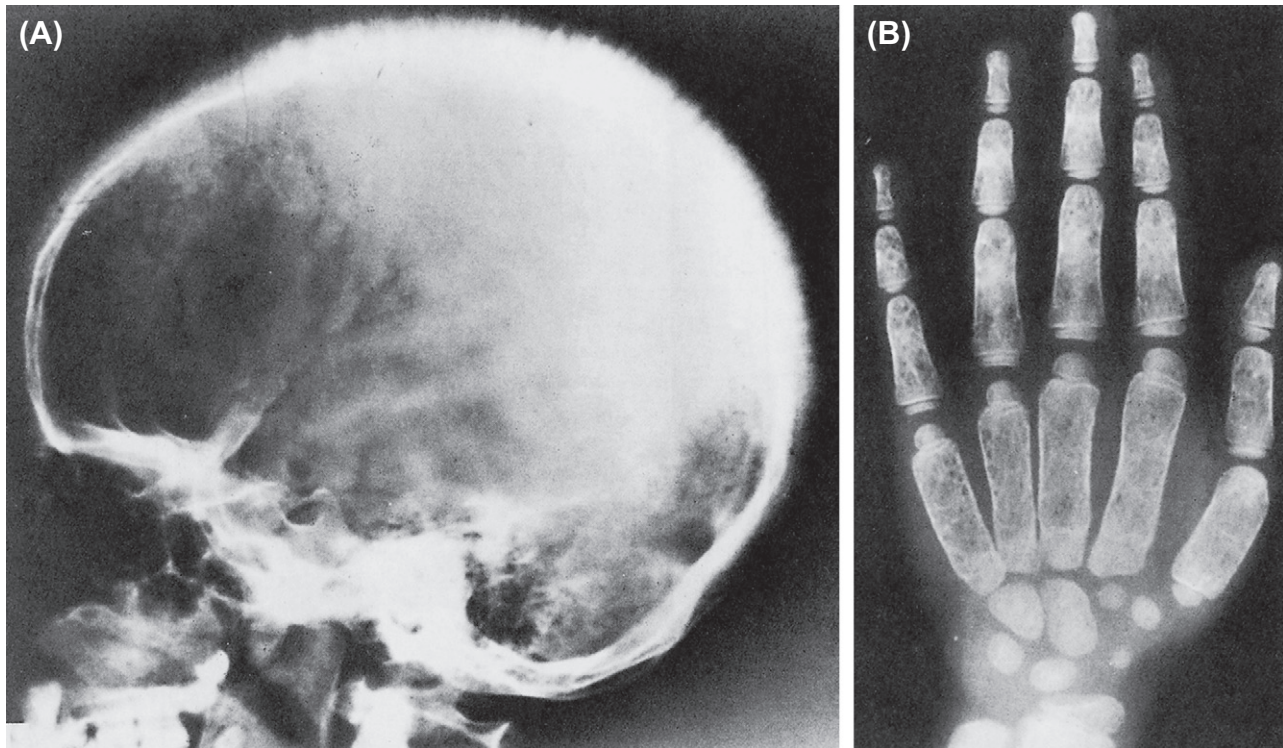
## 71.9.6 Clinical Features

The hematological characteristics of heterozygotes and homozygotes for the main types of globin gene disorders are listed in Table 71-9.

**71.9.6.1  $\alpha$ -Thalassemia.** Four clinical types are seen, depending on the number of  $\alpha$ -genes affected. In order of decreasing severity, these are Hb Bart's hydrops fetalis syndrome (all four genes affected), Hb H disease (three genes affected), heterozygous  $\alpha^0$ -thalassemia and homozygous  $\alpha^+$ -thalassemia (two genes affected), and heterozygous  $\alpha^+$ -thalassemia (one gene affected).

- Hb Bart's hydrops fetalis syndrome. This is the most severe clinical form of  $\alpha$ -thalassemia, resulting from





**FIGURE 71-11** Radiographic changes in homozygous  $\beta$ -thalassemia. (A) Thickened parietal calvaria with outer table destruction and “hair-on-end” appearance. Note absent pneumatization of maxillary sinuses and coincidental epidermoidoma. (B) Widened medullary cavities, cortical thinning, and coarse trabeculation secondary to intramedullary hyperplasia. (Courtesy of Dr John Dorst.)

homozygous  $\alpha^0$ -thalassemia (genotype:  $-/-$ ), sometimes called homozygous  $\alpha$ -thalassemia 1 or hydrops fetalis with Hb Bart's. In the usual case, over 80% of the hemoglobin in the fetal blood is Hb Bart's ( $\gamma_4$ ), which has a very high oxygen affinity, causing severe tissue hypoxia; the remainder Hb H ( $\beta_4$ ) and Hb Portland ( $\zeta_2\gamma_2$ ), the only functional Hb keeping the fetus alive. The resulting severe progressive anemia leads to massive organomegaly and heart failure. This condition is found usually in Asian infants who are spontaneously aborted or die of severe hydrops shortly after birth, although a few cases have been described in which the neonate has survived following intrauterine transfusion therapy and such patients require lifetime transfusion therapy unless they can be cured by stem cell transplantation (18). Prenatal diagnosis is always indicated because of the serious maternal complications that may occur during the pregnancy.

- Hb H disease. Hb H disease (genotypes:  $-/\alpha$ ,  $-/\alpha^T\alpha$ ) is mostly found in Southeast Asians, Greeks, and Italians. The anemia varies with an average range of 8–10 g of hemoglobin per 100 ml of blood, and reticulocytes make up 5–10% of red cells. Splenomegaly and, occasionally, hepatomegaly are found. The red cells are microcytic (decreased MCV) and their hemoglobin content is decreased [decreased mean corpuscular hemoglobin (MCH)], but the concentration of hemoglobin per cell is normal (normal MCHC). On the peripheral smear, poikilocytosis, polychromasia,

and target cells are seen. The  $\beta_4$  tetramer (Hb H) inclusions are seen easily following incubation with 1% brilliant cresyl blue, or after splenectomy they can be seen occasionally with methylene blue reticulocyte stain or Wright's stain see (Figure 71-8C). The  $\alpha/\beta$  ratio is 0.3–0.4. This imbalance causes 20% or higher levels of Hb Bart's at birth and Hb H levels of 4–30% after the switch from  $\gamma$ - to  $\beta$ -chain synthesis is complete. Both tetramers precipitate, causing inclusion body hemolytic anemia. Deficient  $\alpha$ -chain synthesis causes a drop in Hb A<sub>2</sub> ( $\alpha_2\delta_2$ ) levels to 1–1.5%. Deficient  $\alpha$ -chain synthesis is secondary to a deficiency of  $\alpha$ -globin mRNA caused by deletion of three of the four  $\alpha$ -genes. Usually, one of the parents of such a patient has  $\alpha^0$ -thalassemia trait ( $-/\alpha\alpha$ ) and the other has  $\alpha^+$ -thalassemia trait with a gene deletion ( $-/\alpha\alpha$ ) or with a nondeletion mutation ( $-/\alpha^T\alpha$ ). Hb H patients with nondeletional  $\alpha^+$ -thalassemia exhibit symptoms more severe than those with deletional  $\alpha^+$ -thalassemia, and may require recurrent blood transfusions and splenectomy. In rare cases, the interaction of  $\alpha^0$ -thalassemia with a severe type of nondeletion  $\alpha^+$ -thalassemia allele can lead to Hb H hydrops fetalis syndrome, e.g. the interaction of the  $--SEA$  allele with Hb Adana (16).

- $\alpha^0$ -Thalassemia. Heterozygous  $\alpha^0$ -thalassemia individuals (genotype:  $-/\alpha\alpha$ ) are usually of Asian or Mediterranean descent. They are relatively asymptomatic, but have a mild microcytic anemia (10–12 g of hemoglobin per 100 ml of blood) and mild poikilocytosis



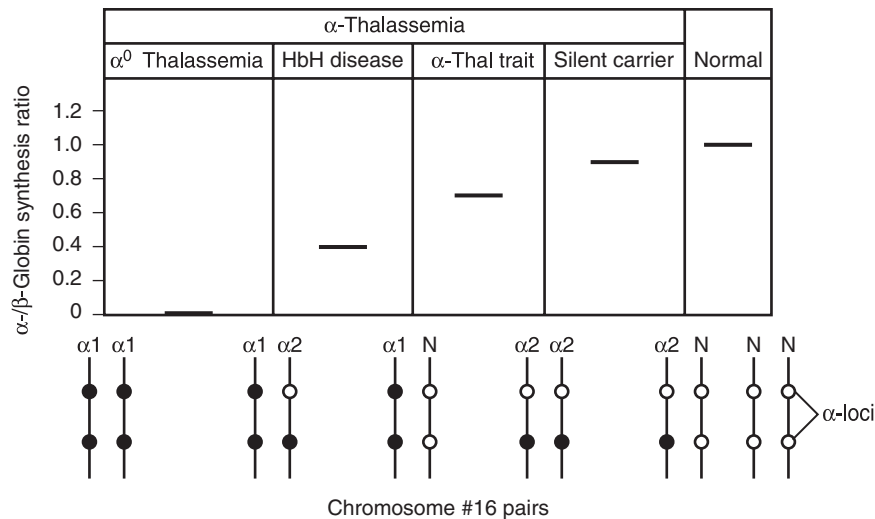
**TABLE 71-9 Characteristics of the Main Types of Globin Gene Disorders**

Hematologic Features			
Disorders	Heterozygotes	Homozygous State	DNA Analysis by PCR Methods
$\alpha^+$ -Thalassemia			
Deletion ( $-\alpha$ )	0–2% Hb Bart's at birth, minimal hematologic changes	5–10% Hb Bart's at birth, low MCH and MCV	Gap-PCR: $-\alpha 3.7$ , $-\alpha 4.2$ alleles
Nondeletion ( $\alpha^T\alpha$ )	Similar to above, but hematological changes may be more severe	Hb H disease in some cases	Selective PCR of $\alpha 1$ or $\alpha 2$ , then ASO, RE-PCR or sequencing
Nondeletion ( $\alpha^{CS}\alpha$ )	0–2% Hb Bart's at birth, 0.5–1% Hb Constant Spring	Slightly more severe than heterozygous $\alpha^0$ -thalassemia	Selective PCR of $\alpha 1$ or $\alpha 2$ , then ASO, RE-PCR or sequencing
$\alpha^0$ -Thalassemia ( $-$ )	5–10% Hb Bart's at birth, low MCH and MCV; normal Hb A <sub>2</sub>	Hb Bart's hydrops fetalis; 80% Hb Bart's, 20% Hb Portland at birth	Gap-PCR: $-(\alpha)20.5$ , $--MED$ , $--SEA$ , $--THAI$ , $--FIL$ alleles; MLPA for all alleles
$\alpha^+$ -Thalassemia/ $\alpha^0$ -Thalassemia		Hb H disease; 20–40% Hb Bart's, at birth; Hb H hydrops fetalis with a few severe $\alpha^T\alpha$ alleles	As above
$\beta^0$ -Thalassemia	Low MCH and MCV; Hb A <sub>2</sub> 3.5–7.0%	Thalassemia major; Hb F 98%; Hb A <sub>2</sub> 2%	Nondeletion alleles: as for $\beta^{++}$ -thal Deletion alleles: Gap-PCR, MLPA
$\beta^+$ -Thalassemia			
Severe	Low MCH and MCV; Hb A <sub>2</sub> 4.5–7.0%	Thalassemia major; Hb F 70–95%	ASO, ARMS, sequencing
Mild	Low MCH and MCV; Hb A <sub>2</sub> 3.5–7.0%	Thalassemia intermedia; Hb F 20–40%	ASO, ARMS, sequencing
Silent	Normal MCH and MCV; Hb A <sub>2</sub> 2.8–3.8%		ASO, ARMS, sequencing
Hb Lepore	Low MCH and MCV; Hb Lepore 8–20%; low Hb A <sub>2</sub>	Thalassemia major/intermedia; Hb F 80%, Hb Lepore 20%	Gap-PCR, MLPA
$\delta\beta$ -Thalassemia	Low MCH and MCV; normal Hb A <sub>2</sub> , Hb F 4–18% (heterocellular)	Thalassemia intermedia; 100% Hb F	Gap-PCR, MLPA
HPFH			
Deletion	Normal indices; Hb F 15–35% (pancellular)	Normal; 100% Hb F	Gap-PCR, MLPA
Nondeletion	Normal indices; Hb F 1–20%	Not described	Sequencing, Xmn I for $-158 G\gamma C \rightarrow T$
Hb S	Hb A, Hb A <sub>2</sub> (3–4%), Hb S (35–40% for $\alpha\alpha/\alpha\alpha$ ; 30–35% for $-\alpha/\alpha\alpha$ ; 24–28% for $-\alpha/\alpha$ )	Hb S, Hb F (1–15%) Hb A <sub>2</sub>	RE-PCR (Dde I), ASO, ARMS
Hb S/ $\beta$ -thalassemia		$\beta^0$ : severe sickle-cell anemia; $\beta^{+}$ : less severe sickle-cell anemia $\beta^{++}$ : mild sickle-cell anemia	As above Hb S and $\beta$ -thal
Hb E/ $\beta$ -thalassemia		Thalassemia major or intermedia; 60–70% Hb E; 30–40% Hb F	ASO, ARMS, sequencing
Hb EA Bart's disease		Similar to Hb H disease; Hb E 13%, Hb A, Hb Bart's 3%	As for Hb E + $\alpha$ -thalassemia
Hb EF Bart's disease		Severe thalassemia intermedia; Hb E 80%, Hb F 15%, Hb Bart's 5%	As for Hb E + $\alpha$ -thalassemia

and anisocytosis. At birth, Hb Bart's may reach 5% in cord blood. The  $\alpha/\beta$  synthesis ratio is 0.6–0.75 (Figure 71-12). The diagnosis of  $\alpha^0$ -thalassemia trait should be considered when the MCV and MCH are low, the MCHC is relatively normal and the patient is not iron deficient, and the Hb A<sub>2</sub> is normal. This phenotype is also associated with that of homozygous  $\alpha^+$ -thalassemia (genotypes:  $-\alpha/-\alpha$ ,  $-\alpha/\alpha^T\alpha$ ,  $\alpha^T\alpha/\alpha^T\alpha$ ) and the two conditions can only be distinguished by DNA analysis techniques. Homozygous  $\alpha^+$ -thalassemia is

associated with mild elevation of Hb Bart's (>2%) in 2–5% of newborns.

- $\alpha^+$ -Thalassemia. Heterozygous  $\alpha^+$ -thalassemia (genotypes:  $-\alpha/\alpha\alpha$  or  $\alpha\alpha/\alpha^T\alpha$ ) is effectively a silent carrier state because the reduction of a mRNA is insufficient to produce significant globin chain imbalance ( $\alpha/\beta=0.8-0.9$ ) (see Figure 71-12). The hematological indices in carriers are only slightly altered and overlap considerably with those of normal individuals. A reliable diagnosis can only be made by DNA analysis.



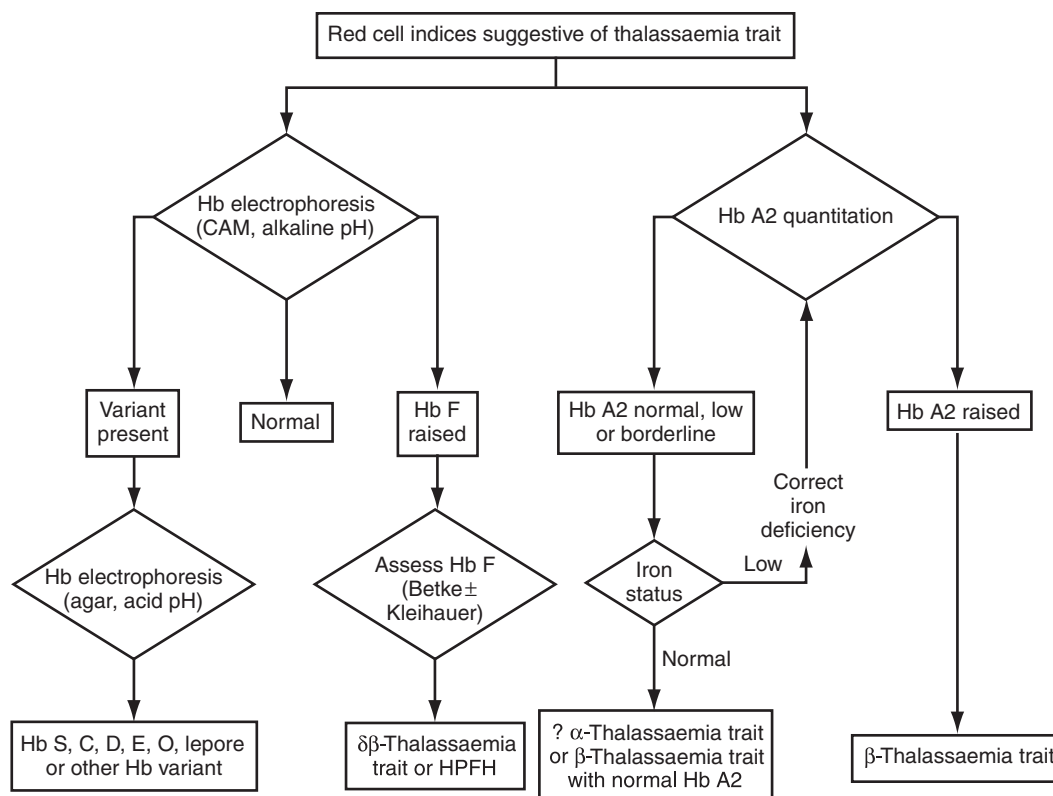
**FIGURE 71-12** Chain synthesis ratios and proposed genotypes in the different  $\alpha$ -thalassemia states.  $\alpha$ -Genes: o, normal; •, abnormal. Genotypes:  $\alpha_1$ ,  $\alpha^0$ -thalassemia;  $\alpha_2$ ,  $\alpha^+$ -thalassemia, N, normal. (Modified from Nathan, 1972, with permission.)

**71.9.6.2  $\beta$ -Thalassemia.** In contrast to the  $\alpha$ -thalassemia states in which there are four levels of severity, the  $\beta$ -thalassemias can be considered as having two degrees of severity.  $\beta$ -Thalassemia major (Cooley anemia) results from two  $\beta$ -thalassemia genes at the  $\beta$ -globin locus;  $\beta$ -thalassemia trait results from a single  $\beta$ -thalassemia gene. A milder form of homozygous  $\beta$ -thalassemia known as thalassemia intermedia also exists, the disorder resulting from a wide range of genotypes and covering a broad spectrum of phenotypes.

- $\beta$ -Thalassemia major.**  $\beta$ -Thalassemia major is a severe disease. At birth, affected infants are relatively normal because the change from  $\gamma$ -chain synthesis to  $\beta$ -chain synthesis has not been completed (see Figure 71-5). However, by 6 months of age the infant develops a severely microcytic hemolytic anemia with aniso- and poikilocytosis, polychromasia, and teardrop red cells (see Figure 71-8F). The failure in  $\beta$ -globin production due to absent or greatly decreased  $\beta$  mRNA leads to imbalance in  $\alpha$ - and  $\beta$ -globin synthesis. Subsequent precipitation of free  $\alpha$ -chains results in inclusion bodies that damage the erythrocyte membrane and lead to destruction of nucleated red cells in the marrow. The reticulocyte count is usually no greater than 5–10% because of massive destruction of erythroid precursors in the marrow. To maintain an adequate hemoglobin level, transfusions are usually required every 4–8 weeks. Affected children develop hepatosplenomegaly secondary to extramedullary hematopoiesis, and a characteristic Asian facial appearance due to excessive intramedullary hematopoiesis. The bones have expanded marrow cavities resulting in pathologic fractures and a “hair-on-end” appearance on skull films (see Figure 71-11). Other complications include cholelithiasis, susceptibility to infections, secondary

hypersplenism, and delayed growth and maturation. The major causes of mortality are hemochromatosis and overwhelming infections following splenectomy, the former due to excessive iron deposition as a result of blood transfusions and increased gastrointestinal absorption. Excess iron deposited in the heart, pancreas, liver, and other organs damages tissue and leads to cardiac failure, arrhythmias, diabetes mellitus, and liver failure. Without regular transfusion, patients usually die before the age of 20 years. Given optimal transfusion and iron chelation therapy, many patients now survive beyond the age of 40 years.

- $\beta$ -Thalassemia intermedia.** Patients with  $\beta$ -thalassemia who have a milder clinical course and are not fully transfusion dependent but have a more severe condition than the carrier states for  $\beta$ -thalassemia are classified as having  $\beta$ -thalassemia intermedia. Transfusions are not usually required in these patients even though  $\alpha/\beta$  synthesis ratios are similar to those observed in Mediterranean homozygotes. The genetic interactions leading to the phenotype of  $\beta$ -thalassemia intermedia are very heterogeneous and include homozygosity or compound heterozygosity for mild  $\beta$ -thalassemia mutations, co-inheritance of  $\alpha^0$ -thalassemia trait or homozygous  $\alpha^+$ -thalassemia and the inheritance of HPFH determinants. Inheritance of single  $\beta$ -thalassemia genes can also cause thalassemia intermedia. In the majority of cases this is related to an increased  $\alpha$ -globin production from the co-inheritance of one ( $\alpha\alpha\alpha/\alpha\alpha$ ), or in most cases, two extra  $\alpha$ -genes, either as two triplicated  $\alpha$ -genes alleles ( $\alpha\alpha\alpha/\alpha\alpha\alpha$ ), or more rarely, as a quadrupled  $\alpha$ -gene allele ( $\alpha\alpha\alpha\alpha/\alpha\alpha$ ).
- $\beta$ -Thalassemia trait.** Individuals with  $\beta$ -thalassemia trait (heterozygous  $\beta$ -thalassemia) are usually asymptomatic. The classical picture is mild anemia (10–11 g hemoglobin per 100 ml blood) with decreased MCV



**FIGURE 71-13** Approach to screening for thalassemia trait. Note that, since this approach was designed as a guide for screening of adults, other causes of microcytosis, such as lead poisoning in children, are not considered. (Modified from Pearson et al., 1973, with permission.)

(55–70) and MCH (1 pg 6–22 pg) and Hb A<sub>2</sub> raised above 3.5%. Microcytosis, anisocytosis, poikilocytosis, and targetting and stippling of the red cells can be seen on the blood smear see (Figure 71-8E). It should be noted that a small number of  $\beta$ -thalassemia heterozygotes may have normal or borderline-raised Hb A<sub>2</sub> levels. These carriers fall into two groups, those with a reduced MCV and those with a normal MCV. The first category consists of patients heterozygous for some of the mild  $\beta^+$ -thalassemia mutations, such as IVS 1-6 T→C and CAP+1 A→C, or individuals with both  $\beta$ -thalassemia and  $\delta$ -thalassemia traits. Carriers for the  $\delta\beta$ -thalassemias also have this phenotype but are distinguished by their marked increase in Hb F. The second category of atypical carriers are said to have silent  $\beta$ -thalassemia as they have normal MCV and MCH values with a borderline/normal Hb A<sub>2</sub> and normal Hb F level. They are defined by a slight imbalance in the  $\alpha/\beta$  chain ratio and are identified by DNA analysis, the mutations being designated as  $\beta^{++}$ -types. The most common silent  $\beta$ -thalassemia mutation is -101 C→T, found in Mediterranean individuals and Asian Indians (17).

### 71.9.7 Differential Diagnosis

The basic hematological tests required for carrier detection are the measurement of the MCV, the MCH value

and the quantity of Hb A<sub>2</sub> and Hb F. In addition, the hemoglobin pattern needs to be examined, and traditionally, electrophoresis methods have been used for this purpose. However, if HPLC is used to quantitate the Hb A<sub>2</sub> and Hb F level, it will also detect most of the common, clinically relevant hemoglobin variants, such as Hb S, Hb C, Hb D-Punjab, Hb O-Arab and Hb E at the same time.

In the general practice of medicine many patients present with a mild microcytic anemia. Nearly all have iron deficiency anemia or a type of thalassemia trait. In heterozygous thalassemia, the peripheral smear may be more abnormal than that of iron deficiency, and the MCV and MCH are decreased; but the MCHC is normal in contrast to the decreased MCHC seen in advanced iron deficiency anemia. The MCV in thalassemia traits also tends to be lower in relation to the red cell count than the MCV in iron deficiency. This difference is the basis of the Mentzer index (MCV/red cell count (RBC)). MCV/RBC values of less than 11.5 suggest thalassemia trait, while values greater than 13.5 suggest iron deficiency anemia. A much more definitive measurement of iron deficiency is to determine the ferritin level or to determine the erythrocyte zinc protoporphyrin (ZnPP) level. Iron deficiency is indicated by a lower-than-normal ferritin level and a higher-than-normal ZnPP value.

The first step in carrier identification is to measure the Hb A<sub>2</sub> level in patients with microcytosis (Figure 71-13).

Patients with microcytosis and normal Hb A<sub>2</sub> should have serum iron or ferritin determinations; a low value suggests iron deficiency anemia, while a normal iron or ferritin value suggests  $\alpha$ -thalassemia trait. When microcytosis and an increased Hb A<sub>2</sub> (3.5–6%) with a normal or slightly increased Hb F (2–5%) are found, a diagnosis of  $\beta$ -thalassemia trait is made. Confirmation of the diagnosis is obtained by family studies and DNA analysis. With the flowchart in [Figure 71-4](#) only the very rare cases of silent  $\beta$ -thalassemia trait,  $\beta$ -thalassemia co-inherited with  $\delta$ -thalassemia, and the extremely rare condition of  $\epsilon\delta\beta$ -thalassemia will be missed. Note that it is common for patients with a clearly raised Hb A<sub>2</sub> level to co-inherit  $\alpha$ -thalassemia, and thus antenatal patients with  $\beta$ -thalassemia trait should be screened for  $\alpha^0$ -thalassemia trait by DNA analysis when they are from Southeast Asia or Mediterranean regions where  $\alpha^0$ -thalassemia is common.

In the rare cases showing normal or low MCH and MCV values, normal or reduced Hb A<sub>2</sub> levels and a high Hb F (5–30%)  $\delta\beta$ -thalassemia trait or HPFH is indicated. These two conditions are distinguished by analyzing the Hb F distribution in the red cells and/or by DNA analysis. The  $\delta\beta$ -thalassemia trait usually has a heterogeneous distribution of Hb F (generally between 4 and 18%) while HPFH has a homogenous (pancellular) distribution (15–35%) see ([Table 71-9](#)).

### 71.9.8 Molecular Diagnosis

Mutation identification by DNA analysis techniques is required after differential diagnosis in several instances, for example, to distinguish between homozygous  $\alpha^+$ -thalassemia and  $\alpha^0$ -thalassemia trait, to distinguish between  $\delta\beta$ -thalassemia trait and HPFH with co-inherited  $\alpha$ -thalassemia, and to give a definitive diagnosis of Hb D-Punjab in antenatal patients following a presumptive diagnosis by HPLC or electrophoresis. Genotype analysis is also necessary for prenatal diagnosis by fetal DNA analysis and for solving complex difficult diagnostic cases due to the interaction of several alleles or mutations with atypical phenotypes. The major PCR-based approaches for diagnosing thalassemia mutations are summarized in [Table 71-9](#).

The technique of Gap-PCR is the most widely used method for the diagnosis of the common  $\alpha$ -thalassemia deletion alleles, and multiplex assays have been developed to identify the two most common  $\alpha^+$ -thalassemia deletion genes, the  $-\alpha^{3.7}$  and  $-\alpha^{4.2}$  alleles, and five  $\alpha^0$ -thalassemia deletion mutations, the  $_{-FIL}$ ,  $_{-THAI}$ ,  $_{-MED}$ ,  $-(\alpha)^{20.5}$  and the  $_{-SEA}$  alleles ([19](#)). The many rare  $\alpha^0$ -thalassemia deletion alleles cannot be identified by Gap-PCR because the breakpoint DNA sequences have not been characterized for primers to be synthesized; however they can be detected, although not definitively identified, by the technique known as multiplex ligation-dependent probe amplification (MLPA) ([20](#)).

The diagnosis of nondeletion  $\alpha^+$ -thalassemia mutations is more complicated than the diagnosis of  $\beta$ -thalassemia mutations as it requires specific preamplification of one of the two  $\alpha$ -globin genes before a PCR-based diagnostic can be applied. Known mutations may be diagnosed by simple quick techniques, such as restriction enzyme polymerase chain reaction (RE-PCR) for the Hb Constant Spring mutation, or by the amplification refraction mutation system (ARMS) PCR for regionally specific mutations in Southeast Asia ([21](#)). However, for unknown mutations, DNA sequencing of each  $\alpha$ -globin is the gold standard technique used by most laboratories.

For the identification of  $\beta$ -thalassemia point mutations, many different PCR-based approaches have been developed over the past 20 years, from simple methods of hybridization of labeled oligonucleotides to highly technological methods using primer extension microarrays. However, the simplicity of the mutation spectrum in countries without a large multiethnic immigrant population makes it easy to screen for most  $\beta$ -thalassemia mutations with simple cheap techniques if the ethnic origin of the patient is known. The most widely used techniques are based on the principles of ASO hybridization or allele-specific priming, such as reverse dot blotting or ARMS-PCR ([22](#)). Each population at risk has its own particular spectrum of  $\beta$ -thalassemia mutations enabling prenatal diagnosis to be carried out by screening for the common mutations in that particular ethnic group. The common mutations (at frequency of 1% or more) for the major ethnic groups (Mediterranean, Indian, Chinese, and African) are listed in [Table 71-10](#). The ARMS-PCR technique has been developed to identify all these common mutations ([23](#)). For countries with large multiethnic populations, the number of mutations required to be diagnosed is much greater and a general method for both known and unknown mutations such as DNA sequencing is more useful. Microarray technology holds the promise of being able to screen for all the 1000 thalassemia and Hb variant point mutations simultaneously, and several groups have developed arrays to screen a DNA sample for up to 23  $\beta$ -thalassemia point mutations in one analysis ([24,25](#)). Large  $\beta$ -thalassemia deletion mutations, including  $\delta\beta$ -thalassemia and HPFH deletion alleles, can be identified by gap-PCR if the deletion breakpoints have been characterized, or simply detected by MLPA if they are unknown ([26](#)).

**71.9.8.1  $\delta\beta$ -Thalassemia.** Homozygous  $\delta\beta$ -thalassemia is a mild disorder and one of the many genotypes that result in thalassemia intermedia. Homozygotes have 100% Hb F and lack Hb A and Hb A<sub>2</sub>. The mild anemia and hemolysis are due to increased  $\gamma$ -chain synthesis, which makes the imbalance between synthesis of  $\alpha$ -chains and non- $\alpha$  chains less than that seen in other  $\beta$ -thalassemias. Patients with heterozygous  $\delta\beta$ -thalassemias have mild microcytosis and 4–18% Hb F on electrophoresis see ([Table 71-9](#)). They are classified



**TABLE 71-10 The Distribution of the Common  $\beta$ -Thalassemia Mutations and Their Relative Gene Frequencies**

Mutation	Mediterranean			Indian		Chinese		African
	Italy	Greece	Turkey	Pakistan	India	China	Thailand	African-American
-88 C→T					0.8			21.4
-87 C→G	0.4	1.8	1.2					
-30T→A			2.5					
-29 A→G						1.9		60.3
-28 A→G						11.6	4.9	
CAP+1 A→C					1.7			
CD 5 (-CT)		1.2	0.8					
CD 6 (-A)	0.4	2.9	0.6					0.8
CD 8 (-AA)		0.6	7.4					
CD 8/9 (+G)				28.9	12.0			
CD 15 G→A				3.5	0.8			
CD 16 (-C)				1.3	1.7			
CD 17 A→T						10.5	24.7	
CD 24T→A								7.9
CD 39 C→T	40.1	17.4	3.5					
CD 41/42 (-TCTT)				7.9	13.7	38.6	46.4	
CD 71/72 (+A)						12.4	2.3	
IVS I-1 G→A	4.3	13.6	2.5					
IVS I-1 G→T				8.2	6.6			
IVS I-5 G→C				26.4	48.5	2.5	4.9	
IVS I-6 T→C	16.3	7.4	17.5					
IVS I-110 G→A	29.8	43.7	41.9					
IVS II-1 G→A	1.1	2.1	9.7					
IVS II-654 C→T						15.7	8.9	
IVS II-745 C→G	3.5	7.1	2.7					
619 bp deletion				23.3	13.3			
Others	4.1	2.2	9.7	0.5	0.9	6.8	7.9	9.6

CD, Codon; IVS, intervening sequence; bp, base pairs.

into two groups: the  $(\delta\beta)^0$ -thalassemias in which the Hb F is composed of both  $G_\gamma$  and  $A_\gamma$  chains (13 deletion mutations), and the  $(A_\gamma\delta\beta)^0$ -thalassemias in which the Hb F contains only  $G_\gamma$  chains see (Figure 71-11) due to  $A_\gamma$ -globin gene being deleted (15 deletion mutations).

**71.9.8.2 Hb Lepore Thalassemia.** As previously mentioned, Hb Lepore is a variant hemoglobin containing a  $\delta\beta$ -fusion chain. Four different Hb Lepore variants have been described (Hollandia, Boston/Washington, Baltimore and Leiden), differing in the point at which the  $\delta\beta$ -fusion occurs. Hb Lepore has an electrophoretic mobility similar to that of Hb S, and it forms 5–15% of the total hemoglobin of heterozygotes see (Table 71-9). It can be easily identified by IEF gel electrophoresis in which it focuses to a characteristic position between Hb A and S. Decreased Hb Lepore synthesis may be secondary to instability of the  $\delta\beta$ -fusion mRNA. Heterozygotes are clinically similar to  $\beta^0$ -thalassemia heterozygotes and Hb Lepore homozygotes, or Lepore/ $\beta^0$ -thalassemia genetic compounds are similar to  $\beta^0$ -thalassemia homozygotes with a severe thalassemic picture ranging from transfusion-dependent disease to thalassemia intermedia when ameliorated by other factors such as  $\alpha$ -thalassemia.

**71.9.8.3  $\epsilon\gamma\delta\beta$ -Thalassemia.** Infants heterozygous for this rare condition are born with a severe hemolytic, hypochromic anemia and microcytosis and may require blood transfusions. The condition improves at 3–6 months after birth and adults have a phenotype similar to heterozygous  $\beta$ -thalassemia but with a normal Hb  $A_2$  level. Seventeen large deletion mutations have been described, which delete all the functional globin genes ( $\epsilon$ ,  $G_\gamma$ ,  $A_\gamma$ ,  $\delta$ , and  $\beta$ ) and three have been described in which the deletion leaves the  $\beta$ -globin gene intact but not expressed. Mapping of the deleted DNA of the latter cases shows that in all three cases the locus control region (LCR) located 5' to the  $\epsilon$ -gene has been removed. The absence of this segment inactivates all the intact globin genes following the 3' end of the deletion. The homozygous condition is presumed to be incompatible with fetal survival.

**71.9.8.4  $\delta$ -Thalassemia.** Individuals with heterozygous and homozygous  $\delta^0$ -thalassemia have, respectively, decreased and absent Hb  $A_2$ . However, anemia and changes in peripheral smears are not seen because of the normal low level of  $\delta$ -chain production see (Figure 71-5). At least 26 different  $\delta$ -thalassemia alleles have been described. Their only clinical significance is when they are co-inherited with

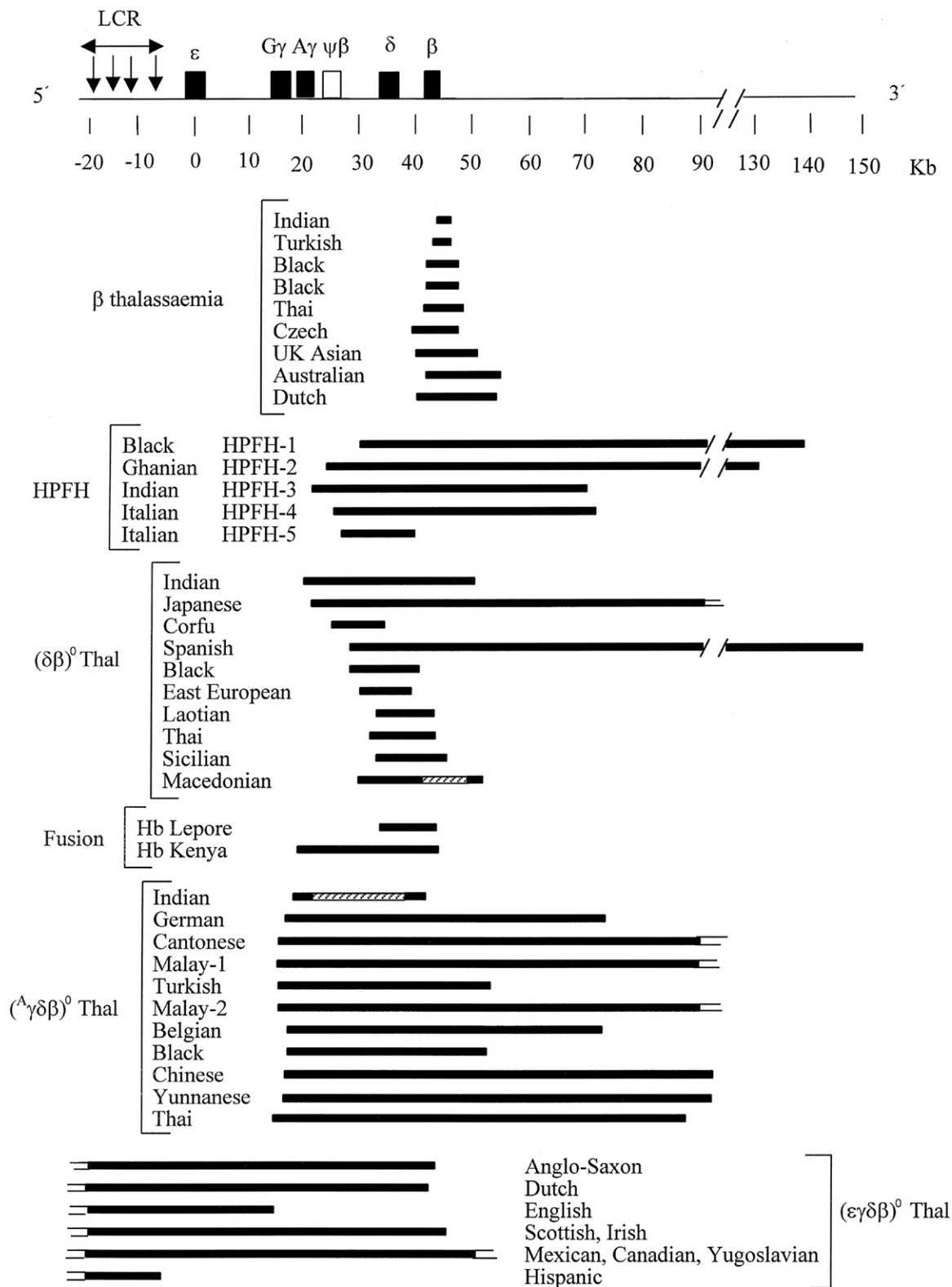


FIGURE 71-14 Deletion mutations in the β-globin gene cluster.

heterozygous β-thalassemia and complicate the diagnosis because of a normal Hb A<sub>2</sub> level of 2–2.5%.

**71.9.8.5 Hereditary Persistence of Fetal Hemoglobin.** HPFH is a disorder in which Hb F is increased above the normal adult level and there are no morphological

changes to the red cells. The disorder is caused by at least 25 different mutations, either large deletions in the globin gene cluster (Figure 71-14) or point mutations in the γ-gene promoter regions. HPFH heterozygotes differ from thalassemia heterozygotes in that they have no imbalance

between the synthesis of  $\alpha$  and non  $\alpha$ -chains (i.e.  $\gamma$  and  $\beta$ -chains) and thus are characterized by an asymptomatic heterozygous state without microcytosis. The elevated Hb F ranges from 3 to 35%, depending on the type of the mutation. The proportion of  $\gamma$ -chain type ( $G_\gamma$  versus  $A_\gamma$ ) varies among patients with different HPFHs and usually, but not always, the Hb F is homogeneously distributed within red cells, in contrast to db and other thalassemias. In a few cases, HPFH heterozygotes have two populations of cells: one contains Hb F; the other lacks Hb F. These patients are said to have heterocellular HPFH, as opposed to the bulk of patients who have pancellular HPFH.

There are seven types of deletional HPFH (including the deletion that gives rise to the fusion globin Hb Kenya) and at least 14 different nucleotide changes responsible for nondeletional HPFH. The  $\beta$  and  $\delta$ -genes are deleted in the deletional HPFH types, but both are present and active in the nondeletional HPFH cases. In these latter cases, point mutations between -202 and -110 upstream of the  $A_\gamma$  and  $G_\gamma$  globin genes have been found.

The differences between HPFH and  $\delta\beta$ -thalassemia are subtle, but in  $\delta\beta$ -thalassemia the clinical picture and blood smears are somewhat more abnormal and the Hb F has a more heterogeneous cellular distribution. HPFH homozygotes have mild hypochromia, microcytosis, and morphologic changes in the red cells; 100% of the hemoglobin is F, and there is no anemia. In some of these cases,  $\alpha/\gamma$  chain synthesis ratios of 1.5 occur, similar to the  $\alpha/\beta$  ratios seen in milder  $\beta$ -thalassemia trait. It has been hypothesized that a suppression region for  $\gamma$ -chain synthesis is located between the  $A_\gamma$  and  $\delta$  loci and that HPFH, but not  $\delta\beta$ -thalassemia mutations, inhibits its function. However, suppression and activation regions in the  $\beta$ -globin gene cluster appear multiple in number, and a clear picture explaining the difference between the deletions of the two disorders has not emerged.

## 71.9.9 Interaction of Thalassemia with Hemoglobin Variants

**71.9.9.1  $\alpha$ -Thalassemia/Hb Q-Thailand ( $\alpha 74$  Asp  $\rightarrow$  His).** The Hb Q-Thailand mutation is linked to  $\alpha^+$ -thalassemia allele: the mutation is located in the  $\alpha 1$ -gene on a chromosome in which the  $\alpha 2$ -gene is deleted ( $-\alpha^Q$ ). Thus, the quantity of Hb Q-Thailand in heterozygotes is 30–35%, higher than normal for an  $\alpha$ -chain variant. The genetic compound of  $\alpha^0$ -thalassemia ( $-$ ) and Hb Q-Thailand is found Thai, Chinese, and Japanese families. The clinical picture is similar to that of Hb H disease, but there is an absence of Hb A synthesis.

**71.9.9.2  $\alpha$ -Thalassemia/Hb G-Philadelphia ( $\alpha 68$  Asn  $\rightarrow$  Lys).** Hb G-Philadelphia is linked with an  $\alpha^+$ -thalassemia allele in some ethnic groups. Heterozygotes for Hb G-Philadelphia, the most common  $\alpha$ -chain variant in individuals of African origin, show a trimodal distribution of Hb G (22, 30, and 41% of the total hemoglobin). This is because the Hb G-Philadelphia mutation is

located on either a normal  $\alpha$ -gene allele ( $\alpha^G\alpha$ ) or an  $\alpha^+$ -thalassemia allele ( $-\alpha^G$ ). Individuals with 22% Hb G-Philadelphia have three normal  $\alpha$  genes in addition to one  $\alpha^G$  gene ( $\alpha\alpha/\alpha^G\alpha$ ). Those with 30% Hb G-Philadelphia have one normal  $\alpha$ -gene allele and one  $\alpha^+$ -thalassemia allele containing just a single  $\alpha$ -gene: the Hb G-Philadelphia mutation may be on the  $\alpha^+$ -thalassemia allele ( $\alpha\alpha/-\alpha^G$ ) or on the normal allele ( $-\alpha/\alpha^G\alpha$ ). Those with 41% Hb G have homozygous  $\alpha^+$ -thalassemia, with one chromosome containing a single  $\alpha^G$  gene and another with a single  $\alpha^A$  gene ( $-\alpha/-\alpha^G$ ).

**71.9.9.3  $\alpha$ -Thalassemia/Hb S.** Sickle-cell heterozygotes show a trimodal distribution of the percentage Hb S because of the effect of  $\alpha^+$ -thalassemia. AS individuals who are normal with respect to  $\alpha^+$ -thalassemia ( $\alpha\alpha/\alpha\alpha$ ) have 36–40% Hb S, those with  $\alpha^+$ -thalassemia trait ( $\alpha\alpha/-\alpha$ ) have 30–34% Hb S, and those homozygous for  $\alpha^+$ -thalassemia ( $-\alpha/-\alpha$ ) have 24–28% Hb S.

**71.9.9.4  $\alpha$ -Thalassemia/Hb E.** In Southeast Asia, Hb E heterozygotes who inherit  $\alpha$ -thalassemia also show a reduction in the percentage Hb E in a manner similar to AS individuals as described previously. AE individuals who are normal with respect to  $\alpha^+$ -thalassemia ( $\alpha\alpha/\alpha\alpha$ ) have 25–30% Hb S, those with  $\alpha^+$ -thalassemia trait ( $\alpha\alpha/-\alpha$ ) have 21–25% Hb S, and those homozygous for  $\alpha^+$ -thalassemia ( $-\alpha/-\alpha$ ) or heterozygous  $\alpha^0$ -thalassemia ( $-\alpha/\alpha$ ) have 19–21% Hb S. All these combinations are asymptomatic disorders. However,  $\alpha$ -thalassemia genotypes can interact with Hb AE and Hb EE genotypes to produce symptomatic disorders. The most commonly observed disorders are Hb AE Bart's disease and Hb EF Bart's disease (Table 71-6).

Hb AE Bart's disease results from the interaction of an Hb H disease genotype with the heterozygous state for Hb E. The disorder is characterized by the presence of Hb A, Hb E (13–15%), and Hb Bart's on hemoglobin analysis. The clinical manifestations are similar to Hb H disease, with patients having a variable degree of anemia and splenomegaly. Two common subtypes of Hb AE Bart's disease have been observed:  $\alpha^0$ -thalassemia/ $\alpha^+$ -thalassemia ( $-\alpha/-\alpha$ ) with heterozygous Hb E ( $\beta^A/\beta^E$ ) and  $\alpha^0$ -thalassemia/Hb Constant Spring ( $-\alpha^{CS}\alpha$ ) with heterozygous Hb E ( $\beta^A/\beta^E$ ). The latter disorder was found to have a more severe clinical syndrome.

Hb EF Bart's disease results from the interaction of an Hb H disease genotype with homozygous Hb E. The disorder is characterized by the presence of Hb E, Hb F, and Hb Bart's on hemoglobin analysis. Hb E constitutes approximately 80%, Hb F 10%, and Hb Bart's 10%. Patients with this condition have severe thalassemia intermedia, with a Hb level ranging from 6 to 10g/dl and markedly reduced MCV and MCH values. Four compound genotypes for Hb EF Bart's disease have been identified: (i)  $\alpha^0/\alpha^+$ -thalassemia ( $-\alpha/-\alpha$ ) plus homozygous Hb E ( $\beta^E/\beta^E$ ), (ii)  $\alpha^0$ -thalassemia/Hb Constant Spring ( $-\alpha^{CS}\alpha$ ) plus homozygous Hb E ( $\beta^E/\beta^E$ ), (iii)  $\alpha^0/\alpha^+$ -thalassemia ( $-\alpha/-\alpha$ ) plus Hb E/ $\beta^0$ -thalassemia, and (iv)  $\alpha^0$ -thalassemia/Hb

Constant Spring ( $--/\alpha^{CS}\alpha$ ) plus Hb E/ $\beta^0$ -thalassemia. To differentiate among these genotypes, family studies and further investigation by DNA analysis are required.

**71.9.9.5  $\beta$ -Thalassemia/Hb S.** Hb S/ $\beta$ -thalassemia is characterized by microcytic red and target cells with occasionally sickled forms. Hemoglobin electrophoresis reveals 60–90% Hb S, 0–30% Hb A, 1–20% Hb F, and an increased Hb A<sub>2</sub> level. The percentages of Hb S and Hb A vary depending on whether the  $\beta$ -thalassemia gene is  $\beta^+$  or  $\beta^0$  type. Coexisting  $\alpha$ -thalassemia increases the Hb concentration, the MCV, and MCH.

The clinical course of sickle-cell  $\beta$ -thalassemia is very variable, ranging from a disorder identical with sickle-cell anemia to a completely asymptomatic condition. The Hb concentration varies from 5 g/dl to a level within the normal range. The heterogeneity is mostly due to the type of  $\beta$ -thalassemia mutation that is co-inherited. It tends to be very mild in Africans because of the likelihood of the co-inheritance of one of the three mild  $\beta^+$  mutations commonly found in this racial group ( $-88, C \rightarrow T$ ;  $-29, A \rightarrow G$ ; CD24,  $T \rightarrow A$ ). However, those patients who inherit a  $\beta^0$ -thalassemia allele exhibit a clinical disorder very similar to sickle-cell anemia.

**71.9.9.6  $\beta$ -Thalassemia/Hb C.**  $\beta$ -Thalassemia/Hb C is mostly seen among individuals of African origin. The disorder is clinically heterogeneous because of the different types of  $\beta$ -thalassemia alleles involved. Hb C/ $\beta^+$ -thalassemia in Africans has hematologic features similar to those of  $\beta$ -thalassemia trait because  $\beta^+$ -thalassemia mutations are the mild types, as described previously for Hb S/ $\beta^+$ -thalassemia. However, Hb C/ $\beta^+$ -thalassemia with a severe type of mutation and Hb C/ $\beta^0$ -thalassemia produce a more severe phenotype, characterized by an anemia of 8–12 g/dl and splenomegaly. The clinical picture is a slightly more severe form of Hb C disease (homozygous Hb C), with which individuals have a mild hemolytic anemia (10–15 g/dl) and moderate splenic enlargement. Hb C is less soluble than Hb A in both the oxygenated and deoxy forms, resulting in the formation of crystals in high concentrations. Hb CC red cells become dehydrated, dense, and abnormally rigid, resulting in a shortened life span. AC individuals have no clinical manifestations and normal hematological values, although their red cells are slightly dehydrated and dense. Diagnostically, Hb C/ $\beta^0$ -thalassemia can be difficult to distinguish from Hb C disease (Hb CC) because of overlap of the hematological findings and the fact that, apart from HPLC, the commonly used methods of hemoglobin analysis do not distinguish Hb A<sub>2</sub> from Hb C. A raised Hb A<sub>2</sub> level by HPLC, family studies, or DNA analysis is required.

**71.9.9.7  $\beta$ -Thalassemia/Hb E.** Hb E/ $\beta$ -thalassemia is a common disease in Thailand and parts of Southeast Asia. It results in a variable clinical picture similar to that of homozygous  $\beta$ -thalassemia, ranging from a condition indistinguishable from thalassemia major to a mild form of thalassemia intermedia. The severest conditions are

found in individuals with Hb E and  $\beta^0$ -thalassemia, who usually have about 50–70% Hb F, the remainder being Hb E. Hemoglobin levels may be as low as 4–5 g/dl, and the clinical management of these patients is similar to that for those with thalassemia major. Compound heterozygotes for Hb E and  $\beta^+$ -thalassemia usually have a milder disorder and produce variable amounts of Hb A, with the mildest phenotype resulting from mild  $\beta^+$ -type mutations such as  $-28 (A \rightarrow G)$  and codon 19 ( $G \rightarrow A$ ), although some patients with severe  $\beta^+$ -type mutations such as IVS I-5 ( $G \rightarrow C$ ) and IVS II-654 ( $C \rightarrow T$ ) can produce symptoms as severe as Hb E/ $\beta^0$ -thalassemia (27). As with homozygous  $\beta$ -thalassemia, the genetic factors that account for a mild phenotype in some, but not all patients, are mild  $\beta^+$ -type mutations, the co-inheritance of  $\alpha$ -thalassemia, and the homozygosity for the XmnI restriction site due to the  $C \rightarrow T$  polymorphism at position  $-158 5'$  to the  $\gamma$ -globin gene.

**71.9.9.8  $\delta\beta$ -Thalassemia/Hb S.** Hb S/ $\delta\beta$ -thalassemia is a milder form of sickle-cell disease than sickle-cell anemia, because the high percentage of Hb F (15–25%) produced by the  $\delta\beta$ -thalassemia allele protects against red cell sickling by reducing the Hb S concentration and inhibiting its polymerization. Hb S/ $\delta\beta$ -thalassemia has been described in Sicilian, Italian, Greek, Arab, and African-American individuals. Patients have a mild anemia with a Hb concentration in the range of 10–12 g/dl, a significantly reduced MCH and MCV, Hb S, Hb F, and a normal or low Hb A<sub>2</sub> level.

**71.9.9.9 HPFH/Hb S.** Patients doubly heterozygous for Hb S and HPFH are either asymptomatic or have an extremely mild form of sickle-cell disease. There is usually no anemia and patients have very few episodes suggesting sickle-cell crises, although occasional mild bone pain has been reported. The condition is found in individuals of African origin (with either the black HPFH1 or Ghanaian HPFH2 deletions), and also in Asian Indians (with the Indian HPFH3 deletion). Patients have near-normal red cells with 20–30% Hb F in a pancellular distribution. The condition is difficult to distinguish diagnostically from Hb S/ $\delta\beta$ -thalassemia without DNA analysis because many patients have reduced red cell indices due to co-inherited  $\alpha^+$ -thalassemia.

## 71.9.10 Management of Thalassemia

**71.9.10.1 Prevention.** The hemoglobin disorders are the most common clinically serious single gene disorders in the world. About 250 million people are carriers and about 300,000 affected homozygotes for thalassemia and sickle-cell disease are born each year, the majority in countries with limited resources. Because the treatment for affected patients is still not perfect and very expensive, from the public health perspective the most important means of controlling thalassemia is prevention by antenatal screening and prenatal diagnosis. Most countries have implemented prevention programs,



based on the identification of couples at risk by carrier screening, the provision of information by nondirective counseling, and the option of prenatal diagnosis to allow the termination of an affected fetus if required. Such programs have been extremely successful in the populations at risk in the Mediterranean area, leading to a marked decline in the incidence of thalassemia major.

In England, the Department of Health has recently implemented the NHS Sickle Cell and Thalassemia Screening Programme in order to improve the provision of screening services. This consists of two linked screening programmes for hemoglobinopathies: sickle cell screening of all newborns and sickle cell and thalassemia screening during pregnancy. The antenatal screening program has two approaches, each with its own screening algorithm, depending on whether the local population to be covered by the screening laboratory is defined as low prevalence or high prevalence (affected births greater than 1.5 per 10,000 pregnancies). The screening program is designed to detect the main hemoglobinopathy carrier states that combine to give rise to the risk of an affected fetus:  $\alpha^0$ -thalassemia,  $\beta$ -thalassemia,  $\delta\beta$ -thalassemia, and Hb's C, D-Punjab, E, Lepore, and S (28).

From 1975 to 1980, prenatal diagnosis of various thalassemias was done exclusively by the analysis of the relative synthesis of globin chains in fetal blood. The procedure usually resulted in adequate fetal blood samples (>90%) with few technical errors. However, the method is associated with a 6% risk of fetal loss and cannot be performed until 18 weeks of gestation, which means a long wait for the mother for a result and, if indicated, a relatively difficult elective abortion. Despite the disadvantages inherent in the method, it proved very successful with over 13,000 cases being carried out in over 20 diagnostic centers worldwide (1) until molecular diagnostic techniques were introduced.

In 1978, prenatal diagnosis of sickle-cell disease by restriction endonuclease mapping of fetal DNA from amniotic fluid cells was shown to be possible, quickly followed by the application of the same approach to  $\beta$ -thalassemia, although this was still a second-trimester procedure. However, in 1982, the first case of a first-trimester fetal diagnosis using chorionic villus DNA was achieved and this approach quickly became the method of choice for prenatal diagnosis worldwide. Chorionic villi were a better source of fetal DNA than amniocytes and the first-trimester diagnosis enables an easier therapeutic termination if required. The application of PCR methods decreased diagnostic turn around times dramatically and also increased the accuracy of the diagnosis. Commonly used diagnostic techniques for prenatal diagnosis include ASO hybridization with dot blots or reverse dot blots, restriction enzyme digestion of amplified product, allele-specific priming techniques, denaturing gradient gel electrophoresis, gap-PCR, and direct nucleotide sequencing of amplified globin gene product (29). For the future, microarray technology offers the

most potential for the mass screening of mutations combined with automation, but may not find many applications for prenatal diagnosis because of the relatively high costs of this technology (30).

Recent developments in prenatal diagnosis have been directed toward noninvasive methods of fetal sampling and preimplantation diagnosis (PGD). The use of fetal nucleated red blood cells that circulate in the blood of pregnant women was the first approach to be explored. Prenatal diagnosis of sickle-cell disease and  $\beta$ -thalassemia was achieved in 1986 by the analysis of DNA from single cells after enrichment and positive identification for removal from a slide by microdissection. However, this approach has not been repeated with great success and remains technically very difficult (31). Current research is targeting better specific fetal cell markers and other ways of isolating single fetal cells, such as noncontact laser capture microdissection. One successful application of this technique has been the prenatal detection of Hb Bart's hydrops fetalis syndrome by antibody staining of fetal erythrocytes in maternal blood for  $\alpha$ - and  $\zeta$ -globins, as these cells from an affected fetus cannot express  $\alpha$ -globin (32).

The discovery of fetal DNA in maternal plasma offers a much simpler and more robust approach to noninvasive prenatal diagnosis, as the DNA is cleared very rapidly from the maternal plasma post partum and enrichment steps are desirable but not absolutely required (33). The use of this DNA poses a significant technical challenge, as the cell-free DNA is very fragmented (less than 300 bp in size) and is present in much lower amounts compared to the maternal cell free DNA and therefore highly sensitive detection methods are required. The technique is being used for the prenatal diagnosis of sex-linked diseases and for determining the fetal RhD blood group type (34). However, for the hemoglobinopathies the rate of progress has been much slower, and for several years the technique was only applied for prenatal diagnosis of the thalassemias (and other recessive disorders) by the exclusion of the paternally derived allele (35). The use of linked markers offers the potential to diagnose both maternal and paternal alleles, but probably the most exciting development with this approach is the application of the new technologies of digital PCR and next-generation sequencing techniques for prenatal diagnosis by measuring the balance of the mutant and normal alleles (36).

PGD for  $\beta$ -thalassemia is now offered by a growing number of diagnostic centers around the world. The technique represents a "state-of-the-art" procedure that potentially avoids the need to terminate affected pregnancies. The approach is especially useful for couples who have already had one or more elective terminations, and also for couples for whom religious or ethical beliefs will not permit the termination of pregnancy. The two main approaches are by polar body DNA analysis and by the analysis of DNA from a blastomere

biopsy from a cleavage stage embryo (37). The PCR techniques must be able to diagnose the required genotype in single cells reliably and accurately, in order to avoid the problem of allele drop out, which could lead to a misdiagnosis. PGD is a technically challenging, multistep, and expensive procedure, with a low overall success rate and is not likely to become a routine alternative to conventional prenatal diagnosis for couples in most countries. However, a small number of centers around the world are now set up to carry out this procedure and PGD has been used successfully for both  $\alpha$ -thalassemia (38) and  $\beta$ -thalassemia (39). One specific use of this approach is to allow the birth of a normal child that is HLA identical to a sibling affected with  $\beta$ -thalassemia, thus permitting a possible cure by stem cell transplantation (40).

**71.9.10.2 Therapy.** Advances in the management of thalassemia major have greatly improved the prognosis for patients with this disease, although its management remains complex, difficult, and very expensive. Regular transfusion and optimum iron chelation therapy enables patients to survive at least to the third decade with a normal lifestyle in countries able to afford the treatment program, and survival to the fourth decade is now common. However, in the third world countries where thalassemia reaches its highest frequency, widespread adoption of this expensive treatment is not possible and the problem of thalassemia remains almost untouched. Revised guidelines for the clinical management of thalassemia have recently been published and can be downloaded from the Thalassemia International Federation Web site ([www.thalassemia.org.cy](http://www.thalassemia.org.cy)) (41).

**Anemia.** Treatment for severe  $\beta$ -thalassemia is primarily symptomatic. If anemia is severe enough, transfusions are required to maintain adequate levels of hemoglobin. Thalassemia major children require regular transfusions to maintain the pretransfusion hemoglobin at a target between 9.0 and 10.5 g per 100 ml. This approach may require 2–3 units every 2–4 weeks in adults. If started early in life this hypertransfusion regimen prevents all the bone malformations and abnormalities characteristic of untransfused thalassemia major children. Patients are transfused with washed or frozen red cells uncontaminated by white cells and plasma proteins to avoid the development of severe reactions to transfusion caused by leukocyte sensitization.

**Iron Accumulation.** Hypertransfusion is associated with substantial iron loading. Chronic iron overload can cause cardiac, liver, and endocrine problems, and eventually death. Iron chelation therapy is used to prevent this side effect of chronic transfusion. Transfused patients who do not receive or do not accept chelation therapy accumulate in excess of 70 g of iron by the second decade of life. This is almost a lethal dose and cardiac failure due to cardiac hemosiderosis remains the leading cause of death in thalassemia patients. The first drug available for treatment of iron overload was

desferrioxamine (Desferal). This drug is not orally absorbed and is injected subcutaneously into the abdominal wall by overnight infusion by a pump; this treatment is cumbersome and cannot be tolerated by many patients, resulting in poor compliance. It is an extremely costly regimen and completely beyond the means of less developed countries.

The cost, inconvenience, and difficulty of desferrioxamine therapy have driven the search for cheaper orally administered iron chelating drugs. A number of potential oral iron chelators have been identified but only two, the hydroxypyridone compound known as L1 or deferiprone and the tridentate bis-hydroxyphenyl-tiazole compound known as Deferasirox (ICL670, Exjade), are now in widespread clinical use following extensive clinical trials. These studies have shown that deferiprone is about 70% effective as desferrioxamine at iron excretion and results in a decrease of serum ferritin levels, although a number of treatable side effects have been observed, including nausea, agranulocytosis, and neutropenia (42). Deferiprone is not as effective in controlling hepatic iron concentration but is more effective than desferrioxamine in the removal of myocardial iron (43) and treatment is associated with reduced cardiac morbidity (44). Deferiprone has been licensed for use in 25 countries as a cheap, orally active alternative to desferrioxamine, but is not licensed in North America.

Desferrioxamine and deferiprone can be used in combination therapy to achieve levels of iron excretion that cannot be achieved by either drug alone. Combination therapy decreases the number of desferrioxamine infusion days, thus improving compliance and quality of life and appears to be effective, well tolerated, and acceptable to patients (45). Trials evaluating combined therapy versus desferrioxamine monotherapy have shown a significant improvement in myocardial T2\* levels and combination therapy should be considered for patients who comply poorly with desferrioxamine monotherapy or high-risk cases with particularly low myocardial T2\* values or with very high levels of iron overload (46).

The second oral chelator to achieve approval, Deferasirox, is a member of a new class of tridentate iron chelators, the bis-hydroxyphenyl-triazoles. Deferasirox (ICL670, Exjade), is a once-daily administered oral iron chelator, which has shown to be effective in both adults and children in extensive clinical trials. Studies have shown it is as effective as desferrioxamine but without the adverse effects of deferiprone (47). Common adverse events were generally mild, including transient gastrointestinal events and skin rashes (48). The drug is now licensed for use in most countries for use in children aged 2 years and older with transfusional iron overload. However, the cost of therapy using deferasirox is very high, with the cost per patient per year calculated to be as high as \$80,000, which poses problems for the developing world where the majority of patients live (49).

Deferiprone is much less expensive than deferasirox, but must be taken several times a day and requires more safety monitoring (50).

Other side effects of transfusions include hepatitis and cytomegalovirus infections. Isoimmunization to minor blood groups may occur, but careful selection of donors may decrease this risk. Sensitization to white cell or plasma antigens may be decreased by using blood with the white cells removed. Urticaria may be treated with antihistamines, as well as epinephrine, prior to and during transfusion. Febrile reactions may require antipyretics and occasionally, if severe, steroids. Recombinant  $\alpha$ -interferon is the only effective therapy for hepatitis C infection, being effective only in about 25% of infected patients. However, when combined with ribavirin, a guanosine analog, it produces sustained viral clearance in up to 45% of younger thalassemia major patients. Finally, the increased rate of erythropoiesis can lead to increased folic acid requirements. If folate deficiency occurs, it may cause increased anemia; however, deficiency is easily avoided by daily oral administration of folic acid.

**71.9.10.3 Infection.** Splenectomy may be avoided by hypertransfusion therapy; many patients reaching adolescence who received early hypertransfusion therapy do not require it. However, splenectomy may be necessary to alleviate hypersplenism with worsening of the anemia or pain due to progressive splenomegaly or infarction of the spleen. Splenectomy should be delayed until the age of 5 years because, as discussed previously, splenectomized children, especially those under 6 years of age, are at risk for life-threatening infections. Pneumococcal vaccine, as well as prophylactic penicillin, should be used in such children, and suspected infections should be treated aggressively.

**71.9.10.4 Bone Marrow Transplantation.** Bone marrow transplantation offers the only cure for  $\beta$ -thalassemia major. Allogeneic bone marrow transplantation using related donors has been used in a large number of patients, predominantly in Europe, with studies showing approximately 80% of patients surviving long term and of these, nearly 90% being cured (3). Better outcomes are associated with adequate iron chelation and the absence of significant hepatic disease, although survival and disease-free survival rates still vary considerably between different centers. The post-transplant complications involve acute and chronic graft-versus-host disease and the persistence of host hematopoietic cells (mixed chimerism). The major limitation of bone marrow transplantation is the lack of an identical HLA-identical sibling donor for most affected patients. Future and experimental approaches to marrow transplantation in patients with thalassemia include transplantation from matched unrelated donors, cord blood transplantation, and in utero transplantation. Good success rates have been reported for a limited number of transplantations from carefully matched unrelated donors (51) and umbilical cord blood

transplantation from a related donor offers a good possibility of success with a lowered risk of graft-versus-host disease (52).

In theory, gene therapy using autologous bone marrow has the potential to cure thalassemia major permanently without the limitations of finding a matched donor or the risk of graft-versus-host disease. In practice, progress on gene therapy for  $\beta$ -thalassemia has been slow as globin gene vectors with the required regulatory elements have proved challenging to design and produce (53). However, a major advance was achieved when a lentiviral vector was used to correct mouse  $\beta$ -thalassemia (54) and current research is focussed on enhancing the safety and efficiency of lentivirus vectors for correcting of the molecular defect in hematopoietic stem cells or the use of homologous recombination instead of gene transfer. A clinical trial using a lentiviral vector is in progress in France while other trials are in development (55).

**71.9.10.5 Augmentation of Fetal Hemoglobin.** As in sickle-cell syndromes, a number of pharmacological agents are being used to elevate hemoglobin F in patients with homozygous  $\beta$ -thalassemia with the aim of increasing Hb F to levels high enough to prevent all complications of the disease. These agents, including 5-azacytidine, hydroxyurea and butyrate compounds, induce Hb F synthesis by different mechanisms (56). Although a significant amelioration of the anemia has been achieved in some thalassemia patients, the effects of treatment have been more modest compared to the treatment of sickle-cell disease, and better agents that can induce higher levels of Hb F are still needed.

The first trials with both hydroxyurea and butyrate derivatives showed only modest increases in hemoglobin without any apparent beneficial effects, apart from the case of one patient with Hb Lepore/ $\beta$ -thalassemia. Further studies with hydroxyurea treatment of patients with thalassemia intermedia and Hb E/ $\beta^0$ -thalassemia showed a small but significant increase of both Hb F and hemoglobin concentration (57), which could be increased further with a combination therapy of hydroxyurea and sodium phenylbutyrate. However, the treatment of patients with thalassemia major appears to be less effective (58). Although it has been reported that some patients have responded with a sufficient rise in Hb level to shift the patient into stable transfusion-free condition, the majority do not (59). A study of the treatment with hydroxyurea of Iranian thalassemia major patients has revealed that the responsive patients were those with mutations linked to specific  $\beta$ -gene haplotypes, in particular the ones linked to the -158  $G\gamma$  C→T nondeletion HPFH allele (the XmnI polymorphism) (60). More long-term in vivo trials on Hb F inducers are required to investigate dosing regimens and combinations of drugs, together with the identification of specific  $\beta$ -thalassemia mutations that may be particularly responsive to therapy.



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### Biography



**Dr John Old** is a consultant clinical scientist in the Molecular Haematology department of the John Radcliffe Hospital in Oxford, UK, and holds the honorary post of Reader in Haematology at Oxford University. He is the Director of the National Hemoglobinopathy Reference Service, established in 1982 by the Department of Health to carry out first trimester prenatal diagnosis for the hemoglobinopathies, and diagnose difficult diagnostic cases by DNA analysis. He is registered as a clinical scientist with the Health Professions Council and is a Fellow of the Royal College of Pathologists.

After completing a PhD in biochemistry at Liverpool University in 1974, he worked as a research scientist in Professor Sir David Weatherall's laboratory at the University of Oxford for 8 years, investigating the molecular basis of the thalassemias and the diagnosis of globin gene mutations, before changing direction to set up a molecular diagnostic laboratory for hemoglobinopathies and develop new diagnostic techniques for carrier detection and prenatal diagnosis. He has collaborated with many international laboratories on thalassemia research and diagnostic techniques, including assisting centers in India and Cyprus to set up national screening and prevention programmes for  $\beta$ -thalassemia, and has published 165 papers and articles on the subject of the hemoglobinopathies. He has also held many professional appointments, including Vice-President of the Royal College of Pathologists, a Director of the Board of Clinical Pathology Accreditation (UK) Ltd and a Council Member of the Health Professions Council.

# CHAPTER

# 72

## Other Hereditary Red Blood Cell Disorders

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### 72.1 HEMOLYSIS DUE TO HEREDITARY RED BLOOD CELL ENZYME DISORDERS

The majority of erythrocyte enzyme abnormalities associated with hemolysis is due to gene mutations that cause deficiency of an enzyme critical for red blood cell (RBC) function. The enzyme deficiency in these cases may be due to decreased enzyme synthesis, the presence of an enzyme with abnormal kinetics, or the presence of a physically unstable enzyme whose function changes as cells age (which is important since mature, anucleate erythrocytes are incapable of new protein synthesis). In rare cases hemolysis is not due to an enzyme deficiency but rather a consequence of increased enzyme activity that secondarily has deleterious effects on cell metabolism (e.g. adenosine deaminase (ADA) excess). In addition to enzymopathies that cause hemolysis, some enzyme deficiencies have no adverse effects on RBC function (e.g. enolase, lactic dehydrogenase, and 6-phosphogluconate dehydrogenase deficiency), or if they occur in patients with hemolytic anemia, it is not clear that the enzyme deficiency and hemolysis are causally related (e.g. glutathione peroxidase and glutathione reductase deficiency). The major causes of hemolysis due to hereditary RBC enzyme defects are abnormalities in the hexosemonophosphate (HMP) shunt and glutathione metabolism, glycolytic enzyme deficiencies, and abnormalities in purine and pyrimidine metabolic enzymes (Figure 72-1).

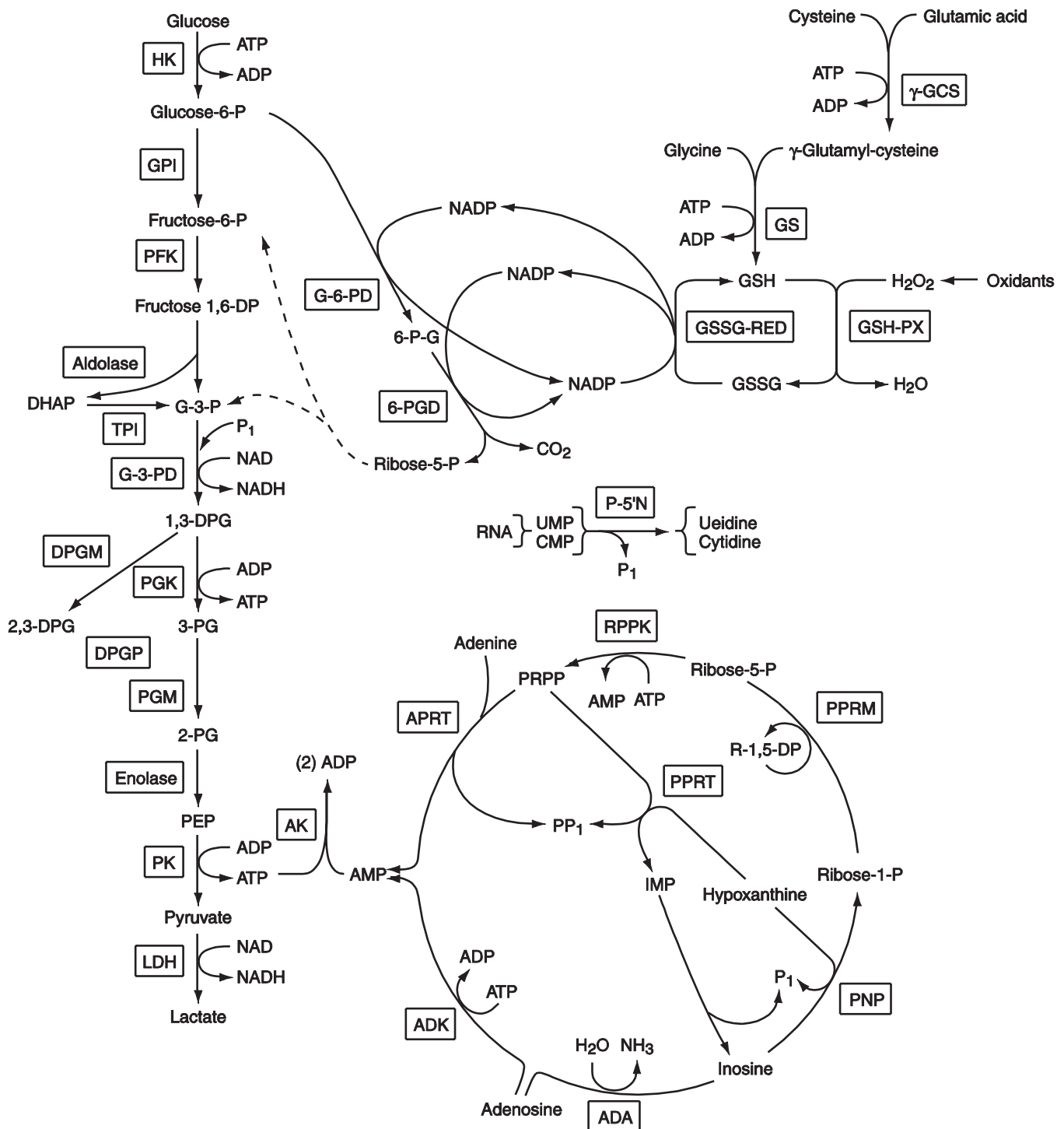
#### 72.1.1 Hemolysis due to Disorders of the Hexosemonophosphate Shunt and Glutathione Metabolism Pathway

The HMP shunt pathway metabolizes 5–10% of the glucose utilized by RBCs and this is critical for protecting RBCs against oxidant injury. The HMP pathway is the only source of reduced nicotinamide adenine dinucleotide phosphate (NADPH), a cofactor important in

glutathione metabolism. RBCs contain relatively high concentrations of reduced glutathione (GSH), a sulfhydryl containing tripeptide ( $\gamma$ -glutamylcysteinylglycine) that functions as an intracellular reducing agent, protecting cells against oxidant injury. Oxidants, such as superoxide anion ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ), are produced by exogenous factors (i.e. drugs, infection) and also are formed within red cells as a consequence of reactions of hemoglobin with oxygen. Under conditions where these oxidants accumulate within the RBC, cellular proteins are altered thereby leading to lethal cell injury. Under normal circumstances this does not occur since GSH, in conjunction with the enzyme glutathione peroxidase (GSH-Px), rapidly inactivates these oxidants. During the detoxification process, however, GSH itself is converted to oxidized glutathione (GSSG), and GSH levels fall. To sustain protection against persistent oxidant injury, GSH levels must be maintained, and this is accomplished by glutathione reductase (GSSG-Rx), which catalyzes the reduction of GSSG to GSH. This reaction requires the NADPH generated by glucose-6-phosphate dehydrogenase (G6PD), the first enzymatic reaction of the HMP shunt. Thus, it is the tight coupling of the HMP shunt and glutathione metabolism that is responsible for protecting intracellular proteins from oxidative assault. Almost all hemolytic episodes related to altered HMP shunt and glutathione metabolism are due to G6PD deficiency. Rare cases of hemolysis associated with decreased GSSG-Rx activity, GSH-Px deficiency, and deficiencies of GSH synthetic enzymes have been described.

##### 72.1.1.1 Glucose-6-Phosphate Dehydrogenase Deficiency.

**72.1.1.1.1 Prevalence and Geographic Distribution.** This is the most common RBC enzyme abnormality associated with hemolysis (1–4). This RBC enzyme disorder affects millions of people throughout the world,



**FIGURE 72-1** Glycolysis. Enzymes: HK, hexokinase; GPI, glucosephosphate isomerase; PFK, phosphofructokinase; TPI, triosephosphate isomerase; G3PD, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; DPGM, 2,3-diphosphoglycerate mutase; DPGP, 2,3-diphosphoglycerate phosphatase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; LDH, lactate dehydrogenase. Substrates: 1,3-DPG, 1,3-diphosphoglycerate; G-3-P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; HMP shunt and glutathione metabolism. Enzymes: G-6-PD, glucose-6-phosphate dehydrogenase; 6-PGD, 6-phosphogluconate dehydrogenase; GSSG-Red, glutathione reductase; GSH-Px, glutathione peroxidase; GS, glutathione synthetase; γGCS, gamma-glutamyl-cysteine synthetase. Substrates: G-3-P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; R-5-P, ribose-5-phosphate; 6-PG, 6-phosphogluconate; GSH, reduced glutathione; GSSG, oxidized glutathione. Purine and pyrimidine metabolism. Enzymes: ADA, adenosine deaminase; ADK, adenosine kinase; AK, adenylylate kinase; APRT, adenine phosphoribosyl transferase; RPPK, ribopyrophosphoryl kinase; PPRM, pyrophosphoribosyl mutase; PPRT, pyrophosphoribosyl transferase; PNP, purine nucleoside phosphorylase; P-5'N, pyrimidine 5' nucleotidase. Substrates: AMP, adenosine monophosphate; IMP, inosine monophosphate.



the highest frequency occurring in Mediterranean countries, Africa, and Asia. Approximately 10–15% of African-Americans are deficient in erythrocyte G6PD activity, and this is similar to the 20% incidence reported in Nigeria. In contrast, the incidence of G6PD deficiency in Mediterranean individuals is much more variable, ranging from approximately 2% in Sicily to over 20% in the Greek Island of Rhodes. As many as 60–70% of Kurdish Jews may be affected and in the Asian male population, the incidence of G6PD deficiency is estimated to be 14% in Cambodia, 6% in South China, 3% in India, and less than 0.1% in Japan.

It is intriguing that the worldwide distribution of G6PD deficiency is similar to that for malaria, and it has long been considered that the gene for G6PD deficiency may be protective against malaria (5). Partial indirect support for this is that G6PD deficiency in Sardinia is more common at sea level compared to higher elevations, and this also parallels the endemicity of malaria. In addition, it has been observed that female heterozygotes for G6PD deficiency (who therefore have normal and G6PD-deficient RBC) have more malaria parasites in normal erythrocytes compared to their own G6PD-deficient cells. The reason for this balanced polymorphism is unknown and remains a subject for interesting speculation.

**72.1.1.1.2 Biochemistry.** Several hundred different variants of G6PD have been described, each of which is thought to differ in their biochemical properties (e.g. kinetic activity, electrophoretic mobility, pH optima, and/or affinity for substrate and/or cofactor). The World Health Organization (WHO) has classified the different G6PD variants on the basis of enzyme deficiency and severity of hemolysis (6):

Class I: severe deficiency with chronic hemolytic anemia;

Class II: severe deficiency with intermittent hemolysis;

Class III: moderate deficiency with intermittent hemolysis;

Class IV: no deficiency or hemolysis.

The normal wild-type enzyme is designated G6PD<sup>B</sup> and is found in almost all Caucasians and a majority of black people. It has normal catalytic activity and is not associated with hemolysis (class IV). In addition, there are over 400 different biochemical variants that are designated by geographic or trivial names. Commonly encountered variants include the following:

- (1) G6PD<sup>A+</sup> is found in 20% of African-Americans. It has normal catalytic properties and does not cause hemolysis (class IV). It differs from G6PD<sup>B</sup> in that it has a much faster electrophoretic mobility. The structure of G6PD<sup>A+</sup> differs from that of G6PD<sup>B</sup> by the substitution of one amino acid, an asparagine for aspartate.
- (2) G6PD<sup>A-</sup> is the most common variant associated with mild to moderate hemolysis (class III), and is found in 10–15% of African-Americans. Its electrophoretic

mobility is identical to that of G6PD<sup>A-</sup>. This is an unstable enzyme and its catalytic activity is decreased in older RBCs. Hence, this variant is designated G6PD<sup>A-</sup> compared with G6PD<sup>A+</sup>.

- (3) G6PD<sup>Mediterranean</sup> is the most common abnormal variant found in Caucasians, particularly those whose origins are in the Mediterranean area and the Middle East. The electrophoretic mobility of G6PD<sup>Mediterranean</sup> is identical to that of G6PD<sup>B</sup>, but its catalytic activity is markedly reduced and hemolysis can be severe (class II).

- (4) G6PD<sup>Canton</sup> is a variant seen in Asians. Its biochemical properties are very similar to those of G6PD<sup>Mediterranean</sup>.

Advances in molecular biology have enhanced our understanding of the abnormalities of G6PD deficiency (4,6). In view of the numerous biochemical variants that have been described, it is no surprise that over 100 different point mutations and deletions in the G6PD gene have been identified (7,8). However, large deletions or frameshift mutations have not been identified, suggesting that complete absence of G6PD might be lethal. The mutations on the gene do not appear to be random and most of the variants associated with chronic hemolysis (class I) have abnormalities in the NADP or glucose-6-phosphate binding site of the gene.

An interesting example of how molecular biology has enhanced our understanding relates to G6PD<sup>A-</sup>. This once was thought to represent a single unstable variant found in black people throughout the world. However, molecular analysis now has demonstrated that G6PD<sup>A-</sup> may have more than one genotype. In all cases there is a mutation at nucleotide 376 (A→G), which also is the nucleotide substitution characteristic of G6PD<sup>A+</sup>. In addition, however, the G6PD<sup>A-</sup> variants have a second mutation, and in the majority of cases it is at nucleotide 202 (G→A), while in some the second substitution is at nucleotide 680 (G→T) or at nucleotide 968 (T→C). Thus, the G6PD<sup>A-</sup> variant, once thought to be a single homogeneous mutation in Africans, now turns out to represent at least three different genotypes (6). Moreover, a number of G6PD variants originally described in non-Africans are now found to have one of the known G6PD<sup>A-</sup> mutations (Table 72-1). For example, G6PD<sup>Betica</sup>, a Spanish variant, and G6PD<sup>Matera</sup>, an Italian variant, have demonstrated base substitutions at nucleotides 202 and 376, identical to the more common G6PD<sup>A-</sup> variant. They are examples, therefore, of G6PD<sup>A-</sup>. There are several other variants that appear clinically heterogeneous but have been found to be genetically more uniform. For example, G6PD<sup>Mediterranean</sup> involves many different ethnic groups, although most subjects have the same genetic defect, a single base substitution (C→T) at nucleotide 563. Moreover, just as in the case of G6PD<sup>A-</sup>, many of the known biochemical variants turned out to have the same molecular defect as G6PD<sup>Mediterranean</sup> (Table 72-1).

**TABLE 72-1 Biochemical, Epidemiological, and Clinical Features of Select G6PD Variants**

AG6PD Variant Classification	Nucleotide Substitution		Amino Acid Substitution		Population	WHO Classification
A <sup>-</sup>	202	G→A	68	Val Met	Africa, Italy, Spain;	3
Alabama	376	A→G	126	Asn→Asp	Canary Islands, Mexico	
Betica						
Ferrara						
Septic						
A <sup>+</sup>	376	A→G	126	Asn→Asp	Africa	4
A <sup>-</sup>	376	A→G	126	Asn→Asp	Africa, Spain; Canary Islands	3
	680	G→T	227	Asn→Asp		
A <sup>-</sup>	376	A→G	126	Asn→Asp		3
Betica	968	T→C	387	Arg→Cys		
Selma						
Mahidol	487	G→A	163	Gly→Asp	Southeast Asia, China, Taiwan	3
Mediterranean	563	C→T	188	Ser→Phe	Italy, Greece, Saudi Arabia	2
Birmingham					Iran, Iraq, Israel, Egypt	
Cagili						
Dallas						
Panama						
Sassari						

Data from Glader (2004) modified from Beutler (1991, 1994) and Miwa and Fujii (1996).

**72.1.1.1.3 Pathophysiology.** As normal RBCs age, the intracellular activity of G6PD<sup>B</sup> decreases. Despite this loss of enzyme activity, normal old RBCs contain sufficient G6PD activity to generate NADPH and thereby maintain GSH levels in the face of oxidant stress. The defect in Africans with G6PD deficiency is due to increased enzyme instability of G6PD<sup>A-</sup> such that young RBCs have relatively normal enzyme activity, whereas older red cells are severely G6PD deficient. The clinical correlate of this is that hemolysis in patients with G6PD<sup>A-</sup> generally is mild and is limited to the older deficient erythrocytes. In contrast, the enzymatic defect in G6PD<sup>Mediterranean</sup> is due to a much greater enzyme instability, and RBCs of all ages are grossly deficient. Consequently, the entire RBC population of individuals with G6PD<sup>Mediterranean</sup> is susceptible to oxidant-induced injury, and this can lead to severe hemolysis.

The clinical observation that the severity of hemolysis due to G6PD deficiency is worse in males compared to females is consistent with the fact that the gene for G6PD is located on the X chromosome. Males have only one type of G6PD, whereas females may have two different biochemical types of enzyme. According to the Lyon hypothesis, however, only one X chromosome is active in any given somatic cell (9). Thus, although females may have two different G6PD variants, any given RBC contains only one type of G6PD. Depending on the degree of lyonization, the mean RBC enzyme activity in females who carry a gene for G6PD deficiency may be normal, moderately reduced, or grossly deficient. The G6PD-deficient cells in females, however, are as vulnerable to hemolysis as are the enzyme-deficient RBCs in males.

**72.1.1.1.4 Clinical Features.** With the most prevalent G6PD variants (G6PD<sup>A-</sup> and G6PD<sup>Mediterranean</sup>),

severe hemolysis occurs only after exposure to certain offending agents. The steady state is not associated with anemia or any alteration in blood morphology. Sudden destruction of the older, more deficient erythrocytes is triggered by drugs having a high redox potential and by selected infectious or metabolic perturbations. The characteristics of an acute hemolytic episode are sudden pallor, jaundice, dark urine with or without abdominal and back pain, and an abrupt decrement of 3–4 g/dl in hemoglobin concentration. In response to anemia, RBC production increases and an increase in reticulocytes is apparent within 5 days after the onset of hemolysis. In cases of G6PD<sup>A-</sup> the anemia is self-limited because the older, vulnerable population of erythrocytes is replaced by younger RBC with sufficient G6PD activity to withstand an oxidative assault. In contrast, the hemolysis occurring with G6PD<sup>Mediterranean</sup> is more severe because this variant enzyme has a very short intraerythrocytic half-life, and thus a larger population of circulating erythrocytes is vulnerable to injury. For practical purposes, the most important consequences of G6PD deficiency are limited to the RBCs. In rare cases of G6PD deficiency in Caucasians, there is a concurrent decrease in the enzyme activity of leukocytes, but this usually is of little clinical significance.

**72.1.1.1.5 Drugs.** The discovery of G6PD deficiency as a cause of hemolysis originally followed the observation that some African-American soldiers developed hemolysis after receiving primaquine phosphate for malaria prophylaxis (10). Subsequently, numerous other drugs have been implicated as causative agents and the common denominator of these drugs is that they interact with hemoglobin and oxygen, thus accelerating the intracellular formation of H<sub>2</sub>O<sub>2</sub> and other oxidizing radicals.

**72.1.1.1.6 Infections.** Hepatitis, salmonellosis, and pneumonia are established inciting causes of hemolysis in G6PD deficiency, but virtually any type of infection can lead to accelerated destruction of G6PD-deficient red cells. A reasonable explanation for this relationship between infection and hemolysis is that oxidants generated by phagocytosing macrophages diffuse into the extracellular medium and pose an oxidative threat to G6PD-deficient erythrocytes.

**72.1.1.1.7 Favism.** The fact that exposure to the fava bean (*Vicia fava*, broad bean) is toxic and potentially fatal for some individuals allegedly has been known since the time of Pythagoras. Hemolysis following exposure to fava beans occurs in G6PD-deficient Caucasians and Asians, but rarely ever is seen in African individuals. It occurs primarily in children. Hemolytic episodes can occur after exposure to fava bean pollen, after ingesting fresh fava beans, and following consumption of dried beans. It also can occur in babies who nurse following maternal exposure (11). It is thought that unstable pyrimidine glycosides (isouramil and divicine) in fava beans are the active oxidants responsible for hemolysis (12). Favism does not occur in all susceptible G6PD individuals, and it is thought that an additional genetic factor is involved, presumably related to how fava bean oxidants are metabolized.

**72.1.1.1.8 Neonatal Jaundice.** Hyperbilirubinemia is a well-documented consequence of G6PD deficiency (class II) in Caucasian and Asian neonates. This hyperbilirubinemia often occurs in the absence of any obvious exogenous oxidant stress, and there often is no evidence of hemolysis. It is now recognized that very severe hyperbilirubinemia in neonates with G6PD deficiency often occurs in those who also carry the gene for Gilbert syndrome (13,14). African-American infants with G6PD<sup>A-</sup> appear to have a much reduced incidence and severity of hyperbilirubinemia, although significant hemolysis and severe hyperbilirubinemia can occur (15). Of interest, black infants in Africa have an increased incidence of neonatal hyperbilirubinemia, also associated with kernicterus and severe neurologic injury or death. Since black Africans and African-Americans have the same G6PD<sup>A-</sup> genotypes, the adverse outcomes in Africa are thought to relate to local customs and differences in oxidant exposure. This is also seen with other ethnic groups. The incidence of hyperbilirubinemia in G6PD-deficient infants born in Australia to Greek immigrants is lower than that noted in deficient infants in Greece (16). Herbs used in traditional Chinese medicine, clothing impregnated with naphthalene, and chemicals taken by the mother in late gestation all have been implicated as the inciting oxidant in selected cases of G6PD deficiency.

**72.1.1.1.9 Diagnosis.** The diagnosis of G6PD deficiency is made by a spectrophotometric assay or screening test that utilizes hemolysate as a source of enzyme. Regardless of the specific test used, however, false-negative reactions may occur if the most enzymatically

deficient RBCs have been removed by hemolysis. This generally is not critical in testing male Caucasians and Asians, but can be a problem in diagnosing people of African heritage, especially during the reticulocytosis following acute hemolysis. In these cases it sometimes is necessary to wait until the hemolytic crisis is over and reevaluate for G6PD deficiency in 2–3 months when the RBC mass has been repopulated with cells of all ages.

**72.1.1.1.10 Treatment.** RBC transfusions are appropriate in those rare circumstances in which signs and symptoms of anemia indicate severe cardiovascular compromise. In the usual case, however, therapeutic endeavors are directed toward minimizing potential sources of oxidant stress. The most common drugs implicated are seen in Table 72-2. Most individuals with G6PD deficiency have an almost normal RBC survival and hemolytic anemia occurs only with oxidant stress, whereas there are rare individuals with G6PD variants who manifest chronic hemolysis. Common clinical characteristics of these patients with class I type of G6PD deficiency include mild anemia, neonatal jaundice, gallstones, and splenomegaly. These individuals always have evidence of hemolysis (reticulocytosis, hyperbilirubinemia).

**72.1.1.1.11 Screening.** Generalized screening for G6PD deficiency does not exist because the most common variants responsible for acquired hemolytic anemia pose little health hazard. However, recent data from the USA Kernicterus Registry indicate that over 30% of kernicterus cases are associated with G6PD deficiency (17). These observations thus have raised the question of whether G6PD testing should be part of newborn screening programs (18). In the United States, the only required newborn screening for G6PD deficiency occurs in the District of Columbia. It could be argued that certain Mediterranean and Middle East populations might benefit from knowing their G6PD status, and could thereby avoid obvious oxidant exposures. Routine blood bank screening appears to be unwarranted and G6PD deficiency is not considered a major problem in transfusion medicine. However, in premature infants, simple transfusions with G6PD-deficient red cells have been associated with hemolysis and severe hyperbilirubinemia requiring exchange transfusion. Moreover, massive intravascular hemolysis has occurred in an Indian neonate following an exchange transfusion with G6PD-deficient blood (19). In view of these occurrences, it has been recommended that in areas where G6PD deficiency (presumably class II variants) is common, donor blood should be screened for the enzyme before transfusing premature infants or using the blood for a neonatal exchange transfusion. Of interest, hemolysis has occurred in bone marrow transplant recipients who have received their stem cells from a G6PD-deficient donor (20).

**72.1.1.2 GSSG-Rx Deficiency.** Partial deficiency of erythrocyte GSSG-Rx activity, although not associated with hemolysis, has been described in a variety of hematologic disorders, malignancies, liver disease, and

**TABLE 72-2 Compounds Associated with Hemolysis in G6PD Deficiency**

<b>Drugs and Chemicals Unsafe for Class I, II, and III G6PD Variants</b>	
Furazolidone (Furoxone)	Sulfacetamide
Methylene blue	Sulfamethoxazole (gantanol)
Nalidixic acid (Neggram)	Sulfanilamide
Naphthalene (Mothballs)	Sulfapyridine
Nitrofurantoin (Furadantin)	Thiazolesulphone
Phenazopyridine (Pyridium)	Toluidine blue
Phenylhydrazine	Trinitrotoluene (TNT)
Primaquine	
<b>Drugs and Chemicals Safe for Class II and III G6PD Variants<sup>a</sup></b>	
Acetaminophen	Probenecid
Ascorbic acid	Procainamide
Aspirin	Pyrimethamine
Chloramphenicol	Quinidine
Chloroquine	Quinine
Colchicine	Streptomycin
Diphenhydramine	Sulfamethoxypyridazine
Isoniazid	Sulfisoxazole
Menadione sodium bisulfite	Trimethoprim
Phenacetin	Tripeleminamine
Phenylbutazone	Vitamin K
Phenytoin	

<sup>a</sup>In most cases safety for class I G6PD variants is not known.  
Data from Glader (2004) modified from Beutler (1991, 1994).

malnutrition. However, it now appears that most GSSG-Rx deficiencies reported to date are a reflection of riboflavin deficiency since flavin adenine dinucleotide (FAD) is an essential cofactor for maximal GSSG activity. Only one clear case of hemolysis associated with GSSG-Rx deficiency has been described (21).

**72.1.1.3 GSH-Px Deficiency.** Rare cases of hemolysis in association with moderate deficiency of erythrocyte GSH-Px activity have been described in adults, children, and newborn infants. However, the general consensus now is that GSH-Px deficiency may not be a cause of hemolysis or other hematologic problems. Many healthy normal individuals, particularly those of Jewish or Mediterranean ancestry, have reduced GSH-Px activity without evidence of hemolysis (22). Moreover, low GSH-Px activity, in the absence of hemolysis, also has been observed in normal people from New Zealand with selenium (Se) deficiency. (Se is an integral part of GSH-Px.) In view of these observations, it is unlikely that GSH-Px deficiency is a cause of significant hemolysis.

## 72.1.2 Deficiencies of Glutathione Synthetic Enzymes

Glutathione is actively synthesized in RBCs and has an intracellular half-life of only 4 days, in part due to cellular efflux of GSSG. RBCs are capable of de novo GSH synthesis and this is accomplished by two critical enzymes (Figure 72-1). Gamma-glutamyl-cysteine synthetase ( $\gamma$ GCS) catalyzes the first step in GSH synthesis, the formation of gamma-glutamyl-cysteine from glutamic acid

and cysteine. Glutathione synthetase (GS) catalyzes the formation of GSH from glutamyl-cysteine and glycine. In many tissues, but not RBC, these two enzymes are part of the gamma-glutamyl cycle, which is involved with the synthesis and degradation of GSH and also thought to have a role in amino acid transport across cell membranes. Hereditary hemolytic anemia, characterized by reduced GSH content, has been reported in patients with deficiencies of both gamma  $\gamma$ GCS and GS activity.

**72.1.2.1 Gamma-Glutamyl-Cysteine Synthetase Deficiency.** This rare hemolytic anemia was first described in two adults who were brother and sister (23). Both these patients had a lifelong history of anemia, intermittent jaundice, cholelithiasis, and splenomegaly. They also manifested severe neurologic dysfunction, and a generalized aminoaciduria (24). This disorder appeared to be an autosomal recessive condition and, in the family studied, presumed carriers had reduced gamma  $\gamma$ GCS activity, although erythrocyte GSH levels were normal. Hemolytic anemia was seen only in the homozygous state where erythrocyte GSH levels are approximately 5% of normal, and there was markedly reduced  $\gamma$ GCS activity. Subsequent to the initial case report, an additional six patients have been identified (13,25–27). In all these cases hemolysis was the only clinical manifestation and no neurologic abnormalities have been noted since the original case report over 30 years ago (28,29).

**72.1.2.2 GS Deficiency.** This disorder has been described in association with hemolysis in over 70 patients (27,29,30). There are three types of GS deficiencies with different clinical manifestations. In the mild



**TABLE 72-3 Features of Glycolytic and Nucleotide Enzymopathies**

Enzymopathy	Approximate Fraction of Enzymopathies (a)	Mode of Inheritance (b)	Effects of (c)
HK	<1%	AR	Mild/severe CNSHA
GPI	3–5%	AR	Moderate/severe CNSHA
PFK	<1%	AR	Mild CNSHA +/-myopathy
Aldolase	<1%	AR	Mild/moderate CNSHA, psychomotor retardation
TPI	<1%	AR	Moderate/severe CNSHA, severe neurologic deficits
PGK	<1%	X-linked	Mild/severe CNSHA, psychomotor retardation, seizures, extra pyramidal disease
PK (48)	80–90%	AR	Moderate/severe CNSHA
P5'N	2–3%	AR	Moderate CNSHA
ADA excess	<1%	AD	Mild CNSHA
AK	<1%	AR	CNSHA

AR, autosomal recessive; AD, autosomal dominant; CNSHA, chronic nonspherocytic hemolytic anemia.

Data from Glader (2004). Approximate estimates derived from several sources (Beutler 1979; Tanaka and Zerez 1990; Mentzer 2003).

type, significant enzyme deficiency is limited to erythrocytes. The defect is due to mutations that affect the stability of the enzyme. Other nucleated cells that also have this defect can compensate for this instability because they are capable of new protein synthesis. In contrast, anucleate red cells are unable to synthesize more enzyme and GSH levels are depleted. The major manifestations of this deficiency are splenomegaly, intermittent jaundice, and compensated hemolytic anemia. In moderate cases, GS deficiency is generalized to other tissues in addition to red cells. Clinical problems include hemolytic anemia, persistent metabolic acidosis presenting in the newborn period, and overproduction of 5-oxoproline with oxoproluria. In the most severe cases there also are organic neurologic defects and mental retardation (29,31,32). Deficiency of GS appears to be an autosomal recessive disorder. Relatives of affected patients have reduced enzyme activity, but erythrocyte GSH content is normal and there are no hematologic problems. The possibility of this disorder is suspected in patients with hemolytic anemia and markedly reduced RBC GSH content. A case of a newborn with GSS deficiency was recognized when tandem mass spectrometry-based newborn screening revealed greater than a 10-fold elevation of 5-oxoproline in dried blood (33). Virtually no GS activity is detected in homozygous or compound heterozygous deficient individuals. In those GS-deficient individuals with oxoprolinemia, oral bicarbonate administration is necessary to control the acidosis. The gene for GS is located on chromosome 20q11. In one study of 41 patients, 27 different GS mutations were identified and almost all were missense or splice mutations (29).

### 72.1.3 Hemolysis Associated with Glycolytic Enzyme Abnormalities

Since mature RBCs lack mitochondria, glycolysis is the only metabolic pathway capable of adenosine triphosphate (ATP) synthesis (Figure 72-1). The cause of

hemolysis in RBC glycolytic defects is not known, but is thought to be due to decreased function of some vital ATP-dependent process. Hemolytic anemias due to glycolytic enzymopathies are relatively rare, affecting only a few thousand individuals in the world (1,34,35). Abnormalities in virtually every glycolytic enzyme have been described, although 80–90% of all cases associated with hemolysis are due to pyruvate kinase deficiency (Table 72-3). In some of these disorders, the enzyme deficiency is not limited to RBCs but also includes leukocytes, platelets, muscle, and/or neural tissue. Most glycolytic enzymopathies manifest an autosomal recessive pattern of inheritance. Heterozygotes almost always are hematologically normal, although their RBCs contain less than normal levels of enzyme activity. It once was thought that hemolysis occurred only in those individuals who were homozygous for the enzyme deficiency. However, true homozygotes for a given mutant enzyme now are known to be less common, and usually restricted to consanguineous kindred. The vast majority of cases of hemolytic anemia due to glycolytic enzyme deficiencies are a consequence of double heterozygosity for two different enzyme variants, and this accounts for the diverse biochemical and clinical heterogeneity of the red cell glycolytic enzymopathies. The one exception to this autosomal inheritance is phosphoglycerate kinase (PGK) deficiency, which is an X-linked disorder. Clinical manifestations of hemolysis include chronic anemia, reticulocytosis, and some degree of hyperbilirubinemia. The magnitude of anemia frequently is increased during viral infections and this can be due to increased hemolysis, or transient aplastic crises associated with viral infections. Most children with RBC glycolytic defects have a history of neonatal jaundice, many may require an exchange transfusion, and rarely kernicterus has been reported. Unlike hemolysis associated with G6PD deficiency, the jaundice noted in pyruvate kinase (PK)-deficient infants invariably is associated with anemia and often

with splenomegaly. There are no specific morphologic abnormalities to suggest an RBC glycolytic enzymopathy. The possibility of a glycolytic enzymopathy exists when chronic hemolytic anemia cannot be explained by the more common causes of hemolysis (i.e. hereditary spherocytosis (HS) or hemoglobinopathies). Screening tests are readily available, but are of limited value, frequently yielding false-negative results. An inherent problem in the diagnosis of glycolytic enzymopathies is that the most severely affected cells usually are removed *in vivo* (i.e. hemolyzed). For this reason, it sometimes is helpful to study parents and other family members for the presumed heterozygous state of the enzyme deficiency. In some cases DNA-based molecular diagnostic tests may be available.

Therapy for RBC glycolytic defects is similar to that of other chronic hemolytic anemias. Severely anemic patients often benefit from splenectomy. However, the response to splenectomy is only partial and, in most cases, hemolysis usually continues, although RBC transfusion requirements decrease. Cholelithiasis is a constant problem and all patients with glycolytic enzymopathies should have periodic gallbladder ultrasound examinations, even after the spleen is removed. The most common of these rare disorders are summarized in the following paragraphs and in Table 72-3.

**72.1.3.1 PK Deficiency.** PK catalyzes one of the two glycolytic reactions responsible for ATP production. There are two different PK-encoding genes in humans. One of these is PKLR (which is expressed in the liver and RBCs) and this resides on chromosome 1. The second is PKM2 (which is expressed in leukocytes and muscle) and this resides on chromosome 15. PKLR has been cloned and sequenced (36). The hemolytic anemia associated with PK deficiency is due to mutations of the PKLR gene and over 100 different mutations have been identified (37,38). Most of these are due to point mutations, the large majority occurring at nucleotide 1529 (39). A significant fraction of affected Caucasians (30–40%) have at least one mutation at nucleotide 1529 (G→A) resulting in an amino acid change at the 510 position (Arg→Gln). The variable phenotypic expression of PK deficiency undoubtedly reflects the heterogeneity of these different PK mutants. Hemolysis is seen in the homozygous state, but more commonly, it occurs in the presence of double heterozygosity for two different abnormal variants. Simple heterozygotes for abnormal PK variants are hematologically normal.

Most cases of PK deficiency have been reported from Northern Europe, the United States, and Japan; however, the disorder occurs worldwide (35,38,40). Using molecular biology techniques with four common PK mutations, it has been estimated that the prevalence of PK deficiency is 51 cases per million (0.01%) white population (41). A particularly high frequency exists among the Pennsylvania Amish, in whom the disorder can be traced to a single immigrant couple (42).

The degree of hemolysis in PK deficiency varies greatly and often is severe enough to require frequent or regular RBC transfusions. Almost all affected patients demonstrate a beneficial response to splenectomy. The anemia that persists following splenectomy generally is well tolerated and the need for transfusions is markedly reduced. In rare patients with severe hemolysis stem cell transplantation (BMT) has been successfully employed (43).

In families with a child with PK deficiency, the issue of prenatal diagnosis in subsequent pregnancies is a matter of concern. Biochemical techniques have been used successfully to assay for red cell PK deficiency at 30 weeks gestation (44). However, biochemical enzyme assays have limited utility in the prenatal diagnosis of PK deficiency, and direct DNA analysis is preferred whenever possible (39). In particular this should be helpful in diagnosing those fetuses with the 1529A mutation, thought to account for 45% of cases of PK deficiency in non-Gypsy Caucasians. The large number of cases of PK deficiency in Gypsies is associated with a unique deletional mutation that also could be used for molecular diagnosis.

**72.1.3.2 Glucose Phosphate Isomerase Deficiency.** This enzyme deficiency, first reported by Baughan et al. in 1968, is now recognized as the second most common glycolytic enzymopathy associated with hemolysis. Since the first description of the disorder more than 50 cases have been reported (1,35,45). Just as for PK deficiency, many more cases probably exist but are not published or listed in any rare disease registry. It is estimated that 0.2% of North Americans are heterozygous for a glucose phosphate isomerase (GPI) mutant. The gene for GPI is located on chromosome 19; it has been isolated and sequenced. Several different gene mutations of GPI deficiency associated with hemolysis have been identified (45,46). In most cases associated with hemolysis, the defect is due to an unstable enzyme. The clinical manifestations of this disorder are mostly limited to the RBCs, although neurologic impairment has been described in some cases (45). Just as is the case for PK deficiency, hemolysis occurs in GPI homozygotes or compound heterozygotes. Hemolytic anemia due to GPI deficiency has been reported as a cause of neonatal hydrops fetalis (47). Usually there is a partial response to splenectomy. Prenatal diagnosis of GPI by enzymatic assay previously has been demonstrated (48,49); however, in a family with a known mutation direct molecular analysis is preferable.

**72.1.3.3 Hexokinase Deficiency.** Deficiency of red cell hexokinase activity is a rare cause of hemolysis that now has been identified in over 20 individuals (50,51). Splenectomy ameliorates but does not cure the hemolytic process. There are several hexokinase genes (HK1–HK4) in humans. The HK1 gene codes for the hexokinase found in erythrocytes, and this gene maps to chromosome 10 (52).

**72.1.3.4 Phosphoglycerate Kinase Deficiency.** The inheritance of this disorder is unique among the glycolytic

enzymopathies in that it is X-linked (53,54). Biochemical variants of PGK deficiency manifest abnormal kinetics and/or enzyme instability. Several different PGK mutations have been identified (55–57). Females are mosaics with normal and PGK-deficient red cells, and there may be variable degrees of hemolysis. In males with PGK deficiency, hemolysis can be severe. Leukocyte PGK activity also is low but there is no evidence that these individuals have leukocyte dysfunction or increased infections. Of interest, however, the most severely PGK-deficient individuals with hemolysis also have neurologic abnormalities (i.e. seizures, mental retardation, aphasia, or movement disorders) (23). Deficient male infants experience apparent normal development until 3–4 years of age, when motor regression, expressive aphasia, and emotional lability become apparent. Seizures and progressive extra pyramidal disease follow late in the first decade. Most PGK variants manifest both hemolysis and neurologic disease (PGK-Uppsala, PGK-Tokyo, PGK-Matsue, PGK-Michigan); however, there are exceptions. PGK-Shizuoka is characterized by hemolysis and muscle disease, while PGK-San Francisco has only hemolysis without other symptoms (56).

**72.1.3.5 Phosphofructokinase Deficiency.** Phosphofructokinase (PFK) in RBCs is a tetrameric protein made up of two types of subunits: muscle or M type subunits, and liver or L type subunits (58). The M type subunit is encoded by a gene on chromosome 1 (56). The L type PFK deficiency subunit is encoded by a gene located on chromosome 21. The RBC contains equal amounts of M and L subtypes and all possible tetrameric variations are present ( $L_4$ ;  $L_3 M_1$ ;  $L_2 M_2$ ;  $L_1 M_3$ ;  $M_4$ ). The clinical manifestations of this enzyme deficiency relate to the specific type of structural enzyme abnormality in PFK. In all cases associated with hemolysis, red cells show only partial PFK deficiency, with about 50% normal enzyme activity, although there is a profound enzyme deficiency in muscle and this is associated with a severe myopathy (59). In these cases of PFK deficiency, RBCs lack M subunits although the L subunits are present (60). Hemolysis is thought to occur because tetramers of the L type PFK subunits are unstable and their enzymatic function is inhibited under normal intracellular conditions. Of interest, a second syndrome is recognized in which the degree of PFK deficiency is similar but there is little if any hemolysis and no muscle disease. This latter condition is due to a deficiency in the L subunits.

**72.1.3.6 Aldolase Deficiency.** Aldolase catalyzes the conversion of fructose-1,6-diphosphate to dihydroxyacetone and glyceraldehyde-3-phosphate. It is a tetrameric protein and three tissue isozymes (A, B, and C) have been identified. Type A aldolase, the main isozyme in RBCs and muscle has been characterized and cloned. Aldolase deficiency as a cause of hemolytic anemia is very rare, and has been identified in only four kindred. The first case was documented in a child whose parents were first cousins. In addition to mild hemolytic anemia, the child had

hepatomegaly associated with increased glycogen deposition and psychomotor retardation (61). A second report of aldolase deficiency, in a Japanese family, described more severe hemolytic anemia (hemoglobin 6 g/dl; 7–8% reticulocytes) without attendant hepatomegaly or developmental delay (62,63). In a third case, a 4½-year-old boy in Germany was identified with aldolase deficiency (64). He had a history of neonatal jaundice, recurrent episodes of jaundice beyond the newborn period, and anemia requiring red cell transfusions in the first year of life. In addition, however, he had a myopathy characterized by severe muscle weakness, exercise intolerance, and laboratory evidence of rhabdomyolysis in association with fever and an upper respiratory infection. In a fourth reported case, a young girl of Sicilian extraction had a transfusion-dependent hemolytic anemia requiring splenectomy at 40 months of age (65). She also had a myopathy with recurrent and progressive episodes of rhabdomyolysis. She died because of hyperkalemia and rhabdomyolysis during a febrile illness associated with gastrointestinal (GI) hemorrhage at 54 months of age. Molecular analysis in each of these cases has identified several different missense or nonsense mutations, each resulting in a thermolabile unstable enzyme (64–67). The mechanism of hemolysis in these cases is uncertain. Deficient red cells accumulate proximal glycolytic intermediates, especially fructose-1,6-diphosphate.

**72.1.3.7 Triose Phosphate Isomerase Deficiency.** Triose phosphate isomerase (TPI) deficiency is encoded by a gene on chromosome 12. Only one isozyme of TPI is produced and, thus, enzyme deficiency is shared by several tissues. At least 30 cases of TPI deficiency with hemolytic anemia have been described. Several different point mutations in the TPI gene have been identified (68,69). All clinically affected patients have been homozygotes or, less commonly, compound heterozygotes. Of the known mutations, one in particular accounts for the molecular defect in almost all studied families with TPI deficiency. This variant is due to a mutation at codon 104 (GAG→GAC), which results in a single amino acid substitution (glu→asp) and an unstable enzyme (70).

The most unique feature of this enzymopathy is an associated severe neurologic disorder characterized by spasticity, motor retardation, and hypotonia. These neurologic abnormalities usually become manifest after 6 months of age, and most affected patients die before they are 5 years old. An interesting report of TPI deficiency with chronic hemolytic anemia has been described in a 13-year-old boy and his 23-year-old brother. Noteworthy was the observation that the 13-year-old boy had hyperkinetic tortion dyskinesia, but his 23-year-old brother had no neurological abnormalities. It is interesting to note that the frequency of the heterozygous state for TPI deficiency is relatively high (0.1–0.5% in whites, 5.5% in blacks) (71). The rare occurrences of homozygous TPI deficiency may reflect incompatibility with fetal life.

Prenatal diagnosis of TPI deficiency initially relied on biochemical analyses of cultured fetal amniocytes, and in

one published report this was successful (72). Now, the large number of cases associated with the codon 104 mutation has allowed the use of chorionic villus biopsy samples and molecular techniques for prenatal diagnosis (73).

## 72.2 PURINE AND PYRIMIDINE ABNORMALITIES ASSOCIATED WITH HEMOLYSIS

Many of the enzymes utilized for purine and pyrimidine synthesis are present in erythrocytes (Figure 72-1), although mature RBCs do not synthesize DNA and are incapable of *de novo* purine or pyrimidine synthesis. Abnormal activity of these erythrocyte enzymes, however, often is observed with inborn errors of metabolism.

### 72.2.1 Pyrimidine 5'Nucleotidase Deficiency

Pyrimidine 5'nucleotidase (P5'N) is an enzyme that degrades pyrimidine nucleotides to inorganic phosphate and the corresponding pyrimidine nucleoside (74). Deficiency of P5'N is now recognized as one of the more common hereditary enzymopathies associated with hemolysis (75–77). It is found throughout the world, although there may be a predilection for individuals of Mediterranean and African ancestry. It is generally agreed that P5'N deficiency is inherited as an autosomal recessive disorder. Family members are hematologically normal, although they manifest reduced P5'N activity consistent with biochemical heterozygosity. The disorder is characterized by mild to moderate anemia, reticulocytosis, and hyperbilirubinemia. RBC morphology is unique in that marked basophilic stippling is present. Splenomegaly usually is observed and occasionally there is hepatomegaly. There are no enzymatic abnormalities in platelets or leukocytes. The most reasonable explanation for hemolysis in P5'N deficiency is that retained aggregates of ribosomes produce direct membrane injury, akin to that observed with Heinz bodies. Basophilic stippling of RBCs is the morphologic equivalent of partially degraded ribosomes (78). The enzyme activity in affected patients is less than 5% of that detected in normal RBCs of comparable age. Treatment for this disorder is supportive and RBC transfusions are required rarely. Splenectomy is not effective. However, since cholelithiasis is common, patients should have periodic ultrasound examinations for gallstones.

Of interest, the normal P5'N enzyme is readily inactivated by heavy metals such as lead, and it has been proposed that the basophilic stippling in lead poisoning is secondary to acquired P5'N deficiency (79). *In vitro* studies support the hypothesis that a consequence of mild lead intoxication is acquired P5'N inhibition (80). Moreover, cases of acute lead poisoning associated with hemolytic anemia are associated with basophilic stippling, decreased P5'N activity, and increased pyrimidine nucleotides, all characteristics of hereditary P5'N deficiency.

### 72.2.2 Adenosine Deaminase Excess

In contrast to other enzyme disorders that cause hemolysis this condition is due to a 60-fold to 100-fold excess of enzyme activity. In contrast to other enzymopathies, ADA excess is inherited in an autosomal dominant pattern (74,81). This rare cause of congenital hemolytic disease has been described in several families. Clinical features include mild to moderate anemia, reticulocytosis, and hyperbilirubinemia. The increased ADA production is associated with an elevated level of structurally normal ADA mRNA; however, no specific DNA mutation has been identified. The function of this enzyme is to catalyze the irreversible deamination of adenosine to inosine, a critical step in the purine salvage pathway. In patients with increased ADA activity, erythrocyte ATP content is reduced, presumably because the high ADA activity effectively competes with other adenosine-dependent reactions. There are no distinguishing clinical, hematologic, or morphologic features to aid in the diagnosis of this disease. The specific diagnosis is confirmed by demonstrating increased ADA activity in a hemolyzate. Usually no specific therapy is indicated since most patients have very mild anemia. It is noteworthy that a much smaller increase in enzyme activity (1.5- to 4.0-fold) has been observed in Diamond–Blackfan anemia (DBA) (see later).

### 72.2.3 Adenylate Kinase Deficiency

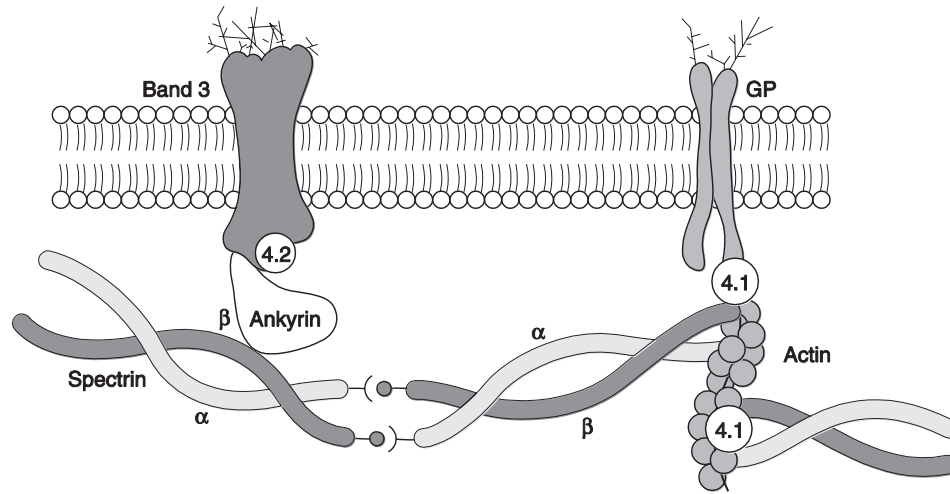
Adenylate kinase (AK) reversibly catalyzes the interconversion of adenine nucleotides and thereby salvages adenosine monophosphate (AMP) generated in a variety of RBC reactions. Hereditary nonspherocytic hemolytic anemia has been reported in 11 families with erythrocyte AK deficiency (38,62,77,82,83). In most cases AK deficiency is associated with moderate to severe hemolytic anemia. In some cases there also is psychomotor impairment (83,84).

In one report, there was congenital hemolytic anemia and a marked reduction in erythrocyte AK activity in one patient, although the proband's brother, who was hematologically normal, also had no detectable erythrocyte AK activity (85). The significance of this observation is not known. The AK enzyme has been studied at a molecular level in almost all cases, and each family has its own unique mutation, predominantly a missense mutation.

## 72.3 HEMOLYSIS DUE TO HEREDITARY RED BLOOD CELL MEMBRANE DISORDERS

The RBC membrane structure is a bilayer of phospholipids intercalated with molecules of unesterified cholesterol and glycolipids (Figure 72-2). The phospholipids are asymmetrically organized, with the choline phospholipids (phosphatidyl choline and sphingomyelin) primarily





**FIGURE 72-2** Structure of the RBC membrane.

on the outside half of the bilayer and the amino phospholipids (phosphatidyl ethanolamine and phosphatidyl serine) on the inside half. The membrane also contains proteins that are asymmetrically organized. All the glycoproteins are exposed on the outer membrane surface, and these are red cell antigens and receptors (i.e. glycophorins) or transport proteins (i.e. the anion transport channel). These integral membrane proteins penetrate or span the lipid bilayer, interact with the hydrophobic lipid core, and are tightly bound to the membrane. A separate protein network forms the membrane cytoskeleton that interacts with both the integral membrane proteins and the lipid bilayer. This cytoskeleton is composed of spectrin, ankyrin, protein 4.1, pallidin (protein 4.2), and actin. The biochemical and genetic characteristics of these RBC membrane proteins are depicted in [Table 72-4](#). Spectrin is made up of  $\alpha\beta$  heterodimers, which, at the head end, interact with other spectrin  $\alpha\beta$  dimers to form heterotetramers ( $\alpha\beta$ )<sub>2</sub>. These spectrin tetramers are the major structural subunits of the membrane skeleton. At the distal end, spectrin tetramers interact with protein 3 (the anion exchange channel) via ankyrin, and this interaction is important for attachment of the cytoskeleton to the membrane. Also at the distal end, spectrin tetramers interact with actin via protein 4.1. In addition, there are other membrane protein interactions not directly involving spectrin. The horizontal protein interactions, mainly the interactions of spectrin dimers, are responsible for the lateral deformability of the cytoskeleton. Thus, these protein interactions are responsible for the overall stability of the membrane, thereby allowing the RBC to be deformable during its life span within the circulation. Hereditary RBC membrane disorders are due to alterations in the quantity, quality, or both of these individual proteins and their interactions with each other. HS is due to an uncoupling of the cytoskeleton from the lipid bilayer, thereby leading to membrane instability with loss of lipids and some integral membrane proteins. Hereditary elliptocytosis (HE) disorders are due to defects in

spectrin dimer interactions, thereby leading to disruption of the skeletal lattice and RBC fragmentation. Several excellent reviews of the molecular advances in these diseases are available ([86–88](#)).

### 72.3.1 Hereditary Spherocytosis

**72.3.1.1 Prevalence and Demography.** This is the most common hereditary hemolytic anemia occurring in Caucasians ([89–91](#)). Approximately one per 5000 individuals of Northern European extraction is affected. It also occurs in other ethnic groups, but at a much lower frequency.

**72.3.1.2 Genetics.** In at least 75% of cases, HS is an autosomal dominant disorder and one parent (and often other family members) usually has the disease. The remaining 25% of HS cases are thought to be autosomal dominant disorders with decreased penetrance, autosomal recessive disorders (supported by the reports of families in which apparently normal parents have had more than one affected child), or new mutations (supported by the fact that approximately 50% of the offspring of individuals with sporadic HS also have the disease).

**72.3.1.3 Biochemistry and Pathophysiology.** The basic defect in HS is due to an alteration in the RBC cytoskeleton, which normally stabilizes membrane lipids and integral proteins. Most cases are related to spectrin deficiency, and the degree of spectrin deficiency correlates closely with the severity of disease. Mutations of  $\alpha$  spectrin are associated with recessive HS while mutations of  $\beta$  spectrin occur in families with autosomal dominant HS. Mutations in the ankyrin gene lead to secondary defects in spectrin assembly and thereby decreased RBC spectrin content. Genetic defects in band 3 or pallidin also lead to abnormal spectrin interactions and the clinical picture of HS. Individuals with autosomal recessive HS have only 40–50% the normal amount of spectrin (relative to band protein 3), while red cell spectrin levels range from 60 to 80% of normal in the autosomal dominant form of

**TABLE 72-4 Characteristics of Red Blood Cell Membrane Cytoskeletal Proteins**

SDS-Page Band	Protein Involvement Localization Anemias	MW (kDa) in Hemolytic	Total (%)	Chromosome
1	$\alpha$ -Spectrin HE, HS	240	16	1 q22–q23
2	$\beta$ -Spectrin HE, HS	220	14	14 q23–q24.2
2.1	Ankyrin HS	210	4.5	8 p11.2
3	Anion exchange HS	90–100	27	17 q21–qter
4.1	Protein 4.1 HE	80	5	1 p33–p34.2
4.2	Pallidin HS	72	5	15 q15–q21
5	Actin–	43	5.5	7 pter–q22
6	G3PD–	35	3.5	12 p13.31–p13.1
7	Stomatin HSt	31	2.5	

HS, hereditary spherocytosis; HE, hereditary elliptocytosis; HSt, hereditary stomatocytosis; G3PD, glyceraldehyde-3-phosphate. Data from Glader (2004), modified from Palek and Jarolim (1995).

HS. Numerous mutations have been observed in each of the genes involved in membrane protein synthesis (Table 72-4). Most of these mutations are private in that they are unique to a given family, and thus not useful for genetic screening.

The major physiologic abnormality in HS is RBC membrane instability. Erythrocytes exit from the bone marrow as normal biconcave disks, but the intrinsic membrane instability leads to loss of lipids because the spectrin-deficient cytoskeleton is unable to provide adequate support for the lipid bilayer (Figure 72-3). The loss of membrane surface area transforms red cells from biconcave discs to spherocytes. An important physiologic consequence of this is that cellular deformability of spherocytes is decreased and this leads to their destruction by splenic macrophages.

**72.3.1.4 Clinical Features.** In most cases, the degree of anemia in HS is minimal and patients have no major limitations. Jaundice is common in the newborn period, and 30–50% of adults with HS have a history of jaundice during the first week of life. The magnitude of hyperbilirubinemia sometimes may be such as to require exchange transfusion. Beyond the neonatal period jaundice is rarely intense. An increase in scleral icterus and a darker urine color commonly are seen in children with nonspecific viral infections. From a clinical perspective, it has been useful to classify HS according to the severity of disease (92–94).

*Moderate HS*—accounts for 60–75% of cases; usually manifests autosomal dominant pattern of transmission, although new mutations can occur; recognized as a chronic hemolytic disorder with characteristic RBC spherocytes on the peripheral blood smear; associated with mild to moderate anemia, modest splenomegaly, and intermittent jaundice.

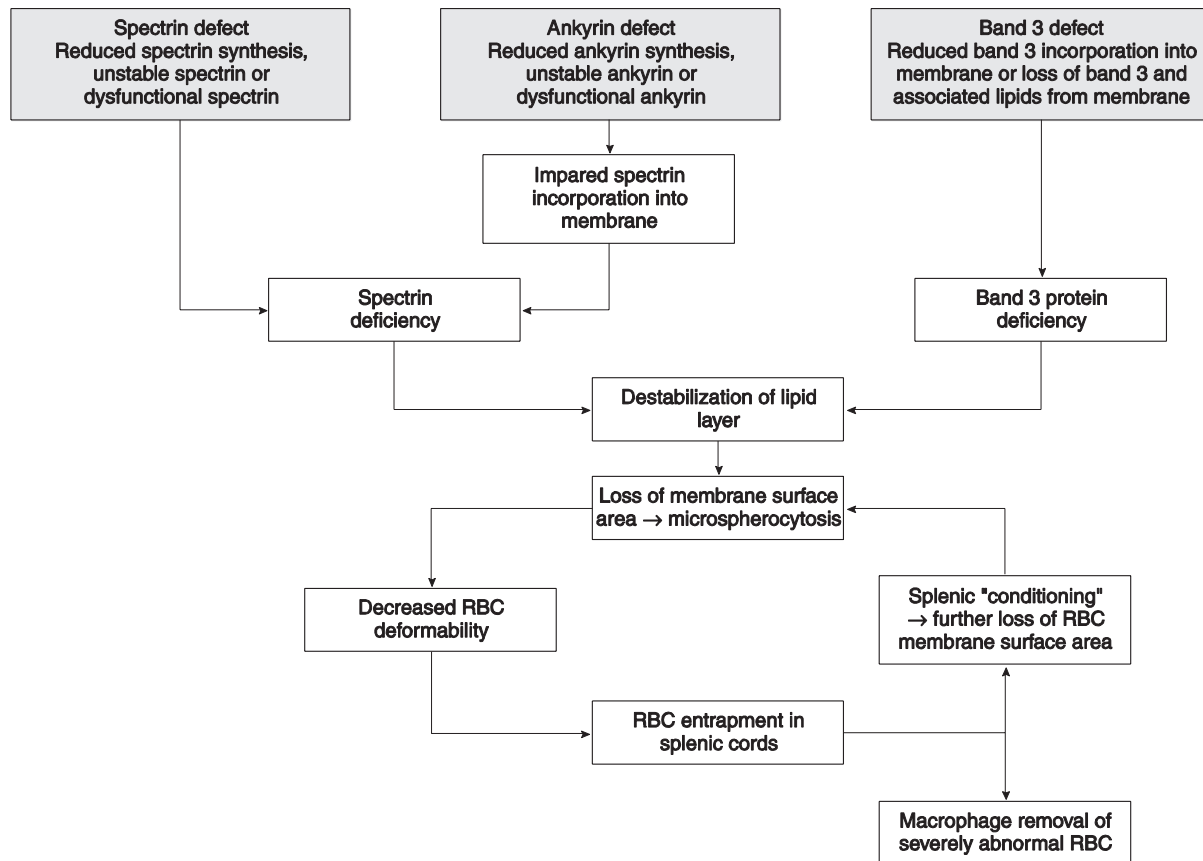
*Mild HS*—accounts for 20–30% of cases; autosomal dominant inheritance; generally no anemia because the bone marrow is able to compensate for increased RBC destruction; little or no splenomegaly; few spherocytes on blood smear; usually asymptomatic and often not diagnosed until later in life during a hemolytic or aplastic episode triggered by infection.

*Severe HS*—accounts for 5% of cases; almost always a recessive pattern of inheritance; characterized by severe hemolytic anemia, requiring RBC transfusions.

In addition to the previous categories there exists the HS silent carrier state in families with autosomal recessive HS. In most of these cases, the parents of an affected patient have no signs of HS or only a mild increase in the reticulocyte count.

The two major complications seen in HS are episodic worsening of anemia and the development of gallstones. Exacerbations of anemia occur in almost all HS patients. Often this is due to increased hemolysis associated with nonspecific viral infections. Hypoplastic crises due to human parvovirus infection also occur, and can be a medical emergency because of the rapid decrease in hemoglobin, but crises do not occur more than once. Cholelithiasis is common in HS just as in other chronic hemolytic disorders. Bilirubin gallstones are uncommon before 5 years of age, but the incidence increases markedly with age, and are present in 40–80% of adults who have not been splenectomized. In patients with mild HS, cholelithiasis may be the first sign of an underlying red cell disorder. It is of interest that the coinheritance of the gene for Gilbert syndrome is associated with a five-fold increase in gallstone formation (95).

**72.3.1.5 Laboratory Features.** Characteristics of HS are mild to moderate anemia, reticulocytosis, and the presence of spherocytes (RBC lacking central pallor) in



**FIGURE 72-3** The pathophysiology of hereditary spherocytosis is a consequence of spectrin, ankyrin, or band 3 abnormalities, which lead to membrane instability, loss of lipid microvesicles, decreased red cell surface area, reduced cellular deformability, and stagnation of RBC in the splenic cords. (From Glader, B. E. *Wintrobe's Clinical Hematology*; Lippincott Williams and Wilkins: Philadelphia, 2004.)

the peripheral smear. The loss of membrane surface area is associated with RBC dehydration as manifested by an elevated mean corpuscular hemoglobin concentration (MCHC) greater than 35%. The commonly used test for diagnosing HS is the incubated osmotic fragility test, which indirectly detects spherocytes by measuring RBC lysis in sodium chloride solutions of varying concentrations. A newer test employs flow cytometry to measure eosin-5-maleimide (EMA) binding to erythrocytes, reflecting relative amounts of band 3 and Rh-related proteins (96). The purported advantage of the EMA binding flow test is that there are no false-positive results with immune-mediated or non-membrane-associated hemolytic disease. However, a disadvantage of the EMA procedure is that the test is nonspecific, also detecting other erythrocyte abnormalities, especially those associated with abnormal band 3, including congenital dyserythropoietic anemia (CDA), and abnormalities of erythrocyte hydration.

**72.3.1.6 Therapy.** Definitive therapy for HS is splenectomy, which removes the site of RBC destruction (93). Following splenectomy, the hemoglobin concentration almost always increases, reticulocytes decrease, bilirubin levels return to normal, and RBCs have a relatively normal life span. The one exception to this favorable response to splenectomy is seen in patients with the

autosomal recessive form of HS, who may continue to hemolyze. Most hematologists now recommend that HS patients with moderate degrees of hemolysis have a splenectomy after 5–10 years of age. If patients have not developed gallstones before splenectomy, it is unlikely they will develop bilirubin stones at a later date. Splenectomy is not indicated before age 5 years, since a functioning spleen is important in young children for protection against infections due to encapsulated bacteria. Children are given pneumococcal vaccine before splenectomy and placed on prophylactic penicillin therapy for at least 2 years following surgery.

### 72.3.2 Hereditary Elliptocytosis

This is an autosomal dominant disorder that occurs in one per 2000–4000 people in all racial and ethnic groups, but in the United States it appears to be much more common among African-Americans (86,90,91). In areas where malaria is endemic, HE is considerably more frequent, having a prevalence of 0.6% in equatorial Africa. Like HS, HE is transmitted as an autosomal dominant trait and in most cases is associated with no or minimal clinical abnormalities. Spectrin abnormalities are found in the vast majority of patients with HE, and almost all these are due to  $\alpha$ -spectrin mutations (86,90).

Most of the  $\alpha$ -spectrin mutations result in abnormal spectrin dimer–dimer interaction. Three distinct HE syndromes are summarized here.

Common HE is a dominantly inherited condition characterized by many elliptocytes in the peripheral blood smear. The clinical severity of common HE is extremely variable, ranging from an asymptomatic condition (80%), to a moderate hemolytic anemia (20%). It is due to the presence of at least one  $\alpha$  spectrin mutation. In those cases associated with hemolysis, it is now recognized that a second mutation is present that allows expression of the variant  $\alpha$  spectrin (97). Individuals with common HE usually are discovered accidentally when elliptocytes are identified during routine evaluation of the peripheral blood film. These people usually are not anemic, reticulocytes are normal or only slightly elevated, and there is no splenomegaly. However, the peripheral blood smear is striking, containing 15–100% elliptocytes. In the minority of common HE cases associated with hemolysis, the peripheral blood smear reveals numerous poikilocytes as well as elliptocytes, the spleen may be enlarged, and gallstones occur with increased frequency.

HE with infantile poikilocytosis is a clinical manifestation of HE that occurs almost exclusively in black families. Affected young infants have moderately severe hemolytic anemia and hyperbilirubinemia in the newborn period, the latter often-necessitating exchange transfusion (98). The blood smear is characterized by marked red cell fragmentation and poikilocytosis in addition to elliptocytosis. Beyond the newborn period hemolysis gradually lessens, and by 6–12 months of age the clinical and hematologic features convert to those of mild HE with the disappearance of the bizarre RBC forms and the emergence of normal elliptocytes.

Homozygous (doubly heterozygous) HE is associated with a moderate to severe hemolytic anemia due to homozygosity or compound heterozygosity for two common  $\alpha$ -spectrin mutations. Morphological characteristics include marked poikilocytosis, microelliptocytosis, and red cell fragmentation. In many cases both parents have nonhemolytic HE, or since many individuals with a single  $\alpha$ -spectrin mutation are asymptomatic, there may be no parental anemia or RBC abnormalities. These patients also have enlarged spleens, and usually benefit from splenectomy. In contrast to HS, however, the response to splenectomy usually is incomplete and hemolysis persists.

Hereditary pyropoikilocytosis (HPP) is an unusual and severe recessively inherited hemolytic anemia, characterized by red cell fragments, microspherocytes, and poikilocytes seen on the peripheral smear. HPP is the result of a double heterozygous state for an  $\alpha$ -spectrin mutation and a defect involving spectrin synthesis. Just as in other hemolytic variants of HE, spectrin dimers are increased relative to tetrameric spectrin, but the total spectrin content also is decreased (99). Most patients

with this interesting variant are black. Moreover, at least one parent or sibling invariably has a milder form of HE. Splenectomy is followed by an increase in hemoglobin concentration and a decrease in reticulocytes. Most impressive are changes in red cell morphology, which include extreme poikilocytosis, microspherocytosis, microelliptocytosis, membrane budding, and cell fragments. The mean cell volume (MCV) may be extremely low (30–50fl). The MCHC is normal. The osmotic fragility is increased.

### 72.3.3 Hereditary Stomatocytic Disorders

Stomatocytes are erythrocytes with a central slit or stoma instead of a circular area of pallor when examined on dried smears; they are uniconcave rather than biconcave, giving them a bowl-like appearance. A few stomatocytes may be observed in blood smears prepared from normal individuals, as well as from patients with acute alcoholism and hepatobiliary disease. Large numbers of stomatocytes are associated with very rare hereditary disorders of red cell cation permeability leading to increased or decreased red cell water content (100–102). It is important to recognize these disorders since venous thrombosis and pulmonary embolism are complications of splenectomy and thus this procedure is contraindicated in these conditions.(103)

### 72.3.4 Congenital Bone Marrow Failure Syndromes

**72.3.4.1 Diamond–Blackfan Anemia (Congenital Hypoplastic Anemia).** This congenital red cell aplastic anemia was first recognized 50 years ago (104,105). The anemia is not due to inadequate erythropoietin (EPO) production, since circulating EPO levels actually are elevated in DBA patients. Rather, it is due to an intrinsic defect of erythroid progenitors resulting in impaired erythroid production. Under in vitro conditions that stimulate normal differentiation erythroblast growth, little or no colony formation is seen when DBA marrow cells are studied, and there is evidence of increased apoptosis. (106).

This is a relatively rare disorder, although several hundred individuals have been identified in the medical literature, and the number of recognized persons now is increasing with the recent establishment of registries in Europe and North America (107–110). Several excellent reviews of this disorder are available (111–113). It is characterized by a lifelong anemia that usually presents in the first 2–3 months of life, and over 90% of patients are diagnosed within the first year of life. It occurs equally in both sexes and has been identified in all ethnic groups, although the majority of reported cases have been in whites. Approximately 35% of affected children manifest one or more congenital abnormalities (112). The most common abnormality is short stature.



Also common are thumb abnormalities including triphalangeal thumbs, bifid thumbs and hypoplastic thumbs. A short webbed neck, cleft palate, high-arched palate, congenital heart disease, hypertelorism, strabismus, and structural abnormalities of the kidney are other stigmata frequently encountered. It is noteworthy that many of these congenital abnormalities also are found in Fanconi aplastic anemia (see later discussion), but in DBA only RBC production is impaired and there is no evidence of increased chromosome fragility.

Although most DBA cases are sporadic (75%), it generally is thought that this is a genetic disorder since 10–15% of cases occur in kindred that have had more than one affected family member (109). Both autosomal dominant and autosomal recessive modes of inheritance have been postulated. It has been observed in cousins, identical twins, full siblings, and, in several instances, in half-siblings where the father or mother has had a different mate. It also has been observed to occur in a parent and child, and several large kindreds with autosomal dominant transmission over three generations have been observed (114,115).

The observation of DBA in a girl with a balanced reciprocal X:19 translocation was the initial finding leading to the identification of a specific gene (116). Further studies localized 19q13 as the putative site for at least one DBA gene, now known to be a gene (RPS19) for directing the production of a ribosomal protein. Mutations in RPS19 occur in 25% of unrelated DBA patients. It is now recognized that DBA is a disorder of ribosome biogenesis (117–119). Nine ribosomal protein gene mutations have been identified to date and these account for 50% of recognized DBA cases (120).

The diagnosis of DBA is suggested by anemia with reticulocytopenia presenting in infancy. The bone marrow reveals decreased erythroid precursors while myelopoiesis and megakaryopoiesis are normal. Circulating RBCs have many fetal-like characteristics and these abnormal features persist throughout life, independent of the patient's therapy or clinical state. These RBC are macrocytic relative to the patient's age and the fetal hemoglobin concentration often is elevated. A majority of DBA patients (greater than 75%) have increased erythroid activity of adenosine deaminase. Elevated RBC ADA activity also has been observed in some healthy relatives of DBA individuals (121). It has been observed that this biochemical abnormality in family members cosegregates with the DBA gene region on chromosome 19q13. Taken together, these findings suggest there probably is a silent carrier state for DBA (112,122).

Corticosteroids correct the anemia in many DBA patients (112,123). In some cases, trivial prednisone doses are necessary to sustain a remission, while in other patients remission is maintained without steroids. However, the clinical course can change abruptly at any time. Those who fail steroids rarely respond to other pharmacologic agents and most require RBC transfusions.

Consequently, iron overload is a serious problem in transfusion-dependent DBA patients and lifelong chelation therapy is necessary. For this reason, just as for transfusion-dependent children with various thalassemia syndromes, BMT from a human leukocyte antigen (HLA) compatible sibling is considered as an alternative therapy for this disorder. This approach generally is reserved for DBA children who demonstrate an absolute dependence on RBC transfusions for 4–5 years. Several patients now have been transplanted with a successful outcome (63,107). A concern in evaluating HLA-matched family members as possible bone marrow donors is the problem of identifying those who may be silent carriers. This requires assessing donor erythrocyte ADA activity and, if available, testing for known DBA mutations. The use of unrelated donors for stem cell transplants in DBA has been discouraged because of the poor outcomes (112). However, the recent availability of new transplant conditioning drugs resulting in markedly increased survival rates, may lead to a larger use of unrelated donors for certain DBA patients (123).

The prognosis for the majority of DBA patients who respond to steroids is generally quite good. Affected women are known to have become pregnant and delivered normal infants, although pregnancy occasionally exacerbates the magnitude of anemia. Of concern, however, acute leukemia, myelodysplastic syndrome, and other malignancies (especially osteosarcoma) may be delayed complications of this disorder (112). In view of the rarity of DBA, the number of leukemia cases described to date suggests this is more than a chance event.

**72.3.4.2 Fanconi Anemia (Constitutional Hypoplastic Anemia).** Aplastic anemia is characterized by anemia, thrombocytopenia, and leukopenia with reduced or absent hemopoietic precursor cells in the bone marrow. Most commonly, this is an acquired disorder occurring in previously healthy individuals. In the majority of cases it is due to an immunologic assault on marrow stem cells. There also are constitutional or hereditary forms of aplastic anemia, the most common of which is Fanconi anemia (FA) (120,124–126). This is an autosomal recessive disorder with a heterozygote frequency of 1 in 300. The disorder occurs in all races and ethnic groups.

In addition to the hematologic problems, FA is associated with a variety of physical abnormalities for a majority of children (127). Common findings include skin hyperpigmentation, increased cafe-au-lait spots or both (62%); short stature (59%); skeletal abnormalities, particularly of the thumb (48%); hypogonadism in males (42%); renal anomalies (24%); microcephaly or micrognathia (26%); low birth weight (14%); retardation (13%); ear abnormalities, with or without deafness (10%) and, rarely, congenital heart disease and GI tract abnormalities.

The onset of pancytopenia usually occurs during the first decade of life, although it may appear later. The clinical manifestations begin insidiously with

thrombocytopenia, leukopenia or both that evolves into mild pancytopenia, and gradually becomes more severe over months to years. The anemia typically is macrocytic and the fetal hemoglobin concentration is increased.

A characteristic feature of FA is increased chromosomal breaks in cultured skin fibroblasts and in peripheral blood lymphocytes stimulated in vitro with clastogenic drugs. Cells from FA patients are particularly sensitive to certain crosslinking agents that damage DNA and produce chromosomal abnormalities. This particular sensitivity is the basis of the current diagnostic test, which employs diepoxybutane (DEB) as the clastogenic agent to induce chromosomal breakage in peripheral blood lymphocytes (128). The DEB breakage studies do not discriminate FA heterozygotes from the normal population. The test also does not distinguish severe and mildly affected FA patients. Since approximately one-third of all FA patients have no congenital abnormalities, a DEB-chromosomal breakage study should be done on any new patient thought to have acquired aplastic anemia. Moreover, since FA patients have a predilection for developing leukemia (see later discussion) any new acute myeloid leukemia (AML) patient should be screened because acute leukemia may be the first manifestation of FA. This is important because FA patients have a very poor tolerance of standard antileukemia chemotherapy.

There are many theories about the pathophysiology of FA, including increased sensitivity to oxygen free radicals and defects in DNA repair; however, the precise reason for this increased susceptibility of DNA injury remains unknown (124,129). It is of major significance that the increased chromosomal breakage induced by alkylating agents is corrected to normal when FA fibroblasts are fused in vitro with normal cells. Moreover, since fusion of cells between different FA patients occasionally corrects the breakage sensitivity in hybrid cells, this suggests a different cause of increased chromosomal fragility in each of these cells. These observations now have resulted in the characterization of 11 different FA complementation groups and 13 genes have been characterized FANC-A, B, C, D1, D2, E, F, G, I, J, L, M and N (120). FANC-A accounts for 65% of FA cases, FANC-C 12%, FANC-G 12% cent, and the other known genes responsible for 11% of cases. An important function of FA proteins is to maintain chromosomal stability, but it is not clear how this occurs.

In a majority of children with FA there is a beneficial response to androgen therapy and most children who respond require only small doses to maintain a remission. After a few years, refractoriness to androgens develops and most patients ultimately succumb to progressive bone marrow failure and its complications. In about 20% of cases, patients develop cancer, and in some older patients this may be the initial presentation without a preceding pancytopenic phase. In most cases the malignancies are due to myelodysplasia or acute myelogenous leukemia. A smaller number, 5%, develop

solid tumors (GI tract, liver, and gynecologic). These malignancies tend to occur in older FA patients, more commonly female, sometimes without an antecedent history of aplastic anemia.

The most appropriate therapy for an FA patient with significant aplasia is BMT from an HLA compatible sibling. The best time to perform this procedure is not clear but, in order to avoid HLA sensitization, it probably should be done before platelet transfusion therapy is needed. Since this is an autosomal recessive disorder, it is critical to check potential stem cell donors to determine whether they have FA. This testing must include DEB chromosome breakage studies and chromosome analysis. A unique complication of transplantation in FA patients is severe skin and GI toxicity due to the conditioning drugs (i.e. cyclophosphamide), presumably a consequence of chromosomal fragility in these other tissues. The overall survival from marrow transplantation using HLA-matched related donors approaches 75% at 5 years, while the outcome for matched unrelated donors has been very poor. However, with the development of new transplant conditioning regimens, the outcomes using unrelated donors have improved significantly (130,131). Utilizing preimplantation genetic diagnosis it is possible to select an embryo that is both HLA compatible and does not have FA (132). The decisions regarding stem cell transplant for FA, and when to undergo the procedure, continue to be defined as new data appear.

A long-term complication after successful hematopoietic engraftment is the later occurrence of malignancies; in one report there was a 24% projected incidence of having cancer 8 years after BMT. This increase reflects the fact that correction of the hematologic problem leads to longer survival, but genetic instability in other tissues allows for the expression of cancer. Gene therapy in the future may be used for this disorder and published studies indicate it is possible to insert the cloned FANC-C and FANC-A gene into hematopoietic FA stem cells and provide protection against clastogenic agents (133).

## 72.4 MEGALOBlastic ANEMIAS

Megaloblastic anemias are characterized by abnormal differentiation of bone marrow erythroblasts and ineffective erythropoiesis. This results in a macrocytic anemia and the peripheral blood smear typically manifests hypersegmented neutrophils with many having more than five lobes. In all cases, megaloblastic erythropoiesis is a consequence of impaired DNA synthesis. Most genetic causes of megaloblastic anemia are due to abnormalities of vitamin B<sub>12</sub> metabolism (134).

Dietary vitamin B<sub>12</sub> (cobalamin), found in eggs, milk products, meats, and vegetables, is released from complex food forms by proteolytic enzymes in the stomach. Once in the duodenum, vitamin B<sub>12</sub> binds to intrinsic factor (IF), a protein secreted by gastric parietal cells. Subsequently, the IF-B<sub>12</sub> complex binds to specific

receptors in the distal part of the ileum, IF is split off, and B<sub>12</sub> is transported into intestinal cells. Next, cobalamin binds to a transport protein, transcobalamin II (TC-II), which carries the B<sub>12</sub> to the liver, bone marrow, and other tissue storage sites. Serum also contains two other B<sub>12</sub> binding proteins, transcobalamin I and III (TC-I and TC-III). The latter two forms of transcobalamin have no specific transport role but are known to reflect vitamin B<sub>12</sub> tissue stores. In fact, almost all B<sub>12</sub> in plasma is bound to TC-I and TC-III and thus the measurement of serum B<sub>12</sub> concentration reflects the storage of this vitamin. One important metabolic function known to be impaired in human B<sub>12</sub> deficiency is methylmalonyl-CoA, an important reaction in propionic acid metabolism. When this reaction is impaired, there is both methylmalonic acidemia and methylmalonic aciduria, and testing for the latter is a good screening test for B<sub>12</sub> deficiency. A second important reaction requiring B<sub>12</sub> is homocysteine:methyltetrahydrofolate methyltransferase. In this reaction a methyl group from methyltetrahydrofolate is transferred to homocysteine with the formation of methionine and tetrahydrofolate. The latter is a required cofactor for thymidine synthesis, partially explaining why DNA synthesis is defective in vitamin B<sub>12</sub> deficiency. Megaloblastic anemia due to vitamin B<sub>12</sub> deficiency can occur because of an abnormality in any one of the steps in B<sub>12</sub> absorption, transport, or metabolism. Acquired causes include loss of IF following gastrectomy or loss of absorptive sites after extensive ileal resection, tapeworm infestation, or intestinal bacterial overgrowth (from broad spectrum antibiotic therapy). The remainder of this section focuses on those megaloblastic anemia conditions due to B<sub>12</sub> deficiency that are considered genetic disorders.

### 72.4.1 Classical Pernicious Anemia

This disorder is the most common form of B<sub>12</sub> deficiency and usually occurs in middle-aged and elderly adults. It is characterized by macrocytic anemia and neurologic disease. The neurologic problems include decreased vibratory and position sensation of the lower extremities, paresthesias, dementia, and/or depression. The cause of pernicious anemia (PA) is impaired IF production and is associated with gastric atrophy, achlorhydria, and antibodies to both gastric parietal cells and IF. The disorder traditionally has been diagnosed by low serum vitamin B<sub>12</sub> levels and an abnormal Schilling test that measures B<sub>12</sub> absorption with and without added IF. The Schilling test is not available nowadays. Currently, the most direct functional evidence of decreased cobalamin is suggested by increased levels of cobalamin intermediates, homocysteine and methylmalonic acid. There may be an ethnic predisposition to develop PA for Northern Europeans, in particular Scandinavians; however, the disorder is known to occur worldwide in many different ethnic groups. It is not clear that this is a genetic disease,

although 20% of PA patients also have an affected relative with the same disease.

### 72.4.2 Congenital Intrinsic Factor Deficiency

This rare megaloblastic anemia is first recognized between 1 and 4 years of age. It is due to a congenital absence of functional IF (135). There are none of the other gastric abnormalities or other antibody features of classic PA. Infants may also manifest failure to thrive and, occasionally, abnormal neurologic findings. The occurrence of multiple affected siblings suggests autosomal recessive inheritance; no abnormalities have been found in parents or other relatives. Reports indicate that hereditary cobalamin deficiency is due to mutations in the gastric IF gene (GIF) (136). Treatment is parenteral vitamin B<sub>12</sub> for life.

### 72.4.3 Impaired Vitamin B<sub>12</sub> Absorption (Imerslund–Grasbeck Syndrome)

Imerslund (137) and Grasbeck (138) first described children with familial malabsorption of vitamin B<sub>12</sub> due to impaired ileal uptake of cobalamin, presumably due to an abnormality of the receptor for B<sub>12</sub> absorption. There are no gastric abnormalities and IF production is normal. This disorder is now referred to as Imerslund–Grasbeck syndrome (IGS) (139). This is a relatively rare condition, found particularly in Finland, Norway, and the eastern Mediterranean region. It is known to be transmitted as an autosomal recessive disorder and most children show marked failure to thrive in the first 2 years of life. Characteristically there is megaloblastic anemia with low serum B<sub>12</sub> levels. Of interest, however, mild proteinuria also is present in many patients. It is now recognized that this disorder is due to mutations in the cubulin (CUB) or amnionless (AMN) genes (140,141). The protein products of these two genes form a heterodimer called cubam that is responsible for the internalization of the IF-cobalamin complex (142). Therapy is parenteral B<sub>12</sub> given every 2–3 months for life.

### 72.4.4 Transcobalamin II Deficiency

This autosomal recessive disorder is due to a deficiency of the major transport protein for vitamin B<sub>12</sub>. However, serum vitamin B<sub>12</sub> levels are normal since TC-I and TC-III are not affected. Approximately 30 cases of transcobalamin II deficiency have been reported. In rare instances, TC-II may be detected immunologically, although it demonstrates defective binding to vitamin B<sub>12</sub>. Generally, this disorder manifests itself in the first weeks of life. Characteristically there is a failure to thrive, diarrhea, vomiting, glossitis, neurologic abnormalities, and megaloblastic anemia. The diagnosis of this disorder is suggested by the presence of severe megaloblastic anemia with normal serum B<sub>12</sub> and folate levels with no evidence

of any other inborn errors of metabolism. The serum B<sub>12</sub> levels must be kept high to utilize cobalamin. Hence, the therapy for this disorder is large parenteral doses of vitamin B<sub>12</sub> given twice a week for life. These frequent and large doses of cobalamin appear to overcome the transport deficiency. Most children with this disorder die if not treated in infancy.

### 72.4.5 Hereditary Orotic Aciduria

This autosomal recessive disorder usually appears in the first year of life and is characterized by growth failure, developmental retardation, megaloblastic anemia, and increased urinary excretion of orotic acid. This defect, the most common metabolic error in the *de novo* synthesis of pyrimidines, affects nucleic acid synthesis. The usual form of hereditary orotic aciduria is caused by a deficiency (in all body tissues) of both orotic phosphoribosyl transferase and orotidine-5-phosphate decarboxylase, two sequential enzymatic steps in pyrimidine nucleotide synthesis. This disorder may present as severe megaloblastic anemia with normal serum B<sub>12</sub> and no evidence of TC-II deficiency. A presumptive diagnosis is made by finding increased urinary orotic acid. Confirmation of the diagnosis, however, requires assay of the transferase and decarboxylase enzymes in the patient's erythrocytes. The heterozygous state is characterized by intermediate levels of enzyme activity in otherwise healthy individuals.

## 72.5 DYSERYTHROPOIETIC ANEMIAS

CDA refers to a class of hemolytic disorders characterized by unique morphologic abnormalities in marrow erythroblasts (multinuclearity, abnormal nuclear fragments and intrachromatin bridges between cells) (101,143–145). Clinically these disorders are characterized by variable degrees of anemia despite the fact that there is markedly increased marrow erythroid activity (i.e. ineffective erythropoiesis). CDA has been reported in newborn infants with hydrops fetalis (145–147). Three major types of CDA (types I, II, and III) have been defined.

### 72.5.1 Type I Congenital Dyserythropoietic Anemia

This is a very rare autosomal recessive disorder in which the onset of anemia and/or jaundice may be noted at any age. Affected patients can manifest slight icterus, moderate splenomegaly, and a mild to moderate macrocytic anemia. (145,148,149). The reticulocyte count is less than expected for the degree of anemia. In more than half of cases anemia is first recognized in the neonatal period. A characteristic feature is RBC macrocytosis persisting beyond the newborn period with MCVs ranging between 100 and 120 fl. Peripheral RBC morphology is characterized by large poikilocytosis and ovalocytes,

similar to that seen in megaloblastic anemias. White blood cells and platelets are normal. Indirect bilirubin levels are slightly elevated, haptoglobin levels are low and transferrin may be saturated with iron (serum iron level approximates the TIBC concentration). Bone marrow aspiration reveals erythroid hyperplasia with dyserythropoietic features characterized by interchromatin bridges between cells.

In contrast to Type II CDA, there are no serologic features of this dyserythropoietic anemia (see later discussion). Treatment of this disorder has been unsuccessful with the usual hematinics, including all types of vitamins, metals, and steroids. However, some success in increasing the hemoglobin has been achieved with  $\alpha$ -interferon. Splenomegaly is common, although splenectomy has not been helpful. Gallstones have been a problem in some patients. The most important long-term complication may be hemosiderosis caused by increased intestinal absorption of iron and ineffective erythropoiesis, combined with mild hemolysis. In some cases there are associated bone abnormalities of the extremities (syndactyly of the fingers and toes, hypoplasia or absence of fingers or toes, and vertebral abnormalities resulting in scoliosis) (149).

The gene for CDA I has been mapped to chromosome 15q15.1 (150) cloned (*CDAN1*), and many mutations have been identified (151). The role of codanin-1 encoded by *CDAN1* is unknown.

### 72.5.2 Type II Congenital Dyserythropoietic Anemia

This is the most common variant of CDA and several hundred cases of this autosomal recessive disorder are recognized (152). The gene for CDA II was mapped to 20q11.2 (153) and is now known to be associated with several different mutations in *SEC23B* (154,155). Abnormalities in this gene are thought to disturb normal protein transport through membrane vesicles, and thus may account for the cellular abnormalities noted in CDA II.

Characteristic laboratory features include anemia, normal-sized RBC, a relatively low reticulocyte count for the degree of anemia, elevated bilirubin, and decreased serum haptoglobin. The magnitude of anemia in type II CDA usually is greater than that seen in type I CDA. The bone marrow reveals increased red cell precursors with many (up to 50%) abnormal late erythroblasts as manifested by binuclearity, multinuclearity, and abnormal lobulation. The original pathognomonic findings in CDA type II were serologic in that the patient's red cells would lyse in the presence of acidified serum. Unfortunately, this test, originally used to screen for paroxysmal nocturnal hemoglobinuria (PNH), is no longer available. The combination of erythroblast multinuclearity and the sensitivity of circulating RBC to lysis by acidified normal serum was the reason that type II CDA also was known by the acronym HEMPAS (hereditary erythroblastic



multinuclearity with a positive acidified serum test (156). Glycosylation of membrane proteins is impaired, and there is also an accumulation of RBC glycolipids. The cause of both of these abnormalities is thought to be a deficiency of *N*-acetylglucosaminyltransferase II, an RBC enzyme responsible for membrane protein glycosylation.

Patients with severe anemia require blood transfusions, and in these cases splenectomy is helpful as manifested by a decreased need for transfusions. Iron overload occurs from both transfusions and increased intestinal absorption (even in untransfused patients), and in some patients iron chelation therapy may be necessary. Hyperbilirubinemia and cholelithiasis are variable features, which are now known to be five times more common in CDA II patients who also have inherited the mutation for Gilbert syndrome (157).

### 72.5.3 Type III Congenital Dyserythropoietic Anemia

This is the least common CDA variant. These patients have RBC macrocytosis and a mild to moderate degree of anemia. Transfusions usually are not required, icterus is minimal, and iron overloading does not occur. In contrast to CDA I and CDA II, this disorder is inherited as an autosomal dominant defect. Bone marrow examination shows erythroid hyperplasia, with many multinucleated erythroblasts containing up to 12 nuclei. The gene for type III CDA has been mapped to 15q21 (158).

## 72.6 SIDEROBLASTIC ANEMIAS

Sideroblastic anemias are due to acquired and hereditary disorders of heme synthesis (159–161). Most commonly these are acquired disorders occurring in middle age and later life, and they can be due to drugs, alcohol, myeloproliferative disorders, or they can be idiopathic. The inherited sideroblastic anemias are rare genetic conditions presenting in childhood.

In all sideroblastic anemias, regardless of etiology, a common feature is impaired heme synthesis leading to retention of iron within mitochondria. The bone marrow aspirate characteristically demonstrates nucleated red cells with iron granules in a perinuclear distribution (i.e. aggregates of iron in mitochondria). These unusual cells, known as ringed sideroblasts, are found only in pathological states, and are distinct from the sideroblasts in the marrows of normal subjects (i.e. RBC precursors that contain diffused cytoplasmic ferritin granules).

The different types of congenital sideroblastic anemias (CSAs) are summarized (162). Three of the most common are X-linked sideroblastic anemia, Pearson syndrome and thiamine-responsive megaloblastic anemia.

### 72.6.1 X-Linked Sideroblastic Anemia

This CSA usually occurs in males, although skewed lyonization has resulted in affected females. This disorder

is due to abnormalities of the erythrocytic isozyme for 5-aminolevulinic acid synthetase (ALAS2), the rate-limiting enzyme reaction in heme synthesis. An important cofactor for ALAS is pyridoxal phosphate. The gene for the erythrocyte-specific ALAS (ALAS2) is located on the X chromosome, and almost 50 different mutations have been identified in 80 unrelated individuals. Of interest, several of these mutations occur near the binding site for pyridoxal phosphate (160,161).

The anemia in this disorder is characterized by hypochromic microcytic RBC mixed with normal red cells, thus giving an overall picture of a dimorphic population of erythrocytes. Severe anemia can be recognized in infancy or early childhood, whereas milder cases may not become apparent until early adulthood or later. Reports of elderly women with late onset of X-linked sideroblastic anemia, presumably are a consequence of acquired skewing with age (163). Patients present with pallor, icterus, moderate splenomegaly, hepatomegaly or both. The severity of the anemia varies such that some patients require no therapy while others need regular RBC transfusions. A subset of patients manifests a hematologic response to pharmacologic doses of pyridoxine. In a few severe RBC transfusion-dependent cases, BMT has been done utilizing fully matched siblings as donors (164). Iron overload as manifested by elevated serum ferritin, elevated serum iron, and increased transferrin saturation is a major complication of this disorder. In some cases where there is little or no anemia, there still may be clinical evidence of iron overload (i.e. diabetes mellitus, liver dysfunction, etc.). The coinheritance of the gene for hereditary hemochromatosis has been shown to exacerbate the iron problems associated with sideroblastic anemia (165).

### 72.6.2 Pearson Syndrome

This unique variant of CSA is characterized by the early onset of transfusion-dependent anemia, neutropenia and thrombocytopenia (161,166). In addition to the usual marrow abnormalities of sideroblastic anemia, these children also have vacuolization of RBC and myeloid precursors, and pancreatic fibrosis. The anemia is normocytic or macrocytic. It is now known that this syndrome is due to mitochondrial DNA deletions in many different tissues (167,168). In the few children who survive the hematologic consequences of this mitochondrial DNA disorder, they often later develop Kearns-Sayer syndrome, due to an identical mitochondrial DNA deletion (169).

**72.6.2.1 Thiamine-Responsive Megaloblastic Anemia.** This CSA is associated with diabetes and deafness. The characteristic hematologic findings are megaloblastic erythroid development in the marrow with ringed sideroblasts. The disorder is associated with mutations in the high-affinity thiamine transporter

gene (*SLC19A2*). Of interest the anemia can respond to pharmacologic doses of thiamine.

## 72.7 METHEMOGLOBINEMIA

Methemoglobin (MHb) is an oxidized derivative of hemoglobin in which iron (normally  $\text{Fe}^{2+}$ ) is in the oxidized or ferric ( $\text{Fe}^{3+}$ ) state. When MHb levels are increased, oxygen transport is impaired because MHb does not complex with oxygen. Under normal physiologic conditions, small amounts of hemoglobin are continually being oxidized by endogenous agents including oxygen itself (auto-oxidation). Usually, however, MHb levels are less than 1% of the total hemoglobin because RBCs contain NADH-cytochrome  $\text{b}_5$  reductase, an enzyme that catalyzes the reduction of MHb. The cardinal clinical manifestation of methemoglobinemia is cyanosis without evidence of cardiac or respiratory disease (normal physical examination, chest radiograph, electrocardiograph, and arterial  $\text{PO}_2$ ). Blood appears dark in color but, in contrast to deoxygenated blood, mixing with air does not change the color to bright red. This is the basis of a simple screening test to detect MHb: a drop of blood is placed on filter paper and then allowed to dry while the filter paper is waved in air. Blood that is not saturated with oxygen turns red, while MHb remains brown. Cyanosis is apparent at MHb levels of 1.5 g/100 ml (10% of total hemoglobin), but symptoms due to decreased oxygen transport are generally not apparent until 30–40% of hemoglobin is oxidized to MHb. Levels greater than 70% are incompatible with life. Methemoglobinemia usually is not associated with anemia, hemolysis, or other hematologic abnormalities. Two forms of methemoglobinemia are seen clinically: acquired or toxic methemoglobinemia (common), and hereditary methemoglobinemia (rare). Acquired methemoglobinemia occurs in normal individuals exposed to increased concentrations of chemicals that oxidize hemoglobin iron (certain organic nitrates, amyl nitrate, aniline derivatives, lidocaine, prilocaine, dapsone, etc.). Hereditary methemoglobinemia is due to inherited hemoglobin M disorders or deficiency of NADH-cytochrome  $\text{b}_5$  reductase.

### 72.7.1 Hemoglobin M Disorders

These are rare autosomal dominant defects due to amino acid substitutions in  $\alpha$ ,  $\beta$ , or  $\gamma$  globin chains of hemoglobin (170). As a result of these substitutions, heme iron is more stable in the ferric than the ferrous state and RBC MHb reductive capacity cannot compensate for this instability of ferrous heme. Both  $\alpha$  and  $\gamma$  globin M hemoglobins are manifest at birth. However, methemoglobinemia disappears in the first few months of life in neonates with Hgb M disorders due to  $\gamma$  globin mutations. Conversely, M hemoglobin disorders due to  $\beta$  chain mutations usually do not appear until 3–5 months of age when  $\beta$  chain synthesis predominates. Heterozygotes for hemoglobin M disorders have increased MHb levels and

some degree of cyanosis but otherwise are asymptomatic. No therapy is indicated and none is possible. The homozygous state is incompatible with life. The presence of M hemoglobins can be detected by electrophoresis of a hemolysate in which the hemoglobin is converted to MHb (by potassium ferricyanide).

### 72.7.2 NADH-Cytochrome $\text{b}_5$ Reductase Deficiency

This is an autosomal recessive disorder. Heterozygotes are asymptomatic although intermittent methemoglobinemia occurs when exposed to certain oxidant drugs. Homozygous deficient patients often have 15–40% MHb and this can increase in the presence of certain oxidants. These patients are cyanotic, but otherwise are usually asymptomatic. Three different types of hereditary NADH-cytochrome  $\text{b}_5$  reductase deficiency are recognized. Type I deficiency is limited to RBC and is manifested by methemoglobinemia only. Type II deficiency is due to a widespread enzyme deficiency and is characterized by mental retardation in addition to methemoglobinemia. Type III deficiency occurs in RBC, leukocytes and platelets, but the only clinical manifestation is methemoglobinemia. The gene for NADH-cytochrome  $\text{b}_5$  reductase is located on chromosome 22. At least 18 different NADH-cytochrome  $\text{b}_5$  reductase mutations, mostly missense, have been described in three types of enzyme deficiency (171,172).

The diagnosis of NADH-cytochrome  $\text{b}_5$  reductase deficiency is made by direct assay of the enzyme. Most individuals with this deficiency usually require no treatment. Occasionally, therapy is given for cosmetic reasons to decrease cyanosis. This is readily accomplished with daily oral administration of methylene blue or ascorbic acid. Methylene blue, the most effective of the two, will produce blue urine, but this is harmless. In NADH-MHb reductase deficient patients who are symptomatic (i.e. more than 25% MHb), intravenous methylene blue (4 mg/kg as a 1% solution) can be given for prompt relief.

Erythrocytes contain a second enzyme, NADPH-MHb reductase, which by itself is unable to reduce MHb and individuals lacking this enzyme do not have methemoglobinemia. However, this enzyme is important in the treatment of methemoglobinemia. In the presence of certain redox compounds such as methylene blue, NADPH-MHb reductase rapidly reduces MHb to ferroheme. The response to methylene blue is both therapeutic and diagnostic. A rapid decrease in MHb occurs within 1–2 h if the cause of methemoglobinemia is due to an acquired toxic agent or a deficiency of NADH-MHb reductase. Failure to improve following administration of methylene blue suggests one of the M hemoglobins. It should be pointed out that G6PD deficiency per se is not a cause of methemoglobinemia, but rather it may cause a poor response to methylene blue because it is the primary source of NADPH.

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### Biography



**Bertil Glader** received his PhD at the University of Illinois (Physiology, 1967) and MD from Northwestern University (1968). He was a pediatric intern at Stanford University (1968–1969). Following internship he served in the US Army as a research hematologist in the Department of Medicine at the Walter Reed Army Institute of Research (1969–1972). Subsequently he completed residency and hematology/oncology fellowship at the Children's Hospital Medical Center in Boston (1972–1974). He next was on the Pediatric faculty at Harvard (1974–1977), holding joint appointments in Medicine (Hematology/Oncology) at the Children's Hospital Medical Center and the Sidney Farber Cancer Center. Since 1977 he has been at the Stanford University School of Medicine where he is a Professor of Pediatrics (Hematology/Oncology) and Pathology. He also is also an attending Hematologist at the Lucile Salter Packard Children's Hospital at Stanford. He is the medical director of the RBC Special Studies Laboratory of the Stanford Clinical Laboratories. He is a co-editor of the textbook Wintrobe's Clinical Hematology. His clinical and research interests relate to acquired and genetic hematologic problems, with special focus on inherited red blood cell disorders.

# CHAPTER

# 73

## Hemophilias and Other Disorders of Hemostasis

*Jordan A Shavit and David Ginsburg*

### 73.1 OVERVIEW OF HEMOSTASIS AND THROMBOSIS

Maintenance of the integrity of the vascular tree is critical to all higher organisms with closed circulatory systems. A complex, highly regulated system has evolved for this purpose, consisting of extensive interactions between the endothelial cell lining of the blood vessel, the blood platelet, and an intricate cascade of plasma proteins (1,2). This complex system can be conceptually divided into three major limbs, as illustrated in [Figure 73-1](#). Although depicted as discrete compartments, multiple connections link these pathways to each other and to other homeostatic processes, including inflammation and tissue remodeling.

The first response to vessel injury often involves the platelet limb of hemostasis ([Figure 73-1](#)). Exposure of specific ligands in the injured vessel wall leads to the adhesion of blood platelets at the site of injury, subsequent platelet activation recruiting additional platelets, and activation of the plasma coagulation cascade. The coagulation cascade can also be triggered directly by tissue injury, exposing tissue factor (TF) to the plasma milieu ([Figure 73-2](#)).

These processes result in a temporary plug to the injured vessel composed of a mixture of fibrin meshwork and activated platelets. A complex response then ensues, resulting in breakdown and removal of the blood clot and repair of the injured tissue. The final regulatory cascade limb of hemostasis, referred to as the fibrinolytic system, serves to dissolve the blood clot, limiting the spread of the thrombotic process and speeding its resolution. The fibrinolytic and coagulation cascades are both characterized by sequential interactions between a series of plasma proteases and their specific cofactors and inhibitors. A number of regulatory feedback loops within the system serve to dampen the coagulation cascade, most notably the protein C anticoagulant pathway ([Figure 73-3](#)).

A shift in the delicate balance between the complex interacting limbs of the clotting cascade can result in pathologic thrombosis or hemorrhage. Considerable

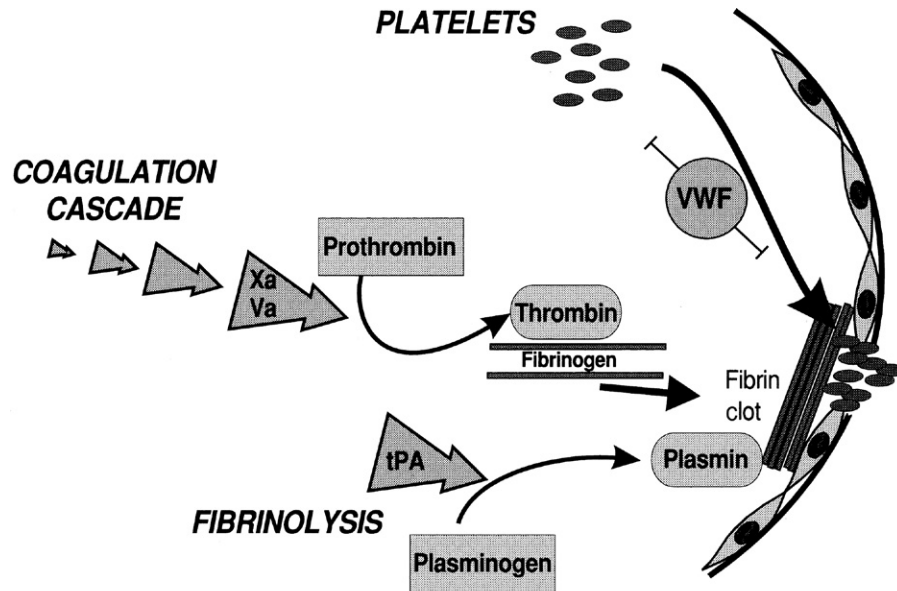
insight into the functions of the various components of this system has come from characterization of the associated human genetic diseases. In addition, subtle variation at several of the genetic loci discussed in this chapter can also contribute to susceptibility to atherosclerosis, arterial thrombosis and venous thromboembolism, the leading causes of death and morbidity in developed countries. This chapter briefly reviews the biology of the plasma coagulation cascade, platelets, and the fibrinolytic system, as background for a discussion of the inherited bleeding and thrombotic disorders resulting from genetic defects in the components of these systems.

### 73.2 THE COAGULATION CASCADE

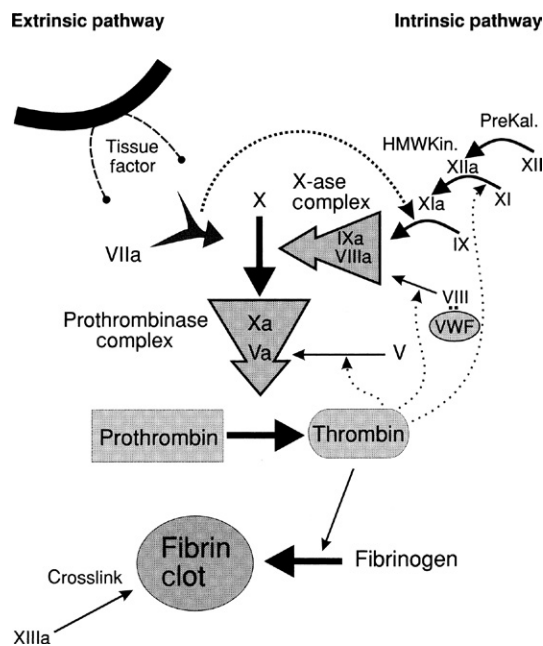
The coagulation cascade was historically one of the first biologic systems associated with human disease to be studied in detail at the biochemical level (3). This was due in large part to the ready availability of the relevant proteins through blood. Investigations over many years identified an ordered cascade consisting of a plasma protease activating an inactive zymogen target to an active protease form, which subsequently acts on the next step in the cascade ([Figure 73-2](#)). The components of the coagulation cascade were historically assigned Roman numerals, generally in the reverse order of their activation in the cascade ([Table 73-1](#)). Factors V through XIII are commonly referred to by their Roman numeral, whereas factors I through IV have more common names (fibrinogen, prothrombin, tissue thromboplastin (a combination of TF and phospholipids), and calcium, respectively). The term “factor VI” was abandoned when it was shown to simply represent the activated form of factor V (factor Va).

Although the clotting cascade was traditionally divided into the “intrinsic” and “extrinsic” pathways, the biologic significance of this distinction has been questioned. The extrinsic pathway is now viewed as the major mechanism of coagulation activation under most circumstances. The intrinsic pathway ([Figure 73-2](#)) begins with



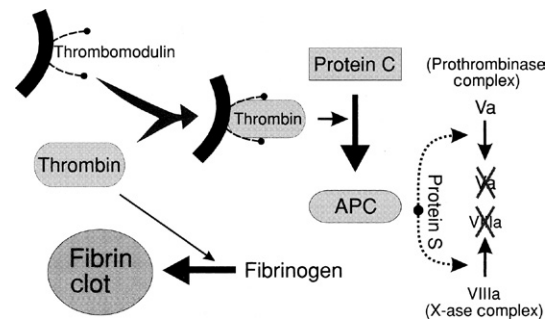


**FIGURE 73-1** Overview of hemostasis. The platelet, coagulation cascade, and fibrinolysis limbs of hemostasis are illustrated schematically. Xa, factor Xa; Va, factor Va; VWF, von Willebrand factor; tPA, tissue plasminogen activator. See text for description.



**FIGURE 73-2** The coagulation cascade. See text for description. VWF, von Willebrand factor; HMWKin, high-molecular-weight kininogen; PreKal, prekallikrein. Other clotting factors are indicated by their Roman numeral designations.

the activation of factor XII (FXII) to FXIIa on surfaces such as glass, a property exploited in several laboratory tests. FXIIa, in the presence of high-molecular-weight kininogen then activates FXI to FXIa. FXIa activates FIX, feeding into the common portion of the coagulation cascade. The activated partial thromboplastin time (aPTT), commonly used in the clinical laboratory, primarily measures the activity of the intrinsic pathway. The absence of bleeding symptoms in patients with deficiencies of FXII



**FIGURE 73-3** Protein C anticoagulant pathway. When thrombin is bound to thrombomodulin, its protease specificity is altered and no longer efficiently generates a fibrin clot from fibrinogen. Instead, thrombomodulin-bound thrombin now specifically cleaves protein C to generate activated protein C (APC). APC proteolytically inactivates factor Va and factor VIIIa, aided by its cofactor, protein S. Va, active form of factor V; VIIIa, active form of factor VIII. Factors Va and VIIIa are critical components of the prothrombinase and Xase complexes, respectively.

or high-molecular-weight kininogen, despite marked prolongation of the aPTT (see later), suggests that these factors are not important physiologic activators of the coagulation cascade. By contrast, deficiency of FXI, the next step in the intrinsic pathway, is clearly associated with significant bleeding. This paradox appears to be explained by the demonstration of FXI activation by thrombin and autoactivation by FXIa. These reactions are thought to form an amplification loop, leading to additional activation of FIX by FXIa (4,5).

The coagulation cascade is now thought to be activated primarily through the so-called extrinsic pathway (now often termed “initiation” (6)), binding of FVIIa to TF on a cell membrane surface, with subsequent activation of FIX to IXa or of FX directly. FIXa and FXa

**TABLE 73.1** Components of the Classic Coagulation Cascade

Factor Number	Synonym	Chromosomal Localization
I	Fibrinogen	4q28
II	Prothrombin	11p11-q12
III	Tissue thromboplastin (TF and phospholipids)	1p21.3 (TF)
IV	Calcium	—
V	Proaccelerin	1q23
VI	Activated form of factor V (FVa; FVI no longer used)	—
VII	Proconvertin	13q34
VIII	Antihemophilic factor	Xq28
IX	Christmas factor	Xq27.1-q27.2
X	Stuart–Prower factor	13q34
XI	Plasma thromboplastin antecedent	4q35
XII	Hageman factor	5q33-qter
XIII	Fibrin stabilizing factor	6p25-p24 (A subunit)
	Plasma transglutaminase	1q31-q32.1 (B subunit)

are serine proteases, each of which forms a distinct membrane-bound complex with a specific cofactor, FVIIIa in the case of FIXa and FVa in the case of FXa. Factors V and VIII are highly homologous, nonenzymatic proteins that serve as essential cofactors for their cognate proteases. The FVIIIa/IXa complex, sometimes referred to as the X-ase (or tenase) complex, specifically cleaves FX to generate its active form, FXa. In turn, FXa interacts with FVa to form an enzyme complex referred to as “prothrombinase,” which specifically activates prothrombin to thrombin. Initiation generates a small amount of activated thrombin, which then feedback activates the intrinsic pathway (now often termed “amplification” and “propagation” (6)) by activating factors XI, VIII and V, leading to a “thrombin burst.” This excess of thrombin, the final protease in the cascade, acts directly on fibrinogen to generate the fibrin polymer that forms the structural basis of the blood clot.

TF expression is induced on endothelial cells and monocytes by inflammatory mediators such as tumor necrosis factor and endotoxin. In addition, cells in the subendothelium and tissues surrounding blood vessels express large amounts of TF. Thus, vascular injury initiates the cascade by exposing the coagulation proteins, including FVIIa, to TF. Although a low level of FVII is constitutively present in the circulation, it is inactive until it encounters TF. In the prothrombin time (PT) assay, coagulation is initiated by the addition of a crude brain extract that contains large amounts of TF on a lipid membrane surface. This assay is most sensitive to abnormalities in the vitamin K-dependent enzymes including thrombin, FVII, FIX, and FX. These factors share a common post-translational processing event that modifies selected glutamic acid residues to  $\gamma$ -carboxyglutamic acid. Vitamin K is an essential cofactor for this reaction

and thus vitamin K deficiency, or antagonism of vitamin K by warfarin, leads to bleeding as a result of the deficient function of these proteins.

### 73.2.1 Protein C Anticoagulant Pathway and Other Inhibitors of Coagulation

Figure 73-3 illustrates a negative feedback regulatory loop in the coagulation cascade, based on the activation of the anticoagulant serine protease, protein C, by thrombin. Abnormalities in this pathway are associated with several common inherited thrombotic disorders (see later). Although thrombin is normally a procoagulant protein, when bound to the transmembrane protein thrombomodulin, thrombin is converted from a procoagulant to an anticoagulant by changing its protease target specificity. Thrombomodulin-bound thrombin cleaves protein C to form activated protein C (APC). Accelerated by its cofactor protein S, APC proteolytically inactivates FVa and FVIIIa, thereby damping down the clotting cascade. Thus, activated thrombin can feed back on itself to turn off the activation of additional thrombin. As one might predict, deficiencies of protein C, protein S, and thrombomodulin are all associated with inherited thrombotic disorders. A remarkably common mutation in FV (factor V Leiden) renders FVa resistant to inactivation by APC, also resulting in a mild prothrombotic disorder.

Several important inhibitors also contribute to the regulation of the coagulation cascade, including TF pathway inhibitor, protein C inhibitor, and two specific inhibitors of thrombin, antithrombin III (AT3) and heparin cofactor II. AT3 also inhibits FXa and several other clotting cascade proteases. The inhibitory activity of AT3 is dramatically stimulated by heparin, providing the primary mechanism for the latter's anticoagulant function. Inherited deficiency of AT3 is associated with thrombosis (see later).

### 73.2.2 Platelets

Defects affecting the platelet limb of hemostasis can also result in abnormal bleeding. Platelets provide the most immediate response to vascular injury by forming a cellular plug at the site. Deficiencies in platelet number (thrombocytopenia) or platelet function (qualitative platelet disorders) are associated with abnormal bleeding. The major ligand facilitating binding of the platelet to the vessel wall is the multimeric plasma protein, von Willebrand factor (VWF) (Figure 73-1). Defects in VWF result in a platelet-like bleeding disorder, von Willebrand disease (VWD) (see later).

### 73.2.3 The Fibrinolytic System

The final limb of the hemostasis tree depicted in Figure 73-1 is provided by the fibrinolytic system. This proteolytic cascade results in dissolution of the fibrin clot. The

final enzyme in this pathway is plasminogen, a serine protease zymogen that in its active form (plasmin) specifically degrades fibrin and may also contribute to tissue remodeling through proteolysis of other matrix components. Plasminogen is activated by plasminogen activators (PAs), which include urokinase-type and tissue-type PA (uPA and tPA, respectively). Plasmin, as well as both PAs, are inhibited by specific serine protease inhibitors,  $\alpha_2$ -antiplasmin and PA inhibitor-1, respectively. Deficiencies of these inhibitors are associated with abnormal bleeding, whereas deficiency of plasminogen has recently been uncovered as the explanation for a rare autosomal recessive disorder, ligneous conjunctivitis (see later).

### 73.2.4 Inherited Disorders of the Coagulation Cascade

Inherited bleeding disorders due to deficiencies of factors within the coagulation cascade (Table 73-1) generally result in similar phenotypes. Hemorrhage into deep tissues, particularly the joints (hemarthroses), as well as increased bleeding following surgery or trauma, is characteristic. The pattern of bleeding can often be distinguished clinically from that associated with defective platelet function, as outlined in Table 73-2. Bleeding associated with coagulation cascade disorders is generally delayed compared to that of platelet defects. The latter is more often from mucosal surfaces, particularly the nose, oral cavity, and gastrointestinal tract, in contrast to the deep tissue hemorrhage characteristic of abnormalities in the coagulation cascade. These clinical differences can be quite useful in establishing a differential diagnosis. The PT and aPTT are typically used as screening tests for coagulation cascade defects and are generally normal in inherited platelet disorders. The bleeding time, although often unreliable and of questionable value as a screening test (7,8), should generally be prolonged in platelet disorders and normal in coagulation cascade defects. If the initial PT and aPTT screening tests are abnormal

or there are other reasons for clinical suspicion, additional workup, including specific factor assays, may be indicated. For most clotting factors, activity is measured in units, where 1 unit represents the activity present in 1 mL of normal plasma. Values may also be expressed as a percentage of the activity present in an equal volume of normal plasma. Pooled plasma from a large number of normal donors is generally used as a reference standard. Specialized platelet aggregation studies can be performed in suspected cases of platelet dysfunction. There is currently no reliable screening laboratory test for defects in the fibrinolytic system.

### 73.2.5 Hemophilias

The term *hemophilia* is generally reserved for two specific inherited X-linked disorders, FVIII deficiency (hemophilia A or classic hemophilia) and FIX deficiency (hemophilia B or Christmas disease), although FXI deficiency is sometimes referred to as hemophilia C. Other clotting factor deficiencies, particularly von Willebrand disease (VWD), are sometimes included in this general disease category. The latter is also characterized by decreased FVIII levels, as a result of the close association between these two proteins.

**73.2.5.1 Hemophilia A.** Hemophilia A is the most common inherited severe bleeding disorder. Its X-linked pattern of inheritance was first recognized by Jewish scholars in the second century AD, who exempted a male infant from circumcision if his mother and mother's sisters had other sons who had died of bleeding following their circumcisions (3,9). The syndrome was rediscovered in the late eighteenth and early nineteenth centuries and is often referred to as "classical hemophilia" (9–11). A number of cases of hemophilia occurred among several branches of the royal families of Europe, and for many years it was unclear whether these represented hemophilia A or B (12). However, advanced technologies including next-generation sequencing led to the recent identification of a causative mutation in the *FIX* locus (13).

FVIII is a nonenzymatic cofactor that is assembled on membrane surfaces together with FIXa to form the "X-ase" complex (Figure 73-2) that subsequently activates the zymogen FX to the active protease FXa. The central nature of this step in the cascade and the critical role of FVIII were largely revealed by the severe clinical manifestations of FVIII deficiency.

**73.2.5.1.1 Clinical Features.** The clinical manifestations of hemophilia A vary considerably from a severe bleeding disorder presenting at birth to a very mild condition that may be totally asymptomatic or only diagnosed very late in life (11,14,15). The severity of the disease can be predicted quite accurately from the level of residual FVIII activity (Table 73-3). Patients with very low or no FVIII activity (less than 1%) are affected with severe hemophilia A, whereas those with FVIII levels of 1–5% have moderate and those with 5–25% mild

**TABLE 73.2 Clinical Findings in Inherited Bleeding Disorders**

Parameters	Platelet-Type Defects	Coagulation Cascade Defects
Timing of bleeding	Early after trauma or spontaneous	Delayed
Sites	Skin and mucous membranes, petechiae, ecchymoses	Deep tissue hematomas, including joint, muscle and retroperitoneum
Inherited disorders	von Willebrand disease, Glanzmann thrombasthenia, Bernard–Soulier syndrome, other inherited platelet defects	Hemophilia A and B, factor V deficiency, deficiency of factors XI, VII, II, or X; afibrinogenemia

disease. FVIII levels above 25% are generally associated with a normal phenotype (Table 73-3). FVIII activity is measured in a clotting assay that titrates the capacity of a test sample to complement the defect in FVIII-deficient plasma, with 100%, or 1 U/ml, representing the comparable activity of normal plasma. In addition to this functional assay for FVIII activity, FVIII protein antigen can be measured using specific antibodies, although this assay is not generally available on a routine clinical basis. It is important to point out that FVIII and VWF circulate together as a tight complex in plasma, with VWF constituting approximately 98% of the mass of the complex. Thus, antibodies raised against this complex, historically referred to as FVIII-related antigen (FVIII:Ag), actually detect the VWF protein rather than FVIII and should be more correctly referred to as VWF:Ag. Unfortunately, many laboratories still use the old nomenclature, leading to considerable confusion. This test, (VWF:Ag or FVIII:Ag) is a standard part of the VWD workup. Although generally decreased in VWD, plasma VWF:Ag should be normal in hemophilia A. As noted earlier, the bleeding time is also generally normal in hemophilia A. FVIII deficiency can prolong the PT and aPTT, although the aPTT is usually more severely affected.

Hemophilia A should be suspected in any male with a severe congenital bleeding disorder and also in older males with mild bleeding. The diagnosis can usually be readily established by screening with the PT and aPTT, followed by the specific FVIII assay, with the level expected to correlate directly with the clinical course (Table 73-3). Although FVIII deficiency due to an acquired autoantibody inhibitor is not uncommon, these diagnoses can readily be distinguished in the laboratory by routine mixing assays between the test and control plasmas. The FVIII level can also be low in VWD. It is particularly important to distinguish hemophilia A from variants of VWD, especially type 2N (see later).

TABLE 73.3 Clinical Classification of Hemophilia A and B		
Classification	FVIII or FIX Activity (%)	Clinical Manifestations
Severe	<1	Spontaneous hemorrhage beginning in early infancy. Frequent hemarthroses; hemarthroses and other serious hemorrhages requiring factor replacement
Moderate	1–5	Hemorrhaging following trauma; occasional spontaneous hemorrhage
Mild	5–25	Bleeding generally only following significant trauma or surgery
	>25	No significant bleeding

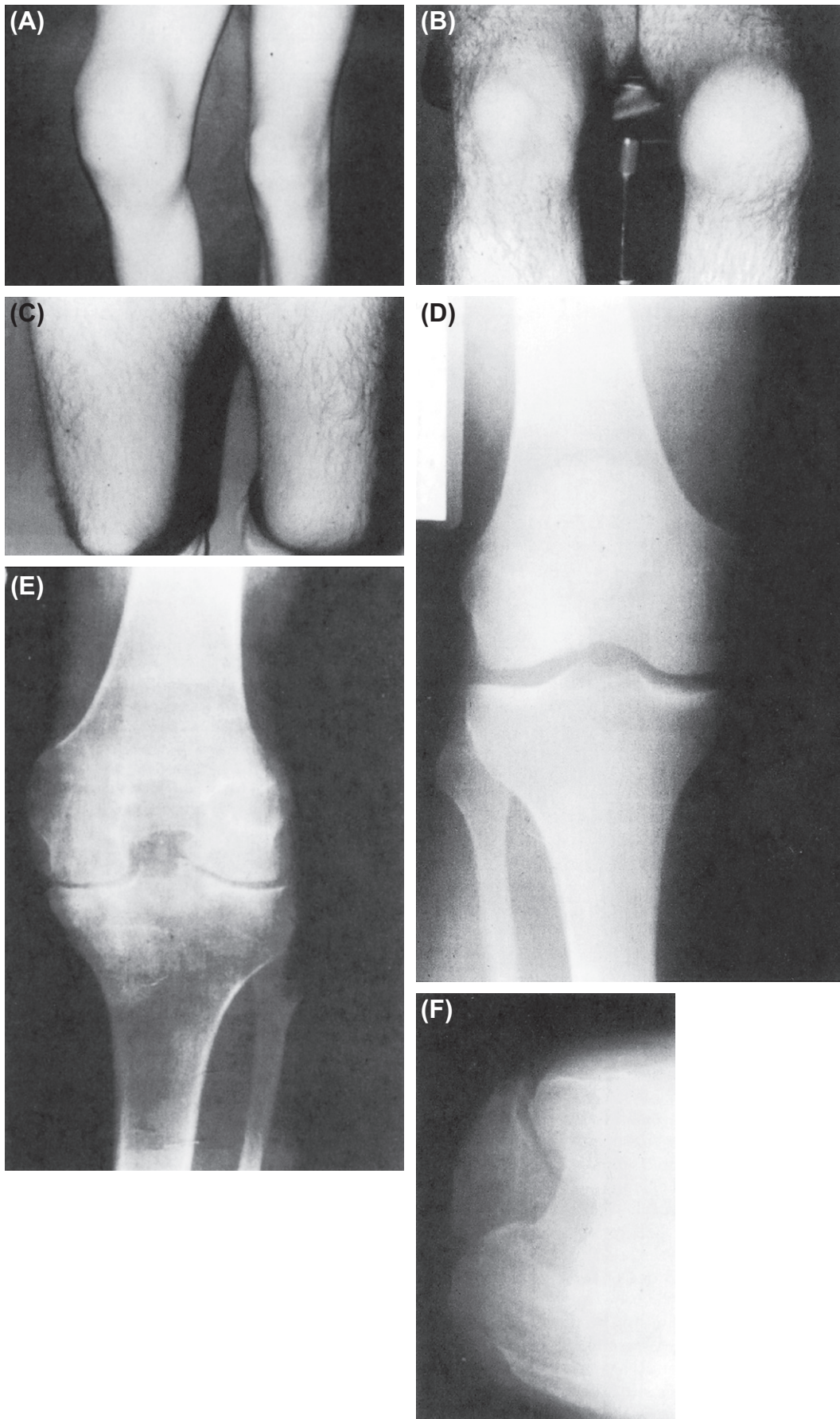
The typical clinical presentation in hemophilia A patients includes joint and muscle hemorrhages, easy bruising, and excessive, sometimes fatal hemorrhage after trauma or surgery. Hemarthroses are most common in the large joints, including the knees, elbows, ankles, shoulders, and hips. Although joint hemorrhage is generally triggered by stress or trauma, the precipitating event may not be recognized. Hemorrhage into joints is unusual until the child begins to walk, and the disease may go undiagnosed until that time. However, many severe hemophiliacs are diagnosed around the time of birth due to hemorrhage from the umbilicus or following circumcision.

Hemarthroses generally begin with mild pain and some limitation of motion. After several hours, pain will increase and joint swelling and warmth will be noted. Damage to the joint results from a chronic inflammatory reaction, and the resulting synovitis renders the joint more susceptible to repeat hemorrhage, leading to accumulated damage (Figures 73-4 and 73-5). Repeated bleeding into chronically inflamed joints results in the development of “target joints,” which are particularly susceptible to recurrent hemorrhage. The radiographic findings associated with the hemophilia joint are characteristic, although not significantly different between hemophilia A and B. Hemophilic arthropathy, with loss of cartilage and limited motion, often leads to permanent disability and may require joint replacement in selected cases. Such surgery can be accomplished safely under the cover of FVIII replacement (see later).

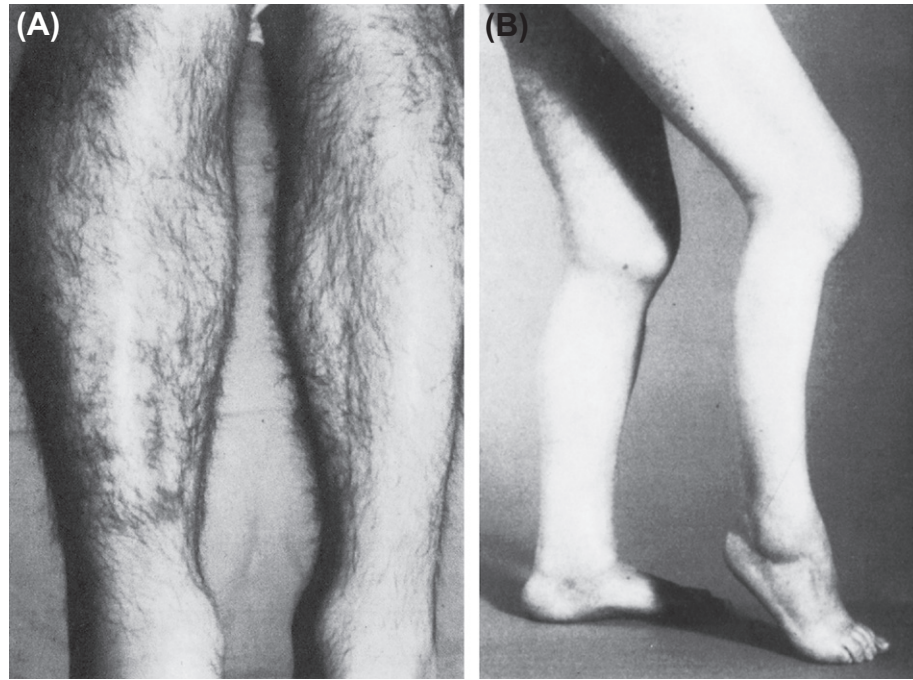
Other forms of internal bleeding may be more immediately life threatening. Large, intramuscular hematomas can result in compression, sometimes leading to specific compartment syndromes. Bleeding into other sensitive structures can lead to airway obstruction or other complications. Perhaps the most urgent type of bleeding is intracranial, accounting for about 25% of deaths among hemophiliacs prior to acquired immunodeficiency syndrome (AIDS). Another unique clinical problem seen in the hemophilias is the development of pseudotumors, cystic lesions arising from hematomas filled with a brownish necrotic core of debris surrounded by a thick fibrous wall. These lesions may also be complicated by infection. Hematuria frequently develops in patients with severe hemophilia during their lifetime. Although workup for structural defects is indicated, this is usually negative. A common complication is the development of ureteral obstruction by clots.

Modern treatment with FVIII replacement has resulted in a dramatic improvement in life expectancy from 11.4 years during the early 1900s to between 60 and 70 years in 1980. The AIDS epidemic has had a major impact on the hemophilia community and for a period during the 1980s to 1990s it was a central clinical manifestation of this disease. Most hemophiliacs receiving blood products prior to 1984 were exposed to the human immunodeficiency virus (HIV), with 60–70% of US and Western





**FIGURE 73-4** (A) Acute hemarthrosis. (B) Chronic hemophilic arthropathy particularly of the left knee demonstrating enlargement of femoral condyles with associated quadriceps wasting (C). Radiographs of right (D) and left (E) knees of the same individual illustrating rarefaction, enlargement of condyles and intercondylar notch and loss of cartilage along with degeneration of the patellofemoral surface (F).



**FIGURE 73-5** (A) Hematoma in calf. If inadequately treated, ischemic necrosis and subsequent fibrosis will ensue to give equinus deformity (B).

European severe hemophiliacs treated at that time subsequently becoming HIV infected (10,11,14,15). Many of these patients have developed overt AIDS, approximately two-thirds of whom have died. As a result, the median life expectancy for hemophiliacs between 1981 and 1990 dropped to 49 years (14). With improved screening and treatment of blood-derived factor, as well as recombinant factor products, this is no longer a major clinical issue.

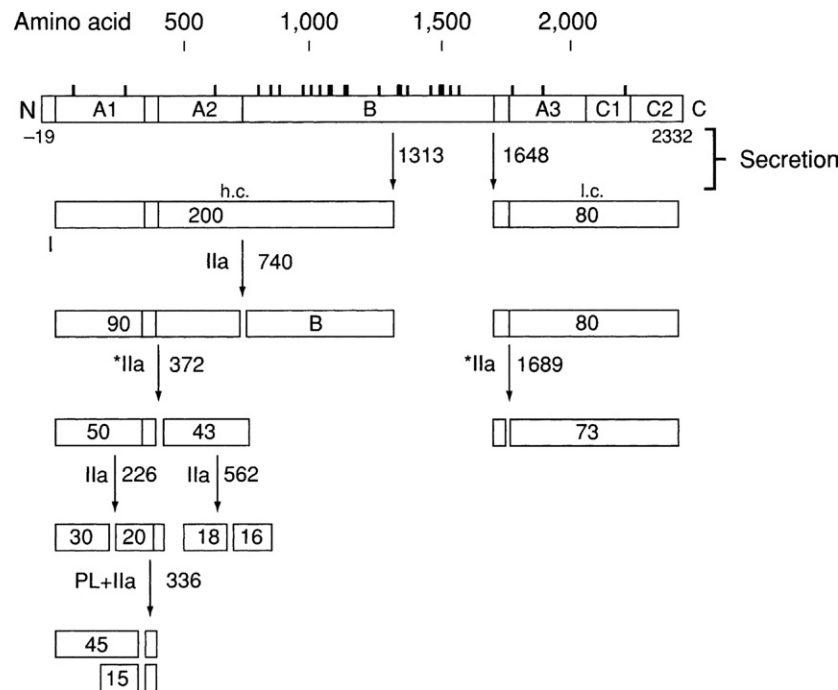
**73.2.5.1.2 Genetics.** The FVIII gene is located near the tip of the long arm of the X chromosome (Xq28). It is a large gene spanning 186 kilobases (kb), constituting 0.1% of the X chromosome, and is dispersed across 26 exons corresponding to a 9-kb mRNA (16). The FVIII protein sequence demonstrates a repeated structure, with three A domains, duplicated C domains, and a large central B domain, the last of which is encoded in a single exon (17–19) (Figure 73-6). The FVIII sequence and gene structure are highly homologous to FV (20). The B domain in both proteins appears to be a dispensable connector, since recombinant FVIII or FV from which the B domain has been deleted still maintains relatively normal function (21–23). The B domain is excised during FVIII activation, resulting in a heavy chain consisting of the A1 and A2 domains, and a light chain containing the A3, C1, and C2 domains, held together in a calcium-dependent fashion. Cleavage at arginine 1689 by thrombin liberates FVIII from VWF, allowing FVIII to bind to a phospholipid surface and interact with FIXa to form the X-ase complex (10).

A broad spectrum of genetic defects within the FVIII gene has been defined, resulting in a range of hemophilia phenotypes determined solely by the amount of residual

FVIII activity (10,24). The frequency of hemophilia A in most populations is estimated at approximately 1:5000 males (15). As predicted by Haldane, about one-third of cases appear to be new mutations, although this will likely decrease as the widespread availability of recombinant factor products leads to survival well beyond the reproductive years. Homozygous hemophilia A in females is predicted to occur at a frequency of less than 1:25,000,000 and such cases are indeed quite rare. Female hemophilia A may also result from hemizyosity for a FVIII mutation as a result of extreme inequality of lyonization or a translocation or other X-chromosome defect interfering with X inactivation. Finally, the type 2N VWD variant may also closely mimic hemophilia A, but with autosomal recessive inheritance (see later).

A large number of hemophiliacs have been studied at the DNA level (25) and a regularly updated database of known mutations can be accessed at <http://hadb.org.uk>.

Approximately 5% of patients carry deletions within the FVIII gene, generally leading to complete loss of protein expression. A variety of other defects, including frameshifts, nonsense mutations, and missense mutations, have also been described. In addition, several unique mechanisms for mutation have been identified in the FVIII gene. Two patients have been shown to have new LINE element insertions disrupting the FVIII gene (26,27). In addition, another novel mutation mechanism accounts for a large proportion of severe hemophilia A patients. Naylor and coworkers (28) initially observed an inability to amplify polymerase chain reaction (PCR) products crossing intron 22 from mRNA templates obtained from a large subset of severe hemophiliacs. Gitschier and coworkers (29) subsequently



**FIGURE 73-6** Domain structure and processing of factor VIII. The structural domains of factor VIII are depicted as A1, A2, B, A3, C1, C2. Vertical ticks indicate potential N-linked glycosylation sites. Thrombin cleavage within the B domain generates a 200-kDa heavy chain (h.c.) and an 80-kDa light chain (l.c.). Additional thrombin (IIa) cleavage sites are shown. PL indicates phospholipids and asterisks indicate thrombin cleavage sites required for activation of factor VIII. (From *Annu. Rev. Med.* 1992; 43, 325, with permission.)

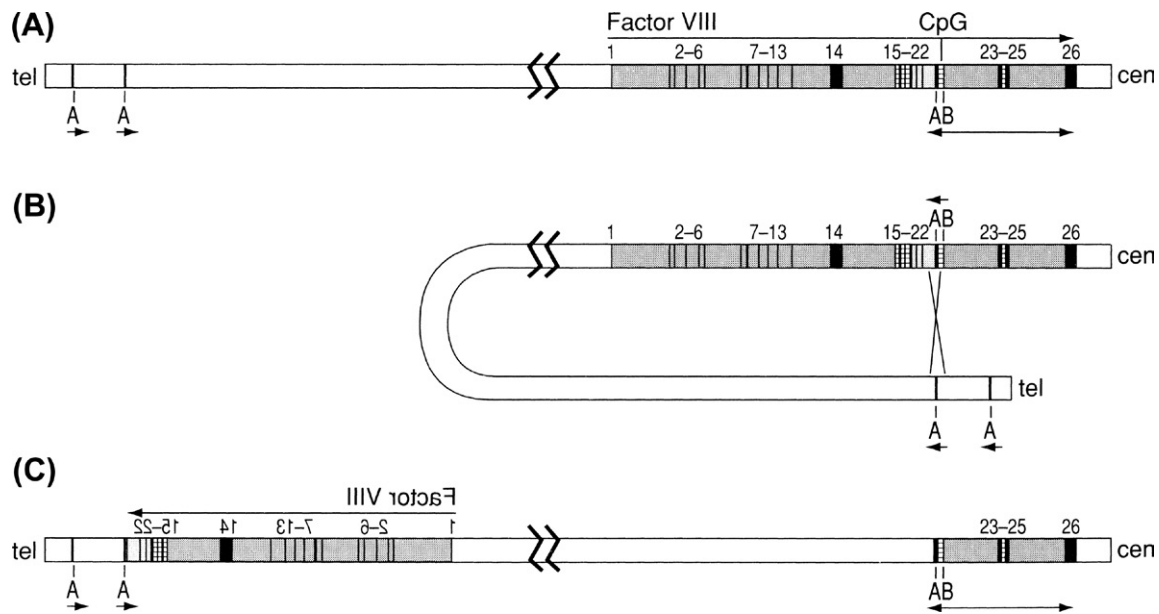
demonstrated the occurrence of a large gene inversion in these patients, explaining the PCR results. This inversion occurs via a recombination between a small gene called gene A, located within intron 22 and one of two additional copies of gene A located upstream of the gene, toward the tip of the X chromosome. The resulting inversion (Figure 73-7) disrupts the FVIII gene and removes the C-terminus of the protein encoded by exons 23–26. Two mRNAs are transcribed from the inversion, one beginning in gene A and including exons 23–26, and the derivative of the original FVIII transcript, now containing only exons 1–22. This model explains the original observations reported by Naylor et al. (28) and leads to severe hemophilia A, with no detectable FVIII activity. Remarkably, this recurrent mutation is responsible for approximately 45% of severe hemophilia A (30). A very similar mutation mechanism was identified more recently, involving a repeated sequence in intron 1 of the factor VIII gene (31). This similarly results in a large gene inversion, which is also a recurrent source of mutation in hemophilia A, accounting for about 5% of severe cases.

About 20–30% of hemophilic patients develop clinically significant antibodies against FVIII (FVIII inhibitors), which severely complicate therapy. Inhibitor development is more frequent among patients with large gene deletions, or the factor VIII gene inversion, compared to those with other types of mutations. Patients with missense mutations that result in the synthesis of residual endogenous protein, even although nonfunctional, may develop immune tolerance, accounting for considerably lower inhibitor prevalence (as low

as 5%) (32). Currently, investigation into the etiology as well as novel therapy for inhibitors is a major focus of hemophilia research. This includes examination of associations with various unlinked polymorphisms, HLA genotype, and FVIII mutation (33,34).

**73.2.5.1.3 Clinical Management.** Treatment for hemophilia A rests on replacement of the deficient FVIII activity. Although long-term continuous FVIII replacement would most likely be curative for this disorder, this approach is currently not feasible, given the limited supply of material and its high cost. Hemophilia A was initially treated with unpurified plasma, which contains 1 U/ml of FVIII activity. The cryoprecipitate fraction of plasma was subsequently shown to be enriched for FVIII activity, thus permitting administration of a smaller volume. Over the past three decades, highly purified FVIII concentrates have become the mainstays of therapy for hemophilia A. Standard commercial concentrates are prepared from plasma that has been generally pooled from 2000 to 200,000 donors. Unfortunately, the purification processes used until the mid-1980s failed to inactivate common viral contaminants, including hepatitis B and C, cytomegalovirus (CMV), and HIV. As a result, all these infections were nearly uniform among patients heavily treated during the late 1970s and early 1980s. Following the identification of HIV, methods were developed to inactivate all these viruses by detergent or heat treatment. Monoclonal antibody purification procedures further improved the quality of these products. As a result, all currently available FVIII concentrates are thought to be free of most viral hazards, although there





**FIGURE 73-7** Model of factor VIII gene inversion. (A) Structure of the normal factor VIII gene. tel, telomere; cen, centromere. A, the two copies of the A gene upstream of the factor VIII gene and the single copy within intron 22. B, another small locus that shares the A gene promoter. The arrows indicate the directions of transcription. (B) Model for homologous recombination between the intron 22 copy of the A gene and one of the two upstream copies. The indicated crossover results in an inversion of the sequence between the two recombined A genes (C). (From Nat. Genet. 1993, 5, 236, with permission.)

remains concern about slow viruses or other unknown infectious agents (10,11,15). Recombinant FVIII production was a major goal driving the cloning of the *FVIII* gene in 1984 (18,35,36), finally reaching the clinic with approval of a commercial product in 1994. Although originally assumed to be free of viral contamination, the first-generation recombinant products may have occasionally transmitted several viruses of minor or no pathologic significance, including the B19 and TT viruses, raising the concern of the potential for transmission of the Creutzfeldt-Jakob disease agent, or other as yet unknown pathogenic viruses (15,37). The source of the virus appears to have been plasma-derived albumin used as a stabilizer, and this risk seems to be reduced or eliminated in the second-generation recombinant products, which are albumin-free (37).

Newly diagnosed and previously untreated patients are generally managed with recombinant FVIII or another highly purified plasma-derived product, with most clinicians preferring the former. There are also a number of newer recombinant products including B-domain-deleted FVIII, which is synthesized more efficiently and appears to have equivalent efficacy in vivo. A third-generation product manufactured and formulated in the absence of any human or animal protein is also available (15). Currently, manufacturers are working on the production of long-acting factor products to reduce dosing frequency through PEGylation of recombinant factor (33,38-40).

The type and level of treatment are dictated by the clinical situation. Many hemophiliacs are followed in comprehensive hemophilia treatment centers where they receive multidisciplinary care. A dose of FVIII of

approximately 50 U/kg will generally raise the FVIII level from 0 to 100%. The half-life of FVIII is approximately 8 h, requiring repeated doses every 8-12 h or treatment by continuous infusion for full replacement. Therapy is monitored by following the FVIII activity level. A FVIII level of 50% or greater should achieve normal hemostasis, even under extreme conditions. Full replacement doses are generally administered at the time of a major surgical procedure or trauma, with a gradual taper at an empirically determined rate following the procedure. Less severe trauma or surgical challenge is treated with less aggressive replacement. Patients often administer FVIII at home following minor trauma or at the first sign of hemarthrosis symptoms. Early clinical experience suggested that low-level, long-term prophylaxis could convert a severe hemophiliac to a moderate hemophiliac and dramatically improve long-term prognosis for both joint and other complications (10,11,41). This was borne out in a prospective, randomized clinical trial, although there was a nonsignificant trend to increased inhibitor incidence in the prophylaxis arm (42). Prophylaxis is now the standard approach for the treatment of newly diagnosed pediatric cases in most centers, with patients performing their own prophylactic infusions two to three times per week. Hemophiliacs can also be treated with desmopressin (DDAVP), which will result in a two- to fivefold increase in FVIII and VWF levels that can last a number of hours (43). This treatment is often sufficient to achieve adequate hemostasis in mild or moderate hemophiliacs. However, in severe hemophiliacs, DDAVP generally has minimal or no effect. Oral  $\epsilon$ -aminocaproic acid (EACA) and local methods are often adequate for



dental and other superficial procedures. However, major surgery or trauma often requires aggressive support with FVIII concentrates.

Approximately 20% of patients develop antibody inhibitors to FVIII that can dramatically complicate therapy. These antibodies can reach extremely high titers, and infusion of sufficient FVIII to overwhelm the inhibitor may often be impractical. A variety of strategies have been used to treat these inhibitors, including immunosuppression and various regimens to induce tolerance. Infusion of porcine FVIII is sometimes effective, as well as other specific factor concentrates that may “bypass” FVIII in the cascade and achieve improved hemostasis (10,11,15). Recently, recombinant FVIIa has been licensed for use in hemophilia patients with inhibitors. This product is rapidly becoming the treatment of choice in the setting of life-threatening hemorrhage in inhibitor patients, although its use is limited by high cost, which can exceed \$50,000 per episode (15,44).

While advanced recombinant products have virtually eliminated known infectious risks, the suggestion has been raised that these same products may result in an increased risk of inhibitor development (33). Certain FVIII products have been suggested to specifically increase risk. Alternatively, the absence of VWF in recombinant products has been proposed to increase FVIII exposure to the immune system. However, whether one or more recombinant factor products do indeed lead to increased inhibitor formation, and if so the underlying mechanism(s), remain controversial (40,45,46).

Gene therapy may offer an improved treatment for hemophilia A in the future. Tolerance of wide levels of FVIII without major negative sequelae and the need for only small increases in FVIII activity to achieve major clinical improvement make hemophilia A an excellent candidate for a gene therapy approach. At this time eight trials for either hemophilia A or B involving 50 patients have shown no significant toxicity using various vectors, including plasmid, retrovirus, adenovirus and adenovirus-associated virus (AAV) targeting liver, skeletal muscle and autologous fibroblasts. Complications have included modest and transient elevations in factor level and development of inhibitors, and thus there are major technical obstacles to be overcome (47–49). New methods have included various strategies tested in mouse models, such as expression of FVIII in hematopoietic stem cells, thus resulting in platelet delivery to sites of injury (49). A recent, and probably the first truly successful, human trial for hemophilia B using AAV is discussed below (50).

The modern molecular genetics of hemophilia A provide powerful tools for genetic counseling and prenatal diagnosis. Diagnosis of the common FVIII inversion is now routinely performed in a number of DNA diagnostic laboratories (30). This test will define the molecular defect in approximately 45% of severe cases and can serve as a diagnostic marker in other family members

at risk. Of particular importance for genetic counseling, it appears that the FVIII inversion generally only occurs during a male meiosis, possibly due to inhibition of inversion by the pairing of Xq homologs, which occurs during a female meiosis (30,51). Thus, if a new hemophiliac case has the inversion, the mother is generally a carrier, with the inversion having occurred in the germ line of the maternal grandfather. Somatic mosaicism is surprisingly frequent in hemophilia A, occurring in up to 13% of families, although more common in families with point mutations, particularly CpG transitions (52).

Efficient screening of all 26 FVIII exons and associated splice junctions can identify the molecular defect in the vast majority of hemophilia A cases not due to FVIII gene inversions. Such direct DNA sequence screening is available through several commercial DNA diagnostic laboratories. In rare patients for whom the precise mutation cannot be identified by direct DNA analysis, accurate genetic diagnosis usually can still be achieved by linkage analysis, which is routinely available through a number of DNA diagnostic labs. A number of intragenic polymorphisms are available that should lead to an informative analysis in over 90% of families. In questionable or uninformative cases, a fetal blood sample can be collected and plasma FVIII activity measured directly, providing definitive prenatal diagnosis. Although the ratio of FVIII to VWF is generally decreased in female hemophilia A carriers and was previously routinely used for carrier detection, the accuracy of this testing is limited and variable among individual laboratories.

**73.2.5.2 Hemophilia B.** The clinical features of hemophilia B closely resemble hemophilia A. Hemophilia B is also an X-linked recessive disorder with the defect due to deficiency of coagulation factor IX (FIX). As shown in Figure 73-2, FIX and FVIII interact to form the X-ase complex, which converts FX to FXa. Thus, it is perhaps not surprising that a deficiency in either of these factors results in a similar hemorrhagic phenotype. The FIX gene is also located on the long arm of the X chromosome, approximately 15 Mb from the FVIII gene. Hemophilia B, sometimes referred to as Christmas disease, is approximately one-fifth to one-tenth as common as hemophilia A, with an estimated prevalence of approximately 1:30,000 males (15).

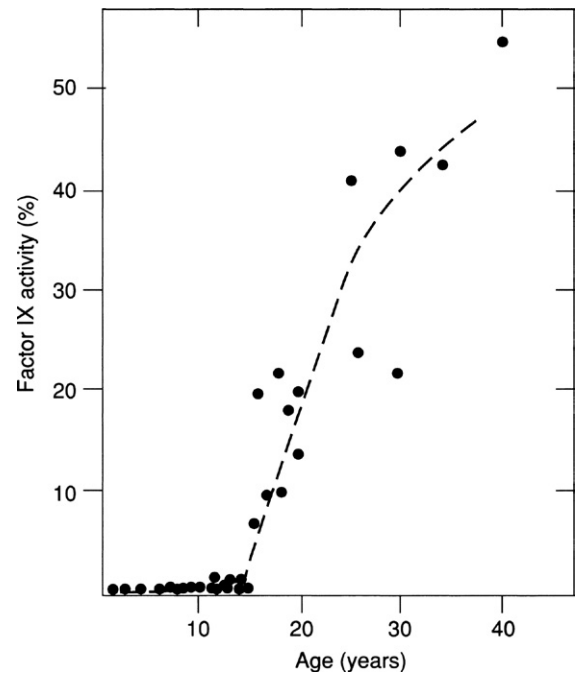
**73.2.5.2.1 Clinical Features.** Hemophilia B was first distinguished from hemophilia A in 1952 by the demonstration of two distinct complementation groups through blood mixing experiments and given the name “Christmas Disease” after one of the first patients (3,9). As in hemophilia A, the clinical severity of hemophilia B can be closely correlated with the level of residual FIX activity with levels of less than 1% associated with severe disease and of 1–5% with moderate disease (Table 73-3). Hemorrhages are again typically in deep tissues, often involving joints and resulting in chronic hemarthroses. Intracranial hemorrhage is a major cause of death in hemophilia B.

**73.2.5.2.2 Genetics.** Hemophilia B also demonstrates typical X-linked recessive inheritance. Rare cases of females affected with this disease generally demonstrate extreme lyonization or an abnormality of an X chromosome. Although carriers usually demonstrate reduced plasma levels of FIX (in the range of 50%), these values are unreliable for carrier detection given the wide variation in FIX levels among normal females. More accurate classification of carriers and prenatal diagnosis in an “at-risk” pregnancy can usually be provided by linkage analysis or direct mutation detection. Where such testing is not possible, definitive prenatal diagnosis can also be established by fetal blood sampling.

The FIX gene is 34kb in length and contains 8 exons. The gene is located approximately 15 Mb toward the centromere from the FVIII gene. The gene encodes a 461-amino acid precursor protein. FIX is a member of the serine proteinase family and shows significant homology to other members. A signal peptide is followed by a propeptide domain that is post-translationally modified by the addition of  $\gamma$ -carboxyl groups to glutamic acid, a step dependent on vitamin K and required for function. FIX shares this feature with a group of vitamin K-dependent coagulation enzymes, including factors II (prothrombin), VII, and X, as well as protein C, protein S and protein Z (53). The regulation of FIX gene transcription has been extensively studied. A unique variant of hemophilia B has provided important insights into the regulation of FIX expression. This variant termed FIX Leyden, is associated with very low levels of FIX and severe hemophilia, but promptly improves at puberty with a rise of FIX levels to near the normal range (54) (Figure 73-8). A number of point mutations in the promoter region of the FIX gene, most within a 40-bp segment surrounding the major transcription start site, have been identified in these patients, contributing to the understanding of FIX gene regulation (54–56).

A large number of gross gene deletions, small insertions/deletions and point mutations have been identified in more than 2800 hemophilia B patients. A complete database of known mutations is maintained by a consortium of investigators in this field and can be accessed at <http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html>.

As for FVIII, inhibitor antibody formation is observed primarily in patients with large gene deletions, to a lesser extent with nonsense mutations, and rarely in patients with missense mutations (32). Point mutations occur throughout the coding sequence and also have been identified in positions interfering with splicing or other processing steps. The study of FIX gene mutations has provided valuable information about the general mechanism and rate of mutation in human populations. Approximately 1/3 of FIX gene mutations have been shown to occur at CpG dinucleotides (57) and approximately 1/3 of severe patients appear to represent new mutations, as also observed in hemophilia A and consistent with the original hypothesis of Haldane for X-linked lethal disorders. Analysis of a large cohort of hemophilia



**FIGURE 73-8** Factor IX Leyden. Factor IX activity levels in plasma as a function of age. Factor IX Leyden is associated with a severe hemophilia B phenotype that markedly improves after puberty. (From N. Engl. J. Med. 1982; 306, 788, with permission.)

B patients identified an overall human mutation rate of  $2.14 \times 10^{-8}$  per base per generation (or 128 mutations per human zygote), with an estimated 1% of these (approximately 1.3 per zygote) being detrimental (58).

Although no common recurrent mutation mechanism (as occurs in hemophilia A) has been identified in hemophilia B, the smaller size of the FIX gene makes direct DNA sequence analysis for mutation detection more straightforward. Such testing is available through several clinical DNA diagnostic laboratories. In addition, numerous polymorphisms have been identified within the FIX gene and can be used for linkage analysis for those cases in which a responsible mutation is not readily identified. DNA testing can thus be used for prenatal diagnosis or to definitively establish carrier status in at-risk females.

**73.2.5.2.3 Clinical Management.** Similar to hemophilia A, the treatment of hemophilia B is based on replacement with highly purified FIX concentrates or with recombinant FIX products. Management is generally similar to that of hemophilia A, and many of the experimental approaches discussed earlier for hemophilia A are being attempted for hemophilia B as well, including long-acting recombinant factor and inhibitor management. As with FVIII, the management of inhibitors to FIX is complex, although also improved by the availability of recombinant FVIIa (44). One particularly innovative approach is the oral ingestion of bioencapsulated FIX (expressed in plants) to induce tolerance in mice (46).

Although less common than hemophilia A, the much smaller size of the FIX gene has made hemophilia B an

attractive target for experimental gene therapy. A handful of human trials have been conducted to date, with only modest and transient increases observed in plasma FIX levels (48,49). Many of the experimental approaches discussed earlier for hemophilia A are being attempted for hemophilia B as well, including long-acting recombinant factor and inhibitor management, as well as gene therapy (see earlier). Two particularly exciting advances have been reported recently. The first was successful somatic cell genome editing using zinc finger nucleases. This achieved FIX expression levels of 1–5% in a mouse model of hemophilia B (59). Second was the first truly successful human gene therapy for hemophilia (50), with FIX levels maintained at 2–11% for 6–16 months with minimal to no side effects. Past clinical trials had demonstrated only transient correction possibly because of immune-mediated clearance of the viral gene therapy vector (49).

### 73.2.6 VWD

VWD is due to either a quantitative or a qualitative defect in VWF. In contrast to the hemophilias, where straightforward correlation between the level of factor activity and clinical phenotype can be made (Table 73-3), the clinical manifestations of VWD are bewilderingly complex, with extensive phenotypic and genotypic heterogeneity. Although VWD is considerably more frequent than hemophilia, the most common forms are generally quite mild and frequently go undiagnosed.

VWD was first described by Eric von Willebrand in 1926, who originally gave it the name “hereditary pseudohemophilia.” Considerable confusion existed for many years between this disorder and hemophilia A, since FVIII procoagulant activity is frequently also reduced in VWD. In addition, the antigen recognized by antibodies raised against the partially purified FVIII complex, composed primarily of VWF, was initially referred to as FVIII antigen. These antibody-based assays are now known to predominantly measure VWF. Thus, this factor, although normal in hemophiliacs, is generally decreased in VWD. VWD is also distinguished from hemophilia A by its generally autosomal dominant mode of inheritance.

**73.2.6.1 Clinical Features.** Multiple clinical subtypes of VWD have been described and an NHLBI expert panel recently formalized evidence-based guidelines for diagnosis and therapy (60). Traditionally, the most common variant of VWD, type 1, is associated with simple quantitative deficiency of VWF, with levels in the range of 3–50% of normal. Type 1 VWD appears to account for about 70% of clinically significant VWD (61,62), and inheritance is generally autosomal dominant with incomplete penetrance. The NIH Expert Panel (60) recommended that a definitive diagnosis of VWD generally be restricted to patients with VWF levels <30% of normal, given the significant overlap of levels of 30–50% with the lower range of the normal distribution. However, this does not preclude a diagnosis of VWD in patients

with 30–50% VWF levels, in the presence of additional supportive clinical or family history evidence.

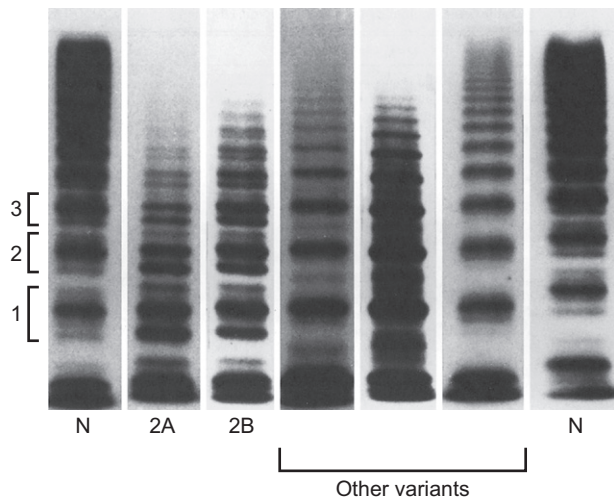
VWF is a multimeric plasma glycoprotein that plays two major roles in hemostasis (63,64). First, it serves as an adhesive link between platelets and the blood vessel wall at sites of vascular injury (Figure 73-1). Second, it serves as the carrier for FVIII in plasma, with deficiency of VWF generally resulting in a corresponding decrease in the FVIII level. Mild VWD is generally associated with only modest decreases in FVIII, which should not contribute significantly to the bleeding tendency. The major cause of bleeding in mild VWD is related to the deficiency in platelet adhesive function. This bleeding resembles that associated with thrombocytopenia or a mild platelet functional defect. In contrast to the deep tissue bleeding of hemophilia, VWD patients suffer prolonged cutaneous or mucosal bleeding, such as spontaneous nosebleeds or gastrointestinal bleeding. More serious systemic bleeding is unusual but can complicate major surgery or trauma. Increased or prolonged menstrual bleeding is also a frequent complication in female patients who, as a result, are more likely to have the diagnosis established.

Bleeding time is a crude indicator of hemostatic function, determining the time to cessation of bleeding, following placement of a uniform superficial wound in the forearm. The bleeding time is typically prolonged in VWD and platelet disorders, but normal in hemophilia, although this test is notoriously unreliable and nonreproducible. False-positive and false-negative results are frequently observed. Aspirin can prolong the bleeding time in normal people as well as VWD patients. Although this has been suggested as a provocative test for VWD, the overall clinical utility of the bleeding time has been questioned (7,8). Automated platelet function testing devices are on the market to replace the bleeding time, but their clinical utility has not yet been clearly demonstrated (65).

The standard laboratory workup for VWD consists of bleeding time, and a series of coagulation tests, often referred to as the “VWD workup” (61). The latter includes FVIII activity, VWF antigen level (previously referred to as FVIII-related antigen or FVIII:Ag), and a ristocetin cofactor assay of VWF functional activity. All these three tests are generally proportionately decreased in typical type 1 VWD. In hemophilia, only FVIII activity is decreased. To properly subclassify the type of VWD, an additional test must be performed to examine the multimeric structure of plasma VWF. VWF multimer analysis is generally performed by agarose gel electrophoresis (Figure 73-9), although other techniques, including crossed immunoelectrophoresis, are sometimes used. In type 1 VWD, although VWF is quantitatively decreased, the multimer pattern is normal.

A number of less common variants of VWD have been distinguished, including types 2A, 2B, 2N, and 2M. Their features are summarized in Table 73-4. Type 2A VWD is associated with selective loss of large and intermediate-size VWF multimers, the forms of VWF that generally





**FIGURE 73-9** VWF multimer analysis. VWF multimers from plasma of patients with various qualitative variants of VWD are shown here, as analyzed by agarose gel electrophoresis. The brackets to the left encompass three individual multimer subunits including the main band and its associated satellite bands. The finer subclassification of VWD variants is often based on variations in these satellites. N, normal sample, 2A and 2B, type 2A and 2B VWD, respectively. (From Berkowitz, S. D.; Ruggeri, Z. M.; Zimmerman, T. S. *von Willebrand Disease. In Coagulation and Bleeding Disorders. The Role of Factor VIII and von Willebrand Factor*; Zimmerman, T. S., Ruggeri, Z. M. Eds.; Marcel Dekker, Inc.: New York, 1989, pp 215–259, with permission.)

exhibit the greatest adhesive function. Type 2B VWD is associated with spontaneous or increased binding of VWF to platelets, with subsequent clearance from circulation of the largest multimers, as well as platelets, resulting in thrombocytopenia and a multimer pattern similar to type 2A VWD. Type 2M VWD is often misdiagnosed as type 1 VWD, which has resulted in patient reclassification after analysis by VWF gene sequencing (66). In contrast to type 1 VWD, which has a VWF activity to antigen ratio close to 1, type 2M is characterized by a ratio less than 0.5–0.7, in the presence of normal VWF multimers (60).

Type 2N VWD, or VWD Normandy, is a unique variant associated with decreased VWF binding to FVIII (61,67). VWF function is otherwise normal. Homozygotes or compound heterozygotes for this condition can have moderately decreased FVIII levels closely resembling mild to moderate hemophilia A. However, this disorder is distinguished from hemophilia A by autosomal recessive inheritance and the decreased ability of plasma VWF to bind FVIII. The latter is also often reflected in a poor response to purified FVIII concentrates; thus differentiation of type 2N VWD from hemophilia A is of particular clinical importance.

A large number of other variants have been reported, many as isolated cases. These are generally distinguished by subtle abnormalities in the VWF multimer structure. A database of known VWD mutations and polymorphisms is maintained by a consortium of investigators in this field: <http://www.vwf.group.shef.ac.uk/>.

Type 3 VWD, the most severe form, is associated with extremely low or undetectable levels of VWF and generally also very low levels of FVIII (61). This disorder often appears to be autosomal recessive in inheritance, with parents unaffected or showing only borderline evidence for type 1 VWD. In other families, one or both parents may be affected with characteristic type 1 VWD. Type 3 VWD is associated with severe bleeding, both the mucosal type typical of type 1 VWD as well as the deep tissue bleeding characteristic of FVIII deficiency. Thus VWD testing should be included in the analysis of patients with severe FVIII deficiency, to avoid confusion between type 3 VWD and hemophilia A.

**73.2.6.2 Genetics.** The VWF gene is composed of 52 exons spanning approximately 180 kb on the short arm of chromosome 12 (68). There is also a nonprocessed partial pseudogene, localized to chromosome 22, that duplicates approximately the middle third of the VWF gene (exons 23–34). VWF encodes a 2813-amino acid protein composed of a number of internally repeated, homologous domains (Figure 73-10.)

A large number of genetic defects responsible for VWD have been reported. Gene deletion appears to be an uncommon mechanism, with only a limited number of cases identified. These deletions can include the entire VWF gene. Gene deletions, nonsense mutations, and frameshifts have been identified in a number of cases of type 3 VWD. A frameshift mutation in exon 18 is particularly common in Scandinavia and has been shown to be the defect in the original family described by von Willebrand (69,70). Several large-scale, multicenter sequencing efforts have provided new insight into the molecular genetics of type 1 VWD (71–73). VWF gene sequencing in approximately 330 type 1 families identified candidate mutations in 67% of index cases. Seventy-five percent were missense mutations scattered throughout the gene, and the rest were small insertions/deletions, splicing mutations, nonsense mutations, or promoter mutations (summarized in (74)).

Mutations have also been identified accounting for the majority of type 2A VWD, restricted primarily to the A2 repeat within exon 28 (Figure 73-10). These mutations have been shown to produce type 2A VWD via two distinct mechanisms (75). In group 1, a mutation results in the misfolding of the protein and retention in the ER, with multimerization accounting for the dominant negative effect. In group 2, multimers containing mutant subunits are secreted but show increased susceptibility to proteolysis in plasma, resulting in degradation of the largest multimers to smaller forms. The end result in both groups is similar, with loss of large multimers and a variable quantitative decrease in VWF. The plasma protease responsible for accelerated cleavage of type 2A VWF (76) is now known to be ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type 1 motif, 13). ADAMTS13 appears to be responsible for the physiologic processing of wild-type VWF



**TABLE 73.4 Summary of VWD Subtypes**

Subtype	Frequency	Clinical Features	Diagnosis	Molecular Basis
Type 1	1–10:1000; most common VWD variant (>70% of VWD)	Mild to moderate bleeding; autosomal dominant; incomplete penetrance (approximately 60%)	VWF:Ag, VWF:RCo, and FVIII all proportionately decreased (20–50 U/dl); normal multimer distribution	Some cases are heterozygotes for type 3 VWF
Type 3	1–5:10 <sup>6</sup>	Severe bleeding disorder; autosomal recessive inheritance	Markedly decreased or undetectable VWF:Ag, VWF:RCo, and FVIII	VWF gene deletions; nonsense mutations; frameshift mutations; other cis defects in mRNA expression; some cases are homozygous for type 1 defect
Type 2A	Approximately 10–15% of clinical VWD cases	Mild to moderate bleeding disorder; autosomal dominant, more complete penetrance than type 1; generally poor response to DDAVP	Variably decreased VWF:Ag, VWF:RCo, and FVIII; absent high and intermediate size VWF multimers with prominent satellite bands	Missense mutations clustered within VWF A2 domain; two subgroups: group 1, defect in intracellular transport; group 2, proteolysis in plasma after secretion
Type 2B	Uncommon variant (<5% of clinical VWD)	Mild to moderate bleeding disorder; autosomal dominant, more complete penetrance than type 1; ? DDAVP contraindicated	Variably decreased VWF:Ag, VWF:RCo, and FVIII; loss of large multimers; enhanced RIPA; thrombocytopenia	Missense mutations clustered in VWF A1 domain resulting in increased or spontaneous binding to platelet GPIb
Type 2M	Uncommon variants	Mild to moderate bleeding disorder; autosomal dominant; may respond to DDAVP	Variably decreased VWF:Ag, VWF:RCo, and FVIII; disproportionately low VWF:RCo relative to VWF:Ag; normal multimers	Missense mutations clustered in VWF A1 domain resulting in decreased platelet adhesion
Type 2N (FVIII binding defects; VWD Normandy)	Allele frequency may be as high as 1% in some populations	Variable bleeding disorder; homozygotes (or compound heterozygotes) may present as autosomal hemophilia A	Variable VWF:Ag and VWF:RCo; disproportionately low FVIII; generally normal multimers; decreased or absent VWF binding to FVIII	Missense mutations within the N-terminus of mature VWF, which interfere with FVIII binding
Platelet-type VWD (“pseudo-VWD”)	Rare (case reports)	Similar to type 2B VWD	Can be distinguished from type 2B by mixing studies with normal platelets and plasma	Missense mutation within GPIb $\alpha$ -chain resulting in increased or spontaneous binding to VWF

GP, glycoprotein; RIPA, ristocetin-induced platelet aggregation; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, VWF antigen; VWF:RCo, VWF ristocetin cofactor activity.

Modified from (253), with permission.

and deficiency of this protease is the cause of thrombotic thrombocytopenic purpura (TTP) (see later) (76–83).

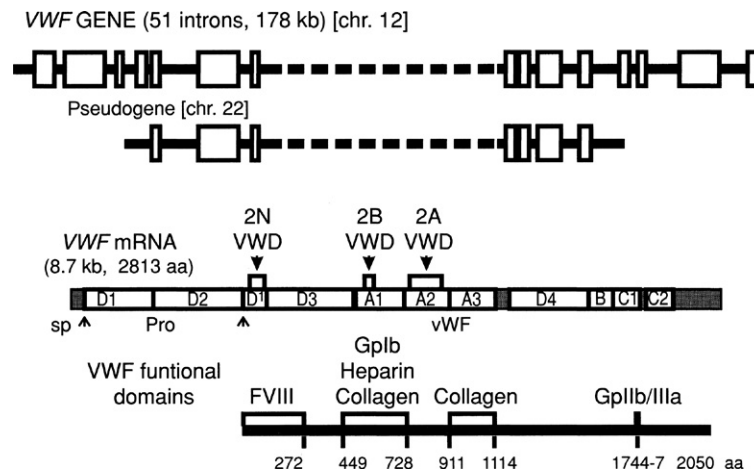
Type 2B VWD has been shown to be due to a limited number of mutations confined to the A1 repeat of VWF, corresponding to the binding domain for its major platelet receptor, GPIb (Figure 73-10). All these mutations appear to be “gain-of-function” mutations resulting in spontaneous or increased binding of VWF to platelets. Finally, a group of mutations has also been identified near the N-terminus of VWF, a region previously shown to contain the FVIII binding domain, which results in the type 2N variant of VWD, associated with decreased FVIII binding (61).

Type 2M is usually due to mutations in exon 28, which includes the A1 domain, the primarily functional

domain of VWF necessary for the ristocetin cofactor assay. These are usually missense mutations or in-frame deletions, and are autosomal dominant with a high degree of penetrance (84).

Although several examples have been reported (85), the frequency of new mutation in VWD appears to be low, consistent with the presumably modest decrease in reproductive fitness. It has also been proposed that mild VWD might actually provide some protection against pathologic thrombosis, including myocardial infarction (86). Consistent with this idea, high VWF levels are associated with venous and arterial thrombosis, at least in part through elevated FVIII levels (87–89).

Penetrance is decreased in type 1 VWD (approximately 60%) (90) and appears to be more complete in



**FIGURE 73-10** Structure of the VWF gene, mRNA, and protein. The VWF gene and pseudogene are depicted at the top with boxes representing exons and the solid black line, introns. The VWF mRNA encoding the full prepro-VWF subunit is depicted in the middle as the stippled bar and lettered boxes. The location of signal peptide (sp) and propeptide (pro-) cleavage sites are indicated by arrowheads below the bar, and the lettered boxes denote regions of internally repeated sequence. The clusters of mutations responsible for type 2A VWD, type 2B VWD, and FVIII binding defects (type 2N) are indicated above the bar. The approximate localizations for known VWF functional domains within the mature VWF sequence are indicated at the bottom. Numbers underneath the domains refer to amino acid residues within the mature VWF subunit. aa, amino acids, kb, kilobase pairs, chr., chromosome. (From Blood 1992; 79, 2507, with permission.)

type 2A and 2B VWD, although the latter has not been carefully studied. A number of factors contribute to the variable penetrance and expressivity of VWD. ABO blood group has been shown to modify VWF levels significantly and to account for about 30% of the genetic variance in plasma VWF antigen. VWF levels in type O individuals are on average 30–40% lower than those in type A, B, or AB (91,92). Families with higher heritability and lower VWF levels are more likely to have identifiable VWF gene mutations (93). Environmental effects also modify VWF levels, including estrogens, stress, underlying vascular disease, and thyroid dysfunction.

A novel locus modifying plasma VWF levels in the mouse results from altered lineage-specific expression of a glycosyltransferase, leading to aberrant glycosylation of VWF and accelerated clearance from plasma (94). Although this identical mutation is unlikely to occur in humans, similar polymorphic variation in post-translational glycosylation may account for significant alterations in plasma VWF levels and is likely the mechanism underlying the effect of ABO blood group on plasma VWF (95). Quantitative trait locus analysis using various mouse genetic backgrounds has identified additional polymorphisms, in the VWF gene and at other loci, contributing to the wide variation in plasma VWF levels among inbred mouse strains (96–98). There is some orthology between non-VWF loci in mice and humans, providing additional evidence that modifiers may be shared between the two species (96,98). Indeed, recent genome-wide association studies have confirmed ABO and VWF, as well as several other novel loci, in the control of human plasma VWF levels (99,100).

The true prevalence of VWD is controversial. Large screening studies have suggested that mild VWD may

affect about 1% of individuals in several populations (101,102). However, there is clearly overlap in VWF levels between normal and VWD patients and these prevalence figures and the criteria for a type 1 VWD diagnosis have been questioned (103). Prospective analyses of large primary care populations suggest that the prevalence of symptomatic disease is closer to 0.1% (104,105). A precise determination of prevalence must await more accurate and reproducible criteria for the laboratory diagnosis of mild VWD, possibly applying DNA diagnostic tools.

**73.2.6.3 Management.** The treatment of choice for mild type 1 VWD is the vasopressin analog DDAVP. Administration of DDAVP results in a two- to three-fold increase in VWF levels, lasting up to 6–12 hours (43,63). Although tachyphylaxis occurs after repeated use, administration every 12–24 hours is frequently sufficient to maintain adequate hemostasis. For most patients with baseline VWF levels in the 25–50% range, a two- to threefold increase is sufficient to bring VWF into the normal range. Adequate hemostasis can generally be obtained, even during major surgery. It is important to document an appropriate response to DDAVP by a test challenge prior to therapeutic use. The dose of DDAVP is generally 0.2–0.3 µg/kg, administered over approximately 20 mins by intravenous (IV) bolus. A high-concentration intranasal form of DDAVP has also been used successfully in patients, with considerably improved convenience and patient acceptance (43,63,106). As noted earlier, DDAVP is also an effective treatment in mild hemophilia A. DDAVP is generally ineffective in type 2A VWD, although some patients have been noted to respond, and is often considered contraindicated in type 2B VWD, where the release of abnormal VWF may lead to worsening thrombocytopenia and exacerbation of bleeding. Patients with type 2M

may or may not respond, but a therapeutic trial is warranted. DDAVP is of limited value in type 2N, although a therapeutic trial can be attempted. DDAVP is generally ineffective in type 3 VWD. Dental procedures in VWD patients can often be managed with oral EACA and local therapy.

In patients who are unresponsive to DDAVP or in whom DDAVP is contraindicated, the treatment of choice is factor replacement. Although recombinant VWF is used in the production of recombinant FVIII, it is removed during the purification process. A recombinant VWF product is currently in preclinical testing (107). Although effective, VWF replacement in the form of either fresh frozen plasma or cryoprecipitate is rarely used today, as standard treatment should generally be restricted to virally inactivated products. Several specific FVIII concentrate preparations containing large quantities of VWF of normal multimer structure, such as Humate-P, have been shown to be therapeutically effective (63). Most investigators view these preparations as the treatment of choice in patients unresponsive to DDAVP. It is important to emphasize that most standard plasma-derived FVIII concentrates do not generally contain useful VWF multimers, and many of the monoclonal antibody-purified preparations, as well as recombinant FVIII, are nearly devoid of VWF.

Prenatal diagnosis is not generally performed in the mild variants of VWD, given the minor bleeding usually associated with these conditions. For several of the subtypes, particularly type 2B VWD, a limited number of mutations account for the majority of patients and thus DNA analysis could be useful as a diagnostic test. Complete sequence analysis of the VWF gene, including the exons containing most type 2A, 2B, 2M and 2N mutations, has recently become available through several clinical laboratories. Testing of patient plasma VWF for reduced FVIII binding activity is also offered through specialized reference coagulation laboratories. As noted earlier, type 2N VWD is an important consideration in the differential diagnosis of mild to moderate hemophilia A, particularly if inheritance is not entirely consistent with an X-linked pattern. Prenatal diagnosis has been successfully performed by linkage analysis in a limited number of type 3 VWD families (108).

### 73.2.7 Pseudo-von Willebrand Disease

A unique platelet disorder has been described, termed pseudo-VWD or platelet-type VWD, which closely mimics type 2B VWD (109). This disorder is also associated with increased spontaneous interaction between plasma VWF and platelets and clearance of the complexes from plasma, resulting in thrombocytopenia and loss of large VWF multimers. However, mixing studies with normal platelets and plasma demonstrated that the defect in this disorder resides in the platelets rather than in VWF. The defect has been shown to be a gain-of-function

mutation in the GPIb receptor for VWF on the platelet surface (110,111), resulting in a phenotype similar to the gain of function within the VWF GPIb binding domain responsible for type 2B VWD. Replacement of VWF is ineffective in this disorder and treatment with DDAVP would appear to be contraindicated. Platelet transfusion is the treatment of choice, when treatment is necessary.

### 73.2.8 Factor XI Deficiency

After the classic hemophilias, factor XI deficiency is the most common inherited defect in the coagulation cascade (112). Factor XI deficiency is autosomal recessive and is particularly common among Ashkenazi Jews, with a heterozygote frequency of 9% in this population. One-half of all identified FXI deficiency cases occur in this population, with a much lower incidence in other groups. A number of point mutations in the FXI gene have been identified in affected patients, with two mutations accounting for greater than 90% of disease alleles among Ashkenazi Jews (113). Detailed haplotype analysis demonstrated an ancient founder allele for the common Glu117Stop mutation in this population (allele frequency 0.022), which apparently arose before the separation of Ashkenazi and non-Ashkenazi Jews. The Phe-283Leu mutation also common in this population (allele frequency equals 0.025) appears to have arisen more recently (114). A Cys38Arg founder mutation has also been identified in the French Basque population, with an allele frequency of 0.01 (115).

In contrast to hemophilia A or B, the plasma level of FXI does not always closely agree with the severity of bleeding. Occasionally, heterozygotes may also have significant bleeding. There does appear to be a correlation of phenotype with genotype, however, with the presence of hemorrhagic complications frequently breeding true in families. Significant bleeding complications are most often associated with FXI levels of less than 10%. Bleeding is most common after surgical procedures, including dental extractions. Hemarthroses are rare. Deficiency is often picked up by a screening aPTT, with specific FXI plasma activity assays available in specialized clinical laboratories. FXI deficiency has also been reported in several patients with Noonan's syndrome (116). Reported association of FXI deficiency with other clotting factor disorders, including hemophilia A and B and VWD, is probably due to the additive symptoms resulting from coincident occurrence of these relatively common defects. Dental and other minor bleeding can generally be managed with oral antifibrinolytic agents. When necessary, replacement with fresh frozen plasma can be used, preferably with a virally inactivated product. A FXI concentrate has been used in Europe, but may be associated with thrombosis. Patients who develop inhibitors can be treated with recombinant FVIIa (115).

### 73.2.9 Deficiency in Contact Activation Factors

Inherited deficiencies of FXII (Hageman factor), prekallikrein (Fletcher factor), and high-molecular-weight kininogen (Fitzgerald factor) have all been reported. These factors comprise the “contact” phase of coagulation, which also intersects with the fibrinolytic cascade and the kinin and complement systems. These disorders are primarily a laboratory curiosity, as they are generally not associated with clinically significant bleeding. Although a possible association between thrombosis and FXII deficiency has been proposed, this has not been confirmed in a large study (117). Diagnosis usually results from incidental identification of a markedly prolonged aPTT (more than 100 seconds) on a routine screening test. Inheritance of all three of these disorders is generally autosomal recessive, although possible autosomal dominant cases have also been reported. Differentiation among these three disorders requires specialized assays for the corresponding factors.

### 73.2.10 Disorders of Fibrinogen

The three fibrinogen genes,  $\alpha$ ,  $\beta$ , and  $\gamma$ , are located in a cluster on chromosome 4. Defects in any one of the three chains can result in markedly decreased fibrinogen (hypofibrinogenemia) or absent fibrinogen (afibrinogenemia) (118,119). Inheritance of afibrinogenemia is usually autosomal recessive, with consanguinity noted in many affected families. Over 150 families with congenital afibrinogenemia have been reported to date and a recent report suggests that over 80% of the mutations lie in the fibrinogen  $\alpha$ -gene, with the remainder most likely located within the closely linked  $\beta$ - or  $\gamma$ -genes. Although only a limited number of cases have been studied, the spectrum of mutations include a large gene deletion eliminating most of the fibrinogen  $\alpha$ -gene, as well as splice, frameshift, and nonsense mutations (118). Despite the central position of fibrinogen within the hemostatic system, symptoms can be surprisingly mild. Although the blood is unclottable *ex vivo*, bleeding generally appears to be less severe than in hemophilia A, a puzzling observation, given its downstream location in the cascade. Bleeding can be severe, however, and intracranial bleeding is the leading cause of death. Hemarthroses are also seen, as are mucosal and deep tissue bleeding. Pregnancies in afibrinogenemic females generally result in fetal loss. Reports of paradoxical thromboembolism have been noted in patients following replacement of fibrinogen. All the screening clotting tests, including PT, aPTT, and thrombin clotting time (TCT), are markedly prolonged, and the direct fibrinogen assays available in the clinical laboratory demonstrate absent fibrinogen. Bleeding time is also prolonged, probably due to a defect in platelet aggregation associated with the absence of fibrinogen. Hypofibrinogenemic patients rarely have significant

bleeding, unless the fibrinogen level is considerably less than 50 mg/dL. Therapy has generally been cryoprecipitate or fresh frozen plasma, although a fibrinogen concentrate is available (120).

Qualitative abnormalities in fibrinogen also occur and are termed “dysfibrinogenemia.” Defects in all three chains have been reported, resulting in abnormalities at all the possible stages of fibrinogen function, including cleavage by thrombin, polymerization, stabilization by FXIII, and fibrinolysis. Inheritance is generally autosomal dominant, indicating a dominant negative effect of the mutant subunit, since hypofibrinogenemic patients with 50% normal levels are generally asymptomatic. Clinical symptoms are varied, and about 40% of identified patients are asymptomatic, with 30% demonstrating hemorrhage and 10–15% thrombotic complications. A few patients have been seen with both hemorrhage and thrombosis. Both types of complications are generally mild, but fatal thrombosis or hemorrhage does occur. Recurrent spontaneous abortions and abnormal wound healing have also been described. The diagnosis is usually made on the basis of abnormal screening tests, with the TCT the most sensitive. Some dysfibrinogenemias result in shortening of the TCT, which may correlate with thrombosis. Fibrinogen concentration can be normal or low. Abnormalities in fibrinogen level must be distinguished from a wide variety of acquired disorders that alter fibrinogen level as well as acquired qualitative abnormalities. Many of these are associated with malignancy or autoimmune disease.

### 73.2.11 Factor XIII Deficiency

Factor XIII (FXIII) is a plasma and platelet-associated protein that cross-links the fibrin clot, significantly enhancing its stability. Clots not cross-linked by FXIII are much more easily lysed by the fibrinolytic system. Homozygous FXIII deficiency is rare with frequency estimated at less than 1:10<sup>6</sup>. Inheritance is autosomal recessive and can be due to defects either in the A subunit on chromosome 6 or in the B subunit on chromosome 1, with the vast majority in the former (119,121). Plasma levels in patients are generally less than 1%, and the disorder frequently presents with umbilical cord bleeding shortly after birth. Soft tissue hemorrhage and postsurgical traumatic bleeding are common and are classically described as being delayed by 1–2 days, although early bleeding can also be seen. Poor wound healing and abnormal scar formation are often noted. Intracranial hemorrhage is a particularly common complication and has been reported to occur in up to 25% of patients. All routine laboratory clotting tests are normal. The diagnosis is established by a urea clot solubility test since FXIII-deficient clots are readily lysed in 5 M urea, whereas normal clots are resistant. Treatment is with fresh frozen plasma or cryoprecipitate, which can be given as infrequently as every 4–6 weeks, since FXIII has an unusually



long plasma half-life (9–19 days). Concentrates of FXIII have been available in Europe, and are now available in the United States. Recombinant FXIII-A is now in phase III trials with promising results, although transient non-neutralizing inhibitors have been observed (121).

### 73.2.12 Defects in Other Coagulation Cascade Proteins

Prothrombin (factor II, FII) deficiency is quite rare, with only approximately 40 cases reported (119,122,123). Inheritance is generally autosomal recessive; defects in the prothrombin gene (chromosome 11), affecting most of the molecule, have been identified. Severity of bleeding is correlated with the level of prothrombin activity and varies from mild to severe. Residual activity has been seen in all patients and it has been speculated that complete deficiency of prothrombin is incompatible with life. Indeed, the generation of prothrombin-deficient mice by gene targeting (124,125) revealed a uniformly lethal phenotype in homozygous deficient animals. Compared to the much milder presentation of afibrinogenemia, these observations are consistent with the critical role of thrombin in platelet activation as well as fibrinogen cleavage, and potentially other processes. PT and aPTT are prolonged and TCT is normal. A specific factor assay is also available. Prothrombin complex concentrates are available and fresh frozen plasma can also be used for treatment.

Factor V (FV) deficiency is a rare autosomal recessive disorder, with an estimated frequency of 1:1,000,000 (119,122,126,127). The clinical presentation is very similar to that of hemophilia A and the disorder was originally referred to as “parahemophilia.” FV is found both in plasma and platelets and the relative contributions of these two pools to hemostasis are unclear. Some investigators have suggested a close correlation of bleeding symptoms with the platelet level of FV, and platelet transfusions have appeared to be effective therapy in some patients. However, standard therapy is fresh frozen plasma, as FV-containing concentrates are not currently available and FV is lost in the preparation of cryoprecipitate. Clinical symptoms are generally associated with levels of less than 1–20%, ranging again from severe to mild. PT and aPTT are prolonged and a specific FV assay is available. Bleeding time is also prolonged and may relate to the platelet FV pool. An unusual autosomal dominant variant of FV deficiency leading to moderate bleeding, known as FV Quebec, is associated with decreased platelet FV and a normal plasma pool (128). However, subsequent studies in these patients indicate that the reduced platelet FV is the result of a generalized accelerated proteolysis of  $\alpha$ -granule contents that is not restricted to FV alone (129), because of increased levels of urokinase-type plasminogen activator (PLAU) (130). These patients were recently shown to have a tandem duplication of the *PLAU* gene (131).

Residual low levels of plasma FV activity can be detected in nearly all FV-deficient patients, although a few examples of apparently complete deficiency have been reported, including a patient who is apparently homozygous for a frameshift mutation (132). Based on the observations that complete FV deficiency is uniformly lethal in mice, including death during midembryogenesis in a subset of animals, the occurrence of a similar early intrauterine loss in homozygous (or compound heterozygous) null human patients has been hypothesized to account for the milder defects observed in the majority of clinically ascertained patients (133). Consistent with this hypothesis, levels of plasma FV <0.1% of normal are sufficient to partially rescue the lethal hemorrhage in FV deficient mice (134), as are low levels of FV restricted to the platelet pool (135).

FVII deficiency has an incidence of 1:500,000 and has been associated with missense mutations in the FVII gene (chromosome 13) (119,122,136). Although hemorrhage is most likely with FVII of less than 10%, correlation with level is variable. Levels of less than 1% are associated with a bleeding pattern similar to that of classic hemophilia. The aPTT is generally normal and PT prolonged. A specific factor assay is available. Plasma or prothrombin complex concentrates are effective, although the treatment of choice is now recombinant FVIIa.

Factor X deficiency is a rare disorder with an incidence estimated at 1:1,000,000 (137). Analyses of patient DNAs have identified specific point mutations and deletions within the FX gene, located on chromosome 13 (119,122,137). Inheritance is autosomal recessive and the clinical presentation in severely deficient patients (activity less than 1%) is very similar to that of hemophilia. As in hemophilia, significant bleeding is rare, with levels above 10–15%. PT and aPTT are typically prolonged, and a specific factor assay is available. Treatment is with fresh frozen plasma or prothrombin complex concentrates. Recently, a FX concentrate that also contains some FIX has been developed (137).

A number of rare cases of multiple clotting factor defects have been reported. Inherited defects in the  $\gamma$ -carboxylase or vitamin K reductase pathways have been shown to account for combined defects in the synthesis of all the vitamin K-dependent factors, including FVII, FIX, FX, and prothrombin (138). Similar patterns are seen with vitamin K deficiency or with warfarin treatment. Several missense mutations in the  $\gamma$ -glutamylcarboxylase gene have now been identified in pedigrees with this disorder (138,139). This latter disorder is termed vitamin K clotting factor deficiency (VKCFD) with mutations in the  $\gamma$ -carboxylase enzyme termed VKCFD1. Recently, a second subtype of this disorder termed VKCFD2 has been shown to be due to mutations in the vitamin K2,3-epoxide reductase (VKOR) enzyme, which was first identified through a study of these patients (140). The same gene (VKORC1) was also independently identified through a direct expression cloning approach (141). This same

gene turns out to be responsible for warfarin resistance in mice and rats, where genetic mapping studies identified a syntenic region, contributing to the positional cloning. Polymorphic variations in VKORC1, as well as a cytochrome P450 gene, have also been identified as important determinants of warfarin sensitivity in human patients, accounting for 30–40% of the variance in warfarin dosing (142,143). Use of this pharmacogenetic information to guide therapy in patients has been suggested (144,145), although an improvement in patient outcome has not yet been demonstrated and such testing is not widespread in clinical practice (146).

An autosomal recessive disorder resulting in combined deficiency of factor V and factor VIII is particularly prevalent in non-Ashkenazi Jews (147) but has also been identified in a number of other populations. The gene responsible for this disorder was identified by positional cloning (148) and shown to encode the endoplasmic reticulum (ER)/Golgi intermediate compartment protein LMAN1 (also known as ERGIC-53). Complete deficiency for LMAN1 results in a coordinate decrease in both FV and VIII via partial block to the export of these specific proteins from the ER. Over 100 cases have now been identified with approximately 3/4 of studied patients found to be homozygous or compound heterozygous for null mutations in the *LMAN1* gene. The remaining subset of patients has a mutation in a second gene, *MCFD2* (149). *MCFD2* and *LMAN1* form a complex, which appears to function as a specific cargo receptor for FV and FVIII.

### 73.2.13 Platelet Disorders

#### 73.2.13.1 Inherited Disorders of Platelet Function.

A number of inherited disorders lead to congenital bleeding by interfering with the platelet limb of coagulation. All these disorders present similar clinical phenotypes, characterized by predominantly cutaneous mucosal bleeding and prolonged bleeding from minor injuries (Table 73-2). Petechiae and purpura are occasionally seen. Deeper tissue bleeding and hemarthroses are less common than in the coagulation cascade disorders, although serious central nervous system (CNS) hemorrhage can occur, particularly subarachnoid bleeding. By far the most common genetic cause of platelet-type bleeding is VWD, with the remaining inherited platelet disorders quite uncommon. A number of genetic platelet defects have been characterized in detail in the mouse (150,151), some of which may have relevance for human diseases.

**73.2.13.2 Bernard–Soulier Syndrome.** Bernard–Soulier syndrome is a rare disorder associated with platelet-type bleeding, unusually large platelets, and thrombocytopenia. The main functional defect in the platelet is loss of VWF-dependent platelet adhesion. Platelets in these patients have been shown to be missing the GPIb/IX/V complex on the platelet surface, the major receptor for VWF. Inheritance is generally autosomal recessive and frequently

associated with consanguinity. Most patients appear to have mutations within the GPIb $\alpha$  gene, although mutations in the GPIb $\beta$  and GPIX genes have also been identified (152,153).

**73.2.13.3 Glanzmann Thrombasthenia.** Glanzmann thrombasthenia is autosomal recessive in inheritance and, along with Bernard–Soulier syndrome, is the most clearly defined of the platelet functional defects at the molecular level (153,154). Platelets in this disorder show a profound defect in platelet aggregation. The clinical presentation is similar to that of other platelet functional defects. This disorder is due to absence or dysfunction of the GPIIb-IIIa ( $\alpha_{IIb}\beta_{III}$ ) integrin receptor on the platelet surface. This receptor plays a major role in platelet aggregation by binding to RGDS sites in fibrinogen and possibly other plasma proteins, including VWF. Inheritance is autosomal recessive; a variety of defects have been identified in both the *GPIIb* (*ITGA2B*) and *GPIIIa* (*ITGB3*) genes (153,154). Glanzmann thrombasthenia is sometimes divided into type I, characterized by complete absence of surface GPIIb/IIIa; type II, with GPIIb/IIIa antigen decreased to 10–20% of normal; and type III, with normal GPIIb/IIIa antigen but abnormal function. Type III appears to be due primarily to missense mutations in GPIIIa, whereas a variety of deletions and point mutations (many resulting in frameshifts or aberrant splicing) have been identified in type I patients, involving either the *GPIIb* or *GPIIIa* genes (155).

**73.2.13.4 Other Platelet Disorders.** A large number of rare congenital platelet disorders can be grouped together under the designation of storage pool deficiencies. A deficiency in any of several specific types of platelet storage granules can be seen, often requiring electron microscopic study for diagnosis. The gray platelet syndrome (GPS) results from the absence of normal  $\alpha$ -granules in megakaryocytes and platelets, although other granules appear normal. The inheritance is autosomal recessive, with only a few cases reported, occurring in both males and females. Recently, mutations in *NBEAL2* were identified as the cause of GPS (156–158). *NBEAL2* contains a domain that has been implicated in vesicular trafficking, suggesting a potential role in the development of platelet  $\alpha$ -granules.

Deficiency of dense bodies is also seen in some hereditary platelet disorders. Several of these conditions are associated with other clinical findings, including Hermansky–Pudlak syndrome, which is associated with ocular or oculocutaneous albinism. This disorder is particularly frequent in a restricted region of Puerto Rico. Abnormalities of this class of granules are also seen in other genetic syndromes, including Chediak–Higashi syndrome, thrombocytopenia and absent radii (TAR) syndrome, and Wiskott–Aldrich syndrome. In addition to the routine platelet studies described earlier, decreased serotonin uptake can often be documented in these conditions, serving as a marker for dense granule formation. Rare patients have also been described with defects in

platelet activation, affecting either the arachidonate or cyclooxygenase pathways. The precise molecular defects and the genetics of these latter disorders have not been clearly defined (153). A number of other rare disorders exist, both with and without additional constitutional phenotypes (159).

Treatment of all qualitative platelet disorders is primarily supportive, with platelet transfusion administered as necessary. Antiplatelet drugs such as aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) should be avoided. Bleeding symptoms associated with selective serotonin reuptake inhibitors (SSRIs) may be secondary to depletion of this mediator from platelet granules, resulting in platelet dysfunction, and may act synergistically with NSAIDs (160). Estrogen and/or progesterone treatment is sometimes helpful for menstrual bleeding. Dental hygiene is important and antifibrinolytics are often useful to cover dental procedures. Iron deficiency anemia frequently develops from chronic low-grade mucosal blood loss. DDAVP may be of benefit in some patients, although the mechanism is not well understood. Given its relative lack of toxicity, a therapeutic trial with DDAVP is a reasonable consideration.

**73.2.13.5 Defects of the Fibrinolytic System.** Review of the complex coagulation regulatory system depicted in Figure 73-1 would suggest that abnormal overactivity of the fibrinolytic system could also result in pathologic bleeding (161). This is indeed the case. Although relatively rare, hereditary deficiencies of the fibrinolytic inhibitors  $\alpha_2$ -antiplasmin (162,163) and plasminogen activator inhibitor-1 (PAI-1) (164,165) have both been reported. These disorders would be predicted to result in overactivity of endogenous plasmin, resulting in accelerated clot lysis. Inheritance is autosomal recessive and is associated with the expected increased fibrinolytic activity and a mild to moderate bleeding defect, usually following minor trauma or surgery. Description of these defects provided the first strong indication that the major function of the plasminogen activation system is in the regulation of blood coagulation. Treatment is generally with one of the orally available antifibrinolytic agents that inhibit plasmin function such as EACA or tranexamic acid. The latter was available only in Europe and Canada until recently. Rare patients with abnormally elevated levels of tPA associated with a bleeding tendency have been reported (166), although some of these cases may represent deficiency of PAI-1 or another primary vascular defect (161).

Mild bleeding is also seen as a component of other genetic disorders. Abnormal bleeding into the skin is commonly observed in several types of Ehlers–Danlos syndrome. Hereditary hemorrhagic telangiectasia frequently is associated with nose bleeds and gastrointestinal (GI) blood loss. Earlier reports of an apparent association between VWD and hereditary hemorrhagic telangiectasia appear to have been ascertainment bias (167).

**73.2.13.6  $\alpha_1$ -Antitrypsin Pittsburgh.**  $\alpha_1$ -Antitrypsin Pittsburgh is an unusual disease observed in only two patients (168,169), but it is a highly instructive example of a dominant “gain of function” as a mechanism for human disease. This disorder is due to a single point mutation within the  $\alpha_1$ -antitrypsin gene resulting in substitution of an arginine for methionine at the P1 position (Met358).  $\alpha_1$ -Antitrypsin is a highly abundant plasma serine protease inhibitor (serpin) that inhibits the serine protease elastase. Deficiency of  $\alpha_1$ -antitrypsin results in a common genetic disease associated with emphysema and liver disease. The effect of the  $\alpha_1$ -antitrypsin Pittsburgh mutation is to convert  $\alpha_1$ -antitrypsin from an inhibitor of elastase to an efficient inhibitor of thrombin, an activity not exhibited in native  $\alpha_1$ -antitrypsin. The mutant  $\alpha_1$ -antitrypsin thus resembles AT3 in its activity. AT3 is the primary physiologic regulator of thrombin activity and deficiency of AT3 results in a hereditary prothrombotic condition (see later). Since  $\alpha_1$ -antitrypsin is present in considerably higher plasma concentrations, the  $\alpha_1$ -antitrypsin Pittsburgh mutation results in very high levels of AT3 activity. In addition,  $\alpha_1$ -antitrypsin is an acute-phase reactant and is markedly elevated during inflammatory challenges. The clinical presentation is a severe bleeding disorder that occurs at infectious illness, due to the resulting induction of  $\alpha_1$ -antitrypsin and the associated pathologic AT3 activity. The original patient died as a result of such a fatal hemorrhagic episode (168). The second patient enjoyed a considerably milder clinical course, possibly because of a counterbalancing inhibition of protein C (169). Although this mutation has not been transmitted, inheritance would be assumed to be a complete autosomal dominant, given the unique “gain-of-function” effect. Few human genetic diseases so clearly and elegantly demonstrate this important molecular mechanism.

### 73.3 INHERITED DISORDERS PREDISPOSING TO THROMBOSIS

Pathologic thrombosis is a major cause of morbidity and mortality in the United States. In addition, inherited abnormalities in coagulation balance may contribute to the development of atherosclerosis, leading to stroke and myocardial infarction. The multifactorial genetic determinants of atherosclerosis risk are just beginning to be elucidated.

Venous thrombosis, most commonly in the lower extremities, affects approximately 1 in 1000 individuals in the United States per year and is responsible for approximately 300,000 hospital admissions annually. The occurrence of venous thrombosis in patients under the age of 45, recurrent unexplained thromboses, or positive family history are all suggestive of an inherited predisposition to thrombosis or “thrombophilia.” Indeed, a positive family history can be elicited in

approximately 40% of young patients (170,171). Five well-defined genetic risk factors attributable to coagulation protein loss or gain of function have been associated with inherited thrombophilia: AT3 deficiency, protein C deficiency, protein S deficiency, the prothrombin G20210A mutation and factor V Leiden (172). The last variant is particularly common, and is found in 20–50% of patients with spontaneous venous thrombosis. However, no genetic susceptibility can be identified in 50% or more of patients and it is likely that a number of additional genetic risk factors for thrombosis remain to be discovered.

### 73.3.1 Antithrombin III Deficiency

AT3 is a member of the serpin gene family and a potent inhibitor of several serine proteases in the coagulation cascade including thrombin, factors IX, X, XI, and XII (1,2). The anticoagulant heparin acts by greatly potentiating the activity of AT3 against FIX, FX and thrombin. This function is presumably carried out by natural glycosaminoglycans *in vivo*. The hemostatic balance is very sensitive to variations in AT3. Levels in the range of 50% of normal are associated with a significantly increased risk of thrombosis, accounting for the autosomal dominant nature of this disorder. Although AT3 deficiency is generally defined as a level of less than 50% of normal, significant overlap can be observed between genetically deficient patients and normal individuals. The prevalence of AT3 deficiency is estimated at approximately 1 in 500–5000 (170,173,174).

**73.3.1.1 Clinical Features.** AT3 deficiency is generally subdivided into types I and II. Type I, or classic, AT3 deficiency is due to an absolute quantitative deficiency of the protein with corresponding decreases in antigen levels as well as functional activity by several assays. Type II deficiency can be associated with normal levels of antigen but decreased functional activity, generally due to point mutations within specific important functional domains such as the heparin-binding domain. Inheritance is autosomal dominant, although the penetrance of thrombosis is incomplete (see later). Although thrombosis can be spontaneous, it is often associated with other known risk factors such as pregnancy, contraceptive use, surgery, or trauma. The most common sites of thrombosis are in the deep veins of the leg and mesenteric veins; however, thrombosis at unusual sites can also occur. Recurrent thrombotic episodes are common, as are progression to pulmonary emboli. Arterial thromboses are rare, but have been reported in some severe cases. The typical age of onset of thrombosis is in the third to fourth decade of life. Although presentation in childhood and infancy has been reported, thrombosis before puberty is unusual.

Laboratory diagnosis is generally established by measuring AT3 antigen and functional activity. The

latter is usually measured as heparin cofactor activity, the most efficient initial screening test, which should detect defects of most types. A variety of acquired disorders can also result in decreased AT3 levels and should be distinguished from the inherited disorder. These include acute thromboses, chronic liver disease, nephrotic syndrome, and disseminated intravascular coagulation (DIC). Estrogens can also mildly reduce AT3 levels. Acute treatment with heparin also results in decreased AT3, presumably based on accelerated clearance. Thus, patients presenting with thrombosis should not undergo laboratory evaluation while on heparin. Because of these many confounding factors, an abnormal AT3 level should be confirmed with at least one additional measurement before establishing the diagnosis. Evaluation of other family members can also be helpful in supporting the diagnosis. The degree of thrombosis is often milder in patients with type II defects, presumably due to residual activity of the abnormal protein. Rare individuals with homozygous type II AT3 deficiency have been reported (170,173).

As noted earlier, the penetrance of thrombosis in heterozygous AT3 deficiency is incomplete. A review of published cases in the literature suggests penetrance of approximately 50%, although this figure may still represent an overestimate, due to greater ascertainment of more highly penetrant families. In contrast, careful analysis of a single large pedigree suggested that an unbiased penetrance figure is probably closer to 20% (175). This issue is of major importance in deciding whether to treat asymptomatic patients. Although the risk of thrombosis in asymptomatic AT3 carriers is probably greater than that in protein C, protein S and factor V Leiden (discussed later), the risk still is probably less than the major complication rate of long-term anticoagulation with warfarin (which may be as high as 1% per year). Most experts do not recommend routine long-term anticoagulation of asymptomatic carriers (170,173,176–178). One study suggests a risk for thrombosis of approximately 1.5% per year in AT3 carriers (176), 10–20 fold higher than the corresponding risk in normal individuals (177). However, overall, AT3 deficiency is associated with a normal survival and a low risk of fatal thromboembolic events (178). For this reason, anticoagulant prophylaxis is generally reserved only for individuals with additional significant risk factors or in particularly high-risk situations.

Although acute thromboses may be treated with thrombolytic therapy using guidelines similar to those for other cases of thrombosis, treatment is generally with standard or low-molecular-weight heparin (179). Difficulty in achieving adequate heparinization, or heparin resistance, may occur as a result of decreased AT3 and may initially suggest the diagnosis. AT3 concentrates have been used in selected clinical settings, particularly when standard anticoagulation is contraindicated. The



available products are of high purity and are not thought to carry viral risk. These purified concentrates are preferable to crude plasma products. The long half-life of AT3 (48 h) makes it feasible to raise levels above 80–100%. Standard long-term management of symptomatic AT3 deficiency generally relies on anticoagulation with warfarin. As noted earlier, asymptomatic patients are generally not treated with anticoagulants, given the significant long-term hemorrhagic risk, unless undergoing a procedure or treatment that significantly increases their risk of thrombosis. Pregnancy presents a particularly difficult management issue, given the teratogenic effects of warfarin (170,179). For the first time since the development of warfarin decades ago, several new oral anticoagulants have been approved, with varying availability in Europe and North America. These include small molecules that directly inhibit either thrombin or FXa at fixed doses without a need for routine laboratory monitoring (180), and that appear to exhibit fewer drug interactions than warfarin. However, these agents currently lack antidotes for emergent reversal of anticoagulation, and predominantly renal excretion complicates management in the setting of renal failure. The precise role of these new drugs, relative to warfarin, in adults as well as children remains to be fully defined.

**73.3.1.2 Genetics.** A single copy of the AT3 gene is located on human chromosome 1. A large number of molecular defects scattered throughout the gene have been catalogued ([www1.imperial.ac.uk/departments-of-medicine/divisions/experimentalmedicine/haematology/coag/antithrombin](http://www1.imperial.ac.uk/departments-of-medicine/divisions/experimentalmedicine/haematology/coag/antithrombin)), including a number of deletions, frameshift mutations, and nonsense mutations all resulting in type I AT3 deficiency, as well as missense mutations leading to type II. Structure/function studies of type II mutations have often demonstrated the molecular mechanism to be a specific defect in heparin binding or disruption of the active site, interfering with protease inhibition.

The diagnosis of AT3 deficiency is generally based on clinical AT3 antigen and functional assays, as described earlier. Although DNA diagnosis can be achieved on a research basis, this procedure is not routinely performed, given the large number of different mutations that have been identified. Although prenatal diagnosis by linkage or direct mutation detection is possible, it is generally not performed, given the usually mild nature of this disorder.

### 73.3.2 Protein C Deficiency

Griffin and coworkers (181) first identified protein C deficiency in 1981 in a family with recurrent thrombosis. Affected family members exhibited protein C antigen levels of less than 50% of normal. A large number of protein C-deficient patients have since been reported and their defects biochemically characterized.

Protein C is a protease zymogen that is activated when cleaved by thrombin bound to thrombomodulin on the endothelial cell surface. APC proteolytically inactivates factors Va and VIIIa on the platelet surface, thus downregulating thrombin generation by turning off the prothrombinase and X-ase complexes. In this way, thrombin autoregulates its own activity by activating the protein C anticoagulant pathway (Figure 73-3). The presence of thrombomodulin on the surface of vascular endothelial cells may serve to ensure vascular patency through this mechanism. Protein C deficiency removes this important balance, resulting in hypercoagulability.

**73.3.2.1 Clinical Features.** Protein C deficiency is an autosomal dominant disorder with prevalence estimated at approximately 1:500 (170,173,182,183). Estimates of the penetrance of protein C deficiency vary from as low as 20–30% to as high as 75%. In addition, the same mutation may vary widely in its expression in different families. Heterozygous deficiency results in protein C levels in the range of up to 50% of control, although there is considerable overlap with the lower portion of the normal range (183). Clinical presentation is similar to AT3 deficiency, with venous thrombosis usually presenting in the third or fourth decade, and rarely before puberty. A number of cases of homozygous protein C deficiency have been reported, associated with the severe form of neonatal purpura fulminans, which is fatal if not treated promptly (170,173).

**73.3.2.2 Genetics.** A large number of mutations in the protein C gene on chromosome 2 have been reported (184). Similar to AT3 deficiency, heterozygous protein C deficiency is divided into type I, or classic, associated with both low protein C antigen as well as functional activity, and type II, associated with normal antigen and decreased functional activity. The diagnosis of protein C deficiency is generally based on functional and antigen tests, rather than DNA diagnosis, given the large number of mutations that have been identified. Most type I patients have nonsense or frameshift mutations, or in some cases missense mutations.

Forty-three percent of the identified point mutations occur at CpG dinucleotides (184). The wide range in the degree of symptoms, even among families segregating the same mutation, has been puzzling. It is now clear that at least some of these cases can be explained by cosegregation of another prothrombotic mutation (see Factor V Leiden later). Protein C is a vitamin K-dependent protease that requires post-translational modification by  $\gamma$ -carboxylation at its N-terminus for full functional activity. Thus, warfarin treatment reduces functional activity, and to a lesser extent immunologic activity, making it difficult to diagnose this disorder during warfarin therapy.

**73.3.2.3 Management.** Anticoagulant management of protein C deficiency is similar to that described for AT3

deficiency (170,173). However, it is important to pre-treat for sufficient time with full heparinization before beginning warfarin anticoagulation, and to avoid large loading doses of warfarin. Abrupt initiation of treatment with a vitamin K antagonist has been associated with a dramatic thrombotic complication in the skin, referred to as “warfarin-induced skin necrosis.” This problem is thought to result from an imbalance between the anticoagulant effect of warfarin and the earlier, more rapid, inhibition of protein C. The latter effect results in an initial worsening of the relative protein C deficiency until sufficient inhibition of the coagulation factors II, VII, IX, and X has occurred to produce a net anticoagulant effect. If this complication should develop, treatment with fresh frozen plasma or protein C concentrate is indicated. Although purified protein C concentrates are not always routinely available, they have been used effectively in the treatment of children with homozygous protein C deficiency.

### 73.3.3 Protein S Deficiency

Protein S is a cofactor for the anticoagulant activity of APC (Figure 73-3). Thrombophilia due to heterozygous deficiency appears to be similar in frequency to protein C deficiency, and severe thrombosis has been reported in a few patients with apparent homozygous deficiency (170). It now appears that many cases previously ascribed to protein S deficiency may actually have been due to the APC resistance defect resulting from factor V Leiden. Some cases of protein S deficiency also appear to be associated with arterial thrombosis, a complication not generally seen in AT3 or protein C deficiency. However, this association is still controversial. Approximately 60% of protein S antigen in plasma is in a complex with the complement component, C4B-binding protein. Only the free fraction is functionally active as a cofactor. This interaction complicates the interpretation of protein S levels, as most screening tests measure only total protein S antigen. Type I deficiency is associated with a decrease in both total and free protein S antigen. Type II deficiency is characterized by normal free protein S antigen, but low protein S activity (APC cofactor activity). Type III is associated with a selective decrease in free protein S. The protein S gene and a homologous pseudogene are both located on human chromosome 3. Although a number of specific mutations leading to protein S deficiency have been identified, DNA testing is still limited to the research setting.

A study of a large cohort of well-established protein S-deficient patients and first degree relatives demonstrated that low free protein S level is the most reliable screening test for protein S deficiency. Patients carrying a protein S deficiency mutation showed a fivefold increased risk of thrombosis (185).

Clinical features of heterozygous protein S deficiency are similar to those of AT3 and protein C deficiency, with

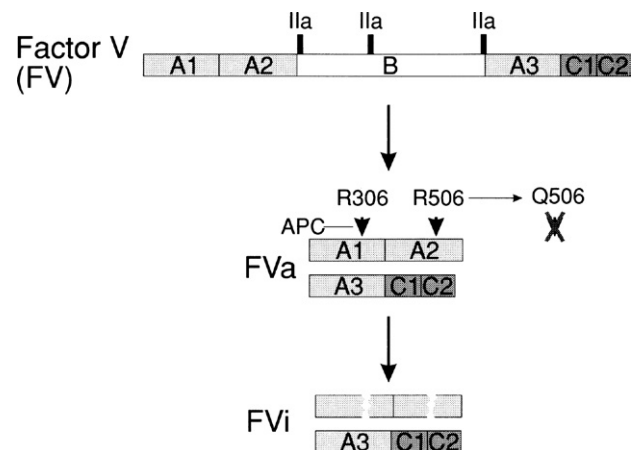
onset typically in the third or fourth decade. Acquired abnormalities in protein S can be associated with oral contraceptives, pregnancy, DIC, and acute thrombosis. In addition, C4B-binding protein is an acute phase reactant whose elevation in inflammatory states can be associated with a decline in free protein S activity. It is also important to note that protein S measurements can be difficult to interpret in patients on warfarin, since protein S also requires  $\gamma$ -carboxylation. Long-term treatment is similar to that described earlier for AT3 and protein C deficiency.

### 73.3.4 Factor V Leiden

Until the mid-1990s, deficiencies of AT3, protein C, and protein S were the only known thrombophilic risk factors. However, measurement of these factors generally detected an abnormality in only 5–10% of patients admitted with unexplained thrombosis (186).

Dahlbäck and colleagues (187,188) first reported a poor anticoagulant response to APC in three families with familial thrombophilia. These workers developed an assay based on prolongation of the PTT by the addition of APC. Affected individuals and their families showed resistance to this effect of APC. Inheritance of this APC resistance appeared to be autosomal dominant.

The responsible mutation was subsequently identified as a G  $\rightarrow$  A substitution at nucleotide position 1691 of factor V (189), resulting in the substitution of glutamine for arginine 506 (R506Q), a predicted protein C cleavage site within factor V (Figure 73-11). The R506Q mutation, commonly denoted as factor V Leiden, is found in 85–100% of individuals with APC resistance, depending



**FIGURE 73-11** Domain structure and processing of factor V. Thrombin cleavage sites (IIa) are indicated. FVa, the active form of factor V; FVi, factor V which has been inactivated through cleavage by activated protein C (APC). The APC cleavage sites at Arg306 (R306) and Arg506 (R506) are indicated. The common factor V Leiden mutation, substitution of glutamine for Arg506 (Q506), results in APC resistance.

on the specific assay used. APC resistance in the absence of factor V Leiden also appears to confer an increased risk for thrombosis, due in some cases to a lupus anticoagulant or other acquired disorder, although the cause often remains unidentified. Mutations at a second APC cleavage site within factor V (Arg306) have also been reported, including factor V Hong Kong (R306G) (190) and factor V Cambridge (R306T) (191). The R306G mutation appears to have a prevalence of ~4% in parts of Asia (190). Analysis of recombinant factor VIII indicates that mutations at the homologous APC cleavage sites in factor VIII do not produce APC resistance in the standard clinical assay (192) and no such patients have been reported.

Factor V Leiden has been identified in diverse populations, although it is particularly prevalent in Europe. Allele frequency among Europeans ranges from 2 to 7%, with the highest frequency observed in Greece. Haplotype analysis suggests a single founder for this mutation, who was estimated to have lived 21,000 to 34,000 years ago (193). This mutation appears to be quite uncommon in African and Asian populations (194).

Individuals homozygous for factor V Leiden have a high risk of thrombosis, with lifetime penetrance estimated at ~80%, compared to 10% in heterozygotes (170,173,195). Thromboses may appear at slightly younger ages in homozygotes (195), although this has not been confirmed in recent large cohort studies, where the major increase in thrombosis for both genotypes was not seen until after age 60 (196,197).

Oral contraceptives and hormone replacement therapy both confer an increased risk of thrombosis in factor V Leiden patients (170,198), although there is currently insufficient data to support routine genetic screening prior to initiating treatment (199). Together with the prothrombin G2021A mutation (see later), factor V Leiden is a major risk factor for thromboembolic complications during pregnancy and was observed among 44% of such patients in one study (200). The presence of both mutations resulted in a marked increase in the risk of thrombosis. Overall, the risk of thrombosis during pregnancy among factor V Leiden carriers was estimated at 0.2%, 0.5% for prothrombin G2021A, and 4.6% among patients with both mutations. However, no clinical benefit has yet been demonstrated for routine mutation screening (201), and current clinical guidelines only recommend prophylactic anticoagulation if there is a family or prior history of venous thromboembolism or homozygosity for one of these mutations (202,203).

The thrombosis associated with factor V Leiden appears to be restricted to the venous circulation with only limited controversial evidence for arterial thrombosis or increased risk of myocardial infarction. The prospective physicians health study of nearly 15,000 men confirmed an approximately 3.5-fold risk of venous

thrombosis in factor V Leiden carriers with no evidence of increased risk of myocardial infarction or stroke (204).

Given the remarkably high prevalence of factor V Leiden, its relatively low penetrance for thrombosis, and the lack of a significant effect on life expectancy (205) coupled with the significant morbidity of long-term anticoagulation with currently available drugs, no specific treatment can be recommended at this time for the asymptomatic patient, even those homozygous for the mutation. Patients who develop symptomatic thrombosis are currently treated using the same guidelines applied to the general patient population (206–208). Indeed, current evidence suggests that the risk of recurrent deep venous thrombosis is similar among carriers of factor V Leiden and patients without this mutation, as well as in patients with the prothrombin G2021A, or even in patients with both FV Leiden and prothrombin G2021A (209). Some experts recommend more aggressive anticoagulation in patients carrying more than one prothrombotic mutation or in patients also exposed to another risk factor such as pregnancy (210,211). However, this approach is controversial (208). It is likely that the approach to treatment will be refined over the coming years with the identification of additional high-risk groups and further definition of interactions between genetic and environmental risk factors.

APC resistance can be diagnosed by an APC resistance coagulation assay, which is available in many specialty coagulation laboratories. In addition, direct DNA mutation testing for factor V Leiden is clinically available. Given the lack of any specific changes in therapy, routine screening is not currently recommended (212). However, it is likely that such testing may eventually be shown to be of benefit in selected subgroups of patients. The remarkably high prevalence of factor V Leiden in European populations and its origin from a single founder mutation argue strongly against a significantly adverse effect on life expectancy and also suggest the existence of a positive evolutionary selective advantage for factor V Leiden carriers. A significant survival advantage for factor V Leiden patients in the setting of severe sepsis has been reported (213), suggesting that an infectious pathogen may provide balancing selection accounting for the exceptionally high prevalence of this polymorphism in some human populations. A reduced risk of intrapartum bleeding complications in factor V Leiden patients might also confer a survival advantage (214). In addition, factor V Leiden patients appear to have lower rates of pulmonary embolism, suggesting they may have an advantage of more stable thrombi (215,216).

### 73.3.5 The Prothrombin G20210A Mutation

Analysis of the prothrombin gene as a candidate for venous thrombosis predisposition identified a common mutation in the 3' untranslated region (G20210A) with

an allele frequency of approximately 1% in European populations. This mutation appears to be associated with elevated prothrombin levels, although elevated prothrombin level itself has not been found to be a risk factor for venous thrombosis (217). The G20210A mutation is at the 3' terminus of the 3' untranslated region and has been reported to increase the efficiency of prothrombin mRNA 3' end processing (218), although subsequent studies suggest other mechanisms for mRNA accumulation, including linkage disequilibrium to another nearby single nucleotide polymorphism (SNP) altering splicing efficiency (219).

The G20210A mutation leads to an approximately one- to sixfold increased risk of thrombosis. As in factor V Leiden, the thrombotic risk seems to be primarily restricted to venous thrombosis. However, an elevated risk for myocardial infarction in young women has been reported (220). The overall thrombosis risk conferred by prothrombin G20210A appears to be somewhat lower than that of factor V Leiden, which in turn appears to be somewhat less than that of AT3 deficiency (207).

### 73.3.6 Other Miscellaneous Prothrombotic Disorders

A number of other conditions have been associated with thrombosis but are not generally considered in a routine evaluation. These include rare qualitative abnormalities in fibrin function referred to as dysfibrinogenemias. This heterogeneous group of disorders can present either with a bleeding diathesis or with recurrent thrombosis (see earlier). Deficiency of FXII (Hageman factor) was thought to be associated with thrombosis, but this has not been confirmed (117). With the recent discoveries of highly prevalent mutations in factor V and the prothrombin gene leading to predisposition to thrombosis, increased analysis of other clotting factor genes has identified new potential prothrombotic risk factors, although none are well defined at this time. A high level of factor XI has been reported as a risk factor for thrombosis, with doubling of the risk at levels present in the upper 10% of the population. Although the genetic basis for this variation in factor XI remains unknown, if confirmed, this additional factor could be an important genetic contribution to thrombophilia (221). A similar association of elevated factor IX levels with thrombosis risk has also been reported (222). Recently, a novel FIX variant (FIX Padua, R338L) was discovered in a patient with thrombosis who had an eightfold increase in FIX activity (223). However, follow-up studies have not yet identified other individuals with this variant outside of the proband's family. In several studies, plasma factor VIII level has been found to be an important determinant of thrombosis risk. In one analysis, elevated factor VIII was calculated to account for 16% of the population-wide attributable risk for thrombosis, compared to 25% for factor V Leiden, 4% for prothrombin 20210A, 2%

for protein C deficiency, and <1% for protein S and AT3 deficiency (224). Patients with elevated plasma factor VIII may be at particularly high risk for recurrence (225). Family studies suggest that elevated factor VIII levels are at least in part genetically determined (226,227) and do not appear to be linked to the factor VIII locus itself. A recent report suggests a potential role for imprinted loci on chromosomes 5 and 11 (228), although the responsible genes remain to be identified. As noted earlier, abnormalities in fibrinogen, particularly the dysfibrinogenemias, can also be associated with thrombosis (170).

Premature atherosclerosis and venous and arterial thromboses are well-known complications of the autosomal recessive genetic disorder, homocystinuria due to cystathionine synthase deficiency. These patients appear to have a particularly increased risk of venous thrombosis when factor V Leiden has been coinherited (229). Even modest elevations in plasma homocysteine, which may result from heterozygous deficiency or other dietary or genetic influences, may be a significant independent risk factor for the development of atherosclerosis (230). Mild homocystinemia may also be an independent risk factor for venous thrombosis (231), although this hypothesis is controversial since homocysteine lowering therapy has not been effective for the prevention of recurrent thrombosis (232,233). A very common polymorphism in the MTHFR gene (C677T), leading to a thermolabile enzyme, exhibits an allele frequency as high as 38% in some populations, with homozygous frequencies as high as 12% (234). Homozygosity for the 677T MTHFR allele may be associated with elevated plasma homocysteine levels, particularly in the setting of folate deficiency. However, it does not appear to confer an increased risk for ischemic heart disease or venous thrombosis (235,236), even when coinherited with factor V Leiden (237,238).

### 73.3.7 Plasminogen Deficiency

Inherited defects of fibrinolysis are a rare cause of familial thrombosis. Although a number of cases of partial plasminogen deficiency have been reported, the association with thrombosis is controversial (161). Plasminogen deficiency has been classified as type I deficiency, with reduced antigen and functional activity and type II deficiency in which antigen is normal but functional activity is proportionately decreased. Type II deficiency due to an Ala600Thr mutation is very common in the Japanese population with an allele frequency of about 1%. This mutation was initially identified in a patient with thrombosis (239). However, other family members were unaffected. Although a number of other missense mutations within the plasminogen gene have been identified, partial deficiency of plasminogen does not appear to be a significant risk factor for thrombosis, with similar prevalence observed among thrombosis patients and controls (240,241).



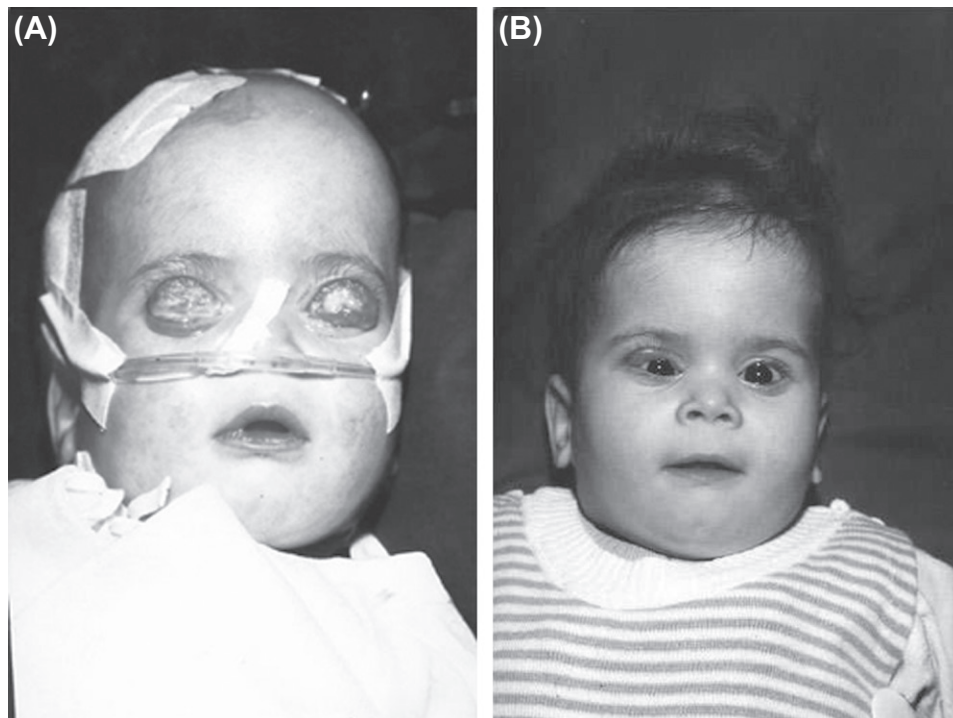
Complete plasminogen deficiency in knockout mice (242,243) results in severe and widespread chronic thrombosis. Failure to observe a similar disorder in humans led to the presumption that such a condition would be lethal. However, homozygosity for null mutations in the plasminogen gene was subsequently shown to be the molecular basis for a rare ophthalmologic disorder, ligneous conjunctivitis (244) (reviewed in (161)). This disorder usually presents first in early infancy as a chronic, pseudomembranous conjunctivitis, although symptoms can be delayed into adulthood. The membranes that form over the eyes have a woody consistency and may follow minor trauma or infection (Figure 73-12). These membranes can occur on other mucosal surfaces in the oropharynx, trachea, or female genital tract. Congenital hydrocephalus is also noted in a subset of patients. Inheritance is autosomal recessive. In 1997, Schuster et al. (244) reported two patients with ligneous conjunctivitis who were each homozygous for a different mutation in the plasminogen gene, associated with undetectable plasminogen activity and antigen. Five additional patients were subsequently reported, with the range of mutations and phenotypes observed suggesting a correlation between residual plasminogen activity and severity of disease (245). Treatment of one of the original patients, who was homozygous for a nonsense mutation (Glu460Ter), with a plasminogen concentrate prepared from human plasma produced a dramatic clinical response (246). Remarkably, there have been no reports of thromboembolic complications

in ligneous conjunctivitis patients or their families, suggesting that the primary role of plasminogen in humans is in the clearance of extravascular fibrin. A careful reanalysis of plasminogen-deficient mice identified ocular lesions very similar to human ligneous conjunctivitis, but that were undetected in the initial evaluation of these animals (247).

No genetic deficiencies of tPA or uPA have been reported in humans. Several families have been described with reduced fibrinolytic potential that could be due to either over production of PAI-1 or deficiency in tPA (170). Although laboratory tests for plasminogen are available, routine screening in families with thrombosis is not generally indicated.

### 73.3.8 Thrombotic Thrombocytopenic Purpura (TTP)

TTP is a catastrophic, multisystem disorder characterized by the formation of platelet and VWF-rich microthrombi in vessels of multiple organs, leading to the classic pentad of microangiopathic hemolytic anemia, thrombocytopenia, fever, and varying degrees of renal and CNS dysfunction (80,248). Not all of these manifestations are present in all patients, although thrombocytopenia and hemolytic anemia are nearly universal. Typical age of onset is in the second or third decade, although this disorder can be seen at any age and there is usually no clear precipitating event. Untreated, mortality is greater than 90%, although with plasma exchange this figure falls



**FIGURE 73-12** Ligneous conjunctivitis. (A) The initial appearance of a child with ligneous conjunctivitis resulting from homozygous plasminogen deficiency. (B) The same child after 7 months of replacement with Lys-plasminogen. (Adapted from N. Engl. J. Med. 1998; 339, 1679, with permission.)

to 10–20 (248,249). Most adult cases are sporadic and associated with the presence of acquired autoantibodies against a plasma metalloprotease that cleaves VWF (78,83). Absence of this VWF-cleaving protease results in accumulation of unusually large multimeric forms of VWF, which are thought to trigger the pathologic platelet thrombi responsible for TTP (80,248). A rare familial form of TTP, also referred to as Upshaw–Schulman syndrome, typically presents at birth or in early childhood and is usually highly responsive to the infusion of normal plasma (248). The gene responsible for this rare autosomal recessive disorder was identified by positional cloning as the metalloprotease, ADAMTS13 (81). It was independently identified at the same time by others as the VWF-cleaving protease previously associated with this disorder (77,79,82). A range of mutations scattered throughout the ADAMTS13 gene have now been identified and appear to account for all cases of familial TTP, with no evidence to date for locus heterogeneity (80,81). Patients with the familial form of TTP can generally be managed with simple plasma infusion, which presumably replaces the missing enzyme. Plasma exchange in adult patients with acquired disease may serve to partially remove autoantibodies, in addition to protein replacement (248,249).

### 73.3.9 Interactions among Multiple Genetic Defects

The discovery of the very common factor V Leiden mutation has also shed light on the penetrance of other less common familial hypercoagulable states. The risk of thrombosis varies considerably among protein C-deficient families. In some families, inheritance appears to be recessive and in others, dominant. In some cases, the same mutation has been found in families of both types. Koeleman and coworkers (250) assessed the effect of factor V Leiden on the penetrance of protein C deficiency-related thrombosis. These workers observed a prevalence of the factor V Leiden mutation of 19% among symptomatic protein C-deficient patients, compared to a 2–4% incidence in the control population. In six large pedigrees, 73% of patients inheriting both gene defects experienced thrombosis, compared to 31% of patients with only the protein C defect and 13% of the patients with only factor V Leiden. Similar interaction of the factor V Leiden mutation with protein S deficiency has also been noted (251), as has an increased risk of thrombosis associated with coinheritance of factor V Leiden and the prothrombin G20210 gene mutation (see earlier).

Thus, venous thrombosis, like many other common, complex disorders, can be viewed as the product of interactions between a number of genetic and environmental factors. Factor V Leiden, the prothrombin 22010 mutation, and likely a number of other common, yet to be identified genetic factors combine with environmental factors such as pregnancy, immobility, and underlying

malignancy to determine the overall thrombosis risk for each individual. The strong foundation already in place for the basic biochemistry and physiology of blood coagulation should greatly facilitate deciphering the interactions between these multiple factors, eventually leading to accurate predictions of risk and individually tailored therapy. In this way, the treatment of thrombosis may serve as a valuable paradigm for other complex genetic disorders.

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# Rhesus and Other Fetomaternal Incompatibilities

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## 74.1 HISTORICAL BACKGROUND OF RH ALLOIMMUNIZATION

The first recorded case of hemolytic disease of the newborn was described in 1609 by Louyse Bourgeois, a French midwife. The first of a pair of twins was hydropic and died shortly after birth. The second was well at birth, but became deeply jaundiced and died a few days later. In 1932, Diamond et al. (1) showed that hydrops fetalis, icterus gravis, and kernicterus were different presentations of a single, as-yet unidentified, disease characterized by hemolytic anemia, extramedullary erythropoiesis, hepatosplenomegaly, and erythroblastosis, which they termed “erythroblastosis fetalis.”

Darrow (2), in 1938, suggested that fetal hemoglobin crossed the placenta and stimulated maternal production of a fetal hemoglobin antibody, which crossed back into the fetal circulation, causing destruction of the fetal red blood cells (RBCs). The involvement of the Rh antigen in erythroblastosis fetalis was discovered in 1939 by Levine and Stetson (3). The mother of a stillborn baby, who had been given a postpartum transfusion of blood donated by her husband, experienced a severe transfusion reaction. Antibody was detected in her serum, which agglutinated RBCs from 80% of randomly selected type O donors. The antibody specificity was identical to that of antibodies raised in rabbits injected with erythrocytes from rhesus monkeys, hence “antirhesus” or “anti-Rh” (4). The cause of fetal hemolysis was the production of maternal anti-RhD alloimmune antibodies was proved by Chown in 1954 (5,6). An RhD-negative primigravida, with no anti-D at the time of delivery, gave birth to an anemic infant with erythroblastosis and hepatosplenomegaly. The result of the direct antiglobulin test was negative for fetal erythrocytes, and at delivery, the mother appeared to be weakly RhD positive. Chown showed that many of her circulating erythrocytes were RhD positive and of fetal origin,

suggesting that a very large fetomaternal hemorrhage had occurred. Within 20 days after delivery, the woman produced a strong anti-D reaction and her subsequent pregnancies were severely affected by erythroblastosis fetalis.

## 74.2 ETIOLOGY OF ALLOIMMUNIZATION

In 1953, Chown demonstrated that the cause of Rh immunization was the passage of Rh-positive fetal erythrocytes into the maternal circulation. The risk of Rh alloimmunization is 16% if the Rh-positive fetus is ABO-compatible with its mother. If it is not Rh compatible, then the risk approximates 2%. The risk increases if invasive measures such as amniocentesis, chorionic villus sampling, or fetal blood sampling is undertaken to evaluate for a fetomaternal hemorrhage (7).

Approximately 50% of Rh-positive newborns with Rh hemolytic disease of the newborn are mildly affected. These newborns usually do not require treatment. However, 25% will be born near term in stable condition. Whereas 10% of these newborns may become extremely jaundiced and either succumb to kernicterus or be left with severe neurosensory defects such as spastic choreoathetosis and mental retardation. The remaining fetuses will become hydropic in utero. Half of these will occur prior to 34 weeks of gestational age. Before treatment for Rh alloimmunization was made available, the perinatal mortality rate approximated 50% (7).

## 74.3 DETECTION OF FETOMATERNAL HEMORRHAGE

In the first trimester, fetal cells will be detected in only 3% of normal pregnancies, rising to 12% and 45% in the second and third trimesters and 65% after delivery. Approximately 4% of women will have a transplacental

hemorrhage greater than 0.2 mL following first-trimester termination of pregnancy (8).

The acid elution test of Kleihauer et al. (9) depends on the resistance of fetal hemoglobin to acid elution. After acid elution, staining of a blood smear with eosin will show the fetal erythrocytes as dark cells on a background of white maternal “ghost” cells. The technique will detect as few as one fetal cell per 200,000 maternal cells, and has been used to determine the size and frequency of fetomaternal hemorrhage at different stages of pregnancy. However, since this test relies upon the detection of fetal erythrocytes instead of Rh-positive cells, a reading of up to 2% of cells with fetal hemoglobin can be considered a false positive since it is not abnormal for an adult to circulate 2% of cells with fetal hemoglobin (10).

Therefore, the detection of fetal hemoglobin in maternal circulation does not necessarily imply that a fetomaternal hemorrhage occurred. It has been demonstrated that anywhere from 0.2% to 7% of adult erythrocytes contain hemoglobin F. It is thought that this proportion is genetically determined. One of the drawbacks to the Kleihauer–Betke test is that it is imprecise and can lead to undertreatment as well as overtreatment. In some, undertreatment may explain why some women still develop Rh disease despite the administration of Rh immunoglobulin. With this in mind, more rigorous tests such as flow cytometry should be considered (10).

## 74.4 RH BLOOD GROUP SYSTEM

Individuals are classified as Rh positive or Rh negative, depending on the presence or absence of the major D antigen on the RBC surface. The RhD epitope is the most immunogenic of the Rh system. The next most antigenic, Rhc, is more than 20-fold less potent. For this reason, the terms rhesus positive, Rh positive, and RhD positive have become synonymous in clinical practice. While the introduction of RhD immunoprophylaxis has reduced the incidence of RhD alloimmunization, the incidence of disease due to other antigens is increasing. Furthermore, none of the antigens is present on rhesus monkey erythrocytes.

Two other major antigens, the C/c and E/e series, play important clinical roles. One significant difference between the D and CcEe antigens is that there is no serologically defined “d” antigen. Although hematologists will refer to an individual as DD, Dd, or dd, the designation “d” indicates the absence of the “D” antigen. In the case of Cc and Ee, both the uppercase and lowercase letters indicate the presence of a serologically definable antigen. The genes encoding these three antigens are inherited together. This suggested to earlier investigators that there would be either one single gene encoding all the C/c, D/d, E/e epitopes or three genes closely linked on the chromosome, so that recombination rarely takes place between them. Virtually all normal erythrocytes carry the antithetical antigens C

and/or c and E and/or e. The erythrocytes of individuals who lack these antigens have multiple membrane abnormalities, suggesting that the Rh antigens are key to membrane stability. The RhD-negative phenotype occurs in approximately 15% of the white population. Thirty-five percent of Basques are RhD negative, whereas only 1% of North American Indians and 7% of African-Americans are RhD negative. The incidence in Asiatic Chinese and Japanese is almost zero.

### 74.4.1 Nomenclature of the Rh Antigen System

Theories about the molecular basis of the Rh blood group system have been proposed and then discarded. The Fisher–Race system is based on the original Fisher theory that the Rh gene locus consists of three genes with antithetical alleles C/c, D/d, and E/e. In this scheme each haplotype (i.e. each chromosome) is designated as carrying one of each of the antithetical triplets. For example, the three most common are CDe, cde, and cDE. This simple and relatively straightforward scheme was opposed by Wiener, whose theory suggested that all the Rh alleles are epitopes of a single protein, and therefore, the product of a single gene. In the nomenclature based on this theory, separate terms are used for the whole complex of antigens (so, for example, CDe becomes R1), and for each component factor (Rh0 = D, rh' = C, rh1 = Ce, etc.). Although this terminology is more complex and less specific, and despite a recommendation by WHO in 1977 that it should be abandoned in favor of the Fisher–Race system, it is still widely used by hematologists.

### 74.4.2 Molecular Basis of Rh Antigens

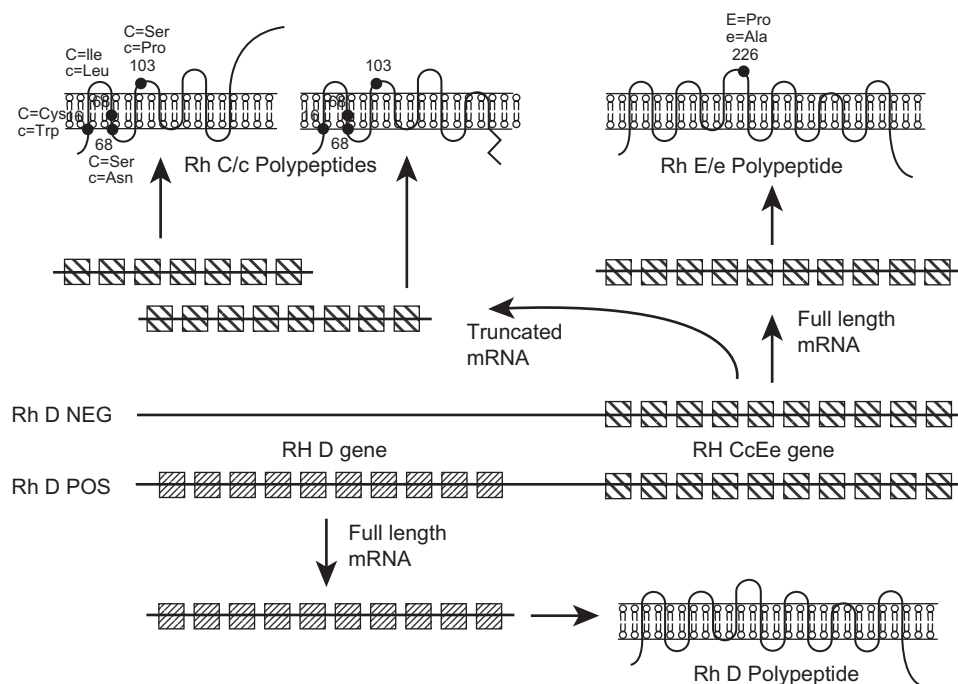
Studies of the molecular basis of the Rh antigens at the protein level were complicated by loss of Rh antigenic reactivity after membrane solubilization or immunoblotting.

Moore et al. (11) and Gahmberg (12) independently identified proteins of 28–32 kDa that could be immunoprecipitated using anti-Rh antibodies. A team led by Cartron at Institut National de Transfusion Sanguine (INTS) in Paris, France virally transformed B cells from donors with circulating Rh antibodies to develop cell lines secreting monoclonal antibodies specific for RhD, c, and E antigens. These monoclonal antibodies allowed the number of Rh antigen sites per cell to be determined. There are approximately 105 Rh antigens to each erythrocyte, with C/c, D, and E each contributing about one-third of the total (13–15). The Rh polypeptides could then be immunopurified by the addition of large quantities of the monoclonal or polyclonal anti-D to erythrocyte membranes that had been surface labeled with 125I (16,17). Rh polypeptides were also isolated from surface-labeled RhD-positive erythrocytes by hydroxyapatite chromatography and electrophoresis of

SDS-solubilized membrane skeleton vesicles (18), leading to a nearly 200-fold purification. From these studies, the total number of Rh polypeptides per erythrocyte was calculated to be approximately 60,000. The 28- to 32-kDa Rh polypeptides were suspected to consist of several species, and one-dimensional SDS polyacrylamide gel electrophoresis showed that there was a small difference in the mobilities of the different polypeptides. The RhD polypeptide migrated with an apparent molecular weight of 31.9 kDa, whereas c and E were 33.1 kDa. When the isolated Rh polypeptides were digested and analyzed by electrophoresis, variations in the degradation patterns indicated that RhD is distinct from the C/c and E/e polypeptides. Antibodies raised to denatured, purified RhD polypeptide were found to cross-react with the Rhc and Rhe polypeptides (19). Digestion of intact erythrocytes with phospholipase A2 and papain caused degradation of the RhD polypeptide but not the RhC/c or -E/e polypeptides (20). These studies, therefore, showed that the Rhc, -D, and -E polypeptides are very similar although each is a distinct protein (21). The c and E polypeptides were found to be nearly identical, whereas the D polypeptide was found to be related but less similar (Figure 74-1).

Almost all our current knowledge of this group of proteins has come from cloning studies of the Rh cDNA. Although antibodies to RhD and other Rh antigens were widely available, they were only specific for the Rh antigens. They were not suitable for the identification of Rh

polypeptides produced from cDNA expression libraries. Oligonucleotide probes for isolating Rh cDNAs were designed from partial amino acid sequence data of the isolated polypeptides. A clone specific for one species of Rh polypeptide was isolated independently in 1990 by the Paris group (22) and a group in Bristol (23), both having used the polymerase chain reaction (PCR) with oligonucleotide primers designed from segments of the N-terminal amino acid sequence to amplify cDNA templates prepared from either thalassemic spleen erythroblasts or peripheral reticulocytes. These PCR products were then hybridized to cDNA libraries to identify specific clones of approximately 1.4 kb. Both groups used the same commercially acquired cDNA library, prepared from the marrow of the same donor. The sequence of the open reading frames reported by each group was identical. The isolated cDNA localized to chromosome 1p34.3–p36.1 by in situ hybridization, which was consistent with previous linkage evidence that localized the RH gene locus to chromosome 1 (24). This first cDNA clone proved to encode both the C/c and E/e proteins. In 1992, the Paris group reported the isolation of the RhD polypeptide cDNA from a cDNA library (25). The coding sequence of this clone exhibited 3.5% divergence at the amino acid level. To confirm that this clone encoded the RhD polypeptide, they demonstrated that its gene was present only in RhD-positive individuals. Restriction fragment length polymorphism analysis of DNA from individuals with



**FIGURE 74-1** The genetic basis of the rhesus blood group system. RhD protein is encoded by a single gene that is deleted on RHD-negative chromosomes. The RhCcEe proteins are encoded by a single gene that is highly homologous to the RHD gene. Translation of a full-length mRNA transcript produces the RhEe polypeptide, which is of similar size and structure to the RhD polypeptide. The RhEe polymorphism is due to a single-point mutation, which changes amino acid 226. RhC polypeptides are produced from smaller splicing isoform transcripts of the same gene and are therefore smaller peptides than their RhD or RhEe counterparts. The RhCc polymorphism is due to four-point mutations, which make four amino acid substitutions. Associated with these substitutions are two further silent point mutations.

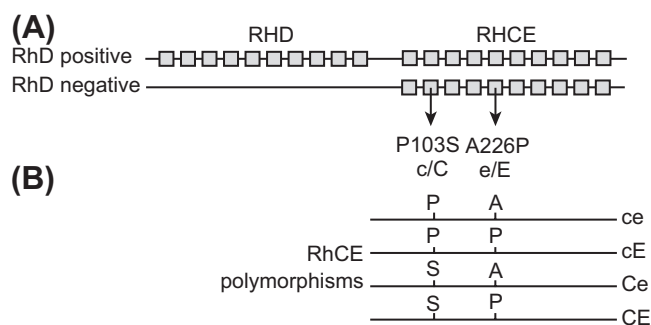
RhD-positive and RhD-negative erythrocytes shows that RhD-positive individuals have two Rh polypeptide genes and that RhD-negative individuals have only one (25). It therefore appears that the RH gene locus consists of two highly homologous, closely linked genes, one of which encodes both C/c and E/e proteins. The other gene encodes the RhD protein and is absent in RhD-negative individuals. Rh antigens are expressed on the erythrocyte surface only in the presence of RhAG. Both Rh and RhAG proteins have 12 transmembrane  $\alpha$ -helices through the erythroid cell membrane, joined by six exoloops and five endoloops with N- and C-termini within the cytoplasm (26,27). The Rh accessory proteins form a group of glycoproteins that are associated with the Rh protein family, and together the association is called the “Rh complex.”

The Rh polypeptides are 30-kDa unglycosylated proteins that can undergo reversible palmitoylation. It is likely that they play a fundamental role in the physiology of RBC membranes that is unrelated to their antigenicity. Similar observations have been made in the cases of other blood group antigens that are often functionally important structures. The multiple membrane-spanning domains of the Rh polypeptides suggest a transporter protein, as a channel, an exchanger or a pump, but the function of the polypeptide or the ligand has not yet been conclusively demonstrated. It has recently been postulated that they may have a function similar to methylamine permease (Mep) transporters (28), that they may be related to the superfamily of ammonium transporters (29), and that the Rh glycoproteins function as dual directional ammonium transporters (30). However, the homology is about 20%, the Mep/Amt family of transporter proteins is present in bacteria, yeast, and plants but not in vertebrates, and there are conserved sequences within the Rh family not found in the Mep/Amt family. So, it remains unclear what may be transported across the membrane, if indeed anything at all. The study of uptake and excretion of metabolic molecules such as

ammonium across normal and Rh-null erythrocyte membranes may help elucidate their biological function in mammals.

Erythrocytes from donors of all the common Rh phenotypes are normal, suggesting that the C/c or E/e polymorphisms have no effect on the function of the protein and that erythrocytes can function entirely normally without expression of the D polypeptide. Comparison of distantly related proteins isolated from erythrocytes of several nonhuman species show conservation of the fatty acylation characteristic, suggesting a common functional significance. The membrane defects seen on erythrocytes from individuals with the Rh-null phenotype provide clues to the functional roles of the Rh polypeptides. Rh-null patients have a mild to moderate chronic hemolytic anemia. Rh-null erythrocytes are pleomorphic but always have some degree of stomatocytosis and spherocytosis, and have increased sensitivity to osmotic lysis. Rh-null membranes have characteristically hyperactive membrane ATPases, reduced cation and water contents, and a deficiency in membrane cholesterol (31). Physiologic roles in membrane stability and volume regulation have been suggested for the Rh polypeptides, but specific details have yet to be elucidated.

Certainly, the principal clinical interest in the Rh polypeptides is in their roles as antigens. The RhD protein expresses the D antigen. The RhCE protein expresses both the C (or c) and E (or e) antigens on the same protein, the C/c antigens being present on the second exoloop and the E/e antigens on the fourth exoloop (32,33). Many of the current data on the primary structures of these polypeptides have come from cDNA studies (see previous discussion). They are 417 amino acids long, there being only about 8% difference in the sequence between RhD and RhCE, or by only 30–35 amino acids (Figure 74-2). Despite such homology, the two proteins do not share antigens unless there is hybrid rearrangement between the two genes (34). These antigens appear early during erythropoietic development and early in fetal life.



**FIGURE 74-2** The genetic basis of the RhCcEe polymorphisms. (A) The RhCE proteins are encoded by a single gene, which is highly homologous to RHD. (B) Common RhCE polymorphisms arise from point mutations within RHCE. Single amino acid substitutions (P, proline; S, serine; A, alanine) give rise to the antigenic differences on the exoloops observed between the various isoforms. Numbers start from first methionine residue.

### 74.4.3 Structure, Evolution, and Molecular Diversity of Rh Gene Family

RH genes (RHD, RHCE) are highly homologous and RHAG is a close ancestral cousin. RHD codes for Rh30 proteins carrying the RhD antigen. RHCE codes for Rh30 polypeptides coexpressing the Ce, ce, cE, and CE antigens. Both genes comprise 10 exons encompassing 69kb of DNA. They reside in tandem on chromosome 1 ordered 5'-RHCE-RHD-3' (24,35). The two genes are opposite in orientation (tail-to-tail joining, 3' ends of the genes face each other) (36). RHAG is also composed of 10 exons encompassing 32 kb on chromosome 6, and has a very similar intron-exon structure to the RH genes.

Although initially thought to be erythroid specific and the genetic heritage of higher vertebrates, RHAG



homologs have been found in widely diverse organisms, such as the marine sponge, nematodes, mice, and nonhuman primates. The genetic and phenotypic polymorphism observed within the Rh gene family has arisen from a combination of inter- and intragenic duplication, rearrangement, allelism, and alternative splicing. It is thought that an ancient gene duplication event (250–346 million years ago) diverged RH from RHAG (37). A more recent gene duplication (5–12 million years ago) led to the human RHD and RHCE genes. Primitive organisms have fewer copies of the Rh genes, suggesting that the Rh gene family has expanded through duplication. Variant Rh antigens arise from alterations in the RHD/RHCE (38).

Molecular biodiversity underpins the variety of Rh antigenicity seen within and without ethnic communities. The genetic rearrangements have arisen by whole gene deletion, microconversion or macroconversion events, and antithetical missense mutations. Of all the known variations (and potential variants), the D-negative phenotype is clinically the most important. The loss of D antigenicity can arise from silencing of the D epitope. The incidence of the RhD-negative phenotype (dd) in whites is 15%, the vast majority arising from a total deletion of RHD (36,39). Among the Asian and some African races, other mechanisms, such as replacement of as many as eight of the central exons by RHCE, of point mutations and base pair deletions, are known to act. The incidence of RhD negativity in African-Americans is 7%, and this falls to almost zero among the Asiatic Chinese and Japanese. Although rare and occurring in a population where dd homozygotes are uncommon, knowledge of these alternate mechanisms for D antigen silencing indicates caution when applying a PCR-based diagnosis for prenatal genotyping.

#### 74.4.4 Prenatal Rh Genotyping

The tandem arrangement of the RHD and RHCE genes on chromosome 1 may contribute to the complexity of the Rh blood group. This arrangement may explain the evolution of aberrant RH alleles caused by replacement of RHD or RHCE gene fragments with their counterparts resulting in the formation of RHD-CE-D or RHCE-D-CE alleles. Template mutations caused by gene conversions have also been noted. Single-point mutations have also produced other variants on these genes. These variants have been known to cause problems with genotyping assays. Most of the aberrant RHD alleles code variant RhD polypeptides on which not all RhD epitopes are expressed while causing new Rh antigens to appear. Theoretically, these variants might lead to alloimmunization in Rh-negative mothers. The relative frequency of these mutations is rare.

The significance of these RH alleles for genotyping is that they may increase the false-positive or false-negative results. A fetus, which is Rh negative, may be classified

as Rh positive when the RH-specific PCR assays are performed. It is entirely possible that when a serologically Rh-negative mom is tested, she may test positive because of these aberrant sequences, which in turn may lead to false-positive fetal genotyping.

The fetus of a sensitized RhD-negative mother and a father who is heterozygous for the D antigen has a 50% chance of being RhD positive and at risk of allo-immune intrauterine hemolysis. Prenatal determination of the fetal Rh serotype or genotype will reveal whether the fetus is at risk. A significant benefit of identifying an Rh-negative fetus is that it does not need to be subjected to invasive testing. Much of the development in prenatal Rh genotyping has been focused on finding less-invasive methods to identify the RhD-negative fetus.

#### 74.4.5 Molecular Diagnosis

Until recently one of the two approaches—fetal blood sampling or serial amniocentesis—was used to identify the at-risk RhD-positive fetus where the father is heterozygous RhD positive. Fetal blood sampling for serologic Rh typing has 1–2% risk of fetal loss, and 40% risk of fetomaternal hemorrhage, which may cause Rh sensitization. Serial amniocenteses for quantification of bilirubin in amniotic fluid are less accurate because it is unable to distinguish an RhD-negative fetus from a mildly affected RhD-positive fetus. In addition, it exposes the mother of an RhD-negative fetus to multiple invasive procedures. With each subsequent procedure, the risk of catastrophic sensitization increases. Before the cloning and sequencing of the RhCcEe and RhD cDNAs, prenatal determination of fetal RhD status could be performed only on fetal blood samples or fetal erythrocytes obtained from chorionic villus sampling. The small quantity of fetal erythrocytes obtained from chorionic villus samples makes standard agglutination techniques unreliable. Therefore, immunofluorescence or immunorosette techniques are used (40,41). This approach was not widely adopted because it was time consuming and cumbersome, and because of the risk of false-negative results, where the yield of fetal cells was very low. In addition, chorionic villus sampling causes detectable fetomaternal hemorrhage in half the cases, which, if the fetus is RhD positive, may accelerate hemolytic disease and may lead to fetal death as early as 20 weeks.

Since the cloning of RHCE and RHD, it is now possible to Rh genotype the fetus by PCR using a small amount of its DNA from amniocytes, chorionic villi, and fetal cells/free fetal DNA in the maternal blood. When designing, using, or relying on a PCR-based Rh genotyping, it should be borne in mind that (1) although the majority of RhD-negative individuals have an RHD gene deletion, some RhD variants have diminished or absent antigenicity with intact, albeit altered, RHD; (2) as the prevention of RhD alloimmunization through anti-D immunoprophylaxis programs becomes more effective,

so will Rh alloimmunization due to the other Rh antibodies gain in importance.

The perfect strategy for prenatal determination of fetal RhD genotype would be to design a pair of PCR primers that would amplify a specific region of the RHD gene with no cross-hybridization, with either the RHCE gene or to any other gene. A PCR product would therefore be generated from RhD-positive and not from RhD-negative DNA. To demonstrate that the absence of an RHD-specific product from RhD-negative DNA is not due to laboratory or experimental error, a second control amplification should be performed in duplex with the RHD-specific reaction. Although this could be of any other conserved sequence, it is more elegant to use a sequence specific to the RHCE gene. The principal difficulty in designing RHCE- and RHD-specific PCR primers is the high degree of homology between the RHCE and RHD genes. In exons 1–9, there are isolated nucleotide differences between RHCE and RHD, making the design of specific PCR primers difficult. Exon 10 contains a region of divergence. In this region, there exists a copy of the Alu repeat motif, which is found in a large number of genes and other noncoding sequences.

The first report of the use of PCR for prenatal determination of fetal RhD used RhD-specific primers designed from the 5' end of exon 10 of RHD (16). This primer pair was originally used by the Paris group to demonstrate that their novel RH cDNA encoded the RhD polypeptide. The 5' primer lay within a region of 100% homology between RHCE and RHD. It was the 3' primer, designed to an RHD-specific region, which gave the specificity to the reaction. A control primer pair, designed from sequences in exon 7, amplified a 134-bp product from both RHCE and RHD to act as the experimental control in a duplex reaction. This original report was of 15 cases of amniocentesis, in which the fetal RhD type was also determined by fetal blood sampling. It was followed by a report of the use of prenatal determination of fetal RhD type in six clinical cases (42). Larger studies of the reliability of this system in correctly determining RhD type in a wide range of serotypes using lymphocyte DNA from blood donors were performed by Simsek et al. (43) and Bennett et al. (44). The Simsek group found five discrepancies in 200 cases using the original primers, but was able to correctly predict the RhD serotype in every case using an alternative PCR approach designed by Arce et al. (45). This group had found that the intron between exons 4 and 5 of the RHD is 600 bp smaller than the equivalent intron in RHCE. PCR primers were therefore designed for the 3' end of exon 4 and the 5' end of exon 5 to amplify intron 4, which would generate a 1200-bp product from the RHCE gene and a 600-bp product from the RHD gene. This approach is elegant in that the same pair of primers is designed from both RHD and RHCE genes. Using this primer pair, Simsek et al. (43) were able to correctly predict the RhD serotype in all 200 donors. In a similar study of 248 donors, Bennett

et al. (44) correctly predicted RhD serotype in all cases, using the original exons 7/10 primer sets.

PCR is notorious for its false-positive results caused by contamination, and due care must be taken to eliminate contamination by RhD-positive DNA, which might occur at any point from collection of the sample to performance of the test. However, in a test where the “negative” result so heavily biases management, we must be mindful of false negatives too. In a study of 135 clinical cases, in which fetal RhD type was determined using the original exons 7/10 PCR primers following amniocentesis, Lighten et al. (46) made two errors where RhD-positive fetuses were thought to be RhD negative, resulting in a mild disease in one fetus and hydrops and death in the other. When both fetuses were retested after birth, they were both RhD positive by PCR. Although this represents an error rate of only 1.4%, the consequences of incorrect determination of fetal RhD type, whether due to laboratory error or to genetic variation, can be so great that most workers would now agree that two independent primer sets should be used. This approach has been successfully combined in a single multiplex reaction, and should give clinicians greater confidence in prenatal RhD determination by PCR.

Inaccuracies of prenatal RhD determination may be due not only to experimental error in the PCR but also increasingly importantly to genetic variation at the RH gene locus. Some individuals produce an altered RhD protein (partial D) within their erythrocytes. This comes about by genetic rearrangements within RHD exons. The RBCs of such individuals can induce allo-antibodies in RhD-negative individuals and, conversely, they themselves can make alloanti-D if transfused with RhD-positive erythrocytes. So, as a donor, they must be classed RhD positive; as a recipient, they are RhD negative. A fetus carrying a partial D antigen should be classed as RhD positive, but if the mother is known to have a partial D phenotype, she must be treated as RhD negative with respect to the pregnancy. A fetus with a partial D phenotype appears unlikely to suffer severe intrauterine hemolysis (47). Similarly, mothers with a partial D phenotype alloimmunized to the D antigen appear less likely to severely affect their RhD-positive fetuses (48). With respect to fetal partial D in an RhD-negative mother, however, identifying the presence of RHD is enough, initially, to flag the clinical case as one of potential alloimmunization; prognostication is another matter.

By contrast, in some racial groups (Africans and Asians in particular), it is not enough to identify the presence of RHD as they carry genetic mutations that render an intact RHD nonfunctional. A fetus may have the RHD gene but may, at the same time, be RhD negative and not warrant any further invasive testing. As knowledge and awareness of these variations increase, and as mixed-race couplings and molecular analyses become more common, this group of conditions will gain in importance. Under these circumstances, knowledge of

the ethnic origins of the parents and both their blood group phenotype and genotype will dictate the molecular testing most appropriate for the individual fetus.

**74.4.5.1 Noninvasive Prenatal Diagnosis.** Historically, fetal DNA was obtained from amniocytes obtained via amniocentesis or chorionic villus sampling. Both these techniques are invasive and do carry a risk of spontaneous abortion. The risk of a maternal hemorrhage from these procedures approximates 20%. Because of these risks, a better source of fetal DNA was needed (49). Because RhD-negative individuals are commonly deleted for the RHD gene, it is an attractive possibility that fetal RhD-positive DNA might be detected from within a background of maternal RhD-negative DNA using PCR. Unlike the case with single-gene disorders, where the carrier or affected mother would also have the mutated gene represented in her genome, the RhD-negative maternal DNA should not be a template for PCR, which should only amplify a product if there is fetal RhD-positive DNA present. Free DNA of fetal origin has been shown to circulate in whole maternal blood (17) and the maternal plasma and serum (50), or it can be extracted from the cellular fraction of maternal blood. It was hypothesized that fetal RhD-positive DNA might be detected from any of these fractions, allowing the RhD type of the fetus to be determined without exposing the mother to the risk of invasive procedures.

Approximately 3% of cell-free DNA in the maternal plasma during the first trimester is of fetal origin. As the pregnancy progresses, this figure rises to 6% by the third trimester (49). Free fetal DNA has been detected as early as 4 weeks of gestational age (49). Cell-free DNA in plasma of pregnant women consists of longer fragments than those found in nonpregnant women. Fetal DNA fragments are generally shorter than maternal fragments. Thus, enrichment of the proportion of DNA derived from the fetus is theoretically possible by exploiting these differences in fragment sizes, but complete separation has not been practical. Currently, the only diagnostic tests available on free fetal DNA targets genes which are not present in the mother. Examples include fetal sex determination via a gene present only on the Y-chromosome and detection of fetal blood grouping in women whose erythrocytes lack the target antigen (7).

Lo et al. (51) first described the use of PCR to identify fetal RhD type from maternal blood without any prior attempt to separate the fetal from the maternal cells. Using a slightly longer version of the original Cartron exon 10 primers in a heminested reaction, they were able to detect RhD-positive DNA in pregnancies where the fetus was RhD positive and the mother was RhD negative. There was, however, an unacceptable false-positive rate which may be secondary to the fact that the concentration of fetal DNA in maternal plasma is very low (7). Over time, this was improved upon by stricter anti-contamination measures (52) and the use of real-time fluorogenic PCR analysis (53). Recently, the same group

published an important clinical paper (54), in which they studied 57 RhD-negative pregnant women, 12 in their first trimester of pregnancy, 30 in their second trimester, and 15 in their third trimester. Thirty-nine fetuses were RhD positive and 18 were RhD negative. In the samples obtained from women in their first trimester, two contained no RhD DNA but the fetuses were RhD positive; the results in the other 10 samples were concordant (seven were RhD positive and three RhD negative). However, among the maternal plasma samples collected in the second and third trimesters of pregnancy, the results of RhD PCR analysis of maternal plasma DNA were completely concordant with the results of serologic analysis of the fetus. It is unclear from this report, however, how many of the 47 multigravidae in their cohort were already sensitized to the D antigen. This will have a bearing on the overall efficacy of this test as their earlier work suggested the possibility of enhanced clearance in sensitized mothers (55).

Fetal Rh genotyping using cell-free fetal DNA circulating in maternal plasma appears to be the most cost effective and readily acceptable for clinical practice (56). The methods which have been shown to be reliable for determining the D phenotype from fetal DNA in maternal plasma use real-time quantitative PCR with Taqman chemistry. These primers and probes detect exons 4, 5, and 10 of RHD but not RHCE (7). Some authors have cautioned premature reliance on this technology (57,58). However, others such as Finning et al. show 100% accuracy in fetal RhD genotyping from maternal blood (59). This has led to the introduction of this test into clinical practice since May 2001, at the International Blood Group Reference Laboratory (IBGRL) in Bristol, UK. Using currently to predict the fetal RhD status in allo-immunized women, the group is expanding its research focus to study mass-testing of plasma from all D-negative pregnant women (60). The usefulness of cell-free fetal DNA for determining the fetal RH genotype has been confirmed by the demonstration that fetal Rh D, C, c, and E genotypes can now all be predicted from maternal plasma (61).

**74.4.5.2 Preimplantation Genetic Diagnosis.** A subgroup of sensitized RhD-negative women with heterozygous RhD-positive partners, who have experienced repeated pregnancy losses or serial intrauterine transfusions in previous pregnancies, are unable to cope with psychological stress of future affected pregnancies. For these patients, preimplantation determination of embryonic RhD type after in vitro fertilization and before embryo transfer would allow RhD-positive pregnancies to be avoided in the future. To perform molecular diagnosis of the RhD blood type from the DNA present in a single human diploid cell, DNA amplification by “nested PCR” is required. This consists of two rounds of amplification. First, a low number of cycles is used with an “outer” set of oligonucleotide primers. The second PCR is then performed on a small aliquot from the first

reaction, using a higher number of amplification cycles with primers internally nested to those in the first reaction. This increases both the specificity and efficiency of the PCR. A primer pair for the outer reaction common to both RHD and RHCE genes within exon 7 was created (62). To account for a single-base-pair difference between the two genes in the region of the 5' primer, this oligonucleotide was synthesized with a single "redundancy" and so consisted of a mixture of two molecules. The use of a common primer pair rather than two sets of primers for "duplex PCR" in the outer reaction of the nested PCR reduces the incidence of inconsistent locus-specific amplification. To differentiate the two genes, an inner reaction was used, taking advantage of four oligonucleotide differences between the two amplified fragments. One inner primer pair was designed to anneal perfectly to the RHD sequence, and a second primer pair annealed perfectly to the RHCE sequence at different but overlapping sites, producing an amplification product of a different size from that of the RHD gene. This strategy, similar to the competitive oligonucleotide priming (COP) PCR used for allele-specific amplification at a single locus, should eliminate the risk that an RhD-positive embryo would be missed.

Numerous other minor antigens are found on the fetal erythrocyte surfaces. These will increase in incidence as RhD alloimmunization become less frequent due to anti-D prophylaxis. Examples of these antibodies known to cause hemolytic disease in the newborn include anti-c, anti-E, anti-Kell, and anti-Duffy. Interestingly, alloimmunization can occur not only against fetal red cell antigens, but also against fetal platelet antigens. Alloimmune thrombocytopenia (AITP) can cause significant fetal morbidity, and is covered in detail in the following section.

## 74.5 ALLOIMMUNE THROMBOCYTOPENIA

AITP is analogous to rhesus alloimmunization, but in this case, the mother produces IgG class antibodies that cross the placenta and affect fetal platelets, leading to thrombocytopenia, and a risk of both antenatal and intrapartum hemorrhage. AITP occurs in approximately 1:350–1:1000 (63). AITP carries the risk of severe hemorrhage, with intracranial bleeding occurring spontaneously in utero in as many as 20% of cases. Neonatal mortality occurs in 1–7% of affected infants. Those who survive are at risk for severe neurological sequelae. These sequelae include mental retardation, cerebral palsy, cortical blindness, and seizures (63). Cases are therefore discovered antenatally when ultrasound identifies signs of intracranial bleeding, or at the birth of an already affected infant. Maternal platelet antibody typing is not performed routinely as part of antenatal care, so women at risk are only identified when they have an affected fetus. Given that screening is not routinely performed,

antenatal treatment is usually provided for those with an affected child. Various in utero treatments have been attempted, including intravenous  $\gamma$ -globulin or steroids and fetal platelet transfusions. However, recent data has demonstrated that intravenous immunoglobulin (IVIG) treatment without invasive testing has been shown to be safe and effective, at least for those with a previous affected child (49).

Platelet alloantigens are located on the glycoproteins on platelet membranes. Conventionally, these antigens are named according to the International Platelet Antigen Working Party nomenclature (64): human platelet alloantigens (HPA), their chronological numbering based on the order of their discovery. Letters "a" and "b" are assigned to the high- and low-frequency alleles, respectively. Excluding alloantigens that are limited to single families and those where only one allele has been identified so far, five platelet alloantigens have been identified to date. HPA-1 and HPA-4 are both epitopes represented on the platelet glycoprotein IIIa, HPA-2 is present on glycoprotein Ib, HPA-3 is represented on platelet glycoprotein IIb, and the weakly antigenic HPA-5 is expressed on platelet glycoprotein Ia.

The molecular basis of HPA-1–5 has been identified and, in each case, is due to a single-nucleotide substitution leading to a single amino acid change in the glycoprotein. The platelet membrane protein GPIIIa gene, which carries the HPA-1 (and HPA-4) polymorphism, was cloned in 1989. The HPA-1b allele differs from the HPA-1a allele by having a C–T substitution at nucleic acid position 196 of the cDNA. This replaces the amino acid leucine in the HPA-1a protein with proline in the HPA-1b isoform. This change also creates a recognition site for the restriction endonuclease Nci I, which has been used as the basis for HPA-1 typing of adults. PCR primers have been designed that span the polymorphic site. The 170-bp products from both HPA-1a and HPA-1b DNA are indistinguishable. To differentiate them, digestion with Nci I is performed, which cuts HPA-1b DNA into two smaller fragments of 106 and 64 bp. A single 170-bp fragment after Nci I digestion indicates an HPA-1a homozygote, and two smaller fragments of 106 and 64 bp indicates an HPA-1b homozygote. A mixture of three fragments indicates a heterozygote. If, however, the restriction enzyme partially digests the target DNA, an HPA-1b homozygote (phenotypically HPA-1a negative) may be incorrectly typed as HPA-1a/b heterozygote (phenotypically HPA-1a positive). An alternative strategy is hybridization of PCR products to allele-specific oligonucleotide probes (44). These are short, single-stranded DNA molecules that are complementary to the polymorphic site. One perfectly matches the HPA-1a DNA; the other contains the C–T substitution and, therefore, matches the HPA-1b DNA. The hybridization conditions are optimized such that only the oligonucleotide with 100% matching will hybridize to the PCR product. Oligonucleotide A will only hybridize to HPA-1a



homozygotes, oligonucleotide B will only hybridize to HPA-1b homozygotes, and both oligonucleotides will hybridize to heterozygote DNA. Each of the polymorphisms giving rise to the HPA-2, -3, and -4 also generates restriction polymorphisms, allowing similar methods of diagnosis.

AITP is regarded as the platelet equivalent of Rh hemolytic disease of the newborn, but in fact, there are differences. Most significantly, in over half the cases, AITP presents in the first pregnancy (65), and maternal antibody levels correlate poorly with disease severity (66,67). The maternal alloantibodies are IgG class antibodies, commonly against HPA-1a in whites (55), and associated with HLA-B8, HLA-DR3, and HLA-DR52 (68–70). After crossing the placenta, the fetal platelet-specific IgG antibodies cause platelet destruction and thrombocytopenia.

In an alloimmunized HPA-1a-negative woman with a heterozygous partner, there is a 50% chance that the fetus will be unaffected. Fetal genotyping by molecular techniques is now possible using amniocytes, chorionic villi, and single cells of blastomeres (71–74).

## 74.6 MANAGEMENT OF ALLOIMMUNIZATION

Prevention is by far the most important aspect of management, because sensitization, or active (as opposed to passive) immunization, triggers the immunologic memory and leads to lifelong alloimmunization.

### 74.6.1 Prevention

Prevention of alloimmunization can be achieved as follows:

- (1) Preventing conception of a fetus with target antigens, either by cessation of reproduction or by selecting sperm from men negative for the offending antigens. The latter could be a social process, for example, changing partners, or a medical one, that is, donor insemination. Neither of these methods has proved to be effective or popular.
- (2) Preventing implantation of embryos positive for the antigen. This has become possible with preimplantation diagnosis.
- (3) Preventing sensitization to the RhD antigen by passive immunization with human anti-D immunoglobulin. The large-scale introduction of this in 1968–1970, following the pioneering work of the Liverpool group (75) and others, has led to a dramatic fall in fetal and perinatal deaths from this cause; it has been a major success in public health.

All pregnant women should be blood-typed and investigated for atypical antibodies in early pregnancy, preferably at the initial prenatal visit. Those who are RhD negative and anti-D negative are at risk of being

sensitized by fetomaternal hemorrhage if the fetus is RhD positive, and this risk is highest at delivery. Patients, who are weak rhesus positive, are not at risk for rhesus alloimmunization and do not require rhesus immunoglobulin. For those who are positive and require it, anti-D given intramuscularly within 72 h of delivery prevents 90% of cases of alloimmunization (75). In the United Kingdom, 100 µg is given, whereas in the United States and elsewhere, 250–300 µg is recommended. The 100 µg dose prevents immunization by up to 4–5 mL RhD-positive RBCs, that is, about 99% of all fetomaternal hemorrhages. At delivery, maternal blood is Kleihauer tested to estimate the volume of fetomaternal hemorrhage, but some consider this unnecessary if 250–300 µg anti-D are given. However, 0.2% of women have fetomaternal hemorrhages larger than 15 mL RBCs, and these would not receive an adequate dose of anti-D and be at risk of sensitization.

Fetomaternal hemorrhage can also be caused by any other events producing placental separation, for example, threatened or complete miscarriage, termination of pregnancy, ectopic pregnancy, antepartum hemorrhage, external cephalic version, abdominal trauma, and invasive procedures such as amniocentesis (76) and particularly chorion villus sampling (77). Women should receive prophylactic anti-D on these occasions, the dose in the United Kingdom being 50 µg before 20 weeks' gestation and 100 µg after 20 weeks (78).

Despite these measures RhD immunization still occurs, probably as a result of spontaneous fetomaternal hemorrhages. Many centers now give routine antenatal prophylaxis of 100 µg anti-D to all nonimmunized RhD-negative women at 28 weeks gestation, as well as at delivery. The American Association of Blood Banks recommends that a repeat antibody screen be obtained prior to the administration of antenatal rhesus immunoglobulin at 28 weeks. Maternal sensitization can occur by 28 weeks. An opportunity to detect hemolytic disease may be missed if such a screen is not performed. A small number of RhD immunizations will continue to occur, as well as those such as anti-c or anti-Kell, which are not preventable. For these patients, methods of treatment must be maintained and improved.

### 74.6.2 Treatment

**74.6.2.1 Red Blood Cells.** A variety of treatment strategies have been developed, both ante- and postnatally. The relatively nonspecific postnatal treatments were the first to be used and are still very important. Early delivery removes the fetus from the adverse environment containing the hostile maternal antibodies. However, this increases the risks due to prematurity, and therefore, the need for neonatal intensive care, which has seen major advances in the past 30 years. Exchange transfusion was one of the most important developments. Its aims are threefold: (1) to lower the unconjugated bilirubin level

and prevent kernicterus, (2) to wash out the maternally derived anti-D and reduce further hemolysis, and (3) to treat anemia by replacing RhD-positive RBCs with RhD-negative RBCs, which will not be hemolyzed. Phototherapy is also a means of lowering unconjugated bilirubin and reduces the need for exchange transfusion.

Postnatal management reduces mortality and morbidity, such as kernicterus, but has complications. Furthermore, the severely affected fetus that might die in utero before a reasonable chance of extrauterine survival, for example, at about 28 weeks' gestation, still requires treatment. Methods of antenatal treatment are needed for these fetuses to help them survive in utero, preferably until they are mature enough for a complication-free postnatal course.

Neutralization of maternal antibodies, or "desensitization," has been attempted by using Rh haptens and RhD-positive RBC membranes. Results have been inconsistent, but on the whole disappointing (79) and none of these methods has entered clinical practice. Maternal anti-RhD antibody levels can be reduced by plasmapheresis, and this has been used in some centers (80), but there is no good evidence that it is effective in improving severe alloimmunization.

Several methods have been used to prevent or modify fetal hemolysis. Corticosteroids and promethazine hydrochloride have been shown to lack beneficial effect. Therapeutic success has been claimed for high-dose maternal intravenous IgG infusions (81). As yet, there are no randomized controlled trials and good results have not been reproduced in other centers. This treatment also has the disadvantage of being extremely expensive.

Fetal RBC production is enormously increased in severe alloimmunization, but in theory, it could be limited by a deficiency in hematinics. Supplementation could therefore improve the erythropoietic response, but a study of the administration of vitamin B12 and folic acid did not result in improved outcome (82). Furthermore, hydropic fetuses do not seem to be deficient in vitamin B12 or folic acid (Rodeck and Nicolaides, unpublished observations). Erythropoietin has been used to manage several types of anemia by increasing RBC production, but it has not been tried in fetal hemolytic disease. As fetal erythropoietin levels are already high, it is unlikely to be successful.

Another approach to the treatment of alloimmune hemolysis is to replace the fetuses' RhD-positive RBCs by transplanting, in utero, RhD-negative erythropoietic stem cells. In theory, transplantation at a gestational age before the development of fetal immune competence, thought to occur at 14–18 weeks, could lead to the induction of tolerance and a state of stable erythropoietic chimerism. The fetus should then have a population of RhD-negative RBCs that would not be destroyed and would prevent death from severe anemia. This has been attempted by giving RhD-negative bone marrow cells into the fetal peritoneal cavity between 12 and 16 weeks

(82) and intravascularly by fetoscopy at 17 weeks into the umbilical vein (83), but without success.

By far, the most successful in utero treatment of severe Rh alloimmunization has been fetal transfusion of RhD-negative RBCs. Fetal anemia can be indirectly assessed by ultrasonography and Doppler examination, less indirectly by amniotic fluid analysis for bilirubin content (84), and directly by fetal blood sampling. Intrauterine therapy is indicated when fetal blood sampling shows that the fetal hematocrit is low or if there is hydrops fetalis. The red-cell transfusion can be administered intraperitoneally, or preferably intravascularly.

The fetal peritoneal cavity was the most widely used route for almost 20 years (85). It relies on the donor red cells being absorbed into the fetal circulation via the subdiaphragmatic lymphatics and the thoracic duct. However, the most severely affected fetuses, with ascites, were not able to absorb the blood and benefited least (86). A significant advantage of this technique is that fetal hemoglobin levels can be obtained before and after the transfusion. The volume of blood needed for transfusion can be calculated using the pretransfusion hematocrit, estimated fetoplacental volume, and the hematocrit of the donor blood (87). Another advantage is that it can be used at very early gestations, is unhampered by fetal lie and position, and can be combined with intravascular transfusion for an extended therapeutic effect (88–90).

In comparison to intraperitoneal transfusion, intravascular transfusion is more successful at reversing hydrops given that hydrops reduces the ability of the lymphatic system to absorb RBCs. Intravascular transfusion was attempted in a few cases in the 1960s by cannulating fetal vessels after exposure by laparotomy. The fetal mortality and maternal morbidity of these procedures were prohibitive, and fetal intravascular transfusion did not become a therapeutic reality until the fetoscope was used (91). The umbilical vein, at either the placental insertion of the cord (92) or its intrahepatic part (93,94), is the preferred target. The donor blood should be fresh (less than 24 h old) O RhD-negative adult blood, screened for transmissible infectious agents, cross-matched with the mother's blood, white cell depleted and packed to a hematocrit of approximately 80% to minimize the volume of donor blood that must be transfused. The volume to be transfused depends on the pretransfusion fetal hematocrit, donor blood hematocrit, and the estimated fetoplacental blood volume (92). The first repeat transfusion usually occurs within a fortnight, and subsequent transfusions are scheduled based on the rate of fall of the fetal hematocrit.

In experienced centers, the fetal/neonatal survival rate should be 90–95%. Intrauterine transfusion is generally considered a safe procedure. However, some complications do occur. The most common complication is fetal bradycardia. Fetal distress may occur secondary to cord accidents (cord rupture, spasm, tamponade (from a hematoma)), or hemorrhage from the puncture site.

Other complications can include preterm rupture of membranes or preterm labor (87). Neonates treated with intrauterine transfusions still need intensive care postnatally. In one series, neonates with Rh disease treated with intrauterine transfusions required less phototherapy but required more top-up blood transfusions. The effect of intrauterine RBC transfusion on the length of phototherapy is most likely due to the fact that less hemolysis occurs because fetal erythrocytes are replaced by adult (donor) erythrocytes, which are less likely to hemolyze. The increased need for top-up transfusions postnatally may be due to decreased erythropoiesis secondary to the intrauterine transfusions (95). Indeed, the management of the neonate is much easier because they are usually delivered at about 37 weeks, and the need for exchange transfusion is minimal owing to the presence of 100% RhD-negative cells, suppression of endogenous RhD-positive cells, insignificant hemolysis, and only minor degrees of hyperbilirubinemia.

**74.6.2.2 Platelets.** Passive prevention of platelet alloimmunization, as for RhD, is not possible. The management of an affected neonate includes IVIG treatment, and the use of washed maternal/donor platelets. In contrast, the antenatal management of AITP is less clear.

If platelet antibodies are detected in a current pregnancy, it is not clear whether the fetus will be affected and require treatment. Nearly 50% of clinically significant cases of AITP are diagnosed during the first pregnancy (96). Pregnant women who have previously given birth to an affected infant with intracranial hemorrhage have a high risk of recurrence, but the majority of at-risk fetuses have siblings with a history of severe thrombocytopenia but without internal bleeding. It is difficult to assess the risk of intracranial hemorrhage to the fetus, so early invasive testing may have to be considered to obtain a fetal platelet count (97,98), or to group the fetus if the father is heterozygous (99). Rarely, platelet alloimmunization may be present in a first-affected pregnancy with fetal hydrocephalus due to intracranial hemorrhage caused by severe thrombocytopenia (100).

The optimal treatment for a patient with a severely affected fetus is controversial. Noninvasive therapies aimed at treating the fetus, such as high-dose maternal intravenous IgG and/or steroids, or both, are of uncertain efficacy (6,55,66,101). On the other hand, invasive treatment, such as fetal platelet transfusion, is of unquestionable efficacy but carries considerable risk because the short half-life of platelets means that frequent transfusions (every 7–10 days) are required (102). The main concern with fetal platelet transfusion is the fetal loss rate, particularly from exsanguination in the presence of severe thrombocytopenia. In one series involving 12 patients, the procedure-related fetal loss rate was 1.2% but per pregnancy, the rate was 8.3% (96). Because of the small numbers of patients seen at each center, multicenter collaboration will be necessary to evaluate different treatment protocols. In the meantime,

preimplantation diagnosis may also become a possibility for these patients (103).

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## Biography



**Dr Lau** graduated from the University of Arizona with a Bachelor's degree in Molecular and Cellular Biology (with Honors). He attended the University of Arizona College of Medicine. During that time, he was commissioned as an Ensign in the United States Navy Reserve. He completed an Internship in Ob/GYN at Naval Medical Center San Diego. Prior to internship, he was commissioned as a Lieutenant in the United States Navy. He completed his residency at the Uniformed Services Residency in Obstetrics and Gynecology (National Naval Medical Center and Walter Reed Army Medical Center). He served a tour of duty as an OB/GYN at Naval Hospital Camp Lejeune (North Carolina). Subsequently, his next tour was at Naval Hospital Lemoore (California) where he served as Department Chair. He was awarded the Navy and Marine Corps Commendation Medal (two awards) as well as the Army Commendation Medal. In addition, he has served as a representative to the Patient Safety and Quality Improvement at the American Congress of Obstetrics and Gynecology. He is now completing a Maternal Fetal Medicine Fellowship at Cedars-Sinai Medical Center. His research interests include the use of ultrasound in first trimester fetal diagnosis as well as means of improving patient safety and satisfaction during labor and delivery.

# CHAPTER

# 75

## Leukemias, Lymphomas, and Other Related Disorders

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### 75.1 INTRODUCTION

Hematologic malignancies, such as leukemia and lymphoma, are cancers of the blood producing cells. Although in rare cases associated with an inherited mutation and a genetic syndrome such as Fanconi anemia, Bloom syndrome, or ataxia–telangiectasia (AT), these disorders usually occur as sporadic cancers. In other words, they arise from a mutation in a previously normal somatic cell. Leukemia and lymphoma are not genetic diseases in the classic sense, but nonetheless can be called genetic diseases as the primary underlying etiology has been shown to be a DNA mutation, especially chromosomal rearrangements.

Normal hematopoiesis is a highly regulated differentiation and maturation process from a hematopoietic progenitor stem cell to a highly specialized cell of myeloid or lymphoid lineage. If an aberration in the regulatory pathways confers a proliferative advantage to a mutant cell, the end result may be the clinical presentation of leukemia or lymphoma. Leukemia is characterized by a progressive expansion of immature myeloid or lymphoid cells in the bone marrow, whereas lymphoma is featured by an abnormal lymphoid proliferation commonly in lymph nodes, but also in the spleen, tonsils, or skin. In the earlier classifications, such as the French–American–British cooperative Group (FAB classification) in leukemia, and the European and American classification of lymphoid neoplasm (REAL) (1,2), hematopoietic malignancies were classified based on the morphologic features of the abnormal cell population in combination with the clinical presentation. With the expanding knowledge of oncogenesis, in particular the genetic mechanisms in leukemia and lymphoma, the World Health Organization (WHO) developed and revised a universally accepted

classification system of myeloid and lymphoid neoplasms in 2001 and 2008, respectively (3,4), according to the lineage of cell origin and the combination of morphologic, immunophenotypic, genetic, and clinical criteria. Many new entities of myeloid and lymphoid leukemia and lymphoma, with unique genetic background and clinical features have been included to define disease entities of clinical significance. The 2008 WHO classification consists of five major groups of myeloid leukemia and six types of tumors of the lymphoid tissues. Genetic abnormalities play an important role as diagnostic criteria for further sub-classification of some neoplasms, in particular of acute myeloid leukemia (AML) (for a detailed classification, see References (4–6)). The new classification defines individual disease groups with a homogenous genetic background and clinical relevance.

### 75.2 GENERAL PATTERNS OF CHROMOSOME ABERRATIONS AND GENOMIC ABNORMALITIES IN LEUKEMIA AND LYMPHOMA

Cytogenetic evaluation of cells from patients with hematologic malignancies has resulted in major advances in our understanding of the specificity of some of the abnormalities observed in tumor cells (7). Chromosomal abnormalities in cancer are non-random and acquired somatic mutations. They occur in the tumor cells only, and represent clonal abnormalities. These abnormalities are not detected when cancer cells are killed and patients go into complete remission; however, they will reappear in relapse. Balanced or unbalanced chromosome translocations, inversion and insertion, loss and gain of chromosomes, and deletions and

amplifications of parts of chromosomes are the common forms of chromosome abnormalities in cancer. The incidence of chromosome abnormalities varies based on disease status and types, from 10 to 25% in low-grade myelodysplastic syndrome (MDS) and myeloproliferative neoplasm (MPN) to 100% in chronic myeloid leukemia (CML) (by definition) (8–10). Balanced chromosome translocations are common in de novo acute myeloid and lymphoid leukemia, and in lymphoma, whereas losses or gains of chromosome materials are frequent in de novo MDS and in therapy-related MDS and AML. In lymphoid leukemia and lymphoma, chromosome translocations, such as t(8;14) in Burkitt leukemia and lymphoma, and t(7;11) in T-cell leukemia and lymphoma, usually result in the conjunction of the promoters and enhancers of the immunoglobulin and T-cell receptor genes with an oncogene or anti-apoptosis gene, leading to a deregulation (i.e., over-expression) of an intact onco-protein (7,11). In myeloid leukemia, and some lymphoid leukemia, that is, acute lymphoblastic leukemia (ALL), chromosome translocation, and insertion or inversion, such as t(8;21) in AML and t(9;22) in CML, result in novel chimeric fusion genes with novel or aberrant function. Chromosome translocation and/or inversion in myeloid and lymphoid leukemia and lymphoma frequently involve the genes that are functionally related to transcriptional regulation, cell cycling, apoptosis regulation, and cytokine signaling pathways (12,13). Loss or deletion of chromosome material may lead to the loss of relevant tumor suppressor genes, whereas gain or duplication are associated with amplification of oncogenes, sometimes in the form of heterogeneous staining regions or double minutes.

Recently, using array comparative genomic hybridization (CGH) and genomic microarray techniques, additional genomic abnormalities have been discovered in almost all leukemia and lymphoma samples analyzed (14,15). Genomic imbalances detected by array techniques are usually subtle and even cryptic, below the sensitivity of conventional cytogenetic analysis. In particular, microarray studies have identified novel genomic aberrations, such as copy-neutral loss of heterozygosity (CN-LOH) in various leukemia and lymphoma with normal karyotypes, and thus have provided new genetic markers for clinical usage and cancer research, because these subtle chromosome lesions commonly contain one or a few critical genes, such as oncogenes and tumor suppression genes that are involved in leukemogenesis and lymphomagenesis (16). Newer techniques, such as next-generation sequencing of leukemia samples will facilitate further delineation of the genetic mechanisms that lead to leukemia and lymphoma.

### 75.3 SIGNIFICANCE OF DETECTING ACQUIRED CHROMOSOME AND GENE ABNORMALITIES IN LEUKEMIA AND LYMPHOMA

Chromosome abnormalities and somatic gene mutations are the hallmarks of many leukemia and lymphoma, such

as the t(9;22) in CML and t(8;14) in Burkitt leukemia and lymphoma. The close association of specific chromosome abnormalities with particular types of human leukemia and lymphoma has been well established in the past several decades, and are very helpful in confirming the diagnosis (17). For instance, the detection of t(9;22) by cytogenetic, or fluorescence in situ hybridization (FISH) analysis will enable a precise diagnosis of CML, and will rule out any other MPN, regardless of morphological features in bone marrow specimens (18). In non-Hodgkin lymphoma (NHL), the detection of t(11;14) in lymph nodes, bone marrow, or peripheral blood samples strongly favors mantle cell lymphoma (MCL), rather than chronic lymphocytic leukemia (CLL), despite any unusual morphological or immunophenotypical features of the tumor cells. Because of its unique association with disease, detecting certain chromosome abnormalities greatly helps in selecting appropriate treatment, such as t(15;17) in acute promyelocytic leukemia (APL) and t(9;22) in CML. Once the t(15;17) is detected in APL by cytogenetic, FISH, or PCR techniques, treatment using all-trans retinoic acid (ATRA) will efficiently induce leukemia cells to further differentiate and then apoptose (19). In CML with t(9;22), Imatinib (also called Gleevec) treatment can induce most CML patients into clinical, hematological, cytogenetic, and molecular remission in a short period (20,21). Moreover, chromosome abnormalities are the most significant prognostic factors for patients with acute leukemia and lymphoma. Patients with AML and t(8;21) and inv(16)/t(16;16), involving the alpha and beta subunits, respectively, of the core-binding factor (CBF) have a very favorable prognosis, whereas patients with AML and abnormalities of chromosome 7 or a complex karyotype show a significantly dismal clinical course (8,9,22). Patients with ALL and t(9;22), and patients with NHL and MYC translocations have much more aggressive clinical courses and unfavorable prognosis (23–26). In addition, detecting a clone with a specific chromosome abnormality such as chromosome translocations enables scientists to clone the genes that are involved in leukemo- and lymphomogenesis, which may provide further therapeutic targets.

The value of performing cytogenetic analysis is reflected by the incorporation of genetic information in the 2001 and 2008 WHO classification systems of myeloid and lymphoid neoplasms. With the new classification system, cytogenetic analysis is required in the complete evaluation of a patient with a newly diagnosed or suspected AML (6). Therefore, a cytogenetic evaluation should be performed for every patient with a suspected or confirmed hematological malignant disease to aid in the correct diagnosis of the malignancy and to assess the patient's prognosis. Furthermore, any abnormality noted at the time of diagnosis can be used as a biological marker to monitor the response to therapy or to detect residual disease in follow-up specimens.



## 75.4 APPLICATION OF CYTOGENETIC, FISH, AND MICROARRAY TECHNIQUES IN DIAGNOSIS OF LEUKEMIA AND LYMPHOMA

The principles and methods used for conventional cytogenetic analysis of malignant disease are very similar to those used for constitutional abnormalities, which have been outlined in detail in Chapter 22. Cancer cytogenetic analysis can be performed on almost any tissue with actively dividing (malignant) cells. For leukemia studies the specimen is usually a bone marrow aspirate although a bone biopsy can also be processed successfully. Alternatively, in patients with circulating immature cells, a sample of peripheral blood can be analyzed. For lymphoma studies, the most appropriate tissue is a biopsy of the affected tissue such as lymph node, spleen, or tonsil. However, even when lymphoma cells are extensively present in the bone marrow, cytogenetic evaluation of a bone marrow aspirate rarely proves to be informative for the study of lymphoma, particularly in low-grade B-cell lymphoma, such as follicular lymphoma and CLL (27).

A typical cytogenetic analysis includes the complete analysis of at least 20 banded metaphase cells. However, this may not always be possible and depends on the cellularity, mitotic index, and quality of the specimen. An analysis of fewer than 20 cells is still informative when a clonal abnormality is detected. According to the International System of Human Chromosome Nomenclature (28), a chromosomal abnormality is considered to be clonal if a structural abnormality or gain of a chromosome is identified in two or more cells. Chromosome loss can occur as a technical artifact during metaphase cell preparation; thus, a loss of a chromosome is considered to be clonal when it occurs in three or more cells. Although chromosome analysis has a low resolution and requires dividing cells, and is very labor consuming, it provides a picture of all chromosome abnormalities in a cell, and frequently will detect novel disease-specific chromosome abnormalities.

The field of cytogenetic analysis was significantly advanced through the development of molecular cytogenetics, that is, FISH in the 1980s (29,30). FISH uses a directly or indirectly fluorescence-labeled single-stranded DNA probe that will anneal to the complementary DNA sequences of a target specimen in interphase or metaphase cells affixed to microscope slides. The source of these tumor cells can be fresh or fixed bone marrow or peripheral blood smears, touch preparations, or paraffin-embedded sectioned tissue. The test probe can be gene or locus specific, chromosome and/or arm-specific, or repetitive sequences specific for individual centromeres or telomeres of each chromosome. FISH can be used not only on metaphase cells but in interphase cells as well, thus enhancing the detection of small non-dividing tumor populations. Moreover, FISH can be simultaneously performed with immunophenotyping techniques

in defining the cell lineages and differentiation of tumor cells with specific chromosome and gene abnormalities. The high sensitivity and specificity of FISH, and the speed with which the technique can be completed have made FISH a powerful tool with numerous applications, such as the detection of numerical and structural chromosomal abnormalities. In many cases, FISH has clarified the nature of chromosomal abnormalities that were ambiguous with banding analysis alone. Using FISH, and the related techniques of multicolor spectral karyotyping (M-FISH or SKY), karyotypic abnormalities can be detected in most leukemia specimens. Nevertheless, FISH is complementary to conventional cytogenetic analysis because it only detects the abnormalities the probes target and hybridize, and it will miss other chromosome abnormalities not targeted by the FISH probes.

The development of CGH and microarray techniques has provided us with more powerful tools to study comprehensive genomic and expression profiling in leukemia and lymphoma at the whole genomic level. The resolution of CGH and microarray is much higher than chromosome analysis in detecting genomic and chromosomal abnormalities leading to copy number alterations in hematological neoplasms. Traditional CGH and array-based CGH use a competitive DNA hybridization of normal and tumor tissues with different-color fluorescence labeling (31). Single nucleotide polymorphism (SNP) microarray can detect genomic imbalances such as loss or gain of gene copy numbers, and CN-LOH, so-called acquired uniparental disomy, due to incomplete chromosome segregation or to mitotic recombination (15). Various cryptic copy number imbalances and CN-LOH have been found in AML, MDS, MPN, ALL, and lymphoma (14,16,32). Novel gene deficiencies have been defined in microarray studies and further characterized by molecular analysis, such as *JAK2* mutations in MPN, *IKZF1* and *PAX5* deletions and mutations in ALL, and *TET2* mutations in MDS and AML (13,33–36). Recently, the application of microarray analysis in leukemia and lymphoma has been extended from basic and translational research to clinical diagnostic laboratories, and certain novel genomic abnormalities discoveries by microarray have been validated to be significant in disease diagnosis and prognosis, and will soon become important new biomarkers in clinical patient care (18,37).

Gene expression profiling analyses by use of microarrays in AML, ALL, MDS, MPN, and lymphoma have provided important information regarding gene expression changes in the pathogenesis of leukemia and lymphoma, and are helpful in the diagnosis and prognosis of lymphoma and leukemia (38). The differences in gene expression profiling can readily distinguish AML from ALL, and can further subgroup diffuse large B-cell lymphomas that have different prognoses and treatment responses (39,40). Gene expression profiling is particularly prognostic in pediatric ALL patients in terms of risk classification and outcome prediction. In combination

with minimal residual disease (MRD) measurement, gene expression profiling helps identify pediatric ALL patients with a higher chance of relapse-free survival. More recently, microRNA expression profiling studies have discovered different microRNA expression patterns in many leukemia and lymphoma, such as AML, ALL, and CLL, and high-grade lymphoma (41–43). MicroRNA expression patterns differ between the normal cells and blast cells, and between AML and ALL, and correlate with cytogenetic abnormalities and molecular markers in AML (44–46). Moreover, microRNA expression in cytogenetically normal AML has been associated with the clinical outcome and with target genes encoding proteins involved in specific innate-immunity pathways (47). The results from microRNA expression profiling are indicative of epigenetic regulation of the targeted genes in leukemia and lymphoma, and are useful in making diagnosis and establishing prognosis (42). Furthermore, microRNA array analysis may highlight novel treatment options by restoring lost tumor suppressor microRNA or silencing highly expressed microRNAs using antagomirs (48).

## 75.5 MYELOPROLIFERATIVE NEOPLASM

### 75.5.1 Chronic Myeloid Leukemia

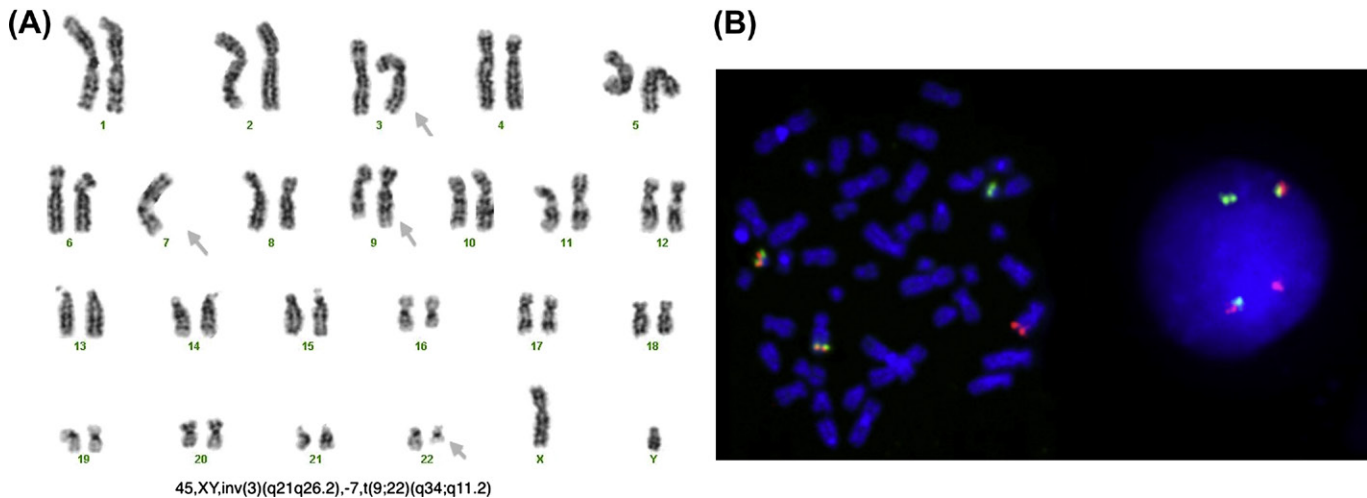
CML that is characterized by t(9;22) and the *BCR/ABL1* fusion is one of the most common leukemia in adults, characterized by the proliferation of multiple myeloid lineages, and serves as the best model for our understanding of the mechanisms of genetic abnormalities in leukemogenesis (10,49–51). CML was the first cancer in which a unique chromosome abnormality, namely the Philadelphia (Ph) chromosome, was identified (52). It was the second cancer to be associated with a recurring chromosome abnormality, that is, t(9;22), shortly after the t(8;21) was discovered in AML (53,54). CML was also the first disease in which a molecular rearrangement was recognized as resulting in a novel fusion gene and a chimeric protein that was fundamental to leukemogenesis (55–58). Furthermore, CML serves as the first cancer with a rationally designed drug treatment that directly targets the molecular consequence responsible for the pathogenesis of the disease (59). Therefore, CML is a validated research model in cancer biology, normal and abnormal hematopoiesis and lineage commitment, and in targeted drug development and therapy (21,60–62).

The t(9;22) is the hallmark of CML as recognized by the 2008 WHO classification of hematopoietic and lymphoid neoplasms and can be detected by conventional cytogenetic analysis in more than 90% of CML patients (Figure 75-1A) (7,63). It is present in all myeloid lineage cells, including granulocytes, monocytes, erythroid precursors, megakaryocytes, and in all B and some T lymphocytes (64,65). In the remaining 2–10% of the patients with CML, a variant of the t(9;22), such as a

three- or four-way translocation involving a third chromosome or more, can be observed. In a few cases, the t(9;22) is cryptic due to an insertion of the *BCR* gene into the *ABL1* gene or vice versa, which can be confirmed by FISH analysis on metaphase cells or PCR studies (Figure 75-1B). The t(9;22) also is one of the recurring chromosome abnormalities in ALL, particularly in adult patients; the consequence of the t(9;22) is the chimeric mRNA and protein with the fusion between the 3' end of the first exon of the *BCR* gene and the 5' end of the second exon of the *ABL* gene on the derivative chromosome 22 (Ph chromosome) (66,67). The breakpoints in *ABL* are always proximal to the second exon in both CML and ALL, whereas the breakpoints in *BCR* are variably different between CML and ALL. In CML, the breakpoints in *BCR* are more telomeric in the major breakpoint cluster region (micro-bcr (m-bcr), between exons 12 and 15) or m-bcr region (between exons 19 and 21), giving rise to the P210 and P230 fusions, respectively. In contrast, the breakpoints in the *BCR* gene in ALL are far more proximal to exon 2 in a minor breakpoint cluster region (m-bcr), producing the P190 fusion (68). Thus, the location of the breakpoints and the amount of the *BCR* gene that are included in the *BCR/ABL* fusion determine whether the leukemia will be CML or ALL, both showing similar but not identical *BCR/ABL* fusion proteins.

The *ABL* gene is also involved in another translocation, with the *TEL* (*ETV6*) gene on 12p13. *ABL* is a member of the family of non-receptor protein tyrosine kinases, and in its wild-type form it has no transforming activity. Thus, oncogenic transforming properties must be provided by its fusion partners, that is, *BCR* and *TEL*. The *BCR* gene is a member of the Rho family of small GTPases, GAP proteins (69). It is critically involved in activating the *ABL* tyrosine kinase in the *BCR/ABL* fusion protein (70). The first 63 amino acids of *BCR* include a coiled-coil motif, which is able to induce homodimerization and tetramerization of *BCR/ABL*; the *TEL* protein also contains protein dimerization domains. Thus, oligomerization of *ABL* by *BCR* or *TEL* activates the *ABL* tyrosine kinase, induces a microfilament-binding function, and results in transformation. Both p210BCR/*ABL* and p190BCR/*ABL* display deregulated constitutive tyrosine kinase activity; p210BCR/*ABL* is located exclusively in the cytoplasm, where it complexes with various cytoskeletal proteins and constitutively interferes with a variety of cytoplasmic and cytoskeletal signaling pathways. The fusion protein p210BCR/*ABL* is also a powerful anti-apoptotic molecule in mammalian cells, and is directly implicated in the defective responses of both immature and differentiated primary myeloid cells to growth factor deprivation (71,72).

Typically, before the introduction of Imatinib treatment, the chronic phase of CML usually lasted about 3–5 years, and then evolved to an advanced stage. The disease evolution was accompanied in more than 60% of



**FIGURE 75-1** A. Karyotype of a metaphase cell from a male patient with CML in the blast phase. The karyotype is 45, XY, inv(3)(q21q26.2), -7, t(9;22)(q34;q11.2). Gain of additional chromosome aberrations, in addition to t(9;22), is frequently observed in CML during the accelerated and blast phases. B. FISH analysis of cells from the same patient reveals the *BCR/ABL* fusions in metaphase and interphase cells. The probes for *ABL* and *BCR* are labeled with SpectrumOrange® and SpectrumGreen®, respectively. Normal chromosomes 9 and 22 show a single red and green signal, whereas the derivative chromosomes 9 and 22 display a fusion (yellow) signal pattern containing the fused *BCR* and *ABL* probes. Abnormal interphase cells show the typical pattern with two fusion (yellow) and one red and one green signal.

patients with additional chromosome aberrations, which can be reliably identified by cytogenetic analysis in many cases. The most common additions are gain of chromosome 8 (33%), followed by an additional Ph chromosome (30%), i(17q) (20%), +19 (12%), loss of the Y chromosome (8% in males), trisomy 21 (7%), and loss of chromosome 7 (5%) (73,74). They may appear individually or in combination. In certain situations, the appearance of additional abnormalities, such as +8 and i(17q), is a strong indicator of an occult disease progression, often several months earlier than morphological evidence on bone marrow examination or clinical symptoms. Molecular analysis has identified abnormalities of various genes in CML in the accelerated and blast phases (75). These include *TP53* (17p13), *RB1* (13q14), *MYC* (8q24), *p16INK4a* (*CDKN2*) (9p21), and *RAS* (11p15) (75,76). However, it is unclear what chromosome or gene abnormalities are responsible for CML progression.

In the early 1990s, a tyrosine kinase inhibitor, that is, Imatinib was introduced as the frontline drug for CML and has revolutionized the treatment of CML (21). It has significantly improved the outcome of patients with CML, with 97% complete hematologic response and 87% complete cytogenetic remission in a 5-year follow-up study (20). The natural course of CML has now been completely changed because no CML patients with complete cytogenetics remission and an excellent molecular response with more than 3 log reduction of the *BCR/ABL* fusion mRNA has evolved to the accelerated or blast phase during the 5-year Imatinib treatment. Furthermore, novel tyrosine kinase inhibitors, that is, Nilotinib and Dasatinib that have stronger inhibition than Imatinib, have showed significant improvement in patients with resistance to Imatinib treatment (20,77).

In up to 15% of patients with CML on Imatinib, acquired chromosomal abnormalities with no Ph chromosome may appear that either herald disease progression or that may be of unclear clinical significance. The most common clonal abnormalities are +8, -7, -Y, or del(20q) in Ph-negative cells (78,79). The development of new abnormalities has been noted both in patients with prior therapy (IFNa) and in patients treated only with Imatinib (80). Some of these patients had clinical features of a MDS (81,82). Although loss of the Y chromosome is frequently observed in bone marrow cells in elder patients (83), it is likely that the appearance of Ph-negative aberrant clones, particularly -7 and +8, in CML remission after TK inhibitor treatment is a reflection of an early malignant myeloid change at least in some patients; repeating cytogenetic analysis, not just FISH tests at regular intervals (yearly), is warranted to monitor all CML patients in remission (73,84).

### 75.5.2 JAK2 and Other Gene Mutation in Classic Myeloproliferative Neoplasms

In the 2008 WHO classification of leukemia and lymphoma, there are three additional classic MPN diseases, that is, polycythemia vera (PV), essential thrombocythemia (ET), chronic idiopathic myelofibrosis (CIMF), all with features of over-proliferation of one or more myeloid lineages in bone marrow and extra-medullary areas (18). Cytogenetically abnormal clones are present in <5% of ET to >40% in CIMF. Typical abnormalities include del(5q), -7/del(7q), +8, +9, del(11q), del(13q), and del(20q). However, none of these chromosome abnormalities are specific for any of MPN because they are also detected in other myeloid disorders, including MDS and AML.



In 2005, a *JAK2* mutation was identified in these three classic MPN disorders (85–87). *JAK2* belongs to the Janus kinase subfamily and contains a central Src homology 2 (SH2) domain, and two C-terminal domains: a tyrosine kinase domain JH1 (also termed PTK or TyrKc domain) and a tyrosine kinase-like domain JH2 (also termed STYKc). *JAK2* is a protein tyrosine kinase of the non-receptor type that associates with the intracellular domains of cytokine receptors and is the predominant JAK kinase activated in response to several growth factors and cytokines such as IL-3, GM-CSF, and erythropoietin; it has been found to be constitutively associated with the prolactin receptor and is required for responses to gamma interferon (88). The most common *JAK2* mutation is the dominant gain-of-function V617F mutation in the JH2 kinase-like domain. This mutation leads to deregulation of the kinase activity, and thus to constitutive tyrosine phosphorylation activity. The incidence of the *JAK2* mutation is almost 100% in PV, and about 50% in patients with ET and CIMF (5,18). Although *JAK2* mutations are not specific for any of the MPN subgroups and can occur in some patients with MDS with marked thrombocytosis, detecting a *JAK2* mutation helps to prove clonality and to distinguish malignant proliferation from a reactive process. The V617F mutation seems to occur exclusively in hematopoietic malignancies of the myeloid lineage. Other mutations in MPN are *JAK2* exon 12 in PV, *MPL* W515L/K in ET and CIMF, and *TET2* and *ASXL1* (18) (see Section 75.6) in a significant low incidence. In general, these mutations do not seem to be the initial clonogenic event in the development of MPN, and not to correlate with survival or transformation of MPN.

In certain myeloid and lymphoid neoplasms with eosinophilia, abnormalities of the platelet-derived growth factor receptor alpha and beta (*PDGFRA* and *PDGFRB*) genes and fibroblast growth factor receptor 1 (*FGFR1*) have been implicated through various chromosome deletions or translocations, such as t(5;12) (q33;p13), fusing *TEL* (*ETV6*) and *PDGFRB* in chronic myelomonocytic leukemia (CMML), and a cryptic interstitial deletion of the *CHIC2* locus at 4q12, leading to a fusion between *FIP1L1* and *PDGFRA* (65,89) (Table 75-1). Multiple chromosome translocations and deletions have been described in this group with eosinophilia, and some are cryptic and can be detected only by FISH or PCR techniques (Table 75-1). It is important to detect these chromosome abnormalities because patients with *PDGFRA/B* rearrangements are responding well to the treatment with Imatinib and other tyrosine kinase inhibitors.

## 75.6 MYELOYDYSPLASTIC SYNDROMES

The MDS are a heterogeneous group of clonal hematological stem cell disorders characterized by chronic cytopenias, such as anemia, neutropenia, or thrombocytopenia,

and accompanied by abnormal cellular maturation, with an increased risk of development of AML (90). The bone marrow is usually hypercellular due to over-proliferation of myeloid cells, whereas anemia in the peripheral blood is a consequence of inefficient maturation and an early apoptosis (3). Among MDS patients, single or multiple myeloid lineages may be involved, affecting erythroid, granulocytic, or megakaryocytic lineages. The number of blasts in bone marrow and/or peripheral blood samples is variable. In some patients MDS can also develop after cytotoxic chemotherapy and/or radiation therapy for a primary cancer, such as lymphoma, and breast cancer, or after immunosuppression treatment for non-malignant disorders, such as rheumatoid arthritis, or following organ transplantation (6).

Clonal chromosome abnormalities in bone marrow samples can be detected in 40–70% of patients with primary MDS at diagnosis, in up to 90% of therapy-related MDS/AML, and in 100% of MDS with isolated del(5q) which is commonly seen in older female patients with refractory anemia with low blast counts, and normal or elevated platelet counts (90,91). In contrast to AML with the common balanced chromosome translocations, MDS generally shows genomic imbalances, that is, gain or loss of chromosomes and/or chromosome arms (92,93). The most common chromosomal deletion, losses, or gains in primary MDS include del(5q) (25%), -7/del(7q) (20%), +8 (15%), and del(20q) (5%) (Table 75-1) (94–97). As indicated earlier, these common chromosome abnormalities are not specific for MDS as they are also commonly detected in other myeloid disorders, that is, AML and MPN. However, the presence of some of these chromosome abnormalities, along with a refractory anemia, is considered presumptive evidence for MDS, even if there is no morphological evidence of dysplasia in the bone marrow cells (6). Del(5q) and -7/del(7q) (up to 40–50% each) are particularly characteristic in therapy-related MDS associated with alkylating agents and/or radiation therapy (98). Only a few chromosome translocations, for example, t(3;21), inv(3)/t(3;3), t(6;9), and t(11;16) are observed in primary MDS and in therapy-related MDS (t-MDS). Recently, genomic microarray studies have discovered multiple lesions through the whole genome analysis in MDS, including many small cryptic genomic imbalances, such as deletions and duplications, and CN-LOH in low- and high-grade MDS with and without clonal cytogenetic abnormalities. Some of these novel genomic abnormalities detected by microarray in MDS have been implicated in disease progression and the pathobiology of MDS (99–102).

### 75.6.1 Del(5q)

Deletion of the long arm of chromosome 5 [del(5q)] is one of the most common chromosomal abnormalities in MDS, ranging from 10 to 20% in de novo MDS to 40% in t-MDS/AML. An isolated del(5q) has been included in the WHO classification system of MDS to



**TABLE 75-1 WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues****CHRONIC MYELOPROLIFERATIVE DISEASES**

Chronic myelogenous leukemia  
 Chronic neutrophilic leukemia  
 Chronic eosinophilic leukemia/hypereosinophilic syndrome  
 Polycythemia vera  
 Chronic idiopathic myelofibrosis  
 Essential thrombocythemia  
 Chronic myeloproliferative disease, unclassifiable

**MYELOYDYSPLASTIC/MYELOPROLIFERATIVE DISEASE**

Chronic myelomonocytic leukemia  
 Atypical chronic myeloid leukemia  
 Juvenile myelomonocytic leukemia  
 Myelodysplastic/myeloproliferative disease, unclassifiable

**MYELOYDYSPLASTIC SYNDROMES**

Refractory anemia  
 Refractory anemia with ringed sideroblasts  
 Refractory cytopenia with multilineage dysplasia  
 Refractory anemia with excess blasts  
 Myelodysplastic syndrome, unclassifiable  
 Myelodysplastic syndrome associated with isolated del(5q) chromosome abnormality

**ACUTE MYELOID LEUKEMIA (AML)**

Acute myeloid leukemia with cytogenetic abnormalities  
   AML with t(8;21)(q22;q22), (*RUNX1/ETO*)  
   AML with inv(16)(p13;q22) or t(16;16)(p13;q22), (*CBFB/MYH11*)  
   Acute promyelocytic leukemia (AML with t(15;17)(q22;q12), (*PML/RARA*) and variants)  
   AML with 11q23 (*MLL*) abnormalities  
 Acute myeloid leukemia with multilineage dysplasia  
 Acute myeloid leukemia and myelodysplastic syndrome, therapy related  
   Alkylating agent related  
   Topoisomerase II inhibitor related  
 Acute myeloid leukemia not otherwise categorized  
   AML, minimally differentiated  
   AML without maturation  
   AML with maturation  
   Acute myelomonocytic leukemia  
   Acute monoblastic and monocytic leukemia  
   Acute erythroid leukemia  
   Acute megakaryoblastic leukemia  
   Acute basophilic leukemia  
   Acute panmyelosis with myelofibrosis  
   Myeloid sarcoma  
 Acute leukemias of ambiguous lineage

**PRECURSOR B- AND T-CELL NEOPLASMS**

Precursor B-lymphoblastic leukemia/lymphoma  
 Precursor T-lymphoblastic leukemia/lymphoma

**MATURE B-CELL NEOPLASMS**

Chronic lymphocytic leukemia/small lymphocytic leukemia  
 B-cell prolymphocytic leukemia  
 Lymphoplasmacytic lymphoma/Waldenström macroglobulinemia  
 Splenic marginal zone lymphoma  
 Hairy cell leukemia  
 Plasma cell neoplasms  
   Plasma cell myeloma  
   Plasmacytoma  
 Monoclonal immunoglobulin deposition diseases

**TABLE 75-1 WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues—cont'd****MATURE B-CELL NEOPLASMS—cont'd**

Heavy chain diseases  
 Extranodal marginal zone B-cell lymphoma (MALT lymphoma)  
 Nodal marginal zone B-cell lymphoma  
 Follicular lymphoma  
 Mantle cell lymphoma  
 Diffuse large B-cell lymphoma  
 Mediastinal (thymic) large B-cell lymphoma  
 Intravascular large B-cell lymphoma  
 Primary effusion lymphoma  
 Burkitt lymphoma/leukemia  
 Lymphomatoid granulomatosis

**MATURE T-CELL AND NK-CELL NEOPLASMS**

T-cell prolymphocytic leukemia  
 T-cell large granular lymphocytic leukemia  
 Aggressive NK-cell leukemia  
 Adult T-cell leukemia/lymphoma  
 Extranodal NK/T-cell lymphoma, nasal type  
 Enteropathy-type T-cell lymphoma  
 Hepatosplenic T-cell lymphoma  
 Subcutaneous panniculitis-like T-cell lymphoma  
 Blastic NK-cell lymphoma  
 Mycosis fungoides/Sézary syndrome  
 Primary cutaneous CD30-positive T-cell lymphoproliferative disorders  
   Primary cutaneous anaplastic large cell lymphoma  
   Lymphomatoid papulosis  
   Borderline lesions  
 Angioimmunoblastic T-cell lymphoma  
 Peripheral T-cell lymphoma, unspecified  
 Anaplastic large cell lymphoma

**HODGKIN LYMPHOMA**

Nodular lymphocyte-predominant Hodgkin lymphoma  
 Classical Hodgkin lymphoma  
 Nodular sclerosis Hodgkin lymphoma  
 Mixed cellularity Hodgkin lymphoma  
 Lymphocyte-rich classical Hodgkin lymphoma  
 Lymphocyte-depleted Hodgkin lymphoma

**IMMUNODEFICIENCY-ASSOCIATED LYMPHOPROLIFERATIVE DISORDERS**

Lymphoproliferative diseases associated with primary immune disorders  
 Human immunodeficiency virus–related lymphomas  
 Post-transplant lymphoproliferative disorders  
 Methotrexate-associated lymphoproliferative disorders

**HISTIOCYTIC AND DENDRITIC CELL NEOPLASMS**

Histiocytic sarcoma  
 Langerhans cell histiocytosis  
 Langerhans cell sarcoma  
 Interdigitating dendritic cell sarcoma/tumor  
 Follicular dendritic cell sarcoma/tumor  
 Dendritic cell sarcoma, not otherwise specified

**MASTOCYTOSIS**

Mastocytosis  
 Cutaneous mastocytosis  
 Systemic mastocytosis  
 Mast cell sarcoma  
 Extracutaneous mastocytoma

define the 5q- syndrome which primarily occurs in older females with a very favorable prognosis and an excellent response to treatment with lenalidomide (103–105). In the 5q- syndrome, del(5q) occurs as a single abnormality, whereas it is more frequently included in a complex karyotype in high-grade MDS, such as RAEB. The deletion size of 5q in MDS is variable (106,107). Several genomic mapping studies have showed that deletion of 5q33.1 is uniquely involved in the 5q- syndrome with a good prognosis, whereas a deletion of 5q31 is frequently observed in t-MDS and more aggressive disease (108–110). More than two dozen candidate genes that are located in the critical deletion region on 5q31 were screened for mutations in many primary myeloid leukemia cells. Nevertheless, none of these candidate genes showed any inactivating mutations in the remaining alleles. Thus, it has been proposed that haploinsufficiency or epigenetic regulation in conjunction with del(5q) could be involved in the pathobiology of MDS (111).

Recently, the RPS14 protein that is required for the processing of 18S pre-ribosomal RNA was implied as a candidate gene for the 5q- syndrome through gene expression profiling, knockdown experiments using RNA interference, and forced expression studies (112). Down-regulation of *RPS14* in CD34+ bone marrow cells blocks the differentiation of erythroid cells, and increases apoptosis in differentiating erythroid cells in vitro (113–115). The ribosomal processing defect caused by haploinsufficiency of RPS14 in the 5q- syndrome is highly analogous to the functional ribosomal defect seen in Diamond–Blackfan anemia. It has been shown that loss of *RPS14* cooperates with the haploinsufficiency of microRNAs miR-145 and miR-146a near the *RPS14* gene; both miR-145 and miR-146a are abundant in hematopoietic stem and progenitor cells (116). The Toll-interleukin-1 receptor domain-containing adaptor protein and tumor necrosis factor receptor-associated factor-6 are respective targets of these microRNAs, implying inappropriate activation of innate immune signal pathways in MDS with del(5q).

### 75.6.2 Del(7q)/-7

Deletion of the long arm or loss of a whole chromosome 7 occurs in about 10% of de novo MDS and in up to 50% of t-MDS alone or as part of a complex karyotype (92,98). In many patients with t-MDS/AML, del(7q) or -7 coexist with del(5q). Notably, loss of chromosome 7 is also the most common chromosome abnormality in children with MDS, and in inherent bone marrow failure syndromes. Similar to the status of del(5q), several critical deletion regions were defined in the long arm of chromosome 7, and various candidate genes were analyzed for an inactivating mutations in the remaining alleles; none was positive (117). Patients with abnormalities of chromosome 7 are at high risk of disease progression and have a poor prognosis (97).

### 75.6.3 Trisomy 8

Trisomy 8 is seen in <10% of patients with MDS, often as a part of a complex karyotype. CD34 cells from MDS patients with trisomy 8 express early apoptotic markers but avoid programmed cell death by up-regulation of anti-apoptotic proteins, including MYC and CD1, and thus may result in a selective advantage over normal hematopoietic precursors (118).

### 75.6.4 Del(20q)

Del(20q) of various size occurs in <5% of MDS and is associated with the morphological features of hypogranulated and vacuolized neutrophils and neutrophil erythrophagocytosis (4). Del(20q) is associated with a favorable prognosis if detected as the sole chromosomal abnormality. In chromosome analysis, the del(20q) usually involves a large segment of the long arm (119,120). Genomic SNP microarray analysis defined two common deletion regions of 2.5 and 1.8 Mb at 20q11.23 and 20q13.12, respectively, in a series of MDS patients with a cytogenetic del(20q) (121). Recently, L3MBTL1 polycomb protein, a candidate tumor suppressor located within the critical deletion region on 20q myeloid disorders, has been shown to interact with CDC45, MCM2-7, and PCNA, components of the DNA replication machinery, and is required for normal replication fork progression. Depletion of L3MBTL1 from human cells causes replicative stress, DNA breaks, and activation of the DNA damage response. Thus, haploinsufficiency of L3MBTL1 likely contributes to the development of 20q deletion-associated hematopoietic malignancies, such as MDS, AML, and MPN, by inducing replicative stress, DNA damage, and genomic instability, and by promoting erythroid differentiation (122,123).

### 75.6.5 Prognostic and Diagnostic Significance of Chromosome Abnormalities in MDS

As indicated above, the most common chromosome aberrations in MDS are not specific for any MDS groups; they are also present in other myeloid disorders. However, these chromosome abnormalities are prognostically associated with survival and progression of MDS to AML. The prognostic significance of chromosomal abnormalities in MDS is incorporated into several prognostic scoring systems, such as the International Prognostic Scoring System, the WHO Prognostic Scoring System, and the MD Anderson Cancer Center MDS model (124–128). Patients with a favorable outcome have normal karyotypes, -Y alone, del(5q) alone, or del(20q) alone; those with a poor outcome have complex karyotypes ( $\geq 3$  abnormalities) or chromosome 7 abnormalities, and patients at an intermediate risk have other abnormalities. The median survival times of patients

within these three groups were 3.8, 0.8, and 2.4 years, respectively, and the median intervals for 25% of the patients to undergo evolution to AML were 5.6, 0.9, and 1.6 years (124). In addition, patients with abnormalities involving 3q26.2 and/or over-expression of *EVI1*, and trisomy 11 appear to have a significantly inferior survival (95,129–133). In combination with other clinical and pathological features, detecting chromosome abnormalities provides critical risk-stratification guidelines in deciding appropriate treatment protocols and determining disease prognosis in patients with MDS (130).

The diagnosis of MDS is not always straightforward in some cases. Cytogenetic analysis will provide presumptive evidence in establishing a diagnosis of MDS with detection of certain MDS-related chromosome abnormalities, when patients with refractory cytopenia suspected to have MDS lack diagnostic morphologic features in bone marrow (3,6). These unique chromosome aberrations include unbalanced abnormalities of loss or deletion of chromosome arms in 5q, 7q, 9q, 11q, 12p, 13q, 17p and *idic(Xq)*, and balanced translocations and inversion, such as *t(1;3)*, *t(2;11)*, *t(3;21)*, *inv(3q)*, *t(6;9)*, and *t(11;16)* (Table 75-1) (3). Therefore, all patients with unexplained anemia and pancytopenia should be studied by cytogenetic analysis of bone marrow samples.

### 75.6.6 Gene Mutation in MDS

In addition to the deletions and gains of chromosome material described above, various mutations in several genes, such as *TET2* and *KRAS*, have been detected in MDS. These gene mutations are often related to epigenetic regulation through DNA methylation, histone acetylation, and phosphorylation of tumor suppressor genes and oncogenes. DNA methylation is a mechanism of disease progression to AML and is a prognostic marker and predictor of treatment response in patients with MDS (134,135). In several large series of studies, these gene mutations defined some unique groups of MDS, and were associated with various prognoses. Mutations in *RUNX1*, *TP53*, and *NRAS* are most strongly associated with a poor prognosis, and an increased blast component in bone marrow. Recently, DNA hypomethylating agents that inhibit DNA methyltransferases (DNMTs) have demonstrated activity in MDS and have been incorporated in the initial therapy of many patients with MDS. Together, chromosome abnormalities (particularly deletions) and gene mutations provide insight into the pathobiology of MDS, its progression to AML, and possible new strategies for treatment (102,136,137).

**75.6.6.1 *TET2*.** Ten–eleven translocation (*TET*) family genes encode for proteins that participate in the epigenetic control of DNA expression through demethylation in epigenetic programming, embryonic stem cell maintenance, and early development (138). Somatic mutations, such as frame shift, nonsense, and missense

mutation in *TET2*, occur in 20–25% of myeloid disorders, including MDS, AML, and MPN (37,139,140). *TET2* mutations are particularly common in about 50% of CMML, a mixture of myelodysplastic and myeloproliferative disease with a propensity to progress to AML. Loss-of-function mutations of *TET2* result in increased methylation and silencing of genes that are normally expressed. Recently, mouse model studies have showed that inactivation of *TET2* enhances hematopoietic stem cell (HSC) self-renewal and promotes the development of myeloproliferative disorders (140–142). Inactivation of *Tet2* in mouse perturbs both early and late steps of hematopoiesis including myeloid and lymphoid differentiation in a cell-autonomous manner, endows the cells with competitive advantage, and eventually leads to the development of malignancies. *TET2* mutations are associated with a favorable prognosis in MDS (139). Interestingly, *TET2* mutations can coexist with other pathogenetically relevant mutations in MDS and AML, such as *KIT* D816 V (130).

**75.6.6.2 *RUNX1/AML1*.** *RUNX1/AML1* encodes the DNA binding unit of the heterodimeric CBF that is a critical regulator of definitive hematopoiesis. *RUNX1* mutations, including amplification and point mutations, occur in 10–15% of de novo MDS, and in 15–30% of t-MDS/AML (143,144). Mutations of *RUNX1* are more frequent in high-grade MDS, and often coexist with activating *RAS* mutations, and *-7/del(7q)*. *RUNX1* mutations are associated with poor prognosis and a short overall survival (145).

**75.6.6.3 *TP53*.** The *TP53* tumor suppressor gene encodes an important checkpoint protein that mediates cell cycle arrest in response to cellular stresses and DNA damage. In 10–30% of MDS, *TP53* mutations are present at the time of diagnosis (146,147). Abnormalities in *TP53* are more common in patients with t-MDS (126,148). In addition to various missense mutations in one allele, loss of wild-type *TP53* allele, often seen as a consequence of abnormalities of 17p within a complex karyotype with *del(5q)*, is associated with resistance to treatment and rapid progression to AML, regardless of the IPSS risks score (126,149,150).

**75.6.6.4 *RAS*.** The *RAS* proto-oncogenes, including *HRAS*, *KRAS*, and *NRAS*, play an important role in signal transduction and hematopoiesis (151–154). Mutations of *RAS* have been identified in 10–35% of MDS and AML patients (155). The most common mutations in MDS are in the *NRAS* gene, followed by mutations in *KRAS* and *HRAS*. *RAS* mutations in MDS are associated with loss and deletion of chromosome 7, and with higher incidence of transformation to AML, and a short survival.

**75.6.6.5 *ASXL1*.** The *ASXL1* (additional sex comb-like 1) gene at 20q11.1 belongs to the enhancer of trithorax and polycomb genes that both activate or repress *Hox* genes depending on cellular context. It contains several nuclear receptor binding motifs and

a carboxy-terminal plant homeodomain (PHD) (156). Inactivating mutations involving exon 12 of *ASXL1* are detected in 11–20% of MDS, and in other myeloid disease, such as AML, and MPN (91,157). Most mutations are frameshift mutations and others are missense mutations. *ASXL1* mutations are associated with a shorter overall survival in MDS and a quicker progression to AML (158).

**75.6.6.6 IDH.** Cytosolic and mitochondrial isocitrate dehydrogenases *IDH1* and *IDH2* catalyze oxidative decarboxylation of isocitrate and are involved in cellular defense of oxidative damage (159). Mutations in *IDH1* and *IDH2* oncogenes have recently been reported in 5–15% of MDS and AML (160). *IDH1* mutations are common in AML with mutated *NPM1* without *FLT3* mutations (see below), whereas *IDH2* mutations occur frequently in older patients and are associated with distinctive gene and microRNA expression profiles (159,160). The mutations result in DNA hypermethylation and alteration of gene expression, and are associated with a poor prognosis (161).

## 75.7 ACUTE MYELOID LEUKEMIA DE NOVO

With initial banding analyses, clonal chromosome abnormalities were detected in about 50–80% of patients with AML (7,9). The most frequent abnormalities are translocations t(15;17), t(8;21), inv(16), and a gain of chromosome 8 (Table 75-1). Specific structural rearrangements are closely associated with a particular subtype of AML as defined by the FAB Cooperative Group (FAB classification) (1). For most cases of AML, myeloid blast forms must account for at least 20% of the total cellularity of the bone marrow biopsy sample (4,6). Exceptions to this include leukemia with certain genetic abnormalities or myeloid sarcoma, which are considered diagnostic of AML without regard to the blast count, that is, t(8;21)(q22;q22)/*AML1-ETO*, inv(16)(p13.1q22)/t(16;16)(p13.1;q22)/*CBFB-MYH11*, and t(15;17)(q22;q21.1)/*PML-RARA*. In addition, four more chromosome abnormalities-associated subgroups were defined as AML with balanced chromosome translocations, that is, t(9;11)(p22;q23)/*AF9-MLL*, t(6;9)(p23;q34)/*DEK-NUP214*, inv(3)(q21q26.2)/t(3;3)(q21;q26.2); *RPN1-MDS1/EV11*, and t(1;22)(p13;q13)/*RBM15-MKL1*. These seven subtypes of AML are collectively identified in the 2008 WHO classification system as AML with recurrent genetic abnormalities. In addition, several other rare recurring chromosome translocations are typical in AML, including the t(8;16)(p11;p13) in both de novo and therapy-related AML involving *MOZ*, on chromosome 8 and the *CREBBP* gene at 16p13 (162), and t(8;22)(p11;q13) involving *MOZ* and *p300*. *CREBBP* and *p300* are highly homologous proteins both of which function as histone acetylases (163).

Over 30 recurrent translocations involving 11p15, all involving the *NUP98* gene, have been reported. Twenty-eight partner genes to *NUP98* have been identified including a number of homeobox genes such as *HOXA9* at 7p15, *PMX1* at 1q24, and *HOXD13* at 2q31 (7). Thus, *NUP98*, along with *MLL*, *ETV6* (*TEL*), and *RUNX1/AML1*, is among the rare genes that have numerous genetic partners with vastly different functions (164). Each of these balanced translocations or inversions results in a fusion gene encoding a chimeric protein that participates in leukemogenesis, but is not necessarily sufficient for the development of AML by itself.

### 75.7.1 t(8;21)(q22;q22) and the *AML1/ETO* Fusion

t(8;21)(q22;q22) was first identified in 1972, just before the t(9;22) (54). It occurs in about 8–10% of all karyotypically studied AML and in about 40% of all abnormal patients with acute myeloblastic leukemia with maturation (AML-M2) (8). The t(8;21) is the most frequent abnormality in children with AML, being reported in about 15% of karyotypically abnormal cases (22,153). t(8;21) is often accompanied by the loss of a sex chromosome (in 56%) and del(9q) (in 25%) (165). It results in the juxtaposition of 5' *AML1/RUNX1* and 3' *ETO* (also called *RUNX1T1*), leading to a fusion gene and a fusion or chimeric protein (166,167). The fusion gene is transcribed on the der(8) chromosome from telomere to centromere.

The *AML1* gene belongs to a family of transcription factors with homology to the pair-rule *Drosophila* gene, Runt. The *ETO* gene, also called *Nervy* in *Drosophila* because it is active in the brain, encodes a zinc-finger protein. The fusion gene includes the DNA binding runt homology region of the *AML1* gene and virtually the entire *ETO* gene (168,169). Genomic breakpoints in both *AML1* and *ETO* are clustered in several intron regions and co-localize with topoisomerase II cleavage sites, and DNA hypersensitive sites, implying that these chromatin structural elements are important in the formation of the t(8;21) (170). *AML1* heterodimerizes with the *CBFB* to form a transcription factor, the CBF. The *CBFB* gene is located at 16q22 and involved in the inv(16)/t(16;16). The *AML1/CBFB* transcription factor is critical to hematopoietic stem and progenitor cell growth, differentiation, and function. It binds directly to an enhancer core motif that is present in the transcriptional regulatory regions of a number of genes, including interleukin (*IL*)-3, *GM-CSF*, the *CSF1* receptor, myeloperoxidase, and neutrophil elastase (169,171). Mutant mice with loss of *AML1* or *CBFB* gene function are deficient in hematopoiesis, and die during embryogenesis of intracranial hemorrhage, indicative of the critical role of the *RUNX1/CBFB*-regulated target genes in the development of all blood lineages (171–173).



Transformation by the *AML1/ETO* fusion likely results from transcriptional repression of normal *AML1* target genes via aberrant recruitment of nuclear transcriptional co-repressor complexes to the *ETO* component of the fusion gene (174). Recent studies of the *AML1/ETO* protein fusion showed that it also involves abnormal histone deacetylation, and experiments in cell culture with histone deacetylase (HDAC) inhibitors led to the differentiation of leukemia cells with t(8;21) (175,176). In addition, the *AML1/ETO* fusion protein can directly generate dysplastic hematopoietic progenitors and activate expression of other genes (174). Thus, leukemogenesis by *AML1/ETO* probably results from both altered transcriptional regulation of normal *RUNX1/AML1* target genes and activation of new target genes that block programmed cell death and/or cellular differentiation pathways (174).

The t(8;21) identifies a morphologically and clinically distinct subset of AML (4). The median age in adults is approximately 25 years. The leukemia blasts have features of maturation with Auer rods in pink cytoplasm, and they express CD19 and CD56 in most patients. Clinically, patients with t(8;21) respond well to conventional chemotherapy, in particular to high dose cytarabine treatment, and this leukemia is characterized by a favorable prognosis in both adults and children with the overall 10-year survival of 61 and 80%, respectively (8,9,177). However, gain-of-function mutations of *KIT* involving exons 8 and 17 have recently been detected in AML with t(8;21) and inv(16)/t(16;16), and in mastocytosis, as well as in gastrointestinal stromal tumors and germ cell tumors (178). *KIT* is a member of the type III tyrosine kinase family and encodes a 145-kd transmembrane glycoprotein. Mutations of *KIT* have been shown to have adverse impact on the prognosis of CBF leukemia, with a significantly shorter event-free survival and overall survival rate in patients with *KIT* mutations compared with wild-type *KIT* patients (178).

*AML1* has been shown to be involved with more than 50 other translocation partners, including the t(3;21), which was first identified in CML patients and then in patients with MDS or therapy-related AML/MDS (153). The translocation involves genes at 3q26, *EAP/MDS1/EV11*, in a complex series of alternative fusions (153). Other common partner genes of *AML1* include *MTG16* at 16q24 and *TEL* at 12p13. Except for the fusions with *TEL*, *AML1* provides the 5' part of the fusion and the patients have myeloid leukemia. In contrast, when *TEL* is 5', the leukemia has a lymphoid phenotype (12). In addition, loss-of-function mutations in *AML1* are also responsible for the inherited leukemia syndrome, and familial platelet disorder with propensity to develop AML, and is common in various MPN and MDS (143,144).

### 75.7.2 Inv(16)(p13q22)/t(16;16)(p13;q22) and the CBFB/MYH11 Fusion

The inv(16) and t(16;16) is a clinical cytogenetic rearrangement that results in myelomonocytic (M4)

leukemia with abnormal eosinophils, and is observed in about 7% of newly diagnosed AML (179,180). AML with inv(16)/t(16;16) typically demonstrates monocytic and granulocytic differentiation with abnormal eosinophils in the bone marrow. Arthur and Bloomfield (1983) described a deleted number 16 [del(16)(q22)] in patients whose bone marrow contained an excess of eosinophils (8–54%) (181). We described patients who had M4 leukemia with eosinophils that showed unique morphologic changes, including large and irregular basophilic granules; one-third lacked increased eosinophils because the marrow had fewer than 5% eosinophils (182). In a most comprehensive study to date, inv(16) was observed in 4% of all AML cases where the cytogenetic data were available (8,9). This relatively common (25% of our AML-M4 patients have this aberration) but subtle chromosome aberration was probably undetected in the past, in part because of poor morphology. In children with AML the frequency of inv(16) ranges from 6 to 11%, and is seen in 19–36% of all patients with M4 (22). This chromosome abnormality has clinical implications because the median survival time was more than 65 weeks for AML patients with abnormal 16s, compared to 29 weeks for those with a normal 16 (182). This good response has been confirmed in a number of other studies (8,9,183).

The inversion and/or translocation results in a fusion between the *CBFB* at 16q22 and the myosin heavy-chain gene (*MYH11*) at 16p13 (184–186). Both genes are transcribed from centromere to telomere. The critical fusion is 5' CBFB-3' MYH11. As described earlier, CBFB forms a heterodimer with *AML1*, which directly binds to an enhancer core motif that is present in the transcriptional regulatory regions of a number of genes that are critical to myeloid cell growth, differentiation, and function. It is astonishing that both genes are involved in translocations that are common in AML, although the leukemia cells with t(8;21) and inv(16)/t(16;16) display different morphological features. Eosinophilia is seen in both *AML1/ETO* and *CBFB/MYH11*, suggesting that there may be common pathways that lead to leukemia with the t(8;21) and inv(16) (4). The CBFB protein increases the efficiency of heterodimerization and the affinity of *AML1* binding to DNA. The portion of *AML1* involved in the fusion contains the DNA-binding and heterodimerization motifs. Thus in the t(8;21), the fusion protein contains not only these motifs but also most of the *ETO* protein. Dimerization with CBFB is apparently unaffected. In the inv(16), *CBFB* is fused to *MYH11* creating the *CBFB/SMMHC* fusion protein. *MYH11* also has a dimerization motif; presumably CBFB binds to CBFA2, but it has an additional dimerization partner at the C-terminus binding to *SMMHC*. This *MYH11* protein contains a repeated alpha helical structure involved in myosin filament interactions and may be important in dimerization of the fusion protein in leukemia cells. The *CBFB/MYH11* fusion protein interferes

and represses the function of the CBF transcription factor, and in cooperation with other genetic mutations, such as *c-KIT* and *RAS* mutations, leads to leukemia.

Patients with *inv*(16)/*t*(16;16) have a good response to intensive chemotherapy with a rate of complete remission and 10-year overall survival of 92 and 55%, respectively (8). As described above, gain-of-function mutations of *KIT* will negatively affect on the prognosis of patients with *inv*(16)/*t*(16;16).

### 75.7.3 *t*(15;17)(q22;q12) and the PML/RARA Fusion

A structural rearrangement involving chromosomes 15 and 17 in APL was first recognized in 1977 (187,188). This rearrangement is unique to APL and is the basis for the diagnosis of APL in the WHO classification (3,4,6). The translocation results in a fusion between the *PML* (promyelocytic leukemia) gene on chromosome 15 and the retinoic acid receptor alpha (*RARA*) gene on chromosome 17 (189). The critical junction is located on the *der*(15) and consists of the 5' portion of *PML* fused to virtually the entire *RARA* gene, beginning with the *RARA* response element (190–193).

In the normal situation, *RARA* and retinoic receptor X form a heterodimer that binds to the appropriate DNA RA response elements of target genes. In the absence of ligands, the target genes are in the inactive state and a complex of repression proteins, including nuclear corepressor (NCOR), SIN3, and HDAC, binds to the heterodimer and prevents transcription. Ligands, such as retinoic acid or its derivatives, when present, bind the receptor at physiologic levels and, probably as a result of a conformational change in the heterodimer, displace the repressor complex and recruit transcriptional coactivators, including CREBBP/p300 and SRC1. This leads to transcriptional activation of target genes and differentiation of normal hematopoietic precursors. The *PML/RARA* fusion protein is insensitive to the normal ligands and recruits DNMT enzymes and HDACs to the target site. Transcriptional silencing results from the DNA methylation, hypoacetylation, and recruitment of methyl-CpG binding proteins and NCOR complexes (194,195). The *PML/RARA* fusion protein shows reduced sensitivity to retinoic acid in terms of dissociation of NCOR, a ubiquitous nuclear protein that mediates transcriptional repression. This could lead to persistent transcriptional repression, thereby preventing differentiation of promyelocytes. This effect can be overcome by ATRA, which acts as a ligand for the fusion protein, resulting in release of the HDAC repression complex from the *PML/RARA* fusion protein, with the resultant target gene transcription and maturation and then apoptosis of the APL cells (189,195,196). The fusion protein forms unusual *PML* bodies in the cytoplasm compared with no such bodies with normal *PML*; treatment with ATRA results in normalization of this pattern.

As with other genes discussed, *RARA* is involved in three other translocations with genes *PLZF* (11q23), *NPM1* (5q32), and *NUMA1* (11q13), and with *STAT5B* in a rearrangement within 17q21 (Table 75-1). Recently, a novel translocation *t*(4;17)(q12;q21) was cloned in a patient with JMML, which results in the fusion of the *FIP1L1* and *RARA* gene. These variant translocations are associated with atypical APL. Cells expressing *PLZF/RARA* fusion proteins are not sensitive to ATRA-induced differentiation, although they contain identical *RARA* domains to those of *PML/RARA*. This has been explained by demonstrating that *PLZF* itself functions as a transcriptional repressor through its interaction with the same complex of repression proteins, and this interaction is not affected by ATRA (189,195). Treatment with HDAC inhibitors, however, induces differentiation of *PLZF/RARA* cells in vitro. Therefore, the common mechanism of APL is probably recruitment of HDACs and the transcriptional repression apparatus to the fusion proteins, with the resultant loss of regulation of the target genes.

With the introduction of ATRA for treatment, APL became the first malignancy in which genotype-specific therapy was used. This treatment leads to maturation of leukemic cells in virtually all *t*(15;17) patients, but unfortunately the malignant clone persists and the patients relapse (197). ATRA is combined with cytotoxic drugs to eradicate the leukemic clone. This leads to a remarkable improvement in the overall survival of APL patients. However, patients with some of the variant translocations do not respond to ATRA, and so the precise karyotypic or molecular diagnosis is critical for proper treatment. More recently, treatment in newly diagnosed APL patients with arsenic trioxide (ATO), in combination with ATRA and chemotherapy, achieves an overall survival rate of 91%, in comparison to 60–80% with ATRA and chemotherapy in a 5-year follow-up study (19). Arsenic can directly bind the C3HC4 zinc-finger motif in the RBCC domain of *PML* and *PML-RARA*, resulting in their homodimerization and multimerization with enhanced interaction with the SUMO E2 conjugase Ubc9, facilitating subsequent sumoylation, ubiquitination, and proteasomal degradation (198). Thus, in APL, the combination of ATO and ATRA and chemotherapy has dramatically enhanced therapeutic efficacy and become a frontline treatment option in this otherwise very aggressive disease.

### 75.7.4 11q23 Aberrations and the MLL Gene

The close association of translocations—or, less often, deletions—of the long arm of chromosome 11 (11q) and acute monoblastic leukemia (M5) was observed in multiple studies, especially in children with monoblastic leukemia (199,200). The use of various DNA probes to map the rearrangements of the long arm of chromosome

11 has resulted in the cloning of several different breakpoints in band 11q23, one of which accounts for about 95% of the translocation breakpoints in this band. These translocations have a number of unique clinical features: (1) More than 100 different recurring translocations that involve 11q23, of which 64 partner genes have been cloned in acute leukemia, especially the monoblastic and myelomonocytic types (201). (2) These translocations occur in both myeloid and lymphoid leukemia. The most common translocations in AML are t(9;11), t(6;11), and t(11;19) (q23;p13.1); in ALL they are t(4;11) and t(11;19) (q23;p13.3) (17,202–206). (3) Translocations involving 11q23 have an unusual age distribution, accounting for about three-quarters of the chromosomal abnormalities observed in leukemia cells of infants under 1 year of age. These leukemias are either myelomonocytic or monocytic AML or ALL (207–209). (4) These translocations are seen in leukemias that develop in patients with a primary cancer who previously received treatment with drugs targeting topoisomerase II (206,210,211).

The gene involved in virtually all 11q23 translocations is *MLL*, for myeloid–lymphoid or mixed lineage leukemia (212,213). The *MLL* gene contains regions of homology to the *Drosophila trithorax* gene, especially in the zinc-finger region and the most 3′ segment; as a consequence, it is also called *Htrx*, *HRX*, or *ALL1* (212–214). The breakpoint in *MLL* in virtually all patients occurs in an 8.3-kb breakpoint cluster region just 5′ of the zinc fingers; the conserved junction is on the der(11) chromosome (205). This region contains a number of Alu repeat sequences, as well as one in vivo topoisomerase II cleavage site and an adjacent DNase I hypersensitive site (215–217). By mapping the translocation breakpoints, we and others have shown that in de novo leukemia the breaks appeared to be located preferentially in the 5′ half of the *MLL* breakpoint cluster region, whereas treatment-related leukemias and infant leukemias break more frequently in the 3′ half of the breakpoint cluster, which contains the scaffold attachment region (218). As in *Drosophila*, where the gene is called *trithorax*, *MLL* appears to regulate a number of *HOX* genes, especially in the *HOXA* cluster as well as *MEIS1* by directly binding to promoter sequences (219). These genes, in turn, regulate the expression of genes related to formation of somites during embryonic development, as well as hematopoiesis.

The mechanisms that target *MLL* to specific promoters remain unclear, although at present, we understand some of the functional domains of *MLL*. The *MLL* protein is proteolytically cleaved into two fragments in the cytoplasm (220,221). The N-terminal portion, containing the AT hook domains, the DNMT binding region, and the PHD fingers, functions as a transcriptional repressor through recruitment of HDACs (222). The C-terminal fragment contains the trans-activation domain and the SET domain. When the N-terminal fragment heterodimerizes with the C-terminal fragment

the complex has a transcriptional activation effect. The activation effects are thought to involve histone modifications and chromatin remodeling through recruitment of histone acetyltransferases and by the intrinsic histone methyltransferase, that is, histone H3 lysine 79 (H3K79) activity of the SET domain to interact with multiple *MLL* fusion partners including AF9, ENL, AF10, and AF17 (223–228). At least four groups of *MLL* partner genes with various putative functions have been identified, that is, nuclear proteins with putative DNA-binding functions AFF1 (AF4), MLLT3 (AF9), MLLT10 (AF10), MLLT1 (ENL), and OCEL1 (ELL); histone acetyltransferases CREB binding protein (CBP) and EP300 (P300) (226,229); FOXO (AFX) and MLLT4 (AF6) with the coil–coil oligomerization domains, and the Septin family genes SEPT2, SEPT5, SEPT6, SEPT9, and SEPT11 that interact with cytoskeletal filaments during the mitosis. The unifying features of many of the partner genes are their function in regulation of the rate of transcriptional elongation (211). Some, such as ELL, are involved directly in regulating and reducing “pausing” of the transcription complex; others, such as ENL and its homologous protein, AF9, recruit many factors required for the *MLL* complex including other partner proteins, such as AF4 and AF10. *MLL* expression appears to be closely regulated by a number of miRNAs, including miR17-92 (46) and miR196b (45).

The clustering of the breaks makes it possible to use a single cDNA probe spanning the breakpoint and a single restriction enzyme digest to detect the rearrangement in virtually any patient on Southern blot analysis. For the common breakpoints in which the other partner chromosome has been cloned, one can also use RT-PCR (230). In fact, multiplex PCR has been developed to screen for the common translocations simultaneously (231). Partial tandem duplication (PTD) within the *MLL* gene has been described as a novel genetic alteration in 90% of AML with trisomy 11 and in 11% of patients with AML and normal karyotype, and it was shown that this rearrangement is associated with a poor prognosis (232,233). PTD within *MLL* are found in blood and bone marrow from normal individuals as well (234,235). However, whether the origin and the composition of PTD within *MLL* in these individuals are the same as in AML patients or are distinct remains a matter of controversy.

Regardless of myeloid or lymphoid lineage involvement, leukemia patients with 11q23/*MLL* rearrangements have a very dismal prognosis, particularly infant ALL patients with the t(4;11) (8,23,24,236–238). However, patients with t(9;11)(p22;q23) tend to have an intermediate response to standard therapy (8). In the 2008 WHO classification of hematopoietic and lymphoid tissues, the t(9;11) is listed as one of the subcategories of AML with recurring genetic abnormalities (Table 75-1) (4). Patients with the t(9;11)(p22;q23) had rates of complete remission and 10-year survival of 84 and 39%, respectively (8,22). In children AML with 11q23/*MLL*



rearrangement, t(6;11), t(10;11), and t(11;19) are associated with a very poor prognosis, whereas patients with t(1;11) and t(9;11) display a better outcome (8,22,236).

### 75.7.5 Inv(3)(q21q26.2)/t(3;3)(q21;q26.2) with Thrombocytosis and Over-Expression of EVI1

The t(3;3) and inv(3) are one of the rare recurring chromosome abnormalities, occurring in only 1% of AML cases (8,239). It is detected in de novo AML, in CML in blast crisis, and in therapy-related MDS/AML (Table 75-1). Abnormalities involving 3q26.2 in AML are associated with thrombocytosis in the peripheral blood and increased atypical megakaryocytes in the bone marrow (132,239).

The inv(3)/t(3;3) results in the activation of the *EVI1* gene. Activation of *EVI1* in hematopoietic cells can occur by juxtaposition of the gene to enhancer elements of the ribophorin (*RPN1*) gene located at 3q21 in inv(3) and t(3;3) (240). The genomic breakpoints in *EVI1* are either 5' of the gene in the t(3;3) or 3' of the gene in the inv(3) (241). *EVI1* is a zinc-finger transcription factor that contains a seven-zinc-finger domain at the N-terminal end, a three-finger domain in the central part of the molecule, and an acidic domain distal to the second group of zinc fingers (153). Depending on its binding partners, *EVI1* can act as a transcriptional activator to promote the proliferation of HSCs through binding to GATA2 or as a transcriptional repressor inhibiting erythroid differentiation when it binds to GATA1. Abnormal over-expression of *EVI1* can be detected in patients with myeloid leukemia and a normal karyotype, suggesting that inappropriate activation of this gene occurs through various mechanisms (133,242). All patients with inv(3q) or t(3;3) are associated with a poor prognosis. As the most common additional aberration to inv(3q)/t(3;3), loss of chromosome 7 contributes to the adverse prognostic impact on relapse-free survival and overall survival (8).

### 75.7.6 t(6;9)(p22;q34) and the DEK/NUP214 Fusion

t(6;9)(p22;q34) is detectable in less than 1% of newly diagnosed AML and is characterized by increased basophilia, single or multi-lineage dysplasia in adult patients, variable FAB morphology, and a high incidence (70%) of FMS-like tyrosine kinase 3 (*FLT3*) gene internal tandem duplications (ITD) (4,8,22,243). The translocation results in the juxtaposition of *DEK* on chromosome 6 with *NUP214* (also known as *CAN*) on chromosome 9. *NUP214* is an essential component of the nuclear pore complex at the nuclear envelope and is required for proper nucleocytoplasmic transport. The *DEK* protein is a major component of the chromatin to modify the DNA structure by introducing supercoils. This results in the creation of a nucleoporin fusion protein that acts

as a transcription factor and also alters nuclear transport. Notably, the *NUP214* gene is fused to *ABL1* in episomal amplification in T-cell ALL (see below in T-ALL). Patients with the t(6;9)(p23;q34) have a poor outcome with standard therapy. In a recent analysis of 5876 younger adults with newly diagnosed AML, the complete remission rate and 10-year survival were 88 and 27%, respectively in the patients with t(6;9) (8). The poor prognosis is likely also due to the high incidence of *FLT3*-ITD mutations (243).

### 75.7.7 t(1;22)(p13;q13) and the RBM15-MKL1 Fusion

t(1;22) is a very rare entity (<0.5%) exclusively in newly diagnosed AML (8,244). It typically presents with megakaryoblastosis occurring in infants, and is associated with AML-M7 in young children, most often without Down syndrome and prior MDS or transient myeloproliferation (245). This translocation involves the RNA-binding motif protein-15 (*RBM15*, also called *OTT*) at 1p13 and a DNA-binding motif protein known as megakaryocyte leukemia-1 *MKL1* (also known as *MAL1*) at 22q13. *RBM15* belongs to the *SPEN* (Split-end) family of proteins that function as repressors in various signaling pathways and may bind to RNA through interaction with spliceosome components (246). *MKL1* is involved in the normal production of platelets (247). The *RBM15-MKL1* fusion protein shares many features with the *MLL* fusion in infant leukemia, and is assumed to participate in the modulation of chromatin organization through the binding of AT-rich DNA sequences, recognized by the AT hook motif in *MLL* fusions and by the scaffold attachment factor box in the *OTT-MAL* fusion, in *HOX*-induced differentiation, or extracellular signaling pathways (248). The prognostic significance of t(1;22) in AML treated with modern therapy is not clear (244).

### 75.7.8 Mutations of NPM1, FLT3, and CEBPA in AML with Normal Karyotypes

As noted earlier, recurring chromosome translocations, inversions, and chromosomal gains and losses are detectable in 50–80% of de novo AML and up to 92% of t-AML/MDS. The remaining patients with AML will have a normal karyotype by conventional cytogenetic analysis. This is a very heterogeneous group of patients with variable age, morphological features, and clinical course. In general, AML patients with a normal karyotype have an intermediate prognosis (8,177).

Using genomic microarray analysis, and other molecular techniques, many novel gene mutations have been identified in AML with normal karyotypes (NK-AML) and are associated with various prognoses. Identification of these mutations provides new insights into the pathogenesis of this group of NK-AML, and it also may



provide novel therapeutic targets (164). PTD of *MLL* is detected in 5–11% of NK-AML and up to 90% of AML with trisomy 11, and is associated with reduced remission duration (249). DNA methyl transferase and/or HDAC inhibitors may have some promise in NK-AML with *MLL* PTD. ITD and tyrosine kinase domain (TKD) mutations of *FLT3* occur frequently in AML with t(6;9) and in APL with t(15;17), and are particularly common in NK-AML (25–34%); they are associated with reduced remission duration and survival in adult NK-AML, especially in the absence of the wild-type allele (164). *FLT3* is a member of the class III receptor tyrosine kinase family, and plays an important role in proliferation, survival, and differentiation of hematopoietic progenitor cells. Brain and acute leukemia, cytoplasmic (*BAALC*), located at 8q22.3, encodes for a cytoplasmic protein with yet unknown function, and its high-expression adversely impacts outcome in adult NK-AML. In contrast, mutations in CCAAT/enhancer binding and protein alpha gene (*CEBPA*) are detected in about 15–18% of NK-AML, and are associated with improved remission duration and survival (164). More recently, patients with *CEBPA* double mutations including both frameshift mutations in the N-terminal and in-frame insertions or deletions in C- and N-terminals, frequently occurring on different alleles, have defined a distinctive disease entity with a very favorable prognosis. *NPM1* encodes for nuclear-cytoplasmic shuttling phosphoprotein with pleiotropic functions. *NPM1* mutations are detected in 25–35% of AML, and in 45–64% of NK-AML, and are associated with good prognosis, in particular in the absence of *FLT3*-ITD (250). AML with *NPM1* mutations show a distinct gene expression profile that is characterized by up-regulation of *HOX* genes (251). In addition, mutations of *ERG*, *EVII*, *MN1*, and *WT1* are associated with a poor prognosis in AML (4). Both AMLs with *NPM1* and *CEBPA* mutations are included as a provisional disease entity in the 2008 WHO classification, and detection of *NPM1*, *CEBPA*, and *FLT3* mutations has entered clinical practice and affects diagnosis, risk assessment, and guidance of therapy (164).

## 75.8 AML AND MDS ASSOCIATED WITH PRIOR CYTOTOXIC TREATMENT

A distinctive disorder of bone marrow morphology and function that terminates in MDS or in AML has been recognized as a late complication of cytotoxic therapy used in the treatment of both malignant and non-malignant diseases (252,253). Characteristic non-random chromosome abnormalities are commonly observed in the bone marrow cells of patients with t-MDS/t-AML. These abnormalities differ in their type and frequency from those noted in AML developing *de novo*. In general, two distinct subtypes exist based on their prior treatment for primary cancer or disease, clinical presentation, and chromosome abnormal patterns, although the 2008 WHO

classification of myeloid neoplasms and acute leukemia no longer includes subcategories of these diseases based on previous treatment types (4). In patients with previous treatment with alkylating agents and/or irradiation, both of which are mutagenic, the disease occurs with latency of 4–6 years after the treatment for primary disease, and presents with an insidious MDS phase prior to t-AML. Abnormalities of chromosomes 5 and/or 7 are most common occurring in almost 70% of t-MDS/t-AML patients. Analysis of the data from patients with a deletion of the long arm of chromosome 5 has identified a critically deleted region at 5q31 that was consistently observed in every patient (98,107). Genes for a number of growth factors or growth factor receptors have been mapped to this band. A number of candidate tumor suppressor genes have been screened, but at present none has been shown to play a role in leukemogenesis (see Section 75.6.1) (110).

The other subgroup of t-AML/MDS, cancer patients have been treated with drugs that inhibit the ligase function of topoisomerase II (epipodophyllotoxins, anthracyclines, etc.), resulting in DNA with double-strand breaks. The leukemias that develop as acute disease with no previous MDS stage in these patients have a different karyotypic pattern, namely translocations involving the *MLL* gene at 11q23 and the *AML1* gene at 21q22 (94,252,254–258). Virtually all patients with t(11;16) (q23;p13) that involves the *MLL* and *CREBBP* genes have treatment-related hematologic disorders induced by exposure to topoisomerase II inhibitors (210,259). The genomic breakpoints in t-AML often coincide with topo II cleavage sites, DNase I-hypersensitive (HS) sites, and scaffold attachment regions, implying that these chromatin structural elements may influence the location of these translocation breakpoints (215–217,260–262). Therapy-related APL with t(15;17)(q22;q21) is particularly associated with exposure to mitoxantrone and epirubicin; both preferentially induce topoII-mediated DNA damage in a “hotspot region” within the *PML* gene. Whether DNA cleavage at this site can be the initiating event for all *PML* translocations is unclear, but some treatment-related leukemia cases had breakpoints within a few hundred nucleotides of the cleavage site (218,263,264). DNA topoisomerase II inhibitors can be found in some fruits and vegetables, soybeans, tea, cocoa, wine, pesticides, and specific derivatives of benzene (260). In fact, some bioflavonoids (quercetin, ellagic acid, genistein), which are advocated for adult consumption because of their supposedly cancer preventive properties, are potent topoisomerase II inhibitors. Nearly 80% of infants diagnosed with leukemia show an abnormality involving the *MLL* gene; it cannot be excluded that exposure of pregnant women to substances that are identified as topoisomerase II inhibitors might be critical in the development of acute leukemia in infancy (265). In fact, Greaves’ group performed genomic cloning of the t(4;11) in three children with ALL (266) They were able to obtain genomic DNA from the Guthrie filters of

newborn blood from these children. Using the appropriate genomic probes for the leukemic junction, they could show that the t(4;11) was present in all children at birth, indicating that leukemia developed in utero (267).

## 75.9 B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

Although the correlation of cytogenetic changes with morphology in AML led to the identification of the specific associations described above, this correlation was not useful in B-cell ALL, except for the t(8;14) and its variants in L3, B-cell ALL. However, with the widespread use of precise immunophenotyping, the correlation of certain chromosome rearrangements with specific immunologic subsets of ALL has been established (4).

ALL is the most frequent leukemia in children, with 85% being of the B lineage. A better understanding of the complex interrelation of the disease and patient characteristics with therapeutic intervention changed the prognosis in children with ALL. Long-term event-free survival, which is virtually synonymous with cure, which before 1965 was only 5%, now exceeds 80% (23,268,269). This is not the case in adult patients, where cure rates are only 20–40%. These striking differences in treatment response, as well as the new information regarding the clinical outcome of different genetic abnormalities in patients, support the theory that childhood and adult ALL represent two different subsets of the disease. For many years, metaphase chromosomes from ALL patients had poor morphology with indistinct bands, making an accurate analysis difficult. Thus, there were fewer reports of chromosome patterns in ALL than in AML. It was rigorously demonstrated for the first time at the Third International Workshop on Chromosomes in Leukemia (1981) that the karyotype is an important independent prognostic factor in ALL; this was confirmed in children by two major studies conducted in 1997 (61,269,270). The most useful prognostic indicators in ALL are age, white blood cell (WBC) count, immunophenotype, MRD detection, and karyotype (25,271–274). The incidence of chromosome abnormalities are sharply different in childhood and adult ALL, that is t(4;11) is most common in infant younger than 12 months, and t(12;21) and hyperdiploid karyotypes are common in pediatric ALL, and they are rare in adult ALL, whereas t(9;22) is frequently observed in adult patients and is uncommon in the childhood patients (275–278). In general, a hyperdiploid clone, t(12;21), and t(1;19) are associated with a favorable prognosis, and t(9;22) and some gene mutations and deletions, that is *CRLF2* and *PAX5*, are predictable for a poor prognosis (33,36,101,237,277,279–284).

### 75.9.1 t(9;22)(q34;q11.2)

The Ph chromosome is the most frequent rearrangement in 25% of adult ALL. However, it occurs in only

2–5% of childhood ALL (23,283). At the cytogenetic level, the breakpoints appear identical to those in CML; molecular analysis indicates that the breakpoint in the *BCR* gene on chromosome 22 is more proximal in about 50% of adult and 80% of childhood patients with Ph+ ALL. We also successfully used FISH in combination with morphological observation for distinguishing Ph-positive ALL from the lymphoid blast phase of CML on the basis of lineage involvements of cells: patients with CML had a multilineage disease, and in ALL it was lymphoblast restricted (64). There are two distinct molecular subgroups of ALL with t(9;22). In approximately 30–50% of adult patients, the molecular rearrangement is identical to that observed in CML, that is the t(9;22) produces a chimeric gene that encodes for a 210-kd fusion protein, and the Ph chromosome is present in both myeloid and lymphoid cells, suggesting that they actually are cases of CML in lymphoid blast crisis. In the remaining patients, the breakpoint in the *BCR* gene occurs in the m-bcr, and results in smaller fusion proteins (185–190 kd) (285–287). The Ph chromosome is restricted to myeloid cells, and disappear in hematologic remission, indicative of the origin of the Ph translocation in a more restricted, perhaps a committed B-lymphoid, progenitor (288). Both the 210- and 185–190-kd fusion proteins participate in constitutive signaling via the RAS pathway of signal transduction. Transgenic mice transplanted with p185 *BCR/ABL1* develop tumors more rapidly than those transplanted with p210 *BCR/ABL1* (287,289,290).

Children with Ph-positive ALL respond poorly to available therapy at diagnosis or in relapse (291). One exception is a subset of children with Ph-positive ALL and low leukocyte counts at presentation or good initial responses to prednisone therapy who appear curable with intensive chemotherapy alone (25,283). A similarly poor prognosis was reported in adults where, despite high remission rates comparable to those of Ph-negative ALL, remission duration and survival times were short (284). In the MRC/ECOG clinical trial, the 5-year event-free and overall survival rates in Ph-positive ALL patients were 16% and 22%, respectively, in comparison with 36% and 41% in those without the t(9;22) (101). The use of tyrosine kinase (TK) inhibitors, for example, Imatinib, has revolutionized the treatment of CML with a superior outcome of 97% complete hematological remission and 87% of complete cytogenetic remission in a 5-year follow-up study (20). Imatinib, and the second generation TK inhibitors, such as dasatinib and nilotinib, have been used as the front-line treatment options in Ph-positive ALL, in combination with chemotherapy, and have showed a significant improvement in complete remission (95%) and overall survival (over 50% with 3-years follow-up), although an improved treatment regimen is needed to overcome the frequent resistance to Imatinib and to improve the remission durability (292).

### 75.9.2 t(12;21)(p13;q22)

Molecular analysis has proved indispensable for identifying certain prognostic and therapeutically important genetic subtypes of ALL, for example, cases defined by the *TEL/AML1* (*ETV6/RUNX1*) fusion gene owing to the cryptic translocation t(12;21)(p13;q22) (293–298). The t(12;21) is present in about 25% of childhood ALL (274). However, because of the marked similarity of chromosomal segments involved in this rearrangement, it is essentially undetectable by conventional cytogenetic analysis. Only FISH or PCR techniques can reliably detect it (294,299–301).

The t(12;21) results in fusion of the 5' HLH domain of *ETV6* with the 3' DNA-binding and transactivation domain of *AML1/RUNX1* (295). The *ETV6* protein contains a helix–loop–helix (HLH) protein dimerization domain and an ETS DNA binding domain, and it functions as a transcriptional repressor (280,302). The *RUNX1* protein (*AML1/CBFA2*) is a member of a family of transcription factors with homology to the pair-rule *Drosophila* gene, *runt*. *RUNX1* heterodimerizes with *CBFB* (also known as *PEBP2 beta*) and form a transcription factor *CBF* [see AML with t(8;21) and inv(16)/t(16;16)]. The *ETV6/RUNX1* inhibits transactivation of gene expression by the normal *RUNX1* protein, which requires the HLH domain of *ETV6* (303,304). In more than a half of ALL patients with the t(12;21), the *ETV6* allele on the other chromosome 12 homolog is also deleted, indicative of a tumor suppressor effect of *ETV6* (304).

The *TEL/AML1* fusion sequences were found at birth in six of nine patients with childhood ALL (2–5 years of age) (305). These findings indicate that not only translocations had occurred in utero, but also cells with the translocation had proliferated at birth to be detectable with RT-PCR. As noted for other translocations, a number of years passed before the development of a fully malignant phenotype. Analysis of *TEL/AML* genomic sequences suggests that these events arise from error-prone non-homologous end-joining repair of double-strand breaks in DNA (306–308).

Patients with ALL and the t(12;21) generally have a favorable prognosis. Patients with the *ETV6* rearrangement had a much higher 5-year event-free survival than those without the rearrangement. In the MRC/ECOG study, the event-free and overall survival rates at 5 years of 368 childhood ALL patients with the t(12;21) were 89% and 96%, respectively (101,294).

### 75.9.3 t(4;11)(q21;q23)

The most common translocation involving chromosome band 11q23 is t(4;11)(q21;q23). It is observed in more than 60% of infants, 2% of children, and 3–6% of adults with ALL (309). The association of t(4;11) with neonatal or early-childhood ALL is particularly interesting in

view of the low incidence of ALL in this age group; acute leukemias in this age group are usually of the myeloid type and, as noted earlier, usually involve the *MLL* gene at 11q23 (200,214). Children with a t(4;11) have very high leukocyte counts (median WBC 214000/mm<sup>3</sup>), which is a poor prognostic factor (25,310). Although the morphology of some cells often appears lymphoid, other features are more suggestive of a monocytic leukemia. Thus, these cells appear to be very early precursor cells that have dual lineage capabilities. The t(4;11) results in a fusion between *MLL* on 11q23 with *AF4* at 4q21 (203,207). This translocation can be detected by RT-PCR in virtually all t(4;11) patients (238). Using molecular FISH probes for *MLL* to screen cells from infant leukemias, 73–80% were found to have *MLL* rearrangements including t(4;11), t(11;19), and t(9;11) (311). In a clinical trial of the Children Oncology Group, the 5-year event-free survival for infant patients with these three *MLL* translocations was significantly lower than that of infants lacking an *MLL* rearrangement or other *MLL* rearrangements: 22–30% vs. 53–61% (24,238). Because of the dismal clinical outcome for both adults and children with t(4;11), intensive therapy and/or allogeneic HSC transplantation might be beneficial.

### 75.9.4 t(1;19)(q23;p13.3)

Another recurring chromosome abnormality is t(1;19)(q23;p13.3), which has been identified in about 5–6% of all childhood ALL and in 30% of childhood ALL with pre-B cell phenotype (312,313). The t(1;19) occurs either as a reciprocal translocation, t(1;19)(q23;p13.3) or, more often, as an unbalanced form, that is, der(19)t(1;19)(q23;p13.3) (314). There is no difference in clinical and laboratory features and event-free survival for these two subtypes. This breakpoint has been cloned and fused the *PBX* gene on chromosome 1 and the *TCF3* (*E2A*) gene on chromosome 19 at 19p13.3 (312,315,316).

The *TCF3* gene encodes for two transcription factors (E12 and E47), which bind to enhancer elements in the *IGK* gene and to the regulatory elements of other genes. *PBX1* at 1q23 is a homeobox (*HOX*) gene that encodes for DNA-binding transcription factors and regulates developmental processes and hematopoiesis (313). *TCF3/PBX1* fusion mRNAs contain the transcriptional activating domain of E12/E47 and the DNA-binding and protein dimerization domains of *PBX1*. The fusion protein may lead to the transactivation of a number of genes that are not normally expressed in lymphoid tissues, such as over-expression of WNT-16 transcripts (63,317,318).

t(1;19) was associated early treatment failure and poor prognosis. However, recent studies have showed that this adverse prognosis can be overcome by more intensive chemotherapy. In the MRC/ECOG study, the event-free and overall survival rates at 5-years were 80% and 84%, respectively, very comparable to those (84% and 93%) with a hyperdiploid karyotype (101). In fact,



t(1;19), t(12;21) and hyperdiploidy are all associated with a favorable prognosis (25).

### 75.9.5 Hyperdiploid Karyotype

The leukemic cells of some patients with ALL are characterized by a gain of many chromosomes and fewer structural abnormalities. Chromosome numbers usually range from 50 to 60, and a few patients have up to 65 chromosomes. Although identical karyotypes are unusual, certain additional chromosomes are commonly seen. Gain of chromosome 21 is the most common aberration, following by +4, +6, +10, +14, +17, and +18. It is interesting that some of these chromosomes, particularly chromosomes 10, 18, and 21, are also seen as additional in patients with near haploidy, with chromosome numbers of 26–36 (median 28) (319). Structural abnormalities, such as duplication of the long arm of chromosome 1, iso-chromosome of 17q, as well as t(9;22) and t(1;19) may occur in a hyperdiploid clone (up to 25%). In general, patients with a hyperdiploid clone have a very favorable overall survival, which is often coincident with clinical features of a good prognosis, including age between 1 and 9 years, low WBC count, and favorable immunophenotypes (non-T or B markers), that is early pre-B or pre-B. Gain of chromosomes 4 and 10 appears to be the most influent chromosome aberrations that predict a good prognosis, whereas other structural aberrations did not appear to affect event-free survival except for a poor outcome associated with t(9;22) and iso-chromosome 17q (23).

### 75.9.6 Hypodiploid Karyotype

A hypodiploid clone with fewer than 46 chromosomes can be detected in about 5–6% of ALL patients. There are three distinct subgroups of hypodiploidy in ALL, that is near-haploidy (23–29 chromosomes), low hypodiploidy (33–39 chromosomes), and high hypodiploidy (42–45 chromosomes) (320). The near-haploid clone is often accompanied by a doubling of the chromosome complement resulting in a hyperdiploid population with a gain of two copies of certain chromosomes, which is distinguishable from the more common hyperdiploid clone with a single gain of certain chromosomes. In some patients, cytogenetic analysis may detect the doubling clone only. ALL patients with hypodiploid clones generally have a poor prognosis, especially patients with near-haploid and low-hypodiploid clones (320). In this regard, it is critical to avoid confusing the doubled (tetraploid) populations of a hypodiploid clone with a true hyperdiploid population, as the prognosis is sharply different between these groups.

### 75.9.7 9p Abnormalities in ALL

Deletions of 9p occur in about 5–10% of childhood and adult ALL patients as a single abnormality or as a part

of a complex clone with other numerical and structural abnormalities. Del(9p) can be present in various forms on cytogenetic analysis, such as del(9p), add(9p), der(9)t(v;9)(v;p), or a dicentric chromosome with 9q (23).

Application of genomic microarray analysis has identified alternations in genomic imbalances, and genes that regulate B-lymphoid development in over 60% of B-ALL (34). A mono-allelic deletion of the *PAX5* gene at 9p13.2 was detected in 28% of ALL patients with a cryptic or a large deletion involving 9p (321). Deletion or mutation of the *PAX5* gene results in reduced level of the *PAX5* protein or the generation of hypomorphic alleles, thereby leading primarily to loss of function. A somatically acquired mutation in the Janus kinase (*JAK2*) gene on chromosome 9p24 has been reported in 18% of patients with Down syndrome associated ALL, but is not commonly found in ALL patients without Down syndrome or in patients with Down syndrome associated acute megakaryoblastic leukemia (33,322,323). Notably, this *JAK2* mutation (R683) is not the same mutation as that seen in MPN, that is the V617 mutation. Deletion of 9p is an unfavorable risk factor associated with a high rate of relapse for pre-B-ALL in children and similar to those with poor prognosis t(9;22)/BCR-ABL positive ALL (324,325).

### 75.9.8 Over-Expression of the *CRLF2* Gene at Xp22.3/Yp11.3 in B-ALL

Using FISH and genomic SNP array techniques, the *CRLF2* (cytokine receptor-like factor 2) gene at the tip of the short arm of the sex chromosomes, that is Xp22.3 and Yp11.3, was found to be deregulated in up to 7% of all pediatric B-ALL and in more than 60% of Down syndrome-associated B-ALL (33). *CRFL2* rearrangements seem to be mutually exclusive with the common chromosome aberrations in ALL. Deregulation of the *CRLF2* gene in ALL results from different mechanisms. A cryptic chromosome translocation of t(X;14) or t(Y;14) results in over-expression of the *CRLF2* due to the juxtaposition of the enhancer of the *IgH* gene to the *CRLF2* gene (326). An interstitial deletion involving the *P2RY8* gene of the PAR1 (pseudoautosomal region 1) at Xp22.3/Yp11.3 leads to the juxtaposition of the promoter of the *P2RY8* gene to the *CRLF2* gene (33,327). An activating point mutation (F232C) of the *CRLF2* has been identified in some patients with B-ALL (328).

Deregulation of the *CRFL2* gene in B-ALL is associated with the *JAK2* mutations (276,287). In ALL, *JAK* mutations including *JAK2* R683G are common (11%), and over 50% of B-ALL patients with *CRLF2* rearrangements have *JAK* mutations. In Down syndrome-associated ALL, nearly all patients with *JAK2* mutation have *CRLF2* over-expression. In vitro assays determined that *CRLF2* over-expression or *JAK2* mutation alone will not confer cytokine-independent growth to hematopoietic progenitor cells. However, transduction of both



mutations together will result in the transformation of Ba/F3-EpoR cells to cytokine-independent growth (33). The JAK/STAT5 phosphorylation is induced as a consequence of the *CRLF2* rearrangements and *JAK2* mutations in ALL. The transformation can be abrogated by pharmacologic JAK inhibitors (33,34).

Over-expression of the *CRLF2* gene is associated with an extremely poor treatment outcome with a high rate of relapse in pediatric B-ALL (327,329,330). Thus, the *CRLF2* rearrangements and *JAK2* mutations in ALL provide novel diagnostic, risk stratification, and therapeutic opportunities in ALL.

## 75.10 RECURRING CHROMOSOME AND GENOMIC ABNORMALITIES IN T-CELL ALL

Compared with the high frequency (80–90%) of chromosome abnormalities in B-cell ALL, an abnormal karyotype is present in about 50–70% of patients with T-ALL (274,331). Recently, gene expression profiling and genomic SNP microarray studies have identified various novel genomic alterations and defined several distinct genetic subgroups of T-ALL that correspond to T-cell development (332,333). The most common recurrent cytogenetic abnormalities occur in various translocations with the alpha/delta TCR loci (*TRA/TRD*) at 14q11.2, the beta locus (*TRB*) at 7q34, and the gamma locus (*TRG*) at 7p14 (Table 75-2). The common partner genes are *TAL1/SCL* (1p32), *HOX11L2/TLX3* (5q35), *HOXA* (7p15), *MYC* (8q24.1), *TAL2* (9q31), *HOX11/TLX1* (10q24), *RBTN1/LMO1* (11q15), *RBTN2/LMO2* (11p13), *CCND2* (12p13), *TCL1* (14q32), and *LYL1* (19p13) (Table 75-2) (201,334–339). With few exceptions, the involved gene on the partner chromosome encodes a cell cycle inhibitor or a transcription factor whose expression is deregulated or activated as a result of juxtaposition with the regulatory regions of one of these TCR loci. Some non-TCR loci translocations are also common in T-ALL, such as the *SIL-TAL1*, *PICALM-AF10*, *MLL-ENL*, *HOX11L2-BCL11B*, *ETV6-JAK2* fusion, and the *NUP214-ABL1* episomal amplification (339,340). Other abnormalities include genomic micro-deletions, such as deletion of the short arm of chromosome 9 at the *CDKN2A* tumor suppressor locus containing the *p16/INK4a* and *p14/ARF*, both control cell cycle progression and *P53* mediated apoptosis. Gene mutations, involving *NOTCH*, *JAK1*, and *FLT3*, are also found in T-cell ALL. The different chromosome and genomic abnormalities in T-ALL likely reflect different pathways and their cooperation in leukemogenesis in T-ALL (341). Notably, in many cases, these chromosome abnormalities are cryptic and can be detected only by molecular cytogenetic techniques, that is, FISH or PCR. SKY was used to perform a study on samples from 15 T-ALL patients (342). Although no recurring translocations were identified, the cytogenetic findings in some

patients were clarified. Only five patients had a normal karyotype.

The t(10;14)(q24;q11.2) and its variant t(7;10)(q34;q24) are detected in 5–10% of patients with T-ALL or T-cell lymphomas (343). The t(10;14) and t(7;10) result in the translocation of *TLX1*(*HOX11*) to *TRA/D* and *TRB*, respectively, leading to over-expression of *TLX1*. However, *TLX1* over-expression in leukemic blasts has also been observed in the absence of 10q24 rearrangement in up to 50% of T-ALL cases. Therefore,

**TABLE 75-2** Recurring Structural Rearrangements in Malignant Myeloid Diseases

Disease	Chromosome Abnormality	Involved Gene
Chronic myeloid leukemia	t(9;22)(q34;q11.2)	<i>ABL/BCR</i>
CML blast phase	t(9;22),+8,+Ph,i(17q)	<i>ABL/BCR</i>
Chronic myelomonocytic leukemia	t(5;12)(q33;p13)	<i>PDGFRB/TEL</i>
Acute myeloid leukemia		
AML-M2	t(8;21)(q22;q22)	<i>ETO/AML1</i>
APL-M3, M3V	t(15;17)(q22;q12)	<i>PML/RARA</i>
	t(11;17)(q23;q12)	<i>PLZF/RARA</i>
	t(11;17)(q13;q12)	<i>NUMA/RARA</i>
	t(5;17)(q31;q12)	<i>NPM/RARA</i>
AMMoL-M4Eo	inv(16)(p13;q22) or t(16;16)(p13;q22)	<i>MYH11/CBFB</i>
AMMoL-M4/AMoL-M5	t(6;11)(q27;q23)	<i>AF6/MLL</i>
	t(9;11)(p22;q23)	<i>AF9/MLL</i>
	t(10;11)(p13;q14)	<i>AF10/CALM</i>
	t(10;11)(p13;q23)	<i>AF10/MLL</i>
	t(11;17)(q23;q25)	<i>MLL/IAF17</i>
	t(11;19)(q23;q13.1)	<i>MLL/IELL</i>
	t(8;16)(p11;p13)	<i>MOZ/CBP</i>
AML-M7	t(1;22)(p13;q13)	<i>OTT/MAL</i>
AML	t(3;3)(q21;q26) or inv(3)(q21;q26)	<i>RPN1/EV1</i>
	t(6;9)(p23;q34)	
	t(7;11)(p15;p15)	
	inv(11)(p15q22)	
	t(9;12)(q34;p13)	
	t(12;22)(p13;q13)	
	t(16;21)(p11;q22)	
	–7 or del(7q)	
	–5 or del(5q)	
	del(20q)	
	del(12p)	<i>TEL, ?p27<sup>KIP1</sup></i>
Therapy-related AML	–7 or del(7q) and/or –5 or del(5q)	
	t(11q23)	<i>MLL</i>
	t(3;21)(q26;q22)	<i>EAP/MDS1/EVI1-AML1</i>

AML, acute myeloid leukemia; AMMoL, acute myelomonocytic leukemia; AMoL, acute monoblastic/monocytic leukemia; APL, acute promyeloid leukemia; CML, chronic myelogenous leukemia.

other mechanisms, such as disruption of gene silencing, may lead to aberrant expression of the gene as well. The gene expression pattern of *TLX1*-expressing lymphoblasts is similar to that of early cortical thymocytes. Thus, the lack of expression of anti-apoptotic genes during this stage of thymocyte development (and in *TLX1*-expressing lymphoblasts) may explain why pediatric and adult patients with this type of lymphoblast have a highly favorable outcome. Somatic inactivating mutations or deletions in the plant homeodomain finger 6 (*PHF6*) locus located on the X chromosome at Xq26.3 were detected in 16% of pediatric and 38% of adult primary T-cell ALL (344), which is exclusively in T-ALL samples from male patients. *PHF6* is an X-linked tumor suppressor in T-ALL and loss of *PHF6* is associated with aberrant expression of the *TLX1* (*HOX11*) and *TLX3* (*HOX11L2*) transcription factor oncogenes.

Another recurring rearrangement in T-cell neoplasia is the *inv*(14)(q11.2q32) or its variant *t*(14;14)(q11;q32), particularly in T-cell prolymphocytic leukemia (T-PLL) and phytohemagglutinin-stimulated lymphocytes from patients with AT, as well as in the leukemic cells of AT patients in whom this disease evolved (345). Japanese adult T-cell leukemia-lymphoma patients have a high incidence of 14q11 breaks. Thus, in some of these T-cell diseases breaks occur in either 14q11 or 14q32, or in both bands in the same patient; in B-cell disorders, however, breaks occur essentially only in 14q32, and they rarely involve 14q11 (346). One of the first translocations to be cloned in T-cell leukemia was the *t*(8;14)(q24;q11). The breakpoint involves *MYC* at 8q24, and the *TRA* gene. The break in *MYC* is 3' of the third exon and *MYC* remains on chromosome 8; in *TRA* the break is just 5' of a *Ja* segment (*JaD*) (347). This translocation is comparable to these involving the immunoglobulin light-chain genes in B-cell lymphomas in which *MYC* also remains on chromosome 8. *TRA* is also involved with translocations affecting 14q32 and the heavy chain gene in the *inv*(14) (345). Another translocation of special interest is the *t*(1;14)(p33;q11). The translocation juxtaposes the *TAL1* (*SCL*) gene at 1p33 with the *TRD*, and it occurs in about 3% of T-ALL patients (345). Probes for *TAL1* detect a 90-kb deletion involving the 5' region of the *TAL1* gene in 25% of patients (348). *TAL1* is never expressed in lymphoid cells and the translocation or deletion results in its inappropriate expression in these cells (349). Analysis of mRNA from T-ALL samples has revealed ectopic expression of *TAL1* in 35% of patients whose cells have neither a translocation nor a deletion. Thus, *TAL1* is expressed in over 60% of T-ALL patients, which makes it a very critical gene in this leukemia (349). Another common translocation involves either 11p13 or 11p15 and *TRA*; the genes on 11p are *LMO1* (formerly Rhombotin, *RBTN1*) at 11p15 and *LMO2* (*RBTN2*) at 11p13. Like the *TAL1* gene, *LMO1* and *LMO2* are not expressed in normal T-cells (336).

The extra-chromosomal episomal *NUP214-ABL1* amplification is a cryptic abnormality, and occurs in about 6% of T-ALL cases. The extra-chromosomal amplifications contained *ABL1*, *LAMC3*, and *NUP214* genes, localized within a 500-kb region on 9q34, and result in the *NUP214-ABL1* fusion genes. The copy number of the episome may vary from cell to cell, increasing due to unequal segregation during cell division. FISH analysis using probes for *ABL* is the only reliable means to reveal a variable spectrum of multiple uncountable copies of the *ABL* locus in interphase nuclei and metaphase cells. *ABL* FISH signals are not attached to any metaphase chromosomes or exceptionally intrachromosomal amplifications exist as small homogenous staining region. In many patients, the *NUP214-ABL1* amplification is a part of complex karyotypes, including some of the recurrent translocations seen in T-ALL. The 9q34 amplification often coexists with a 9p deletion involving *CDKN2A* and *CDKN2B* and ectopic expression of *TLX1* or *TLX3* (180). The *NUP214-ABL1* fusion is associated with aggressive disease and poor outcome in childhood T-ALL. Like *BCR-ABL1*, the *NUP214-ABL1* fusion is a constitutively active tyrosine kinase with transforming activity in vitro. However, because of the heterogeneity in the genetic lesions observed in many cases, the response to Imatinib in *NUP214-ABL1* positive T-ALL patients should be evaluated with caution. *ABL1* quantitative RT-PCR may be easily applied to monitor MRD.

### 75.10.1 *NOTCH1* Activating Mutations and Its Pathway in Leukemogenesis of T-ALL

About 5% of T-cell ALL patients have the translocation *t*(7;9)(q35;q34.3) between the *TCRB* at 7q35 and *NOTCH1* (*TAN1*) on chromosome 9 (350–352). In contrast to the translocations discussed above, rearrangements involving *NOTCH* disrupt the coding portion of the *NOTCH* gene, resulting in a truncated form of the *NOTCH* protein. This truncated form of protein produces T-cell malignancies when introduced into murine bone marrow (353). *NOTCH* family proteins are trans-membrane receptors, and *NOTCH1* signaling is required for commitment to the T-cell lineage and for the proliferation of T-cell progenitors in the thymus (354,355). Point mutations, insertions, and deletions of *NOTCH1* have been identified in more than 50% of all T-ALL (354,356), although the precise mechanisms by which *NOTCH1* causes T-cell ALL are not yet fully elucidated. Deletion of the *NOTCH1* promoter can also result in activation of the *NOTCH1* gene from cryptic initiation sites. Spontaneous deletion of the *NOTCH1* locus is seen in approximately 75% of *IKZF1*-deficient T-cell ALL cases (357). Patients with T-cell ALL demonstrating a *NOTCH1* mutation or a related mutation in *FBXW7* may represent a subset with superior event-free and overall survival rates when compared with other

patients with T-cell ALL (180,274,358,359). Functional analysis showed that gain-of-function mutations in *NOTCH* result in T-ALL in mice (352).

Both children and adults with T-cell ALL are generally treated with high-risk protocols and often have comparably favorable outcomes. In contrast to B-ALL, cytogenetic abnormalities in T-ALL are not strongly associated with a specific prognosis. In childhood T-ALL, the outcome is similar between patients with an abnormal karyotype and patients with a normal karyotype. However, T-cell ALL with low expression of both *ERG* and *BAALC* genes by RT-PCR is associated with significantly lower relapse rates compared with other T-cell ALL (17 vs. 60%) and higher 4-year overall survival (69 vs. 30%), and patients with *NOTCH* activating mutations have a rapid early response to treatment (274).

### 75.11 LEUKEMIA IN CHILDREN WITH DOWN SYNDROME

Children with Down syndrome have about a 20-fold increased risk of developing acute leukemia, 50% of which will be acute megakaryoblastic leukemia. About 10% of infants with Down syndrome develop transient myeloproliferative disease (TMD), a profound leukocytosis with blasts indistinguishable from leukemic megakaryoblasts. TMD usually disappears spontaneously during the first months of life. However, approximately 25% of those who present with TMD will develop acute megakaryoblastic leukemia in the first 4 years of life (360). Most Down syndrome children with AML have an abnormal karyotype, most commonly trisomy 8 (25%) or, less commonly, trisomies 19 or 21–13% and 17%, respectively (361). The role of the extra copy of chromosome 21 in the development of leukemia is unknown. A gain of chromosome 21 is the most common numerical change in both ALL and AML in children and has led to the hypothesis that over-expression of genes present on chromosome 21 may alter expression of genes on other chromosomes that subsequently affect hematopoiesis (270). In fact, increased levels of chromosome 21-encoded tumor invasion and metastasis factor (*TIAM1*) mRNA were reported in the bone marrow of children with Down syndrome during the acute phase of AML-M7 (362). Another hypothesis suggests that disomic homozygosity for a predisposing mutated allele of a putative suppressor gene on chromosome 21 may play a role in leukemogenesis. In support of this, in cases of TMD, maternal age was lower (about 27 years) compared with true leukemia (about 40 years) (361). Cavani et al. (1998) analyzed cytogenetic heteromorphisms and 26 polymorphic DNA markers from the long arm of chromosome 21 and showed an increased number of pericentromeric crossovers between the non-disjoined chromosomes in Down syndrome patients with AML-M7, compared to other Down syndrome cases (363). Recently, acquired mutations of *GATA1*, a transcription

factor that regulates growth and maturation of hematopoietic cells located on chromosome band Xp11, were detected in the majority of patients with Down syndrome-associated acute megakaryoblastic leukemia and almost all patients with TMD (191). Studying fetal tissue it was shown that *GATA1* mutations can occur prenatally and are likely early steps in leukemogenesis requiring additional genetic events and/or environmental exposures for the full development of leukemia (364). Whether *GATA1* mutation types and truncated protein expression level are associated with a risk of progression to acute leukemia in Down syndrome patients is controversial. The precise pathway by which *GATA1* contributes to leukemia in patients with Down syndrome remains under investigation.

### 75.12 PRENATAL ORIGINS OF CHILDHOOD LEUKEMIA

Recognition of the modest concordance of leukemia in monozygotic twins and analysis of DNA from blood spots on Guthrie cards from children who later developed leukemia indicate that the chromosome translocations resulting in abnormalities, such as *MLL* fusions, the *TEL/AML1* fusion in ALL, the *AML1/ETO* fusion in AML, and hyperdiploid clones in pediatric ALL, arise prenatally in utero at various frequencies (266,306). Using RT-PCR, Mori et al. detected *TEL/AML1* and *AML1/ETO* fusion transcripts in cord blood samples proposing that the abnormalities arise during fetal hematopoiesis (308). The frequency of detection of disease associated transcripts was approximately 100-fold greater than the incidence for development of leukemia, supporting the requirement of secondary, complementary mutations (308). As mentioned above, similar findings were identified when *GATA1* was studied in fetal tissue (364). Notably, pre-leukemia clones such as the *TEL/AML1* fusion occur in utero at a high rate in excess of clinical leukemia, and the concordance of infant ALL in monozygotic twins is not very high (around 10–20%) (307,365). Thus, all the data indicate that additional genetic mutations, most likely postnatal, are required for the development of an overt leukemia.

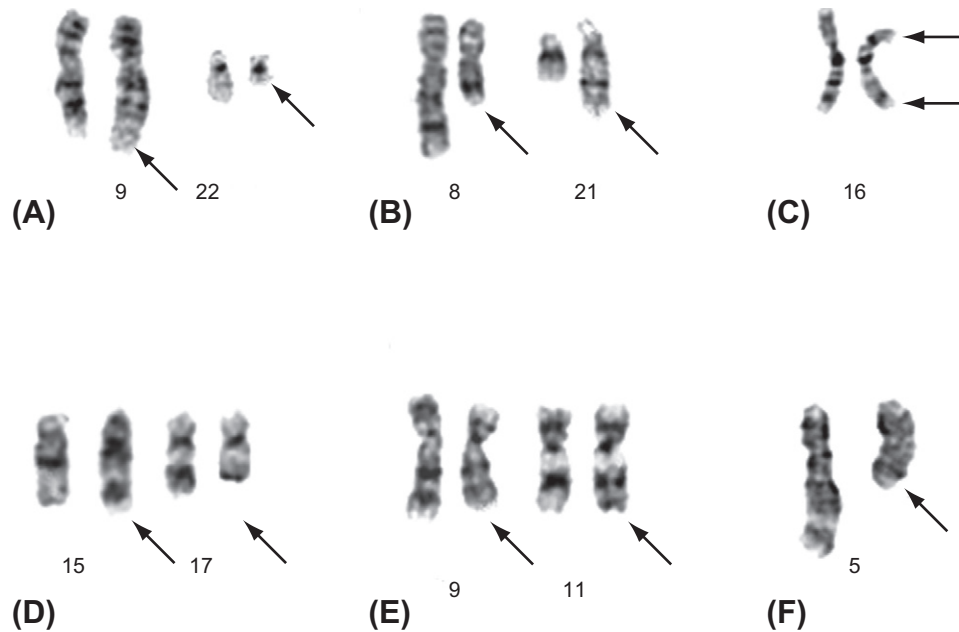
### 75.13 NON-HODGKIN LYMPHOMAS

The same explosion of information regarding the genes that are altered in the chromosome abnormalities described for the myeloid disorders has occurred in mature lymphoid disorders as well. There are some similarities, but also some major differences in the genetic (chromosome) changes that occur in these two lineages. In contrast to myeloid leukemia that are characterized with novel chimeric fusion genes and functions from chromosome translocations, lymphoid disorders are associated with over-expression of an intact oncogene, such as *MYC* or anti-apoptosis, that

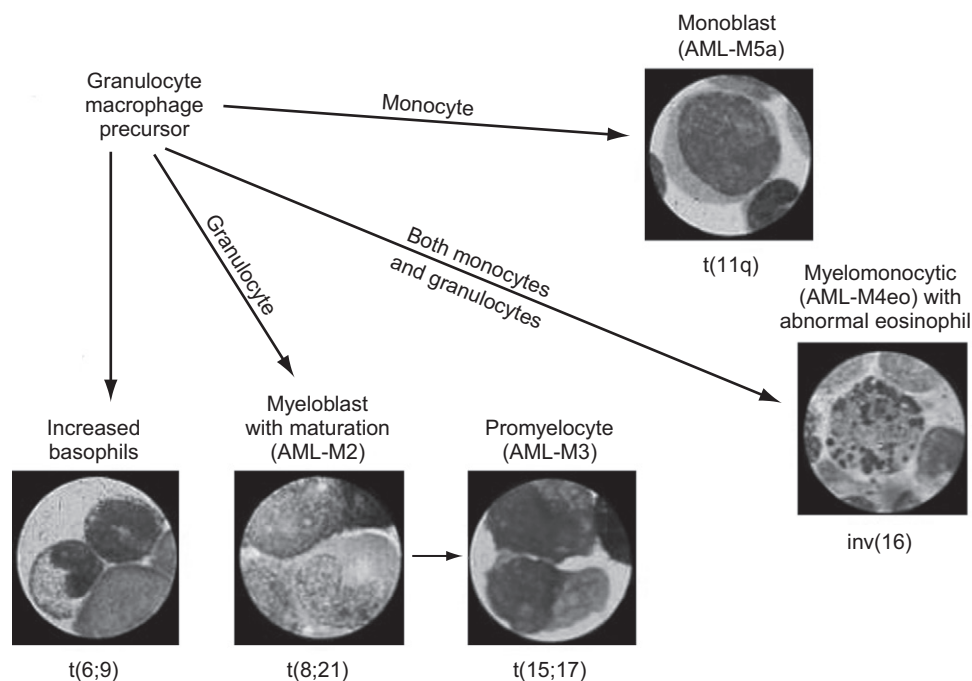
is, *BCL2*, due to this juxtaposition to the promoters and/or enhancers of immunoglobulin or T-cell receptor genes in various translocations. This section reviews the consistent translocations seen in mature B-cell NHL, and Burkitt lymphoma, and in some T-cell disorders (Figures 75-2–75-4).

### 75.13.1 t(8;14) and Its Variants, and the IGH-MYC Fusion in Burkitt and Other High Grade NHL

Some 80–90% of NHLs display chromosomal aberrations and, more importantly, many of the recurring



**FIGURE 75-2** Partial karyotypes from trypsin-Giemsa-banded metaphase cells depicting recurring chromosomal rearrangements observed in myelogenous leukemia. **A**, t(9;22)(q34;q11) in chronic myelogenous leukemia (CML). **B**, t(8;21)(q22;q22) in acute myeloblastic leukemia with maturation (AML-M2). **C**, inv(16)(p13q22) in acute myelomonocytic leukemia associated with marked eosinophilia (AMMoL-M4Eo). **D**, t(15;17)(q22;q11-12) in acute promyelocytic leukemia (APL). **E**, t(9;11)(p22;q23) in acute monoblastic leukemia (AMoL-M5). **F**, del(5)(q13q33) in therapy-associated AML (t-AML). The rearranged chromosomes are identified with arrows.



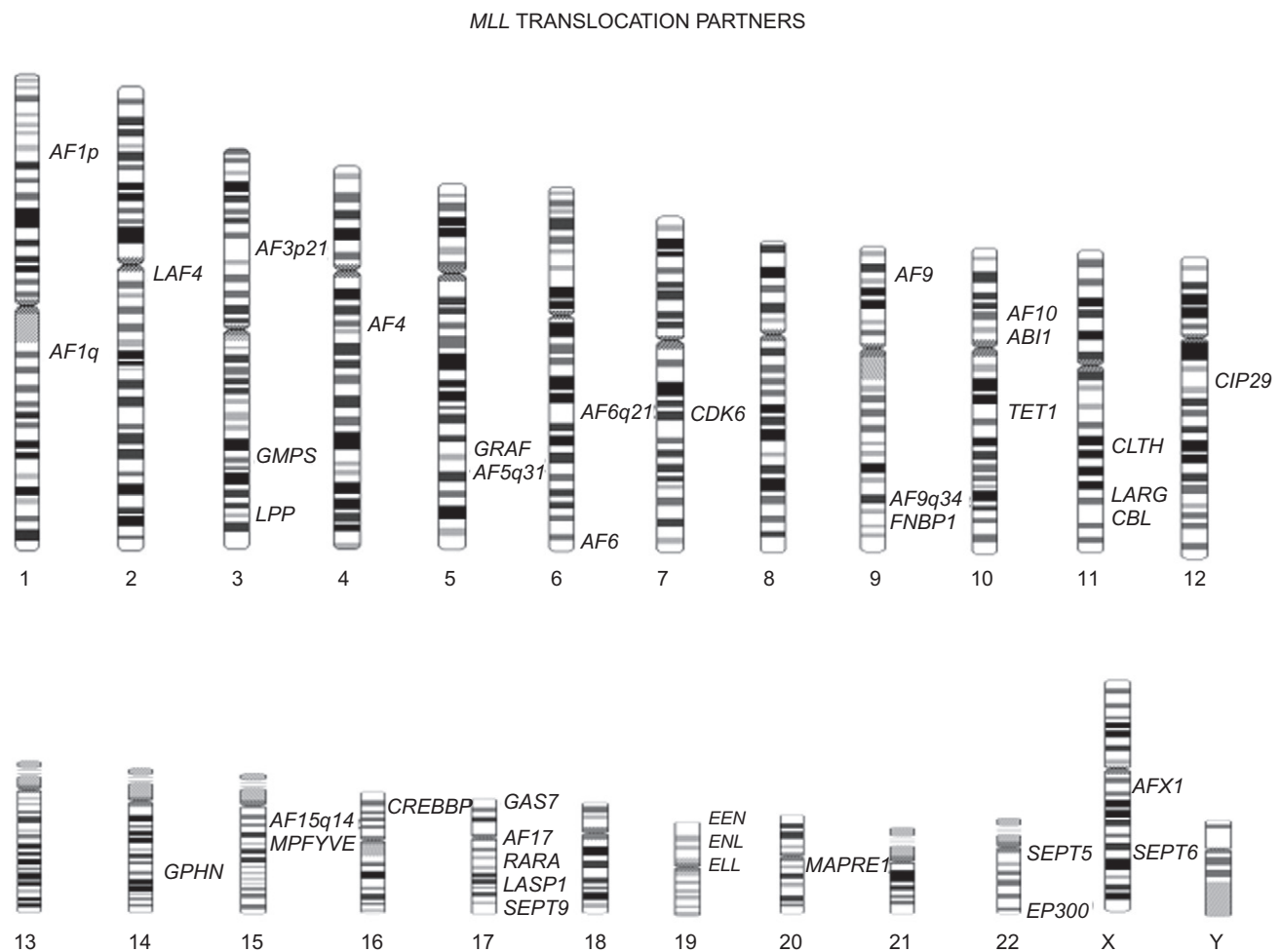
**FIGURE 75-3** Relationship of the subtypes of AML and the particular chromosome abnormality associated with each subtype. Each circle contains a photomicrograph illustrating the special features of the leukemic cells from the bone marrow of untreated patients; the particular chromosomal rearrangements associated with that type of leukemia are listed under the photomicrograph.



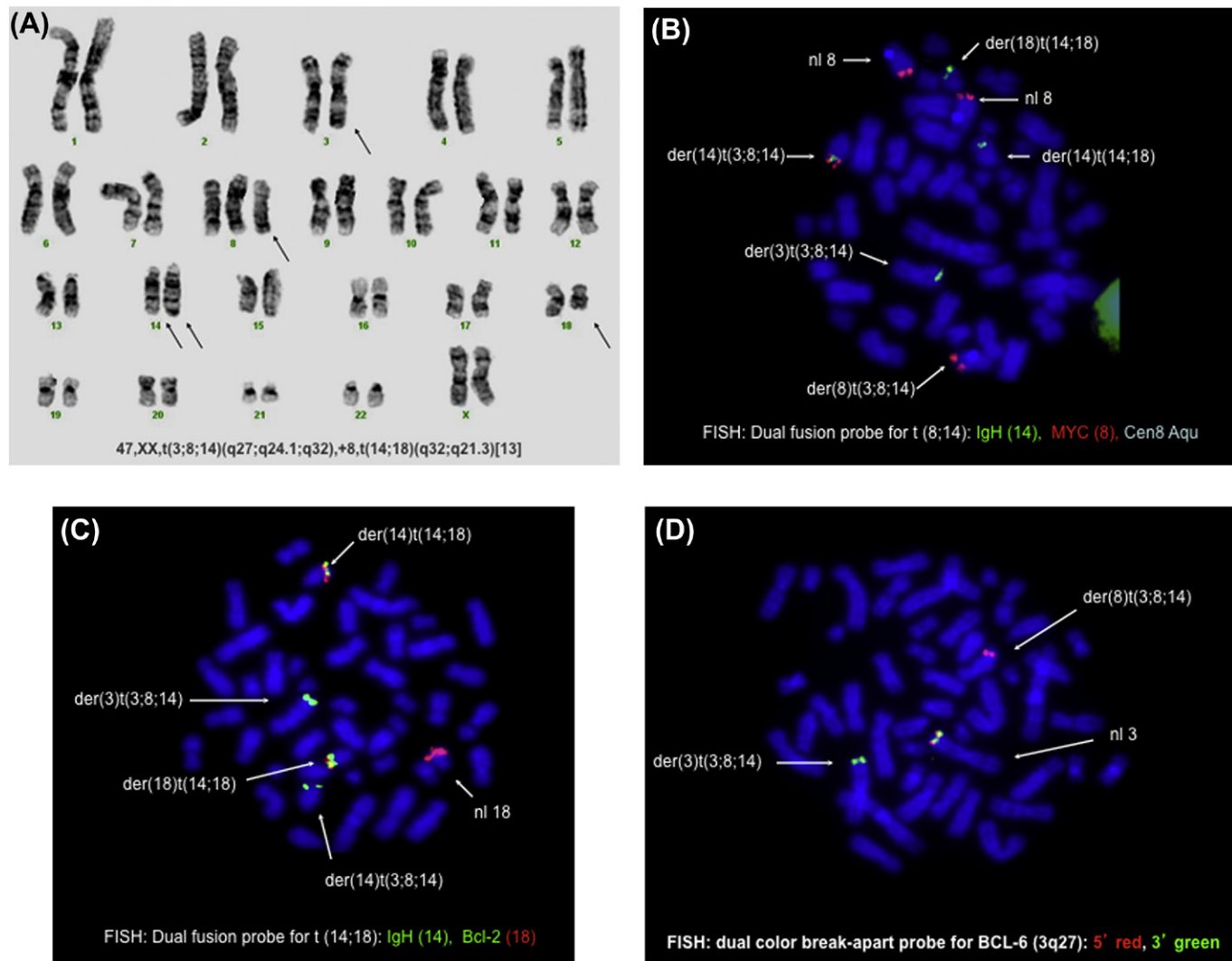
abnormalities correlate with histology and immunophenotype and may be independent prognostic factors (7). In 1972, Manolov and Manolova were the first to discover that cells of Burkitt lymphomas had an additional band at the end of the long arm of one chromosome 14 (14q+). In 1976, Zech and co-workers first observed that the end of one chromosome 8 was consistently absent, and they suggested that the missing part of chromosome 8 was translocated to 14, that is,  $t(8;14)(q24;q32)$  (Fig. 75.5a). The  $t(8;14)$  was also the first structural chromosomal abnormality in any hematological disease or carcinoma to be characterized at the molecular level (366,367). In fact, the  $t(8;14)$  and its variants are diagnostic for classic Burkitt leukemia and lymphoma, and is associated with a good prognosis in this entity when treated with intensive chemotherapies. It is found in both endemic African and sporadic Burkitt lymphoma, the latter being Epstein-Barr virus negative, and in immunodeficiency-associated Burkitt lymphoma. The  $t(8;14)$  is found in 75–85% of Burkitt lymphomas, and variant translocations,  $t(2;8)(p12;q24)$  and  $t(8;22)(q24;q11)$ ,

are described in 15–25% of cases (Table 75-2). All three translocations involved chromosome 8 with a break in the same band, 8q24. The reciprocal translocation fuses the protooncogene *MYC* (the cellular homolog of the avian myelocytomatosis virus) on chromosome 8 (band q24) to one of the immunoglobulin genes (heavy-chain, and K and L light-chain genes) which are located at the breakpoints on one of the three chromosomes, 14q32, 2p12, and 22q11, respectively (366). The 8q24 breakpoints demonstrate considerable variability at the molecular level, extending from over 300 kb centromeric to at least the same distance telomeric to *MYC* (368). Clustering of breakpoints has been described, and the location appears to differ in endemic, sporadic, and HIV-associated Burkitt lymphoma. Regardless of the molecular breakpoints, the consequence of these translocations is the deregulated expression of a full-length *MYC* protein, although the precise mechanisms by which deregulation occurs is unclear.

*MYC* acts as a transcription factor and plays a central role in a number of cellular processes, including



**FIGURE 75-4** The location of translocation breakpoints that involve *MLL* is indicated by colored bands. The involvement of *MLL* was determined by FISH, Southern blot analysis, or RT-PCR. Symbols to the right of the band indicate that the translocation has been cloned and the partner gene has been identified and named. (From Rowley JD [1999] *The role of chromosome translocations in leukemogenesis. Semin Hematol* 36[Suppl 7].)



**FIGURE 75-5** Cyto-genetic and FISH analysis reveal a complex karyotype with t(3;8;14) and t(14;18) in a lymph node specimen from a female patient with a high grade B cell lymphoma. A. Karyotype of a metaphase cell shows 47, XX,t(3;8;14)(q27;q24.1;q32),+8,t(14;18)(q32;q21.3). A three-way translocation among chromosomes 3, 8, and 14 involves the *BCL6* at 3q27, *MYC* at 8q24.1, and *IGH* at 14q32. These analyses indicate a so-called "double-hit lymphoma" with some overlapping features of Burkitt lymphoma and diffuse large B cell lymphoma, involving the *MYC*, *IGH*, *BCL2*, and *BCL6* genes. B. Using a triple-color FISH probe set for *MYC* (red), *IGH* (green), and chromosome 8 centromere (aqua), FISH analysis confirms the translocation between the *MYC* and *IGH* (fusion) with a red/green fusion on derivative chromosome 14, and the *IGH* split signal pattern due to the t(14;18), with a green signal on the derivative chromosome 18. FISH also confirm the presence of a third normal chromosome 8. C. FISH analysis using a *IGH/BCL2* dual fusion probe set displays the dual fusions of the *IGH* and *BCL2* genes resulting from the t(14;18), and confirms *IGH* involvement in t(3;8;14) with a split green signal on the derivative chromosome 3. D. Using a dual color break-apart probe set for the *BCL6* gene, FISH analysis confirms a split of the *BCL6* probe between derivative chromosomes 3 and 8. Thus, FISH using multiple probes confirms the involvement of *IGH*, *MYC*, *BCL2*, and *BCL6* in this sample.

proliferation and apoptosis (369). It is also a key component of the gene regulatory network in B cells, with DNA binding sites at the promoter regions as well as many non-coding regions. *MYC* participates in regulating chromatin dynamics by directly binding to several proteins that control epigenetic modifications of both histone and the DNA methylation state, and is also involved in the regulation of microRNA expression (370). Recently, gene expression profiling analyses have revealed a unique transcriptional and genomic profiling in Burkitt lymphoma with a high level of expression of *MYC* target genes, the expression of a subset of germinal-center B-cell genes, and the low-level of

expression of major-histocompatibility-complex class I genes and nuclear factor-kB target genes (371). Moreover, molecular classification of Burkitt lymphomas further confirmed an overall superior survival in patients with Burkitt lymphoma who had received intensive chemotherapy (372).

The t(8;14) and particularly other *MYC* abnormalities are also observed in other lymphoma, such as diffuse large B-cell lymphoma, DLBCL with overlapping features of Burkitt lymphoma, and follicular lymphoma, MCL, and T-cell leukemia/lymphoma and CLL (373,374). In all these diseases, *MYC* abnormalities are prognostic and associated with a more aggressive

phenotype, resistance to standard chemotherapy, and a higher mortality (375). So-called double-hit lymphomas often contain both *MYC* translocations and one or two other typical lymphoma-associated translocations, such as t(14;18)/*BCL2*, t(11;14)/*BCL1*, and t(3;14)/*BCL6* (Figure 75-5B–D) (376). In contrast to Burkitt lymphoma with a relatively simple karyotype of t(8;14) and its variants, these lymphomas often display a complex karyotype with multiple chromosomal abnormalities on cytogenetic analysis (377). Gene expression profiling analysis has further defined three subgroups of DLBCL, that is activated B-cell-like associated with poor prognosis, germinal B-cell-like with favorable prognosis, and others (378). The expression profile of a small group of DLBCL was similar to those of classic Burkitt lymphoma. Recently, next-generation sequencing analyses have identified that somatic mutations of the *MLL2* gene that encodes a histone methyltransferase occur in 32% of DLCL and 89% of follicular lymphoma. Moreover, 11–14% of DLCL and follicular lymphoma showed mutations in *MEF2B*, a calcium-regulated gene that cooperate with CREBBP and EP300 in acetylating histones (379,380).

Translocations involving chromosome band 3q27 are found in 8–12% of NHLs, in particular in high-grade lymphoma. Molecular characterization showed that the 3q27 breakpoints cluster in a 4-kb region of the gene called *BCL6* (*LAZ3*) (381). *BCL6* encodes a transcriptional regulator that is involved in germinal center formation and in the generation and maintenance of both T and B cells during immune responses. In many cases, *BCL6* occurs as a late and secondary genetic abnormality, in addition to t(14;18) or t(8;14), as discussed in those double-hit lymphoma. Similar to *MYC* in t(8;14) in Burkitt lymphoma, the translocation of 3q27/*BCL6* results in the deregulated expression of a full-length *BCL6* protein.

### 75.13.2 t(14;18)/IGH-BCL2 Fusion in Follicular Lymphoma and Diffuse Large B-Cell Lymphoma

Another common recurring translocation in NHL, t(14;18) (q32;q21), was first identified by Fukuhara and co-workers, in 1979, in poorly differentiated lymphocytic lymphoma (3). It has been detected in 80–90% of follicular lymphomas and in about 20% of diffuse B-cell lymphomas. The translocation results in the juxtaposition of the *BCL2* gene on chromosome 18 to the immunoglobulin locus, with consequent deregulated expression of an intact *BCL2* protein. The cloning of this gene led to the discovery of a new class of oncogenes, which, instead of promoting proliferation, contributed to the development of a neoplastic state by preventing programmed cell death. Breakpoints cluster in at least two sites in *BCL2*; the major cluster is in the 3' untranslated region (UTR) of the third exon.

The increased production of *BCL2* protein leads to prolongation of cell survival and the accumulation of follicular B cells (315,382). The *BCL2* rearrangement appears to occur in a very early multilineage progenitor stem cell (383). There are conflicting reports about the prognostic significance of having t(14;18), but several studies suggest that the level of *BCL2* expression and the site of *BCL2* breakpoint might be important (375). This translocation has been found in lymphoid hyperplasia and even in normal individuals (at low percentages and only by molecular and not standard cytogenetic techniques); it has therefore been postulated that it causes only proliferation and prolonged cell survival, and that additional genetic changes are required for neoplastic transformation (384). The long chronic phase (up to 10 years) before the inevitable terminal phase supports this hypothesis. It is important to understand that the detection of low-level *BCL2*-*IGH* rearrangement does not in itself necessarily reflect the presence of malignant cells in the sample, nor that the B-cell proliferation is a (transformed) follicular lymphoma.

### 75.13.3 t(11;14)/IgH-BCL1 Fusion in Mantle Cell Lymphoma

More recently, the importance of several other translocations has been recognized. One of these, the t(11;14) (q13;q32), has been shown to be associated with a relatively new pathologic entity known as mantle cell lymphoma (MCL), which was previously classified as low-to-intermediate-grade small cell lymphoma. Cloning of t(11;14) translocation breakpoints showed juxtaposition of the *IGH* gene (JH segment) to a putative oncogene, *BCL1*. The breakpoint was originally cloned by Tsujimoto and colleagues in 1984, who called the locus at 11q13, *BCL1*; no gene was identified initially, but several other groups analyzing other genes found that one of the cyclins (*CCND1*), also called *PRAD1* because it was isolated from a parathyroid adenoma breakpoint, was a partner in the translocation (385). Genomic 11q13 breakpoints are heterogeneous, extending over several hundred kilobases, but three clusters have been described. Besides MCL, the t(11;14) has also been reported in 3% of multiple myelomas (MMs) and up to 20% of PLL, but the breakpoint cluster in these diseases is located in the region containing the IgH constant segments, and thus appears to be distinct from that observed in MCL (386). *CCND1* is involved in cell cycle progression from the G1 to the S phase by forming a complex with CDK4 and activating the RB1-E2F1 pathway (see Section 75.13.6). It is likely that deregulated expression of the *BCL1* (*CCND1/PRAD1*) gene disrupts cell cycle control and cellular proliferation. Due to a large variation of breakpoints in *CCND1*, FISH using a cocktail of several overlapping BCA probes is by far the most sensitive method of t(11;14) detection in MCL. It was shown that FISH can detect t(11;14) in every patient with MCL, regardless

of their morphological or clinical presentation, whereas RT-PCR and conventional cytogenetics are successful in 50–60% and 70–75%, respectively (387). The detection of t(11;14)/IGH-CCND1 fusion is clinically significant because MCL has an aggressive clinical course and the morphology and immunophenotypes of MCL cells is variable, making it difficult to separate from CLL and other low-grade B-cell lymphoma and leukemia.

#### 75.13.4 Common Chromosome and Genomic Abnormalities in Chronic Lymphocytic Leukemia

CLL is the most common leukemia in the United States and Europe, accounting for about 30% of all leukemias. It is virtually unknown in people less than 30 years old, but after the age of 70 its incidence is about 50 cases per 100,000. It is considered to be a monoclonal neoplastic proliferation of small lymphocytes that in 95% of cases are of B-cell origin. There appears to be a genetic susceptibility to CLL because CLL is rare both in Asia and in Africa. The ethnic origin per se, however, does not influence the biology or clinical behavior of the disease. The ethnic variation appears to be maintained even after population migration to regions of high incidence; in the United States, CLL is much rarer in people from the Far East and in Japanese immigrants than in Caucasians. A recent intriguing finding is that in families in which both a child and a parent have CLL, the disease develops approximately 20 years earlier in the younger generation, a phenomenon called anticipation (388). This strongly supports the importance of genetic factors in the pathogenesis of CLL. The disease has a prolonged natural history, sometimes measured in decades; cells presumably gradually acquire sequential genetic abnormalities, rendering them more malignant.

The early studies of the cytogenetic pattern in CLL showed a normal karyotype in most samples, but it is now clear that the incidence of genetic abnormalities in CLL is critically dependent on the techniques used for their detection (27). Using immunostimulatory synthetic oligodeoxynucleotides containing unmethylated CpG motifs, such as DSP30 (CD40 ligand) as B-cell mitogen in combination with cytokine stimulation, that is interleukin 2 in cell culturing, abnormal karyotype can now be detected in more than 80% of CLL bone marrow or peripheral blood samples (389,390). Likewise, by using FISH screening of interphase cells, approximately 85% of CLL patients have a genetic abnormality (391). Current practice in the diagnosis of CLL now includes an interphase FISH analysis with a cocktail of probes for the following loci: p53(17p13), ATM(11q22.3), 13q14, chromosome 12 centromere, and IGH/14q32. In our experience, this increases the abnormal detection rate from approximately 25% (by cytogenetic analysis) to approximately 85%. Conventional and molecular cytogenetic methods have shown that the most common genetic abnormalities in CLL (up to 55% of all CLL

cases) are deletions or translocations of 13q14.3 (391). The presence of del(13q), detected by conventional chromosome analysis, as a sole abnormality is consistent with a good prognosis (median survival 133 months) (391). A detailed molecular analysis of this area, which is telomeric to the retinoblastoma gene *RB1*, has narrowed the interval to a minimally deleted region of no more than 10 kb, from which two candidate tumor suppressor genes, *LEU1* and *LEU2*, have been cloned (392,393). However, no mutations have been found in either gene in CLL (394). A third gene, *LEU5*, has homology to some zinc-finger proteins involved in cancer, but no alterations have been found in CLL (392). Recently, genomic microarray and gene expression profiling studies in CLL have defined two distinct subgroups of del(13q) in CLL based on the size of the deletion and the involvement of microRNAs (see Section 75.13.5) (395–397).

Trisomy 12, the most common abnormality detected by classic cytogenetic studies, is in fact only present in 16% of cases when using FISH. The gain of chromosome 12 as a sole abnormality or in the presence of del(13q) is associated with a good to moderate prognosis (median survival 114mos) (398). The mechanism by which trisomy 12 contributes to leukemogenesis in CLL is still unknown, although it cannot be excluded that amplification of one or more relevant genes may be responsible for the more malignant phenotype (391). About 7% of CLL patients have deletions of the *TP53* gene at 17p13.3, and these have been associated with poor survival (median survival 32mos) and drug resistance (391,399). Deletions of chromosome band 11q22 are noted in 18% of cases of CLL using FISH. This abnormality is associated with a moderate to poor outcome (median survival 79mos) (391). The *ATM* (ataxia–telangiectasia mutated) gene is located within the minimal region of loss of 11q22.3, and its inactivation may interfere with normal lymphoid function (400). Notably, both deletion of *P53* and *ATM* in CLL are often observed as secondary abnormalities during disease progression. Loss of material on the long arm of chromosome 6 at 6q23 has been found in 6% CLL patients, and it may contain an as-yet unidentified tumor suppressor gene (391). In the past, numerous CLL cases have been reported to carry t(11;14) (q13;q32), but these cases are now considered to represent a distinctly different lymphoid malignancy, namely MCL (398). Another translocation involving 14q32 is the t(14;19) (q32;q13). The breakpoint junction in CLL patients with this translocation was cloned and the gene on chromosome 19 was called *BCL3* (401). Rearrangements of the *BCL2* gene are found to be rare in CLL, but this gene is overexpressed in more than 85% of cases. Because *BCL2* is a potent inhibitor of apoptosis it may also contribute to CLL pathogenesis.

#### 75.13.5 MicroRNA and Epigenetic Dysregulation in CLL

MicroRNAs are short 19–25 nucleotide (nt) RNA that are processed from much longer primary transcripts



(called pri-miRNA with hundreds to thousands of nucleotides) and arise from hairpin loop structures (called pre-miRNA, 60–110 nts) after successive enzymatic maturation steps in the nucleus and in the cytoplasm (402). miRNAs bind primarily to the 3' UTRs of their target transcripts and thus reduce the levels of their target transcripts and the amount of protein encoded by the transcripts. It has been shown that miRNAs are involved in many important biological processes, including development, differentiation, metabolism, cell cycle regulations, and apoptosis, and in various cancers (44).

As described above, a homozygous or heterozygous deletion of the long arm of chromosome 13 is among the most common genomic abnormalities in CLL. Recently, miR-15a and miR-16-1 located in the minimally deleted regions at 13q14.3 have been found to be expressed highly in CD5 positive cells and reduced in CLL, high-grade B-cell lymphoma, myeloma, and other cancer cells (44,403,404). Both microRNAs are located in a region of exons 2 and 5 of the LEU2 gene, and it is proposed that the loss of both microRNAs is an early event in the pathogenesis of CLL (218). Both miR-15a and miR-16-1 negatively regulate BCL2 at a post-transcriptional level, and thus induce apoptosis in leukemia cells (405). Furthermore, a unique microRNA profiling for CLL was established that is associated with poor prognostic factors, such as IG Vh mutations and ZAP70 expression, and with disease progression in CLL (406). Higher miR-15a and miR-16-1 expression is associated with de novo aggressive CLL, and can affect chemotherapy potency and lead to drug resistance. There are many novel and promising discoveries of microRNA in CLL and other cancers, and some of these new biomarkers have become therapeutic targets on clinical trials (48,397).

### 75.13.6 Multiple Myeloma

As in CLL, the application of molecular cytogenetic tools, such as FISH and microarray techniques, has led to the discovery of numerous chromosomal abnormalities in MM, its precursor monoclonal gammopathy of undetermined significance (MGUS), and plasma cell leukemia (407). These chromosome and genomic abnormalities apparently occur stepwise following two different pathways involved in the pathogenesis of MGUS and MM (see below). In MM, abnormalities of the *IGH* locus in various chromosome translocations are found in about 40% of cases. *IGH* translocations deregulate the expression of oncogenes located near the translocation breakpoints, and are detectable by interphase FISH analysis in 50% of patients with MGUS, 60–75% of patients with MM, and more than 80% of patients with plasma cell leukemia (407). Five recurrent chromosome translocations have been observed and are associated with various prognoses. The t(11;14)(q13;q32) is found in 16% of cases, and results in the IgH/CCND1 fusion. The t(4;14)(p16;q32) is noted in about 15% of patients and deregulates the expression of the fibroblast growth

factor receptor 3 gene (*FGFR3*) and *MMSET* on the der(4) chromosome. The t(14;16)(q32;q23) is present in about 5% of cases and involves the juxtaposition of *IGH* and *MAF* (v-maf musculoaponeurotic fibrosarcoma oncogene homolog). The t(6;14)(p21;q32) is detected in about 3% of MM patients and leads to CCND3 overexpression. The t(4;14) and t(14;16) are associated with a poor clinical outcome, whereas the t(11;14) and t(6;14) confer a more favorable prognosis. Approximately 50% of patients with MM have a hyperdiploid clone with gain of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21. These gains can be seen alone or more frequently in combination. A hyperdiploid karyotype in MM is associated with longer survival as compared to translocations involving 14q32 (408). Interestingly, samples with a hyperdiploid karyotype very rarely have rearrangements of *IGH* suggesting that there are two mutually exclusive pathways for the development of MM. Loss of chromosome 13 or a del(13q) is the most frequently observed chromosomal loss in MM (40–50%) and confers a poor prognosis, in particular when detected by metaphase analysis (407). Interestingly, microarray analyses have revealed that *miRNA-15a* and *miRNA-16* display a range of expression patterns in MM, independent of the chromosome 13 status, indicating that genes other than *miR-15a* and *miR-16* may explain the prognostic significance of 13q14 deletions.

Recently, gene expression profiling analysis in MM showed dysregulated and/or increased expression of cyclin D1, D2, or D3 in all of the above genetic pathways virtually in all MM and MGUS patients (407,409). Cyclin D1 is bi-allelically dysregulated in a majority of hyperdiploid myeloma patients. Increased expression of one of the cyclin D proteins facilitates activity of CDK4 or CDK6, which then phosphorylates and inactivates RB so that E2F can facilitate G1>S cell cycle progression. During the progression of MGUS to MM, activating mutations of K- or N-RAS play a critical role, in cooperation with loss or deletion of chromosome 13. Subsequently, additional oncogenic events occur including MYC dysregulation, bi-allelic deletion of p18 and loss or mutation of p53 resulting in more advanced stages (410). Thus, MM actually covers several subgroups that have differences in early or initiating events, global gene expression patterns, bone marrow dependence, clinical features, prognosis, and response to therapy (411).

### 75.13.7 t(2;5)(p23;q35) and the *ALK/NPM1* Fusion in Anaplastic Large T-Cell Lymphoma

t(2;5) is one of a few recurring chromosome translocation identified cytogenetically in mature T-cell lymphoma. Patients bearing this translocation have a specific histologic pattern of diffuse pleomorphic large cell lymphoma often called malignant histiocytosis, phagocytic large T-cell lymphoma, Ki-1 positive lymphoma, or now anaplastic large cell lymphoma (ALCL) (412). The

lymphoma cells are CD30 positive with variable morphologic features. The genes involved in this translocation are the nucleophosmin gene (*NPM1*) on 5q35 and the anaplastic lymphoma kinase gene (*ALK*) on 2p23. *NPM1* is a ubiquitously expressed nucleolar protein that shuttles between the nucleus and cytoplasm. It is an important protein that is implicated in multiple functions, including ribosomal protein assembly and transport, control of centrosome duplication, and regulation of the tumor suppressor ARF. *ALK* encodes a tyrosine kinase of the insulin receptor superfamily that is normally expressed in the neural cells of intestine, testis, and brain but is silent in normal lymphoid cells (413–415). The *NPM1/ALK* fusion protein contains the *NPM1* oligomerization motif and the *ALK* catalytic domain; it is constitutively activated through autophosphorylation, and mediates malignant cell transformation in vitro and in vivo by activating downstream effectors, including STAT3. The fusion protein *NPM1/ALK* is able to transform NIH3T3 cells suggesting that activation of this kinase is the primary event in the transformation (416). The t(2;5) involving the *NPM1* gene causes cytoplasmic rather than nuclear localization of the *ALK* protein, which can be detected by immunohistochemistry studies. In addition to t(2;5), more than eight other chromosome translocations and inversions, such as t(1;2) (q25;p23), all resulting in up-regulation of *ALK*, have been reported in ALCL. Recently, *ALK* has been found to be a novel dependence receptor and is inactive in the absence of engaging ligand(s). Its expression results in enhanced apoptosis, whereas *ALK* activation, via a ligand-mediated engagement or as result of *ALK* fusion proteins, decreases apoptosis (413,415). Notably, the *NPM1/ALK* fusion induces *MYC* expression in all pediatric ALCL with t(2;5) and *ALK* expression, indicating that *MYC* may be a downstream target of *ALK* signaling (417). Patients with *ALK* expression and t(2;5) and other translocations have a significantly better prognosis than those without these genetic abnormalities.

## 75.14 MOLECULAR ANALYSIS OF RECURRENT CHROMOSOME ABNORMALITIES

### 75.14.1 How and When Consistent Translocations Occur

We do not know how consistent structural rearrangements occur, but there are at least two possibilities. The rearrangements may be random, but selection may act to eliminate the vast majority that do not provide the cell with a proliferative advantage. Alternatively, certain changes may occur preferentially and thus may be the ones we see. The detection of cells which may harbor gene rearrangements typical for leukemia or lymphoma, in individuals without any sign of malignancy, has become one of the most intriguing findings of recent

years (167,384). It should be noted, however, that in a majority of cases described a highly sensitive (one cell per  $10^6$ – $10^8$  of total number of cells) nested RT-PCR or nested PCR was used. In addition, a study has shown that *BCR/ABL*, *AML1/ETO*, and *DEK/NUP214* (a nucleoporin gene) genes are generated spontaneously in hematopoietic cell lines in culture after exposure to ionizing radiation. However, these cells fail to proliferate in a malignant fashion and they die. Earlier studies showed an association of chromosome rearrangements in tumor cells with fragile sites affecting one of the chromosome bands broken in the tumor cells; however, the contribution of fragile sites to tumorigenesis is very unclear (418). The recognition of leukemia/lymphoma associated fusion transcripts in healthy individuals has been an important discovery, but much remains to be learned about its biologic significance.

Genomic analysis of translocation breakpoints has shown that some occur within *Alu* sequences (419,420), whereas others occur near topoisomerase II sites or DNaseI hypersensitive sites (421–424). A number of chromosome translocation breakpoints, for example, t(12;21), identified in ALL patients have been mapped near extended tracts of alternating purine and pyrimidine residues (425). These segments are known to form a helical structure known as Z-DNA. These regions might be susceptible to DNA recombination events because these repeat regions are susceptible to “slippage” during DNA replication both in vitro and in vivo (426,427).

Many of the chromosome rearrangements in B- and T-cell tumors involve sequences used in the normal recombination mechanisms of the V-D-J segments of the immunoglobulin and T-cell receptor genes. The presence of heptamer and nonamer sequences in the non-immunoglobulin gene involved in the translocation, for example, *MYC* and *BCL2*, has been reported (428,429). However, myeloid cells do not express recombinase-activating gene (*RAG*), and so this mechanism cannot explain translocations in these cells.

An equally important question is, when in the multistage process of malignant transformation of a particular cell do translocations or other chromosomal aberrations occur? Some chromosomal changes occur as part of the further evolution of the malignant phenotype, for example, blast crisis of CML, and are therefore relatively late events. But what about the occurrence of the t(9;22) in CML, for example? Does the translocation occur in a single normal cell, which becomes the progenitor of the leukemic clone, or is there expansion of a clone, possibly a pre-leukemic one, in which a translocation occurs in one of these already abnormal cells? In 1985, Fialkow and Singer have presented detailed evidence supporting the latter proposal. It was shown that some *BCR/ABL* transcripts detected in normal leukocytes and in the non-CML cell lines have an aberrant structure owing to incorrect

junctions between *BCR* and *ABL* exons; such genes would encode truncated BCR/ABL proteins that would presumably not be able to promote a growth advantage (430).

From all the data discussed in this chapter, it is clear that additional mutations as yet unidentified are generally needed to transform a minimally affected cell into a truly malignant expansion cell. There are several lines of evidence for this proposal. First, Adams and Cory constructed transgenic mice whose cells all had a vector containing the *MYC/IGH* junction from a murine plasmacytoma (431). All cells contained this construct; however, the B-cell tumors that occurred in every animal were clonal. Second, rare families with an inherited predisposition to develop AML exist (432). Third, molecular studies of childhood ALL showed that specific chromosome translocations may originate prenatally, although frank leukemia develops months or years later (307).

We have already discussed possible causes for mutagenic genetic events leading to leukemia development, and it is very likely that different causal mechanisms are at play in different patients. It is also important to consider genetic predisposition, which is poorly understood in the hematologic malignancies.

### 75.14.2 Specificity of Chromosome Rearrangements

The evidence presented in this chapter clearly demonstrates the remarkable specificity of certain chromosome rearrangements for particular subtypes of tumor, especially leukemia or lymphoma. The mechanism(s) by which this specificity is achieved is(are) unknown; however, a number of investigators have shown that certain proteins required for the promotion of gene expression are synthesized in a very cell-type specific manner. These proteins are only present in the appropriate cell type, and therefore the particular gene is activated only in that cell type. The chromosome rearrangements affecting *MYC* in B- and T-cell tumors strongly support the interpretation that the specificity resides in the gene that is uniquely active in a particular cell type (433). Thus the immunoglobulin genes are highly regulated in B cells and can, therefore, serve as the switch or activator mechanism for *MYC* in B cells; on the other hand, *TCRA* is an active gene in T cells with a strong enhancer and promoter and is clearly an activator for *MYC* in T cells. A reasonable paradigm is that translocations bring together in an inappropriate manner a growth factor, a growth factor receptor gene, or a transcription factor adjacent to an active cell-specific gene. It should be emphasized that some of these genes, which have been called proto-oncogenes, were identified as oncogenes in viruses that cause tumors. However, these genes have not been conserved through evolution from yeast and *Drosophila* to the chicken, mouse and humans solely to cause cancer.

### 75.14.3 Biologic Consequences of Consistent Chromosome Abnormalities

Cloning of many of the chromosome translocation breakpoints and the identification of the involved genes has had a major impact on our understanding of at least one critical event in the transformation of a normal cell to a leukemic one (434). The translocations in the lymphoid leukemias and lymphomas that involve the immunoglobulin genes in B-lineage tumors and the T-cell receptor genes in T-lineage tumors result in inappropriate expression of the other gene in the translocation, with no alteration in its protein structure. In contrast, all of the translocations cloned to date in the myeloid leukemias result in a fusion mRNA and a chimeric protein. This same situation is true for the t(1;19), t(4;11), t(11;19), and t(12;21) in ALL.

Cloning of the translocation breakpoints has led to the identification of a large number of new genes. It has been pointed out repeatedly that most of the genes cloned from the breakpoints in acute leukemia have been transcription factors, for example, *MYC*, *AML1* (*RUNX1*), and others. The *MLL* gene, however, appears to be involved in maintaining the chromatin structure of a gene in a more open configuration to allow transcription. These are so-called architectural genes, whose function involves modifying the chromatin conformation of genes, rather than altering the level of gene expression. Moreover, many of the translocation partners of *MLL* are normally part of the same large protein complex containing *MLL*, which is bound to DNA. This complex is involved in the regulation of the rate of mRNA transcription from the target gene. Thus, to have a critical partner present in a translocation appears to lead to a substantial growth advantage for cells containing the *MLL* fusion gene. Our new sophistication regarding the genetic changes in hematologic malignant diseases provides us with some very critical new diagnostic tools (12). Standard Southern blot analysis of tumor DNA can reveal clonal genomic rearrangements of genes using the appropriate probes. PCR can increase the sensitivity of detection of these aberrations; sometimes the sensitivity is too great to be clinically applicable. The development of gene expression array and genomic SNP microarray technology provides a powerful tool to analyze a tumor sample for measuring gene expression dynamics at various disease stages, for novel genomic alternations including gains and losses of genes, and for CN-LOH (31,38). cDNA and microRNA expression microarray technology classifies tumors based on their gene expression profiles. This allows for the characterization of tumors by the biologic pathways that are disrupted and or deregulated as opposed to primary DNA abnormalities. In some cases expression profiles could be used to identify patients who were likely to fail therapy or develop secondary disease (435,436). Genomic SNP microarray

techniques have greatly enabled us to detect subtle genomic imbalances and CN-LOH in various tumor cells, such as *PAX5* and *IKZF1* deletions in ALL.

Our increasing precision in identifying the genetic changes in the malignant cells comes at a most opportune time, because physicians will soon be in a position to use targeted therapy aimed at the specific genetic defect in the malignant cells. To use this targeted therapy effectively requires a precise genotype of the malignant cells. Two very powerful examples of targeted therapy are the use of ATRA in APL and of Imatinib (Gleevec) in CML. Although a number of genes will be affected in various genetic alterations leading to a tumor cell, those involved in chromosomal changes may be among the easiest to monitor and the most effective to treat.

#### 75.14.4 Cooperation of Multiple Genetic Abnormalities in Leukemogenesis and Lymphomagenesis

As described above, there are many acquired recurring chromosomal abnormalities and gene mutations in leukemia and lymphoma. Some of these aberrations are unique with a strong association with clinical and morphological features. From various transgenic mouse models, it is assumed that most of these genetic abnormalities alone are not sufficient in inducing leukemia and lymphoma. Coexistence of different genetic changes, such as chromosome translocations that usually result in loss of function or mutations of transcription factors and gene mutations involving tyrosine kinases, suggests that these aberrations cooperate in oncogenesis. As described in the earlier sections in AML, the t(8;21) or inv(16)/t(16;16) and *c-KIT* mutations are common in CBF leukemia, and *FLT3* mutations in AML with other recurring chromosome translocations. In patients with Down syndrome, Look et al. reviewed the interactive mechanism of the *GATA1* mutations with trisomy 21 in leading to acute megakaryoblastic leukemia. Gilliland et al. proposed a hypothesis of “two-hit” model that at least two classes of mutations are involved to transform a normal HSC into a clonal AML. Class I mutations are activating mutations that confer proliferative and/or survival advantage to hematopoietic progenitors, but do not affect differentiation (437–439). It commonly involves genes of tyrosine kinases pathways, such the *BCR/ABL1* fusion in CML, and the *TEL/PDGFRB* in CMML, *JAK2* point mutations in MPN, and *KIT* and *FLT3* activating mutations in AML, and genes in the *RAS/BRAF* pathway in AML and MDS. These constitutively activated tyrosine kinase genes provide several therapeutic targets with tyrosine kinase inhibitors, such as Imatinib in CML. Class II mutations result in loss of function of those genes that are transcriptional factors and tumor suppressor genes, and serve primarily to impair hematopoietic differentiation and subsequent apoptosis, such as the *AML1/ETO* and *CBFB/MYH11*

fusion in CBF leukemia, the *PML/RARA* fusion in APL, translocations involving *MLL* and *EVII1*, mutations in *CEBPA* and *NPM1* in AML and MDS, and the *TEL/AML1* fusion in ALL. This hypothesis has been validated in acute leukemia evolving from MPN. In MPN, leukemia cells carry various mutations, such as *JAK2* and *PDGFRA/B*. The clinical course is usually mild with a long latency to evolving to acute disease. To generate overt leukemia, a second class of genetic alterations must produce lineage-specific blocks in differentiation, such as translocations and deletion and duplications that involve genes encoding chimeric transcription factors produced by chromosomal translocation. The most common cooperation mutations in myeloid leukemia are mutations between *AML1* and *RAS*, *NPM1* and *FLT3*, and *MLL* and *BRAF* (440–442).

The last few years has revealed many mechanisms related to the epigenetic regulation of genes, such as microRNAs (discussed in the CLL section) plus histone modification at both the DNA and RNA level (42). Many of the partner genes of common AML translocations such as *ETO* and *PML* are repressed through their association with HDACs and their repression passes to the partner fusion gene *AML1/RUNX1* or *RARA* which has critical function related to cell growth and differentiation (194,442,443). The next revolution is likely to involve more RNA species about which we are ignorant, as well as a more sophisticated understanding of the role of protein modification in the cell. Finally, we pay little attention to lipids, to the regulation and interaction of cell organelles, of scaffold proteins, and their essential roles in protein transport and intra- and intercellular signaling. Although we have made enormous progress in the last five or six decades, we have a long way still to go before we will be equipped to monitor the critical changes in each tumor that will allow us to really practice personalized medicine. Using novel techniques, such as microarray of genomic DNA, gene expression and microRNA, and next generation sequencing, many novel genetic abnormalities will be found in leukemia and lymphoma, as well as in other solid tumors of epithelial cell origin (135,379,415). We will better understand the genetic mechanism of oncogenesis and thus provide more powerful and personalized treatment options.

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Leukemias, Lymphomas, and Other Related Disorders  
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### Biographies



**Dr Janet D Rowley** is Blum-Riese Distinguished Service Professor of Medicine, Molecular Genetics and Cell Biology, and Human Genetics, Pritzker School of Medicine, University of Chicago. She earned a Bachelor of Philosophy degree (1944), a Bachelor of Science degree (1946) and a Doctor of Medicine degree (1948) from the University of Chicago.

A faculty member at the University of Chicago since 1962, Dr Rowley is internationally renowned for her studies of chromosome abnormalities in human leukemia and lymphoma. In 1972, she discovered the first consistent chromosome translocation in any human cancer, the t(8;21) in AML. In a landmark paper in 1973, Dr Rowley described the t(9;22) in CML. Subsequently, she identified more than a dozen different recurring translocations in children and adults with leukemia and lymphoma. Her discoveries have resulted in more accurate diagnostic techniques and the development of effective treatment protocols targeted to particular genetic abnormalities, such as Imatinib for CML.

Dr Rowley is a member of the National Academy of Sciences, the Institute of Medicine, the American Philosophical Society, and the American Academy of Arts and Sciences. She was awarded the 2011 Ernest Beutler Lecture and Prize from the American Society of Hematology, the Seventh Annual AACR Award for Lifetime Achievement in Cancer Research in 2010, the 2009 Presidential Medal of Freedom, the 1998 Albert Lasker Clinical Research Award, the 1998 National Medal of Science, and the 1989 Charles S. Mott Prize from General Motors Cancer Research Foundation.



**Dr Yanming Zhang** attended a medical school in China in the early 1980s, and received his MD degree in 1994 in Germany. He did postdoctoral research training in cytogenetics in non-Hodgkin lymphoma in Kiel, Germany from 1995 to 1998, and continued on characterizing genomic breakpoints and the mechanisms of chromosome translocations in AML under Dr Janet D Rowley's guiding at the University of Chicago between 1999 and 2002. Later Dr Zhang finished a 2-year clinical cytogenetics fellowship training that was accredited by the American Board of Medical Genetics, and became a board certified clinical cytogeneticist. From 2005 to 2010, he was Assistant Professor of Medicine, and served as Assistant and Associate Director with Dr Michelle M Le Beau as Director in the Cancer Cytogenetics Laboratory at the University of Chicago. Since 2011, Dr Zhang is Associate Professor of Pathology at Northwestern University, Medical Director of newly established Cytogenetics Laboratory at Northwestern Memorial Hospital, and a member of R. Lurie Comprehensive Cancer Research Center at Northwestern University. Dr Zhang's research interests are to clarify the mechanisms of recurring chromosome translocations in leukemia and lymphoma and to study genomic imbalances in cancer using genomic microarrays and other molecular techniques.

# CHAPTER

# 76

## Immunologic Disorders

### *Autoimmunity: Genetics and Immunologic Mechanisms*

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### 76.1 INTRODUCTION

Autoimmunity is a breakdown of the normal mechanisms that maintain immunologic homeostasis in the immune response to specific antigens. The pathology of autoimmune disease is multifactorial, including both genetic and environmental factors affecting the onset, maintenance, and progression of the disease (1–6). Autoimmune diseases result from the recognition of self antigen(s) and the subsequent attack of self-tissues, usually by the adaptive immune response. Autoimmune pathology can be caused by T lymphocytes, natural killer (NK) cells, and/or antibodies and can be modulated by hormonal effects. One of the unifying themes of many autoimmune disorders is that they are associated with specific alleles and genotypes of the highly polymorphic major histocompatibility complex (MHC) where, in humans, the human leukocyte antigen (HLA) loci reside. Since the completion of the human genome project in April 2003 (<http://www.genome.gov/10001772>), a plethora of new information about the genetics of autoimmune disease has allowed the identification of a number of new genes involved in autoimmunity. The aim of this chapter is to present an update of recent genetic associations of autoimmunity with common genetic variants obtained through the genome-wide association studies (GWAS) and how these data have changed our knowledge of selected diseases, which historically showed an association with HLA (7,8).

### 76.2 THE IMMUNE RESPONSE

#### 76.2.1 The Adaptive Immune System

In general, the mechanisms that produce tissue damage and clinical disease in autoimmune disorders are the same as those that mediate protection from pathogens. The immune system consists of a system of interacting molecules and cells that function to (1) recognize intracellular and extracellular threats (primarily pathogens); (2) alert and activate lymphocytes, monocytes and NK cells for specific response to these threats; (3) commit the responding cells to an appropriately regulated response to the threat; (4) amplify the response, if necessary, by recruitment of additional cells, including polymorphonuclear (PMN) leukocytes and macrophages, as well as additional lymphocytes and monocytes; and (5) ultimately, terminate the specific immune response after the threat has resolved.

The major components of the human adaptive immune response consist of antigen presenting cells (APC), T lymphocytes, B lymphocytes, HLA molecules, peptides, and costimulatory molecules. An immune response occurs when a peptide bound to an HLA molecule is recognized by a T-cell receptor (TCR), a phenomenon known as *antigen presentation*. The specific peptide, HLA molecule, and T cell together determine the nature of the response. There are two general categories of HLA molecules that perform this antigen presentation function: class I molecules (e.g.

A, B, and C) and class II molecules (e.g. DR, DQ, and DP), each class having a different tissue distribution.

X-ray crystallographic structural studies have revealed that the outer domains of both class I alpha-chain molecules and the class II alpha-beta heterodimers form a peptide-binding groove, consisting of a beta-pleated sheet floor and two alpha-helical walls (9,10). Typically, the peptides bound in class I clefts are octamers or nonamers with the ends of the peptide buried within pockets of the cleft (11). CL-II peptides bound in the class II groove are somewhat longer, usually 12–14-mers, with the ends protruding from the cleft. The extensive polymorphism in HLA molecules lies primarily in the amino acids forming the floor and sides of the groove. The topography of the groove is unique for each allelic group of HLA molecules. Because only certain amino acid residues can “fit” into certain pockets based on size and charge, the peptides that can be bound by any given HLA molecule are limited. Some polymorphic residues within the HLA binding groove bind to the peptide, whereas others bind to the T-cell receptor (12). The structure and the capacity of HLA molecules to bind different peptides differ widely among different individuals owing to the extensive genetic polymorphism of the HLA genes, accounting for the association between genetic polymorphisms and variable immune response (13–16). Thus, it is the specific combination of a peptide–HLA complex that provides the genetically controlled determinant of antigen-specific recognition.

It is the complex of specific peptide bound within a given HLA antigen that is recognized by the TCR. The TCR of CD4 T cells generally recognize peptide in the context of class II molecules to provide T-cell help (Th). These T helper cells can be of at least two different types. In general, Th1 cells provide help to other T cells (CD8) to affect cell killing. Th2 cells provide help to B cells in the production of antibody. By virtue of the cytokines they release, Th1 cells are predominantly inflammatory, whereas Th2 cells can often inhibit the inflammatory response. However, Th2 cells can contribute to an overproduction of antibody, which is a pathologic feature of some autoimmune diseases. In contrast, the TCR of CD8 T cells generally recognize peptide in the context of class I molecules. These cells perform target cell killing and are known as cytotoxic T lymphocytes (CTL). In addition, a subpopulation of both CD4 and CD8 T cells can carry out a regulatory (suppressive) role, in which case they are referred to as T-regulatory cells (TR or Tregs). These latter cells are CD4+CD25+FOXP3+ or CD8+CD25+/-FOXP3+. The Tregs (2–10% of the circulating T cells) are essential in the maintenance of normal tolerance to self antigens. Dysregulation of the Tregs can lead to profound autoimmunity (17–19).

In the adaptive immune system, a specific immune response is initiated when a peptide bound to an HLA class II molecule on the surface of an APC, such as a macrophage or dendritic cell, is recognized by the T-cell receptor on a CD4 T lymphocyte, which is specific for the peptide–HLA molecule complex. T-cell activation

results from this antigen-specific recognition event, provided a costimulatory interaction between B7 molecules (also known as CD80 and CD86) on the APC and CD28 on the T cell has also occurred (see Section 76.4.2).

## 76.2.2 The Innate Immune System

NK cells are one of the first lines of defense of the innate immune system, functioning before initiation of the adaptive immune response, as well as when the latter system is subverted, as happens in some viral infections and cancer and likely in autoimmunity (20). Regulation of NK cytolytic activity is a function of engagement of one or more NK surface receptors that may be activating or inhibitory. Two of the primary sets of receptors present on human NK cells are called the killer immunoglobulin-like receptor (KIR) and the CD94/NKG2 lectin-like receptors (21–23). They each are comprised gene families. The KIR genes (15 genes, 2 pseudogenes) located on chromosome 19q13.4 have enormous genetic diversity generated by a combination of variable gene content on any given haplotype, alternative splicing, and allelic polymorphism, and they recognize a limited set of HLA Class I molecules or epitopes. The more ancestral CD94/NKG2 genes (four genes plus NKG2D) located on chromosome 12p12.3–p13.2 have less diversity but an unconventional array of HLA Class I-like ligands. The ligands for the KIR are the HLA classical Class I molecules A, B, C and the nonclassical Class I, HLA-G. HLA Class II does not appear to be involved. The CD94/NKG2 and NKG2D ligands are generally HLA Class Ib molecules such as HLA-E or stress inducible nonclassical HLA Class I-like molecules such as MICA and MICB [MHC Class I-related chain] proteins (24). In the innate immune system, *de novo* recognition of self antigens can occur when cryptic epitopes become exposed due to cell death/apoptosis (25) and can lead to autoimmune disease (see Section 76.3).

## 76.3 AUTOIMMUNE PATHOLOGY

Autoimmunity is thought to involve the activation of a self-peptide-specific T cell or NK cell, leading to the development of autoreactive CTLs, the inappropriate activation of macrophages, and the release of cytokines as well as inappropriate T-cell help, resulting in the formation of antibodies to self antigens. Autoimmune tissue damage is mediated by autoantibodies, autoreactive T cells, or NK cells. Autoantibody-mediated disorders are distinguished by the different types of antigens that are recognized and the different classes of immunoglobulin that result in disease. Allergic (atopic) reactions are mediated by the production of IL-4 by CD4 Th2 cells, leading to the production of immunoglobulin E by B cells, in response to extrinsic (not self) antigens. The IgE can bind to and activate mast cells via the Fcε R1, releasing histamines, and other mediators and causing clinical symptoms, such as asthma or allergic rhinitis (hay fever). Some autoimmune diseases result from the production of IgG antibodies in response to self



**TABLE 76-1** Selected Autoimmune Diseases and Associated HLA Alleles

Disease	Associated HLA Alleles
<b>Organ-specific (endocrine)</b>	
Type 1 diabetes	DRB1*03:01–DQB1*02:01; DRB1*04:01–DQB1*03:02 <sup>a</sup> DRB1*03:01–DQB1*02:01; DRB1*04:01–DQB1*03:02 <sup>a</sup>
Graves' disease	DRB1*03:01–DQB1*02:01
Hashimoto's thyroiditis	DRB1*11–DQB1*03:01
Idiopathic Addison's	DRB1*03:01–DQB1*02:01 disease
<b>Organ-specific (other)</b>	
Inflammatory bowel	DRB1*01:03–DQB1*05:01; disease DRB1*01:03–DQB1*03:01
Crohn's disease	DRB1*11:01
Ulcerative colitis	DRB1*11:01
Myasthenia gravis	DRB1*03:01–DQB1*02:01
Multiple sclerosis	DRB1*15:01–DQB1*06:02
Psoriasis vulgaris	C*06
Pemphigus vulgaris	DRB1*04:02–DQB1*03:02; DRB1*14:01–DQB1*05:03
Narcolepsy	DQB1*06:02
Celiac disease	DQA1*05:01–DQB1*02:01 (in cis or in trans; see text)
Dermatitis herpetiformis	DQA1*05:01–DQB1*02:01
<b>Rheumatologic diseases</b>	
Rheumatoid arthritis (RA)	DRB1*04–DQB1*03:02 <sup>b</sup> ; DRB1*01– DQB1*05:01; DRB1*10– DQB1*05:01
Pauciarticular juvenile RA	DRB1*08–DQB1*04:02; DRB1*11– DQB1*03:01, DPB1*02:01
Ankylosing spondylitis	B*27
Reactive arthropathy,	B*27 including Reiter's disease
Systemic lupus	DRB1*03:01–DQB1*02:01 <sup>c</sup>
erythematosus (C4A null allele)	DRB1*15:01–DQB1*06:02

<sup>a</sup>Not all DRB1\*04 alleles confer equal risk for type 1 diabetes; DRB1\*04:03 and \*04:06 are protective, whereas the highest risk DRB1\*04 alleles are 04:01,04:02, and 04:05. The highest risk genotype is DRB1\*03:01–DQB1\*02:01/DRB1\*04–DQB1\*03:02 (see text).

<sup>b</sup>Not all DRB1\*04 alleles confer equal risk for RA; DRB1\*04:01 and 04:04 and 04:05 appear to confer the greatest risk. These alleles also appear to be associated with the severity of disease, unlike the disease-associated DRB1\*01 and DRB1\*10 (see text).

<sup>c</sup>The DRB1\*03:01 association with SLE is likely due to linkage disequilibrium with the C4A null allele on B\*08-DRB1\*03:01 haplotypes (see Section 76.7.9).

antigens, such as cell-surface receptors. The autoantibody can disrupt the normal function of the receptor by causing uncontrolled activation (agonist) or by blocking receptor function (antagonist) and signaling. In Graves' disease, autoantibodies to the thyroid-hormone-stimulating receptor cause activation and hyperthyroidism, whereas in myasthenia gravis, autoantibodies to the acetylcholine receptor disrupt function and cause progressive muscle weakness. Some antibody-mediated autoimmune diseases, such as pemphigus vulgaris (PV), involve the production of IgG in response to cell- or matrix-associated antigens (the skin matrix protein, desmoglein 3 for PV), leading to tissue damage (blistering of skin and mucosal membranes for

PV). Another form of antibody-mediated disease, such as systemic lupus erythematosus (SLE), reflects the production of antibodies to soluble antigens subsequent to exposure to cryptic autoantigens. In these diseases, the tissue damage is caused by responses triggered by immune complexes. A selected set of autoimmune diseases and their associated HLA alleles are listed in Table 76-1 and the putative autoantigens and pathogenic immunological mechanisms for some of these diseases are listed in Table 76-2.

In most autoimmune diseases, the environmental trigger or the eliciting antigen is not known. In some autoimmune diseases, infection with specific pathogens may represent an environmental trigger, eliciting an autoimmune response in genetically predisposed individuals. There are several hypothetical mechanisms by which infectious agents could break tolerance and induce auto-reactive responses, such as molecular mimicry, release of cryptic self antigen, and activation of nontolerized cells, among others. Although epidemiologic studies may ultimately reveal the role of an infectious environmental trigger, direct evidence for the initiation of autoimmunity by an infectious agent is still lacking for most human autoimmune diseases.

## 76.4 AUTOREACTIVE T CELLS AND THE FAILURE OF TOLERANCE

The maturation of T cells in the thymus involves positive selection as well as negative selection (deletion of self-reactive T-cell clones). The presence of autoreactive T cells in autoimmune disorders indicates that this latter process is not complete. Current consensus is that only the highest affinity self-reactive T cells are deleted in the thymus. Thus, self-reactive T cells with lower affinities may escape negative selection, as well as T cells specific for self peptides that are not expressed in the thymus. These autoreactive T cells may, under normal conditions, be prevented from mediating autoimmune disease by never encountering the relevant antigen or by being in a state of Treg-induced quiescence or by some other unknown regulatory mechanisms. But environmental triggers, such as infection, may disrupt these mechanisms in genetically predisposed individuals.

### 76.4.1 Thymic Education: Positive and Negative Selection

The diversity of TCR specificities creates a broad functional potential for antigenic recognition, an essential component of the adaptive immune response. By chance, TCR specificities, which are potentially reactive with self antigens, are also created during random TCR gene rearrangement, creating the potential for autoreactive T cells. In the normal course of T-cell maturation, however, several TCR selection steps are designed to protect the individual from expression of such autoreactive T cells. This pathway of T-cell development is called

**TABLE 76-2 Selected Autoimmune Diseases and Autoantigens**

Disease	Immunologic Mechanism	Putative Autoantigen	Clinical Consequences
Graves' disease	Antibody to cell surface receptor (agonist)	Thyroid stimulatory hormone receptor (TSH)	Hyperthyroidism
Myasthenia gravis	Antibody to receptor (antagonist)	Acetylcholine receptor	Progressive muscle weakness
Pemphigus vulgaris	Antibody to skin protein	Epidermal cathedrin (desmoglein 3)	Blistering of skin and mucosal membranes
Type 1 diabetes	T-cell mediated	Insulin, GAD (glutamic acid decarboxylase), Insulin antibodies (IA-2), Islet cell antibodies (ICA)	$\beta$ -cell destruction, insulin insufficiency
Rheumatoid arthritis	T-cell mediated	Unknown synovial antigen	Inflammation and destruction of joints
SLE (systemic lupus erythematosus)	Deposition of immune	DNA, histones, small nuclear ribonucleoproteins (SnRNP), small cytoplasmic ribonucleoproteins (ScRNP), ribosomes	Arthritis, glomerulonephritis, vasculitis
Narcolepsy	Autoantibodies	Hypocretin, Tribbles Homolog 2 (TRIB2)	Excessive sleepiness

*thymic education* because it occurs predominantly in the thymus, the site of T-cell maturation.

After expression of rearranged TCR genes, immature T lymphocytes in the thymus are capable of receiving antigen-specific signals from HLA–peptide complexes expressed on APCs. Early in the maturation process, such T cells encounter HLA–peptide complexes presented by thymic epithelial cells arrayed in the thymic cortex. In a process known as *positive selection*, T cells at this immature stage receive growth and differentiation signals as a result of TCR ligation. T cells with rearranged TCR capable of reacting with self HLA and self peptide complexes continue to develop and mature (26). T cells with TCR that do not recognize self HLA–peptide complexes die within the thymus. This positive selection of T cells based on HLA–peptide recognition is responsible for the phenomenon known as *MHC restriction*, wherein T cells specific for a given peptide recognize that peptide only in the context of a particular HLA molecule. Thus, following TCR alpha and beta gene rearrangement, CD4, CD8 double-positive, immature T cells are positively selected to become single positive and restricted to either specific class I molecules (CD8 T cells) or specific class II molecules (CD4 T cells).

After positive selection, the T cells traffic to the thymic medullary areas, where they encounter HLA–peptide complexes expressed on a different type of APC, namely, hematopoietically derived monocytes and dendritic cells similar to the types of mature APCs used by the peripheral immune system to respond to antigenic challenge. In the thymic medulla, these immature T cells can proceed through one of the four developmental routes. One, when they encounter very strong antigenic signals from the HLA–peptide complex, an apoptosis (programmed death) response is triggered that results in the deletion or negative selection of these strongly autoreactive cells. Two, they can undergo receptor editing to display a receptor that is not, or is less strongly, self reactive. Three, they can become tuned or anergic, so they are less responsive to self antigens. Four,

T cells specific for self peptides that are not expressed in the thymus can exit the thymus as immunologically ignorant but potentially autoreactive and approximately 20–50% of the resulting TCRs are dangerously self reactive, requiring peripheral control (26). Experimental animal models of immune maturation have demonstrated that failure of this negative selection maturation step results in several types of organ-specific and systemic autoimmunity. A complex set of poorly characterized developmental regulatory genes controls this T-cell maturation pathway, some of which may contribute to human autoimmunity and aberrant immune response (27,28).

### 76.4.2 Breakdown of Immunologic Homeostasis or Equilibrium

The clonal deletion of autoreactive T cells in the thymus is only one of the mechanisms for preventing autoimmunity. In general, the adaptive immune response to a specific antigen is a dynamic and regulated process, reflecting the ability of the vertebrate immune system to maintain a “precarious equilibrium between the extremes of reactivity and quiescence” (reviewed in Reference (29)). The immune system has several fail-safe mechanisms to prevent aberrant or unregulated TCR activation, as well as several feedback mechanisms designed to guide pathways of T-cell commitment to specific effector functions. As a counter balance to immune activation, the immune system has several mechanisms for downregulating lymphocytes, and terminating an ongoing immune response. The disruption of these terminator pathways can result in uncontrolled immune amplification and autoimmune disease. Some mechanisms for lymphocyte regulation are mediated through cell surface intermolecular interactions, analogous to pathways used for activation. One of the most important of these pathways is the interaction between CTLA-4 molecules expressed on T lymphocytes and the CD80/CD86 molecules (B7) expressed on antigen presenting cells. The CTLA-4-B7 recognition

system acts as a counter balance to the CD28-B7 recognition system involved in T-cell activation. When the CTLA-4 protein is expressed on T lymphocytes and is activated by contact with the B7 molecule on APCs, it recruits phosphatases to the site of TCR activation complexes, which intersect and interrupt the kinase-mediated activation pathways, resulting in a negative, rather than a positive, signaling pathway (30). The lymphoid protein phosphatase (LYP), encoded by the gene PTPN22, is known to associate with the negative regulatory kinase Csk and to downregulate T-cell activation. The R620W polymorphic variant at this locus has been shown to be associated with a number of autoimmune diseases (see Section 76.7.7).

Another form of immune downregulation is mediated by the TNF cytokine family and by the Fas–Fas ligand set of cell surface ligands, leading to death of the target cells. These terminator effector pathways induce apoptosis, by triggering signaling cascades that activate death-mediating molecules such as caspases and that inhibit protective molecules such as BCL-2 (31). Yet another set of signal transducing cell surface molecules that can maintain homeostasis are the inhibitory and activating receptor systems on NK cells. In general, receptor systems on lymphoid and myeloid cells can result either in activation or inhibition depending on the structure of the cytoplasmic domain and the signals transduced when the receptor is engaged by the ligand. An appropriate balance between activation and inhibition signals seems to be required for normal immune modulation, by regulating initiation, amplification, and termination of specific responses. The absence of inhibitor signaling, as demonstrated in knockout mice with targeted disruption of inhibitory receptors, can result in autoimmune diseases and unregulated inflammatory responses (29).

## 76.5 GENETICS OF AUTOIMMUNE DISEASES

Familial clustering and comparisons of disease concordance among monozygotic and dizygotic twins clearly indicate that a variety of autoimmune diseases have a strong genetic component. Unlike the case of monogenic diseases caused by mutations, the alleles and genotypes that predispose to most autoimmune diseases are present in normal healthy individuals and generally only a minority of individuals with the high-risk genotypes actually gets the disease. Most autoimmune diseases are thought to be polygenic with a significant environmental component. In principle, polymorphism in any of the genes that encode elements in the T-cell activation pathways described earlier could play a role in genetic predisposition to specific autoimmune diseases. Linkage analysis studies have been used until recently to study possible transmission of two or more linked genes on a chromosome. Studies of autoimmune disease in families show some familial aggregation but the linkage analysis is generally of a low power. A linkage analysis study is feasible only if the genetic component of the disease is strong and samples are available from relevant family members.

Several historic studies indicate many autoimmune disorders that show linkage to and/or association with genes in the MHC and specific alleles of the HLA loci. The HLA region on the short arm of chromosome 6 (6p21.3), spans 3.6 Mb and contains approximately 200 genes, many of which are involved in immune function (32–34). Both the HLA class I and class II genes encode highly polymorphic cell surface molecules that bind and present processed antigens in the form of peptides to T lymphocytes. Recognition by the T cell of the HLA–peptide complex, along with a costimulatory signal, results in T-cell activation. The class I molecules HLA-A, -B, and -C are found on the surface of most nucleated cells, presenting peptides primarily derived from endogenously synthesized proteins (e.g. self, viral, and tumor peptides) to CD8 T cells. These heterodimers consist of an HLA-encoded alpha chain associated with a monomorphic polypeptide, beta-2 microglobulin, encoded on chromosome 15.

The HLA class II molecules consist of HLA-encoded alpha and beta chains associated as heterodimers on the cell surface of antigen presenting cells such as B cells, macrophages, and dendritic cells. Class II molecules HLA-DR, DQ, and DP serve as receptors for processed peptides, derived predominantly from membrane and extracellular proteins (e.g. self and bacterial peptides) and are presented to CD4 T cells. Both the HLA-DQ and DP regions contain one functional gene for each of their alpha (DQA1 and DPA1) and beta (DQB1 and DPB1) chains, as well as the pseudogenes DQA2, DQB2, DPA2, and DPB2. The HLA-DR region, however, contains one functional gene for the alpha chain (DRA), but either one or two functional genes for the beta chain, depending on the haplotype. All individuals express a DRB1 encoded polymorphic polypeptide that is found on the cell surface in association with the monomorphic alpha chain. The other functional class II DRB genes DRB3, DRB4, and DRB5 encode a beta chain that forms a second cell surface heterodimer with the DRA-encoded alpha chain. In general, the DRB3 locus is found on haplotypes where DRB1 is \*03 (comprising the serologic subtypes DR17 and DR18), \*11, \*12, \*13, or \*14; the DRB4 locus is found on haplotypes where DRB1 is \*04, \*07, or \*09, and the DRB5 locus is found on haplotypes where DRB1 is \*15 or \*16 (corresponding to the serological subtypes DR15 and DR16 of serologic type DR2). DRB1\*01, \*08, and \*10 haplotypes typically have only the DRB1 locus.

Many other genes with important immune functions are found within the MHC. In the class III region, between the HLA-DR region (class II) and the HLA-B locus (class I), are found the complement genes encoding C2 and C4, as well as the TNF-alpha and -beta loci. The MICA and MICB loci (see Section 76.7.9), are located just centromeric of HLA-B. The TAP1 and TAP2 loci are located between the DQ and DM loci.

The TAP loci encode the peptide transporter molecules involved in loading the newly synthesized class I molecules in the ER with peptides derived from proteolysis of proteins in the cytoplasm by the proteasome. The LMP2 and LMP7 genes, located near the TAP loci, encode the  $\gamma$ -interferon-inducible subunits of the proteasome. HLA-E and HLA-G are class I loci, whose polymorphism and tissue distribution are both much more limited than those of the classical HLA-A, B, and C genes. The HLA-G genes appear to be particularly important in pregnancy, where they are expressed at the maternal–fetal interface of the cytotrophoblast (35). There are many other genes within the MHC, whose function has not yet been elucidated. In general, these loci are all much less polymorphic than the HLA class I and class II loci.

## 76.6 HLA ALLELIC DIVERSITY AND POPULATION GENETICS

The HLA class I and class II genes are the most polymorphic coding sequences in the human genome. The total number of HLA alleles for all the loci, was of 6403 as April 2011 (The International Immunogenetics Project (IMGT)/HLA Database). Virtually all of this extensive sequence diversity is localized, for the class I loci, in the second and third exons, and for the class II loci, in the second exon. For the class I molecule, the peptide-binding groove is formed by a single chain with the beta-pleated sheet floor and two alpha-helical walls being encoded by the second and third exons. The class II polymorphic second exons encode the outer domains of the alpha and beta chains, which, together form the characteristic peptide-binding groove.

The patterns of allelic sequence diversity for both the class I and class II loci are highly unusual; some alleles differ in the polymorphic exons by as much as 15% and the sequence variation is distributed as a patchwork of localized polymorphic sequence motifs. The allelic diversity at these loci is thought to have been generated by recombinational mechanisms such as gene-conversion-like events or, to a lesser extent, by reciprocal recombination (33). Thus, new alleles appear to have been created by shuffling these discrete polymorphic sequence motifs. In addition, point mutation has contributed to sequence diversity at the HLA loci.

Although a very large number of alleles (e.g. >400 for HLA-DRB1) can be found in the global human population, a much smaller number (e.g. 30–50 for HLA-DRB1) is present in most individual populations. Many populations who have gone through bottlenecks or founding events (e.g. Native Americans) show more limited allelic diversity. In general, different populations tend to have different distributions of alleles as well as exhibit different patterns of linkage disequilibrium. Strong linkage disequilibrium is a striking feature of the genetics of the HLA region and can create difficulties in moving from an observed disease

association with an allele at one locus, to identifying a causal locus. In all populations, particular haplotypes consisting of specific alleles at the linked HLA loci are found much more frequently than would be expected at random. The linkage disequilibrium for this haplotype extends to the DPB locus, about 3Mb centromeric of HLA-A. Although a variety of evolutionary forces can create linkage disequilibrium, selection for particular combinations of HLA alleles has been suggested as the primary cause for these extended haplotypes. This strong linkage disequilibrium makes it difficult to assess which allele or which combination of alleles on a disease-associated HLA haplotype is responsible for observed correlations with disease. But the alleles found in linkage disequilibrium differ among various populations, disease association studies in many different ethnic groups can prove valuable in identifying the contributions of individual alleles.

## 76.7 GENETIC SUSCEPTIBILITY TO AUTOIMMUNE DISEASE

Linkage of genes within the HLA region to several different diseases has been demonstrated by cosegregation studies in families, or with nonparametric approaches such as haplotype sharing among affected sib pairs. The genetic region identified by linkage studies can be large, on the order of 5 cM (5% recombination). Higher resolution mapping of disease genes can be provided by disease association studies because these depend on strong linkage disequilibrium between the genetic marker and the disease allele. In general, the association of a marker with a given disease implies that the genetic marker is significantly <1 cM away from the disease gene. In case-control association studies, the frequency of a given genetic marker among unrelated patients is compared with the frequency in matched controls. Although many parameters influence linkage disequilibrium, such as population history, population admixture, the age of the marker and of the disease alleles, and so on, the strength of linkage disequilibrium is generally inversely related to physical distance. Thus, a strong association with a given disease, particularly if it is found in different ethnic groups, suggests either that the associated marker locus is very close to the disease locus or that it may itself confer susceptibility to the disease.

HLA alleles that are positively associated with disease are referred to as *susceptible*, whereas negatively associated alleles are termed *protective*. In heterozygote conditions in which one haplotype is susceptible and one is protective, protection is generally dominant. Table 76-1 lists a selected set of autoimmune diseases and their associated HLA alleles. Specific HLA alleles may enhance the autoimmune response by influencing immunogenicity thereby predisposing to autoimmunity by influencing the expressed repertoire of T cells.

These disease associations with specific HLA alleles have been demonstrated in numerous case-control



studies, as well as family studies in a large number of different populations (reviewed in Reference (36)). Most of these diseases are associated with alleles in the HLA class II region, but several diseases are associated with specific class I alleles. Some of the more common diseases associated with specific HLA alleles are listed below along with newer data of other genes that may also play a role in the disease process.

### 76.7.1 Ankylosing Spondylitis (AS)

There is a well-known correlation of HLA-B\*27 and AS. HLA-B\*27 is more common in northern Europeans and is rare in Africans and Australian Aborigines. Most studies reveal that 80–95% of AS patients are HLA-B\*27 positive. In white Europeans, the relative risk (RR) or odds ratio (see Section 76.7.8) for AS and B27 is well over 100. There are over 80 B27 alleles (see the Anthony Nolan Trust database, <http://hla.alleles.org>), some of which are sufficiently prevalent that a relative strength of their association with AS can be compared. There appears to be a hierarchy of associations with some B27 alleles and AS (reviewed in Reference (37)). HLA-B\*27:04 shows a stronger association than B\*27:05, B\*27:02, or B\*27:07, which in turn show a stronger association than B\*27:06 or B\*27:09. The reported total genetic risk for HLA-B\*27 and AS is 20–50%. Several theories have been proposed (reviewed in Reference (3)) to explain the HLA-B\*27 association with AS: HLA-B\*27 may present a microbial peptide that elicits a T cell response that cross-reacts with a self antigen; the HLA-B\*27 may misfold and thereby accumulating in the endoplasmic reticulum causing a cytokine response; and B\*27 heavy chains may be expressed at the cell surface as homodimers disrupting the normal HLA class I mediated response.

HLA-B\*27 may be a dominant genetic component but the presence of HLA-B\*27 alone is insufficient for the disease to develop. Thus, susceptibility to AS cannot be fully explained by associations with B\*27. GWAS indicate significant associations of non-MHC genes (37–40) including the interleukin-1 gene cluster, interleukin-23 receptor (IL23R) and endoplasmic reticulum-associated aminopeptidase 1 (ARTS1) genes (41–46) with AS. The IL1 gene complex is on chromosome 2 and encodes for IL-1 alpha, IL-1 beta, IL-1 receptor antagonist, and six other genes, IL-1F5 through IL1F10. A meta-analysis (47) confirmed the association of IL-1A with susceptibility to AS. A strong association was seen with three IL-1A SNPs (44). The IL23R is encoded on chromosome 1p31.3 and has been implicated in other diseases such as psoriasis and irritable bowel syndrome (IBD), both of which frequently occur in patients with AS. The IL23R finding strongly implicates the TH17 lymphocyte system in AS pathogenesis. IL17R induces the differentiation of naïve CD4+ T cell into TH17 helper cells. One particular SNP showed a strong association with AS, which was independent of an association with IBD (44,48). ARTS1 is encoded by three genes on chromosome 5. The

two functions of ARTS1 to cleave cytokine receptors (IL-6 receptor A, tumor necrosis factor R1 and IL-1 R2) from the cell surface and to process peptides to an optimal length for presentation by class I molecules (44). Four SNPs in ARTS1 have been found associated with AS. These studies have revealed possible pathways that regulate the disease that may lead to the development of approaches for therapeutic intervention such as targeting the TH17 system.

### 76.7.2 Behcet's Disease (BD)

BD is characterized by recurrent inflammatory attacks affecting the mouth, genital, mucosa, eyes, and skin. BD is prevalent in countries along the ancient silk routes and into Middle Eastern countries. HLA-B\*51 and HLA-A\*26 have been found associated with BD in various ethnic groups including Turkish and Japanese populations (49–51). Studies have also shown an independent MHC class I association telomeric to HLA-B (49). Kurata et al. (52) identified two SNPs in the region from HLA-A to HLA-E that were associated with BD independent of HLA-B\*51 and A\*26. Although HLA-B\*51 appears to be a strong genetic component in BD, it accounts for <20% of the genetic risk, indicating other genetic factors may be involved. GWAS and meta-analysis have identified common variants in IL 10 and at the IL23R-IL12RB2 locus (50,52). Several SNP haplotypes of the IL-10 gene promoter have been reported to be associated with regulation of the gene's expression. Genetic variants contributing to low IL-10 expression resulting in an inflammation-prone state in concert with the presentation of an unknown peptide by the HLA antigens may increase susceptibility to BD.

### 76.7.3 Multiple Sclerosis (MS)

MHC class II and more recently class I associations with MS have been reported as well as non-MHC components. In northern European populations, the RR for HLA-DR2 and MS is modest, on the order of 3. The same DR2 extended haplotype (DRB1\*15:01, DQB1\*06:02, DQA1\*01:02, DRB5\*01:01) (53,54) that confers increased risk for MS is highly protective (see Section 76.7.4) for type 1 diabetes (T1D). In African-American populations, the DRB1\*15:01 has been shown to have an association independent of DQB1\*06:02 (54–56). In Chinese populations, an SNP in an intron of HLA-DPB1 has been shown to have a strong association with MS susceptibility (55,57). Extended haplotype analysis has been handicapped by the very strong linkage disequilibrium across the MHC region. Conditioning studies of an SNP putative independent cluster in the class II and class I regions emerge with HLA-B\*44:02 giving a strong signal (58). A putative role for the class I molecules is to facilitate the transendothelial migration of antigen-specific CD8+ T cells into the brain (59).

Additional disease-associated genes have been discovered through the GWAS including two very interesting genes, protoglycan 5 (GPC5) and PARK2 (Parkin) (60). GPC5 is a member of the glypican family that plays an important role in the control of cell division. Since GPC5 is expressed in neurons, it may be important in interactions with growth factors, chemokines, and other extracellular matrix proteins. PARK2 is a part of the E3 ubiquitin ligase complex that mediated the targeting of substrate proteins for proteasomal degradation. It is abundant in the brain and mutations have been associated with juvenile-onset Parkinsonism (61). Three other SNPs have been found associated with MS along with other autoimmune diseases suggesting that they may play roles in the pathogenesis of different disorders (62).

#### 76.7.4 Type 1 Diabetes (T1D)

For some diseases, there is a hierarchy of risk associated with different alleles and genotypes, rather than a single predisposing allele. For T1D, the RR for the heterozygous genotype DR3/DR4-DQB1\*03:02 is about 30–40, whereas the RR for DR3/DR3 and DR4/DR4-DQB1\*03:02 homozygotes is around 5–8 and DR1/DR4-DQB1\*03:02 and DR4-DQB1\*03:02/DR8 heterozygotes also confer lesser but significant risk. The DR4-associated susceptibility to T1D reflects contributions both from the DQB1 locus, where \*03:02 but not \*03:01 is associated with disease, as well as from the DRB1 locus where \*04:01, \*04:02, and \*04:05 are disease associated. The DRB1\*04:03-DQB1\*03:02 haplotype is negatively associated with T1D. This pattern of disease association, involving specific combinations of HLA alleles, found either in *cis* in haplotypes (i.e. susceptible DRB1 and DQB1 alleles carried on one haplotype) or in *trans* in genotypes (i.e. susceptible allele of DRB1 carried on one haplotype and susceptible allele of DQB1 carried on the other), has been observed in other diseases as well.

Some associations are negative, suggesting a protective role for the associated alleles or haplotypes, such as DR2 and T1D. As noted earlier, the RR for the DR2 haplotype common among whites, DRB1\*15:01-DQB1\*06:02, is around 0.05. Other DR2 haplotypes are not negatively associated with T1D. On the basis of the disease association observed with various DR2 haplotypes (e.g. DRB1\*15:01-DQB1\*04:02 is not protective), the dominant protection conferred by the DRB1\*15:01-DQB1\*06:02 haplotype appears to be attributable primarily to the DQB1\*06:02 allele. It has been noted that this protection may be attributed to the volume of the P6 pocket and the specificity of the P9 pocket of the class I peptide-binding groove (63). DNA-based typing methods have made it possible to evaluate the role of individual polymorphic amino acid residues in HLA disease associations. Comparing a sequence that is disease

associated with closely related sequences that are not, can point to specific polymorphic sequence motifs and, given the known structure, to specific pockets in the peptide-binding groove as functionally important in disease susceptibility or resistance. The role of DQB1 position 57 in T1D susceptibility based on the negative association of Asp-57 with disease is well known (64), but the susceptibility of a given haplotype cannot be predicted by the presence of an individual amino acid residue. Clearly, there are some haplotypes that contain DQB1 Asp-57 (e.g. DRB1\*08:01-DQB1\*04:02 in whites or DRB1\*04:05-DQB1\*04:02 in Asians) that are positively associated with T1D (65).

#### 76.7.5 Pemphigus Vulgaris (PV)

Pemphigus is an autoimmune disease, in which antibodies specific for desmogleins (Dsg3) cause loss of keratinocyte cell adhesion and blisters. The onset and progression of the disease depends on multiple factors including drugs, diet, UV radiation and genetic background. A strong genetics background to PV has been shown in certain ethnic groups, such as Ashkenazi Jews and those of Mediterranean and south Asian origin. Associations between HLA class II alleles and PV have been reported (66–69). HLA-DRB1\*04:02 has been found associated with PV in Jewish populations, whereas HLA-DQB1\*05:03, HLA-DRB1\*14:04 and HLA-DRB1\*14:01 have been found associated in non-Jewish populations (67).

One theory is that this disease is caused by Th2 cells that induce the production of Dsg-specific autoantibodies. It is assumed that the MHC molecule forms a complex with peptides from Dsg3 (70). The negative charge of residues of DR 70 and 71 residues in DRB1\*04:02 and the DQB 57 residue of DQB1\*05:03, for example, confers selective binding to self peptides that have a positive charge at the P4 pocket in the peptide-binding groove. Similarly, the HLA-DRB1\*14:01 allele can present the same Dsg3 peptides. Thus, these alleles are the restricting elements of the T cell response to Dsg3 in PV patients. Antibodies specific for Dsg have been characterized and cloned from patients with PV (71). The variable heavy-chain gene usage complementarity determining region 3 (H-CDR3) was determined to be the most pathogenic. This H-CDR3 is critical in the antibody function and antigen specificity, suggesting that agents interfering with these areas may block pathogenic antibodies.

#### 76.7.6 Narcolepsy

Some autoimmune diseases still show stronger association with HLA than with genes identified by the GWAS. Narcolepsy is a sleep disorder characterized by excessive daytime sleepiness, cataplexy, and rapid eye movement during sleep. Narcolepsy has been shown to be strongly associated with the HLA-DRB1\*15:01-DQB1\*06:02 haplotype (72,73). Almost all Japanese patients with

narcolepsy carry this haplotype compared to only 10% of the general Japanese population. These results suggest that this haplotype alone is not responsible for the development of narcolepsy. The GWAS revealed six genes that were associated with narcolepsy (73). TCRA/DAD1 (T cell receptor- $\alpha$ ) was also identified as a new susceptibility gene in a second study (74). Another gene NFATC2 (nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 2) also shows a significant association with narcolepsy and its expression level is lower in narcoleptic patients. These two genes and HLA all being associated with narcolepsy further support their involvement in the autoimmune disease. Hor et al. (75) found association with a protective variant gene near DQ on the HLA-DQB1\*02-DRB1\*03 haplotype and also on the DRB1\*13:01-DQB1\*06:03 haplotype. More recently the best biological marker for narcolepsy has been found to be a deficiency in the hypothalamic neuropeptide hypocretin-1 leading the hypothesis that narcolepsy is caused by an autoimmune attack targeting hypocretin-producing neurons (76,77). Comparisons between different ethnic groups have indicated that the susceptibility locus is closer to DQB1 locus (75). These findings suggest that HLA class II regions show the strongest association in narcolepsy.

### 76.7.7 Rheumatoid Arthritis (RA)

One of the prominent features of RA is the presence of rheumatoid factor (RF), which is an autoantibody to the Fc portion of IgG and present in most RA patients. Recently, antibodies to citrullinated proteins have been detected in about 80% of RA patients with 98% specificity (78 and references therein). Citrullination is mediated by peptidylarginine deiminases, of which, PADI4 has been identified as an RA-susceptibility gene (79). Citrullinated peptides bind with high affinity to DRB1\*04:01, perhaps explaining some of the HLA association seen with this disease (80). More recently, a variant allele (R620W) of PTPN22, an intracellular tyrosine phosphatase, has been shown to be highly associated with seropositive RA (as well as with T1D and SLE) (81–83).

When patients with citrullinated protein antigen positive forms of RA are studied, the top signal maps to DQA1\*03:01 and the top SMP is located ~2.5 kb upstream from HLA-DRA. Other independent SNP signals are located within the class II region are located between HLA-DQA1 and DQA2; within the HLA-DOB locus, and in the intron of the HLA-DRA locus. Seropositive homozygous patients have an OR equal to 4.57 compared with heterozygotes. No association or interaction with HLA-DRB1 has been seen, suggesting that these are independent susceptibility loci. MICA and MICB have also been implicated in RA. Because they are present on the synovial cells, they can activate autologous T cells through their NKG2D ligand (84).

Specific HLA class II alleles are associated with RA, with risk in white populations increased 5–10-fold for individuals with specific HLA-DRB1\*04 genes, such as DRB1\*04:01 and DRB1\*04:04 and \*04:05. In addition, DRB1\*01 and \*10 also show a weak association in some studies. However, the same alleles are prevalent in the normal population, so that screening for disease susceptibility based on the presence of these alleles is not highly predictive and is not clinically indicated. The absolute risk to an individual positive for one of these specific DR4 susceptibility alleles is, on average, only 8% (range 5–12%), compared with a population prevalence of approximately 1% (85). In addition, there is marked clinical heterogeneity in the spectrum of RA associated with these HLA genes. Although 80–90% of patients with long-standing erosive forms of RA are positive for the DR\*4 susceptibility alleles, only 55–65% of newly diagnosed RA patients carry the same alleles. This observation has been the subject of considerable clinical interest because it appears to indicate that patients diagnosed with RA represent a spectrum of clinical heterogeneity that is caused, in part, by an underlying genetic heterogeneity. Indeed, further studies have shown that the DR4 susceptibility genes are most prevalent in patients with RF and severe erosive RA, and that other class II susceptibility genes, such as DRB1\*01:01, are markers for a broader clinical spectrum that includes seronegative and nonerosive forms of RA (86).

This genetic distinction between severe erosive RA and other forms of polyarthritis has potential clinical utility for prognosis. When individuals newly diagnosed with RA are analyzed, the presence of DRB1\*04 RA susceptibility alleles (e.g. DRB1\*04:01 or \*04:04) predicts progression to severe erosive disease. In studies of patients who met clinical criteria for RA but did not yet have erosive disease (X-ray evidence of joint erosions), the presence of DRB1\*04 susceptibility alleles was correlated with the onset of erosive disease within two years (87–89). Patients who lacked these DR4 susceptibility genes had a high frequency of nonerosive polyarthritis, and a low frequency of erosive and progressive RA. In other studies using unselected RA patients, there was little additional prognostic value shown for use of these genetic tests in patients who already had erosive disease, indicating that the positive predictive value of DRB1\*04 analysis is probably limited to use in early patients, who do not yet have severe disease (90).

Therefore, although the association between HLA-DR genes and RA suggests a role for HLA molecules in disease susceptibility, the current practical clinical utility lies in the association between specific RA susceptibility alleles and prognosis for progressive erosive forms of disease. HLA-DRB1\*04 genetic analysis is potentially useful in this regard as a means to select patients for alternative forms of therapy. In a randomized clinical trial comparing an aggressive form of multidrug therapy for RA with a single-drug regimen,

there was a marked distinction in clinical outcome that corresponded to the HLA-DRB1\*04 genetic analysis (91). The DRB1\*04-negative patients, as expected, had overall milder disease course over the 2-year clinical trial, and they responded well to both arms of the clinical trial. Patients treated with the single-drug regimen responded to therapy with an excellent clinical outcome (83% response). In contrast, the DRB1\*04-positive patients who were randomized to the single-drug regimen did very poorly, with a marginal (32%) clinical response. The DRB1\*04-positive patients who received the multidrug treatment, however, had a 94% response rate, comparable to the DR4-negative groups (88%). Thus, the use of HLA-DR4 genetic typing to influence the selection of “aggressive” vs standard therapies in RA for newly diagnosed Caucasoid patients who do not yet have joint erosions may be a valuable adjunct to medical management in this disease.

### 76.7.8 Risk Estimates

As noted earlier, a variety of diseases has been associated with specific HLA class I or class II alleles. Risk estimates for the disease-associated marker can be calculated based on the distribution of the marker in patients and controls. The mathematical definitions of various parameters used to estimate genetic risk are shown in Table 76-3 (92). The odds ratio (or RR) gives the probability that someone with the specific marker will get a given disease relative to someone without the marker. The absolute risk refers to the probability that someone with the marker will get the disease; this estimate is related to the disease incidence. The etiologic fraction (or population attributable risk) is yet another risk parameter, measuring what

proportion of the disease risk can be attributed to this gene.

Studies of multiplex families or of affected sib pairs also allow one to estimate the proportion of the total genetic risk attributable to a given genetic region defined by a specific marker; this approach involves calculating a value ( $\lambda_s$ ) from the ratio of expected to observed affected sib pairs that share zero haplotypes (93). For insulin-dependent diabetes mellitus (IDDM), for example, the observed proportion of affected sib pairs sharing zero HLA haplotypes is about 0.06; the expected proportion is 0.25, yielding a  $\lambda_s$  for HLA of about 4.1. For IDDM, the familial clustering or sibling risk ratio (total  $s$ ), the ratio of sibling risk to population prevalence is about 15. This familial clustering ratio may include components of both genetic risk and shared environmental factors. A value of about 50% for the proportion of familial clustering attributable to the HLA region (95) can be calculated, making certain assumptions, from these estimates of  $s$ . By comparison, the value of  $s$  for seropositive, erosive RA is between 5 and 10, whereas for SLE or MS, it is about 20 (94).

### 76.7.9 The Role of Non-HLA Genes within the MHC in Disease

A large number of non-HLA genes within or near the MHC appear to be responsible for several presumed HLA-associated diseases. Some of these diseases have no obvious immunopathology. For example, hereditary hemochromatosis, an autosomal-recessive disease of excessive iron storage associated with HLA-A3, is now known to result from a mutation in the HFE locus. HFE is a class-I-like gene telomeric to the MHC that is involved in iron transport (96). Its product normally associates with beta-2 microglobulin, but a common HFE polymorphism can abrogate its ability to bind, thereby making the molecule nonfunctional (33). There are also several non-HLA genes within the MHC that have an immunologic function that have been implicated in disease susceptibility. Polymorphisms in the promoter of the TNF-alpha locus have been associated with several autoimmune as well as infectious diseases (97,98). These polymorphisms are thought to affect the level of TNF-alpha produced in response to certain stimuli. A second RA-susceptibility locus, located within the MHC, is found in I kappa BL. A single nucleotide polymorphism (SNP 96452 T/A) in the promoter region disrupts a putative binding motif for the transcriptional repressor delta EF1 and may influence transcription of I kappa BL (99). Null alleles at the C4 locus (a component in the complement cascade) appear to contribute to several diseases, including SLE. As noted earlier, it is likely that the well-known association of SLE with the B8-DR3 haplotype is due to strong linkage disequilibrium with the C4A null allele.

The role of the recently identified MICA and MICB (for MHC class-I-associated chain) loci, located about

**TABLE 76-3** Definition of Risk Estimates

		Marker	
		+	–
Disease	0	<i>a</i>	<i>b</i>
	–	<i>c</i>	<i>d</i>

*a* = number of patients with marker.

*b* = number of patients without marker.

*c* = number of controls with marker.

*d* = number of controls without marker.

$$\text{Odds ratio (relative risk)}^a = \frac{ad}{cb}$$

$$\text{Absolute risk} = \frac{a/(a+b)}{c/(c+d)} \times \text{disease incidence.}$$

$$\text{Etiologic fraction} = \frac{[a/(a+b) - c/(c+d)]}{1 - [c/(c+d)]} \text{ (population attributable risk).}$$

<sup>a</sup>The relative risk used by epidemiologists for prospective studies differs slightly from the odds ratio used in case-control studies and is defined as  $a(ac)/b(bd)$ . For rare diseases in which *a* and *b* are small relative to *c* and *d*, this expression approximates the odds ratio.



45kB centromeric of HLA-B, in disease susceptibility is still unclear. There are 76 alleles at MICA and 31 alleles at MICB (<http://hla.alleles.org/classo.html>, April 2011). These genes encode class-I-like glycoproteins that serve as ligands/restriction elements for the TCR of gamma-delta T cells but certainly also function as ligands for the NKG2D-activating receptor on NK cells. These receptors on NK cells can either activate NK cells directly or act as costimulatory molecules for T-cell activation independent of the TCR. It has been suggested that certain alleles of MICA and/or MICB may predispose to certain HLA-associated diseases, such as Behçet's disease or to CD. This latter disease is an interesting candidate because it appears to involve the action of gamma-delta T cells in the gut. However, the issue of linkage disequilibrium with disease-associated B locus alleles (e.g. B\*51) in the case of Behçet's disease, or DR-DQ haplotypes (e.g. DRB1\*03-DQB1\*02) in the case of CD, will have to be examined carefully to assess the true role of polymorphism at these loci in disease susceptibility.

#### 76.7.10 The Role of NK Cell Receptor Genes in Autoimmune Disease

Autoimmune disease has also been associated with the KIR/NKG2/CD94 genes, which are receptors found on NK cells whose ligands are HLA glycoproteins or other class-I-like molecules. Activating killer cell immunoglobulin-like receptor (KIR), especially KIR2DS1 and KIR2DS2, has been implicated in autoimmune disease such as RA and psoriatic arthritis (PsA) (100). Because the affinity of the HLA-C group ligand binding is higher for the inhibitory KIR2DL1 and KIR2DL2/3 receptors than it is for the activating KIR2DS1 and KIR2DS2 receptors, disease appears to ensue when the balance is disrupted. For PsA, it appears that disease susceptibility is a function of the sum total of the activating and inhibitory KIR-ligand combinations that are present, where genotypes corresponding with strong activating potential increase the risk of developing disease. Thus, the particular array of KIR or KIRs present on any given cell will dictate the response of that cell, and the sum total of the population of cells will dictate the susceptibility to PsA.

#### 76.7.11 The Role of Non-MHC Disease Genes

Although the HLA loci and, possibly, other genes within the MHC play a critical role in many autoimmune and inflammatory diseases, other genes also contribute significantly to disease risk. Linkage studies using segregation analyses (parametric) and haplotype sharing among affected sib pairs (nonparametric) have been carried out for a variety of diseases, notably T1D MS, RA, and inflammatory bowel disease, using genome-wide scans with microsatellite markers and haplotype maps (18,94,101–104). These studies have identified several

regions of significant linkage, including the MHC, for all of these diseases, although not all studies for the same disease have implicated the same chromosomal locations (105). In general, the genes responsible for the observed disease linkage to a chromosomal region have not yet been identified and extensive association studies with candidate genes and anonymous genetic markers mapping in these regions are under way in many labs. For example, many associations and some linkage studies carried out in several populations suggest that the insulin locus (chromosome 11p15) and the CTLA4 locus (chromosome 2q33) are involved in susceptibility to T1D. Polymorphism in the CTLA4 locus has been associated with other autoimmune diseases as well (106). The functional polymorphism (R620W) in the PTPN22 gene that encodes the lymphoid protein tyrosine phosphatase (LYP) has been shown to be associated with a variety of autoimmune diseases such as T1D (6), RA, and SLE (81,94). Unlike the variant encoded by the more common allele (R620), the LYP variant (W620) associated with autoimmunity does not bind the negative regulatory kinase, Csk.

#### 76.7.12 Celiac Disease (CD): An Instructive Example

CD is an inflammatory autoimmune disease of the intestinal mucosa, elicited by ingestion of wheat gluten or gliadin. In terms of elucidating immunologic mechanisms, CD is one of the most informative of the HLA-associated diseases because the pathogenic antigen is known and antigen-specific T cells can be recovered from the gut and analyzed *in vitro*. In terms of identifying the molecule responsible for the observed disease association with the DRB1\*03:01-DQA1\*05:01-DQB1\*02:01 and DRB1\*07:01-DQA1\*02:01-DQB1\*02:01 haplotypes, the genetic association studies on CD have also been instructive. The strongest associations with CD are with the *cis* DRB1\*03 haplotype (DRB1\*03:01-DQA1\*05:01-DQB1\*02:01) and with the *trans* genotype DRB1\*11/DRB1\*07 (DRB1\*11-DQA1\*05:01-DQB1\*03:01/DRB1\*07:01-DQA1\*02:01-DQB1\*02:01). A unifying interpretation of this association pattern is that the molecule responsible for the disease association is the DQ heterodimeric molecule with the alpha chain encoded by DQA1\*05:01 and the beta chain encoded by DQB1\*02:01 (106,107). This molecule is encoded in *cis* by the DRB1\*03 haplotype and is formed as a trans-complementing heterodimer in a DRB1\*011/DRB1\*07 heterozygote. This model represents strong evidence that the HLA polymorphism associated with CD is not simply a genetic marker in linkage disequilibrium with some other disease gene but is causally related to disease susceptibility.

T cells have been isolated from the gut of CD patients that recognize specific peptides in the context of the DQ2 molecule. The sequence analysis of peptides bound to the

molecule encoded by DQA1\*05:01 and DQB1\*02:01 has been determined, and the binding motif has been identified (109). Surprisingly, this motif, which contains glutamic acid, is not found in gliadin and other components of wheat gluten. The resolution to this apparent paradox was the discovery of an enzyme, tissue transglutaminase, present in the gut. This enzyme converts glutamine to glutamic acid in gliadin, creating peptides that are then capable of being bound by the DQ2 molecule and presented to T cells. Increased levels of tissue transglutaminase have been observed in the jejunal biopsies of patients and antibodies to this enzyme are a hallmark of CD (110). Thus, the genetics of CD can be best explained by preferential binding and presentation of a modified gliadin peptide by the DQ molecule encoded by the disease-associated alleles DQA\*05:01 and DQB1\*02:01. The demonstration of the role of this DQ molecule in CD does not mean, however, that other genes within and outside the MHC do not also contribute to genetic susceptibility for this disease. As noted earlier, multiple genes, as well as environmental factors, are likely to be involved in all of the many diseases associated with specific HLA alleles.

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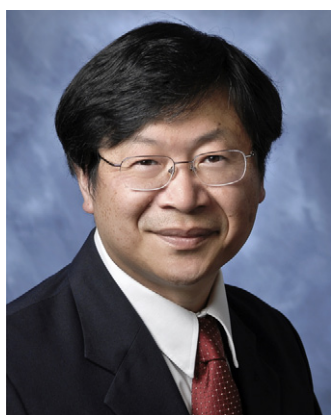
### Biographies



**Dr Nancy L Reinsmoen** received her BA from Luther College, Decorah, Iowa and MS and PhD in Pathobiology and Immunogenetics from the University of Minnesota, Minneapolis, MN. During her 26 years at the University of Minnesota, she held positions as Senior Scientist in the Cellular Histocompatibility Laboratory and Director of the Transplant Immunology Laboratory in the Department of Surgery, where she also held academic appointments of Assistant Professor and Associate Professor. Subsequently, she was appointed as Director of the Clinical Transplantation Immunology at Duke University, Durham, NC and was a tenured full Professor in the Department of Pathology. Dr Reinsmoen's current position is Director of the HLA and Immunogenetics Laboratory at Cedars-Sinai Medical Center, Los Angeles, CA and Professor of Pediatrics, David Geffen School of Medicine, University of California, Los Angeles. Dr Reinsmoen has held several national positions including President of the American Society for Histocompatibility and Immunogenetics (ASHI) and member of Board of Directors for the United Network for Organ Sharing (UNOS). Currently, she is Chair of the Histocompatibility Committee for UNOS. She has received several awards including, most recently, the ASHI Distinguished Scientist Award. She has authored over 190 original manuscripts, chapters, and invited review papers.



**Dr Kai Cao** received both her MD and MS in dermatology and immunogenetics from China Medical University, Shenyang, China. She had 1-year scholarship in the Laboratory of Immunogenetics at the National Cancer Research Institute in Genoa, Italy. Then, she has worked in the National Histocompatibility Laboratory of the American Red Cross and the Histocompatibility Laboratory at the University of Maryland Medical System for 8 years, where she held positions as Senior Scientist and Supervisor. Subsequently she worked at the C.W. Bill Young/Department of Defense Marrow Donor Program at the Naval Medical Research Center and Georgetown University School of medicine, where she held positions as laboratory Supervisor and General Supervisor. Dr Cao's current position is Associate Director at the HLA laboratory of Cedars-Sinai Medical Center in Los Angeles, CA. She has been a member of the American Society of Histocompatibility and Immunogenetics (ASHI), the International Society for Heart and Lung Transplantation (ISHLT) and the American Society of Transplantation (AST). During her 20 years working in the field of Histocompatibility and Immunogenetics, Dr Cao has discovered numerous new HLA gene sequences which were registered in the International ImMunoGeneTics Project (IMGT) database and has authorized over 40 original manuscripts and chapters.



**Dr Lai** graduated from Soochow University, Taipei, Taiwan in 1984 with a bachelor's degree in Chemistry. He completed his Master of Science degree in Clinical Science, Bioanalysis option in 1995 at California State University, Dominguez Hills. In 2004, he earned his doctoral degree from University of California, Los Angeles, Department of Pathology and Laboratory Medicine. Dr Lai completed his Clinical Molecular Genetics fellowship at the University of California, Los Angeles in 2007. He did his Histocompatibility and Immunogenetics director training at Cedars-Sinai Medical Center, Los Angeles, CA, 2007–2011. Dr Lai is board certified in Histocompatibility and Immunogenetics.

Dr Lai is a Diplomat of the American Board of Histocompatibility and Immunogenetics and is an active member of many professional societies. Dr Lai serves on the committee of American Society for Histocompatibility and Immunogenetics. In addition, he is an Inspector for American Society for Histocompatibility and College of American Pathologists. He has more than 15 peer reviewed articles on variety of subjects such as virology, genetics and histocompatibility. Dr Lai is currently a Clinical Associate PhD in the HLA and Immunogenetics Laboratory at Cedars-Sinai Medical Center.

# Systemic Lupus Erythematosus

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## 77.1 INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototype autoimmune disease characterized by autoantibody production, immune complex deposition, and heterogeneous clinical manifestations. Epidemiologic studies on the sibling risk ratio, familial aggregation of SLE and the disease concordance rate in monozygotic twins support a strong genetic contribution to the development of SLE (1). The inheritance of SLE is complex, following a polygenic model that common variations in a number of genes are involved, each contributing modestly to disease risk (2). As a genetically complex disease, SLE typically exhibits genetic heterogeneity, which has been demonstrated by genetic association analyses stratified by specific clinical features and ethnic backgrounds. In addition, *epistasis*, that is, interactions between genes or between genes and environmental factors, should be considered as potentially important determinants of disease risk.

Numerous genetic studies on SLE have been performed. Before 2007, the main approaches to explore genes predisposing to disease risk are (1) targeted and genome-wide linkage studies using multiplex families and (2) candidate gene association studies, usually performed using unrelated cases and ethnically matched control individuals. In the past four years (2007–2011), genome-wide association (GWA) studies that represent an important step beyond the two aforementioned methods have revolutionized the search for genetic influences on SLE susceptibility with more than 30 disease-associated loci identified and confirmed in large datasets. Efforts in delineating functional variants underlying SLE-associated signals and rare variants that may have strong impact in risks for SLE are underway. Results from these studies will help elucidate the molecular mechanisms and cellular pathways modulated by robust genetic associations with SLE, and should provide new targets for more focused therapies.

## 77.2 HISTORICAL BACKGROUND, DEFINITION, AND CLASSIFICATION

The milestones in the history of SLE have been reviewed. The term *lupus*, meaning wolf in Latin, was used in medieval times to describe erythemic ulcerations that can eat away the face. The ability of lupus to cause multisystem disease was described approximately 130 years ago. During the past 30 years (1980–2011), clinical and laboratory definitions of this disease have been characterized and refined. A set of classification criteria for SLE was developed and revised by the American Rheumatism Association (now the American College of Rheumatology) exhibiting 96% sensitivity and 96% specificity (3). Subsequently, the 11 classification criteria for SLE were updated as shown in Table 77-1 (4). Individuals who fulfill four or more of 11 criteria are classified as SLE patients. The criteria were designed for classification of the disease in studies involving SLE patients rather than as a diagnostic tool for individual patients.

## 77.3 PATHOGENIC AUTOANTIBODIES AND IMMUNE COMPLEXES

The wide array of clinical features observed in SLE patients is often mediated by pathogenic autoantibodies and their immune complexes. Autoantibodies may start appearing in symptom-free individuals by years before the diagnosis of SLE, followed by a gradual acquisition of more autoantibodies and clinical symptoms (5). SLE patients usually have high-affinity immunoglobulin G (IgG) autoantibodies to self-antigens, which are different from normal individuals who have a repertoire of IgM autoantibodies displaying low affinity to a multitude of antigens, including self-antigens. Characteristics of autoantibodies present in SLE patients often resemble protective IgG antibodies induced in immune responses against foreign antigens. These autoantibodies have been shown to be the products of clonally expanded B cells, which

**TABLE 77-1 1997 Revised American College of Rheumatology Criteria for SLE Classification<sup>a</sup>**

Criterion	Definition
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds.
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging, atrophic scarring may occur in older lesions.
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation.
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician.
5. Arthritis	Nonerosive arthritis involving two or more peripheral joints, characterized by tenderness, swelling or effusion.
6. Serositis	(a) Pleuritis—convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion; OR (b) Pericarditis—documented by ECG or rub or evidence of pericardial effusion.
7. Renal disorder	(a) Persistent proteinuria >0.5 g per day or >3+ if quantitation not performed; OR (b) Cellular casts—may be red cell, hemoglobin, granular, tubular, or mixed.
8. Neurologic disorder	(a) Seizures—in the absence of offending drugs or known metabolic derangements (e.g. uremia, ketoacidosis, or electrolyte imbalance); OR (b) Psychosis—in the absence of offending drugs or known metabolic derangements (e.g. uremia, ketoacidosis, or electrolyte imbalance).
9. Hematologic disorder	(a) Hemolytic anemia—with reticulocytosis; OR (b) Leukopenia—<4000/mm <sup>3</sup> total on two or more occasions; OR (c) Lymphopenia—<1500/mm <sup>3</sup> on two or more occasions; OR (d) Thrombocytopenia—<100,000/mm <sup>3</sup> in the absence of offending drugs.
10. Immunologic disorder	(a) Anti-DNA: antibody to native DNA in abnormal titer; OR (b) Anti-Sm: presence of antibody to Sm nuclear antigen; OR (c) Positive finding of antiphospholipid antibodies based on: (1) an abnormal serum level of IgG or IgM anticardiolipin antibodies; (2) a positive test result for lupus anticoagulant using a standard method; or (3) a false-positive serologic test for syphilis known to be positive for at least 6 months and confirmed by <i>Treponema pallidum</i> immobilization or fluorescent treponemal antibody absorption test.
11. Antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with “drug-induced lupus” syndrome.

<sup>a</sup>The proposed classification is based on 11 criteria. For the purpose of identifying patients in clinical studies, a person shall be said to have SLE if any four or more of the 11 criteria are present, serially or simultaneously, during any interval of observation.

are descendants of the initial antigen-activated B cells. In addition, these autoantibodies often have somatic mutations in the variable regions of their immunoglobulin genes that enhance the binding affinity for their selected autoantigens. The constant regions of these autoantibodies also play a role in disease. Those autoantibodies with isotypes that fix complement well are more likely to be pathogenic. The production of different autoantibodies appears to correlate with heterogeneous clinical manifestations of lupus. For example, elevated serum IgG antibodies to double-stranded DNA (dsDNA) are associated with immune-complex-mediated glomerulonephritis (6), antibodies to phospholipid with thrombosis and recurrent fetal loss (7), and antibodies to ribonucleoprotein (Ro) with photosensitive skin rash and congenital heart block (7,8).

Many autoantibodies associated with SLE recognize altered autoantigens displayed on the membranes of cells undergoing apoptosis, leading to the current thinking that these autoantibodies are induced by increased rates of apoptosis, and/or abnormal clearing of apoptotic cells (9,10). As apoptosis occurs in normal physiologic conditions to maintain tissue homeostasis, the loss of tolerance to autoantigens in apoptotic blebs (such as nucleosome, phospholipid, and Ro) may reflect defects in immunoregulation of apoptosis resulting in survival of autoreactive B and T lymphocytes. Environmental insults such as ultraviolet (UV) light can induce apoptosis of keratinocytes, leading to redistribution of several autoantigens to the

surface of cells in membrane-encased blebs. These redistributed autoantigens, including DNA/histone complexes (nucleosomes or chromatin), Ro particles, and phosphatidylserine (the negatively charged inner-membrane component), are normally sequestered from the immune system, but become available when presented on cell surfaces during apoptosis. In addition, these autoantigens may be altered by processes occurring during apoptosis that make them more immunogenic, thus inducing autoantibody production, as shown in normal mice following systemic exposure to irradiated apoptotic cells (11). Defective clearance of apoptotic cells in individuals with a single gene deficiency in classical complement components (C1q or C4) can cause lupus-like disease in normal mice (12,13), and almost all humans with homozygous C1q deficiency develop lupus (14).

## 77.4 THE GENETICS OF HUMAN SLE

### 77.4.1 Genome-Wide Linkage Studies

Several different study designs that involve sibling pairs (for whom, parents may or may not be available) or extended pedigrees with several generations have been used for linkage scans to explore susceptibility loci for SLE. A total of 12 genome-wide scans and 8 targeted linkage analyses have established nine loci (1q23, 1q31–32, 1q41–43, 2q37, 4p16, 6p11–21, 10q22–23, 12q24, and 16q12–13) reaching the threshold for significant



**TABLE 77-2 Confirmed Linkage Regions in SLE**

Cytogenetic Location	Candidate Genes	Studied Population	References
1q23	<i>FCGR2A, FCGR3A</i>	EA, AA	(16,17,94,95)
1q31–32	<i>CR2, CFH/CFHR</i>	AA, EU, EA, As	(17,18,87,91,92)
1q41–43		EA, HS	(19,20)
2q37	<i>PDCD1</i>	EU	(21,22)
4p16		EA	(23,24)
6p11–21	HLA haplotypes, <i>C4, TNFA</i>	EA, AA, HS	(25)
10q22–23		EA	(16)
12q24		EA, HS	(26)
16q12–13		EA, AA, HS	(27,28)

AA, African–American; As, Asian; EA, European American; EU, European; HS, Hispanic; SLE, systemic lupus erythematosus.

linkage to SLE (15), which have also been replicated in independent populations (16–28). Confirmation of significant linkage of a locus offers strong evidence for the existence of putative susceptibility gene(s), which leads to further narrowing of the linked loci to localize susceptibility genes. These promising linkage loci and the susceptibility gene(s) identified within these regions are summarized in Table 77-2.

**77.4.1.1 Linkage Analysis Using Pedigree Stratification Approach.** The extremely diverse and variable clinical manifestations of SLE both in individual patients and over time have promoted a hypothesis that genetic factors contribute to this disease heterogeneity. There is likely to be a set of genes which are overrepresented in families with particular clinical manifestations. Stratification of multiplex pedigrees by clinical symptoms is a rational approach to increase power for detecting linkage if phenotypic heterogeneity is affected by genetic heterogeneity (29). This approach has been applied successfully in the Oklahoma collection, which has yielded 10 publications reporting strong evidence for linkage (Table 77-3) (30–39). However, reduced sample size after phenotype stratification and the high probability of false-positive results from repeatedly testing subgroups highlight the importance of independent confirmation of the findings. Rather than stratifying on the presence or absence of a single manifestation, a principal component approach using seven major SLE-related phenotypes plus age at SLE onset and race was used to conduct multivariate analysis of the genome scan data. This approach has identified several loci exhibiting suggestive evidence for linkage to SLE-related traits (40). Familiality of thrombocytopenia, discoid rash, neurologic disorder (defined as seizure or psychosis), hemolytic anemia, co-occurring neurologic disorder plus hemolytic anemia and age at SLE diagnosis have been observed in 159 sibling pairs affected with SLE (41). Linkage to familial subphenotypes of SLE may improve homogeneity and facilitate the localization of susceptibility genes predisposing to particular clinical manifestations.

**77.4.1.2 Meta-analysis of Genome-Wide Scans.** Although a number of genomic regions harboring SLE susceptibility genes have been established in individual

**TABLE 77-3 Linkage Loci Identified in Oklahoma Collection using Pedigree Stratification Approach**

Stratifying Manifestation	Linkage Region	Studied Population	References
Discoid lupus erythematosus	11p13	AA	(30)
Self-reported RA	5p15.3	EA, AA, HS	(31,32)
Renal disorder	10q22.3	EA	(33)
	2q34–35	AA	
	11p15.6	AA	
Neuropsychiatric disorder	4p16	EA	(34)
Hemolytic anemia	11q14	AA	(35)
Thrombocytopenia	1q22-23	AA	(36)
	11p13	AA	
Anti-dsDNA antibodies	19p13.2	EA	(37)
	18q21.1	AA	
Nucleolar antinuclear antibody	11q14	AA	(38)
Vitiligo	17p13	EA	(39)

AA, African–American; EA, European American; HS, Hispanic.

genome-wide scans, many other regions have yielded inconsistent results across studies. Additionally, studies from a single center may be limited with regard to the acceptable power recommended for declaring significant linkage. Thus, the genome scan meta-analysis method, which is designed to deal with variation in study designs, analysis methods and marker densities used in different single center scans, has been proposed as a valid and robust tool for combining numerous linkage results (42). The two genome meta-analyses have reported several linked regions that may harbor SLE susceptibility genes, including three linkage intervals (6p21–6p22, 16p12–16q13 and 20p11–20q13) that appeared in both analyses. Some regions (such as 3p14–3q12, 10p14–10q11 and 12q24–12qter) appeared only in one analysis (43,44). Some of loci linked to SLE and/or SLE subphenotypes have been validated in subsequent fine-mapping studies leading to the identification of robust disease-associated loci; for example, *ITGAM* in 16p12–16q13, and *PDHX/CD44* in 11p13 as described below.

**77.4.1.3 Recent GWAS Findings Related to Linkage Results.** Success in the initial identification of chromosomal regions containing SLE susceptibility genes was achieved through linkage studies, but the utility of linkage studies in precisely localizing causal variants is limited owing to the inability to map genetic variants of small phenotypic effect sizes. Recently, GWAS technologies that allow efficient and high-throughput genotyping for single nuclear polymorphisms (SNPs) have enabled genome searches for variants predisposing to SLE.

**77.4.1.3.1 6p21 and HLA Genes.** Before the advent of GWAS, one of the most extensively studied regions linked to SLE susceptibility was human leukocyte antigen (HLA), which is encoded by the major histocompatibility complex (MHC) locus. The classical MHC encompasses approximately 3.6 Mb on 6p21.3 and is divided into the class I, class II and class III regions (reviewed in Chapter 76). Given that long-range linkage disequilibrium (LD) and MHC-related genes exist outside this classically defined locus, the concept of the extended MHC (xMHC) spanning nearly 7.6 Mb of the genome has been established. It consists of five subregions: the extended class I subregion (*HIST1H2AA-MOG*; 3.9 Mb), classical class I (*C6orf40-MICB*; 1.9 Mb), classical class III (*PP1P9-NOTCH4*; 0.7 Mb), classical class II (*C6orf10-HCG24*; 0.9 Mb), and the extended class II subregions (*COL11A2-RPL12P1*; 0.2 Mb) (45). Of the 421 genes within this extended region, 60% are expressed and approximately 22% have putative immunological function.

Until 2005, most published disease association studies of HLA were restricted to a subset of ~20 genes in small case-control panels of predominant European ancestries. These genes include the classical HLA loci (*HLA-A*, *-B*, *-C*, *-DRB*, *-DQA*, *-DQB*, *-DPA*, *-DPB*), *TNFA*, *LTA*, *LTB*, *TAP*, *MICA*, *MICB* and the complement loci (*C2*, *C4A*, *C4B* and *CFB*) (reviewed in Fernando and Vyse (46)). A recent pooled analysis which was undertaken to consolidate and evaluate the current literature base regarding HLA genetics with SLE over the past 30 years (1970–2008) has highlighted the importance of variants within *HLA-DR3*- and *HLA-DR2*-bearing haplotypes in SLE susceptibility (47). The strongest associations are found with the *HLA-DR3* haplotypes, *HLA-B8-DRB1\*0301* and *HLA B18-DRB1\*0301* [odds ratio (OR): 1.5–2.5]; the *HLA-DR2/DRB1\*1501* haplotype exhibits OR of 1.7. The remaining association signals largely arise from class II alleles *HLA-DRB1*, *HLA-DQA1* and *HLA-DQB1*, some of which exhibit evidence of population specificity (47). For instance, *HLA-DRB1\*1503* (a *HLA-DR2* subtype) has been found in African-Americans only although there are several studies in African-Americans revealing no association of *DR2* or *DR3* alleles with SLE susceptibility (48,49). *HLA-DRB1\*1602* has been observed in Mexican Mestizo, Thai, and Bulgarian populations; while *HLA-DRB1\*0401* has been seen largely in Mexican Mestizo and Hispanic populations (47).

Two further class II alleles, *HLA-DQA1\*0401* and *HLA-DQB1\*0402*, reside on a *DR8* haplotype, which is uncommon in European populations (47). These studies have shown inconsistent results, but may help elucidate the risk factors for SLE within the HLA region among multiple populations.

There is strong and complicated LD between genetic markers across the HLA region; thus, highly correlated variants spanning multiple genes are inherited together in a single block. Intense efforts are underway to dissect the independent effects of tightly linked loci. The increased knowledge of the human genome structure and technological advances in the efficient high-throughput genotyping have led to the availability of dense marker sets across HLA locus. Together with collaborative efforts allowing the analysis of several thousands of individuals, these marker sets have enabled extensive fine mapping of HLA variants associated with SLE. Recent GWAS in populations of European and Asian ancestries have shown consistent evidence that the strongest contribution to risk for SLE susceptibility resides in the HLA region and consists of multiple genetic effects (50–55). Of interest, these studies not only confirmed predominant association signals at the class II region, but also highlighted the importance of class III genes in SLE susceptibility. For example, one SNP (rs3131379) of the HLA class III locus *MSH5* exhibited the highest association in a GWAS conducted in 2008 (51). Using a relatively high-density SNP map of HLA, data from several studies in SLE patients has refined these associations (56,57). A mapping study in 314 European SLE families reported two distinct and independent signals (56): one from a small 180-kb class II region tagged by *HLA-DRB1\*0301* allele; and the other observed at an SNP marker (rs419788) in the class III gene *SKIV2L* [superkiller viralicidic activity 2-like (*Saccharomyces cerevisiae*)]. Examination of LD structure around this marker (rs419788) showed that this class III signal could also be limited to a 40-kb interval containing the genes *CFB* (complement factor B), *RDBP* (RD RNA binding protein), *DOM3Z* [dom-3 homolog Z (*Caenorhabditis elegans*)] and *STK19* (serine-threonine kinase 19). *CFB* encodes complement factor B, which is a vital component of the alternate complement pathway (58). The functions of *RDBP*, *SKIV2L*, *DOM3Z* and *STK19* are not well characterized although their products have been reported to play a role in mRNA processing (59). Moreover, transcripts of *RDBP* and *SKIV2L*, in particular, are detectable in T, B lymphocytes and dendritic cells (56). It is notable that this study shows evidence against an independent effect from *TNF-308G/A* promoter polymorphism in SLE, which is inconsistent with results from another meta-analysis study (60). A recent collaborative study has demonstrated HLA associations in multiple immune-mediated diseases, exhibiting complex and multilocus effects (57). In the SLE cohort of European ancestry, the most highly associated SNP (rs1269852) is

detected at class III region between *TNXB* and *CREBL1* genes, which has strong but not complete LD with the *HLA-DRB1\*0301* allele ( $r^2=0.78$ ). Other class III association signals include peaks centered around the *NOTCH4* gene and those on either side of the *RCCX* module (which contains *C4A* and *C4B* genes along with three neighboring genes). The influence of copy number variation (CNV) at the complement *C4/RCCX* locus in relation to the association signals revealed in this study remains to be established (57).

Despite the fact that association of the HLA region with SLE susceptibility has been well confirmed using GWAS and high-density SNP studies, there appear to be limitations such as the inability of these studies to capture CNV and/or to pinpoint the causal gene variants within this complicated region. Alternative methods, including comparative genomic hybridization array and multiplex ligation-dependent probe amplification, are therefore being employed to more accurately assess the effect of the structural variation in disease susceptibility. Next-generation sequencing of associated intervals/haplotypes in large transethnic cohorts will contribute greatly to further refine the HLA association signals in SLE.

**77.4.1.3.2 16p12–16q12 and *ITGAM*.** The product of *ITGAM* is integrin  $\alpha M$  which combines with integrin  $\beta_2$  to form a leukocyte-specific integrin. The  $\alpha M\beta_2$  integrin regulates leukocyte adhesion and emigration through interactions with a myriad of ligands that are potentially relevant to SLE (such as ICAM-1, ICAM-2, C3bi and fibrinogen) (61). The expression level of  $\alpha M\beta_2$  integrin is elevated in neutrophils from SLE patients with active disease activity, which correlates to endothelial injury (62). Other functions of  $\alpha M\beta_2$  integrin involved in autoimmunity processes are its importance in clearing immune complexes and in suppressing Th17 cell differentiation (63,64). Two independent GWAS performed in European populations have reported four SNPs associated with SLE in or very near the gene encoding integrin  $\alpha M$  (*ITGAM*, or *CD11b*) (50,51), which is located within the previously identified linkage interval of 16p12.3–16q12.2. Consistent with these results, a fine-mapping study showed that a nonsynonymous variant (rs1143679; R77H), with an effect on structural and functional changes of integrin  $\alpha M$ , contributed to SLE susceptibility (65). In a subsequent meta-analysis, this association and the role of rs1143679 were confirmed in various ethnicities, including Americans of European, Hispanic or African ancestries, as well as Mexican and Colombian populations (66). Despite the low frequency of the risk allele in Asian populations, it also displayed significant associations with SLE risk as well as with severe manifestations (such as lupus nephritis, neurological, hematological and immunological disorders) in Hong Kong Chinese and Thai individuals (67). However, the correlation of this variant with different clinical manifestations will need further replication using enlarged samples.

**77.4.1.3.3 11p13 between *PDHX* and *CD44*.** Initial GWAS in SLE have not only identified several novel susceptibility loci, but also generated a number of candidate loci not reaching genome-wide significance ( $p < 5 \times 10^{-8}$ ). For example, putative association at 11p13 from an European SLE GWAS (52) has been confirmed in a recent replication study using >15,000 multiethnic case-control samples (68). One intergenic SNP rs2732552 displays robust association with SLE in European, African-American and Asian populations, while another intergenic SNP rs387619 only has association in a European population (68). This region that has also been identified in a linkage study of multiplex SLE families with thrombocytopenia (36) lies between two immune-related genes *PDHX* and *CD44*. *CD44* encodes a cell-surface glycoprotein expressed on most immune cell types that plays an important role in lymphocyte activation, recirculation, apoptosis, hematopoiesis and tumor metastasis (69,70). Although no direct evidence shows SNPs within the *CD44* gene are associated with SLE susceptibility (68,71), there are several reports regarding levels of alternative spliced mRNA transcripts and CD44 protein related to the disease, supporting a role for *CD44* involved in SLE pathogenesis. Li et al. has reported that T cells from SLE patients display elevated levels of CD44 and enhanced adhesion and chemotactic migration compared with T cells from normal individuals or RA patients (72). CD44 is also highly expressed in T cells infiltrating in the kidneys of patients with lupus nephritis when compared to patients with renal allografts undergoing rejection (72). Additionally, a recent study has demonstrated that T cells from SLE patients overexpress CD44v3 and CD44v6 transcript isoforms, and the expression levels are correlated with disease activity (73).

## 77.4.2 Candidate Gene and GWAS

Linkage studies have been successful in identifying chromosome regions linked to SLE, but progress toward further localizations of underlying gene variants has met with limited success. The identified linkage intervals are usually large and contain many potential candidate genes, while in some instances, important SLE susceptibility genes (such as *IRF5*, IFN regulatory factor 5) are not located within established linkage regions. Major advances in gene discovery are summarized here, highlighting the success of both insightful candidate gene approaches and recent GWAS.

**77.4.2.1 Candidate Gene Studies in SLE.** Candidate gene studies are commonly used to assess whether a test genetic marker is present at a higher frequency among patients with SLE than in ethnically matched healthy control individuals. Some candidate genes are chosen based on data from linkage studies, and others on the basis of their functional relevance to the disease pathogenesis. In the majority of cases, the genetic marker under investigation is the SNPs. An SNP observed with



greater-than-expected frequency in individuals with disease is either a functional, disease-causing variant (a direct association) or a nonfunctional variant that exhibits strong LD with the functional variant (an indirect association) (74). Many loci have been implicated in risk for SLE over the years 1981–2010 (reviewed in Tsao & Deng (75)). However, owing to the variations in sample size, ethnicity and number of SNPs studied among individual reports, conflicting results often appear which lead to a limited number of confirmed SLE susceptibility genes. This situation changed dramatically with the advent of GWA studies.

**77.4.2.2 Overview of GWA Studies in SLE.** Since 2007, six GWAS and a series of subsequent targeted replication studies in SLE have been published (50–55,76), which have expanded the number of established genetic associations with SLE to more than 30 (Table 77-4). Such associations are consistent with the common disease–common variant (CD/CV) hypothesis, which assumes that genetic variants present in more than 1–5% of the population confer an important contribution to common diseases (77). The identified SLE-associated variants each have a modest magnitude of risk with an OR in the range of 1.1–2.3 (Table 77-4), accounting for a fraction of the overall genetic risk for SLE. However, they indicate that many different pathways, processes and cell types are involved in generating the SLE phenotype (Figure 77-1), refining our understanding of SLE pathophysiology to the molecular level. Most of these susceptibility loci can be assigned into three key biological pathways: (1) clearance and processing of immune complex; (2) Toll-like receptor (TLR)/type I interferon (IFN) signaling in innate immune response; and (3) lymphocyte activation and regulation in adaptive immune response. Of note, certain loci and genes seem to be shared between SLE and other autoimmune disorders, including rheumatoid arthritis (RA), type 1 diabetes (T1D), Grave's disease (GD), multiple sclerosis (MS), and psoriasis (PSO), implying that common immunological mechanisms exist among some of these disease processes. In the following paragraph, we will elaborate the confirmed genetic risk loci identified to date, placed in the context of major SLE disease pathways where possible. In addition, we will highlight the recent findings that are particularly informative and discuss similarities and differences of these genetic factors between ethnic populations to gain an emerging landscape of SLE genetics.

**77.4.2.3 Complement and Immune Complexes Clearance.** Defects in apoptotic cell clearance, processing and presentation to lymphocytes lead to initiation of autoimmune response, promoting the development of SLE (78). Associations of several genetic loci have supported the importance of this theme in SLE pathogenesis: complement component genes, a cluster of Fcγ receptor genes, *CRP*, and *ITGAM*.

Complement is critical for the opsonization and clearance of autoantibody containing immune complexes (79).

The relationship between complement and SLE pathogenesis has long been noticed because low levels of complement are common immunologic features of SLE, particularly during disease flares (80,81). Complete deficiencies of the classical complement pathway genes (*C2*, *C4A*, *C4B* and *C1Q*) and complement *C3*, although rare, confer a strong genetic risk to SLE (82–85). Common CNVs in *C4A* (but not *C4B*) have been associated with SLE that a low copy number of *C4A* increases risk while a high copy number decreases risk for SLE (86). Deletion of genes encoding two regulators of the alternative complement pathway, *CFHR3* and *CFHR1* (complement regulator factor H-related 3 and 1), which may contribute to dysregulated complement activation, has been associated with increased risk of SLE in European American, African-American and Asian populations (87). In addition, common SNP of *C1Q*, *C3* or *CR2* (complement receptor 2) gene, which either confers lower serum levels of C1q or C3, or alters transcriptional activity of *CR2*, is also associated with risk of developing SLE in various ethnic groups (88–92). It appears that multiple genetic mechanisms, including gene deletion, structural variations, and SNPs, which result in low levels of these complement components, contribute to risk for SLE.

Chromosome 1q23 contains five genes (*FCGR2A*, *FCGR3A*, *FCGR2C*, *FCGR3B* and *FCGR2B*) encoding the low-affinity Fcγ receptors (FcγRs), which play critical roles in regulating a variety of humoral and cellular immune responses including immune complex clearance and antibody-dependent cellular cytotoxicity (93). Several functional variants in these genes, which may alter the binding affinities of the encoded receptors leading to a less-efficient clearance of ICs, have been reported to confer risk for SLE and/or lupus nephritis among multiple populations: a nonsynonymous variant of *FCGR2A* (H131R) (94), a nonsynonymous mutation in the mature sequence for *FCGR3A* (F158V) (95), and a nonsynonymous variant of *FCGR2B* (I187T) (96–98). However, the presence of high sequence homology between the *FCGR* genes, together with the presence of known segmental duplication in this region may preclude the assessment of specific SNPs in *FCGR* gene complex on the currently available GWAS arrays. Additionally, the presence of structural variation is another complicating factor for this locus. Association between SLE and a decreased copy number of *FCGR3B* is observed in multiple ethnic groups (99–103), which correlates with levels of protein expression and immune complex clearance (104). Further interpretations of the relative contribution of various *FCGR* variants to SLE must be made in the context of LD involving multiple functional variants.

**77.4.2.4 TLR/IFN Signaling in Innate Immune Response.** Innate immune system activation including increased type I interferon (IFN-α) production has been implicated in SLE pathophysiology, which is supported by a range of studies (105–107). The overproduction of



TABLE 77-4 SLE Risk Loci Identified through GWAS in Various Ethnic Groups							
Study	Platform	Initial Scan	Replication	Strong Evidence		Good Evidence	
		Subjects (n)	Subjects (n) <sup>a</sup>	(p < 5 × 10 <sup>-8</sup> )		(p < 1 × 10 <sup>-5</sup> )	
		Case/Control	Case/Control	Gene	OR	Gene	OR
European							
Hom et al. (50)	Illumina 550K	1311/3340	793/857	HLA-DR3 <sup>b</sup>	ND	NA	NA
				IRF5/TNPO3 <sup>b</sup>	ND		
				STAT4 <sup>b</sup>	ND		
				C8orf13-BLK	1.4		
				ITGAM-ITGAX	1.3		
Harley et al. (51)	Illumina 300K	720/2337	1846/1825	HLA region <sup>b</sup>	1.4–2.3	PTPN22 <sup>b</sup>	1.5
				IRF5/TNPO3 <sup>b</sup>	1.3–1.6	FCGR2A <sup>b</sup>	1.4
				STAT4 <sup>b</sup>	1.5	NMNAT2	1.2
				ITGAM	1.3–1.7	ATG5	1.2
				KIAA1542	1.3	UBE2L3	1.2
				PXK	1.3	SCUBE1	1.3
				XKR6 <sup>c</sup>	1.2–1.3		
				BLK	1.2		
				LYN	1.3		
Kozyrev et al. (76)	Affy100K	279/515	2003/1968	BANK1	1.4	NA	NA
Graham et al. (52)	Affy 5.0	431/2155	740 trios <sup>d</sup>	HLA region <sup>b</sup>	ND	ITGAM	ND
				IRF5/TNPO3 <sup>b</sup>	ND		
				STAT4 <sup>b</sup>	1.5		
				BLK	1.3		
				TNFAIP3	2.3		
Gateva et al. (53) <sup>e</sup>	Illumina Infinium II	1310/7859	1963/4329	HLA-DRB1 <sup>b</sup>	2.0	IRAK1-MECP2 <sup>b</sup>	1.1
				IRF5 <sup>b</sup>	1.9	UBE2L3	1.2
				STAT4 <sup>b</sup>	1.6	ATG5	1.2
				PTPN22 <sup>b</sup>	1.4	PXK	1.2
				TNFSF4 <sup>b</sup>	1.2	IKZF1	1.2
				IL10 <sup>b</sup>	1.2	SLC15A4	1.1
				ITGAM	1.4		
				BLK	1.4		
				TNFAIP3	1.7		
				KIAA1542	1.2		
				TNIP1	1.3		
				PRDM1-ATG5	1.2		
				JAZF1 <sup>c</sup>	1.2		
				UHRF1BP1 <sup>c</sup>	1.2		
Asian							
Han et al. (54)	Illumina 610	1047/1205	3152/7050	HLA region <sup>b</sup>	1.9	NA	NA
				IRF5 <sup>b</sup>	1.4		
				STAT4 <sup>b</sup>	1.5		
				TNFSF4 <sup>b</sup>	1.4–1.5		
				BLK	1.3–1.4		
				PRDM1-ATG5	1.3		
				TNFAIP3	1.7		
				HIC2-UBE2L3	1.3		
				IKZF1	1.4		
				RASGRP3 <sup>c</sup>	1.4		
				SLC15A4 <sup>c</sup>	1.3		
				TNIP1	1.3		
				ETS1	1.4		
				LRCC18-WDFY4	1.2		
Yang et al. (55)	Illumina 610	320/1500	3300/4200	HLA region <sup>b</sup>	1.8–2.0	IRF5 <sup>b</sup>	1.5
				STAT4 <sup>b</sup>	1.7	TNFAIP3	1.9
				ETS1	1.3	BLK	1.6
				WDFY4	1.3		

GWAS, genome-wide association studies; NA, not applicable; ND: not determined; OR, odds ratio; SLE, systemic lupus erythematosus.

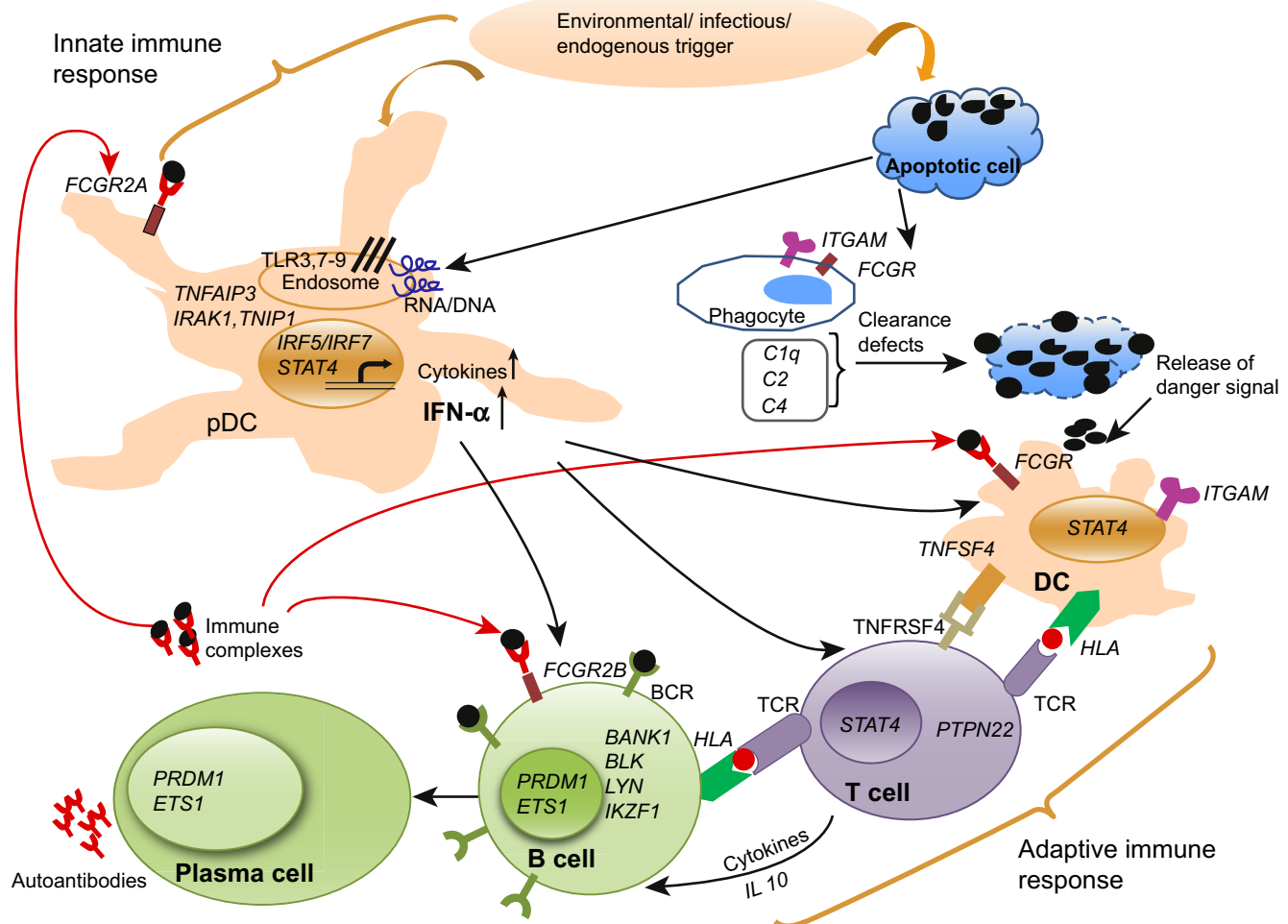
<sup>a</sup>Replication followed by initial GWA scan to calculate joint *P* values in a single publication.

<sup>b</sup>Loci identified through candidate gene studies and GWAS.

<sup>c</sup>Have not been replicated independently at the time of writing.

<sup>d</sup>Replication conducted in 740 SLE trio pedigrees.

<sup>e</sup>It is a large-scale replication study using a similar cohort to the Hom et al. (50).



**FIGURE 77-1** A model of SLE-associated genetic variants in the immune response. This model is highlighted by the identified SLE susceptibility loci, based on current understandings of important immunological pathways involved in SLE pathogenesis. Clearance and processing of immune complexes: environmental triggers that induce apoptosis and release of nuclear antigens can stress phagocytes (including macrophages and neutrophils), causing defective clearance of nuclear antigens. TLR/IFN signaling in innate immune response: environmental triggers including UV light, demethylating drugs and viruses can yield stimulatory DNA or RNA that activates TLRs, resulting in secretion of type I IFN. Lymphocyte activation and regulation in adaptive immune response: an adaptive immune response begins with the presentation of nuclear antigens to dendritic cells, leading to the generation of autoantibodies and immune complexes that in turn amplify both innate and adaptive immune responses. Abbreviations: BCR, B-cell receptor; DC, dendritic cell; IFN, interferon; IL, interleukin; pDC, plasmacytoid dendritic cell; SLE, systemic lupus erythematosus; TCR, T-cell receptor; TLR, Toll-like receptor.

IFN- $\alpha$  can promote dendritic cells maturation, autoreactive B- and T-cells activation, and proinflammatory cytokines and chemokines expression (108). The major source of excess IFN- $\alpha$  production in SLE is induced by immune complexes containing self-antigens and nucleic acids, which bind to endosomal Toll-like receptors (TLR7 and TLR9) and further activate transcription factors of the IFN pathway (such as IRF5/7, NF- $\kappa$ B and STAT4). Several specific genes with influence on IFN pathway and related innate immune signaling functions have been identified to associate with SLE susceptibility, which will enhance our understanding of genetic contribution to this dysregulated pathway predisposing to SLE.

**77.4.2.4.1 IRF5.** Interferon regulatory factor 5 (IRF5; encoded by *IRF5*), a pivotal transcription factor in the type I IFN pathway, regulates the expression of IFN-dependent genes, inflammatory cytokines and

genes involved in apoptosis. *IRF5* is one of the most strongly and consistently SLE-associated genes outside the MHC region, conferring a modest risk with an OR  $\geq 1.5$  (Table 77-4). Four functional *IRF5* variants, including a 5-bp indel (insertion/deletion) near the 5' untranslated region (UTR), rs2004640 in the first intron, a 30-bp indel in the sixth exon and rs10954213 in the 3' UTR, have been identified in association studies derived from multiple ethnic groups (109–117). Alleles of these functional variants in different combinations defined various haplotypes that are associated with increased, decreased, or neutral levels of risk for SLE. The SLE-risk haplotypes of *IRF5* have functional consequences, including increased expression of *IRF5* mRNA and interferon-inducible chemokines, as well as elevated IFN- $\alpha$  activity (118,119). Indeed, a required role for *IRF5* in mediating lupus pathogenesis

is demonstrated in murine models of lupus-like disease using *Irf5*<sup>-/-</sup> MRL/lpr mice (120), or *Irf5*-deficient and *Irf5*-sufficient *FcγRIIB*<sup>-/-</sup> Yaa mice (121).

**77.4.2.4.2 STAT4.** *STAT4* encodes the signal transducer and activator of transcription 4 protein (STAT4), which transmits signals from the receptor for type I IFN, IL-12 and IL-23, and can contribute to autoimmune responses by affecting the functions of several innate and adaptive immune cells (122). The SNP (rs7574865) in the third intron of *STAT4* is first identified for association with SLE in several case-control studies, exhibiting an OR of 1.5–1.7 (123–126), and has been subsequently confirmed by GWAS using populations of European or Asian ancestry (51–55). This rs7574865 risk variant is associated with a more-severe SLE phenotype characterized by disease-onset at a young age (<30 years), a high frequency of nephritis, the presence of antibodies toward dsDNA (124,126,127), and/or an increased sensitivity to IFN- $\alpha$  signaling in peripheral blood mononuclear cells (PBMCs) (128). Fine-mapping studies have led to the identification of several markers that are independently associated with SLE and/or with differential levels of *STAT4* expression (127,129,130), and a 73-kb-risk haplotype common to European Americans, Koreans and Hispanic Americans (130). Of note, individuals carrying one or more risk alleles of both *IRF5* and *STAT4* variants have an increased risk for SLE (127), suggesting multiple genes in the type I IFN pathway may interact predisposing to SLE.

**77.4.2.4.3 PHRF1/IRF7.** Two independent studies of European populations have reported an SLE-associated SNP (rs4963128) in a gene of unknown function named PHD and RING-finger domains 1 (*PHRF1*, also known as *KIAA1542*) (51,53). The observed association might be attributable to its close proximity with interferon regulatory factor 7 (*IRF7*), because the strong LD ( $r^2=0.94$ ) between this disease-associated SNP and a 3' UTR *PHRF1* SNP (rs702966) is within 0.6-kb flanking region of the *IRF7* gene (51). *IRF7*, similar to *IRF5*, is a transcription factor that can activate transcription of IFN- $\alpha$  and IFN- $\alpha$ -inducible genes downstream of endosomal TLRs. Supporting *IRF7* as an SLE susceptibility gene comes from two studies: (1) SLE patients carrying *PHRF1* risk alleles and expressing SLE-associated autoantibodies to dsDNA or anti-Sm exhibit elevated serum IFN- $\alpha$  activity (131), and (2) the major allele of a nonsynonymous SNP (Q412R) in *IRF7* confers elevated IFN-stimulated response in vitro and predisposes to SLE in Asian, European American and African-American populations (132). However, a complete assessment of the *PHRF1/IRF7* locus with dense genetic markers and/or sequencing is still pending to localize all possible causal variants.

**77.4.2.4.4 TNFAIP3 and TNIP1.** *TNFAIP3*, the tumor necrosis factor alpha inducible protein 3 gene, encodes the zinc finger A20 protein which is a ubiquitin-modifying enzyme critical for termination of NF- $\kappa$ B responses downstream of signal transduction through

tumor necrosis factor receptor (TNFR), TLR, IL1R (interleukin 1 receptor) and nucleotide-binding oligomerization domain containing 2 (NOD2) (133–135). Reduced A20 expression predisposes to autoimmunity as is shown in mice with B-lymphocyte-specific A20 ablation, which exhibits elevated numbers of germinal center B cells, autoantibodies and glomerular immunoglobulin deposits (136). In humans, GWA studies have suggested *TNFAIP3* as a susceptibility locus for SLE (52,54,55). Independent genetic associations of the *TNFAIP3* region with SLE in European population have been localized to a region 185 kb upstream of *TNFAIP3*, which was also associated with RA (137,138), a region 249 kb downstream of *TNFAIP3* (139) and a 109-kb haplotype spanning the *TNFAIP3* coding region, which harbors a putative causal variant in exon 3 (rs2230926, Phe127Cys) (52,140). By fine mapping and genomic resequencing in ethnically diverse populations, Adrianto et al. have further characterized the *TNFAIP3*-risk haplotype and identified a TT>A dinucleotide (deletion T followed by a T–A transversion) as the best candidate polymorphism responsible for the association between *TNFAIP3* and SLE in subjects of European and Korean ancestries (141). The TT>A dinucleotide variant, 42 kb downstream of the *TNFAIP3* promoter, is located in a region of high conservation and regulatory potential that may influence *TNFAIP3* expression by altering the binding of a nuclear protein complex composed of NF- $\kappa$ B subunits. *TNFAIP3*-interacting protein 1 (encoded by *TNIP1*) is involved in inhibition of NF- $\kappa$ B activation by interacting with A20 (encoded by *TNFAIP3*). Recent GWAS have revealed association of *TNIP1* with SLE in both Chinese and European-derived populations (53,54).

**77.4.2.5 Regulators of Lymphocytes in Adaptive Immune Response.** SLE is characterized by a loss of T- and B-cell tolerance, accounting for the formation of autoantibodies. GWAS have identified multiple SLE susceptibility genes involved in T- and B-cell signal-transduction pathways, which illustrate the importance of the differentiation, activation or function of various lymphocytes participating in the SLE pathogenesis.

**77.4.2.5.1 PTPN22.** *PTPN22* encodes tyrosine-protein phosphatase nonreceptor type 22, a lymphoid-specific phosphatase that inhibits T-cell activation. The 620Trp allele of the nonsynonymous SNP rs2476601 (Arg620Trp) is associated with a risk of developing multiple autoimmune diseases, including SLE, RA, GD and T1D, providing evidence for shared mechanisms despite their diversely different clinical presentations (142). The association between rs2476601 and SLE has been confirmed in European (51,53), but not in Asian GWAS (54,55), possibly attributable to a high variability in 620Trp allele frequencies among populations (European: 2–15%, Asian: nearly absent). The substitution of Arg with Trp at the amino acid 620 increases the intrinsic lymphoid-specific phosphatase activity of *PTPN22* that reduces the signaling threshold for T-cell receptor

(TCR) and promotes the development of autoimmunity (143). Supporting this notion, the Arg263Gln variant of *PTPN22* that reduces the phosphatase activity of *PTPN22* and increases the threshold for TCR signaling has been associated with protection against SLE in European populations (144). A connection between *PTPN22* and the type I IFN pathway has been suggested on the basis of elevated serum IFN- $\alpha$  activity in SLE patients carrying the 620Trp allele (145).

**77.4.2.5.2 *TNFSF4*.** TNF ligand superfamily member 4 (encoded by *TNFSF4*, also known as OX40L) expressed on antigen-presenting cells interacts with its receptor, TNF receptor superfamily member 4 (OX40L receptor), present on cell surfaces of activated T cells to induce the production of costimulatory signals. OX40L-mediated signaling induces B-cell activation and differentiation as well as IL-17 production, but inhibits the generation and function of IL-10-producing CD4<sup>+</sup> type 1 regulatory T cells (146). An SLE-risk haplotype defined by tag SNPs in the upstream region of *TNFSF4* is identified using both case-control and family-based European-derived samples, which correlates with increased expression of OX40L (147). Increased OX40L levels may predispose to SLE either by augmenting the interaction between T cells and antigen-presenting cells, or by influencing the functional consequences of T-cell activation via the OX40L receptor (147). Subsequently, associations between the *TNFSF4*-tagging SNPs and an increased risk for SLE have been confirmed in a GWAS of Chinese populations, a European replication study (53,54), and four independent SLE datasets from Germany, Italy, Spain and Argentina (148). These consistently observed robust associations warrant further studies to localize causal variants, and to understand how these *TNFSF4* polymorphisms affect the pathogenesis of SLE.

**77.4.2.5.3 *BLK*, *BANK1* and *LYN*.** *BLK* encodes B-lymphocyte-specific tyrosine kinase (Blk), a member of the Src family kinases, which functions in intracellular signaling and regulates the proliferation, differentiation and tolerance of B cells. Two SLE-associated SNPs of *BLK* have been identified in GWAS of European populations (50,51): one is rs13277113, located in the intergenic region between *FAM167A* and *BLK*, of which A allele is associated with reduced expression of *BLK* but increased expression of *FAM167A* in SLE patients; the other is an intronic SNP of *BLK*, rs2248932, located 43 kb downstream of rs13277113. Both SNPs have been subsequently confirmed for association with SLE in Asian populations (55,149,150).

*BANK1* encodes an adaptor/scaffold protein primarily expressed in B cells. *BANK1* protein regulates direct coupling between the Src family of tyrosine kinases and the calcium channel IP3R, and facilitates the release of intracellular calcium, altering the B-cell activation threshold (151). It is also identified as a binding partner of tyrosine-protein kinase Lyn (encoded by *LYN*), which plays an essential and rate-limiting role in mediating B-cell

inhibition by phosphorylation of CD22 and recruitment of SHP-1 (Src homology domain (SH)-1-containing tyrosine phosphatase) (152). GWAS in European populations have led to the identification of *BANK1* and *LYN* variants associated with SLE (51,76). Three functional *BANK1* variants including a nonsynonymous SNP in the IP3R binding domain (rs10516487; Arg61His), a branch point-site SNP (rs17266594; located in an intron), and another nonsynonymous SNP in the ankyrin domain (rs3733197; Ala383Thr), predispose to the sustained activation of B-cell receptors and the subsequent B-cell hyperactivity commonly observed in SLE (76). With the exception of the rs10516487 SNP of *BANK1*, which showed a weak association with SLE in an Asian GWAS (55), the remaining SNPs of *BANK1* and *LYN* have not been confirmed in Asian GWAS, partly owing to the low frequencies of the SNPs in Asian population (54,55). It is tempting to postulate that multiple B-cell-specific gene variants may interact, contributing to aberrant regulation of B-cell signaling commonly observed in SLE patients.

**77.4.2.5.4 *ETS1* and *PRDM1*.** *ETS1* encodes *ETS1* protein, a member of the ETS family of transcription factors, which inhibits the function of PR domain zinc finger protein 1 (encoded by *PRDM1*, also known as *BLIMP1*) that negatively regulates B-cell and T-helper-17-cell differentiation (153,154). Of interest, *PRDM1/ATG5* has been identified as a risk locus for SLE in both European and Asian GWAS (51,53,54), while genetic associations within *ETS1* region have been reported only by GWAS in Asians (54,55). The SLE-risk allele A of the *ETS1* variant (rs1128334; located in the 3' UTR) is associated with decreased *ETS1* expression levels in PBMCs from normal healthy controls (55). The importance of *ETS1* in SLE pathogenesis is further supported using *Ets1*-deficient mice that developed a lupus-like disease characterized by high titers of autoantibodies and local activation of complement (155).

**77.4.2.5.5 *IKZF1*.** DNA-binding protein Ikaros (encoded by *IKZF1*) is a member of a family of lymphoid-restricted zinc-finger transcription factors that regulates lymphocyte differentiation and proliferation, as well as self-tolerance through regulation of B-cell-receptor signaling (156). *IKZF1* is identified as a novel SLE susceptibility locus in a Chinese GWAS (54), and in large replication studies of European-derived populations (53). Decreased expression of *IKZF1* mRNA has been observed in PBMCs from patients with SLE (157), however, the role for *IKZF1* in the pathogenesis of SLE requires further study.

**77.4.2.5.6 *IL10*.** *IL10* encodes interleukin-10 (IL-10), which inhibits T-cell function by suppressing the synthesis of proinflammatory cytokines such as TNF $\alpha$ , IL-1, IL-6, IL-8, and IL-12 (158), inhibits antigen-presenting cells by downregulating MHC-II and B7 expression (159), and promotes B-cell-mediated functions, enhancing survival, proliferation, differentiation and antibody production (158). Of interest, increased IL-10 production by peripheral blood B cells and monocytes is observed in patients



with SLE and associated with disease activity (160), which could explain B-cell hyperactivity in SLE. A study of the molecular mechanisms underlying this increase in IL-10 production led to the identification of *IL10* haplotypes (defined by three SNPs in the *IL10* promoter region) that associated with variability in IL-10 production (161). Associations between these *IL10* SNPs and SLE susceptibility have been reported in European, Hispanic American and Asian populations (162–164). A large-scale replication study in populations from the USA and Sweden has confirmed *IL10* as an SLE susceptibility locus (53).

**77.4.2.6 X Chromosome Genes.** The striking ratio of approximately 9 females to 1 male for SLE susceptibility might be attributed to direct or indirect effects of sex hormones and/or X-linked genes. The first experimental evidence for sex chromosome related to SLE risk came from a report that showed increased autoimmune disease in mice with XX sex chromosome complement, as compared to XY, regardless of the gonadal sex (testes or ovaries) (165). In support of this idea, Scofield and colleagues found overrepresentation of Klinefelter's syndrome (men with more than 1 X chromosome; 47,XXY) among men with SLE and possibly decreased risk of SLE in X0 Turner's syndrome women (166,167). Recent studies have reported the genetic contributions for SLE on the X chromosome genes including *IRAK1*, *MECP2* and *TLR7*.

**77.4.2.6.1 *IRAK1* and *MECP2*.** *IRAK1* encodes a serine–threonine protein kinase named IL-1 receptor-associated kinase 1, which regulates multiple pathways in both innate and adaptive immune responses by linking several immune-receptor complexes to TRAF6 (TNF receptor-associated factor 6) (168). Studies by Jacob et al. provide an important insight into *Irak1* function in murine models of SLE as *Irak1* could play a role in regulation of nuclear factor  $\kappa$ B (NF $\kappa$ B) in TCR signaling and TLR activation, as well as in the induction of IFN- $\alpha$  and IFN- $\gamma$  (169). Additionally, they tested the candidacy of *IRAK1* as an X chromosome-encoded risk factor for SLE in humans. In studying ~5000 subjects of four different populations, five SNPs spanning *IRAK1* gene showed disease association in both adult-onset and childhood-onset SLE (169). Located in the region of LD with *IRAK1* is another potential risk gene for SLE, methyl-CpG-binding protein 2 (*MECP2*), which has a critical role in the transcriptional suppression of methylation sensitive genes (170). A large replication study in a European-derived population confirmed the importance of this region (*IRAK1*–*MECP2*) to SLE although further work is required to identify the causal variants (53).

**77.4.2.6.2 *TLR7/TLR8*.** Toll-like receptor 7 (*TLR7*), and its functionally related gene *TLR8* encode proteins that play critical roles in pathogen recognition and activation of innate immunity (171). They recognize endogenous RNA-containing autoantigens and induce the production of IFN- $\alpha$  (172). There is compelling evidence supporting the contribution of *TLR7* to the development of SLE. Transgenic mice with a twofold

overexpression of *Tlr7* accelerate to develop spontaneous autoimmunity (173), whereas *Tlr7*-deficient mice have ameliorated lupus disease, decreased lymphocyte activation, and decreased serum IgG (174). In addition, inhibitors of *Tlr7* can reduce a number of lupus-associated phenotypes both in the MRL and NZB/W lupus-prone strains (175). However, the finding that a genomic duplication of *Tlr7* associated with an increased severity of lupus-like disease in murine models was not translated to humans with SLE. A CNV study in multiple ethnic groups showed that the CN of *TLR7* varied in both SLE patients and normal controls, but it was not significantly increased among SLE patients compared to controls (176). Fine mapping the genomic region containing *TLR7* and *TLR8* led to the identification of a functional polymorphism in 3' UTR of *TLR7* that conferred elevated expression of *TLR7* and predisposed to the development of SLE in Asians (177). Similar studies in other ethnic populations are underway to elucidate variants within the *TLR7/TLR8* region for risk of SLE.

**77.4.2.7 Other Loci.** GWAS in multiethnic groups have revealed several SLE-associated loci appear to be unique to a specific ethnic group, such as *PXK* (PX domain containing serine/threonine kinase), *XKR6* (XK, Kell blood group complex-related family member 6), *JAZF1* (juxtaposed with another zinc finger gene 1) and *UHRF1BP1* (UHRF1-binding protein 1) in Europeans (51,53), but *RASGRP3* (RAS-guananyl-releasing protein 3) and *WDFY4* (WDFY family member 4) in Asians (54,55). Functions of these novel genes are neither fully characterized nor obviously connected to the known pathways contributing to SLE. To elucidate the underlying mechanisms by which these genes increase the risk for SLE will help to establish novel relationships to the disease pathogenesis.

**77.4.2.8 SLE Subphenotypes with Currently Known Susceptibility Loci.** SLE is a genetically complex disease with heterogeneous clinical manifestations. Following the GWAS that have greatly expanded the number of established SLE-risk loci, recent studies have begun to characterize cumulative associations of multiple risk loci and their relationship to SLE subphenotypes. Anti-double-stranded DNA (anti-dsDNA) autoantibody, an SLE-related autoantibody with diagnostic and clinical importance, is observed in 40–60% of SLE patients. A GWAS performed in European-derived population has identified robust genetic associations with SLE based on anti-dsDNA autoantibody production (178). SNPs of *STAT4*, *IRF5*, *ITGAM* and *HLA* are strongly associated with anti-dsDNA<sup>+</sup> patients, but fewer and weaker associations are observed for anti-dsDNA<sup>−</sup> patients. SNPs in *BANK1*, *PHRF1* and *UBE2L3* show associations only in anti-dsDNA<sup>+</sup> patients. These data suggest that many established SLE-susceptibility loci may confer disease risk through their roles in autoantibody production. By analyzing 22 previously established SLE-susceptibility loci, Taylor and his colleagues defined a genetic risk score (GRS) for SLE as the number of risk alleles

with each weighted by the SLE risk OR (179). Additionally, they studied associations of the individual risk allele and the GRS with clinical manifestations for patients. Their analysis categorizes SLE subphenotypes into three groups: those associated with GRS (cumulative risk loci) including age at diagnosis, anti-dsDNA autoantibody, oral ulcers, immunological and hematologic disorders; those associated with single-risk loci including renal involvement and arthritis; and those with no known genetic associations such as serositis, neurologic disorder, photosensitivity, malar and discoid rash (179).

**77.4.2.9 Shared Loci between SLE and Other Autoimmune Disease.** Paralleling the GWAS of mapping SLE-risk loci are the success in identifying genetic associations among other autoimmune diseases including RA, systemic sclerosis (SSc), primary antiphospholipid syndrome (APS), Behcet's disease (BD), T1D, inflammatory bowel disease (IBD), Crohn's disease (CD), ulcerative colitis (UC), GD and PSO. The identification of risk loci shared between SLE and other autoimmune disorders provides the possibility that common immunological mechanisms exist among some of these disease processes (Table 77-5). For example, a cluster of

genes involved in T-cell activation may increase susceptibility to autoimmune disease generically: *HLA class II* with multiple autoimmune diseases (180) (reviewed in Chapter 76); *PTPN22* with SLE, RA, GD, SSc and T1D (143,181–183); *TNFSF4* with SLE and SSc (184). More recently, the newly developed “ImmunoChip” array contains 184 loci with >200,000 SNPs representing genetic associations identified from one or more of 12 different inflammatory phenotypes, including SLE, RA, T1D, CD, UC, PSO, primary biliary cirrhosis, autoimmune thyroid disease, MS, celiac disease, IgA deficiency, and ankylosing spondylitis. The availability of this new platform will accelerate the identification of shared variants between multiple autoimmune diseases, and loci that promote disease-specific phenotypes.

## 77.5 GENE EXPRESSION PATTERNS ASSOCIATED WITH SLE

The association between type I IFN pathway activation and SLE has been demonstrated by a number of studies, showing (1) upregulated IFN-inducible gene expression patterns (known as IFN signature) in SLE blood cells (106,185–187), (2) increased serum IFN- $\alpha$  activity (188), and (3) elevated serum levels of IFN-regulated chemokines (189,190). The activation of type I IFN pathway appears to be associated with disease flares at a single time point of cross-sectional studies (191,192). However, longitudinal studies reveal that the IFN signature does not fluctuate with marked clinical changes (193,194), suggesting that upregulation of IFN-inducible gene expression is not a dynamic component of the SLE disease process, but a relatively stable characteristic (193). It is likely that a robust IFN signature provides a pro-flare environment but is insufficient to trigger rapid disease exacerbation (194). There may be additional factors precipitating disease flares in the context of IFN-mediated biochemical changes. Recent advances in SLE genetics have helped define specific SLE-associated genetic variants with functional relationships to IFN production or responses in patients. Given the proposed pivotal role of IFN in SLE, therapeutics targeting the IFN pathway is in development for the treatment of SLE, including ongoing clinical trials to test monoclonal antibodies that bind to IFN- $\alpha$  (195,196).

## 77.6 PREGNANCY COUNSELING OF SLE PATIENTS

In general, pregnancies in SLE do not exacerbate the disease. Flare rates of SLE during pregnancy are approximately 25%, which is similar to flare rates in nonpregnant SLE patients (197,198). Preexisting clinically active disease and nephritis are the main features that predict maternal complications (197,198). Preeclampsia/eclampsia as a pregnancy complication occurs in approximately 10% of lupus nephritis patients (198). Mutations in

**TABLE 77-5 SLE Loci Shared with Other Autoimmune Diseases**

Function	Gene	Location	Disease
Clearance of immune complex	<i>FCGR2A</i> <sup>a</sup>	1q23	T1D, UC
	<i>FCGR3A</i> <sup>a</sup>	1q23	RA
Regulation of Innate Immunity			
TLR/IFN signaling	<i>IRF5</i>	7q32	RA, IBD, SSc
	<i>STAT4</i> <sup>a</sup>	2q33	RA, SS, SSc, pAPS
TNF/NF- $\kappa$ B signaling	<i>TNFAIP3</i>	6q23	RA, T1D, PSO, Celiac disease
	<i>TNIP1</i>	5q33	PSO
Activation of Lymphocyte			
T-cell signaling	<i>HLA Class II</i>	6p21.3	RA, SSc, Graves' disease, IBD, T1D
	<i>PTPN22</i> <sup>a</sup>	1p13	RA, T1D, SSc, Graves' disease, CD
	<i>TNFSF4</i>	1q25	SSc
B-cell signaling	<i>BANK1</i> <sup>a</sup>	4q24	SSc, RA
	<i>BLK</i> <sup>a</sup>	8p23	SSc, pAPS
	Intergenic ( <i>PRDM1</i> )	6q21	RA, CD
	Intergenic ( <i>IKZF1</i> )	7p12	CD
Cytokine	<i>IL10</i>	1q31–q32	UC, T1D, BD

These loci were identified through GWAS, GWA meta-analysis studies, candidate gene studies or replication papers.

GWAS, genome-wide association studies; IBD, inflammatory bowel disease; IFN, interferon; NF- $\kappa$ B, nuclear factor  $\kappa$ B; pAPS, primary antiphospholipid syndrome; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, primary Sjögren's syndrome; SSc, systemic sclerosis; T1D, type 1 diabetes; TLR, toll-like receptor; TNF, tumor necrosis factor; UC, ulcerative colitis.

<sup>a</sup>All genetic variants at the shared loci are common across the listed diseases.

complement regulatory proteins, including membrane cofactor protein, and complement factor I predispose to preeclampsia in SLE (199). Increased synthesis of complement (C3, C4) by the liver during pregnancy can mask the drop in complement levels that indicate an impending disease flare. Changes in levels of estrogen, prolactin, and other hormones that have complex interactions with the immune system may affect the disease course. It is recommended that patients with SLE attempt pregnancy only during periods of disease quiescence, and if renal, cardiac and pulmonary functions are not impaired.

Although there is no known teratogenic effect of SLE itself, maternal transfer of autoantibodies can cause damage to a developing fetus. Antiphospholipid antibodies and lupus anticoagulant are prevalent in lupus patients and have been implicated in recurrent fetal loss and thrombosis (200). Maternal transfer of antiphospholipid antibodies can cause fetal loss and intrauterine growth retardation (201). A few SNPs in *BLK* and *STAT4* genes that predispose to SLE also predispose to APS (without SLE) (202). Transfer of maternal anti-Ro (SS-A) and/or anti-La (SS-B) antibodies can cause injury to the atrioventricular node of a fetal heart, leading to the development of congenital heart block (CHB) during the eighteenth through twenty-fourth weeks of gestation (203). Because only 2% of pregnant women with antibodies to SSA/SSB deliver babies with CHB, it has been thought that maternal genetic predisposition is also required, and studies have suggested that several genes in the HLA region are involved (204). However, the development of CHB in a fetus grown in a surrogate mother who was anti-Ro-positive suggests that in some cases, the antibody itself can be pathogenic (203). Buyon reviewed studies of mothers of children with CHB and concluded that mothers who have both anti-Ro (SS-A) and anti-La (SS-B) antibodies are at higher risk for infants with CHB than mothers with either autoantibody alone (201). It is not unusual for mothers of infants with neonatal lupus to be asymptomatic. In a neonatal lupus research registry, 35% of mothers (39 of 110) were asymptomatic at the time CHB was identified in their infants. After a mean follow-up of 8 years, 14 of the 39 mothers (36%) developed symptoms of a rheumatic disease, while the majority (64%) remained asymptomatic (201). Therefore, pregnant women with SLE should be assessed for the presence and the titer of antiphospholipid antibodies (especially of the IgG class, with a history of intravascular thrombosis or prior fetal loss) because of risk for fetal loss, as well as for presence of anti-Ro (SS-A) and anti-La (SS-B) antibodies because of the small risk for CHB. Genetic determinants for the ability to make antiphospholipid and anti-SSA/SSB antibodies autoantibodies are only partially understood. The majority of association studies between MHC class II alleles and the production of specific autoantibodies to Ro, La, and phospholipid have shown that (i) the presence of both anti-Ro (SS-A) and anti-La (SS-B) is associated with HLA-B8, DR3, DRw52, and DQw2 (201), (ii) the

presence of anti-Ro (SS-A) alone is mainly associated with DR2 and DQw1 (201,205), and (iii) the presence of various antiphospholipid antibodies is associated with DR4, DR7, DRw53, and DQB1\*0302 (206). Results from these studies have not reached a point where they can be used to successfully predict fetal risk for neonatal lupus or for CHB. Thus, genetic counseling for families of SLE patients cannot offer precise risk figures yet.

## 77.7 CONCLUDING REMARKS

In the past few years (2007–2011), collaborative efforts among investigators in the field of lupus genetics have made it possible to conduct large-scale, case-control association studies using both the candidate gene approach and GWAS, establishing over 30 confirmed loci including many novel loci. Despite the tremendous progress, there remain several challenges for the future studies. (1) The disease-associated variants identified in current GWAS are almost common SNPs (frequency > 5%) and not all of them are likely to be the causal variants. Next-generation sequencing of an associated region will be needed to reveal unknown variants, including rare SNPs with a prevalence <1% and other polymorphisms (including insertion/deletion, copy number, and repeat element variations) which could also contribute to SLE. (2) It is important to note that many of the GWAS associations detected so far have been in European or Asian populations. Additional studies using large samples of diverse ethnic populations, particularly African and Hispanic ancestries, are also required. This will help clarify the basis for disparities of SLE association between different populations and provide a better understanding of the genetic basis of SLE. (3) GWAS have not only highlighted the importance of shared biological pathways in the pathogenesis of SLE and some of other autoimmune diseases but also implicated the involvement of previously unrecognized pathways. Understanding the contribution of each associated genetic locus in the context of complex molecular pathways and networks that lead to higher order clinical disease will improve current medical practice in providing pregnancy counseling, personalized care and treatments for patients affected with SLE in the near future.

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Immunologic Disorders.

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**Yun Deng, MD** is a postdoctoral scholar of the Department of Medicine, Division of Rheumatology at UCLA. Her current research includes the identification of susceptibility genes for systemic lupus erythematosus (SLE) and elucidating the molecular mechanisms underlying these identified genetic risk factors that affect disease pathophysiology.



**Bevra H Hahn, MD** is a professor of Medicine, Vice-Chair of the Department of Medicine, and Chief of Rheumatology at UCLA. She is past-president and a Master of the American College of Rheumatology. Dr Hahn provides clinical consultations for patients with rheumatic diseases, particularly SLE. Her current research includes investigations in genetics, pathogenesis, and treatment of SLE, as well as clinical trials in new therapies for accelerated atherosclerosis.



**Betty P Tsao, PhD** is a professor of Medicine of the Department of Medicine, Division of Rheumatology at UCLA. Her current research includes the identification of human chromosomal regions containing susceptibility genes for systemic lupus erythematosus (SLE). Genetic susceptibility is the single greatest risk for SLE. Her laboratory has gathered approximately 300 families that contain two or more members with SLE. Recently, she has shown that the human chromosome 1q regions are likely to contain multiple genes predisposing to SLE, and discovered that Asian males, in particular, with a variant form of the X-linked gene “Toll-like receptor 7 (TLR7)” are at increased risk of developing SLE. Dr Tsao is currently studying the role of X-linked genes in gender bias of SLE, functional consequences of SLE-associated variants, and the genome-wide association studies of Asians in SLE.



# Rheumatoid Disease and Other Inflammatory Arthropathies

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## GLOSSARY

**Anticitrullinated peptide antibody (ACPA)** – Citrulline is a nonstandard amino acid derived by posttranslational deimination of arginine by the enzyme peptidylarginine deiminase. Compared to rheumatoid factor, ACPA are more specific (95%) and equally sensitive (67%) serological biomarkers of RA.

**Enteropathic arthritis** – Inflammatory arthritis related to inflammatory bowel disease (e.g. Crohn's disease or ulcerative colitis).

**Endoplasmic reticulum aminopeptidase 1 (ERAP1)** – (a.k.a. ARTS-1 (Type 1 tumor necrosis factor receptor shedding aminopeptidase regulator)) A gene with a highly significant association with HLA-B27 positive ankylosing spondylitis. Protein for which it codes trims peptide antigens to optimal length for binding to HLA class 1 molecules.

**Felty syndrome** – Disease complex of rheumatoid arthritis, lymphadenopathy, neutropenia, and splenomegaly.

**Fibroblast-like synoviocyte (FLS)** – Cells within the synovial intimal lining which are key players in the inflammation and joint destruction in rheumatoid arthritis.

**Genome-wide association studies (GWAS)** – These studies involve systematic genotyping of a large number of SNPs with minor allele frequency >1% across the entire genome; comparison between patients with disease and healthy controls aims to identify genetic loci over- or underrepresented in the case cohort.

**Receptor activator of nuclear factor- $\kappa$ B (RANK)** – Receptor on surface of osteoclasts for **receptor activator of nuclear factor- $\kappa$ B ligand (RANKL)**. RANKL binding to RANK induces differentiation of osteoclast precursors into osteoclasts, and activates mature osteoclasts.

**Rheumatoid factor (RF)** – Antibody against the Fc portion of IgG.

**Seronegative spondyloarthropathy (SpA)** – Group of inflammatory conditions including ankylosing spondylitis, reactive arthritis, enteropathic arthritis, and psoriatic arthritis. They are characterized by a number of common features including inflammation of the

entheses; prominent axial and asymmetric lower limb peripheral large-joint arthritis; extra-articular features including uveitis; the formation of new bone at the site of inflammation; and association with the MHC class I gene HLA-B\*27. AS is the hallmark SpA.

**Severe combined immunodeficiency (SCID)** – A group of rare genetic diseases in which both B cells and T cells are deficient resulting in marked susceptibility to recurrent and overwhelming infection.

**Shared epitope (SE)** – All rheumatoid-associated DR molecules (encoded by DRB1\*0401, \*0404, \*0405, \*0408, \*0101, \*0102, \*10, and \*1402) share an identical or similar sequence between amino acids 67 and 74, <sup>67</sup>LLEQRRAA<sup>74</sup>. This sequence codes for a highly conserved sequence along the  $\alpha$ -helix derived from the DR $\beta$  chain, which forms one side of the antigen-binding site of the DR molecule.

**Tumor necrosis factor  $\alpha$  (TNF $\alpha$ )** – A cytokine with a central role in inflammation.

## 78.1 INTRODUCTION

The inflammatory arthropathies described in this chapter are multifactorial polygenic disorders. In recent years, the application of genome-wide association studies (GWAS) has played an important part in identifying many of the genes involved. Associations with human leukocyte antigens (HLAs) were first described in 1973 although the precise mechanisms by which these cause disease are still unclear. The association with protein tyrosine phosphatase PTPN22 is now well established as the second strongest with rheumatoid arthritis (RA) and is particularly a good example of an association that is common to several autoimmune conditions. There is now convincing evidence for the involvement of more than 30 genes in RA and at least 14 in ankylosing spondylitis (AS). Many of these are key factors in the regulation of inflammatory and immunological responses, as expected, but others have less-obvious biological expectations. The association of RA

with HLA-DRB1 alleles is robust and its interaction with smoking as an environmental factor is strongly suggested. The association of AS with several genes in the IL-17 producing (Th17) lymphocyte subset has already marked this as a potential therapeutic target (1). The highly significant association of AS with ERAP1 has provided an important example of gene–gene interaction in susceptibility to a complex disease since the association is entirely restricted to those individuals with AS who also carry HLA-B\*27 (2). This association has also been evaluated at the level of protein structure and function. ERAP1 variants associated with protection against AS have reduced ability to trim peptide antigens to optimal length for binding to HLA class 1 molecules (3). This raises the possibility that inhibitors of ERAP1 could be protective against AS.

## 78.2 RHEUMATOID ARTHRITIS

Clinical descriptions of RA appeared fairly late in the medical literature and it was only distinguished from gout and osteoarthritis by Garrod 200 years ago. Classical RA is characterized by a destructive inflammatory arthritis affecting the synovial joints, but a wide spectrum of severity exists. The substantial variation in clinical features and severity of the disease underlines the likelihood that its etiology is also heterogeneous, including the genetic factors that are involved. Familial clustering of cases is well recognized, with a generally accepted fivefold excess sibling recurrence risk over the general population risk ( $\lambda_s \sim 5$ ) although this varies with the severity of the disease in the proband.

Typically, RA presents as a distal small-joint arthropathy of the hands (Figure 78-1) and feet that may initially involve relatively few joints. Subsequently, the more proximal load-bearing joints become involved, potentially leading to severe functional disability. Any synovial joint, including the temporomandibular and cricoarytenoid joints, may be affected. Overall the disease is approximately three times more common in women than

men, but this varies with the age of onset. Thus, at the age of 30 years, women are nearly 10 times more frequently affected than men, while there is no gender difference in incidence by the age of 65 years. RA may present in many different ways. In about two-thirds of cases, there is pauciarticular, insidious onset (typically in the hands and feet) with cumulative joint involvement over the course of months/years. This may be difficult to distinguish from other self-limiting causes of synovitis in the early stages of the disease. In contrast, the onset is explosive in a small minority of cases who develop widespread symmetrical polyarthritis over a few days or even overnight. Despite this dramatic onset, a proportion of such cases eventually show complete resolution of synovitis and little joint destruction a year or two later. Systemic features, including fever, weight loss, and malaise, occasionally dominate the onset of RA, particularly in middle-aged men, prompting extensive investigation to exclude alternative causes, such as deep-seated infection or malignancy. Limb girdle symptoms similar to polymyalgia rheumatica may be prominent, particularly in those with later-onset disease. Occasionally, patients present with “palindromic rheumatism;” this consists of short-lived attacks (~48 h) of mono-, oligo- or polyarticular synovitis that initially resolve completely but recur at intervals and may eventually persist. Positive tests for rheumatoid factor (RF) and anticitrullinated protein antibodies (ACPAs) are predictive of progression to persistent rheumatoid disease.

Extra-articular features are common in RA. These include skin nodules, cachexia, and normochromic, normocytic anemia. Mild peripheral neuropathy with glove-and-stocking sensory loss is more common than mononeuritis multiplex caused by rheumatoid vasculitis of the vasa nervorum. Major rheumatoid vasculitis affects men more commonly than women and can cause life-threatening cutaneous ulceration, myocardial infarction, or bowel ischemia. Serositis is relatively common in RA; pericardial effusions are often asymptomatic and have been reported in up to 30% of those with RA on echocardiography. Rarely, constrictive pericarditis may result, and the heart valves are occasionally affected, particularly in those with nodular disease. Corticosteroids may be required to treat symptomatic pleurisy or pericarditis. About 20% of patients develop secondary Sjögren syndrome with keratoconjunctivitis sicca and/or xerostomia. The eyes are commonly affected by episcleritis, which usually requires no treatment, but, less commonly, there may be scleritis, which may require systemic corticosteroids or immunosuppression. Lymphadenopathy is common, but <1% of patients develop Felty syndrome (RA, lymphadenopathy, neutropenia, and splenomegaly). This disorder rarely develops <10 years from the onset of RA (4). Patients with Felty syndrome probably suffer joint disease of similar severity to non-Felty patients, but have more extra-articular manifestations. Familial recurrence of RA is more common where the proband has Felty syndrome.



**FIGURE 78-1** Classical rheumatoid deformity of the hands with ulnar deviation and subluxation at the metacarpophalangeal joints.

Historically, despite active treatment with standard disease-modifying drugs, <50% of those with RA have been able to work full-time after 10 years of disease. Mortality is also increased by about 50%, reducing life expectancy by about 11 years (mainly as a result of increased cardiovascular disease and infections). New approaches to treatment with antitumor necrosis factor biologics have had a dramatic effect on joint disease in RA that may eventually be reflected in better mortality statistics too.

### 78.2.1 Pathology

Despite intensive research, the pathophysiology of RA remains incompletely understood. Animal models of the disease have provided some useful insights, including the central role of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) in rheumatoid inflammation. Such models are imperfect, however, and much useful information has been gleaned from careful studies of the disease in humans and from dissecting the genetic basis of the disease in GWAS.

There is convincing experimental and clinical evidence of involvement of many cell types, including macrophages, B lymphocytes, T lymphocytes, and synovial fibroblasts, all of which are overrepresented in RA synovium. In RA, the normal synovial lining expands from 1–3 to 10–15 cell layers, which is predominantly composed of macrophages and synovial fibroblasts; the additional influx and proliferation of immune and resident synovial cells also contribute to synovial hyperplasia. The most abundant stromal cells are the fibroblast-like synoviocytes (FLS) that appear abnormally resistant to apoptosis and accumulate in the RA synovium. Activated FLS are detectable early on in rheumatoid disease and although multiple factors from both the innate (primarily TLR2) and adaptive (e.g. TNF $\alpha$ , IL-1, IL-6 and IL-17) immune systems support FLS activation, at least part of FLS activation appears to be independent of the surrounding inflammatory tissue (5). FLS activation leads to the upregulation of numerous chemokines (MIP-1 $\alpha$ ), cytokines, matrix metalloproteinases, and adhesion molecules (integrins, VCAM-1 and cadherins) required for the recruitment of inflammatory cells and their destructive effects in the joint (6). In particular, the production of interleukin (IL)-15 by FLS induces the production of other cytokines, such as TNF $\alpha$  and IL-17, by T cells through direct cell–cell contact. This further stimulates the expression of IL-15 and IL-6 by FLS, creating a feedback loop that favors persistent inflammation (7). Microarray analysis has suggested two subgroups of FLS distinguished by their gene expression signatures. FLS from highly inflamed areas have a TGF $\beta$ -activin A-inducible signature characteristic of myofibroblasts (which have a particular propensity for chemokine and cytokine production), while FLS from less-inflamed synovial tissue have a predominance of insulin-like growth factor-regulated genes (8).

Classification of RA synovial tissue histomorphology has been attempted according to differential synovial infiltration by leukocyte populations. Most patients with RA have diffuse sublining infiltration consisting of scattered CD4 T lymphocytes and monocytes, but a significant proportion (up to 25% in some series (9)) develop more discrete synovial aggregates with T- and B-cell compartmentalization. Intriguingly, these aggregates can progress into ectopic lymphoid structures resembling germinal centers with characteristic follicular dendritic cell networks and proximity to high endothelial venules. A recent study using a severe combined immunodeficiency (SCID) mouse model suggested that these follicles are functional, not only expressing activation-induced cystidine deaminase (AID), the key enzyme in somatic hypermutation and class-switch recombination of immunoglobulin genes, but also supporting the production of ACPA (10). Unfortunately, differences in clinical study design and classification of aggregates have thus far led to contradictory findings on the correlation of lymphoid neogenesis with disease- or treatment-response phenotypes in RA. Indeed, lymphoid neogenesis may simply represent a bystander effect in any form of local inflammation (and interestingly such aggregates may also be found in patients with psoriatic arthritis, which is not held to be an autoantibody or B-cell-mediated disease). Despite this, some homogeneity does indeed exist and the synovium as a potential biomarker for disease severity or treatment response remains a tantalizing possibility.

The hypertrophic synovium develops into invasive pannus, eroding the articular cartilage and bone, particularly at the points of synovial attachment, causing loss of joint space, instability, and deformity. Accumulation of osteoclasts at sites of bone erosion is characteristic. These multinucleated cells express receptor activator of nuclear factor- $\kappa$ B (RANK) and are derived from CD14-positive cells of monocyte/macrophage lineage under the influence of macrophage/monocyte colony-stimulating factor, RANK ligand (RANKL), and inflammatory cytokines. Increased numbers and activity of osteoclasts are both hallmarks of inflammatory bone loss. The inflammatory milieu within the RA joint also serves to augment not only osteoclast precursor recruitment from bone marrow but also their subsequent differentiation into mature osteoclasts. Mature osteoclasts secrete hydrochloric acid to dissolve inorganic bone matrix, while the bone matrix proteins are degraded by proteolytic enzymes (e.g. matrix metalloproteinases and cathepsin K). The main sources of RANKL are osteoblasts, FLS and activated T cells and its expression in these cells is upregulated by proinflammatory cytokines in synovial tissue, including IL-1, IL-6, IL-17 and TNF $\alpha$ . Such cartilage and bone loss is manifested radiologically by juxta-articular osteoporosis, narrowing of the joint space and the development of erosions and joint deformity (Figure 78-2); however, the relationship between synovial inflammation and articular erosion is complex and variable. Occasionally,





**FIGURE 78-2** Radiographic appearances of advanced rheumatoid arthritis.

patients with prolonged synovitis do not erode, while erosions may be apparent in others at the time of presentation. Erosive disease is more common in those who are HLA-DRB1\*04 positive.

Angiogenesis is a key event in the maintenance of synovial inflammation, delivering nutrients and immune cells to the site of inflammation. Despite new vessel formation, inflamed synovial tissue is invariably hypoxic: synovial proliferation leads not only to increased metabolic demand but also regional hypoperfusion as tissue hyperplasia increases the distance between proliferating cells and infiltrating vessels. The resultant low tissue oxygen tension drives the transcription of hypoxia-inducible factor (HIF) regulated genes, not least vascular endothelial growth factor (VEGF), the most potent proangiogenic molecule.

The complex pathophysiology of RA has been amply demonstrated in recent years by the efficacy of several different immunomodulatory drugs that target discrete elements of the immune system. These include biologic agents targeting cytokines, such as TNF $\alpha$  (therapeutic monoclonal antibodies and a recombinant TNF receptor/Ig fusion protein) and the IL6 receptor; T-cell activation (recombinant CTLA4/Ig fusion protein); and B cells (anti-CD20 agents).

### 78.2.2 Diagnostic Criteria

RA is regarded by many as a heterogeneous group of conditions with overlapping phenotypes. Over the years, many attempts have been made to define the disease

**TABLE 78-1** 2010 American College of Rheumatology/European League against Rheumatism Classification Criteria for Rheumatoid Arthritis (15)

**Score  $\geq 6/10$  required for classification as definite RA:  
Add scores A–D**

**A. Joint involvement**

1 large joint **0 points**; 2–10 large joints **1 point**; 1–3 small joints **2 points**; 4–10 small joints **3 points**;  $>10$  joints including at least 1 small joint **5 points**.

**B. Serology**

Negative RF and negative ACPA **0 points**; Low positive RF or low positive ACPA **2 points**; high positive ( $>3$  times upper limit of normal) RF or high positive ACPA **3 points**.

**C. Acute-phase reactants**

Normal CRP and ESR **0 points**; Abnormal ESR or CRP **1 point**.

**D. Duration of symptoms**

$<6$  weeks **0 points**;  $\geq 6$  **1 point**.

Adapted from Aletaha, D.; Neogi, T.; Silman, A.; et al. 2010 Rheumatoid Arthritis Classification Criteria: An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum.* 2010, 62, 2569–2581.

more accurately using classification criteria, including those developed by the American College of Rheumatology (or its antecedents and collaborators) in 1958, 1987 and 2010. Comparisons between population studies are therefore somewhat complicated by differences in the diagnostic criteria that have been used. The 2010 American College of Rheumatology/European League Against Rheumatism classification criteria (Table 78-1) have a high degree of sensitivity and specificity. They have been modified particularly to detect early disease more effectively than the previous criteria because it is now recognized that early treatment of RA can prevent irreversible joint damage (11). The diagnosis is not difficult in typical cases once the disease has been present for several months, but in early disease, a confident diagnosis may be difficult, particularly where there is palindromic onset, limited synovitis or normal inflammatory markers. As many as one-fifth of patients presenting at an early synovitis clinic may turn out not to have RA after 1 year or so (12,13). The recognition that ACPA occur with high frequency in RA has led to their development as diagnostic aids in early inflammatory arthritis. ACPA have a similar sensitivity to RF for RA [ $\sim 80\%$ ] but are more specific [ $\sim 98\%$ ]. Both RF and ACPA may precede the development of clinical disease by months or years (14).

### 78.2.3 Differential Diagnosis

The diagnosis of RA is relatively straightforward except in the early phase, when distinction from self-limiting arthropathies (e.g. reactive arthritis, viral arthropathies, and crystal arthropathies) may be more difficult.



Distinction from the seronegative arthropathies, notably psoriatic arthritis and reactive arthritis, may sometimes be problematic. Generalized nodal osteoarthritis is occasionally similar and may coexist with RA; typically it affects the proximal and distal interphalangeal joints but usually spares the metacarpophalangeal joints and wrists. Osteoarthritis affecting the knees and hips is very common and is partly genetic in origin; large-joint osteoarthritis requiring joint replacement has an excess sibling recurrence risk of 2.3 (16).

Crystal arthritis is common and may be difficult to distinguish from RA, particularly in the elderly. For example, gout is polyarticular in onset in 10% of cases and quite commonly is not associated with elevated uric acid levels in the acute phase. On the other hand, its typical presentation with monoarticular lower limb arthropathy is quite distinct (50% of initial attacks involve the great toe, “podagra”). The diagnosis is correctly established by demonstrating intracellular needle-shaped crystals of monosodium urate in aspirates from affected tissues that are strongly negatively birefringent under polarized light. It is strongly familial, and its genetic etiology is reviewed in Chapter 95. Pyrophosphate arthropathy may cause a “pseudorheumatoid” pattern of chronic arthropathy, as well as the more widely recognized acute episodes of “pseudogout,” which can also punctuate the more chronic forms of pyrophosphate arthropathy. The diagnosis of crystal arthritis is best established by aspiration of the affected joint and demonstration of the relevant intracellular crystals under polarized light. Familial forms of chondrocalcinosis, characterized by early-onset pyrophosphate arthropathy, often have a dominant inheritance pattern. Some are linked to chromosome 5p (17), where gain-of-function mutations in ANKH, encoding a transmembrane transporter of inorganic pyrophosphate, have been described. Polymorphic variants of ANKH are also associated with sporadic forms of pyrophosphate arthritis (18). Pyrophosphate arthropathy is also a classic component of hemochromatosis (Chapter Iron Metabolism and Related Disorders), in which abnormal iron handling causes its deposition in numerous tissues, including the liver, myocardium, and synovium. The defective HFE gene is a member of the immunoglobulin superfamily and has a very high mutant allele frequency (~0.1) in northern Europe. Premature degenerative arthritis, particularly in atypical sites, such as the metacarpophalangeal joints, wrists, and ankles, should raise suspicions, especially if articular calcification is present. The diagnosis is best established by demonstrating saturation of transferrin and elevated ferritin levels. Elevated ferritin levels alone may be spurious, since ferritin, an acute-phase reactant, is commonly elevated in inflammatory states including RA.

#### 78.2.4 Population Prevalence

Depictions of RA in art and literature seem to be lacking until about 200 years ago in Europe. As a consequence,

there has been speculation that the disease appeared relatively late in Europe and may even have been imported from the New World. RA has rarely been identified convincingly in the archeological record, but it may be mistaken for other diseases. Nonetheless, there is some evidence that the disease existed in Egypt in the third millennium B.C. and also in Roman Britain (19).

The disease has a peak incidence in the age of 40–50, but may present at almost any age. There is an excess of incidence in the puerperium, and it has been suggested that oral contraceptives may confer some protection. Although most women also experience remission in pregnancy, unfortunately, recrudescence in the puerperium is almost invariable and may be severe.

The prevalence of RA in many populations worldwide is remarkably similar (~1%), but with some important exceptions that may give clues to the etiology of the disease. Environmental and/or genetic factors could account for the variations in the prevalence observed in certain ethnic groups (20,21). For example, the disease is rare in most of sub-Saharan Africa (prevalence = 0.3%) but common in certain American Indian groups (5–7%). South African blacks in a rural environment exhibit the same low prevalence of the disease as in most of sub-Saharan Africa. This contrasts starkly with the high frequency of the disease in their urban counterparts, similar to that in South Africans of European descent, which strongly suggests an environmental influence. By contrast, the high frequency of RA in the Chippewa and Yakima Indians of North America may be due to the high frequency of certain HLA class II alleles (see Section 78.2.5.2.1) associated with RA: HLA-DRB1\*04 and the rare HLA-DRB1\*1402 allele, respectively (22,23). In most populations that have been studied, there are strong associations with various HLA-DRB1\*04 alleles (relative risk ~5) (21,24,25). The HLA-DRB1\*04 series, originally defined serologically as the HLA-DR4 transplantation antigen, can be subdivided into numerous HLA-DRB1 alleles, not all of which are associated with rheumatoid. Thus, HLA-DRB1\*0402 (originally defined as Dw10 in the mixed lymphocyte reaction) is the dominant HLA-DRB1\*04 subtype in Jews, but since DRB1\*0402 is not associated with RA, there is no apparent association of RA with DRB1\*04 overall in Jews, as discussed in Ref. (26). In contrast, associations are seen with HLA-DRB1\*01 and HLA-DRB1\*10 in Jews, which are relatively weaker associations in other populations where the association with HLA-DRB1\*04 is more obvious. The rarity of HLA-DRB1\*04 in Nigeria could account for the low incidence of RA in this population, but by contrast, in nearby Gambia, where HLA-DRB1\*04 is relatively common (~15%), RA is still very rare (our own survey in the 1990s revealed only one case in 2000 individuals). This suggests that either non-HLA genetic effects and/or environmental factors are also important. Indeed, it has been speculated that chronic stimulation of the immune system by tropical infections, including malaria, may in

some way be protective against the development of autoimmunity (27). The lack of geographic case clustering is in stark contrast to other forms of inflammatory arthritis, such as Lyme disease (28) or postenteric reactive arthritis (see Section 78.3.3), in which there is a clear evidence for an infective trigger. No convincing epidemiologic data have yet been provided to support a role for infection although it is widely believed that microbial agents are involved (29). Several viruses, including Epstein–Barr virus, rubella, and parvovirus B19, can cause acute and occasionally more persistent arthropathies, but neither viruses nor bacteria have been consistently isolated from the joints of affected individuals.

## 78.2.5 Genetics

Concordance rates in monozygotic twins vary between studies from 12% to 30%, which is not surprising in view of the different methodologies used. In the United Kingdom in the 1960s, concordance in identical twins with seropositive erosive disease was estimated as 30% (30). This contrasts with a lower rate of 12% obtained in a nationwide survey of twins in Finland that included individuals with less-severe disease (31). A more recent UK study (32) found 15% concordance in identical twins and demonstrated that this was highest in those twin pairs that were HLA-DRB1\*04 positive (33). In all of these studies, the rates for monozygotic twin concordance were four to five times higher than for dizygotic twins, highlighting the likely importance of genetic factors. There seems little doubt that nongenetic factors are important, not only in the development of RA but also in its progression. On the basis of these relatively small studies, broad sense heritability has been estimated to be about 55% (34).

**78.2.5.1 Sibling Recurrence Risk.** Sibling recurrence risk varies according to the severity of the disease in the proband. In Lawrence's UK studies in the 1960s (30), the excess risk to the sibs of individuals with mild nonerosive seronegative disease was barely greater than the general population frequency ( $\lambda_s$  1.1). This is increased to sixfold in the siblings of individuals with seropositive, erosive disease, where as many as 15% of individuals will have an affected first-degree relative (35). Family recurrence risks of RA are also around seven times greater when the proband has Felty syndrome and are probably also increased when the index case has early-onset disease. The observation that the sibs of those with RA who share both HLA haplotypes identical by descent with the proband are at particular risk (36) is especially interesting as this group may be more likely than others to be HLA-DRB1\*04 homozygous (37,38). The excess sibling recurrence risk over the general population risk ( $\lambda_s$ ) is critically dependent on the criteria used in diagnosis. Thus mild, nonerosive disease is relatively common in the community at large and sib recurrence risks are relatively low. In contrast, seropositive, erosive rheumatoid

disease (that is typically followed up in hospital clinics) is much less common in the community and has higher sib recurrence risks. For this type of chronic, erosive disease,  $\lambda_s$  is probably nearer 12 to 14 and the genetic contribution is correspondingly higher.

**78.2.5.2 HLA and RA.** RA is clearly linked to the major histocompatibility complex (MHC) (39), as demonstrated in many populations (Table 78-2). The application of DNA-based approaches amply confirmed these linkages (40–43) and has now been applied at the genome-wide level for non-MHC loci. HLA class I associations with RA are invariably weaker than those in the HLA class II region, particularly with HLA-DRB1 alleles (21,24,25,44). With the application of high-density mapping techniques, it is now clear that there are important associations not only with HLA-DRB1 but also with the HLA-B and HLA-DP loci (45).

**78.2.5.2.1 Associations with the Classic Serologic HLA-DR (HLA-DRB1) Specificities.** Although these associations were originally defined by serology, they have been amply replicated and refined latterly at the DNA level. Associations with HLA-DRB1\*04 are well established in most ethnic groups globally, typically with a relative risk of approximately 5. Some important exceptions to this general rule have been observed, particularly among Jews (26), some Indian populations (46), Chileans (47), and the Yakima American Indians (23), in whom alternative associations have been found with DRB1\*01, DRB1\*09, and DR6 (DRB1\*1402). The association with HLA-DRB1\*09 originally described in Chileans has also been found as a minor association in the UK (48). Although the association with DRB1\*04 predominates in most populations, other weaker associations are not uncommon. Thus, associations with HLA-DRB1\*10 have been observed in Spaniards (49,50), Indians (51), and Jews (52), and with HLA-DRB1\*01 (DR1) in Indians (46,51), Jews (52,53), and southern European populations, in particular (54). While the strongest associations in the United Kingdom are with HLA-DRB1\*04, weaker associations can also be detected with both DRB1\*01 and DRB1\*10 (44,55).

**78.2.5.2.2 Differential Association of the DR4 (HLA-DRB1\*04) Allelic Subtypes with RA.** Not all DR4 haplotypes are equally associated with RA, despite the fact that SNP analysis indicates a high degree of conservation in the DNA flanking the HLA-DRB1 locus that encodes the polymorphic DR $\beta$ 1 chain (56). Thus, HLA-DRB1\*0401, \*0404, \*0408, and \*0405 are positively associated with RA, but \*0402, \*0403 and \*0407 are negatively associated (44,57,58). In the United Kingdom, there is a hierarchy of HLA-DRB1 susceptibility alleles (Table 78-3). The most widely held explanation for this observation (shared epitope hypothesis) is that there is a conserved functional epitope in the HLA-DR molecules positively associated with RA (encoded by DRB1\*0401, \*0404, \*0405, \*0408, \*0101, \*0102, \*10, and \*1402).

TABLE 78-2 HLA Sharing in Rheumatoid Arthritis—Affected Sib-Pairs

Ethnic Group	Criteria	Haplotype Sharing		
		0	1	2
French	1958 ARA C/D	0	1	0
Mixed	1958 ARA C/D	3	11	7
American	1958 ARA C/D	0	4	1
North Sweden	1958 ARA C	2	8	4
American	Seropositive erosive	2	0	1
Caucasoid	1958 ARA C/D	2	7	4
Italian	1958 ARA n/s	1	0	1
Canadians	1958 ARA C/D	1	0	2
Australasian	1958 ARA C/D	4	9	4
German	1958 ARA C/D	0	0	1
Norwegian	1958 ARA C/D	1	4	2
American	1958 ARA C/D	3	6	3
Dutch	1958 ARA C/D	1	8	0
North Sweden	1958 ARA C/D	1	1	0
UK white	1958 ARA n/s	5	20	17
Southern Irish	1958 ARA C/D/Pr/Po	6	12	16
UK white	1958 ARA D/Pr	6	24	23
Mixed	1958 ARA C/D	1	7	7
Dutch	1958 ARA D	0	0	2
American	1958 ARA C/D	4	8	4
North Indian, Hindu	n/s	0	3	6
English	1958 ARA C/D	3	6	6
American	1958 ARA C/D	4	15	18
Egyptian	1958 ARA n/s	0	7	10
<b>German</b>	1958 ARA C/D	7	4	1
<b>Total</b>		<b>57</b>	<b>165</b>	<b>140</b>
<b>Percentage</b>		<b>16</b>	<b>45</b>	<b>39</b>
<b>Expected Percentage</b>		<b>25</b>	<b>50</b>	<b>25</b>

ARA, American Rheumatism Association; C, classical; D, definite; ns, not specified; Po, possible; Pr, probable. Original studies in this table are referenced elsewhere.

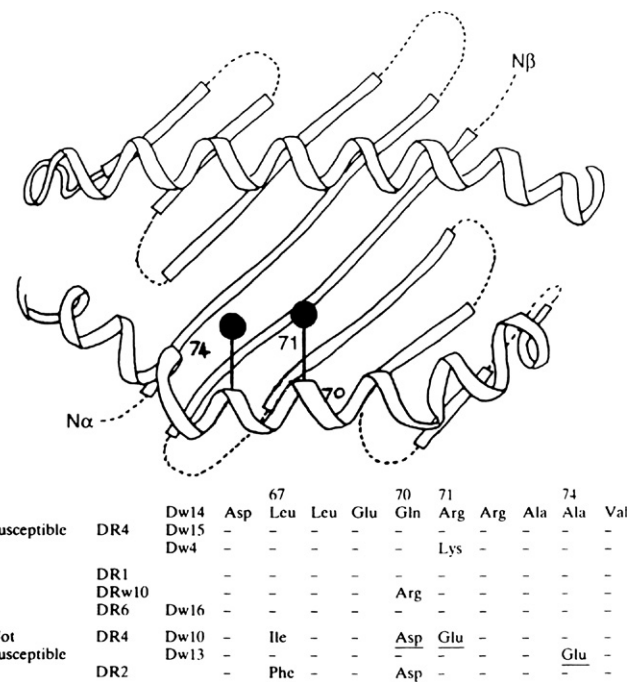
TABLE 78-3 Hierarchy of DRB1 Allelic Associations with Rheumatoid Arthritis Using Relative Predispositional Effects Analysis

Allele		
*0401	↑	<10 <sup>-38</sup>
*0404,8	↑	<10 <sup>-43</sup>
*0405	↑	<10 <sup>-8</sup>
*10	↑	<10 <sup>-3</sup>
*0101,2	↑	<10 <sup>-2</sup>
*0403	↘	0.02

This analysis included 576 HLA-DRB1 alleles from the patient group and 1000 alleles from ethnically matched controls.

Adapted from Hall, F. C.; Weeks, D. E.; Camilleri, J. P., et al. Influence of the HLA-DRB1 Locus on Susceptibility and Severity in Rheumatoid Arthritis. *Q. J. Med.* 1996; 89, 821–829.

In particular, a highly conserved sequence between amino acids 67 and 74 along the  $\alpha$ -helix derived from the DR $\beta$  chain, which forms one side of the antigen-binding site of the DR molecule, is incriminated in susceptibility as shown in Figure 78-3. All the rheumatoid-associated DR molecules share an identical or similar sequence,



**FIGURE 78-3** Antigen-binding site of an HLA-DR molecule. Charged amino acids at position 71 in Dw10 (DRB1\*0402) and 74 in Dw13 (DRB1\*0403 and \*0407) abolish susceptibility to rheumatoid arthritis; noncharged substitutions at position 71 in Dw4 (DRB1\*0401) do not.

<sup>67</sup>LLEQRRAA<sup>74</sup>; with the exception of DRB1\*0401, where the substitution of a basic lysine for arginine at position 71 is a relatively conservative change. This hypothesis probably accounts for the lack of association with HLA-DRB1\*04 in Israeli Jews, in whom \*0402 is the predominant DR4 subtype. This allele encodes a quite distinct <sup>67</sup>ILEDERAA<sup>74</sup> sequence in the DR4 molecule, including two acidic substitutions, compared to HLA-DRB1\*0404/8 (57). It has therefore been speculated that the capacity of the HLA-DR molecules to bind a potentially arthritogenic peptide may trigger an autoimmune response within the synovial joint, leading to the chronic process of inflammation and destruction that ensues (24,25,58,59). Crucially, this same <sup>67</sup>LEDERAA<sup>74</sup> motif is also found in the uncommon variant of HLA-DR1 (HLA-DRB1\*0103), which is not associated with RA (44). It should be noted that the shared epitope has been associated predominantly with ACPA positivity. In addition, there is a pronounced and dose-dependent risk of smoking in individuals carrying the shared epitope in developing ACPA-positive, but not ACPA-negative, RA (60). It has been postulated that this gene–environment interaction in RA may be explained by smoke-induced citrullination of pulmonary peptides resulting in increased binding affinity to shared epitope MHC class II receptors, and thereby T-cell activation (60).

Associations with other MHC genes have been suggested by some studies. For example, it has been suggested that TNF polymorphisms might influence responses to anti-TNF therapies (61). It is extremely difficult to separate possible effects from the TNF locus and those arising from HLA-DRB1 haplotypes because of the linkage disequilibrium between the two loci (62). To achieve this, association studies must be both adequately powered and also very carefully matched for controls (e.g. for HLA-DRB1 status). We have previously suggested that there may be an extended TNF haplotype marking a 126-kb region centromeric to TNF that is particularly interesting (63). This region contains the gene AIF1, encoding allograft inflammatory factor that has previously been implicated in inflammatory states (64); however, such suggestions require very careful validation in carefully controlled, high-density genetic mapping. The longstanding suggestion that numerous HLA alleles, particularly in the MHC class II region, are involved in RA (65) has been confirmed by recent high-density mapping studies of the MHC. It is now clear that contributions arise not only from HLA-DRB1 but also from HLA-B and HLA-DP (45). It has also been possible to refine the precise epitopes within each of these HLA molecules that are associated with susceptibility. This is analogous to the complex HLA associations that have also been described with type 1 diabetes mellitus (66).

**78.2.5.2.3 Non-Inherited Maternal HLA Antigens and RA.** Despite the relatively strong association between RA and HLA-DRB1 alleles, at least 15% of patients do not carry the conserved DRB1 epitope. It has

been proposed that, in these circumstances, some form of immunogenetic modulation may occur as a result of exposure of the host immune system to the noninherited HLA-DRB1\*04 from the mother in utero (67). HLA-DRB1\*04 appears to be overrepresented among non-inherited maternal HLA antigens in patients with RA (68). It has also been demonstrated that noninherited maternal HLA-DR antigens can exert a protective effect. HLA-DR antigens containing the protective sequence <sup>67</sup>ILEDERAA<sup>74</sup> are substantially underrepresented in the mothers of those with RA. The functional significance of these observations remains to be established. It could reflect an impact on the shaping of the T-cell receptor repertoire or an influence on tolerance mediated through exposure of the developing immune system to maternal antigens resulting from long-term microchimerism (69).

**78.2.5.2.4 Associations between HLA and Disease Severity.** HLA-DR associations with RA are dependent on the method of ascertainment. Community-based surveys pick up many milder cases compared with hospital-based studies, and interesting differences are apparent in their respective HLA associations. Community-ascertained case studies find relatively weak or absent association with HLA-DR4 (70,71) in contrast to hospital-based studies, in which about 70% of patients are DR4 positive. The progression of erosions has been correlated with presence of DRB1\*04 and DRB1\*0401 (72), and twin concordance is greatest in DR4-positive identical twins (33). Patients with Felty syndrome have a particularly strong association with DR4 (up to 95%), and the DRB1\*0401 allele (>50%) in particular (73). In populations where the DRB1\*0401 is uncommon (Greeks, Chinese, and Japanese), Felty syndrome is either rare or absent. There is also an excess of DRB1\*04 homozygotes in hospital patients with the disease (37,38) that is particularly obvious in Felty syndrome (73).

**78.2.5.3 Non-HLA Genetic Contribution to RA.** The heritability of RA attributable to HLA is no greater than 40% of the whole genetic component (74–76), and in recent years, much research has been focused on identifying the remainder of this genetic contribution. Initial studies using whole genome linkage approaches had only limited success but more recent GWAS have proved very effective, identifying more than 30 genetic regions likely to be involved in the disease. It appears that there are no other major genetic effects of the size of the MHC and that the majority of loci have small effect sizes with odds ratios (ORs) ≤ 1.05.

Genome-wide linkage scans using microsatellite markers in affected sib-pair families from Europe, Japan (77), United Kingdom, and North America unsurprisingly reported varied results. The first systematic linkage study, reported by Cornélis et al. (40), identified numerous microsatellites exhibiting nominal evidence of linkage, including two (IDDM6 and IDDM9) that had previously shown suggestive evidence of linkage to insulin-dependent diabetes mellitus. The first large-scale



systematic genome scan from North America also reported overlapping linkages with other autoimmune disorders (41), but by far, the most striking linkage was with HLA, as in other similar studies. Most of these linkages were not confirmed subsequently in a UK study of 183 multicase families (43). However, there is a weak association of IDDM6 haplotypes on chromosome 18 with RA (78). A replication study from North America subsequently confirmed some of these loci in a combined analysis of 512 families (42). Evidence for linkage was seen at 1p13, 1q43, 6q21, 10q21, 12q12, 17p13, and 18q21 as well as HLA ( $5 < 10^{-12}$ ).

Two particularly striking successes have been achieved at least in part using this approach. First, susceptibility to RA was identified in the 1p13 region with a missense mutation in a hematopoietic-specific protein tyrosine phosphatase (“Lyp”) encoded by PTPN22. The risk allele PTPN22 C1858T (R620W), present in 10–17% of the white population, disrupts its interaction with a cytoplasmic tyrosine kinase and thereby alters its normal function as a negative regulator of T-cell activation (79). It is possible that there are cis-regulatory elements in PTPN22 that regulate its expression in RA (80); however, others have suggested that there is no evidence of any other effects arising from the PTPN22 locus independent of C1858T (81). It is particularly interesting that this gene is also implicated in the etiology of several other autoimmune diseases, including autoimmune thyroid disease, type 1 diabetes and SLE (82). It is also noteworthy that the association seems to be restricted to patients with RF-positive disease (83,84). Studies in the murine equivalent have shown increased T-cell activation and CD4 and CD8 accumulation in the tissues of the affected animals and the development of spontaneous germinal centers. B-cell function may also be affected (85). Second, Suzuki et al. (86) identified a linkage interval on 1p36 in RA containing four plausible candidate genes, encoding peptidylarginine deiminases, which posttranslationally modify arginine to citrulline. RA is strongly associated with the development of ACPA even before the onset of clinical disease, suggesting that citrullination could be an important etiologic factor. Strong evidence for association with PADI4 in Japanese ( $P = 0.000008$ ) has been presented although the association appears very much weaker in other populations (87,88). It has recently been suggested that PADI4 polymorphisms particularly predispose male smokers to RA (89).

**78.2.5.4 Genome-Wide Scans.** With the introduction of GWAS, the number of genetic regions implicated in susceptibility to RA has grown rapidly. The landmark study conducted by the Wellcome Trust Case Control Consortium (WTCCC) in 2007 examined cohorts of patients with a variety of common diseases, including 2000 RA patients, and 3000 controls (90). In addition to confirming the association of RA with HLA-DRB1 and PTPN22, nine novel SNPs with nominal association to the disease, and many others with modest association,

were identified. There is now compelling evidence for the involvement of dozens of regions in RA from GWAS, and meta-analysis of such data has substantiated 35 RA-risk loci in Caucasians of European origin, including HLA-DRB1, PTPN22, CCR6, TNFAIP3, and STAT4 (91). The increased power afforded by meta-analysis has also led to identification of novel RA loci, such as CD40 (92), which was later validated in a UK study (93). Recently, the Gene Relationships Across Implicated Loci (GRAIL) method has been developed and employed to confirm many genetic associations including CD28 (94). GRAIL involves statistical mining of published abstracts to identify and prioritize genes from related biological pathways (95).

On the basis of the GWAS data, it has been demonstrated that certain combinations of alleles compound RA risk, similar to the case with HLA compound heterozygosity (e.g. OR ~21 in ACPA-positive individuals with the genotype HLA-DRB1 + PTPN22 + STAT4 + TRAF1/C5) (96). In addition, differential genetic associations within seropositive disease are emerging and may provide insights into pathogenesis; for example, PTPN22 was recently found to associate primarily with ACPA positivity, while HLA-DRB1 is associated with both RF and ACPA (97).

Most of the identified genetic associations are with seropositive RA; seronegative RA may represent a pathogenetically distinct disease and further studies on this cohort are warranted. Similarly, the majority of studies have been conducted in populations of European origin. To address this, a meta-analysis and replication study was performed in the Japanese, which identified nine novel associations reaching genome wide significance ( $p < 5 \times 10^{-8}$ ), including PTPN22, CD83, NFKBIE, ANXA3, ARID5B, B3GNT2, CSF2, PDE2A-ARAP1, and PLD4 (98). Although a multiancestry comparative analysis of Japanese and European RA heritability indicated a high degree of overlap, the differential contribution of PADI4 to RA risk (see Section 78.2.5.3) demonstrates the need for GWAS in all ethnic populations (98).

It is interesting that many genetic variants conferring RA risk are associated with multiple autoimmune or inflammatory diseases, e.g. STAT4 (SLE, type 1 diabetes, inflammatory bowel disease), TRAF/C5 (SLE, JIA), and the chromosome 6q23 locus (SLE, type 1 diabetes, celiac disease) (99). A recent meta-analysis identified eight non-HLA-risk loci common to both celiac disease and RA, bringing the number of shared loci to 14 (100). Seven of these genes are also common to type 1 diabetes (101). The themes of these common risk alleles include innate immunity (e.g. TRAF/C5), T-cell differentiation (STAT4), and T-cell signaling (PTPN22) (102). It has been postulated that the unique and dominant HLA associations result in presentation of disease-specific autoantigens to T cells; the common non-HLA risk determinants may in turn influence the response of the immune cells to these autoantigens (100). In many cases, additional work is

required, including fine mapping or deep resequencing of the genetic regions of interest, to precisely identify the causative allelic variants underlying the SNP associations (99). An example is the association of RA with SNP rs6920220 on chromosome 6q23, positioned between the TNFAIP3 and OLIG3 genes (103). Subsequent fine mapping determined that there are actually three RA risk alleles within this region, one of which confers protection (104). The functional and pathogenic significance of these allelic variants also require further study.

Despite these advances, more than 50% of the genetic contribution to RA remains unexplained. This may be due to as yet unidentified rare genetic variants with large effect, or large numbers of risk alleles of small effect not yet identifiable reliably by current methods (98). The expectation that copy number variants (CNVs) would represent a significant proportion of human disease risk was recently largely discounted; a large GWAS covering approximately half of the genomic CNVs >500 bp found no association with eight common diseases (105). Further large-scale association studies with saturation mapping of the relevant loci are currently nearing completion and should further advance the field in the near future.

**78.2.5.5 Genetic Models and Pathologic Mechanisms.** The shared epitope hypothesis put forward to explain the disparate HLA-DR associations with RA (106) implies the presentation of a single or limited range of potentially arthritogenic peptides by antigen-presenting cells within the joint to a subset of T lymphocytes capable of initiating a specific inflammatory response. This would most obviously produce a dominant model of susceptibility, but several studies have cast doubt on this, suggesting that the involvement of HLA genes fits best with a recessive model (75,107). The hypothesis of HLA-DQ-mediated susceptibility would fit with this model (65) as would the role of the HLA genes in shaping the T-cell receptor repertoire by positive and negative thymic selection. Another possibility invokes molecular mimicry between the shared epitope on rheumatoid-associated DR molecules with potential triggering pathogens although firm evidence for this has not been forthcoming, and such cross-reactive immune responses have not routinely been observed.

Early reports using PCR to detect expanded T-cell populations, particularly in the rheumatoid synovial

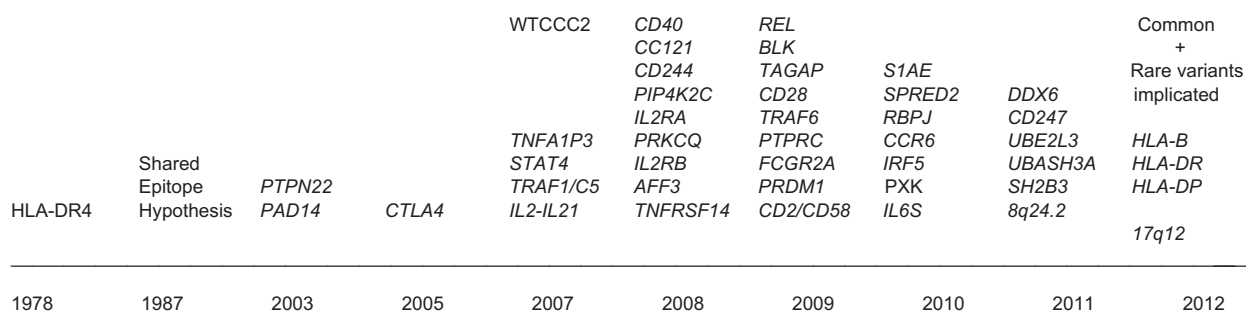
compartment, were somewhat suggestive of an antigen- or superantigen-driven response (108). Although numerous subsequent studies have sought such oligoclonal expansions in patients with RA, there seems little consistency between individual studies to support such an idea (24,109,110). This contrasts with some interesting data in reactive arthritis and psoriatic arthritis (111,112).

The association with HLA-DRB1 variants and RA suggests that engagement of specific immune responses is important at some stage in the development of the disease. It is well known that additional signaling through costimulatory molecules in this “immunologic synapse” is critical to this process. Interactions between the CD80/86 molecules on antigen-presenting cells and their potential ligands on T cells (CD28 or cytotoxic T lymphocyte associated antigen-4 (CTLA-4)) play an important role in determining activation. Variation in CTLA-4 is involved in susceptibility to autoimmune diabetes mellitus and thyroiditis. A large collaborative study from Sweden and North America suggests that there is also a weak association (OR 1.2) with RA (113). It is certainly of interest in this context that the fusion protein CTLA4Ig has impressive disease-modifying effects in RA (114). Other components of the immunologic synapse are potential candidates in the etiology of the disease. For example, CD28 is also associated with RA (94) and there is weak evidence that the inhibitory product of the programmed cell death 1 gene (PDCD1) may also be involved (115).

A list of the major genetic effects in RA identified to date is shown in Figure 78-4. Major challenges remain in the identification of all the major genetic effects in RA, particularly those attributable to rare alleles. Even weak genetic influences could herald the identification of crucial pathogenic pathways and the means to modulate them.

## 78.2.6 Management

About half of patients presenting with early undifferentiated polyarthritis have self-limiting disease, but only 15% of patients fulfilling criteria for RA go into remission (116). In those with persistent arthritis, agents that modify the course of the inflammation are required. These include sulfasalazine, methotrexate, antimalarials,



**FIGURE 78-4** Progress in the identification of genes involved in rheumatoid arthritis.

gold salts, D-penicillamine, azathioprine, and leflunomide (often used in combination). Corticosteroids (either in low doses orally or as intramuscular pulses) may be used in the early phases of the disease to suppress inflammation and erosive damage as second-line drugs are commenced (117). From the early 1990s, the treatment of the more severe forms of RA changed dramatically with the introduction of anti-TNF biologic agents (118,119). In clinical trials recruiting patients with very active disease, these drugs routinely induced remission in a quarter of patients within 3 months. Further, they could be shown to completely suppress the progression of erosions. Quite suddenly, it became unacceptable for rheumatologists to aim for anything less than complete suppression of the disease. This stimulated the use of combinations of the older disease modifying anti-inflammatory drugs, such as methotrexate, sulfasalazine and leflunomide, and if these failed to suppress the disease adequately, escalation to biologic therapies would follow. This has had a profound knock-on effect in the clinical practice of rheumatology with the development of sophisticated clinical and imaging tools to assess the degree of residual disease activity and the presence of potentially destructive synovitis. Patients are now treated much more aggressively early in the disease to prevent irreversible structural joint damage. Good responses to anti-TNF therapy can be anticipated in the majority of patients with ACR 20%, 50% and 70% responses being achieved within 3 months by 60%, 40% and 20% of patients with substantial disease activity before treatment ( $\text{DAS28} \geq 5.1$ ). In those failing anti-TNF therapy, other options with broadly comparable efficacy include rituximab, an anti-CD20 monoclonal antibody B-cell depleting agent (120), tocilizumab (anti-IL6 receptor monoclonal antibody), and abatacept (recombinant CTLA4/immunoglobulin fusion protein).

Even with the improved therapeutic armory, effective management of RA still requires a coordinated approach by a multidisciplinary team because of the chronic nature of the condition. The primary objectives are the relief of pain and preservation of function. A combination of drugs, physical therapy, orthotics, and appliances to prevent or accommodate increasing long-term disability will be required. Surgical intervention is frequently needed at some stage in the course of the disease, but requires careful planning, particularly in patients with more severe forms of the disease, who may require many procedures.

The development of reliable methods of total joint replacement, particularly in the large weight-bearing joints of the hip and knee, has otherwise been the most important single development in the treatment of patients with RA. Good results from both hip and knee replacement can be anticipated in more than 90% of patients, and, typically, the prosthesis will last for 15 years or more. Subluxation of the cervical spine is relatively common, particularly in patients with severe erosive disease, and may require surgical stabilization. The median standard mortality ratio for RA is approximately

1.5 compared with the general population (121), mainly from infection and cardiovascular disease. Only half of this can be attributed to classic risk factors such as smoking, hyperlipidemia and hypertension, all of which should be tightly controlled.

### 78.3 SERONEGATIVE SPONDYLOARTHROPATHIES

The term *seronegative spondyloarthropathy* (SpA) refers to a group of inflammatory conditions characterized by inflammation of the entheses (sites of mechanical stress where ligaments or fibrocartilage interface with bone, e.g. sacroiliac joints). In contrast to RA, RF and ACPA are absent. These two characteristics differentiate these conditions from the somewhat more common RA, which is characterized pathologically by synovitis rather than enthesitis and in which RF is present in 85%.

Other features of SpA include an association with the MHC class I gene HLA-B\*27; characteristic distribution of joint involvement with prominent axial, sacroiliac, and asymmetric lower limb peripheral large-joint arthritis; characteristic extra-articular features, particularly uveitis; and the formation of new bone at the site of inflammation, eventually leading to ankylosis. By contrast, seropositive RA is associated with the class II HLA-DRB1 genes, typically causes a small-joint symmetrical polyarthritis, has a different range of extra-articular features, and causes erosion of cartilage and bone rather than ankylosis.

Several forms of SpA are described:

1. Ankylosing spondylitis
2. Reactive arthritis
3. Enteropathic arthritis (associated with inflammatory bowel disease)
4. Psoriatic arthritis.

Some patients with SpA fail to meet the criteria for any of these individual diseases and are said to have “undifferentiated spondyloarthritis.” The features that distinguish these conditions from one another are presented in Table 78-4.

#### 78.3.1 Diagnostic Criteria

A variety of diagnostic criteria have been proposed for AS (122–124), reactive arthritis (125), and psoriatic arthritis (126,127). Criteria for AS have historically relied heavily on the presence of radiographic sacroiliitis for diagnostic specificity. As there is a mean delay of 9 years between the onset of symptoms and the development of radiographic changes, the sensitivity of these criteria in early disease is poor (128). These criteria have also been criticized for being too restrictive as they exclude a significant group of patients with clear features of SpA, but who do not clearly fit into any of the currently defined disease groups. In response to these shortcomings, the

**TABLE 78-4 Clinical Features of the Spondyloarthropathies**

	Ankylosing Spondylitis	Reactive Arthritis	Psoriatic Spondyloarthritis	Enteropathic Spondyloarthritis
Sex	M > F	M > F	F > M	M = F
Age of onset (yr)	15–35	Any age	Any age	Any age
Uveitis	++	++	+	+
Conjunctivitis	—	++	—	—
Urethritis	—	++	—	—
Skin involvement	—	++	++	—
Mouth ulcers	—	++	—	+
Sacroiliitis	+++	++	++	++
Peripheral arthritis	Lower > upper	Lower > upper	Upper > lower	Lower > upper
Spinal symmetry	+++	+	+	++
Enthesopathy	++	++	++	++
Aortitis	+	+	?+	+
HLA-B*27 (%)	95	80	50	50
Risk for B*27-positive	2–8	10–20	Unknown	Unknown individual (%)
Self-limiting	—	++	—	—

—, rarely; ++, occasional; ++, frequent; ++++++, always.

Assessment of Spondyloarthritis International Society (ASAS) has developed criteria based on clinical features and magnetic resonance imaging (MRI), which are much more sensitive at picking up early disease (129). As discussed later, a combination of simple screening questions designed to identify inflammatory back pain combined with HLA-B\*27 testing and the use of MRI to detect sacroiliitis, can have a considerable impact on the diagnosis of early disease. Early diagnosis is of increasing importance since the advent of anti-TNF treatment, which can have life-changing efficacy in those with AS.

## 78.3.2 Ankylosing Spondylitis

**78.3.2.1 Epidemiology.** The most widely used diagnostic criteria for AS are the modified New York criteria (Table 78-5). Those are heavily reliant on the presence of radiographic evidence of sacroiliitis, which may take several years to develop. MRI allows much earlier detection of sacroiliac joint abnormalities, particularly active inflammation, and is the imaging technique of choice for early diagnosis (130). AS typically develops in early adulthood, with more than 90% of cases diagnosed before the age of 40 years. There is typically considerable delay in diagnosis, particularly in females among whom atypical patterns of joint involvement appear to be more common. Overall, men are also more commonly affected than women (ratio ~2.8:1). Estimates of the prevalence of AS in western Europe and North America vary between 0.05% and 1.4%, depending on study methodologies. Recent studies from Berlin suggest a prevalence of around 0.5%.

What is not in doubt is that the prevalence of AS roughly parallels the prevalence of the main susceptibility factor, HLA-B\*27, in different populations (126). Thus a high prevalence of AS is found in some populations,

**TABLE 78-5 Revised New York Criteria for Ankylosing Spondylitis<sup>a</sup>**

Low back pain ≥3 months' duration (improved by exercise and not relieved by rest)
Limited back movement (sagittal and coronal)
Reduced chest expansion (compared to age- and sex-matched values)
Bilateral sacroiliitis (grade ≥2)
Unilateral sacroiliitis (grade ≥3)

<sup>a</sup>Ankylosing spondylitis is diagnosed if significant radiographic evidence of sacroiliitis is present along with one or more clinical criteria.

Modified from van der Linden, S.; Valkenburg, H. A.; Cats, A. Evaluation of Diagnostic Criteria for Ankylosing Spondylitis: A Proposal for Modification of the New York Criteria. *Arthritis Rheum.* 1984, 27, 361–368.

including North American Indians (B27 prevalence, 18–50%), Norwegian Lapps (25–30%), and Alaskan Eskimos (25–40%). Populations with a low prevalence of HLA-B\*27, including most sub-Saharan African ethnic groups, Australian Aborigines, and South American Indians, have correspondingly low prevalences of AS. However, the relationship is not simple since other environmental and genetic factors play a significant role in the etiology of the disease. HLA-B\*27 itself is polymorphic, and there is considerable interest in the possibility that allelic differences may be responsible for different degrees of disease susceptibility associated with the B27 subtypes (see Section 78.3.2.4.1).

**78.3.2.2 Pathology.** As in all spondyloarthropathies, the basic pathologic lesion of AS is enthesitis. Enteses represent specialized areas of bone adapted to cope with stress loading at interfaces with ligaments or fibrocartilage. Enteses have large vascular beds and are the site of relatively high connective tissue metabolic activity, and are thus vulnerable to the effects of inflammation. Plasmacytic and lymphocytic infiltrates are seen. Localized



osteitis and osteoporosis occur initially. Later granulation tissue forms, fibrosis occurs, and reactive new bone formation begins. This process may continue until ankylosis occurs across the involved joint. Analogous changes occur at the attachment of the joint capsule to periarticular bone.

Although entheses and fibrocartilagenous joints are primarily affected, inflammation sometimes also involves synovial joints. In some patients, AS may even present as peripheral arthritis involving synovial joints. Eventually approximately 20% of cases develop significant hip arthritis, and involvement of the zygapophysial joints is universal. Synovial tissue from these joints shows changes similar to RA, although typically less severe. There is villous hypertrophy, synovial cell hyperplasia, and lymphocytic, plasmacytic, and histiocytic infiltration. The cellular infiltrate is diffuse, but also shows some perivascular aggregation. Although the disease is characterized systemically by raised immunoglobulin (Ig)A levels, plasma cells in the synovium secrete principally IgG. Attention has recently been drawn to the high proportion of CD4 T cells expressing the killer immunoglobulin-like receptor KIR3DL2 and their production of IL-17 (231). The synovial fluid contains fewer polymorphs and more lymphocytes than rheumatoid synovial fluid (131).

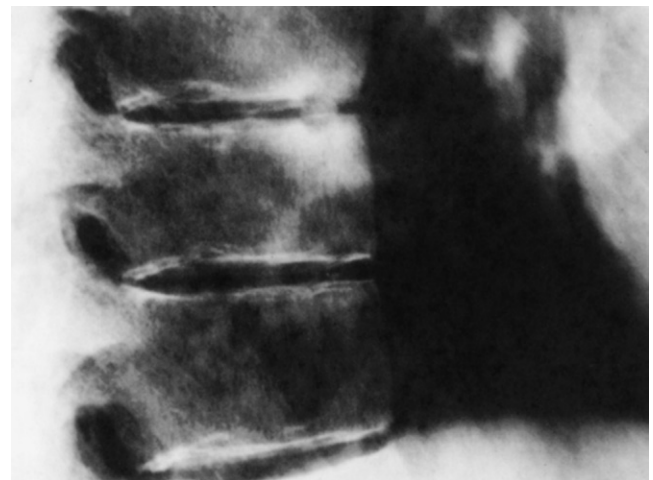
The earliest changes detectable by imaging are bone marrow edema of the sacroiliac joints (Figure 78-5) and osteitis at the vertebral corners of the spine (Figure 78-6) on fat-suppressed MRI, which reflect underlying inflammation. On plain radiographs, the earliest changes are sclerosis and erosions in the juxta-articular bone, but these may take years to develop. In the sacroiliac joints, erosions are seen as irregular variations in the width of the sacroiliac joint space and loss of clear definition of the joint line. This is usually most obvious in the inferior iliac aspect of the synovial component of the joint. Late

disease is characterized by new bone formation underneath inflamed periosteum; this is reflected on plain radiographs by “squaring” of the vertebral bodies (loss of the normal concave anterior surface) and the presence of syndesmophytes. In the sacroiliac joints, radiographs demonstrate periarticular sclerosis and later ankylosis. MRI scans may also show fibrosis and fatty replacement of the periarticular bone marrow but are inferior to plain radiographs in demonstrating the extent of new bone growth (syndesmophytes) (Figure 78-7).

**78.3.2.3 Other Clinical Features.** Osteoporosis is common in AS, often from an early stage (132–134). Spinal fractures are common in advanced disease (135), reflecting both increased bone fragility and reduced flexibility. The eye is the most common extraskelletal site of involvement in AS. Anterior uveitis (involving the structures anterior to the lens—the iris and ciliary body) occurs in around 40% of cases of AS and 5–10% of cases of reactive arthritis (136). It runs a relapsing/remitting course, is usually easily treated with cycloplegics and topical steroids, and is rarely sight-threatening unless neglected.



**FIGURE 78-5** Tilted coronal STIR MRI of sacroiliac joints showing bone marrow edema (high signal) before (A) and after (B) anti-TNF therapy.



**FIGURE 78-6** “Shiny corners” demonstrated on sagittal STIR MRI of the thoracolumbar spine due to osteitis in ankylosing spondylitis.



**FIGURE 78-7** Florid syndesmophytes contributing to the appearance of a “bamboo spine” in long-standing AS.

Idiopathic anterior uveitis is also strongly associated with HLA-B\*27 (95%) independent of AS.

Other uncommon overt extraskelatal manifestations of AS include cardiac and proximal aortic involvement by fibrosis and endarteritis. This rarely results in clinically significant reduction in left ventricular function. Heart block and aortic regurgitation due to dilation of the aortic valve ring are described and may be dramatic in onset. Standardized mortality rates are increased by about 50% in AS, much of which is due to the increased risk of cardiovascular disease. Pulmonary involvement is predominantly mechanical because of the fusion of the costovertebral joints and thoracic kyphosis, reducing vital capacity. AS is occasionally complicated by upper lobe pulmonary cavitation or fibrosis (~1% of cases), which is usually of little clinical significance but may need to be distinguished from pulmonary tuberculosis or aspergillosis.

**78.3.2.4 Genetic Studies.** A major role for genetic factors in the etiology of AS is emphasized by the high recurrence risks for AS among close relatives of patients (126,137). Several relatively small twin studies have suggested a significant genetic component to AS. The largest study, undertaken in the UK, estimated monozygotic twin concordance at 75% compared to only 12.5% of dizygotic twins. Even in B27-concordant dizygotic twins, concordance for AS was only 27%. Variance modeling using these data suggests that broad sense heritability for AS is in excess of 92%, and that HLA-B\*27 accounts for less than half of the genetic contribution (138). Environmental factors are probably important but are very common or ubiquitous, therefore playing little part in determining population variance. It has become increasingly apparent that the genetic contribution to AS is polygenic. At least two HLA genes have already been clearly demonstrated to increase susceptibility to AS. HLA-B\*27 was the first in 1973 (139,140). Subsequently, HLA-B\*60 has also been shown to be associated with AS in both B27-positive and -negative individuals (141,142), although this has not been a universal finding (143,144). Our results from a large UK survey suggest that HLA-B\*60 is associated with a two- to threefold excess risk (145).

**78.3.2.4.1 HLA-B\*27.** In the United Kingdom, more than 90% of patients with AS carry HLA-B\*27 (relative risk ~160), but only 2–8% of B27-positive individuals develop AS. The incidence of reactive arthritis (see Section 78.3.3) following bacterial enteritis may be as high as 20% in B27-positive individuals, who are also at increased risk of subsequent AS. The fact that relatively few B27-positive individuals develop B27-related diseases probably reflects the role of other genes, rather than the environmental exposure.

There are at least 70 different HLA-B\*27 alleles, differing from one another by between one and eight amino acids, which have evolved from the ancestral HLA-B\*2705 subtype (146). Different ethnic groups

have distinct subtype distributions. In most white populations, more than 90% of B27 alleles are HLA-B\*2705 and the remainder almost entirely HLA-B\*2702.

Association between AS and most HLA-B\*27 subtypes has been described, at least sporadically, (particularly HLA-B\*2702, \*2704, and \*2705), although systematic studies have not always been possible because of the rarity of some subtypes, such as HLA-B\*2701, \*2707, and \*2708. There has been particular interest in two subtypes (B\*2703 and \*2709) that appeared to be less obviously associated with AS. Early suggestions that the HLA-B\*2703 allele might not be associated with AS were based on the relatively high frequency of this allele in The Gambia where, nonetheless, AS is extremely rare (147,148), although sporadic cases of the disease in B\*2703-positive individuals have been reported in neighboring Senegal. Approximately 50% of the B27-positive individuals in The Gambia carry the most common white subtype, HLA-B\*2705, which is positively associated with disease in all other populations studied so far. Despite this, AS is vanishingly rare in The Gambia, perhaps suggesting the influence of other protective genetic or environmental effects in this population. In Europe there is good evidence from Sardinia that HLA-B\*2709 is protective against AS (149).

As a diagnostic test, HLA-B\*27 has a low positive predictive value, unless the patient already has a moderately high prior probability of disease. When the pretest probability of spondylitis is 0.5, the presence of B27 increases the likelihood of the disease to 0.92, whereas a negative result reduces it to 0.08 (150). When the prior probability is low (e.g. in population screening programs), the main use of the test is its negative predictive value. In the clinical situation of early SpA with a suspicious history and examination but normal radiographs, a positive B27 test increases the probability from as low as 0.12 to only slightly greater than chance (0.62), adding very little to the clinical decision-making process (151). Rudwaleit et al. have developed a very useful algorithm for the diagnosis of early AS, based on simple clinical questions to identify inflammatory back pain, HLA-B\*27 typing and MRI of the spine (Table 78-6) (152). The other setting in which B27 tests may be useful, is in determining the likelihood of the offspring of patients developing AS themselves. The pretest probability here is only 0.1, and positive testing for B27 increases this to only 0.2. A negative result makes it highly unlikely that the individual will develop disease and may in some circumstances be reassuring and justified.

Many theories have been proposed to explain the association with HLA-B\*27, but none is universally accepted (153). Doubts about whether HLA-B\*27 itself was directly involved or whether it was a marker for a nearby linked gene have been resolved. Transgenic HLA-B\*27-positive rats have proved susceptible to an SpA-like condition, albeit one in which inflammation of the bowel, psoriasisform skin lesions, and orchitis are prominent in

**TABLE 78-6** Assessment of SpondyloArthritis International Society (ASAS) Classification Criteria for Axial Spondyloarthritis 2009 (152)

**In patients with age at onset of symptoms <45 years and back pain  $\geq 3$  months**

**EITHER**

1. Sacroiliitis (active inflammation on MRI/X-ray changes per modified New York criteria)
2. **PLUS** at least one SpA feature from:
  - a. Inflammatory back pain
  - b. Arthritis
  - c. Heel enthesitis
  - d. Uveitis
  - e. Dactylitis
  - f. Psoriasis
  - g. Inflammatory bowel disease
  - h. Good response to NSAIDs
  - i. Family history of SpA
  - j. HLA-B\*27
  - k. Elevated CRP

**OR**

1. Positive for HLA-B2\*7 **PLUS** at least 2 SpA features

Adapted from Rudwaleit, M.; van der Heijde, D.; Landewe, R., et al. The Development of Assessment of SpondyloArthritis International Society Classification Criteria for Axial Spondyloarthritis (Part II): Validation and Final Selection. *Ann. Rheum. Dis.* **2009**, *68*, 777–783.

addition to peripheral arthritis (154). Although these rats only develop disease if a relatively high copy number ( $>10$ ) of the B\*27 transgene is present, no other gene is inserted and control animals do not express disease with high copy numbers of other HLA class I alleles (155). Some doubts remain about the relevance of the model to human disease, but further insights could come from study of this or other animal models (156). For example, the genetic background of the transgenic animal influences susceptibility; Dark Agouti strains of rat transgenic for HLA-B\*27 are susceptible, but this is lost when they are backcrossed with other strains. Rats bred in sterile conditions also fail to develop the disease, consistent with the hypothesis that exposure to common bacteria or even gut commensal organisms might trigger disease. It has also been suggested that cell-surface expression of HLA-B\*27 may not be necessary for the development of SpA since  $\beta_2$ -microglobulin knockout mice ( $\beta_2m^{-/-}$ ) can develop disease ( $\beta_2m$  is necessary for the stability of the B27 heavy chain (HC), with which it noncovalently associates) (157). Suggestions that presentation of B27-derived peptides by HLA class II molecules could account for this observation seem unlikely because class II knockouts can also get disease. Molecular mimicry has been expounded as a plausible theory for many years as the description of antibodies cross-reacting between *Klebsiella* species and HLA-B27. Initial reports of an increase in *Klebsiella* carriage in the stools of patients with active AS have not been reproduced, and such

cross-reactive antibodies seem unlikely to account for the tissue specificity of AS. Most likely, the role of HLA-B\*27 in antigen presentation plays an important part. In common with other HLA class I molecules, HLA-B27 binds antigenic peptides (typically 9-mers) derived from self-proteins and presents them to CD8-positive T lymphocytes for immune surveillance. Viral and tumor antigens are particularly detected in this way. Precisely how HLA-B\*27-associated antigens might trigger the disease is not clear, but CD8-positive T cells with specificity for organisms have been identified in the joints of patients with reactive arthritis (158). In contrast to other class I molecules, HLA-B27 can exist in atypical forms. B27 can form homodimers ( $B27_2$ ) that are not associated with  $\beta_2m$ , through a disulfide bond between Cys67 residues (159). How these homodimers induce AS remains unknown. They are known to be expressed at the cell surface, and may be involved in aberrant antigen presentation or in triggering the innate immune system (160). Conditions that may increase the chances of  $B27_2$  formation include peptide deficiency; excess HCs, as seen in the B\*27 transgenic rat (161); or  $\beta_2m$  deficiency, seen in the B\*27 transgenic  $\beta_2m^{-/-}$  mouse (162).

The possibility that other genes within the MHC, in addition to HLA-B\*27 and HLA-B\*60, play a role remains somewhat controversial. Weak associations with HLA-DRB1\*01 (OR 1.5; 95% confidence interval, 1.1–2.0), and the possibility that there is an extended B\*27 haplotype containing another relevant locus have been suggested (163,164); however, subsequent data from large-scale GWAS do not lend much support.

**78.3.2.4.2 Other genes.** Since genetic predisposition to AS is polygenic and B27 accounts for less than half of heritability, it is reasonable to screen the remainder of the genome for susceptibility candidates. In 1998, we published our initial genome screen on 120 affected sib-pairs in 105 families (165,166). Linkage with HLA was confirmed, but six other regions outside the MHC showed some evidence of linkage, with LOD scores of  $>1.00$ . The peak non-MHC linkage was on chromosome 16q (LOD = 2.6). Nominal linkage to the CYP2D6 microsatellite on chromosome 22 was found, and subsequent linkage and association studies with intragenic CYP2D6 markers have also proved positive (167). This gene (otherwise known as *debrisoquine hydroxylase*) is one of the cytochrome P-450 enzyme complex genes. The poor metabolizer phenotype is found in 6% of the UK population, but how this might contribute to the etiology of AS is unclear.

Initial attempts to identify non-MHC genes using linkage analysis of affected sib-pair pedigrees met with only limited success. Subsequent meta-analysis of all published studies of linkage analysis was undertaken, which confirmed highly significant linkage to the MHC ( $p < 3 \times 10^{-7}$ ) but also suggested linkage ( $p < 7.4 \times 10^{-4}$ ) to regions on chromosome 6q, 10q and 16q. Several other regions including the IL-1 gene cluster on chromosome



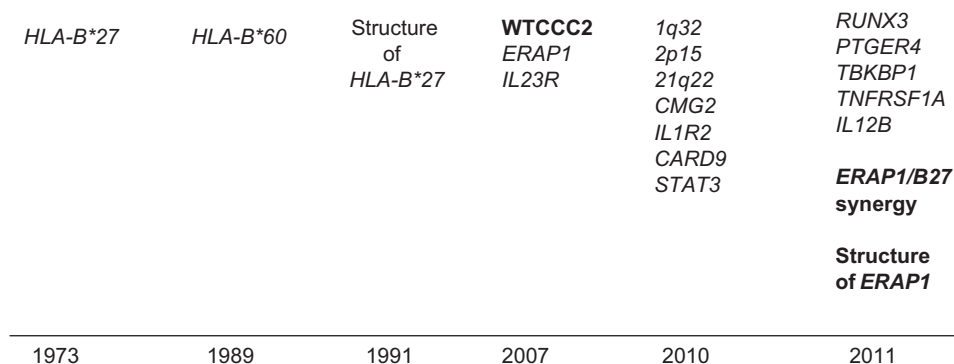
2 showed moderate evidence of linkage. The  $\lambda$  (LOCUS) value for the MHC was 4.5, suggesting that it accounted for around 34% of the familiarity of AS.

GWAS have rapidly supplanted linkage analysis in identifying genetic effects in AS, building on the work of the WTCCC in other polygenic diseases including RA. In AS, two major international consortia have been established for these studies with sufficient power to reliably identify genes increasing the risk of AS by around 5–10%. First, the Triple A (Australo-Anglo-American) Spondyloarthritis Consortium (TASC) and second, the International Genetics of AS (IGAS) consortium have generated samples of up to 13,000 cases for analysis. This has resulted in major genetic discoveries.

In 2007, TASC and WTCCC reported the results of a limited gene-targeted GWAS of 15,000 nonsynonymous single nucleotide polymorphisms (ns SNPs) in 1000 cases (103). The association with the MHC was confirmed (OR ~ 120,  $p < 10^{-120}$ ) as expected. Outside the MHC, the most striking association was with ERAP1, encoding the endoplasmic reticulum aminopeptidase 1 ( $p \sim 10^{-6}$ ), which was subsequently replicated in an independent data set. Numerous SNPs in ERAP1 were associated with AS; rs30187 encoding K528R was associated with an OR of 1.3 ( $p \sim 10^{-9}$ ). This association has been robustly replicated in several further studies (2,168–170), including a relatively large GWAS in Han Chinese ( $n = 3937$ ) in which the strongest association was also with rs30187 ( $p < 7 \times 10^{-4}$ ) (171). In this study, the other interesting observation was of weak association ( $p < 1.7 \times 10^{-3}$ ) with IL23R, encoding the IL23 receptor expressed by a subset of CD4 lymphocytes producing the cytokine IL17, known as T helper 17 (Th17) cells. IL23R is of particular interest because it is also associated with Crohn's disease and psoriasis, two conditions that are known to be overrepresented in patients with AS. This association was replicated in an independent data set and subsequently in an independent UK study and meta-analysis of previously published studies (168,170,172). The coding SNP rs11209026 is most strongly associated with AS (OR = 0.61,  $p < 10^{-10}$ ) as in Crohn's disease.

A subsequent GWAS reported by TASC in 2010 confirmed the association with the MHC, ERAP1 and IL23R but, in addition, identified several new associations (170). This study was more heavily powered than previous studies, comparing 2053 unrelated subjects with AS with 5140 ethnically matched controls. Cases were genotyped for 370,000 SNPs on the Illumina HumMap 370 genotyping platform and independent replication of positive results was undertaken in a further 898 cases and 1518 controls. Strong association was observed with two “gene deserts” on chromosomes 2p15 ( $p < 10^{-19}$ ) and 21q22 ( $p < 10^{-9}$ ) and also the genes ILIR2 (the decoy receptor for IL1) and ANTXR2 (encoding a receptor for anthrax toxin, but also functioning as a capillary growth factor). The region of chromosome 2p15 was again replicated in Han Chinese (171) with the marker rs10865331 most strongly associated ( $p = 2 \times 10^{-8}$ ).

Several major breakthroughs in our understanding of the genetic basis of AS have recently been made. These relate in particular to the likely role of ERAP1, the key role of the Th17 lymphocyte development pathway and the overlap between AS and other inflammatory disorders, including psoriasis and inflammatory bowel disease. In 2011, the TASC and WTCCC published the results of another large GWAS, in which a discovery set of 3023 cases of European ancestry were genotyped on the Illumina 660W-Quad microarray and compared with 8779 controls. A limited replication study of the top 50 “hits” was then undertaken in an independent set of 2111 cases and 4483 controls (2). The major findings are included in Figure 78-8. Of particular interest was the observation that the association with ERAP1 was limited to the B27-positive subset of patients, contrasting with the B27-negative minority (~9%) in whom no such association was apparent. Other associations, such as IL23R, were not influenced by HLA-B\*27 status of the patients. It is therefore clear that there is synergy between HLA-B\*27 and ERAP1 in susceptibility to AS. This most likely reflects a role for ERAP1 in trimming peptide antigens in the endoplasmic reticulum to optimal length (eight or nine amino acids) for binding to HLA class I molecules, including HLA-B27. The solution of



**FIGURE 78-8** Manhattan Plot showing association of AS with ERAP1 in (A) all patients, (B) B27-positive patients, and (C) B27-negative patients.



the crystal structure of ERAP1 at around the same time presented a structural basis for the understanding of this function of ERAP1. Furthermore, we were able to show that particular variants associated with protection against AS, such as K528R (rs30187), showed significantly reduced peptide processing characteristics (3). At least in theory, this would suggest that small molecule inhibitors of ERAP1 could be of value in the treatment or prevention of AS. The role of Th17 lymphocytes in AS is highlighted by the associations of a number of genes involved in this pathway (2,173). These include not only IL23R (expressed on Th17 lymphocytes) but also IL12B (encoding the common p40 chain of IL23 and IL12) and STAT3 (a key transcription factor for Th17 lymphocytes). Similar associations are seen in both psoriasis and inflammatory bowel disease, highlighting the shared genetic predisposition of these conditions (Chapters 150 and 68). Other associations include the transcription factor RUNX3, which is involved in lymphocyte differentiation, which is driven by IL-7. In this respect, it is interesting that we have also observed a moderate association with the IL-7 receptor ( $8 \times 10^{-5}$ ). The latest TASC/WTCCC study also shows a strong association with PTGER4 (prostaglandin E receptor 4), which is also associated with Crohn's disease. Prostaglandin E2 acts through this receptor to induce the production of IL23 and IL17, both of which are elevated in AS. Synovial levels of PTGER4 are increased in spondyloarthropathies. Of interest,  $\beta$ -glucan, a component of certain fungal and bacterial cell walls, induces PGE2 through interactions with components of the innate immune system, including CARD9 that is also associated with AS (174). This would be consistent with the hypothesis that AS might be induced by ubiquitous organisms carrying  $\beta$ -glucan by stimulation of the Th17 pathway. PTGER4 is also part of the anabolic bone response to stress. Its expression is increased in the synovium of those with spondyloarthropathies and this could potentially explain the new bone formation at sites of mechanical stress (entheses) in AS. These associations and doubtless many more will be further refined by the current IGAS consortium "ImmunoChip" experiment nearing completion in 2012. This will study known and candidate genes of inflammatory/immunological importance in 13,000 individuals globally and is likely to increase the number of target genes and pathways for study substantially.

**78.3.2.5 Management.** Pain relief and minimization of disability are the major aims of treatment. Physical exercises to maintain flexibility of the axial skeleton and good posture must be undertaken regularly, if necessary, under the supervision of a physiotherapist, since the benefit of intensive exercise regimens in hospital has been well recorded. Because of the likelihood of pronounced costovertebral joint involvement, lung function is often markedly reduced, and smoking should be strongly discouraged. Nonsteroidal anti-inflammatory drugs (NSAIDs) are usually highly effective although there is

only very weak evidence to suggest that they have any effect on the underlying rate of paraspinal ossification. Continuous treatment may be more effective at preventing new bone formation than on-demand therapy (175). Sulfasalazine and methotrexate are valuable for patients with active peripheral joint involvement but have no discernable effect on the spinal disease. Likewise, corticosteroids administered systemically are usually ineffective for spinal disease. In contrast, local injection of corticosteroids may be very valuable for the management of peripheral joint synovitis and occasionally for costovertebral joint disease and sacroiliitis. Management of active axial inflammation refractory to NSAIDs has been revolutionized by the demonstration that anti-TNF agents are highly effective in this condition (176). Whether these expensive drugs also retard the progression of joint ankylosis is as yet unknown. Promising results were demonstrated in early trials of anti-IL17 therapy in active AS, but larger studies are awaited. Uveitis is common (up to 40%) and is usually responsive to topical corticosteroids and mydriatics. Osteoporosis is recognized as a relatively early complication of the condition and may contribute to spinal deformity if wedge fractures occur. Bone densitometry measurements in the lumbar spine may be misleading because of the presence of syndesmophytes, which may cause spuriously high values. Joint surgery, particularly hip and knee arthroplasty, may be required in about 10% of patients but is particularly likely in those with juvenile-onset disease. Finally, a range of aids for daily living may be required, ranging from prismatic spectacles to allow severely kyphotic individuals to see where they are going, through to adaptations to cars (particularly additional mirrors for all-around vision) and wheelchairs for those with extreme disability and handicap.

### 78.3.3 Reactive Arthritis (Previously Reiter Syndrome)

Classic reactive arthritis (previously Reiter syndrome) represents the triad of arthritis, conjunctivitis, and urethritis, developing after a triggering bacterial infection; however, incomplete forms of this triad are more common.

**78.3.3.1 Clinical Features.** Reactive arthritis usually develops between 1 and 3 weeks after a precipitating infection. The arthritis typically affects a few large lower limb joints asymmetrically. Diffuse swelling of fingers and toes, referred to as "sausage digits," is typical of reactive or psoriatic arthritis. Axial skeletal involvement (particularly sacroiliitis), affects up to 50% of those affected, particularly those who are HLA-B\*27 positive. Plantar fasciitis and Achilles tendonitis are common. Mild conjunctivitis commonly precedes the arthritis. Mucocutaneous involvement may include mouth ulcers, sterile urethritis, circinate balanitis, and keratoderma blennorrhagica (a rash resembling pustular psoriasis).

The illness can be very severe and associated with marked systemic features of fever, malaise, and weight loss. It runs a variable course depending on the nature of the triggering organism and host genetic factors. Many cases are extremely mild and last only a few days. For those with more sustained synovitis, the average duration is <5 months. About 15% proceed to chronic disease and about one quarter may develop recurrent episodes (177). Chronic arthritis is much more likely in patients who are HLA-B\*27 positive, 20% of whom may ultimately develop AS. Patients with concurrent human immunodeficiency virus (HIV) infection tend to develop more severe arthritis and are probably at greater risk of disease because of increased exposure to the relevant genital tract pathogens.

**78.3.3.2 Epidemiology and Etiology.** While environmental triggers and genetic susceptibility contribute to reactive arthritis, it is not known how these interact to cause arthritis. Between 68% and 85% of cases of reactive arthritis occur in B27-positive individuals, and, in epidemics of arthritogenic bacterial infections, approximately 20% of B27-positive individuals will develop reactive arthritis.

Although the triggering bacteria cannot be cultured from the affected joints, there is reasonable evidence for the presence of bacterial proteins and nucleic acid in the joints (178). Cellular immunity to triggering bacteria has been demonstrated among both CD4- and CD8-positive B27-restricted T cells (158). These T cells are of the helper T cell type 1 subset of lymphocytes (179), which are thought to protect against intracellular infection. This seems appropriate as most arthritogenic pathogens are intracellular organisms, and it is consistent with theories suggesting a primary role for persistence of bacterial fragments within joints as the cause of reactive arthritis. The occurrence of reactive arthritis in face of CD4 lymphocyte depletion in HIV infection suggests that these cells are not critical in the development of the illness, in contrast with the situation in RA (in which the disease often improves as the CD4 lymphocyte count falls). Other theories implicate B27 itself as the autoimmune target, due to either molecular mimicry or loss of tolerance resulting from chemical alteration of the molecule. Evidence that B27 may be associated with lesser protection against invasion by, and less-efficient clearance of, enteric bacteria provides a further possible explanation for B27-related arthritis.

**78.3.3.3 Management.** Pain relief from the musculoskeletal manifestations of reactive arthritis is the primary objective in non-persistent disease. Nonsteroidal anti-inflammatory drugs are commonly used but have only a moderate beneficial effect. Local steroid injections are appropriate for peripheral arthritis and enthesopathy and may need frequent repetition. Sulfasalazine, methotrexate, and azathioprine have demonstrated beneficial effects and are appropriate to use in cases in which a protracted or recurrent course seems likely. Anti-TNF therapy may be effective in refractory cases. In cases of

presumed urogenitally acquired reactive arthritis, appropriate cultures should be taken for *Chlamydia* and *Neisseria gonorrhoeae*, as well as syphilis and HIV serology. Short courses of antibiotics are appropriate to clear any triggering infection, but there is also some evidence that longer courses of antibiotics may improve the late outcome of the arthritis (180).

### 78.3.4 Enteropathic Arthritis

Arthritis may complicate up to 10% of cases of inflammatory bowel disease. It may affect the axial skeleton and/or peripheral joints and is approximately twice as common in Crohn's disease as in ulcerative colitis. Axial arthritis occurs in 10–15% of individuals with inflammatory bowel disease, but the association of sacroiliitis with HLA-B\*27 may be weaker (50–60%) in this group of patients than in those suffering from AS without inflammatory bowel disease or psoriasis (~95%). These figures are heavily dependent on the means of ascertainment. For example, using a UK database of patients with known AS, we found the prevalence of HLA-B\*27 to be 83% in those with spondylitis and bowel disease (181). It seems possible that limited sacroiliitis (frequently asymptomatic) may be relatively common in inflammatory bowel disease, but HLA-B\*27 increases the likelihood of developing more extensive axial disease.

Peripheral arthritis exists in two distinct clinical forms that are also immunologically distinct (182). Both forms occur about twice as commonly with Crohn disease as ulcerative colitis. The type 1 arthropathy (self-limiting, pauciarticular, and associated with flares of the bowel disease) is positively associated with HLA-B\*27, HLA-B\*35, and HLA-DRB1\*0103 but the type 2 arthropathy (polyarticular, symmetrical, persistent, and not related to activity of the bowel disease) is associated with HLA-B\*44 (183). In view of the fact that AS has a high frequency of subclinical ileitis (184), it is interesting that peripheral arthropathy may predate the onset of clinically apparent inflammatory bowel disease by months or occasionally years. In type 1 arthropathy, the knees and ankles are most frequently affected, whereas in type 2 arthropathy, the metacarpophalangeal joints, wrists, and knees are most commonly affected.

### 78.3.5 Psoriatic Arthritis

Arthritis associated with psoriasis exhibits a considerable variety of clinical patterns and was only distinguished as a separate entity relatively recently (185). The arthropathy ranges through forms resembling RA to classical SpA. The extent to which these different expressions of psoriatic arthritis are under genetic controls is debatable since they do not appear to breed true in families with psoriatic arthritis (186).

**78.3.5.1 Epidemiology.** Psoriasis itself occurs in 1–2% of whites with an equal gender frequency. Common

forms of arthritis may thus occur fortuitously with psoriasis, without there being any causal relationship. Psoriatic arthritis typically occurs in 7% of patients with psoriasis although frequencies of up to 42% have been reported in some series (187,188). Arthritis is particularly common where there is nail involvement, but it may predate the onset of skin lesions. Although it usually starts in the third or fourth decade, pediatric onset is also quite common (see Section 78.4).

**78.3.5.2 Pathogenesis.** Inappropriate activation of the immune system appears to underlie both the skin and joint disease of psoriasis. In the psoriatic epidermis, keratinocytes proliferate and mature rapidly, leading to deficient adhesion of corneocytes that causes the characteristic scales of the psoriatic skin plaques. Significant infiltration of both the skin and joint synovium by T cells suggests a role for specific antigen presentation. Exacerbation of the skin lesions by CD4 depletion and HIV and/or acquired immunodeficiency syndrome has been observed, while specific T-cell-targeted therapy (e.g. cyclosporin A) may be effective. Furthermore, the efficacy of T-cell-targeted biologic therapies (alefacept directed against CD2 and efalizumab against LFA-1) in around 50% of patients supports this concept although the latter has been withdrawn from the market over safety concerns. An important role for TNF $\alpha$  in skin and joint lesions is emphasized by the efficacy of anti-TNF biologics although this is a complex relationship as anti-TNF agents have also been reported to precipitate flares of skin disease.

In the synovial membrane, there is evidence of new blood vessel growth, and substantial increase in the amounts of transforming growth factor- $\beta$ , platelet-derived growth factor, vascular endothelial growth factor, and angiopoietins has been observed (189).

**78.3.5.3 Genetics.** The genetic basis of psoriasis is presented in detail in Chapter 150. The heritability of psoriatic arthritis is substantial with a recurrence risk among first degree relatives ( $\lambda_1 = 30$ –55) and siblings ( $\lambda_s = 30$ ) estimated to be at least threefold higher than in psoriasis (190,191). Evidence from linkage and association studies led to the designation of the major psoriasis and psoriatic arthritis susceptibility locus within the MHC as PSORS1 (MIM\*177900). It has been shown to be primarily associated with type 1 psoriasis (onset <40 years of age), as no association was found in patients with psoriatic arthritis and late-onset psoriasis (192). The precise location of the PSORS1 risk allele is debated and is difficult to establish because of the extensive linkage disequilibrium within the MHC region; possibilities include HLA-Cw\*0602 (193) and the SNP rs10484554\*T (194).

Several other HLA associations have been reported in the literature; however, dissecting the contribution to psoriatic arthritis risk from the contribution to psoriasis risk is complex. A recent study, including a significant cohort of psoriatic arthritis patients, analyzed HLA associations by logistic regression. Alleles specifically associated with arthritis independently of cutaneous disease

included HLA-B\*27, B\*08, and B\*38 (195). Subgroup analysis demonstrated association of HLA-C\*06 and HLA-B\*27 with peripheral arthritis. HLA-B\*27 was strongly associated with the development of sacroiliitis, and B\*08, B\*38, and B\*39 were also associated with spondyloarthritis (195). Sacroiliitis is a common finding in patients with psoriatic arthritis; our survey of 105 unselected hospital outpatients showed abnormalities on MRI in one-third, but these were frequently asymptomatic (196). In psoriatic spondyloarthritis, the association with HLA-B\*27 is less strong (50–60%) than in the idiopathic forms of AS (95%), and we postulate that it correlates with more severe forms of spinal involvement.

Non-HLA genes within the MHC region, which may contribute to psoriatic arthritis risk include MICA\*002 (197), and the TNF promoter polymorphisms TNF-238A and TNF-857T (190), although the evidence is contradictory (190,198). An exploratory GWAS and replication study identified a single nucleotide polymorphism (SNP) rs2395029, which confers psoriatic arthritis risk with a high OR (3.2). This SNP lies within the MHC class I gene HCP5 positioned between the MICA and MICB loci (194).

GWAS have demonstrated significant associations with non-MHC gene loci including IL23R, IL12B, TNIP1, and TRAF3IP2 (190,199). Significant associations have also been linked with regions on chromosome 4q27 containing the IL2 and IL21 genes, and to chromosome 15q21 (194). Suggestive associations that have not reached significance, although have been replicated, include IL13, IL21, NFKBIA, NFAIP3, NOS2, and FBXL19 (190). These associations hint at pathogenic mechanisms underlying PsA as they include genes encoding Th2 (IL13) and Th17 (IL12B, IL23R) cytokines and signaling pathways including multiple associations with NF- $\kappa$ B signaling (TNIP1, TRAF3IP2, NFKBIA, TNFAIP3, NOS2, FBXL19).

Children of affected fathers are more likely to develop psoriasis or psoriatic arthritis (16.2%) than children of affected mothers (8.3%) (200,201). Evidence for imprinting is supported by the association of psoriatic arthritis with a region on chromosome 16q, which only confers risk when conditioned on paternal inheritance (202). Prognostically, males with axial psoriatic arthritis are more severely affected than females, in the absence of any differences in HLA distribution (203). A higher risk of progressive or erosive arthritis is found in patients carrying HLA-B\*39, HLA-DRB1\*04, HLA-DQ3 without HLA-DR7, HLA-B\*27 plus HLA-DR7, or the AA variant of the IL4 SNP rs1805010, while patients with the combination HLA-Cw\*6 plus HLA-DRB1\*07 have a better prognosis (204).

Approximately 75% of the genetic risk of psoriatic arthritis remains unidentified and further studies are required to identify risk alleles with smaller effect sizes, or indeed rare genetic variants conferring high risk (199,204). The emerging field of epigenetics will help to

elucidate how heritable pathways affecting gene expression may influence clinical risk and disease phenotype.

**78.3.5.4 Clinical Manifestations.** Classically, psoriatic arthritis has been divided into five subsets depending on clinical and radiographic criteria: (i) “classical” psoriatic arthritis, confined to the distal interphalangeal joints of the hands and feet (5%); (ii) arthritis mutilans (5%); (iii) symmetrical polyarthritis, resembling RA (15%); (iv) asymmetrical oligoarthritis (70%); and (v) spondyloarthritis (5%) (205). Recent studies have demonstrated that the pattern of onset of peripheral arthritis does not predict outcome in most cases, supporting the development of classification criteria, in which all patients with peripheral arthritis are pooled together (206).

Dactylitis (sausage digits) is characteristic of reactive or psoriatic arthritis, occurring in more than one-third of patients. Achilles tendonitis and plantar fasciitis (both forms of enthesitis) are also common. Dystrophic nail changes, including pitting, ridging, discoloration, and onycholysis, are associated particularly with distal interphalangeal joint disease. Overall, approximately 80% of patients with psoriatic arthritis have nail changes, compared with only 20% of psoriatics without arthritis.

Psoriatic arthritis is often a benign illness, but severe joint damage is not uncommon. Patients may present with severe acute monoarthritis and marked constitutional symptoms, mimicking sepsis or reactive arthritis. Typically, an increasing number of joints become involved with time although there is little relationship between this and the severity of the skin disease. Arthritis mutilans, although rare, causes severe joint destruction and disability. Axial disease is common but highly variable and tends to cause less functional impairment than in idiopathic AS. Compared to the “pure” form of AS, psoriatic spondyloarthritis also tends to be less symmetrical; radiographs may show non-marginal origin of syndesmophytes (origin not from the anterolateral border of the vertebral end plate); radiographic changes may skip vertebral segments; atlantoaxial involvement is more common; and unilateral sacroiliitis is not unusual. In reality, these divisions into subgroups are somewhat arbitrary; of those patients with peripheral joint disease, probably one-third have evidence of axial disease on MRI, although this is commonly asymptomatic (196).

**78.3.5.5 Management.** Nonsteroidal anti-inflammatory drugs similar to those used in RA are helpful for the peripheral joint arthritis. Intra-articular corticosteroids can be useful in peripheral arthritis and enthesitis, but oral corticosteroids have been associated with severe flares of cutaneous disease on withdrawal and are therefore not widely used. Anti-TNF biologic agents are highly effective in both the peripheral and axial joint manifestations of the disease although etanercept is considerably less effective at treating the skin manifestations (207,208).

Slow-acting disease-modifying antirheumatic drugs, such as sulfasalazine, methotrexate, and leflunomide,

may be effective in some cases, but cyclosporin A, despite its proven efficacy in skin disease, is relatively ineffective on the joints. In contrast to their helpful effects in peripheral arthritis, these agents are generally ineffective for psoriatic spondylitis.

## 78.4 JUVENILE IDIOPATHIC ARTHRITIS

Historically, this has been a somewhat ill-defined entity, a situation exacerbated by the differences in nomenclature in Europe and North America. Until recently, the term juvenile chronic arthritis has been used in Europe, whereas in North America, these disorders were often called juvenile chronic RA (in contrast to the use of this term in Europe specifically for seropositive juvenile-onset RA). Further confusion may arise from the use of the eponymous term Still disease for juvenile idiopathic arthritis (JIA). In 1897, Sir George Frederick Still distinguished juvenile forms of arthritis from adult RA, commenting on the fever found in the systemic form. Subsequently, in 1959, Ansell and Bywaters distinguished these childhood forms on the basis of their mode of onset. Thereafter, in general, “Still disease” was reserved for the systemic onset form of the disease although it has also been sometimes loosely applied to juvenile arthritis overall. These problems of classification can make comparisons between studies difficult. It has also complicated analysis of the genetic component of these disorders since many studies have not adequately distinguished between the various forms.

Childhood arthropathy has many potential causes, and it is important to exclude sepsis and viral infection, in particular, before concluding that the child has JIA. A World Health Organization/International League Against Rheumatism report proposed a widely accepted classification based on clinical patterns of disease (209) that defines seven subtypes of JIA:

- Systemic onset (11%)
- Oligoarthritis and extended oligoarthritis (50%)
- RF-positive polyarthritis (3%)
- RF-negative polyarthritis (17%)
- Enthesitis-related arthritis (10%)
- Psoriatic arthritis (7%).

Specific diseases associated with joint inflammation, such as SLE, rheumatic fever, septic arthritis, and neoplasia, are excluded from this classification.

### 78.4.1 Systemic-Onset JIA (Still Disease)

Still disease is the form of childhood arthropathy that carries the most adverse prognosis, with a significant mortality of 10%. It is characterized by systemic features including quotidian fevers, evanescent rash, lymphadenopathy, hepatosplenomegaly, arthropathy, and polyserositis (210). These features, coupled with a pronounced neutrophilia ( $>13 \times 10^9/L$  in 75% or more of patients),



may suggest infection, particularly when systemic features predate the arthropathy, which may happen by weeks or months. Other causes of fever and serositis should be considered, such as familial Mediterranean fever, particularly in patients of the appropriate ethnic background. Other important differential diagnoses include infection and malignancy. The disease has been reported in most populations worldwide and affects the sexes equally. The age of onset is variable but is usually between 4 and 6 years. It can also occur in adults, in whom it frequently causes diagnostic problems, particularly if systemic features predominate.

While some patients exhibit complete remission within 2 years of onset and others have repeated cycles of activity, the majority follow a chronic course (211). Some of the systemic features, including the fever and malaise, may respond well to short-term nonsteroidal anti-inflammatory drugs. For patients with more severe disease or poor prognostic signs such as active fever and high physician's global score, the systemic features may require moderate- to high-dose corticosteroids (including intravenous methylprednisolone). Anti-IL1 biologic therapy (e.g. Anakinra) may also be indicated in these patients and is frequently dramatically effective (212).

Multiple intra-articular injections of corticosteroids can be very effective in managing mild joint disease, but in patients with persistent or severe polyarticular involvement, disease-modifying antirheumatic drugs may be necessary. Weekly methotrexate (either orally or subcutaneously) has proved particularly beneficial compared to other second-line antirheumatic agents (e.g. gold salts, D-penicillamine, sulfasalazine), which have, in general, been disappointing. For resistant articular disease, biologic therapies, including anti-TNF (213), but particularly anti-IL1 and anti-IL6 agents, may be effective (212).

## 78.4.2 Oligoarticular JIA

Oligoarticular JIA affects young girls at least six times more frequently than boys, with a peak incidence at under 3 years of age, although the disease may also present for the first time in teenage life. The prevalence is between 20 and 30 per 100,000 and most ethnic groups are affected (214). By definition, children with this form of JIA have four or fewer joints affected within the first 6 months of disease, although in as many as one-third, the disease may subsequently extend to polyarticular involvement (210).

**78.4.2.1 Clinical Features.** Constitutional symptoms are not prominent in contrast to Still disease. Although joint pain is usually an obvious feature, the presentation is sometimes less obvious. Thus, a parent may notice a swollen joint in the absence of symptoms or observe nonspecific features, such as poor behavior or cessation from walking. Almost two-thirds of patients have

only one joint involved, and more than 90% have no more than two joints involved, in the first 6 months. Those children who remain oligoarticular for 5 years are unlikely subsequently to develop more extensive disease.

Levels of acute-phase reactants, such as C-reactive protein, may be normal or only slightly risen. If there is marked elevation, a search for alternative explanations is merited. This group of patients is classically associated with the presence of antinuclear antibodies in the serum (40–75%), usually in low titer (<1:640). Positive antinuclear antibodies indicate a higher risk of developing chronic anterior uveitis, which is the most serious potential complication of early-onset oligoarticular JIA (215), although all children with JIA are at risk. It causes blindness in up to 10% of patients, but this is avoidable if the appropriate screening and treatment steps are exercised. Ocular disease affects up to 57% of patients with oligoarticular JIA, usually with anterior uveitis although isolated posterior disease rarely may be present. Only a few of those with iritis (5%) have polyarticular disease at onset. JIA-related uveitis is usually asymptomatic and the onset of ocular features may be delayed. Therefore, slit-lamp examination is mandatory in all patients at diagnosis and regularly for many years thereafter (Guidelines for Screening for Uveitis in Juvenile Idiopathic Arthritis (JIA), BSPAR and the RCPOphth 2006). The uveitis is chronic, lasting between 2 and 15 years, and most commonly affects both eyes.

The joint disease associated with oligoarticular JIA typically resolves within 5 years, but chronic arthritis may sometimes recur, even many years later. Those children whose arthropathy extends to become polyarticular (about 20% overall) account for the majority exhibiting significant functional handicap at 15 years.

**78.4.2.2 Management.** It is important to keep the joints as normal as possible while the arthropathy is in its active phase. This can usually be accomplished by the use of nonsteroidal anti-inflammatory drugs and intra-articular corticosteroids (best administered under general anesthetic in young patients). In general, there is a good response, and second-line drugs, such as methotrexate, are reserved for patients with persistent or severe disease. Physical methods of treatment to prevent contracture and preserve muscle strength are beneficial. Surgery is rarely needed except in those cases in which severe contractures develop or abnormalities of bone growth occur secondary to epiphyseal involvement. Anti-TNF biologics are potentially very effective in resistant cases. Topical corticosteroids and mydriatics are effective in 40% of patients with uveitis, but intraocular steroids or systemic corticosteroids are frequently required to prevent the formation of posterior synechiae between the lens and the iris. In some cases, immunosuppressive drugs such as azathioprine, chlorambucil, and cyclosporin A may be necessary to control the uveitis. The ocular disease is

potentially sight-threatening and demands regular ophthalmologic review.

### 78.4.3 Polyarticular JIA

Polyarticular JIA is much less clearly defined than the oligoarticular and systemic-onset forms. It is apparent that some patients whose disease begins with limited joint involvement subsequently progress to a more widespread form of arthropathy with a correspondingly poorer outcome (these patients are included under the extended oligoarticular disease subset). If this occurs, it is invariably within the first 5 years of disease. Other patients have polyarticular symptoms from the outset, but these appear to represent a relatively heterogeneous group with the exception of the subgroup with juvenile-onset seropositive RA (3% of all JIA). The prognosis of this specific subset is similar to the adult form of RA, with similar immunogenetic associations. Indeed, the HLA associations are even more striking in the juvenile form of the disease, and the association with the HLA-DRB1\*0401/\*0404 genotype is particularly striking (33).

### 78.4.4 Genetics of JIA

It has been estimated that JIA has a prevalence of between 20 and 120 per 100,000 and an annual incidence of 10–20 per 100,000. The prevalence appears to be similar in Europe and North America in general although a threefold excess risk to Native American children compared with their white counterparts in British Columbia has been reported. No differences have been observed in the prevalence in North American black and white children, but, by contrast, the condition appears to be rare in China. The available family studies provide support for a genetic component to JIA, but many of these studies predate the more accurate classification of JIA into various subgroups. Thus, Lawrence (216) found an excess concordance in monozygotic twins (40%) versus dizygotic twins (9%), but these studies included only 16 twin pairs. Subsequently Moroldo et al. (217) studied 71 sib-pairs affected by juvenile chronic arthritis and found substantial concordance (70–80%) for the type of disease between the sibs. There is a slight excess recurrence in sibs for systemic-onset JIA, and a genetic component to the disease arising from HLA-linked genes has been suggested although these results are inconsistent.

HLA-DR alleles have been estimated to confer almost 20% of the total sibling recurrence risk in JIA (218) although the risks associated with HLA alleles differ between the JIA subtypes. HLA-DRB1\*08 (a rare allele in most white populations) is consistently found in between 25% and 50% of patients with oligoarticular JIA (relative risk ~12). HLA-DRB1\*11 (relative risk ~5) is also increased, and some studies have suggested a weak association with HLA-DRB1\*13. Our results suggest that

HLA-DRB1\*13 is most closely associated with the presence of uveitis (OR 3.4) in oligoarticular disease (219). HLA-DRB1\*04, which is associated with RA in adults is associated with juvenile seropositive polyarthritis (OR 3.2), but is protective against many other JIA subtypes (220). DRB1\*07 is associated with decreased risk of JIA (220). There is considerable evidence for a marked compound heterozygote effect with predisposing DRB1 alleles (e.g. HLA-DRB1\*08/DRB1\*11) (221–223), analogous to the situation in RA with DRB1\*0401/\*0404 (see Section 78.2.5). Gender has a significant influence on the age that the HLA risk is conferred (224). HLA analysis has been particularly useful in distinguishing a subset of children with pauciarticular disease, typically boys older than 9 years, in whom limited joint involvement with an associated enthesopathy is the first sign of spondyloarthritis (see Section 78.3.2). This variant is strongly associated with HLA-B\*27 and is known to be associated with an excess of family members with sacroiliitis. Many of these children go on to develop AS although others appear to continue with a more peripheral form of enthesopathy without AS.

Other HLA alleles also contribute to risk. HLA-DQA1\*0103 is associated with oligoarticular disease and HLA-DQA1\*05 with oligoarticular and systemic-onset arthritis. HLA-DQA1\*02 is protective against oligoarticular and seropositive polyarthritis (220). DPB1\*0201 confers risk of early-onset arthritis (221). Previously reported HLA-DRB1/DQB1 risk associations were found to be solely due to the effect of the DRB1 locus (221).

It has been suggested that particular combinations of HLA-A\*2, DPB1\*0201, and other DRB1 susceptibility alleles (\*08, \*11, \*13) are associated with onset of oligoarticular disease under 3 years of age (224). The DRB1\*1501–DQA1\*0102–DQB1\*0602 haplotype protects against oligo- and polyarticular JIA (221).

Conducting studies of sufficient power to examine the risk contribution of non-HLA alleles is made difficult by the rarity of JIA and the phenotypic heterogeneity of the disease. While studies encompassing JIA as an umbrella disease may mask genetic associations with individual JIA subsets, subset subanalysis may fail to detect genetic risk alleles with small effects (225). Therefore, approaches to identifying candidate genes have included investigating loci associated with other autoimmune diseases (226).

More than 100 non-MHC risk alleles have been examined (225), and although many were found to be positively associated, only a small number of these have been replicated to date. The PTPN22 and IL2RA gene associations have reached genome-wide significance. PTPN22 is involved with T-cell signaling and confers risk of oligo- and polyarticular arthritis, while IL2RA, which codes for the high-affinity IL2 receptor alpha chain, is particularly associated with ANA positivity, female gender, and oligoarthritis (227,228). Other significant associations

include genes implicated in T-cell signaling and activation (STAT4, VTCN1), innate immunity (TNFA, TNFAIP3, TRAF1/C5, MIF, SCL11A1) and cartilage homeostasis (WISP3). Many of these genes are associated with risk of other autoimmune conditions such as RA (229). For example, the ERAP1 and IL23R gene variants, which are associated with AS in adults, have been found to increase risk of enthesitis-related arthritis (230) and juvenile psoriatic arthritis, respectively. These findings await replication (230).

The strong association of oligo- and polyarticular JIA with HLA class II alleles suggests pathogenic involvement of CD4 T cells (227). No etiologic agents have yet been identified in this disorder although autoantigens present in the eyes and the joints are strongly suggested as being responsible for the chronic inflammatory process in oligoarticular disease. The non-HLA associations implicate Tregs, which may influence the balance between pro- and anti-inflammatory processes, as well as innate immune cells, which may contribute to cytokine production and CD4 T-cell activation. The distinct HLA and non-HLA genetic association between the JIA subsets implies differences in pathogenic mechanisms (227).

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# CHAPTER

# 79

## Amyloidosis and Other Protein Deposition Diseases

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### 79.1 INTRODUCTION

Amyloidosis is the classic example of protein deposition disease. However, there are many forms of amyloidosis and, by definition, these represent only those diseases associated with extracellular deposits of  $\beta$ -structured protein fibrils (1–4). We now know that there are diseases characterized by protein deposits that may be extracellular or intracellular, and the deposits may contain either fibrillar or nonfibrillar protein aggregates.

Each type of protein deposition disease, whether fibrillar or nonfibrillar, is characterized by a specific protein that forms the major constituent of the pathologic deposits. In the case of the amyloidoses, there are at least 26 proteins that can form extracellular  $\beta$ -structured fibril deposits, and each represents a separate disease entity (5). Some of these diseases are systemic (involving several organ systems) and some are localized (limited to one organ). The systemic forms of amyloidosis may be sporadic or acquired (e.g. immunoglobulin AL amyloidosis); secondary to other conditions (e.g. reactive AA amyloidosis) or hereditary (e.g. familial amyloidotic polyneuropathy (FAP)/transthyretin (TTR) amyloidosis). The localized forms of amyloidosis may also be sporadic or acquired (e.g. AL amyloid in the upper respiratory tract, urinary tract or plasmacytomas and, most important, Alzheimer disease); secondary to other conditions (e.g. infectious Creutzfeldt–Jakob disease) or hereditary (e.g. many of the corneal dystrophies, familial Alzheimer disease, Gerstmann–Sträussler–Scheinker pri-onosis). Amyloid deposits composed of several specific proteins can produce systemic or localized, sporadic or secondary, and hereditary or nonhereditary pathologic conditions. Each of these diseases probably shares certain pathogenic mechanisms that lead to  $\beta$ -fibril formation but there must also be specific factors that dictate where, when and how amyloid deposits occur in each of these disease entities. A similar story can be constructed for the non-fibrillary protein deposition conditions, which have

only recently come to attention. These include systemic immunoglobulin light chain deposition disease and the localized, mainly intracellular, protein deposits such as  $\alpha$ -synuclein (Lewy bodies), tau (Pick bodies), huntingtin and neuroserpin. All of these protein deposits are associated with one or more disease entities. However, they do not form the  $\beta$ -structured fibrils that we call amyloid.

In this chapter, we are concerned with those forms of amyloidosis and other protein deposition diseases that give hereditary syndromes. First, however, we should consider the more common types of amyloidosis because they can tell us much about the mechanisms of fibrillogenesis, and they often must be included in the differential diagnosis of hereditary amyloidosis.

Immunoglobulin light chain (LC) amyloidosis (AL) is the most common type of amyloidosis (6). It is a sporadic (acquired) disease that increases in incidence with age. It has no definite hereditary features other than increased incidence in families with a history of multiple myeloma. AL amyloidosis is a monoclonal plasma cell disease in which proteolysed fragments of immunoglobulin LC protein ( $\kappa$  or  $\lambda$ ) are deposited as  $\beta$ -structured fibrils (7). These deposits are extracellular and occur in major organs such as kidney, heart, liver and spleen, and also in the blood vessel walls of most organs. This is the most aggressive form of amyloidosis, with median 1-year survival after diagnosis of approximately 18 months for nontreated patients, 5-year survival of 20%, and 10-year survival of only 5% (8). It may cause carpal tunnel syndrome and sensorimotor polyneuropathy, and, therefore, is often mistaken for TTR amyloidosis (FAP). The cardiomyopathy of some forms of hereditary apolipoprotein A-I (apo A-I) amyloidosis may also be mistaken for AL amyloidosis. The prognosis is markedly different.

Much is known about the pathogenesis of AL amyloidosis, and this is significant for the consideration of amyloid fibrillogenesis in general. For example, X-ray diffraction analysis has shown the high degree of  $\beta$ -pleated

sheet structure in the immunoglobulin LC domains (9). This would be expected to be an important factor in fibrillogenesis. However, the greater incidence of  $\lambda$  LC amyloidosis than  $\kappa$  LC amyloidosis suggests that there are more cryptic structural factors that influence fibrillogenesis than just the overall  $\beta$  structure that is shared by both  $\kappa$  and  $\lambda$  light chains. The selective deposition as fibrils of one specific monoclonal light chain in the presence of a plethora of polyclonal immunoglobulins also testifies to the importance of structure in fibrillogenesis. There is tremendous interest in the physical properties of peptides that contribute to  $\beta$  structure and fibril formation (10). However, it should be remembered that, while structure of a peptide may be the basis for amyloid fibril formation, there must be specific *in vivo* metabolic processes that lead to the end result.

Reactive (secondary) AA amyloidosis appears to be much less common than it used to be. This may be the result of improved treatment of the infections (tuberculosis) and inflammatory (rheumatoid arthritis) diseases that predispose to this form of amyloidosis. Research on AA amyloidosis has shown that increased synthesis of the fibril precursor protein, serum amyloid A (SAA) and certain tissue factors (amyloid-enhancing factor) are important for fibrillogenesis (11). The primary structure of the precursor protein is also important and, in the case of inbred mice, explains the species specificity of amyloid fibril formation (12). Of particular interest to the geneticist is the AA amyloidosis associated with autosomal recessive febrile illnesses, such as familial Mediterranean fever (FMF) (13), and autosomal dominant febrile syndromes, such as TRAPS (tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) receptor-associated periodic syndromes) (14), Muckle-Wells syndrome (15) and familial cold urticaria (16). The amyloidosis is not inherited: it is a reactive form of amyloidosis, a result of chronic overproduction of the amyloid precursor protein SAA. The fact that different mutations in the FMF gene (MeV) (17,18), in the TNF- $\alpha$  receptor gene (19), and in the CIAS1 gene (20) result in varying degrees of amyloid induction points to other, unknown factors in fibrillogenesis.

## 79.2 HEREDITARY SYSTEMIC AMYLOIDOSIS

### 79.2.1 Transthyretin Amyloidosis

First and foremost among the genetically determined forms of amyloidosis and other protein deposition diseases are the TTR amyloidoses (21) (Table 79-1). This condition was originally named FAP by Andrade, who in 1952 first described this peculiar syndrome of sensorimotor neuropathy with varying degrees of organ involvement (heart, kidney, eye) (22). This form of amyloidosis is now known to be the most widespread and prevalent of the hereditary amyloidoses. It is transmitted as an autosomal dominant trait owing to mutations in the plasma protein TTR (formerly named prealbumin) (23). There are more

than 100 mutations in TTR known to cause the disease, and all are single amino acid substitutions except one, which is loss of one amino acid residue ( $\Delta$ Val122) (21).

TTR is a plasma transport protein for thyroxine and retinol-binding protein/vitamin A (24). It is synthesized in the liver as a single polypeptide chain of 127 amino acid residues (25,26). The gene has been localized to 18q23 (27,28). A murine gene knockout model shows that TTR is not essential for life, although expression in embryos and adults suggests that it is normally important in both fetal development and adult life (29).

Mutations in TTR presumably alter the molecular structure, and this leads to aberrant degradation and aggregation of the protein as amyloid fibrils (Figure 79-1). TTR has extensive  $\beta$ -pleated sheet structure, and this makes it a good candidate for fibril formation (30). Indeed, TTR amyloid fibril deposits are commonly found in the hearts of aged humans who do not have any mutant form of the protein (31,32). It seems logical, therefore, that mutations in TTR are not necessary for amyloidosis but, instead, move the age of onset to earlier in adult life. TTR amyloidosis is an adult-onset disease in which the genetic mutation has been passed on to the next generation before serious clinical disease presents itself.

Clinically, TTR amyloidosis usually starts as a small fiber peripheral neuropathy affecting the extremities and autonomic functions (22,33–35). It often presents as paresthesias or dysesthesia in the lower limbs but may start as carpal tunnel syndrome, alternating diarrhea and constipation, impotence in males, restrictive cardiomyopathy or, in rare forms, central nervous system (CNS) syndromes due to leptomeningeal amyloid infiltration (36–38). Vitreous opacities due to amyloid fibril deposition are seen in one-quarter to one-third of kindreds with mutant forms of TTR (21,39) (Figure 79-2). In fact, the presence of vitreous amyloid in a patient essentially limits the diagnosis to some form of TTR amyloidosis. Progression of the neuropathy is cephalad and may lead to sensory neuropathy over much of the body, plus loss of motor function that precludes ambulation. Even paralysis of the recurrent laryngeal nerve has been seen with this type of amyloidosis. Death used to be the result of infection and inanition in many persons affected with FAP. Now, restrictive cardiomyopathy and those syndromes associated with seriously compromised cardiac function (e.g. renal failure) are the most common cause of demise.

Disease onset varies from the third decade of life to advanced age, with examples of incomplete penetrance in a number of kindreds with different TTR mutations (40,41). In general, it would seem that the disease is more rapidly progressive in individuals affected at an early age, with time from diagnosis to death being less than a decade. In older-onset syndromes (past age 50), it is not unusual to observe periods from diagnosis to death of 15–20 years. In these cases, the disease usually involves slowly progressive restrictive cardiomyopathy and lesser degrees of peripheral neuropathy.

TABLE 79-1 TTR Amyloidoses

Mutation	Clinical Features <sup>a</sup>	Geographic Kindreds
Cys10Arg	Heart, eye, PN	United States (PA)
Leu12Pro	LM	United Kingdom
Val14Leu	PN	Italy
Asp18Glu	PN	South America, United States
Asp18Gly	LM	Hungary
Asp18Asn	Heart	United States
Val20Ile	Heart, CTS	Germany, United States
Ser23Asn	Heart, PN, eye	United States
Pro24Ser	Heart, CTS, PN	United States
Ala25Ser	Heart, CTS, PN	United States
Ala25Thr	LM, PN	Japan
Val28Met	PN, AN	Portugal
Val30Met	PN, AN, eye, LM	Portugal, Japan, Sweden, United States (FAP I)
Val30Ala	Heart, AN	United States
Val30Leu	PN, heart	Japan
Val30Gly	LM, eye	United States
Phe33Ile	PN, eye	Israel
Phe33Leu	PN, heart	United States
Phe33Val	PN	United Kingdom, Japan
Phe33Cys	CTS, eye, heart, kidney	United States
Arg34Ser	PN	United States
Arg34Thr	PN, heart	Italy
Arg34Gly	Eye	United Kingdom
Lys35Asn	PN, AN, heart	France
Lys35Thr	Eye	United States
Ala36Pro	Eye, CTS	United States
Asp38Ala	PN, heart	Japan
Trp41Leu	Eye, PN	United States
Glu42Gly	PN, AN, heart	Japan, United States, Russia
Glu42Asp	Heart	France
Phe44Ser	PN, AN, heart	United States
Ala45Thr	Heart	United States
Ala45Asp	Heart, PN	United States
Ala45Ser	Heart	Sweden
Gly47Arg	PN, AN	Japan
Gly47Ala	Heart, AN	Italy, Germany
Gly47Val	CTS, PN, AN, heart	Sri Lanka
Gly47Glu	Heart, PN, AN	Turkey, United States, Germany
Thr49Ala	Heart, CTS	France, Italy
Thr49Ile	PN, heart	Japan
Thr49Pro	Heart	United States
Ser50Arg	AN, PN	Japan, France/Italy
Ser50Ile	Heart, PN, AN	Japan
Glu51Gly	Heart	United States
Ser52Pro	PN, AN, heart, kidney	England
Gly53Glu	LM, heart	Basque
Gly53Ala	LM	United Kingdom
Gly53Arg	LM	United States
Glu54Gly	PN, AN, eye	England
Glu54Lys	PN, AN, heart, eye	Japan
Glu54Leu		United Kingdom

Continued

TABLE 79-1 TTR Amyloidoses—cont'd

Mutation	Clinical Features <sup>a</sup>	Geographic Kindreds
Leu55Pro	Heart, AN, eye	United States, Taiwan
Leu55Arg	LM	Germany
Leu55Gln	Eye, PN	United States
Leu55Glu	Heart, PN, AN	Sweden
His56Arg	Heart	United States
Gly57Arg	Heart	Sweden
Leu58His	CTS, heart	United States (MD) (FAP II)
Leu58Arg	CTS, AN, eye	Japan
Thr59Lys	Heart, PN, AN	Italy
Thr60Ala	Heart, CTS	United States (Appalachian)
Glu61Lys	PN	Japan
Glu61Gly	Heart, PN	United States
Phe64Leu	PN, CTS, heart	United States, Italy
Phe64Ser	LM, PN, eye	Canada, England
Ile68Leu	Heart	Germany
Tyr69His	Eye	United States
Tyr69Ile	Heart, CTS, AN	Japan
Lys70Asn	Eye, CTS, PN	United States
Val71Ala	PN, eye, CTS	France, Spain
Ile73Val	PN, AN	Bangladesh
Ser77Tyr	Kidney	United States (IL, TX), France
Ser77Phe		France
Tyr78Phe	PN, CTS, skin	Italy, France
Ala81Thr	Heart	United States
Ala81Val	Heart	United Kingdom
Ile84Ser	Heart, CTS, eye, LM	United States (IN), Hungary (FAP II)
Ile84Asn	Heart, eye	United States
Ile84Thr		Germany, United Kingdom
His88Arg	Heart	Sweden
Glu89Gln	PN, heart	Italy
Glu89Lys	PN, heart	United States
His90Asp	Heart	United Kingdom
Ala91Ser	PN, CTS, heart	France
Glu92Lys	Heart	Japan
Val94Ala	Heart, PN, AN	Germany, United States
Ala97Gly	Heart, PN	Japan
Ala97Ser	PN, heart, AN	Taiwan, United States
Ile107Val	Heart, CTS, PN	United States
Ile107Met	PN, Heart	Germany
Ile107Phe	PN, AN	United Kingdom
Ala109Ser	PN, AN	Japan
Leu111Met	Heart	Denmark
Ser112Ile	PN, heart	Italy
Tyr114Cys	PN, AN, eye, LM	Japan
Tyr114His	CTS	Japan
Tyr116Ser		France
Ala120Ser	Heart	Afro-Caribbean
Ala120Thr	PN, CTS	Japan
Val122Ile	Heart	United States
ΔVal122	Heart, PN	United States (Ecuador)
Val122Ala	Heart, eye, PN	United States

<sup>a</sup>AN, autonomic neuropathy; CTS, carpal tunnel syndrome; LM, leptomeningeal; PN, peripheral neuropathy.



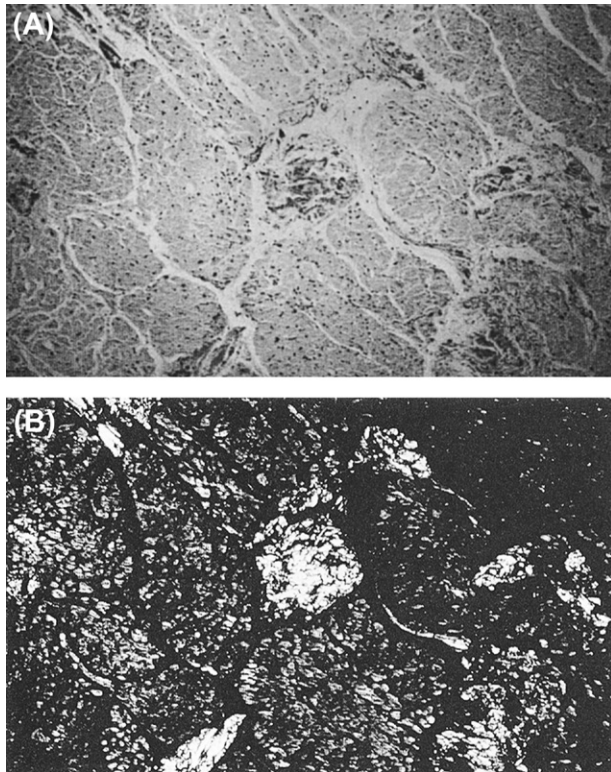
The Val30Met form of TTR amyloidosis is the most common type of FAP. It was originally described in patients in northern Portugal, but has been found in kindreds in northern Sweden, Japan, Cypress, Turkey and Greece, and, of course, in many American families of European descent (22,33,41–43). Haplotype analysis

has shown that the Portuguese, the Swedish and many of the Japanese kindreds probably have the mutant gene that originated in northern Portugal (44). Similar studies reveal that some English, Turkish and Japanese kindreds probably have the Val30Met gene from other mutation events (45).

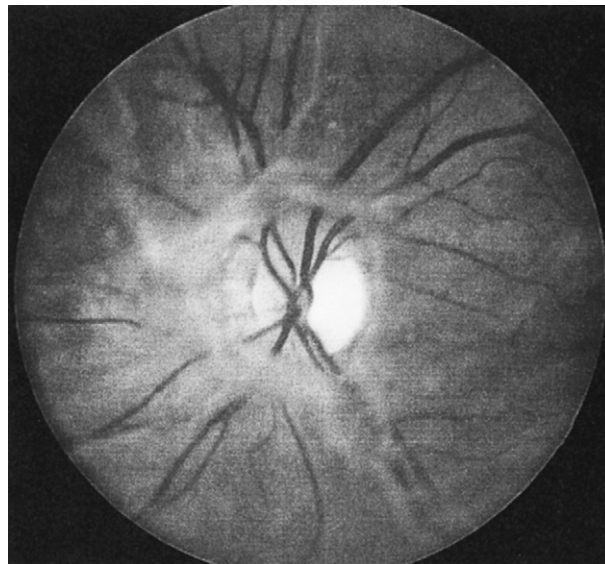
Other widespread forms of TTR amyloidosis include the Thr60Ala mutation, which was first discovered in a large kindred in the Appalachian region of the United States (46,47) but now has been shown to have originated in Ireland (48). There are kindreds of Irish ancestry with this mutation in Australia and New Zealand. The TTR Leu58His mutation was discovered in a number of kindreds in the eastern United States (Maryland and Pennsylvania) (34,49). It has now been found in Germany in the birthplace of the ancestors of the American kindreds (50). Both the Thr60Ala and Leu58His mutations are associated with relatively late-onset disease with progressive cardiomyopathy.

The Ser77Tyr TTR amyloidosis has also been found in many countries, including the United States, Germany and France (51,52). Haplotype studies indicate that a French kindred probably had a separate mutation event (53). Other TTR amyloidosis kindreds are relatively restricted geographically. The Indiana/Swiss Ile84Ser amyloidosis is present in mid-America, Switzerland and one kindred in Hungary (35,39,54,55). The Cys10Arg mutation has been found in one kindred in the United States (56). The  $\Delta$ Val122 TTR mutation, which was discovered in an Ecuadorian family, has now been found in Spain, perhaps indicating the country of origin (57).

Perhaps the greatest number of carriers of a TTR mutation that causes amyloidosis is represented by the Val122Ile mutation, which is present in 3–4% of African Americans (58–60). This mutation, which has



**FIGURE 79-1** Amyloid deposits in the myocardium stain with Congo red (A) and give characteristic green birefringence when viewed by polarization microscopy (B).



**FIGURE 79-2** Amyloid in the vitreous of the eye, which causes progressive loss of vision, can be seen as fluffy deposits on funduscopy examination.

now been discovered in those parts of western Africa from which most American blacks originated, causes restrictive cardiomyopathy in later adult life, usually after the age 60. There is often minor associated neuropathy, and the disease is frequently not recognized clinically. Instead, the patient is often thought to have heart failure due to previous hypertensive or coronary vascular disease.

**79.2.1.1 Clinical Variations in FAP.** Although the TTR amyloidoses are all systemic diseases, the marked variation in organ system involvement leads to syndromes that obscure the underlying diagnosis (this is particularly true for certain mutations in TTR that are associated with relatively similar clinical presentations). The classic perception of FAP is of a syndrome of sensorimotor and autonomic neuropathy with malfunction of the gastrointestinal tract, orthostatic hypotension, varying degrees of restrictive cardiomyopathy and nephropathy. This clinical presentation is typical of the Portuguese FAP, Val30Met (22), as well as Ser77Tyr (61) and a majority of the other mutations. In early studies, it was recognized that certain mutations resulted in disease with the initial presentation of carpal tunnel syndrome (FAP II), which is characteristic of the Leu58His mutation in the Maryland kindreds (34) and the Ile84Ser mutation in the Indiana kindred (35). It has now been appreciated that peripheral neuropathy may be a minor aspect of the amyloidosis. This is particularly true of the Thr60Ala (46), Ile68Leu (62), Ile84Ser (35), Ile84Asn (63), Ala97Gly (64), Ile107Val (65) and Val122Ile (58,60) mutations. The Leu111Met mutation originally described by Frederiksen and colleagues (66) in Denmark is present in a kindred with restrictive cardiomyopathy and no signs of peripheral neuropathy (67). Of particular interest are those TTR mutations that cause extensive leptomeningeal vascular amyloid deposition—Leu12Pro, Asp18Gly (38), Val30Gly (36,37) and Phe64Ser (68,69)—and are associated with intracranial hemorrhage, either subarachnoid or intracerebral. In the case of two of these mutations (Asp18Gly and Val30Gly), neurodegeneration with dementia is a prominent feature of the syndrome (36,38). Other clinical variations have been seen. In one kindred with Tyr69His only vitreous amyloid was seen (70), whereas in another Tyr69His kindred, patients suffer with the consequences of leptomeningeal amyloidosis, and a Japanese kindred (Tyr114His) had only carpal tunnel syndrome as evidence of amyloidosis (71). Thus, although multi-organ involvement with TTR amyloidosis may produce syndromes that are relatively easy to diagnose, the many variations in clinical presentation obviously make this group of diseases underappreciated.

**79.2.1.2 Genetics.** The TTR amyloidoses show an autosomal dominant inheritance. Most affected individuals are heterozygous for a point mutation, indicating that the disease is the result of “gain of function.” This gain of function, however, would appear only to

be the ability of the mutant TTR to participate in the synthesis of amyloid fibrils. A few TTR mutations have been found in the homozygous state. Homozygosity for TTR Val30Met is associated with clinical disease that is not distinguished from that seen in heterozygotes (40). The same would appear to be true for the Val122Ile TTR mutation, which is typically expressed as restrictive cardiomyopathy in African Americans after the age 60 (60,72). Homozygosity for TTR Leu58His, however, has been reported to give earlier onset and more aggressive disease (73). Studies have suggested that inheritance of the mutant Val30Met gene from an affected mother results in earlier onset than inheritance from an affected father (74). Reduced penetrance of TTR amyloidosis is a confounding factor in diagnosis, and has often resulted in a misdiagnosis of immunoglobulin amyloidosis, in which peripheral neuropathy and cardiomyopathy are often seen. Reduced penetrance is particularly high in the northern Sweden population with TTR Val30Met (40,41) but is also seen in the United States with the same mutation, with the Thr60Ala and Cys10Arg mutations, and with several other mutations (56). On the other hand, extensive study of the Indiana/Swiss kindred with Ile84Ser has not identified an escapee over several generations (75).

TTR mutations associated with amyloidosis have been found in most countries of the world (see Table 79-1). Although larger representations of mutations have been from a few countries, including Italy, Germany, Japan, France and the United States, this is probably largely a reflection of scientific interest and medical sophistication in these countries. Over the last 20 years in which the disease has been studied at the molecular biology level, all of the mutations that have been discovered in the United States have been traced to immigrant populations. To date, no TTR mutation has been found in a Native American. No TTR mutation associated with amyloidosis has been found in Australian Aborigines or in Polynesians, and only the Val122Ile mutation has been described in individuals from Africa. This will undoubtedly change with the advancement and availability of DNA diagnostics. Several of the TTR mutations found in the United States have been traced to ancestors in Europe: the Leu58His TTR to Germany (50), the Val30Met to Portugal and Sweden (76), the Thr60Ala to Ireland (48) and the Ser77Tyr to Germany. The ΔVal122 TTR, originally discovered in a kindred in Ecuador, has now been reported from Spain (57).

Haplotype analysis based on polymorphisms in TTR have produced convincing evidence that the Val30Met mutation of the Portuguese was shared with the people of northern Sweden and with a number of kindreds in Japan; however, haplotype analysis indicates that at least two other mutation events occurred to produce the Val30Met mutation in English, Turkish and Japanese

families (44,45,77). There are several CpG dinucleotide sequences in TTR, which may explain the increased incidence of some TTR mutations, including Val30Met and Val122Ile (44). However, the large number of TTR mutations that have been discovered, and the fact that some mutations, such as Ser77Tyr, are seen on more than one haplotype and would not be predicted to have an increased mutation rate, indicate a high rate of retention of spontaneous mutations. Factors that might add to this observation include the following:

1. TTR amyloidosis is an adult-onset disease and appears to have minimal effect on procreation.
2. TTR, as shown by the murine knockout model, appears not to be necessary for survival and normal development (29).
3. Any loss of function by TTR mutation, such as reduced thyroxine binding or vitamin A transport, is compensated for by other biologic systems.
4. The identification of many TTR mutations may be enhanced by the fact that almost any alteration in structure of this heavily  $\beta$ -structured molecule will lead to abnormal metabolism and amyloid formation. There are only 12 reported mutations in TTR that have not been associated with the development of amyloidosis, whereas over 100 mutations can cause disease (21).

## 79.2.2 Other Systemic Amyloidoses

**79.2.2.1 Apolipoprotein A-I Amyloidosis.** Apo A-I amyloidosis was originally designated FAP III (78). Although the first kindred with this type of amyloidosis had a clinical syndrome that included peripheral neuropathy and renal failure, the presence of hepatic amyloidosis and gastric ulcers in a large number of affected individuals indicated that it was different from FAP I and FAP II, which were subsequently shown to be related to mutations in TTR. This disparity was confirmed by the demonstration that the amyloid in FAP III was the result of a mutation in apo A-I in which an arginine was substituted for glycine at position 26 of the mature protein (79). Subsequently, a number of mutations in apo A-I have been identified that are associated with systemic amyloidosis (80–85). Most are single nucleotide point mutations, but single nucleotide deletions with shift of reading frame and codon insertions have also been reported (Table 79-2). This is an autosomal dominant disease, with all affected individuals being heterozygous; however, the small numbers of kindreds with this disease have precluded an assessment of degree of penetrance. The gene for apo A-I is on chromosome 11 and codes for a mature protein of 243 amino acid residues that is the major protein constituent of plasma high-density lipoprotein. Amyloid deposits contain only the first 83 to 93 N-terminal residues of apo A-I and, as native apo A-I has been shown to have a predominantly

$\alpha$ -helical structure, major rearrangement of the amino-terminal peptide must occur during catabolic processing so that  $\beta$ -pleated sheet amyloid fibrils are formed (86). One study has shown increased catabolism of mutant apo A-I Gly26Arg, and this may be a significant factor in the pathogenesis of the amyloidosis (87). The first mutations in apo A-I associated with amyloidosis were found in the amino-terminal portion of the protein, the portion that forms amyloid fibrils. The clinical syndrome usually involves renal and hepatic amyloidosis. Only the Gly26Arg mutation is associated with neuropathy, and this has not been found in all kindreds with this mutation. Subsequently, mutations in the carboxyl-terminal portion of apo A-I were discovered and were shown to cause a different syndrome characterized more by cardiac, dermal and laryngeal amyloid deposition (Figure 79-3) (88–92). These mutations indicate that incorporation into the amyloid fibril is not required to cause amyloid formation, and, indeed, alteration in metabolism is probably a major factor in pathogenesis.

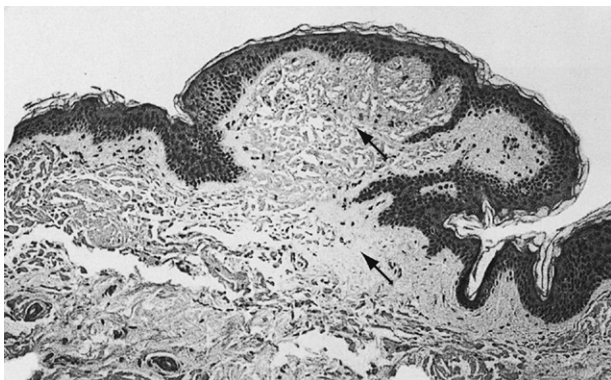
**79.2.2.2 Gelsolin-Related Amyloidosis.** FAP IV was the designation given to the Finnish type of amyloidosis originally reported by Meretoja (93,94). It is an adult-onset disease with lattice corneal dystrophy occurring as early as the second decade of life, followed by neuropathy of the cranial nerves, thickening and later laxity of facial skin, and a mild sensory neuropathy in the limbs (95). It is a systemic disease with deposits of amyloid also occurring in the heart and gastrointestinal tract. Analysis of the deposits has shown that the fibril protein is a 71-amino acid residue internal fragment of gelsolin. The disease in the original Finnish kindreds has been shown to be related to the substitution of asparagine for aspartic acid at position 187 of the mature protein (96–98). The disease has now been reported in American families, in The Netherlands and in Japan (99,100). Most affected individuals are heterozygous for the Asp187Asn mutation, but a few homozygous individuals have been identified. A separate mutation at the same codon of gelsolin (Asp187Tyr) has been found in kindreds from Denmark and Czechoslovakia with similar syndromes (Table 79-2) (99).

Gelsolin is a calcium-binding protein that is involved in the fragmentation of actin filaments (101,102). It is coded by a single gene on chromosome 9 (9q32–q34), with two forms resulting from alternative splicing (101). Cytoplasmic gelsolin has an important role in cytoskeletal reorganization, whereas plasma gelsolin binds actin and presumably functions to clear actin from the plasma. It is the plasma protein that is most likely the precursor of amyloid fibrils. In general, gelsolin amyloidosis is not associated with shortened longevity, although homozygosity for the Asp187Asn mutation is associated with severe renal disease (103). The lattice corneal dystrophy is often not of great clinical significance and, if necessary, may be treated with corneal transplantation.



**TABLE 79-2 Mutant Proteins Other Than TTR Associated with Autosomal Dominant Systemic Amyloidosis**

Protein	Mutation	Clinical Features	Geographic Kindreds
Apolipoprotein A-I	Gly26Arg	PN <sup>a</sup> , nephropathy	United States
	Trp50Arg	Nephropathy	United Kingdom
	Leu60Arg	Nephropathy	United Kingdom
	Leu64Pro	Nephropathy	United States, Italy
	Del 60–71 ins Val/Thr	Hepatic	Spain
	Del 70–72	Nephropathy	South Africa
	Asn74Lys(fs) <sup>b</sup>	Nephropathy	Germany
	Leu75Pro	Hepatic	Italy
	Leu90Pro	Cardiomyopathy, cutaneous, laryngeal	France
	Ala154(fs) <sup>b</sup>	Nephropathy	Germany
	Leu170Pro	Laryngeal	Germany
	Arg173Pro	Cardiomyopathy, cutaneous, laryngeal	United States
	Leu174Ser	Cardiomyopathy	Italy
	Ala175Pro	Laryngeal	United Kingdom
	Leu178His	Cardiomyopathy, laryngeal	France
Gelsolin	Asp187Asn	PN <sup>a</sup> , lattice corneal dystrophy	Finland, United States, Japan
	Asp187Tyr	PN <sup>a</sup>	Denmark, Czech Republic
Cystatin C	Leu68Gln	Cerebral hemorrhage	Iceland
Fibrinogen A $\alpha$ chain	Val522Ala(fs) <sup>b</sup>	Nephropathy	United States
	Glu524Glu(fs) <sup>b</sup>	Nephropathy	France
	Thr525(fs) <sup>b</sup>	Nephropathy	China
	Glu526Val	Nephropathy	United States
	Thr538Lys	Nephropathy/Neuropathy	China
	Glu540Val	Nephropathy	Germany
	Pro552His	Nephropathy	Afro-Caribbean
	Arg554Leu	Nephropathy	Mexico
	Del 1636–1650 ins CA 1649–1650	Nephropathy	Korea
Lysozyme	Tyr54Asn	Cardiomyopathy, gastrointestinal	United States
	Ile56Thr	Nephropathy, petechiae	United Kingdom
	Phe57Ile	Nephropathy	Canada
	Trp64Arg	Nephropathy	France
	Asp67His	Nephropathy	United Kingdom
	Trp112Arg	Nephropathy, gastrointestinal hemorrhage	Germany
Apolipoprotein A-II	Stop78Gly	Nephropathy	United States
	Stop78Ser	Nephropathy	United States
	Stop78Arg	Nephropathy	United States
	Stop78Leu	Nephropathy	Spain

<sup>a</sup>PN, peripheral neuropathy.<sup>b</sup>(fs), frame shift.**FIGURE 79-3** Apolipoprotein A-I amyloid deposits in the skin are characteristic of the Leu90Pro and Arg173Pro mutations.

**79.2.2.3 Lysozyme Amyloidosis.** Six mutations in lysozyme have been shown to cause systemic amyloidosis (Table 79-2) (52,104,105). In one family, a petechial skin rash was a presenting feature, with subsequent renal failure (Ile56Thr) and in another petechial skin rash was associated with cardiomyopathy (Tyr54Asn). Other mutations—Asp67His, Trp64Arg, Phe57Ile and Trp112Arg—are also associated with renal amyloidosis. Lysozyme is a bacteriolytic enzyme that is synthesized by polymorphonuclear leukocytes and macrophages. The gene on chromosome 12 codes for a 14,500-Da protein. The entire mature protein is incorporated into amyloid fibrils, but only the mutant form has been found in all tissues examined, which have been from heterozygous individuals (106).



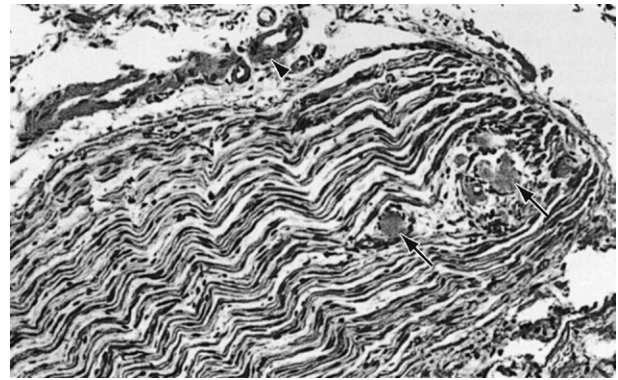
**79.2.2.4 Cystatin C Amyloidosis (Hereditary Cerebral Hemorrhage with Amyloidosis).** A mutant form of cystatin C, a serine protease inhibitor, has been shown to cause leptomeningeal vascular amyloidosis, which is associated with repeated intracranial hemorrhage (107). The disease is systemic, with amyloid deposits in other organs such as the spleen. It is found principally in Iceland, is autosomal dominant, and usually occurs in the third or fourth decade of life. The amyloid deposits are composed of cystatin C lacking the first 10 amino acids of the mature protein (108). A substitution of glutamine for leucine at position 58 of the amyloid subunit peptide determines the expression of the disease (109). The gene for cystatin C is on chromosome 20 (110).

**79.2.2.5 Fibrinogen A $\alpha$  Chain Amyloidosis.** Nine mutations in the gene for fibrinogen A $\alpha$  chain have been found to be associated with amyloidosis expressed as nephropathy (Table 79-2) (111,112). Five are point mutations that give single amino acid substitutions, and two are single nucleotide deletions that cause a shift in the reading frame of the messenger RNA and aberrant peptides with premature termination (113,114). A recently described deletion/insertion mutation associated with renal amyloidosis in a pediatric patient appears to have occurred de novo. All mutations are in the protease-sensitive carboxyl-terminal portion of the A $\alpha$  chain and result in proteolytic peptides containing from 49 to 83 amino acid residues (115). The clinical syndrome is characteristic, with the development of hypertension followed by proteinuria and then progressive azotemia. Life can be prolonged for several years by dialysis, but hepatic and splenic involvement will occur. Renal transplantation has been shown to be followed by amyloid deposition in the transplanted organ within 1 to 10 years. Families in the United States, Canada and Germany have been described with these mutations. The gene for fibrinogen A $\alpha$  chain is on chromosome 4 and codes for a mature protein with a molecular weight of 66,000 Da (116). Fibrinogen is synthesized exclusively by the liver and demonstrates a modest acute-phase response to injury.

**79.2.2.6 Apolipoprotein A-II Amyloidosis.** In 1973, Weiss and Page (117) described the autopsy pathology of two sisters with renal amyloidosis. Subsequent study of affected offspring revealed that the amyloid was composed of Apo A-II that was 21 amino acid residues longer than the wild-type protein, the result of mutation in the A-II stop codon (Stop76Gly) (118). The clinical syndrome is characterized by hypertension followed by slowly progressive renal failure. Other stop mutations (Stop76Ser, Stop76Arg, Stop76Leu) have now been described in families with similar clinical disease (119,120).

## 79.2.3 Diagnosis

The diagnosis of systemic hereditary amyloidosis is rarely made in a timely fashion. The systemic amyloidoses can give a number of symptom complexes (congestive



**FIGURE 79-4** Amyloid deposits within peripheral nerves (arrows) are often not evenly distributed and may be missed on standard nerve biopsy. Amyloid in the walls of perineural blood vessels (arrowhead) may reveal the diagnosis.

heart failure, renal failure, polyneuropathy, diarrhea) that reflect the clinical presentation of several more common and mundane diseases. Therefore, a heightened awareness of the various forms of hereditary amyloidosis is very important to early diagnosis and institution of appropriate therapies. There are a number of features of hereditary amyloidosis that should raise suspicion. A progressive small fiber sensorineuropathy with abnormalities of autonomic function is often seen with the hereditary TTR amyloidoses. In such cases, a peripheral nerve biopsy should always be examined pathologically with the diagnosis of amyloidosis in mind, as amyloid deposits are often not appreciated on routine histologic stains (Figure 79-4). The possible diagnosis of amyloidosis should be considered in any subject who presents with restrictive cardiomyopathy with or without orthostatic hypotension. Amyloid cardiomyopathy may give an anginal syndrome and often causes fatigue or dyspnea on exertion as signs of heart failure. Typical congestive features of heart failure, with peripheral edema, ascites and pleural effusion, may not be seen until late in the course of the disease. The most valuable tests for this condition are electrocardiography, which typically shows low voltage often with a pseudo-anteroseptal myocardial infarction pattern; and echocardiography or cardiac magnetic resonance imaging, which reveal a thickened interventricular septum and left ventricular posterior wall and enlarged left atrium, if the restrictive hemodynamics have been present for a long time. Chronic renal failure with or without heavy proteinuria should raise the suspicion of amyloidosis, as should unexplained impotence in males and chronic constipation alternating with diarrhea in either males or females. The presence of vitreous opacities affecting vision in any subject with heart failure or renal failure should raise the possibility of TTR amyloidosis (39). As with most genetically determined diseases, family history can be very important in making the diagnosis. However, family history may be misleading, as the diagnosis of amyloidosis has often been confused with other diseases in previous generations, and many forms

of the amyloidoses, despite being autosomal dominant, fail to show complete penetrance.

If a patient with amyloidosis has a family history of similar disease, or if there is a strong suspicion of hereditary amyloidosis, DNA analysis is usually the best way to confirm this suspicion (121). The diagnosis of amyloidosis has first to be made by tissue biopsy of an affected organ, and although immunohistochemistry may be helpful in identifying the type of amyloid involved, the results of immunostaining are not always reliable. Most new patients who present with hereditary amyloidosis are not aware of the gene or specific mutation that is the cause of their disease, even if they have a positive family history. In this case, it is important for the diagnostician to narrow the field of differential diagnoses by analyzing the symptom complex, the ethnic origin of the patient or his or her ancestors, the age of onset of the disease and the duration of disease before death. Table 79-1 lists the known disease-associated mutations in TTR and identifies where in the world significant kindreds have been identified. Unless a patient's syndrome matches to that of a previously identified kindred with a specific mutation and geographic localization, it is probably not cost-effective to pursue direct DNA analysis for specific mutations. There are over 100 mutations in TTR that cause systemic amyloidosis, and although there is a polymerase chain reaction (PCR)-based test using specific restriction enzymes, allele-specific PCR, or induced mutation restriction analysis for each, it is not feasible to perform all of these tests to find the causative mutation. Instead, since automated DNA sequencing of TTR exons is now commercially available, this form of DNA diagnosis has become the most efficient means to detect amyloid-associated mutations. Single-strand conformation polymorphism remains a useful screening procedure for new TTR mutations and for testing large numbers of subjects for a specific mutation (65). At the protein level, TTR variants can be detected by isoelectric focusing, but this technique is difficult and not generally available (122). The same is true for immunoprecipitation of TTR and the identification of mutant forms by mass spectrometry (123). DNA testing for the other forms of hereditary amyloidosis, including apo A-I, lysozyme, fibrinogen A $\alpha$  chain and apo A-II, presents similar problems. None of these tests is readily available for the various mutations, and the clinician needs to consult one of the few laboratories dedicated to the study of amyloidosis. It is now relatively easy for the patient or physician to obtain the necessary information from the Internet (<http://www.mayoclinic.com/health/amyloidosis/DS00431>; <http://www.iupui.edu/~amyloid>; <http://www.bu.edu/amyloid/>; <http://www.amyloidosisupport.com/index.html>; <http://www.amyloidosisupport.com/>).

#### 79.2.4 Management

There are three aspects to management of the patient with hereditary amyloidosis. First, the availability of

DNA analysis allows the patient to have the benefit of a definitive diagnosis and avoid extensive, costly and time-consuming medical evaluations searching for other diagnoses. Unfortunately, many patients with hereditary amyloidosis have already undergone cardiac catheterization, coronary angiography, multiple organ biopsy and even psychiatric evaluation before the diagnosis of amyloidosis has been entertained. Once a mutated allele associated with a specific form of amyloidosis has been identified, DNA testing becomes available for at-risk individuals in the family, and these individuals may benefit from the results of this testing. Testing, of course, needs to be accompanied by proper counseling as to the genetics of the specific disease and the prognosis based on current knowledge. The identification of specific mutations associated with amyloidosis has made prenatal testing available; however, to date only prenatal testing for TTR mutations has been reported (124,125). Either amniotic fluid or chorionic villus sampling is adequate for this first-trimester test, and results can be obtained within 48h if the specific mutation in the parent is known.

A second aspect of the management of amyloidosis is the nonspecific therapies that might improve quality and length of life. For instance, a number of commonly used drugs aggravate the heart failure associated with restrictive cardiomyopathy. Treatment for chronic diarrhea, gastroparesis, urinary retention and dysesthesias may help quality of life. Cardiac pacing and renal dialysis both may prolong life.

The third aspect of patient management is centers on specific therapy or the lack thereof. In the last 20 years, approximately 2000 liver transplantations have been carried out as a specific treatment for TTR amyloidosis (126,127); (<http://www.fapwtr.org/ram1.htm>). Liver transplantation results in rapid loss of variant TTR from the plasma, as TTR is synthesized predominantly in the liver. The greatest success has been in patients with the Val30Met TTR mutation. The most immediate effect has been improvement of gastrointestinal function and possibly hypotension. There has been a report of improvement in nerve myelination in patients who have had liver transplantation early in the course of their disease (128,129). Unfortunately, there is now definite evidence that progression of systemic amyloidosis occurs in some patients who have had liver transplantation. This has been found to be true in patients with the Glu42Gly, Thr60Ala, Ile84Asn and Cys10Arg mutations. Biochemical analysis of amyloid deposits in tissues of patients who died 3–5 years after liver transplantation have shown that normal TTR can continue to form amyloid after variant TTR has initiated the process but is no longer present (130,131). Renal transplantation in patients with fibrinogen A $\alpha$  chain amyloidosis usually results in amyloid deposition in the transplanted organ within 1–10 years. However, combined kidney and liver transplantation has shown good results, and it has been suggested that since normal fibrinogen is not found in the amyloid fibrils,

liver transplantation may be a cure for the disease. One patient is still alive after more than 12 years without disease progression and recently liver transplantation done prior to end-stage renal disease requiring concurrent kidney transplantation has been done (132,133). A few liver transplantations have been done in patients with apo A-I amyloidosis, and may delay progression of disease (134). Apo A-I is synthesized in the liver and in the gastrointestinal tract, and it is not known whether both the organs or just the liver is the source of the plasma precursor of the amyloid fibril deposits. A number of cardiac transplants have been done for patients with apo A-I cardiomyopathy and appear justified since this form of amyloidosis is very slowly progressive. Renal transplantations have also been done for patients with apo A-II amyloidosis since the renal disease is also slowly progressive (135).

To date, research on gene therapy for the hereditary amyloidoses has not been fruitful. With these diseases, the challenge is to find a means to stop the production of a protein that is not wanted, instead of producing an enzyme or other protein that is necessary for normal life, as is the object in most of the autosomal recessive diseases. For TTR amyloidosis small organic molecules that thermodynamically stabilize the TTR tetramer are being tested for treatment of the amyloidosis. Also, antisense oligonucleotides specific for TTR have been shown to decrease hepatic production of TTR in mice transgenic for Ile84Ser TTR and promise a possible specific therapy for this amyloidosis (136).

## 79.3 HEREDITARY LOCALIZED AMYLOIDOSIS

There are a number of localized forms of hereditary amyloidosis, each characterized by a specific protein that, because of a mutation in structure or a genetically determined alteration in metabolism, gains the features to participate in amyloid fibril formation. In most cases, the localized nature of the amyloid deposition is the result of synthesis of the fibril precursor protein that is restricted to the specific organ or tissue involved. There are, however, other factors necessary for the generation of amyloid deposits, and these must be present in proximity to the site of synthesis of the amyloid precursor protein. In most forms of localized amyloidosis, the fibril subunit protein is a proteolytic fragment of a larger precursor protein. Therefore, proteases that generate these fragments must be present and functional in the tissue where amyloid fibril deposition occurs. The same must be true for the various forms of proteoglycans, which are invariably part of amyloid deposits. As with the systemic forms of amyloidosis, once the amyloid fibrils are formed they must be resistant to removal by any localized tissue mechanisms.

### 79.3.1 Alzheimer Disease

By far, the most common form of localized amyloidosis is seen in Alzheimer disease. Amyloid deposits,

which are localized to cerebral cortical tissues and blood vessels, contain a 39- to 42-amino acid residue internal fragment from the C-terminal portion of a precursor protein expressed in the CNS (137). This protein,  $\beta$ -amyloid precursor protein ( $\beta$ -APP), is coded by a single gene on chromosome 21 and has several alternatively spliced transcripts, the largest encoding a protein of 770 amino acid residues (138). A few forms of familial Alzheimer disease are the result of point mutations in the  $\beta$ -APP gene that causes early-onset autosomal dominant dementia (139–142). A larger number of familial forms of Alzheimer disease are the result of mutations in the presenilin genes (PS1 and PS2) localized to chromosomes 14 and 1, respectively (143–145). The majority of cases of Alzheimer disease, however, is age related and does not show simple mendelian genetics. A current hypothesis for pathogenesis is that mutations associated with hereditary forms of Alzheimer disease accelerate the age of onset and progression of amyloid plaque formation, which is a normal aging phenomenon and may be modulated by such factors as apolipoprotein E.

### 79.3.2 Gerstmann–Sträussler–Scheinker Disease

Gerstmann–Sträussler–Scheinker disease (GSS) is another hereditary form of amyloidosis localized to the CNS (146). In this neurodegenerative disease, the amyloid deposits are derived from the prion protein, which is also associated with Creutzfeld–Jakob disease and other transmissible forms of spongiform encephalopathy (147,148). Variant forms of GSS are associated with missense mutations or numbers of octapeptide repeats in the prion gene localized to chromosome 20 (149). Pathogenesis is hypothesized to be related to transition in the tertiary structure of a protease-sensitive form of prion protein to a protease-resistant form that is capable of forming  $\beta$ -pleated sheet fibrils. Considerable variability in the clinical expression of the prion diseases is seen, and pathologic manifestations may include varying degrees of spongiform encephalopathy, cortical amyloid deposits and amyloid angiopathy. However, all are adult-onset diseases with autosomal dominant inheritance, manifested as progressive neurodegeneration.

### 79.3.3 British Dementia

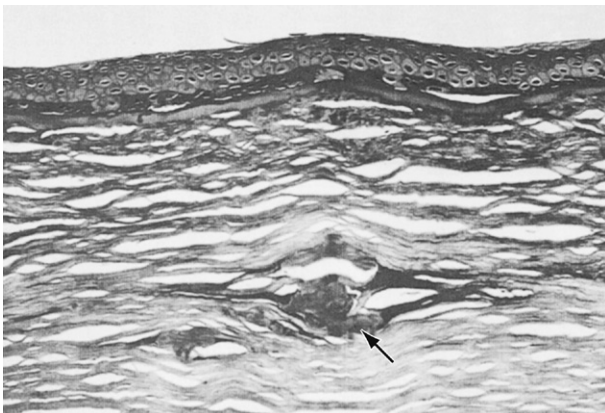
Another form of localized amyloidosis limited to the CNS is the autosomal dominant familial British dementia, characterized by progressive dementia, spasticity and cerebellar ataxia (150). In this condition, cerebral amyloid angiopathy, non-neuritic and perivascular plaques, and neurofibrillary tangles represent the pathology. The disease is the result of a mutation in the stop codon for the *BRI* gene, which is localized to chromosome 13



(151). Owing to a single base substitution in the stop codon, a larger 277-residue protein is produced. A carboxyl-terminal fragment containing 34 amino acid residues of the mutated protein is the ABRI (Amyloid BRI) subunit. A similar syndrome with dementia in a Danish kindred is associated with a 10-nucleotide duplication proximal to the stop codon of the *BRI* gene. This results in a new C-terminal peptide that is the precursor of amyloid fibrils (152).

### 79.3.4 Corneal Dystrophies

A number of corneal dystrophies are examples of localized amyloidosis that are genetically determined (153) (Figure 79-5). Because of the localized nature and small amount of amyloid in the corneal tissues, the chemical nature of the amyloid deposits remained unknown until recently, when genetic studies and molecular biology techniques identified causative genes and mutations. Lattice corneal dystrophy (type 2), which is related to mutations in the gelsolin gene, was the first corneal amyloid to be chemically characterized, but this is a systemic disease and the protein structure was determined from fibrils isolated from major organs (97). A number of point mutations in the Big-h3 gene, localized to chromosome 5q31, have been found to cause varying phenotypes of corneal dystrophy (154,155). Mutations in the M1S1 gene (chromosome 1p), which encodes a gastrointestinal tumor-associated antigen, have also been found to cause corneal amyloid deposition (156). Although it is now possible to separate several of the various types of corneal dystrophy by DNA analysis, chemical characterization of the deposited amyloid fibril proteins has been delayed by the small amount and localized nature of the deposits. Given the unique environment of the cornea, characterization of the proteolytic processing and factors involved in deposition of amyloid fibrils in these diseases may be very revealing for amyloid fibrillogenesis in general.



**FIGURE 79-5** Amyloid deposits (arrow) in familial corneal dystrophy.

### 79.3.5 Other Localized Amyloidoses

There are numerous other forms of localized amyloidosis that are expressed in a familial manner. In two of these, the amyloid subunit protein has been identified. Isolated atrial amyloid contains a 28-amino acid residue carboxyl-terminal peptide of atrial natriuretic peptide (157,158). In most cases, deposition of this form of amyloid in the cardiac atria is age related and associated with chronic congestive heart failure. The familial atrial standstill syndrome is associated with this type of amyloid deposition and is inherited as an autosomal dominant condition (159). Amyloid that is deposited in medullary carcinoma of the thyroid is derived from procalcitonin (160). In the familial form of medullary carcinoma of the thyroid, an autosomal dominant condition that may be associated with other endocrinopathies, the amyloid deposition may be a result of overproduction of the amyloid precursor procalcitonin, as no structural defect or mutation in the amyloid precursor protein has been identified (161).

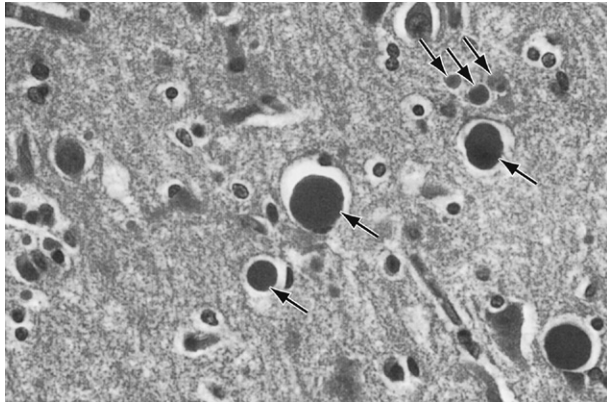
## 79.4 HEREDITARY NON-AMYLOID PROTEIN DEPOSITION DISEASE

While the number of amyloid proteins continues to grow, attention is now being directed toward diseases in which protein deposits occur intracellularly and the protein may or may not form fibrillar structures. Some of these diseases are hereditary and, as with most inherited forms of amyloidosis, are autosomal dominant. Included in this group are Huntington disease, frontotemporal dementia linked to chromosome 17, and familial Parkinson disease.

In Huntington disease, the disorder is associated with expanded CAG repeats in the Huntington gene (chromosome 4). Intranuclear deposits of the expressed protein with polyglutamine sequences are found in affected individuals, but the role of these protein deposits in the pathogenesis of the neurodegeneration is not known. Pick disease is characterized by presenile dementia with circumscribed cerebral atrophy. In this disease, protein deposits (Pick bodies) within the cytoplasm of cortical neurons contain abnormal forms of the microtubule-associated protein tau (162). Familial forms of Pick disease are associated with mutations in the tau gene (chromosome 17) and appear to change the ratio of expressed isoforms of tau protein (145). The role of protein deposition in the pathogenesis of the disease is still not known. Autosomal dominant Parkinson disease is associated with intracellular deposits that contain  $\alpha$ -synuclein. In a few families with hereditary Parkinson disease, mutations in the  $\alpha$ -synuclein gene (chromosome 4) have been found to be the cause of the disease (163).

Recently, families with progressive myoclonus epilepsy and dementia have been found to have intraneuronal





**FIGURE 79-6** Intraneuronal nonamyloid deposits contain mutant neuroserpin in a patient with hereditary myoclonus epilepsy and dementia.

cytoplasmic inclusions of neuroserpin, a serine protease inhibitor (Figure 79-6). Two mutations in the neuroserpin gene (chromosome 3q26) have been found to cause these syndromes (164,165). Mutation in the ferritin light polypeptide gene also causes intracellular protein deposits associated with dementia. Both of these syndromes are examples of pathology associated with protein deposition without actual amyloid  $\beta$ -fibril formation. Although the significance of such protein deposits in the pathogenesis of dementia is not known, identification of variant forms of intracellular proteins in these neurodegenerative diseases will certainly add to our knowledge of neuronal function (166).

## 79.5 CONCLUSION

In conclusion, it is best to view the hereditary amyloidoses as diseases associated with the deposition of specific proteins, which may be structurally normal or abnormal depending on the etiologic factor in each case. In addition, protein deposition diseases may occur without amyloid fibril formation, but with similar clinical results. Study of these hereditary diseases can tell us much about normal cell formation and physiology, and may lead to therapies for many pathologic conditions.

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### Biography



**Professor M D Benson** received a BA (1961), MS-Pathology (1964) and MD (1965) from the University of Vermont. He received professional training at Bellevue Hospital (Columbia University College of Physicians and Surgeons) and Boston University.

He joined the faculty of Indiana University in 1976 as Associate Professor of Medicine and became Professor of Medicine in 1981 and Professor of Medical Genetics in 1984.

Professor Benson is an internationally recognized expert in the field of amyloidosis. He has discovered the origin of several forms of hereditary amyloidosis, including those related to mutations in apolipoprotein-A1 and fibrinogen Aa-chain. His research has revealed structural changes of proteins, which are related to the transition of proteins the  $\beta$ -structure that is characteristic of amyloid fibrils. These studies have included basic research on proteins implicated in amyloidosis of human immunoglobulin (AL), reactive (SAA) and hereditary (TTR) forms of amyloidosis. His research has also lead to significant discoveries in the area of Alzheimer disease, a form of amyloidosis localized to the central nervous system.

Professor Benson was Chairman of the department of Medical and Molecular Genetics from 1996 to 1999. He is a member of the American Association of Immunologists, American Society of Clinical Investigation, Association of American Professors, American Society of Human Genetics, American Rheumatism Association, the Central Society for Clinical Research and the International Amyloid Society.

In 2003, *The Pasteur-Weizmann Board and the Servier Institute* presented in Paris, their prestigious inaugural award, “Pasteur-Weizmann/Servier International Prize in Biomedical Research” to *Dr Benson and his group*. This award recognized his pioneering research on “Amyloid” protein deposits. ([article link in French](#))

Dr. Benson and colleagues had also discovered a mutation in the Alzheimer amyloid protein, leading to the development of a widely used mouse model of Alzheimer disease.

**Interests**—Amyloidosis, with particular emphasis on the systemic forms including Immunoglobulin (Primary), Reactive (Secondary) and Hereditary Amyloidosis. Present interests include clinical studies of patients with all forms of amyloidosis, in addition to basic studies in pathology, biochemistry, genetics and molecular biology. Current research projects include studies on regulation of gene expression, synthesis and metabolism of plasma proteins, and tertiary structure of proteins. Studies of Alzheimer disease include DNA testing and structural characterization of amyloid plaque proteins. These studies have led to interest in other late-onset neurodegenerative diseases. Transgenic animal models are being used to study pathogenesis of human disease. This is a new area which undoubtedly will lead to new direction in research.

# CHAPTER

# 80

## Immunodeficiency Disorders

*Rochelle Hirschhorn, Kurt Hirschhorn and Luigi D Notarangelo*

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### 80.1 INTRODUCTION

The immunodeficiency diseases are part of a spectrum of conditions involving defects in host defense against infections. Adequate host defense is dependent on the interaction between phagocytic cells, immunocompetent cells and their products, and the complement system. Diseases involving disorders of phagocytes and deficiencies of complement components (innate immunity) are discussed in subsequent chapters. Here, we will review disorders that affect the development and/or function of T and B lymphocytes and of natural killer (NK) cells, which play a central role in our ability to resist infection. It is the occurrence of infections with unusual frequency that draws our attention to the possibility that a patient may be suffering from a primary immunodeficiency disorder.

T and B lymphocytes are key components of the adaptive immune system. Adaptive immune responses are characterized by specificity and memory. The specificity is conferred by the expression of receptors (T cell receptor, B cell receptor) that recognize antigens. Furthermore, upon encounter with a foreign antigen, T and B lymphocytes produce memory cells that are capable of mediating prompt and more powerful responses upon subsequent challenge with the same antigen. On one hand, immune responses mediated by B lymphocytes include production of specific antibodies. On the other hand, cytotoxic T cells contribute to killing of virus-infected cells, whereas helper T lymphocytes produce cytokines that promote antibody production and inflammatory responses.

NK lymphocytes lack antigen-specific receptors and hence are not a component of the adaptive immune system; however, they play an important role in antiviral and anti-tumor immune responses. Several forms of immune deficiency due to impaired development and/or function of T lymphocytes are also characterized by defects of NK cells; therefore, these conditions are discussed in this chapter. Another important function of the adaptive immune system is the ability to distinguish self- versus non-self antigens, so that exposure to foreign antigens results in a protective immune response, whereas self-antigens are tolerized. In a number of immune deficiency diseases, this self/non-self discrimination is

compromised, leading to autoimmunity. Therefore, the discovery of autoimmune phenomena along with infection represents another clue that may help suspect an immune deficiency disease.

Primary immunodeficiencies are classified according to the underlying defect (1). In particular, in this chapter we will review:

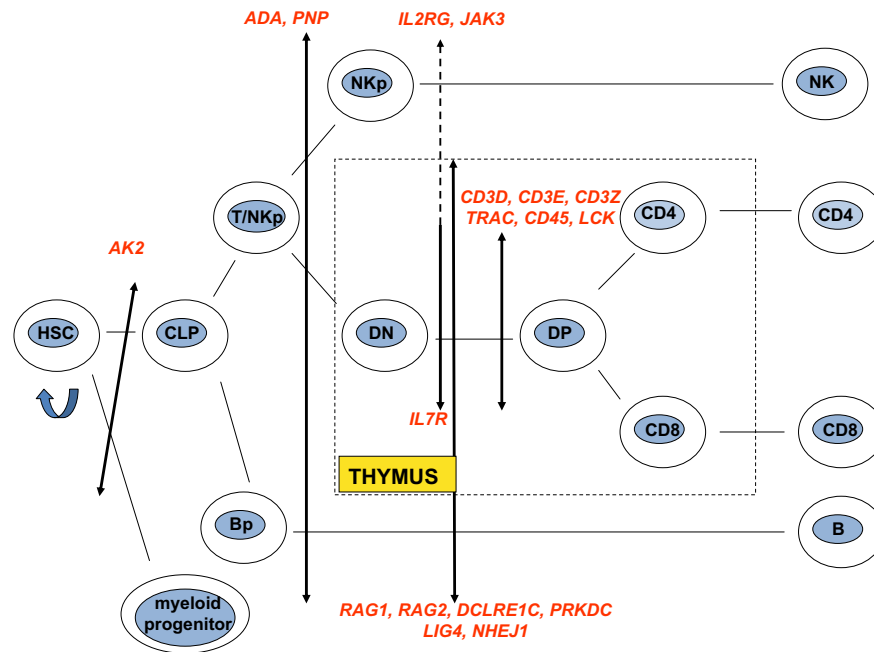
- (1) Combined Immunodeficiencies: These are disorders with abnormal development and/or function of T lymphocytes. In most of these cases, antibody responses are also compromised, either because of an associated developmental defect of B lymphocytes, or because of the impaired function of helper T lymphocytes;
- (2) Antibody deficiencies: These disorders include defects that affect development and/or function of B lymphocytes, without affecting T lymphocytes;
- (3) Disorders of immune dysregulation, in which there is an impairment of immunological tolerance or an inability to extinguish immune responses in the face of defective cytolytic function;
- (4) Immunodeficiency syndromes: These include a heterogeneous group of disorders, in which immune deficiency is an important component, but other extra-immune manifestations also exist.

### 80.2 SPECIFIC INHERITED IMMUNODEFICIENCY DISORDERS

#### 80.2.1 Combined Cellular and Humoral Immune Defects

**80.2.1.1 Severe Combined Immunodeficiency Disease.** Severe combined immunodeficiency disease (SCID) is characterized by profound defects of both cellular and humoral immunity and includes a heterogeneous group of genetically determined disorders that affect T cell development (1,2). In some cases, development and/or function of B lymphocytes and/or NK cells are also compromised (Figure 80-1); the presence or absence of these lymphocyte subsets represents the basis of immunological classification of SCID.





**FIGURE 80-1** Schematic representation of genetic defects (in red and italic) that have been identified in patients with Severe Combined Immune Deficiency. ADA: adenosine deaminase; AK2: adenylate kinase 2; CD3D: CD3 delta; CD3E: CD3 epsilon; CD3Z: CD3 zeta; DCLRE1C: Artemis; IL2RG: Interleukin-2 receptor gamma chain; IL7R: Interleukin-7 receptor alpha; JAK3: Janus-associated kinase 3; LIG4: DNA ligase 4; NHEJ1: Cernunnos; PNP: purine nucleoside phosphorylase; PRKDC: DNA-PK catalytic subunit; TRAC: T cell receptor alpha constant. B: B lymphocyte; Bp: B cell progenitor; CLP: common lymphoid progenitor; DN: CD4<sup>+</sup> CD8<sup>-</sup> double negative thymocyte; DP: CD4<sup>+</sup> CD8<sup>+</sup> double positive thymocyte; HSC: hematopoietic stem cell; NK: Natural Killer lymphocyte; NKp: Natural Killer cell precursor; T/NKp: common progenitor to T and NK lymphocytes.

SCID has a prevalence of 1:50,000–1:100,000 live births and is the most rapidly progressive and devastating of the primary immunodeficiency syndromes. SCID may be inherited as an autosomal or X-linked recessive. This syndrome was originally called “essential lymphocytophthisis,” “thymic alymphoplasia,” or “Swiss-type agammaglobulinemia.” Patients with this syndrome within the first few months of life present with severe, persistent or recurrent infections due to bacterial, viral, and fungal pathogens. Opportunistic infections (*Pneumocystis jiroveci* pneumonia, *Cytomegalovirus* infection) are common and are a frequent cause of interstitial pneumonia and chronic diarrhea, leading to failure to thrive (3–5). Candida infection is almost universal. A morbilliform exanthematous rash or exfoliative dermatitis may develop. This may reflect graft-versus-host disease (GvHD) due to transplacental passage of maternally-derived T lymphocytes or use of unirradiated blood products. In patients carrying hypomorphic mutations in SCID-causing genes, development of a skin rash may also reflect the presence of autologous and oligoclonal T cells that infiltrate and attack the skin. This condition is also known as Omenn syndrome, and is characterized by erythroderma, lymphadenopathy, liver and spleen enlargement, severe infections, eosinophilia, and increased serum IgE.

Other manifestations of GvHD in patients with SCID and maternal T cell engraftment are represented by hepatosplenomegaly, diarrhea and eosinophilia. On the other hand, it should be noted that maternal T cells are detected in as many as 50% of all cases of SCID (6), and

yet only a fraction of them show clinical manifestations of GvHD (7). Immunization with live vaccines (e.g. rotavirus, oral poliovirus vaccine, Bacillus Calmette–Guerin (BCG)) can have disastrous consequences in infants with SCID and should not be performed. The course of SCID, even without iatrogenic complications, is rapidly progressive, associated with wasting and runting, and, if untreated, fatal by 2 years of age.

Virtually all parameters of cellular and humoral immunity are abnormal. Patients usually have absent tonsils, small or absent lymph nodes, and absent thymic shadow. Lymphopenia is detected in approximately 70% of the patients. Enumeration of lymphocyte subsets may provide useful information on the underlying genetic defect (5). In vitro proliferative responses to mitogens are absent or markedly reduced. Immunoglobulins are usually absent, although passively transferred maternal immunoglobulin (Ig) of the IgG class can be present as long as the first 6 months of life. Failure of development of serum IgA and/or IgM (which do not cross the placenta) can often provide a sensitive marker for immunoglobulin abnormality early in life. Even in patients with a normal number of B lymphocytes, there is inability to mount specific antibody responses. Pathologically, there is a lack of normal lymph node architecture and a small, dysplastic or fetal thymus.

Recently, a newborn screening test for SCID has become available. The test is based on polymerase chain reaction (PCR)-mediated amplification of T cell receptor excision circles (TRECs) from a dried blood spot collected at birth (8,9). TRECs are a by-product of T cell receptor

(TCR) gene rearrangement and persist in newly developed T lymphocytes that express the  $\alpha\beta$  form of the TCR (10), but are diluted off with subsequent proliferation of cells in the periphery. Therefore, enumeration of TREC levels provides important information on thymic function.

Recently, it has become clear that, as in most other inborn errors, there is significant clinical variation in phenotypic manifestations, including age at diagnosis, which may range from the classically described infantile onset to as late as early-adult onset (11). This variation is most likely due to different mutations in the responsible genes, leading to greater or lesser expression of the gene product. Autoimmunity and increased risk of malignancies are frequently observed in patients with delayed-onset of the disease.

Treatment of SCID is based on hematopoietic stem cell transplantation (HSCT). Optimal results (>90% survival) are obtained if the transplant is performed from an HLA-matched sibling (12,13). Alternatively, HSCT can be performed from a matched unrelated donor or from a haploidentical parent; in the latter case, mature T lymphocytes must be removed from the graft to avoid GvHD. Excellent results (>95% survival) have been reported if HSCT for SCID is performed at <3.5 months of life (13), emphasizing the significance of newborn screening for SCID. Regular administration of Igs, use of prophylactic antibiotics and contact precautions should be used while waiting for HSCT. Infections should be sought and treated aggressively. When indicated, transfusion of blood products should be irradiated to prevent transfusional GvHD, and should be from CMV-negative donors to avoid the risk of viral transmission.

Alternative forms of treatment include enzyme replacement therapy and gene therapy, which have been successfully used in some forms of SCID, as described below.

**80.2.1.2 Pathophysiology of SCID.** SCID includes a heterogeneous group of genetically determined conditions that result in severe and early blocks in the development of T lymphocytes; some of these forms also affect differentiation of B and NK lymphocytes. These blocks correspond to critical steps in T cell development that mark fundamental mechanisms in T cell biology.

Hematopoietic stem cells (HSCs) give rise to common lymphoid progenitors (CLPs), from which all major lymphocyte subsets (T, B, NK cells) derive. Upon seeding to the thymus, CLP receive instructive signals through Notch that promote commitment to the T cell lineage. These signals require the presence of thymic stromal cells. Furthermore, early thymic progenitors undergo significant expansion in response to cytokines, in particular interleukin-7 (IL-7).

Genetic defects that compromise survival of lymphoid progenitors or their ability to respond to IL-7 are responsible for some forms of SCID. Rearrangement of the TCR loci is a key step in T cell development, and is accomplished through the process of V(D)J recombination, whereby Variable (V), Diversity (D) and Joining (J) elements of the TCR are brought together. V(D)J recombination is initiated

by the recombinase activating gene (RAG) proteins RAG1 and RAG2, that recognize recombination signal sequences (RSS) that flank V, D, and J elements. The RAG proteins introduce double strand breaks in the DNA; this is followed by formation of a complex that brings together V, D and J elements. Next, proteins of the non-homologous end joining (NHEJ) pathway mediate repair of the double strand DNA breaks, resulting in sealing of the coding ends, and deletion of the intervening DNA sequence that is circularized as TREC. As a result of this process, expression of a pre-TCR (composed of TCR $\beta$  chain and a pre-T $\alpha$  chain) is allowed on the cell surface, in combination with CD3 chains, thereby permitting delivery of signals that promote progression to later stages in T cell development. Defects of the RAG genes, of genes involved in NHEJ and of CD3 chains all result in SCID by arresting T cell development at an early stage. In contrast, genetic defects that affect late steps of T cell development and/or that interfere with the function of T cells without preventing generation of mature T lymphocytes do not cause SCID, but rather are classified as “combined immunodeficiencies.”

## 80.2.2 X-linked SCID

The most common form of SCID affects males and is inherited as an X-linked recessive disorder. Typically, these patients have low numbers of T and NK cells, and a normal number of B lymphocytes (T<sup>-</sup> B<sup>+</sup> NK<sup>-</sup> SCID). In spite of the presence of B lymphocytes, they have low immunoglobulins and fail to produce specific antibodies. Female carriers are clinically unaffected, but show skewed X-chromosome inactivation in T cells and NK lymphocytes (14). Furthermore, unswitched (IgM<sup>+</sup>) B lymphocytes from female carriers display a random pattern of X-chromosome inactivation, whereas switched (IgM<sup>-</sup>) B cells display skewed X-inactivation (15). Altogether, these data strongly indicated that T and NK lymphocyte precursors that express the normal copy of the gene have a strong selective advantage and that a similar advantage is also observed in the B cell compartment at later stages of maturation.

The gene responsible for X-linked SCID was mapped to Xq13 in 1987 (16). In 1992, the *IL2RG* gene, encoding for the  $\gamma$  chain of the interleukin-2 receptor (IL-2R $\gamma$ ) was cloned (17); one year later it was mapped to Xq13 and mutations of this gene were identified in patients with X-linked SCID (18,19). IL-2 is a potent stimulator of immune responses, and it binds to a heterotrimeric receptor composed of  $\alpha$ ,  $\beta$ , and  $\gamma$  chains. Although it was originally thought that X-linked SCID was caused by mutations of IL-2R $\gamma$  that prevented IL-2 signaling, it was soon discovered that the IL-2R  $\gamma$  chain is also a component of other interleukin receptors, including IL-4R, IL-7R, IL-9R, IL-15R and IL-21R (18,20–24). For this reason, the IL-2R $\gamma$  chain was renamed as common  $\gamma$  chain, or  $\gamma_c$ . Studies in mice have shown that IL-7 plays a critical role in T cell development (25,26), whereas IL-15 is essential to promote differentiation of NK lymphocytes

(27,28). Thus, the T<sup>-</sup> B<sup>+</sup> NK<sup>-</sup> phenotype of patients with X-linked SCID results primarily by the lack of signaling through IL-7R and IL-15R.

A variety of mutations in the *IL2RG* gene have been identified in patients with X-linked SCID (29). Phenotypic variability has been reported, including milder cases (30), normal thymic morphology (31), residual development of T and/or NK lymphocytes (29,31–33), total absence of B cells (34), and presentation resembling hemophagocytic lymphohistiocytosis (35). The development of autologous T lymphocytes has also been observed in a patient with X-SCID after spontaneous somatic gene reversion. The reversion event occurred in a lymphoid progenitor, rather than a stem cell, and the patient, therefore, displayed a partial reconstitution of the T-cell compartment that lasted for only a few years (36).

Approximately one third of the cases of X-linked SCID represent new mutations; this is consistent with what observed in other X-linked disorders. Identification of the disease-causing mutation in a patient allows prenatal diagnosis and carrier detection (37). In some cases, maternal germ line mosaicism has been demonstrated, emphasizing the need to search for mutations in the offspring, even when the mother of an affected child is not identified as a carrier (38). HSCT is the therapy of choice, as it is for all forms of SCID. Successful in utero HSCT has been reported in a few cases (39,40), however this treatment carries the avoidable risk of GvHD. Reconstitution of T cell immunity is typically achieved in infants with X-linked SCID and persists long-term, even after unconditioned T-cell depleted haploidentical HSCT (41). Reconstitution of B cell function is more problematic (42), unless donor B cell engraftment is achieved. This defect reflects failure of autologous, genetically-defective B lymphocytes, to respond to IL-21 and undergo plasma cell differentiation (43). Moreover, initial reconstitution of NK cell count after HSCT for X-linked SCID is often followed by progressive decline of these cells. Whether NK cell deficiency contributes to the higher risk of warts due to papillomavirus infection that has been reported after HSCT for X-linked SCID remains to be established (44). Gene therapy has been successfully used in infants with X-linked SCID (45–47); however, five of 20 infants with X-linked SCID treated by gene therapy have developed leukemic proliferation. This reflected insertional mutagenesis, with integration of the transgene near oncogenes (especially LMO-2), thereby inducing their transcriptional activation (48,49).

### 80.2.3 Autosomal Recessive SCID

**80.2.3.1 Adenosine Deaminase Deficiency.** In 1972, Giblett et al. (50) described two children who were presented with autosomal recessive combined immunodeficiency with both lacking the enzyme adenosine deaminase (ADA). This represented the first molecular defect defined in immunodeficiency disorders (51).

ADA deficiency is the second most common form of SCID, accounting for 20–30% of autosomal recessive SCID or 10–20% of all SCID (52). Most patients present with a syndrome indistinguishable from classic SCID. They are considered to have absence of both T and B cells but may initially retain B cells. The most severely affected ADA SCID cases can present with prominence of the costochondral junctions on physical examination, and cupping and flaring on radiography. The spectrum of ADA deficiency is also known to encompass a later onset, milder immunodeficiency disorder with persistence of some function and longer life span, to a syndrome of late-adult-onset disease. These later onset cases often initially present with recurrent sinopulmonary infections and may develop autoimmune phenomena, hyper-IgE, and asthma. However, in retrospect, lymphopenia was present from infancy. The rare adult-onset cases have been marked by warts and herpetic manifestations.

A variety of mutations have been identified in patients with ADA deficiency (51,53–56), and there is relatively good correlation between mutation, biochemical phenotype in vitro, biochemical phenotype in vivo (metabolites and enzyme activity), and clinical picture (57,58). In particular, hypomorphic mutations (including some splice site mutations) are often responsible of milder and delayed presentations. A number of children have also been found who lack ADA in red blood cells, but show sufficient residual activity in their lymphocytes to sustain normal immune function (55,59,60). Somatic mosaicism has been reported as a modulator of phenotype, resulting in milder disease (61–63). These observations indicate that the normal cells have a selective advantage, an important point with respect to prognosis for gene therapy.

Prenatal diagnosis by amniocentesis or chorionic villus sampling, as well as carrier detection, can be achieved by enzymatic or, when the mutation is known, by molecular methods (64).

Children with ADA deficiency show accumulation of adenosine and deoxyadenosine (the substrates of the deficient enzyme) and of their phosphorylated derivatives, in various tissues. These compounds, and especially, deoxy-ATP (dATP) are toxic to highly proliferating cells, such as T lymphocytes, and cause apoptosis and tissue damage. Moreover, there is also secondary inhibition of SAH hydrolase in ADA deficiency. Accumulation of toxic metabolites may also cause myeloid dysplasia and bone marrow hypocellularity (65), pulmonary alveolar proteinosis (66), hepatic failure (67), osteoblast insufficiency (68), and neurological abnormalities such as developmental delay, sensorineural hearing defects, motor dysfunction and behavioral problems (3,69,70). Accumulation of deoxyadenosine has been reported to result in increased chromosome breaks (51), and this may explain the occurrence of a rare tumor, multicentric dermatofibroma protuberans, in several ADA-deficient patients (71). Finally, abnormalities of

purine metabolism interfere with the function of regulatory T (Treg) cells, thereby contributing to autoimmune manifestations (72).

The therapy of choice for this disorder is HSCT. Best results, with complete immunological reconstitution, resolution of the metabolic abnormalities and 88% survival rate, are obtained if an HLA-matched family donor is available. In contrast, HSCT from haploidentical parents or unrelated donors is associated with higher morbidity and mortality (12,73,74). Neurological abnormalities, including sensorineural hearing abnormalities, mental retardation, motor dysfunction and behavioral problems, may persist even after successful HSCT (75).

A modified preparation of calf intestinal ADA coupled to polyethylene glycol (PEG-ADA) has been injected into over 180 ADA-deficient patients worldwide, resulting in sufficiently high circulating enzyme levels to more completely reduce toxic metabolites, restore immunofunction in most of the patients, and result in a 20-year survival rate of 78% (73,76).

Clinical response to enzyme replacement therapy (ERT) has been generally good, as measured by normal growth and development, ability to attend school, absence of opportunistic infections, and normal recovery from varicella and other viral diseases. Immune reconstitution, as measured by lymphocyte counts and in vitro function, has been less complete, and approximately 20% of patients, predominantly with infantile-onset disease, show slower and lesser improvement.

Half of the patients treated by ERT continue to require immunoglobulin replacement therapy (73). Moreover, gradual decline in lymphocyte counts, abnormalities of lymphocyte function and impaired production of T and B lymphocytes have been documented (77–79).

Virtually all patients on PEG-ADA develop antibody against the bovine ADA protein that in approximately 10% of cases is neutralizing, requiring increase of the dosage or discontinuation of ERT in an attempt to achieve desensitization (80,81). In some cases development of neutralizing antibodies was associated with irreversible loss of efficacy, severe autoimmune complications and death (73). Furthermore, deaths due to liver cancer, late infections and chronic lung disease have been documented in patients receiving ERT (73), and the very-long-term effect of this therapy remains to be determined (53).

The most recent approach to therapy is gene therapy. The early trials were based on introduction of the ADA complementary DNA into patient's T cells by means of a retroviral vector (82,83). Subsequent attempts targeted patient's cord blood stem cells (84), but use of ERT in these patients prevented clear assessment of efficacy of gene therapy. Following the demonstration that transduced T lymphocytes may have a selective advantage, multilineage engraftment of gene-corrected cells, metabolic correction, and improvement of immune function have been achieved in ADA-deficient patients who were

not treated with PEG-ADA and received genetically modified CD34<sup>+</sup> cells after preparative nonmyeloablative chemotherapy (85,86). In a series of 15 patients treated in Milan (Italy), all of which are currently alive, progressive immune reconstitution has been observed, with restoration of T cell immunity with no need for ERT in 13 of them. One patient continued to experience severe autoimmunity and required immune suppression (87). Encouraging results have been also obtained in smaller series in London and in the United States (74,88). Immune reconstitution after gene therapy for ADA deficiency is associated with shortening of telomeres in T lymphocytes, but not in bone marrow cells, indicating that homeostatic proliferation of T cells contributes to immune reconstitution and is not associated with stem cell senescence after gene therapy (88a). Contrary to the experience in X-linked SCID (see above), none of the patients treated by gene therapy for ADA deficiency worldwide have developed leukemic proliferation as a result of insertional mutagenesis.

**80.2.3.2 FOXN1 Deficiency.** The *FOXN1* genes encodes for a transcription factor that is required for the development and maturation of the thymus stroma and eccrine glands (89). Biallelic *FOXN1* mutations cause an autosomal recessive SCID phenotype in which lack of circulating T lymphocytes is associated with alopecia (90). There is complete blockage of CD4<sup>+</sup> cell development; a low number of oligoclonal CD8<sup>+</sup> T cells may be detected (91). This disease is the human equivalent of the *nude* phenotype in mice. In one affected fetus, immune deficiency was also associated with anencephaly and spina bifida (92). Treatment is based on thymus transplantation (93). However, HSCT from an HLA-identical sibling may also be used; in this case, reconstitution of CD4<sup>+</sup> cells is accomplished through homeostatic proliferation of T lymphocytes contained in the graft (94).

**80.2.3.3 Reticular Dysgenesis.** Reticular dysgenesis is a form of autosomal recessive SCID characterized by the association of severe lymphopenia, agranulocytosis and sensorineural deafness (95,96). The disease is due to defects in adenylate kinase 2 (AK2) that regulates the levels of ADP and AMP in mitochondria. Another isoform of adenylate kinase (AK1) is expressed in most tissues, but not in T lymphocytes and neutrophils. Consequently, AK2 deficiency causes disruption of mitochondrial membrane potential and apoptosis in these cells, resulting in SCID and agranulocytosis (97,98). AK2 is also expressed in the stria vascularis in the inner ear (98), and this may account for sensorineural deafness in patients with reticular dysgenesis. Patients with reticular dysgenesis are also prone to myelodysplasia (99). Treatment is based on HSCT, but results are less satisfactory than in other forms of SCID, and use of intensive conditioning is typically required to achieve engraftment (100).

**80.2.3.4 IL-7 Receptor Deficiency.** IL-7 serves an essential role in the proliferation and differentiation of early T cell progenitors. The IL-7R is a heterodimer



composed of the IL-7 $\alpha$  chain and the  $\gamma$ c. Mutations of the *IL7R* gene (encoding for IL-7 $\alpha$ ) result in SCID with absence of T cells, and normal development of B and NK lymphocytes (T<sup>-</sup> B<sup>+</sup> NK<sup>+</sup> SCID) (101,102). A variety of mutations have been reported in the *IL7R* gene (103). However, development of oligoclonal, activated, autologous T cells resulting in Omenn syndrome has been also reported (104). HSCT allows full immune reconstitution, even if donor cell engraftment is restricted to T lymphocytes, with persistence of autologous B and NK cells.

**80.2.3.5 JAK3 Deficiency.** Cytokine receptors that contain the common gamma chain ( $\gamma$ c) recruit tyrosine kinases to deliver intracellular signaling. In particular, in all these receptors, the  $\gamma$ c is physically and functionally coupled to the Janus associated kinase 3 (JAK3) (105), whose gene has been mapped to chromosome 19p12. JAK3 deficiency is inherited as an autosomal recessive trait and results in an SCID phenotype that is indistinguishable from that of X-linked SCID, with absence of T and NK lymphocytes (106,107), reflecting lack of IL-7 and IL-15-mediated signaling, respectively. Identification of the mutation allows prenatal diagnosis (108). Phenotypic variability has been reported with development of autologous T cells (109), which in some cases partially retained JAK3 function, allowing survival into late adolescence or young adulthood (110). Successful immune reconstitution is typically achieved after HSCT (111), but warts may occur even long-term after transplantation (44). Furthermore, insufficient humoral immune reconstitution has been frequently observed in patients who attain donor T cell engraftment, but autologous B cell reconstitution, after unconditioned haploidentical HSCT (43).

**80.2.3.6 RAG-1 and RAG-2 Deficiency.** RAG deficiency represents an important cause of autosomal recessive SCID. Null mutations in the *RAG1* and *RAG2* genes impede V(D)J recombination, and hence result in complete lack of circulating T and B lymphocytes, with normal number of NK cells (112). Hypomorphic mutations in the same genes may also cause distinct phenotypes, including Omenn syndrome (113,114) and leaky SCID (with the presence of autologous T lymphocytes, but without the distinctive phenotypic features of Omenn syndrome). Even when present, autologous T lymphocytes are oligoclonal, and fail to respond normally to antigenic stimulation. More recently, it has been demonstrated that hypomorphic RAG mutations may cause a milder clinical phenotype, with delayed-onset infections, autoimmunity and granuloma formation (11). In one patient, RAG mutations were associated with idiopathic CD4 lymphopenia and preserved diversity of T cell repertoire (115). Treatment is based on HSCT, however, immunosuppression is typically needed pre-transplant in patients with Omenn syndrome to control the autoimmune and inflammatory manifestations of the disease. Furthermore, use of

chemotherapy is often required in Omenn syndrome to facilitate engraftment.

**80.2.3.7 Artemis Deficiency.** Artemis participates at NHEJ pathway of DNA double strand break repair, by opening the hairpins that seal the coding ends at V, D, and J elements that have been targeted by the RAG proteins. As the other components of the NHEJ pathway, Artemis plays a role in DNA repair that goes beyond V(D)J recombination. Accordingly, the clinical phenotype of Artemis deficiency is characterized by T<sup>-</sup> B<sup>-</sup> NK<sup>+</sup> SCID associated with several non-immunological problems, such as oral and genital ulcers, teeth abnormalities, dystrophic features of the gut mucosa (116,117). Increased cellular radiosensitivity in patients with Artemis deficiency reflects the important role of the protein in DNA repair (116). A partial form of the disease, with residual expression and function of the protein, is characterized by reduced number of circulating T and B lymphocytes, and unincreased risk of lymphoma (118).

The Artemis protein is encoded by the *DCLRE1C* gene, located on chromosome 10p (116,119). Artemis deficiency is inherited as an autosomal recessive trait and is particularly frequent among Navajo, Apache, and other Athabascan Indians (120). Treatment is based on HSCT, however, a high rate of complications (growth failure, diarrhea due to gut mucosa dystrophy) has been reported (121).

**80.2.3.8 Other Forms of T<sup>-</sup> B<sup>-</sup> NK<sup>+</sup> SCID Associated with Increased Cellular Radiosensitivity.** Autosomal recessive SCID associated with radiation sensitivity may also be due to defects of other genes involved in the NHEJ pathway. DNA ligase IV deficiency may present with a variable phenotype, characterized by microcephaly, increased sensitivity to radiations and radiomimetic agents, increased risk of bone marrow failure and of leukemia, and a variable degree of immunodeficiency (122). In some cases, the immunodeficiency may be so severe to cause a T<sup>-</sup> B<sup>-</sup> SCID phenotype (123–125).

Deficiency of Cernunnos (also known as XLF), another component of the NHEJ pathway, causes immunodeficiency with microcephaly and increased risk of bone marrow failure and myelodysplasia (126,127). The immunological phenotype is often characterized by the residual presence of T and B lymphocytes. Cernunnos is encoded by the *NHEJ1* gene.

One patient has been reported with autosomal recessive T<sup>-</sup> B<sup>-</sup> NK<sup>+</sup> SCID due to deficiency of the DNA protein kinase catalytic subunit (DNA-PKcs), encoded by the *PRKDC* gene (128).

All these forms of radiation-sensitive SCID can be successfully treated with HSCT; however, attention must be paid to reducing the risk of toxicity related to the use of chemotherapeutic agents during conditioning.

**80.2.3.9 Defects of CD3 Chains.** During T cell development, the pre-TCR and the TCR are expressed on the cell surface in association with the CD3 $\gamma$ ,  $\delta$ ,  $\epsilon$  and  $\zeta$  chains. Mutations of the *CD3G* gene (encoding for

the CD3 $\gamma$  chain) result in a variable degree of immunodeficiency with autoimmunity, but do not cause SCID (129). In contrast, null mutations in the *CD3D*, *CD3E* and *CD3Z* genes (that encode for CD3 $\delta$ , CD3 $\epsilon$  and CD3 $\zeta$  chains, respectively) impede signaling and cause autosomal recessive T<sup>-</sup> B<sup>+</sup> NK<sup>+</sup> SCID (130–134). Interestingly, somatic mutations of the *CD3Z* gene have been associated with partial postnatal development of T lymphocytes (133).

**80.2.3.10 T Cell Receptor Alpha Constant (TRAC) Chain Deficiency.** A homozygous nucleotide substitution affecting splicing of the TRAC transcript was identified in two affected individuals from apparently unrelated families with autosomal recessive immunodeficiency characterized by increased susceptibility to infections, autoimmunity (vitiligo, autoimmune hemolytic anemia) and allergy (eczema, eosinophilia) (135). The mutation was predicted to result in a TCR $\alpha$  chain protein lacking the transmembrane and cytoplasmic domains, which are essential for surface expression of the TCR $\alpha\beta$  complex. Consistent with this, circulating T lymphocytes expressed TCR $\gamma\delta$  or had an aberrant phenotype, with low expression of CD3 molecules and trace amounts of TCR $\alpha\beta$ . Proliferative responses to mitogens were variably reduced. In contrast, immunoglobulin levels were normal, and the patients were able to mount antibody responses to vaccine antigens. More recently, however, biallelic LCK mutation has been reported in a child with recurrent infections and autoimmunity. Expression of CD4 and CD8 protein on the surface of CD3<sup>+</sup> cells was markedly reduced, and *in vitro* T cell proliferation in response to CD3 stimulation was severely compromised (139a).

**80.2.3.11 CD45 Deficiency.** Deficiency of the CD45 protein tyrosine phosphatase causes impaired intracellular signaling in developing T lymphocytes. The disease is inherited as an autosomal recessive trait and is a rare cause of SCID (136,137). One patient was a compound heterozygote for a deletion and a point mutation at a splice site, while another was homozygous for a six-base pair deletion.

**80.2.3.12 p56/lck Deficiency.** One infantile-onset patient with SCID was found to have diminished expression of the protein tyrosine kinase p56/lck, possibly associated with a splicing abnormality in the gene (138). An adult with CD4 lymphopenia has been described with diminished expression of this protein, but no genetic abnormality has been found (139).

**80.2.3.13 Coronin-1A Deficiency.** Coronin-1A is a negative regulator of the Arp2/3 complex and of actin cytoskeleton reorganization. A biallelic dinucleotide deletion in the *CORO1A* gene has been identified in a patient with an atypical T<sup>-</sup> B<sup>+</sup> NK<sup>+</sup> SCID phenotype (140). *In vitro* proliferative responses to mitogens were reduced, but not absent, and the thymic shadow was visible at chest X-ray. The clinical history was characterized by recurrent infections, thrush, vaccine-induced varicella, and diarrhea due to rotavirus infection. In addition, there

was delay in language and motor development. The child survived with supportive medication until 4 years of age, when she received unrelated cord blood transplantation leading to immune reconstitution (141). The mechanism underlying the SCID phenotype is unclear. Several mouse models of Coronin-1A deficiency exist, which interfere with survival and/or egress of thymocytes.

## 80.2.4 Other Combined Immunodeficiency Diseases

Several genetic defects of T cell development are known, which allow for residual T cell development and function. The partial preservation of T lymphocyte development and function may account for delayed clinical onset and higher chance of survival beyond infancy even in the absence of HSCT. Nonetheless, clinical manifestations are often severe, and similar to those observed in patients with SCID, although autoimmunity is more frequent. HSCT is the treatment of choice, however, the presence of autologous T cells with residual function makes use of conditioning regimen necessary to achieve donor cell engraftment.

**80.2.4.1 Purine Nucleoside Phosphorylase Deficiency.** Genetic deficiency of purine nucleoside phosphorylase (PNP) results in a defect predominantly of cellular immunity and was the second specific genetic molecular defect described that results in immunodeficiency. PNP reversibly catalyzes the phosphorylation of the purine nucleosides guanosine, inosine, deoxyguanosine, and deoxyinosine and is thus the next enzyme in the purine salvage pathway following ADA. Therefore, PNP deficiency causes accumulation of all four nucleoside substrates (inosine, guanosine, deoxyinosine, and deoxyguanosine). Deoxyguanosine triphosphate (deoxy-GTP) is the major phosphorylated metabolite accumulated by PNP-deficient children, and like deoxy-ATP, is also an allosteric inhibitor of ribonucleotide reductase and could account for the specific effects of PNP deficiency on thymocyte development (142). Because the block is near the terminal portion of the major common pathway to uric acid, patients with complete PNP deficiency have low serum uric acid and excrete diminished amounts of uric acid.

PNP deficiency is rarer than ADA deficiency and is also inherited as an autosomal recessive trait. The PNP gene has been mapped to chromosome 14q13.1 (143). Missense mutations account for the majority of the mutations reported, however, nonsense mutations and splice site mutations have been also described (144).

The clinical onset of the disease may vary from 6 months to over 6 years of age, and is marked by severe infections, often sustained by opportunistic pathogens. Autoimmunity (especially autoimmune hemolytic anemia and other cytopenias) and progressive neurological abnormalities (including spastic tetraplegia, ataxia, and multifocal leukoencephalopathy) are very common (145).

Bone marrow dysplasia has been also observed (146). It is apparent that all the patients have eventually had severe T-cell dysfunction, as measured by marked reduction of T-cell numbers, reduced response to mitogens and allogeneic cells, and severe recurrent fungal and viral infections. In many children, preservation of humoral immunity has been demonstrated, as measured by normal numbers of B cells in the blood and of plasma cells in lymphoid tissue, normal to elevated immunoglobulin concentration in vivo, and normal antibody production.

HSCT is the therapy of choice, but persistence and progression of neurological defects may be observed even in patients who attain immune reconstitution.

**80.2.4.2 ZAP-70 Kinase Deficiency.** The Zeta Chain-Associated Protein of 70 kDa (ZAP-70) is a tyrosine kinase that phosphorylates the CD3 chains and participates at TCR-mediated signaling (147). Patients with Zap-70 deficiency have a virtual lack of CD8<sup>+</sup> lymphocytes; the number of CD4<sup>+</sup> cells is preserved, but they are unable to proliferate in response to mitogens and antigens (148–151).

They proliferate in response to stimulation with calcium ionophores and phorbol esters, which bypass the membrane-proximal steps in T cell activation. Inability of the T cells to respond to antigens in patients with ZAP-70 deficiency causes increased susceptibility to severe infections, resulting in a clinical phenotype that is indistinguishable from that of SCID. Heterogeneity of the clinical phenotype has been observed, with some patients showing delayed clinical onset and others presenting with features of atopic dermatitis, eosinophilia and increased serum IgE (152,153).

## 80.2.5 Major Histocompatibility Complex Class II Deficiency

The positive selection of CD4<sup>+</sup> thymocytes requires the interaction of thymocytes expressing a functional TCR with thymic epithelial cells (and dendritic cells) expressing self-antigens bound to HLA class II molecules, respectively.

Patients with MHC-II deficiency lack expression of HLA class II (D, DR, DQ, and DP) antigens. This group of patients is genetically heterogeneous, as evidenced by the existence of at least four complementation groups, two of which account for the majority of patients (154).

The genetic defects accounting for MHC-II deficiency do not involve the HLA II genes, but rather a set of genes that encode for transcriptional activators of HLA class II genes. In particular, MHC-II deficiency may be due to mutations of MHC class II transactivator *CIITA* (155), or of *RFX5* (156–158), *RFXAP* (159), and *RFXANK* (160) genes, which encode for the subunits of RFX that binds to the HLA promoter. The disease is more common in northern Africa; it is inherited as an autosomal recessive trait and is characterized by a marked reduction of the number of circulating CD4<sup>+</sup> cells. Patients

lack antigen-specific T-cell proliferative responses and show impaired specific antibody production; however, the T cells respond to polyclonal mitogens, such as PHA (161). There is increased risk of infections, including *P. jiroveci* pneumonia and severe bacterial and viral diseases. Patients are also unusually susceptible to *Cryptosporidium* disease, leading to protracted diarrhea and sclerosing cholangitis. Mutations in *CIITA* and in *RFXANK* have been described in cases of milder immunodeficiency (162,163). In most cases, patients die early in childhood, however, survival into adolescence and young adulthood has been also reported, especially if patients are maintained on immunoglobulin replacement therapy and antibiotic prophylaxis (164). Treatment with HSCT may be effective, but is often problematic, because it does not correct the defective expression of MHC-II antigens on thymic epithelial cells (164,165).

**80.2.5.1 Defective Expression of MHC Class I Antigens.** Lack of expression of MHC class I antigens impairs positive selection of CD8<sup>+</sup> T lymphocytes in the thymus. The disease is inherited as an autosomal recessive trait. It is genetically heterogeneous, and may be caused by mutations in the genes that encode for TAP1, TAP2 or Tapasin (166–168). TAP1 and TAP2 are subunits of a peptide transporter system that carries antigenic peptides to the lumen of the endoplasmic reticulum, where they associate with and stabilize class I MHC molecules complexed to  $\beta_2$ -microglobulin prior to transport to the lymphocyte surface. Tapasin links TAP to the HLA class I heavy chain.

The clinical phenotype of MHC class I deficiency is variable, but is often characterized by chronic inflammatory lung disease leading to bronchiectasis, nasal polyps, and vasculitic skin manifestations that may evolve into necrotizing granulomas, especially on the legs (169). The reasons underlying heterogeneity of the severity of the clinical phenotype are unclear, but are unlikely to depend on the type of the mutation, since variability of phenotype has also been reported in patients sharing the same mutation.

As a result of defective expression of HLA class I molecules, the number of CD8<sup>+</sup> lymphocytes is significantly reduced. However, some CD8<sup>+</sup> cells may develop; in particular, development of CD8<sup>+</sup> lymphocytes expressing the TCR $\gamma\delta$  form of the T cell receptor is not blocked. The cytolytic activity of NK cells is also maintained, and this may explain why patients with MHC class I deficiency are not uniquely susceptible to viral infections. On the other hand, the impaired expression of HLA class I antigens would not permit engagement of killer inhibitory receptors (KIRs) regulating the function of NK cells. Hence, NK cells from the patients could mount cytotoxic responses to autologous cells, contributing to the inflammatory and destructive lesions observed in the lungs and the skin. Increased surface expression of KIRs and of CEACAM1 in patients with TAP and Tapasin deficiency reduces this risk (170).



**80.2.5.2 CD8  $\alpha$  Chain Deficiency.** Homozygosity for a Gly111Ser missense mutation in the  $\alpha$  chain of CD8 was found to cause complete deficiency of CD8<sup>+</sup> lymphocytes (171). Variable severity of the clinical phenotype has been reported: one patient died of pulmonary complications after recurrent bacteria infections (171), whereas another patient has maintained good lung function, in spite of frequent infections (172).

**80.2.5.3 MST1/STK4 Deficiency.** The MST1 kinase (also known as STK4) is the human ortholog of the Hippo protein, a key component of a novel pathway that controls cell growth and apoptosis in *Drosophila*. Mutations of MST1 in humans cause an autosomal recessive form of combined immunodeficiency with marked and progressive loss of naïve T cells, recurrent bacterial and viral infections and autoimmune manifestations (173,174). A modest degree of intermittent neutropenia and heart defects have been also observed. Lymphocytes and neutrophils show increased loss of mitochondrial membrane potential, and MST1-deficient T cells show reduced expression of the transcription factor FOXO1, as well as of IL7R and BCL2. Altogether, these abnormalities cause reduced survival of naïve cells and neutrophils.

**80.2.5.4 DOCK8 Deficiency.** The dedicator of cytokinesis 8 (DOCK8) protein is a member of the DOCK family of intracellular signaling proteins that serve as guanine exchange factors for the Rho/Rac family GTPases. DOCK8 plays a critical role in cell adhesion, migration and signaling. Individuals with biallelic inactivating DOCK8 mutations present with signs of combined immunodeficiency: recurrent sinopulmonary infections, severe cutaneous viral infections due to HPV, HSV and VZV, and molluscum contagiosum. Furthermore, they show severe atopic dermatitis and are at increased risk for tumors. T and B cell lymphopenia, with marked reduction of naïve cells, defective in vitro lymphocyte proliferation (especially of CD8<sup>+</sup> cells), poor antibody production, low levels of IgM and markedly increased levels of IgE are immunological hallmarks of the disease (175,176). Many patients carry large intragenic deletions (175,176). DOCK8 deficiency affects generation and persistence of memory T cells (177) and impairs migration of dendritic cells during immune responses (178). Furthermore, studies in mice suggest that DOCK8 deficiency may also cripple germinal center formation and generation of long-lived plasma cells (179). Loss of naïve T cells is associated with markedly reduced levels of TRECs in young infants, suggesting that the disease might also be identified through newborn screening for SCID (180). In most patients, the clinical course is dismal, however, immune reconstitution can be achieved by HSCT (181,182).

**80.2.5.5 Calcium Flux Defects.** TCR cross-linking results in downstream signaling events leading to activation of phospholipase C $\gamma$ , generation of inositol 1,4,5-triphosphate and release of endoplasmic Ca<sup>2+</sup> stores.

This is followed by influx of extracellular Ca<sup>2+</sup> through calcium-release activated calcium (CRAC) channel.

Autosomal recessive mutations in the *ORAI1* gene were identified in a family with combined immunodeficiency associated with severe impairment of calcium influx and of NFAT-dependent gene activation (183–185). The ORAI1 protein was then found to be an essential component of the CRAC channel (186). The stromal interacting molecule 1 (STIM1) is located in the endoplasmic reticulum and senses release of Ca<sup>2+</sup> from endoplasmic stores. Through its C-terminal domain, STIM1 interacts with and activates ORAI1, thereby promoting opening of CRAC channels. Following the discovery of *ORAI1* mutations, genetic defects of *STIM1* were also identified in patients with combined immunodeficiency and calcium flux defects (187).

Deficiencies of ORAI1 and of STIM1 in humans are associated with a unique clinical phenotype that is characterized by immunodeficiency, nonprogressive muscular hypotonia and anhidrotic ectodermal dysplasia (with a defect in dental enamel calcification and inability to sweat), as well as, in the case of STIM1 deficiency, autoimmunity and lymphoproliferative disease (184,187–189). In one case, STIM1 deficiency was associated with early-onset and fatal Kaposi syndrome (190). Patients with ORAI1 or STIM1 deficiency have a normal number of circulating T, B and NK lymphocytes; however, there is an increased proportion of T cells expressing activation markers. Furthermore, there is defective proliferation to mitogens and impaired induction of the nuclear factor of activated T cells (NF-AT) transcription factor upon T cell activation (183,184). The latter abnormality accounts for reduced cytokine secretion upon T cell activation. NK cytotoxic activity is also defective (191). Abnormalities of regulatory T cells have been identified in patients with STIM1 deficiency and may contribute to autoimmune manifestations (189).

**80.2.5.6 Defects of CD40 Ligand and CD40.** CD40 ligand (CD40LG) is transiently expressed as a homotrimeric complex on the surface of activated CD4<sup>+</sup> lymphocytes and of platelets. It interacts with CD40, that is constitutively expressed on the membrane of B lymphocytes and of dendritic cells. In addition, CD40 is also expressed on the surface of activated endothelial and epithelial cells. CD40LG/CD40 interaction during cross talk between T and B lymphocytes is one of the signals that promote B cell activation and proliferation. Along with appropriate cytokines, it induces class switch recombination and somatic hypermutation. On the other hand, interaction between T lymphocytes and dendritic cells via CD40LG/CD40 is important for T cell priming and generation of memory T cell responses (192). Accordingly, defects of CD40LG and CD40 result in a combined immunodeficiency, characterized by a normal number of T and B lymphocytes, but impaired antigen-specific T cell responses and an aberrant distribution of immunoglobulin isotypes, with predominance of serum



IgM and severely diminished levels of other Ig isotypes (hyper-IgM phenotype).

The disease is most often seen as an X-linked form, reflecting mutations of the *CD40LG* gene that has been mapped to Xq26 (193,194). The *CD40LG* gene is also known as tumor necrosis factor soluble factor (superfamily), member 5 (TNFSF5). A variety of mutations have been identified in the *CD40LG* gene. The clinical phenotype is marked by infections with bacteria, *Pneumocystis*, CMV, *Parvovirus*, *Cryptosporidium* and *Histoplasma* (195,196). Sclerosing cholangitis secondary to *Cryptosporidium* infection and neuroendocrine or hepatobiliary carcinomas have been reported in a significant number of patients (197,198). Treatment consists of gammaglobulin replacement, the aggressive use of antibiotics (195,196), G-CSF in cases of persistent neutropenia (199) and, in some patients, HSCT (200).

An autosomal recessive variant of the disease is also known, and is due to mutations of *CD40*. Only few cases have been reported (201,202). The clinical and immunological phenotype, and management of the disease are similar to what described above for *CD40LG* deficiency (203).

### 80.3 PRIMARY B-CELL DEFICIENCIES (HUMORAL IMMUNE DEFECTS)

Disorders of humoral immunity, or antibody deficiency disorders, can be of infantile or late onset, and may involve absence of all classes or only specific classes of immunoglobulins, with or without the absence of B cells of different maturation stages. The primary clinical manifestation in all of these disorders is recurrent infections with common pyogenic bacteria such as *Haemophilus influenzae* and *Streptococcus pneumoniae*. The response to most viral, fungal, or mycobacterial infections may be relatively normal, reflecting the retention of normal T-cell immunity. However, in patients with common variable immunodeficiency, diarrhea, chronic pulmonary disease, and autoimmune phenomena are more prominent features, and variable degrees of cellular immune defects may be present. Treatment of most humoral deficiencies consists of regular infusions of immunoglobulins via intravenous or subcutaneous route, which results in marked reduction of bacterial infections.

While the most common form of agammaglobulinemia is inherited as an X-linked recessive disorder (Bruton agammaglobulinemia), several rarer autosomal recessive forms, have also been identified, and in most cases their genetic basis has been defined. A recent comprehensive review on primary B cell immunodeficiencies was published by Conley et al. (204).

#### 80.3.1 X-Linked Agammaglobulinemia

Infantile X-linked (Bruton) agammaglobulinemia is the classical example of an isolated B-cell defect

(205), and was first described in 1952, when Colonel Bruton reported the case of an 8-year-old boy with recurrent pneumococcal infections, who lacked the  $\gamma$ -globulin fraction on serum electrophoresis (206). The clinical course of these patients is such that they are usually healthy during the first months of life, probably reflecting the presence of adequate amounts of placentally transferred maternal IgG. They then begin to suffer from multiple recurrent infections, usually with pyogenic organisms, while infections with most viruses and gram-negative bacteria are not strikingly increased. Upper and lower respiratory tract and skin infections are particularly common, however, invasive infections including sepsis and meningitis may also occur. Although the use of antibiotics has lessened mortality from acute infections, without immunoglobulin replacement therapy, chronic pulmonary disease, bronchiectasis, and respiratory failure will develop in these patients. These children are more susceptible to infection with enteroviruses, particularly ECHO and poliomyelitis viruses. Paralytic polio can follow administration of live polio vaccine, and a dermatomyositis-like syndrome apparently due to ECHO virus can evolve, even in patients treated with gamma globulin. Mycoplasma and ureaplasma may cause arthritis and meningitis. Intestinal *Giardia lamblia* infection may cause protracted diarrhea and malabsorption. Atypical clinical presentations, including adult onset (207,208), as well as phenotypic variability within families (209,210) have been reported.

Patients with XLA have a severe deficiency of serum IgG, IgM, and IgA, and of circulating B lymphocytes. In the bone marrow, there is a block at the pro-B to pre-B cell stage of differentiation, corresponding to the stage when signaling through the pre-B cell receptor (pre-BCR) would normally occur to promote progression in B cell development. The diagnosis is often suggested by the presence of hypoplastic tonsils and small lymph nodes. Lymph node biopsies show lack of B cell follicle, consistent with the severe reduction of B lymphocytes. T-cell immunity is normal in affected children, and childhood exanthems are handled normally.

Inheritance is that of a classical X-linked disorder. As in any lethal X-linked disorder, one third of patients should reflect new mutations, and therefore a family history may not be obtained.

In 1993, it was demonstrated that XLA is due to mutations of the gene encoding for an intracellular tyrosine kinase, named Bruton's tyrosine kinase, BTK (211,212). The *BTK* gene has been mapped at Xq21.3–22. The BTK protein is expressed throughout B cell differentiation, but is also expressed in platelets and myeloid cells. BTK interacts with various proteins, including phospholipase C- $\gamma$ 2 (PLC- $\gamma$ 2) and the adapter molecule BLNK. Activation of pre-B cells through the pre-BCR, and of B cells through the BCR results in tyrosine phosphorylation of BTK and calcium flux (213).

More than 600 mutations have been described in the *BTK* gene (214,215), most of which result in lack of BTK protein expression. Analysis of BTK expression in monocytes or platelets by flow cytometry may help identify patients with XLA (216) and can also be used to identify heterozygous carriers of XLA. However, detection of the BTK protein does not rule out the disease, since the protein may be expressed in patients with missense mutations. One mutation has resulted in selective anti-polysaccharide antibody deficiency (217).

Several patients have been reported with X-linked agammaglobulinemia associated with growth hormone deficiency, originally described by Fleisher et al. (218). At least some of these cases have mutations in *Btk* (219). Similarly, a significant proportion of male patients diagnosed as having common variable agammaglobulinemia (see later) with diminished B-cell numbers have been shown to have mutations in *Btk* and are therefore variants of X-linked agammaglobulinemia (220).

Analysis of BTK expression can also be used for carrier detection. In this case, two populations of monocytes and platelets (expressing and non-expressing BTK) can be detected, reflecting X-chromosome inactivation (221,222); however, this approach may fail to identify carriers of heterozygous missense mutations. Ultimately, diagnosis of XLA is based on demonstration of a *BTK* mutation, and this may also permit carrier detection and prenatal diagnosis.

Treatment of XLA is based on replacement therapy with intravenous immunoglobulins (IVIG) or subcutaneous immunoglobulins.

### 80.3.2 Autosomal Recessive Agammaglobulinemia

*BTK* gene mutations account for approximately 85% of all cases of agammaglobulinemia (204). The remaining 15% affect both males and females. Mutations in autosomal genes that encode for components of the pre-BCR or for proteins of the pre-BCR signaling pathway have been identified in these patients. In particular, six different genes have been identified, each of which, when mutated, causes autosomal recessive agammaglobulinemia:

- $\mu$  heavy chain (*IGHM*) (223), whose mutations are the second most common cause of congenital agammaglobulinemia (224)
- $\lambda 5/14.1$  (*IGLL1*) (225)
- Ig $\alpha$  (*CD79A*) (226,227)
- Ig $\beta$  (*CD79B*) (228)
- BLNK (B-cell linker protein) (229)
- p85 $\alpha$  subunit of PI-3 kinase (230)

In patients with autosomal recessive agammaglobulinemia, the arrest in B cell development is more severe and complete than in patients with XLA (204). However, the clinical manifestations and the treatment are similar.

Infantile agammaglobulinemia must be differentiated from transient hypogammaglobulinemia of infancy. Normally, after birth, the total IgG initially falls precipitously, then slowly begins to rise after a nadir at 5–6 months of age, reflecting the catabolism of maternal IgG counterbalanced by the slow increase in the child's own synthesis of IgG. Simultaneously, IgA and IgM concentrations begin to increase. In transient hypogammaglobulinemia, the expected rises can be delayed until 3 years of age. Sequential measurements demonstrating increases in IgA and IgM, as well as determination of the number of B cells, may help differentiate this temporary disorder from congenital agammaglobulinemia. When present, a family history of maternal male relatives or previous male sibs with persistent agammaglobulinemia, or demonstration of parental consanguinity may aid in the diagnosis, suggesting an X-linked or an autosomal recessive form of congenital agammaglobulinemia.

### 80.3.3 B Cell-intrinsic Defects of Class Switch Recombination

Maturation of antibody responses is marked by class switch recombination (CSR) and by somatic hypermutation (SHM). CSR is the process through which B lymphocytes express immunoglobulin isotypes other than IgM or IgD. In contrast, SHM introduces mutations in the variable regions of rearranged immunoglobulin genes, thereby resulting in improved affinity of antibodies for the respective antigens. Both CSR and SHM occur primarily during the germinal center reaction and require cell membrane as well as soluble signals. Interaction between CD40LG (expressed by activated CD4<sup>+</sup> cells) and CD40 (expressed by B lymphocytes) promotes activation of the NF- $\kappa$ B signaling pathway and induction of the expression of the *AICDA* gene, encoding for Activation-dependent cytidine deaminase (AID). This enzyme deaminates cytidine to uridine at the switch regions located 5' to the heavy chain constant region genes, as well as within the variable regions of immunoglobulin genes. Another enzyme (uracil N-glycosylase, UNG) removes the uridine residues, leaving abasic sites that are cleaved by a DNA endonuclease. DNA repair mechanisms then intervene bringing together two different switch regions, thus allowing CSR.

*AICDA* (231,232) and *UNG* (233) mutations in humans are responsible for autosomal recessive hyper-IgM syndrome, a condition characterized by the presence of increased (or normal) serum levels of IgM with a marked decrease in the other immunoglobulin isotypes. Clinically, patients show increased susceptibility to recurrent pyogenic infections, to autoimmune disease associated with IgM antibodies and to malignant lymphoproliferation of IgM-producing B cells (234). Enlargement of tonsils and lymph nodes is very common. B cells are normal in number but qualitatively abnormal

in that there is a lack of switched and of memory B lymphocytes. SHM is severely compromised in patients with AID deficiency and follows an abnormal pattern in patients with UNG deficiency.

Treatment is based on regular administration of immunoglobulins and prompt recognition and therapy of infections.

### 80.3.4 Common Variable Immunodeficiency

Shortly after Bruton's description of the first case of congenital agammaglobulinemia in 1952, other authors reported the occurrence of severe hypogammaglobulinemia in male and female adults (235,236). Variability of clinical and laboratory findings in such patients was appreciated (237), and for this reason the disease was named "common variable immune deficiency" (CVID). Although the onset may occur at any age, the majority of the patients have a disease onset in late adolescence or adulthood (238,239). Patients presenting at less than 4 years of age are more likely to suffer from some genetically determined immunodeficiency and are excluded from the definition of CVID in several classification. Respiratory tract infections, diarrhea and autoimmune disorders (especially cytopenias) are the most common findings of CVID at presentation. Other clinical manifestations that are common during the course of the disease include: malabsorption (often associated with giardiasis), intestinal nodular lymphoid hyperplasia, lung lymphocytic hyperplasia, thyroid abnormalities, and neoplasia (especially of the gut). Lymphadenopathy and splenomegaly are also common. In addition to low IgG serum levels, CVID patients also have reduced levels of IgA; IgM concentration may be low or within the normal range. B cells are present in most, but not all patients; the majority of them lack CD27<sup>+</sup> memory B cells (239a).

The genetic basis of CVID remains ill-defined. Many cases are sporadic presentations, however, families with an apparent autosomal dominant or, less commonly, an autosomal recessive inheritance have been described. Mutations in the *ICOS* gene, encoding for the inducible co-stimulator molecule expressed by activated T lymphocytes in the germinal center, have been reported in few cases of autosomal recessive CVID (240). Heterozygous and homozygous mutations affecting *TAC1*, which belongs to the TNF receptor family (also known as *TNFRSF13B*), have been reported in 10% of patients with CVID, but also in 1% of healthy controls (241–245). With few exceptions, these are considered disease-predisposing rather than disease-causing mutations. CVID patients with *TAC1* gene abnormalities have a higher risk to develop autoimmunity and splenomegaly. Biallelic mutations in the gene encoding another TNF-R family member, *BAFF-R*, have been described in two siblings from a single family (246). In a few CVID patients,

autosomal recessive mutations have been identified in the genes encoding CD19 (247,248) or another component of the CD19 complex, CD81 (249). Single cases of CD20 deficiency (250) and of CD21 deficiency (251) have been described. Finally, two siblings with hypogammaglobulinemia, poor antibody response to T-dependent antigens, T cell dysfunction, and EBV viremia have been recently reported to carry homozygous mutations in the CD27 gene (252). With the exception of *TAC1* variants, all other disease-causing mutations reported above represent rare causes of CVID. Therefore, the genetic basis of the disease remains undefined in the majority of CVID patients. Association with certain HLA haplotypes has been reported (253–256), but the precise gene involved remains unknown. Recent genome-wide association studies have suggested that some copy number variants are more common in CVID (257). Treatment is based on regular administration of immunoglobulins, antibiotic prophylaxis, and use of immunosuppressive or immunomodulatory drugs in patients with autoimmune manifestations. Surveillance against lymphoma and tumors of the gastrointestinal tract should be also performed.

### 80.3.5 Selective IgA Deficiency

Selective IgA deficiency is the most commonly observed immunodeficiency, with an incidence of 0.1%–0.2% in normal blood donors, although it is rare in Orientals. There is a 14-fold increase in the incidence of IgA deficiency among the first-degree relatives of these normal individuals (258,259). In most such individuals, this deficiency is not associated with any disease. A high incidence of IgA deficiency has also been reported in patients with recurrent infections, autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus, malabsorption syndrome (sometimes with gluten sensitivity), childhood asthma, and other atopic diseases. Normal blood donors are by definition healthy adults, and therefore individuals with an increased predisposition to infection beginning in childhood would not be included. Familial occurrence of IgA deficiency has been reported on numerous occasions, with patterns of inheritance consistent with both an autosomal dominant and autosomal recessive mode of inheritance. Association with MHC haplotypes has been reported (260–262). Discordance of IgA-deficient identical twins has been described, indicating a strong environmental component for the determination of serum IgA. In several cases, evolution of selective IgA deficiency toward CVID has been reported; furthermore, coexistence of CVID and IgA deficiency in the same family is also possible.

IgA deficiency is also found in one third to one half of individuals with a wide variety of chromosome 18 abnormalities and is typically present in patients with ataxia telangiectasia (see later). As a note of warning, IgA-deficient individuals often have antibodies to IgA

and can have a severe transfusion reaction when given normal whole blood, plasma, or preparation of immunoglobulins containing significant amounts of IgA.

### 80.3.6 Selective IgG Subclass Deficiency

Selective deficiency of specific IgG subclasses due to either regulatory or structural mutations could, in theory, lead to inability to cope with a limited spectrum of infectious agents. This hypothesis is based on the observation that certain antibody activities occur largely within specific subclasses of IgG. However, compensatory mechanisms often exist, such that IgG subclass deficiency per se may not represent a clinically significant immunodeficiency, unless impaired production of specific antibodies are demonstrated, in association with recurrent sinopulmonary infections. Deletions and duplications at the duplications at the Ig heavy chain locus on chromosome 14 have been reported in 5%–10% of Caucasian subjects, and may contribute to IgG subclass deficiency (263); however, in most cases, there are no major structural changes of the locus. Alternatively, the condition may reflect impaired regulation of the immune system, including primary imbalance of T-cell subsets (264). The subject has been reviewed (265,266).

## 80.4 IMMUNODEFICIENCIES WITH IMMUNE DYSREGULATION

So far, we have reviewed disorders in which defects in the development and/or function of T and/or B lymphocytes cause immunodeficiency with increased susceptibility to infections. In other conditions, however, abnormalities of lymphocyte function cause immune dysregulation, with increased risk to develop autoimmune diseases and/or inability to extinguish immune responses, leading to uncontrolled inflammation and autoimmunity.

### 80.4.1 Autoimmune Polyendocrinopathy–Candidiasis–Ectodermal Dystrophy

Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) syndrome represents the prototypic disorder of impairment in the mechanisms that govern T cell central tolerance. The disease is due to mutations in the gene *AIRE* (autoimmune regulator) (267,268) that encodes a transcription factor expressed by thymic medullary epithelial cells where it induces expression of tissue-specific antigens that are presented in association with MHC class II molecules to nascent T lymphocytes (269). High-affinity recognition of self-peptides leads to deletion of self-reactive T cells. APECED is inherited as an autosomal recessive trait and is characterized by autoantibodies to multiple endocrine organs, ectodermal dysplasia, susceptibility to candidiasis, an increased incidence of oral squamous cell carcinoma, and fulminant autoimmune hepatitis. It is

clinically heterogeneous, and there are numerous different mutations in the gene.

### 80.4.2 Immune Dysregulation–Polyendocrinopathy–Enteropathy–X-linked Syndrome

Treg lymphocytes play a critical role in the periphery by suppressing self-reactive T lymphocytes that have escaped central tolerance (270). Generation of Treg cells is under control of another transcription factor, FOXP3 that includes a forkhead domain. Mutation of this gene, which maps on the X-chromosome (Xp11.23) cause a severe X-linked syndrome combining autoimmunity with immune dysregulation (271–273). Clinically the syndrome, called immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX), is characterized by skin manifestations (up to generalized erythroderma), intractable enteropathy, autoimmunity (often including early-onset diabetes mellitus), other endocrine disorders, and cytopenias. Lymphoproliferation is also very common. The onset is typically in the first month of life, but delayed presentations have been also observed. A similar phenotype has been observed in the scurfy mouse (274), a mouse model with a spontaneous mutation in the ortholog gene. Patients with IPEX require immune suppression, but the only definitive treatment currently available is allogeneic HSCT (275).

### 80.4.3 Immune Dysregulation due to Defects of IL-2-mediated Signaling

Treg cells express the high-affinity IL-2 receptor, and signaling through IL-2 is important to maintain high levels of expression of FOXP3. Autosomal recessive mutations in the *CD25* gene, which encodes for the  $\alpha$  chain of the IL-2R, cause immune dysregulation associated with lymphoproliferation (276,277).

Interaction of IL-2 with its high-affinity receptor activates the JAK–STAT pathway. An important role in this process is played by the transcription factor STAT5B, which is also activated in response to other receptors, including the growth hormone receptor (GHR). Biallelic mutations of the *STAT5B* gene in humans cause an immunodeficiency characterized by susceptibility to infections, growth failure with growth hormone insensitivity, and autoimmunity (278,279).

### 80.4.4 Autosomal Lymphoproliferative Syndrome

Another syndrome with excessive lymphoproliferation is due to diminished apoptosis of lymphocytes and is associated with autoimmune phenomena (e.g. hemolytic anemia, thrombocytopenia), marked lymphadenopathy, and splenomegaly. The disease is characterized by an



expansion of CD3<sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup> double-negative (DN) T cells that express the  $\alpha\beta$  form of the T-cell receptor (280). Most patients are heterozygous for a mutation of apoptosis-inducing FAS (CD95, APO-1) (281,282), although homozygotes with a more severe disease have been reported. There is variable penetrance, partly dependent on the site of the mutation in the gene (283,284). Patients with FAS defects have a higher risk of developing lymphoma.

Other rare causes of ALPS are represented by mutations of FAS ligand (285,285a), FADD (286), caspase 8 (287) and caspase 10 (288), all of which are involved in the FAS-mediated signaling pathway leading to apoptosis.

The apoptosis of chronically activated T lymphocytes can also be induced through FAS-independent mechanism involving the release of cytochrome *c* and the activation of caspase 9. This “intrinsic” (or mitochondrial) pathway of apoptosis is elicited in response to cell damage and cytokine (e.g. IL-2) deprivation. Gain-of-function mutations of *N-RAS* may disrupt this pathway and cause ALPS (289). Somatic mutations of *K-RAS* may also cause ALPS (290,291), but this variant does not result in an increase in the number of DN T lymphocytes.

#### 80.4.5 Defects of Lymphocyte Cytotoxicity

Cytotoxic T lymphocytes and NK cells play an essential role in the defense against viruses, by promoting killing of virus-infected cells through two key mechanisms: FAS-mediated apoptosis and release of cytotoxic molecules such as granzyme through pores in the membrane that are generated by perforin. Defects in the cytolytic machinery of cytotoxic T cells and NK lymphocytes cause increased susceptibility to severe viral infections, especially to Epstein-Barr virus (EBV), but also to cytomegalovirus (CMV) and other viruses. Furthermore, persistence of the virus may promote continuous activation of the immune system, with release of inflammatory cytokines (especially IFN- $\gamma$ ) that cause tissue damage. Several genetic defects have been identified in humans that compromise lymphocyte-mediated cytotoxicity.

#### 80.4.6 Familial Hemophagocytic Lymphohistiocytosis

Familial hemophagocytic lymphohistiocytosis (FHL) includes several genetically determined disorders of lymphocyte cytotoxicity. In patients with FHL, the inability to clear viral infections causes persistent activation of CD8<sup>+</sup> and NK lymphocytes, and results in high levels of IFN- $\gamma$  and of pro-inflammatory cytokines (TNF $\alpha$ , IL-6) (292). Clinical features of FHL include episodes of high fever, pancytopenia, liver and spleen enlargement and neurological signs. At present, five

different forms of FHL have been identified, and the defective gene is known in four of them; in all cases, the disease is inherited as an autosomal recessive trait. Perforin deficiency (also known as FHL2) accounts for approximately 30% of all cases of FHL (293). It is caused by mutations of the *PRF1* gene, which prevent expression and/or correct assembly of perforin multimers that form pores through which cytolytic enzymes are released into target cells.

In other forms of FHL, the genetic defect interferes with the formation and intracellular trafficking of cytolytic granules. This is an ordered process through which cytoskeleton rearrangement guides transport of endosomal cytolytic granules to the lytic synapse that forms between cytotoxic lymphocytes (or NK cells) on one hand, and the target cell on the other. During this process, the small GTPase Rab27a promotes docking of the lytic granules, Munc 13-4 induces priming of the cytolytic granules, and syntaxin-11 and Munc 18-2 (also known as syntaxin-binding protein) promote the fusion of the lytic granules with the cell membrane. Mutations of the *UNC13D* gene, encoding for Munc13-4, cause FHL3 (294,295), the most common form of FHL (30–40% of all cases). FHL4 is caused by mutations of the *STX11* gene, encoding for syntaxin 11 (296). FHL5 has been recently shown to be caused by mutations of the *STXBP2* gene, encoding for the Munc18-2 protein. Both missense and nonsense mutations in this gene have been identified (297,298), and there is evidence of a genotype–phenotype correlation, with severe mutations being associated with early onset and more severe clinical course (297). Finally, mutations of the *ITK* gene, encoding for the IL-2-inducible tyrosine kinase that modulated the strength of signaling through the TCR, have been associated with increased risk of EBV-driven lymphoproliferative disease, Hodgkin lymphoma, hypogammaglobulinemia and hemophagocytic lymphohistiocytosis. The condition is inherited as an autosomal recessive trait and is also characterized by a reduced number of natural killer T (NKT) cells and of naïve CD4<sup>+</sup> lymphocytes (299).

FHL is fatal in the absence of treatment, which is based on the prompt recognition and therapy of underlying infections, use of potent immunosuppressive drugs and chemotherapy (300). The only curative approach is represented by HSCT, and excellent results have been obtained with use of reduced-intensity conditioning regimens (301).

In some other disorders, defective lymphocyte-mediated cytotoxicity is associated with extra-immune manifestations related to defective intracellular trafficking of other proteins. In particular, mutations of the *RAB27A* gene cause Griscelli syndrome type 2, an autosomal recessive disease in which features of FHL associate with partial albinism, with clumping of pigment in hairshafts (302).

Chediak-Higashi syndrome associates features of FHL with partial albinism, peripheral neuropathy and

the presence of giant lysosomes, which can be readily identified in leukocytes. The disease is due to mutations of the *CHS1* gene, also known as *LYST* (lysosomal trafficking regulator), which encodes for a protein involved in the sorting of proteins to secretory endosomes (303,304). This defect affects not only cytotoxic lymphocytes (accounting for FHL-like clinical features), but also melanocytes, which are unable to transfer melanin to keratinocytes and other epithelial cells, thus accounting for albinism. Neutropenia is often present and may contribute to increased susceptibility to bacterial infections.

Hermansky–Pudlak syndrome type 2 is an autosomal recessive disease caused by mutations of the *AP3B1* gene, which encodes for the  $\beta$  component of the AP-3 complex, involved in sorting of transmembrane proteins to secretory lysosomes (305). Clinical manifestations include partial albinism, nystagmus, prolonged bleeding, recurrent bacterial and viral infections, and increased risk of hemophagocytic lymphohistiocytosis (306). Pigmentary abnormalities, neutropenia, platelet dysfunction (with absence of dense granules and reduced platelet aggregation) and defective cytotoxic activity account for the clinical manifestations of the disease.

### 80.4.7 X-Linked Lymphoproliferative Syndrome

An X-linked recessive lymphoproliferative syndrome has been described in several kindreds (307). In most cases, development of lymphoproliferative disease reflects inability to contain infection with EBV. Two genetically distinct forms of the disease are known. Males with X-linked lymphoproliferative disease type 1 (XLP1) are uniquely susceptible to life-threatening complications of EBV infections, including fatal infectious mononucleosis, hemophagocytic lymphohistiocytosis, aplastic anemia, and EBV-related lymphoma. A proportion of the males who survive primary EBV infection, develop hypogammaglobulinemia (307) and may be misdiagnosed as having CVID (308). XLP1 is due to mutations of the *SH2D1A* gene that encodes for an SH2 domain-containing adapter molecule, also known as SAP (SLAM-associated protein) (309,310). Various molecules of the SLAM family are expressed on the surface of cytotoxic T lymphocytes and NK cells. Through SAP, they trigger activatory signaling pathways that ultimately lead to the killing of EBV-infected cells (311). In the absence of SAP, these activatory pathways are inhibited, and EBV infection remains uncontrolled. SAP is also important for the function of follicular helper T cells that promote maturation of antibody responses (312). Consistent with this, XLP1 males often develop hypogammaglobulinemia with lack of memory B lymphocytes. Finally, XLP1 is also associated with impaired development of NKT lymphocytes (313). The condition can be corrected by transplantation of bone marrow (314) and by cord blood stem cell infusion (315).

A minority of patients with XLP carry defects in another gene (*BIRC4*) that encodes for the X-linked inhibitor of apoptosis (316). Consistent with this, lymphocytes from patients with this disease (XLP2) show increased susceptibility to activation-induced apoptosis. As compared to XLP1, patients with XLP2 have a higher incidence of HLH (with or without EBV infection), but do not show increased risk of lymphoma. Hypogammaglobulinemia is often present (317,318).

## 80.5 IMMUNODEFICIENCY SYNDROMES

A number of congenital immunodeficiencies are also characterized by extra-immune clinical manifestations, reflecting a broader expression of the gene whose defects are responsible for the disease. Here, we will review those immunodeficiency syndromes that also involve defects of lymphoid development and/or function.

### 80.5.1 Immunodeficiency with Hyper-IgE (Job's Syndrome)

In 1972, Buckley described a novel immunodeficiency syndrome characterized by recurrent staphylococcal abscesses, markedly elevated serum IgE, coarse facies, and a history of pruritic dermatitis (319). The original patients were fair-skinned, red-headed girls with eczema and recurrent “cold” staphylococcal abscesses of the skin, subcutaneous tissue, lymph nodes, lung, liver, and abdominal cavity. The severe recurrent staphylococcal abscesses often begin in infancy and involve skin, lungs, joints, and other sites, with virtually universal development of pneumatocèles. The abscesses are tender and warm, although systemic toxicity is less than expected. Occurrence of boils in these patients has also led to use the eponym of Job's syndrome (320). Infections with other bacterial and fungal agents can occur. All patients at some time have pruritic dermatitis, but the distribution and characteristics of the lesion are different from classical atopic dermatitis. Eosinophilia has been a consistent finding. Abnormalities of teeth, bones, and connective tissue have been described (321). A neutrophil or monocyte chemotactic defect is not a necessary part of this syndrome and is an inconstant finding. Variable abnormalities in cellular immunity are seen, manifested usually as skin test anergy and diminished proliferation in vitro to antigens and allogeneic cells. Both males and females have been affected with equal frequency, and members of succeeding generations have also been affected, consistent with an autosomal dominant mode of inheritance. Sporadic presentations are also common. This multisystem disorder is due to heterozygous dominant-negative mutations of the gene encoding the transcription factor STAT3 (322,323). Most of the HIES-causing mutations of STAT3 are missense or in-frame deletions in the SH2 or DNA-binding domains. The mutations tested are intrinsically loss-of-function but exert inhibit STAT3

function in a dominant-negative manner in heterozygous cells (322). STAT3 is directly involved in signaling from a multitude of hematological and extra-hematological receptors, including those using the common  $\beta$  chain gp130. STAT3 is required for the development of IL-17 CD4<sup>+</sup> T cell-mediated responses, due to the requirement of STAT3 for cellular responses to IL-6, IL-21 and IL-23, which induce development of IL-17-producing T cells (Th17). The development of Th17 cells is thus profoundly impaired in patients with HIES (324–326). IL-17 is essential for the defense against *Candida*, and Th17 cytokines induce production of antimicrobial peptides by bronchial epithelial cells (327), thus explaining the increased occurrence of *Candida* and staphylococcal infections in patients with HIES. The molecular mechanisms underlying elevated IgE levels in patients with HIES remain unknown.

Autosomal recessive forms of HIES have been also described. DOCK8 deficiency is associated with severe abnormalities of lymphocyte function, resulting in a combined immunodeficiency (see above).

A patient with autosomal recessive HIES due to deficiency of TYK2 (a member of the JAK family of tyrosine kinases that activate STAT proteins) has been also described (328). The clinical and immunological phenotype was characterized by atopy, susceptibility to cutaneous staphylococcal diseases and high serum concentrations of IgE. This patient was also susceptible to intramacrophagic bacteria, such as BCG and *Salmonella* in particular, and viruses, which are not typically observed in patients with HIES due to *STAT3* mutations. Recently, another Turkish patient with TYK2 deficiency has been described, who suffered from disseminated BCG infection, neurobrucellosis, and cutaneous herpes zoster infection, in the absence of atopy, elevated IgE or staphylococcal infections (329). Thus, the clinical and immunological phenotype of TYK2 deficiency remain controversial and may not necessarily be part of the HIES spectrum.

### 80.5.2 DiGeorge Syndrome

DiGeorge syndrome is a congenital disorder in which there are abnormalities of structures derived from the third and fourth pharyngeal pouches, including the thymus and parathyroids. The absence of the parathyroid glands often results in neonatal tetany. Patients have characteristic facies with micrognathia, low-set malformed “pixie” ears, cleft palate, short philtrum of the lip, and antimongoloid slant of the eyes. There are often associated abnormalities of the aortic arch (commonly truncus arteriosus communis), and the cardiac manifestations may overshadow the other features of this disorder (330). Although the thymic shadow is absent radiographically, some ectopic thymic tissue may be identified at autopsy. This variability in the degree of thymic hypoplasia presumably explains the variability

in the extent of the T-cell defect in different patients. Patients are not usually profoundly lymphopenic, but cellular immune function is usually diminished, with reduced number of T cells and impaired response to mitogens or antigens. These patients classically have multiple *Candida* and viral infections, while antibody function is usually normal. B-cell percentages are elevated. Therapy is difficult to evaluate critically since, in some patients, immune function gradually improves, presumably reflecting growth of thymic remnants. However, approximately 1% of the patients with DiGeorge syndrome show extreme lymphopenia and present with SCID-like clinical features. For these patients with “complete DiGeorge syndrome,” treatment is based on thymus transplantation (331,332).

Many cases of DiGeorge syndrome show a deletion of 22q11.2 (333), either sporadic or as a result of an unbalanced translocation, often familial. It has become clear that DiGeorge syndrome is a developmental field defect associated with a contiguous gene deletion of variable size, sometimes visible by cytogenetic techniques but more generally detectable by fluorescent in situ hybridization using probes from 22q11.2 (334,335). Smaller deletions result in the velocardiofacial syndrome or only in the conotruncal defects of the heart. Other cases have a deletion of chromosome 10p14–p13 (336–338). Numerous genes have been isolated from the deleted region in chromosome 22, but none has been definitively shown to be responsible for the syndrome (339), although a possible candidate gene is *TBX1* since its deletion causes abnormalities in mice that resemble those in affected humans (340). The mechanisms of the various-sized deletions have been shown to be due to unequal crossing over at regions of low-copy repeats (341–343).

### 80.5.3 Wiskott–Aldrich Syndrome

The Wiskott–Aldrich syndrome is a rare X-linked recessive disorder characterized by thrombocytopenia, eczema, and recurrent infections, usually with polysaccharide-containing pyogenic bacteria, but also with other bacteria, viruses, and fungi. If untreated, the majority of patients die in childhood (344). The most common cause of death is infection, commonly of the respiratory system, followed by bleeding, most often into the central nervous system. Lymphoid malignancies occur frequently (over 12% of patients). Thrombocytopenia is usually observed at birth and is often exacerbated during periods of infection. Platelets are small in size, respond abnormally to aggregating agents and have a diminished half-life. Splenectomy usually results in increase in platelet number and size, a normal half-life of autologous platelets, and a normal aggregating response to epinephrine (345), but is associated with an increased risk of fatal bacterial infections. The eczema usually appears by 1 year of age and may be superinfected. The recurrent infections are associated with variable defects

in humoral and cellular immunity. Immunoglobulins are both catabolized and synthesized more rapidly than normal, and the most common resulting pattern of serum immunoglobulin is elevated serum IgA and IgE with low IgM. Serum isohemagglutinins are absent or very low. Patients are unable to mount an antibody response to polysaccharide antigens (e.g. pneumococcal vaccine or A and B blood groups) but can generate relatively normal antibody responses to protein antigens. B cells are normal in number, but *in vitro* synthesis of immunoglobulin is variably abnormal, depending on the stimulant utilized. Cellular immunity can also be abnormal. Patients are generally anergic to skin test antigens, and T lymphocytes do not proliferate normally *in vitro* in response to antigens or allogeneic cells. Autoimmune phenomena (e.g. hemolytic anemia) are common (346).

Wiskott–Aldrich syndrome has a crude incidence of approximately four per one million male births, however under-reporting is likely. The WAS gene has been mapped to Xp11.22–11.23 (347), and has then been cloned (348). The WAS protein (WASp) is involved in cytoskeleton reorganization, and mediated actin polymerization in response to activating signals (349). In particular, the WASp GTPase binding domain (GBD) interacts with Cdc42, thus linking cellular activation to WASp recruitment. Furthermore, the C-terminal domain of WASp binds to the Arp2/3 complex, thereby facilitating actin polymerization. WASp also interacts with tyrosine kinases involved in mediating growth factor signals (349,350). The N-terminal domain of WASp interacts with the WASp-interacting protein (WIP), and this interaction stabilizes WASp. A wide diversity of WAS mutations has been observed in affected patients, with some genotype/phenotype correlation (351). In particular, missense mutations in exons 1 and 2 of the WAS gene that affect interaction with WIP are associated with reduced, but detectable, levels of WASp and often result in a milder phenotype, with isolated thrombocytopenia (352). In some families, the thrombocytopenia may even be intermittent (353).

Patients with WAS should receive immunoglobulin replacement therapy and antimicrobial prophylaxis. Definitive treatment is based on HSCT, and improved outcome has been reported in recent years (354).

Activating mutations in the GBD domain of WASp lead to a very distinct phenotype, characterized by congenital X-linked neutropenia and myelodysplasia (355,356).

Mutations of the *WIPF1* gene, encoding for WIP, result in an immunodeficiency syndrome that resembles WAS, but is inherited as an autosomal recessive trait (356a). In this disease, lack of WIP results in impaired stability, and hence decreased levels, of WASp.

## 80.5.4 Immunodeficiencies with Associated Chromosome Breakage

**80.5.4.1 Ataxia Telangiectasia.** Ataxia telangiectasia (AT) is characterized clinically by the occurrence of

progressive cerebellar ataxia, ocular and cutaneous telangiectases, frequent and severe sinopulmonary infections, a very high incidence of neoplasia, and variable abnormalities of both cellular and humoral immunity (357). Although initial investigations indicated that this is a genetically and clinically heterogeneous group of disorders with between five and nine complementation groups (358), it is now clear that all patients with classical AT have mutations in the same gene (see later).

The disease is usually first recognized as the child attempts to walk. The ataxia and dysarthria are progressive, and additional neurologic abnormalities develop, including choreoathetosis, myoclonic jerks, nystagmus, and oculomotor apraxia. Increased infections become evident during the first year of life but are not usually prominent until 3–8 years of age. The telangiectases usually appear between 2 and 8 years of age. Progeric changes develop in the adult and include premature graying of the hair, early loss of subcutaneous tissue, sclerodermoid changes, vitiligo, and café au lait spots.

The most prominent and consistent immunologic abnormality is absent or deficient serum and secretory IgA and serum IgE and the presence of a low-molecular-weight IgM. The latter may result in factitiously high IgM measurements by radial immunodiffusion. Autoantibodies are common. Diminished *in vitro* cellular immune responses are also common, and the thymus often has a fetal-like histologic pattern. Endocrine abnormalities involving several organs are also frequent. Some patients have hyperinsulinism, insulin resistance, and hyperglycemia. Many patients show hypogonadism with absent or hypoplastic ovaries in females. Hepatic abnormalities also occur, and elevated  $\alpha$ -fetoprotein is common. Patients usually die before early adulthood as a result of the recurrent respiratory infections or a lymphoproliferative neoplasm.

AT is one of the chromosome instability syndromes (359), and patients' cells develop chromosome abnormalities at high frequency. The chromosome abnormalities typically involve the translocation of the long arm of chromosome 14 with the breakpoint at 14q11–12 and 14q32, as well as chromosome 7 (7p13 and 7q35). These positions correspond to the loci for immunoglobulin heavy chains and the various genes for the T-cell receptor. In addition to showing an increased rate of "spontaneous" chromosome rearrangements, cells from patients with AT are also more sensitive to ionizing radiation and radiomimetic chemicals. *In vivo*, patients react adversely to standard radiotherapy and may die in the course of treatment. The gene (*ATM*) has been cloned, and disease-causing mutations have been identified in patients with AT (360,361). The gene product is a large phosphoprotein that is a member of a family of genes involved in response to DNA-damaging agents and that shares a PI3-kinase domain (362) and exhibits kinase activity (363). It also plays a role in immunoglobulin class switch recombination (364).



AT has an incidence of approximately 25 per million and appears to be inherited as an autosomal recessive. The disorder occurs at higher frequency among Moroccan Jews. Obligate heterozygotes have been reported to be at increased risk for development of neoplasia (365,366). Detection of heterozygotes is now available by mutation analysis. Numerous different mutations of all types have been reported (367,368). Autoimmunity and oculocutaneous telangiectases have been reported in some heterozygotes.

**80.5.4.2 Nijmegen Breakage Syndrome.** The Nijmegen breakage syndrome (NBS) is an autosomal recessive disorder characterized by chromosomal instability, microcephaly, immunodeficiency, increased incidence of cancer and sensitivity to ionizing radiation. The patients show no neurologic abnormalities or telangiectasia. The disease was mapped to 8q21 (369), and then the gene was cloned by positional cloning (370) and by isolating a member (p95) of a DNA repair complex (hMre11/hRad50) mapping to the same region (371). The NBS gene encodes for a protein nibrin, and multiple mutations have been identified.

**80.5.4.3 Ligase 4 Deficiency.** DNA ligase 4 is another DNA repair protein and is involved in NHEJ. Mutations of the *LIG4* gene are associated with a spectrum of clinical manifestations that include microcephaly, facial dysmorphisms, bone marrow failure, growth and developmental delay, cellular radiosensitivity leading to increased risk of leukemia and other malignancies, and a variable degree of immunodeficiency (122,372). In some cases, impaired NHEJ activity causes severe defects of V(D)J recombination, and is associated with T- B- SCID (see above).

**80.5.4.4 AT-like Syndrome.** Two families with cerebellar degeneration, immunodeficiency, chromosomal instability, and increased susceptibility to ionizing radiation but no telangiectasia were found to have normal amounts of ATM protein. In light of the similarity to Nijmegen syndrome, genes for other proteins of the hMre11/hRad50 complex were examined, and mutations were identified in the gene *MRE11* (373).

**80.5.4.5 Bloom Syndrome.** Immune defects have also been reported in Bloom syndrome (359), in which chromosomal instability (in the form of increased sister chromatid exchange and homologous chromosomal translocations), an increased rate of infection, decreased IgM, and diminished delayed hypersensitivity have been described. The gene responsible, termed *BLM*, is a helicase (374). Numerous mutations have been found (375). A syndrome resembling Bloom syndrome, with moderately severe immunodeficiency, has been associated with a mutation in the gene for DNA ligase 1, a DNA repair gene (376). The syndromes are inherited as autosomal recessives.

**80.5.4.6 Immunodeficiency–Centromeric Instability–Facial Anomalies Syndrome.** Another syndrome has been described with immunodeficiency associated with

instability of the centromeric heterochromatin of chromosomes 1, 9, and 16 and with facial anomalies, leading to the acronym ICF syndrome (377). The defect was predicted by studies showing that the affected chromosome regions are hypomethylated (378). About 50% of the patients have been shown to carry mutations in the DNA methyl transferase 3B (*DMMT3B*) gene (379–381). More recently, it has been shown that ICF may also be due to mutations in the zinc-finger- and BTB (bric-a-bric, tram-track, broad complex)-domain-containing 24 (*ZBTB24*) gene (382).

## 80.5.5 Skeletal Abnormalities with Immunodeficiency

Short-limbed dwarfism is associated with more than one form of immunodeficiency (383).

McKusick et al. (384) described the phenotype of 77 Amish children in 53 sibships affected with a form of short-limbed dwarfism associated with cartilage–hair hypoplasia (CHH). In addition to the cartilage and hair abnormalities, affected individuals typically could not extend their elbows, had hyperextensibility of fingers and wrists, and often had a marked sternal deformity. Subsequent studies have demonstrated in vitro immune defects, primarily of cellular but also of humoral immune function (385–387). The syndrome has been described in high frequency in the Finnish population. In some cases, the immunodeficiency is very severe, and resembles SCID (388,389) or may present with Omenn syndrome (390). Patients with CHH are at higher risk of complications after varicella (391) and have an increased incidence of neoplasia, primarily lymphoma and basal cell carcinoma (392). The gene, which has been mapped to 9p13 (393), has been cloned and encodes for the RNA component of the mitochondrial RNA-processing endoribonuclease (RMRP). Mutations affecting coding region and the promoter have been reported, and the type and level of RMRP functional impairment appear to predict the phenotype (394,395). HSCT is effective in the most severe forms of the disease (389).

Schimke immuno-osseous dysplasia is a form of spondyloepiphyseal dysplasia associated with focal glomerulosclerosis and T-cell immunodeficiency (396). The kidney disease is progressive and leads to renal failure. The disease is due to mutations of the *SMARCA1* gene, encoding for a chromatin remodeling protein (397). Incomplete penetrance is associated with environmental and genetic disturbances of gene expression (398).

## 80.5.6 X-linked Hypohidrotic Ectodermal Dysplasia with Immunodeficiency

Hypomorphic mutations of the *IKBKG* gene, encoding for the IKK- $\gamma$  chain, (also known as NF- $\kappa$ B essential regulator, NEMO) result in X-linked EDA-ID (399). Defective NF- $\kappa$ B signaling in males with EDA-ID causes

impaired induction of AID expression in germinal center B cells, and hence results in impaired class switch recombination and a hyper-IgM phenotype. Furthermore, activation of the NF- $\kappa$ B signaling pathway is also important to induce IL-12 production by dendritic cells in response to CD40 stimulation. Defective production of IL-12 and of IFN- $\gamma$  in patients with NEMO mutations causes increased occurrence of mycobacterial infections. In addition, impaired activation of the NF- $\kappa$ B signaling pathway in response to other receptors is responsible for extra-immune manifestations of the disease, including ectodermal dystrophy (with sparse hair, lack of sweat glands, and conical shaped teeth) and, in some patients, lymphedema and osteopetrosis; however, some patients lack manifestations of ectodermal dystrophy (400). Genotype–phenotype correlation has been identified and may explain the complexity of phenotypes (401). Interestingly, null mutations in the same gene cause incontinentia pigmenti in heterozygous females and lead to death in utero in males.

An autosomal dominant form of EDA-ID due to gain-of-function mutation of the *IKBA* gene (encoding for IKB- $\alpha$ ) has been also reported (402,403). The mutation prevents phosphorylation and ubiquitylation-mediated degradation of the protein, and hence results in decreased activation of NF- $\kappa$ B. As compared to NEMO deficiency, the disease is also characterized by defective T cell activation and impaired memory T cell generation.

### 80.5.7 Warts–Hypogammaglobulinemia–Infections–Myelokathexis Syndrome

Immunodeficiency with neutropenia, hypogammaglobulinemia, and extensive warts due to human papilloma virus infection has been shown to be due to mutations in *CXCR4*, the gene for a chemokine receptor (404). The disease follows an autosomal dominant pattern of inheritance and is due to heterozygous nonsense or frameshift mutations that affect the C-terminus of the protein, in a region corresponding to its intracytoplasmic tail. The mutant CXCR4 is expressed at the cell surface, but is not internalized following binding of its ligand CXCL12 (SDF-1). Continuous signaling through the chemokine is therefore delivered. Abnormally sustained CXCR4 signaling causes retention of neutrophils in the bone marrow, where they accumulate and die (myelokathexis).

## 80.6 CONCLUSION

It should be clear from the previous descriptions that, as in other genetic diseases, there is a great deal of heterogeneity in the immunodeficiency disorders. Such heterogeneity can be due to different alleles at the same locus or mutations at different loci resulting in similar phenotypes. Not only is there expected genetic heterogeneity within each general phenotype, but due to the

dependence of many of the symptoms on chance exposure to environmental agents, there is a superimposed significant degree of nongenetic individual variation. In fact, a number of individuals demonstrating immunodeficiency, sometimes indistinguishable from the genetic diseases, develop their conditions as a result of such nongenetic problems as severe viral infections, malignancies, acquired immunodeficiency (e.g. AIDS), or therapy with immunosuppressive and cytotoxic agents. Additionally, a number of inborn errors of metabolism, including storage diseases, urea cycle defects, and organic acidurias and aminoacidurias, as well as several chromosomal disorders, such as those involving chromosome 18 and Down syndrome, demonstrate a variety of immunologic defects leading to increased susceptibility to infection. In great part, the difficulty of accurate classification, with a few notable exceptions, is due to our general lack of understanding of the fundamental molecular defects responsible for many of these conditions. In addition, the dependence of current classification systems on the developmental model of stem cell, B-cell, or T-cell defects, while initially highly useful, has become somewhat naive and, therefore, constricting. The continuous advance in our understanding of the interaction and interdependence of the components of the immune system with each other, and with even more cells and molecules, makes it clear that many modifications of the definitions of immunodeficiencies will come about. No doubt these discoveries, especially as they become increasingly understood on a molecular basis, will bring about the definition of many new defects associated with host defense problems. A number of syndromes that include various forms of immunodeficiency have been described, but the genes responsible have not been elucidated.

It is not only our hope but also our sincere conviction that the increased application of modern biochemical and molecular methodology, including next-generation sequencing, will lead to a clearer understanding of the genetics and the fundamental defects of primary immunodeficiency. It is only with such understanding that more rational counseling and therapy will develop. As so often was true in the past for other fields, a dissection of these genetic defects will inevitably continue to take the field of clinical and cellular immunology from its previous state of descriptive phenomenology into the realm of a proper science, a process that began with our understanding of the immunoglobulins and antibody diversity, as well as the specificity of T cells, and has begun to blossom in the past few years with the molecular definition of several of the primary immunodeficiency diseases.

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# CHAPTER

# 81

## Inherited Complement Deficiencies

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### 81.1 OVERVIEW

The complement system is a group of 14 proteins comprising the complement cascade and more than 10 regulatory proteins. Seven receptors mediate the biological functions of the complement ligands. Most complement proteins are produced in the liver, although C1q, properdin and C7 are produced predominantly by myeloid cells and factor D is produced by adipocytes and is also known as adipsin (1–5). Many other cells produce small amounts of complement components after pro-inflammatory stimuli and this is thought to magnify the local response (6). The major functions of the complement system are host defense, protection of endothelial surfaces and waste clearance. Each of these functions depends on a slightly different subset of complement proteins and the roles of the specific proteins have been largely elucidated through the study of inborn errors affecting the individual components.

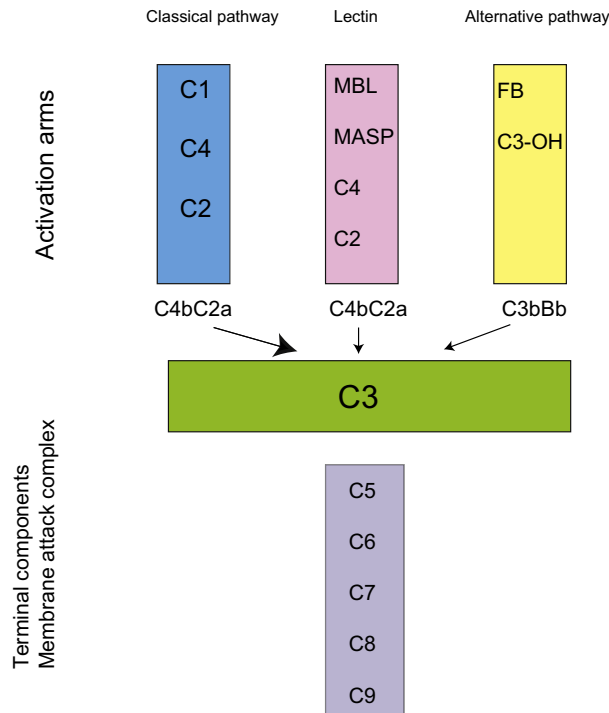
Complement nomenclature follows certain patterns with the classical pathway components generally indicated with an upper case C followed by a number that roughly correlates with the position in the cascade (C4 appears out of order). Alternative pathway members are generally referred to as a “factor” and are designated with a letter (factor B, factor D, factor H). As protein fragments are cleaved off, they are given lower case letter identifiers with the “a” most often designating the smaller fragment (the exception is C2a which is larger than C2b). In some cases, the two fragments can be further cleaved and those smaller fragments are named with additional lower case letters. When a cleavage product is inactive, it is preceded by the letter “i”. Protein complexes with enzymatic activity are termed convertases, i.e. C3 convertase and C5 convertase.

### 81.2 INTRODUCTION TO THE COMPLEMENT SYSTEM

The complement cascade originally evolved to opsonize pathogens and enhance their phagocytosis. The most evolutionarily ancient members of the complement cascade are factor B and C3, the minimal components needed to effect opsonization. As the adaptive immune system evolved, the complement system coevolved such that it now interfaces with nearly all aspects of host defense. A model for the organization of the complement cascade has three activation arms: the classical pathway, the lectin activation pathway and the alternative pathway (Figure 81-1). These three pathways cleave the central protein, C3, and allow it to bind to the nearest surface, usually a pathogen. The lectin activation pathway and the alternative pathway are truly part of the innate immune system but the classical pathway is activated by antigen–antibody complexes and therefore is dependent on the adaptive immune system. C3 constitutes a powerful opsonin and markedly enhances phagocytosis of the pathogen. Cleavage of C3 leads to activation of the terminal components which catalyze the formation of a pore in the membrane.

### 81.3 THE CLASSICAL PATHWAY

The classical pathway is activated primarily by immune complexes. A conformational change occurs when antibody binds antigen, which renders the antibody molecule capable of interacting with C1 (7,8). Only IgG and IgM activate complement and IgM is much more efficient than IgG. In addition, not all isotypes of IgG are equivalent. IgG3 is the most efficient followed by IgG1 and IgG2. IgG4 is not able to activate complement. A single molecule of IgM is sufficient to activate complement while many molecules of IgG bound to a particle are



**FIGURE 81-1** The three activation arms of the complement system. Activation of C3 is important for opsonization and B-cell costimulation. These two major functions of host defense are dependent on C3 and its appropriate activation. The terminal components or membrane attack complex are required for lysis of bacteria.

required to activate complement. In addition to immune complexes, the classical pathway is activated by apoptotic debris and a variety of other proteins and nucleic acids. Once activated, C1, C4 and C2 interact to produce a C3 cleaving enzyme: C4aC2b. Cleavage products resulting from this activation pathway lead to inflammation with erythema (vasodilatation) and edema (vascular leak). Neutrophils become activated and are therefore primed to cross the vascular endothelium to enter into tissues to initiate the attack on the pathogen.

### 81.4 THE ALTERNATIVE PATHWAY

The alternative pathway can, in some circumstances, be activated directly by immune complexes, but it is generally activated through the recognition of oligosaccharide and charge differences common to pathogens. It is a process belonging to of the innate immune system. The alternative pathway exploits the instability of the native C3 molecule and, on activator surfaces, nucleates a complex of C3bBb which cleaves additional C3 to opsonize bacteria and initiate activation of the terminal components. Note that the classical pathway provides an important substrate for the alternative pathway, C3b.

The basis of this pathway is a spontaneous hydrolysis of C3, which occurs in the serum at a rate of 0.2–0.4% per hour (9). The hydrolyzed C3 undergoes a conformational change that facilitates interaction with factor B. When factor B is bound to hydrolyzed C3 can it is cleaved by factor D. C3bBb is the alternative pathway C3 converting enzyme,

which is stabilized by properdin and cleaves additional C3 into C3b and C3a (10). The regulation of this pathway is largely through the effect of factor H on our surfaces. A nonactivator surface (usually our own cells) binds factor H avidly due rich sialic acid residues (11–13). In contrast, on activator surfaces (pathogens), factor H cannot displace factor B from C3b and the alternative pathway activation is allowed to proceed. Activator surfaces are often coated with mannose or *N*-acetyl glucosamine.

### 81.5 THE LECTIN ACTIVATION PATHWAY

The lectin activation pathway is the most recently identified activation arm of the complement cascade and also is a part of the innate immune system. The critical protein, mannose binding lectin (MBL), recognizes oligosaccharides specific to pathogens in a manner similar to that of factor H discussed above (14–16). Mammalian glycoproteins are generally decorated with galactose and sialic acid, not recognized by MBL. In contrast, MBL avidly binds to oligosaccharides associated with bacteria, yeast and parasites such as mannose, *N*-acetyl glucosamine, fucose and glucose (15). MBL also binds to agalactosyl IgG with high affinity (17). This unusual IgG is produced primarily at times of inflammation and this antibody would therefore amplify the complement response at sites of inflammation by activating through both the classical pathway and the lectin activation pathway.

MBL undergoes a conformation change after engaging carbohydrate, leading to activation of MASP2. MASP2 cleaves C4, which binds to a nearby surface as C4b (18). The remaining protein–protein interactions are identical to those in the classical pathway.

### 81.6 THE MEMBRANE ATTACK COMPLEX

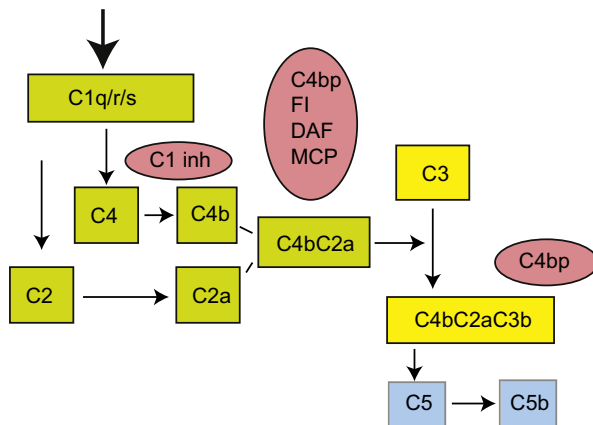
Once C3 is cleaved by any of the activation arms described above, it becomes a part of the next enzymatic complex and cleaves C5. The large cleavage fragment of C5 becomes attached to the surface of the pathogen. C5b binds to C6 and C7 and inserts into a lipid membrane (19,20). C5b also binds directly to C8, which then becomes incorporated into the complex. Once this happens, the membrane integrity is compromised. The addition of C9 leads to the formation of true pore (21).

### 81.7 REGULATION OF COMPLEMENT ACTIVATION

The regulators of complement are divided into fluid phase regulators and membrane bound regulators (Table 81-1). C1 inhibitor is perhaps the most clinically important of the regulatory proteins and it is a fluid phase regulator. C1 inhibitor is a serine protease, which inhibits the low-level auto-activation of C1 (Figure 81-2) (22). Immune complex activation of C1 is preserved, although chronic

TABLE 81-1 Complement Regulatory Proteins		
Protein	Localization	Function
C1 inhibitor	Serum	Binds to C1r and C1s and dissociates the C1 complex
C4-binding protein	Serum	Cofactor for factor I cleavage of C4b
Factor I	Serum	Cleaves C3b and C4b
Factor H	Serum	Defines activator surface
S-protein	Serum	Inhibits the insertion of the membrane attack complex into the cell membrane
Decay accelerating factor (DAF)	Ubiquitous-cell membrane	Dissociates both C3 and C5 convertases
Membrane cofactor protein	Hematopoietic cells except erythrocytes	Cofactor for C3b cleavage by factor I
C8 binding protein	Most hematopoietic cells	Binds to C8 and prevents interaction with C9
CD59	Hematopoietic cells, endothelial cells, epithelial cells, glomerular cells	Inhibits the membrane attack complex

Classical pathway activation

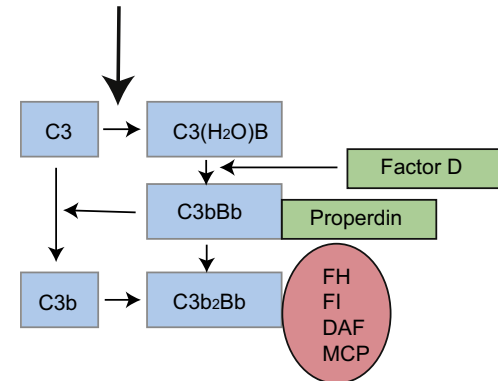


**FIGURE 81-2** The regulation of the classical pathway. The classical activation pathway is schematized with the relevant regulatory proteins shown as red ovals.

consumption of downstream proteins limits the efficiency of the classical pathway. C1 inhibitor directly binds to C1s and C1r, leading to dissociation from C1q. C1 inhibitor has a similar function in the lectin activation pathway. Relevant for the clinical manifestations of C1-inhibitor deficiency are its roles in the coagulation pathway. C1 inhibitor has important roles inhibiting factor XII (Hageman factor) and prekallikrein (23,24).

The other important fluid phase regulator is C4 binding protein, which displaces C2a and inhibits cleavage of C3 (25,26). Factor I and factor H regulate the alternative pathway (Figure 81-3; Refs (27–30)). Factor I inactivates

Alternative pathway activators



**FIGURE 81-3** The regulation of the alternative pathway. The alternative activation pathway is schematized with the relevant regulatory proteins shown as a red oval.

C3b and its activity is enhanced by factor H. Factor H identifies nonactivator surfaces through the recognition of mammalian oligosaccharides and displaces Bb from C3b on those surfaces. These two regulators are critical in the prevention of significant spontaneous activation of the alternative pathway. When an activator surface is available, factor H releases Bb and a more intensive and sustained activation of the alternative pathway occurs. Factor I also acts to inhibit C4b from the classical pathway (28,31). Finally, S-protein (distinct from protein S) binds terminal complement components in the fluid phase.

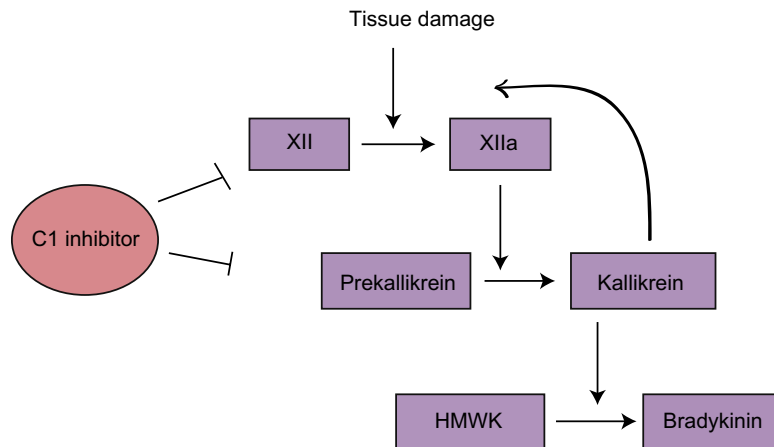
The membrane bound regulators of complement consist of decay accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46), C8 binding protein and CD59 (28,32–37). DAF dissociates the C3 convertase and MCP serves as a cofactor for factor I cleavage of C3b and C4b. CD59 inhibits the membrane attack complex and inhibits C9 binding. C8 binding protein functions similarly, but acts via binding to C8. Several receptors terminate complement function and have a regulatory function. CR1 binds C3b and C4b and serves as a cofactor for factor I mediated cleavage. CR2 has a similar role supporting cleavage of C3b (Figure 81-4).

## 81.8 INHERITED COMPLEMENT DEFICIENCIES

### 81.8.1 Overview

Most inherited complement deficiencies are autosomal recessive and except where noted, the mutations are diverse (Table 81-2). Properdin deficiency is X-linked and C1-inhibitor deficiency is autosomal dominant (hemizygous). The defects associated with susceptibility to atypical hemolytic uremic syndrome (aHUS) are particularly diverse, with activating and inactivating mutations, as well as recessive and dominant patterns of inheritance. Inherited complement component disorders are typically associated with a CH50 or AH50





**FIGURE 81-4** The role of C1 inhibitor. C1-inhibitor deficiency compromises the natural inhibitory pathways regulating bradykinin production. Although the complement activation leads to the elaboration of peptides with anaphylatoxic abilities, the main mediator of angioedema is thought to be bradykinin.

**TABLE 81-2** Inherited Complement Deficiencies

Deficiency	Chromosomal Location	Number of Cases Reported	Clinical Features, Diagnostic Strategy
C1q	1p36.12	10–100	SLE, infections, CH50 near zero
C1r/s	12p13	10–100	SLE, infections, CH50 near zero
C4	6p21.3	10–100	SLE, infections, CH50 near zero
C2	6p21.3	Many	SLE, infections, some asymptomatic, CH50 near zero
C3	19p13.3–13.2	10–100	Infections frequent and severe, glomerulonephritis, HUS, CH50 near zero
Factor D	19p13.3	<10	Neisseria, AH50 near zero
Factor B	6p21.3	<10	Neisseria, HUS, AH50, C3 level
Properdin	Xp11.3–11.23	>100	Neisseria, AH50 diminished
C5	9q33	10–100	Neisseria, CH50 near zero
C6	5p13	>100	Neisseria, CH50 near zero
C7	5p13	>100	Neisseria, CH50 near zero
C8	1p32, 9q34.3	>100	Neisseria, CH50 near zero
C9		Many	Neisseria, CH50 diminished
Factor I	4q25	10–100	Neisseria, HUS, C3 may be diminished, many require mutation analysis
Factor H	1q32	10–100	Neisseria, HUS, C3 may be diminished, many require mutation analysis
MCP	1q32	<10	HUS, mutation analysis required
C1 inhibitor	11q12–13.1	Many	Angioedema, C1 antigen and functional levels
CR3/CR4	16p11.2	>100	Leukocyte adhesion deficiency, flow cytometry
CD59	11p13	<10	Hemolysis, flow cytometry

of near zero and identification of the specific deficiency requires a labor intensive serum mixing approach. Specific complement deficiencies are generally defined at this level and mutation analysis is not routinely performed. Exceptions to this are the defects in the regulatory proteins. Because these have a less profound effect on the CH50 and AH50 and they cannot be identified in mixing studies, direct mutation testing is generally the diagnostic maneuver of choice. C1-inhibitor deficiency is an exception to this, as functional testing is widely available and is quite reliable.

## 81.8.2 Defects in Proteins Involved in the Activation of C3

**81.8.2.1 C1 Complex Deficiencies.** C1 is a multi-subunit protein. C1q binds to antibody and C1r and C1s are the enzymatic components, responsible for cleavage. C1q, C1r and C1s deficiencies present nearly uniformly with early onset systemic lupus erythematosus (SLE) (38–40). The SLE is typically severe and arises in childhood. The autoantibody profile is similar to that seen in other SLE patients although anti-dsDNA antibodies may be less

common (41,42). An additional phenotype is recurrent bacterial infections, most often systemic infections with encapsulated organisms. All three deficiencies are inherited in an autosomal recessive pattern.

**81.8.2.2 C4 Deficiency.** There are two distinct genes for C4 termed C4A and C4B. They are highly homologous although C4A binds more avidly to protein while C4B binds more avidly to carbohydrate. Within each C4 locus, there can be deletions or duplications or simple inactivating mutations (43,44). Partial C4 deficiencies are extremely common. One to two percent of the general population and up to 15% of patients with SLE have complete C4A deficiency. One to two percent of the population has complete C4B deficiency and up to 15% of patients with invasive bacterial disease are C4B deficient (45). Complete C4 deficiency due to four inactive alleles is quite rare and the majority of patients with complete C4 deficiency have SLE (46–48). Some complete C4-deficient individuals can be asymptomatic but many have recurrent infections with encapsulated organisms. The SLE and infection phenotypes can be separate or occur together in an individual.

**81.8.2.3 C2 Deficiency.** C2 deficiency is the most common of the inherited complement component deficiencies in Caucasians with a frequency of 1:10,000. The common Caucasian mutation is a 28bp deletion. Most C2 deficient individuals are asymptomatic although their susceptibility to infection is thought to be increased. Twenty to forty percent of C2-deficient individuals will develop SLE (49–51). Anti-Ro antibodies are common in C2-deficient patients with SLE although anti-dsDNA antibodies are infrequent (52). In spite of SLE being the most common phenotype, the most common cause of death among C2 deficient patients is sepsis (49). Other systemic infections such as meningitis, pneumonia, epiglottitis and peritonitis have been seen and the most common organisms have been *Streptococcus pneumoniae* and *Haemophilus influenzae*.

**81.8.2.4 C3 Deficiency.** C3 deficiency is the least common of the complement cascade protein deficiencies and it has the most severe phenotype by far (53–57). All patients have a profound predisposition to infection and the infections are sometimes characteristic of neutrophil dysfunction (abscesses), humoral deficiencies (sinopulmonary disease) and complement deficiencies (sepsis, meningitis). Membranoproliferative glomerulonephritis occurs in one-third of the cases of C3 deficiency (50,51). One other feature of C3 deficiency deserves mention. During infections, a vasculitic rash may appear and symptoms of serum sickness may occasionally be seen. These unusual findings are due to the lack of immune complex solubilization by C3. C3 deficiency is rare, with fewer than 30 cases reported in the literature. There is a founder effect among the Afrikaans-speaking population (56). Hypomorphic C3 mutations have been identified in some patients with autoimmune disease but the phenotype and prevalence are not clear (58,59).

Activating mutations of C3 can be associated with aHUS. These patients have been infrequent but have all had mutations that inactivated the interaction of C3 with MCP, a loss that leads to chronic activation and consumption of C3. These patients have had diminished serum C3.

**81.8.2.5 MBL Deficiency.** MBL deficiency is common with 2–7% of people affected (60). There are common structural mutations that destabilize the higher order complexes (61) and several promoter mutations that lead to impaired production (61). Combinations of mild mutations can lead to complete loss of function. The consequences of MBL deficiency are controversial with data supporting an increased susceptibility to infection and other studies finding no effect (61–64). MBL deficiency may be a mild risk factor for infection but is unlikely to have a significant effect. Similarly, an effect on susceptibility to autoimmune disease has been proposed; however, the effect has not been consistently identified and if there is an effect, it must be small.

**81.8.2.6 MASP2 Deficiency.** MASP2 protein deficiency is seen in 5–10% of the population (65). The common polymorphism, D105G, leads to compromised interactions with MBL and ficolin. Additional polymorphisms have been identified in various populations but their functional effect has not yet been defined. Like MBL deficiency, it may represent a risk for infection but the effect is modest (64). There is no strong evidence for an association of MASP2 deficiency and autoimmune disease.

**81.8.2.7 Factor B Deficiency.** A single case of complete factor B deficiency in a patient with meningococcemia has been reported (66). The disorder was suspected after studies demonstrated a low C3 and an absent AH50. No mutation was characterized; however, abnormal protein was seen on electrophoresis. Heterozygous gain-of-function mutations of factor B are found in approximately 2–4% of patients with aHUS.

**81.8.2.8 Factor D Deficiency.** Neisserial infections are the most common manifestation of factor D deficiency (50,51,67). Factor D deficiency should be suspected when the AH50 activity is absent.

**81.8.2.9 Properdin Deficiency.** Properdin deficiency is the only X-linked complement deficiency and the identified mutations have been diverse, with some affecting protein function and some having a null phenotype. Approximately half of the properdin deficient individuals present with meningococcal disease (50,51,68–71). There is a high fatality rate in meningococcal disease in properdin deficient patients. There may be a founder effect in Tunisian–Jewish people; however, properdin deficiency is seen on all ethnic backgrounds.

### 81.8.3 Defects in Proteins in the Membrane Attack Complex

**81.8.3.1 C5 Deficiency.** C5 deficiency is associated with susceptibility to meningococcal and gonococcal disease.

C5 deficiency is found on a variety of ethnic and racial backgrounds and the mutations are diverse.

**81.8.3.2 C6 Deficiency.** C6 deficiency is one of the most common complement disorders and occurs more frequently in African Americans and in people of South Africa. C6 deficiency is associated with meningococemia, meningococcal meningitis and disseminated gonococcal disease (50,51,72,73). There are two notable mutations associated with variants of C6 deficiency. In one case, a splice defect leads to a smaller than usual protein, C6SD (74). This protein functions less efficiently than wild-type C6; however, it is not clear whether bearing C6SD leads to compromised host defense. The other variation is combined C6 and C7 deficiency (75).

**81.8.3.3 C7 Deficiency.** C7 deficiency is not particularly common and the most common presentation has been neisserial disease (50,51,76). There may be a founder affect among Moroccan Jewish kindreds (77) but generally the mutations are diverse (76).

**81.8.3.4 C8 Deficiency.** C8 is composed of three chains:  $\alpha$ ,  $\beta$ ,  $\gamma$ . The  $\alpha$  and  $\gamma$  chains are covalently attached and bind to the  $\beta$  chain. C8 $\beta$  deficiency is more common in Caucasians whereas C8 $\alpha$ - $\gamma$  deficiency is more common among African Americans and Japanese (78–81). The majority of the C8 $\beta$  mutations are due to a single base-pair transition leading to a premature stop codon (80,82). The majority of the C8 $\alpha$ - $\gamma$  mutations are due to a 10-bp insertion leading to a stop codon (78). All types of C8 deficiency are associated with susceptibility to neisserial disease (50,51). Meningococcal meningitis, meningococemia and disseminated gonococcus have been seen.

**81.8.3.5 C9 Deficiency.** C9 deficiency is seen with high frequency in Japan and Korea (83–85). Approximately 0.05% of the population in Japan is C9 deficient. It is more difficult to diagnose than most of the other complement cascade protein deficiencies because the CH50 is diminished but not absent (86). As is true for the other terminal complement component deficiencies, C9 deficiency is associated with neisserial disease although the penetrance appears to be less than that seen in other terminal component deficiencies (87). The common Japanese mutation is a nonsense mutation in exon 4 (88).

## 81.8.4 Defects in Regulatory Proteins

**81.8.4.1 C1-Inhibitor Deficiency.** C1-inhibitor deficiency is associated with recurrent episodes of submucosal or subcutaneous edema (89). It is unusual in that it is most classically due to a heterozygous mutation and is inherited in an autosomal dominant fashion, although homozygous deficient patients exist (23,90–93). Serum levels are typically slightly less than 50% of normal due to accelerated consumption and in those cases where serum protein levels are normal, the functional level is slightly less than 50% of normal. Approximately half of the patients present in childhood. Rare asymptomatic

subjects have been identified. The frequency of angioedema episodes is highly variable, as is the severity. The extremities, face, genitalia and gastrointestinal tract are most often involved. Appendectomy and exploratory laparotomy for abdominal pain due to intestinal swelling are frequently performed (89). Upper airway swelling can lead to respiratory arrest. This complication can occur in as many as 2/3 of patients with C1-inhibitor deficiency, although improved management has made it less common. Prior to modern management, slightly over 10% of patients underwent a tracheostomy as a result of airway episodes.

The angioedema typically progresses for 1 to 2 days and resolves in another 2 to 3 days. Many people cannot identify precipitants, but common triggers are illness, hormonal fluctuations, trauma and stress. The C1-inhibitor promoter is androgen responsive, which is why men can be less severely affected than female patients (94–96). It is also the mechanism underlying the oldest therapeutic modality, attenuated androgens.

**81.8.4.2 C4 Binding Protein Deficiency.** A single kindred with C4 binding protein deficiency has been described (97). Angioedema, vasculitis and arthritis were seen.

**81.8.4.3 Factor I Deficiency.** Factor I deficiency has two phenotypes and the genotype–phenotype relationship is not fully understood. When factor I is lacking, C3 cleavage occurs unchecked and a secondary deficit in C3 occurs. Both the CH50 and AH50 are depressed but not absent and C3 antigen levels are low (98–101). Neisserial disease and infections with *S. pneumoniae* and *H. influenzae* have been described (50,51). The infection phenotype is most often associated with autosomal recessive mutations.

The second phenotype is renal disease, either aHUS or membranoproliferative glomerulonephritis II (102–104). These cases of factor I deficiency are difficult to identify because traditional complement studies are often normal. The factor I level is typically normal as the mutations inactivate critical binding sites. The fenestrated endothelium of the glomerulus is rich in polyanions, where complement can be activated in the absence of regulatory proteins. Mutations most often affect the catalytic domain (105).

**81.8.4.4 Factor H Deficiency.** Infections, aHUS, and macular degeneration are the main phenotypes seen in factor H deficiency (106–109). There is a strong genotype–phenotype correlation and factor H mutations are often heterozygous mutations. The infections are due to consumption of C3 and a secondary partial C3 deficiency (110). C3 levels are typically diminished and the CH50 and AH50 are low. Factor H levels may be diminished. The mutations associated with this phenotype are diverse. The other two phenotypes are less likely to be associated with low factor H levels, and mutation testing is typically required. Kindreds with membranoproliferative glomerulonephritis have been

identified with factor H deficiency and an acquired phenocopy is due to autoantibodies directed to factor H (111). The most common phenotype is now known to be aHUS. Factor H defects were found to be the underlying basis for 15–30% of patients with a HUS (112). Mutations typically occur at the C-terminus. Both autosomal recessive and heterozygous mutations have been seen. The disease is typically early onset and severe with recurrences in many cases (113).

A common tyrosine–histidine polymorphism of factor H (the His402 variant) was identified as a risk factor for macular degeneration (106,114). The retinal deposits called drusen contain factor H and terminal complement components. This polymorphism affects protection of the endothelium allowing smoldering complement activation and gradual damage.

**81.8.4.5 Membrane Cofactor Protein (CD46) Deficiency.** MCP deficiency is associated with a later onset of aHUS compared with factor H and factor I deficiencies (113,115–117). MCP mutations are thought to account for approximately 10% of all aHUS (112). In contrast to factor H and factor I deficiencies, renal transplantation can be successful because MCP is expressed on the kidney; however, relapses have been described (118,119). Traditional complement analyses are normal.

**81.8.4.6 CD59 Deficiency and Paroxysmal Nocturnal Hemoglobinuria.** A single patient with CD59 deficiency has been described and the major manifestation was chronic hemolytic anemia and recurrent stroke (120). CD59 confers protection from intravascular complement-mediated lysis and is expressed on hematopoietic cells and endothelial cells.

Paroxysmal nocturnal hemoglobinuria is due to acquired somatic mutations of PIG-A or PIG-M (121). These proteins are required for GPI anchored proteins such as C8 binding protein, DAF and CD59 (122). The diagnosis of PNH is made by flow cytometry for CD59 or CD55 (DAF).

**81.8.4.7 DAF Deficiency (CD55).** DAF deficiency is also termed the Inab blood group phenotype (123–125). DAF deficiency has no clear phenotype. Although, red cells may be slightly more sensitive to lysis (126).

**81.8.4.8 CR3/CR4 Deficiency.** This disorder is a defect in the three  $\beta$ 2-integrin adhesion molecules. This deficiency is more often called leukocyte adhesion deficiency type I (LAD type I). Mutations in the common  $\beta$  chain (CD18) lead to failure to express adequate  $\alpha$  chains (127,128). This disorder will be discussed in more detail in the chapter on immunodeficiencies.

## 81.9 MANAGEMENT OF COMPLEMENT DEFICIENCIES

The management of complement deficiencies is dependent on the type of defect. With few exceptions, there are no trials supporting the management strategies offered

here. The management approaches offered in this chapter represent possible interventions based on current literature. As this is a rapidly moving field, it is wise to seek out expert advice when confronted with a complement deficient patient.

### 81.9.1 Early Classical Component Deficiencies

C1, C4 and C2 deficiencies are all associated with bacterial infections and SLE. The treatment for SLE in these patients does not differ from standard approaches, although the more severely affected patients could in theory be treated with a liver transplant.

To mitigate the susceptibility to infection, patients are often vaccinated to raise the titers of antibodies to encapsulated organisms. For early complement component deficiencies the major risks seem to be *S. pneumoniae* and *H. influenzae* (49–51). Vaccines to these entities exist and there is reason to believe that having high titers of antibody may offer protection. Alternatively, antibiotics may offer protection from serious infection. In one study, half of the C2-deficient patients had serious infections such as sepsis and infection was the leading cause of death, accounting for over 10% of the deaths in this cohort (49). One-quarter of the patients had meningitis. Patients on immunosuppressive medication for rheumatologic disorders may require yet more vigilance and protection.

### 81.9.2 C3 Deficiency

The infections seen in C3-deficient patients are the most severe of any of the complement deficiencies and management must address loss of opsonization, loss of B-cell costimulation and loss of immune-complex solubilization (110). Immune globulin replacement is sensible to compensate for the compromised B-cell function and prophylactic antibiotics could ameliorate the risk of infection. The membranoproliferative glomerulonephritis seen in C3-deficient patients has no specific intervention, although renal transplantation has been attempted. The recurrence risk has not been characterized; however, one would anticipate some recurrence risk. Those patients with aHUS are often treated as other patients with HUS acutely. Fresh frozen plasma (FFP) can be administered to replace C3. Liver transplantation has not been performed but could be considered given the severity of the disease.

### 81.9.3 Factor D and Properdin Deficiency

Patients with factor D and properdin deficiency are susceptible to neisserial disease as well as *S. pneumoniae* and *H. influenzae*. Vaccination to achieve high titers of antibody to those entities could theoretically provide benefit. Traditionally, prophylactic antibiotics have been



used for some patients in an effort to prevent infections (129–131).

#### 81.9.4 Defects Associated with a Risk of Neisserial Infections

Terminal component deficiencies are all associated with an increased risk of neisserial disease. Meningococcal disease is by far the most common, but disseminated gonococcal infections have been described. Vaccination every 3 years with the meningococcal vaccine decreases the frequency of meningococcal episodes but does not eliminate them (132–135). The frequency is decreased to 20% of what non-vaccinated individuals experienced. No study has examined prophylactic antibiotics and it may be that careful monitoring and hyper-vaccination may be sufficient. Factor H and factor I deficiencies can also be associated with susceptibility to neisserial infections. There are no data to support a specific treatment strategy but the interventions described for patients with terminal components deficiencies could be considered.

#### 81.9.5 C1-Inhibitor Deficiency

Current management is evolving rapidly due to the recent release of C1-inhibitor products and a kallikrein inhibitor. Management differs in other countries but in the United States current management options include attenuated androgens to raise expression of the intact gene, C1 inhibitor and ecallantide (a kallikrein inhibitor). Each have demonstrated efficacy. Each may be used to treat acute episodes but ecallantide has not been approved as a preventive strategy. Although attenuated androgens such as danazol and oxandrin can be used to treat acute episodes, their effect is slow compared with C1-inhibitor products or ecallantide. For high-risk surgical procedures, C1 inhibitor is preferred although FFP may be used if C1 inhibitor is not available.

#### 81.9.6 Defects Associated with aHUS

As is done for typical acute hemolytic uremic syndrome, some patients receive pheresis and FFP replacement (115,136,137). In the longer term, one study found that factor H replacement prevented episodes, suggesting that FFP alone might be of benefit as prophylaxis for those patients with defects in soluble proteins. In the case of MCP, where the affected protein is membrane bound, it is less clear that pheresis and FFP would provide benefit, but it could potentially act to clear inciting agents or complement activation products (138). For patients with end-stage renal disease, the recurrence of disease in patients with soluble factor deficiencies is unacceptably high and renal transplantation is not recommended (118,119). In contrast, renal disease in MCP typically does not recur in the transplanted kidney. A combined liver and kidney transplant has been performed successfully, although

the preparative regimen was complex (139). A monoclonal antibody directed at C5 (eculizumab) was recently shown to be of benefit on aHUS and may become a useful adjunct in the transplant setting (140).

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### Biography



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# CHAPTER

# 82

## Disorders of Leukocyte Function

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### 82.1 INTRODUCTION

Over the years 1950–2010, the field of phagocytic disorders has attained major biologic and clinical significance and a great deal of literature has accumulated on these disorders, which are frequently hereditary. Recently, owing to the development of exciting new advances in molecular biology and cellular physiology of signal transduction, it has been possible to identify the genetic defects involved in many of these phagocyte disorders. Moreover, through immunopharmacologic intervention, bone marrow transplantation (BMT), and the prospect of gene therapy, we have begun to at least partially correct genetic defects in cell activation pathways in phagocytes. Prenatal diagnosis employing first restriction fragment length polymorphism (RFLP), polymerase chain reaction (PCR) techniques and, more recently, scanning by high-resolution melting analysis and direct DNA sequencing, along with the assessment of granulocyte function in fetal blood have made these diseases potentially preventable, treatable in utero, or shortly after birth.

### 82.2 HISTORICAL BACKGROUND

In the late 1800s, Metchnikoff first established that the essential and primary element in typical inflammation consists of a reaction of the phagocyte against a harmful agent (1). Earlier, it was believed that phagocytes were harmful to the host and that they contributed to the untoward consequences of bacterial infection. After establishing that phagocytes were helpful in defense against bacteria, Metchnikoff predicted that defects in phagocyte function might predispose the host to an increase in the numbers and toxicity of infection with microorganisms. The past 60 years of clinical and laboratory definition of phagocyte disorders have proved this

hypothesis as patients with phagocyte function defects, with either quantitative or qualitative disorders, tend to have recurrent severe or even fatal bacterial and fungal infections.

Kostmann (2) identified an autosomal-recessive quantitative phagocyte disorder resulting in severe congenital granulocytopenia ( $<1500/\text{mm}^3$ ). Patients with Kostmann disease (also called infantile genetic agranulocytosis or severe congenital neutropenia (SCN)) usually have severe, chronic, and eventually fatal bacterial infections starting early in life. Evidence suggests that some cases are transmitted as an autosomal-dominant trait, whereas others are sporadic or rarely X-linked. There are now at least 15 gene defects known to cause SCN, but in about 30% of cases, the genetic etiology is still unknown.

Quie et al. (3) provided the first evidence of an inborn error of phagocyte bactericidal activity in a disease, then termed fatal chronic granulomatous disease (CGD). The authors found that granulocytes obtained from patients with CGD were able to ingest bacteria normally, but were unable to kill the internalized organisms. This disease, which often resulted in fatal granulomatous infection of childhood (4), most frequently occurred in boys, suggesting an X-linked mode of inheritance (5). Of additional interest was the observation that granulocytes from the mothers (presumed carriers of an X-linked disorder) were intermediate in their bactericidal capacity. Not only did these observations establish the first intrinsic defect of phagocyte function, but also the intermediate bactericidal defect in maternal granulocytes was consistent with the Lyon hypothesis. Baehner and Nathan (6) demonstrated a primary metabolic abnormality in granulocytes from CGD patients using a yellow dye, nitroblue tetrazolium, which turns to blue formazan in the reduced state

when superoxide is generated. Normal granulocytes stimulated to ingest and kill bacteria reduced the dye, whereas similarly stimulated granulocytes obtained from patients with CGD were unable to reduce the dye into blue formazan. Again, maternal granulocytes were found to be intermediate in dye reduction capacity. Hohn and Lehrer (7) reported that CGD granulocytes had defective NADPH oxidase activity. Segal et al. (8) showed that membrane cytochrome  $b_{245}$  (also called  $b_{558}$ ), a component of the NADPH oxidase, was missing in X-linked CGD granulocytes. Evidence existed, however, that some of the patients with CGD had an autosomal-recessive trait with intact membrane cytochrome  $b_{558}$  on granulocytes (9). The defective genes resulting in autosomal-recessive CGD have now been identified. The X-linked CGD gene coding for the cytochrome  $b_{558}$  heavy chain (p91phox), a component of the NADPH oxidase complex, is located on the short arm of the X chromosome; the autosomal-recessive genes encoding other components of the NADPH oxidase complex, including cytosolic factors p67phox and p47phox and cytochrome  $b_{558}$  light chain (p22phox), are located in chromosomes 1, 7, and 16, respectively (10). A small G protein, p21 Rac, is also involved in the activation of NADPH oxidase (11). Defects in any component of the NADPH oxidase complex can impair phagocyte oxidative bactericidal activity, resulting in CGD. These results suggested that a biochemical event under genetic control was the underlying basis for the bactericidal defect. In addition, the fact that phagocyte bactericidal activity can be individually controlled by a specific gene distinct from other phagocyte functions, such as adhesion, movement, and ingestion, suggests that these other phagocyte activities might also be individually controlled by specific genes.

From 1950 to 2010, these hypotheses have been proved true. An entire spectrum of phagocyte disorders, including adhesion, movement, ingestion, and bactericidal defects, has been recognized and their genetic bases are determined. Serious phagocyte function defects such as seen in leukocyte adhesion disorder (LAD) and CGD may occur as frequently as 1 in 250,000 people. Other minor phagocyte defects, such as myeloperoxidase (MPO) deficiency, have an even higher incidence of approximately 1 in 4000 to 1 in 2000. These minor disorders, however, do not often result in severe infections; for example, MPO deficiency is often asymptomatic, but can cause abnormal laboratory testing. This chapter highlights the current status in this exciting field and points out the possibilities for prenatal diagnosis, immunopharmacologic correction, and gene therapy based on the genetic defects, as well as the biochemical and physiologic aberrations of phagocytes. To understand the individual phagocyte defects, a comprehensive understanding of normal phagocyte development, kinetics, and function is essential.

## 82.3 PHAGOCYTE DEVELOPMENT, KINETICS, AND FUNCTIONS

### 82.3.1 Development

There is evidence that human myelopoiesis occurs early in fetal life—as early as 6–8 weeks of gestational age (12,13). Phagocyte progenitor cells arise from pluripotential hematopoietic stem cells in the bone marrow. These precursors can give rise to mature phagocytes, including polymorphonuclear granulocytes and mononuclear phagocytes, under the influence of various colony-stimulating factors: stem cell factor, granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and interleukin (IL)-3. These growth and differentiation factors are derived mostly from the stromal cells in the bone marrow, but some are produced by myeloid cells in the reticuloendothelial system (14). The development of granulocytes from hematopoietic stem cells to common myeloid progenitors, then to granulocyte–monocyte progenitors, and finally to mature granulocytes and monocytes is controlled by transcriptional factors (15). The differentiation of myeloid progenitors into mature phagocytes probably results in part from the acquisition of specific factor receptors on the cells at different phases of differentiation and maturation (reviewed by Yang and Hill (16,17)). Interaction of lectins in the stromal cells with receptors on phagocyte precursors may also be involved in the process of growth and differentiation (14). Phagocytes obtained from a 20-week gestational age fetus reveal active phagocytic and respiratory burst activity. Phagocytes in a full-term newborn, however, do not have complete functional activity. Neonatal granulocytes and monocytes have defects in bone marrow storage pools, cellular activation, and chemotaxis (18–22). Studies with adult phagocytes have indicated that a variety of cytokines and glycoproteins can modulate phagocyte function. These results suggest that intact phagocyte function is due to intrinsic maturation, but is also modulated by environmental factors that alter the granulocyte's biochemical and physiologic status. It is necessary to differentiate intrinsic defects from extrinsic environmental abnormalities.

### 82.3.2 Kinetics of Granulocytes

Granulocytes are produced in the bone marrow and released into the blood and tissues, where they act as the first line of defense in host resistance and wound healing (13). The total granulocyte pool is divided into two compartments: the bone marrow and the circulating pools. The bone marrow provides the environment for the proliferation of myeloblasts, promyelocytes, and myelocytes, as well as the maturation of metamyelocytes and band-form granulocytes. The latter two populations represent the main marrow storage pool of granulocyte precursors, containing  $6-8 \times 10^9$  cells/kg. The recycling



and the proliferation and maturation phases of granulocytes in bone marrow require 2–3 days and 7–10 days, respectively (16,17,23). In the blood, granulocytes are in the circulating pool ( $0.3\text{--}0.4 \times 10^9$  cells/kg) and marginating pool ( $0.4 \times 10^9$  cells/kg). Endotoxin, corticosteroids, and complement fragments (C3e, C3d,g) cause accelerated release of mature granulocytes into the circulation. Adrenergic neurotransmitters such as epinephrine and norepinephrine can promote granulocyte mobilization from the marginating pool into the circulatory pool. Granulocytes in the circulation have a short circulating transit time of about 6–9 h. Certain patients with LAD have a longer circulating time (24). In contrast, patients after multiple blood transfusion may have a shorter circulating time of <1 h (25). In spite of this short half-life, granulocyte numbers in the blood are normally maintained between 3000 and 6000 cells/mm<sup>3</sup>.

### 82.3.3 Kinetics of Monocytes/Macrophages

Monoblasts are the first recognizable cells in the monocyte series in the bone marrow (26,27). However, few monoblasts are found in the bone marrow as the storage pool of these cells is relatively small. The transit time from monoblast to monocyte takes about 6 days (28). Promonocytes are the prominent cells of the monocyte series in the bone marrow, with a major storage pool containing  $6 \times 10^8$  cells/kg (29). Promonocytes mature into monocytes in the bone marrow and are released into the blood within 2 days. Circulating monocytes in the blood make up 3–8% of the total leukocytes ( $300\text{--}600$  cells/mm<sup>3</sup>, or  $0.3 \times 10^8$  cells/kg), and three to four times more monocytes marginate in the blood. These monocytes leave the circulation with a transit time of 1–3 days (23,28,29). Every day, approximately 108 monocytes are released into the bloodstream and leave for the tissues. These monocytes become transformed into a variety of tissue macrophages, depending on conditions in different tissues (26,27). They then remain in tissues as macrophages for months or even years. The monocyte–macrophage system is also widely distributed in a number of organs as microglial cells in the brain, Kupffer cells in the liver, osteoclasts in the bone marrow, Langerhans cells in the skin, mesangial cells in the kidney, and resident macrophages in the lungs, spleen, thymus, lymph nodes, pleuroperitoneal cavity, and joint spaces. Immature monocytes can also develop into dendritic cells that distribute to all tissues and act as professional antigen-presenting cells (30). During acute inflammation, the transit time of monocytes from the bone marrow to the inflammatory tissue may be shortened from 2 days to 6–8 h. In contrast to granulocytes, which do not replicate in the circulation or in tissues, both circulating monocytes and tissue macrophages are able to replicate rapidly. In chronic inflammation, tissue macrophages may transform into

giant cells or epithelioid cells in the presence of tumor necrosis factor (TNF)- $\alpha$  and interferon- $\gamma$  (IFN- $\gamma$ ) (28,29), especially in a variety of granulomatous disorders. Defects in IFN- $\gamma$  receptor result in the absence of granuloma formation in normally avirulent mycobacterial infections (31).

### 82.3.4 Functions of Granulocytes

Granulocytes can become activated in the blood circulation and in interstitial tissues. Activation of granulocytes requires several integrated signal pathways. A small G protein, Rac2, is specifically involved in triggering respiratory burst activity (11,32). A GTPase, Cdc42, together with Wiskott–Aldrich syndrome protein (WASp) activates actin assembly (33). Another small G protein, Ras, regulates phosphatidylinositol-3-kinase activities for coordination of granulocyte migration (34). Moreover, different G proteins can be coupled to different receptors and mediate the degranulation of granulocytes (32). The differentiation and activation of granulocytes are also regulated by mitogen-activated protein kinases such as p38 and MEK signaling (35,36). Adhesive glycoproteins on granulocytes, including selectins such as L-selectin, as well as integrins such as the complement receptors CR3 and CR4, promote granulocyte adhesion (37,38). Selectins are involved in granulocyte rolling in the blood circulation, whereas integrins mediate granulocyte extravasation (37,38). In response to chemoattractants, C5a, IL-8, platelet-activating factor, leukotrienes, and bacterial formylated peptides (fMLP), granulocytes reorganize their cytoskeleton and migrate into tissues and inflammatory sites, where they interact with and ingest target organisms, especially if the microbes are coated with immunoglobulin or complement. This interaction of granulocytes with microbes is followed by ingestion, respiratory burst activity, and degranulation. Reactive oxygen intermediates released from the respiratory burst, and lysosomal enzymes and cationic proteins released from degranulation of primary and secondary granules, respectively, contribute to oxygen-dependent and oxygen-independent bactericidal activities, resulting in extracellular and intracellular killing of microorganisms. Moreover, oxygen radicals are involved in nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation for defense against bacterial infection (39).

There is also evidence that granulocytes have an important role in inflammatory and degenerative reactions arising during tissue injury, tumor invasion, viral infection, autoimmune disorders, aging, and atherosclerosis. Enhancement of granulocyte adhesiveness to endothelium may result from thermal injury, hypoxia, bacterial sepsis, and immune complexes. On traumatic injury, platelets adhere and aggregate on the vascular endothelium and subsequently release chemotactic factors such as leukotriene B<sub>4</sub>, transforming growth factor- $\beta$  (TGF- $\beta$ ), and platelet-derived growth factors. This

results in a rapid recruitment of granulocytes and fibroblasts that have a central role in tissue inflammation and wound healing (16). Thermal injury, hypoxia, and bacterial sepsis may cause adult respiratory distress syndrome, probably as a result of enhanced and sustained granulocyte adhesiveness followed by release of toxic granulocyte-derived mediators (16,40). Immune complexes, either alone or accompanied by complement activation, may also promote granulocyte influx, adhesiveness, respiratory burst activity, and degranulation, contributing to vasculitis and autoimmune inflammatory reactions. Hyperreactive oxygen metabolites produced by the respiratory burst are able to damage DNA and cause endothelial damage, resulting in aging and atherosclerosis. Cationic proteins released during degranulation of granulocytes are also involved in the cytotoxicity. Interferon-like substances released by granulocytes have been implicated in the inhibition of virus replication. Furthermore, granulocytes have been shown to mediate antibody-dependent cellular cytotoxicity (ADCC) against tumors and viruses, especially in the presence of cytokines such as interferon and GM-CSF. Defects or abnormal activation in any aspect of these granulocyte responses may result in immunodeficiency diseases or serious inflammatory disorders. For patients with life-threatening granulocyte function deficiency, transfusion of granulocytes may be required. In contrast, patients with inflammatory disorders, such as ulcerative colitis, may require granulocyte/monocyte apheresis (41).

### 82.3.5 Functions of Monocytes/Macrophages

Monocytes may develop into different forms of dendritic cells and macrophages, possessing diverse morphologies and functions depending on environmental and immunologic factors. Mononuclear phagocytes, similar to polymorphonuclear granulocytes, can move toward foreign invaders and denatured tissues and destroy them by ingestion, degranulation, and respiratory burst activity. On maturation into macrophages, cells of the monocyte series show an increase in size, adhesiveness, and functional receptors (e.g. CR1, CR3 FcRI and FcRII), resulting in enhancement of phagocytosis (29,42). Studies have shown that cells of the monocyte series have relatively low microbicidal activity compared to granulocytes. Tissue macrophages usually lose their MPO, which mediates a potent microbicidal activity by catalyzing halides into hypohalites. Loss of MPO may be a developmental milestone resulting in harmless contact between cells of the monocyte series and other immune cells required for antigen presentation. Increases in lectin-like receptors, as well as receptors for lipoproteins, are found mainly on macrophages but not on monocytes. Defects in the former may impair nonimmunologic reticuloendothelial clearance, whereas dysregulation of lipoprotein uptake by macrophages may give rise to atherosclerosis.

Apart from the professional function of “phagocytosis,” cells of the monocyte series have a central role in antigen presentation as well as modulation of inflammation and tissue repair. Dendritic cells that derived from monocytes or other lineage cells are particularly effective in antigen presentation to T cells (43,44). They are termed professional antigen-presenting cells (30). Monocytes and their derived dendritic cells bearing Toll-like receptors (TLRs) are responsible for bacterial and viral recognition and signal transduction, as these cells serve as a link between innate and adaptive lymphocyte differentiation and activation (45–47). Monocytes recruited into the inflammatory area are able to release monokines, including IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12, IFN- $\alpha/\beta$  and TNF- $\alpha$ , to enhance lymphocyte infiltration and to present antigens to lymphocytes, resulting in lymphocyte transformation (46,48–51). On activation, monocytes ingest particles, produce reactive oxygen molecules, and release procoagulant factors and cytokines, as well as increase the expression of adhesive molecules and class II human leukocyte antigens (HLA). Procoagulant factors are involved in coagulation and homeostasis. Granules of the monocyte series contain lysozyme, proteases, elastase, and collagenase, which augment tissue damage. In contrast, granules containing  $\alpha_1$ -protease inhibitor,  $\alpha_2$ -macroglobulin, and IL-1 inhibitors may offer a mechanism to protect against tissue damage. Cytokines from cells of the monocyte series act in an endocrine (affecting distant cells by blood transportation), a paracrine (affecting adjacent cells), and an autocrine (affecting themselves) manner (23). For instance, IL-1 has an endocrine effect on the regulation of body temperature and sleep pattern in the hypothalamus. Paracrine cytokines from the monocyte series include GM-CSF, G-CSF, monocyte colony-stimulating factor (M-CSF), erythropoietin, TNF- $\alpha$ macroglobulin, and IL-1 inhibitors may offer a mechanism to protect against tissue damage. Cytokines from cells of the monocyte series act in an endocrine (affecting distant cells by blood transportation), a paracrine (affecting adjacent cells) and an autocrine (affecting themselves) manner (23). For instance, IL-1 has an endocrine effect on the regulation of body temperature and sleep pattern in the hypothalamus. Paracrine cytokines from the monocyte series include GM-CSF, G-CSF, M-CSF, erythropoietin, TNF- $\alpha$ , and TGF- $\beta$ , which are all known to be involved in immune regulation. Some of the cytokines also have an autocrine effect on the monocyte series themselves. For example, ligation of M-CSF to its receptor (a c-fms proto-oncogene product) on monocytes induces monocyte differentiation, activation, and proliferation. Furthermore, cells of the monocyte series are the only cell population in the phagocyte series that express both major histocompatibility complex (MHC) class I and MHC class II antigens. Antigens are taken up by the monocyte/macrophage series and processed via biochemical and physical reactions. These antigens are presented in a peptide form associated with MHC molecules, which

lymphocytes recognize as antigen–MHC molecular complexes. Cells of the monocyte series bearing the antigen–MHC class I complex can be recognized and killed by cytotoxic T cells, whereas those bearing antigen–MHC class II molecules interact with helper T cells, resulting in clonal expansion of antigen-specific lymphocytes. Cytokines such as IL-2, IL-4, IL-5, IL-13, or IFN- $\gamma$  from lymphocytes act in a paracrine manner to amplify monocyte functions. Eicosanoids such as prostaglandin E<sub>2</sub>, cytokines (e.g. TNF- $\alpha$ , TGF- $\beta$ ), and  $\alpha_2$ -macroglobulin from the monocyte series have an immunosuppressive effect. Dysregulation of the antigen-presenting process may cause immunodeficiency and autoimmune diseases.

## 82.4 GRANULOCYTE FUNCTION DISORDERS

### 82.4.1 Congenital Granulocytopenia (Growth and Differentiation Defects)

Granulocytopenia may result from a decrease in bone marrow production or an increase in peripheral destruction of cells by autoantibodies or the reticuloendothelial system. Congenital granulocytopenias are due to a decrease in bone marrow production resulting from defects in the growth and differentiation of granulocytes. Empirically, a blood granulocyte level  $<1500/\text{mm}^3$  is defined as granulocytopenia, and  $500/\text{mm}^3$  is usually considered severe granulocytopenia. It should be noted, however, that a baseline blood granulocyte level  $<1500/\text{mm}^3$  is not applicable to all populations. Approximately 25–50% of African descent and some ethnic groups in the Middle East have neutropenia not associated with an increased risk of infection called *benign ethnic neutropenia*. The white blood cell (WBC) count response to intense exercise, which reflects neutrophil demargination in response to catecholamine release, has also been found to be lower in people of African descent than in those of European descent (52). Importance of the different baseline value lies in the fact that it can lead increased blood testing, treatment interruptions and discontinuation. A single nucleotide polymorphism (SNP) that is associated with race is DARC rs2814778. DARC encodes the Duffy antigen receptor for chemokines and the common African variant confers protection from *Plasmodium vivax* infection and has a low WBC count (53). This variant explained approximately 20% of population variation in WBC.

### 82.4.2 Granulocytopenia

**82.4.2.1 Clinical Features.** Patients with congenital granulocytopenia usually have recurrent infections such as omphalitis, septicemia, and abscess formation early in life. Those who survive infancy frequently show progressive periodontitis.

**82.4.2.2 Genetics and Pathogenesis.** Several congenital (primary) granulocytopenias have been described, including SCN, cyclic neutropenia, and other syndromic

neutropenias (Table 82-1). Genetic agranulocytosis (Kostmann syndrome) was first identified as an autosomal-recessive disorder by Kostmann (2), but it took 57 years to find the genetic defect in the families described by Kostman (54). Reticular dysgenesis is due to failure of bone marrow production of blood stem cells, resulting in pancytopenia (55,56). The Shwachman syndrome is characterized by pancreatic insufficiency, granulocytopenia, and failure to thrive (57). Cyclic granulocytopenia occurs in cycles, with periods of normal granulocyte counts of approximately 3 weeks, followed by granulocytopenia secondary to failure of maturation lasting approximately 1 week (58). It is now known that a variety of genes that can regulate granulocyte growth and differentiation at different levels can also affect the production of mature granulocytes.

**82.4.2.3 Diagnosis and Differential Diagnosis.** The diagnosis of congenital granulocytopenia is based on a history of recurrent infections early in life associated with persistent granulocytopenia of  $<1500/\text{mm}^3$ . Some patients with congenital granulocytopenia may, however, have a benign or cyclic course and remain asymptomatic until childhood. For instance, patients with cyclic granulocytopenia have a variable course, from spontaneous recovery to recurrent infections associated with periodontitis. Shwachman syndrome is often confused with cystic fibrosis because of malabsorption and recurrent infections. In addition, nutrition, infections, drugs, irradiation, malignancy, hypersplenism, and even metabolic disorders may also cause granulocytopenia, so that one needs to exclude the possibility of a secondary disorder before making a diagnosis of congenital granulocytopenia.

**82.4.2.4 Management.** Most patients with genetic granulocytopenia tend to present with severe or fatal infections early in life. Bone marrow or stem cell transplantation from HLA-matched donors or T-cell-depleted haploidentical donor marrow may be able to rescue these patients if the diagnosis is established as early as possible. For those who survive into infancy or childhood with chronic and recurrent infections, the administration of growth factors such as GM-CSF, G-CSF, and a chimeric protein conjugating IL-3 with GM-CSF may be of benefit (50,59,60). Some patients responded to the treatment with growth factors, but certain patients have not shown any effect. For nonresponders, BMT or human umbilical cord blood stem cell transplantation may be required. Transfusion of irradiated granulocytes may be lifesaving in patients with overwhelming infections. In addition, early intervention with antibacterial and antifungal prophylaxis may be important as patients with granulocytopenia are more susceptible to infection with extracellular organisms, including bacteria and fungi.

### 82.4.3 Leukocyte Adhesion Disorders

Leukocyte trafficking from the bloodstream to the injured tissues is a multistep process (61). First, the leukocytes

**TABLE 82-1 Congenital Neutropenias**

<b>Severe Congenital Neutropenia (SCN)</b>				
<b>Gene</b>	<b>Inheritance</b>	<b>Protein Function</b>	<b>Disease</b>	<b>References</b>
ELA2	AD/sporadic	Azurophilic granule serine protease	SCN/cyclic neutropnia	(272) (273) (274)
HAX1	AR	Mitochondrial HCLS1-assoc.protein	SCN with neurological symptoms	(54) (275)
GFI1	AR	Myeloid/stem cell transcription factor controls ELA2 expression	Neutropenia, lymphopenia	(276)
WAS	X-linked	Activating mutations in Wiskott Aldrich protein	X-linked neutropenia	(277) (278)
G6PC3	AR	Glucose-6-phosphatase catalytic subunit 3	Neutropenia, dysmorphic features, organ malformations	(279)
SLC37A4	AR	Glucose-6-phosphate transporter	SCN with type-1 glycogen storage disease	(280) (281)
<b>Syndromes with Neutropenia</b>				
CHS1/LYST	AR	Intracellular trafficking	Chediak–Higashi: enlarged lysosomes, neutropenia hypopigmentation	(91) (92) (93)
AP3B1	AR	Intracellular trafficking	Type 2 Hermansky–Pudlak syndrome Hypopigmentation, decreased cytotoxicity	(209)
RAB27A	AR	Intracellular trafficking	Griscelli syndrome Hemophagocytic syndrome	(215)
p14/ROBLD3	AR	Endosomal adapter	Hypopigmentation, lymphoid immunodeficiency, growth failure	(282)
VPS13B	AR	Intracellular trafficking	Cohen syndrome: intermittent neutropenia with skeletal abnormalities, psychomotor retardation	(283)
RMRP	AR	RNase MRP	Cartilage hair dysplasia: hypoplastic hair, skeletal dysplasia, immunodeficiency	(284)
AK2	AR	Mitochondrial phosphotransferase	Reticular dysgenesis: SCN with severe combined immunodeficiency hearing loss,	(285) (286)
CXCR4	AR	Chemokine receptor	Warts, hypogammaglobulinemia immunoideficiency, myelokathexis	(85)
SBDS	AR	Ribosomal protein	Schwachmann–Diamond syndrome: skeletal, pancreatic, hepatic cardiac disease	(287)
TAZ1	X-linked	Phospholipid transacylase	Barth syndrome: skeletal and cardiac myopathy	(288)
mtDNA	Mitochondrial	mtDNA	Pearson syndrome: bone marrow failure, neuromuscular and exocrine pancreatic insufficiency	(289) (290) (291)

tether to the activated vascular endothelial cells expressing selectins. The leukocytes then roll on the vascular endothelium. This rolling is mediated by fucosylated ligands. The rolling slows down the leukocytes allowing tight binding to the endothelium and eventual emigration into the tissues.  $\beta 2$  integrins expressed on the leukocyte surface mediate the tight binding to the endothelium. Rolling adhesion also enables the adherent leukocytes to become activated by locally presented chemokines. Leukocyte activation leads to further integrin (e.g. LFA-1, CD11a/CD18 and Mac-1, CD11b/CD18) expression. Leukocyte integrins interact with immunoglobulin-like counterreceptors on endothelial cells (ICAM-1, ICAM-2) to strengthen the adhesion and promote the transmigration of cells from the circulation into the underlying tissues. Leukocyte adhesion deficiency disorders are characterized by the inability of leukocytes to emigrate from

the circulation to the sites of injury. LAD-I is caused by mutations in the gene encoding the common  $\beta$ -chain of the  $\beta 2$  integrin family (or CD18). LAD-II deficiency in fucosylation of, among other molecules, the adhesion molecules is required for leukocyte rolling. LAD-III is caused by the deficient cytokine activation of the adherent leukocytes leading to diminished integrin expression.

The first LAD was characterized as the deficiency in a family of surface glycoproteins called the CD11/CD18 complex (62). The CD11/CD18 family of glycoproteins share an identical  $\beta$  chain (CD18, 95 kDa) and separate  $\alpha$  chains: CD11a (180 kDa), CD11b (165 kDa), and CD11c (150 kDa). CD11a/CD18, also called leukocyte function antigen-1 (LFA-1), is present on all leukocytes and facilitates lymphocyte adhesion, natural killing, T-cell cytotoxicity, and helper activity. CD11b/CD18, also called Mac-1 or CR3 and found on granulocytes, monocytes,



and macrophages, is a receptor for iC3b and mediates phagocyte adhesion-related functions and ADCC. CD11c/CD18, also called CR4, is found on monocytes and granulocytes and mediates functions similar to those mediated by CR3. Granulocytes from patients with CD11/CD18 complex deficiency, called LAD-I, show poor adhesion-related functions, including adhesion to endothelial cells, chemotaxis, and ADCC, as well as iC3b-mediated phagocytosis, particle-induced respiratory burst activity, and degranulation (63). In contrast, LAD-II is characterized by defective expression of selectin leukocyte ligand, resulting in poor homotypic aggregation and migration (64,65). LAD-II granulocytes are activated normally with upregulation of integrin molecules, but not with certain fucosylated antigens, such as the sialyl Lewis X blood group (sLex) and cutaneous lymphocyte antigens, which are missing on leukocytes (66). Another LAD called LAD-III has been deciphered involving a signal defect in Rap1 activation that results in poor integrin expression (67).

**82.4.3.1 Clinical Features.** Patients with LAD-I frequently have delayed separation of the umbilical cord, perirectal abscesses, and recurrent staphylococcal and gram-negative bacterial infections. Patients surviving infancy often have progressive periodontitis and gingivitis. Lymphocyte function in LAD-I patients is also impaired as CD18 is actively involved in specific interactions between lymphocytes and antigen-presenting cells. The severity of the clinical course is variable. Severe LAD-I often leads to death in infancy, whereas patients with moderate LAD-I survive into adulthood. The severity of infections is related to the degree of CD18 deficiency: severe deficiency is caused by severely diminished (1% or less of normal expression) of CD18, and in moderate disease, the patients demonstrate residual (2.5–10%) CD18 expression (Fischer A et al. *Immunol Rev* 1988). Patients with LAD-II also have peripheral blood neutrophilia, recurrent infections, and periodontitis. They do not have delayed separation of the umbilical cord or impaired lymphocyte functions, and the infections are usually milder. They do, however, demonstrate mental retardation and craniofacial abnormalities, a leukocyte locomotion defect, and low leukocyte adhesion to endothelial cells, along with severe reduction in leukocyte rolling. Some blood group antigens dependent on fucosylation are decreased or absent; LAD-II patients have the very rare Bombay blood group phenotype (absence of the fucosylation-dependent H antigen) and are Lewis a and Lewis b-negative (66,68). Patients with LAD-III were first described as LAD-I variants who suffered from a bleeding tendency in addition to recurrent bacterial infections, resulting from an activation defect in all major integrin family members expressed on leukocytes and platelets (67). Leukocytes from LAD-III patients demonstrate severely diminished adhesion and normal rolling like in LAD-I, but also have platelet aggregation deficiency.

**82.4.3.2 Genetics.** LAD-I (OMIM 116920) is inherited in an autosomal-recessive manner. Crowley et al. (69)

first reported its recessive inheritance and association with a missing glycoprotein. Parents and heterozygous siblings of patients express about half-normal amounts of these adhesive glycoproteins on their phagocytes. The disorder is the result of a deficiency in the synthesis of the CD18 molecule, resulting in defects in CD11–CD18 coupling as well as translocation of the complex onto the cell surface (63). Data from molecular studies have demonstrated that the gene coding for the synthesis of CD18 (or Integrin  $\beta$ 2; ITGB2) is located on chromosome 21q22.3 (70). LAD-II (OMIM 266265; or Congenital Disorder of Glycosylation, Type IIc, CDG2C) is caused by mutations in the SLC35C1 gene encoding a Golgi ADP-fucose transporter (71). It is inherited in an autosomal-recessive manner, resulting in deficiency of the selectin-binding ligand, sLex antigen. LAD-III is caused by a homozygous mutation in the FERMT3 (or KINDLIN3) gene (72). In LAD-III, the function of integrins on both leukocytes and platelets is disrupted because of the “inside-out signaling” defects in cytokine activation-dependent alterations of surface integrins that enable high-avidity binding to ligands on endothelium and platelets (73).

**82.4.3.3 Diagnosis and Differential Diagnosis.** Granulocytes and monocytes obtained from patients with LAD-I do not adhere well on plastic or glass plates or umbilical cord endothelial cells. A definite diagnosis of the disorder is based on flow cytometric analysis of cell membrane glycoproteins, using specific monoclonal antibodies. The patient’s phagocytes have normal HLA and CR1 antigen expression, but low to very low levels (0–10%) of CD11/CD18 glycoproteins on the cell surface (63). Leukocytosis with a predominant granulocytosis in the range of 20,000–100,000/mm<sup>3</sup> is common. The granulocytosis is most likely due to invasion by microorganisms, which result in complement activation and the production of other hemopoietic stimulants. This leads to an increase in bone marrow production and the egress of granulocytes into the blood circulation, where they cannot adhere or marginate on the endothelial layer (16). The granulocytosis, with almost all mature granulocytes, should be differentiated from a leukemoid reaction, in which a shift to the left in granulocyte morphology is prominent. Phagocytes obtained from patients with LAD-I reveal not only abnormal adhesion but also abnormal chemotaxis and phagocytosis, as adhesion is a prerequisite step for movement and engulfment. Thus, patients with LAD-I have quite similar clinical symptoms and findings to those with chemotactic or phagocytic defects. In general, however, no granulocytes are seen within their infections, so that there is no pus.

Differential diagnosis between LAD-I and LAD-II can be made by flow cytometric analysis of CD18 expression and by the assay of granulocyte adhesion to activated endothelial cells. Granulocytes from patients with LAD-I do not express CD18 on the cell surface. Granulocytes from LAD-II patients have normal CD18 expression but abnormal adhesion to IL-1-activated endothelial cells

(65,66), and their red blood cells have the characteristic Bombay (hh) phenotype. Antibodies directed against sLe<sup>x</sup> (CD15), such as the 7C3 antibody, can be used for analysis of sLe<sup>x</sup> expression (66). Leukocytes with LAD-III have impaired Rap1 activation associated with defective expression of all major adhesion molecules, and a bleeding tendency in the presence of normal platelet numbers (67).

**82.4.3.4 Management.** Patients with LAD-I can be effectively treated by BMT, the success rate for which has increased to around 70% (74). The possibility of employing gene therapy in the treatment of this disorder is currently being explored (75). Patients with moderate LAD-I phenotype usually respond well to prophylactic antibiotic therapy. Patients with LAD-II tend to have less-severe recurrent infections. They have prominent periodontitis but do not suffer intractable infections when appropriate antibiotic treatment is employed. These patients also have mental retardation that might dominate the phenotype past childhood. BMT or gene therapy has not proved to be a suitable approach in LAD-II. Fucose supplementation, however, might work in patients with the mutations leading to decreased fucose affinity transporter (76). Similarly, only a few LAD-III patients have been reported. LAD-III patients need antibiotic prophylaxis as well as blood transfusion as it is associated with a severe bleeding tendency and repeated infections. BMT is the only curative therapy.

## 82.4.4 Disorders of Granulocyte Chemotaxis

Granulocyte chemotaxis involves an organized ligand-receptor signal coupling response. A defect existing at any level in the response can affect granulocyte locomotion and chemotaxis, so that extensive heterogeneity among these disorders exists.

**82.4.4.1 Clinical Features.** In general, these patients suffer from cutaneous abscesses, sinopulmonary infections, chronic candidiasis, and other tissue infections. Patients with phagocyte chemotactic deficiency can be classified into five different categories based on the level of the defect.

### 82.4.4.2 Genetics.

**82.4.4.2.1 Defects at the Ligand Level.** A major physiologic ligand inducing phagocyte chemotaxis in human beings is C5a, a fragment of complement activation. Patients with a familial deficiency of complement components—C1, C2, C4, properdin, factor B, C3, and C5—may be unable to produce adequate amount of C5a. These complement components are genetically controlled by an autosomal-recessive or -codominant inheritance, except for properdin deficiency, which is an X-linked disease. Leiner disease is characterized by seborrheic dermatitis, recurrent gram-negative bacterial infections, intractable diarrhea, and failure to thrive (77). Patients with this syndrome are reported to have normal C5 levels,

but have C5 dysfunction associated with a humoral chemotactic defect (78). Patients with Leiner disease show spontaneous remission after 2 months of age. This disease has been found in both twins and consecutive family members, so that a genetic disease has been proposed. The fact that this disease is limited to breast-fed babies may, however, suggest a multifactorial disorder.

In the autosomal-recessive Papillon-Lefevre syndrome, loss-of-function mutations in cathepsin C result in periodontal disease and palmoplantar keratosis. Loss of cathepsin C leads to the failure to activate neutrophil serine proteases that, in turn, prevent the proteolytic degradation and inactivation of MIP-1 $\alpha$ . The loss of proteolytic cleavage of the chemokine MIP-1 $\alpha$  results in the dysregulation of the local inflammatory response and tissue destruction (79–81).

### 82.4.4.2.2 Defects in Cell-Surface Receptors.

Defects in chemotaxis at the membrane receptor level include the previously mentioned deficiency of the adhesive molecules (CD11/CD18 complex deficiency). Granulocytes with adhesive-glycoprotein deficiency are unable to adhere to endothelium or interstitial tissue, so that they show poor chemotaxis both in vivo and in vitro. Delayed separation of the umbilical cord, followed by the onset of recurrent tissue infections in which pus cells are absent, is characteristic of these patients. Juvenile periodontitis usually occurs in adolescents who have impaired chemotaxis and localized periodontitis and extraoral infection (82,83). Limited evidence suggests that this syndrome is due to a defect in a 108-kDa surface glycoprotein and transmitted by an autosomal-codominant trait (82).

WHIM syndrome is an autosomal-dominant disorder of Warts, Hypogammaglobulinemia, Infections, and Myelokathexis (84). WHIM patients have numerous warts on hand, feet and trunk as well as genital and anal condyloma acuminata, and cervical and vulval premalignant dysplasia due to papilloma virus (HPV) infections. The marked hypogammaglobulinemia results in recurrent upper respiratory infections, such as sinusitis, tonsillitis, otitis media, pneumonia. Myelokathexis refers to the retention of white cells in the marrow causing neutropenia, the bone marrow is hypercellular but there is an associated neutropenia. WHIM syndrome is caused by heterozygous gain-of-function mutations in the gene encoding CXCR4 chemokine receptor (85). WHIM syndrome is the first example of a human disease caused by dysfunction of a chemokine receptor. The neutropenia of WHIM syndrome is unique in that it responds to acute infection, and G-CSF, GM-CSF, epinephrine, or glucocorticoids and the neutrophil counts may reach normal levels within hours, hence the infections are usually not life threatening.

**82.4.4.2.3 Defects in the Mechanics of Cell Movement.** Granulocyte chemotactic deficiency due to cytoskeleton organization defects is seen in actin dysfunction. Granulocyte actin dysfunction, an autosomal-recessive disorder, usually results in a chemotactic defect and a defect in ingestion, as both require cytoskeleton

reorganization and actin polymerization. Miller et al. (86) described a primary disorder of granulocyte movement with what appeared to be a hereditary basis. Three children in two families presented with a symptom complex of congenital ichthyosis and *Trichophyton rubrum* infections. Granulocytes obtained from the patients and their fathers showed abnormal directed movement but normal random motility. On further investigation, it was found that each father had been troubled with chronic cutaneous candidiasis throughout most of his life. These findings suggested a familial chemotaxis defect with autosomal-codominant traits.

**82.4.4.2.4 Defects Associated with Impaired Degranulation.** Granulocytes with specific granule deficiency, an autosomal-recessive disease, have a chemotactic receptor recycling defect resulting in a chemotactic defect (87). Granulocytes from patients with Chédiak–Higashi syndrome (CHS) have a chemotactic defect based on altered microtubule function and membrane regulation in which elevated cyclic AMP (cAMP) is prominent (88). Evidence showed that the CHS protein is widely distributed in the cytosol but not the membranes (89), suggesting that the CHS protein may act to regulate lysosomal fusion and membrane regeneration. This disease is transmitted as an autosomal-recessive trait, and is caused by homozygous or compound heterozygous mutation in the lysosomal trafficking regulator gene (LYST on chromosome 1q42.1–1q42.2 (90–93)). There are also some other syndromes similar to CHS demonstrating hypopigmentation and leukocyte function deficiency. Patients with Griscelli syndrome (GS) have a defect in myosin V, a molecule involved in organelle transportation (94). These patients also demonstrate partial albinism and cellular deficiency. The gene encoding the myosin V protein is located on chromosome 15q21 (94,95). Some forms of oculocutaneous albinism, may also have neutropenia (96): Type 2 Hermansky–Pudlak syndrome (HPS) is an autosomal-recessive disorder characterized by oculocutaneous albinism, bleeding disorders, recurrent infections, and neutropenia. The disease is caused by mutations in the AP3B1 gene encoding for the beta3A subunit of the adapter protein 3 (AP-3) complex. Because the expression of the beta3A subunit is normally ubiquitous, its deficiency leads to a phenotype in all cells with intracellular granules, such as neutrophils, natural killer cells, cytotoxic T lymphocytes, platelets, and melanocytes (97).

**82.4.4.2.5 Extrinsic Suppressive Factors.** Many extrinsic suppressive factors, such as immune complexes (98), steroids, and immunoglobulin (Ig)A paraproteins (99), can interfere with phagocyte chemotaxis. A common and life-threatening condition, sepsis, can lead to the suppression of neutrophil migration, and this “neutrophil paralysis” is directly related to the severity of the disease (100,101). The hyper-IgE syndrome (HIES or Job syndrome) (102) is usually associated with a granulocyte chemotactic defect (103) and high levels of IgE, altered T-cell activity (104), and low IFN- $\gamma$  and IL17 production

(105,106) The granulocyte chemotactic defect appears to have both intrinsic and extrinsic components although these have not been identified yet (107). HIES is inherited as an autosomal-dominant trait in certain families although most of the cases are sporadic (108–111). HIES is caused by mutations in STAT3 (112–116). Mutations in HIES are always heterozygous missense mutations or in-frame deletions; no homozygous mutations or nonsense mutations are seen. Homozygous Stat3-deletion is incompatible with life in mouse experiments, whereas the heterozygous littermates have no phenotype (117), suggesting that the cause of HIES is not haploinsufficiency, but a dominant-negative effect. The likely explanation of the dominant phenotype is that the STAT proteins work as dimers, and the nonfunctional mutant protein heterodimerize with the wild-type protein inhibiting its function. The HIES is a multisystem disorder affecting dentition, facial features, and immunity (108,109). The disease has also been reported in association with osteogenesis imperfecta (118). There is an autosomal-recessive HIES that is caused by mutations in DOCK8 (119), and TYK2 (120,121); despite the name, these patients have an entirely different disorder with no somatic (no bony and connective tissue problems) who also have recurrent viral infections that are not characteristic of the classical autosomal-dominant HIES. Granulocyte motility has not been studied in these patients.

**82.4.4.3 Diagnosis and Differential Diagnosis.** Patients with chemotactic disorders usually have similar clinical manifestations, with recurrent cutaneous abscesses, mucocutaneous candidiasis, pneumonia, and otitis media. These disorders may be assessed using an in vivo Rebuck skin window test (122), or by an in vitro Boyden chamber chemotaxis (103). A chemotactic defect due to deficiency of cell-surface receptors can be detected by flow cytometric analysis of adhesion molecules or by protein electrophoretic analysis of glycoproteins. CHS can be diagnosed by clinical features as this syndrome has a defect in degranulation, resulting in giant granules in granule-containing cells, partial albinism, and neurologic deficits (88). Chemotaxis defects due to extrinsic suppressing factors can be individually diagnosed based on individual deficits. For instance, patients with leprosy, cirrhosis, and sarcoidosis have been reported to have chemotactic defects based on the presence of a chemotactic factor inactivator. Several bacteria have been shown to produce inhibitors or enzymes capable of destroying complement-derived chemotactic factors, including *Capnocytophaga* (123) and group A and B streptococci (124). In addition, patients showing intractable eczema associated with recurrent cold abscesses should be tested for serum IgE to confirm the diagnosis of the HIES. A high titer of specific IgE directed against *Candida albicans* and *Staphylococcus aureus* is also suggestive of the disorder.

The HIES (Job syndrome) must be differentiated from atopic dermatitis associated with secondary infections



(125). Patients with HIES (102) frequently have cold abscesses in addition to intractable eczema and hyper-IgE (116,126,127). There are characteristic abnormalities in facial features in the patients (105). Secondary infections associated with atopic dermatitis are usually more superficial and show erythema and swelling, and are painful but not cold. Moreover, HIES (Job syndrome) patients often have severe sinopulmonary infections.

**82.4.4.4 Management.** Although complete correction of a granulocyte chemotactic defect has not been accomplished, partial correction in certain patients has been carried out in vitro and in vivo. C5 dysfunction in Leiner syndrome was reported to be corrected by replacement with fresh plasma that contained normal C5. Development of periodontitis and loss of teeth can be minimized in Papillon-Lefevre syndrome with close dental care and prophylactic antibiotics. The treatment of keratoderma is usually based on topical anti-inflammatory emollients and keratolytic agents. Severe pyogenic complications such as liver abscess are possible (128). Vitamin C has been shown to enhance chemotaxis of granulocytes obtained from patients with CHS (129). Studies from our laboratory have shown that IFN- $\gamma$  can enhance chemotaxis of granulocytes obtained from patients with HIES in vitro (130). A small limited clinical trial in four patients with HIES (131) suggested some beneficial clinical effects of IFN- $\gamma$ . Clearly, controlled clinical trials of this recombinant human cytokine are indicated in this disorder. High doses of vitamin C or E have also been reported to be beneficial in some patients. Other strategies, such as cromoglycate and intravenous immunoglobulin, also have been tried in some patients with this disease (132,133). We have also had a Job patient treated with peripheral blood stem cell transplantation for lymphoma, which corrected several of the immunologic features of the disease (134), but it failed in another case (135). More recently, Goussetis et al. (136) reported two unrelated boys with sporadic HIES complicated by high-grade non-Hodgkin lymphoma, who were cured with myeloablative HLA-matched sibling BMT. The autosomal-recessive Hyper-IgE or DOCK8-deficiency is successfully treated with BMT (137–139).

## 82.4.5 Disorders of Phagocytic Uptake

The mechanism by which phagocytes ingest microorganisms involves two distinct stages: adhesion and internalization. Adhesion of targets to phagocytes is through receptors such as Fc and complement receptors, as well as lectin-like receptors (16,23). As described earlier, membrane adhesive glycoproteins, including CR3 and the fibronectin receptor, have been shown to promote the adhesion process. Internalization results from a zipper process, in which circumferential attachment of phagocyte receptors to the ligands (targets) induces engulfment.

**82.4.5.1 Clinical Features.** Disorders of phagocytic uptake are usually secondary to infections, drugs, alcohol, and systemic diseases. Primary defects in phagocytic uptake are rare. Patients with LAD have defects in particulate-mediated phagocytosis (63). Granulocytes from a patient with actin dysfunction failed to polymerize actin, resulting in chemotactic and phagocytic defects (140).

**82.4.5.2 Genetics of Phagocyte Actin Dysfunction.** A congenital phagocytic defect occurs in actin dysfunction, a hereditary disorder with impaired actin polymerization during phagocytosis and movement. Granulocytes obtained from the patient's parents show retarded actin polymerization (141), although not the profoundly impaired actin polymerization seen in the patient, suggesting that this disease is an autosomal-recessive disorder. Some believe that this disorder may actually have been a case of LAD-I. WASP deficiency causes macrophage deficiency at several levels, including morphology (142), adhesion defects (143–146) chemotactic defects (147), and phagocytic defects (148–151).

**82.4.5.3 Diagnosis and Differential Diagnosis.** Most phagocytic defects are secondary to endogenous cytokines, exogenous drugs, and infections. Before making a diagnosis of an intrinsic phagocytic defect, one should rule out extrinsic defects, as well as other phagocyte function defects. A  $2 \times 2$  factorial design can be used to make a differential diagnosis. The patient's and control's granulocytes are incubated with bacteria opsonized with autologous or heterologous serum to differentiate intrinsic phagocytic defect from serum factor abnormalities. Latex-coated particles, as well as antibody- and complement-coated targets, are traditionally utilized to test phagocytosis under a microscope. Using a fluorescein isothiocyanate (FITC)-labeled technique, phagocytosis can be assessed with a flow cytometer. FITC-labeled bacteria ( $1 \times 10^7$  cfu/mL), which are heat-killed (70°C for 60 min) and labeled with 0.03% FITC (Sigma Co.) at room temperature for 2 h, are incubated with normal or patient's granulocytes ( $2 \times 10^6$  cells/mL) in the presence of 0.1 volume of human serum at 37°C for 30 min. Results are read with a flow cytometer after washing twice and fixing in 1% paraformaldehyde.

**82.4.5.4 Management.** Primary defects in phagocytic uptake are rare. Lack of evidence of the underlying defect in the minimal number of cases with primary phagocytic defects limits the approach to treatment in disorders of phagocytic uptake. Studies with normal granulocytes have recently shown that several cytokines and glycoproteins enhance motility and phagocytic uptake (152–154). Such cytokines and glycoproteins may be candidates for treatments in these phagocytic disorders. Treatment of patients with secondary phagocytic defects is based on the individual deficits. For example, patients with phagocytic defects due to opsonin deficiency, such as antibody, can be infused with intravenous gamma globulin. For those with a relative cytokine



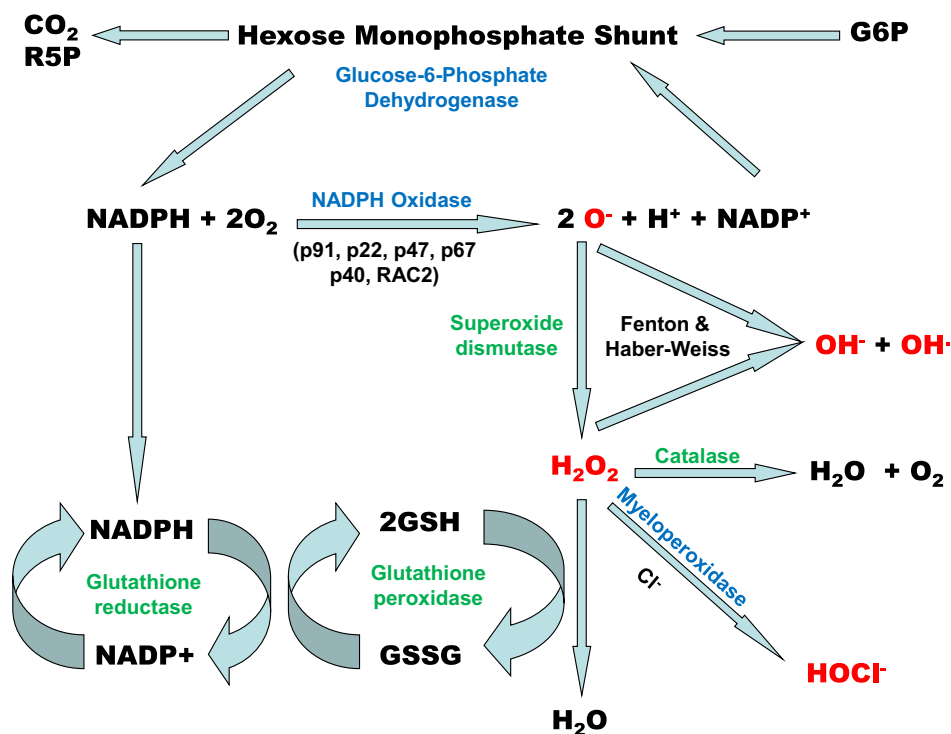
(e.g. IL-12, IFN- $\gamma$ ) deficiency, specific correction with recombinant cytokine may reverse the immune dysfunction (155,156). In addition, as with other granulocyte function disorders, patients with phagocytic defects may sometimes acquire life-threatening infections, so that antibiotics or granulocyte transfusion may be required in certain situations.

### 82.4.6 Disorders of Oxygen-Dependent Microbicidal Activity

In response to soluble or insoluble stimuli, granulocytes are induced to increase chemokinesis, adhesion, chemotaxis, and phagocytic uptake. In addition, these stimuli simultaneously cause respiratory burst activation and degranulation. Granulocytes with defects in respiratory burst activity usually have normal adhesion, chemotaxis, and phagocytic uptake, but abnormal intracellular microbicidal activity. These disorders include CGD, glucose-6-phosphate dehydrogenase (G6PD) deficiency, MPO deficiency, and glutathione

synthetase deficiency. Respiratory burst activity associated with phagocyte activation is elicited by NADPH oxidase, which is composed of a flavoprotein, a ubiquinone, and cytochrome b558 (Figure 82-1). The NADPH oxidase, in conjunction with certain cytosolic factors, serves to transport an electron from NADPH to  $O_2$ , resulting in  $O_2$ -formation (reviewed in Yang and Hill (23)). Defects in any component of the oxidase (seen in patients with CGD), as well as defects associated with generation of the cofactor NADPH (seen in severe leukocyte G6PD deficiency) or impaired NADPH regeneration (seen in glutathione synthetase deficiency), may cause a deficiency in oxygen-dependent microbicidal activity.

**82.4.6.1 Clinical Features.** Patients with defects in oxygen-dependent bactericidal activity suffer from recurrent cutaneous, organ, and tissue abscesses and recurrent sinopulmonary infections. Granulomas may form at the site of tissue infections, resulting in obstructive disorders involving the gastrointestinal and urinary systems. Lymphadenitis and osteomyelitis are not uncommon.



**FIGURE 82-1** Respiratory burst pathways in the phagocyte system. The hexose monophosphate shunt is a process that generates NADPH and pentoses (Ribulose-5-Phosphate or R5P) from glucose-6-phosphate (G6P). G6P dehydrogenase is part of the hexose monophosphate shunt, and a critical step for NADPH generation. NADPH oxidase generates superoxide anions ( $O_2^-$ ) from NADPH and oxygen. The neutrophil NADPH oxidase function requires six protein subunits (p91, p22, p47, p67, p40, and RAC2), mutations in five of which are known to cause chronic granulomatous disease. Upon phagocyte activation, cytosolic factors are translocated into the plasma membrane components composed of p91phox and p22phox. Hydrogen peroxide ( $H_2O_2$ ) is generated from superoxide anion by superoxide dismutase. Myeloperoxidase produces hypochlorous acid (HOCl) from  $H_2O_2$  and chloride anion ( $Cl^-$ ). Reduction of  $H_2O_2$  can also be mediated by iron (or copper) in the Fenton reaction generating hydroxyl radicals ( $\cdot OH$ ). The Haber-Weiss reaction (superoxide anion plus hydrogen peroxide) produces  $OH^-$  and  $\cdot OH$ . Antioxidative systems are shown in green. Antioxidative systems are critical as reactive oxygen species damage not only pathogens but cellular DNA and membranes as well. The reactive oxygen species are shown in red. Critical enzymes required for primary phagocyte defects are highlighted in blue. GSH is reduced glutathione, GSSG reduced glutathione.

Infections are most often due to catalase-positive microorganisms.

#### 82.4.6.2 Genetics.

**82.4.6.2.1 Genetics of CGD.** The phagocyte-specific NADPH oxidase (NOX2) is a member of a ubiquitous family of NADPH oxidases (NOX1-5, DUOX1-2). The neutrophil NADPH oxidase is composed of two membrane and four cytosolic components. Studies in a cell-free system indicate that superoxide production requires the participation of both membrane-bound oxidase (NADPH oxidase) and cytosolic factors. Two-thirds of CGD cases are transmitted in an X-linked recessive pattern owing to the absence or inadequate function of cytochrome b558 heavy chain (p91phox, encoded by CYBB), a component of the NADPH oxidase complex (also known as NOX2) located on the short arm of the X chromosome (Xp21.1). The rest are inherited by an autosomal-recessive mechanism and are due to the absence of cytochrome b558 light chain (p22phox, encoded by CYBA) or one of the cytosolic factors necessary for NADPH oxidase activation. The gene coding for cytochrome b558 light chain (p22phox) is located on chromosome 16q24, whereas those encoding the cytosolic factors p47phox and p67phox are located on chromosomes 7q11.23 (neutrophil cytosolic factor 1 or NCF1) and 1q25 (neutrophil cytosolic factor 2 or NCF2), respectively (10). The cytosolic p40phox and Rac2 GTPase are also required for the neutrophil NADPH oxidase function. p40phox stimulates phagocytosis-induced NADPH oxidase activity via a phox homology (PX) domain that binds to phosphatidylinositol-3-phosphate (PtdIns(3)P) that accumulates on phagosomes. Rac2 (22q13) is involved in the control of the neutrophil actin cytoskeleton, cell migration, in addition to the NADPH oxidase (157). Mutations in p47phox are found in about 30% of CGD patients. Mutations in p22phox and p67phox each result in <5% of CGD cases. Only one CGD patient is described with a mutation in NCF4 (neutrophil cytosolic factor 4; 22q13) that encodes p40phox (158). Rac2 mutations have not been reported as a cause of CGD, but one patient with mutation in Rac2 was reported (159,160); this patient presented with severe bacterial infections and poor wound healing and had a partial oxidase defect, impaired leukocyte migration, adhesion, degranulation and superoxide anion production, a clinical picture somewhat resembling that of leukocyte adhesion deficiency.

The nature (e.g. missense, nonsense, frameshift, deletion, splicing; (161,162)) and exact location of the mutations in the various components of the NADPH oxidase is critical. A recent study demonstrated a strong genotype–phenotype correlation in CGD as the residual NADPH oxidase activity determines the severity of morbidity and mortality (163). As the clinical presentation is varied and dependent on the degree of NADPH oxidase system deficiency, some CGD results in a less-severe phenotype presenting later in life. These variant CGDs

are more difficult to diagnose, but this form is becoming more readily recognized based on improved flow cytometry and DNA-based testing methods (164,165). It is also important to note that the phagocyte NADPH oxidase (NOX2) is not neutrophil specific as the NADPH oxidase is also expressed in monocytes, macrophages, B cells and dendritic cells, and the deficiency in these cells is not understood. It appears that some mutations in p91phox might mainly manifest as macrophage deficiency leading to X-linked predisposition to mycobacterial infection (166).

**82.4.6.2.2 Genetics of Leukocyte G6PD Deficiency.** G6PD deficiency is the most common enzyme deficiency in humans, with over 140 different mutations described (167,168). The most common mutations (seen in patients with Africans ancestry) lead a mild-to-moderate enzyme deficiency (20–60% activity) and to intermittent hemolysis usually secondary to infections or drugs. Few patients with severe forms of G6PD deficiency present with a CGD-like clinical picture. Profound deficiency of G6PD leads to a defect in NADPH production, causing impaired respiratory burst activity (Figure 82-1). The gene coding for this G6PD enzyme is located on Xq28 (169). The G6PD enzyme is a heterodimer, each monomer (molecular weight, 56,000) being associated with an NADP<sup>+</sup> molecule. This enzyme has a widely variant polymorphism in different populations in the world. Studies in blacks and whites have shown that whites with G6PD deficiency more often have affected leukocytes (170,171). G6PD deficiency in leukocytes is encoded by the same gene as that in erythrocyte precursors and other tissues. G6PD activities in leukocytes are usually higher than those in erythrocytes, so that patients with G6PD-deficiency-associated hemolytic anemia usually have normal leukocyte function. Only leukocytes with <20% (particularly <5%) of normal G6PD activity show abnormal bactericidal activity. Whether there are unknown factors in leukocytes from different races that could affect G6PD activity remains to be determined as patients with G6PD deficiency associated with the CGD-like syndrome have been reported in white, but not in black or Mongolian populations.

**82.4.6.2.3 Genetics of Glutathione Synthetase Deficiency.** As shown in Figure 82-1, glutathione levels are modulated by NADPH, glutathione reductase, and glutathione synthetase. Granulocytes with glutathione reductase or glutathione synthetase deficiency have a normal early respiratory burst, whereas subsequently continuous production of toxic oxygen products, which are normally handled by glutathione, results in auto-oxidative damage and defective microbicidal activity (172) (reviewed by Yang and Hill (173)). About 30 different mutations in the GSS gene have been identified. Genotype–phenotype correlations studies demonstrated that the mildly affected (based on enzymatic activity) patients have isolated hemolytic anemia, this is compounded with metabolic acidosis in moderately

affected patients, whereas the severely affected patients also have progressive dysfunction of the central nervous system. Excretion of 5-oxoproline in the urine is present in almost all patients (174). Both glutathione reductase and glutathione synthetase deficiencies are transmitted by autosomal-recessive inheritance. The gene coding for glutathione reductase is mapped to chromosome 8p21.1 (175,176). The gene encoding human glutathione synthetase maps to chromosome 20q11.2 (177). Patients with glutathione reductase deficiency can have hemolytic crises and usually abnormal phagocyte bactericidal activity. In contrast, patients with glutathione synthetase deficiency show abnormal bactericidal activity, in addition to having erythrocytes susceptible to hemolysis. Patients with the latter disease are more susceptible to bacterial infections because phagocyte activation is associated with an increase in intracellular hydrogen peroxide, with a low level of glutathione synthetase resulting in auto-oxidative damage to the cell.

**82.4.6.2.4 Genetics of MPO Deficiency.** Granulocytes in MPO deficiency have normal production of superoxide, but defective generation of hypochlorite ion. Patients with this disorder are not uncommon (1 in 2000 to 1 in 4000 in the general population), but are frequently asymptomatic or at most have delayed granulocyte killing activity and recurrent candidal infections (173,178). The MPO gene is located on chromosome 17q23.1 (179) that encodes the azurophilic granule-localized MPO. MPO uses heme as a cofactor to oxidize chloride to the +1 state forming HOCl. Candidal infections have been observed in MPO-deficient patients with diabetes mellitus, although the frequency is <5% of reported MPO-deficient patients (180). Some studies also demonstrated that complete MPO deficiency has a higher incidence of malignancies (180). MPO deficiency is inherited as an autosomal-recessive trait (181–183). A study of the MPO protein messenger RNA and complementary DNA simultaneously indicates the existence of diverse abnormalities in patients with MPO deficiency (184). Certain patients have MPO-expressing defects originating from a regulatory mutation that induces the MPO gene to switch off at an early stage of phagocyte differentiation (185).

**82.4.6.3 Diagnosis and Differential Diagnosis.** Patients with CGD have normal phagocytic uptake but abnormal oxygen-dependent intracellular killing, resulting in recurrent abscesses, lymphadenitis, and granuloma formation. Catalase-positive microorganisms, such as *S. aureus*, *Escherichia coli*, *Serratia marcescens*, *Aspergillus*, and *Candida*, are the common pathogens in patients with CGD and CGD-like diseases. In contrast, organisms such as streptococci seldom cause infection as the hydrogen peroxide generated by these organisms can interact with Cl<sup>-</sup> in the presence of MPO from phagocytes to form hypochlorite ion (OCl<sup>-</sup>), resulting in killing of bacteria. The laboratory diagnosis of CGD is usually made by assessing the phagocyte respiratory burst by

flow cytometric analysis using dihydrorhodamine123 (DHR) fluorescence (148). Nitroblue tetrazolium dye reduction should no longer be used for CGD diagnosis because of this tests failure to adequately screen for autosomal-recessive or variant-linked CGD.

A decrease in luminol-dependent chemiluminescence associated with elevated lucigenin-mediated chemiluminescence can be used to screen for patients with MPO deficiency, as lucigenin-mediated chemiluminescence is used to reflect superoxide production and luminol-mediated chemiluminescence is used to reflect MPO activity (186). In addition, assessment of granulocyte bactericidal activity using a bacterial colony formation assay is also applicable (3). The flow cytometric DCFH or DHR assay that is used for diagnosing CGD is positive in complete MPO deficiency, but eosinophils from MPO-deficient individuals retain eosinophilic peroxidase, and therefore, generate a normal DHR signal (187).

**82.4.6.4 Management.** The cellular and molecular bases of disorders related to oxidative microbicidal activity have been extensively explored (Figure 82-1). Pharmacologic approaches to treating CGD have been tried for three decades. Most of these therapies have been partially successful or controversial (188). Chronic prophylaxis with trimethoprim–sulfamethoxazole at 5 mg/kg of trimethoprim daily decreases the numbers of bacterial infections (189). In contrast, there is concern about an increased risk of fungal infections, especially *Aspergillus*. Fortunately, the data from Margolis et al.'s study (189) did not warrant this concern. Other troublesome infections for the CGD patients are due to fungi, especially *Aspergillus* lung and bone infections that usually require several months of antifungal therapy. An antifungal drug, itraconazole, has been used for *Aspergillus* prophylaxis and is reported to be effective (190). More recently, other azols, such as voriconazole, have utilized but are much more expensive.

Studies have also shown that recombinant IFN- $\gamma$  in a low dose (50  $\mu$ g/m<sup>2</sup>) administered subcutaneously three times a week significantly reduces serious infections in patients with CGD (191–193). Initial in vitro and in vivo studies in CGD patients suggested that phagocytes that do not normally release superoxide in response to stimuli did release small amounts of superoxide after treatment with IFN- $\gamma$  (191,193). This enhancement of superoxide generation by IFN- $\gamma$  treatment could not be confirmed in a large controlled trial, but there was a 70% reduction in infectious complications among the participants treated with this agent (192). BMT in CGD patients has been reported to result in long-term chimeric engraftment with a decrease in infectious complications (194). Despite important advances in supportive therapy, CGD remains a potentially lethal disease. The only curative approach, which is being utilized more frequently with success, is allogeneic stem cell transplantation. A recent approach with donor lymphocyte infusion after nonmyeloablative allogeneic peripheral blood stem cell transplantation

for CGD appears to be promising, showing fewer graft-versus-host reactions (195). Following detection of the genes coding for CGD (196,197), attempts at gene therapy for the disease have been made with vector gene transfer (198,199), but the first corrections have been short lived. Newer attempts demonstrated clinical benefits, such as successful clearance of *Aspergillus* infections, but one of the two patients developed a myelodysplastic syndrome and died (200–202). The latest gene therapy trial treated three gp91mutant patients and achieved long-term correction of neutrophil oxidase function in two (203).

Glutathione synthetase deficiency can be partially corrected by administration of vitamin E (204). Patients with MPO deficiency are usually asymptomatic; however, for those developing chronic candidiasis, ketoconazole or fluconazole can be used.

### 82.4.7 Disorders of Oxygen-Independent Bactericidal Activity

Oxygen-independent microbicidal defects can arise from the absence of granules or from defective degranulation. Several patients with an absence of specific granules have been described as specific granule deficiency. Studies with granule-deficient granulocytes show impaired bactericidal activity in addition to impaired chemotaxis (87). HPS, Chediak–Higashi syndrome and GS are all lysosomes and lysosome-related organelle deficiencies (205). All these diseases can also lead to neutropenia and are listed in Table 82-1. The reason for granulocyte dysfunction in these patients is the failure of granule constituents to fuse with the phagosome. The defective protein in Chediak–Higashi syndrome is involved in the lysosomal fusion, resulting in defects of both degranulation and membrane regeneration (89,206). This subsequently decreases oxygen-independent bactericidal activity and the expression of adhesion molecules and chemotactic factor receptors on the cell surface. CHS has a defect in transportation of granules, affecting all granule-containing cells, such as melanocytes, neurons, and granulocytes. This syndrome is characterized by partial albinism, granulocytopenia, neurologic deficits, recurrent infections, and poor ADCC against tumor cells. There are also defects in neutrophil chemotaxis and bactericidal activity. Another similar granule-transporting defect showing hypopigmentation and immunodeficiency, called GS, was first described in 1978 (207). It is now known to involve a defect of one of the two genes called MYO5A (myosin V) and RAB27A that control movement of melanosomes. MYO5A is expressed in the nervous system, but RAB27A is expressed in the skin and immune cells (208). Most GS cases are reported from Turkish and Mediterranean populations. Mutations in eight genes are known to cause HPS (HPS1–8). HPS2 is the only one HPS gene, in which mutations lead to immune deficiency (209). The secretory lysosomes of HPS-2 mutant cells fail to polarize upon activation (210).

**82.4.7.1 Genetics.** Patients with specific granule deficiency have autosomal-recessive inheritance caused by mutations in CCAAT/enhancer-binding protein epsilon (C/EBPE) gene (211–213). Heterozygous siblings or parents usually have a normal phenotype. Similarly, patients with CHS have an autosomal-recessive trait. Affected victims have parents with normal phagocyte function, but there may be a history of intermarriage. The human CHS gene maps to chromosome 1q42–q43 (90,214). GS involves albinism, granulocytopenia, and immunodeficiency encoded on chromosome 15q21 (95). The first genetic defect identified in GS (type 1; MIM 214450) was the gene, which encodes the actin-associated myosin Va motor protein (MYO5A). Subsequently, RAB27A was cloned for GS type 2 (MIM 607624; (215)). These two genetic defects have similar skin manifestations but distinct pathologic findings in the neurologic system (208). Griscelli syndrome type 3 (GS3) (MIM 609227) is caused by mutations in Melanophilin (MLPH; (216)).

**82.4.7.2 Diagnosis and Differential Diagnosis.** Diagnoses of the granule deficiency syndromes are based primarily on clinical manifestations as well as granulocyte morphology, with absence of specific granules (217). Furthermore, granulocytes from patients with specific granule deficiency do not release certain constituents from specific granules, and show a defect in membrane recycling of adhesion molecules and chemotactic factor receptors. The constituents inside specific granules, such as transferrin and vitamin-B12-binding protein, can be detected by enzyme-linked immunosorbent assay and radioimmunoassay, respectively (87). Three intracellular organelle trafficking defects have recently been identified, including CHS, GS, and oculocutaneous albinism (89,94). Patients with these syndromes all have hypopigmentation (albinism) and granulocytopenia. In addition to these symptoms, patients with CHS have recurrent infections, neurologic deficits, neoplastic transformation, altered membrane fusion associated with giant azurophilic granules, and defective chemotaxis, degranulation, and bactericidal activity (129). GS involves albinism and immunodeficiency, including neutropenia, thrombocytopenia, and T- and B-cell defects. Defects in MYO5A, known as GS-I, cause neurologic pathology in addition to skin hypopigmentation and immunodeficiency, and defects in RAB27A, known as GS-II, cause partial albinism and immunodeficiency but not neural deficits (208). Molecular diagnosis of CHS and GS may be available as the genes have been mapped to chromosomes 1q42–43 and 15q21, respectively (89,94). The third form of GS (GS-III) is restricted to hypopigmentation without immunodeficiency (216).

**82.4.7.3 Management.** There is no specific treatment for patients with deficiency of specific granules as only a limited number of cases have been described. Palliative treatment with antibiotic prophylaxis and granulocyte transfusion may be indicated. Bactericidal



defects in CHS have been shown to be improved following the administration of large doses of vitamin C. This may reduce the intracellular cAMP level, resulting in improvement in chemotaxis and bactericidal activity (129). Experimental evidence suggests that administration of G-CSF (10 µg/kg) can improve CHS-associated granulocytopenia, chemotaxis, and phagocytosis in an animal model (218). Currently, BMT may be the best way to treat patients with this syndrome although BMT only treats the hematologic and immunological defects, the neurological problems remain (219). In the future, the possibility of gene therapy for CHS and GS must be considered as the genes for these disorders have been well characterized as described earlier.

## 82.5 MONOCYTES/MACROPHAGES FUNCTION DISORDERS

Monocytes/macrophages share the same ancestor cells as granulocytes, so that most of the functional defects present in granulocyte function disorders occur in monocytes/macrophages, including LAD, phagocyte actin dysfunction, respiratory burst defects such as CGD, degranulation defects such as CHS, and specific granule deficiency. Monocytes/macrophages are also involved in antigen presentation, especially dendritic cells derived from monocytes that have TLRs on their surfaces that are used to recognize microbes and mediate signals. These serve as the link between innate and adaptive immunity (43,45,220). Primary macrophage function defects are relatively rare; most of the macrophage function defects are secondary. The function of monocytes/macrophages can be compromised by environmental agents, drugs, infections, and metabolic disorders resulting in functional defects. For instance, smoke, asbestos, silica, hyperbaric oxygen, and air pollution can impair macrophage function. In addition, monocytes/macrophages can be seriously impaired in patients with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS). Differential induction of cytokines may contribute to the resistance or susceptibility of macrophages to infections with intracellular organisms (156). Monokine enhancement of HIV infection and defective antigen presentation by monocytes have been implicated in patients with both AIDS and tuberculosis. CD40-ligand-mediated monocyte chemotactic proteins and RANTES production by monocytes and dendritic cells may contribute to suppression of HIV infections (221,222). Deficiencies of certain cytokines and their receptors, such as IL-12, IL-12 receptor, and IFN-γ receptor (IFN-γR), can cause impaired intracellular bactericidal defects resulting in mycobacterial infections and repeated salmonellosis (31,155,223,224). Monocytes obtained from patients with chronic mucocutaneous candidiasis show impaired chemotactic activity owing to defects in lymphocyte-derived cytokines. Similarly, monocytes obtained from a patient with common variable hypogammaglobulinemia

have been shown to have impaired antigen presentation to T cells (225), which could be a primary or secondary monocyte function defect. In summary, four types of primary macrophage function defects have been identified, including osteoporosis, TLR signaling deficiency, IFN-γR deficiency, and familial lysosomal storage diseases.

### 82.5.1 Clinical Features

Monocytes, dendritic cells, and macrophages have different functions in different tissues. Thus different monocyte/macrophage function defects have quite different clinical features. Osteopetrosis is due to dysfunction of osteoclasts (specialized macrophages), resulting in impaired resorption of mineralized cartilage and bone remodeling. The abnormal encroachment of sclerotic bone can compromise the nerve foramen and bone marrow space, resulting in neurologic and hematopoietic defects. Although monocytes from osteopetrosis patients may show normal or minimally impaired phagocyte function, BMT can rapidly reverse abnormal osteosclerotic changes and reconstitute immune function, which suggests that this specialized macrophage (osteoclast) defect may result in this disease (226). Pulmonary alveolar proteinosis (PAP) is characterized by pulmonary surfactant accumulation leading to respiratory distress and potentially respiratory failure (227). Surfactant catabolism requires the activation of alveolar macrophages by GM-CSF. PAP can be secondary but may also result from a primary genetic defect of the GM-CSF receptor α gene on the X chromosome (228–230).

Monocytes and dendritic cells have at least 10 TLRs (43,45,220). Different TLRs can recognize different microorganisms or microbial products, and mediate signals for innate immunity and adaptive T-cell differentiation (43,45,47). Patients with defects in different TLRs may be prone to certain types of infections. In an animal model, deficiency of TLR-4 results in altered allergic-type reactions (231). A defect in IL-12 production by the monocyte-series cells leads to impaired IFN-γ production, resulting in mycobacterial and *Salmonella* infections (155,223). The clinical features are also similar to those in patients with IFN-γR deficiency. The atypical tuberculosis in IL-12 or IL-12 receptor deficiency tends to be accompanied by granuloma formation (155,223). In contrast, patients with IFN-γR deficiency usually have poorly formed granulomas (31,224).

Another group of hereditary macrophage function defects include the metabolic storage disorders, especially those with lysosomal enzyme deficiencies. The accumulation of lipid or polysaccharide in the macrophages may lead to the development of foam cells and defective catabolic functions. The fact that metabolic storage diseases have been successfully treated by BMT (232) suggests that the macrophage catabolic defect may have a critical role in the pathogenesis of these diseases.

A number of primary immunodeficiencies that mainly manifest in other cells also affect monocytes and macrophages. Primary defects in neutrophils, such as CGD and leukocyte adhesion deficiency, often affect monocytes and macrophages. Some NADPH oxidase mutations, for example, might not even present as CGD, but as a monocyte macrophage defect (166,233). Chemotaxis defects such as Papillon–Lefèvre syndrome involve both myeloid lineages. Crohn’s disease is traditionally thought as an autoimmune disease, but some research suggests that CD could be primary immunodeficiency of macrophages: macrophages fail to attract neutrophils to mucosal injuries to ensure local acute inflammatory response and immunity leading to the development of chronic, granulomatous disease (234).

### 82.5.2 Genetics

Osteopetrosis is a hereditary disease transmitted by an autosomal-recessive trait (osteopetrosis congenita), which occurs in infants and children, but some cases are mediated by an autosomal-dominant trait (osteopetrosis tarda), which occurs in adults. The healthy bone is maintained by a dynamic balance of bone formation and resorption. The mesenchymal osteoblasts synthesize the bone and the hematopoietic-derived osteoclasts are responsible for bone resorption. The autosomal-recessive osteopetroses can be divided into two groups, one with a deficient number of osteoclasts and another with normal or increased number of nonfunctional osteoclasts. AR osteopetrosis patients die before the age of 6 years, mostly due to recurrent infections. This disorder is more commonly seen in the offspring of consanguineous parents. Five genes are known to be causative for AR osteopetrosis. The underlying gene in most patients is TCIRG1, which encodes the 116-kD subunit of the vacuolar proton pump (235), but mutations in the CLC-7 chloride channel (236), OSTM1 (Gray-lethal; (237,238)), PLEKHM1 (239), TNFSF11 (encoding RANKL (receptor activator of NF- $\kappa$ B ligand); (240)) or mutations in TNFRSF11A (encoding RANK (241)) all lead to osteopetrosis. Osteopetrosis due to mutations in RANK also leads to hypogammaglobulinemia (241). A rare X-linked recessive anhidrotic ectodermal dysplasia with osteopetrosis and immunodeficiency is caused by mutations in IKBKG gene (encoding NEMO, [MIM 300248]; (242)). An autosomal-dominant form of anhidrotic ectodermal dysplasia with T-cell immunodeficiency is caused by gain-of-function mutation of IKBA, resulting in impaired activation of NF- $\kappa$ B (243).

Studies in a mouse model of osteopetrosis indicate that a defect in the M-CSF gene contributes to abnormal bone resorption and macrophage differentiation (244). The M-CSF gene maps to chromosome 1p21 in humans, but was excluded as the disease-causing gene in patients with autosomal-dominant osteopetrosis (245). A gene close to the M-CSF gene in the 1p21 region is

proposed (245). Prenatal diagnosis by fetal sonography may be made through the recognition of sclerotic, dense, narrow, and radiopaque bones. Osteopetrosis can also accompany leukocyte adhesion deficiency type III caused by mutations in KINDLIN3 (FERMT3; (246)). Carbonic anhydrase II (CAII) deficiency syndrome is characterized by osteopetrosis (OP), renal tubular acidosis (RTA), and cerebral calcifications (247).

Primary immunodeficiency with pneumococcal and streptococcal infections due to TLR signal deficiency has been reported (248). A mutation of the interleukin-receptor-associated kinase-4 (IRAK-4) that is responsible for TLR activation can lead to repeated bacterial infections and poor antibody responses (249). The bridge between the TLRs and IRAK signaling complex is the myeloid differentiation factor (MyD)88. The clinical features of myeloid differentiation factor (MyD)88 deficiency are indistinguishable from IRAK-4 deficiency (250,251). All human TLRs other than TLR3 use both MyD88 and IRAK-4. Several IRAK members are responsible for IL-1 and TLR signaling, but it remains unclear whether all the IRAK family members are involved in TLR signaling and in the host defense against bacteria or viruses.

Macrophage phagosomes are both the site of elimination of mycobacteria and the site of mycobacterial replication. Mendelian susceptibility to mycobacterial diseases (MSMD) are caused by deficiencies in the IL-12/IL-23/IFN- $\gamma$  cytokines and their signaling pathways. IL-12 is produced by antigen-presenting cells in response to infection. IL12B encodes the p40 subunit shared by IL-12 and IL-23. IL12RB1, encodes the beta 1 subunit shared by the IL-12 and IL-23 receptors (IL-12Rb1). IL-12 induces IFN- $\gamma$  production, among others. IFNGR1, encodes the ligand-binding chain of the IFN- $\gamma$  receptor (IFN- $\gamma$  R1); IFNGR2 encodes the associated chain of the IFN- $\gamma$  receptor (IFN- $\gamma$ R2); STAT 1 encodes the signal transducer and activator of transcription-1 (STAT 1) in the IFN- $\gamma$  receptor signaling pathway. Interestingly, the autosomal-recessive form of STAT1 deficiency leads to susceptibility to both mycobacteria (IFN- $\gamma$ -mediated immunity) and viruses (IFN- $\alpha/\beta$ -mediated immunity), whereas the autosomal-dominant STAT1 deficiency leads to MSMD only. NEMO is a regulatory subunit of the NF- $\kappa$ B (NF $\kappa$ B) inhibitor kinase complex, and required for IL12 production, NEMO deficiency is an X-linked MSDS. Mutations in IFN- $\gamma$ R1 (OMIM 107470; (252,253)), IFN- $\gamma$ R2 (OMIM 147569; (254)), STAT1 (OMIM 600555; (255)), IL-12p40 (OMIM 151561; (155,256)), IL-12Rb1 (OMIM 601604; (257)), and NEMO (OMIM 300248) genes lead to susceptibility to mycobacterial and *Salmonella* infection. Tyrosine kinase 2 (TYK2) deficiency (OMIM 611521) leads to sensitivity to mycobacterial and *Salmonella* infections in addition to other viral, bacterial and fungal infection and elevated serum IgE (120); this likely represents an autosomal-recessive form of HIES.

Macrophage function defects in certain lipid storage diseases due to lysosomal enzyme deficiencies, such as Niemann–Pick disease, Gaucher disease,  $G_{M1}$  gangliosidosis type I, and Fabry disease, usually involve macrophage catabolic defects, with the formation of histiocytic foam cells containing mucolipids (e.g. sphingolipids) in the reticuloendothelial system, vessels, and visceral organs. Similarly, mucopolysaccharidoses, such as Hurler, Hunter, and Sanfilippo syndromes, have macrophage catabolic defects in addition to dystrophic storage in connective tissues. Mucopolysaccharidoses are also commonly transmitted by autosomal-recessive inheritance, except for Hunter syndrome (iduronate sulfatase deficiency), which is X-linked (Chapter 102).

### 82.5.3 Diagnosis and Differential Diagnosis

Patients with osteopetrosis are usually characterized by having a generalized increase in bone density throughout the body, failure to thrive, and hypocalcemia with low serum phosphate due to impaired bony resorption, as well as a degree of anemia, thrombocytopenia, and leukopenia. A defect in macrophage function could contribute to recurrent infections suffered by these patients in addition to abnormal bony resorption.

Patients with a mutation in IRAK4 and MyD88 are susceptible to bacterial infections, mostly by gram-positive bacteria (e.g. *Streptococcus pneumoniae* and *S. aureus*), with no decreased resistance to fungi, parasites, and viruses (250). Interestingly, those IRAK4 and MyD88 deficient patients who survive childhood improve with age.

A patient with defective TLR signaling associated with recurrent pneumococcal infections revealed impaired IL-6 and IL-12p40 induction by TLR agonists (248).

Patients with IL-12, IL-12 receptor or IFN- $\gamma$ R deficiencies tend to have a similar clinical picture, with disseminated BCG, atypical mycobacterial, and *Salmonella* infections (31,155,223). Patients with IL-12 deficiency can be diagnosed by a decrease in IL-12 production by peripheral mononuclear leukocytes after mitogen stimulation. IL-12 receptor deficiency can be diagnosed by flow cytometric analysis of IL-12 receptor expression on T cells and NK cells. Patients with IFN- $\gamma$ R deficiencies cannot be simply assessed by flow cytometric analysis of IFN- $\gamma$ R expression as many different variant forms have been identified (31,224,253,258). Defective CD64 expression, TNF- $\alpha$  production, and Jak-STAT phosphorylation may be better diagnostic criteria. Of course, direct gene sequence analysis or studies of polymorphism may offer better diagnosis and carrier detection and prenatal diagnosis (258).

The diagnosis of patients with lipidoses and mucopolysaccharidoses is usually based on clinical features,

with characteristic dysmorphism of the face, chest, and extremities, as well as mental retardation and hepatosplenomegaly. The presence of urinary mucopolysaccharides is characteristic. A definitive diagnosis should be based on specific enzyme assays employing cultured fibroblasts and leukocytes. Heterozygous carriers in some of these storage diseases can be detected by showing half of the normal amount of the lysosomal enzyme activity in cultured fibroblasts or leukocytes. Prenatal diagnosis using cultured amniotic cells obtained between 18 and 20 weeks' gestation is also available.

### 82.5.4 Management

Most of the primary macrophage function defects can be effectively treated by BMT, which provides normal macrophage precursors. In certain diseases, exogenous replacement of defective enzyme may be useful. For instance, Hurler syndrome, which is associated with a deficiency of  $\alpha$ -L-iduronidase, can be partially corrected by replacement with the normal enzyme. Takahashi et al. (259) showed that osteopetrosis in a mutant mouse model lacked functional M-CSF activity, which could be partially corrected with exogenous M-CSF. This is not the case, however, in humans with autosomal-dominant osteopetrosis (245). Patients with TLR signaling defects might possibly be treated through early administration of proinflammatory cytokines, such as IL-6 or IL-12, as these patients tend to have poor IL-6 and IL-12 induction (248,249).

Patients with IL-12 deficiency may be treated with exogenous IL-12 or IFN- $\gamma$  supplementation. Those with IL-12 receptor deficiency can be treated with exogenous IFN- $\gamma$  as this promotes intracellular killing of mycobacteria (155,223). Patients with IFN- $\gamma$ R deficiency frequently show persistent infections with atypical mycobacteria and disseminated BCG infections, which do not respond to antibiotics or IFN- $\gamma$  and TNF (31). On the basis of genetic polymorphism, carrier detection and prenatal diagnosis may be available (258), and gene therapy may be possible in the future.

Dietary calcium restriction and high-dose calcitriol have been used to stimulate host osteoclasts to resorb bone. High-dose corticosteroids, erythropoietin and parathyroid hormone can improve hematopoiesis and bone resorption, and hematopoietic stem cell transplantation is an option for osteopetrosis (260). Exogenous RANKL-induced formation of functional osteoclasts from their monocytes, suggesting that they could, theoretically, benefit from exogenous RANKL administration (240).

### 82.5.5 Prevention of Phagocyte Function Disorders

**82.5.5.1 Primary Prevention.** Phagocyte function disorders occur in X-linked, autosomal-recessive, and autosomal-dominant patterns of transmission or

sporadically. Most are X-linked or autosomal recessive. Primary prevention for such disorders is based on carrier detection. Young people should receive health examinations (including a complete family pedigree) before marriage and be aware of the risks of intermarriage. Carrier detection should be undertaken in major X-linked and autosomal-inherited disorders when available. Several techniques can be used to detect the carrier state: analysis of random X-chromosome inactivation, analysis of chromosome breakage or deletion, RFLP, single-stranded conformational polymorphism followed by direct sequencing, direct measurement of gene products, and cell function assays (261–263). There are several phagocyte disorders for which carrier detection is currently available. Carrier detection in the X-linked form of CGD can be established with a functional assay such as dihydrorhodamine fluorescence (264). LAD can be detected by flow cytometric analysis of the gene product (CD18) using monoclonal antibodies directed against CD11 or CD18 (63). Heterozygous siblings or parents show about half-normal amounts of the surface glycoproteins. Similarly, parents of affected offspring with IL-12 receptor  $\beta$ 1 deficiency reveal half-normal amounts of cell-surface receptor expression, indicating the possibility of carrier detection by flow cytometry. Heterozygotes in actin dysfunction syndrome show retarded actin polymerization as determined by flow cytometric analysis of NBD-phalloidin staining (141). Heterozygotes which carry a defective lysosomal enzyme gene encoding for a macrophage storage disease can be detected by measuring enzyme activity in cultured fibroblasts and leukocytes. It is likely that future studies will develop additional simple and rapid ways to detect the carrier states of other granulocyte function disorders. As the specific genes for these disorders are pinpointed, identification of the carrier state can be carried out using specific probes, sequence analysis, or PCR techniques, followed by gene polymorphism determination.

**82.5.5.2 Secondary Prevention.** Recent advances in molecular, biochemical, hematologic, and obstetric techniques have made early diagnosis and intervention for some of the congenital phagocyte disorders available. Chorionic villus sampling at 9–10 weeks of gestational age and fetal blood sampling at 16–20 weeks of gestational age, as well as sampling of peripheral blood from suspected patients and their family members, provide tissue for chromosome analysis, RFLP, single-stranded conformational polymorphism followed by direct sequencing, biochemical analysis, gene product measurement, and cell function assays (261–263,265,266). The gene coding for CGD is not critical for the development of the granulocytes, so that nonrandom X-inactivation does not occur in affected granulocytes. Employing the nitroblue tetrazolium dye reduction test preferably, or dihydrorhodamine fluorescence, CGD can be diagnosed prenatally at approximately 16–20 weeks' gestation (264).

Some individuals with the X-linked form of CGD arising from gene deletion may be detected prenatally using a DNA probe (10,262,267). The specific gene responsible for this disorder has also been determined, and prenatal diagnosis for the different genotypes of CGD has been made possible (261,267,268). Granulocytes as well as other granule-containing cells from patients with CHS have giant lysosomal granules, the characteristic morphology of which can be used to detect the syndrome in fetal cord blood samples at 18–20 weeks of gestational age. Similarly, fetal cord blood sampling may also provide the diagnosis of disease or carrier detection in LAD by measurement of the gene product (CD18 complex) in midtrimester. Leukocytes from cord blood or fibroblasts from amniotic cells can be cultured for detecting metabolic storage diseases by measuring suspected lysosomal enzyme activity. PCR (269) permits a prenatal diagnosis of  $\alpha$ 1-antitrypsin deficiency using fewer than 100 amniocytes or from small samples of fetal blood (270). An accurate prenatal diagnosis may be made in 1 day with this technique (267,268). Prenatally detected phagocyte function disorders may be managed by therapeutic abortion. Gene therapy may become available even for intrauterine therapy for fetuses with CGD or other genetic leukocyte function disorders. Recently, RD114 envelope pseudotyped vectors capable of transducing more than 95% of CD34+ stem cells have been developed (271).

**82.5.5.3 Management of Disease Complications (Tertiary Support).** Patients with phagocyte function disorders should receive physical, psychosocial, and rehabilitative support. Physicians should coordinate the support systems to assist in preventing infections, the development of physical dysfunction, and psychosocial problems. As discussed, BMT may occasionally be curative. Alternatively, antibiotic prophylaxis, chest physiotherapy, and surgical drainage may reduce sequelae. Regular visits to a dentist familiar with the problems suffered by patients with phagocyte function disorders are essential, especially for those with LAD. Patients with phagocyte abnormalities who suffer from recurrent infections since infancy usually develop psychological problems secondary to physical changes (skin scarring, coarse facies, hearing loss, dental decay, and/or pulmonary dysfunction), loss of schooling, or the recurring need for hospitalization or intravenous antimicrobial therapy. Caring for patients with phagocyte function disorders requires teamwork involving family, medical, and social support. The exciting prospect for these patients and their physicians is the future possibility of early diagnosis and genetic therapy to correct these disorders.

## CROSS REFERENCES

Mucopolysaccharidoses; Oligosaccharidoses, Disorders allied to the Oligosaccharidoses.



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### Biographies



**Harry R. Hill** Dr Hill is a Professor of Pathology, Pediatrics and Internal Medicine. He serves as the Head of the Division of Clinical Immunology at the University of Utah School of Medicine. Dr Hill is a Senior Vice President at ARUP, Inc., and the Executive Director of the ARUP Institute for Clinical and Experimental Pathology, which is the research arm of ARUP Laboratories with 90 scientists and 55 medical directors. After receiving his MD from Baylor School of Medicine, he received pediatric and immunology training at the University of Washington. Dr Hill then trained in immunology, infectious diseases, and clinical laboratory medicine at the University of Minnesota. He was also an Epidemic Intelligence Service officer with the CDC at the Streptococcal Disease Laboratory in Fort Collins, Colorado for 2 years. He was NIH-funded for work on group B, and more recently group A streptococcal disease for 30 years and has over 389 peer-reviewed articles, textbook chapters, and reviews. His clinical work involves seeing adult and pediatric patients with primary immunodeficiencies. Current research efforts are directed at the discovery of new molecular causes of primary immune deficiencies and the development of rapid diagnostic tests for these disorders.



**Kuender D. Young** Dr Kuender D. Yang from Taiwan has been devoted to the study of infections and immunity since 1980's. He did his doctoral dissertation on fibronectin-mediated signal transduction in phagocyte defense against bacterial infections (Yang et al., *J. Infect. Dis.* 1990, 161, 236–241; *J. Cell Physiol.* 1994, 158, 347–353) with Professor Harry R. Hill in the Departments of Pediatrics and Pathology at the University of Utah School of Medicine. He also did fellowship work in Molecular Pharmacology at the Dana Farber Cancer Institute, Harvard Medical School (Mentors: Don Kufe, MD and Richard M. Stone, MD; 1991–1992), and published a study on retinoic acid induction of PKC $\beta$  for monocytic differentiation in Blood (Yang et al., *Blood* 1994, 83, 490–496). Dr Yang was promoted to a full professor in 1997 at Tri-service General Hospital, Taipei, Taiwan. Since 1997, Dr Yang has joined Chang Gung University and been studying the immunopathogenesis of common emerging infections, including enterovirus 71, SARS, dengue hemorrhagic fever and novel swine H1N1 influenza, in Taiwan. He has published over 200 refereed articles. He has recently focused on studying genomic and epigenomic mechanisms of dengue hemorrhagic fever and perinatal programming of adult diseases, particularly allergic diseases, funded by the National Science Council, National Health Research Institute and National Genomic Research Program of Taiwan.



**Attila Kumánovics** Attila Kumánovics is a molecular genetic pathology fellow at the University of Utah. He received his MD from the University of Pécs School of Medicine in Pécs, Hungary and his residency training in clinical pathology and fellowship training in diagnostic immunology at the University of Utah School of Medicine in Salt Lake City, Utah, USA. He did research at the Howard Hughes Medical Institute and University of Texas Southwestern School of Medicine in Dallas, Texas, USA, and at the University of Utah School of Medicine in Salt Lake City, Utah, USA. His clinical and research interest is in the genetics of the immune system. He first studied the genes and genomics of the major histocompatibility complex and now concentrates on primary (genetic) immunodeficiencies.

# CHAPTER

# 83

## Genetic Disorders of the Pituitary Gland

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### 83.1 INTRODUCTION

The pituitary gland is composed of two embryologically, morphologically, and functionally distinct units, the anterior pituitary (adenohypophysis) and the posterior pituitary (neurohypophysis). Disease processes usually involve only one of these units unless both are affected because of their anatomic proximity or because of hypothalamic involvement. Thus, disorders of anterior and posterior pituitary functions are discussed separately in this chapter.

### 83.2 ANTERIOR PITUITARY

#### 83.2.1 Normal Pituitary Development and Function

The anterior pituitary is derived from an epithelial invagination of the roof of the posterior pharynx, Rathke's pouch. This mass of cells migrates upward toward the base of the brain to meet an outpouching of the third ventricle, the future posterior pituitary. The pituitary gland comes to lie in a bony cavity of the sphenoid bone known as the sella turcica. It is separated from the brain superiorly by the diaphragma sella, an extension of the dura mater. The pituitary stalk, composed primarily of neurohypophyseal tissue surrounded by nerves and blood vessels, passes through the diaphragma sella, connecting the gland with the hypothalamus. This intimate vascular connection between the hypothalamus and the pituitary allows sensitive hypothalamic control of pituitary function.

The anterior pituitary gland contains a number of distinct cell types responsible for the secretion of the pituitary hormones: growth hormone (GH), thyroid stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL), and melanocyte-stimulating hormone (MSH). The secretion of each of these is under the direct control of the hypothalamus. This hypothalamic control of pituitary secretion is mediated by a variety of hypothalamic-releasing hormones, which stimulate the secretion of the specific pituitary hormones (e.g. thyrotropin releasing hormone (TRH) stimulates TSH secretion), and hypothalamic inhibitory hormones, which inhibit the secretion of specific pituitary hormones (e.g. somatostatin (SST) inhibits GH secretion). The interplay between the specific releasing and the inhibitory hormones directly controls the secretion of each of the pituitary hormones. In turn, the secretion of the hypothalamic inhibitory and releasing hormones by the hypothalamus is modulated by a variety of humoral and central nervous system (CNS) factors. The pituitary hormones, once released into the plasma, exert their effects on their targets, either a specific endocrine gland (e.g. thyroid) or a variety of end organs. Because of the complexity of this hypothalamopituitary axis, a variety of pathogenetic mechanisms can operate at each level of the system, resulting in a widely heterogeneous group of disorders with similar symptoms of pituitary insufficiency (Table 83-1).

Isolated deficiencies of TSH, ACTH, LH, and FSH have been reported in Table 83-1. As the clinical symptoms produced are the result of the target organ hormone deficiency (e.g. hypothyroidism, hypoadrenalism, or



**TABLE 83-1 Selected Genetic Disorders that May Affect Pituitary Development or Function<sup>a</sup>**

Name of Disorder	OMIM	Mode	Locus	Gene/OMIM	Endocrine Features
Adrenal hypoplasia, congenital	300200	XL	Xp21.3-p21.2	NROB1 aka DAX1/300473	Primary adrenal insufficiency, XY sex reversal, hypogonadotropic hypogonadism
Adrenocorticotrophic hormone (ACTH) deficiency, isolated	201400	AR	1q23-q24	TBX19/604614	Central adrenal insufficiency
Alopecia, neurologic defects, and endocrinopathy syndrome	612079	AR	7q32.1	RBM28/612074	Pituitary hypoplasia, hypogonadotropic hypogonadism, central adrenal insufficiency
Anencephaly	206500	AR	?	?	Abnormal development of hypothalamus and pituitary
Autoimmune polyendocrinopathy syndrome, type 1	240300	AD	21q22.3	AIRE/607358	Involves parathyroid glands and/or adrenals; also pituitary, pancreas, gonads
Axenfeld–Rieger syndrome	180500	AD	4q25-q26	PITX2/601542	Pituitary abnormalities and growth hormone deficiency
Borjeson–Forssman–Lehman syndrome 301900 XL Xq26.3 PHD finger protein 6 Hypogonadism, hypopituitarism, Lehmann syndrome (PHF6, 300414) obesity	301900	XL	Xq26.3	PHF6/300414	Hypogonadism, hypopituitarism, obesity
Cerebellar ataxia and hypogonadotropic hypogonadism	212840	AR	?	?	Hypogonadotropic hypogonadism
CHARGE syndrome	214800	AD	8q12.1	CHD7/608892	Short stature, hypopituitarism
		S?	7q21.11	SEMA3E/608166	
Chorioretinal dystrophy, spinocerebellar ataxia, and hypogonadotropic hypogonadism (Boucher–Neuhauser syndrome)	215470	AR	?	?	Hypogonadotropic hypogonadism, hypothalamic dysfunction
Chromosome 18p deletion syndrome	146390	AD, S	18p	Contig. gene deletion	Hypopituitarism, hypogonadism, gonadal dysgenesis
Chromosome 18q deletion syndrome	601808	S	18q-	Contig. gene deletion	Growth hormone deficiency, hypothyroidism, hypogonadotropic hypogonadism
Cleft lip/palate with abnormal thumbs and microcephaly	216100	AR	?	?	Growth hormone deficiency
Cohen syndrome	216550	AR	8q22-q23	VPS13B/607817	Obesity, delayed puberty, growth hormone deficiency
Combined pituitary hormone deficiency disorders—see Table 83-2					
Diabetes insipidus, neurohypophyseal	125700	AD AR	20p13	AVP/192340	Central diabetes insipidus
Ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome	604292	AD	3p27	TP63/603273	Growth hormone deficiency, hypogonadism, central diabetes insipidus
Endocrine–cerebro–osteodysplasia	612651	AR	6p12.3	ICK/612325	Adrenal and pituitary hypoplasia
Fanconi anemia	227650	AR	Multiple	Heterogeneous/227650	Growth hormone deficiency, hypogonadism
FSH deficiency	229070	AR	11p13	FSHB/136530	Primary amenorrhea, hypogonadotropic hypogonadism
Growth hormone pathway disorders—see Table 83-3					
Hemochromatosis, type 1	235200	AR	6p21.3	HFE/235200	Diabetes mellitus, hypogonadotropic hypogonadism
Hemochromatosis, type 2A	602390	AR	1q21	HJV/608374	Hypogonadotropic hypogonadism
Hemochromatosis, type 2B	613313	AR	19q13	HAMP/606464	Hypogonadotropic hypogonadism
Hemochromatosis, type 3	604250	AR	7q22	TFR2/604720	Hypogonadotropic hypogonadism
Hemochromatosis, type 4	606069	AD	2q32	SLC40A1/604653	Hypogonadotropic hypogonadism
Holoprosencephaly 1	236100	AR	21q22.3	HPE1 locus	Absent pituitary gland, hypopituitarism
Holoprosencephaly 2	157170	Ad	2P21	SIX3/603714	Hypopituitarism
Holoprosencephaly 3	142945	AD	7q36	SHH/600725	Pituitary hypoplasia
Holoprosencephaly 7	610828	AR	9q22.3	PTCH1/601309	Hypopituitarism
Holoprosencephaly 8	609408	?	14q13	?	Pituitary hypoplasia

**TABLE 83-1 Selected Genetic Disorders that May Affect Pituitary Development or Function<sup>a</sup>—Cont'd**

Name of Disorder	OMIM	Mode	Locus	Gene/OMIM	Endocrine Features
Holoprosencephaly 9	610829	AR	2q14	GLI2/165230	Hypopituitarism
Hypothyroidism, congenital, nongoitrous, 4	275100	AR	1p13	TSHB/188540	Congenital isolated central hypothyroidism
Hypogonadism	152780	AD	19q13.32	LHB/152780	Male hypogonadism
Hypogonadotropic hypogonadism, isolated	146110	AR?	4q21.2	GNRHR/138850	Hypogonadotropic hypogonadism
		AR	8p21-p11.2	GNRH1/152760	
		AR	19p13.3	KISS1R/604161)	
		AR?	9q34.3	NELF/608137	
		?	8q12.1	CHD7/608892	
		AR?	12q13-q21	TAC3/162330	
		AR?	4q25	TACR3/162332	
		?	10q24	FGF8/600483	
Kallmann syndrome 1	308700	XL	Xp22.3	KAL1/308700	Hypogonadotropic hypogonadism with anosmia
Kallmann syndrome 2	147950	AD	8p11.2-p11.1	FGFR1/136350	
Kallmann syndrome 3	244200	?	20p13	PROKR2/607123	
Kallmann syndrome 4	610628	?	3p21.1	PROK2/607002	
Kallmann syndrome 5	612370	?	8q12.1	CHD7/608892	
Kallmann syndrome 6	600483	?	10q24	FGF8/600483	
Lissencephaly, X-linked, 2	300125	XL	Xp22.13	ARX/300382	Abnormal genitalia, hypothalamic dysfunction
Martolf syndrome	212720	AR	1q41	RAB3GAP2/609275	Growth hormone deficiency, hypogonadotropic hypogonadism
McCune–Albright syndrome	174800	SM	20q13.2	GNAS/139320	Activating mutation; classic triad: café au lait spots, precocious puberty, polyostotic fibrous dysplasia; also hyperthyroidism, Cushing syndrome, acromegaly, hyperprolactinemia
Microphthalmia, syndromic 3	206900	AD	3q26.3-q27	SOX2/184429	Pituitary anomalies and hypogonadotropic hypogonadism
Microphthalmia, syndromic 6	607932	AD	14q22-q23	BMP4/112262	Pituitary anomalies and hypopituitarism
Moebius syndrome 1	157900	AD	13q12.2-q13	MBS1 locus	Hypogonadotropic hypogonadism
Neoplastic disorders—see Table 83-5					
Obesity, adrenal insufficiency, and red hair	176830	AR?	2p23.3	POMC/176830	Adrenal insufficiency due to lack of ACTH
Obesity, morbid, with hypogonadism	164160	AR?	7q31.3	LEP/164160	Hypogonadotropic hypogonadism
Obesity, morbid, with hypogonadism	601007	AR?	1p31	LEPR/601007	Hypogonadotropic hypogonadism
Pallister–Hall syndrome	146510	AD	7p13	GLI3/65240	Hypopituitarism, hypothalamic hamartoblastoma
Precocious puberty	176400	AD	19p13.3	KISS1R/604161	Isosexual precocious puberty
Pseudohypoparathyroidism Type 1A	103580	AD	20q13.2	GNAS/139320	Pseudohypoparathyroidism with multiple hormone resistance, severity determined by imprinting
Solitary median maxillary incisor	147250	AD	7q36	SHH/600725	Growth hormone deficiency, central incisor
Stratton–Parker syndrome	185120	?	?	?	Growth hormone deficiency
Thyroid hormone resistance (generalized)	188570	AD	3p24.3	THRB/190160	Resistance to thyroid hormone in both pituitary and peripheral tissues; clinically euthyroid
	274300	AR	3p24.3	THRB/190160	
Thyroid hormone resistance (pituitary)	145650	AD	3p24.3	THRB/190160	Selective pituitary insensitivity to thyroid hormone resulting in specific hyperthyroidism due to action of thyroid hormone in peripheral tissues
Thyrotropin releasing hormone deficiency	275120	AR	3q13.3-q21	?	Hypothalamic hypothyroidism, short stature
Thyrotropin releasing hormone resistance	188545	?	8q23	TRHR/188545	Congenital hypothyroidism due to TRH resistance

Continued

**TABLE 83-1 Selected Genetic Disorders that May Affect Pituitary Development or Function<sup>a</sup>—Cont'd**

Name of Disorder	OMIM	Mode	Locus	Gene/OMIM	Endocrine Features
Thyrotropin resistance	275200	AR	14q31	TSHR/603372	Congenital hypothyroidism
Trichomegaly with mental retardation, dwarfism, and pigmentary degeneration of retina	275400	AR	?	?	Short stature, growth hormone deficiency, hypogonadotropic hypogonadism
Wolfram syndrome 1	222300	AR	4p16.1	WFS1/606201	Diabetes insipidus, diabetes mellitus, optic atrophy, deafness
Wolfram syndrome 2	604928	AR	4q22-24	CISD2/611507	

AR, autosomal recessive; AD, autosomal dominant; XL, X-linked; SM, somatic mosaicism; S, sporadic; TRH, thyrotropin-releasing hormone.

A “?” indicates information not available or not confirmed.

Online Mendelian Inheritance in Man, OMIM (TM). McKusick–Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), (accessed October 2010–March 2011). World Wide Web URL: <http://www.ncbi.nlm.nih.gov/omim/>.

<sup>a</sup>Genetic disorders affecting genes in the growth hormone pathway, causing combined pituitary hormone deficiency, or associated with endocrine neoplasia are treated separately (Tables 83-2, 83-3, and 83-5 respectively).

hypogonadism), they are discussed in the corresponding endocrine organ chapters (Chapters 84, 87, and 88). This chapter focuses primarily on developmental disorders of the pituitary and disorders of the GH axis.

**83.2.1.1 The Role of Transcription Factors in Pituitary Development.** A complex cascade of transcription factors guides the development of the anterior and posterior pituitary. Transcription factor signaling in both the developing oral ectoderm (eventually to become Rathke’s pouch) and the ventral diencephalon is important for initiation of pituitary development as well as migration of the nascent anterior pituitary to its final location. Important factors in early pituitary development include PITX1/2 (OMIM 602149/601542), HESX1 (OMIM 601802), SHH (OMIM 600725), and LHX3/4 (OMIM 600577/602146), while genes such as *PROP1* (OMIM 601538) and *POU1F1* (also called PIT1, OMIM 173110) play an important role in the differentiation of anterior pituitary hormone-secreting cells. In general, disruptions of genes involved in early pituitary development result in more severe defects and often brain anomalies (e.g. holoprosencephaly due to SHH mutations, Table 83-1), while defects in genes involved in later aspects of pituitary development lead to varying combinations of pituitary hormone deficiencies (Table 83-2). For an excellent review of transcription factor signaling in pituitary development, see (1,2).

Two key transcription factors in the development and function of the anterior pituitary are *PROP1* and *POU1F1*. *PROP1* appears early in anterior pituitary development and is important for differentiation of anterior pituitary cell lineages. *PROP1* forms a complex with  $\beta$ -catenin and this complex both represses *HESX1* expression and activates *POU1F1* expression, leading to progression of cellular determination (1).

*POU1F1* is a member of the POU family of transcription factors that regulate mammalian development (Figure 83-1). The POU family is so named because the first three members identified were *POU1F1* and

*OCT1* of mammals and *Unc-86* of *Caenorhabditis elegans*. *POU1F1* contains two protein domains, termed POU-specific and POU-homeo, which are both necessary for high-affinity DNA binding on genes encoding GH and PRL. *POU1F1* is also important for regulation of the genes encoding PRL and the TSH $\beta$  subunit by TRH and cyclic AMP.

**83.2.1.2 The Growth Hormone Gene.** GH is a multifunctional hormone, produced in the pituitary, which promotes postnatal growth of skeletal and soft tissues through a variety of effects (Figure 83-1). Controversy remains about the contribution of direct and indirect actions of GH. GH receptors (GHRs) have been documented in a number of cell types. A major portion of the effects of GH are mediated through the actions of GH-dependent insulin-like growth factor 1 (IGF1, also called somatomedin C). IGF1 is produced in many tissues, primarily the liver, and acts through its own receptor (IGF1R, OMIM 147370) to enhance the proliferation and maturation of many tissues, including bone, cartilage, and skeletal muscle. In addition to promoting tissue growth, GH has also been shown to exert a variety of other biological effects, including lactogenic, diabetogenic, lipolytic, and protein-anabolic effects, as well as sodium/water retention.

At the cellular level, a single GH molecule binds two GHR molecules, causing them to dimerize. Dimerization of the two GH-bound GHR molecules leads to signal transduction, through the JAK-STAT pathway. It has been suggested that the diverse effects of GH may be mediated by a single type of GHR molecule, which can possess different cytoplasmic domains and/or phosphorylation sites in different tissues. When activated by JAK2, these differing cytoplasmic domains can lead to distinct phosphorylation pathways, one for growth effects and others for various metabolic effects.

The gene encoding GH (GH1, OMIM 139250) and those genes encoding chorionic somatomammotropin hormones (CSH1/2, OMIM 150200/118820) reside in a 50,000 base-pair (50kb) gene cluster on human

**TABLE 83-2 Selected Genetic Defects Causing Combined Pituitary Hormone Deficiency (CPHD)**

Anterior Pituitary Phenotype												
Disorder	OMIM	Mode	Locus	Gene	GH	PRL	TSH	LH	FSH	ACTH	Morphology	Posterior Pituitary
CPHD 1	613038	AR AD	3p11	POU1F1 aka PIT1/173110	–	–	–	+	+	+	Absent, hypoplastic, or normal	No
CPHD 2	262600	AR	5q	PROP1/601538	–	–	–	–	–	± <sup>a</sup>	Absent, hypoplastic, or enlarged <sup>b</sup>	No
CPHD 3	221750	AR	9q34.3	LHX3/600577	–	–	–	–	–	±	Hypoplastic or enlarged	No
CPHD 4	262700	AD	1q25	LHX4/602146	–	?	–	–	–	–	Hypoplastic	Ect
Microphthalmia, syndromic, 5	610125	?	14q21-q22	OTX2/600037	±	±	±	±	±	±	Hypoplastic or normal	No
Septo-optic dysplasia	182230	AR AD	3p21.2-p21.1	HESX1/601802	–	?	±	±	±	+	Hypoplastic or normal	Ab
Panhypopituitarism, X-linked	312000	XL	Xq27.2-q27.3	Chromosome duplication	–	?	–	?	?	+	Hypoplastic	No
Mental retardation, X-linked, with panhypopituitarism	300123	XL	Xq26.3	SOX3/313430	±	±	±	±	±	±	?	Ab

CPHD, combined pituitary hormone deficiency; AR, autosomal recessive; AD, autosomal dominant; XL, X-linked; GH, growth hormone; PRL, prolactin; TSH, thyroid stimulating hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; ACTH, adrenocorticotropic hormone; –, deficient; +, present; ±, variable.

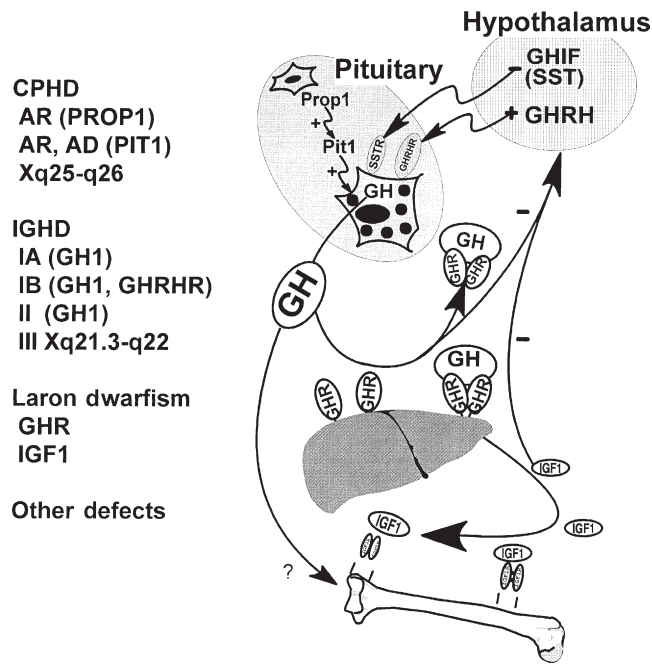
A “?” indicates that information is unavailable as to the presence or absence of the condition.

Online Mendelian Inheritance in Man, OMIM (TM). McKusick–Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), accessed October 2010–March 2011. World Wide Web URL: <http://www.ncbi.nlm.nih.gov/omim/>.

<sup>a</sup>ACTH deficiency may evolve with age in patients with PROP1 deficiency.

<sup>b</sup>Longitudinal studies in patients with PROP1 mutations suggest pituitary morphology changes over time (see text) (53).





**FIGURE 83-1** Growth hormone axis. Defects at each of the steps of the axis have now been described which result in proportionate short stature. GH, growth hormone; GHRH, growth hormone-releasing hormone; GHRHR, growth hormone-releasing hormone receptor; IGF1, insulin-like growth factor 1; IGF1R, insulin-like growth factor 1 receptor; SST, somatostatin.

chromosome 17. The GH2 gene (OMIM 139240) also resides in this cluster and encodes a protein (GH2) that is expressed in the placenta rather than in the pituitary, and differs from the primary sequence of GH by 13 amino acids. The CSH1/2 genes encode proteins of identical sequences, whereas the CSH pseudogene (CSHP1) encodes a protein differing by 13 amino acids and contains a mutation that should alter its pattern of messenger RNA (mRNA) splicing and thus the primary sequence of the resulting protein. The extensive homology (92–98%) between the immediate flanking, intervening, and coding sequences of these five genes suggests that this multigene family arose through a series of gene duplication events. These duplications are thought to have arisen from successive unequal recombinations between Alu family repeats. This method of gene amplification is analogous to the Alu–Alu recombinations that cause deletions of the human  $\beta$ -globin and low-density lipoprotein receptor genes.

Expression of the GH1 gene is controlled by *cis* and *trans* factors. The former include the CAT and TATA promoter components located, respectively, 85 and 30 bp upstream from the GH1 gene's origin of transcription. In addition, there are *cis* sequences that bind the POU1F1 transacting factor. Thus, POU1F1 binds to and activates the promoters of the GH1 and PRL genes, and also affects differentiation of thyrotropes.

**83.2.1.3 Disorders of Pituitary Function.** Hereditary disorders of both pituitary hypofunction and hyperfunction have been described (Tables 83-1–83-3, 83-5).

Similar to genetic disorders of the other endocrine glands, diseases involving hormone deficiency states are much more frequent and better delineated than hereditary forms of hormone excess. Pituitary deficiency disorders may involve a single tropic hormone (isolated or monotropic deficiency) or a combined pituitary hormone deficiency (CPHD, deficiency of two or more pituitary hormones) and may result from disturbances in any part of the hypothalamohypophyseal-target organ complex (Tables 83-1-83-3). Theoretically, a syndrome of pituitary hormonal insufficiency might result from developmental, degenerative, or receptor lesions of the hypothalamus, deficiencies of the hypothalamic-releasing hormones or their receptors, developmental or degenerative lesions of the pituitary gland, deficiencies or structural abnormalities of the pituitary hormones, or defects in target organ responsiveness to hormonal action. Each of these mechanisms has now been described in patients with pituitary insufficiency, resulting in the marked genetic heterogeneity that has been observed in pituitary disorders (3).

### 83.2.2 Developmental Anomalies and Syndromes

### 83.2.2.2.1 Congenital Absence or Hypoplasia of the Pituitary.

Complete absence of the anterior pituitary gland results in severe neonatal adrenal insufficiency, hypothyroidism, and hypoglycemia and, if untreated, usually results in neonatal death. This syndrome probably goes unrecognized in most cases, unless the adrenal insufficiency is diagnosed early and pituitary function is then studied, or postmortem examination includes a detailed examination of the pituitary fossa. The sella turcica is usually small and no trace of anterior pituitary tissue is found at autopsy. Posterior pituitary tissue may be present or absent. There is atrophy of the adrenals with absence of the fetal zone, presumably secondary to ACTH deficiency.

Clinical features include early lethargy, cyanosis, convulsions, circulatory collapse, and hypoglycemia. Neonatal jaundice has been documented and may occur with most forms of neonatal hypopituitarism. The thyroid and gonads may be hypoplastic and the penis is usually small. Survival past the neonatal period will result in severe dwarfism, hypogonadism, and cretinism, but early total hormone replacement therapy should result in complete phenotypic reversal. Thus, baseline endocrine tests should be performed immediately followed by magnetic resonance imaging (MRI) of the brain in neonates with hypogonadism and unexplained hypoglycemia, so that the irreversible neurological and developmental consequences of panhypopituitarism can be prevented (4).

Sobrier and colleagues described a consanguineous family in which two siblings displayed a complete absence of the anterior pituitary revealed by a deficit in

**TABLE 83-3 Genetic Disorders of the Growth Hormone (GH) Pathway**

Phenotype	Disorder	OMIM	Mode	Locus	Gene/OMIM	Endogenous GH	Response to GH Therapy
Isolated GH deficiency	IGHD IA	262400	AR	17q22-q24	GH1/139250	Absent	Often temporary due to antibody development
	IGHD IB	612781	AR	17q2-q24	GH1/139250	Decreased	Present
			AR	7p15-P14	GHRHR/139191	Decreased	Present
	IGHD II	173100	AD	17q22-q24	GH1/139250	Decreased	Present
	IGHD III	307200	XL	Xq21.3-q22	BTK/300300	Decreased	Present
	Mental retardation X-linked with isolated GH deficiency	300123	XL	Xq26.3	SOX3/313430	Decreased	Present
	Kowarski syndrome ("bioinactive" GH)	262650	AR	17q22-q24	GH1/139250	Present (but nonfunctional)	Present
Short stature with or without GH deficiency	Short stature, idiopathic, autosomal	604271	AR	3q26.3	GHSR/601898	Present or absent	Present in at least one patient
GH resistance	Laron dwarfism 1	262500	AR	5p13-P12	GHR/600946	Normal or increased	Absent or decreased (may respond to exogenous IGF1)
	GH insensitivity with immunodeficiency	245590	?AR	17q11.2	STAT5B/604260	Normal or increased	Absent or decreased (may respond to exogenous IGF1)
	IGF1 deficiency	608747	AR	12q22-Q24.1	IGF1/147440	Normal or increased	Absent or decreased (may respond to exogenous IGF1)
	IGF1 resistance	270450	AR	15q25-Q26	IGF1R/147370	Normal or increased	Absent or decreased (may respond to exogenous IGF1)

IGHD, isolated growth hormone deficiency; combined pituitary hormone deficiency; AR, autosomal recessive; AD, autosomal dominant; XL, X-linked. Online Mendelian Inheritance in Man, OMIM (TM). McKusick–Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), (accessed October 2010–March 2011). World Wide Web URL: <http://www.ncbi.nlm.nih.gov/omim/>.

**TABLE 83-4 Summary of GH1, GHR, and GHRHR Mutations Causing GHD<sup>a</sup>**

Mutation Type	Number of Mutations		
	GH1	GHR	GHRHR
Missense/nonsense	17	43	9
Splicing	16	16	4
Regulatory	7	0	1
Small deletions	4	7	1
Gross deletions	9	3	0
Complex rearrangements	1	1	0
Total	54	70	15

<sup>a</sup>HGMD Professional Database 9/3/10 (<http://www.hgmd.cf.ac.uk/ac/index.php>).

all anterior pituitary hormones (5). One patient, who also had retinal coloboma, carried a HESX1 defect in the homozygous state. HESX1 mutations in humans have been associated with various pituitary hormone deficiencies usually combined with optic nerve anomalies (see Sections 83.2.2.6 and 83.2.3).

A syndrome of congenital hypopituitarism with severe hypoglycemia and microphallus, which resembles congenital absence of the pituitary clinically, has been described. Plasma PRL was normal or elevated and there was a TSH rise following TRH administration, indicative of a hypothalamic defect. All these cases were sporadic. Hypoplasia or ectopia of the pituitary gland has also been described with pituitary insufficiency. It is impossible to state whether these latter disorders are simply less complete forms of pituitary agenesis or secondary to distinct pathogenetic mechanisms.

Yagi et al. described three brothers with congenital CPHD and central diabetes insipidus (DI). MRI indicated absence of the pituitary stalk, severe hypoplasia of the anterior pituitary, and an absent or ectopic posterior pituitary. Also, Shashi and colleagues described two brothers who had features of orofaciocigital (OFD) syndrome types II and VI and Pallister–Hall syndrome (PHS), both of whom had congenital absence of the pituitary gland, and suggested that this represented yet another pleiotropic effect of the OFD syndrome.

**TABLE 83-5 Selected Neoplastic Syndromes Affecting the Pituitary Gland**

Disorder	OMIM	Mode	Locus	Gene/OMIM	Endocrine Features
Carney complex type 1	160980	AD	17q23–q24	<i>PRKAR1A</i> /188830	Pituitary adenomas, primary adrenocortical nodular hyperplasia
Carney complex type 2	605244	AD	2p16	?	Pituitary adenomas, primary adrenocortical nodular hyperplasia
Familial isolated pituitary adenoma (FIPA)	102200	AD	11q13	<i>AIP</i> /605555	Pituitary adenomas
Multiple endocrine neoplasia, type 1	131100	AD	11q13	<i>MEN1</i> /131100	Pancreatic, parathyroid, and pituitary adenomas
Multiple endocrine neoplasia type 4	610755	AD	12p13	<i>CDKN1B</i> /600778	Pituitary adenomas

Online Mendelian Inheritance in Man, OMIM (TM). McKusick–Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), Accessed October 2010–March 2011. World Wide Web URL: <http://www.ncbi.nlm.nih.gov/omim/>.

Ferrier and Stone described two sisters who had severe growth failure, hypoglycemia, and mental retardation, and who manifested evidence of relative GH, TSH, and ACTH deficiency (OMIM 262700; Table 83-2). These individuals differed from the usual form of hereditary CPHD in that both had a very small sella turcica located in a morphologically abnormal sphenoid bone. The authors postulated that the association of CPHD, mental retardation, and abnormal sella turcica may represent a distinct syndrome inherited as an autosomal recessive trait. Two sisters with a similar syndrome of CPHD with a poorly developed sella turcica were later described by Sipponen and colleagues. On autopsy, no pituitary gland could be found in one child and only a rudimentary, partly ectopic pituitary in the other. Thus, there are some questions as to whether this represents a distinct syndrome or is simply a less severe form of congenital absence of the pituitary.

Hypoplasia of the pituitary with ectopic posterior pituitary has also been reported as part of a constellation of alopecia, neurological defects, and endocrinopathy (ANE syndrome), with central hypogonadism and central hypothyroidism (6). The patients were found to have a homozygous mutation of *RBM28*, which encodes an 85 kDa protein that forms part of snRNP complexes. Rough endoplasmic reticulum (ER) structure and ribosome density were both abnormal in these patients, although the exact mechanism by which deficiency of this protein causes the ANE syndrome is unclear.

**83.2.2.2 Anencephaly.** Anencephaly is associated with the complete absence of a normal hypothalamus, absence or severe hypoplasia of the posterior pituitary, and variable degrees of anterior pituitary hypoplasia (OMIM 206500, 301410; Table 83-1). These infants have hypoplastic adrenals and often die from adrenal insufficiency. Plasma GH levels have been found to vary from low normal to deficient in anencephalic infants. The intravenous insulin tolerance test has failed to evoke elevations in plasma GH in anencephalic infants. Administration of lysine–vasopressin caused active GH release, however, and there was a large increase in serum TRH. Thus, the anterior pituitary appears to be capable of releasing GH

and TSH when directly stimulated, but anterior pituitary function mediated by hypothalamic-releasing hormones appears to be totally deficient. These endocrinologic data are supported by histologic observation of TSH-, FSH/LH-, and GH-secreting cells in the pituitaries of these infants.

**83.2.2.3 Holoprosencephaly.** The holoprosencephalies form a spectrum of developmental anomalies associated with impaired midline cleavage of the embryonic forebrain, aplasia of the olfactory bulbs and tracts, and midline dysplasia of the face, ranging from cyclopia to cleft lip and palate with hypotelorism (OMIM 236100, 157170; Table 83-1). Anomalies of the pituitary gland have been described in all forms of holoprosencephaly, ranging from malformation of the gland to its complete absence. In individuals with absence of the pituitary, the adrenals are also hypoplastic and there may or may not be thyroid hypoplasia as well. Hypoplastic or aplastic pancreas, testes, and ovaries have also been described. ACTH deficiency and neurohypophyseal DI (NDI) have been documented. All degrees of insufficiency may occur in the holoprosencephalies, and the degree of pituitary dysfunction appears to be unrelated to the severity of the facial deformity. The pituitary insufficiency is presumably secondary to a developmental anomaly of the hypothalamus. The holoprosencephalies are a genetically heterogeneous group of disorders associated with 7q, trisomy 13, deletion of the short arm of chromosome 18, simple autosomal dominant or recessive inheritance, and a variety of other chromosomal anomalies (see Chapter 115). Mutations have been described in a number of genes, including *SHH*, *ZIC2*, *SIX3*, and *TGIF* (7). Twelve loci have been defined on 11 chromosomes (8).

**83.2.2.4 Transsphenoidal Encephalocele.** A number of cases of transsphenoidal encephalocele associated with variable degrees of hypothalamopituitary dysfunction have been reported. In transsphenoidal encephalocele, a defect exists in the sphenoid bone and the encephalocele usually extends into the epipharynx. Associated features include an epipharyngeal or nasopharyngeal mass, hypotelorism, midfacial or midline craniocerebral anomalies, and optic nerve abnormalities. The facial appearance is

characterized by a broad nasal root. Hormonal deficiencies have been reported in these patients, including GH, TSH, ACTH, LH, FSH, PRL, and ADH deficiencies. In individual patients, pituitary function has ranged from normal, to an isolated hormone deficiency such as isolated GH deficiency (IGHD), to CPHD. In one autopsied proband, degeneration of the hypothalamus and agenesis of the supraoptic nuclei were found. Duplication of the pituitary and agenesis of the corpus callosum have also been described. Double pituitary glands have been described with a variety of craniofacial malformations. Diagnosis of this syndrome is important, as iatrogenic hypopituitarism has been described following extirpation of a nasopharyngeal mass containing anterior and posterior pituitary tissue in a case whose sphenoidal defect was not recognized preoperatively. Although the majority of cases of transsphenoidal encephalocele are sporadic, two sibs with transsphenoidal pituitary herniation, midfacial anomalies, and CPHD, who were the offspring of a consanguineous mating, have been described, suggestive of autosomal recessive inheritance in some families.

**83.2.2.5 Empty Sella Syndrome.** Primary empty sella is caused by a defect in the diaphragma sellae, with extension of the subarachnoid space into the sella turcica, resulting in a diffusely enlarged sella. Although patients typically have normal pituitary function, it can be familial and associated with other anomalies, as well as short stature and pituitary deficiency. Yokoyama and colleagues found a duplication of the q13.3–q21.2 region of the X chromosome in patients with GH deficiency and empty sella syndrome.

**83.2.2.6 Septo-Optic Dysplasia.** Septo-optic dysplasia (SOD, OMIM 182230) is a rare malformation of the anterior midline structures of the brain (Table 83-1). The classic triad includes optic nerve hypoplasia, midline brain defects (most commonly absent or cavum septum pellucidum or agenesis of the corpus callosum), and hypopituitarism, sometimes with hypoplasia of the anterior pituitary and/or ectopic posterior pituitary. Severely affected neonates may present with signs of hypopituitarism including hypoglycemia and prolonged jaundice; patients may also have defective vision, behavioral delay, hypotonia, and seizures. In mildly affected cases, the child may present with proportionate short stature and pendular nystagmus, with or without amblyopia. Ophthalmologic examination reveals bilateral hypoplasia of the optic nerves, with small optic discs and irregular field defects. Absence of the septum pellucidum can be diagnosed prenatally by ultrasound examination (9). Any or all of the three major defects (brain, optic tract, pituitary) may be present. Morishima and Aranoff concluded that only 30% of fully evaluated patients had evidence of all three components. Intelligence may be normal or mildly to moderately affected. The pituitary insufficiency, which may vary from IGHD to CPHD, is probably secondary to a diencephalic malformation resulting in deficiency of one or more of the hypothalamic-releasing hormones. Sexual precocity has been reported, as

has hypersecretion of other pituitary hormones, including GH, ACTH, and PRL. Autopsy has shown absence of the posterior pituitary and diffuse lesions of the hypothalamus, optic nerves, corpus callosum, and olfactory tract, as well as defects in the cerebral cortex. A variety of other anomalies, including hand and facial defects, have occasionally been described. This syndrome should be considered in any child with hypopituitarism and nystagmus or abnormalities of the optic disc. Almost all of the cases described to date with SOD and growth retardation have been sporadic, with no evidence of ocular anomalies or hypopituitarism in their parents or sibs. Dattani and colleagues (3) demonstrated homozygosity for a missense mutation in the HESX1 gene in a brother and a sister with agenesis of the corpus callosum and panhypopituitarism. Subsequently, other patients with SOD and pituitary defects have been found to have HESX1 mutations, although it appears to be a relatively rare cause (fewer than 1% of patients with SOD and hypopituitarism) (10). HESX1 is described further in Section 83.2.3.

SOX2 and SOX3 are HMG-box containing transcription factors expressed in the developing brain. Duplications of SOX3 (OMIM 313430) have been associated with X-linked hypopituitarism and mental retardation; some of these patients have been reported to have abnormalities of the corpus callosum but not the optic tract. On the other hand, patients with SOX2 (OMIM 184429) mutations may have severe optic manifestations including microphthalmia or even anophthalmia in association with hypopituitarism (11,12). Birrell and colleagues have reported two patients with Borjeson–Forssman–Lehmann syndrome and CPHD who also had optic nerve hypoplasia, which may represent a variant of SOD. The patients had a missense mutation in the PHF6 gene, which caused an early stop codon (K8X) (13).

**83.2.2.7 Cleft Lip and Palate.** Midline cleft lip and palate can be associated with GH deficiency, DI, holoprosencephaly, and optic nerve hypoplasia with absence of the septum pellucidum (14). Functional pituitary insufficiency has been described in a number of individuals with cleft lip and palate who did not have other facial or neurologic abnormalities. Pituitary insufficiency may vary from complete CPHD associated with congenital aplasia of the pituitary to IGHD. Rudman and colleagues studied 200 children with isolated clefts and found 4% with GH deficiency (40 times higher than the frequency in children without clefts). Short children with cleft lip and palate and growth retardation should thus be subjected to a complete pituitary evaluation. One can speculate that this disorder is simply the mild end of the spectrum of the holoprosencephaly–SOD range of hypothalamic anomalies associated with pituitary insufficiency (also see Section 83.2.2.13). Thus, any individual with cleft lip and/or palate and hypopituitarism deserves brain imaging.

**83.2.2.8 Solitary Maxillary Central Incisor.** Solitary maxillary central incisor may occur as an isolated defect



or as a milder manifestation of hereditary holoprosencephaly. In one series of seven patients with a single maxillary central incisor in both deciduous and permanent dentition and short stature (OMIM 147250, [Table 83-1](#)), five had documented GH deficiency. Two who were treated with GH had a good growth response. No other pituitary hormone deficiencies were found. The patients had normal facies or only mild midline facial anomalies, and there was no evidence of hypothalamic or optic defects.

A number of other cases of single central maxillary incisor in association with short stature have been reported. Boudaille et al. discovered hypopituitarism associated with mental retardation, dysmorphic features, and solitary maxillary incisor. A deletion of 18p has been reported in several cases. This observation suggests that the cause of the pituitary insufficiency in other cases of 18p- and pituitary dwarfism may be secondary to midline brain and hypothalamic defects.

Single central maxillary incisor has also been described in association with precocious puberty and a hypothalamic hamartoma, and in a dominant ectodermal dysplasia syndrome consisting of mild short stature, sparse scalp hair, skin pigmentation, and “hypoplastic” thumbs. Thus, this dental defect should alert one to the possibility of midline CNS defects and pituitary deficiency. Hall and colleagues described 21 cases of solitary median maxillary central incisor syndrome, pointing out that this syndrome is a possible predictor of holoprosencephalies of varying degrees in the proband, and related family members. A missense mutation in the sonic hedgehog (SHH) gene was found in eight members of a single family without holoprosencephaly, three of whom had a single central maxillary incisor ([15](#)). An SHH missense mutation was also described in a holoprosencephaly family in which the mother has a single central maxillary incisor and mild hypotelorism ([16](#)).

**83.2.2.9 Pallister–Hall Syndrome.** Hall and colleagues described a neonatally lethal malformation syndrome consisting of hypothalamic hamartoblastoma, hypopituitarism, postaxial polydactyly, and imperforate anus (OMIM 146510; [Table 83-1](#)). Variable features include laryngeal cleft, abnormal lung lobulation, renal agenesis and/or renal dysplasia, short fourth metacarpals, nail dysplasia, multiple buccal frenula, hypoadrenalism, microphallus, congenital heart defects, and intrauterine growth retardation. The hypothalamic tumor was apparent on the inferior surface of the cerebrum and extended from the optic chiasma to the interpeduncular fossa. The tumor replaced the hypothalamus and other nuclei that originate in the embryonic hypothalamic plate. It was composed principally of cells resembling primitive undifferentiated germinal cells. The olfactory bulbs and tracts were short and thick, suggesting a relationship to the holoprosencephaly syndromes. An anterior pituitary gland was absent in all cases and the posterior pituitary was absent in the majority. The associated adrenal

hypoplasia, small thyroid, and microphallus are presumably secondary to pituitary insufficiency.

Most babies with PHS die in infancy probably as a result of secondary adrenal insufficiency. Graham and colleagues reported the survival of one child who was promptly treated for pituitary insufficiency in infancy and then had a hypothalamic tumor surgically resected at 1 year of age. Thus, medical and surgical treatment of this syndrome is possible. Prenatal diagnosis may also be accomplished by measurement of decreased levels of maternal estriol, prenatal ultrasonography for the fetal adrenal, renal, or brain abnormalities, and measurement of amniotic fluid disaccharides, which would be decreased with an imperforate anus. Feuillan et al. studied hypothalamic function in 14 patients with PHS, and found that 6/7 (86%) children and 6/8 (75%) adults had decreased spontaneous GH secretion; most of the patients were not assessed with formal GH stimulation tests, and some of the adult patients had normal stature, suggesting that they may not have had true GH deficiency. Only one patient had an abnormal pituitary–gonadal axis (manifested as precocious puberty) and none had adrenal or thyroid dysfunction ([17](#)).

Familial cases of PHS exhibit autosomal dominant inheritance; sporadic cases also occur. This disorder is not always lethal in infancy, as a father of two affected siblings had mild manifestations of the disease, and two other unrelated patients with the full-blown syndrome were reported at 2 and 17 years of age. Overlap of this syndrome with Smith–Lemli–Opitz syndrome type II, OFD syndrome type VI, the holoprosencephaly–polydactyly syndrome, and McKusick–Kaufman syndrome have been suggested. However, molecular studies have disproved these comparisons. Kang et al. have shown that Greig cephalopolysyndactyly syndrome (GCPS) maps to the same region of chromosome 7p as does PHS. They reported two PHS families with frameshift mutations in *GLI3*, whereas haploinsufficiency of the *GLI3* gene has been noted with GCPS. A boy with PHS, with short stature, and GH neurosecretory dysfunction was found to have deficient spontaneous GH secretion despite a normal response to pharmacologic stimulation. The patient responded positively to long-term GH treatment ([18](#)). Johnston and associates screened 46 patients with PHS and 89 patients with GCPS for *GLI3* mutations. They detected 47 pathologic mutations (among 60 probands), and when these mutations were combined with previously published mutations, 2 genotype–phenotype correlations were evident. GCPS was caused by many types of alterations, including translocations, large deletions, exonic deletions and duplications, small in-frame deletions, and missense, frameshift/nonsense, and splicing mutations. In contrast, PHS was caused only by frameshift/nonsense and splicing mutations. Among the frameshift/nonsense mutations, they found a clear genotype–phenotype correlation. Mutations in the first third of the gene (from open reading frame nucleotides 1–1997) caused GCPS, and

mutations in the second third of the gene (from nucleotides 1998–3481) caused primarily PHS. Surprisingly, there were 12 mutations in patients with GCPS in the 3' third of the gene (after open reading frame nucleotide 3481), and no patients with PHS had mutations in this region (19). Thus, there is a clear genotype–phenotype correlation for GLI3 mutations.

**83.2.2.10 CHARGE syndrome.** CHARGE is an acronym that was coined to describe a nonrandom association of anomalies: Coloboma of the eye; Heart disease; choanal Atresia of the choanae; Retarded growth, development, and/or CNS anomalies; Genital hypoplasia; and Ear anomalies or deafness (OMIM 214800; Table 83-1). Other prominent features include facial palsy, micrognathia, cleft palate, swallowing difficulties, tracheoesophageal fistula, and a “wedge-shaped” audiogram.

Hypogonadotropic hypogonadism is a frequent feature, and in boys may manifest as cryptorchidism, microphallus, and/or hypospadias. Girls typically will not have symptoms of gonadotropin deficiency until later in childhood when they may present with delayed puberty. Although growth retardation also occurs frequently in patients with CHARGE association, GH is usually normal on testing. However, August et al. documented both GH and gonadotropin deficiencies in a girl with the association, who also had a delayed peak TSH response to TRH. Davenport and colleagues found GH borderline responses to arginine and insulin in one patient. Arhinencephaly and holoprosencephaly have been reported suggesting a hypothalamic cause for the pituitary deficiencies. Zuppinger and associates described an unusual patient with coloboma of the right choroid and optic nerve, cleft lip, GH deficiency, DI, and central hypothyroidism. None of the other features of the CHARGE association were present. Finally, James et al. (20) have reported a patient with CHARGE association accompanied by both hypogonadotropic hypogonadism and central adrenal insufficiency.

Visser and colleagues reported a 2.3Mb de novo overlapping microdeletion on chromosome 8q12 in two individuals with CHARGE syndrome (21). Sequence analysis of genes located in this region detected mutations in CHD7 in 10/17 (59%) of individuals with CHARGE syndrome who did not have microdeletions, thus accounting for the disease in most affected persons. CHD7 (OMIM 608892) is a DNA-binding protein involved in remodeling of chromatin and regulation of gene expression. Although dominant inheritance occurs, most mutations are de novo. The phenotype of CHARGE syndrome due to CHD7 mutations is highly variable, possibly due to somatic and/or germline mosaicism possibly with other modifying factors (22). An individual with CHARGE syndrome with an apparently balanced chromosome 6 and 8 translocation has been reported. Martin et al. reported a patient with CHARGE syndrome and a balanced translocation between chromosomes 2 and 7 (23). Lalani et al. performed further characterization of

this translocation and identified a breakpoint at 7q21.11. They identified the gene SEMA3E within 200kb of the breakpoint, and confirmed a mutation in SEMA3E in a second unrelated patient with CHARGE syndrome. SEMA3E encodes semaphorin 3E, one of a group of proteins involved in processes such as cell migration, axonal guidance, and heart development. Animal homologs of SEMA3E are expressed in the nasal placode, otic placode, and semicircular ducts, consistent with tissues involved in CHARGE (24).

**83.2.2.11 Chromosomal Syndromes.** Growth hormone deficiency has been described with deletions of chromosomes 18q (OMIM 601808), 18p (OMIM 146390), and 20p (Table 83-1). In addition, deletions of 17q (the GH gene cluster region) have been documented in IGHD type IA (see later discussion). Because the gene for GHRH has been mapped to 20p, the GH deficiency in 20p deletion could result from either the deletion of the *GHRH* gene or a developmental anomaly of the hypothalamus. The deletion in one such 20p deletion patient, however, did not include the *GHRH* locus. A patient with 18p deletion has been reported with hypopituitarism and solitary central maxillary incisor, suggesting that the pituitary insufficiency in 18p deletion may be due to a structural malformation of the hypothalamus. GH deficiency has been rarely associated with 47,XXY; 49,XXXXY; and ring 5. A boy with moderate intellectual disability associated with distinctive hand malformations (hypoplastic and angel-shaped middle phalanges) was found to have GH deficiency associated with mosaic deletion of 13q31.1–13q32.3 (25).

**83.2.2.12 Borjeson–Forssman–Lehmann Syndrome.** The X-linked Borjeson–Forssman–Lehmann syndrome is characterized by short stature, hypogonadism, hypotonia, severe mental deficiency, and coarse facial appearance with a prominent brow ridge and large ears in affected males (OMIM 301900; Table 83-1). Carrier females show a wide range of expression. A variety of other ocular and skeletal anomalies may also occur. Normal stimulated GH secretion in one patient has been reported. Robinson et al. documented markedly deficient GH responses to arginine and L-dopa, as well as low IGF1 levels in a severely affected male and both of his mildly affected twin sisters. The growth deceleration in this syndrome, however, may not begin until 8–10 years of age, and therefore the pituitary deficiency may be progressive. This syndrome was initially mapped to Xq26–q27; the gene for this disorder, PHF6, has now been identified (26). Birrell and colleagues (13) have reported a case of a patient with BFLS and deficiency of GH, ACTH, TSH, and gonadotropins associated with optic nerve hypoplasia, who had a premature stop codon in PHF6. Assessment of skewing of X-inactivation in suspected female carriers may identify those with PHF6 mutations (27).

**83.2.2.13 Ectrodactyly–Ectodermal Dysplasia–Clefting Syndrome.** One patient with the ectrodactyly–ectodermal dysplasia–clefting syndrome (EEC)

associated with GHD has been described (OMIM 129900; Table 83-1). Additionally, two brothers with EEC were reported to have pituitary deficits; both had hypogonadotropic hypogonadism and one also had central hypothyroidism. At least three loci have been found for EEC including EEC 1 on 7q, EEC 2 on chromosome 19 and EEC 3 on 3q27. Mutation analysis of the p63 gene, which maps to 3q27, revealed several mutations responsible for EEC 3 (28).

**83.2.2.14 Fanconi Anemia.** Fanconi syndrome is an autosomal recessive disorder characterized by chronic pancytopenia with bone marrow hypoplasia, abnormal pigmentation, upper limb malformations, kidney anomalies, growth retardation, small genitalia, and increased frequency of chromosomal breaks in cultured lymphocytes (OMIM 227650, 227645, 227646, 300514, 600901, 603467, 605724, 609054, 609053; Table 83-1). Nilsson found that 38/68 (56%) of published cases of Fanconi anemia had stunted growth and 24/68 (35%) had genital anomalies. Cussen reported a child with Fanconi anemia, who appeared to be a pituitary dwarf, and pointed out that small pituitary glands, adrenocortical atrophy, and atrophic testes have been described in this syndrome. A number of investigators have now documented GH deficiency in patients with Fanconi anemia, most of whom had no other endocrine deficiencies. A survey of 54 patients, belonging to a number of different complementation groups, in the International Fanconi Anemia Registry revealed that 81% had some type of endocrine disorder, with 44% having abnormal GH stimulation testing and 36% having hypothyroidism; gonadotropins were not assessed in this study (29). Recently, five patients with Fanconi anemia were found to have GH deficiency that responded well to GH therapy, in association with a finding of interrupted pituitary stalk on MRI in all five patients. Three of the five (60%) patients had abnormal TSH levels and two had gonadotropin deficiency (30). These findings suggest that the underlying genetic defect of Fanconi anemia may also affect pituitary development. GHD has also been reported in a patient with Fanconi syndrome with proximal tubule dysfunction (31).

**83.2.2.15 Gonadal Dysgenesis.** Although GH secretion has been reported to be normal in most patients with gonadal dysgenesis, pituitary insufficiency has now been reported in several patients (32). Although the most likely explanation for these cases is a chance association, a hypothalamic disturbance in gonadal dysgenesis has been postulated. Ross and colleagues studied GH secretion in 30 patients with Turner syndrome and found no differences in mean GH concentration or peak amplitudes throughout the day and night between patients less than 8 years of age and controls. Patients over 9 years of age had lower mean GH levels and peak amplitudes. Reduced plasma IGF1 levels and delayed bone age were found in patients of all ages. Deletion of the SHOX gene on the end of Xp is thought to explain a good portion of

the short stature seen in Turner syndrome, as well as with dyschondrosteosis (see Chapters 44 and 158). Despite the presence of normal GH secretion in most Turner syndrome patients, they can have a variable response to GH therapy, and Turner syndrome is now an approved indication for GH therapy.

**83.2.2.16 Hemochromatosis.** Male hypogonadism and pituitary hemosiderosis have long been considered integral features of hemochromatosis (OMIM 235200, Table 83-1). Abnormalities of gonadotropin, cortisol, GH, PRL, and TSH secretion have all been reported. Stocks and Martin demonstrated that functional pituitary insufficiency of varying degrees occurs in 60% of patients with hemochromatosis. Signs and symptoms of gonadal dysfunction included depressed sexual function, testicular atrophy, absent urinary gonadotropins, decreased plasma levels of LH, and low plasma testosterone levels, indicating that the hypogonadism in hemochromatosis is secondary to a deficiency of pituitary gonadotropin. In 7/15 (47%) patients, there was no plasma GH response to hypoglycemia, 6/15 (40%) had an absent or decreased plasma cortisol response to hypoglycemia, and 2/15 (13%) were hypothyroid. The degree of pituitary insufficiency was not related to the severity of the liver disease, nor to the degree of abnormality in either iron or estrogen metabolism.

This pituitary deficiency state appears to be secondary to iron deposition in the anterior pituitary. The testes of hypogonadal patients with hemochromatosis usually show evidence of secondary atrophy without iron deposition, documenting the hypogonadotropic nature of the hypogonadism in this disease. Most studies have reported testicular atrophy with low levels of gonadotropins, and lack of response to gonadotropin-releasing hormone. However, in a 78-year-old man with hemosiderosis secondary to sideroblastic anemia and multiple transfusions, Williams and Frohman documented hypothyroidism and hypogonadism that was hypothalamic in origin (see Chapter 101 for a discussion of the genetics of hemochromatosis).

**83.2.2.17 Neurofibromatosis Type 1.** A variety of endocrine disturbances have been reported in patients with neurofibromatosis type 1 (NF1, OMIM 162200; Table 83-1). Short stature is common, and in one study 50% of patients who attained their adult height during the study were significantly below their predicted adult height. While some studies have found GHD even in the absence of suprasellar tumors and/or neurosurgery or radiation, other studies have found GHD only in patients who had previously undergone intervention for tumor (33). The cause of the short stature in those patients who are not GH deficient is unclear. Precocious puberty can be seen in children with NF1, presumably due to hypothalamic involvement. GH hypersecretion has also been reported in some cases of NF1 (34,35).

**83.2.2.18 Reiger Syndrome.** Reiger syndrome is an autosomal dominant disorder associated with



malformation of the iris, pupillary anomalies, and hypoplasia of the teeth, with or without maxillary hypoplasia. Sadeghi-Nejad and Senior reported a large family in which three and possibly five individuals had both Rieger syndrome and IGHD (OMIM 180500; Table 83-1). Sibs of the proband had Rieger syndrome with normal pituitary function, but GH was not deficient in any member of the family who did not have Rieger syndrome. Affected individuals had insulin hypersensitivity, but normal plasma insulin responded to arginine and glucose. One subject who was treated with GH exhibited substantial enhancement of his rate of growth. It is postulated that the basic pathogenetic mechanism in this autosomal dominant disorder is maldevelopment of the neural crest, resulting in ocular, dental, and hypothalamic abnormalities. The homeobox gene *PITX2* (also called *RIEG*) has been identified as one cause of Rieger syndrome, which is genetically heterogeneous. The mouse homolog gene *Pitx2* is expressed in Rathke's pouch, and mice heterozygous for a *Pitx2* knockout have pituitary hypoplasia. In addition, expression of GH, TSH, and GHRH are decreased in the pituitaries of such mice, and expression of GNRHR and gonatropins is almost absent. Thus, *Pitx2* appears to be an important regulator of pituitary development (36).

**83.2.2.19 Moebius Syndrome.** Moebius syndrome (congenital paralysis of the facial nerves) has been reported in association with various types of pituitary dysfunction. Hashimoto and colleagues described a 17-year-old with Moebius syndrome, GHD, probable hypogonadotropic hypogonadism, and hypoplastic optic discs (OMIM 157900; Table 83-1). Multiple cases of Moebius syndrome in association with isolated hypogonadotropic hypogonadism have also been reported; this association may represent a distinct subtype of Moebius syndrome (37).

**83.2.2.20 Syndromes Reported with Growth Hormone Deficiency.** A number of other syndromes have had occasional reports of associated GH deficiency: Stratton-Parker syndrome (38), Kearns-Sayre syndrome (39), Costello syndrome (40,41), Williams syndrome (42), Langer-Giedion syndrome (43), Duchenne muscular dystrophy (44,45), and amelogenesis imperfecta (46). It remains to be seen how many of these observations are simply chance occurrences.

### 83.2.3 Combined Pituitary Hormone Deficiency

Hereditary forms of pituitary insufficiency represent a genetically heterogeneous group of disorders that can result from interruptions at any point in the hypothalamic-pituitary-IGF1-peripheral tissue axis (Tables 83-2 and 83-3). Most of these disorders result from disruption of normal pituitary development. CPHD is defined as deficiency of any two or more of the pituitary hormones (most commonly GH plus at least one

other hormone). The clinical features of hereditary CPHD are identical to those of the nongenetic forms of the disease and are dependent on which of the hormones are deficient. Deficiency of GH results in proportionate short stature, increased subcutaneous adipose tissue, and characteristic high-pitched voice. Gonadotropin deficiency results in sexual immaturity with primary amenorrhea in females, small testes and phallus in males, and lack of secondary sexual characteristics in both sexes. TSH deficiency most often causes mild hypothyroidism. ACTH deficiency may contribute to severe hypoglycemia in infancy and childhood and may be life threatening. There is both inter- and intrafamilial variability in associated hormonal deficiencies.

**83.2.3.1 CPHD Due to Mutations in *POU1F1*.** In Snell (dw) and Jackson (dw) dwarf mice, mutations in *pit1*, a tissue-specific pou-domain transcription factor, lead to the absence of somatotroph, lactotroph, and thyrotroph cells. Multiple mutations in the human gene *POU1F1* have been found in humans in a subtype of CPHD associated with GH, PRL, and TSH deficiency (omim 613038, Table 83-2). Clinically, some patients present with all hormone deficiencies and growth failure at a young age, while others present with GH deficiency initially and develop TSH deficiency later in their childhood. The appearance of the pituitary on MRI can be either normal or hypoplastic (47). Currently, at least 19 different *POU1F1* mutations are cataloged in the human gene mutation database (HGMD, available online at <http://www.hgmd.cf.ac.uk/hgmd0.html>).

Both autosomal recessive and autosomal dominant inheritance patterns have been reported. The first and second autosomal recessive *POU1F1* mutations were reported in two consanguineous Japanese families. In one family, a C to T transition in codon 172 encoded a CGA (arginine) to TGA (stop) substitution. Both parents were heterozygous, and the affected child was homozygous for the mutation. In the second family, an A to G transition in codon 143 encoding a CGA (arginine) to CAA (glutamine) was found. The patient was shown to be homozygous for this mutation, but the parents and two unaffected siblings were shown to be heterozygous. Both mutations occur in the POU-specific domain and are believed to affect DNA binding.

The third and fourth *POU1F1* mutations were found in two Dutch families who had postnatal growth failure with complete deficiencies of GH and PRL, whereas the T4 levels were low or normal prior to or following GH replacement. Subjects having normal T4 levels were homozygous for a G to C transversion in codon 158 encoding a GCA (alanine) to CCA (proline) substitution. This mutation interferes with the formation of *POU1F1* homodimers and dramatically reduces the ability of the altered *POU1F1* to activate transcription. Additional *POU1F1* mutations with autosomal inheritance have since been reported; the majority are in the POU-specific domain and appear to affect DNA binding



and/or transactivation of transcription. Other autosomal recessive mutations cause premature termination of the protein, creating a nonfunctional product. Compound heterozygotes have also been reported in several instances (47–51).

The first dominant-negative *POU1F1* mutation identified was a G to T transversion in codon 271 that encodes a GGG (arginine) to TGG (tryptophan) substitution. This mutation functions as a dominant inhibitor of *POU1F1* action by some as-yet unknown mechanism. Three unrelated patients have been reported to be heterozygous for this mutation. Two of the patients were evaluated as adults and found to have pituitary hypoplasia and deficiencies of GH, PRL, and TSH. The third patient was identified at only 2 months of age and found to have a normal pituitary and normal basal levels of TSH but a delayed TSH response in a TRH stimulation test. The authors suggest that as *POU1F1* may be necessary for anterior pituitary cell survival, the affected patient will develop hypoplasia and TSH deficiencies with age. The second dominant-negative *POU1F1* gene mutation was a T to C transition in codon 24 that encodes a CCT (proline) to CTT (leucine) substitution. This proline residue resides within the major transactivating domain of *POU1F1* and is highly conserved in different species. The mechanism by which this mutation exerts its dominant-negative effect is also not known.

**83.2.3.2 CPHD Mutations Due to *PROP1* Mutations.** Ames dwarf (*df/df*) mice have CPHD and hypoplastic anterior pituitaries that lack somatotropes, lactotropes, and thyrotropes as well as GH transcripts. The genetic defect associated with the Ames dwarf phenotype perturbs the Prophet of Pit 1 (*Prop1*) gene, which encodes a pituitary-specific homeodomain factor. Sequence analysis of the *Prop1* cDNA in the *df/df* mouse revealed a T to C transition in codon 83 that causes a Ser to Pro amino acid change in the first  $\alpha$ -helix of its homeodomain. Although *Prop1* is required for expression of *Pit1*, how *Prop1* regulates *Pit1* expression remains uncertain.

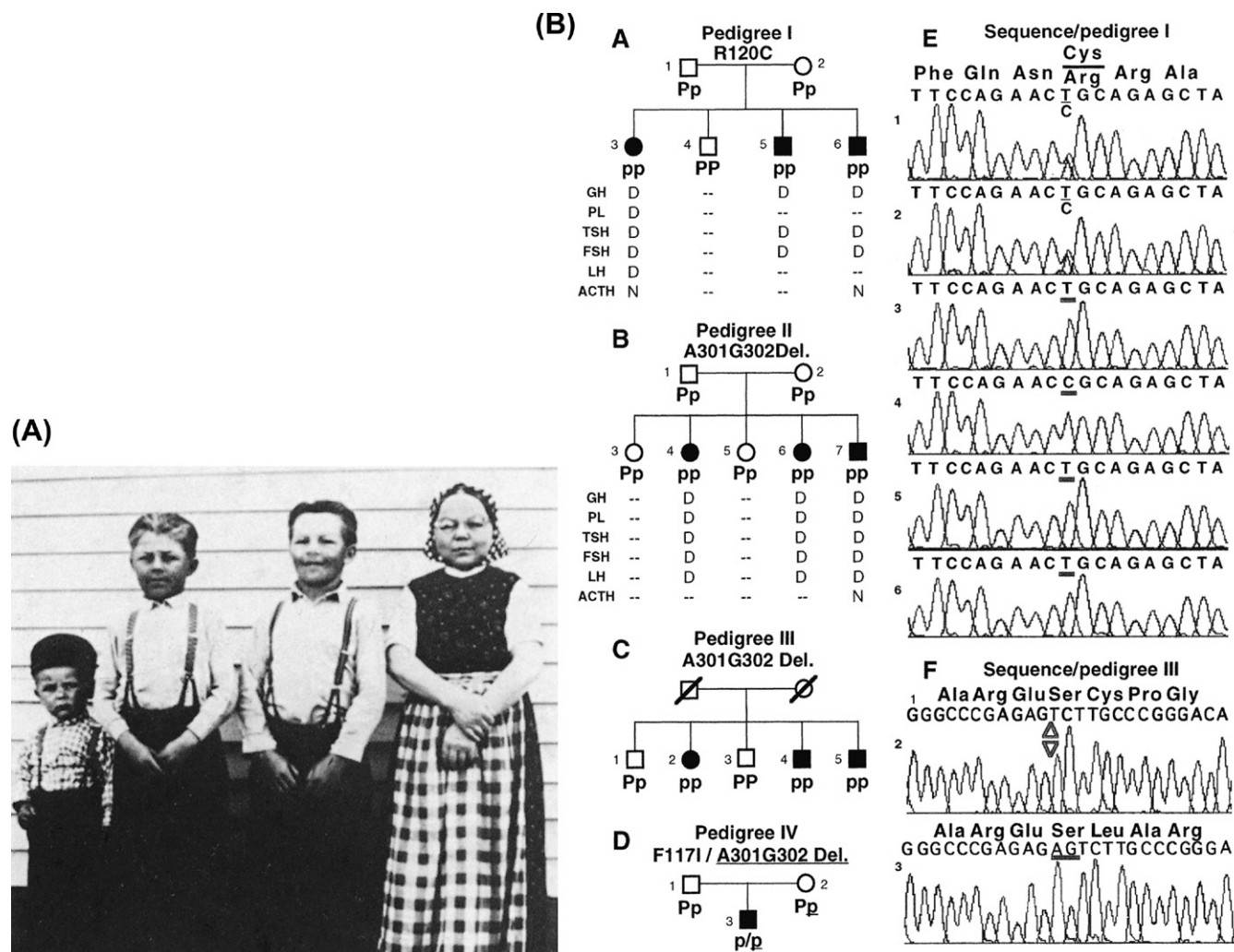
McArthur and colleagues restudied that the Hutterite family illustrated in Figure 83-2 and demonstrated that the tropic hormone loss was sequential. In one sibship, GH and gonadotropin deficiencies occurred in the first decade, with subsequent loss of TSH, and finally, ACTH in the third decade. Subsequently, these patients have been shown to have *PROP1* mutations (Figure 83-2). Since that time, it has become apparent that development of anterior pituitary hormone deficiencies in patients with *PROP1* mutations follows a predictable sequence: GH, TSH, FSH, LH, and ACTH. PRL deficiency is variable. ACTH deficiency may not develop until the fourth or fifth decade of life (52–54). Most patients present with growth failure in childhood; however, some patients may present initially with evidence of central hypothyroidism. Longitudinal imaging studies of patients with *PROP1* mutations show that some patients develop a

pituitary mass, which eventually regresses, while others have anterior pituitary hypoplasia. It has been hypothesized that the pituitary mass arises from the intermediate lobe of the pituitary but this has not been confirmed (53,55,56). There has been one report of a patient with a R120C mutation in *PROP1* who achieved a normal final height; the patient presented with absent puberty. In another family with the more common 101delAG mutation, one affected patient required GH to reach a normal final height, while other affected family members achieved a height in the low normal range with no or inadequate treatment. These cases emphasize the wide range of variable expressivity that is seen in patients with *PROP1* mutations and the need for careful and repeated assessment of pituitary function to detect changes over time (53,57).

Multiple *PROP1* gene mutations have now been identified. The first mutation, identified by Wu et al. in 1998, was a C to T transition in codon 120, which encoded a TGC (Arg) to CGC (Cys) substitution in the third helix of the homeodomain. This mutation greatly reduced DNA binding. The same authors also found a 2-bp deletion in codon 101 (101delAG) that caused a frameshift and resulted in a premature stop at codon 109 (Figure 83-2B, panel F3). This protein product had absent DNA binding. Subsequently, the 101delAG mutation has been found to be a recurring mutation and is estimated to occur in about 55% of familial and 12% of sporadic CPHD cases. The region of the 101delAG mutation contains three tandem repeats (GAGAGA) and appears to be a hot spot for mutations in *PROP1*. Multiple other missense and splicing mutations have been found, most of which occur in the homeodomain region and appear to affect DNA binding (58).

**83.2.3.3 CPHD Due to LIM-Homeobox Gene Mutations.** Mutations in *LHX3* have been associated with a syndrome of CPHD and rigid cervical spine. These patients have deficits in all pituitary hormones except ACTH and have limited head rotation (see Table 83-2). On imaging studies, both pituitary hypoplasia and enlargement have been seen. One missense mutation has been shown to impair activation of gene transcription by the *LHX3* gene product, while another mutation deletes the DNA binding domain and thus also results in an inability to induce transcription (59,60). Kristrom et al. described six unrelated patients with CPHD and limited neck rotation who also had scoliosis and congenital hearing loss. All were found to have the same splice-acceptor site mutation. CT imaging of the neck showed abnormalities of the occipito-atlanto-axial joints, which explained the limited neck rotation (61). Pfaeffle et al. reported four novel mutations, including one (W224Ter) which was associated with CPHD only with normal neck rotation (62).

Machinis et al. reported a consanguineous French family in which two sibs with short stature were found



**FIGURE 83-2** (A) Autosomal recessive CPHD. Three affected Hutterite siblings on the right and their cousin on the left. (B) Pedigrees of CPHD kindreds (left) including B–C, that of the Hutterite family shown in (A) and the DNA sequences of *PROP1* genes (right) including B–F, that of the Hutterite family shown in (A). (From Wu, W., Cogan, J. D., Pfaffle, R. W., et al. *Mutations in PROP1 Cause Familial Combined Pituitary Hormone Deficiency*. *Nat. Genet.* 1998, 18, 147–149.)

to have GH, TSH, and ACTH deficiencies (63). MRI showed that both had small sella turcica, persistent craniopharyngeal canals, hypoplastic anterior hypophyses with associated pointed cerebellar tonsils (as seen in the Chiari malformation), and ectopic posterior hypophyses. The two probands had ectopic posterior pituitaries, which were not found in other relatives. A heterozygous germline mutation of the LIM-homeobox transcription factor *LHX4* was found that cosegregated with the syndrome in this family. Mice null for *Lhx4* have pituitary hypoplasia due to increased cell death (64). Subsequently, additional studies have shown that pituitary deficiencies in patients with *LHX4* mutations may vary, ranging from deficiency of GH only to deficiency of GH, TSH, ACTH, and gonadotropins in varying combinations, even within the same family. Anterior pituitary morphology may range from hypoplastic to normal to enlarged, while the posterior pituitary may be either normally sited or ectopic. Some patients/families may have additional brain abnormalities including abnormal

corpus callosum, Chiari malformation, or abnormal cerebellar tonsils (65–67).

**83.2.3.4 CPHD Due to *HESX1* Mutations.** Like *POU1F1* and *PROP1*, *HESX1* is a transcription factor important in the development of the pituitary. It is a member of the Paired class of homeodomain proteins and functions as a transcriptional repressor. Its role in pituitary development was first discovered in mice; mice null for the mouse homolog *Hesx1* were found to have a phenotype similar to SOD in humans, with abnormal forebrain development, absent or poorly developed eyes, and midline abnormalities including hypothalamic defects and abnormal development of Rathke's pouch. These findings led to the analysis of the human *HESX1* gene in patients with SOD, and a homozygous mutation in the homeodomain region was found in two sibs with agenesis of the corpus callosum and panhypopituitarism. The unaffected parents were heterozygous, consistent with autosomal recessive inheritance. Subsequently, some heterozygous patients have been found to be mildly

affected, with phenotypes ranging from IGHD to CPHD to GHD with optic nerve hypoplasia (68). The mutations found to date appear to affect DNA binding and/or interactions of the HESX1 protein product with other partner proteins (69). Specifically, the I26T mutation impairs the ability of HESX1 to recruit corepressors, with a phenotype of panhypopituitarism associated with anterior pituitary hypoplasia and posterior pituitary ectopia, but without optic nerve defects or other midline brain defects typically seen with SOD (70). Conversely, a mutation, which causes increased binding leading to increased repression of PROP1, has also been identified to cause SOD with GHD (71). A Japanese patient with CPHD with a hypoplastic anterior pituitary, ectopic posterior lobe, and left optic nerve hypoplasia was found to have a heterozygous frameshift mutation (306/307ins AG) in exon 2 of HESX1, which introduced a premature stop codon soon after the mutation site. Therefore, this mutation would be predicted to generate a protein lacking the carboxyl-terminal homeobox (DNA-binding) domain (72).

**83.2.3.5 CPHD Due to OTX2 Mutations.** Defects in OTX2, another pituitary transcription factor, were first identified in patients with microphthalmia syndrome, but it was quickly realized first that some of these patients have pituitary defects as well and that patients can have CPHD due to OTX2 without eye abnormalities. The number and combination of pituitary hormone deficiencies is highly variable. OTX2 is known to interact with HESX1 and affect its expression (73,74).

**83.2.3.6 CPHD Due to X-Linked Mutations.** There are pedigrees in which CPHD appears to be inherited as an X-linked recessive trait. A review of the older reported pedigrees reveals several families with only male siblings affected, compatible with either autosomal recessive with sex-limited expression or X-linked recessive inheritance. A family with four cases of X-linked recessive CPHD was found by Lagerstrom-Fermer and colleagues to show the linkage with markers in the Xq25-q26 region (peak lod score of 4.12) (see OMIM #307200; see Tables 83-2 and 83-3). Further examination revealed an extra copy of the marker DXS102, suggesting a duplication in this region. Solomon et al. identified a pedigree with mental retardation and CPHD and localized the defect to a duplication in the region Xq26.1-q27.3, then narrowed the critical region to a 3.9Mb interval at Xq27.2-q27.3 (75,76). This critical region includes SOX3, an HMG-box protein. A mutation in SOX3 has been shown to cause mental retardation and GHD in a large pedigree (77) and SOX3 has been shown to be essential for pituitary development and function in a mouse model (78). Woods and colleagues found families in which both overdosage and underdosage of SOX3 were associated with similar phenotypes, consisting of infundibular hypoplasia and hypopituitarism, but not necessarily mental retardation (79).

## 83.2.4 Disorders of the Growth Hormone Pathway

The various disorders of GH secretion and function can be classified based on (1) the level of the defect, (2) mode of inheritance, (3) number of hormone deficiencies, (4) complete or partial absence of GH versus defective GH, and (5) receptor function or resistance. This section will focus on those disorders isolated to the GH pathway (Table 83-3).

Proportionate short stature may result from a wide variety of endocrinologic, metabolic, nutritional, emotional, and genetic disorders. Pituitary deficiency has long been recognized as a cause of proportionate short stature, and it is now apparent that these disorders represent a heterogeneous group of disorders secondary to a variety of genetic and acquired defects in GH secretion or action. Defects have been described at all levels of the hypothalamopituitary-IGF1-chondro-osseous end-organ axis (Figure 83-1; Table 83-3). Delineation of the distinct disorder in each patient has implications for genetic counseling; now that both recombinant GH and IGF1 have become available for treatment, an exact diagnosis has therapeutic significance.

The frequency of GH deficiency (either isolated or concomitant with other pituitary hormone deficiencies) is estimated to range from 1/4000 to 1/10,000 in various studies. Most cases are sporadic and are assumed to arise from cerebral insults or defects, including cerebral edema, chromosome anomalies, histiocytosis, infections, radiation, SOD, trauma, or tumors affecting the hypothalamus or pituitary. Hypothalamic or pituitary anomalies are detected by MRI in ~12% of patients who have IGHD.

Estimates of the proportion of GH-deficient cases having an affected parent, sibling, or child range from 3% to 30% in different studies. This familial clustering suggests that a significant proportion of cases may have a genetic basis. The genetics and molecular pathophysiology of familial IGHD are discussed in the following sections.

Adequate amounts of GH are needed throughout childhood to maintain normal growth. Newborns with GH deficiency are usually of normal length and weight. In those with IGHD, skeletal maturation is usually delayed. Truncal obesity, facial appearance younger than expected for chronological age, delayed secondary dentition, and high-pitched voice are often present. Puberty may be delayed until the late teens, but fertility is usually normal. Fine, wrinkled skin appearing similar to that of premature aging is seen in affected adults. In patients with CPHD, the retardation of growth and skeletal maturation is often more severe and spontaneous puberty may not occur.

**83.2.4.1 GHRHR Gene Defects.** The defect in the “little” (lit) mouse is a point mutation in the GHRH receptor (*GHRHR*) gene that results in an Asp to Gly substitution at residue 60. A nonsense mutation has been



reported in the human *GHRHR* gene in two first cousins of a consanguineous Indian Muslim family with profound IGHD (OMIM 139191; Table 83-3). Both cousins were homozygous for a G to T transversion in exon 3, which converted a Glu to Stop in their *GHRHR* genes. Subsequently, the same mutation has been identified in an isolate from the Indus valley of Pakistan. A second mutation has been identified in a large isolate from Brazil diagnosed with an autosomal recessive form of IGHD. Affected subjects were found to be homozygous for a G to A transition of the first base of IVS1, which is predicted to alter splicing, and results in an inactive protein product.

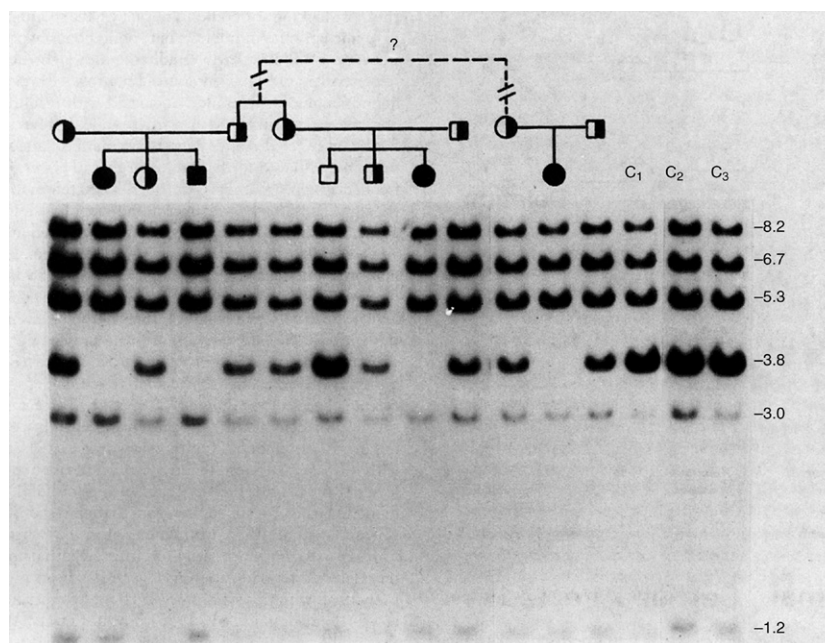
Mutations in *GHRHR* are emerging as a relatively common cause of inherited autosomal recessive IGHD. The first human *GHRHR* mutations were discovered in families with a history of parental consanguinity. More recently, kindreds in which IGHD subjects are compound heterozygotes for two distinct mutations have been reported, suggesting that mutant *GHRHR* alleles may need to be considered even in sporadic IGHD cases. Mutations in the *GHRHR* gene are usually associated with anterior pituitary hypoplasia (APH, defined as pituitary height more than 2 SDS below normal). Alba and colleagues demonstrated that pituitary height may fall within two SDS from the norm in patients with severe IGHD due to a homozygous *GHRHR* mutation, and that pituitary size may vary within patients with identical mutations who belong to the same family (80).

**83.2.4.2 Familial IGHD.** From 3% to 30% of GHD cases have an affected parent, sib, or child. This familial clustering suggests that a significant proportion of cases

may have a genetic basis. Familial IGHD is associated with at least four Mendelian disorders (Table 83-3). These include two forms that have autosomal recessive (IGHD IA and IB) inheritance, as well as autosomal dominant (IGHD II) and X-linked (IGHD III) forms. A variety of molecular defects have been detected that cause these disorders (Table 83-4). IGHD IA and IB as well as IGHD II are caused by defects in *GH1*. There are 56 *GH1* mutations documented to date in the HGMD. These defects will be reviewed under the type of inheritance (IGHD I-III) that they cause.

**83.2.4.3 IGHD IA.** The most severe form of IGHD, called IGHD IA, has an autosomal recessive mode of inheritance (OMIM 139250, Table 83-3). Affected individuals occasionally have short lengths at birth and hypoglycemia in infancy, but uniformly develop severe dwarfism by 6 months of age. In response to replacement therapy with exogenous GH, IGHD IA subjects have a strong initial anabolic and growth response that is frequently followed by the development of anti-GH antibodies in sufficient titer to block the response to GH replacement. These features led Illig and colleagues to hypothesize that IGHD IA caused complete prenatal and postnatal deficiency of endogenous GH secretion, resulting in an immune response to exogenous GH.

**83.2.4.4 Deletions.** Initially, all individuals with IGHD IA were found to be homozygous for *GH1* gene deletions (Figure 83-3; Table 83-4), and they developed anti-GH antibodies with treatment. Subsequently, additional cases with *GH1* gene deletions have been described who also have complete GH deficiency but respond well to GH replacement. Thus, the clinical outcomes of subjects



**FIGURE 83-3** IGHD type IA. Autoradiogram patterns of DNA from three Swiss families and three controls. Note absent bands in homozygous affected patients. (From Phillips, J. A. III; Hjelle, B. L.; Seeburg, P. H., et al. *Molecular Basis for Familial Isolated Growth Hormone Deficiency*. Proc. Natl. Acad. Sci. USA. 1981, 78, 6372–6375.)



with the same molecular findings (homozygosity for *GH1* gene deletions) vary, making the presence of anti-GH antibodies an inconsistent finding in IGHD IA cases.

At a molecular level, Southern blot analysis showed deletions of ~6.7, 7.0, or 7.6 kb (kilobase), with most (~75%) being 6.7 kb. DNA sequence analysis of the fusion fragments associated with *GH1* gene deletions has shown that homologous recombination between sequences flanking the *GH1* gene causes these deletions (Figure 83-3). This same mechanism has been shown to cause deletions of the beta-globin and low-density lipoprotein receptor genes in some subjects with beta thalassemia or familial hypercholesterolemia, respectively. In the past, *GH1* gene deletions were detected using polymerase chain reaction (PCR) amplification of the homologous regions flanking the *GH1* gene and the fusion fragments associated with *GH1* gene deletions. Inasmuch as the fusion fragments associated with 6.7 kb deletions differ in the size of fragments produced by certain restriction enzymes, homozygosity, or heterozygosity for these deletions can easily be detected by enzyme digestion of PCR products. More recently, multiplex ligation-dependent probe amplification (MLPA) has been used to detect *GH1* and other selected deletions. MLPA is a variation of PCR that enables multiple targets such as different exons of a target gene to be amplified with only a single primer pair. By comparing, the peak pattern obtained on a given sample with that obtained on reference samples, the relative quantity of each amplicon and exon can be determined (81).

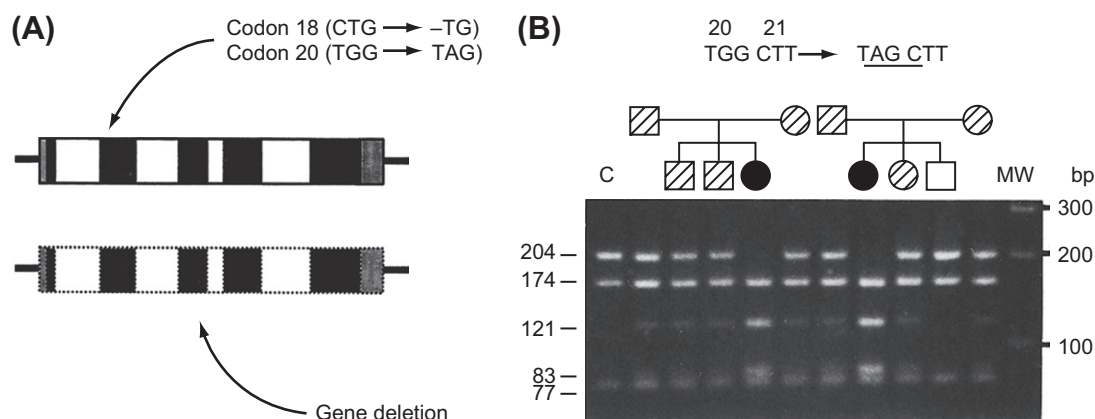
A variety of studies suggest that 13–15% of subjects with severe IGHD ( $\geq -4.5$  SD in height) have *GH1* gene deletions. Recently, frameshift and nonsense mutations have also been found in subjects with the IGHD IA phenotype, so that this disorder may best be described as complete GH deficiency due to *GH1* gene defects rather than to gene deletions alone.

**83.2.4.5 Deletion/Frameshift Mutations.** Two affected sibs with IGHD IA have been reported who

are compound heterozygotes for deletion and frameshift mutations of the *GH1* gene. Southern blot analysis showed them to be heterozygous for the 6.7 kb *GH* gene deletion. DNA sequence analysis of cloned copies of the *GH1* gene showed deletion of a cytosine at position 371 (Table 83-4). This single-base deletion results in a frameshift within the signal peptide-coding region and prevents the synthesis of any mature GH protein. The patients presented with severe growth failure and, after an initial growth response to treatment with exogenous GH, developed high titers of anti-GH antibodies.

**83.2.4.6 Nonsense Mutation.** A G to A transition in codon 20 that encodes a tryptophan (TGG) to a stop (TAG) codon substitution of the GH signal peptide has been reported in a consanguineous Turkish family with IGHD IA (Figure 83-4A). This results in the termination of translation after residue 19 of the signal peptide and no production of mature GH. Patients homozygous for this mutation have no detectable GH and produce anti-GH antibodies in response to exogenous GH treatment. Interestingly, this mutation generates a new *AluI* site that can readily be screened by PCR amplification of the GH gene, followed by *AluI* digestion and gel electrophoresis (Figure 83-4B).

**83.2.4.7 IGHD IB.** The second autosomal recessive form of IGHD is type IB (OMIM 139250; Tables 83-3 and 83-4). This disorder is associated with proportionate short stature, increased subcutaneous fat, and typical pinched facies, with high forehead, wrinkled skin, and high-pitched voice (Figure 83-5A; Tables 83-3 and 83-4). Plasma GH is deficient, but is clearly detectable after provocative stimuli. Deficient growth velocity responds quickly and consistently to GH therapy and patients do not develop high titers of blocking antibodies. They may have spontaneous hypoglycemic episodes in infancy but this is not a problem after early childhood, although they maintain hypersensitivity to exogenous insulin into adulthood. As adults, abnormal glucose tolerance associated with insulinopenia is a characteristic feature; both



**FIGURE 83-4** (A) Schematic representation of the *GH1* gene showing the locations of various IGHD IA mutations. (B) Detection of a G to A transition that encodes a Trp to Stop substitution at codon 20 by digestion with *AluI* restriction enzyme and gel electrophoresis. Note, when this mutation is present a new *AluI* recognition site occurs and the normal 204 bp fragments are cleaved to 121 and 83 bp.

quickly revert to normal following GH therapy. Puberty occurs spontaneously, but is frequently delayed to the late teens or early 20s. Puberty frequently appears abruptly during the first few months of GH therapy. Thus, in the prepubertal individual, IGHD cannot be clinically distinguished from a combined deficiency of GH and gonadotropins until at least the early 20s. LHRH stimulation studies may distinguish between these two disorders.

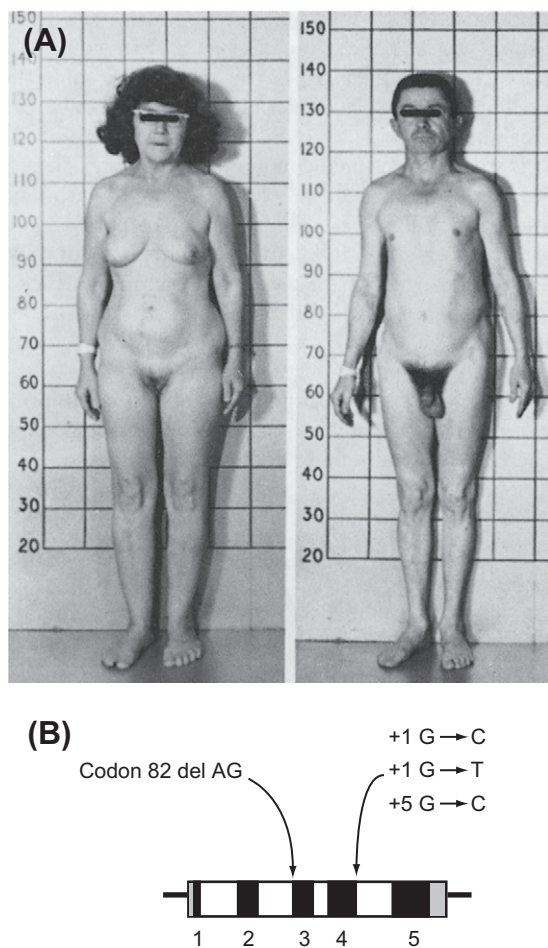
Autopsy studies in three cases of IGHD IB have all revealed the presence of typical somatotrophic cells in the pituitary and, in the one case assayed, the presence of significant amounts of immunoreactive GH. Although the relative numbers of somatotrophic cells in the pituitary differed between the three cases studied, they clearly demonstrated the pituitary's ability to synthesize GH. Administration of GHRH to two sibs with IGHD IB resulted in a significant rise in plasma GH levels. Sporadic cases of IGHD have been found to vary in their response to GHRH, suggesting that IGHD can occur secondary to either hypothalamic or pituitary defects. To date, the *GH1* gene has been present in all individuals with this syndrome who have been studied. Phillips and

colleagues found that in the majority of families with IGHD IB, the disease did not cosegregate with RFLPs at the GH gene cluster. Thus, the defects in IGHD IB may include deficiencies of GHRH synthesis or secretion or defects in GHRHR (see following). Further locus heterogeneity must be excluded.

*GH1* gene defects have been found in three families originally diagnosed with IGHD IA owing to the severity of growth retardation and apparent lack of endogenous GH as determined by RIA. They have been reclassified as having IGHD IB because of the nature of their *GH1* gene defects and their failure to produce anti-GH antibodies in response to treatment with endogenous GH (Fig. 83-5B; Table 83-3).

**83.2.4.8 Splicing Mutations.** A G to C transversion on the first base of the donor splice site of intron IV was detected in a consanguineous Saudi Arabian family with IGHD IB (Figure 83-5B; Tables 83-3 and 83-4). The effect of this mutation on mRNA splicing was determined by transfecting the mutant gene into cultured mammalian cells and sequencing the resulting GH complementary DNAs (cDNAs). The mutation was found to cause the activation of a cryptic splice site 73 bases upstream of the exon IV donor splice site. This altered splicing results in the loss of amino acids 103–126 of exon IV and creates a frameshift that altered the amino acids encoded by exon V. Such changes in the amino acids encoded by exons IV and V may not only affect the stability and biologic activity of the mutant GH protein but, as studies with bovine GH mutants have shown, may also derange intracellular targeting of GH protein products to the secretory granule. A G to T transversion has been identified at the same site in another consanguineous Saudi family (Figure 83-5B). Analysis of GH mRNA transcripts from the lymphoblastoid cells of affected patients confirmed that the G to T transversion had the same effect on splicing as the G to C transversion. Both of these mutations destroy an HphI site that enables their detection by restriction digestion of PCR products containing the GH mutation followed by gel electrophoresis. Patients homozygous for these different defects in two different families responded well to exogenous GH treatment and did not make anti-GH antibodies, suggesting that GH proteins may be released that protect against an immune response to exogenous GH.

A G to C transversion of the fifth base of intron IV of *GH1* was identified in a highly consanguineous family diagnosed with IGHD IB (Figure 83-5B). This mutation created a new Mae II site, which was used to screen all family members for the mutation. RT-PCR analysis of GH mRNA transcripts from the lymphoblastoid cells of an affected patient demonstrated that the mutation destroyed the intron IV donor splice site and had the same overall effect on splicing as the +1G to C transversion described previously. Analogous splicing mutations occur in the beta-globin gene, causing milder forms of beta thalassemia, as well as in many other genes.



**FIGURE 83-5** (A) Autosomal recessive IGHD type 1B in two siblings who are the offspring of a consanguineous mating. (B) Schematic representation of the *GH1* gene showing the locations of various IGHD IB mutations.

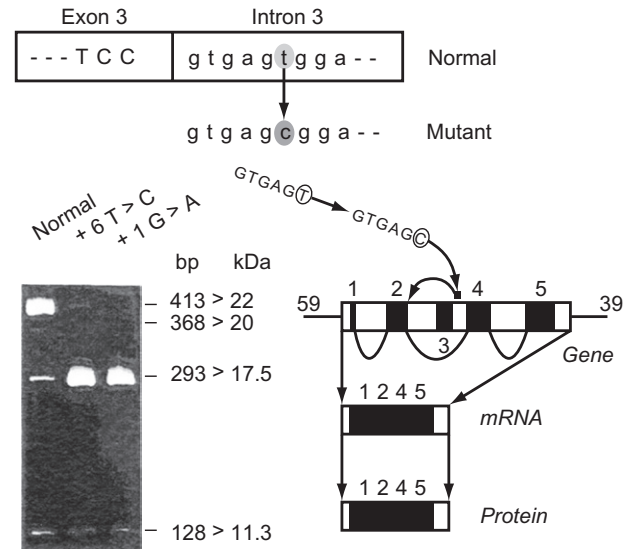
**83.2.4.9 Deletion/Frameshift Mutations.** A patient with severe growth retardation diagnosed with IGHD was found to be a compound heterozygote for mutations at the *GH1* gene locus. PCR followed by restriction enzyme digestion showed the patient to be heterozygous for the 6.7kb deletion. DNA sequence analysis of the GH gene revealed a 2-bp deletion in exon III of the second GH1 allele (Figure 83-5B; Table 83-4). This 2-bp deletion results in a frameshift within exon III and generates a premature stop codon at the position of amino acid residue 132 in exon IV. The patient had a positive response to GH replacement therapy and did not produce anti-GH antibodies, again suggesting that some GH-related protein is produced.

#### 83.2.4.10 IGHD II.

**83.2.4.10.1 Introduction.** IGHD II is an autosomal dominant disorder that is usually caused by heterozygosity for splicing mutations that increase GH1 exon 3 skipping (Figure 83-6). IGHD II cases vary in severity but respond well to exogenous GH because they do not typically develop neutralizing antibodies. The first IGHD II mutations identified were point mutations at the 5' or donor splice site of IVS3 of GH1 that caused skipping of exon 3 by impairing U1 snRNP recognition and spliceosome assembly (Figure 83-6). Other IGHD II mutations occur at the 3' splice site in IVS2 (82,83).

**83.2.4.10.2 Theoretical Basis of IGHD II.** Why these splicing mutations cause IGHD II remains poorly understood. Haploinsufficiency is unlikely because individuals who are heterozygous for GH1 deletions do not have GHD. A gain of function effect for the 17.5 kD isoform is also unlikely given its inactivity on GH receptors. A dominant-negative mechanism has long been suggested because of the autosomal dominant mode of inheritance and the cosegregation of GH1 and the low serum GH responses of IGHD II cases in response to GHRH.

There have been a variety of hypotheses for the dominant-negative mechanism of the 17.5 kD isoform (84–87). First, since loss of exon 3 results in an unpaired cysteine, Cogan and colleagues hypothesized that this could form aberrant intermolecular disulfide bonds that disrupt secretion of 22 kD GH. However, Lee et al. showed that replacing the free cysteine with an alanine did not correct the secretory defect in GH4C1 cells (87). Second, Lee et al. reported that the 17.5 kD isoform decreased the stability of the 22 kD isoform in neuroendocrine cells by 50% (87). While this supports a dominant-negative effect, a 50% reduction in stability does not explain IGHD II, since patients have little or no detectable GH on GHRH stimulation. Third, the 17.5 kD isoform inhibits secretion of the 22 kD isoform in secretory cell lines (87), without affecting PRL secretion or overall cell function or viability. Fourth, overexpression of the 17.5 kD isoform in COS7 cells disrupted the Golgi apparatus and, in contrast to previous results, impaired overall protein trafficking, specifically inhibiting secretion of PRL and trafficking of TRH receptor to

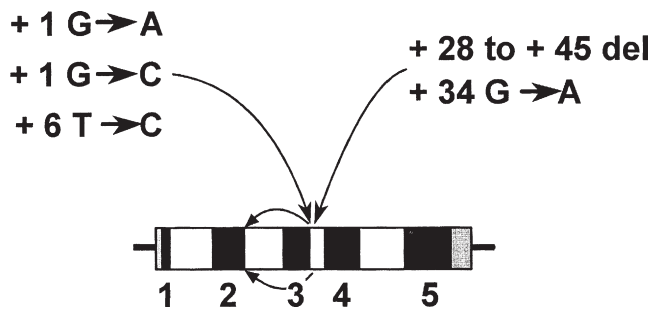


**FIGURE 83-6** IGHD II is caused by exon 3 skipping. Note the effects of IVS 3 mutations on cDNA (left) and mRNA (right).

the cell membrane (86). Collectively, these data suggest that the 17.5 kD isoform impairs 22 kD GH secretion by inhibiting protein trafficking. However, none of these hypotheses fully explained the complete deficiency of GH secretion that is seen in IGHD II cases.

To determine why GH1 mutations that give rise to products lacking exon 3 cause IGHD II, McGuinness et al. used cell culture and transgenic approaches (88). GC cell lines were generated that express either wild-type GH (WT-hGH-GC) or a GH1 mutation encoding a G to A transition at the 5' splice site of IVS3 ( $\Delta$ exon3hGH-GC) (88). WT-hGH-GC cells grew normally and produced equivalent amounts of human and rat GH packaged in dense-core secretory vesicles (SVs). In contrast,  $\Delta$ exon3hGH-GC cells showed few SVs but instead accumulated secretory product in amorphous cytoplasmic aggregates. They produced much less rat GH and grew more slowly than WT-hGH-GC cells. WT-hGH-GC cells co-transfected with a GH-eGFP construct that co-packages with GH in SVs showed normal morphology and individual SV movements, tracked with confocal and TIRF microscopy in living cells. In contrast, co-expression of  $\Delta$ exon3hGH with GH-eGFP in GC cells abolished the vesicular trafficking of GH-eGFP, which accumulated in motionless aggregates. When  $\Delta$ exon3hGH was targeted to somatotrophs in transgenic mice, it induced a syndrome of autosomal dominant GHD, with mild to severe pituitary hypoplasia and dwarfism evident at weaning in the most severely affected lines (Figure 83-7). Pituitary somatotrophs in  $\Delta$ exon3hGH transgenic mice showed gross disruption of cellular morphology, with very few GH SVs detectable by immunogold EM. In  $\Delta$ exon3hGH transgenic mice, hypothalamic GHRH expression was upregulated, while somatostatin expression was reduced, consistent with profoundly reduced GH feedback. The least affected transgenic line showed milder pituitary



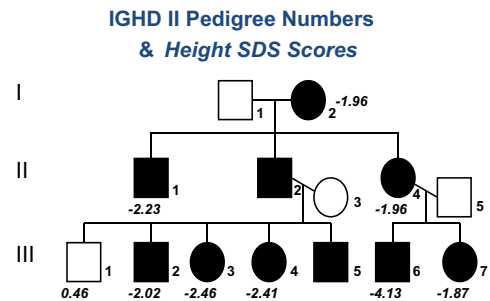


**FIGURE 83-7** Model of constitutive (A) and aberrant (B) GH1 splicing. Wild-type mice express the 22 kD isoform of GH and have normal pituitary morphology with abundant secretory granules and normal phenotype (A).  $\Delta$ exon3 mice (see text) express the 17.5 kD isoform of GH and show replacement of secretory granules by amorphous aggregates (B). This results in somatotroph death with pituitary hypoplasia and dwarfism. (From Ryther, R. C.; McGuinness, L. M.; Phillips, J. A. III, et al. *Disruption of Exon Definition Produces a Dominant-Negative Growth Hormone Isoform that Causes Somatotroph Death and IGHD II*. *Hum. Genet.* **2003**, 113, 140–148.)

deficits relatively specific for GH, while the severely affected lines with higher transgene copy numbers showed early, widespread pituitary damage, macrophage invasion, and multiple hormone deficiencies (88).

**83.2.4.10.3 Transgenic Model of IGHD II.** In the  $\Delta$ exon3 mice, the 17.5 kD isoform progresses through the regulated secretory pathway to form complexes with the WT 22 kD isoform (88). Since the 17.5–22 kD complexes are stable but cannot exit efficiently via SVs, both proteins accumulate in the Golgi and the ER. This triggers a response to the misfolded protein and the complexes are transported into the cytosol (Figure 83-7). McGuinness et al. conclude that when the ratio of 17.5:22 kD isoforms exceeds the degradative capacity of the proteasome pathway, toxic aggregates accumulate in the cytosol, ER, and Golgi. For cells producing GH in culture, autolytic cell death occurs. Interestingly, this is greatly accelerated in  $\Delta$ exon3 mice by trophic drive from GHRH to expand the (defective) somatotroph population and by macrophage invasion that destroys defective cells (88). Mutations identified in ESE1, ESE2, and ISE also increase exon 3 skipping and synthesis of the 17.5 kD isoform that causes IGHD II (89). Ryther et al. also found that artificial SE mutations that weaken exon 3 recognition lead to the production of variable amounts of the 17.5 kD isoform (89). They hypothesize that variations in the severity of IGHD II correlate with the relative levels of the 22 and 17.5 kD isoforms and explain the clinical variability seen in IGHD II.

**83.2.4.10.4 Correlation of IGHD II Severity with Mutant/Normal Transcript Ratios.** We hypothesized that the relative ratios of mutant/normal transcripts in patient-derived cultured lymphocytes (CLs) from a pedigree with an exon 3 skip mutation (Figure 83-8) would correlate with their IGHD II severity. This hypothesis is based on the 17.5-kDa transcript having a dominant-negative effect, and increased levels of expression have

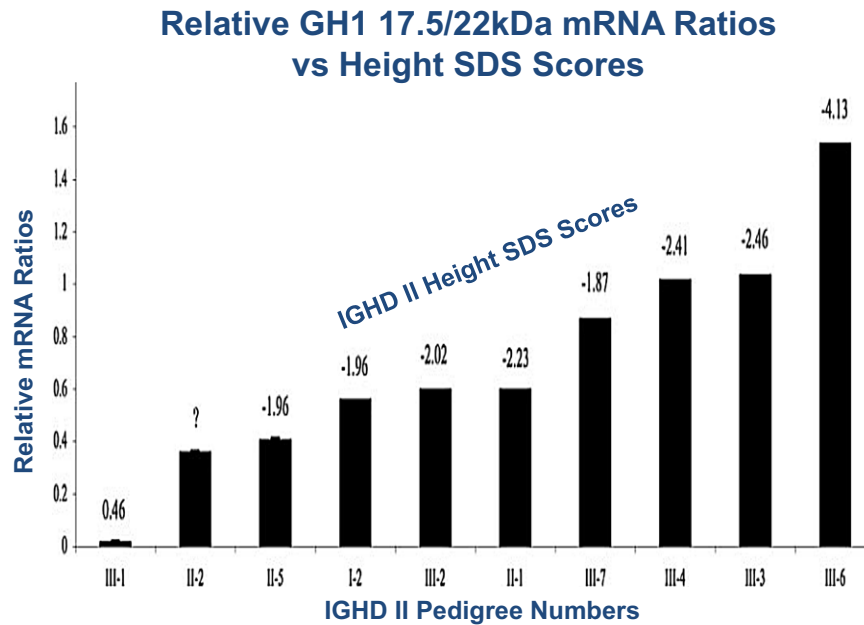


**FIGURE 83-8** Pedigree of the kindred with the E3 + 1 G/A dominant mutation that causes exon 3 skipping. Solid circles and boxes represent affected individuals. The letter “T” represents individuals whose CL-derived RNA was tested for GH1 transcripts by real-time PCR. Numbers below the boxes and circles represent Ht SDS before treatment. (From Hamid, R.; Phillips, J. A. III; Holladay, C., et al. *A Molecular Basis for Variation in Clinical Severity of Isolated Growth Hormone Deficiency Type II*. *J. Clin. Endocrinol. Metab.* **2009**, 94, 4729–4734. Copyright 2009, The Endocrine Society.)

been shown to be cytotoxic to pituitary cells. As shown in Figure 83-9, the relative ratio of 17.5/22-kDa transcripts statistically correlated with Ht SDS ( $P = 0.001$ ), indicating that expression levels of both the mutant and normal *GH1* allele are important in the pathogenesis of IGHD II. These results also suggest that more than ~80% of the variation in Ht SDS is attributable to differences in the isoform ratios. In agreement with this, we note that III-6 who has the highest mutant/normal ratio also has the lowest Ht SDS (Figure 83-9) and GH peak as well as a hypoplastic anterior pituitary (data not shown). In contrast, I-2 and II-5, who have among the lowest mutant–normal ratios of all the family members with IGHD II, also have Ht SDS of –1.96 despite having never been treated with GH replacement (90).

**83.2.4.10.5 Variable Expressivity of IGHD II.** Variable expressivity is regularly seen in IGHD II pedigrees that contain affected individuals whose heights vary, and even without treatment, some may be of normal height. One hypothesis to explain this is that *GH1* mutation that causes IGHD II is not the only determinant of the final height, and thus other “modifying” genes may play an important role in the final phenotype. Knowledge about these modifying genes is still lacking. Our data suggest that one of the important modifiers of IGHD II severity may be variations in the ratios of *GH1* transcripts from both the normal and the mutant IGHD II alleles. Reports that variation of expression of the alleles modifies disease phenotype are rare but are noted for a small number of autosomal dominant diseases (91–93). Observing this variation raises the question of what regulates *GH1* transcript/protein expression. Our segregation studies show that expression levels for the same mutant and normal *GH1* alleles are not allele specific because sibs with disparate Ht SDS inherited the same parental mutant and normal *GH1* alleles. Our segregation data suggest that transcript levels and ratios can differ between sibs such as III-6 and III-7, presumably due to differences





**FIGURE 83-9** (A) 17.5-kDa (gray) and 22-kDa (black) GH1 transcript levels as measured by relative real-time PCR analysis. (B) The relative transcript ratios of 17.5-kDa/22-kDa in CL cell lines derived from the kindred presented in Figure 83-8. Numbers above the columns in panel B represent Ht SDS. Question mark indicates that Ht SDS of II-2 is not known. Family member II-4 was not included in the figure because we could not calculate his Ht SDS due to missing height information (his relative transcript ratio was 0.1, as would be expected for a normal person); additionally, because this person married into the kindred, we felt that he would not be an appropriate control. Each sample was done in triplicate, and analysis was repeated three times. Error bars represent SEM. (From Hamid, R.; Phillips, J. A. III; Holladay, C.; et al. *A Molecular Basis for Variation in Clinical Severity of Isolated Growth Hormone Deficiency Type II*. *J. Clin. Endocrinol. Metab.* **2009**, 94, 4729–4734. Copyright 2009, The Endocrine Society.)

in *trans*-acting factors and/or altered splicing efficiency rather than *cis*-acting factors (90).

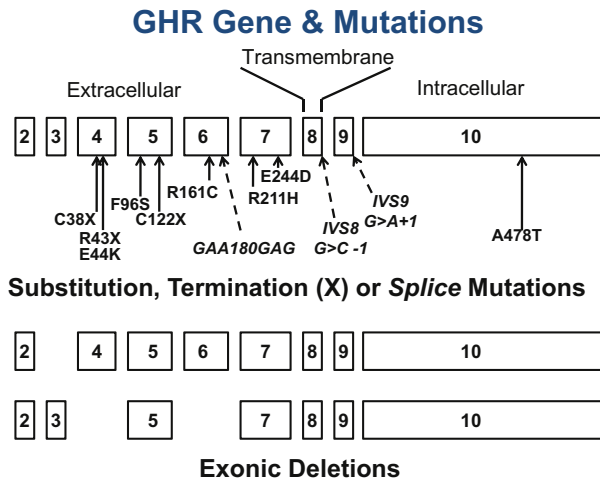
#### 83.2.4.10.6 Experimental Treatment of IGHD II.

Allele-specific degradation of mRNAs encoding the 17.5 kD isoform is a possible way to approach for therapy for individuals with IGHD II. Because numerous *in vivo* (88,89) and *in vitro* (86,87) experiments suggest that normal GH secretion depends, in part, on avoiding increased levels of the 17.5 kD isoform, Ryther et al. concluded that therapies specifically targeting the 17.5 kD isoform might be useful in treating IGHD II. To exclusively target skipped transcripts for degradation, Ryther et al. utilized RNA interference (RNAi) employing silencing RNAs (siRNAs) (Figure 83-10) (94). The exon 2–4 junction was selected as the siRNA target because it is the only sequence unique to transcripts encoding the 17.5 kD GH1 isoform. Plasmids expressing this siRNA (17.5-si) or a random dsRNA sequence (control-si) were cotransfected with either wt GH1 or GH1 enhancer mutants (ESEM and ISEM1) that produce more skipped transcripts. Splicing patterns and actin expression levels were analyzed by RT-PCR. Interestingly, the 17.5-siRNA construct reduced the skipped transcripts by more than 95%, while having no effect on either the FL or cryptic mRNAs (Figure 83-10B). Similar analyses with dsRNA identical to the 17.5-si sequence showed a greater than 90% decrease in skipped mRNAs without affecting the FL or cryptic mRNAs (data not shown). We conclude that siRNAs can specifically degrade 17.5 kD isoform

mRNA, suggesting that this strategy could be effective in preventing production of sufficient dominant-negative 17.5 kD mRNA to cause IGHD II.

**83.2.4.10.7 Summary.** GH1 mutations that cause exon 3 skipping include those in the donor splice site of IVS 3, as well as exon splice enhancers (ESEs) and intron splice enhancers (ISEs). These heterogeneous that perturb splicing result in a 17.5 kD isoform that causes IGHD II in humans and dose-dependent disruption of GH SVs in GC cells and transgenic mice (Figure 83-7) (89). Ryther et al. found that dual SEs are required to ensure the exon 3 definition that produces 22 kD GH (94). Artificial SE mutations that weaken exon 3 recognition lead to the production of variable amounts of the 17.5 kD isoform. We hypothesize that variations in the severity of IGHD II correlate with the relative amounts of 22 and 17.5 kD. Interestingly, Ryther et al. found that siRNAs specific for mRNAs encoding the 17.5 kD isoform could be used to specifically degrade these transcripts in transfected cells (94). If siRNAs could be used in patients to destroy deleterious transcripts such as those causing IGHD II, this approach might provide a potential new avenue of therapeutic intervention for individuals with IGHD II and other dominant and dominant-negative disorders.

Mullis et al. found marked clinical variability in the severity of the IGHD II phenotype, depending on the GH1 gene alteration (95). On follow-up, a number of these patients developed other pituitary hormone



**FIGURE 83-10** Destruction of the 17.5kD messenger by allele-specific siRNA. (A) GH1 constructs expressing wt or ESE mutations were co-transfected with 17.5 kD or control siRNAs. (B) Note knock-down of 17.5kD transcripts in presence of 17.5kD siRNAs. (From Ryther, R. C.; Flynt, A. S.; Harris, B. D., et al. *GH1 Splicing Is Regulated by Multiple Enhancers Whose Mutation Produces a Dominant-Negative GH Isoform that can be Degraded by Allele-Specific Small Interfering RNA (siRNA)*. *Endocrinology*. 2004, 145, 2988–2996.)

deficiencies, mainly ACTH and TSH, especially those that developed a more hypoplastic pituitary gland with time. It is possible that destruction of pituitary somatotrophs by the dominant-negative mechanism above leads to destruction of other cell lines, but this remains to be proved.

**83.2.4.11 IGHD III.** Fleisher et al. described a kindred in which two brothers and their two maternal uncles had a syndrome consisting of hypogammaglobulinemia and IGHD (OMIM 307200; [Table 83-3](#)). They had proportionate short stature, retarded bone age in childhood, delayed onset of puberty, lack of plasma GH response to insulin–arginine stimulation, low bio- and immunoassayable IGF1, and normal TSH, ACTH, FSH, and LH secretion. Recurrent sinopulmonary infections were a problem in two patients, which were abated by parenteral globulin therapy. Three of the patients had pan-hypogammaglobulinemia and absence of circulating B cells, whereas the other patient had normal serum IgA and IgM levels and decreased levels of circulating B cells. All had an absence of specific in vitro antibody production after antigenic stimulation and a failure of in vitro immunoglobulin production. Two of the patients had normal-appearing tonsils. T-cell function and numbers were normal. One of the patients was treated with exogenous GH and developed detectable circulating B lymphocytes, as well as higher levels of IgA, IgM, and IgE than his affected relatives.

Affected individuals in some kindreds have agammaglobulinemia associated with their IGHD, but others do not. This suggests that contiguous gene defects of Xq21.3-q22 may occur in some cases. Interestingly, other cases of IGHD have been found to have an interstitial

deletion of Xp22.3 or duplication of Xq13.3-q21.2, suggesting that multiple loci may cause IGHD III.

**83.2.4.12 Kowarski Syndrome (Bioinactive Growth Hormone).** A number of patients have been described with the clinical features of IGHD who achieved normal plasma immunoactive GH levels following stimulation, but low levels of IGF1 (OMIM 139250; [Table 83-3](#)). Less GH was detected by radioreceptor assay than by radioimmunoassay. Following GH administration, however, they generated normal IGF1 levels and had a significant increase in their growth rates. TSH and ACTH secretion was normal. In view of their clinical syndrome of IGHD, normal plasma GH, low basal IGF-levels, and their normal response to exogenous GH, they appear to secrete a biologically inert GH. As their endogenous GH reacted normally in the immunoassay, this appears to represent a cross-reactive material (CRM) positive mutation.

Valenta et al. reported a similar patient with short stature and normal levels of immunoreactive GH but decreased radioreceptor activity. This patient responded well to exogenous GH, but his plasma IGF1 level was normal. When analyzed by column chromatography his GH exhibited unusual patterns, with most of it migrating as large tetramers or dimers. However, there is a question as to whether his growth failure occurred before age 8 years.

Takahashi et al. identified a C to T transition in codon 77 that results in an Arg to Cys substitution in the GH1 gene of a subject diagnosed with bioinactive GH (OMIM 139250; [Table 83-3](#)). The patient was heterozygous for the mutation, and isoelectric focusing of his serum showed an abnormal GH peak in addition to a normal peak. Surprisingly, his father was also heterozygous for the C to T transition but was of normal height and had normal isoelectric focusing results. The disparate findings in the father and his affected child were not explained.

Besson et al. identified a missense mutation (G705C) in the GH1 gene of a Serbian patient with short stature ([96](#)). This mutation was found in the homozygous state and led to the absence of the disulfide bridge Cys-53 to Cys-165. Thus, GH-C53S is a bio-inactive GH at the physiological range and the disulfide bridge Cys-53 to Cys-163 appears to be required for mediating the biological effects of GH.

By contrast, a number of patients have been described with “invisible GH,” that is, individuals with normal growth, deficient GH secretion as measured by radioimmunoassay, but normal GH concentrations as measured by radioreceptor assay. Thus, these individuals appear to secrete a mutant molecule with normal biological activity which is “invisible” to GH radioimmunoassay. Bis-tritzer et al. postulated that this unusual molecule could be expressed from the *GH-V* gene.

**83.2.4.13 Growth Hormone Secretagogue Receptor (GHSR) Defects.** GHSR (OMIM 601898) is a G-protein-coupled receptor highly expressed in the pituitary and hypothalamus. First recognized as a receptor

for exogenous stimulators of growth hormone secretion, its endogenous ligand Ghrelin (GHRL, OMIM 605353) was identified in 1999. Ghrelin has pleiotropic effects on many body systems and is an orexigenic hormone, but it is also known to release GH, suggesting that it or its receptor GHSR might be involved in short stature. In 2006, Pantel et al. identified a GHSR mutation, A204E, in two unrelated families with apparent idiopathic short stature (ISS). The pedigrees suggested autosomal dominant inheritance with more severe expression in one patient who was homozygous. This amino acid is conserved across all species studied so far and the substitution indicates a significant change in charge. In vitro studies showed that the mutation selectively impaired the constitutive activity of GHSR (97). Subsequently, the same group identified a patient who was a compound heterozygote for two mutations in GHSR. The heterozygous parents were unaffected suggesting autosomal recessive inheritance. The patient had laboratory studies consistent with IGHD and responded well to GH. The mutations also showed reduced constitutive activity of the receptor (98).

**83.2.4.14 Growth Hormone Resistance.** The GHR is localized to human chromosome 15p13.1-p12 and to mouse chromosome 15. The human *GHR* gene spans at least 87kb, making it over 40 times larger than the *GH1* gene, and contains 9 exons (numbered 2–10), whereas exons 2–9 are small, ranging from 66 to 179 bp, exon 10 is large and spans about 3400 bp. The signal peptide and extracellular portions of the GHR that correspond to the GHBP are encoded by exons 2 and 3–7, respectively. The transmembrane and cytoplasmic portions of the GHR are encoded by exons 8 and 9–10, respectively. Currently, 70 mutations have been described in GHR (Table 83-4).

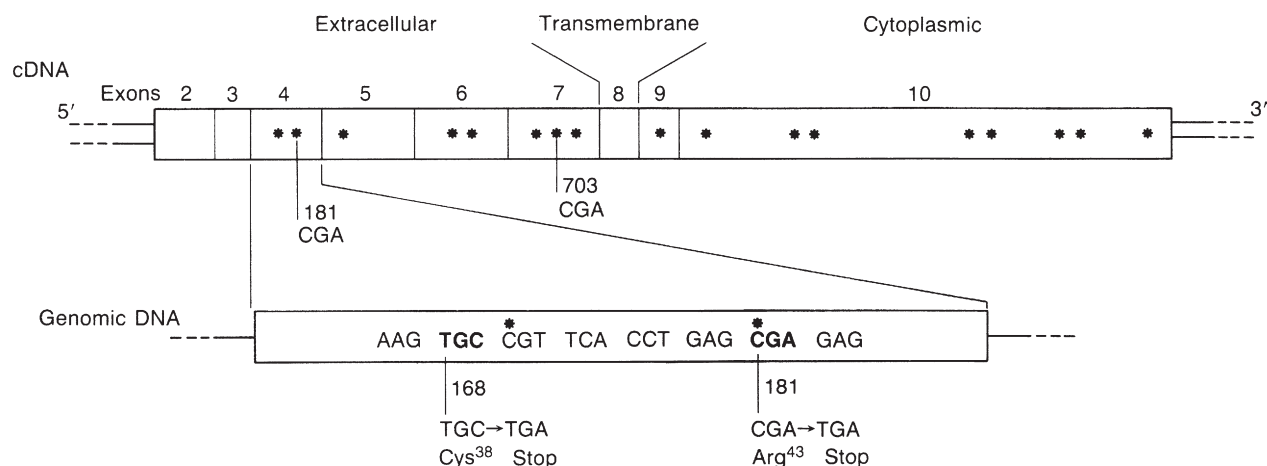
**83.2.4.14.1 GHR Expression.** The extracellular domain of the GHR is normally found in serum as GHBP. The binding affinity of GHBP for GH is about the same as that of intact GHR. The GHBP is thought to arise by alternative splicing of GHR mRNA or proteolysis of the mature GHR peptide. Evidence for alternative

splicing was provided by the detection of multiple GHR transcript sizes by Northern blot analysis. The binding complex formed between GH and a slightly truncated form of GHBP (residues 1–238) is a heterotrimer consisting of one molecule of GH plus two molecules of the truncated GHBP. In this novel process, the second truncated GHBP molecule can bind to GH only if the first truncated GHBP molecule is already bound.

The transmembrane domain of the GHR is encoded by exon 8 and contains 30 residues (Figure 83-11). This portion of the receptor functions as an anchor to position the extracellular and cytoplasmic portions of the GHR molecule correctly in relation to the cell membrane.

The cytoplasmic domain of the GHR is encoded by exons 9 and 10 and contains residues 277–620 (Figure 83-11). This segment of about 350 amino acids functions in signal transduction. The box 1 site, amino acids 276–287, is constitutively associated with janus kinase 2 (jak2); box 2 (amino acids 325–338) is required for full activation of Jak2. The GHR also interacts directly with Stat5.

**83.2.4.14.2 GHR Gene Defects.** Laron dwarfism type 1 is an autosomal recessive disorder caused by target resistance to the action of GH (Figure 83-1). Laron and colleagues described a syndrome with the clinical features of pituitary dwarfism, associated with high plasma concentrations of immunoreactive GH (Figure 83-12; OMIM 262500; Table 83-3). Although their patients were all oriental Jewish, this autosomal recessive syndrome has since been described in numerous other ethnic groups. These individuals have the clinical appearance of patients with IGHD to an exaggerated extent, with severe growth retardation, severely pinched facies, high-pitched voices, and small male genitalia. Males have delayed puberty. Birth weight is normal, but birth length may be reduced. Motor development may be delayed and some are mildly retarded. Teething and fontanelle closure are delayed. Their hands and feet are small and, like pituitary dwarfs, they are obese and their body proportions are childlike.



**FIGURE 83-11** Schematic diagram of the GHR gene showing the sequence and location of point mutations. (From Phillips, J. A. III. *Molecular Biology of Growth Hormone Receptor Dysfunction*. Acta Paediatr. 1992, 363, 127–131.)



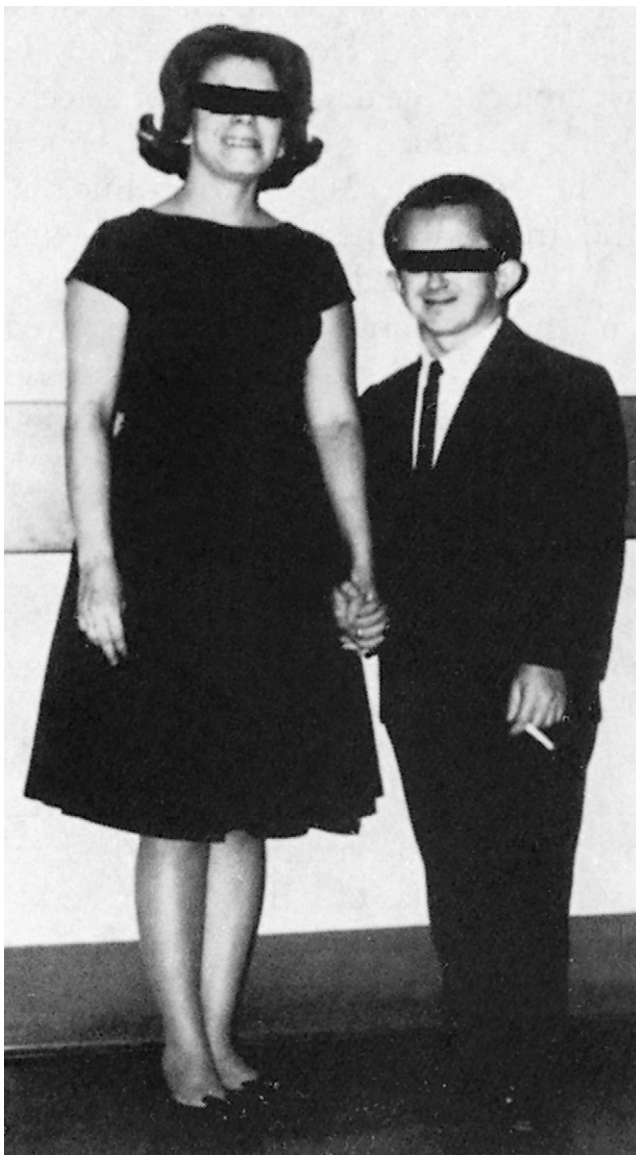
They may have spontaneous hypoglycemic episodes in infancy and usually have insulinopenia in response to glucose and arginine. ACTH, TSH, gonadotropin, and vasopressin secretion is normal. Fasting plasma GH concentrations are usually elevated, but may fluctuate from normal levels to over 100 ng/mL in the same patient. There is further elevation of plasma immunoreactive GH concentration following insulin-induced hypoglycemia and arginine infusion. Plasma IGF1 levels are low and, unlike those in GH-deficient patients, do not respond to GH administration. Furthermore, they are relatively unresponsive to the metabolic and growth-promoting effects of GH. It was first thought that this disorder was due to the synthesis of a structurally altered GH molecule which was immunologically active but biologically inert. Jacobs et al. used a receptor assay to show that the GHR is functionally defective, and Eshet et al. showed that GH

binding was absent in the livers of patients with Laron syndrome. The absence of both GHR activity and GHBP in Laron syndrome suggested that the GHR and GHBP might be identical or closely related. Further proof that Laron syndrome is due to GHR defects is the effectiveness of IGF1 in inducing GH-like metabolic responses in patients with the syndrome. Currently 61 GHR mutations are cataloged in the HGMD. Selected examples and their effect on GHR expression will be reviewed.

**83.2.4.14.3 GHR Deletions.** The first examples of GHR mutations reported were deletions of portions of the gene encoding the extracellular domain (Figure 83-11). Southern blotting showed altered restriction patterns of the GHR genes from patients with Laron syndrome who had no detectable GHBP and very low levels of IGF1. These and other studies using exon-specific probes were interpreted as showing deletions of exons 3, 5–6, and part of 4 from the GHR gene. How these two noncontiguous deletions arose remains unclear. Whereas 2/9 (22%) of patients studied by Godowski and associates had GHR deletions, Parks detected GH receptor deletions in 5/9 (56%) of the Laron syndrome probands studied (personal communication). The heights of patients with Laron syndrome ranged from –7 to –8.5 SD in those with GHR deletions and –5 to –9 SD in those with point mutations. Five families with GH insensitivity syndrome and partial deletions of the GHR gene have recently been reported in individuals with diverse ethnic backgrounds (99). A small (22 bp) deletion in GHR resulting in a frameshift and truncation of the GHR protein after amino acid 449 has been reported to result in loss of Stat5 signaling with a phenotype of Laron syndrome (100).

**83.2.4.14.4 GHR Point Mutations.** Multiple point mutations have been detected within the GHR gene (Figure 83-11). Amselem et al. detected a T to C substitution that converts the 96th residue of the extracellular domain from phenylalanine to serine. Duquesnoy et al. demonstrated that cells transfected with this mutant cDNA lacked GH binding activity. Interestingly, GH binding activity was found in the lysosomal fraction and the mutant proteins were located in the cytosol. These findings suggest that cells do not correctly transport the GHR with serine at position 96, and demonstrate the importance of phenylalanine at position 96, which is conserved in related cytokine receptors. Other missense mutations that affect protein trafficking and binding affinity as well as receptor expression and dimerization have been reported (101).

In a patient of northern European origin, a cysteine (TGC) to stop codon (TGA) mutation was detected at codon 38 in exon 4 (Figure 83-11). An arginine (CGA) to stop codon (TGA) mutation was found at codon 43 in exon 4 of two Mediterranean patients who were products of consanguineous marriages. Both stop codons truncate the GHR protein and delete most of its GHBP domain and all of its transmembrane and intracellular domains. These findings are consistent with the



**FIGURE 83-12** A woman with IGHD and her husband with Laron dwarfism.



lack of GHBP in each of the patients with Laron syndrome. The mechanism of the CGA to TGA mutation is consistent with deamination of 5-methylcytosine that preferentially occurs in CpG dinucleotides. Such dinucleotides often represent “hot-spots” for CG to TG or for CG to CA mutations, and 17 occur within the GHR gene (Figure 83-11). Two of these, at nucleotides 181 and 703, occur in CGA codons that could yield stop codons. An ATG to TTG mutation which abolishes the translation initiation codon and thus results in complete absence of GHR protein has also been reported (101). Up to 5–8% of patients with ISS may have GHR mutations.

**83.2.4.14.5 GHR Splicing Defects.** Rosenbloom et al. identified 20 patients with Laron dwarfism type 1 in an inbred population of Spanish extraction in southern Ecuador. These patients were –6.7 to –10 SD below the mean height and had limited elbow extension, blue sclerae, short limbs, hip degeneration, acrohypoplasia, and normal or superior intelligence. To determine the associated defect in the GHR gene, Berg et al. used denaturing gradient gel electrophoresis to analyze each exon of the GHR gene. Unusual fragments derived from exon 6 showed abnormal mobility, suggesting a 20- to 32-bp deletion. Genomic DNA sequencing showed an A to G substitution in the third position of codon 180, which is 24 nucleotides from the 3′ end of exon 6. Although this mutation does not cause an amino acid substitution, it produces a consensus 5′ or donor splice sequence within exon 6. Using allele-specific oligonucleotide probes, 45/46 Ecuadorian patients with Laron syndrome were found to be homozygous for this defect. This substitution produces a near-consensus donor splice site within exon 6 that results in the deletion of eight amino acids, owing to abnormal splicing of the 3′ end of exon 6. Deletion of these residues is thought to reduce the function of the GHR molecule. Interestingly, some individuals who are homozygous for this mutation apparently retain some GHR activity, suggesting that some normal splicing can occur. A point mutation in intron 9, which results in exon 9 skipping and creation of a stop codon in exon 10, has been reported. This splice defect results in a truncated protein which is missing most of the intracellular domain and causes GH resistance by a dominant-negative suppression of GHR signaling. Patients with this mutation also had elevated GHBP levels.

**83.2.4.14.6 GHRd3 Isoform.** The GHRd3 isoform results from genomic deletion of exon 3 and is polymorphic. GHRd3 appears to be sufficient for normal growth (102). In children with ISS and short stature due to SGA, the GHRd3 polymorphism has been associated with improved response to exogenous GH (103). Such data could eventually be used to individualize therapy.

**83.2.4.14.7 Clinical Relevance.** Significant genetic heterogeneity has already been demonstrated in GHR gene defects in Laron syndrome. Most of the differences seen in clinical severity may be due to this heterogeneity.

The height abnormalities of patients studied ranged from –5, –8, –10 SD in those homozygous for point mutations, deletions, and splicing abnormalities, respectively. Whether the clinical differences seen in blue sclerae, hip degeneration, and intelligence are due to the severity of the syndrome or to specific GHR alleles remains to be determined. From mutations found in familial GHD and beta thalassemia, it could be predicted that clinically milder and atypical cases of Laron syndrome will be found that have GHR abnormalities. Combinations of different mutations in single patients, referred to as genetic compounds, could be another source of variation in clinical severity. Indeed, a number of children with idiopathic short stature and low serum GHBP had heterozygous mutations in the GHR gene, resulting in partial resistance to GH. It has been estimated that GHR mutations account for up to 5% of all idiopathic short stature patients.

**83.2.4.15 Laron Dwarfism II (Post-Receptor Defects).** Lanes and colleagues described an adolescent male with proportionate dwarfism, a normal plasma GH response to stimulation and elevated IGF1 by bioassay, radioreceptor assay, and radioimmunoassay (OMIM 245590; Table 83-3). Bone age was clinically retarded, but by age of 15 his sexual development was well established; 24-h GH secretion was normal, as were his ACTH, TSH, and gonadotropin functions. In view of his elevated IGF1 and clinical hypopituitarism, peripheral unresponsiveness to IGF1 at either the receptor or the postreceptor level was postulated. Cultured fibroblasts from such a patient had a 50% decrease in IGF1 binding compared to controls. Patients with Laron dwarfism II have elevated serum GH, normal GHBP levels, and respond well to treatment with IGF1, indicating that their growth deficiency is due to a post-GHR defect (Figure 83-1). Woods et al. described a patient with severe growth failure, sensorineural deafness, and mental retardation who was found to be homozygous for a partial deletion of the IGF1 gene. RT-PCR analysis confirmed the deletion of exons 4–5, which would result in a severely truncated mature IGF1 peptide. Interestingly, this patient had only a slightly delayed bone age indicating that GH directly stimulates bone maturation.

After binding to the GH receptor, GH initiates signal transduction through a number of pathways, including the JAK-STAT pathway. Postreceptor defects in GH responsiveness have been described secondary to a STAT5b mutation (104,105).

IGF1 receptor (IGF1R) mutations have been described in patients with intrauterine growth retardation and poor postnatal growth (106). Patients with a deletion of the distal long arm of chromosome 15, where the IGF1R gene is located, have growth retardation, but a cause and effect relationship could not be established in vitro. It is likely that both IGF1R and postreceptor defects will be found in other children with combined intrauterine and postnatal growth retardation.

**83.2.4.16 African Pygmies.** Peripheral unresponsiveness to GH administration in the presence of normal concentrations of immunoreactive plasma GH and normal bioassayable IGF1 activity has been documented in the African Pygmies. This population, who inhabit the rain forests of equatorial Africa, resemble pituitary dwarfs in size and skeletal proportions, but do not have the truncal obesity, peculiar facies, and wrinkled skin of pituitary dwarfism. Following insulin-induced hypoglycemia and arginine infusion, plasma GH levels are normal but, like type I IGHD, they are insulinopenic and hypersensitive to the effects of exogenous insulin. They are completely unresponsive to the lipolytic, insulinotropic, and nitrogen-retaining properties of GH. Deficiency of IGF1 with normal IGF2 has been noted in these pygmies. Decreased serum GHBP and IGF1 levels were found to be significantly decreased in two Pygmy populations in the Philippines (107) and decreased IGF1 was described in the Central African Republic pygmies. Normal levels of serum IGF1 and 2, IGFBP3, GHBP, and GH in the African pygmies, compared to a neighboring population of normal size, have been reported by Geffner et al. Using HTLV-II transformed-lymphoblast cell lines from Efe pygmies, they demonstrated complete resistance to the growth-promoting actions of IGF1 and GH but normal stimulation by insulin, suggesting that non-responsiveness to IGF1 may be the cause of the short stature. In addition, no difference in the distribution of IGF1 alleles could be detected between pygmies and non-pygmy black Africans. Thus, it is not yet clear as to whether the GH unresponsiveness in the pygmies is secondary to a deficiency of IGF1 or to an IGF1 receptor or postreceptor defect.

## 83.2.5 Disorders of the Gonadotropin Pathway

**83.2.5.1 Hypogonadotropic Hypogonadism.** Kallmann syndrome (KS) (OMIM 308700) is a syndrome of hypogonadotropic hypogonadism coupled with anosmia, caused by failure of migration of GNRH neurons from the developing olfactory lobe to the hypothalamus. X-linked, autosomal dominant, and autosomal recessive forms are recognized (Table 83-1). The X-linked form is the most common and is caused by mutations in KAL1 (308700) which encodes anosmin-1, a extracellular matrix protein which acts as a chemoattractant for migrating neurons (108,109). Anosmin-1 also interacts with fibroblast growth factor signaling, and *FGFR1* (fibroblast growth factor receptor 1, OMIM 136350) has been shown to be the causative gene in autosomal dominant KS (OMIM 147950) (110). Multiple other genes have now also been implicated (Table 83-1). Interestingly, one of these genes, *CHD7*, has also been implicated in idiopathic hypogonadotropic hypogonadism as well as in CHARGE association (see above). Kim et al. analyzed the *CHD7* gene in 50 patients with KS (defined as HH

with anosmia or hyposmia) and 51 with IHH. They found mutations in three patients with KS and four with IHH. Interestingly, the same mutation (IVS8+5G>A) was found in one KS patient and one IHH patient, and this mutation has previously been reported in a patient with CHARGE syndrome. The authors propose that KS/IHH may represent a mild variant of CHARGE syndrome, with phenotypic expression modified by additional genes or epigenetic factors (111).

**83.2.5.2 Central Precocious Puberty.** Onset of puberty is complex process, in which onset of pulsative hypothalamic GNRH secretion is a key step. One regulator of this process is signaling of kisspeptin through its receptor (KISS1R, OMIM 604161; also called GPR54). Teles et al. have reported a case of central precocious puberty caused by an activating mutation of KISS1R (inactivating mutations may cause IHH, see above and Table 83-1) (112).

## 83.2.6 Disorders of Pituitary Hypersecretion and/or Neoplasia

Genetic disorders of pituitary hyperfunction are far less common than those of pituitary insufficiency (see Tables 83-1 and 83-5). At least four distinct syndromes have been recognized: multiple endocrine neoplasia, type 1 (MEN1), and type 4 (MEN4); Carney complex (CNC); and familial isolated pituitary adenoma (FIPA) (113). The most common form of hereditary pituitary neoplasia is MEN1; but most of these adenomas are PRL-secreting or nonfunctioning and only about 10% are GH secreting (somatotropinomas). In both CNC and IFS, the tumors are always somatotropinomas. Although multiple cases of familial acromegaly and of the amenorrhea-galactorrhea syndrome have been described in certain kindreds with no evidence of other endocrine involvement, these disorders may well represent limited forms of MEN1.

**83.2.6.1 Familial Isolated Pituitary Adenoma.** Although the majority of cases of acromegaly are sporadic, many families have been reported in which multiple members are said to be affected. Familial cases of prolactinoma have also been described. Initially, it was thought that familial cases involved only one type of tumor (usually GH secreting); however, it is now recognized that affected families may manifest many different types of tumors (114). Prolactinomas are most common, followed by somatotropinoma, followed by nonfunctioning tumors. Cushing's disease and thyrotropinomas occur but are very rare. Kindreds have been described in which all affected family members have the same type of tumor, while other kindreds have multiple different types. Genetic anticipation occurs with tumors appearing earlier in succeeding generations (114).

Approximately 15% of kindreds with FIPA have mutations in the aryl hydrocarbon receptor interacting protein gene (AIP, OMIM 605555) (115). AIP mutations

were first described by Vierimaa et al., in two kindreds with familial somatotropinomas (116). AIP appears to function as a tumor suppressor gene in pituitary tissues and it appears that a second hit in pituitary tissue is needed for an adenoma to occur, which would explain the relatively low penetrance in families with AIP mutations (115).

In an interesting historical corollary, Chahal et al. were able to extract DNA from teeth of the “Irish Giant,” a famous patient with gigantism who died in 1783 and whose skeleton was preserved in the Hunterian Museum in London, UK. They were able to demonstrate that the patient had a 910C-T mutation in his AIP gene. They used microsatellite studies to demonstrate that four contemporary kindreds in Ireland with FIPA and the same AIP mutation share a common ancestry with the Irish Giant and likely share the mutation through the founder effect (117).

Although AIP mutations explain a significant percentage of FIPA, a majority of cases do not have those mutations. There are at least nine other loci which show linkage to FIPA in affected kindreds without AIP mutations (118).

**83.2.6.2 Multiple Endocrine Neoplasia Type 1.** MEN1 (OMIM 131100) is a familial disorder characterized by multiple tumors or hyperplasia of the endocrine glands, most commonly parathyroid, pancreas, and pituitary (see also Chapter 85). The clinical manifestations of pituitary disease are dependent on the type of tumor; prolactinomas are most common with nonfunctioning and GH-secreting adenomas following. In any patient with a pituitary neoplasm, an effort should be made to rule out the involvement of the other endocrine organs, in both the patient and their close relatives.

The MEN1 syndrome is inherited as an autosomal dominant trait, with marked intrafamilial variable expressivity. The MEN1 gene (OMIM 613733) encodes a nuclear protein, menin, and a large number of inactivating mutations have been recognized. Currently, 333 mutations in the MEN1 gene are cataloged in the HGMD.

**83.2.6.3 Multiple Endocrine Neoplasia Type 4 (MEN4).** Multiple endocrine neoplasia type 4 (MEN4) (OMIM 610755) is a neoplastic syndrome similar to MEN1. It is also autosomal dominant, but clinical features vary somewhat, consisting primarily of pituitary tumors and hyperparathyroidism (119). Pellegata et al. identified mutations in the cyclin-dependent kinase inhibitor p27 (CDKN1B, OMIM 600778), another tumor suppressor gene (119).

**83.2.6.4 Carney Complex.** Carney et al. described a syndrome consisting of mucocutaneous and cardiac myxomas, spotty skin pigmentation, schwannomas, and endocrine overactivity. Pituitary gland adenomas occur in >20% of patients and are always GH secreting (120). The syndrome has been linked to two separate loci, 17q and 2p. The 17q gene associated with CNC encodes the

protein kinase A regulatory subunit 1 (PRKAR1A), inactivation of which leads to enhanced activity of the GH-releasing hormone-induced signal transduction pathway (121,122). This pathway exerts proliferative effects on somatotropes and is identical to that activated by somatic mutations in the Gs alpha gene, which has been associated with up to 40% of (nonfamilial) somatotropinomas and the developmental mutations occurring as a mosaic in patients with McCune–Albright syndrome, in which somatotrope hyperplasia and adenoma formation frequently occur. There are at least 32 mutations in PRKAR1A cataloged in the HGMD. CNC type II refers to those patients with linkage to chromosome 2p, in which the molecular defect is still not known.

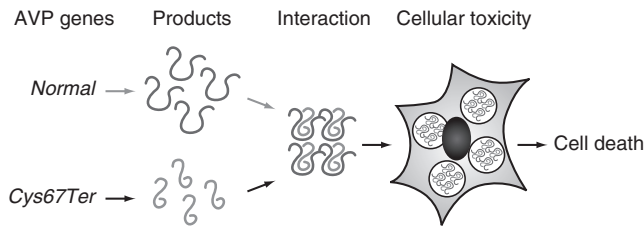
### 83.3 POSTERIOR PITUITARY: GENETIC DISORDERS OF AVP DEFICIENCY

The posterior pituitary gland (neurohypophysis), which is derived from an invagination of the hypothalamus, is embryologically and functionally distinct from the anterior pituitary. The primary function of the neurohypophysis is the storage and secretion of the hormones arginine vasopressin (AVP, OMIM 192340) and oxytocin (OXT, OMIM 167050), which are synthesized by neurons in the supraoptic and paraventricular nuclei of the hypothalamus. Both hormones are synthesized and processed in the neuronal cell body and then transported down the neuronal axons (supraopticoneurohypophyseal tracts) to the posterior pituitary in SVs. The hormones are stored in the posterior pituitary and released into the circulation following appropriate stimuli; for AVP this includes hyperosmolarity and hypovolemia (123).

AVP is encoded by the AVP gene (OMIM 192340) on chromosome 20 as a prohormone. This protein includes the AVP moiety (encoding nine amino acids), a signal peptide, neurophysin II, and copeptin (Figure 83-13). The signal peptide guides the prohormone to the ER, where folding occurs. The prohormone then exits the ER to the Golgi apparatus where packaging into SVs occurs. Conversion to the active hormone AVP takes place in the SVs (123).

NDI is a syndrome characterized by polyuria, polydipsia, and dehydration secondary to a deficiency of AVP. This syndrome is characterized by acute thirst, especially for cold water, enormous daily urinary output (3–15 L/day), and persistent nocturia. If water is withheld, the patient rapidly loses weight and develops hypernatremic dehydration. It is distinguished from diabetes mellitus by the presence of dilute urine without glycosuria. A variety of acquired lesions of the hypothalamus, such as neoplasia, basilar skull fractures, granulomatous diseases, vascular lesions, meningitis, and encephalitis, can result in AVP deficiency. In ~50% of NDI cases, however, no obvious primary lesion can be found. It is quite likely that many of these idiopathic cases represent sporadic cases of the genetic form of the disease. Hereditary forms account for fewer than 10% of all cases of DI; of the





**FIGURE 83-13** Schematic representation of the AVP or AVP-neurophysin II gene. The AVP gene encodes the following: (1) a prepropeptide precursor that includes a signal peptide (SP) whose initiation codon (ATG) is shown; (2) the nonapeptide AVP; (3) the AVP carrier protein neurophysin II (NPII); and (4) a glycoprotein copeptin (CP) whose function is unknown.

hereditary forms, nephrogenic DI is actually more common. We will focus on NDI here.

### 83.3.1 Autosomal Dominant Neurohypophyseal Diabetes Insipidus

In 1841, Lacombe first documented a familial form of DI; he described excessive thirst and polyuria in five males and three females in two generations of a family. Numerous other families with multiple affected members have since been described. The signs and symptoms of this autosomal dominant disorder are quite similar to those of the acquired forms of DI. There is, however, a great deal of intrafamilial variability in the clinical severity and age of onset of the disease. In one large family, urinary output varied from 3–4 to 15–20 quarts per day among affected relatives. Some, but not all, affected individuals have an increase in fluid requirements during febrile episodes, exercise, or pregnancy. In most cases, the onset of the disease occurs in infancy, but symptoms may not occur until late childhood or adolescence. Symptoms may abate in old age. In many of the affected families, the condition is regarded as an unpleasant family habit, rather than a disease. Apart from drinking enormous quantities of water, the disease does not impair health or well-being, unless free access to water is denied. Typical imaging studies of the brain show absence of the pituitary bright spot on MRI; however, the bright spot may still be present in young children.

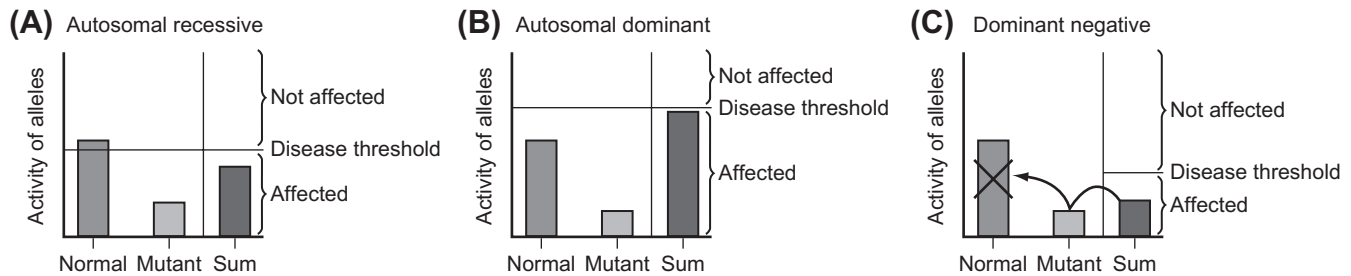
Plasma levels of AVP are very low or undetectable in these cases. AVP secretion may be normal for the first few years of life, but then decreases rapidly. Autopsy studies of individuals with autosomal dominant NDI (ADNDI) or sporadic idiopathic forms of the disease have found a severe reduction in the number of neurosecretory neurons in both supraoptic and paraventricular nuclei of the hypothalamus. There is associated gliosis, and in the paraventricular nucleus the small- to medium-sized neurons may be normal or reduced in number. The posterior pituitary gland has been found to be normal in size or small in these cases, but no neurosecretory material was observed on special staining. Although these individuals appear to have no neurosecretory neurons

in their hypothalamic nuclei and have all the signs and symptoms of AVP deficiency, OT secretion appears to be normal in most. Several females with ADNDI, including one in whom a marked deficiency of neurosecretory cells was documented, have undergone normal pregnancies and deliveries and have successfully nursed their children. Thus, they appear to secrete OT, despite the deficiency of neurosecretory neurons. Several patients with DI have been reported, however, who have had difficulty in expelling the fetus and placenta during labor and/or inability to secrete milk, suggesting that OT deficiency might also exist. In one such family, plasma levels of OT and its carrier protein were normal, but did not increase normally following estrogen administration.

**83.3.1.1 Animal Models of ADNDI.** Neurohypophyseal DI is an autosomal recessive trait in the Brattleboro rat. Although these animals have an absolute deficiency of AVP in their hypothalamus and posterior pituitary, unlike in the human disease the rats have hypertrophy of the hypothalamus and pituitary system. The neurons in the supraoptic nucleus are extremely well developed but lack neurosecretory granules. Similar, but less marked, changes are seen in the paraventricular nuclei, and the posterior lobe of the pituitary is three to four times heavier than normal. Heterozygous animals have a reduced concentration of AVP in the hypothalamus and pituitary and have deficient secretion and release of the hormone. In vivo, ADH and its neurophysin carrier are absent, but markedly reduced levels of ADH mRNA are present in the hypothalamus. The molecular defect in the Brattleboro rat has now been found to be a single deletion of a G residue in the segment of the AVP gene which encodes for the neurophysin carrier protein. The basic defect in the autosomal recessive variety of DI in the rat is decreased synthesis of active hormone with compensatory hypertrophy of the secretory neurons. The differences in the genetics and pathogenesis of DI between humans and rats support the general rule of recessive inheritance of peptide hormone deficiency syndromes.

Russell and colleagues established murine knock-in models of two different naturally occurring human mutations that cause ADNDI (124). A mutation in the signal peptide of AVP, ala-1 thr (A-1T; OMIM 192340.0003), is associated with a relatively mild phenotype or delayed presentation in humans. This mutation caused no apparent phenotype in mice. In contrast, heterozygous mice expressing a mutation that truncates the AVP precursor, Cys98Stop (C98X; also C67X in alternate numbering system; OMIM 192340.0005), exhibited polyuria and polydipsia by 2 months of age; these features of DI progressively worsened with age. Studies of the paraventricular and supraoptic nuclei revealed induction of the chaperone protein BiP (OMIM 138120) and progressive loss of AVP-producing neurons relative to oxytocin-producing neurons. In addition, AVP gene products were not detected in the neuronal projections, suggesting retention of wild-type and mutant AVP precursors





**FIGURE 83-14** (A) Pedigree of ADNDI family obtained from affected family members, showing affected individuals as solid squares (males) and solid circles (females). Symbols filled with a question mark indicate subjects not formally tested or whose phenotype is uncertain. (B) DNA sequence analysis of DNAs from a mother and her three children all of whom have autosomal dominant neurohypophyseal DI (see pedigree above). Note the G to A transition at codon 19 of the signal peptide, which corresponds to –1 of AVP and encodes a GCG (Ala) to ACG (Thr) substitution.

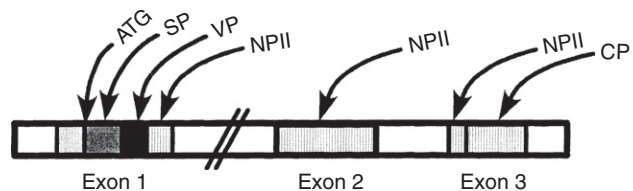
within the cell bodies. This murine model recapitulated many features of the human disorder and demonstrated that expression of the mutant AVP precursor leads to progressive neuronal cell loss, presumably through a dominant-negative effect (see below).

**83.3.1.2 Molecular Pathophysiology of ADNDI.** A major function of ADH is to promote resorption of water by the kidney when plasma osmolality increases. Once secreted, AVP travels through the circulation to the kidney, where it binds to AVP receptor 2 (AVPR2). Through a cAMP mediated signal cascade, this binding causes aquaporin 2 (AQP2) to be translocated to the luminal membrane of the renal tubular cell, resulting in resorption of water. Defects in both of these genes have been described, resulting respectively in X-linked nephrogenic DI or autosomal dominant nephrogenic DI (OMIM 304800, 125800). Mutations causing ADNDI may affect the signal peptide, AVP, or neurophysin II regions of the gene. A majority of mutations lead to ADNDI by affecting protein folding and transport in the ER. For example, mutations in the signal peptide region may affect signal peptide cleavage and mutations in neurophysin II affect protein folding. Mutations in the AVP region of the gene may cause either folding problems (resulting in a dominant phenotype) or receptor binding defects (resulting in a recessive phenotype, see following). For example, a G to A transition which changes the last codon of the signal peptide portion of the AVP gene affects signal peptide cleavage and causes NDNDI (Figure 83-14). At least 60 mutations in the AVP gene have been reported, of which a majority occur in exon 2 which encodes neurophysin 2 (123). In vitro studies demonstrate that abnormally folded precursors are retained in the ER, and long-term cell culture studies and animal models suggest that this accumulation leads to cytotoxic effects which ultimately results in degeneration of the affected neurons (124–126). This at least partially explains the delayed onset of ADNDI in most kindreds, a similar mechanism to other dominant-negative disorders such as IGHD Type 2 (discussed earlier in this chapter). However, in a mouse model similar to that used by Russell et al., Arima et al. found that polyuria progressed

**TABLE 83-6 Summary of AVP Mutations Causing Neurohypophyseal Diabetes Insipidus<sup>a</sup>**

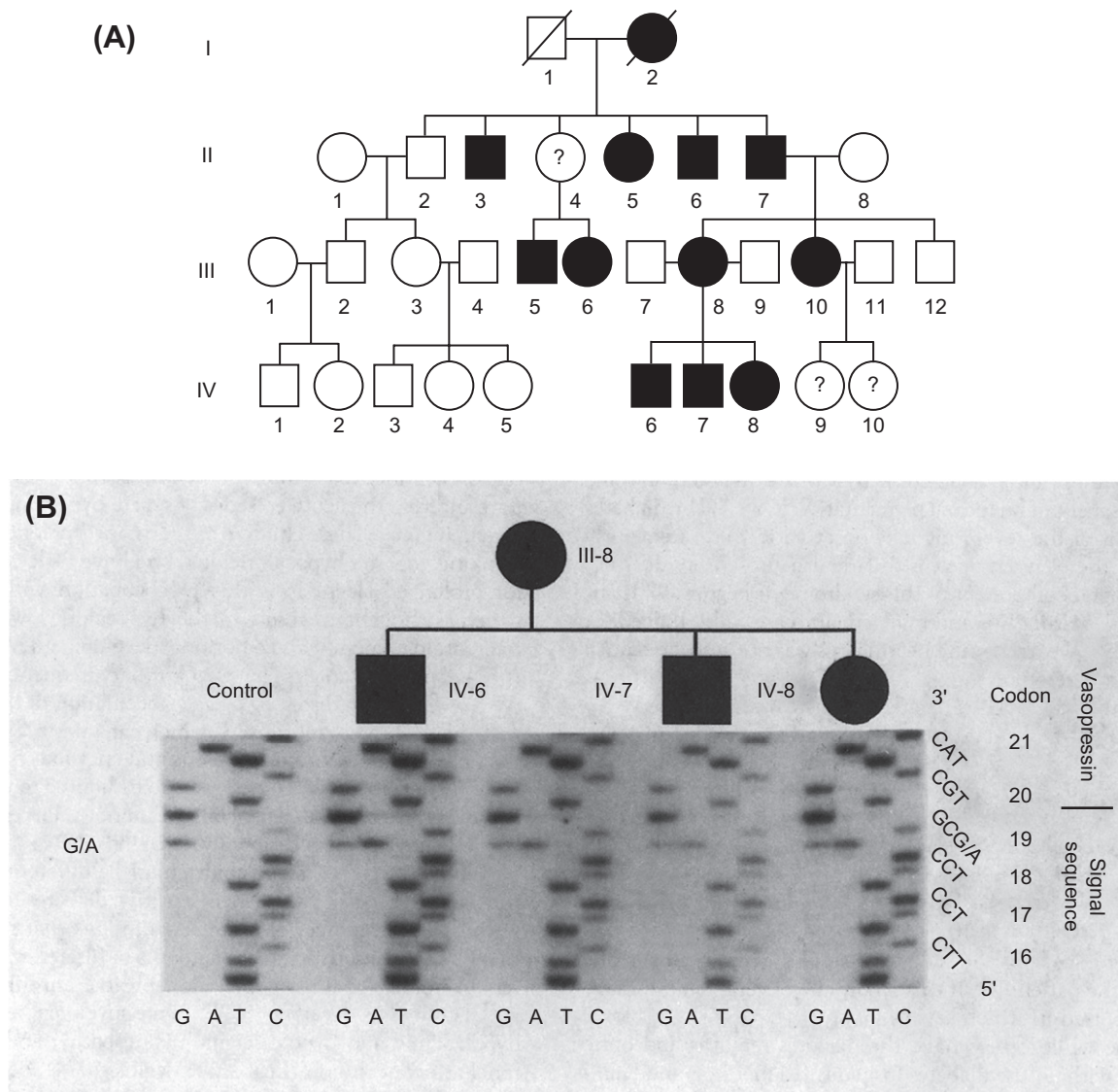
Mutation Type	Number of Mutations
Missense/nonsense	53
Splicing	0
Regulatory	0
Small deletions	5
Small insertions/deletions	2
Complex rearrangements	0
Total	60

<sup>a</sup>From HGMD Professional Database on 9/3/10 <http://www.hgmd.cf.ac.uk/ac/index.php>.



**FIGURE 83-15** In the pathogenesis of FNDI, normal and Cys67Ter AVP products interact to prevent AVP secretion. These products also accumulate within, damage, and cause loss of AVP secretory cells. (From Phillips, J. A. III. Dominant-negative diabetes insipidus and other endocrinopathies. *J. Clin. Invest.* **2003**, 112, 1641–1643.)

even before neuronal loss occurred, suggesting that cellular dysfunction due to accumulation of abnormal proteins in the ER may also be a cause of progressive ADNDI (124,127). The cause of variation in age of onset between and within kindreds remains to be discovered; however, it is plausible that it could be at least partially explained by variations in modifying genes (such as those regulating ER protein quality control) and/or by epigenetic phenomena. See Figure 83-15 for an explanation of the dominant negative-mechanism of central DI due to AVP gene mutations, and Table 83-6 for a summary of known mutations. See Figure 83-16 for a comparison of the general mechanisms of autosomal recessive, autosomal dominant, and dominant-negative genetic disorders. Phillips tabulated 21 disorders caused by mutations in



**FIGURE 83-16** Relationships of levels of gene expression, reflected as the activity of single homologous alleles (left) and the sum of the activities of the two homologous alleles (right). The dashed line represents the minimal sum of allelic activity required to prevent autosomal recessive (A), autosomal dominant (B), and dominant-negative (C) disorders. (From Phillips, J. A. III. *Dominant-Negative Diabetes Insipidus and Other Endocrinopathies*. J. Clin. Invest. **2003**, 112, 1641–1643.)

18 genes which demonstrated the dominant-negative phenomenon and were instances of hormone deficiency (128).

In families with ADNDI, genetic testing for AVP mutations may be helpful in pre-symptomatic patients and in those patients with equivocal water deprivation testing (129).

### 83.3.2 Other Forms of Familial Neurohypophyseal DI

In the 1940s, Forssman et al. described a large kindred with apparent X-linked familial NDI (FNDI). Patients had partial response to vasopressin. Subsequently, however, the same kindred was relocated and found to have a form of nephrogenic DI with a

confirmed AVPR2 gene mutation (130). In 1996, however, Habiby et al. described a kindred with apparent X-linked FNDI with linkage to Xq28. Mutations in either AVPR2 or AVP were not found and the etiology remains unknown.

Two kindreds with autosomal recessive FNDI have been reported. Each kindred (one an Arab family living in Texas, one a Palestinian family) had the same P26L mutation and microsatellite analysis suggested a founder effect. The presentation was essentially the same as in ADNDI, with the exception that the patients in the second kindred presented in the first few weeks of life. All responded well to desmopressin therapy. The mutant AVP is secreted but has a 30-fold decrease in V2 receptor binding and a 10-fold decrease in the ability to stimulate adenylate cyclase (131).

### 83.3.3 Wolfram Syndrome

This autosomal recessive syndrome is also known as the DIDMOAD syndrome and consists of *DI*, Diabetes Mellitus, Optic Atrophy, and neurosensory Deafness (OMIM 222300; Table 83-1). The diabetes in Wolfram syndrome (WFS) is caused by degeneration of the insulin producing cells of the pancreas and is a constant feature of the syndrome. Functionally, the diabetes is similar to Type 1 diabetes, but there is no autoimmunity and the diabetes tends to be milder with less tendency toward ketoacidosis. Optic atrophy is typically the next sign to appear (occurs in 100% of patients), and may be present at the time of diabetes diagnosis. The optic atrophy is primary and it is characterized by white discs and, in some instances, peripheral retinal pigmentation. Bilateral neurosensory deafness is an integral component of the WFS and occurs in about 60% of patients; it begins as a high-frequency hearing loss and may remain quite mild. Hearing loss may not be suspected until audiograms are performed. Neurohypophyseal DI occurs in more than one-third of patients with this syndrome (132). Several families have been reported in which several members have the full-blown syndrome, whereas others have diabetes mellitus and optic atrophy without DI. AVP deficiency has been documented in affected patients following a hyperosmolar stimulus; normal free water resorption resumes following DDAVP administration. Postmortem examination of two siblings with this syndrome revealed degeneration of the hypothalamic nuclei, more severe in the paraventricular than supraoptic nuclei, and atrophy of the posterior lobe of the pituitary, adrenal cortex, pons, and substantia nigra.

A number of other associated abnormalities have been described in certain families, including ataxia, autonomic dysfunction with a neurogenic bladder, sideroblastic anemia, and hyper alaninuria. The progression over time of simple optic atrophy and diabetes mellitus to full-blown WFS with neurosensory hearing loss, atonic bladder, and ataxia in the patient appears to be due to the pleiotropic effects of a single mutant gene and represents one distinct syndrome (132).

WFS has been linked to 4p16. The gene, *WFS1* (OMIM 606201) has been identified and a variety of mutations have been described in Wolfram patients (133). *WFS1* codes for a novel transmembrane protein called wolframin, an endoglycosidase H-sensitive glycoprotein, which localizes primarily to the ER of a variety of neurons (hippocampus CA1, amygdaloid areas, or olfactory tubercle), inner ear cells, and pancreatic beta cells (134–136). In the ER, wolframin helps regulate calcium homeostasis and the unfolded protein response (137–139). Yamada et al. showed that wolframin deficiency increases ER stress resulting in eventual apoptosis of pancreatic beta cells (140). Khanim et al. identified *WFS1* mutations in 90% of the patients they studied. Most were compound heterozygotes with private mutations distributed

throughout the gene with no obvious hotspots (135). Smith et al. found that most causative changes identified in the *WFS1* gene occurred in exon 8 (141). Giuliano et al. screened 19 patients with WFS and 36 relatives from 17 French families for mutations in the *WFS1* gene (142). *WFS1* mutations were identified on both alleles in 16/19 (84%) patients and on one allele of three patients, showing that *WFS1* is the major gene involved in WFS in the French population. Genotype–phenotype correlations suggested that the presence of inactivating mutations on both alleles may be associated with an early onset of diabetes mellitus.

A second autosomal recessive form of WFS (*WFS2*, OMIM 604928) with linkage to 4q22–q24 has been established in several families (143). Amr et al. identified a single homozygous mutation in the zinc finger protein, CDGSH iron sulfur domain protein (*CISD2*, OMIM 611507). *CISD2* is expressed in brain and pancreas and localizes to the ER, where it also appears to regulate calcium homeostasis (144). *CISD2* null mice have a phenotype of premature aging, neurologic deterioration, and glucose intolerance consistent with some features of WFS (145).

Rotig et al. suggested that some cases of the syndrome of early-onset diabetes mellitus, optic atrophy, and deafness may have their basis in a mitochondrial mutation. They described a patient with this disorder in association with a 7.6-kb heteroplasmic deletion of mtDNA. Pilz et al. described a 19-year-old male with long-standing diabetes mellitus, optic atrophy, grand mal seizures, and unilateral sensorineural hearing loss who had the most common mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy, which was inherited from his mother. They suggested that the DIDMOAD phenotype is a mitochondrial disorder in some cases and is likely to have a heterogeneous etiology. *CISD2* null mice have mitochondrial dysfunction and degeneration suggesting that mitochondrial dysfunction may be a final common pathway for both forms of WFS as well as other similar syndromes. Barrett et al. investigated a cohort of 50 WFS patients for evidence of a distinct mitochondrial haplotype and mitochondrial DNA rearrangements and found no evidence supporting a role for mtDNA in WFS (146).

## 83.4 GENETIC TESTING

Tests for many of the genetic disorders described are available either commercially or on a research basis. For up-to-date information on availability of testing for specific disorders, consult the GeneTests website (<http://www.geneclinics.org>).

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# CHAPTER

# 84

## Thyroid Disorders

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The codes in parenthesis will refer to OMIM database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed>; search OMIM).

### 84.1 INTRODUCTION

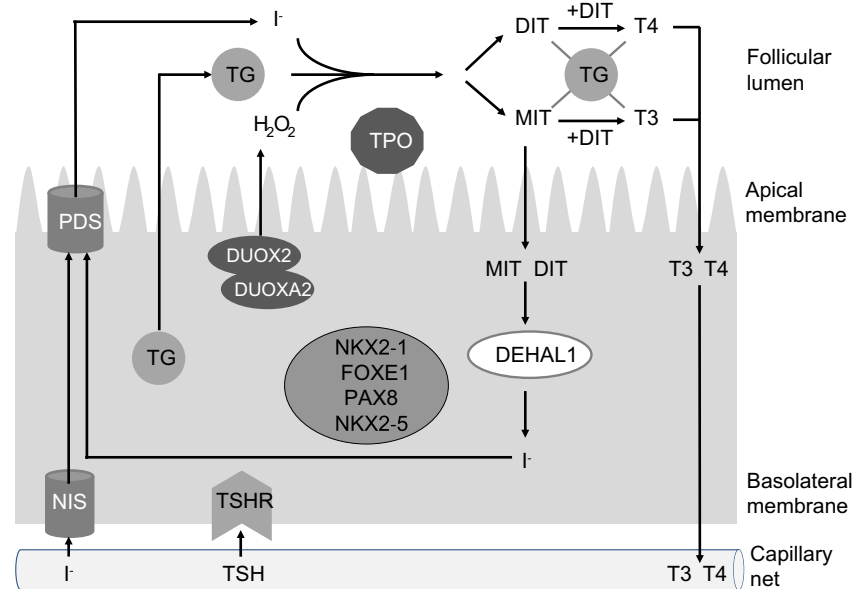
The mammalian thyroid gland consists of two endocrine cell types: (1) Thyroid follicular cells represent the large majority of endocrine cells of the thyroid. They form the monolayered spherical functional units of the mature thyroid tissue, the thyroid follicles and are responsible for thyroid hormone synthesis, storage and secretion. (2) Parafollicular C-cells represent less than 1% of cells in the human and produce calcitonin, a hormone involved in calcium homeostasis. C-cells are scattered as single cells or small-cell groups between the thyroid follicles and the dense capillary net in the thyroid interstitium (1,2).

The mammalian thyroid follicular precursor cells derive from the foregut endoderm and form the median anlage of the thyroid while C-cells are of neuroectodermal origin and are localized within the paired ultimobranchial bodies of the caudal pharyngeal pouches. After a migratory process, the median anlage fusions with the paired ultimobranchial bodies at the definitive pretracheal position. Thyroid development is accomplished with the onset of thyroid hormone synthesis at the end of the first trimester in the human embryo (2–5). Abnormal thyroid gland development or thyroid dysgenesis is the most common cause of permanent thyroidal congenital hypothyroidism in the human in iodine sufficient areas of the world.

The thyroid hormones are thyroxine (tetraiodothyronine, T<sub>4</sub>) and triiodothyronine (T<sub>3</sub>). These iodothyronines

are synthesized by fixation of four respectively three iodide atoms on thyronine residues resulting from condensation of two tyrosine amino acids. The only limiting substrate of thyroid hormone synthesis is iodide. Iodine deficiency remains worldwide still the most important reason for hypothyroidism. Thyroid hormone synthesis is characterized by several consecutive steps: (1) iodide uptake and concentration in the thyroid follicular cells, (2) thyroglobulin (TG) synthesis, (3) organification of iodide, and (4) iodotyrosine deiodination/iodide recycling. Inborn errors of thyroid hormone synthesis, called thyroid dysmorphogenesis, occur at any step of thyroid hormone synthesis and usually cause familial goitrous congenital hypothyroidism (6–8).

The most important factors controlling thyroid hormone synthesis are iodine availability and the hypothalamic–pituitary thyroid axis. Hypothalamic thyrotropin-releasing hormone (TRH) regulates pituitary thyrotroph synthesis and secretion of thyroid-stimulating hormone/thyrotropin (TSH). The activity of the hypothalamic–pituitary thyroid axis is under control of a negative feedback mechanism of circulating thyroid hormones. Secreted and circulating TSH binds to the TSH receptor (TSHR) localized at the basolateral membrane of the thyroid follicular cells. Receptor binding activates virtually every step in thyroid hormone synthesis and release mediated through the membrane-bound adenylate cyclase-cAMP second messenger system. In summary, TSH stimulates iodine uptake, TG synthesis, organification, stored TG endocytosis and thyroid hormone secretion (2,7–10). Developmental defects of the pituitary gland as well as inborn errors of the TRH



**FIGURE 84-1** Key Steps of Thyroid Hormone Synthesis. Thyroid hormone synthesis takes place at the apical membrane of the thyrocyte within the extracellular follicular lumen. Substrates for thyroid hormone synthesis are the amino acid tyrosine (not represented), iodide ( $I^-$ ), the glycoprotein TG and hydrogen peroxide ( $H_2O_2$ ). Iodide is incorporated into TG to form monoiodotyrosine (MIT) and diiodotyrosine (DIT) after oxidation and covalent binding to tyrosyl residues of the TG molecule. In a second step, thyroid hormones are formed by coupling of MIT and DIT to T3, or of two DITs to T4 or thyroxine. Four different mechanisms are involved in thyroid hormone synthesis. (1) Active  $I^-$  uptake from the bloodstream at the basolateral membrane by the sodium/iodide symporter (NIS, encoded by the *SCL5A5* gene) and  $I^-$  release into the follicular lumen at the apical membrane by pendrin (PDS, encoded by the *SCL26A4* gene). (2) TG synthesis and secretion into the follicle. (3)  $H_2O_2$ -generation by dual oxidase 2 (DUOX2), the dual oxidase maturation factor 2 (DUOXA2), and iodide oxidation and coupling by thyroid peroxidase (TPO). (4) Thyroid hormone secretion and iodotyrosine deiodination/iodide recycling by the iodotyrosine deiodinase (DEHAL1, encoded by the *IYD* gene). All these processes are regulated by TSH, while thyrocyte development and differentiation is coordinated by the thyrocyte enriched transcription factors NK2 homeobox 1 (NKX2-1), paired box gene 8 (PAX8), forkhead box E1 (FOXE1) and NK2 homeobox 5 (NKX2-5).

receptor and *TSH* may cause central or hypothalamic-pituitary hypothyroidism, while defects in the *TSHR* cause TSH resistance (Figure 84-1).

The thyroid gland secretes about 80% T4 and 20% T3 directly into the blood, where they are bound to three binding proteins: thyroxine binding globulin (TBG), transthyretin (TTR) and albumin (ALB). TBG has the highest affinity for T4 and T3, and carries the majority of T4 in serum. Saturation of the single binding site for T4/T3 on TBG is tightly regulated by TSH to adjust the concentration of unbound T4 (free T4) within narrow limits (11). Inherited defects in the thyroid hormone transport proteins do not cause altered metabolic state or thyroid disease. However, they produce alterations in thyroid hormone concentrations leading to inappropriate treatment if unrecognized (12).

Only recently, it has been shown, that thyroid hormone transporters are necessary for the intracellular availability of thyroid hormones to have access to the deiodinases and then nuclear thyroid hormone receptors (TRs) in the cell (13,14). The clinical importance of the specific thyroid hormone transporter monocarboxylate transporter 8 (MCT8) has been shown in patients with MCT8 mutations suffering from X-linked mental retardation, now referred as Allan–Herndon–Dudley syndrome (AHDS) (15).

Once intracellularly present, free T4, which is a pro-hormone of T3, is enzymatically deiodinated into the

active T3 by the selenoproteins deiodinase 1 (D1, mainly expressed in liver, kidney and thyroid) and deiodinase 2 (D2, mainly localized in brain, pituitary gland, placenta and brown adipose tissue). The inner-ring deiodinase 3 (D3) located predominantly in skin, brain and placenta converts T4 into inactive reverse T3, and T3 into inactive diiodothyronine (T2) (10,16,17).

So far no genetic defects have been identified in deiodinases 1–3 (D1–3), but recently missense mutations in a crucial cofactor for selenoprotein synthesis (SECISBP2) have been associated with mild alterations of thyroid hormone concentrations (18).

The major cellular actions of thyroid hormones are mediated by nuclear TRs via transcriptional regulation. Two *TR* genes, *alpha* and *beta*, encode five T3-binding receptor isoforms (alpha1, alpha2, beta1, beta2 and beta3). T3 binds with 10- to 15-fold higher affinity to TRs than T4 and is mainly responsible for mediating thyroid hormone action. T3 derives from circulating T3 monodeiodinated from T4 in peripheral tissues, from a limited amount (10–15%) of circulating T3 secreted by the thyroid gland, and from intracellular T3 derived directly from intracellular monodeiodination of T4 (19,20).

The transcriptional activity of TRs is regulated at multiple levels, especially by the developmental- and tissue-dependent expression of TR isoforms, and by nuclear co-regulatory proteins. In addition to TR-mediated gene

**TABLE 84-1 Genetic Disorders of Thyroid Function, Thyroid Hormone Transport, Metabolism and Action (Numbers Refer to OMIM Database Codes for Associated Disease)**

<b>Congenital Hypothyroidism</b>	
<i>Central: Hypothalamic–pituitary hypothyroidism</i>	
Pituitary dysgenesis	
Thyroid-stimulating hormone releasing hormone receptor deficiency (TRHR)	#275120
Thyroid-stimulating hormone beta subunit deficiency (TSHB)	#275100
<i>Thyroidal: Dysgenetic or dyshormonogenetic hypothyroidism</i>	
Thyroid dysgenesis	#218700
NK2 homeobox 1 (NKX2-1, TTF1) (Brain–lung–thyroid syndrome)	#610978
Paired box gene 8 (PAX8)	#218700
Forkhead box E1 (FOXE1, TTF2) (Bamforth–Lazarus syndrome)	#241850
NK2 homeobox 5 (NKX2-5)	#225250
<i>Resistance to TSH</i>	
Thyroid-stimulating hormone receptor (TSHR)	#275200
Gs alpha (GNAS) (Pseudohypoparathyroidism type 1a)	#103580
<i>Thyroid dyshormonogenesis</i>	
Sodium/iodide symporter (SCL5A5, NIS)	#274400
Thyroid peroxidase (TPO)	#274500
Dual oxidase 2 (DUOX2)	#607200
Dual oxidase maturation factor 2 (DUOX2A2)	#274900
Pendred syndrome (SCL26A4, Pendrin)	#274600
Thyroglobulin (TG)	#274700
Iodotyrosine deiodinase (IYD, DEHAL1)	#274800
<i>Peripheral: Disorders of thyroid hormone transport, metabolism or action</i>	
<i>Disorders of thyroid hormone transport proteins</i>	
Thyroxine binding globuline (TBG)	+314200
Transthyretin (TTR)	#145680
Albumin (ALB) (Familial dysalbuminemic hyperthyroxinemia)	+103600
<i>Disorders of thyroid hormone membrane transporters</i>	
Monocarboxylate transporter 8 (MCT8) (Allan–Herndon–Dudley syndrome)	#300523
<i>Disorders of iodothyronine deiodinase function</i>	
Selenocysteine insertion sequence binding protein 2 (SECISBP2)	#609698
<i>Thyroid hormone resistance</i>	
Thyroid hormone receptor beta (TRbeta) (generalized/pituitary)	#188570/#145650
<b>Genetic hyperthyroidism</b>	
Thyroid-stimulating hormone receptor (TSHR)	#609152
Gs alpha (GNAS) (McCune–Albright syndrome)	#174800
<b>Syndromes associated with hypothyroidism</b>	
Down syndrome	#190685
Turner syndrome	
Williams–Beuren syndrome	#194050
DiGeorge syndrome	#188400
<b>Genetic basis of autoimmune thyroid disease</b>	
<i>Autoimmune thyroid disease</i>	
Hashimoto disease/Graves' disease	%140300/%275000
<i>Autoimmune polyendocrine syndromes</i>	
APS type I/APS type II	#240300/%269200
IPEX syndrome	#304790
<b>Genetic basis of thyroid carcinoma</b>	
<i>Medullary thyroid carcinoma</i>	
Familial medullary thyroid carcinoma	#155240
Multiple endocrine neoplasia type 2A/2B	#171400/#162300
<i>Follicular thyroid carcinoma</i>	
Papillary carcinoma	#188550
Follicular carcinoma	%188470

expression, there is increasing evidence for non-genomic effects of thyroid hormones in target cells that occur independently of nuclear TR binding (19,20). Inherited defects of TRs result in thyroid hormone resistance (21).

Optimal action of thyroid hormones in the human organism in iodine sufficient areas are dependent on the integrity of the entire hypothalamic–pituitary–thyroid tissue axis, normal thyroid hormone transport in blood, uptake into the cell and intracellular deiodination and binding to TRs. Table 84-1 gives a summary of genetic disorders of thyroid function and thyroid hormone transport, metabolism and action.

## 84.2 CONGENITAL HYPOTHYROIDISM

The essential role of thyroid hormones for brain development and skeletal maturation and growth has been clearly demonstrated and congenital hypothyroidism is one of the most frequent preventable causes of mental retardation. Neonatal screening programs have been established in the seventies of the last century in USA, Canada and Europe, and early diagnosis and substitutive treatment of congenital hypothyroidism has been shown to be highly sensitive, specific and cost-effective for prevention of congenital hypothyroidism-associated mental retardation (22–25).

Genetic defects leading to congenital hypothyroidism may occur at three levels: the hypothalamus and pituitary gland, the thyroid gland, or the peripheral tissue level. Possible abnormalities are listed in Table 84-1. Permanent congenital hypothyroidism affects about 1:3000–1:4000 newborns. Thyroidal congenital hypothyroidism outweighs by far the rare central or hypothalamic-pituitary congenital hypothyroidism cases. Girls are more commonly affected than boys (female to male ratio 2:1 to 4:1) and are more common in Hispanic infants (1:2000) than in white infants, and is thought to be less common in black infants (1:11,000) (6,26–29).

### 84.2.1 Hypothalamic-Pituitary Congenital Hypothyroidism

**84.2.1.1 Developmental Defects of the Pituitary.** Defects of pituitary development result in various forms of impaired secretion of pituitary hormones. Usually the clinical picture is characterized by multiple hormone deficiencies including TSH. However, the time course of manifestations of the different hormone deficiencies is highly variable and therefore congenital hypothyroidism may be the first and leading symptom of defective hypothalamic-pituitary development. Hypothalamic-pituitary disorders associated with hypothalamic-pituitary anomalies and disorders and with panhypopituitarism are reviewed elsewhere (see Chapter 83).

**84.2.1.2 Pituitary Hormone and Hormone Receptor Defects.**

**84.2.1.2.1 Thyrotropin-Releasing Hormone Receptor Deficiency (OMIM #275120).** To date no mutations have been described in the human *TRH* gene.

The human *TRH receptor* gene is localized on chromosome 8q23 (30). Targeted disruption of the *TRH receptor* gene in mice led to an unexpected mild phenotype of central hypothyroidism and hyperglycemia (31). Resistance to thyrotropin-releasing hormone has been reported in two families so far (32,33). In both families, the index cases bearing a compound heterozygous and a homozygous loss-of-function mutation, respectively, presented with growth retardation, clinical signs of hypothyroidism at 9 and 11 years of age without mental retardation, and showed absence of TSH and prolactin response after TRH stimulation test. The patients were not detected by TSH-based neonatal screening. In both families, the heterozygous family members were asymptomatic and had normal TSH and prolactin response to TRH.

**84.2.1.2.2 Familial-Isolated TSH Deficiency (OMIM #275100).** Isolated TSH deficiency due to *TSH-beta* subunit defects is a rare autosomal recessive cause of central congenital hypothyroidism. The first reported family was described in detail on clinical grounds by Miyai et al. (34) in 1971. The *TSH-beta* gene was cloned in 1988 (35), and allowed genetic investigations of the first described family, detecting the first mutation in the *TSH-beta* gene (36). Since then 11 different homozygous or compound heterozygous *TSH-beta* mutations have been identified so far. The 313delT mutation is the most prevalent mutation, was identified in different populations and is derived from a common ancestor (37). It causes severe congenital hypothyroidism. It involves a 1-bp deletion from codon 105 of the *TSH-beta* gene, converting a cysteine to a valine residue (C105V) and yielding an additional 8-amino acid non-homologous peptide extension on the mutant protein, which has lost biological activity (38). The hallmark of patients with *TSH-beta* mutations is congenital hypothyroidism not detected by TSH-based neonatal screening due to low or undetectable TSH levels in combination with low circulating thyroid hormones. TSH levels do not increase after TRH stimulation testing, while all other pituitary hormone axes respond normally to specific provocative tests excluding multiple pituitary hormone deficiency. The clinical phenotype of patients with homozygous or compound heterozygous *TSH-beta* mutations is variable, but severe forms of congenital hypothyroidism with mental retardation are commonly encountered if diagnosis and thyroid hormone substitution is delayed. Heterozygous family members are symptom free (34,38–41).

### 84.2.2 Thyroidal Congenital Hypothyroidism

In iodine sufficient areas the most common etiology of thyroidal congenital hypothyroidism is thyroid dysgenesis, representing 80–85% of all cases of permanent congenital hypothyroidism. Thyroid dysgenesis includes a spectrum of developmental abnormalities of the thyroid gland ranging from (1) “agenesis” or athyreosis (20–30%) due to a defect in survival of the thyroid follicular cell precursors, (2) ectopic thyroid gland (50–60%) mostly located in a sublingual position as a result of



**TABLE 84-2 Spectrum of Thyroid Morphology and Associated Malformations in Genes Causing Thyroid Dysgenesis or Resistance to TSH in the Human**

Mutated Gene	Thyroid Morphology				Associated Malformations
	Ectopy	Athyreosis	Hypoplasia	Normal Thyroid	
NKX2-5		One patient described			Cardiac defects (f)
FOXE1			One patient described	One patient described	Cleft palate, "Spiky" hair
PAX8					Kidney agenesis (f)
NKX2-1		Two patients described			Choreoathetosis, Lung disease (f)
TSHR		Apparent athyreosis			None

Gray cells represent expected phenotype.

f, facultative-associated malformation.

premature arrest of its migratory process or (3) hypoplasia of an orthotopic gland (5%). The term "apparent athyreosis" has been created to describe patients with no functional thyroid tissue on thyroid scans but demonstration of a thyroid gland remnant in ultrasound studies as well as measurable serum thyroxine and TG concentrations, suggesting presence of hypo-functional remnant tissue (26,42–44) (Table 84-2).

In 10–15% of patients, thyroidal congenital hypothyroidism is the consequence of defects of thyroid hormone synthesis (dyshormonogenesis) characterized by a normal or enlarged thyroid gland in normal position. Defects can occur in each step of thyroid hormone synthesis: iodide transport, TG synthesis or oxidation and coupling and are further detailed below (10,29,45).

While dyshormonogenetic cases are inherited in an autosomal recessive way, thyroid dysgenesis might be considered as a non-genetic condition to its mainly sporadic occurrence. In addition, the female predominance as well as the discordance of monozygotic twins argues against a classic Mendelian inheritance of thyroid dysgenesis. However, recent work has revealed some arguments for genetic basis of congenital hypothyroidism in some cases: First, although representing only a minority of cases of thyroid dysgenesis, familial cases were observed in a significantly higher proportion (>15-fold) than would be expected by chance alone. Second, an increased frequency of minor thyroid abnormalities in first-degree relatives of patients with thyroid dysgenesis has been described. Third, thyroid dysgenesis is associated with an increased incidence of associated extra-thyroidal malformations. A fourfold increase in congenital malformations was found in a population based study of 1420 infants with congenital hypothyroidism (8.4%) compared with the control infant population (1 to 2%) (26,27,42,46–49).

Nevertheless, non-Mendelian mechanisms need to be considered to explain thyroid dysgenesis in most cases. A multigenic origin of thyroid hypoplasia and hemiagenesis has been shown in transgenic mouse models, but discordance in monozygotic twins excludes multigenic origin of thyroid dysgenesis as the exclusive mechanism of its etiology in the human. Early somatic mutations or epigenetic modifications could play a role in thyroid dysgenesis development, in analogy with other human diseases. Recently,

Deladoëy et al. (50) proposed a two-hit model in analogy with focal hyperinsulinism: a germ line mutation (first hit) and a somatic mutation or epigenetic modification (second hit) of genes involved in critical steps of thyroid development. The hypothesis of a combination of germ line and somatic gene modifications is appealing for thyroid dysgenesis etiology, but needs to be confirmed (51).

**84.2.2.1 Thyroid Dysgenesis (OMIM #218700).** Based on the phenotype of transgenic mouse models mutational screening of cohorts of patients with thyroid dysgenesis led to the identification of mutations in genes involved in thyroid development: *PAX8* (1998), *FOXE1* (1998), *NKX2-1* (deletion 1998, mutation 2002) and *NKX2-5* (2006) (52–57). However, mutations in these four genes are only found in a small percentage of thyroid dysgenesis patients, even when screening large cohorts for each of the genes. So far, *NKX2-1* mutations with about 50 published cases are the most frequent cause of thyroid dysgenesis. *PAX8* mutations have been reported in about 30 patients and mutations in *FOXE1* and *NKX2-5* in less than 10 families so far. Further, Castanet et al. (58) showed by linkage and mutational analysis in 19 multiplex families genetic heterogeneity of thyroid dysgenesis: first, the LOD (logarithm of the odds) scores failed to prove linkage between *NKX2-1*, *PAX8*, *FOXE1*, and *TSHR* and thyroid dysgenesis, and second, no mutations in any of the four genes were found in 5 of the 19 families implicating novel yet unknown genes (59,60).

**84.2.2.1.1 NKX2-1 (Brain–Lung–Thyroid Syndrome) (OMIM #610978).** Based on the knowledge of NK2 homeobox 1 (*NKX2-1*, formerly *TTF1*) expression in the developing thyroid, lung and hypothalamus, the first patient with a *NKX2-1* deletion has been described in 1998 with the combination of infant respiratory distress syndrome at term, congenital hypothyroidism and hypotonia evolving to developmental delay (54). Heterozygous mutations of the *NKX2-1* gene have been first described in 2002, recapitulating the clinical triad of brain–lung–thyroid disease (55,57). Further, the neurological phenotype was described more precisely, revealing choreoathetosis or benign hereditary chorea (BHC) as a constant and specific sign for *NKX2-1* gene anomalies (61) (see Chapters 61, 117, and 118).

A recent review of 6 own and 40 published patients revealed that the clinical spectrum varies between the

complete triad of “brain–lung–thyroid syndrome” (50%), brain and thyroid disease (30%), to isolated BHC (13%), which represents the mildest expression of the syndrome. Thyroid dysgenesis ranges from hypoplasia (about 35%) to normal morphology (more than 50% of patients). Isolated hyperthyrotropinemia is more frequent than overt hypothyroidism at birth and later in childhood accounting for about 60% of patients. In addition, the severity of symptoms varies widely, even in families with the same disease-causing mutation. *NKX2-1* defects occur either as sporadic cases or as familial cases inherited in an autosomal dominant way (62).

Lung disease, if present at birth, manifests as surfactant deficiency syndrome and evolves in a subgroup of patients to interstitial lung disease with relevant mortality. Recently, it was shown that interstitial lung disease in patients with *NKX2-1* mutations was associated with altered surfactant protein metabolism (63).

BHC is the most constant sign of the triad that develops between 1 and 5 years of age and is nonprogressive in later life.

#### **84.2.2.1.2 Paired Box Gene 8 (OMIM #218700).**

Heterozygous *PAX8* mutations have been described since 1998 with a spectrum ranging from athyreosis to normal morphology; however, most patients displayed hypoplasia (56,64,65). Thyroid function also exhibits a large range from euthyroid patients to severe hypothyroidism, even within the same family. Failure of normal postnatal growth led to secondary hypoplasia in one family. Decreased iodide trapping and pathologic perchlorate test was described in the same family, suggesting thyroid dysmorphogenesis rather than thyroid dysgenesis as a basis of congenital hypothyroidism (64). Kidney agenesis or malformations are a facultative associated malformation in patients with *PAX8* mutations. So far 10 *PAX8* mutations are described.

**84.2.2.1.3 *FOXE1* (Bamforth–Lazarus Syndrome) (OMIM #241850).** Homozygous *FOXE1* (formerly *TTF2*) mutations have been shown to be the genetic basis for Bamforth–Lazarus syndrome, a rare syndrome characterized by cleft palate, athyreosis, spiky hair, choanal atresia and bifid epiglottis (52). In two families with a total of four patients athyreosis was found, while recently one patient was described with normally sized and located thyroid gland without radioactive iodide uptake on scintigraphy and very low TG levels and another patient with severe thyroid hypoplasia. All patients showed severe hypothyroidism. *FOXE1* mutations were not found in large cohorts of isolated cleft palate patients (66–69). Recently, the length of the alanine stretch within the normal *FOXE1* gene has been shown to influence the risk for thyroid dysgenesis, introducing a possible new role of *FOXE1* as a modifier gene of thyroid dysgenesis development (70).

#### **84.2.2.1.4 *NK2 Homeobox 5* (OMIM #225250).**

A higher prevalence of congenital heart disease has been

documented in children with congenital hypothyroidism than in the general population (48). Such an association suggested a possible pathogenic role of genes involved in both heart and thyroid development. The *NKX2-5* gene encodes a homeodomain-containing transcription factor with a major role in heart development. *NKX2-5* mutations have been shown to occur in 3% of patients with congenital heart disease without thyroid phenotype. Recently, four patients were described with heterozygous *NKX2-5* mutations with thyroid ectopy and athyreosis and severe hypothyroidism. Only one patient showed minor mitral valve insufficiency, the others having no cardiac phenotype (53,71,72). The exact prevalence and importance of this transcription factor in thyroid dysgenesis remain to be further characterized.

#### **84.2.2.2 Resistance to TSH.**

##### **84.2.2.2.1 Inactivating *TSHR* Mutations (OMIM #275200).**

The *TSHR* is localized in the basolateral membrane of thyroid follicular cells. The *TSHR* is a member of the G-protein-coupled seven transmembrane receptor family (73). The transmembrane domain interacts with G-proteins to activate the intracellular downstream cAMP pathway. The concentration of cAMP in the cytosol regulates proliferation and functional activity of thyroid follicular cells and the expression of thyroid specific genes as TPO, NIS and TG (74). The extracellular domain is responsible for high affinity binding of TSH. The *TSHR* gene is localized on chromosome 14q31 and consists of 10 exons. The extracellular aminoterminal domain is encoded by the first nine exons, while the transmembrane domain is encoded by the 10th exon (75).

TSH resistance was first described in a patient with congenital hypothyroidism, normal thyroid volume, but lacking uptake of radioiodide after exogenous TSH application (76). The first mutations in the *TSHR* gene was a compound heterozygous mutation found in three siblings with high TSH levels but normal T3 and T4 (hyperthyrotropinemia) (77). Since this initial report about 40 mutations have been described. The consecutive molecular mechanisms responsible for loss-of-function of the *TSHR* range from reduced cell-surface expression, probably due to misfolded protein structure and retention in the endoplasmic reticulum, reduced TSH binding, to impaired activation of the receptor or coupling to the intracellular G-proteins.

Congenital hypothyroidism due to mutations in the *TSHR* shows considerable disease heterogeneity, ranging from severe congenital hypothyroidism with severe thyroid gland hypoplasia to isolated hyperthyrotropinemia with normal thyroid volume. The severity of the disease is associated with genotype–phenotype correlations but is also dependent on biallelic versus monoallelic mutations in the same pedigree. Complete and moderate TSH resistance is mostly due to biallelic mutations inherited in an autosomal recessive manner and localized in exon 10 encoding the transmembrane domain (44,78–80), while mild resistance may occur by dominantly inherited monoallelic mutations (81).

The prevalence of *TSHR* gene mutations is higher than expected. In a Japanese cohort of patients with congenital hypothyroidism detected by TSH-based neonatal screening, *TSHR* gene mutations were found in 4.3% of patients with severe CH and 9.4% of patients with mild CH (82). Further, in adolescents with hyperthyrotropinemia but exclusion of autoimmune thyroiditis specific antibodies 11–29% of patients had monoallelic *TSHR* gene mutations in two Italian cohorts (83,84).

While severe cases always need T4 substitutive therapy for congenital hypothyroidism, controversy exists for proven partial TSH receptor defects. Several patients diagnosed at adult age with hyperthyrotropinemia remained euthyroid throughout life without any treatment, suggesting that elevated TSH could overcome the partial receptor defect (81,85,86). For such cases, detailed biochemical and molecular diagnostics is required to exclude mild forms of thyroid dysmorphogenesis that require treatment.

**84.2.2.2.2 Inactivating G-Protein Mutations (OMIM #103580).** A second form of TSH resistance with mild hypothyroidism can occur in pseudohypoparathyroidism type 1a due to inactivating mutations in the *GNAS* gene, which encodes the Gs alpha subunit of the G-protein. Patients usually present with short stature, round facies, shortening of the metacarpals and metatarsals, and obesity collectively termed as Albright hereditary osteodystrophy (10,45,87) (see Chapter 85).

**84.2.2.3 Thyroid Dysmorphogenesis.** Defects in any of the proteins indispensable for thyroid hormone synthesis lead to thyroid dysmorphogenesis and account for about 10–15% of congenital hypothyroidism with an incidence of 1:30,000–40,000 newborns (29,88,89). In these patients thyroid development and differentiation is normal; however, one of the critical steps of the synthetic machinery of the thyroid follicular cells is impaired. These include defects in (1) iodine uptake into thyroid follicular cells due to mutations in the *sodium/iodide symporter* (*NIS*, *SLC5A5*); (2) organification defects due to mutations in the *TPO*, *DUOX2* and *DUOX2* genes constituting the peroxidase enzyme system, or mutations in the *Pendrin* gene (*SLC26A4*); (3) defects in TG synthesis, storage or release; and (4) defective IYD (*DEHAL1*) activity leading to failure of thyroid follicular cell iodide recycling.

Patients with thyroid dysmorphogenesis are usually detected by neonatal screening suffering from congenital hypothyroidism, and present a goiter. As inheritance is autosomal-recessive, family history for congenital hypothyroidism and/or goiter is often positive (29,45). However, patients with different forms of thyroid dysmorphogenesis may develop mild hypothyroidism only during childhood or adolescence with or without goiter or present euthyroid goiter later in life.

For accurate clinical diagnosis of different forms of thyroid dysmorphogenesis, the kinetics of iodide uptake and release can be traced by administration of

radioiodide. After  $^{131}\text{I}$  administration an analysis of the thyroidal iodide concentration in relation to that in serum and the degree of iodine bound to protein can be obtained (45,90).

**84.2.2.3.1 Iodide Transport Defect (OMIM #274400).** Iodide uptake from the bloodstream into the thyroid follicular cell is the first step in thyroid hormone synthesis. Iodide is concentrated up to 40-fold by an active transport through the basolateral membrane. The iodide uptake is under close stimulatory regulation by TSH, especially in the context of low-iodine diet, and can be competitively inhibited by anions, such as thiocyanate or perchlorate, which are also used diagnostically (91,92).

The *sodium/iodide symporter* (*NIS*, *SLC5A5*) is a specialized plasma membrane glycoprotein that mediates active iodide transport at the basolateral membrane and is responsible for active iodide trapping from the bloodstream not only in the thyroid but also in other *NIS*-expressing tissues like mammary gland, salivary glands, gastric mucosa or placenta (91). Rat and human *NIS* genes were cloned in 1996 (93,94). Human *NIS* is located on chromosome 19. Expression of *NIS* is the limiting step for the onset of thyroid function in the human embryo at 12 weeks of gestation and the molecular explanation for accidental radioablation of the fetal thyroid by scintigraphy of pregnant women with radioactive iodide after the first trimester (4).

Iodide transport defect (ITD) is an uncommon form of dysmorphogenetic congenital hypothyroidism caused by *NIS* mutations transmitted according to an autosomal recessive pattern. Diagnostic criteria for ITD include a variable degree of congenital hypothyroidism and goiter, low or absent radioiodide uptake by the thyroid and other *NIS*-expressing organs (e.g. the salivary glands and gastric mucosa), and a low iodide saliva-to-plasma ratio (91,92,95). The first *NIS* mutation causing ITD was described in 1997 by Fujiwara et al. (96).

In a recent review of 31 patients with *NIS* mutations (97), a wide spectrum of severity and onset of hypothyroidism was observed. Genotype–phenotype correlations, however, revealed genotype specific onset of hypothyroidism at birth, during infancy or after the first year of life. Interestingly, patients with neonatal onset of hypothyroidism had the lowest residual radioiodide uptake on thyroid scintigraphy, compared to patients with infancy or childhood onset of hypothyroidism. This observation suggests that genotype-specific residual *NIS* activity in the thyroid gland may be a major determinant of the onset of hypothyroidism and the reason that patients suffering from milder forms of ITD will not be detected by neonatal screening (97).

**84.2.2.3.2 Iodine Organification Defects.** After uptake into the follicular cell at the basolateral membrane by *NIS* and efflux into the thyroid follicle at the apical membrane by *pendrin*, nearly 100% of the rare element iodide is rapidly bound in organic form (“organified”) (7,8,45).

This process takes place at the apical membrane of the thyroid follicle and is mediated by the enzymes TPO, DUOX2 in close contact with TG (98,99). For functional maturation of DUOX2, an endoplasmic reticulum resident DUOX2 is essential (100).

Iodine organification consists of oxidation of iodide to an active intermediate and iodination of tyrosyl residues of TG to form the iodotyrosines MIT and DIT. Then, these iodinated tyrosyl residues are linked to form T4 after coupling of two DITs and T3 after coupling of one MIT and one DIT. The enzyme TPO catalyzes iodination and coupling. For this it requires (1) hydrogen peroxide, which is provided by DUOX2 and possibly further NADPH oxidases and (2) iodine acceptors, which are the TG-bound tyrosyl residues.

Iodine organification defects (IODs) represent the most frequent cause of thyroid dysmorphogenesis and are either caused by defective TPO function or deficiency of hydrogen peroxide generation by DUOX2 or DUOX2A (45,101). In a recent study of 183 infants with congenital hypothyroidism, 14% of patients were diagnosed with an IOD (102).

IODs are classified as total (TIOD) or partial (PIOD) based on a perchlorate ( $\text{ClO}_4^-$ ) discharge test during thyroid scintigraphy with radioactive iodine. After administration,  $^{131}\text{I}$  is rapidly concentrated and organified in the thyroid follicle. Subsequent administration of perchlorate competitively inhibits further  $^{131}\text{I}$  uptake. In case of a normal organification process, radioactive iodide is iodinated and remains protein bound. As a consequence, less than 10% of the radioactive iodide is washed out. In TIOD, over 90% and in PIOD more than 10% of  $^{131}\text{I}$  is washed out after perchlorate administration, indicating that the  $^{131}\text{I}$  was efficiently concentrated within thyroid follicular cells but not organified sufficiently.

**84.2.2.3.3 TPO Deficiency (OMIM #274500).** TPO is a heme-containing enzyme at the apical membrane and is only active in the presence of hydrogen peroxide. TPO mediates organification of iodide and coupling of MIT and DIT. The human TPO gene is localized on chromosome 2p25, and consists of 16 coding exons. The nucleotide sequence was reported in 1987 (103). The first patient with a mutation in the TPO gene was a 12-year-old boy with compressive goiter due to noncompliance with substitutive therapy necessitating partial resection. He was diagnosed with overt hypothyroidism at the age of 4 months with congenital hypothyroidism. Biochemical studies of the resected thyroid tissue revealed absence of TPO activity. Subsequent DNA analysis identified a frame shift mutation (104). So far, about 60 mutations have been described and the majority of mutations are localized in exon 7, 8 and 9 of the gene, encoding the catalytic heme-binding domain of the protein. Patients with homozygous or compound heterozygous inactivating TPO mutations mostly show TIOD with goitrous congenital

hypothyroidism (105,106). However, recently few patients with PIOD have been described (107,108).

**84.2.2.3.4 Defects in DUOX2 (OMIM #607200) and DUOX2A (OMIM #274900).** Dual oxidase 2 (DUOX2, formerly referred as THOX2) and Dual oxidase maturation factor 2 (DUOX2A) form a heterodimeric NADPH oxidase complex at the apical membrane of the thyroid follicular cells. Together, they are the core of the  $\text{H}_2\text{O}_2$  thyroidal generating system, feeding TPO with  $\text{H}_2\text{O}_2$  for iodination and coupling. Human DUOX2 and DUOX2A are contiguous genes on chromosome 15q15.3. DUOX2 was cloned in 1999 and consists of 33 coding exons, while DUOX2A was identified only recently and consists of 6 exons (100,109,110). The existence of a NADPH oxidase dependent  $\text{H}_2\text{O}_2$  generating system was proposed as early as 1973 by Niepomniszcze et al. (111). They described an adult patient presenting with large goiter and IOD. After thyroidectomy, they observed restitution of iodination capacity of the thyroid tissue in vitro by addition of  $\text{H}_2\text{O}_2$ .

The first mutations in the DUOX2 gene were described in 2002 by Moreno et al. (112) by screening nine patients with molecularly unexplained IOD. They identified one homozygous mutation leading to severe congenital hypothyroidism and TIOD in one patient, while three patients were found harboring heterozygous mutations presenting with transient congenital hypothyroidism (112). Thus, in contrast to other dysmorphogenetic defects with autosomal recessive inheritance, haploinsufficiency of DUOX2 can result in transient congenital hypothyroidism. So far about 20 mutations have been described rendering the genotype–phenotype correlation more complex. Several authors have shown PIOD and transient congenital hypothyroidism in patients with compound heterozygous mutations. It has been postulated that dietary intake of iodine as well as residual function of the mutated DUOX2 protein might have modulated the phenotype of complete DUOX2 defects (113–115).

The first homozygous nonsense mutation in DUOX2A providing evidence for its critical role in thyroid hormonogenesis has been described in 2008 by Zamproni et al. (116). They screened the DUOX2A gene for mutations in 11 patients (10 Caucasian and 1 Chinese) with congenital hypothyroidism due to PIOD but negative for mutations in TPO and DUOX2. The index patient was of Chinese origin suffered from mild CH and PIOD. Pedigree analysis demonstrated recessive inheritance because heterozygous carriers had normal thyroid function including negative results in neonatal TSH screening. The Y246X mutation might be frequent in Chinese population, as 1 of 92 controls was identified as carrier (116).

**84.2.2.3.5 Pendred Syndrome (OMIM #274600).** The syndrome of familial profound congenital hearing loss associated with large multinodular goiter was first described by Pendred in two sisters in



1896 (117). The full triad of the syndrome is characterized by goiter of variable degree, PIOD and deaf mutism due to a specific malformation of the inner ear with enlarged vestibular aqueduct. Pendred syndrome is the only form of thyroid dyshormonogenesis with associated malformation. While the dysplastic cochlea with sensorineural deafness is a constant and leading clinical sign of the syndrome, goiter may be missing. Hypothyroidism is not a specific sign of the triad. Pendred syndrome is one of the most prevalent forms of syndromic deafness, with an estimated incidence of 10/100,000 individuals (118).

Pendred syndrome is caused by mutations in the *SLC26A4* gene encoding Pendrin, which was cloned in 1996. The *SLC26A4* gene is localized on chromosome 7 and has 21 exons. Pendrin expression is found in the thyroid, the inner ear and the kidney. Pendrin is localized at the apical membrane of thyroid follicular cells (119–123). The role of Pendrin in the thyroid is still in debate. In heterologous *in vitro* models, it has been shown that Pendrin facilitates iodide efflux from the thyroid follicular cells into the thyroid follicle at the apical membrane (124). However, there is no data available confirming the role of pendrin for apical iodide transport *in vivo* so far.

Thyroid dysfunction of Pendred syndrome consists of goiter in the context of PIOD. Goiter is of variable degree appearing during childhood. Thyroid function can be mildly hypothyroid or euthyroid even in patients with biallelic mutations. Most frequently, hypothyroidism is only seen in areas of low nutritional iodine intake (125,126). A hallmark of the syndrome is the large inter- and intrafamilial variability of the phenotype (127,128). To date, about 150 mutations have been reported in the *SLC26A4* gene. Several mutations are recurring in different ethnic groups: In Japanese and Korean patients, the H723R mutation accounts for about 50%, while in Europe mutations L236P, T416P and IVS8+1G→A are most frequently found (129,130).

As sensorineural deafness associated with enlarged vestibular aqueduct and not hypothyroidism is the leading clinical signs of the syndrome. Magnetic resonance imaging studies of the inner ear have been recommended in association with molecular genetic analysis for diagnosis of true Pendred syndrome (131) (see Chapter 142).

**84.2.2.3.6 Defective TG Synthesis and Transport.** TG is a homodimeric glycosylated iodoprotein with a molecular weight of approximately 650,000 Da. It is exclusively synthesized in thyroid follicular cells and released by exocytosis at the apical membrane into the thyroid follicle. It represents the most abundant protein of the thyroid tissue. Together with iodide and peroxide it is one essential substrate for thyroid hormone synthesis. TG contains tyrosine residues, which are the core matrix for organification and storage of MIT, DIT, T3 and T4. TSH stimulates colloid endocytosis at the apical membrane and TG proteolysis within the cytoplasm (132). The resulting T3 and T4 are released into

the bloodstream, while MIT and DIT are deiodinated by the IYD.

The human TG gene maps on chromosome 8q24.2 and contains 48 exons (133–135). TG is composed of three repetitive units and of an acetylcholinesterase-like domain at the carboxy-terminal domain, which is required for dimerization and intracellular transport (136).

Mutations in the TG gene are inherited in an autosomal recessive way. They occur in homozygous or compound heterozygous states. The first mutation in the TG gene was reported in 1991 (137). So far about 50 mutations have been identified in the last twenty years. Mutations of the TG gene can cause congenital hypothyroidism by three different ways: TG deficiency with little or no TG detected in thyroid tissues of patients (137,138), TG transport defect with accumulation of TG in the endoplasmic reticulum (139), and abnormally structured TG with defective iodotyrosine coupling and T4 deficiency (140). Interestingly, truncated TG protein might be secreted and could be sufficient for partial thyroid hormone synthesis as shown *in vitro* (135).

The majority of patients presents with severe congenital hypothyroidism with goiter at birth or appearing during infancy. Goiter can be detected already *in utero* (141). Typically, TG levels in blood are very low and show an absent rise of TG after stimulation with recombinant TSH, if applied. T4 is usually decreased, while T3 can be low or normal. The perchlorate test is negative in contrast to iodide organification defects. However, the phenotypic spectrum ranges from the severe cases to euthyroid goiter (141–144).

**84.2.2.3.7 IYD Defect (OMIM #274700).** The thyroid follicular cell recycles the iodide that is released from TG in form of MIT and DIT, while T3 and T4 are released into the blood. MIT and DIT are deiodinated by the IYD system. Failure to deiodinate thyroid MIT and DIT results in severe iodine deficiency due to severe iodine wastage, since these non-deiodinated iodotyrosines diffuse out of the thyroid and are excreted in urine. IYD defect was clinically described by several authors in 1950 (145,146). Patients showed hypothyroidism and goiters and suffered from cretinism. Radioiodide studies showed (1) an accelerated uptake of radioiodide (2) negative perchlorate test, (3) spontaneous release of labeled iodide and (4) high doses of labeled MIT and DIT instead of free labeled iodide in serum and in urine. About 25 pedigrees have been clinically described with a spectrum of cretinism, simple goiter or hypothyroidism and goiter (147). The IYD gene (*DEHAL1*) is localized on chromosome 6p24, and has 6 exons. The iodotyrosine deiodinase belongs to the NADH oxidase/flavin reductase superfamily (148). In 2008, Moreno et al. (149) identified the first homozygous mutations of the IYD/*DEHAL1* gene in four patients of different European countries. None of the patients was detected by neonatal screening but were diagnosed clinically with severe hypothyroidism appearing from infancy to

8 years of life (149). All had goiter, and two suffered from mental retardation. A further family with an *IYD/DEHAL1* mutation has been described (150). Interestingly, heterozygous family members were euthyroid and had no goiter.

### 84.2.3 Disorders of Thyroid Hormone Transport, Metabolism and Action

**84.2.3.1 Disorders of Thyroid Hormone Transport Proteins.** After hormone secretion from thyroid follicular cells into the blood, thyroid hormones are present in a free form and in a bound form. The bound form of thyroid hormones represent a large extra thyroidal pool of T3 and T4, while free T3 and T4 are transported into the cell, bind to the nuclear receptor and result in thyroid hormone dependent processes. Thyroid hormone serum transport proteins protect the body from fluctuations of thyroid hormone secretion. Approximately 0.03% of T4 and 0.3% of T3 are free or unbound in serum (12). The three serum thyroid hormone-binding and -transporting proteins are TBG, TTR and ALB. TBG binds 75% of T4 and T3 in serum, while TTR and ALB are responsible for 20% and 5% of thyroid hormone transport, respectively (11).

Disorders of serum thyroid binding proteins result in alteration of measured thyroid hormone concentrations but do not alter thyroid hormone dependent metabolic state of the body and do not cause thyroid disease. Thus, patients are clinically euthyroid but present with altered total T3 and T4 levels in the context of normal TSH. Often patients are treated inappropriately for biochemically diagnosed congenital hypothyroidism without clinical disease (45).

**84.2.3.1.1 Defects of TBG (OMIM +314200).** Inherited TBG defects may result in three different forms: complete TBG deficiency, partial TBG deficiency and TBG excess. The *TBG* gene is localized on chromosome Xq21 and is composed of five exons (151). Inheritance of *TBG* defects follows an X-linked pattern with complete phenotype in males or females with Turner syndrome.

Complete TBG deficiency has been first described in 1959 (152). Male patients are clinically euthyroid but are diagnosed on the basis of low total T3 and total T4 values in the context of normal TSH. TBG measurements reveal undetectable or very low TBG levels, while in carrier females, TBG levels are decreased by about 50%. Patients may be diagnosed by T4-based neonatal screening, but not if screening is based on TSH levels only. Based on neonatal screening data, the incidence varies from 1:1500 newborn in Japan to about 1:10,000 in Caucasians (153,154). Partial TBG deficiency is the most common form of TBG defects, with 1:4000 newborn. TBG levels are decreased but measurable, with milder disorders of T4 and T3 levels in affected males. A rare form of TBG defects is TBG

excess with a variable incidence in different countries (1:6000 to 1:40,000). Affected patients are characterized by clinically euthyroid state but increased total T4 in the context of normal free T4 and T3. TBG levels are increased by up to four times (12,155). So far 28 mutations have been associated with the three different forms of *TBG* defects (156).

**84.2.3.1.2 TTR Defects (OMIM #145680, #105210).** The first clinically euthyroid patient with increased TTR levels was described by Moses et al. (157). He was diagnosed based on elevated total T4 in the context of normal free T4, free T3, TSH, TBG and ALB. The *TTR* gene is localized on chromosome 18q11.2 and consists of 4 exons (11). Mutations in the *TTR* gene can cause changes in TTR concentration as well as decreased or increased affinity to T3 and T4. As TTR affinity to thyroid hormones is about 100 times less than for TBG, only mutations with increased affinity will be diagnosed as euthyroid hyperthyroxinemic patients (45,158). Some mutations of the *TTR* gene are associated with familial amyloidotic polyneuropathy or cardiopathy and with acquired and hereditary forms of system amyloidosis (159,160).

**84.2.3.1.3 Familial Dysalbuminemic Hyperthyroxinemia (OMIM +103600).** ALB represents about 50% of blood serum proteins in the human. ALB is carrier for steroids, fatty acids and thyroid hormones. The human *ALB* gene is localized on chromosome 4q11 and consists of 15 exons (11). Mutations in the *ALB* gene cause familial dysalbuminemic hyperthyroxinemia (FDH). It is the most common form of inherited euthyroid hyperthyroxinemia in Caucasians. FDH is transmitted in an autosomal dominant way. Patients are characterized by high total T4 due to increased affinity of ALB to T4 (161,162). In contrast to *TBG* and *TTR* defects, free T4 levels measured by standard clinical laboratory methods may also be falsely elevated. T3 levels are rarely increased (163). Genetic testing has been proposed to prove the disease as all FDH associated mutations have been identified in the 218 residues so far (163).

**84.2.3.2 Defects of Thyroid Hormone Membrane Transporters (OMIM #300523).** The effects of thyroid hormone on development and metabolism are exerted at the cellular level. Metabolism and action of thyroid hormones take place intracellularly, which require transport of the hormone across the plasma membrane. This process is mediated by thyroid hormone membrane transporter proteins. Many thyroid hormone transporters have been identified at the molecular level, although a few are classified as specific thyroid hormone transporters, including MCT8, MCT10 and organic anion-transporting polypeptide 1C1 (13,14). So far, mutations have only been described in the *MCT8* gene, which is located on the chromosome Xq13.2, consists of 6 exons and encodes for a protein with 12 predicted transmembrane domains. The hallmark of *MCT8* gene mutations is the combination of thyroid and neurological dysfunction

(15,164). Patients present during infancy with central hypotonia, poor head control, evolving to spastic quadriplegia, nystagmus and severe mental retardation with absence of speech. There are no other signs of hypothyroidism. The key laboratory finding is elevated T3 concentration in the context of low T4 and free T4. TSH levels are in the upper normal range. *MCT8* mutations have been associated with Allan–Herndon–Dudley syndrome and a Pelizaeus–Merbacher-like disease (165,166). So far, mutations have been found in over 50 families. Patients with unexplained mental retardation and abnormal thyroid function tests with low fT4, elevated T3 and normal to elevated TSH should be screened for *MCT8* mutations (14). Although females are heterozygous for the disease and asymptomatic, women are at risk to develop hypothyroxinemia during pregnancy. Therefore, heterozygous women should be monitored for hypothyroxinemia to avoid fetal and neonatal hypothyroidism and substituted if necessary to prevent cognitive delay (167).

**84.2.3.3 Defects in Iodothyronine Deiodinase Cofactors (OMIM #609698).** Intracellular metabolism of T4, which serves as prohormone and availability of the active T3 is regulated by the three selenoprotein iodothyronine D1–3. The three deiodinases serve as subunits of larger, functional enzyme complexes. The activating D1 and D2 can intracellularly increase the T3 level, while D3 is inactivating T4 to reverse T3 (10,16,17). The gene for D1 (*DIO1*) is located on chromosome 1, the genes for D2 and D3 (*DIO2* and *DIO3*) are found on chromosome 14 (16). Several human cases have been reported with suspected D1 deficiency (168,169). The patients were euthyroid with elevated levels of T4, free T4 and reverse T3, and low normal to normal levels of T3. However, no mutations have been found so far in *DIO1* or *DIO2*. Dumitrescu et al. (18) reported of three siblings with clinical evidence of abnormal thyroid hormone metabolism, presenting with high serum T4 and TSH concentrations, low T3 concentrations and markedly elevated rT3 concentrations. Their fibroblasts showed decreased D2 enzymatic activity but no *DIO2* mutation was found. Systematic linkage analysis of genes involved in D2 synthesis and degradation led to the identification of an inherited selenocysteine incorporation defect, caused by a homozygous missense mutation in the *selenocysteine insertion sequence binding protein 2* (*SECISBP2*) gene which serves as cofactor for deiodinase synthesis. An unrelated child with a similar phenotype was compound heterozygous with respect to mutations in *SECISBP2* (18). While these patients all presented clinically only with retarded growth, more recently, subjects with further defects in the *SECISBP2* gene were described with a much broader extra thyroidal phenotype due to reduced synthesis of most of the 25 known human selenoproteins, resulting in azoospermia, muscular dystrophy, photosensitivity, impaired T lymphocyte proliferation, abnormal mononuclear

cell cytokine secretion and enhanced insulin sensitivity (170).

**84.2.3.4 Thyroid Hormone Resistance (OMIM #188570, #145650).** In the nucleus, thyroid hormones are effective by binding to the TR. TRs belong to the nuclear receptor family and so far five isoforms have been identified (TR alpha 1 and 2, TR beta1, 2 and 3); however, the TR alpha 2 has no binding capacity for T3. TRs are mediating thyroid hormone effects by binding to thyroid-response elements in the promoter region of thyroid hormone responsive genes. Consequently, gene expression of thyroid responsive genes is up- or down-regulated. The expression of the different isoforms is tissue-specific (19,20,171).

The first family with resistance to thyroid hormone (RTH) has been described in 1967 by Refetoff et al. (172). This characterizes a syndrome with reduced responsiveness of target tissues of thyroid hormone. Mutations in the *TR beta* gene have been associated with RTH by Sakurai et al. (173). Since then, up to 1000 cases have been described in the literature with a wide range of clinical symptoms, 349 were reviewed in detail (21).

The clinical presentation is heterogeneous. Some patients have no or minor symptoms, others can present with symptoms of hypo- or hyperthyroidism. The characteristic diagnostic features of RTH are: (1) elevated serum levels of fT3 and fT4, (2) normal or slightly increased TSH levels that respond to TRH, (3) absence of signs and symptoms of hyperthyroidism and (4) goiter. Further clinical signs associated with RTH are attention-deficit hyperactivity disorder (ADHD) and learning disorders, mental retardation, recurrent ear, nose, throat infections, hearing loss and short stature during childhood, but not affecting adult height (174,175). Historically, two major forms have been described: generalized RTH (GRTH), and selective pituitary RTH (PRTH). GRTH patients are completely or partially compensating for decreased tissue responsiveness by increasing T4 synthesis. They are clinically euthyroid or hypothyroid. PRTH patients present with signs of hyperthyroidism. However, more recent data and genotype–phenotype correlation analyses found both, the two different forms in the same families with identical mutations. Thus, the two forms represent rather the wide clinical spectrum of RTH than two distinct forms (176,177).

So far, mutations were only identified in the *TR beta* gene, which is localized on chromosome 3, but none in the *TR alpha* gene. Inheritance is autosomal dominant. Most of the mutations cluster in the three domains of the T3-binding site of the TR beta molecule, while no mutations have been identified so far in the DNA-binding domain. As mutant TRs are forming homodimers with wild-type TRs, they are exerting a dominant negative effect on the normal TR loss of T3 binding, or reduced release of corepressor binding, as shown by in vitro experiments (178). In about 10% of families RTH occurs also in the absence of mutations in the *TR beta* gene (179).

### 84.3 GENETIC HYPERTHYROIDISM

#### 84.3.1 Activating TSHR Mutations (OMIM #609152)

While inactivating mutations of the *TSH receptor* gene (TSHR) cause congenital hypothyroidism, activating mutations can cause nonautoimmune hyperthyroidism. The three distinct forms of genetic hyperthyroidism are sporadic congenital hyperthyroidism caused by de novo germ line mutations, familial nonautoimmune hyperthyroidism due to autosomal dominant inheritance of germ line mutations, and autonomous adenomas caused by de novo somatic mutations (180). The first patient with sporadic congenital hyperthyroidism due to a de novo activating TSHR gene was reported in 1995 by Kopp et al. (181). Patients present at birth or during infancy with clinical signs and laboratory findings of hyperthyroidism: prematurity, low birth weight, goiter and craniosynostosis. In contrast to neonates with transient autoimmune hyperthyroidism due to placental transmission of activating TSHR autoantibodies from mothers with Graves' disease, patients with activating mutations have no antibodies and the disease persists beyond 3–6 months of life (181,182). Familial hyperthyroidism with autosomal dominant inheritance due to a gain-of-function mutation of the TSHR gene was first described by Duprez et al. (183) and so far 27 families have been reported. The onset of hyperthyroidism and goiter is highly variable, neonatal expression has been described. Both forms can be treated by antithyroid drugs, but often definitive cure is only possible by total thyroidectomy or radioablation. While in the two former forms all cells of the thyroid are hyperfunctioning, the monoclonal expansion of one mutated cell results in the development of an autonomous hyperfunctioning adenoma (45). The rest of the thyroid tissue is usually inactive. As gene expression profiling shows common characteristics, the different phenotypes of genetic hyperthyroidism are the result of the differences in intensity and onset of constitutive activity of the mutated receptor and its cellular distribution (184).

#### 84.3.2 Activating G-Protein Mutations (OMIM #174800)

McCune Albright syndrome is classically defined by the clinical triad of fibrous dysplasia of bone, café au lait skin spots and endocrinopathies, including precocious puberty, growth hormone and/or prolactin secreting pituitary adenomas, autonomous adrenal hyperplasia and hyperthyroidism (185–188). The disease results from somatic mosaicism for mutations of the *GNAS* gene. In patients with hyperthyroidism, the mosaic pattern involves the thyroid follicular cells leading to a constitutive activity of the Gs alpha protein. A review of patients with McCune Albright syndrome described the spectrum thyroid disorders ranging from nodular goiter with and

without overt hyperthyroidism, diffuse goiter with and without overt hyperthyroidism and hyperthyroidism without goiter. The thyroid hyperactivity is manifest in infancy or early childhood (188).

### 84.4 THYROID DISEASE ASSOCIATED WITH CHROMOSOMAL ABNORMALITIES AND CONTIGUOUS GENE DELETION SYNDROMES

Thyroid disorders have been reported to have a prevalence rate of 3–54% in individuals with Down syndrome (OMIM #190685) (189). The population-based incidence of medically treated thyroid disease in children with Down syndrome was 10.8% in a retrospective cohort study, with a 73-% increase after release of American Academy of Pediatrics guidelines in 2001 (190). The spectrum of thyroid disorders ranges from congenital hypothyroidism, autoimmune (Hashimoto) thyroiditis, to compensated hypothyroidism (hyperthyrotropinemia). The incidence of congenital hypothyroidism in newborns with Down syndrome is not precisely known, but was found to be as high as 3.5% compared with 0.5% in the general population (191). Cumulative percentage of patients developing hypothyroidism was 15% until 8 years, and 30% at 20 years. Hypothyroidism before 8 years was nonautoimmune, while most patients older than 8 years developed autoimmune thyroiditis (189). Routine screening for thyroid disease annually is recommended for children with Down syndrome (192) (see Chapter 43).

Individuals with Turner syndrome have an increased risk for autoimmune thyroiditis (193). In a longitudinal study 24% of 84 children with Turner syndrome developed hypothyroidism after a mean duration of 8 years (194). Annual screening for hypothyroidism is recommended from the age of 4 years on (193).

Patients with Williams–Beuren syndrome (OMIM #194050) and DiGeorge syndrome (OMIM #188400) have a high prevalence of thyroid hypoplasia (50–70%) and compensated hypothyroidism (25–30%) (195,196).

### 84.5 GENETIC BASIS OF AUTOIMMUNE THYROID DISEASE

#### 84.5.1 Familial Graves' Disease and Hashimoto Thyroiditis (OMIM %275000, %140300)

The two major autoimmune thyroid diseases (AITD) include Graves' disease and Hashimoto thyroiditis (see Chapter 76). Both disorders are characterized by infiltration of the thyroid tissue by T- and B-cells reactive to thyroid antigens, and the presence of autoantibodies against one or several specific thyroid proteins. Graves' disease is characterized by production of activating autoantibodies against TSH receptor (thyroid-stimulating immunoglobulins) by B lymphocytes. The thyroid-stimulating antibodies



bind to the TSH receptor, activate the TSH–cAMP cascade and finally causing hyperthyroidism. Hashimoto thyroiditis is characterized by lymphoid infiltration by autoreactive B- and T-cells leading to apoptosis, progressive autoimmune thyrocyte depletion, fibrosis and hypothyroidism. Autoantibodies against TPO and TG are a diagnostic feature of the disease (197,198). Both diseases arise due to complex interactions between environmental and genetic factors leading to the breakdown of self-tolerance.

Familial occurrence of AITD is well described, and both disorders may occur in the same family (197,199). Nearly one half of the first-degree relatives of patients with Graves' disease and Hashimoto thyroiditis have evidence of thyroid autoimmunity, with or without evidence of thyroid dysfunction. The ratio of the prevalence of the diseases in the siblings of affected individuals compared with the prevalence in the general population has been estimated to be 17 for AITD (11.6 for Graves' disease and 28.0 for Hashimoto disease) (197). Twin studies further support the inherited susceptibility to AITD, the concordance rate for Graves' disease and Hashimoto disease in monozygotic twins being as high as 35% and 55%, respectively (200,201).

The mechanisms of thyroid autoimmunity in Graves' disease and Hashimoto thyroiditis and the five- to 10-fold higher prevalence in females remain unclear. AITD susceptibility genes have been identified. They can be divided into immune modulating genes such as *HLA-DR*, *CD40*, *CTLA-4* and *PTPN22*, and thyroid specific genes like *TG* and *TSHR*. Amino acid substitutions in the *HLA-DR*, *TG* and the *TSHR* gene were found to predispose to AITD (197,202–204).

### 84.5.2 Autoimmune Polyglandular Syndrome (OMIM #240300, #269200, #304790)

The autoimmune polyglandular syndromes (APSs) are a group of diseases characterized by the presence of a combination of multiple autoimmune disorders. AITD occurs frequently in these patients (205–208).

APS-1 also called autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) is a rare monogenic disease caused by mutations in the *autoimmune regulator* (*AIRE*) gene. *AIRE* is assigned to chromosome 21q22.3 (209,210). To date, about 45 mutations have been described in *AIRE* inherited in an autosomal recessive manner. The decreased expression of the *AIRE* transcription factor results in the loss of central tolerance to self-autoantigens. Autoreactive T-cells target specific tissues like skin, parathyroid, adrenal glands and others. The diagnosis is made in the context of a combination of mucocutaneous candidiasis, hypoparathyroidism and Addison's disease. AITD is a common additional endocrinopathy in these patients occurring in about 10% (207,208).

APS-2 is the most common APS, also referred to as Schmidt or Carpenter syndrome. The combination of

Addison's disease with either AITD (Hashimoto thyroiditis or Graves' disease) or type 1 diabetes mellitus is diagnostic. Further non-endocrine autoimmune diseases associated with APS-2 are celiac disease, vitiligo, alopecia, pernicious anemia and myasthenia gravis. In contrast to APS-1, the inheritance of APS-2 is complex. Genetic association with HLA haplotypes (*HLA-DR3* and *HLA-DR4*) and polymorphisms of genes encoding immunologically relevant proteins (*MICA5*, 1, *PTPN22*, *CTLA4*, *VNTR*) have been described. The sum of these genetic factors confers a general susceptibility for the disease and the clinical spectrum of targeted organs. Circulating organ-specific autoantibodies are usually present even in the absence of overt clinical disease; 15% of relatives have autoantibodies detectable and manifest unsuspected illness, most commonly AITD (206–208).

IPEX syndrome is a rare X-linked condition of male infants resulting in multiple autoimmune disorders. The first symptom is severe chronic diarrhea due to autoimmune enteropathy. AITD occurs in the first year of life in 90% of patients (211). It is a result of absence or dysregulation of regulatory T-cells, caused by mutations in the *forkhead box P3* (*FOXP3*) gene (212). Children usually die within the first 2 years of life.

## 84.6 GENETIC BASIS OF THYROID CARCINOMA

### 84.6.1 Medullary Thyroid Carcinoma (OMIM #171400, #155240, #162300)

Medullary thyroid cancer (MTC) accounts for only 3–10% of all thyroid carcinomas, but up to 13% of deaths related to thyroid malignoma. MTC derives from calcitonin-producing parafollicular C-cells. A premalignant tumor stage is characterized by diffuse C-cell hyperplasia, followed by malignant transformation into MTC with or without metastases. Tumor size correlates with basal calcitonin levels, a reflection of tumor burden. In about 75% of cases, MTC is sporadic, unifocal with a peak incidence in the fifth and sixth decades. In 25% of patients, MTC is familial, inherited in an autosomal dominant trait, and is characterized by early onset in the first decade in the context of multiple endocrine neoplasia type 2 (MEN 2) (213–216). Three distinct but overlapping forms of MEN 2 can be distinguished: MEN 2A is characterized by the unique combination of MTC, pheochromocytoma and parathyroid hyperplasia/adenoma, MEN 2B comprises MTC, pheochromocytoma, mucosal and intestinal ganglioneuromatosis and Marfanoid habitus, and the familial medullary thyroid carcinoma (FMTC) is diagnosed in families with four or more cases of MTC in the absence of pheochromocytoma, parathyroid hyperplasia/adenoma or ganglioneuromatosis. In all three subtypes, MTC has a nearly 100% penetrance and is the first tumor of the syndrome to manifest (213–215).

The common genetic basis of the MEN 2 subtypes has been unraveled in 1993 by identifying gain-of-function germ line point mutations of the *rearranged during transfection* (*RET*) protooncogene in patients with MEN 2A, MEN 2B and FMTC (217,218). The *RET* gene was described in 1985 by Takahashi et al. (219). It is localized on chromosome 10q11.2 and contains 22 exons. The *RET* protooncogene belongs to the platelet-derived growth-factor (PDGF) family of protein tyrosine kinases. Its extracellular cysteine-rich domain is encoded by the first 11 exons, while the intracellular tyrosine kinase domain and the C-terminal tail are encoded by exons 12–21 (220). Most *RET* mutations are clustered in specific regions of the gene, coding for the extracellular cysteine-rich region (exon 10 and 11) and the intracellular tyrosine kinase domains 1 + 2 (exons 13–16) of the *RET* protein. *RET* mutations in MEN 2A and FMTC are inherited in an autosomal dominant manner. In contrast, *RET* mutations causing MEN 2B occur de novo (221,222). Inactivating mutations have been associated with Hirschsprung disease but it may also occur in patients with specific activating mutations in MEN 2A and FMTC (215,223,224).

Genetic testing for *RET* mutations has rapidly replaced biochemical screening by calcitonin measurements as first-line screening in asymptomatic kindred of MEN 2A and FMTC families in the last decade. It is currently the most specific and sensitive genetic screening in any of the hereditary cancer syndromes, and allows diagnosis and treatment in a preclinical disease stage for MEN 2A and FMTC, while MEN 2B patients need to be diagnosed on clinical grounds (213,215,225). Further, between 4 and 10% of apparently sporadic cases of MTC carry heritable disease-causing mutations in *RET* and *RET* gene mutation testing is widely recommended in all MTC patients (213,215,226).

Striking genotype–phenotype correlations characterize the MEN 2 subtypes and allow genotype-based prediction of MTC aggressiveness (227,228): MEN 2B is in 95% of cases caused by mutations in codon 918.

These patients develop the most aggressive form of MTC with metastatic disease as early as in the second year of life (221,222). In contrast, MEN 2A and FMTC show less aggressive MTC-phenotypes. Mutations at codon 634 represent the most frequent *RET* mutation (>60%) and result in the most aggressive MEN 2A phenotype (229,230). In 94 children with the c634 mutation undergoing total thyroidectomy until 20 years of age, 65% showed MTC and 16% metastatic MTC (230). Less frequent mutations in other codons typically show a slower development of MTC.

Genetic testing identifies at risk children in MEN 2A and FMTC families and allows “prophylactic” or early total thyroidectomy at a preclinical stage of disease. The strong genotype–phenotype correlations led to a refinement of the timing of total thyroidectomy based on codon-specific MTC aggressiveness initially into three risk categories (213). The current consensus guidelines have been published in 2009 by the American Thyroid Association (Table 84-3) and classify *RET* mutations into four risk levels (215). Risk levels also define the optimal age at prophylactic total thyroidectomy, the only curative approach for the thyroid disease. Patients with MEN 2B should be thyroidectomized during the first year of life, patients with MEN 2A or FMTC should undergo thyroidectomy according to codon-specific risk level. In general early total thyroidectomy at 5 years of age is a safe approach in any MEN 2A or FMTC patient unless baseline calcitonin is raised. In these cases immediate total thyroidectomy should be performed (213,215,230,231).

Although long-term results on prognosis are lacking, available data show that children with MEN 2A and FMTC undergoing prophylactic thyroidectomy until 5 years, respectively 8 years of age on the basis of genetic testing had no or lower incidence of recurrent or persistent MTC during follow-up than children operated at an older age (230,232). In contrast, as MEN 2B is due to de novo mutations, patients are often diagnosed with an advanced stage of MTC. Consequently they often suffer

**TABLE 84-3 American Thyroid Association (ATA) Medullary Thyroid Cancer Guideline 2009 (215)**

MTC Aggressiveness	ATA Risk Level	RET Genotype Mutated Codon	Phenotype	Earliest Age of MTC Onset	ATA Guideline for Prophylactic Total Thyroidectomy
Highest	D	918, 883, 804 + 805, 804 + 806, 804 + 904	MEN 2B	Infancy	<1 year
High	C	634	MEN 2A	1 year	<5 years
Moderate	B	609, 611, 618, 620, 630, 631, 633, 804 + 778	MEN 2A, FMTC	5 years	<5 years for higher risk mutations, alternatively*
Low	A	321, 531, 532, 515, 533, 600, 603, 606, 635, 649, 666, 768, 777, 790, 791, 804, 819, 833, 844, 866, 891	FMTC	>5 years	5 years, alternatively*

\*Prophylactic total thyroidectomy may be delayed in the setting of normal annual (1) serum calcitonin, (2) neck ultrasound, (3) less aggressive MTC family history, and (4) family preference.

from persistent or recurrent disease postoperatively with considerable mortality during childhood (221,222).

### 84.6.2 Thyroid Follicular Carcinoma (OMIM #188550, %188470)

Thyroid follicular cell tumors mostly occur sporadically, but familial forms do exist. Carcinoma of the thyroid in children and adolescents has little risk of mortality but high risk of recurrence. Younger patients present with a more advanced stage of disease (233,234). The clear association between radiation exposure and subsequent development of papillary thyroid carcinoma has been shown by Winship and Rosvall in 1961 (235).

Different genetic alterations are involved in thyroid tumorigenesis. Hyperfunctioning autonomous thyroid adenomas are considered to be clonal. The main mechanism responsible is the constitutive activation of the cAMP-dependent mitogenic cascade through somatic activating mutations of the *TSH receptor* or the *Gs* protein (184,236). In a series of 33 cases studied by Parma et al. (184), 82% were found to harbor a somatic *TSH receptor gene* mutation and 6% a *GNAS* gene mutation.

In a retrospective series from 1970–2000 of 56 patients between 4 and 20 years the histologic subtype of the thyroid tumor was pure papillary carcinoma in 66%, follicular variant of papillary in 29% and pure follicular carcinoma in 5% (233). No dedifferentiated tumors were observed. In adult patients papillary carcinoma, follicular carcinoma, poorly differentiated carcinoma and anaplastic carcinoma represent about 80, 15, 1 and 2% of all thyroid carcinomas, respectively (237). The distinct forms of thyroid carcinomas are characterized by different genetic alterations in signaling pathways. Papillary thyroid carcinomas are essentially characterized by mutations of the *BRAF* and *RAS* genes and *RET/PTC* rearrangement—all effectors of the MAPK signaling pathway—leading to constitutive activation of the pathway. The prevalence of *BRAF* is low in children while the prevalence of *RET/PTC* is significantly higher in pediatric PTC and children exposed to radiation after the Chernobyl nuclear accident than in adults (238–242). Follicular thyroid carcinoma is mainly associated with mutations of the *RAS* gene and the *PAX8-PPARGamma* rearrangement, while in poorly dedifferentiated and anaplastic thyroid carcinomas mutations in *RAS*, *BRAF*, *TP53* and *CTNNB1* are found (237,243).

Familial non-medullary thyroid cancer is rare. It can be classified in two groups: First, as non-syndromic forms occurring as (1) isolated familial papillary thyroid carcinoma (FPTC), (2) FPTC associated with renal cell carcinoma and (3) familial multinodular goiter. Second, as syndromic forms in the context of familial tumor syndromes such as (1) familial adenomatous polyposis syndrome and Gardner syndrome, (2) PTEN-hamartoma

tumor syndrome including Cowden syndrome, (3) Peutz-Jegher syndrome, (4) Carney complex and (5) Werner syndrome (244,245).

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### Biographies



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His fields of special interests are fetal/perinatal endocrinology and in developmental biology and genetics, thyroid and endocrine pancreas development.

His training took place in Paris and during postdoctoral fellowships in the Department of genetics and microbiology, Centre Médical Universitaire, Genève, Switzerland and at Harvard Medical School, Department of Neurobiology (Prof. Potter) and Section of Immunology and Immunogenetics, Elliot P. Joslin Research Laboratory (Prof. Eisenbarth), Joslin Diabetes Center, Boston, MA, USA.

He has published more than 130 peer-reviewed papers in his field and was offered in 2003 the Jacques Raymond Ducharme award in pediatric endocrinology of the Sainte Justine hospital Montréal, Canada and in 2006 a French national award in medicine: “les victoires de la médecine” for the work on neonatal diabetes mellitus. Pr Polak was awarded the prestigious European Society for Pediatric Endocrinology research award in 2012.



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# CHAPTER

# 85

## Parathyroid Disorders

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### 85.1 CALCIUM HOMEOSTASIS

The parathyroid gland plays a key role in calcium homeostasis by sensing a decrease in ambient calcium concentrations and responding appropriately by synthesizing and secreting more parathyroid hormone (PTH) (1). PTH acts on the kidney to enhance renal calcium reabsorption and promote conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D]. This active metabolite of vitamin D increases gastrointestinal absorption of calcium, induces osteoclast differentiation, and with PTH stimulates skeletal resorption, leading to increased extracellular calcium. The parathyroid gland responds to the normalization of the ambient calcium concentrations by reducing PTH release. The calcium-sensing receptor (CASR) is a plasma membrane G-protein coupled receptor (GPCR) that is expressed in the PTH-producing cells of the parathyroid gland and the cells lining the renal tubule (2,3). By virtue of its ability to sense small changes in circulating calcium concentrations ([Ca<sup>2+</sup>]<sub>o</sub>) and to couple this information to intracellular signaling pathways (thereby modifying PTH secretion and renal cation handling), the CASR plays an essential role in maintaining mineral ion homeostasis. Earlier studies showed that a cluster of missense polymorphisms—A986S, R990G, Q1011E—located in the cytoplasmic tail of the CASR is associated with interindividual population differences in [Ca<sup>2+</sup>]<sub>o</sub> (4,5). Different haplotypes are associated with primary hyperparathyroidism and the frequency of kidney stones (6). Recent genome-wide association studies in ~33,000 individuals of European and Indian Asian ancestry confirmed that the blood calcium concentration associated most significantly with SNPs in the CASR gene (7,8).

PTH also modulates the extracellular concentration of other ions, the most important of which is phosphate.

PTH-induced lysis of skeletal mineral [largely hydroxyapatite or Ca<sub>10</sub>(PO<sub>4</sub>)<sub>6</sub>(OH)<sub>2</sub>] increases extracellular phosphate as well as calcium levels. A compensatory decrease in extracellular fluid phosphate is brought about by the action of PTH-mediated inhibition of renal phosphate reabsorption, thereby inducing phosphaturia.

In target tissues, PTH itself binds to another GPCR, PTHR1, which also binds the related ligand, PTH-related peptide (PTHrP). The binding of PTH to its cognate receptor in bone and kidney is known to stimulate adenylate cyclase on the inner surface of the plasma membrane and induces increased phosphatidylinositol turnover (9). The adenylate cyclase product, cyclic AMP (cAMP), activates protein kinase A (PKA), while the products of phospholipase C activity, inositol-(1,4,5)-trisphosphate (IP<sub>3</sub>), and diacylglycerol (DG), mobilize intracellular Ca<sup>2+</sup> stores and activate protein kinase C (PKC). Thus, a cascade of intracellular signaling events is involved in target cell responses to PTH.

### 85.2 PRIMARY HYPERPARATHYROIDISM

#### 85.2.1 Clinical Features

Primary hyperparathyroidism is a common cause of hypercalcemia in adults. Multichannel autoanalyzer screening led to the recognition of its true incidence, presently estimated at about 1 per 1000 (10). The important laboratory features of primary hyperparathyroidism are elevated serum calcium, decreased fasting serum phosphate, increased serum PTH, and hypercalciuria. Although the disorder can present at any age, it occurs most frequently in the sixth decade. More women than men are affected, the ratio being about 3:1. When found

in children, primary hyperparathyroidism is likely to be a component of a familial endocrinopathy and a sex bias is not observed.

In primary hyperparathyroidism due to glandular hyperplasia, the hypercalcemia usually results from excessive PTH secretion due to increased parathyroid gland mass. In adenomatous disease, it is more often a change in control of PTH secretion by the parathyroid due to a resetting of the response to ambient calcium concentrations (11). Approximately 85% of cases involve a single benign adenoma, while hyperplasia (multiple hypercellular glands) is present in 15%. Parathyroid carcinoma is seen in less than 1% of cases, and ectopic secretion of PTH by nonparathyroid tumors is rare.

In primary hyperparathyroidism, other organ systems can be involved. These include the kidneys, skeleton, gastrointestinal tract, and central nervous system. Nephrolithiasis is the most important manifestation clinically, with kidney stones occurring in 20% of patients. Formally a classic sign, overt bone disease (osteitis fibrosa cystica, brown tumors, or pathological fractures) is uncommon. Gastrointestinal manifestations (nausea, vomiting, or constipation) are common but nonspecific; pancreatitis and peptic ulcer disease are now rare. In contrast, decreased bone mineral density (BMD), measured by dual-energy X-ray absorptiometry, is a more common mode of presentation. Because primary hyperparathyroidism may contribute to osteoporosis and increased fracture risk, a BMD measurement ("T-score") of  $-2.5$  or less at any site is now a criterion for parathyroid surgery (12). Even mild elevations of calcium can be accompanied with weakness and a feeling of lassitude. Major mood disturbances and psychotic behavior are associated with more severe hypercalcemia. Hypercalcemia and hypercalciuria lead to increased urinary frequency, and progressive nocturia is a common symptom. In more severe cases, dehydration and secondary electrolyte disturbances may also be present.

Hypercalcemia and elevated serum PTH levels are the biochemical hallmark of primary hyperparathyroidism. It is recommended that more than one measurement of these parameters be made, as values may be within the normal range early in the disease. Changes in serum proteins affect the ionized (biologically active) calcium fraction and total calcium values should be corrected for the albumin concentration or direct measurement of the ionized calcium made. Serum PTH should be assayed by a two-site immunoradiometric assay, which detects the intact molecule, preferably one of the new generations of assays detecting "bio-intact" (PTH 1-84) hormone. Elevated PTH levels occur in 90% of primary hyperparathyroid patients, but a high normal PTH level in the face of hypercalcemia indicates lack of suppression and is consistent with primary hyperparathyroidism. In interpreting a PTH result, it should be kept in mind that intact PTH levels increase in normal subjects after age 45, largely due to declining renal function. Serum phosphate

is usually at the lower end of the normal range because of the phosphaturic action of PTH. Hypophosphatemia occurs in about 25% of patients. To be diagnostically useful, serum phosphate measurements should be made in the fasting state to avoid postprandial fluctuations. Mild hyperchloremic acidosis is common because of the effects of PTH on renal chloride and bicarbonate handling. Urinary calcium is frankly elevated in one-third of patients. The effect of PTH on the renal 25-hydroxy-vitamin D-1 $\alpha$ -hydroxylase enzyme is reflected in serum 1,25(OH) $_2$ D levels at the upper end of the normal range or frankly elevated in one-third of cases.

## 85.2.2 Genetics

A single case report documented an R83X mutation in the *PTH* gene (MIM #168450, <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>), predicting a truncation after amino acid 52 of the mature PTH polypeptide (13). The patient exhibited hypercalcemia, skeletal changes of primary hyperparathyroidism but undetectable circulating intact PTH. While the patient's leukocyte DNA was heterozygous for the mutation, the wild-type *PTH* allele had been deleted in the tumor. Hence, the truncated mutant PTH that retained the biological active amino-terminal part was secreted from the gland and was active at PTH target tissues. Most, if not all, parathyroid adenomas and carcinomas are monoclonal cell expansions (14). Alterations in two genes, cyclin D1 (14) and multiple endocrine neoplasia type 1 (MEN1) (15), have been implicated in the development of some sporadic parathyroid adenomas. One study reported mutations in the target of the Wnt pathway,  $\beta$ -catenin, encoded by the *CTNNB1* gene (MIM #116806), in 3 of 20 parathyroid adenomas (16). However, this has not been replicated in other studies and  $\beta$ -catenin's involvement in the initiation or progression of parathyroid adenomatosis remains to be established (17). Cytogenetic loss of 13q14, containing the *RB* gene and other potential tumor suppressor genes, has also been implicated in parathyroid carcinoma (18,19). Abnormalities involving several other chromosomal regions have been observed in parathyroid adenomas (11), but the genes remain to be identified.

Ten percent of primary hyperparathyroidism cases are hereditary, occurring as an isolated form or associated with other abnormalities. Genetic linkage studies have confirmed the hereditary nature of these syndromes and in some cases identified specific mutations responsible for parathyroid hyperfunction (20–22). The entities discussed here are familial hypocalciuric hypercalcemia (FHH)/neonatal severe hyperparathyroidism (NSHPT), MEN1 and MEN2, the hyperparathyroidism-jaw tumor syndrome (HPT-JT), and familial isolated hyperparathyroidism (FIHP) (Table 85-1). The most common of these syndromes is MEN1, but the population frequencies are not well defined.

TABLE 85-1 Familial Hypercalcemic Syndromes <sup>a</sup>						
	FHH	NSHPT	MEN1	MEN2A	HPT-JT	FHPT
OMIM #	145980	239200	131100	171400	145000	145001
Mode of inheritance	AD	AR	AD	AD	AD	AD
Genetic locus	3q13.3-21	3q13.3-21	11q13	10q11.2	1q21-31	2p13.3-14
Mutated gene	CASR	CASR	MEN1	RET	HRPT2	Not known
Gene product	CASR	CASR	MENIN	RET	Parafibromin/CDC73	—
Associated conditions	Chondrocalcinosis Pancreatitis	—	Pancreatic islet and pituitary tumors; other neuroendocrine tumors (see Table 85-3)	MTC and pheochromocytoma (see Table 85-4)	Jaw fibromas; renal and uterine tumors	—

<sup>a</sup>FHH, familial hypocalciuric hypercalcemia; NSHPT, neonatal severe primary hyperparathyroidism; MEN, multiple endocrine neoplasia; HPT-JT, hyperparathyroidism-jaw tumor; FHPT, familial hyperparathyroidism; AD, autosomal dominant; AR, autosomal recessive.

**85.2.2.1 Genetic Counseling.** Individuals with idiopathic primary hyperparathyroidism should be carefully investigated for a hereditary syndrome if there is any suggestion of a positive family history. In sporadic cases, individuals should be advised that there is a low but non-negligible recurrence risk, since there is a small but significant genetic component to this multifactorial disease and it may be impossible on occasion to exclude a hereditary syndrome.

Surveys of recurrent FIHP have identified mutations of *CASR*, *MEN1*, and *HRPT2* genes (23–26). In these surveys, there are many families without evidence of any mutation and the possibility of unknown causative genes has not been excluded. In some families, FIHP is linked to a 1.7Mb region on chromosome 2p13.3-14 but the causative gene has yet to be identified (27). In any new family, first-degree relatives should be screened by measuring serum calcium and PTH, and affecteds should be counseled and considered for molecular testing.

**TABLE 85-2 Familial Hypocalciuric Hypercalcemia, Neonatal Severe Hyperparathyroidism, and Autoimmune Hypocalciuric Hypercalcemia Clinical Phenotypes**

Familial (benign) hypocalciuric hypercalcemia
Mild hypercalcemia and hypermagnesemia
PTH inappropriately normal
Parathyroidectomy ineffective in normalizing hypercalcemia
Renal calcium/creatinine clearance ratio <0.01
Renal concentrating ability normal
Neonatal severe hyperparathyroidism
Marked symptomatic hypercalcemia
Bony changes of hyperparathyroidism
Neurodevelopmental deficits if untreated
Parathyroidectomy recommended
Less severe forms now documented
Autoimmune hypocalciuric hypercalcemia
Phenocopy of FHH
Inactivating CASR autoantibodies

## 85.3 FAMILIAL HYPOCALCIURIC HYPERCALCEMIA

Almost 50 years ago a syndrome was described that was called familial benign hypercalcemia, which emphasizes the asymptomatic nature of the lifelong hypercalcemia. The syndrome was named as familial hypocalciuric hypercalcemia (FHH; MIM #145980) because of the abnormal renal calcium handling in affected family members (2).

The hypercalcemia of FHH is mild to moderate (Table 85-2). It is usually no more than 10% above the upper limit of normal, although occasional families may exhibit higher calcium concentrations. Fatigue, weakness, mental disturbance, and polydipsia/polyuria, common in primary hyperparathyroidism, are noted occasionally and are of lesser severity. In some FHH kindreds, affected individuals may have pancreatitis or gall stones, and an increased incidence of diabetes mellitus and cardiovascular disease has been suggested. Nephrolithiasis and peptic ulcer are not part of the syndrome.

In patients without increased parathyroid activity, skeletal radiographs and BMD are normal and susceptibility to fracture is not increased (28). Bone turnover is also normal although it may be mildly increased in a few patients. There is an increased incidence of chondrocalcinosis but this is not debilitating. Circulating levels of vitamin D metabolites and intestinal absorption of calcium are typically normal.

The degree of hypercalcemia in the majority of FHH patients is similar to that of mild primary hyperparathyroidism. In contrast to typical primary hyperparathyroidism, however, FHH is associated with increased serum magnesium. FHH patients demonstrate serum concentrations of PTH that are inappropriately normal, given the degree of hypercalcemia. While the majority of primary hyperparathyroid patients have elevated intact PTH, diagnostic difficulties can arise in differentiating FHH from the 10% of primary hyperparathyroid patients with PTH levels at the upper limit of normal.

Comparative review of diagnostic indices suggests that no single serum measurement may be adequate for differentiating primary hyperparathyroidism from FHH, although most cases will show a discordance between calcium and PTH concentrations (29). Helpful features in the differential diagnosis may be family history, serum magnesium, and assessment of renal calcium handling.

An important characteristic of FHH is the unusually high renal tubular reabsorption of calcium and magnesium in the face of hypercalcemia. In the majority of FHH patients, the renal calcium/creatinine clearance ratio is less than 0.01, while it is higher in patients with primary hyperparathyroidism and other hypercalcemic disorders (Figure 85-1). It should be noted that this lower limit for the calcium/creatinine clearance ratio is based on analyte concentrations measured in international (molar) units. It is also important to note that there are FHH families in which some affected members have increased calcium excretion or hypercalciuria (30).

In contrast to primary hyperparathyroidism, urinary concentrating ability in FHH is normal (2). The renal CASR may mediate the polyuria and diminished urinary concentrating ability typically seen with hypercalcemia in primary hyperparathyroidism. The CASR in the apical membrane of inner medullary collecting duct cells modulates vasopressin-controlled water permeability in that portion of the kidney.

Before identification of FHH as a distinct entity, affected patients thought to have primary hyperparathyroidism would undergo parathyroidectomy. In most instances, these patients remained hypercalcemic, and the present consensus is that surgery is to be avoided if the patient is asymptomatic. Moreover, the enhanced renal

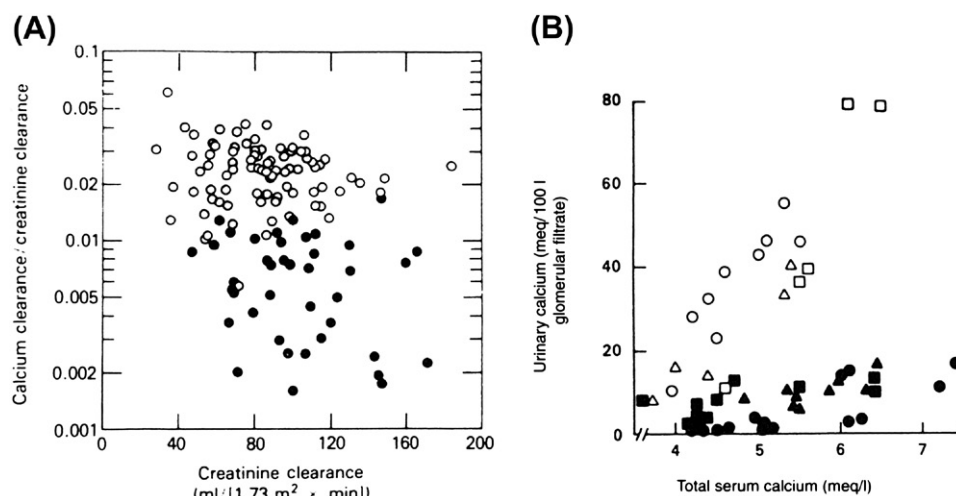
tubular reabsorption of calcium persists following induction of hypoparathyroidism by total parathyroidectomy, illustrating the fact that the abnormal renal handling of calcium in FHH is independent of PTH (see Figure 85-1).

## 85.4 NEONATAL SEVERE HYPERPARATHYROIDISM

Primary hyperparathyroidism occurs very rarely in childhood. Thirty years ago it was noted that three patients from two kindreds had severe (primary) hyperparathyroidism in the neonatal period. These and other identified cases of NSHPT (MIM #239200) involved multiglandular parathyroid hyperplasia rather than a single adenoma. Affected children under the age of 6 months develop severe, symptomatic hypercalcemia with the florid rickets and other bony changes of hyperparathyroidism. Delay in effective treatment can lead to a devastating neurodevelopmental disorder, if it is not fatal. Some forms of neonatal hyperparathyroidism, particularly *de novo* mutations of the *CASR* gene or secondary hyperparathyroidism, present with milder, less symptomatic disease that can be transient, so the decision to pursue a surgical solution requires frequent monitoring of serum calcium and clinical course.

### 85.4.1 Genetics

FHH is inherited in an autosomal dominant manner with almost 100% penetrance, but variable expressivity. The population prevalence of FHH is ill-defined, but it accounts for only a few percent of cases of asymptomatic hypercalcemia. This may reflect the infrequency of



**FIGURE 85-1** Altered renal handling in FHH patients. (A) Urinary calcium to creatinine clearance ratio in FHH patients (●) relative to values for primary hyperparathyroid patients (○). Eighty percent of FHH patients have a clearance ratio of less than 0.01, while primary hyperparathyroid patients rarely have such a low value. (From Marx, S. J.; Attie, M. F.; Levine, M. A., et al. *The Hypocalciuric or Benign Variant of Familial Hypercalcemia: Clinical and Biochemical Features of Fifteen Families*. Medicine (Baltimore). 1981, 60, 397–412, with permission.) (B) The abnormal renal handling of calcium is independent of PTH. This is shown by the difference in relationship between urinary calcium excretion in surgically aparathyroid FHH patients (●, ▲, ■) and hypoparathyroid patients (○, △, □). (From Attie, M. F.; Gill, J. Jr.; Stock, J. L., et al. *Urinary Calcium Excretion in Familial Hypocalciuric Hypercalcemia*. J. Clin. Invest. 1983, 72, 667–676.)



its recognition rather than the rarity of the disorder itself. The FHH locus was first mapped to 3q21-24 by linkage analysis. The trait in most FHH families map to the long arm of chromosome 3 (FHH1, MIM #145980), but in one was mapped to 19p13 (FHH2, MIM #145981). In a family from Oklahoma with atypical features (osteomalacia and increasing circulating PTH with age) and one other family, the condition mapped to 19q13.3 (FHH3, MIM #600740)(31). Thus, FHH is genetically heterogeneous.

In families with FHH and NSHPT and evidence of consanguinity, haplotype mapping of chromosome 3q markers closely linked to the FHH locus was consistent with NSHPT being the homozygous expression of the same disorder. The *CASR* locus was shown to reside on human chromosome 3q and the gene was further mapped to 3q13.3-21 by fluorescence in situ hybridization (FISH) (3). Mutation of the *CASR* gene is the most frequent cause of FHH and NSHPT.

### 85.5 *CASR* MUTATIONS IN FAMILIAL HYPOCALCAEMIC HYPERCALCAEMIA AND NEONATAL SEVERE HYPERPARATHYROIDISM

Almost 20 years ago, three different missense mutations (R185Q, E297K, R795W) in the 1078 amino acid receptor protein encoded by the *CASR* gene were identified in three unrelated FHH families. Since then, more than 100 additional inactivating mutations have been identified (Figure 85-2). The majority are missense with others being nonsense, insertion, and deletion/insertion. Two different missense mutants within the hydrophobic core of the NH<sub>2</sub>-terminal signal peptide cause marked impairment in cotranslational processing with little of the mutant *CASR* polypeptide entering the endoplasmic reticulum (32). In one case, an insertion of an Alu sequence in the *CASR* gene was shown and in another, a splice-site mutation was characterized (33). Several missense mutations are recurrent, and independent inactivating mutations to two different amino acid substitutions have been identified at some positions. The mutations are not evenly distributed but are clustered in two regions: the NH<sub>2</sub>-terminal 300 amino acids of the extracellular domain (ECD), and an approximately 340 amino acid stretch (residues 549–886) in the transmembrane and intracellular domains (see Figure 85-2). The *CASR* is constitutively dimerized in the endoplasmic reticulum and likely functions as a dimer at the plasma membrane (34). Heterodimerization of mutant and wild-type receptors may occur and some mutant receptors act in a dominant-negative way on the wild-type receptors.

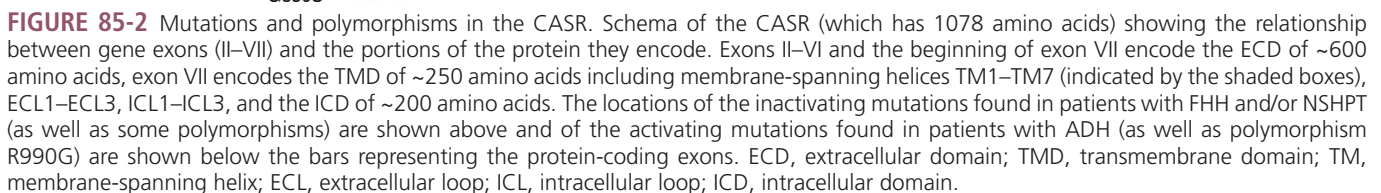
Functional studies have been reported for some mutations and show that disruption of function may occur in a number of ways (30). Some mutated receptors fail to reach the cell surface. Others are targeted to the plasma membrane but show reductions in apparent affinity for

[Ca<sup>2+</sup>]<sub>o</sub>. And yet other forms of the receptor with mutations involving membrane-spanning segments show abnormal conformational changes following ligand binding. Mutations within the intracellular loops of the *CASR* may directly interfere with G-protein interaction. Finally, some receptors with de novo point mutations exert dominant-negative effects associated with milder (less severe) neonatal hyperparathyroidism. A more detailed review of *CASR* structure and function is given by Hendy and colleagues (3) and Hu and Spiegel (35), or can be found online in the *CASR* Locus-Specific Mutation Database (*CASRdb*; [www.casrdb.mcgill.ca](http://www.casrdb.mcgill.ca) (36)).

Parental transmission effects may occur in FHH as a result of maternal–fetal interdependence of calcium metabolism. The fetal calcium concentration is normally elevated above the maternal concentration, driven by PTHrP-stimulated placental calcium transport. The *CASR* in the placenta plays an important role in regulating this. Disruption of *CASR*, as seen in heterozygous or homozygous knockout mice, results in a further increase of fetal calcium concentrations.

In humans with FHH, neonatal hypercalcemia may be more severe than would be otherwise expected, much as if a form of secondary hyperparathyroidism existed in utero. This may be more prominent if the trait is inherited from the father or arises de novo, since the normal fetal calcium concentration set by the mother is sensed as low by the FHH fetal parathyroid gland (37,38). This may also occur in the heterozygous offspring of surgically hypoparathyroid NSHPT mothers. In most cases, the parathyroid gland overactivity is self-limited and the hypercalcemic hyperparathyroid state subsides after the first few months of life. Neonates with de novo *CASR* mutations that appear similar by in vitro functional analysis may, nonetheless, present with markedly different degrees of severity (39,40). Thus, factors such as vitamin D status (37) or general health of the mother may also play a role by modulating *CASR* expression from the normal allele. It is known that the active vitamin D metabolite, 1,25(OH)<sub>2</sub>D, and cytokines are important regulators of *CASR* gene expression (41–43).

Generally, late-onset primary hyperparathyroidism is not a part of the FHH syndrome and histologically the parathyroid glands removed from FHH patients are normal or mildly hyperplastic. This contrasts with the marked hypercellularity of the glands removed from NSHPT patients and *Casr* knockout mice. However, some FHH kindreds do include family members with hyperparathyroidism and it may be that certain *CASR* mutations, even in the heterozygous state, create a propensity to parathyroid cell proliferation (44). The apparent link between parathyroid calcium sensing and parathyroid cell proliferative pathways suggests that somatic alterations in the *CASR* gene could be tumorigenic. Although somatic *CASR* mutations are not a significant factor in parathyroid tumorigenesis, more than half of the parathyroid glands of patients with



primary and severe uremic secondary hyperparathyroidism show reduced CASR expression (11). Thus, mutations in growth-regulating genes may secondarily alter the calcium set-point by decreasing expression of the CASR. Evidence for this comes from a mouse model in which a cyclin D1 (*CCND1*, MIM #168461) transgene is under the control of the *PTH* gene regulatory region (45). Parathyroid gland CASR expression is reduced; the calcium set-point is shifted to the right; parathyroid enlargement occurs; and serum calcium and PTH levels are increased. In addition, the regulator of G-protein signaling 5 (*RGS5*) that inhibits signaling by the CASR is overexpressed in neoplastic parathyroid tissues (46). Therefore, reduced expression and/or activity may contribute to altered CASR signaling in parathyroid tumors.

Vitamin D metabolites have marked antiproliferative effects on the parathyroid gland. Patients with inherited disorders in which there is homozygous inactivation of the vitamin D receptor (*VDR*) or its ligand (and the corresponding mouse models of these disorders) manifest marked parathyroid hyperplasia (47). However, like the *CASR* gene, somatic mutation of the *VDR* gene, or the 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase gene (*CYP27B1*) that locally produces the ligand, 1,25(OH) $_2$ D, does not contribute to parathyroid tumorigenesis, but parathyroid *VDR* expression is reduced in both primary and secondary hyperparathyroid patients. Vitamin D response elements that mediate upregulation of the *CASR* are present in both promoters of the gene (41). Thus, the reduced *CASR* expression may in part be secondary to decreased *VDR* expression. Additional evidence for the involvement of both the *VDR* and the *CASR* in controlling parathyroid function and/or growth comes from studies showing association of *VDR* and *CASR* gene polymorphisms (48) with primary and/or secondary uremic hyperparathyroidism.

*CASR* mutations have been found in two-thirds of FHH kindreds. In the remaining one-third, the molecular etiology is unknown. The disease may be due to mutations in portions of the *CASR* gene not yet examined. Secondly, a gene other than the *CASR* could be involved. Finally, an FHH phenocopy exists in those patients with anti-*CASR* autoantibodies associated with autoimmune disorders such as sprue or autoimmune thyroid disease (49,50). The anti-*CASR* antibodies interfere with elevated [Ca $^{2+}$ ]-mediated suppression of PTH release and perturb [Ca $^{2+}$ ]-sensing in the kidney, thereby closely mimicking FHH.

### 85.5.1 Genetic Counseling

FHH is relatively benign, but prospective parents may wish to determine the status of their spouse because NSHPT is not benign. Screening for a *CASR* mutation will confirm inheritance and allow unambiguous testing of family members with an equivocal phenotype, if a causative mutation is identified.

## 85.6 MUCOLIPIDOSIS AND NEONATAL HYPERPARATHYROIDISM

Mucopolipidosis II (ML II), or I-cell disease (MIM #252500) is a rare autosomal recessive lysosomal enzyme targeting disorder (see Chapter 103). ML II can present in the neonatal period with radiological and biochemical signs of hyperparathyroidism. It was suggested that the enzyme targeting defect of ML II interferes with transplacental calcium transport leading to a calcium-starved fetus with resultant secondary hyperparathyroidism to maintain fetal extracellular calcium concentrations (51,52).

## 85.7 MULTIPLE ENDOCRINE NEOPLASIA

The MEN syndromes are dominant conditions characterized by multiple tumors involving different endocrine glands. Familial recurrence of tumors in the parathyroid glands, the pancreatic islets, and the anterior pituitary is typical of MEN1 (MIM #131100), also known as Wermer's syndrome. In MEN2 (MIM #171400), also known as Sipple's syndrome, medullary thyroid carcinoma (MTC) occurs with pheochromocytoma and hyperparathyroidism. Although MEN1 and MEN2 syndromes are usually distinct and separate, occasionally patients develop tumors associated with both MEN1 and MEN2. There are rare examples of such cases in which mutations in both the *MEN1* and the *RET* genes have been identified (53,54). All forms of MEN are inherited in an autosomal dominant fashion, but sporadic occurrences are recorded. In some cases, the distinction may be difficult to make because of variability of expression or a negative family history.

## 85.8 MULTIPLE ENDOCRINE NEOPLASIA TYPE 1

### 85.8.1 Clinical Features

Primary hyperparathyroidism occurs in 95% of MEN1 cases and is the first endocrinopathy to be detected in 90% of affected patients (Table 85-3). The hypercalcemia is generally mild. The mean age of onset is 20 years in the familial disorder vs. 55 years in sporadic primary hyperparathyroidism, and there is no sex bias (22). MEN1 patients should be followed carefully for the onset of symptoms and complications, at which time total parathyroidectomy should be considered. The life-long hypocalcemia is treated effectively with oral calcitriol [1,25(OH) $_2$ D $_3$ ] (15).

Pancreatic islet cell tumors develop frequently. The most common are gastrinomas, giving rise to the marked gastric acid production and recurrent peptic ulceration of the Zollinger–Ellison syndrome. This is the major cause of morbidity and mortality in MEN1 patients. Insulinomas and asymptomatic pancreatic polypeptidomas are also frequent, while glucagonomas and VIPomas are less

**TABLE 85-3 Characteristic Tumors of the MEN1 Syndrome**

Parathyroids
Pancreatic islets
Gastrinoma
Insulinoma
Glucagonoma
VIPoma
Ppoma
Pituitary (anterior)
Prolactinoma
Somatotrophinoma
Corticotrophinoma
Nonfunctioning
Associated tumors
Adrenal cortical
Carcinoid
Lipoma
Angiofibromas
Collagenomas

common. Anterior pituitary tumors occur in one-third of MEN1 patients and, of these, most are prolactinomas. However, somatotrophinomas, corticotrophinomas, and nonfunctioning pituitary tumors are also common. Other associated tumors include carcinoid, adrenal cortical carcinoma, thyroid follicular carcinoma, facial angiofibromas, collagenomas, and lipomas.

### 85.8.2 Genetics

More than two decades ago, the *MEN1* locus was mapped to chromosome 11q13 by linkage analysis in families and loss of heterozygosity (LOH) studies in MEN1-associated tumors. These studies indicated that MEN1 encodes a tumor suppressor, consistent with the Knudson two-hit model of tumorigenesis.

The *MEN1* gene was identified by positional cloning, and now more than 500 independent germline and somatic mutations have been found (55,56). Somatic mutations occur to a variable extent in parathyroid adenoma, gastrinoma, insulinoma, lung carcinoid, and anterior pituitary tumors. Many of the mutations lead to a truncated inactivated form of menin, the *MEN1* gene product (Figure 85-3). The lack of menin, caused by the loss of both alleles, leads to tumor development. Ten percent of the *MEN1* mutations occur de novo (55,56), and 25% of MEN1 patients do not have mutations in the coding region of the *MEN1* gene (57). Whether they have unidentified mutations in gene regulatory or untranslated regions remains to be seen. There is no apparent correlation between genotype and phenotype, and there is a wide diversity of mutations seen throughout the 1830bp coding region.

Human menin is a 610-amino acid protein without homology to known proteins. Two novel nuclear localization signals reside in the COOH-terminal portion and

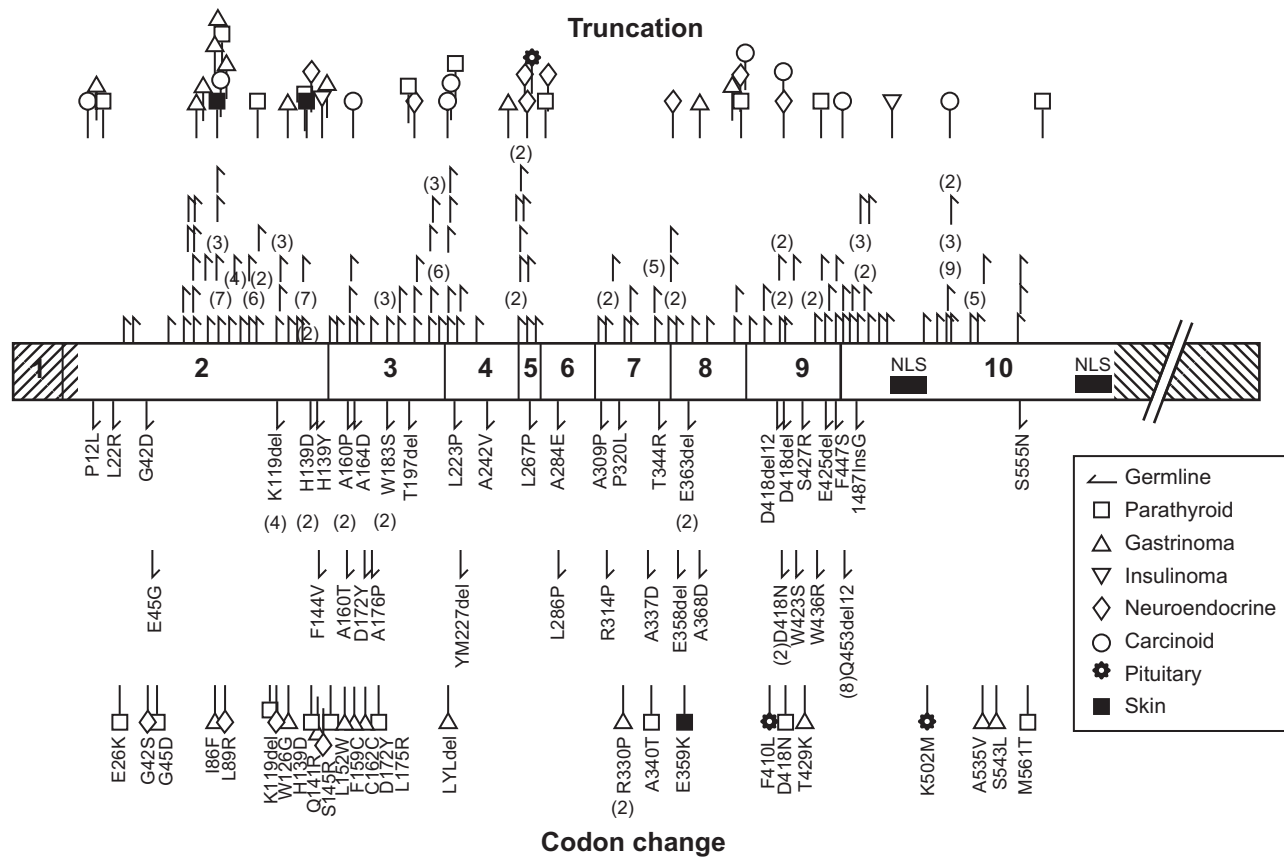
menin is located predominantly in the nucleus. How loss of menin leads to tumorigenesis only in certain tissues, given that it is ubiquitously expressed, is unclear (58). Some MEN1 missense mutants of menin are selectively targeted to and rapidly degraded by the ubiquitin-proteasome pathway and the chaperone heat shock protein 70 (Hsp70) and the ubiquitin ligase, COOH terminus of Hsp70 interacting protein (CHIP) are important for this (59,60).

Menin is a scaffold protein and is a component of complexes important for chromatin remodeling, for example, the mixed lineage leukemia (MLL) complex. MLL has histone methyltransferase activity that controls *Hox* gene expression important for early development, and cyclin-dependent kinase inhibitors such as p18(Ink4c) and p27(Kip1) important for cell cycle control (61–63). It may be involved in telomere biology (64,65) and DNA replication and repair (66,67). Menin interacts with several transcription factors including Smads, JunD, and NF- $\kappa$ B and modulates their activities (58). It is a Smad3-interacting protein and inactivation of menin blocks transforming growth factor- $\beta$  (TGF- $\beta$ ) and activin signaling antagonizing their growth inhibitory properties in anterior pituitary cells (68,69). In cultured parathyroid cells, menin inactivation achieved by menin antisense oligonucleotides leads to loss of TGF- $\beta$  inhibition of cell proliferation and PTH secretion (70). Moreover, TGF- $\beta$  did not affect (decrease) the proliferation and PTH production of parathyroid cells from MEN1 patients (70,71). Loss of various TGF- $\beta$  signaling pathway components by genetic or epigenetic means is common in cancer and is likely to be important in MEN1 tumorigenesis (72).

Menin serves as a critical oncogenic cofactor of MLL fusion proteins in acute leukemias (73,74). Recently, the first crystal structure of a menin homolog from the sea anemone, *Nematostella vectensis*, has been reported (75). The protein core of menin comprises three tetratricopeptide motifs that are flanked by two  $\alpha$ -helical bundles and covered by a  $\beta$ -sheet motif. There is a large central cavity that constitutes the binding site for MLL.

Mice, heterozygous for genetic ablation of the *Men1* gene, develop and grow normally and only later in life express endocrine tumors similar to human MEN1 patients (76,77). Hence, humans and mice are haplo-sufficient for menin. In mice, homozygous deletion of *Men1* is an embryonic lethal. The affected fetuses die at midgestation with defects in multiple organs. They also show craniofacial hypoplasia, suggesting that menin might be implicated in bone development (78). Menin is required for the commitment of pluripotential stem cells into the osteoblast lineage (79,80). In this process, menin interacts physically and functionally with bone morphogenetic protein (BMP)-2 regulated Smads, such as Smad1 and Smad5, and the key osteoblast regulator, Runx2 (81). These interactions are lost as the committed osteoblasts differentiate further, at which time





**FIGURE 85-3** Distribution of germline and somatic mutations of the *MEN1* gene. Mutations shown above the exons are mainly nonsense or insertion/deletion causing a frameshift and introduction of a premature stop codon, and some cause errors in the splicing of the primary transcript. Mutations shown below the exons are missense or deletions, which do not change the reading frame. Hatched zones are untranslated regions of the mRNA, and NLS indicates nuclear localization sequence. (From Marx, S. J.; Agarwal, S. K.; Kester, M. B. et al. *Multiple Endocrine Neoplasia Type 1: Clinical and Genetic Features of the Hereditary Endocrine Neoplasias*. Rec. Prog. Horm. Res. 1999, 54, 397–439.)

menin interacts with Smad3, mediating the negative regulation of Runx2 by TGF- $\beta$  (81). Menin also suppresses osteoblast maturation, in part, by inhibiting the differentiation-promoting actions of JunD (82). Tissue-specific inactivation of menin in the mouse in neural crest cells (that contribute to several cell types including the mesenchymal stem cells that become osteoblasts) leads to perinatal death and cranial bone defects with decreased p27(Kip1) expression (83). Adult mice lacking the *Men1* gene only in osteoblasts demonstrate reduced bone mass (84).

## 85.9 MULTIPLE ENDOCRINE NEOPLASIA TYPE 2

There are three distinct variants of this autosomal dominant disorder (Table 85-4). The most common is MEN2A (MIM #171400), in which MTC, which is seen in 95% of cases, occurs with pheochromocytoma (50%) and parathyroid tumors (20%). In MEN2B (MIM #162300), representing 5% of all MEN2 cases, hyperparathyroidism is rare, and MTC and pheochromocytoma occur together with developmental abnormalities such as Marfanoid habitus, mucosal neuromas,

**TABLE 85-4** Clinical Features of the MEN2 Variants

Type	Tumor	Associated Abnormalities
MEN2A (#171400)	Medullary thyroid carcinoma Pheochromocytoma Parathyroid	
MTC-only (#155240)	Medullary thyroid carcinoma	
MEN2B (#162300)	Medullary thyroid carcinoma Pheochromocytoma	Mucosal neuromas Marfanoid habitus Medullated corneal nerve fibers Megacolon

medullated corneal fibers, and autonomic ganglion dysfunction leading to multiple diverticula and megacolon. In familial MTC (MIM #155240), the medullary thyroid tumor is the sole disease phenotype. Hirschsprung disease and cutaneous lichen amyloidosis have also been described in MEN2.

### 85.9.1 Clinical Features

The clinical diagnosis of MTC is established by identifying hypercalcitoninemia ( $>90$  pg/ml), either in the basal state or following stimulation by intravenous pentagastrin (0.5 mg/kg) infusion. In the early stages, medullary thyroid tumors metastasize to cervical lymph nodes. Later, metastases may be found in mediastinal lymph nodes, lung, liver, trachea, adrenal gland, esophagus, and bone. Even when metastatic, however, MTC usually follows a relatively indolent course. In some, unfortunately, MTC pursues an aggressive course with widespread metastases and death. Total thyroidectomy with complete lymph node resection is the recommended treatment for MTC, followed by thyroxine replacement therapy (85).

Pheochromocytomas secreting norepinephrine and epinephrine occur in over half of all patients and constitute a major cause of morbidity and mortality. In MEN2, the incidence of bilateral involvement is 70%, which is much higher than the 10% bilaterality in non-MEN2 patients. Bilateral adrenalectomy may be the appropriate treatment for pheochromocytoma in MEN2A, even if radiological evidence suggests a unilateral tumor.

Primary hyperparathyroidism with elevated serum calcium and PTH occurs in 20% of MEN2A cases. This is due to parathyroid hyperplasia or multiple parathyroid adenomas. The incomplete suppression of PTH in response to a calcium infusion test and the observation of parathyroid hyperplasia at the time of thyroidectomy in MEN2A patients without clearcut hypercalcemia both suggest that parathyroid abnormalities are probably present in most cases.

### 85.9.2 Genetics

The *MEN2* gene maps to chromosome 10cen-10q11.2. This region contains the REarranged during Transfection (RET) proto-oncogene encoding a tyrosine kinase receptor (TKI) with cadherin-like, cysteine-rich ECDs and a tyrosine kinase intracellular domain (86). RET point mutations have been identified in all variants of MEN2 (87) ([www.arup.utah.edu/database/MEN2/MEN2\\_welcome.php](http://www.arup.utah.edu/database/MEN2/MEN2_welcome.php)). In MEN2A and FMTC, missense mutations predominantly occur in one of five cysteine codons (609, 611, 618, and 620 in exon 10 and 634 in exon 11). In 85% of MEN2A kindreds, this involves a RET codon 634 mutation (e.g., C634R), and in more than 50% of FMTC cases, a codon 618 mutation is found. Mutations in codons 768 (exon 13) and 804 (exon 14 within the intracellular tyrosine kinase domain) and in other parts of the protein occur in some FMTC kindreds (87). More than 95% of MEN2B patients have an M918T mutation in exon 16, at a site where the gene encodes the substrate recognition pocket of the tyrosine kinase catalytic core of the RET product (86). The receptor is a target for small-molecule inhibitors (88). Clinical trials of inhibitors of TKIs have shown positive responses in 30–50% of metastatic MTC patients (89).

The *RET* gene is a candidate for involvement in non-familial hyperparathyroidism. Although MEN2-type RET mutations—predominantly the M918T mutation of MEN2B—have been implicated in the pathogenesis of some sporadic MTCs and pheochromocytomas, no mutations have been found in sporadic parathyroid tumors. Mutations of the RET receptor ligand, glial cell-derived neurotrophic factor, do not appear to play a role in the genesis of MEN2 neoplasms, or in parathyroid adenomas.

### 85.9.3 Genetic Counseling

Testing for MEN2-type mutations is recommended in the diagnosis and treatment of patients with MEN2 and their families (85,90). Once a mutation has been identified, the 50% of related at-risk family members who do not have the mutation can be spared further biochemical screening. For carriers, total thyroidectomy may be recommended and constitutes a preventive cure (85). The earliest age of metastasis in MEN2A is 5 years; in MEN2B, it has occurred by 2 years of age, and therefore thyroidectomy should be pursued earlier (90). In at-risk patients, screening for excess catecholamine production should be performed annually.

## 85.10 MULTIPLE ENDOCRINE NEOPLASIA TYPE 4

Ten percent of MEN1 patients do not have detectable mutations of the *MEN1* gene and thus may have mutations in other genes. Attention was directed toward the *CDNK1B* gene that encodes the 196 amino acid CDKI, p27(Kip1), because of the finding that a naturally occurring rat model denoted as MENX that expressed a recessive MEN-like trait had a homozygous 8-bp frameshifting insertion at codon 177 of p27 with a missense peptide that terminated at codon 218 (91). The p27 protein was absent in rat tumor cells. In one family with MEN1 affected members were heterozygous for a germline *CDNK1B* W76X mutation (91). Other cases of mutations of p27 as well as other CDKIs, p15, p18, and p21, have been identified (92–95). It is to be emphasized, however, that such mutations are rare (~2%) in *MEN1* mutation-negative *MEN1* cases (96–100). MEN due to *CDKN1B* mutation has been designated as MEN4 (MIM #610755) to distinguish it from MEN1, MEN2A, or MEN2B (previously known as MEN3). Although parathyroid involvement is present in all MEN4 cases described to date, presentation of other MEN1-type tumors varies widely and the clinical phenotype is not clear-cut (101–103).

## 85.11 HYPERPARATHYROIDISM-JAW TUMOR SYNDROME

The HPT-JT syndrome is a multisystem disorder characterized by early onset of recurrent parathyroid tumors,

fibrous ossifying tumors of the mandible and maxilla, and tumors of kidney, uterus, and other organs (Table 85-5). Inherited in an autosomal dominant fashion, the syndrome shows a considerable range of expression (104). In a large group of families, this condition was linked to 1q21-31(105) and mutation analysis led to the identification of the responsible gene, *HRPT2*, which encodes a novel transcription factor, parafibromin/Cdc73 (106). Subsequently, studies of familial and sporadic parathyroid carcinoma suggest that the two-hit hypothesis of Knudson applies to the *HRPT2* gene and parathyroid carcinogenesis.

### 85.11.1 Clinical Spectrum

Patients with HPT-JT may be markedly hypercalcemic in childhood and adolescence, and there is a greater risk of hypercalcemic crisis than in the other variants of familial hyperparathyroidism. The condition may be associated with crippling skeletal disease and death. The incidence of parathyroid carcinoma is much higher than in sporadic hyperparathyroidism or MEN syndromes, potentially affecting more than 15% of hyperparathyroid patients carrying a mutant allele.

In contrast to the glandular hyperplasia found in other forms of inherited hyperparathyroidism, there is usually a solitary, enlarged cystic gland. After removal of a solitary tumor, the patient becomes normocalcemic, but double adenomas have been reported and recurrence is well documented.

TABLE 85-5 Screening for Manifestations of HPT-JT		
Tumor	Test(s)	Frequency
Parathyroid glands	Serum Ca, PTH <sup>a</sup> Neck ultrasound <sup>b</sup> <sup>99m</sup> Tc Sesta MIBI scan	Annually
Jaw fibromas	Panoramic X-ray	5 years
Renal lesions <sup>c</sup>	Abdominal MRI, renal ultrasound	5 years
Uterine <sup>d</sup>	Transvaginal ultrasound, endometrial biopsy	5 years

The age at which screening should be started has not been established, but affected children have been reported.

<sup>a</sup>Serum calcium and PTH may not always be sufficiently sensitive, and neck ultrasound may be added to the screening protocol. This is particularly important for carriers who have undergone partial resection and remain at risk for recurrence.

<sup>b</sup>Ultrasound-guided fine needle aspiration biopsy and analysis of the aspirate for PTH and/or thyroglobulin may be helpful in establishing the tissue of origin for any echogenic masses identified on ultrasound.

<sup>c</sup>Renal ultrasonography may be the first choice if access to abdominal MRI is limited.

<sup>d</sup>Screening for uterine disease should be guided by menstrual and reproductive history, as well as physical examination. If the history is negative and the preliminary ultrasound is normal, then a longer interval may be appropriate. Adapted from Bradley, K. J.; Hobbs, M. R.; Buley, I.D., et al. Uterine Tumours Are a Phenotypic Manifestation of the Hyperparathyroidism-Jaw Tumour Syndrome. *J. Intern. Med.* 2005, 257, 18–26.

Fibro-osseous jaw tumors are frequent, appearing as cystic lesions on radiographs. They are restricted to maxilla and mandible and their natural history is independent of the hyperparathyroidism. The tumors may be small and asymptomatic or they may be large, destructive, and recurrent. They consist of woven trabecular bone on a stromal background but the aberrant cell type has not been identified. However, the tumors lack osteoclasts and differ markedly from the brown tumors of longstanding untreated hyperparathyroidism, which also occur in the jaw but are often found elsewhere in the skeleton along with other signs of florid hyperparathyroidism.

Associated renal lesions include Wilms' tumor hamartomas, and polycystic kidneys. Uterine tumors include adenosarcoma, but benign adenofibromas and leiomyomas are more commonly reported. Benign or not, such tumors may have a significant impact on reproductive fitness (107).

Renal and uterine anomalies tend to occur in familial clusters, and are more common in patients who have jaw fibromas. A variety of other tumors have been described, but most are also seen in the general population, including carcinoma of the pancreas, prostate, thyroid, colon, and breast. Whether there is a predisposition to common cancers in some or all families is not known.

In a number of kindreds, only hyperparathyroidism is observed, adding to the genetic heterogeneity characteristic of FIHP (*HRPT1*; MIM #145000). FIHP is not the common presentation (24,25) for patients with an *HRPT2* mutation, but may account for a significant fraction of all affected families (108,109).

### 85.11.2 Genetics

HPT-JT and its variant forms are transmitted in an autosomal dominant manner with incomplete, age-dependent penetrance. Although the variability of the phenotype is reduced within families that share a common mutation and similar genetic background, genotype–phenotype correlations have not yet emerged. The *HRPT2* locus located at 1q25-q32 encodes a single-copy gene (*CDC73*) of 17 exons that produces a mature transcript of 2.7 kb and predicts a protein originally named parafibromin of 531 amino acids (110). The COOH-terminal half of parafibromin has homology with the yeast cell division cycle protein 73 (*Cdc73*) (106). Hence dual designations of the gene, *HRPT2/CDC73* (MIM #607393) and the protein, parafibromin/Cdc73, may be found in the current literature. Parafibromin is a ubiquitously expressed and predominantly nuclear protein (111): a nuclear localization signal resides at amino acids 125–139 (112,113) and a nucleolar localization signal is at amino acids 76–92 (114,115). Parafibromin is a component of the Polymerase Associated Factor 1 complex that interacts with RNA polymerase II and is involved in transcript elongation and mRNA 3' end processing (116,117). Like menin, the product of the *MEN1* gene,

parafibromin can function as a tumor suppressor or an oncogene in a cell context-dependent manner. For both, their binding proteins determine their particular functional roles in transcription that can direct opposing responses either promitogenic or antiproliferative. For example, the protein tyrosine phosphatase, SHP2, encoded by the *PTPN11* gene, dephosphorylates parafibromin (118) converting it from a transrepressor of c-myc (119) and cyclin D1 (111) into an activator of promitogenic Wnt signaling by interacting directly with  $\beta$ -catenin (120).

Most of the mutations identified to date (>110; two-thirds, germline; one-third somatic) involve truncation or premature stops (121). Nearly half of the mutations are found in exon 1, while most of the remainder are found in exons 2 and 7. Exons 3–6, 8, and 14 are also mutation sites, but mutations in the 3' half of the cDNA sequence occur much less frequently. Mutations are only detected in about half of HPT-JT families, even when there is evidence linking the family disorder to chromosome 1q31.2. Moreover, de novo mutations have been identified and parental mosaicism is reported (109).

### 85.11.3 HRPT2 in Parathyroid Carcinoma

Somatic *HRPT2* mutations are not found in sporadic typical benign parathyroid adenomas (122). In parathyroid carcinomas, LOH at chromosome 1q, particularly in tumors from HPT-JT kindreds, has been well characterized, supporting a tumor suppressor function for parafibromin (106). Studies of sporadic parathyroid carcinomas show somatic mutations of the *HRPT2* gene in tissue, while carcinomas in HPT-JT kindreds show tissue LOH in affected carriers (108,123,124). Of particular note is the finding that patients with apparently sporadic parathyroid carcinomas harbor germline mutations (108,124). Parathyroid carcinoma is potentially resectable when contained within the gland, but difficult to control when it has metastasized, so it would be recommended that all patients with newly diagnosed parathyroid carcinoma should have a careful review of family history, and should be offered *HRPT2* mutation screening, if there are family members at risk (125). Parafibromin immunostaining (see below) may serve as a clinical screen for tumors resulting from a germline *HRPT2* mutation that will help direct molecular testing for patients and families most at risk. Guidelines have been proposed and screening of asymptomatic carriers is now recommended (see Table 85-5).

Distinction between parathyroid carcinoma and adenoma is difficult using histopathological criteria (20,126). Features of parathyroid cancers, parenchymal mitoses, trabeculated parenchyma including thick fibrous bands, and capsular and vascular invasion may also be found in atypical adenomas. Capsular and lymphovascular invasion is the most specific histopathological feature of parathyroid carcinoma. Therefore, identification of

useful immunohistochemical tumor markers has become of primary importance. To date, parafibromin, either alone or in combination with other markers, is one of the most promising. An initial report (127) found that loss of nuclear parafibromin expression identified parathyroid malignancies with near absolute sensitivity and specificity with more recent reports observing somewhat lower specificity and sensitivity while still finding it to be a useful marker (128–130). Combination with staining for other markers has been proposed to offer advantages over staining with parafibromin alone, for example, loss of *RB1* and overexpression of galectin-3 (131). The combined loss of parafibromin staining and downregulation of *CASR* expression indicates increased malignant potential in parathyroid carcinoma patients (132).

## 85.12 OTHER HEREDITARY HYPERPARATHYROIDISM

FIHP (or HRPT1; MIM #145000) has been defined as hereditary primary hyperparathyroidism without the association of other disease or tumors. A small proportion of these cases have now been shown to be variants of the other monogenic diseases—FHH, MEN1, or HPT-JT—but in other cases additional genes as yet unknown are likely to be involved. As pointed out above in some kindreds, FIHP is linked to a region on chromosome 2p but the causative gene has yet to be identified (27).

## 85.13 DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS OF HYPERCALCEMIA

With the frequent detection of hypercalcemia in biochemical screening programs, the differential diagnosis of primary hyperparathyroidism from other conditions causing hypercalcemia has become increasingly important. Causes of nonparathyroid hypercalcemia include humoral hypercalcemia of malignancy, vitamin D or A intoxication, milk-alkali syndrome, granulomatous disorders (especially sarcoidosis), immobilization of patients with a preexisting high bone turnover state, thyrotoxicosis, Paget's disease, and treatment with thiazide diuretics or lithium.

Malignancy is a common cause of hypercalcemia. Although the hypercalcemia can occur through several different mechanisms, increased osteoclastic bone resorption is often involved. PTHrP is the major causative agent in the humoral hypercalcemia of malignancy—especially in squamous cell carcinomas (e.g., lung, head and neck, esophagus), cancer of the breast, kidney, and ovary, and T-cell lymphomas—although many other circulating factors may contribute.

Study of the relatives of patients with hypercalcemia can contribute to establishing the diagnosis in 10–15% of all cases of primary hyperparathyroidism that prove to be hereditary. The finding of another relative with hypercalcemia furnishes evidence of primary



hyperparathyroidism, if FHH is not suggested by a relatively low urinary calcium/creatinine clearance ratio or definitively diagnosed by the identification of a mutation of the *CASR* gene. The finding of a hypercalcemic relative also requires investigation of the patient for manifestations of the MEN or HPT-JT syndromes. The diagnosis of FIHP is made by exclusion of FHH, MEN, and HPT-JT. With the development of improved hormone immunoassays, it is now less likely that an MEN1 family, for example, would be classified as FIHP. Genetic testing may be helpful in that identification of a mutation in the *MEN1* gene should clinch the diagnosis of an MEN1 variant, alerting the surgeon to the likelihood of parathyroid hyperplasia, and prompting the physician to search for other endocrine abnormalities.

### 85.13.1 Management

Parathyroidectomy by an experienced surgeon is the treatment of choice (133). In cases of multiglandular disease, total parathyroidectomy is the definitive treatment and the patient is maintained on lifelong calcium and vitamin D supplementation. Some centers perform either a subtotal parathyroidectomy (removal of 3½ glands), or a total parathyroidectomy with autotransplantation of parathyroid tissue into the nondominant forearm (133). However, persistent or recurrent hypercalcemia may ensue if the transplanted tissue resumes its autonomous growth, necessitating further surgery.

Criteria for surgery in hyperparathyroidism have been established by a consensus conference at the National Institutes of Health (12). Asymptomatic patients who are treated conservatively with twice yearly serum calcium and urinary calcium excretion determinations and yearly bone densitometry generally do well and the progression of the disease is slow. Medical therapy (estrogens, progestins) is, at present, usually unsatisfactory if there is symptomatic disease, and is reserved for patients unable to undergo surgery. Bisphosphonate may be a useful alternative to parathyroidectomy in asymptomatic primary hyperparathyroidism among those with low BMD (134). In another study, primary hyperparathyroid patients with serum calcium levels above 2.8 mmol/l underwent intravenous infusions of pamidronate 1 month before parathyroidectomy. There is a short and limited calcium-lowering effect of bisphosphonate treatment, so it cannot be considered as an alternative to surgery (135). In a third study, the successful use of short-term intravenous pamidronate to treat the extreme hypercalcemia of NSHPT and halt hyperparathyroid-driven skeletal demineralization in preparation for parathyroidectomy (so-called rescue therapy) has been described (136).

Calcimimetics, orally active compounds that increase the sensitivity of the parathyroid *CASR* to extracellular calcium thereby reducing PTH secretion, offer a non-surgical approach for management of hyperparathyroid

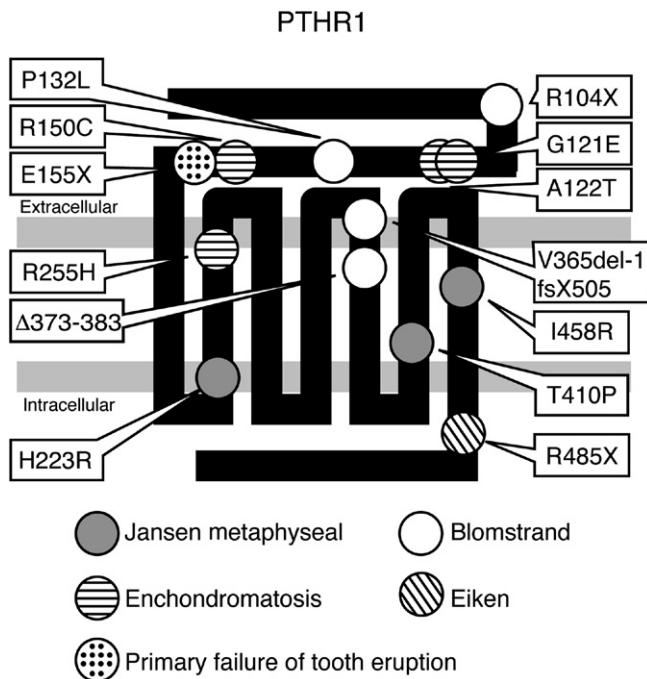
states (137). The calcimimetic, cinacalcet, is approved clinically in North America for secondary hyperparathyroid patients with chronic kidney disease on dialysis and patients with parathyroid carcinoma (and those with primary hyperparathyroidism in Europe). Cinacalcet was found to reduce hypercalcemia in two-thirds of patients with inoperable parathyroid carcinoma in an open-label, single-arm study and may be an option for treatment of such patients (138). Also several off-label uses of cinacalcet have been reported (139). In an infant with neonatal hyperparathyroidism secondary to a de novo heterozygous *CASR* mutation, treatment with cinacalcet decreased PTH secretion and serum calcium levels and mitigated the need for parathyroidectomy (38).

### 85.14 JANSEN METAPHYSEAL CHONDRODYSPLASIA (PSEUDOHYPERPARATHYROIDISM)

Direct evidence that the parathyroid hormone receptor, PTHR1, mediates the calcium homeostatic actions of PTH and the skeletal actions of PTHrP comes from the study of Jansen metaphyseal chondrodysplasia (JMC; MIM #156400). This condition is inherited in an autosomal dominant fashion, although most reported cases are new mutations. Important features include short-limbed dwarfism secondary to severe growth-plate abnormalities, asymptomatic hypercalcemia, and hypophosphatemia (140). A milder form of metaphyseal chondrodysplasia with nephrolithiasis and suppressed serum PTH, but normal height and serum calcium, may also be seen (141). There is increased bone resorption similar to that in primary hyperparathyroidism and urinary cAMP levels are elevated, but circulating PTH and PTHrP levels are low or undetectable. Although the PTHR1 is found widely in fetal and adult tissues, it is most abundant in three sites—kidney, bone, and metaphyseal growth plates. Molecular analysis shows that the altered mineral ion homeostasis and growth-plate abnormalities of JMC are the result of heterozygous gain-of-function mutations (Figure 85-4) in the *PTHR1* gene—that maps to 3p21.1-22—giving rise to constitutively active receptors (141). Diagnosis is based on the combination of radiological assessment and biochemical profile.

### 85.15 PRIMARY HYPOPARATHYROIDISM

Primary hypoparathyroidism is caused by a group of heterogeneous conditions in which hypocalcemia and hyperphosphatemia occur as a result of deficient PTH secretion (142). Hypoparathyroidism most commonly occurs as a result of surgical excision of, or damage to, the parathyroid glands. Genetic forms of hypoparathyroidism due to decreased secretion of PTH are listed in Table 85-6.



**FIGURE 85-4** Schematic representation of the human PTHr1. The locations of the H223R, T410P, and I458R activating mutations identified in patients with Jansen metaphyseal chondrodysplasia, the R104X, P132L, V365del-1fsX505, and Δ373–383 inactivating mutations found in patients with Blomstrand chondrodysplasia, the R485X Eiken syndrome mutation, the G121E, A122T, R150C, and R255H enchondromatosis mutations, and the E155X primary failure of tooth eruption (PFE) mutation are indicated. Splice-site mutations that would result in predicted mutant C351fsX485 and E182fsX203 proteins have been identified in additional PFE cases.

**TABLE 85-6** Forms of Hypoparathyroidism Having a Genetic Basis

Isolated
Autosomal dominant
PreproPTH signal peptide mutation
CASR activating mutation
GCM2 mutation (dominant negative)
Autosomal recessive
PreproPTH RNA splice-site mutation
GCM2 mutation
X-linked
Congenital multi-system syndromes
DiGeorge and Velocardiofacial (22q11)
Barakat/HDR
Kenny-Caffey
Metabolic disease
Mitochondrial neuromyopathies
Long-chain hydroxyacyl-CoA dehydrogenase deficiency
Heavy-metal storage disorders
Autoimmune disease
Autoimmune polyendocrine syndrome type 1 (APS-1 or APECED)
PTH resistance syndromes
Pseudohypoparathyroidism
Blomstrand chondrodysplasia and related PTH receptor defects
Hypomagnesemia

**TABLE 85-7** Clinical Features of Hypocalcemia

Neuromuscular Irritability
Paresthesias
Laryngospasm
Bronchospasm
Tetany
Seizures
Chvostek's sign
Trousseau's sign
Prolonged Q–T interval on ECG

### 85.15.1 Clinical Features

The signs and symptoms of hypoparathyroidism include evidence of latent or overt enhanced neuromuscular excitability due to hypocalcemia (Table 85-7). The effect may be aggravated by hyperkalemia or hypomagnesemia, but there is wide variation in the severity of the symptoms. Patients may complain of circumoral numbness, paresthesias of the distal extremities, or muscle cramping progressing to carpopedal spasm or tetany. Laryngospasm or bronchospasm and seizures may also occur. Other less specific manifestations include fatigue, irritability, and personality disturbance. Severe hypocalcemia may be associated with a prolonged Q–T interval on electrocardiography, which reverses with treatment. More extensive cardiomyopathic changes are occasionally seen, particularly in adults. These include chest pain, elevated enzymes (CPK), left ventricular impairment, and T-wave inversion, suggestive of a myocardial infarction (143,144). Patients with chronic hypocalcemia may have calcification of the basal ganglia or more widespread intracranial calcification, detected by skull radiograph or CT scan. Also seen are extrapyramidal neurological symptoms (more often with intracranial calcification), subcapsular cataracts, band keratopathy, and abnormal dentition.

Increased neuromuscular irritability may be demonstrated by eliciting a Chvostek or Trousseau sign. The Chvostek sign is prolonged reflex contraction of the facial muscle in response to a digital tap on the cheek just anterior to the ear. As with other hyperreflexias, up to 20% of normal individuals may demonstrate a slight positive reaction. The Trousseau sign is carpopedal spasm induced by inflation of a blood pressure cuff covering the upper arm to 20 mm Hg above systolic blood pressure for 3 min. A positive response reflects the heightened irritability of nerves undergoing pressure ischemia.

In hypoparathyroidism, serum calcium concentrations are decreased and serum phosphate levels are increased. Serum PTH is low or undetectable. (The important exception is PTH resistance—pseudohypoparathyroidism (PHP)—discussed further later.) Usually, serum 1,25(OH)<sub>2</sub>D is low, but alkaline phosphatase activity is normal. Despite an increase in fractional excretion of calcium, intestinal calcium absorption and bone resorption

are both suppressed. The renal filtered load of calcium is decreased, and the 24-h urinary calcium excretion is reduced; nephrogenous cAMP excretion is low and renal tubular reabsorption of phosphate is elevated. After par-enteral administration of biologically active PTH (the Ellsworth–Howard test), the plasma and urinary cAMP, and phosphate excretion increase, differentiating hypoparathyroidism from PHP. However, this test is rarely used in clinical practice now, since diagnosis is more readily established by less invasive means.

Decreased parathyroid gland reserve can be detected by an ethylenediaminetetraacetate infusion study. However, this test is usually not required and should be performed in a metabolic center with careful monitoring and observation.

### 85.15.2 Genetics

Idiopathic hypoparathyroidism is a feature common to a variety of syndromes and inherited disorders. Although most cases are sporadic, familial occurrence is known. This may occur as an autoimmune disorder either alone or with other endocrine deficiencies as in a pluriglandular autoimmune syndrome or associated with diverse developmental abnormalities such as lymphedema, nephropathy, nerve deafness, or cardiac malformation. It also occurs as an isolated finding.

## 85.16 FAMILIAL ISOLATED HYPOPARATHYROIDISM

Familial isolated hypoparathyroidism (FIH) may show autosomal dominant, autosomal recessive, or X-linked inheritance.

In a few instances of autosomal dominant disease, a mutation in the *PTH* gene has been found. In one family presenting reduced hormone production and chronic hypocalcemia, a missense mutation (C18R) in the signal sequence of the preproPTH precursor was identified and the mutant shown to be defective *in vitro* in processing of preproPTH to proPTH, although as patients had one normal gene copy the autosomal-dominant mode of inheritance was unexplained. Further studies in transfected cells showed that the mutant was trapped in the endoplasmic reticulum promoting ER stress and apoptosis (145). In another family, a different mutation in the signal sequence segregated with affected status. This mutation may prevent proper cleavage of the signal peptide during processing of the nascent protein. In one family with autosomal recessive hypoparathyroidism, a donor splice site mutation at the exon 2/intron 2 junction of the *PTH* gene was identified. The mutation leads to exon skipping and loss of exon 2 containing the initiation codon and signal sequence of preproPTH mRNA. In two multigeneration families with X-linked recessive hypoparathyroidism exhibiting neonatal onset of hypocalcemia and parathyroid agenesis, the trait was

mapped to a 906-kb region on distal Xq27 that contains three genes including *SOX3* but no intragenic mutations were found (MIM #307700) (146). A deletion/insertion [del(X)(q27.1)inv ins(X)(q27.1;p25.3)] mutation was identified that was speculated to exert a position effect on *SOX3* expression and affect embryonic development of the parathyroid glands (147).

Gain-of-function mutations in the *CASR* gene have been identified in several families previously diagnosed with autosomal dominant hypocalcemia (ADH) (MIM #601198), autosomal dominant hypoparathyroidism (MIM #241400), and hypocalcemic hypercalciuria (MIM #146200). In the parathyroid gland, the activated *CASR* suppresses PTH secretion and in the kidney, it induces hypercalciuria, which further contributes to the hypocalcemia. In many cases of ADH, a family history is clear, but *de novo* mutations are surprisingly common (148,149). Mosaicism for *de novo* mutation in an otherwise healthy parent has been described (150), and may explain some cases of apparently recessive disease. Importantly, it has serious implications for counseling parents about the risk of recurrence. More than 50 unique activating mutations (almost all missense), of which one-third are recurrent, have been identified, and appear almost equally divided between the amino-terminal third of the ECD and the transmembrane domain (TMD) (Figure 85-2). Of special interest is the cluster of ECD mutations (from A116T to C131W), which cause a leftward (activating) shift in receptor sensitivity, suggesting that this region is critical for receptor activation. This cluster overlaps the two cysteine residues—C129 and C131—involved in the interface of the mature protein dimer. Further details can be found in the locus-specific database—<http://www.casrdb.mcgill.ca/> (3).

Some patients with *CASR*-activating mutations have presented with symptoms of Bartter's syndrome (151,152). Study of these particular mutations confirms that one action of *CASR* in the cortical thick ascending limb is sodium chloride wasting, which can be physiologically tied to increasing losses of calcium and magnesium in the urine.

Parental transmission effects may occur in hypocalcemic disorders associated with activating mutations of the *CASR*, as a result of the maternal–fetal interdependence of calcium metabolism. If affected, a neonate would be at risk for hypocalcemia and seizures during the immediate postnatal period. Conversely, unaffected infants could manifest transient secondary hyperparathyroidism with associated bone demineralization. Therefore, the *CASR* mutational analysis can be useful in monitoring the neonatal course of infants born to a mother with ADH (153).

Recessively inherited FIH may occur with mutations of the glial cell missing-2 gene (*GCM2*; MIM #603716). The *GCM2* gene localizes to chromosome 6p24.2 and encodes a transcription factor. It is expressed in the PTH-secreting cells of the developing parathyroid glands and is critical for their development in terrestrial vertebrates (154–157). In turn, the

transcriptional activator, musculoaponeurotic fibrosarcoma oncogene family B (MafB), that is expressed in the bilateral parathyroid/thymus common primordia is essential for the expression of GCM2 (158). GCM2 transactivates the *CASR* gene promoter (159,160) and in combination with MafB transactivates the *PTH* gene promoter (158). A patient with neonatal hypoparathyroidism was found to be homozygous for a partial deletion of the *GCM2* gene acquired from both parents (161), and a pair of siblings with homozygous mutations has been reported (162). Additional studies have identified other inactivating *GCM2* mutations in cases with autosomal recessive FIH (159,163). On the other hand, heterozygous mutations that cause dominant-negative *GCM2* mutants have recently been identified in patients with autosomal dominant hypoparathyroidism (159,164,165). Nevertheless, it appears that the prevalence of genetic defects affecting *GCM2* function is not high in FIH, as a recent study investigating 20 unrelated cases with this disorder (10 familial and 10 sporadic) failed to identify any *GCM2* mutations segregating with the disease and/or leading to loss of function (166).

## 85.17 HYPOPARATHYROIDISM WITH MULTIPLE MALFORMATIONS

### 85.17.1 DiGeorge and Velocardiofacial Syndromes

Hypoparathyroidism due to parathyroid hypoplasia is a frequent feature of 22q11.2 microdeletions, the common cause of the DiGeorge syndrome (MIM #188400) (167). This syndrome complex arises from a failure of the third and fourth pharyngeal pouches to develop, leading to agenesis or hypoplasia of the parathyroid glands, thymus, and the anterior heart field. Patients with DiGeorge syndrome may typically present with neonatal hypocalcemic seizures due to hypoparathyroidism, severe infections due to thymic hypoplasia, and conotruncal heart defects (168). Because a microdeletion is involved, the identification of novel developmental genes in the 22q11 region has been keenly pursued. Mouse models with *Tbx1* transcription factor haploinsufficiency were used to establish the essential contribution of this factor to conotruncal development (169) and to place it in developmental context during organogenesis (170,171). It is likely, however, that full expression of DiGeorge syndrome involves loss of other contiguous genes (172). Thus, 22q11 deletions are responsible for a wider spectrum of clinical conditions that includes isolated congenital heart disease and velocardiofacial (VCF) syndrome (167). Associated craniofacial abnormalities include cleft palate, pharyngeal insufficiency, and mildly dysmorphic facies. In the VCF syndrome, anatomical anomalies of the pharynx are prominent, and hypernasal speech due to abnormal pharyngeal musculature with or without

cleft palate is typical. In most patients, some degree of intellectual deficit is present and there is a strong predisposition to psychiatric illness (schizophrenia or bipolar disorder) in adolescents and adults (173,174). Further information, both clinical and educational, can be found at web sites devoted to this condition (e.g., <http://www.vcfsef.org/> and <http://www.22q.org/>).

The 22q11.2 defect is one of the most common of microdeletions (1 in 3000 to 1 in 6000 livebirths), and it may go clinically unrecognized in its milder or incomplete forms. Most cases are the result of sporadic new mutations, but familial patterns are evident in up to 10%. Although many cases of DiGeorge syndrome occur *de novo*, autosomal dominant inheritance is not unknown. In utero influences may be important determinants of the clinical picture, since there are instances of monozygotic twins with discordant phenotypes. Phenocopies occur with diabetic embryopathy, fetal alcohol syndrome, and retinoid embryopathy. In rare instances, it has been shown that a phenotypically normal parent can transmit a microdeletion to an offspring. Such parents have been found to carry a duplication of the 22q11 on the second chromosome, and the combination of duplication and deletion alleles in a parent generates a balanced state that has been termed “gene dosage compensation” (175,176).

Although the hypoparathyroidism affects about half of all carriers, it is usually not severe, and frequently treatment following neonatal hypocalcemia can be tapered or stopped in older children. However, the hypoparathyroidism may also remain asymptomatic until adolescence or emerge at times of stress, such as corrective cardiac surgery or severe infection, suggesting that continued surveillance of parathyroid gland reserve is important (177–179).

Currently, diagnosis of 22q11.2 microdeletion requires specific cytogenetic studies—usually with locus-specific FISH testing or PCR-based techniques. These tests reliably pick up many of the larger common deletions that involve regions of low-copy number repeats. However, array-based analyses suggest that 15–30% of microdeletions that are atypical are being missed (167), and it seems likely that microarray methods will eventually become the preferred diagnostic approach. Because the clinical picture is so variable and the prevalence so high, testing for 22q11.2 microdeletion should be considered in the workup for any new hypoparathyroid case for which another cause is not found.

A small but significant minority (~10%) of patients will have associated autoimmune disease, perhaps driven in part, by the thymus-based defect in T cell function (167,180). Among the more common (non-endocrine) conditions are arthritis, celiac disease, and autoimmune hematologic disease, particularly idiopathic thrombocytopenic purpura. Autoimmune thyroid disease, with either hypo- or hyperparathyroid states, has been reported (180,181). It has been suggested that the later-onset hypoparathyroid disease may be partly autoimmune in



origin, not developmental. A survey of 59 Norwegian patients showed discordance of adult onset disease with neonatal hypoparathyroidism, but a significant correlation with parathyroid autoantibodies and the presence of autoimmune disease (180).

### 85.17.2 Barakat/HDR Syndrome

Hypoparathyroidism is a part of the Barakat or Hypoparathyroidism, nerve Deafness, and Renal dysplasia (HDR) syndrome (MIM #146255). Deletions of two non-overlapping regions of chromosome 10p contribute to a DiGeorge-like phenotype (the DiGeorge critical region II on 10p13-14) and the HDR syndrome (10p14-10pter). The latter is due to haploinsufficiency of the *GATA3* gene (MIM #131320), which encodes a dual zinc finger transcription factor (182) that is essential for normal embryonic development of the parathyroids, auditory system, and kidney. Since the original description, several additional *GATA3* loss-of-function mutations have been described in HDR patients (183–185). Heterozygous *Gata3*-deficient mice develop parathyroid abnormalities as revealed when challenged with a diet low in calcium and vitamin D that are due to dysregulation of the parathyroid-specific transcription factor *Gcm2*. *Gata3*<sup>-/-</sup> embryos at E12.5 lack *Gcm2* expression and have gross defects in the third and fourth pharyngeal pouches, including absent parathyroid-thymus primordia (186). *GATA3* transactivates the *GCM2* promoter and with *GCM2* forms part of a transcriptional cascade essential for the differentiation and survival of parathyroid progenitor cells.

### 85.17.3 Kenny–Caffey and Sanjad–Sakati Syndromes

In another congenital disorder, the Kenny–Caffey syndrome, hypoparathyroidism is found variably associated with the typical picture of growth retardation, osteosclerosis, cortical thickening of the long bones, and delayed closure of the anterior fontanel. Both dominant and recessive modes of inheritance have been observed (MIM #127000 and MIM #244460, respectively). The Sanjad–Sakati syndrome (MIM #241410) is a similar recessive condition characterized by congenital hypoparathyroidism, seizures, growth and developmental retardation, and characteristic dysmorphic features, including deep set eyes, depressed nasal bridge with beaked nose, long philtrum, thin upper lip, micrognathia, and large floppy ear lobes. Radiographs show medullary stenosis reminiscent of Kenny–Caffey syndrome. Linkage studies localized recessive Kenny–Caffey and Sanjad–Sakati syndromes to 1q42-43, and causative mutations in the tubulin chaperone E, *TBCE*, gene, were identified in this hypoparathyroidism, retardation, and dysmorphism syndrome (187). This highlighted the role of *TBCE* that binds microtubules and proteasomes and protects against misfolded protein stress (188) in parathyroid development (189).

## 85.18 HYPOPARATHYROIDISM DUE TO METABOLIC DISEASE

### 85.18.1 Mitochondrial Myoneuropathies

Hypoparathyroidism is also a variable component of the neuromyopathies caused by mitochondrial gene defects. Among these are the Kearns–Sayre syndrome (ophthalmoplegia, retinal degeneration, and cardiac-conduction defects) (MIM #530000), the Pearson marrow pancreas syndrome (lactic acidosis, neutropenia, sideroblastic anemia, and pancreatic exocrine dysfunction) (MIM #557000), and mitochondrial encephalomyopathy (MIM #540000). The molecular defects range from large deletions and duplications of the mitochondrial genomes in a number of tissues to single base-pair mutations in one of the transfer RNA genes found only in a restricted range of cell types (MIM #590050). The role of these mitochondrial mutations in the pathogenesis of hypoparathyroidism remains to be clarified, but hypomagnesemia due to renal wasting can present with symptoms similar to hypocalcemia and the two may co-exist in these disorders.

### 85.18.2 Long-Chain Hydroxyacyl-CoA Dehydrogenase Deficiency

Long-chain hydroxyacyl-CoA dehydrogenase deficiency (MIM #600890) is an inborn error of oxidative fatty acid metabolism that may be accompanied by hypoparathyroidism. Whether the parathyroid disease is directly related to the enzyme deficiency or secondary to the accompanying mitochondrial disease needs further study.

### 85.18.3 Inherited Disorders Leading to Metal Storage

Parathyroid insufficiency and symptoms of hypocalcemia are occasionally seen in inherited metabolic disorders leading to excess storage of iron (thalassemia, Diamond–Blackfan anemia, hemochromatosis) or copper (Wilson disease). In most instances, there is similar dysfunction in other endocrine glands, and the parathyroid disease is usually mild. Nonetheless, recognition of the hypoparathyroid state may help explain otherwise nonspecific symptoms and aid in overall management of these multisystem diseases.

## 85.19 AUTOIMMUNE HYPOPARATHYROIDISM: ACQUIRED AND INHERITED DISORDERS

Antibodies directed against parathyroid tissue have been detected in up to 38% of patients with isolated hypoparathyroid disease, and over 40% of patients having hypoparathyroidism combined with other endocrine

deficiencies (190). Subsequently, a survey of a parathyroid expression library led to the identification of one protein selectively associated with the autoimmune process, the NACHT leucine-rich-repeat protein 5 (NALP5). Elevated antibody titers occur in half the patients with autoimmune hypoparathyroidism, with or without other autoimmune disease, but uncommonly in other conditions without hypoparathyroidism (191).

Antibodies against the ECD of the parathyroid CASR were originally reported in more than half of patients with either type 1 autoimmune polyglandular syndrome (APS-1, also known as autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy or APECED; MIM# 240300) (192) or acquired hypoparathyroidism associated with autoimmune hypothyroidism. This finding was confirmed in a subsequent study of 51 cases of idiopathic hypoparathyroidism, but there was a 13% positive rate in controls (193). However, larger studies of APS-1 patients failed to reveal a significant preponderance of positives (194–196). Although some have argued that CASR antibody assays are clinically indicated in acquired hypoparathyroidism (197), others have demurred, and it remains to be seen whether the autoantibodies are of primary or secondary importance (190,198). In contrast, there is now good evidence that autoantibodies can be functional activators of CASR and thereby induce hypoparathyroidism (199). Unfortunately, there is no convincing test for this state, outside of an *in vitro* demonstration that patient serum activates CASR transfected into HEK cells. In some hypoparathyroid patients, both autoimmune parathyroid destruction and suppression by CASR activation may co-exist (196).

In APS-1, the most common associated manifestations are hypoparathyroidism with mucocutaneous candidiasis and Addison's disease. Additional features include pernicious anemia, chronic active hepatitis, alopecia, keratitis, gonadal failure, thyroid disease, pancreatic insufficiency, and diabetes mellitus (192). The phenotype is highly variable and patients may not express all elements of the basic triad, leading to the suggestion that the criteria used for molecular screening be relaxed (200). The disease usually presents in infancy with chronic oral thrush, followed by hypoparathyroidism in the first decade, and then adrenocortical failure in the third. Interestingly, there is nearly 100% penetrance of hypoparathyroidism in females, but less than 60% in males, even though the adrenal hypofunction affects both sexes equally (195). Moreover, patients who develop the adrenal hypofunction first are less likely to be male and may never develop hypoparathyroidism. The responsible gene, called the autoimmune regulator (AIRE), maps to chromosome 21q22 and encodes a transcriptional regulator (201). In the absence of AIRE protein, tissue-specific self-antigens are not expressed in the thymus and multiorgan autoimmunity develops, because negative selection of the T cells bearing the autoantigens is disrupted (202). Many patients with APS-1 can be shown to have autosomal

recessive inheritance of the AIRE defect. In families with autosomal recessive mutations of AIRE, obligate heterozygotes may also have common autoimmune disorders but APECED is not seen (203). A phenocopy leading to acquired APS-1 may occur when the AIRE gene is silenced by thymic neoplasia (204). APS-1 has been associated with more than 200 mutations of the AIRE gene, and updates can be found in the online mutation database (<http://bioinf.uta.fi/AIREbase/>).

## 85.20 PSEUDOHYPOPARATHYROIDISM

Several clinical disorders characterized by end-organ resistance to PTH are described collectively by the term pseudohypoparathyroidism (PHP) (205,206). They are associated with hypocalcemia, hyperphosphatemia, and increased circulating PTH, but target tissue unresponsiveness to the hormone manifests as a lack of increased cAMP excretion in response to PTH administration. The biochemical characteristics of these disorders are contrasted with those of hypoparathyroidism in Table 85-8.

### 85.20.1 Albright Hereditary Osteodystrophy

Seventy years ago Fuller Albright first recognized that the likely cause of the hypoparathyroid state in PHP is a constitutive absence of target tissue responsiveness. In many patients, end-organ resistance is accompanied by a specific pattern of physical findings, called Albright hereditary osteodystrophy (AHO). Typically, patients have short stature, round facies, brachydactyly, obesity, and ectopic soft tissue or dermal ossification(s) (osteoma cutis) (Figure 85-5). In the calvaria, this may manifest as hyperostosis frontalis interna (207). Intracranial calcification(s), cataracts and band keratopathy, subcutaneous calcifications, and dental hypoplasia are also common, but are likely to be the consequences of long-standing hypoparathyroid hypocalcemia (Table 85-9). The brachydactyly may be asymmetric or not, and may involve one or both hands or feet, but the pattern is quite distinctive (208,209). The shortening tends to involve the first distal phalanx, leading to a thumbnail (or first toenail) that is wider than it is long. The fourth and fifth metacarpals (or metatarsals) are frequently shortened out of proportion to the others and the second metacarpal is often spared. Radiographic analysis of the hands (pattern profiling) is helpful in assessment of the brachydactyly (see Figure 85-5).

Although affected patients are generally short as adults, their bone age as children may be advanced and growth accelerated. Patients with AHO are probably predisposed to hypertension, conductive and sensorineural hearing loss, cord compression due to spinal anomalies, and movement disorders due to basal ganglia calcification. The features of AHO may be subtle in infancy or early childhood; in a few, there is little to see even in adulthood.

**TABLE 85-8 Biochemical Characteristics of Hypoparathyroidism and Pseudohypoparathyroidism**

	Response to PTH Infusion						Multiple Endocrine Defects
	Serum PO <sub>4</sub>	PTH	25(OH)D	1,25(OH) <sub>2</sub> D	U <sub>cAMP</sub>	U <sub>PO4</sub>	
Hypoparathyroidism	↑	↓	→	↓	→	→	Yes/no <sup>a</sup>
Pseudohypoparathyroidism							
Type 1a	↑	↑	→	↓	↓	↓	Yes
Type 1b	↑	↑	→	↓	↓	↓	No/yes <sup>b</sup>
Type 1c	↑	↑	→	↓	↓	↓	Yes
Type 2	↑	↑	→	↓	→	↓	No

↑, increased; ↓, decreased; →, normal.

<sup>a</sup>Depending upon the etiology.

<sup>b</sup>Variable defects of the thyroid and somatotropin axes are seen.

The round facies, short neck, and low, flat nasal bridge are often accompanied by central obesity. A recent study showed that the obesity phenotype occurs primarily in those patients who also have multiple hormone resistance, that is, PHP1a (see below), but according to data from mice, hypothalamic mechanisms, rather than hypothyroidism, are the primary underlying cause (210).

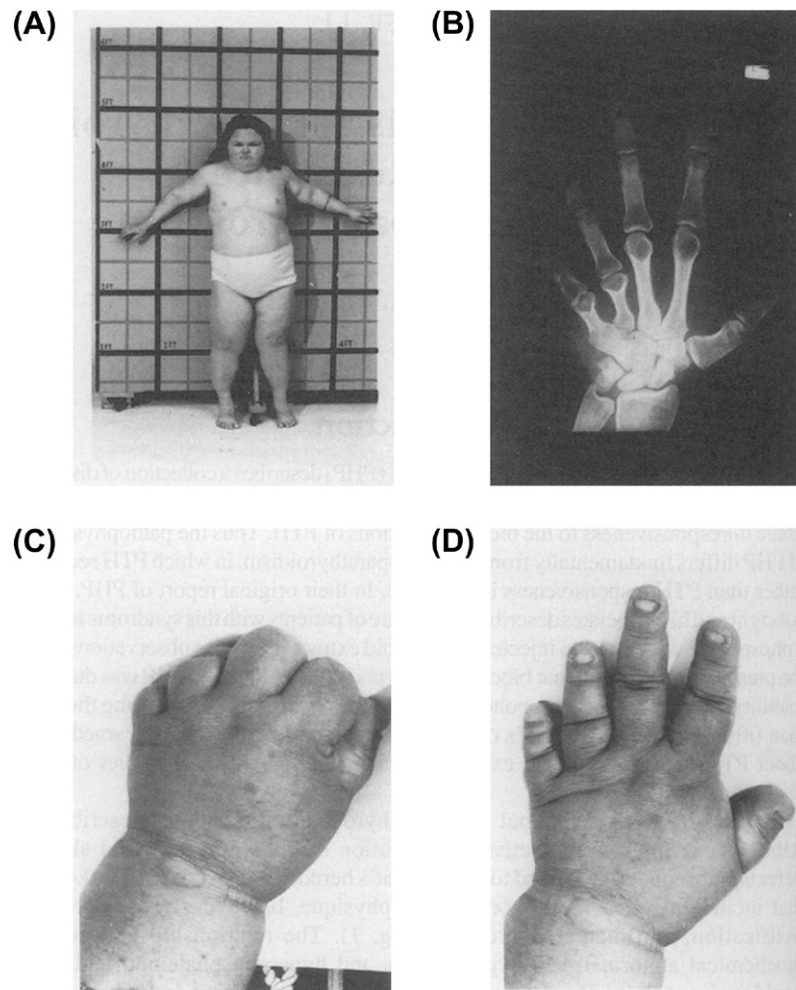
Patients with brachydactyly–mental retardation (BDMR), and other features closely resembling AHO have been found to carry microdeletions of chromosome 2q27 (BDMR; MIM #600430). Genes important for skeletal and neurological development lie within this region. Haploinsufficiency of *HDAC4* (MIM #605314), encoding a histone deacetylase that regulates gene expression during the development of many tissues including the bone, is responsible for the BDMR in those patients (211). Isolated brachydactyly type E (BDE; MIM #113300) has been associated in sporadic cases with mutations in *HOXD13* (MIM #142989) (212), and recently mutations in the *PTHLH* gene (MIM #168470) on 12p11.2 that encodes PTHrP have been implicated. In one family with autosomal BDE, a *cis*-regulatory site downregulates *PTHLH* in translocation t(8;12)(q13;p11.2) and downregulates its targets *ADAMTS-7* and *ADAMTS-12*, leading to impaired chondrogenic differentiation (213). Affected individuals of one large family with BDE, short stature and learning difficulties had an ~900 bp microdeletion encompassing *PTHLH* (214). Additional individuals with BDE and short stature from other different kindreds were found to have missense (L44P and L60P), nonstop (X178WextX\*54), and nonsense (K120X) mutations (214).

### 85.20.2 PHP1a

PHP1a patients, characterized by AHO, PTH resistance, and evidence of target organ resistance to other hormones, are usually found to have a reduction in the activity of the G $\alpha$  subunit, which is part of the membrane-associated heterotrimeric G-protein complex, transducing signals between G-protein coupled receptors and adenylate cyclase. Adenylate cyclase catalyzes the

synthesis of the second messenger cAMP, and, therefore, PHP-1a patients tend to have a deficiency in cAMP generation, particularly in certain tissues (215). This deficiency is clearly evident when measuring cAMP excretion in response to PTH administration.

The *GNAS* gene (MIM #168470) encoding the G $\alpha$  protein maps to 20q13.2-13.3 and has at least four alternative transcriptional start sites (Figure 85-6) and an antisense transcript, *NESPas* (216,217). The three upstream exons and the preceding promoter regions are genetically imprinted, that is, methylated in an allele specific manner. The promoter of the G $\alpha$  transcript, which uses exon 1, is unmethylated. Unlike the other alternative *GNAS* products, G $\alpha$  expression is biallelic with the exception of a small set of tissues, where G $\alpha$  is derived predominantly from the maternal allele (218–220). This tissue-specific monoallelic G $\alpha$  expression affects penetrance of the PHP phenotype. The maternal transmission of the hormone resistance in PHP1a can be explained by silencing of the paternal G $\alpha$  allele, which would otherwise allow expression of 50% of G $\alpha$  protein. Thus, full expression of a coding *GNAS* mutation, which occurs in maternally transmitted cases, leads to AHO plus hormone resistance (PHP1a), whereas a paternally transmitted mutation causes AHO alone (pseudopseudohypoparathyroidism; PPHP) (209). Despite clinical evidence supporting imprinting in portions of the kidney tubule, it has been difficult to confirm this experimentally in humans (221). The imprinting of *GNAS* is complex and involves multiple differentially methylated regions (DMR) (216). Moreover, it is tissue-specific and may vary with developmental stage, although key imprinting of the 1A DMR is a primary event that occurs during gametogenesis and is maintained thereafter (222). Ablation of the G $\alpha$  ortholog in mice (*Gnas*) has confirmed that maternal, but not paternal, transmission of the deleted allele results in PTH resistance. The homozygous deletion of *Gnas* is embryonic lethal. Comparison of G $\alpha$  expression in mice with maternally vs. paternally disrupted G $\alpha$  expression also demonstrated that G $\alpha$  expression is predominantly maternal in the renal cortex, but not in renal medulla.



**FIGURE 85-5** Features of Albright Hereditary Osteodystrophy (AHO). (A) Young woman with short stature (approximately third centile), disproportionate shortening of the limbs, generalized obesity, and round, flattened face. (B) Radiograph of the hand showing the shortened fourth and fifth metacarpals. (C) Fist with the characteristic “dimples” over the third, fourth, and fifth digits replacing the knuckles formed by the distal head of normally sized metacarpal bones (Archibald sign). (D) Brachydactyly of the hand, with the short fourth and fifth digits, the greatly foreshortened terminal first digit, and very short, wide thumbnail (potter’s thumb). (From Levine, M. A. In *The Genetics of Osteoporosis and Metabolic Bone Disease*; Econs, M. J., Ed.; Humana Press: New York, 2000; pp 179–209.)

A variety of inactivating mutations in the portion of the *GNAS* gene encoding  $G\alpha$  have been identified in PHP1a patients (223,224). The spectrum includes missense mutations, point mutations impairing efficient and accurate splicing, and small insertion/deletion mutations. A 4-bp deletion in exon 7 ( $\Delta$ GACT 188/190) has been observed in multiple unrelated cases, suggesting that this may be a hot spot (223). Several other mutations have also been observed in more than one kindred, indicating that additional susceptibility regions may exist. The identification of de novo germline mosaicism (225) is consistent with the view that most sporadic cases harbor new mutations, but the separation of such sporadic cases from familial ones, in which there is suppression of phenotype due to imprinting, may be difficult without detailed molecular studies.

PHP1a cases have been described in which no mutations of the *GNAS* gene have been found by nucleotide sequence analysis of exons encoding  $G\alpha$ . This may be

because the mutation is in a regulatory region of the gene not yet examined, or it may be that a large deletion prevents amplification of the mutant allele for subsequent analyses. In cases without identified *GNAS* coding mutations, an assessment of  $G\alpha$  bioactivity in erythrocytes is helpful in ruling out regulatory region mutations or large deletions. A 30-kb deletion spanning exons 1–5 has been identified recently by using comparative genome hybridization in a patient with PHP1a in whom coding mutations had been ruled out but a marked reduction of erythrocyte  $G\alpha$  activity demonstrated (226,227). Typically, PHP1a is associated with multiple hormone resistance, including thyroid stimulating hormone (TSH) and gonadotropins, causing hypothyroidism and gonadal failure, respectively. Because the hypothyroidism may express before hypocalcemia is observed, early surveillance of thyroid function is warranted. However, thyroid replacement from birth does not appear to prevent the mental deficit typical of PHP1a. In women, the



**TABLE 85-9 Incidence of Signs and Symptoms in PHP with AHO**

	Percentage
Body habitus	
Short stature	80
Obesity	50
Craniofacial	
Round face	92
Lenticular opacities <sup>a</sup>	44
Strabismus	10
Dental hypoplasia <sup>a</sup>	51
Basal ganglia calcification <sup>a</sup>	50
Thickened calvaria	62
Mental deficit	75
Brachydactyly	
Brachymetacarpia	68
Brachymetatarsia	43
Brachyphalangia	50
Other connective tissue features	
Decreased bone density	15
Ectopic ossification	56
Subcutaneous calcification <sup>a</sup>	55

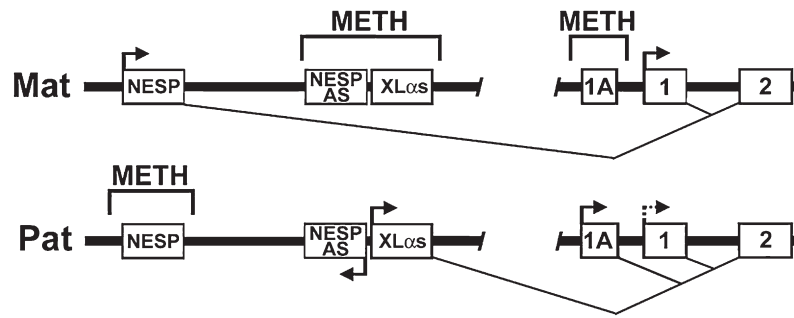
<sup>a</sup>Features common to other forms of chronic hypoparathyroid hypocalcemia. Taken from Drezner, M. K.; Neelon, F. A. Pseudohypoparathyroidism. In *The Metabolic Basis of Inherited Disease*; Scriver, C.R., Beaudet, A. L., Sly W. S., Valle, D., Eds.; McGraw-Hill: New York, 1995, pp 1508–1527.

hypogonadism is partial so that oral contraceptives may help regulate the menstrual cycle. Estrogen can antagonize bone resorption leading to an exacerbation of hypocalcemia, but placental 1,25-(OH)<sub>2</sub>D synthesis likely obviates this effect altogether in pregnancy, so women are frequently normocalcemic at that time. Abnormalities of the somatotropin axis have also been reported, with documentation of subnormal growth hormone release following stimulation by L-arginine or growth hormone releasing hormone (228,229). The effect of growth hormone replacement has been recently investigated in a small group of pre-pubertal PHP-1a patients (230). This study concluded that the treatment is potentially effective but has to be initiated as early as possible. The tissue-specific silencing of the paternal *Gsα* allele also plays a key role in the development of the additional hormone resistance phenotypes, as monoallelic *Gsα* expression has been demonstrated in the thyroid, the ovaries, and the pituitary (218–220,231). Recent studies have revealed that obesity also develops primarily in patients who inherit the inactivating *Gsα* mutations from their mothers (232). *Gsα* is not imprinted in the white adipose tissue (233), but the investigations of mice in which *Gsα* is ablated conditionally in the brain showed that *Gsα* is also monoallelic in certain parts of the hypothalamus (210), thus explaining the imprinted mode of inheritance of the obesity phenotype. Likewise, it has been recently noted that cognitive impairment, a typical AHO feature, also develops primarily after maternal inheritance of the inactivating *Gsα* mutation (234).

### 85.20.3 PHP1b

PHP1b is typically not associated with AHO or a generalized reduction in *Gsα* expression. PHP1b patients show a defect in renal PTH signaling, but an apparently normal response to PTH in bone. Affected individuals are, therefore, functionally hypoparathyroid but show normal skeletal architecture and development. Due to unimpaired PTH responsiveness in bone, signs of hyperparathyroid bone disease (osteitis fibrosa cystica) are occasionally observed, complicating the picture. Biochemical abnormalities suggestive of TSH resistance are also seen in some patients (231), and abnormalities of renal uric acid handling have been documented (235,236). Because the hormone resistance is mostly limited to PTH, it was thought at one time that these findings could be explained by a defect in the type-1 parathyroid hormone receptor (*PTHR1*, MIM #168468), but sequencing in PHP1b patients shows no mutations in protein-coding exons or gene promoter regions of the gene, and studies of PHP1b families show no linkage to *PTHR1*.

Most cases of PHP1b are sporadic, but a familial form of PHP-1b with an apparent autosomal dominant mode of inheritance also exists (AD-PHP-1b). In four AD-PHP1b kindreds, linkage to chromosome 20q13.3 was established, the same region which includes the *GNAS* locus. In these families, the pattern of transmission suggests paternal imprinting, and inheritance is therefore the same as for PHP1a (217). Another 13 PHP1b subjects, some of whom had bone responsiveness to PTH, were studied. All lacked methylation of the alternate exon 1A (also known as A/B), an epigenetic defect that is postulated to inhibit expression of the functional exon 1-containing *Gsα* transcript in renal tissues only (Figure 85-6). Thus, the loss of methylation of the maternal as well as paternal *Gsα* alleles, causing PTH resistance specifically in renal proximal tubule cells (222). Genetic analysis indicated that mutations in a regulatory region some distance from the *GNAS* coding exons were likely to account for the unique imprinting defect(s) associated with PHP1b (237). A search for the mutation revealed the presence of a 3-kb microdeletion that segregated with the disease in 12 kindreds with AD-PHP1b and also occurred in four sporadic cases (238). The deletion, flanked by direct repeats, removes three exons of the *STX16* gene which encodes syntaxin-16 (Figure 85-7). In addition, another deletion within *STX16* that removes exons 2–4 was later identified in a single kindred with AD-PHP1b (239). In all these cases, maternal, but not paternal, inheritance of the *STX16* deletion led to PTH resistance. Because *STX16* is apparently not imprinted (239), loss of one copy of this gene is not predicted to underlie the PHP1b pathogenesis. Instead, these deletions presumably disrupt a *cis*-acting element that controls imprinting at *GNAS* exon 1A. In two other PHP1b kindreds, nearly identical deletions of the NESP55 DMR



**FIGURE 85-6** The normal allele-specific methylation and expression patterns of the four alternate first exons of *GNAS*, which splice onto exon 2 to produce transcripts encoding NESP55 (NESP),  $XLaS$ , a transcript of unknown function (exon 1A), and  $Gs\alpha$  (exon 1). NESP55 and  $XLaS$  promoters are oppositely imprinted: NESP55 is expressed from the maternal allele and its promoter region is methylated on the paternal allele, whereas  $XLaS$  is expressed from the paternal allele and its promoter is methylated on the maternal allele. The  $XLaS$  promoter also generates paternally expressed antisense transcripts, and the first antisense exon is shown (NESPas). Exon 1 appears to be paternally imprinted in some tissues (e.g., renal proximal tubule cells) indicated by the dashed arrow. NESP55 protein is unrelated to  $Gs\alpha$ , and its entire coding region is located within its first exon. In contrast,  $XLaS$  and  $Gs\alpha$  proteins have identical COOH-terminal domains (encoded by exons 2–13), while their unique NH<sub>2</sub>-terminal domains are encoded within their respective first exons. Exon 1A does not have a translational start site. It is proposed that loss of exon 1A imprinting leads to decreased  $Gs\alpha$  expression in renal proximal tubules and is the cause of PHP1b. (From Liu, J., et al. *GNAS: Normal and Abnormal Functions*. Endocrinology. 2004, 145, 5459–5464.)

including exons 3 and 4 of the antisense transcript segregated with the disease (240). In this instance, however, the 1A DMR was not the only region to lose the differential methylation required to allow maternal expression of  $Gs\alpha$  in the kidney. Maternal methylation was also lost in the regions of the  $XLaS$  and NESPas promoters. Another kindred with these widespread epigenetic defects of *GNAS* has recently been described (241). The affected individuals in this kindred carried a maternally inherited deletion that removed antisense exons 3 and 4 with flanking intronic regions but not the NESP55 exon.

Sporadic PHP1b cases also show broad *GNAS* epigenetic defects that involve exon 1A (242). In some of these cases, paternal uniparental disomy of different chromosome 20 segments have been reported as the likely cause of PHP1b (243–246). The cause of the epigenetic defects and PTH resistance, however, remains unknown for most cases of sporadic PHP1b. Based on one report, no marked clinical differences could be established according to the pattern of *GNAS* epigenetic defects, although serum PTH levels were significantly higher in females with broad *GNAS* methylation defects than females with isolated loss of 1A methylation (247).

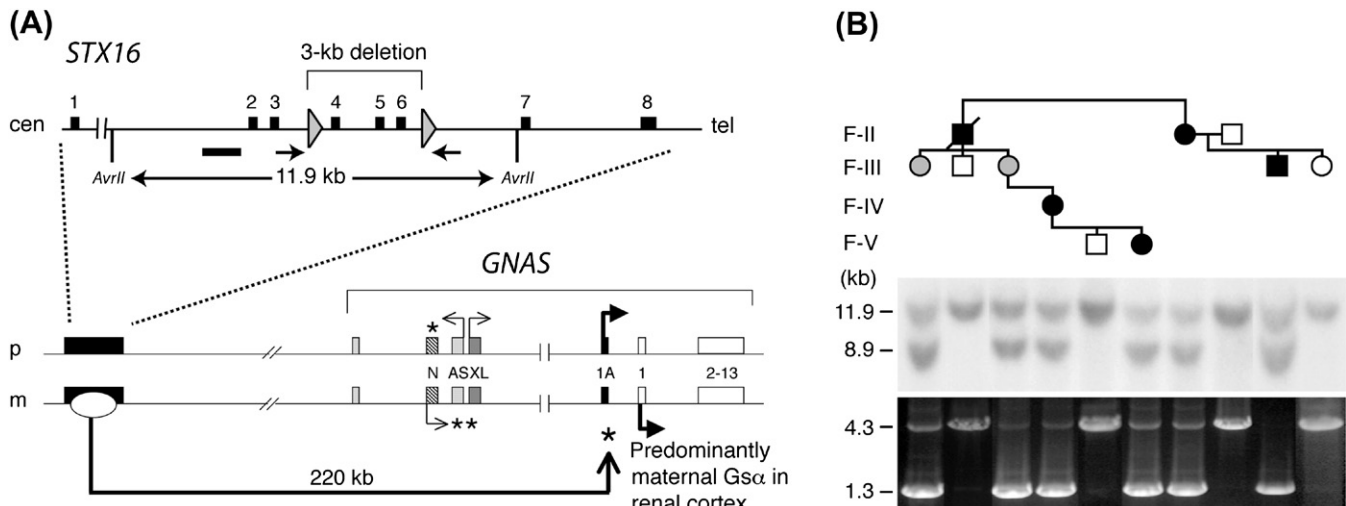
In contradistinction to the classical understanding that AHO features are unique to PHP1a, some recent studies have identified patients with PTH resistance and AHO features who show *GNAS* epigenetic defects rather than  $Gs\alpha$  coding mutations (226,248,249). Thus, there may be some overlap between the clinical and molecular features of PHP1a and PHP1b. It is possible that the AHO features observed in patients with *GNAS* epigenetic defects result from a genetic mechanism that is similar to the mechanism underlying the hormone resistance in PHP1a patients, that is, due to monoallelic  $Gs\alpha$  expression in additional tissues.

A PHP1b family with a novel  $Gs\alpha$  mutation, deletion of isoleucine-382 in the carboxyl terminus (leading to uncoupling from the PTHR and isolated PTH resistance), shows transmission through three generations, consistent with paternal imprinting (250). However, such mutations within  $Gs\alpha$  coding exons are rare.

#### 85.20.4 PHP1c and PHP2

Patients with PHP1c have multiple hormone resistance but normal  $Gs\alpha$  activity. The defect may be in other components of the receptor-adenylate cyclase system, such as the catalytic unit, but some PHP1c cases have been reported to carry  $Gs\alpha$  coding mutations (251). These mutations render the  $Gs\alpha$  protein unable to mediate cAMP generation in response to receptor activation but do not affect basal adenylate cyclase stimulating activity or the ability to be activated by non-hydrolyzable GTP analogs (251–253). Thus, some forms of PHP1c appear to be an allelic variant of PHP1a. Finally, patients with PHP2 have a normal urinary cAMP response to PTH but an impaired phosphaturic response. The defect could be in the cAMP-dependent protein kinase (PKA), one of its substrates or targets, or in a component of the PTH-PKC signaling pathway.

A recent study (254) has discovered a heterozygous mutation of the gene encoding the regulatory subunit of PKA (*PRKAR1A*) in three patients with multiple hormone resistance and acrodysostosis (MIM #101800), a form of skeletal dysplasia that includes severe brachydactyly and other skeletal findings that resemble AHO. This mutation (p.R368X), which leads to the truncation of the COOH-terminal 14 residues, impairs cAMP binding to the regulatory subunit, thereby blocking the activation of PKA (254). In addition to acrodysostosis, patients carrying this mutation display evidence for target organ resistance to PTH, thyrotropin, growth



**FIGURE 85-7** (A) The syntaxin-16 (*STX16*) gene, which is not imprinted, is located some 220 kb upstream of *GNAS* exon 1A. A heterozygous 3 kb deletion that is flanked by two direct repeats of 391 bp (triangles), within the maternal *STX16* gene, is hypothesized to disrupt a putative element that controls the normal establishment and/or maintenance of exon 1A methylation. The epigenetic change and/or associated derepression of maternal 1A transcript expression is postulated to silence maternal *Gsα* protein expression in renal proximal tubule cells. N, NESP55; AS, antisense; \*, normally methylated. (B) Identification of the 3-kb microdeletion in a PHP-1b kindred. (Top) Filled symbols, affected individuals; open symbols, healthy individuals; gray symbols, unaffected obligate gene carriers. (Middle) Genomic DNA digested with *AvrII* and subjected to Southern blot analysis. The position of *AvrII* sites flanking the normal restriction fragment of 11.9 kb and of the probe is indicated in Panel A. (Bottom) PCR amplification of wild-type (4.3 kb) and mutant (1.3 kb) alleles from genomic DNA. (Redrawn from Bastepe, M., Frohlich, L. F., Hendy, G. N., et al. Autosomal Dominant Pseudohypoparathyroidism Type 1b Is Associated with a Heterozygous Microdeletion That Likely Disrupts a Putative Imprinting Control Element of *GNAS*. *J. Clin. Invest.* 2003, 112, 1255–1263.)

hormone releasing hormone, and gonadotropins, but these findings are accompanied by elevated basal plasma and urinary cAMP levels and with an apparently normal urinary cAMP response to exogenous PTH administration.

### 85.21 OTHER PHENOTYPES ASSOCIATED WITH *GNAS* MUTATIONS

In contrast to the PHP phenotype associated with inactivating *GNAS* mutations, a different form of sporadic bone disease, (polyostotic) fibrous dysplasia, results from de novo *GNAS* mutations that cause constitutive *Gsα* activity (255). A more severe form of this disease (panostotic fibrous dysplasia) with hyperphosphatasia and hyperphosphaturic rickets has also been described. Patients carrying these activating mutations are mosaic for mutant and wild-type cells, indicating that the mutation is acquired during postzygotic development. These mutations affect the arginine residue at position 201 (exon 8) and, rarely, the glutamine at 227 (exon 9), and inhibit the intrinsic GTP hydrolase activity of *Gsα* and, thereby, lead to constitutive activity. Such constitutively activating mutations of *GNAS* are also found in a variety of endocrine and non-endocrine tumors, such as growth hormone-secreting adenomas. A missense mutation in exon 13 (A366S) results in a *Gsα* protein that is unstable at 37°C, but constitutively active at lower temperatures. Affected patients have PHP due to PTH resistance and precocious puberty (testitoxico-sis) due to hormone-independent constitutive activation

of luteinizing hormone receptors at lower ambient temperatures in the testes. Another *Gsα* mutant carrying Ala-Val-Asp-Thr amino acid repeats in the guanine-binding domain has been recently described in a patient with neonatal diarrhea and PTH resistance (256). In this instance, the mutant protein is unstable and localized to the cytoplasm rather than plasma membrane, which explains the hormone resistance. On the other hand, this mutation increases the rate of GDP–GTP exchange and, thus, confers overactivity. The increased activity of *Gsα* seems to be evident during the neonatal period in the gut, where the mutant localizes to the plasma membrane, thus explaining the diarrhea phenotype. Undoubtedly, other patterns of altered hormone–receptor interaction due to a *GNAS* mutation await discovery.

Inactivating *GNAS* mutations have also been identified in patients with congenital osteoma cutis and progressive osseous heteroplasia (POH), suggesting that these connective tissue conditions are another variant in the phenotypic spectrum of *GNAS*-related disease (257). No genotype–phenotype correlation has been revealed regarding these disorders, as the same mutation can be associated with either typical AHO features or severe ossifications that involve deep connective tissues and skeletal muscle (258). Nonetheless, most patients with POH inherit the *GNAS* mutation from their fathers or acquire this mutation de novo on the paternal *GNAS* allele. This parent-of-origin-specific inheritance of POH was established by analyzing 18 unrelated kindreds with this disorder (259). In a single three-generation kindred, the inheritance of the mutation from males led to POH,

while the inheritance of the same mutation from females led to typical AHO. It thus appears likely that alterations in the activity of a paternally expressed *GNAS* product, such as XL $\alpha$ s, contribute to the pathogenesis of POH.

### 85.21.1 Differential Diagnosis and Genetic Counseling

Patients with dysmorphic features resembling AHO may require careful endocrinologic workup to confirm and delineate the form of PHP that is present. Similar studies of family members may also be warranted, since the biochemical and clinical features vary within families. If PHP1a with AHO is established, genetic counseling may aid in understanding the multisystemic nature of the disorder, particularly in relation to the patient's growth and development, and later-onset connective tissue complications. For either PHP1a or PHP1b, extensive counseling may be required to adequately explain the various implications of paternal imprinting for the parent-specific recurrence risks in offspring. Germline mosaicism has been reported (224), which is clearly important in assessing risks for recurrence in future sibs of a singleton family. Given the recently described complexities in the molecular, biochemical, and physical features of PHP1a and PHP1b, molecular testing is critical for achieving a clear diagnosis and validating the inheritance pattern in any given family.

## 85.22 THE PARATHYROID HORMONE RECEPTOR AND SKELETAL DYSPLASIAS

Polymorphisms in the *PTHr1*, MIM #168468, are associated with adult height and BMD (260), emphasizing the role that the receptor and its ligands play in endochondral bone formation. Inactivating or loss-of-function mutations in the *PTHr1* have been implicated in the molecular pathogenesis of Blomstrand's lethal chondrodysplasia (BLC, MIM #215045) and other skeletal dysplasias and dental abnormalities (140). The rare BLC disease is characterized by advanced endochondral bone maturation, short-limbed dwarfism, abnormal breast and tooth morphogenesis, and fetal death, thus mimicking the phenotype of *Pthr1*-less mice. The majority of BLC cases were born to phenotypically normal consanguineous parents suggesting an autosomal recessive mode of inheritance. Mutant *PTHr1*s identified in BLC fetuses (Figure 85-4) fail to bind ligand or stimulate cAMP or inositol phosphate production. The Blomstrand disease has been recently subdivided into type I, which refers to the severe (classical) form, and type II, which refers to a relatively milder variant, and the difference between severities is attributed to complete or incomplete inactivation of the *PTHr1*, respectively (261,262). A milder form of recessively inherited skeletal dysplasia, known as Eiken's syndrome (MIM #600002), has been linked to mutations of *PTHr1* (263), suggesting a wider range

of skeletal phenotypes to this gene. Dominantly acting heterozygous *PTHr1* mutations have been identified in endochondromas of patients with endochondromatosis (Ollier's disease, MIM #166000), a familial disorder with evidence of autosomal dominance characterized by multiple benign cartilage tumors, and a predisposition to malignant chondrosarcomas (264,265). As many patients with Ollier's disease do not apparently have *PTHr1* mutations, the condition may be genetically heterogeneous (266). Dominantly inherited symmetrical enchondromatosis is associated with duplication of 12p11.23 to 12p11.22 that includes the *PTHr1* gene encoding PTHrP, suggesting that abnormal *PTHr1* signaling may underlie this unusual form of endochondromatosis (267). In addition, some cases of autosomal dominant nonsyndromic primary failure of tooth eruption (PFE) are due to loss-of-function mutations in the *PTHr1* that are dominantly acting leading to haploinsufficiency of the receptor (268,269).

## 85.23 NHERF1 MUTATIONS AND RENAL RESPONSIVENESS TO PARATHYROID HORMONE

In the renal proximal tubule, the sodium–hydrogen exchanger regulatory factor 1 (NHERF1) bridges the *PTHr1* and sodium phosphate cotransporter, NPT2a. In a group of patients with nephrolithiasis or bone demineralization, three mutations in NHERF1 were found in seven patients with low tubular maximal reabsorption of phosphate over glomerular filtration rate (TmP/GFR) (270). In cultured renal cells, the mutants demonstrated increased cAMP production and reduced phosphate transport in response to PTH.

## 85.24 HYPOMAGNESEMIA

In humans, hypomagnesemia leads to a suppression of PTH release and some degree of peripheral resistance. Although the exact molecular mechanism underlying the suppression of the parathyroid gland in hypomagnesemia is unknown, it is important to recognize that laboratory testing in cases of hypocalcemia with reduced PTH should include measurement of serum magnesium, particularly in newborns (271).

## 85.25 MANAGEMENT OF HYPOPARATHYROIDISM

### 85.25.1 Calcium and Vitamin D

The goal of treatment in hypoparathyroid states is to raise the serum calcium sufficiently to alleviate acute symptoms of hypocalcemia and prevent the chronic complications (142). The calcium concentration required for this purpose is generally the low–normal range. Acute or severe symptomatic hypocalcemia is best treated with



intravenous calcium infusion. Initial doses of 2–5 mM of elemental calcium as the gluconate salt can be given over a 10- to 20-min period, followed by 2 mM elemental calcium per hour as a maintenance dose, to be adjusted according to symptoms and biochemical response. Care must be taken to ensure that the infusion does not extravasate, and ionized or total calcium levels should be monitored frequently on a stat basis. Doses in children 5–14 years of age need to be adjusted for body weight, while neonates and infants require age-specific dosing schedules. In adults, intravenous vitamin D therapy is not needed. Hyperphosphatemia, alkalosis, and hypomagnesemia should be corrected concomitantly if present. Post-surgical hypocalcemia is now rarely severe and usually transient with appropriate management (272). However, the occasional patient can represent a significant problem, particularly if the indication for surgery is chronic hyperparathyroidism, and the post-operative hypoparathyroid state is permanent (273). The long-term effects of standard therapy remain a concern (274).

The mainstay of chronic treatment is oral calcium and vitamin D, which should be started as soon as possible to allow reduction and discontinuation of the intravenous calcium. Oral calcium comes in several forms, but calcium carbonate is generally the least expensive. A total of 20–80 mM elemental calcium daily (2–8 g calcium carbonate per day) is generally effective, but should be given in divided doses and adjusted on the basis of gastro-intestinal tolerance, relief of hypocalcemic symptoms, and appropriate biochemical response. Vitamin D is preferably administered as calcitriol (0.25–1.0 mg per day) but, if cost is a factor, pharmacological doses of cholecalciferol or ergocalciferol or calcidiol may be less expensive and equally efficacious. Cholecalciferol and ergocalciferol have the longest duration of action and can result in sustained toxicity. It is, therefore, appropriate to institute a starting dose of 25,000 IU/day and titrate upward (to 100,000 IU/daily) with assessment of serum and urinary parameters afterward with follow-up at 6 and 12 months, even if the patient is relatively asymptomatic. In any event, serum calcium and 24-hour urinary excretion should be carefully monitored when therapy is started and continued until the patient is stabilized. Hypercalciuria that occurs as treatment is initiated, even prior to the normalization of the serum calcium, may warrant an ongoing assessment of nephrocalcinosis, most sensitively detected by renal ultrasound. Consequently, only a low-normal serum calcium may be attainable, but many patients feel well enough that there is no need to entirely normalize the serum calcium. That way, the risk of renal failure due to chronic hypercalciuria—especially problematic in patients with CASR activating mutations (148)—is minimized. Nevertheless, a significant number of patients report problems with easy fatigue and exhaustion, and mood disturbances (e.g., depression, anxiety, hostility, and paranoid ideation) not in keeping with the degree of hypercalcemia, suggesting

that there may be non-calcitropic effects of PTH not remedied by maintenance of normocalcemia alone (274).

In PHP, monitoring serum PTH levels during treatment is critical with the aim of normalizing or reducing PTH levels as much as possible. This is done to avoid the long-term elevation of circulating PTH, that would likely cause bone resorption. Also, hypercalciuria as a result of the calcitriol and calcium treatment is a lesser concern because PTH actions in the distal tubule are functional, preventing the loss of calcium in the urine. Of note, calcitriol (and not other forms of vitamin D) should be used for the treatment, because the PTH resistance in the proximal tubule does not allow for the efficient synthesis of 1,25(OH)<sub>2</sub>D from 25-hydroxyvitamin D.

### 85.25.2 Hormone Replacement Therapy

Hormone replacement has been advocated as a potentially superior form of treatment for decades but only recently have preparations of recombinant human hormone—teriparatide (PTH 1-34) and full length parathyroid hormone (PTH 1-84)—become widely available. Preliminary studies on 27 adults suggested that hypercalciuria was less of a problem in hypoparathyroid subjects receiving twice daily subcutaneous injections of PTH 1-34 (275). Similar trials have been reported in hypoparathyroid children, with a reduction in calcitriol requirements (276,277). Teriparatide administered every other day in hypoparathyroid adults has been shown to reduce calcium and calcitriol requirements over a 24-month period (278), while PTH 1-84 is associated with a reversal in abnormal bone remodeling, without major adverse effects (279). Although both hormonal agents are well tolerated acutely, long-term exposure of animals to very high doses has been associated with tumor formation (280). While there is no evidence of increased risk associated with replacement doses, caution has been advised particularly in children. In hypoparathyroidism due to activating CASR mutations, such potential risks need to be balanced against ongoing risks of progressive renal damage associated with excessive hypercalciuria, exacerbated at times by the standard calcium and vitamin D therapy. Although the evidence is limited, it appears that hormone replacement is a safe and beneficial option in some of the affected children (281,282). However, better criteria for recommending a switch to recombinant human hormone use are needed, along with more data on long-term safety (283).

### 85.25.3 Calcilytics

Calcilytics—drugs that antagonize the CASR and promote PTH secretion—are a promising alternative for disorders with intact but hypofunctioning parathyroid glands (284). When administered to healthy adults, the CASR antagonist, ATF936, causes a dose-dependent rise in serum PTH immunoreactivity that constitutes proof

of principle (285), but studies have not been reported in hypoparathyroid subjects.

### 85.25.4 Other Therapies

If the serum calcium attainable with oral calcium and calcitriol is below the normal range and the patient remains symptomatic, then a trial of a thiazide diuretic may be considered, with the aim of reducing the hypercalciuria to raise the serum calcium further. The argument for efficacy seems greatest for responsive forms of autosomal dominant hypocalcemia due to activating CASR mutations, since the thiazide-sensitive transporter, SLC12A3 (MIM #600968), is a downstream target for suppression by activated CASR in the kidney. For reasons that are not clear, however, thiazides work well in some patients (286) but not others.

As the serum calcium is normalized, elevated serum phosphate concentrations generally decline but phosphate-binding gels such as aluminum hydroxide are occasionally helpful in reducing hyperphosphatemia at the beginning of therapy.

Patients with intracranial calcifications may experience seizures related to chronic neuropathic changes and it may be necessary to add appropriate anti-epileptic medication(s). In all chronically hypocalcemic patients, ocular assessments should be performed periodically.

## CROSS REFERENCES

Autoimmunity: Genetics and Immunologic Mechanisms; Vitamin D Metabolism of Action; Disorders Predisposing to Bone Fragility and Decreased Bone Density; Chondrodysplasias; Abnormalities of Bone Structure.

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## WEBSITES

- Online Mendelian Inheritance in Man. <http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim>.
- Calcium-Sensing Receptor Database. <http://www.casrdb.mcgill.ca/>.
- VCFSF Velo-Cardio-Facial Syndrome Educational Foundation, Inc. <http://www.vcfsef.org/>.
- The International 22q11.2 Deletion Syndrome Foundation, Inc. <http://www.22q.org/>.
- BIOINF AIREbase Mutation registry for Autoimmune Polyendocrinopathy with Candidiasis and Ectodermal Dystrophy (APECED). <http://bioinf.uta.fi/AIREbase/>.
- Gene Tests. <http://www.ncbi.nlm.nih.gov/sites/GeneTests/>.

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# CHAPTER

# 86

## Diabetes Mellitus

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### 86.1 INTRODUCTION

Diabetes mellitus is a diagnostic term for a group of disorders characterized by abnormal glucose homeostasis resulting in elevated blood sugar. There is variability in its manifestations, wherein some individuals have only asymptomatic glucose intolerance, while others present acutely with diabetic ketoacidosis, and still others develop chronic complications such as nephropathy, neuropathy, retinopathy, or accelerated atherosclerosis. It is among the most common of chronic disorders, affecting up to 5–10% of the adult population of the Western world. Its prevalence varies over the globe, with certain populations, including some American Indian tribes and the inhabitants of Micronesia and Polynesia, having extremely high rates of diabetes (1,2). The prevalence of diabetes is increasing dramatically and it has been estimated that the worldwide prevalence will increase by more than 50% between the years 2000 and 2030 (3).

It is clearly established that diabetes mellitus is not a single disease but a genetically heterogeneous group of disorders that share glucose intolerance in common (4–7). The concept of genetic heterogeneity (i.e. that different genetic and/or environmental etiologic factors can result in similar phenotypes) has significantly altered the genetic analysis of this common disorder. Diabetes and glucose intolerance are not diagnostic terms, but, like anemia, simply describe symptoms and/or laboratory abnormalities that can have a number of distinct etiologies.

### 86.2 DIFFICULTIES IN GENETIC STUDIES OF DIABETES

The geneticist is confronted with a number of obstacles in attempting to unravel the genetics of diabetes. These include differences in the definition of affected

individuals, modification of the expression of the diabetic genotype by environmental factors, and variability in the age of onset of the disease. One of the major sources of confusion in the study of diabetes mellitus has been the definition of an “affected” individual. Some investigators have called an individual diabetic only if they have clinical symptoms of the disease, whereas others have accepted a mildly abnormal glucose tolerance test. There is marked clinical variability in diabetes. The phenotypic expression of the diabetes genotype (or genotypes) appears to be modified by a variety of environmental factors, including diet, obesity, infection, and physical activity, as well as sex and parity. Obese individuals with type 2 diabetes may lose all signs of the disorder, clinical as well as chemical, if their weight returns to normal. Because of the marked variability in the age of onset of the disease, at any given time only a fraction of those individuals possessing the diabetic genotype may be recognized. Therefore, it may be impossible to say at any specific time whether a clinically unaffected individual carries the diabetic genotype. Thus, longitudinal studies are required to detect those genetically affected family members who will eventually manifest clinical disease.

The high prevalence of diabetes in the population presents additional difficulties for the geneticist. Are relatives affected because they have the same genotype, because they share the same environment, or because they have a chance occurrence of a common disorder? Furthermore, the diabetic syndromes are sufficiently common that two genetically different forms may occasionally occur in the same family. The most important impediment to genetic analysis has been a lack of knowledge concerning the basic defect(s) in each of the disorders leading to diabetes. Because of this, there is no certain method for detecting all individuals with disease-predisposing genotypes

before their clinical manifestations, that is, individuals who possess the diabetic genotypes but have no signs of abnormal carbohydrate metabolism. Despite all these obstacles, major strides have been made in delineating the genetic basis of the diabetic syndromes. The main force behind these advances has been genome-wide association studies (GWAS) conducted in thousands to tens of thousands of subjects, typically in cross-sectional case/control cohorts, targeting either type 1 or type 2 diabetes. Such large sample sizes have been able to overcome clinical heterogeneity and have yielded dozens of susceptibility loci for both forms of diabetes.

## 86.3 DIABETES IN FAMILIES AND TWINS

### 86.3.1 Familial Aggregation of Diabetes

Many authors have shown that diabetics have an “increased family history” of the disease (4). In most reports, the frequency of diabetics with positive family histories of the disease ranges from 25% to 50%. Since the frequency of a positive family history of diabetes in nondiabetic individuals has usually been found to be below 15%, this family history information has been used to support the hypothesis that diabetes mellitus is a hereditary disorder. These types of data, however, are not very powerful. A more accurate method of assessing familial aggregation is to compare diabetes prevalence in specific relatives of affected individuals with that found among similar relatives of nondiabetic controls. Pincus and White (8) were the first to use this method in the study of diabetes, when they statistically established the increased prevalence of the disease among the relatives of diabetics. These findings have since been confirmed by many other investigators (4). Using more sensitive markers of the diabetic genotype, such as oral, intravenous, and cortisone-induced glucose tolerance tests, it has been found that the prevalence of affected individuals among the relatives of diabetics is even higher (usually ranging between 10% and 30% of the parents, sibs, or close relatives, as compared to a prevalence of 1–6% of the relatives of nondiabetic individuals). Thus the prevalence of both clinical diabetes and abnormal glucose tolerance is significantly greater among the close relatives of diabetics than among similar relatives of nondiabetic individuals.

### 86.3.2 Early Twin Studies

Familial aggregation of a trait may be caused by either genetic or environmental factors and twin studies have been used to confirm the importance of genetic factors in the etiology of diabetes. Using clinical diabetes as the criteria for affected, most investigators have reported a concordance rate for monozygotic (MZ) twins between 45% and 96% and for dizygotic twins between 3% and 37%. When type 2 diabetes is considered separately

and glucose tolerance tests are performed in the “non-diabetic” MZ cotwins, the concordance rate is usually above 70%. Thus the concordance of diabetes mellitus in MZ twins is significantly greater than in dizygotic twins, suggesting an important genetic component to disease etiology. The available data suggest that dizygotic twin risk appears to be approximately equivalent to that of siblings, arguing that whatever environmental factors contribute are present in the majority of a given population and suggesting that there is not a large contribution from unique family environments. As will be discussed later, the MZ concordance rates are very different for type 1 and type 2 diabetes.

## 86.4 GENETIC HETEROGENEITY IN DIABETES

Although the evidence from studies of familial aggregation and twins leaves no doubt as to the importance of genetic factors in the etiology of diabetes, for many years there was little agreement as to the nature of the genetic factors involved. This confusion can, in large part, be explained by the genetic heterogeneity that is now known to exist in diabetes. In 1967, the hypothesis of genetic heterogeneity was proposed based on several lines of evidence. Indirect evidence included (1) the existence of distinct, mostly rare genetic disorders (now numbering over 80) that have glucose intolerance as one of their features; (2) genetic heterogeneity in diabetic animal models; (3) ethnic variability in prevalence and clinical features; (4) clinical variability between the thin, ketosis-prone, insulin-dependent juvenile onset diabetic (type 1) and the obese, nonketotic insulin-resistant adult-onset diabetic (type 2); and (5) physiological variability (i.e. the demonstration of decreased plasma insulin in juvenile versus the relative hyperinsulinism of maturity-onset diabetics). In addition, some direct evidence for heterogeneity came from clinical genetic studies that suggested that juvenile and adult-onset diabetes differed genetically within families (9).

### 86.4.1 Rare Syndrome Associations

There are more than 80 distinct genetic disorders associated with glucose intolerance and, in some cases, clinical diabetes (Table 86-1). Although individually rare, these syndromes demonstrate that mutations at many different loci can produce glucose intolerance. Furthermore, they illustrate the wide variety of pathogenetic mechanisms that can result in glucose intolerance. These mechanisms range from absolute insulin deficiency due to pancreatic degeneration (in such disorders as hereditary relapsing pancreatitis, cystic fibrosis, and polyendocrine deficiency disease) to relative insulinopenia (in the growth hormone deficiency syndromes), to inhibition of insulin secretion (in the hereditary pheochromocytoma syndromes associated with elevated catecholamines), to various deficits in the interaction of insulin and its receptor (in the



**TABLE 86-1 Genetic Syndromes Associated with Glucose Intolerance and Diabetes Mellitus**

Syndromes	Types of DM	Associated Clinical Findings	Pattern of Inheritance	McKusick
<b>Syndromes associated with pancreatic degeneration</b>				
Congenital absence of the pancreas	Type 1 (congenital)	IUGR, poor adipose and muscle, malabsorption, dehydration	AD, ?AR	260370
Immunodysregulation, Polyendocrinopathy, and enteropathy, X-linked (IPEX)	Type 1 (congenital)	IUGR, dehydration, ±fatal secretory diarrhea	XR	304790
Congenital pancreatic hypoplasia	Type 1 (infancy)	IUGR, pancreatic exocrine deficiency	?AR	260370
Permanent neonatal diabetes mellitus with pancreatic and cerebellar agenesis	Type 1 (infancy)	IUGR, cerebellar hypoplasia/agenesis, optic nerve hypoplasia, beaked nose, dysplastic ears, triangular face, decreased subcutaneous fat	AR	609069
Hereditary pancreatitis	IGT, Type 1	Abdominal pain, chronic pancreatitis, portal and splenic vein thrombosis	AD	167800
Cystic fibrosis	IGT, Type 1	Malabsorption, chronic respiratory disease	AR	219700
Polyendocrine deficiency disease (Schmidt syndrome)	Type 1	Autoimmune endocrine disease, hypothyroidism, hypoadrenalism, female predominance, usually onset in middle age	?AR, AD	269200
IgA deficiency, malabsorption and diabetes	Type 1	IgA deficiency, malabsorption	?AD	137100
Hemochromatosis (includes juvenile hemochromatosis (HFE2), HFE3 and HFE4)	Type 2	Hepatic, pancreatic, skin, cardiac, and endocrine complications of iron storage	AR	235200
Thalassemia	IGT→Type 2	Anemia, iron overload	AR	602390
α-1-antitrypsin deficiency	IGT	Emphysema, cirrhosis	AR	604250
Cystinosis, Nephropathic	Type 1	Failure to thrive, Fanconi syndrome and renal failure, photophobia and decreased visual acuity because of corneal crystals, rickets, hepatosplenomegaly, hypopigmentation, primary hypothyroidism	AR	606069
Tropical calcific pancreatitis	Type 1	Juvenile-onset pancreatitis, insulin-dependent but ketosis-resistant diabetes, pancreatic cancer	AR (may require additional mutations in other genes as well)	141900
<b>Disorders causing neonatal diabetes without pancreatic degeneration</b>				
Transient neonatal diabetes	Neonatal→Type 2	Transient neonatal diabetes that resolves at a median age of 3 months, many develop type 2 diabetes later in life, IUGR, macroglossia, abdominal wall defects, brain anomalies, congenital heart disease depending on the gene involved and whether hypomethylation of other genes occurs	AR AD or AR AD Imprinting (hypomethylation) abnormalities of chr. 6q24	107400 219800
Permanent neonatal diabetes	Neonatal	Permanent diabetes beginning in early infancy, IUGR, DEND (developmental delay, epilepsy and neonatal diabetes) associated with KCNJ11 mutations	AR AD AD AD or AR	608189

**TABLE 86-1 Genetic Syndromes Associated with Glucose Intolerance and Diabetes Mellitus—cont'd**

Syndromes	Types of DM	Associated Clinical Findings	Pattern of Inheritance	McKusick#
Neonatal diabetes with congenital hypothyroidism	Neonatal	Congenital hypothyroidism and diabetes	AR	610199
Neonatal diabetes with cerebellar agenesis	Neonatal	Cerebellar agenesis and diabetes, dysmorphic facies	AR	609069
<b>Hereditary endocrine disorders with glucose intolerance</b>				
Isolated growth hormone deficiency	Type 2	Proportionate dwarfism	AD	173100
			AR	262400
Hereditary panhypopituitary dwarfism	Type 2	Proportionate dwarfism hypogonadism ± TSH and ACTH deficiency	AR	262600
			XR	312000
Johanson-Blizzard syndrome	Type 1	Hypoplastic nasal alae, deafness, hypothyroidism, growth retardation, mental retardation, malabsorption	AR	243800
Laron dwarfism	Type 2	Proportionate dwarfism	AR	262500
Hyperinsulinemic hypoglycemia, familial	Type 2	Hyperinsulinism, hypoglycemia in infancy can evolve into late glucose intolerance	AR	256450
			AD	601820
				602485
				609975
				609968
				606762
				610021
Pheochromocytoma	IGT	Hypertension, tremor, paroxysmal sweating	AD	171300
Multiple endocrine neoplasia Type 1	IGT	Pituitary (acromegaly), parathyroid (renal stones), pancreatic adenomas (peptic ulcer)	AD	131100
<b>Inborn errors of metabolism with glucose intolerance</b>				
Alaninuria (Stimmler syndrome)	Type 1 (infancy)	Mental retardation, microcephaly, IUGR, dwarfism, enamel hypoplasia, high blood pyruvate, lactate and alanine	?AR	202900
Proprotein convertase 1 deficiency	Type 2	Early-onset obesity, malabsorption, diarrhea, intestinal villous atrophy, reactive hypoglycemia, hypocortisolemia, hypogonadotropic hypogonadism, and primary amenorrhea	AR	600955
Thiamine-responsive megaloblastic anemia	Type 2	Megaloblastic anemia responsive only to thiamine, sideroblasts, sensorineural ringed syndrome deafness, hoarseness, progressive optic atrophy, situs inversus, septal defects, generalized puffiness, aminoaciduria	AR	249270
Aceruloplasminemia	Type 1	Blepharospasm, retinal degeneration, ataxia, chorea, torticollis, progressive dementia, mild anemia; onset between age 30–50 years	AR	604290

Leprechaunism (point mutations in insulin receptor gene)	Insulin-resistant	IUGR and growth retardation, large hands, feet and genitals, acanthosis nigricans, decreased subcutaneous fat, hirsutism	AR	246200
Seemanova syndrome	Insulin-resistant	Obesity, MR, delayed puberty, macroorchidism, acanthosis nigricans, curly hair	AD	100600
SHORT syndrome	Insulin-resistant	Short, hyperextensibility, ocular depression, Rieger anomaly, delayed teething, lipodystrophy	AR	269880
Rabson-Mendenhall syndrome	Insulin-resistant	Unusual facies, enlarged genitals, precocious puberty, acanthosis nigricans, hirsutism, pineal hyperplasia	AR	262190
Acanthosis nigricans insulin-resistant diabetes syndromes Type A	Insulin-resistant decreased receptors	Acanthosis nigricans, ovarian dysfunction, hirsutism, accelerated growth	AD	610549
Type A with acral hypertrophy and cramps	Insulin-resistant (postreceptor defect)	Large hands, acanthosis muscle cramps, enlarged kidneys, polycystic ovaries	?AR	
Type A with brachydactyly and dental anomalies	Insulin-resistant	Acanthosis nigricans, bitemporal narrowing, acral hypertrophy, decreased body fat, brachydactyly, dental anomalies	?AR	
Type A with muscle cramps and coarse facies	Insulin-resistant (postreceptor defect)	Coarse facies, muscular women, acanthosis nigricans, headaches, facies muscle cramps, hyperprolactinemia, no ovarian dysfunction	AD	
Type B	Insulin-resistant (circulating inhibitor)	Acanthosis nigricans, immunological disease	?	
<b>Hereditary neuromuscular disorders associated with glucose intolerance</b>				
Anosmia-hypogonadism syndrome (Kallmann syndrome)	IGT or Type 1	Anosmia, hypogonadotropic, hypogonadism, hearing loss, ±cleft lip and palate	XR	308700
			?AR	244200
			?AD	147950
				612370
Fryns syndrome	Type 1	MR, craniofacial dysmorphism, hypogonadism, seizures		610628
				600483
				229850
Muscular dystrophies	IGT→Type 2	Muscular dystrophy	AD	158900
			AR	253600
			XR	310200
Congenital Myopathy with Fiber-Type Disproportion	IGT→Type 2	Hypotonia, weakness, joint contractures	AR	255310
Huntington disease	IGT→Type 2	Chorea, dementia	AD	143100
Machado-Joseph disease	Type 2	Ataxia	AD	109150
Herrmann syndrome	Type 2	Photomyoclonus, deafness, nephropathy, dementia	AD	172500
Myotonic dystrophy	Type 2	Myotonia, muscular dystrophy, cataracts, hypogonadism, frontal balding, and ECG changes	AD	160900
				602668
Diabetes mellitus—optic atrophy, diabetes insipidus—deafness syndrome (Wolfram, DIDMOAD syndrome)	Type 1	Optic atrophy, diabetes insipidus, deafness, neurologic symptoms	AR	222300
			?Mitochondrial	604928 598500

**TABLE 86-1 Genetic Syndromes Associated with Glucose Intolerance and Diabetes Mellitus—cont'd**

Syndromes	Types of DM	Associated Clinical Findings	Pattern of Inheritance	McKusick#
Friedrich ataxia	Type 1 or Type 2	Spinocerebellar degeneration, visual field defects, hypertrophic cardiomyopathy	AR	229300
Ataxia telangiectasia	Type 2	Conjunctival and cutaneous telangiectasia, nystagmus, hypogonadism, cerebellar ataxia, recurrent infection, malignancies	AR	208900
Ramon syndrome	Type 1	Gingival fibromatosis, cherubism, MR, epilepsy, JRA, vascular skin lesions	AR	266270
Pseudo-Refsum syndrome	Type 2	Muscle atrophy, ataxia, retinitis pigmentosa	AD	158500
Stiff Person syndrome	Type 1	Fluctuating muscle rigidity with painful spasm, characteristic EMG, autoimmune disease of nervous and endocrine system	?AD/multifactorial (most sporadic)	184850
Hereditary motor and sensory neuropathy, Okinawa type	Type 2	Neurogenic atrophy, sensory involvement, painful muscle cramps, fasciculations, areflexia, elevated CK levels, hyperlipidemia	AD	604484
Roussy-Levy syndrome	Type 2	Ataxia, areflexia with amyotrophy	AD	180800
<b>Progeroid syndromes associated with glucose intolerance</b>				
Cockayne syndrome	IGT	Dwarfism, progeria, MR, deafness, blindness	AR	216400 133540
Acrogeria, Grotton type (Metageria)	Type 2	Early atherosclerosis, tall and thin, birdlike facies and aged appearance, normal sexual development, atrophic mottled skin, telangiectasia, little subcutaneous fat	?AR	201200
Werner syndrome	Type 2	Premature aging, cataracts, arteriosclerosis	AR	277700 604611
Mulvihill-Smith syndrome	Type 1	Premature aging, pigmented nevi, lack of facial subcutaneous fat, microcephaly, short stature, sensorineural hearing loss, mental retardation, immunodeficiency, tumors, severe insomnia, and cognitive decline	AD	176690
<b>Mitochondrial syndromes</b>				
Ballanger-Wallace syndrome	Type 1/Type 2	Deafness, cardiomyopathy, retinopathy		520000
MELAS syndrome	Type 1/Type 2	Myopathy, encephalopathy, lactic acidosis, strokelike episodes		540000



Kearns-Sayre syndrome	Type 1/Type 2	Short stature, microcephaly, sensorineural hearing loss, progressive external, ophthalmoplegia, retinopathy muscle weakness, cerebellar ataxia, dementia	Mitochondrial deletions	530000
Rotig syndrome	Type 1/Type 2	Proximal tubulopathy, cerebellar ataxia, myopathy, skin abnormalities		560000
Pearson Marrow-pancreas syndrome	Type 1	Sideroblastic anemia, exocrine pancreatic dysfunction, failure to thrive, metabolic acidosis	Mitochondrial deletions	557000
Mitochondrial myopathy, lipid type	Type 1/Type 2	Myopathy, cerebellar ataxia		500002
<b>Syndromes with glucose intolerance secondary to obesity</b>				
Achondroplasia	IGT	Disproportionate dwarfism, relative obesity	AD	100800
Bardet-Biedl syndrome	IGT→Type 2	Mental retardation, pigmentary hypogonadism, and obesity	AR (but may require more than one gene)	209900 600151 600374
Alstrom syndrome	IGT→Type 2	Features similar to Bardet-Biedl syndrome but no polydactyly	AR	203800
Hyperostosis frontalis interna	Type 2	Hyperostosis frontalis interna, obesity, hypertrichosis, galactorrhea, hyperprolactinemia, menstrual irregularity, and hyperphosphatasemia	AD? (most sporadic, majority female)	144800
Prader-Willi syndrome	Type 2	Obesity, short stature, acromicria, MR, disproportionate dwarfism	15q abnormal (deletion or altered imprinting)	176270
<b>Miscellaneous syndromes associated with glucose intolerance</b>				
Christian syndrome	IGT, Type 2	Short stature, ridged metopic suture, mental retardation, fusion of cervical vertebrae thoracic hernivertebrae, scoliosis, sacral hypoplasia, abducens palsy, carrier females may have type 2 DM or IGT	XR	309620
Williams syndrome	IGT→Type 2	Short stature, elfin facies, stellate iris, hypercalcemia, supraaortic stenosis, mental retardation, hoarse voice, anxiety	Contiguous gene deletion	194050
Epiphyseal dysplasia and infantile onset diabetes mellitus (Wolcott-Rallison syndrome)	Type 1 (congenital)	Epiphyseal dysplasia, tooth and skin defects	AR	226980
Symmetric lipomatosis	IGT→Type 2	Diffuse symmetric lipomas of neck and trunk, stiff skin, muscle cramps, decreased sensation, heating loss, urolithiasis, hypertension, peptic ulcers	AD	151800

**TABLE 86-1 Genetic Syndromes Associated with Glucose Intolerance and Diabetes Mellitus—cont'd**

Syndromes	Types of DM	Associated Clinical Findings	Pattern of Inheritance	McKusick#
Pyogenic sterile arthritis, pyoderma gangrenosum	Type 2	Cortisol-responsive pyogenic arthritis, pyoderma gangrenosum, and acne severe cystic acne, proteinuria	AD	604416
Woodhouse-Sakati syndrome	Type 2	Unusual facies, hypogonadism, absent breast tissue, sparse hair, mental retardation, sensineural deafness and ECG abnormalities	AR	241080
Bloom syndrome	Type 2	Prenatal onset growth retardation, microcephaly, facial telangiectasia, spotty hypo- and hyperpigmentation, photosensitivity, hypertrichosis, learning disability and/or mild mental retardation, life-threatening infections, neoplasia	AR	210900
AREDYLD	Type 2 (Lipoatrophic)	Prognathism, peculiar shape of nose, pronounced antitrageal incisura, hypotrichosis	AR	207780
Dunnigan-Type familial partial lipodystrophy	Type 2	Loss of subcutaneous fat from limbs, hypertension, dyslipidemia, premature CAD	AD	151660
Mandibuloacral dysplasia with type A lipodystrophy	Type 2	Partial lipodystrophy of extremities, mandibular hypoplasia, acroosteolysis, skin atrophy, alopecia in males, hyperlipidemia	AR (Allelic to Dunnigan-type familial partial lipodystrophy)	248370
Lipodystrophy, familial partial, type 3 Berardinelli-Seip syndrome	Insulin-resistant type 2	Acanthosis nigricans, insulin resistance, Congenital lipodystrophy, advanced bone age, overgrowth, hepatosplenomegaly, mild MR, lytic cystic lesions in appendicular bones, hypertriglyceridemia	AD AR	604367 269780
Lipodystrophy, familial partial, type 1	Type 2	Loss of subcutaneous adipose tissue in limbs, increase on trunk, xanthomata, CAD, hypertension, pancreatitis	AD or X-linked dominant?—only females reported	608600 604367
Mandibuloacral dysplasia with type B	Type 2	Mandibular hypoplasia, bird- like facies, generalized lipodystrophy lipodystrophy including face and neck, acroosteolysis, skin atrophy and sparse hair	AR	608612
Congenital malabsorptive diarrhea, type 4	Type 1	Congenital diarrhea	AR	610370
<b>Cytogenetic disorders associated with glucose intolerance</b>				
Down syndrome	IGT	MR, short stature, typical facies	Trisomy 21	
Klinefelter syndrome	IGT→Type 2	Hypogonadism, tall stature, MR	47, XXY	
Turner syndrome	IGT→Type 2	Short stature, gonadal dysgenesis, web neck	45, XO	

AD, autosomal dominant; AR, autosomal recessive; Type 1, Type 1 insulin-dependent diabetes mellitus; IGT, impaired glucose tolerance; IUGR, intrauterine growth retardation; JRA, juvenile rheumatoid arthritis; MR, mental retardation; Type 2, Type 2 non-insulin-dependent diabetes mellitus.

nonketotic insulin-resistant states, such as myotonic dystrophy and the lipotrophic diabetes syndromes), to relative insulin resistance (in the hereditary syndromes associated with obesity). Even within these individual categories, further division can be made, either by mechanism or by genetic criteria. For example, the lipotrophic syndromes—characterized by the total or partial absence of adipose tissue, hyperlipidemia, insulin resistance, nonketotic diabetes mellitus, increased basal metabolic rate, and hepatomegaly—can be further subdivided into a recessive, several dominant, and nongenetic forms (10) (MIM#’s 604367, 608594, 608600, 269700, 207780). Even within a more restricted phenotype, such as that of type 1 diabetes, these disorders demonstrate formal linkage heterogeneity. Thus neither the diabetes insipidus optic atrophy (Wolfram) syndrome, nor Friedreich ataxia, both of which clearly cause an insulin-dependent form of diabetes, are linked to the HLA region on chromosome 6 (11–13). In fact, frataxin, the gene for Friedreich ataxia, is located on the long arm of chromosome 9 (14), and the genes (WFS1 and CISD2 [WFS2]) causing Wolfram syndrome are located on the short and long arms of chromosome 4, respectively (15,16).

There are a variety of syndromes that are characterized by marked insulin resistance. The pathophysiology of the resistance of many of these disorders has been defined by studies of the insulin receptor and its interactions, with some disorders characterized by decreased receptor number, others by decreased receptor affinity, and still others by humoral antagonists to the receptor (17,18). A number of distinct molecular defects in the insulin receptor gene have been described in leprechaunism (Donohue syndrome), Rabson Mendenhall syndrome, and type A acanthosis nigricans syndrome (see MIM ID \*147670 for a description of the various mutations; (19, 20)). It is of interest that these affected individuals are often homozygous for a mutant allele or are compound heterozygotes. Relatives who are heterozygous for these defects have been found to have hyperinsulinemia without hyperglycemia (21). Even within what is felt to be one genetic entity, multiple endocrine neoplasia syndrome type 1, an autosomal dominant disorder characterized by pituitary, parathyroid, and pancreatic adenomas, a variety of different hormonal mechanisms can result in insulin antagonism (e.g. eosinophilic adenomas of the pituitary may secrete growth hormone, adenomas of the adrenal gland can secrete cortisol, and non-beta islet cells of the pancreas can produce glucagon) (22). Individually, each of the hormones is an insulin antagonist and their excess can lead to marked glucose intolerance. Thus, each of these many different genetic diseases are capable of resulting in carbohydrate intolerance through a variety of different pathogenetic mechanisms.

More recently, a distinct form of diabetes, presenting in very young infants, has also been found to be genetically heterogeneous (23). In one form of neonatal diabetes, transient diabetes develops at birth or shortly

thereafter but resolves after a few months. Such transient neonatal diabetes can result from mutations in at least three genes (ZFP57, ABCC8, KCNJ11) or from altered imprinting of a region of chromosome 6q24. Depending on the specific gene and mutation, the diabetes can be accompanied by intrauterine growth retardation, macroglossia, abdominal wall defects, congenital heart disease, and/or brain malformations. In addition, mutations in ABCC8, KCNJ11, INS, GCK, GLIS3, and PTF1A can produce permanent forms of neonatal diabetes, with or without additional abnormalities. Identifying the specific genetic mutation becomes critical in these conditions, as this will determine the most appropriate way to treat the diabetes.

These rare syndromes suggest that a similar degree of heterogeneity, both genetic and pathogenetic, may exist in “idiopathic” diabetes mellitus.

### 86.4.2 Heterogeneity Between Type 1 and Type 2 Diabetes

As summarized in Table 86-2, a number of lines of clinical and genetic evidence have led to the eventual separation of type 1 and type 2 diabetes as clearly distinct groups of disorders. Clinical differences that tended to run true in families provided some of the first evidence (24–28). In addition, the extensive MZ twin studies by Pyke and his coworkers in England strongly supported the separation of juvenile insulin-dependent and maturity non-insulin-dependent diabetes (29). Among 200 pairs of MZ (identical) twins, concordance for diabetes was shown to be less than 50% for type 1 diabetes, but close to 100% for type 2 diabetes. This suggested that there are a large group of individuals with type 1 diabetes in whom nongenetic as well as genetic factors may play a role in the development of clinical disease.

Physiological studies further supported the separation of type 1 and 2 diabetes. The absolute insulinopenic response of juvenile-onset diabetics versus the relative hyperinsulinemic response of maturity-onset diabetes parallels the therapeutic observation of the absolute insulin requirement of the juvenile (type 1) diabetic, which contrasts with the ability to manage most adult cases with oral hypoglycemics and/or diet (type 2 diabetes).

Immunologic studies pinpointed the importance of immune mechanisms in the etiology of type 1 but not type 2 diabetes. Direct evidence for an autoimmune role in the pathogenesis of type 1 diabetes came from the discovery of organ-specific cell-mediated immunity to pancreatic islets, and then the successful demonstration of antibodies to the islet cells of the pancreas (30). While these antibodies were first detected only in insulin-dependent diabetics with coexistent autoimmune endocrine disease, it soon became apparent that they were common (60–80%) in most newly diagnosed juvenile diabetics. Studies on islet cell antibodies (ICA) supported the differentiation of insulin-dependent from

**TABLE 86-2 Separation of Type 1 from Type 2 Diabetes**

Other Nomenclature	Type 1 IDDM (Juvenile-Onset Type)	Type 2 NIDDM (Maturity-Onset Type)
(1) Clinical	Thin Ketosis prone Insulin required for survival Onset predominantly in childhood and early adulthood	Obese Ketosis resistant Often treatable by diet or drugs Onset predominantly after 40
(2) Family studies	Increased prevalence of juvenile or type 1	Increased prevalence of maturity or type 2
(3) Twin studies	<50% concordance in monozygotic twins	Close to 100% concordance in monozygotic twins
(4) Insulin response to a glucose load	Flat	Variable
(5) Associated with other autoimmune	Yes	No endocrine diseases and antibodies
(6) Islet cell antibodies and pancreatic	Yes	No cell-mediated immunity
(7) HLA associations and linkage	Yes	No

non-insulin-dependent diabetes, as autoantibodies were present in 30–40% of the former group (even after onset) as opposed to 5–8% of the latter. Many (probably the majority) of the non-insulin-dependent, yet antibody positive, patients appear to become insulin dependent with time. They have flat insulin responses to a glucose load, and they also have the HLA-associated DR3 and DR4 antigens (31). This has suggested that etiologically, these cases belong to the insulin-dependent category (i.e. they are just in a transitional state on the way to eventual insulin dependence and share the same underlying pathogenic mechanisms as type 1 diabetes). Thus immunologic studies have served both to separate disorders (juvenile versus adult) and combine others (insulin dependent and non-insulin dependent, yet antibody positive).

Finally, the clear and consistent findings of HLA associations with juvenile insulin-dependent, but not maturity-onset non-insulin-dependent diabetes, became a major argument for etiologic differences between these two disorders; approximately 95% of type 1 diabetes patients have DR3 or DR4 or both (reviewed in (4,32)).

Based to a large extent on the evidence reviewed previously, the American Diabetes Association's current classification divides diabetes mellitus into four major subcategories: type 1 diabetes (insulin-dependent diabetes), type 2 diabetes (non-insulin-dependent diabetes), gestational diabetes, and other specific types of diabetes (33). The major defining characteristics of each of these categories are summarized in Table 86-3.

There is, however, some evidence that families of either type 1 or type 2 diabetes have more of the other type of diabetes than do families in the general population (34). Part of this overlap may be attributed to the insulin-independent phase of the insulin-dependent type (the frequency of which is still being defined), but it may also be the result of even further etiologic heterogeneity (see later discussion). In addition, the age of onset and other clinical differences can be important. For example, a distinct form of non-insulin-dependent diabetes that has been termed "maturity-onset diabetes of the young" (MODY) has been described (35) (discussed later).

The delineation of this entity clearly demonstrated that age of onset is a useful clinical criterion for classification purposes. Similarly, there is evidence that the age of onset may still be helpful as an additional classification criterion in type 1 diabetes, in that those individuals with both DR3 and DR4 alleles have the youngest age of onset. It has also been recognized that adults may develop type 1 diabetes, often with a less dramatic presentation than in children; this has been termed latent autoimmune diabetes of adults (LADA).

### 86.4.3 Type 1 (Insulin-dependent) Diabetes Mellitus

Type 1 diabetes mellitus is characterized by low levels or absence of endogenous insulin production (see Table 86-3). This is secondary to destruction of the insulin-producing beta cells of the pancreas and is the single characteristic that most decisively separates type 1 and type 2 diabetes. It is estimated that 5–10% of all US patients with diabetes have type 1 diabetes, and that the US incidence in children aged 0–14 years is in the range of 12 per 20/100,000 (36). The incidence appears to vary dramatically worldwide, from an estimated low of 1 per 100,000 children in parts of Asia and South America to greater than 40 per 100,000 in some regions in Scandinavia (36–39). In most regions of the world, the incidence appears to be increasing (38,40–43). The increasing incidence suggests that some environmental risk factor(s) for type 1 diabetes are becoming more prevalent, but the nature of these environmental risks is still under investigation.

### 86.4.4 Natural History and Pathophysiology of Type 1 Diabetes

Patients with a new diagnosis of type 1 diabetes typically present acutely ill, with severe dehydration, ketoacidosis, and marked hyperglycemia. The history is generally that the individual was well until perhaps a week or two before presentation, when increased thirst and urination were noted. Occasionally an account



**TABLE 86-3 Etiologic Classification of Diabetes Mellitus**

Class Name	Former Terminology	Characteristics
Type 1 diabetes <sup>a</sup>	Insulin-dependent diabetes (IDDM)	β-cell destruction, usually leading to absolute insulin deficiency
Immune mediated	Juvenile diabetes	Dependent on injected insulin to prevent ketosis and sustain life
Idiopathic	Juvenile-onset diabetes (JOD)	Onset predominantly in youth but can occur at any age Associated with HLA DR3 and DR4 Islet cell antibodies are frequently present before and at diagnosis
	Ketosis-prone diabetes	
	Brittle diabetes	
Type 2 diabetes <sup>a</sup>	Non–insulin-dependent diabetes (NIDDM)	May range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance
	Adult-onset diabetes	Insulin levels may be normal, elevated, or depressed
	Maturity-onset diabetes (MOD)	
	Ketosis-resistant diabetes	
	Stable diabetes	Not insulin-dependent or ketosis-prone under normal circumstances, but may use insulin for treatment of hyperglycemia or during stress conditions Onset predominantly after age 40, but can occur at any age Approximately 60% of patients are obese Hyperinsulinemia and insulin resistance characterize some patients
Gestational diabetes (GDM)	Gestational diabetes	Glucose intolerance that has its onset during pregnancy; (GDM) virtually all patients return to normal glucose tolerance following parturition Conveys increased risk for progression to diabetes
Other specific types includes: Genetic defects of β-cell function	Secondary diabetes	In addition to the presence of the specific condition, hyperglycemia at a level diagnostic of diabetes is also present
Genetic defects in insulin action		
Diseases of the exocrine pancreas		
Endocrinopathies		
Drug- or chemical-induced infections		
Uncommon forms of immune- mediated diabetes		
Other genetic syndromes sometimes associated with diabetes		

<sup>a</sup>Patients with any form of diabetes may require insulin treatment at some stage of their disease. Such use of insulin does not, of itself, classify the patient. Data from National Diabetes Data Group, 1979 (485), Expert Committee on Classification, American Diabetes Association, 2003 (33).

is given of a viral upper respiratory tract infection or other mild infectious illness shortly before the increased thirst and urination began. As a result, for many years, type 1 diabetes was thought to be an acute onset disorder. However, with the appreciation of the autoimmune nature of the beta cell destruction, this assumption began to be questioned.

Several studies followed nondiabetic identical cotwins and triplets of type 1 diabetic probands with serial testing for the presence of circulating autoantibodies (44,45) and, not surprisingly, many of these twins and triplets ultimately developed type 1 diabetes. What was

not anticipated, however, was that anti-ICA were often detectable months to many years before the time when these cotwins became overtly diabetic. These studies demonstrate that the development of type 1 diabetes actually occurs gradually. A variety of abnormalities in immune function and insulin release precede the “abrupt” development of the diabetic syndrome in patients genetically predisposed to type 1 diabetes (46). Eisenbarth (47) proposed dividing the development of type 1 diabetes into six stages: (1) genetic susceptibility; (2) triggering events; (3) active autoimmunity; (4) gradual loss of glucose-stimulated insulin secretion;

(5) appearance of overt diabetes, with some residual insulin secretion; and (6) complete beta cell destruction. The autoimmune destruction of pancreatic beta cells progresses slowly over time, and it is not until the majority of these cells have been destroyed that clinically apparent diabetes occurs. At the onset of type 1 diabetes, as little as 10% of the beta cells remain and, within several years, essentially all beta cells are destroyed (46). Maclaren (48) also pointed out that the pace of these events may well relate to the age of onset and the underlying genetic heterogeneity.

Nonobese diabetes (NOD) mice and biobreeding (BB) rats are excellent models of the autoimmune form of type 1 diabetes (see recent reviews in (49–51)). Data from these animal models indicate that T lymphocytes are important in the pathogenesis of islet T-cell destruction, as activated T lymphocytes from acute-diabetic BB rats can transfer diabetes to other animals (52). Similar evidence for the importance of T lymphocytes in human diabetes comes from the studies of pancreatic transplantation between identical twins (53,54). When pancreata were transplanted from nondiabetic twins to their diabetic MZ cotwins without immunosuppression, islet cell destruction with massive T-cell infiltration and relapse of the diabetes occurred within weeks. Thus, the basic defect in type 1 diabetes appears to be extrinsic to the pancreas and related to the activation of T lymphocytes, which then mediates the destruction of the islets (46).

The mechanisms resulting in the autoimmune destruction of the islets are complex. Although both T cells and the cytokines they excrete are directly involved in the destruction, it has become increasingly clear that it is not just autoreactive T cells that are important. Macrophages, natural killer cells, dendritic cells and altered Treg cell function have also been implicated (55). In light of the complex processes involved, it has been difficult to identify what the initiating abnormality is that triggers the autoimmune process. Given the many immune-related genes that are now implicated in type 1 diabetes, it is likely that the combination of a number of alterations in immune function is necessary to initiate beta cell destruction.

Because it is estimated that at diagnosis of type 1 diabetes about 10% of beta cells are still alive and capable of producing insulin, some patients go through a transient “honeymoon” phase in the early months after diagnosis, during which their requirement for exogenous insulin may decrease or even disappear. These remaining beta cells are lost within the first months to years following diagnosis, however, and all patients ultimately will require lifelong treatment with insulin.

With the recognition that there were functional beta cells still present at the time of diagnosis, treatment with immunosuppressants was attempted to see if type 1 diabetes could be reversed. Although it was possible to produce a remission in some patients who were

treated with cyclosporine within the first few months after diagnosis, the effect was short lived in most cases and overt diabetes returned as soon as cyclosporine was discontinued (56,57). More recent attempts to produce remission have utilized more narrowly targeted agents, such as humanized anti-CD3 or anti-CD20 monoclonal antibodies. While none of the subjects treated with these agents went into remission, there was evidence of preservation of C-peptide levels and in some cases a reduced insulin requirement was observed (58–60). The observation that these effects may persist for several years after short-term treatment is encouraging (60,61). At present, while research continues, there is no proof that it will be possible to cure diabetes once beta cell destruction has progressed to the point of overt diabetes. Therefore, attention has also been focused on attempts to intervene in high-risk individuals who have not yet progressed to clinical diabetes (see later discussion).

#### 86.4.5 Penetrance of Type 1 Diabetes

When the mode of inheritance is unknown, the only estimate we have for penetrance comes from identical twin concordance data. The largest twin data set (the British diabetic twin study) reported concordance for type 1 diabetes of some 50% of cases (29,62). However, it is clear that this sample is an unrepresentative one, with only a fraction of the twins in the British Isles identified, and thus there is a presumed bias toward concordant pairs (63). Studies of less-biased, but much smaller, samples reported concordances of approximately 20% (64). Finally, in 1988, a prospective study of twins from the British group yielded a concordance estimate of about 36%, an estimate similar to that in a recent (2009) study from Sweden (65,66). While the best estimate is that perhaps only one-third of all persons with the genes for type 1 diabetes actually develop clinical disease, one recent study has suggested that the concordance may be higher if twins are followed up long term (67).

Whichever estimate is used, however, it is clear that MZ twin concordance is substantially less than 100%. This reduced penetrance indicates that what is inherited in type 1 diabetes is disease susceptibility; other factors, presumably environmental, are required to convert genetic susceptibility into clinical disease. This view is supported both by the observation of the time of onset of type 1 diabetes clusters in families and twin pairs (68,69) and the epidemiological, experimental, animal, and clinical evidence for viral infections as a supervening factor in at least some cases (70–72) (see later discussion). However, environmental influence is not necessarily the only explanation, as the somatic recombination that occurs within the immune system is also a potential explanation for the reduced penetrance (see later discussion).

### 86.4.6 The HLA Region and Type 1 Diabetes

With the discovery of HLA antigen associations with type 1 diabetes, the genetic region that provides the major (but by no means only) genetic susceptibility to type 1 diabetes was located. The earliest studies implicating the HLA region in type 1 diabetes susceptibility demonstrated an increased frequency of the class I antigens B8 and B15 in white type 1 diabetes subjects (73–75). Subsequently, a large number of studies found an increased frequency of the class II HLA antigens DR3 and DR4 among white type 1 diabetes patients (76,77). Because of the linkage disequilibrium within the HLA region, the associations of B8 and B15 were thought to result from these alleles occurring with a high degree of frequency on DR3-containing (in the case of B8) and DR4-containing (in the case of B15) haplotypes. The type 1 diabetes association is unusual among HLA disease associations, because it involves two antigens, HLA-DR3 and DR4. In addition, the relative risk for type 1 diabetes in individuals who carry both DR3 and DR4 (compound heterozygotes) is greater than those homozygous for either DR3 or DR4 (76–78). This finding of the increased risk of the DR3/DR4 heterozygote was the first suggestion that more than one gene may predispose to type 1 diabetes.

Approximately 95% of all type 1 diabetes (in white populations) have HLA-DR3, DR4, or both, compared with about 50% of individuals in the nondiabetic population (32). There are also more subtle relative increases in HLA-DR1 (especially among those who have only one copy of DR3 or DR4); conversely, DR2 and DR5 are decreased in individuals with type 1 diabetes (79,80). HLA-DR3 and DR4 (as defined serologically) are not pathognomonic of type 1 diabetes; nearly half the US population has either DR3 or DR4 (only 1–3% have both), yet only a small percentage (about 0.5%) of these individuals will develop type 1 diabetes. However, if one's sibling has type 1 diabetes, the chance of a DR3 or DR4 individual developing type 1 diabetes rises sharply (12–24%). These observations suggested that DR3 and DR4 as defined serologically could not adequately explain the risk for type 1 diabetes present in the HLA region. Either the serologic DR typing was not sensitive enough to detect the type 1 diabetes-specific forms of DR3 and DR4 or other genes were responsible for at least some of the type 1 diabetes susceptibility associated with the HLA region.

With the advent of molecular HLA typing, it became clear that both these possible explanations are indeed important in understanding the role of the HLA region in type 1 diabetes susceptibility. Initially, when the complexity of the HLA class II region was uncovered, attention focused on the hypothesis that another locus in tight linkage disequilibrium with DR was actually responsible for type 1 diabetes susceptibility. Studies demonstrated that the HLA class II region consists of at least three

genetic loci: DR, DQ, and DP, each of which codes for a slightly different glycoprotein consisting of two peptide chains, alpha and beta. Because of this variability, there are differences at the DNA level between diabetics and nondiabetics, even when they share the same serologic DR type.

Given the extensive linkage disequilibrium, identifying which class II locus was primarily responsible for type 1 diabetes susceptibility was challenging. Studies in white populations initially implied that the primary locus for type 1 diabetes susceptibility was the DQ beta gene, with the DQB1\*0302 allele being highest risk. However, subsequent studies in other ethnic groups led to the realization that other genes within the class II region clearly are of importance as well. Thus, for example, in Mexican American type 1 diabetes patients, DRB1\*0402-DQB1\*0302 and DRB1\*0405-DQB1\*0302 (European white haplotypes) are strongly associated with risk, whereas DRB1\*0408-DQB1\*0302 and DRB1\*0411-DQB1\*0302 (Native American haplotypes) are actually protective against type 1 diabetes, even though they all contain the DQB1\*0302 allele (81). In most, but not all populations that have been studied, the HLA haplotypes that are commonly associated with risk for type 1 diabetes are DRB1\*0301-DQA1\*0102-DQB1\*0201 and DRB1\*0401-DQA1\*0301-DQB1\*0302, whereas other haplotypes such as DRB1\*1501-DQA1\*0102-DQB1\*0602 are strongly protective against type 1 diabetes (82,83). Such data suggest that at a minimum, the DRB1, DQA1, and DQB1 loci are implicated in diabetes susceptibility. Additionally, the DPB1 locus has also been shown to play a role in type 1 diabetes susceptibility in both white and Mexican American populations (84,85). It thus now appears that many of the class II loci are involved in type 1 diabetes susceptibility, with associations reported with the DQ beta region (DQB1\*0302), DQ alpha region (DQA1\*0301) and the DP beta region (DPB1\*0301), as well as with DR itself (84,86,87).

The HLA region is even more complex, however, extending across approximately 7.6Mb and containing more than 250 genes (88). Because of the extensive linkage disequilibrium across the HLA region, it has proven to be very difficult to determine the loci that actually contribute to type 1 diabetes risk versus those loci that demonstrate associations only as a result of linkage disequilibrium. It is now clear that the HLA class II region is not the only portion of the HLA region conferring risk to type 1 diabetes. Data have been reported suggesting that genes in many other HLA regions may also be involved in disease susceptibility (86,89–96). There is quite strong evidence implicating the HLA-A and B class I genes (87,97). Most recently, a conditional meta-analysis was performed using data from the Wellcome Trust Case-Control Consortium and International Type 1 Diabetes Consortium and it implicated the genes TCF19, POU5F1, CCHCR1, and PSORS1C1 within the class I region (98).

While it is still unclear which of these putative associations will ultimately be confirmed, the observed increased risk for type 1 diabetes in DR3/4 heterozygotes compared with either DR3/3 or DR4/4 homozygotes means that the susceptibility to type 1 diabetes associated with DR3 and DR4 are different. This is reinforced by the observation that familial aggregation of type 1 diabetes suggests that DR3 susceptibility acts in a recessive manner, with most DR3-carrying type 1 diabetes patients also having a second high-risk HLA haplotype (containing either DR3 or DR4). DR4-related type 1 diabetes susceptibility, on the other hand, appears to act in a dominant manner, as demonstrated by the observation of many DR4-carrying type 1 diabetes patients who do not carry a second high-risk haplotype (99).

There is also evidence for phenotypic heterogeneity between type 1 diabetes associated with HLA-DR3 and that associated with HLA-DR4 (Table 86-4) (reviewed in (4)). The DR3 form of the disease is characterized by a greater persistence of pancreatic ICA and antipancreatic cell-mediated immunity, but a relative lack of antibody response to exogenous insulin. This form apparently has onset throughout life and probably accounts for a significant fraction of older-onset type 1 diabetes. In the older age groups, this form of type 1 diabetes may present as latent autoimmune diabetes (LADA) and may be treated without insulin for a significant period, but the presence of ICA presages eventual insulin dependence (100). Type 1 diabetes associated with DR4 is not as strongly associated with autoimmune disease or ICA, but this form is accompanied by an increased antibody response to exogenous insulin (101,102). The relation between HLA-DR4 and insulin immunogenicity also can be seen before the initiation of exogenous insulin therapy, with the occurrence of insulin antibodies before disease onset (103–105). DR4-associated type 1 diabetes also appears to have an earlier age of onset, exhibits seasonality, and may be related to viral infections.

With multiple genes participating, it is clear that the mechanisms by which the HLA region produces susceptibility must be complex. Based on our current understanding of the functioning of the various HLA genes, the alleles associated with increased risk of diabetes likely alter the way that the class I and class II receptors bind peptide antigens and interact with antigen-presenting cells (APCs), CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T cells (106). However, a more complete understanding of these mechanisms must await further clarification of both the number and identity of the HLA-linked genes that account for type 1 diabetes risk.

### 86.4.7 Non-HLA Region Genes and Type 1 Diabetes

Estimates of the proportion of genetic susceptibility to type 1 diabetes for which the HLA region accounts vary, but even the highest estimates are in the range of 60–70%, clearly indicating that other, non-HLA loci must also exist that play a role in type 1 diabetes (99,107,108). Over the past several decades, numerous other regions have been implicated in type 1 diabetes susceptibility, but until the advent of the GWAS era, the data in support of most loci remained fairly tentative.

**86.4.7.1 Candidate Genes and Type 1 Diabetes.** Before GWAS, the best substantiated locus was IDDM2, near the insulin gene on the short arm of chromosome 11. This region was first implicated in the early 1980s, when a polymorphic region 5' to the insulin gene (INS) was discovered (109). The polymorphism results from the presence of a variable number tandem repeat (VNTR) region near the 5' regulatory region of INS. A number of population studies reported an association between type 1 diabetes and class I alleles, which have smaller numbers of tandem repeats in this region as compared to the class III alleles (110,111). Although it is now appreciated that classical linkage methods are

**TABLE 86-4 Heterogeneity within Type 1 Diabetes**

Evidence	DR3	DR4	Combined Form (DR3/DR4)
Linkage disequilibrium	A1, B8	B15, DQβ1*0302	Penetrance in MZ twins
Insulin antibodies	Nonresponder (low antibody titers)	High responder (high antibody titers)	Occurrence in familial cases
Islet cell antibodies	Persistent	Transient	
Insulin autoantibodies	Less frequent	Increased frequency	Highest titers
Antipancreatic cell-mediated immunity	Increased	Not increased	
Thyroid autoimmunity in	Yes	Less frequent Type 1 DM	
Associated with other autoimmune	Yes	No endocrine diseases	
IgA deficiency in	Increased	Not increased Type 1 DM	
Age of onset	Any age	Younger age	Youngest
Ketoacidosis at	Lesser frequency	Greater frequency clinical onset	
Levels of C-peptide	Preserved longer	Absent after shorter duration	Lowest



rarely informative when analyzing loci contributing a comparatively small amount to disease susceptibility, when these association studies were followed by family studies that failed to demonstrate linkage, there was some controversy as to whether the association was real or not. With the advent of family-based association methodologies, a role for IDDM2 was better substantiated and this locus became the second (after HLA) well accepted in type 1 diabetes susceptibility locus (110–112).

For many years, however, questions remained about how this region results in diabetes susceptibility, until it was shown that it affects expression of insulin mRNA in the fetal thymus and thus influences the development of tolerance to antigenic determinants of insulin (113,114). Class I alleles, which contain smaller numbers of repeats and are associated with increased risk for diabetes, have been observed to result in lower insulin gene mRNA expression in the thymus. The other common allele size, known as Class III alleles, has greater numbers of repeats within the VNTR and has been associated with decreased diabetes risk and increased thymic mRNA levels. Recently Cai et al. (115) showed that the mechanism for this altered expression involves the autoimmune regulator (AIRE) protein, which interacts with the INS VNTR to regulate insulin expression. The correlation between VNTR length and thymic expression is not absolute, however. Vafiadis and coworkers (116) observed that in a minority of subjects, silencing of the Class III allele occurs, resulting in thymic expression from the other insulin allele. Such silenced Class III alleles predispose to type 1 diabetes, presumably because they lead to overall reduced mRNA levels. At the level of the VNTR, the mechanism responsible for silencing has not yet been identified but is hypothesized to result from altered imprinting.

CTLA4, cytotoxic T-lymphocyte-associated protein 4, is a member of the immunoglobulin superfamily, and was tested as a candidate gene within the IDDM12 linkage region on chromosome 2q33, which was homologous to the *Idd5* locus in the mouse. Association with type 1 diabetes was demonstrated in several ethnic groups, as well as associations with several other autoimmune disorders, especially thyroid disease (117–126). CTLA4 is a negative immunoregulatory molecule involved in T-cell activation and expansion that appears to be important in normal Treg function (127,128).

PTPN22 encodes the lymphoid-specific protein tyrosine phosphatase (LYP), which is another inhibitor of T-cell activation (83,129,130). The 1858 T allele, which replaces arginine with a tryptophan residue at codon 620, was originally reported to be associated with type 1 diabetes by Bottini et al. (131). Like CTLA4, it has also been implicated in other autoimmune diseases, including rheumatoid arthritis, autoimmune thyroid disease, lupus, celiac disease, and autoimmune vasculitis (122,132–135). The associated allele results in a gain of

function (136), suggesting that inhibitors of LYP could be of therapeutic benefit.

IL2RA, interleukin 2 receptor A gene (also known as CD25), was first reported to be associated with type 1 diabetes by Vella et al. (137) and subsequently replicated (138–140). Like CTLA4 and PTPN22, IL2RA is associated with risk for other autoimmune diseases including Graves' disease, multiple sclerosis, and juvenile idiopathic arthritis (141–143). The mechanism by which IL2RA results in risk for autoimmune disease is still under study, but the allele associated with protection against type 1 diabetes results in higher CD25 levels on CD4+ memory cells and such cells are likely more responsive to IL-2 and to TCR-mediated activation (144).

#### 86.4.8 Identifying Type 1 Diabetes Genes by Genome-Wide Linkage Scans

With the application of systematic linkage mapping to type 1 diabetes, a variety of other candidate gene regions were mapped. Some of the linkage regions corresponded to previously known loci, including HLA and INS. Studies of candidate genes within other regions, including CTLA4 (located within the IDDM 12 region on chromosome 2q and SUMO4 in the IDDM5 region on chromosome 6q), appear to explain the observed linkage peaks (118,121,145–150). Data suggest that SUMO4 contributes to type 1 diabetes susceptibility in Asians, but it is less clear whether this gene is important in Caucasian populations (140,149–151). Data for other regions have been equivocal and many may ultimately turn out to have been false positives.

#### 86.4.9 Type 1 Diabetes Genes Identified by Genome-Wide Association Studies

GWAS have resulted in the identification of a substantial number of genes for type 1 diabetes (Table 86-5). This has been facilitated by cooperation between multiple centers, both in the acquisition of large numbers of cases and controls and in data sharing to confirm association signals. The first genome-wide association study for type 1 diabetes was published in 2006 reporting an association with the innate immunity viral RNA receptor gene region, IFIH1 (152). This was followed rapidly by a number of other GWAS (153–156) that increased the number of loci demonstrating association. Additional loci have been identified by GWAS meta-analyses using very large combined study populations (one with more than 3500 cases and 4500 controls (157) and a second with more than 7500 cases and 9000 controls (158)). Many of the GWAS and meta-analysis findings have also been confirmed in independent samples, and as of early 2011, there are more than 50 loci with quite convincing evidence for association with type 1 diabetes (Table 86-5; see also <http://www.t1dbase.org> (159)).

TABLE 86-5 Susceptibility Genes for Type 1 Diabetes						
Locus: Nearest Gene(s)	Chromosomal Location	Marker(s)	Variant Type(s)	Source of Initial Discovery	Diabetes Effect: OR (95% CI)	Other Genom Wide Associat Disorder(s)
PTPN22	1p13.2	rs2476601	Arg620Trp	Candidate gene study in North American Caucasians	2.05	Crohn's, Graves', SLE, Vitiligo
RGS1	1q31.2	rs2816316	8 kb upstream	Candidate gene study following up a Celiac GWAS hit European Caucasian	0.89	Celiac, MS
CD55, IL10	1q32.1	rs3024505	1 kb downstream of IL10	GWAS meta-analysis in European & North American Caucasians	0.84	Crohn's, SLE, UC
AFF3	2q11.2	rs9653442	66 kb upstream,	GWAS meta-analysis in European & North American Caucasians	1.11	JIA, RA
IFIH1	2q24.2	rs1160542	73 kb upstream		0.81–0.86	Graves', SLE
		rs1990760	Ala946Thr	GWAS analysis in European Caucasians		
STAT4	2q32.2	rs7574865	Intronic	Candidate gene study following up a RA & SLE GWAS/linkage hits European Caucasians	1.1–1.11	RA, SLE, SSC, pSS
CTLA4	2q33.2	rs6752770 rs3087243	Immediately downstream	Candidate gene study	0.82–0.88	Celiac, RA
CCR5	3p21.31	rs11711054 rs333	66 kb upstream, 32-bp insertion–deletion variant	Candidate gene study in European Caucasians; multiple prior small studies with equivocal results	0.85	Celiac
	4p15.2	rs10517086	Intergenic	GWAS meta-analysis in European & North American Caucasians	1.09	
IL2	4q27	rs2069762, rs2069763, rs4505848	Immediately upstream, Leu-38Leu, 240 kb downstream	GWAS meta-analysis in European & North American Caucasians	0.89 1.13	UC
HLA-DQB1, HLA-B, HLA-DRB1, HLA-C	MHC			Candidate gene studies in multiple populations	3.05	
BACH2	6q15	rs11755527	Intronic	Candidate SNP follow-up of suggestive GWAS associations in European & North American Caucasians	1.13	
	6q22.32	rs9388489	Intergenic	GWAS meta-analysis in European & North American Caucasians	1.17	

TNFAIP3	6q23.3	rs10499194, rs2327832, rs6920220	188 kb upstream, 216 kb upstream, 182 kb upstream	Candidate gene study following up an RA GWAS in North American Caucasians	0.9 1.09	Celiac, RA, SLE, U
TAGAP	6q25.3	rs1738074	5' untranslated region	Candidate gene study following up a Celiac GWAS in European Caucasians	0.92	Celiac
SKAP2	7p15.2	rs7804356	Intronic	GWAS in North American Caucasians	0.88	
IKZF1	7p12.2	rs10272724	4 kb downstream	Candidate gene study following up an acute leukemia GWAS & borderline T1D GWAS in European Caucasians	0.87	
	7p12.1	rs4948088	Intergenic	GWAS meta-analysis in European & North American Caucasians	0.77	
GLIS3	9p24.2	rs7020673	Intronic	GWAS meta-analysis in European & North American Caucasians	0.88	
IL2RA	10p15.1	rs11594656, rs12722495	18 kb upstream, intronic	Candidate gene study in European & North American Caucasians	0.84–0.87 0.62–0.63	MS, RA, Vitiligo
PRKCQ	10p15.1	rs11258747, rs947474	Arg616Arg, 79 kb downstream	GWAS meta-analysis in European & North American Caucasians	0.69 0.88–0.91	
ZMIZ1	10q22.3	rs1250550, rs1250558	Intronic	GWAS meta-analysis in European & North American Caucasians		IBD
RNLS	10q23.31	rs10509540	11 kb downstream	GWAS meta-analysis in European & North American Caucasians	0.75	
INS	11p15.5	rs689	5' untranslated region	Candidate gene studies in multiple populations	0.42	
CD69	12p13.31	rs4763879	Intronic	GWAS meta-analysis in European & North American Caucasians	1.09	
CYP27B1	12q13.3	rs10877012	Immediately upstream of gene	Candidate gene study in European Caucasian families	1.22	MS
ERBB3	12q13.2	rs2292239	Intronic	GWAS meta-analysis in European & North American Caucasians	1.31	
SH2B3	12q24.12	rs3184504,	Trp262Arg	GWAS in European Caucasians	1.28	Celiac, MS

TABLE 86-5 Susceptibility Genes for Type 1 Diabetes—cont'd						
Locus: Nearest Gene(s)	Chromosomal Location	Marker(s)	Variant Type(s)	Source of Initial Discovery	Diabetes Effect: OR (95% CI)	Other Genom Wide Associat Disorder(s)
GPR183	13q32.3	rs9585056	122 kb upstream	Candidate gene study in European Caucasians following up rat expression data & borderline T1D GWAS in European Caucasians	1.15	
	14q24.1	rs1465788	Intergenic	GWAS meta-analysis in European & North American Caucasians	0.86	
	14q32.2	rs4900384	Intergenic	GWAS meta-analysis in European & North American Caucasians	1.09	
DLK1	14q32.2	rs941576	105 kb downstream	GWAS meta-analysis in European & North American Caucasians	0.90	
RASGRP1	15q14	rs17574546, rs7171171	45 kb upstream, 50 kb upstream	Family-based GWAS meta-analysis in North American Caucasians	1.21	
CTSH	15q25.1	rs3825932	Intronic	GWAS meta-analysis in European & North American Caucasians	0.86	
CLEC16A	16p13.13	rs12708716	Intergenic	GWAS meta-analysis in European & North American Caucasians	0.81	MS, SLE
IL27	16p13.13	rs12927773	Intergenic	GWAS in European Caucasians	0.81	Celiac
	16p11.2	rs4788084	22 kb upstream	GWAS meta-analysis in European & North American Caucasians	0.86	Crohn's, IBD
	16q23.1	rs7202877	Intergenic	GWAS meta-analysis in European & North American Caucasians	1.28	
GSDMB, ORMDL3	17q12	rs2290400	Intron of GSDMB	GWAS meta-analysis in European & North American Caucasians	0.87	Crohn's, UC
	17q21.2	rs7221109	Intergenic	GWAS meta-analysis in European & North American Caucasians	0.95	



PTPN2	18p11.21	rs45450798, rs478582	Intronic	GWAS in European Caucasians	1.28 0.83	Celiac, Crohn's
CD226	18q22.2	rs763361	Ser307Gly	GWAS meta-analysis in European & North American Caucasians	1.16	MS
TYK2	19p13.2	rs2304256	Val362Phe	GWAS meta-analysis in European & North American Caucasians	0.86	
PRKD2	19q13.32	rs425105	Intronic	GWAS meta-analysis in European & North American Caucasians	0.86	
FUT2	19q13.4	rs602662	Gly258Ser	GWAS meta-analysis in European & North American Caucasians		Crohn's
SIRPG	20p13	rs2281808	Intronic	GWAS meta-analysis in European & North American Caucasians	0.90	
UBASH3A	21q22.3	rs3788013	Intronic	Family-based GWAS in North American Caucasians	1.13	Vitiligo
AIRE	21q22.3	rs760426	Intronic	Candidate gene causing APECED	1.12	
	22q12.2	rs5753037,	Intergenic	GWAS meta-analysis in European & North American Caucasians	1.10	Crohn's, IBD
IL2RB	22q12.3	rs3218253	Intronic	GWAS meta-analysis in European & North American Caucasians		RA
C1QTNF6	22q13.1	rs229527, rs229541	Gly21Val, 7 kb upstream	GWAS meta-analysis in European & North American Caucasians	1.11–1.12	Vitiligo
TLR7, TLR8	Xp22.2	rs5979785	Intergenic	GWAS meta-analysis in European & North American Caucasians	0.86	Celiac
GAB3	Xq28	rs2664170	Intronic	GWAS meta-analysis in European & North American Caucasians	1.16	

Adapted from <http://t1dbase.org/> accessed 3/20/2011.

GWAS identify single nucleotide polymorphisms (SNPs) that demonstrate association with a disorder or trait of interest. Occasionally, the associated SNP results in a functional change and is directly responsible for the observed association. More commonly, however, it is unlikely that the associated SNP is actually causing disease risk. In such situations, the SNP association directs research to a specific chromosomal segment, but identifying which genes within the associated regions and which variants within specific genes are responsible for type 1 diabetes susceptibility continues to be a challenge. Many of the GWAS loci contain genes that are involved in immune function, which are good candidates. In addition, a number of the same regions have been identified in GWAS performed for other autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, celiac disease, Graves' disease, Crohn's disease, and vitiligo. Based on such observations, it is likely that some loci are associated with a generalized risk for autoimmunity, whereas other loci may provide the specificity that determines exactly which autoimmune disease will present clinically. Understanding the ways in which the various loci interact to produce type 1 diabetes will only be possible after the actual susceptibility genes and functional variants have been definitely identified.

What also remains to be clarified is how many genes are involved in type 1 diabetes susceptibility. As is true with all complex genetic disorders, the genes and loci known from candidate gene studies and GWAS still do not account for all the observed heritability of the disease. One possible explanation for this "missing heritability" is that rare variants that are not detectable using current analytic methods and sample sizes may contribute to disease. On the assumption that such rare variants will only be identifiable from sequencing, exome and whole-genome sequencing projects are now underway. While it is expected that these approaches will increase the proportion of heritability that can be explained, other factors, including gene–gene interactions, epigenetic factors, copy number variations, noncoding RNAs that affect gene expression, and gene–environment interactions, may also be implicated.

#### 86.4.10 The Role of Environmental Factors in Type 1 Diabetes

The possibility that there are important environmental components to the etiology of type 1 diabetes is raised by the MZ twin data, which show a type 1 diabetes concordance of approximately 20–40%. As discussed previously, the lack of 100% concordance in MZ twins does not absolutely require the involvement of environmental factors; immunologic gene rearrangements could also provide an explanation for such a reduced penetrance (160–162). Yet the possibility of environmental factors having a significant role must be thoroughly investigated,

especially as regards the implications for preventive strategies. Environmental effects on diabetes development have been postulated going back to the 1920s, based on the observation of seasonal variations in the diagnosis of diabetes in children and young adults (163). As the pathogenetic processes that lead to type 1 diabetes appear to be complex and may take years from initiation to completion, environmental agents could function as initiating factors (i.e. factors that begin the etiologic processes that eventually terminate in type 1 diabetes), or, alternatively they could act mainly as precipitating factors (i.e. factors that convert preclinical diabetes into clinical disease). In either role (or both), what is clear is that environmental factors must act on genetically susceptible individuals for type 1 diabetes to occur. Several classes of environmental agents have been implicated in the etiology of type 1 diabetes.

**86.4.10.1 Infectious Agents.** A viral etiology for diabetes has been suggested for many years, with case reports of diabetes following an episode of an infectious disease dating back to the 1800s (164,165). The current evidence for a role of viral agents comes from several sources, including case reports, epidemiological studies, clinical studies, and evidence from animal and human models.

Anecdotally, a "viral-like illness" is known to precede the onset of many cases of type 1 diabetes (166). Several lines of epidemiological evidence are also consistent with an infectious etiology. For example, it has been noted that trends in age at onset of diabetes are consistent with a viral etiology (69). The total number of infections during the preceding year has been shown to correlate with type 1 diabetes risk (167).

Another suggestion that environmental agents play a role in the etiology of type 1 diabetes comes from studies of time of clinical disease onset in pairs of siblings with type 1 diabetes. At least one study suggested that sibling pairs are more likely to have their onset of diabetes within a year of one another than would be expected by chance (69). Similarly, the period of discordance for type 1 diabetes in MZ twins where both ultimately develop type 1 diabetes has been reported to be less than 3 years in 60% (168).

There is also limited evidence for an infectious agent's role (e.g. mumps, coxsackie) from seroepidemiological studies (i.e. studies that compare viral and bacterial antibody titers in type 1 diabetics and nondiabetic controls) (71,72,166). While some studies have suggested an association, there have also been many negative studies. Green and colleagues (169) performed a systematic review of all case-control studies examining Coxsackie viruses and found a range of odds ratios from 0.2 to 22.3 for type 1 diabetes mellitus in serology-positive vs. serology-negative subjects; 7 of 13 studies had point estimates significantly greater than 1.0 ( $P < 0.05$ ). Additionally, studies have utilized molecular techniques to determine the prevalence of viral DNA. For example,

Nairn and colleagues (170) reported detection of enterovirus RNA in 27% of children with new-onset type 1 diabetes as compared to only 4.9% of controls and a recent meta-analysis of multiple studies has provided further support (171).

The effect of childhood immunization on type 1 diabetes susceptibility has also been controversial. Although a study of the incidence of type 1 diabetes in relationship to the introduction of the measles-mumps-rubella vaccination and the subsequent disappearance of mumps in Finland suggested that elimination of natural mumps infection has decreased the incidence of type 1 diabetes (172) and measles vaccination by itself correlated with a lower risk of type 1 diabetes (167), the observation that an increasing incidence of type 1 diabetes has paralleled the expansion of childhood immunization has resulted in postulation that one or more of these vaccines may increase diabetes risk. While a number of large-scale studies appear to have disproven this hypothesis (173–175), it remains a concern for many, particularly among parents' groups.

If indeed viral infections do play a role in the development of overt diabetes, the question that must be asked is what mechanisms are involved. The insulinitis that has been noted in early type 1 diabetes could be consistent with viral infection of the pancreas, and autopsy studies have clearly documented pancreatic beta cell damage in children dying from overwhelming viral infections (176). Coxsackie B-specific antigens have specifically been found in the islets of Langerhans, and the coxsackie B4 virus itself has been isolated from the pancreas of a child dying of acute-onset type 1 diabetes (177) as well as from pancreata removed from diabetic patients undergoing organ transplantation (178). Several types of viruses are known to be capable of infecting human pancreatic beta cells in vitro, and data suggest that coxsackie virus B groups, rubella virus, and possibly cytomegalovirus are capable of producing pathologic beta cell changes in vivo.

Molecular mimicry has also been postulated to be a mechanism by which viral infection can impact the development of type 1 diabetes (179). There are regions of sequence homology between glutamic acid decarboxylase (GAD) and the p2C protein of coxsackie B virus, and between regions of both GAD and tyrosine phosphatase IA-2 and the VP7 protein of rotavirus (180,181). IA-2 also demonstrates significant sequence homologies with a variety of other viral genomes, including dengue, CMV, measles, and hepatitis C (182). Such observations raise the possibility that viral infection can trigger an anti-beta cell autoimmune response by such molecular mimicry. This possibility is further supported by the observation that the homologous peptides from VP7 and IA-2 both bind to HLA-DR4 (\*0401), whereas peptides from p2C and GAD both bind to DR3 (182,183). Antibodies to GAD species and to the p2C protein have also been shown to cross-react

in some, but not all, studies. Some authors have argued that molecular mimicry with viral proteins may actually play more of a role in the activation of preexisting auto-reactive T cells than in de novo stimulation of an autoimmune response (184). In this case, a viral infection could act to precipitate the development of diabetes by acutely exacerbating a previously existing low-grade autoimmune process.

The best human models of infectious agents in type 1 diabetes come from studies of individuals with the congenital rubella syndrome and from serial studies of children with viral infections who subsequently develop type 1 diabetes. The incidence of type 1 diabetes and other autoimmune diseases among children and young adults with the congenital rubella syndrome is markedly increased over that in the general population and may be as high as 15–40%. Those cases of congenital rubella with type 1 diabetes have an increased frequency of HLA-DR3 and DR4 and a decreased frequency of HLA-DR2, much as in non-rubella type 1 diabetes cases (185). A significant proportion of patients with congenital rubella syndrome have T-cell subset abnormalities, and a variety of autoimmune antibodies, including anti-thyroid microsomal, anti-thyroglobulin, and anti-islet cell and islet-cell surface antibodies, suggesting an autoimmune etiology for their type 1 diabetes (185,186). Rubella virus has been isolated from the pancreas of several cases with congenital rubella syndrome (187), and at least one case is known of insulinitis and beta cell destruction in an infant with congenital rubella infection who died of acute diabetes (188). This evidence suggests that rubella can indeed infect and damage the beta cell, and that the diabetes seen in congenital rubella syndrome could be due either to initiation of an immune process by the rubella virus, or directly to persistent pancreatic rubella infection.

**86.4.10.2 Dietary Agents and Molecular Mimicry.** A continuing area of interest regarding environmental triggers for type 1 diabetes has been dietary exposure. In the 1990s several studies reported an association of the introduction of cow's milk and cessation of breast feeding with type 1 diabetes risk (189–193). Elevated antibodies to several cow's milk proteins have also been found in children with type 1 diabetes (193,194). When it was reported that one epitope of bovine serum albumin, a 17-amino-acid residue peptide (ABBOS) cross-reacts with p69, a beta cell surface protein, the hypothesis was raised that early introduction of cow's milk into the infant diet could result in initiation of autoimmune injury to the beta cell via this molecular mimicry (195,196). In addition to bovine albumin, the hypothesis has been put forward that bovine insulin in cow's milk could trigger development of anti-insulin antibodies (197). However, several subsequent large studies of infant nutrition have not supported the association of autoimmunity to cow's milk and type 1 diabetes risk and the likelihood that cow's milk has a significant effect on risk now appears

small (198–200). Despite these studies, the possible role for dietary exposure in diabetes risk remains a topic of study. Dietary exposures may, for example, influence the development of type 1 diabetes in individuals who are genetically at high risk (201,202). To date, however, the data appear to argue more strongly for infectious triggers than for dietary ones.

### 86.4.11 Genetic Counseling in Type 1 Diabetes

Because the mode of inheritance in type 1 diabetes is not straightforward, most genetic counseling for type 1 diabetes is based on empirical risk estimates, which have been developed from both population-based and family-based epidemiological studies (Table 86-6). These recurrence risks are frequently reassuring to families, as the risks are often less than the family has feared, particularly for siblings of the type 1 diabetes patient. The empirical risk of recurrence for type 1 diabetes is dependent on the relationship of the individual in question to the affected family member. For siblings the empirical risk is approximately 5–10%. If the father is affected, the risk to his offspring is 4–6%, compared with 2–3% if the mother is affected (203,204). For further refinement of sibling risks, HLA testing and autoantibodies can be used to determine haplotype sharing with the diabetic sib (205). If two HLA haplotypes are shared, the risk increases to 16–17%, and is 20–25% if the haplotypes contain both DR3 and DR4. Siblings who share one haplotype have a risk in the range of 5–7%, whereas the risk is approximately 1–2% if no haplotypes are shared (4). It is important to realize that

the sibling of an individual with type 1 diabetes still has a risk for type 1 diabetes that is increased above that of the general population, even when the sib shares no HLA haplotypes in common with the diabetic in the family.

There is some question regarding the benefit of performing genotyping or autoantibody testing for the siblings of an individual with type 1 diabetes when at present it will not lead to any alteration in management. While testing may be performed as part of research studies examining possible prevention strategies, at present there are no proven therapies for individuals who are found to be at high risk. Particular attention must be paid to the potential negative effects of stigmatization and the risks of the child being treated as though he is ill. The concept that HLA testing at best identifies someone more susceptible to developing type 1 diabetes but in no way guarantees that she will become diabetic must be stressed in discussions with the family. The potential for other negative effects, such as possibly being ruled ineligible for health, life, and/or disability insurance because of the presence of a “preexisting” condition must also be discussed whenever a family is contemplating more refined testing. Such risks have been reduced in the US with the enactment of the Genetic Information Nondiscrimination Act of 2008 (GINA), but still remain of concern (206).

What is clear is that at present genotyping is not appropriate as a clinically applied screening tool for the general population. Approximately 50% of the nondiabetic population has the same HLA-DR types as patients with type 1 diabetes. Thus, at least 98% of the people with DR3 or DR4 will never develop type 1 diabetes. For every 1000 persons with HLA-DR3 or DR4 in the population, only two to four will develop type 1 diabetes in their lifetimes (see Table 86-6). While GWAS have identified many more genetic loci that contribute to risk, the overall ability to predict individuals who will go on to develop type 1 diabetes remains far from ideal (207).

In order for risk prediction to become appropriate, a mechanism for intervening to delay or prevent diabetes onset is needed. A few decades ago, there was great hope that such interventions were close to reality. A variety of clinical trials were undertaken to test various methods of intervention to prevent or delay the onset of type 1 diabetes, including the Diabetes Prevention Trial (DPT-1) (USA), Type 1 Diabetes Prediction and Prevention Project (DIPP) (Finland), European Nicotinamide Diabetes Intervention Trial (ENDIT), European Paediatric Pre-diabetes Subcutaneous Insulin Trial (EPP-SCIT), and the Finnish Trial to Reduce IDDM in the Genetically at Risk Study (TRIGR) (208–212). Unfortunately, many of these efforts have been proved to be ineffective and the future of type 1 diabetes prevention is less hopeful. A few prevention studies continue, however, including ones involving oral insulin (NCT00419562; NCT00336674) and teplizumab (NCT01030861) (<http://www.clinicaltrials>).

**TABLE 86-6 Risks for Type 1 Diabetes**

<b>Population risks</b>	
Overall	1/500
<b>HLA-DR related</b>	
No high risk allele	1/5000
One high risk allele (i.e. DR3/x or DR4/x)	1/400
HLA-DR4 subset defined by molecular techniques	1/300
HLA-DR3/3 or DR4/4	1/150
HLA-DR3/4	1/40
<b>Risks in relatives</b>	
<b>Siblings</b>	
Overall	1/14
<b>HLA haplotypes shared with diabetic sibling</b>	
0 haplotypes shared	1/100
1 haplotypes shared	1/20
2 haplotypes shared	1/6
3 haplotypes shared and DR3/4	1/5–1/4
Monozygotic Twin of Diabetic	1/3
<b>Offspring</b>	
Overall	1/25
Offspring of affected female	1/50–1/40
Offspring of affected male	1/20



[gov/ct2/home](http://gov/ct2/home)). More trials are underway in newly diagnosed diabetic subjects (reviewed in (213)).

**86.4.11.1 Screening for Other Autoimmune Disorders.** Diabetes is not the only autoimmune disorder for which relatives of an individual with type 1 diabetes are at risk. Family members, as well as the diabetic patient, are at an increased risk for autoimmune thyroid disease (Hashimoto thyroiditis, Graves' disease), pernicious anemia secondary to autoimmune gastritis, autoimmune adrenal disease (Addison's disease), myasthenia gravis, vitiligo, and celiac disease (214,215). A study looking at individuals with type 1 diabetes and their relatives found that 21% of the diabetics and 22% of their first-degree relatives had evidence of autoimmune disease (216). Of patients with persistent ICA, 57% had other autoimmune conditions compared with 15% of those not found to have persistent ICA (216). About 75% of the autoimmune disease in relatives occurred in families in which there was a proband with autoimmune disease, indicating that there may be increased genetic susceptibility to other autoimmune disorders in certain type 1 diabetes families.

The most common form of autoimmune disease in families with type 1 diabetes is thyroid disease (216–218). Although the proportion of type 1 diabetes patients with clinical or subclinical thyroid disease has been reported to be as high as 35%, the actual proportion is thought to be closer to 15–20% (216,219). In contrast, the prevalence of autoimmune thyroid disease in nondiabetic whites is thought to be 4.5% (218). The prevalence of clinical or subclinical autoimmune thyroid disease in first-degree relatives of individuals with type 1 diabetes is estimated to be 15–25% (216,219). As is true with autoimmune thyroid disease in the general population, female family members have higher rates of thyroid and gastric autoimmunity than do males.

Other autoimmune disorders are also seen with increased frequency in type 1 diabetic individuals and their relatives. Autoimmune gastritis, as evidenced by the detection of gastric parietal cell autoantibodies or pernicious anemia, is seen in 5–12% of individuals with type 1 diabetes and 2.5–6% of their first-degree relatives (216,219–221). The prevalence of adrenal autoantibodies is 1–3% in individuals with type 1 diabetes compared with up to 0.6% in nondiabetics (216,218).

It is particularly important for the relatives of patients with type 1 diabetes to be made aware of this increased risk for autoimmune disease, since approximately 40% of all families that include an individual with type 1 diabetes will have at least one other family member with latent or clinical autoimmune disease (216). Although most physicians know of the association of type 1 diabetes with other autoimmune disease, the fact that close relatives are also at risk is not as well appreciated in the medical community. Since many of these autoimmune disorders can have relatively insidious onsets, with fairly nonspecific symptoms, making the relatives and their

physicians aware of the increased risk may lead to earlier diagnosis and treatment.

Given this increased risk for autoimmune diseases, periodic screening of individuals with type 1 diabetes and all their first-degree relatives is warranted, particularly for thyroid dysfunction (via standard tests such as obtaining T4 and TSH levels) and for vitamin B12 deficiency, which if untreated, leads to pernicious anemia.

**86.4.11.2 Pregnancy and Type 1 Diabetes.** There is a markedly increased risk of congenital anomalies in the offspring of women with type 1 diabetes (222,223). In the general population, the risk to have a child with a birth defect is 2–3%, whereas for women with type 1 diabetes, the risk is increased threefold i.e. 6–10% (224–226). The malformations seen in infants born to diabetic women tend to be more severe than those seen in infants of nondiabetic women and include abnormalities of the skeletal, renal, cardiac, and central nervous systems (Table 86-7) (225,227–231). Virtually all anomalies occur with increased frequency in infants of diabetic mothers but those that have the highest relative risks are caudal regression, renal agenesis, transposition of the great vessels, ventricular septal defects, atrial septal defects, situs inversus, focal femoral hypoplasia/unusual facies, and neural tube defects (anencephaly and meningomyelocele). Although these malformations are generally not specific for diabetes, caudal regression is seen much more often in infants of diabetic mothers than in the general population. The relative risk for caudal regression in the offspring of a diabetic woman has been estimated to be as high as 200 (228). The relative risks for the other defects are not as high, due in large part to their higher incidence in the general population (227).

The disruption of embryogenesis leading to the abnormalities occurs before the eighth week of pregnancy (i.e. often before a woman realizes that she is pregnant) (232). There is evidence to suggest that elevated glycosylated hemoglobin (HbA1c) levels are associated with a high risk for malformations, and vigorous control of

**TABLE 86-7** Congenital Malformations in Infants of Diabetic Mothers

Malformation	Ratio of Incidences <sup>a</sup>
Caudal regression	200–600
Spina bifida, hydrocephalus, and other CNS defects	2
Cardiac defects (including transposition of the great vessels, ventricular septal defects, atrial septal defects)	4
Anal/rectal atresia	3
Agenesis	6
Cystic kidney	4
Duplicated ureter	23
Situs inversus	84

<sup>a</sup>In diabetic vs nondiabetic pregnancies.

Data from Mills JL (486) and Soler et al. (230).

blood glucose levels before conception has been shown to significantly reduce the incidence of congenital malformations (233–236). Although it is beneficial to optimize diabetes control even in women who present when they are already pregnant, postconceptional intervention is less likely to reduce the malformation risk (232,237). Beginning in early adolescence, diabetic women of child-bearing age should be made aware of the risk of congenital malformations and counseled that planning their pregnancies is essential so that optimal metabolic control of their disease can be achieved before conception and continued throughout their pregnancy.

Because of the increased risk for major structural malformations, prenatal diagnostic tests should be recommended for all pregnant women who have type 1 diabetes. These should be performed during the second trimester (usually between 16 and 20 weeks gestation), providing women with abnormal results the opportunity to obtain genetic counseling regarding the anomaly (i.e. prognosis and treatment options) and to make informed decisions regarding pregnancy options. For women who have normal results, the information obtained via prenatal diagnosis can be very reassuring and help alleviate anxiety for the remainder of the pregnancy. Ultrasonography can be used to evaluate fetal growth and to rule out major fetal structural anomalies such as renal agenesis, neural tube defects, and caudal regression. Fetal echocardiography, performed at 16–22 weeks following the first day of the last menstrual period, enables prenatal diagnosis of major structural cardiac malformations. Elevations of maternal serum alpha-fetoprotein (MSAFP) are associated with open neural tube defects such as anencephaly and meningomyelocele (238,239); thus, MSAFP, ideally incorporated into triple marker screening (with  $\beta$ -HCG and estriol), is recommended for all pregnant diabetics. Because MSAFP levels are altered in pregnant diabetics compared to nondiabetics, tables specific for diabetic women must be used when calculating their MSAFP values and it is therefore important that the laboratory performing the assay be made aware that the patient is diabetic (240–242). There is good evidence from the general population that folic acid supplementation, begun before conception, is helpful in decreasing the risk for neural tube defects (243–246). Although studies looking specifically at infants of diabetic mothers have not been reported, folic acid supplementation before conception should be strongly considered, as the potential benefits (i.e. reducing the risk of neural tube defects and possibly cardiac defects as well) outweigh any known risks (247).

## 86.5 TYPE 2 DIABETES MELLITUS

### 86.5.1 Introduction

Type 2 diabetes is characterized by a relative disparity between endogenous insulin production and insulin requirements, leading to an elevated blood glucose. In

contrast to type 1 diabetes, there is always some endogenous insulin production in type 2 diabetes; many type 2 patients have normal or even elevated blood insulin levels. The disease usually occurs in persons over the age of 40 years, although occurrence in childhood is increasingly recognized, and the onset may be insidious, or even clinically inapparent. The hyperglycemia of type 2 diabetes can often be controlled by diet or oral hypoglycemic agents, although exogenous insulin may be required. Type 2 diabetes continues to increase in frequency and is considered a rising epidemic worldwide (248). It has been estimated that one in every three individuals (one in two minorities) born in the United States in the year 2000 will develop diabetes in their lifetime (249), mainly due to the epidemic of obesity.

The primary pathogenetic lesion in type 2 diabetes has yet to be discovered. Primary insulin resistance of the peripheral tissues has been suggested by many as the initial event. Similarly, insulin secretion abnormalities have been argued as the primary defect in type 2 diabetes. It is likely that both phenomena are important in the development of type 2 diabetes, and genetic defects predisposing to both are likely to be important contributors to the disease process. In the most commonly accepted pathogenetic model, insulin resistance initially develops and induces compensatory insulin hypersecretion to maintain normoglycemia. Diabetes develops only when the pancreatic beta cells become, over time, unable to secrete enough insulin to overcome peripheral insulin resistance. Type 2 diabetes is a progressive disorder in that beta cell function declines with increasing duration of diabetes. GWAS in recent years appear to suggest that failure of insulin secretion is the key event that initiates type 2 diabetes; most diabetes loci discovered by GWAS appear to compromise insulin secretion or pancreatic beta cell development.

**86.5.1.1 Evidence of a Genetic Contribution to Type 2 Diabetes.** Several lines of evidence suggest the importance of genetic susceptibilities underlying the development of type 2 diabetes (250). Genetic epidemiological studies provide convincing descriptive data including population and ethnic differences, studies of familial aggregation, familial transmission patterns, and comparisons of twin concordance rates. Animal models of type 2 diabetes and studies of specific genetic syndromes that feature glucose intolerance provide further data supporting the etiologic role of genetic factors in the pathogenesis of type 2 diabetes. Finally, the genetic etiologies for type 2 diabetes have also been supported by association and linkage studies of genetic markers in populations and families.

**86.5.1.2 Evidence from Animal Models.** Relevant animal models provide the opportunity to study genes and pathophysiological mechanisms that may have application to human diabetes. Variability in blood glucose levels occurs between different strains of inbred mice and rats (251). Among the more intensively studied

mouse models of type 2 diabetes are the ob/ob (obesity and hyperglycemia) and db/db (diabetic obese) mice (252,253). The diabetes and obesity seen in conjunction with these two mutations are modified by the genetic background of the strain of mouse in which they occur (254,255). In the ob/ob mouse, the obesity and diabetes phenotype results from a nonsense mutation that generates a stop codon in the gene for the hormone leptin. The db/db mouse model has resistance to the ob gene product because of mutation in the leptin receptor (256,257). As with leptin, mutations in the leptin receptor are extremely rare in humans (258).

Several other animal models have been proposed as being more relevant to the human condition. These include the C57BL/6 J mouse strain (without leptin or leptin receptor mutations) (251,259–261), the NSY mouse (262), the TSOD mouse (263), which manifests both obesity and diabetes, and the SHR/N-cp rat (264,265). Multiple genes appear to be involved in causing diabetes in these models, similar to the apparent multigenic etiology of type 2 diabetes in humans. Investigation in these rodent models has only very rarely implicated loci that may affect type 2 diabetes in humans (266).

**86.5.1.3 Evidence from Population Studies.** Population-based studies of the distribution of a phenotypic trait can be helpful as a first step in evaluating whether the trait is likely to be controlled by a “major gene” or by multiple factors (either genetic or environmental). Several studies suggest that in populations with a high prevalence of type 2 diabetes, the distribution of glucose tolerance may be bimodal; that is, fasting glucose levels appear to be distributed around two distinct mean values. For example, in the Pima Indians, the Oklahoma Seminoles and several South Pacific populations, the distribution of glucose tolerance values in adults is consistent with an underlying bimodal distribution (259,267–269). This is usually interpreted as suggesting that there is a major gene that influences glucose tolerance, although these data are also consistent with more than one major gene. However, in most populations, blood glucose values in the population appear to be distributed unimodally. This is likely due to the heterogeneous nature of most other populations under study.

**86.5.1.4 Evidence from Twin Studies and Family Studies.** Studies in twins and families have long suggested a “genetic,” or at least a strong familial component, to the susceptibility to type 2 diabetes. MZ twin studies demonstrate very high concordance for type 2 diabetes in the twin pairs (62), yet the overall familial aggregation of clinical disease or glucose levels is not consistent with a single, simple mode of inheritance (270). Genetic heterogeneity would seem the most likely explanation and is supported by recent GWAS findings. In addition, exposure to environmental factors is known to be important as well. The identical twin data, with 70–90% concordance in MZ twins, suggest that, in the urbanized Western world, the environment is sufficiently

constant (and diabetogenic) such that genetic susceptibility is the primary determinant for the development of type 2 diabetes. In studying a specific phenotype hypothesized to be related to type 2 diabetes development, Tremblay and coworkers (271) found tentative evidence for genetic factors influencing sensitivity of insulin levels with physical training in response to short-term exercise in male MZ twin pairs.

**86.5.1.5 Difficulties in Studying the Genetics of Type 2 Diabetes.** Type 2 diabetes and other common chronic diseases present a number of difficult analytic challenges to the geneticist. The late and variable age of onset of type 2 diabetes, probably resulting from interactions of both genetic and environmental factors, can result in an underestimation of the number of individuals who are genetically susceptible to type 2 diabetes. This is a particularly vexing problem for family studies, in which linkage of type 2 diabetes with genetic markers is often the goal. While there is typically no confusion about the status of an affected living individual, unaffected individuals who carry the requisite gene(s), but who have not yet lived long enough to express diabetes, will not be recognizable. In addition, at the time a family is studied, many affected members in the older generations will be deceased and may have had their diabetes diagnosed (or not diagnosed) years ago, using perhaps less-than-optimal diagnostic criteria. The late age of onset also means that some individuals who are genetically “affected” will die of other causes before developing diabetes.

Another difficulty in studying the genetics of type 2 diabetes is the strong environmental component involved in many forms of diabetes. In industrialized or “westernized” countries, high MZ twin concordance rates suggest that the environment is sufficiently uniform (and diabetogenic) such that most individuals with the genetic predisposition will develop diabetes. On the other hand, in nonwesternized countries, studies of the genetics of type 2 diabetes are far more difficult to carry out. Many people with the requisite genes will simply never have the opportunity to manifest clinical disease under existing environmental conditions.

Studies in migrant populations that have had a rapid change in diet and/or exercise levels give some indication of the strength of the environmental component in the etiology of type 2 diabetes. For example, among the Nauruans of the South Pacific, documented prevalence of diabetes has increased from low rates to more than 50% of the adult population in a time period of about 30 years (272). Similar increases in prevalence with westernization have been noted in other populations as diverse as the natives of Australia, Africa, and Near Eastern immigrants to Israel, Japanese immigrants to the United States, and certain Native American populations (273–276).

Perhaps the most problematic aspect of studying the genetics of type 2 diabetes is the likely extensive etiologic

heterogeneity that underlies this disease. Genetic defects could (and probably do) influence any of the many steps involved in glucose regulation. Each of these defects, either alone or in concert with other defects, could result in type 2 diabetes. While such etiologic complexity by no means precludes genetic investigations, extensive etiologic heterogeneity implies that to understand particular pathogenetic mechanisms, one must be able to measure physiological “defects” at a more specific level than the gross phenotype of glucose intolerance.

### 86.5.2 Genetics of Intermediate Phenotypes for Type 2 Diabetes Mellitus

The study of physiological traits associated with type 2 diabetes within families can be useful in several levels, including dissection of disease heterogeneity. First, it may allow characterization of early stages of, and variability in, the natural history of the disease. It also allows for comparison between families, which may be helpful in separating etiologic subtypes. Finally, it can lead to better studies of mode of inheritance and linkage to genetic markers, as more of the genetically “affected” individuals in the pedigree will be identified. Another advantage of the study of intermediate phenotypes is that they may more closely reflect underlying genetic defects that predispose to disease.

The first physiological studies in families with type 2 diabetes were conducted using glucose tolerance as the phenotype. Even with this relatively crude measure of glucose metabolism, there was evidence that, in normal healthy subjects, glucose and insulin responses have an appreciable genetic component (277). In their studies of large pedigrees with type 2 diabetes, Beaty and Fajans (278) also assessed the role of genetic determinants of fasting blood sugar levels. Their data were consistent with a role for additive genetic factors, although a large proportion of the intrafamilial variability could not be explained by genetic factors. Familial studies of liability for hyperglycemia in Pacific Nauruans have also been interpreted as consistent with the effect of a major gene (279). However, in studies of Japanese Americans, Williams and colleagues (280) concluded that heritability of fasting blood glucose within families was low, and they could find no evidence for a major gene. Similar results were reported from a study of families in Jerusalem (281). In contrast, a study among healthy female twin pairs found a high heritability (proportion of total trait variance that is due to genetic factors) of 0.75 for fasting glucose (282). In studies of Danish twins, the heritability of abnormal glucose tolerance was found to be 0.61; the heritabilities for women and men of fasting glucose were 0.12 and 0.38 and of 2-h postload glucose were 0.38 and 0.43, respectively (283,284).

Insulin sensitivity/resistance is known to be heritable, evidenced by the observation of reduced insulin sensitivity in nondiabetic relatives of subjects with type 2

diabetes (285,286). Fasting insulin level is a simple surrogate measure of insulin sensitivity/resistance. Studies in the Mexican American population, a high-risk population with a high prevalence of type 2 diabetes, have demonstrated a genetic “dosage effect” on fasting insulin levels (287). An increase in fasting insulin levels was a function of whether an individual had 0, 1, or 2 diabetic parents, suggesting that insulin resistance is familial. Reported estimates for the heritability of fasting insulin range from 0.17 (288) to 0.38 (289) to 0.53 (290,291). In addition to genetic differences between populations studied, this wide range may reflect the fact that fasting insulin is determined not only by insulin sensitivity but also by insulin secretion and insulin clearance, each of which appears to be genetically determined (289).

Insulin sensitivity may also be more directly quantified by physiological phenotyping procedures used in research settings, such as the euglycemic hyperinsulinemic clamp, the frequently sampled intravenous glucose tolerance test, or the insulin suppression test (292). In an elegant study in Pima Indians (another high risk group), Lillioja and associates (293) demonstrated using the euglycemic clamp that in vivo insulin action has a familial component. Glucose uptake at maximally stimulating insulin concentrations showed a high degree of familiarity that was independent of age, sex, or degree of obesity. To control for familial correlations in dietary intake, subjects were placed on a standard diet for at least 7 days. Thus, the “familial” component, which was estimated to explain 38% of the variance in insulin action, appeared to be due to genetic rather than environmental similarities. Other studies utilizing the euglycemic clamp, widely considered to be the “gold standard” in quantification of insulin sensitivity, found heritabilities of 0.37–0.55 (289,294–296). The heritability of insulin sensitivity, quantified by the frequently sampled intravenous glucose tolerance test ranged from 0.28 to 0.44 (278,297,298).

Besides insulin resistance, an abnormality in insulin secretion is present in people with type 2 diabetes. Genetic/environmental influences on the insulin response to glucose were studied at the Karolinska Hospital in Sweden (299,300). In studying insulin release after a glucose infusion in family members, as well as fasting and stimulated glucose and insulin levels, these researchers first concluded that their data showed considerable intrafamilial correlation and was consistent with a major recessive gene common in the Swedish population (with a gene frequency perhaps as high as 20%) (299). More recent studies of insulin release and sensitivity in these families still suggest that these variables are genetically regulated, although the evidence for a major gene is no longer as convincing (300). The studies of Haffner and colleagues (301) in both Mexican American and non-Hispanic whites have also provided evidence that insulin secretion is likely genetically influenced, as a family history of diabetes was associated with decreased insulin secretion in response to an oral glucose load, as well as



with fasting insulin levels. Other studies have also looked at physiological abnormalities of insulin secretion in relatives of diabetics (302). For example, O’Rahilly and colleagues (303) studied the normal pulsatile release of insulin in first-degree relatives of type 2 diabetes subjects. Compared to controls, the first-degree relatives lacked the normal oscillations in insulin secretion following an intravenous glucose challenge. Since these relatives had mild glucose intolerance and high normal fasting glucose levels, this lack of pulsatile insulin release may be the first expression of type 2 diabetes in these high-risk relatives (304). Heritability estimates for insulin secretion range from 0.25 to 0.57 as determined from insulin measurements during oral glucose tolerance testing (283,284,305) and 0.23 to 0.84 as determined from intravenous glucose tolerance tests (277,296,297,306–308).

The genetic basis of the previously mentioned intermediate traits is supported by the fact that linkage signals for these traits have been detected in genome-wide scans. The Insulin Resistance Atherosclerosis (IRAS) Family Study found linkage of both fasting glucose and fasting insulin with a locus on chromosome 17p (309). The same study found linkage peaks for insulin secretion on chromosomes 4q, 11q, and 12q; insulin sensitivity index was linked to chromosome 15p (310). The insulin secretion linkage to 4q was replicated by the HERITAGE Family Study, which also identified another locus for insulin secretion on 10p (311). The Finland-United States Investigation of Non-insulin Dependent Diabetes Mellitus Genetics (FUSION) study also found linkage of insulin secretion to 10p, as well as 9p (312).

### 86.5.3 Evidence Supporting Heterogeneity in Type 2 Diabetes

When surveys of glucose tolerance have been performed in populations of European ancestry, the number of individuals found to have latent (subclinical) diabetes has been approximately equal to that with known diabetes. Among the Eskimos, however, clinical diabetes is extremely rare, but abnormal glucose tolerance tests have been found to be very common (313). Thus, abnormal glucose tolerance in the Eskimo appears to be a biochemical trait that rarely leads to clinical diabetes. The maximum plasma insulin response to an oral glucose challenge in healthy Navajo and Pima Indians was over three times as great as that observed in Western Europeans (314,315). In addition, the insulin output of type 2 diabetics was also clearly different in the American Indians than in the Europeans. Physiological studies of Asian Indians with type 2 diabetes suggest that they are more insulin resistant than are whites with type 2 diabetes, even when the degree of obesity is comparable (316). These data raise the possibility that there may be distinct subtypes of type 2 diabetes in different populations or ethnic groups and that it is possible that these differences are genetically determined.

Even early clinical genetic studies suggested heterogeneity within type 2 diabetes. When Kobberling (317) divided his adult-onset probands into low, moderate, and markedly overweight categories, he found a significantly higher frequency of affected siblings in the light-proband category (38%) and a significantly lower frequency in the heavy-proband category (10%). Irvine and colleagues (318) also suggested a difference between nonobese and obese insulin-dependent probands. They observed a different clinical range of diabetes in the relatives of the nonobese and obese probands.

Fajans and coworkers (319) have demonstrated metabolic heterogeneity in nonobese latent diabetes. These investigators were able to divide their latent diabetic patients into two broad groups: those with an insulinopenic form of glucose intolerance and those with high levels of plasma immunoreactive insulin. The high responders and low responders remained consistent and distinct over many years of follow-up, suggesting that they represented different metabolic disorders.

There is remarkable variability in the physiological abnormalities seen in patients with type 2 diabetes, ranging from structural and numeric abnormalities of pancreatic alpha and beta cells to abnormalities in pancreatic insulin secretion and decreased insulin sensitivity in the pancreas and peripheral tissues. There is considerable evidence for even further physiological heterogeneity in type 2 diabetes (320). For example, among patients with mild type 2 diabetes or impaired glucose tolerance are individuals with early insulin responses that range from supernormal to subnormal. Similar variability has been documented for the late insulin response in such patients (320). Because so much variability is seen in individuals with presumably early states of diabetes, it strongly suggests that type 2 diabetes is not caused by a single defect. Genetic studies, as discussed later, support this notion.

### 86.5.4 Genetic Approaches in Type 2 Diabetes

In dealing with heterogeneity in type 2 diabetes, there are several possible research strategies that can potentially be employed. In general, there are three options: (1) start with observable physiological differences and then work backward to determine if these differences can be explained by different genetic defects (working from the phenotype down); (2) start with a candidate gene or allele proposed to be related to diabetes, establish a genetic relationship, and work forward (working from the genotype up) to determine if specific physiological traits are associated with this gene or gene defect; and (3) use genome-wide linkage or GWAS approaches to identify chromosomal regions likely to contain diabetes-related traits or clinical diabetes itself and then assess if there are detectable physiological differences between those individuals (or families) displaying linkage and/or linkage disequilibrium and those individuals or families

that do not appear to be linked to the particular genomic locus. In actuality, research often involves the sequential application of all these approaches, as is well demonstrated by the investigations of MODY.

### 86.5.5 Maturity Onset Diabetes of the Young

MODY was originally described in 1964 (321) and was clearly identified as an autosomal dominant subtype of type 2 diabetes in the 1970s (322). In addition to the criteria for the diagnosis of diabetes, the MODY diabetic must meet the following additional criteria: (1) age of onset for at least one family member under 25 years; (2) correction of fasting hyperglycemia for at least 2 years without insulin; and (3) nonketotic diabetes (322). Using these criteria, many families with clearly dominant inheritance have been identified. However, there is considerable clinical heterogeneity within MODY, which is now appreciated to be due in large measure to genetic heterogeneity. In the French population, using rather stringent criteria, it is estimated that MODY may account for as much as 10–15% of familial diabetes cases, but less of general or later-onset type 2 diabetes (323). Although MODY is a relatively rare disorder, accounting for approximately 2–5% of all type 2 diabetes cases, it has taken on great importance in the past decade because of the lessons it has taught about the loci involved in type 2 diabetes and genetic heterogeneity.

The first MODY locus was identified by Bell and colleagues (324), with the demonstration of linkage of MODY with the adenosine deaminase (ADA) locus on the long arm of chromosome 20 in one large MODY family (the RW pedigree). Interestingly, clear delineation of linkage was only possible after exclusion of certain branches of the RW pedigree from analysis, following appreciation of the fact that non-MODY type 2 diabetes was occurring in these branches. The responsible gene is HNF-4 $\alpha$  (325); to date only a handful of MODY families have mutations in HNF-4 $\alpha$ , suggesting that this locus (MODY1) accounts for a minority of MODY subjects.

Not long after linkage to chromosome 20 was reported, linkage in other MODY families was reported with the glucokinase gene (GCK) on chromosome 7p (326,327). Unlike the ADA locus on chromosome 20, which was tested simply as a polymorphic marker in a systematic mapping approach, GCK was tested as a candidate gene because of its role in glucose homeostasis (323,328,329). Most MODY patients have a decreased insulin response to glucose, suggesting a primary pancreatic beta cell defect (330); thus the GCK was an excellent candidate for genetic investigations. Following the demonstration of linkage, actual mutations within the coding region were identified (331–333). Mutations in GCK account for a major portion of MODY pedigrees; as high as 60% of French MODY families have GCK mutations (334).

To date, mutations in six genes have been identified that produce a MODY phenotype, with another five being proposed (Table 86-8). Mutations in HNF-1 $\alpha$  (MODY3) are the most common form of MODY. While in most forms of MODY diabetes is the only identified abnormality, mutations in HNF-1 $\beta$  are also associated with serious renal defects and sometimes also with genital anomalies (Mullerian aplasia) (335–338). Heterozygous mutations in IPF1 cause MODY, whereas homozygous mutations produce pancreatic aplasia or hypoplasia and neonatal diabetes requiring insulin treatment (339,340). The severity of diabetes varies depending on which gene is altered; defects in the hepatocyte nuclear factor genes result in much more serious, often insulin-requiring, diabetes than is seen with GCK mutations. Unlike classical type 2 diabetes, the predominant abnormality leading to diabetes resulting from MODY gene mutations lies in insulin secretion, with insulin resistance not being a significant factor.

Heterozygous GCK mutations cause a relatively mild form of diabetes or only impaired glucose tolerance in most affected individuals. The prevalence of frank diabetes compared with impaired glucose tolerance is less than 50% (341). They rarely require insulin and usually do not develop vascular complications (342). Mutations in GCK are thought to alter the set point of the beta cell so that a higher circulating glucose level is necessary to trigger insulin secretion (343). The much rarer homozygous GCK mutations result in permanent neonatal diabetes requiring insulin therapy (344).

Even with the identification of six (and perhaps eleven) genetically distinct forms of MODY, there may be additional MODY loci to be found. Approximately 15–20% of MODY in England and France, and an even higher proportion in Japan, does not result from mutations in any of the known MODY genes (345). Given that several genes are able to cause MODY, it is not surprising that even greater genetic heterogeneity has been observed within “classical” type 2 diabetes. Just as different MODY defects appear to cause varying degrees of diabetes severity and complications, the clinical and physiological differences among type 2 diabetes patients may well result from genetically separate forms of diabetes.

### 86.5.6 Candidate Genes and Type 2 Diabetes

A large number of candidate genes have been tested for possible roles in the etiology of type 2 diabetes with mostly negative results. Many associations initially reported were not reproduced in other studies; this may be a result of false-positive studies, false-negative studies, or true variability in association among different populations, wherein a variant may alter diabetes risk differently depending on the genetic and/or environmental characteristics of the particular population (120). Those genes with the strongest evidence of association

TABLE 86-8 Genes that Can Cause Maturity-Onset Type Diabetes			
Locus	Chr	Gene	Function
MODY 1	20	Hepatocyte nuclear factor-4 $\alpha$ (HNF4, TCF14)	Expressed in liver, kidney, intestine, and pancreatic islets Member steroid/thyroid hormone receptor superfamily A key regulator of pancreatic gene expression, it is an upstream activator of HNF-1 $\alpha$ expression
MODY 2	7	Glucokinase (GCK)	First step in glycolysis; phosphorylates glucose/glucose-6-P; functions as glucose sensor in beta cells by controlling glucose entry to glycolysis; expressed in beta cells and liver
MODY 3	12	Hepatocyte nuclear factor 1 $\alpha$ (HNF 1A; TCF1)	Transcription factor in liver, kidney and $\beta$ -cell Weak activator of transcription of the rat insulin gene; impaired dimerization may be the cause of beta cell dysfunction; associated with a more severe insulin secretory defect than seen in MODY2
MODY 4	13	Insulin promoter factor (IPF1)	Key regulator of islet peptide hormone expression and also responsible for development of the pancreas, likely by determining maturation and differentiation of pancreatic precursor cells in the developing gut. Heterozygous mutations cause MODY; homozygous mutations cause pancreatic agenesis
MODY 5	17	Hepatocyte nuclear factor-1 $\beta$ (HNF1B; TCF2)	Transcription factor in liver, kidney and $\beta$ -cell Associated with renal abnormalities (nephron agenesis, hypoplastic glomerulocystic disease, severe nephropathy leading to renal failure and genital anomalies)
MODY 6	2	Neurogenic differentiation 1 or beta cell E-box trans-activator 2 (NEUROD1; BETA2)	Transcription factor required for normal development of pancreatic beta islets; Transcriptional activator of insulin gene
MODY 7	2	Kruppel-like factor 11	Transcription factor that activates the insulin gene
MODY 8	9	Carboxyl-ester lipase	Major component of pancreatic juice. Mutation leads to diabetes and pancreatic exocrine dysfunction, suggesting a link between exocrine and endocrine pancreatic function
MODY 9	7	Paired box gene 4	Transcription factor involved in $\beta$ -cell development
MODY 10	11	Insulin	Rare missense mutations in the insulin gene have led to MODY in a few families. Some affected members had mild diabetes initially controlled by oral agents, followed by insulin
MODY 11	8	B-lymphocyte specific tyrosine kinase	Expressed in $\beta$ -cells, where it colocalizes with insulin. Overexpression in islets enhanced insulin secretion in high glucose conditions

MODY, maturity-onset diabetes of the young.

are peroxisome-proliferator-activated receptor gamma (PPARG), the pancreatic beta cell inwardly rectifying potassium channel Kir 6.2 (KCNJ11), transcription factor 2, hepatic (TCF2, also known as HNF1B), and wolfram syndrome 1 (WFS1). Of note, rare, severe mutations in all four of these genes lead to syndromic diabetes, and two of these genes (PPARG, KCNJ11) code for proteins that are targets of oral antidiabetic medications. PPARG codes for the nuclear hormone receptor transcription factor PPAR- $\gamma$ , which is highly expressed in adipose tissue and plays a role in adipocyte differentiation and insulin sensitivity. A common variant, Pro12Ala, is associated with diabetes, with increased risk conferred by the more frequent proline allele (odds ratio 1.2). While the effect on diabetes is modest, the frequency of the proline allele may translate to a large population-attributable risk, which has been estimated as high as 25% (346,347). PPARG is the target of the thiazolidinedione class of oral antidiabetic agents used in type 2 diabetes.

The pancreatic beta cell potassium channel encoded by KCNJ11 codes for Kir6.2, which complexes with the product of ABCC8 (also known as SUR1, sulfonylurea receptor-1) to form the pancreatic beta cell potassium channel whose function governs insulin secretion. This channel is closed in response to the ATP generated when glucose enters glycolysis in pancreatic beta cells; the closure of this channel leads to exocytosis of insulin. Sulfonylureas bind to and close these channels, triggering insulin release. Rare mutations in KCNJ11 cause monogenic syndromes; activating mutations can cause transient or permanent neonatal diabetes, and inactivating mutations can cause persistent hyperinsulinemic hypoglycemia of infancy. In terms of common type 2 diabetes, the lysine allele of the common Glu-23Lys variant in KCNJ11 has been found to confer an odds ratio of 1.4 for diabetes (348). This allele has also been associated with impaired insulin response during oral glucose tolerance testing, supporting its effect on insulin secretion.

The most recently identified candidate genes for type 2 diabetes were previously known for their role in monogenic diabetes syndromes, TCF2 with MODY 5 and WFS1 with Wolfram syndrome (also known as DIDMOAD, diabetes insipidus, diabetes mellitus, optic atrophy, and deafness). While rare missense variants in these genes cause these syndromes, noncoding variants in or near these genes contribute to risk of common type 2 diabetes (349–351), most likely via an effect on beta cell development or survival (352). TCF2 is a transcription factor that may play a role in pancreatic beta cell development. Expressed in the brain and pancreas, WFS1 codes for an integral membrane glycoprotein that localizes primarily in the endoplasmic reticulum, where it regulates calcium fluxes.

Of note, the odds of diabetes in carriers of particular variants in these genes ranges from 1.1 to 1.2 (348,352,353); such moderate effects are detectable only

by sufficiently powered studies. This likely contributed to the limited success of the candidate gene approach.

### 86.5.7 Identifying Type 2 Diabetes Genes by Genome-Wide Linkage Scans

Given that candidate gene studies did not appear to explain the majority of genetic susceptibility to type 2 diabetes, there was great hope that the advent of genome-wide linkage analyses would finally allow the major loci involved in type 2 diabetes to be identified. While some potential loci have come from these approaches, the yield has been much less than was initially anticipated. To date, dozens of genome-wide linkage scans have been performed to identify loci for type 2 diabetes and have led to the identification of only two diabetes genes. Many other loci for type 2 diabetes have been suggested, with the strongest evidence across studies for loci on chromosomes 1q and 20q.

The first locus reported from a genome scan was NIDDM1 on chromosome 2 (354). This study was performed in Mexican American sib pairs and subsequent searches in non-Hispanic populations were unable to confirm this locus (355–358). However, Horikawa and coworkers (359) reported the identification of calpain 10 (CAPN10) as the gene on distal chromosome 2 responsible for the NIDDM1 linkage previously reported. Their studies suggested that a particular haplogenotype (termed 112/121, composed of three intronic variants) accounts for type 2 diabetes susceptibility attributable to this locus. Since the original publication, a large number of reports examining association of CAPN10 variants with type 2 diabetes and related insulin and glucose traits have been published, with many, but not all, suggesting a role for CAPN10 in diabetes pathogenesis. Notably, comprehensive meta-analyses support association of particular intronic variants with type 2 diabetes (360,361). Whether these intronic variants are functional or are linked to functional variation elsewhere in the gene is unknown. No CAPN10 variants have been identified as associated with type 2 diabetes by GWAS.

In 2006, TCF7L2 (transcription factor 7 like 2), the locus with the strongest effect on the risk of type 2 diabetes, was discovered by investigators who were following up a linkage signal found on chromosome 10 in Icelandic individuals (362). Each risk allele at this locus confers a 1.4 odds of type 2 diabetes in Europeans; the 7% of Europeans with two risk alleles have double the diabetes risk compared to the 55% of individuals with no risk alleles (363). A large number of subsequent studies reproduced association of TCF7L2 variation with type 2 diabetes, not only in Europeans but also in other racial/ethnic groups (364). The actual variants that influence diabetes may differ by racial groups (365,366). TCF7L2 diabetes alleles are associated with reduced insulin secretory response to oral or intravenous glucose in nondiabetic individuals (367–370). TCF7L2,



a nuclear receptor for beta-catenin, is a component of the Wnt signaling pathway; as such, it may regulate cell proliferation, motility, cancer, myogenesis and/or adipogenesis (371). TCF7L2 transactivates the insulin and proglucagon genes, the latter codes for both glucagon and the incretin hormone glucagon-like peptide-1 (GLP-1), which potentiates glucose-stimulated insulin secretion. Impairment of these systems is a likely mechanism whereby variants in TCF7L2 reduce insulin secretion (372–374).

Another locus of interest from the genome scans is located on the long arm of chromosome 1. This locus is near a “diabesity” locus mapped in the Pima (375). Linkage of 1q with type 2 diabetes and related phenotypes has been demonstrated in numerous populations, including whites and African Americans, French whites, United Kingdom whites, Old Order Amish, Chinese, Hispanics, Pima Indians, and Utah whites (311,356,375–381). Notably, the region of the rat genome that is syntenic to human 1q has been linked with glucose tolerance and fasting insulin level in genome scans in the Goto Kakizaki diabetic rats (382,383). The fact that this is an often-replicated locus for type 2 diabetes led to the formation of the International 1q Consortium, whose goal is to identify the responsible gene(s). Recently, the Consortium fine-mapped a 23-Mb region of 1q, finding signals in the NOS1AP and ASH1L/PKLR regions that subsequently did not replicate association with type 2 diabetes (384). A weak effect of variation in NOS1AP was subsequently found in a Chinese study (385).

Linkage has also been reported between type 2 diabetes and the MODY1 region on chromosome 20 in a number of white populations (386–389), as well as Japanese (390). Linkage of 20q with diabetes was not demonstrated in other studies (391). Linkage of this region of 20q with fasting insulin was also reported in a Chinese population (392). While Hani and coworkers (393) reported an HNF-4 alpha mutation in one family with late-onset type 2 diabetes, other HNF-4 alpha mutations have only been found in classical MODY pedigrees. It thus appears that the type 2 diabetes locus on chromosome 20 is likely separate from HNF-4 alpha. Fine mapping of the 20q region in Japanese cohorts also did not implicate HNF-4 alpha in type 2 diabetes (394,395).

The genome-wide linkage scans performed to date in type 2 diabetes and other multifactorial disorders have yielded few susceptibility genes. Even when linkage to a chromosomal region is firmly established, determining which gene and which sequence variant within or nearby that gene are responsible for diabetes susceptibility has been challenging. The advent and application of GWAS has been much more productive. Virtually none of the type 2 diabetes loci discovered by GWAS have corresponded to linkage signals (396), suggesting that linkage might be explained by rare variants of high effect (or other types of genetic variation), rather than common variation typically covered by GWAS.

### 86.5.8 Type 2 Diabetes Genes Identified by Genome-Wide Association Studies

GWAS have resulted in the discovery of a dramatic number of genes for type 2 diabetes (Table 86-9). This was facilitated by cooperation between multiple centers, both in the acquisition of large numbers of cases and controls and in data sharing to confirm association signals. The first wave of GWAS in type 2 diabetes consisted of five studies published in 2007, all of which were conducted in European-origin subjects (Finnish, French, British, Swedish, Icelandic) (397–401). The loci identified included polymorphisms in or near HHEX/IDE, SLC30A8, CDKAL1, CDKN2A/2B (2 independent signals), and IGF2BP2. FTO was identified as a locus for body mass index (BMI) and type 2 diabetes via the GWAS that did not match diabetes cases and controls for BMI (398,401) and confirmed in other GWAS (402,403). In 2008, three of the groups behind the initial studies, the Wellcome Trust Case Control Consortium (401), the Diabetes Genetics Initiative (397), and the Finland-US Investigation of NIDDM (FUSION) study (398), collaborated to conduct a GWAS meta-analysis as the Diabetes Genetics Replication and Meta-analysis (DIAGRAM) study, which discovered an additional six loci, JAZF1, CDC123/CAMK1D, TSPAN8/LGR5, THADA, ADAMTS9, NOTCH2 (404). This year also saw the first type 2 diabetes GWAS conducted in Asian populations (Japanese, Korean, Chinese, Singaporean), which identified KCNQ1, subsequently found to be associated with diabetes in Europeans as well (405,406). In Asians, KCNQ1 is the locus with the strongest effect size, comparable to TCF7L2 in Europeans. In 2009, a multistage GWAS in French, Danish, and Finnish subjects identified a variant near the IRS1 gene as associated with type 2 diabetes (407). Multiple studies following up GWAS signals for fasting glucose identified six additional type 2 diabetes loci, ADCY5, DGKB, GCK, GCKR, MTNR1B, and PROX1 (408–411). In 2010, a GWAS in Taiwanese subjects added two more genes, SRR and PTPRD (412), while two additional European meta-analyses identified 13 additional loci, RBMS1/ITGB6, BCL11A, DUSP9, KLF14, CENTD2, HMG2, HNF1A, ZBED3, CHCHD9, ZFAND6, PRC1, and a second signal in KCNQ1 (413,414). A GWAS in Japanese subjects identified C2CD4A/4B and UBE2E2 as additional diabetes loci; only the latter was also associated with diabetes in Europeans (415). A different SNP downstream of C2CD4B had previously been associated with fasting glucose (408), and the diabetogenic SNP was later found to affect glucose-stimulated insulin secretion (416). A GWAS from the Asian Consortium of Diabetes discovered a new locus near SPRY2 and identified two new signals in the CDC123/CAMK1D and C2CD4A/4B regions (417).

It is important to note that for most of these loci, the causal gene and variant have not been established; the

TABLE 86-9 Susceptibility Genes for Type 2 Diabetes							
Locus: Nearest Gene(s)	Chromosomal Location	Marker(s)	Variant Type(s)	Source of Initial Discovery	Genome-Wide Associated Trait(s)	Diabetes Effect: OR (95% CI)	Protein
ADAMSTS9	3p14.3-p14.2	rs4607103	38 kb upstream	European GWAS meta-analysis	T2DM	1.09 (1.06–1.12)	ADAM metalloproteinase; peptidase; thrombospondin type 1 motif
ADCY5	3q13.2-q21	rs2877716, rs11708067	Intronic	European fasting measures GWAS meta-analysis, 2-h measures GWAS meta-analysis	FG, HOMA-B, 2-h G, T2DM, birth weight	1.12 (1.09–1.15)	Adenylate cyclase
BCL11A	2p16.1	rs243021	99 kb downstream	European GWAS meta-analysis	T2DM	1.08 (1.06–1.10)	B-cell CLL/lymphoma 11A (zinc finger protein)
C2CD4A/4B	15q22.2	rs7172432	Intergenic	Japanese GWAS	T2DM	1.13 (1.09–1.18)	C2 calcium-dependent domain containing protein
CDC123/CAMK1D	10p13	rs12779790	Intergenic	European GWAS meta-analysis	T2DM	1.11 (1.07–1.14)	Cell division cycle 123 homolog (Saccharomyces cerevisiae); calmodulin-dependent protein type 1D
CDKAL1	6p22.3	rs7754840, rs10946398	Intronic	Multiple European GWAS	T2DM	1.12 (1.08–1.16)	CDK5 regulatory unit associated protein
CDKN2A/2B	9p21	rs10811661	125 kb upstream	Multiple European GWAS	T2DM	1.20 (1.14–1.25)	Cyclin-dependent kinase 2A/2B
CENTD2	11q13.4	rs1552224	5' UTR	European GWAS meta-analysis	T2DM	1.14 (1.11–1.17)	Centaurin
DGKB/TMEM195	7p21.2	rs2191349	Intergenic	European fasting measures GWAS meta-analysis	FG, T2DM	1.06 (1.04–1.08)	Diacylglycerol kinase beta-90 Transmembrane protein
DUSP9	Xq28	rs5945326	8 kb upstream	European GWAS meta-analysis	T2DM	1.27 (1.18–1.37)	Dual specificity phosphatase 90
FTO	16q12.2	rs8050136, rs9939609	Intronic	Multiple European GWAS	BMI, T2DM	1.15 (1.09–1.22)	Fat mass and obesity-associated protein

GCK	7p15.3-p15.1	rs4607517, rs1799884 (-30G>A)	36 kb upstream, promoter	European fasting measures GWAS meta-analysis	FG, HbA1c, T2DM	1.07 (1.05–1.10)	Glucokinase (Hexokinase 2)
GCKR	2p23	rs780094, rs1260326	Leu446Pro, intronic	European fasting measures GWAS meta-analysis, 2-hr measures GWAS meta- analysis	FG, FI, 2-hr G, HOMA-IR, T2DM	1.06 (1.04–1.08)	Glucokinase (hexokinase 2) regulator
HHEX/IDE	10q23/10q23-q25	rs1111875	7.7 kb downstream	French GWAS	T2DM	1.13 (1.08–1.17)	Hematopoietic cell-specific homeobox Insulin-like growth factor enzyme
HMGA2	12q15	rs1531343	43 kb upstream	European GWAS meta-analysis	T2DM	1.10 (1.07–1.14)	High mobility group AT-hook domain
HNF1A	12q24.2	rs7957197	20 kb downstream	European GWAS meta-analysis	T2DM	1.07 (1.05–1.10)	HNF1 homeo-
TCF2 (HNF1B)	17cen-q21.3	rs757210, rs4430796	Intronic	Candidate gene	T2DM	1.12 (1.07–1.18)	Hepatocyte growth factor 1
IGF2BP2	3q27.2	rs4402960	Intronic	Multiple European GWAS	T2DM	1.17 (1.10–1.25)	Insulin-like growth factor 2 binding protein
IRS1	2q36	rs2943641	502 kb upstream	French, Danish GWAS	T2DM	1.19 (1.13–1.25)	Insulin receptor substrate 1
JAZF1	7p15.2-p15.1	rs864745	Intronic	European GWAS meta-analysis	T2DM	1.10 (1.07–1.13)	Juxtaposed to another finger p
KCNJ11/ ABCC8	11p15.1	rs5219	Glu23Lys in KCNJ11	Candidate gene	T2DM	1.15 (1.09–1.21)	K inwardly- rectifying chan- nel subfamily J, me- mber 11 ATP-binding site, sulfonyl- urea receptor C (CFTR) member 8
KCNQ1	11p15.5	rs2237892	Intronic	Japanese, Korean, Chinese GWAS	T2DM	1.40 (1.34–1.47)	K voltage-gate- d channel subfamily A member 1
KCNQ1	11p15.5	rs231362	Intronic	European GWAS meta-analysis	T2DM	1.08 (1.06–1.10)	K voltage-gate- d channel subfamily A member 1
KLF14	7q32.3	rs972283	47 kb upstream	European GWAS meta-analysis	T2DM	1.07 (1.05–1.10)	Kruppel-like factor 14

[illegible]

Locus: Nearest Gene(s)	Chromosomal Location	Marker(s)	Variant Type(s)	Source of Initial Discovery	Genome-Wide Associated Trait(s)	Diabetes Effect: OR (95% CI)	Protein
MTNR1B	11q21-q22	rs10830963	Intronic	European GWAS meta-analysis	FG, HOMA-B, HbA1c, T2DM	1.09 (1.06–1.12)	Melatonin r1B
NOTCH2	1p13-p11	rs10923931	Intronic	European GWAS meta-analysis	T2DM	1.13 (1.08–1.17)	Neurogenic notch h log prot (Drosop
PPARG	3p25	rs1801282	Pro12Ala	Candidate gene	T2DM	1.14 (1.08–1.20)	Peroxisome prolifera activate receptor
PRC1	15q26.1	rs8042680	Intronic	European GWAS meta-analysis	T2DM	1.07 (1.05–1.09)	Protein reg cytokine
PROX1	1q32.2–q32.3	rs340874	2 kb upstream	European fasting measures GWAS meta-analysis	FG, T2DM	1.07 (1.05–1.09)	Prospero box 1
SLC30A8	8q24.11	rs13266634	Arg325Trp	French GWAS	T2DM, FG, HbA1c	1.12 (1.07–1.16)	Solute carri family3 transpo member
TCF7L2	10q25.3	rs7903146, rs7901695	Intronic	Icelandic linkage region	T2DM, FG, HbA1c	1.37 (1.28–1.47)	Transcriptio factor-7 (T-cells HMG-bo
THADA	2p21	rs7578597	Thr1187Ala	European GWAS meta-analysis	T2DM	1.15 (1.10–1.20)	Thyroid ade associat
TLE4 (CHCHD9)	9q21.31	rs13292136	234 kb upstream	European GWAS meta-analysis	T2DM	1.11 (1.07–1.15)	Transducin- enhance split 4 (l homolo Drosop



TP53INP1	8q22	rs896854	Intronic	European GWAS meta-analysis	T2DM	1.06 (1.04–1.09)	Tumor protein p53 inducible protein
TSPAN8/LGR5	12q14.1-q21.1/12q22-q23	rs7961581	Intergenic	European GWAS meta-analysis	T2DM	1.09 (1.06–1.12)	Tetraspanin, a membrane protein containing four extracellular protein-protein-receptor domains
WFS1	4p16	rs1801214, rs10010131	Intronic	Candidate gene	T2DM	1.13 (1.07–1.18)	Wolfram syndrome (wolframin)
ZBED3	5q13.3	rs4457053	41 kb upstream	European GWAS meta-analysis	T2DM	1.08 (1.06–1.11)	Zinc finger, BED domain-containing protein
ZFAND6	15q25.1	rs11634397	1.5 kb downstream	European GWAS meta-analysis	T2DM	1.06 (1.04–1.08)	Zinc finger, FANCD1 domain-containing protein
SPRY2	13q13.1	rs1359790	193 kb downstream	Asian GWAS	T2DM	1.15 (1.10–1.20)	Sprouty homolog 2 (Drosophila)
SRR	17p13	rs391300	Intronic	Taiwanese GWAS	T2DM	1.28 (1.18–1.39)	Serine racemase
PTPRD	9p23–p24.3	rs17584499	Intronic	Taiwanese GWAS	T2DM	1.57 (1.36–1.82)	Protein tyrosine phosphatase receptor type D
UBE2E2	3p24.2	rs7612463	Intronic	Japanese GWAS	T2DM	1.19 (1.12–1.26)	Ubiquitin-conjugating enzyme E2E2 (Ube2E2)
RBMS1/ITGB6	2q24.2	rs7593730	Intron 3 of RBMS1	European GWAS meta-analysis	T2DM	0.90 (0.86–0.93)	RNA binding motif single-strand binding protein 1, interacting with RNA and beta-actin

FG, fasting glucose; T2DM, type 2 diabetes mellitus; HbA1c, hemoglobin A1c; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-B, homeostasis model assessment of  $\beta$ -cell function; 2-h glucose level on oral glucose tolerance test.

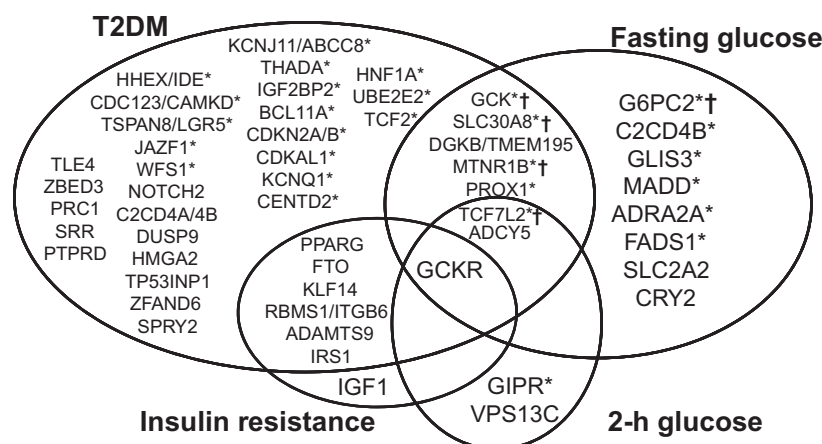
associated variants may be in linkage disequilibrium with functional variants in the nearest gene (typically used to label the locus) or elsewhere. As displayed in Table 86-9, in most cases the associated variants are in introns or intergenic regions; only a handful of functional missense variants have been discovered. Even in the latter cases, complex scenarios are possible, for example, the Glu-23Lys variant in *KCNJ11* is strongly linked to the Ser-1369Ala variant in *ABCC8*, with both genes coding for components of the ATP-sensitive potassium channel that is critical to glucose-stimulated insulin secretion (418). Evidence suggests that the presence of both variants may lead to deficient insulin secretion (419). Also, intronic variants may prove functional, as suggested by the association of the *TCF7L2* SNP rs7903146 with open chromatin and greater enhancer activity (420).

GWAS for fasting glucose and 2-h glucose identified associations with genes previously or later found to also be associated with type 2 diabetes (e.g. *TCF7L2*, *ADCY5*, *GCKR*, see Figure 86-1); however, they also identified several genes associated with glucose only, suggesting that some genes influence glucose in the physiological range without contributing to diabetes risk (408,421). Genes with similar effect on fasting glucose, such as *ADCY5* and *MADD*, each of which has an effect of approximately 0.5 mg/dL per risk allele, may have very different effects on diabetes risk (only *ADCY5* is associated with diabetes) (408). On the other hand, a gene with a small effect on diabetes (e.g. *MTNR1B*, odds ratio 1.2 per allele) may have a large effect on fasting glucose (1.2 mg/dL per allele), whereas a gene with a strong effect on diabetes (e.g. *TCF7L2*, odds ratio 1.4 per allele) may have a smaller effect on fasting glucose (0.4 mg/dL per allele) (408). These facts point to the complexity of glucose regulation and how genes that affect glucose may or may not influence the risk of diabetes.

A similar situation is found in GWAS of hemoglobin A1c (HbA1c), which reflects average glycemia over a

2- to 3-month period. Hemoglobin A1c, used to monitor treatment response in diabetes for many years, has been recently advocated as a diagnostic tool (422). A GWAS for HbA1c found association with loci previously implicated in fasting glucose (*G6PC2*) and fasting glucose and type 2 diabetes (*SLC30A8*, *GCK*) (423). This GWAS also identified a novel locus *HK1* (hexokinase 1 expressed mainly in red blood cells), which is not associated with fasting or 2-h glucose or type 2 diabetes (423,424). Subsequent meta-analyses added *TCF7L2* and *MTNR1B* as diabetes loci that were associated with HbA1c (425,426), however, several other loci were identified that appear to influence HbA1c via nonglycemic pathways, such as anemia and iron storage (424,426). Notably, loci associated with HbA1c in studies of type 1 diabetes were not associated with HbA1c in cohorts with type 2 diabetes (427).

Type 2 diabetes is thought to arise from concurrent insulin resistance and deficient insulin secretion. Several studies, typically in nondiabetic subjects, have examined the type 2 diabetes loci for association with indexes of insulin resistance and insulin secretion. Most studies derived these traits from fasting insulin and glucose measures, via homeostatic model assessment (HOMA) indexes of insulin resistance (HOMA-IR) and beta-cell function (HOMA-%B) or via indexes derived from oral glucose tolerance testing. A minority of studies employed more sophisticated methods of quantifying insulin-related traits, such as euglycemic or hyperglycemic clamps or frequently sampled intravenous glucose tolerance tests. The overwhelming finding of these studies is that the majority of type 2 diabetes loci are associated with impaired beta-cell function, thought to act via compromised development of beta cells or defective insulin responses to glucose or to the incretin hormones (Figure 86-1). Only a few genes appear to act via insulin resistance. This was unexpected, as insulin resistance was known to be a heritable trait, and may reflect a different genetic architecture of insulin resistance (e.g. more



**FIGURE 86-1** Established loci for type 2 diabetes, fasting glucose, 2-h glucose, and insulin resistance. Those loci also associated with insulin secretion (apparent effects on beta cell development/mass or glucose/incretin-stimulated insulin release) and hemoglobin A1c are indicated with \* and †, respectively.

influenced by rare variants). On the other hand, insulin resistance may not be well reflected by the quantitative traits commonly examined (428). Alternatively, insulin resistance may be more influenced by environmental and lifestyle factors, such as diet and weight gain. The predominance of diabetes genes associating with beta cell function suggests a model wherein genetically determined robustness of the beta cell's ability to maintain compensatory insulin secretion in the face of insulin resistance is the main gateway by which subjects develop type 2 diabetes. Those individuals who maintain sufficient insulin secretion do not develop diabetes, even if substantial insulin resistance is present. Consistent with this is the fact that obesity and insulin resistance are more prevalent in the population than type 2 diabetes.

The explosion of GWAS publications had led some to question their value. As of early 2011, the over 40 genes described as associated with type 2 diabetes appear to explain only 10% of the inheritance of this disease. The unexplained approximately 90% has been called the “missing heritability.” A number of factors may ultimately account for the missing heritability. First, GWAS chips capture (via linkage disequilibrium), mainly common (frequency >5%) variation. Depending on the racial group studied, they capture 70–80% of such variation. Missed common variation notwithstanding, those loci that are identified may be in linkage disequilibrium with functional variants elsewhere; identifying those variants may explain more of the heritability. Many believe that a substantial portion of the missing heritability will ultimately be explained by rare variation with large effect sizes. Already, rare variants are being examined in diabetes genes such as TCF2, with some manifesting association with type 2 diabetes (429). Current whole-exome sequencing efforts (1000 Genomes Project, [www.1000genomes.org/page.php](http://www.1000genomes.org/page.php)) are anticipated to capture rare variation. Other factors that may account for missing heritability include the effects of gene–gene interactions, gene–environment interactions, epigenetic factors (e.g. DNA methylation and histone modification), prenatal programming, and non-SNP variation (e.g. copy number variation). Noncoding RNAs that affect gene expression may be responsible for observed associations, as suggested in the CDKN2A/2B locus (430). It is clear that GWAS, while increasing the number of diabetes genes 20-fold, are only the beginning of a complete understanding of diabetes genetics.

The diabetes genes discovered by GWAS have yet to translate to applications in clinical care. Several studies evaluating the utility of genetics to predict who may develop diabetes in the future, typically utilizing a risk score based on the total number of risk alleles, have found little incremental predictive value when added to traditional risk factors (e.g. age, BMI, gender, family history) (431–434). There is some evidence that genetic information in the prediction of type 2 diabetes may be more effective in younger adults (435).

Ultimately, the major contribution of GWAS to diabetes care may be in the development of new therapies. GWAS have led to novel insights in the pathophysiology of diabetes, and may have already identified key pharmacological targets. Two of the earliest diabetes genes, PPARG and KCNJ11, encode drug targets. It is highly likely that the many additional genes harbor one or more potential targets. Assisted by advances in technology, GWAS have discovered diabetes loci much more quickly than molecular, physiological, and pharmaceutical research are able to develop these into new drug therapies. Within the next decade, these arenas should be able to utilize genetic information to produce new treatments for diabetes. Another frontier that is likely to advance as a result of the many new genes is pharmacogenetics: use of genetic information to predict treatment response and adverse effects. Several studies have already documented effects of diabetes alleles on treatment response (436–439). In the future, once larger numbers of disease-response-predicting alleles have been elucidated, genetic testing before starting therapy may become common practice.

### 86.5.9 Mitochondrial Mutations and Maternal Transmission

In early studies of type 2 diabetes and MODY-like families, Dorner and colleagues (440,441) reported evidence that diabetes occurred more frequently on the maternal than on the paternal side of families ascertained through a diabetic proband. A study of French white type 2 diabetes patients demonstrated a significant excess of diabetic mothers and maternal relatives (aunts and uncles) compared with fathers and paternal relatives (442). Pettitt and coworkers, studying the inheritance of diabetes in Pima Indian type 2 diabetes families, also have evidence that supports the importance of maternal diabetes in determining the risk for diabetes in the offspring (443). In these studies, 45% of the offspring of women diabetic before pregnancy were themselves diabetic by age 20–24 years, compared with 1.4% and 8.6% of the offspring of nondiabetic and “prediabetic” women (women who became diabetic later), respectively. The paternal diabetic status appeared to contribute little additional risk to the offspring, after correcting for maternal diabetes and other risk factors (443). Significant excess maternal transmission of diabetes and an insulin-resistant phenotype characterized by heart disease, stroke, and hypertension, was also described in a Mexican-American type 2 diabetes population (444), although these findings were not observed in a similar population (445). Finally, Lin and coworkers (446) have described a maternal excess of diabetes inheritance in a Taiwanese population, with an odds ratio for reporting maternal diabetes of 2.64 (95% CI: 1.12–5.71) in diabetic patients compared with nondiabetic subjects.

Mitochondrial mutations could explain this excess maternal transmission. Indeed, inheritance of mitochondrial gene mutations leading to defects in glucose tolerance has been identified. The first mitochondrial mutation causing diabetes was shown to be caused by inheritance of deletions or duplications at a common breakpoint in the mitochondrial genome; however, this was shown in only one pedigree (447,448). A more widespread mutation at nucleotide 3243, a conserved position in the mitochondrial gene for tRNA Leu(UUR), has been reported by multiple groups in diabetes with maternal transmission history and associated hearing loss. This mutation alters the dihydrouridine loop in this tRNA, leading to impairment of mitochondrial transcription termination. This may cause defects in mitochondrial translation and protein synthesis. This same mutation is also responsible for the more dramatic MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) (448,449). Individuals with this mutation have been characterized as having insulin secretory defects along with an increased prevalence of sensorineural deafness or maternally inherited diabetes and deafness (MIDD) (450,451,452). Additionally, some of these individuals with the nucleotide 3243 mutation have been islet cell antibody positive type 2 diabetes patients who went on to require exogenous insulin therapy, due to beta-cell destruction and/or failure (453). The reason for the marked phenotypic variability seen in association with the 3243 mutation is still unclear. Although it has been proposed that the degree of heteroplasmy may explain the phenotypic variation, this appears to be an inadequate explanation. It is possible the mitochondrial–nuclear gene interactions are important (454). Like MODY, mitochondrial mutations are likely to account for only a small proportion of type 2 diabetes cases; Smith and colleagues (455) estimated that the 3243A–G mutation accounts for 0.5–2.8% of diabetes. Variants in nuclear-encoded mitochondrial genes also do not appear to play a significant role in typical type 2 diabetes (456). A number of other rare mutations in the mitochondrial genome have also been shown to cause diabetes, often but not always with associated hearing loss (457–460).

Several other hypotheses have been generated to explain this excess maternal transmission. These include: (1) a metabolic (teratogenic) effect of a diabetic or subclinical diabetic (“prediabetic”) environment during pregnancy; (2) the involvement of an imprinted gene that is only expressed when it is passed through a female meiosis; and (3) reporting bias. One of the GWAS-discovered loci for type 2 diabetes, KLF14, exhibits imprinted expression from the maternal allele, with maternal expression of the risk allele responsible for the effect on diabetes risk (413). Parent-of-origin effects have also been observed at the KCNQ1 locus (461). Reporting bias seems an unlikely explanation, however, as this phenomenon has also been observed in rats. Results from crosses between Goto-Kakizaki rats, which exhibit spontaneous type 2

diabetes, and outbred nondiabetic Wistar rats have demonstrated an effect of maternal inheritance on diabetes in offspring of the first generation (383). These hypotheses are not exclusive and one or more may be interacting to cause the observed excess maternal transmission of disease.

Several reports have identified low birthweight as a risk factor for type 2 diabetes and insulin resistance, suggesting a mechanism that may support maternal transmission (462–465). Decreased numbers of beta cells and impaired beta cell function have been associated with low birthweight, but a lack of correlation between low birthweight and the subsequent development of diabetes suggests that additional genetic or environmental factors are necessary for the development of type 2 diabetes (466). Furthermore, there were no differences in the birthweights of individuals with type 2 diabetes, impaired glucose tolerance, or normoglycemic subjects when the offspring of hyperglycemic and normoglycemic mothers were considered separately (466). Thus, these data do not provide conclusive evidence for a fuel-mediated teratogenic mechanism since diabetic mothers were no more likely than nondiabetic mothers to have babies of low birthweight.

Further research examining mitochondrial genes, sex-influenced autosomal loci, and environmental factors is warranted.

### 86.5.10 Genetic Counseling for Type 2 Diabetes

For the most part, we must depend on empirical recurrence risks for genetic counseling. For relatives of an individual with type 2 diabetes, the empirical recurrence risk to first-degree relatives is of the order of 10–15% for clinical diabetes and 20–30% for impaired glucose tolerance. In general, this increased risk appears to be for type 2 diabetes, not for type 1 diabetes, although as discussed previously, in some studies there is a somewhat increased risk for both forms of diabetes (467). For MODY diabetes, in whom diabetes is an autosomal dominant disorder, the risk to siblings and offspring is 50%.

**86.5.10.1 Screening and Prevention for Type 2 Diabetes.** Screening of first-degree relatives of type 2 diabetes can be accomplished by periodic glucose tolerance testing. In scenarios where this is not feasible, easily obtained clinical parameters can be entered into prediction tools to calculate the current (468) or future (469) risk of diabetes. Those relatives with impaired glucose tolerance should be advised to attain ideal body weight through diet and exercise, as this will improve glucose tolerance. This should be strongly encouraged, with the goals being to delay or prevent progression to frank diabetes and minimize the cardiovascular risks associated with impaired glucose intolerance. Indeed, lifestyle modification in subjects with impaired glucose tolerance has been demonstrated to reduce the incidence of type 2



diabetes by approximately 60% (470,471). Screening and intervention for other risk factors for cardiovascular disease (e.g. hypertension and hyperlipidemias) is also important, as a family history of diabetes is a significant risk factor for CAD.

Screening and implementation of appropriate life style modifications must begin early in life. While type 2 diabetes (with the exception of MODY) is traditionally thought of as an adult-onset disorder, there is a rapidly growing epidemic of adolescent and childhood onset of type 2 diabetes (472), particularly in children of Mexican American and African American descent (472,473). While the etiology is still under investigation, it appears that overeating and a sedentary lifestyle, in the context of a family history of type 2 diabetes, markedly increase risk.

Further refinement of genetic risk currently is only possible in MODY families and in those rare forms of type 2 diabetes due to mutant insulin, insulin receptor variants, or mitochondrial mutations. In such families, individuals at risk can potentially be identified at any age at which DNA can be obtained (e.g. even in childhood or prenatally). The issues that must be considered before going to DNA studies are complex, however. Particularly with forms of diabetes that have a later (e.g. adult) onset, the benefit of childhood carrier detection is not clear, and there is the risk of stigmatization as well as the risk of adversely impacting insurability. Therefore the pros and cons of DNA analysis should be discussed carefully before any testing. As discussed above, it is currently premature to utilize genes for typical type 2 diabetes as predictive tools, although private companies have already made this available to the public.

**86.5.10.2 Type 2 Diabetes and Pregnancy.** As detailed previously in the section discussing pregnancy and type 1 diabetes, it is well known that women with diabetes have a higher rate of pregnancy complications than do nondiabetics. Although attention is usually focused on the pregnant patient with type 1 diabetes, women with type 2 diabetes are also at increased risk for complications during pregnancy. In general, the more severe the diabetes, the poorer the pregnancy outcome. Women with type 2 diabetes have a significantly increased risk of delivering a child with congenital malformations (226,227,474,475), but the risk is probably less than that for women with type 1 diabetes. Becerra and coworkers (474) reported that the relative risk for major malformations for women with type 1 diabetes was 7.9 (95% CI: 1.9–33.5) as compared to nondiabetic women, whereas the relative risk for women with type 2 diabetes who required insulin treatment during pregnancy was 3.4 (95% CI: 1.0–11.7). Towner and colleagues (475) reported a major malformation frequency of 8.9% in women with type 2 diabetes. The spectrum of malformations was similar to that seen in association with type 1 diabetes. Of importance is that the risk of malformations was correlated with the degree of maternal glycemic control, suggesting

that preconceptional optimization of metabolic control is as important in women with type 2 diabetes as in those with type 1 diabetes.

With the trend toward delaying pregnancy into the 30s and 40s, coupled with the increasing proportion of the US population that is of Hispanic ethnic background, type 2 diabetes in pregnancy will be seen with increasing prevalence and may, without appropriate intervention, become an increasingly important cause of congenital anomalies (475,476). Women with type 2 diabetes should be counseled about the risks associated with diabetes in pregnancy and encouraged to maximize diabetes control before conception.

Prenatal diagnostic tests should also be recommended for all pregnant women who have type 2 diabetes, similar to those recommended for type 1 diabetes. These tests include ultrasonography to evaluate fetal growth and to rule out major fetal structural anomalies such as renal agenesis, neural tube defects, and caudal regression; fetal echocardiography (at 18–22 weeks' gestation) to identify major structural cardiac malformations; and MSAFP screening to screen for neural tube defects. Since there is evidence that in the general population folic acid supplementation begun before conception is helpful in decreasing the risk for neural tube defects (243–246), folic acid supplementation before conception should be strongly considered as the potential benefits (i.e. possibly reducing the risk for neural tube defects) outweigh any known risks.

## 86.6 FINAL CONSIDERATIONS AND SPECULATIONS

### 86.6.1 Evolutionary Aspects

Heterogeneity within both the insulin-dependent and non-insulin-dependent types of diabetes appears extensive. An important question arises from the population genetic viewpoint as to why these genes should be so prevalent. These diabetic disorders, whose susceptibility appears to be primarily genetically determined, are deleterious, and thus reproductive fitness should be impaired. As regards type 2 diabetes, a possible explanation is the concept of a “thrifty” genotype, as first proposed by Neel (477). He proposed that the diabetic genotype somehow allowed more efficient utilization of foodstuffs by the body in periods of famine to which primitive humans were often exposed. Such a thrifty gene would therefore have a selective survival advantage and would tend to increase in frequency. However, in the modern western world, with its continuous abundance of calories, such a gene would lead to diabetes and obesity. Neel's hypothesis has received support by observations in both humans and animals. The extremely high frequency of diabetes and obesity in populations such as the Pima Indians (478) and Pacific Islanders (479,480), and their apparent increase with modernization and urbanization, are

entirely consistent with the thrifty genotype hypothesis. Direct support comes from studies that have shown that heterozygotes for rodent diabetes–obesity genes exhibit a much better ability to survive fasting than normal rodents (480).

What might be the selective advantage of the genes that predispose to type 1 diabetes? Since type 1 diabetes is a disorder in which autoimmunity and immune response genes are implicated, a possible role in the resistance to infectious agents has been proposed. However, one should realize that the problem of the selective advantage of type 1 diabetes is much greater than for type 2 diabetes. Before the onset of insulin therapy, type 1 diabetes was usually a lethal disorder, at least in genetic terms (i.e. failure to reproduce). Since susceptibility seems to be provided even by single alleles of HLA-linked genes, this negative selection is much greater than that for recessive genetic disorders such as sickle cell anemia or Tay-Sachs disease, where negative selection operates only on those homozygous for the disease genes. Thus, one would suppose that the positive selective advantage would of necessity be dramatic and that the positive selection should have continued into modern human history. Otherwise the incidence of the disorder would have been decreasing dramatically before the advent of insulin therapy. Yet no such positive selective advantage has been discerned, at least postnatally. Evidence has been developed that indeed suggests a potential selective advantage mechanism for type 1 diabetes in utero. What has been observed in some studies is preferential transmission of diabetogenic HLA haplotypes, not only to affected offspring but also to unaffected offspring (481,482). Furthermore, the available evidence suggests that this possibly occurs via in utero selection (482,483). These data may thus provide an explanation for the maintenance of the high population frequency for this previously frequent genetically lethal disease. In addition, the suggestion that this prenatal selection could occur via immunologically mediated events raises the theoretical possibility that an additional consequence of these events, in fetuses that survive, might be immune changes that presage the eventual development of type 1 diabetes (270,484).

### 86.6.2 Counseling Summary

Given these recent advances in our knowledge of the genetics and heterogeneity of diabetes, what is the genetic counseling we can provide at this time to our diabetic patients? First, as in all genetic counseling, an accurate diagnosis must be made. On clinical grounds one can distinguish between type 1 typically juvenile-onset insulin-dependent type diabetes, type 2 maturity-onset non-insulin-dependent type diabetes, and MODY type diabetes. In distinguishing among these phenotypes, one already has important counseling information. As discussed previously, in a given family the increased risk for

diabetes over the general population is in general only for the specific type of diabetes that has already occurred in the family, not for all diabetes. Thus, if the index case presenting for counseling is a juvenile insulin-dependent diabetic, the increased risk for that patient's relatives is for type 1 diabetes. If the index case is a non-insulin-dependent diabetic, the increased risk for the patient's relatives is, for the most part, for type 2 diabetes only. Associated abnormalities or diseases may suggest one of the rare genetic syndromes that include diabetes, where the risk of recurrence is dependent on the specific diagnosis.

Once we have accurately characterized the clinical phenotype of the patient, how do we then proceed? At this stage, we must fall back for the most part on observed empirical recurrence risks (i.e. data concerning the actually observed recurrence of these disorders in a large number of families). Even these empirical recurrence risks have limitations, since for the most part they have been reported only from white populations. Even with the reservation that these empirical risks can be safely applied only to the populations from which they were derived, the most reassuring aspect of the data is the overall low absolute risk for the development of clinical diabetes in first-degree relatives, especially for insulin-dependent diabetes.

The heterogeneity that has so far been discovered among typical diabetes mellitus probably represents just the tip of the iceberg. But even this currently demonstrable heterogeneity has immediate relevance to current research efforts into the pathogenesis and therapy of the diabetic state. The susceptibility to a given environmental agent may very well depend on the heterogeneity elucidated by these studies. There may also be heterogeneity in the diabetic complications associated with genetically distinct forms of diabetes, having implications for disease management. Only when each of the many disorders resulting in diabetes mellitus and/or glucose intolerance are delineated will specific prognostication and therapy be possible for all diabetic patients.

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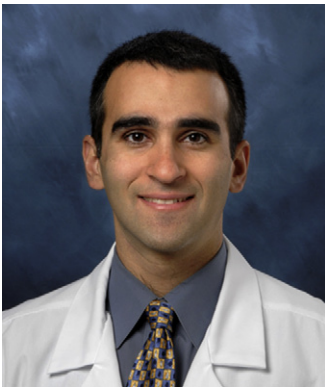
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- T1DBase <http://t1dbase.org/>.  
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 Juvenile Diabetes Research Foundation International <http://www.jdrf.org/>.

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# CHAPTER

# 87

## Genetic Disorders of the Adrenal Gland

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### 87.1 CLINICAL ASPECTS: NORMAL AND ABNORMAL ADRENAL FUNCTION

#### 87.1.1 Endocrine Functions of the Adrenal Cortex

The adrenal cortex produces numerous steroids, secreted in widely varying amounts, each of which has a different potency of hormonal action (Figure 87-1). According to their primary effect, these are classified as mineralocorticoids (MCs), glucocorticoids (GCs), or sex steroids.

Aldosterone is the end product of the MC pathway. It is the most potent steroid affecting active transport of sodium ions across membranes and thus in maintaining electrolyte balance. The primary site of action of aldosterone is at the renal distal convoluted tubules and the cortical collecting ducts, where it promotes reabsorption of the crucial 2% of sodium to be regained from the renal filtrate.

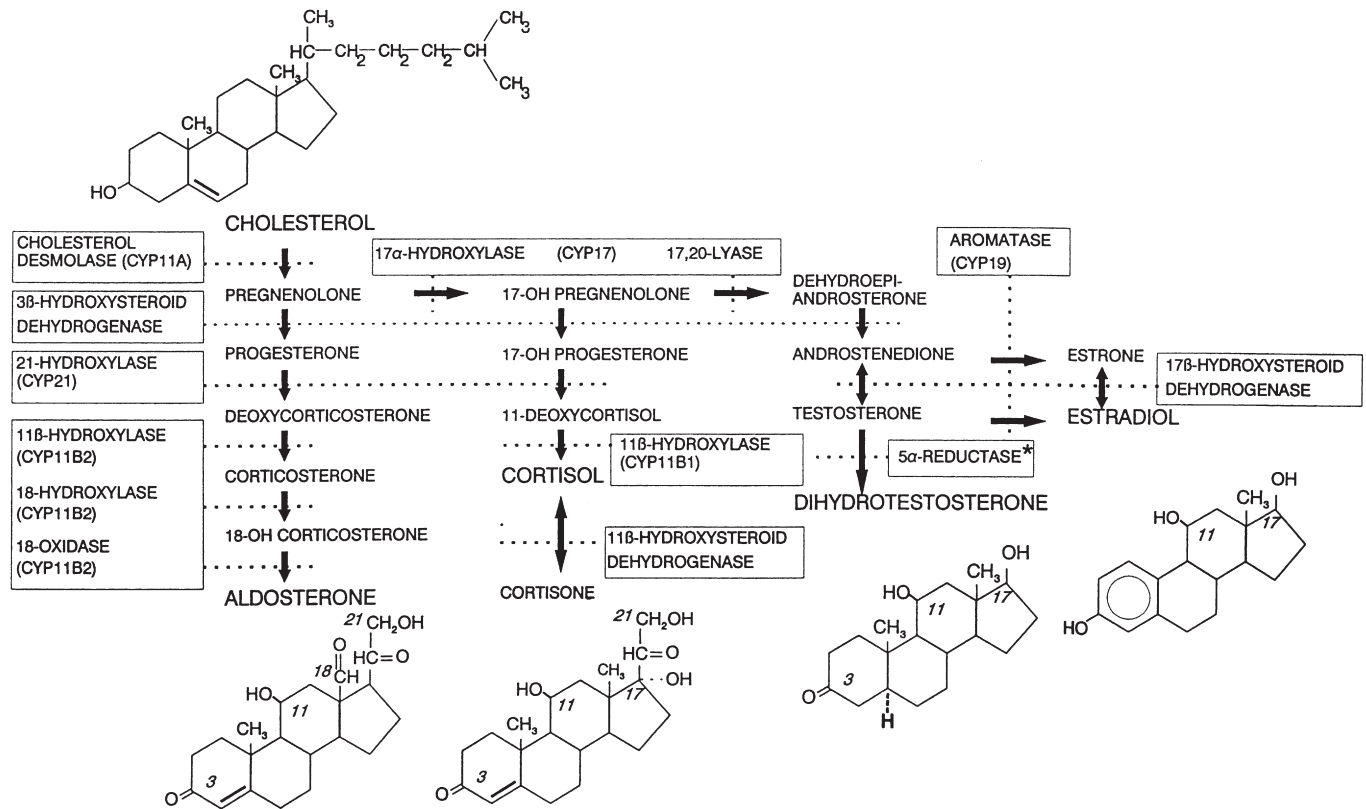
Cortisol, the principal GC in humans and most mammalian primates (1) is quantitatively the main secretory product of the adrenal cortex. It is indispensable for proper carbohydrate metabolism and in ensuring systemic capacity to withstand events of stress such as infection and trauma. Several studies have defined important mediating roles for GCs in the functioning of the immune response (2–5).

The major sex steroids secreted by the adrenal cortex are dehydroepiandrosterone (DHEA) and its sulfate; androgenic steroids and estrogens are normally very minor products. In late childhood, there is a significant rise in adrenal

sex steroid production, termed the adrenarche, leading to further complex interactions with the hypothalamus–pituitary axis and the gonads in preparation for puberty and the transition to sexual maturity (6). This remains the least understood aspect of the function of the adrenal cortex.

#### 87.1.2 Regulation of Steroid Synthesis

The adrenal cortex is divided histologically into three regions: the outer zona glomerulosa, the wide middle zona fasciculata, and the more compact inner zona reticularis adjoining the adrenal medulla. Differences in the production of MCs, GCs, and sex steroids by the adrenal cortex are determined by local activities of certain adrenal enzymes, which correspond in large part with this zonation (7). Synthesis of the MC aldosterone is dependent on enzymatic activity limited to the zona glomerulosa, while production of cortisol and androgens requires enzymes found in the middle and inner zones, the zona fasciculata and zona reticularis. Adrenal steroid synthesis depends predominately on the tropic effects of the anterior pituitary peptide, adrenocorticotrophic hormone (ACTH). ACTH exercises acute and chronic effects on adrenocortical cell processes (8), resulting in an acute response that produces up to 10-fold amplification of the rate of steroidogenesis within 2–6 min. Corticotropin releasing hormone (CRH), produced in the hypothalamus, and vasopressin located in the posterior pituitary synergistically stimulate ACTH production (9–12). The



**FIGURE 87-1** Schema of adrenal steroidogenesis. (From New, M., White, P. In: Genetic and Molecular Biological Aspects of Endocrine Disease; Balliere Tindall: London, 1995, pp: 526.)

most direct result of the intracellular changes induced by ACTH in the adrenal cortex is the increased availability of cholesterol as substrate to cholesterol desmolase, the first enzyme in steroid synthesis. The rate-limiting step in steroidogenesis is cholesterol transport to the matrix side of the inner mitochondrial membrane, which is regulated by the steroidogenic acute regulatory protein (StAR). StAR acts by increasing the flow of cholesterol into the mitochondria. StAR acts on the outer mitochondrial membrane to promote sterol translocation to P450<sub>scc</sub>, and the importation of StAR into mitochondria terminates its action (13). Cholesterol for steroid biosynthesis is provided by the cytoplasmic pool of free cholesterol, which is supplied primarily by the action of cholesteryl ester hydrolase. This kinase-dependent enzyme hydrolyzes cholesterol from cholesteryl esters in cytoplasmic lipid droplet stores.

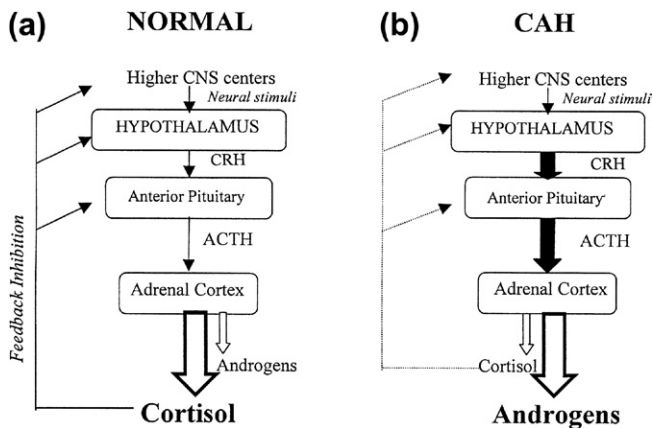
Continued adrenal steroid demand depletes this intracellular source, at which stage uptake of plasma low-density lipoprotein (LDL) provides further quantities of cholesterol and, after prolonged stimulation, cholesterol biosynthesis within the cell becomes significant.

Continued elevation of ACTH also produces progressively wider effects within the cell, increasing mRNA transcription and protein translation rates for each of the enzymes in the cortisol synthetic pathway (14), the enzymes governing lipoprotein endocytosis and

intracellular movement of cholesterol (with up-regulation of the cell surface lipoprotein receptor), and, given sufficiently prolonged stimulation, eventually for the enzymes of cholesterol synthesis as well (8,15).

### 87.1.3 Cortisol and Adrenocorticotrophic Hormone

The synthesis of cortisol is modulated directly by the net circulating levels of ACTH. ACTH, a peptide with a relatively short plasma half-life, is one of a group of trophic hormones cleaved from a high-molecular-weight precursor, proopiomelanocortin. ACTH is stored in the corticotroph cells of the anterior pituitary. Pulsatile release of ACTH from this cell population is in turn modulated by CRH. The hypothalamic–pituitary–adrenal axis forms a regulated system (Figure 87-2). Negative feedback control is exerted by cortisol, and the central nervous system (CNS) determines the hypothalamic setpoint for the expected plasma cortisol level. Net ACTH release has basal, diurnal, and stress-induced components. Plasma cortisol levels lower than the hypothalamic–pituitary setpoint will increase the rate and intensity of ACTH secretory pulses. Adrenal enzyme deficiencies causing impaired synthesis and decreased secretion of cortisol thus lead to chronic elevations of ACTH with overstimulation and consequent hyperplasia of the adrenal cortex.



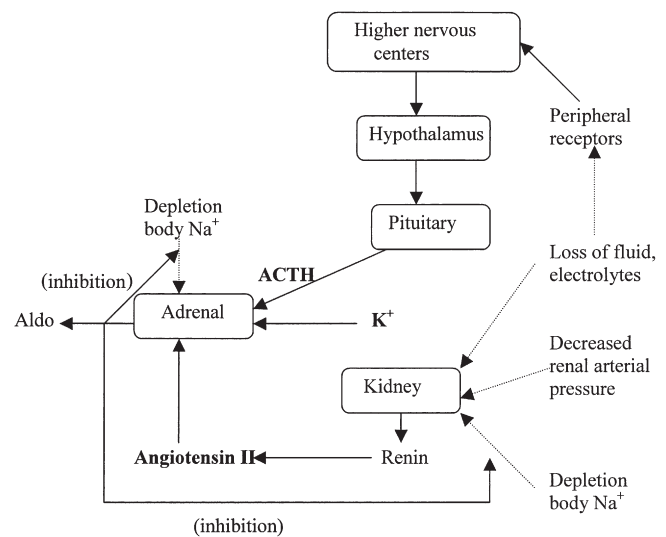
**FIGURE 87-2** Feedback in the hypothalamic-pituitary-adrenal axis in (a) the normal individual and in (b) the patient with classic congenital adrenal hyperplasia.

### 87.1.4 Aldosterone and Renin-Angiotensin

The primary regulation of aldosterone synthesis is via the renin-angiotensin system (RAS) (16), which is responsive to the state of electrolyte balance and plasma volume. The enzyme renin, which arises from the renal juxtaglomerular apparatus, cleaves the decapeptide angiotensin I from angiotensinogen, a plasma  $\alpha_2$ -globulin. Angiotensin I is then further converted enzymatically by passage through the lungs to the octapeptide angiotensin II. Angiotensin II is a potent vasoconstrictor that directly stimulates aldosterone secretion by the zona glomerulosa (17). Aldosterone secretion is also stimulated directly by high serum  $K^+$  concentration, less sensitively by low serum  $Na^+$  concentration, and by ACTH (17). ACTH has a permissive role through its general effect on adrenocortical function but, in addition to this, the zona glomerulosa is transiently very sensitive to ACTH, especially when the late aldosterone synthetic stages specific to this zone have been potentiated by chronic angiotensin II stimulation or electrolyte imbalance ( $K^+$  loading/ $Na^+$  restriction) (18). In addition, cis-regulatory elements appear to influence transcription of aldosterone synthase (CYP11B2), the enzyme that catalyzes the final steps in aldosterone synthesis, but further studies will be necessary to define these factors (19). Thus, the four major factors affecting aldosterone synthesis by the zona glomerulosa are angiotensin II,  $K^+$ ,  $Na^+$ , and ACTH (Figure 87-3).

### 87.1.5 The Adrenal Cortex as Two Glands

Proper adrenal development in the fetus is essential for fetal maturation and survival in the perinatal period (20). While the adrenal medulla cells originate in the neuroderm, primitive cells of the adrenal cortex are first identified in the fourth week of gestation arising from the mesoderm. In the following weeks, the migrating cells organize into two separate zones: the fetal zone and the definitive (adult) zone (21). The fetal zone is much larger



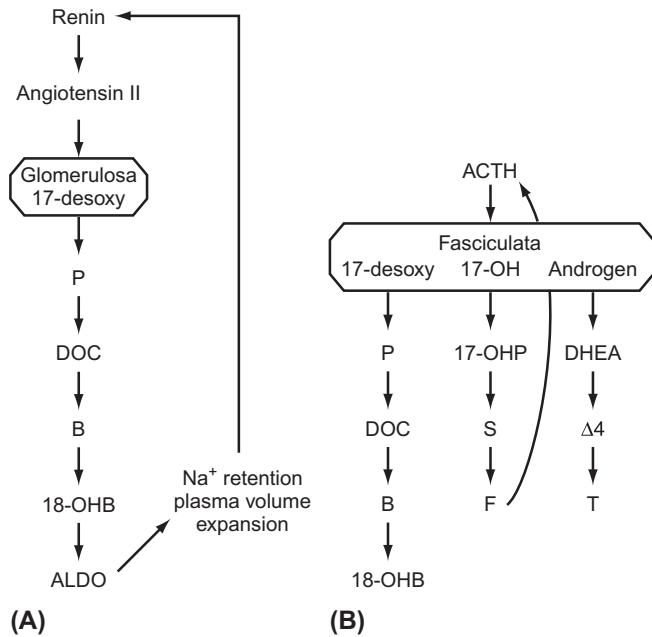
**FIGURE 87-3** Regulation of aldosterone production by the four major factors: angiotensin II, plasma potassium ion ( $K^+$ ), ACTH (adrenocorticotropin), and sodium ( $Na^+$ ). (From New, M. I.; Peterson, R. E. *Disorders of Aldosterone Secretion in Childhood*. *Pediatr. Clin. North Am.* 1966, 13, 43–58.)

than the very compact definitive zone and is responsible for the majority of fetal steroidogenesis, including DHEA sulfate. ACTH is the primary trophic regulator of the fetal adrenal cortex; however, growth, transcription, and placental factors are also important in development of the cortex. Remodeling of the adrenal cortex occurs in the neonatal period when the fetal zone atrophies and the three zones of the adult cortex develop (22).

The zonal theory of the adrenal cortex indicates that the zona glomerulosa and the zona fasciculata are histologically distinct cell populations. New and Seaman (23) formulated the idea that the adrenal behaves hormonally and biochemically as two separate glands with respect to regulation and secretion. Steroidogenesis in the fasciculata is regulated primarily by ACTH (in the hypothalamic-pituitary-adrenal axis), the ACTH stimulating secretion of cortisol, deoxycorticosterone (DOC), corticosterone, and androgens. Steroidogenesis in the glomerulosa is regulated primarily by angiotensin II (in the RAS) and potassium; angiotensin stimulating aldosterone secretion through ACTH presumably exerts only a secondary influence in this zone (Figure 87-4). The zona fasciculata does not exhibit the enzymatic activity necessary for the terminal step of aldosterone synthesis, whereas the zona glomerulosa lacks the  $17\alpha$ -hydroxylase activity required for the production of  $17$ -hydroxycorticoids and androgens.

### 87.1.6 Adrenal Steroids in Development

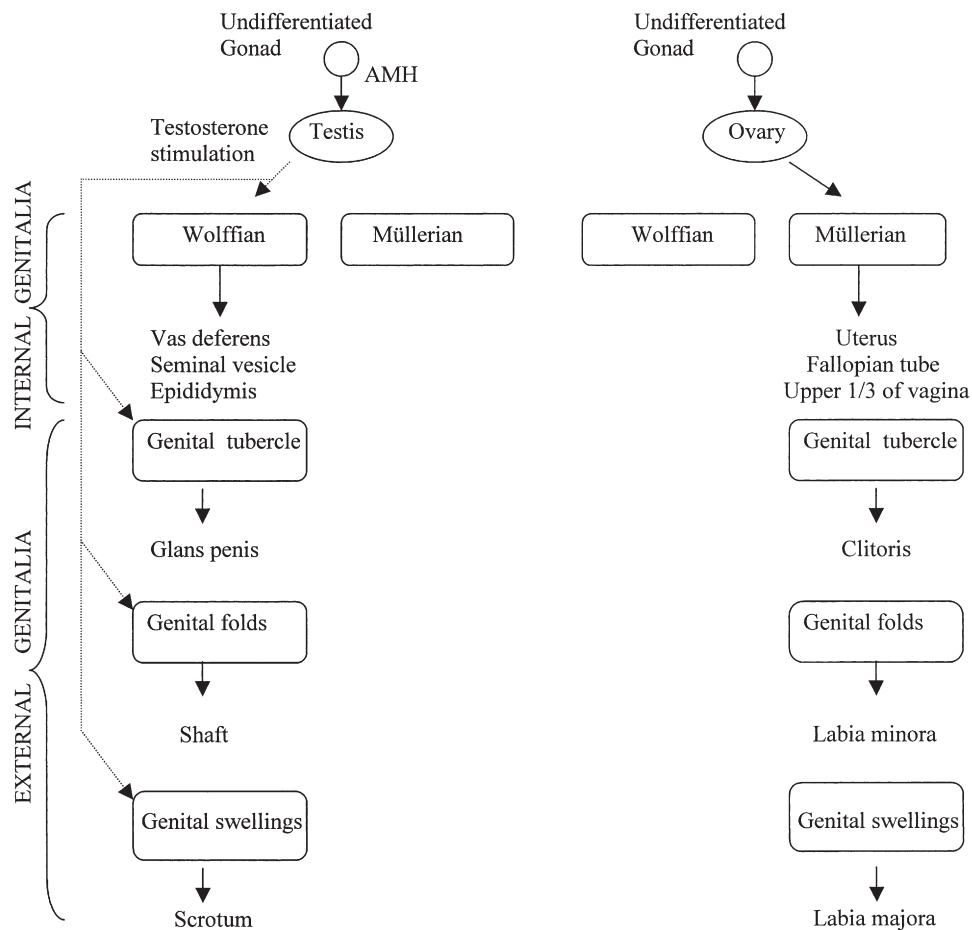
Normal male genital differentiation in embryonic and fetal life is dependent on two functions of the fetal testes (24): (1) the secretion of sufficient quantities of testosterone to direct the formation of the internal male genital structures (i.e. the epididymis, vas deferens, seminal vesicles, and



**FIGURE 87-4** Regulation of adrenocortical steroid production considering (a) the zona glomerulosa (ZG) and (b) the zona fasciculata (ZF) as two separate glands in two separate negative (inhibitory) feedback loops. (From Reference (69).)

ejaculatory ducts) from the Wolffian (mesonephric) ducts and (2) the secretion of a nonsteroidal factor to suppress development of the müllerian ducts into the female internal structures (i.e. the fallopian tubes, uterus, cervix, and upper vagina) (Figure 87-5). This factor, anti-müllerian hormone (AMH), also termed müllerian inhibiting substance (MIS) or factor (MIF), is a glycoprotein dimer (25) known to belong to a superfamily of peptide growth/differentiation factors, including the activins and transforming growth factor- $\beta$  (TGF- $\beta$ ) (26). In the fetal testis, AMH is first synthesized by differentiating Sertoli cells (27) at 6–7 weeks of gestation and thus precedes testosterone secretion by the Leydig cells, which begins at about the eighth week. Since there is no anomalous production of AMH in the gonadally normal female, females suffering even the most extreme virilization from adrenal androgen excess have normal development of the internal genital structures. The rescue of childbearing capacity in these gonadally intact patients is thus a prime goal of treatment in congenital adrenal hyperplasia (CAH).

Testosterone is also required for suppression of the breast anlage and indirectly for normal formation of the male external genitalia. To act on the tissues of the external genital primordium, testosterone must first undergo peripheral conversion to dihydrotestosterone (DHT). The



**FIGURE 87-5** Fetal sex differentiation. (From Reference (167).)



differentiation promoted by DHT includes formation of the scrotum from the genital swellings, midline closure of the genital folds, elongation into the body of the phallus, and extension of the urogenital sinus by fusion along the ventral groove to form a penile urethra (28).

Pertaining to the virilizing forms of CAH, progressive differentiation toward the male type in genetic females has been given a five-stage classification by Prader (29,30). Raised androgen levels from abnormal fetal adrenal function (virilizing adrenal hyperplasia) or from the mother (an androgen-producing tumor or exogenous androgen administration during pregnancy) do not affect genital development in the male fetus because of the already high levels of androgens produced by the testes. The androgen levels, however, are high enough to cause significant masculinization of the external genitalia in females. Virilized genitalia of the female fetus have now been reported to also result from changes in steroid conversion consequent to a defect in the placental aromatase enzyme (31,32). To date, there have been seven males and seven females reported with aromatase deficiency (33). In pregnancies at risk of a female child affected with virilizing adrenal hyperplasia, successful suppression of fetal adrenal androgen production has resulted by giving the mother GCs able to cross the placental barrier (see discussion of CAH prenatal diagnosis and treatment, in Section 87.8).

### 87.1.7 Pathogenesis of Congenital Adrenal Hyperplasia

CAH refers to the histological alterations consequent to elevated ACTH and chronic glandular overactivity resulting from the inability of the adrenal cortex to achieve normal plasma cortisol levels in response to (normal) ACTH stimulation (see Figure 87-2). With the exception of lipoid CAH, CAH arises biochemically from reduced or absent enzymatic activity at one of the steps of steroid synthesis. Each deficient enzymatic step produces characteristically abnormal adrenal hormone and precursor levels. The varying patterns of imbalance give rise to a wide range of clinical effects manifesting in metabolic disturbances and developmental abnormalities.

The following enzymatic defects of steroidogenesis and their associated clinical syndromes have been described (34–37):

1. 21-Hydroxylase deficiency (classic salt wasting, classic simple virilizing, and nonclassic)
2. 11 $\beta$ -Hydroxylase deficiency (classic and nonclassic)
3. 3 $\beta$ -Hydroxysteroid dehydrogenase (3 $\beta$ -HSD) deficiency (classic and nonclassic)
4. 17 $\alpha$ -Hydroxylase/17,20-lyase deficiency
5. Congenital lipoid adrenal hyperplasia (StAR deficiency)

The 21- and 11 $\beta$ -hydroxylase deficiencies, occurring distal to the common precursor stages, cause channeling of these precursor steroids into the androgen pathway,

resulting in virilization of females and hyperandrogenic effects in both sexes. In the 3 $\beta$ -HSD defect, there is glandular production only of  $\Delta^5$  steroids, which are relatively inactive. While lack of testicular production of  $\Delta^4$  androgens produces pseudovaginal hypospadias in the male, enormously high-secreted levels of DHEA may undergo peripheral conversion to more potent androgens, which are then able to cause external virilization in females. In 17 $\alpha$ -hydroxylase deficiency, there is extremely high production of 17-deoxysteroids: corticosterone (B), which has some GC function, and 11-deoxycorticosterone (DOC), which causes hypertension. Blocked formation of C<sub>19</sub>/C<sub>18</sub> steroids in both the 17 $\alpha$ -hydroxylase/17,20-lyase deficiency and the isolated 17,20-lyase defect (in which cortisol production is intact) causes undervirilization in males and sexual infantilism in females. Congenital lipoid adrenal hyperplasia results from a complete inability to synthesize steroids causing lipid accumulation in adrenal cells, and along with the very serious effects of adrenal insufficiency, undervirilization occurs in genetic males.

### 87.1.8 Classic and Nonclassic Forms

Total or near-total blocks in the activity of these enzymes result in genital ambiguity and represent the classic form of CAH (36). Improved biochemical assessment of adrenal function now allows for the identification of lesser enzyme defects also, which cause milder endocrine disturbance and absence of genital ambiguity. Called nonclassic forms, such partial defects have been confirmed for steroid 21-hydroxylase (38), steroid 11 $\beta$ -hydroxylase (39), and 3 $\beta$ -HSD (40). As might be expected, nonclassic defects are much more common than occurrences of the corresponding classic defects.

## 87.2 STEROID 21-HYDROXYLASE DEFICIENCY

Decreased cortisol synthesis owing to impaired steroid 21-hydroxylation is the most common biochemical cause of CAH. The decreased plasma cortisol induces pituitary ACTH secretion, stimulating the adrenal cortex and causing elevated adrenal production both of cortisol precursors and of androgens that do not require 21-hydroxylase for their biosynthesis. Early clinical studies showed increased amounts of pregnanetriol, the principal metabolite of 17 $\alpha$ -hydroxyprogesterone (17-OHP), 17-ketosteroids (17-KS; 17-oxosteroids), resulting from the metabolism of DHEA,  $\Delta^4$ -androstenedione ( $\Delta^4$ -A), and testosterone, present in the urine of patients with 21-hydroxylase deficiency. Radioimmunoassay (RIA)-based laboratory procedures for the determination of hormone levels in serum are simple, reliable, and allow for a more accurate diagnosis than could be provided formerly by methods assessing urinary hormone and metabolite levels.

### 87.2.1 Classic 21-Hydroxylase Deficiency

**87.2.1.1 Simple Virilizing Form.** The prominent feature of 21-hydroxylase deficiency is progressive virilization with advanced somatic development. The classic disorder is of the simple virilizing type in about one-fourth to one-third of cases. Developmental genital anomalies manifest in females as varying degrees of genital ambiguity, which should flag the diagnosis in the female. Because genital formation in males is normal, before the wide implementation of the newborn screening program for CAH, the disease often went unrecognized until signs of androgen excess such as accelerated height and precocious sexual hair appeared later in childhood.

Adrenocortical cell differentiation and the formation of the fetal zone occur early in embryogenesis, and it is clear that genital development in the fetus takes place under the influence of active adrenal steroid synthesis. Thus, in the female, the extent of masculinization of the external genitalia ranges from mild clitoral enlargement, through varying degrees of fusion of the labioscrotal folds (posterior to anterior), to the profound morphological anomaly of a penile urethra.

Genetic sex, gonadal differentiation, and internal genital morphogenesis are normal in 21-hydroxylase deficiency. Since there is no anomalous secretion of AMH in the female, the müllerian ducts develop normally into uterus and fallopian tubes. Wolffian duct stabilization and differentiation proceed under the control of high intraluminal levels of gonadal androgens in the male; this process appears to be unaffected by elevated adrenal androgens, and there is no observable Wolffian development in females with 21-hydroxylase deficiency CAH.

The most common cause of ambiguous genitalia in the newborn female is 21-hydroxylase deficiency, and because affected females have the capacity for an entirely normal female sex role including childbearing, it is very important to recognize this disorder in newborns. Although the male is not jeopardized by inappropriate sex assignment, premature masculinization, short stature, and accelerated physical development cause problems of social and physical adjustment. In addition, continued adrenal androgen excess may suppress the pituitary–gonadal axis, preventing maturation of the testes, resulting in infertility (41,42). In both sexes, there is early fusion of the epiphyses, causing short adult stature.

**87.2.1.2 Salt-Wasting Form.** Two-thirds to three-fourths of classic cases (i.e. presenting at birth) of 21-hydroxylase deficiency present with renal salt wasting, primarily from deficient aldosterone synthesis (43), defined by hyponatremia and hyperkalemia, inappropriately high urinary sodium, and low serum and urinary aldosterone with concomitantly high plasma renin activity (PRA). The increase in the proportion of salt wasting cases in recent years can be attributed to better case identification and patient survival. In addition to inadequate secretion of aldosterone or other

salt-retaining steroids, other precursors with natriuretic action produced in excess may counter the marginally competent sodium-conserving mechanism of the immature newborn renal tubule (44–46). Salt loss in infancy from an aldosterone biosynthetic defect may improve with age (39,47,48), and possible adjustments in sodium intake and MC replacement in patients labeled neonatally as salt wasters can be made on the basis of careful monitoring of PRA.

Although correlation of the severity of salt wasting with the extent of virilization has been claimed (49), the degree of genital ambiguity in a female newborn does not indicate the form. Even mildly virilized newborn females with 21-hydroxylase deficiency may be salt wasters and should be observed carefully for signs of adrenal insufficiency in the first weeks of life.

With a few exceptions (48,50,51), the presence or absence of salt wasting in 21-hydroxylase deficiency has been seen consistently within families, and subsequent affected offspring have thus been expected to have the same form of the disease as the index case. However, discordance for salt wasting has been found in several families among siblings whose molecular studies confirmed identical mutations (52–54).

**87.2.1.3 Nonclassic 21-Hydroxylase Deficiency.** An attenuated, late-onset form of adrenal hyperplasia was first suspected during the early 1950s by gynecologists in clinical practice who used GCs on an empirical basis to treat women with physical signs of hyperandrogenism, including infertility. The first biochemical documentation of an apparent 21-hydroxylase defect was by Baulieu and coworkers in 1957 (55). During the next two decades, glucocorticoid treatment of virilized women became commonplace, and in cases of a good response it was assumed that the androgens were of adrenal origin.

Diagnosis of 21-hydroxylase defects on the basis of serum steroid measurements became feasible with the development of a specific RIA for 17-OHP (56), the steroid immediately proximal to the adrenal 21-hydroxylating step in the cortisol pathway, and thus the index compound for the 21-hydroxylase defect. Based on RIA assays, family members of CAH patients were found to have serum elevations of 17-OHP above the intermediate levels observed for most heterozygotes for a severe 21-hydroxylase-deficiency gene (57). Many family studies on classic 21-hydroxylase deficiency followed the initial report by Dupont and coworkers (58) of genetic linkage of CAH (21-hydroxylase deficiency) with human leukocyte antigen (HLA). Through such studies, the existence of nonclassic 21-hydroxylase deficiency alleles became apparent (59–62). Linkage of nonclassic 21-hydroxylase deficiency to HLA was established (63,64), confirming that this disorder was allelic with the classic defect (59,65). It is now known that mutations in the gene for 21-hydroxylase associated with the nonclassic defect are distinct from those found in the classic forms and often differ by ethnicity (54,66).

Clinical symptomatology of nonclassic 21-hydroxylase deficiency is variable and may present at any age. Nonclassic 21-hydroxylase deficiency can result in premature development of pubic hair in children; to our knowledge, the youngest such patient was noted to have pubic hair at 6 months of age (67). In a review of 23 cases presenting to The New York Presbyterian Hospital–Weill Medical College of Cornell University for evaluation of premature pubarche, seven children demonstrated a 17-OHP response to ACTH stimulation consistent with the diagnosis of nonclassic 21-hydroxylase deficiency, a prevalence of 30% in this preselected group of pediatric patients at high risk (68). Other investigators found four of 48 children with premature adrenarche to be affected with nonclassic 21-hydroxylase deficiency, demonstrating an ACTH-stimulated 17-OHP response greater than that of obligate heterozygote carriers of the 21-hydroxylase-deficiency gene (69,70). Elevated adrenal androgens promote the early fusion of epiphyseal growth plates; children with this disorder commonly have advanced bone age (67) and accelerated linear growth velocity (71), although they are ultimately shorter than the final height prediction based on mid-parental height and on linear growth percentiles before the apparent onset of excess androgen secretion.

Severe cystic acne refractory to oral antibiotics and retinoic acid has been attributed to nonclassic 21-hydroxylase deficiency (72,73). In one study comparing the responses of 11 female patients with acne and 8 (female) control subjects to a 24-h infusion of ACTH, elevated urinary excretion of pregnanetriol suggestive of a partial 21-hydroxylase deficiency was found in six patients (74). In another study of 31 young female patients with acne and/or hirsutism tested with low-dose ACTH stimulation after overnight dexamethasone suppression, no case of 21-hydroxylase deficiency was found (75). Male-pattern baldness has been noted in other cases as the sole presenting symptom in young women with nonclassic 21-hydroxylase deficiency.

Menarche in females may be normal or delayed, and secondary amenorrhea is a frequent occurrence (62,67). A sector of female nonclassic 21-hydroxylase deficiency patients represent a subgroup of women affected with polycystic ovarian disease (76–78). Initial-phase adrenal sex-steroid excess, disrupting the usual cyclicity of gonadotropin release and/or with direct effects on the ovary, is probable in the pathophysiology of this syndrome, ultimately leading to the formation of ovarian cysts, which then may continue autonomously to produce androgens.

Retrospective analysis identified partial 21-hydroxylase defects in 16 of 108 women (14%) presenting to New York Presbyterian Hospital–Weill Medical College of Cornell University for evaluation of hirsutism and oligomenorrhea (79). The prevalence of nonclassic 21-hydroxylase deficiency as an etiology of these endocrine complaints in women in other published series

ranges from 1.2% to 30% (76–78,80–82). The wide range of frequencies in these reports may relate to differences in the ethnic makeup of the groups studied since the disease frequency is ethnic specific.

Although the androgen profiles in serum and urine in either the basal or the ACTH-stimulated state may not be markedly different overall from those demonstrated by women with the syndrome of polycystic ovaries from other causes, the serum 17-OHP response on ACTH stimulation clearly differentiates the patients with an adrenal 21-hydroxylase defect (69,79). In one report, of six women with nonclassic 21-hydroxylase deficiency who underwent sonography or laparoscopic visualization of the ovaries, four had polycystic ovaries (69). Thus, even sonograms of the ovary do not distinguish women with excess androgens due to polycystic ovarian disease from those with nonclassic 21-hydroxylase deficiency. The response of the hypothalamic–pituitary–gonadal axis to luteinizing hormone-releasing hormone (LHRH) has been observed to be variably abnormal in virilized women with nonclassic 21-hydroxylase deficiency (83). An ACTH stimulation test is necessary to differentiate polycystic ovarian disease from nonclassic 21-hydroxylase deficiency after LHRH testing of pituitary gonadotropin secretion.

In boys, early beard growth, acne, and growth spurt may be detected. In cases of pubic hair growth and enlarged phallus from an androgen excess condition in boys, a reliable indication of an adrenal (as opposed to testicular) source of androgens is the proportionately small size of the testes that results from suppression of the hypothalamic–pituitary–gonadal axis. In men, signs of androgen excess are difficult to appreciate; the manifestations of adrenal androgen excess may be limited to short stature or oligozoospermia and diminished fertility from this same adrenal sex-steroid induced gonadal suppression reversible with GC treatment (41,42,84,85). Certain individuals—males and females—affected with nonclassic 21-hydroxylase deficiency have no overt symptoms of disease while demonstrating biochemical abnormalities comparable with patients. Longitudinal follow-up evaluation of these cases (usually detected as part of a family study) often shows signs of hyperandrogenism to wax and wane with time.

## 87.2.2 Epidemiology

**87.2.2.1 Classic 21-Hydroxylase Deficiency.** From the first separation of the “adrenogenital syndrome” into different enzyme defects, steroid 21-hydroxylase deficiency has been recognized to be the predominant form. It accounts for 90–95% of all cases of CAH. The incidence of classic 21-hydroxylase deficiency reported in numerous studies based on case presentation shows considerable variation. Currently, the combined figures from the largest newborn screening programs in different populations show an overall worldwide frequency of

1 per 13,000–15,000 live births (86), NNSIS 2009 National Newborn Screening Information System. (Available at <http://www2.uthscsa.edu/nnsis>).

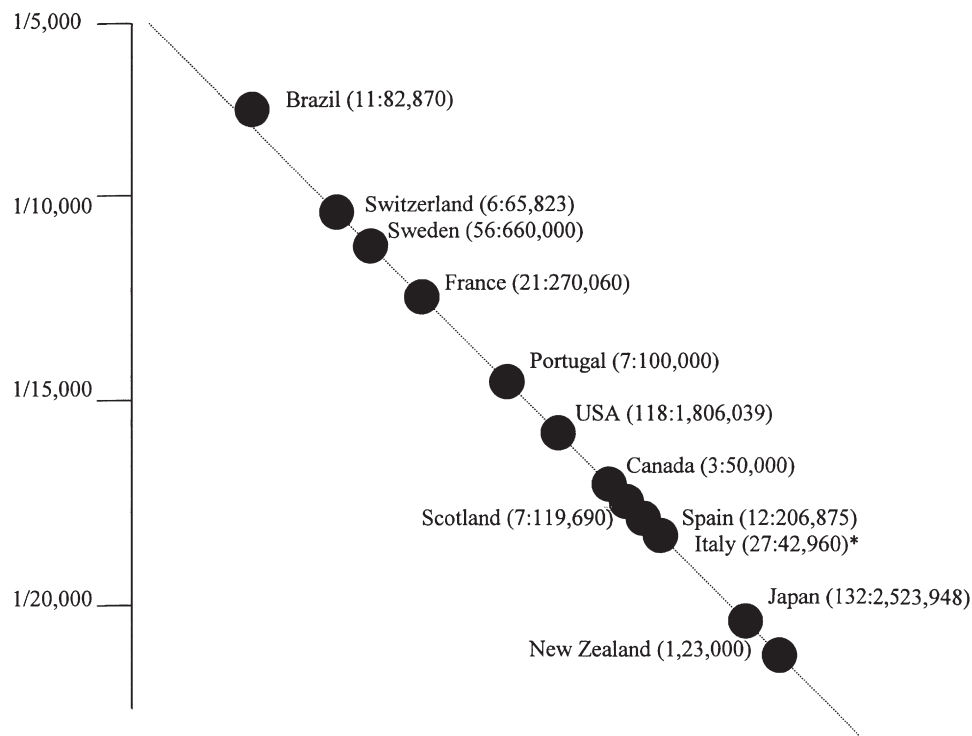
In North America, the estimated incidence of virilizing CAH in the earliest regional (Maryland) study, which in fact was the first to establish that the disease shows autosomal recessive transmission, was very low, clearly due to incomplete case identification (the underrepresentation of males was recognized) (87). Subsequent regional studies by case presentation reported confirmed figures of 1 per 15,000, although suggested closer to one per 10,000 in one study (50) and one per 26,292 specifically for the salt-wasting form in another (88). Retrospective analysis of CAH births in the province of Manitoba for the period 1968–1988 revealed an incidence of 1 per 14,500 (similar to screening studies) but a skewed female/male sex ratio of 2.2:1, suggesting probable deaths of undiagnosed males (89). Newborn screening was operational in eight states (AK, GA, IL, IA, MA, NC, TX, WA) in the United States for the calendar year 1990, and the statistics from these programs gave a combined incidence of one in 16,116 (90). A recent report on the screening results of 1.9 million Texas newborns revealed an incidence of 1 in 16,008, with a ratio of salt wasting to simple virilizing of 2.7:1 (91). Currently all 50 states are participating in newborn screening for CAH.

European case survey studies have given regional incidences ranging from one in 13,000 to one in 23,040 in France (92,93), one in 21,000 in the Republic of Ireland

(94), one in 20,900 in Scotland (95), one in 18,500 and one in 15,500 in Switzerland (29,96), one in 12,100 in Wales (97), and one in 8990 in the Tyrol (98). The incidences reported from a few metropolitan area studies suggest better case identification: one in 9800 from Munich (99) and one in 5040 from Zurich (100). Combined data from more recent and ongoing neonatal screening programs in France show a sex ratio (M/F, 1:1.3) representing a deficit in the number of male patients detected (101). Incidences currently given by screening programs in some European and other countries are shown in Figure 87-6.

An initial case survey study in Japan had once suggested a somewhat lower incidence than in white populations, one in 43,674 (102). Nationwide screening in Japan has now determined that the incidence overall does not differ significantly from that in other countries (see Figure 87-6), and in fact for two of 11 regions an incidence significantly higher than in other areas was found (103).

Two isolates show unusually high incidences: Yup'ik Eskimo (Inuit) people of the Kuskokwim river delta in southwestern Alaska and the mixed colonial/indigenous population of the island of La Réunion in the Indian Ocean. On La Réunion, frequency observed by screening was one in 2220 in 1988 and one in 3540 cumulatively over a more than 10-year period (104). The high frequency of severe salt-wasting CAH among the Yup'ik (105,106) appears to result from a single mutation established in this group (107).



**FIGURE 87-6** Incidence of classic 21-hydroxylase deficiency as given by some current screening programs internationally. (Data from (86); Balsamo, A.; Cacciari, E.; Piazzzi, S.; et al. Congenital Adrenal Hyperplasia: Neonatal Mass Screening Compared with Clinical Diagnosis Only in the Emilia-Romagna Region of Italy 1980–1995. *Pediatrics* 1996, 98, 362–367.)



**87.2.2.2 Nonclassic 21-Hydroxylase Deficiency.** The high frequency of nonclassic 21-hydroxylase deficiency was first determined in a numerical analysis of genetic and hormonal data on a collection of pedigrees from a number of ethnic groups (108), and it was confirmed by a follow-up study using a more rigorous computer-based analysis by the method of commingling distributions (109). The gene frequency for nonclassic 21-hydroxylase deficiency was highest in Ashkenazi Jews and was also high in Hispanics, Yugoslavs, and Italians. Disease frequencies were 0.037 (one in 27) for Ashkenazi Jews, 0.019 (one in 53) for Hispanics, 0.016 (one in 63) for Yugoslavs, 0.003 (one in 333) for Italians, and 0.001 (1/1000) for other whites (Figure 87-7).

### 87.2.3 Molecular Genetics and 21-Hydroxylase Deficiency

The adrenal steroid 21-hydroxylase enzyme is a microsomal cytochrome P450. The members of this functionally diverse group of heme proteins are grouped into a number of families that all accept electrons from a NADPH-dependent cytochrome P450 reductase (110,111). The adrenal 21-hydroxylase is the unique known occupant of its family, XXI. Following the cytochrome P450

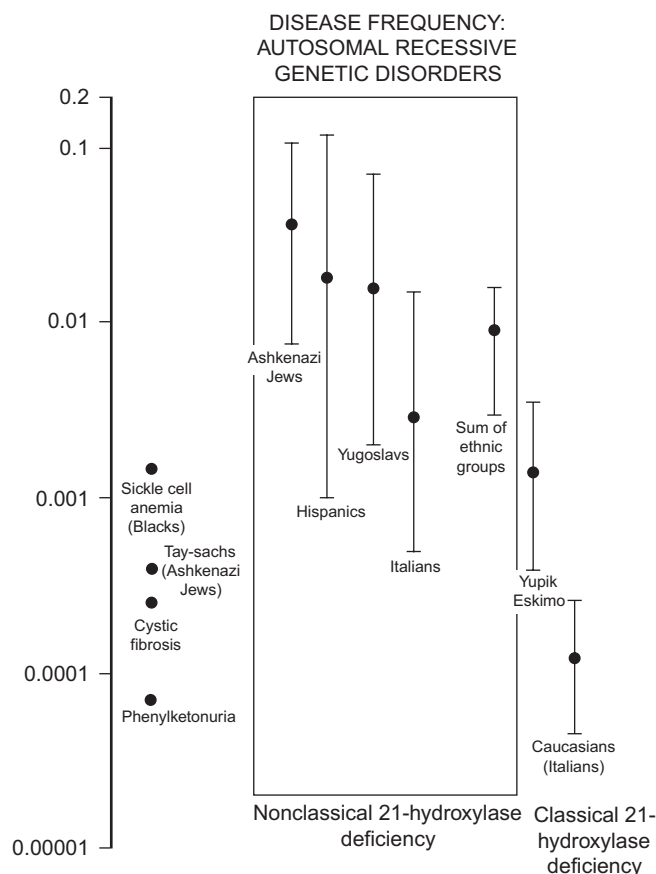
nomenclature, the enzyme and the gene encoding the enzyme are termed *CYP21A2*. This gene *CYP21A1P* is situated within the human major histocompatibility complex, HLA, on the short arm of chromosome 6, in a region exhibiting the highest gene density of all known chromosome regions to date.

The *CYP21A2* gene is mapped to 6p in a tandem paired arrangement with several other genes—two related active genes, *C4A* and *C4B*, the genes for the two isoforms of component C4 of serum complement; and a homolog to the *CYP21A2* gene, *CYP21A1P*, a pseudogene that retains 96–98% similarity in base sequence, but contains deleterious mutations generating a nonfunctional protein product. Flanking *C4A* and *C4B* in the 5' region are genes *RP1*, encoding a nuclear protein, and *RP2*, a pseudogene of *RP1*. On the opposite strand, extracellular matrix protein tenascin *TNXB* and *TNXA* overlap with the last exon of *CYP21A2* and *CYP21A1P* (112). The *C4* genes are both moderately allelic, expressing a number of serum variants (allotypes) for each isoform, which show fine differences in electrophoretic mobility, while generally the two *C4* isoforms (corresponding to the Rogers and Chido antigens) differ in hemolytic activity, the *C4B* about four times greater. Comparison of the nucleotide sequences of *CYP21A2* and *CYP21A1P* (113,114) shows that any possible expression of *CYP21A1P* is precluded by a number of irregularities in the open reading frame.

The *CYP21A2* and *CYP21A1P* genes are about 5kb long, the *C4A* gene about 21kb and the *C4B* gene in two size variants 21kb and 14.6kb long. They occupy an 80-kb segment in the order *C4A*–*CYP21A1P*–*C4B*–*CYP21A2*, all oriented with the sense strand reading left to right. This configuration is itself hypothesized to have come about by a past recombination event or events in mammalian evolution occurring for an original single *C4*\*–*CYP21A2*\* pair and the duplication going to fixation.

Two types of genetic instability apparently exist to generate mutations at the rates maintaining the high incidence of steroid 21-hydroxylase deficiency. The first is created by the symmetry of the arrangement, which predisposes it to misalignment and unequal crossing over in meiosis, producing imbalanced complementary chromosomes, one carrying a further duplication and the other with a deletion of one type of gene or (most often) a *C4*–*CYP21A2* unit. In the second, homologous genes are subject to a less well-understood process involving the nonreciprocal transfer of (generally shorter) DNA sequences from *CYP21A1P* to *CYP21A2*, called gene conversion (115).

Both classic and nonclassic 21-hydroxylase deficiency are inherited in a recessive manner as allelic variants. Classic 21-hydroxylase deficiency results from the presence of two severely affected alleles and nonclassic 21-hydroxylase deficiency results from the presence of either two mild 21-hydroxylase deficiency alleles or one severe and one mild allele.



**FIGURE 87-7** Incidence of nonclassic 21-hydroxylase deficiency in comparison with some other disease incidences. (From Reference (108).)

**87.2.3.1 Human Leukocyte Antigen Linkage.** Genetic linkage between HLA and 21-hydroxylase was reported by Dupont and coworkers in 1977 (58,116–119). This linkage, initially useful in family studies, has been supplanted by molecular genetic studies.

The initial molecular genetic analysis of 21-hydroxylase deficiency made use of the known associations of the disorder with specific HLA antigens B and DR and serotypes of C4, the fourth component of serum complement. In particular, salt-wasting 21-hydroxylase deficiency is often (15–20% of all alleles) associated with an extended HLA haplotype, A3, Bw47, DR7, which is half-null for C4 (i.e. only one serum isoform instead of two normally present) (120–122). Using a probe for 21-hydroxylase, DNA samples obtained from normal individuals and from steroid 21-hydroxylase deficiency patients with HLA types Bw47/- and Bw47/Bw47 were tested. Southern blotting after digestion with several restriction endonucleases showed that normal DNA samples yielded two fragments hybridizing with the probe at equal intensity. By contrast, DNA samples from the HLA-Bw47 heterozygous 21-hydroxylase-deficient individuals showed a reduced intensity of one band and the DNA sample from the HLA-Bw47 homozygous individuals showed a total absence of that band (123). Additionally, the hormonally normal status of individuals missing the CYP21A1P (originally termed the CYP21A gene) fragment after hybridization and Southern blotting suggested the gene was not necessary for adrenal steroid 21-hydroxylating function (124,125).

The deletions within the HLA-A3; Bw47; DR7 and HLA-A1; B8; DR3 haplotypes were further characterized: the HLA-A1; B8; DR3 haplotype was found to be missing the C4A gene next to the absent CYP21A1P, and the HLA-A3; Bw47; DR7 haplotype to be missing C4B, next to the absent B gene (CYP21A2) (124,126,127). Physical mapping of this region showed that each repeat unit C4A + CYP21A1P and C4B + CYP21A2 spans about 30 kb and that within each unit the C4 and CYP21 are only 3 kb apart.

The HLA-A1; B8; DR3 haplotype is not uncommon, occurring in about 5% of all normal chromosomes (128) and has itself been negatively associated with 21-hydroxylase deficiency. Disproportion in length between a single C4–CYP21A2 compared with the duplicated units on most other haplotypes may discourage recombination and lead to increased stability of this and other haplotypes such as the “Sardinian” haplotype without duplication of C4 and CYP21A2 genes, conjectured to be an ancestral type (129).

Further examination of the DNA sequence of HLA-A3; Bw47; DR7 identified a crossover site for the past recombination event within the CYP21A2 gene, such that misalignment between CYP21A2 and CYP21A1P produces a nonfunctional chimeric gene. Of significance for Southern blotting restriction fragment length polymorphism (RFLP) studies, chimeric fragments may also

be created with lengths established by the distance to the first restriction sites upstream and downstream of the splice, and these can coincide in some instances with standard fragment lengths, wrongly suggesting the presence of DNA that is actually deleted. Proper interpretation of such banding patterns may present considerable difficulty (130–133). A more accurate analysis can be made with the use of multiple and widely spaced restriction digests; however, the use of RFLP and dot blot analysis has been replaced in favor of newer polymerase chain reaction (PCR) techniques. Rapid allele-specific PCR techniques have been proved to be less time consuming and more sensitive, but must be used in conjunction with Southern blot analysis in order to distinguish between deletions and gene conversions (134). Pulsed field gel electrophoresis and multicolor fluorescence in situ hybridization (FISH) have also been used to identify deletions and duplications (135,136). Recently, a new rapid analysis method has been developed to detect point mutations and large deletions by PCR (137).

Duplication/deletion events have created the rearrangements observed in the HLA-A1; B8; DR3 and the A3; Bw47; DR7 haplotypes (126). The HLA-Bw47 type is extremely rare in mixed white populations. In the northern United Kingdom, where it has a higher natural frequency, deletion/duplication events are also found to account for a higher proportion of cases of 21-hydroxylase deficiency CAH, in the range of 30–35%.

The haplotype HLA-B14, DR1 has been found to be associated with from 36% (138) to over 85% (139) of cases of nonclassical 21-hydroxylase deficiency in different groups according to ethnic composition. In individuals with this haplotype, an extra C4B gene (122,124,140) and an extra CYP21A1P or CYP21A1P-like gene (124,141) have been identified. The mutations present in the active CYP21A2 gene on this haplotype have since been identified (142), and expression studies have confirmed that the enzyme expressed has 20–50% of normal activity (143).

**87.2.3.2 Deletions and Gene Conversions.** HLA genotyping is a possible means of diagnosis but has now been superseded by direct molecular analysis of the 21-hydroxylase locus. Allele-specific amplification of the CYP21A2 gene utilizing PCR is the preferred method of mutation analysis (134).

In most populations, CYP21A2 mutations in the form of deletions generally appear to comprise about 20% of classic 21-hydroxylase deficiency alleles (144–147), the majority of which have associated deletions of a C4B gene. The majority of deletions stretch from a region between exons 3 and 8 of CYP21A1P to a corresponding base pair in CYP21A2. Since the hybrid genes contain one or more mutations associated with the pseudogene, they are nonfunctional (133).

Most haplotypes carrying CYP21A2 alleles that cause classic CAH do not have associated RFLPs; thus, the presence of these mutations cannot be detected by Southern

blot hybridization. Fine analysis of these mutations has in many cases identified mutated sequences corresponding to the base sequences at the homologous positions in the pseudogene (Table 87-1). These are apparently the result of gene conversions. All the deleterious mutations resident on the pseudogene have by now been identified, singly or in combination, in mutant *CYP21A2* genes.

An early-identified conversion was the point mutation changing isoleucine-172 to asparagine, possibly affecting interactions between the P450 protein and the membrane of the endoplasmic reticulum (148). Another point mutation produces a stop codon (nonsense mutation) and premature termination at position 318 (149), whereas another more extended conversion spanning the third to the sixth exon was also identified (150). All patients with the nonsense mutation have salt-wasting disease, whereas the patients with the asparagine-172 mutation retain limited ability to synthesize aldosterone (manifested as simple virilizing disease, often with elevated plasma renin-to-aldosterone ratio). Substitution of valine-281 to isoleucine produces a partially functional mutant cytochrome P450c21 and is the basis of non-classical 21-hydroxylase deficiency associated with the haplotype HLA-B14, DR1 (142). In the larger rearrangement involving exons 3–6, transfer of the 8-bp deletion from *CYP21A1P* shifts the reading frame of translation and prevents synthesis of a functional protein; thus, it is associated with salt-wasting disease (150).

Gene deletions, nonsense and frameshift mutations, and certain amino acid substitutions result in salt-wasting alleles, one nonconservative substitution causes a simple-virilizing allele, and conservative substitutions are associated with a nonclassic allele. Conversely, some patients with documented episodes of salt-wasting in infancy develop the ability to synthesize adequate amounts of aldosterone later in life (151). This recovery might result from increased expression of a poorly active P450c21 or from individual variations in level of some other P450 enzyme, distinct from P450c21, with some 21-hydroxylase activity (152,153).

Although patients with 21-hydroxylase deficiency are often compound heterozygotes, the patient's phenotype is usually determined by the least severely affected allele. Thus, there is generally a good correlation between the severity of the clinical disease and the discrete mutations observed (154,155). Several studies evaluating the phenotype–genotype relationship in 21-hydroxylase deficiency, however, have demonstrated that there is often a divergence in phenotypes within mutation-identical groups, for reasons not yet known (134,156). Some patients who bear the V281L mutation associated with nonclassic CAH on one allele and a deletion on the other have, in fact, by hormonal diagnosis simple virilizing or salt-wasting classical 21-hydroxylase deficiency (54). Two HLA-identical brothers were reported who have mutations in intron 2 and exon 10 of *CYP21A2*, but one was shown to have salt-wasting CAH, whereas the other

retained the ability to synthesize aldosterone and was diagnosed to be a simple virilizer (53). The most recent analysis of 182 unrelated families from Finkelstein et al. demonstrated that the genotype–phenotype concordance is 90.5% for salt-wasting CAH, 85.1% for simple-virilizing CAH, and 97.8% for nonclassic CAH (157). Rocha et al. recently showed that CAG repeats in the androgen receptor has a great influence on variability in virilization of external genitalia of CAH women (158). Further studies in promoters, surrounding genes, transcription factors, transport proteins, and other modifiers may reveal a cause for the discordance in phenotype.

De novo mutations have been identified consistent with the operation of the two mechanisms above (159–162); thus, these mechanisms appear to explain the high frequency of 21-hydroxylase deficiency and will probably continue to generate numbers of mutations far in excess of the few random cytochrome P450c21 mutations that appear to have occurred.

### 87.3 STEROID 11 $\beta$ -HYDROXYLASE DEFICIENCY

Abnormal adrenal steroid secretion attributable specifically to impeded 11 $\beta$ -hydroxylation was first reported by Eberlein and Bongiovanni (163). The variable hormonal profiles of patients with 11 $\beta$ -hydroxylase deficiency eventually led to the identification of two 11 $\beta$ -hydroxylase isoenzymes encoded by two genes, *CYP11B1* and *CYP11B2* (164–166). *CYP11B1* encodes 11 $\beta$ -hydroxylase enzyme, which catalyzes the final step in cortisol synthesis and is consequently regulated by ACTH. The final steps in aldosterone biosynthesis are catalyzed by aldosterone synthase, which is encoded by *CYP11B2* and is regulated by angiotensin II and potassium.

The characteristic steroid profile of 11 $\beta$ -hydroxylase deficiency (P450c11) owing to mutations in *CYP11B1* shows elevated 11-deoxycortisol (compound S) and DOC in the serum, with marked urinary elevation of the corresponding tetrahydro metabolites, THS and THDOC, and complete absence of any 11-oxygenated C<sub>19</sub> or C<sub>21</sub> steroids in the blood or urine.

Hypertension with hypokalemic alkalosis is the single clinical feature distinguishing 11 $\beta$ -hydroxylase from 21-hydroxylase deficiency, yet it is not uniformly present. DOC has been thought to be the causative agent of hypertension (167,168), but this is not certain. While it has moderately potent MC effects, causing sodium retention, plasma volume expansion, and suppression of PRA, DOC may be elevated in 11 $\beta$ -hydroxylase-deficiency patients who are normotensive (169) or only mildly elevated in hypertensive patients (170,171). In addition, neither does intravenous DOC infusion uniformly induce hypertension in control subjects (172) nor does suppression of DOC always lead to remission of hypertension in 11 $\beta$ -hydroxylase deficiency patients (173). MC excess and hypertension is not necessarily proportional to the

TABLE 87-1 Steroidogenic Enzymes: Gene Mutation Table

	Exon/Mutation Name/Type	Gene AA	Intron Comments	Reference
CYP21A2	E1	1. Insertion (conversion)	+L9	Normal polymorphism
	E1	2. Nonsense mutation		(150) (Lajic and Wedell, 1996)
	E1	3. Frameshift	W22+ 1nt	Insertion of 1 nucleotide
	E1	4. Missense mutation	P30L	Nonclassical (NC) phenotype
				(Tusie Luna et al., 1991) (conversion)
	E1	5. Missense mutation	P30Q	SW allele
	E1	6. Frameshift mutation	Y47 Δ 1nt	Deletion of thymidine at nt 141 leads to L51X
				(Krone et al., 1999)
	I1	7. Aberrant splicing of intron 1	W23X nt 295AØG	
				(Lajic and Wedell 1996)
	E2	8. Missense mutation	G90V	Spanish patient
	I2	9. Abberant splicing of	387* GØA	Intron 2 splice donor site; intron 2 Chinese patient
				(Lee et al., 1998)
	I2	10. Aberrant splicing of	656*	Part of intron (end intron 2 conversion) A/CØG 19 bases retained in mRNA processing
				Higashi et al., 1988
	E3	11. Nonsense mutation	Y97X	
				(Krone et al., 1998)
	E3	12. Missense mutation	P106L	NC allele
				(154)
	E3	13. 8 nt-deletion (conversion)	G110Δ8 nt	Frameshift: 20-AA + stop
				(150)
	E3	14. Missense mutation	R132C	
				(Minuto et al., 2011)
	E3	15. Missense mutation	H119R	NC
				(Concolino et al., 2009)
	E4	16. 1-nt deletion	C169Δ1 nt	Frameshift
				(Witchel et al., 1999)
	E4	17. Missense mutation	I172N	Affects anchoring in (conversion) membrane
				(148)
	E4	18. Missense mutation	I49C	
				(Minuto et al., 2011)
	E5	19. Missense mutation	G178A	SW allele
				(Lobato et al., 1999)
	E5	20. Three-base deletion	ΔE 196	Deletion of nucleotides 1158–1160
				(Nikoshkov et al., 1998)
	E5	21. Missense mutation	I194N	
				(Concolino et al., 2009)
	E6	22. Cluster (conversion)	I236N V237E M239K	2 more charges added in region with multiple charged residues
				(Higashi et al., 1988)
	E6	23. Missense mutation	R224W	NC
				(Concolino et al., 2008)
	E7	24. Missense mutation	V281L	Major NC mutation HLA-B14; DR1 associated
				(142) (Conversion)
	E7	25. Missense mutation	V281G	
				(156)
	E7	26. Missense mutation	G291S	AA substitution CØT at conserved positio
				(154)
			398*	At position +9 of intron (secondary effect?)
	E7	27. Missense mutation	G291C	
				(Lobato et al., 1999)
	E7	28. Missense mutation	L300F	
				(156)
	E7	29. Nonsense mutation	W302X	Finnish patient
				(Levo and Partanen, 1997)
	E7	30. Single base	F306 + 1nt	Frameshift: insertion (conversion) +T at codon 305–7
				(Harada et al., 1987)
	E7	31. Missense mutation	M283V	
				(Minuto et al., 2011)
	I7	32. Loss of splice donor	1784* GØC	Aberrant splicing site at Intron 7 Found in one SW patient
				(Wedell and Luthman, 1993)
	I7	33. Loss of splice donor	1785*TØG	Aberrant splicing site at Intron 7 Found in one NC patient
				(Ordóñez-Sánchez et al., 1998)
	E8	34. Nonsense mutation	R316X	Chinese patient
				(Lee et al., 1998)
	E8	35. Nonsense mutation	Q318X	Conversion
				(149)



**TABLE 87-1 Steroidogenic Enzymes: Gene Mutation Table—cont'd**

	Exon/Mutation Name/Type	Gene AA	Intron Comments	Reference
	E8 36. Frameshift	S330 $\Delta$ 10 nt	Chinese patient	(Lee et al., 1998)
	E8 37. Missense mutation	R339H	NC allele	(Helmberg et al., 1992c)
	E8 38. Missense mutation	R354H	0% activity in transfected cells	(Lobato et al., 1999)
	E8 39. Missense mutation	R354C		(156)
	E8 40. Missense mutation	R356W	Radical AA substitution may impair redox interactions	(Chiou et al., 1990) (conversion)
	E8 41. Missense mutation	R356P	May impair redox interactions	(Lajic et al., 1997)
	E8 42. Missense mutation	R356Q	May impair redox interactions	(Lajic et al., 1997)
	E9 43. Missense mutation	E380D		(Kirby-Keyser et al., 1997)
	E9 44. Duplication	V397 + 16nt	Frameshift, Chinese patient	(Lee et al., 1998)
	E9 45. Nonsense mutation	W405X		(Wedell and Luthman, 1993)
	E9 46. Missense mutation	D407N	NC	(Concolino et al., 2008)
	E10 47. Missense mutation	G424S	Brazilian patient	(Billerbeck et al., 1999)
	E10 48. Missense mutation	P453S+(P453) and non-conserved (P105) residue	NC allele plus P105L AA substitution of conserved	(Owerbach et al., 1992; (154)
	E10 49. Frameshift mutation	P475 $\Delta$ 1nt		(Ordonez-Sanchez et al., 1998)
	E10 50. Missense mutation	R483P		(Wedell and Luthman, 1993b)
	E10 51. Compound frameshift	R483 $\Delta$ 1 nt	Replaces last 11 AA and extends mutation protein by a further 45 AA	(154)
	E10 52. Missense mutation	E431K		(Minuto et al., 2011)
	E10 53. 2-base deletion	g.2511_2512	Frameshift del GC	(Minuto et al., 2011)
CYP11B1	E1 1. Nonsense mutation	Q19X	Caucasian patient	(Merke et al., 1998)
	E1 2. Single base deletion	32 $\Delta$ 1nt	Frameshift	(Curnow et al., 1993)
	E1 3. Missense mutation	P42S	Compound heterozygote	(176)
	E2 4. 28-nt deletion	105 $\Delta$ 28 nt	Deletion of codon 105 to first nt to 113	(Skinner et al., 1996)
	E2 5. 5-nt duplication	dup5 nt		(Skinner et al., 1996)
	E2 6. Nonsense mutation	W116X	Japanese (consanguineous)	(201)
	E2 7. 5-nt duplication	121-2dup	Frameshift Asian (Kenya) patient	(202)
	E2 8. Missense mutation	V129M	Compound heterozygote and 28-nt deletion	(203)
	E2 9. Missense mutation	W116G	Classic	(342)
	E2 10. Missense mutation	M88I	Nonclassic	(342)
	E3 11. Missense mutation	N133H	Compound heterozygote	(176)
	E3 12. Nonsense mutation	K174X	Premature termination	(Curnow et al., 1993)
	E3 13. Missense mutation	A165D	Classic	(342)
	E3 14. Missense mutation	P159L	Nonclassic	(342)
	E3 15. Missense mutation	R141X		(206)
	E4 16. Nonsense mutation	W247X	Premature termination	(Curnow et al., 1993)
	E4 17. Six base deletion	K254_A259 del	Frameshift	(342)
	E5 18. Missense mutation	G267R		(Skinner et al., 1996)
	E5 19. Missense mutation	G267D		(Skinner et al., 1996)
	E5 20. Missense mutation	T318T	G to C in the last codon of exon 5. mRNA not detected in vitro	(Chabre et al., 2000) (splicing)
	E5 21. Missense mutation	T318M	Parents Yemenite (first cousins)	(Curnow et al., 1993)
	E5 22. Missense mutation	T318R	Caucasian patient	(Merke et al., 1998)
	I5 23. Aberrant splicing	G to A	African-American	(Merke et al., 1998)
	E6 24. Missense mutation	T319M		(176)
	E6 25. Missense mutation	A331V		(203)
	E6 26. Nonsense mutation	Q338X	Parents both Indian Sikh	(Curnow et al., 1993)
	E6 27. Nonsense mutation	Q356X	Number of other genetic defects; African-American	(Curnow et al., 1993)
	E6 28. Nonsense mutation	Q356X	Premature termination	(Skinner et al., 1996)

Continued

**TABLE 87-1 Steroidogenic Enzymes: Gene Mutation Table—cont'd**

	Exon/Mutation Name/Type	Gene AA	Intron Comments	Reference
	E6 29. Nonsense mutation	E371G		(203)
	E6 30. Missense mutation	R366C	Nonclassic	(342)
	E7 31. Missense mutation	R374Q	Split codon (exon6—exon 7) Lebanese patient	(Curnow et al., 1993)
	E7 32. Missense mutation	R384G		(Nakagawa et al., 1995)
	E7 33. Missense mutation	R384Q		(Curnow et al., 1993)
	E7 34. Small deletion	R394Δ1		(327)
	E7 35. 2 nt insert (frame-shift)	394 + GA	Turkish Jewish patient	(Helmberg et al., 1992a)
	E8 36. Nonsense mutation	Y423X	Premature termination and compound heterozygote	(176)
	E8 37. Missense mutation	R427H		(Skinner et al., 1996)
	E8 38. Missense mutation	V441E		(Curnow et al., 1993)
	E8 39. Missense mutation	R448C		(203)
	E8 40. Missense mutation	R448H	In heme-binding region; Moroccan Jewish/Berber	(191)
	E8 41. Small insertion	464+CTG		(203)
	E8 42. Missense mutation	T401A	Nonclassic	(342)
	E8 43. Missense mutation	G411D		(206)
	I8 44. Splicing mutation	+4 AØG	Exon 8 is skipped	(Chabre et al., 2000)
	E9 45. Missense mutation	C494F		(206)
CYP11B2	E1 1. 5(4+1)-nt deletion	Δ12–14	North American family Aldosterone synthase deficiency type I	(191)
	E3 2. Missense mutation	D147E		(217)
	E3 3. Missense mutation	K152N		(217)
	E3 4. Deletion	ΔR173	Aldosterone synthase deficiency II	(216)
	E3 and 7 5. Double missense	R181W plus	In Iranian Jews Aldosterone synthase V386A deficiency II	(212) Mutation
	E3 and 7 6. Missense mutations	R181W plus V386A	Aldosterone synthase deficiency II	(213)
	E3 7. Missense mutation	T185I		(216)
	E3 and 7 8. Missense mutations	R173K, E198D, and V386A	Homozygous in exons 3 and 7; Aldosterone synthase deficiency type I	(194)
	E4 9. Missense mutation	I248T		(217)
	E4 10. Nonsense mutation	E255X	Aldosterone synthase deficiency type I	(218)
	E4 11. Missense mutation	W260X	Premature stop	(340)
	E4 12. Insertion mutation	G206WfsX51	Insertion leading to stop	(340)
	E4 13. Deletion mutation	L496SfsX169	Deletion leading to 169 extra amino acids	(340)
	E5 14. Missense mutation	S315R		(340)
	E5 and 7 15. Missense mutations	T318M plus V386A		(215)
	E6 16. Single base deletion of C	372ΔC		(215)
	E6 17. Missense mutation	R374W		(340)
	E7 18. Missense mutation	R384P	Aldosterone synthase deficiency type I	(219)
	E8 19. Missense mutation	L461P	Aldosterone synthase type I deficiency	(220)
CYP17	E1 1. Nonsense mutation	W17X	Japanese patients	(Yanase et al., 1988)
	E1 2. Missense mutation	R35L	Isolated 17, 20-lyase deficiency Turkish patient	(257)
	E1 3. Deletion of 1 of 2	F53 or F54	Japanese patients adjacent identical codons	(Yanase et al., 1989)
	E1 4. Missense mutation	Y46S	Caucasian patients	(Imai et al., 1993)
	E1 5. Nonsense mutation	W74X	Isolated 17,20-lyase deficiency	(Biaison-Lauber et al., 1997)
	E1 6. Deletion	Δ70–77	24-nt deletion Italian patient	(257)
	E1 7. Nonsense mutation	R96W	Italian patients	(257)
	E1 8. Missense mutation	M1T		(261)
	E2 9. Missense mutation	S106P	Guamanian patient	(Lin et al., 1991)

TABLE 87-1 Steroidogenic Enzymes: Gene Mutation Table—cont'd

	Exon/Mutation Name/Type	Gene AA	Intron Comments	Reference
	E2 10. Insert (codon	I112	Caucasian patient	(Imai et al., 1993) duplicated)
	E2 11. 7 nt duplication	120+ 37nt	Japanese patient	(Yanase et al., 1990)
	E2 12. 1 nt Δ	131	Deletion of C leads to stop codon at 442	(Yamaguchi et al., 1998)
	I2 13. Intron 2 donor site	+5 GØT	Frameshift leads to premature stop codon	(Suzuki et al., 1998) mutation
	E3 14. Extensive internal	Exon 2 to Deletion of 518 nt; and Exon 3 DNA insert insertion of 469 nt	Italian patient deletion and foreign	(Biason et al., 1991)
	E3 15. Missense mutation	N177D	Isolated 17,20-lyase deficiency	(257)
	E3 16. Nonsense mutation	E194X	Caucasian patient	(Rumsby et al., 1993)
	E4 17. Nonsense mutation	R239X	Caucasian patient	(Ahlgren et al., 1992; Rumsby et al., 1993)
	E5 18. Deletion mutation	Δ330, 331	2-nt deletion Japanese patients	(Monno et al., 1993)
	E6 19. Nonsense mutation	Y329X	1-nt insertion leading to frameshift	(257)
	E6 20. Missense mutation	P342T	Caucasian patient	(Ahlgren et al., 1992)
	E6 21. Missense mutation	R347H	Isolated 17,20-lyase deficiency	(252)
	E6 22. Missense mutation	R358Q	Isolated 17,20-lyase deficiency	(252)
	E6 23. Missense mutation	Y329D		(261)
	E6 24. Missense mutation	R362C		(261)
	E7 25. Missense mutation	W409R		(261)
	I7 26. Splice donor site	+5GØA	Frameshift, leads to premature mutation of intron 7 stop codon at 410	(262)
	E8?? 27. Missense mutation	H373L	Japanese patient	(Monno et al., 1993)
	E8 28. Missense mutation	F417C	Isolated 17,20-lyase deficiency	(Biason-Lauber et al., 1997)
	E8 29. Nonsense mutation	R440H	German patient (heme-binding region)	(276)
	E8 30. Nonsense mutation	I443X	GΔ at codon 438, isolated 17,20-lyase deficiency	(Oshiro et al., 1995)
	E8 31. Nonsense mutation	Q461X	Swiss patient	(258)
	E8 32. 4-nt duplication	480 dup	Frisian/Mennonite sect (New World)	(Yanase et al., 1988)
	E8 33. 9 nt Δ	Δ D487, S488 and F489	Thai patient	(Fardella et al., 1993)
	E8 34. Missense mutation	R496C	Swiss patient isolated 17,20-lyase deficiency	(258)
	E8 35. Missense mutation	R496H	Isolated 17,20-lyase deficiency	(257)
	E8 36. Missense mutation	P428L		(261)
HSD3B2	E2 1. Missense mutation	A10E	French-Canadian patients	(Alos et al., 2000)
	E2 2. Missense mutation	A10V	Egyptian patient	(227)
	E2 3. Missense mutation	G15D		(Rheume et al., 1995)
	E2 4. Missense	T259R	Severe salt wasting	(343)
	E3 5. Missense mutation	A82T	In two remote kindreds	(228)
	E3 6. Missense mutation	N100S	Non-salt-wasting allele	(Mebarki et al., 1995)
	E3 7. Missense	L6F	Consanguineous Pakistani	(343)
	I3 8. Point mutation in	6651* intron 3	US patient (mild form)	(Rheume et al., 1997)
	E4 9. Missense mutation	L108W		(Sanchez et al., 1994)
	E4 10. Missense mutation	G129R	US patient (mild form)	(Chang, 1994; Rheume et al., 1994)
	E4 11. Missense mutation	E142K		(Simard et al., 1993)
	E4 12. Missense mutation	P155L	French patient	(227)
	E4 13. Nonsense mutation	W171X	Unrelated kindreds	(Rheume et al., 1992)

Continued

**TABLE 87-1 Steroidogenic Enzymes: Gene Mutation Table—cont'd**

	Exon/Mutation Name/Type	Gene AA	Intron Comments	Reference
(US, Switzerland)				
	E4 14. Missense mutation	L173R		(229)
	E4 15. Single-base insert	186+C	Unrelated kindreds	(Rheume et al., 1992)
(US, Switzerland)				
	E4 16. Missense mutation	P186L	Japanese patient	(Sanchez et al., 1994)
	E4 17. Missense mutation	L205P	Japanese patient	(Katsumata et al., 1995); (227)
	E4 18. Missense mutation	S213G	US patient (mild form)	(Chang et al., 1993a)
	E4 19. Missense mutation	K216E	US patient (mild form)	(Chang et al., 1993a)
	E4 20. Missense mutation	P222Q	Algerian patient	(227)
	E4 21. Missense mutation	P222H	Brazilian patient	(Marui et al., 1998), (227)
	E4 22. 27-nt deletion	687* Δ27 nt	Deletion of last nt of codon 229, all of codons 230–237, and first 2 nts of codon 238. Sri-Lanka patients	(227)
	E4 23. Missense mutation	L236S	French patient	(227)
	E4 24. Missense mutation	A245P	Turkish patient	(Simard et al., 1993)
	E4 25. Compound missense	V248N frameshift mutation	US patient	(Chang et al., 1993b)
	E4 26. Missense mutation	Y253N	Dutch patient	(Simard et al., 1993)
	E4 27. Missense mutation	Y254D		(Sanchez et al., 1994)
	E4 28. Missense mutation	T259M	Brazilian patients	(Tajima et al., 1995; (227)
	E4 29. 2-base pair deletion	273 Δ2 nt	Frameshift mutation	(Simard et al., 1994)
	E4 30. Nonsense mutation	867* ΔG	Deletion of G in codon 290	(227)
	E4 31. Missense mutation	G294V		(227)
	E4 32. Missense mutation	X373C	95 extra amino acids	(341)
	E4 33. Missense mutation	P222T	Salt wasting	(341)
StAR	E1 1. Frameshift	+A163*	Insertion of A at nucleotide 163	(288)
	E2 2. Frameshift	23X	Deletion of G at nucleotide 189, leads to premature stop at codon 23	(289)
	E2 3. Frameshift	46X	Insertion of G at nucleotide 246, leads to premature stop at codon 46	(289)
	E2 4. Frameshift	257/+G/258*	Insertion of G between nucleotide 257 and 258	(35)
	I2 5. Splice donor site	+T 3nt <sup>E2/I2</sup>	Insertion of T in intron 2, 3 bp mutation from exon 2 splice donor site	(Okuyama et al., 1997)
	E4 6. Frameshift	548/+TT/549*	Insertion of TT between 548 and 549	(35)
	E4 7. Frameshift	Δ146–150*	Deletion of 13 nt at nt 564, leads to premature stop at codon 181	(289)
	E4 8. Missense mutation	N148K	Sex reversal from male to female	(339)
	E4 9. Insertion mutation	P129fs	Consanguineous Turkish	(339)
	E4 10. Missense mutation	Q128R	Consanguineous Turkish	(339)
	I4 11. Splice acceptor site	TØA 11 bp <sup>I4/I5</sup>	TØA 11-bp upstream from intron 4/exon 5 junction	(Tee et al., 1995)
	E5 12. Missense mutation	E169G		(35)
	E5 13. Missense mutation	E169K		(35)
	E5 14. Missense mutation	R182L	Patients with Palestinian ancestry	(35); (228)
	E5 15. Nonsense mutation	R193X		(35)
	E5 16. Nonsense mutation	Q212X		(289)
	E5 17. Frameshift	Δ2T593*		(35)
	E5 18. Frameshift	ΔC650*		(35)
	E5 19. Foreign DNA insertion		Truncated protein	(35)
	E5 20. Frameshift	+T643*		(288)
	E5 21. Missense mutation	R217T	Exon 5/Splice donor site mutation	(Katsumata et al., 1999)
	E5 22. Missense mutation	V187M	Pakistani	(Baker et al. 2006)



**TABLE 87-1 Steroidogenic Enzymes: Gene Mutation Table—cont'd**

Exon/Mutation Name/Type	Gene AA	Intron Comments	Reference
E5 23. Missense mutation	R188C	Consanguineous Thai	(Baker et al. 2006)
E5 24. Missense mutation	R182H		(338)
E6 25. Missense mutation	A218V		(35); (289); Katsumata, 1999)
E6 26. Frameshift	+A667*		(288)
E6 27. Frameshift	238 $\Delta$ 1 nt	Leads to premature stop at codon 320	(289)
E6 28. Missense mutation	G221D		(Sahakitrungruang et al. 2010)
E7 29. Nonsense mutation	W250X		(Korsch, 1999)
E7 30. Nonsense mutation	Q258X	Japanese patients	(288); (291)
E7 31. Frameshift	272 $\Delta$ R	Deletion of nucleotides 940–942	(35)
E7 32. Frameshift	947/+A/948*		(35)
E7 33. Missense mutation	L275P		(35)
E7 34. Missense mutation	L260P		(Flück et al. 2005)
E7 35. Missense mutation	F267S		(Sahakitrungruang et al. 2010)

degree of hypokalemia (173) and cases showing lack of suppression of PRA (considered a hallmark of this defect) have been noted (39,174). More recently, the role of DOC metabolites such as 18-hydroxy-DOC has been considered, but the specific adrenocortical factor—if any—operative in the hypertension seen in this disorder remains to be established (173,175).

As in 21-hydroxylase deficiency, excess fetal androgen production causes prenatal virilization of females, resulting in ambiguous external genitalia with normal female internal reproductive organs. In newborn males with 11 $\beta$ -hydroxylase deficiency, the external genitalia may be normal, but in either sex, virilization ensues postnatally if the disorder is untreated. There is no direct correlation between the degree of virilization and hypertension (173).

### 87.3.1 Nonclassic 11 $\beta$ -Hydroxylase Deficiency

Mild, late-onset, and even cryptic forms of 11 $\beta$ -hydroxylase deficiency have been reported (39,176–183). Nonclassic 11 $\beta$ -hydroxylase deficiency has been diagnosed in normotensive children with mild virilization (184) or precocious pubarche (183) and in adults with signs of hyperandrogenemia (176) as well as a woman with infertility (185). Despite a hormonal profile consistent with 11 $\beta$ -OHD, mutations in the *CYP11B1* gene may not always be present (176). As in 21-hydroxylase deficiency, this clinical variability may represent allelism at the 11 $\beta$ -hydroxylase structural gene locus, but biochemical investigations of obligate heterozygote parents did not find a consistent defect either in the baseline state or with ACTH stimulation with some, but not all, showing an elevated 17-OHP-to-cortisol or 11-deoxycortisol-to-cortisol ratio (186).

**87.3.1.1 Epidemiology.** Steroid 11 $\beta$ -hydroxylase deficiency has been found in roughly 5% of cases of CAH (96,187), a figure that probably applies worldwide. In Israel, however, 20% of CAH cases identified over a period of 16 years were attributable to 11 $\beta$ -hydroxylase deficiency (188), for a frequency of approximately 1 per 60,000 live births with a corresponding heterozygote frequency of 1 in 123. Many of the families in which 11 $\beta$ -hydroxylase deficiency occurred could claim forebears from a cluster of old Jewish settlements in north Africa (some dating from before AD 70) and a founder effect originating from within one of these communities or introduced from indigenous Berber people is most likely (189). Turkish Jews, who may formerly have engaged in increased intermarriage, are also known to have higher incidences of the 11 $\beta$ -hydroxylase deficiency (173,184,190) and were also thought likely to carry a recurring mutation. Molecular genetic studies have confirmed the presence of a single *CYP11B1* mutation in each of these groups (191,192). Unlike the common nonclassic form of 21-hydroxylase deficiency, the nonclassic form of 11 $\beta$ -hydroxylase deficiency is very rare.

### 87.3.2 Aldosterone Synthase Deficiency

Aldosterone synthase (*CYP11B2*) not only has 11 $\beta$ -hydroxylase activity but also converts corticosterone to 18-hydroxycorticosterone by 18-hydroxylase and subsequent 18-oxidation of 18-hydroxycorticosterone to aldosterone by 18-oxidase (see Figure 87-1). Defects in aldosterone synthase activity are termed aldosterone synthase deficiency I or II depending on the production of 18-OH corticosterone, which is increased only in type II. Aldosterone synthase deficiency type I occurs less frequently than type II, typically results in

little or no aldosterone production, and is phenotypically more severe than aldosterone synthase deficiency type II (193,194). Defects in aldosterone synthase activity are inherited as autosomal recessive conditions.

Formerly, biochemical studies by Hall and his group on bovine enzymes had indicated that 11 $\beta$ -hydroxylase and two closely related activities, originally termed corticosterone methyloxidase I (18-hydroxylase) and corticosterone methyloxidase II (18-oxidase), all resided in one protein (195); thus, corticosterone methyloxidase deficiency I and II was first investigated as a possible allelic variant of 11 $\beta$ -hydroxylase deficiency. In 1996 Ulick suggested changing the nomenclature to aldosterone synthase deficiency type I and II since it was known that one enzyme, aldosterone synthase (P450c11Aldo), catalyzed all three final steps in aldosterone biosynthesis (196).

Aldosterone synthase deficiency types I and II cause a salt-wasting syndrome that presents with hyponatremic hyperkalemia and dehydration in an infant. Both types of aldosterone synthase deficiencies cause an increase in plasma levels of DOC and corticosterone. The diagnosis of aldosterone synthase deficiency type I can be determined by measuring increased precursor to product ratios of corticosterone/18-hydroxycorticosterone, whereas the diagnosis of aldosterone synthase deficiency type II is established by measuring increased precursor to product ratios for 18-oxidase activity, that is, the 18-hydroxycorticosterone/aldosterone ratio in serum, or in the urine the ratio of their major respective metabolites, 18-hydroxytetrahydro-11-dehydrocorticosterone (18-OH-THA) to tetrahydroaldosterone.

### 87.3.3 Molecular Genetics

The molecular genetic basis of steroid 11 $\beta$ -hydroxylase deficiency and of two related disorders, aldosterone synthase deficiency I/II and dexamethasone-suppressible hyperaldosteronism (DSH), has been elucidated (197).

CYP11B1 and CYP11B2, encode two mitochondrial cytochrome P450 isoforms (93% amino acid sequence similarity), cytochrome P450c11 and cytochrome P450c11Aldo, with important differences in function consistent with the separate cell expression patterns and distinct regulation of the genes. CYP11B1 is expressed in all zones of the adrenal cortex and its regulation is ACTH dependent, whereas CYP11B2 is expressed exclusively in the zona glomerulosa and is regulated by angiotensin II and potassium ion. Cytochrome P450c11 and cytochrome P450c11Aldo show considerable sequence similarity with cholesterol side-chain cleavage enzyme (cytochrome P450scc), also located in the mitochondrial inner membrane; they are grouped together in the same family, family XI (111,198).

The structural gene for the 11 $\beta$ -hydroxylase enzyme (CYP11B1) was cloned and sequenced and then localized to the long arm of chromosome 8q22 (164,199).

CYP11B2 was eventually mapped to chromosome 8q24.3 (166,199). The two genes are separated by about 40 kb of DNA.

Mutations in the CYP11B1 gene cause steroid 11 $\beta$ -hydroxylase deficiency, which affects cortisol synthesis and produces virilizing CAH with hypertension (see Table 87-1), and mutations in the CYP11B2 gene cause aldosterone synthase deficiency types I and II. A third clinical condition involving these genes is a hypertensive disorder called DSH, or GC-remediable aldosteronism (GRA). DSH is hormonally characterized by ACTH-dependent aldosterone synthesis, which is the result of the creation of a chimeric gene by fusion of the CYP11B1 and CYP11B (197,200).

**87.3.3.1 CYP11B1.** The first mutations in the CYP11B1 gene were sought in cases of steroid 11 $\beta$ -hydroxylase deficiency occurring in Israel, where the defect is more prevalent than elsewhere. In a number of cases in Israel, family origins were traced back to Moroccan Jewish settlements, among which it was assumed that a founder mutation occurred or was introduced (189). Genetic analysis of these families discovered the recurrence of a single mutation in the CYP11B1 gene, a point mutation in exon 8 of the gene (9 exons), changing the specification of codon 448 from arginine to histidine (191). The second CYP11B1 mutation to be identified was in a pedigree of Turkish Jewish origin. This mutation, a 2-base insert in exon 7, shifts the reading frame to code for an incorrect 77-amino acid continuation (resulting in a mutant protein slightly shorter than the normal 503 residues), obliterating the heme-binding domain, and thus makes a completely dysfunctional product. Further studies analyzing DNA from individuals of diverse ethnic backgrounds identified a series of eight distinct mutations—four missense mutations, a frameshift mutation (a single-base deletion) and three point mutations specifying premature termination. Expression (by transfection assay) of the Moroccan mutation and the four other missense mutations produced mutant proteins that were in all cases devoid of activity. It was noted that of the 10 mutations reported in these three studies, seven are in exons 6 to 8, but if this was nonrandom, it was not known whether it was conditioned by the location of functionally important amino acid residues in this region of the enzyme polypeptide or whether there was any tendency for mutations to develop latent in specific base sequences in this region of the gene.

Many reports have since appeared in the literature, many of which have identified patients with homozygous mutations (201–205). In the first of such reports, a Japanese patient was found to be homozygous for a point mutation changing the specification of codon 116 from tryptophan to termination (W116X). The researchers assayed this mutation in a cell expression system and found predictably no enzymatic activity in the mitochondria, and they sequenced the patient's CYP11B2 gene and found it entirely normal (201). In

another report, CYP11B1 exons were selectively amplified by PCR and then screened individually for sequence changes by single-strand conformation polymorphism (SSCP) analysis, which revealed an unusual pattern for the exon 2 segment. Cloning and sequencing identified a 5-base duplication between codons 121 and 122, and further analysis confirmed that it occurred in both alleles in the patient. Causing a frameshift and early termination (exon 3), this mutation was thought to explain the phenotype, but exon 8 (whose SSCP segment also differed from controls) was found to carry two additional point mutations. Although presumed to be irrelevant to the present case, it was speculated whether these exon 8 mutations might have occurred previously and what their independent effects would be. While the first corresponds to a conservative substitution, valine to alanine (V348A), the second mutation (also confirmed to be on both alleles) corresponds to substitution of a histidine for a highly conserved arginine near the heme-binding domain (R427H) and would very likely compromise enzymatic activity (202). To date, more than 50 different mutations in the CYP11B1 gene have been identified. A recent multicenter study identified seven new mutations in the CYP11B1 gene. Three mutations (p.W116G, p.A165D and p.K254\_A259 del) were associated with classic disease and four mutations (p.P159L, p.M881, p.R366C and p.T401A) were associated with nonclassic disease. These findings doubled the number of mild CYP11B1 gene mutations previously described associated with mild or nonclassic 11 $\beta$ -hydroxylase deficiency. The data was validated with in vitro expression studies (342).

**87.3.3.2 CYP11B2 (Aldosterone Synthase Gene).** Isolated defects in the terminal steps of aldosterone synthesis produce a salt-wasting syndrome in infancy (197,206). Early clinical studies in aldosterone synthase deficiency were carried out on 12 patients from an isolated community of Iranian Jews who presented with a wide range of clinical severity (207). Later a genetic study designed to examine DNA from these patients using a probe for CYP11B1 was undertaken. With this probe, a specific banding pattern was found to correlate with the presumptive genotypes of affected and carrier family members, suggesting the presence of a mutation within or very closely neighboring the CYP11B1 gene locus (208).

Initial characterization of CYP11B2 showed the gene to have an intact open reading frame (209) and marked divergence of 5' regulatory sequences relative to CYP11B1 (165,209). Evidence for expression of the CYP11B2 gene was first found in a cDNA library derived from human aldosteronoma tissue, which was then screened and cloned corresponding to the transcript of the CYP11B2 gene found (210). It was then shown that CYP11B2 was expressed in normal adrenal glands and that it was in fact the gene encoding the required enzyme for the completion of aldosterone synthesis (211). This reopened analysis of the Iranian salt-wasting families with aldosterone

synthase defects: all of the affected members of these kindreds were found to be homozygous for a double mutation in CYP11B2: R181W and V386A (212,213). When these were individually expressed in a cell culture system, the R181W mutant enzyme showed reduced 18-hydroxylase, undetectable 18-oxidase, and intact 11 $\beta$ -hydroxylase activity. In addition, there was V386A (this amino acid is normal at this position in CYP11B1) reduced 18-hydroxylating activity—consistent with aldosterone synthase deficiency type II. Family members homozygous for either mutation alone were asymptomatic. No mutations were found in any CYP11B1 genes (214). Characterization of the CYP11B2 genes in DNA from three other Iranian Jewish families found the identical mutations (213).

Recently, additional mutations in patients presenting with aldosterone deficiency type II have been identified: an amino acid substitution (T318M) (215), a deletion of codon 173 in exon 3 (216), and a gene conversion in which 500bp of exons 3 and 4 in CYP11B2 were changed to the corresponding sequence of CYP11B1 (217). To date there have been three patients with aldosterone synthase deficiency type II who did not carry any detectable mutation in their entire CYP11B2 alleles (206).

A total of 10 mutations causing aldosterone synthase deficiency type I have been detected in the CYP11B2 gene (193,194,218–221), with five new mutations reported in 2010 by Nguyen (222). One study reported an individual homozygous for two deleterious mutations and a polymorphism, whose hormonal phenotype was consistent with aldosterone synthase deficiency type I, but whose in vitro transfection data was typical of the aldosterone synthase deficiency type II (194). The unexplained discrepancy between the in vitro activity and the clinically observed phenotype demonstrates the need for further phenotype/genotype studies.

**87.3.3.3 CYP11B1–CYP11B2 Chimeric Gene.** The existence of a familial form of hyperaldosteronism has been known for some years in which the prompt sustained reduction of aldosterone levels by the GC dexamethasone is the basis for diagnosis and treatment and has an autosomal dominant mode of transmission. This particular type of aldosteronism, known as DSH or GC-remediable hyperaldosteronism (GRH or GRA, as noted previously), also has as a specific biochemical index: abnormal serum and urinary elevations of 18-hydroxy and 18-oxocortisol, steroids normally synthesized in very limited amounts by the adrenal cortex. The molecular genetic basis for DSH in all reported cases is the creation of a fusion gene, because of unequal crossing over, between CYP11B1 and CYP11B2, in addition to a normal copy of each flanking the chimeric gene. This mutation combines an ACTH-responsive regulatory region with coding sequences for partial or full (depending on the precise location of the crossover) steroid 18-hydroxylating and 18-oxidating functions (200,212,223). The chimeric

gene has 5' and 3' ends that correspond to CYP11B1 and CYP11B2, respectively, and the points of transition are all located between intron 2 and exon 4 (197). This results in the expression of a hybrid cytochrome P450 at high levels in the zona fasciculata that synthesizes aldosterone from corticosterone in a pattern following ACTH cyclicity and at levels inducing the pathology, as well as synthesizing 18-oxocortisol from cortisol normally present, producing the quantitative abnormality of a 17 $\alpha$ , 18-dihydroxysteroid. Since a functional excess is involved, it is sufficient that the fusion gene be present on one chromosome, hence the autosomal dominant mode of expression.

The situation of CYP11B genes has some similarity to that of CYP21 genes (homology: CYP21/21P 97% and CYP11B1/B2 93%; spacing on the chromosome: CYP21P–CYP21 30kb and CYP11B2–CYP11B1 30–45 kb), but crossover events for CYP11B genes appear to be much less frequent. The creation of a chimeric gene is not a priori the only type of event able to generate the hormonal features and inheritance pattern of DSH. Plausibly nonreciprocal transfers, whether of an ACTH-regulatory segment from the *CYP11B1* to the *CYP11B2* gene or conversely of a coding segment specifying 18-oxidating activity from the *CYP11B2* to the *CYP11B1* gene, could occur.

## 87.4 3 $\beta$ -HYDROXYSTEROID DEHYDROGENASE DEFICIENCY

The enzyme 3 $\beta$ -HSD is necessary for the synthesis of all adrenal and gonadal steroids beyond the relatively inactive  $\Delta^5$  precursors. In addition to 3 $\beta$ -hydroxysteroid dehydrogenation, the enzyme also performs subsequent 3-oxosteroid isomerization, and is alternately termed  $\Delta^5$ , $\Delta^4$ -isomerase. First described in 1962 by Bongiovanni, 3 $\beta$ -HSD deficiency appears to have a monogenic autosomal recessive mode of transmission based on pedigree analysis (224–226).

As gonadal 3 $\beta$ -HSD enzymatic activity is also reduced (although not always equally with the adrenal defect), gonadal androgen production is deficient. In genetic males, incomplete genital development results in sexual ambiguity at birth. By contrast, in affected females, the very high levels of circulating DHEA—and perhaps some peripheral conversion of DHEA to more potent androgens—may produce a limited androgen effect (restricted to clitoral enlargement and rarely, labial fusion).

A high ratio of  $\Delta^5$  to  $\Delta^4$  steroids, characterized specifically by elevated serum levels of the  $\Delta^5$ -steroids pregnenolone, 17-hydroxypregnenolone, and DHEA, and increased excretion of the  $\Delta^5$  metabolites pregnanetriol and 16-pregnenetriol in the urine, is diagnostic for this enzyme disorder.

Deficient aldosterone production in cases of a complete or near-complete 3 $\beta$ -HSD enzyme block results in salt wasting; in other cases, the ability to conserve sodium has been intact (85,224–236). Thus, in 3 $\beta$ -HSD

deficiency, as in 21-hydroxylase and 11 $\beta$ -hydroxylase deficiency, there is a phenotypic spectrum for each clinical feature, and salt wasting may be present to some degree (226). As with the two other common defects, it is not possible to judge the degree of severity of the 3 $\beta$ -HSD enzyme defect based on the appearance of the external genitalia at birth.

### 87.4.1 Nonclassic 3 $\beta$ -HSD Deficiency

As with 21-hydroxylase, nonclassic 3 $\beta$ -HSD is an attenuated enzyme defect with no major developmental abnormalities (79). Nonclassic 3 $\beta$ -HSD patients generally present with premature adrenarche, hirsutism, or oligomenorrhea (237). With postadrenarchal or peripubertal onset (234) it appears to affect the fasciculata–reticularis zones, thus the spared functioning of the glomerulosa ensures adequate salt retention (225). Sixty-minute ACTH testing with serum sampling at 0 and 60 min after administration of Cortrosyn (synthetic ACTH<sub>1–24</sub>, 0.25 mg IV) will reveal a 3 $\beta$ -HSD defect when serum  $\Delta$ -17-hydroxypregnenolone ( $\Delta^5$ -17P), DHEA levels, serum ratios ( $\Delta^5$ -17P), DHEA levels, serum ratios  $\Delta^5$ -17P: 17-OHP, and  $\Delta^5$ -17P:F are all more than 2 SD above the normal mean values (226). There are slight differences between investigators in the hormonal evaluation and definition of this enzyme defect (238).

Because several studies have not identified any mutations in the gene for 3 $\beta$ -HSD in nonclassic patients (239,240), it has been suggested that some cases may be an acquired defect or secondary to an intra-adrenal androgen excess (241,242). Further investigation is needed before nonclassic 3 $\beta$ -HSD deficiency may be classified as a genetic disorder.

**87.4.1.1 Epidemiology.** Severe defects of 3 $\beta$ -HSD (genital ambiguity at birth in both sexes, with or without salt wasting) are uncommon, and no geographic clustering or ethnic predominance has been discerned.

**87.4.1.2 Molecular Genetics.** The 3 $\beta$ -HSD enzyme is one of a diffusely defined group of NADH/NADPH-dependent enzymes performing reversible dehydrogenations on various organic compounds, with limited regions of substrate and cofactor-binding amino acid sequence similarity. The 3 $\beta$ -HSD enzyme is strongly NAD preferring. In experimental systems the 3 $\beta$ -HSD function requires the presence of NAD<sup>+</sup>, whereas the attendant  $\Delta^5$ , $\Delta^4$ -isomerization of 3-oxosteroids proceeds with the cofactor in either the oxidized (NAD<sup>+</sup>) or the reduced (NADH) state.

Two genes for 3 $\beta$ -HSD have been cloned and sequenced, HSD3B1 and HSD3B2, which encode two types of 3 $\beta$ -HSD enzyme, the type II enzyme expressed specifically in adrenal cortex and gonads (under appropriate separate regulation in each gland/cell type) and the type I enzyme, which is also the placental form, ubiquitously expressed in extraglandular tissues. There may be other isoforms of 3 $\beta$ -HSD not yet characterized (a type



III and IV have been found, for instance, in the rat, and a type III in the mouse) with other particular patterns of distribution and expression, such as in liver or adipose tissue.

HSD3B1 and HSD3B2 share more than 90% sequence homology and have been localized to chromosome 1p13.1 (243–245). All mutations causing  $\beta$ -HSD deficiency have been identified in the HSD3B2 gene only, and approximately 30 mutations have been identified to date. In 1995, Simard et al. identified 14 unique point mutations in the HSD3B2 gene in a study of 16 severely affected patients. There was no detectable  $\beta$ -HSD2 activity in the salt-wasting patients, while the enzymatic activity in non-salt-wasting patients due to missense mutations ranged from 1% to 10% (246). Gene conversion with  $\beta$ -HSD pseudogenes may account for some of the identified mutations.

## 87.5 STEROID 17 $\alpha$ -HYDROXYLASE/17,20-LYASE DEFICIENCY

A single microsomal P450 enzyme (P45017) catalyzes both the conversion of MCs to GCs by 17 $\alpha$ -hydroxylase activity and the conversion of GCs to sex steroids via 17,20-lyase activity. A 17 $\alpha$ -hydroxylase/17,20-lyase defect reduces conversion of steroid precursors to 17-hydroxy ( $C_{21}$ ) and consequently 17-keto ( $C_{19}$ ) steroids; adrenal secretion of GCs and sex steroids is thus diminished, and there is overproduction of 17-deoxysteroids. Plasma DOC and especially corticosterone are elevated. The defect was first identified by Biglieri and colleagues (247) in a genetic female and was first reported in a genetic male by New (248).

Corticosterone secreted in significant excess (producing plasma levels up to 60 times normal) appears to provide marginally sufficient GC activity for survival. The hypertension and hypokalemia are attributed to chronically elevated DOC and corticosterone. Untreated 17 $\alpha$ -hydroxylase deficiency in females at pubertal age results in primary amenorrhea and lack of development of secondary sex characteristics. Male undervirilization is evident at birth and includes incomplete Wolffian duct development (while müllerian structures are absent because of normal testicular production of AMH). Lack of androgens embryonically also fails to suppress the breast anlage in males, and gynecomastia is a prominent feature at puberty. Plasma gonadotropins are very high in both sexes postpubertally. While studies have shown renin levels to rise soon after GC treatment has begun and DOC levels to fall, it may take many months for normal aldosterone levels and proper glomerulosa function to be established (249).

### 87.5.1 Isolated 17,20-Lyase Deficiency

17,20-Lyase and 17 $\alpha$ -hydroxylase activities reside in the same protein (250) and is catalyzed on the same active

site (251); however, cases of isolated 17,20-lyase deficiency have been reported (252,253). Deficiency of 17,20-lyase causes a defect in the synthesis of  $C_{19}$  sex steroids in the adrenals and gonads (254,255). Affected individuals have phenotypically normal female genitalia at birth, regardless of the genetic sex, and usually present at adolescence with primary amenorrhea or lack of sexual development. Urinary pregnanetriolone, a metabolite of 17-OHP, is increased and increases further after ACTH and human chorionic gonadotropin (hCG) stimulation, the latter observation indicating concordance for the gene defect in both adrenal gland and testes (256). Testosterone or DHEA excretion does not rise appreciably. While 17 $\alpha$ -hydroxylase activity is often diminished in these patients, there is adequate activity to prevent MC hypertension and cortisol insufficiency.

In 1997, Geller et al. identified mutations (R347H and R358Q) in two patients with isolated 17,20-lyase deficiency (252). Using transfection and expression studies, the mutations were shown not to impair the affinity for 17-hydroxypregnenolone, but to inhibit electron donation that is crucial for lyase but not 17 $\alpha$ -hydroxylase activity (251). Both these CYP17 mutations were located in a region necessary for binding P450 oxidoreductase, which is responsible for electron donation to all microsomal cytochrome P450 enzymes. In addition, a mutation (F419C) preventing phosphorylation of P450c17 was identified in another patient, which also affects the ability of electron transfer and thus lyase function (257).

**87.5.1.1 Epidemiology.** Steroid 17 $\alpha$ -hydroxylase/17,20-lyase enzyme deficiency is generally rare. The total number of cases of 17 $\alpha$ -hydroxylase/17,20-lyase deficiency CAH recorded in 1992 was approximately 140–150 (258,259). A possibly greater incidence is suggested by Kater and Biglieri (260). As reflected in clinical case reports, defects in this enzyme, whether manifesting as combined 17 $\alpha$ -hydroxylase/17,20-lyase deficiency or as isolated 17,20-lyase deficiency, have appeared sporadically. Molecular studies have so far characterized over 70 mutations (257,261–264). One particular mutation appearing in widely dispersed families in North America belonging to the Anabaptist sect called Mennonites was first identified in the Friesland region of The Netherlands, where the sect first began (265–267). Biason-Lauber et al. (257) identified a homozygous mutation, R96W, in two unrelated Italian families, which may suggest a founder effect in the Italian population. In the Brazilian population 82% of mutant alleles are due to two mutations, suggesting a founder effect as well (263). Other populations with frequently reoccurring mutations that have been described are patients in Japan (268) and patients from East Asia (269–271).

**87.5.1.2 Molecular Genetics.** The structural gene for the microsomal cytochrome 17 $\alpha$ -hydroxylase and 17,20-lyase enzyme (P450c17) is termed CYP17 (198). This gene is on chromosome 10 (272), in the region 10q24-q25 (273), and exists in a single copy. The CYP17 gene

was shown to be identically transcribed in the adrenal gland and testis (274).

It was established early on such that the mutational pattern for CYP17 did not appear to include gross deletions or rearrangements in or near the gene locus (275). This was the second form of CAH after steroid 21-hydroxylase deficiency to have defects identified at the molecular level (258,259). To date there are more than 70 CYP17 locus mutations that have been characterized (see Table 87-1). It appears that the mutations occurring in this gene are random and not the results of any predisposing mechanism (276).

## 87.6 CONGENITAL LIPOID ADRENAL HYPERPLASIA

Deficient uptake of cholesterol into the mitochondria blocks conversion of cholesterol to pregnenolone at the rate-limiting step in steroid synthesis, leading to profoundly impaired production of all steroids. The condition was first described by Prader, who termed it lipid adrenal hyperplasia because of the characteristic appearance of the cholesterol-laden adrenocortical tissue (277). Gonadal hypogenesis or agenesis, severe fluid and electrolyte disturbances, and susceptibility to infection and addisonian pigmentation are seen; affected individuals often do not survive beyond infancy. Early reports include histologic examinations (278–280) and clinical descriptions (281), including one instance of a less severe phenotype (282). A case report reviewing 32 known cases in the literature describes a patient diagnosed in the newborn period who was successfully treated with MC and GC replacement therapy for 18 years (283).

### 87.6.1 Steroid Acute Regulatory Protein

The enzyme for cholesterol side-chain cleavage, known as cytochrome P450<sub>scc</sub>, or cholesterol desmolase, and encoded by CYP11A is the first enzymatic step in steroid synthesis, catalyzing the conversion of cholesterol to pregnenolone. The system of cholesterol side-chain cleavage consisting of P450<sub>scc</sub> and electron transfer proteins including adrenodoxin and adrenodoxin reductase was the most likely candidate for the cause of lipid CAH in initial studies. Southern blotting of DNA digests from affected individuals and family members found no gross abnormalities in the CYP11A gene (284). Mutations have been found, however, in the gene coding for the StAR, which is located on chromosome 8p11.2 (285,286). StAR is a phosphoprotein that is rapidly inducible by cAMP. While the mechanism of StAR is not thoroughly understood, its action facilitates transport of cholesterol into the mitochondria.

Two separate lesions, or events, described by Bose et al. (35) account for the variety of clinical observations in patients with congenital lipid adrenal hyperplasia, including the lack of virilization in the male and

spontaneous feminization in genetic females. The first event is the obvious absence of StAR-dependent steroidogenesis in the testes and adrenal glands. The second event is based on the observation of small amounts of steroid that can be made through basal StAR-independent steroidogenesis, which then stimulates trophic hormone secretion. These hormones stimulate the uptake of more extracellular cholesterol, which accumulates as lipid droplets, eventually damaging the cells and destroying any ability of steroidogenesis. Thus virilization does not occur in the male patient because the fetal testes are severely affected because of high levels of steroid synthesis. In contrast, in females, the ovaries produce no steroids until puberty, which occurs spontaneously and at which time follicular gonadotropin production through StAR-independent steroidogenesis enables spontaneous feminization.

**87.6.1.1 Epidemiology.** Congenital lipid adrenal hyperplasia is the rarest form of CAH. It has a significant mortality because of complete adrenal insufficiency. It appears to occur with lesser severity and more frequency among Japanese, Koreans (287), and Palestinian Arabs (288). Recent studies estimate the carrier rate in Japan for the most common StAR mutation seen in Asian populations (C898T) to be between 1 in 200 and 1 in 300 people (35,289).

**87.6.1.2 Molecular Genetics.** The StAR protein is a 30-kDa phosphoprotein first identified by Orme-Johnson in mouse adrenocortical cells (290). The synthesis of StAR protein occurs in the cytosol and is dependent on a protein kinase A signaling pathway. The first mutations in the StAR protein were C-to-T transitions that resulted in the premature insertion of a stop codon. Western analysis confirmed the mutant protein following cloning and expression of the cDNA (291). A report by Bose et al. (288) describes 15 patients with congenital lipid adrenal hyperplasia from 10 countries. In all three of the Japanese patients, four of the six unrelated alleles had the mutation, C898T, perhaps representing a founder effect. Similarly, in six patients of Palestinian origin, seven of the nine unique alleles had the R182L mutation in exon 5. Among the 15 mutations identified, A218V and L275P retained minimal activity in transfection studies, while the others had essentially no detectable activity. One patient was a compound heterozygote for A218V and L275P and had some StAR activity.

Until recently lipid CAH was known as a severe disorder characterized by significantly diminished or absent synthesis of all adrenal and gonadal steroids. In 2006, Baker et al. described three children from two families who presented with adrenal insufficiency at 2–4 years of age and where the males had normal genitalia. These patients were homozygous for StAR mutations V187M and R188C and in functional studies they retained approximately 20% of wild-type activity of the StAR protein (292). Hence a new disorder was described, termed nonclassic lipid CAH. In a more recent report

by Sahakitrungruang et al. four phenotypically different patients with partial loss-of-function mutations were described. The first three patients were phenotypic 46,XY males with various degrees of genital undermasculinization and adrenal insufficiency; the fourth patient was a 46,XX phenotypic female with late-onset adrenal crisis who was menstruating but had anovulatory cycles. Patient 1 was homozygous for the mutation 5033C>T, changing arginine 188 to cysteine (R188C). The mutation was previously described by Baker et al. (292). Patient 2 was a compound heterozygote for 6732T>C, changing leucine 260 to proline (L260P), and 6753T>C, changing phenylalanine 267 to serine (F267S). L260P was described in Swiss patients with classic lipoid CAH (293); F267S is a novel mutation. Patient 3 was compound heterozygous for R188C and tryptophan 250 stop mutation (W250X). Homozygous W250X was previously found in a Serbian 46,XY phenotypic female with classic lipoid CAH (294). Patient 4 was homozygous for the novel mutation 5780G>A, changing glycine to aspartic acid (G221D). In vitro studies demonstrated varied degrees of StAR activity, correlating to the severity of the phenotype. These recent reports of nonclassic StAR indicate that the disorder represents a spectrum of phenotypic severity with varied degrees of StAR activity.

It is of interest to note that most mutations found in StAR occur in the carboxy-terminal 40% of the protein, and that the mutations have recently been found to result in altered folding of the protein. Thus, the importance of the C-terminal portion may be significant in elucidating the mechanism of the StAR protein (295,296).

## 87.7 TREATMENT

### 87.7.1 Endocrine Therapy

The fundamental aim of endocrine therapy in CAH is to provide replacement of the deficient hormones. Since 1950, when Wilkins and coworkers discovered the efficacy of cortisone therapy for CAH due to 21-hydroxylase deficiency, GC therapy has been the key-stone of treatment for this disorder. GC administration both replaces the deficient cortisol and reduces ACTH release and overstimulation of the adrenal cortex, suppressing excessive adrenal androgen production. Proper GC replacement therapy in 21- and 11 $\beta$ -hydroxylase deficiency ameliorates the noxious effects of oversecreted adrenal androgens, averting further virilization, slowing accelerated growth and bone age advancement to a more normal rate, and allowing a normal onset of puberty. GC treatment also leads to remission of hypertension in 11 $\beta$ - and 17 $\alpha$ -hydroxylase deficiency by diminishing oversecretion of hormonal precursors with MC activity. Excessive GC administration should be avoided since this produces cushingoid facies, growth retardation, and inhibition of epiphyseal maturation. In the enzyme deficiencies impairing MC synthesis, the inclusion of

a salt-retaining steroid in the replacement therapy is required to maintain adequate sodium balance.

Hydrocortisone (cortisol) is most often used; it is the physiologic hormone and does not introduce the complication of adjustment for potency, biologic half-life, or altered profile of steroid action. Oral administration is the preferred and usual mode of treatment; it has conventionally been believed that better suppression of adrenal androgen production is achieved with divided doses, although this has been questioned (297); 10–20 mg/m<sup>2</sup> hydrocortisone divided equally in two daily doses by tablet is adequate for the otherwise healthy child. In non-life-threatening illness or stress, increased dosage of two to three times the maintenance regimen is indicated for a few days. Each family must be given injection kits of hydrocortisone (25 mg for infants, 50 mg for young children, and 100 mg for older patients) for emergency use. In the event of a surgical procedure, a total of 5–10 times the daily maintenance dose (depending on the nature of the operative procedure) may be required over the first 24 h and can then be rapidly tapered.

If there is poor response to hydrocortisone at the standard dose, dosage may be increased to 20–30 mg/m<sup>2</sup>/day, or the regimen may be changed to either one of the hormone analogs, prednisone (17 $\alpha$ ,21-dihydroxypregna-1,4-diene-3,11,20-trione) or dexamethasone (9 $\alpha$ -fluoro-16 $\alpha$ -methylprednisolone). These agents are more potent and are longer acting, although their relative GC and MC effects differ and the smaller amounts used make dosage adjustment more critical. Because of differences in hepatic metabolism and variability in the plasma half-life of 11-oxosteroids, prednisolone (11 $\beta$ -hydroxyprednisone) is found in some patients to be more effective than prednisone in the replacement of cortisol function. Classic 21-hydroxylase patients with salt losing additionally require MC replacement. The cortisol analog (21-acetyloxy)-9 $\alpha$ -fluorohydrocortisone (Florinef; 9 $\alpha$ -FF), is used for its potent MC activity. In an adrenal crisis, a patient unable to ingest medication or take fluids is administered high doses of hydrocortisone intravenously, along with liberal infusions of isotonic saline.

Increasing attention has focused on the role of the RAS in 21-hydroxylase deficiency CAH. Although aldosterone levels may not be deficient in simple virilizing cases, it is well known that PRA is elevated in non-salt-wasting, as well as salt-wasting 21-hydroxylase deficiency (298,299). In spite of this observation, it has not been customary to attempt to correct PRA in the therapeutic management of non-salt-wasting 21-hydroxylase deficiency. Inclusion of a salt-retaining steroid in the steroid regimen for non-salt-wasting patients with elevated PRA was found to improve hormonal control (299). Rösler showed that the PRA in 21-hydroxylase deficiency patients was closely correlated with the ACTH level. Thus, when PRA was normalized by the administration of 9 $\alpha$ -FF, the ACTH level fell and excessive

adrenal androgen secretion diminished. In addition, it was found that the GC dose could often be decreased in these patients and that normalization of PRA often resulted in improved statural growth, a finding borne out in subsequent reports (300).

Steroid RIAs have been useful not only for the initial diagnosis of CAH but also for improved monitoring of hormonal control once therapy has been instituted. Serum 17-OHP and  $\Delta^4$ -A levels provide a sensitive index of biochemical control in steroid 21-hydroxylase deficiency (301,302). In females and prepubertal males, but not in newborn and pubertal males, the serum testosterone level is also a useful index. The combined determinations of PRA, 17-OHP, and serum androgens, as well as the clinical assessment of growth and pubertal status, must all be considered in adjusting the dose of GC and salt-retaining steroid for optimal therapeutic control. Combinations of hydrocortisone and  $9\alpha$ -FF have proved to be highly effective (302).

Measurement of PRA can be used to monitor efficacy of treatment not only in 21-hydroxylase deficiency, but also in other salt-losing forms of CAH (StAR and  $3\beta$ -HSD deficiencies). It is also useful as a therapeutic index in those forms of CAH with MC excess and suppressed PRA (11 $\beta$ -hydroxylase and 17 $\alpha$ -hydroxylase deficiencies). In poor control, PRA is elevated in the salt-losing forms and suppressed in the MC-excess forms.

### 87.7.2 Fertility in Nonclassic 21-Hydroxylase Deficiency

Since the presumptive identification of the first nonclassic patients some 50 years ago, it has been recognized that infertility in women may be reversed during GC therapy (55,303). GC treatment is effective in suppressing adrenal androgen production and, with time, clinical signs of androgen excess show improvement. Given the 9-month life expectancy of established hair follicles, remission of hirsutism generally takes at least 1–2 years. A precise timetable to regression of each clinical sign has yet to be established, but Riddick and Hammond (304) reported that five patients with postmenarchal onset of 21-hydroxylase deficiency resumed regular menses and demonstrated adequate suppression of 17-KS and pregnanetriol within 2 months after beginning therapy with GCs alone. Birnbaum and Rose (303) found that of 18 infertile women with acne and/or facial hirsutism and hormonal criteria consistent with 21-hydroxylase deficiency, five conceived after 2 months, and one patient after 7 months of prednisone treatment alone; four more women conceived within 2 months of the addition of clomiphene to the therapeutic regimen. Hormonal profiles after initiation of therapy were not reported in this study. Oligospermia and subfertility have been reported in men with nonclassic 21-hydroxylase deficiency (305,306) and reversal of infertility with GC treatment has been reported (41,307,308). In a study of response to ACTH

stimulation in a population of men with infertility and idiopathic oligospermia, none of the 50 subjects tested by Ojeifo and colleagues (308) demonstrated a 17-OHP response consistent with the diagnosis of nonclassic 21-hydroxylase deficiency.

**87.7.2.1 Sex Assignment.** Sexual ambiguity at birth characteristic of male or female undervirilization is a common presenting sign of CAH. In these cases, rational and judicious choice of sex assignment is a critical aspect of treatment, since the decision of sex assignment has obvious lifelong implications. Determination of genetic sex by karyotype or buccal smear and the accurate diagnosis of the specific underlying enzymatic defect are essential in assessing a patient's potential for future sexual activity and fertility.

In cases of female undervirilization due to 21- or 11 $\beta$ -hydroxylase deficiency, female sex assignment is appropriate. When medical treatment is begun early in life, the initially large and prominent clitoris shrinks slightly and, as the surrounding structures grow normally, it becomes much less prominent, so that surgery may not be required. When the clitoris is conspicuously enlarged or when the abnormal genitalia interfere with parent–child bonding, surgical revision to correct the appearance of the clitoris should be performed. Definitive vaginoplasty should be performed by an experienced gynecologic or urologic surgeon (309). An early one-stage vaginal and perineal reconstruction has been developed (310) that dispenses with a second-stage procedure.

Because of the normal internal genitalia, gonadal structure and karyotype in these patients, normal puberty, fertility, and childbearing are possible when there is early therapeutic intervention. In view of this potential for normal female sexual development, it is unfortunate when, as a result of a hasty delivery room examination of the virilized external genitalia, affected females are improperly assigned and reared as males.

In cases of male undervirilization due to enzyme deficiencies impairing androgen synthesis, a sex assignment consistent with the genetic sex (i.e. a male sex assignment) is not always optimal. We consider certain physiologic capacities integral to “normal” male sexual development: a capacity for urinating in a standing position in prepuberty and a capacity for sexual intercourse. Virilization of the genitalia in these children is frequently so extremely and irrevocably incomplete that the anatomy precludes normal male functioning. In these patients, relatively normal sexual development as a female and normal, albeit infertile, sexual activity is possible, so that sex assignment as a female may be preferable. Impaired androgen synthesis also limits the synthesis of estrogens; thus, whatever be assignment of sex, administration of sex steroids will usually be required to induce development of appropriate sex characteristics at puberty—either estrogens if the patient is to be reared as a female or androgens if the patient is to be reared as a male.



Because of the wide individual variability in the presentation of ambiguous genitalia, there are no all-inclusive rules for sex assignment of these patients solely based on genetic sex or type of enzyme deficiency.

### 87.7.3 Psychoendocrine Treatment

Psychologists and psychiatrists well acquainted with these endocrine disorders provide a vital component of the treatment regimen, as one of the major goals of therapy is to ensure that gender role, gender behavior, and gender identity are isosexual with the sex of assignment (311,312).

## 87.8 CONGENITAL ADRENAL HYPERPLASIA: PRENATAL DIAGNOSIS AND TREATMENT

The affected, untreated genetic female with classic virilizing CAH (predominantly the result of a 21-hydroxylase defect, but can also be from an 11 $\beta$ -hydroxylase defect) experiences virilization of the external genitalia because of excess androgen production by the fetal adrenals early on in the course of gestation. While the postnatal manifestations of CAH are hormonally controlled, surgical correction has been the effective treatment of the genital malformation present at birth. The potent semisynthetic steroid dexamethasone, which is able to transit the placental barrier without complete metabolic inactivation, has been found to be effective in suppressing fetal adrenal activity in the affected female fetus and to reduce significantly the degree of virilization exhibited at birth when administered to the pregnant mother at low/moderate doses. Prenatal treatment of 21-hydroxylase deficiency has now been utilized for approximately 30 years. In the majority of prenatally treated cases, reduction of the genital defect has obviated the need for surgical correction in infancy, sparing the baby girl the trauma of genital surgical correction and psychological damage from the genital ambiguity.

Specific prenatal treatment protocols for CAH have been developed in conjunction with the increasing capabilities of prenatal diagnosis. The earliest form of prenatal diagnosis was direct hormonal assay of the amniotic fluid for 17-OHP (313). Hormonal levels of 17-OHP are clearly elevated in cases of the severe, salt-wasting form of 21-hydroxylase deficiency CAH. In simple virilizing and nonclassic CAH, however, 17-OHP levels are normal, rendering this hormonal test useless for the diagnosis of CAH in these forms (314). Linkage with HLA permitted presumptive diagnosis in all forms of 21-hydroxylase deficiency CAH by serotyping of cultured fetal cells from the amniotic fluid, when the HLA types of parents and family index case had been determined (315). Molecular analysis, using DNA obtained by amniocentesis or from chorionic villus sampling (CVS), initially relied on the high degree of polymorphism of the HLA loci for

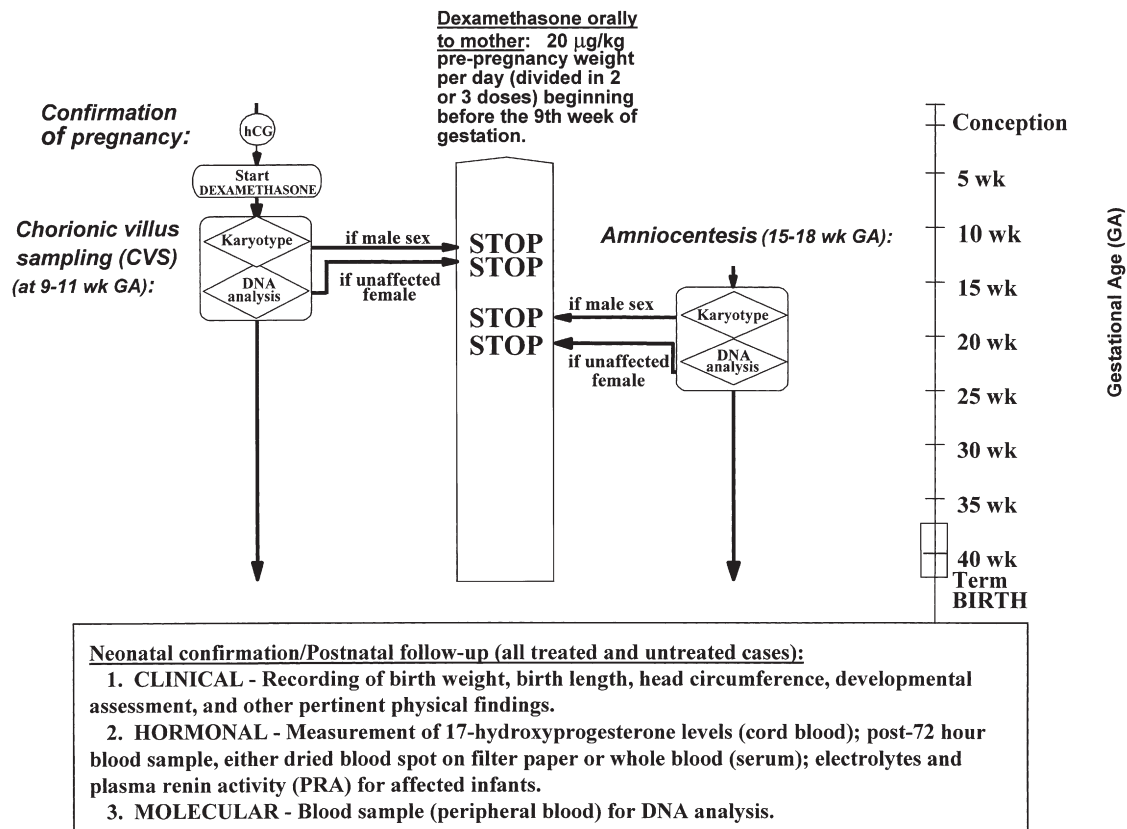
informative DNA results. The accuracy of these results depended on nonrecombination with the marker and 21-hydroxylase loci (316) and thus resulted in many diagnostic errors because of recombination or haplotype sharing between parents. Currently, direct molecular analysis of the 21-hydroxylase locus by allele-specific PCR is used effectively for prenatal diagnosis (134).

The approach we have evolved for prenatal treatment and diagnosis is illustrated in Figure 87-8 (317,318). A couple is determined to be “at risk” for producing CAH-affected offspring usually because of affectation of a prior-born offspring (the index case). In some counseled families the index case may be more distantly related.

Under ideal circumstances, preconceptional genetic evaluations will be undertaken. Basic genetic evaluation consists of 21-hydroxylase genotyping of both parents and the index case. It is of paramount importance that there is the earliest confirmation of pregnancy so that the course of treatment may be started before the ninth week of gestation to prevent virilization of an affected female. The starting treatment regimen is dexamethasone administered orally to the mother at a dose of 20  $\mu$ g/kg maternal prepregnancy weight per day (to a maximum of 1.5 mg) in two or three divided doses. Early start of treatment is essential to anticipate the critical period of genital differentiation in the embryo. Treatment (blind to the status of the fetus) is continued until the diagnostic procedures have been performed and analysis is complete—9–11 weeks for CVS and 15–18 weeks for amniocentesis, plus the time for cell culturing and DNA analysis. Treatment is then stopped in the case of a male fetus (one in two) or unaffected or carrier status of a female fetus (three in eight), but it is continued to term for an affected female fetus (one in eight). Cessation of treatment will be sooner in the case of genetic male fetuses because of the shorter time required for sex chromosome determination.

When properly administered, this regimen of dexamethasone treatment prevents ambiguous genitalia in the affected female. The average Prader score in a large study of prenatally treated fetuses was 1.7, compared with the average score of 3.9 for untreated affected females. In most cases dexamethasone has been well tolerated by the mother, showing no or only minor maternal side effects (317,319,320). In only a few cases has there been frank maternal intolerance of the steroid (321–323). Notably, however, some mothers, even after experiencing side effects, have indicated that they would nonetheless repeat the treatment because of the good result for the affected baby girl (324).

To date, the largest human studies have shown no affected or unaffected fetuses treated interim or to term (317–319,325) for prevention of virilization in CAH to have any congenital malformation related to dexamethasone. In a study by Carlson and colleagues of over 400 pregnancies, 36 mothers carrying affected females were treated full term with dexamethasone. No significant or enduring side effects were noted in fetuses, and



**FIGURE 87-8** Algorithm for prenatal diagnosis and treatment of 21-hydroxylase deficiency.

newborns did not significantly differ in birthweight from untreated newborns (317). In a recent update from the same group, data from almost 600 pregnancies at risk for CAH was analyzed. Of the fetuses 80 were prenatally treated until term and 27 who were male received dexamethasone for a short period. The newborns in the dexamethasone-treated group did not differ in weight, length or head circumference from untreated, unaffected siblings. No significant or enduring side effects were noted in either the mothers or the fetuses. Greater weight gain in treated versus untreated mothers did occur, as well as the presence of striae and edema. Excessive weight gain was lost after birth. No differences were found regarding gestational diabetes or hypertension (326). Data from other groups on long-term follow-up evaluation of CAH-affected and unaffected children exposed to dexamethasone prenatally has shown them to be developing normally and within expected limits, both physically and mentally (317,319,325).

Principles of prenatal diagnosis and treatment for 21-hydroxylase deficiency were also applied to several cases of affected female fetuses with 11 $\beta$ -hydroxylase deficiency (327,328). The treatment was successful, as the newborns were born with normal female external genitalia. Prenatal treatment is therefore safe and effective.

Preimplantation genetic diagnosis (PGD) has been utilized in many monogenic recessive disorders, including CAH. PGD identifies genetic abnormalities in

preimplantation embryos before embryo transfer, so only unaffected embryos established from in vitro fertilization are transferred. There are reports of successful PGD in families whose offspring is at risk for CAH (329), and we know that families are seeking PGD with greater frequency.

The novel approach of noninvasive prenatal diagnosis of CAH is currently being investigated. As discussed, masculinization of the genitalia in a female fetus affected with CAH owing to 21-OHD and 11 $\beta$ -OHD can be treated prenatally with dexamethasone administered to the mother. Because CAH is an autosomal recessive disorder, the risk is 1/4 of the fetus being affected with the disease and 1/8 of the fetus being a female with ambiguous genitalia. Therefore seven of eight pregnancies will receive unnecessary treatment until the sex and the affection status of the fetus are known. Treatment with dexamethasone must begin before the ninth week of gestation, yet chorionic villous sampling can only be done at the 9–11th week, with genotype results available 2–3 weeks later. Noninvasive prenatal diagnosis would eliminate unnecessary treatment and invasive procedures such as CVS and amniocentesis. Lo et al. in 1997 discovered the presence of fetal DNA in the maternal circulation (330). Fetal DNA has been extracted and enriched with high accuracy and yield in fetal Rh factor identification (331) and aneuploidy detection (332,333). Identification of the SRY sequence in maternal blood, performed

in multiple academic centers and by several methods, has also achieved excellent accuracy in several studies (334,335). In noninvasive prenatal diagnosis of CAH, by extracting fetal DNA from the maternal blood at 6–7 weeks gestation, the SRY sequence is to be identified, and if the fetus is female (SRY sequence not identified), DNA analysis of the extracted fetal DNA is to be conducted. There has been only one case reported of prenatal diagnosis in CAH (336); however, studies are underway to further develop this innovative method.

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## Biographies



**Dr Maria New** is a Professor of Pediatrics and of Genetics and Genomic Sciences, as well as the Director of the Adrenal Steroid Disorders Program at Mount Sinai School of Medicine in New York City. She is also the Associate Dean for Clinical Research at the Florida International University Herbert Wertheim College of Medicine. Dr New has edited or coedited 12 medical textbooks and published more than 600 peer-reviewed articles. She has conducted pioneering research in the area of congenital adrenal hyperplasia and other adrenal steroid disorders. In addition, Dr New discovered a new form of hypertension, apparent mineralocorticoid excess, which opened a new field of receptor biology. She was also the first to describe dexamethasone-suppressible hyperaldosteronism, another form of low-renin hypertension. In 1999, she reported what may be the first example of a transcription factor defect in humans.

Dr New's contributions have been recognized by her being selected as one of the few pediatricians in the National Academy of Sciences. She has received numerous honors including the Rhone-Poulenc Rorer Clinical Investigator Award from the American Endocrine Society and the 2003 Fred Conrad Koch Award, the highest award given by the American Endocrine Society. In 2010, she received the Van Wyk Prize, the highest award given in pediatric endocrinology.

Dr New received her Bachelors degree from Cornell University and her Doctor of Medicine degree from the University of Pennsylvania, where she was awarded the Distinguished Graduate Award.



**Dr Lekarev** is a Clinical Instructor of Pediatrics and Pediatric Endocrinology at the Mount Sinai School of Medicine. Her areas of interest include congenital adrenal hyperplasia and other adrenal steroid disorders, disorders of sexual development, and noninvasive prenatal diagnosis of genetic disorders. Dr Lekarev graduated cum laude with honors from Barnard College of Columbia University with a bachelor of arts in linguistics. She received her medical degree at the University of Medicine and Dentistry of New Jersey. She completed an internship in pediatrics at the Robert Wood Johnson Medical Center and went on to complete her residency in pediatrics at Tufts-New England Medical Center. Dr Lekarev completed her fellowship in pediatric endocrinology at the Mount Sinai School of Medicine.



**Dr Lin-Su** is a Clinical Associate Professor in Pediatric Endocrinology at Weill Medical College of Cornell University. She is also the Medical Director of CARES Foundation, a non-profit organization committed to improving the lives of families and individuals affected by congenital adrenal hyperplasia (CAH). She is certified in Pediatrics and Pediatric Endocrinology by the American Board of Pediatrics and has received awards from the National Institutes of Health for her rare disease and health research work. Extensively published, her work has appeared in numerous peer-reviewed journals and textbooks. She graduated cum laude with honors from Yale University and received her medical degree at Cornell University Medical College. She completed her pediatric residency and pediatric endocrinology fellowship training at New York Presbyterian Hospital, Cornell.

# CHAPTER

# 88

## Disorders of the Gonads, Genital Tract, and Genitalia

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### 88.1 INTRODUCTION

Disorders of gonadal differentiation resulting from mutant genes are the focus of this chapter. Disorders resulting from abnormalities of sex chromosomes are alluded to only briefly, because they are considered in detail elsewhere in this volume (Chapter 44). Additional clinical aspects of both Mendelian and cytogenetic causes of sex differentiation have been discussed elsewhere by this author (1,2). Disorders of adrenal biosynthesis (21 and 11 $\beta$ -hydroxylase) are discussed in Chapter 87.

### 88.2 46,XX DISORDERS OF SEXUAL DEVELOPMENT

#### 88.2.1 Gonadal and Genital Embryology

Primordial germ cells (PGCs) originate in the endoderm of the yolk sac and migrate to the genital ridge to form the indifferent gonad, which is initially indistinguishable in 46,XY and 46,XX embryos. PGCs migrate from the yolk sac around 28 days (post-conception), reaching the gonadal ridge by 37 days, or 5 days after formation of the genital ridge. Indifferent gonads develop into testes if the embryo—or more specifically the gonadal stroma—is 46,XY. This process begins about 43 days after conception (15 mm crown rump length). Testes become morphologically identifiable 7–8 weeks after conception (9–10 weeks gestational or menstrual weeks).

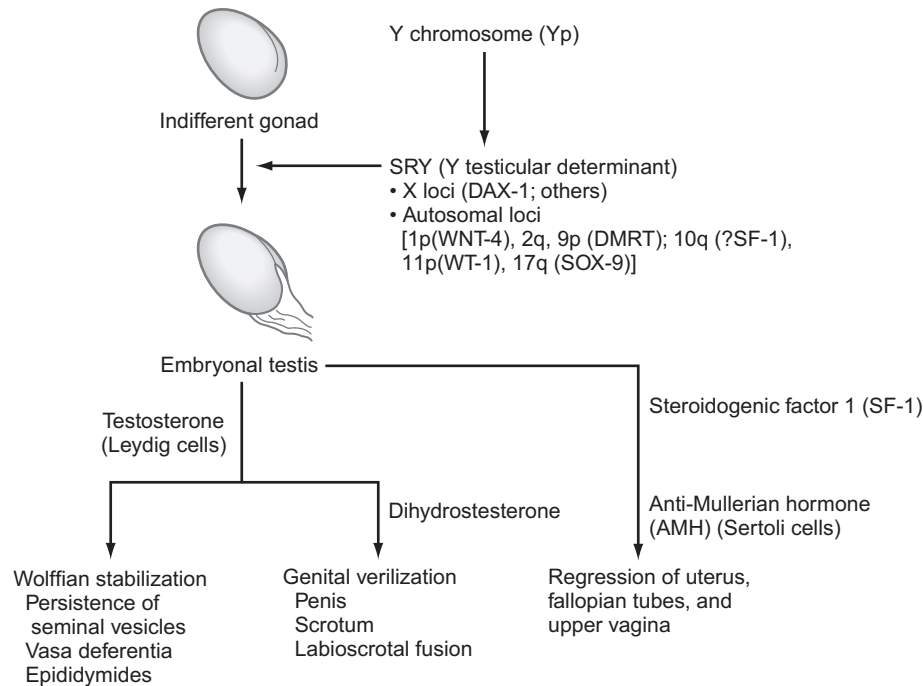
Sertoli cells are the first cells to become recognizable in testicular differentiation, organizing the surrounding cells into tubules. Both Leydig cells (3) and Sertoli cells (4) differentiate prior to testicular morphogenesis, consistent with their directing gonadal development rather than the converse. These two cells secrete hormones that direct different aspects of male differentiation (Figure 88-1).

Fetal Leydig cells, which are first recognized at 55 days, produce by 63 days an androgen (testosterone) that

stabilizes Wolffian ducts and permits differentiation of the vasa deferentia, epididymides, and seminal vesicles. Following conversion by 5 $\alpha$ -reductase to dihydrotestosterone (DHT), external genitalia are virilized. These actions can be mimicked by the administration of testosterone to female or castrated male mammalian embryos. Fetal Sertoli cells produce anti-Müllerian hormone (AMH, previously called Müllerian inhibitory substance or MIS), a glycoprotein that diffuses locally to cause regression of Müllerian derivatives (uterus and fallopian tubes). AMH also has functions related to gonadal development, given that if AMH is chronically expressed in XX transgenic mice oocytes fail to persist, tubule-like structures develop into gonads, and Müllerian differentiation is abnormal (5). Wolffian differentiation (vasa differentiation, epididymides, seminal vesicles) requires testosterone.

In the absence of a Y chromosome, the indifferent gonad develops into an ovary (6,7), at least embryologically. Transformation into fetal ovaries begins at 50–55 days of embryonic development. By 20 weeks of embryonic life, fetal ovaries contain up to 7 million germ cells, the vast majority undergoing atresia and only 400 actually ovulated as adults. Even in 45,X embryos, oocytes differentiate only to undergo atresia at a rate more rapid than that occurring in normal 46,XX embryos. Thus, genes on the X necessary for normal ovarian development actually control ovarian maintenance. These genes are localized to specific regions of the X, and must be accompanied by various autosomal genes to generate normal ovaries. See Genetic Control of Sex Differentiation below.

Ductal and external genital development occur independent of gonadal differentiation because in the absence of testosterone and AMH, external and internal genitalia develop in female fashion. Absent AMH, Müllerian ducts form the uterus and fallopian tubes, and Wolffian ducts regress. Such changes occur in normal XX embryos, as well as in XY animals that were castrated as embryos prior to testicular differentiation.



**FIGURE 88-1** Schematic diagram illustrating embryonic differentiation in the normal male. (Modified from Simpson, J. L., Elias, S. *Disorders of Sex Differentiation: Gonadal Abnormalities and Hypogonadotropic Hypogonadism*. In *Genetics in Obstetrics and Gynecology*, 3rd ed.; WB Saunders: Philadelphia, 2003.)

## 88.2.2 Genetic Control of Sex Differentiation

Both sex chromosomes (X and Y) and autosomes contain loci that must remain intact for normal sexual development. Knowledge and location of these loci facilitate clinical diagnosis. The initial step in elucidating genetic control distinguishing male from female sex came with recognition that a Y was required for (normal) sexual development.

**88.2.2.1 Genital Ridge.** In the mouse, a number of genes (*Emx2*, *Gata4*, *Lim1*, *Lhx9*) are expressed in the genital ridge. If not present (gene knockout), absence of gonads and internal ducts results. Although expected, perturbations of these genes have not yet been documented in the human genital ridge or in liveborns. However, we shall see that *Wt1* and *Sf-1* knockouts produce such a phenotype and humans with 46,XY disorders of sexual development (46,XY DSD) have shown perturbations of *Wt1* and *Sf1* DSD.

**88.2.2.2 Testicular Development: Loci on Y Chromosome, and Autosomes.** That 46,X,i(Yq) individuals were female in appearance demonstrated nearly 40 years ago that the major testicular determinants (testis-determining factors) were localized to the Y short arm (Yp). Based on (80%) 46,XX male subjects (46,XX sex reversal) arising from interchange of not just the expected homologous pseudoautosomal regions (PARs) of Xp and Yp, but also a greater region encompassing the proximal non-PAR, the pivotal gene on Yp was shown to be *SRY* (sex-determining region Y). In confirmation, 10–15% of sporadic XY sex reversal (female phenotype) show point mutations within

*SRY* (8,9). The *SRY* gene is composed of two open reading frames, 99 and 273 amino acids in length. Based on females with XY gonadal dysgenesis (10,11), the key sequence involves a high-mobility group (HMG) box that has characteristics in common with other DNA-binding and DNA-bending sequences (homeodomains). When XY gonadal dysgenesis is associated with a point mutation in *SRY*, perturbation almost always involves the HMG box. *SRY* is evolutionarily conserved, being present in all male (XY) mammals. *SRY* is expressed before testicular differentiation is manifested (12) and transgenic XX mice with *SRY* predictably show testicular differentiation (13). However, *SRY* alone does not suffice for testicular development nor is it obligatory. This role is played by *SOX9*, located on human 17q24.1–q25.1. *SOX9* has a DNA-binding box and can thus interact with *SRY*.

As already predicted, certain genes (e.g. *NR5A1*) are necessary to establish the genital ridge in anticipation of migration by PGCs. In mice, *Sry* apparently must also be activated by *Gata4*, *Fog2/Nr5a1* and *Wt1*. Murine *Sry* and presumably human *SRY* then upregulates *SOX9*. Upon reaching a given threshold, *SOX9* is believed to sustain itself by a positive regulatory loop involving *FGF9* and *PGD2*. *NR5A1* (SF1) appears to play a synergistic role (14). Haploinsufficiency of the *SOX9* region causes XY sex reversal (male genotype to female phenotype) despite presence of *SRY* (15). Specifically, a region up to 1 Mb upstream of *SOX9* is necessary to produce a male phenotype even if *SOX9* is intact (16). This became evident when deletion (960 kb) of a region 517–1477 kb upstream of

SOX9 led to acampomelic dysplasia and lack of male development in a 46,XY child (17) who had SRY and SOX9. Duplication of SOX9 results in the converse (XX to male phenotype) despite absence of SRY (18). In mice, Qin and Bishop (19) showed definitively that SOX9 is sufficient to direct testicular differentiation in the absence of Sry.

In aggregate, male differentiation must involve derepression by SRY of an otherwise constitutively repressed autosomal testicular-determining autosomal region synonymous with or near SOX9 on 17q24. There exist other less pivotal regions that if deleted impede testicular development in humans. These include 9p24.3 (a region that codes for DMRT) (20–26), 10q26 (a region that encodes), SF1 (NR5A1) for which EMX is a candidate gene (27,28), and 2q33 (29). In addition to duplications of SOX9 and 22q11.2 (30–32), duplication of 1p has resulted in XX sex reversal (XX male) (33). 1p contains Wnt-4, which if overexpressed upregulate DAX1, a dosage-sensitive Xp region (see below). On Xp lies DAX1, a region of significance because it is dosage-sensitive and located on Xp (34). When duplicated, XY individuals undergo sex reversal to female phenotype (the converse of duplication of SOX9 in XX individuals) (34).

**88.2.2.3 Male Internal Ducts.** Testicular Sertoli cells secrete AMH which provided the AMH receptor (AMHR) is intact precludes Müllerian differentiation in males. These processes require several other genes (NR5A1 or SF1, GATA4, WT1, WNT1, SOX8), based on perturbations of these genes resulting in failure of uterine development in females or persistence in 46,XY males. SF1 (NR5A1) especially seems pivotal.

Irrespective of whether Müllerian suppression occurs in 46,XY, Wolffian differentiation can occur if testosterone is produced. Existence of syndromes in which Wolffian derivatives are absent indicates that other genes are required, the best known of which is cystic fibrosis (CF) (CFTR). If CFTR is perturbed, congenital absence of the vas deferens occurs (unilateral CAVD; bilateral CBAVD).

**88.2.2.4 Y Chromosome and Spermatogenesis.** About 15% of azoospermic men have Y-deletions, and about 5–10% of oligospermic men have deletions. Several Y-loci are involved, but their number and interrelationships are still uncertain. The most popular model assumes three loci on Yq: AZFa, the rarest and whose deletion is associated with the absence of spermatogenesis and stem cells; AZFb, whose deletion produces maturational arrest and which corresponds to the locus RNA-binding motif; AZFc, which if deleted is associated with azoospermia and severe oligospermia. AZFc contains the locus DAZ (deleted in azoospermia). Through intracytoplasmic sperm injection, men with DAZ deletions can sire offspring. The Yq deletion is predictably transmitted to all male offspring but none of the females (35).

**88.2.2.5 Gonadoblastoma Y.** Loss of the fluorescent (and presumably contiguous euchromatic nonfluorescent) portion of Yq surprisingly protects against the

germ cell neoplasia that is prevalent in XY women. XY women with deletion of Yq fail to develop neoplasia characteristics of those without deletion. This locus is termed gonadoblastoma Y (GBY) (36,37).

An attractive candidate gene is testis-specific protein Y (TSPY), a multicopy gene (38) upregulated in gonadoblastomas (37) and normally expressed in spermatogonia of normal testes (39).

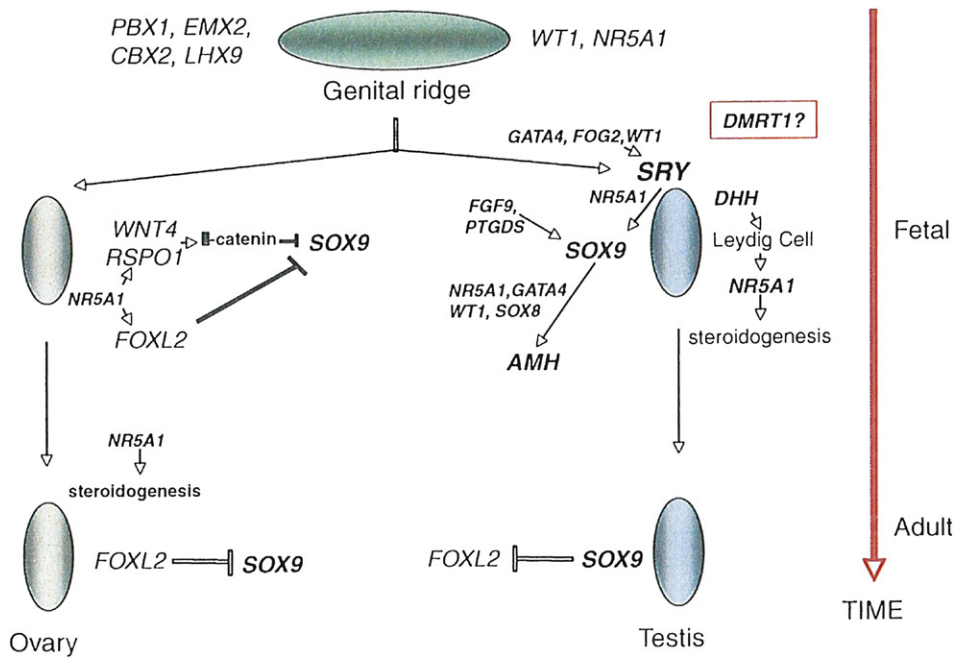
**88.2.2.6 Ovarian Development: Ovarian Maintenance Regions on the X Chromosome.** Pathogenesis of germ cell failure in humans involves as noted increased germ cell attrition. Loci on both the X chromosomes and autosomes are required for normal ovarian differentiation and oogenesis. If two intact X chromosomes are not present, ovarian follicles in 45,X individuals usually degenerate prematurely, if not by birth then at the time of expected adolescence. Genes on the second X chromosome thus could be said to be responsible for ovarian maintenance rather than primary ovarian differentiation. The specific mechanism appears to involve expression of WNT4 and RSPO1, both autosomal loci. If SRY is lacking, SOX9 remains repressed (40), preventing male differentiation. Continued repression of SOX9 is accomplished by FOXL2 (41). FOXL2 is thus essential for ovarian development. Many other genes—X chromosome and autosomal—are, however, necessary for normal ovarian development to proceed normally.

**88.2.2.6.1 Pivotal Regions on the X Short Arm (Xp) and X long Arm (Xq).** Approximately half of 46,X,del(Xp)(p11) individuals show primary amenorrhea and gonadal dysgenesis; other individuals menstruate and usually show breast development. Figure 88-2 shows the author's tabulation of the phenotypes based solely on terminal deletions detectable by karyotype (6 Mb resolution).

In the X short arm, the region of greatest importance involves Xp21–22.1 or 22.2. Women with these deletions usually show complete or premature ovarian failure (POF). Women who have more distal Xp deletions [del(X)(p21.1 to p22.1.22)] and, hence, retain a greater portion of the X menstruate more often than 45,X women; however, many are infertile or have secondary amenorrhea. Women with deletions of Xp are usually short in stature. Given that del(Xp) women may menstruate yet still be short, regions on Xp responsible for ovarian and statural determinants must differ (2,42–46). Both mother and daughter may show the same Xp deletion, for del(X)(p11) as well as for del(X)(p21 or 22) (47–49). Of clinical relevance is that phenotype may vary among relatives in the same family who have the same X-deletion or rearrangement. Interstitial deletions involving Xp11–22 and Xp11.4–22.3 (50,51) have also been reported.

Almost all terminal deletions originating at Xq13 are associated with primary amenorrhea, lack of breast development, and complete ovarian failure (see Figure 88-2) (43,52–54). Xq13 is thus pivotal for ovarian maintenance.





**FIGURE 88-2** The molecular and genetic events in mammalian sex determination and differentiation. (From McElreavey, K., Bashamboo, A. *Genetic Disorders of Sex Differentiation*. Adv. Exp. Med. Biol. 2011, 11, 707, 91–99.)

Del(X)(q21 → 24) individuals menstruate more often than those with more proximal deletions, suggesting that these women might have retained a region containing an ovarian maintenance gene that is deleted in del(X)(q13). (The designation POF2 has been applied to a presumptive locus in the Xq13–21 region, but this label and other similar gene designations for POF may be simplistic.) In terminal Xq deletions at Xq25 or 26, the more common phenotype is not primary amenorrhea but POF (47,53–55). These distal Xq deletions are often familial (56). More distal deletions arise at Xq27 or 28. The “locus” in this more distal deletion has been labeled POF1.

Xq deletions at Xq27 or 28 exert less severe effect on stature than do proximal deletions. Turner stigmata seem no more common than in the general population.

**88.2.2.7 Ovarian Genes on Xp and Xq.** Although gene designations have as noted been assigned to the chromosomal regions on Xp and Xq (POF1, POF2), the actual genes remain unclear. Array CGH copy number variants (CNVs; microdeletions smaller than detectable by conventional karyotype) in women with ovarian failure have also not consistently correlated with that expected if genes were restricted to regions assigned for POF1 and POF2. Knauff et al. (57) found CNVs (262kb resolution deletions) in Xq21.3 to be associated with POF, in a region where PCHCH1X and TGHF2LX are located. However, no deletions were found in other regions considered on the basis of cytogenetic studies to be pivotal. Quilter et al. (58) found that 48% of their cases showed a CNV microduplication or deletion with many X regions involved, whereas Dudding et al. (59) found only a 800-kb duplication at Xq13.3 and a Xp22.3 duplication among 50 POF cases (4%). Aboura et al. (60) reported a statistically significant

association for a 0.217-kb gain on Xq28, but the same gain was found in 5/67 controls. No significant X-deletions were found in 90 POF cases. Discrepancies among these CNV studies are most likely explained by failure to perform the parental array studies that are necessary to exclude paternal transmission of a polymorphic CNV of no likely clinical significance.

Genome-wide linkage analysis has shown association between Xp21.3 (LOD score 3.1) and the age of menopause (61).

Ochalski (62) recently proposed a list of genes having potential relevance to ovarian development and function. Several candidate genes on the X deserve specific comment.

#### 88.2.2.7.1 Xp.

##### 88.2.2.7.1.1 USP9X (Ubiquitin-Specific Protease 9).

This gene maps to Xp11.4 (63) and is expressed in multiple tissues. The *Drosophila* ortholog of USP9X is required for eye development and oogenesis. The role USP9X plays in human gonadal development is still unclear, but its location in the important Xp11.4 region is tantalizing.

##### 88.2.2.7.1.2 Zinc Finger X. Zinc finger X (Zfx) is a

candidate gene for short stature and ovarian failure (64). Mice null for Zfx are small, less viable, less fertile, and characterized by diminished germ cell number in ovaries and testes (65). Their external and internal genitalia are otherwise normal.

##### 88.2.2.7.1.3 Bone Morphogenetic Protein 15. Con-

sidered the strongest candidate gene on Xp is bone morphogenetic protein 15 (BMP15), a member of the transforming growth factor-beta (TGFβ) superfamily. TGF genes direct many developmental pathways through

binding and activating transmembrane serine/threonine kinase receptors. BMP is specifically involved in folliculogenesis and embryonic development, being expressed in gonads.

BMP15 is located on chromosome Xp11.2 and consists of only two exons. Before informative human cases were reported, animal studies had suggested that perturbations of BMP15 could be important in the ovarian failure. Heterozygous Inverdale sheep carrying a mutation in the BMP15 gene shows an increased ovulation rate, with twin and triplet births; primary ovarian failure occurs in ovine homozygotes. BMP15 knockout mutant female mice are subfertile, showing decreased ovulation rates, reduced litter size and decreased number of litters per lifetime (66). In humans, BMP was implicated in POF first by Di Pasquale and coworkers (67) who reported a heterozygous Y235C missense mutation in the second exon of the BMP15 gene in each of two sisters having ovarian failure. The proband had streak gonads and elevated follicle-stimulating hormone (FSH) (80 MIU/mL); the younger sib had one episode of vaginal spotting but otherwise similar. The mother was homozygously normal at this allele, Y235C transmitted from their father. The authors presented in vitro evidence for a dominant-negative mechanism.

#### 88.2.2.7.2 Xq.

**88.2.2.7.2.1 XIST.** Xq13 contains the X-inactivation center and XIST. Loss of germ cells may or may not be the direct result of perturbation of XIST, despite disturbances of X-inactivation per se clearly leading to ovarian failure. The concept of a defined “critical region” necessary for ovarian development receives less attention than in the past, but this does not exclude the region being rich in pivotal genes.

**88.2.2.7.2.2 DIAPH2 (Diaphanous).** This candidate gene lies in the Xq21–24 region. Human DIAPH2 is the homolog of *Drosophila melanogaster* diaphanous (dia). In *Drosophila*, dia is a member of a family of proteins that help establish cell polarity, govern cytokinesis, and reorganize the actin cytoskeleton. In flies, mutations cause sterility in both male and female subjects (68). Xq21/autosome translocation was found to have disrupted the last intron of DIAPH2 in a human patient (69).

**88.2.2.7.2.3 XPNPEP2.** Bione and Toniolo (70) and Prueitt and colleagues (71) found disruption of XPNPEP2 in an Xq/autosome. The proband had POF.

**88.2.2.7.2.4 Angiotensin II (Type 2) (ATZ) Receptor.** Perturbed in a diverse group of disease states, this gene is expressed in fetal tissue. Atretic granulosa cells express the gene in rodents, and thus a plausible relationship could be made between this gene and POF.

Another attractive reason is its location on Xq22–23, a region of known significance (so called POF2). Katsuya (72) studied two families in each of which sibs each had POF; no AT2 mutations were found.

**88.2.2.7.2.5 FMR1.** Fragile X syndrome is a common form of X-linked mental retardation caused by mutation of the FMR1 gene, located on Xq27. The molecular basis involves repetition of the triplet repeat CGG. If more than 200 repeats exist, transcriptional silencing of an RNA-binding protein occurs. This disorder is discussed in detail in Chapter 107. The normal number of CGG repeats is usually 29 or 30. Men or heterozygous women with 55–199 repeats are said to have a permutation (73). During female (but not male) meiosis, the number of triplet repeats may increase (expand). A woman with an FRAXA permutation may have an affected son if the number of CGG repeats on the oocyte of the X she transmits to her male offspring expands during meiosis to greater than 200. That boy will then show mental retardation, characteristic facial features, and large testes (see Chapter 107). Expansion will not occur if there are less than 55 CGG repeats, although the precise threshold remains arguable. Women may also show mental retardation if an X expands, but the phenotype is less severe than in men. About 15–20% of women with an FMR1 premutation (55–100 CGG repeats) develop POF (74).

Schwartz and colleagues (75) found oligomenorrhea in 38% of permutation carriers versus in 6% controls. Allingham-Hawkins and coworkers (76) analyzed 1268 controls, 50 familial POF cases and 244 sporadic POF cases. In this international collaborative survey (76), 63 of 395 premutation carriers (16%) underwent menopause before 40 years of age; the frequency in controls was 0.4%. In a survey of Atlanta women, Sullivan and colleagues (73) found 12.9% of permutation carriers ( $N = 250$ ; >59 repeats) had POF versus 1.3% (2/157) of controls. The number of CGG repeats significantly correlated with the risk of POF but only within selected ranges. Only a slightly increased risk exists with 40–79 repeats, whereas the risk was much higher with 80–99 repeats; there was no further increased risk after >100 repeats. The reason for the plateau is not clear, but consistent with women having the full mutation (>200 CGG) not showing POF (76).

FMRI testing should probably be part of the work-up for POF, and it is so recommended in Europe (77). If oocyte or ovarian slice cryopreservation becomes more feasible, population screening might even be justified for fertility preservation.

Although located in the same region of Xq, FMR1 cannot logically correspond exactly to the region which when deleted causes only ovarian failure in del(Xq). However, On the other hand, terminal deletions at Xq27 (karyotype or array CGH) have been observed in females with fragile X syndrome. This may be the result of skewed X-inactivation such that the sole active X is heterozygous for an FMR1 premutation transmitted from a phenotypically normal but heterozygous mother (78).

**88.2.2.8 Short Stature Homeobox.** Short stature homeobox (SHOX) exists in the PAR1 that involves Xp22 and Yp11.3. SHOX has been proposed as

regulating skeletal growth, reasoning based on short stature existing in Turner syndrome and tall stature in polyploidy X (47,XXY). Consistent with this, SHOX escapes X-inactivation as does all of PAR1. SHOX mutations and deletions have been associated with short stature (79,80), Leri-Weill syndrome (81) and Langer mesomelic dysplasia (82); duplications have also been observed. Tachdjian et al. (83) studied three POF cases known by conventional karyotype to have an Xq deletion, one having del(X)(q21.31) also had an array CGH 620-kb duplication (Xp22.3 → PAR1). Although postulated, a relationship between POF and specifically SHOX seems unlikely. Location of SHOX in the PAR makes it unlikely to have ovarian (or testicular determinants) value. Moreover, we have noted above that cases of 46,X,del(Xp) need not necessarily be short, nor the converse.

**88.2.2.9 Autosomal Ovarian Genes.** Disturbance of many autosomal genes adversely affects normal ovarian development. Some perturbed genes could have a function restricted to oogenesis or ovarian development, whereas in others, ovarian disturbance is only one component of a pleiotropic effect. A host of candidate autosomal genes exist. Genes considered established include FSH receptor (FSHR), luteinizing hormone receptor (LHR), factor in germline alfa (FIGLA) and newborn ovary homeobox (NOBOX). None (save FMR1 and in Finland FSHR) appear to explain more than the occasional case of ovarian failure. See Ovarian Dysgenesis/POF.

### 88.2.3 Nomenclature

The nomenclature used in previous editions to describe clinical disorders of sexual differentiation is time-honored and descriptive. However, some perceive pejorative connotations, and media have often sensationalized affected individuals. For this reason, a revised classification has been proposed (84) (Table 88-1). A problem with this classification is that collapsing heterogeneous disorders into only a few overarching categories unavoidably pools (“lumps”) disparate disorders. Comparisons to older literature containing almost uniquely informative cases also become difficult. In this chapter, we shall attempt to utilize both traditional and more recently “recommended” (84) nomenclature.

**TABLE 88-1 Proposed Revised Nomenclature: DSD**

Traditional	Proposed
Male pseudohermaphroditism	46,XY DSD
Female pseudohermaphroditism	46,XX DSD
True hermaphroditism	Ovotesticular DSD
XX male or XX sex reversal	46,XX testicular DSD
XY female or XY sex reversal	46,XY complete gonadal dysgenesis

### 88.2.4 46,XX Males (XX Sex Reversal)

A systematic review of autosomal causes of ovarian failure will be discussed below, at which time many other genes will receive comment. In the previous section, we reasoned that SRY was the Y-testicular determinant on the basis of analysis of 46,XX (sex-reversed) males. These males have bilateral testes (85,86), and usually have small testes. Androgen deficiency is evident, resembling 47,XXY Klinefelter syndrome. Facial and body hair are decreased, and pubic hair is distributed in the female pattern. About one third have gynecomastia. The penis and scrotum may be small but usually well differentiated (85,86). Wolffian derivatives are normal. Seminiferous tubules are decreased in number and size. Peritubular and interstitial fibrosis is present, Leydig cells are hyperplastic or hypoplastic, and spermatogonia are usually detectable.

In contrast to 47,XXY men, however, virilization may be apparently normal in 46,XX men. Except for sterility, some cases are thus indistinguishable from normal men. Penile differentiation is usually normal; in sporadic cases, genital ambiguity is not uncommon in familial cases bespeaks a different etiologic basis for XX males with genital ambiguity.

Among 46,XX males not having genital ambiguity, 80% show SRY as noted. The cytologic basis is unequal crossing over between Xp and Yp during paternal meiosis. The normal Yp–Xp interchange extends beyond the PAR to include SRY, which is closer to the centromere. The atypical recombination results in SRY becoming translocated from the paternal Y to the paternal X.

In the 20% of nonfamilial XX males lacking SRY, other mechanisms must be postulated. Plausible hypotheses include mosaicism or a mutant autosomal gene (87), the latter seemingly more likely to this author. Indeed, kindreds exist in which there are multiple 46,XX males (88–91) as well as family members having both 46,XX males and 46,XX true hermaphrodites (88,92–94).

### 88.2.5 46,XY Females (XY Sex Reversal) (46,XY Complete Gonadal Dysgenesis)

Gonadal dysgenesis may occur in phenotypic women having apparently normal male (46,XY) chromosomal complements. This phenotype is predictable because loss of testicular tissue before 7–8 weeks would be expected to produce such a phenotype, as originally shown by Jost (6) in rabbits. Indeed, analysis of these women has been pivotal in elucidating the genetic control of sex differentiation. From the clinical perspective, a mutant gene must be systematically excluded.

The various types of XY gonadal dysgenesis are all characterized by structurally normal female external genitalia, vagina, uterus, and fallopian tubes. In at least some cases, the gonads of human XY women were unequivocally ovaries (95–97). This is unequivocally



shown by the extraordinary report of Dumic et al. (97) in which a proband had 46,XY complete gonadal dysgenesis but her mother showed only 46,XY or 45,X cells in lymphocytes, skin fibroblasts and ovary. These cases contrast with the usual XY gonadal dysgenesis secondary sexual development fails to occur at puberty (absent the exceptional case just cited). Height is normal, and in prototypic cases, somatic anomalies are not present. As in 46,XX gonadal dysgenesis without somatic anomalies, FSH and LH are elevated, and estrogens are decreased. In the absence of somatic abnormalities indicative of a syndrome, women with XY gonadal dysgenesis may be indistinguishable from those with XX gonadal dysgenesis on the basis of physical examination.

XY gonadal dysgenesis does contrast, however, with XX gonadal dysgenesis in one very important way. Approximately 20–30% of XY gonadal dysgenesis patients develop a dysgerminoma or gonadoblastoma (98) presumably as a result of presence of the GBY locus (see above). Neoplasia (gonadoblastoma or dysgerminoma) may arise in the first or second decade, necessitating prophylactic gonadal extirpation. Whether this applies to all XY females discussed in this section is unclear, but prudence dictates that one should make this assumption. Laparoscopic removal of gonads and sometimes a gonadoblastoma or dysgerminoma are possible (99,100). Importantly, however, the uterus and fallopian tubes should not be removed because the patient may wish to use her uterus to carry a pregnancy achieved through donor oocytes or donor embryos. Successful pregnancies are documented.

**88.2.5.1 Absence or Mutation in SRY.** Aa alluded to already, deletions or perturbations of SRY precludes testicular differentiation and leads to XY sex reversal. Approximately 10–15% of sporadic XY gonadal dysgenesis cases show perturbations of SRY (8,9). The mutation typically usually involves the HMG box (10,11).

**88.2.5.2 X-Linked XY Gonadal Dysgenesis.** XY gonadal dysgenesis may also segregate in the fashion expected of an X-linked recessive or sex-limited autosomal dominant disorder (1,10,101–104). If on the X, the locus involved appears distinct from DAX1. Contemporary genome-wide molecular studies have not been conducted in order to determine if autosomal regions are perturbed.

**88.2.5.3 DAX1 Duplication.** XY sex reversal may be due to duplication of an X-linked gene initially called dose-sensitive sex reversal region and now DAX1. The locus lies on Xp21. One of 27 “46,XY sex reversal women” studied by Bardoni and colleagues (34) showed duplication of Xp21.2622.1. DAX1 is upregulated by Wnt-4. Overexpression presumably occurred in a case in which a 46,XY female had 1p duplication (33).

**88.2.5.4 MAP3K1.** Pearlman et al. (105) reported mutations in MAP3K1 in two families each showing a range of abnormal sexual phenotypes in XY individuals. Phenotypes included not only XY sex reversal (female)

but also phenotypic males with simple hypospadias. In one family, a splice acceptor mutation (c.634-8T>A) was segregating and in the second missense Gly616Arg. Of 11 sporadic XY females surveyed for MAP3K1 mutations, two missense mutations were found. It was speculated that the disparate phenotypes indicated that MAPK directs the balance between male-determining loci (SOX9 and FGF9) and female-determining loci (WNT4 and CTNNB1, the latter upregulating FOXL2).

**88.2.5.5 CBX2/M33.** An extraordinary 46,XY individual having ovaries (oocytes), uterus and female external genitalia was reported by Biason-Lauber (95). Compound heterozygosity existed for CBX2, a gene located on 17q25.3 and homologous to M33. In mice, Cbx2/M33 suppresses Hox genes during embryogenesis. Given that HOX genes are not typically invoked as pivotal for gonadal differentiation, the relationship between the observed phenotype and mutation is still tenuous. Half of XY mice undergoing M33 knockouts show female phenotype, as did the sole human case.

**88.2.5.6 Desert Hedge Hog.** Desert Hedge Hog is a signaling protein localized in 12q12 → 13. Umehara and coworkers (106) studied a 46,XY phenotypic woman with neuropathy and female external genitalia; it was stated that there was a “blinded vagina with an immature uterus.” A testis was present on one side and a streak gonad on the other. A diagnosis of “partial gonadal dysgenesis” was made, but this author would apply the term mixed gonadal dysgenesis. Irrespective of this, a homozygous mutation was found at the initiating codon of exon 1, resulting in no gene product.

**88.2.5.7 SF1 (NR5A1) Deficiency of Steroidogenic Factor-1 (SF-1) (NR5A1).** Steroidogenic factor-1 (SF-1) plays a potentially pivotal role in the hypothalamic–pituitary–gonadal axis. The Sf-1 gene product is encoded by FTZ1, located on human 9q33. FTZ1 has two zinc finger motif proteins. The first human case of presumptive SF-1 deficiency due to mutant FTZ-1 was reported by Achermann and coworkers (107). A 46,XY phenotypic woman showed primary adrenal failure, female external genitalia, streak gonads and normal Müllerian derivatives responsive to hormones. The proband proved heterozygous for a 2-bp substitution at codon 35 (G35E) in SF-1. The assumption of compound heterozygosity was made, although a second mutation could not be detected; SRY, steroidogenic acute regulatory (StAR) protein, and DAX1 were normal. That the mutated amino acid involved glycine in the last amino acid of the first zinc finger of SF-1 suggests disturbance of a DNA-binding site.

The second XY SF-1 sex reversal case involving SF1 was homozygous for Arg92Gln (108). Two normal family members were heterozygous. A further case (109) had adrenal insufficiency and seemingly normal ovarian differentiation showing that a Y chromosome does not obligatorily prevent ovarian development. Of clinical significance, a uterus is present in genetic males (46,XY)



with SF-1 deficiency, forming the basis for believing that SF-1 regulates repression of AMH. SF-1 heterozygous mutations have been observed in XY sex reversal or genital ambiguity cases who do not show adrenal insufficiency (110a,111a,112a).

**88.2.5.8 GXY Gonadal Dysgenesis and Campomelic Dysplasia (SOX9).** XY gonadal dysgenesis is associated, we have noted, with campomelic dysplasia (CPD) and SOX9. CPD is caused by haploinsufficiency of SOX9 and presents clinically as a de novo autosomal dominant mutation. The perturbation may be missense or nonsense mutations or deletions and sometimes visible chromosomal abnormalities (113). About 75% of XY cases are sex-reversed females. However, perturbations of SOX9 need not manifest solely as XY sex reversal, nor do all SOX9 mutations causing XY sex reversal have characteristic skeletal anomalies (114). (If bowing of long bones—campomelia—is not present, the term acampomelia is applied.) The variable phenotype presumably reflects existence of modifying genes, loss of a contiguous gene or mutations elsewhere in the 1-Mb SOX9 promoter region (115).

**88.2.5.9 XY Gonadal Dysgenesis and Wilms' Tumor Oncogenes (WT1).** Recognition that perturbations of WT-1 (Wilms' tumor oncogene), a gene on 11p13, were relevant to sex differentiation began when the association of nephropathy, genital ambiguity, and Wilms' tumor was observed in a male child. This constellation of features became known as Denys-Drash syndrome (116). Mental retardation, aniridia, and Wilms' tumor were then shown to be associated with deletions of 11p13, the location of the WT1 gene (117). The molecular basis is most often a heterozygous mutation in exons encoding a zinc finger motif (118). The mutant protein is believed to exert a dominant-negative effect given manifestation in heterozygotes.

In Denys-Drash syndrome, phenotypic males show genital ambiguity (male pseudohermaphroditism); however, a few individuals are 46,XY women (sex-reversed). Gonadal development thus ranges from streak gonads through dysgenetic testes to 46,XY ovotesticular DSD (true hermaphroditism) (119). Mueller (118) stated that half the phenotypic females with WT1 mutations were actually 46,XY. The manner by which mutations of WT1 produce genital abnormalities is unclear, but presumably related to WT1 having many isoforms (8) and certain molecular peculiarities. These include alternative translation initiation sites, alternative differential splicing in exon 5 and alternative donor-acceptor sites at the exon 9/intron 9 border. The latter results in differential splicing of the three amino acids lysine (K), serine (S), threonine (T), or KTS (120,121). The ratio of KTS (+) and KTS (–) transcripts differs in testes and ovary (122). This same pathway appears to be involved in Fraiser syndrome (119), characterized by a renal parenchymal disease in addition to XY sex reversal. In Frasier syndrome, the mechanism

of gene action involves an imbalance of KST+ and KST– transcripts (123).

WT1 is believed to act upstream of SRY. In mice, the WT1 null mutation results in failure of either males (46,XY) or females (46,XX) to develop gonads (and kidneys) (124). Both SRY and WT-1 are found in the testes, but WT-1 is expressed earlier. Thus, WT-1 is required upstream of SRY (i.e. before SRY is expressed) (8).

**88.2.5.10 XY Gonadal Dysgenesis and Alpha Thalassemia X Chromosome.** ATX (alpha thalassemia X chromosome) is a member of the DNA helicase family (125,126). Localized to Xq12–q21.31, mutation of ATX causes mental retardation,  $\alpha$ -thalassemia, abnormal facies (e.g. upturned nose, carp-shaped mouth), and male pseudohermaphroditism. The molecular perturbation generally is a truncated protein (127,128). The genital phenotype may be hypospadias or micropenis, or it may extend to sex reversal (female external genitalia) (125–127,129,130). Lack of Müllerian derivatives suggests that AMH was expressed early in embryogenesis prior to when embryonic testes failed to persist.

**88.2.5.11 XY Sex Reversal and Autosomal Deletions (2q, 9p, 10q).** In considering the genetics of sex determination, we have already alluded to several other autosomal regions that deleteriously affect male sex differentiation if deleted. Best known is 9p, deletion of which has on multiple occasions produced XY gonadal dysgenesis (20–26,131). 9p24.3 contains a domain homologous to key sex-determining genes in *Caenorhabditis elegans* (mab3) and *D. melanogaster* (doublesex or dsx). The human locus is dmrt1 (doublesex and mab3-related transcription factor 1). Calvari and colleagues (132) concluded that del(9)(p24.3) was a frequent cause of sry-positive 46,XY gonadal dysgenesis; of 11 deletions, 3 showed complete deletion of one “dmrt1” allele. Raymond and coworkers (133) found far fewer 9p deletions, even when investigating two 9p24.3 domains (dmrt1 and dmrt2). In sequencing 87 unexplained XY sex reversal patients, only 1 mutation was found in dmrt1 and 0 in dmrt2. A later study by the same group found no deletions using a dmrt1 fish probe (134). It was postulated that both dmrt1 and dmrt2 must be deleted to cause XY sex reversal (133). Necessity for two genes implies a quantitative threshold for dmrt gene activity, only below which testicular differentiation is adversely affected. This would imply a variable phenotype, which indeed occurs in del(9) (26).

Slavotinek and coworkers (29) reported sex reversal associated with deletion of 2q33. Waggoner and colleagues (135) reported sex reversal associated with deletion of 10q26, the chromosome on which SF-1 is located.

**88.2.5.12 Leydig Cell Hypoplasia LHR.** In the absence of Leydig cells, 46,XY individuals have female external genitalia, no uterus, and bilateral testes devoid of Leydig cells. Epididymides and vasa deferentia are present, and serum LH is elevated. Affected siblings have been reported and prenatal consanguinity observed. Thus,

autosomal recessive inheritance was accepted long before the LHR gene was localized to 2q. In 46,XY cases, Leydig cells presumably fail to develop because LH cannot exert its normal effect during embryogenesis. Embryonic testes presumably still secrete AMH, explaining the absence of a uterus.

The LHR gene consists of 11 exons and 699 amino acid residues. These exons comprise intracellular domains, intracellular and extracellular loops, transmembrane domains, and extracellular domains. Inactivating mutations lead to complete resistance to LH and XY women, whereas partial resistance leads to men with a small penis or hypospadias (136).

Approximately 12 different LHR mutations have been found in 46,XY women (137). Kremer and coworkers (138) reported two siblings of consanguineous parents having homozygosity for a missense mutation (codon 593). Deletions, point mutations, and stop codons have also been recognized (136,139). Sinha (140) reported homozygous in-frame 27-bp insertion in exon 1 in two 46,XY phenotypic female sibs. Given that in several others this mutation has been present in compound heterozygosity, it is likely to be nonfunctional.

46,XX cases are typically detected in families in which their 46,XY sibs who are female in appearance present with primary amenorrhea.

Of additional interest, activating LHR mutations cause precocious puberty in boys, but activating LHR receptor mutations do not exert the same effect in girls.

**88.2.5.13 Germ Cell Failure in Both Sexes (XY and 46,XX).** In several sibships, both males (46,XY) and females (46,XX) have shown germ cell failure. No other organ systems were affected with regard to differentiation. Affected females show streak gonads, whereas males show germ cell aplasia (Sertoli cell only syndrome). In two families, parents were consanguineous, and in neither were somatic anomalies observed (141,142).

These families demonstrate that a single autosomal gene may be capable of deleteriously affecting germ cell development in both sexes, presumably acting at a site common to early germ cell development (e.g. migration) or to the genital ridge. Several of the genes expressed in the genital ridge (see Genetic Control of Sexual Differentiation) could be candidates. Disturbances involving migrating PGCs are another possibility (See Figure 88-1).

In some families, germ cell absence in both 46,XY and 46,XX sibs coexist with distinctive patterns of somatic anomalies. It is reasonable to assume that these families display a different disorder. Al Awadi and coworkers (143) reported germ cell failure and an unusual form of alopecia. Scalp hair persisted in the midline, but no hair was present on sides (“manelike”). Mikati and colleagues (144) reported germ cell failure, microcephaly, short stature, mental retardation, and unusual facies (e.g. synophrys, abnormal pinnae, micrognathia, and loss of teeth). The sibs reported by Al Awadi and coworkers (143) were Jordanian; those reported by Mikati and

colleagues (144) were Lebanese. In both families, parents were consanguineous.

**88.2.5.14 46,XY Gonadal Regression or Testicular Regression (Agonadia) (46,XY).** In this condition, historically labeled agonadia, gonads are completely absent, external genitalia are abnormal but usually female-like, and no more than rudimentary Müllerian or Wolffian derivatives present. External genitalia usually consist of a phallus about the size of a clitoris, underdeveloped labia majora, and often nearly complete labioscrotal fusion. Less often, external genitalia are nearly female in appearance. Approximately half show somatic anomalies: craniofacial, vertebral, and mental retardation (121).

Given the usually observed 46,XY complement, pathogenic explanations have focused on loss of testes early in embryogenesis. In 46,XY individuals, this must take into account not only absence of gonads (presumptively testes) but also abnormal external genitalia and lack of internal genital ducts. The most frequent hypothesis is transient presence of fetal testes (hence testicular regression), sufficiently long to initiate male differentiation and suppress Müllerian differentiation but not sufficiently long to complete male differentiation. Consistent with this hypothesis, SRY is present in XY gonads.

46,XX cases have been described in which the phenotype seems similar—absent gonads, abnormal external genitalia, rudimentary Müllerian or Wolffian derivatives (145). Invoking testicular regression is obviously not logical in 46,XX cases. One plausible hypothesis is defective connective tissue (field defect). Given existence of both heritable tendencies (146) and frequent coexistence of somatic anomalies, such a hypothesis seems reasonable. This explanation applies also to 46,XY cases having somatic anomalies. Indeed, 46,XY agonadia has been observed in the CHARGE association (147). In one case (148), heterozygous SF-1 mutation was reported.

#### 88.2.5.15 Other Malformation Syndromes.

Table 88-5 lists other malformation syndromes that show XY sex reversal. These syndromes include XY gonadal dysgenesis and ectodermal anomalies (149); XY gonadal dysgenesis in the genital–palato–cardiac (Gardner–Silengo–Wachtel) syndrome (150); and XY gonadal dysgenesis, spastic paraplegia, optic atrophy, and microcephaly (151).

### 88.2.6 DSD Causing 46,XY or 45,X/46,XY Genital Ambiguity (Male Pseudohermaphroditism) (46,XY DSD or Mixed Gonadal Dysgenesis)

If individuals with a Y chromosome have external genitalia that fail to develop as expected for normal males, a variety of disorders could be responsible. The term 46,XY DSD or male pseudohermaphroditism is generally applied. In the disorders discussed in this section, external genitalia are usually sufficiently ambiguous

**TABLE 88-2 Malformation Syndromes in Which Male Pseudohermaphroditism (46,XY DSD) Is One Component**

Syndrome	OMIM Number	Prominent Features	Etiology
Ablepharon-macrostomia	200110	Absent eyelids, eyebrows, eyelashes, external ears; fusion defects of the mouth; ambiguous genitalia; absent or rudimentary nipples; parchment-like skin; delayed development of expressive language	Autosomal recessive
Aniridia–Wilms' tumor association	194070	Moderate to severe mental deficiency, growth deficiency, microcephaly, aniridia, nystagmus, ptosis, blindness, Wilms' tumor, ambiguous genitalia, gonadoblastoma	Chromosomal or autosomal dominant
Antley–Bixter	210750	Craniosynostoses, synostoses, radius, and humerus, Bowing of ulna and femur, arachnodactyly, joint contractures	Autosomal recessive? (P450 oxidoreductase deficiency)
Asplenia, cardiovascular	208530	Hypoplasia or aplasia of the spleen complex cardiac malformations, abnormal lung lobulation, anomalous position and development of the abdominal organs, agenesis of corpus callosum, imperforate anus, ambiguous genitalia, contractures of the lower limb	Autosomal recessive
Beemer	209970	Hydrocephalus, dense bones, cardiac malformation, bulbous nose, broad nasal bridge, ambiguous genitalia	Autosomal recessive
Jacobsen	147791	Trigonocephaly, flat and broad nasal bridge, micrognathia, carp mouth, hypertelorism, low-set ears, severe congenital heart disease, anomalies of limbs, external genitalia	Autosomal dominant or Chromosomal
Denys–Drash	194080	Wilms' tumor, nephropathy, ambiguous genitalia with 46,XY karyotype	Unknown
Fraser	219000	Cryptophthalmia, defect of auricle, hair growth on lateral forehead to lateral eyebrow, hypoplastic nares, mental deficiency, partial cutaneous syndactyly, urogenital malformation	Autosomal recessive
Lethal acrodysgenital dysplasia	270400	Failure to thrive, facial dysmorphism, ambiguous genitalia, syndactyly, postaxial polydactyly, Hirschprung disease, cardiac and renal malformations	Autosomal recessive
Rutledge	270400	Joint contractures, cerebellar hypoplasia, renal hypoplasia, ambiguous genitalia, urologic anomalies, tongue cysts, shortness of limbs, eye abnormalities, heart defects, gallbladder agenesis, ear malformations	Autosomal recessive
SCARF	312830	Skeletal abnormalities, cutis laxa, craniosynostosis, ambiguous genitalia, psychomotor retardation, facial abnormalities	Uncertain
Short rib polydactyly, Majewski-type	263520	Short stature; short limbs; cleft lip and palate; ear anomalies; limb anomalies, including preaxial and postaxial polysyndactyly; narrow thorax; short horizontal ribs; high clavicles; ambiguous genitalia	Autosomal recessive
SLO	270400	Microcephaly, mental retardation, hypotonia, ambiguous genitalia, abnormal facies	Autosomal recessive (deficiency 7-OH cholesterol dehydrogenase)
Trimethadione, teratogenicity	N/A	Mental deficiency, speech disorders, prenatal onset growth deficiency, brachycephaly, midfacial hypoplasia, broad and upturned nose, prominent forehead, eye anomalies, cleft lip and palate, cardiac defects, ambiguous genitalia	Teratogenicity
VATER association	192350	Vertebral, anal, tracheoesophageal, and renal; anomalies, subjects with ambiguous genitalia as part of the cloacal anomalies	Unknown (if valid entity); alleged progestational teratogenicity unproved

Updated from Simpson, J. L.; Elias, S. *Genetics in Obstetrics and Gynecology*, 3rd ed.; WB Saunders: Philadelphia, 2003.

**TABLE 88-3 Autosomal Genes Causing XX Ovarian Dysgenesis/Premature Ovarian Failure**

FSH $\beta$ deficiency
Inactivating FSHR
Inactivating LHR
Inhibin A (INH A)
17 $\alpha$ -Hydroxylase/17,20 desmolase deficiency (CYP17)
Progesterone receptor membrane component 1
Forkhead transcription homeobox (FOXL2)/ Blepharophimosis-ptosis-epicanthus
NOBOX
Growth differentiation factor 9 (GDF9)
BMPR1B
FIGLA
POU5F1
PTH-responsive B1 (PTHB1)
ADAMTS
Fragile X syndrome (CGG <sup>N</sup> )
Myotonic dystrophy (DM)
Galactosemia (GALT)
Carbohydrate-deficient glycoprotein (GD9) due to phosphomannomutase deficiency (PMM2)
Autoimmune regulation/autoimmune polyendocrinopathy– candidiasis–ectodermal dystrophy (AIRE/APECED)
Ovarian leukodystrophy (eukaryotic translation initiation factor EIF2B)
Symphangism and Noggin (NOG)
Perrault syndrome
Cerebellar ataxia with XX ovarian dysgenesis
FOXO3A
FOXO1A
LIM DNA-binding proteins (LHX8)
NANOS3
G-protein receptor 3 (GPR3)
KIT
RET finger protein-like 4
MSH5 and DMC1
AMH and AMHR type II
Germ cell failure in both sexes
46,XX gonadal regression (agonadia)
Other nuclear genes
Mitochondrial genes
Malformation syndromes with XX gonadal dysgenesis

to generate confusion concerning the sex of rearing, and, hence, the term is not applied typically to simple hypospadias.

**88.2.6.1 Teratogenic Forms.** If given in sufficiently high doses in the first trimester to a woman pregnant carrying a male fetus, certain drugs would be expected to interfere with male genital development. These include cyproterone acetate, flutamide, and finasteride. The mechanism of action of cyproterone involves blocking the androgen receptor (AR), as does flutamide to a lesser extent. A third drug is finasteride, which inhibits 5 $\alpha$ -reductase. Potential consequences of perturbing these functions are obvious—46,XY DSD or even complete sex reversal. However, case reports apparently do not

exist. Still, these drugs are approved in various countries for treatment of hirsutism or even contraception, so the potential for teratogenic male pseudohermaphroditism exists. Absence of case reports may reflect uncommon exposures in pregnant women or absence of effect due to low-exposure doses.

Controversy persists concerning whether administration of progestins or progesterones during pregnancy can produce hypospadias. This author's opinion is that evidence is considerably against these agents adversely affecting genital development (152,153). Certainly in doses resulting from inadvertent exposure to progestins in hormonal contraception, this is the case.

**88.2.6.2 Mixed or Partial Gonadal Dysgenesis (45,X/46,XY Mosaicism).** A variety of phenotypes are associated with 45,XY/46,XY mosaicism ranging from almost normal men with cryptorchidism or penile hypospadias to those with genital ambiguity to those who are phenotypically normal women (1,154). Based on cohort studies of 45,X/46,XY mosaicism unexpectedly detected in utero (prenatal genetic diagnosis) in unbiased fashion, 90% of cases with this mosaicism are normal males (155). The phenotype of cases ascertained postnatally differs from that of those ascertained prenatally. Differing phenotypes are presumed to reflect differing tissue distributions of the various cell lines, but this is still not unproved. The Y chromosome may be structurally unstable (e.g. dicentric), as a result of which a 45,X line may arise secondarily following loss of a structurally abnormal Y.

**88.2.6.2.1 45,X/46,XY Unambiguous Female External Genitalia.** 45,X/46,XY individuals may be short, have Turner stigmata and thus be clinically indistinguishable from 45,X individuals. Other 45,X/46,XY cases are normal in stature and show no somatic anomalies. As in other types of gonadal dysgenesis, external genitalia, vagina, and Müllerian derivatives remain unstimulated because of the lack of embryonic sex steroids. Breasts fail to develop; pubic and/or axillary hairs are scanty. If breast development occurs in a 45,X/46,XY individual, an estrogen-secreting tumor such as gonadoblastoma or dysgerminoma should be suspected (156). Virilization has been reported and is speculated to be on the basis of gonadotropin stimulation and androgen-producing interstitial cells in streak gonads (157).

Although streak gonads of 45,X/46,XY individuals may be histologically indistinguishable from those of 45,X individuals, gonadoblastomas or dysgerminomas develop in about 15–20% of 45,X/46,XY individuals (98,156). As noted earlier, the GBY locus (GBY chromosome) on Yq predisposes to neoplasia in phenotypic XY women if not deleted by a chromosomal rearrangement. If GBY is deleted, the risk of neoplasia is not apparently increased in XY sex-reversed females (158).

Gonadal extirpation is recommended for all 45,X/46,XY individuals having female external genitalia,



**TABLE 88-4 Mendelian Disorders Associated with Ovarian Failure**

Disorder	OMIM Number	Somatic Features	Ovarian Anomalies	Etiology
Cockayne syndrome	216400	Dwarfism, microcephaly, mental retardation, pigmentary retinopathy senility. Sensitivity to ultraviolet light	Ovarian atrophy and fibrosis	Autosomal recessive and photosensitivity, premature
Martsolf syndrome		Short stature, microbrachycephaly, cataracts, abnormal facies with relative prognathism due to maxillary hypoplasia	"Primary hypogonadism"	Autosomal recessive
Nijmegen syndrome	251260	Chromosomal instability, immunodeficiency, hypersensitivity to ionizing radiation, malignancy	Ovarian failure (primary)	Autosomal recessive (7;14 rearrangement)
Werner syndrome	277700	Short stature, premature senility, skin changes (scleroderma)	Ovarian failure	Autosomal recessive
Rothmund–Thompson syndrome	268400	Skin abnormalities (telangiectasia, erythema, irregular pigmentation), short stature, cataracts, sparse hair, small hands and feet, mental retardation, osteosarcoma	Ovarian failure (primary hypogonadism or delayed puberty)	Autosomal recessive
Carbohydrate-deficient glycoprotein syndrome, type 1 (phosphomannomutase deficiency)		Neurologic abnormalities (e.g., unscheduled eye movements), ataxia, hypotonia/hyporeflexia strokes, joint contractures	Ovarian failure (hypogonadism) (287)	Autosomal recessive
Ataxia telangiectasia	208900	Cerebellar ataxia, multiple telangiectasias (eyes, ears, flexor surface of extremities), immunodeficiency, chromosomal breakage, malignancy, X-ray hypersensitivity	"Complete absence of ovaries," "absence of primary follicles"	Autosomal recessive
Bloom syndrome	210900	Dolichocephaly, growth deficiency, sun-sensitive facial erythema, chromosomal instability (increased sister chromatid exchange), increased malignancy	Ovarian failure	Autosomal recessive

Updated from Simpson, J. L.; Elias, S. *Genetics in Obstetrics and Gynecology*, 3rd ed.; WB Saunders: Philadelphia, 2003.

probably arguably even if GBY is absent. Neoplasia may develop in the first or second decade of life; thus, surgery should not await expected puberty. If breast development and pubarche are present, one should assume a hormone-producing tumor (gonadoblastoma or dysgerminoma) because the expected streak gonads should not be hormone producing. Gonadectomy can usually be accomplished by laparoscopy (99,100). The uterus should be retained because pregnancy may be desired through donor oocytes or donor embryos.

**88.2.6.2.2 45,X/46,XY Ambiguous External Genitalia.** In individuals who have ambiguous external genitalia and a 45,X/46,XY complement, a uterus is usually (90%) present. Many investigators believe that the phenotype is invariably associated with 45,X/46,XY mosaicism, although only 45,X or only 46,XY cells may be demonstrable. The term asymmetric or mixed gonadal dysgenesis is applied to individuals having one streak gonad and one dysgenetic testis.

The presence of a uterus is an important diagnostic sign because that organ is absent in almost all genetic (Mendelian) forms of male pseudohermaphroditism (46,XY DSD) (see later). If an individual has ambiguous external genitalia, bilateral testes, and a uterus, it is reasonable to infer that individual actually has 45,X/46,XY mosaicism, irrespective of whether both cell lines can be demonstrated cytogenetically.

**88.2.6.2.3 45,X/46,XY with Nearly Normal Male External Genitalia.** 45,X/46,XY mosaicism may be detected in individuals with nearly normal male external genitalia. Given 90% of 45,X/46,XY fetuses ascertained at amniocentesis having a normal male phenotype at birth (159), this is the most common phenotype for this mosaicism. That 45,X/46,XY neonates with genital ambiguity are recognized more often simply reflects biases of ascertainment.

45,X/46,XY individuals having almost normal male external genitalia do not seem to develop neoplasia

**TABLE 88-5 The Spectrum of 46,XY Sex Reversal (XY Females)**

XY gonadal dysgenesis without somatic anomalies
– Perturbations of SRY (HMG box)
– Duplication Xp (DAX1)
– X-linked recessive/sex-limited autosomal dominant
– Forms without detectable molecular perturbation or heritability
XY gonadal dysgenesis and Wilms' tumor oncogene (WT-1)
– Denys–Drash syndrome
– Frasier syndrome
XY gonadal dysgenesis and campomelic dysplasia (SOX9)
XY gonadal dysgenesis/ATX chromosome
XY gonadal dysgenesis in other malformation syndromes
– Ectodermal anomalies (Brosnan)
– Genital–palato–cardiac (Gardner–Silengo–Wachtel)
– Spastic paraplegia–optic atrophy–microcephaly (Teebi)
XY gonadal dysgenesis with autosomal deletions
– Del (2a)
– Del (9p)
– Del (10q)
XY gonadal dysgenesis with autosomal duplications
– 1p
– 22q
Germ cell failure in both sexes (46,XY cases)
XY gonadal regression (Agonadia)
Steroid biosynthetic defects
– StAR deficiency
– 17 $\alpha$ -Hydroxylase/17,20 desmolase deficiency (CYP17)
– P450 oxidoreductase (POR) deficiency
– 3 $\beta$ -OI dehydrogenase/3 $\beta$ -hydroxysteroid dehydrogenase deficiency

as often as do 45,X/46,XY individuals with female or frankly ambiguous genitalia. Gonadal extirpation need not be necessary if a male sex of rearing is chosen, for gonads can be assessed periodically by ultrasound or palpation if within the scrotum (98).

**88.2.6.3 Genital Ambiguity or Sex Reversal Multiple Malformation Syndromes.** Genital ambiguity is a component of many multiple malformation syndromes (160), some of which have known enzymatic defects [e.g. Smith–Lemli–Opitz (SLO) syndrome (161) and Antley–Bixler syndrome (18a)]. Among the more common are the Meckel–Gruber syndrome (162), SLO (161) syndrome, brachioskeletal–genital syndrome (163), esophageal–facial–genital syndrome, and genitopalatocardiac syndrome (150) (Gardner–Silengo–Wachtel). These disorders are usually autosomal recessive or X-linked recessive (Table 88-2). The many other syndromes are associated only with cryptorchidism nonambiguous hypospadias (164) will not be discussed here.

In SLO syndrome, the mutant gene has disturbed conversion of 7-hydroxycholesterol to cholesterol (165,166). The most common molecular perturbation is a defect in exon-intronic splicing. 46,XY individuals show genital abnormalities (hypospadias or male

pseudohermaphroditism). In Ontario, the incidence was estimated at one per 22,700 among individuals of European ancestry (167). A broad phenotypic spectrum exists (19), and traditionally two clinical types have been differentiated. In type I, simple hypospadias is most common, whereas in type II, external genitalia may be female (sex reversal) (161). The feasibility of postnatal as well as prenatal treatment with a high-cholesterol diet is being explored. Given its low molecular weight, cholesterol crosses the placenta readily. During pregnancy, maternal serum estriol is typically very low to nondetectable (168), making detection during maternal serum analyte screening feasible. Presence of the novel compounds dehydro-estriol and dehydropregnanetriol in maternal urine also indicates a SLO fetus, because in normal pregnancies these compounds are undetectable (169).

Puffenberger et al. (170) reported TSPY-like (TSPYL) 1 mutations in XY males with sudden infant death, testicular dysgenesis, and genital ambiguity; TSPYL is involved in nucleosome assembly. 46,XX cases do not show sexual abnormalities.

In genitopalatocardiac (Gardner–Silengo–Wachtel) syndrome, 46,XY individuals show phenotypic variability, not only in external genitalia but also in gonads (150). Complete sex reversal can occur in 46,XY individuals, who may have ovaries. See Simpson (44) for further discussion.

**88.2.6.4 Disorders of Androgen Synthesis (Testosterone Biosynthetic Defects).** 46,XY DSD may result from deficiencies of various adrenal or gonadal enzymes: 3 $\beta$ -ol-dehydrogenase, the bifunctional enzyme 17 $\alpha$ -hydroxylase/17,20 desmolase, 17-ketosteroid reductase, and the enzymes necessary to convert cholesterol to pregnenolone (congenital adrenal lipid hyperplasia) (Figure 88-3). The common pathogenesis involves low testosterone, specifically levels inadequate to virilize external genitalia. Deficiencies of 21- or 11 $\beta$ -hydroxylase, the most common causes of female pseudohermaphroditism, do not cause male pseudohermaphroditism; males (46,XY) deficient of these enzyme in fact lead to precocious masculinization.

Adrenal biosynthetic defects should be suspected whenever levels of testosterone or its metabolites are decreased. Diagnosis may be difficult during infancy because neonatal testosterone levels are physiologically low. Provocative tests (e.g. human chorionic gonadotropin (hCG) stimulation) may be necessary.

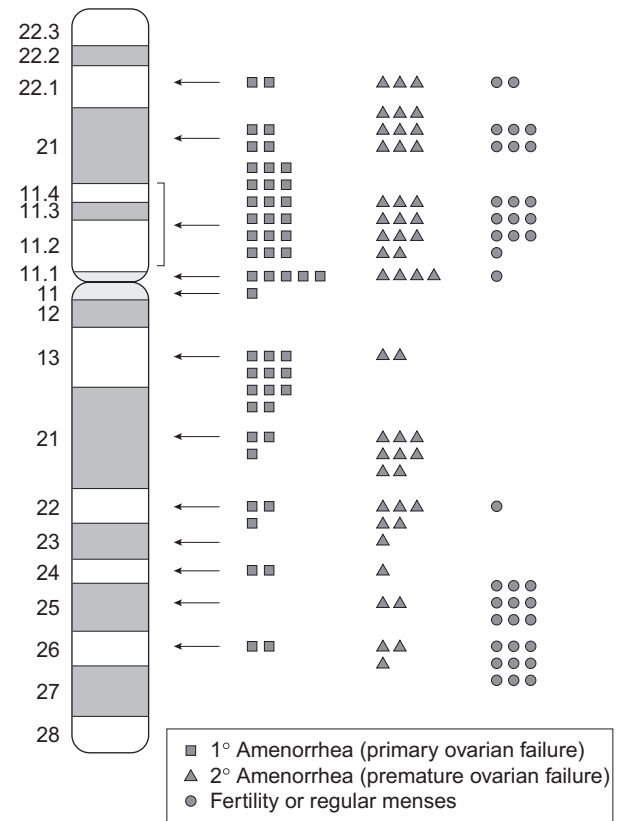
In a European cohort of 98 families with 46,XY DSD in which the proband had low testosterone (171), Morel et al. found molecular perturbations in 35 cases for HSD17 $\beta$ 3, 28 for HSD3 $\beta$ 2, 15 for StAR, 5 for CYP11A, 10 for CYP17, and 5 for POR. In only three cases could a molecular explanation not be found, one with 17 $\beta$ 3 deficiency and two with adrenal lipid hyperplasia. Genetic consequences of mutations involving each of these genes will be discussed below.

**88.2.6.4.1 Congenital Adrenal Lipoid Hyperplasia due to Deficiency of StAR.** In this autosomal recessive disorder (congenital adrenal lipoid hyperplasia), external genitalia are ambiguous or female like (see Chapter 87). Sodium wasting is severe, and adrenals are characterized by foamy appearing cells filled with cholesterol (172,173). Hyperpigmentation is observed, and respiratory distress common (25%) in neonates. Cholesterol accumulation has long been assumed to reflect inability of cholesterol to be converted to pregnenolone (see Figure 88-3). Levels of C18, C19, and C21 steroids are almost undetectable in plasma and urine. If targeted molecular diagnosis cannot be made, prenatal genetic diagnosis may still be possible on the basis of decreased levels of certain hormones in amniotic fluid (174).

Congenital adrenal lipoid hyperplasia in humans has usually proved to be the result of perturbation of the gene encoding StAR protein. The adjective “acute” reflects ability to respond rapidly (“acutely”) to corticotropin stimulation, specifically by producing a 30-kD mitochondrial protein in adrenal cells. The StAR protein facilitates entry of cholesterol into mitochondria, needed to accomplish the above function. Because StAR delivers precursors for cholesterol side chain cleavage, its perturbation has obvious downstream consequences on hormone action in gonads and adrenals. Salt wasting is severe. Mapped to 8p11.2, StAR spans 8kb and consists of seven exons (175). Many reported cases are of Japanese and Korean descent. In these populations, the mutant allele is most often (20%) a stop codon: Gln258X. In Arabs, the mutation is likely to be Arg182Leu. Different mutations have been found in other ethnic groups. Founder effects are presumably operative in these different ethnic groups (175). In Palestinians, del 201–202CT is the common explanation (88a).

**88.2.6.4.2 Congenital Adrenal Lipoid Hyperplasia due to P450scc Deficiency (CYP11A).** Congenital adrenal lipoid hyperplasia has been found in infants lacking a perturbation of StAR or SF-1. The defect here involves deficiency of P450scc, required for enzymatic steps by which cholesterol is cleaved to generate pregnenolone (Figure 88-3). Presumptive CYP11A deficiency with genital abnormalities was first found in a heterozygous mutation and of uncertain but plausible significance (93a,176a). Compound heterozygosity was later shown in two affected infants (93a,177a). Unlike StAR mutations, adrenal problems are not observed (salt wasting).

**88.2.6.4.3 Deficiency of 3 $\beta$ -Hydroxysteroid Dehydrogenase (3 $\beta$ -ol-Dehydrogenase Deficiency) (HSD3 $\beta$ 2).** In this enzyme deficiency, synthesis of both androgens and estrogens is decreased (see Figure 88-3). The major androgen produced is dihydroepiandrosterone (DHEA). A relatively weaker androgen than testosterone, DHEA alone is not capable of adequately virilizing the male fetus; thus, genital ambiguity occurs. The phallus is small, the urethral opening proximal on the penis, and labioscrotal fusion incomplete. Testes and Wolffian

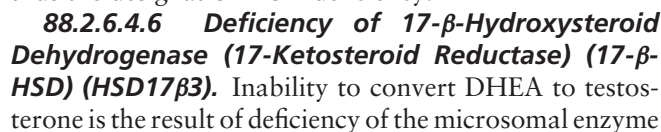


**FIGURE 88-3** Schematic diagram of the X chromosome showing ovarian function as a function of nonmosaic terminal deletion based on pooled data. In familial aggregates, all affected cases are included because their phenotypes are not always concordant. In some cases, patients are described as having POF, but no information is provided on fertility; in the absence of explicit information, it is assumed no pregnancy has occurred. In some younger patients (e.g. >14 years but <20–25 years), there has been little opportunity to demonstrate pregnancy, nor is there assurance regular menses will continue. Nonetheless, they are designated as having “regular menses/fertility.” (Data from Simpson, J. L.; Rajkovic, A. *Ovarian Differentiation and Gonadal Failure*. Am. J. Med. Genet. 1999, 89, 186–200.)

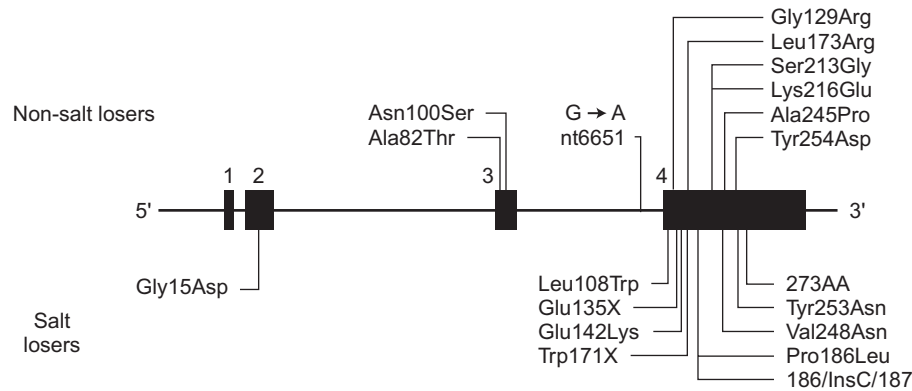
ducts differentiate normally. The diagnosis is usually established in neonates on the basis of disproportionately increased serum DHEA following ACTH stimulation. In addition to genital abnormalities, 3 $\beta$ -ol-dehydrogenase deficiency is associated with severe sodium wasting. This is predictable, given that both aldosterone and cortisol are decreased.

Of the five 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) genes, only type II is expressed in adrenal and gonads. External genitalia of males with type II 3 $\beta$ -HSD deficiency are thus incompletely developed. Mutations are scattered among the four exons, most commonly aggregated in the fourth exon (Figure 88-4) (178,179).

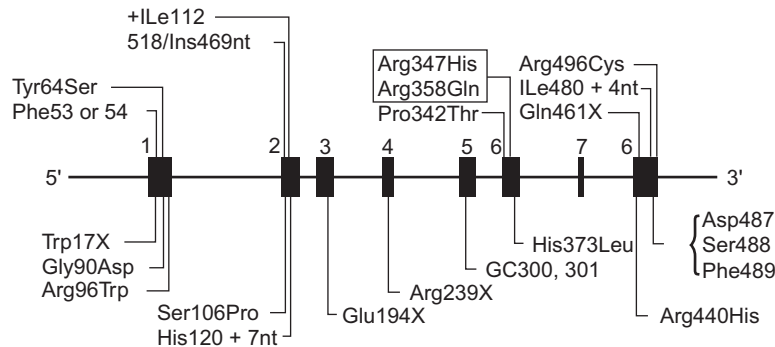
Like 46,XY cases, 46,XX individuals with this enzyme deficiency also show genital ambiguity with virilization due to elevated DHEA and androstenediol. Those androgens are relatively weaker than testosterone but nonetheless sufficiently potent to virilize.







**FIGURE 88-5** Diagram of the 3 $\beta$ -hydroxysteroid dehydrogenase type II (HSD3B2) gene with selected mutations that result in 3 $\beta$ -HSD deficiency. The numbered solid boxes indicate the exons. Missense mutations causing amino acid substitutions in the enzyme are indicated by the three-letter abbreviation for the wild-type amino acid, followed by the amino acid number in the enzyme and then the three-letter abbreviation for the substituted amino acid. X indicates a nonsense (stop) mutation. Mutations with less than 1% 3 $\beta$ -HSD activity are indicated below the gene and cause salt loss. Missense and splicing mutations, indicated above the genes, result in 2–4.7% enzymatic activity and are associated with the non-salt-losing phenotype. (Data from Grumbach, M. L.; Leuan, A. H.; Conte, F. A. *Disorders of Sex Differentiation*. In Williams' Textbook of Endocrinology, 10th ed.; WB Saunders: Philadelphia, 2003; pp 842–1002.)



**FIGURE 88-6** Diagram of selected mutations in the CYP17 gene (17 $\alpha$ -hydroxylase/17,20-lyase deficiency). The exons are the numbered black boxes. Missense mutations causing amino acid substitutions in the enzyme are indicated by the three-letter abbreviation for the wild-type amino acid, followed by the amino acid number in the enzyme and the three-letter abbreviation for the substituted amino acid. X indicates a nonsense (stop) mutation. All these mutations cause 17 $\alpha$ -hydroxylase deficiency. Missense mutations at codons 347 and 358 (indicated by the box) have been associated with "isolated" 17,20-lyase deficiency. (Data from Grumbach, M. L.; Leuan, A. H.; Conte, F. A. *Disorders of Sex Differentiation*. In Williams' Textbook of Endocrinology, 10th ed.; WB Saunders: Philadelphia, 2003; pp 842–1002.)

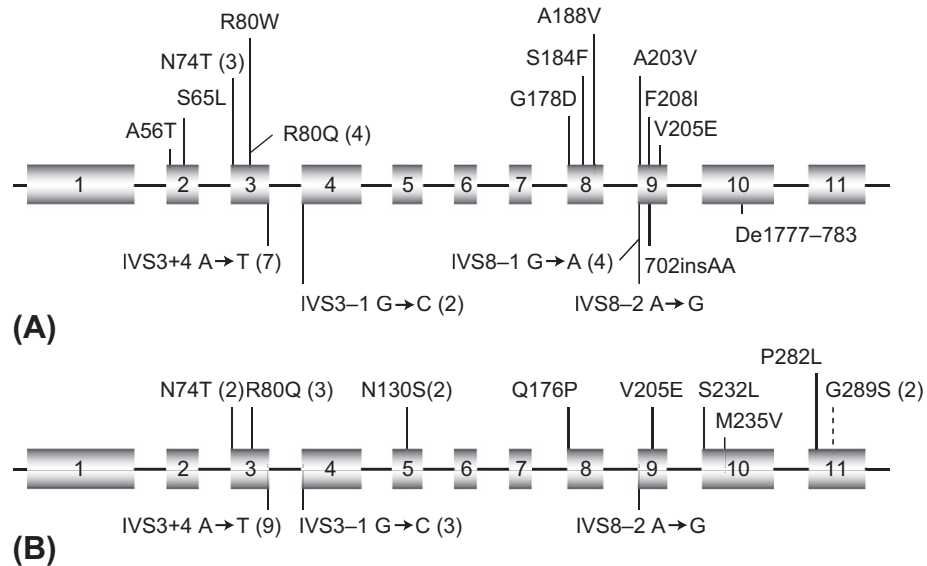
17-ketosteroid reductase, also called 17- $\beta$ -hydroxysteroid dehydrogenase or 17- $\beta$ -ol dehydrogenase (see Chapter 87). There are five 17- $\beta$ -HSD isozymes; deficiency due to HSD17 $\beta$ 3 mutations is causative in this condition. This enzyme reaction is reversible, the multiple designations connoting both oxidative and reductive steps.

Plasma testosterone is usually decreased, whereas androstenedione and DHEA are increased. Affected men show ambiguous or female-like external genitalia, bilateral testes, and Wolffian derivatives, but no Müllerian derivatives. Breast development may or may not be present, apparently depending upon the estrogen to testosterone ratio. Pubertal virilization may be greater than in other enzyme deficiencies, and sometimes gynecomastia is not even evident (191). This is considered the result of androstendione being converted to testosterone by 17- $\beta$ -HSD enzymes other than the 17- $\beta$ -HSD3 (HSD3) enzyme typically utilized. HSD3 deficiency shares with

5 $\alpha$ -reductase deficiency the characteristic of virilization at puberty. Inacio et al. (192a) reported that 3 of 11 cases in a Brazilian cohort chose female to male sex reversal at puberty.

Located on chromosome 9q22, 17HSD3 or HSD3 consists of 11 exons (Figure 88-6). The gene is micro-somal rather than mitochondrial, consistent with its action involving gonads rather than adrenals. Molecular perturbations typically involve single amino acid substitutions (193,194), especially exon 3. Also frequent are disruptions of the splice junction involving intron 3 and mutations in exons 8, 9 and 10. However, mutations encompass all exons (see Figure 88-6).

Among 87 cases reported in 2001, Simard and coworkers (179,195) tabulated 18 missense, 2 frameshift, and 4 splice junction mutations. Most missense mutations have no residual activity, but this is not true for the most common single mutation, a Arg80Gln or R80Q missense mutation



**FIGURE 88-7** Representation of the HSD17B3 mutations identified as of 2003 in homozygotes from 32 families (A) and compound heterozygotes from 14 families (B) suffering from Type III 17 $\beta$ -HSD/17-KSR deficiency. The location of the neutral polymorphism G2895 in the HSD17B3 gene is represented by a broken line. The number in parenthesis indicates the number of families in which a specific mutation has been found. (Data from Simard, J.; Moisan, A. M.; Michel, L. C.; et al. Males with 17 $\beta$ -Hydroxysteroid Dehydrogenase Deficiency. *Endocrinol.* 2003, 13, 195–200.)

in Gaza Arabs (196,197). This mutation, in exon 3, reduces enzyme activity to 20% of normal (179,193,195). The phallus is typically bound by chordee, and only 4–8 cm long. Gynecomastia is common. R80Q has also been found in Portuguese, Spanish, Dutch, and Brazilian patients, said to be consistent with its introduction by the Phoenicians.

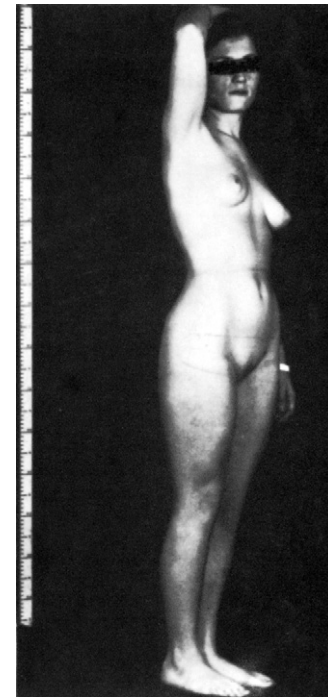
46,XX women with mutations at both alleles are asymptomatic (195), consistent with the 17- $\beta$ -HSD3 expression being limited to the testes.

#### 88.2.6.4.7 Deficiency of 5 $\alpha$ -Reductase (SRDA1).

Genetic men may show ambiguous external genitalia at birth but at puberty undergo virilization like normal men. They demonstrate phallic enlargement, increased facial hair, muscular hypertrophy, and voice deepening but no breast development. External genitalia consist of a phallus that resembles a clitoris more than a penis, and a perineal urethral orifice. Usually, there is a separate, blindly ending perineal orifice that resembles a vagina (pseudovagina) (Figure 88-10).

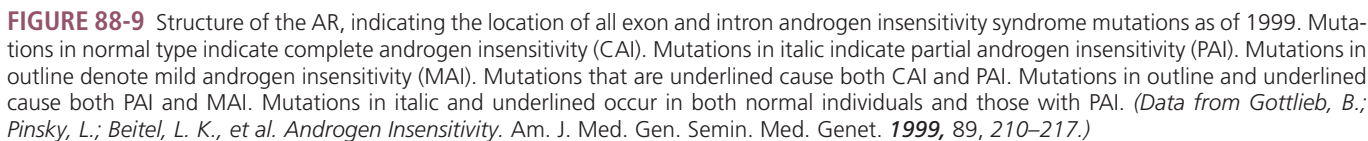
Once called pseudovaginal perineoscrotal hypospadias (PPSH), this disorder was shown in 1971 to be inherited in autosomal recessive fashion (198,199). The responsible enzyme was later shown to be 5 $\alpha$ -reductase (192,200,201), which converts testosterone (T) to DHT. That intracellular 5 $\alpha$ -reductase deficiency results in the PPSH phenotype is consistent with virilization of the external genitalia during embryogenesis requiring only DHT; Wolffian differentiation requires only testosterone. By contrast, pubertal virilization can be accomplished without DHT.

Two 5 $\alpha$ -reductase (SRD5) genes exist. Type I (SRD5A1) is located on chromosome 5; type II (SRD5A2) is located on 2p23. Only type II is expressed in gonads; thus, deficiency of only this product causes



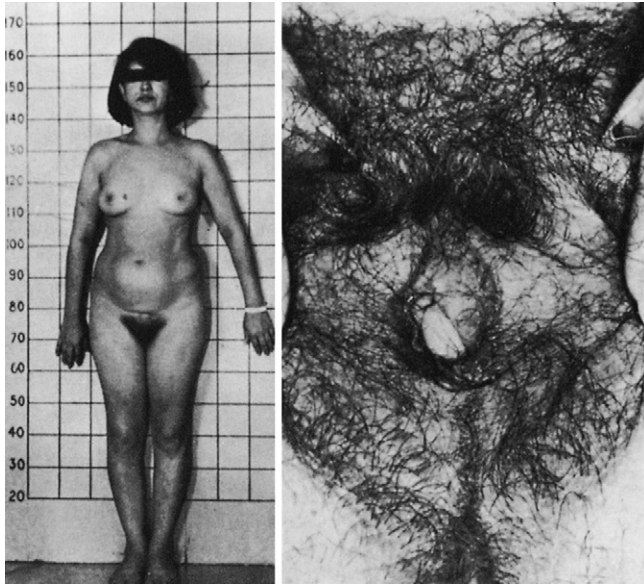
**FIGURE 88-8** Photograph of a 46,XY individual with complete androgen insensitivity. (From Simpson, J. L. *Male Hermaphroditism: Genetics and Clinical Delineation*. Hum. Genet. 1978, 44, 1–49.)

male pseudohermaphroditism. SRD5A2 consists of five exons (202). Missense mutations are common (203), and different ethnic groups typically show different mutations. These are scattered among the five exons, presumably reflecting founder effects (Figure 88-11). Within a given ethnic group, a sentinel mutation is usually observed, typically homozygous and presumably



46,XX women homozygous for SRD5A1 mutations are fertile and show normal ovarian function (207). Breast development is normal. Limb and pubic hair may be reduced, and menarche delayed.

**Syndrome.** In these forms of DSD, testosterone is produced but cellular response and androgen action are lacking. The result is that 46,XY individuals develop phenotypically as females. In complete androgen insensitivity syndrome (CAIS; complete testicular feminization), 46,XY individuals show bilateral testes, female external genitalia a blindly ending vagina, and no Müllerian derivatives (Figure 88-7). The incidence is one per 10,000–20,000. Clinical findings are predictable, given that the underlying pathogenesis is known to involve inability to respond to testosterone. Testosterone levels are in the range expected of 46,XY males. Testes synthesize estrogens in unimpeded fashion, their action not counterbalanced as usual in males by functionally active testosterone. Affected individuals manifest breast development and pubertal feminization. AMH is concurrently synthesized



**FIGURE 88-10** Photograph of a 46,XY individual with incomplete (partial) androgen insensitivity. (From Jones, H. W. Jr.; Park, I. J. *A Classification of Special Problems in Sex Differentiation*. Birth Defects Orig. Artic. Ser. 1971, 7 (6), 113–121.)

normally as predicted by Sertoli cells. The body likewise responds appropriately to AMH, as a result of which there is no uterus. LH is disproportionally increased, presumably reflecting hypothalamic unresponsiveness to testosterone.

In CAIS, some affected individuals may show excellent breast development, an often overemphasized description in textbooks (see [Figure 88-7](#)). Actually, most patients are similar in appearance to women in the general population. Breasts contain normal ductal and glandular tissue, but areole are often pale and underdeveloped. Pubic hair and axillary hair (terminal) are usually sparse (e.g. only vellus hair); scalp hair is normal. Presence of some pubic and axillary hair is usually less common than some axillary hair. This should not dissuade one from the diagnosis of CAIS. The vagina terminates blindly, foreshortened but still usually adequate for coitus. Occasionally, the vagina is only 1–2 cm long or represented merely by a dimple. Use of progressive dilators or even surgery to create a neovagina may be necessary. Neither uterus nor fallopian tubes are ordinarily present, but fibromuscular remnants, rudimentary fallopian tubes, or rarely, even a rudimentary uterus may persist.

Testes are usually normal in size. They may be located in the abdomen, inguinal canal, labia, or anywhere along the path of embryonic testicular descent. If present in the inguinal canal, testes may produce inguinal hernias. Height is slightly increased compared with normal women, but unremarkable compared to 46,XY males. In a Brazilian cohort (25 subjects) (208), adult heights ranged from  $-1.35$  to  $0.59$  SD using normal male standards, i.e. taller than 46,XX females and shorter than normal 46,XY males.



**FIGURE 88-11** Photographs of the external genitalia of an individual with PPSH. At puberty, phallic enlargement occurred and breast development did not. Individuals with this phenotype have  $5\alpha$ -reductase deficiency. (From Opitz, J. M.; Simpson, J. L.; Sarto, G. E.; et al. *Pseudovaginal Perineoscrotal Hypospadias*. Clin. Genet. 1972, 3, 1–26.)

The frequency of gonadal neoplasia is increased due to intra-abdominal location of testes, but before 25–30 years of age, the risk of malignancy is low. Orchiectomy is not necessarily indicated prior to spontaneous pubertal feminization. If herniorrhaphy is required before puberty, however, most surgeons perform orchiectomy simultaneously. Laparoscopy is preferable for removing inguinal testes, but open laparotomy is required for retroperitoneal testes. There may also be some psychological benefits to prepubertal orchiectomies. Benign tubular adenomas (Pick adenomas) are common in postpubertal patients, probably stimulated by increased LH secretion.

Some individuals with androgen insensitivity show clitoral enlargement and labioscrotal fusion; the term incomplete or partial androgen insensitivity (PAIS) (incomplete testicular feminization) is then applied. An even milder end of the spectrum consists of men manifesting only gynecomastia or only oligospermia/azoospermia (mild androgen insensitivity syndrome or MAIS). Complete, partial, and mild androgen insensitivity are all inherited in an X-linked recessive fashion and are due to different mutations of the same AR gene located on the X long arm.

**88.2.7.2 Partial Androgen Insensitivity and Mild Androgen Insensitivity.** At puberty, certain 46,XY individuals with androgen insensitivity feminize (i.e. breast development), but their external genitalia are characterized by phallic enlargement and partial labioscrotal fusion ([Figure 88-9](#)). Defined as partial or incomplete androgen insensitivity (PAIS) (or incomplete testicular feminization), the phenotype shares many features with CAIS: bilateral testes with Leydig cell hyperplasia, absence of Müllerian derivatives, pubertal breast development, lack of pubertal virilization, normal (male)



plasma testosterone, and failure to respond to androgen. Of historical note, PAIS encompasses several entities once considered to be distinct, erstwhile syndromes reported by Lubs and colleagues (209), Gilbert-Dreyfus and coworkers. Differences between these entities reflect molecular heterogeneity (164,210–212). However, family members with the same mutation have shown different sex of rearing have also been reported (213).

PAIS must be excluded before a male sex of rearing can be assigned because ability to respond to exogenous androgens is necessary. Identifying or excluding a specific molecular perturbation can be especially useful if the molecular defect in a relative is already known.

**88.2.7.3 Molecular Studies in CAIS and PAIS.** Perturbation of the AR gene located on Xq11 is responsible for the phenotypes described (CAIS and PAIS). As an X-linked recessive condition that is genetically lethal, one third of all cases would be predicted to be new (sporadic) mutations. Indeed, 8 of 30 (30%) patients studied by Hiort and coworkers (214) were the result of a new mutation. A few heterozygous women show decreased pubic hair and delayed puberty.

The gene is approximately 90,000bp long but codes for fewer than 2000 amino acids. There are eight exons (Figure 88-8). Exon 1 confers regulatory function. The DNA-binding domain of amino acids 552–616 is encoded by exon 2 and part of exon 3. The latter part of exon 3 and part of exon 4 encode bipartite nuclear localization (617–636 amino acids). The C-terminal region of 250 amino acids extends over exons 4–8 and constitutes the androgen-binding domain (211). The gene contains two regions of homopolymeric amino acid “repeats” of varying size: one polyglutamine (9–36 amino acids) and the other polyglycine (10–31 amino acids). Many different mutations have been reported (210,215), tabulated in a registry maintained at McGill University (e-mail: [mc33@musica.mcgill.ca](mailto:mc33@musica.mcgill.ca) or Website: <http://www.mcgill.ca and rogenb>).

Mutations can be found in most cases of CAIS, and in about 75% of PAIS (208). Exon 1 encompasses half of the AR gene, but has the fewest recognized mutations (10%). More mutations have been found in exons 2 and 3 (the DNA-binding domain). The phenotype may be either CAI or PAI. The most common sites of mutation lie in exons 5–8, the androgen-binding domain. Large deletions and mutations resulting in premature termination (stop codon) predictably cause CAIS (215,216). However, most mutations are missense mutations, and these may produce CAIS, PAIS, or MAIS (see below) in seemingly random fashion. Presumably, some point mutations are compatible with production of limited quantities of functional AR, although the receptor may be unstable or characterized by poor binding (216). That a given mutation may be associated with either PAIS or MAIS (12) could suggest modifying genes.

**88.2.7.4 Mild Androgen Insensitivity Syndrome.** In MAIS, phenotypic expression is usually limited to

impaired spermatogenesis. Gynecomastia, impotence, and poor virilization may also occur (217–219). Molecular/phenotypic correlation is imprecise, the same mutation being observed in either MAIS or PAIS (220).

### 88.2.8 Ovotesticular DSD (True Hermaphroditism)

In 46,XY, ovotesticular DSD cases have both ovarian and testicular tissues, and genital ambiguity is typical. This disorder probably accounts for fewer than 5% of all DSD cases, the exception occurring in South Africa where approximately half of all DSD cases (221) have this condition. Almost all cases in Southern Africa are black.

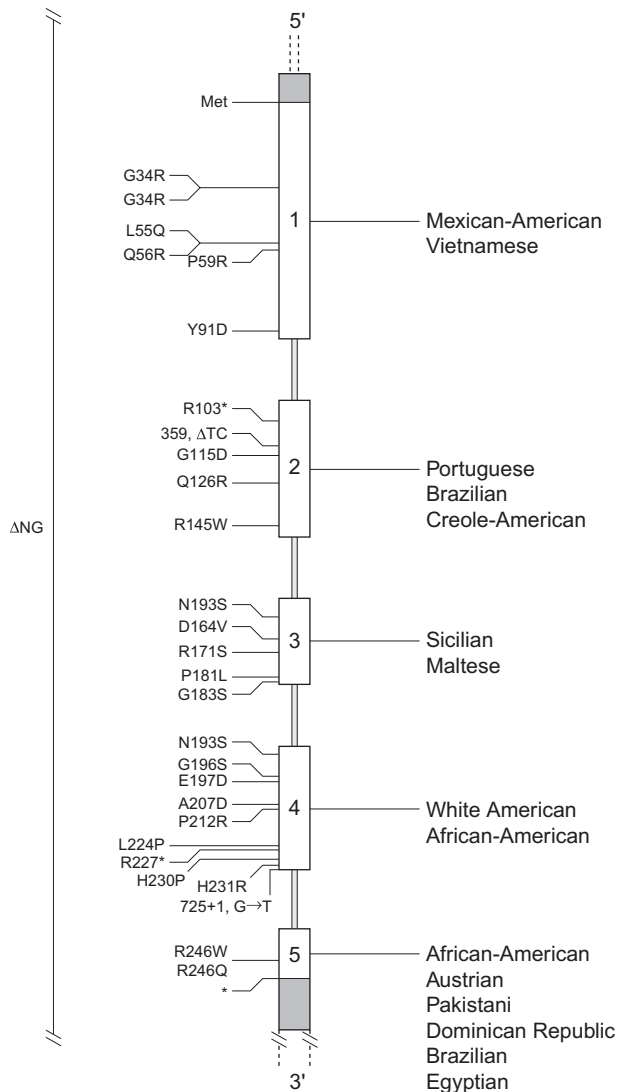
Most cases have a 46,XX chromosomal complement; however, 46,XX/46,XY, 46/XY, 46,XX/47,XXY and other complements exist (222–224).

Gonads may exist as a separate ovary and a separate testis or, more often, one or more ovotestes (222). Phenotype logically should reflect chromosomal constitution in mosaic (or chimeric) cases (224), but this has actually never been proved. Irrespective of this, it will suffice here to generalize concerning the overall phenotype of true hermaphrodites.

**88.2.8.1 Phenotype.** Before the era of routine medical intervention, two thirds of cases were raised as boys (1). Wiersma found that of 125 cases in South Africa reported in 2010, no patient showed normal male or female external genitalia (225). All except two were raised as males. Only 9 of 125 showed separate urethral and vaginal openings. Paradoxically, breast development was typical at puberty, even with predominantly male external genitalia. This may have reflected atrophy of testicular tissue due to intra-abdominal location.

As noted, gonads may consist of two ovotestes: one ovary and one contralateral testis or one ovotestis and a contralateral ovary or testis. Gonadal tissue may be located in the ovarian, inguinal, or labioscrotal regions. A testis or an ovotestis is more likely to be present on the right than on the left. Spermatozoa are rarely present (226); however, ostensibly normal oocytes may exist even in ovotestes. Gonads were palpable in only 59 of the 125 South African cases of Wiersma et al. (221,225) and of these 59 only 17 bilateral. In 66 cases, gonads were in the pelvis. Ovotestes are the predominant gonadal form in South Africa and these were almost always pelvic in location in the series of Wiersma and their tissue distribution was typically bipolar (ovarian cranial and testicular caudal), albeit with interdigitation. In 89% of ovotestes studied by Wiersma, a mantle of ovarian tissue surrounded a core containing stroma and intermingled ovarian and testicular tissue. The clinical significance of gonadal intermingling is that extirpating gonadal tissue of a single type is not simple surgically.

Van Niekerk, whose elegant book in 1974 analyzed a presumably different South African cohort, reported



**FIGURE 88-12** Mutations in the steroid 5 $\alpha$ -reductase 2 gene and protein. A schematic diagram of the 5 $\alpha$ -reductase gene is shown in the middle. On the left are the location of 28 different mutations, and the predominant ethnic groups on the right.  $\Delta$ NG (far left) represents the deletion of the gene in the New Guinea cohort. (Data from Wilson, J. D.; Griffin, J. E.; Russell, D. W. *Steroid 5 $\alpha$ -Reductase 2 Deficiency*. *Endocr. Rev.* 1993, 14, 577–593.)

at the time that the greater the proportion of testicular tissue in an ovotestis, the greater the likelihood of gonadal descent. In 80% of his cases, the testicular and ovarian components of ovotestes were juxtaposed end to end (Figure 88-12) (227), seemingly different from the recent experience of Wiersma. Van Niekerk collected cases from the region surrounding Pretoria, whereas Wiersma (221,225) is active in Durban. Thus, different tribal ancestry may exist. Van Niekerk noted that ovotestis may be detectable by inspection or palpation; testicular tissue is softer and darker than ovarian tissue. Ultrasound or magnetic resonance imaging (MRI) is now obligatory if the undesired portion of an ovotestis is to be extirpated.

Gonadal neoplasia and breast carcinoma have been reported (98,156,224). Gonadal neoplasia may reflect risks associated with intra-abdominal location of testicular tissue. This is consistent with data of Kuhnle and coworkers (228), who estimated that neoplasia arises in 25% of 46,XY cases but in only 3% of 46,XX cases. The preponderance of gonads existing in the form of ovotestes in South Africa is less pronounced elsewhere. Vilain (229) stated that the distribution worldwide is 34% ovotestes/ovary, 29% ovotestes/ovotestes and 25% ovary/testes.

A uterus is usually (90%) present based on cases reported worldwide and based on Van Niekerk's sample (227). Sometimes, the uterus is bicornuate or unicornuate (224). However, in his South African series, Wiersma reported that only 73 of 125 had a "uterine structure." Absence of a uterine horn usually indicates ipsilateral testis or ovotestis. The fimbriated end of the fallopian tube is often occluded ipsilateral to an ovotestis. Squamous metaplasia of the endocervix may occur (227). Menstruation is common and may be manifested as cyclic hematuria. Presence of a uterus in true hermaphroditism is diagnostically useful, particularly in the rare 46,XY case. Of individuals with genital ambiguity having a Y chromosome, only 46,XY hermaphrodites and 45,X/46,XY mosaics have a uterus.

Approximately a dozen true hermaphrodites have become pregnant (230,231), usually (but not always) after removal of testicular tissue. Excluding a single 46,XX/46,XY case (232a), all pregnant individuals have been 46,XX. Their offspring seem no more likely to be abnormal than in the general population, although Kuhnle and colleagues (223) stated that all offspring had been boys.

Diagnosis is typically made when genital ambiguity is observed and only after male and female pseudohermaphroditism are excluded. If a female sex of rearing has been chosen, extensive surgery may or may not be necessary. If a male sex of rearing is chosen, genital reconstruction and selective gonadal extirpation are invariably indicated.

**88.2.8.2 Etiology.** 46,XX/46,XY true hermaphroditism is rare and usually caused by chimerism. The presumptive explanation is chimerism, the presence in a single individual of two or more cell lines by definition each derived from different zygotes. Some 46,XY cases may also be unrecognized chimeras (144). 46,XX/47,XXY individuals are probably more likely to have resulted from nondisjunction or anaphase lag.

In 46,XX true hermaphrodites, possible explanations include translocation during paternal meiosis of SRY from the Y to an X; translocation of SRY from the Y to an autosome; or activation (depression) of autosomal genes. The former can be excluded as uncommon because very few 46,XX cases receive DNA sequences from their father's Y (233). Rarity of X–Y interchange in true hermaphrodites contrasts with its frequent occurrence in

46,XX men, 80% of whom show SRY as a result of such an interchange. A few 46,XX ovotesticular cases do show a mutant SRY, and somatic mutation can result in gonadal mosaicism associated with true hermaphroditism (234).

Autosomal genes seem to be a more likely explanation for 46,XX true hermaphroditism. This hypothesis is supported by existence of families characterized by either multiple siblings with XX true hermaphroditism or families in which both 46,XX males and 46,XX true hermaphrodites occur in the same kindred. (For references, see prior section on 46,XX Males (XX Sex Reversal)). In these kindreds, the 46,XX men usually show genital ambiguity, unlike the typical 46,XX male who shows normal male external genitalia. Familial XX true hermaphrodites seem more likely to be characterized by bilateral ovotestes and absence of the uterus than nonfamilial true hermaphroditism (224); both gonads in familial true hermaphroditism tend to be morphologically similar and ovotestes. This suggests a central basis for gonadal perturbation (224). Given that differing gonadal composition per side logically should more likely be the result of chimerism. Derepression of a normally dormant autosomal gene conferring male differentiation could provide an explanation for unscheduled (testicular) gonadal development in 46,XX individuals. We have already observed instructive cases resulting in 46,XX sex reversal (female genotype to male phenotype) in which duplication of SOX9 has not been well studied in true hermaphrodites that causes 46,XX sex reversal in the absence of SRY.

### 88.2.9 46,XX Ovarian Dysgenesis/POF

Turner syndrome and monosomy X are discussed in detail elsewhere in this volume (Chapter 44). In this chapter, we have also alluded to the pathogenesis of germ cell failure in monosomy X and X-deletions. Here we shall consider mutant autosomal genes causing ovarian dysgenesis in 46,XX individuals (Table 88-3). The archetypal form of “XX gonadal dysgenesis” is characterized by streak gonads not associated with somatic anomalies. Inheritance has long been accepted as autosomal recessive (235). Affected individuals are normal in stature (103) and Turner stigmata are absent. However, “XX gonadal dysgenesis” is very heterogeneous, and this diagnosis will increasingly become more specific. At present, however, only about 10–20% of cases have a precise diagnosis. To a large extent, this reflects limited diagnostic evaluation, in particular failure to perform molecular studies or any evaluation beyond routine cytogenetics.

Of clinical relevance is frequent variable expressivity. In many families, one sibling has had bilateral streak gonads, whereas another ovarian hypoplasia yet not streak gonads per se (1,235–237). Any mutant gene responsible for “XX gonadal dysgenesis” that is capable of variable expression may thus be responsible for isolated cases of “POF.”

**88.2.9.1 FSH  $\beta$ .** FSH is composed of a unique  $\beta$  subunit and an  $\alpha$  subunit shared in common with thyroid-stimulating hormone, LH, and hCG. Cellular action requires a G-protein (GP) FSHR, the gene for which is present on chromosome 2.

Mutations in FSH- $\beta$  are rare, but reported. Two affected women showed neither thelarche nor the menarche. Matthews and colleagues (238) described a homozygous 2-bp deletion (GT) in exon 3 at codon 61. Layman and coworkers (239) reported a compound heterozygote: one allele was characterized by a deletion in exon 3, codon 61, whereas the other was a missense mutation in exon 3, codon 51.

**88.2.9.2 Inactivating FSHR.** FSHR mutations are common in Finland but rare elsewhere. In a landmark study, Aittomaki and colleagues (236,237) meticulously searched Finnish hospitals and cytogenetic laboratories to identify 75 women having 46,XX primary or secondary amenorrhea, based on serum FSH > 40 MIU/mL. The gene was first localized to chromosome 2p and then a missense mutation in exon 7 of FSHR (C566T or Ala566 Val) found in six families (236,237). This mutation lies in the extracellular portion of this GP. Women heterozygous for the mutation did not show decreased fertility.

The Ala566 Val mutation is uncommon outside Finland. No mutations in FSHR were found in North American women having either 46,XX hypergonadotropic hypogonadism (239) or POF (240). Similar findings were reported in 46,XX POF or primary amenorrhea cases from Germany (241), Brazil (242), and Mexico (243). Compound heterozygosity for FSHR mutations has been observed, namely genotypes Ile-160Thr/Arg573Cys and Asp224Val/Leu602Val (244).

**88.2.9.3 Inactivating LHR.** Most reported LHR mutations are 46,XY and have resulted in XY sex reversal (137) (page XXX). However, LHR mutations in 46,XX women cause the phenotype XX gonadal dysgenesis.

All 46,XX cases with LHR mutations have been recognized in sibships ascertained through affected 46,XY siblings, who presented with Leydig cell hypoplasia and XY sex reversal. LHR is 75 kD in length and consists of 17 exons. Located on 2p near the locus for FSHR, the first 10 exons in LHR are extracellular, the 11th transmembrane and the last 6 intracellular. Most mutations have been detected in the transmembrane domain of this GPR.

Latronico and coworkers (136) reported a 22-year-old woman who presented with primary amenorrhea due to an LHR mutation. In that family, three 46,XY sibs also had the same homozygous C544X mutation, which resulted in a truncated protein consisting of five rather than seven transmembrane domains. The 46,XX sib had breast development but only a single episode of menstrual bleeding at age 20 years; LH was 37 MIU/mL, and FSH was 9 MIU/mL. The mutation reduced the signal transduction activity of the LHR gene. In another 46,XX case, Latronico and colleagues (136) observed secondary amenorrhea; LH and

FSH were 10 and 9 MIU/mL, respectively. A homozygous mutation for Ala593Pro was found.

Other 46,XX women with LHR mutations may show oligomenorrhea. Ovulation does not occur, even though gametogenesis proceeds until the preovulatory stage. This is consistent with mouse knockout models (245).

Interestingly, activating LH receptor mutations have little effect in women despite activating LHR mutations causing precocious puberty in males (137).

**88.2.9.4 Inhibin A.** INHs are heterodimeric glycoproteins that consist of an  $\alpha$  subunit and either of two  $\beta$  subunits ( $B_A$  or  $B_B$ ), producing  $INH\alpha$  or  $INH\beta$ , respectively. These genes exert negative feedback inhibition on FSH. INHs are opposed by activins, which enhance FSH secretion. Given that INH is synthesized by granulosa cells, it is not surprising that in POF serum INH is low given FSH concomitantly elevated. Elevated FSH and low INH thus indicate reproductive aging. Perturbation of INHs could plausibly cause ovarian failure.

Several studies have claimed shown associations between POF and a specific INHA missense mutations or polymorphisms—G769A (Ala57Thr) (246,247). Studying patients from New Zealand, Shelling and colleagues (248) found G769A in 3 of 43 POF patients (7%) versus only 1 of 150 normal controls (0.7%). However, the mother of one of the three G769A individuals had the same perturbation and was clinically normal. Marozzi and coworkers (249) found G769A in 7 of 157 Italian POF individuals, 3 of 12 primary amenorrhea cases, and 0 of 36 early menopausal (40–45 years) women. Familial POF cases were relatively more likely to have G769A than sporadic cases. Dixit and colleagues (250) found G769A in 9 of 80 Indian POF cases; no mutations in  $INH\beta$  or  $INB\beta$  were found. Also studying an Indian cohort, Prakash et al. (251) found the variant in heterozygous form in 3 of 30 cases of primary amenorrhea, 3 of 20 with secondary amenorrhea, and 2 of 50 controls. Thus, normal individuals have had the G769A transition, and in fact individuals with G769A may be normal even if another G769A family member has POF; thus, G769A does not obligatorily confer ovarian failure at least in heterozygotes. More recent reports by Shelling and his group (252) have expanded rationale for function but not defined mutation(s) unequivocally causing POF (247,253–255).

**88.2.9.5  $17\alpha$ -Hydroxylase/17,20 Desmolase Deficiency (CYP17).** In discussing 46,XY DSD, we described 46,XY individuals with a CYP17 defect. Less commonly, 46,XX individuals have been reported with a CYP17 defect, presenting with primary amenorrhea or POF (256). Thus, deficiency of  $17\alpha$ -hydroxylase/17,20 desmolase should be considered an uncommon cause of 46,XX hypergonadotropic hypogonadism. Hypertension may coexist but less commonly than in 46,XY cases. Ovaries in affected cases are hypoplastic and sometimes have a streak-like appearance. Oocytes appear incapable of reaching diameters of more than 2.5 mm (190).

Stimulation with exogenous gonadotropins can produce oocytes capable of fertilization in vitro (257).

**88.2.9.6 Aromatase Mutations (CYP19) (46,XX) (CYP19).** Conversion of androgens ( $\Delta^4$ -androstenedione) to estrogens (estrone) requires cytochrome P450 aromatase (CYP19), an enzyme that is the gene product of a 40-kb gene located on chromosome 15q21.1 (241a). The gene consists of 10 exons.

46,XX aromatase deficiency may present with primary amenorrhea in phenotypic women. Ito and coworkers (258) reported an aromatase mutation (CYP19) in a 46,XX 18-year-old Japanese woman having primary amenorrhea and cystic ovaries. The patient was a compound heterozygote, having two different point mutations in exon 10. The mutant protein had no activity. Conte and colleagues (110) also reported aromatase deficiency in a 46,XX woman presenting with primary amenorrhea, elevated gonadotropins, and ovarian cysts. Compound heterozygosity for two different exon 10 mutations was found. One was a C1303T transition leading to cysteine rather than arginine, whereas the other was a G1310A transition leading to tyrosine rather than cysteine.

A different phenotype was reported by Mullis and coworkers (259). Clitoral enlargement occurred at puberty, and there was no breast development. Multiple ovarian follicular cysts were present. FSH was elevated; estrone and estradiol were decreased. Estrogen and progesterone therapy resulted in a growth spurt, decreased FSH, decreased androstenedione and testosterone, breast development, menarche, and decreased follicular cysts. Compound heterozygosity was found.

Placental aromatase deficiency can cause 46,XX DSD (female pseudohermaphroditism), as a result of POR deficiency.

Aromatase deficiency in 46,XY males leads to increased testosterone, macrorchidism and atherosclerosis but not 46,XY DSD (260).

**88.2.9.7 Progesterone Receptor Membrane Component 1.** This X-linked gene was interrogated in 67 POF cases, finding 1 heterozygous mutation (H165R) (261). The change occurred in a domain necessary for nontranscriptional regulation of cytochrome P450, potentially a functional effect.

**88.2.9.8 FOXL2/Blepharophimosis–Ptosis–Epicanthus.** In Genetic Control of Sex Differentiation, we discussed the pivotal role FOXL2 plays in ovarian development. Encoded on 3q21–24, the protein of FOXL2 must be expressed in order to maintain suppression of SRY and allow ovarian differentiation to proceed. FOXL2 and other forkhead DNA-binding proteins are crucial in signal induction. Blepharophimosis–ptosis–epicanthus (BPE) syndrome is an autosomal dominant malformation syndrome in which FOXL2 is perturbed. One of the two types of this syndrome (type II) is characterized by POF (262). FOXL2 is expressed in eyelids and ovaries (263). In four families, FOXL2 mutations



co-segregated with BPE and POF. Nonsense mutations reported include stop codons as well as a 17-bp duplication that resulted in a frameshift and, hence, truncated protein.

In the absence of somatic features, FOXL2 mutations are uncommon explanations for POF. De Baere and colleagues (264) found no FOXL2 mutations in 30 POF patients lacking eyelid abnormalities; Harris and coworkers (265) found two mutations among 70 cases. In 1 of the 70 cases of Slovenian origin, a deletion (A221–A230) removed 10 of the 14 alanines in the poly A tail (266). In a patient of New Zealand origin, a missense mutation (Tyr258Asp) was found.

**88.2.9.9 Newborn Ovary Homeobox.** NOBOX is a homeobox gene, an example of the genes that bind DNA and function as transcription factors to direct differentiation. NOBOX (NOBOX gene) encodes a homeobox transcriptional regulator. NOBOX is oocyte specific, expressed from the primordial follicle through metaphase II. Female null mice (knockout) show ovarian failure, whereas males are normal. One earlier study failed to show NOBOX perturbations in 30 Japanese women (63), but our group found two novel missense mutations (Arg355His and Arg360Gln) among 96 Caucasian POF cases (267) and Arg355His was present in a conserved region. Electrophoretic mobility shift arrays using Arg355His DNA showed disrupted binding of the NOBOX homeodomain to DNA, thus capacity for exerting a dominant-negative effect.

**88.2.9.10 Growth Differentiation Factor 9.** GDF9 is a member of the TGF $\beta$  family, like BMP15 (which is also called GDF9b). GDF9 can form dimers with BMP15. Expressed in oocytes, GDF9 is an attractive candidate gene. Various heterozygous mutations have been detected in some European and Asian samples (135,255,268,269), but not in others (270–272). A deleterious heterozygous change requires a dominant-negative effect, plausibly given dimerization and presumably analogous to deleterious effect of BMP15 mutations. If missense mutations like a hydrophobic amino acid replacing a hydrophilic amino acid are causative, GDF9 perturbations could account for substantial number (1–4%) of POF cases.

**88.2.9.11 BMPR1B.** Homozygous deletion (del 359–366) of BMPR1B, another autosomal TGF $\beta$  superfamily gene, was reported by Demirhan et al. (273) in a 16-year-old female with ovarian failure and acromesomelic chondrodystrophy. Although murine knockouts for this gene are infertile (274), heterozygous mutations of the BMPR1B homolog in sheep can lead to increased fertility (gain of function) (275). This is similar to the phenotype experienced by sheep heterozygous for BMP15 (FecX) mutations (276).

**88.2.9.12 Factor in Germline Alfa.** This gene on 2p13.3 is a germ cell-specific basic helix-loop-helix transcription factor. It regulates expression of zona pellucida genes. FIGLA is expressed in the embryonal ovary and in FIGLA knockout mice. Primordial follicles are either

not formed or all lost soon after birth. Zhao et al. (277) studied 100 Han Chinese with POF and found 3 variants in 4 women. A missense mutation (A49) was found in 2 cases: a 15–36 deletion (p.G6fsX66) in one that results in a frameshift and dysfunctional haploinsufficiency and a 419–421 deletion (140 del N). Functional studies of the latter (140delN) demonstrated that FIGLA binding to the TCF3 helix-loop-helix was impeded. This gene is attractive for interrogation in other ethnic groups.

**88.2.9.13 POU5F1.** This transcription factor gene located on 6p21.31 is significantly downregulated in NOBOX knockout mice, which lack ovaries. Thus, POU5F1 becomes a potential candidate gene, a downstream target of NOBOX. Wang et al. (278) sequenced 175 Chinese POF cases and found one non-synonymous variant (Pro13Thr), a heterozygous hydrophobic to hydrophilic substitution.

**88.2.9.14 PTH-Responsive B1.** PTHB1 was reported to be associated with POF in a small study (24 cases and 24 controls) (279). The use of a gene association of genome-wide association study (GWAS) formal for a sample size this small is considered inadequate. Sequencing data are awaited.

**88.2.9.15 ADAMTS.** Located on 5q14.1 → q15, this gene was found to be associated with POG in the discovery set of a GWAS performed on 99 Dutch POF cases and 181 controls (280). However, the finding was not confirmed in the replication set. Given this considerably underpowered GWAS, conclusions concerning the role this transcription factor plays in POF remain uncertain.

**88.2.9.16 Myotonic Dystrophy (CTG<sup>N</sup>).** Myotonic dystrophy is an autosomal dominant disorder characterized by muscle wasting (e.g. head, neck, extremities), frontal balding, cataracts, and male hypogonadism (80%) attributable to testicular atrophy (see Chapter 122). Female hypogonadism is much less common than male hypogonadism if increased at all. Despite frequent citations in texts, ovarian failure in myotonic dystrophy appears not well documented.

Pathogenesis of myotonic dystrophy involves nucleotide expansion of CTG repeats in the 3' untranslated region of the causative gene, which is located on chromosome 19. Normally, 5–27 CTG repeats are present. Heterozygotes usually have at least 50 repeats; severely affected individuals show 600 or more. As in FRAXA (FMR1) (see below), response to ovulation induction regimes is poor. Sermon and colleagues (281) reported fewer embryos per cycle than in standard assisted reproductive technologies (ARTs); thus, pregnancy rates in preimplantation genetic diagnosis were thought to be decreased. However, more recent reports show better results (232), perhaps indistinguishable from the general population of infertile women undergoing ART.

**88.2.9.17 Galactosemia.** Discussed in Chapter 93, galactosemia is due to galactose 1-phosphate uridyl transferase (GALT) deficiency. Ovarian failure may occur. Kaufman and coworkers (282) reported POF in 12 of 18 galactosemic women, and Waggoner and colleagues (283)

reported ovarian failure in 8 of 47 (17%) women with galactosemia. Pathogenesis presumably involves galactose toxicity after birth, given that elevated fetal levels of toxic metabolites should be cleared rapidly in utero by maternal enzymes. Consistent with this idea, a neonate with galactosemia showed normal ovarian histology (112).

There remains little reason or evidence to believe that galactosemic heterozygotes show POF. Not all homozygotes for human galactosemia are abnormal, nor are transgenic mice in which GALT is inactivated (knockout) (284).

**88.2.9.18 Carbohydrate-Deficient Glycoprotein (Phosphomannomutase Deficiency, PMM2).** In type 1 carbohydrate-deficient glycoprotein deficiency, mannose 6 phosphate cannot be converted to mannose 1 phosphate. This lipid-linked mannose-containing oligosaccharide necessary for secretory glycoproteins cannot be synthesized. The gene is located on 16p13, and the usual molecular perturbation is a missense mutation (285). In addition to characteristic neurological abnormalities (286), ovarian failure is frequent. FSH is elevated, secondary sexual development fails to occur, and ovaries lack follicular activity (287,288).

**88.2.9.19 Autoimmune Regulation/Autoimmune Polyendocrinopathy–Candidiasis–Ectodermal Dystrophy.** The AIRE gene located on 21q22.3 is responsible for the condition titled by the physical features listed above. In addition to the abnormalities defined by the appellation, other findings include alopecia, vitiligo, keratopathy, malabsorption, hepatitis, and mucocutaneous candidiasis. Ovarian hypoplasia exists in 55% of APECED cases in the third decade (289). Many different AIRE perturbations have been found in this autosomal dominant disorder, a pleiotropic condition (289) of varied expressivity. These include nonsense mutations and frameshifts, the latter confirming causation is not in doubt (289).

There is no evidence that any particular mutation leads to ovarian failure as distinct from other autoimmune phenomenon in the disorder's spectrum.

AIRE mutations have not yet been sought in women with POF who do not have non-ovarian features.

**88.2.9.20 Ovarian Leukodystrophy (Eukaryotic Translation Initiation Factor EIF2B).** Potentially, part of the same clinical constellation as cerebellar ataxia disorders having ovarian failure (see below) is a condition termed ovarian leukodystrophy. Characterized by MRI-detectable “vanishing white matter” leading to variable but progressive neurological degeneration, ovarian failure of varied expressivity coexists (290,291). As a result of mutation occurring in EIF2B, denatured stress-related proteins accumulate, of potential relevance to oogenesis given ubiquitous oocyte degeneration. In ovarian leukodystrophy, Fogli et al. (292) found variants in EIF2B2, EIF2B4 and EIF2B5. However, 0 of 93 cases with only isolated POF showed perturbations (293).

**88.2.9.21 Symphalangism and Noggin.** NOG is located on 17q22 and is responsible for the autosomal dominant disorder proximal symphalangism (SYM1).

Characteristic features include ankylosis of the proximal interphalangeal joints, carpal–tarsal fusion, brachydactyly and deafness. NOG is expressed in the ovary and is an antagonist of BMPs 4 and 7 (294). The latter are members of the TGF family of genes, some (e.g. BMP15) already considered as candidate genes for POF.

One SYM1 case has shown POF, and as predicted, an NOG mutation was found (295). NOG perturbations have not been sought in isolated POF subjects.

**88.2.9.22 Perrault Syndrome.** XX gonadal dysgenesis with neurosensory deafness is termed Perrault syndrome (296), an autosomal recessive disorder (297–299). Endocrinologic features seem identical to those of XX gonadal dysgenesis without deafness.

Candidate genes are likely to merge from the connexin family, for an attractive gene knockout model exists in connexin 37 (300). Null mice show gonadal failure due to arrest at the antral stage of oogenesis. The connexin gene family is responsible for many forms of congenital deafness in humans.

**88.2.9.23 Cerebellar Ataxia with XX Ovarian Dysgenesis.** Ataxia and hypergonadotrophic hypogonadism were first associated by Skre and colleagues (301), who described cases in two families. In one family, a 16-year-old girl was affected, whereas in the other family, three sisters were affected. In the sporadic case and in one of the three sisters, ataxia was observed shortly after birth; in the two other sisters, age of onset was later during childhood. Cataracts were present in all the cases reported by Skre et al. (301).

Hypergonadotrophic hypogonadism and ataxia were later reported by De Michele and colleagues (302), Gottschalk and colleagues (303), Fryns and coworkers (304), Nishi and colleagues (298), and Amor and coworkers (305). The nature of the ataxia differed among patients. Clinical findings similar to those of Skre and colleagues (301) were reported by De Michele and coworkers (302), Nishi and colleagues (298), and Amor and coworkers (305); ataxia was usually not progressive. Mitochondrial enzymopathy was found by De Michele and colleagues (306), but mitochondrial studies were apparently not performed by others. Cataracts were observed only by Skre and coworkers (301), and amelogenesis only by Linssen and colleagues (307). Neurosensory deafness reminiscent of Perrault syndrome was reported by Amor and colleagues (305) and Linssen (307), who wondered if this condition could be synonymous with Perrault syndrome. Mental retardation is also variable (305).

Overall, genetic heterogeneity must be assumed in the hypergonadotrophic hypogonadism disorders showing cerebellar ataxia. A single mutant gene is unlikely to explain every single case, but not every family need be unique.

**88.2.9.24 Forkhead Transcription 3A (FOXO3A).** Forkhead transcription genes other than FOXL2 cause ovarian follicular depletion in murine knockout models (135).

This holds true for FOXO3A, whose gene product regulates G1/S transition in granulosa cells. Of 60 POF cases (30 New Zealand; 30 Slovenia), 2 showed potentially significant FOXO3A mutations, neither present in controls (308). One mutation was a single heterozygous mutation in a Slovenian woman that resulted in a nonconservative amino acid charge (Ser421Leu); the mutation is potentially capable of inducing a conformational protein change. The other mutation was Arg506His, found in a New Zealand woman. This conservative change seems less likely to exert an untoward effect.

**88.2.9.25 FOXO1A.** Another forkhead transcription gene is FOXO1A. Watkins et al. (308) found a single conservative change (P84L) in FOXO1A among the 90 POF cases. The patient was Slovenian in ancestry.

**88.2.9.26 LIM DNA-Binding Proteins (LHX8).** LIM homeobox genes encode DNA-binding proteins. LIM family members contain two tandemly repeated domains that have cysteine-rich, double zinc finger motifs. LHX8 transcripts localize to mouse oocyte germ cells through antral follicles. Null mice lack germ cells (309). Qin et al. (310) sequenced all 8 LHX8 exons in 95 Caucasian women with POF. No novel single nucleotide polymorphisms (SNPs) were found.

**88.2.9.27 NANOS3.** NANOS3 is an RNA-binding protein. In mice, female and male knockouts are both infertile; female KO mice show no other phenotypic effect (311). Thus, human NANOS3 is a candidate gene for POF.

Human NANOS3 gene consists of two exons and is expressed in germ cells. In our study of 80 Chinese and 88 American Caucasians with POF, the only NANOS3 sequence variant found was a synonymous nucleotide substitution that was already known in the general population (312).

**88.2.9.28 GPR3 and GPs.** GPs are regulatory proteins. FHS, LH and other protein hormones are ligands for specific cell surface GPRs, leading to intracellular signal transduction. Widely disparate stimuli (e.g. hormones, light) also exert conformational changes on GPRs to allow binding to GPs. The oocyte-specific G-stimulating protein-coupled receptor GPR3 is known to have a role in maintaining meiotic arrest in the mouse oocyte. Female mice lacking GPR3 develop premature ovarian aging as a result of spontaneous resumption of meiosis in antral follicles, independent of the LH surge (313). Oocyte attrition results.

Located on chromosome 1, GPR3 consists of two exons. Our group interrogated GPR3 for perturbations in 82 Caucasian women with POF; none (0) of the 82 showed perturbations of significance (314). A single woman showed heteroduplex formation as a result of a heterozygous nucleotide substitution, C to A at position 51 (c.51C > A). However, this substitution does not alter the amino acid sequence, and had already been registered in the SNP database. GPR3 mutations have thus not yet

been shown to be a common explanation for POF in North American Caucasians.

**88.2.9.29 KIT.** KIT is an autosomal (4q12) gene that encodes for the tyrosine kinase transmembrane regulator for mast/stem cell growth factor (187). The c-kit receptor and its ligand (KL) constitute two murine loci known to be characterized by decreased germ cells: white spotting (W) and steel (L). Thus, human KIT is a good candidate gene for POF. Shibamura et al. (315) studied 40 women with unexplained POF, sequencing the entire coding region. One synonymous mutation was found but this is not a plausible disease-causing perturbation.

**88.2.9.30 RET Finger Protein-Like 4.** RING finger-like protein (RFLP) is expressed in oocytes and in mice exclusively in that organ. The gene encodes an E3 ubiquitin protein ligase which helps regulate protein degradation. Human RFLP4 is located on 19q13.4, and has been shown to interact with oocyte proteins of the ubiquitin-protease degradation pathway (316). In the context of a review in which details were not provided, Suzumori et al. (176) stated no mutations were found in Japanese POF patients with “46,XX POF.”

**88.2.9.31 MSH5 and DMC1.** Various pleiotropic syndromes that are associated with chromosomal abnormalities (e.g. ataxia telangiectasia and Bloom syndrome) have long been known to result in ovarian failure. Ataxia telangiectasia and Bloom syndrome (Table 88-4) are examples. Thus, genes perturbing meiosis are logical candidates for non-syndromic POF. The family of mismatch genes are pivotal in repairing DNA damage. Mutations lead to hereditary nonpolyposis colon cancer, as an example. Mandon-Pepin et al. (317) sought mutations in 44 POF women for SMC1, MSH4, MSH5 and SPO11. A heterozygous mutation (2547C > T) for MSH5 was found in one woman. Another woman showed homozygosity 3351 > AC in DMC1.

**88.2.9.32 AMH and AMHR Type II.** In addition to its role in Müllerian duct regression in males, MIS (AMH) is an oocyte inhibitor in the rat. Murine knockout models show early depletion of primordial follicles (318). Apparently, AMH plays a permissive or synergistic role in gonadal development as well. In humans, Wang et al. (289a) failed to find plausible perturbations in 16 POF cases.

**88.2.9.33 PTEN.** PTEN is a tumor suppressor gene, which as a regulator of cell growth could logically be related to POF. Shimizu et al. (319) failed to find perturbations in 20 women with idiopathic POF.

**88.2.9.34 Germ Cell Failure in Both Sexes.** See Section 88.2.5.13.

**88.2.9.35 46,XX Gonadal Regression (Agonadia).** Discussed above in the context of XY sex reversal (Section 88.2.5.13), agonadia in 46,XY individuals is characterized by absent gonads, abnormal external genitalia, and all but rudimentary Müllerian and Wolffian derivatives. Almost all affected individuals are 46,XY, for which reason a full description of the phenotype was



discussed previously in the context of 46,XY sex reversal. However, 46,XX gonadism is reported as well.

Mendonca and colleagues (320) reported gonadal regression without somatic anomalies in phenotypic sibs having unlike chromosomal complements (46,XY and 46,XX). Sporadic XX cases were reported by Duck and colleagues (145) and Levinson and coworkers (131). Kennerknecht and colleagues (321) reported gonadal regression, hypoplasia of the pulmonary artery and lung, and dextrocardia in XX and XY siblings.

Existence of these rare 46,XX case illustrates that the terms gonadism or gonadal regression are preferable to the appellation testicular regression.

**88.2.9.36 Malformation Syndromes with XX Gonadal Dysgenesis.** XX gonadal dysgenesis is found in several rare malformation syndromes. All are presumed autosomal recessive on the basis of multiple affected siblings: XX gonadal dysgenesis, microcephaly and arachnodactyly (322); XX gonadal dysgenesis and epibulbar dermoids (323); and XX gonadal dysgenesis, short stature, and metabolic acidosis (324). None have been elucidated fully.

**88.2.9.37 Other Nuclear Genes.** Over 200 murine genes cause male or female infertility (245) and often are expressed only in germ cells. Null (knockout) models in mice show germ cell deficiency or absence. The human homologs of these genes are attractive candidate genes for POF. Many remain to be interrogated for mutations in women with POF.

Attractive candidate genes include SOHLH1, SOHLH2, OBOX, NR6A1, TAF4B and BAX. These govern different mechanisms of gene action, some related to gonadotropins.

Continued interrogation of many genes is in order because it seems unlikely that any given gene will be responsible for more than perhaps 1–2% of POF. Whole genome associations using SNPs or CNVs are in progress and should identify chromosomal regions of interest. Novel genes can be anticipated. Nontraditional (non-Mendelian) mechanisms involving CNVs leading to nonhomologous allele recombination could also play a role.

**88.2.9.38 Mitochondrial Genes.** Perturbations of mitochondrial DNA cause a number of disorders, typically involving muscular and neurological systems. Perturbations of mitochondrial genes are good candidates for POF because the mature oocyte has the greatest number of mtDNA copies of any human cell. One gene is already proved so.

In progressive external ophthalmoplegia (PEO) proximal myopathy, sensory ataxia and parkinsonism occurs. This disorder results from a mutation in the mitochondrial gene polymerase gamma. Among seven families studied by Luoma et al. (325), POF co-segregated with PEO in three. The mutation Y955C was found in two of the three families. This tyrosine to cytosine change involves a highly conserved region, making a functional effect highly plausible. In the third family, compound

heterozygosity (N468D/A1105T) was observed in an affected woman. In another report, Pagnamenta et al. (326) studied individuals in three generations were affected with both PEO and POF. Y955C co-segregated with PEO.

## 88.2.10 Anomalies Limited to Internal Genital Ducts (Müllerian or Wolffian Derivatives)

**88.2.10.1 Imperforate Hymen.** The central portion of the hymen is ordinarily patent (perforate), permitting egress of mucus and blood. If the hymen is imperforate, mucus and blood from endometrial sloughing accumulate. Hydrocolpos or hydrometrocolpos results. The hymen is thin and distensible, unlike other types of vaginal obstructions with thick obstructions [e.g. transverse vaginal septum (TVS), vaginal agenesis or cervical atresia (CA)]. Vulvar distension thus may arise in imperforate hymen, uniquely suggesting the cause of hydrometrocolpos.

Affected siblings have been reported (327,328). Stelling and colleagues (329) reported concordant monozygotic twins; one twin had an affected daughter, who also had pyloric stenosis and possibly hip dislocation.

In the original description of Schinzel–Giedion syndrome, “hymenal atresia” was described (291a).

**88.2.10.2 TVS and McKusick–Kaufman Syndrome.** TVS are about 2 cm thick and usually located near the junction of the upper third and lower two-thirds of the vagina (1,330–332). Septa may, however, vary in location among different individuals (B in Figure 88-13). The uterus appears absent on vaginal exam, but can often be detected by rectal examination and should be evident by imaging (e.g. ultrasound or MRI). Gonads (ovaries) and external genitalia (female) are normal.

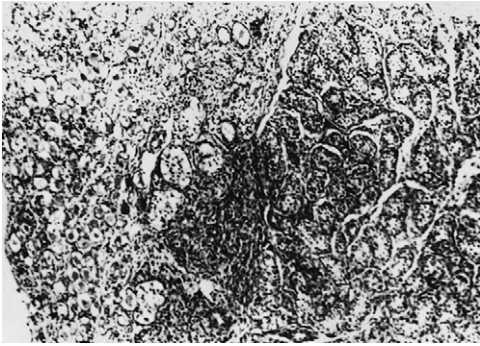
If existing in septa, perforations are typically central in location. If no perforation exists, mucus and menstrual fluid accumulate to produce hydrocolpos or hydrometrocolpos. Geneticists should appreciate that hydrometrocolpos is not a singular anatomic condition but rather merely a nonspecific physical feature that can arise as a result of having several different anatomic disturbances (e.g. imperforate hymen, TVS, vaginal atresia (VA), CA, and even incomplete Müllerian fusion (IMF)) (see Figure 88-14). Clinical presentation is similar in all except imperforate hymen. In imperforate hymen, a distended vulva and membrane are observed. In VA, TVS, and CA, the obstruction is thicker and no bulging is evident.

In the Amish, an autosomal recessive gene is responsible for TVS (333,334), and appears to be synonymous to that causing McKusick–Kaufman syndrome (MKS). The eponym was derived from recognition of an association between TVS with polydactyly and cardiac defects (333,335). Whether all cases of TVS—Amish and non-Amish—are caused by the MKS gene is uncertain but unlikely. Distinct genes could also exist in Amish



and non-Amish. Familial aggregates of MKS have been observed in the Italian and Puerto Rican populations (336), and in these families, VA seems relatively more common than TVS.

Making the hypothesis that only a single pleiotropic gene (MKS) exists, Chitayat and colleagues (336) analyzed 54 Amish individuals. Hydrometrocolpos was estimated to be present in 95%, polydactyly in 93%, and

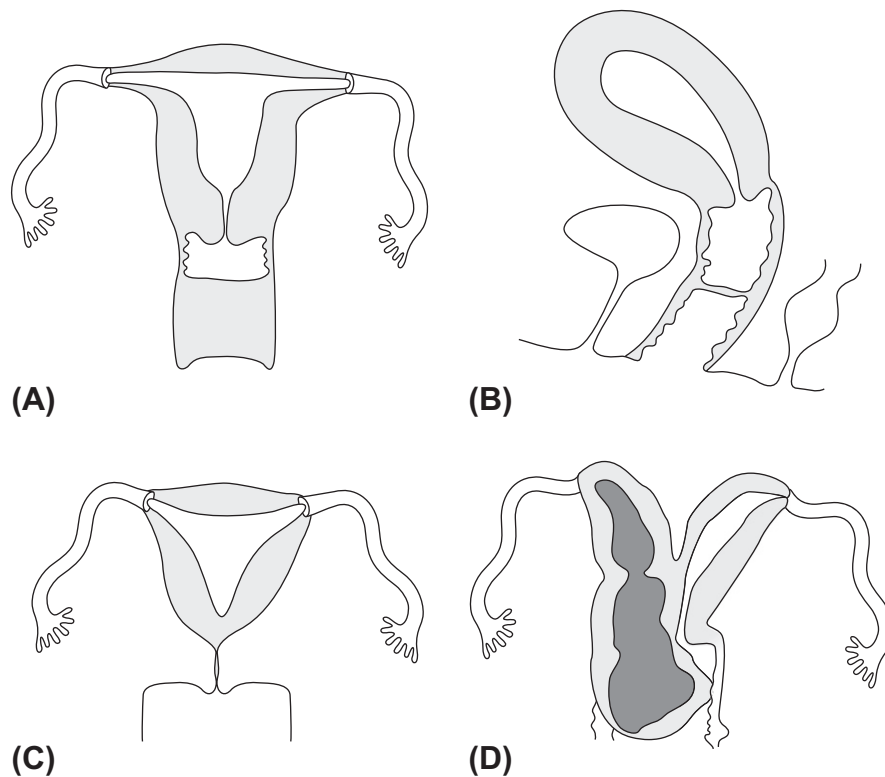


**FIGURE 88-13** Photomicrograph of the left ovotestis of patient no. 2 of van Niekerk complement. Numerous primordial follicles are present in the smaller left portion; infantile testicular tissue is evident on the right. (From van Niekerk. *True Hermaphroditism—Clinical, Morphologic, & Cytogenetic Aspects*. Harper & Row: Hagerstown, 1974.)

cardiovascular malformations in 9%. In these cases, hydrometrocolpos was usually the result of TVS. Stone and coworkers (337) estimated penetrance to be 70% for hydrometrocolpos in women, 60% for polydactyly in either sex, and 15% for cardiovascular defects in either sex. Based on these estimates, 9% of men and 3% of women would have the MKS gene in a clinically nonpenetrant form.

The MKS gene product is a chaperonin, representative of that class of proteins that facilitates protein folding in conjunction with adenosine triphosphate hydrolysis (338). The MKS gene is localized on 20p12. Causation was deduced on the basis of H84Y/A242S compound heterozygotes co-segregating with the disorder in a large Amish pedigree (338). Each of these two chaperonin sequence differences (H84Y and A242S) is present in 1 per 100 Amish controls, a frequency (1%) in Amish consistent with a heterozygote frequency of that magnitude. Neither sequence was found in 100 non-Amish controls. Three Amish compound heterozygotes were unaffected, suggesting nonpenetrance higher than the 3–9% estimated on clinical grounds by Stone and coworkers (337).

Five other MKS probands showed no perturbations in the MKS chaperonin. In these cases, it was wondered if the diagnosis of Bardet–Biedl syndrome might not be more appropriate, given that Slavotinek and coworkers (339) showed that Bardet–Biedl syndrome can result



**FIGURE 88-14** Schematic diagram contrasting salient features of various anatomic defects capable of producing hydrometrocolpos or hydrocolpos. A bulging hymen would indicate imperforate hymen, but any of the following four conditions may appear similar. (A) VA. (B) TVS. (C) CA. (D) IMF with obstruction due to blindly ending hemiuterus. The distended hemiuterus can obliterate the vagina, making it difficult to identify the contralateral hemiuterus containing a cervix. TVS can exist at various sites. (Modified from Simpson, J. L. *Genetics of the Female Reproductive Ducts*. Am. J. Med. Genet. Semin. Med. Genet. 1999, 89, 224–239.)

from disturbance of the MKS gene. An atypical non-Amish patient of northern European descent with a frameshift mutation showed a hypoplastic clitoris with prominent labia majora, small ears, uterine and skeletal anomalies, and atrioventricular septal defects.

**88.2.10.3 Longitudinal Vaginal Septa.** Vaginal septa may be longitudinal as well as transverse (coronal or sagittal). Diagnosis of longitudinal vaginal septa (LVS) assumes absence of IMF (e.g. uterus didelphys), a condition in which the fusion defect extends inferiorly to produce a vaginal septum. In contrast to IMF (see below), isolated LVS need not necessarily be midline in position.

Usually, isolated LVS cause no clinical problems. If septa impede the second stage of labor, ligation and division may be necessary.

Heritable tendencies have not been reported in isolated LVS, but systematic studies have not been conducted. LVS has been observed in genetic syndromes, including Johanson–Blizzard syndrome (340) and the syndrome described by Edwards and Gale (341). The latter is an autosomal dominant syndrome characterized by LVS, hand anomalies and bladder neck anomaly.

**88.2.10.4 Vaginal Atresia.** In VA, the lower portion of the vagina, typically one fifth to one third of the total length, is replaced by 2–3 cm of fibrous tissue (see Figure 88-13). External genitalia are otherwise normal for women, except for absence of the hymen. Embryonic origin is presumed to involve failure of the urogenital sinus to contribute the caudal portion of the vagina. Superiorly, there lie a well-differentiated upper vagina, cervix, uterine corpus, and fallopian tubes. Diagnosis requires ultrasound, MRI, or rectal examination to verify presence of Müllerian derivatives, specifically the cervix and uterus. Women who lack not only most of the vagina but also the uterus do not have VA but rather Müllerian aplasia (MA) (see immediately below).

VA is clinically and embryologically distinct from TVS, even though in both conditions the upper vagina and uterus are intact. In TVS, an intact lower vagina exists, whereas in VA, it does not. In TVS, a thick intervening septum separates the lower from the upper vagina. In VA, fibrous tissue varies in amount and may sometimes replace nearly the entire vagina, beginning inferiorly near the perineum and extending superiorly to the cervix. Some confusion has arisen because in certain MKS cases, VA rather than TVS was observed (336). Although this could be taken as evidence that VA and TVS are the same condition, misclassification or genetic heterogeneity are equally plausible explanations.

No familial aggregates of isolated VA have been reported. However, VA is not uncommon in the context of multiple malformation syndromes (2,332). Of note is the syndrome described by Winter and colleagues (246). This autosomal recessive disorder is characterized by VA, renal hypoplasia or agenesis, and middle ear anomalies (e.g. malformed incus, fixation of the malleus and incus) (246,342). Other syndromes in which VA has been

reported include Antley–Bixler, Apert, Bardet–Biedl, Ellis–Van Creveld, Fraser (cryptophthalmos), Laurence–Moon, Pallister–Hall and Robinow syndromes (2,332).

**88.2.10.5 Müllerian Aplasia.** Aplasia of the Müllerian ducts leads to absence of the uterine corpus, uterine cervix, and upper (superior) vagina. This condition is the embryologic complement of VA, in the latter the lower vagina is absent but the upper vagina is present. In MA, the vagina is derived exclusively from urogenital sinus invagination. In MA, the ovaries are normal and, hence, secondary sexual development normal. Because most of the vaginal length is contributed by Müllerian derivatives, the vagina may be greatly shortened to only 1–2 cm. Vaginal length may or may not be adequate for coitus. By definition, there is no well-differentiated uterus, but bilateral remnants may and usually does persist in the form of cords. When gynecologists invoke the term “absence of the vagina” (in an otherwise normal woman) or “congenital absence of the uterus and vagina,” the diagnosis is usually MA (considered rigorously, the only other condition in the differential diagnosis is CAIS). The eponymic Rokitansky–Kuster–Hauser syndrome is considered synonymous with MA.

Affected sibships are well documented (343–345). Discordant monozygotic twins have also been recognized (346), indicating that a single autosomal recessive gene cannot explain all cases. Consanguinity does not appear to be increased. Carson and coworkers (347) studied 23 probandae and found no affected relatives. Similarly, van Lingen and colleagues (348) observed only one set of affected siblings among 35 cases. In the 1970s, sex-limited autosomal dominant inheritance was invoked by Shokeir (349) following the study of 16 Saskatchewan families. However, no subsequent studies have confirmed those findings.

Women with MA can still have genetic offspring, using ART. Because such women have normal ovaries, their oocytes can be stimulated and aspirated. Fertilization can then be achieved in vitro using the sperm from the husband or a donor, followed by transfer of the fertilized embryos into the (surrogate) uterus of a woman placed in hormonal synchrony with the probanda whose oocytes were aspirated. The surrogate woman then carries the pregnancy, but resulting offspring have the genetic constitution of the (MA) woman and her husband. Information on inheritance of this once genetically lethal disorder can thus be derived. Surveying United States ART programs, Petrozza and colleagues (350) identified 34 women with MA whose oocytes were used to produce a pregnancy carried in the uterus of a surrogate. Of the 17 resulting female offspring, none had MA; one male child had a middle ear defect and hearing loss. MA can thus be presumed to be polygenic/multifactorial in etiology. Using denaturing gradient gel electrophoresis, in the 1990s, various studies sought and found no molecular abnormalities in a number of candidate genes. However, these studies could probably completely exclude only

large deletions and studying WT1 (348) and PAX2 (351) no perturbations were found. This also held for AMH and AMHR (352). The N314 allele of GALT (352,353) was not increased. Perturbations of HOXA13 cause the autosomal dominant condition hand-foot-genital (HFG) syndrome (see later), but HOXA13 mutations have not been found in isolated MA. More recent and more detailed studies include those of Cheroki et al., who excluded 25 cases with WNT4, RAR-Gamma and RXR-alpha perturbations (354). Burel (355) found no perturbations in Hox genes (A7–A13) or PBX in a sample of six cases. Sultan et al. (356) studied 28 cases for mutations in WNT4, finding 1 heterozygous missense mutation (L12P).

All these studies involve very small sample sizes and all involve only European populations. Several groups have performed array CGH, (354,357–362) showing a variety of microdeletions and microduplications (i.e. less than the 5 Mb required for recognition by high-resolution CTG-banding studies). These include 1q21.1, 16p11.2, 17q12 and 22q11.2, all common variants. Familial transmission likely indicative of benign polymorphisms was not usually excluded, unfortunately because these regions are among those most commonly detected in array CGH studies irrespective of indication for performing (e.g. postnatal infant with developmental delay, fetus with ultrasound-detected anomalies). Del22q11.2 is probably most likely to play a role in certain cases, whether de novo or familial (if paternal). Microdeletions and microduplications of these regions are also found in normal individuals; thus, ascribing a causative role in MA is hazardous. An illustrative study receiving attention is that of Gervasini (363), who in a study of 30 MA cases found a duplication of Xq21 only in 5 MA cases (2 sporadic and 3 familial). Especially intriguing was a family of 2 affected MA sibs who received a 17-kb duplication from their father. Two sibs having a uterus failed to inherit the microduplication. The PAR duplicated contained SHOX, a homeobox gene that escapes X-inactivation and has been related to short stature if deficient and to Leri–Weill syndrome and Langer mesomelic dysplasia if duplicated (81,82). However, array CGH findings in MA were not confirmed in the much more robust study of Sandbacka et al. (364) of 101 cases. This study noted that all CNVs reported by Gervasini are in fact recorded in the Database of Genomic Variants and considered without phenotypic effect.

A molecular perturbation has been found in MA only in a very atypical case. Biason-Lauber et al. (365) found perturbations of WNT4 in many typical cases of MA in which virilization and adrenal insufficiency existed. The relevance to isolated MA is unclear. Gervasini et al. (363) found no perturbations in 12 MA cases who also had hyperandrogenism. Wnt4 acts before AMH and is required for initial Müllerian development (366), however, so plausibility could exist.

**88.2.10.6 MA and Malformation Syndromes.** MA may be one component of malformation syndromes, tabulated in Table 88-6 and discussed elsewhere by Simpson (2) and Simpson and Elias (2). The acronym MURCS has been applied when MA also coexists with renal and skeletal anomalies as well as facial clefts and cardiac anomalies (367). MA is not uncommon in the fascio-auricular-vertebral syndrome (Goldenhar syndrome) (368), limb/pelvis/uterus hypoplasia syndrome (369,370), and thalidomide embryopathy (371) and thrombocytopenia-absent radius syndrome (372). In the other disorders cited in Table 88-6, MA is uncommon.

**88.2.10.7 Incomplete Müllerian Fusion.** During embryogenesis, the paired Müllerian ducts fuse and canalize at the 150- to 200-mm stage, thereafter forming the upper vagina, uterus, and fallopian tubes. When the two ducts fail to fuse and canalize, IMF exists. Specific terms are applied to various subtypes that exist, as discussed elsewhere (1,332). Failure of fusion of Müllerian ducts may result in two hemiuteri, for example, each associated with no more than one fallopian tube. If one Müllerian duct fails to contribute to the definitive uterus, a rudimentary horn results. If one uterine horn is atretic, ipsilateral renal agenesis is especially common.

Many familial aggregates of IMF have been reported, including multiple affected siblings, as well as affected mother and daughter (373,373a,374–381). In the same kindred, affected relatives may show different forms of IMF (373a). The single formal genetic study involved only 24 index cases (373), finding 1 of 37 (2.7%) sisters to have a clinically symptomatic uterine anomaly. Such a recurrence risk for first-degree relatives is consistent with predictions based on polygenic/multifactorial etiology (2–3%). No molecular studies have been conducted in isolated IMF, but below we will note that perturbation of HOXA13 causes HFG syndrome. Several other candidate genes are worth exploring, such as Wnt7a, which is required in mice for completion of Müllerian differentiation (382).

**88.2.10.8 IMF and Malformation Syndromes.** More than a dozen malformation syndromes have IMF as one component (2,382). In Fryns syndrome, Halal syndrome, and hydrolethrus syndrome, IMF is consistently observed. IMF is less commonly observed in the other disorders listed in Table 88-7.

**88.2.10.9 HFG Syndrome.** HFG syndrome is an autosomal dominant disorder characterized by IMF, skeletal anomalies, and urologic anomalies. Skeletal anomalies include short first metacarpals, small distal phalanges on the thumbs, short middle fifth phalanges, and fusion of various wrist bones (306,380). The great toe is short because of its shortened metatarsal; the distal phalanx is small and pointed. Urinary system abnormalities include urinary incontinence in women, a ventrally displaced urethral meatus (men and women), and malposition of the ureteral orifices

**TABLE 88-6 Malformation Syndromes with Müllerian Aplasia<sup>a</sup>**

Syndrome	OMIM Number	Somatic Anomalies	Etiology
Fraser	219000	Cryptophthalmia, nose and external ear anomalies, stenotic larynx, skeletal defects, syndactyly, renal agenesis, large clitoris, and labia majora, mental retardation	Autosomal recessive
Meckel–Gruber	249000	Microcephaly, posterior encephalocele, eye anomalies, cleft palate, polydactyly, polycystic kidneys	Autosomal recessive
MURCS association	601076	Renal aplasia, cervicothoracic somite dysplasia, Klippel–Feil anomaly, deafness, short stature	Unknown; probably heterogeneous
Thalidomide teratogenicity		Nasal hemangioma, neurosensory hearing loss, ear anomalies, limb reduction defects, visceral anomalies	Teratogen
Urogenital adysplasia, hereditary (hereditary renal adysplasia; bilateral renal agenesis)		Oligohydramnios, flattened (Potter) facies, pulmonary hypoplasia, unilateral or bilateral absent kidneys, limb deformities	Autosomal dominant
Winter	247990	Lacrimal duct stenosis, external and middle ear anomalies, renal agenesis	Autosomal recessive
Kumar		Skeletal anomalies	Unknown

<sup>a</sup>Several of these syndromes could be heterogenous, that is, more than one entity. In particular, Klippel–Feil and Müllerian aplasia above could be a distinct entity, as could deafness and Müllerian aplasia.

From Simpson, J. L. Genetics of the Female Reproductive Ducts. *Am. J. Med. Genet.* 1999, 89, 224–239.

in the bladder wall of women (380). These urologic anomalies are distinct from those associated with isolated IMF, which more often is associated with the absence of one kidney, pelvic kidney, or duplication or absence of ureters. Vertebral anomalies in IMF also differ in type from those in HFG.

First reported by Stern and colleagues (383), many familial aggregates of HFG have been reported (306,380,384–387). The disorder is caused by perturbation of HOXA13, localized to 7p14–p15. Studying a member of the original HFG family reported by Stern and colleagues (383), Mortlock and Innis (388) found an HOXA13 nonsense mutation that resulted in a highly conserved tryptophan being converted to a stop codon, truncating the protein by 20 amino acids. In one of three HFG individuals studied by Goodman and coworkers (386), an HOXA13 mutation again involved a stop codon. In a second family, an expansion of the polyadenosine (poly A) tail was noted, suggesting a dominant-negative mechanism.

Goodman and colleagues (386) later studied six additional families, two previously not studied and four previously reported. In three families, nonsense mutations resulted in a truncated protein. In another family, originally described by Hennekam (389), the N-terminal polyalanine tract was again expanded, suggesting a dominant-negative effect. In a fifth family, a missense mutation was present, altering an invariant asparagine residue in the homeodomain recognition helix that is necessary to target DNA. In a final family, no HOXA13 mutation was found. In 2002, Utsch and colleagues (390) reported poly A expansion in a five-generation HFG family, giving further support to a dominant-negative mechanism in some cases.

In conclusion, perturbation of HOXA13 clearly causes HFG. Pathogenesis could be interpreted as resulting from a null allele (haploinsufficiency) if we are to assume that the polyalanine expansion mitigates against action of HOXA13. HOXA13 is clearly integral for both skeletal development and Müllerian fusion.

**88.2.10.10 Persistent Müllerian Derivatives in Males.** The uterus and fallopian tubes (Müllerian derivatives) may persist in ostensibly normal (46,XY) males. External genitalia, Wolffian (mesonephric) derivatives, and testes develop as expected for males, and pubertal virilization occurs.

Testes are presumed to differentiate normally, but they may not remain normal. One or more testes may be present in the intra-abdominal or inguinal region, and they undergo secondary degeneration. About 5% of individuals develop a seminoma or other germ cell tumor. Testes in persistent Müllerian derivatives (PMDs) are also abnormally mobile (3) as a result of not being anchored properly to the processus vaginalis. Increased mobility could predispose to testicular torsion and secondary testicular degeneration, which are frequent in PMD (63). Infertility is common for these and perhaps other reasons.

Two genes are integral for precluding Müllerian development in men. One gene codes for AMH (formerly called MIS); the other gene codes for the AMHR. Josso and colleagues have studied AMH and AMHR in over 100 PMD families; in 15%, no mutation was identified (391). Mutations are equally likely to involve AMH or the AMHR. In a 2005 report, 82 cases of PMD in men were analyzed by Josso and coworkers (392). Of these,



**TABLE 88-7 Selected Multiple Malformation Syndromes in Which Incomplete Müllerian Fusion**

Syndrome	OMIM Number	Somatic Anomalies	Etiology
Bardet–Biedl	209900	Retinal pigmentary degeneration (retinitis pigmentosa), polydactyly, obesity, mental deficiency	Autosomal recessive
Beckwith–Wiedemann	130650	Macroglossia, omphalocele, macrosomia	Autosomal dominant, after uniparental disomy
Donohue (leprechaunism)	24200	Elfin facies with thick lips; large, low-set ears; prominent breasts and external genitalia; hirsutism; abnormal carbohydrate metabolism; failure to thrive; motor and mental retardation	Autosomal recessive
Fraser	219000	Cryptophthalmia, external ear and nose anomalies, laryngeal stenosis, syndactyly, skeletal defects, renal agenesis, large clitoris and labia majora, mental retardation	Autosomal recessive
HFG	140000	Metacarpal and metatarsal anomalies, malformed thumbs, displaced urethral meatus, urinary incontinence	Autosomal dominant
Johanson–Blizzard	243800	Deafness, hypoplastic alae nasi, primary hypothyroidism, mental retardation	Autosomal recessive
Laryngeal atresia	607132	Hydrocephaly, complete or partial laryngeal obstruction, tracheoesophageal fistula or atresia, renal hypoplasia, varus deformity of feet	Unknown
Meckel–Gruber	24900, 603194 607301	Microcephaly, posterior encephalocele, eye anomalies, cleft palate, polycystic kidneys, polydactyly	Autosomal recessive
Roberts	268300	Sparse, silvery blond hair; midfacial hemangioma; cleft lip with or without cleft palate; limb reduction defect; intrauterine growth retardation	Autosomal recessive
Cavalcanti		Tibial aplasia, triphalangeal thumb, micratia, scaliosis, club foot (Müllerian aplasia also reported)	Unknown
Rudiger	268650	Bifid uvula, coarse facies, absent ear cartilage, hydronephrosis secondary to ureterovesical stenosis, short digits	Autosomal recessive
Thalidomide teratogenicity		Nasal hemangioma, neurosensory hearing loss, ear anomalies, limb reduction defects, visceral anomalies	Teratogen
Trisomy 18		Prominent occiput, malformed ears, micrognathia, short sternum, cardiac defects, horseshoe kidney, overlapping fingers, intrauterine growth retardation, severe development retardation	Chromosomal aneuploidy
Trisomy 13		Microcephaly, microphthalmia, malformed ears, cleft lip and palate, cardiac anomalies, polydactyly, intrauterine growth retardation, severe developmental retardation	Chromosomal aneuploidy
Urogenital dysplasia, hereditary (hereditary renal agenesis)		Oligohydramnios, flattened (Potter) facies, pulmonary hypoplasia, unilateral or bilateral absent kidneys, limb deformities	Autosomal dominant

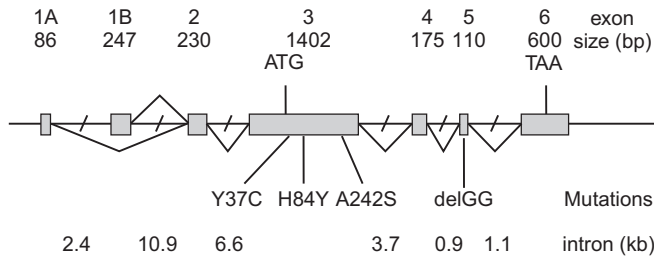
From Simpson, J. L. Genetics of the Female Reproductive Ducts. *Am. J. Med. Genet.* 1999, 89, 224–239.

38 showed an AMH mutation, 33 an AMHR mutation, and 11 neither (392).

**88.2.10.10.1 AMH Mutation.** Located on 19p13.3, AMH consists of five exons (Figure 88-15). The 3' end is guanine cytosine rich. The AMH gene product can be measured by enzyme-linked immunosorbent assay, but results are informative only before sexual maturation. AMH production is suppressed after puberty. When AMH is not detected, a mutation in the structural gene can usually be demonstrated. Although Imbeaud and coworkers (393) initially failed to detect recurrent AMH mutations in 19 PMD families (molecular heterogeneity),

recurrent mutations were later found (394). In a 2005 tabulation, 38 different mutations were found in 46% of families studied (392). Most mutations were homozygous and found in individuals of North African (Arab) or Mediterranean descent.

**88.2.10.10.2 AMH Receptor.** The AMHR II gene is located on 12q13.12. It consists of 11 exons (Figure 88-15) and is 8700 bp in length. AMHR II mutations have now been found throughout the gene. Compared with AMH-negative cases, AMH-positive cases are relatively less likely to be found in north African Arab populations.



**FIGURE 88-15** Diagram of MKS structure and mutations. Exons are shown as rectangles with alternate splicing of exons 1a and 1b as indicated. The sequence alterations of the Old Order Amish chromosome are H84Y and A242S. (Data from Stone, D. L.; Slavotinek, A.; Bouffard, G. G., et al. *Mutation of a Gene Encoding a Putative Chaperonin Causes McKusick-Kaufman Syndrome*. *Nat. Genet.* 2000, 25, 79–82.)

If AMH is elevated, an AMHR mutation should be suspected. Most mutations found are missense, generally occurring throughout the gene. Mutations in exon 1 and in the 3' portion of exon 5 are most common. The most frequent single perturbation is deletion of 27 bp in exon 10 (del 6331–6357). When present, the deletion exists in homozygous form in 42% of case; compound heterozygosity exists in 58%, the deletion coupled with a missense mutation (394). For diagnosis, Belville and coworkers (394) recommend polymerase chain reaction to detect the 27-bp mutation, followed by sequencing the entire AMHR gene if the deletion is not present in homozygous form.

Women (46,XX) with AMHR mutations undergo puberty normally (394). Actually, this is a mild surprise because transgenic XX mice that chronically express AMH show gonadal abnormalities (5). Granulosa cells also normally produce AMH (395,396), for which reason a role for AMH in gonadal development seems plausible.

**88.2.10.11 CAVD and CF.** Almost all men with CF are infertile, specifically as a result of CAVD (CBAVD). At least 70% if not almost all men with CBAVD have one or more mutations in CFTR. The most common CF mutations causing CAVD are  $\Delta F508$  and W128X.

In addition to the missense and nonsense mutations in CFTR causing classic CF with pancreatic and pulmonary pathologies, a deleterious polymorphism of specific relevance to CBAVD exists when 5-thymidines (5-T) are present in a particular location in intron 8 (397). The 5-T polymorphism (allele) results in very low (10%) transcription of CFTR from that chromosome (cis), owing to improper exon–intron splicing and subsequent loss of exon 9. The 7-T polymorphism has less effect, and 9-T none. If 5-T “polymorphism” is homozygous, CBAVD results. Classic CF (pulmonary and pancreatic diseases) does not result because the 10% CFTR gene product is sufficient for normal lung and pancreas function.  $\Delta F508/5-T$  compound heterozygosity leads to CBAVD. However, if 5-T exists cis with R117H, that chromosome produces no CFTR. Thus,  $\Delta F508/R117H + 5-T$  results in classical CF with pancreatic and pulmonary symptoms.

For purposes of genetic counseling, CAVD cases should be assumed to involve compound heterozygosity for two mutant CF alleles or one CF allele and the 5-T variant. It

should be assumed that both CF alleles are dysfunctional, even if one or both are not diagnostically evident. The exception arises if unilateral renal aplasia is present (398).

## 88.2.11 Miscellaneous Disorders

**88.2.11.1 Absence of Testes (Anorchia).** Males (46,XY) with unilateral or bilateral anorchia have unambiguous male external genitalia, normal Wolffian derivatives, no Müllerian derivatives and no detectable testes (1,111). Testicular tissue is not detected in the scrotum, the inguinal canal, or the entire path along which the testes descend during embryogenesis. Splenic–gonadal fusion can mimic the disorder. Unilateral anorchia is asymptomatic and not extraordinarily rare. Bilateral anorchia is far less common. Somatic abnormalities do not ordinarily coexist.

Despite the absence of testes (anorchia), the phallus is well differentiated. Pathogenesis presumably involves atrophy of fetal testes after 12–16 weeks' gestation, by which time male genital differentiation has occurred. Vasa deferentia terminate blindly, often in association with spermatic vessels.

Heritable tendencies exist (399), but recognition of monozygotic twins discordant for bilateral anorchia (400) has long indicated that genetic factors are not paramount. Pathogenesis could involve in utero torsion of the testicular artery, which is known to have a heritable tendency (401,402).

Anorchia is a component of several malformation syndromes in which the external genitalia are unequivocally male. These include Cross syndrome (403), the OEIS syndrome (omphalocele, extrophy, imperforate anus, and spine deformity) (177,404), Saldino syndrome (405) (cone-shaped epiphyses), and sirenomelia. (Anorchia as the sole anomaly has also been observed in an unusual family in which an SF1 mutation was segregating.) Family members with this mutation showed a wide variety of phenotypes, ranging from XY sex reversal to hypospadias to anorchia. Philibert et al. (148) sought SF1 mutations in various conditions and found R114Q in a boy whose only abnormality was anorchia.

**88.2.11.2 Absence of Ovary and Fallopian Tubes.** Otherwise normal females may show absence of one ovary, usually in conjunction with ipsilateral absence of the fallopian tube (406,407). Pathogenesis probably involves congenital absence of a vessel or the occurrence of a vascular accident (torsion). These hypotheses are analogous to those invoked for anorchia.

**88.2.11.3 Accessory or Malpositioned Gonads.** Two ovaries or more than two testes may exist in a single individual (408,409).

Polyorchidism is far more common than polyovaries, with more than 100 cases of polyorchidism being reported (408,410). In half of patients, the testes are scrotal; in other cases, maldescent may exist, sometimes with intra-abdominal gonadal location. Gonads may also be located near the kidneys (411) or fused to the spleen (412). No familial aggregates have been reported.

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# CHAPTER

# 89

## Cancer of the Breast and Female Reproductive Tract

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### 89.1 HISTORICAL BACKGROUND: MAPPING BREAST CANCER GENES

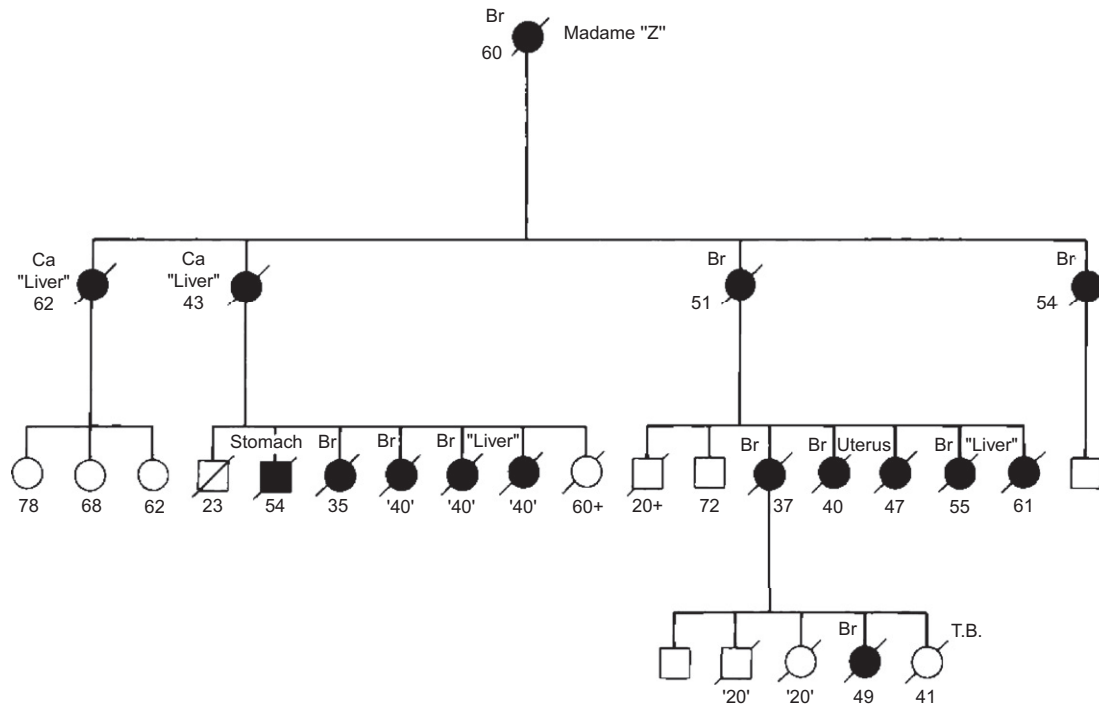
It has long been clear that the resolution of many controversies—the overall scale of the genetic contribution to breast cancer, the number of major genes implicated and their modes of inheritance, the distinctions between different clinical types of breast cancer family, and the relationship between genetic and environmental factors in breast cancer etiology—could come only through identification and characterization of the relevant genes. Despite many serious attempts at linkage analysis and a number of tentative claims (1), no real progress was made until 1990. That year, the association between germline mutations in p53 and at least some instances of Li-Fraumeni cancer family syndrome was reported (2,3). Some months later, a LOD score of +5.98 was found for the CMM86 locus on chromosome 17 at q21 in a subset of breast cancer families characterized by mean age of onset before 45 years (4). The latter association had been found through a systematic screen of mapped polymorphic markers, because unlike polyposis coli or retinoblastoma, no helpful cytogenetic clues to the location of genes implicated in human breast cancer were forthcoming.

### 89.2 DISCOVERY OF BRCA1

Support for a candidate gene locus on 17q came promptly (5) when three of five large breast/ovarian cancer families were shown to give substantial positive LOD scores with CMM86; thereafter, an international consortium effort was launched, using six 17q markers to improve the accuracy of mapping the breast cancer locus and to establish the characteristics of 17q-linked cancer families. These families all came from pedigrees that were reminiscent of “Madame Z,” the original hereditary breast-ovary family described by Dr Broca in 1898 (Figure 89-1). In April 1993, results were reported for 214 families by Easton and colleagues (6) who indicated that the gene

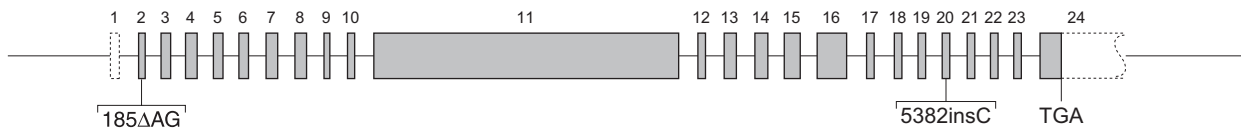
termed “BRCA1” lay approximately 20 cm centromeric of CMM86 and that the great majority of families with breast/ovarian cancer—but only some 45% of families with breast cancer without ovarian cases—were linked to it. There was much stronger evidence for linkage in families with mean age of onset younger than 45 years and evidence against linkage in families that included one or more male breast cancers. These findings gave enormous impetus to subsequent attempts to clone BRCA1. They also provided important insights into the genetic heterogeneity of familial breast cancer: first, by distinguishing male breast cancer families from the rest, and second, by suggesting that within the BRCA1-linked set are subsets characterized by very different relative risks (RRs) of breast and ovarian cancers (7). Nevertheless, Easton and colleagues highlighted some of the inherent difficulties of classic genetic studies in this situation. Only large multi-case families can provide much linkage information, and these may be atypical, particularly with respect to penetrance of the trait. Unaffected family members are usually treated as uninformative, which may also distort estimates of penetrance. Furthermore, even a single sporadic case of cancer in a large family may cast doubt as to linkage, and because sporadic cases are likely to occur more frequently in older subjects, this may contribute to the disparity in LOD scores for families with different mean age of onset.

The cloning of BRCA1 was accomplished through a long and labor-intensive search of yeast artificial chromosomes and cosmids from the appropriate region. Ultimately, Skolnick’s group and their collaborators at the University of Utah and Myriad Genetics identified mutations that cosegregated with the disease in affected members of five of eight breast cancer kindreds, only four of which had substantial positive LOD scores for 17q12 markers, although the others showed haplotype sharing at this locus for at least three affected members (8). The gene implicated (Figure 89-2) is a large one, with

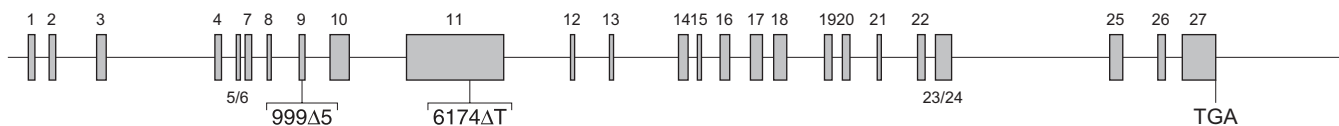


**FIGURE 89-1** Illustration of the family tree Madame "Z". The original hereditary breast ovary family described by Dr Paul Broca in France in 1852. Most commentators believe it was his wife's family.

#### BRCA1



#### BRCA2



**FIGURE 89-2** Exon/intron maps of the human BRCA1 and BRCA2 genes. Exon 1 is not transcribed in either genes. Some of the mutations identified in early studies are indicated below the gene map. Note that already two recurring mutations (deletion of AG in exon 2 and insertion of C in exon 20) were recognizable. (From Simard, J.; Tonin, P.; Durocher, F., et al. *Common Origins of BRCA1 Mutations in Canadian Breast and Ovarian Cancer Families*. Nat. Genet. 1994, 8, 393–399.)

24 exons spanning some 100kb of genomic DNA and encoding a protein of 1863 amino acids.

The initial stages of the search for BRCA2 have been described earlier. Once linkage to the proximal region of 13q had been established, this again became a mapping race. The gene was identified, and a partial sequence, including documentation of some mutations, was published by Stratton's group from the Institute of Cancer Research, London (9). A more complete sequence and further mutations were reported by the Myriad Genetics team a few months later (10).

On the basis that most families with breast and ovarian cancers are linked to BRCA1, Easton and colleagues (6) used the excess of observed over expected breast cancers in relatives of ovarian cancer patients (and vice versa) to

calculate the population frequency of relevant BRCA1 mutations. If all the excess is attributable to high-penetrance BRCA1 mutations, the overall frequency of these is in the order of 0.0007 (i.e. about one in 700 women worldwide is a heterozygous carrier). A similar result was derived by Peto and colleagues (11) from the frequency of identified mutations in breast cancer patients, the estimated penetrance, and the efficiency of mutation detection. It may appear to be very low compared with the figures quoted earlier, which were derived from segregation analyses, but there is an important distinction between mutations at any one locus and the cumulative frequencies of all mutations at any loci that may predispose to breast or ovarian cancer. Furthermore, in most calculations of gene frequency from epidemiologic data,



there is an inverse relationship between penetrance and frequency. Thus, if some BRCA1 mutations carry lifetime risks for cancer substantially lower than 80%, their frequencies may be considerably higher than the above-mentioned figure suggests (12).

In addition to breast and ovarian cancers, early studies indicated that BRCA1 mutations confer lifetime risks for colon and prostate cancers that are some three to five times higher than the population average (13). Subsequently, these findings have been only partially confirmed: the pancreatic cancer risk is significantly elevated in BRCA2 as is the prostate cancer risk, but other cancers, including pancreatic, cervical, and uterine cancers, have been reported as overrepresented among mutation carriers. However, the numbers of family members included in the initial surveys only permitted reliable calculations to be made in respect to common tumors (14–16). In the past few years, the colon cancer risk has been largely dismissed (17), and there has been a significant controversy over the potential contribution to endometrial cancer, specifically the papillary serous endometrial cancer (18). Most of them now agree that endometrial cancer is not an independent cancer risk in BRCA carriers and that the excess endometrial cancer is due to the use of tamoxifen (19).

### 89.3 DISCOVERY OF BRCA2

A collection of 22 breast cancer families with at least one affected male relative was assembled, also through the auspices of the international consortium. Formal exclusion of linkage to BRCA1 was reported by Stratton and colleagues (20) (aggregate LOD of  $-16.63$ ). A few months later, results were published (9) about a systematic linkage screen on 15 large breast cancer kindreds not linked to BRCA1 (including six with affected male relatives) that localized a second breast cancer susceptibility locus (BRCA2) to human chromosome 13 at q12–13, proximal to the retinoblastoma gene. All but one of the families with affected male relatives gave some evidence of linkage (multipoint LODs  $+0.15$  to  $+3.70$ ) but so also did four of the five families with breast and ovarian cancer patients (multipoint LODs  $+0.86$  to  $+3.70$ ). Five of the families included both affected male relatives and patients with ovarian cancer. The conclusion was that BRCA2 is implicated in a substantial proportion of those families with breast cancer not linked to BRCA1. The conclusion also indicated that involvement of BRCA2 carries a high risk for female breast cancer—penetrance figures of up to 87% by age 80 have been presented, although this is probably a high estimate (13,21–23) and appreciable, though lower, risks for male breast and ovarian cancers. Other cancers overrepresented in families with BRCA2 include prostate, pancreatic, and ocular melanoma (24,25). The gene was cloned in December 1995 (9,10) and shows only minimal homology with BRCA1.

#### 89.3.1 Identification and Characterization of BRCA1 and BRCA2

BRCA2 is even larger than BRCA1, with 27 exons encoding a protein of 3418 amino acids. Both are transcribed in several tissues, most abundantly in developing thymus and testis and also in the breast and ovary. There is only minimal homology between them and little homology to any other known genes, apart from a ring finger domain at the N-terminus of BRCA1 and a heptad repeat element in the middle of the protein, which might enhance dimerization. Both are common characteristics of DNA-binding proteins such as transcription factors. Neither feature is found in BRCA2. Both BRCA1 and BRCA2 are involved in multiple functions, including homologous recombination (HR), DNA repair, and transcriptional regulation (26–28). There is also evidence that BRCA1 is an important link in the signal chain that starts with recognition of DNA damage (sensed by ATM) and leads to cell cycle arrest at the G2/M checkpoint (29,30). BRCA1 is a 220-kD nuclear phosphoprotein. DNA checkpoint-associated kinases such as CHK2, ATM and RAD51 phosphorylate various specific residues of BRCA1 when the cell has undergone DNA damage, making the cells more or less sensitive to environmental stressors (31).

As in the case of BRCA1, there are truncating mutations throughout the BRCA2 gene, and these include small deletions, insertions, and nonsense base substitutions in coding regions, splice sites, and regulatory elements. In BRCA1, large genomic deletions covering several exons account for a substantial proportion of germline mutations, particularly in the Dutch population (32). As in the case of BRCA1, certain recurring founder BRCA2 mutations have been identified in particular populations. These include 6174delT in Ashkenazi Jews and 999del5 in Icelanders (33,34).

Data on BRCA1 and BRCA2 mutations are listed on a National Human Genome Research Institute Web site, the Breast Cancer Information Core, but information on mutations and particularly variants of uncertain clinical significance is not consistently and uniformly added to the database. Initially, in several genetically homogeneous populations, recurrent mutations were identified in BRCA1, BRCA2, or both (Figure 89-2). These include the Ashkenazi Jewish mutations, the Icelandic BRCA2 999del5, and the major BRCA1 gene deletions in Dutch families already mentioned, which make up the majority of mutations detected within those populations. There are also at least four recurring BRCA1 mutations in Norwegian families (35,36) and at least two (BRCA1 2800delAA and BRCA2 6503 delTT) characteristics of Scottish/Irish families, including many now living in North America (37), but now there have been recurrent mutations detected worldwide. Though not of the same frequency, recurrent “founder” mutations are seen in numerous population isolates, including as divergent populations as west Africa, Japan, Pakistan, and Italy (Table 89-1).

**TABLE 89-1** Significant BRCA1/2 Founder Mutations in Populations Worldwide

Population	Mutation BRCA1	Mutation BRCA2
Ashkenazi Jews	185delAG 5832insC	
Icelanders		6174delT 995delG
Norwegians	1675delA 816delGT 3347delAG 1135insA	
Finns	IVS11 + 3A>G	9345 + 1G>A C7708T T8555G
Swedes	3171ins5	
French	3600del11	
Dutch	2804delAA IVS12-1643del3835	5579insA 6503delTT
Italians (Calabria)	5083del19	
Italians (Sardinia)		8765delAG
French Canadians (Quebec)	C4446T	8765delAG 3398delAAAAG
Hispanics (south California)	R1443X S995X 2552delC	
Hispanics (Columbia)	3450delCAAG A1708E	3034delACAA
Afro-Americans	943ins10 1832del5 5296del4	IVS13 + 1G>A
South Africans	E881X	
Iraqi/Iranian Jews	Tyr978X	
Chinese	1081delG	
Japanese	Q934X L63X	5802delAATT
Malaysians	2846insA	
Filipinos		4265delCT 4859delA
Pakistanis	5454delC S1503X R1835X	

Frequency of founder mutations and proportion of breast and ovarian cancers related to BRCA mutations vary widely. Ethnic and geographic isolation increases likelihood of founder mutations.

Reprinted with permission from: Ferla, R., et al. Founder Mutations in BRCA1 and BRCA2 Genes. *Ann. Oncol.*, 2007, 18, Suppl 6, vi93–vi98.

Interestingly, when appropriate studies have been undertaken, it appears that common mutations in different families usually entail preservation of a shared haplotype, as defined by a number of polymorphic intragenic and close-flanking markers, even when the families concerned appear to be from widely separated geographic regions and communities (38). This not only suggests founder effects rather than mutational “hot spots” as the basis for the recurrent mutations but also points to a high degree of genetic stability at this locus, over many centuries, with a remarkably low rate of new mutations.

There have been only a few case reports published in the world on confirmed de novo mutations but have been recorded in both *BRCA1* and *BRCA2* (39).

In the general population, it is estimated that about 1 in 500 people (of either gender) carry a *BRCA1/2* mutation (40) (Table 89-3). Due to population genetic effects, geographically or socially isolated populations or mass genocide resulting in a narrow reproductive pool, certain recurrent mutations are fairly frequent in some populations (Table 89-1) (41,42). Example populations include eastern European Ashkenazi Jewish, French Canadian and Icelandic populations where the prevalence of *BRCA* mutations can be as high as 1 in 45 (34). Common recurrent mutations also have been described in Polish and Filipino populations (43). Afro-American and Afro-Caribbean populations have not been as extensively studied, but the *BRCA1* 943ins10 mutation is a very old west African founder mutation (44) and has been tracked through the West Indies and the United States (45). *BRCA* carrier frequency is 32% among breast/ovary cancer families in Japan, equal to Ashkenazi Jewish women, although Pakistan has the highest rates of breast and ovarian cancers in Asia, also due to recurrent founder *BRCA* mutations (41,46). The frequency of mutations found among Israeli Ashkenazi Jewish women with ovarian cancer, even without any family history, is more than 30% (47).

## 89.4 PATHOLOGY OF FAMILIAL BREAST CANCERS

One of the early noted features of familial breast cancers is that they tend to show an excess of the unusual medullary histologic type, although allowance must be made for the early age of onset that characterizes both familial breast cancers and those with medullary features on histology (36,48,49). One comprehensive survey (50) has found that when adjusted for age of onset, breast cancers arising in members of families with strong evidence of genetic predisposition are not overall strikingly different from those that occur in the population at large. Most are invasive ductal tumors of “no special type,” according to the World Health Organization breast cancer classification. However, there are significant differences within the general category of familial cancers. Initial histologic observations in tumors in probable *BRCA1* mutation carriers (defined by genetic linkage) were predominantly aneuploid, with a DNA index in the hyperdiploid range and with a high S-phase fraction, indicating rapid proliferation. In contrast, tumors from non-*BRCA1*-linked families showed a significant excess of cases with tubular or lobular features on histology, suggesting that there may be a separate genetic basis for a subset of breast cancers belonging to these (related) pathologic types. A review of the pathology of breast tumors from *BRCA1* and *BRCA2* mutation carriers has been conducted under the auspices of the Breast Cancer Linkage Consortium. The above-mentioned findings were essentially confirmed

and extended. Cancers associated with germline BRCA1 mutations tend to have the appearances of high grade (e.g. extreme pleomorphism, high mitotic index and S-phase fraction, and little tubule formation). The majority are estrogen receptor (ER)-negative, but in contrast to most high-grade sporadic tumors, they also tend to show low expression of Erb-B2 (Her-2, neu). This is referred to as the “basal phenotype.” BRCA2 tumors show similar features, but the majority (80%) are hormone-positive (51). Likewise, BRCA1 tumors (but not those with BRCA2 mutations) tend to show substantial lymphocytic infiltration. Both categories were noted to show continuous “pushing” margins, apparently compressing rather than invading adjacent tissues (21,52,53). On the basis of histologic appearances, an experienced pathologist may suggest an underlying BRCA mutation even in the absence of a known family history. Cytokeratin 5 and 14 have been shown to have high predictive value for underlying germline BRCA1 mutation (54). It has been suggested that since BRCA1 tumors are so often ER-negative, a cell of origin effect is reflected and it is intrinsic. Since the 20% of ER-positive tumors seen in BRCA1 carriers tend to occur in older women, these cancers may be incidental or “phenocopies” and not a consequence of the BRCA1 deficiency (55,56). Tung and colleagues have shown that loss of heterozygosity for BRCA1 is associated with about half of ER-negative sporadic breast cancers but 97% of BRCA1 carriers, regardless of whether the cancer was ER-positive or ER-negative, and that there was significant skewing toward loss of the wild-type (wt) allele, suggesting that the mutant allele has a role in the ER-positive tumors as well (57).

As in the Marcus study (50), familial breast cancers not attributable to BRCA1/2 mutations tend to have distinctly favorable prognostic features, being generally of low histologic grade and including well-differentiated tubular and lobular categories (53). In keeping with the epidemiologic and histologic evidence for fundamental differences among the various categories of familial breast cancer, molecular analysis at the level of both DNA structure and gene expression shows that tumors associated with germline mutations in BRCA1 or BRCA2 are distinct from each other and from non-BRCA tumors (58,59).

### 89.5 BRCA1 AND BRCA2 MUTATION ANALYSIS

Because BRCA1 and BRCA2 are so large, searching for mutations is a major undertaking. The procedure involves amplification of the exons and the intronic splice junction sites with suitably chosen polymerase chain reaction (PCR) primer pairs. The original BRCA1 study (8) used 25 such pairs, but more than 50 have now been documented (60), including 24 for exon 11, which comprises approximately 60% of the coding sequence. For BRCA2, the number of primer sets required is even greater. Ideally, the PCR products should be no more than 300 bases

long. They may be sequenced directly or tested for the presence of a mutation by an indirect approach such as the single-strand conformational polymorphism (SSCP) assay, denaturing gradient gel electrophoresis, and heteroduplex analysis or mismatch cleavage (chemically enhanced or by bacterial resolvase). None of these indirect methods can guarantee detection of all mutations (61,62). SSCP was, for several years, the most widely favored approach because of its relative simplicity, but it may be insensitive to single-base substitutions, especially if the change occurs near the middle of a large PCR fragment. Many laboratories now resolve PCR fragments by denaturing high-performance liquid chromatography on resin-based columns, which provides a sensitive, rapid, and inexpensive alternative to gel electrophoresis (63).

Protein truncation assays (64) are favored in several laboratories. Because BRCA1 and BRCA2 complementary DNA (cDNA) can be generated from blood lymphocytes, it is technically feasible to use a prokaryotic translation “cassette” to express the sequence in vitro and hence to demonstrate alterations in the length of a specific protein fragment. When only genomic DNA is available, the same technique can be applied to exon 11 (which encodes over half the protein for both genes) plus, for BRCA2, exon 10, which is also large (65). A number of permutations and variations of these basic techniques are currently in use in both research and diagnostic settings throughout the world, but the most efficient analytical process appears to be automated direct sequencing of all the exons and splice sites for both genes. This can detect the causal BRCA1 or BRCA2 mutation in more than 80% of all multicase families with breast cancer or breast and ovarian cancers, but the capital investment required to undertake such a screen on very large number of samples would probably be prohibitive, except on a commercial scale.

Large deletions in BRCA1, which may represent 10% or more of all mutations in some populations (32), can be detected by multiplex ligation-dependent probe amplification (MLPA) and related techniques (66,67). In a study of high-risk BRCA mutation-negative families, defined as four or more breast or ovarian cancers but wt BRCA1/2 results, Walsh found that 12% harbored a large genomic rearrangement missed by sequencing (68). This led to heightened pressure to overturn the BRCA1 and BRCA2 patent held by Myriad Genetics Corporation but only resulted in the laboratory offering additional testing for genomic rearrangements via quantitative PCR rather than MLPA. This is referred to as BART™, for BRCA rearrangement test.

The cost and the technical effort involved usually mean that strict criteria must be applied before a BRCA1 or BRCA2 mutation screen is embarked on. When a family belongs to a population group where “founder” mutations account for a substantial proportion of hereditary cases, an initial analysis limited to the recurring mutations is an attractive option. A reasonably robust



estimate of the likelihood that a germline mutation is present in one or the other gene can be derived simply from the (verified) family history. Thus, for example, if there are six or more individuals with female breast cancer, or just a single case of breast and ovarian cancers in the same patient, there is greater than 50% chance that a BRCA1 or BRCA2 germline mutation is present (69). This approach has been refined, for example, by development of a computer model, BRCAPRO (70), and empirically by Evans and colleagues (71), whose scoring system is based on the number, nature, and ages of onset of tumors in a given family. Both methods predict the likelihood of a BRCA1 or BRCA2 mutation and can be used to prioritize families for molecular testing. With increased volume of testing and testing of less “classic” hereditary breast–ovarian syndrome pedigrees, more mutations have been discovered than by the original high-risk cohorts. The generally accepted current indications for BRCA testing are noted in Table 89-2. Current testing guidelines vary slightly by professional society and country, but insurance companies in the United

States most often cite the National Comprehensive Cancer Network (NCCN) ([http://www.nccn.org/professionals/physician\\_gls/f\\_guidelines.asp](http://www.nccn.org/professionals/physician_gls/f_guidelines.asp)). Because of the availability of more targeted mutation testing at reduced cost for high-risk populations such as Ashkenazi Jewish individuals, testing should proceed in a stepwise manner based on the burden of the family history (Figure 89-3). In the future, particularly for BRCA1, initial screening may be in the molecular pathology laboratory on breast tumors, regardless of patient’s age or family history, in the same way that mismatch repair (MMR) gene defects are now screened for in colon cancers.

### 89.5.1 The Myriad Patent

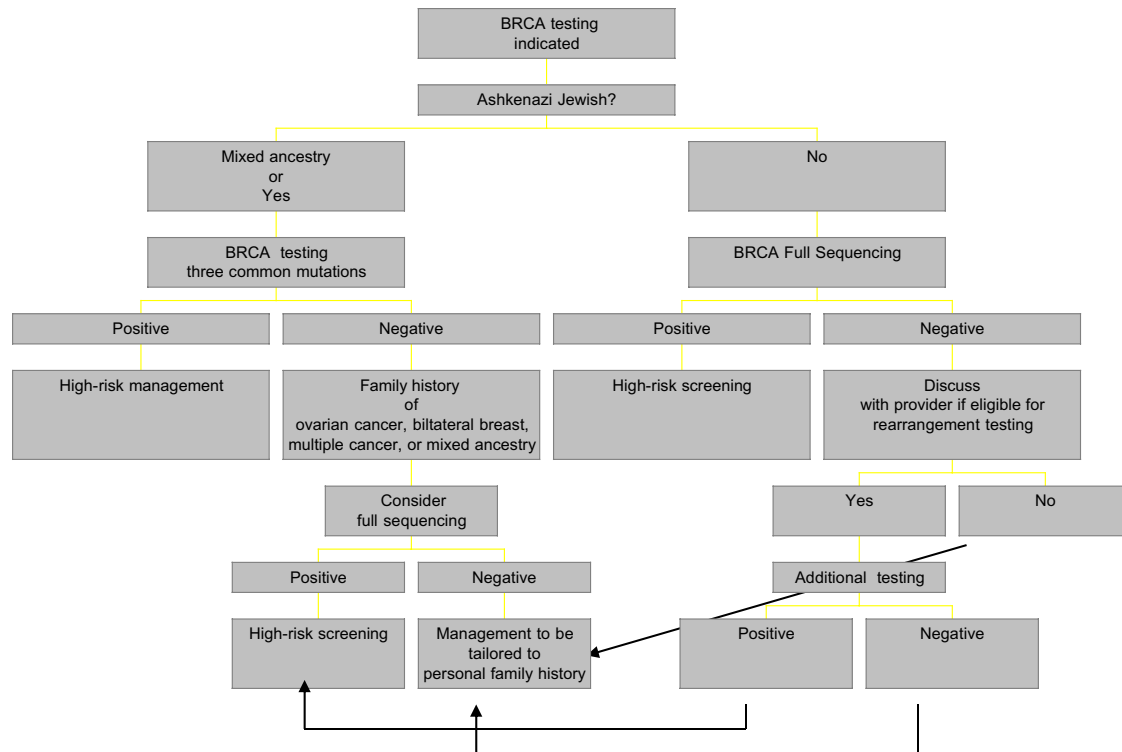
The construct of patenting a gene is one that garners great debate and fervor on both sides of the argument. Thousands of gene patents have been issued in the United States for the process relating to the sequencing of a particular gene. Though some require nominal licensing fees for use of the primers, most are open source. The notorious exceptions to this are the BRCA1 and BRCA2 gene patents held by Myriad Genetics Laboratory. Myriad has held exclusive rights to the commercial sequencing of the BRCA1/2 genes in North America and Europe since 1994. In May 2009, a group of plaintiffs led by the American Civil Liberties Union and the Public Patent Foundation challenged various aspects of the patent in a New York District Court case *Association for Molecular Pathology et al vs. US Patent and Trademark office*. In a landmark decision in March 2010, Judge Sweet overturned the Myriad patents, stating that isolated DNA was not materially different from that which exists in nature. Myriad immediately filed an appeal and there has been no practical changes in clinical practice in terms of availability of commercial testing in any other laboratory or changes in cost of testing (which at the end of 2011 approaches \$4000 for sequencing of both genes, with an additional cost of \$700 for testing of genomic rearrangements). In July 2011, a three-judge Federal Circuit Court reinstated the Myriad patent with a 2:1 opinion that cDNA in particular differs significantly from DNA in nature and is therefore eligible for patent under article 101 of the Patent Act, reversing the lower courts invalidation of product claims for BRCA genes and fragments. The judges, however, affirmed the lower courts ruling that Myriad’s methods for comparing mutant to normal sequences of the BRCA genes were *not* subject to patent protection. Both parties have since submitted petitions for further review, which had been denied by the Circuit Court of Appeals. It has been decided that the case will be heard by the US Supreme Court. Though the case is critical in terms of the broader issues in terms of whole-genome sequencing and feasibility of multiplex arrays in clinical testing, the patents on the BRCA genes will begin to expire in 2014.

**TABLE 89-2** Personal and Family History Indications for BRCA1/2 Testing

- Women with a Personal History of Breast Cancer with One or More of the Following:
  - Diagnosis at age  $\leq 45$
  - Diagnosis at age  $\leq 50$ , with  $\geq 1$  first-, second-, or third-degree relatives with breast cancer at age  $\leq 50$
  - Bilateral breast cancer or two breast primaries particularly if the first diagnosed at age  $\leq 50$
  - Diagnosis at any age, with  $\geq 2$  first-, second-, or third-degree relatives with breast and/or epithelial ovarian/fallopian tube/primary peritoneal cancer at any age
  - A male relative with breast cancer
  - A personal history of epithelial ovarian/fallopian tube/primary peritoneal cancer
- For an individual of ethnicity associated with higher mutation frequency (e.g. Ashkenazi Jewish, Dutch, Icelandic, Swedish, and Hungarian), no additional family history is necessary to test for the founder mutations
  - Triple-negative breast cancer before the age of 60 years
- Personal history of epithelial ovarian/fallopian tube/primary peritoneal cancer even if no other family history
- Personal history of male breast cancer even without any other family history
- Family history only:
  - First- or second-degree blood relative, meeting any of the above criteria
  - Third-degree blood relative with breast cancer and/or ovarian/fallopian tube/primary peritoneal cancer, with  $\geq 2$  close blood relatives with breast cancer (at least 1 with breast cancer at age  $\leq 50$ ) and/or ovarian cancer

NCCN guidelines are considered the most authoritative in the United States ([www.nccn.org/guidelines](http://www.nccn.org/guidelines), version 1.2011), but all US insurance companies have differing criteria for eligibility of testing. Indications noted above are minimum criteria recognized by most family cancer clinics and insurers. Refs: (12,149,274–276).





**FIGURE 89-3** Flowchart demonstrating stepwise approach for BRCA1/2 testing. Ashkenazi Jewish individuals are used as an example founder population, but any other high-frequency founder group would follow the same testing. Until genomic rearrangement testing is standard on all comprehensive tests, BART™ must always be considered as additional testing, though the yield is low in Caucasian populations. (From Morris, J. L.; Gordon, O. K. *Positive Results: Making the Best Decisions when You're at High Risk for Breast or Ovarian Cancer*. Prometheus Books: Amherst, NY, 2010. Reprinted with permission of Prometheus Books; [www.prometheusbooks.com](http://www.prometheusbooks.com))

### 89.5.2 Genetics and the Jews

The first major Jewish dispersion out of the land of Israel occurred after the Babylonian conquest and with it the fall of the First Temple in the year 586 BCE. This dispersion was to Mesopotamia, to the land now known primarily as Iraq. After several generations, some Jews returned to the land of Israel, but after the revolt against the Persians in the fourth-century BCE, many migrated further north toward the Caspian Sea and began the further gradual migration into Europe. Those who migrated north and west became the Ashkenazi Jews. The Jews who remained in Mesopotamia became the Persian Jews.

The next major upheaval and Jewish migration was the destruction of the Second Temple in the year 70 CE. At this time, the Romans shipped about 80,000 Jews as slaves to what is now Spain, south of Cordova. The descendants of these slaves became the Sephardic population. Until these Jews were expelled from Portugal and Spain in fifteenth century, there was no intermarriage between Sephardic Jews and Jews in other parts of the world.

The oldest of these founder mutations is 187delAG on *BRCA1* (previously designated 185delAG). It has been traced back between 2500 and 3000 years to around the time of the destruction of the First Temple (72). This mutation was carried north by the migration from

Mesopotamia through Europe. It occurs in about 1% of Jews in the United States and Europe. This mutation, interestingly, is also prevalent in certain Hispanic populations in northern Mexico and the southwest United States, areas that were settled by Spanish merchants. Historians believe that many of these settlers were *Conversos*, Jews who converted to Catholicism in response to the Inquisition and introduced this founder mutation into that community (73).

The second mutation, 6174delT in *BRCA2*, is the most prevalent of the three Jewish *BRCA* mutations, occurring in about 1.4% of Ashkenazi Jews. It does not appear in either Persian or Sephardic Jews. Because it does not appear in Persian Jews, the mutation must have occurred after the resettlement of the Ashkenazi Jews to Europe. This mutation likely originated in eastern Europe about 700 years ago, based on the mitochondrial DNA of carriers (72).

The third mutation is also on the *BRCA1* gene, although it is present in a far smaller percentage of Jews. Only about one-tenth of 1% of Ashkenazi Jews carry this third mutation known as 5382insC. This mutation is linked to another event in Jewish world history because it is also common among high-risk, non-Jewish women of eastern Europe, Russia, and Poland, in particular. Some call it the “pogrom” mutation and believe it was introduced into the Jewish community as a result of

pregnancies following conquests between the thirteenth and the nineteenth centuries. But like the 187delAG mutation that is common in certain North American Hispanic communities, it could be the reverse. Jewish individuals who converted to Catholicism may have perpetuated this mutation in the non-Jewish Polish population.

Together, these three mutations are found in 2.5% of so-called Ashkenazi Jewish women and men, in Israel, the United States, and Europe. The high rate of mutation carriers in the Jewish community is as a result of the cultural practice of a closed reproductive pool. Approximately 90% of American Jews are eastern European or Ashkenazi. Among those, 95% with a *BRCA* mutation carry one of these three founders, while about 5% of American Jews with a *BRCA* mutation carry one of the 2000 mutations, most of which are individually rare. These other mutations have been introduced because intermarriage and conversion have become more common, as well as due to the rare spontaneous new mutation.

## 89.6 RARE FAMILIAL SYNDROMES ASSOCIATED WITH INCREASED RISK FOR BREAST OR OVARIAN CANCER

The highly distinctive features of certain familial syndromes associated with cancer of the breast or ovary have served to emphasize genetic predisposition to these tumors, although the conditions (Table 89-3) are individually rare. Li–Fraumeni syndrome (LFS), for example, probably accounts for less than 5% of all heritable breast cancer cases, and the same is true of Cowden’s syndrome. Lynch syndrome may be the most common, though very rarely features breast cancer, and Peutz–Jeghers syndrome (PJS) very rare.

### 89.6.1 Li–Fraumeni Cancer Family Syndrome

The familial association of early-onset breast cancer with childhood soft tissue sarcomas, adrenal carcinomas, leukemias, or brain tumors (74,75) is certainly very striking and, compared with many other genetic syndromes, not rare. Historically, the fact that the tumors of close relatives may be managed at different specialist centers has perhaps militated against more widespread recognition of the hereditary nature of the condition: affected families have often been found by systematic surveys, for example, of childhood brain tumors or sarcomas, rather than by direct referral to a geneticist from a breast cancer surgeon or pediatric oncologist (76).

When the association between LFS and constitutional mutation in the *p53* gene was reported in 1990 (2,3), it seemed that this particular aspect of familial breast cancer was adequately explained. A very similar spectrum of tumors is also observed in *p53*-deficient transgenic mice (77). However, in a considerable number of “classic”

Li–Fraumeni families, *p53* mutations can be extremely difficult to find (76,78). Some germline mutations involve parts of the gene outside the “hot-spot” regions of exons 5–8, including splice sites and regulatory elements, and partial or whole-gene deletions (79,80). The mutation may be in another gene, *CHEK2*, that participates in an analogous DNA damage response/cell cycle arrest pathway (81).

The clinical definition of the LFS has been widened to encompass germ cell tumors and Wilms’ tumor. Other cancers that may also arise from constitutional *p53* mutations include those of lung, prostate, and pancreas, as well as malignant melanoma. Williams and Anderson (82) concluded that more than 90% of “gene carriers” in Li–Fraumeni kindreds would develop a cancer by age 70, but this study was based on families ascertained, at least to some extent, on the basis of high penetrance.

**TABLE 89-3 Cancer Syndromes with Clinical Features**

Syndrome	Features	Gene
Cowden	Multiple hamartomas of skin and oral mucosa, trichilemmoma, 35% have gastrointestinal (GI) hamartomas, limited malignant potential, autosomal dominant inheritance, breast cancer, uterine cancer, and 10% of both sexes develop thyroid cancer	PTEN
Li–Fraumeni	Classically, early-onset breast cancer in affected females and an excess of childhood sarcomas, acute leukemias, brain tumors, or adrenal cancers. Melanoma, Wilms’ tumor, and germ cell tumors may also be part of the syndrome, which can be widened to include “Li–Fraumeni-like” families. Multiple primary tumors are characteristic	<i>p53</i> <i>CHEK2</i>
Muir–Torre variant of HNPCC	Skin lesions (keratoacanthomas and sebaceous adenomas) associated with ovarian, uterine, colorectal, and duodenal cancers and some excess of breast, bladder, and esophageal cancers	MLH1 MSH2 MSH6 PMS2 EpCAM
PJ	Hyperpigmentation, mainly in and around the mouth. Multiple hamartomatous polyps, particularly of the small intestine, breast, and ovarian sex cord tumor (SCT)	STK11

All individuals with a personal or family history of breast cancer and other malignancies should have a clinical examination to evaluate for the possibility of Cowden syndrome or PJS. Trichilemmomas are pathognomonic for Cowden. Individuals with sebaceous adenomas and/or keratoacanthomas and endometrial or colon cancer should be assessed for Muir–Torre. These cutaneous lesions alone, however, are not an indication for testing.

More recently, *TP53* mutations have been found in families not meeting the original strict clinical diagnosis criteria, and a Li–Fraumeni-like syndrome with more broadly inclusive criteria has emerged (83). These are referred to as the Eeles, Birch, and Chompret criteria (Table 89-4). The breast cancer risk in those with LFS is highly elevated, perhaps as high as 50–60% by age 45 (84,85). Mutations in *TP53* have been identified in 4% of women diagnosed with breast cancer at younger than 30 years, half of which were *new* rather than inherited mutations (84). In 2009, Gonzalez reported on

525 diagnostic samples evaluated for p53 mutations. About half of those tested who met classic LFS were found to have a detectable mutation. Breast, brain, sarcoma, and adrenocortical carcinoma (ACC) were the core cancers seen in all positive cases. However, the Chompret criteria (very young age of onset or multiple primaries in proband) was also sensitive in predicting mutations (79).

So, though a number of historic studies have reported that only 1% or less of unselected breast cancers can be attributed to constitutional p53 mutations, it is clear that this figure is considerably higher for patients who have suffered two primary tumors (e.g. including breast or germ cell cancer), particularly if one of these presented at an early age, even if there is no obvious LFS in the patient's family history, or if the proband has breast cancer at younger than 30 years, ACC, or a choroid plexus tumor (2,79,80).

**TABLE 89-4 Testing Criteria for LFS**

Classic LFS Criteria:
– Combination of an individual diagnosed at age <45 with a sarcoma
AND
– A first-degree relative diagnosed at age <45 with cancer
AND
– An additional first- or second-degree relative in the same lineage, with cancer diagnosed at age <45 or sarcoma at any age
Li–Fraumeni-like syndrome criteria:
Birch:
• Combination of an individual diagnosed with a childhood tumor or sarcoma, brain tumor, or adrenocortical carcinoma diagnosed at age <45
AND
• A first- or second-degree relative with a typical LFS tumor at any age
AND
• Another first- or second-degree relative with cancer diagnosed at age <60 (76)
Chompret:
• An individual with a tumor belonging to the LFS tumor spectrum (soft tissue sarcoma, osteosarcoma, premenopausal breast cancer, brain tumor, adrenocortical carcinoma, leukemia, or lung bronchoalveolar cancer) before the age of 46 years
AND
• At least one first- or second-degree relative with a LFS tumor before the age of 56 years or multiple tumors
OR
• Personal history of multiple tumors (except multiple breast tumors), two of which belong to the LFS tumor spectrum and the first of which occurred before the age of 46 years
• Personal history of adrenocortical carcinoma or choroid plexus tumor
Eeles:
• Two first- or second-degree relatives with LFS-related malignancies at any age
Early-onset breast cancer:
– Individual with breast cancer at age <30, with a negative <i>BRCA1</i> and <i>BRCA2</i> test, especially with a family history of sarcoma, brain tumor, or adrenocortical carcinoma

Criteria have widely varying sensitivity, with Chompret being the most predictive and Eeles the least. Cancers associated with LFS include, but are not limited to, premenopausal breast cancer, bone and soft tissue sarcomas, acute leukemia, brain tumor, adrenocortical carcinoma, colon cancer, early-onset of other adenocarcinomas, or other childhood cancers. Refs: (2,79,80,83–85).

### 89.6.2 Diffuse Hereditary Gastric Cancer Syndrome

E-cadherin gene mutations are often found in sporadic cancers and are frequently used to distinguish histologically lobular breast cancer from ductal carcinoma. Hereditary diffuse gastric cancer is caused by germline mutations in *CDH1*, and women carriers are at greatly increased risk for lobular breast cancer. The gene is inherited as an autosomal dominant disorder and carries an almost 90% risk for diffuse gastric cancer (*limitis plastica*) and up to 40% risk for breast cancer in women and a 67% risk for gastric cancer in men (86,87). Pedigrees with lobular carcinoma of the breast and “stomach cancer” should be assessed for the possibility of an E-cadherin mutation. However, adenocarcinoma of the stomach is the most common form of gastric cancer and is not associated with *CDH1* mutations but is rather associated with *Helicobacter pylori* infections and chronic reflux.

### 89.6.3 Peutz–Jeghers Syndrome

PJS is a multiple cancer susceptibility syndrome that also has clinical features. PJS, which is due to mutations in the serine-threonine kinase 11 (*STK11*) gene, causes multiple colon hamartomas, distinctive pigmented macules of the mucocutaneous tissue, plantar and palmar volar surfaces, and a predisposition to cancer of the small and large intestines, breast, and pancreas. *STK11* is a tumor suppressor gene localized to 19p13.3 and is also known as *LKB1* (88). In a recent meta-analysis review of 20 studies, the most common malignancy seen was colorectal, followed by cancer of the breast and the small bowel and stomach. Other gynecologic malignancies seen are ovarian cancer, which characteristically is a stromal sex-cord tumor and not an epithelial tumor (as in *BRCA*) and cervical cancer. In this meta-analysis of the worldwide experience, the average age of cancer was 42 years,

and women had much higher overall RR for malignancy (89). The frequency estimations widely vary from 1 in 8000–200,000, in part because the clinical syndrome is often overlooked in the differential diagnosis of early-onset breast cancer. Notably, early and bilateral breast cancer is the hallmark of PJS. STK11 is the only gene associated with PJS, and molecular testing can now identify virtually all mutations, thus making genotyping very useful to confirm or exclude a suspected diagnosis (88).

#### 89.6.4 Cowden's Syndrome/PTEN

Cowden syndrome (CS) is a cancer predisposition syndrome arising from mutations in the *PTEN* gene, associated with increased risk for breast, thyroid, and uterine cancers. This condition, like all the others discussed thus far, is inherited as an autosomal dominant disorder. Distinctively, it is associated with the development of hamartomas and benign tumors in the skin and the internal organs and hence the alternative name “multiple hamartoma syndrome” (90). An increased incidence of breast cancer in affected women has long been demonstrated and, indeed, overlaps with two other allelic inherited disorders, Lhermitte–Duclos disease and Bannayan–Zonana syndrome (also known as Bannayan–Riley–Ruvalcaba syndrome), suggesting that all three carry significant risks for malignancy of thyroid, brain, and endometrial, as well as breast (91,92). The causal locus for all three conditions was subsequently mapped to chromosome 10q23, and germline mutations were then identified in a number of affected families, in a gene variously known as PTEN (phosphatase and tensin homolog) or MMAC1 (mutated in multiple advanced cancers) or TEP1 (tumor growth factor  $\beta$ -regulated and epithelial cell-enriched phosphatase) (93,94). The lifetime risk for breast cancer is estimated at 25–50%, the most common malignancy in CS (95), and for endometrial at 5–10% (96).

Diagnostic criteria are sorted into pathognomonic features, major and minor criteria (Table 89-5). Recently, after analysis of over 3000 pediatric and adult patients referred for PTEN testing, a Cleveland Clinic PTEN scoring system was created. This weighted system recommends testing for scores above 10, or a pretest probability of a mutation of 3% (97). Diagnosis is made with a single pathognomonic feature (Lhermitte–Duclos disease or the mucocutaneous lesions: facial trichilemmomas, acral keratosis, or papillomatous papules). Major criteria must include macrocephaly (defined as a head circumference greater than 97th percentile) and endometrial, breast, or non-medullary thyroid cancer. Combinations of minor criteria exist, but the likelihood of detecting a mutation is very low unless major criteria are met (98). Nevertheless, it is quite probable that the diagnosis is overlooked in some breast cancer families because the defining signs and symptoms can be subtle. Breast cancer in patients with Cowden's syndrome may be preceded by a long history of benign proliferative disease (91,99).

**TABLE 89-5** Diagnostic Criteria for Cowden Syndrome

Major Criteria for Cowden Syndrome	Minor Criteria for Cowden Syndrome
<ul style="list-style-type: none"> <li>Breast cancer</li> </ul>	<ul style="list-style-type: none"> <li>Other thyroid lesions (such as adenoma, nodule, goiter)</li> </ul>
<ul style="list-style-type: none"> <li>Mucocutaneous lesions</li> </ul>	<ul style="list-style-type: none"> <li>Mental retardation</li> </ul>
<ul style="list-style-type: none"> <li>One biopsy-proven trichilemmoma</li> </ul>	<ul style="list-style-type: none"> <li>Autism spectrum disorder</li> </ul>
<ul style="list-style-type: none"> <li>Multiple palmoplantar keratosis</li> </ul>	<ul style="list-style-type: none"> <li>Single GI hamartoma or ganglioneuroma</li> </ul>
<ul style="list-style-type: none"> <li>Multifocal or extensive oral mucosal papillomatosis</li> </ul>	<ul style="list-style-type: none"> <li>Fibrocystic disease of the breast</li> </ul>
<ul style="list-style-type: none"> <li>Multiple cutaneous facial papules (often verrucous)</li> </ul>	<ul style="list-style-type: none"> <li>Lipomas</li> </ul>
<ul style="list-style-type: none"> <li>Macular pigmentation of glans penis</li> </ul>	<ul style="list-style-type: none"> <li>Fibromas</li> </ul>
<ul style="list-style-type: none"> <li>Macrocephaly (megalencephaly) (i.e. &gt;97th percentile, 58 cm in adult women and 60 cm in adult men)</li> </ul>	<ul style="list-style-type: none"> <li>Renal cell carcinoma</li> </ul>
<ul style="list-style-type: none"> <li>Endometrial cancer</li> </ul>	<ul style="list-style-type: none"> <li>Uterine fibroids</li> </ul>
<ul style="list-style-type: none"> <li>Non-medullary thyroid cancer</li> </ul>	
<ul style="list-style-type: none"> <li>Multiple GI hamartomas or ganglioneuromas</li> </ul>	
Individual with a personal history of:	
<ul style="list-style-type: none"> <li>Bannayan–Riley–Ruvalcaba syndrome OR</li> </ul>	
<ul style="list-style-type: none"> <li>Adult Lhermitte–Duclos disease (cerebellar tumors) OR</li> </ul>	
<ul style="list-style-type: none"> <li>Autism spectrum disorder and macrocephaly OR</li> </ul>	
<ul style="list-style-type: none"> <li>Two or more biopsy-proven trichilemmomas OR</li> </ul>	
<ul style="list-style-type: none"> <li>Two or more major criteria (one must be macrocephaly) OR</li> </ul>	
<ul style="list-style-type: none"> <li>Three major criteria, without macrocephaly OR</li> </ul>	
<ul style="list-style-type: none"> <li>One major and three or more minor criteria (may include an additional major criteria in place of one of the three minor criteria) OR</li> </ul>	
<ul style="list-style-type: none"> <li>Four or more minor criteria</li> </ul>	

The combination of mucocutaneous lesions and breast cancer should always prompt evaluation for Cowden syndrome. The online scoring system is a helpful tool in determining pretest probability of a mutation. Tan, M.H., et al. A Clinical Scoring System for Selection of Patients for PTEN Mutation Testing Is Proposed on the Basis of a Prospective Study of 3042 Probands. *Am. J. Hum. Genet.*, 2011, 88(1), 42–56.

Refs: (90,92,97,98).

The PTEN gene behaves as a classic tumor suppressor. The wt allele is generally lost in tumor tissue from mutation carriers, and unlike BRCA1 or BRCA2, somatic mutations in PTEN are found in a substantial proportion of sporadic breast, prostate, endometrial, and other cancers (100,101). It has nine coding exons, and both germline and somatic mutations appear to be distributed throughout the length of the gene (94). Various deletions have been reported (102) and 2% of patients with a clinical diagnosis of Cowden syndrome have mutations in the promoter region of the PTEN gene (103). Most recently, a promoter mutation in KILLIN gene, which is adjacent to PTEN and also regulates PTEN function, was found among a subset of patients with CS and Cowden-like



syndrome. *PTEN* and *KILLIN* share the same transcription start site but are transcribed in opposite directions, and germline methylation of the 10q23.31 bidirectional promoter CpG island silences *PTEN* or *KILLIN*, or the translation of both (104). These patients appear to have an even greater risk for breast cancer and a significant risk for renal carcinomas.

### 89.6.5 CHEK2

*CHEK2* produces a checkpoint kinase protein, activated in response to radiation and other agents that cause breaks in the DNA. *CHK2* (or *CHEK2*) functions broadly comparable to *ATM*, has been implicated in some instances of LFS and is considered a low-penetrance breast cancer gene. *CHEK2* was dubbed the breast-colon gene as it appeared to increase the risk for breast and colon cancers in both men and women, as well as prostate cancer in men (105). A common deleterious variant in *CHEK2*, 1100delC, is predicted to result in a truncated version of the repair protein. This variant is found in approximately 1.4% of Caucasians of northern European descent (106) and is associated with a twofold to threefold increased risk for breast cancer (CHEK consortium 2004). Whereas, one variant, T59K, appeared to be associated with cancer (particularly breast cancer) in Icelandic patients (107). *CHEK2* is now considered a common “moderate” risk gene for breast cancer. Several additional studies implicate a mutation in *CHEK2* as a significant risk for bilateral breast cancer and may increase cancer risk from the effects of ionizing radiation such as those found in radiation therapy and computed tomography scans (108). Multiple studies have found no interaction between *CHEK2* variants and *BRCA1* and *BRCA2* mutations (106,109), though it is likely to interact with other genes because the cancer risk varies depending on the extent of the family history, with at-risk children of mothers with bilateral breast cancer having higher lifetime risk than those with no family history.

### 89.6.6 PALB2

*PALB2* is a partner and localizer of *BRCA2*. The *PALB2* gene was originally identified as producing a *BRCA2*-interacting protein and thought to be a modifier gene in families with *BRCA2* mutations. Additionally, it is called *FACN* and functions within the Fanconi anemia pathway, with biallelic mutations resulting in a similar phenotype as biallelic *BRCA2* carriers (38). However, several mutations in *PALB2* have been associated independently with an increased risk for breast and pancreatic cancers. A founder mutation, 1592delT, has been reported in Finland among high-risk families who are *BRCA*-negative (families without any *BRCA* mutations). The cancer risk varies among studies, from moderate-like in *CHEK2* to mimicking *BRCA*, with up to a 40%

lifetime breast cancer risk (110). Monoallelic germline mutations have been implicated in both familial breast and pancreatic families (111,112). There has been a significant disparity over the proportion of familial breast and pancreatic cancers accounted for by *PALB2*, and it may be an ethno-regional issue. In a large series from the University of Washington, 3.2% of subjects unselected for ethnicity but *BRCA*-negative were found to carry a mutation, but none of 172 Ashkenazi Jewish individuals has the mutation. The age and distribution of cancers (female and male breast and pancreatic cancers) were similar to *BRCA2* (113). Hofstadter also found about a 2% frequency among breast/pancreatic families, with frequency not enriched by pancreatic history (114). However, in a Dutch series of 110 *BRCA1/2*-negative hereditary breast-ovary families, one recurrent mutation was found in a male breast cancer, and the authors concluded that *PALB2* does not explain *BRCA*-negative hereditary breast-ovarian cancer (HBOC) (115). *PALB2* is now commercially available for testing and should be certainly considered for hereditary pancreatic cancer families as well as patients with breast and pancreatic family history, though it remains to be clarified whether there are particular ethnic groups in whom it is not likely to be causative.

## 89.7 LOW-PENETRANCE BREAST CANCER GENES

Estimates of genetic risk for breast cancer derived from segregation analysis show that only a minority of women at increased risk belong to families in which the incidence of disease is so high as to generate a striking “cancer family” pedigree (6,116). About two-thirds probably carry mutations that confer a lifetime risk of approximately 25–40%. If such a mutation is in an autosomal gene, it is most unlikely to generate a spectacular cancer pedigree as the chance of an affected woman having an affected mother or sister (in an average-sized family) will be less than 50% (11). In the early 2000s, a great number of association studies were published as candidates for polymorphisms conferring low levels of risk or protection from breast cancer. For example, a review of reported studies in this field identified approximately 13 polymorphisms in 10 genes that had been associated with breast cancer at a 5% significance level (117) and that one *BRCA1* variant (Gln356Arg) may actually be protective against breast cancer (118). The many polymorphisms within *BRCA1* and *BRCA2* are obvious candidates for low-penetrance cancer genes, but no convincing evidence has been presented for such a role. Most of these associations have not been sustained by larger associations or genome-wide studies.

Ataxia-telangiectasia (A-T) is an autosomal recessive disorder, characterized by immunologic defects, ocular and cutaneous telangiectasia, and progressive cerebellar ataxia from early childhood (119). Homozygotes have

an increased susceptibility to cancers, particularly leukemias and lymphomas (120,121). The primary defect seems to be in one of the pathways of DNA repair following ionizing radiation or radiomimetic agents, and cells from homozygotes are abnormally sensitive to ionizing in vitro (122). The A-T locus was mapped to chromosome 11q22–23, and the gene (ATM) was cloned in 1988, one of the earliest cancer genes identified (123). Reference has already been made to the evidence that BRCA1, BRCA2, and certain other genes involved in the detection of—and cellular response to—DNA damage may form an interactive chain, with ATM as an important member. Although A-T homozygotes are rare (about 1 in 40,000 individuals at birth), heterozygotes comprise about 1% of the population. Several epidemiologic studies in the United States, the United Kingdom, and Scandinavia have suggested that female heterozygotes have a risk for breast cancer three to five times greater than the age-matched controls (124,125), but this has not been upheld in a large, collaborative, multicenter survey (126). Overall, therefore, it seems that the gene may be responsible for a limited number of early-onset cancers but not for many striking family clusters.

It is far from clear whether increased radiosensitivity plays any part in the cancer susceptibility of A-T or CHEK2 heterozygotes and, in particular, whether the radiation dose associated with mammography (about 2 mGy per film in traditional nondigital mammograms) might be sufficient to exacerbate the situation. On the one hand, data from atomic bomb survivors suggest that doses of around 100 mGy are required to demonstrate any increase in breast cancer risk and that sensitivity is greatest around adolescence, declining considerably by age 40. On the other hand, conventional in vitro tests of sensitivity to radiation and other clastogens give some indication that A-T heterozygotes are abnormally susceptible to chromosome breakage, and a threshold radiation dose is difficult to define (127,128). Studies, using blood lymphocytes arrested in the G2 phase of the cycle, appear to demonstrate radiosensitivity in about 10% of unselected individuals, whereas approximately 40% of breast cancer patients fall into the abnormally sensitive range (129). The relatively high frequency of the sensitive phenotype suggests that A-T heterozygosity accounts for very little of it. Nevertheless, radiation sensitivity, as measured by this assay, is inherited as a Mendelian dominant or codominant trait (130). Furthermore, one study (131) found that breast cancers arising in patients who show high G2 sensitivity tend to show favorable prognostic features reminiscent of the characteristic of non-BRCA1 and -BRCA2 familial breast cancers (52). The (currently unknown) gene or genes responsible may well contribute substantially to the total genetic risk for breast cancer. However, systematic studies to try and determine how much breast cancer in the population is due to ATM mutations have been conflicting. The WECARE—Women's Environment, Cancer, and

Radiation Epidemiology—study is currently ongoing and addresses the question of whether routine low-level radiation exposure as in mammography increases breast cancer risk in ATM carriers and other specific populations (132).

## 89.8 ASSOCIATION STUDIES AND GENOMICS OF BREAST CANCER

Since 1997, there have been numerous association studies identifying polymorphisms in genes involved in breast cell proliferation, hormone receptor response and metabolism. For example, in one study (133), a polymorphism of the Cyp17 gene was significantly more common in breast cancer patients than in controls. Subsequent surveys found no such association for breast cancer in women, although it does appear to hold true for male breast cancers (134) and may be attributed to variation in activity of the steroid-metabolizing enzyme encoded by CYP17, with consequent small changes in estrogen level (133). Polymorphisms in CYP17 have been incorporated into the proprietary breast cancer risk algorithm known as OncoVue™, which combines the effects of multiple single nucleotide polymorphisms (SNPs) and stratifies risk based on menopausal status (135).

Many published studies have investigated possible interactions between major susceptibility genes such as p53, BRCA1, or BRCA2, and minor modifying genetic characters that could influence the penetrance of the cancer trait or its severity (e.g. age of onset and types of cancer observed). An SNP in the RAD51 gene was over-represented among women with breast cancer who carried the Ashkenazi Jewish BRCA2 mutation, compared with unaffected carriers; no such association was found for BRCA1 mutations, nor for CHK2 or ATM variants (136,137). RASSF1A is a tumor suppressor that regulates cell cycle progression and apoptosis. It is methylated in 50% of breast cancers. More recently, in a group of 138 BRCA1/2 carriers, the A133 polymorphism in RASSF1A has been shown to be associated with earlier age of onset of breast cancer (138). There have been a large number of investigations into the role of one specific variant in RAD51, as it relates to sporadic breast cancer and role as a modifier of BRCA-related breast cancer. There are much conflicting data in the literature regarding the impact of an SNP of RAD51, 135G>C (rs1801320). There have been several recent meta-analyses, in both BRCA carriers and the other in sporadic breast cancer. In sporadic cancer, the 135 variant was not associated with increased risk alone, but may be in a recessive model and not in a heterozygous state (139). Zhou conducted an even larger meta-analysis and found an increase in risk overall, but among the subset analysis, only the homozygous variant had an influence on risk in BRCA2 carriers (103).

However, to date, none of these SNP associations have provided any meaningful clinical utility (140,141), even

though the RAD51 SNP was briefly commercially available as a BRCA breast cancer modifier test (DeCode™ Genetics, 2009). The CIMBA and EMBRACE consortium have been investigating genome-wide associations of breast cancer penetrance in BRCA carriers. A cohort of 2825 BRCA1 carriers underwent haplotype analysis, and specific haplotypes in ATM, CTIP, NBSI and TOPBP1 were associated with increased breast cancer risk, whereas BRIP1 and RAD50 with decreased risk. There was no association with BRCC36, ABRA1, MRE11A, PALB2, RAD51 and RAP80 with regard to breast cancer, but ABRA1, BRCC45 and RAP80 haplotypes were associated with ovarian cancer risk. Authors conclude that proteins that interact with BRCA1 modify the cancer risk in BRCA1 carriers (142).

Easton et al. have been pioneers in genome-wide studies of breast cancer, and along with researchers from Iceland, they identified seven SNPs through a genome-wide association study (GWAS) of 225,000 European Caucasian women (143). Additional studies are regularly published as the mapping becomes more refined and different cohorts are evaluated. The risk in most women is felt to be the cumulative effect of multiple, common genetic changes, each one of which has a small impact on breast cancer risk (144). Only a single common coding variant in CASP8, which was originally found via a candidate gene association study, was also seen as a risk allele in Easton's GWAS (145).

In an effort to estimate individualized risk, Pharoah modeled that these original seven SNPs would only have a significant impact on 63 of every 10 million women: 56 of whom will have all of the low-risk alleles (SNP changes associated with lower breast cancer risk) and seven of the 10 million women will have all of the high-risk alleles. Pharoah and Easton estimated that those with all of the low-risk changes will have a lifetime breast cancer risk of 4.2% and those with all of the high-risk changes will have a lifetime risk of 23% (using 9.4% as the population risk percent). All others will have some combinations of high- and low-risk alleles—2187 possible combinations of these SNPs—and it is not clear what risk those combinations confer (141). The goal is that when the majority of protective and risk SNPs are identified, a true genomic prediction of risk will be attainable. Nonetheless, commercial companies are already marketing tests based on some or all the SNPs that have already been identified.

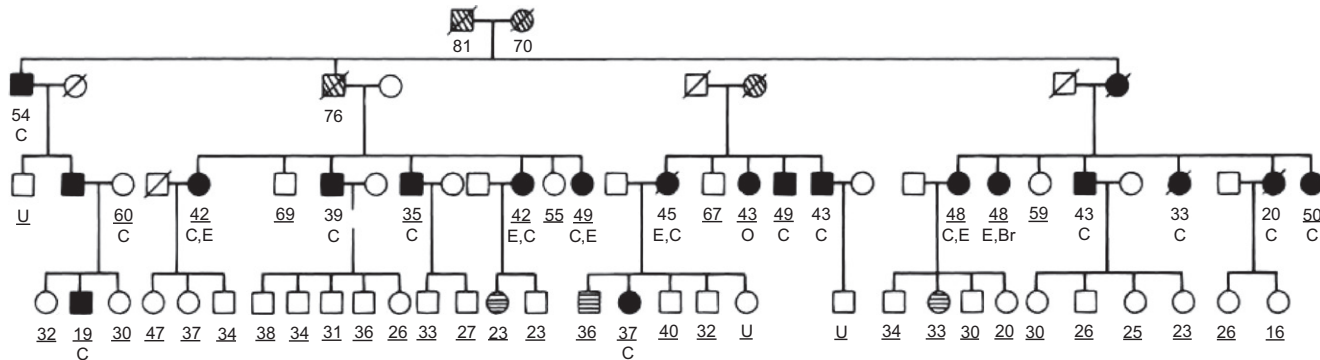
There are multiple SNP-based genomic tests currently available. Some are based on various combinations of SNPs and other tests combine SNP information with other risk factor data, such as family history and reproductive history (135,146). These models are being validated in terms of improvements in predicting individual risk above the Gail model alone, which looks only at age, family history, and reproductive risk factors (147–149). More recently, Mealiffe assessed the validity of the seven common SNPs noted in the Caucasian

GWAS, mentioned noted above, combined with the standard Gail-calculated breast cancer risk in several 1000 cases and controls from the Women's Health Initiative Study. Subjects were white, non-Hispanic postmenopausal women. Their results, like others, found that the SNP risk score was nearly independent of the Gail score, which has also been seen by Jupe et al. using other SNPs. Based on the assumption that the Gail risk and each of the SNPs are independent, the combination of the SNP and Gail scores resulted in a slight improvement in risk prediction, with greatest improvements for those whose Gail score alone was in the intermediate risk category, defined as an empiric 5-year risk of 1.5–2.0% (150). Similar results were found in a much larger analysis, with the authors concluding the reclassification of a substantial proportion of subjects from an intermediate risk category to a higher or lower level of risk (151).

## 89.9 HEREDITARY NONPOLYPOSIS COLORECTAL CANCER SYNDROME (OR LYNCH SYNDROME)

Several human homologs of the yeast or bacterial MMR genes (152) have now been mapped, sequenced, and implicated in familial cancer syndromes. These genes, designated hMLH1, hMSH2, hMSH6, hPMS1, and hPMS2, are located on chromosomes 3p21–23, 2p15–16, 2p, 2q31–33, and 7p22, respectively (153). The characteristic homozygous mutant phenotype is instability of microsatellite repeats owing to incomplete editing of replication errors, hence the term replication error repair or microsatellite instability (MSI) syndromes. Such instability is found only in tumors in which the wt allele has been inactivated. Thus, the mutations appear to be recessive at the cellular level. However, in contrast to many other situations in which a recessive tumor suppressor is involved, loss of heterozygosity (LOH) at the relevant locus is rare (154).

Most individuals carrying a mutation in one of these genes have been identified by screening for the hereditary nonpolyposis colon cancer (HNPCC) phenotype, originally subdivided by Lynch into types I and II. Type II is now synonymous with Lynch syndrome or HNPCC and encompasses other malignancies—particularly endometrial and ovarian—in addition to colon cancer, in which the tumors are predominantly in the right colon, in contrast to sporadic cancers that occur predominantly in the left colon. There is a clear excess of other cancers, notably endometrial, which is often the presenting cancer in female carriers (Figure 89-4). However, because the original families are large and were selected for a high incidence of cancers, some of the tumors noted in the pedigrees may be unconnected with the constitutional mutations demonstrated. In particular, whether breast cancer occurs more frequently than would be expected from the relevant population incidence is still under debate, and although overall breast cancer does not appear as a consistent cancer in



**FIGURE 89-4** Pedigree showing features of HNPCC (Lynch type 2 family cancer syndrome), associated with mutation in the replication error repair gene hMSH2 on chromosome 2p. Filled symbols = persons with cancer. Horizontal stripes indicate persons with colorectal adenoma. Letters signify tumor sites: C, colon or rectum; E, endometrium; U, unknown; O, ovary; and Br, breast. (From Peltomäki, P.; Aaltonen, L. A.; Sistonen, P., et al. *Genetic Mapping of a Locus Predisposing to Human Colorectal Cancer*. Cell **1993**, 75, 1215–1225.)

HNPCC, there are clearly some families with a significant overrepresentation of breast cancers (155,156).

In unselected series, very little evidence has been obtained of MSI in either breast or gynecologic tumor, contrasting with rates of approximately 15% reported for “sporadic” colon cancers (157,158). MSI is an inconsistent feature of MMR-related hereditary endometrial and ovarian cancers, making it a poor pathologic screen for HNPCC (159). Immunohistochemistry (IHC) testing is a widely and commercially available antibody assay that can identify intact protein in paraffin-embedded tumor blocks. In colon tumors, MSI and loss or absence of IHC staining are strongly correlated (160) and have largely replaced MSI as first-tier screening for MMR gene defects. Significant attention has focused on the universal screening of colon tumors for evidence of MMR defects to identify germline mutations in individuals who might otherwise not come to clinical attention and it has been endorsed under the Center for Disease Control’s independent panel, the Evaluation of Genomic Applications in Practice and Prevention Working Group recommendation statement (161,162).

### 89.10 ENDOMETRIAL CANCER

As already discussed, the association of colon and endometrial cancers is a striking feature of both HNPCC and Cowden’s syndrome (94,163). Endometrial cancer is often the presenting cancer in women with HNPCC, with upward of 60% lifetime risk and earlier age of onset than the colon cancer (164). The original Bethesda guidelines for screening of HNPCC in 1996 recommended screening six patient populations, two of which were gynecologic: early-onset endometrial cancer and two Lynch cancers and synchronous ovarian and endometrial cancers. Subsequent findings suggested that endometrial cancers are associated particularly with mutations in hMSH6 (165), but more comprehensive investigations have demonstrated both gynecologic malignancy risk and colon cancer risk in MSH6 mutations, with some

families demonstrating substantial endometrial and ovarian cancers (166). Endometrioid adenocarcinomas of the endometrium and synchronous endometrial and ovarian cancers are a hallmark of HNPCC-related gynecologic tumors, and although they tend to occur earlier than sporadic endometrial cancer (mean age of 42 years in the International Collaborative Group on HNPCC’s cohort), the tumors are otherwise generally well differentiated and tend to be early stage when diagnosed (167). Hampel and colleagues evaluated the feasibility of screening all the endometrial cancers with MSI testing and comparing that to IHC testing as a screen. They found that among 543 newly diagnosed cases of endometrial cancer, at least 1.8% had a confirmed mutation in one of the four MMR genes. Seven of 10 did not meet any criteria for HNPCC testing and six were older than 50 years. In addition, a number of subjects had missense mutations; thus, their functional status could not be confirmed (159). In a study of early-onset endometrial cancers (before age 45) and synchronous primary endometrial and ovarian cancers, 12% of patients had a mutation identifiable in MLH1, MSH2 or MSH6, and methylation was seen in three patients with MHL1-absent stain, but no mutation was detected, supporting the universal screening for all the early-age or synchronous tumors with IHC (168).

PMS2 mutations are emerging as another potentially significant proportion of HNPCC cases, but the families described vary immensely in terms of spectrum of malignancy and ages of onset, from much milder presentations overall to a few outliers with profound cancer family histories (169). Finally, epithelial cell adhesion molecule (EpCAM) gene is upstream of MSH2, and in recent years, it has been shown to be a novel mutational mechanism causing Lynch syndrome by epigenetic inactivation of MSH2. It has been shown recently that loss of protein expression by IHC testing of the EpCAM protein correlates with a heterozygous deletion of the gene (170), and this may lead to changes in pathology department screening to include IHC for EpCAM in MSH2-absent tumors.



Small excesses of endometrial cancer have also been recorded in epidemiologic studies among relatives of patients with ovarian or breast cancer (171,172). For most of the published studies, it is not possible to quantify the contribution of families with HNPCC or Cowden's syndrome to the data sets, and endometrial cancer has been reported in cohorts of BRCA1 and BRCA2 mutation carriers, with controversy whether those genes also confer an increased risk (173,174). For example, endometrial cancer was recorded in several of the Icelandic families with BRCA2 999del5 mutations (175), and in an Israeli series of uterine papillary serous carcinoma, a distinct and aggressive type of endometrial cancer, four of 20 women were BRCA carriers (18), and it has been hypothesized that perhaps some of the primary peritoneal cancer seen in BRCA carriers who had previously undergone prophylactic oophorectomy arose from the fallopian tube still implanted within the uterine wall (176). Because of this, for a number of years, recommendations had shifted to consider total hysterectomy at the time of salpingo-oophorectomy. Several large series of endometrial cancers, even papillary serous endometrial cancer, did not show an excess of BRCA mutations (19,177).

Endometrial cancer risk is also important because one option being considered in an attempt to reduce the risk for breast cancer in BRCA1 and BRCA2 family members is prophylactic tamoxifen (178). Endometrial cells respond to tamoxifen by enhanced proliferation, and there is evidence of an increased incidence of endometrial cancer in patients exposed to the drug (179–181).

Other tumors of the uterus (principally sarcomas) are rare and not known to have any underlying hereditary basis.

### 89.11 FAMILIAL OVARIAN CANCER

Family clustering of ovarian cancer, in the absence of any other cancers, is rare. Much more common is the association with breast cancer, and even large “ovarian-only” cancer families ultimately demonstrate breast cancer when the current generation used oral contraception or underwent preventive surgery (83). There is no evidence for any major gene predisposing strongly to ovarian cancer, other than BRCA1 and BRCA2 and rarely in HNPCC (without the more prevalent colon and endometrial cancers). In families with PJS, the ovarian tumors are not epithelial tumors but gonadal sex cord tumors. Among families with breast and ovarian cancers, constitutional mutations in BRCA1 outnumber those in BRCA2 by about 4:1. Together, constitutional mutations at these loci account for at least 5% and possibly as much as 15% of all ovarian cancers in most developed countries (7,116,182).

It is clear that the great majority of familial tumors are serous adenocarcinomas, carrying a very poor prognosis (183,184). Borderline tumors do not appear to form part of the BRCA1 or BRCA2 mutation phenotype, nor do

germ cell tumors. Although there are occasional reports of familial clusters of teratoma, arrhenoblastoma, or dysgerminoma, the question of whether these are truly genetic remains open. In a few instances, constitutional mutations in p53 may be suspected (185). A most important finding, in relation to management of familial ovarian cancer, is that in BRCA1 and BRCA2 mutation carriers, there is a substantial risk that the primary ovarian tumor may arise in the fallopian tube or diffusely within the peritoneum (186,187).

The mean age of onset for familial ovarian cancer is lower than that for sporadic cases, but the effect is less clear-cut than that for breast cancer, and familial ovarian cancers rarely occur in patients younger than 40 years (174). Estimates of age-specific penetrance for breast or ovarian cancer in BRCA1 and BRCA2 mutation carriers are shown in Table 89-5. The ovarian cancer risks associated with germline mutations in BRCA2 are, in general, less than half of those applying to BRCA1 mutation carriers. As discussed earlier, there initially appeared to be a phenotype/genotype correlation, with an “ovarian cancer cluster region” being located in the central portion of the gene (188), but this has also been largely discounted more recently (189,190).

Alternative explanations for differential risks for breast or ovarian cancer in BRCA1 or BRCA2 families include the effects of modifying genes and environmental factors (7). The use of oral contraception may have played a part in shifting the risk from ovarian cancer toward breast cancer over the course of the past 20 years (191), and this may be relevant in the few large families with ovarian cancer mentioned earlier, in which breast cancers have occurred only recently. Nevertheless, there remain striking and unexplained instances of BRCA1 mutation-bearing families with multiple cases of ovarian cancer but no (or few) breast cancer, although the germline mutations are identical to those carried by several other families with more typical distribution of tumors. Further work, including, for example, twin studies, is urgently required to cast light on this clinically important issue.

The lifetime risk for ovarian cancer in individuals with a MMR gene defect is estimated at 11% (168). Synchronous colon and ovarian cancers should always prompt consideration of Lynch syndrome. However, as colon cancer frequently metastasizes to the ovary, pathologic confirmation of multiple primaries is crucial in risk assessment. Screening ovarian tumors for IHC analysis of the MMR genes is possible, but the reliability of IHC in this cell type is unknown, and as discussed earlier, MSI testing is insensitive in HNPCC-related ovarian cancer (159).

#### 89.11.1 Low-Penetrant Genes for Ovarian Cancer

Epithelial ovarian cancer has a major heritable component, even stronger than that for breast cancer, but the aforementioned single genes (BRCA1/2, MLH1, MSH2,

MSH6, PMS2) explain less than half of the familial risk (87). Women with a first-degree relative with ovarian cancer have a threefold increased risk of developing the disease (192), though probably about half of this observed risk is explained by BRCA and MMR gene mutations. Genome-wide studies have thus attempted to identify new regions of risk and low-penetrant polymorphisms that predispose to ovarian cancer. In a three-stage genome-wide study conducted with the Ovarian Cancer Association Consortium throughout the United Kingdom, the United States, and Australia, 12 polymorphisms reached significance within the 9p22.2 region, but only one, rs381413, retained significance after multivariate regression analysis (193). In subsequent studies, candidate loci nearby on 9p13, rs8170 and rs236356 met significance, which are within the MERIT40 gene that interacts with BRCA1, and therefore functional plausibility (194). Nine additional SNPs with  $p$  values  $<10^{-4}$  and two approaching genome-wide significance on 2q31 and 8q24 have been identified in the consortium cohort, all in serous histology subtypes. There is support of functional role in a number of genes in these regions, including HOXD1, MYC, TIPARP, SKAP1 and ESR1 on 8q24 and 2q31 and BNC2 on 922 (195,196).

**89.11.1.1 KRAS Variant.** Micro RNA (miRNA) is a class of 22 nucleotide noncoding RNAs that are evolutionarily conserved and negatively regulate gene expression by binding to partially complementary sites in 3'-untranslated regions of target messenger RNA. miRNAs are aberrantly expressed in almost all cancers and function as novel class of tumor suppressors and oncogene regulators (197). SNPs within the miRNA or the miRNA-binding sites can act as biomarkers of cancer risk. The rs61764370 germline KRAS variant disrupts *let-7* binding to KRAS and increases levels and has been shown to be disproportionately present in various cancers including lung, colon and ovarian. A case-control study examined the prevalence of KRAS variant in several 100 unselected ovarian cases and 31 HBOC, BRCA-negative families and found an RR of 1.7 in sporadic cases and an odds ratio (OR) of 2.46 for ovarian cancer among carriers of the variant. This led to a commercial SNP test called "PreOvar™," which is currently available in the United States (198,199). Because this variant did not show significance in GWAS, an analysis was performed on 8669 ovarian cases and controls from 19 case-control sets and powered to detect an RR over 1.3 for sporadic cases and 2.0 for familial cases. This provides substantial argument that there is no evidence for association between rs61764370 and epithelial ovarian cancer in univariate analysis (200), though the investigators of the initial study dispute this and the test remains available.

## 89.12 CERVICAL CANCER

The major etiologic factor in both squamous and adenocarcinoma of the cervix is undoubtedly human papillomavirus (HPV), particularly types 16, 18, 31 and 33

(201,202). Predisposition to the effects of the virus can be associated with overt immunologic deficiencies (203), which may be genetic or acquired (e.g. renal transplant recipients). There is also some evidence of interaction between specific papillomavirus types and certain histocompatibility antigens, suggesting a possible genetic basis for susceptibility to cervical cancer (204). For example, the HLA-B7 allele presents an immunogenic octapeptide epitope of HPV type 16 that shows frequent variation to a nonimmunogenic form. The latter variant has been found most commonly in tumors from HLA-B7-positive women and may contribute to the adverse prognosis of cervical cancer associated with the B7 allele (205).

Although initial surveys of cancers arising in BRCA1 mutation-bearing families did not detect a significant excess of cervical tumors, the Breast Cancer Linkage Consortium reported an increase in risk (16), but this has not been reported subsequently.

There is a suggestion of an excess of cervical tumors among families with LFS (206,207), but this does not appear to be a consistent finding and certainly does not constitute a measurable familial component in the incidence of cervical cancer. Several SNPs have been associated with cervical cancer but none meaningfully validated or reproduced to date. In a small Monte Carlo model study, candidate genes with a multilocus approach showed an epistatic effect between CD28 and IFNG among a comparison of SNPs in 12 genes (208). As mentioned above in the discussion of TP53 mutations, because of the suggestion of increased risk for cervical cancer among women with LFS, Hu et al. investigated functional polymorphisms in 577 family trios and noted a TP53Arg variant associated with susceptibility to cervical cancer, particularly in those infected with HPV type 16 (209).

In the rare inherited dyskeratosis syndrome, squamous carcinoma of the cervix, vagina, or vulva can occur, but such conditions are believed to make only a minuscule contribution to the overall frequency of these tumors (210).

## 89.13 IMPLICATIONS OF FAMILIAL BREAST AND OVARIAN CANCERS FOR CLINICAL PRACTICE

From having been a neglected area, within the past 15 years, the issue of hereditary predisposition to common cancers has come to occupy a prominent place in the public eye. It is widely discussed in the popular media, and growing numbers of women who are concerned about their family histories of breast or ovarian cancer are seeking medical help (211). Many "cancer family" clinics have been established in response to this demand (212,213). Penetrance estimates vary widely and by study design and population investigated. Table 89-6 provides penetrance estimates for breast and ovarian cancers, by decade of age until age 70, based on a meta-analysis of the 10 most robust studies over the past 15 years.

**TABLE 89-6 Meta-analysis of Cumulative Risk for Breast and Ovarian Cancers by Decade of Age**

(A)										
Current Age (years)	Risk (%) of Developing <i>Breast Cancer</i> by Decade of Age									
	30 Years		40 Years		50 Years		60 Years		70 Years	
	BRCA1	BRCA2	BRCA1	BRCA2	BRCA1	BRCA2	BRCA1	BRCA2	BRCA1	BRCA2
20	1.8	1	12	7.5	29	21	44	35	54	45
30			10	6.6	28	20	44	35	54	45
40					20	15	38	30	49	42
50							22	18	37	32
60									19	17

(B)										
If You Are (in years):	Risk (%) of Developing <i>Ovarian Cancer</i> by Decade of Age									
	30 Years		40 Years		50 Years		60 Years		70 Years	
	BRCA1	BRCA2	BRCA1	BRCA2	BRCA1	BRCA2	BRCA1	BRCA2	BRCA1	BRCA2
20	1	0.19	3.2	0.7	9.5	2.6	23	7.5	39	16
30			2.2	0.52	8.7	2.4	22	7.4	39	16
40					6.7	1.9	20	7	38	16
50							15	5.2	34	14
60									22	9.8

Risk for breast (A) and ovarian (B) cancers for an unaffected woman carrying a BRCA1 or BRCA2 mutation beginning at age 30 until age 70. Ten studies included in this meta-analysis by Chen and Parmigiani include the original Breast Cancer Linkage Consortium risk figures and other studies that predict risk until age 80, but tables are truncated at age 70. Chen, S.; Parmigiani, G. Meta-analysis of BRCA1 and BRCA2 Penetrance. *J. Clin. Oncol.*, 2007, 25(11), 1329–1333. (From Morris, J. L.; Gordon, O. K. *Positive Results: Making the Best Decisions when You're at High Risk for Breast or Ovarian Cancer*; Prometheus Books, Amherst, NY, 2010. Reprinted with permission of Prometheus Books; [www.prometheusbooks.com](http://www.prometheusbooks.com).)

### 89.13.1 Management Options

**89.13.1.1 Enhanced Surveillance.** The US NCCN and the UK NICE guidelines on the surveillance for BRCA1/2-related hereditary breast–ovary syndrome are for the semiannual imaging of the breast with magnetic resonance imaging (MRI) and mammogram—alternating each on an annual basis beginning at age 25, clinical breast examination twice yearly and twice yearly transvaginal ultrasound (TVU) and CA-125 levels ([www.nccn.org](http://www.nccn.org)) (214). Surveillance for ovarian cancer is considered as an intermediary until definitive prophylactic preventive surgery is undertaken, given the limitations to screening efficacy for ovarian/tubal/peritoneal cancer.

Evidence is now clear that annual screening is of less benefit to carriers of BRCA1 mutations than to women with a family history of breast cancer but without demonstrable mutations. The former are more likely to have high-grade tumors that carry a substantial risk of progression even if detected while small and node-negative (215,216). This is entirely in keeping with the finding that non-BRCA1 and non-BRCA2 familial breast cancers tend to have good prognostic features (53). As mentioned previously, the issue of radiation risk from mammography is currently unresolved and may be a significant factor if some of the cancer-predisposing mutations increase sensitivity to ionizing radiation. p53 is a case in point, as radiation-damaged cells in the absence of functioning p53 fail to arrest cycling during the repair phase or to undergo apoptosis when the damage is irrecoverable (217). Nevertheless, there is no direct evidence that the doses of radiation

associated with mammography (even repeated at regular intervals) have any measurable clinical consequence, but because of recent mathematical modeling study that very early initiation of mammography (at younger than 30 years) among BRCA carriers may be associated with excess cancers, guidelines are currently cautious regarding mammography at younger than 30 years in BRCA carriers (218). MRI of the breast is the mainstay of surveillance for high-risk women, with initial studies of BRCA carriers comparing mammogram, ultrasound, and MRI demonstrating much higher sensitivity for detection of invasive cancer (219). Subsequent studies confirm that MRI (with gadolinium contrast enhancement) outperforms mammography in the detection of very small, noncalcified lesions, but it is less well suited to recognition of ductal carcinoma in situ (DCIS) (220). Therefore, MRI may be of particular value for surveillance of women with BRCA1 mutations, in whom DCIS is often absent. NCCN and American Cancer Society surveillance recommendations now consider it standard of care for BRCA carriers to have annual mammogram and annual MRI (alternating every 6 months) after the age of 25 years (221). Not all women can tolerate the close confinement of the MRI scanner, and the examination is likely to remain very expensive. In 2009, the American College of Radiology modified guidelines for MRI to include any woman with an empiric lifetime risk of greater than 20% or who have extremely dense breast tissue via mammography. This initiated a deluge of screening breast MRI for a very heterogeneous group of women—it remains to be seen what is the optimal group of patient characteristics suited for various

imaging modalities. Among general practitioners, however, despite widely available standard of care recommendations for screening of BRCA and other hereditary carriers, actual practice ranges very broadly (222).

For women at increased risk for ovarian cancer, early detection (or, better still, prevention) is of paramount importance because prognosis is very closely related to stage at diagnosis. In most centers, the mainstay of ovarian cancer screening is regular (semiannual) ultrasound examination with a transabdominal or transvaginal probe in combination with CA-125 levels.

Glycoprotein markers in the plasma, such as CA-125, and, more recently, HE-4 are of great value in following the course of disease and the patient's response to chemotherapy in women affected with ovarian cancer. However, as a screen, CA-125 and every other serum marker evaluated to date have been inadequate. A single CA-125 test alone is not effective at detecting ovarian cancer, but serial testing and the use of receiver operating characteristic (ROC) curves have been much more promising (223). Furthermore, because normal levels fluctuate with the menstrual cycle, and other diseases can cause CA-125 levels to rise, including endometriosis, benign ovarian cysts, early-stage pregnancy, and pelvic inflammatory disease, the false-positive rate in premenopausal women is 3%, which can be a source of alarm. Multi-center trials are ongoing in assessing the combination of CA-125 and HE-4 as a stand-alone screening assay for BRCA carriers, HNPCC carriers, and women with significant family history ([NCTrials.gov](http://NCTrials.gov)) (224).

The US National Cancer Institute has begun a long-term trial on the effectiveness of TVU and the CA-125 test, alone and in combination, for detecting ovarian cancer in high-risk women (225). A large population screening UK study investigated the use of ultrasound, alone and in combination with CA-125, for detecting ovarian cancer in postmenopausal women of average risk for ovarian cancer (226). Though 50% of cancers detected were of early stage, the remainders were still stage III and stage IV and continued to have high false-positive rates, commiserate with prior studies.

Despite careful application of the best available technology, however, there is as yet no direct evidence that screening programs for hereditary ovarian cancer are having any impact on morbidity or mortality. The alternative of prophylactic bilateral salpingo-oophorectomy, at around age 40 for BRCA1 carriers, and closer to menopause for BRCA2 carriers and for women at substantial familial risk for ovarian cancer, appears to be a much better option (227,228). Nevertheless, prophylactic oophorectomy is not a risk-free procedure (229) and can be associated with some negative psychological effects, including loss of libido. There has been concern about the long-term consequence of premature menopause in women who undergo risk-reducing oophorectomy, but the results of PROSE (Prevention and Observation of Surgical Endpoints) trial demonstrate a reduction in

all-cause mortality for women with BRCA1 and BRCA2 mutations who undergo oophorectomy (230).

**89.13.1.2 Preventive Medication.** Not only is the risk for a first breast cancer markedly elevated but also the risk for a second primary cancer in the contralateral breast after an initial unilateral cancer diagnosis in a BRCA mutation carrier is up to 29.5% at 10 years (231). There is good evidence that the nonsteroidal antiestrogen tamoxifen, given from 2 to 5 years to women who have had a stage I breast cancer, reduces the risk for a second primary breast cancer by approximately 35% (232). This is in keeping with a huge body of evidence implicating estrogen in the etiology of breast cancer. However, the question of whether tamoxifen may be protective in women at genetically increased risk for breast cancer is not a simple one (233,234). Not only is there concern about the possible induction of endometrial cancer but there is also debate about the duration of any protective effect because resistance to tamoxifen tends to develop within a few years in established cancers (235). The same time course may not apply when the drug is used prophylactically, but if intervention has to begin by about age 30, it may be unrealistic to expect benefit to be sustained for decades, even though it has been shown to persist for 10 years in the early cancer trials cited earlier. Pure antiestrogens are improved alternatives to tamoxifen as adjuvant therapy for breast cancer, and cross-resistance has not been reported. However, they lack the advantages of tamoxifen, for example, in protection against osteoporosis, and actually accelerate bone loss (236,237).

The US National Surgical Adjuvant Breast and Bowel Project double-blind P-1 trial of prophylactic tamoxifen showed a highly significant reduction in risk for invasive breast cancer in those on the active drug (179). Initial results from the United Kingdom (International Breast Cancer Intervention Study) and the Italian trials were less encouraging (238). However, with longer follow-up, all trials combined showed a significant protective effect of tamoxifen or raloxifene (almost 40% reduction in breast cancer incidence, compared with placebo). This reduction applied only to ER-positive tumors but was not restricted to any particular age group (178,239). Aromatase inhibitors demonstrate improved efficacy over tamoxifen adjuvant treatment of breast cancer and a primary prevention trial in women defined as high risk based on age, Gail risk greater than 1.66% over 5 years, or personal history of atypical hyperplasia or DCIS, but specifically excluding BRCA carriers. Results of the exemestane (aromatase inhibitor) MAP.3 trial in postmenopausal women at empiric risk for breast cancer above the 1.6% year risk demonstrated substantial efficacy, but BRCA carriers were specifically excluded from this study (240).

The use of the combined oral contraceptive pill and hormone replacement therapy is often raised as an issue of concern in the family cancer clinic. Most of the data on the oral contraceptive pill (234,241) relate to



preparations no longer in use, which contained higher doses of estrogen than does the modern pill. Regardless, there is overwhelming evidence that the use of combined oral contraceptives reduces the risk for ovarian cancer among carriers of BRCA1 and BRCA2 mutations by as much as 50% (191). This may well be at the expense of some small increase in risk for breast cancer (242,243).

Hormone replacement therapy can be dramatically beneficial for some women suffering from severe menopausal symptoms. Particularly when prophylactic oophorectomy has been carried out (see later), estrogen replacement, in a dose just sufficient to relieve symptoms and for as short a period as seems clinically appropriate, is justified and is unlikely to add measurably to the cancer risk (243). Long-term therapy, especially with combined estrogen and progesterone, is probably contraindicated in those who do not undergo preventive surgery (17,234,244).

**89.13.1.3 Risk-Reduction Surgery.** Without dispute, the most effective cancer prevention strategy for individuals at high risk is with prophylactic surgery. Prophylactic mastectomy reduces breast cancer risk in BRCA mutation carriers by at least 95% and has been reported to reduce risk to less than 1% in one recent study of unaffected women (245). In addition, although the impact of a prophylactic contralateral mastectomy on survival may depend on the overall survival from the initial ipsilateral breast cancer, in women already affected with cancer, contralateral mastectomy has been shown to reduce contralateral breast cancer incidence by up to 97% among BRCA mutation carriers (246), therefore having an effect on morbidity. Prophylactic salpingo-oophorectomy reduces the risk for ovarian/fallopian tube/peritoneal cancer by 80–90+ percent in BRCA mutation carriers and has been associated with significant reductions in both breast cancer- and gynecologic cancer-specific mortality among this group.

Large published series of total prophylactic mastectomy for familial risk record reductions in cancer risk of more than 90%, with follow-up periods in excess of 10 years (83,247), even though in this original data, a variety of surgical techniques were utilized and women were characterized as high risk by family history or LCIS (lobular carcinoma *in situ*) and not by BRCA status. As in the case of prophylactic oophorectomy, unsuspected tumors are sometimes found in breasts removed prophylactically from women at high genetic risk (248).

The question of total bilateral mastectomy often arises at the time of first breast cancer diagnosis in a known BRCA1 or BRCA2 mutation carrier, or when there is a strong family history, even in the absence of molecular confirmation. Certainly, the long-term risk for contralateral breast cancer after conventional management of the first tumor is high—from 3–6% per year (36,40). Additionally, although the outcome of conservative (breast-conserving) management of the presenting tumor is generally satisfactory, and the incidence of

a second primary breast cancer appears to be very low in the first two after cytotoxic chemotherapy for a first cancer, the required radiation therapy can significantly impair future reconstructive options and there is controversy over whether there is an increased incidence of ipsilateral second primary cancers in BRCA carriers who have had breast conservation and radiation therapy (249,250–252).

Historically, prophylactic mastectomy was viewed with considerable unease in most cultures (253), though when undertaken, the procedure is usually very successful in improving the quality of life (254). Variations in the uptake rate of prophylactic surgeries are thought to be influenced by a number of factors, including differences in healthcare costs and access to care and differences in providers' recommendations. Interestingly, Metcalfe and colleagues found lower rates of uptake of prophylactic surgeries among women who had access to a universal healthcare system (in which preventive surgery and reconstruction are available at no cost) than among women from the United States, most of whom rely on private health insurance (231).

Of the 1383 women without breast cancer from nine different countries, only 248 (18%) had undergone prophylactic mastectomy at a mean of 3.9 years after genetic testing, while 52.1% of all women in their study had prophylactic salpingo-oophorectomy. The authors noted large differences in the uptake of preventive surgeries by country. The United States had the highest uptake of prophylactic mastectomy at 36.3%, compared to Canada (22.4%), Poland (2.7%), and Israel (4.2%). A similar trend was noted for the uptake of prophylactic salpingo-oophorectomy, with the United States having the highest rate of uptake at 71.1%.

In another study, Metcalfe and colleagues demonstrated that family history is a strong predictor of the uptake of cancer-preventive procedures among women with BRCA mutations (245,255). In their study of 517 BRCA mutation carriers, they found that women with a mother with breast cancer trended toward having a higher uptake of prophylactic mastectomy than those without, although the result was not statistically significant ( $p = 0.01$ , OR = 1.7). In addition, they found that women with a sister with breast cancer were more than twice as likely to have a prophylactic mastectomy than those without ( $p = 0.003$ , OR = 2.4), and women with a mother or sister with ovarian cancer were more likely to have prophylactic salpingo-oophorectomy than those without ( $p = 0.04$ , OR = 1.6) (245,255).

Early published "Decision Analyses" attempting to predict the effects of screening and intervention in familial breast and ovarian cancers had been based on unproven assumptions about the relative efficacies of the different options available and have not always allowed for differences in outcome between carriers of BRCA1 or BRCA2 mutations, or none (256,257). A more recent study of this type, which utilizes a Monte Carlo survival analysis

that takes greater account of accrued clinical evidence, suggests that prophylactic surgery offers appreciable survival advantages over surveillance for young mutation carriers, with maximum benefit to BRCA1 carriers if prophylactic mastectomy and salpingo-oophorectomy are complete by age 40 and with little additional benefit for very young prophylactic mastectomy (age 25 versus 40) (228,258). The combination of prophylactic mastectomy and prophylactic salpingo-oophorectomy at age 40 improves survival more than either intervention alone, with a 24% survival gain for BRCA1 mutation carriers and an 11% gain for BRCA2 mutation carriers (258).

Oophorectomy not only provides substantial protection against ovarian cancer but also reduces the risk for breast cancer by almost 50% over the succeeding 5 years if completed by age 40. The level of protection was even greater on longer follow-up and was not reversed by the use of hormone replacement therapy (17,259–261). The explanation for this protective effect of oophorectomy is uncertain, given that a high proportion of BRCA1 breast cancers are ER-negative, but the findings are very clear and are in keeping with the protective benefit seen by the use of tamoxifen as well. There is speculation that all breast cancers may initially be estrogen-responsive, such that the reduction in hormonal stimulation to the breast from oophorectomy and selective ER modulators like tamoxifen have a chemostatic effect on breast proliferation (262). Primary peritoneal cancer, histologically indistinguishable from serous ovarian cancer, has been recorded in a substantial number of patients following prophylactic oophorectomy, which therefore does not confer total protection (184). There is continuing debate over the origin of such tumors, seedlings from microscopic ovarian cancers undetected at prophylactic oophorectomy or a “field change” in the peritoneal epithelium, which is embryologically identical to the outer layer of ovarian cells. The fact that in some instances a primary peritoneal tumor has presented 10 years after oophorectomy perhaps favors the latter explanation, as does the evidence from X-inactivation analysis, that such tumors are often multifocal (263). The incidence of this outcome is uncertain. A figure of 2.8% is quoted by Piver and colleagues (184). A series of studies (17,259,264) suggested that the risk is 1–2%, with a follow-up period of 2–8 years from the date of oophorectomy, but in a more recent meta-analysis by Rebbeck, the risk reduction for ovarian cancer was only 80%. One of the largest studies in the analysis though included patients who had only been treated with oophorectomy and not salpingo-oophorectomy, which was the routine practice for risk reduction before 2000, when the risk for fallopian tube cancer became clear (265). In the larger of these series, unexpected ovarian cancer was diagnosed in several patients at the time of “prophylactic” surgery, and this experience has been replicated in many other centers, and risk for incidental malignancy detected at time of prophylactic surgery is quoted to be between

3 and 6% (264–266). Hence, prophylactic oophorectomy is now referred to as risk-reduction bilateral salpingo-oophorectomy. The American Society of Clinical Oncology and Gynecology Oncology Group recommend preventive surgery be completed as a staging procedure with peritoneal biopsy and pelvic washings, usually by or accompanied by a gynecologic oncologist. Tubal ligation, without oophorectomy, has been found to reduce the subsequent risk for ovarian cancer among BRCA1 (but not among BRCA2) mutation carriers, although the mechanism of this effect is unclear (267).

## 89.14 IMPACT OF DEFINITIVE GENETIC DIAGNOSIS ON CLINICAL PRACTICE

### 89.14.1 Clinical Considerations

BRCA testing has exploded over the past few years due to improved reimbursement by insurance companies, more liberal testing criteria, and increased awareness among the clinicians and the general public. Myriad Genetics Laboratory, holders of the BRCA1/2 patent in North America and the United Kingdom, reports the number of BRCA tests increasing from 50,000 to more than 500,000 over the past decade (Myriad Laboratory, personal communication). Because of that, BRCA is often thought to be synonymous with hereditary breast cancer. However, more than half of pedigrees consistent with hereditary breast cancer will test negative for a BRCA mutation. Intense investigations over the past decade have failed to uncover a “BRCA3” that has implications for more than just a few isolated families. As reviewed previously, PALB2 and CHEK2 can present as hereditary breast cancer with fairly penetrant pedigrees but probably do not account for more than a few percent of BRCA-negative families. Because *de novo* TP53 changes can be responsible for breast cancers in young women, TP53 gene testing should always be considered in a woman with very early-onset breast cancer who tests negative for a BRCA mutation (Table 89-3).

Integration of genetic testing in the initial treatment plan of the patient is also increasing for hereditary breast and gynecologic cancers. Point-of-care testing at the time of breast cancer diagnosis is becoming much more common, so that women can make definitive surgical decisions and avoid the radiation therapy or the need for suboptimal reconstruction at a later time.

### 89.14.2 Prospects for Targeted Treatment and Preventive Therapy

An understanding of the biologic functions of these genes, and how these functions are disrupted by mutation, will be the key to the development of specific corrective or compensatory strategies, based on new pharmacologic agents. Careful evaluation for underlying genetic susceptibility as well as particular histologic profiles of cancer

becomes an increasingly important task as we move swiftly into the era of molecular diagnosis.

The very apparent differences in survival in ovarian cancer patients with BRCA mutations were postulated to be due to differential response to chemotherapeutic agents. Because most chemotherapeutic agents work by directly or indirectly damaging DNA, the role of BRCA mutations has been of interest for several years in targeting therapy (268) (in vitro assays of cultured breast cancer cells demonstrated substantial differential sensitivity to taxanes, a standard chemotherapeutic agent in the adjuvant treatment of breast cancer). BRCA null cells were 1000 times more resistant to taxanes and extremely sensitive to alkylating agents such as platinum drugs (269). Although this has led to generalized support for BRCA status to be used as a stratifier for treatment (264), this has not translated into clinical treatment changes until recently, where trials have begun comparing platinum drugs with poly(ADP-ribose) polymerase 1 (PARP1) inhibitors for ovarian cancer as well as *cis*-platinum in BRCA-related breast cancers as well as triple-negative sporadic breast cancer (42A).

**89.14.2.1. PARP1 Inhibitor.** The concept of synthetic lethality was described 50 years ago and is when two mutations are benign individually but result in cell death when combined (270). To repair both endogenous and exogenous DNA damage, cells utilize a range of DNA repair mechanisms. They are largely divided into single-strand break (SSB) and double-strand break repair mechanisms. The SSB mechanics include base excision repair, nucleotide excision repair and MMR. HR and nonhomologous end joining are DNA double-strand repair mechanisms. When the DNA repair pathway is compromised as with BRCA1/2 deficiency, this feature may be exploited as a cancer therapeutic with a PARP inhibitor (269).

When DNA SSBs encounter DNA replication forks, they can cause the fork to stall or collapse, which then creates subsequent double-strand breaks. Normally, these double-strand breaks are repaired quickly by the HR pathway. The hypothesis for the drug target known as a PARP1 inhibitor proposed that BRCA null (–/–) tumor cells have defective HR pathway. When tumors are treated with a PARP inhibitor, the cells must rely on alternative, less precise mechanisms to repair the breaks, which result in genomic instability and cell cycle arrest (271). In two recent phase II multicenter studies of the effectiveness of olaparib, an orally active PARP inhibitor, patients were randomized to one of two doses of olaparib, after having been on at least three different prior chemotherapies. Subjects had to have measurable disease and had been treated with at least three different chemotherapies. In the higher dose, the arm given 400 mg had a 41% objective response rate with minimal toxicity (272,273). Phase III trials are underway using PARP1 inhibitors alone in less heavily treated patients and in combination with single agents.

Developing assays to delineate molecular biomarkers of response to PARP inhibitors in “sporadic” tumors with acquired defects in the HR system is critically important as there are a large number of patients with breast cancers who show a high degree of “BRCAness” but for whom no germline mutations are detected.

This is the entry into an exciting era into personalized therapy where cancers are treated on the basis of their underlying molecular characteristics and no longer as one disease. Ultimately, this may translate into preventive therapy as well, but this is a far more challenging prospect because of the heterogeneous nature of risk, and more importantly the unknown long-term consequences of a DNA damage repair mechanism being “inhibited” in all tissues.

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### Biography



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# CHAPTER

# 90

## Disorders of the Body Mass

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### 90.1 OVERVIEW

There are numerous human conditions that produce both elevated and reduced body mass, and extremes at both ends of the body-size spectrum are associated with increased relative risk of mortality (1). This chapter will be devoted to increased body mass associated with excess fat stores (obesity). Obesity-associated comorbidities include significantly increased risks for diabetes, cardiovascular disease, the “metabolic syndrome,” cancer (2), respiratory disease (asthma, sleep apnea), infertility, degenerative joint disease, depression, anxiety, and discrimination both in social life and in the workplace. It is likely that diabetes-related morbidities will contribute most to the disabilities related to obesity (3). The *metabolic syndrome*, defined as the combined presence of obesity, hyperinsulinemia, hypertension, and hyperlipidemia, is increasing in prevalence in parallel with obesity in adults (4) and in youth (5,6). For the first time in known history, the life expectancy in the United States may potentially decline because of, in large part, the obesity epidemic (7). The etiology of human obesity is undoubtedly multifactorial, reflecting complex interactions between genetic background, environmental conditions, and developmental processes.

#### 90.1.1 Definition

Centers for Disease Control and Prevention (CDC) defines individuals as obese whose body mass index (BMI) (weight in kilograms divided by the square of the height in meters;  $\text{kg}/\text{m}^2$ ) exceeds the age- and sex-specific 95th percentile. Those who have a BMI between the 85th and 95th percentiles are overweight and are at increased risk for obesity-related comorbidities. High BMI correlates well with excess body fat in all age groups and in both genders, with the exception of persons with very high muscle mass (e.g. “body builders”). A cutoff of

$>30 \text{ kg}/\text{m}^2$  is accepted as defining obesity in adult Americans. For growing children, age- and sex-specific BMI standards are used (Figure 90-1).

In the past, the CDC recommended that the term obesity be avoided, and that the following terms be used: At risk for overweight—BMI between the 85th and 95th percentile for age and gender; overweight—BMI higher than 95th percentile for age and sex. But in 2010, based on the recommendations of an AMA expert panel, it was decided that the terminology for children would be the same as that used in adults, i.e. children whose BMI ranges from the 85th to 95th percentile for age and sex would be classified as overweight and those who exceed the 95th percentile will be classified as obese (8).

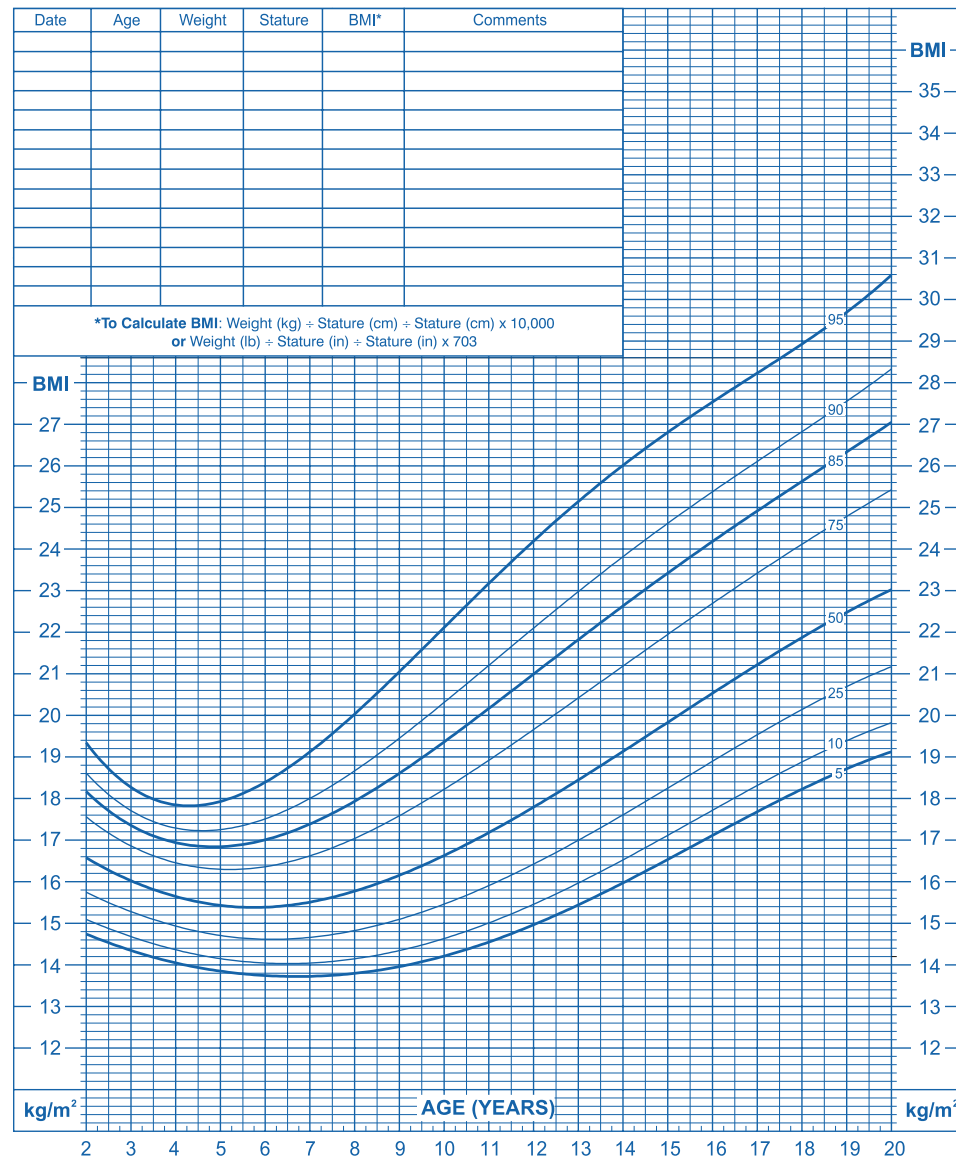
The regulation of body fat stores is complex, and is controlled by the interaction of environment and genetic background. The great importance of environmental factors on body size is underscored by the marked increase in obesity prevalence over the past 30 years, a time period whose brevity most certainly did not allow for a significant change in the gene pool. The prevalence of obesity is increasing at a dramatic rate. This is illustrated in a dramatic animation of US obesity prevalence by states compiled by the CDC (<http://www.cdc.gov/obesity/data/trends.html>). The prevalence is significantly higher, and the trend is even more pronounced, in American minority groups, particularly in females. This is not only occurring in adults (9) (Figure 90-2), but in youth as well (5) (Table 90-1). There is some indication that the trend may be flattening in some groups, but in others, the prevalence of overweight and obesity continues to rise. This pattern has been observed throughout the United States (10–12), has accelerated over the past 15 years, and is mirrored by a rise in the prevalence of type 2 diabetes mellitus in adults and youth. International trends parallel those of the United States (13,14). The prevalence in a given population, such as China, must be determined

## 2 to 20 years: Boys

### Body mass index-for-age percentiles

NAME \_\_\_\_\_

RECORD # \_\_\_\_\_



Published May 30, 2000 (modified 10/16/00).

SOURCE: Developed by the National Center for Health Statistics in collaboration with the National Center for Chronic Disease Prevention and Health Promotion (2000). <http://www.cdc.gov/growthcharts>



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**FIGURE 90-1** Childhood BMI curves, age 2–20 years. A. BMI for age, boys. <http://www.cdc.gov/growthcharts/data/set1clinical/cj41c021.pdf>.

using population-specific standards in order to avoid underestimating the scale of the problem (15).

The impact of environment on the epidemic of obesity includes unfavorable trends in food intake and physical activity, as well as barriers to reversing these trends (16). The impact of environment on body size is also underscored by the development of obesity in patients who have survived leukemia (17,18), or who have suffered hypothalamic damage (19), especially patients treated for craniopharyngioma (20). In addition, a human virus has been implicated in the etiology of animal obesity (21,22) and human obesity (23).

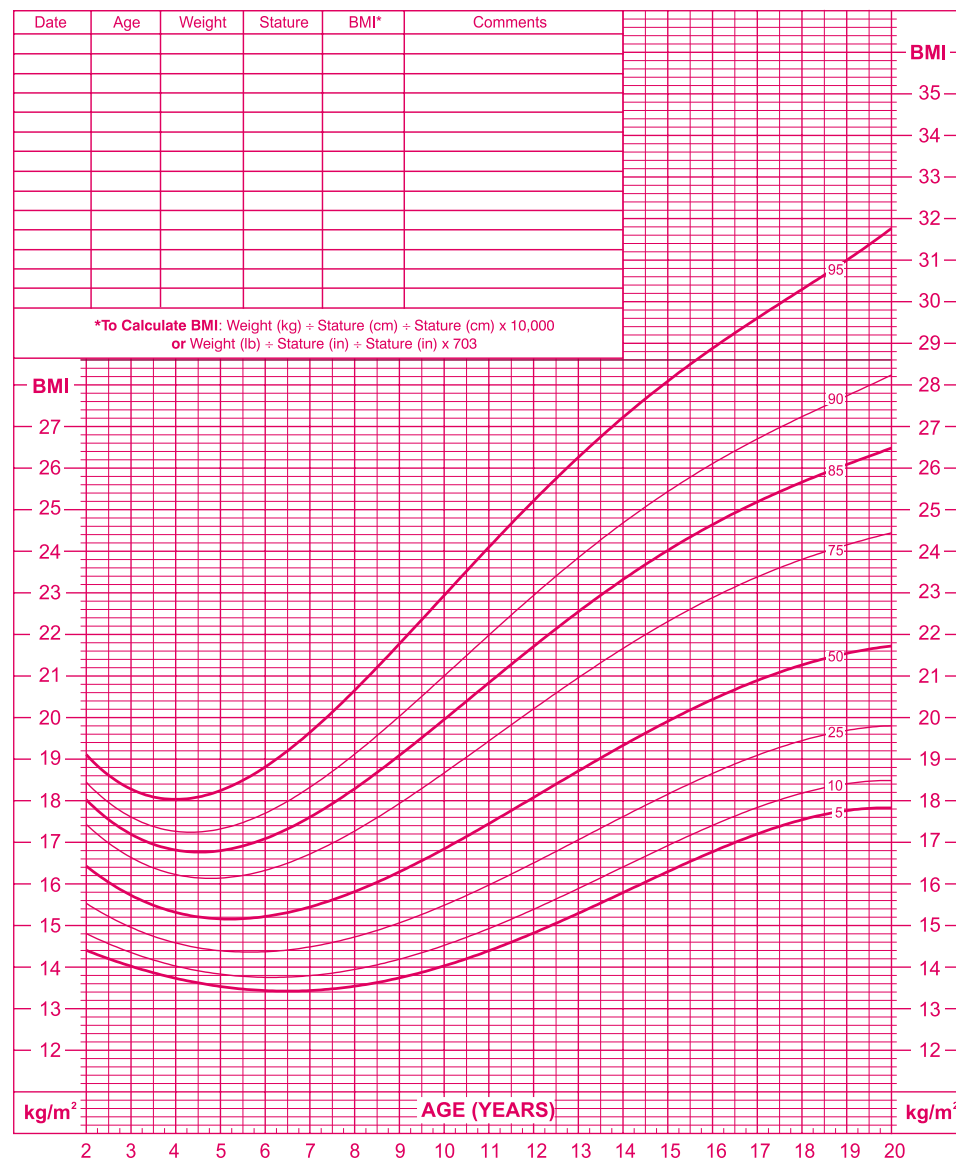
While this pronounced secular trend is almost certainly environmental, it is also clear that there is a significant heritable component in obesity risk, and that this genetic component explains up to 80% of the variation in BMI within a given environment. This genetic risk of obesity may have been acquired during the stage of human evolution when acquisition of food was achieved through strenuous activity (hunting, digging, etc.), and periods of prolonged fasting and famine were constant threats, that genotypes were enriched to favor energy storage. In the current environment of plentiful and easily accessible high-calorie food, these so-called “thrifty

## 2 to 20 years: Girls

### Body mass index-for-age percentiles

NAME \_\_\_\_\_

RECORD # \_\_\_\_\_



Published May 30, 2000 (modified 10/16/00).  
SOURCE: Developed by the National Center for Health Statistics in collaboration with the National Center for Chronic Disease Prevention and Health Promotion (2000).  
<http://www.cdc.gov/growthcharts>



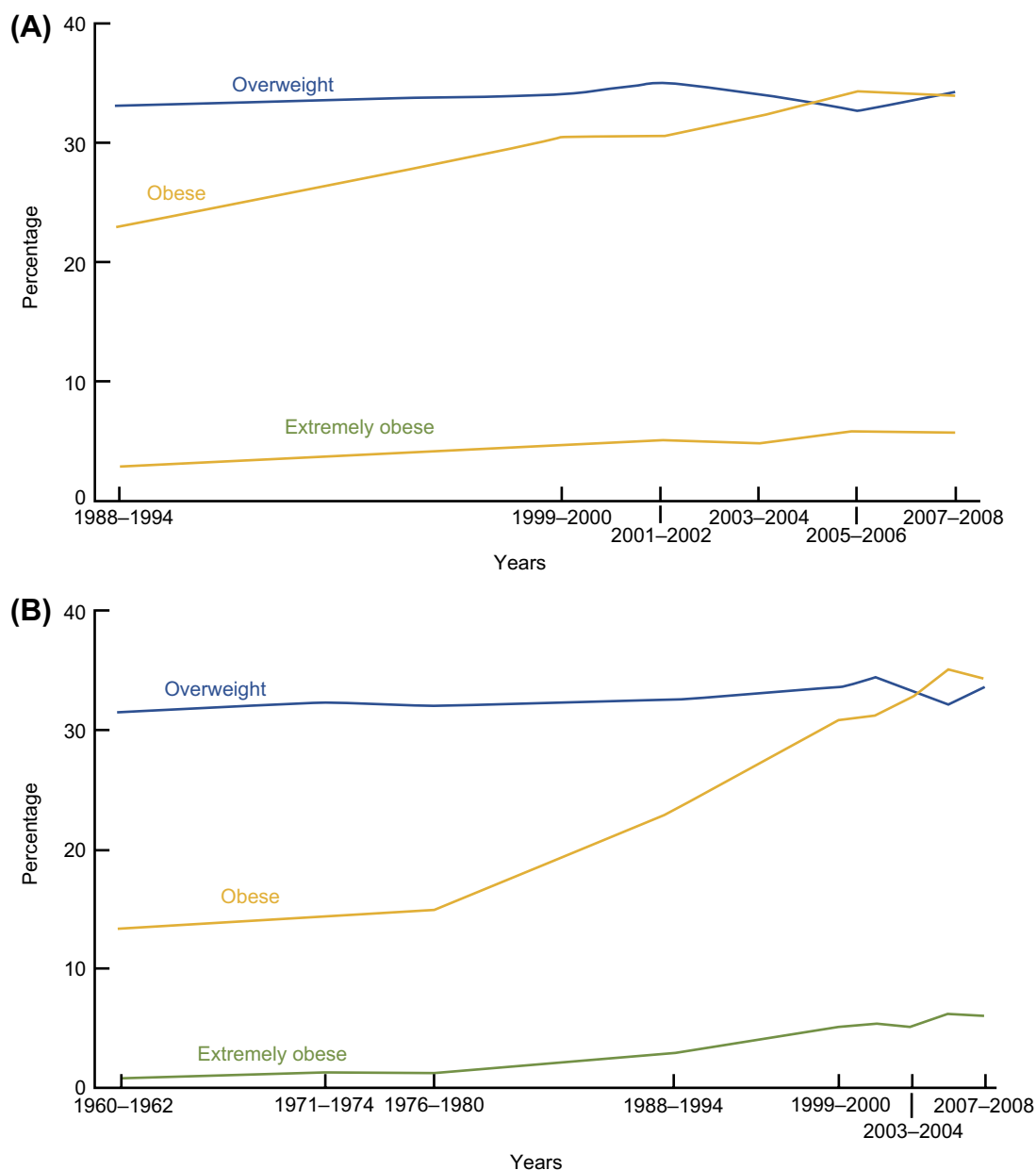
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**FIGURE 90-1** cont'd. B. BMI for age, girls. <http://www.cdc.gov/growthcharts/data/set1clinical/cj41c022.pdf>.

genotypes” are highly prevalent and, unfortunately, are now detrimental. This evolutionary pressure to prevent weight loss has led to the “absence of protection” (against weight gain) model of genetic control of obesity. Another model, termed the “Central Resistance” model, suggests that human genetic background would provide equal defenses against weight gain or loss, but that additional genetic or acquired defects impair the homeostatic control (24). In addition to absolute difference in BMI, body composition and the distribution of body fat also play an important role in the development of obesity-related morbidities (25) and variation in body

fat distribution also includes a genetic component (26). Body fat may be preferentially located in the abdomen (android or central obesity pattern) or surrounding the hips and thighs (gynoid obesity pattern). The android obesity pattern is more strongly associated with dyslipidemia, hypertension, and glucose intolerance (27), and is associated with increased intra-abdominal or visceral fat stores. This is the complex of features known as the *metabolic syndrome*. Thus, even at the same level of overweight, an individual with a greater amount of visceral fat is more likely to have or develop serious health consequences associated with obesity.





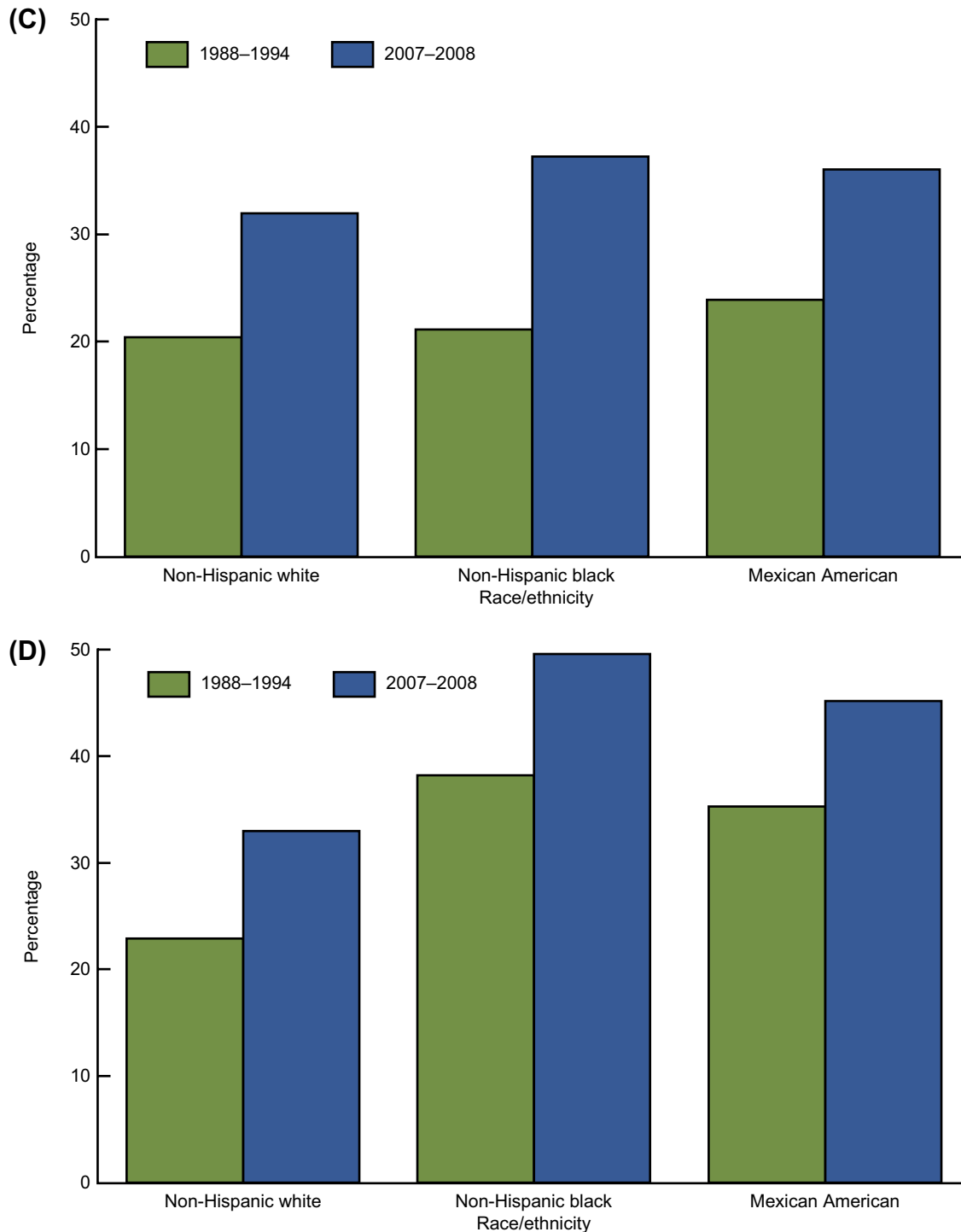
**FIGURE 90-2** A. Trends in overweight, obesity, and extreme obesity among adults aged 20 years and over: United States, 1988–2008. B. Trends in overweight, obesity, and extreme obesity among adults aged 20–74 years: United States, 1960–2008.

### 90.1.2 Heritability of BMI

The important influence of heredity on human body size has been demonstrated in multiple studies of dizygotic and monozygotic twins, and of adopted individuals and their biological siblings (28). Studies of twin pairs have consistently demonstrated higher concordance for body size among monozygotic than dizygotic twins. In a study of 1974 monozygotic and 2097 dizygotic pairs, concordance for body size at six different degrees of overweight (15, 20, 25, 30, 35, and 40% overweight) at approximately 20 years of age were 1.9–3.6-fold higher for monozygotic than dizygotic pairs (29). In a study of adult adoptees and their biological siblings, both full and half sibs, there was

a significant correlation of BMI in biological sibs across the entire distribution of body sizes. This correlation was much stronger for the full sibs (30). In a study of pediatric twins, there was a genetic contribution to percentage body fat (PBF) measured by bioelectric impedance that was distinct from the genetic contribution to BMI. This contribution accounted for 75–80% of the variation in PBF, and 62.5% of the total genetic variation in PBF was due to genes that influenced PBF but not BMI (31).

In *phenotypic* analyses of large unrelated populations, there is statistical evidence for recessive gene effects on body size variables, though the specific genes were not identified. Some examples include BMI in Muscatine,



**FIGURE 90-2** cont'd. C. Prevalence of obesity among men aged 20 years and older, by race and ethnicity: United States, 1988–1994 and 2007–2008. D. Prevalence of obesity among women aged 20 years and over, by race/ethnicity: United States, 1988–1994 and 2007–2008. All figures available in Reference (9).

Iowa (32), BMI in Caucasian and African-American families (33), abdominal visceral fat in Québec (34), relative fat pattern in Utah pedigrees (35), and obesity in American Pima Indians (36).

Studies to identify factors contributing to the genetic epidemiology of childhood obesity have been undertaken using the population of Muscatine, Iowa. This population

has been studied extensively with longitudinal follow-up of body size and relative fatness, cardiovascular risk factors, blood lipid levels, blood pressure, and other phenotypic features for over 40 years. The data obtained from the children in this community established normal ranges for tracking of height, weight, skin fold thickness, blood pressure, cholesterol and triglyceride levels (37).

**TABLE 90-1** Prevalence of Overweight (BMI >95th percentile) for American Children 6–19 years of Age

Years	Age 6–11 years	Age 12–19 years
1963–1970 <sup>a</sup>	4.2	4.6
1971–1974	4.0	6.1
1976–1980	6.5	5.0
1988–1994	11.3	10.5
1999–2000	15.1	14.8
2001–2002	16.3	16.7
2003–2004	18.8	17.4
2005–2006	15.1	17.8
2007–2008	19.6	18.1

<sup>a</sup>1963–1965 data are for 6–11-year-old children, and 1966–1970 data are for 12–17 (not 12–19)-year-old children. Data obtained from References (412) and (5).

The relationship between ponderosity and increased coronary risk factors was established for school children and their family members (38). As an extension of this study, it was demonstrated that there was increased familial cardiovascular mortality among the obese school children (39,40). A longitudinal study of trends in BMI in this population indicated that both genetic and environmental factors were involved in the variability of BMI (32). The data indicated strong support for a single autosomal-recessive locus with a major effect that accounted for almost 35% of the variation in adjusted BMI. Polygenic loci accounted for an additional 42% of the variation. Therefore, only 23% of the variation was not explained by genetic factors and was presumed to represent the environmental influence. The most recent report from the Muscatine Studies show that childhood BMI is the most predictive phenotype for adult obesity (41).

In other communities, longitudinal studies have also demonstrated familial aggregation of obesity and cardiovascular risk. These include the Bogalusa Heart Study (42,43), the San Antonio Family Heart Study (44), the Heritage Study (45), the Québec Family Study (46), and studies of American Pima Indians (47).

Human obesity in rare instances may be associated with defects at a single genetic locus. These include the Prader–Willi (48) and Bardet–Biedl/McKusick–Kaufman Syndromes (49), Alström Syndrome (50), and interstitial deletion of chromosome 18 (q12.2q21.1) (51). The mechanisms by which these genetic defects produce the obesity phenotype are not completely known. Several rare single gene defects produce obesity through better understood mechanisms (see Section 90.3).

An extremely important factor in maintenance of body weight is the relationship between body weight and total energy expenditure (52). The trend toward returning to a specific set point for body weight is powerful, and results from not only a reduction in total energy expenditure in response to weight loss but also an increase in energy expenditure with weight gain. There are no doubt genetic

factors controlling this set point (described in Section 90.2). Sustained weight gain is also associated with an increase in urinary norepinephrine excretion and in serum triiodothyronine concentrations, with the reverse patterns seen with sustained weight loss. The percentage of changes in these parameters correlated with the percentage of changes in energy expenditure (53). Changes in carbohydrate metabolism were also noted. In subjects with sustained weight gain, trends toward insulin resistance were more apparent in those who were obese than in never obese subjects, suggesting to the investigators that there is a threshold effect of total body fat on insulin sensitivity (53). Ethnic variability in resting energy expenditure has been demonstrated, being higher in white than in black prepubertal girls (54), and higher in white than in black prepubertal children, independent of PBF and sex (55). There is ethnic variability in insulin sensitivity and atherogenic risk among adolescents of comparable BMI. Black-obese adolescents have a more diabetogenic insulin secretion and sensitivity profile than white-obese adolescents. On the other hand, the white-obese youth had more visceral adiposity and atherogenic risk factors than their black peers with similar BMI (56). Genetic factors are likely to have major influence on the lower physical activity and resting energy expenditure observed in infants who later become obese children (57). A somewhat genetically isolated population, the Old Order Amish, that is known to have high levels of physical activity has a lower rate of obesity despite high caloric consumption (58).

## 90.2 GENETIC ARCHITECTURE OF OBESITY

The detailed genetic architecture of obesity risk has not yet been precisely defined. Even the true magnitude of the heritability of obesity is not yet settled and estimates range from as low as 20% to as much as 80% (59). A relatively small percentage (5% or less) of obesity cases is due to monogenic or syndromic obesity (60), but most cases are due to complex interaction between multiple genes and environmental factors. Whether the genetic component of this risk is due to multiple common genetic variants of small effect (common disease common variant hypothesis) or whether the effect is due to multiple rare variants or even a few rare variants of large effect has not yet been clarified. But whatever the nature of this genetic influence, it does appear to become more prominent as the prevalence and severity of obesity increases in a given population, indicating that genes influencing obesity may be more aggressively expressed in an obesogenic environment (61).

It is also becoming apparent that individual genomes differ from each other much more than previously assumed; for example, it has been estimated that as compared to the reference haploid genome, each individual human genome on average contains some three and a half million single nucleotide variants (SNV) and about

1000 copy number variants (CNV) of >450 bp, many of which are rare in the population from which the individual was sampled and are unique to the individual's family or clan. Each individual genome is truly unique, not just in terms of sequence variants in individual genes, but in terms of the complex interaction between multiple genes and gene networks. Our models of genetic risk, therefore, need to be modified to take the interplay of multiple variants within each unique individual genome into consideration (62).

In the following pages, we will outline the known monogenic and syndromic forms of obesity, their animal models and human correlates, and the results of recent genome-wide scans and linkage studies of common (polygenic) obesity, keeping in mind that the boundaries between these categories are not sharply defined. Genes and chromosomal regions involved in monogenic forms of obesity are also involved in polygenic common obesity and all forms of obesity are ultimately the product of complex interactions between multiple genes and environmental factors. Thus, the same mutation that causes morbid obesity in one individual may have a significantly attenuated effect in another individual with a different individual genetic background, environmental exposures and family and clan genomic structure.

### 90.2.1 Animal Models and Human Correlates (Spontaneous Mutations)

In rodents, multiple examples of spontaneous single gene mutations producing obesity are known, and form the basis for a candidate gene approach to identify the genes responsible for human obesity (Table 90-2). Several human counterparts of these rodent obesity syndromes have been identified, and will be described in Section 90.3.

**90.2.1.1 Leptin and Its Receptor.** The prototypic obese mice with single gene defects are the obese (*ob/ob*, *Lep<sup>ob</sup>*) and diabetes (*db/db*, *Lepr<sup>db</sup>*) autosomal-recessive mutations. If present on the same genetic background

strain, they cause the identical phenotypes of severe hyperphagia, obesity, diabetes, defective thermogenesis, and infertility due to hypogonadotropic hypogonadism. Parabiosis experiments showed that *ob/ob* animals were unable to produce a circulating factor regulating food consumption, and that *db/db* mice were unable to respond to this factor despite producing it in excessive quantities (63). The phenotype of the *ob/ob* mouse is partially obliterated by adrenalectomy (except for the thermogenic defect) and these adrenalectomized obese mice have significantly increased sensitivity to corticosterone (64). Mice with electrolytic lesioning of the ventromedial hypothalamus (VMH) were found to be resistant to the circulating factor thought to be absent in *ob/ob* mice, but lacked the other phenotypic features of *ob/ob* and *db/db* such as diabetes, defective thermoregulation, and infertility (65). This led to the theory that the VMH was only one of the several sites of action of this circulating "satiety" factor. The phenotypic expression of the *ob/ob* and *db/db* genotypes is highly dependent upon the background strain; for example, the BL/Ks background is associated with more severe diabetes than the BL/6 background (65).

The mutant gene responsible for the phenotype in *Lep<sup>ob</sup>* mice encodes the protein leptin (66,67), which is deficient in these animals. The main site of expression of *ob* (*Lep*) mRNA is white adipose tissue (67). The *Lep* sequence is highly conserved among vertebrates, and the human gene maps to chromosome 7p31. Leptin is a cytokine-like hormone secreted mainly by white (not brown) adipose tissue (67,68), with myriad effects including modulation of satiety and basal energy expenditure, and sexual maturation. Treatment of *ob/ob* mice with recombinant leptin or through leptin gene therapy (69,70) corrects the obesity/diabetes phenotype.

Recombinant leptin treatment causes some weight reduction in normal mice and rats, but has no effect in *db/db* mice (71,72). Recombinant leptin also corrects the infertility in *ob/ob* mice (73), and stimulates early

**TABLE 90-2 Rodent Obesity Mutations, Human Regions of Synteny, and Human Homologs**

Mutation	Gene	Mode of Inheritance (Autosomal)	Rodent Chromosome	Human Syntenic Region	Human Mutation Described	Mutant Protein
Mouse						
Agouti	<i>Ay</i>	Dominant	2	20q11	No <sup>a</sup>	ASP
Diabetes	<i>db</i>	Recessive	4	1p31	Yes	Lepr
Fat	<i>fat</i>	Recessive	8	4q21	No <sup>b</sup>	Carboxypeptidase E
Obese	<i>ob</i>	Recessive	6	7q31	Yes	Leptin
Tubby	<i>tub</i>	Recessive	7	11p15	No <sup>c</sup>	Phosphodiesterase
Rat						
Fatty	<i>fa</i>	Recessive	5	1p31	Yes	Lepr
Corpulent	<i>faK</i>	Recessive	5	1p31	Yes	Lepr

ASP, Agouti signaling protein; Lepr, leptin receptor; CPE, carboxypeptidase E.

<sup>a</sup>ASP prevents binding of  $\alpha$ MSH to its receptor MC4R. Several dominant MC4R mutations are associated with human obesity.

<sup>b</sup>CPE is required for normal prohormone processing by prohormone convertase 1 (PC1). PC1 mutations are associated with human obesity.

<sup>c</sup>Phenotype is similar to human Bardet-Biedl and Usher Syndromes.



reproductive function despite slower growth in normal mice (74). Leptin acts as a “metabolic gate” allowing for pubertal maturation in the rat (75), similar to the effect seen in normal mice. In normal rats, the effects of recombinant leptin are enhanced by VMH injection relative to peripheral infusion (76), and leptin induces rapid modulation of synaptic transmission in isolated arcuate nucleus slices from rat hypothalami (77). In rats with streptozocin-induced diabetes, the iatrogenic leptin deficiency is reversed, and even overcompensated, by leptin treatment. This effect is independent of the amount of weight regained or the resulting blood glucose concentrations (78).

Leptin exists in protein-bound and free states in the plasma, the latter being the predominant form in obese subjects (79). The demonstration of high leptin levels in the plasma of obese individuals correlating with BMI and %BF, along with elevated leptin mRNA in adipose tissue, lead to the hypothesis that obese humans are, to some degree, leptin resistant (80). Leptin levels in plasma are normally higher in women than in men (81–83), and they correlate with %BF rather than genetic background in identical twins discordant for obesity (84). Circulating leptin concentration, when normalized for total fat mass, decreases in males as they progress to late pubertal stages, whereas the reverse trend is seen in females (85). Plasma levels decrease significantly after 60 years of age in both sexes (86). Fasting and sustained weight loss result in a reduction in plasma leptin, however, high-fat diet and energy expenditure *per se* have no effect (83,87). As expected, leptin levels are low in women with anorexia nervosa (88), high in patients with the Prader–Willi Syndrome (PWS) (89), and relatively high in newborns (90), but correlate with %BF in all groups. There is a normal oscillation and diurnal rhythm of leptin levels (91), which shows a blunted pattern in women athletes with amenorrhea relative to athletes with normal menses (92). This perhaps provides a correlation to the defect in sexual maturation seen in *ob/ob* mice. Exposure of cultured human hepatocytes to high concentrations of leptin results in attenuation of insulin-induced activities including downregulation of gluconeogenesis (93), leading to the possibility that leptin is involved in the pathogenesis of the diabetes associated with human obesity.

The gene encoding human leptin has been studied extensively, but with the exception of leptin deficiency caused by rare *LEP* gene mutations (94), its importance in altered human satiety and abnormal body size determination has not been clearly demonstrated. Studies of this gene in large panels of obese and/or diabetic individuals have failed to demonstrate mutations (95–97). Linkage of obesity to this genetic region was not demonstrated in Pima Indians (98), nor in Mexican-Americans with NIDDM (99), and Mexican-American obese sib pairs from Starr County, Texas (100). However, some investigators have shown a suggestion of linkage of polymorphic markers near *LEP* to extreme obesity

(101,102), to obesity-related traits and insulin precursors in Mexican-American families with a type II diabetic proband (103), and to lower leptin levels in obese subjects (104). A leptin gene polymorphism is associated with hypertension, independent of obesity, in a Japanese population (105).

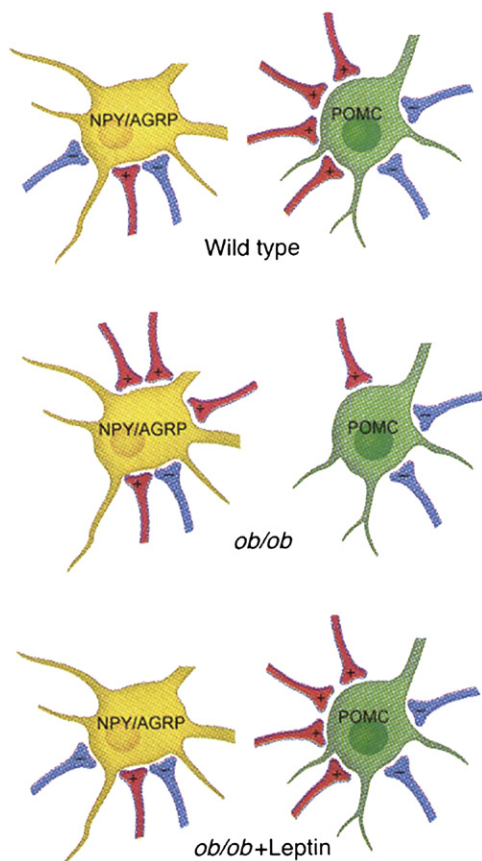
The cloning of the leptin receptor gene *Lepr* (106) from mouse choroid plexus led to characterization of the mutations causing the mouse *db/db* phenotype, and its rat homologs, the Zucker fatty (*fa/fa*) and obese Koletsky phenotypes (107–111). Thus, mutation of the same gene, *Lepr*, occurred spontaneously in three different strains and produced strikingly similar phenotypes. Multiple splice variants of *Lepr* exist (112–114), and are expressed in various tissues within and outside of the central nervous system (CNS), including lung, liver, skeletal muscle and kidney (106), the major site of leptin clearance (115). One of these encodes a circulating (short) form of the leptin receptor, which transports leptin across the blood–brain barrier producing a saturable system of transport into the CNS (79). If excess leptin is secreted, such as in obese individuals with a large fat mass, plasma levels of free leptin will increase significantly because of the saturable transport of the system. The production of this short or soluble form of the receptor is regulated independently by many different physiological conditions, and serves to modulate the amount of free leptin available to the transmembrane signaling long form of the receptor (116).

The cascade of effects downstream of leptin binding to its receptors is not completely understood, but involves activation of members of the STAT family of transcriptional activators (117,118). Both insulin and leptin work in parallel in many of these pathways. Treatment of *fa/fa* (leptin receptor defective) rats with a thermogenic mixture of sympathomimetic amines (ephedrine and methylxanthines) can reverse or prevent their obesity (119,120). Such findings suggest that the downstream effects of leptin include actions on the sympathetic nervous system. This has been confirmed in studies of rodents as well as humans (121,122). Mice that are heterozygous for either the *ob* or the *db* mutation have body composition and leptin homeostasis phenotypes that are intermediate between homozygous wild-type and mutant animals (123).

Other downstream effects of leptin include inhibition of the neurons that produce neuropeptide Y (NPY), a hypothalamic neurotransmitter that can cause obesity through appetite stimulation (described in more detail below), and which is found at high levels in the hypothalami of *ob/ob* mice (124). Leptin also stimulates neurons that increase production of POMC, which is known to inhibit feeding. The NPY and POMC neurons in the arcuate nuclei of mice are rapidly altered by leptin administration. Genetically engineered wild-type and leptin-deficient (*ob/ob*) mice, who express two different green fluorescent proteins in the two neuronal types,

were administered systemic leptin treatment. In the *ob/ob* mice, who normally had increased NPY excitatory synapses and decreased inhibitory POMC synapses, leptin treatment caused a rapid change in this pattern. Within 6 h, before any effect on food intake or weight was observed, the numbers of excitatory and inhibitory synapses became indistinguishable from those of the wild-type, untreated mice (Figure 90-3) (125).

In addition to the effects on feeding, leptin receptor-mediated effects alter bone metabolism, the immune system, angiogenesis, and the cardiovascular system (126,127). Thus, it is clear that animals or humans with defective leptin receptors would require therapeutic intervention aimed at a number of organ systems in order to correct the defect. Multiple factors exist that result in leptin resistance at the tissue level in obese subjects, including interactions with transcription factors that are associated with obesity-induced endoplasmic reticulum (ER) stress. These factors attenuate leptin signaling, and may, in fact, promote more weight gain and adiposity in obese individuals (128).



**FIGURE 90-3** Changes in hypothalamic synapses of *ob/ob* mice after leptin treatment. This diagram demonstrates the difference in the number of excitatory (red) and inhibitory (blue) synaptic inputs onto NPY (coexpressing AgRP) (yellow) and POMC (green) neurons in wild-type and *ob/ob* mice. Leptin treatment to *ob/ob* mice rapidly reversed the number of inputs onto NPY and POMC to wild-type levels. NPY, neuropeptide Y; AgRP, Agouti-related protein; POMC, proopiomelanocortin. Pinto et al. (125), Figure 94-4C, page 114.

In population genetic studies, the *LEPR* region has shown no relation to body size variables in the Pima Indians (98), but a possible correlation was seen with acute insulin response in the same group (129). In the Québec Family Study, results of sib-pair linkage analysis with 137 adult sibships suggested linkage of body fat and insulin levels to polymorphic markers on chromosome 1p32–p22, the cytogenetic location of *LEPR* (130). These markers spanned the regions syntenic with mouse *db* as well as one of the 10 mouse quantitative trait loci (QTLs) related to susceptibility to diet-induced obesity, termed *Do1* (131). Polymorphisms within the *LEPR* introns (130) and exons (132) have been tested for their relationship to obesity in humans. Among these, the sequence variations at coding exons 2 (Lys109Arg), 4 (Lys204Arg and Gln223Arg), and 12 (Lys656Asn) are the most likely to have effects on receptor function. These amino acid substitutions are at positions that are conserved in rat, mouse, and humans. In addition, the codon 223 and 656 variants cause changes in charge (neutral to positive, and positive to neutral, respectively). Of 20 nondiabetic American Pima Indians chosen for extremes in body size, seven *LEPR* sequence variations were identified, including Lys109Arg and Gln223Arg. Three of these variants were within noncoding regions of the gene and these were found exclusively in obese Pima Indians (133). Lys109Arg and Gln223Arg were associated with BMI and fat mass in Caucasians, but not in African-Americans in the HERITAGE Family Study (134). The Lys656Asn variant was significantly linked to BMI in the Muscatine (135), and Gln223Arg was linked to fat mass in the Québec Family Study (136). On the other hand, in adult subjects from Baltimore, Gln223Arg and Lys656Asn were not associated with obesity traits (137), and the codon 109, 204, 223, and 656 changes were not positively associated with juvenile-onset obesity in 56 Danish men (138). However, a meta-analysis of *LEPR* polymorphisms failed to confirm this as an important population-wide locus (139).

Severe early-onset obesity caused by a mutant leptin receptor was originally identified in three female siblings (140). These homozygotes had failure of pubertal development, and reduced growth hormone and thyroid stimulating hormone secretion. Despite absence of leptin signaling, and massively elevated plasma leptin concentrations, leptin mRNA levels in adipose tissue, assessed by quantitative reverse transcription polymerase chain reaction, were as expected for their BMI (141). This argues against a direct negative feedback loop in regulation of leptin gene expression in humans.

**90.2.1.2 Tubby.** The *tubby* (*tub*) mutation in mice is an autosomal-recessive trait, which is associated with less-severe obesity and insulin resistance than *ob* or *db*, is not associated with significant hyperphagia, causes variable degrees of hyperlipidemia depending on background strain, and is more severe in males than females. The phenotype also includes retinal degeneration and sensorineural

hearing loss similar to the human Usher, Alström, and Bardet–Biedl syndromes (BBS) (50,142,143). The mapping (144) and cloning (145,146) of *tub* has led to its characterization as a gene which is expressed mainly in the hypothalamus and encodes a protein with phosphodiesterase-like sequences, possibly affecting cellular apoptosis. Further studies have identified and characterized several Tubby-like proteins (TULPs), mutations of which in mammals can cause retinal degeneration, and/or hearing loss (147), as well as alterations in neural tube formation (148). The exact biological functions of these proteins remain unknown, however, their roles in complex cellular functions are being characterized. They are membrane-bound transcriptional activators that translocate to the nucleus, and interact in the G-protein system of intracellular signaling (149). It appears that TUB is the only member of this protein family that is implicated in obesity. It is highly concentrated in the nuclei of the hypothalamus that are involved in energy regulation (148). Human *tub* maps to chromosome 11p15. Linkage of human obesity to this cytogenetic region has not been demonstrated, nor have *tub* mutations.

**90.2.1.3 Fatty.** In the fatty (*fat/fat*) mouse, the recessively inherited mutation causes hyperinsulinemia without hyperglycemia, and postpubertal obesity that is less severe than that seen in *ob/ob* or *db/db* mice. The “hyperinsulinemia” is actually due to hyperproinsulinemia. The molecular defect is in the gene encoding carboxypeptidase E (CPE), an endoprotease required for normal processing of prohormones to active hormones, including proinsulin to insulin (150), and for transport and processing of propeptides in the granules of the regulated secretory pathways of the CNS. The primary example of such a peptide is proopiomelanocortin (POMC), which is the precursor for adrenocorticotrophic hormone (ACTH), melanocyte-stimulating hormone (MSH), beta-endorphin, and beta-lipoprotein. POMC has a “sorting signal” consisting of two acidic N-terminal residues, which bind to the two basic residues of CPE. Proinsulin and proenkephalin also attach to this CPE-binding site (151). POMC and proinsulin are then cleaved by prohormone convertase 1 (PC1).

The role of CPE mutations (*fa*) in the expression of the human obesity phenotype is unknown; however, a correlate has been described in which there are mutations of the *PC1* gene (152). This patient has obesity along with impaired processing of insulin leading to diabetes and hyperproinsulinemia, and ACTH deficiency due to impaired processing of POMC. Polymorphic genotypes of the *PC1* gene and neighboring anonymous DNA markers, however, do not confer susceptibility to obesity, NIDDM, or gestational diabetes (153), but may play a role as one of the important polygenes in these syndromes.

**90.2.1.4 POMC.** Since POMC processing defects lead to obesity, it is not surprising that the POMC locus itself is important in body size determination. Two siblings have

been identified with compound heterozygous POMC gene mutations. They had early-onset severe obesity, congenital ACTH deficiency, and red hair pigmentation (154). Both mutations alter the cleavage of POMC, and blood levels of POMC cleavage products (ACTH,  $\alpha$ MSH) were subnormal. The heterozygous parents were unaffected. In genome-wide searches, the region containing the POMC locus is linked to serum leptin levels and fat mass in Mexican-Americans (155,156), and to serum leptin levels in African-Americans (157) and French Caucasians (158). However, POMC coding region and promoter region sequence variants were not related to obesity in Caucasians with juvenile-onset obesity (159). A similar negative association with either extreme of body size was seen when the POMC coding region was screened in patients who were obese, underweight, or with anorexia nervosa (160).

**90.2.1.5 Agouti.** The *yellow* mutation of Agouti mice is a dominant trait which causes yellow coat color (rather than the wild-type hairs which are banded black and yellow), obesity and diabetes. This was the first rodent obesity gene to be cloned, and subsequently over 30 Agouti *yellow* alleles have been identified, which vary in their phenotypic expression. The molecular defect in the most dominant form *Ay* is a deletion in the 5' regulatory region, which brings a different promoter into contact with the coding region. This results in excessive production of Agouti signaling protein (ASP) through ectopic overexpression of normal Agouti mRNA in many tissues, rather than its normal site of expression which is limited to the skin (161). Other Agouti alleles also result from mutations that cause abnormal promoters to be utilized, such as *Aiapy* which has an insertion of retroviral DNA between exons C and D, directing use of an aberrant promoter. This mutation is paternally imprinted. The *Aiy* and *Asy* mutations have insertions upstream of the first coding exon acting as ubiquitous promoters. When transgenic mice overexpress ASP only in the hair follicles of skin, obesity and diabetes are not observed, leading to the hypothesis that these effects are due to a paracrine rather than an endocrine effect of ASP (161). The large number of these dominant Agouti alleles makes it likely that this locus has a propensity toward spontaneous mutations, making it an interesting candidate gene for studies of the genetics of obesity.

ASP and another similar peptide, Agouti-related peptide (AgRP) act as antagonists of native ligand binding to melanocortin receptors 1–4 (MC1R through MC4R), each of which are expressed differentially in specific tissues. AgRP binds specifically to the MC4R in the CNS, and ASP binds to both CNS MC4R and to skin MC1R (162). Transgenic mice with a knockout (KO) mutation of the CNS-expressed MC4R (MC4R-KO) have features of the yellow Agouti phenotype. Both *Ay* and MC4R-KO mice have high levels of NPY expression in an abnormal CNS site, the dorsal medial hypothalamus, which may be an etiologic factor for the obesity phenotype in this



syndrome (163). Mice with MC4R haploinsufficiency have an obese phenotype that is intermediate between wild-type and null mice (164).

**90.2.1.6 Melanocortin Receptors.** Although ASP mutations have not yet been described in humans, several MC4R mutations are now known to be associated with obesity. Two frameshifts, a 4-bp deletion at codon 211 and a 4-bp insertion at nucleotide 732 of the coding sequence, are associated with dominantly inherited obesity (165,166). A novel in-frame deletion of codons 88–92 has been described in one obese female (167). Several other MC4R mutations have been identified among obese cohorts (168,169). Up to 10% of obese subjects in Germany have MC4R mutations (168). The prevalence rate appears to be lower in the United States (170). Functional analyses of several mutations have confirmed their deleterious effects on receptor function (169–171). The codon 88–92 deletion prevents ligand binding (167). The dominant nature of these mutations is presumed to be due to haploinsufficiency as it is in the transgenic mouse model. In one child, homozygous for a 2-bp deletion causing premature termination of MC4R, the obesity phenotype was more severe and of earlier onset than in his heterozygous siblings and parents (172). Interestingly, this child did not have any significant differences in insulin or glucose levels during an oral glucose tolerance test, when compared with 39 BMI/age/sex-matched control children.

In the Québec Family Study, linkage and association analyses showed that both MC4R and MC5R genotypes were related to obesity phenotypes (173). The melanocortin receptor MC3R mutation I183N, identified in a father and daughter with high PBF, alters agonist-induced G-protein activation (174). It does not exert a dominant negative effect on the wild-type receptor, thus is likely to cause its phenotypic effect through haploinsufficiency. In mice with MC3R KO, there is a decrease in food intake and normal energy expenditure, but they have twice the fat mass of their wild-type littermates (174).

## 90.2.2 Energy Expenditure

**90.2.2.1 Hormonal and Genetic Control.** The genetic control of energy expenditure and heat production is an area of intense investigation as many of these metabolic and neuronal pathways are involved in expression of the genetic defects described above. Defects specific to this system are also important as independent contributors to obesity. In rodents, the generation of heat by brown adipose tissue (BAT) is related to the pathways controlling body fat content and distribution. BAT generates heat through its capacity for uncoupling of mitochondrial respiration from oxidative phosphorylation via several mitochondrial uncoupling proteins (UCPs) (175–177). BAT as well as white adipose tissue (WAT) contains the beta adrenergic receptors ( $\beta$ ARs). The  $\beta$ ARs mediate lipolysis in BAT and WAT, as well as mitochondrial

UCP-mediated thermogenesis in BAT. The  $\beta$ 3-AR is thought to be adipose specific, is the predominant subtype in rodent BAT, and can be chronically stimulated by its specific agonists, as opposed to the  $\beta$ 1- and  $\beta$ 2-ARs which become refractory to chronic stimulation (178). In transgenic mice deficient in BAT through targeted disruption of the *UCP-1* gene, obesity develops in the absence of hyperphagia (179). The BAT-deficient mice have enhanced susceptibility to diet-induced obesity and its comorbidities (insulin resistance, hyperglycemia, and hyperlipidemia) (180), which are not reversed by leptin treatment (181). Alternatively, constitutive transgenic overexpression of UCP-1 in both BAT and WAT of mice cause significant reduction in subcutaneous fat, both in normal and genetically obese (*A<sup>y</sup>*) mice (182). Consumption of a high-fat diet can alter UCP gene expression in some mouse strains but not others, indicating variable polygenic control of body composition and thermogenesis (183). In normal rats, UCP protein and mRNA are regulated in a tissue-specific and subtype-specific manner by fasting and by leptin treatment. In some circumstances, the effects on specific UCP subtype protein and mRNA are disparate (184).

In normal mice, there is strain-specific variation in the prevention of high-fat-diet-induced obesity by  $\beta$ 3-AR agonists, which is related to the ability to recruit BAT in WAT anatomic sites (185). Targeted disruption of the  $\beta$ 3-AR gene results in a much milder phenotype than seen in the BAT-deficient mice. They have only modestly increased fat stores (females greater than males), but also have upregulated levels of  $\beta$ 1 but not  $\beta$ 2-AR mRNA in adipose tissue (in BAT more so than in WAT) suggesting crosstalk among the receptor subtypes (186). In WAT of *ob/ob* mice, lipolysis and adenylyl cyclase activation by the  $\beta$ ARs is deficient. Whereas the  $\beta$ 3-AR is the main activator of cyclase activity in WAT of lean mice, it is only partially responsible for adenylyl cyclase activity in *ob/ob* mouse WAT (187,188). In normal rats, the interaction between the sympathetic nervous system, leptin, UCPs, and regulation of fat cell energy utilization is complex. The in vivo modulation of leptin and UCPs appears to depend on sympathetic nerve activity, plasma insulin and glucose, and hemodynamic factors (189).

Variation of the human  $\beta$ 3-AR (ADRB3) gene sequence has been studied extensively, again with disparate results. The human gene has been cloned and its product exhibits properties pharmacologically identical to rodent  $\beta$ 3-ARs (190). In omental fat tissue from obese individuals, there is increased catecholamine-induced lipolysis relative to cells from nonobese subjects, and this effect is mainly due to increased  $\beta$ 3-AR function (191). An ADRB3 missense mutation (Trp64Arg) has been associated with earlier onset of NIDDM and lower resting metabolic rate in Pima Indians (192), abdominal obesity, insulin resistance, and BMR in Finns (193,194), and an increased capacity to gain weight in a French cohort (195). There is no association between this polymorphism and NIDDM



susceptibility in at risk family members (196), obesity phenotypes in the Québec Family Study or in the Swedish Obese Subjects Cohorts (197), obesity and NIDDM susceptibility in Nauruans (198), obesity in Japanese men (199), BMI gained or response to a hypocaloric diet in morbidly obese Finns (200), or obesity in a population of healthy British men (201).

A polymorphism of *UCP-1* (202) is reportedly associated with resistance to weight loss with a low-calorie diet in the French (203), and this polymorphism may have an additive effect with the *ADRB3* polymorphism on rate of weight gain in morbid obesity (204). From a physiologic standpoint, there is no evidence quantifying the amount of BAT in humans, and studies of brown adipocytes from nonhuman primates indicate a lack of functional  $\beta$ 3-ARs (205). In addition, pharmacological characterization of the Trp64Arg-mutant  $\beta$ 3-AR shows no difference in its response to agonists when compared with wild-type receptors in CHO cells (206). In 204 Japanese subjects, the Trp64Arg variant allele was not associated with differences in BMI, plasma glucose or insulin, or family history of obesity or diabetes. However, those with the variant had lower resting autonomic nervous system activity than those without it (207). This conflicting evidence, combined with the mild phenotype in  $\beta$ 3-AR KO mice, would rule against this locus having a major gene effect on obesity. However, a minor effect, when combined with other defects affecting body fat deposition, may contribute to the obesity phenotype.

UCP activity is stimulated by a number of physiologic factors, including cold,  $\beta$ ARs, and fatty acids (208–211). When activated, the protein allows hydrogen ions to “leak” through the respiratory chain of the inner mitochondrial membrane, which abolishes the gradient of hydrogen ions required for ATP synthesis from stored nutrients (209). Mammalian UCP-1 is expressed mainly in BAT, which is present to a significant degree in humans only in the newborn period. The human *UCP-1* gene maps to chromosome 4q31 (210). Human *UCP-2* and *-3* are also expressed in WAT and skeletal muscle (78,208,209), and the genes map to a duplicated locus as adjacent genes on chromosome 11q13 (212,213). *UCP-3* is preferentially expressed in skeletal muscle (212), and has both long and short transcripts (214). In American Pima Indians, BMI is negatively correlated with both the *UCP-3* long and short forms, but not with *UCP-2*. Metabolic rate during sleep correlated positively with the long form of *UCP-3* (214). In the skeletal muscle of *UCP-3* KO mice, there was reduced mitochondrial uncoupling activity. However, there was no effect seen on body weight regulation, exercise tolerance, fatty acid oxidation, or cold-induced thermogenesis (215).

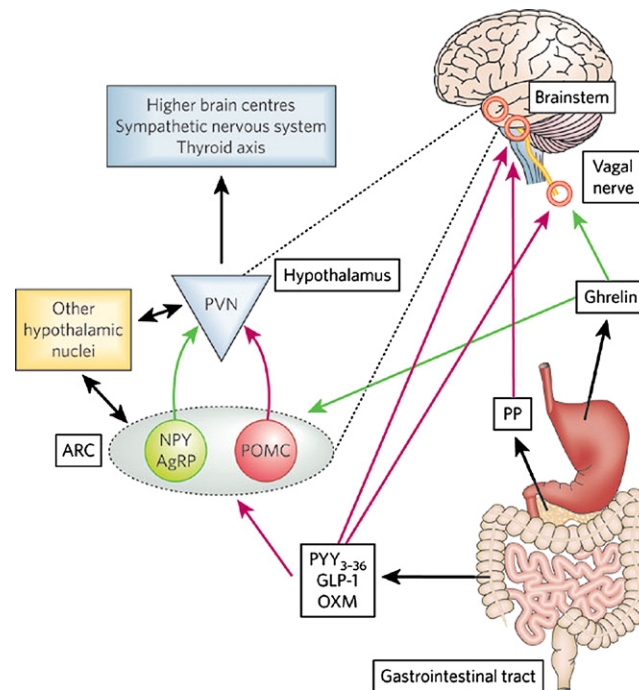
A *UCP-1*-associated Bcl-I RFLP may be associated with increased body size (202). Seven sequence variants of the *UCP-1* gene were identified in Danish subjects, but genetic variation in the coding region of the gene did not contribute to obesity in this population (216). There is

no association between *UCP-2* genotypes and obesity in French Caucasians (217). Allele frequencies of a *UCP-2* gene polymorphism at codon 55 (A55V) were similar in African-Americans and Caucasian Americans (218), but its association with body size was not characterized. A 45-bp insertion polymorphism of the 3′ flanking region of the *UCP-2* gene is correlated with high BMI and %BF in children (219). This insertion/deletion polymorphism results in mRNA species of different lengths, and the insertion variant is less stable. The insertion variant is in linkage disequilibrium with a pair of linked promoter region sequence variants (T/A at –2723 and G/A at –866). The minor allele –866A is associated with a decreased risk of obesity in middle-aged subjects from Austria (220). Regarding *UCP3*, an exon 5 C–T substitution that does not change the codon 210 Tyrosine residue was associated with significantly lower resting energy expenditure in African-American but not White women (221). There were no effects of genotypes of three other *UCP-3* variants, nor of two *UCP-1* and two *UCP-2* variants. In a review of over 40 studies of UCP gene variants in humans, *UCP-1* was not felt to contribute significantly to variability in BMI. However, *UCP-2* and *UCP-3* effects on BMI are accepted. As they are adjacent genes, it is likely that their genetic variants are in linkage disequilibrium, and the effects of one gene on BMI may reflect variants in the other (222).

### 90.2.3 Control of Feeding

Energy intake and expenditure is under the control of complex interactions between peripheral signaling and effector systems (e.g.  $\beta$ ARs and UCPs discussed above) and neuroendocrine systems (162,223). There is great complexity in these interactions (224), and the details of these pathways are under widespread investigation. Some of the pathways are illustrated in Figure 90-4. The contribution of signals from the gastrointestinal tract represents the more acute responses to feeding, rather than the chronic responses to energy stores (225). Among these are ghrelin from the stomach, insulin and glucagon from the pancreas, and PYY and glucagon-like peptides from the intestinal tract. The factors important in determining an individual’s set point for food intake may change as individuals become obese because of attenuation of leptin signaling induced by increasing adiposity (128).

**90.2.3.1 Ghrelin.** Ghrelin was first discovered in the 1970s as a growth hormone secretagogue. Its major role, however, is not modulation of growth hormone secretion from the anterior pituitary, but rather its role in increasing energy stores is felt to be more important, physiologically (226). It is secreted mainly by the stomach, with minor contributions from the brain, kidney, and placenta. These other sources may compensate for gastric secretion as patients having gastrectomies only have 65% reductions in blood levels. Serum levels are inversely correlated with BMI but are paradoxically elevated in children with the



**FIGURE 90-4** Interactions that regulate food intake and body fat mass. Dashed lines: inhibitory effects. Solid lines: stimulatory effects. Y1R and Y2R, neuropeptide Y (NPY) receptor subtypes; MC4R, melanocortin 4 receptor; GHsR, growth hormone secretagog receptor (ghrelin receptor); AgRP, Agouti-related protein; POMC, proopiomelanocortin;  $\alpha$ -MSH,  $\alpha$ -melanocyte-stimulating hormone; LEPR, leptin receptor; INSR, insulin receptor. (With permission from Kevin G. Murphy and Stephen R. Bloom. *Gut Hormones and the Regulation of Energy Homeostasis*. *Nature* **444**, 854–859.)

PWS (227). Ghrelin levels are high in anorexia nervosa, and levels drop after food intake in lean subjects. However, in obese subjects, the levels do not drop after eating (228). Ghrelin binds to specific receptors in the hypothalamus on NPY/AgRP neurons, and acts to some degree as a stimulator of hunger (225). Despite evidence that suggests a potentially strong influence of ghrelin on body size, ghrelin-deficient mice created by gene KO are the same as their wild-type littermates in terms of size, growth rate, food intake, response to fasting, body composition, and reproduction (229). In humans, variation in the ghrelin gene was associated with higher BMI and lower insulin response to glucose in a group of 70 tall obese children (230). Other evidence points to a genetic contribution of ghrelin to body size, including a study showing that ghrelin levels at baseline and in response to overfeeding, are more similar in monozygotic than dizygotic twins (231).

**90.2.3.2 Central Control of Feeding and Energy Expenditure.** The hormone leptin is made almost exclusively in peripheral (mainly adipose) tissues, and acts centrally in the hypothalamus by modifying two major effector systems. Low plasma concentrations of leptin and insulin (e.g. during weight loss) increase food intake and decrease energy expenditure by stimulating NPY synthesis, and perhaps by inhibiting sympathetic activity and other catabolic pathways. High leptin and insulin concentrations (e.g. during feeding and weight gain) decrease food intake and increase energy expenditure through release of melanocortin and corticotropin

releasing hormone (CRH), among others (232). The list of neuropeptides which are known to alter energy balance is growing rapidly. Some of these are listed in Table 90-3.

**90.2.3.3 Neuropeptide Y.** NPY is a neurotransmitter that is abundant in the hypothalamus, and may be important in mediating obesity. Hypothalamic levels of NPY and its message are elevated in *ob/ob* mice, *db/db* mice, and *fafa* rats. Central administration of NPY to lean rats causes hyperphagia, obesity, insulin resistance and increased triglyceride deposition in WAT. These effects are prevented by adrenalectomy before NPY treatment (233). In a special strain of *ob/ob* mice that are deficient in NPY, the obesity and hyperphagia are less severe, infertility is less marked, and diabetes is seen later and is milder than in *ob/ob* mice with intact NPY (234). Otherwise, normal mice homozygous for NPY KO mutations have normal fed and fasting plasma levels of leptin, thyroxine, corticosterone, and sex steroids, indicating NPY is not vital for normal pituitary function (235). They also have lower seizure thresholds and higher ethanol consumption and tolerance. However, after a fasted state, NPY KO mice have less food intake than WT mice, have anxiogenic-like behavior, and hypoalgesia (236). NPY receptor 1 KO mice have marked obesity (159), but NPY receptor 5 KO mice have a less severe obesity phenotype (237).

In human studies, iv NPY administration increases sleep and decreases ACTH release in males (238).

**TABLE 90-3 Central Nervous System Proteins (Neuropeptides) Involved in Energy Homeostasis**

Neuropeptide	Regulation by Leptin or Insulin
<b>Orexigenic (stimulates feeding)</b>	
Neuropeptide Y <sup>a</sup>	Decreased
Agouti-related peptide <sup>a</sup>	Decreased
Melanin concentrating hormone	Decreased
Orexin A and B (Hypocretin 1 and 2)	Decreased
Galanin	Decreased
<b>Anorexigenic (inhibits feeding)</b>	
Leptin	
α-Melanocyte-stimulating hormone <sup>a</sup>	Increased
Corticotropin releasing hormone <sup>a</sup>	Increased
Thyrotropin releasing hormone <sup>a</sup>	Increased
Cocaine- and amphetamine-regulated transcript (CART) <sup>a</sup>	Increased
Interleukin-1β <sup>a</sup>	Increased
Urocortin <sup>a</sup>	
Glucagon-like peptide 1	
Oxytocin	
Neurotensin	
Serotonin	
Somatostatin	

<sup>a</sup>Effects both energy intake and expenditure that change energy stores.

<sup>b</sup>β-endorphin treatment stimulates feeding in many animal models, but β-endorphin knockout male mice have hyperphagia and obesity.

<sup>c</sup>Most ghrelin is secreted from the stomach, but some is produced in the hypothalamus.

Information obtained from Schwartz et al. (232), and Sahu (413).

Genetic studies of the human genes encoding NPY have identified no deleterious mutations. Linkage analysis of the *NPY* gene showed evidence of linkage to body size in Mexican-American obese sib pairs (239), but not in French Caucasian obese families (240).

The NPY Y1 and Y5 receptors are transcribed from a common promoter region in opposite directions, suggesting they evolved from a gene duplication event (241). Linkage and association studies with these genes have failed to identify an effect on body size in several populations (239,240,242).

**90.2.3.4 Orexins.** Orexins A and B are stimulators of feeding that are secreted as preprohormones by the hypothalamus. They act through binding to orexin receptors, which are of the classical G-protein-coupled seven transmembrane domain class (243). Orexin KO mice have narcolepsy (244), a finding that may correlate with NPY-induced sleep in humans, described above. Human orexin receptors are localized in the pituitary gland (245) and in pheochromocytomas (246). Mutational analyses and linkage studies of the genes encoding these peptides have not yet been reported in humans with altered body size.

### 90.2.3.5 The Hypothalamic–Pituitary–Adrenal (HPA) Axis in Feeding and Body Size Determination.

It has been demonstrated in multiple animal models that adrenalectomy obliterates or attenuates the obesity syndromes expressed in genetically obese rats and mice, in diet-induced obesity, and in hypothalamic obesity (233). Replacement of only trace quantities of glucocorticoid to these adrenalectomized animals causes prompt return of the phenotypes indicating heightened sensitivity to the steroid. In humans and animals with hypercortisolemia, obesity is prominent. However, in adrenalectomized animals, the effects seen could result from a combination of glucocorticoid deficiency alone or in combination with the secondary elevation of hypothalamic CRH and pituitary ACTH production. Obese Zucker (*fa/fa*) rats have no difference in hypothalamic levels of CRH or in magnitude of CRH response to stressors when compared to lean littermates. However, basal secretion of CRH is less in the obese rats, and CRH response to adrenalectomy is more pronounced in the obese rats. Corticosterone infusion is more effective in suppressing CRH levels in adrenalectomized obese rats than in adrenalectomized controls (247), leading to the hypothesis that *fa/fa* rats have an abnormality of the HPA axis at a site proximal to that which mediates glucocorticoid responsiveness. Central administration of CRH as well as a related hormone, urocortin (248,249), mimics the events seen in the “stress response” including anorexia. This treatment prevents excessive weight gain in *fa/fa* rats when compared with pair fed *fa/fa* controls (250,251).

Leptin treatment of normal mice and rats causes a blunted HPA axis response to stress, perhaps via decreased release of CRH (252). In *A<sup>y/a</sup>* obese mice, the effects of POMC and leptin pathway activation are additive, yet independent (253). Full-length *LEPR* message has been identified in human adrenal cortex, and to a lesser degree in adrenal medulla. In cultured adrenal cells, leptin inhibited ACTH-stimulated cortisol, aldosterone, and dehydroepiandrosterone secretion (254). In addition, some individuals may have heightened sensitivity to glucocorticoid because of a sequence variant of the glucocorticoid receptor gene, which has been associated with higher BMI and lower bone density in the Rotterdam Study (255). These data suggest that therapeutic manipulations of the HPA axis could alter the phenotype of certain genetically obese individuals.

One of the most interesting developments in this area is the role of adipose tissue in the synthesis and degradation of cortisol through the action of 11β-hydroxysteroid dehydrogenase (11β-HSD). 11β-HSD2 converts cortisol to its inactive metabolite cortisone. In the kidney, this prevents activation of the mineralocorticoid receptor by cortisol, which is present in plasma in much higher concentrations than its native ligand, aldosterone. Cortisol has equal affinity for the receptor, and aldosterone is not a substrate for the enzyme. If 11β-HSD2 is deficient,

the syndrome of apparent mineralocorticoid excess occurs (256). Glycyrrhetic acid in licorice causes salt retention and hypertension because it inhibits  $11\beta$ -HSD2 activity (257). Topical administration of glycyrrhetic acid in females caused reduction in the underlying femoral subcutaneous fat (258), presumably by causing a local reduction in adipose tissue cortisol. Chronic ingestion of licorice was associated with a reduction in fat mass in 15 normal-weight young adults, without a change in BMI or in caloric intake (259).

$11\beta$ -HSD1 (cortisone reductase) converts cortisone back into cortisol in the liver. Defects in  $11\beta$ -HSD1 activity have been implicated in several clinical settings (260,261). There is mounting evidence that increased  $11\beta$ -HSD1 activity in visceral adipose tissue contributes to obesity and the metabolic syndrome. Transgenic mice that overexpress  $11\beta$ -HSD1 in adipose tissue develop the typical metabolic syndrome, with visceral obesity exaggerated by a high-fat diet, insulin-resistant diabetes, hyperphagia, hyperleptinemia, and hyperlipidemia (262). Patients with hypothalamic obesity may have increased  $11\beta$ -HSD1 activity (263), and adipose tissue of individuals with idiopathic obesity has been shown to have higher than expected  $11\beta$ -HSD1 activity (264,265).

The human gene encoding  $11\beta$ -HSD1 contains two polymorphic intronic microsatellites, and one intronic polymorphism useful for linkage and association studies. In a study of 439 normal adults from Glasgow, and of 557 Danish military draftees (234 with juvenile-onset obesity and the rest normal controls), there was no significant association of  $11\beta$ -HSD1 genotypes with BMI in either group, but there were weak associations between  $11\beta$ -HSD1 activity and waist-to-hip ratio (266). In American children, the intronic ins4436A polymorphism was associated with BMI and insulin resistance (267).

#### 90.2.4 Adipose Tissue Development and Function

Adipose tissue is a biologically active organ, involved not only with energy storage and release but also with autocrine and paracrine effects that are widespread (268). These effects are manifested in endocrine/hormonal function, immune function including its role in cardiovascular risk, and the CNS communication through leptin, tumor necrosis factor alpha (TNF $\alpha$ ), interleukin 6 (IL6), adiponectin, resistin, and other adipokines. Leptin and adiponectin are thought to be insulin sparing in their effects, whereas TNF $\alpha$ , IL6, and resistin are thought to contribute to the insulin resistance phenotype associated with obesity (268,269).

Adipocytes play a pivotal role in the maintenance of energy balance. The roles of BAT, WAT, sympathetic nervous system signals, and UCPs were discussed in Section 90.2.2. The development of mature adipose tissue from

preadipocytes is accompanied by a marked increase in the expression of adipose-related transcripts such as leptin and UCPs. The terminal differentiation of adipocytes is controlled by several transcription factors, one of the more important being peroxisome proliferator-activated receptor- $\gamma$ 2 (PPAR- $\gamma$ 2). After differentiation, the fat cells produce many products other than metabolic fuels that are secreted into the circulation. Prominent examples of these include the cytokines leptin (discussed in Section 90.2.1) and TNF $\alpha$ . In addition, adipocyte macrophage colony-stimulating factor has been shown to be important in stimulating adipocyte hyperplasia (270). Hormone-sensitive lipase (HSL) stimulates breakdown of triglycerides into fatty acids, which have been implicated in the development of insulin resistance. The gene encoding human *HSL* maps to chromosome 19q13, and contains a dinucleotide repeat polymorphism within one of its introns. This polymorphism was associated with obesity and type 2 diabetes in a French population (271).

**90.2.4.1 PPARs.** Members of the PPAR family of nuclear receptors regulate adipocyte differentiation. PPARs are divided into subtypes  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ , each of which is expressed in a tissue-specific manner. The PPAR- $\gamma$  class is expressed in adipose tissue, and has two isoforms,  $\gamma$ 1 and  $\gamma$ 2, which are differentially spliced products of the same gene. The PPAR- $\gamma$ 2 isoform is the longer protein, and is adipose specific (272). When PPAR- $\gamma$ 2 binds its ligands (prostaglandin derivatives, fatty acids, and thiazolidinediones), it forms a heterodimer with the retinoid X receptor that activates a cascade of fatty acid metabolism and preadipocyte differentiation (272–274). This effect is adipose-depot specific (273), and can even occur in fibroblasts (274). Homozygous *PPAR $\gamma$*  KO mice have a prenatal lethal phenotype due to placental dysfunction. Heterozygous mice seem to be protected from the development of insulin resistance, and have reduced fat mass, smaller adipocyte size, and hypersecretion of leptin (275). Mice that have a muscle-specific KO of *PPAR $\gamma$*  had normal responsiveness to thiazolidinediones, as well as normal glucose and insulin levels, despite excess adiposity with reduced caloric intake. However, they have whole-body insulin resistance when tested with a hyperinsulinemic euglycemic clamp, due to localized insulin resistance in nonmuscle tissues (liver, and perhaps, fat) (276).

The human gene encoding PPAR- $\gamma$ 2 has been cloned, and maps to chromosome 3p25 (277). Several sequence variants have been described, and as with other candidate genes for obesity, studies in human populations have revealed differing results. A Pro115Gln variant was shown to accelerate differentiation of murine fibroblasts into adipocytes when it was over expressed (278). This variant was identified more frequently in German obese subjects than nonobese subjects (278). However, this variant was not found in a different study of German subjects (279), nor in Danish men (280). Another more widely studied variant, Pro12Ala, has also shown both



positive and negative correlations with obesity. It was initially studied and ruled out as a candidate gene for lipotrophic diabetes (277). *PPAR-γ2* Pro12Ala occurred with the same gene frequency in obese and lean German subjects (279). Association studies with two different Caucasian populations revealed a positive relationship between Pro12Ala and high BMI (281). In French subjects, studies of this variant suggested a role for it in weight control and lipid homeostasis, but not in the etiology of type 2 diabetes (282). Among patients with type 1 and type 2 diabetes, the variant had no effect on lipids or BMI (283). In Mexican-Americans from the San Antonio Heart Study, presence of the Pro12Ala allele was positively associated with BMI and serum leptin levels (284). A large study of Danish men underscores the conflicting nature of these study results. Obese Danish men who were homozygous for Pro12Ala had higher BMI and higher weight gain than wild-type carriers. However, homozygotes for Pro12Ala in the control group had a lower BMI and less weight gain than the wild-type carriers (280).

**90.2.4.2 TNFα.** TNFα is one of the several cytokines, including leptin, interleukin 1 and 6, interferon, and TNFβ, known to have profound effects on lipid metabolism. It not only is secreted by adipose tissue but also has direct effects on adipocyte metabolism (285). These effects are mostly catabolic, including suppression of many lipogenic enzymes, and development of insulin resistance (285). TNFα inhibits human adipose cell insulin signaling (286) and causes apoptosis in human adipose cells (287), and in rat brown adipocytes (288). TNFα stimulates leptin release from cultured human adipocytes (289), and levels of the soluble TNFα receptor (sTNFα-R55) are positively correlated with plasma insulin and leptin levels in patients with type 2 diabetes and in controls (290). TNFα infusion in humans induces an increase in serum leptin levels in humans (291). KO mice that are deficient in the adipocyte fatty-acid-binding protein aP2 are equally susceptible to diet-induced obesity as are control mice. However, the aP2 null mice fail to express TNFα, and also failed to develop the insulin resistance seen in the obese control mice (292).

TNFα KO mice did not have a significant change in body size by 28 weeks of age, but there was an increase in insulin levels, and a decrease in triglyceride, leptin, and glucose levels (293). In mice with gold thioglucose-induced obesity, TNFα deficient animals had lower levels of glucose and insulin, and lower insulin and glucose responses to a glucose tolerance test than the obese wild-type animals (293). In KO mice simultaneously deficient for both the p.55 and p.75 types of the TNFα receptor, obesity and diabetes were present. However, KO of either receptor alone did not produce this phenotype, and *db/db* mice lacking p.55 were still diabetic and insulin resistant (294).

The human TNFα gene maps to chromosome 6p21, within the region containing the class 3 major

histocompatibility locus and the steroid 21-hydroxylase genes (295). Polymorphic dinucleotide repeat markers that map near TNFα were genotyped in American Pima Indian families. The marker closest to TNFα was linked to %BF in a sib-pair analysis, and was associated with BMI by analysis of variance (296). However, screening of the TNFα coding region and promoter identified only a single polymorphism, and this was not associated with obesity in this population. A G–A substitution at position –308 in the TNFα promoter, which causes a restriction fragment length polymorphism (RFLP) with the enzyme NcoI, was associated with a higher rate of transcription of TNFα in vitro. In Spanish overweight individuals, this RFLP was associated with %BF, insulin sensitivity, and leptin levels, despite no differences in BMI and plasma TNFα levels (297).

**90.2.4.3 Adiponectin.** Adiponectin is a peptide secreted by adipose tissue, and is preferentially secreted by mature adipocytes over preadipocytes (298). It was originally termed Acrp30 (adipocyte complement-related protein of 30 kDa), because of its homology to complement factor C1q. It was also known as adipocyte most abundant gene transcript 1 (apM1), having been isolated as the most abundant mRNA in human adipose tissue (299). Plasma levels are strongly positively correlated with insulin sensitivity, and it has putative antiatherogenic properties (298). Plasma levels are the reverse of those of leptin—adiponectin concentrations are lower in obese than lean subjects (300). Its gene expression in and secretion from 3T3-L1 adipocytes is reversibly inhibited by interleukin 6, an adipocytokine that is elevated in states of insulin resistance (301). In mice, intravenous injection of adiponectin was followed by a rise in CSF levels, consistent with transport to the CNS. Intracerebroventricular administration of adiponectin in these animals caused a decrease in body weight, due to an increase in energy expenditure (302).

The adiponectin gene spans a 17-kb region on human chromosome 3q27, and contains three exons and two introns (303). Several polymorphisms of the gene have been detected and analyzed in human subjects (303,304). A conservative G/T substitution in exon 2 did not correlate with plasma adiponectin levels or with the presence of obesity in Japanese subjects (304), but was associated with serum cholesterol and waist circumference in obese Swedish subjects (305). In the latter study, the frequencies of the G or T allele did not differ between obese and lean subjects. In obese subjects undergoing gastric banding surgery, genotype haplotypes of two linked polymorphisms 11,377 C/G and 11,391 G/A correlated with adiponectin levels at baseline (306).

**90.2.4.4 Visfatin.** Visfatin is one of the members of the adipokine group. It was formerly known as pre-B-cell colony-enhancing factor (PBEF), and also small-molecule insulin mimetic compound 2 (307,308). This nonpeptidic activator of insulin receptor action is secreted by visceral fat as well as lymphocytes. Plasma levels in

humans and in mice correlate positively with the quantity of visceral fat but not subcutaneous fat (309). Treatment of mice with this compound reduces food intake and prevents high-fat-diet-induced obesity (310). When mice with diabetes are treated with recombinant visfatin, their diabetes improves whether they have type 2 or streptozocin-induced diabetes. Homozygous visfatin KO is a prenatal lethal mutation, and heterozygous mutants have a diabetic tendency but no change in body fat (309). Studies of variation in the human gene for visfatin may provide insight into the link between visceral adiposity and type 2 diabetes.

### 90.2.5 Gene Targeting and Effects on Body Fat/Feeding

The widespread use of gene targeting to study a myriad of genes has been applied extensively to the study of obesity. Many examples of these animal models have been described above. These, along with other relevant animal models are summarized in Table 90-4, and they demonstrate both monogenic and polygenic effects on body size variables.

### 90.2.6 Polygenic Models

The polygenic mouse models of obesity have allowed identification of multiple autosomal and nonautosomal QTLs within individual strains, which modify obesity, plasma cholesterol levels, specific deposition of body fat depots (311–314) and propensity toward development of high leptin levels and obesity on a high-fat diet (315,316). To underscore the complexity of the contribution of genetic background to autosomal-recessive obesity, obese Zucker (*fa*)/NKY13H intercross F2 rats have at least three QTLs unrelated to *Lepr* that control BMI and glucose homeostasis (317). In addition, heterozygosity for *Lep<sup>ob</sup>* and *Lep<sup>db</sup>* in mice is known to affect body composition and leptin homeostasis (123). Polygenic models may more closely resemble the human obesity phenotypes, however, the single gene defects producing recessive traits, dominant traits, promoter alterations, and those subject to parental imprinting must continue to be considered candidates for genetic effects in human obesity (224).

### 90.2.7 Gene–Environment Interactions

There are a number of examples of environmental factors influencing genotype expression. Consumption of a high-fat diet can alter *UCP* gene expression in some mouse strains, indicating variable polygenic control of body composition and thermogenesis (183). In normal mice, there is strain-specific variation in the prevention of high-fat-diet-induced obesity by  $\beta$ 3AR agonists, which is related to the ability to recruit brown adipose tissue in white adipose tissue anatomic sites (185). In

**TABLE 90-4 Transgenic Models for Altered Body Size or Body Fat**

<b>Increased</b>
MC4R-KO (164)
5-HT2c Receptor KO (414)
CRH overexpression (415)
BAT ablation (416)
$\beta$ 3-AR KO (+/–) (186)
GLUT4 overexpressed in fat (417)
NPY receptor 1 KO (159)
Nhlh2 gene KO (418)
11 $\beta$ -HSD1 KO (262)
MCH overexpression (413)
$\beta$ -endorphin KO (413)
MC3R KO (174)
Muscle PPAR $\gamma$ -KO (276)
<b>Decreased</b>
Dopamine D1Receptor KO (419)
UCP-1 overexpression (182)
Tyrosine phosphatase 1B KO (420)
GLUT4 KO (421)
MCH KO (422)
LPL overexpression in muscle (417)
PKA RII $\beta$ KO (423)
PPAR $\gamma$ KO heterozygotes (275)
MCH or MCH-R ablation (413)
<b>No change on normal diet</b>
CRH KO (424)
NPY KO (235)
TNF $\alpha$ KO (293)
UCP-3 KO (215)
Ghrelin KO (229)
Visfatin partial KO (309)

MC4R, melanocortin receptor 4; KO, knockout; 5-HT2c, 5-hydroxy-tryptophan 2c; CRH, corticotropin releasing hormone; BAT, brown adipose tissue;  $\beta$ 3-AR, beta 3-adrenergic receptor; GLUT4, glucose transporter 4; NPY, neuropeptide Y; Nhlh2, one of two helix–loop–helix transcription factors expressed in the developing mouse nervous system; UCP, uncoupling protein; MCH, melanin concentrating hormone; LPL, lipoprotein lipase; PKA RII $\beta$ , protein kinase A regulatory subunit II beta; PPAR, peroxisome proliferator-activated receptor; TNF, tumor necrosis factor; 11 $\beta$ -HSD1, 11beta-hydroxysteroid dehydrogenase type 1; MCH, melanin concentrating hormone; MCH-R, MCH receptor; MC3R, melanocortin 3 receptor; PPAR $\gamma$ , peroxisome proliferator-activated receptor gamma.

humans, physical activity may counteract the obesity related to specific genotypes of the beta 2 adrenergic receptor  $\beta$ 2AR gene (318). The  $\beta$ 3AR (319) and lipoprotein lipase (*LPL*) genes (320) may be involved in similar interactions. Both  $\beta$ 2AR- and  $\beta$ 3AR-mediated effects involve interactions with UCPs in their production of thermogenesis and response to high-fat diets (321). Other loci that may impact individual responses to increased energy intake include *ApoE*, *ApoA-II*, and the LDL receptor (322). These and other as-yet uncharacterized interactions would be important in predicting the responses to prevention strategies or treatments for obesity.

### 90.2.8 Nonmammalian Models

While the above discussion has focused on rodent models, nonmammalian animal models are also playing an increasing role in the investigation of obesity and related traits. These nonmammalian animal models have several advantages over rodents, including shorter generation times, ease of breeding, large population size and the existence of tools for the creation and screening of mutants and phenocopies on a large scale. Some highlights of genetic findings in nonmammalian models are listed below.

**90.2.8.1 *Drosophila*.** Mutations in the Laminin A (*LanA*) gene, a fruit fly gene that is closely related with the human *LAMA5* gene, lead to a decrease in triacylglycerol storage, body weight and total protein content (323). The human *LAMA5* gene maps to a well-replicated obesity-linkage region on chromosome 20q13.2–q13.3 and variants may play a role in body composition and lipid phenotypes (324). Other studies indicate that members of the syndecan gene family may play a role in energy metabolism and regulation of body weight in *Drosophila* and that variants in homologous human genes may influence the same phenotypes in humans (325). Further studies are needed to confirm if these genes play a role in human obesity and to elucidate the mechanisms involved in any such role.

**90.2.8.2 Zebrafish.** Zebrafish are being used to study the effects of KO or knockdown of various genes involved in obesity and metabolic regulation (326) and may provide a means of interrogating known obesity-related genes as well as the discovery of novel genes in the future.

**90.2.8.3 *Caenorhabditis elegans*.** *C. elegans* provides an excellent model for the study of the basic biology of obesity and energy regulation (327). Genes identified in human genetic studies can be knocked down or mutated in *C. elegans* to study their physiology, and conversely, targeted mutagenesis and screening of mutants can reveal novel genes involved in energy metabolism and the regulation of adiposity (328). For example, the *Indy* (I am not dead yet) gene was shown to regulate lifespan and adiposity phenotypes in *Drosophila* and *C. elegans* and this finding was later replicated in rodents using a mouse KO (329).

## 90.3 MENDELIAN DISORDERS ASSOCIATED WITH INCREASED BMI IN HUMANS

Increased risk of obesity can, in relatively rare cases, be secondary to single gene disorders of large effect. While these disorders are uncommon, they do shed light on some of the complex hormonal and neural networks that regulate adiposity. Their murine models, physiological role and known counterparts in humans have been discussed in detail in Section 90.2.1. Here

we will briefly review known human genetic defects and their clinical features.

### 90.3.1 Leptin Deficiency

Missense and nonsense mutations in the leptin gene are an extremely rare cause of morbid obesity. Leptin deficiency is inherited in an autosomal-recessive manner and leads to early-onset extreme obesity characterized by intense hyperphagia. Resting and free-living energy expenditure is normal. Patients also have hypogonadotropic hypogonadism with delayed spontaneous puberty, immune deficiency and may have other neuroendocrine defects (e.g. hypothyroidism) (330). They respond dramatically to treatment with recombinant human leptin, with resolution of hyperphagia and associated phenotypes (331,332). Recently, a patient with a leptin mutation and relatively mild obesity and hypogonadotropic hypogonadism has been described, raising the possibility that milder clinical forms of leptin deficiency may also exist (333).

**90.3.1.1 Leptin Receptor Mutations.** Human leptin receptor deficiency was initially reported in a single family with very severe early-onset obesity and growth retardation secondary to impaired growth hormone secretion. Since then, it has been found that mutations in the leptin receptor may be present in up to 3% of the cases of morbid obesity (334). These subjects presented with early-onset hyperphagia and morbid obesity without significant elevation of leptin levels or developmental abnormalities or dysmorphism.

**90.3.1.2 MC4R.** Defects in the melanocortin 4 receptor may be the most common form of monogenic obesity in humans. These mutations appear to be codominant, with more severe obesity seen in homozygotes. Prevalence of MC4R mutations ranges from 1% to 6% in morbidly obese patients (335), but has not been confirmed in all populations (336,337). Functional studies of these mutants have confirmed the deleterious effects in many cases (167,171). Nonpathogenic mutations and mutations that exert only a mild effect on BMI are more common than mutations associated with morbid obesity, making genetic diagnosis relatively complicated in these cases (335).

**90.3.1.3 POMC Mutation.** POMC is processed to produce the hormones ACTH, MSH alpha, beta and gamma, and beta-endorphin. Defects in POMC are an extremely rare cause of obesity, ACTH deficiency and red hair (154) though mutations that were not associated with red hair have also been reported (338,339).

**90.3.1.4 PC1 Mutation.** PC1 cleaves prohormones before processing by carboxypeptidase. A mutation in PC1 has been described a patient with obesity, elevated proinsulin, hypocortisolism, hypoglycemia, hypogonadotropic hypogonadism and elevated POMC (152). In at least one case, a mutation in PC1 was also associated with neonatal diarrhea and malabsorption (340).

**90.3.1.5 Cohen Syndrome.** Mutations in the *VPS13B* gene (vacuolar protein sorting 13, yeast, homolog B), located on chromosome 8q22, are associated with Cohen syndrome. Cohen syndrome is characterized by truncal obesity, thin extremities and short stature and is inherited in an autosomal-recessive manner (341).

**90.3.1.6 Alstrom Syndrome.** Alstrom syndrome (ALMS) is an autosomal-recessive ciliopathy that is characterized by truncal obesity, short stature, retinal cone dystrophy, progressive hearing loss, dilated cardiomyopathy, type 2 diabetes and progressive pulmonary, hepatic, and renal dysfunction. The involved gene, *ALMS1*, is located on chromosome 2p13 and encodes a large protein that is involved in ciliary function (342). How and why a ciliopathy causes obesity is the focus of intense research and may shed new light on the regulation of adiposity and energy metabolism in humans (343).

**90.3.1.7 Others.** Individual cases of mutations in several other genes involved in the CNS appetite regulation and the development of neuronal circuits have been described in patients with morbid obesity, e.g. in *SIM1* (single-minded, *Drosophila*, homolog of, 1) gene (344) and *NTRK2* (neurotropic tyrosine kinase receptor 2) gene (345).

## 90.3.2 Syndromic Obesity

Syndromic obesity describes obesity that is associated with well-defined combinations of other phenotypes such as mental retardation, dysmorphic features and other developmental abnormalities. Examples of such syndromes with their known genetic cognates include the following.

**90.3.2.1 Prader-Willi Syndrome.** PWS is the most common obesity syndrome with an incidence of 1/15,000–1/25,000 live births. It is caused by loss of expression of paternally expressed genes in the imprinted (differentially methylated) 15q11–13 region. This loss may be secondary to deletions of the paternal 15q11–13 region (70–75% of cases), maternal uniparental disomy of chromosome 15 (20–25%), microdeletions in the imprinting center at the *SNURF-SNRPN* gene locus (<3%) and paternal translocations (<1%) (346). Diagnostic testing is best performed by analyzing the methylation status of the PWS region using methylation-specific PCR. The clinical features of PWS include low birth weight, severe hypotonia and feeding difficulties in early infancy, followed by hyperphagia and obesity starting in early childhood. Other characteristic features include hypogonadism, short stature, small hands and feet, facial dysmorphology (narrow bifrontal diameter, almond-shaped eyes, triangular mouth) and a distinctive behavioral phenotype (347). The 15q11–13 region in an imprinted region in which at least 10 genes are only expressed from the paternal chromosome and these were therefore considered candidate genes for PWS (348).

Some of the variations in clinical phenotypes between different PWS patients are due to variation in the number of deleted or silenced genes in this region. But while several genes contribute to the phenotype, deletion of genes involved in the production and processing of small nucleolar RNAs, particularly snoRNA SNORD116 cluster (*HBII-85*) appear to be sufficient to cause the core phenotypes of PWS (349).

**90.3.2.2 Bardet-Biedl Syndrome.** BBS is a rare syndrome characterized by early-onset obesity, rod-cone dystrophy, dyslexia, learning disabilities, postaxial polydactyly, hypogonadism and progressive renal disease. The McKusick-Kaufman syndrome is an allelic form of BBS. It was initially thought to be a classic autosomal-recessive disorder, but the genetics of BBS turn out to be much more complex, with at least 15 different genes now identified as being associated with this syndrome (350,351). Some behave in an autosomal-recessive manner, but others are “triallelic,” requiring a homozygous recessive mutation in one gene and an additional mutation at a second locus. Most of these genes are involved in ciliary function and the syndrome is now considered ciliopathy that overlaps clinically with ALMS (352). Its recurrence risk depends on the specific locus containing genetic mutation of each pedigree (353).

**90.3.2.3 WAGR Syndrome.** Wilms' Tumor, aniridia, genitourinary abnormalities and mental retardation (WAGR) syndrome is associated with heterozygous interstitial deletions of chromosome 11p-13. This syndrome included obesity in some but not all patients and comparison of the deleted regions between obese and nonobese patients indicated that obesity is associated with deletion of the brain-derived neurotrophic factor (*BDNF*) gene (354). Polymorphisms in the same gene have since been found to be associated with obesity in GWAS cohorts. This gene is expressed in the CNS and appears to be involved in the regulation of appetite.

**90.3.2.4 16p11.2 Deletions.** Deletions in this region are associated with obesity and in some cases with autism. This region includes the gene *SH2B1*, which codes for an adapter protein involved in tyrosine kinase signaling including leptin and insulin signaling (355).

## 90.3.3 Polygenic Obesity

In most cases, the inherited risk of obesity appears to be complex and is very likely polygenic in origin. It remains a matter of dispute if this risk is due to rare variants of large effect or common variants of small effect. At the dawn of the genomic era, it was generally assumed that common variants, each of small effect, are responsible for the main genetic contribution to risk of obesity. But very large genome-wide association studies (GWAS) have explained a relatively small proportion of the genetic risk of obesity and some researchers have proposed that this may be due to the fact that rare variants of large effect that arise relatively recently in extended families or clans



are responsible for a large proportion of the hereditary risk of obesity (62).

**90.3.3.1 Linkage and Candidate Gene Studies of the Genetics of Human Obesity.** Starting in the 90s, several research groups attempted to identify genes associated with obesity using candidate gene and linkage studies. Genes thought to be involved in appetite regulation, partitioning of body fat, energy storage, energy expenditure and other physiologic processes that could play a role in obesity were investigated to see if variants in their sequences were linked to risk of obesity. Linkage studies using minisatellites and other markers were used to identify genomic regions that appeared linked to BMI variation. Among the regions and genes found to be possibly linked to obesity were a region on chromosome 3 that included the adiponectin gene as well as other genes that were previously not known to be involved in the control of body composition or energy metabolism (e.g. *PSARL* and *VPS8*) (356). Overall, the results of these studies were somewhat disappointing and did not explain most of the genetic risk of obesity.

**90.3.3.1.1 GWAS in Humans.** With advancements in technology, it has become feasible to genotype hundreds of thousands to several million SNPs in individual microarrays and to test their association with obesity and related traits. The first large GWAS in humans led to the identification of *FTO* (Fat Mass and Obesity associated gene) as a gene that is associated with variation in BMI in almost every population studied to date (357). Since then, large GWAS and meta-analyses have identified dozens of new genes and intergenic loci associated with BMI, while also replicating many of the genes identified earlier by candidate and linkage studies (Table 90-5) shows many of the genes found to be associated with obesity in GWAS. We will discuss a few of these in greater detail:

**90.3.3.1.2 *FTO*.** *FTO* (Fat mass and obesity associated) is a large gene, over 400 kb in size, and several different SNPs in the first intron have been robustly associated with obesity in at least 22 different population groups (358). The effect size is modest (3 kg increase in weight in individuals homozygous for the risk allele) but the risk allele is prevalent at a level of almost 50% in the Caucasian population, so the gene may still contribute to obesity in a very large number of individuals. When it was initially discovered, nothing was known about its function, let alone its role in the regulation of BMI. Since then, it has been discovered that the *FTO* gene product is an oxoglutarate-dependent demethylase that is able to bind and demethylate single-stranded DNA and RNA. How this relates to regulation of body weight remains unknown at this time.

The first complete *Fto* null mouse (*Fto*<sup>-/-</sup>) was reported in 2009. The homozygote displayed a phenotype that includes growth retardation, decreased fat mass and lean mass, increased metabolic rate and increased food

intake after correction for lean body mass. Heterozygotes, interestingly, are resistant to high-fat-diet-induced obesity (359). A different mutation that causes partial loss-of-function of the *FTO* gene is associated with decreased fat mass and increased energy expenditure, but no hyperphagia or growth retardation is seen (360). The *FTO* gene is widely expressed in the body, with high levels of expression in the CNS and particularly in the hypothalamus. Overexpression of *FTO* in the arcuate nucleus in mice leads to a reduction in daily food intake, while knocking down its expression leads to increased food intake (361).

The effects of an *FTO* mutation in humans have been studied in one extended family and all affected homozygotes suffered from a polymalformation syndrome that includes postnatal growth retardation, microcephaly, severe brain deficits, dysmorphic facies, cardiac abnormalities and cleft palate and all affected members died within the first 30 months of life. Heterozygotes in the extended family have not been studied in detail, but no overt body weight phenotype has been reported. Thus, *FTO* appears to be essential for the normal development of a number of major organs and a complete lack of *FTO* activity is incompatible with life beyond early childhood.

The physiological role of *FTO* in body-weight regulation remains unknown at this time. The *FTO* protein may play a role in appetite regulation as well as resting energy expenditure. As it is capable of demethylating both DNA and RNA (362), it may be involved in epigenetic modulation of DNA as well as in the regulation of various RNAs.

**90.3.3.1.3 Peroxisome Proliferator-Activated Receptor Gamma Gene (*PPARγ*).** A polymorphism (Pro115Gln) in this gene was found to be associated with adipocyte differentiation and greater cellular accumulation of triglyceride (278). Another polymorphism (Ala allele of Pro12Ala polymorphism) was associated with lower BMI, increased insulin sensitivity and higher plasma HDL levels (363). Associations of these and other (*PPARγ*) polymorphisms with obesity and other metabolic syndrome phenotypes were replicated in several populations (364,365), but explained only a tiny fraction of the inherited risk of obesity.

**90.3.3.1.4 Beta 2- and Beta 3-Adrenergic Receptor Genes (*ADRB2*, *ADRB3*).** As described in Section 90.2.2.1, polymorphisms in this adrenergic receptor gene were associated with BMI and fasting fatty acid levels in some populations in linkage and candidate gene studies (366,367). The beta 2- and beta 3-adrenergic receptor genes were also implicated in onset of obesity as well as blood pressure elevation in other studies (368) but these associations have not replicated in all studies (369,370). Some evidence suggests an additive effect of the Beta 3 adrenergic gene and UCP-1 gene variants on body-size phenotypes (371,372).

**90.3.3.1.5 *MC4R*.** The melanocortin 4 receptor is an integral component of the leptin-melanocortin

TABLE 90-5

Chromosomal Location	Suspected Gene(s)	Function	Reference
1p31	NEGR1(Neural growth regulator 1)	Axonal growth promoter	(425)
1q25	SEC16B, RASAL2	Endoplasmic reticulum/protein export, GTPase activator	(425)
1q41	LYPLAL1, ZC3H11B	Lipase, zinc finger pseudogene	(26)
1q43-44	SDCCAG8	Centrosomal protein	(426)
2p25	TMEM18	Cell migration modulator	(427)
3q27	Near ETV5	Transcription factor	(425)
4p13	GNPDA2	Glucosamine-6-phosphate isomerase	(427)
6p12	TFAP2B	Transcription factor	(26)
6p22.2–p21.3	PRL	Prolactin	(428)
8p23.1	MSRA	Reduction of methionine sulfoxide/repair oxidative damage	(26,426)
10p12	PTER	Phosphodiesterase related	(428)
11p11.2	MTCH2	Mitochondrial carrier protein	(427)
12q13	FAIM2, BCDIN3D	Neuronal membrane protein/apoptosis inhibition. Methyltransferase	(425)
14q31	NRXN3	Neurexin; CNS cell adhesion	(428,429)
16p11.2	SH2B1	Kinase signaling pathways	(425,427)
16q22–q23	MAF	Transcription factor; pancreas development and insulin gene transcription	(428)
16q22.2	FTO	Nucleic acid demethylase; other functions?	(357) Multiple others
18q11.2	NPC1	Niemann–Pick disease gene; intracellular cholesterol trafficking	(428)
18q22	MC4R	Melanocortin 4 receptor, appetite regulation. Associated with monogenic obesity	(430) Multiple others
19q13.11	KCTD15	Potassium channel	(425,427)

Human genetic loci associated with GWAS or epigenetics studies. Based on Reference (375), Figure 90-1.

regulatory pathway and disruptions in this receptor are the most common monogenic cause of severe obesity. GWAS scans reveal that polymorphisms in and around this gene are also associated with BMI in diverse populations (373,374). It is likely that variations in the functionality of this receptor lead to alterations in satiety and a change in body weight because of increased appetite.

**90.3.3.1.6 UCP Genes.** UCPs are mitochondrial proteins that help to dissipate the proton gradient at the inner mitochondrial membrane, decreasing the generation of ATP. It has been postulated that polymorphisms in the various uncoupling proteins (UCP1, 2 and 3) can lead to alteration in energy expenditure and thus in the accumulation of fat. Members of this gene family were initially implicated in obesity using candidate gene approaches (177) and these associations have been replicated in several populations.

**90.3.3.1.7 Others.** Other genes including TNF $\alpha$  gene (TNFA), angiotensin-converting enzyme gene (ACE), G-protein beta3 subunit gene (GNB3), leptin gene (LEP), leptin receptor (LEPR), SLC6A14, GAD2, TMEM18, INSIG2 and ENPP1 have been linked to the risk of obesity in different studies (375,376). Their exact role remains unclear in many cases and their combined contribution to the obesity epidemic is relatively small. Many examples of human loci identified in GWAS and

imprinting or epigenetics studies as correlated with body size variables are listed in Table 90-5.

### 90.3.3.2 Addressing the Human Obesity Problem: Predictive Factors, Prevention Strategies, and Treatments.

**90.3.3.2.1 Prediction of Adult Obesity during Childhood.** It is well known that blood pressure, blood lipid levels, and obesity in childhood tracks into adulthood (377). Thus childhood obesity itself is a predictor of adult obesity (378), and of higher-than-expected adult morbidity and mortality (379). These late effects may be unrelated to the presence of overweight in adulthood (380). The presence of a specific growth pattern termed early adiposity rebound may predict adult obesity in young children (381). Early adiposity rebound is associated with parental obesity but not with socioeconomic status or dietary variables (382). Whereas most of the comorbidities of obesity occur in adults, they are definitely present in youth in their presymptomatic or early symptomatic forms (38,42,383,384), and when present in children, they are associated with increased cardiovascular morbidity and mortality in their relatives (38–40). The prevalence of clinically significant obesity-related morbidities in youth is definitely on the rise (385), and predicts earlier onset of more severe problems in adolescents, especially among African-Americans (386), and in young adults (387). The alarming increase in type 2 diabetes among

children and adolescents is directly related to the obesity epidemic (388).

Many investigators have established the importance of parental obesity as a predictive factor for childhood obesity. In a study of the influence of different aspects of the home environment on obesity in nearly 3000 children <8 years of age, maternal obesity was the most significant predictor of childhood obesity (389). High birth weight is a significant predictor of later obesity, and the most important factor contributing to high birth weight is maternal diabetes and, to a lesser degree, maternal obesity (390). The relative risk of developing obesity in young adulthood is higher for young children if they have obese parents, and higher for older children if they themselves are obese (391) (Table 90-6).

The influence of lack of physical activity on the development of obesity is reflected in the NHANES data, especially from the most recent survey. Lack of physical activity is directly related to television viewing, and hours of television are significantly correlated to weight gain during the growing years (392). Reduction in television viewing significantly reduced the rate of weight gain in a study of third-grade children, when compared to third graders with no intervention (393).

**90.3.3.2.2 Prevention Strategies.** Obviously, community-wide efforts need to be directed toward increasing physical activity, and changing dietary habits. Reduction of dietary calories and fat, and increasing dietary fiber are recommended (394). The CDC has developed a resource guide for prevention of obesity and other chronic diseases that cover many aspects of nutrition, physical activity, and other lifestyle interventions (395). Counseling obese parents about the risk of childhood obesity in their children must be practiced by health care providers. Enhancement of physical education programs must be instituted by the school systems. It has been established that breast-fed infants are less likely to develop adult obesity than bottle-fed infants (396), adding obesity prevention to the list of benefits of breast feeding, which health care providers should convey.

**90.3.3.2.3 Treatments.** Despite the identification of specific single gene defects and metabolic abnormalities

resulting in obesity, novel efficacious therapeutic interventions have not been forthcoming.

**90.3.3.2.4 Medications.** Current medical therapeutic options for treatment of obesity are not very promising, and the principals of therapy are flawed. Therapy must be long-term and ongoing, as is the case with treatment of hypertension and diabetes. Current obesity drugs are not approved for prolonged use, or for use in youth, have not been extensively tested for safety over long periods of time, may only benefit a minority of patients (12,397), and are associated with serious cardiovascular side effects (398). The latter has resulted in two such agents being taken off the market. The major classes of drugs are those that reduce food intake (monoamine oxidase inhibitors, sympathomimetic drugs), those that increase energy expenditure (ephedrine, caffeine), and those that inhibit fat absorption. The use of agents to induce dietary fat malabsorption has been only minimally successful, and is associated with significant intestinal discomfort if not accompanied by a low-fat diet. Of course, the diet itself may be nearly equally effective over the long-term.

The use of recombinant leptin in humans resulted in a modest and highly variable loss of weight (loss of fat mass), which was dose related, and occurred in both lean and obese subjects (399). Leptin treatment in the rare leptin-deficient patients produced a rapid reduction in weight and increase in energy expenditure, and antibodies to leptin developed after 2 months (400).

In an animal study, mice that were treated with fatty acid synthase inhibitors had a reduction in food intake and in body weight (401). This treatment was effective when given either systemically or intracerebroventricularly. It inhibited NPY expression in the hypothalamus, as well as leptin expression in WAT.

**90.3.3.2.5 Surgery.** Bariatric surgery perhaps provides the best weight loss results (402), but both short- and long-term risks are present. Bariatric surgical procedures fall into two categories—those reducing gastric volume, such as vertical banded gastroplasty or adjustable gastric band, and those that also result in moderate selective malabsorption such as gastric bypass. Bypass results in more rapid and more significant weight loss. As a result, there is a recent trend toward gastric bypass as the preferred procedure. However, there is an altered gut hormone response to feeding, potential for deficiencies of vitamin B12, folate, and iron leading to anemia, as well as secondary hyperparathyroidism, presumably due to reduction of calcium absorption due to loss of gastric acidity. Vertical banded gastroplasty has been associated with loss of bone density without hyperparathyroidism (403). Several studies confirmed that gastric bypass results in reduction in diabetes and hypertension (404,405), but the risk for metabolic bone disease, vitamin deficiencies, and intestinal complications is high (406–409). Surgery to remove fat (liposuction), if used alone, is not a long-term solution. However, it may

**TABLE 90-6 Odds Ratios for Obesity in Young Adulthood According to Obesity Status in Childhood and Parents' Obesity Status**

Age (yr)	Obese as Child Yes versus No	Number of Obese Parents	
		1 versus 0	2 versus 0
1–2	1.3	3.2	13.6
3–5	4.7	3.0	15.3
6–9	8.8	2.6	5.0
10–14	22.3	2.2	2.0
15–17	17.5	2.2	5.6

Data were obtained with permission from Reference (391).

be a useful cosmetic adjunct in patients who are successful with diet and exercise, or surgical gastroplasty.

### 90.3.3.2.6 Future Directions in Treatment.

Of course, the most significant impact on the obesity epidemic will have to be achieved through patient education and prevention strategies. Many resources offering guidance in these areas are available (410) (see also [www.cdc.gov](http://www.cdc.gov) and [www.surgeongeneral.gov](http://www.surgeongeneral.gov)). New medical treatments, including potent agonists for the  $\beta$ 3-AR and the MC4R, are under development. Presymptomatic genotyping using microarray technologies may be able to identify those individuals genetically predisposed to obesity, allowing prevention strategies to be initiated early. Gene or hormone replacement therapy may become available for those rare patients with single gene defects. Direct electrostimulation of specific hypothalamic nuclei has been successful in increasing metabolic rate and fat utilization, and decreasing feeding in rats (411). Similar techniques are used in humans to treat tremors, and perhaps could be applied to obesity treatment.

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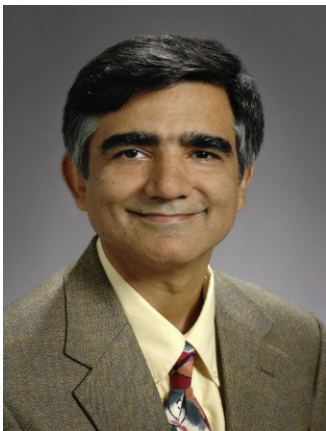


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### Biographies



**Patricia A Donohoue** attended medical school at The Ohio State University, and completed her internship in Pediatrics there at what is now known as Nationwide Children's Hospital. She completed her second and third years of pediatric residency at Rainbow Babies and Children's Hospital, Case Western Reserve University. Her Pediatric Endocrinology Fellowship was at Johns Hopkins under the mentorship of Professor Claude Migeon and the late Professor Neil Van Dop. She stayed there as a junior faculty member for 4 years before moving to the University of Iowa. At Iowa, she continued her NIH-funded laboratory research on the genetics of steroid 21-hydroxylase deficiency, and then focused her interest on other inherited defects of the hypothalamic–pituitary–adrenal axis, and eventually on the genetics of obesity. After 18 years at the University of Iowa, she moved to the Medical College of Wisconsin in Milwaukee in 2008, where she is a Professor of Pediatrics and Section Chief of Pediatric Endocrinology and Diabetes.



**Omar Ali, MD** attended medical school at King Edward Medical College in Lahore, Pakistan, and completed his residency in pediatrics at Jersey City Medical Center. He then did a 1-year fellowship in community pediatrics at the Children's Hospital of Pittsburgh. He worked in general pediatrics for the next 12 years, but then decided to do a fellowship in Pediatric Endocrinology at UCLA-Mattel Children's Hospital under Dr Hassy Cohen. Subsequently, he joined the Medical College of Wisconsin in 2006. He is an Assistant Professor of Pediatrics in the Section of Pediatric Endocrinology and Diabetes, and Director of the Pediatric Endocrinology Fellowship Program. His research interest is the genetics and epigenetics of obesity and the metabolic syndrome, and he is currently working on grants funded by the NIH, the National Children's study and the Children's Research Institute.

# CHAPTER

# 91

## Genetic Lipodystrophies

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### 91.1 INTRODUCTION

Lipodystrophies are disorders of adipose tissue characterized by selective loss of body fat and a predisposition to develop insulin resistance and its complications (1). The prevalence of metabolic and other manifestations of insulin resistance, such as impaired glucose tolerance, diabetes, hyperinsulinemia, dyslipidemia, hepatic steatosis, acanthosis nigricans, polycystic ovarian disease and hypertension, varies among the different subtypes of lipodystrophies and is generally determined by the extent of adipose tissue loss (1). The loss of adipose tissue is readily apparent in females, and thus the diagnosis of lipodystrophies is relatively easy in them but it may be missed in affected males as many normal men look muscular and have much less body fat than women. Lipodystrophies can be classified into genetic syndromes and acquired disorders due to various causes (1,2). The inherited lipodystrophy syndromes can be subclassified into autosomal-recessive or autosomal-dominant disorders and a classification is given in Table 91-1 (2). The precise mode of inheritance still remains to be characterized for some disorders.

A great deal of progress has been made recently in elucidating the genetic basis of many types of inherited lipodystrophies (1,2); however, the defective genes for some disorders still remain to be discovered. The discoveries of the molecular mechanisms underlying various types of genetic lipodystrophies has added to our understanding of adipocyte biology and has revealed potential pathways, which may be implicated in development of insulin resistance in these disorders as well as in other common disorders of adipose tissue such as generalized and regional obesity (3).

### 91.2 MECHANISMS OF INSULIN RESISTANCE AND ITS COMPLICATIONS IN LIPODYSTROPHIES

The underlying mechanisms causing insulin resistance in patients with lipodystrophies may be similar to those implicated in obesity. For example, in patients with

severe forms of obesity and lipodystrophies, there may be limitation in further storage of triglycerides in adipose tissue resulting in diversion of dietary and endogenously synthesized triglycerides to aberrant sites, such as the liver and skeletal muscles resulting in insulin resistance (4). The frequency of hepatic steatosis differs in patients with various lipodystrophies. For example, nearly all patients with congenital generalized lipodystrophy (CGL) have some degree of hepatic steatosis since birth whereas only a few patients with familial partial lipodystrophies (FPL) may develop hepatic steatosis (5,6). Accumulation of triglycerides in skeletal muscle has not been studied systematically. Slight increase in skeletal muscle triglyceride was noted even in patients with CGL (7). The ectopic triglyceride accumulation may induce hepatic and skeletal muscle insulin resistance; however, the precise molecular mechanisms by which this occurs have not been elucidated. Hypertriglyceridemia and lowering of high-density lipoprotein (HDL) cholesterol in patients may be related to severity of hepatic steatosis. Clinically, a dramatic reduction in insulin resistance as well as hepatic steatosis is observed when patients reduce energy intake because of either exogenous leptin therapy or otherwise (8,9).

The mechanisms of diabetes mellitus seem to be similar to that noted in patients with type 2 diabetes. Marked islet amyloidosis and pancreatic  $\beta$  cell atrophy was observed on autopsy of two lipodystrophic patients with diabetes, one with CGL and another with Familial Partial Lipodystrophy, Dunnigan variety (FPLD) (6,10). Diabetes in patients with lipodystrophies is usually ketosis resistant and many patients require extremely high doses of insulin to achieve good glycemic control (8). Whether the resistance to ketosis is due to residual endogenous insulin secretion or due to reduced flux of free fatty acids, the substrates for production of ketones, is not clear.

Extremely high serum levels of insulin may be implicated in other clinical manifestations noted in patients with lipodystrophies such as acanthosis nigricans, acromegaly features (enlargement of mandible, hands

**TABLE 91-1 Classification of Genetic Lipodystrophies**

<b>A. Autosomal-Recessive Syndromes</b>	
1.	Congenital generalized lipodystrophy (CGL; Berardinelli–Seip Syndrome)
a.	CGL Type 1: <i>AGPAT2</i> (1-acylglycerol-3-phosphate O-acyltransferase 2) mutations
b.	CGL Type 2: <i>BSCL2</i> (Berardinelli–Seip congenital lipodystrophy 2) mutations
c.	CGL Type 3: <i>CAV1</i> (Caveolin 1) mutation
d.	CGL Type 4: <i>PTRF</i> (polymerase I and transcript release factor) mutations
e.	Other varieties
2.	Mandibuloacral dysplasia (MAD)-associated lipodystrophy
a.	Partial lipodystrophy (Type A pattern): <i>LMNA</i> (lamin A/C) mutations
b.	Generalized lipodystrophy (Type B pattern) <i>ZMPSTE24</i> (zinc metalloproteinase) mutations
c.	Other varieties: Unknown
3.	Autoinflammatory syndromes
a.	JMP syndrome: <i>PSMB8</i> (proteasome subunit, beta-type, 8) mutations;
b.	CANDLE syndrome: <i>PSMB8</i> mutations
4.	Familial Partial Lipodystrophy (FPL): <i>CIDEA</i> (cell death-inducing DNA fragmentation factor a-like effector c) mutation
5.	SHORT (Short stature, Hyperextensibility or inguinal hernia, Ocular depression, Rieger anomaly and Teething delay) syndrome: Unknown
6.	Mandibular hypoplasia, Deafness, Progeroid features (MDP)-associated lipodystrophy: Unknown
7.	Neonatal Progeroid (Weidemann–Rautenstrauch) Syndrome: Unknown
<b>B. Autosomal-Dominant Syndromes</b>	
1.	Familial Partial Lipodystrophy (FPL)
a.	FPL, type 1, Kobblerling variety: Unknown
b.	FPL, type 2, Dunnigan variety (FPLD): <i>LMNA</i> (lamin A/C) mutations
c.	FPL type 3: <i>PPARG</i> (peroxisome proliferator-activated receptor- $\gamma$ ) mutations
d.	FPL type 4: <i>AKT2</i> (v-AKT murine thymoma oncogene homolog 2) mutation
e.	FPL, type 5: <i>PLIN1</i> (perilipin 1) mutations
f.	Other varieties: Unknown
2.	Atypical progeroid syndrome: <i>LMNA</i> mutations
3.	Hutchinson–Gilford progeria syndrome: <i>LMNA</i> mutations
4.	SHORT syndrome: Unknown

and feet), generalized organomegaly and clitoromegaly as well as polycystic ovarian disease. These may occur because of “specificity spillover phenomenon” and actions of high levels of insulin through other receptors such as those for insulin-like growth factors, I and II. The precise molecular mechanisms involved in various clinical manifestations are expected to be revealed in the near future as we make progress in understanding biological processes related to the genes implicated in causing genetic lipodystrophies.

### 91.3 GENETIC LIPODYSTROPHIES

Most of the patients with genetic lipodystrophies reveal a classical autosomal-recessive (congenital generalized or mandibuloacral dysplasia (MAD)-associated) or autosomal-dominant inheritance pattern (FPL). However, for the SHORT-syndrome-associated lipodystrophy, both autosomal-recessive and autosomal-dominant inheritance patterns have been reported. In the following section, each type of inherited lipodystrophy is discussed.

#### 91.3.1 Autosomal-Recessive Types

**91.3.1.1 Congenital Generalized Lipodystrophy (Berardinelli–Seip Syndrome).** This autosomal-recessive disorder (Online Mendelian Inheritance in Man #269700)

was reported originally by Berardinelli from Brazil approximately 57 years ago (11). Shortly thereafter, Seip from Norway provided detailed description of the phenotype (12). The diagnosis of CGL is made usually at birth or shortly thereafter. Approximately 300 cases have been reported from all over the world. It is more commonly reported from the racial and ethnic groups such as from Brazil and Lebanon, where consanguinity is common (13,14). If the reported cases represent only one-fourth of the actual cases, the estimated prevalence of this disorder is about 1 in 10 million.

**91.3.1.1.1 Clinical and Biochemical Characteristics.** The affected newborns appear “strikingly muscular” because of almost complete absence of adipose tissue. They grow at an accelerated rate and their bone age may be advanced. Children display markedly increased appetite. Slight enlargement of the hands, feet and mandible (“acromegaloid” features) are apparent (Figure 91-1A). Umbilical hernia or enlargement of the umbilicus is observed commonly. Acanthosis nigricans usually develops late in the childhood or adolescence and may affect extensive area of the body such as the trunk, hands, elbows, knees and ankles besides the common sites at the neck, axillae and groins.

Hepatomegaly is usually noticed during infancy and is due to hepatic steatosis. A few patients develop cirrhosis and its complications later on in life. Many patients





**(A)** **(B)**  
**FIGURE 91-1** CGL. Panels A and B show anterior and posterior views, respectively, of a 37-year-old female of African-American origin with congenital generalized lipodystrophy type 1, showing generalized lack of fat, extreme muscularity and acromegaly features. She developed diabetes mellitus at the age of 17 years. Acanthosis nigricans was present in the neck, axillae and groin. She had a homozygous mutation (IVS4-2A>G resulting in prematurely truncated protein Gln196fsX228) in the *AGPAT2* gene.

develop splenomegaly. In postpubertal females, mild hirsutism, clitoromegaly, oligoamenorrhea and polycystic ovaries are common. Most affected women are unable to get pregnant but successful pregnancy has been reported. Affected men usually have normal reproductive ability. Many patients develop focal lytic lesions in the appendicular bones after puberty, commonly involving the humeri, but also sometimes affecting the femur, radius, ulna, carpal, tarsal and phalangeal bones (15). These lytic lesions may be related to the lack of bone marrow fat and inability to replace hematopoietic marrow with adipose tissue during childhood and adolescence. Hypertrophic cardiomyopathy and mild mental retardation have been reported in some patients (16–19).

Patients have marked fasting and postprandial hyperinsulinemia suggestive of severe insulin resistance. Extreme hypertriglyceridemia predisposes patients to recurrent episodes of acute pancreatitis. Levels of HDL cholesterol also tend to be low. Diabetes is usually noted during the pubertal years; however, the onset of diabetes

has occurred as early as 6 weeks of life (18). In some neonates, diabetes occurs transiently during period of stress or infection only to recur later in life. Patients require high doses of insulin to control diabetes.

Consistent with the near total absence of body fat, the serum levels of adipocytokines, such as leptin and adiponectin, are low (20). It is likely that hypoleptinemia contributes to excessive appetite and metabolic complications in patients with CGL.

#### 91.3.1.1.2 Genetic Defect and Pathophysiology.

Using genome-wide linkage analysis, we reported the first locus for CGL on human chromosome 9q34 (21) and described genetic heterogeneity in 17 pedigrees. Subsequently, another locus was found on chromosome 11q13 (13). Positional cloning approach led us to identify mutations in the 1-acylglycerol-3-phosphate-O-acyltransferase 2 (*AGPAT2*) gene in affected patients from pedigrees linked to 9q34 (17). The defective gene on chromosome 11q13 was found to be a novel gene, called Berardinelli-Seip Congenital Lipodystrophy 2 (*BSCL2*) and the product of the gene was named Seipin, after Seip (13). Recently, candidate gene approach has led to the identification of two additional CGL loci, caveolin 1 (*CAV1*) (22) and polymerase I and transcript release factor (*PTRF*) (23).

The *AGPAT*s belong to the acyltransferase family of enzymes and 11 isoforms, each encoded by a different gene are known currently (24–26). The *AGPAT*s catalyze an important step in the biosynthesis of glycerophospholipids and triglycerides. These enzymes acylate lysophosphatidic acid (1-acylglycerol-3-phosphate) to phosphatidic acid (1,2 diacylglycerol-3-phosphate) (24,27) (Figure 91-2). Each isoform may have substrate specificity for different fatty acids but precise nature of it remains unclear. These isoforms may also differ in subcellular localization and may play different roles in mitochondrial or microsomal synthesis of triglyceride or phospholipids. There may also be heterogeneity as far as expression of these isoforms in various tissues, particularly among adipose tissue depots localized at different regions of the body.

Of the two major isoforms of *AGPAT*, 1 and 2, we reported that *AGPAT2* is highly expressed in the omental adipose tissue (17), but *AGPAT1* showed an equal expression in the liver and its expression was nearly twofold in the skeletal muscle. Thus, it can be hypothesized that mutations of *AGPAT2* gene may cause lipodystrophy either by reducing triglyceride deposition in the adipocytes or by affecting adipocyte function resulting from the lack of synthesis of phospholipids. *AGPAT2* consists of 278 amino acids and shares two highly conserved motifs, Asn-His-x-x-x-Asp (amino acids 97–103, with x denoting any amino acid) and Glu-Gly-Thr-Arg (amino acids 172–175) with other acyltransferases. These motifs are critical for the enzymatic activity (24,28). Besides the well-conserved motifs, we have recently reported that carboxy-terminal residues are also important determinants of the *AGPAT2* enzymatic

activity (29). Using *in vitro* expression of wild-type and mutant forms of AGPAT2 in Chinese Hamster Ovary cells, we found that null mutants had markedly reduced activity to convert lysophosphatidic acid to phosphatidic acid and the missense mutants had mild to moderate reduction in activity (29). However, because of reduced sample size, compound heterozygous mutations in many patients, and the fact that most of the CGL patients do present with near-total absence of body fat, we were unable to find a genotype–phenotype relationship.

The *BSCL2* encodes a 398-amino-acid transmembrane protein, seipin, which does not reveal any striking homology to any other protein. It contains a CAAX motif at the C-terminal and a glycosylation site, Asn-Val-Ser, at position 88–90. We previously reported a weak but not insignificant homology of seipin to the linker region of the protein called midasin (30). Interestingly, missense mutations in the glycosylation motif of seipin have been reported to cause distal hereditary motor neuropathy and Silver syndrome most likely by aggresome formation (31). Recent data suggest the role of seipin in lipid droplet formation and in adipocyte differentiation (32–34).

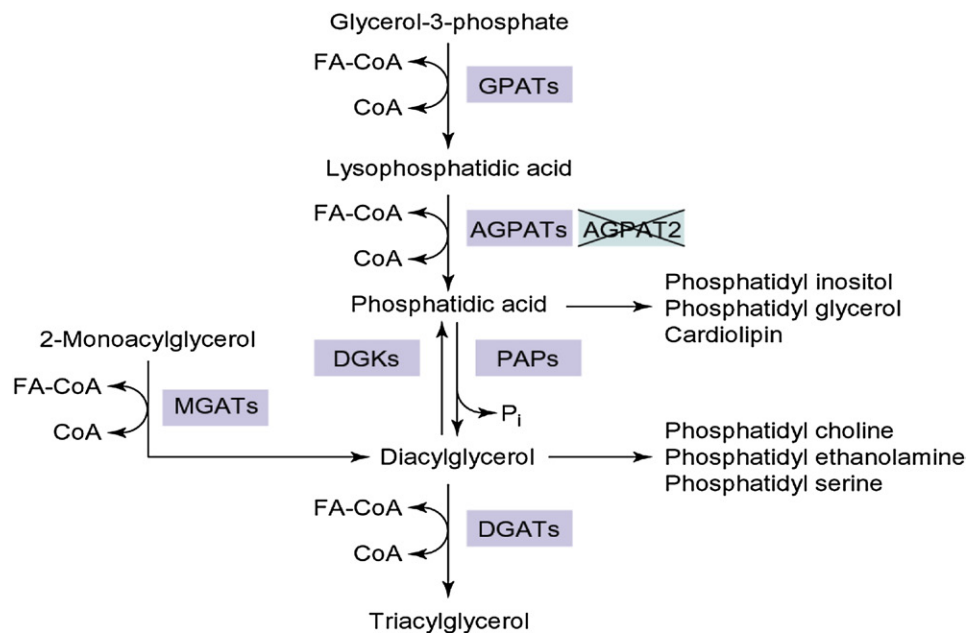
Caveolin 1 is expressed in caveolae, which are specialized microdomains on cell membranes of adipocytes (35). It contributes to lipid droplet formation by binding

fatty acids and translocating those to lipid droplets. PTRF is also known as cavin, and plays a role in biogenesis of caveolae. PTRF regulates expression of caveolins 1 and 3 (23).

#### 91.3.1.1.3 Genotype and Phenotype Variation.

There are phenotypic differences in the two most prevalent types of CGL, called type 1 (*AGPAT2* related) and type 2 (*BSCL2* related) (17–19). Patients with CGL, type 2 have increased prevalence of cardiomyopathy and mild mental retardation (17–19), while focal lytic lesions in appendicular skeleton are more prevalent in CGL type 1 (36). In both the types, metabolically active adipose tissue, which is found in most subcutaneous (sc) areas, intra-abdominal and intrathoracic regions, and bone marrow, is nearly completely lost (36). The mechanical adipose tissue depots located in the palms, soles, under the scalp, retro-orbital and periarticular regions are spared in CGL, type 1 but severe loss of fat also occurs from these depots in patients with CGL, type 2 (5,36,37). Serum leptin levels also tend to be lower among those with type 2 CGL compared to those with type 1 CGL but interestingly, they have increased serum adiponectin levels (18).

CGL, type 3 has been reported in only one Brazilian girl who had a homozygous *CAVI* mutation and had



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**FIGURE 91-2** Pathways for biosynthesis of triacylglycerol: glycerol-3-phosphate (G3P) is the initial substrate for acylation at sn-1 position by the enzyme glycerol-3-phosphate acyltransferase (GPAT), to form 1-acylglycerol-3-phosphate or lysophosphatidic acid (LPA). LPA is further acylated at sn-2 position by 1-acylglycerol-3-phosphate acyltransferase (AGPAT, aka LPAAT) to form phosphatidic acid (PA). In the next step, phosphate group is removed by phosphatidate phosphohydrolase (PAP) to produce diacylglycerol (DAG). DAG is further acylated at sn-3 position by diacylglycerol acyltransferase (DGAT) to produce triacylglycerol (TG). In addition, TG can be synthesized via the acylation of 2-monoacylglycerol by the enzyme monoacylglycerol acyltransferase (MGAT), which is highly expressed in small intestine. DAG kinase (DGK) can phosphorylate DAG to synthesize PA. PA and DAG are also substrates for the synthesis of glycerophospholipids such as phosphatidylinositol (PI), cardiolipin, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS). (Reproduced with permission from Elsevier. Agarwal, A. K.; Garg, A. *Congenital Generalized Lipodystrophy: Significance of Triglyceride Biosynthetic Pathways*. Trends Endocrinol. Metab. 2003, 14 (5), 214–221.)

short stature and presumed Vitamin D resistance (22). Type 4 CGL due to *PTRF* mutations has been reported in approximately 21 patients. Peculiar finding in these patients include congenital myopathy, percussion-induced myoedema, pyloric stenosis and atlantoaxial instability. Of particular concern are arrhythmias including prolonged QT interval and exercise-induced ventricular tachycardia, and sudden death (23,38,39). Patients with *CAV1* and *PTRF* mutations have well-preserved mechanical and bone marrow fat (22,36,38,40). There are a few patients with CGL who do not have mutations in the four known loci and may lead us to yet more novel loci.

In our experience as well as of that of others, nearly all patients of African origin harbor the founder mutation (IVS4-2A>G) in *AGPAT2*, whereas patients of Lebanese origin have a founder mutation (659delGTATC) in *BSCL2* (18) (Figures 91-3 and 91-4). Patients of European and Asian descent have been reported to harbor mutations in either of the two genes. CGL, type 3 has been reported from Oman in several pedigrees.

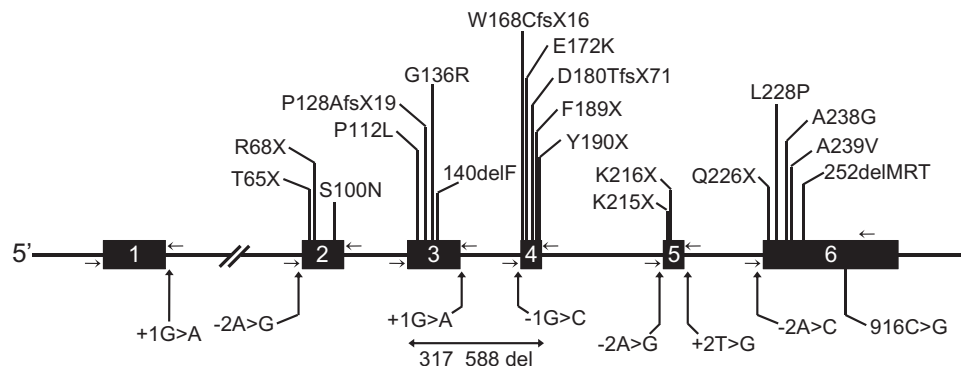
#### 91.3.1.1.4 Molecular and Differential Diagnosis.

Patients with CGL can be easily diagnosed at birth or

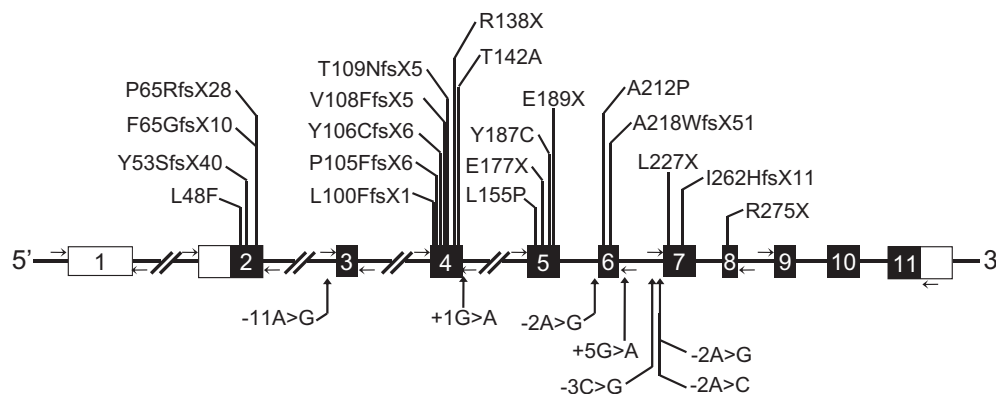
immediately thereafter. The two main types of CGL can be distinguished based on their clinical features. Diagnosis at the molecular level may be needed in some but it raises economic and ethical issues. Couples with a child affected with CGL may use molecular diagnosis to know their risk of having another child with CGL. Finally, molecular diagnosis can be used for prenatal screening.

Many different syndromes can present phenotype similar to CGL such as acquired generalized lipodystrophy with onset during early infancy, leprechaunism, atypical Werner syndrome, pubertal-onset generalized lipodystrophy due to rare *LMNA* mutations and neonatal progeroid syndrome.

**91.3.1.2 Mandibulo-Acral Dysplasia (MAD)-Associated Lipodystrophy.** MAD (OMIM #248370) is a rare autosomal-recessive disorder characterized by hypoplasia of the mandible and clavicles, and acro-osteolysis (resorption of the terminal phalanges) (41,42). Patients also have delayed closure of cranial sutures, joint contractures, mottled cutaneous pigmentation and short stature. Features of accelerated aging called “progeroid features” such as bird-like facies, high-pitched voice and



**FIGURE 91-3** Structure of *AGPAT2* gene and various mutations found in the affected individuals with CGL. Boxes represent exons and small arrows show location of primers used for exons amplifications. (Reproduced in a modified form with permission from Agarwal, A. K.; Barnes, R. I.; Garg, A. *Genetic Basis of Congenital Generalized Lipodystrophy*. *Int. J. Obesity* 2004, 28, 336–339.)



**FIGURE 91-4** Structure of *BSCL2* gene and various mutations found in the affected individuals with CGL. Boxes represent exons and small arrows show location of primers used for exons amplifications. (Reproduced in a modified form with permission from Agarwal, A. K.; Barnes, R. I.; Garg, A. *Genetic Basis of Congenital Generalized Lipodystrophy*. *Int. J. Obesity* 2004, 28, 336–339.)





**FIGURE 91-5** MAD. A. Lateral view of a 20-year-old Hispanic female with MAD due to homozygous Arg527His missense mutation of the *LMNA* gene. She had reconstructive surgery for micrognathia. She had loss of fat from the extremities leading to a muscular appearance and prominent subcutaneous veins and had excess fat accumulation in the face and neck region. Note the mottled skin pigmentation over the trunk. B. Hands show rounding of the finger tips and clubbing appearance due to acro-osteolysis of the terminal phalanges. There is atrophy of the skin on the dorsum of hands especially over the interphalangeal joints and metacarpophalangeal joints.

ectodermal defects, such as skin atrophy, pigmentation, alopecia, and nail dysplasia are also seen in some patients. Only a few patients have had hypogonadism and sensorineural deafness. Approximately 40 patients with MAD have been reported in the literature (Figure 91-5).

Patients with MAD display two patterns of lipodystrophy: type A pattern with partial loss of sc fat from the extremities and type B pattern with more generalized loss of sc fat involving the face, trunk and extremities (41). Hyperinsulinemia, insulin resistance, impaired glucose tolerance, diabetes mellitus and hyperlipidemia have been reported but are usually mild to moderate in severity (41).

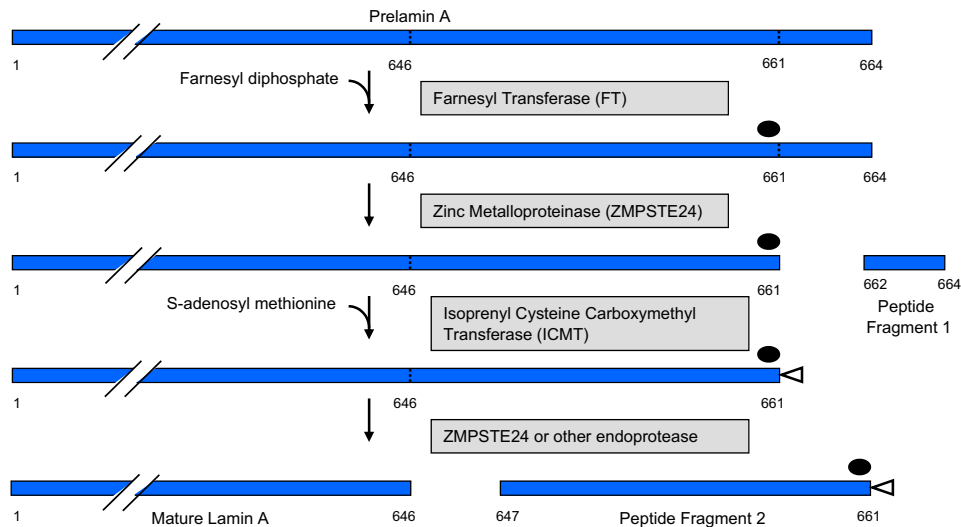
**91.3.1.2.1 MAD-Associated Partial Lipodystrophy due to *LMNA* Mutations.** Novelli et al. (42) reported a homozygous Arg527His mutation in *LMNA* gene in MAD patients with type A (partial) lipodystrophy of Italian origin. The heterozygotes had no phenotypic manifestations of MAD. *LMNA* encodes lamins A and C by alternative splicing in exon 10 (43). Prelamin A has a CAAX motif at its C-terminal, which undergoes posttranslational modification involving an endoprotease called *zinc metalloproteinase* (ZMPSTE24) to form

mature lamin A (Figure 91-6). The lamins belong to the intermediate filament family of structural proteins. Lamins A and C form hetero- or homodimeric coiled-coil structures and are integral components of the nuclear lamina; a polymeric structure intercalated between chromatin and the inner membrane of the nuclear envelope (44,45). The mechanisms by which this *LMNA* mutation causes predominantly skeletal and cutaneous dystrophy but less-severe lipodystrophy in patients with MAD, remain unclear. By now, a total of 30 patients with MAD due to various *LMNA* mutations have been reported (41,42, 46–48). Some patients have more-severe progeroid manifestations, such as those seen in Hutchinson–Gilford progeria syndrome (HGPS) such as alopecia, loss of eyebrows, delayed sexual maturation and premature loss of teeth. Most of the *LMNA* mutations causing MAD are located in the C-terminal region affecting exons 8–10. How these specific *LMNA* mutations cause resorption of bones such as mandible, clavicles and terminal phalanges remains unclear.

**91.3.1.2.2 MAD-Associated Generalized Lipodystrophy due to Zinc Metalloproteinase (*ZMPSTE24*) Mutations.** On the basis of the critical role of ZMPSTE24 in posttranslational maturation of prelamin A to its mature form lamin A and the phenotype of lipodystrophy and skeletal abnormalities in ZMPSTE24-deficient mice (49,50), we sequenced *ZMPSTE24* gene in MAD patients who did not have mutations in *LMNA* gene. We found compound heterozygous mutations in the *ZMPSTE24* gene in a Belgian woman with MAD (51). She also had progeroid features and generalized lipodystrophy (51). She died prematurely at age 24 years of complications of chronic renal failure due to collapsing variety of focal segmental glomerulosclerosis (51). Given the role of ZMPSTE24 in posttranslational proteolytic processing of prelamin A, cellular accumulation of prelamin A and/or lack of mature lamin A may be responsible for phenotypic features (52). A recent study suggests toxic effects of prelamin A accumulation to be responsible for the phenotype in ZMPSTE24-deficient mice (53). In our cohort of patients with MAD, some patients do not have any variants in either *LMNA* or *ZMPSTE24* genes, suggesting additional as yet unmapped loci (51).

**91.3.1.2.3 Genotype and Phenotype Variation.** About 30 patients have been reported to have MAD due to *LMNA* mutations, but only eight MAD patients have *ZMPSTE24* mutations. Several MAD patients have been reported from Italy and all of them have a founder *LMNA* Arg527His mutation (42). MAD patients with *LMNA* or *ZMPSTE24* mutations share features of mandibular and clavicular hypoplasia and acro-osteolysis but patients with *ZMPSTE24* mutations are premature at birth, have early onset of skeletal defects including acro-osteolysis, have more progeroid appearance and develop sc calcified nodules on the phalanges. We have reported focal segmental glomerulosclerosis in two





**FIGURE 91-6** Posttranslational processing of prelamins A and B. Prelamin A contains 664 amino acids and a conserved CAAX motif at the carboxy terminal. The cysteine residue in the CAAX motif is first farnesylated by the farnesyl transferase (shown by filled circles) followed by first proteolytic cleavage of the three extreme C-terminal residues by zinc metalloproteinase (ZMPSTE24). Then, the farnesylated cysteine residue is methylated by isoprenylcysteine carboxyl methyl transferase (ICMT) using S-adenosyl methionine as the methyl donor (shown by unfilled triangle). Thereafter, ZMPSTE24 or other endoprotease cleave the 15 amino acids from the C-terminal forming the mature lamin A with 646 residues. (Reproduced with permission from Elsevier Inc. Garg, A.; Misra, A. *Lipodystrophies: Rare Disorders Causing Metabolic Syndrome*. Endocrinol. Metab. Clin. North Am. 2004, 33, 305–331.)

patients with ZMPSTE24 deficiency; kidney pathology has not been reported in those with *LMNA* mutations (51). Interestingly, the remaining patients, who do not harbor *LMNA* or ZMPSTE24 variants, do not seem to develop clavicular resorption or acro-osteolysis.

#### 91.3.1.2.4 Molecular and Differential Diagnosis.

Ascertaining molecular diagnosis may help predict peculiar clinical features noted in different genetic varieties of MAD. Patients with MAD have several overlapping clinical features with HGPS and those with atypical Werner syndrome (all due to heterozygous mutations in *LMNA*) and should be differentiated from such patients. Patients with several other genetic syndromes, such as, Hajdu-Cheney, congenital insensitivity to pain with anhidrosis, Haim-Munk, and Papillon-Lefevre syndromes present with acro-osteolysis as well and need to be differentiated from patients with MAD.

#### 91.3.1.3 Autoinflammatory Syndromes.

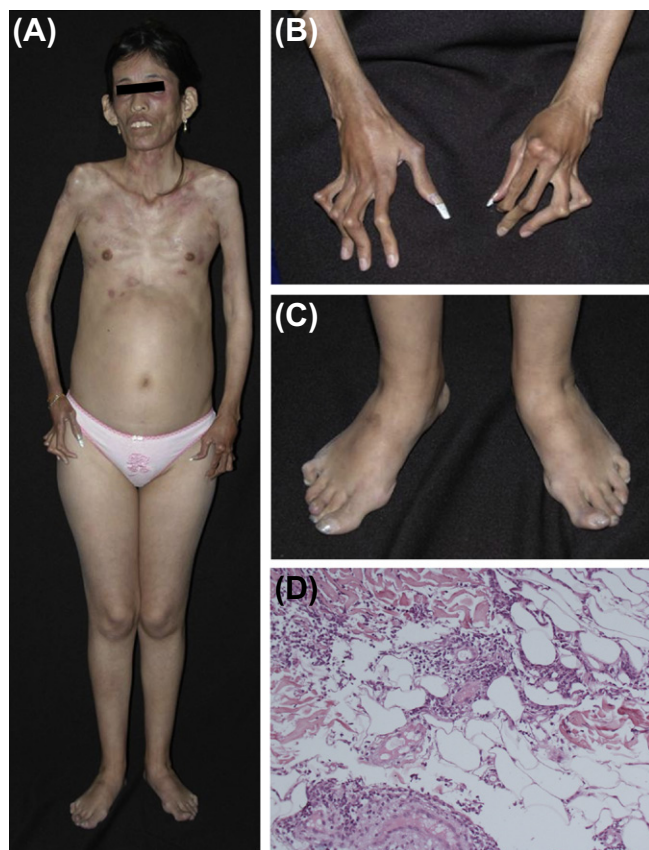
**91.3.1.3.1 Joint Contractures, Muscle Atrophy, Microcytic Anemia and Panniculitis-Induced (JMP) Lipodystrophy.** Recently, an autosomal-recessive, auto-inflammatory, JMP syndrome was reported by us in a patient from Portugal and two siblings from Mexico (54). These patients started developing progressive panniculitis-induced lipodystrophy during childhood. Previously, three patients with very similar features were reported from Japan (55,56). Other reported features of JMP syndrome include intermittent fever, hypergammaglobulinemia, elevated erythrocyte sedimentation rate, hepatosplenomegaly and calcification of basal ganglia (Figure 91-7).

Genome-wide linkage analysis was conducted by us in the two pedigrees with JMP syndrome and we looked for regions of extended homozygosity in the affected

patients. These studies linked JMP syndrome to chromosome 6. Sequencing of immune response genes in this region led us to a homozygous, missense (Thr75Met) loss of function, mutation in proteasome subunit, beta-type, 8 (*PSMB8*) gene in affected patients from both the pedigrees (57). *PSMB8* encodes the  $\beta 5i$  subunit of the immunoproteasome (58). Immunoproteasomes are responsible for proteolysis of antigens presented by major histocompatibility complex class I molecules and result in generation of immunogenic epitopes. We believe that the mutation in *PSMB8* may trigger autoinflammatory response which results in panniculitis and other autoinflammatory manifestations. Two other groups from Japan have recently confirmed our findings and have reported another missense mutation, Gly201Val, in *PSMB8* in patients with autoinflammation and lipodystrophy (59,60).

**91.3.1.3.2 Chronic Atypical Neutrophilic Dermatitis with Lipodystrophy and Elevated Temperature (CANDLE) Syndrome.** This is a relatively new syndrome reported by two groups in a total of five patients. Clinical manifestations include onset of recurrent fever and annular violaceous plaques during infancy which result in loss of sc fat from the face and upper limbs (61,62). Patients develop violaceous swelling of the eyelids, hepatosplenomegaly, arthralgias, hypochromic anemia, raised erythrocyte sedimentation rate and basal ganglia calcifications. On the basis of limited data, the mode of transmission seems to be autosomal recessive. Recently, homozygous Cys135X and Thr75Met mutations in *PSMB8* have been reported in patients with CANDLE syndrome (63).

**91.3.1.4 Familial Partial Lipodystrophy (FPL) due to CIDEC Mutation.** A single patient with



**FIGURE 91-7** JMP syndrome. A. 26-year-old Hispanic female with more marked loss of sc fat from the face, neck, chest and upper extremities than from the abdomen, hips and lower extremities, which were spared. The breasts were atrophic and the neck and chest showed many discrete, small, erythematous nodular skin lesions. B. View of the hand showing flexion contracture at the wrist. The metacarpophalangeal joints were hyperextended and the proximal and distal interphalangeal joints showed variable flexion contractures. C. She had mild contractures of the foot joints. D. Hematoxylin and eosin stain of the skin and subcutaneous tissue biopsy specimen showing lymphocytic infiltrate permeating deep dermal collagen and subcutaneous adipose tissue, with a perivascular component (lower left part of field) consistent with panniculitis. No vasculitis or fat necrosis is seen (200 $\times$ ). (Figures A and D are reproduced with permission from the Endocrine Society. Garg, A.; Hernandez, M. D.; Sousa, A. B.; Subramanyam, L.; de Villarreal, L. M.; Dos Santos, H. G.; Barboza, O. *An Autosomal Recessive Syndrome of Joint Contractures, Muscular Atrophy, Microcytic Anemia and Panniculitis-Associated Lipodystrophy*. J. Clin. Endocrinol. Metab. 2010, 95, E58–E63.)

autosomal-recessive FPL has been reported to harbor a homozygous missense mutation in cell death-inducing DNA fragmentation factor  $\alpha$ -like effector c (*CIDE*) (64). The sc adipose tissue of the patient had multilocular, small lipid droplets in adipocytes as has been seen previously in the knockout mouse model (64,65).

**91.3.1.5 SHORT Syndrome-Associated Lipodystrophy.** The acronym of SHORT syndrome is based on several clinical features such as short stature, hyperextensibility of joints and/or inguinal hernia, ocular depression, Reiger anomaly and teething delay. Only about 30 patients have been reported to have SHORT

syndrome and both autosomal-recessive (66,67) and -dominant (68–70) modes of transmission have been reported (66–68). Features of Reiger anomaly include eye abnormalities such as hypoplasia of iris stroma, prominent Schwalbe ring, iridocorneal synechiae, micro- or megalocornea and teeth anomalies such as hypodontia, microdontia, enamel hypoplasia and atypical teeth. Some patients also manifest intrauterine growth retardation with slow postnatal weight gain, delayed speech development with normal intellect, frequent childhood illnesses, small head circumference, bilateral clinodactyly and sensorineural hearing loss.

Different patterns of fat loss have been reported. In many patients, lipodystrophy affects the face, upper extremities and sometimes the trunk, with relative sparing of the lower extremities. On the other hand, a few pedigrees with autosomal-dominant SHORT syndrome had lipodystrophy affecting only the face, gluteal region and elbows (69,70). Diabetes occurs as early as the second and third decade of life. The genetic basis remains unknown.

**91.3.1.6 Mandibular Hypoplasia, Deafness, and Progeroid Features—Associated Lipodystrophy (MDP Syndrome).** We recently reported this new syndrome distinct from MAD as these patients did not have acroosteolysis (71). All males with MDP syndrome had undescended testes and hypogonadism. One adult female showed lack of breast development. The molecular basis of this syndrome remains unknown.

**91.3.1.7 Neonatal Progeroid Syndrome (Wiedemann–Rautenstrauch Syndrome).** This is an autosomal-recessive syndrome with a total of approximately 25 reported cases (72–74). The clinical features are evident at birth and include a triangular, old-looking face with relatively large skull (progeroid appearance), prominent veins on the scalp, sparse scalp hair, large anterior fontanelle and generalized lipodystrophy. Interestingly, sc fat in the sacral and gluteal areas is spared and can be prominent (74,75). Approximately, half of them die before the age of 6 years but patients surviving up to the age of 16 years have been reported (74–77). The genetic basis remains to be elucidated.

## 91.3.2 Autosomal-Dominant Types

**91.3.2.1 Familial Partial Lipodystrophy (FPL).** Patients with FPL have variable loss of body fat from the extremities as well as from the truncal region. They follow an autosomal-dominant inheritance pattern. Recognition of affected men is very difficult because even many normal adult men appear muscular. Consequently, nearly all the reported pedigrees have been ascertained from female probands. The diagnosis can be suspected in patients with early onset of diabetes and hypertriglyceridemia in the second or third decades who do not have generalized obesity and display marked loss of fat from the extremities, especially from the gluteal region. There are several distinct subtypes of FPL as described below.



**FIGURE 91-8** FPL. A 38-year-old white woman with FPL, Dunnigan variety due to heterozygous missense mutation in the *LMNA* gene. She had loss of fat from the extremities and trunk beginning at puberty and had excess fat accumulation in the face and neck region. She has had surgical removal of fat from the chin, neck, axillae and mons pubis previously. She had acanthosis nigricans in the axillae and groins. (Reproduced with permission from Elsevier Inc. Garg, A.; Misra, A. *Lipodystrophies: Rare Disorders Causing Metabolic Syndrome*. Endocrinol. Metab. Clin. North Am. 2004, 33, 305–331.)

**91.3.2.1.1 Familial Partial Lipodystrophy, Dunnigan Type due to *LMNA* Mutations.** This lipodystrophy (OMIM #151660) was initially described by Ozer et al. (78) in a brief abstract form and later Dunnigan et al. provided a detailed phenotypic description (79). Initial reports of this syndrome described only affected females but subsequently, approximately 300–500 men and women have been reported to have FPLD (80,81). Nearly all of them are of European origin, but Asian Indian and African-American patients have also been reported (Figure 91-8).

**91.3.2.1.1.1 Clinical and Biochemical Characteristics.** It is difficult to recognize affected subjects before puberty as they have normal body fat distribution. The onset of lipodystrophy occurs during childhood or at the time of puberty and sc adipose tissue from the extremities and trunk is lost gradually. The loss of sc fat is more evident from the anterior truncal region than the posterior region (82). Concurrent with the onset of lipodystrophy, excess fat deposition occurs at the chin (“double chin”),

supraclavicular area, and face (“Cushingoid appearance”) and sometimes over the dorsocervical region (“Buffalo hump”). Magnetic resonance imaging reveals particularly prominent intermuscular (in-between the muscular fasciae) fat deposition at the level of thighs and pelvis (81). In many patients, excess fat accumulates in the intra-abdominal and intrathoracic regions. FPLD is easier to diagnose in women but the diagnosis in affected men is challenging. Some affected men do show a muscular phenotype despite no resistance exercise training and show accumulation of fat in the face and neck. About one-fifth to one-third of the patients have acanthosis nigricans, hirsutism, menstrual abnormalities, and polycystic ovaries (82).

Women seem to be particularly severely affected by the metabolic complications of insulin resistance than men (80). The prevalence of diabetes among affected women is more than 50% in pedigrees collected by us versus only about 20% in the men. Diabetes usually develops after the second decade and multiparity and excess fat deposition in the nonlipodystrophic regions, such as the chin, can be predisposing factors for diabetes (83). Affected women also have higher prevalence of hypertriglyceridemia, low serum-HDL-cholesterol concentrations, and atherosclerotic vascular disease, including coronary heart disease (80,84). Some patients also develop cardiac conduction system disturbances resulting in atrial fibrillation and congestive heart failure, manifestations of associated cardiomyopathy (85).

#### **91.3.2.1.1.2 Genetic Defect and Pathophysiology.**

Using genome-wide linkage analysis approach in five large informative pedigrees with FPLD, we reported the FPLD locus on chromosome 1q21–22 (86). Subsequently, screening for candidate genes in the 5-Mb interval, Cao and Hegele (87) reported a missense mutation, Arg482Gln, in lamin A/C (*LMNA*) gene in a Canadian pedigree. Since then, several missense mutations in *LMNA* gene have been reported in patients with FPLD, most of them substituting C-terminal residues (83,85,87–89). Interestingly, other heterozygous or homozygous mutations in *LMNA* have been implicated now in various other disorders such as idiopathic cardiomyopathy, limb-girdle, Emery–Dreifuss and congenital muscular dystrophies, Hutchinson–Gilford progeria syndrome, Charcot–Marie tooth neuropathy, MAD and atypical Werner syndrome (42,90–95). Some patients manifest overlapping clinical features of some of these disorders, which led us to propose that *LMNA* mutations cause a multisystem dystrophy syndrome affecting adipose tissue, cardiac, skeletal muscle, nerve, cutaneous and skeletal tissue (85). However, how specific mutations in *LMNA* cause adipocyte loss from certain areas of the body remains unknown. It is probably those defective interactions of lamins A and C with chromatin or other nuclear lamina proteins during the cell division leading to apoptosis and premature cell death of adipocytes. The gain of fat in nonlipodystrophic regions may be merely a compensatory phenomenon.



**91.3.2.1.1.3 Genotype and Phenotype Variation.**

Most of the missense mutations in patients with FPLD have been reported in exon 8, encoding the globular C-terminal (tail) portion of the protein (83,85,87–89,96). Arginine residue at position 482 seems to be a hot spot with approximately 75% of the patients harboring a mutation resulting in substitution of this residue to tryptophan, glutamine or leucine (83). We have reported an atypical, less-severe FPLD in a pedigree with Arg582His mutation in exon 11 (which can only affect lamin A and not lamin C) (97). We and others have found a strong association between FPLD and cardiomyopathy manifesting as congestive heart failure, atrial fibrillation and other conduction system disturbances requiring pacemaker implantation in patients with exon 1 mutations (Arg28Trp, Arg60Gly, Arg62Gly and Asp192Val) (85). Some patients manifest overlapping features of mild proximal myopathy or muscular dystrophy with slightly increased serum creatine kinase levels (85,98).

**91.3.2.1.1.4 Molecular and Differential Diagnosis.**

Since the clinical diagnosis is difficult in affected men and prepubertal boys and girls, molecular diagnosis may be helpful in them. Genotyping for mutations may be particularly important for identifying those with exon 1 *LMNA* mutations at high risk of cardiomyopathy and conduction system disturbances (85). Whether molecular diagnosis in children and adolescents can help in prevention of diabetes and other metabolic complications later in life by maintaining an active life style with intense physical activity and prevention of undue weight gain remains to be proven.

Many syndromes can present challenge in diagnosis of FPLD such as Cushing's syndrome, truncal obesity, multiple symmetric lipomatosis due to alcohol intake, acquired generalized lipodystrophy, and lipodystrophy in HIV-infected patients. Patients with FPL due to *PPARG* mutation generally have milder loss of fat from the limbs compared to those with FPLD. Subcutaneous fat from the trunk is not affected in those with *PPARG* mutations.

**91.3.2.1.2 Familial Partial Lipodystrophy due to Peroxisome Proliferator-Activated Receptor- $\gamma$  (*PPARG*) Mutation.** Candidate gene approach in many patients affected with FPL, who did not harbor a mutation in *LMNA* gene, resulted in identification of a heterozygous missense mutation, Arg397Cys, in the *PPAR $\gamma$*  gene in a 64-year-old woman with diabetes, hypertriglyceridemia, hypertension and hirsutism, who had lipodystrophy of the face and extremities (OMIM #604367) (99). Subsequently, ~30 more FPL subjects with variable presence of disorders associated with insulin resistance such as diabetes, hypertension and hypertriglyceridemia with heterozygous *PPARG* mutations have been reported (100–102). The age of onset of lipodystrophy appears to range from second decade to later during adulthood but the pattern of progression of fat loss remains to be defined. All affected subjects have been reported to have lost sc fat from the distal extremities; however, facial fat

has been variously reported to be reduced, normal or increased (99–101).

The *PPAR $\gamma$*  protein plays a central role in adipogenesis. It is a ligand-inducible nuclear transcription factor, which forms a heterodimer with the retinoid X receptor and induces transcription of adipocyte-specific genes in response to activation by hormones or other agonists (103,104). *PPAR $\gamma$*  is highly expressed in the adipose tissue, further suggesting its important role in adipose tissue function. Thus, lipodystrophy in FPL patients with missense mutations in *PPAR $\gamma$*  may be due to reduced adipogenesis. Why only selective adipose tissue depots are affected in patients with *PPARG* mutations remains unclear.

**91.3.2.1.3 Familial Partial Lipodystrophy due to v-AKT Murine Thymoma Oncogene Homolog 2 (*AKT2*) Gene Mutations.** Recently, George et al. (105) reported a heterozygous missense mutation, Arg274His, in *AKT2* gene in four subjects from a family who had insulin resistance and diabetes mellitus. The proband was a 35-year-old Caucasian female who developed diabetes mellitus at age 30 years, whereas her mother and grandmother harboring the same mutation developed diabetes during late thirties and a maternal uncle, a middle-aged person, had no diabetes but had hyperinsulinemia. Three of the four affected subjects had hypertension. The proband also had reduced body fat and partial lipodystrophy affecting mainly her extremities (Stephen O'Rahilly, personal communication), however, in depth characterization of the phenotype related to body fat distribution has not been conducted.

*AKT2* belongs to the family of phosphoinositide-dependent serine/threonine kinases and is also known as protein kinase B (PKB) (106). The three isoforms of *AKT* share more than 80% amino acid identity. *AKT1* is almost ubiquitously expressed, whereas *AKT2* is predominantly expressed in insulin-sensitive tissues and *AKT3* in the testes and brain. As compared to the overexpression of wild-type *AKT2* in 3T3-L1 mouse preadipocytes, the mutant form, Arg274His, showed markedly reduced lipid accumulation (105). Previously, a knockout mouse model has shown features of lipodystrophy, insulin resistance and diabetes with increasing age (107). Thus, taken together, lipodystrophy in patients with *AKT2* mutations may be related to reduced adipocyte differentiation and to dysfunctional postreceptor insulin signaling.

**91.3.2.1.4 Familial Partial Lipodystrophy due to Perilipin 1 (*PLIN1*) Gene Mutations.** Gandotra et al. (108) recently reported two heterozygous frameshift mutations in *PLIN1* in three families with FPL of French ancestry. A total of five patients had mutations in *PLIN1*; and all of them had fatty liver, hypertriglyceridemia and hyperinsulinemia. Three of them had diabetes mellitus. HDL-cholesterol levels were reduced in four of them. Lipodystrophy was most striking in the lower limbs and femorogluteal depot. Acanthosis nigricans was present in



all the probands and two of them also had a cushingoid appearance.

Histology of the sc adipose tissue from four patients revealed reduced size of adipocytes and increased macrophage infiltration and adipose tissue fibrosis. Retrovirally transfected preadipocytes (3T3L-1) with mutant *PLIN1* showed smaller lipid droplets compared with those transfected with the wild-type *PLIN1*. Perilipin is the most abundant protein coating lipid droplets in adipocytes. It is essential for formation and maturation of lipid droplets, storage of triglycerides and release of free fatty acids from droplets. Recent data suggest that *PLIN1* mutants fail to bind to AB-hydrolase containing 5 (*ABHD5*), which results in constitutive coactivation of adipose triglyceride lipase and increased basal lipolysis (109).

**91.3.2.1.5 Other Types of Familial Partial Lipodystrophy.** It appears that four loci for FPL, *LMNA*, *PPARG*, *AKT2* and *PLIN1* may not be able to explain the genetic basis of all the patients with FPL and there is likelihood of additional loci (99,100). In-depth characterization of the clinical phenotype related to the pattern of loss of fat in FPL patients with mutations in different genes may be helpful in identification of different phenotypes without resorting to molecular diagnosis.

**91.3.2.2 Atypical Progeroid Syndrome due to *LMNA* Mutations.** Approximately 30 patients with partial or generalized lipodystrophy, insulin resistant diabetes and progeroid features have been reported to harbor missense mutations in the *LMNA* gene (94,111,112). They also present with mottling, pigmentations and sclerosis of skin, hepatic steatosis, cardiomyopathy and valvular lesions, short stature, and deafness. Lack of breast tissue is prominent in many females while some women report premature ovarian failure.

**91.3.2.3 Hutchinson–Gilford Progeria Syndrome.** Approximately 100 patients have been reported with HGPS so far. These patients appear normal at birth but soon thereafter develop features of early aging even as neonates (113). These features include, severe alopecia, graying of hair, micrognathia, beaked nose, shrill voice, and extensive wrinkling of the skin due to loss of underlying adipose tissue, poor sexual development, joint contractures and severe atherosclerosis (113). Many of them died between the ages of 6 and 20 years. Most of the HGPS patients have synonymous heterozygous mutation Gly608Gly of the *LMNA* gene (93). This mutation presents a cryptic splice site, which leads to a mutant form of prelamin A with 50 carboxy-terminal amino acid deleted. The mutation occurred *de novo* in all patients and was demonstrated to be of paternal origin in some of the patients (93). Only one patient had a missense Gly608Ser mutation (93,114,115). Although at birth, the children with HGPS look normal, progressive and generalized loss of body fat occurs later and they develop severe lipodystrophy with increasing age. The precise pattern of body fat loss in patients with HGPS remains to be studied.

### 91.3.3 Other Extremely Rare Types of Genetic Lipodystrophies

An autosomal-dominant variety of generalized lipodystrophy with acromegaloid features and onset after 18 years of age was reported in a pedigree from Brazil (116). We have also observed lipodystrophy of the lower extremities in two siblings with unbalanced translocation involving chromosome 8p and 10p.

## 91.4 LONG-TERM COMPLICATIONS

Patients with lipodystrophies are predisposed to long-term complications of diabetes, i.e. nephropathy, retinopathy and neuropathy. A few patients with CGL have developed end-stage renal disease requiring renal transplantation (117). Blindness due to retinopathy has also been seen in some. Many patients develop recurrent attacks of acute pancreatitis due to extreme hypertriglyceridemia. In some patients, hepatic steatosis can result in further liver dysfunction such as steatohepatitis and cirrhosis. Patients with CGL, type 4 have been reported to have died suddenly most likely due to arrhythmias (39). Some patients with MAD die during childhood of unknown reasons and while some others die during early adulthood due to complications of renal failure due to focal segmental glomerulosclerosis (118,119). Accelerated atherosclerosis causing coronary heart disease, peripheral vascular disease and cerebrovascular accidents has been noted in patients with FPL, particularly among women (6,80,84,120). Other patients with HGPS are also prone to develop severe atherosclerosis.

## 91.5 TREATMENT OPTIONS

Management of severe insulin resistance and its complications, such as severe diabetes, hypertriglyceridemia, hepatic steatosis, polycystic ovarian disease and acanthosis nigricans, in patients with lipodystrophies is challenging. Because many patients may have abnormally elevated serum alanine aminotransferase and aspartate aminotransferase levels indicating liver dysfunction, the choice of therapeutic agents may differ. Briefly, various therapeutic options available are as follows.

### 91.5.1 Diet and Physical Activity

Metabolic complications such as dyslipidemia, diabetes and hepatic steatosis can be mitigated with reduced energy intake and increased physical activity. Patients with partial lipodystrophies must avoid gain of excess fat in nonlipodystrophic regions to reduce their risk of developing metabolic complications. However, appetite is difficult to control in young patients with generalized lipodystrophies. Children also require energy for growth and development and thus diet should allow for adequate growth in young patients. For patients with extreme

hypertriglyceridemia, low-fat diet is appropriate to avoid formation of chylomicrons. Whether extremely low-fat diets will be beneficial for CGL patients remains to be investigated. To reduce risk of atherosclerosis, attempt should be made to reduce low-density-lipoprotein cholesterol levels (or non-HDL cholesterol levels in hypertriglyceridemic patients) to minimum by limiting intake of saturated and trans fats as well as cholesterol (121).

### 91.5.2 Drug Therapy

Diabetes control can be achieved with oral hypoglycemic drugs or insulin. Metformin is particularly attractive since it improves insulin sensitivity, reduces appetite and can induce ovulation in patients with polycystic ovarian disease. However, efficacy of metformin therapy has not been systematically studied in patients with lipodystrophies. On the other hand, thiazolidinediones such as rosiglitazone and pioglitazone, which are PPAR $\gamma$  agonists, may be particularly attractive for FPL patients with *PPARG* mutations; however, limited experience in two such patients resulted in equivocal results (101). In most patients with severe insulin resistance, high doses of insulin remain the mainstay of therapy. Rigorous effort to improve glycemic control is needed to manage hypertriglyceridemia.

If hypertriglyceridemia persists despite good glycemic control, fibric acid derivatives (PPAR $\alpha$  agonists) such as gemfibrozil, fenofibrate or clofibrate are helpful in reducing serum triglycerides. For those unable to tolerate fibrates or with liver dysfunction,  $\omega$ -3 polyunsaturated fatty acids from fish oils should be used in high doses (5–10 g/day). In some patients, a combination of low-dose statins and fibrates can be tried for maximal reduction of non-HDL cholesterol levels. Niacin should be avoided as it can induce insulin resistance and can exacerbate hyperglycemia (122). In women, oral estrogens should also be avoided as they can induce extreme hypertriglyceridemia and acute pancreatitis (6).

Recently, sc recombinant leptin therapy has been reported to be efficacious in improving hyperglycemia, dyslipidemia and hepatic steatosis in patients with severe lipodystrophies and hypoleptinemia (8,123–125). Leptin therapy reduces appetite and results in substantial weight loss, which seems to be a primary mechanism for improving metabolic complications (8). However, leptin therapy is still investigational.

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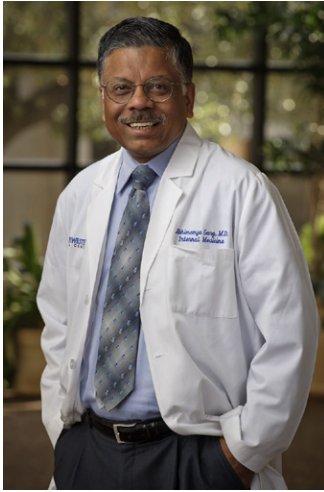


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### Biography



**Abhimanyu Garg, MD** is a professor of Internal Medicine and is Chief, Division of Nutrition and Metabolic Diseases at UT Southwestern. He holds a Distinguished Chair in Human Nutrition Research. Dr Garg's research has focused on the problems of nutrition for patients with diabetes and management of dyslipidemia in patients with type 2 diabetes. In addition, he has contributed to the understanding of the relationship of body fat distribution and insulin resistance. He has carefully characterized the phenotype of various disorders of adipose tissue, called lipodystrophies, which has led to discovery of many novel genes for these disorders. Dr Garg's group identified deficiency of AGPAT2 enzyme, which is critical for triglyceride and phospholipid biosynthesis, as the cause of congenital generalized lipodystrophy, type 1. His group also linked peroxisome proliferator-activated receptor- $\gamma$  gene, the key adipocyte differentiation transcription factor, to familial partial lipodystrophy. His team has also identified the second locus for mandibuloacral dysplasia, i.e. zinc metalloproteinase (ZMPSTE24), that is responsible for posttranslational processing of prelamin A to its mature form lamin A. Recently, he discovered mutation in proteasome subunit, beta-type 8 (*PSMB8*) as the underlying basis of a novel autoinflammatory lipodystrophy syndrome. He demonstrated that patients with generalized lipodystrophy have profound leptin deficiency and proposed that leptin deficiency might contribute to the metabolic complications in the disorder. This led him to initiate a collaborative trial that demonstrated dramatic improvement in hyperglycemia, dyslipidemia, and fatty liver with leptin therapy.

## Amino Acid Metabolism

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The inborn errors of amino acid metabolism are a family of genetic conditions in which an enzyme deficiency typically results in the accumulation of a ninhydrin-positive amino acid. They are conceptually identical to disorders caused by enzyme defects that result in the accumulation of the organic acid intermediates (see Chapter 97). As our understanding of the dynamics of amino acid metabolism grows, simplistic notions of metabolic pathways solely as conduits for elimination of excess substrate have been abandoned. We now realize that most, if not all, of these reactions are highly regulated and integrated into the total fabric of metabolic homeostasis in which amino acids play an important role. Nevertheless, such considerations are largely beyond the scope of this concise overview and are covered in more detail in the work by Scriver and colleagues (1), the standard reference work on these and other inborn errors of metabolism and one that explores the physiologic interactions more fully.

This chapter strives to highlight the clinical, biochemical, molecular, and pathologic features of these inborn errors of metabolism. Although approached in a reductionist manner, providing quick reference for a geneticist with a patient carrying the diagnosis, we realize that in practice, the undiagnosed patient presents to the physician with symptoms in search of an explanation. Based on the clinical features described in the following sections, the physician must decide whether specific diagnostic tests, plasma amino acids, urine organic acids, and ammonia for acutely ill patients, or urinary amino acids for those with more insidious clinical symptoms, should be performed. A deeper understanding of many amino acid-related disorders necessitates the inclusion in this sixth edition of a new section about serine biosynthetic disorders as well as appreciable expansions of the sections regarding cobalamin defects, proline metabolism, citrin deficiency, and many more.

In addition, we have updated this chapter with results of clinical trials, as advances in therapeutics for many

of the aminoacidopathies have allowed for significant changes in patient management and outcomes. Much of the current information on the molecular aspects of these disorders can be accessed through the Online Mendelian Inheritance in Man database (OMIM; <http://www.ncbi.nlm.nih.gov/omim>) or the GeneTests database (<http://www.ncbi.nlm.nih.gov/sites/GeneTests/>).

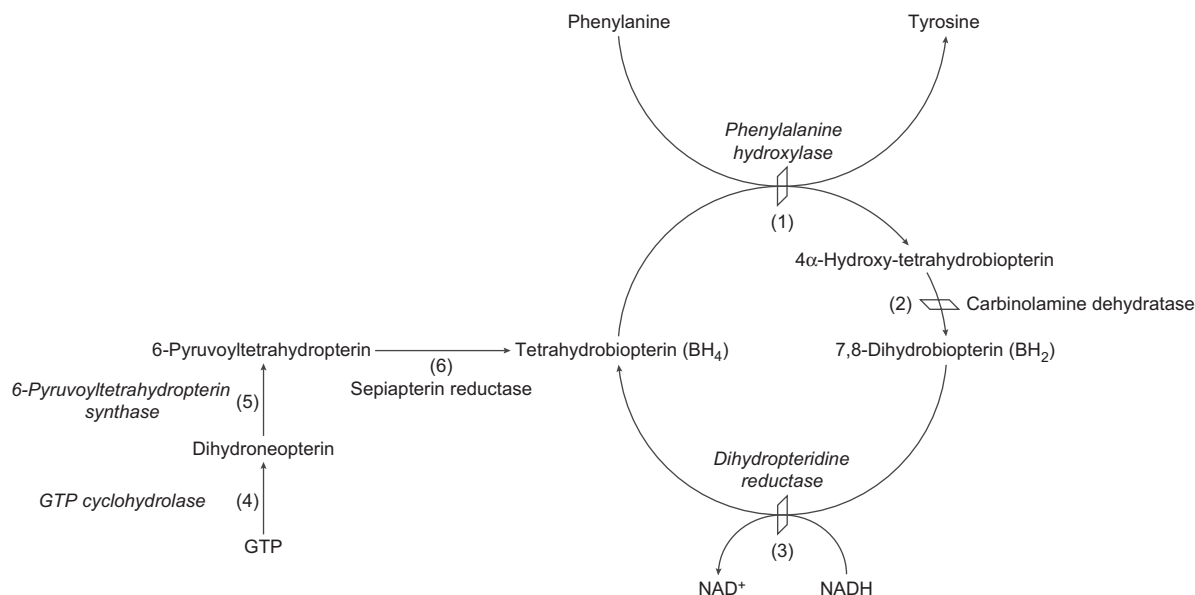
## 92.1 DISORDERS OF PHENYLALANINE METABOLISM

### 92.1.1 The Hyperphenylalaninemias

Folling, a Norwegian chemist, first recognized the existence of a disorder of phenylalanine metabolism in 1934 (2). He found phenylpyruvic acid and phenylacetic acid in the urine of mentally retarded patients in an institution for the retarded. This condition was eventually called phenylketonuria (PKU) and is a cornerstone of research in clinical and biochemical genetics. We now recognize several genetic entities that can cause an elevation in blood phenylalanine and mimic the clinical phenotype of PKU. Each disorder interferes with the hydroxylation responsible for the conversion of phenylalanine to tyrosine. Each disorder causes an elevation in phenylalanine concentration in the blood; collectively, they are referred to as the hyperphenylalaninemias. These disorders are reviewed in detail by Scriver and Kaufman (3) and Blau and colleagues (4).

### 92.1.2 Phenylalanine Hydroxylase Reaction

Phenylalanine is hydroxylated to tyrosine by the enzyme phenylalanine hydroxylase (PAH). The reaction requires molecular oxygen, and tetrahydrobiopterin is the active cofactor (5). The tetrahydrobiopterin is generated de novo in humans from guanosine triphosphate (GTP) by a complex series of enzymatic reactions (Figure 92-1).



**FIGURE 92-1** Regulation of PAH activity. (1) Phenylalanine is converted to tyrosine by the holoenzyme, PAH. PAH requires tetrahydrobiopterin (BH<sub>4</sub>) as an active cofactor and is recycled by the sequential actions of (2) PCD and (3) DHPR. BH<sub>4</sub> is synthesized *in vivo* through a complex series of steps that involve (4) GTP-CH, (5) PTPS, and (6) SR. Genetic defects at any of these steps except for (6) may be associated with hyperphenylalaninemia.

The products of the PAH reaction are tyrosine and an oxidized biopterin, 4α-hydroxy-tetrahydrobiopterin. Tetrahydrobiopterin is regenerated by the sequential actions of carbinolamine dehydratase and dihydropteridine reductase (DHPR). In humans, the conversion of phenylalanine to tyrosine occurs primarily, if not entirely, in the hepatocytes. The functional enzyme exists as a homodimer or homotetramer and has catalytic, regulatory, and tetramerization domains (6).

### 92.1.3 Classic PKU

Classic PKU (OMIM 261600) occurs when an individual expresses 5% or less of normal PAH activity in the hepatic tissue. The enzyme deficiency was first documented by Jervis (7) and subsequently by others. If left untreated, patients with classic PKU develop mental retardation, eczema, hypopigmentation, and neurologic symptoms. Knox (8) reviewed the intellectual results of untreated children and adults with PKU and concluded that most would have an intelligence quotient (IQ) in the severely retarded range. On screening approximately 250,000 blood samples submitted to the Massachusetts State Health Laboratory for syphilis testing, Levy and colleagues (9) found only three persons with an elevated blood phenylalanine level, and each was retarded. Thus, normal intelligence is infrequently achieved in persons with PKU who remain untreated.

The characteristic “mousey” odor present in some untreated persons with PKU is due to the excretion of phenylacetic acid. The hypopigmentation of the skin and hair occurs because of competitive inhibition of tyrosine hydroxylase by the increased concentration of phenylalanine. The inhibition of tyrosine hydroxylase activity

prevents the conversion of tyrosine to dopa and the subsequent formation of melanin. Thus, affected and untreated individuals have been described as having blue eyes, blonde hair, and pale skin.

Neurologic symptoms in untreated persons with PKU include hypertonicity, irritability, agitated behavior, tremors, hyperactivity, and occasional seizures; some are considered autistic. The precise mechanisms that cause the defective myelin formation, neurologic abnormalities, and mental retardation are unknown.

In most of the developed countries, infants are being screened for PKU by measurement of blood phenylalanine during the newborn period, using either a fluorometric or a microbiologic assay. Patients with classic PKU eventually demonstrate a persistent elevation in phenylalanine levels, greater than 1000–1200 μM (16–20 mg%, normal < 80 μM or 1.3 mg%), in their blood while receiving a normal dietary intake of protein. Their plasma tyrosine will be within the normal range, and hydroxyphenylacetic acid and phenylpyruvic acid will eventually be detectable in the urine. Plasma studies have now completely supplanted those in the urine.

Most infants with an elevated blood phenylalanine level detected through a screening program do not have classic PKU; rather, they have a transient or a mild form of hyperphenylalaninemia, which is usually benign and does not require therapy.

Most newborn screening programs had used a cutoff value of 120 or 240 μM (2 or 4 mg%) as an indicator of a “positive” test. With the advent of screening by tandem mass spectrometry, by which both phenylalanine and tyrosine can be measured with great accuracy, the phenylalanine to tyrosine ratio is becoming an increasingly



important measurement, maintaining a high degree of sensitivity (even with early discharge) while increasing specificity (10). The newborn screening samples for healthy infants are typically collected on the day of discharge from the hospital. In the recent past, this usually occurred at 3–5 days of age, and because the blood phenylalanine inevitably rose above  $240\ \mu\text{M}$  in infants with PKU by day 3 of life, it was a reliable and sensitive assay. With the recent changes in healthcare policy in most western countries, infants are being discharged earlier than 3 days, sometimes at less than 24 h of age. Because of the advances in screening algorithms and technology, repeat screening at 4 weeks of age is no longer required, even with early postnatal testing (11).

Classic PKU is inherited in an autosomal recessive manner and occurs in approximately 1:10,000 births in populations of western European origin. In these populations, the PAH-deficient alleles can be considered a polymorphism because the gene frequency ( $q$ ) exceeds 0.01 and the heterozygote frequency ( $2pq$ ) is 0.02 or greater. The highest frequency for PKU is in Turkey, with an incidence of 1 in 2600 newborns; Scotland has a frequency of 1 in 5300 newborns, with most other European countries reporting a frequency of 1 in 10,000 for newborns. China has an incidence of 1 in 16,000; PKU in Japan is relatively rare, with a reported frequency of 1 in 143,000 births. PKU is similarly rare in Ashkenazi Jews, Finns, and blacks.

More than 350 mutations have been identified to be associated with the PKU phenotype. Most of these mutations have been identified in exons 6–12 (see data in OMIM, Scriver (1), and the PAH database (<http://www.pahdb.mcgill.ca/>)).

No single mutation accounts for the majority of cases in persons with the PKU phenotype. Clustering of specific mutations occurs within certain ethnic groups or certain geographic regions. For instance, most affected French Canadians have the p.M1V mutation. Affected persons from eastern Europe commonly bear the p.R408W mutation. In the data available from the Chinese population, a restricted number of mutations have been identified, most commonly associated with a common haplotype (haplotype 4) (12).

For the white population of northern European origin, most PKU patients were initially identified as having mutations on a background of chromosomes of similar haplotype, either haplotype 2 or haplotype 3. Direct identification of mutations found a plethora of alterations rather than a single common mutation. Recurrent mutations found in PAH are p.M1V, p.R261Q, p.R408W, p.Y414C, c.1315+1G>A, and c.1066-11G>A. One study was able to confirm the genotype of both alleles in 35% of PKU patients from the Midwest region of the United States (13). In general, there appears to be a good correlation between the severity of protein change, determined by mutant genotype and the degree of phenylalanine intolerance. However, the exceptions are frequent enough that routine genotyping of patients does not appear to be clinically useful (14,15). A

great source of variability in cognitive outcome may lie in interindividual variability in phenylalanine transport from the blood into the brain. Rare individuals have biochemically classic PKU yet normal intelligence, in spite of high plasma phenylalanine levels because of lower levels of phenylalanine transport into the brain (16).

Heterozygote detection using plasma phenylalanine and tyrosine levels, under standard conditions, has been possible but imprecise (17). Realistically, carrier testing is not frequently performed due to the existence of newborn screening for ascertainment of subsequent children and limited clinical utility of PAH genotyping.

Accurate diagnosis of infants identified from newborn screening programs is critical to establish appropriate therapeutic regimens. Most infants with an elevated blood phenylalanine above the screening laboratories' selected threshold will not have PKU. False-positive tests generally occur in infants who are premature, receive hyperalimentation, or have liver disease, or the condition is of a transient nature and occurs for unknown reasons. Fewer than 30–50% of infants would be confirmed as having persistent hyperphenylalaninemia. We recommend the following as a practical approach in the evaluation of infants with a positive screening test.

- (1) A single value of  $120\text{--}360\ \mu\text{M}$  and/or an elevated phenylalanine to tyrosine ratio: repeat the screening test.
- (2) A single value above  $360\ \mu\text{M}$ : quantify blood phenylalanine and tyrosine and
  - (a) if phenylalanine and phenylalanine to tyrosine ratio are all still elevated, then evaluate for persistent hyperphenylalaninemia (step 3)
  - (b) if tyrosine is elevated, then evaluate the causes of hypertyrosinemia
  - (c) if phenylalanine and tyrosine have rapidly normalized, then consider maternal hyperphenylalaninemia.
- (3) Persistent hyperphenylalaninemia (greater than  $360\ \mu\text{M}$ ) and normal tyrosine: repeat quantification of plasma phenylalanine: collect urine for quantification of pterin metabolites and quantify DHPR in a whole blood sample or from a dried blood spot.

We believe that the above-mentioned procedure is a rational and convenient approach to diagnosis within the capability of programs that screen newborns for PKU. More elaborate studies of mutation analysis of the PAH gene or liver biopsies to measure the activity of the PAH enzyme are not required for routine diagnosis and decisions for appropriate therapy. The quantification of urinary pterin metabolites and blood DHPR should be performed in one of the selected laboratories that routinely handle a large volume of samples for diagnostic purposes.

Dietary therapy has proved effective in preventing mental retardation in patients with PKU. In long-term studies in the United Kingdom, Canada, and the United States, the restriction of dietary phenylalanine within 30 days of birth and continuing for 12 years has resulted in

intelligence comparable to, or slightly below, that of normal sibs (18,19). It is currently believed that restriction of dietary phenylalanine should begin as soon after birth as possible and should continue throughout life. Recent studies suggest a reversible diminution in performance in almost all PKU patients when plasma phenylalanine rises (20), particularly in language skills (21). In some cases, permanent loss of intellectual achievement results from dietary termination. The diet should be managed by a competent team consisting of a nutritionist, a physician, and a person with skills in family dynamics to ensure dietary compliance. It had been recommended that blood phenylalanine levels of 120–600  $\mu\text{M}$  (2–10 mg%) were satisfactory for achieving normal development in patients with PKU. It is now believed that blood phenylalanine levels should be maintained below 360  $\mu\text{M}$  (6 mg%) (11,22). Although desirable, many families are unable to achieve such levels consistently.

Newborns with persistent hyperphenylalaninemia of greater than 360–600  $\mu\text{M}$  (6–10 mg%) should be treated. The exact quantity of phenylalanine in the diet needs to be individually determined because the variation in genotype, among other factors, influences phenotype. Most affected infants can be placed on a special commercial formula deficient in phenylalanine and specifically designed for children with PKU to lower their blood phenylalanine below predetermined therapeutic levels. At that point, the formula needs to be supplemented with an infant formula or milk to supply a phenylalanine concentration of approximately 60–90 mg/kg/day. This quantity of phenylalanine needs to be individually adjusted to maintain the blood phenylalanine at 60–360  $\mu\text{M}$  (1–6 mg%) on a consistent basis. Once stabilized, blood values determined once a month are recommended for monitoring dietary compliance and making nutritional adjustments. Most children and adults require a daily phenylalanine intake of 200–500 mg/day from all food sources to maintain blood phenylalanine within the therapeutic range. A lifetime commitment to the use of a special formula, a vegetarian-style diet, and the use of low-protein foods are required for the prevention of neurologic sequelae from persistent hyperphenylalaninemia. Compliance of families and patients, particularly during the teenage years, may be a problem.

The treatment of PAH deficiency is continuing to evolve with development of novel therapies. Supplementation with tetrahydrobiopterin ( $\text{BH}_4$ ) improves phenylalanine tolerance in many patients, particularly in patients with milder mutations, but responsiveness to  $\text{BH}_4$  cannot be reliably predicted from the mutations in a given patient (23,24). Supplementation with  $\text{BH}_4$  not only can improve protein activity with  $\text{K}_m$  mutations but also can act as a chemical chaperone to improve folding of some types of mutant PAH (25). Multicenter, randomized, double-blind, placebo-controlled clinical trials have been performed, which showed a percentage of PKU patients

receiving a sapropterin hydrochloride (6R-tetrahydrobiopterin) concentration of 20 mg/kg/day, demonstrating increased phenylalanine tolerance of 17.7 mg/kg/day compared to baseline (26). While  $\text{BH}_4$  is most effective in patients with hyperphenylalaninemia or mild PKU and therefore will have the least impact on their treatment, a small percentage of classic PKU patients respond to treatment with increased phenylalanine tolerance. The degree of response, however, is slight and patients continue to require phenylalanine-restricted diets with synthetic protein supplementation. Enteric enzyme therapy with modified PAH or bacterial phenylalanine ammonia-lyase is being developed, converting phenylalanine to innocuous substances in the body (27). When it is perfected, gene therapy will be the ultimate cure for PAH deficiency.

Children born to mothers with PKU have a high risk of having mental retardation or congenital defects. Approximately 90% of offspring born to mothers with classic PKU who have not received dietary restriction of phenylalanine during their pregnancy will be affected with mental retardation, microcephaly, and impaired growth (28,29). Fourteen percent of these children will have cardiac malformations (30).

Ensuring preconceptional treatment is a significant public health issue for women with PKU. Failure to resolve the issue and to allow women with PKU to give birth to children who are mentally damaged could largely reverse the major societal gains that have been achieved during the past 40 years. Women with PKU can have normal children if satisfactory dietary intervention can maintain blood phenylalanine levels at less than 360  $\mu\text{M}$  (6 mg%), both before and during pregnancy (31–33). For pregnant women who have remained on their diet before conception and who continue to maintain satisfactory blood phenylalanine values of less than 360  $\mu\text{M}$  during pregnancy, a successful outcome can be expected. For those women who have been less diligent in maintaining their diet or who have been untreated for a number of years, returning to a phenylalanine-restricted diet can be difficult and requires considerable medical and social support to assist them in achieving satisfactory phenylalanine values (34). Mild hyperphenylalaninemia with untreated plasma phenylalanine no greater than 360  $\mu\text{M}$  (6 mg%) does not appear to pose a risk to the fetus and does not require treatment (35).

Commercial formulas are available to help the older patients return to diet. Additional supplements of tyrosine and possibly branched-chain amino acids (BCAAs) may be required during pregnancy. Administration of these supplements requires careful amino acid monitoring of plasma and frequent adjustments of formula composition and food selection.

The problem of maternal PKU emphasizes the need of governmental and regional programs to maintain contact with women who have hyperphenylalaninemia and to

ensure that these women are aware of the risks of pregnancy and of the personal discipline required to achieve a child free of the teratogenic effects of hyperphenylalaninemia in utero. Programs responsible for newborn screening and the management of persons with PKU should ensure that appropriate resources are in place to counsel, support, and manage women with PKU who may elect to become pregnant.

### 92.1.4 Defects in Biopterin Metabolism

Not all children with persistent hyperphenylalaninemia have a primary defect in the PAH gene. Approximately 1% of infants ascertained with persistent phenylalanine levels above 600  $\mu$ M (10 mg%) will suffer from neurologic deterioration, despite adequate and timely restriction of phenylalanine intake. Most of these children lack adequate levels of tetrahydrobiopterin, owing either to a defect in dihydrobiopterin biosynthesis or to a deficiency of DHPR (see [Figure 92-1](#)) (36). Tetrahydrobiopterin is an indispensable cofactor for PAH activity and serves as an essential cofactor for tyrosine and tryptophan hydroxylase reactions. Decreased activity of these two enzymes results in low levels of two neurotransmitters in the brain: dopamine and serotonin (37), resulting in neurologic damage, mental retardation, and, in many instances, death. Disorders that involve biopterin synthesis or regeneration have been called “atypical or malignant PKU (or hyperphenylalaninemia).”

In vivo synthesis of the active cofactor, BH<sub>4</sub>, is accomplished by the sequential activity of GTP cyclohydrolase (GTP-CH), 6-pyruvoyltetrahydropterin synthase (PTPS) and sepiapterin reductase (SR). Approximately 60% of patients with documented disorders of biopterin metabolism have a deficiency of PTPS (38). Most of the remainder have defects in the recycling of BH<sub>2</sub> to BH<sub>4</sub> by DHPR and pterin-4 $\alpha$ -carbinolamine dehydratase (PCD) (see [Figure 92-1](#)).

### 92.1.5 PTPS Deficiency

PTPS deficiency (OMIM 261640) is now typically identified by newborn screening programs when infants with hyperphenylalaninemia are found to have abnormal biopterin metabolites in their urine. The urine and cerebrospinal fluid (CSF) contain a high ratio of neopterin to biopterin. Levels of CSF neurotransmitter metabolites homovanillic acid (HVA, derived from catecholamines) and 5-hydroxyindoleacetic acid (5-HIAA, derived from serotonin) are low. The inability to form BH<sub>4</sub> from GTP results in impairment of PAH activity, with a resultant elevation in blood phenylalanine levels.

Infants with PTPS deficiency develop neurologic symptoms despite normalization of blood phenylalanine levels. They may be microcephalic and small for gestational age. They will typically develop increased tone in the arms and legs, poor truncal tone, irritability, difficulty

swallowing, hyperthermia, seizures, and delayed mental development. A parkinsonian movement disorder may be apparent in some children.

Treatment of PTPS deficiency should consist of daily replacement of BH<sub>4</sub> (2–5 mg/kg/day), levodopa, 5-OH-tryptophan, and carbidopa (39). Although most children with PTPS deficiency improve with treatment, many have sustained permanent central nervous system (CNS) damage by the time diagnosis had been correctly established and treatment begun. The damage may even be prenatal (40). Treatment efficacy must be monitored continually and dosage of replacement medicines adjusted. Rare patients with hyperphenylalaninemia due to PTPS deficiency have normal neurotransmitter metabolite levels and can be treated with BH<sub>4</sub> alone (40).

PTPS deficiency is inherited as an autosomal recessive disorder. The enzyme has been documented to be deficient in the hepatic tissue of affected patients (41,42) and in erythrocytes (43). The enzyme is a tetramer of identical subunits; heterozygotes may have less than 50% of activity due to heteropolymeric inactivation of the complete enzyme by the mutant. A number of independent mutations have been identified in patients with deficiency of this enzyme (44).

### 92.1.6 GTP-CH Deficiency

GTP-CH is the rate-limiting first step of BH<sub>4</sub> biosynthesis. GTP-CH deficiency (OMIM 128230, 233910) can occur as a dominant or recessive condition. The dominant condition is one cause of dopa-responsive dystonia, another being tyrosine hydroxylase deficiency or Segawa syndrome. Patients with dominant GTP-CH deficiency do not have hyperphenylalaninemia; rather, after a period of normal gross motor development, they develop appendicular dystonia with gait disturbance and hyperreflexia in childhood, typically by age 6. Normal intelligence is retained. The dystonia responds dramatically following daily treatment with small doses of levodopa. Later development of parkinsonism and extrapyramidal symptoms may result in misdiagnosis as “juvenile Parkinson’s disease,” but the disorder is distinguished from true Parkinson’s by lack of dyskinesia, on–off effect, or resistance to levodopa therapy. The recessive form of GTP-CH deficiency is typically picked up by newborn screening with hyperphenylalaninemia, although exceptions exist (45–47).

Patients with recessive GTP-CH deficiency demonstrate much more neurologic dysfunction than the dominant form, with early-onset developmental delay, seizures, tremors, truncal hypotonia with appendicular hypertonia, and autonomic dysfunction (38,48,49). Similar to 6-PTPS deficiency, the recessive mutations in GTP-CH lead to a severe deficiency in the synthesis of BH<sub>4</sub> from GTP (see [Figure 92-1](#)). In addition to persistent hyperphenylalaninemia, both biopterin and neopterin levels are low in body fluids such as urine, plasma,

and CSF. A normal neopterin to biopterin ratio is preserved. CSF neurotransmitter metabolite analysis typically also shows low HVA and 5-HIAA levels. Treatment of recessive GTP-CH deficiency consists of supplementation with oral BH<sub>4</sub> in addition to levodopa/carbidopa and 5-hydroxytryptophan.

### 92.1.7 DHPR Deficiency

A deficiency of DHPR (OMIM 261630) is the second most common defect of biopterin metabolism. DHPR deficiency accounts for approximately 0.5% of infants documented to have persistent hyperphenylalaninemia.

DHPR is required for the regeneration of BH<sub>4</sub> from BH<sub>2</sub>. A deficiency of DHPR can be expected to produce a secondary deficiency of PAH, tyrosine hydroxylase, and tryptophan hydroxylase, with the metabolic phenotype of hyperphenylalaninemia and CNS deficiency of catecholamines and serotonin. Folate metabolism is also altered because DHPR is responsible for maintaining folate in its active tetrahydrofolate form (50).

The clinical consequences of DHPR deficiency are serious. Affected children develop seizures, microcephaly, and progressive neurologic degeneration despite apparent satisfactory management of their hyperphenylalaninemia, if phenylalanine restriction is the only form of therapy (51). Oculogyria, diurnal variation, hypersalivation, and hyperthermia, which are symptoms of neurotransmitter deficiency, also manifest. They are also at risk of sudden death, possibly because of unregenerated BH<sub>2</sub> accumulation and folate deficiency. They have low levels of HVA and 5-HIAA in the CSF and urine (42).

The disorder is inherited in an autosomal recessive manner. A number of different mutations have been identified, which include point mutations, small deletions, and insertions (52,53). The identification of heterozygotes for DHPR deficiency in families with an affected child can be achieved by direct enzymatic assay using erythrocytes or leukocytes (54,55).

The diagnosis of DHPR deficiency can be established by either molecular sequencing of the gene or more rapidly via direct measurement of DHPR activity in leukocytes or erythrocytes. The most efficient manner is by enzyme assay using a dried blood spot on filter paper (56) as part of the routine evaluation of all infants with hyperphenylalaninemia. Although in some infants, alterations in urinary biopterin profiles are a clue to the diagnosis, it is estimated that one-third of the infants with DHPR deficiency will be missed if urine biopterin levels alone are relied on for detection (41,42).

Children with DHPR deficiency can benefit from therapy, especially if the condition is detected early and treated aggressively but remain significantly delayed in development (57). It is important to restrict the phenylalanine levels in the diet to achieve near-normal blood levels and to supply adequate precursors of the neurotransmitters. In addition to neurotransmitter replacement, as

described for PTPS deficiency, folinic acid 12.5 mg/day is recommended (58). Some may also improve following treatment with BH<sub>4</sub> (59).

### 92.1.8 PCD Deficiency

PCD and DHPR are required for the conversion of oxidized BH<sub>2</sub> back to active BH<sub>4</sub>. Deficiency of PCD (OMIM 264070) results in neonatal-onset hyperphenylalaninemia that is transient, even with a normal diet. Clinically, most patients with PCD have normal growth and development, though some have been reported with transient hypotonia (60). Patients with PCD deficiency excrete 7-biopterin, also known as primapterin (47). CSF neurotransmitter metabolite levels are normal.

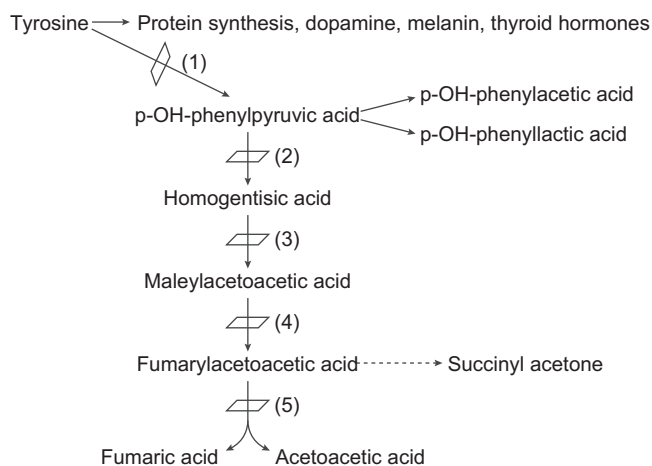
### 92.1.9 SR Deficiency

Although SR deficiency does not cause hyperphenylalaninemia, it is still included here as an inborn error of BH<sub>4</sub> synthesis. The mechanism for absence of hyperphenylalaninemia is the existence of aldose reductase, carbonyl reductase, and dihydrofolate reductase in the liver but not the CNS, allowing phenylalanine hydroxylation via peripheral conversion of sepiapterin to BH<sub>4</sub> (39,61). Phenylalanine loading tests in patients with SR deficiency are abnormal, however, and CSF neurotransmitter analysis demonstrates low levels of HVA and 5-HIAA, while neopterin and BH<sub>4</sub> levels may be normal. SR deficiency is one of the rare forms of BH<sub>4</sub> biosynthetic deficiency, with less than 40 cases currently well described. While most patients present with the typical dopa-responsive dystonia phenotype of the other neurotransmitter synthetic deficiencies, others have presented with somnolence, psychomotor retardation, and gait disturbance later in childhood (62,63). Unfortunately, SR deficiency does not respond well to BH<sub>4</sub> supplementation; rather, the treatment of the disorder centers around provision of L-dopa/carbidopa and 5-hydroxytryptophan.

## 92.2 DISORDERS OF TYROSINE METABOLISM

Approximately one-half of the available tyrosine in humans is formed from the oxidation of phenylalanine. Tyrosine is essential for the synthesis of protein, the synthesis of thyroid hormones, the formation of melanin pigment, and the production of dopamine and catecholamines. Tyrosine is metabolized through a series of oxidative steps to form acetoacetic and fumaric acids (Figure 92-2). In the presence of excess dietary protein, the rate-limiting enzyme for the pathway is tyrosine aminotransferase (TAT). Enzyme deficiencies within the catabolic pathway may cause accumulation of tyrosine or a tyrosine product. Severe liver disease may elevate plasma tyrosine levels as well. The only common disorder of tyrosine metabolism is transient neonatal tyrosinemia, a





**FIGURE 92-2** The tyrosine metabolic pathway. Enzymes involved are (1) TAT; (2) *p*-OH-phenylpyruvic acid dioxygenase; (3) homogentisic acid oxidase; (4) maleylacetoacetic acid isomerase; and (5) fumarylacetoacetic acid hydrolase.

problem of delayed developmental synthesis or stability of *p*-hydroxyphenylpyruvic acid (pHPPA) dioxygenase. The other known disorders are genetically determined but are uncommon. These disorders are reviewed by Mitchell and colleagues (64,65).

### 92.2.1 Transient Neonatal Tyrosinemia

In the 1960s, it was estimated that 30% of premature infants and 10% of full-term infants would develop neonatal tyrosinemia. Neonatal tyrosinemia does not occur because of a genomic mutation in the tyrosine catabolic but is a result of an infant's interaction with the environment. Gestational age rather than birth weight was the most consistent predisposing factor. It is more common in Alaskan Eskimos and Inuit Indians, but this may be due to ascorbate deficiency, rather than a genetic predisposition. Neonatal tyrosinemia, per se, is not believed to be associated with clinical symptoms, but some studies suggested reduced intellectual performance.

The incidence of significant neonatal tyrosinemia has decreased in recent years because of changes in feeding practices of newborns. The major environmental factors associated with neonatal tyrosinemia are prematurity, a diet containing protein concentration of greater than 3 g/kg/day, and deficient intake of vitamin C. Cow's milk is less widely used and would typically supply a protein concentration of 5 g/kg/day or greater. Modern infant formulas supply a protein concentration of 2–3 g/kg/day, similar to breast milk feeding, and are supplemented with vitamin C.

Transient neonatal tyrosinemia is due to decreased activity of pHPPA dioxygenase, an ascorbate-dependent enzyme. The amount of enzymatic activity in the hepatic tissue increases with increasing gestational age, often reaching normal levels only after birth. Thus, young infants may have insufficient pHPPA dioxygenase to metabolize the pHPPA formed from tyrosine. Tyrosinemia, if significant,

may be treated by lowering the dietary protein to 2 g/kg/day to decrease the formation of pHPPA from tyrosine and administering 100 mg of ascorbic acid per day to stabilize those enzyme molecules that have been synthesized. Most infants with neonatal tyrosinemia will promptly lower their plasma tyrosine concentration to the normal range (less than 0.1 mM) with these two maneuvers.

### 92.2.2 Hepatorenal Tyrosinemia

Hepatorenal tyrosinemia (OMIM 276700), or tyrosinemia I, is a rare and potentially lethal inborn error of metabolism caused by a deficiency of fumarylacetoacetate hydrolase (FAH). This enzyme catalyzes the conversion of fumarylacetoacetate to fumarate, a Krebs cycle intermediate, and acetoacetate, a ketone body. FAH deficiency leads to accumulation of fumarylacetoacetate, its precursor maleylacetoacetate, and a decarboxylation product succinylacetone, all of which are toxic to the liver and kidneys (see Figure 92-2). Succinylacetone is also a potent inhibitor of  $\delta$ -aminolevulinic acid (ALA) dehydratase, which leads to reduction in heme synthesis. Clinical symptoms of tyrosinemia I span a continuum of severity. The most severe cases can present in the neonatal period and typically prior to the sixth month of age, with irritability, poor feeding, and failure to thrive. Sometimes the characteristic “boiled cabbage” odor is present. Laboratory investigations will identify elevations in liver transaminases, prolonged prothrombin and partial thromboplastin bleeding times, and marked elevation in serum  $\alpha$ -fetoprotein (AFP, usually >100,000 ng/mL) that are hallmarks of the hepatotoxic effects of fumarylacetoacetate and its adducts. Without treatment, the infantile-onset patients will rapidly develop fulminant hepatic necrosis; cholestatic jaundice; renal Fanconi syndrome; and excruciating, recurrent, porphyria-like neurologic pain crises. Caused by reduced heme synthesis, the pain crises typically occur following intercurrent illnesses, starting with irritability and progressing to severe pain; the child assumes a position of hyperextension of the trunk and neck. These painful crises may last from 1 day to as long as a week. Other children may develop weakness or paralysis, requiring ventilatory support. Autonomic dysfunction with ileus, hypertension, and tachycardia may occur. Neurologic crises, especially those with paralysis, have an associated mortality rate of 10%. It is believed that these crises are similar to what occurs in acute intermittent porphyria with decreased  $\delta$ -ALA dehydratase activity and accumulation of  $\delta$ -ALA in blood. Unfortunately, there is no absolute correlation between blood levels of  $\delta$ -ALA and clinical symptoms.

Early mortality, typically in the first decade, occurs due to liver failure, hepatocellular carcinoma, or neurologic crisis (66). At the other end of the severity continuum is a “chronic form,” with slower progression of growth failure, hepatic nodular cirrhosis, renal tubular acidosis with generalized aminoaciduria and hypophosphatemia,

and episodic neurologic crises. The hepatic nodules often contain regenerating hepatocytes with spontaneous reversion to the wild-type *FAH* genotype (67). Patients with the chronic form typically develop rickets and nephrocalcinosis due to hyperphosphaturia and have a high risk of developing hepatocellular carcinoma in childhood or adolescence.

The observed biochemical abnormalities include elevated urinary levels of succinylacetone, *p*-hydroxyphenyllactic, and *p*-hydroxyphenylacetic acids. Affected children have an increased concentration of tyrosine (two to three times normal) and methionine in the plasma owing to the reduced activity of pHPPA dioxygenase and methionine adenosyl transferase activity, respectively. Serum AFP increases over time as liver damage progresses.

Newborn screening programs have begun to adopt succinylacetone measurement from dried blood spots to screen for tyrosinemia I, especially because tyrosine levels were neither sensitive nor specific for the detection of the disease. Earlier reports indicate that succinylacetone is a much better marker; the New York state newborn screening program screened approximately 500,000 newborns over a 2-year period, identifying two newborns with the disorder, with three false-positives. In the two true-positive cases, it was noted that their initial tyrosine levels would not have been flagged as abnormal (68).

The prevalence of tyrosinemia I in most parts of the world is low. It occurs in higher frequency in the Scandinavian countries, apparently from genetic drift; a modest number of cases have been reported in Finland. The highest frequency is along the St. Lawrence Waterway in Canada, where one in 20 individuals is a carrier owing to founder effect. A single mutation in the *FAH* gene (IVS12+5G>A) accounts for 90% of the abnormal alleles within this population (69). This same mutation has been identified in patients from northern Europe. Other mutations are present in lower frequency, with some evidence for ethnic prevalence: c.1009G > A in Scandinavia, p.W262X in Finland, and p.D233V in Turkey (70).

Heterozygotes for tyrosinemia I are asymptomatic and have normal plasma amino acid levels and normal values of succinylacetone in the urine and blood. Heterozygote detection can be accomplished by assaying *FAH* activity in cultured skin fibroblasts, lymphocytes, or red blood cells (71), or by identifying the specific mutation in the family by molecular analysis. Prenatal diagnosis can be performed by molecular analysis if the mutations are known, by measurement of *FAH* activity in cultured amniotic fluid cells or chorionic villus cells (71), or by documentation of increased succinylacetone in the amniotic fluid. The existence of a pseudodeficiency allele for *FAH*, although rare, needs to be considered when using prenatal diagnosis or testing for carriers (72).

There exist three approaches in the management of children with hepatorenal tyrosinemia: (1) metabolic

inhibition of the proximal segment of the tyrosine catabolic pathway, (2) dietary intervention with the reduction of phenylalanine and tyrosine, and (3) liver transplantation.

The primary focus of treatment in infants identified via newborn screening should be on prompt initiation of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC; nitisinone), a potent inhibitor of pHPPA dioxygenase. NTBC effectively creates an enzymatic block proximal to the *FAH* deficiency, abolishing the generation of toxic fumarylacetoacetate, maleylacetoacetate, and succinylacetone. Treatment with NTBC, at a dose of 1 mg/kg/day, results in an immediate reduction in AFP levels, improvement in  $\delta$ -ALA dehydratase activity, normalization of coagulation times and urinary succinylacetone levels, and prevention of neurologic crises (73). Transient thrombocytopenia and neutropenia may occur, and plasma tyrosine levels will increase to 400–700  $\mu$ M. Due to risk of corneal tyrosine crystallization with levels exceeding 500  $\mu$ M, similar to the process occurring in oculocutaneous tyrosinemia (see below), any treatment with NTBC must be paired with dietary restriction of phenylalanine and tyrosine to keep tyrosine levels below 400  $\mu$ M. While published data regarding long-term efficacy of combined NTBC and low phenylalanine/tyrosine diet are sparse, these studies indicate that the best predictors of symptom-free outcome are initiation of treatment prior to 6 months of age and normalization of AFP levels (74,75).

When NTBC and dietary phenylalanine and tyrosine restriction are instituted in children identified symptomatically, they demonstrate dramatic reversal of symptoms, improved hepatic and renal function, and prevention of neurologic crises. However, the risk of developing hepatic cirrhosis, nodules, and carcinoma is not completely eliminated, especially in children diagnosed and treated after their second birthday (75). Prior to the development of NTBC, dietary therapy alone was used, which improved the clinical symptoms but did not prevent progression of the disease; liver and renal involvement would worsen, with eventual hepatic failure or the development of hepatic carcinomas, or both.

Patients treated with NTBC and diet need to be monitored with frequent phenylalanine/tyrosine levels to ensure adequate natural protein intake while preventing corneal tyrosine precipitation. Periodic measurements of liver transaminases, prothrombin and partial thromboplastin times, urinary succinylacetone, AFP levels, and liver imaging are necessary to monitor treatment efficacy, liver synthetic function, and development of hepatocellular carcinoma. If the AFP does not decrease after initiating therapy or increases at a later time, the patient needs to be evaluated for hepatic malignancy and transplantation (76).

For children who develop hepatic failure or hepatocellular carcinoma, orthotopic liver transplantation is

the remaining treatment modality, as NTBC alone is not effective in these circumstances (76). Although liver transplantation in children is not benign, it has offered improved survival and reduced morbidity to patients undergoing the procedure. Donor selection, operative technique, postoperative care, and immunosuppressive regimens have been developed, which have significantly reduced the rate of perioperative complications. Liver transplantation for children with tyrosinemia type 1 reverses the symptoms associated with acute hepatic failure, neurologic decompensations, and renal dysfunction. However, ongoing excretion of succinylacetone post-transplant has been reported (77).

A great deal has been learned about the pathogenesis and potential therapies for tyrosinemia from the study of knockout mice. Succinylacetone can react with amino acid side chains, particularly lysine, to form stable adducts that interfere with protein function. In addition to  $\delta$ -ALA dehydratase, succinylacetone inhibits DNA ligase I, which may explain the chromosomal breakage observed in hepatocytes of patients with tyrosinemia and contribute to carcinogenesis (78). Fumarylacetoacetate appears to induce apoptosis (79). Hepatocytes expressing FAH can completely repopulate the liver of the FAH-deficient mouse when NTBC is withheld because of the selective growth advantage of normal cells (80). Perhaps of import for humans, even when treatment with NTBC was started prenatally, the mice still developed hepatocellular carcinoma (81).

### 92.2.3 Oculocutaneous Tyrosinemia

Oculocutaneous tyrosinemia (OMIM 276600) results from a deficiency of TAT, which catalyzes the transamination of tyrosine to pHPPA. It has also been called tyrosinemia II, TAT deficiency, and keratosis palmoplantaris with corneal dystrophy and by the eponym of the Richner-Hanhart syndrome.

A typical patient with this disorder presents after the first year of life with eye irritation or skin lesions. Ocular symptoms typically include photophobia, conjunctival redness, and pain. These changes occur due to the deposition of tyrosine crystals within the corneal stroma with subsequent erosions and scarring.

The skin lesions consist of blisters on the fingertips and the thenar eminences. These progress to form painful hyperkeratotic plaques that involve the entire surface of the palms and the soles of the feet. The major microscopic changes found on skin biopsies are not diagnostic of the condition but show hyperkeratosis, parakeratosis, and acanthosis.

Mental retardation has been a common theme in many reported patients, particularly in those from a single, isolated rural area. These early patients were reported to have approximately 40% incidence of developmental delay. It is unclear whether the neurologic symptoms are a true manifestation of oculocutaneous tyrosinemia or

whether the mental retardation has been the symptom that caused a biochemical derangement to be sought, causing an ascertainment bias.

Deficiency of TAT causes accumulation of tyrosine within the biologic fluids. Plasma tyrosine values have ranged from 400 to 3000  $\mu$ M when first diagnosed. Other amino acids in the blood are reportedly normal, with the possible exception of a mild elevation in phenylalanine levels. Urinalysis shows a marked increase in tyrosine excretion and, paradoxically, an increased excretion of pHPPA, *p*-hydroxyphenyllactate, and *p*-hydroxyphenylacetate. These latter compounds apparently arise by the ability of mitochondrial aspartate aminotransferase to convert tyrosine to pHPPA.

TAT is present solely in the cytoplasm of hepatocytes and is the rate-limiting enzyme of tyrosine degradation; its transcription is regulated by hormone treatment and diet. A number of mutations in TAT have been identified in oculocutaneous tyrosinemia (82).

The treatment of oculocutaneous tyrosinemia is aimed at reducing plasma tyrosine levels. If tyrosine levels can be reduced to approximately 500  $\mu$ M, symptoms should resolve. It has been suggested, from the evaluation of patients with oculocutaneous tyrosinemia, that a plasma level of 600  $\mu$ M is a reasonable goal (83); however, data from children treated for tyrosinemia I with NTBC would indicate that a level of less than 500  $\mu$ M offers greater protection from corneal symptoms. Commercial formulas that are low in phenylalanine and tyrosine are available. These formulas can be used to replace milk and other major protein sources in the diet and, when coupled with a vegetarian-style diet, are effective in reducing plasma tyrosine and in reversing ocular and skin symptoms.

### 92.2.4 Alkaptonuria

Alkaptonuria is a rare autosomal recessive disorder of homogentisic acid metabolism caused by a deficiency of homogentisic acid oxidase (84–86). Alkaptonuria is more frequent in Slovakia and the Dominican Republic (87). A number of mutations have been identified (88,89). Although rare, studies on alkaptonuria are of great historical significance in the field of metabolism and genetics. Sir Archibald Garrod developed his ideas of inherited metabolic disorders from his studies of alkaptonuria.

The presenting symptom of alkaptonuria is that of urine that turns dark brown or black on prolonged exposure to oxygen or rapidly after the addition of an alkali. Alkaptonuria is a genetic cause of ochronosis, in which a slate blue or gray pigmentation of the sclera of the eyes and the cartilage of the ears becomes evident by the second or third decade of life and cartilage becomes progressively black. Although the disorder is often diagnosed in childhood, it may not be detected until adulthood, when arthritis appears or dark pigmentation of the cartilage is

noted. In addition to arthritis, with progressing age, individuals with alkaptonuria frequently develop degenerative disk disease and back pain, aortic and mitral valve stenosis, coronary artery calcifications, nephrolithiasis, and tendons that tear easily (86).

Oxidation of homogentisic acid leads to formation of a pigmented polymer, the structure of which has not been elucidated. The pigment is preferentially deposited in the cartilage, leading to arthritis, mostly of the hip and knee, occurring earlier in men. There is degeneration and calcification of the intervertebral disks of the lumbar spine, followed by fusion. The radiographic appearance is nearly pathognomonic. The mechanism of damage to the cartilage is not known. Ochronosis also occurs in the cardiac valves, and there is a high percentage of alkaptonuric patients older than 50 years with aortic valve thickening, stenosis, and insufficiency (90). There is currently no cure for alkaptonuria. Some have advocated the consumption of up to 1 g of ascorbate per day to diminish pigment deposition in the cartilage by increasing renal excretion of homogentisic acid or its oxidized metabolite (87,91). However, this approach seems to have little clinical effect (86). NTBC (nitisinone), an inhibitor of pHPPA dioxygenase, decreases the production of homogentisic acid and has been proposed as a potential therapy but would probably require reduction of tyrosine intake in the diet similar to tyrosinemia type I (86).

### 92.2.5 Other Disorders of Tyrosine Metabolism

A number of other abnormalities of tyrosine metabolism have been reported but those are very rare, fail to cause disease in many homozygous individuals, or cause disease without obvious biochemical abnormalities. These disorders are noted here (for a more extensive discussion, see Reference (64)).

Deficiency of 4-hydroxyphenylpyruvate dioxygenase is associated with the condition known as tyrosinemia type III. Tyrosine levels are high in the blood, and 4-hydroxyphenylpyruvate, 4-hydroxyphenyllactate, and 4-hydroxyphenylacetate excretion is increased in the urine. It is unclear whether this biochemical abnormality results in disease or is associated with mental retardation (92).

Hawkinsinuria, named from the first family in whom it was described, is a disorder that leads to the accumulation of an intermediate in the tyrosine catabolic pathway. The exact enzymatic and genetic mechanism is not understood but is thought to be a defect in a partial reaction of the pHPPA dioxygenase reaction. The symptoms vary from none to severe acidosis and failure to thrive and may relate to the protein content of the diet. The condition responds to dietary protein limitation despite the absence of elevations in plasma tyrosine levels. It appears to become less threatening as the patient matures. The

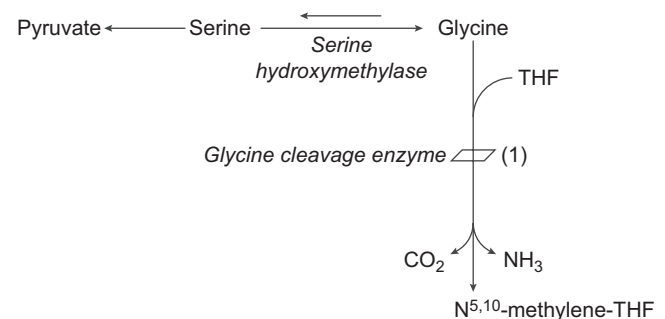
mode of inheritance has not been identified, but it may be autosomal dominant (93).

## 92.3 DISORDERS OF GLYCINE METABOLISM

Glycine is the smallest and the most ubiquitous of the naturally occurring amino acids. It is not an essential amino acid in either infants or adults. Its major synthetic sources are probably through the one-carbon metabolic pathway and recovery from protein catabolism. Glycine may account for 25% of the composition of many proteins and is involved in the synthesis of creatine, glutathione, heme, and porphyrins. Glycine may also be used in the formation of glycogen and synthesis of purine.

The major reaction of glycine is its reversible conversion to serine, catalyzed by serine hydroxymethyltransferase (Figure 92-3). This enzyme requires tetrahydrofolate as a cofactor; the energetics favor the formation of glycine. An alternative and major route for the conversion of glycine to serine is through the glycine cleavage reaction. In this system, glycine reacts with tetrahydrofolate and is decarboxylated and deaminated to form N<sup>5,10</sup> methylenetetrahydrofolate, which is combined with a second molecule of glycine to form serine in a reaction catalyzed by serine hydroxymethyltransferase. The glycine cleavage system takes place only in the mitochondria by a complex four-protein system that is responsible for the decarboxylation. This four-protein system is similar in structure to the pyruvate-dehydrogenase complex and contains (1) a P-protein that is pyridoxal-dependent, the glycine decarboxylase; (2) an H-protein that is a hydrogen carrier protein, utilizing lipoic acid as a cofactor; (3) a T-protein that is required for binding of tetrahydrofolate; and (4) an L-protein that is lipoamide dehydrogenase. Mutational alterations in any of these four proteins can theoretically lead to the syndrome of nonketotic hyperglycinemia; mutations in three of the four have, in fact, been described. Disorders of glycine metabolism are reviewed by Hamosh and Johnston (94).

Disorders of metabolism resulting in hyperglycinemia were originally divided into two groups: ketotic and



**FIGURE 92-3** Abbreviated pathway for some of the metabolic reactions related to glycine. The known genetic defect is due to the deficiency of the (1) glycine cleavage complex causing nonketotic hyperglycinemia.



nonketotic. The term “ketotic hyperglycinemia” was coined by Childs and colleagues (95) to describe infants with metabolic acidosis, ketonuria, and hyperglycinemia. It was subsequently determined that children with this syndrome have disorders of organic acid metabolism, with secondary perturbations in glycine metabolism. These are principally children with propionic, methylmalonic, and isovaleric acidemia. These disorders are reviewed in Chapter 91. The recognition of nonketotic hyperglycinemia occurred in a group of children who typically present with severe neurologic symptoms soon after birth; absence of acidosis and organic acidemia; and elevated concentrations of glycine in plasma, CSF, and urine.

### 92.3.1 Glycine Encephalopathy

Glycine encephalopathy (OMIM 605899), classically known as nonketotic hyperglycinemias, represents several distinct genetic abnormalities that present with similar clinical findings (reviewed in Reference (94)). The estimated frequency of glycine encephalopathy is 1:12,000 to 63,000 live births (96). The classic infant with glycine encephalopathy appears normal at birth but soon develops poor feeding, listlessness, hypotonia, lethargy, respiratory failure, and seizures. While not specific for this disorder, burst-suppression pattern is usually seen on electroencephalography. Onset of symptoms typically occurs before 1 week of age and often in the first few days of life. The neurologic progression is inexorable, and these children develop intractable seizures, hypertonicity, and failure of intellectual development. Dysphagia leads to high risk of aspiration pneumonia. Most of these infants die of neurologic complications at younger than 1 month of age or, if they do survive, from infections before 2 years of age. Few live beyond this age and those who do demonstrate little to no cognitive development, require enteral feeding and have extremely refractory seizure disorders. Men may fare slightly better than women (97).

Rare cases of neonatal “transient nonketotic hyperglycinemia” have been described in which the intellectual outcome can be normal (98–100). There is currently no way to distinguish these rare babies from the much more common cases with an extremely poor outcome, making decisions about initiation or withdrawal of mechanical ventilation in the newborn period difficult (96).

Milder forms of glycine encephalopathy do exist. Some young children may present with mental retardation with slowly progressive neurologic features and seizures. Some families have had children who have a clinical course most similar to a neurodegenerative disease, with its onset between 6 months and 1 year of age. Still older persons have been described who present in adolescence with weakness and spasticity and whose condition may be confused with Friedreich’s ataxia or even Charcot-Marie-Tooth disease (101). However,

mutations in the glycine cleavage complex have not yet been demonstrated in these late-onset cases. The critical finding in establishing these individuals as having a form of glycine encephalopathy is dependent on documentation of elevated glycine in plasma and CSF. Plasma glycine levels are three to five times normal, whereas those in the CSF are increased 15–30 times, and the CSF to plasma glycine ratio is greater than 0.04 (102). Although elevated plasma glycine is invariant in older infants, it may sometimes be normal or near-normal soon after birth. Administration of valproate as an anticonvulsant may cause elevated glycine in plasma, serving to confuse the diagnosis.

The metabolic abnormality in glycine encephalopathy is a defect in the conversion of glycine to serine. The major route for this conversion is through the glycine cleavage reaction. Liver tissue from patients with the disorder is unable to cleave the C-1 of the glycine molecule to form carbon dioxide in the presence of tetrahydrofolate (103,104). Approximately 80% of cases of glycine encephalopathy are due to deficiency of glycine carboxylase (P-protein) (105,106), 10–15% of cases from aminoacyl protein (H-protein) deficiency (107,108), and the remainder of cases in the tetrahydrofolate-binding protein (T-protein) (109). No defect has yet been identified in the lipoamide dehydrogenase (L-protein).

No consistently effective treatment is available to reverse the neurologic damage that occurs in nonketotic hyperglycinemia. Low-protein diets, sodium benzoate, folic acid, and benzodiazepines have not proved effective (108). Strychnine has been given to a number of patients, with reports of improvement in muscle tone, but no improvement has been documented in their neurologic status (108). Some patients have partially responded to dextromethorphan (8–12 mg/kg/day) and high-dose sodium benzoate (500–600 mg/kg/day) (97). Dextromethorphan is a noncompetitive antagonist of the NMDA type of glutamate receptors, which may be stimulated by glycine (110).

Nonketotic hyperglycinemia is inherited as an autosomal recessive condition. Men and women are equally affected, and an increased incidence of consanguinity has been noted among parents. Genetic heterogeneity exists based on variation in clinical presentation and on known deficiencies of the P, H, and T enzymes of the glycine cleavage reaction. The glycine cleavage reaction is not detectable in normal white cells or fibroblasts, but it may be usefully studied in transformed lymphocytes (111). There is no detectable alteration in plasma glycine levels of obligate heterozygotes.

In patients with defined mutations, prenatal diagnosis may be carried out using molecular analysis of the gene from amniocytes or chorionic villi. The identification of a common p.S564L mutation in the P-protein in Finns and a p.H42R mutation in the T-protein in Israeli-Arabs makes prenatal diagnosis and heterozygote detection straightforward in these populations (112). Analysis of



Cystathionine is cleaved by  $\gamma$ -cystathionase to form cysteine and  $\alpha$ -ketobutyrate. Cystathionine is not known to function in other reactions. This enzyme also requires pyridoxal phosphate as a cofactor. Cysteine may undergo a number of enzymatic reactions. It is an important amino acid in proteins, playing a key role in folding and stabilization; as noted, it may be an essential or a conditionally essential amino acid in the neonate. It is a component of glutathione, a compound important in maintaining the redox state of the cell and in stabilizing the cell membranes. It also participates in the formation of coenzyme A and is metabolized to organic sulfate through sulfite. The final step is catalyzed by sulfite oxidase, a molybdenum-dependent enzyme.

Deficiency of  $\gamma$ -cystathionase produces cystathioninuria, an apparently benign autosomal recessive disorder. Deficiency of methionine adenosyltransferase causes hypermethioninemia, which can be inherited as an autosomal dominant or recessive trait. In the benign autosomal dominant form, mutations in one monomer of the homotetrameric enzyme interfere with function, resulting in moderate hypermethioninemia. Although most individuals are normal, patients with the autosomal recessive form due to functional null mutations have severe hypermethioninemia and may have demyelination in the brain and cognitive impairment (120).

The most common and important genetic defects of the sulfur amino acids produce an accumulation of homocysteine. Classified as the hyperhomocysteinemias, they are the result of enzymatic defects in either CBS (catabolic pathway) or the remethylation of homocysteine to methionine, encompassing disorders of folate and B<sub>12</sub> metabolism. Disorders causing elevations in urinary homocysteine are also termed homocystinuria, the most common cause of which is CBS deficiency.

### 92.4.1 CBS Deficiency

Before the recognition of homocystinuria due to CBS (OMIM 236200) deficiency as a defined entity, affected patients were confused with those with Marfan syndrome because of similarities in the clinical phenotype, including a thin habitus, scoliosis, pectus excavatum, dislocated lenses with myopia, and, occasionally, some degree of arachnodactyly. A significant number of patients have mental retardation. In 1962, and during the immediate years thereafter, homocystinuria was recognized as a discrete entity and the enzymatic defect defined (121). The separation of homocystinuria from Marfan syndrome represented one of the earliest and best clinical examples of the concept of phenocopies (122).

The classic description of the patient with homocystinuria is the young teenager who is tall and slender, and has scoliosis, long extremities, a high arched palate, light-colored dry hair, pale skin with livedo reticularis, and bilateral dislocation of lenses with myopia. Radiographs of the skeletal system demonstrate osteoporosis. A mild

to moderate degree of mental retardation may be present. This description undoubtedly represents the extreme clinical phenotype, because most patients with homocystinuria do not fit this classic description. It is striking that the disorder was first described in a more severely mentally retarded population, in which the classic phenotype was not noted.

Since the observation by Hooft and colleagues (123) that some patients with CBS deficiency may respond to pyridoxine (vitamin B<sub>6</sub>) therapy, two major subgroups have traditionally been defined: pyridoxine-responsive homocystinuria and pyridoxine-nonresponsive homocystinuria. The clinical manifestations of the two groups are different, with those who are pyridoxine-responsive tending to have a later onset, a milder phenotype and a better prognosis. Somewhat fewer than 50% of all patients with CBS deficiency respond to 25–500 mg of pyridoxine per day, with significant reduction in their plasma homocysteine concentration (117).

Ninety percent of patients with CBS deficiency have dislocated lenses; in these patients, 90% of lenses are dislocated downward in contrast to patients with Marfan syndrome, in whom the lenses typically dislocate upward. This may be observed in patients as young as 2 years. Half of the pyridoxine-nonresponsive patients have documented lens abnormalities by 6 years of age and half of the responsive patients have dislocation by 10 years of age. Myopia always accompanies lens subluxation, and occasionally, secondary glaucoma occurs. Retinal degeneration has been reported. Lens dislocation occurs because of a disruption of the zonular fibrils, which are predominantly composed of fibrillin-1 (mutated in Marfan syndrome), a cysteine-rich protein. Homocysteine may bind the cysteine residues in fibrillin-1, disturbing proper microfibril formation.

Mental retardation is a frequent observation in affected patients. The median IQ for pyridoxine-responsive patients has been estimated at 78, with 22% having IQs greater than 90, as compared with pyridoxine-nonresponsive patients, with a mean of 56 and only 4% with IQs greater than 90. Abnormal electroencephalograms (EEGs) and seizures are occasionally noted, and there is an impression of significant psychiatric disturbances in approximately one-half of the patients. Personality and behavior disorders tend to predominate in the pyridoxine-nonresponsive group. Rare cases have developed leukodystrophy with spasticity or an extrapyramidal movement disorder. When treatment is initiated early (such as in patients detected by newborn screening) in pyridoxine-responsive patients, the intellectual outcome is normal or near-normal (117).

Pancreatitis in CBS deficiency has been reported (124). In addition, spontaneous pneumothorax (125), which not coincidentally is a complication of Marfan syndrome, also occur in the disorder. Homocysteine inhibits tyrosinase, potentially explaining the hypopigmentation that patients frequently have (126).

Osteoporosis can usually be documented by age 20 in at least 50% of patients. It has been suggested that the osteoporosis may be a contributing cause in patients in whom scoliosis develops and is certainly contributory to those patients who demonstrate vertebral collapse. Homocysteine appears to interfere with collagen cross-linking (127).

The major life-threatening symptom in patients with classic homocystinuria is the propensity toward vascular thrombosis. Twenty-five percent of untreated patients would have had a thromboembolic event by the age of 20, earlier in pyridoxine-nonresponsive cases. Mudd and colleagues (117) have estimated that the risk of a significant thromboembolic event in a young adult with untreated homocystinuria is approximately 4% per year. The arterial walls show marked fibrous intimal thickening and abnormalities of the media. In vitro, homocysteine stimulates smooth muscle cell proliferation (128). In addition to the arterial vascular changes, homocysteine causes hypercoagulability. There is an increased risk of thromboembolism if there are further genetic risk factors, such as factor V Leiden, a common form of activated protein C resistance (129).

Patients may be suspected of having homocystinuria on the basis of clinical phenotype, the presence of dislocated lenses in a child, or the occurrence of a thromboembolic phenomenon in a child or young adult. Some cases are detected as a consequence of metabolic evaluation in a retarded individual. Homocystine in the urine is easily detected by an amino acid analysis. In the plasma, methionine is elevated in the range of 500–2000  $\mu\text{M}$  and homocysteine in the range of 50–200  $\mu\text{M}$ . The documentation of elevated methionine concentration in plasma is necessary for the presumptive diagnosis of CBS deficiency in the absence of a direct enzymatic assay; molecular genetic sequencing is used to confirm the disorder.

CBS deficiency must be distinguished from other defects that can cause homocystinuria, including problems in remethylation to methionine, a deficiency of  $N^5$ -methyltetrahydrofolate:homocystine methyltransferase, a deficiency of  $N^5$ , $^{10}$ -methylene tetrahydrofolate reductase, or a defect in cobalamin metabolism that results in a deficiency of methylcobalamin. These latter disorders typically have low or low-normal plasma levels of methionine. Homocystine is also seen in the urine of some patients with cystathioninuria owing to bacterial contamination and the synthesis of homocysteine from cystathionine. By contrast, some patients who have homocystinuria may be missed by urinary screening if they have been taking vitamin supplements containing pyridoxine.

Therapy is available for patients with CBS deficiency. In 25–50% of patients, homocysteine concentration in blood can be lowered to a significant degree by taking an oral dose of pyridoxine (50–500 mg/day). Lowering the plasma homocysteine concentration may offer considerable protection to most of these patients from

thromboembolic events. The clinical data suggest that patients with the lowest levels of plasma homocysteine would have the lowest incidence of thrombotic episodes. Folic acid depletion has been noted in a number of patients with CBS deficiency, and it is supplemented routinely (1000  $\mu\text{g/day}$ ) along with pyridoxine. This phenomenon may relate to the ability to recycle homocysteine to methionine, a reaction that is both vitamin  $B_{12}$ - and folate-dependent. Betaine (up to 150 mg/kg/day) promotes the remethylation of homocysteine to methionine, a far less toxic or perhaps nontoxic compound, and therefore is used to lower homocysteine levels still further.

A low-methionine diet has been used to prevent the accumulation of homocysteine. This approach is most effective when used in young infants found to have homocystinuria through newborn screening programs or early diagnosis. Patients identified later on are rarely able to assume this degree of discipline. Special low-methionine formulas are available for dietary management. These formulas are supplemented with a small amount of milk and L-cysteine to meet the methionine and L-cysteine requirements and to maintain plasma methionine levels to a near-normal range. Preliminary evidence indicates that methionine-restricted diets are beneficial to children who are non-pyridoxine-responsive.

To assist in the prevention of thromboembolism in patients who have persistent homocystinemia, dipyridamole combined with aspirin is recommended. Although short of unequivocal statistical proof, the prevailing impression is that these drugs are helpful. Because of the increased risk of thromboembolism, estrogen-containing oral contraceptives are probably best avoided. Pregnancy, or surgical procedures conducted with adequate hydration do not appear to significantly increase the risk of complications. Although there may be an increased rate of fetal loss, the offspring of homocystinuric women appear to be normal.

Deficiency of CBS is inherited as an autosomal recessive condition. The estimated incidence varies from one in 50,000 to one in 400,000, with the highest incidence in Italy and Ireland. More than 158 different mutations in CBS have been described, with most being quite rare (141; <http://cbs.lf1.cuni.cz/mutations.php>). The p.G307S substitution is most common in the Irish and results in a pyridoxine-nonresponsive phenotype, whereas the p.I278T mutation is the most common in Italians and confers pyridoxine responsiveness. These two mutations may represent up to 50% of those seen in some populations of northern European origin (130). Other recurrent mutations that predict pyridoxine responsiveness when found in the homozygous state include p.A114V, p.R266K, and p.R336H. Recurrent mutations predicting nonresponsiveness when found in the homozygous state are p.T191M and p.C165Y (116).

Most newborn screening programs for homocystinuria screen for hypermethioninemia. However, this



strategy results in most cases of pyridoxine-responsive CBS deficiency being missed (131). Homozygotes for the p.I278T mutation usually present with thromboembolic events in their third decade, and while they have elevated plasma homocysteine levels, they often have normal intelligence and absent ocular/skeletal symptoms (132). In countries like Ireland, where most cases are pyridoxine-nonresponsive and the incidence is 1:65,000, newborn screening can be quite effective (133).

Obligate heterozygotes have been documented to have less than normal activity when CBS activity has been measured in extracts of cultured fibroblasts, stimulated lymphocytes, long-term cultured lymphocytes, and liver tissue. In general, this activity has been less than 50% of the mean control specific activity observed in each tissue. It has been implied that the decrease in activity below 50% of normal is due to impaired activity of hybrid molecules, when the normal subunits of the enzyme are combined with mutant ones (dominant negative effect). Because of the rarity of the mutant gene in the population and the wide variation in normal enzymatic activity, it is difficult to reliably distinguish heterozygotes. Similarly, methionine loading followed by sensitive measurements of total homocysteine will not detect many obligate heterozygotes. Obligate heterozygotes may not have a significantly increased risk for vascular thrombosis, although a small increase in risk cannot be excluded with the available data.

Prenatal diagnosis of CBS deficiency is feasible by molecular analysis or enzymatic assay of cultured amniotic fluid or chorionic villus cells. CBS-deficient mice have been generated. The homozygotes die within 5 weeks. The heterozygotes have a twofold elevation in total homocysteine (134).

### 92.4.2 $N^{5,10}$ -methylenetetrahydrofolate Reductase Deficiency

The active cofactor necessary for the conversion of homocysteine to L-methionine is  $N^5$ -methyltetrahydrofolate. This cofactor is resynthesized from  $N^{5,10}$ -methylenetetrahydrofolate by the enzyme  $N^{5,10}$ -methylenetetrahydrofolate reductase (MTHFR). A small number of children have been reported with a deficiency of this enzyme (OMIM 236250). Patients deficient in the synthesis of  $N^{5,10}$ -methylenetetrahydrofolate accumulate homocysteine and have low or low-normal plasma methionine levels (135).

Affected patients show a spectrum of clinical symptoms. Young infants have been identified with severe neurological symptoms of hypotonia, poor feeding, failure to thrive, seizures, lack of neurocognitive development, and severe apnea. Most have died at younger than 1 year of age due to central respiratory failure. Several infants have responded well to high-protein intake, with or without supplementation with methyl-donor medications such as folinic acid and betaine. Older children

have been identified with MTHFR deficiency after presenting to medical attention for mental retardation, acute psychosis, muscle weakness, ataxia, marfanoid habitus, spastic paraparesis, or thromboembolic events (136). Adults have presented with gait disturbance. Some have shown improvement in symptoms when supplemented with pharmacologic doses of folate.

The disorder is inherited as an autosomal recessive condition. There is evidence that obligate heterozygotes can be distinguished by measurement of the enzymatic activity. Prenatal diagnosis has been accomplished. A number of mutations have been identified in these cases, and there is reasonable correlation between residual enzyme activity and clinical severity (135). However, there can be substantial variability within families, with one asymptomatic adult sibling of an affected patient having been reported (137).

### 92.4.3 Thermolabile $N^{5,10}$ -methylenetetrahydrofolate Reductase and Mild Homocysteinemia

Very little homocysteine exists in an unbound form in the blood. Between 70 and 80% of homocysteine is protein-bound, whereas the rest exists as homocysteine (homocysteine homodimer) and mixed disulfides. The most practical measurement to make is that of total homocysteine, requiring prior reduction of disulfide bonds. The 95th percentile in normal individuals for plasma total homocysteine is approximately 15  $\mu\text{mol/L}$ . Women have a lower homocysteine level than men, and the level increases with age, decreased vitamin intake, and decreased glomerular filtration rate (138).

Epidemiologic studies have shown that elevated homocysteine is associated with an increase in atherosclerosis, coronary artery disease, cerebrovascular disease, and venous thromboembolism. The exact mechanisms are not understood, but the available evidence suggests increased endothelial damage, oxidation of lipids, and proliferation of smooth muscle cells (139). It has been estimated that a 5  $\mu\text{M/L}$  increase in homocysteine confers the same risk for coronary artery disease as a 20 mg/dL increase in cholesterol—a 1.5-fold increase in the risk for coronary artery disease and cerebrovascular disease (140). Just as in CBS deficiency, the risk of thromboembolism due to mild homocystinuria is increased with other risk factors, such as activated protein C resistance due to the factor V Leiden mutation (141).

On a population basis, there is an inverse relation between folate intake and total homocysteine. There is lesser relationship between homocysteine and vitamin B<sub>6</sub> or B<sub>12</sub> intake. Folate and vitamin B<sub>12</sub> lower fasting homocysteine levels, whereas vitamin B<sub>6</sub> lowers the homocysteine level after a methionine load. Elevated fasting and post-methionine load homocysteine levels are independently associated with vascular disease (142);

thus, either measurement alone is insufficient to evaluate for hyperhomocysteinemia.

Most folate-responsive mild homocysteinemia is due to homozygosity for a common C677T polymorphism in methylene tetrahydrofolate reductase that produces a thermolabile enzyme (tMTHFR). Approximately 10% of European Caucasians are homozygous for tMTHFR. The enzyme tMTHFR has been epidemiologically associated with an increased risk for coronary artery and peripheral vascular disease (143–147). The only potential benefits of tMTHFR to those who carry it is that it may reduce the risk of acute lymphocytic leukemia (148) and colorectal cancer (149).

Maternal folate deficiency is clearly associated with fetal neural tube and cardiac defects, but the role of homocysteine in the pathogenesis of these birth defects is not clear (150). There is mounting evidence that elevated homocysteine may be teratogenic. Because of differing genetic backgrounds and amounts of folate intake in different studies, it is difficult to compare the often contradictory epidemiologic studies. In any case, it is doubtful that the full effects of folate supplementation on the reduction of neural tube defects can be explained solely on the basis of reduction of homocysteine levels. In chick embryos, administration of homocysteine causes neural tube and cardiac defects that are preventable by adding folate (151). In humans, homozygosity for tMTHFR in the fetus is associated with an increased risk for neural tube defects when the maternal folate intake is low (152). Increased maternal homocysteine may also confer an increased risk for congenital heart disease (153) and placental abruption (10,154). The tMTHFR homozygosity may be associated with an increased risk of cleft palate (155) but not cleft lip +/- cleft palate (156).

Regardless of the mechanism of action in the prevention of birth defects or the exact risk for vascular disease associated with mild homocysteinemia, consumption of adequate folate appears prudent for all ages. The exact daily requirement of folate to prevent homocysteinemia is not known, but 0.4 mg/day has been adequate in most studies. This dose is too small to exacerbate the neurologic symptoms of vitamin B<sub>12</sub> deficiency, which is the major fear impeding full folate supplementation of food in the United States (157).

#### 92.4.4 Homocystinuria due to a Deficiency of Methylcobalamin

Vitamin B<sub>12</sub> is an essential vitamin in humans. A complex series of transport and conversion reactions are required to produce biologically active forms. In the small intestine, ingested hydroxycobalamin is bound to intrinsic factor and transported into the enterocyte. It is released into the portal circulation bound to transcobalamin II. This complex interacts with a specific receptor on the cell surface and is endocytosed. The endocytic vesicles fuse with the lysosome, and the binding proteins

are hydrolyzed. Hydroxycobalamin undergoes mediated transport from the lysosome into the cytosol. In the cytosol, it is either converted to methylcobalamin or transported into the mitochondria, where it is reduced and adenylated to form adenosylcobalamin.

Methylcobalamin is the active cofactor for the conversion of homocysteine to L-methionine by the enzyme N<sup>5</sup>-methyltetrahydrofolate:homocysteine methyltransferase, also known as methionine synthase. A deficiency of methylcobalamin, by any number of mechanisms, can result in homocystinuria, homocystinemia, hypomethioninemia, and significant clinical symptoms. Adenosylcobalamin is an essential cofactor for methylmalonyl-CoA mutase, the enzyme deficient in typical methylmalonic acidemia (see Chapter 97). Isolated deficiency of adenosylcobalamin synthesis produces methylmalonic acidemia. Combined deficiencies of both methylcobalamin and adenosylcobalamin metabolism produce disorders, with elevations in homocysteine and methylmalonic acid, although the biochemical abnormalities are less severe than those in CBS or methylmalonyl-CoA mutase deficiency.

Precellular defects, such as intestinal malabsorption of cobalamin and severe transcobalamin II deficiency, are clinically milder than those of intracellular cobalamin metabolism. Affected individuals usually have megaloblastic anemia, developmental delay, or failure to thrive. Defects in the intracellular cobalamin pathway are designated by their complementation group (Cbl A through G) and are inherited as autosomal recessive traits. Cbl A and B cause isolated deficiency of adenosylcobalamin synthesis and consequently methylmalonic acidemia without hyperhomocysteinemia (see Chapter 97).

The Cbl G defect (OMIM 250940) is due to a deficiency of methionine synthase, which uses N<sup>5</sup>-methyltetrahydrofolate as a methyl donor to create methionine from homocysteine (158). For methionine synthase to remain active, its cobalamin core must remain in the reduced cob(I)alamin state. However, at physiologic conditions, the cobalt core will spontaneously oxidize to the cob(II)alamin state. To regenerate the active enzyme, the methionine synthase reductase enzyme utilizes S-adenosylmethionine to reduce methionine synthase back to the +1 oxidation state and remethylate the enzyme. A deficiency of methionine synthase reductase results in the Cbl E defect (OMIM 236270) (159). Clinically, both disorders typically present in the same way, with developmental delay, hypotonia, ataxia, cerebral atrophy, seizures, thromboembolic events, nystagmus, or blindness in the first several years of life. One patient with Cbl G defect presented in adulthood and was initially misdiagnosed as a case of multiple sclerosis (160). Biochemically, patients with Cbl E and G defects have megaloblastic anemia, elevated plasma total homocysteine and hypomethioninemia without methylmalonic aciduria. Therapy with intramuscular hydroxocobalamin (minimum 1000 µg/day) and oral folic acid (1000 µg/day), betaine (up to 250 mg/kg/day), and L-methionine

is able to correct anemia, reduce plasma homocysteine levels and normalize plasma methionine, but often times, the patients remain developmentally delayed. Early diagnosis and initiation of treatment, even prenatally, may help prevent irreversible neurologic damage (119,161).

Patients with Cbl C (OMIM 277400), Cbl D (OMIM 277410), and Cbl F (OMIM 277380) defects are unable to convert hydroxycobalamin to either methylcobalamin or adenosylcobalamin, leading to combined homocysteinemia and methylmalonic acidemia. Cbl C defect is the most common inborn error of cobalamin metabolism. Most patients are now identified by newborn screening, typically with elevated C3-acylcarnitine and C3:C2 ratio. Confirmatory testing will show elevated plasma total homocysteine, methylmalonic acid, and propionylcarnitine levels; normal vitamin B<sub>12</sub> levels; and hypomethioninemia. Plasma-free carnitine levels may also be depleted. It is important to note that there are Cbl C cases, even severe ones, that have not been detected by newborn screening, so the disorder must be suspected in any neonate with lethargy, poor feeding, or failure to thrive. Older children will present symptomatically with developmental delay, hypotonia, or seizure disorder; nystagmus, reduced visual acuity, gait disturbance, myelopathy and psychosis have also been described as presenting symptoms (119,162). Megaloblastic anemia or pancytopenia is usually present as well. Additional findings may include congenital heart disease, cerebral atrophy, hydrocephalus, and renal failure (163). Patients may have dysmorphic features (164). Treatment with intramuscular injections of hydroxocobalamin (1000 µg/day, although doses as high as 20,000 µg/day have been utilized (165)), betaine, and folic acid can normalize levels of methionine and reduce hyperhomocysteinemia and methylmalonic acidemia. A low propiogenic amino acid diet and levocarnitine supplementation may also be used to mitigate the toxic effects of methylmalonic acid. Response to treatment is variable. Patients with milder disease typically have normalized levels of homocysteine and methylmalonic acid, and those treated from birth often have normal growth and development. Biochemical analytes in patients with severe Cbl C defect will improve following treatment, but even those identified by newborn screening and treated immediately will manifest developmental delay, nystagmus, and seizures. Those who are identified and treated later in childhood do improve but are neurologically impaired; neurologic deterioration can occur in spite of therapy (163,166).

The Cbl C defect is caused by recessive mutations in the *MMACHC* gene; the protein product appears to encode an enzyme that removes the axial cyano group from exogenous cobalamin and may be involved in its trafficking as well. Recurrent mutations observed in *MMACHC* include the “European” c.271dupA frameshift-premature truncation, whose haplotype was observed in many ethnicities; the c.331C > T “Acadian” mutation; the c.394C > T “East Indian/Middle Eastern”

nonsense mutation; and the c.328\_331delAACC frameshifting deletion, very common in Mexican and Central American countries (167).

There have been 10 patients reported with Cbl D defect. Encoded by the *MMADHC* gene, the Cbl D protein appears to be a homolog of an ATPase portion of an ABC transporter. The biochemical phenotype is variable and dependent on the site of the mutation; three patients termed “Cbl D variant 2” demonstrated isolated methylmalonic acidemia and had mutations affecting the N-terminal portion of the protein; three patients termed “Cbl D variant 1” had isolated hyperhomocysteinemia, with mutations at the C-terminal end; and four “classic” patients had elevations in both methylmalonic acid and homocysteine bore mutations, resulting in a nonfunctional protein. Based on these observations, the putative function of the Cbl D N-terminal domain is to assist in adenosylcobalamin synthesis and that of the C-terminal domain to assist in methylcobalamin synthesis (168). Regardless of the biochemical derangements, the symptoms of Cbl D deficiency are significant: seizures, developmental delay, and encephalopathy were reported from all three variants; visual disturbances and megaloblastic anemia were observed in the classic and variant 1 forms. One patient was described as having “poor social skills and behavioral problems,” while another presented with acute psychosis. The classic form and variant 1 have been treated with intramuscular hydroxycobalamin, betaine, aspirin, and folic acid, with biochemical improvement, although one neonate died following a thrombotic event. Hydroxocobalamin and levocarnitine have been used for variant 2.

The Cbl F defect arises from deficient efflux of cobalamin out of the lysosome following hydrolysis from transcobalamin II. Multipoint linkage analysis of 12 patients with Cbl F defect led to the identification of *LMBD1*, a lysosomal transmembrane protein, as the protein deficient in Cbl F. Common presenting symptoms of patients with Cbl F defect were prenatal-onset growth retardation with postnatal failure to thrive, stomatitis, feeding difficulties, developmental delay, and cardiac abnormalities. Three of the 12 patients were identified from newborn screening with elevated propionylcarnitine in newborn blood spots. Following treatment with intramuscular hydroxocobalamin, three patients were completely asymptomatic. Only one of these three patients was identified from newborn screening. Death in two patients following cardiac surgery, and in one hydroxycobalamin-treated patient from sudden unknown causes, was observed. Ongoing issues faced by survivors were short stature, developmental delay, and poor attention span (169).

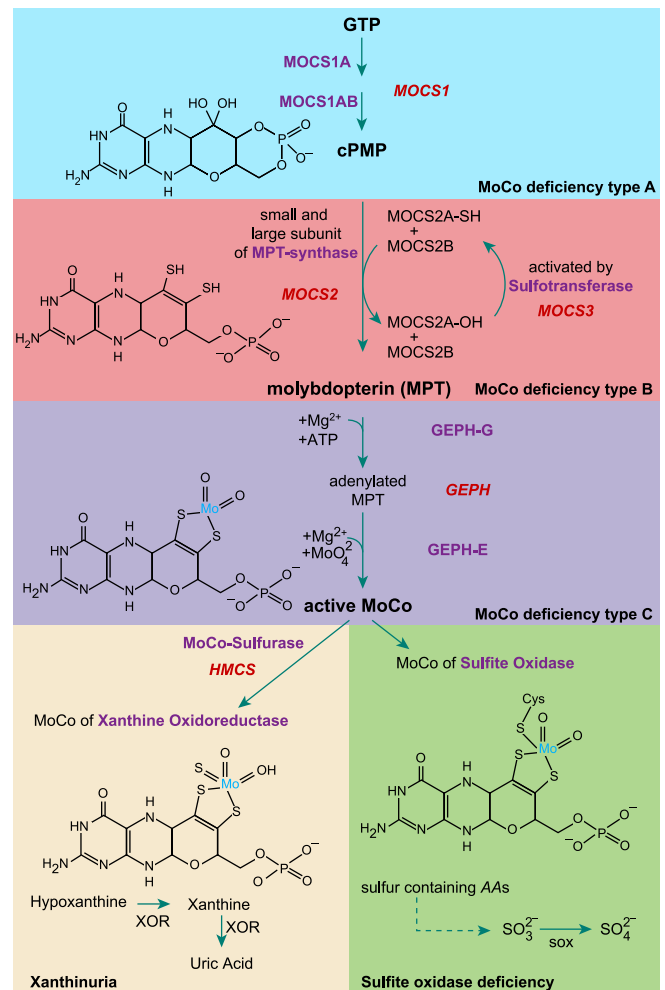
#### 92.4.5 Sulfite Oxidase Deficiency

Sulfite oxidase (see Figure 92-4) deficiency is a rare neurodegenerative disorder, typically presenting during

the neonatal period (170,171). Infants initially may have only feeding difficulties, but they rapidly develop abnormal tone, severe irritability, and refractory generalized and myoclonic seizures. Ectopia lentis, similar to that seen in patients with homocystinuria, may be present at birth but is often evident only after several months. The brain rapidly becomes atrophic, secondary to spongy degeneration and gliosis. Most of these children succumb in early infancy, but a few have survived several years, with severe neurologic impairment. One milder case has been reported, with an initial presentation at 17 months, owing to an incomplete enzymatic defect (172). The urine of the affected individuals contains increased quantities of sulfite, thiosulfate, and *S*-sulfocysteine, whereas the sulfate content is markedly diminished. Sulfite oxidase deficiency is an autosomal recessive disorder with genetic heterogeneity. Although a handful of cases of isolated sulfite oxidase deficiency (OMIM 272300) due to a defect in the apoenzyme have been identified, the majority of cases have been attributed to a deficiency of a novel molybdenum-containing pterin cofactor (OMIM 252150). The molybdenum cofactor is also required for the activities of xanthine oxidase and aldehyde oxidase. Molybdenum cofactor deficiency itself displays locus heterogeneity, with recessive mutations described in *MOCS1*, *MOCS2*, *MOCS3*, or *GEPH* genes, resulting in the disorder (173,174) (Figure 92-5). Prenatal diagnosis can be performed with sequencing of known mutations in the causative gene or with biochemical assays from chorionic villi (175).

Clinically, patients with molybdenum cofactor deficiency are identical to those with isolated sulfite oxidase deficiency, except for hypouricemia and xanthinuria that may result in renal calculi. In addition, urothione, a precursor of the pterin moiety, is undetectable in the urine. The pathogenesis of sulfite oxidase deficiency is unclear; a deficiency of sulfate could disturb the synthesis of sulfated glycosaminoglycans. However, sulfite, normally present only in small quantities, is a highly reactive compound. Modification of the sulfhydryl group of cysteine residues, for example, could prevent normal protein cross-linking, similar to a hypothesized effect of homocysteine.

Treatment in one patient with *MOCS1* deficiency utilizing daily intravenous infusions of cyclic pyranopterin monophosphate (cPMP, 80–160 g/kg) to bypass the enzymatic block resulted in near-normalization of markers related to sulfite oxidase (sulfite, *S*-sulfocysteine, and thiosulfate) and xanthine oxidase deficiency (xanthine and uric acid) within a week and a marked reduction of seizures within 2 weeks. At the last reported follow-up, the patient was free of seizures and thriving but developmentally delayed, with spastic quadriplegia and hyper-tonicity. Her brain magnetic resonance imaging (MRI) demonstrated cerebral atrophy and had the characteristic cystic appearance. Because the patient demonstrated these abnormalities prior to treatment, the authors suggested that earlier recognition by newborn screening and initiation of cPMP would lead to improved developmental



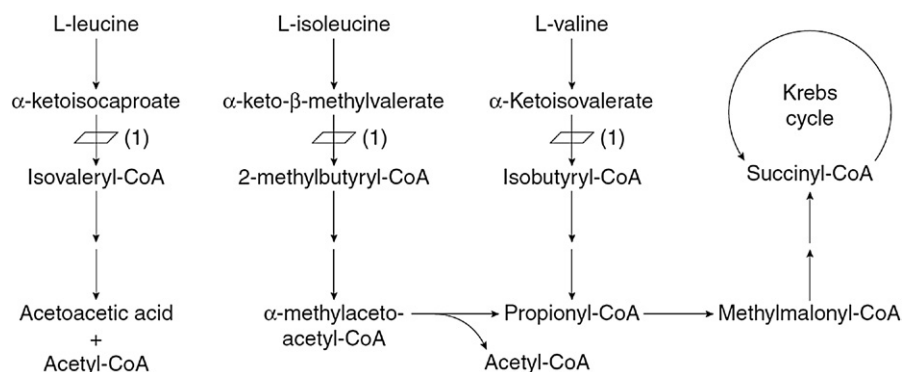
**FIGURE 92-5** Diagram for the synthesis of the molybdenum cofactor from GTP. Genetic defects in *MOCS1* result in MoCD type A, which mechanistically is the only subtype amenable to cPMP treatment. Mutations in *MOCS2* and *MOCS3* give rise to MoCD type B, and mutations in *GEPH* are responsible for MoCD type C. (Used with permission from Reiss, J.; Hahnewald, R. Molybdenum Cofactor Deficiency: Mutations in *GPHN*, *MOCS1*, and *MOCS2*. Hum. Mutat. 2011, 32, 10–18.)

outcomes in *MOCS1*-deficient patients. Larger-scale clinical trials of cPMP are underway (176,177).

## 92.5 DISORDERS OF THE BCAAS

Leucine, isoleucine, and valine are essential amino acids in humans. Each is required for normal nutrition and growth in children and to maintain protein synthesis in adulthood. The term “branched chain” is designated to these amino acids because of their chemical structure; each contains a methyl group that branches from the main aliphatic carbon chain. As a group, they play an important regulatory role in body nitrogen, carbohydrate, and ketone body homeostasis, but their fate is to be used primarily for protein synthesis; the quantity of amino acids not used for protein synthesis undergoes a series of irreversible oxidative steps to form organic acids that eventually enter the tricarboxylic acid cycle or are used for gluconeogenesis.





**FIGURE 92-6** Abbreviated pathway for the metabolism of the BCAAs. The known enzyme defect affecting the amino acid metabolism is (1) branched-chain ketoacid dehydrogenase, causing MSUD.

Two major groups of clinical disorders are associated with deficiencies in the steps of BCAA metabolism. One group of disorders leads to the accumulation of one or more amino acids and includes, primarily, maple syrup urine disease (MSUD) (reviewed in References (178,179)). Disorders more distal in the catabolic pathway are associated with the accumulation of organic acids and are termed “the organic acidemias” (see Chapter 97). The most common disorders of organic acid metabolism include (1) isovaleric acidemia, (2) methylmalonic acidemia, and (3) propionic acidemia. A significant number of other organic acidemias exist within this catabolic pathway; this group is reviewed in Chapter 97.

### 92.5.1 Maple Syrup Urine Disease

The steps in the metabolism of leucine, isoleucine, and valine are similar (Figure 92-6). There is an initial cytosolic transamination reaction, with  $\alpha$ -ketoglutarate serving as the amino group acceptor, forming, respectively, 2-oxoisocaproic acid, 2-oxo-3-methylvaleric acid, and 2-oxoisovaleric acid. The three 2-oxoacids then enter the mitochondrion via a common transporter.

The second reaction is an oxidative decarboxylation of the branched-chain  $\alpha$ -ketoacids. It is likely from biochemical data and from the *in vitro* study of human mutations that a single branched-chain dehydrogenase is used by the branched-chain 2-oxoacids for the reaction. Branched-chain ketoacid dehydrogenase is a large multienzyme complex similar in both structure and reaction mechanism to the pyruvate and  $\alpha$ -ketoglutarate dehydrogenases. It is located on the outer face of the inner mitochondrial membrane. It consists of four separate proteins:  $E_1\alpha$  and  $E_1\beta$ , which form a decarboxylase; a transacylase ( $E_2$ ); and a lipoamide oxidoreductase ( $E_3$ ). The  $E_3$  subunit of this complex is the same protein that forms a part of the pyruvate and  $\alpha$ -ketoglutarate dehydrogenase complexes.

The decarboxylase component requires, and is stabilized by, thiamine. Maximum activity of the entire

complex is dependent on lipoic acid, flavin adenine dinucleotide, nicotinamide adenine dinucleotide (NAD), and coenzyme A. The decarboxylase is carefully regulated by the opposing actions of a specific kinase and phosphatase. The kinase phosphorylates the  $E_1\alpha$ -subunit, inactivating the complex, and is itself inhibited by the precursors of the reaction (branched-chain  $\alpha$ -ketoacids); it is reversed by the action of the phosphatase, which is inhibited by the products of decarboxylation, as well as by a specific inhibitor protein. The complexity of the enzyme system allows at least seven separate proteins from different genetic loci to affect the integrity of the entire complex. The potential exists for many mutational events to affect the activity of this reaction.

A genetic deficiency of the activity of the dehydrogenase complex prevents further oxidation of the  $\alpha$ -ketoacids formed from their respective amino acids. This deficiency is responsible for the clinical syndrome of MSUD (OMIM 248600), one of the earliest described and best known of inborn errors of amino acid metabolism (180). Affected persons accumulate 2-oxoisocaproic acid, 2-oxo-3-methylvaleric acid, and 2-oxoisovaleric acid. These  $\alpha$ -ketoacids may be reversibly reaminated by transamination to again form their respective BCAAs. Thus, patients with a dehydrogenase deficiency accumulate the branched-chain  $\alpha$ -ketoacids along with the amino acids leucine, isoleucine, allo-isoleucine, and valine.

There is a continuum of clinical heterogeneity for MSUD, varying from the severe classic form to rather mild forms of the illnesses. The degree of severity would be related to the residual enzymatic activity of the dehydrogenase complex that exists under physiologic conditions for the substrates. Infants affected with the classic form of MSUD appear normal at birth but begin to have symptoms that occur within the first few days or weeks of life, possibly prior to notification of abnormal newborn screening results. These symptoms are associated with severe acidosis from buildup of 2-oxoacids; as valine and isoleucine are carbon sources for gluconeogenesis, hypoglycemia occurs because the enzymatic block compromises generation of gluconeogenic precursors.

Symptomatic infants soon demonstrate neurologic deterioration manifesting as flaccidity, a high-pitched cry, hypertonicity, and opisthotonic posturing. Cerebral edema is often clinically apparent and may be demonstrable, along with abnormal T2-weighted enhancement of the basal ganglia, on imaging studies during an acute episode (181). The odor of maple syrup can usually be detected in the urine, scalp, cerumen, and skin of the affected infants. Many such infants fail to survive the newborn period, and those who do survive usually demonstrate residual neurologic damage. Those in whom the onset is more indolent may have symptoms of poor feeding, vomiting, lethargy, ataxia, coma, and seizures. The prognosis for these individuals is improved if treated appropriately.

In addition to the classic form of MSUD, there are more mildly affected cases. Children with an intermediate form have ataxia, failure to thrive, and retarded development. Those with the intermittent form may present, even in adulthood, with episodes of ataxia and ketoacidosis, often brought on by intercurrent infections or excessive protein intake. A few patients have been identified with a thiamine-responsive variant, demonstrating improvement in the clinical course and increased (but still subnormal) tolerance for protein, when treated with pharmacologic doses of thiamine. Individuals ingesting fenugreek as a tea or spice may emit a maple syrup-like odor, causing great alarm, but this is a harmless phenomenon (182).

The diagnosis of patients with the classic form of MSUD is not difficult. When symptomatic as infants, these patients have an abnormal urine organic acid chromatogram quite characteristic of the disorder and containing high levels of the accumulated 2-oxoacids. Quantification of amino acids in plasma documents the marked increase in leucine, isoleucine, and valine, and the presence of allo-isoleucine. Allo-isoleucine in plasma may be considered almost pathognomonic of MSUD, appearing rarely in severe ketosis. This unusual amino acid is a diastereomer of isoleucine at the  $\beta$ -carbon, formed from keto-enol tautomerization of 2-oxo-3-methylvalerate that randomizes the  $\beta$ -carbon chirality.

The milder forms of MSUD may require a higher index of suspicion to diagnose, especially because they may not be detected by newborn screening methods utilizing only measurement of BCAAs (183). Patients with “intermittent MSUD” may only be clinically ill when affected with an intercurrent viral illness or when exposed to a high-protein intake. At such times, they may have symptoms of nausea, vomiting, lethargy, ataxia, and mild acidosis. Some of these patients show a persistent elevation of BCAAs and the presence of allo-isoleucine in their plasma; others may only demonstrate these changes when ill. Most only excrete abnormal quantities of 2-oxoacids when clinically symptomatic.

Management of a child with the classic form of MSUD can be frustrating. Many of these children either have

died as infants or have significant neurologic and intellectual impairment, despite special dietary intervention. Those who survive the neonatal period typically have an IQ averaging 70. Those with less severe decompensations generally have better outcomes, as do patients with milder forms (184,185). If aggressive management is started in the first few days of life, the outcome can be much better (186). Practically speaking, this requires prenatal diagnosis or newborn screening, with a short time from testing to referral for care.

Patients with MSUD tend to do well between acute intercurrent illnesses but are vulnerable to rapid deterioration when any situation arises, causing them to become anorexic and dehydrated, or are subjected to severe endogenous protein catabolism. The onset of an acute febrile illness is hazardous; the patient who cannot sustain oral intake with high-carbohydrate fluids, has a diminished state of consciousness, or both must be brought immediately to the hospital for intravenous or nasogastric nutritional support.

Therapy for the acute presentation or for acute deterioration involves the exclusion of exogenous sources of protein and high-glucose and high-calorie intravenous solutions to minimize the hypoglycemic drive to protein catabolism. Hemodialysis, continuous venovenous hemodialysis, continuous hemofiltration, or peritoneal dialysis may be necessary to remove very high levels of leucine, which is toxic to the CNS and well reabsorbed by the kidneys (187). To reduce fatigue and work of breathing, mechanical ventilation should be initiated if severe metabolic acidosis is present. Early readdition to the diet of the 17 nonbranched-chain amino acids may hasten protein synthesis and the return of the offending amino acids toward normal levels. For those with prolonged restriction of enteral intake, special intravenous amino acid products are available. As BCAA levels approach normal ranges, they must be reintroduced into the diet or hyperalimentation solution as deficiency of BCAAs can lead to skin breakdown and trigger additional catabolic release of BCAAs (188).

Longer-term care of patients with MSUD involves the use of high-calorie diets with limiting amounts of leucine, isoleucine, and valine. Commercial products tailored for these requirements are available. The diets are necessary to maintain BCAA levels at or slightly below the lower range of normal. Thiamine (10–100 mg/day or more) may be trialed to assess the patient for responsiveness, as it helps some patients to stabilize the dehydrogenase complex and allows greater branched-chained amino acid dietary tolerance.

The milder forms of MSUD can normally be managed successfully by dietary intervention. Uniformly excellent clinical results are achieved with this group of patients. Nevertheless, these patients remain vulnerable to acute catabolic episodes; instances of death during adolescence of otherwise normal individuals have been reported (189). Affected women withstand pregnancy well if kept

under good metabolic control, and the small number of children born so far have been normal without evidence of BCAA teratogenicity.

Orthotopic liver transplantation can be carried out for severe MSUD, restoring the functional activity of the missing enzyme. Even as long-term survival is improving with refined surgical techniques, less toxic immunosuppressive regimens, and better postoperative care, the choice to proceed with this procedure must still be balanced with the risks entailed with perioperative complications and chronic rejection (190).

MSUD is inherited as an autosomal recessive condition. The classical form is rare, with an incidence no greater than one in 100,000 live births. Neonatal screening programs have detected a worldwide incidence of one in 185,000. This estimate may be low because it relies on the detection of elevations of BCAAs in the blood or urine samples. At birth, patients with MSUD may have normal levels of BCAAs. With the advent of commercially available genetic testing for all subunits of the BCAA dehydrogenase, enzymatic testing is now rarely used. Prenatal diagnosis and carrier testing are also conducted using sequencing as well because measurement of amino acids or ketoacids in amniotic fluid may not be elevated, even in unborn children with MSUD. Complementary DNA (cDNA) clones for four of the major proteins of the complex  $E_1\alpha$ ,  $E_1\beta$ ,  $E_2$ , and  $E_3$  have been isolated and localized to chromosomes 19q13.2, 6p21, 1p31, and 7q31, respectively. Western and Northern blot analyses have been used to separate the disorder into at least five molecular phenotypes (191,192). Missense, insertion, deletion, splice junction, and apparent regulatory mutations have been identified in the  $E_1\alpha$ ,  $E_1\beta$ , and  $E_2$  genes. No apparent increased incidence of MSUD has been detected in specific ethnic groups, and most of the mutations have been unique to a given family. However, an increased incidence does exist, by founder effect, in the Mennonite population of Pennsylvania; these kindreds have a tyrosine 393 to asparagine missense mutation of the  $E_1\alpha$  gene (193). Because thiamine is bound to the  $E_1\alpha$  subunit, it is surprising that the mutations identified in two thiamine-responsive patients have been in the  $E_2$  gene (194,195).

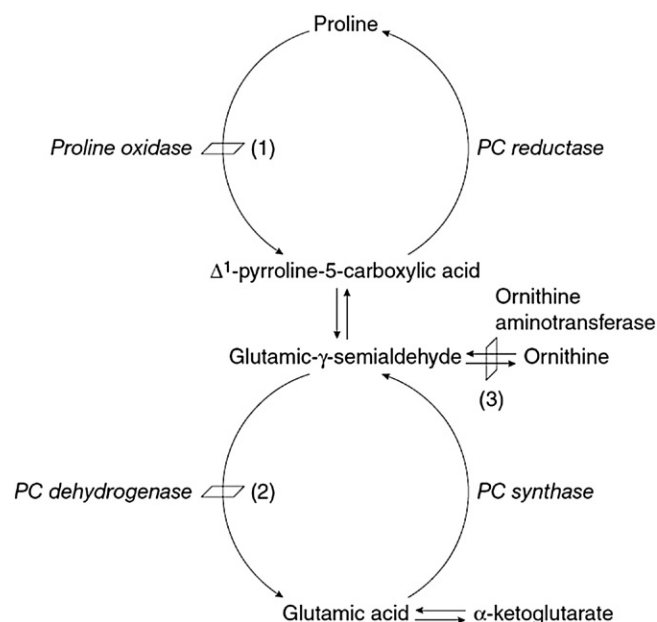
A few individuals with deficiency of the  $E_3$  component have been reported (196). These patients have a clinical presentation similar to that of MSUD and they also have elevations in 2-oxoglutarate and lactate because  $E_3$  is an integral component of the pyruvate and 2-oxoglutarate decarboxylase complexes. The prognosis for this disorder is understandably poor, but at least one patient has responded to therapy with lipoic acid (197).

## 92.6 DISORDERS OF PROLINE AND HYDROXYPROLINE

Proline, a nonessential amino acid, is synthesized from glutamic acid and ornithine through the intermediary

$\Delta^1$ -pyrroline-5-carboxylic acid (PC). The conversion of PC to proline is catalyzed by  $\Delta^1$ -pyrroline-5-carboxylic reductase (P5C). The intermediate in the reaction, PC, is in equilibrium with its noncyclic isomer glutamic- $\gamma$ -semialdehyde via a reversible intramolecular imine/cyclization reaction. The catabolism of proline is almost a reversal of its synthesis but is mediated by separate enzymes. Proline oxidase, also known as proline dehydrogenase, converts proline to PC. PC dehydrogenase further oxidizes the glutamic- $\gamma$ -semialdehyde to glutamic acid. A small amount of glutamic- $\gamma$ -semialdehyde may be converted to ornithine through ornithine aminotransferase. These reactions are summarized in Figure 92-7. Proline oxidase/proline dehydrogenase (PRODH) is a mitochondrial-bound enzyme found only in the kidney, liver, heart, and brain, whereas PC dehydrogenase is more widely distributed and is detectable in cultured skin fibroblasts and blood leukocytes.

Hydroxyproline is formed by posttranslational oxidation of proline residues primarily in collagen, via actions of the prolyl 3-hydroxylase and prolyl 4-hydroxylase complexes. Impaired proline 3-hydroxylation leads to severe, potentially neonatal lethal recessive osteogenesis imperfecta phenotypes (198,199). The catabolism of hydroxyproline is similar to that of proline. Hydroxyproline is first converted to  $\Delta^1$ -pyrroline-3-hydroxy-5-carboxylate by “hydroxy-L-proline oxidase” distinct from proline oxidase.  $\Delta^1$ -Pyrroline-3-hydroxy-5-carboxylate is then oxidized to 4-erythro-hydroxy-L-glutamate by PC dehydrogenase.



**FIGURE 92-7** Biosynthesis and degradation of proline. (1) Proline oxidase deficiency and (2) PC dehydrogenase deficiency are responsible for HPI and HPPII, respectively. (3) Ornithine aminotransferase, the enzyme deficient in gyrate atrophy of the retina, is shown here and in Figure 92-8.

Genetic deficiencies of proline oxidase and PC dehydrogenase exist and are responsible for type I hyperprolinemia (HPI) and type II hyperprolinemia (HPII), respectively. A deficiency of the hydroxy-L-proline oxidase is responsible for hyperhydroxyprolinemia. All are inherited as autosomal recessive conditions. Disorders of proline and hydroxyproline metabolism are reviewed by Phang and colleagues (200).

### 92.6.1 Type I Hyperprolinemia

HPI (OMIM 239500) is caused by deficiency of the *PRODH* enzyme. The original families with HPI were ascertained because of kidney disease, deafness, and neurologic disorders (201). Patients with HPI have increased plasma concentrations of proline (approximately 1000  $\mu$ M) and excrete proline, hydroxyproline, and glycine in the urine. Heterozygotes may have plasma proline values above the normal range. Maternal hyperprolinemia appears to have no effect on the developing fetus (202). Subsequent families with hyperprolinemia were reported as unaffected without clinical problems, and the status of *PRODH* deficiency as a truly pathogenic condition was called into question. The observation of hyperprolinemia in patients with DiGeorge/Velocardiofacial syndrome, which is typically caused by deletions of 22q11, led to the mapping of the *PRODH* gene locus to 22q11.2. Plasma proline levels were found to inversely correlate with intelligence in patients with deletions in 22q11. Schizophrenia, which is a known manifestation in adults with deletion 22q11 syndrome, was also found to correlate with hyperprolinemia (203). *PRODH* sequence variants have been implied as potential risk factors for the development of schizophrenia in conjunction with other modifying genes (204–206). Together with data indicating subtle neurologic dysfunction in *PRODH*-deficient *Drosophila* and *Mus* animal model (207,208), the evidence is emerging that HPI may not be as benign as previously believed.

### 92.6.2 Type II Hyperprolinemia

The clinical significance of HPII (OMIM 239510), caused by a deficiency of the P5C enzyme, is uncertain. The initial reports of the condition were of children with mental retardation and very high plasma proline levels (1500–3000  $\mu$ M), exceeding those of HPI patients. In addition, patients with HPII have substantially high urinary levels of proline, hydroxyproline, glycine, and PC. A study performed on a very large, consanguineous Irish traveler kindred of 312 patients with a proband having severe psychomotor retardation, generalized tonic-clonic seizures, and typical biochemical profile of HPII demonstrated no correlation between HPII and intelligence (209). In the same kindred, there was an association between HPII and seizure disorder. In addition, presumed carrier individuals were identified with

slightly elevated plasma proline without urinary abnormalities. There was no correlation between proline levels in the presumed carriers with only plasma protein elevations in the presence of seizures or psychomotor retardation. Heterozygotes do show partial activity of the dehydrogenase in cultured fibroblasts and blood leukocytes (210). Mutations in the  $\Delta^1$ -pyrroline-5-carboxylate dehydrogenase gene have been shown to be the cause of HPII (211).

### 92.6.3 Hyperhydroxyprolinemia

Hyperhydroxyprolinemia (OMIM 23700) is a rare condition. Similar to HPII, it is considered harmless. Hyperhydroxyprolinemia was originally detected in a child with mental retardation, but subsequent patients have been identified who are mentally and physically normal. Hyperhydroxyprolinemia has been detected in infants through newborn screening programs, and the infants are free of symptoms on long-term follow-up (212). A homozygous woman was found to have normal children (213).

Hyperhydroxyprolinemia is characterized by a markedly elevated concentration of hydroxyproline in the plasma (0.15–0.5 mM; normal less than 0.01 mM). Free hydroxyproline may be much increased in the urine of affected persons, but such concentrations rarely reach a level that interferes with the renal iminoglycine transport system. Thus, the urinary phenotype of hyperhydroxyprolinemia may not include an increased excretion of proline and glycine, as in hyperprolinemia. Heterozygotes do not have elevated levels of hydroxyproline in the plasma and do not excrete increased quantities of hydroxyproline in the urine but may be detectable after a hydroxyproline load. Specific enzyme assays have not been performed to confirm the deficiency. Loading studies using oral hydroxyproline have implied an enzymatic block between hydroxyproline and  $\Delta^1$ -pyrroline-3-hydroxy-5-carboxylic acid, (105,213) suggesting the existence of a separate hydroxyproline oxidase. Subsequent *in vivo* studies confirmed the existence of hydroxy-L-proline oxidase (200).

### 92.6.4 $\Delta^1$ -Pyrroline-5-Carboxylate Synthase Deficiency

$\Delta^1$ -Pyrroline-5-carboxylate synthetase (P5CS) deficiency (OMIM 612652) was reported in two Algerian siblings from a consanguineous union who presented in infancy with failure to thrive, hypotonia, joint laxity, pes planus, hip dislocation, clonic seizures, severe developmental delay, and dysmorphic features including short neck with arachnodactyly. The younger sister had neonatal generalized tonic-clonic seizures. Other clinical findings included skin hyperelasticity, bilateral cataracts, and progressive neurodegeneration beginning after 5 years of age, characterized by loss of independent ambulation, appendicular



dystonia, and peripheral axonal neuropathy. Biochemically, they demonstrated slight hyperammonemia (60–120  $\mu\text{M}$ ); low plasma ornithine, citrulline, arginine, and proline; and normal urinary amino acid levels in the fasted state. Postprandially, levels of ammonia, ornithine, citrulline, and arginine transiently improved or normalized. A pathogenic homozygous p.R84Q mutation in the *P5CS* gene was found, demonstrating a unique inborn error of metabolism in the proline synthetic pathway (214). In a consanguineous Maori family of four affected children with similar skin and joint laxity, joint dislocations, microcephaly, failure to thrive, hypotonia, severe developmental delay, and pyramidal symptoms, a pathogenic homozygous p.H784Y mutation was found in the *P5CS* gene. Biochemically, these patients did not have fasting hyperammonemia and their plasma ornithine, citrulline, and arginine levels were low-normal (215).

The biochemical abnormalities observed in the first family were theorized to be secondary to the pleomorphic effects of cellular proline deficiency arising from deficient *P5CS* activity preventing *de novo* synthesis of proline from glutamate. The dermatologic manifestations of skin hyperelasticity and joint laxity were hypothesized to be as a result of impaired synthesis of collagens, which have a high concentration of proline residues. Similarly, the cataracts were thought to be caused by impaired crystallin synthesis in lens epithelium. Deficiency of proline in the CNS was thought to be the etiology of the severe developmental delay and neurodegeneration. Finally, the biochemical observation of reduced key urea cycle intermediates was explained as a result of deficient *de novo* synthesis of ornithine due to *P5C* deficiency. Without ornithine, the urea cycle cannot progress, resulting in hyperammonemia; the provision of ornithine and arginine with meals in these patients allowed for transient enhancement of urea cycle function (214). The absence of biochemical abnormalities in the second kindred was explained by normal proline biosynthesis from glutamate in their culture fibroblasts (215).

### 92.6.5 Prolidase Deficiency

Special exopeptidases are required for the catabolism of proline-containing dipeptides. X-proline and X-hydroxyproline (where X is any other amino acid) dipeptides are hydrolyzed by prolidase, whereas proline-X and hydroxyproline-X are substrates for prolinase. No patients have yet been identified with prolinase deficiency. Two forms of prolidase are encoded by different genes with different substrate specificities.

Prolidase deficiency (OMIM 170100) is a rare autosomal recessive disorder due to diminished prolidase I activity and has been reviewed by Hechtman (216). Approximately 45 cases have been reported. The disorder demonstrates considerable inter- and intrafamilial variability with respect to clinical manifestations and age of onset.

The earliest age of onset was 3 months and the oldest 22 years, but asymptomatic, prolidase-deficient adults have been occasionally identified (217). The most prominent and consistent finding is recurrent, poorly healing, ulcerative skin lesions. Although ulcerations can occur anywhere, they are usually restricted to the lower extremities. In addition to dermatologic abnormalities, approximately 50% are mentally retarded to varying degrees. About 30% have splenomegaly and 45% have recurrent upper respiratory tract infections. Mild dysmorphic features are frequently noted in adults, including frontal bossing, a low frontal hairline, saddle nose, and mild prognathism. Persons with prolidase deficiency excrete large quantities of iminodipeptides in the urine. The diagnosis may be confirmed by enzymatic assay of erythrocytes or cultured skin fibroblasts. Heterozygotes have half-normal activity.

The pathogenesis of the cutaneous and systemic manifestations of prolidase deficiency is poorly understood. Prolidase I has been cloned and mapped to chromosome 19q12-q13.11. Other than supportive care, no consistently effective therapy has been found (218).

## 92.7 DISORDERS OF THE UREA CYCLE AND ORNITHINE

The urea cycle is a six-step pathway that results in the conversion of two molecules of ammonia and one molecule of bicarbonate to urea. It is the only major metabolic pathway for the removal of waste nitrogen formed from protein turnover or ingestion and occurs primarily, if not exclusively, in the liver. High levels of ammonia in the blood and tissue result in toxicity to the CNS. The primary effect of elevated ammonia appears to be the uptake of fluid into astrocytes, causing cerebral edema. Ammonia metabolism and the urea cycle disorders are reviewed by Brusilow and Horwich (219).

Complete deficiency of any of the enzymes 1–5 (Figure 92-8) leads to severe hyperammonemia during the neonatal period, with symptoms of lethargy, poor feeding, coma, and often death. Ammonia levels often exceed 1000  $\mu\text{M}$  (normal less than 50). Partial enzyme deficiencies may delay onset of clinical symptoms until later in life and may be associated with lower ammonia levels. The presentation could be indolent or an acute catastrophic collapse in conjunction with some infectious event or parturition. With episodes of hyperammonemia, nausea and vomiting may be the initial or the only symptoms. Many patients who have suffered severe hyperammonemic crises experience sequelae of psychomotor delay, learning disabilities, attention deficit disorder, or other neurologic abnormalities.

The enzymatic basis of hyperammonemia can usually be inferred from determination of the plasma amino acid levels, acid–base balance, urinary organic acids, and urinary orotic acid. Specific confirmation by enzymatic assays is desirable whenever possible. Therapy is both generic and disorder-specific and is detailed in the next section.

Hyperammonemia with generalized aminoacidemia and relatively normal acid-base balance is the typical biochemical presentation. When the enzyme deficit is severe, plasma citrulline levels are practically undetectable. It is distinguished in the acute presentation from ornithine transcarbamylase (OTC) deficiency by normal or reduced levels of orotic acid in the urine. Treatment is the same as that for urea cycle disorders (see later).

More than 220 mutations have been described in the *CPS1* gene, of which 90% are unique, “private” mutations; as noted above, sequencing of *CPS1* and *NAGS* is the only way to distinguish the two disorders apart since clinical presentations and biochemical profiles are very similar (228).

One or more polymorphisms in the *CPS1* gene that reduce activity modestly may diminish the arginine biosynthetic reserve and be associated with a poorer outcome in patients with conditions dependent on nitric oxide synthesis for their mitigation (229).

### 92.7.3 OTC Deficiency

OTC is a mitochondrial matrix enzyme and catalyzes the condensation of carbamoylphosphate and ornithine to form citrulline. The gene encoding OTC is at Xp21.1. Patients with OTC deficiency (OMIM 300461), like other inborn errors of metabolism, represent a range of disease severity correlating with degree of residual enzymatic function.

Children with severe OTC deficiency are usually hemizygous males who manifest symptoms in infancy, often in the first 48–72 h of life. The catabolic processes that occur in the neonatal period from stress and dehydration, compounded with the inability to detoxify ammonia liberated by protein catabolism, led to increasing accumulation of blood ammonia. Infants with progressive hyperammonemia are initially irritable and feed poorly. These symptoms may be accompanied with vomiting, which further exacerbates catabolism. Tachypnea and metabolic alkalosis occur as a result of initial stimulation of the brainstem respiratory centers. Eventually, profound hyperammonemia (often exceeding 1000  $\mu\text{M}$ ) leads to encephalopathy and seizures. Many infants who reach this degree of hyperammonemia succumb or suffer profound brain injury if resuscitated. Patients with partial deficiency of OTC may present during the neonatal period or later in infancy, with similar symptoms of altered level of consciousness and vomiting; they are often incorrectly diagnosed with viral encephalitis or aseptic meningitis if ammonia levels are not examined during episodes of lethargy. Inciting events for hyperammonemia for partial deficiencies are usually intercurrent illnesses that precipitate protein catabolism; they may also develop symptoms when solid foods are introduced into the diet, increasing their protein intake beyond what their limited OTC enzymatic activity can handle. Heterozygous girls can also develop symptoms of hyperammonemia. If a large proportion of their hepatocytes inactivate the X-chromosome bearing the wild-type OTC gene, their symptoms can be as severe as the neonatal-onset hemizygous males (230). At least one patient with arginase deficiency became hyperammonemic at the time of her menstrual period (231), and a similar phenomenon in some female OTC carriers would not be unexpected. Additionally, asymptomatic heterozygotes

are known to develop hyperammonemic crises following childbirth, a result of protein catabolism incited by fasting and rapid involution of the myometrium (232). Finally, OTC deficiency can manifest in adulthood not only with hyperammonemic crises but also with psychiatric disturbance akin to delirium or psychosis. Most surviving patients with OTC deficiency are heterozygous women or hemizygous men with milder missense mutations, allowing residual enzymatic function. Older survivors, even “asymptomatic” heterozygotes, often report spontaneous aversion to dietary protein.

Biochemically, symptomatic OTC patients develop hyperammonemia, generalized aminoaciduria, and aminoacidemia, with particular elevations in plasma glutamine, alanine, lysine, and glutamate. The citrulline concentration is reduced and may be undetectable. In contrast to CPS-I and NAGS deficiencies, excretion of orotic acid in the urine of patients with OTC deficiency is markedly elevated. This occurs because the accumulated carbamoylphosphate is shunted to pyrimidine metabolism through CPS-II (see Figure 92-8). CPS-II is a different enzyme from CPS-I; it is located in the cytoplasm and requires glutamate as a cofactor. Patients with partial OTC deficiency and intermittent symptoms may have normal orotic acid excretion and plasma citrulline concentration when they are clinically well.

OTC is found primarily in the liver and is not detectable in the cultured fibroblasts or leukocytes. In severely affected men, the enzyme is virtually absent, with less than 1% of normal activity. In symptomatic women, enzymatic activity has been shown to vary from 10 to 40% of normal.

The gene for OTC has been shown to be X-linked by pedigree data, by the demonstration of two populations of hepatocytes in female carriers, and by genetic linkage and in situ hybridization using cloned cDNA. Most patients with suspected OTC deficiency are now confirmed utilizing sequencing of the OTC gene. Historically, carrier status in females was confirmed utilizing the “allopurinol challenge test,” proposed by Brusilow and colleagues (233). The drug inhibits orotidine metabolism and allows the detection of orotic acid and orotidine in the urine of female carriers under standard conditions. OTC carriers will have higher levels than detected in noncarriers. This same test was also used to detect males with partial OTC deficiency and intermittent symptoms and biochemical abnormalities.

Direct mutation permits prenatal diagnosis in most pregnancies of women who are known carriers of OTC. Numerous point mutations have been described in patients with severe disease, as well as those who are more mildly affected. In one study, 7% of deficient males but 80% of OTC-deficient women had new mutations (234). However, even if the mother does not have the mutation in the somatic cells, recurrence can occur because of gonadal mosaicism (235).

Outcome in the acute form of this disease is uniformly poor. Those who survive the acute hyperammonemia as newborns are usually left with severe neurologic impairment and are subject to recurrent episodes of hyperammonemia, one of which is usually fatal (236). Milder manifestations are more successfully managed by the methods outlined later. Because of the inevitability of severe brain damage, patients diagnosed presymptomatically and those rescued successfully from coma eventually become candidates for preemptive orthotopic liver transplantation. Efforts to develop gene therapy, often in conjunction with hepatocyte transfer, are ongoing.

#### 92.7.4 Citrullinemia Caused by Deficiency of Argininosuccinic Acid Synthetase

The condensation of citrulline, previously transported out of the mitochondrion, and aspartate to form argininosuccinic acid (ASA) is catalyzed by ASA synthetase (see Figure 92-8). Deficiency of this enzyme (OMIM 215700) leads to citrullinemia, citrullinuria, hyperammonemia, and orotic aciduria. The disorder, once called citrullinemia, is now known as citrullinemia type I or ASA synthetase deficiency owing to the recent characterization of “citrullinemia type II,” a disorder of mitochondrial aspartate transport (covered below). The diagnosis of citrullinemia type I is not difficult because elevated amounts of citrulline are found in both plasma and urine.

The clinical picture of patients with citrullinemia type I varies in severity according to residual enzymatic activity; some patients have so benign a course that no disorder is suspected. Age of presentation varies from the neonatal period to adulthood and presenting symptoms mirror those of OTC deficiency (covered above).

ASA synthetase deficiency is inherited in an autosomal recessive manner. Molecular genetic testing is used to confirm the diagnosis and is also used for prenatal diagnosis. Prenatal diagnosis can also be accomplished by measurement of amniotic fluid citrulline, which is usually elevated in the unborn affected. More than 80 mutations have been identified (237). With the exception of the IVS6-2A>G mutation in the Japanese and the p.G390R panethnic mutation, none are common or recurrent (238). Newborn screening by tandem mass spectrometry has successfully identified patients affected by this disorder, including the cases on the mildest end of the spectrum.

#### 92.7.5 Argininosuccinic Aciduria

ASA is cleaved into two smaller molecules, arginine and fumarate, in an equilibrium reaction catalyzed by ASA lyase. The enzyme is active in the liver, brain, and kidney. In the latter organ, at least, working in concert with

ASA synthetase, it is responsible for synthesizing most of the arginine used for biosynthetic purposes. Deficiency of ASA lyase leads to marked elevations in ASA in the plasma, urine, and CSF, and in a variety of tissues. The enzyme is also measurable in the red blood cells and fibroblasts.

Symptoms associated with ASA lyase deficiency (OMIM 207900), like the other urea cycle disorders presented, range in severity. Profoundly deficient cases present in the neonatal period with lethargy, vomiting, and poor feeding, and cannot be clinically differentiated from other neonatal-onset urea cycle disorders. Without treatment, the hyperammonemia leads to cerebral edema, coma, seizures, and eventually death. The disorder can also present later in childhood with hyperammonemia following an intercurrent illness or with nonspecific developmental delay, behavioral problems, and learning disabilities. All patients who are arginine-deficient appear to have at least some degree of trichorrhexis nodosa, a condition that causes friable, nodular hair. Features of ASA lyase deficiency that distinguish it from other disorders previously presented include a propensity toward hepatic fibrosis and hypertension. In addition, a higher proportion of patients with this condition have neurobehavioral dysfunction, including attention-deficit hyperactivity disorder, seizure disorder, learning disability, and developmental delay compared to other urea cycle disorders (239). While not definitively proven, nitric oxide deficiency and accumulation of toxic, oxidative adducts of argininosuccinate like guanidinosuccinate have been advanced as explanations for the additional symptoms seen in ASA lyase deficiency (239). However, normal psychomotor development is possible with early supplementation of arginine and prevention of hyperammonemia.

Biochemically, patients with this condition have less profound elevation in plasma citrulline (100–300  $\mu$ M) compared to those with citrullinemia type I. Alanine and glutamine elevation may be present, reflective of hyperammonemia. ASA and its anhydrides will also be present in the amino acid chromatogram.

ASA lyase deficiency is inherited in an autosomal recessive manner. Molecular sequencing for the *ASL* gene is clinically available and allows diagnostic, carrier, and prenatal testing. Sequencing of the ASA lyase cDNA has demonstrated remarkable similarity to the lens structural protein  $\delta$ -crystallin. Duck crystallin has ASA lyase activity. The significance of these provocative findings for both biochemistry and evolution still remain to be determined. Elevated citrulline in dried blood spots is the basis for newborn screening for this condition.

#### 92.7.6 Hyperargininemia

Like the previous two disorders, a defect in arginase, the final enzyme in the urea cycle that catalyzes the hydrolysis



of arginine to urea and ornithine, is named after the substrate that accumulates as a result of its deficiency. Arginase is most active in the liver but is expressed at lower levels in a variety of other tissues, including red blood cells. Red blood cells may be used to measure the enzyme for diagnosis (240). A second isozyme of arginase, encoded by a separate gene, is localized to the mitochondrion, is more widely distributed than its more abundant namesake and appears to normally function in the synthetic pathways of ornithine, polyamines, and nitric oxide (241,242).

Clinical symptoms of liver arginase deficiency (OMIM 207800) are more subtle and occur at a later age than those of the other urea cycle defects. Recognized episodes of hyperammonemia are the presenting symptoms in only a minority of patients. At the age of 1–3 years, linear growth slows and spasticity begins to develop several years later. Soon, previously normal cognitive development slows or stops and the child begins to lose developmental milestones. If untreated, arginase deficiency usually progresses to severe spasticity, loss of ambulation, complete loss of bowel and bladder control, and severe intellectual disability. Argininemia is the only urea cycle defect in which cortical and pyramidal tract signs predominate. However, because of early recognition and initiation of treatment, more than 80% of reported patients are still alive and some are older than 30 years. It seems likely that the mild clinical course in these patients is due to the presence of residual activity as a result of the existence of the second arginase isozyme, whose activity appears augmented as a consequence of hyperargininemia. Although most patients are severely retarded, therapy is beneficial, preventing symptoms and preserving cognition if begun early and preemptively.

An increased concentration of arginine is found in the plasma and CSF of all patients, but urinary amino acid excretion may be normal in some patients most of the time and in all patients some of the time. The pattern of urinary amino acid excretion may be confused with cystinuria. Patients with argininemia have elevated levels of body fluid guanidinoacetate, an intermediate of creatine biosynthesis generated from donation of the guanidinium group of arginine to glycine. Guanidinoacetate and other guanidinium compounds have been shown to be neurotoxic (243) and may be responsible for the pyramidal signs and neurodegeneration seen not only in argininemia but also in patients with guanidinoacetate methyltransferase deficiency.

Arginase deficiency is inherited in an autosomal recessive manner; parents exhibit half-normal arginase activity. Molecular genetic testing allows clinical and prenatal diagnosis. A number of different mutations have been found in the affected individuals but none in sufficient frequency to be readily useful clinically (242). Arginine levels in blood are likely to be elevated in most newborns with this disorder, and screening by tandem mass spectrometry can detect these individuals presymptomatically.

### 92.7.7 Citrullinemia Caused by Citrin Deficiency

Significant strides have been made in characterizing the natural history of this disorder, caused by a deficiency of citrin, a mitochondrial aspartate/glutamate antiporter encoded by the *SLC25A13* gene (244). Citrin deficiency appears to have a bimodal age of onset, with a neonatal presentation (neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD)) and an adult-onset presentation (termed “citrullinemia type II” (CTLN2)). Infants with NICCD are sometimes identified via newborn screening and display markers of neonatal cholestasis, with elevated phenylalanine or methionine. Newborns may also screen positive for galactosemia if galactose is used as the marker for the disorder and not galactose-1-phosphate uridylyltransferase activity (245). Clinically, infants with NICCD demonstrate some combination of failure to thrive, poor feeding, prolonged jaundice, and cataracts. Laboratory investigations indicate signs of cholestasis and liver synthetic dysfunction: liver transaminases, alkaline phosphatase, and  $\gamma$ -glutamyltranspeptidase levels are high, as are coagulation times, conjugated bilirubin and bile acid levels. Low-level hyperammonemia may be present, usually not exceeding  $70\text{ }\mu\text{M}$ . Plasma citrulline levels are elevated (median  $300\text{ }\mu\text{M}$ ) but not as high as classical ASA synthase-deficient patients. Plasma amino acids are also indicative of liver dysfunction and cholestasis, with tyrosine, methionine, and threonine elevations approximately two to four times the upper limit of normal (246). Liver biopsies performed on infants with NICCD have demonstrated micro- and macrovesicular steatosis in addition to fibrosis. With the exception of several patients whose liver synthetic function deteriorated to require orthotopic liver transplantation (247), most infants with NICCD experience resolution of the cholestasis and liver dysfunction, entering a “compensated” oligosymptomatic phase. After their first birthday, they develop peculiar dietary habits, eschewing high-carbohydrate foods and preferring high-protein and high-fat foods, especially beans and peanuts (248). During this phase, patients may complain of nonspecific constitutional symptoms such as nausea, fatigue, reduced appetite, headaches, and abdominal pain.

Symptoms of CTLN2 generally begin in early adulthood, although age of first presentation has ranged from 11 to 79 years, and resemble the symptoms of later-onset urea cycle disorders (249). Most suffer from sudden-onset altered mental status, disorientation, lethargy, and restlessness, and progress to coma. Others have been reported to have intermittent “psychosis,” delusions, delirium, and seizures. Death may occur from irreversible cerebral edema induced by hyperammonemia. CTLN2 patients continue to demonstrate a preference for foods rich in protein and fat, as intake of alcohol or a carbohydrate-rich meal will precipitate symptoms. Biochemically, plasma ammonia, citrulline, and arginine

levels are elevated, while hyperbilirubinemia, hypertyrosinemia, and hypermethioninemia seen in NICCD are absent. For unknown reasons, the amount of hepatic ASA synthase protein declines even without ASA synthase mutations or reduced messenger RNA levels. Pancreatitis, hyperlipidemia, and hepatocellular carcinoma have been reported in a minority of CTLN2 patients (246). Although most NICCD infants go on either to develop full CTLN2 or to the oligosymptomatic phase, a small percentage stay healthy as adults. This is evidenced by the drop in prevalence from 1:17,000 live births in Japan for NICCD to 1:100,000–230,000 for CTLN2 (250). Furthermore, the ratio of males to females affected with NICCD is approximately 1:1, consistent with its autosomal inheritance, but the ratio of males to females with CTLN2 increases to 2.4:1, indicating the existence of currently unknown factor(s) that partially protects women from the latter phenotype (250).

While molecular sequencing of SLC25A13 is clinically available, perhaps serum pancreatic secretory trypsin inhibitor (PTSI) can be utilized as a screening test where NICCD or CTLN2 is suspected, as nearly all affected individuals where PTSI was checked demonstrated elevated levels of this marker. Most patients with SLC25A13-associated disorders are of East Asian origin, especially Japanese, Southern Chinese, Taiwanese, and Korean descent, but the disease is not restricted solely to these ethnicities (154).

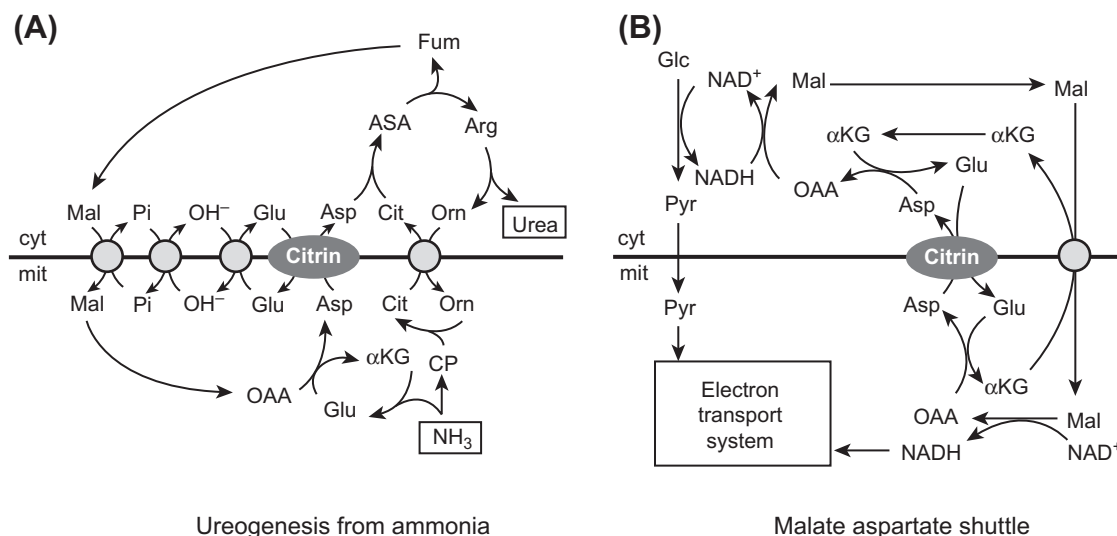
The pathogenesis of the disorder appears to be as a result of aspartate's participation in multiple biochemical pathways. Without proper citrin function, endogenous aspartate becomes sequestered within the mitochondria (Figure 92-9). Because the ASA synthase reaction takes place within the cytosol, ureagenesis is impaired as cytosolic aspartate deficiency prevents production of ASA and results in accumulation of citrulline

and ammonia. In addition, because of the key role played by aspartate via the malate:aspartate shuttle in transporting cytosolic NADH across the mitochondrial inner membrane, citrin deficiency results in accumulation of NADH in the cytosol. The abnormal NADH:NAD<sup>+</sup> ratio subsequently impairs gluconeogenesis, glycolysis, and alcohol metabolism. These mechanisms offer a partial etiology for citrin-deficient patients' preference for protein, augmenting exogenous aspartate intake in order to replenish both the cytosolic arm of the urea cycle and malate:aspartate shuttle. They also explain citrin-deficient patients' avoidance of carbohydrate and alcohol to avoid exacerbation of existing cytosolic NADH:NAD<sup>+</sup> imbalance (251).

Infants with NICCD should be treated with a combination of medium chain triglyceride-containing formula with higher fat and protein content, supplementation with fat-soluble vitamins, and elimination of galactose from the diet. CTLN2 patients can be treated with a similar high-fat, high-protein diet, arginine supplementation, and avoidance of alcohol and high-carbohydrate foods. This combination aims to reduce the cytosolic NADH:NAD<sup>+</sup> ratio and can treat hyperammonemia, hypertriglyceridemia, and steatosis. Intravenous resuscitation for CTLN2 patients runs counter to emergency treatment for most other inborn errors, as infusions of glucose and glycerol have been reported to precipitate sudden hyperammonemia and coma (250). Ultimately, orthotopic liver transplantation has been performed in CTLN2 patients, resulting in abolition of hyperammonemic crises and resolution of the dietary protein/fat preference.

### 92.7.8 Treatment of Urea Cycle Disorders

The two linchpins of therapy for urea cycle disorders are the diminution of the amount of ammonia that must



**FIGURE 92-9** The function of the citrin protein in relation to the (A) urea cycle and (B) the malate aspartate shuttle. In each instance, it is the proper efflux of aspartate from the mitochondria to the cytosol that allows each cycle to proceed. (With permission from Saheki et al. 2010.)

be detoxified and the removal of the ammonia that is formed. Treatment can be divided into that for acute life-threatening hyperammonemia and that used chronically between episodes to keep ammonia levels at near-normal values. The details of these approaches are well described (219). Ammonia is derived from the amino acids of protein, either ingested or endogenously catabolized as part of normal turnover or breakdown and heightened by intercurrent events. Over the short term, increased catabolism presents a far greater threat and must be more feared than small variations in exogenous protein intake. Any more than 12–15 h of anorexia and caloric deprivation is likely to trigger protein catabolism and hyperammonemia.

Severe hyperammonemia ( $>400$ – $500\mu\text{M}$ ) will require hemodialysis to remove ammonia in a timely fashion to reduce the likelihood of permanent brain injury. However, there may already be irreversible brain damage by the time treatment is initiated, especially in cases of neonatal hyperammonemic coma. In the face of significant cerebral edema and an increased head circumference, the parents should be counseled about the neurologic outcome before any specific therapy for hyperammonemia is begun.

During hyperammonemic crisis, maintenance of fluid intake with high carbohydrate-containing liquids is essential to reduce glucagon-mediated protein catabolism and subsequent ammonia release. If unsuccessful, earlier rather than later intravenous intervention is recommended to prevent severe hyperammonemia. Provision of sufficient calories in the form of high dextrose infusion rate (greater than  $6\text{ mg/kg/min}$  glucose infusion rate) and intralipid infusion will remove the hypoglycemic stimulus to protein catabolism. In the first 24–48 h of hyperammonemic crisis, exogenous protein can be excluded to reduce potential sources of nitrogen, but afterward protein should be reintroduced ( $0.5$ – $1\text{ g/kg/day}$ ) to prevent additional catabolism and ammonia release induced by prolonged removal of protein.

A major breakthrough in the therapy for these disorders occurred when Brusilow and colleagues (219) devised a number of means to divert ammonia from urea production and to reduce plasma ammonia. These involve the recognition that arginine may become an essential amino acid and that indirect removal of amino acids in the process of organic acid detoxification will divert nitrogen away from the urea cycle. The amino acid product of the first two steps of the urea cycle catalyzed by CPS-I and OTC is citrulline; the next two steps catalyzed by ASA synthetase and ASA lyase convert citrulline to arginine. Hydrolysis of arginine produces ornithine, needed to react with carbamoylphosphate, which carries the first of the two ammonia molecules destined to be excreted as urea. All patients with deficiencies of the first four enzymes of the urea cycle may be deficient in arginine, and

arginine supplementation is an appropriate part of the therapy. In fact, during periods of hyperammonemic crisis, intravenous arginine ( $250\text{ mg/kg/day}$  as a constant drip) in conjunction with high caloric infusion can significantly bring down ammonia levels as intravenous solutions of nitrogen-scavenging medications are being procured and prepared. In patients with CPS-I or OTC deficiency, the substitution of citrulline for arginine is desirable; citrulline is converted efficiently to arginine and in the process removes another nitrogen molecule from the ammonia pool. In the case of ASA synthetase and ASA lyase deficiencies, higher amounts of arginine reduce blood and body ammonia levels and promote their conversion to the more readily excreted compounds citrulline and argininosuccinate. The use of arginine in these two conditions, but ASA lyase deficiency in particular, effectively eliminates episodes of hyperammonemia.

The organic anions benzoate and phenylacetate (or phenylbutyrate) are known as “ammonia-scavenging” medications because they are excreted as benzoylglycine (hippurate) and phenylacetylglutamine, respectively. Resynthesis of glycine requires one molecule of ammonia and two molecules of glutamine. Administration of either compound or both in stoichiometric amounts diverts ammonia from the urea cycle, and in patients with urea cycle defects, it reduces the accumulation of ammonia and other metabolites proximal to the enzymatic deficiency. An intravenous preparation of sodium benzoate and phenylbutyrate (Ammonul; Hyperion Therapeutics) is used for patients with deficiencies of NAGS, CPS, OTC, ASA synthetase, and ASA lyase in severe hyperammonemic crisis.

Maintenance therapy for a patient with a urea cycle disorder follows the same principles as those for acute treatment. To reduce ammonia-generating substrate, dietary intake of protein should be limited to  $1$ – $2\text{ g/kg/day}$  for infants and  $0.5$ – $1.0\text{ g/kg/day}$  for older children. The quality of the dietary protein may be enhanced by the use of an essential amino acid preparation, if required. Surprising flexibility in protein intake is permitted, provided plasma levels of amino acids approximate the normal range and hypoaminoacidemia is not permitted to function as a stimulus to endogenous protein catabolism. Second, urea cycle intermediates distal to the enzymatic block should be given to replenish the urea cycle and allow additional ammonia detoxification. For CPS and OTC deficiencies, either oral arginine hydrochloride or citrulline  $150\text{ mg/kg/day}$  can be administered; for ASA synthetase and ASA lyase deficiencies, arginine hydrochloride concentration up to  $700\text{ mg/kg/day}$  should be given to normalize plasma arginine levels. Finally, oral sodium benzoate ( $250\text{ mg/kg/day}$ ) or sodium phenylbutyrate ( $250\text{ mg/kg/day}$ ) is used as an ammonia-scavenging medication. Their use has greatly diminished the frequency and intensity of intercurrent hyperammonemic episodes and has been

associated with a markedly improved prognosis for patients with these disorders. Valproic acid should not be used as an anticonvulsant in patients with urea cycle defects because this medication provokes severe hyperammonemia in almost all cases.

Patients with severe urea cycle defects, such as males with complete OTC deficiency, who have not suffered significant neurologic damage, are candidates for liver transplantation. Liver transplantation is an effective “cure” for the metabolic disorder, especially as surgical techniques, postoperative care, and antirejection regimens have undergone considerable refinement to reduce the attendant morbidity and mortality from the procedure (252,253).

Mouse models, occurring either naturally or as a result of gene “knockout” experiments, are available for all six steps of the urea cycle and should prove useful in the development of gene and other therapies for the urea cycle defects.

### 92.7.9 Lysinuric Protein Intolerance

Lysinuric protein intolerance (LPI, OMIM 222700) is a deficiency of the  $\gamma^+$ LAT-1 light chain subunit of the cationic amino acid transporter  $\gamma^+$ L, impairing transport of positively charged amino acids such as lysine. This disorder is reviewed in depth by Sebastio (254). LPI has severe, multisystemic effects owing to deficient egress of lysine, ornithine, and arginine from the epithelial basolateral membranes. Infants present usually after weaning of breastfeeding or formula, with failure to thrive, vomiting, diarrhea, and poor feeding. Lethargy, altered level of consciousness, and vomiting may occur after ingestion of high-protein foods. In fact, LPI patients will self-select their diets to avoid foods rich in protein. Hepatosplenomegaly also occurs and may lead to misplaced suspicion of a lysosomal storage disorder, although typically accompanied by hematologic derangements similar to hemophagocytic lymphohistiocytosis (255,256). Other chronic, life-threatening complications of LPI include progressive alveolar proteinosis leading to respiratory failure and renal manifestations such as proteinuria, microscopic hematuria, Fanconi syndrome, hypertension, and eventual, dialysis-dependent, end-stage renal disease (254). Many patients experience developmental delay, and older patients may manifest psychiatric symptoms.

Biochemically, LPI patients have low plasma lysine, ornithine, and arginine levels. There is elevated excretion of these dibasic amino acids in the urine, but cystine excretion is normal. Hyperammonemia results most likely from insufficient reabsorption of ornithine and arginine from the renal tubular epithelium, leading to hepatocellular depletion of these amino acids and inadequate ureagenesis. Orotic aciduria is also present (257). Systemic depletion of dibasic amino acids results in protein malnutrition and failure to thrive. Trapping

of arginine in leukocytes and in the epithelium of the kidneys and lungs is theorized to result in nitric oxide overproduction, leading to apoptosis and release of inflammatory mediators. It is derangement of tightly regulated nitric oxide production that is thought to lead to the progressive renal and pulmonary failure, along with the bizarre hematologic manifestations of the disease.

Treatment for LPI is complex and requires multidisciplinary care. Reduction of ammonia levels with protein restriction, citrulline supplementation, and sodium benzoate is but one facet. Oral L-lysine supplementation should be provided (258). Treatment of pulmonary alveolar proteinosis, renal disease, and hemophagocytic lymphohistiocytosis should be handled by pulmonologists, nephrologists, and hematologists. For patients with short stature and growth hormone deficiency, administration of human growth hormone may be considered.

More than 50 mutations in the *SLC7A7* gene, which encodes  $\gamma^+$ LAT-1, have been identified in LPI. LPI, although a panethnic disorder, is more common in Finland, where the g.IVS6-2a>t splice site mutation is present in most cases due to founder effect (259,260).

### 92.7.10 Hyperinsulinemia-Hyperammonemia Syndrome

This condition (OMIM 606762) is caused by dominant mutations in the glutamate decarboxylase gene that result in increased enzymatic activity. The enzyme, which catalyzes the conversion of glutamate to  $\alpha$ -ketoglutarate, is allosterically activated by leucine and inhibited by GTP. Release of  $\alpha$ -ketoglutarate indirectly stimulates insulin release from pancreatic  $\beta$ -cells by closing the SUR/KIR6.2 potassium efflux channel. Activating mutations increase sensitivity of the enzyme to leucine or reduce the inhibitory effect of GTP, leading to constitutive conversion of glutamate to  $\alpha$ -ketoglutarate and subsequent hyperinsulinism (261,262). Affected patients present often in the neonatal period with recurrent episodes of hyperinsulinemic hypoglycemia, sometimes misinterpreted as seizures, delaying the diagnosis and increasing the probability of permanent brain injury. The hypoglycemia can be provoked by both fasting and ingestion of a protein-rich meal. Hyperammonemia is usually two to four times upper limit of normal, increments minimally following ingestion of protein, and is usually not accompanied by elevations in plasma glutamine (263). *In vitro* studies in animal models indicate that the source of ammonia is not from urea cycle impairment in the liver, as originally thought, but rather from the increased production from the kidney. The disorder is readily treatable with diazoxide, which suppresses insulin secretion; restriction of protein may help in some cases to prevent postprandial hypoglycemia (264).



### 92.7.11 The Hyperornithinemias

Two inborn errors of metabolism produce elevated levels of plasma ornithine: (1) gyrate atrophy of the retina with hyperornithinemia and (2) hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome. These disorders are reviewed by Valle and Simell (265).

### 92.7.12 Gyrate Atrophy with Hyperornithinemia

Gyrate atrophy of the retina (OMIM 258870) is usually associated with a 10- to 20-fold elevation in plasma ornithine and no hyperammonemia. The disorder primarily affects vision, typically with no associated problems in intellectual development. Visual loss begins to occur by the end of the first decade (myopia and decreased night vision). By the second decade, there is reduced peripheral vision. Cataracts develop by the second or third decade, and most patients are blind by the fifth decade of life. The progression can be more rapid and may accelerate during puberty. There is quite a bit of variability even among patients homozygous for the recurrent L402P Finnish mutation (266). The retinal atrophy begins in the periphery, with loss of the pigment epithelium. The fundal picture is characterized by sharply defined margins of choroidal atrophy, slowly progressing toward the posterior pole of the eye. Some patients have mild proximal muscle weakness and type 2 muscle fiber atrophy with tubular aggregates in the fibers. Ten percent of patients have a clinically significant peripheral neuropathy, but 50% have abnormal electrophysiologic testing (267). Abnormalities in the brain on MRI and abnormal EEGs are common, indicating potential dysfunction of the CNS even in cognitively normal patients (268). A cohort of mentally impaired gyrate atrophy patients were discovered to have abnormally low plasma creatine and guanidinoacetate levels, with severely reduced creatine peaks on brain magnetic resonance spectroscopy (269). Systemic creatine deficiency in gyrate atrophy is a secondary phenomenon resulting from the inhibitory effect of elevated ornithine levels on the first step of creatine synthesis, the conversion of arginine and glycine to ornithine and guanidinoacetate.

Gyrate atrophy is an autosomal recessive disorder caused by a deficiency of ornithine  $\delta$ -aminotransferase (OAT; see Figure 92-8), which transaminates ornithine to glutamate-5-semialdehyde. While the disorder occurs in all ethnicities, it is most common in the Finnish population, with an incidence of 1 in 50,000.

Certain patients with gyrate atrophy have experienced reduction in plasma ornithine levels following administration of pyridoxine or pyridoxal phosphate, but its effects on retinal atrophy and vision loss are not certain. Similarly, creatine supplementation is able to correct the systemic phosphocreatine deficiency but does not

appear to slow the progression of vision loss (270). On the other hand, restriction of dietary ornithine and arginine has successfully delayed chorioretinal degeneration (271,272). An OAT knockout mouse develops retinal degeneration and should help elucidate the pathogenesis and allow testing of potential therapies (273).

### 92.7.13 HHH Syndrome

The HHH syndrome (OMIM 238970) occurs as a result of deficiency of the mitochondrial ornithine translocase encoded by the *SLC25A15* gene (274). The transporter brings cytosolic ornithine into the inner mitochondrial matrix, where the ornithine is transcarbamylated to citrulline. Without proper function of ornithine translocase, the urea cycle has insufficient ornithine substrate to proceed with ureagenesis and ammonia detoxification. Consequently, patients with HHH syndrome develop hyperammonemia (at most three times upper limit of normal) and elevated plasma ornithine (200–700  $\mu$ M, normal < 135  $\mu$ M). The “milder” hyperammonemia seen in HHH syndrome compared to other urea cycle disorders is probably due to the existence of a second ornithine transporter that is able to partially compensate for the lack of the first. The gene for the second transporter (*SLC25A2*) is encoded by a homologous, intronless gene that apparently arose from *SLC25A15* by retrotransposition (275,276). The homocitrullinuria is thought to arise from transcarbamylation of lysine, which is similar in chemical structure to ornithine, with the exception of an additional methylene group in its side chain. Orotic aciduria is also present due to shunting of unused carbamoyl phosphate into the pyrimidine biosynthetic pathway.

The clinical symptoms of HHH syndrome are variable, with neonatal hyperammonemic coma, infantile failure to thrive, adult-onset fulminant liver failure, and stroke-like episodes, all described as initial presentations (277,278). Much like ASA lyase deficiency and argininemia, neurocognitive impairment occurs with high frequency even in those without hyperammonemia under good metabolic control, although normal cognition is possible (279). Developmental delay, seizure disorder, and pyramidal signs occur in a large proportion of patients (278,280). In addition, hepatic synthetic function is also frequently impaired, manifesting as elevated transaminases and prolonged coagulation times. Despite the hyperornithinemia, HHH syndrome patients do not develop the retinal changes associated with ornithine aminotransferase deficiency.

Molecular genetic sequencing is available to confirm the diagnosis of HHH syndrome; over 30 mutations have been ascertained. Currently, the only recurrent ancestral mutation is the p. $\Delta$ F188 in-frame deletion that is found in the French Canadian population. Although the mutation results in minimal expression of functional protein secondary to proteolysis via the endoplasmic reticulum-associated degradation system, the clinical

manifestations of even homozygous p.ΔF188 patients are very heterogenous (280).

Treatment is symptomatic and involves limiting protein intake, supplementation with citrulline, and diverting ammonia from the urea cycle using sodium benzoate and sodium phenylbutyrate.

## 92.8 DISORDERS OF SERINE METABOLISM

Serine is a nonessential polar amino acid with multiple important functions in the body. Catalyzed by serine hydroxymethyltransferase, one-carbon units in the form of  $N^{5,10}$ -methylenetetrahydrofolate are generated from serine for use in purine and pyrimidine biosynthesis and methylation reactions. Sphingolipids and phospholipids, important cell membrane components, are derived from serine. The enantiomer, L-serine, found in proteins and lipids, is converted to the facultative neurotransmitter D-serine via the action of the serine racemase enzyme. L-serine is also an important neurotrophic factor in the CNS, an observation that led to the description of three inborn errors of serine synthesis.

### 92.8.1 Disorders of Serine Synthesis

In addition to dietary protein and catabolism from phospholipids, serine is synthesized endogenously from two sources: the first, from glycine in the reverse reaction catalyzed by serine hydroxymethyltransferase, and the second, from 3-phosphoglycerate. The latter pathway appears to be the main source of serine, especially in the CNS, where transport of L-serine and glycine across the blood–brain barrier is limited. From those observations and the discovery that mice engineered with knockouts of serine biosynthetic enzymes display an embryonic lethal phenotype, inborn errors of serine synthesis involving the three-step pathway were discovered and further characterized.

### 92.8.2 3-Phosphoglycerate Dehydrogenase Deficiency

Cases of 3-phosphoglycerate dehydrogenase (3-PGDH) deficiency (OMIM 601815) were first discovered in two siblings from consanguineous first-cousin parents who had a similar pattern of microcephaly, failure to thrive, irritability, feeding difficulties, severe developmental delay, persistently adducted thumbs, hypertonicity, and infantile-onset seizures. Brain MRI had cortical atrophy and abnormally reduced myelination. Biochemical testing demonstrated low plasma serine and glycine that normalized postprandially; the deficiency was much more pronounced in the CSF. A deficiency of 3-PGDH enzyme was demonstrated in cultured fibroblasts (281). Additional patients were subsequently reported with similar symptoms and biochemical findings. The seizure

disorder in 3-PGDH deficiency is one that is typically difficult to control with antiepileptic medications and has been described as “West syndrome” (282,283). Treatment with supplemental L-serine (500–600 mg/kg/day) and glycine (200–300 mg/kg/day) has been reported to reduce or even abolish the seizures and EEG abnormalities, although the effects on psychomotor development were much more disappointing (284). In addition, some 3-PGDH-deficient patients have demonstrated slowing of head growth with high-dose (1400 mg/kg/day) L-serine therapy, suggesting toxicity of excessive serine supplementation. One infant with 3-PGDH deficiency was diagnosed prenatally, and L-serine therapy was administered to the mother, allowing the birth of a normocephalic infant girl whose treatment was continued postnatally. Subsequently, she had normal psychomotor development and no neurologic symptoms (285). More recently, an attenuated form of 3-PGDH deficiency was described in two siblings with moderate developmental delay (IQ 50–55); absence seizures; hyperactivity; and absence of microcephaly, hypertonicity, growth failure, or brain MRI abnormalities (286). Their fasting plasma glycine levels were normal, while plasma serine levels were just below the lower limit of normal. The older sibling, however, had severely low CSF serine levels, with normal CSF glycine, and both patients responded to lower-dose L-serine supplementation (120–140 mg/kg/day) with resolution of absence seizures, though IQ did not improve. Sequence analysis of the *PHGDH* gene in the few affected families indicate that the p.V490M mutation seems to be recurrent across ethnicities; other reported mutations include p.R135W, p.V261M, p.A373T, and p.G238fsX (287). Homozygous p.G377S mutations were found in the siblings with attenuated 3-PGDH deficiency (286).

### 92.8.3 Phosphoserine Aminotransferase Deficiency

Phosphoserine aminotransferase (PSAT) deficiency (OMIM 610992) has been reported in two siblings. The older brother presented at 9 weeks of age with postnatal-onset microcephaly, intractable seizures, cerebral atrophy, hypoplastic cerebellar vermis, and delayed white-matter myelination. Plasma and CSF serine and glycine levels were below normal limits. Treatment with serine and glycine did not improve seizure control and eventually he died at 7 months of age. The younger sister was identified with the deficiency at birth because of low serine and glycine in plasma and CSF; treatment with serine was started immediately and she did not develop seizures. Brain MRI at 4 months was normal. At the last follow-up, she was 3 years old and demonstrated normal growth and development. Enzymatic analysis of PSAT was unreliable as the older sibling had normal PSAT enzymatic activity. The siblings were found to have compound

heterozygous frameshift (c.delG107) and missense (c.299A>C) mutations in the *PSAT1* gene; the missense mutation was confirmed deleterious by sequence alignment and enzymatic analysis of in vitro-expressed mutant enzyme (288). Based on the lack of neonatal symptoms but divergent outcomes of the siblings, the authors concluded that measurement of CSF serine and glycine is important in patients with neonatal seizures to allow early identification and subsequent treatment of serine biosynthetic deficiencies.

### 92.8.4 Phosphoserine Phosphatase Deficiency

Phosphoserine phosphatase (PSPH) deficiency (OMIM 172480) was identified in one patient with concurrent Williams syndrome. There was prenatal-onset microcephaly and intrauterine growth retardation, and based on the facial appearance, a diagnosis of Williams syndrome was suspected and confirmed. He demonstrated postnatal microcephaly, psychomotor delay, and failure to thrive, with gastroesophageal reflux. Plasma serine levels were reduced or low-normal while fasting and normalized following meals, while CSF serine levels were clearly low. L-serine 300 mg/kg/day was administered while allowed for slight acceleration of head growth, but head circumference remained below the third percentile and height/weight percentiles did not improve. Fibroblast PSPH enzymatic activity was 25% of normal controls and compound heterozygous p.D32N/M52T *PSPH* mutations were demonstrated (289,290). Although the *PSPH* gene and the Williams syndrome critical region are both located on chromosome 7, they are separated by 16.5 Mb and the two findings are unrelated (287).

## 92.9 DISORDERS OF AMINO ACIDS WITHOUT KNOWN CLINICAL CONSEQUENCES

A number of disorders included in the earlier editions of this text have been found to be unassociated with definable diseases and were ascertained only because children with neurologic or developmental problems were studied by amino acid screening methods. Subsequent to their description, studies of normal older siblings with the same disorder, studies of patients ascertained through newborn screening, and the inconsistent clinical findings in the index cases led inexorably to the conclusion that the biochemical abnormality was not the cause of the clinical problem. Disorders in this category include histidinemia, due to histidase deficiency (291); hyperlysinemia, due to deficiency of the bifunctional enzyme with both lysine-ketoglutarate reductase and saccharopine oxidoreductase activities (292); sarcosinemia due to sarcosine dehydrogenase deficiency (293); and cystathioninemia due to  $\gamma$ -cystathionase deficiency (117).

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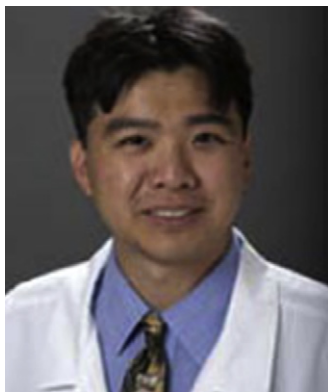
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### Biography



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# CHAPTER

# 93

## Disorders of Carbohydrate Metabolism

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### 93.1 INTRODUCTION

Inborn errors of carbohydrate metabolism discussed in this chapter include disaccharidase deficiencies, disorders of monosaccharide metabolism, glycogen storage diseases (GSDs), and gluconeogenic disorders. Additional information may be sought in *The Online Metabolic and Molecular Basis of Inherited Disease* by Scriver and coworkers (1).

### 93.2 DISACCHARIDASE DEFICIENCIES

The major sources of dietary carbohydrate in humans are starch and the disaccharides lactose and sucrose. In adults, starch constitutes 60% of the carbohydrate ingested, however, in newborns and young infants, the primary carbohydrate is lactose (milk sugar). Sucrose consumption varies widely with the choice of infant formula and other eating habits. The normal digestive process involves the splitting of disaccharides by intestinal hydrolytic enzymes (lactase, sucrase, isomaltase, and maltase) into monosaccharides before absorption (Figure 93-1).

Defective intestinal digestion of dietary sugars leads to symptoms of flatulence, abdominal cramps, diarrhea, and perianal irritation. Levels of enzymes involved in the hydrolysis of disaccharides may be depressed on either a genetic or an acquired basis. The latter situation results from damage to the brush border cells of the small intestine, consequent to infection or other injuries. When enzymatic hydrolysis is impaired, ingested disaccharide accumulates and provides a growth medium for intestinal bacteria that produce carbon dioxide, hydrogen gas, and organic acids. The stools tend to be sour, foamy, loose, and watery with an acidic pH. A diagnosis of disaccharidase deficiency may be suspected from the history of symptoms developing in association with the ingestion of a particular sugar and a laboratory finding of disaccharides in the urine. Direct confirmation may

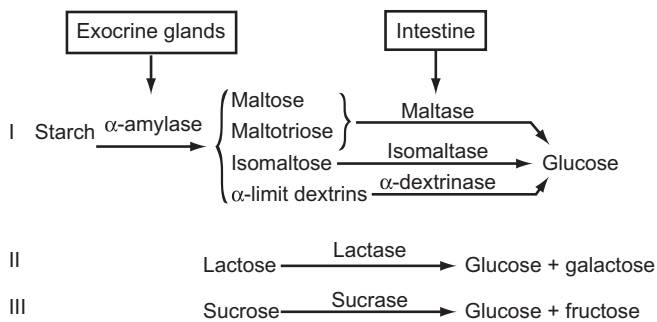
be obtained by enzyme assays in peroral small bowel biopsy. Indirect confirmation of the diagnosis can be made by a disaccharide tolerance test. Disaccharidase deficiency is suggested if the blood glucose curve is flat upon ingestion of the suspect disaccharide and the breath hydrogen concentration is increased. Genetic testing provides an accurate method for determining disaccharidase deficiency (2).

#### 93.2.1 Lactase Deficiency

Congenital lactase deficiency is a rare autosomal recessive inherited disorder that appears to have a higher frequency in the Finnish population (3). Affected infants present with a severe gastrointestinal problem characterized by watery diarrhea as soon as milk or lactose-containing foods are introduced, then dehydration, acidosis, and weight loss follow.

Late-onset (adult-type) lactase deficiency, also known as hypolactasia or lactase nonpersistence, differs from that encountered in infants at the age of onset, clinical severity and molecular defect (4). Symptoms usually do not occur until adult life but occasionally may start at an earlier age. This form of lactase deficiency is common in individuals from Mediterranean countries, African-Americans, Asians, Native Americans and Eskimos (5). Treatment for patients with lactase deficiency usually consists of a diet free of lactose or inclusion of exogenous lactase. In infants, a lactose-hydrolyzed human milk has been used (6). Treatment usually results in a rapid clinical response.

On the molecular level, both adult-type hypolactasia and congenital lactase deficiency stem from variants affecting the lactase gene localized on chromosomes 2q21–22; however, the molecular mechanisms causing the two disorders are different. Congenital lactase deficiency appears to be caused by mutations that directly affect the lactase polypeptide (7). In a study of 32 Finnish patients with congenital lactase deficiency, five mutations in the lactase gene were identified, the most prevalent of



**FIGURE 93-1** Digestion of carbohydrates.

which was the nonsense mutation p.Cys1390X in exon 9 accounting for 90% of affected alleles (4). In adults with hypolactasia, the lactase enzyme appears identical to that in adults with higher lactase levels, as is the lactase gene sequence. Evidence suggests that the variation of lactase level in adults is controlled by polymorphic elements cis-acting to the lactase gene, which necessitate transcription factor binding (7,8). In the normal process of human development lactase activity declines. However, mutations have resulted in lactase persistence, which is inherited as an autosomal dominant condition. Often attributed to evolutionary circumstances, the frequency of these mutations appears to be different in various populations (8,9). Unlike many factors that result in lactase nonpersistence, lactase persistence relies almost singly on the continuation of synthesis. The biological simplicity of lactase persistence may have led to the observed evolutionary variability (10).

### 93.2.2 Sucrase–Isomaltase Deficiency

The mode of inheritance of this condition appears to be autosomal recessive. An incidence of 0.2% has been reported for North Americans and 10% for Greenland Eskimos (11). Clinical manifestations tend to be more severe in younger children and depend on the amount of ingested sucrose, which usually comes from fruits and sweetened foods. The disease is caused by mutations in the gene coding for sucrase–isomaltase. Different mutations lead to either defects in the intracellular processing of the enzyme or functionally alter the enzyme. The degree of severity is a spectrum from complete absence of sucrase to trace levels of operating isomaltase (10). Studies have shown sucrase–isomaltase deficiency can also be noninvasively determined by <sup>13</sup>C-sucrose-labeled breath tests (12). Effective treatment for sucrase–isomaltase deficiency includes Sucraid (sacrosidase) enzyme replacement therapy and a diet with severely limited sucrose intake (13).

## 93.3 GLUCOSE–GALACTOSE MALABSORPTION

This is a rare disorder in which acute, profuse, watery diarrhea develops in newborn infants following initial feeding. Intestinal disaccharidase activities are normal.

Fructose is absorbed normally, but glucose and galactose are not. There is no significant rise in blood glucose levels following an oral glucose–galactose tolerance test. However, a breath hydrogen test reveals glucose–galactose malabsorption (14). The stool usually contains large amounts of reducing sugars (>2g%). Diarrhea stops when free glucose and galactose are removed from the diet. Nephrocalcinosis and nephrolithiasis have been reported. All patients have mild defects in renal tubular reabsorption of glucose. The basic defect is in the transport protein (Na<sup>+</sup>/glucose cotransporter) localized in the brush border membrane of the small intestinal epithelial cells (15). The protein is encoded by the *SGLT1* gene that is localized on chromosome 22q. Pedigree analysis of a consanguineous Swedish family going back 10 generations suggests autosomal recessive inheritance (16). More than 40 mutations have been identified (17). Certain mutations appear to be recurrent in specific populations like the Old Order Amish, suggesting the role of founder effect (18). A novel homozygous nonsense mutation p.Arg267X that results in hypercalcemia, nephrocalcinosis and proximal tubular dysfunction has been reported (19).

### 93.3.1 Glucose Transport Defects

Each of the 14 glucose transporter proteins that have been identified to date targets specific tissues in order to facilitate the diffusion of glucose across the plasma membrane (20). The genes for these transporter proteins have also been localized (21). Of known clinical significance are GLUT1, GLUT2 and GLUT10.

GLUT1 aids in the diffusion of glucose across the blood–brain barrier. The classic presentation for GLUT1 deficiency includes epilepsy, developmental delay, acquired microcephaly, spasticity, and ataxia. The expanded phenotype includes paroxysmal movement disorders and atypical childhood absence epilepsy, although there has been one case without epilepsy (22). The accompanying severe seizure disorder can be partially or fully remedied with a ketogenic diet (23). Low CSF glucose values (relative to blood glucose) and low CSF lactate levels are virtually diagnostic (24,25). The gene for GLUT1 is located on chromosome 1p.34.2. GLUT1 defects are normally inherited in an autosomal dominant manner, with de novo mutations producing haploinsufficiency and conferring symptomatic heterozygosity, but recent studies have shown that the defects may also be inherited in an autosomal recessive manner (26,27). Molecular analyses have identified a wide spectrum of heterozygous mutations, including nonsense, missense, insertion, deletion, splice-site mutations and microdeletions (23). More than 200 cases have been identified (28).

GLUT2 defects cause Fanconi–Bickel syndrome. More than 100 cases have been reported, and 34 different mutations have been identified, with none of them being

particularly frequent (29). It is characterized by proximal renal tubular dysfunction, as well as hepatic dysfunction, which result in glycogen accumulation in the liver. The affected patients present with rickets, hepatomegaly, and growth failure associated with impaired glucose and galactose tolerance, increased renal clearance of glucose, amino acids, protein, phosphate, and uric acid as well as glycogen accumulation in the liver (21). Due to the latter, Fanconi–Bickel syndrome has also been classified as a type of glycogen storage disease (see glycogen storage disease type XI). GLUT3 transports glucose in human neural, brain, and muscular tissues (30). GLUT4 is the insulin-responsive glucose transporter involved in the signaling pathway that regulates glucose metabolism in muscle cells and adipocytes, and the gene is localized in 17p. States of insulin resistance such as in obesity, type II diabetes, and long-term fasting, trigger a drop in GLUT4 transcription and expression in adipocytes (31). GLUT5 has been identified as a key molecule in fructose transportation and GLUT9 has been classified as a urate transporter (20).

GLUT10, instead of transporting glucose like the other GLUT transporters, functions as a mitochondrial transporter for the oxidized form of vitamin C (32). Loss of GLUT10 function causes arterial tortuosity syndrome, an autosomal recessive disorder characterized by tortuosity, elongation, stenosis, and aneurysm formation in the major arteries (33). It is expected that we will learn more about the roles of other glucose transporters in carbohydrate metabolism through research.

## 93.4 DISORDERS OF GALACTOSE METABOLISM

### 93.4.1 Galactose Metabolism

Galactose, a component of lactose, is an important nutrient for newborn infants and young children. In human breast milk, the lactose content is about 7 g/dL, and in cow's milk the concentration is approximately 5 g/dL. In the newborn infant, lactose may provide as much as 40% of the caloric intake but only 3–4% in the adult due to proportionally lower milk intake. Galactose is also a constituent of many glycoproteins, glycolipids, and mucopolysaccharides.

The principal pathway for the metabolism of galactose has been designated as the Leloir pathway (Figure 93-2). Galactose is phosphorylated to galactose-1-phosphate by the enzyme galactokinase. Galactose-1-phosphate is exchanged for the glucose-1-phosphate moiety of uridine diphosphate glucose (UDPG) to form uridine diphosphate galactose (UDPGal) by galactose-1-phosphate uridyl transferase (transferase or GALT). The glucose-1-phosphate released leads into the glucose pathway. The UDPGal formed is converted to UDPG by the enzyme UDPGal-4-epimerase (epimerase). The sum of these three enzymatic reactions involving galactokinase, transferase, and epimerase is

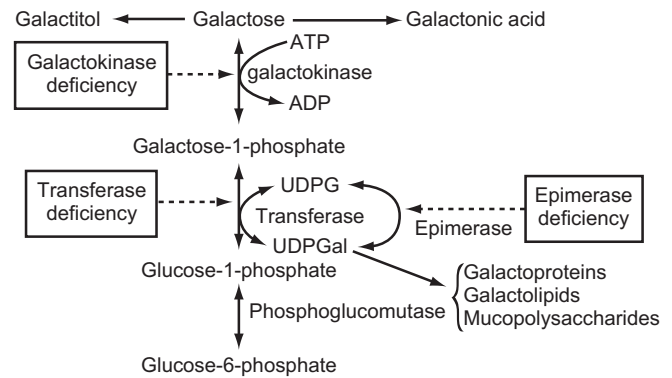


FIGURE 93-2 Pathways of galactose metabolism.

Galactose + adenosine triphosphate (ATP) = Glucose-1-phosphate + adenosine diphosphate (ADP).

UDPGal is also used for the synthesis of galactose-containing complex carbohydrates. A small amount of galactose is converted to galactitol by aldose reductase and to galactonic acid by galactose dehydrogenase.

These three galactose enzymes in the major pathway are widely distributed in tissues, including erythrocytes, leukocytes, liver, kidney, brain, cultured skin fibroblasts, chorionic villi, and amniotic fluid cells. The gene loci in humans for galactokinase, transferase, and epimerase are on chromosomes 17, 9, and 1, respectively (34–36).

Deficiency in the activity of each of the three enzymes results in metabolic disorders known as galactokinase deficiency, galactose-1-phosphate uridyl transferase deficiency (galactosemia), and UDPgalactose-4-epimerase deficiency. All three disorders can be identified by newborn screening based on increased amounts of galactose or galactose-1-phosphate in the blood spots (Guthrie cards), provided that there is normal amount of lactose intake in the newborn's formula or breast feedings. The identification of the specific defect is based on enzyme assays in erythrocytes, providing that the newborn did not receive a blood transfusion before the collection of the blood sample. Often, the Beutler spot test has been employed by newborn screening laboratories for the detection of the transferase defect (37). Some screening laboratories employ automated transferase activity analysis. As of recently, all three galactose enzymes can now be simultaneously analyzed through a novel multiplex enzyme assay utilizing ultraperformance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) (38).

### 93.4.2 Galactokinase Deficiency

**93.4.2.1 Clinical Aspects.** Galactokinase deficiency was first reported by Gitzelmann (39). The patient was a 42-year-old man originally described as having galactose diabetes at 9 years of age. Additional patients were reported subsequently. The major clinical manifestations

are cataracts and pseudotumor cerebri, both appearing early in life. In contrast to the transferase defect, hepatomegaly, jaundice and mental retardation are not usually the features of this disorder, yet isolated reports of one or more of these findings have been made in patients with galactokinase deficiency. It has been suggested that carriers of galactokinase deficiency may be at risk for presenile cataracts.

**93.4.2.2 Biochemical Aspects.** Ingestion of lactose-containing diets will raise blood galactose concentrations to values as high as 100mg/dL in patients with galactokinase deficiency (40). As a consequence, galactose appears in the urine. Galactitol and galactonic acid are produced in increased amounts owing to diversion of galactose into these secondary pathways. It is thought that the accumulation of galactitol is the cause of cataract formation and cerebral edema. In contrast to classic galactosemia (transferase deficiency), aminoaciduria and proteinuria are usually absent. The diagnosis of galactokinase deficiency can be confirmed by measurement of the enzyme activity in erythrocytes. One should be aware that erythrocyte galactokinase activity is much higher in newborns and decreases with age. The enzyme is not stable in erythrocytes stored at room temperature, but it is stable in washed red blood cells frozen for more than 1 week.

**93.4.2.3 Treatment.** Early treatment is important to prevent progression of cataract formation. Treatment consists of exclusion of lactose and other sources of galactose from the diet.

**93.4.2.4 Genetic Aspects.** Galactokinase deficiency is transmitted as an autosomal recessive trait. Parents of affected children exhibit intermediate values of erythrocyte galactokinase activity. Based upon the results of newborn screening programs, the frequency of occurrence has been estimated at about 1:250,000. A galactokinase variant with lower than normal activity (Philadelphia) has been described among asymptomatic black Americans and black Africans (41,42). Over 30 mutations have been identified among patients with galactokinase deficiency, most of them being private mutations. Only three GALK1 mutations have been found to be frequent in certain populations: p.Pro28Thr (a founder mutation in the Romani population); p.Gln382X (occurs frequently in the Costa Rican population); and p.Ala198Val (named the “Osaka variant,” occurs in the Japanese and Korean populations) (43,44). The carrier rate for galactokinase deficiency in the Romani population, particularly those from the former Yugoslavia and present-day Bosnia and Herzegovina, is about 5%. Galactokinase activity is present in cultured amniotic fluid cells; this provides a means for prenatal diagnosis in addition to molecular testing. Families at risk should receive genetic counseling, and pregnant mothers at risk should be advised to restrict intake of lactose and other sources of galactose to protect an affected fetus. Newborn screening and a preemptive adoption of the galactose-free diet is highly

recommended for children whose parents carry the galactokinase deficiency gene, especially those of the Romani population (45).

### 93.4.3 Galactose-1-Phosphate Uridyl Transferase Deficiency (Galactosemia)

**93.4.3.1 Clinical Aspects.** Galactosemia was probably first described in 1908 by von Reuss (46), but it was not until 1956 that Kalckar and his associates (47) established the defect in activity of the enzyme galactose-1-phosphate uridyl transferase. The deficiency is polymorphic with the most common variant, the Duarte allele, differing significantly in prognosis and clinical outcome from classic galactosemia (48). In the classic form, untreated patients show distinctive manifestations early in life. The infant appears normal at birth, and symptoms usually do not develop until milk feedings are given. Food may be refused; vomiting is common, and diarrhea occurs occasionally. Other manifestations include lethargy, hypotonia, jaundice, hepatomegaly, and susceptibility to infection with Gram-negative organisms. In untreated patients, cataracts become evident, and physical and mental retardation occur. The clinical course of many infants is fulminant, and death occurs early from inanition, infection and hepatic failure. In some patients, the course is much milder and may even escape early detection. Late clinical manifestations have been reported in both untreated and treated galactosemia patients (49). These include hypergonadotropic hypogonadism in about 80% of the affected women, speech defects in about half, and, to a lesser extent, neurologic sequelae (50). Although many female patients develop ovarian hypofunction, gonadal function in adult males appears to be normal. It appears that small amounts of transferase activity in some female patients with galactosemia may preserve normal gonadal function; those with the p.Ser135Leu genotype are shown to have a lower likelihood of ovarian dysfunction (50,51). Galactosemic men and women have had normal offspring (52,53). Difficulties in school are frequently encountered.

Contrastingly, those presenting with the heterozygous Duarte variant (D/G) of galactosemia typically have 25% of normal GALT enzyme activity, little to no symptoms, slightly elevated metabolites and no need for intervention (54). Other variants, as they typically have a certain level of enzyme activity, are similar in clinical presentation and usually require no intervention.

**93.4.3.2 Biochemical Aspects.** Classic galactosemia (G/G) is defined as GALT activity below 5% and gal-1-P buildup greater than 20mg/dL accompanying with severe mutations, although a few less serious mutations may also be included (48). Galactosemia may be suspected on clinical grounds, but laboratory confirmation is essential. Diagnostic tests, which are based on response to galactose ingestion, should not be employed because they are hazardous for the infant patients. Direct enzyme



assay using erythrocytes can be readily carried out to confirm the diagnosis. On a galactose-containing diet, affected individuals excrete large amounts of galactose, galactitol, and galactonic acid in the urine. Gross generalized aminoaciduria and proteinuria are also evident. The erythrocyte galactose-1-phosphate level is elevated. It is believed that this compound produces hepatic damage, whereas galactitol accounts for the formation of cataracts. Decreased levels of UDPGal, a product of the transferase reaction, have been observed in erythrocytes, cultured skin fibroblasts, and liver autopsy samples of patients with galactosemia with complete absence of erythrocyte transferase activity (55). On the other hand, patients with small but detectable erythrocyte transferase activity were found to have normal levels of UDPGal. The absolute values of UDPG and UDPGal for erythrocytes were different depending on the method used for analysis (56). However, the relative decrease of UDPGal levels in erythrocytes and cultured skin fibroblasts of galactosemia patients is a consistent finding (57). Molecular genetic testing, in addition to confirming galactosemia, helps identify which variant of the disease is present. This is especially important in differentiating the Duarte allele (D1) from the Los Angeles allele (D2), as they have the same missense mutation (58).

There are many reliable methods for the measurement of erythrocyte transferase activity; affected individuals exhibit either little or no activity in their red blood cells. Blood transfusions in patients interfere with the interpretation of the test because of the transferase activity present in the donor cells. Under this circumstance, studies on both parents to determine heterozygosity can be helpful in reaching a presumptive diagnosis. Neonatal screenings for galactosemia have been carried out in many countries effectively, reducing morbidity and mortality. Methods depend on the measurement of galactose and/or galactose-1-phosphate by microbiologic assays (59), by galactose dehydrogenase (60), by measurement of transferase activity with a fluorescent spot test (61), or modified by automated fluorescent analysis. The latter is based on enzyme coupling reactions through phosphoglucomutase and glucose-6-phosphate dehydrogenase in the production of NADPH. The assay measuring free galactose will detect both galactokinase and transferase defects, whereas the enzyme assay is limited to recognition of the transferase defect. When the screening method incorporates both galactose dehydrogenase and alkaline phosphatase for the measurement of galactose-1-phosphate, an elevated galactose-1-phosphate level will be found not only in patients with transferase deficiency but also in epimerase deficiency. A novel method utilizing high-performance liquid chromatography with nonradioactive UV efficiently and accurately detects galactose-1-phosphate uridyltransferase in erythrocytes (62).

Aside from classic galactosemia, variant galactosemia includes any mutation that causes the GALT enzyme to function partially. The Duarte homozygote has 50% of

normal transferase activity similar to carriers for galactosemia but with a different electrophoretic pattern. Owing to the high frequency of the Duarte variant, the Duarte-galactosemia compound heterozygotes (D/G) with about 25% of normal activity are often identified on confirmation of the neonatal screening programs for galactosemia, which utilizes the transferase assays. Some of the values are near the low-end levels for affected galactosemia patients. It is not proper to rely solely on molecular mutation data for the identification of Duarte-galactosemia compound heterozygotes because the galactosemia allele can have both classic galactosemia and Duarte mutations (63). Identification by biochemical means (both quantitative activity measurement and electrophoretic pattern) is recommended. For genetically heterogenous patients, especially those with less common genotypes, the liquid chromatography–tandem mass spectrometry-based assay for GALT enzyme activity effectively produces quantitative measurements (64).

**93.4.3.3 Treatment.** Treatment is directed toward minimizing the accumulation of galactose and its metabolites in body tissues by excluding milk and milk-containing products from the diet. Various milk substitutes are available (casein hydrolysates, soybean formulas). Although a galactose-free diet is the basis of treatment, supplementary measures are often required in the neonate to correct secondary manifestations, such as hyperbilirubinaemia, hypoprothrombinaemia, sepsis with Gram-negative organisms, and anemia. The infections often respond poorly to antibiotic therapy unless restriction of galactose is also carried out. The effects of dietary treatment are dramatic, with immediate reversal of the acute manifestations. Galactose restriction, with adequate calcium supplementation, is compatible with good general health and normal patterns of physical development. As a group, patients treated at infancy can achieve low to normal intelligence scores, but in spite of dietary restriction of lactose, many patients still have learning and/or behavioral problems in later life. A recent study showed that children with the Duarte variant need not follow a restrictive diet because, although they present with elevated levels of several galactose metabolites, they have normal levels of erythrocyte galactose-1-phosphate (65).

**93.4.3.4 Genetic Aspects.** Galactosemia has been found in all races, but the incidence appears to be higher among Caucasians than among Asians. The frequency of classic galactosemia based on newborn screening in the United States is approximately 1:47,000 (66). The disorder is transmitted as an autosomal recessive trait.

Several biochemical variants of erythrocyte transferase have been described. Some have been associated with disease, and others have not. The most common and understood variant of transferase-deficiency galactosemia is attributed to the Duarte allele (D<sub>1</sub>), which is allelic to the less-frequent Los Angeles allele (D<sub>2</sub>) (61). Both variant enzymes have identical electrophoretic

patterns, but they differ in the extent of their erythrocyte activities. The Duarte variant exhibits low activity, with homozygotes having about half-normal activity, while the Los Angeles variant has a slightly higher than normal activity. The Duarte and Los Angeles variants have the same molecular mutation in cDNA p.Asn314Asp, and the difference in activity is attributed to intron changes (58). The Los Angeles variant carries a silent c.1721C>T mutation in exon 7. Both Duarte and Los Angeles variants have an extended sequence of 28 adenine nucleotides in intron 10, instead of 17 adenine nucleotides in the corresponding sequence of the normal allele. The frequency of Duarte heterozygosity is about 12–13% in the white population. A third asymptomatic transferase variant designated as the Berne variant exhibits decreased activity and slower electrophoretic mobility than normal (67).

More than 230 mutations have been identified that are associated with transferase deficiency, and this list continues to grow (68). Among white patients, the most common mutation causing classic galactosemia is p.Gln188Arg and the second is p.Lys285Asn (69,70). The p.Gln188Arg mutation is associated with complete absence of transferase activity and poor outcome (71,72). The allele is thought to be present in about 60% of the white North American population (61). P.Ser135Leu is seen in 48% of black patients with classic galactosemia and is associated with milder clinical presentations (51). Another mutation, one which seems to appear only in black patients, is p.Phe171Ser (73). The Ashkenazi population has a high carrier frequency (1:127; 0.79%) of a rare deletion mutation (68). Other variants with mild clinical manifestations, detectable transferase activity, and different molecular mutations have also been reported (74,75).

Prenatal diagnosis of galactosemia is feasible and has been performed successfully in a number of instances by measuring transferase activity in cultured amniocytes and chorionic villi, by galactitol concentration in amniotic fluid, or more recently, by molecular mutation analysis.

#### 93.4.4 Uridine Diphosphate Galactose-4-Epimerase Deficiency

Epimerase deficiency can be classified arbitrarily into two forms: severe and benign. The severe form can be referred to as generalized epimerase deficiency due to insufficient enzyme activity in all tissues. The benign form can be characterized as either peripheral or intermediate deficiency, the former referring to low enzyme activity only in erythrocytes and the latter referring to epimerase activity below 50% of normal levels in all other cells in addition to deficient activity in erythrocytes (76). Inheritance for both types is autosomal recessive. The severe form is rare, with only a few documented cases (77–79). The clinical symptoms are somewhat

similar to those in classic galactosemia: jaundice, weight loss, vomiting, hypotonia, and hepatomegaly. Dietary treatment with limitation on galactose intake has been tried, but the outcome was uncertain.

The benign form of epimerase deficiency is more common and does not appear to be associated with any clinical problems. The patients are usually identified through neonatal screening for galactosemia because of increased erythrocyte galactose-1-phosphate levels (20–50 mg/dL) and a small increase of free blood galactose. No treatment appears to be required. The decrease in epimerase activity in the benign form is confined to the red cells and white cells. Normal activity has been found in liver and cultured skin fibroblasts (80). The deficiency is attributed to the presence of an unstable variant enzyme requiring higher NAD concentration for maximum activity. In the United States, the frequency of erythrocyte epimerase deficiency appears to be higher among blacks (81).

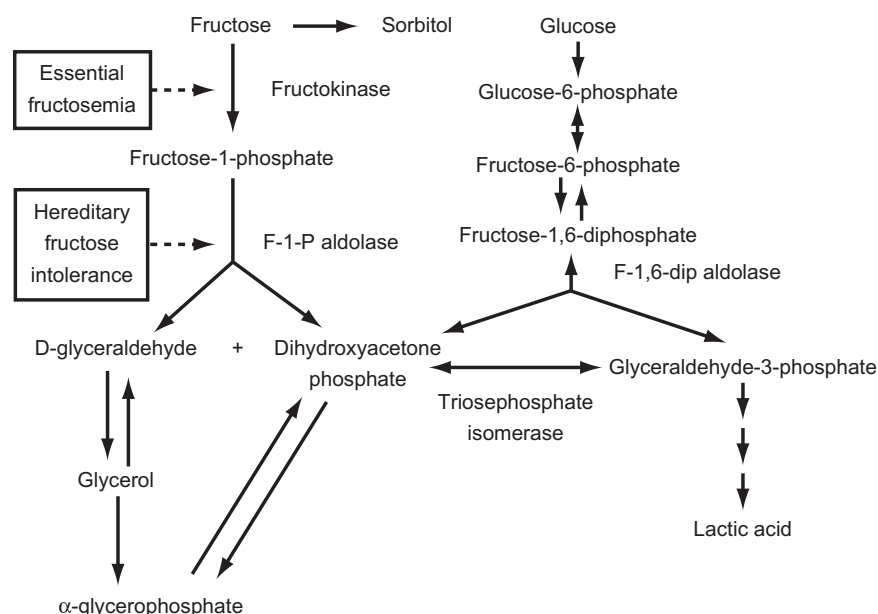
The cDNA for epimerase was cloned and the gene was localized to chromosome 1p36–p35 (82). Several mutations have been identified among patients with epimerase deficiency (83). The pathogenesis of p.Val95Met mutation in the severe form of epimerase deficiency has been explored in the yeast expression system, and accumulation of galactose-1-phosphate may account for the toxicity (84).

## 93.5 DISORDERS OF FRUCTOSE METABOLISM

### 93.5.1 Fructose Metabolism

Fructose is a monosaccharide found in honey, fruits, vegetables, and plants. In combination with glucose, it forms the disaccharide sucrose. It also exists in a number of oligosaccharides, such as raffinose (a trisaccharide) and stachyose (a tetrasaccharide). Stachyose is found abundantly in legumes. Ingested sucrose is hydrolyzed by intestinal sucrase to glucose and fructose. The oligosaccharides raffinose and stachyose, which also contain galactose and glucose, are not digested in humans.

The liver plays a dominant role in the metabolism of fructose; other organs metabolize fructose but to a lesser extent (Figure 93-3). The overall process results in conversion of the sugar to glycolytic intermediates, leading to the formation of either glucose or lactic acid. In the liver, fructose is phosphorylated to fructose-1-phosphate (F-1-P) in the presence of fructokinase. The enzyme is also present in kidney and in intestinal mucosa. Fructokinase is not present in muscle, adipose tissue and blood cells, and in these tissues, fructose is phosphorylated to fructose-6-phosphate by hexokinase. In the liver, F-1-P is further metabolized to D-glyceraldehyde and dihydroxyacetone phosphate by F-1-P aldolase or aldolase B. Aldolase B differs from aldolase A and C in that the latter isozymes act principally on fructose-1,6-diphosphate. In the seminal vesicles, the lens of the eye, and peripheral



**FIGURE 93-3** Pathways of fructose metabolism.

nerves, fructose can be metabolized to sorbitol. In normal subjects, *in vivo* radioisotope studies have shown that fructose is converted to glucose solely by way of F-1-P (85). Sorbitol does not appear to be an intermediate. In two patients with hereditary fructose intolerance (HFI) due to F-1-P aldolase deficiency, it was estimated that 12–20% of fructose was metabolized by way of fructose-6-phosphate.

In humans, deficiencies in hepatic fructokinase and F-1-P aldolase have been described. Inactivity of fructokinase is responsible for essential fructosuria (fructosemia), whereas deficiency of F-1-P aldolase results in HFI. Fructose-1,6-diphosphatase deficiency is sometimes included among the disorders of fructose metabolism, but it seems more appropriate to list it as one of the gluconeogenic disorders described later in this chapter.

### 93.5.2 Essential Fructosuria

The incidence of this benign condition is estimated as 1:130,000 in the general population (86). However, essential fructosuria usually causes no symptoms and the incidence may be somewhat higher. Transmission follows an autosomal recessive pattern. The genetic defect is a deficiency of hepatic fructokinase. Ingested fructose is not well metabolized by the liver and reaches to high levels in the blood with overflow into the urine. The presence of the sugar in the urine can readily be demonstrated. No treatment is necessary.

### 93.5.3 Hereditary Fructose Intolerance

**93.5.3.1 Clinical Aspects.** The clinical manifestation in individuals with HFI may vary with the age at which fructose or sucrose is introduced into the diet and with

the quantity of sugar ingested. In some individuals, symptoms are subtle. In infants, ingestion of fructose may produce findings similar to those found in galactosemia and seizures. Because many formulas contain sucrose, the opportunities of an affected infant for exposure to fructose are increased accordingly. In older children and in adults with HFI, ingestion of fructose, sucrose or sorbitol causes abdominal pain and lowers the blood glucose level precipitously. Pallor, vomiting, sweating, and even coma may occur. It is typical for these individuals to develop a strong aversion for all sweets and to be free of dental caries.

**93.5.3.2 Biochemical Aspects.** The biochemical defect in HFI is a deficiency of liver F-1-P aldolase (aldolase B). Enzyme activity is usually less than 10% of normal when F-1-P is used as the assay substrate, and it is between 10 and 50% of normal when fructose-1,6-diphosphate is the substrate. The enzyme deficiency can also be demonstrated in intestinal mucosa. Blood cells cannot be used for diagnosis because the enzyme is not present in leukocytes or erythrocytes.

Whereas the diagnosis of HFI can be suspected on clinical grounds, laboratory confirmation is essential. Untreated patients ingesting fructose in their diet may excrete large amounts of this sugar in their urine and also show a gross generalized aminoaciduria. A fructose tolerance test, administered with caution, can be a useful first step in facilitating diagnosis. Assay of the enzyme requires either a biopsy sample of liver or intestinal mucosa. Administration of fructose, either orally or parenterally, is followed by a fall in the blood glucose level and serum inorganic phosphate (presumably due to its utilization in the formation of F-1-P). The uric acid level in blood rises from rapid degradation of purine nucleotides to uric acid. The hypoglycemia is related to

inhibition of glycogenolysis by F-1-P. Molecular diagnosis is possible (see section on genetic aspects).

HFI may be confused biochemically with tyrosinosis in early infancy insofar as elevation of blood tyrosine and methionine levels have been observed in some cases, presumably because of liver damage. The gross generalized aminoaciduria is akin to that seen in galactosemia patients and may result from toxic action of F-1-P on the proximal renal tubules. Observation of fructosuria, however, serves to distinguish HFI from galactosemia or tyrosinosis.

**93.5.3.3 Treatment.** The clinical manifestations in young infants with HFI may be severe, and prompt elimination of fructose and sorbitol from the diet is important. Major sources of fructose include cane sugar, honey, fruits, and formula using sucrose as the source of carbohydrate. The prognosis for treated patients is good. Liver and kidney damage is reversed, and neurologic residuals are uncommon. The use of fructose infusion as a source of calories in hospitalized adult patients must be approached with caution until it is known that the patient does not have HFI.

**93.5.3.4 Genetic Aspects.** The frequency of occurrence of HFI in the general population is not known because many patients with HFI may not be recognized. An incidence of 1:20,000 has been reported for Switzerland (87). The defect is inherited as an autosomal recessive trait. Heterozygote detection, which has now been accomplished through DNA testing and P-magnetic resonance spectroscopy, was previously complicated by relative inaccessibility of tissue for enzyme assay and the inability to differentiate normals from heterozygotes by parenteral loading with fructose (86).

The gene for aldolase B is located on chromosome 9q21.3–q22.2 and consists of nine exons that code for 363 amino acids (88). At least 40 mutations (e.g. missense, nonsense, deletions, splice-site) were identified among HFI patients, two of which are upstream of the protein-coding region (89). Three mutations: p.Ala149Pro, p.Ala174Asp, and p.Asn334Lys account for 87% of alleles in the European HFI population and for 68% in the North American population. One recently discovered mutation, IVS1+1G>C, has a higher allele frequency (6%) in African-American and Hispanic HFI patients (89). A patient with partial activity was found to have a nonsense mutation in exon 3 and a missense mutation in exon 5 (90). A study in Napoli, Italy, identified six patients (homozygous p.Ala150Pro and p.Ala175Asp) with the same deletion at exon 5 (91). Therefore, it is possible to use DNA analysis as a way to confirm the diagnosis instead of enzyme assay in liver biopsy samples (92).

## 93.6 DISORDERS OF PENTOSE METABOLISM

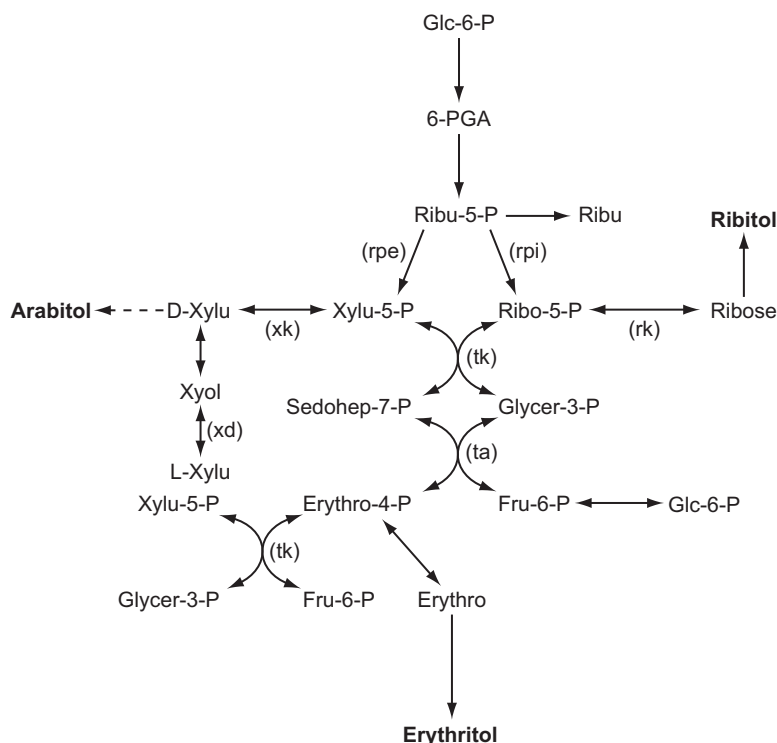
It is well recognized that about 90% of glucose metabolism in animal tissues is through the glycolytic pathway

and the remaining 10% through the hexose monophosphate pathway. The hexose monophosphate shunt leads to the formation of pentoses as well as providing NADPH (Figure 93-4). One of the metabolites, ribose-5-phosphate, is used in the biosynthesis of ribonucleotides and deoxyribonucleotides. Through the transketolase and transaldolase reactions, the pentose phosphates can be converted back to fructose-6-phosphate and glucose-6-phosphate. Sugar alcohols (e.g. erythritol, ribitol, arabitol) are formed through the respective dehydrogenases. The X-linked glucose-6-phosphate dehydrogenase deficiency, the most prominent genetic disorder in humans, is a well-known cause of hemolytic anemia. The prevalence of the deficiency, given the enzyme is present at reduced levels and not completely absent (incompatible with life), is attributed to the mutation's role in protecting against malaria (93). The mutation has also been linked to prevention of coronary heart disease (93,94). The congenital abnormalities in pentose metabolism resulting in clinical manifestations have been recently uncovered and include transaldolase deficiency and ribose-5-phosphate isomerase deficiency.

### 93.6.1 Transaldolase Deficiency

The first case of transaldolase (TALDO) deficiency was described by Verhoeven et al. in 2001 in which a female infant was born from consanguineous Turkish parents (95). The patient presented with hepatosplenomegaly in her early life. Biochemical abnormalities reveal elevated levels of arabitol, ribitol, and erythritol in the urine. Liver function enzymes and intellectual development were normal. At 10 years of age, the patient was found to have a cirrhotic liver, height at the 10th percentile and weight for height at the 2nd percentile, persistent thrombocytopenia, and elevated sugar alcohols, as previously found in both urine and plasma. Indirect assays of transketolase and transaldolase in cultured lymphoblast homogenates pointed out the defect in the transaldolase reaction with absence of fructose-6-phosphate and glucose-6-phosphate from the incubations. Molecular study revealed a homozygous deletion of 3 bp of the transaldolase gene from this patient. A second case was a neonate who died from severe hepatopathy and cardiomyopathy at the age of 18 days (96). At this time, only 10 affected individuals from six families have been identified, all of whom presented with liver disease (97). The disease has broad phenotypic heterogeneity, ranging from fetal hydrops to slow-progressing liver cirrhosis. In all 10 patients, the leading symptoms reported during the neonatal period were bleeding tendencies with thrombocytopenia, abnormal liver function tests, hepatosplenomegaly, hemolytic anemia, and dysmorphic features (down-slanting palpebral fissures, low-set ears, and cutis laxa). Interestingly, mental development and motor development were normal in majority of the patients who were assessed (three patients died before 6 months of age). The biochemical





**FIGURE 93-4** The pentose pathways. Erythro, erythrose; Erythro-4-P, erythrose-4-phosphate; Fru-6-P, fructose-6-phosphate; Glc-6-P, glucose-6-phosphate; Glycer-3-P, glyceraldehydes-3-phosphate; 6-PGA, 6-phosphogluconic acid; Ribo, ribose; Ribo-5-P, ribose-5-phosphate; Ribu, ribulose; Ribu-5-P, ribulose-5-phosphate; rk, ribokinase; rpe, ribulose-5-phosphate epimerase; rpi, ribose-5-phosphate isomerase; Sedohep-7-P, sedoheptulose-7-phosphate; ta, transaldolase; tk, transketolase; xd, xylitol dehydrogenase; xk, xylulokinase; Xylol, xylitol; Xylu, xylulose; Xylu-5-P, xylulose-5-phosphate. (Modified from Huck, J. H. J.; Struys, E. A.; Verhoeven, N. M., et al. *Profiling of Pentose Phosphate Pathway Intermediates in Blood Spots by Tandem Mass Spectrometry: Application to Transaldolase Deficiency*. Clin. Chem. **2003**, 49, 1375–1380.)

profiles of all affected patients were similar: each included elevated urine erythritol, arabitol, ribitol, sedoheptitol, perseitol, sedoheptulose, mannoheptulose, and sedoheptulose-7P (97). Another hallmark metabolite, erythronic acid, was recently identified by urine NMR spectroscopy (98). Diagnostic confirmation can be completed by sequence analysis of the transaldolase gene as well as by measurement of TALDO activity in fibroblasts, lymphoblasts, or liver tissue.

### 93.6.2 Ribose-5-Phosphate Isomerase Deficiency

The first and only known case to date of ribose-5-phosphate isomerase (RPI) deficiency was reported and detailed by Huck et al. (99). The affected male patient had psychomotor retardation from early childhood, developing epilepsy at 4 years of age. Thereafter, a slow neurologic regression developed with prominent cerebellar ataxia, some spasticity, optic atrophy, and a mild sensorimotor neuropathy. Magnetic resonance imaging of the brain at ages 11 years and 14 years showed extensive abnormalities of the cerebral white matter. Proton magnetic resonance spectroscopy of the brain revealed elevated levels of ribitol and D-arabitol. These pentitols were also increased in urine and plasma, similar to what is found in patients with transaldolase deficiency.

Enzyme assays in cultured fibroblast homogenates showed deficient activity in ribose-5-phosphate isomerase. Molecular analysis showed a single base-pair deletion and a single transition.

### 93.6.3 Essential Pentosuria

Essential pentosuria is a benign disorder encountered principally in Ashkenazi Jews and is inherited as an autosomal recessive trait. The urine contains L-xylulose, which is excreted in increased amounts (1–4g daily) because of a block in the conversion of L-xylulose to xylitol due to xylitol dehydrogenase deficiency (100). The condition is usually discovered accidentally, and no treatment is required.

## 93.7 GLYCOGEN STORAGE DISEASES

### 93.7.1 Glycogen Metabolism

Glycogen is the principal storage form of carbohydrate in animal cells; it is present in virtually every type of tissue. Glycogen is a polymer composed of highly branched chains of glucose molecules. The glucose units are linked in 1–4 positions, whereas the branch points are attached in 1–6 linkages. Glycogen molecules are relatively large spherical structures, and their aggregations

are easily recognizable by electron microscopy in cell cytoplasm. Liver has the highest glycogen content of all tissues, usually 3–5 g/100 g wet weight. Skeletal muscle normally contains 0.5–1.0 g/100 g. The glycogen content of liver increases following carbohydrate-rich meals and decreases during periods of fasting. During a fast, liver glycogen is degraded to glucose, which is released into the circulation to maintain glucose homeostasis.

Glycogen synthesis appears to begin with autoglucosylation of the glycoprotein primer glycogenin. The process starts with the attachment of one to two glucose units to a tyrosine residue of the glycogenin. Subsequently, a divalent cation-dependent autoglucosylation reaction occurs, resulting in the addition of six to seven glucosyl residues to the glycogenin. This molecule—glycogenin plus eight glucose residues—serves as the glucan primer for the next step. However, in order for the glucan primer to be utilized, it requires the glycogenin to form a complex with a different enzyme, glycogen synthase. Glycogen synthase is stimulated by insulin and acts as the rate-limiting enzyme of gluconeogenesis. When complexed with the glycogenin primer, glycogen synthase elongates the glucan primer using units from UDP-glucose in the presence of  $Mn^{2+}$ . In order for the chain to continue growing, the glycogen synthase must gradually detach while brancher enzyme completes the process by branching the glycogen molecule.

The following two enzymatic reactions are particularly important in glycogen synthesis:



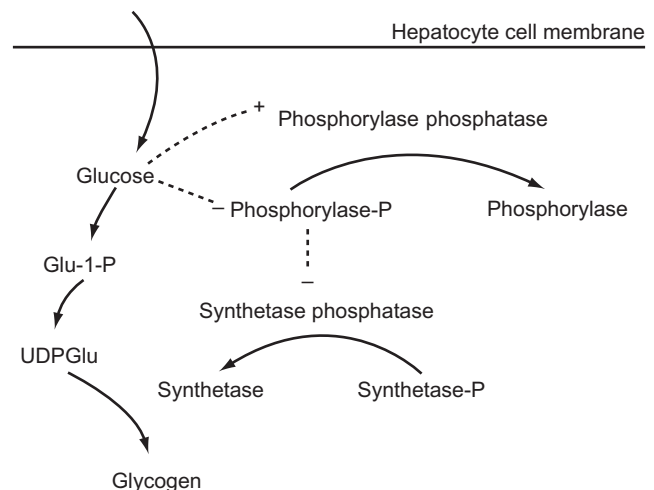
Reaction 1 involves the formation of UDPG and pyrophosphate (PP) from glucose-1-phosphate and uridine triphosphate (UTP) by the enzyme uridine diphosphate glucose pyrophosphorylase. Reaction 2, catalyzed by glycogen synthase, involves the addition of one glucosyl unit to the glycogen molecule taken from uridine diphosphate glucose, leaving uridine diphosphate (UDP). Brancher enzyme adds new branch positions to the chains.

The regulation of glycogenolysis in liver is complex (Figure 93-5). The most clearly defined mechanism involves activation of the enzyme adenyl cyclase by the hormones glucagon and epinephrine. This increases the cyclic adenosine monophosphate (cAMP) level in the cytosol, which activates protein kinase, phosphorylase kinase (PhK) and phosphorylase in rapid sequence by phosphorylation of these enzymes. Phosphorylase acts upon the terminal units of the glycogen chains liberating glucose-1-phosphate. Debrancher enzyme removes branch points and liberates free glucose.

Several other factors have been shown to affect the activity of phosphorylase, in addition to glucagon and epinephrine. Both vasopressin and angiotensin I activate phosphorylase without increasing cAMP. Ionic calcium enhances and potassium ion inhibits phosphorylase activation. Insulin acts at several levels to inhibit

phosphorylase activity. An amylase that is present in hepatocytes removes oligosaccharide chains, 3–5 units long, from glycogen. Lysosomal acid alpha glucosidase (acid maltase) breaks down glycogen and provides an alternate pathway for glycogen catabolism. The starch binding domain containing protein 1/genthiolin 1 (stbd1) is a recently identified transporter protein important in glycogen metabolism due to its role in attaching to glycogen thereby aiding in its anchorage to various cellular locations (101). The rate of glycogen synthesis increases when the concentration of glucose and insulin rise in the blood and the glucagon level falls. Glycogen synthase is the rate-limiting enzyme in glycogen synthesis, and it is very highly regulated. Insulin stimulates the activity of glycogen synthase by decreasing its level of phosphorylation, either involving activation of p13 kinase through activation of insulin receptor, or involving lowering the level of cAMP in liver, thereby decreasing the level of phosphorylase b and releasing the inhibition of the protein phosphatase 1. A fall in blood glucagon concentration also leads to deactivation of phosphorylase and inhibition of glycogenolysis. In addition to glycogenolysis, gluconeogenesis also plays an important role in maintaining blood glucose levels (102). A substantial amount of glucose production comes from gluconeogenesis during fasting conditions.

The disorders of glycogen metabolism, generally named GSDs, result from deficiencies of various enzymes or transport proteins in the pathways of glycogen metabolism (for review, see Reference (102)). GSDs were originally identified numerically: from GSD type Ia (G6Pase deficiency) to GSD type VI (hepatic phosphorylase deficiency). Now, there are more than 12 known forms of glycogenoses. The authors prefer to designate them according to the enzyme deficiency, and have arbitrarily divided GSDs into three groups: those predominantly affecting the liver, those predominantly affecting muscle tissues and those primarily involving the heart. There are



**FIGURE 93-5** Key enzymes involved in the regulation of glycogenolysis.

disorders where several of these organs and others are involved. Diagnostic procedures for GSDs include carbohydrate and glucagon tolerance tests, measurement of tissue glycogen content, enzyme assays, and DNA mutational analysis. Although GSDs are usually recognized in early childhood, some forms have been found in adults.

## 93.8 GSDS PRIMARILY INVOLVING THE LIVER

### 93.8.1 GSD Type Ia (Glucose-6-Phosphatase Deficiency, von Gierke Disease)

**93.8.1.1 Clinical Aspects.** GSD type I has two biochemical subtypes—GSD Ia and GSD Ib—each derived from deficiencies in particular components of the glucose-6-phosphatase (G6Pase) complex. The G6Pase complex is comprised of the catalytic subunit (G6Pase, glucose-6-phosphatase alpha, G6Pase- $\alpha$ ; responsible for GSD Ia) and glucose-6-phosphate translocase (G6PT; responsible for GSD Ib) (103).

GSD type Ia, described in 1929 by von Gierke, was the first abnormality of glycogen metabolism to be recognized (102). The deficient enzyme, G6Pase, is a key protein in both glycogenolysis and gluconeogenesis that aids in the conversion of glucose-6-phosphate to glucose. Clinical manifestations usually appear in the first 6 months of life. The infant comes to medical attention either because of marked hepatomegaly or because of symptoms of hypoglycemia. Brief periods of fasting (3–4 h) cause irritability secondary to hypoglycemia, usually without convulsions, and tachypnea due to acidosis. Perspiration and heat intolerance accompany a high metabolic rate. Epistaxis and bruising are common. Bowel movements tend to be loose. Renomegaly is characteristic, but the spleen and heart are of normal size. The more severely affected infants and children are prone to severe lactic acidosis during minor infections, and, in the past, the mortality rate during childhood was high. Untreated children grow slowly, and their sexual maturation is delayed.

In GSD I, uric acid production and excretion are increased. Consequently, hyperuricemia and gouty arthritis are common. Renal stones and nephrocalcinosis associated with distal renal tubular acidosis are also common. Patients with GSD I have much higher recurrent nephrolithiasis rate than the general population, as well as higher levels of hypocitraturia than other individuals who suffer from stone formation (104). A type of glomerulosclerosis, similar to that seen in diabetes mellitus with hyperfiltration and microalbuminuria at onset of the renal dysfunction, has been recognized. Diffuse interstitial nephritis and renal failure are late complications.

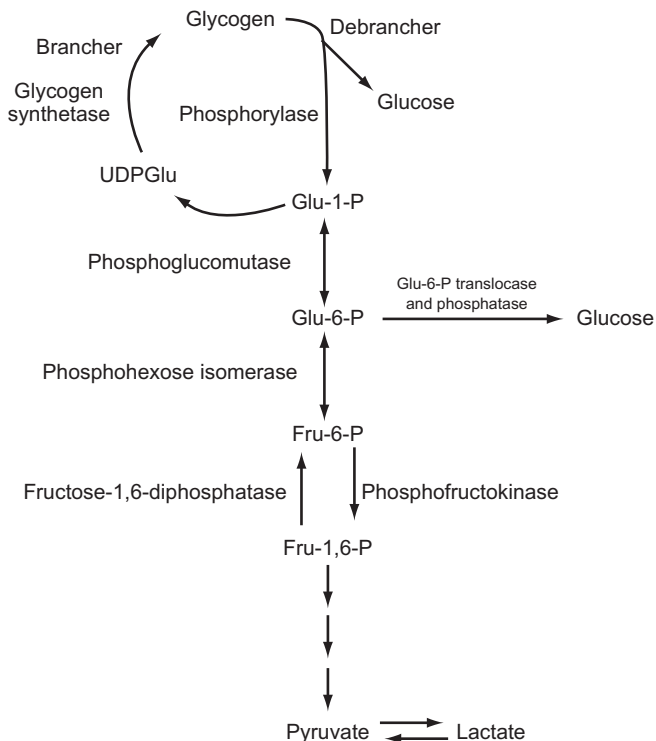
Cholesterol, mainly low-density lipoprotein cholesterol and triglyceride levels are invariably elevated in blood and occasionally result in cutaneous xanthoma.

Hepatic adenoma formation appears to be almost universal by adolescence. Hemorrhagic necrosis of the hepatic adenomas may cause severe acute abdominal pain and anemia. Several patients have died with hepatocellular carcinoma, suggesting that the adenomas are premalignant lesions. Factors that result in the adenoma to carcinoma transformation include genetics and metabolic control. A recent study showed no significant contrast in metabolic balance between GSD Ia patients who developed adenomas and those who did not (105). However, the presence of adenomas did correlate with significantly elevated serum interleukin 8 in GSD Ia patients, consistent with the hypothesis that chronic injury and elevation of hepatic chemokines play a role in adenoma formation (106).

Other complications include osteoporosis with risk for bone fractures, neurologic deficits (apparently from hypoglycemia), seizures, iron refractory anemia, pancreatitis, thyroid abnormalities and pulmonary hypertension. There is considerable variability in the severity of manifestations in GSD I. A small number of individuals are mildly affected and some are discovered as adults with hepatomegaly and gout. Virtually all women have ultrasound findings consistent with polycystic ovaries. However, the other clinical features of polycystic ovary syndrome such as acne and hirsutism are not noted. Type I GSD also does not appear to affect ovulation and fertility: successful pregnancy has been noted in many individuals with GSD I, although symptoms may be exacerbated due to the pregnancy-related increase in renal perfusion and maternal volume (107). In addition, hypoglycemia may also become more difficult to control. Increased vaginal spotting has also been known to occur secondary to the bleeding abnormalities.

**93.8.1.2 Biochemical Aspects.** The enzyme G6Pase is associated with a complex of translocases, or transport proteins (108). The active site of the enzyme faces the lumen of the endoplasmic reticulum in hepatocytes. The enzyme complex appears to consist of the enzyme protein situated in close proximity to three translocases (T1, T2, and T3), which transport glucose-6-phosphate, phosphate and glucose, respectively, across the membrane of the endoplasmic reticulum. The clinical and biochemical features of GSD Ia result from deficiency of the catalytic subunit of the G6Pase enzyme. The absence or defective function of T1 (also called glucose-6-phosphate translocase or G6PT) results in GSD Ib, which is described later. Defects involving the other proteins of the complex are not well characterized, but may appear clinically similar to deficiency of the enzyme itself.

The production of glucose by glycogenolysis and gluconeogenesis is impaired in GSD Ia (Figure 93-6). Normal plasma glucose concentrations cannot be maintained in the postprandial state, and the resultant hypoglycemia inhibits insulin and enhances pancreatic glucagon secretion. The decrease in the insulin to glucagon ratio in blood stimulates glycogen breakdown in the liver, and in



**FIGURE 93-6** Glycogen metabolism and enzyme deficiency (Glu-6-P translocase and phosphatase) in GSD type I.

the absence of G6Pase, lactic and pyruvic acids are produced in excess, instead of glucose.

The biochemical abnormalities in blood are characteristic in GSD type I. Blood obtained after a brief (3–4 h) fast reveals hypoglycemia (10–40 mg/dL) and elevated lactic acid levels (50–100 mg/dL). In this condition, lactate serves as an important substitute fuel for the brain, and symptoms of neuroglycopenia may be minimal or absent despite hypoglycemia. Hyperlipidemia (primarily hypertriglyceridemia and hypercholesterolemia) and hyperuricemia are almost always present. The plasma may be “milky” in appearance as a result of a striking elevation of triglycerides. Uric acid production is stimulated by glucagon secretion, which causes catabolism of hepatic ATP to uric acid. Glucagon administration causes little if any rise in blood glucose but a marked rise in blood lactate and uric acid levels. Intravenous administration of galactose or fructose causes a rise in blood lactate but no or minimal rise in blood glucose. Other types of GSD such as debrancher and phosphorylase deficiencies, although in some ways clinically similar to GSD type I, have normal blood lactate values in the fasting state.

Fructose-1,6-diphosphatase deficiency is similar clinically to GSD type I. Blood lactate levels are elevated during fasting, and glucagon administration causes a rise in blood glucose but not in lactate. Cholesterol and triglycerides levels are usually normal. The diagnosis of type I disease can be suspected on the basis of clinical presentation and abnormal plasma lactate and lipid values. In addition, administration of

glucagon or epinephrine causes little or no rise in blood glucose but increases lactate levels significantly. Before the glucose-6-phosphatase and glucose-6-phosphate translocase genes were cloned, a definitive diagnosis required a liver biopsy to demonstrate the deficiency. Gene-based mutation analysis now provides a noninvasive way of diagnosis for most patients with types Ia and Ib disease (108).

Histologic findings in the liver in GSD type I include distended hepatocytes filled by glycogen and fat. Despite hepatomegaly, liver enzymes are usually normal or near normal.

**93.8.1.3 Treatment.** The main goal of treatment is the maintenance of euglycemia. Meeting this goal suppresses lactic acid production, decreases blood lipid and uric acid levels and markedly enhances growth. Lactic acid production can be suppressed by administering glucose enterally at a rate of ~8–9 mg/kg/min in an infant, and 5–7 mg/kg/min in an older child. Frequent feedings of carbohydrate during the daytime and supplementation of the diet with uncooked cornstarch (approximately 1.75–2.5 g/kg/6 h, day and night) have been very successful in maintaining euglycemia and promoting growth, especially in older children. Feeding of pancreatic enzymes along with cornstarch has been recommended for infants (who may not adequately digest the starch). New starch products that are thought to be longer acting, better tolerated and more palatable are currently in development. In a short-term double-blind cross-over pilot study comparing uncooked, physically-modified cornstarch to traditional cornstarch, longer duration of euglycemia and better short-term metabolic control in the majority of GSD I patients treated with the new starch was noted (109). Larger studies are needed to confirm the efficacy of this new product.

An alternative nighttime regimen is the infusion of a concentrated glucose solution or high-carbohydrate formula overnight through nasogastric tube or by gastrostomy to maintain euglycemia. Dangers associated with overnight tube feeding include hypoglycemia secondary to accidental interruption of the infusion, and aspiration from regurgitation of the infusate. These treatment regimens produce a marked improvement in strength and growth. Allopurinol may be needed to control hyperuricemia. The hyperlipidemia can be reduced with lipid-lowering drugs such as HMG-CoA reductase inhibitors and fibrates. Angiotensin-converting enzyme (ACE) inhibitors, like captopril, seem to be beneficial in treating microalbuminuria, an early indicator of renal dysfunction in GSD I patients. Citrate supplement may be beneficial in preventing or ameliorating nephrocalcinosis and the development of urinary calculi (110).

Liver transplantation can be lifesaving and can eliminate the problems of recurrent adenomas with the risk for malignant transformation and metabolic derangements such as hypoglycemia, lactic acidosis, and hyperlipidemia. Small hepatic adenomas (<2 cm in diameter)



may be treated with percutaneous ethanol injection or transcatheter arterial embolization.

**93.8.1.4 Genetic Aspects.** G6Pase deficiency is inherited as an autosomal recessive disease. The incidence is estimated to be in the order of 1:150,000 births but may be higher in some population groups. The gene for G6Pase is called G6PC1 and has been mapped to chromosome 17q21 (102). DNA isolated from peripheral blood leukocytes is adequate for molecular diagnosis. The mutations p.Arg83Cys and p.Gln347X are common mutations in Caucasians. Chorionic villus biopsy and amniocentesis have been used for prenatal diagnosis (111). The molecular basis for GSD I has been studied in detail and reviewed (108).

## 93.8.2 GSD Type Ib

**93.8.2.1 Clinical Aspects.** GSD type Ib is caused by deficiency of G6PT, the transport protein portion of the G6Pase complex. The diagnosis of GSD Ib is usually made during infancy, but it has been also recognized during adult life. GSD Ia and Ib are similar clinically, except that neutropenia and neutrophil dysfunction are characteristic of Ib. The neutrophil abnormalities result in frequent infections, oral mucosal lesions, gingivitis and chronic inflammatory bowel disease. Severely reduced chemotaxis and phagocytosis, deficient superoxide anion generation, and altered calcium metabolism have been demonstrated in GSD Ib neutrophils.

**93.8.2.2 Biochemical Aspects.** Some individuals with all the clinical and biochemical manifestations of GSD I have normal G6Pase activity when their liver tissue is assayed after freezing; but when the assay is done on fresh tissue, no enzyme activity is detected. This enzyme is thought to be located on the luminal surface of hepatic microsomes. A specific transport protein is required for the passage of G6P into the microsome lumen. As elucidated in the GSD Ia Biochemical Aspects section, GSD Ib is due to lack of this transport protein, T1 or G6PT. When microsomal membranes are disrupted chemically or by freezing, the enzyme is exposed and expressed in the assay procedure. The lack of enzyme activity in unfrozen liver specimens occurs because G6P does not have access to the enzyme under these conditions. The mechanism for the neutropenia and neutrophil dysfunction in patients with GSD Ib is attributed to premature apoptosis (112). This is thought to be due to lack of NADPH resulting in a diminished capacity to (re) generate reduced glutathione (113,114). Cells would thus be rendered more susceptible to oxidative damage and undergo apoptosis. More recently another mechanism, a defect in neutrophil glycosylation, has been identified as a cause for the neutrophil dysfunction in GSD Ib (115).

**93.8.2.3 Treatment.** The bone marrow in patients with GSD Ib contains abundant neutrophils, and the neutropenia can be improved with the administration of granulocyte-colony stimulating factor (G-CSF). Prolonged

treatment with G-CSF results in a marked reduction in the frequency of infection and partial resolution of inflammatory bowel disease. Derivatives of 5-aminosalicylic acid (5-ASA) may be a helpful adjunct for treating the inflammatory bowel disease in GSD Ib patients; renal function needs to be monitored (112). In case, if patients do not respond well to G-CSF or 5-ASA therapies for the gastrointestinal manifestations, adalimumab may be an effective therapeutic option (116). Liver transplantation may be beneficial for indications as it is in GSD Ia (117). Neutropenia, however, typically persists in type Ib patients after liver transplantation, necessitating continuous treatment with G-CSF in some instances.

**93.8.2.4 Genetic Aspects.** The inheritance pattern is autosomal recessive. The gene for GSD Ib, SLC37A4 encoding G6PT, has been mapped to 11q23.3 (118). Two mutations, c.1042delCT and p.Gly339Cys, are prevalent in Caucasian patients, whereas p.Trp118Arg appears to be most common in Japanese patients. At this time, over 85 mutations have been identified, including a novel missense mutation described in a case report that appears to be associated with early-onset neutropenia (119,120). Carrier detection and prenatal diagnosis are possible with the use of molecular techniques.

Successful pregnancies with careful management of varying mild complications have been noted in patients with GSD Ib (121).

## 93.8.3 GSD Type Ic

Several patients with clinical features similar to those of GSD Ib, including neutropenia, have been reported to have a defect in the microsomal transport of phosphate and pyrophosphate (labeled T2). This defect, once characterized as a separate disease by the name of GSD Ic, is now known to be allelic to GSD Ib, alluding to the discovery that the G6PT gene functions as both a G6P and a phosphate transporter (102,122). This binary function explains how several patients with GSD Ic have had mutations in the G6PT reported (102).

## 93.8.4 GSD Type III (Amylo-1,6-Glucosidase (Debrancher) Deficiency, Limit Dextrinosis, Cori Disease, Forbes Disease)

**93.8.4.1 Clinical Aspects.** GSD type III (GSD III) is caused by a deficiency of glycogen debrancher enzyme (GBE), a single polypeptide that has both amylo-1,6-glucosidase (EC 3.2.1.33) and 4- $\alpha$ -glucanotransferase (EC 2.4.1.25) activities (123). GDE has three main domains, which include the transferase domain, glucosidase domain, and a carbohydrate-binding domain (CBD). Mutations in each domain affect the corresponding function of the overall enzyme, however, mutations in the CBD tend to prove the most severe (123). Inherited deficiencies of debrancher enzyme have been reported to involve both liver and muscle (cardiac

and skeletal) (GSD IIIa) and liver only (GSD IIIb). It is estimated that 75–85% of those with GSD III have type IIIa, approximately 15–25% have type IIIb. Debranching enzyme and phosphorylase are responsible for complete degradation of glycogen. When debranching enzyme is defective, glycogen breakdown is incomplete, and an abnormal glycogen accumulates that has short outer chains and resembles limit dextrin.

The clinical manifestations of GSD III can be recognized in infancy. Abdominal distention due to hepatomegaly is moderate to marked; splenomegaly is minimal or moderate. Hypoglycemia is present and often accompanied by ketonuria, especially after overnight fasting. Unlike in GSD I, there is no bleeding tendency, heat intolerance, loose stools, rapid breathing, or enlargement of the kidneys in GSD III.

These children usually survive childhood without difficulty, although growth may be slow. Hepatomegaly decreases with maturity, adenoma formation is infrequent, but cirrhosis, portal hypertension, hepatocellular carcinoma, and liver failure may occur.

In patients with muscle involvement (type IIIa), muscle weakness is usually minimal during childhood but can become severe during the third or fourth decade of life, as evidenced by slowly progressive weakness and muscle wasting (124). Electromyographic changes are consistent with a widespread myopathy, and nerve conduction may be abnormal (125). Ventricular hypertrophy is frequent, but overt cardiac dysfunction is rare. A recent case study of a GSD III patient presenting with premature coronary artery disease showed that patients with GSD III may have a higher susceptibility to cardiac complications and therefore may need to be screened and subjected to preventative measures (126). There are also reports of sudden cardiac death, likely due to cardiac arrhythmias. Myocardial fibrosis has also been reported in a 32-year-old patient with GSD III using cine cardiovascular magnetic resonance (127). In some patients, hepatic symptoms may be so mild that the diagnosis is not made until adulthood, when neuromuscular disease becomes manifest. Multi-parametric functional NMR imaging and spectroscopy have been used as noninvasive methods for quantification of muscle-wasting myopathy in GSD III (128). Some individuals with GSD III may be at an increased risk of osteoporosis (129). The etiology is unclear but likely related to the underlying myopathy. Polycystic ovary appears to be a common finding in female patients; fertility, however, does not seem to be reduced (130). There have been reports of successful pregnancy outcome in GSD III (131).

**93.8.4.2 Biochemical Aspects.** Defects in either enzymatic subunit—4- $\alpha$ -glucanotransferase or amylo-1,6-glucosidase—of debrancher enzyme result in glycogen accumulation in liver and many other tissues, including leukocytes and erythrocytes. In the fasting state, blood glucose levels are sustained primarily by gluconeogenesis

and by limited glycogen degradation. A moderate elevation of serum transaminase activity is common in GSD III. Hyperlipidemia may be present but hyperuricemia is usually absent (132). Blood lactate levels are normal in the fasting state, and there is no significant rise in blood glucose or lactate values following glucagon administration. Creatine phosphokinase activity in blood is high in GSD IIIa and reflects debrancher enzyme deficiency in muscle. However, if this appears normal it does not exclude a diagnosis of GSD IIIa. The histology of the liver is characterized by a universal distention of hepatocytes by glycogen and by the presence of fibrous septa. The fibrosis and the paucity of fat distinguish type III from type I glycogenosis. The fibrosis can range from minimal periportal fibrosis to micronodular cirrhosis.

Debrancher deficiency can be confirmed by demonstrating the absence of enzyme activity in liver and muscle in GSD IIIa and only in liver in GSD IIIb. Cultured fibroblasts may be used to confirm this deficiency. Erythrocytes and leukocytes have also been used for diagnosis (133).

**93.8.4.3 Treatment.** Therapy is directed toward preventing hypoglycemia and reducing the breakdown of muscle protein for gluconeogenesis by frequent feeding, a high-protein diet and the avoidance of prolonged periods of fasting. Treatment regimens that are employed for type I GSD have been used for type III. Cornstarch therapy reduces the demand for gluconeogenesis. Limitation of fructose and galactose intake is not necessary as in the case of GSD I although restriction of dietary fat seems prudent. A high-protein diet with particular attention to overnight carbohydrate and protein administration can markedly improve growth and has been advocated to treat or prevent myopathy. Successful liver transplantation for hepatic failure has been reported.

**93.8.4.4 Genetic Aspects.** The type III glycogenoses are inherited as autosomal recessive traits. The gene for debranching enzyme, amylo-1,6-glucosidase, is located on chromosome 1p21. At least 50 different mutations that cause type III disease have been identified (123,134). Two mutations (c.18\_19delGA (p.Gln6HisfsX20) and c.16C>T (p.Gln6X)), both located in exon 3 at amino acid codon 6, are exclusively found in the subtype IIIb (132,135). Various mutations have been recognized in specific populations: c.2309-1 G>A (11.8% of non-Hispanic Caucasians), c.1384delG (about half of the Hispanic alleles), and several founder mutations in patients from north Africa (Sephardic Jews), the Faroe Islands, Italy, the Mediterranean region, and Turkey (123). A study of 15 Irish patients with GSD III from eight different families recently revealed a variety of novel mutations including p.Thr512fs, p.Ser736fs, p.Ala1400fs, p.Lys1407fs, p.Tyr519X and p.Asp627Tyr, in addition to highlighting how the severity of the symptoms reflects the allelic composition (136). Carrier detection and

prenatal diagnosis are possible with DNA-based linkage or mutation analysis.

### 93.8.5 Brancher Deficiency (GSD Type IV, Amylopectinosis, Amylo-1,4-1,6-Transglucosidase Deficiency, Anderson's Disease)

**93.8.5.1 Clinical Aspects.** Brancher deficiency is rare. Affected infants begin to show evidence of liver dysfunction within the first year of life, and death by 2–4 years appears to be the usual outcome. Features of hepatic failure and portal hypertension appear including growth failure, jaundice, hepatosplenomegaly, and prominent abdominal venous pattern. Hypotonia is common, but hypoglycemia is not a feature. Incidences of nonprogressive liver involvement have also been reported.

A neuromuscular form of type IV GSD has also been reported. Patients with this disease may present (1) at birth with fetal akinesia deformation sequence, fetal hydrops, arthrogryposis, and eventual death due to cardiac or respiratory complications (137), (2) at birth with severe hypotonia, muscle atrophy, and neuronal involvement and during the neonatal period with death (138), (3) in late childhood with myopathy or cardiomyopathy (139,140), or (4) as adults with diffuse central and peripheral nervous system dysfunction accompanied by accumulation of polyglucosan bodies in the nervous system (so-called adult polyglucosan body disease) (141).

**93.8.5.2 Biochemical Aspects.** An abnormal form of glycogen, with long-chain and infrequent branch points, accumulates in many cell types, including hepatocytes, skeletal and myocardial muscle cells, fibroblasts, leukocytes, and nerve cells. Hepatic cirrhosis is presumed to result from the collection of the abnormal type of glycogen. Carbohydrate and glucagon tolerance tests are normal, provided that liver failure is not severe at the time of testing.

Deficiency of brancher enzyme can be demonstrated by enzyme assay in liver tissue, in leukocytes, and in cultured fibroblasts. The histologic findings in the liver are characterized by both micronodular cirrhosis and faintly stained basophilic inclusions in the hepatocytes. The inclusions consist of coarsely clumped, stored material that is periodic acid-Schiff-positive and partially resistant to diastase digestion. Electron microscopy shows, in addition to the conventional glycogen particles, an accumulation of fibrillar aggregations typical of amylopectin. This disease must be differentiated from other forms of liver failure in infancy, such as neonatal viral hepatitis, biliary atresia, polycystic disease of liver and kidney, Wilson's disease, and 1-antitrypsin deficiency. Heterozygotes appear to be detectable by enzyme assay of cultured skin fibroblasts.

**93.8.5.3 Treatment.** There is no specific treatment for type IV GSD. For progressive hepatic failure, liver

transplantation has been performed. However, caution should be taken in selecting patients for liver transplantation because a nonprogressive hepatic form of the disease exists, and extrahepatic manifestations of the disease may manifest after transplantation in other forms.

**93.8.5.4 Genetic Aspects.** This disorder is inherited as an autosomal recessive trait. The enzyme is normally present in cultured amniotic fluid cells, and prenatal diagnosis has been accomplished (142). The glycogen branching enzyme (GBE) gene is located on chromosome 3p12, according to OMIM. Both hepatic and neuromuscular forms are caused by mutations in the same GBE gene, *GBE1* (102). There have been over 34 mutations and 17 polymorphisms found in the *GBE1* gene. The large number of mutations explains the phenotypic variety. Two missense mutations typically cause a milder form of GSD IV, while large deletions and protein-truncating mutations typically cause the severe congenital form (137). A number of the mutations occur in or near exon 12, suggesting that this region is a mutation “hotspot” for the disease (143).

### 93.8.6 GSD Type VI (Hepatic Phosphorylase Deficiency, Hers Disease)

**93.8.6.1 Clinical Aspects.** Hepatic phosphorylase deficiency was first identified by Henri-Géry Hers in 1959 when he saw three patients presenting with hepatomegaly and glycogen storage in the liver. Although the activities of the G6Pase catalytic subunit and debranching enzyme appeared normal, the activity of liver phosphorylase remained highly restricted (144). In 1960, Stetten and Stetten labeled this deficiency of hepatic phosphorylase “Hers Disease,” or GSD type VI (145).

In early childhood, patients with GSD VI usually present with hepatomegaly and growth retardation. The clinical course can vary from mild to severe; laboratory findings include hypoglycemia, hyperlipidemia, and hyperketosis. Lactic acid and uric acid appear normal. There is no involvement of the heart or skeletal muscles. Around puberty, the hepatomegaly begins to improve with age and usually disappears. Clinical heterogeneity exists; Beauchamp et al reported a variety of cases with a more severe clinical course including significant hepatomegaly, recurrent severe hypoglycemia and postprandial lactic acidosis (146). A case of a GSD VI female presenting with a hepatic tumor, which is usually only seen in patients with GSD Ia and III, suggests that screening for adenomas should be done for patients with GSD VI (147). Hepatocellular carcinoma was reported upon histological examination of the explanted liver of a GSD VI female who underwent liver transplantation due to rapidly growing hepatocellular adenomas (148).

**93.8.6.2 Biochemical Aspects.** Phosphorylase exists in three isoforms: the muscle (M), the liver (L), and the brain (B). Each isoform is encoded by genes designated as *PYGM*, *PYGL*, or *PYGB*, which are located on

chromosome 11, 14 and 20, respectively. Mutations of the *PYGM* gene are associated with GSD V (McArdle's Disease), a condition caused by muscle phosphorylase deficiency (see later). Mutations in the *PYGL* gene, on the contrary, elicit GSD VI (Hers Disease) or liver phosphorylase deficiency. Thus far, the *PYGB* gene has not been tied to any form of GSD.

Deficiency of phosphorylase (or one of the enzymes that leads to its activation) obstructs glycogen degradation in the liver. The absence of severe fasting hypoglycemia may be explained by the fact that phosphorylase activity is usually only partially deficient and that hepatic gluconeogenesis is intact. However, there are cases with severe fasting hypoglycemia. Laboratory studies reveal mild to moderate elevations of serum transaminase values, ketonemia and hyperlipidemia. Fasting blood lactic and uric acid levels are normal. Blood lactate values may triple following meals and after glucose, galactose, and fructose loading. Glucagon administration is reported to cause minimal glycemic response in patients with phosphorylase deficiency.

**93.8.6.3 Treatment.** Presentation of symptoms determines treatment protocol. Although most patients do not require a specific treatment, a high-carbohydrate diet and frequent feeding are effective in preventing hypoglycemia.

**93.8.6.4 Genetic Aspects.** The liver phosphorylase gene (*PYGL*) is on chromosome 14q21–22 and has 20 exons. Many mutations are known in this gene; several are missense mutations. One missense mutation of the *PYGL* gene, p.Gly233Asp, was identified in a Chinese patient, and a splice-site mutation in intron 13 was determined in the Mennonite population (149).

## 93.8.7 GSD Type IX (Phosphorylase Kinase Deficiency)

**93.8.7.1 Clinical Aspects.** Physical features of liver phosphorylase kinase (PhK) deficiency are usually recognizable within the first 2 years of life and include short stature and abdominal distention from moderate to marked hepatomegaly (102). The clinical severity of liver PhK deficiency varies considerably. Hyperketotic hypoglycemia, if present, is usually mild but can be severe in some cases. Ketosis may occur even when glucose levels are normal. In some children, there may be mild delays in gross motor development and hypotonia. Liver fibrosis can occur and progress to cirrhosis in rare cases, particularly in patients with *PHKG2* mutations (150). Liver adenoma appears to be very rare. Cognitive and speech delays have been reported in a few individuals, but it is not clear whether these delays are caused by PhK deficiency or whether they are coincidental (151,152). Polycystic ovaries are common in females with liver PhK deficiency (130). Renal tubular acidosis has been reported in a few cases (151,152). Cardiac manifestations have not been reported in liver PhK deficiency. Unlike in GSD I, lactic

acidosis, bleeding tendency, and loose bowel movements are not characteristic, although may be present. Although growth is retarded during childhood, normal height and complete sexual development are eventually achieved (153,154). As with debrancher deficiency, abdominal distention and hepatomegaly usually decrease with age and may disappear by adolescence. Most adults with liver PhK deficiency are asymptomatic, although further long-term studies are needed to fully assess the impact of this disorder in adults (154,155).

**93.8.7.2 Biochemical Aspects.** PhK is composed of four copies each of the four following subunits:  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ . The  $\gamma$  subunit provides the catalytic activity, which is regulated by phosphorylation of the  $\alpha$  and  $\beta$  subunits. The  $\delta$  subunit is calmodulin. Several isoforms of each have been identified. The  $\alpha$  subunit is encoded by the *PHKA1* gene in muscle and the *PHKA2* gene in liver; the  $\beta$  subunit is encoded by the *PHKB* gene and is alternatively spliced in liver and muscle; the  $\gamma$  subunit is encoded by *PHKG1* in muscle and *PHKG2* in liver, and the  $\delta$  subunit (calmodulin) is encoded by the *CALM1*, *CALM2*, and *CALM3* genes in both (152,156). PhK activity is reduced in the liver, erythrocytes, and leukocytes of most individuals with liver PhK deficiency. X-linked liver PhK deficiency, caused by mutations in the *PHKA2* gene, is subdivided into two biochemical subtypes: XLG1, with measurable deficiency of PhK activity in blood cells and liver in vitro, and XLG2 with normal PhK activity in blood cells and variable activity in liver in vitro, but presumably deficient in vivo. While not fully understood, it has been suggested that mutations affecting the regulation of the enzyme, such as missense mutations, result in XLG2, while mutations affecting the amount of protein, such as nonsense mutations, are more likely to result in XLG1 (157). Mutations causing these two subtypes also cluster in different functional domains of the protein (158).

Liver tissue histology shows steatosis, glycogenosis, and may show mild periportal fibrosis, and cirrhosis in rare cases. At diagnosis, individuals with liver PhK deficiency usually have elevated liver transaminases, mildly elevated triglycerides and cholesterol, normal uric acid and lactic acid concentrations, and normal glucagon response (142,156).

**93.8.7.3 Treatment.** In individuals with liver PhK deficiency, hypoglycemia can be prevented by frequent daytime meals that are high in complex carbohydrates and protein. In patients with normal renal function, protein can comprise 15–25% of total calories consumed. Cornstarch can be given with symptom-dependent dosage and timing (0.6–2.5 g/kg every 6 h). Hypoglycemia, if present, should be treated with oral intake. If not tolerated, intravenous glucose should be administered (156).

Growth may be improved by carbohydrate supplementation, presumably by reducing the demand for gluconeogenesis and increasing insulin secretion. Because of



liver enlargement, activities that might lead to abdominal trauma should be approached with caution.

**93.8.7.4 Genetic Aspects.** Liver PhK deficiency can be caused by mutations in the *PHKA2*, *PHKG2*, and *PHKB* genes. Mutations in *PHKA2*, which maps to Xp22.2–Xp22.1, cause X-linked liver PhK deficiency, also known as X-linked glycogenosis. As the most common form of PhK deficiency and one of the most common liver glycogenoses, it accounts for about 75% of PhK cases. The severity of clinical symptoms associated with X-linked liver PhK deficiency is highly variable and can range from mild to severe (152). Typically, female carriers of *PHKA2* mutations are asymptomatic but, depending on the pattern of X-inactivation, some may have symptoms. *PHKG2*, located on 16p12.1–p11.2 causes an autosomal recessive form of liver PhK deficiency that tends to be more severe, progressing to liver cirrhosis in some cases (151). Autosomal recessive liver PhK deficiency is also caused by mutations in the *PHKB* gene on chromosome 16q12–q13. Patients with mutations in this gene typically have a mild clinical course. Although *PHKB* mutations result in PhK deficiency in muscle as well as liver, symptoms of muscle involvement are mild or absent (152,159). The incidence of these disorders is estimated to be approximately 1:100,000.

## 93.9 OTHER LIVER GLYCOGENOSES

### 93.9.1 GSD Type 0 (Liver Glycogen Synthase Deficiency)

Although liver glycogen synthase deficiency (GSD 0) does not involve glycogen storage, it is often included in discussions of GSD. Case reports are few, but the clinical picture is of symptomatic hypoglycemia and ketosis in the fasting state, usually without hepatomegaly (160). Patients present in early infancy with early morning drowsiness and fatigue and sometimes with convulsions associated with hypoglycemia and hyperketonemia. Fasting hypoglycemia is accompanied by low plasma lactate and alanine levels and elevated plasma ketones. These changes are promptly relieved with carbohydrate intake, and postprandial hypoglycemia is common. The hypoglycemia may be very mild or severe enough to cause convulsions. A marked rise in blood lactate follows loading with glucose or galactose. A minimal rise in blood glucose is expected following glucagon administration in the fasting state. Rare patients presenting with hyperglycemia and glucosuria have also been reported (161).

Glycogen is present in hepatocytes but usually in low to normal concentrations. The glycogen content in muscle is normal. In affected individuals, glycogen synthase is absent from liver but present in muscle. Although hypoglycemia during fasting can continue to be a problem, the long-term prognosis is relatively good. Common features of liver glycogen synthase deficiency include short stature and osteopenia.

GSD 0 can be treated effectively with frequent high-protein feeds, starting in the early morning hours and continuing during the daytime, and supplemental feeds of uncooked cornstarch at bedtime to prevent nocturnal hypoglycemia and ketosis. Several isozymes of glycogen synthetase are known. There is a 70% homology of cDNA sequence encoding the liver and muscle enzymes. The gene for muscle glycogen synthase (*GYS1*) has been localized to 19q13.3. The gene for liver glycogen synthase (*GYS2*) was found at 12p12.2. Several mutations of this gene have been identified in patients with GSD 0. GSD 0 is transmitted as an autosomal recessive condition.

### 93.9.2 GSD Type XI (Fanconi–Bickel Syndrome)

**93.9.2.1 Clinical Aspects.** This rare syndrome is recognized on the basis of rickets, hepatomegaly, and growth failure associated with impaired glucose and galactose tolerance, increased renal clearance of glucose, amino acid, protein, phosphate, and uric acid. In the first year of life, the affected child presents with failure to thrive, rickets, and a protuberant abdomen due to hepato- and renomegaly (162). Developmental delay during puberty is most often acute and it is common to see short stature or dwarfism in adult patients. In addition to storing excess fat in the abdomen and shoulders, patients with GSD XI often present with a “moon-shaped” face (163). Those with this deficiency may encounter fractures as a result of early-onset, generalized osteopenia. Late-onset hypophosphatemic rickets and osteoporosis are common characteristics. Furthermore, intestinal malabsorption and diarrhea may occur. One case revealed abnormal neonatal screening for galactosemia with elevated blood galactose accompanied with retarded growth, moderate hepatomegaly, and delayed bone age, with the first radiologic signs of rickets (164).

**93.9.2.2 Biochemical Aspects.** The disease may be confused with type I GSD, as a Fanconi-like syndrome can develop in type I patients (165). The pathogenesis of this disorder was unknown until 1997 when mutations in the facilitative glucose transporter 2 (*GLUT2*) were identified (see section on glucose transport defects) (166). *GLUT2* is responsible for shuttling glucose in and out of hepatocytes, pancreatic  $\beta$  cells and the basolateral membranes of intestinal and renal epithelial cells (167,168). Affected patients have either low or normal fasting plasma glucose levels and normal fasting lactate values. After oral galactose administration, there is an excessive rise in blood lactate. The glycemic response to glucagon is variable. Histologic findings show increased glycogen content in liver.

**93.9.2.3 Treatment.** Symptomatic treatment with phosphate and bicarbonate resulted in marked growth improvement. Rickets improves with oral phosphate and vitamin therapy in pharmacologic doses (169).

**93.9.2.4 Genetic Aspects.** The gene responsible for GSD XI is the *SLC2A2* (or *GLUT2*) gene, however,

one case without SLC2A2 involvement suggests that either there may be another mutation present in the promotor or intronic region of the *SLC2A2* gene or there are other gene mutations to consider (170). One patient was found to have a 3-bp deletion of the *GLUT2* gene (164). A 4-year-old girl in India presenting with Fanconi-Bickel syndrome revealed a homozygous deletion insertion (162). Pregnancy has been reported in two women with the *GLUT2* defect, while one suffered only a few minor pregnancy complications like gestational diabetes and the other underwent daily hemodialysis, each successfully carried and delivered healthy babies (171,172).

### 93.9.3 Glucose Phosphate Isomerase (Phosphoglucose isomerase, Glucose-6-Phosphate Isomerase) Deficiency

The features of this disorder include severe nonspherocytic hemolytic anemia, hepatomegaly and muscle weakness. Liver and erythrocytes contain excessive amounts of glycogen. Glucose phosphate isomerase activity is decreased in many tissues, including erythrocytes and leukocytes. Two patients with GPI deficiency had additional features, including mental retardation, muscle weakness, and/or hepatomegaly. They had excessive amounts of glycogen in the muscle, liver, and erythrocytes. There was no hypoglycemia. Frequent meals with a diet low in carbohydrate normalized the liver size in one patient (173,174).

## 93.10 GSD PRIMARILY INVOLVING MUSCLE

### 93.10.1 GSD Type II (Lysosomal Acid $\alpha$ -Glucosidase Deficiency, Pompe Disease, Acid Maltase Deficiency, $\alpha$ -1,4-Glucosidase Deficiency)

**93.10.1.1 Clinical Aspects.** Acid  $\alpha$ -glucosidase (GAA) deficiency was first recognized in infants with hypotonia and cardiomegaly without murmur. Subsequently, a range of phenotypes—each including myopathy but differing in age at onset, organ involvement, and clinical severity—has been identified. In the infantile form, glycogen accumulates in almost all tissues. The involvement of cardiac, skeletal, and smooth muscle, and neural cells is the most significant for the affected infant. Manifestations usually appear within the first 6 months of life and include hypotonia and developmental delay, macroglossia, moderate hepatomegaly, marked cardiomegaly, and congestive heart failure. Skeletal muscle may be unusually firm to palpation, with a marked hypertrophy of calf muscles. Affected infants have a “floppy baby appearance.” Infantile Pompe, a generally rapidly progressive condition, leads to death by 1–2 years of age.

Characteristic electrocardiograph abnormalities include shortened PR interval, increased QT dispersion, and large left ventricular voltages. Histologic examination reveals glycogen accumulation in virtually every cell type. The excess glycogen is found within membrane-bound structures (lysosomes) as well as free in the cytoplasm.

Juvenile and adult-onset (late-onset) Pompe disease may manifest by gradually increasing muscle weakness during childhood or as late as the seventh decade of life. Transaminase and creatine kinase levels in serum may be elevated. Skeletal muscle weakness, decreased exercise tolerance, and decreased respiratory reserve are the usual features. These patients often present with a proximal limb girdle muscle weakness. The muscles most susceptible to weakness are those of the pelvic girdle, paraspinal region, and diaphragm (175,176). Hepatomegaly, macroglossia, and cardiomegaly are typically absent in this form. With disease progression, patients become confined to wheelchairs and require artificial ventilation. Respiratory insufficiency is the primary presentation in some patients and many succumb to its complications. The age of death varies from early childhood to late adulthood, depending on the rate of disease progression and the extent of respiratory muscle involvement (177). Basilar artery aneurysms with rupture are another cause for mortality. Other features of the disease include ptosis, lingual weakness and dilatation of the ascending aorta (178).

**93.10.1.2 Biochemical Aspects.**  $\alpha$ -1,4- and  $\alpha$ -1,6-glucosidase activities are associated with a single lysosomal enzyme protein. These activities promote hydrolysis of glycogen to glucose, and absence of these enzymes results in glycogen accumulation within lysosomes.

In the infantile form acid  $\alpha$ -1,4-glucosidase is significantly reduced or absent in affected tissues (e.g. skin, muscle, liver and cultured fibroblasts). In general, higher residual activity is found in patients with the juvenile and adult forms of Pompe disease. Blood-based assays (dried blood spots, leukocytes, or blood mononuclear cells) can also be used for the determination of the enzyme activity. The results of enzyme assay in these blood-based assays can only be interpreted reliably with addition of acarbose in the assay to eliminate nonspecific glucosidase activity (179). Blood-based assays have the advantage of a rapid turn-around time. Skin fibroblast assay is usually preferred to muscle biopsy because it is a less invasive procedure. Muscle biopsy can yield faster results and provide additional information especially in cases where the evaluation is for a limb girdle muscle dystrophy. A major limitation of a muscle biopsy in late-onset patients is the variable pathology: as glycogen accumulation varies in different muscles and within muscle fibers, muscle histology and glycogen content can vary depending on the site of muscle biopsy. In the case of infants, it should be noted that muscle biopsy carries

a very high anesthesia risk. Glucose tetrasaccharide has been shown to be elevated in urine and plasma of the affected patients, particularly the infantile form (180). It is a valuable noninvasive biomarker in diagnosis and monitoring response to therapy in Pompe disease.

**93.10.1.3 Treatment.** Enzyme replacement therapy via a bimonthly 20 mg/kg body weight intravenous infusion of alglucosidase alfa, a recombinant acid  $\alpha$ -glucosidase, is available as a definitive treatment for Pompe disease (181,182). In clinical trials, alglucosidase alfa has been shown to improve ventilator-free survival, cardiomyopathy and growth in patients with infantile-onset Pompe disease as compared to an untreated cohort (183). Stabilization of pulmonary disease and improvement in skeletal muscle function has been noted in patients with late-onset Pompe disease: a randomized study with alglucosidase alfa treatment significantly improved patients' walking test abilities and stabilized pulmonary function (184,185). Early treatment is necessary for maximum efficacy: delays in treatment have resulted in more dismal outcomes (186–189). Newborn screening has resulted in early identification of cases in Taiwan as well as significant outcome improvement due to early start ERT in comparison with babies clinically diagnosed at a later age (190).

A high-protein diet may be useful in Pompe disease. Nocturnal ventilatory support in late-onset patients improves the quality of life and is beneficial during a period of respiratory decompensation. Across the disease spectrum, early diagnosis is crucial in allowing for timely intervention. The availability of the lower-risk blood-based assays is a step toward achieving this goal.

**93.10.1.4 Genetic Aspects.** The infantile and late-onset forms are inherited as autosomal recessive disorders. In one Dutch study, infantile, juvenile, and adult-onset Pompe disease population ratios were 1:101,000, 1:720,000, and 1:53,000, respectively (note wide confidence intervals) (191). Infantile-onset disease is thought to have a high frequency of 1:14,000 in African-Americans, whereas adult-onset has the high frequency of around 1:60,000 in Caucasian populations (192,193). In Taiwan, it is estimated that 1:50,000 children have GSD II (194). The successful newborn screening program in Taiwan, which was first piloted in 2005, detected that 1:34,348 (6:206,088) newborns have early-onset Pompe disease and 1:26,466 have late-onset (190,195). Asian populations have a high percentage (3.3–3.9%) of alpha glucosidase pseudodeficiency, complicating newborn screening and requiring removal of hemoglobin through precipitation (196). The c.[1726A; 2065A] pseudodeficiency allele is responsible for low GAA activity occurrence in typical homozygous individuals and can cause a newborn screening test to appear falsely positive (197).

The gene for GAA has been assigned to chromosome 17q25. More than 289 mutations have been identified, and genotype–phenotype correlation may help to predict the clinical course (198,199). A splice-site mutation

(IVS1-13T>G) is seen in up to 60% of patients with adult-onset Pompe disease. P.Gly828\_Asn882del and p.Glu176ArgfsX45 mutations are both relatively prevalent in the Dutch population and are linked to the infantile form (200). Infantile Pompe disease in the Chinese population of Taiwan is greatly attributed to the p.Asp645Glu mutation (201). P.Arg854Ter is responsible for infantile onset in African-Americans (192). Prenatal diagnosis is possible using cultured amniotic fluid cells or chorionic villus biopsy by measuring the enzyme activity or by mutation analysis.

## 93.10.2 GSD Type V (Muscle Phosphorylase Deficiency, McArdle Disease Myophosphorylase Deficiency)

**93.10.2.1 Clinical Aspects.** Glycogen muscle phosphorylase (MP) deficiency usually becomes manifest in late adolescence or in the second decade of life. However, there are several studies showing that the disease can manifest later on, even as old as 74 years of age (202,203). Other studies reveal an early-onset, fatal form of McArdle disease, which was characterized by hypotonia, overall muscle weakness, and intensifying respiratory complications (204–207). The major symptoms are pain and stiffness of muscles during exercise, which may be due to excessive skeletal muscle recruitment (208). Strenuous activity can result in myoglobinuria. Several instances of acute renal failure due to rhabdomyolysis have been reported. Muscle groups that are stressed may acutely become swollen and tender. In later life, muscle weakness may become chronic. Asymptomatic individuals with absent MP activity have been recognized because of elevated levels of creatine kinase in serum (209).

Due to provocation of symptoms, patients with McArdle disease are advised against short, intense bursts of energy expenditure (sustaining heavy loads) as well as mild yet prolonged expenditure (walking up steep inclines). Contrastingly, everyday activities that require moderate expenditure such as walking on even pavement pose little obstacles for patients as long as they carry along at a pace comfortable for them. A phenomenon known as the “second wind” is commonly reported in patients with McArdle: if a patient momentarily halts the strenuous task at first onset of muscular pain, he or she can continue the activity with greater ease shortly thereafter. The explanation for this anomaly is attributed to increased oxidative capacity, which can be enhanced by the glucose infusion during exercise (210). GSD V, a suspected cause of statin-induced myopathy, intimates the necessity for further evaluation of patients with this presentation (211).

It has been recently suggested that there may be a link between GSD V and cognitive impairment (212).

**93.10.2.2 Biochemical Aspects.** The clinical manifestations result from deficient energy production in

muscle. During muscle activity, glycogen is normally broken down, providing energy for muscle contraction. Glycogen degradation is blocked in MP deficiency, and the defect in glycolysis is reflected in the absence of lactate production by muscle during ischemic exercise. Studies using  $^{31}\text{P}$  nuclear magnetic resonance show a rapid exhaustion of phosphocreatinine during ischemic exercise and indicate the importance of normal glycogen metabolism in muscle activity. A marked deficiency of NADH generation during exercise has also been demonstrated. Ammonia, inosine, hypoxanthine, and uric acid levels in blood increase after muscular activity, apparently due to accelerated degradation of muscle purine nucleotides, ATP, ADP, and AMP. These changes are also seen in GSD types III and VII.

Muscle biopsy for measurement of glycogen content, enzyme, and mutation analysis have been the standards for diagnosis of GSD type V. Muscle histology in GSD V shows damaged fibers, increased glycogen content, and absence of MP histochemical activity. MP activity may be present in regenerating fibers owing to the presence of a fetal-type isozyme. This isozyme has been demonstrated in cultured muscle cells from affected individuals. Phosphorylase activity in liver is normal in GSD type V.

**93.10.2.3 Treatment.** In general, avoidance of strenuous exercise can prevent major episodes of rhabdomyolysis, however, regular and moderate exercise is recommended to improve exercise capacity. Sucrose or glucose given before exercise can markedly improve tolerance in these patients (213). A high-protein diet may increase muscle endurance, and creatine supplementation has been shown to improve muscle function in some patients (214). However, a recent study showed that high-dose creatine supplementation could in fact increase muscle pain, thereby impairing activities of daily living (215). Free fatty acids provide an essential yet limited amount of muscle fuel, therefore therapies that increase the maximum rate of free fatty acid oxidation and consequently boost muscle performance are needed (216). In general, avoidance of strenuous exercise prevents the symptoms. Longevity does not appear to be affected.

**93.10.2.4 Genetic Aspects.** GSD V is inherited as an autosomal recessive trait. The gene for myophosphorylase, PYGM, is located on chromosome 11q13. A number of mutations in the PYGM gene have been uncovered (217). It should be noted that in more recent publications, the new numbering system, based on the first methionine as codon 1, has been used (218). The most common mutation in patients in the United States is a nonsense mutation that changes an arginine to a stop codon (p.Arg50X) (217), and the most common mutation in the Japanese is deletion of a single codon (p.Phe710del). In a population of British Caucasian patients with GSD V, 96% had at least one of two mutated alleles: pArg50X and pGly205Ser (219). These features allow DNA-based diagnosis and carrier detection in these three populations. In patients with

myophosphorylase deficiency, Martinuzzi and coworkers found an association between increased clinical severity and the D allele of the ACE insertion/deletion (I/D) polymorphism (220). This may explain some of the phenotypic variability of the disorder.

### 93.10.3 GSD Type VII (Muscle Phosphofructokinase Deficiency, Tarui Disease)

**93.10.3.1 Clinical Aspects.** Phosphofructokinase (PFK) deficiency is associated with several different clinical pictures. Most commonly, the clinical features are very similar to those of McArdle disease. In the classical form of PFK deficiency, exercise tolerance is limited by skeletal muscle cramps and pain. Symptoms usually begin during childhood, but the diagnosis is often made during adolescence or later. Strenuous exercise causes muscle pain, malaise, nausea, and myoglobinuria. Other clinical variants that have been reported include: (1) infantile hypotonia, limb weakness and a rapidly progressive myopathy leading to early death (age 4 years) (221–224), (2) infantile congenital myopathy and arthrogryposis with early mortality, (3) infantile presentation of hypotonia, mild developmental delay, and seizures, (225,226) adulthood presentation of a slowly progressive, fixed muscle weakness as opposed to cramps and myoglobinuria (227–229), and (5) presentation as hereditary nonspherocytic hemolytic anemia without muscle symptoms (230). Late-onset PFK deficiency may lead to thickening of the mitral valve as a result of glycogen build-up (231).

Characteristics that may differentiate type VII from type V are as follows: (a) earlier age of onset of exercise intolerance and more acute symptoms than in type V; (b) a rise in serum bilirubin levels and reticulocyte counts that marks the onset of compensated hemolytic anemia; (c) hyperuricemia is common and more aggravated by muscle employment than in GSD V or III (232); (d) muscle fibers contain an abnormal polysaccharide that is periodic acid-Schiff-positive but resistant to diastase digestion (233,234); and (e) exercise is particularly intolerable after carbohydrate-rich meals (235).

**93.10.3.2 Biochemical Aspects.** Laboratory studies show elevation of muscle enzyme values (aldolase, creatine kinase, lactate dehydrogenase, and oxaloacetate transaminase) and uric acid in the serum. The reticulocyte count is mildly increased, but the hemoglobin level may be normal. Muscle fibers show increased glycogen content and vacuolar and degenerative changes. PFK activity is absent in muscle and reduced in erythrocytes. The PFK molecule is a tetramer. Three subunits exist: muscle (M), liver (L) and platelet (P). The muscle isozyme is composed of four identical (M) subunits, whereas erythrocyte PFK is made up of M and L subunits. In PFK deficiency, the M subunit is absent and the residual PFK



activity in erythrocytes reflects the presence of the L subunit, which is about 50% of normal.

**93.10.3.3 Treatment.** Until recently, the only means of managing PFK deficiency was the avoidance of strenuous exercise. Patients should avoid drugs such as statins. When undergoing anesthesia, precautionary measures to avoid malignant hyperthermia should be taken. Unlike in GSD V, carbohydrate meals and glucose infusions can worsen symptoms in GSD VII. The problem is two-fold in PFK deficiency: glucose cannot be utilized in type VII muscle due to enzymatic block, and the administered glucose actually lowers blood levels of fatty acids—the primary source of muscle fuel. Of late, significant clinical improvement was seen in a child with the severe infantile form of PFK deficiency following the initiation of a ketogenic diet (236).

**93.10.3.4 Genetic Aspects.** PFK deficiency is rare. It is inherited as an autosomal recessive disorder. The gene for the M subunit is on chromosome 1. Around 20 mutations have been reported, including frameshift, missense, and splicing defects (237). There does not appear to be a prevalence of PFK deficiency in any particular ethnic group, although two common mutations—a nucleotide deletion and an exon 5 splicing defect—frequently affect those of Ashkenazi Jewish descent. In this population, 95% of Tarui disease cases are attributed to these two mutations (238). The gene for the L subunit is located at 21q22.3.

### 93.10.4 Muscle Phosphoglucomutase Deficiency

Glycogen accumulation in skeletal muscle has been reported in several infants and children. The clinical picture is variable. One infant had features associated with Reye's syndrome, such as nonketotic dicarboxylic aciduria and hypoglycemia associated with systemic carnitine deficiency. However, a case of adult-onset muscle phosphoglucomutase appeared in a 38-year-old male patient who presented with muscle weakness, clubbed fingers, and hypesthesia of distal extremities (239). Another 35-year-old male with muscle phosphoglucomutase deficiency was identified by his exercise-induced muscle weakness, normal lactate levels, episodic rhabdomyolysis, and elevated ammonia release when given a forearm-exercise test (240).

### 93.10.5 Muscle Phosphorylase Kinase Deficiency

**93.10.5.1 Clinical Aspects.** Introduced in the section GSD Type IX, muscle PhK deficiency, caused by mutations in the X-linked *PHKA1* gene, presents from childhood to adulthood with variable symptoms including muscle cramps and myoglobinuria on exercise, and progressive muscle weakness and atrophy (156). An adult male with asymptomatic myopathy and cognitive

impairment has also been reported. However, it is not known whether the cognitive impairment in this patient is due to the muscle PhK deficiency or has another cause (241). Electromyography and forearm ischemic exercise test are usually normal. Involvement of heart and liver has not been reported in individuals with *PHKA1* mutations. Infants with PhK deficiency in muscle and fatal arthrogryposis and severe hypotonia, without organomegaly, have also been reported. Molecular analysis has not been performed and thus it is unknown whether the PhK deficiency in these cases was caused by mutation of gene encoding a PhK subunit expressed in muscle (such as *PHKA1* or *PHKG1*) or has another cause.

**93.10.5.2 Biochemical Aspects.** The PhK enzyme is composed of four subunits (see liver phosphorylase kinase deficiency). In muscle, the alpha subunit is encoded by the *PHKA1* gene on chromosome Xq13, the beta subunit is encoded by the *PHKB* gene which is alternatively spliced in liver and muscle and located on chromosome 16q12–q13, the gamma subunit is encoded by the *PHKG1* gene on chromosome 7p12–p21, and the delta subunit is calmodulin. In muscle PhK deficiency, subsarcolemmal glycogen accumulation and marked reduction of PhK activity are observed in muscle biopsy samples. Serum creatine kinase may be above normal.

**93.10.5.3 Genetic Aspects.** Mutations causing skeletal muscle PhK deficiency have only been found in the *PHKA1* gene. However, mutations in this gene are found in only a small number of individuals with muscle PhK deficiency suggesting other genetic or metabolic causes, or false positive results (242). Rare cases of infants with severe isolated cardiomyopathy and PhK deficiency in heart muscle have been reported. Mutations in the *PRKAG2* gene, encoding the regulatory subunit of AMP-activated protein kinase have been identified in some of these individuals (243,244). No mutations in genes encoding any of the PhK subunits have been found in these cases (see glycogen storage diseases mimicking hypertrophic cardiomyopathy).

**93.10.5.4 Treatment.** Individuals with muscle PhK deficiency may benefit from physical therapy and nutritional consult to optimize glucose concentrations based on level of activity. Malignant hyperthermia precautions should be taken.

### 93.10.6 Other Muscle Glycogenoses

Six additional enzyme defects produce muscle glycogenoses, namely, deficiencies in phosphoglycerate kinase, phosphoglycerate mutase, lactate dehydrogenase, fructose-1,6-bisphosphate aldolase A, pyruvate kinase, and  $\beta$ -enolase. All six enzyme defects cause symptoms and signs of muscle energy impairment similar to those of the types V and VII GSD. The failure of blood lactate to increase in response to exercise is a useful diagnostic test and can be used to differentiate muscle glycogenoses

from disorders of lipid metabolism, such as carnitine palmitoyl transferase II deficiency and very long chain acyl-CoA dehydrogenase deficiency, which also cause muscle cramps and myoglobinuria. Muscle glycogen levels may be normal in the disorders affecting terminal glycolysis, and definite diagnosis is made by assaying the muscle enzyme activity. There is no specific treatment. Avoidance of strenuous exercise prevents acute attacks of muscle cramps and myoglobinuria.

### 93.10.7 Muscle Glycogen Synthase Deficiency

GSD due to muscle glycogen synthase (glycogen synthase I, GYS1) deficiency has been reported in four children, three from one family. The first child, an 8-year-old south Indian boy of consanguineous parents, presented with muscle glycogen synthase deficiency when he collapsed during muscular exertion and died soon thereafter due to cardiac failure. Mutational analysis revealed a double base pair deletion in exon 2 of GYS1 and autopsy revealed oxidative compensation and a large population of mitochondria (245). In the second family, the three children (two boys and one girl) of consanguineous Syrian parents all had cardiac involvement. The oldest of the three siblings collapsed at age 10.5 years from sudden cardiac death, the second boy exhibited muscle fatigability, heart arrhythmia, hypertrophic cardiomyopathy, and hypotension during exertion, and the youngest showed slight resting cardiac malfunction. Although glycogen was lacking in the muscle biopsies, the two surviving children showed normal glucose tolerance (246).

## 93.11 GSD PRIMARILY WITH CARDIAC INVOLVEMENT

### 93.11.1 GSDs Mimicking Hypertrophic Cardiomyopathy

**93.11.1.1 Clinical Aspects.** Glycogen can amalgamate in heart and skeletal muscle due to malfunctioning of lysosomal associated membrane 2 protein (LAMP2, classified as Danon's disease) and AMP-activated kinase gamma 2 protein (PRKAG2). Patients with LAMP2 deficiency suffer with chest pain, heart palpitations, syncope, and cardiac arrest usually between 8 and 15 years of age. Although patients with PRKAG2 defects experience similar cardiac symptoms, they usually occur at the much later average age of 33 years. Earlier age of presentation has been reported in a study where the range of onset for patients with PRKAG2 gene defects spanned from 9 to 55 years of age (247). In addition, a congenital, rapidly fatal, fetal symptomatic onset presentation with hypertrophic cardiomyopathy with Wolff-Parkinson-White (WPW) syndrome, caused by PRKAG2 mutations, has been noted (243).

**93.11.1.2 Biochemical Aspects.** Electrophysiologic abnormalities in sarcomere proteins, namely ventricular preexcitation and conduction defects, differentiate hypertrophic cardiomyopathy caused by LAMP2 and PRKAG2 mutations from other causes (248–251). PRKAG2 functions as an enzymatic regulator of glucose absorption, glycolysis, and fatty acid metabolism. The deficiency causes myocytes to have abnormally high numbers of vacuoles replete with glycogen. In a yeast model, mutational analysis of the homologous gene *Snf4* revealed overactive AMP kinase, possibly promoting glycogen buildup (250). In humans, this phenomenon is explained by the AMPK feedback loop being constantly activated, which in turn consistently draws more energy metabolites than needed, resulting in excess glycogen storage.

**93.11.1.3 Treatment.** The two deficiencies have differing prognoses: LAMP2 deficiency tends to lead to worsening heart complications and eventual cardiac arrest in young adult patients, whereas life with PRKAG2 deficiency is sustainable given pacemaker implantation and vigilant arrhythmia monitoring. The several infants with severe congenital PRKAG2 deficiency generally succumbed to the acute hypertrophic cardiomyopathy.

**93.11.1.4 Genetic Aspects.** Mutations responsible for Danon's disease are X-linked while those causing PRKAG2 deficient are autosomal dominant. Two mutations causing the congenital form of PRKAG2 that are incompatible with life (p.Arg531Gln and p.Arg384Thr) cause higher levels of AMPK phosphorylation and therefore higher basal levels of activity, although AMP and ATP binding is reduced. Other mutations responsible for adult-onset hypertrophic cardiomyopathy (p.Arg302Gln, p.Thr400Ans, and p.Asn488Ile, p.His487Tyr) and WPW syndrome cause less disruptive molecular changes (243,244). In three of five cases originating from different geographic regions, three equivalent but separate heterozygous p.Arg531Gln missense mutations were discovered in PRKAG2. This mutation is responsible for severe clinical manifestations including: fetal onset (all three patients experienced prenatal bradycardia, necessitating cesarean section preterm delivery; one had prenatal cardiomegaly), extreme cardiomegaly, and infantile-fetal outcome. The three patients with the p.Arg513Gln mutation died due to hemodynamic and respiratory failure, with additional complications including hypertrophic nonobstructive cardiomyopathy and conduction abnormalities seen in WPW syndrome (243). It should be noted, however, that patients with nonfamilial WPW syndrome may not always have the PRKAG2 genetic defect (252).

### 93.11.2 Animal Models of GSD

Several GSDs with inheritance patterns similar to those in humans have been identified in animals. These include GSD type Ia in mice (253,254) and dog (255), GSD II ( $\alpha$ -glucosidase deficiency) in mice (256), Japanese quail

(257) and Australian cattle, GSD III in dog (258), GSD IV in cat (259), GSD V in cattle and sheep (260,261), GSD VII in dog (262), liver PhK deficiency in rats (263), muscle PhK deficiency in mice (264), PhK deficiency in rats (265), and PRKAG2 defects in mice (266).

## 93.12 GLUCONEOGENIC DISORDERS ASSOCIATED WITH LACTIC ACIDOSIS

### 93.12.1 Metabolism

The maintenance of carbohydrate homeostasis in the human body is a complex process and involves the interaction of many factors. In fasting conditions, blood glucose is derived mainly from glycogen breakdown (glycogenolysis) and from the conversion of lactic acid and certain amino acids to glucose (gluconeogenesis).

Gluconeogenesis takes place primarily in the liver and kidneys. The metabolic process is in part under endocrine control. During a prolonged fast, the levels of glucocorticoids may be increased. These hormones increase the synthesis of pyruvate carboxylase, G6Pase, and aminotransferases, participants in the gluconeogenic pathway. Gluconeogenic amino acids, such as alanine, aspartic acid, and glutamic acid are converted to pyruvate, oxaloacetate, and alpha-ketoglutarate, respectively, and subsequently feed into the pathway culminating in the formation of glucose (Figure 93-7).

During fasting, epinephrine and glucagon increase, accelerating the glycogenolytic process through the activation of adenyl cyclase. At the other end of the glycolytic pathway, pyruvate kinase is inactivated by a

cAMP-dependent protein kinase, diminishing the conversion of triose phosphate to pyruvate and lactate.

In addition to pyruvate kinase, gluconeogenesis is also tightly regulated by other key enzymes that are sensitive to allosteric control. Pyruvate carboxylase, which plays a primary role in the conversion of pyruvate to oxaloacetate, is activated by acetyl CoA. When excess acetyl CoA builds up in cells, glucose synthesis is enhanced. Phosphoenolpyruvate carboxykinase is also necessary for phosphoenolpyruvate formation from oxaloacetate.

Other key enzymes in regulating the gluconeogenic pathway are fructose-1,6-diphosphatase and G6Pase. Fructose-1,6-diphosphatase is stimulated by citrate and inhibited by AMP. G6Pase and fructose-1,6-diphosphatase are known to be present in liver and intestinal tissues, whereas pyruvate carboxylase and phosphoenolpyruvate carboxykinase have been shown to be present also in other tissues, including cultured skin fibroblasts. Genetic defects have been encountered in each of these enzymes in humans. Hypoglycemia and lactic acidosis are the most common findings. Lactic acidosis, secondary to hypoxia, shock or sepsis is not an uncommon complication in sick infants and children. Mitochondrial damage observed in Reye's syndrome leads to lactic acidosis. Whatever the cause, persistent lactic acidosis usually needs prompt medical attention. The ratio of blood lactate to pyruvate can be useful in the differential diagnosis of lactic acidosis. A normal ratio of 10:20 with high lactate content suggests the possibility of defects in pyruvate metabolism, whereas a high ratio of more than 20:30 suggests defects in the tricarboxylic acid cycle or mitochondrial disorders, many of which can be diagnosed by molecular mutations or enzyme assays.

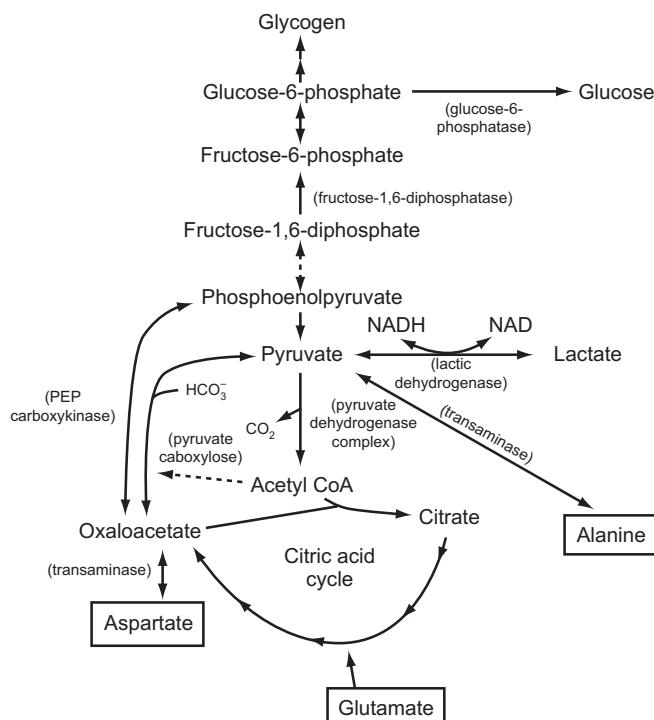


FIGURE 93-7 The gluconeogenic pathways.

### 93.12.2 Fructose-1,6-Diphosphatase Deficiency

**93.12.2.1 Clinical Aspects.** Symptoms usually begin in early infancy with hypoglycemia, hyperlactic acidemia, and ketoacidosis (267). Hypoglycemia occurs when glycogen reserves are limited or exhausted. The onset of symptoms often follows an infection. In some patients, the onset is delayed and the clinical picture is similar to that of ketotic hypoglycemia. With age, the frequency of hypoglycemia attacks decreases. The most common physical finding is hepatomegaly, resulting from fatty metamorphosis. Despite the metabolic defect, growth and intellectual development may proceed normally. Fructose-1,6-diphosphatase deficiency may be confused with GSD type I because of similarity of clinical features and laboratory findings of hypoglycemia and increased blood lactic acid. It differs from HFI in that patients usually have no aversion to sweets as well as normal renal tubular and liver functions. Confirmation of diagnosis is based on the measurement of fructose-1,6-diphosphatase activity in liver tissue or mutation analysis.

**93.12.2.2 Biochemical Aspects.** Fructose-1,6-diphosphatase is the key regulator of gluconeogenesis, during which it catalyzes the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate. This enzyme is expressed in several tissues with maximum activity in the liver and kidney. Prolonged fasting in the affected patient induces severe hypoglycemia, lactic acidosis, and hyperalaninemia. The ingestion of glycerol, fructose, and alanine produces hypoglycemia, an increase in lactate, and a fall in serum inorganic phosphate. Serum uric acid may also increase. The glycemic response to glucagon and galactose administration has been variable. Lactate, pyruvate, beta-hydroxybutyrate, alpha-ketoglutarate, and glycerol-3-phosphate accumulate in the urine (268). Fructose-1,6-diphosphatase is normally present in liver, kidney, and intestinal tissue. It has also been found in muscle, platelets, and lymphocytes; however, the usefulness of these cells for diagnostic purposes has not been established. Enzyme assay in cultured monocytes rather than isolated leukocytes was shown to be of accurate diagnosis of fructose-1,6-diphosphatase deficiency (269).

Successful pregnancy outcome has been reported in one case of a woman with fructose-1,6-diphosphatase deficiency. After the birth of her three children, this patient began to experience auditory and cognitive difficulties (270).

**93.12.2.3 Treatment.** Treatment consists of frequent feedings and avoidance of extended fasting periods in order to prevent hypoglycemia and limiting fructose and sorbitol intake. It has been suggested that folic acid may increase synthesis of fructose-1,6-diphosphatase (271).

**93.12.2.4 Genetic Aspects.** Fructose-1,6-diphosphatase deficiency is a rare condition inherited as an autosomal recessive trait. In parents of affected children, intermediate values of enzyme activity were found in the liver (272,273) and in cultured lymphocytes (274). The cDNA for fructose-1,6-diphosphatase has been cloned, and the gene is localized to chromosome 9q22–q22.3 (275). Several mutations have been identified in affected patients (275,276).

### 93.12.3 Pyruvate Carboxylase Deficiency

**93.12.3.1 Clinical Aspects.** Clinical manifestations usually appear soon after birth. Metabolic acidosis due to lactic acid, failure to thrive, hypotonia, anorexia, and hyporeflexia have been observed. Death within 1–2 months of age may occur. Patients surviving the initial problems exhibit retarded growth, seizures, hypotonia, and continuing metabolic acidosis. Three forms have been described thus far: Type A (infantile or North American form), Type B (neonatal or French form) and Type C (benign form). These phenotypes constitute a continuum spanning from the most severe (Type B) to the least severe form (Type C). Laboratory findings include severe lactic acidosis, ketonemia, with normal

lactate/pyruvate ratio (exception is type B) and, in some cases, hyperammonemia, citrullinemia, hyperlysinemia and hypoglycemia (277). Mosaicism may correlate with longer life span (278).

**93.12.3.2 Biochemical Aspects.** Pyruvate carboxylase (PC) is a biotin-containing enzyme that catalyzes the formation of oxaloacetate in the presence of an allosteric activator, acetyl CoA, from pyruvate. In this process called anaplerosis, the synthesis of oxaloacetate functions to replenish the stores of intermediates depleted during metabolism but required for the citric acid cycle (CAC). Not only is PC critical in this reaction, it plays a critical role in conjunction with oxaloacetate in gluconeogenesis, glycogen synthesis, lipogenesis, glycerogenesis, amino acid and neurotransmitter synthesis, and glucose-dependent insulin secretion (277). The enzyme deficiency has been demonstrated in liver tissue, leukocytes, and cultured skin fibroblasts. The enzyme activity is intermediate in known carriers (277,279). The enzyme is also present in cultured amniotic cells, and prenatal diagnosis has been accomplished (277).

**93.12.3.3 Treatment.** Drastic supportive measures are usually needed. Metabolic acidosis (lactic acidosis) should be treated promptly. Peritoneal dialysis, intravenous fluids with glucose, and general hydration may be helpful in reversing the clinical consequences of the metabolic defects (280). Biotin, thiamine and lipoic acid have been used in some patients, as well as inclusion of glutamine and aspartic acid with questionable benefit (281). Anaplerotic treatment research focuses on restoration of necessary intermediates in the CAC that are undermined by the detrimental effects of pyruvate carboxylase deficiency, and with restorative treatment, the cycle can continue creation of ATP (277,282). Orthotopic liver transplantation has been successful in a couple of cases (280). Fasting and the ketogenic diet should be avoided.

**93.12.3.4 Genetic Aspects.** PC deficiency is inherited as an autosomal recessive disorder. The frequency of occurrence is not known. In two patients who presented with hyperammonemia, there was no cross-reacting material, whereas other patients had shown immunoprecipitated proteins (283). The cDNA for PC has been cloned, and the gene is located on chromosome 11q13.4–q13.5 by somatic hybridization (280,284). At this time, 15 mutations have been identified (285). In the study done by Wang et al., the genotype–phenotype correlations were characterized: type A was caused by mutations p.Arg62Cys, p.Arg631Gln, p.Ala847Val, p.Val145Ala, p.Arg451Cys, p.Ala610Thr and p.Met743Ile; type B was caused by a variety of missense, deletion, and splice donor site mutations; and type C was caused by either a heterozygous p.Ser266Ala mutation with a p.Ser705X mosaicism or heterozygous p.Thr569Ala and p.Leu1137ValfsX1170 mutations (278). In one study, individuals of Ojibwa and Cree backgrounds tended to have a homozygous p.Ala610Thr missense



mutation (founder effect) and two brothers of Micmac origin presented with a p.Met743Ile transversion mutation (286).

### 93.12.4 Phosphoenolpyruvate Carboxykinase Deficiency

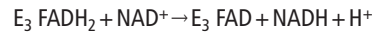
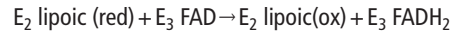
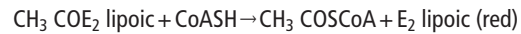
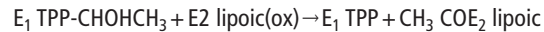
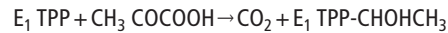
Phosphoenolpyruvate carboxykinase (PEPCK) deficiency is a rare disease and there appear to be two isoforms: cystolic (PEPCK 1) and mitochondrial (PEPCK 2). Only a few patients have been described. The patients with PEPCK 1, influenced by induction of catabolic states and repressed by anabolic states, can suffer with hepatomegaly, hypoglycemia, hyperinsulism, hypertriglyceridemia, and hypercholesterolemia (287,288). Those with PEPCK 2 suffer with hypotonia, hypoglycemia, lactic acidemia, and failure to thrive (288). In one patient, the activity of PEPCK in cultured skin fibroblasts was found to be 17% of the controls in whole cells and 6% in isolated mitochondria (289). Both human hepatic mitochondrial and cystolic PEPCK enzyme cDNA have been isolated (290).

### 93.12.5 Deficiencies of Pyruvate Dehydrogenase Complex

**93.12.5.1 Clinical Aspects.** The extremely variable clinical expression in this group suggests both clinical and biochemical heterogeneity. Patients have been described with one or more of the following findings: microcephaly, slow mental and physical development, seizures, hypotonia, optic atrophy of variable degree, cerebellar ataxia, and severe lactic acidosis. Neurological manifestations can be categorized into two groups: the first, abnormal brain development seen in both males and females, and the second, brain lesions and epilepsy seen only in male patients (291). Neonatal form of PDHC deficiency is severe, and patients usually die within a few months of life. In some patients, postmortem examination revealed specific CNS pathology of Leigh's disease (292). Partial deficiency of the enzyme complex has also been observed in patients with spinocerebellar degeneration and in Friedreich's ataxia (293). Patients with pyruvate dehydrogenase complex (PDHC) deficiency usually do not have hypoglycemia.

**93.12.5.2 Biochemical Aspects.** PDHC comprises three principal enzymes (E1, pyruvate dehydrogenase or pyruvate decarboxylase; E2, dihydrolipoyl transacetylase; and E3, dihydrolipoyl dehydrogenase), and five different coenzymes (thiamine pyrophosphate, lipoic acid, coenzyme A, flavin adenine dinucleotide, and nicotinamide adenine dinucleotide). In addition, a specific kinase for inactivation and a phosphatase for activation of the pyruvate dehydrogenase have been demonstrated, as well as protein X (294). The E1 component is a tetramer containing a 41-kDa and 36-kDa subunit.

The sequence of the PDHC reactions can be simplified as follows (295):



Pyruvate dehydrogenase is composed of four subunits: two E1 alpha and two E1 beta. The alpha subunit is X-linked. Because of the complexity of this enzyme system, it is important to have reliable methods for the enzyme assays to pinpoint the basic defect. PDHC activity is present in cultured skin fibroblasts, and therefore, these cells are a good source for enzyme diagnosis. Caution is needed to avoid mycoplasma contamination because an unusually large amount of pyruvate dehydrogenase activity has been demonstrated in contaminated cultures (296).

In patients with PDHC deficiency, the levels of pyruvate, lactate, and alanine are increased in blood and urine. In milder cases, random samples of blood or urine may show normal amounts of lactate. Oral glucose loads or high carbohydrate intake may induce elevations in blood lactic acid and aggravate the symptoms. Two brothers with pyruvate dehydrogenase deficiency showed absent to trace amounts of both the E1 alpha and beta subunits in liver, skeletal muscle, and heart (297). The lymphocytes had enzyme activity about 10% of normal controls, whereas the activity in cultured skin fibroblasts was normal. Both patients had abnormal neuromuscular development and lactic acidosis. Isolated dihydrolipoyl dehydrogenase (lipoamide dehydrogenase) deficiency has been reported in which patients presented lactic acidosis and ketosis with neurologic abnormality. However, this deficiency appeared to be more common among the Ashkenazi Jewish population (298). A recent study shows how the reactive oxygen species generated by the mutations responsible for lipoamide dehydrogenase deficiency may in fact explain certain disease characteristics as well as proffer the prospect of antioxidant therapy (299).

**93.12.5.3 Treatment.** In general, administration of thiamine or lipoic acid, or both, has not been helpful in most cases. Intravenous glucose should be administered with caution because it can cause severe lactic acidosis. It is recommended that patients should follow a ketogenic diet, high in fats and low in carbohydrates (300). Although this diet may be helpful in saving the patient's life, it has not prevented mental deterioration in severe cases. In patients identified as having dihydrolipoyl dehydrogenase deficiency, favorable responses to lipoic acid treatment (301) and to branched-chain amino acid restriction have been observed (302).

**93.12.5.4 Genetic Aspects.** Because several enzymes and cofactors are involved, the specific biochemical and genetic causes of pyruvate dehydrogenase deficiency

should be demonstrated so as to ascertain the particular mode of inheritance. The frequency of occurrence of PDHC deficiencies may be more than as is now realized. Any patient who has died as a result of lactic acidosis should be considered a possible victim of the condition. Prenatal diagnosis, at least in some cases, should be possible because the enzyme system is present in cultured amniotic cells. Isolation of cDNA clones for various enzyme components of the PDHC has been recently reported. Pyruvate dehydrogenase itself has a tetrameric structure with alpha and beta subunits. The gene for E1 alpha subunit, *PDHA1*, is located on chromosome Xp22.1 (303,304). The most common defects are in the alpha subunit. Several mutations have been described in affected male and female patients (305,306). A mutation in the mitochondrial targeting sequence was identified in an affected family (307). The patient having a mutation (p.Phe205Leu) in the E1 alpha subunit responded to thiamin treatment because the enzyme showed impaired binding of the thiamin diphosphate cofactor (308). One boy with a c.857C>T (p.Pro250Leu) mutation had hypotonia and psychomotor retardation since infancy. Another boy with a c.367C>T (p.Arg88Cys) mutation showed symptoms, including seizures, starting at the toddler age and progressed to dystonia and myopathy with normal psychomotor development by 7 years (309). The gene for the beta subunit of E1 is located on chromosome 3p21.1–1p4.2 (311). Mutations (homozygous p.Met101Thr, and heterozygous p.Arg105Gln and p.Met101Val) on the beta subunit of E1 have caused a couple of patients to present with Leigh's disease (310). Mutations for E3 binding protein (protein X) of the PDHC have been characterized in two siblings in each of two unrelated families from Kuwait (312). A common mutation of lipoamide dehydrogenase deficiency (p.Gly194Cys) was found in both Arab Muslim and Ashkenazi Jewish patients (313).

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## CROSS REFERENCES

Diagnostic Molecular Genetics; Neonatal Screening; Enzyme Replacement and Pharmacologic Chaperone Therapies for Lysosomal Storage Disease; Gene Therapy; Cardiomyopathies; Peroxisomal Disorders; The Muscular Dystrophies.

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### Biographies



**Dr Priya S Kishnani, MD**, throughout her career primarily focused on the translation of laboratory science into the clinical arena, especially in the area of such therapeutic interventions as enzyme replacement therapy and small molecules. The care, treatment and natural history of individuals with lysosomal storage disorders (LSDs), glycogen storage diseases (GSDs), Down syndrome (DS) and other inborn errors of metabolism remain her passions. Her areas of publication include treatment strategies, examination of long-term complications and the results of a number of clinical trials for multiple disorders. She has a long-standing research and clinical interest in Pompe disease and, since 1999, has been the principal investigator for several clinical trials involving Pompe disease which resulted in FDA approval of Myozyme as the first treatment for this otherwise lethal neuromuscular disease in 2006. She has placed an emphasis on continued care for these chronic conditions via a multidisciplinary team approach. At Duke University Hospital where Dr Kishnani serves as a professor of Pediatrics and the Chief of Medical Genetics, her primary clinical responsibilities involve the care for individuals with GSDs (with a research interest in Types I, II, III and IX), LSDs, other inborn errors of metabolism and Down syndrome.



**Professor Yuan-Tsong (Y-T) Chen** received his MD degree from National Taiwan University (Taipei) and PhD from Columbia University (USA). He is currently a Distinguished Research Fellow of the Institute of Biomedical Sciences, Academia Sinica, Taiwan, and professor of Pediatrics at Duke University Medical Center (USA). Professor Chen is a physician/scientist, recognized for his work on human genetic disorders. His translational research leads to the development of new standard therapies for two devastating inherited metabolic diseases: a simple and effective cornstarch therapy for severe hypoglycemia in glycogen storage diseases and an enzyme replacement therapy, the first ever treatment, for a debilitating, progressive and often fatal myopathy called Pompe disease. Professor Chen has also identified the genetic basis of and developed DNA-based diagnosis for several major heritable diseases, and more recently, uncovered genes/SNPs associated with drug-induced Stevens–Johnson syndrome and warfarin sensitivity. Professor Chen is an elected member of Academia Sinica and of the Academy Sciences for the Developing World.



# CHAPTER

# 94

## Congenital Disorders of Protein Glycosylation

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### 94.1 NORMAL PROTEIN GLYCOSYLATION

Congenital disorders of glycosylation (CDGs) are a rapidly growing family of genetic diseases due to defects in the synthesis of the glycan moiety of glycoproteins and glycolipids. This chapter is limited to genetic defects of protein glycosylation (1–3).

There are two main types of protein glycosylation: N-glycosylation and O-glycosylation. N-glycosylation comprises an assembly part and a processing part, and extends over three cellular compartments: the cytosol, the endoplasmic reticulum (ER), and the Golgi. The assembly part of the N-glycosylation starts on the cytosolic side of the ER with the transfer of N-acetylglucosamine (GlcNAc) phosphate from UDP-GlcNAc to membrane-bound dolichyl monophosphate (Dol-P), forming GlcNAc-pyrophosphatedolichol (GlcNAc-PP-Dol). One GlcNAc and five mannose (Man) residues are subsequently attached to this lipid-linked monosaccharide in a stepwise manner. The donor of these mannoses is a nucleotide-activated sugar, GDP-Man, which is synthesized from fructose 6-phosphate, an intermediate of the glycolytic pathway. The lipid-linked heptasaccharide  $\text{Man}_5\text{GlcNAc}_2$  is translocated by a flippase across the ER membrane and, at the luminal side, is elongated by the attachment of four more mannose residues and, subsequently, of three glucose residues. The four mannosyltransferases and three glucosyltransferases involved require dolichyl phosphate-bound monosaccharides (Dol-P-Man and Dol-P-Glc). The completed  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  oligosaccharide is then transferred to selected asparagine residues of the nascent proteins by the oligosaccharyltransferase complex.

The processing part of the N-glycosylation starts in the ER by trimming off of the glucoses (catalyzed by glucosidases I and II) and one mannose (catalyzed by mannosidase I). The residual glycoprotein intermediate is directed to the cis-Golgi, where the processing pathway branches. A

minor branch targets glycoproteins to the lysosomes (after the action of a GlcNAc-phosphotransferase and removal of the GlcNAc residues, leaving high-mannose glycoproteins capped with Man 6-P). The main branch leads to further trimming of mannoses (leaving a trimannosyl core) and addition of respectively GlcNAc, galactose, and eventually, sialic acid in the medial- and trans-Golgi, respectively. Another modification of many N-glycoproteins in the Golgi is the attachment of fucose to the GlcNAc residue that is linked to asparagine. O-glycosylation (O-glycans attached to the hydroxyl group of threonine or serine of proteins) has no processing part and thus consists only of assembly. Contrary to N-glycosylation, this assembly mainly occurs in the Golgi. O-glycan structures show a greater diversity than N-glycans. Examples of important O-glycans are O-N-acetylgalactosaminylglycans (mucin-type glycans), O-xylosylglycans (glycosaminylglycans), and O-mannosylglycans.

Genetic defects of protein glycosylation can be divided into three classes: disorders of protein N-glycosylation, disorders of protein O-glycosylation, and combined disorders of protein N- and O-glycosylation.

In 2008–2009, a novel nomenclature of the CDG was introduced, namely, the official gene symbol (not in italics) followed by “-CDG” (4,5). A list of approved gene names can be found at <http://www.genenames.org>. The novel nomenclature is used in this chapter.

### 94.2 CONGENITAL DISORDERS OF PROTEIN N-GLYCOSYLATION

Eighteen disease-causing defects are known in protein N-glycosylation: 14 assembly defects (in pre-ER and ER) and 4 processing defects (in ER and Golgi) (Table 94-1). The four most frequent disorders (PMM2-CDG, MPI-CDG, ALG6-CDG and ALG1-CDG) are described in more detail, as well as the first N-glycan processing defect (MGAT2-CDG) and the first deafness CDG (RFT1-CDG).

**TABLE 94-1 Genetic Protein N-Glycosylation Disorders**

Disorder	Affected Protein	Defective Gene	OMIM Number
PMM2-CDG	Phosphomannomutase 2	<i>PMM</i>	212065
MPI-CDG	Phosphomannose isomerase	<i>MPI</i>	602579
ALG6-CDG	Glucosyltransferase 1	<i>ALG6</i>	603147
ALG3-CDG	Mannosyltransferase 6	<i>ALG3</i>	601110
ALG12-CDG	Mannosyltransferase 8	<i>ALG12</i>	607143
ALG8-CDG	Glucosyltransferase 2	<i>ALG8</i>	608104
ALG2-CDG	Mannosyltransferase 2	<i>ALG2</i>	607906
DPAGT1-CDG	UDP-GlcNAc:DoI-P-GlcNAc-P-transferase	<i>DPAGT1</i>	608093
ALG1-CDG	Mannosyltransferase 1	<i>ALG1</i>	608540
ALG9-CDG	Mannosyltransferase 7-9	<i>ALG9</i>	608776
ALG11-CDG	Mannosyltransferase 4-5	<i>ALG11</i>	613611
RFT1-CDG	Flippase of Man <sub>5</sub> GlcNAc <sub>2</sub> -PP-DoI	<i>RFT1</i>	612015
TUSC3-CDG	Oligosaccharyltransferase tusc3	<i>TUSC3</i>	611093
MAGT1-CDG	Magnesium transporter 1	<i>MAGT1</i>	300716, 300853
GCS1-CDG	Glucosidase 1	<i>GCS1</i>	606056
MAN1B1-CDG	Mannosidase 1B1	<i>MAN1B1</i>	614202
MGAT2-CDG	N-acetylglucosaminyltransferase 2	<i>MGAT2</i>	212066
ST3GAL3-CDG	Beta-galactoside-alpha-2,3-sialyltransferase 3	<i>ST3GAL3</i>	611090

### 94.2.1 PMM2-CDG (Phosphomannomutase 2 Deficiency)

This is, by far, the most frequent protein N-glycosylation disorder (approximately 700 patients known) (6). The clinical spectrum is very broad: The nervous system is affected in all patients, and most other organs are involved in a variable way. The neurologic picture comprises alternating internal strabismus and other abnormal eye movements, axial hypotonia, psychomotor retardation, ataxia, and hyporeflexia. After infancy, symptoms include retinitis pigmentosa, often stroke-like episodes, and sometimes, epilepsy. As a rule, there is no regression. During the first years of life, there are variable feeding problems (e.g. anorexia, vomiting, diarrhea) that can result in severe failure to thrive. Other features are a variable dysmorphism (e.g. large, hypoplastic/dysplastic ears), abnormal subcutaneous adipose tissue distribution (e.g. fat pads, inverted nipples), mild to moderate hepatomegaly, skeletal abnormalities, and hypogonadism. Some infants develop pericardial effusion or cardiomyopathy, or both. At the other end of the clinical spectrum are patients with a very mild phenotype (no dysmorphism, slight psychomotor retardation). Patients often have an extroverted and happy appearance. There is a substantially increased mortality rate in the first years of life due to severe infection or vital organ involvement. Neurotechnical investigation reveals (olivoponto)-cerebellar hypoplasia, variable cerebral hypoplasia, and peripheral neuropathy. Liver pathology is characterized by fibrosis and steatosis, and electron microscopy shows myelin-like lysosomal inclusions in hepatocytes but not in Kupffer cells.

Phosphomannomutase 2 deficiency is a (cytosolic) defect in the second step of the mannose pathway (transforming mannose 6-phosphate into mannose

1-phosphate), which normally leads to the synthesis of guanosine diphosphate (GDP)-mannose. This nucleotide sugar is the donor of mannose used in the ER to assemble the dolichyl-pyrophosphate oligosaccharide precursor. Deficiency of GDP-mannose causes hypoglycosylation (and hence, deficiency, increase and/or dysfunction) of numerous glycoproteins, including serum proteins, lysosomal enzymes, and membranous glycoproteins.

The diagnosis of PMM2-CDG (and of congenital disorders of N-glycosylation in general) is usually made by isoelectrofocusing (IEF) and immunofixation of serum transferrin. Normal serum transferrin is mainly composed of tetrasialotransferrin and small amounts of mono-, di-, tri-, penta- and hexasialotransferrins. The partial deficiency of sialic acid (a negatively charged and an end-standing sugar) in CDG causes a cathodal shift. Two main types of cathodal shift can be recognized: type 1 is characterized by an increase of both disialo- and asialotransferrin and a decrease of tetra-, penta-, and hexasialotransferrins; in type 2, there is also an increase of the tri- and/or monosialotransferrin bands. In PMM2 deficiency, a type 1 pattern is found. First, a shift due to a transferrin protein variant has to be excluded (IEF after neuraminidase treatment, study of the parents, study of another glycoprotein). Recently, capillary zone electrophoresis of total serum has been introduced for the diagnosis of CDG.

In addition to the aforementioned serum glycoprotein abnormalities, laboratory findings include elevation of serum transaminase levels, hypoalbuminemia, hypocholesterolemia, and tubular proteinuria. To confirm the diagnosis, the activity of PMM2 should be measured in leukocytes or fibroblasts. Prenatal diagnosis is possible by enzymatic analysis of amniocytes and chorionic villus cells; this should be combined with mutation analysis of the *PMM2* gene.

Treatment is only symptomatic. Low-dose acetylsalicylic acid can be effective in the prevention of recurrent strokes.

PMM2-CDG is an autosomal-recessive disease. At least 100 mutations have been identified (mainly missense mutations). The most frequent mutation causes the R141H protein change.

### 94.2.2 MPI-CDG (Phosphomannose-Isomerase Deficiency)

Some 45 patients have been reported or are known to the author with phosphomannose-isomerase deficiency (MPI-CDG). It is mainly a hepatic-intestinal disease. Together with ALG8-CDG, it is the only known N-linked CDG without or with only minor neurologic involvement. Symptoms started between 1 month and 11 months of age and consisted of various combinations of recurrent vomiting, abdominal pain, protein-losing enteropathy, recurrent thromboses, gastrointestinal bleeding, liver disease and symptoms of hypoglycemia. Several patients have died (7).

The defect is in the first step in the biosynthesis of the nucleotide sugar GDP-mannose. The substrate of the enzyme, fructose 6-phosphate, does not accumulate because it is an intermediate of the glycolytic pathway. The blood biochemical abnormalities are indistinguishable from those found in PMM2-CDG. The diagnosis is confirmed by finding a decreased activity of phosphomannose isomerase (PMI) in leukocytes or fibroblasts or mutations in the MPI gene.

This is the only known CDG that is efficiently treatable. The treatment is simple and consists of oral mannose (1 g/kg body weight per day, divided in 4–6 doses). The rationale for this treatment is that hexokinases phosphorylate mannose to mannose 6-phosphate, thus bypassing the defect. Inheritance of MPI-CDG is autosomal recessive.

### 94.2.3 ALG6-CDG (Glucosyltransferase I Deficiency)

This is the second most common protein N-glycosylation disease (approximately 50 patients identified or known). As in PMM2-CDG, patients show hypotonia, strabismus, and seizures, but psychomotor development is less retarded, there is less dysmorphism, and usually, there is no retinitis pigmentosa or cerebellar hypoplasia. Long-term outcome is unknown because most reported patients have been children (8).

Glucosyltransferase I deficiency is a defect in the attachment of the first glucose (of three) to the dolichol-linked  $\text{Man}_9\text{-GlcNAc}_2$  ER intermediate. It causes hypoglycosylation of serum glycoproteins because non-glycosylated oligosaccharides are a suboptimal substrate for the oligosaccharyltransferase. For an unknown reason, some of the glycoproteins have unusually low blood

levels (particularly factor XI, and coagulation inhibitors such as antithrombin and protein C). The reason why the clinical picture in these patients is much milder than that of PMM-deficient patients may be because a deficiency in glucosylation of the dolichol-linked oligosaccharides does not affect the biosynthesis of GDP-mannose and, hence, does not affect the biosynthesis of GDP-fucose or the biosynthesis of glycosylphosphatidylinositol-anchored glycoproteins.

This disease illustrates that even in cases of mild psychomotor retardation without any specific dysmorphism, IEF of serum sialotransferrins has to be performed. When a type 1 pattern is found, PMM2 and PMI deficiency have to be considered first. If these enzymes show normal activities, the next step is analysis of the dolichol-linked oligosaccharides in fibroblasts. In ALG6-CDG, the major fraction of these oligosaccharides consists of nine mannose and two N-acetylglucosamine residues without the normally present three glucose residues. Glucosyltransferase I activity has to be measured in fibroblasts and/or mutation analysis performed.

No efficient treatment is available.

Inheritance is autosomal recessive.

### 94.2.4 ALG1-CDG (Mannosyltransferase I Deficiency)

The 10 reported patients showed epilepsy, severe psychomotor retardation, and a number of variable features such as fetal growth retardation, microcephaly, feeding difficulties, hypotonia, facial dysmorphism, liver dysfunction, cardiomyopathy, nephrotic syndrome, hypogonadism, abnormal visual evoked potentials, cerebellar hypoplasia, cortical atrophy, and depletion of  $\beta$  cells (9).

Lipid-linked oligosaccharide analysis showed an accumulation of  $\text{GlcNAc}_2\text{-PP-Dol}$  in the patients' fibroblasts.

No efficient treatment is available.

Inheritance is autosomal recessive.

### 94.2.5 RFT1-CDG (Flippase of $\text{MAN}_5\text{GLCNAC}_2\text{-PP-DOL}$ Deficiency)

This is a defect in the flippase that transfers  $\text{Man}_5\text{-GlcNAc}_2\text{-PP-Dol}$  from the cytoplasmic to the luminal side of the ER. The six reported patients showed a severe neurological syndrome with pronounced psychomotor retardation, important hypotonia, drug-resistant epilepsy, and sensorineural deafness (10). This is the first CDG showing deafness as a consistent feature.

Dolichol-linked oligosaccharide synthesis showed an accumulation of the aforementioned intermediate in fibroblasts.

No efficient treatment is available.

Inheritance is autosomal recessive.

### 94.2.6 MGAT2-CDG (N-Acetylglucosaminyltransferase II Deficiency)

Four patients have been reported with this first-identified N-glycan processing defect. Besides neurologic involvement (e.g. psychomotor retardation, epilepsy, behavioral disturbances), they presented with craniofacial dysmorphism, skeletal abnormalities, gastrointestinal disturbances, and growth retardation (11).

Laboratory investigation showed dissociation of serum transaminase activities (increased glutamic oxaloacetic transaminase and normal glutamic pyruvic transaminase), a feature which is also seen in other CDG-II group patients. IEF of serum transferrin showed a type 2 pattern (with nearly absent tetrasialo-transferrin) and structural analysis of the transferrin oligosaccharides, a monoantennary N-acetyllactosamine-type glycan. Thus, contrary to the structurally normal sialotransferrins in the CDG-I group or assembly disorders, the sialotransferrins of this and some other CDG-II diseases have abnormal glycan structures.

No efficient treatment is available.

Inheritance is autosomal recessive.

## 94.3 CONGENITAL DISORDERS OF PROTEIN O-GLYCOSYLATION

Eleven defects have been identified: one in O-N-acetylglactosaminylglycan synthesis (GALNT3-CDG or familial hyperphosphatemic tumoral calcinosis), four in O-xylosylglycan synthesis (B4GALT7-CDG or progeroid variant of Ehlers–Danlos syndrome, EXT1-CDG and EXT2-CDG (hereditary multiple exostoses) and CHSY1-CDG

or Tentamy preaxial brachydactyly syndrome), one in O-xylosyl/O-N-acetylglactosaminylglycan synthesis (SLC35D1-CDG or Schneckenbecken dysplasia), three in O-mannosylglycan synthesis (POMT1-CDG or muscular dystrophy-dystroglycanopathy types A1, B1 and C1, POMT2-CDG or muscular dystrophy-dystroglycanopathy types A2, B2 and C2, and POMGNT1-CDG or muscular dystrophy-dystroglycanopathy types A3, B3 and C3), and two in O-fucosylglycan synthesis (LFNG-CDG or spondylocostal dysostosis type 3, and B3GALTL-CDG or Peters plus syndrome) (Table 94-2). Except for the progeroid variant of Ehlers–Danlos syndrome, these are well-known diseases that very recently have been recognized as glycosylation disorders. They are hereby shortly summarized.

### 94.3.1 GALNT3-CDG (Familial Hyperphosphatemic Tumoral Calcinosis)

Patients suffer from recurrent, painful calcified subcutaneous masses that can attain up to 1 kg in weight (12). This can be complicated by secondary infections and incapacitating mutilations.

The basic defect is in isoform 3 of the N-acetylglactosaminyltransferase (GalNAcT3) family, which comprises more than 20 members. This enzyme mediates O-glycosylation of fibroblast growth factor 23 (FGF23), thereby protecting it from degradation through proteolysis. FGF23 is a phosphatonin that functions by decreasing the expression of the renal phosphate transporter. Mutations of GalNAcT3 result in decreased FGF23 activity and thus in increased phosphate reabsorption and hyperphosphatemia.

No efficient treatment is available.

Inheritance is autosomal recessive.

**TABLE 94-2 Genetic Protein O-Glycosylation Disorders**

Disease	Affected Protein	Defective Gene	OMIM Number
<i>Defect in O-N-Acetylglactosaminylglycan Synthesis</i>			
GALNT3-CDG	N-acetylglactosaminyltransferase 3	<i>GALNT3</i>	211900
<i>Defects in O-Xylosylglycan Synthesis</i>			
B4GALT7-CDG	Beta-1,4-galactosyltransferase 7	<i>B4GALT7</i>	130070
EXT1-CDG	Exostosin 1	<i>EXT1</i>	130700
EXT2-CDG	Exostosin 2	<i>EXT2</i>	130701
CHSY1-CDG	Chondroitin sulfate synthase	<i>CHSY1</i>	605282
<i>Defect in O-N-Acetylglactosaminylglycan and O-Xylosylglycan Synthesis</i>			
SLC35D1-CDG	UDP-glucuronic acid/UDP-N-acetylglactosamine dual transporter	<i>SLC35D1</i>	269250
<i>Defects in O-Mannosylglycan Synthesis</i>			
POMT1-CDG	O-mannosyltransferase 1	<i>POMT1</i>	236670, 613155, 609308
POMT2-CDG	O-mannosyltransferase 2	<i>POMT2</i>	613150, 613156, 613158
POMGNT1-CDG	O-mannose beta-1,2-N-acetyl-glucosaminyltransferase	<i>POMGNT1</i>	253280, 613151, 613157
<i>Defects in O-Fucosylglycan Synthesis</i>			
LFNG-CDG	O-fucose-specific beta-1,3-N-acetylglucosaminyltransferase	<i>LFNG</i>	609813
B3GALTL-CDG	O-fucose-specific beta-1,3-N-glucosyltransferase	<i>B3GALTL</i>	261540



### 94.3.2 EXT1/EXT2-CDG (Hereditary Multiple Exostoses)

These are characterized by osteochondromas of the ends of long bones. The tumors are often present at birth, their growth slows during adolescence and stops in adulthood. Only a small percentage of these lesions show malignant degeneration. Compression of peripheral nerves and blood vessels may cause complications (13).

The basic defect is in the Golgi-localized EXT1/EXT2 complex, which has both glucuronyltransferase and N-acetyl-D-hexosaminyltransferase activities involved in the polymerization of heparan sulfate. Evidence has been presented linking the development of the exostoses to activation of Indian hedgehog signaling during embryonic chondrocyte differentiation.

This is the only known CDG with autosomal-dominant inheritance.

No basic treatment is available.

### 94.3.3 SLC35D1-CDG (Schneckenbecken Dysplasia)

This is a severe skeletal dysplasia comprising mainly platyspondyly, extremely short long bones, and small ilia with snail-like appearance (14).

The solute carrier encodes an ER UDP-glucuronic acid/UDP-N-acetylgalactosamine dual transporter needed for chondroitin sulfate biosynthesis.

Inheritance is autosomal recessive.

No efficient treatment is available.

### 94.3.4 POMT1/POMT2-CDG (O-Mannosyltransferase 1 Deficiency, O-Mannosyltransferase 2 Deficiency)

These are rare neuronal migration disorders characterized by brain and eye dysgenesis associated with congenital muscular dystrophy. Male patients often have testicular defects. They are very severe diseases that usually run a fatal course before the age of 1 year. Psychomotor development is absent. The brain lesions consist of cobblestone lissencephaly, agenesis of the corpus callosum, cerebellar hypoplasia, hydrocephaly, and sometimes, encephalocoele (15).

In this disorder, there is an aberrant glycosylation of  $\alpha$ -dystroglycan, an external membrane protein expressed in muscle, brain and other tissues. Most glycans of this heavily glycosylated protein seem to be O-linked through mannose, and they control the interaction with extracellular matrix proteins. Disrupted glycosylation of  $\alpha$ -dystroglycan (and probably other glycoproteins) results in loss of this interaction, and hence, in progressive muscle degeneration and abnormal neuronal migration (overmigration) in the brain. A minority of patients with this syndrome show mutations in the *POMT1* gene coding for protein

O-mannosyltransferase-1. This enzyme catalyzes the first step in the synthesis of the O-mannose-linked core Gal $\beta$ 1-4GlcNAc $\beta$ 1-2Man-O-Ser/Thr.

No efficient treatment is available.

Inheritance is autosomal recessive.

### 94.3.5 POMGNT1-CDG (O-Mannose Beta-1,2-N-Acetylglucosaminyltransferase Deficiency)

This is a neuronal migration or congenital muscular dystrophy syndrome similar to POMT1/POMT2-CDG but less severe. As in the latter syndromes, skeletal muscle shows almost no  $\alpha$ -dystroglycan by Western blotting and immunohistochemical analysis with an antibody that recognizes a glycosylated epitope (15).

These patients show mutations in the gene encoding protein O-mannosyl- $\beta$ 1,2-N-acetylglucosaminyltransferase 1, catalyzing the second step in the synthesis of the O-mannosylglycan core.

No efficient treatment is available.

Inheritance is autosomal recessive.

### 94.3.6 LFNG-CDG (Spondylocostal Dysostosis Type 3)

Patients show a severe vertebral phenotype with malsegmentation due to disruption of somitogenesis. Somites are precursors of the axial skeleton and of the associated musculature (16).

This is a notch pathway defect in Lunatic Fringe, an O-fucose-specific  $\beta$ 1,3-N-acetylglucosaminyltransferase. Of note is that spondylocostal dysostosis type 1 and type 2 are also due to defects in the notch pathway.

No efficient treatment is available.

Inheritance is autosomal recessive.

### 94.3.7 B3GALT1-CDG (Peters Plus Syndrome)

Patients show peculiar eye malformations including corneal opacities and iridocorneal adhesions besides growth retardation and variable abnormalities in other organs (17).

Mutations are in a  $\beta$ 1,3-glucosyltransferase that adds glucose to O-linked fucose. This disaccharide modification is specific to thrombospondin type 1 repeats, found in extracellular proteins that function in cell-cell and cell-matrix interactions.

No specific treatment is available.

Inheritance is autosomal recessive.

## 94.4 CONGENITAL DISORDERS OF PROTEIN N- AND O-GLYCOSYLATION

Eighteen disorders have been reported (Table 94-3). The five most frequent are described here.

**TABLE 94-3 TA Genetic Protein Combined N- and O-Glycosylation Disorders**

Disease	Affected Protein	Defective Gene	OMIM Number
DPM1-CDG	GDP-Man:Dol-P-mannosyltransferase 1	<i>DPM1</i>	608799
DPM3-CDG	GDP-Man:Dol-P-mannosyltransferase 3	<i>DPM3</i>	612937
MPDU1-CDG	Dol-P-Man utilization 1	<i>MPDU1</i>	609180
B4GALT1-CDG	Beta-1,4-galactosyltransferase 1	<i>B4GALT1</i>	607091
GNE-CDG	UDP-GlcNAc epimerase/kinase	<i>GNE</i>	600737, 605820
SLC35A1-CDG	CMP-sialic acid transporter	<i>SLC35A1</i>	603585
SLC35C1-CDG	GDP-fucose transporter	<i>SLC35C1</i>	266265
DK1-CDG	Dolichol kinase	<i>DK1</i>	610768
SRD5A3-CDG	Steroid 5-alpha-reductase	<i>SRD5A3</i>	612379
DHDDS-CDG	Dehydrodolichyl-PP synthase	<i>DHDDS</i>	613861
COG7-CDG	COG complex 7	<i>COG7</i>	608779
COG1-CDG	COG complex 1	<i>COG1</i>	611209
COG8-CDG	COG complex 8	<i>COG8</i>	611182
COG4-CDG	COG complex 4	<i>COG4</i>	613489
COG5-CDG	COG complex 5	<i>COG5</i>	613612
COG6-CDG	COG complex 6	<i>COG6</i>	606977
ATP6V0A2-CDG	Vesicular H <sup>+</sup> -ATPase $\alpha$ 2	<i>ATP6V0A2</i>	219200, 278250
SEC23B-CDG	COPII component SEC23B	<i>SEC23B</i>	224100

#### 94.4.1 GNE-CDG (Hereditary Inclusion Myopathy, Nonaka Myopathy)

This myopathy has an adult onset with progressive distal and proximal muscle weakness. A peculiar feature is that it spares the quadriceps muscles. Muscle histology shows rimmed vacuoles on Gomori's trichrome stain, small fibers in groups and tubulofilaments without evidence of inflammation (18). The gene encodes a bifunctional enzyme that catalyzes the first two (and rate-limiting) steps in sialic acid biosynthesis.

Trials with oral sialic acid precursors are underway in a mouse model.

Inheritance is autosomal recessive.

#### 94.4.2 SRD5A3-CDG (Steroid 5-Alpha-Reductase Deficiency)

Fifteen patients belonging to seven families have been reported. Besides psychomotor retardation, the most prominent symptoms are cerebellar (atrophy, vermis malformations), ophthalmological (hypoplasia or coloboma of the iris, retina, choroid, optic disc; nystagmus, optic atrophy, microphthalmia, glaucoma, cataract) and cutaneous (ichthyosis, erythroderma, dry skin, atopic dermatitis) (19). This is the second reported defect in the synthesis of dolichol phosphate, more specifically in the conversion of pre-dolichol to dolichol.

No therapy is currently available.

Inheritance is autosomal recessive.

#### 94.4.3 COG7-CDG (COG Complex 7 Deficiency)

The conserved oligomeric Golgi (COG) complex is an eight-subunit (COG1-8) peripheral Golgi membrane hetero-oligomeric protein complex. It is organized into

lobes A (COG2-4) and B (COG5-7) with COG1 and COG8 bridging these lobes. This complex is thought to play a critical role in vesicle tethering processes involving retrograde Golgi transport of resident proteins responsible for glycan biosynthesis. Defects have been reported in COG1 (COG1-CDG), COG7 (COG7-CDG), COG8 (COG8-CDG), COG4 (COG4-CDG), COG5 (COG5-CDG) and COG6 (COG6-CDG). The seven reported COG7-deficient patients were all of North African origin. They had a lethal disorder (six of them died in their first year of life) and showed in addition hyperthermia, ventricular/atrial septum defect, and cholestatic liver disease (20). Studies of fibroblast glycoproteins showed a partial N- and O-glycosylation defect caused by a decreased transport of CMP-sialic acid and UDP-galactose into the Golgi, and a reduced activity of two glycosyltransferases involved in the galactosylation and sialylation of O-glycans. The six patients with early lethality were homozygous for the same intronic mutation in *COG7*.

No therapy is currently available.

Inheritance is autosomal recessive.

#### 94.4.4 ATP6V0A2-CDG (Autosomal Recessive Cutis Laxa Type IIA, Wrinkly Skin Syndrome)

These patients have generalized cutis laxa at birth that becomes less obvious with age.

They also present with increased joint laxity, ophthalmological abnormalities (mainly strabismus, myopia or amblyopia, and sometimes corneal dystrophy), microcephaly, and delayed motor development that improves with age (21).

The defect is in the  $\alpha$ 2 subunit of the vesicular H<sup>+</sup>-ATPase pump. Tropoelastin aggregates are found in their Golgi apparatus.

No therapy is currently available.

### 94.4.5 SEC23B-CDG (Congenital Dyserythropoietic Anemia Type II)

Congenital dyserythropoietic anemia type II (CDAIL) has recently been shown to be due to mutations in the gene coding for SEC23B, a COPII component (22).

The disorder is limited to the erythroblastic lineage most probably because in the other tissues, a SEC23A isoform compensates for the SEC23B deficiency.

No efficient treatment is available.

Inheritance is autosomal recessive.

## 94.5 PERSPECTIVES

The field of CDG continues to grow at a rapid rate. Some 50 CDG have been unraveled in the course of 30 years. Major developments are to be expected in the following areas: defects in dolichol metabolism, defects in lipid glycosylation, defects in organ-specific glycosylation, and defects in other multifunctional proteins, i.e. proteins that are involved not only in glycosylation but also in other functions. Advances in treatment are eagerly awaited since an efficient treatment is available for only one CDG (MPI-CDG). Efforts should be directed, in particular, toward a therapy for the important and often devastating disorder, PMM2-CDG. Since about 1% of the human genome is devoted to glycosylation, it is evident that we know only a small minority of existing CDG. The search for novel CDG will, at the same time, greatly booster our knowledge of the many unknown aspects of this most important protein (and lipid) modification.

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### Biography



Jaak Jaeken, MD, PhD, is an Emeritus Professor of Pediatrics at the University of Leuven, Belgium. He is a specialist in metabolic diseases and his main interest is in congenital disorders of glycosylation (CDG) which he first described in 1980. He is actively involved in the study of all aspects of this rapidly growing family of metabolic diseases with some 55 diseases actually known. Since about 1% of the human genome is devoted to glycosylation, it is to be expected that the majority of CDG has still to be discovered. Jaak Jaeken received in 1999 the degree of Doctor Honoris Causa from the University of Zurich, and in 2000 from the University of Havana.



# CHAPTER

# 95

## Purine and Pyrimidine Metabolism

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### GLOSSARY

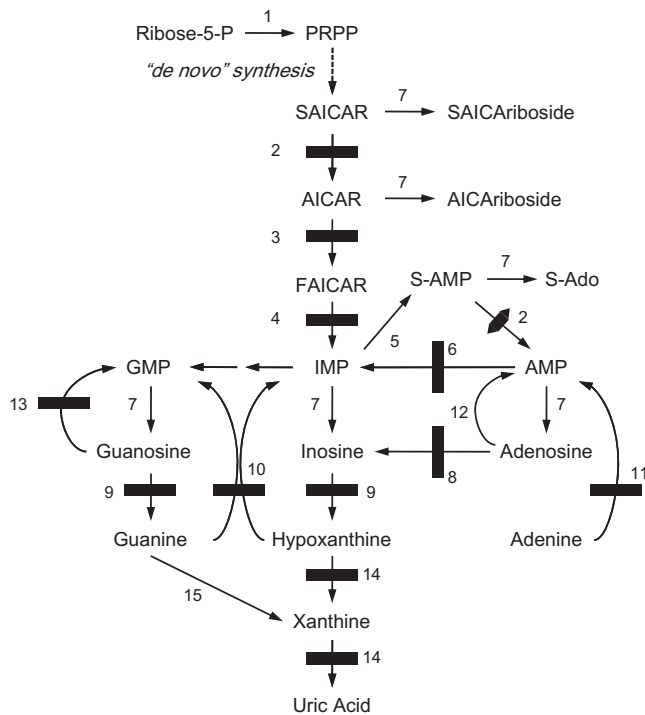
**GWAS** – Genome-wide association study is a method to identify genes or genomic variations associated with traits such as diseases, drug reactions, physical measures, and laboratory test values. In GWAS (sometimes called whole-genome association study or WGA), the associations between a trait and more than 100,000 single-nucleotide polymorphisms (SNPs) spanning the whole genome are statistically analyzed. Careful collection of samples and data from many individuals, powerful and high-speed genotyping machines, and computer programs are the keys of the study.

### 95.1 INTRODUCTION

The study of disorders of purine and pyrimidine metabolism began in the nineteenth century. In 1848, A.B. Garrod (1) found that gouty arthritis is associated with uric acid. In the following century, his son, A.E. Garrod (2), who first introduced the concept of an “inborn error of metabolism,” listed gout among the first diseases that implicated inborn factors. In 1964, Lesch-Nyhan syndrome was reported as an inherited disease and a deficiency of hypoxanthine–guanine phosphoribosyltransferase (HGPRT or HPRT) was discovered a few years later (3). Subsequently, the following purine enzyme

abnormalities were found: adenine phosphoribosyltransferase deficiency (4,5), phosphoribosyl pyrophosphate (PRPP) synthetase superactivity (6), adenosine deaminase (ADA) deficiency (7), purine nucleoside phosphorylase deficiency (8), enzyme deficiencies associated with xanthinuria (9,10), adenylosuccinate lyase deficiency (11), myoadenylate deaminase deficiency (12), ATIC deficiency (13), thiopurine methyltransferase deficiency (14), and deoxyguanosine kinase deficiency (15). The following pyrimidine enzyme abnormalities were found: UMP synthase deficiency (16), dihydropyrimidine dehydrogenase deficiency (17), dihydropyrimidinase deficiency (18),  $\beta$ -ureidopropionase deficiency (19), pyrimidine 5'-nucleotidase deficiency (20), cytosolic 5'-nucleotidase superactivity (21), thymidine phosphorylase deficiency (22), dihydroorotate dehydrogenase deficiency (23), cytidine deaminase deficiency (24), and thymidine kinase 2 deficiency (25).

In the 1980s and 1990s, molecular biology techniques were used to identify the mechanisms for the reported enzyme deficiencies at the DNA and RNA levels, and the precise changes in the genes were identified. In the 2000s, in addition to the previous candidate gene approach, genome-wide approaches were used to discover the genetic causes of gout and hyperuricemia. Thus, the uromodulin (*UMOD*) gene abnormality was discovered



**FIGURE 95-1** Pathways of purine metabolism. AICAR, aminoimidazole carboxamide ribotide; AMP, adenosine monophosphate; FAICAR, formylaminoimidazolecarboxamide ribotide; GMP, guanosine monophosphate; IMP, inosine monophosphate; P, phosphate; PRPP, phosphoribosyl pyrophosphate; S-Ado, succinyladenosine; SAICAR, succinylaminoimidazolecarboxamide ribotide; S-AMP, adenylosuccinate; XMP, xanthosine monophosphate. 1, PRPP synthetase; 2, adenylosuccinate lyase (adenylosuccinase); 3, AICAR transformylase; 4, IMP cyclohydrolase (3 and 4 form ATIC); 5, adenylosuccinate synthetase; 6, AMP deaminase; 7, 5'-nucleotidase(s); 8, adenosine deaminase; 9, purine nucleoside phosphorylase; 10, hypoxanthine-guanine phosphoribosyltransferase; 11, adenine phosphoribosyltransferase; 12, adenosine kinase; 13, guanosine kinase; 14, xanthine oxidase (dehydrogenase); 15, guanine deaminase. Enzyme defects are indicated by solid bars.

by linkage analysis (26,27) and many genes including *SLC22A12*, *SLC22A11*, *SLC2A9*, *ABCG2*, *SLC17A1*, *WDR1*, *GCKR*, *PDZK1*, *R3HDM2-INHBC* region, *RREB1*, and *LRP2* were found to be associated with serum urate concentrations by genome-wide association studies (GWASs) (see Section 95.2.2.4). The latter genes, many of which code for either the transporters in renal tubules or related proteins, are considered to constitute polygenic factors for hyperuricemia.

Monogenic disorders of purine metabolism (Figure 95-1) are known to be responsible for a wide variety of clinical conditions, including kidney stones of various compositions (5,9,28), immunodeficiency diseases (7,8), exercise intolerance (12), and a number of devastating neurologic syndromes (3,10,11,13,29). Inborn errors of pyrimidine metabolism have been found to be responsible for megaloblastic anemia (16), hemolytic anemia (20), and various mental and neurologic disorders (18,19,21,30,31,32).

Thiopurine methyltransferase (33) and dihydropyrimidine dehydrogenase (34) deficiencies have provided

major contributions to the emerging field of pharmacogenomics. In recent years, deficiencies of enzymes for purine and pyrimidine metabolism have been identified in mitochondrial disorders (15,25,35).

For ADA deficiency, enzyme replacement therapy and somatic gene therapy are very effective at improving the severe immunodeficiency symptoms of patients (see Section 95.2.7.5).

In this chapter, a survey of the partly genetically determined disorder, gout, is followed by overviews of the inborn errors of metabolism that have been discovered in the purine and pyrimidine metabolic pathways.

## 95.2 PURINE METABOLISM

### 95.2.1 Metabolic Pathways

Purine nucleotides are essential cellular constituents that are involved in energy transfer, metabolic regulation, and the synthesis of DNA and RNA. Purine metabolism can be divided into three pathways (see Figure 95-1):

The biosynthetic pathway, often termed the *de novo* pathway, starts after the formation of the high-energy compound PRPP, and leads to the synthesis of inosine monophosphate (IMP) in 10 steps (not shown in detail in Figure 95-1). From IMP, two reactions lead to the formation of adenosine monophosphate (AMP). Subsequently, adenosine diphosphate (ADP) and adenosine triphosphate (ATP), and their deoxy counterparts (not shown in Figure 95-1) are formed. Two other reactions convert IMP into guanosine monophosphate (GMP), from which guanosine diphosphate (GDP), guanosine triphosphate (GTP), and their deoxy counterparts (not shown in Figure 95-1) are formed.

The purine catabolic pathway starts from GMP, IMP, and AMP, and produces uric acid, a poorly soluble compound that tends to crystallize once its plasma concentration surpasses 6.5–7.0 mg/dL (0.38–0.47 mmol/L).

The purine salvage pathway uses the purine bases guanine, hypoxanthine, and adenine, which are provided by food intake or the catabolic pathway, and reconverts them into GMP, IMP, and AMP, respectively. Salvage can also occur by the phosphorylation of nucleosides such as adenosine.

### 95.2.2 Gout

Gout is a common disorder caused by the formation of urate crystals in the body. These may result in crystal-induced arthritis, tophi formation, urolithiasis, and kidney disease. Urate crystals may form in the body when the concentration of urate exceeds the limit of solubility (~7.0 mg/dL in plasma at 37°C). Most mammalian species possess the urate-degrading enzyme urate oxidase and, hence, have rather low plasma concentrations of urate. The human genome contains the sequence for urate oxidase, but the gene has stopped functioning

owing to at least three deleterious mutations (36). Due to this deficiency in urate oxidase, humans tend to display plasma urate levels above the limit of solubility, and some individuals with serum urate concentrations  $>7.0$  mg/dL develop gout.

**95.2.2.1 Epidemiology.** Hyperuricemia (usually defined as a urate serum concentration  $>7.0$  mg/dL in men and  $>6.0$  mg/dL in women or  $>7.0$  mg/dL in both genders) does not necessarily indicate the presence of gout; however, the higher the serum urate concentration, the higher the incidence of gout (37). Serum urate concentrations are affected by various factors such as ethnicity, sex, age, social status, habits, and body weight. The prevalence of gout or hyperuricemia was reported to have recently increased in the United States (38,39), Europe (40,41), Saharan Africa (42), and Asian countries (43,44). The average serum urate concentration in men is  $\sim 1.0$ – $1.5$  mg/dL higher than in women. In men, the serum urate concentration increases during puberty and stays at the same level thereafter, whereas it increases after the menopause in women. Therefore, gout is primarily a disease affecting adult males and postmenopausal adult females. The proportion of women among patients with gout is  $\sim 5\%$ , although this statistic varies between reports.

Genetic and environmental factors are associated with hyperuricemia and gout. There is ample evidence indicating that various environmental factors, such as alcohol ingestion and the consumption of meat and seafood, are associated with gout (45,46). The heritability of serum urate levels has been estimated to be 0.7–0.8, and a recent GWAS has successfully identified some of these genetic factors (see Section 95.2.2.4).

**95.2.2.2 Clinical Features.** *Presentation.* Patients with gout usually experience three stages: asymptomatic hyperuricemia, followed by intermittent episodes of gout, and, finally, chronic tophaceous gout. After several years of asymptomatic hyperuricemia, the first episode of gouty arthritis often occurs suddenly, usually in healthy men.

Acute gouty arthritis, often called a gouty attack, is the most prominent clinical feature of the second stage. It is characterized by (a) sudden onset; (b) the presence of aura (abnormal sense around the affected joint) before the initiation of inflammation; (c) monoarthritis; (d) marked inflammation accompanied by redness, warmth, swelling, and severe pain; (e) peak inflammation within 24 h after onset; and (f) almost complete relief of inflammation within 7 days. In  $\sim 50$ – $70\%$  of cases, the first gouty attack occurs in the first metatarsophalangeal joint (podagra), but other common sites include the insteps, ankles, heels, knees, wrists, finger joints, and elbows. Later, the arthritis may become polyarticular, last for more than a week, not subside completely, and be accompanied by fever. Laboratory findings include elevated C-reactive protein, sedimentation rate, and white blood cell count.

In the intermittent stage of gout, that is during the intercritical periods between attacks, an almost complete

remission of symptoms is observed. The second attack usually occurs within 6 months to 2 years of the first. If the hyperuricemic state is not corrected, the frequency of attacks increases.

If gout remains untreated for several years, chronic tophaceous gout may develop. In untreated patients, visible tophi appear at an average of 11.6 years (range 3–42 years) after the initial attack. The interval between gouty attacks becomes unclear and patients suffer almost continuous pain although the pain is less pronounced than that from acute gouty arthritis. Tophi result from the deposition of monosodium urate monohydrate crystals. In the chronic stage of gout, tophi are increasingly detected in various parts of the body, except the brain. Tophi are most often detected in the helix of the ear, toes, knees, hands and fingers, the ulnar surface of the forearm, and the olecranon bursa. In rare cases, tophi that have formed in the spinal canal, bone marrow, and vocal cords have caused neurologic symptoms, bone fractures, and hoarseness, respectively. Tophi can cause changes that are visible on radiographs; bony erosions due to tophi typically show a punched-out appearance. An overhanging edge of bone is also typical of the changes caused by tophi that can be seen on a radiograph. In severe cases, evidence of massive bone destruction can be seen. If left untreated, tophi may destroy joints, and patients may eventually become crippled although such a progression of the disease is very rare in developed countries where patients can receive proper treatment for hyperuricemia.

Renal disease in patients with gout can be caused by several different mechanisms. Urate nephropathy is a disorder caused by the deposition of urate crystals in the interstitial tissue of the kidneys, whereas uric acid nephropathy is caused by the deposition of uric acid crystals, from urine, in the collecting tubules, renal pelvis, and ureter. Due to the difference in pH (the pH of urine is lower), different types of crystals form in tissue fluid and urine. In addition, other factors such as hypertension and the use of anti-inflammatory drugs can cause renal insufficiency in patients with gout. Clinically, gouty nephropathy is characterized by mild proteinuria and a reduced ability to concentrate urine. If patients receive proper treatment, severe renal insufficiency does not occur in most cases. Patients with familial juvenile hyperuricemic nephropathy (FJHN) are an exception because terminal renal insufficiency may occur even when patients receive proper treatment (Section 95.2.2.4).

*Diagnosis.* Diagnosis of acute gouty arthritis is not difficult when the patient has experienced more than one episode of arthritis. The characteristics of this condition, listed as (a)–(f) in the previous section, enable clinicians to make a correct diagnosis. The most confirmatory test for the diagnosis of acute gouty arthritis is the detection of urate crystals in neutrophils in fluid aspirated from the inflamed joints. Crystals can be detected with a standard microscope; however, the detection of needle-shaped,

negatively birefringent crystals by a polarizing microscope confirms that the component of the crystals is monosodium urate. Although the detection of urate crystals in neutrophils can confirm a diagnosis of gouty arthritis, the finding of urate crystals outside the cells in aspirated synovial fluid and the finding of tophi are also useful in the diagnosis of gout because they show that urate crystals have formed in the body.

Differential diagnoses of acute gouty arthritis include septic arthritis, bacterial cellulitis, and palindromic rheumatism. Inflammation of the first metatarsophalangeal joint, due to hallux valgus, sometimes resembles an acute gouty attack.

**95.2.2.3 Classification.** The plasma urate concentration is determined by its production in the liver and its excretion by the kidneys; hence, hyperuricemia can be caused by two different mechanisms: namely, the overproduction of urate by the liver and the underexcretion of urate by the kidneys. According to these mechanisms, hyperuricemia and gout are classified into three types: an overproduction type, an underexcretion type, and a mixed type.

The overproduction type composes ~10% of all cases. The overproduction and underexcretion types of hyperuricemia may be secondary to another disorder or the administration of drugs. In myeloproliferative disorders and lymphomas, hyperuricemia occurs as a result of urate overproduction. Renal insufficiency and the administration of drugs such as thiazides, loop diuretics, pyrazinamide, cyclosporine A, and low-dose aspirin cause hyperuricemia by provoking the underexcretion of urate. Gout caused by another disorder or by the administration of drugs is called secondary gout, as opposed to primary gout, in which no such conditions are identified.

It is of note that hyperuricosuria can also be associated with hypouricemia, which can be caused by genetic factors.

**95.2.2.4 Molecular Genetics.** *Genetic Basis of Hyperuricemia and Gout.* Although genetic factors play a role in the development of hyperuricemia and gout in many patients, the degree of involvement varies from patient to patient. Mendelian inheritance can be suspected in only a very small proportion of patients with a family history of gout.

A number of inherited enzyme abnormalities result in the early development of the overproduction type of hyperuricemia and gout. Most notably, HGPRT deficiency (3,28) and PPRP synthetase superactivity (6,47), both caused by mutations in two different X-linked genes, lead to marked hyperuricemia and gout (see the corresponding sections in this chapter). In addition, hereditary fructose intolerance and some glycogen-storage diseases, including glucose-6-phosphatase deficiency (glycogen-storage disease type I), debranching enzyme deficiency (type III), muscle phosphorylase deficiency (type V), and muscle phosphofructokinase deficiency (type VII), may also cause episodic or continuous hyperuricemia and, potentially, gout (see Chapter 93) (48).

Recently, genome-wide approaches have been used to identify genes associated with gout and serum urate concentrations; thus, uromodulin gene (*UMOD*)

abnormalities were shown to cause FJHN by linkage analysis (26), followed by a search in the 16p12 chromosomal region for the gene responsible for the disease (27). So far, 64 different mutations have been reported in *UMOD*, most of which cause amino acid substitutions. More than half of these substitutions involve cysteine residues. FJHN is characterized by the gradual deterioration of renal function after adolescence and is accompanied by hyperuricemia and gout (49,50). The disease is inherited as an autosomal dominant trait. In the renal tubules of patients, mutant uromodulin protein accumulates within the endoplasmic reticulum, probably because of a failure to excrete the protein into urine (51,52). The accumulation of uromodulin protein within the tubular epithelia of the thick ascending limb of the loop of Henle, owing to mutations that change the amino acids, is likely to be responsible for the decrease in the excretion of urate in the tubules and for hyperuricemia and renal insufficiency (52). Two additional genes, *REN* (53) and *HNF-1 $\beta$*  (54), have been identified as disease loci for FJHN.

*Genetic Basis of Hypouricemia.* Inherited renal hypouricemia has long been reported (55,56). Serum urate concentrations in typical cases are <2.0 mg/dL. This genetic defect can lead to urolithiasis (57) and acute renal failure after exercise (58). Homozygous mutations in *SLC22A12* (59–61) and homozygous and heterozygous mutations in *SLC2A9* (62), which code for renal transporters, were discovered in subjects with hypouricemia.

Although most cases of gout or hyperuricemia are thought to be multifactorial, the search for the genes associated with such conditions had been unsuccessful because of the lack of power of linkage analysis for multifactorial diseases. Since 2002, GWASs have successfully identified the genes responsible for complex traits (63). This approach was successfully used to identify the genes associated with serum urate concentrations, a typical quantitative trait. In these studies, the associations between the serum urate concentrations and the genotypes of many single nucleotide polymorphisms (SNPs) (typically the number of SNPs examined is ~500,000) were examined in many subjects (typically more than 10,000 subjects). GWASs have identified many genes associated with urate concentrations in serum, including *SLC22A12*, *SLC22A11*, *SLC2A9*, *ABCG2*, *SLC17A1*, *WDR1*, *GCKR*, *PDZK1*, *R3HDM2-INHBC* region, *RREB1*, and *LRP2* (64–67). Many of these genes code for renal transporters or related proteins, and are thought to be associated with the reabsorption or secretion of urate in the microtubules of the kidney (68–72). These results suggest that many of the genetic factors for hyperuricemia involve the excretion of urate in the kidney rather than its overproduction in the liver.

**95.2.2.5 Treatment.** The typical treatment for acute gouty attacks includes rest, elevation of the affected joint, and administration of a nonsteroidal anti-inflammatory drug (NSAID). Indomethacin is an effective drug, but



other NSAIDs are also effective. NSAIDs are used at the highest approved dose of each selected agent and are usually continued until the pain decreases to a low degree. Orally administered glucocorticoids are as effective as NSAIDs (73). Oral colchicine, given at a dose of, at most, 1.8 mg/day, is also effective in the early stage of an acute gouty attack. However, the administration of colchicine every hour until adverse events occur, such as diarrhea, is no longer recommended. Rather, a small dose of colchicine may be used for the treatment of acute gouty attacks or as a prophylactic drug when patients suffer from frequent attacks and when they have higher probabilities of attacks after urate-lowering drugs are initiated.

If only acute gouty attacks are treated, without any correction of the hyperuricemia, chronic tophaceous gout may develop. In severe cases, destruction of the joints may lead to disability and renal insufficiency may also develop; however, this does not necessarily mean that all patients with hyperuricemia should be treated. Asymptomatic hyperuricemia is not usually the target of urate-lowering therapy, unless the urate concentration is very high. If this is the case, or if the patient has experienced urolithiasis, alkalization of the urine to a pH of ~6.5 (6.2–6.8) by the use of either sodium bicarbonate or potassium sodium citrate is recommended, along with adequate water intake. As the need for a urate-lowering drug is likely to be lifelong, the advantages and the disadvantages of its administration should be carefully compared. The benefits of urate-lowering therapy include the prevention of acute gouty attacks, the inhibition of disease progression to chronic tophaceous gout, and the prevention of the development of urolithiasis and renal insufficiency. The disadvantages include the occurrence of adverse events caused by the drugs and the necessity to attend clinics and hospitals. Urate-lowering therapy should be started when the inflammation of an acute gouty attack has subsided almost completely because the initiation of such therapy during an acute gouty attack almost always leads to the exacerbation and prolongation of inflammation. Three types of urate-lowering drugs are available: uricosuric drugs, xanthine oxidase inhibitors, and uricases. Probenecid and sulfinpyrazone are available uricosuric drugs, and allopurinol and febuxostat (74–77) are available xanthine oxidase inhibitors. Rasburicase (78–80), a recombinant uricase that is used to treat tumor lysis syndrome, prevents or improves the hyperuricemic nephropathy in patients receiving chemotherapy for hematologic neoplasms such as leukemia and lymphoma. Pegloticase (81,82), a pegylated porcine-like uricase that is used by injection, has recently been approved for the treatment of gout in the United States. There is no strict rule as to which type of drug should be used, but pegloticase should only be used in limited cases where its rapid and strong urate-lowering effects are required. Xanthine oxidase inhibitors are recommended for patients with overproduction-type gout or who have experienced episodes of urolithiasis; however,

allopurinol should be avoided or given at lower dosages in patients with renal function impairment because it can cause severe side effects in these cases. In addition, allopurinol-induced severe skin diseases, including Stevens–Johnson syndrome and toxic epidermal necrolysis (TEN), are strongly associated with *HLA-B\*5801*, which is found at a high frequency, especially in patients of Han Chinese ancestry (83).

It is important to note that, even if begun when no inflammation is present, urate-lowering therapy may evoke gouty attacks; therefore, a urate-lowering drug should be started at the lowest effective dose, i.e., 500 mg/day for probenecid, 100 mg/day for allopurinol, and 40 mg/day in the USA, 80 mg/day in Europe, or 10 mg/day in Japan for febuxostat. The dose of each drug should be increased gradually until the serum urate concentration decreases to <6.0 mg/dL or until the highest approved dose is reached. During an initial 6-month period of urate-lowering therapy, acute gouty attacks tend to occur more often than before treatment. Colchicine and an NSAID are used to treat gouty attacks during this period. Prophylactic colchicine coverage is sometimes preferred if gouty attacks are highly expected.

Lifestyle alteration is also an important aspect of urate-lowering therapy. Studies based on a large cohort have shown that alcohol consumption, especially of beer, and high levels of meat and seafood intake are associated with gout (45,46). In addition, weight loss by obese patients has an evident hypouricemic effect. Therefore, change of lifestyle is likely to lower serum urate concentrations. Most large cohort studies have revealed that the serum urate concentration is positively correlated with mortality from ischemic heart disease. However, whether hyperuricemia is an independent risk factor (84–89) or whether the association is secondary to other factors, such as body weight, hyperlipidemia, and hypertension (90–92), is still controversial. Nevertheless, regardless of whether hyperuricemia is an independent risk factor, controlled weight reduction is recommended for obese patients with hyperuricemia because it will prolong their lives.

### 95.2.3 PRPP Synthetase: Superactivity and Deficiency Syndromes

PRPP-synthetase is involved in the synthesis of PRPP, which serves as a cosubstrate for several enzymes involved in the metabolism of purines, pyrimidines, and pyridines. In purine metabolism, PRPP serves as a cosubstrate for amido phosphoribosyltransferase, the first step in the de novo synthesis of purines (Figure 95-1). It also serves as a cosubstrate for the recycling enzymes, HPRT and APRT. In pyrimidine metabolism, PRPP serves as a cosubstrate for uridine monophosphate synthetase (Figure 95-4), which generates the precursor for pyrimidine nucleotide synthesis. Finally, PRPP also is used for the production of the pyridine nucleotides cofactors NAD and NADP. There are three PRPP synthetases encoded

**TABLE 95-1 PRPP Synthetase Disorders**

	PRS1 Superactivity	Arts Syndrome	CMTX5	DFN2
Hearing loss	Variable	Yes	Yes	Yes
Neuropathy	Variable	Yes	Yes	No
Optic atrophy	No	Yes	Yes	No
Psychomotor retardation	Variable	Yes	No	No
Recurrent infections	No	Yes	No	No
Uric acid overproduction	Yes	No	No	No
PRS1 enzyme activity	High	Absent	Low	Low
Reported mutations	D52H, N114S, L129I, D182H, A189V, H192L, H192Q	Q133P, L152P	E43D, M115T	D65N, A87T, I290T, G306R

The reported mutations follow standard nomenclature with one-letter abbreviations to designate the amino acid change.

by three different genes, but only the *PRPS1* gene has been linked with human disease. The mutations affecting the PRS1 enzyme provide an example of how defects in a single gene can lead to four very different clinical phenotypes, which are outlined below and summarized in Table 95-1. For a detailed review, see Reference (93).

**95.2.3.1 PRS1 Superactivity.** Excessively high activity of PRS1 was first described as an X-linked disorder associated with hyperuricemia and gout in young adults (94). Subsequent studies revealed that some patients also suffered from childhood-onset neurological disability characterized by psychomotor retardation and sensorineural hearing loss. The mild form of the disorder with only overproduction of uric acid is thought to reflect misregulation of the gene transcript, because *PRPS1* is overexpressed with no defects in the reading frame. The result is higher PRPP levels, which drive PRPP-dependent enzymes at an unusually high rate. Excessive drive of de novo purine synthesis is the cause of hyperuricemia (93).

The more severe form of the disorder with accompanying neurological defects arises from point mutations in the reading frame for the protein. These mutations result in an enzyme with reduced feedback inhibition, but increased lability. The resulting effect on PRPP levels is complex and probably tissue dependent. Some tissues may have abnormally high PRPP levels, while postmitotic cells such as erythrocytes have low PRPP levels. The mechanisms responsible for the neurological defects are not well understood.

**95.2.3.2 Arts Syndrome.** This syndrome was first described in 1993 as an X-linked disorder with infantile-onset psychomotor retardation, sensorineural hearing loss, and optic atrophy (95). Overproduction of uric acid does not occur. In affected males, death due to recurrent respiratory infections occurred before 5 years of age. The syndrome results from mutations leading to the loss of PRS1 function. The mechanisms for neural dysfunction are not known, and effective treatments are lacking currently.

**95.2.3.3 CMTX5 or Rosenberg–Chutorian Syndrome.** Charcot–Marie–Tooth disease consists of a family of inherited disorders with prominent involvement of peripheral nerves (96,97). It is both clinically and etiologically heterogeneous, with type CMTX5 being caused by mutations in *PRPS1*. Patients with CMTX5 have an

X-linked disorder of peripheral neuropathy combined with sensorineural hearing loss beginning at 10–12 years of age. Overproduction of uric acid does not occur. Mutations causing CMTX5 reduce PRS1 enzyme activity, but do not eliminate it. The mechanisms responsible for neuropathy are not known, but may reflect the heavy dependence of myelin on lipid esters of pyrimidine nucleotides such as CDP-choline, CDP-neuraminic acid, and CDP-ethanolamine.

**95.2.3.4 Progressive Deafness DFN2.** Progressive, nonsyndromic, and postlingual hearing loss type DFN2 also results from point mutations in *PRPS1*. These patients do not have the neurological impairments associated with other *PRPS1* mutations, and they do not overproduce uric acid. The mechanisms for hearing loss are not known (98).

## 95.2.4 Adenylosuccinate Lyase Deficiency

Adenylosuccinate lyase (ADSL, also known as adenylosuccinase) catalyzes two steps in the synthesis of purine nucleotides (see Figure 95-1): the conversion of succinyl-aminoimidazole carboxamide ribotide (SAICAR) into aminoimidazole carboxamide ribotide (AICAR), the eighth step of the de novo pathway, and the formation of AMP from adenylosuccinate (S-AMP), the second step in the conversion of IMP into AMP. ADSL deficiency was the first enzyme defect reported in humans in the de novo pathway of purine synthesis (11). It is mostly characterized by a marked psychomotor delay and the accumulation of succinyl-aminoimidazole carboxamide riboside (SAICA-riboside) and succinyladenosine (S-Ado) in body fluids. These succinylpurines are the products of the dephosphorylation, by 5'-nucleotidase(s), of SAICAR and S-AMP, respectively. This disorder is mainly reported in Europe, probably because of the more intensive screening for this deficiency in European countries than in other areas of the world (99–102).

**95.2.4.1 Clinical Features.** Patients present with variable associations of psychomotor delay, epilepsy, autistic features, hypotonia, and secondary feeding problems. In the first reported presentation, often referred to as type I, patients display moderate-to-severe psychomotor

delay, frequently accompanied by epilepsy after the first years, and various behavior disturbances. In one of these families, severe growth retardation with muscular wasting was also observed. Rare patients, referred to as type II, show strikingly less psychomotor delay than type I patients. Other patients have been reported with convulsions starting within the first days to weeks of life (103), while a few patients display only autistic features (104). Fundoscopy, auditory, somatosensory and visual-evoked responses, nerve conduction velocities, and electromyography are normal, even in patients with muscular wasting. Computed tomography and magnetic resonance imaging of the brain often show hypotrophy or hypoplasia of the cerebellum, particularly of the vermis.

**95.2.4.2 Diagnostic Tests.** Diagnosis is based on the presence of SAICA-riboside and S-Ado in urine and cerebrospinal fluid, which are normally nearly undetectable. For systematic screening, a modified Bratton–Marshall test, performed on urine, appears to be the most practical; however, false positive results may be observed in patients who receive antibiotics, particularly sulfonamides (for whose measurement the test was initially devised), or antiepileptic medications. Succinylpurines can also be detected by other techniques, including two-dimensional thin-layer chromatography with staining of imidazole compounds. The final diagnosis requires high performance liquid chromatography (HPLC) with ultraviolet spectral analysis (11) or tandem mass spectrometry.

**95.2.4.3 Biochemical Features.** ADSL catalyzes the nonhydrolytic cleavage of the C–N bond linking the succinate moiety to the nucleotide part of its two substrates, SAICAR and S-AMP, to yield fumarate, and AICAR and AMP, respectively. The reaction is similar to that catalyzed by the urea cycle enzyme argininosuccinate lyase. ADSL is composed of four subunits of ~50 kDa. The human enzyme has been crystallized and its three-dimensional structure has been clarified (PDB ID: 2vol6). Measurements of the activity of ADSL with S-AMP as a substrate show markedly decreased activity down to undetectable activities in the liver and kidneys of all patients investigated. In peripheral blood lymphocytes, ADSL activity is ~40% of normal, while the activity of erythrocyte ADSL is normal in some patients, but decreased in others. As a rule, a partial deficiency of ADSL is measured in cultured fibroblasts.

A comparison of the activities of ADSL with S-AMP and SAICAR as substrates shows that both are lost in parallel to ~30% of normal in fibroblasts from profoundly retarded, type I patients. In contrast, in fibroblasts from a mildly retarded, type II patient, the activity with S-AMP was reduced to 3% of normal, whereas that with SAICAR was 30% of control. These findings provide an explanation for the markedly higher S-Ado/SAICA-riboside ratio in the body fluids of the latter patient.

**95.2.4.4 Pathophysiologic Mechanisms.** It is more likely that the symptoms of ADSL deficiency are due

to the toxic effects of the accumulating succinylpurines rather than impaired purine nucleotide synthesis. Moreover, the observation of generally less severe mental retardation in patients with similar SAICA-riboside levels, but with S-Ado/SAICA-riboside ratios >2, suggests that SAICA-riboside is the offending compound and that S-Ado could protect against its toxic effects.

**95.2.4.5 Molecular Genetics.** The ADSL gene has been mapped to chromosome 22q13.1–13.2 and encodes a protein of 484 amino acids.

Analysis of the first identified family with ADSL deficiency revealed a T to C substitution, resulting in a S438P change (105,106). In accordance with the variability of the clinical symptoms, many mutations including missense single nucleotide changes, nonsense mutations, splicing error mutations, and mutations in the 5'-untranslated region have been reported in apparently unrelated sibships (Adenylosuccinate Lyase Mutation Database, <http://www.icp.ucl.ac.be/adslldb>). The R426H mutation is the most frequently encountered, accounting for approximately one-third of the alleles investigated. Prenatal diagnosis of ADSL deficiency has been performed twice on chorion villi from the mother of a previously diagnosed patient with a C5T mutation on the maternal allele, and a C1185A mutation on the paternal allele (107).

**95.2.4.6 Treatment.** Some therapeutic trials have been performed, including oral supplements of adenine and the oral administration of ribose and uridine, without remarkable effects (108,109).

The prognosis for survival of ADSL-deficient patients is variable. Several of those presenting with early epilepsy have died within the first months of life. In markedly retarded patients, absent or minimal progression of psychomotor development and the persistence of autistic behavior were observed. Mildly impaired patients have reached adult age and are able to work in a protected environment.

## 95.2.5 AICA-Ribosiduria (ATIC Deficiency)

This was the second enzyme defect identified in humans in the de novo pathway of purine synthesis (13). The first patient was a female infant presenting with profound intellectual disability, marked dysmorphic features (prominent forehead and metopic suture, brachycephaly, wide mouth with a thin upper lip, low-set ears, and a prominent clitoris due to a fused labia majora), and congenital blindness. Urine analysis showed a positive Bratton–Marshall test. This led to the identification of the massive excretion of 5-amino-4-imidazolecarboxamide (AICA)-riboside, the dephosphorylated counterpart of AICAR (see Figure 95-1), also termed ZMP. Analysis of the patient's erythrocytes revealed the accumulation of ZMP and the di- and triphosphates ZDP and ZTP, respectively. Incubation of her fibroblasts with AICA-riboside led to the accumulation of AICAR (not observed

in control cells), indicating the impairment of the final steps of de novo purine biosynthesis. An assay of AICAR transformylase/IMP cyclohydrolase (ATIC), the bifunctional enzyme catalyzing the two last steps of the pathway, revealed a profound deficiency of transformylase and a partial deficiency of cyclohydrolase. Sequencing of the *ATIC* gene showed a K426R change in the transformylase region in 1 allele, and a frameshift in the other. A recombinant enzyme carrying the K426R mutation completely lacks AICAR transformylase activity. The discovery of this novel inborn error of purine synthesis reinforces the necessity to perform a Bratton–Marshall test in all cases of unexplained psychomotor delay and/or neurologic symptoms.

## 95.2.6 Myoadenylate Deaminase Deficiency

Adenylate deaminase (AMPD) catalyzes the deamination of AMP into IMP with release of free ammonia. It is one of three enzymes, along with adenylosuccinate synthetase and adenylosuccinate lyase, which forms the purine nucleotide cycle that is proposed to play a role in energy production during periods of high demand. Adenylate deaminase has four isoforms, encoded by three different genes. A muscle-specific form known as myoadenylate deaminase (mAMPD) is encoded by *AMPD1* on chromosome 1. Deficiency of mAMPD was first reported for five patients with exercise-induced cramping and myalgias (12). Later studies revealed mAMPD deficiency in 1–2% of the Caucasian population, most of whom had no symptoms. The large number of asymptomatic patients raised the possibility that the loss of mAMPD is a harmless genetic variant. However, there are three different situations outlined below where mAMPD deficiency may be clinically important. For a detailed review, see (110).

### 95.2.6.1 Primary Myoadenylate Deaminase Deficiency.

**95.2.6.1.1 Clinical Features.** Approximately 200 patients have been reported. Most present in the second to fourth decades of life, with poor exercise tolerance due to cramps or myalgias. Muscle wasting does not occur. Around half exhibit minor increases in serum creatine kinase. Electromyographic abnormalities and overt histological defects suggestive of myopathy usually are absent, although minor abnormalities are sometimes found. Rhabdomyolysis with myoglobinuria occurs only rarely.

**95.2.6.1.2 Pathogenesis.** Symptomatic primary mAMPD deficiency is thought to be inherited as an autosomal recessive disorder due to mutations in *AMPD1*. The majority of symptomatic patients harbor a common homozygous mutation c.34C>T leading to Q12X, which has null activity (111). However, a few other mutations also have been reported.

A causal role for homozygosity of the c.34C>T allele in symptom production has been called into question because this same allele is present in nearly a quarter of

the Caucasian population with 1–2% of the population being homozygous (111,112). Several mechanisms have been proposed to explain the lack of symptoms in this larger population (110). One is that the compensatory mechanisms, such as the upregulation of other AMPD isoforms, may mask symptoms. Another possible mechanism involves alternative splicing. The mutation resides within a miniexon that is alternatively spliced out, leaving a potentially functional variant enzyme. A possible final mechanism is that the mutant allele by itself is not pathogenic, but becomes pathogenic when combined with some other defect.

**95.2.6.1.3 Diagnosis.** Deficiency of mAMPD can be detected by the failure of ammonium production during the ischemic forearm exercise test, which involves inflating a sphygmomanometer cuff around the upper arm to measure arterial pressure. After squeezing the hand manometer for 2 min, the several-fold elevation of venous ammonia seen in normal subjects is absent in affected patients (113). Definitive diagnosis is established by histochemical or biochemical assay of the enzyme from a muscle biopsy specimen. Generally, mAMPD activity is <2% of normal.

### 95.2.6.2 Secondary Myoadenylate Deaminase Deficiency.

**95.2.6.2.1 Clinical Features.** The term “secondary mAMPD deficiency” has been used to describe the reductions in normal *AMPD1* mRNA transcripts and associated mAMPD enzyme activity that have been documented for several clinical disorders (110). This phenomenon has been reported for several neuromuscular disorders such as amyotrophic lateral sclerosis, fascioscapulohumeral myopathy, Kugelberg–Welander syndrome, polyneuropathy, Werdnig–Hoffmann disease, and myophosphorylase deficiency. It also has been reported for some connective tissue disorders. The symptoms of the primary disorder dominate the clinical picture, though synergy with mAMPD deficiency may make the primary disorder appear worse.

**95.2.6.2.2 Pathogenesis.** Most patients with secondary mAMPD deficiency are heterozygous for a mutation in *AMPD1* and transcription from the normal allele is significantly reduced. The mechanisms responsible for the lowered transcript levels are not known. Because *AMPD1* mRNA transcripts are reduced but not absent in secondary mAMPD deficiency, some residual AMPD enzyme activity sometimes can be detected.

**95.2.6.2.3 Diagnosis.** Patients with secondary mAMPD deficiency fail the forearm ischemic exercise test and usually have a negative histochemical reaction for AMPD in muscle biopsies. However, some staining can be observed if incubation times are extended beyond routine measures.

### 95.2.6.3 Coincidental Inherited Myoadenylate Deaminase Deficiency.

**95.2.6.3.1 Clinical Features.** The term “coincidental mAMPD deficiency” has been used to describe the clinical situation wherein homozygous or compound



heterozygous mutations in *AMPD1* are coincidentally found in other neuromuscular or rheumatological disorders (110). The clinical features are dominated by the associated neuromuscular or rheumatological disorder, although they may be worse than usual.

**95.2.6.3.2 Pathogenesis.** The pathogenesis of symptoms in coincidental mAMPD deficiency is heterogeneous, and the exact role for the enzyme deficiency is variable and sometimes uncertain. The term “double trouble” has been used to describe one subset of individuals with metabolic myopathy with complete mAMPD deficiency due to homozygosity for Q12X, along with a second defect in another gene known to cause myopathy (114). These patients may have a more profound phenotype than that observed in either disorder alone. Another subset of individuals has been described where heterozygosity for the Q12X mutation is found along with complete genetic deficiency of other metabolic pathways. Once again, symptoms may be more severe than those typically associated with the isolated metabolic disorder. Finally, the term “synergistic heterozygosity” has been applied to a third subset of individuals who carry heterozygous mutations for more than one step in the same metabolic pathway (115,116). In these patients, the combined partial defects may lead to phenotypes that resemble individuals with more complete deficiencies of the pathways involved.

## 95.2.7 Adenosine Deaminase Deficiency

ADA catalyzes the deamination of adenosine and of its deoxy counterpart deoxyadenosine. A gross deficiency of ADA was first reported by Giblett and coworkers (7) in two unrelated children with profound impairment of cellular (T-cell) and humoral (B-cell) immunity, known as severe combined immunodeficiency disease (SCID). Since that time, several hundred patients with ADA deficiency have been diagnosed. Most cases present soon after birth with a life-threatening susceptibility to infections that, if left untreated, invariably lead to an early death, unless drastic steps are taken such as rearing in strictly sterile conditions from birth onward. The development of bone marrow transplantation, enzyme replacement therapy, and gene therapy has drastically changed the prognosis of ADA deficiency.

**95.2.7.1 Clinical Features.** Symptoms of ADA deficiency vary between patients. The most severe patients present in infancy and usually die early if untreated. Ten to fifteen percent of patients have a “delayed” clinical onset by the age of 6–24 months, and a smaller percentage of patients have a “later” onset, diagnosed from the age of 4 years to adulthood. In addition, “partial” ADA deficiency occurs in apparently healthy individuals who show decreased enzyme activity in erythrocytes, but retain substantial enzyme activity ranging from 5 to 80% of normal in leukocytes and other nucleated cells (117). ADA deficiency is observed in ~15% of all SCID cases and one-third of cases of autosomal recessive SCID. Many

patients show a clinical and radiologic bony abnormality of the thorax, with flaring of the costochondral rib junctions, but this is not a consistent or specific finding. Neurologic abnormalities are found in a few affected children, including spasticity, head lag, movement disorders, and inability to focus. Autoimmunity may occur and hepatic dysfunction has also been reported.

**95.2.7.2 Diagnostic Tests.** ADA deficiency should be suspected in all patients who show recurrent infections during the first year of life. Affected patients display a profound impairment of T-cell function with varying degrees of B-cell dysfunction, which can be evidenced by relatively simple laboratory tests; lymphopenia (usually <500 total lymphocytes/mm<sup>3</sup>) involving B- and T cells, and hypogammaglobulinemia are almost invariably present. Immunoglobulin M deficiency may be detected early, but immunoglobulin G deficiency becomes manifest only after the age of 3 months when the maternal supply has been exhausted. More complex tests show a deficiency of antibody formation following specific immunization and an absence or severe diminution of lymphocyte proliferation induced by mitogens. The disease is progressive because residual B- and T-cell functions, which may be present at birth, disappear later on.

Enzymatic diagnosis of ADA deficiency is mostly performed on red blood cells. In general, disease severity correlates with the loss of ADA activity; in children with neonatal or infant-onset SCID, its residual activity is 0–0.1% of normal, and in subjects with delayed or later onset, 1–5% of control activity is found (118). Only ~15% of patients with the clinical and hematologic picture of inherited SCID are ADA deficient. In the remaining patients, SCID is caused by other mechanisms. In subjects with partial ADA deficiency, the enzyme is deficient in red blood cells, but sufficient residual ADA activity is present in their lymphocytes.

**95.2.7.3 Biochemical Features.** *Properties of Normal and Mutant Enzymes.* ADA is a monomeric enzyme with a molecular weight of ~40 kDa. In several epithelial cell types, it is associated with an ADA-binding cell membrane glycoprotein that seems to play a role in thymocyte proliferation. ADA is present in all cell types, but its activity is highest in the thymus and other lymphoid tissues. The crystal structure of human ADA has been resolved (PDB ID: 3iar).

*Pathophysiologic Mechanisms.* ADA deficiency results in the accumulation of adenosine and deoxyadenosine, the latter derived from DNA catabolism, which are normally nearly undetectable. Several pathogenic mechanisms have been proposed that might act in concert. Inside lymphocytes, excess deoxyadenosine leads to the accumulation of dATP, which is probably the primary lymphotoxic compound in ADA deficiency. Indeed, dATP potently inhibits ribonucleotide reductase, an essential enzyme for the synthesis of DNA and, hence, for lymphocyte development and differentiation. Moreover, dATP has been shown to induce mitochondrial

cytochrome C release, followed by apoptosis in fetal ADA-deficient thymocytes (119,120). On the other hand, deoxyadenosine has been shown to cause the irreversible suicide inactivation of *S*-adenosylhomocysteine hydrolase, an enzyme of the transmethylation pathway. As lymphocyte stimulation has been suggested to require methylation, this inactivation might contribute to the deficiency in lymphocyte function.

**95.2.7.4 Molecular Genetics.** The frequency of the deficiency is estimated at 1 per 100,000–500,000 births. Studies of the *ADA* gene, located on chromosome 20q13.11, have revealed a variety of mutations. Approximately two-third of them are single nucleotide changes, resulting in an inactive or unstable enzyme (121). Missense mutations are distributed throughout the gene, and most arise from codons that contain the CpG dinucleotide. Most patients are compound heterozygotes but others, mainly from consanguineous matings or inbred geographical communities, are homozygous. In recent years, knowledge of the structure of *ADA* and the systematic expression of mutant alleles has revealed that the genotype is an important determinant of the phenotype in *ADA* deficiency, and that the latter is strongly associated with the sum *ADA* activity provided by both alleles (117).

Spontaneous *in vivo* reversion to normal of an inherited mutation in one allele has been reported in a patient with *ADA* deficiency (122).

**95.2.7.5 Treatment.** Treatment of *ADA* deficiency became possible with the advent of bone marrow transplantation. This remains the first choice therapy, provided there is an HLA-identical sibling, HLA-haploidentical parent, or an HLA-matched unrelated donor, and gives a good chance for a complete cure, clinically and immunologically (123). The graft provides stem cells, and hence T- and B cells, which have sufficient *ADA* activity to prevent the accumulation of adenosine and deoxyadenosine. However, survival is much lower with HLA-mismatched transplants (124).

When no histocompatible bone marrow donor is found, enzyme replacement therapy can be used. Effective enzyme replacement therapy is achieved with polyethylene glycol-modified *ADA* (PEG-*ADA*). Covalent attachment of the inert polymer PEG to bovine *ADA* results in a markedly prolonged circulation life and diminished immunogenicity. Weekly to biweekly intramuscular injections of 15–30 U/kg usually results in marked clinical improvements, while *in vitro* immune function also significantly improves (125).

The first approved clinical trial of gene therapy was performed in 1990 in two *ADA*-deficient girls (126). Their peripheral T-cells were collected, cultured with interleukin-2, corrected by the insertion of the *ADA* gene by means of a retroviral vector, and reinfused. Although improvements in their cellular and humoral immune responses were observed (127), the patients continued to receive PEG-*ADA*. This very first attempt showed that the procedure was feasible and safe, and led to the persistence of transduced T-cells for as much as 15–20 years.

From the late 1990s, five different groups began *ex vivo* gene therapy with retroviral vectors using hematopoietic stem cells for SCID-X1 disease and *ADA* deficiency. The immunodeficiency was corrected in 19 of 27 (70%) *ADA*-deficient patients (128–130). Although 5 of 20 patients that received *ex vivo*-transduced autologous hematopoietic stem cells developed T-cell leukemic disease, this genotoxicity was not observed in *ADA*-deficient patients who had been similarly treated (128–130).

## 95.2.8 Adenosine Deaminase Superactivity

A hereditary, ~50-fold elevation of erythrocyte *ADA* has been shown to cause nonspherocytic hemolytic anemia (131). The latter can be explained by the enhanced catabolism of adenine nucleotides, including ATP, owing to the increased activity of *ADA*.

## 95.2.9 Purine Nucleoside Phosphorylase Deficiency

Their discovery of *ADA* deficiency prompted Giblett and colleagues (8) to search for other defects of purine and pyrimidine metabolism in patients with immune disorders. This resulted in the discovery of purine nucleoside phosphorylase (PNP) deficiency in a child with an isolated defect of T-cell function. The disorder is much less frequent than *ADA* deficiency, with 67 patients from 49 families reported to date.

**95.2.9.1 Clinical Features. Presentation.** Recurrent infections are usually of later onset, starting from the end of the first year, up to 5–6 years of age, and are initially less severe than in *ADA* deficiency (121). A strikingly enhanced, life-threatening susceptibility to viral diseases, such as varicella, measles, cytomegalovirus, and vaccinia has been reported, but severe candida and pyogenic infections also occur. One-third of the patients have anemia, while two-thirds display neurologic symptoms, including spastic tetraplegia or diplegia, ataxia, and tremor. Immunologic studies reveal an increasingly severe deficiency of cellular immunity, reflected by a marked reduction in the number of T-cells. B-lymphocyte function can be normal but is deficient in approximately one-third of the patients. Autoimmune disorders are also common, including hemolytic anemia, idiopathic thrombocytopenic purpura, and autoimmune neutropenia.

**Diagnostic Tests.** Patients often display a striking decrease in the production of uric acid; plasma uric acid is usually <1 mg/dL and may even be undetectable. However, in patients with residual PNP activity, uricemia may be at the borderline of normal. The urinary excretion of uric acid is usually also markedly diminished. In contrast, these patients excrete very large amounts of the substrates of PNP: inosine, guanosine, and their deoxy counterparts. Enzymatic diagnosis is mostly performed on red blood cells.

**95.2.9.2 Biochemical Features.** *Properties of normal and mutant enzymes.* Human PNP catalyzes the phosphorolysis of inosine and deoxyinosine, and guanosine and deoxyguanosine into hypoxanthine and guanine, respectively, and ribose-1-phosphate or deoxyribose-1-phosphate. It does not act on adenosine or deoxyadenosine. PNP is active as a 32-kDa monomer, but appears to be a trimer, as confirmed by crystallographic analysis of the human erythrocyte enzyme. Mutant enzymes can be completely inactive or display some residual activity.

*Pathophysiologic Mechanisms.* PNP deficiency provokes an accumulation in body fluids of the four normally nearly undetectable substrates of the enzyme, namely guanosine, inosine (see Figure 95-1), and their deoxy counterparts (not shown in Figure 95-1), the latter are derived from DNA breakdown. Formation of uric acid is thus severely hampered. The profound impairment of cellular immunity that characterizes the disorder has been explained by the greater ability of T-cells as compared with B cells to accumulate dGTP, although other factors also seem to intervene. Formed from deoxyguanosine, dGTP inhibits ribonucleotide reductase, and hence, cell division. As observed for dATP in ADA deficiency, dGTP also probably provokes apoptosis in PNP-deficient lymphocytes.

**95.2.9.3 Molecular Genetics.** PNP deficiency is inherited in an autosomal recessive fashion. Studies of the *PNP* gene, located on chromosome 14q13, have revealed a number of molecular defects, among which an R234P mutation is the most common (132). Genotype–phenotype correlations are hampered by the diversity of mutations and small number of patients.

**95.2.9.4 Treatment.** Most initially diagnosed patients have died from overwhelming viral or bacterial infections, although at a later age than untreated ADA-deficient children. The treatment consisted of bone marrow transplantation and repeated transfusions of normal, irradiated erythrocytes. More recently, successfully matched bone marrow transplantation has been reported (133–135), although without an improvement of the neurologic symptoms (136).

## 95.2.10 Xanthine Oxidase Deficiency

Xanthine oxidase is also termed xanthine dehydrogenase or xanthine oxidoreductase (XOR). XOR deficiency is characterized by xanthinuria, and was the first enzyme defect reported in human purine metabolism (9). Originally described as a benign disorder, it was subsequently found that it could also occur in combination with defects of two other molybdenum-requiring enzymes: aldehyde oxidase and sulfite oxidase (10). Three types of XOR deficiency are now recognized: (a) type I, isolated XOR deficiency; (b) type II, combined XOR and aldehyde oxidase deficiency, which causes equally benign xanthinuria; and (c) combined deficiency of XOR, aldehyde, and sulfite oxidase, which, in contrast to the other two types, provokes a devastating disorder similar to isolated sulfite

oxidase deficiency. It is caused by the failure to synthesize the molybdenum cofactor common to the three oxidases.

**95.2.10.1 Clinical Features.** *Presentation.* Type I and type II XOR deficiency can be completely asymptomatic, although kidney stones are formed in approximately one-third of the cases. Although these are most often not visible on a radiograph, they may appear at any age and cause a variety of symptoms including hematuria, renal colic, and even acute renal failure. Myopathy may be present, precipitated by strenuous exercise and associated with crystalline xanthine deposits in muscle. In combined XOR and sulfite oxidase deficiency, the clinical picture of sulfite oxidase deficiency, which is also found as an isolated defect (137), overrides that of XOR deficiency. The symptoms include neonatal feeding difficulties and intractable seizures, myoclonus, increased or decreased muscle tone, eye lens dislocation, and severe intellectual disability.

*Diagnostic Tests.* In isolated and combined XOR deficiency, <1 mg/dL plasma concentrations of uric acid are detected; they may decrease to virtually undetectable values on a low-purine diet. Urinary uric acid is reduced to a few percent of normal and is replaced by hypoxanthine and xanthine. In combined XOR and aldehyde oxidase deficiency (xanthinuria type II), patients are unable to oxidize allopurinol. In combined XOR, aldehyde, and sulfite oxidase deficiency, the urinary changes of xanthinuria are accompanied by the excessive excretion of sulfite and other sulfur-containing metabolites, e.g., S-sulfocysteine, thiosulfate, and taurine. Enzymatic diagnosis requires liver or small intestine mucosa that normally contains substantial amounts of xanthine oxidase. Sulfite oxidase and the molybdenum cofactor can be assayed in the liver and fibroblasts.

**95.2.10.2 Biochemical Features.** XOR is a homodimer composed of two subunits of ~150 kDa. Each subunit contains a molybdenum center, which contains molybdenum under the form of a molybdenum cofactor, similar in structure to that of several other molybdoenzymes. Mutant XOR has been investigated in a few patients. Despite unmeasurably low activity, immunoreactive protein remains detectable in the duodenal mucosa of some patients, but not of others (138).

*Pathophysiologic Mechanisms.* XOR deficiency results in the near-total replacement of uric acid by hypoxanthine and xanthine as the end products of purine catabolism (see Figure 95-1). This alteration is clearly detectable in urine, in which >90% of oxypurine excretion is normally uric acid. Plasma hypoxanthine levels are, as a rule, not or minimally elevated in various forms of XOR deficiency, owing to its efficient reutilization by HGPRT. Plasma xanthine, normally <1  $\mu$ M, may rise to 10–40  $\mu$ M, which is far below the normal uric acid level because of the markedly higher renal clearance of xanthine.

Concentrations of hypoxanthine and xanthine are ~10-fold higher than normal in the muscle of xanthinuric subjects. The limited solubility of xanthine may explain the observation of numerous birefringent crystals in the

muscles of patients complaining of pain and stiffness. The role of hypoxanthine, which increases during vigorous exercise in normal and xanthinuric subjects, is unclear because it is much more soluble than xanthine.

**95.2.10.3 Molecular Genetics.** The inheritance of type I and type II XOR deficiency and of combined XOR and sulfite oxidase deficiency is autosomal recessive. Studies of the XOR gene, localized on chromosome 2p22, have led to the identification of two mutations in hereditary xanthinuria type I: a nonsense substitution and a termination codon (138). Xanthinuria type II is caused by mutations of the molybdenum cofactor sulfurase gene (139,140). In patients with molybdenum cofactor deficiency, mutations have been found in three genes: *MOCS1* (type A deficiency), *MOCS2* (type B deficiency), and *GPHN* (only 1 case has been reported) (141–143). The products of these three genes are considered necessary for the synthesis of the molybdenum cofactor.

**95.2.10.4 Treatment.** Isolated XOR deficiency is mostly benign, and a low-purine diet should be prescribed and fluid intake increased. However, the prognosis of combined XOR and sulfite oxidase deficiency is very poor. So far, all therapeutic attempts, including low-sulfur diets, the administration of sulfate and molybdenum, and trials to bind sulfite with thiol-containing drugs, have been unsuccessful. Veldman et al. reported that cyclic pyranopterin monophosphate supplementation was effective for molybdenum cofactor deficiency type A (144).

### 95.2.11 Hypoxanthine-Guanine Phosphoribosyltransferase Deficiency

Lesch and Nyhan first reported a classical phenotype that now is known as Lesch–Nyhan disease (LND) in 1964 (29). They described two brothers with marked overproduction of uric acid, a motor disorder resembling cerebral palsy, intellectual disability, and a peculiar behavioral syndrome with severe self-injurious behavior. Three years later, Seegmiller and colleagues found deficiency of the purine salvage enzyme, HPRT as the cause (145). Following discovery of the enzymatic basis, there was increasing recognition of milder phenotypes where some of the classical features of LND were absent or unusually mild (145–147). These patients now are known as Lesch–Nyhan variants (LNV). LND and its variants are inherited in an X-linked recessive manner, with nearly all cases being males. The *HPRT1* gene encoding the enzyme was among the first genes to be cloned for any human disease (148), and it is one of the most intensively studied loci in human genetics (149).

**95.2.11.1 Clinical Features.** *Presentation.* The presentation and clinical features of classical LND are quite stereotyped (150–155). Few abnormalities are identifiable during the early neonatal period, but signs of motor

delay become evident within 2–6 months of age. Between 9 months and 4 years of age, involuntary movements become apparent, with increased muscle tone, spasms, and flailing movements. Many LND patients are initially incorrectly suspected of having cerebral palsy, because the presentation and evolution of early motor abnormalities are quite similar. Although motor delay is the presenting feature for the majority of patients, a few may present instead with other problems as infants. Included are complications of uric acid overproduction such as kidney stones or renal failure.

The presentation and clinical features of LNV are more variable (145,154,156). The most severely affected present in a manner similar to LND. Less severely affected LNV patients may present with poor motor skills although a higher proportion present instead with complications from overproduction of uric acid, such as kidney stones or gout during childhood or early adulthood.

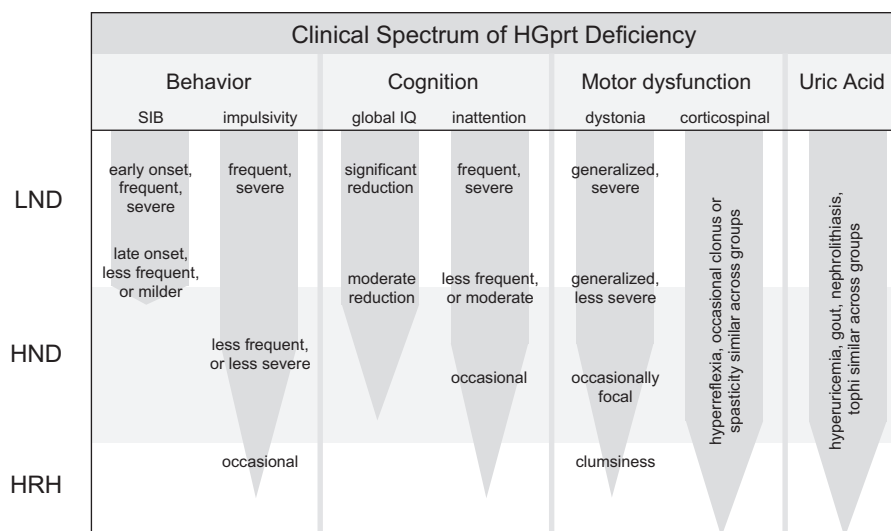
*Uric Acid Overproduction.* Overproduction of uric acid is universal in LND and its variants (Figure 95-2). Uric acid is produced at three to five times of normal rates (157). Elevated serum uric acid levels are nearly universal, although the degree of hyperuricemia may be sufficiently small that it escapes notice (158). In rare cases, the serum uric acid may be normal. The reason serum uric acid may be normal or only slightly elevated despite enormous overproduction involves the remarkable ability of the kidneys to clear it from the blood into the urine.

The many renal complications in LND and its variants are related to the massive amounts of uric acid being filtered by the kidneys into the urine (159–162). A common problem is nephrolithiasis with either large kidney stones or sandy sludge-like material that can develop in any portion of the urogenital system. These may result in flank pain, hematuria, obstructive nephropathy, interstitial nephritis, recurrent urinary infections, and renal failure.

Gout also is a complication of uric acid overproduction. Joint pain due to the inflammatory effects of uric acid precipitation in the joints is most common in the feet and hands, but other joints may be involved. If untreated, tophaceous deposits may accumulate, leading to progressive destruction of the joints.

*Behavioral Abnormalities.* Patients with classic LND have a characteristic behavioral phenotype (163–165). Virtually all patients exhibit severe and recurrent attempts to hurt themselves. This behavior most often emerges between 2 and 4 years of age, although it may be delayed until early adulthood. The most common manifestation involves self-biting. Patients commonly bite their fingers and hands, or they may bite their lips, tongue, and cheeks. Other frequent manifestations include self-hitting, banging the arms or legs on nearby hard objects, head banging, eye gouging, or picking and scratching. Over time, it is not uncommon for individual patients to exhibit multiple different forms of self-injury. Self-injury is not due to loss of sensation or to a need for self-stimulation. Patients





**FIGURE 95-2** Schematic representation of the continuous spectrum of clinical features across LND and its variants. The main clinical problems are listed across the top. Patients are subdivided into three typical groups along the left side. The most severely affected group is LND. The least severely affected group is designated HPRT-related hyperuricemia (HRH). An intermediate phenotype is designated HPRT-related neurological dysfunction (HND). The frequency and severity of each of the clinical problems is depicted by the thickness and taper of the gray bar.

report that they do not wish to hurt themselves, but they are unable to stop.

In addition to self-injury, other difficult behaviors are frequent in LND (164,165). These include attempts at hitting others, spitting toward others, or the use of foul or sexually inappropriate language. These behaviors do not appear to be accompanied by aggressive intent, since patients frequently apologize after the behavior and seem embarrassed by it. Instead, the behaviors may be impulsive or compulsive. Oppositional defiant behavior also is frequent. Although the behavioral phenotype might be expected to lead to poor social relationships, patients with LND are often well liked by their families and associated caregivers because they have very engaging personalities.

Patients with LNV do not exhibit self-injurious behavior or overt problem behaviors seen in classical LND. However, impulsivity and oppositional behavior are not uncommon (Figure 95-2) (156,164).

**Motor Disorder.** The motor disorder of classical LND reflects a combination of problems similar to those seen in dyskinetic cerebral palsy (Figure 95-3) (150,151). All patients have severe generalized dystonia with limited practical use of their limbs. Opisthotonic truncal spasms are common. Dysarthria is severe, although patients usually can make themselves understood to those who know them well. Approximately one-third have superimposed choreoathetosis or ballismus. Approximately one-quarter have corticospinal signs such as spasticity, increased muscle stretch reflexes, or clonus. The motor syndrome is established within the first few years of life. It is not relentlessly progressive.

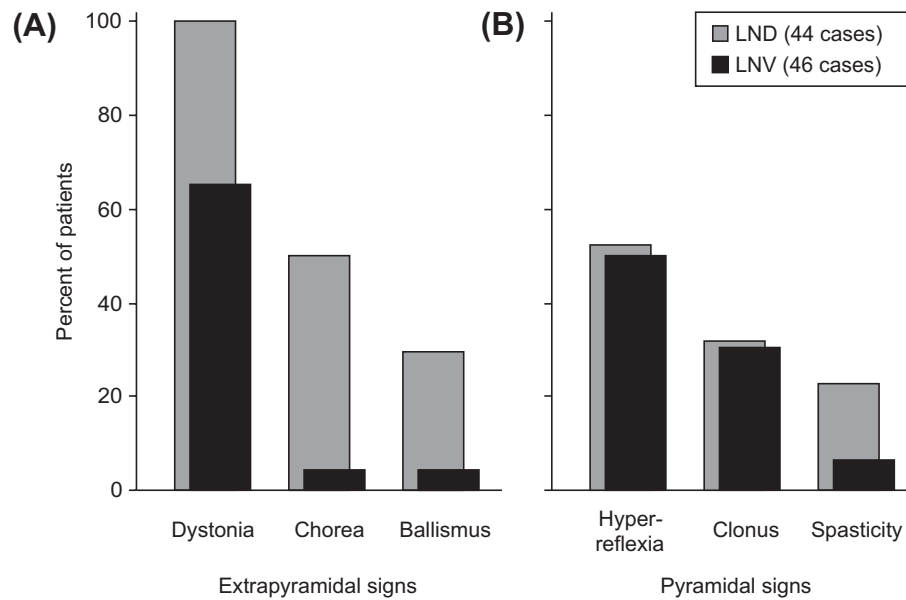
The motor disorder of LNV is more variable (156). The most severely affected LNV patients lack the hallmark behavioral features of LND, but they have a motor

syndrome indistinguishable from classical LND. Moderately affected patients have less disabling dystonia, such that ambulation and self-care may be possible. Less severely affected patients have clumsy motor skills. The least severely affected have normal motor skills.

**Cognitive Abnormalities.** Intellectual disability is nearly universal in classic LND (166–168). Patients sometimes are incorrectly thought to have more severe cognitive impairments because of their difficulties with verbal and other communication, their difficult behaviors, and the limited educational opportunities available to them. However, formal neuropsychological testing typically reveals only mild or moderate intellectual disability, with global IQ scores in the 60–80 range. Intellectual disability also is very common in LNV, although it is less severe than in LND.

**Other Abnormalities.** A variety of other clinical features may also occur in LND and LNV. Feeding difficulties are severe enough to require chronic gastrostomy in approximately a third of those with LND and a few with LNV (151). Many are small for age, and puberty often is delayed. Macrocytic anemia occurs in approximately half of the patients with LND or LNV (169,170). Laryngospasm may cause stridor or recurrent apneas, sometimes severe enough to cause loss of consciousness or even death (171).

**95.2.11.2 Diagnosis. Uric Acid.** The overproduction of uric acid leads to a convenient screening test for LND and its variants, because serum uric acid very frequently is elevated (158). Although hyperuricemia is common, some patients have normal serum uric acid due to high renal clearance. The 24-hour urinary uric acid provides a more sensitive measure, especially when corrected for patient mass by examining the uric acid to creatinine ratio. However, such tests rarely are used for clinical



**FIGURE 95-3** Summary of the motor disorder of LND (gray bars,  $n = 44$ ) and LNV (black bars,  $n = 46$ ) taken from two large studies (151,156). Extrapyramidal (A) and pyramidal (B) features are shown separately.

diagnosis because complete specimens are notoriously difficult to collect.

In addition to the limitations of sensitivity, elevated serum or urinary uric acid is not specific for LND or its variants. Several other disorders may also be associated with hyperuricemia or uricosuria, sometimes reaching or exceeding levels associated with LND or LNV. Thus, evidence for the overproduction of uric acid is helpful as a screening test, but it is not adequate for definitive diagnosis.

**Genetic Testing.** Today, the most widely used method for definitive diagnosis involves documenting a pathological mutation in the *HPRT1* gene (172,173). Mutations are heterogeneous, with more than 400 reported mutations, so sequencing is required. Because mutations are so heterogeneous and often unique, genetic testing often has limited prognostic value for distinguishing among severe or mild phenotypes.

**Enzyme Testing.** Another convenient method for diagnosis involves biochemical measurement of HPRT enzyme activity. Erythrocyte lysates provide a convenient source material for testing, although false negatives and false positives may occur when assays are conducted with artificially high substrate levels (173,174). A more accurate result can be achieved by testing live erythrocytes or fibroblasts with conditions that more faithfully replicate the natural conditions of the enzyme in situ (175–177). There is a rough correlation between the severity of the clinical phenotype and residual enzyme activity, so enzyme testing has some prognostic value (176,177). In classical LND, the live cell assays typically reveal nondetectable enzyme levels. In LNV, some residual enzyme activity usually can be detected. LNV patients with 2–8% activity typically suffer from overproduction of uric

acid with at least some neuromotor dysfunction and/or intellectual disability. LNV patients with more than 8% activity still suffer from overproduction of uric acid, but motor and intellectual functions may appear grossly normal.

**Carrier and Prenatal Diagnosis.** Genetic and enzyme tests both can be used for the detection of asymptomatic female carriers and prenatal diagnosis, although genetic testing is more convenient (178–181). The usual approach is to define the mutation in the affected proband, and then search for the same mutation in others at risk.

**95.2.11.3 Pathogenesis. Molecular Genetic Pathogenesis.** The normal human *HPRT1* gene has nine exons spanning ~45 kb on the long arm of the X-chromosome at Xq26-q27 (149). Because it is on the X-chromosome, males have one copy of the gene but females have two. However, the *HPRT1* gene on the inactivated X-chromosome is transcriptionally silenced by methylation of its CpG clusters, so females also have only one functional copy of the gene.

The mutations responsible for LND and LNV are heterogeneous. As of this writing, more than 400 mutations have been reported (Table 95-1), and new ones are reported yearly. The reported mutations are listed individually at [www.lesch-nyhan.org](http://www.lesch-nyhan.org) (172,173). Approximately, two-thirds of the mutations are single-base substitutions leading to the substitution of a single amino acid, one-quarter are deletions, and the remainder are splice defects, duplications, substitutions, and more complex rearrangements.

Although there is no obvious correlation between individual mutations and specific clinical features, there is a good correlation for the predicted effect of the mutation on HPRT enzyme activity and overall clinical severity

(172,173). The severe LND phenotype typically results from mutations predicted to cause null HPRT enzyme activity such as nonsense mutations, deletions, and other frame-shifting mutations. The milder variants almost invariably occur with single-base substitutions leading to single amino acid substitutions that may permit some degree of residual enzyme activity.

The functional effect of single amino acid substitutions is more difficult to predict. Although early studies suggested that nonconservative amino acid substitutions at the catalytically active site of the enzyme would have the most profound effect on enzyme activity, subsequent studies have demonstrated that mutations outside the active site may have an equally deleterious effect by interfering with protein dimerization, conformational changes required for enzyme action, or protein stability (174,182).

LND and LNV are inherited in an X-linked recessive manner, so virtually all cases are males. However, the classical LND phenotype has been reported for several females (183). All female cases reported to date have had a mutation of one *HPRT1* allele combined with non-random X-inactivation eliminating transcription from the normal *HPRT1* allele, leading to severe enzyme deficiency (172,183).

**Biochemical Pathogenesis.** The *HPRT1* gene encodes a protein of 219 amino acids with a mass of 25 kDa (158). The gene is expressed in all cells of the body at all developmental stages. The HPRT enzyme functions as a dimer or tetramer, and the three-dimensional structure has been resolved by X-ray crystallography from a number of different sources (184–187). HPRT requires magnesium and is one of a small family of enzymes that uses the high-energy bonds of PRPP rather than ATP during catalysis. HPRT is a bifunctional enzyme that converts hypoxanthine to IMP and guanine to GMP.

Mutations resulting in null enzyme activity may cause the deletion of amino acid sequences, early protein truncation, or frame-shifting mutations that yield incorrect amino acid sequences. The effect of point mutations is varied. Biochemical kinetic studies and molecular modeling have suggested that reductions in HPRT activity can be caused by alterations in its kinetic properties toward hypoxanthine, guanine, or PRPP. Other point mutations may disrupt the dimer interface where interactions are required for catalytic activity or protein stability (174,182).

**Pathogenesis of Uric Acid Overproduction.** The pathogenesis of the overproduction of uric acid and its consequences are well understood. First, the absence of HPRT-mediated recycling of hypoxanthine and guanine means that these two bases cannot be reincorporated into the purine pools (188). Both ultimately are catabolized and excreted as uric acid. Second, the synthesis of purines via the de novo pathway is markedly accelerated in the absence of HPRT (157). The mechanism involves accumulation of the PRPP, which serves as a

cosubstrate for both HPRT and the rate-limiting step in the de novo pathway, PRPP-glutamine amidotransferase. When HPRT is missing, the accumulating PRPP drives the de novo pathway at an unusually high rate. Thus, the excessive production of uric acid reflects excessive endogenous purine synthesis together with a failure to recycle hypoxanthine and guanine.

The pathogenesis of the complications of overproduction of uric acid is directly related to its relatively low solubility. Uric acid is near its physiological limit of solubility in the body, and chronic elevations increase its risk of precipitation. One common site of precipitation is the synovial fluids of the joints, which cause an inflammatory reaction and the pain known as gout. A persistent elevation of uric acid leads to macroscopic accumulations known as tophi, which may destroy joints. Tophi may also form subcutaneously outside the joints. Other common sites of precipitation are the kidneys and the urogenital systems, due to constant filtration of serum uric acid into the urinary collecting system. Precipitation of small crystals leads to sludge and sandy material in the urine, or larger kidney stones that may occupy the entire renal medullary collecting system as staghorn calculi.

**Pathogenesis of Neurobehavioral Dysfunction.** The pathogenesis of the neurological and behavioral abnormalities associated with HPRT deficiency is incompletely understood. Many hypotheses have been presented and tested. The earliest focused on the accumulation of some metabolite with putative toxic functions in the nervous system such as uric acid, oxypurines, or PRPP (158). However, there is no experimental evidence for toxicity of any such metabolite. Additionally, there is no evidence for any neurodegenerative process that might be expected from neurotoxicity. Other hypotheses focused on a putative deficiency of the products of HPRT such as IMP or GMP, or more likely their phosphorylated derivatives, such as ATP or GTP (158). Once again, there has been no experimental evidence for deficiency of any such purine metabolites in the brain.

Although the purine metabolic pathways responsible for the neurobehavioral features are not well understood, there is a much better understanding of the brain pathways that are involved. Dysfunction of the basal ganglia, and particularly its dopaminergic connections, has been postulated to be chiefly responsible for the major clinical features including self-injurious behavior, the motor disorder, and intellectual disability (189). Histopathological studies of autopsied brains are largely unrevealing (151,190), although biochemical studies have revealed enormous loss of dopamine in all subregions of the basal ganglia (191–193). Routine clinical neuroimaging studies such as CT and MRI show only minor brain changes (151,194), but PET studies have revealed significant loss of dopamine uptake transporters and the uptake of the dopamine precursor fluoroDOPA (195,196). Detailed studies of a

mouse carrying a deletion mutation of the *HPRT1* gene have similarly revealed significant loss of dopamine and associated markers in the basal ganglia (197,198). *In vitro* tissue culture models based on dopamine neuron-like cells have shown a similar reduction of dopamine and associated markers when HPRT is lost (199–201). All together, these studies have provided strong converging evidence that the loss of HPRT leads to serious dysfunction of dopaminergic neurons. The mechanism by which HPRT deficiency leads to problems in dopamine neurons remains uncertain, but appears to be a metabolic phenomenon rather than a neurodegenerative one (198,202). While the abnormalities of basal ganglia dopamine pathways may be major contributors to the neurobehavioral syndrome of LND and its variants, they do not exclude the possible contributions of other brain regions, such as the corticospinal pathways.

**95.2.11.4 Treatment. Control of Uric Acid.** It is essential to control the excessive production of uric acid to avoid the complications of kidney stones and gout. Effective control requires two ingredients. The first is the use of an inhibitor of xanthine oxidase to suppress the production of uric acid. Allopurinol is used in most cases, although febuxostat also may be used. The second ingredient for managing the overproduction of uric acid is generous hydration at all times, and especially during periods of excess water loss such as particularly hot days, febrile illnesses, or protracted vomiting or diarrhea. The purpose of hydration is to ensure adequate flushing of uric acid and its precursors from the kidneys and through the urogenital system.

Despite vigilant treatment, some patients may still develop kidney stones. In some cases, these may be due to medication noncompliance or inadequate doses of allopurinol leading to uric acid stones. In other cases, recurrent stones may be due to inadequate hydration or overaggressive lowering of uric acid levels, which increases the risk of stones composed of the uric acid precursors, xanthine and hypoxanthine (203). Chemical analysis of the composition of the stones can be helpful in determining the reasons for recurrent stone development and for creating a strategy for stopping it.

**Control of Behavioral Problems.** There are no simple and effective treatments for the self-injurious behaviors. Self-injury is managed by a combination of medications, behavioral interventions, and physical protective devices. There are no oral medications with guaranteed efficacy against self-injurious behaviors, but some may provide at least partial benefits. These include benzodiazepines, gabapentin, and in some cases neuroleptics (163,204,205). Many other medications have been tested with no obvious benefit including serotonin reuptake inhibitors, opiate antagonists, carbamazepine, and levodopa (158). Behavioral interventions emphasize on positive reinforcement of good behaviors and extinguish bad ones through a systematic process of ignoring them, within the limits of safety (206). Negative reinforcement through punishment

sometimes has a paradoxical negative effect of worsening the target behaviors, so this approach is avoided (207). The most effective means for controlling self-injury involves the use of physical protective devices. Self-hitting or biting the fingers and hands can be prevented by straps that limit arm movement, elbow splints that prevent flexing the arm toward the face, or mittens over the hands. The feet and legs may be similarly protected.

The control of self-biting is more difficult when it involves the lips, tongue, or cheeks. Here, the only reliable solution is dental extraction. Many families and providers are reluctant to remove teeth due to permanent disfigurement. However, the consequences of permanent disfigurement from self-biting can be worse, and most families are very relieved when the possibility of self-biting is eliminated by dental extraction. Ultimately, more than half of patients with classic LND have most or all of their teeth removed. When biting begins, lengthy titration periods of oral medications, and lengthy attempts at behavioral intervention are discouraged, because self-biting may be rapid and aggressive, leading to permanent disfigurement in a few minutes or hours.

**Minimizing Motor Disability.** There are no treatments that are fully effective against the motor disability. Medications commonly used to reduce muscle tone include benzodiazepines and baclofen. Assistive devices such as a wheelchair are essential for reducing the impact of motor disability. Wheelchairs often must be customized, so that all sharp or hard objects within reach are covered by soft padding to avoid self-injury.

**Experimental Therapies.** Bone marrow transplantation has been performed for several patients with LND. None of them were benefitted, and the majority died prematurely as a result of complications of the procedure or subsequent immunosuppressive therapy (208,209). The lack of benefit is not surprising, because there is no obvious mechanism whereby the restoration of HPRT enzyme activity in bone marrow can correct the enzyme defect in brain neurons during the developmental period when it is most needed (210). Because of the lack of a rational mechanism together with the unusually high mortality, bone marrow transplantation should not be offered to patients with LND.

Deep brain stimulation also has been performed for several patients with LND (211–214). This technique involves installing an electrode deep into the brain in the region of the globus pallidus, and providing chronic electrical stimulation via an implantable impulse generator. It has proven useful for the treatment of several movement disorders, including Parkinson's disease, tremor, and dystonia. The technique has been reported to reduce the severity of dystonia in LND and suppress self-injury too. However, the results seem to vary from one patient to the next, with dramatic benefits in some and little or no benefits in others. This method seems promising, but further studies are needed to determine the optimal location for stimulation and the optimal stimulation parameters.



### 95.2.12 Adenine Phosphoribosyltransferase Deficiency

Adenine phosphoribosyltransferase (APRT) catalyzes the salvage of adenine into AMP, using PRPP as cosubstrate (see Figure 95-1). Its deficiency was first identified in heterozygotes with partial enzyme deficiency (4). Later, Cartier and Hamet (5) described a 4-year-old child with urinary crystals and stones. These were produced by the conversion of adenine, in the absence of other metabolic routes, into 2,8-dihydroxyadenine (2,8-DHA) by xanthine oxidase. Symptoms are caused by the very poor solubility of 2,8-DHA. More than 300 patients have been diagnosed worldwide, but up to 50% of APRT-deficient subjects may be asymptomatic.

**95.2.12.1 Clinical Features.** *Presentation.* The deficiency may become clinically manifest in childhood, even from birth, but it may also remain silent for several decades. Symptoms include the urinary passage of gravel, small stones, and crystals, frequently accompanied by abdominal colic, dysuria, hematuria, and urinary tract infections. Some patients may present with acute anuric renal failure, whereas others have developed chronic renal failure requiring dialysis and transplantation. The urinary precipitates are composed of 2,8-DHA and are radiolucent.

*Diagnostic Tests.* The observation of brownish spots on diapers or the finding of round, brownish crystals in urine under a light microscope suggest the presence of 2,8-DHA. Notwithstanding its very low solubility, 2,8-DHA can also be identified by HPLC. Confirmation of its presence requires complex analyses, including ultraviolet and infrared spectrography, mass spectrometry, X-ray crystallography, and capillary electrophoresis; therefore, it is usually easier to measure APRT activity. On the basis of the level of residual enzyme activity in red blood cell lysates, two types of APRT deficiency are recognized (see later). In both types, APRT activity is absent in intact cells.

**95.2.12.2 Biochemical Features.** APRT deficiency results in the suppression of adenine salvage (see Figure 95-1), provided by food and the polyamine pathway (not shown in Figure 95-1). Consequently, adenine is oxidized by xanthine oxidase into 2,8-DHA. This compound has solubility in urine, at pH 5 and 37°C, of ~0.3 mg/dL, which is far below that of uric acid.

Human APRT is a dimer with a molecular weight of ~38 kDa. In addition to adenine, many pharmacologic adenine analogs are substrates for APRT, which therefore plays an important role in their conversion into active compounds.

Patients with type I APRT deficiency have no detectable activity in erythrocyte lysates. In patients with type II deficiency, significant residual activity is found, reaching 5–25% of normal when measurements are made at supraphysiologic, saturating concentrations of PRPP. However, kinetic studies reveal that the  $S_{0.5}$  value for PRPP, which is ~3  $\mu$ M for the normal enzyme, is increased to 50–80  $\mu$ M.

This decreased affinity for PRPP results in its near inactivity under physiologic conditions; consequently, APRT activity is not detectable in intact cells such as erythrocytes or fibroblasts. Confirmatory diagnosis of type II deficiency is performed on viable T-cells (215). To date, type II patients have only been found in Japan, where they account for ~80% of the affected subjects.

**95.2.12.3 Molecular Genetics.** The human APRT gene has been mapped to chromosome 16q24.3. In Caucasian and Japanese subjects, a number of polymorphisms are observed. In type I deficiency, >30 mutations have been identified, where ~50% of these are single-base changes. The observation of a number of more common mutations suggests founder effects. All of the type II Japanese patients carry the same T2069C substitution in exon 5, resulting in an M136T change. Approximately 80% of patients are homozygous. Another nonsense mutation, W98X, and a CCGA insertion resulting in a frameshift, account for nearly all of the other cases (216).

Recently, the 3-dimensional structure of human APRT has been determined at 2.1 Å resolution (217). This has provided insights into the mechanisms by which a number of mutations hamper the enzyme's normal activity.

**95.2.12.4 Treatment.** In patients with symptoms, allopurinol should be given, as detailed under "Treatment of Gout," to inhibit the formation of 2,8-DHA. In patients with stones and in those without symptoms, dietary purine restriction and high fluid intake are recommended. Alkalinization of the urine is, however, not advised; unlike that of uric acid, the solubility of 2,8-DHA does not increase up to pH 9. Shock-wave lithotripsy has been beneficial in a small number of patients.

The ultimate prognosis depends on the patient's renal function at the time of diagnosis; late recognition may result in irreversible renal insufficiency that requires chronic dialysis, and early treatment may result in the prevention of stones. Kidney transplantation is sometimes necessary because of severe renal damage (218,219); however, the recurrence of microcrystalline deposits and the subsequent loss of graft function should be prevented with allopurinol. Although a theoretical alternative to allopurinol is febuxostat (72), there have been no reports of the treatment of an APRT-deficient patient with that drug.

### 95.2.13 Thiopurine Methyltransferase Deficiency

Thiopurine S-methyltransferase (TPMT) catalyzes the S-methylation of a number of synthetic purine analogs that contain a thiol group, e.g., 6-mercaptopurine, 6-thioguanine, and azathioprine, which is converted to 6-mercaptopurine in vivo. These drugs are currently used to treat a variety of diseases, including cancer, rheumatoid arthritis, and other autoimmune disorders, and also as immunosuppressants after organ transplantation. The thiopurines are converted through

phosphoribosylation by HGPRT and APRT into active thionucleotides, which exert their therapeutic action by being incorporated into DNA and RNA. Their oxidation by xanthine oxidase, and S-methylation by TPMT, results in their inactivation.

The wide variation in the therapeutic response and occurrence of toxic side effects in individual patients receiving thiopurines led to the identification of TPMT as a determining factor in this variability (14,31). The frequency of variant *TPMT* alleles differs between different ethnic groups; thus, Collie-Duguid et al. found that the frequency of individuals with a *TPMT* variant was 10% (20 of 199) in Caucasians, 2% (2 of 99) in South-west Asians, and 4.7% (9 of 192) in Chinese (220). The composition of the variant alleles also differs between different ethnic groups; thus, *TPMT*\*3A (A154T and Y240C) is the most frequent variant allele among Caucasians, while *TPMT*\*3C (R215H) is the most frequent among Japanese and Chinese patients (220).

The determination of TPMT status before treatment with thiopurines is therefore now recommended and considered one of the best examples of predictive pharmacogenetics (221–223); however, it is not clear whether the enzyme assay or genotyping is preferred (224,225).

#### 95.2.14 Deoxyguanosine Kinase Deficiency

Homozygosity mapping was used to identify the causative gene in three large consanguineous kindreds of Druze origin with the hepatocerebral form of mitochondrial DNA depletion syndrome, characterized by early progressive liver failure, neurologic abnormalities, hypoglycemia, and increased lactate (15). This analysis led to the identification of a deficiency in mitochondrial deoxyguanosine kinase. This enzyme phosphorylates the deoxy counterpart of guanosine (see Figure 95-1) into deoxyGMP. Its deficiency results in an imbalance in the supply of mitochondrial DNA precursors, particularly in the liver and brain that lack a cytosolic form of this enzyme. A single nucleotide deletion (204delA) in the mitochondrial deoxyguanosine kinase gene segregated with the disease in 19 patients from the 3 kindreds (15). Since then, various mutations have been reported. This enzyme deficiency may be frequent since deoxyguanosine kinase gene mutations were found by postmortem search in 4 (14%) of 28 infants with severe progressive liver failure of unknown cause: two homozygotes, one compound heterozygote, and one heterozygote (226).

### 95.3 PYRIMIDINE METABOLISM

#### 95.3.1 Metabolic Pathways

As in the purine nucleotides, the metabolism of the pyrimidine nucleotides can be divided into three pathways (see Figure 95-4):

The biosynthetic pathway, also often termed *de novo* pathway, starts with the formation of the high-energy

compound, carbamoyl phosphate, by cytosolic carbamoyl phosphate synthetase (CPS II). This enzyme is different from mitochondrial CPS I, which catalyzes the first step of ureogenesis. The formation of carbamoyl phosphate is followed by the synthesis of UMP, CMP, their deoxy counterparts, and TMP.

The pyrimidine catabolic pathway starts from (deoxy)CMP, (deoxy)UMP, and TMP. (Deoxy)uridine and thymidine are degraded to finally  $\beta$ -alanine and  $\beta$ -aminoisobutyrate, respectively, which are converted into intermediates of the citric acid cycle.

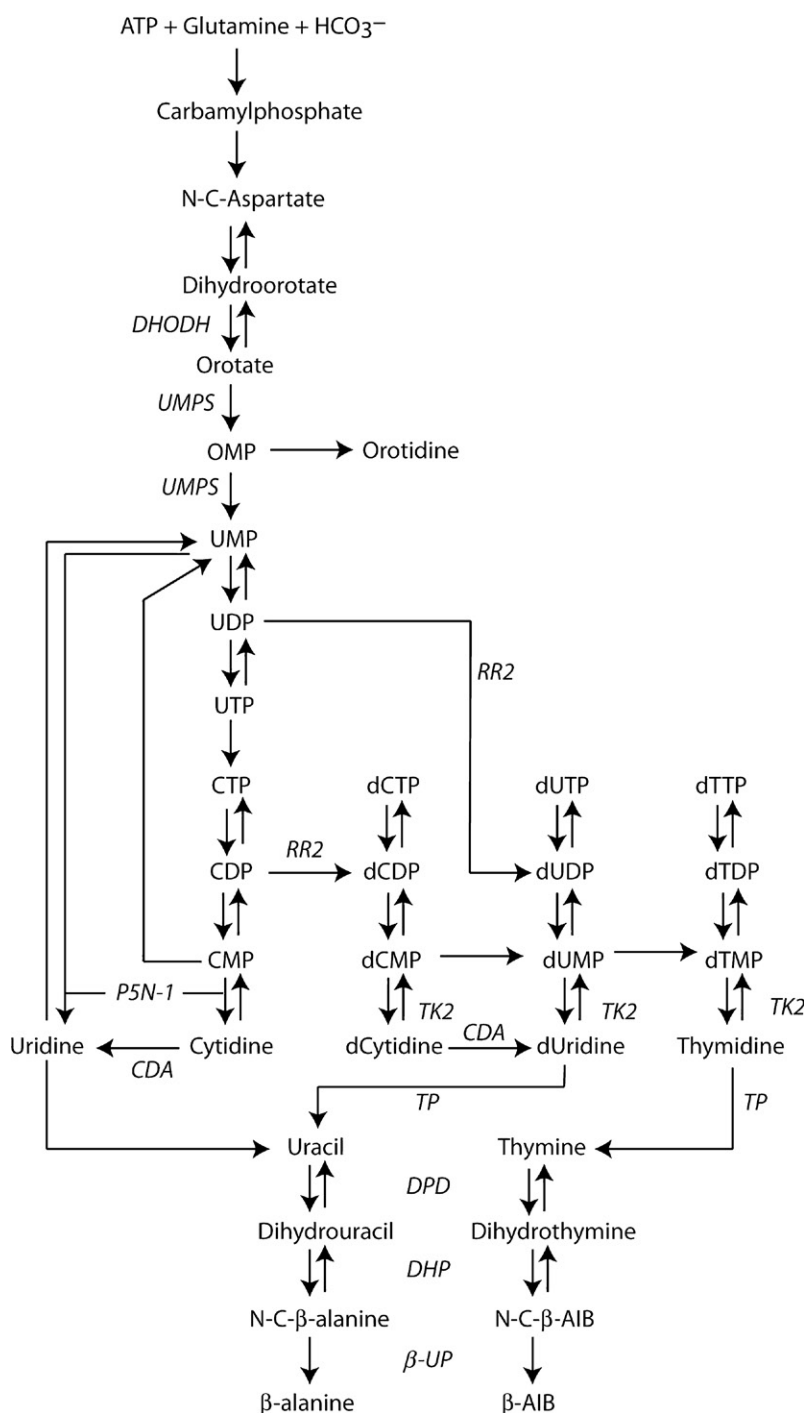
The pyrimidine salvage pathway, composed of kinases, converts the pyrimidine nucleosides, (deoxy)cytidine, (deoxy)uridine, and thymidine, into the corresponding nucleotides, (deoxy)CMP, (deoxy)UMP, and TMP.

#### 95.3.2 Hereditary Orotic Aciduria (UMP Synthetase Deficiency)

Hereditary orotic aciduria was the first inborn error discovered in pyrimidine metabolism (16). It is caused by a deficiency of the last two steps of *de novo* pyrimidine synthesis, orotate phosphoribosyltransferase (OPRT), and orotidine 5'-monophosphate decarboxylase (ODC). Both steps are catalyzed by a single, bifunctional polypeptide termed uridine monophosphate (UMP) synthase (see Figure 95-4). Hereditary orotic aciduria (MIM 258900) is exceedingly rare, with less than 20 cases published over nearly five decades, but early recognition of the disorder is mandatory because it can be easily treated with apparently very good results (227).

**95.3.2.1 Clinical Features.** Patients with hereditary orotic aciduria appear normal at birth but present at a few weeks or months of age with lethargy and failure to thrive, which, untreated, will be followed by developmental delay. Moderate to severe hypochromic anemia is found, with reticulocytes in the normal to low range. Peripheral blood smears show megaloblastosis with severe anisocytosis and poikilocytosis, and moderate hypochromia. Bone marrow examination reveals erythroid hyperplasia and numerous megaloblastic erythroid precursors. In some patients, anemia responded to the treatment with various vitamins, steroids, and hematinics, but characteristically, megaloblastosis persisted. Renal tract obstruction by crystals and infections with various abnormalities of immune function are found in some patients. Unrecognized, the disorder may lead to impairment of growth and cognition.

**95.3.2.2 Diagnostic Tests.** Urinary analysis reveals a massive overexcretion of orotic acid, reaching, in infants, 200- to 1000-fold the normal adult value of 1–1.5 mg per 24 h. This may result in orotic crystalluria, particularly on dehydration. Orotic acid and orotidine can be detected by various techniques including HPLC-UV and HPLC-electrospray tandem mass spectrometry of liquid urine or urine-soaked filter paper



**FIGURE 95-4** Schematic representation of pyrimidine metabolism. Inborn errors of pyrimidine metabolism are depicted in italics. CDA, cytidine deaminase; DPD, dihydropyrimidine dehydrogenase; DHODH, dihydroorotate dehydrogenase; DHP, dihydropyrimidinase; P5N-1, Pyrimidine 5'-nucleotidase type-I; TK2, thymidine kinase 2; TP, thymidine phosphorylase; RR2, ribonucleotide reductase; UMPS, UMP synthase; β-UP, β-ureidopropionase; β-AIB, β-aminoisobutyrate.

strips (228–233). Enzymatic diagnosis can be performed on red blood cells, leukocytes, or fibroblasts. In most patients reported to date, both OPRT and ODC activities were deficient, but remained detectable. This defect is termed type I. To date, no sound data have been published regarding isolated ODC deficiency, referred to as type II. However, pharmacogenetic modeling suggested that patients with orotic

aciduria without megaloblastic anemia are suffering from isolated OPDC deficiency (234).

**95.3.2.3 Biochemical Features.** The two activities of UMP synthase, OPRT and ODC, reside on a single protein containing two domains joined by a linker polypeptide. Limited proteolysis produces separate OPRT and ODC activities, with the former being much more labile than the latter. UMP synthase is composed of two

52-kDa monomers that dimerize and undergo conformational changes to become fully active. The crystal structure of human ODC has been resolved showing plasticity of catalytic residues (235). Studies on patients' cells indicate that UMP synthase deficiency may result from the production of structurally altered proteins that impair the ability of the monomers to form competent, stable dimers.

UMP synthase deficiency provokes, besides massive overproduction of orotic acid, also a deficiency of pyrimidine nucleotides. The overproduction is attributed to the ensuing decrease of the feedback inhibition exerted by the pyrimidine nucleotides, particularly UTP and CTP, on the first enzyme of their *de novo* synthesis, cytosolic carbamoyl phosphate synthetase II (see Figure 95-4). The deficiency of pyrimidine nucleotides leads to impairment of cell division, which results in megaloblastic anemia and in retardation of growth and development.

**95.3.2.4 Molecular Genetics.** UMP synthase is encoded by a single gene, localized on chromosome 3q13, which spans ~15 kb. Three point mutations (R96G, G429R, and V109G) have been identified in two Japanese families (236), among which R96G and V109G are most likely to cause disease. The genetic lesions result in the synthesis of enzymes with reduced stability. Interestingly, deficiency of UMP synthase is estimated to be present in a few percent of Holstein cattle in the United States, in which it seems usually lethal in utero. In the Japanese population, a common polymorphism exists in the OPRT gene with a frequency of 26% (237). This SNP results in an increased activity of OPRT and carriers for this SNP have an increased risk of developing severe grade III–IV diarrhea on treatment with 5-fluorouracil (5FU) (237).

**95.3.2.5 Treatment.** The enzyme defect can be bypassed by the administration of uridine, which is converted into UMP by uridine kinase (see Figure 95-4). An initial dose of 100–150 mg/kg, divided over the day, as a rule induces prompt hematologic response, disappearance of urinary symptoms and infections, and the acceleration of growth. Further dosage has been recommended to be adapted to obtain the lowest possible output of orotic acid. In some cases, normal psychomotor development was achieved, but not in all which may be explained by a delayed onset of therapy.

### 95.3.3 Dihydropyrimidine Dehydrogenase

Dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.2) is the initial and rate-limiting enzyme in the catabolism of pyrimidine bases. It catalyzes the reduction of uracil and thymine to 5,6-dihydrouracil and 5,6-dihydrothymine, respectively (Figure 95-4). Deficiency of DPD (MIM 274270) causes thymine-uraciluria and is typically accompanied by neurological symptoms such as mental and motor delay and convulsions (238,239). However, a consistent phenotype has not yet emerged

(238–240). In addition, DPD plays an important role in the breakdown of the antineoplastic agent 5FU. Patients with a partial or complete enzyme deficiency can suffer from severe and potentially lethal toxicity following 5FU administration (241). Therefore, reliable identification of DPD deficiency is essential to identify cancer patients at risk. Population studies have shown that the prevalence of a partial DPD deficiency in the general population is at least 3–5% (242–244). The carrier frequency in various populations varies considerably (245).

**95.3.3.1 Clinical Features.** The phenotype in patients with DPD deficiency is extremely variable. Many patients are recognized only when they develop a toxic reaction (myelosuppression, diarrhea, fungi infections, and sometimes cardiac toxicity) on 5FU given because of a neoplasm, as they are otherwise completely healthy. Others are detected in metabolic screening because of the search for the cause of developmental delay. Several of these patients have seizures and microcephaly (238,246). Hypotonia and autism or autism spectrum disorders have been described as well (246). Undoubtedly, there is an ascertainment bias and the true clinical phenotype remains to be determined. Patients with DPD deficiency and a marked developmental delay, macrocephaly, and unusual facial morphology may harbor a complete deletion of the gene, and possibly the phenotype is influenced by deletion of neighboring genes as well (247).

The pathological mechanism underlying the various clinical abnormalities is not known. An altered homeostasis of the  $\beta$ -aminoisobutyric acid, a downstream product of thymine, might underlie some of the cerebral dysfunctions often seen in patients with a DPD deficiency (248). The clinical condition of a DPD patient suffering from arthrogryposis multiplex congenita improved considerably when he was treated with both  $\beta$ -alanine and  $\beta$ -AIB (249).

**95.3.3.2 Diagnostics Tests.** DPD deficiency is an autosomal recessive disease characterized by markedly elevated uracil and thymine levels in body fluids such as urine, plasma, and cerebrospinal fluid (231). The concentrations of uracil and thymine in urine range from 47 to 1008 mmol/mol creatinine (controls: 0–30 mmol/mol creatinine) and 7 to 679 mmol/mol creatinine (controls: 0–2 mmol/mol creatinine), respectively. In some cases, 5-hydroxymethyluracil, a metabolite of thymine, is also present in urine and plasma. The pyrimidine catabolites can be detected by various techniques including HPLC, GC–MS analyses of urinary trimethylsilylated organic acid extracts, and HPLC-electrospray tandem mass spectrometry of liquid urine or urine-soaked filter paper strips (228–231,250). The enzyme defect can be demonstrated in the patients' peripheral blood mononuclear cells fibroblasts or liver (251,252).

**95.3.3.3 Biochemical Features.** The activity of DPD is exclusively present in the cytosol and the enzyme is ubiquitously expressed with the highest activity being found in the liver and peripheral blood mononuclear



cells (251). Mammalian DPD appears to be relatively conserved throughout evolution and the high-sequence identities (>92%) between human DPD and that of other mammals, such as bovine and pig, suggest that very similar reaction mechanisms and three-dimensional structures exist in these species (253). The crystal structure of pig DPD revealed that the native enzyme is a homodimer of  $2 \times 111$  kDa and each subunit of 1025 amino acids carries one FAD, one FMN, and four [4Fe-4S] clusters (253). Each subunit appears to be arranged in a highly modular way with five distinctive domains, each carrying a subset of the prosthetic groups. Analysis of the crystal structure of pig DPD suggested that a number of mutations interfered directly or indirectly with cofactor binding or electron transport or affected the structural integrity of the DPD protein (239).

**95.3.3.4 Molecular Genetics.** DPD deficiency is an autosomal recessive disorder and *DPYD* is present as a single copy gene on chromosome 1p21.3 and consists of 23 exons (254). Physically, *DPYD* is at least 950 kb in length with 3 kb of coding sequence and an average intron size of 43 kb (254). The common fragile site *FRA1E* extends over 370 kb within *DPYD* and the region with the highest fragility encompasses exons 13–16 of *DPYD* (255). To date, more than 50 mutations and polymorphisms in the DPD gene have been reported. Analysis of the prevalence of the various mutations among DPD patients has shown that the c.1905+1G>A (IVS14+1G>A) mutation is by far the most common (238,241). This mutation leads to skipping of exon 14, immediately upstream of the mutated splice donor site, in the process of DPD pre-mRNA splicing. A high prevalence of the c.1905+1G>A mutation in the Dutch population was observed, with 1.8% heterozygotes (256). Recently, a deep intronic mutation affecting pre-mRNA splicing in the dihydropyrimidine dehydrogenase gene has been identified (257). Furthermore, the presence of genomic deletions affecting *DPYD* has been demonstrated in 7% (5/72) of all DPD-deficient patients (246). To date, no clear genotype–phenotype correlation has been established, but it is noteworthy that patients with gross deletions in *DPYD* presented with a severe phenotype when compared to that observed in other DPD patients (238,239).

### 95.3.4 Dihydropyrimidinase Deficiency

Dihydropyrimidinase (DHP) is the second enzyme of the pyrimidine degradation pathway and catalyzes the ring opening of 5,6-dihydrouracil and 5,6-dihydrothymine. To date, only 28 patients have been described with a complete DHP deficiency (MIM 222748), including five symptomless individuals who were identified by a screening program for inborn errors of pyrimidine degradation (258). The enzymes of the pyrimidine degradation pathway are also involved in the degradation of the antineoplastic agent 5FU (241). Patients with a DHP

deficiency have developed severe 5FU-associated toxicity (259–261).

**95.3.4.1 Clinical Features.** Clinical features in 20 DHP deficient patients showed a considerable variation in presentation (258). Mental retardation, hypotonia, and seizures were observed in 63%, 45%, and 30% of the patients, respectively. Growth retardation, failure to thrive, microcephaly, and autism were observed less frequently. Forty-five percent of the patients presented with gastrointestinal problems (feeding problems, cyclic vomiting, gastroesophageal reflux, malabsorption with villous atrophy). One patient suffered from a combined deficiency of DHP and a tyrosinemia type II and another patient had both DHP deficiency and congenital microvillous atrophy (262). Phenotypic variability of DHP deficiency was demonstrated in two families in which the index patient was clinically affected whereas the same genotype did not lead to overt symptoms in siblings. It is likely the phenotype is markedly influenced by (epi) genetic and/or environmental factors.

**95.3.4.2 Diagnostic Tests.** DHP deficiency is characterized by markedly elevated dihydrouracil and dihydrothymine levels in body fluids such as urine, plasma, and cerebrospinal fluid (258). The concentrations of dihydrouracil and dihydrothymine in urine range from 100 to 849 mmol/mol creatinine (controls: 0–31 mmol/mol creatinine) and 46 to 622 mmol/mol creatinine (controls: 0–10 mmol/mol creatinine), respectively. The dihydropyrimidines can be detected by various techniques including GC–MS analyses of urinary trimethylsilylated organic acid extracts and HPLC-electrospray tandem mass spectrometry of liquid urine or urine-soaked filter paper strips (229,231,250). Enzyme assay requires liver biopsy, because more accessible tissues do not possess significant DHP activity (263,264).

**95.3.4.3 Biochemical Features.** DHP has been purified and characterized in eukaryotic species and the DHP crystal structures from the yeast *Saccharomyces kluyveri* (<sup>Sk</sup>DHP), the slime mold *Dictyostelium discoideum* (<sup>Dd</sup>DHP), and human DHP have recently been determined (265–269). These studies showed that the native enzyme is composed of four identical subunits containing two zinc ions. In addition, a high structural resemblance between eukaryotic DHP proteins and those of hydantoinases, the bacterial counterparts of DHP as well as the DHP-like noncatalytic proteins involved in neuronal development was observed.

**95.3.4.4 Molecular Genetics.** The gene encoding DHP, *DPYS*, maps to chromosome 8q22 and consists of 10 exons spanning >80 kb of genomic DNA (270). The cDNA coding for human DHP contains an open reading frame of 1560 nucleotides, corresponding to a protein of 519 amino acids with a calculated molecular weight of 56,629 Da (270). *DPYS* analysis has been published in 24 patients showing 21 mutations, including 14 missense mutations, 2 nonsense mutations, 2 deletions, 1 insertion, and 1 splice-site mutation (258,270). Crystal

structures have been shown to be useful in explaining functional compromises of the enzyme (258). Heterologous expression of the mutant enzymes in *Escherichia coli* resulted in mutant enzymes without significant residual activity (258,270).

### 95.3.5 $\beta$ -Ureidopropionase Deficiency

$\beta$ -Ureidopropionase (EC 3.5.1.6). (also termed  $\beta$ -alanine synthase) catalyzes the last step of the pyrimidine degradation pathway, the conversion of *N*-carbamyl- $\beta$ -alanine and *N*-carbamyl- $\beta$ -aminoisobutyric acid into  $\beta$ -alanine and  $\beta$ -aminoisobutyrate, respectively.

To date, only five patients suffering from a complete  $\beta$ -ureidopropionase deficiency have been reported (19,271). However, during a neonatal screening in Japan, five additional asymptomatic children with a putative  $\beta$ -ureidopropionase deficiency were identified (272,273). Acquired (secondary)  $\beta$ -ureidopropionase deficiency has been reported with severe forms of propionic acidemia, due to the inhibition of this enzyme by propionic acid (274).

**95.3.5.1 Clinical Features.** The clinical phenotype of  $\beta$ -ureidopropionase deficiency is characterized by a severe cognitive defect, hypotonia, and delayed motor development and scoliosis, seizures starting in the first months of life, and sometimes dystonia and autism. However, patients that develop normal cognitively have also been found. A single patient had a bladder extrophy with several additional malformations (271).

To date, the mechanism underlying the clinical symptoms observed in our patients with a  $\beta$ -ureidopropionase deficiency is not known. It has been suggested that *N*-carbamyl- $\beta$ -alanine, one of the accumulating substrates, might function as an endogenous neurotoxin (275) as high concentrations of *N*-carbamyl- $\beta$ -alanine inhibited the mitochondrial energy metabolism, which was accompanied by the induction of oxidative stress. The observation, however, that the concentrations of *N*-carbamyl- $\beta$ -alanine were only marginally elevated in CSF of the patients argues against a direct role of *N*-carbamyl- $\beta$ -alanine in the neuropathology. Treatment of a patient with  $\beta$ -alanine for 1.5 years did not result in clinical improvement (276).

**95.3.5.2 Diagnostic Tests.**  $\beta$ -Ureidopropionase deficiency is characterized by markedly elevated *N*-carbamyl- $\beta$ -alanine and *N*-carbamyl- $\beta$ -aminoisobutyric acid levels in body fluids such as urine, plasma, and cerebrospinal fluid (271,273). The concentrations of *N*-carbamyl- $\beta$ -alanine and *N*-carbamyl- $\beta$ -aminoisobutyric acid in urine range from 691 to 745 mmol/mol creatinine (controls: 1–53 mmol/mol creatinine) and 490 to 687 mmol/mol creatinine (controls: 0–11 mmol/mol creatinine), respectively. The *N*-carbamyl- $\beta$ -amino acids can be detected by various techniques including GC–MS analyses and HPLC-electrospray tandem mass spectrometry of liquid urine or urine-soaked filter paper strips (229,250,273).

Enzyme assay requires liver biopsy, because more accessible tissues do not possess significant  $\beta$ -ureidopropionase activity (263,277).

**95.3.5.3 Biochemical Features.** Mammalian  $\beta$ -ureidopropionase appears to have been only relatively conserved throughout evolution, as a comparison of the deduced amino acid sequences of human  $\beta$ -ureidopropionase with that of rat, showed a homology of 84%. Nevertheless, the analysis of a variety of eukaryotic  $\beta$ -ureidopropionases showed that they are functionally related, despite a high degree of structural diversity (278). The native human enzyme is most likely composed of eight identical subunits (279).

**95.3.5.4 Molecular Genetics.** The *UPB1* gene has been mapped to chromosome 22q11.2 and consists of 11 exons spanning ~20 kb of genomic DNA. The cDNA coding for human  $\beta$ -ureidopropionase contains an open reading frame of 1152 nucleotides, corresponding to a protein of 384 amino acids with a calculated molecular weight of 43,158 Da (280). Analysis of *UPB1* in 5 patients showed 2 missense mutations and 2 splice-site mutations (19,271). It may be that missense mutations are more likely to go along with limited neurological problems but numbers are too small to allow for firm conclusions. Heterologous expression of the mutant enzymes in *E. coli* resulted in mutant enzymes without significant residual activity (19,271).

### 95.3.6 Pyrimidine 5'-Nucleotidase Deficiency

Purine and pyrimidine nucleotides are converted to their corresponding nucleosides by a variety of cytosolic and membrane 5'-nucleotidases that may be more specific for one or another type of nucleotide. Several pyrimidine 5'-nucleotidases (P5N) exist that dephosphorylate CMP, UMP, their deoxy counterparts, and TMP to the corresponding nucleosides (Figure 95-4). The deficiency of one of these, pyrimidine 5'-nucleotidase type-I (P5N-1), and isozyme restricted to erythrocytes that catalyze the dephosphorylation of UMP and CMP but not of TMP, was identified in patients with severe chronic hemolytic anemia (MIM 266120). P5N-1 is also involved in the dephosphorylation of nucleoside monophosphates of analogs, such as gemcitabine and AraC (281). To date, ~99 patients have been reported (282,283).

**95.3.6.1 Clinical Features.** Hereditary P5N-1 deficiency is thought to be one of the most common causes of chronic nonspherocytic hemolytic anemia. Patients present with anemia and all consequences of long-term anemia such as hepatosplenomegaly, jaundice, and gallstones. Infrequently, patients show a cognitive delay as well, but it remains uncertain whether this is related. Characteristically, the anemia is nonspherocytic, reticulocytosis may be as high as 45%, and there is prominent basophilic stippling. The basophilic stippling is caused

by the accumulation of high concentrations of pyrimidine nucleotides, mainly CTP and UTP, in the erythrocytes. The mechanism by which the increased pyrimidine nucleotides cause hemolysis remains elusive, but the interference of the accumulating pyrimidine nucleotides with glycolysis, pentose phosphate shunt activity, and altered membrane phospholipid composition has been suggested (282).

**95.3.6.2 Diagnostic Tests.** The diagnosis depends on the demonstration of high concentrations of pyrimidine nucleotides and a reduced P5N-1 activity in red blood cells. Normal red cells contain predominantly purine nucleotides (which have an absorption maximum at ~260 nm) with very low levels of pyrimidine nucleotides (absorption at 280 nm). In P5N-1 deficiency, high levels of pyrimidine nucleotides accumulate in erythrocytes, resulting in a decreased 260:280 absorbance ratio. The analysis of the P5N-1 activity can be performed using a variety of procedures including HPLC-UV. Residual P5N-1 activity has been reported to vary from 1 to 64% and is not correlated with the degree of hemolysis.

**95.3.6.3 Biochemical Features.** Pyrimidine 5'-nucleotidase type-I (P5N-1) is also called cytosolic 5'-nucleotidase III (cNIII) or uridine monophosphate hydrolase type I (UMPH-1) and is a 34 kDa monomeric protein preferentially catalyzing the dephosphorylation of UMP and CMP to their corresponding nucleosides and it is inactive toward purine nucleotides. The activity is dependent on the presence of magnesium ions and it is readily inhibited by heavy metals. Consequently, subjects with chronic low-level lead poisoning may develop severe acquired deficiency of P5N-1, which also results in anemia and basophilic stippling. Clearing of lead returns the nucleotidase activity to normal and resolves the anemia. P5N-1 also displays phosphotransferase activity specific for pyrimidine nucleosides, suggesting an additional role in nucleotide metabolism.

**95.3.6.4 Molecular Genetics.** The gene encoding P5N-1 (*NT5C3*) is localized on 7p15-p14 and consists of 11 exons leading to three alternatively spliced mRNAs (283,284). Since the identification of the P5N-1 gene in 2001, ~24 mutations have been reported in about 40 patients (285). Approximately two-thirds of the mutations are deletions–insertions, generation of nonsense codons, or alternation of splice sites that are most likely causing complete absence of the protein or a truncated version of the protein. Functional analysis of some of the missense mutations showed that the corresponding mutant proteins possessed impaired catalytic activity and/or a reduced thermostability (285,286). Analysis of *NT5C3* in 240 ethnically defined DNA samples revealed 60 polymorphisms and the genetic variation in *NT5C3* has been associated with altered protein function and a potential difference in response to araC and gemcitabine (281).

### 95.3.7 Cytosolic 5'-Nucleotidase Superactivity

An increased activity (6–10-fold) of the cytosolic 5' nucleotidase has been identified in nine patients (21,287) showing a cognitive delay, hyperactivity, and seizures. Routine laboratory tests were unremarkable except for persistent hypouricosuria, explained tentatively by a 30–50% lower concentration of PRPP, as found in patients' fibroblasts, caused by the salvage of the purine bases derived from increased nucleotide catabolism. Decreased PRPP would result in decreased de novo purine synthesis and hence decreased uric acid production. Based on the possibility that increased catabolism might cause a deficiency of pyrimidine nucleotides, patients were treated successfully with oral uridine. Since the initial reports no subsequent patients have been reported and the exact meaning of the findings remains uncertain. A putative nucleotidase responsible for the AMP hydrolyzing hyperactivity has recently been identified (288).

### 95.3.8 Thymidine Phosphorylase Deficiency

Thymidine phosphorylase (TP), also known as “platelet-derived endothelial cell growth factor” (PD-ECGF) and glyostatin, catalyzes the first step in the degradation of the pyrimidine deoxynucleosides thymidine and deoxyuridine (Figure 95-4). Besides natural 2'-deoxynucleosides, TP also recognizes several pyrimidines or pyrimidine nucleosides with antiviral and antitumoral activity, such as 5-fluoro-5'-deoxyuridine (5'DFUR), an intermediate metabolite of capecitabine. TP is found in many normal tissues and cells. Blood platelets are one of the richest sources of TP, which suggests a role for the enzyme in wound healing. Furthermore, TP plays an important role in the female reproductive cycle. TP is upregulated in a wide variety of solid tumors including breast and colorectal cancers. TP promotes tumor growth and metastasis by preventing apoptosis and inducing angiogenesis. Elevated levels of TP are associated with tumor aggressiveness and poor prognosis (22,289). TP is also involved in a wide variety of chronic inflammatory diseases and a deficiency causes mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (MIM 603041) (35,290).

**95.3.8.1 Clinical Features.** MNGIE is characterized by ptosis, progressive external ophthalmoplegia, severe gastrointestinal dysmotility, cachexia, peripheral neuropathy, hearing loss, and skeletal myopathy (290). The initial symptoms are often weight loss and diarrhea, often in the second or third decade (mean 19 years), but much earlier or later has also been reported. Vomiting and periods of pseudo-obstruction occur. The neurological problems are usually relatively mild. The MRI may show a leukoencephalopathy, but this remains usually asymptomatic. The gastrointestinal problems are progressive, and most patients die in the fourth decade.



In patients with MNGIE, the markedly elevated levels of thymidine and deoxyuridine in plasma may cause unbalanced mitochondrial nucleoside and nucleotide pools, which lead to impaired mitochondrial DNA replication and repair. Therefore, therapies that decrease thymidine and deoxyuridine levels may be beneficial to MNGIE patients such as infusion of platelets, dialysis, and allogeneic stem cell transplantation (291–294).

**95.3.8.2 Diagnostic Tests.** In patients with MNGIE, no or a severely reduced TP activity was detected in leukocytes that was accompanied by the presence of strongly elevated levels of thymidine and deoxyuridine in plasma (295–297). In contrast, carriers for mutations in the TP gene, resulting in a substantially reduced activity of TP, did not show the elevated levels of thymidine and deoxyuridine (297). A diagnostic algorithm has been developed based on the analysis of thymidine and deoxyuridine in plasma and the determination of the TP activity in leukocytes, to ensure the proper diagnosis of patients suffering from MNGIE (297). The TP activity can be measured in leukocytes using a non-radiolabel assay, which is based on the separation of thymine and thymidine using reversed-phase HPLC (298). An analysis of thymidine and deoxyuridine concentrations in plasma can be performed using HPLC-UV (297) or HPLC-tandem mass spectrometry (228–230).

**95.3.8.3 Biochemical Features.** TP is a dimer consisting of two identical subunits with a molecular weight of 50 kDa for the monomer. Intracellularly, TP is present in the cytosol and the nucleus. TP catalyzes the conversion of thymidine and 2'-deoxyuridine to their respective bases and 2- $\alpha$ -D-deoxyribose-1-phosphate. Although this reaction is reversible, the most important metabolic function of TP is catabolic. TP also has deoxyribosyl transferase activity by which the deoxyribosyl moiety is transferred from a pyrimidine nucleoside to another pyrimidine base, resulting in the formation of a new pyrimidine nucleoside. Besides natural 2' deoxynucleosides, TP also recognizes several pyrimidines or pyrimidine nucleosides with antiviral and antitumoral activities, such as 5-(E)-(2-bromovinyl)-20-deoxyuridine, 5-trifluorothymidine, 5-fluorouracil, and 5-fluoro-5'-deoxyuridine (289). Specific amino acids in human TP, required for enzymatic activity, have been identified through analysis of the crystal structure and the characterization of mutant proteins (300). There is no correlation between the enzymatic activity of TP and the clinical severity on MNGIE.

**95.3.8.4 Molecular Genetics.** The TP gene (*TYMP*) is located on chromosome 22q13.33 and is composed of 10 exons. Homozygosity for loss-of-function mutations of the TP gene have been identified as the possible cause for MNGIE (35,290,293) making it an autosomal recessively inherited disorder. To date, more than 50 mutations have been identified in *TYMP* in more than 40 families with one or more MNGIE patients (293). No clear genotype–phenotype correlation has emerged.

### 95.3.9 Dihydroorotate Dehydrogenase Deficiency

Dihydroorotate dehydrogenase (DHODH) is the fourth enzyme of the pyrimidine de novo pathway and it catalyzes the conversion of dihydroorotate to orotate. In most eukaryotes, DHODH is localized in mitochondria and the electrons are transferred directly to the respiratory chain via ubiquinone. Inhibitors of DHODH, such as brequinar sodium and leflunomide, have been successfully tested as antiproliferative agents that interfere with neoplastic growth and suppress immunological reactions. DHODH inhibitors have been considered as potential agents for a wide range of parasitic infections (301,302). There have been eight patients published with a DHODH deficiency who showed clinically Miller syndrome (MIM 263750) (23,303).

**95.3.9.1 Clinical Features.** Miller syndrome is characterized by an underdevelopment of the malar bones causing prominence of the eyes, downsloping of the palpebral fissures, and ectropion of the lower eyelids, a small jaw, frequently cleft lip with a cleft palate, and distal limb anomalies (acro-facial dysostosis). The limb anomalies consist of absence or incomplete development of the fifth ray of both hands and feet, and sometimes underdevelopment of the lower arms and lower legs, or underdevelopment of the thumbs is also found. Infrequently, deafness and heart defects are found and sometimes there is a cognitive defect, but most patients develop normally.

**95.3.9.2 Diagnostic Tests.** To date it is not known yet whether patients with a DHODH deficiency present with elevated levels of dihydroorotate or *N*-carbamylaspartate. A dedicated method has been developed for analyses of pyrimidine de novo metabolites using HPLC-electrospray tandem mass spectrometry of liquid urine or urine-soaked filter paper strips (232). The diagnosis is performed using sequence analysis of the DHODH gene.

**95.3.9.3 Biochemical Features.** Mammalian DHODH is a 43 kDa flavoprotein (FMN) localized at the inner mitochondrial membrane, facing the intermembrane space (304). For its activity, mitochondrial DHODH depends on a functional respiratory chain and requires ubiquinone as a direct electron acceptor (305). Mitochondrial import is governed by an uncleaved bipartite sequence at the N-terminus of the protein that consists of a mitochondrial targeting sequence and a membrane stop-transfer sequence that anchors the protein to the inner membrane (304). Structural studies have shown that the truncated enzyme, lacking the bipartite sequence, is fully active (305,306). Detailed analysis of the crystal structure of DHODH has been proven useful for the development of new inhibitors (307).

**95.3.9.4 Molecular Genetics.** The human DHODH gene is located on chromosome 16q22.2 and contains nine exons (23). Collectively, 11 different mutations



have been identified in 6 patients with Miller syndrome by a combination of total exom sequencing and targeted resequencing (23). Ten of these mutations were missense mutations and one was a 1-bp indel that is predicted to cause a frameshift and a termination codon 7 amino acids downstream. Each of the amino acid residues affected by a DHODH mutation was highly conserved among homologs studied to date (23). A polymorphism in the DHODH gene (c.19C>A) has been shown to be associated with toxicity and treatment outcome with leflunomide of patients suffering from rheumatoid arthritis (308,309).

### 95.3.10 Cytidine Deaminase Deficiency

Cytidine deaminase (CDA; MIM 123920) is one of the enzymes of the pyrimidine salvage pathway and it catalyzes the deamination of (deoxy)cytidine into (deoxy)uridine. CDA is able to deaminate, and in this way inactivate, the antitumor agents ara-C and gemcitabine. The pivotal role of CDA in the metabolism of ara-C and gemcitabine is highlighted by the identification of CDA-deficient patients suffering from severe toxicity on treatment with ara-C or gemcitabine (24,310). Although patients with a complete CDA deficiency are rare, it has been suggested that 7% of adult patients with cancer have a partial CDA deficiency and thus an increased risk to develop severe gemcitabine-associated toxicity (310). CDA is also an indispensable enzyme in the metabolism of capecitabine and a high CDA activity has been associated with severe 5FU toxicity (311).

**95.3.10.1 Clinical Features.** Individuals with a CDA deficiency have no known phenotype unless treated with gemcitabine because of cancer such as esophagus carcinoma, lung cancer, pancreas cancer, and also acute lymphoblastic leukemia in children, which may cause marked toxic reactions (312).

**95.3.10.2 Diagnostic Tests.** CDA deficiency (or increased activity) can be established by analysis of the CDA activity in plasma (310,311). In patients suffering from severe toxicities after gemcitabine administration, the mean CDA activity in plasma was ~25% of that observed in patients with no toxicity (310). Analysis of the CDA gene can be performed for the presence of the c.208G>A mutation, for which a clear association with gemcitabine toxicity, has been observed in Japanese patients (313,314).

**95.3.10.3 Biochemical Features.** Human CDA is a homotetramer with a molecular weight of the monomer of 14.9kDa. Each of the four subunits links a zinc atom, which is essential for the enzymatic activity. No cooperativity exists between the subunits and the monomer is inactive. Critical residues involved in the intersubunit interaction have been identified via analysis of the mutant proteins (315). CDA purified from human placenta revealed the presence of five

isoenzymatic forms that differ only in their isoelectric point, which has been ascribed to the existence of two variants with a nonconservative amino acid substitution at codon 27 (316). CDA is readily expressed in human tissues (317).

**95.3.10.4 Molecular Genetics.** The CDA gene is located at chromosome 1p36.12 and the gene is composed of four exons. To date, more than 30 genetic variations have been identified in the CDA gene (312,313,318). The c.208G>A mutation (p.A70T) has been shown to be associated with reduced CDA activity, altered gemcitabine pharmacokinetics, and severe gemcitabine-associated toxicity (24,313,314). Analysis of the prevalence of this mutation in various populations showed that the mutation could only be detected in Japanese and Korean subjects and not in African-, Caucasian- and Chinese-Americans (319). Conflicting data exist as to whether the c.79A>C polymorphism is associated with altered gemcitabine pharmacokinetics (320).

### 95.3.11 Thymidine Kinase 2 Deficiency

Human mitochondrial thymidine kinase 2 (TK2) is a pyrimidine deoxynucleoside kinase, which catalyzes the phosphorylation of pyrimidine deoxynucleosides to their corresponding deoxynucleoside 5'-monophosphates. In resting cells, TK2 plays a key role in the mitochondrial salvage pathway to provide pyrimidine nucleotides for mitochondrial DNA (mtDNA) synthesis and maintenance (321,322). TK2 is also able to phosphorylate a number of chemotherapeutic drugs and antiviral agents (323). This phenomenon might underlie the mitochondrial toxicity associated with prolonged treatment with antiviral nucleoside analogs, such as AZT and FIAU. A deficiency of TK2 is a frequent cause of isolated myopathy or encephalomyopathy in children with mtDNA depletion (mtDNA depletion syndrome type 2; MIM 609560) (25).

**95.3.11.1 Clinical Features.** The mitochondrial depletion syndrome caused by TK2 mutations is usually characterized by neonatal hypotonia and lactic acidosis, and subsequent encephalopathy, liver failure, or renal tubulopathy. In most patients, the disorder is fatal before 6 years of age due to respiratory failure (323). However, the variability can be marked and longer survival, even into the third decade, has been described, and symptoms may be restricted to a pure myopathy.

**95.3.11.2 Pathological Mechanism.** During cell growth, cytosolic dNTPs are synthesized via the S-phase specific de novo synthesis catalyzed by the canonical R1/R2 form of ribonucleotide reductase and the salvage via thymidine kinase 1 (TK1). In postmitotic cells, in which the cytosolic dNTP synthesis is downregulated, mtDNA synthesis relies heavily on the mitochondrial salvage pathway enzymes TK2 and dGK. Therefore, individuals with defects in TK2 show decreased intramitochondrial

TTP pools resulting in imbalance of the TTP/dCTP ratio (324,325). It has been suggested that such mitochondrial nucleotide imbalances could affect the accuracy or the rate of mtDNA synthesis (25,326). The low basal TK2 activity with a high requirement for mitochondrial encoded proteins has been proposed as the main determinant of the muscle involvement in TK-2 deficient individuals (326). In addition, it has been suggested that the onset and organ specificity of TK2 deficiency is dependent on TK1 down-regulation and transcriptional compensation mechanisms to the reduced mtDNA levels (327).

**95.3.11.3 Diagnostic Tests.** The enzyme defect can be demonstrated by analysis of the TK2 activity in mitochondria isolated from muscle or fibroblasts (25,326,328). It has been reported that the activity of TK2 in muscle mitochondria is reduced to 14–45% of the mean value in healthy control individuals (25). Analysis of the TK2 activity in whole cell lysates, containing two interfering deoxyribonucleoside kinases thymidine kinase 1 and deoxycytidine kinase, can be performed using bromovinyl-deoxyuridine, a selective TK2 substrate (329,330).

**95.3.11.4 Biochemical Features.** Human TK2 catalyzes the conversion of thymidine, deoxyuridine, and deoxycytidine into TMP, dUMP, and dCMP, respectively (322). In addition, various nucleoside analogs used as antivirals, can be converted to the corresponding nucleoside monophosphates (322). Although TK2 is predominantly localized into mitochondria, a cytosolic form has been identified as well (331). TK2 is expressed in all tissues in proportion to the mitochondrial content of the cell type. Mature human TK2 consists of 232 amino acids with a subunit molecular weight of 27,487Da (332,333). Human TK2 is active both as a dimer and a tetramer (332,334).

**95.3.11.5 Molecular Genetics.** At present, at least 16 point mutations, deletions and insertions have been reported in TK2 of 20 patients with TK2 deficiency (335). Ten patients proved to be compound heterozygotes and mutations were fairly evenly distributed from exon 3 to exon 10 (335). The effect of several point mutations has been investigated in detail by the expression of mutant proteins followed by the characterization of enzyme function (328,333,335,336). Overall, there was a tendency of a slower progression of manifestations if the mutant TK2 proteins showed some enzyme activity (328,336).

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## CROSS REFERENCE

93. Disorders of Carbohydrate Metabolism.

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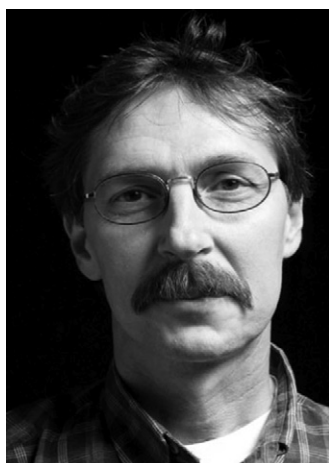
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**André B P van Kuilenburg** received his MSc degree (Honors) in Chemistry (First Class in Biochemistry) in 1986 and his PhD in 1992 from the University of Amsterdam, The Netherlands. In 2003, he was registered as a specialist in clinical genetic laboratory diagnostics with emphasis on clinical biochemical genetics. He is presently working as a clinical biochemical geneticist and principal investigator at the Academic Medical Centre of the University of Amsterdam. His main scientific interests are inborn errors of purine and pyrimidine metabolism, pharmacogenetic aspects associated with inborn errors of the pyrimidine degradation pathway, and biochemical aspects of pediatric oncological diseases. He is the author of 215 scientific papers in peer-reviewed international journals and books. He is a member of the scientific committee of the Purine and Pyrimidine Society and has been elected the secretary of the Dutch Society for Inborn Errors of Metabolism.

# CHAPTER

# 96

## Lipoprotein and Lipid Metabolism

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### ABBREVIATIONS

AD – autosomal dominant  
AR – autosomal recessive  
BA – bile acid  
CHD – coronary heart disease  
CM – chylomicron  
CVD – cardiovascular disease  
HDL – low-density lipoprotein  
HLP – hyperlipoproteinemia  
HTG – hypertriglyceridemia  
IDL – intermediate-density lipoprotein  
LDL – low-density lipoprotein  
LRP – LDL receptor related protein  
MIM – Mendelian Inheritance in Man  
TG – triglyceride  
PL – phospholipid  
VLDL – very-low-density lipoprotein

Alpha, beta, pre-beta and broad-beta lipoproteins: It is possible to classify lipoproteins as “alpha” and “beta” based on their mobility through serum protein electrophoretic gels, according to their position. HDL subfractions have alpha mobility, LDL subfractions have beta mobility, VLDL fractions have pre-beta mobility and the spectrum of remnant triglyceride-rich lipoprotein particles, including IDL, have broad-beta mobility. These electrophoretic phenotypes give rise to names of clinical syndromes of disordered lipoprotein metabolism, such as “abetalipoproteinemia,” “hypo-beta lipoproteinemia” and “hypoalphalipoproteinemia.”

### 96.1 INTRODUCTION

Lipoproteins transport hydrophobic lipids and fat-soluble vitamins through plasma from their site of origin (intestine or liver) to their site of uptake and disposition. Abnormal

levels of certain plasma lipids and lipoproteins increase the risk of cardiovascular disease (CVD) end points, such as myocardial infarction and stroke (1). A wide range of genetic and environmental factors contribute to inter-individual variation in plasma concentrations of lipids and lipoproteins (2). Dyslipidemias that have a monogenic basis typically present earlier in life, while those that present later in life also have genetic determinants but their expression further depends on interactions with nongenetic environmental or lifestyle factors (3). Early diagnosis is central to specific dietary, lifestyle and pharmacological interventions to delay death, disability and medical complications (4). This chapter will review the current understanding of genetic determinants, clinical manifestations and treatment modalities associated with disorders of lipoprotein and lipid metabolism, including disturbances affecting levels of cholesterol (hyper- and hypocholesterolemia), triglycerides (TGs) (hypertriglyceridemia (HTG)) and some related conditions.

### 96.2 PLASMA LIPIDS, LIPOPROTEINS AND APOLIPOPROTEINS

The most important plasma lipids clinically are cholesterol and triglyceride (also called triacylglycerol) (2). Cholesterol is (1) a component of cell membranes; (2) the precursor for steroid hormones and vitamin D, and for oxysterols and bile acids (BAs), both activators of nuclear hormone receptors involved in sterol metabolism; and (3) required for the activation of neuronal signaling molecules (5). Relatively little circulating cholesterol originates from the diet; ~80% is derived from endogenous synthesis in the liver and extrahepatic tissues. Fat or carbohydrate not required by the liver for

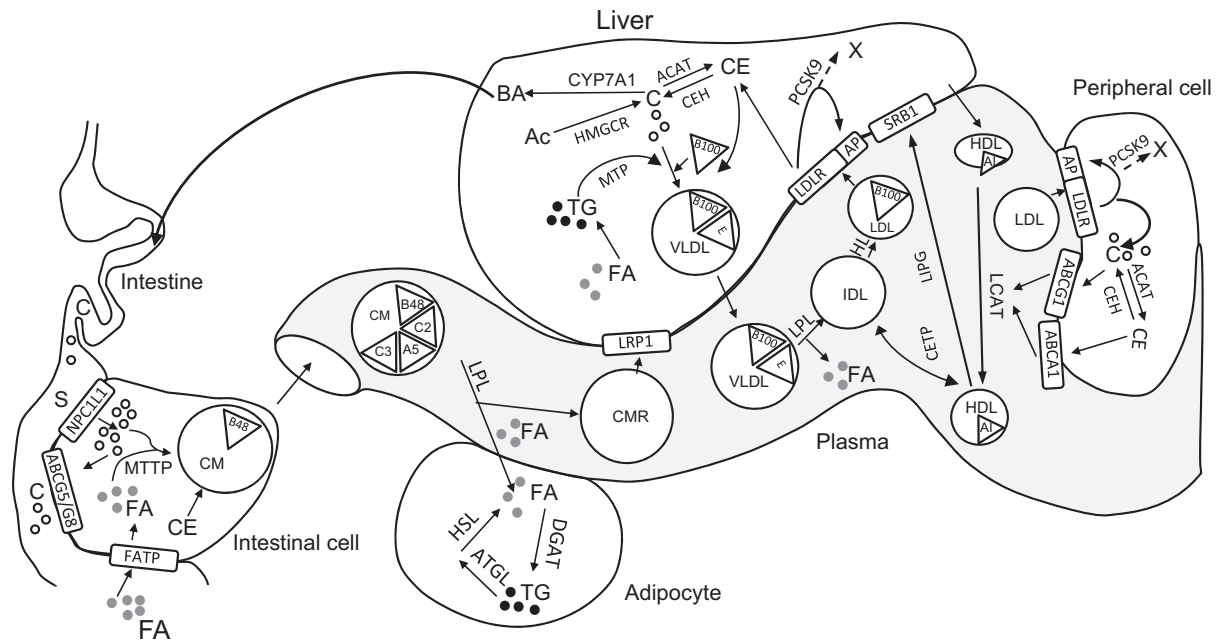


FIGURE 96-1 Lipoprotein metabolism.

TABLE 96-1 Main Lipoprotein Classes

Lipo-protein Class	Size Range (nm)	Density Range (g/mL)	Electrophoretic Mobility	TG (%wt)	PL (%wt)	Free Cholesterol (%wt)	Esterified Cholesterol (%wt)	Protein (%wt)	Main Apolipoproteins
CMs	75–120	0.94	Origin (cathode)	80–95	3–6	1–3	2–4	1–2	A-I, A-IV, A-V; B-48, C-I, C-II, C-III, E
VLDL	30–70	0.94–1.006	Pre- $\beta$	45–65	15–20	4–8	16–22	6–10	B-100, E, C-I, C-II, C-III
IDL	25–50	1.006–1.019	Broad $\beta$ band	25–35	15–25	4–8	24–35	16–20	B-100, E, C-I, C-II, C-III
LDL	18–30	1.019–1.063	$\beta$	4–8	18–24	6–8	45–50	18–22	B-100
HDL	5–12	1.063–1.21	$\alpha$	2–7	26–32	3–5	15–20	45–55	A-I, A-II, E

VLDL, very-low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglyceride; PL, phospholipid; %wt, percent by weight.

energy or synthesis is converted to TG (Figure 96-1) through several biosynthetic pathways. TG is a key energy source that is comprised of three fatty acids (FA), each ester-linked to one of the three carbons on a glycerol backbone. TG is synthesized in intestinal epithelial and liver cells, transported through plasma and after lipolysis at the endothelial surface, delivers free fatty acid (FFA) to peripheral cells for  $\beta$ -oxidation, metabolism or storage. Phospholipids (PL) differ from TG by a phosphate ester linked to the third carbon of glycerol. While PLs are biologically important, they do not directly contribute to the main clinical end points associated with dyslipidemia and will not be further considered.

The insolubility of cholesterol and TG in plasma requires that they are transported within spheroidal macromolecules called lipoproteins, which contain PL, fat-soluble antioxidants and vitamins, and cholesteryl

ester (CE) in their hydrophobic core, as well as free cholesterol (C), PL and apolipoprotein (apo) molecules in their hydrophilic coat. Lipoproteins are distinguished by size, density, electrophoretic mobility, composition and function (2) (Table 96-1). The main TG-carrying lipoproteins are the chylomicron (CM) and very-low-density lipoprotein (VLDL). The main cholesterol-carrying lipoproteins are low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Intermediate-density lipoprotein (IDL), physiologically an evanescent species, contains roughly equimolar amounts of cholesterol and TG.

Apolipoproteins provide plasma lipoproteins with structural stability and solubility (6). They also serve as ligands for receptors and/or activators for enzymes. Some important apolipoproteins are listed in Table 96-2. Apo B-100 (B100) of VLDL, IDL and LDL (Figure 96-1)



**TABLE 96-2 Summary of Main Apolipoproteins**

Name	Peptide Length (Amino Acids)	Molecular Weight (kilodaltons)	Associated Lipoprotein(s)	Function
A-I	243	28	HDL, CM	Structural component of HDL; activates LCAT; SR-B1 ligand; promotes cholesterol efflux
A-II	77	17	HDL, CM	Structural (HDL); activates HL and LPL
A-IV	396	46	HDL, CM	Activates LCAT; modulates LPL; possibly TG transport
A-V	366	44	CM	Modulates LPL
(a)	Variable	250–800	Lp(a)	Unknown
B-100	4536	540	VLDL, IDL, LDL	Structural component of LDL; ligand for LDLR
B-48	2152	264	CM	Defining protein of intestinally derived particles
C-I	57	6.6	CM, VLDL, IDL, HDL	Inhibits interaction with LDLR and LRP1; LCAT activator
C-II	79	8.9	CM, VLDL, IDL, HDL	LPL modulator
C-III	79	8.8	CM, VLDL, IDL, HDL	LPL inhibitor
E	299	19	CM, VLDL, IDL, HDL	LDLR and LRP1 ligand; cell growth and immune response

CM, chylomicron; VLDL, very-low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LCAT, lecithin:cholesterol acyl transferase; HL, hepatic lipase; LPL, lipoprotein lipase; PL, phospholipid; SR-B1, scavenger receptor type B1; LDLR, LDL receptor; TG, triglyceride.

is synthesized in the liver, while apo B-48 (B48) of CM and CM remnants (CMR, [Figure 96-1](#)) is synthesized in the small intestine. Apo B-100 (4356 amino acids) and B-48 (2152 amino acids) are products of the *APOB* gene (7). Apo B-48 mRNA is produced by editing of the apo B-100 mRNA (8), mediated by a 27kDa editase (*APOBEC1*; MIM 600130). Other important apolipoproteins include apo C-II, a cofactor for lipoprotein lipase (LPL), apo E, a ligand for receptor-mediated endocytosis (RME) and apo A-I, an activator of the enzyme lecithin:cholesterol acyl transferase (LCAT) (3).

Thus, lipoprotein metabolism is a complex and partially understood network of assembly, secretion, processing and catabolism ([Figure 96-1](#)). This chapter will focus primarily on genetic disorders that affect the concentrations of two plasma lipoproteins, namely, LDL and HDL, and one plasma lipid, namely, TG, which have been connected to CVD and related disorders.

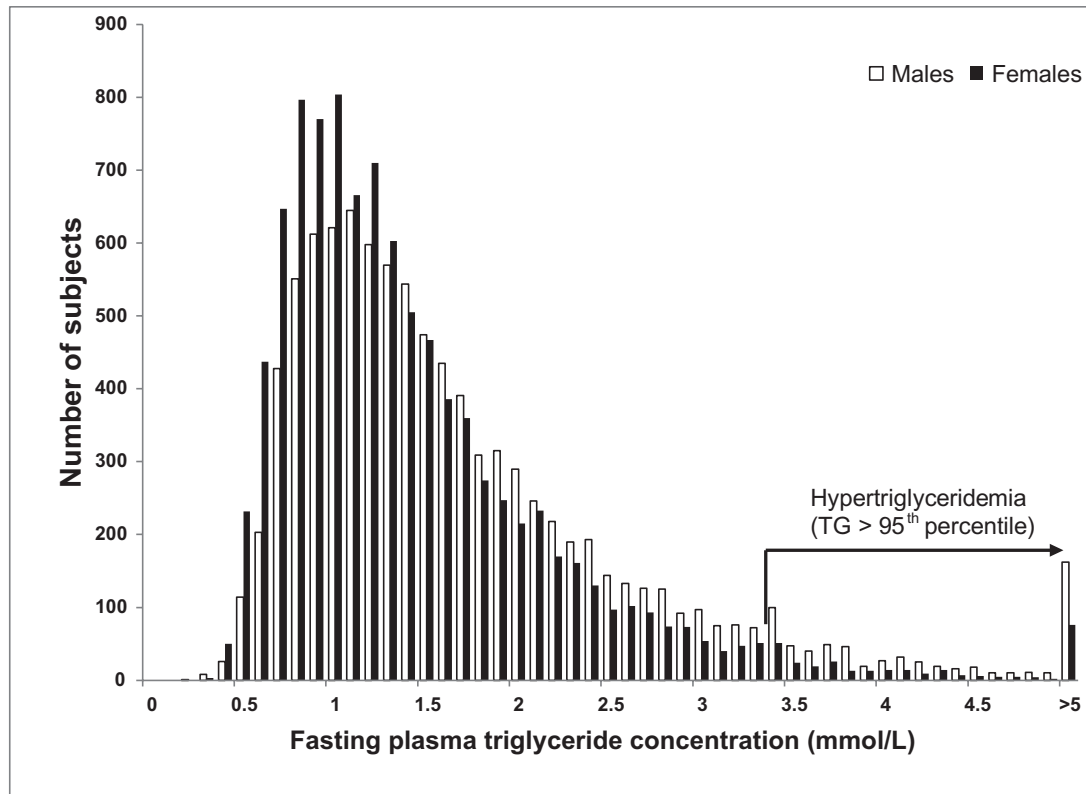
## 96.3 LIPOPROTEIN METABOLISM

### 96.3.1 Low-Density Lipoprotein (LDL) Metabolism

Sterols, such as cholesterol and plant sterols in the intestinal lumen enter enterocytes via the Niemann–Pick C1-like 1 (NPC1L1) transporter (9). Plant sterols and some cholesterol are resecreted by heterodimeric ATP-binding cassette transporter G5/G8 (*ABCG5/G8*) (10). Within enterocytes, free cholesterol (C in [Figure 96-2](#)) is packaged with TG into CMs. Within hepatocytes, cholesterol is recycled or synthesized de novo from acetyl coenzyme A (CoA) (Ac, [Figure 96-1](#)) by a multistep synthetic pathway, with the last committed step catalyzed by 3-hydroxy-3-methylglutaryl CoA reductase (11). Free cholesterol can also be (re-)esterified to CE ([Figure 96-1](#)) by an acyl CoA:cholesterol acyltransferase (ACAT,

[Figure 96-1](#)) (12), for incorporation into cytosolic lipid storage droplets or lipoprotein assembly. Lipoprotein-derived CE can be hydrolyzed to release free cholesterol by lysosomal CE hydrolase (CEH, [Figure 96-1](#)), also called lysosomal acid lipase (LAL), and a distinct neutral CEH hydrolyzes cytoplasmic CE (13). Hepatic free cholesterol can be diverted to the BA ([Figure 96-1](#)) synthetic pathway by hydroxylation, for which cholesterol 7- $\alpha$ -hydroxylase (CYP7A1, [Figure 96-1](#)) is rate limiting (14). Most cholesterol that is transported through the circulation in lipoproteins is esterified, with free cholesterol constituting a relatively minor fraction.

The microsomal TG transfer protein (MTP, [Figure 96-1](#)) in hepatocytes directs the assembly of CE and TG together with apo B-100 and apo E to produce VLDL ([Figure 96-1](#) and [Table 96-1](#)) for secretion into plasma (15). VLDL in plasma is lipolytically remodeled into LDL, which is the main cholesterol-carrying lipoprotein since ~70% of total plasma cholesterol is partitioned into LDL ([Figure 96-1](#)). LDL transports cholesterol from the liver to the periphery (16). LDL is actually a spectrum of particles whose main lipid is CE, and whose defining protein moiety is a single molecule of apo B-100 ([Table 96-1](#)). LDL has a plasma half-life of ~3 days, and is cleared by the binding of apo B-100 to peripheral and hepatocyte low-density lipoprotein receptors (LDLRs) ([Figure 96-1](#)), clustering in coated pits, internalized by RME, assisted by an adaptor protein (LDLRAP) (16). Proprotein convertase subtilisin/kexin type 9 (PCSK9) appears to be secreted into plasma, then reenters the cell, complexes with the LDLR and short-circuits recycling of the receptor from the endosome. After RME, apo B-100-containing lipoproteins are processed through lysosomes, and freed cholesterol enters the cellular pool. As hepatic free cholesterol levels increase, LDLR transcription is suppressed, RME is reduced and plasma LDL rises.



**FIGURE 96-2** Frequency distribution of fasting plasma TG concentrations in a population-based sample of more than 22,000 people from Canada. White bars represent male subjects, black bars represent female subjects. Subjects with plasma TG concentration  $>3.37$  mmol/L are in the 95th percentile, considered the threshold for HTG. The maximum plasma TG concentration in this sample was 45 mmol/L.

### 96.3.2 High-Density Lipoprotein (HDL) Metabolism

“Reverse cholesterol transport” (RCT) describes cholesterol transport in HDL from peripheral cells back to the liver for secretion in bile (17). The initial step in HDL metabolism involves the formation of small lipid-poor nascent HDL particles in the liver and small intestine. Nascent HDL particles (Figure 96-1) attract excess free cholesterol from both extrahepatic cells and other circulating lipoproteins. Within peripheral cells, ACAT and CEH (Figure 96-1) maintain the balance between free cholesterol and CE (18). These small HDL particles, via apo A-I (A1, Figure 96-1), mediate RCT by interacting with ABCA1, which directs transfer of CE, and ABCG1, which directs transfer of free cholesterol, transporters on nonhepatic cells (18). Using apo A-I as a cofactor, LCAT esterifies cholesterol for packaging into HDL, which after remodeling by cholesterol ester transfer protein (CETP) and by endothelial lipase (LIPG) enters hepatocytes via scavenger receptor class B type I (SR-B1) (19). SR-B1 mediates the selective uptake of cholesterol ester and other lipids. The receptor, present on hepatocytes, binds to HDL and other lipoproteins, mediating the transfer of cholesterol from serum HDL to the bile for excretion, completing the cycle of RCT and removal of cholesterol from the body (20). The final step in plasma HDL metabolism involves the clearance of apo A-I and pre  $\beta$ -1 HDL in the kidney and excretion in the urine.

In addition to RCT, HDL might (1) suppress cytokine-induced adhesion of endothelial cells; (2) protect LDL from oxidation; and (3) have anticoagulant effects (21). In research laboratories, HDL particles can be subfractionated according to size and density by ultracentrifugation and gradient electrophoresis (22). They can also be separated according to protein content using immunological assays (23); these specialized methods are beyond the reach of most clinical laboratories.

### 96.3.3 Triglyceride (TG) Metabolism

In the intestine, dietary fat is processed prior to absorption (24). For instance, pancreatic lipase hydrolyzes dietary TG to liberate FFAs that are absorbed both passively and actively. BA (Figure 96-1) from liver is secreted and subsequently reabsorbed through specific mediators (25). BA permits absorption of luminal free cholesterol (Figure 96-1). Hydrolyzed dietary fats enter enterocytes via FA transporters. Within enterocytes, processing by ACAT and TG biosynthetic pathways prepare CE and TG, respectively, for MTP-mediated assembly with apo B-48 and apo E through a vesicular pathway into CM, which is the main lipoprotein carrying fat of exogenous origin secreted into the lymph and plasma (26).

CMs secreted into lymphatics enter the vena cava and circulate until they interact with LPL, whose secretion

depends on lipase maturation factor-1 (LMF1, not shown in Figure 96-1) (27), and which is transported to the intestinal lumen by glycosylphosphatidylinositol-anchored HDL-binding protein 1 (GPIHBP1, not shown in Figure 96-1) (28) and secured to endothelium by proteoglycans. CMs contain apo A-V (A5), apo C-II (C2) and apo C-III (C3), among others. Liberated FFAs incompletely enter peripheral cells.

Within adipocytes, enzymes including acyl CoA: diacylglycerol acyltransferase resynthesize TG, which is hydrolyzed by adipose TG lipase and hormone-sensitive lipase. Within the capillaries of adipose tissue and muscle, CM and VLDL core TG are hydrolyzed to FFA by endothelial-bound LPL (Figure 96-1) using apo C-II as a cofactor (C2, Figure 96-1) (29). FFA is reesterified and stored as TG in fat cells, or oxidized to provide energy in muscle. CM and VLDL are remodeled into the short-lived smaller, denser, more CE-rich CMR (Figure 96-1) and IDL (Figure 96-1), respectively (30). CMR and some IDL are cleared by apo E-mediated endocytosis through hepatic remnant receptors (LRP1, Figure 96-1), contributing to the hepatic CE pool (Figure 96-1). IDL that is not cleared is then hydrolysed by hepatic lipase (HL) (Figure 96-1) making smaller, CE-rich LDL particles (Figure 96-1).

### 96.3.4 Plasma Lipoproteins and Cardiovascular Disease Risk

Plasma lipid and lipoprotein concentrations generally follow a right-skewed normal distribution in the general population (Figure 96-2) (3). Median levels vary by age and sex: in general, older age and male sex are associated with a less favorable lipid profile. There are also differences in median levels across geographical ancestries and these might contribute to differences in CVD risk (1). Epidemiologically, CVD is directly related to plasma levels of total and LDL cholesterol (31) and inversely related to plasma levels of HDL cholesterol (32). In men, a rise in total cholesterol from 5.2 to 6.2 mmol/L (200 to 240 mg/dL) is associated with a threefold increased risk of death from coronary heart disease (CHD) (33). HDL cholesterol <0.9 mmol/L (<35 mg/dL) is an independent CHD risk factor and the most common lipid disturbance seen in CHD patients under 60 years of age (34). While the inverse association between HDL and CVD is indisputable, a causal relationship remains uncertain, in part because not all genetic disorders causing very low HDL are associated with the presence of CVD, in contrast to the uniform presence of CVD in genetic disorders causing high LDL. Plasma LDL cholesterol and apo B concentrations are directly correlated (35), as are plasma HDL cholesterol and apo A-I concentrations (36). The ratio of apo B to A-I has been advocated as the strongest predictor of CHD risk (37) but the ratio of total cholesterol to HDL cholesterol seems equally predictive (38). Although the

relationship between plasma TG and CHD has been confounded by the association of elevated TG with depressed HDL cholesterol, elevated TG, especially nonfasting TG (39) and familial HTG (40) are independently associated with CHD risk (41).

A chronic excess of LDL in plasma alters physiological arterial endothelial dilation, which is an early manifestation of vascular dysfunction (42). LDL that does not undergo regulated RME is taken up in an unregulated manner by scavenger receptors, such as CD36 and SRA-I/II, on arterial wall macrophages (43). Entrapped LDL lipids can become oxidized, generating toxic intermediates that induce cytokine production and chemotaxis of inflammatory cells (44). Arterial wall macrophages can become engorged with cholesterol from LDL, creating foam cells, which are a key component of atherogenic plaques (21). Lipids engulfed by macrophages become oxidized, generating toxic intermediates, which induce cytokine production and inflammatory cell chemotaxis (21,44). Occlusive plaques, often the result of rupture compounded by thrombosis, result in CVD, such as CHD or stroke (21,44).

## 96.4 MONOGENIC DISORDERS OF LIPOPROTEIN METABOLISM

Several important genetic determinants of lipoprotein levels have been identified through studies of monogenic disorders (Table 96-3) (3). Monogenic disorders comprise a rare patient subgroup found at the extremes of population-specific lipoprotein distribution. The molecular basis of many monogenic high and low lipoprotein syndromes was elucidated using biochemical approaches or classic genetic linkage analysis. Studies of these diseases have helped define key pathways, such as RME, through the LDLR and sterol efflux from cells via ATP-binding cassette proteins (45). Several causative genes with rare mutations have resurfaced as loci with common small-effect genetic variants that underlie lipoprotein variation in population studies. Many of these disorders are autosomal recessive, due to homozygous mutations in causative genes. Importantly, some affected patients are compound heterozygotes, a category hereafter implicitly included whenever the term “homozygous” is used.

Some of the classic Fredrickson, also called World Health Organization (WHO), hyperlipoproteinemia (HLP) phenotypes have a monogenic basis (Table 96-4) (3). For instance, HLP type 2A is defined by LDL cholesterol >95th percentile of the normal population distribution. However, ~10% of these subjects have a discrete monogenic syndrome (45), such as heterozygous familial hypercholesterolemia (HeFH), or the phenotypically similar disorders familial defective apo B due to binding defects in the *APOB* gene, and autosomal dominant hypercholesterolemia due to gain-of-function (GOF) mutations in the *PCSK9* gene.

TABLE 96-3 Monogenic Dyslipidemias							
Lipid Phenotype	Disease/Syndrome	OMM Disease ID	Gene Symbol	OMM Gene ID	Protein Name	Refseq Accession Number	Cytol
<i>LDL-C</i>							
Elevated	Familial hypercholesterolemia	143890	<i>LDLR</i>	606945	Low-density lipoprotein receptor (LDLR)	NM_000527.4	19p13
	Familial defective apo B (ADHCHOL2)	107730	<i>APOB</i>	107730	Apolipoprotein (apo) B-100	NM_000384.2	2p24
	AD hypercholesterolemia type 3 (ADHCHOL3)	603776	<i>PCSK9</i>	607786	Proprotein convertase subtilisin/kexin type 9	NM_174936.3	1p32
	Hypercholesterolemia, AR	603813	<i>LDLRAP1 (ARH)</i>	605747	Low-density lipoprotein receptor adaptor protein 1	NM_015627.2	1p33
Depressed	Abetalipoproteinemia	200100	<i>MTTP (MTP)</i>	157147	Microsomal triglyceride transfer protein	NM_000253.2	4q24
	Familial hypobetalipoproteinemia	605019	<i>APOB</i>	107730	Apolipoprotein B-100	NM_000384.2	
	AD hypocholesterolemia	603776	<i>PCSK9</i>	607786	Proprotein convertase subtilisin/kexin type 9	NM_174936.3	1p32
	Chylomicron retention disease	246700	<i>SAR1B (SARA2)</i>	607690	SAR1 homolog B	NM_001033503.2	5q31
<i>HDL-C</i>							
Elevated	CETP deficiency	607322	<i>CETP</i>	118470	Cholesteryl ester transfer protein (CETP)	NM_000078.2	16p11
	SR-B1 deficiency	610762	<i>SCARB1</i>	601040	Scavenger receptor class B, member 1 (SRB1)	NM_005505.4	12q23
	EL deficiency		<i>LIPG (EL)</i>	603684	Lipase, endothelial	NM_006033.2	18q21
Depressed	TD; familial hypoalphalipoproteinemia	205400	<i>ABCA1</i>	600046	ATP-binding cassette, subfamily A member 1	NM_005502.3	9q31
	LCAT deficiency; fish-eye disease	245900	<i>LCAT</i>	606967	Lecithin:cholesterol acyltransferase (LCAT)	NM_000229.1	16q22
	Familial hypoalphalipoproteinemia, primary	604091	<i>APOA1</i>	107680	Apolipoprotein A-I	NM_000039.1	11q23



## TG

Elevated	Chylomicronemia, HLP type 1, LPL deficiency	238600	<i>LPL</i>	609708	Lipoprotein lipase (LPL)	NM_000237.2	8p22
	Chylomicronemia, HLP type 1, apo C-II deficiency	207750	<i>APOC2</i>	608083	Apolipoprotein C-II	NM_000483.3	19q13
	Severe HTG, HLP type 5	144650	<i>APOA5</i>	606368	Apolipoprotein A-V	NM_052968.4	11q23
	Severe HTG, HLP type 5	246650	<i>LMF1</i>	611761	Lipase maturation factor 1	NM_022773.2	16p13
	Severe HTG, HLP type 5	NA	<i>GPIHBP1</i>	612757	Glycosylphosphatidylinositol anchored high-density lipoprotein-binding protein 1 (GPIHBP1)	NM_178172.3	8q24.3

## TG, LDL and HDL

Elevated	HL deficiency	612797	<i>LIPC</i>	151670	Hepatic (triglyceride) lipase (HL; HTGL)	NM_000236.2	15q21
Depressed	Hypobetalipoproteinemia, familial type 2	605019	<i>ANGPTL3</i>	604774	Angiopietin-like 3	NM_014495.2	1p31

## Other

Plant sterols	Sitosterolemia	210250	<i>ABCG5</i>	605459	ATP-binding cassette, subfamily G member 5	NM_022436.2	2p21
Plant sterols	Sitosterolemia	210250	<i>ABCG8</i>	605460	ATP-binding cassette, subfamily G member 8	NM_022437.2	2p21
	Cholesterol ester storage disease	278000	<i>LIPA (LAL)</i>	613497	Lipase A, lysosomal acid, cholesterol esterase	NM_001127605.1	10q23
	Wolman syndrome	278000	<i>LIPA (LAL)</i>	613497	Lipase A, lysosomal acid, cholesterol esterase	NM_001127605.1	10q23
Increased IDL	DBL (HLP type 3)	611771	<i>APOE</i>	107741	Apolipoprotein E	NM_000041.2	19p13

**TABLE 96-4 Classic Hyperlipoproteinemia Phenotypes**

WHO ICD Number	Frederickson HLP Phenotype	MIM Number	Lipids	Lipoproteins	Genetics
E78.3	HLP type 1 Familial chylomicronemia	238600	↑TG	↑CM	Primarily pediatric; young adults; monogenic; AR due to mutant <i>LPL</i> or <i>APOC2</i> ; severe chylomicronemia also seen with homozygous mutations in <i>APOA5</i> , <i>LMF1</i> or <i>GPIHBP1</i>
E78.0	HLP type 2A FH	143890	↑TC	↑LDL	Monogenic, heterozygous form due to mutant <i>LDLR</i> , <i>APOB</i> or <i>PCSK9</i> ; homozygous form due to mutant <i>LDLR</i> or <i>ARH</i>
E78.4	HLP type 2B CHL	144250	↑TC, ↑TG	↑VLDL, ↑LDL	Polygenic—combined SNPs and excess of rare variants in HTG associated genes from GWAS together with LDL-associated SNPs
E78.2	HLP type 3 DBL	107741	↑TC, ↑TG	↑IDL	Polygenic—combined SNPs and excess of rare variants in HTG associated genes from GWAS together <i>APOE</i> E2/E2 homozygosity or mutant <i>APOE</i>
E78.1	HLP type 4 Primary HTG	144600 and 145750	↑TG	↑VLDL	Polygenic—combined SNPs and excess of rare variants in HTG associated genes from GWAS
E78.3	HLP type 5 Mixed hyperlipidemia (MHL)	144650	↑TC, ↑TG	↑VLDL, ↑CM	Polygenic—combined SNPs and excess of rare variants in HTG associated genes from GWAS

WHO, World Health Organization; ICD, International Classification of Diseases; HLP, hyperlipoproteinemia; HTG, hypertriglyceridemia; MIM, Mendelian Inheritance in Man; LPL, lipoprotein lipase; TG, triglyceride; CM, chylomicrons; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; IDL, intermediate-density lipoprotein; AR, autosomal recessive; *LPL*, gene encoding LPL; *APOC2*, gene encoding apolipoprotein (apo) C-II; *APOB*, gene encoding apo B; *PCSK9*, gene encoding proprotein convertase subtilisin/kexin type 9; *ARH*, gene encoding autosomal recessive hypercholesterolemia protein; *USF1*, gene encoding upstream stimulatory factor 1; *APOE*, gene encoding apo E; *APOA5*, gene encoding apo A5; *LMF1*, gene encoding lipase maturation factor 1; *GPIHBP1*, gene encoding glycosylphosphatidylinositol-anchored HDL-binding protein 1; GWAS, genome-wide association study.

The clinical features, molecular genetics, diagnosis and treatment specific to each disorder will be discussed in the following subsections. Later, general approaches to management of dyslipidemia, including diet, lifestyle and pharmacologic classes and individual agents, together with their mechanisms of action, will be discussed in the section on treatment of dyslipoproteinemia.

## 96.5 DISORDERS WITH PRIMARILY ELEVATED LDL CHOLESTEROL

### 96.5.1 Heterozygous Familial Hypercholesterolemia (HeFH)

**96.5.1.1 Clinical Features.** Familial hypercholesterolemia (FH) results from deficiency or defective function of LDLRs (16). FH follows autosomal codominant inheritance, with a very severe phenotype in homozygotes (HoFH), and a dramatic but less severe phenotype in heterozygotes (HeFH). However, the two forms are usually considered separately because HeFH is relatively common, comprising a substantial proportion of adult lipid clinic patients with twice-normal LDL cholesterol levels who can be managed with pharmacological treatment. In contrast, HoFH is essentially a disorder of children and young adults with four times normal LDL cholesterol levels who require extracorporeal plasma exchange or apheresis.

HeFH has been well studied and reviewed (16,46). Its frequency in the general population is 1 in 500, although it is higher within certain ethnic subgroups such as Afrikaners, Quebecois and Christian Lebanese (46b). The defect in HeFH is loss of the capacity of cells to internalize LDL particles. LDL cholesterol residence time in plasma is prolonged, and the particles undergo oxidation and other modifications. Modified LDL particles are taken up in an unregulated manner by macrophages, and this can lead to clinical manifestations. For instance, cholesterol deposits in the eyelids are called “xanthelasmas.” Cholesterol deposits in connective tissues within and surrounding extensor tendons, especially of the hands and Achilles tendons are called “xanthomas.” Deposits along the corneal margin are called “arcus cornealis” or “corneal arcus.” The most life-threatening deposits are within arteries, leading to premature CHD, stroke and peripheral vascular disease. HeFH is a potent risk factor for early CVD and death if undetected and untreated (47). A study of >1000 pediatric HeFH patients found that 89% had a positive family history of premature CVD (48). The Simon Broome Registry (SBR) prospectively evaluated 526 HeFH patients in the prestatin era; the standardized all-cause mortality rate by the fifth decade in HeFH was increased by more than 100-fold compared to normolipidemic subjects (47).

**96.5.1.2 Molecular Genetics.** HeFH patients have one copy of a mutated *LDLR* gene, which is located on

**TABLE 96-5 DLN Criteria for Diagnosis of Heterozygous Familial Hypercholesterolemia**

<i>Family History</i>	
A) First-degree relative with known premature (<55 years men; <60 years women) coronary and vascular disease	1
B) First-degree relative with known LDL-cholesterol >95th percentile and/or	1
i) First-degree relative with tendon xanthomata and/or arcus cornealis	2
ii) Children <18 years with LDL cholesterol >95th percentile	2
<i>Clinical History</i>	
A) Patient has premature (<55 years men; <60 years women) CHD	2
B) Patient has premature (<55 years men; <60 years women) cerebral or peripheral vascular disease	1
<i>Physical Examination</i>	
A) Tendon xanthomata	6
B) Arcus cornealis in a patient <45 years	4
Biochemical results: LDL-C in mmol/L	
>8.5 (320)	8
6.5–8.4 (250–319)	5
5.0–6.4 (190–249)	3
4.0–4.9 (155–189)	1

A “definite” diagnosis of HeFH can be made if the subject scores >8 points or if DNA analysis documents a mutation in the *LDLR* (or other HeFH) gene. A “probable” diagnosis of HeFH can be if the subject scores between 6 and 8 points.

A “possible” diagnosis of HeFH can be made if the subject scores between 3 and 5 points.

From Reference (52).

chromosome 19p13 and comprises 18 exons (16). As of 2011, >1000 mutations in the *LDLR* gene have been documented worldwide in FH patients, and these mutations have been found in all functional domains of the LDLR protein (49). These are found in the Web sites <http://www.ucl.ac.uk/fh-old/> and [http://www.ucl.ac.uk/ldlr/Current/search.php?select\\_db=LDLR&srch=all](http://www.ucl.ac.uk/ldlr/Current/search.php?select_db=LDLR&srch=all). In addition to single nucleotide mutations, insertion–deletions, copy number variations (50) and splicing mutations (51) have also been reported throughout the *LDLR* gene in HeFH patients.

**96.5.1.3 Diagnosis.** The index of suspicion of HeFH would be raised by (1) elevated plasma total or LDL cholesterol found incidentally; (2) a positive family history for premature onset of symptomatic CHD in a first-degree male relative under age 55 and/or first-degree female relative under age 65 and/or possibly very high total and/or LDL cholesterol; and (3) suggestive physical findings. Patients who develop CVD end points at young ages should be carefully evaluated for HeFH.

The Dutch Lipid Network (DLN) (52) and the SBR (47) have suggested diagnostic criteria for HeFH using various clinical, biochemical and molecular genetic attributes (Tables 96-5 and 96-6). More than 80% of individuals with a DLN score >8 had *LDLR* mutations, a cut point now used to specify individuals with “definite” HeFH (52). The SBR guidelines require documentation

**TABLE 96-6 SBR Criteria for Diagnosis of Heterozygous Familial Hypercholesterolemia**

Criterion	Description
<i>a</i>	TC >7.5 mmol/L (290 mg/dL) in adults or >6.7 mmol/L (260 mg/dL) in children <16 years, OR LDL-C >4.9 mmol/L (190 mg/dL) in adults or >4.0 mmol/L (155 mg/dL) in children <16 years
<i>b</i>	Tendon xanthomas in patients or relatives
<i>c</i>	DNA-based evidence of mutation in <i>LDLR</i> (or other FH genes)
<i>d</i>	Family history of MI before age 50 in grandparent, aunt, uncle or before age 60 in parent, sibling or child
<i>e</i>	Family history of raised TC in parent, sibling or child, or TC documented >7.5 mmol/L (290 mg/dL) in grandparent, aunt or uncle
<i>Diagnosis</i>	
A “definite HeFH” diagnosis requires either criteria <i>a</i> and <i>b</i> or criteria <i>c</i>	
A “probable HeFH” diagnosis requires either criteria <i>a</i> and <i>d</i> or criteria <i>a</i> and <i>e</i>	

of tendon xanthomas, which are very specific for HeFH, but are not clinically apparent in ~30% of HeFH subjects, and often not until the fourth decade of life. There appears to be comparable sensitivity and specificity between the DLN and SBR clinical diagnostic criteria, suggesting that either would be helpful in clinical diagnosis (46b). Biochemical screening of relatives of diagnosed HeFH patients, an approach called “cascade testing” (53), appears to be more effective than other detection strategies, such as population-wide LDL cholesterol testing. The attitudes of HeFH family members toward genetic methods of diagnosis appear to be favorable (54).

**96.5.1.4 Molecular Heterogeneity of Autosomal Dominant Hypercholesterolemia.** Part of the gap in detecting mutations in HeFH is due to genetic heterogeneity (45). For instance, a very similar phenotype called HCHOLAD2 results from a missense mutation in *APOB* affecting the LDLR-binding domain of apo B-100, and accounts for 5–10% of patients with the HeFH phenotype. A rare HeFH subtype called HCHOLAD3 results from GOF mutations in *PCSK9*. A similarly rare FH phenotype with autosomal recessive inheritance is called HCHOLAR1, which results from mutations in *LDLRAP1*. These are discussed later.

**96.5.1.5 Treatment.** Once the diagnosis of HeFH has been made, treatment is relatively straightforward. CVD prevention in HeFH requires a global risk reduction program, focusing on modifiable risk factors, including weight control, prudent diet, moderate exercise, smoking cessation and appropriate control of diabetes and hypertension. The dietary protocol in HeFH minimizes cholesterol intake and replaces saturated fats with unsaturated fats. Consumption of plant sterols and stanols can also reduce plasma LDL-C by ~10%. Since HeFH patients

**TABLE 96-7 NCEP ATP III Revised LDL Cholesterol Goals and Cutoff Points for Intervention in Different Risk Categories**

Risk Category	LDL Cholesterol Goal	Initiate Lifestyle Changes	Consider Drug Therapy
<i>High Risk</i> CHD or equivalent (10 year risk >20%) <sup>a</sup>	<2.59 mmol/L (100 mg/dL) Optimal goal	2.59 mmol/L (100 mg/dL) <1.81 mmol/L (70 mg/dL)	2.59 mmol/L (100 mg/dL)
<i>Moderately High Risk</i> 2+ risk factors (10 year risk 10–19%)	<3.34 mmol/L (130 mg/dL)	3.34 mmol/L (130 mg/dL)	3.34 mmol/L (130 mg/dL)
<i>Moderate risk</i> 2+ risk factors	<3.34 mmol/L (130 mg/dL)	3.34 mmol/L (130 mg/dL)	3.34 mmol/L (130 mg/dL)
<i>Lower Risk</i> 0–1 risk factors (10 year risk 0–9%)	<4.12 mmol/L (160 mg/dL)	4.12 mmol/L (160 mg/dL)	4.9 mmol/L (190 mg/dL) Optional 4.12–4.9 mmol/L (160–189 mg/dL)

<sup>a</sup>Calculated according to the Framingham CHD risk equation.  
Modified from Reference (55).

**TABLE 96-8 Canadian Cardiovascular Society Revised LDL Cholesterol Goals and Cutoff Points for Intervention in Different Risk Categories**

Risk Level	Initiate Treatment If	Primary Target (LDL-Cholesterol)	Alternate Target
High Atherosclerosis Most patients with diabetes FRS ≥20% RRS ≥20%	Consider treatment in all patients	<2 mmol/L OR ≥50%↓ LDL-C	Apo B <0.80 g/L
Moderate	LDL-C >3.5 mmol/L OR LDL-C <3.5 mmol/L AND at least one of: FRS 10–19% TC/HDL-C >5.0 Family history of CVD Metabolic syndrome hs-CRP >2 mg/L in Men >50 years Women >60 years	<2 mmol/L OR ≥50%↓ LDL-C	Apo B <0.80 g/L
Low	LDL-C ≥5.0 mmol/L	≥50%↓ LDL-C	

FRS, Framingham risk score 10-year CVD risk; RRS, Reynolds risk score 10-year CVD risk; LDL-C, low-density lipoprotein cholesterol level; apo B, apolipoprotein B level; TC, total cholesterol level; HDL-C, high-density lipoprotein cholesterol level; hs-CRP, plasma C-reactive protein measured using a high-sensitivity assay. Adapted from Reference (124).

would be considered to be at high risk, treatment targets for LDL-C should follow those advised in national clinical treatment guidelines (Tables 96-7 and 96-8) for patients at high risk (55).

Pharmacotherapy is very frequently required in HeFH patients because the plasma LDL cholesterol targets usually cannot be reached with diet and lifestyle changes alone. Currently available agents for management of dyslipidemia are shown in Table 96-9. Statins—HMG-CoA reductase inhibitors—are the agents of first choice in HeFH. Statins deplete liver cholesterol content, which upregulates LDLR expression and results in increased removal of LDL from plasma. Subjects with HeFH have one normal LDLR allele to upregulate. Plasma LDL

cholesterol reductions of ~50% can be achieved with higher dose statin monotherapy (56). HeFH patients generally require more than one medication to reach guideline LDL cholesterol targets. Ezetimibe, a cholesterol absorption inhibitor that is well tolerated, is now increasingly used in combination with statins in individuals who require large absolute and relative reductions in plasma LDL cholesterol, such as those with HeFH (57). When used in combination with a statin, a further decrease in plasma LDL cholesterol of 18–25% was seen with ezetimibe (57). Other agents such as bile acid sequestrants (BAS) and niacin preparations can also be used as part of combination therapy regimens to lower plasma LDL cholesterol in HeFH (58).



**TABLE 96-9** Classes of Available Drugs for Managing Dyslipidemia

Type of Drug	Mechanism of Action	Major Effects	Example(s)	Adverse Reactions
HMG CoA reductase inhibitors (statins)	Inhibits cholesterol synthesis in hepatic cells, resulting in upregulation of hepatic LDL receptors	Lowers LDL-C and TG	Atorvastatin, lovastatin, pravastatin, simvastatin, fluvastatin, rosuvastatin	Raised hepatic enzymes, raised serum creatine kinase, myopathy possibly progressing to rhabdomyolysis
BA-binding resins	Binds intestinal BAs interrupting enterohepatic recirculation, which in turn results in LDL receptor upregulation	Lowers LDL-C, raises TG	Cholestyramine, colestipol, colesevelam	Limited to gastrointestinal tract: gas, bloating, constipation, cramps
Fibric acid derivatives	Probably inhibits hepatic synthesis of VLDL; stimulates LPL	Mainly lowers TG and raises HDL-C, less effect on LDL-C	Gemfibrozil, fenofibrate, bezafibrate, ciprofibrate	Dyspepsia, constipation, myositis, anemia
Nicotinic acid (extended release)	Upregulates hepatic LDL receptors	Lowers TG and LDL-C	Niacin	Flushing, hepatic toxicity
Cholesterol absorption inhibitors	Inhibits intestinal absorption of cholesterol and plant sterols	Lowers LDL-C	Ezetimibe	Myopathy, gastrointestinal upset
Omega-3 fatty acids (fish oils)	Reduce hepatic secretion of VLDL	Lower TG	Omacor (R), Lovaza (R)	Gastrointestinal discomfort; flulike symptoms

HMG CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LDL, low-density lipoprotein; LDL-C, LDL cholesterol level; VLDL, very-low-density lipoprotein; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol level.

Drug treatment of HeFH is very cost-effective: in primary CHD prevention, statin treatment for 10 years saves both lives and money in HeFH men aged 35–44 with no additional risk factors, and in HeFH women aged 35–44 with at least one additional risk factor (59). Cascade testing to detect new HeFH patients followed by statin treatment prevented 26 myocardial infarctions for every 100 persons aged 20–60 treated for 10 years, gaining a mean of 3.3 years of life for each patient so detected and treated (60). The total lifetime cost for screening and testing, lifetime drug treatment and treatment of CHD events was ~US\$9000 per new case detected and the cost per life-year gained was ~US\$10,000 (60), while the cost per death avoided over 10 years using cascade testing followed by statin treatment was ~US\$7000 (61).

A common clinical concern is CHD prevention when HeFH has been diagnosed in children or adolescents (62). A recent approach to treat children with genetically elevated LDL cholesterol has been proposed (Table 96-10). Dietary and lifestyle advice form the therapeutic cornerstone. Drug treatment of pediatric HeFH is an evolving field because there are few long-term data (63). There are advocates for the idea of initiating treatment at a very young age and maintaining treatment over a patient's lifetime (64). BAS have the advantage of not being systemically absorbed, but they are poorly tolerated. Tolerability is also an issue with short-acting niacin preparations. Ezetimibe has theoretical advantages, but is currently not indicated in children or adolescents. The exact age to initiate treatment and the applicability of adult targets in children are uncertain. Referral for specialist opinion remains a very appropriate option for children and adolescents with HeFH.

### 96.5.2 Familial Defective Apo B (Autosomal Dominant Hypercholesterolemia Type 2)

A minority of patients with a clinical and biochemical diagnosis of HeFH actually have autosomal dominant FH type 2 (ADHCHOL2), also called familial defective apo B (FDB) resulting from one of several possible mutations within the LDLR-binding domain of the apo B-100 protein, of which the most recurrent is R3537Q (formerly R3500Q) (65). These apo B-100 mutants exhibit only a small percentage of normal receptor-binding activity. The APOB R3537Q mutation has a population frequency of ~1/1000 in Europeans (66). The severity of the phenotype due to a heterozygous mutation in APOB is often less severe than with a heterozygous mutation in the *LDLR*: heterozygous FDB individuals exhibit LDL cholesterol levels of ~6 mmol/L (~240 mg/dL) (67). Homozygous FDB presents with biochemical, clinical and CAD risk profiles similar to HeFH, while heterozygous FDB is generally less severe than HeFH in all of these respects; for instance, tendinous xanthomas are uncommon in FDB (68). Treatments used in HeFH are also usually effective in FDB (68,69). In contrast to HoFH, homozygous FDB patients respond to statin therapy (67).

### 96.5.3 PCSK9 Gain-of-Function Mutations (Autosomal Dominant Hypercholesterolemia Type 3)

A third molecular form of autosomal dominant hypercholesterolemia (ADHCHOL3) was shown by linkage

**TABLE 96-10 Recommendations for Drug Treatment of High-Risk Children with Dyslipidemia**

1. Consider drug therapy in children 10 years of age (usually wait until menarche for females) and after a 6- to 12-month trial of fat- and cholesterol-restricted dietary management
2. Consider drug therapy if LDL cholesterol level remains >4.90 mmol/L (190 mg/dL) or >4.10 mmol/L (160 mg/dL) and there is a positive family history of premature CVD, or if two other risk factors are present in the child or adolescent after vigorous efforts to modify these risk factors
3. Referral to specialized lipid center may be deemed appropriate
4. Treatment goals:      Minimal LDL cholesterol <3.35 mmol/L (130 mg/dL) Ideal LDL cholesterol <2.85 mmol/L (110 mg/dL)
Other considerations
1. In addition to family history, overweight and obesity should trigger screening with a fasting lipid profile
2. Overweight and obese children with lipid abnormalities should be screened for other aspects of the metabolic syndrome (i.e. insulin resistance and type 2 diabetes, hypertension, or central adiposity).
3. A statin is recommended as first-line treatment when drug treatment is deemed appropriate.
4. For children with high-risk lipid abnormalities, the presence of additional high-risk conditions may also result in adjustment of the LDL cholesterol level for initiation of drug therapy, the target LDL cholesterol level, and may prompt consideration for initiation below the age of 10 years
5. High-risk conditions may include
Male gender
Strong family history of premature CVD or events
Presence of associated low HDL, high triglycerides, small dense LDL
Presence of overweight or obesity and aspects of the metabolic syndrome
Presence of medical conditions such as diabetes, HIV infection, systemic lupus erythematosus, organ transplantation
Presence of hypertension
Current smoking and passive smoke exposure
Presence of novel and emerging risk factors and markers, e.g. elevated Lp(a), C-reactive protein

Adapted from Reference (62).

mapping to be due to heterozygous GOF mutations in *PCSK9*. This disorder displays many features of HeFH (70), although such patients comprise <1% of patients with a clinical diagnosis of HeFH referred to most lipid clinics. They can generally be managed in the same manner as patients with heterozygous *LDLR* mutations (49,71).

## 96.5.4 Homozygous Familial Hypercholesterolemia (HoFH)

**96.5.4.1 Clinical Features.** The classic clinical manifestations of HoFH include early appearance of corneal arcus, cutaneous planar xanthomata over hands and extremities, tuberous xanthomata over elbows, interphalangeal joints, knees, and tendinous xanthomata in the hand extensor and Achilles tendons, although xanthelasmata appear to be more common in HeFH patients (16,72). In HoFH, these signs may become manifest in the first or second decade of life, compared to the third to fifth decade in HeFH patients. HoFH children are predisposed to early atherosclerosis, including arterial plaque formation and coronary ostial stenosis leading to cardiac ischemia (72). With prolonged plasma exchange or LDL apheresis, vascular calcification, especially of the aortic root, has emerged as a significant clinical issue (73). Aortic valvular thickening and aortic root disease can lead to aortic regurgitation (74) or stenosis (72) requiring valve replacement. Death from myocardial infarction occurs in untreated subjects before age 30 (16,47), although disease progression in individual patients is variable (75).

**96.5.4.2 Molecular Genetics.** Children with HoFH inherit two defective copies of the *LDLR* gene and thus lack functional LDLRs, resulting in plasma LDL concentrations elevated on average sixfold above the normal range (76). Parents of HoFH children have HeFH, with one defective and one wild-type *LDLR* allele each. HoFH has a prevalence of 1 in 1,000,000 in most populations, with higher frequencies in founder populations (16).

**96.5.4.3 Diagnosis.** Diagnosis of HoFH is based on a combination of physical and biochemical findings, family history and molecular genetic analysis (77). Clinicians should suspect HoFH in children presenting with these physical signs and a positive family history of HeFH in one or both parents. Conversely, isolated pediatric HoFH may indicate undiagnosed parental HeFH (78). Serum lipid profiling for both parents and their children is indicated. HoFH children have plasma total cholesterol levels >10 mmol/L (>390 mg/dL) and often >18 mmol/L (>700 mg/dL); HoFH should thus be suspected in children when plasma cholesterol levels exceed these thresholds (79). For HeFH in patients <16 years old, the SBR criteria (47) specify a total plasma cholesterol >6.7 mmol/L (>260 mg/dL) or plasma LDL cholesterol >4.0 mmol/L (>155 mg/dL) for a positive diagnosis. In contrast, specific diagnostic guidelines for HoFH have not been validated. Definitive diagnosis requires *LDLR* gene sequencing showing mutations within both alleles or *LDLR* functional assays in cultured fibroblasts showing impaired receptor function (16).

**TABLE 96-11 Emerging Treatments for Dyslipidemia**

Name	Company	Mechanism of Action	Indication	Stage	Biochemical Effect
Lomitapide	Aegerion	MTP inhibitor	Hypercholesterolemia	Phase II	Decreases TGs and VLDL
Mipomersen	ISIS/Genzyme	mRNA inhibitor of APOB	HeFH and HoFH	Phase II	Lowers LDL
Dalcetrapib	Roche	CETP inhibitor	Hyperlipidemia	Phase III	Raises HDL
Anacetrapib	Merck	CETP inhibitor	Hyperlipidemia	Phase III	Raises HDL
Niacin/laripoprant	Merck	Prostaglandin D2 inhibitor	Hyperlipidemia	Phase III	Raises HDL cholesterol, decreases TG
Eprotirome	Karo Bio	Thyromimetic	Dyslipidemia	Phase II	Decreases VLDL, LDL, Lp(a)

MTP, microsomal triglyceride transfer protein; APOB, apolipoprotein B gene; CETP, cholesterol ester transfer protein; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HeFH, heterozygous familial hypercholesterolemia; HoFH, homozygous familial hypercholesterolemia.

**96.5.4.4 Treatment.** Patients with HoFH receive dietary counseling to reduce intake of exogenous cholesterol and saturated fats (80). The current treatment of choice for HoFH is plasmapheresis or LDL-apheresis, which has proved to be highly effective in prolonging end-point free survival (81). LDL-apheresis has the advantages of reduced exposure to blood products, no significant changes in HDL cholesterol levels, and less dramatic volume shifts compared to straight plasma exchange (82). Concurrent high-dose statin therapy may enhance LDL clearance by upregulating the expression of partially functional LDLR, but patients whose mutations leave them with nonfunctional LDLR show little plasma LDL cholesterol response (83). Ezetimibe seems to have some modest efficacy in plasma LDL cholesterol reduction in HoFH (84). Liver transplantation, sometimes together with heart and lung transplantations, and gene therapy have been attempted, with variable results (16,85). Emerging investigational treatments (Table 96-11) for HoFH include biological treatments such as parenteral antisense RNA directed against APOB and the MTP inhibitor class of medications (86).

## 96.5.5 Autosomal Recessive Hypercholesterolemia

**96.5.5.1 Clinical Features.** Autosomal recessive hypercholesterolemia (ARH) has a clinical presentation closely resembling HoFH, with some key differences. For instance, while most physical signs are quite similar, aortic valve stenosis is less common and aortic root disease shows slower progression (87). Age of diagnosis can vary between the first and fifth decades of life, meaning ARH is a potential diagnosis in both pediatric and adult populations. In reported patients, total plasma cholesterol levels have varied between 9.6 and 27.1 mmol/L (370 and 1050 mg/dL), while LDL cholesterol levels have varied between 8.6 and 22.9 mmol/L (330 and 890 mg/dL) (88). Despite the large variability, these parameters are

sufficiently abnormal in ARH patients to satisfy the SBR criteria for HeFH diagnosis; in the context of a severe pediatric clinical presentation ARH closely resembles HoFH (47).

**96.5.5.2 Molecular Genetics.** ARH was mapped by linkage analysis in a family with severe hypercholesterolemia but no mutations in *LDLR* or *APOB*. LDLRAP1 facilitates LDL uptake by hepatocytes by promoting clathrin-pit-mediated LDLR clustering and internalization (89).

**96.5.5.3 Diagnosis.** ARH may be distinguished from severe HeFH or HoFH through sequencing of the *ARH* and *LDLR* genes or through Western blotting of ARH protein in biopsy samples (90).

**96.5.5.4 Treatment.** Treatment of ARH differs substantially from HoFH in that statins reduce plasma LDL cholesterol by 30–60% (91). ARH fibroblasts might employ a different mechanism of LDLR internalization independent of LDLRAP1 protein (89a,92) so that statin-induced LDLR upregulation enhances LDL clearance by these cells. In addition to diet and lifestyle modification, combination therapy including statins, ezetimibe and BAS is typically indicated in these patients (93), without necessarily requiring plasmapheresis or LDL-apheresis.

## 96.6 DISORDERS WITH PRIMARILY DEPRESSED LDL CHOLESTEROL

Abnormally low plasma levels of LDL particles (typically far below 0.5 mmol/L or 20 mg/dL) may be caused by several monogenic disorders. The two best understood are abetalipoproteinemia (ABL) and familial hypobetalipoproteinemia (FHBL), which result from mutations in the *MTP* and *APOB* genes, respectively (69). A rare form of milder FHBL can also result from heterozygous loss-of-function (LOF) mutations in the *PCSK9* gene. Very low plasma TG is also found in patients with chylomicron retention disease (CRD), in addition to low LDL cholesterol, and in some patients with ABL and with FHBL.

### 96.6.1 Abetalipoproteinemia (ABL)

**96.6.1.1 Clinical Features.** At birth, infants with ABL are asymptomatic, but with a high fat diet they can develop gastrointestinal symptoms resembling celiac disease with diarrhea, vomiting and abdominal distension. The gastrointestinal manifestations may later subside, in part because some patients learn to avoid fatty foods (94). Fat-soluble vitamin deficiency develops because plasma transport and delivery depends almost exclusively (for vitamin E and beta-carotene) or in part (for vitamins A, D, and K) on intact synthesis and secretion of apo B-containing lipoproteins. Most reported ABL patients >20 years of age who had not received vitamin supplementation developed neurological and retinal complications. However, there is heterogeneity in disease presentation across patients, which may correlate with the nature and severity of the molecular defect (94).

The initial neurological signs include diminution and loss of deep tendon reflexes followed by progressive sensory loss of proprioception and vibration, development of a spinocerebellar syndrome and muscular weakness. In addition, slowed intellectual development is seen in up to one-third of patients. Neuropathology reveals axonal degeneration of the spinocerebellar tracts and demyelination of the fasciculus cuneatus and gracilis tracts (94). The myopathy results from both neural degeneration and an intrinsic myositis. Although the clinical course is variable without treatment, it leads progressively to impaired mobility, and some patients become wheelchair-bound or bedridden. ABL patients also complain of decreased night and color vision at an early age, followed by a decrease in visual acuity. The visual field shows a concentric contraction. Fundoscopic examination shows pigmentation of the retina. If left untreated, blindness can be present by the fourth decade.

ABL subjects can have hepatosteatosis associated with elevated serum transaminases and sometimes hepatomegaly. Only rarely does this progress to cirrhosis. Acanthocytosis on the peripheral blood film is pathognomonic of ABL. Patients may also have a moderate to severe hemolytic anemia. Abnormalities in coagulation, elevated prothrombin time, caused by deficiency in vitamin K-dependent coagulation factors may lead to bruising or hemorrhage (94). Despite the cholesterol deficiency, steroidogenesis, adrenal function and sexual development are usually normal.

**96.6.1.2 Molecular Genetics.** ABL affects <1 in 100,000 persons. ABL shows autosomal recessive inheritance as obligate heterozygotes have normal plasma lipid levels (94). Missense and truncation mutations in *MTP* (95) that result in nonfunctional protein products cause a near complete lack of circulating apo B-containing lipoproteins in the plasma of homozygotes or compound heterozygotes (96).

**96.6.1.3 Diagnosis.** Most clinical features seen in ABL together with (1) acanthocytosis, (2) the virtual absence

of LDL and apo B containing lipoproteins, and (3) normal LDL cholesterol levels in parents would suggest a diagnosis of ABL. DNA sequence analysis of the *MTP* gene can confirm the diagnosis.

**96.6.1.4 Treatment.** Early diagnosis and treatment are essential to prevent growth retardation and neuro-ophthalmological complications secondary to chronic lipid malabsorption and deficiencies of fat-soluble vitamins. To provide an adequate amount of total calories, the proportion of protein and carbohydrate in the diet must be increased. The lipid-poor diet should also provide the minimal daily requirements of essential FA, often in the form of vegetable oils. Oral medium-chain TGs have been used to provide dietary FA for absorption through the portal circulation, bypassing the MTP defect.

The other mainstay of treatment in ABL is fat-soluble vitamin replacement (94). Vitamin A deficiency is easily compensated for by oral supplementation because after intestinal absorption and transport to the liver, vitamin A has its own lipoprotein-independent transport system, unlike vitamin E. Daily doses two- to fourfold of the normal recommended doses are generally required to normalize the levels of vitamin A or beta-carotene.

Vitamin D deficiency is not classically described in ABL because the metabolism of vitamin D does not depend much on apo B-containing lipoproteins, since there is partial absorption via the portal vein system and specific vitamin D transport proteins. However, the development of rickets and osteomalacia has been reported and therefore prophylactic supplements should be instituted early in life.

ABL subjects require lifetime therapy with vitamin E in large oral doses of 100–300 U/kg/day, which can be absorbed through the portal system; parenteral administration is usually not required. Plasma levels of vitamin E rarely exceed 10–30% of normal even after long-term therapy. Nevertheless, the levels in adipose tissue, hepatic tissue, and erythrocytes almost always increase and sometimes even normalize. Early administration of vitamin E arrests progression of the neuropathy and myopathy (94).

Vitamin K administration either orally or parenterally rapidly corrects defective coagulation.

### 96.6.2 Familial Hypobetalipoproteinemia (FHBL)

**96.6.2.1 Clinical Features.** Homozygous FHBL shares most of the clinical attributes of ABL, with the main distinguishing feature of half-normal plasma concentrations of plasma apo B-containing lipoproteins in heterozygote parents, compared to normal levels in parents of ABL subjects (97). As in ABL, homozygotes may be detected at a young age because of fat malabsorption and reduced plasma total and LDL cholesterol levels.

Heterozygous FHBL patients may be asymptomatic or may experience some of the same symptoms as



homozygous FHBL patients, although these symptoms usually appear during adulthood (96,98). Obligate FHBL heterozygotes have LDL cholesterol concentrations in the lowest tenth percentile, but are typically healthy and usually have little difficulty absorbing fat.

**96.6.2.2 Molecular Genetics.** Homozygous FHBL occurs in <1 in 100,000 persons. FHBL segregates as an autosomal codominant trait. In FHBL, ~60 mutations have been documented in the *APOB* gene (98). These are both truncation mutations and missense mutations that occur outside the LDLR-binding domain of the apo B-100 protein. There is no clear relationship between the type and position of the *APOB* mutation and the severity of the homozygous FHBL phenotype.

**96.6.2.3 Diagnosis.** The clinical features of ABL combined with half-normal LDL cholesterol levels in parents would suggest a diagnosis of homozygous FHBL (97). Sequence analysis of the *APOB* gene can confirm the diagnosis.

**96.6.2.4 Treatment.** The treatment of homozygous FHBL is similar to that of ABL. No specific treatment is indicated for heterozygotes, but dietary supplementation with fat-soluble vitamins, especially vitamin E, is reasonable.

### 96.6.3 PCSK9 Loss-of-Function Mutations

**96.6.3.1 Clinical Features.** While screening individuals with very low LDL cholesterol as a contrast group for sequencing the *PCSK9* gene to detect GOF mutations in individuals with very high LDL cholesterol (70), novel heterozygous mutations were found in the low LDL contrast group (99). These were shown to be LOF mutations in *PCSK9*, indicating that, like mutations in *APOB*, different types of mutations in *PCSK9* produce opposite extremes of the LDL cholesterol phenotype. While heterozygotes for *PCSK9* LOF mutations only had low total and LDL cholesterol and apo B, they had no other specific physical findings. Individuals of several ancestries who had heterozygous LOF mutations in *PCSK9* had significant lifelong protection against atherosclerosis: heterozygous nonsense mutations and nonsynonymous sequence variations in *PCSK9* appear to reduce CVD risk by up to 88% (100). Rare individuals with two LOF *PCSK9* alleles had extremely low but detectable plasma apo B and LDL cholesterol (101), which contrasts with the undetectable levels of these measurements typical of ABL and homozygous FHBL.

**96.6.3.2 Molecular Genetics.** The *PCSK9* gene encodes proprotein convertase subtilisin/kexin 9, a serine protease that enhances LDLR degradation following both biosynthesis and LDL-mediated internalization; *PCSK9* function negatively correlates with LDLR expression and positively correlates with plasma LDL concentration (102). There is no obvious relationship between the type and position of the

mutation in *PCSK9* and the severity of the clinical phenotype.

**96.6.3.3 Treatment.** No specific treatment is considered necessary for patients with LOF mutations in *PCSK9*.

### 96.6.4 Fat Malabsorption Disorders

**96.6.4.1 Clinical Features.** Patients with CRD, also known as Anderson disease, resemble those with ABL with respect to dietary fat malabsorption and its consequences. These patients are able to synthesize the full-length apo B-100 in the liver, but cannot synthesize intestinal apo B-48. The intestinal transcription of apoB-100 mRNA and its editing to apoB-48 mRNA proceed normally. Both apo B and dietary TG are found in enterocytes. Nevertheless, CMs are not secreted (103). Affected children present with steatorrhea and diarrhea within the first few days of life, and failure to thrive becomes apparent after a few months (104). Plasma LDL cholesterol and the BA pool are markedly decreased, while fecal BA content and total stool output are markedly increased (104a,105).

**96.6.4.2 Molecular Genetics.** The causative gene for CRD is *SAR1B*, encoding a member of the small GTPase family called COPII, which governs the intracellular trafficking of proteins in coat protein-coated vesicles. Rare coding sequence mutations were seen in all affected patients (106), indicating that *SAR1B* is the causative gene for a spectrum of related disorders. Mechanistically, it would appear that the apo B-48 isoform interacts with the gene product to engage the COPII transport machinery as part of the mechanisms of CM secretion.

**96.6.4.3 Treatment.** The general treatment regimen follows that recommended for ABL and HHBL (homozygous familial hypobetalipoproteinemia), including fat restriction and fat-soluble vitamin supplements.

### 96.6.5 Primary Bile Acid Malabsorption

The *SLC10A2* gene (MIM 601295) encodes the apical sodium-dependent bile acid transporter, which has an important role in BA reabsorption in the distal ileum (107). Certain splicing and missense mutations in *SLC10A2* have reportedly caused primary BA malabsorption in children who are homozygotes or compound heterozygotes for these rare mutant *SLC10A2* alleles (104a). Patients have low plasma LDL cholesterol, although data on long-term CVD risk are lacking. Steatorrhea and diarrhea improve dramatically through dietary therapy that is low in TG and high in medium-chain FA (104a).

## 96.7 DISORDERS WITH PRIMARILY ELEVATED HDL CHOLESTEROL

A few individuals with HDL cholesterol >95th percentile, also termed “hyperalphalipoproteinemia,” have mutations causing deficiencies of CETP or SR-B1 (molecular

alias *SCARB1*) (108). In addition, some LOF mutations in *LIPG* encoding endothelial lipase (EL) are associated with elevated HDL cholesterol levels.

### 96.7.1 Cholesteryl Ester Transfer Protein (CETP) Deficiency

**96.7.1.1 Clinical Features.** CETP deficiency leads to the accumulation in plasma of CE-rich large HDL, with resulting elevations in plasma concentrations of HDL cholesterol and apo A-I and reductions in plasma LDL cholesterol and apo B (17). There are no specific physical manifestations associated with CETP deficiency. The impact of genetic CETP deficiency on CVD risk is controversial, especially after a clinical trial with torcetrapib, a CETP inhibitor, caused an unexpected increase in cardiac deaths (109). CETP deficiency might be a state of impaired RCT, which may lead to atherosclerosis despite high plasma HDL cholesterol levels (110).

**96.7.1.2 Molecular Genetics.** Genetic deficiency of CETP is caused by mutations in the *CETP* gene (111). Genetic CETP deficiency is relatively common in Japanese subjects with elevated plasma HDL cholesterol: several CETP mutations have been identified in the Japanese population and these appear to be associated with protection from atherosclerosis (17). However, in Caucasian populations, elevated HDL cholesterol due to mutant *CETP* seems to be rare and inconsistently associated with CVD risk (112).

**96.7.1.3 Treatment.** No specific treatment is indicated in CETP deficiency. Global CVD risk should be assessed and preventive treatments used, especially in the presence of multiple risk factors other than low HDL cholesterol.

### 96.7.2 Scavenger Receptor B1 (SR-B1) Deficiency

**96.7.2.1 Clinical Features.** DNA sequencing of individuals with very high plasma HDL cholesterol identified a family with a missense mutation in *SCARB1*, altering the amino acid sequence: P297S (113). Heterozygotes had mean HDL cholesterol that was increased by ~50% over noncarriers. The lipid profile was otherwise normal, as was CVD risk. Platelets from carriers had increased unesterified cholesterol content and impaired function. In addition, adrenal steroidogenesis was attenuated, with decreased urinary excretion of sterol metabolites, decreased response to corticotropin stimulation, and symptoms of diminished adrenal function (113).

**96.7.2.2 Molecular Genetics.** High HDL cholesterol segregated as an autosomal dominant trait in *SCARB1* mutation carriers; the mutant had diminished function in vitro (113). Carriers also had a reduced capacity to efflux cholesterol in vivo.

**96.7.2.3 Diagnosis.** The constellation of increased HDL cholesterol together with abnormalities of platelet

function and adrenal steroidogenesis might indicate an individual whose *SCARB1* gene could be sequenced.

**96.7.2.4 Treatment.** No specific treatment for the lipoprotein disturbance is indicated. Reduction in CVD risk—management of blood pressure, diabetes, and obesity, with smoking cessation—should be strongly considered.

### 96.7.3 Loss-of-Function Variants in *LIPG* Encoding Endothelial Lipase (EL)

*LIPG* LOF mutations were found in asymptomatic individuals with elevated plasma concentrations of HDL cholesterol (114). There are no obvious clinical symptoms or physical findings associated with the biochemical findings; in particular, the association with atherosclerosis or CVD is unclear. Among individuals with elevated HDL cholesterol, in vitro lipase activity assays demonstrated that most were LOF variants with decreased EL activity (114). The *LIPG* N396S LOF variant, which has a population frequency between 1 and 5% and significantly decreased lipase activity, was associated with increased HDL cholesterol. Diagnosis of EL deficiency is based primarily on DNA sequence analysis. No specific treatment for the lipoprotein disturbance is indicated.

## 96.8 DISORDERS WITH PRIMARILY DEPRESSED HDL CHOLESTEROL

Several genetic disorders lead to reduced plasma HDL cholesterol, including mutations in *ABCA1*, *LCAT* and *APOA1*. In addition, glucocerebrosidase mutations in Gaucher disease, type 1 (GD1; MIM 230800), are associated with low HDL (115). Finally, a subtype of Niemann–Pick disease is associated with very low HDL (116), reinforcing the role of Niemann–Pick type C protein (NPC1; MIM 257220) in intracellular cholesterol transport. Criteria for the definition of familial hypoalphalipoproteinemias are (1) a low HDL cholesterol level in the presence of normal VLDL cholesterol and LDL cholesterol levels, (2) an absence of conditions in which low HDL cholesterol may be secondary, and (3) the presence of a similar lipoprotein pattern in a first-degree relative.

### 96.8.1 Tangier Disease

**96.8.1.1 Clinical Features.** Tangier disease (TD) is characterized by the presence of orange tonsils, splenomegaly, discoloration of the rectal mucosa, hepatomegaly, corneal opacities, premature CHD, and peripheral neuropathy that may be either transient and recurring or progressive and debilitating. Biochemically, TD is characterized by a near absence of HDL particles (<0.05 mmol/L or <2 mg/dL) (117) and very low plasma apo A-I levels. HDL catabolism is accelerated and in vitro efflux of cholesterol and PL from fibroblasts is impaired (118). CHD risk appears to be increased in

TD homozygotes and heterozygotes, perhaps because of concurrent presence of low LDL cholesterol levels on occasion (119). Some TD patients also have mild HTG.

**96.8.1.2 Molecular Genetics.** TD is caused by homozygous mutations of the *ABCA1* gene (120), whose product plays a focal role in cholesterol efflux from the cell interior onto nascent HDL particles via apo A-I (121). CE becomes deposited in the reticuloendothelial system. TD mutations probably impair the ability of cell surface *ABCA1* transporters to transfer cholesterol to HDL and the lipid-poor HDL is more prone to catabolism (122).

**96.8.1.3 Treatment.** TD has no specific treatment; currently available HDL-raising drugs are minimally effective (123). Thus a reasonable treatment strategy later in life is to improve the plasma LDL:HDL ratio using LDL-lowering pharmacotherapy in combination with CVD risk factor reduction and prevention through lifestyle and diet modification (124). Treatment is guided by the presence of secondary conditions and complications.

## 96.8.2 Lecithin:Cholesterol Acyl Transferase (LCAT) Deficiency States

**96.8.2.1 Clinical Features.** Two kinds of genetic LCAT deficiencies have been reported. The first is complete LCAT deficiency, which is characterized by anemia, increased proteinuria and renal failure (125). The second is partial LCAT deficiency, also called fish-eye disease (FED) (126). Both disorders are characterized by progressive corneal opacification, very low plasma HDL cholesterol (usually  $<0.25$  mmol/L or  $<10$  mg/dL), and variable HTG. Complete LCAT deficiency is also characterized by abnormal lipid deposition in tissues, normochromocytic anemia, renal disease and variable presentation of early CVD (127).

**96.8.2.2 Molecular Genetics.** Approximately 30 kindreds and numerous LCAT mutations have been reported worldwide: these comprise ~60 patients with complete LCAT deficiency and ~20 patients with FED (128). Deficiency of LCAT prevents the normal esterification of cholesterol that is necessary in the life cycle of the developing HDL particle. There is no consistent relationship between the nature of the LCAT mutation or its position within the coding sequence and the clinical and biochemical subphenotypes of either familial LCAT deficiency or FED. LCAT deficiency is inconsistently associated with an increased risk of CHD (128).

**96.8.2.3 Diagnosis.** In the presence of extremely low plasma HDL cholesterol, additional signs such as corneal clouding, tendon or skin-fold xanthomata and, in some cases, premature CVD during adulthood should trigger consideration of possible LCAT deficiency (129). The diagnosis of LCAT deficiency could be made based on the quantification of plasma cholesterol esterification activity, performed in specialized laboratories; however, the LCAT gene can also be sequenced.

**96.8.2.4 Treatment.** LCAT deficiency has no specific treatment; currently available drugs are minimally

effective at raising HDL cholesterol. A reasonable treatment strategy is to improve the plasma LDL:HDL ratio using LDL-lowering pharmacotherapy in combination with CVD risk factor reduction and prevention through lifestyle and diet and management of complications, such as renal transplantation for end-stage renal disease (129).

## 96.8.3 Apolipoprotein A-I Deficiency

**96.8.3.1 Clinical Features.** Homozygosity for some *APOA1* mutations affects the assembly of HDL, and has been found in patients with very low HDL cholesterol, xanthomas and early CHD (128). Heterozygotes for mutant apo A-I often have less severe reductions in HDL cholesterol ( $<0.25$  mmol/L or  $<10$  mg/dL). Other than corneal opacities and occasional xanthomas, most patients exhibit no clinical sequelae. Certain *APOA1* missense mutations, e.g. apo A-I Iowa (G26R) cause systemic amyloidosis; mutant protein is found within amyloid plaques, but these mutations have no impact on plasma HDL cholesterol levels or CVD risk (130).

**96.8.3.2 Molecular Genetics.** Approximately 30 mutations involving the *APOA1* gene have been reported (128), including (1) large deletions that disrupt *APOA1* plus neighboring genes in the *APOA1/C3/A4/A5* gene cluster on chromosome 11, (2) nonsense or splicing mutations or deletions that affect *APOA1* specifically, and (3) missense mutations affecting the coding sequence of *APOA1*. In general, plasma HDL cholesterol is almost undetectable in persons with deletions or nonsense mutations of the *APOA1* gene. In contrast, HDL cholesterol levels are detectable (usually 0.3–0.6 mmol/L or 10–20 mg/dL) with some missense mutations in *APOA1*. For instance, patients with *APOA1* Milano, inherited as an autosomal dominant trait, have low but detectable HDL levels, but do not have an increased risk of premature CVD (131).

**96.8.3.3 Diagnosis.** The molecular diagnosis can be made by DNA sequence analysis or by plasma apolipoprotein electrophoresis showing absence of the apo A-I band.

**96.8.3.4 Treatment.** Apo A-I deficiency has no specific treatment; currently available drugs are minimally effective at raising HDL. A reasonable treatment strategy is to improve the plasma LDL:HDL ratio using LDL-lowering pharmacotherapy in combination with CVD risk factor reduction and prevention through lifestyle and diet.

## 96.9 DISORDERS WITH PRIMARILY ELEVATED TRIGLYCERIDES

### 96.9.1 Hyperchylomicronemia

**96.9.1.1 Clinical Features.** Hyperchylomicronemia seen in pediatric patients is predominantly due to one of two monogenic disorders affecting the peripheral metabolism of TG-rich, intestinally derived CM particles, namely, LPL deficiency and apo C-II deficiency.



Recurrent pancreatitis is relatively common and is thought to result from episodic pancreatic ischemia secondary to hyperchylomicronemia (132). Patients usually complain of mild to severe abdominal pain with nausea and vomiting. Other physical signs during childhood include failure to thrive, hepatosplenomegaly, lipemia retinalis and eruptive xanthomata over extensor surfaces and buttocks (46a). Presentations during infancy can be heterogeneous and may include other signs such as intestinal bleeding, pallor, anemia, irritability, diarrhea, seizures and encephalopathy (133). Patients typically have extremely elevated fasting TG >10 mmol/L (134) (>900 mg/dL), although pancreatitis usually occurs when TG >20 mmol/L (>1800 mg/dL) (135). LDL and HDL levels are usually subnormal (136). Plasma is milky and turbid, or lipemic, because of the high TG content (134).

**96.9.1.2 Molecular Genetics.** Some patients with plasma TG >95th percentile have rare monogenic disorders resulting from homozygous LOF mutations in *LPL*, *APOC2*, *APOA5*, *LMF1* or *GPIHBP1* genes, respectively (136,137). Complete LPL deficiency is an extremely rare (~1:10<sup>6</sup>) AR disease resulting from homozygosity or compound heterozygosity for mutant LPL (138). More than 100 different dysfunctional *LPL* variants have been reported and almost all reduce or eliminate LPL activity in the homozygous state, preventing hydrolysis and resulting in accumulation of TG-rich lipoproteins (TRLs), primarily CMs (139).

Less frequently, homozygous mutations have been shown in other molecules that interact with LPL, resulting in a comparable clinical phenotype (138). For instance, ~10 homozygous mutations in *APOC2*, which encodes apo C-II, a key cofactor for LPL, have been found in patients with familial chylomicronemia (138).

The physiological role of apo A-V was solidified by work in animal models (140). HTG is seen in some probands with homozygosity for rare LOF mutations in *APOA5* (141), although the phenotype is less severe clinically and biochemically than in patients with homozygous *LPL* or *APOC2* mutations. Apo A-V appears to play a focal role in hydrolysis of TRLs by enhancing LPL activity (142).

Another form of familial lipase deficiency and chylomicronemia results from homozygous mutations in *LMF1*, the human ortholog of the *cld* (combined lipase deficiency) gene in mice (137a). A few probands with chylomicronemia and pancreatitis have homozygous LOF mutations in *LMF1* (27). Studies in mice indicate that *Lmf1* is coexpressed in tissues that express *LpL* or *LipC*; its gene product localizes to the membrane of the endoplasmic reticulum, stimulating maturation of both lipases (27). Thus, human mutations in *LMF1* likely cripple transport of the target lipases.

In addition, some patients with hyperchylomicronemia have homozygous mutations in *GPIHBP1* (143), which encodes glycosylphosphatidylinositol-anchored HDL-binding protein 1. The mouse ortholog *Gpibp1*,

when deleted, also caused severe HTG (28). Recently, *GPIHBP1* was shown to be responsible for entry of LPL into the capillary lumen (144).

Finally, other rare monogenic conditions that do not primarily affect lipoprotein metabolism can result in severe HTG. These include the inherited syndromes of lipodystrophy (145), which have variable patterns of inheritance and a wide range of clinical features, such as variation in the distribution and loss of adipose tissue. Patients with severe inherited lipodystrophy can present with chylomicronemia and pancreatitis (145).

**96.9.1.3 Diagnosis.** Early diagnosis is important to prevent chronic pancreatitis and pancreatic necrosis (146), although often pancreatic function deteriorates very slowly (147). CVD risk may also be increased in these patients (132,148). An infant or child presenting with hyperchylomicronemia syndrome was tested for LPL deficiency by performing an LPL assay using postheparin plasma. Apo C-II deficiency can be diagnosed if reduced LPL activity from postheparin plasma normalized upon addition of exogenous apo C-II (149). However, presently, direct gene sequencing has become the more expedient diagnostic method for both disorders, and also for detecting even rarer homozygous mutations in *APOA5*, *LMF1* or *GPIHBP1*.

**96.9.1.4 Treatment.** Unfortunately, hyperchylomicronemia resulting from deficiency in LPL or apo C-II is challenging to treat with existing pharmacologic approaches. Although much rarer than even LPL or apo C-II deficiency, treating deficiencies in apo A-V, *LMF1* and *GPIHBP1* appears similarly challenging. Fibrates, niacin and fish oil are not particularly effective in improving the biochemical disturbances in these patients (132). The cornerstone of treatment is severe dietary fat restriction, although recommended targets vary from less than 50g/day, or <25% of the total daily caloric intake (132) to less than 20g/day, or under 15% (150). However, such a dietary regime is often very difficult for most patients to follow. Experimental adenoviral-mediated LPL gene therapy is under evaluation for documented LPL deficiency (151).

## 96.10 DISORDERS WITH MULTIPLE LIPOPROTEIN DISTURBANCES

### 96.10.1 Familial Combined Hyperlipidemia

**96.10.1.1 Clinical Features.** The familial combined hyperlipidemia (FCHL) (HLP type 2B) biochemical phenotype is seen in up to 1 in 40 adults and is characterized by elevated total and LDL cholesterol and TG (all >90th percentile for age and sex) in probands and affected relatives, and may be the most common genetical dyslipidemia causing CHD (152). Although defined clinically based on biochemical phenotypes, affected individuals in a few families appear to demonstrate



physical findings that are typical for other dyslipidemias, such as skin or tendinous xanthomas. CHD risk in FCHL is related to the degree of TG elevation (40).

**96.10.1.2 Molecular Genetics.** Of the linkage and association studies performed in FCHL families over the 1980s and 1990s, the most promising gene identified as contributing to susceptibility is *USF1*, an upstream transcription factor with multiple targets in metabolic pathways (153). Recent association studies seem to indicate that FCHL is not a monogenic disorder, but rather a complex trait that results from the accumulation of numerous small-effect common alleles together with a few large-effect rare mutations that are individually associated with elevated TG or LDL cholesterol levels and an important contribution from secondary environmental or nongenetic metabolic factors (154).

## 96.10.2 Hepatic Lipase (HL) Deficiency

**96.10.2.1 Clinical Features.** In HL deficiency, compound heterozygosity for mutant *LIPC* resulted in elevated plasma HDL and apo A-I, and also in increased concentrations of IDL, TG enrichment of LDL and HDL, abnormal catabolism of remnant lipoproteins, and failure to remodel HDL and early CHD (155). This is consistent with the concept that HL contributes to the degradation of TGs in CE-rich particles such as LDL and HDL.

**96.10.2.2 Molecular Genetics.** The deficiency of HL is very rare, with <5 reported kindreds who had affected individuals with homozygous mutations in the *LIPC* gene encoding HL (156).

**96.10.2.3 Diagnosis.** A molecular diagnosis can be made using DNA sequencing. A biochemical diagnosis of HL deficiency can be derived from total lipolytic activity measured in plasma after intravenous injection of heparin.

**96.10.2.4 Treatment.** There is no specific treatment for the elevated levels of compositionally abnormal HDL in HL deficiency. When CVD risk is increased, the abnormal lipoprotein profile can be managed with a diet reduced in total calories and fat content, and a statin plus or minus a fibrate to correct the predominant abnormality of non-HDL lipoprotein fractions. Reduction in CVD risk in general terms—management of blood pressure, diabetes, and obesity, with smoking cessation—should be evaluated on a case-by-case basis.

## 96.10.3 Dysbetalipoproteinemia

**96.10.3.1 Clinical Features.** Remnant lipoproteins, known collectively as  $\beta$ -VLDL and including IDL, have density <1.006 g/ml and migrate in the beta-position on agarose gel electrophoresis (157). Some VLDL remnants are considered to be IDL. The archetypal disorder of IDL excess is called dysbetalipoproteinemia (DBL) or HLP type 3. Remnant lipoproteins are normally rapidly catabolized by apo E-mediated RME to

LDLR, the cell-surface heparan sulfate proteoglycans/LDLR-related protein complex, or heparan sulfate proteoglycans alone. The dyslipidemia in these patients, a combined elevation of total cholesterol and TG, usually becomes manifest in the third decade of life. CVD in these patients involves both multiple vascular beds (158).

**96.10.3.2 Molecular Genetics.** The prevalence of DBL is ~1:10,000 (158). Disease expression requires homozygosity for the *APOE* E2 isoform, which differs from the common E3 isoform by a single amino acid substitution (R158C). Because ~1% of Caucasians are E2/E2 homozygotes, secondary environmental, metabolic or genetic factors are required for disease expression. Recently, part of the genetic component of DBL was shown to be the accumulation of numerous small-effect common alleles and a few large-effect rare mutations that are individually associated with higher TG levels (159). Other rare *APOE* mutations cause this phenotype, and are associated with AD inheritance and variable clinical severity (158).

**96.10.3.3 Diagnosis.** Patients with DBL typically have elevations of cholesterol and TG to about equal concentrations (~7.5 mmol/L or ~300 mg/dL), an elevated ratio of CE to TG in VLDL, characteristic skin findings (tuberous and/or palmar xanthomas) and early atherosclerosis.

**96.10.3.4 Treatment.** The dyslipidemia can be managed with a diet reduced in total calories and fat content, and either a statin or a fibrate to correct the predominant abnormality of non-HDL lipoprotein fractions. Reduction in CVD risk in general terms—management of blood pressure, diabetes, and obesity, with smoking cessation—should be determined on a case-by-case basis.

## 96.10.4 Familial Combined Hypolipidemia

**96.10.4.1 Clinical Features.** A single four-generation family had affected individuals with very low LDL cholesterol, HDL cholesterol and TG, but none of the systemic clinical features of ABL or HHBL, and no clear evidence of resistance to atherosclerosis or of hepatic steatosis. Only one family with this condition has so far been reported (160).

**96.10.4.2 Molecular Genetics.** Whole-genome exome sequencing found that affected subjects were compound heterozygotes for two nonsense variants in *ANGPTL3*, namely, S17X and E129X (160). Relatives heterozygous for either mutation had plasma levels of LDL cholesterol and TG that were intermediate between the levels in persons with neither or both mutations, consistent with a codominant mode of inheritance. In contrast, the level of HDL cholesterol segregated as a recessive trait: relatives who carried both nonsense alleles had significantly lower plasma HDL cholesterol levels than those with one or no mutations.

**96.10.4.3 Treatment.** Given that the primary phenotype was biochemical without obvious clinical sequelae, no specific treatment is indicated for the condition.

## 96.11 OTHER DYSLIPOPROTEINEMIAS

### 96.11.1 Sitosterolemia

**96.11.1.1 Clinical Features.** Sitosterolemia, also known as phytosterolemia, is usually detected in pediatric patients. The defect produces a selective increase in plasma concentrations of plant sterols or phytosterols, including sitosterol and campesterol, which are present at concentrations of 100 and 10 times higher than normal, respectively (161). Plant sterols consumed by humans are not utilized, and their absorption from the intestine is selectively impeded: <5% of dietary plant sterols are absorbed compared to ~50% of dietary cholesterol. Patients with sitosterolemia accumulate plant sterols in many tissues, but have some features in common with HoFH patients, such as elevated plasma cholesterol, xanthomata, and early CVD due to atherosclerosis of the ascending aorta and coronary ostia (161). However, sitosterolemia is distinguished from HoFH by the absence of corneal arcus and by several additional key symptoms including recurrent joint arthritis, splenomegaly, hemolysis and platelet abnormalities (161). The total plasma cholesterol range is elevated, but is usually <10 mmol/L or 390 mg/dL (162).

**96.11.1.2 Molecular Genetics.** This condition results from mutations in the *ABCG5* and *ABCG8* genes (10), which together encode a heterodimeric G5/G8 protein. In normal individuals, the G5/G8 transporter in enterocytes rapidly returns most absorbed phytosterols back to the intestinal lumen before they can reach the systemic circulation. Phytosterols that do enter the circulation are then probably secreted into the bile by hepatocytes using the liver G5/G8 transporter (163). *ABCG5* and *ABCG8* mutations causing dysfunctional G5/G8 transporters result in severely increased plasma phytosterol concentrations; these sterols are incorporated into cholesterol-carrying lipoproteins.

**96.11.1.3 Treatment.** Treatment involves dietary elimination of all sources of noncholesterol sterols, including common foods such as vegetable oils, margarine, nuts, olives, avocados and shellfish (161). BAS and ezetimibe lower both plasma cholesterol and plant sterol levels, while statins produce minimal response because of their selective reduction in plasma cholesterol, each ultimately via hepatic LDLR upregulation (164).

### 96.11.2 Lysosomal Acid Lipase (LAL) Deficiency

**96.11.2.1 Clinical Features.** Wolman disease and cholesteryl ester storage disease (CESD) are characterized, respectively, by the production of nonfunctional and

partially functional LAL (165). CESD is a disease of adolescence and adulthood, while Wolman disease is a neonatal disease often leading to death during the first year of life (166). CESD typically presents with hypercholesterolemia and hepatomegaly that can progress to hepatic fibrosis (166). Wolman disease has a more dramatic presentation during infancy, with clinical features that include hepatosplenomegaly, failure to thrive, steatorrhea, anemia, jaundice and an acutely ill appearance (166,167). Pathological examination shows TG and cholesterol accumulation in liver, spleen, intestinal lining, bone marrow and vasculature, as well as severe adrenal calcification (166,167). Plasma cholesterol and TG levels are not necessarily dramatically elevated in Wolman disease (166).

**96.11.2.2 Molecular Genetics.** LAL is the enzyme responsible for intracellular hydrolysis of TG and CE by many tissues (168). Homozygous or compound heterozygous mutations in the *LIPA* gene encoding LAL underlie lipid storage diseases characterized by intracellular accumulation of unhydrolyzed lipids (168).

**96.11.2.3 Treatment.** While patients with CESD can have increased LDL cholesterol that responds to treatment with statins and ezetimibe (169), there is no specific treatment for Wolman disease. The prognosis for Wolman disease is often unfavorable, although some remarkable results have been obtained showing the feasibility of bone marrow transplantation, enzyme therapy and gene therapy (170). One report showed promise for early umbilical stem cell transplantation as a curative intervention (171).

### 96.11.3 Elevated Lipoprotein(a)

The biochemical and functional complexities of lipoprotein(a) (Lp(a)) have made it hard to understand its role in atherosclerosis (172). Lp(a) comprises LDL with a single molecule of apo B-100 linked to the polymorphic protein apo(a). The structural similarity between apo(a) and plasminogen, together with the LDL moiety suggest a prothrombotic or atherogenic role, or both. Older retrospective case-control studies and more recent population-based prospective genetic epidemiological studies (173) suggest a strong association between Lp(a) concentration and CHD risk. While there are no reported monogenic syndromes of Lp(a) excess, size polymorphism of the *LPA* gene on chromosome 6q26–27 is responsible for much of the interindividual variation in plasma Lp(a) concentration (174).

**96.11.3.1 Treatment.** If elevated Lp(a) is a concern, the focus should be on general CVD risk reduction, including LDL cholesterol reduction with a statin. In addition, niacin-based preparations reduce plasma Lp(a) levels, although there is no randomized clinical trial evidence that reduction to a specific target level reduces CVD end points.

### 96.11.4 Common Polygenic Dyslipidemia

In contrast to rare monogenic syndromes, most adult patients with dyslipidemia do not have a single genetic determinant that fully explains their clinical phenotype (3). While lipid abnormalities tend to be found at higher prevalence in relatives of these patients, there is no clear-cut pattern of inheritance and specific components of the lipoprotein profile may differ in affected relatives.

Recent genome-wide association studies (GWAS) have identified numerous genetic loci associated with plasma lipid levels (175). Some of the genes with common variants that have small effects on complex traits were already known to be important in lipoprotein metabolism, for instance, because they harbor rare large-effect variants in monogenic disorders. Therefore, previously unknown or unappreciated genes identified using this approach may prove to have some role in lipoprotein metabolism.

The role of multiple small-effect genetic variants as determinants of complex lipid and lipoprotein phenotypes was seen in a study of common complex HTG (176). For instance, in “garden variety” HTG, in both individual patients and across cohorts of patients with dyslipidemia, there was an accumulation of small-effect common variants from GWAS (154). Risk alleles for each of these common variants can be tallied to provide propensity or susceptibility scores for individuals. In addition, patients with common HTG are much more likely to have heterozygous rare variants in genes that contain common small-effect variants from GWAS studies (154). These increase the risk of expression but are not an absolute guarantee of developing HTG; the presence of secondary nongenetic or environmental factors

is still required. The clinical value of genetic risk scores for common nonmonogenic dyslipidemia is not yet clear; these may be useful for prognostication or perhaps personalized therapeutic approaches (176).

### 96.11.5 Secondary Dyslipidemias

Common dyslipidemias are often associated with other conditions, such as type 2 diabetes, obesity, alcoholism, hypothyroidism, pregnancy, renal failure, or drug use, and hence are termed secondary. Secondary dyslipidemias often have a strong genetic component, since some exacerbating conditions are frequently but not universally associated, suggesting that subjects who develop secondary dyslipidemia might have a subtle inherited metabolic defect that confers susceptibility. For example, abdominal obesity, metabolic syndrome (Table 96-12) 180 and type 2 diabetes have important effects on lipoprotein metabolism, and both these traits are highly heritable. It is important to distinguish between primary and secondary dyslipidemias, since this may determine the preferred means of intervention. Table 96-13 lists some conditions associated with secondary dyslipidemias.

## 96.12 GENERAL PRINCIPLES IN THE MANAGEMENT OF DYSLIPIDEMIA

CVD in the presence of mild-to-moderate polygenic dyslipidemia is common on a population-wide basis, while the monogenic disorders are relatively rare as a group. Several national guidelines provide advice for appropriate screening (Table 96-14) and management of dyslipidemia in the adult general population that does not specifically apply to the severe monogenic or

**TABLE 96-12 International Diabetes Federation Classification of the Metabolic Syndrome**

Central Obesity	
Waist circumference	
Europeans	Men $\geq 94$ cm; women $\geq 80$ cm
South Asians	Men $\geq 90$ cm; women $\geq 80$ cm
Chinese	Men $\geq 90$ cm; women $\geq 80$ cm
Japanese	Men $\geq 90$ cm; women $\geq 80$ cm
Ethnic South and Central Americans	Use South Asian recommendations until more specific data are available
First Nations	Use South Asian recommendations until more specific data are available
Sub-Saharan Africans	Use European data until more specific data are available
Eastern Mediterranean	Use European data until more specific data are available
Middle East (Arabic) populations	Use European data until more specific data are available
Plus Two of the Following Factors:	
Plasma TGs	$>1.7$ mmol/L ( $>200$ mg/dL)
High-Density Lipoprotein Cholesterol	
Men	$<1.03$ mmol/L ( $<40$ mg/dL)
Women	$<1.3$ mmol/L ( $<50$ mg/dL)
Blood pressure	$>130/85$ mmHg (or treatment for hypertension)
Fasting plasma glucose	$>5.6$ mmol/L ( $>100$ mg/dL)

Adapted from Reference (177).

**TABLE 96-13 Secondary Causes of and Contributors to Dyslipidemia**

Condition/Disorder	Principal Biochemical Disturbance			
	Increased LDL	Increased TG	Decreased HDL	Other
Obesity		X	X	
Metabolic syndrome		X	X	
Diabetes, particularly type 2		X	X	
Positive caloric balance plus poor diet		X	X	
Alcohol consumption		X		HDL can be elevated
Renal disease				
Uremia		X	X	
Nephrotic syndrome	X	X		
Liver disease				
Hepatosteatorsis	X	X		
Primary biliary cirrhosis	X			Elevated LpX species
Hypothyroidism	X			
Pregnancy (mainly third trimester)	X	X		HDL can be elevated
Autoimmune disorders				
Paraproteinemias		X	X	
Systemic lupus erythematosus		X	X	
Medications:				
Corticosteroids	X	X	X	
Thiazide diuretics	X			
Noncardioselective beta-blockers	X	X		
Tamoxifen		X		
Oral estrogens		X		HDL can be elevated
Isotretinoin	X	X	X	
BAS		X	X	
Cyclophosphamide	X	X	X	
Antiretroviral regimens		X	X	
Atypical psychotropic medications	X	X	X	

HDL, high-density lipoprotein; LDL, low-density lipoprotein; LpX, phospholipid-rich lipoprotein X; TG, triglyceride.

**TABLE 96-14 Screening for Dyslipidemia in the General Population**

Men and women $\geq 30$ years of age
All patients with the following conditions, regardless of age:
Evidence of atherosclerosis
Diabetes
Hypertension
Current cigarette smoking
Obesity or metabolic syndrome
Family history of premature CVD ( $< 60$ years in first-degree relatives)
Inflammatory diseases (systemic lupus erythematosus, rheumatoid arthritis, psoriatic arthritis)
Chronic renal disease (estimated glomerular filtration rate (eGFR) $< 60$ mL/min/1.73 m <sup>2</sup> )
HIV infection treated with highly active antiretroviral therapy
Clinical manifestations of hyperlipidemias (xanthomas, xanthelasma, premature arcus cornealis)
Erectile dysfunction
Children with a family history of hypercholesterolemia or chylomicronemia

Adapted from Reference (124).

familial forms of dyslipidemia discussed earlier. The foundation of management for all patients is diet and lifestyle modification (Table 96-15). After a patient has survived a CVD event, or has clinical signs of atherosclerosis, or is asymptomatic but living with a high

**TABLE 96-15 General Lifestyle and Health Behavior Modification in Treatment of Dyslipidemia**

Smoking cessation
Diet (reduced saturated fats and refined sugars)
Weight reduction and maintenance
Exercise (daily; total of 1000 kcal expended per week or 100 min of moderate exercise per week)
Stress management

level of risk due to a high burden of risk factors such as smoking, diabetes, hypertension, obesity and family history, lipid-lowering therapy, specifically statins to reduce LDL cholesterol, can reduce the risk of future CVD events, even when baseline lipid levels are relatively normal.

Numerous national agencies such as the United States' National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP) III (Table 96-7) and the Canadian Cardiovascular Society (Table 96-8) recommend LDL cholesterol as the primary target of therapy to reduce CVD risk. LDL cholesterol goals are recommended for patients stratified into particular risk categories, as determined using risk algorithms such as the Framingham risk score or the Reynolds risk score 126. For instance, subjects with



CHD or a CHD risk equivalent should aim for optimal LDL cholesterol  $<2.0$  mmol/L (or 78 mg/dL) according to Canadian guidelines or  $<1.8$  mmol/L (or 70 mg/dL) according to the updated NCEP ATP III<sup>56</sup> (Tables 96-8 and 96-7, respectively). Treatment of patients at lesser CHD risk proceeds in a graded manner. Intervention to reduce LDL cholesterol includes lifestyle management and, if necessary, pharmacologic therapies.

However, as noted earlier, certain monogenic disorders are associated with dramatically increased CVD risk, placing these individuals into the highest risk strata. These individuals will almost always require comprehensive and assertive management of lifestyle factors, with specific pharmacologic therapies as required for the individual conditions.

### 96.12.1 Dietary Intervention

Dietary interventions include (1) overall reduction in intake and reduced portion sizes, (2) redistribution of relative quantities of sources of calories (e.g. replace “high glycemic index” foods with complex carbohydrates, replace trans and saturated fats with monounsaturated and polyunsaturated fats), (3) addition or enhancement of specific foods that may have functional effects on the lipid profile (e.g. add soluble fiber or plant sterols), and (4) elimination of specific components that perturb the lipid profile (e.g. eliminate alcohol in some patients with elevated TG).

The NCEP ATP III advises that carbohydrate and protein intake should be 55–60% and 15–20%, respectively, whereas total and saturated fat should be  $<25$ –35% and  $<7$ %, respectively, of total calories, with restrictions on dietary cholesterol ( $<200$  mg/day), saturated fat ( $<7$ % of total calories/day), polyunsaturated fat (up to 10% of total calories), monounsaturated fat (up to 20% of total calories) and increased intake of fiber (20–30 g/day, of which half should be soluble fiber). The intake of animal products should be reduced. In obese patients, weight correction through reduction in total caloric intake is mandatory and may normalize the lipid profile. In normal-weight patients an isocaloric replacement of fat with carbohydrates is often effective. Plant sterols and stanols at intakes of 2–3 g daily can lower LDL cholesterol by 6–14%. Alcohol raises plasma TG, especially in people with an underlying predisposition, although it can also raise plasma HDL cholesterol; it is recommended to limit alcohol intake in individuals with moderate to severe dyslipidemia.

In general, the severity of the dyslipidemia dictates the intensity of the intervention. For less severe dyslipidemia, restricting saturated fat while increasing aerobic activity may substantially improve lipid levels. For severe hyperchylomicronemia, recommended fat intake is restricted further to 10–15% of total calories, with reductions in both saturated and unsaturated fat. In an adult, this represents 15–20 g/day of fat. A specialized dietician can be very helpful in these circumstances.

### 96.12.2 Physical Activity

Regular exercise is important in recalibrating the balance between caloric intake and expenditure, thus contributing to weight loss. Exercise also lowers blood pressure and improves insulin resistance; more directly it reduces plasma cholesterol and TG, and raises HDL cholesterol levels. This increases the catabolism of TRLs and tissue lipolysis. Recommendations relating to the intensity of regular exercise depend on the health and fitness of the patient; adults and children need to engage in activities equivalent to a daily total of 30–60 min of moderately intense physical activity three to five times per week.

### 96.12.3 Pharmacological Therapy

In general, drug therapy may be started together with lifestyle interventions in high-risk patients. The patient’s level of risk guides the timing of treatment initiation and also the intensity of the treatment. Moderate- or low-risk patients may be started on medication after a trial period of lifestyle interventions (Table 96-7). The main priority of drug therapy is to achieve the LDL cholesterol target level. Therefore, once drug therapy has been decided upon for a general patient with high CVD risk and mild-to-moderate complex dyslipidemia, an LDL-lowering drug is almost always the first step.

The response to drug therapy should be checked in about 6–8 weeks. If the LDL cholesterol target is not achieved, options include upward titration, switching to a more potent member of the same drug class or addition of a second-line agent. If the patient develops a drug-related adverse event, alternatives include a trial period off the medication to confirm a temporal relationship, empirically switching with the same class in the event that the side effect is agent specific rather than class specific, or switching to a second-line drug, such as a BAS, cholesterol absorption inhibitor, niacin-based therapy or fibrate. In HeFH or FCHL, combination regimens, such as statin plus a BAS, niacin, fibrate or cholesterol absorption inhibitor, are often required to attain target levels.

**96.12.3.1 HMG-CoA Reductase Inhibitors.** HMG-CoA reductase inhibitors (statins) block the rate-limiting step in de novo cholesterol biosynthesis, which depletes intracellular cholesterol and upregulates the LDLR, increasing LDL catabolism and lowering LDL cholesterol levels. Statins also appear to have a minor effect on reducing secretion of apo B-containing lipoproteins. The evidence that CVD end points and mortality are reduced by statins is overwhelming. Considering their very widespread use in cardioprotection, statins are generally well tolerated and only rarely cause myopathy or hepatic toxicity. Their place as front-line drug therapy in patients with mild-to-moderate dyslipidemia is incontrovertible. Available statins include lovastatin, pravastatin, simvastatin, fluvastatin, atorvastatin, rosuvastatin and pitavastatin (Table 96-16).

**TABLE 96-16 Dosing and Efficacy of Currently Available Drugs for the Treatment of Dyslipidemia**

Drug	Class	Mechanism	Dosing	Approximate LDL Cholesterol Reduction (%)
Atorvastatin	Statin	HMG-CoA inhibition	10–80 mg/day	35–50
Fluvastatin	Statin	HMG-CoA inhibition	20–80 mg/day	24–38
Lovastatin	Statin	HMG-CoA inhibition	20–80 mg/day	26–38
Pitavastatin	Statin	HMG-CoA inhibition	1–4 mg/day	22–39
Pravastatin	Statin	HMG-CoA inhibition	10–40 mg/day	25–40
Rosuvastatin	Statin	HMG-CoA inhibition	20–80 mg/day	37–52
Simvastatin	Statin	HMG-CoA inhibition	5–80 mg/day	32–46
Fenofibrate	Fibrate	PPAR- $\alpha$ agonist	48 and 145 mg/day	–10 to 11
Gemfibrozil	Fibrate	PPAR- $\alpha$ agonist	600 mg twice daily	–6 to 11
Cholestyramine	BAS	Interrupts enterohepatic circulation	4–8 g one to two times daily	8–20
Colesevelam	BAS	Interrupts enterohepatic circulation	625 mg two to three times daily	7–18
Ezetimibe	Cholesterol absorption inhibitor	Blocks NPC1L1 transporter at intestinal brush border	10 mg/day	15–24

**96.12.3.2 Bile Acid Sequestrants.** BAS, such as cholestyramine, cholestipol, and colesevelam, are basic anion-exchange resins that bind BA in the intestine, interrupting the enterohepatic BA recirculation, diverting hepatic cholesterol into BA synthesis, and depleting intrahepatic cholesterol. The upregulation of LDLR increases LDL catabolism and decreases LDL cholesterol levels. BAS have a complementary mechanism of action and are additive to the LDL-lowering effects of statins. Despite evidence for reduction in CVD and CHD end points and their long track record of safety, BAS suffer from low compliance because of adverse gastrointestinal effects. Because BAS tend to raise serum TG, they should be avoided in persons with high TG ( $>400$  mg/dL or  $>5$  mmol/L). They are considered second-line agents to be added to maximally tolerated statin doses when LDL cholesterol targets are not reached. They can also be considered in persons who develop statin-related adverse effects.

**96.12.3.3 Cholesterol Absorption Inhibition.** Cholesterol absorption inhibitors work preferentially at the level of the luminal brush border of enterocytes in the upper intestine, reducing cholesterol content of CMs while maintaining the absorption of lipid-soluble vitamins or steroid hormones. Ezetimibe is thought to lower plasma LDL cholesterol by upregulating the activity of hepatic LDLR. Ezetimibe is available at a single dose of 10 mg daily and lowers LDL cholesterol levels by 18–25%, with no effect on other components of the lipid profile. The combination of a statin and ezetimibe can lower LDL cholesterol by ~70%. Compared to BAS and niacin, ezetimibe is well tolerated with minimal side effects. At this time, ezetimibe should still be considered as a second-line drug added to statin therapy when LDL cholesterol targets have not been reached or when statins cannot be tolerated as first-line therapy.

**96.12.3.4 Nicotinic Acid.** Nicotinic acid, or niacin, is a second-line agent for CVD risk reduction in patients with mild-to-moderate common dyslipidemia. It is also an

add-on therapy in higher risk persons who are not at LDL cholesterol target on highest tolerated statin dose and who have a low HDL cholesterol component to their risk profile. Niacin has multiple favorable effects on the lipid profile: doses of up to 2 g daily for extended release preparations or up to 3 g daily of the crystalline form can lower plasma TG by up to 45%, raise plasma HDL cholesterol by up to 25%, and reduce plasma LDL cholesterol by up to 20%. After almost six decades of clinical use, niacin's mechanism of action remains poorly understood, but it may, at least in part, reduce hepatic secretion of VLDL by inhibiting peripheral mobilization of FFA. Older clinical trials suggested a reduction in CVD events related to niacin treatment. However, niacin frequently causes light-headedness, cutaneous flushing and pruritus. These adverse effects can be minimized by initiating therapy at low doses and then gradually increasing the daily dose, along with the concomitant use of aspirin or by the use of extended-release niacin preparations. Other adverse effects include elevated liver enzymes, gastrointestinal upset, worsened glucose tolerance and elevated uric acid.

**96.12.3.5 Fibrates.** Fibrates, such as gemfibrozil, fenofibrate, bezafibrate and ciprofibrate (of which the latter two are not available in the United States) can reduce plasma TG by up to 50%, and can raise plasma HDL cholesterol by up to 20%, although these percentages vary between patients. The complex mechanism of action of fibrates includes modulation of the activity of peroxisome proliferator-activated receptor- $\alpha$ , PPAR- $\alpha$ , in the liver, resulting in the downregulation of the expression of apo C-III and upregulation of apo A-I, fatty acid oxidation, and LPL, thereby increasing fatty acid oxidation with reduction in VLDL production. Because the LDL lowering capacity is very modest and because large recent clinical trials show little to no benefit of fibrates added to statin therapy for CVD risk reduction in patients with normal to modestly increased TG levels (178), fibrate use

is mainly reserved for treatment of patients with severe HTG to reduce the risk of acute pancreatitis. Fibrates can also be considered as an add-on therapy for patients with high CHD risk who require consideration for a second agent because primarily TG remains elevated.

**96.12.3.6 N-3 (Omega) Fatty Acids.** Populations in countries where large quantities of fatty fish are consumed and whose diets are naturally high in alpha-linolenic acid and longer chain omega-3 FA appear to have a decreased CVD risk. N-3 ( $\omega$ 3) FA, such as eicosapentanoic and docosahexanoic acid, are components of both Mediterranean diet and fish oils. Omega-3 FA can reduce hepatic secretion of VLDL and thus reduce TG. Ingestion of 4 g/day of omega-3 FA, with caloric and saturated fat restriction, may reduce plasma TG by up to 20%. However, omega-3 FA are not effective in many patients when used as the sole TG-lowering therapy.

**96.12.3.7 Extracorporeal LDL Reduction in Homozygous Familial Hypercholesterolemia.** Untreated HoFH has been associated with premature mortality since patients have virtually no functional LDLRs to upregulate, so that medications such as statins have little to no effect. The mainstay of treatment in HoFH is one of several extracorporeal approaches to remove the accumulating LDL particles, such as weekly or biweekly serial plasma exchange, plasmapheresis or specific LDL apheresis using size exclusion columns or antibody-based affinity columns (179). These treatments have prolonged the atherosclerosis-free survival of HoFH patients, who now live long enough to have manifestation of unexpected CVD end points, specifically aortic root and valvular calcification, often requiring surgical replacement (73).

## 96.12.4 Emerging Therapies

**96.12.4.1 LDLR-Based Gene Therapy.** Trials of LDLR gene replacement in HoFH have shown variable improvements in the lipoprotein profile (180). Gene delivery systems that are currently under development include several virus-based approaches. To date, however, many inherent problems remain to be solved, and long-term success and efficacy must be further improved before these techniques pass the experimental stage.

**96.12.4.2 Microsomal Triglyceride Transfer Protein (MTP) Inhibition.** The cardioprotective biochemical phenotype in ABL due to mutations in MTP, i.e. extremely low levels of CMs, VLDL and LDL, inspired the development of an orally administered inhibitor of MTP, namely, lomitapide. Early-phase clinical trials in patients with severe elevations in LDL cholesterol, such as those with HoFH, showed that MTP inhibition significantly reduces plasma total and LDL cholesterol, and apo B (86). However, ~25% of patients in short-term studies developed transaminase elevations and accumulation of hepatic fat, although this became less severe with prolonged treatment.

**96.12.4.3 Interference with APOB Synthesis.** The cardioprotective biochemical phenotype in FHBL due to mutations in *APOB*, i.e. extremely low levels of VLDL and LDL, inspired the development of parenteral antisense RNA therapies. Antisense RNA therapy refers to agents that have a range of mechanisms that result in specific targeting and degradation of messenger RNA (mRNA). Instead of pharmacological targeting, these newer methods, including antisense oligonucleotides (ASOs) and ribonucleic acid (RNA) interference (RNAi), reduce translation by selectively degrading mRNAs. The first antisense RNA for the treatment of dyslipidemia is mipomersen, a 20-nucleotide ASO administered subcutaneously using nondaily or weekly dosing regimens. Mipomersen binds *APOB* mRNA sequence and targets the bound mRNA for degradation via the activation of endogenous RNase H enzymes. Mipomersen can reduce both apo B and LDL-C levels by up to 50% in HoFH (181). Common adverse effects include skin site reactions and, to a much lesser degree, fatty liver.

**96.12.4.4 Interference with PCSK9.** The cardioprotective biochemical phenotype and reduction in CVD events seen in individuals with mutations in *PCSK9*, i.e. extremely low levels of LDL cholesterol, inspired the development of drugs to target *PCSK9*. Both RNA interference and monoclonal antibody strategies are being developed.

**96.12.4.5 Cholesteryl Ester Transfer Protein (CETP) Inhibition.** The first CETP inhibitor, torcetrapib, increased HDL cholesterol markedly, but was associated with increased CVD event rates. While this raised concern that perhaps the mechanism itself was fundamentally flawed, further evaluation showed that torcetrapib had off-target effects, particularly an increase in systolic blood pressure and possible increase in serum aldosterone levels. Two newer members of the class, namely, dalcetrapib (182) and anacetrapib (183), affect neither blood pressure nor serum aldosterone. Because of their apparently superior safety profile, fairly comprehensive development and clinical trial programs are in progress for both these newer agents.

**96.12.4.6 Other Emerging Treatments.** Other pharmacological approaches that are under various stages of clinical development include the combination of extended-release niacin with the prostaglandin D2 inhibitor laropiprant, which reduces the incidence of niacin-induced flushing (184), and the thyroid receptor agonist eprotirome, which lowers total and LDL cholesterol and TG (185).

## 96.13 CONCLUSION

There has been considerable recent progress in understanding at least a portion of the molecular bases for severe monogenic dyslipidemias. Clear distinctions at the molecular and clinical levels are emerging between polygenic dyslipidemias that manifest during adulthood

and more severe dyslipidemias that present at younger ages. Monogenic disorders may be suspected in individuals who present with markedly deviated levels of plasma LDL-cholesterol, HDL-cholesterol or TGs or with the signs and symptoms of syndromes described in this review. Genetic analysis is becoming more practical and useful in the diagnosis and management of these patients. Since the molecular genetic bases for some extreme pediatric lipid disorders are being increasingly well understood, there is some hope for future treatments that take advantage of this understanding. Clinicians must determine the proximal, intermediate and long-term risk of complications and adjust their perspective and goals appropriate to the individual patient with dyslipidemia.

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# CHAPTER 97

## Organic Acidemias and Disorders of Fatty Acid Oxidation

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### 97.1 ORGANIC ACIDEMIAS

Most organic acidemias are disorders of amino acid metabolism. They were first described in 1961 as a syndrome of mental retardation, hyperglycinemia and episodic ketoacidosis, neutropenia and thrombocytopenia induced by protein intake or infection. Ultimately, it was realized that this biochemical and clinical phenotype, which had become known as ketotic hyperglycinemia, was actually a manifestation of organic acidemias, and in particular, methylmalonic acidemia, propionic acidemia, and 2-methyl-3-hydroxybutyric acidemia.

Organic acidemias can present in many ways and the indications for organic acid screening are quite diverse. They include (i) features of ketotic hyperglycinemia (see previous discussion); (ii) an unusual body odor; (iii) acute disease in infancy, especially when associated with metabolic acidosis, hypoglycemia, or hyperammonemia; (iv) chronic or recurrent metabolic acidosis, with or without an anion gap; (v) static or progressive extrapyramidal movement disorder in childhood; (vi) Reye syndrome when recurrent, familial, or in infancy; (vii) the combination of ataxia, alopecia, and rash; and (viii) seizure disorder and/or developmental delay of unknown cause. Because most organic acids are effectively cleared from the bloodstream by the kidney, urine is the preferred fluid for analysis.

Diagnosis of these conditions depends on methods to separate and identify organic acids, and the most widely used of these is gas chromatography-mass spectrometry (GC-MS), in which organic acid derivatives are separated by gas chromatography and then passed into a mass spectrometer for identification. Tandem mass spectrometry (MS-MS) is a related technology in which compounds are separated by molecular weight by one mass spectrometer, fragmented as they exit, and identified on the basis

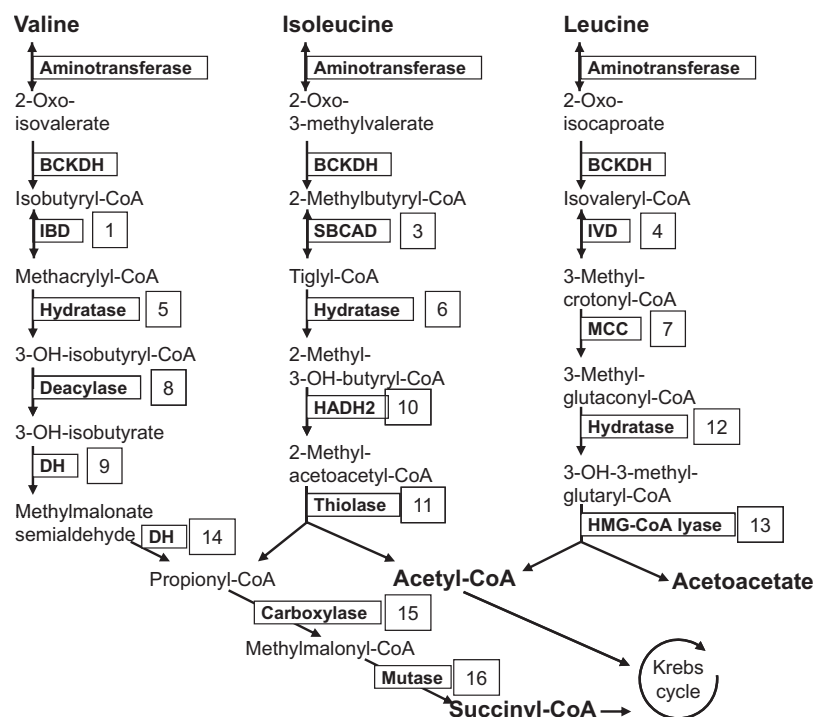
of their fragments by a second mass spectrometer. This technique is used to measure the acylcarnitine esters that accumulate in various fatty acid oxidations and organic acid disorders and serves the basis for effective newborn screening for many of these disorders. The convergence of many metabolic pathways especially onto the distal end of branched-chain amino acid catabolism has led to much confusion in diagnosis over the years. Diseases named for metabolites ultimately proved to be imprecise because of the accumulation of the same metabolites in more than one condition. Thus, it is best to discuss this group of disorders based on the enzymatic and/or molecular defect rather than metabolite accumulation.

Most described organic acidemias are inherited as autosomal recessive traits. Two are X-linked recessive. Prenatal diagnosis is typically accomplished through measurement of enzyme activity in cultured amniotic cells or a chorionic villus sample, or through measurement of diagnostic metabolites excreted by the affected fetus into the amniotic fluid. Molecular diagnosis is also possible when a proband's genetic defect has been identified.

#### 97.1.1 Branched-Chain Organic Acidemias

The largest group of organic acidemias derives from defects in the mitochondrial-based degradation of the branched-chain amino acids leucine, isoleucine and valine (Figure 97-1). The branched-chain amino acids enter mitochondria via a combined, reversible deamination and transport step performed by the vitamin B6-dependent branched-chain amino acid aminotransferases. Subsequently, the branched-chain ketoacids undergo oxidative decarboxylation by a single branched-chain ketoacid dehydrogenase active with all three substrates, another reversible reaction. Deficiency of this





**FIGURE 97-1** Pathways of branched-chain amino acid catabolism. The first two steps in the metabolism of branched-chain amino acids are reversible and their deficiency leads to amino acid rather than organic acid accumulation. BCKDH, branched-chain ketoacid dehydrogenase. Documented steps shown to cause disease in humans are numbered. 1, IBD, isobutyryl-CoA dehydrogenase; 2–4, isobutyryl, short branched-chain, and isovaleryl-CoA dehydrogenase; 5 and 6, methacrylyl-CoA and tiglyl-CoA hydratase, respectively; 7, MCC, methylcrotonyl-CoA carboxylase; 8, 3-hydroxyisobutyryl-CoA deacylase; 9, 3-hydroxyisobutyrate dehydrogenase; 10, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase; 11, mitochondrial acetoacetyl-CoA thiolase; 12, 3-methylglutaconyl-CoA hydratase; 13, 3-hydroxy, 3-methylglutaryl-CoA lyase; 14, methylmalonate semialdehyde dehydrogenase; 15, propionyl-CoA carboxylase; 16, methylmalonyl-CoA mutase.

dehydrogenase leads to maple syrup urine disease and is discussed elsewhere in the book (Chapter 92). The remaining steps of the pathway are unique to the metabolites of each of the amino acids.

#### 97.1.1.1 Isovaleryl-CoA Dehydrogenase Deficiency.

Isovaleric acidemia (IVA) was the first condition to be recognized as an organic acidemia when the odor of sweaty feet that surrounded an infant with episodic encephalopathy was shown to be due to isovaleric acid (1,2). The disorder in leucine degradation is due to deficiency of isovaleryl-CoA dehydrogenase, the mitochondrial enzyme that oxidizes the first irreversible step in this pathway, isovaleryl-CoA to 3-methylcrotonyl-CoA (3).

**97.1.1.1.1 Clinical Course.** Early literature on IVA, an autosomal recessive disorder, emphasized two apparent phenotypes (4). The first was an acute, neonatal presentation with patients becoming symptomatic within the first two weeks of life (1,5–9). Patients appeared initially well, then developed vomiting and lethargy, progressing to coma. The second group presented with relatively nonspecific failure to thrive and/or developmental delay (chronic intermittent presentation) (5,6,10–12). Patients who survived an early acute presentation subsequently were indistinguishable from those with the chronic phenotype, and both groups of patients were prone to intermittent acute episodes of decompensation with minor illnesses (4). In reality, it is now apparent that patients can

fall anywhere on the spectrum of acute to chronic presentation and that there is probably little predictive value to the initial presentation. Moreover, with the application of MS-MS in newborn screening, potentially asymptomatic patients with one recurring IVD gene mutation and a mild biochemical phenotype are being identified in increasing numbers, representing an additional phenotype of IVA (13). This type may represent a biochemical phenotype only without the expression of any clinical symptoms (such as in benign hyperphenylalaninemia) and therefore needs to be differentiated from the classic forms of IVA.

**97.1.1.1.2 Diagnosis.** Diagnosis is most frequently made by newborn screening in countries where tandem MS-MS screening is performed. In symptomatic patients, the diagnosis is suggested by the clinical course and odor, and is confirmed by organic acid analysis or by demonstrating a deficiency of isovaleryl-CoA dehydrogenase in tissues. Odor may be mild or absent, however, and thus the clinical picture may be relatively nonspecific. The organic acids characteristic of the condition are isovalerylglycine and 3-hydroxyisovaleric acid; however, a long list of isovaleryl-CoA derived metabolites has been reported in blood and urine from patients with IVA and can assist in the confirmation of the disorder. Isovaleric acid itself, which is responsible for the odor, is not detected by most analytic methods. Most of the accumulated isovaleryl-CoA is excreted as the nontoxic glycine

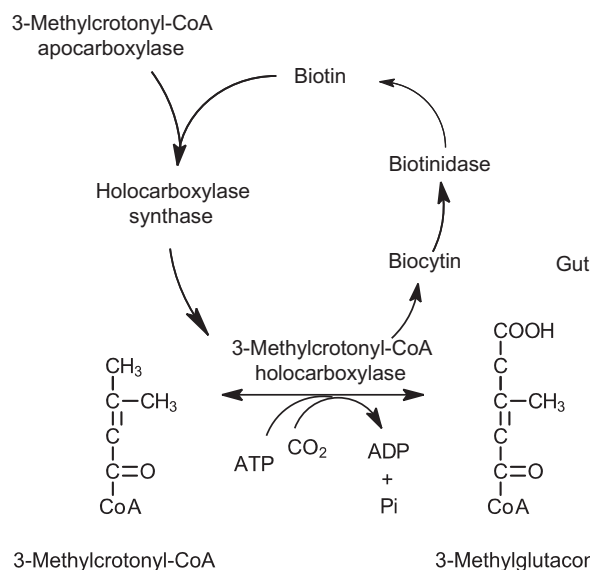
and carnitine esters, but the amounts that accumulate during infection or after a protein load exceed the capacity of the liver to esterify it, and the carbon skeleton then appears in other compounds. The same clinical presentation and odor can be seen in glutaric acidemia type II, but urine organic acid analysis will effectively differentiate the two conditions.

**97.1.1.1.3 Treatment.** There are three goals for therapy of almost any organic acidemia, including IVA (3). The first is prevention of metabolic decompensation by careful clinical observation of the patient. During times of metabolic stress (including illness and fasting) endogenous substrate (leucine in IVA) from protein catabolism adds significantly to the production of metabolic intermediates. Achieving or maintaining anabolism is the main therapeutic approach to counter this problem. Reducing, but not eliminating, natural protein in the diet for 12–24 h may help in this regard, but only if additional other calories to promote anabolism can be given. The second goal is long-term reduction of the production of toxic metabolites from general catabolism through dietary manipulation. Total protein and caloric intake must be adequate to support normal growth in children and maintain an anabolic state, but this may require the use of an artificial protein source restricted to the appropriate amino acid for a portion of the protein requirement. The third goal of therapy is to prevent the accumulation of toxic metabolites by enhancing alternative metabolic pathways that produce alternative nontoxic compounds that are readily excreted. Carnitine is commonly used for this purpose in many of the organic acidemias. In IVA, glycine also effectively conjugates isovaleryl-CoA but is usually not needed to maintain homeostasis.

**97.1.1.1.4 Genetics.** IVA is inherited as an autosomal recessive trait. The gene encoding isovaleryl-CoA dehydrogenase has been cloned and localized to chromosome 15 (15q14–15), and multiple disease-causing mutations have been identified. Nearly half the mutant IVD alleles sequenced from infants diagnosed by newborn screening have been found to contain a common recurring missense mutation (932C>T; A282V) (13). Prenatal diagnosis can be achieved through enzyme measurement in cultured amniocytes, quantitation of isovalerylglycine in amniotic fluid, and molecular analysis when the mutations in the family are known.

## 97.1.2 3-Methylcrotonyl-CoA Carboxylase Deficiency

3-Methylcrotonyl-CoA carboxylase (3MCC) deficiency can be caused by a defect in the gene for this enzyme or as part of a multiple carboxylase deficiency (see later sections). Isolated deficiency, manifesting as isolated 3-methylcrotonylglycinemia was first described in 1970 in a female infant with feeding problems, developmental delay, severe hypotonia, and an odor like that of cat's



**FIGURE 97-2** Relation of carboxylation of 3-methylcrotonyl-CoA to the activities of biotinidase and HCS. HCS also links biotin to apocarboxylases for propionyl-CoA, acetyl-CoA and pyruvate, and biotinidase releases biotin from proteins and enzymes that contain it.

urine. The disorder is due to deficiency of 3-methylcrotonyl-CoA carboxylase, a biotin-containing enzyme that converts its substrate, an intermediate in leucine oxidation, to 3-methylglutaconyl-CoA (Figure 97-2).

**97.1.2.1 Clinical Course.** Early reports on patients with this condition reported episodes of vomiting, hypoglycemia, hepatomegaly, hyperammonemic encephalopathy, metabolic stroke, and hypotonia with developmental delay. Numerous affected babies have now been identified through newborn screening, most, if not all, of whom have remained well (14). Elevated metabolites characteristic of 3MCC deficiency have been identified through newborn screening in infants born to asymptomatic mothers with 3MCC deficiency (15). In conjunction with the identification of asymptomatic siblings of severely affected patients, the clinical relevance of the biochemical abnormalities related to this deficiency must be called into question.

**97.1.2.2 Diagnosis.** Diagnosis is usually made by organic acid analysis, and can be confirmed by enzyme assay in leukocytes or cultured fibroblasts. The most characteristic organic acids are 3-methylcrotonylglycine and 3-hydroxyisovaleric acid, and increased 3-hydroxyisovalerylcarnitine can be demonstrated by MS-MS. Serum carnitine may be low, possibly because of the excretion of 3-hydroxyisovalerylcarnitine. The characteristic but not specific metabolite identified through expanded newborn screening MS-MS is “C5-OH” carnitine. A similar organic aciduria is seen in biotin deficiency and combined carboxylase deficiency, but in these conditions together with 3-hydroxypropionic and methylcitric acids. The relationship of the metabolites to the clinical features of the disease is not well understood.

**97.1.2.3 Treatment.** Acute episodes of vomiting and hypoglycemia should be treated with fluids, glucose, and

electrolytes. The need for long-term restriction of protein or leucine is not established. Biotin supplementation (10–30 mg/day) has been reported but is of unproven effect. Carnitine supplementation may be warranted if serum levels are significantly reduced.

**97.1.2.4 Genetics.** 3-Methylcrotonyl-CoA carboxylase deficiency is transmitted as an autosomal recessive trait. The genes encoding the  $\alpha$ - and  $\beta$ -subunits are located on chromosomes 3 (3q25–27) and 5 (5q12–13), respectively, and disease-causing mutations have been identified in both (14). Prenatal diagnosis, although likely possible, has not been reported.

### 97.1.3 Multiple Carboxylase Deficiency (Biotinidase or Holocarboxylase Synthetase Deficiency)

Multiple carboxylase deficiency is caused by defects in the incorporation of the water-soluble B-complex vitamin biotin into or its release from four apocarboxylases acting on 3-methylcrotonyl-CoA, propionyl-CoA, acetyl-CoA, and pyruvate. Holocarboxylase synthetase (HCS) covalently binds biotin to the apocarboxylases, while biotinidase catalyzes its release from biotinyl- $\epsilon$ -lysine (biocytin) or biotin-containing peptides (Figure 97-2). Both these conditions are inherited as autosomal recessive traits, and frequently cause a syndrome of alopecia, skin rash, and encephalopathy.

**97.1.3.1 Clinical Course.** Biotinidase deficiency usually presents in infancy with a perioral dermatitis that resembles acrodermatitis enteropathica, patchy alopecia, and neurologic features such as ataxia, neurosensory defects, developmental delay, and convulsions, though later presentation with predominantly neurologic symptoms is possible (16). Biotinidase assay is now a part of many newborn screening programs and frequently identifies infants with partial deficiencies. These infants are likely to remain well, but late onset symptoms have been reported. HCS deficiency usually causes earlier and more severe disease, with ketoacidosis, alopecia, and a red and scaly total body eruption (17). Coma, apnea, and death often ensue if therapy is not begun promptly. In both conditions the rash may be complicated by superinfection with *Monilia*. There is sufficient overlap in time and severity of onset; however, accurate differentiation between the two can only be made by enzyme assay.

**97.1.3.2 Diagnosis.** Urine organic acid analysis shows increased 3-methylcrotonylglycine and 3-hydroxyisovaleric acid, together with methylcitric and 3-hydroxypropionic, and acylcarnitine analysis shows 3-hydroxyisovaleryl- and propionylcarnitine. HCS can be assayed in fibroblasts or leukocytes, and biotinidase can be assayed in serum. Dietary biotin deficiency, especially during parenteral hyperalimentation, can be biochemically indistinguishable from biotinidase deficiency (18).

**97.1.3.3 Treatment.** Large doses of biotin (10–30 mg/day) usually produce rapid clinical improvement and

almost complete disappearance of abnormal urine organic acids. The frequency of biotinidase deficiency, the ease with which it can be treated, and the irreversibility of the neurologic sequelae have led to its inclusion in several newborn screening programs, and many affected patients have now been detected and treated successfully before the onset of symptoms (16,19). The need for treatment of partial biotinidase deficiency remains controversial.

**97.1.3.4 Genetics.** Both conditions are transmitted as autosomal recessive traits. The genes that encode biotinidase and HCS have been localized to chromosomes 3 (3p25) and 21 (21q22.1), respectively. Numerous disease-causing mutations have been delineated in each gene (16,17,19,20). Both conditions can be diagnosed in utero by enzyme assay on cultured amniocytes or chorionic villus samples, and by mutation analysis, but prenatal diagnosis of an affected fetus has thus far been reported only in HCS deficiency (21).

### 97.1.4 3-Methylglutaconic Aciduria

Elevation of 3-methylglutaconic aciduria is seen in a heterogeneous group of disorders: 3-methylglutaconyl-CoA hydratase deficiency (Figure 97-1), Barth syndrome (cardiomyopathy due to tafazzin gene defects), mutations in the OPA3 gene (optic atrophy plus or Costoff syndrome), and cardiomyopathy with ataxia (due to mutations in the DNAJC19 gene) (Table 97-1) (22). Occasional patients with significant 3-methylglutaconic aciduria of unknown cause continue to be identified and are referred to as having the type 4 phenotype (23). While all these disorders share a similar biochemical phenotype, they are quite different disorders clinically.

**97.1.4.1 Clinical Course.** Patients with 3-methylglutaconyl-CoA hydratase deficiency have been reported to have a variable phenotype ranging from mild delay (including isolated speech delay) to more severe delay, cerebellar ataxia, and movement abnormalities and even quadriplegia. Older reports are likely to have confused hydratase deficiency with other causes of 3-methylglutaconic aciduria, but the variability persists even in newer reports with documented enzymatic or molecular diagnoses. Thus the full spectrum of this disorder remains to be determined. Barth syndrome is an X-linked disorder

**TABLE 97-1** Classification of Disorders Exhibiting Increased Excretion of 3-Methylglutaconic Acid

Type	Gene Defect
1	3-methylglutaconyl-CoA hydratase
2 (Barth syndrome)	Tafazzin
3	OPA3
4	Unknown
5	DNAJC19

characterized by cardiomyopathy, neutropenia and skeletal myopathy (24). Mitochondria are abnormal with tightly packed cristae and inclusion bodies. The cardiomyopathy may be congenital but can also be absent. In the latter setting, infections due to neutropenia suggest the diagnosis. In one report, the age distribution in 54 living patients was 0–49 years (24). Female carriers are usually healthy. Mutations in the tafazzin gene that encodes a mitochondrial cardiolipin transacylase are responsible for Barth syndrome (25). Secondary effects on the respiratory chain function and increased oxidative stress presumably lead to the pathophysiology in this disease. OPA3 deficiency presents with early-onset bilateral optic atrophy and late development of spasticity, extrapyramidal dysfunction and occasionally cognitive deficit (26). Cardiomyopathy and neutropenia are not present. An Iraqi-Jewish isolate has accounted for most reported cases. The function of the OPA3 gene is unknown, but it encodes at least two transcripts that are targeted to mitochondria (27). Mutations in DNAJC19 cause an autosomal recessive Barth-like syndrome (28). Described in 11 consanguineous Canadian Hutterite families, affected individuals had severe, early onset cardiomyopathy that tended to be progressive. They subsequently exhibited growth failure and cerebellar ataxia. Mild normochromic, microcytic anemia and hepatosteatosis may be present. DNAJC19 is a homolog of a yeast inner mitochondrial cochaperonin.

**97.1.4.2 Diagnosis.** 3-Methylglutaconic acid is identified in urine by standard GC-MS techniques. 3-Methylglutaric acid is also usually present. Levels are the highest in the hydratase deficiency and more modest in the other disorders. The hydratase activity can be measured in fibroblasts, but in practice molecular testing is probably easier clinically. Functional tests for the other disorders are not available, and so molecular testing is the only diagnostic option. In theory, prenatal diagnosis through metabolite analysis of amniotic fluid should be feasible,

but has not been reported yet. Molecular diagnosis can be applied prenatally.

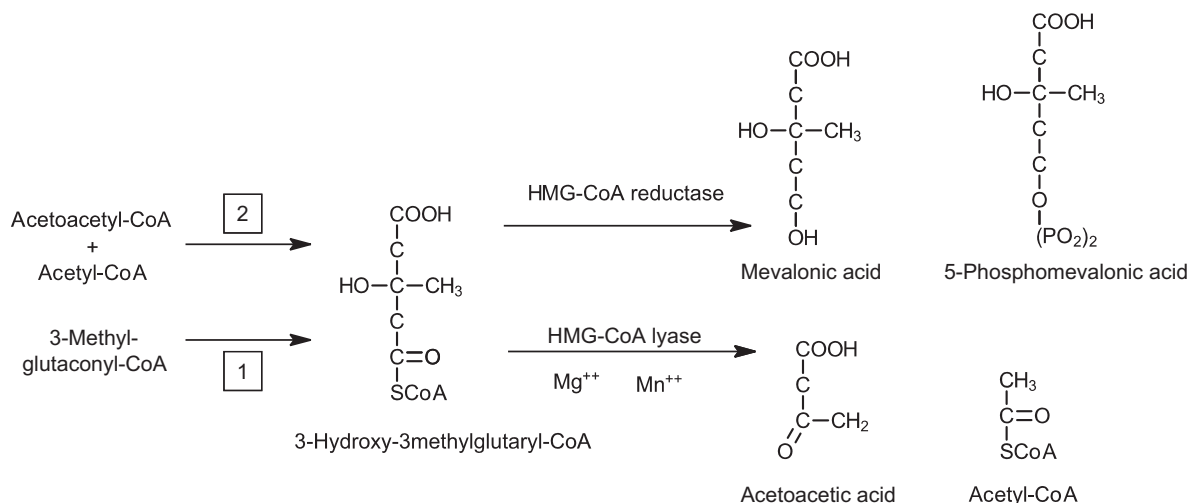
**97.1.4.3 Treatment.** Unfortunately, therapeutic advances for 3-methylglutaconic acidemias have lagged far beyond the growing understanding of their molecular causes. Currently, no effective therapy exists for any of the recognized defects. Leucine restriction has not been shown to affect outcome.

**97.1.4.4 Genetics.** The genes for 3-methylglutaconyl-CoA hydratase, tafazzin, OPA3 and DNAJC19 are on chromosomes 9, X, 19, and 3, respectively. Mutations in all the genes related to disease have been identified. No ethnic predilection has been reported for hydratase or tafazzin deficiencies. OPA3 and DNAJC19 defects have been identified in Iraqi-Jews and Canadian Hutterites, respectively.

### 97.1.5 3-Hydroxy-3-Methylglutaryl-CoA Lyase Deficiency

Hydroxymethylglutaric acidemia is typically an early-onset disease. It is due to a deficiency of 3-hydroxy-3-methylglutaryl-CoA lyase, an enzyme involved in leucine oxidation and in ketone body biosynthesis (Figure 97-3). Identification by newborn screening with MS-MS offers the opportunity to intervene before symptoms appear.

**97.1.5.1 Clinical Course.** The condition most frequently presents in early infancy with hypoglycemia, metabolic acidosis, and hyperammonemia. In 30% of cases onset is between birth and day 5 of life or between 3 and 11 months of age and is frequently lethal (29). Older children present with episodic hypoketotic hypoglycemia, hepatomegaly, and encephalopathy after intercurrent infections. The latter form of the condition is frequently mistaken for Reye syndrome. Neurologic signs, mental retardation, and cerebral atrophy may follow if the condition is not recognized and treated. Rare first presentations in adulthood have been reported.



**FIGURE 97-3** Reaction catalyzed by hydroxymethylglutaryl-CoA lyase. (1) Methylglutaconyl-CoA hydratase. (2) Hydroxymethylglutaryl-CoA synthetase.



**97.1.5.2 Diagnosis.** This condition, and disorders of fatty acid oxidation, should be excluded in any infant or child with hypoketotic hypoglycemia. Organic acid analysis shows large increases in 3-hydroxy-3-methylglutaric, 3-methylglutaconic and 3-hydroxyisovaleric acids, especially after protein intake or in situations that favor the synthesis and utilization of ketone bodies. Note that the same pattern of metabolites is sometimes seen as a nonspecific marker of mitochondrial dysfunction and so follow up testing is mandatory. The diagnosis can be confirmed by demonstrating deficiency of hydroxymethylglutaryl-CoA lyase activity in leukocytes or cultured fibroblasts. Molecular testing is available. 3-Hydroxy-3-methylglutaryl-CoA lyase deficiency can be identified by newborn screening with MS-MS, but the metabolite identified ("C5 hydroxycarnitine") is not specific, so urgent follow-up testing (urine organic acids) is required (30–32).

**97.1.5.3 Treatment.** Episodes of hypoketotic hypoglycemia should be treated with intravenous fluids, electrolytes, and glucose to reestablish an anabolic state. Long-term management is directed at avoiding fasting and the resulting hypoglycemia. Leucine restriction has been suggested but probably has limited effect (29). Carnitine supplementation may be helpful. The possible life-threatening consequences of acute attacks make it critical that parents bring the child to hospital as soon as possible whenever oral intake is reduced.

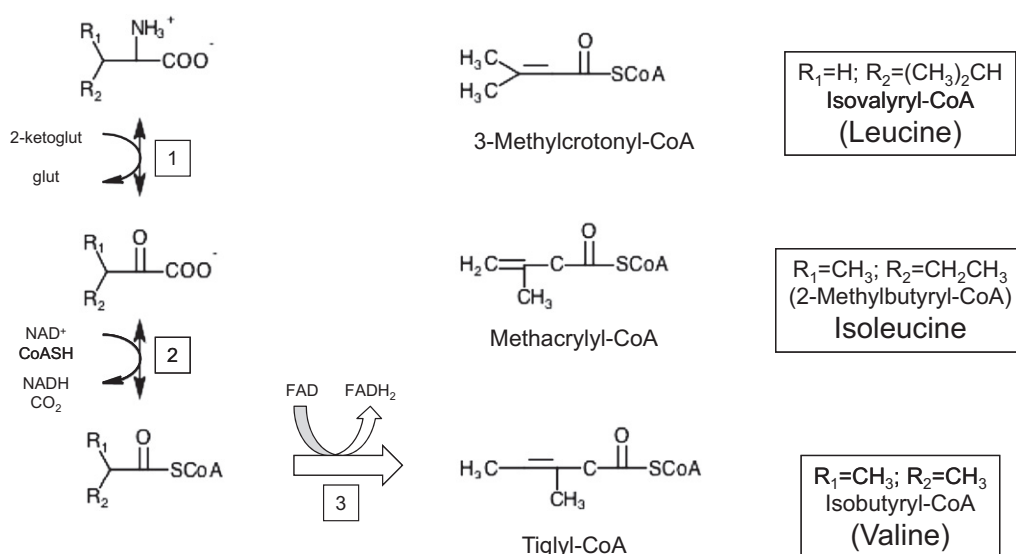
**97.1.5.4 Genetics.** Hydroxymethylglutaric acidemia is inherited as an autosomal recessive trait, and heterozygote detection is possible by demonstrating intermediate lyase activity in leukocytes. The gene encoding HMG-CoA lyase has been cloned and localized to chromosome 1 (1pter-p33) and multiple disease-causing mutations

have been identified (29,33). Prenatal diagnosis has been established by enzyme assay in cultured amniocytes, by metabolite analysis of maternal urine and amniotic fluid, and by mutation analysis on chorionic villus samples.

## 97.1.6 Short/Branched-Chain Acyl-CoA Dehydrogenase Deficiency

This enzyme was originally named 2-methyl branched-chain acyl-CoA dehydrogenase based on the substrate specificity of an enzyme purified from rat liver. At that time, it was not clear whether the same enzyme was active in both isoleucine and valine metabolism. Subsequently, cloning of the human genes showed the two pathways to use distinct enzymes and based on substrate specificity of the expressed human enzymes, the enzymes were designated short branched-chain- and isobutyryl-CoA dehydrogenase (SBCAD and IBD, for the isoleucine and valine enzymes, respectively) (Figure 97-4) (34). Deficiencies of both have now been described.

**97.1.6.1 Clinical Course.** SBCAD deficiency was first described in two patients with rather significant neurologic symptoms (35,36). However, it was soon noted to be present in high frequency in the Hmong population in the United States due to a common founder mutation (37). These individuals, largely identified through newborn screening and family studies, appear to be asymptomatic. Other studies on affected, non-Hmong individuals, again largely identified through newborn screening, corroborate the lack of symptoms (38). Currently, it seems appropriate to consider SBCAD deficiency a biochemical phenotype rather than a clinical disease, although long-term follow-up studies are necessary to



**FIGURE 97-4** Branched-chain acyl-CoA dehydrogenases. The third step in the pathway of all three branched-chain acyl-CoA dehydrogenases is mechanistically shared but performed by three distinct enzymes for each amino acid (3). A common amino transferase (1) and NAD-dependent dehydrogenase (2) generate branched-chain acyl-CoAs unique to each amino acid. Individual defects of the FAD-dependent branched-chain acyl-CoA dehydrogenases are known, and a secondary deficiency of all three is seen in multiple acyl-CoA dehydrogenase deficiency (glutaric aciduria type 2) because of inability to reoxidize  $\text{FADH}_2$ .

define any risks later in life. The major clinical concern is related to the characteristic metabolite (“C5 carnitine”) identified in babies by newborn screening with MS-MS. Identification of 2-methylbutyrylcarnitine and isovalerylcarnitine, isobaric 5 carbon species by MS-MS can be due to either SBCAD or IVD deficiency. Urine organic acid analysis can readily distinguish the two disorders.

**97.1.6.2 Diagnosis.** Most children will be identified through newborn screening with MS-MS. The characteristic metabolite (“C5 carnitine”) can be either 2-methylbutyrylcarnitine or isovalerylcarnitine since they are isobaric. Urine organic acid analysis can readily distinguish the two disorders and should be performed urgently because of the potential for a more severe phenotype due to IVA. Fibroblast or lymphocyte enzyme testing and molecular analysis are possible but of minimal clinical utility.

**97.1.6.3 Treatment.** No treatment is needed based on extensive experience with the Hmong population. In theory, there could be a risk for increased organic acidemia with intercurrent illnesses, so caution by parents at these times seems prudent, and they should seek medical attention if an affected child has protracted vomiting or dehydration.

**97.1.6.4 Genetics.** The SBCAD gene is located on chromosome 10q25–q26. A common c1165A>G mutation in the Hmong population leads both to protein inactivating amino acid substitution and exon skipping due to activation of a cryptic RNA splice site (37). Multiple other private mutations have been described (38).

### 97.1.7 2-Methyl-3-Hydroxybutyryl-CoA Dehydrogenase Deficiency

This enzyme in isoleucine metabolism has received several functional and genetic names since its discovery, which has led to some confusion in the literature. Based on one of the documented activities of the enzyme as a hydroxysteroid (17-beta) dehydrogenase, it has officially been assigned a gene symbol *HSD17B10*, replacing the *HADH2* designation more frequently seen in the inborn errors literature (Figure 97-1) (39). Two clinical phenotypes have been proposed to be the result of two different molecular mechanisms (40).

**97.1.7.1 Clinical Course.** The first patient identified with this deficiency, a male, exhibited a picture of progressive infantile neurodegeneration. He had unexplained metabolic acidosis with hypoglycemia in the newborn period. Modest psychomotor delay was reported in the first year of life, after which he developed choreoathetotic movements and neurointellectual deterioration (41). Metabolic acidosis has not been consistently seen in subsequent patients, but neurodegenerative symptoms have been uniform. Additional reported features in only a handful of patients include myoclonic or other seizures, hypotonia, optic atrophy, pigmentary or nonpigmentary retinopathy, sensorineural deafness, ataxia, dystonia, choreoathetosis, spastic

di-/tetraplegia, cardiomyopathy and mild dysmorphism (42). Cerebral magnetic resonance imaging (MRI) may be normal or exhibit characteristic findings including frontotemporal or frontoparietal atrophy, parieto-occipital periventricular white matter changes, and basal ganglia lesions. These patients have had severe or complete enzyme deficiency. Female patients are typically milder in keeping with X-linked inheritance, but severe disease can occur. X-linked mental retardation associated with choreoathetosis, and abnormal behavior in a four-generation pedigree has been linked to the *HSD17B10* gene but affected patients had near-normal enzyme activity, so an alternative mechanism of pathogenesis was postulated.

**97.1.7.2 Diagnosis.** Urine organic acids are notable for the accumulation of 2-methyl-3-hydroxybutyrate and tiglylglycine but not 2-methylacetoacetic acid. 2-Ethylhydracrylic and 3-hydroxyisobutyric acids have reported to be elevated. Enzyme and molecular testing are available. Prenatal testing has not been reported but should be possible.

**97.1.7.3 Therapy.** No specific therapies are available. Isoleucine restriction is probably not useful. Carnitine supplementation may provide nonspecific benefit related to organic acid conjugation.

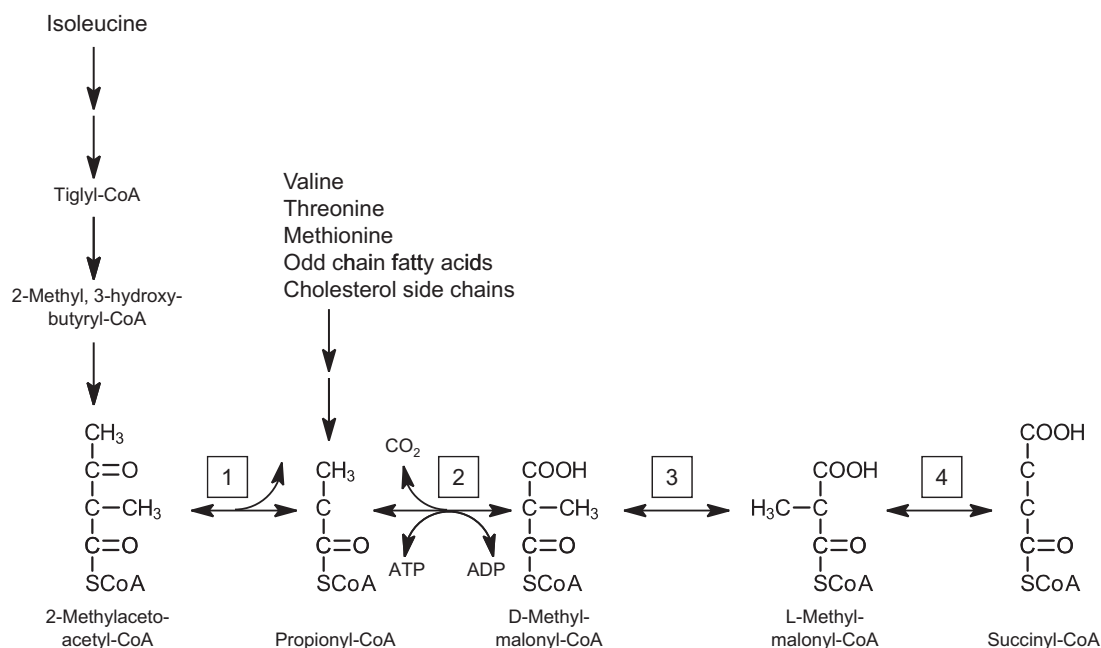
**97.1.7.4 Genetics.** The *HSD17B10* gene is located at Xp11.2. Rare, private mutations inactivating enzyme activity have been reported in males with the full clinical picture (42). In the extended family with normal enzyme activity, a c.574C>A mutation in the codon for R192 does not alter the amino acid at this position but leads to an altered balance of RNA splicing products of unknown significance (40).

### 97.1.8 Mitochondrial Acetoacetyl-CoA Thiolase Deficiency

Mitochondrial acetoacetyl-CoA thiolase deficiency, also called  $\beta$ -ketothiolase, is a mixed disorder of isoleucine catabolism and ketone body production (Figure 97-5) (43). It presents with episodes of acidosis and encephalopathy most frequently occurring in conjunction with upper respiratory tract infections and other minor illness. Accumulation of 2-methyl-3-hydroxybutyric acid in urine is characteristic.

**97.1.8.1 Clinical Course.** The disease can present in the newborn period with hyperammonemia, metabolic acidosis, and severe ketosis, but more often presents beyond the first year of life with fasting or protein-induced episodes of vomiting, hepatomegaly, ketoacidosis, and encephalopathy (44). Mental retardation or death during an episode of ketoacidosis is common, but some patients are relatively free of symptoms. Intrafamilial variation has been reported (45).

**97.1.8.2 Diagnosis.** Organic acid analysis shows increased 2-methyl-3-hydroxybutyric acid, 2-methylacetoacetic acid, and tiglylglycine, but large amounts of normal



**FIGURE 97-5** Formation and metabolism of propionyl-CoA. (1) Acetoacetyl-CoA thiolase. (2) Propionyl-CoA carboxylase. (3) Methylmalonyl CoA racemase. (4) Methylmalonyl-CoA mutase. Adenosylcobalamin is the specific coenzyme for methylmalonyl-CoA mutase.

ketone bodies (3-hydroxybutyric and acetoacetic acids) will often obscure these increases when the patient is acutely ill. Indeed, it may only be possible to detect the diagnostic metabolites when the patient is free of symptoms, or after an oral load (100 mg/kg) of isoleucine. As in propionic and methylmalonic acidemia, glycine is frequently elevated in blood and urine. Enzyme assays to confirm the diagnosis, if necessary, can be performed on fibroblasts or lymphocytes. Mutation analysis is available, but genotype/phenotype correlations have been poor.

**97.1.8.3 Treatment.** Acute episodes should be treated with intravenous glucose and sodium bicarbonate. Long-term treatment with a low-protein diet, combined with the avoidance of fasting, decreases the frequency and severity of episodes of acidosis and permits normal growth and development if irreversible neurologic damage has not already occurred.

**97.1.8.4 Genetics.** The disease is inherited as an autosomal recessive trait. The mitochondrial acetoacetyl-CoA (*ACAT1*) gene is on chromosome 11, and multiple mutations in patients have been described (44). Prenatal diagnosis should be possible by enzyme assay in cultured amniocytes or chorionic villus samples, or by mutation analysis, but has not yet been reported.

### 97.1.9 Isobutyryl-CoA Dehydrogenase Deficiency

Of the three mitochondrial dehydrogenases involved in the third step of leucine, isoleucine, and valine metabolism, isobutyryl-CoA dehydrogenase (IBDH) in the valine pathway was the last to be definitively identified (Figure 97-2) (46). As with deficiency in its companion

enzymes in leucine and isoleucine catabolism, IBDH deficiency is largely identified through newborn screening by MS-MS. While an original symptomatic patient was described, those identified through newborn screening have been mostly well (47).

**97.1.9.1 Clinical Course.** The first patient with IBDH deficiency presented with carnitine and cardiomyopathy at 1 year of age (48). The child responded to carnitine supplementation and has no recurrence of cardiac disease or episodes of metabolic decompensation. Since the identification of the disorder, at least 15 additional patients have been reported, all identified through newborn screening (47,49,50). All but one have remained without symptoms. One patient had recurrent episodes of vomiting and dehydration with intercurrent illnesses (47). Of note, in keeping with observations in the original patient, this infant had significant decreases in plasma free carnitine (to  $<10 \mu\text{M}$ ) during these episodes, suggesting decreased muscle stores or excess renal loss of carnitine. She showed no signs of cardiomyopathy and had normal development at age 5 years.

**97.1.9.2 Diagnosis.** Isobutyrylcarnitine elevation in blood can be documented by tandem mass spectroscopy including newborn screening. Since MS-MS does not distinguish between butyryl- and isobutyryl carnitine, follow-up testing is necessary. Identification of isobutyrylglycine (as opposed to butyrylglycine) in urine differentiates the two disorders, and the diagnosis is then best confirmed by molecular analysis. Enzyme assay on fibroblasts or lymphocytes is possible but not readily available in many clinics.

**97.1.9.3 Treatment.** The need for treatment in IBDH deficiency is unproven. Carnitine losses in two patients

may indicate the need for carnitine supplementation at least when ill, but this remains conjecture. Valine restriction is not warranted.

**97.1.9.4 Genetics.** The gene for IBDH is the *ACAD8* gene located on chromosome 11q25. Multiple private mutations have been identified in patients (47).

### 97.1.10 3-Hydroxyisobutyryl-CoA Deacylase Deficiency

The next step in the valine catabolic pathway associated with a clinical disease is 3-hydroxyisobutyryl-CoA deacylase (Figure 97-1). (Methacrylyl-CoA hydratase deficiency has not been described.) Only two patients have been described with molecularly confirmed diagnoses (51,52). Accumulation of 3-hydroxybutyric acid in urine is not seen. Part of the pathology of this disorder is likely due to the highly reactive chemical nature of accumulated methacrylyl-CoA (51). Patients reported with 3-hydroxyisobutyric acid in urine likely have a different disorder.

**97.1.10.1 Clinical Course.** The first patient with 3-hydroxyisobutyryl-CoA deacylase deficiency presented with vertebral malformations and tetralogy of Fallot along with dysmorphic features (51). There was little growth or development and he died at age 3 months. Agenesis of the cingulate gyrus and corpus callosum was found on autopsy. Enzyme assay confirmed the diagnosis. The second patient appeared well at birth but exhibited neurodegeneration beginning at age 4 months. He had an episode of acute metabolic acidosis at age 14 months during which 3-hydroxyisobutyric acid was not identified in urine. MRI of the brain at that time demonstrated abnormalities in the globus pallidus and the midbrain, with asymmetrical involvement of the cerebral peduncles, although no structural abnormalities were noted. A number of patients have been reported with 3-hydroxyisobutyric acid in urine but no consistent clinical picture suggesting that they have a different disorder(s).

**97.1.10.2 Diagnosis.** 3-Hydroxyisobutyric acid does not accumulate in this disorder, probably because it is in equilibrium with methacrylyl-CoA, which is highly reactive. In the first patient, the alternative metabolites *S*-(2-carboxypropyl)cysteamine and *S*-(2-carboxypropyl)cysteine were seen. These compounds were not reported in the second patient but a hydroxy-C4-carnitine species was elevated. Since mass spectrometry cannot distinguish between hydroxybutyryl- and hydroxyisobutyrylcarnitine, this finding can also be consistent with a disorder of short-chain 3-hydroxyacyl-CoA deficiency. Both patients were shown to have enzymatic deficiency of 3-hydroxyisobutyryl-CoA deacylase on fibroblast assay, but the second patient also had partial deficiencies of respiratory chain complexes I and IV in muscle. Because of the complicated metabolite picture in these patients, it is likely that 3-hydroxybutyryl-CoA deacylase is underdiagnosed

and the full clinical spectrum remains to be described. Patients with 3-hydroxyisobutyric aciduria without deacylase deficiency also have had an inconsistent array of branched-chain organic acids in urine, again pointing to possible heterogeneity in diagnosis (53).

**97.1.10.3 Treatment.** No effective treatment has been reported for the deacylase defect. Experience with other distal disorders in the branched-chain amino acid degradative pathways suggests that valine supplementation is unlikely to be effective. Supplementation with cysteamine to increase conjugation of the accumulating methacrylyl-CoA is an attractive consideration but has not been tested. Carnitine supplementation may provide additional nonspecific conjugation of abnormal intermediates.

**97.1.10.4 Genetics.** The gene for 3-hydroxyisobutyryl-CoA deacylase is designated *HIBCH* and is located on chromosome 2q32.2. Mutation analysis has only been reported on one patient (52). Sequencing of the 3-hydroxyisobutyryl-CoA dehydrogenase gene in one patient with 3-hydroxyisobutyric aciduria failed to identify a mutation and thus the cause for this biochemical phenotype remains unclear (53).

### 97.1.11 Methylmalonate Semialdehyde Dehydrogenase Deficiency

Several reports in the literature describe patients with metabolite accumulation thought to be consistent with methylmalonate semialdehyde dehydrogenase deficiency but only one has subsequently been shown to have mutations in the gene for this enzyme (Figure 97-1) (54).

**97.1.11.1 Clinical Course.** The single patient with this disorder was identified on newborn screening for the unrelated finding of hypermethioninemia (54). Additional metabolic evaluation revealed high levels of urinary 3-hydroxyisobutyric acid and lesser elevations of 2-ethylhydracrylic acid, 3-aminoisobutyric acid, and  $\beta$ -alanine. Valine loading studies induced increased excretion of 3-hydroxyisobutyric acid in urine. A mutation in the *MMSDH* gene was ultimately identified (55). In the same study, three other patients previously suspected as having methylmalonate semialdehyde dehydrogenase deficiency were shown not to have *MMSDH* gene mutations, pointing to the lack of specificity of the metabolic findings. The patient was last reported at age 4 years to be well, so the clinical relevance of the biochemical phenotype is unknown.

**97.1.11.2 Diagnosis.** Based on the single bona fide patient, metabolite detection is unreliable. Patients with significant elevation of 3-hydroxyisobutyric acid in urine of otherwise unknown cause should have sequencing of the *MMSDH* gene.

**97.1.11.3 Treatment.** No treatment is warranted based on the single patient.

**97.1.11.4 Genetics.** The *MMSDH* gene is on chromosome 14q24.3. The single patient described was



homozygous for a single mutation, for which his parents were heterozygous, confirming recessive inheritance.

## 97.2 OTHER ORGANIC ACIDEMIAS

### 97.2.1 Ethylmalonic Encephalopathy

Deficiency of a mitochondrial sulfur dioxygenase leads to a characteristic phenotype known as ethylmalonic encephalopathy. Previously thought to be a disorder in branched-chain amino acid metabolism, the defect rather leads to the accumulation of sulfides within mitochondria, which in turn impairs mitochondrial energy metabolism.

**97.2.1.1 Clinical Course.** Ethylmalonic encephalopathy is characterized by neurodevelopmental delay and regression, prominent pyramidal and extrapyramidal signs, recurrent petechiae, orthostatic acrocyanosis, and chronic diarrhea (56,57). Pyramidal signs, hypotonia, microcephaly, failure to thrive, seizures, and episodic encephalopathy are common. Death usually occurs before age two; however, heterogeneity and longer survival has been reported (58,59). Symmetrical necrotic lesions in the deep gray matter structures are consistent neuropathological features of the disease. Most patients have been of Mediterranean origin. Ethylmalonic encephalopathy is caused by homozygous mutations in the *ETHE1* gene that encodes a mitochondrial sulfur dioxygenase (58). The resultant enzymatic deficiency leads to impaired catabolism of inorganic sulfur (sulfite), accumulation of H<sub>2</sub>S in tissues, and inhibition of the mitochondrial respiratory chain complex IV (60).

**97.2.1.2 Diagnosis.** Ethylmalonic acid accumulation in urine is the hallmark of ethylmalonic encephalopathy, but is nonspecific and can be intermittent in milder cases (58). The compound is likely the product of  $\alpha$ -carboxylation of butyrate elevated secondary to the inhibition of mitochondrial fatty acid oxidation. However, ethylmalonic aciduria is also seen in short-chain acyl-CoA dehydrogenase (SCAD) deficiency and primary respiratory chain deficiencies. Elevation of C4 and C5 carnitines in blood and isobutyrylglycine and 2-methylbutyrylglycine can also be seen in all these disorders. Urinary thiosulfate accumulation may be a more specific marker (60). Ultimately, in the correct clinical

setting, diagnosis by molecular testing should be pursued (61,62).

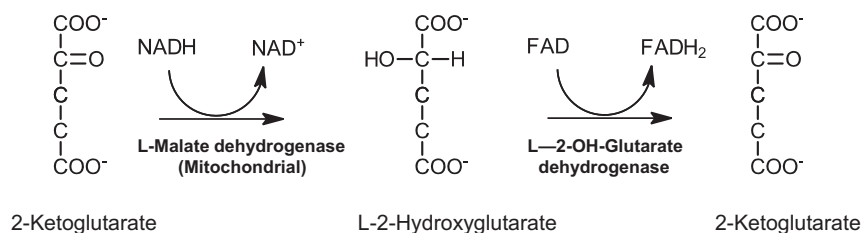
**97.2.1.3 Treatment.** Identification of the enzymatic cause of ethylmalonic encephalopathy has led to new therapeutic options in this previously untreatable disorder. In a single study, metronidazole, or N-acetylcysteine (a precursor of sulfide-buffering glutathione) substantially prolonged the lifespan of *ETH1*-deficient mice, with the combined treatment being additive. The same dual treatment caused marked clinical improvement in five affected children, with no reported adverse side effects (63). Additional studies will be needed to confirm the efficacy of this promising regimen.

**97.2.1.4 Genetics.** The molecular cause of ethylmalonic encephalopathy was first localized to chromosome 19q13 in a genetic mapping study and multiple mutations were ultimately identified in the *ETHE1* gene. All patients have mutations of both alleles, confirming autosomal recessive inheritance. No common mutations have been identified.

### 97.2.2 L-2-Hydroxyglutaric Acidemia

L- and D-2-Hydroxyglutaric acids are chiral enantiomers and require special separation techniques to be differentiated (64). Patients with L-2-hydroxyglutaric acidemia have a defect in the mitochondrial flavoenzyme L-2-hydroxyglutarate dehydrogenase that catalyzes the conversion of L-2-hydroxyglutarate to 2-ketoglutarate (Figure 97-6) (65). They exhibit a variable leukodystrophy. Of note, L-2-hydroxyglutarate does not seem to have a physiologic function in human metabolism (66). Rather it appears to be the product of mitochondrial L-malate dehydrogenase illicitly acting on 2-ketoglutarate. Thus, L-2-hydroxyglutarate dehydrogenase has been called a metabolic repair enzyme, preventing the accumulation of its extremely toxic substrate.

**97.2.2.1 Clinical Course.** The first patient described with this disorder had relatively nonspecific mental retardation (67). Additional patients were subsequently identified with mental retardation, cerebellar symptoms, and movement abnormalities with onset of symptoms in childhood (2). MRI revealed a consistent pattern including subcortical leukoencephalopathy, cerebellar atrophy, and signal changes in the putamina and dentate nuclei.



**FIGURE 97-6** Physiological role of L-2-Hydroxyglutarate metabolism. L-2-hydroxyglutarate dehydrogenase serves to oxidize its highly toxic substrate produced as a secondary reaction product of L-malate dehydrogenase in mitochondria.

Later onset patients have exhibited milder and relatively nonspecific symptoms including autism (68). Intriguingly, patients are at increased risk to develop CNS tumors, although the percentage so affected is still small (~5%) (69). Riboflavin (100 mg daily) and flavin adenine dinucleotide (FAD) (30 mg daily) supplementation have been reported to improve symptoms in separate patients (70,71). Successful pregnancy in an affected woman has been reported (72).

**97.2.2.2 Diagnosis.** 2-Hydroxyglutaric acid is identifiable in urine by routine organic acid analysis, but additional separation techniques are required to differentiate the L and D enantiomers (64). The metabolite is also elevated in brain. L-2-hydroxyglutaric acid is often elevated in glutaric academia type II, but other metabolites permit unequivocal differentiation of these disorders. Lysine is consistently elevated in blood and should not be confused with hyperlysinemia. Characteristic findings on MRI of the brain include a combination of predominantly subcortical cerebral white matter abnormalities and abnormalities of the dentate nucleus, globus pallidus, putamen, and caudate nucleus (73). Enzymatic assay is difficult and thus molecular analysis is probably the definitive diagnostic test of choice (74,75).

**97.2.2.3 Treatment.** Riboflavin (100 mg daily) and FAD (30 mg daily) supplementation have been reported to improve symptoms in separate patients but larger

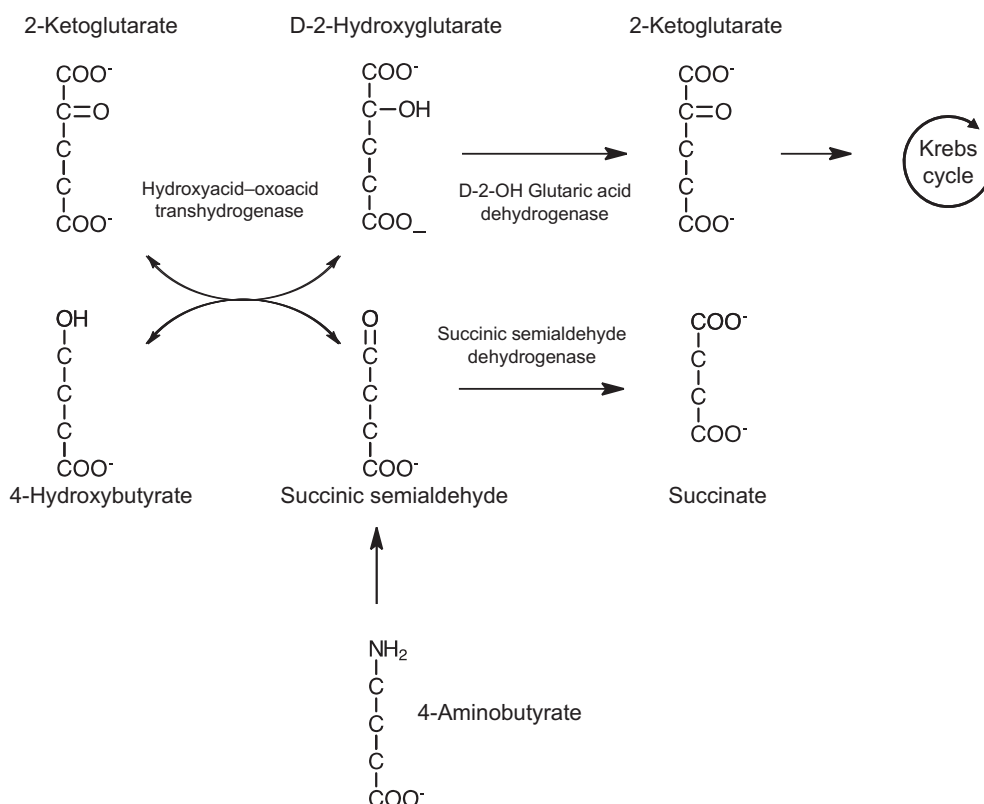
trials have not been performed (70,71). Carnitine may increase organic acid excretion nonspecifically.

**97.2.2.4 Genetics.** The L-2-hydroxyglutaric dehydrogenase (*L2GHDH*) gene is located on chromosome 14q22.1. Multiple private mutations have been reported. Turkish and Iraqi isolates exist (76,77).

### 97.2.3 D-2-Hydroxyglutaric Acidemia

D-2-Hydroxyglutaric acidemia is less frequent than its L-counterpart and has two genetic causes with variable symptoms. D-2-Hydroxyglutaric acid is generated from 2-ketoglutarate by the enzymatic action of hydroxyacid-oxoacid transhydrogenase but the role of D-2-hydroxyglutaric acid in normal metabolism remains undefined (Figure 97-7) (78).

**97.2.3.1 Clinical Course.** Variable clinical symptoms have been described in patients with D-2-hydroxyglutaric aciduria. In one large survey of 17 patients, 10 had a severe early-infantile-onset encephalopathy characterized by dysmorphic features, epilepsy, hypotonia, cerebral visual failure, and little development. In addition, five of these patients had cardiomyopathy (79). Dysmorphic features described include coarse facial appearance, flat face, broad nasal bridge, upturned nose, and simple and anteverted ears (80). Seven patients had a much milder and variable clinical picture, most often characterized by mental retardation, hypotonia, and macrocephaly.



**FIGURE 97-7** D-2-Hydroxyglutarate metabolism. D-2-Hydroxyglutarate is formed as a product of the enzyme hydroxyacid-oxoacid transhydrogenase, but the physiologic role of this metabolite remains unknown.

Asymptomatic patients have been reported (81). Defects in two enzymes have now been reported in patients with D-2-hydroxyglutaric acidemia but clinical correlations have not yet been possible. Both mild and severely affected patients have been shown to have mutations in the gene for D-2-hydroxyglutaric acid dehydrogenase, while others have mutations in the mitochondrial isocitrate dehydrogenase 2 gene (74,82,83).

**97.2.3.2 Diagnosis.** Biochemical findings include elevations of D-2-hydroxyglutaric acid in urine, plasma, and cerebrospinal fluid (CSF).  $\gamma$ -Aminobutyric acid is also elevated in CSF. Urinary citric acid cycle intermediates are variably elevated. Brain MRI in severely affected patients shows mild ventriculomegaly with enlarged frontal subarachnoid spaces and subdural effusions similar to those seen in glutaric aciduria (79). Cerebral maturation is delayed. Subependymal cysts over the head or corpus of the caudate nucleus are seen in patients <6 months of age. Elevated D-2-hydroxyglutaric acid in urine has been reported in patients with proven succinic semialdehyde dehydrogenase deficiency (84).

**97.2.3.3 Treatment.** No specific therapy is currently available for the disorder. Seizures and movement abnormalities should be treated symptomatically.

**97.2.3.4 Genetics.** The *D2HGA* gene for D-2-hydroxyglutaric acid dehydrogenase is on chromosome 2q37.3. The gene for the mitochondrial isocitrate dehydrogenase (*IDH2*) is on chromosome 15q26.1. Mutations in patients are nearly evenly divided between the two loci (74,82,83). *D2HGA* gene mutations are recessive, but *IDH2* mutations appear to be dominant.

## 97.2.4 Propionic Acidemia

Propionic acidemia, one of the more common organic acidemias, was first described in 1968 in an infant with severe metabolic acidosis, and many additional patients have since been reported. Propionyl-CoA is an intermediate in the oxidation of four amino acids (threonine, valine, methionine and isoleucine) as well as odd-chain fatty acids. Propionic acid is also absorbed from the large intestine where it is produced by propiogenic bacteria. Propionic acidemia is due to deficiency of propionyl-CoA carboxylase, a mitochondrial biotin-containing enzyme that catalyzes conversion of propionyl-CoA to D-methylmalonyl-CoA (Figure 97-5). The disorder is extremely variable and identification through newborn screening is possible.

**97.2.4.1 Clinical Course.** The disorder may present in the first week of life with feeding difficulties, lethargy, vomiting, and life-threatening acidosis, hypoglycemia, hyperammonemia, and bone marrow suppression (85,86). Severe hyperammonemia probably contributes appreciably to the encephalopathy of the acutely ill neonate, possibly because propionyl-CoA inhibits the synthesis of N-acetylglutamate, the major allosteric activator of carbamyl phosphate synthetase. Mortality in early-onset

disease is high (87). Equally common is a more chronic course, which presents in the first months of life with poor feeding and episodes of vomiting, infection-induced ketoacidosis, failure to thrive, and osteoporosis severe enough to cause pathologic fractures. Developmental retardation, which is probably due to neonatal hyperammonemia or chronic illness, is common, and metabolic strokes due to acute degeneration of the basal ganglia may occur during or between episodes of ketoacidosis (88). Cardiomyopathy, which may be rapidly fatal, occurs frequently and does not respond to carnitine (89). Pancreatitis is an increasingly recognized complication of the disease (90). Before expanded newborn screening, most patients did not survive beyond the first decade of life, with death often occurring during an episode of ketoacidosis in a chronically malnourished child. Newborn screening via tandem mass spectroscopy reliably can identify propionic acidemia before symptoms occur and a much milder clinical spectrum results. However, the specificity of moderately elevated C3 carnitine levels in newborns remains controversial.

**97.2.4.2 Diagnosis.** Urine organic acids at diagnosis show large amounts of 3-hydroxypropionic and methylcitric acids, often with propionylglycine and tiglylglycine, and abnormal ketone bodies such as 3-hydroxy-n-valeric and 3-keto-n-valeric acids. Acylcarnitine analysis by MS-MS shows increased C3 carnitine, and glycine levels are often elevated in blood and urine. Although usually not necessary, the enzyme defect can be demonstrated in many tissues, including leukocytes and cultured fibroblasts. Molecular testing is clinically available. Prenatal diagnosis has been accomplished with metabolite, enzymatic, and molecular techniques (91).

**97.2.4.3 Treatment.** Acute therapy is directed to treating shock, acidosis, hypoglycemia, and hyperammonemia with fluids, bicarbonate, glucose, and dialysis. Restriction of dietary natural protein (or of propiogenic amino acids) to amounts necessary to support normal growth and development is indicated, and usually results in natural protein intake less than 1 g/kg/day. Specialized formula restricted in isoleucine, valine, methionine, and threonine is used to provide additional protein for growth. Biotin supplementation is not clinically useful, but oral carnitine may be helpful to increase excretion of nontoxic metabolites. N-carbamylglutamate may help resolve hyperammonemia more quickly during episodes of acute metabolic decompensation (92,93). Metronidazole given on an intermittent basis decreases the load of propiogenic bacteria in the bowel. Liver transplant eliminates the risk for episodes of metabolic decompensation and can reverse cardiomyopathy (94,95).

**97.2.4.4 Genetics.** Propionic acidemia is inherited as an autosomal recessive trait. The genes encoding the  $\alpha$ - and  $\beta$ -subunits have been localized to chromosomes 13 (13q32) and 3 (3q13.3–22), respectively, and several disease-causing mutations have been identified in both genes (96–98). No one prominent  $\alpha$ -gene mutation has





atrophy, hyperkinesia, mental retardation, growth failure, central and cortical atrophy of the brain, and basal ganglia atrophy (103).

Mutations in the *CblC* gene cause combined methylmalonic acidemia and homocystinemia. *CblC* deficiency most commonly presents in infancy with severe clinical manifestations including basal ganglia necrosis, microcephaly, failure to thrive, mental retardation, retinopathy, and megaloblastic anemia. Thromboembolic events can occur because of elevated homocystine in the blood. However, patients with milder, late-onset disease have been reported (104). *CblD* mutations can cause combined disease as seen in *CblC*-deficient patients, but variants with isolated methylmalonic acidemia as well as isolated homocystinemia have been identified (105). Patients with defects in the *CblE* and *CblG* groups are deficient only in methylcobalamin biosynthesis, and have homocystinuria without methylmalonic aciduria (106). *CblF* deficiency results in defective transport of B12 out of lysosomes and a combined methylmalonic acidemia and homocystinemia (107). The clinical picture can be severe or mild.

Methylmalonic acidemia is now often identified through newborn screening by MS-MS; however, reports of the clinical efficacy of early detection have been mixed (99,108,109).

**97.2.5.2 Diagnosis.** Urine organic acids show increased methylmalonic acid and, especially in mutase-deficient patients, 3-hydroxypropionic and methylcitric acids, and tiglylglycine (99). The same abnormal ketone bodies noted in propionic acidemia are seen in this condition. Acylcarnitine analysis by MS-MS shows increased C3 carnitine, and glycine is usually elevated in blood and urine. Megaloblastosis is often observed in patients with the *cblC*, *cblD*, and *cblF* defects. The homocystinemia and homocystinuria seen in such patients is accompanied by low methionine and high cystathionine in serum and not, as in cystathionine synthetase deficiency, by high methionine and low cystathionine. Excretion of methylmalonic acid can be seen in dietary B12 deficiency but is not as pronounced as in the inherited disorders unless the vitamin deficiency is very severe (110). Differentiation among the various enzyme deficiencies often requires a combination of fibroblast enzyme analysis,

complementation studies, and molecular sequencing of candidate genes.

**97.2.5.3 Treatment.** As in propionic acidemia, treatment in episodes of acute metabolic decompensation is directed first to treating shock, acidosis, hypoglycemia, and hyperammonemia, followed by restriction of protein (specifically, propiogenic amino acids). Carnitine is used to treat secondary carnitine deficiency. Some patients treated in this manner do well, but many do not and die in early childhood, often during an episode of ketoacidosis (99). Patients with defects in cobalamin metabolism may respond partially or completely to supplementation with intramuscular hydroxycobalamin, but residual metabolic abnormalities are common. Betaine hydrochloride, which promotes conversion of homocysteine to methionine by betaine:homocysteine methyltransferase, can decrease homocystine levels in blood in patients with combined disorders. Liver transplantation in isolated mutase deficiency can reduce metabolic symptoms, and one patient with chronic renal failure was treated successfully with combined liver–kidney transplantation (111,112).

Patients with defects in adenosyl-B<sub>12</sub> biosynthesis often respond to large doses of B<sub>12</sub>. Even with treatment, however, many patients succumb to pancytopenia, renal involvement and neurologic impairment resembling hemolytic–uremic syndrome or to metabolic coma and cardiorespiratory arrest during childhood. Long-term survival does, however, occur, often with neurologic deficits. Prenatal diagnosis by metabolite, enzyme, or molecular analysis should be possible for all of the disorders.

**97.2.5.4 Genetics.** All forms of congenital methylmalonic acidemia are transmitted as autosomal recessive traits (Table 97-2). Multiple mutations in patients with all the disorders have been reported.

## 97.2.6 Glutaryl-CoA Dehydrogenase Deficiency

Glutaric acidemia (type I) was first described in 1975 in two siblings with a progressive movement disorder beginning in early childhood (113). The condition is inherited as an autosomal recessive trait and is due to deficiency of glutaryl-CoA dehydrogenase, a mitochondrial enzyme that converts glutaryl-CoA, an intermediate

**TABLE 97-2 Complementation Groups in Cobalamin Metabolic Disorders**

Metabolite Present	Cbl Complementation Group								
	A	B	C	D	D (v1)	D (v2)	E	F	G
Methylmalonic acid	+	+	+	+		+		+	
Homocystine		+	+	+	+		+	+	+
Enzyme activity	?	Cob(1)alamin adenosyl-transferase	?	?			Methionine synthase reductase	?	Methionine synthase
Gene	MMAA	MMAB	MMACHC	MMADHC			MTRR	LMBRD1	MTR
Chromosome	4q31.1–q31.2	12q24	1p34.1	2q23.2			5p15.3–p15.2	6q13	1q43

in the oxidation of lysine, tryptophan and hydroxylysine, to crotonyl-CoA (Figure 97-9).

**97.2.6.1 Clinical Course.** Most patients with glutaric acidemia are born with relative macrocephaly, and suddenly develop hypotonia and dystonia during or after a relatively minor infection between the ages of 6 months and 3 years (114). Seizures, abnormal movements, hypoglycemia, hepatomegaly, and acidosis may be noted during the episode, and CT or MRI scans show acute striatal degeneration, with shrinkage of the caudate and putamen. Less common is a more chronic course in which dystonia and athetosis develop gradually during the first years of life (115). Fatty changes in the viscera, and gliosis and neuronal loss in the putamen and lateral aspects of the caudate, have been described at autopsy. Perhaps 5% of enzyme-deficient individuals remain asymptomatic. Diagnosis through expanded newborn screening with MS-MS reduces the risk for acute metabolic episodes and chronic neurologic damage (116,117).

**97.2.6.2 Diagnosis.** Type 1 glutaric acidemia is caused by a deficiency of glutaryl-CoA dehydrogenase. In most instances glutaric and 3-hydroxyglutaric acids are increased in urine; acylcarnitine analysis by MS-MS shows increased glutaryl carnitine (C5 hydroxycarnitine). Serum carnitine may be low (116). Some patients have easily detectable abnormal organic aciduria only when they are ill, and a few have so little glutaric aciduria even when ill that they are very difficult to detect with organic acid and/or acylcarnitine analysis. Diagnosis of such patients may require measurement of glutaric and 3-hydroxyglutaric acid in serum or urine by stable isotope dilution GC-MS, assay of enzyme activity in leukocytes or cultured fibroblasts, or mutation analysis. Molecular diagnosis is available. Glutaric aciduria type 2, also known as multiple acyl-CoA dehydrogenase, is a distinct disorder caused by the deficiency of either the electron transfer flavoprotein (the physiologic electron acceptor for glutaryl-CoA dehydrogenase) or its dehydrogenase and is discussed later in this chapter. Glutaric aciduria type 3 appears to be a benign phenotype of no clinical relevance caused by a deficiency of a gene of still unknown function. These individuals do not accumulate 3-hydroxyglutarate, glutaryl carnitine, or glutarylglycine in urine.

**97.2.6.3 Treatment.** Treatment of neurologically impaired patients is not usually effective, but when begun

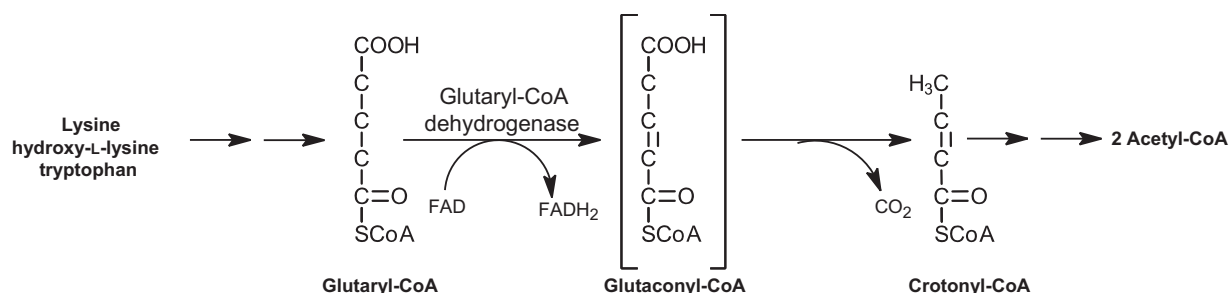
before the onset of symptoms appears to prevent damage in two-thirds of cases (115,116). Treatment involves preventing catabolism during fasting and/or infections with intravenous fluids, electrolytes, glucose and insulin if necessary, and oral carnitine, as well as symptomatic treatment. Natural protein (or lysine) restriction is less well established in preventing symptoms, but should probably be tried. Supplementation with carnitine, creatine, glutamine, riboflavin, coenzyme Q10, pantothenic acid and  $\alpha$ -linolenic acid as a cocktail has been used extensively in the Amish population, but has not been standardized in other ethnic groups (117). Aggressive neurologic management of seizures and spasticity is necessary for patients with striatal damage.

**97.2.6.4 Genetics.** Consistent with inheritance as an autosomal recessive trait, the gene encoding glutaryl-CoA dehydrogenase is located on chromosome 19 (19p13.2). Many disease-causing mutations have been identified, but none is common except in inbred populations (118,119). A common Amish isolate has been described (117). Prenatal diagnosis is possible by enzyme assay in cultured amniocytes or chorionic villus samples, by mutation analysis, or by demonstrating large amounts of glutaric acid in amniotic fluid (118). Glutaric aciduria type 3 in the Amish population is caused by mutations in the C7orf10 gene on chromosome 7p14.

## 97.2.7 Mevalonate Kinase Deficiency

Mevalonic acidemia is caused by a deficiency of mevalonate kinase, an enzyme involved in the biosynthesis of cholesterol and nonsterol isoprenes from 3-hydroxy-3-methylglutaryl-CoA, and is inherited as an autosomal recessive trait (Figure 97-3) (120).

**97.2.7.1 Clinical Features.** Mevalonate kinase deficiency is an extremely heterogeneous disorder (121). The most severely affected patients have had profound developmental delay, distinctive facial dysmorphism, cataracts, lymphadenopathy and hepatosplenomegaly, and have died in infancy. Less severely affected patients have had milder retardation and hypotonia, myopathy, and ataxia. All have had recurrent crises of fever, lymphadenopathy, arthralgia, subcutaneous edema, and a morbilliform rash. Neuroimaging shows



**FIGURE 97-9** Glutaryl-CoA dehydrogenase both dehydrogenates and decarboxylates the substrate. Electrons pass from the FAD of glutaryl-CoA dehydrogenase into the electron transport chain at coenzyme Q, through the ETF and ETF:QO.

progressive atrophy of the cerebellum (122). When less complete, the same enzyme defect causes hyperimmunoglobulinemia D and periodic fever syndrome, characterized by recurrent febrile episodes accompanied by lymphadenopathy, abdominal distress, joint involvement and skin lesions, but without mevalonic aciduria (121).

**97.2.7.2 Treatment.** Treatment has largely been non-specific and ineffective (121). Cholesterol restriction and use of lovastatin can worsen symptoms. Corticosteroids (prednisone 2mg/kg/day) may be helpful to reduce the duration of acute crises. Long-term administration of antioxidants such as vitamin C and ubiquinone has been proposed. A three-year-old boy with severe disease was free of symptoms for 15 months following allogeneic bone marrow transplant (123).

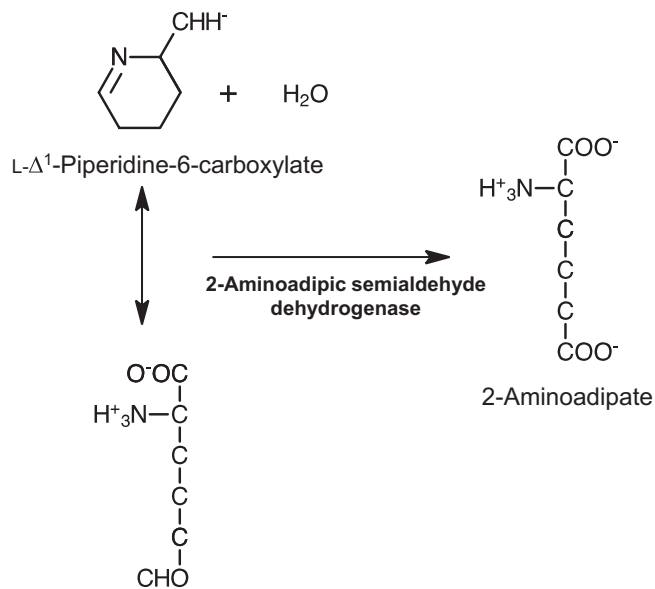
**97.2.7.3 Diagnosis.** Organic acid analysis shows increased mevalonic acid lactone, and mevalonic acid can then be measured in blood and/or urine by stable isotope dilution GC-MS (120). If necessary, enzyme deficiency can be demonstrated in cultured fibroblasts or lymphocytes.

**97.2.7.4 Genetics.** The gene encoding mevalonate kinase has been localized to chromosome 12 (12q24), and although several disease-causing mutations have been identified, none is common (124). Prenatal diagnosis is possible by enzyme assay in chorionic villus samples or by showing increased mevalonic acid in amniotic fluid.

### 97.2.8 L- $\alpha$ -Aminoadipic Semialdehyde Dehydrogenase Deficiency

Pyridoxine-dependent seizures were first described in the medical literature in 1954, but only recently has the cause been identified as a deficiency of the enzyme L- $\alpha$ -aminoadipic semialdehyde dehydrogenase (*ALDH7A1*), also known as antiquinin (Figure 97-10) (125,126). Multiple mutations have now been described in this gene and the clinical phenotype has expanded beyond isolated seizures (127).

**97.2.8.1 Clinical Features.** Pyridoxine-dependent seizures were originally defined as clonic seizures responsive to pyridoxine supplementation in the first month of life, but it is now clear that in atypical cases, they may begin as late as 2 years of life. The electroencephalograph (EEG) is usually abnormal and may show a burst suppression pattern. MRI of the brain may be normal or show a variety of nonspecific abnormalities (128). While seizures are usually isolated, they may occur in the context of electrolyte imbalances, infections, endocrine disturbances (including hypothyroidism and diabetes insipidus), irritability, feeding intolerance, hypotonia, and respiratory distress (127). It should thus be considered in all sick infants with seizures. L- $\alpha$ -aminoadipic semialdehyde is in equilibrium with L- $\Delta^1$ -piperidine 6-carboxylate in cells and when the latter accumulates in



2-Aminoadipic semialdehyde

**FIGURE 97-10** Pyridoxine-dependent seizures are caused by a deficiency in 2-aminoadipic semialdehyde dehydrogenase. Pyridoxine is lost from cells after it reacts nonenzymatically with L- $\Delta^1$ -piperidine-6-carboxylate, which is in chemical equilibrium with 2-aminoadipic semialdehyde.

this defect, it reacts nonenzymatically with and depletes pyridoxine phosphate (127).

**97.2.8.2 Treatment.** Rapid response of seizures to pyridoxine supplementation (50–100mg intravenous (IV)) is usual but may take up to 7 days. Patients can then be maintained on 5–10mg/kg/day per os (PO). Other antiepileptics are usually not effective and generally not necessary to control seizures.

**97.2.8.3 Diagnosis.** Pípecolic acid is elevated in the blood and CSF but is not specific to this disorder (129). Elevation of L- $\alpha$ -aminoadipic semialdehyde and its ratio to creatinine in urine is diagnostic and the level usually drops with treatment (127). CSF and blood levels of threonine, glycine, taurine, and 3-methoxytyrosine may be elevated, mimicking pyridoxine-5'-phosphate oxidase deficiency, while glycine, taurine, and glutamine are often elevated in blood. Definitive testing is through molecular analysis of the *ALDH7A1* gene. Enzyme analysis is not readily available.

**97.2.8.4 Genetics.** *ALDH7A1* is on chromosome 5q31 and the disorder is inherited in an autosomal recessive manner. Sixty-four disease-causing mutations, mostly private, have been published. Genotype/phenotype correlations have been poor (127).

### 97.2.9 Succinic Semialdehyde Dehydrogenase Deficiency

This disorder is a part of the degradation pathway of the neurotransmitter GABA but manifests with increased excretion of the organic acid 4-hydroxybutyrate in urine (Figure 97-7).

**97.2.9.1 Clinical Features.** The most frequent clinical findings in this disorder include mild to moderate global developmental delay, expressive language delay, variable mental retardation, hypotonia, dystonia, seizures, hyporeflexia, ataxia, and behavioral problems (130,131). Age of onset varies from <1 to 21 years of age. MRI of the brain typically reveals an increased T2-weighted signal of the globus pallidus, with variable involvement of white matter and the cerebellar dentate nucleus.

**97.2.9.2 Treatment.** Vigabatrin may improve ataxia and dystonia. It probably does not affect intellectual development and has been reported to both help and aggravate seizures (132,133).

**97.2.9.3 Diagnosis.** 4-Hydroxybutyrate is excreted in urine, but some extraction techniques may lead to loss of this volatile compound. If the diagnosis is suspected, additional specific metabolite analysis is warranted (134). 4-Hydroxybutyrate is also present in high concentrations in CSF. 4-Aminobutyrate and homocarnosine are also elevated in urine and CSF, and glutamine may be low. Enzyme analysis is possible but not often clinically useful. With characteristic metabolites present, it is more expedient to proceed directly to molecular testing.

**97.2.9.4 Genetics.** The gene for succinic semialdehyde dehydrogenase (*ALDH5A1*) is on chromosome 6p22. Multiple mutations have been described in patients with no common mutations (135).

## 97.3 DISORDERS OF FATTY ACID OXIDATION: INTRODUCTION

Mitochondrial fatty acid oxidation is a complex process involving transport of activated acyl-CoA moieties into the mitochondria, and sequential removal of two carbon acetyl-CoA units. It is the main source of energy for many tissues including heart and skeletal muscle and is critically important during times of fasting or physiologic stress. When the body's glycogen stores are depleted, long-chain fatty acids are mobilized from adipose tissue and taken up by liver and muscle cells. While short- and medium-chain fatty acids ( $C_4$  to  $C_{12}$ ) diffuse freely across plasma and mitochondrial membranes, the transport of longer chain species ( $C_{14}$  to  $C_{20}$ ) depends at least in part on active transport, a high-affinity mechanism of major physiological importance in skeletal muscle, liver, and adipocytes (136,137). Two additional enzymatic steps are necessary for the complete oxidation of mono- and diunsaturated fatty acids, 2,4 dienoyl-CoA reductase and an enoyl-CoA isomerase, which allow for the complete oxidation of physiologically abundant fatty acids such as linoleate ( $C_{18:2}$ ) and oleate ( $C_{18:1}$ ) (138,139). Each cycle of the pathway produces a molecule of acetyl-CoA and a fatty acid with two fewer carbons. Under physiological conditions, the latter reenters the cycle until it is completely consumed. In peripheral tissues, the acetyl-CoA is terminally oxidized in the Krebs cycle for ATP production. In the liver,

the acetyl-CoA from fatty acid oxidation can instead be utilized for the synthesis of ketones, 3-hydroxybutyrate, and acetoacetate, which are then exported for final oxidation by brain and other tissues (140). At least 25 enzymes and specific transport proteins are responsible for carrying out the steps of mitochondrial fatty acid metabolism, some of which have only recently been recognized (Figures 97-11 and 97-12) (141–143). Of these, defects in at least 22 have been shown to cause disease in humans (143).

Most patients with fatty acid oxidation defects are now identified through newborn screening by MS-MS of carnitine esters in blood spots. Unscreened patients can present throughout life. In the first week of life, cardiac arrhythmias, hypoglycemia, sudden death, and occasionally with facial dysmorphism and malformations, including renal cystic dysplasia are seen. Symptoms in later infancy and early childhood may relate to the liver or cardiac or skeletal muscle dysfunction, and include fasting or stress-related hypoketotic hypoglycemia or Reye-like syndrome, conduction abnormalities, arrhythmias or dilated or hypertrophic cardiomyopathy, and muscle weakness or fasting- and exercise-induced rhabdomyolysis. Adolescent- or adult-onset muscular symptoms, including rhabdomyolysis, and cardiomyopathy predominate.

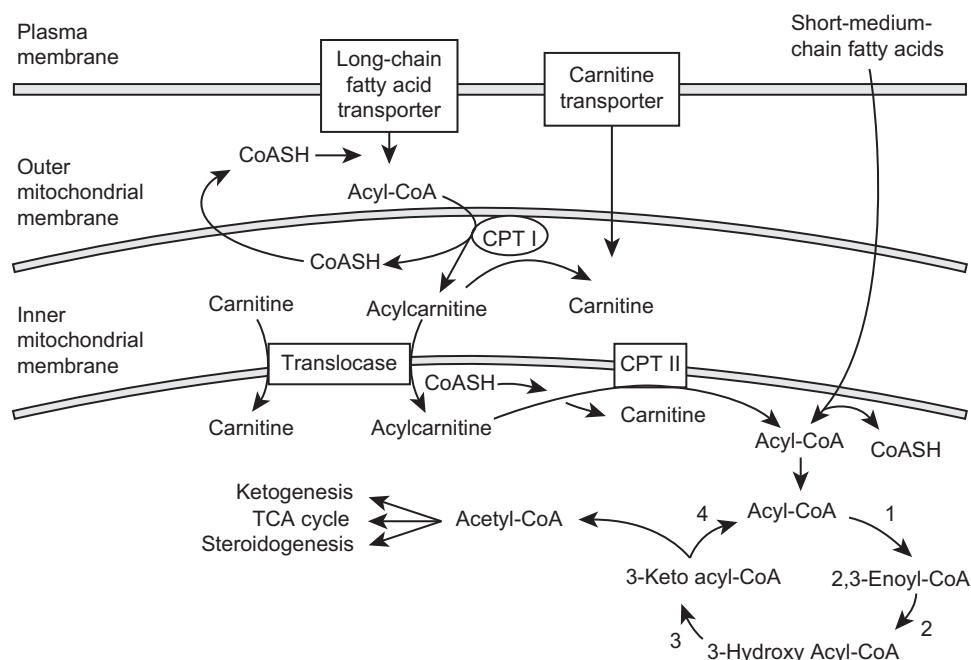
Diagnosis can usually be established even when the patient is asymptomatic, although analysis of samples during acute illness can uncover some mild cases. The most important single diagnostic test is analysis of acyl-carnitine esters in serum, plasma, or dried blood spots by tandem MS, which will identify characteristic compounds in many of these conditions. Other tests that may be useful include urine organic acids and acylglycines, free and total carnitine in serum and urine, and enzyme assays or flux studies in leukocytes or fibroblasts.

Treatment of the acute encephalopathy of hypoketotic hypoglycemia is by intravenous glucose and L-carnitine. Long-term therapy involves replenishing carnitine stores with L-carnitine, and preventing hypoglycemia. In some cases this can be done by providing a snack or glucose polymers before bedtime, but in others requires continuous intragastric feeding. Supplementation with medium-chain triglyceride (MCT) oil provides a fat source that can be utilized by patients with long-chain defects.

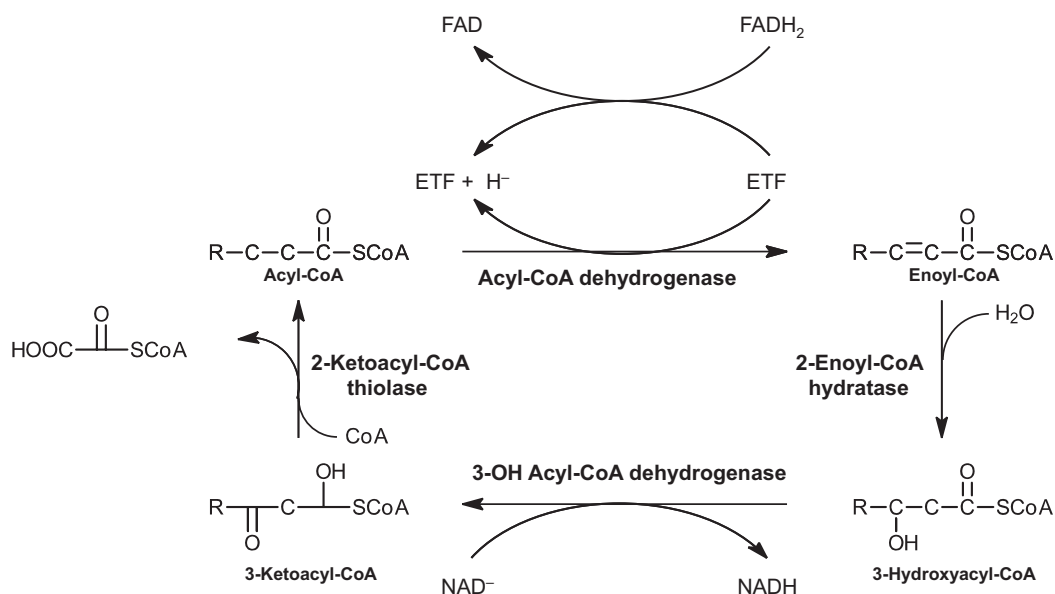
### 97.3.1 Long-Chain Fatty Acid Transport Defect

Two patients affected with a defect of long-chain fatty acid transport at the plasma membrane level have been reported (Figure 97-11) (3). The first patient had a history of recurrent acute liver failure and uninformative or nonspecific findings of biochemical investigations. In some of the episodes, hyperammonemia and encephalopathy were also present, but skeletal or cardiac





**FIGURE 97-11** Fatty acid transport and mitochondrial oxidation. Medium- and short-chain fatty acids do not require an active transport mechanism to reach the mitochondrial matrix. Long-chain fatty acids and carnitine are actively transported across the plasma membrane by tissue-specific transporters. Enzymes of the carnitine cycle (CPT I, translocase, and CPT II) shuttle long-chain fatty acids across the mitochondrial membranes. The fatty acid  $\beta$ -oxidation spiral includes an FAD-dependent acyl-CoA dehydrogenase step (1) followed by a 2,3-enoyl-CoA hydratase reaction (2), the NAD-dependent 3-hydroxyacyl-CoA dehydrogenase step (3) and the thiolase cleavage reaction (4). CPT, carnitine palmitoyltransferase; CoASH, free coenzyme A; TCA, tricarboxylic acid cycle.



**FIGURE 97-12** The fatty acid oxidation spiral. Multiple enzymes with chain-specific substrate utilization optima exist at each step.

myopathy was never observed. Growth and development were normal and between episodes the patient was entirely well. At 5 years of age, however, he underwent orthotopic liver transplantation following a life-threatening episode that evolved into chronic hepatic insufficiency. The second patient presented with fulminant liver failure at 4 years of age and also underwent

liver transplantation (3). Both patients are doing well several years posttransplant.

In cultured skin fibroblasts, the oxidation rates of  $C_{14}$  to  $C_{18}$  fatty acids were reduced with the severity of the defect correlating to the chain length of the substrate, but were normal in digitonin-permeabilized cells. The site of the defect was confirmed by reduced velocity of  $C_{14}$  to

C<sub>20</sub> fatty acid uptake, while the uptake of 2-deoxy-D-glucose, carnitine, and palmityl-L-carnitine were normal. The two index cases did not show correction of the uptake defect after complementation, providing additional evidence that they are affected with the same primary defect. However, any other combinations lead to normalization of oleate uptake, suggesting the possible existence of multiple defects manifesting with impaired long-chain fatty acid uptake in vitro. The gene encoding this transporter(s) has not yet been identified.

### 97.3.2 Carnitine Uptake Defect (Primary Carnitine Deficiency)

Before undergoing  $\beta$ -oxidation, free fatty acids must be activated to their corresponding acyl-CoA thioesters by long-chain specific acyl-CoA synthetases (Figure 97-11) (144). Short- and medium-chain carboxylic acids directly enter the mitochondrial matrix where they are activated. In contrast, long-chain fats are activated in the cytoplasm and require active transport into mitochondria. Transport of long-chain acyl-CoAs requires at least two enzymes, a transporter protein and the use of carnitine as an intermediate carrier molecule. Carnitine is itself transported intracellularly by a specific transporter protein (145). Two carnitine transporters have been described, one specific to the liver and a second with a more ubiquitous distribution including kidney, muscle, and fibroblasts.

Primary carnitine deficiency is caused by a defect in the sodium-dependent high-affinity carnitine transporter in the plasma membrane of muscle and kidney (but not liver) cells, which ultimately limits  $\beta$ -oxidation by reducing the entry of acyl-CoA esters into mitochondria (Figure 97-11). Free fatty acids are transported through the blood after intestinal absorption or mobilization from endogenous stores by the use of albumin as a carrier protein or in the form of triacylglycerols in lipoprotein complexes (146). Transport of free fatty acids intracellularly and through the cytoplasm is probably accomplished by a specific transport process; however, the mechanism of this step is not well characterized (147). Before undergoing  $\beta$ -oxidation, free fatty acids must be activated to their corresponding acyl-CoA thioesters. Long chain-specific acyl-CoA synthetases can be found in various subcellular locations but are thought to arise from a single gene product (144). Short- and medium-chain carboxylic acids directly enter the mitochondrial matrix where they are activated. In contrast, long-chain fats are activated in the cytoplasm and require active transport into mitochondria. Transport of long-chain acyl-CoAs requires at least two enzymes, a transporter protein and the use of carnitine as an intermediate carrier molecule. Carnitine is itself transported intracellularly by a specific transporter protein (145). Two carnitine transporters have been described, one specific to the liver and a second with a more ubiquitous

distribution including kidney, muscle, and fibroblasts. Long-chain acyl-CoAs are conjugated to carnitine by carnitine palmitoyl transferase I (CPT I) (148). This enzyme is located on the inner aspect of the outer mitochondrial membrane. Tissue-specific isoforms of this enzyme exist for muscle, liver and brain (145). Long-chain acylcarnitines are then passed to carnitine palmitoyl transferase II (CPT II) in the inner mitochondrial membrane by a translocase (149). Carnitine is freely filtered by the kidney and must be reabsorbed from the proximal tubules to preserve plasma levels. Lack of carnitine uptake in the kidney and gut causes severe hypocarnitinemia, which responds dramatically to L-carnitine.

**97.3.2.1 Clinical Course.** Patients with carnitine transporter deficiency can present with severe hypoglycemia and dilated cardiomyopathy in infancy or childhood. Alternatively, they may show onset of hypertrophic cardiomyopathy, progressive muscle weakness, and muscle lipid storage with mild elevations of creatine kinase. Carriers of OCTN2 mutations are usually asymptomatic, but hypertrophic cardiomyopathy has been reported in middle-aged individuals. Fetal hydrops secondary to this disorder have been reported (150,151). Multiple reports of asymptomatic, affected mothers have been identified when newborn screening of their affected or carrier offspring have been positive for severely low free carnitine levels (145).

**97.3.2.2 Diagnosis.** Acylcarnitine and organic acid analysis are usually normal, and diagnosis is suggested by finding extremely low levels of carnitine in serum and tissues. In fact, serum carnitine may be 1  $\mu$ mol/L or undetectable (normal = 30–70). If necessary, deficient carnitine uptake by tissues such as cultured fibroblasts can also be demonstrated. Molecular testing of the OCTN2 gene is clinically available.

**97.3.2.3 Treatment.** The response to L-carnitine supplementation is dramatic and life saving; 100 mg/kg/day can be given intravenously in emergency situations, and then administered orally on a long-term basis.

**97.3.2.4 Genetics.** The carnitine uptake defect is inherited as an autosomal recessive trait. The OCTN2 gene encoding the carnitine transporter is on chromosome 5q31, and numerous disease-causing mutations have been described (52,73). No single prominent mutation has been identified. Prenatal diagnosis can be accomplished by showing deficient carnitine uptake in cultured amniocytes or molecular testing when mutations in the proband are known (10).

### 97.3.3 Defects of Fatty Acid Entry into Mitochondria (The Carnitine Cycle)

Short- and medium-chain fatty acids are thought to enter mitochondria directly, but mitochondrial uptake of fatty acids longer than C10–12 requires esterification to an acyl-CoA, and the concerted action of CPTs I and II, and carnitine–acylcarnitine translocase (Figure 97-11). CPT I

in the outer mitochondrial membrane first transfers the acyl moiety from CoA to carnitine, and the translocase moves the acylcarnitine ester across the inner membrane in exchange for free carnitine. CPT II in the inner membrane then reconstitutes the CoA esters, which enter the  $\beta$ -oxidation spiral.

**97.3.3.1 Clinical Course.** Severe deficiency of liver CPT I is rare but more frequent milder variants have been identified in geographically restricted populations. Severe symptoms include episodic hypoketotic hypoglycemia beginning in infancy and multiorgan system failure (152–154). Cardiac symptoms are not present. Creatine kinase levels in blood are elevated in acute episodes. Organic aciduria is not prominent in this disorder, but hyperammonemia may be present. Mild CPT1 deficiency is found in high frequency in first nation populations in Canada and Alaska where it is most frequently identified through newborn screening (155,156).

Deficiency of the carnitine-acylcarnitine translocase (CACT) was initially reported in newborns who had a nearly uniform poor outcome (157–159), presenting with severe hypoketotic hypoglycemia and cardiac arrhythmias and/or hypertrophy (157,158,160,161). All have had a grossly elevated acylcarnitine to free carnitine ratio, while dicarboxylic aciduria was reported in one. Patients with a more benign clinical course have since been identified, who have responded well to modest carnitine supplementation and dietary therapy (145,162). Two affected sibs have been reported, where the younger sib was prospectively treated and has not developed any sequelae 2 years later (163). It appears that these patients have a higher level of residual enzyme activity than the more severely affected patients. Specific diagnosis of this disorder can be made via direct enzyme or molecular analysis.

CPT II deficiency is the most common of this group of disorders. It classically presents in late childhood or early adulthood as episodes of recurrent exercise- or stress-induced myoglobinuria (145,164,165). Episodes can be severe enough to lead to acute renal failure. Patients are typically well between episodes. There is no tendency to develop hypoglycemia. Weakness and muscle pain are reported. The characteristic diagnostic finding in these patients is a low total plasma carnitine level with an increased acylcarnitine fraction and no dicarboxylic aciduria. Long-chain acylcarnitines may be elevated (145). A more severe variant of CPT II deficiency presenting with symptoms similar to severe CACT deficiency has been described (166,167). In these patients, the presenting symptoms were neonatal hypoglycemia, hepatomegaly, and cardiomyopathy. Several polymorphic variants in the CPT gene have been associated with an adverse neurologic outcome in influenza encephalitis in Japan.

**97.3.3.2 Diagnosis.** The serum acylcarnitine profile is usually normal in CPT I deficiency, but acylcarnitine levels are low. CPT II and translocase deficiency can be identified but not distinguished from each other by

biochemical testing, both showing elevated C16 esters. The acylcarnitine profile may be normal in milder disease. Urine organic acids either are normal or show mild dicarboxylic aciduria. Blood amino acids are usually normal. Free carnitine in serum is two to three times normal in CPT I deficiency, and is very low in CPT II and translocase deficiency. All three enzymes can be assayed in fibroblasts and leukocytes.

**97.3.3.3 Treatment.** Acute episodes of hypoketotic hypoglycemia should be treated with intravenous glucose-containing fluids to provide at least 8–10 mg/kg/min of glucose. Treatment of hyperammonemia may require dialysis. Ammonia conjugating agents are usually not needed as the hyperammonemia reverses with correction of the underlying metabolic process. Prevention of fasting is the mainstay of therapy in all three disorders and continuous intragastric feeding may be necessary in severe disease. Carnitine supplementation is not usually effective but should be considered when free carnitine is extremely low. Bezafibrate has been shown to induce fatty acid oxidation in cells and improve flux through fatty acid oxidation in cells from patients with residual CPT2 activity (168). A subsequent small trial (six patients) indicated improvement over a 3-year period of treatment (169). Expansion of this therapy to other long-chain fatty acid oxidation disorders may be possible.

**97.3.3.4 Genetics.** All three enzyme defects are inherited as autosomal recessive traits, and the genes *CPT1A*, *CPT2*, and *SLC25A20* (the gene for the translocase) have been localized to chromosomes 11 (11q13), 1 (1p32), and 3 (3p31.21), respectively. Disease-causing mutations have been identified in all three genes, with a relatively common mutation present in the late-onset muscular form of CPT II deficiency (170), and mild CPT1 deficiency in the Hutterite population (155). Numerous coding polymorphisms of unknown significance have been identified in the CPTII gene. Prenatal diagnosis by mutation analysis or enzyme assay on amniocytes is possible in all three conditions.

## 97.3.4 Defects of the $\beta$ -Oxidation Spiral

Once in the mitochondrial matrix, acyl-CoA esters enter the  $\beta$ -oxidation spiral in which a series of four reactions successively removes two-carbon fragments of acetyl-CoA (Figure 97-12). FAD-dependent acyl-CoA dehydrogenases first oxidize the acyl-CoA to 2,3-unsaturated (enoyl-) derivatives, and these are hydrated to 3-hydroxy esters by hydratases. Oxidation to 3-ketoacyl-CoAs by NAD-requiring hydroxyacyl-CoA dehydrogenases and removal of acetyl-CoA by 3-ketothiolases follow, and the acyl-CoA, now two carbons shorter, reenters the spiral. The acyl-CoA dehydrogenases differ from most other dehydrogenases because they utilize electron transfer flavoprotein (ETF) as a final electron acceptor, and thus can channel electrons directly into the ubiquinone pool of the

electron transport machinery by way of ETF:ubiquinone oxidoreductase (ETF dehydrogenase, ETF:QO) (171).

All the enzymes of  $\beta$ -oxidation have distinct (and often overlapping) substrate chain-length specificities. For instance, different FAD-containing dehydrogenases oxidize very-long-chain (C12–24), long-chain (C6–20), medium-chain (C4–14), and short-chain (C4–6) acyl-CoAs, and similar specificities exist for the hydratases, hydroxyacyl-CoA dehydrogenases, and thiolases. Inherited defects in almost all these enzymes have been described. As a rule, defects in long chain-specific enzymes block  $\beta$ -oxidation more completely and cause more severe clinical diseases than do deficits in the medium- and short chain-specific enzymes. Although most of these conditions were originally thought to be rare, defects in very-long-chain acyl-CoA dehydrogenase (VLCAD), medium-chain acyl-CoA dehydrogenase (MCAD), and long-chain hydroxyacyl-CoA dehydrogenase (LCHAD) are among the most common metabolic defects identified through newborn screening with tandem MS. Patients who were originally reported with long-chain acyl-CoA dehydrogenase (LCAD) deficiency have all in fact been subsequently shown to have defects of VLCAD (172). Thus no bona fide patients with LCAD deficiency are known to exist.

### 97.3.5 Very-Long-Chain Acyl-CoA Dehydrogenase and ACAD9 Deficiency

**97.3.5.1 Clinical Findings.** VLCAD deficiency can present in the newborn period with arrhythmias and sudden death, or with hepatic, cardiac, or muscle presentations later in infancy or childhood (173–177). The hepatic presentation is characterized by fasting-induced hypoketotic hypoglycemia, encephalopathy, and mild hepatomegaly, often with mild acidosis, hyperammonemia, and elevated liver transaminases. Some present with arrhythmias or dilated or hypertrophic cardiomyopathy in infancy or childhood, and some with adolescence onset of exercise- or fasting-induced muscle pain, rhabdomyolysis, elevated creatine phosphokinase, and myoglobinuria. The disorder is inherited as an autosomal recessive trait. Tendency to develop hypoglycemia decreases with age, but low-grade, chronic rhabdomyolysis with acute exacerbations is common.

ACAD9 deficiency has been reported with two distinct phenotypes. In the first publication, patients had severe recurrent hypoglycemia with hepatocellular failure reversible with administration of intravenous glucose. One set of sibs also had cardiomyopathy (178). All reported patients appeared to have null mutations as indicated by lack of enzyme antigen. The deficiency has also been reported in patients with a deficiency of complex I of the respiratory chain apparently because of a second function for ACAD9 as a complex I assembly or stability factor (179,180). All these patients have

had point mutations, a finding that may be integral to determining the clinical picture. In fact, both phenotypes can be explained by the existence of a multifunctional protein complex within mitochondria that contains both the respiratory chain and fatty acid oxidation enzymes providing close physical and functional relationships between the two pathways (181).

**97.3.5.2 Diagnosis.** Analysis of serum acylcarnitines by tandem MS usually shows elevations of saturated and unsaturated C14–18 esters in VLCAD deficiency, even between episodes. Organic acid analysis during acute episodes often shows C6, C8, and C10 dicarboxylic aciduria, but because these acids can also be seen when physiological ketosis is resolving, or following the intake of MCTs, this will not raise suspicion of disease unless C12 and C14 dicarboxylic acids are also present. Free carnitine in serum is usually low. If necessary, enzyme deficiency can be demonstrated in fibroblasts or leukocytes. Molecular testing is readily available. VLCAD deficiency is now most frequently diagnosed by newborn screening with tandem MS. No consistent specific biochemical markers in blood or urine have been identified in patients with ACAD9 deficiency. Liver acylcarnitine profile has been reported to be abnormal with an excess of unsaturated compared to saturated species.

**97.3.5.3 Treatment.** Acute management of VLCAD deficiency involves administration of high infusion of high rates of glucose-containing intravenous fluids to give 8–10 mg/kg/min of glucose. Chronic management is somewhat controversial (182,183). Avoiding fasting and maintaining a high carbohydrate intake are clearly indicated, and continuous intragastric feeding may be necessary to achieve this goal, especially overnight. MCTs, whose oxidation does not involve VLCAD, can be administered to provide calories but should not be used until a diagnosis of MCAD deficiency has been excluded. However, safe fasting intervals, the use of oral carnitine, and substitution in the diet of the experimental medium-chain oil triheptanoin are more controversial. As with CPT2 deficiency, bezafibrate has been suggested as a possible means of increasing activity in patients with partially stable mutations and residual enzyme activity (168). Treatment of ACAD9 deficiency remains uncertain because of its infrequency. Institution of high glucose infusion is warranted if hypoglycemia or elevated liver enzymes are elevated, but the need for chronic management when well has not been demonstrated.

**97.3.5.4 Genetics.** The ACADVL gene has been cloned and localized to chromosome 17 (17p13), and although several disease-causing mutations are known, there is no single prominent mutation. In general, the more severe defects cause the most severe and early presenting clinical disease (5). Prenatal diagnosis is possible through enzyme assay in cultured amniocytes, by demonstrating abnormal metabolism of stable isotopically labeled palmitate by amniocytes (51), and by mutation analysis (4).



The *ACAD9* gene is on chromosome 3q21.3. There have been no reported cases of prenatal diagnosis.

### 97.3.6 Medium-Chain Acyl-CoA Dehydrogenase Deficiency

**97.3.6.1 Clinical Findings.** The most common of the fatty acid oxidation disorders, MCAD deficiency, historically most frequently presented during the first 2 years of life with episodes of fasting-induced vomiting, hepatomegaly, hypoketotic hypoglycemia, and lethargy progressing to coma and seizures (184). Blood levels of ammonia, uric acid, liver transaminases, and creatine phosphokinase may be elevated during acute episodes, and liver biopsy shows microvesicular steatosis. Autopsy shows fatty infiltration of the liver, renal tubules, and heart and skeletal muscle (185,186). The disorder was often misdiagnosed as Reye syndrome or sudden infant death syndrome, because the initial episode was fatal in about 25% of cases. Diagnosis through clinical symptoms is now rare as the disorder is readily identified through newborn screening by tandem MS. Patients thus identified are typically well, although at risk for hypoglycemia with intercurrent illness, and fatalities are a rarity. A few enzyme-deficient individuals born prior to newborn screening have had their first presentation in adolescence or adult life and some have remained asymptomatic (187).

**97.3.6.2 Diagnosis.** Analysis of serum acylcarnitines by tandem MS shows elevations of C8, C8:1, and C10:1 esters even between episodes (188). The same abnormalities are identified through newborn screening. The C6, C8, and C10 dicarboxylic aciduria that occurs during acute episodes often should raise suspicion of the disease and biochemical confirmation can be obtained by measurement of hexanoylglycine and suberylglycine in urine. Phenylpropionylglycine in urine will be elevated if the gut has been colonized by adult-type flora (189), but can be missed by all but the most sensitive techniques. Free carnitine in serum is usually low. Enzyme deficiency can be shown in fibroblasts or leukocytes, but molecular diagnosis is more readily available and often faster.

**97.3.6.3 Treatment.** Treatment of acute episodes in MCAD deficiency is primarily supportive and aimed at quickly reversing the catabolic state that is responsible for stimulating the pathways of lipolysis and fatty acid oxidation (183,190). Hypoglycemia should be corrected with bolus administration of intravenous dextrose. Continuous infusion of dextrose should then be given at a rate that maintains plasma glucose levels at, or slightly above, the normal range in order to stimulate insulin secretion and suppress adipose tissue lipolysis. Specific therapy for the mild hyperammonemia that may be present during acute illness has not usually been required. Cerebral edema has occurred during treatment in some patients with severe coma, possibly as a late reflection of acute brain injury from hypoglycemia, toxic effects of fatty acids, or

ischemia. Recovery from the acute metabolic derangements associated with coma may require more than a few hours, but is usually complete within 12–24 h, except where serious injury to the brain has occurred. Long-term management consists of dietary therapy to prevent excessive periods of fasting that can lead to coma. Overnight fasting in infants should be limited to no more than 8 h. A duration of 12 h is probably safe in children >1 year of age (191). Home blood glucose monitoring is not useful because symptomatic illness can begin before hypoglycemia has occurred. Although it is reasonable to modestly reduce dietary fat, because this fuel cannot be used efficiently in MCAD deficiency, patients appear to tolerate normal diets without difficulty, and severe restriction of fat intake may be unnecessary. Formulas containing MCT oil should be avoided. Although patients with MCAD deficiency and other acyl-CoA oxidation defects have secondary carnitine deficiency, the use of carnitine supplementation in these disorders is controversial (183,190). Some investigators suggest 50–100 mg/day of oral carnitine but its utility is unproven.

**97.3.6.4 Genetics.** The *ACADM* gene is on chromosome 1 (1p31), and MCAD deficiency is inherited as a recessive trait. The vast majority of patients with MCAD deficiency have a single common missense mutation: an A-to-G transition at cDNA position 985, which changes a lysine residue to glutamate at amino acid 329 of the MCAD precursor protein (192). The mutated amino acid is far removed from the catalytic site of the enzyme but appears to make the protein unstable by interfering with intramitochondrial folding and assembly of the nascent peptide (193). Preventing this misfolding offers an opportunity for development of new therapeutic agents for MCAD deficiency (194). The A985G mutation accounts for approximately 90% of the mutant alleles in MCAD deficiency (192). Approximately 70% of patients are homozygous for the A985G mutation. Most of the remaining patients are compound heterozygotes for the A985G allele in combination with one of several rarer mutations. Thus, only a small percentage of MCAD patients do not have at least one A985G allele. The unusually high frequency of a single common mutation has made molecular diagnosis especially valuable in MCAD deficiency. As more information accumulates from patients identified through newborn screening, correlation of phenotype with genotype is becoming clearer. Patients with the common mutation accumulate the highest levels of metabolites in the newborn period and are probably at risk for more severe disease than are many other mutations (195).

### 97.3.7 Short-Chain Acyl-CoA Dehydrogenase Deficiency

**97.3.7.1 Clinical Findings.** A number of patients with SCAD deficiency have been reported (196,197). Reported clinical findings have included episodes of intermittent

metabolic acidosis, neonatal hyperammonemic coma, neonatal acidosis with hyperreflexia, multicore myopathy, and infantile-onset lipid storage myopathy with failure to thrive, and hypotonia. Hypoglycemia is a rare finding in this disorder. The characteristic metabolites of ethylmalonic and methylsuccinic acids of SCAD deficiency were also detected in individuals with normal SCAD activity in fibroblasts (198,199). Subsequently, it was demonstrated that the presence of one of two relatively common variants of SCAD (625 G>A and 511 C>T) predisposes to excessive ethylmalonic acid production. In general, it is clear that the vast majority of patients with complete SCAD deficiency identified through newborn screening have been well, while a variety of symptoms continue to be ascribed to the deficiency in patients identified through clinical testing later in life (197,200). The full clinical spectrum of this deficiency, and the clinical relevance of the common polymorphisms, remains to be defined (201,202).

**97.3.7.2 Diagnosis.** Butyrylcarnitine in blood is elevated in complete SCAD deficiency and less reliably so in the presence of the common polymorphism. Urine ethylmalonic acid can be elevated in both clinical settings but is not specific for SCAD deficiency. Fibroblast enzyme analysis and acylcarnitine profile will identify the deficiency but may be normal in the presence of just the common polymorphisms. Molecular testing is clinically available.

**97.3.7.3 Treatment.** The need for specific dietary or supplement therapy is not supported by current literature (196). Since ethylmalonic and butyric acids are organic acids, it seems prudent to caution parents to be alert for the development of signs of acidosis during intercurrent illness and seek emergency care if they develop.

**97.3.7.4 Genetics.** The *ACADS* gene is located at chromosome 12q24.31. The two common variants (625 G>A and 511 C>T) can be present in as many as 35% of Caucasians. A common inactivating mutation has been described in the Ashkenazi Jewish population. Multiple other private inactivating mutations have been reported, as have combinations of an inactivating mutation with one of the polymorphisms in trans.

### 97.3.8 Trifunctional Protein and Long-Chain 3-Hydroxyacyl-CoA Dehydrogenase Deficiency

Several chain length-specific NAD-dependent 3-hydroxyacyl-CoA dehydrogenases catalyze the oxidation of 3-hydroxyacyl-CoA esters to 3-ketoacyl esters. LCHAD acts on hydroxyacyl-CoAs longer than C8. LCHAD and long-chain enoyl-CoA hydratase activities are carried out on the  $\alpha$ -subunit of the mitochondrial trifunctional protein, and long-chain  $\beta$ -ketothiolase activity is carried out on the  $\beta$ -subunit. LCHAD deficiency can exist alone, or together with deficiency of the other two enzymes.

**97.3.8.1 Clinical Findings.** Patients with a deficiency of this enzyme tend to fall into two clinical subclasses (203–207). One group presents primarily with symptoms of cardiomyopathy, myopathy, and hypoglycemia. Peripheral neuropathy and recurrent myoglobinuria may be present. These patients are deficient in all three enzymatic activities of the trifunctional protein. The other group, deficient only in LCHAD activity, has hepatocellular disease with hypoglycemia with or without pigmentary retinopathy. Cholestasis and fibrosis may also be present (208). Considerable overlap in these groups has been described, however, and LCHAD deficiency has also been reported in patients with recurrent Reye syndrome-like symptoms and in sudden infant death (209). Milder cases with adolescent onset of recurrent rhabdomyolysis have been reported (210). Fetal LCHAD deficiency frequently causes acute fatty liver or HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets) in the (heterozygous) mother during pregnancy, especially when one or both mutant alleles in the fetus is E474Q (211).

**97.3.8.2 Diagnosis.** Acylcarnitine analysis by tandem MS is usually diagnostic including in the newborn period, and shows elevated saturated and unsaturated C16 and C18 hydroxyacylcarnitines. Organic acid analysis often shows elevated C6–14 3-hydroxydicarboxylic acids, but the same abnormalities have been seen in patients with respiratory chain defects and glycogenoses, and are not specific. The enzyme defect can be demonstrated in fibroblasts and leukocytes and, for prenatal diagnosis, in amniocytes.

**97.3.8.3 Treatment.** Therapeutic options and controversies parallel those for VLCAD deficiency (143,182,183, 212). In addition, docosahexaenoic acid, a polyunsaturated C20 acid, has been proposed to slow down the development of retinitis but remains under investigation (213).

**97.3.8.4 Genetics.** LCHAD deficiency, whether isolated or part of trifunctional protein deficiency, is inherited as an autosomal recessive trait, as the genes for both subunits (*HADHA* and *HADHB*) are located on chromosome 2 (2p24.1–23.3). Several disease-causing mutations have been identified, and most affect the  $\alpha$ -subunit. One of these, E510Q (E474Q in the mature subunit), accounts for nearly 90% of mutant alleles in patients of European extraction with isolated LCHAD deficiency (214). Defects in the  $\beta$ -subunit tend to destabilize the trifunctional protein resulting in the multiple enzymatic deficiencies seen in some patients (207,215–217). Prenatal diagnosis can be made by enzyme assay in amniocytes or chorionic villus samples or, when appropriate, by mutation analysis, and on occasion will be indicated to avoid the complications of pregnancy.

### 97.3.9 Short-Chain L-3-Hydroxyacyl-CoA Dehydrogenase Deficiency

Short-chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD) catalyzes the NAD-dependent oxidation of

3-hydroxyacyl-CoA for C4 to C10 substrates. Deficient patients present with hypoketotic hypoglycemia associated with hyperinsulinism, reduced SCHAD activity in fibroblast mitochondria, and mutations in the *HADHSC* gene (218–220). Several other patients with abnormal metabolite or enzymatic studies suggestive of SCHAD deficiency have been reported, but as SCHAD gene sequencing was normal, the nature of their disorders remains unclear.

**97.3.9.1 Diagnosis.** A biochemical diagnosis is based on the findings of marked ketotic C8 to C14 3-hydroxy dicarboxylic aciduria. SCHAD activity in cultured skin fibroblasts may or may not be abnormal. Characteristic metabolic findings in patients with hyperinsulinemic hypoglycemia should trigger gene sequencing studies (218–220).

**97.3.9.2 Treatment.** Patients with hyperinsulinism may exhibit improved glucose homeostasis with diazoxide therapy. No specific metabolic interventions beyond avoidance of fasting and IV hydration with glucose containing solutions during illness have been reported.

**97.3.9.3 Genetics.** The genetic nomenclature for SCHAD is somewhat confusing. The gene defective in patients with hyperinsulinism is referred to as *HADHSC* in the literature but is designated as *HADH* in GenBank. It is located on chromosome 4q22–q26 and mutations in patients have been described. An enzyme designated MSCHAD in the literature is likely different than the one defective in hyperinsulinism patients, but also is identified as *HADH* in GenBank. No gene mutations have yet been identified in patients with metabolic findings of SCHAD deficiency but without hyperinsulinism, suggesting these individuals in fact have a different disorder.

### 97.3.10 Medium-Chain 3-Ketoacyl-CoA Thiolase Deficiency

The first confirmed case to be reported was a Japanese male neonate who died shortly after presenting at 2 days of age with vomiting, dehydration, metabolic acidosis, liver dysfunction, and terminal rhabdomyolysis with myoglobinuria (221). Urine organic acid analysis revealed ketotic lactic aciduria and significant C6 to C12 dicarboxylic aciduria, with strikingly elevated C10 and C12 species. In skin fibroblasts, palmitate oxidation was normal, octanoate oxidation was reduced to 31% of controls, and there was an isolated deficiency of medium-chain 3-ketoacyl-CoA thiolase activity, a result supported by the finding of a reduced protein signal by immunoprecipitation. Additional patients presented with variable but nevertheless typical symptoms of a fatty acid oxidation disorder (fasting intolerance, cardiomyopathy, and sudden death in one case) (222,223). Unfortunately, in vitro functional or molecular confirmation is not readily available.

### 97.3.11 Multiple Acyl-CoA Dehydrogenase Deficiency/Glutaric Acidemia Type II

Electrons from the acyl-CoA dehydrogenases involved in mitochondrial fatty acid and amino acid oxidation are transferred from their FAD coenzymes to coenzyme Q in the respiratory chain via ETF and ETF:QO. Defects in ETF and ETF:QO cause multiple acyl-CoA dehydrogenase deficiency, often called glutaric acidemia type II because of one of the characteristic metabolites that accumulates.

**97.3.11.1 Clinical Findings.** Glutaric acidemia type II was first described in 1976 in a baby who died at 3 days of age with severe hypoglycemia, metabolic acidosis, and the smell of sweaty feet, and many additional patients have since been described. Clinical manifestations are extremely heterogeneous (224); Frerman, 1988 #9910. A neonatal form can be seen with severe hypotonia, dysmorphic features, and cystic kidneys. These infants also exhibit metabolic acidosis and hypoglycemia. Milder variants are common, presenting with nonspecific neurological signs, lipid storage myopathy, fasting hypoketotic hypoglycemia, and/or intermittent acidosis. In some patients, only fasting hypoketotic hypoglycemia and/or intermittent acidosis is seen and can be of late onset (202,224). In these cases, the organic acid profile in times of illness is usually dominated by ethylmalonic and adipic acids, leading to an alternate name of ethylmalonic-adipic aciduria for this disorder. Structural brain abnormalities are common including agenesis of the cerebellar vermis, hypoplastic temporal lobes, and focal dysplasia of the cerebral cortex (225). Neuronal migration abnormalities may be present. Riboflavin-responsive mutations in the *ETFDH* gene have been reported (226).

**97.3.11.2 Diagnosis.** Organic acid analysis usually shows increased ethylmalonic, glutaric, 2-hydroxyglutaric, and 3-hydroxyisovaleric acids, together with C6, C8, and C10 dicarboxylic acids and isovalerylglycine, and acylcarnitine analysis by MS-MS shows glutaryl carnitine, isovalerylcarnitine, and straight-chain esters of chain length C4, C8, C10, C10:1, and C12. Serum carnitine is usually low, and serum sarcosine is often increased in patients with mild disease. Enzyme or immunoblot analyses, if necessary, will show that some patients are deficient in ETF, and that others are deficient in ETF:QO. Molecular testing is typically more readily available.

**97.3.11.3 Treatment.** Patients with complete defects often die during the first weeks of life, usually of conduction defects or arrhythmias, but those with incomplete defects can survive well into adult life. As in other fatty acid oxidation disorders, treatment relies on the avoidance of fasting, sometimes with continuous intragastric feeding, and carnitine to replenish lost stores. Riboflavin is usually given, and appears to have helped some



patients. Carnitine supplementation (100 mg/kg/day) will increase metabolite excretion and should be used.

**97.3.11.4 Genetics.** ETF and ETF:QO deficiencies are both inherited as autosomal recessive traits, and the genes encoding ETF:QO and the  $\alpha$ - and  $\beta$ -subunits of ETF have been mapped to chromosome 4 (4q32>ter), 15 (15q23–25), and 19 (19q13.3), respectively. Disease-causing mutations have been identified in all three genes, but only in the *EFTA* gene is there a common mutant allele (T266M) (227). Severe forms of the disease have been diagnosed in utero by demonstrating increased amounts of glutaric acid in amniotic fluid (228,229), and in some cases renal cysts have been seen in the fetus on ultrasound examination (230).

### 97.3.12 Disorders of Ketone Body Metabolism

**97.3.12.1 Clinical Findings.** Deficiency of the mitochondrial acetoacetyl-CoA thiolase (also known as  $\beta$ -ketoacyl-CoA thiolase) and 3-hydroxy- and 3-methylglutaryl-CoA lyase are shared disorders between ketone body metabolism and isoleucine degradation. They are discussed with the organic acidemias. Succinyl-CoA: 3-ketoacid CoA transferase (SCOT) functions in conjunction with mitochondrial acetoacetyl-CoA thiolase to generate ketones in extrahepatic tissues. SCOT deficiency presents as persistent ketonuria in the first 1–2 years of life, while acetoacetyl-CoA thiolase deficiency presents with variable clinical symptoms and exaggerated ketoacidosis in response to minor physiologic stress (231–233). HMG-CoA synthase deficiency has been reported in eight patients who presented with coma, hypoglycemia and dicarboxylic aciduria with very low ketones (234–236). Their acylcarnitine profiles were reported as normal.

**97.3.12.2 Treatment.** Treatment for most of the ketone body synthesis disorders is essentially supportive. Maintaining adequate hydration during intercurrent illness minimizes symptoms. HMG-CoA lyase deficiency can present with life-threatening hypoglycemia and hyperammonemia and must be treated aggressively (see Organic Acidemias in this chapter).

**97.3.12.3 Genetics.** Multiple mutations in the succinyl-CoA: 3-ketoacid CoA transferase gene (also called 3-oxoacid CoA transferase; OXCT1; chromosome 5) have also been described in patients with enzymatic deficiency. The HMG-CoA synthase gene (*HMGCS2*) is on chromosome 1 and gene mutations in patients have been identified (234–236).

### CROSS REFERENCES

Abnormal Mental Development; Amino Acid Metabolism; Analysis of Genetic Linkage; Diagnostic Molecular Genetics.

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## Biography



Dr Vockley is Professor of Pediatrics, School of Medicine; Professor of Human Genetics, Graduate School of Public Health; and Chief of Medical Genetics, Children's Hospital of Pittsburgh. He received his undergraduate degree at Carnegie-Mellon University in Pittsburgh, Pennsylvania, and received his degree in Medicine and Genetics from the University of Pennsylvania School of Medicine in Philadelphia, Pennsylvania. He completed his pediatric residency at the University of Colorado Health Science Center, and his postdoctoral fellowship in Human Genetic and Pediatrics at Yale University School of Medicine in New Haven, Connecticut. Before assuming his current position in Pittsburgh, Dr Vockley was Chair of Medical Genetics in the Mayo Clinic School of Medicine.

Dr Vockley is internationally recognized as a leader in the field of inborn errors of metabolism. His laboratory has been responsible for identifying multiple new disorders, many of them defects in mitochondrial energy and amino acid metabolisms, and he has published over 140 scientific articles in peer review journals. His current research focuses on the molecular architecture of mitochondrial energy metabolism, in which he is breaking new ground in describing the role of dysfunction of mitochondrial energy metabolism in such common conditions as diabetes, obesity, and Alzheimer disease. Dr Vockley serves on numerous national and international scientific boards including the Advisory Committee (to the Secretary of Health and Human Services) on Heritable Disorders in Newborns and Children where he is chair of the technology committee. He also serves as chair of the Pennsylvania State Newborn Screening Advisory Committee and the American College of Medical Genetics Therapeutics Committee. He is a past president of the International Organizing Committee for the International Congress on Inborn Errors of Metabolism and the Society for the Inherited Metabolic Disorders (SIMD).

Dr Vockley is the cofounder and editor of the North American Metabolic Academy established by the SIMD to help educate the next generation of metabolic physicians in the United States, and serves as associate editor for the journals *Molecular Genetics and Metabolism* and *The Journal of Inherited Metabolic Disorders*. Dr Vockley was recognized in 2002 as the Research Educator of the Year while at the Mayo Clinic. At the University of Pittsburgh, Dr Vockley teaches in both the Medical School and Graduate School of Public Health. Dr Vockley has mentored numerous PhD candidates, postdoctoral fellows, and undergraduate in their research.



# CHAPTER

# 98

## Vitamin D Metabolism or Action

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### 98.1 HISTORY

Rickets and osteomalacia were widespread problems until the discovery of the calciferols (1), after which they were used for prevention and treatment. However, some cases did not respond to the usual doses of calciferols, and multiple genetic and other causes were subsequently recognized. In 1937, Albright reported detailed studies of a child with this problem and suggested a hereditary resistance to the actions of calciferols (2). Rickets resistant to calciferols was subsequently recognized as a common cause of hereditary dwarfism. Most hereditary cases showed biochemical features different from those of calciferol nutritional deficiency, and are now classified as phosphate diabetes or X-linked hypophosphatemia.

In 1961, Prader et al. (3) characterized a distinctive form of hereditary rickets that they called pseudodeficiency rickets. Features that clearly distinguished pseudodeficiency rickets from X-linked hypophosphatemia were hypocalcemia, the potential for complete remission with high doses of calciferols, and an autosomal transmission pattern.

In 1971,  $1\alpha,25(\text{OH})_2\text{D}_3$  was shown to be the active metabolite of vitamin  $\text{D}_3$  that accumulated in the nuclei of target tissues (4–6). This discovery quickly led to the development of methods to measure active metabolites in blood, characterization of defects in  $1\alpha,25(\text{OH})_2\text{D}$  synthesis and action, and an understanding of the roles of  $1\alpha$ -hydroxylated and other analogs for therapy. Fraser et al. (7) showed that pseudodeficiency rickets in one patient was corrected with physiologic doses of  $1\alpha,25(\text{OH})_2\text{D}_3$ ; they suggested that this disorder represented a defect in  $25(\text{OH})\text{D}_3$   $1\alpha$ -hydroxylase enzyme. Subsequently, Brooks et al. (8) described a patient with similar clinical features but high serum levels of  $1\alpha,25(\text{OH})_2\text{D}$  before and during treatment; they suggested that pseudodeficiency rickets be classified as type I (deficient production of  $1\alpha,25(\text{OH})_2\text{D}$ ) or type II (impaired end-organ response to  $1\alpha,25(\text{OH})_2\text{D}$ ).

Recent identification of patients with novel defects in the activation (9) or action of (10) vitamin D have further extended the molecular pathophysiology of this disorder.

### 98.2 NORMAL PHYSIOLOGY OF CALCIFEROLS

#### 98.2.1 Vitamin D Sources

Cholecalciferol (vitamin  $\text{D}_3$ ) (11) is a secosteroid produced via opening of the B-ring of 7-dehydrocholesterol (Figure 98-1). In humans, this reaction is driven by ultra-violet radiation (from sunlight) in the basal layers of the epidermis (12). Skin pigment can decrease the amount of cholecalciferol synthesized in response to UV radiation (13). In addition, with aging, vitamin D synthesis in skin is reduced and requires more prolonged exposure to UV radiation (14). Ergocalciferol (vitamin  $\text{D}_2$ ) is produced by opening the B-ring of ergosterol, a sterol found in plants and fungi. Plants do not contain important amounts of ergocalciferol but this chemical is synthesized in bulk for use as a nutritional supplement. Humans obtain calciferols either endogenously through metabolism of precursors in the skin or exogenously as a dietary component or supplement. The metabolism and actions of vitamin  $\text{D}_3$  and  $\text{D}_2$  are similar in humans but  $\text{D}_3$  is slightly more effective than  $\text{D}_2$  in correcting vitamin D deficiency and maintaining normal circulating levels of  $25(\text{OH})\text{D}$  (15–18).

#### 98.2.2 Vitamin D 25-Hydroxylation

Cholecalciferol and ergocalciferol have little or no direct biological activity on vitamin D target cells or receptors. They must be hydroxylated at positions 25 and  $1\alpha$  to become maximally active (Figure 98-2). The initial hydroxylation at carbon-25 is carried out primarily in

the liver by a microsomal cytochrome P450 mono-oxygenase. The 25-hydroxylation of vitamin D is not highly regulated; the principal determinant of the rate of this reaction is the circulating level of its substrate, vitamin D.

The identity of the microsomal P450 enzyme CYP2R1 as the primary hepatic 25-hydroxylase (19) was confirmed via molecular studies of two brothers with congenital rickets who were previously hypothesized to have a putative deficiency of 25-hydroxylase (9). Both these brothers were found to be homozygous for a transition mutation in exon 2 of CYP2R1 on chromosome 11p15.2, which causes an amino acid substitution that eliminates ability of the enzyme to 25-hydroxylate substrate vitamin D<sub>3</sub> (20). Activity of CYP2R1 is constitutive, and therefore, production of 25(OH)D from precursors D<sub>2</sub> and D<sub>3</sub> is substrate dependent.

In addition to liver, CYP2R1 is also expressed in keratinocytes (21), testis (22) and to a minor degree in numerous other tissues (23). Additional less-important cytochrome P450 enzymes with 25-hydroxylase function are CYP3A4, CYP27A1 and CYP2J2 (24).

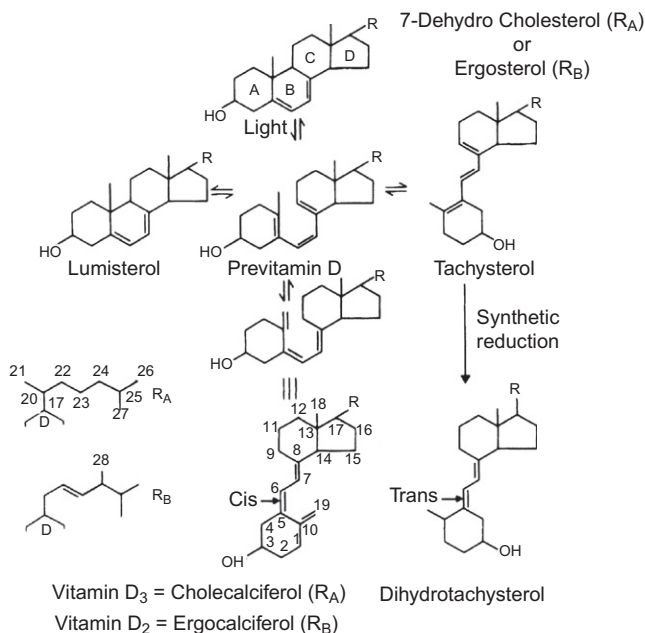
### 98.2.3 1 $\alpha$ -Hydroxylation

The fate of 25(OH)D is determined by two competing enzymatic pathways. Circulating 25(OH)D can undergo transformation to the fully active form of vitamin D, 1,25(OH)<sub>2</sub>D, via a second hydroxylation performed by the 1 $\alpha$ -hydroxylase, CYP27B1 (25), that is present principally in the proximal renal tubule. Alternatively, 25(OH)D can be inactivated to a water-soluble metabolite

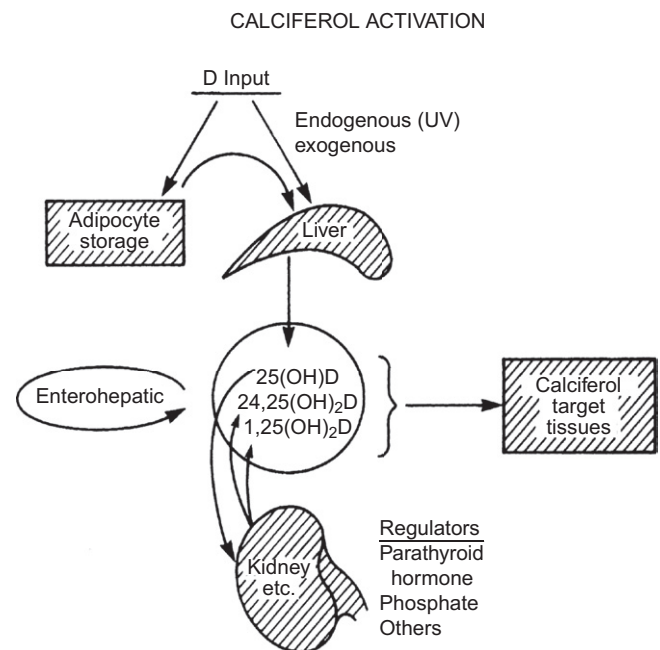
24,25(OH)<sub>2</sub>D by mitochondrial cytochrome P450 enzymes that are present in the kidney (CYP24A1) or liver and intestine (CYP3A4). The renal 1 $\alpha$ -hydroxylase is the gene product of CYP27B1, present on chromosome 12q13, and cloned in 1997 (26). Renal production is the primary source of circulating 1,25(OH)<sub>2</sub>D (27), although many nonrenal tissues also express CYP27B1, as discussed below. Renal 1 $\alpha$ -hydroxylase is the rate-limiting step in the production of serum 1,25(OH)<sub>2</sub>D and the activity of this enzyme is tightly regulated by parathyroid hormone (PTH) and the phosphotonin fibroblast growth factor 23 (FGF23). In addition, hypocalcemia and calcitonin can induce CYP27B1 expression. PTH activates transcription and activity of CYP27B1 in the renal proximal tubule through a cyclic AMP-mediated pathway (28). The enzyme is inhibited by 1,25(OH)<sub>2</sub>D, calcium, phosphate, and FGF23. Following PTH stimulation in the kidney, the nuclear orphan receptor NR4A2 is upregulated, enhancing the PTH-induced increase in CYP27B1 expression; C/ERBP $\alpha$  inhibits this transcriptional enhancement (29).

The renal 25(OH)D 1 $\alpha$ -hydroxylase enzyme contains a cytochrome P450 and reno-redoxin (30,31); dephosphorylation of the reno-redoxin component may mediate activation of the 1 $\alpha$ -hydroxylase by PTH (32).

CYP27B1 is also expressed in many nonrenal tissues, including parathyroids, (33), testis (22), prostate (34), breast (35), colon (36), pancreatic beta cells (37), endometrium (38), cervix (39), ovary (40), adipocyte (41), bone cells (both osteoblasts and osteoclasts) (42), placenta (43) and vascular endothelial (44) and smooth



**FIGURE 98-1** Synthesis of cholecalciferol, ergocalciferol, and dihyrotachysterol. (From Aurbach, G. D.; Marx, S. J.; Spiegel, A. M. *Parathyroid Hormone, Calcitonin, and the Calciferols*. In *Williams Textbook of Endocrinology*, 7th ed.; Wilson, J. D.; Foster, D. W., Eds.; WB Saunders: Philadelphia, 1985; pp 1137–1217.)



**FIGURE 98-2** Metabolic pathways for activation of cholecalciferol or ergocalciferol. (From Aurbach, G. D.; Marx, S. J.; Spiegel, A. M. *Parathyroid Hormone, Calcitonin, and the Calciferols*. In *Williams Textbook of Endocrinology*, 7th ed.; Wilson, J. D.; Foster, D. W., Eds.; WB Saunders: Philadelphia, 1985; pp 1137–1217.)

muscle cells (45). Although  $1,25(\text{OH})_2\text{D}_3$  is produced in these tissues, the contribution to the circulating pool of  $1,25(\text{OH})_2\text{D}_3$  appears negligible, and in these tissues,  $1,25(\text{OH})_2\text{D}_3$  appears to play a paracrine or apocrine role. For examples, locally produced  $1,25(\text{OH})_2\text{D}$  serves as a differentiating factor for many cells. A wide variety of pathologic tissues also express CYP27B1 (including granulomas and most malignancies) and mutations of CYP27B1 in cancer tissues are thought to be one mechanism by which these tissues escape the normal constraints on their growth (46,47). Whereas in the kidney, production of  $1,25(\text{OH})_2\text{D}$  is tightly regulated by PTH and FGF23, in most nonrenal tissues, local factors regulate its production (24).

### 98.2.4 24-Hydroxylation

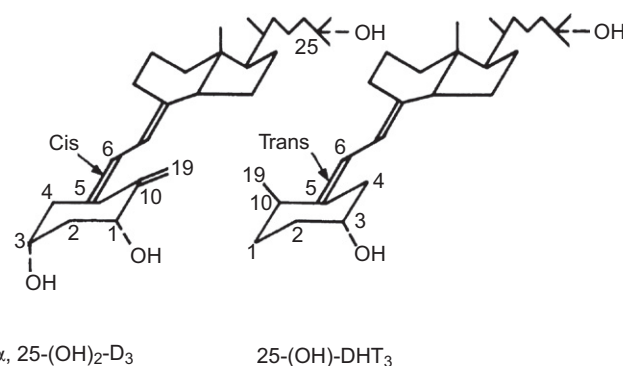
The primary catabolic enzyme that prevents excessive production of  $1,25(\text{OH})_2\text{D}$  is a 25-hydroxyvitamin D-24-hydroxylase, herein referred to as CYP24A1, that is expressed in the kidney. CYP24A1 is a cytochrome P450 enzyme and catalyzes the addition of a hydroxyl group on carbon 24 of the vitamin D secosteroid backbone. The human CYP24A1 gene, located on chromosome 20, was cloned in 1993 (48). CYP24A1 is one of the most inducible genes in body, and transcription can be increased 20,000-fold by  $1,25(\text{OH})_2\text{D}$  (47). This high inducibility of CYP24A1, with consequent rapid inactivation of  $1,25(\text{OH})_2\text{D}$  to the water-soluble inert metabolite  $1,24,25$ -trihydroxyvitamin D, is likely the major reason that vitamin-D intoxication is uncommon after exposure to high doses of vitamin D. The inactivation of  $1,25(\text{OH})_2\text{D}$  consists of five enzymatic steps that involve successive hydroxylation/oxidation reactions at carbons 24 and 23 followed by cleavage of the secosteroid at the C-23/C-24 bond and subsequent oxidation of the cleaved product to calcitroic acid.  $1,25(\text{OH})_2\text{D}$  can also undergo inactivation by CYP3A4-dependent 23- and 24-hydroxylations in the liver and small intestine. Both CYP24 and CYP3A4 can also inactivate  $25(\text{OH})\text{D}$ . CYP3A4 is one of the most polymorphic genes, and plays a critical role in activating and inactivating a large number of endogenous compounds and drugs. Hence, induction of CYP3A4 by drugs such as dilantin and phenobarbital likely accounts for the low circulating levels of vitamin D metabolites in individuals who receive these medications. There are many similarities between the 1-hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1) enzymes, which suggest that they share certain components. Both are mitochondrial enzymes containing cytochrome P450 (49). Both are modulated (though in opposing directions) by cAMP and  $1,25(\text{OH})_2\text{D}$  (50) and both are distributed in similar anatomical sites. The role of 24-hydroxylase has not been fully determined; it may be the only route to a group of calciferols with unique actions not obtainable with  $1,25(\text{OH})_2\text{D}$  (see later discussion in Section 98.3.11), and it might be a critical step in regulating vitamin D action via enhanced production of  $24,25(\text{OH})_2\text{D}$ ,

which reduces availability of substrate  $25(\text{OH})\text{D}$  for activation by the 1-hydroxylase. Moreover, 24-hydroxylase also limits biological activity of  $1,25(\text{OH})_2\text{D}$ : expression of the CYP24A1 gene is induced by  $1,25(\text{OH})_2\text{D}$  in target tissues (50,51), and the 24-hydroxylase shows a preference for  $1,25(\text{OH})_2\text{D}$  rather than  $25(\text{OH})\text{D}$  as substrate. These observations define a system of elegant control, in which the  $1,25(\text{OH})_2\text{D}$  hormone induces the expression of the gene encoding a key effector of its catabolic breakdown. Mouse vitamin D 24-hydroxylase has been cloned; its mRNA is most abundant in the kidney and intestine, with smaller amounts detectable in skin, thymus, bone, lung, spleen, pancreas, and heart (52).

As seen with reduced CYP27B1 expression in cancers (discussed above), increased CYP24A1 expression in cancers has been found (53–55), both leading to reduced tissue production of  $1,25(\text{OH})_2\text{D}$  and reduction in its differentiating effect on cells (53,56,57).

### 98.3 5,6-CIS-TRANS-ISOMERIZATION

$25(\text{OH})\text{D}$  may also be converted to 5,6-trans- $25(\text{OH})\text{D}$ . This pathway was initially documented in plasma from rats that were treated with pharmacologic amounts of vitamin D3 (58). The pathway is of interest because it produces a metabolite analogous to  $25(\text{OH})$ -dihydrotachysterol (Figure 98-3) (dihydrotachysterol is a synthetic calciferol analog). These metabolites have an A-ring rotated  $180^\circ$ , bringing the 3-hydroxyl group into a pseudo- $1\alpha$ -hydroxyl position. This rotation increases the biological activity and potency of the metabolite relative to the parent metabolite. 5,6-trans metabolites might have important roles in states where the renal  $1\alpha$ -hydroxylase enzyme is severely deficient. In vitro studies show that the 5,6-trans metabolites are less-potent promoters of intestinal calcium absorption (by a factor of 0.0003) than  $1,25(\text{OH})_2\text{D}_3$  (59). Numerous synthetic stereoisomers of the A-ring (60) and C-D rings (61) are now under study for potential clinical use as fully active forms of vitamin D that represent novel analogs of  $1,25(\text{OH})_2\text{D}_3$ .



**FIGURE 98-3** Steric conformation of two activated calciferol analogs.  $1\alpha, 25(\text{OH})_2\text{D}_3$  has the 5–6 double bond in cis configuration.  $25(\text{OH})$ -dihydrotachysterol has the 5–6 double bond in trans configuration; the  $180^\circ$  rotation of the A ring (lowest ring in figure) brings the  $3\beta$  hydroxyl group into a pseudo- $1\alpha$  configuration.

### 98.3.1 Vitamin D-Binding Protein in Plasma

All calciferols are fat-soluble and circulate, principally bound to an  $\alpha$ -globulin of 58 kDa (62), made in the liver called vitamin D binding protein (DBP, also known as GC-globulin or group-specific component globulin). The gene for DBP, GC, is located on chromosome 4, in the same gene cluster as the gene for albumin (63). Although GC was sequenced in 1993 (64), it was identified as a protein that binds vitamin D analogs in 1975 (65). The messenger RNA for this DBP, Gc, is homologous to that for albumin and  $\alpha$ -fetoprotein (66). DBP is a major component of plasma protein (normal concentration 10–5 M) and is one of the most highly polymorphic proteins (67), thus making it a useful genetic marker. In fact, DBP isoforms were studied as measures of genetic diversity long before a function in calciferol transport was recognized. DBP contains one high-affinity sterol-binding site with the following order of affinities:  $25(\text{OH})\text{D}_3$ -26,23-lactone >  $25(\text{OH})\text{D}$  =  $24,25(\text{OH})_2\text{D}$  >  $1\alpha,25(\text{OH})_2\text{D}$  >> vitamin D. It is a highly expressed protein with multiple functions, including transport of vitamin-D metabolites, control of bone development, binding of fatty acids, sequestration of actin, and a range of less-defined roles in modulating immune and inflammatory responses. DBP plays an important role in modulating inflammatory and immune responses as a precursor of the inflammation-primed macrophage activating factor, GcMAF (65,68).

### 98.3.2 Calciferol Turnover and Requirements

Most of the body pool of vitamin D is stored in fat, compared with 30% of  $25(\text{OH})\text{D}$  (69) and a small fraction of  $1,25(\text{OH})_2\text{D}$  (70). When present in the circulation, more than 99% of each metabolite is bound to proteins. Normal turnover of vitamin D is approximately 30  $\mu\text{g}$  per day; most of this is cleared through catabolic pathways, with only 1  $\mu\text{g}$  per day cleared as  $1,25(\text{OH})_2\text{D}$  (Table 98-1). Drugs and diseases known to induce CYP3A4 or CYP24A1 (antiseizure medications, bile acids, alcohol, hyperthyroidism) can increase clearance of  $25(\text{OH})\text{D}$  and  $1,25(\text{OH})_2\text{D}$ .

### 98.3.3 $1,25(\text{OH})_2\text{D}$ Receptors

The biological actions of  $1,25(\text{OH})_2\text{D}$  are mediated through two different types of receptors. Conventional vitamin-D effects reflect the genomic actions, which are mediated via binding to the VDR, a high-affinity intracellular nuclear receptor that is analogous to the receptors for other steroid hormones (71). By contrast, other vitamin-D actions are mediated through binding of  $1,25(\text{OH})_2\text{D}$  to a putative plasma membrane receptor (discussed more fully under Section 98.3.5), which affects nongenomic actions of  $1,25(\text{OH})_2\text{D}$ . The genomic vitamin D receptor (VDR) is a 50-kDa protein whose sequence (72) resembles that of other members of the steroid hormone—thyroid hormone—retinoic acid receptor gene superfamily. This superfamily includes the receptors for other steroid hormones, the receptor for triiodothyronine, the receptor for retinoic acid, and a product of the *erb-a* oncogene, which acts as a regulator of gene transcription (73,74). The VDR is highly expressed in small intestine and bone, the major vitamin D target tissues, and is also present in most other tissues, including testis, ovary, pituitary, parathyroid, skin, breast, muscle, lymphocytes, some tumor cell lines (75–80) and cancers. In prostate cancer, high levels of VDR expression in tumor tissue were found to be associated with a more favorable clinical outcome (81). The VDR has a hormone-binding domain at the C-terminus for the binding of  $1,25(\text{OH})_2\text{D}$  and a DNA-binding domain with two zinc fingers at the amino terminus (82). After binding of  $1,25(\text{OH})_2\text{D}$  to the hormone-binding domain of the VDR, the  $1,25(\text{OH})_2\text{D}$ -VDR complex dimerizes with the retinoid X receptor (RXR) and the  $1,25(\text{OH})_2\text{D}$ -VDR-RXR heterodimer then binds to vitamin D-response elements (VDRE) that are present in target genes, thereby regulating gene transcription (83,84). Expression of VDR is induced by cell proliferation, by exposure to  $1,25(\text{OH})_2\text{D}$ , and by the ontogenetic state. For example, the receptor first appears in rat intestine 14 days postnatally and correlates with the onset of  $1,25(\text{OH})_2\text{D}$ -dependent calcium transport therein (85). The affinity of vitamin D compounds for the VDR correlates with their biological potency;  $1,25(\text{OH})_2\text{D}$  is the most potent natural calciferol metabolite, with an ED50

**TABLE 98-1 Serum Levels and Body Pools of Calciferol Metabolites in Adults<sup>a</sup>**

	Concentration in Serum (ng/mL)	Half-Life in Serum (Days)	Pool Size in Body (mg)	Turnover in Body Metabolite (mg/day)
D3	10 <sup>b</sup>	30	1000	30
$25(\text{OH})\text{D}$	25	15	500	15
$1,25(\text{OH})_2\text{D}$	0.03	0.2	0.5	1
$24,25(\text{OH})_2\text{D}$	1	2	10	10

<sup>a</sup>With the exception of serum concentrations, these numbers are based on limited data.

<sup>b</sup>Typical summer mean temperate climate; normal winter mean is below 0.5 ng/ml.

From Marx, S. J. Vitamin D and Other Calciferols. In *The Metabolic Basis of Inherited Disease*, 6th ed.; Scriver, C. R.; Beaudet, A. L.; Sly, W. S.; Valle, D. Eds.; McGraw Hill: New York, 1989; pp 2029.



of approximately 1/1000 that of 25(OH)D in most test systems (86). Because of differing concentration ranges for metabolites (Table 98-1), the receptor may be activated in vivo by a mixture of agonists, including 1,25(OH)<sub>2</sub>D, 25(OH)D<sub>3</sub>, and 5,6-trans-25(OH)D.

The identification of allelic variants of the VDR gene has stimulated interest in examining possible relationships between VDR genotypes and bone mineral density (87). These allelic variants, many of which are associated with polymorphisms for the recognition sites of restriction endonucleases (e.g., BsmI, TaqI, and ApaI), are distributed into five major genotypes in most populations studied (88,89). Although initial studies showed a strong association between VDR gene polymorphisms, bone mass and bone turnover (87), subsequent studies in more diverse populations have shown more conflicting results, and indicate a lesser (or inconsistent) role for VDR alleles in the determination of the heritable component of bone density and strength (reviewed in References (90,91,92a,93). The recognition that vitamin D plays a role in diverse biological processes beyond bone and mineral metabolism, such as regulation of inflammation, neoplasia, and apoptosis, has led to additional studies of possible genetic associations with the VDR. For example, recent analyses show modest (or conflicting) associations between VDR genotype and risk of some cancers (94a), height, and adiposity (95–98). The nonbone effects of vitamin D, such as its antiproliferative action in some tissues and tumors, also appear to be dependent on the presence of VDRs in the target tissue (99). (For review of associations of VDR polymorphisms and diverse diseases, see Reference (100)).

### 98.3.4 Transcriptional Effects of 1,25(OH)<sub>2</sub>D

The best characterized function of the VDR is as a ligand-dependent transcription factor. After 1,25(OH)<sub>2</sub>D binds to the VDR, the VDR forms a stable heterodimer complex with retinoid X receptors. The heterodimer binds to VDREs (83), specific DNA motifs in the promoter region of target genes, and attracts nuclear receptor coactivators and other components necessary to alter gene transcription (101,102). By this mechanism, 1,25(OH)<sub>2</sub>D alters gene transcription of many proteins. Some of the target genes are known. For example, in response to 1,25(OH)<sub>2</sub>D, cholecalciferol (see later discussion in Section 98.3.6) mRNA levels in duodenal mucosa (and in many other tissues) are increased (103), levels of mRNA encoding osteocalcin (also termed bone  $\gamma$ -carboxyglutamic acid-containing, or bone GLA protein) rise in osteoblasts (104), and levels of procollagen type I mRNA fall in parathyroid cells (105) (see later discussion in Section 98.3.6 for more details concerning the proteins encoded by some of these mRNAs). These observations have led to the identification of VDREs in the promoter regions of the osteocalcin gene (positive VDRE) and the PTH gene

(negative VDRE) that are required for these vitamin D effects. In addition to regulating traditional targets genes that are involved with calcium homeostasis, 1,25(OH)<sub>2</sub>D is now known to be an important regulator of cell differentiation and growth (see References (106,107) for review), immune responses (108) and hormone secretion from endocrine tissues such as the pancreas (109).

### 98.3.5 Nongenomic Effects of 1,25(OH)<sub>2</sub>D

1,25(OH)<sub>2</sub>D exerts some cellular effects through a mechanism that does not involve alterations in gene transcription (i.e. nongenomic pathway). Evidence for these mechanisms includes extremely rapid effects (110,111) and dependency on normal receptor genes (112). Nongenomic effects of steroid hormones in target cells are transmitted through membrane receptors that appear to be identical to classical intranuclear steroid hormone receptors (113a). Activation of this signaling pathway leads to stimulation of MAP kinase and involves cross-talk with the nuclear VDR (see References (106,114) for review) as well as interaction of membrane receptors with other proteins (e.g. ion channels) or signal transduction pathways (e.g. protein kinase C, cAMP, Ca<sup>2+</sup>, etc.). The membrane-bound VDR appears to control pathways that are important for both fracture healing and chondrocyte maturation (115,116). One nongenomic receptor has been identified, protein-disulfide isomerase-associated 2 (Pdia3, also called Grp58, ERp60, ERp61, PDI-Q2, and 1,25D3-MARRS (membrane-associated, rapid response steroid binding), a glycoprotein-specific thiol oxidoreductase that is localized to the endoplasmic reticulum (see Reference (117) for review). The gene for Pdia3 is located on chromosome 15. Pdia3 has been shown to mediate 1,25(OH)<sub>2</sub>D effects on the osteoblast in vitro, through activation of protein kinase C (118).

### 98.3.6 Calbindin and Intestinal Transport of Calcium

The most important traditional physiologic action of 1,25(OH)<sub>2</sub>D is stimulation of active calcium transport across the duodenum from lumen to bloodstream. Calcium absorption occurs predominantly in the proximal small intestine (90%), with lesser amounts absorbed through the colon (10%). In the duodenum, calcium transport is active and saturable; in the jejunum and ileum, calcium transport is passive and nonsaturable. When calcium intake is low, the active duodenal transport of calcium predominates. When calcium intake is high, passive absorption is increased (see Reference (119) for review). Calbindin (also called cholecalciferol, or vitamin D-dependent calcium-binding protein) is a vitamin D-dependent protein that binds calcium with high affinity (the calcium-binding regions are homologous to those of the calmodulin family). Calbindins are highly expressed in the intestine and kidney in the chick and mammals,

and are classified in different subfamilies as they differ in the number of  $\text{Ca}^{2+}$ -binding EF-hand sites. There are at least two *calbindin* genes; one codes for a 9-kDa protein (*calbindin-D9k*) concentrated in the duodenum, the other for a homologous 28-kDa (*calbindin-D*) protein concentrated in kidney, brain, bone, and many other tissues. There is no homology between *calbindin-D28k* and *calbindin-D9k*, apart from their calcium-binding domains (EF-hands): *calbindin-D9k* has two EF-hands, and *calbindin-D28k* has six. The *calbindin-D9k* gene is expressed in duodenal cell cytoplasm and is involved in  $1,25(\text{OH})_2\text{D}$ -mediated calcium absorption although the precise mechanism is unknown (120). *Calbindin* in the duodenum might act as a buffer of intracellular calcium (121) or as a regulator of calcium ATPase (122). *Calbindin-D9k* constitutes approximately 2% of duodenal mucosal cell protein (found only in high amounts in the duodenum) in the D-replete state, and is undetectable in duodenum in the D-deficient state.

A widespread tissue distribution of  $1\alpha,25(\text{OH})_2\text{D}$  receptors and of a family of *calbindin* proteins indicates that  $1,25(\text{OH})_2\text{D}$  may act directly in many tissues. Recent experimental evidence indicates that *calbindin-D* can inhibit apoptosis induced in varied cells, including pancreatic beta cells, renal cells, and osteoblasts, partially through its role in buffering intracellular calcium (123). Recent evidence suggests that TRPV5 and TRPV6, apical calcium channels that are colocalized in the duodenum with *calbindin*, are involved in active duodenal calcium absorption (124). There is no known regulation of passive, paracellular calcium absorption (125). Bone volume and stiffness are both increased in *calbindin-D28K* knockout mice, indicating that *calbindin* plays a significant role in skeletal remodeling (126a).

### 98.3.7 $1,25(\text{OH})_2\text{D}$ Actions on Bone

Most of the antirachitic actions of calciferols are secondary to maintenance of calcium and phosphate concentrations in extracellular fluid adequate for bone mineralization (127–129).  $1,25(\text{OH})_2\text{D}$  inhibits proliferation and collagen synthesis in fetal bone and osteoblasts (130). In osteoblast-like cells from adult humans, however,  $1,25(\text{OH})_2\text{D}$  stimulates collagen synthesis (131). Differing effects on alkaline phosphatase have been reported in several systems; however, there is general agreement that in rapidly growing osteoblast-like cells, alkaline phosphatase levels are low and that they rise in response to  $1,25(\text{OH})_2\text{D}$  (132).

In vivo and in organ culture,  $1,25(\text{OH})_2\text{D}$  is a potent activator of osteoclasts. Isolated osteoclasts, however, show no response to  $1,25(\text{OH})_2\text{D}$  (133) and contain no receptors for  $1,25(\text{OH})_2\text{D}$  (134). At least three mechanisms have been suggested to explain the ability of  $1,25(\text{OH})_2\text{D}$  to activate osteoclasts: first,  $1,25(\text{OH})_2\text{D}$  may stimulate differentiation of osteoclast precursors, related to monocytes and macrophages (135,136);

second,  $1,25(\text{OH})_2\text{D}$  may stimulate fusion and metabolism of the immediate precursor of the multinucleated osteoclast (137); and third,  $1,25(\text{OH})_2\text{D}$  might stimulate adjacent cells (138) such as osteoblasts to activate osteoclasts.  $1,25(\text{OH})_2\text{D}$  can activate cells of the osteoblast lineage, leading them to undergo shape changes and secrete collagenase and other enzymes that lyse proteins on the bone surface; they also express a factor that is termed RANK ligand (previously known as osteoclast differentiating factor, ODF). RANKL interacts with a receptor on osteoclast precursors that is identical to the receptor involved in interaction of T cells and dendritic cells called receptor activator of NF-kappa B (RANK). RANKL stimulates activation, migration, differentiation, and fusion of hematopoietic cells of the osteoclast lineage to begin the process of resorption (139,140). RANKL can also bind a protein that is produced by osteoblasts and other marrow cells called osteoprotegerin (OPG) or osteoclastogenesis inhibitory factor (141,142). OPG acts as a “decoy receptor” for RANKL and thereby inhibits osteoclast recruitment and activation (143). Recent studies have documented that transgenic mice, in which OPG has been inactivated have marked osteoporosis (144), and genetic mutations in humans that reduce expression of OPG (145,146) or activate RANK (147,148) are associated with excessive bone resorption. In vivo studies on a mouse VDR knockout model have suggested that vitamin D-mediated osteoclast formation requires the presence of osteoblasts, but normal osteoclasts can form in the absence of VDR, presumably via the influence of other agents (149).  $1,25(\text{OH})_2\text{D}$  also plays a role in osteoblast differentiation (150).

### 98.3.8 $1,25(\text{OH})_2\text{D}$ Actions on Skin and Hair

Receptors for  $1,25(\text{OH})_2\text{D}$  have been identified directly by in vivo autoradiography in basal layers of epidermis and in the outer root sheath cells of the rat hair follicle (151). The VDR is also present in human skin fibroblasts and keratinocytes (14).  $1,25(\text{OH})_2\text{D}$  inhibits proliferation of cultured human fibroblasts and promotes terminal differentiation of keratinocytes (14). These effects of  $1,25(\text{OH})_2\text{D}$  have led to the use of vitamin-D analogs in the treatment of psoriasis, a hyperproliferative disorder of the epidermis (14,152). Topical vitamin-D analogs have been developed to minimize the hypercalcemic response to vitamin-D treatment in psoriasis (153). In vitro studies have shown that  $1,25(\text{OH})_2\text{D}$  suppresses ultraviolet-induced apoptosis in cultured skin keratinocytes (154). Although not yet completely delineated,  $1,25(\text{OH})_2\text{D}$  plays a significant role in hair growth. The mammalian hair cycle requires both the VDR and the hairless (Hr) corepressor, each of which is expressed in the hair follicle. Hr interacts directly with VDR to repress VDR-targeted transcription (155). Lack of VDR or Hr results in premature apoptosis of the hair bulb during

the first catagen stage of the hair cycle, with subsequent loss of hair follicles. In nude mice with congenital alopecia and in cultured hair follicles,  $1,25(\text{OH})_2\text{D}$  stimulated hair growth (156,157). New research has shown that the effect of  $1,25(\text{OH})_2\text{D}$  on hair involves the wnt (158) and hedgehog (159) signaling pathways.

### 98.3.9 $1,25(\text{OH})_2\text{D}$ Actions on Parathyroid Gland

$1,25(\text{OH})_2\text{D}$  inhibits parathyroid cell function in vitro. In particular, it decreases transcription of mRNA for preproparathyroid hormone (105), and inhibits PTH secretion and parathyroid cell proliferation (see Reference (160) for review). The expression of VDR by parathyroid cells is modulated by both extracellular calcium concentration and  $1,25(\text{OH})_2\text{D}$  (161). However,  $1,25(\text{OH})_2\text{D}$  also inhibits parathyroid cell apoptosis (162). In vitro studies have shown that  $1,25(\text{OH})_2\text{D}$  induces expression and transcription of the calcium sensing receptor (CASR) on parathyroid cells, which should make the parathyroids more sensitive to suppression of PTH release by serum calcium (163). In vivo studies of transgenic mice, in which the VDR was specifically deleted in the parathyroid glands have raised new questions about the role of VDR on regulation of PTH secretion, however (164). In these eucalcemic mice, parathyroid expression of the CASR was decreased and basal PTH levels were only moderately increased, suggesting that the VDR has a limited role in parathyroid physiology.

### 98.3.10 $1,25(\text{OH})_2\text{D}$ Actions on Calciferol Metabolism

$1,25(\text{OH})_2\text{D}$  regulates calciferol metabolism at several steps. It can increase the levels of 7,8-didehydrocholesterol in skin (165), and is a potent inhibitor of the renal  $25(\text{OH})\text{D}_3$   $1\alpha$ -hydroxylase enzyme in kidney and in many other tissues (50).  $1,25(\text{OH})_2\text{D}$  also stimulates the clearance of  $25(\text{OH})\text{D}$  and  $1\alpha, 25(\text{OH})_2\text{D}$ ; this regulated clearance is mediated by induction of 24-hydroxylation, (CYP24A1 gene) and 23-hydroxylation (166,167). Other catabolic pathways for  $1,25(\text{OH})_2\text{D}$  are likely in that the CYP24A1 knockout mouse is only hypercalcemic when fed a high-calcium diet and during pregnancy (168).

### 98.3.11 Specific Role of $24,25(\text{OH})_2\text{D}_3$

A growing body of evidence contradicts the notion that  $24,25(\text{OH})_2\text{D}$  has little or no physiological activity. It was reported that  $24,25(\text{OH})_2\text{D}$  showed unique actions on cartilage from fetal or newborn animals, not reproduced by any dosage of  $1,25(\text{OH})_2\text{D}_3$  (169,170). For example, in chicks, normal skeletal development and egg hatchability require both  $1,25(\text{OH})_2\text{D}$  and  $24,25(\text{OH})_2\text{D}$ . In addition,  $24,25(\text{OH})_2\text{D}$  appears to improve fracture repair and increase bone mass in

vitamin D-replete animals. Moreover, other recent studies have revealed that  $24,25(\text{OH})_2\text{D}$  also plays a role in endochondral ossification.  $24,25(\text{OH})_2\text{D}$  regulates the maturation of resting zone chondrocytes via phospholipase D (115,171), which produce the hormone themselves. When resting zone chondrocytes mature into growth zone chondrocytes, they become unresponsive to  $24,25(\text{OH})_2\text{D}_3$  and become responsive to  $1,25(\text{OH})_2\text{D}_3$  (115). Finally, a significant physiological role for  $24, 25(\text{OH})_2\text{D}_3$  has recently been identified in mice that are genetically deficient in  $24,25(\text{OH})_2\text{D}$  because of targeted ablation of the 24-hydroxylase gene. These mice manifest defective formation and mineralization of intramembraneous bone. This effect was previously attributed to elevated levels of  $1,25(\text{OH})_2\text{D}$  rather than low levels of  $24,25(\text{OH})_2\text{D}_3$  (135).

## 98.4 GENERAL FEATURES OF CALCIFEROL DEFICIENCY

### 98.4.1 Clinical Presentation

The general features of vitamin D deficiency (126,172,173) occur in patients with hereditary defects in calciferol metabolism or action.

The clinical features of calciferol deficiency are weakness, bone pain, bone deformity, and fracture. The mineralization defect causes its greatest effects at the epiphyses and metaphyses of the most rapidly growing bones, which develop the characteristic changes of rickets. In the first year of life, the most rapidly growing bones are the skull, ribs, and long bones. Calciferol deficiency at this time leads to widened cranial sutures, frontal bossing, posterior flattening of the skull, bulging of costochondral junctions, and enlargement of the wrists. The rib cage may be so deformed that it contributes to respiratory failure. Dental eruption is delayed, and teeth show enamel hypoplasia. Muscular weakness and hypotonia are severe and result in a protuberant abdomen. Muscular weakness may also contribute to respiratory failure. Linear growth may be adequate but the child may be unable to walk without support. Tetany is unusual as the degree of hypocalcemia is mild and its onset is slow. After age 1, deformities are most prominent in the legs because of their weightbearing function. Anemia is common.

The clinical features of calciferol-deficiency states depend principally on age of onset. Calcium and phosphate levels in fetal plasma are sustained by placental transport from maternal plasma, and this transport is probably not regulated by calciferols (174). A fetus with a hereditary abnormality in calciferol metabolism developing in a mother with normal calciferol metabolism is presumed to have normal calcium and phosphate levels in plasma and bone until birth.

In children, the mineralization defect leads to rickets, with disturbed development and maturation of diaphyses,

metaphyses, and epiphyses. The initial changes of rickets are best visualized at the growth plates of the most rapidly growing bones. In the upper limbs, the distal ulna may show the earliest effects of impaired mineralization. In the older child, the metaphyses about the knees become more useful. Early features of rickets include widening of the epiphyseal plate and a loss of definition of the provisional zone of calcification at the metaphyses. As the disease progresses, the growth plate becomes more disorganized and cupping, splaying, spur formation and stippling appear. The development of the epiphyses may be delayed or the epiphyses may appear small, osteopenic or ill defined. When the mineralization defect occurs in very early childhood, it is likely to cause genu valgum, whereas genu varum is more common when the mineralization defect occurs after the age of 2–3 years of life.

Calciferol deficiency that begins after epiphyseal fusion causes less deformity, and results in a mineralization defect termed *osteomalacia*. In the mature, remodeling skeleton, <5% of the calcium is newly deposited per year. Thus, a mineralization defect in an adult must exist for several years to be clinically manifest. The earliest symptom is bone pain, particularly low in the back. Proximal muscle weakness may be so prominent as to suggest a primary neurologic disturbance.

### 98.4.2 Pathophysiology

Virtually all the features of calciferol deficiency can be understood as direct or remote consequences of the effect of deficient calciferol on duodenal transport of calcium. Malabsorption of calcium causes hypocalcemia. Hypocalcemia and, perhaps, also the effect of deficient calciferol on the parathyroid gland (175) leads to increased secretion of PTH (i.e. secondary hyperparathyroidism). If this process continues for many months, the parathyroid glands enlarge through hypertrophy and hyperplasia. PTH acts on the proximal renal tubule to decrease reabsorption of phosphate and bicarbonate, producing hypophosphatemia and hyperchloremic metabolic acidosis. Hypophosphatemia rather than hypocalcemia appears to be the principal basis for the growth plate defect in rickets. Studies in a series of mouse models (*VDR-null*, diet-induced hypophosphatemia/hypercalcemia and hypophosphatemia secondary to mutations in the *Phex* gene) have demonstrated that normal phosphorus levels are required for growth plate maturation, and have identified phosphate-regulated apoptosis of hypertrophic chondrocytes, via activation of caspase-9, as a putative mechanism (176).

In some patients, severe vitamin D deficiency can be associated with a confusing biochemical profile, particularly when hypocalcemia is severe. In these patients, the very low serum calcium concentrations can inhibit the phosphaturic effect of PTH, resulting in PTH resistance with normal or elevated serum phosphorus levels.

These patients have elevated urinary levels of nephrogenous cyclic AMP but do not demonstrate the expected phosphaturic response (177), a condition that has been termed *pseudohypoparathyroidism type II*. In children, secondary hyperparathyroidism also causes generalized aminoaciduria. The combination of hypocalcemia and hypophosphatemia reduces the rate of mineralization of bone matrix. PTH also acts directly on bone, where it increases osteoclastic reabsorption (but the release of calcium and phosphate from bone does not fully compensate for the hypocalcemia or hypophosphatemia).

The principal goal of therapy is to provide enough calcium in extracellular fluid to allow normalization of bone mineralization and suppression of secondary hyperparathyroidism. Chronic deficiency of calciferol causes deficiency of calcium in the skeleton that can result in removal of calcium from plasma for many months during remineralization. During the early months of treatment with vitamin D or 25(OH)D<sub>3</sub>, the serum contains low calcium, high PTH, low phosphate and normal or high 25(OH)D, all of which promote synthesis of 1,25(OH)<sub>2</sub>D. The prevalence of vitamin D deficiency has increased from the 1990s to the 2000s (178,179), most likely due to a combination of factors including increasing incidence of obesity, reduced outside leisure activity and use of sunscreens.

### 98.4.3 Treatment

During the first 1–4 months of treatment, endogenous production of 1,25(OH)<sub>2</sub>D is regulated at rates greater than normal (46,180–182). A successful response to calciferol therapy is evidenced by reversal of secondary hyperparathyroidism within several weeks; that is, serum PTH and urinary cAMP fall and serum concentrations of phosphate and calcium increase and normalize. Early in treatment, there may be a transient increase in serum alkaline phosphatase, which over several months, normalizes as the bone mineralizes. In children, normal bone mass may be achieved after recovery from calciferol deficiency but in adults, bone mass may remain low (183).

### 98.4.4 Nomenclature of Vitamin D Defects

The calciferols traverse a metabolic pathway that could justify their being described as normal metabolites, vitamins (vitamin D is a dietary factor required in trace amounts in states of limited skin exposure to ultraviolet light) or hormones (1 $\alpha$ , 25(OH)<sub>2</sub>D is secreted into the bloodstream at a regulated rate by the kidney and acts on the intestine and other targets). Previous nomenclature to describe rare inborn errors of calciferol metabolism has been confusing because the terms evolved prior to elucidation of the biosynthetic pathway



**TABLE 98-2 Vitamin D-Dependent Rickets**

OMIM	25(OH)D	1,25(OH) <sub>2</sub> D	i PTH	Inheritance	Gene Defect
VDDR-1	N/I	D	I	A.R	1- $\alpha$ -hydroxylase, CYP27B1 (264700)
VDDR-2	N/I	N/I	I	A.R	Vitamin D receptor, VDR

VDDR, Vitamin D-dependent rickets.

of vitamin D activation. Several terms have included the words “deficiency” or “dependency” (e.g., pseudodeficiency, dependency, and resistance) to describe pathophysiologic states that share many features of vitamin D deficiency (i.e. calcium deficiency, secondary hyperparathyroidism, impaired skeletal mineralization), but in which no actual deficiency of vitamin D (or dietary calcium) is present. Two hereditary syndromes in which vitamin D action is impaired have been recognized. The first is classified as vitamin D-dependent rickets type 1 (VDDR-1), and is due to an inability to convert 25(OH)<sub>2</sub>D to 1,25(OH)<sub>2</sub>D. The second is type 2 vitamin D-dependent rickets (VDDR-2), and is due to generalized resistance to 1,25(OH)<sub>2</sub>D. Although the two syndromes share many clinical features, they differ in the circulating concentration of 1,25(OH)<sub>2</sub>D, the therapeutic response to 1 $\alpha$ -hydroxylated vitamin-D analogs, and obviously in the primary defect in vitamin D metabolism (Table 98-2). Recently, a novel form of inherited pseudo vitamin-D deficiency has been described (9,20), due to homozygous mutation of 25-hydroxylase (CYP2R1).

### 98.5 HEREDITARY VITAMIN D DEPENDENCY TYPE 1 (VDDR-1)-1- $\alpha$ -HYDROXYLASE DEFICIENCY

Hereditary vitamin D-dependent rickets type 1 (VDDR-1) is characterized by deficient conversion of 25(OH)D to 1,25(OH)<sub>2</sub>D and is caused by mutations in the cytochrome P450 enzyme, 25-hydroxyvitamin D3-1  $\alpha$ -hydroxylase (CYP27B1). The complete response of the original index cases to vitamin D suggested vitamin D deficiency, but the continuing requirement for high doses of vitamin D to sustain the remission indicated a vitamin D dependency. Thus, VDDR-1 is also known as “selective and simple deficiency of 1 $\alpha$ , 25(OH)<sub>2</sub>D,” “hereditary vitamin D-pseudodeficiency type 1,” “autosomal recessive vitamin D dependency type 1 (ARVDD-1),” and “hereditary 25D, 1 $\alpha$ -hydroxylase deficiency rickets.” This disorder is a rare cause of rickets, described in approximately 100 patients (3,7,184–201). VDDR-1 occurs with unusual frequency in the French-Canadian population (186). The 1 $\alpha$ -hydroxylase gene, which is located on chromosome 12q13.3, was cloned in 1997 (26), and shortly thereafter was confirmed as the genetic defect in VDDR-1 (202).

#### 98.5.1 Clinical Features

Patients appear normal at birth but problems related to defects in mineral metabolism become obvious at 2–24 months, suggesting that the abnormality in calciferol action was present at the time of birth. Muscle weakness, irritability, tetany or convulsions, and failure to thrive are typical in the first few months of life. If diagnosed later, skeletal fractures and deformity (e.g. frontal bossing, rib cage abnormalities, and long-bone deformities) as well as growth retardation are notable. Laboratory studies reveal hypocalcemia, hypophosphatemia, and elevated serum levels of alkaline phosphatase and PTH. Plasma concentrations of 1,25(OH)<sub>2</sub>D are low or even undetectable (188,197,200,203), despite normal or even modestly increased levels of 25(OH)D (reflecting vitamin D supplementation or decreased metabolism or clearance of 25(OH)D). Plasma amino acids are normal, but generalized aminoaciduria is present. Skeletal radiographs show severe rachitic changes (see Section 98.4). Permanent teeth show enamel hypoplasia (184). If untreated, the natural history is progressive skeletal deformity and short stature. If adequately treated, healing of rickets and normal growth ensue and if treatment is started before the pubertal growth spurt, normal height can be achieved (200). At least two patients have been reported in whom CYP27B1 mutations resulted in partial 1 $\alpha$ -hydroxylase activity (198).

Reduced activity of the renal 25(OH)D<sub>3</sub> 1  $\alpha$ -hydroxylase enzyme accompanies several other hereditary or acquired disorders that are distinct from VDDR-1, and replacement of 1,25(OH)<sub>2</sub>D is an important component of the therapy for these disorders. These disorders typically affect the proximal renal tubule and include X-linked (mutation in *PHEX*), autosomal dominant (mutation in *FGF23*) and autosomal recessive (mutation in *ENPP1* or *DMP1*) hypophosphatemic rickets (204–207), as well as renal tubular acidosis (208–210), Fanconi syndrome (211–213), and tumor-induced osteomalacia (TIO). X-linked and autosomal hypophosphatemic rickets and TIO share a common pathophysiology in which a circulating phosphatonin, typically fibroblast growth factor 23 (FGF23), impairs 1 $\alpha$ -hydroxylase activity and reduces expression of *NPT2a* and *NPT2c* in the proximal renal tubule, leading to renal wasting of phosphate (214). FGF23, FRP4 (frizzled related protein 4), and MEPE (matrix extracellular phosphoglycoprotein), each possesses some or all of the properties associated

with phosphatonin, and were discovered through studies of XLH, ADHR, and TIO.

### 98.5.2 Genetics

VDDR-1 is an autosomal recessive disorder and is characterized by deficient  $1\alpha$ -hydroxylase activity. All patients reported to date, from a variety of ethnic backgrounds, have had mutations in the *CYP27B1* gene, resulting in complete or partial loss of  $1\alpha$ -hydroxylase enzyme function. Numerous different mutations have been found throughout the gene (195,201,215–221). VDDR-1 was initially mapped to chromosome 12q14 by linkage analysis in five French-Canadian families (193). The relatively isolated French-Canadian community of Saguenay in Quebec shows an unusually high prevalence of this disorder, but there appears to be allelic diversity, suggesting that more than one genetic founder must account for the disorder in these patients (220). Prediction of carrier status and early detection in newborns is possible. The  $1\alpha$ -hydroxylase enzyme is a complex system composed of three subunits (ferredoxin reductase, ferredoxin, and cytochrome P450 D1), and heterogeneity, involving mutations of different components, is therefore not surprising.

A spontaneous animal (porcine) model of VDDR-1 has been described (222), with autosomal recessive hypocalcemic rickets that is responsive to physiologic doses of  $1,25(\text{OH})_2\text{D}$  (223). Renal homogenates from homozygous animals lack  $1\alpha$ -hydroxylase and  $24$ -hydroxylase activities (224,225). Several mouse models have been developed through targeted inactivation of the  $25$ -hydroxyvitamin D-1  $\alpha$ -hydroxylase gene (*CYP27B1*) (226,227). These mice develop the typical skeletal and biochemical features of the human disorder, which are completely reversed by chronic administration of the active  $1,25(\text{OH})_2\text{D}$  metabolite. By contrast, feeding mice deficient for the *Cyp27b1* gene a rescue diet (high-calcium and lactose, 2% calcium, 1.25% phosphorus, 20% lactose) that corrects the hypocalcemia and secondary hyperparathyroidism did not entirely correct the skeletal

defects. Treated mice showed decreased bone growth as femur size remained significantly smaller than that of control mice, despite bone histology and histomorphometry confirming that rickets and osteomalacia were cured (228,229).

### 98.5.3 Management

Patients have been treated successfully with all available calciferol analogs, but use of calcitriol is the most straightforward approach to replacement therapy (188,194,230,231). Calcium supplements (elemental calcium at doses of 50 mg/kg per day to children and 1000 mg/kg per day for adults) should also be given. The specific calciferol analog used depends on several factors. The use of calcitriol has a significant theoretical advantage as  $1,25(\text{OH})_2\text{D}_3$  is the deficient hormone. Therefore, physiologic doses of calcitriol are effective. Calcitriol is available orally as a capsule or suspension, and it is generally given twice daily because of its short half-life. Treatment with vitamin D<sub>2</sub> (ergocalciferol) or vitamin D<sub>3</sub> (cholecalciferol) requires pharmacologic doses but is still less expensive than calcitriol. Ergocalciferol and cholecalciferol have significantly longer half-lives than calcitriol, which allows for once-daily dosing. However, calcitriol is the drug of choice because of its short half-life, limited distribution and fat solubility, rapid onset and offset of action, and availability in a form that may be administered intravenously. During the first 3–6 months of treatment, patients will generally require doses of calciferols that are two- to fivefold greater than the dose expected for long-term maintenance (Table 98-3), as the undermineralized skeleton requires unusually large amounts of calcium. During successful treatment with vitamin D, serum  $25(\text{OH})\text{D}$  levels will be markedly elevated (approximately 200–250 ng/mL), but the serum concentration of  $1,25(\text{OH})_2\text{D}$  may remain low or undetectable (197,203). Successful therapy maintains fractional intestinal absorption of calcium near a constant value and serum calcium in the low normal range. Because the fractional absorption of calcium

**TABLE 98-3** Calciferol Doses for Maintenance Treatment of Patients with Hereditary Defects in Calciferol Metabolism

	Dosage in VDDR-1 ( $\mu\text{g}$ per Day)	Dosage in VDDR-2 ( $\mu\text{g}$ per Day)
Vitamin D <sub>3</sub> or D <sub>2</sub>	500–3000	500–? <sup>a</sup>
$25(\text{OH})\text{D}_3$	30–200	30–? <sup>a</sup>
$1\alpha,25(\text{OH})_2\text{D}_3$	0.3–2	5–60 <sup>b</sup>
$1\alpha(\text{OH})\text{D}_3$	0.5–3	5–60 <sup>b</sup>
Dihydrotachysterol	150–1000	2000–20,000 <sup>b</sup>

Dose requirements are uncorrected for body weight and are similar in children and adults.

<sup>a</sup>Patients with milder grades of resistance to  $1\alpha,25(\text{OH})_2\text{D}$  (usually with normal hair) can respond to analogs requiring 1-hydroxylation. Maximal useful doses have not been defined. Serum  $1\alpha,25(\text{OH})_2\text{D}$  must be maintained in the range of 200–1000 pg/mL.

<sup>b</sup>Maximal doses are limited only by cost and patient acceptance; some patients have shown no response to maximal doses tested.

From Marx, S. J. Vitamin D and Other Calciferols. In *The Metabolic Basis of Inherited Disease*, 6th ed.; Scriver, C. R.; Beaudet, A. L.; Sly, W. S.; Valle, D. Eds.; McGraw Hill: New York, 1989; pp 2029.

from the gastrointestinal tract is constant in the absence of mechanisms that regulate synthesis of  $1,25(\text{OH})_2\text{D}$ , calcium homeostasis relies upon the direct actions of PTH on kidney and/or bone. Thus, levels of serum (and urine) calcium can fluctuate greatly in response to changes in the dietary calcium intake of these patients. To avoid erratic changes in serum calcium levels, these patients should receive oral calcium supplements daily to maintain a consistent dietary calcium intake. Treatment must be continued indefinitely.

## 98.6 HEREDITARY VITAMIN D-DEPENDENT RICKETS TYPE 2 (VDDR-2)

### 98.6.1 Introduction

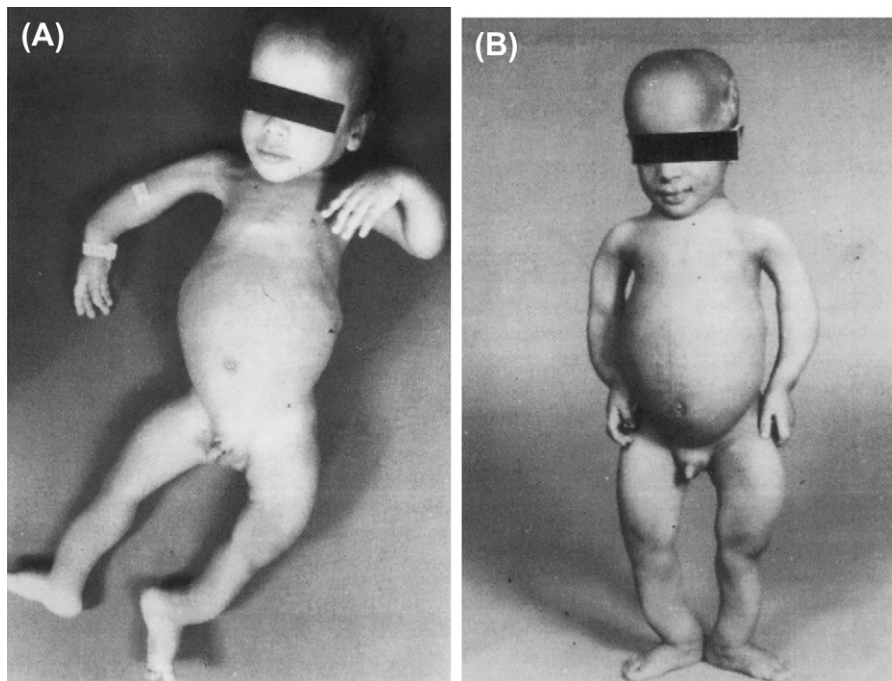
Hereditary vitamin D-dependent rickets type 2 (VDDR-2) is an autosomal recessive disorder, in which hypocalcemia is due to true end-organ resistance to  $1,25(\text{OH})_2\text{D}$ . By analogy to VDDR-1, early reports of affected patients described responsiveness to treatment with high doses of vitamin D, and suggested that children with this disorder had a related form of vitamin D dependency. However, based on later case reports in which about half the patients with this disorder did not respond to any form of vitamin D therapy, the term VDDR-2 seems to be a misnomer. VDDR-2 is therefore more appropriately described by the terms hereditary  $1,25(\text{OH})_2\text{D}$ -resistant rickets (HVDRR) or hereditary resistance to  $1,25(\text{OH})_2\text{D}$ . VDDR-2 is a rare

syndrome, first described in 1978 (8), with over 100 individuals in at least 30 kindreds (10,192,232–236).

### 98.6.2 Clinical Features

The clinical features are almost identical to those that occur in patients with VDDR-1, but about half of patients with VDDR-2 have alopecia (234,237). Patients with VDDR-2 appear normal at birth and develop features of calciferol deficiency over the first 2–8 months of life. Alopecia, generally developing at 2–12 months, may be total or incomplete, and reflects the integrated effects of the VDR and hairless (hr) gene proteins on regulation of the mammalian hair cycle (see Section 98.3.8), as inactivating mutations in either result in alopecia (Figure 98-4). Sometimes, there is selective sparing of the eyelashes. Alopecia seems to be a marker of the more severe forms of the disease, as judged by earlier onset of hypocalcemia, more marked clinical presentation, and poor response to therapy. Other ectodermal defects have been reported in small numbers of cases and have an uncertain relation to the syndrome; these include oligodontia (238), epidermal cysts, and multiple milia (238,239).

In several cases, neonatal development was apparently normal, and hypocalcemia and/or rickets was not evident until late childhood (240) or even adulthood (241) (in the latter case, serum  $1\alpha,25(\text{OH})_2\text{D}$  was not measured, so alternative etiologies for high calciferol dose, such as non-compliance, were not excluded). In each report, the patient did not show alopecia, and each responded to high doses



**FIGURE 98-4** A 5-year-old boy with hereditary resistance to  $1,25(\text{OH})_2\text{D}$ . He was unable to sit without support at age 5. A, Despite being unresponsive to high doses of calciferols, he showed improvement 2 years later following treatment with high doses of calcium orally. B, Although the weakness and deformity improved, alopecia worsened during treatment. (From Sakati, N.; Woodhouse, N. J. Y.; Niles, N., et al. *Hereditary Resistance to 1,25-dihydroxyvitamin D: Clinical and Radiological Improvement during High-Dose Oral Calcium Therapy*. *Horm. Res.* 1986, 24, 280–287.)

of calciferols, indicating a mild variant of the syndrome. Neither showed clear features of a genetic etiology, in that there was no parental consanguinity and no affected sibling.

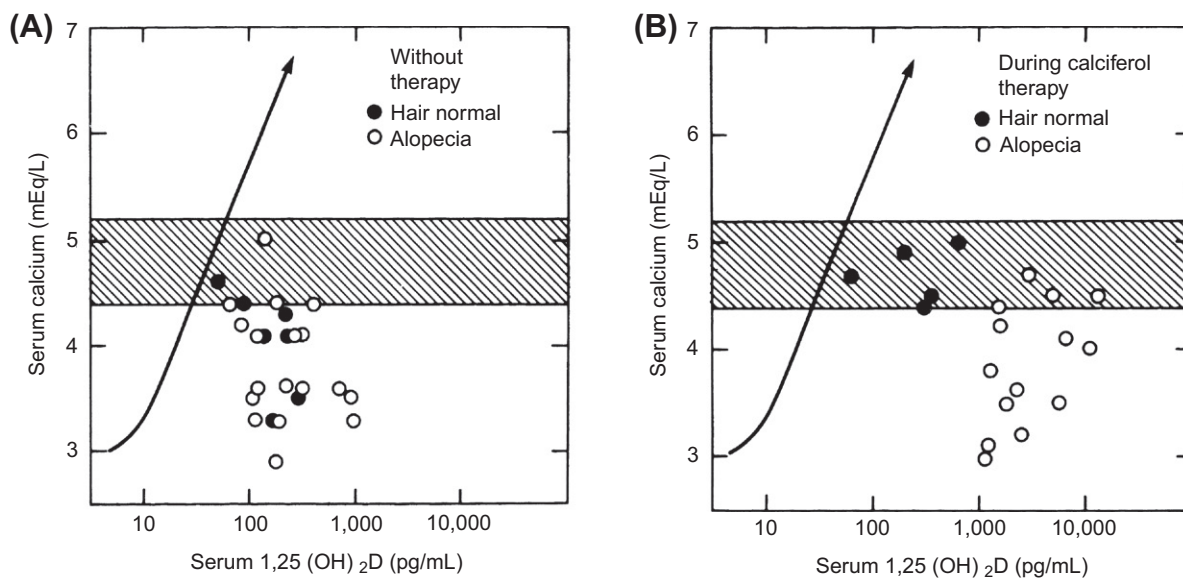
Laboratory studies show low serum concentrations of calcium and phosphorus, high serum levels of alkaline phosphatase and PTH, and generalized aminoaciduria. Unlike patients with VDDR-1, patients with VDDR-2 have elevated serum levels of  $1,25(\text{OH})_2\text{D}$ , in the range of 50–1000 pg/mL (normal in children is 30–100 pg/mL); during calciferol treatment, typical concentrations are 200–10,000 pg/mL (Figure 98-5). Basal and stimulated concentrations of insulin, thyrotropin, prolactin, growth hormone, and testosterone have been normal (242). Skeletal radiographs show severe rachitic changes. Light microscopic examination of a scalp biopsy showed normal numbers and morphology of hair follicles in a patient with total alopecia (243). Bone biopsies have shown normal or increased numbers of osteoclasts (185,244), but their resorptive activity was suggested to be impaired (185). Without therapy, this disorder leads to inanition, severe skeletal deformity, recurrent respiratory infections, and death by age 8 years.

### 98.6.3 Genetics

Inheritance of VDDR-2 is consistent with an autosomal recessive pattern. There is a striking clustering of patients in a broad region centered about the Mediterranean Sea, and this may relate to the very high consanguinity rate in patients with  $1,25(\text{OH})_2\text{D}$  receptor defects in this area (245). In addition, most cases reported from North America and Europe are descendants of families that originated from this area. Other, unrelated cases have been reported from Japan (241). The cloning and

characterization of the human VDR cDNA (72) and gene (246) facilitated molecular studies that have revealed heterogeneous mutations in the VDR in affected patients (246–252). Subsequent generation of mice deficient in VDR by gene targeting confirmed the role of the VDR in the pathogenesis of VDDR-2, and has allowed insights into not only the molecular basis for the actions of  $1,25(\text{OH})_2\text{D}_3$  in bone formation but also in development of the hair follicle (113,235,253). Remarkably, no defects in development and growth were observed before weaning, irrespective of reduced expression of vitamin D target genes. After weaning, however, mice lacking *Vdr* failed to thrive, with appearance of alopecia, hypocalcemia and infertility, and impaired bone formation. Unlike humans with VDDR-2, however, most null mutant mice have a markedly shortened lifespan, and females show uterine hypoplasia with impaired ovarian folliculogenesis. The development of alopecia and uterine hypoplasia are not observed in vitamin D-deficient animals, and thus, establish a critical role for VDR in growth, bone formation, and female reproduction in the postweaning stage (113,253). Recent work indicates that *Vdr-null* mice develop their first coat of hair normally but reinitiation of anagen after the first hair cycle is impaired (214a). Lack of *Vdr* is associated with increased expression of hairless (*Hr*), a transcriptional regulator of hair follicle cycling that appears to interact with *Vdr* to regulate hair follicle cycling (155).

Genetic studies have complemented cellular analyses of  $1\alpha$ ,  $25(\text{OH})_2\text{D}$  receptor action and DNA binding using cultured skin fibroblasts (254,255), keratinocytes (256), bone cells (94,257), and peripheral blood lymphocytes (258) from affected patients. Through a combination of several biochemical and immunological methods,



**FIGURE 98-5** Relationship between serum concentrations of calcium and  $1,25(\text{OH})_2\text{D}$ . Hatched area is normal range for calcium. Solid curve is theoretical normal relationship between calcium and  $1,25(\text{OH})_2\text{D}$ . A, Without calciferol therapy. B, During calciferol therapy. (From Marx, S. J.; Bliziotis, M. M.; Nanes, M. Analysis of the Relation between Alopecia and Resistance to  $1,25$ -Dihydroxyvitamin D. Clin. Endocrinol. 1986, 25, 373–381.)



at least six phenotypically different intracellular receptor defects have been identified:

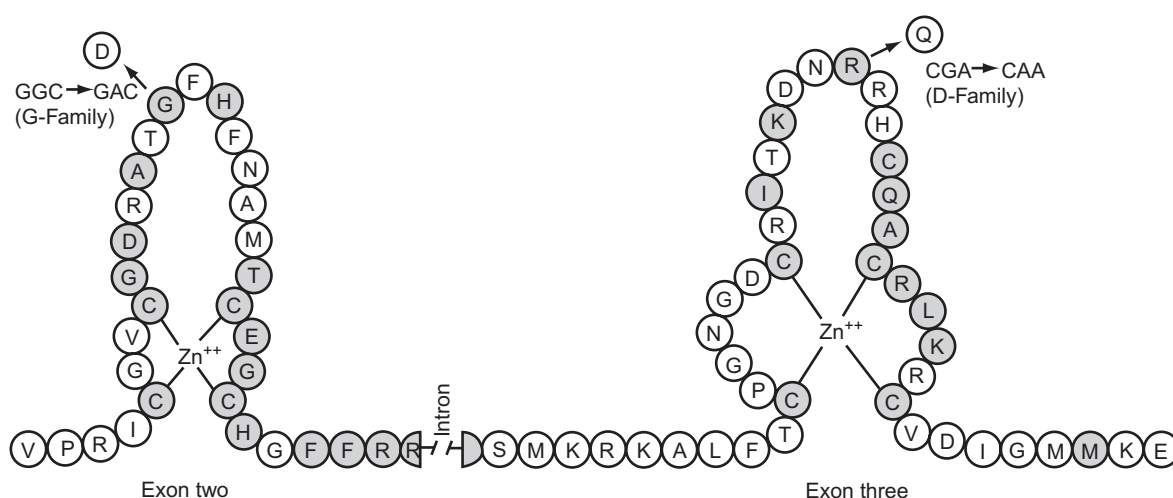
1. Hormone binding negative. This is the most common abnormality observed, and is characterized by expression of normal amounts of receptor molecules that are unable to bind  $1\alpha, 25(\text{OH})_2\text{D}$ . The postulated mechanism, a defect in the hormone-binding domain of the VDR, has been confirmed in affected subjects from three related families, in whom a single C to A nucleotide transversion at base 970 within exon 7 converts the normal codon for tyrosine at position 292 to a premature other termination codon (250,259). This results in a truncated protein that lacks a large portion of the hormone-binding domain. Another patient was described with a point mutation leading to deletion of exon 4 (260).
2. Defect in hormone-binding capacity, in which the number of binding sites is markedly reduced (261).
3. Decreased hormone-binding affinity (233,262).
4. Deficient nuclear localization of receptors with normal or near-normal binding affinity and capacity (254,255).
5. Normal hormone binding but decreased affinity of the VDR–hormone complex for DNA sequences. Four different point mutations in exons 2 and 3 have been identified in affected members of five different kindreds with this type of defect (246,251). Each mutation caused an amino acid substitution in the region of the zinc fingers of the VDR protein, and would be predicted to impair binding of the VDR–hormone complex to DNA sequences (Figure 98-6).
6. Reduced hormone-dependent transactivation and impaired heterodimeric interaction with retinoid X receptor. Two patients have been described with mutations in the hormone-binding domain of the VDR (263).

One patient with VDDR-2 has been reported in whom no mutation in VDR could be found (264). The authors who reported this patient proposed naming the condition VDDR-3 and demonstrated an overexpression of heterogeneous nuclear ribonucleoprotein-like dominant-negative-acting hormone response element (RE)-binding protein (REBiP), a protein known to modulate steroid hormone-mediated transcription (10).

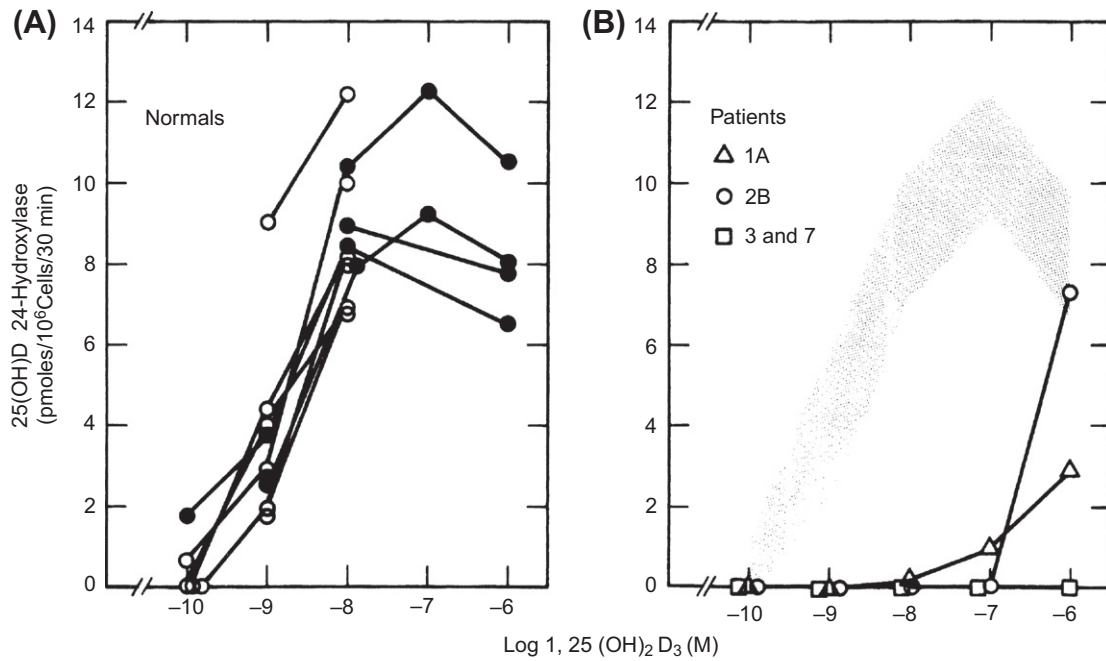
#### 98.6.4 $1,25(\text{OH})_2\text{D}$ Actions in Patients' Cells

Posttranscriptional actions of  $1,25(\text{OH})_2\text{D}$  in cells from these patients have been evaluated. The most extensively tested response to  $1,25(\text{OH})_2\text{D}$  is induction of  $25(\text{OH})\text{D}_3$  24-hydroxylase activity (265,266). In general, patients with milder disease (normal hair, calcemic response to high doses of calciferols) show inducible 24-hydroxylase with supraphysiological concentrations of  $1\alpha, 25(\text{OH})_2\text{D}_3$  (Figure 98-7), but patients with the severest disease (alopecia, no calcemic response to maximal doses of calciferols) show no 24-hydroxylase response to maximal concentrations of  $1\alpha, 25(\text{OH})_2\text{D}$ . Five of six obligate heterozygotes showed no abnormality (267); the sixth showed a 50% decrease in hormone-binding capacity and a similar decrease in maximal induction of 24-hydroxylase. Similar severe defects have been identified with analyses of inhibition of cell growth by  $1\alpha, 25(\text{OH})_2\text{D}$  (in cultured skin fibroblasts and keratinocytes (256) or in peripheral mononuclear cells (258)) and stimulation of 24-hydroxylase and of osteocalcin secretion in osteoblast-like bone cells (94).

A state resembling hereditary resistance to  $1,25(\text{OH})_2\text{D}$  is found in New World primates (marmosets and tamarinds). These animals sometimes develop



**FIGURE 98-6** Amino acid sequence and hypothetical structure of a portion of the receptor for  $1\alpha, 25(\text{OH})_2\text{D}_3$  in normals and in affected members from two kindreds with hereditary generalized resistance to  $1\alpha, 25(\text{OH})_2\text{D}_3$ . Amino acids are represented by a signal-letter code. For example, C depicts cysteine. The deduced amino acids from complementary DNA are shown as two potential zinc-finger arrays. The change in a triplet codon in exon 2 converts a glycine to aspartic acid in one kindred; the mutation in exon 3 converts an arginine to glutamine in another kindred. (Modified from Hughes, M. R.; Malloy, P. J.; Kieback, D. G. et al. Point Mutations in the Human Vitamin D Receptor Gene Associated with Hypocalcemic Rickets. *Science* 1988, 242, 1702–1705.)



**FIGURE 98-7** 25(OH)<sub>2</sub>D<sub>3</sub> 24-hydroxylase in skin fibroblasts preincubated with indicated concentrations of 1,25(OH)<sub>2</sub>D<sub>3</sub>. A, Normal subjects. B, Patients with hereditary generalized resistance to 1,25(OH)<sub>2</sub>D<sub>3</sub>. Patients 1A and 2B each showed a satisfactory calcemic response to high doses of 1,25(OH)<sub>2</sub>D<sub>3</sub>, but patients 3 and 7 showed no calcemic response. (From Gambelin, G. T.; Liberman, U. A.; Eil, C. et al. Vitamin D-Dependent Rickets Type II: Defective Induction of 25-Hydroxyvitamin D<sub>3</sub>-24-Hydroxylase by 1, 25-Dihydroxyvitamin D<sub>3</sub> in Cultured Skin Fibroblasts. *J. Clin. Invest.* 1985, 75, 954–960.)

osteomalacia in captivity and are known to have high nutritional requirements for calciferols (268). New World primates have elevated circulating concentrations of 1,25(OH)<sub>2</sub>D (230,269,270) and deficient hormone-binding affinity (271). These New World primates also exhibit hereditary resistance to several steroid hormones, including glucocorticoids, estrogens and progestins, due to overexpression of two types of proteins that modify hormone-directed transactivation and metabolism, heterogeneous nuclear ribonucleoproteins and heat shock protein-70 heterogeneous (272,273).

### 98.6.5 Treatment

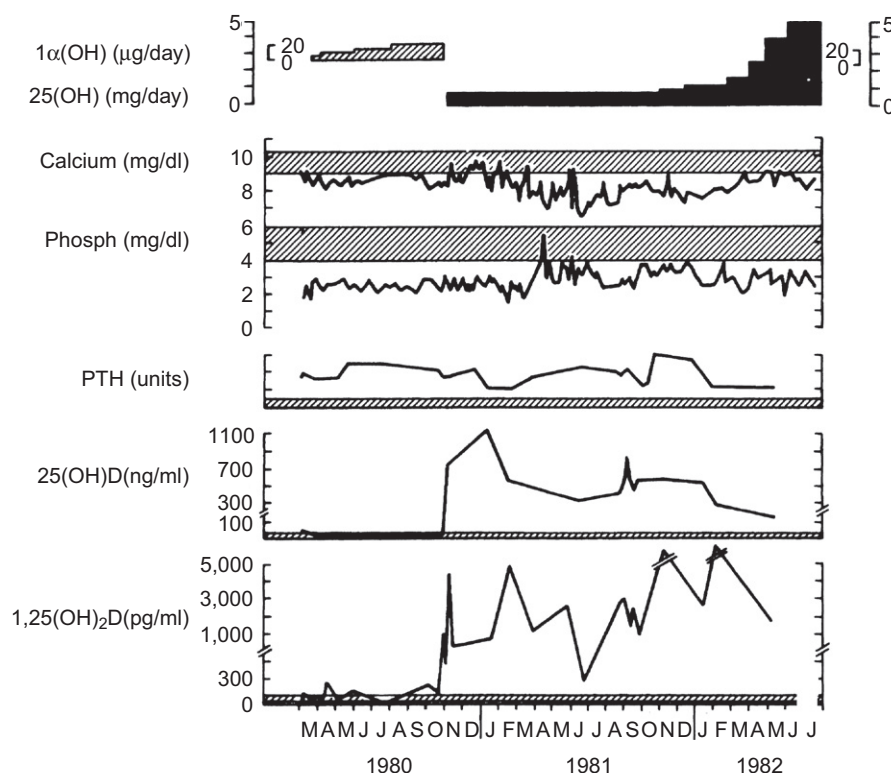
Many cases show complete remission while receiving extraordinarily high doses of calciferols (274) (Table 98-2). Alopecia is one simple predictor of potential for response to therapy (275). Virtually all cases with normal hair can sustain remission when given high doses of analog not requiring 1-hydroxylation. Among cases with alopecia, approximately half are resistant to the highest doses of calciferols achievable; the other half have shown a satisfactory calcemic response, but the dose requirement is typically 10 times higher than in those with normal hair (Figure 98-8). Maintenance treatment is based upon four considerations: (1) some patients can be treated with calciferols (vitamin D<sub>3</sub>, vitamin D<sub>2</sub>) that provide substrate for production of very high serum levels of 1α, 25(OH)<sub>2</sub>D; (2) others may respond only to very high doses of analogs (1α, 25(OH)<sub>2</sub>D<sub>3</sub>, 1α-(OH)D<sub>3</sub>, dihydrotachysterol) that do not require

1α-hydroxylation by the kidney; (3) a minority may not respond to maximal doses of any calciferols; and (4) the role of calcium supplements is different in each of the previous three groups (see discussion below).

Several patients have shown remissions while receiving high doses of vitamin D<sub>2</sub> or 25(OH)<sub>2</sub>D<sub>3</sub> (8,234). In these cases, tissue resistance is only moderate; sufficient 1α, 25(OH)<sub>2</sub>D is produced endogenously from high concentrations of substrate in response to elevated circulating PTH. In this group, calcium supplements may have little or no role as serum concentrations of PTH and 1α, 25(OH)<sub>2</sub>D can compensate for fluctuations in calcium availability.

Patients unable to produce sufficient 1α, 25(OH)<sub>2</sub>D endogenously (because of a requirement for particularly high 1α, 25(OH)<sub>2</sub>D concentrations) may still respond to extraordinarily high doses of analogs that do not require 1α-hydroxylation (i.e. 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1α(OH)D<sub>3</sub>, dihydrotachysterol). Patients in this group requiring therapy that bypasses 1α-hydroxylation should receive fixed calcium supplements (1000 mg per 24 h elemental calcium), for the same reasons as patients with hereditary selective deficiency of 1α, 25(OH)<sub>2</sub>D.

Some patients show no response to maximal doses of any calciferol (Figure 98-8) (185,261,276). Patients with inadequate responses to calciferols can obtain substantial benefit from intravenous infusions of calcium (244,261,277); high-calcium doses (1000mg elemental calcium per 24 h infused over 12 h) can be tolerated even by young children with this disorder. Because normal positive calcium balance during childhood growth is approximately 300mg/24h and deficits prior to



**FIGURE 98-8** Absent calcemic response during a long therapeutic trial with calciferols. Calciferol therapy is in upper panel:  $1\alpha(\text{OH})$  refers to  $1\alpha(\text{OH})\text{D}_3$  or  $1,25(\text{OH})_2\text{D}_3$  and  $25(\text{OH})$  refers to  $25(\text{OH})\text{D}_3$ . Hatched zones indicate normal ranges. Not only is hypocalcemia persistent but also secondary hyperparathyroidism persists (high parathyroid hormone and low phosphate), and very high serum levels of  $1\alpha,25(\text{OH})_2\text{D}$  are documented. (From Marx, S. J.; Liberman, U. A.; Eil, C. et al. *Hereditary Resistance to 1,25-Dihydroxyvitamin D*. *Rec. Prog. Horm. Res.* 1984, 40, 589–615.)

treatment are large, such infusions must be given repeatedly over many months to accomplish significant results. This form of therapy requires methods similar to those used in hyperalimentation programs. Another way to increase calcium input to the bloodstream is to increase net absorption independent of calciferols (239). Unfortunately, the upper limit of tolerance to calcium by mouth is around 6000 mg per day and requires great cooperation; in the absence of calciferol bioeffect, the net calcium retention is approximately 10%. The requirement for intravenous or very high doses of oral calcium confirms the importance of  $1,25(\text{OH})_2\text{D}$ -dependent active transport of calcium in the upper intestine for physiological absorption of calcium.

As in the calciferol-deficiency states, total body calcium requirements are highest at the onset of treatment. Thus, the doses of calcium, the doses of calciferols, and the type of approach judged necessary to initiate therapy (for example intravenous calcium) may not prove to be the same as those required for maintenance therapy.

### 98.7 STATES RESEMBLING HEREDITARY GENERALIZED RESISTANCE TO $1,25(\text{OH})_2\text{D}$

There are multiple forms of hereditary or acquired rickets or osteomalacia in which calciferol metabolism is normal or, if abnormal, only as an appropriate

response to a primary disturbance in mineral flux. Rickets or osteomalacia with high circulating  $1,25(\text{OH})_2\text{D}$  is found in generalized resistance to  $1,25(\text{OH})_2\text{D}$  and in two additional states (calcium deficiency or phosphate deficiency).

#### 98.7.1 Calcium Deficiency

Rickets due to dietary calcium deficiency is unusual under normal dietary conditions, but has been described in infants fed with a low calcium soy-based formula as the main source of nutrition, as well as in children with low dietary calcium intakes both in the developing world (92,278–281) and more recently in the United States (282). The resulting calcipenia leads to secondary hyperparathyroidism, increased urinary phosphate excretion, and subsequent hypophosphatemia. Of course, calcium repletion cures all abnormalities. Osteopetrosis (marble bone disease) is caused by several types of defect (usually hereditary) in osteoclast function. Both in humans and in animal models of this disease, serum  $1,25(\text{OH})_2\text{D}$  is increased and subtle histologic changes of osteomalacia have been noted (283), particularly in patients with “osteopetrorickets” due to mutations in the *TCIRG1* gene that encodes a subunit of the vacuolar proton pump specifically expressed in both osteoclasts and gastric parietal cells (284). Mice deficient in *Tcirg1* have low gastric acid levels (hypochlorhydria) and mild

hypocalcemia, which suggests that gastric acid levels, rather than solely affecting osteoclast activity, help determine proper serum calcium levels, and that osteopetrorickets and osteopetrosis are distinct phenotypes. At least one patient has been treated with a low-calcium diet, plus high doses of  $1,25(\text{OH})_2\text{D}_3$ , with apparent improvement in osteoclast function (285). Other cases of osteopetrosis have not responded to similar treatment, and at least one cellular defect (carbonic anhydrase II deficiency) unlikely to be overcome by  $1,25(\text{OH})_2\text{D}_3$  has been discovered (286).

### 98.7.2 Phosphate Deficiency

Severe deficiency of phosphate can cause rickets with high  $1,25(\text{OH})_2\text{D}$ . In hereditary hypophosphatemic rickets with hypercalciuria, homozygous mutations of *SLC34AC* leads to loss of function of the NaPiIIc sodium-phosphate cotransporter, and the renal loss of phosphate causes osteomalacia and activation of the renal  $1\alpha$ -hydroxylase (287a,175a,288,289). High  $1,25(\text{OH})_2\text{D}$  causes absorptive hypercalciuria; parathyroid function is suppressed, unlike in hereditary generalized resistance to  $1,25(\text{OH})_2\text{D}$ .

Survivors of extreme prematurity can pass through a phase when their growing bones (deprived of the placental pump) are severely deficient in both calcium and phosphate, producing neonatal rickets with high serum  $1,25(\text{OH})_2\text{D}$  (290,291). In this group, immaturity of intestinal responsiveness to  $1,25(\text{OH})_2\text{D}$  may contribute to the disturbance (85).

### 98.7.3 Deficient Bone Mineralization with Normal Calcium and Phosphate in Serum

There are several causes of deficient bone mineralization with otherwise normal calcium and phosphate fluxes. These include fibrogenesis imperfecta ossium, hypophosphatasia (292), the chondrodysplasias (which can disturb epiphyseal function), and skeletal accumulation of aluminum, bisphosphonates, or fluoride (172). Because PTH is not high or phosphate is not low in serum,  $1\alpha,25(\text{OH})_2\text{D}$  is normal in serum.

## 98.8 OTHER HEREDITARY DEFECTS IN CALCIFEROL METABOLISM OR ACTION

### 98.8.1 Calciferol-Deficiency States

Although antigenic variation in vitamin D-binding protein (Gc) is common, no differences in its function for calciferol transport have been identified in humans (293). However, in the chicken, this protein shows 10-fold higher affinity for vitamin D3 metabolites than for vitamin D2 metabolites, and this seems to account

for a higher requirement for vitamin D2 than D3 in this species (287).

Congenital rickets due to genetic defects in 25-hydroxylase deficiency has been reported as an unusual cause of vitamin D deficiency (20).

Deficient 24-hydroxylation of  $25(\text{OH})\text{D}$  was suggested in another patient with hereditary generalized resistance to  $1\alpha,25(\text{OH})_2\text{D}$  (238). That patient showed low concentrations of  $24,25(\text{OH})_2\text{D}$  and showed partial remission of resistance when given  $24,25(\text{OH})_2\text{D}_3$ . These observations remain unexplained, but a persistent abnormality in DNA binding of the  $1\alpha,25(\text{OH})_2\text{D}$  receptors has been documented in cells cultured from this patient (294), suggesting that the receptor defect is her primary problem. Decreased production of  $24,25(\text{OH})_2\text{D}$  could be secondary to the abnormality in  $1,25(\text{OH})_2\text{D}$  action, as one of its actions is induction of  $25(\text{OH})\text{D}_3$  24-hydroxylase.

### 98.8.2 Calciferol Excess States: Idiopathic Infantile Hypercalcemia

Idiopathic infantile hypercalcemia was recognized as a syndrome in the 1950s (295). Most patients present within the first year of life with failure to thrive, dehydration and vomiting, in some accompanied by nephrocalcinosis. PTH levels are suppressed. Since its description, there has been speculation that the cause was an increased sensitivity to vitamin D present in infant formula (296). Recently, through a candidate screening approach, several different inactivating mutations in *CYP24A1* were reported in six patients (from four families in Germany) with autosomal recessively inherited idiopathic infantile hypercalcemia (297). In these patients, four presented ages between 6 and 11 months with failure to thrive and nephrocalcinosis and two with more mild hypercalcemia were asymptomatic, diagnosed by family screening. Interestingly, serum  $1,25(\text{OH})_2\text{D}$  was only frankly elevated in one of these patients. In addition, in this report (297), four infants (5 weeks to 7 months old) with suspected vitamin D intoxication were also described, with similar mutations to those found in the familial cases. Mutations in *CYP24A1* can rarely be seen as a cause of hypercalcemia in adults as well. One of the authors (EAS) and her group found a *CYP24A1* mutation in a 45-year-old man with hypercalcemia (homozygous E143del) who presented at age 19 with hypercalcemia and nephrolithiasis (298). Unlike the infantile cases, in the adult with hypercalcemia and *CYP24A1* mutation, serum  $25(\text{OH})\text{D}$  and  $1,25(\text{OH})_2\text{D}$  were consistently and frankly elevated.

### 98.8.3 Calciferol Sensitivity States: Williams Syndrome

Three groups (299–301) described patients with a constellation of unusual features, including infantile hypercalcemia, prenatal and postnatal growth deficiency, elfin



fancies, mental retardation and supravalvular aortic stenosis, which now is called Williams syndrome (see Chapter 46). It is also called Williams–Beuren syndrome.

### 98.8.4 Clinical Features

The characteristic dysmorphology includes the following: wide slack mouth, malocclusion, prominent upper lip, underdeveloped mandible, depressed nasal bridge, hypertelorism, epicanthic folds, low-set ears, increased bone density, craniostenosis, and osteosclerosis, especially at the base of the skull. Neurologic manifestations include hypotonia, hyperreflexia, and motor retardation. Children are unusually friendly and have been described as having “cocktail party” personalities. Renal involvement is common in Williams syndrome, including nephrocalcinosis and anatomic defects. A variety of clinical manifestations can occur including renovascular hypertension, proteinuria, and an elevated plasma creatinine concentration.

Cardiovascular abnormalities involve local or diffuse stenosis of the medium-sized or large-sized arteries, most commonly in the ascending aorta above the aortic valves (i.e. supravalvular aortic stenosis) or in the pulmonary arteries. Nonetheless, stenosis of the descending aorta, intracranial arteries, and renal arteries has been reported. Overall, unexpected death is rare but is 25-fold to 100-fold higher than in age-matched control subjects. Factors implicated in sudden death have included supravalvular aortic stenosis, severe pulmonary stenosis, and myocardial ischemia secondary to either coronary insufficiency or biventricular outflow tract obstruction with ventricular hypertrophy. Coronary insufficiency appears most likely because of stenosis that results from intimal fibrosis and muscular hypertrophy. Stroke and hypertension occur at younger-than-expected ages. Cases showing portions of the phenotype have sometimes been collected within this syndrome without implying identical pathophysiology for all cases (302). Early studies showed elevated serum and urine levels of calcium during the first year of life and a tendency to develop nephrocalcinosis. Hypercalcemia rarely persists after the first year of life, but persistent hypercalciuria is not uncommon. Although many reports of children with Williams syndrome have failed to document a high frequency of hypercalcemia, in a report of 50 cases from Greece, three had hypercalcemia (303). Serum 25(OH)D and 24,25(OH)<sub>2</sub>D are normal (304), but circulating levels of 25(OH)D show excessive increases after administration of vitamin D (305). Serum 1 $\alpha$ ,25(OH)<sub>2</sub>D levels have been reported to be elevated (306), or suppressed (304,307,308), suggesting more than one mechanism for the hypercalcemia (309).

### 98.8.5 Genetics

Most cases appear to be sporadic, but some have had affected siblings (310) and autosomal-dominant-like pedigree can occur (311). Insights into the molecular basis

for Williams syndrome came from the study of patients with isolated supravalvular aortic stenosis, a distinct autosomal-dominant disorder caused by mutations in the elastin (*ELN*) gene (312). Analysis of patients in whom supravalvular aortic stenosis occurred as part of the Williams syndrome revealed hemizygous chromosomal deletions at chromosome band 7q11.23 (313), which includes the elastin gene (*ELN*). Most deletions are not detected through standard karyotyping but rather through CGH microarray or fluorescent in situ hybridization (FISH) for a 1.5-Mb deletion. The size of the deletion can vary. Williams syndrome is not solely caused by elastin haploinsufficiency; the deletion involves a region that spans more than 25 genes and, hence, is considered a contiguous gene deletion syndrome. The cardiovascular findings, part of the connective tissue pathology, and facial dysmorphology are attributed to the elastin gene haploinsufficiency, while the other features of Williams syndrome are likely due to loss of adjacent genes in the region of chromosome 7q11.2 (314,315). Elastin arteriopathy is generalized; thus, virtually any artery may be affected.

Williams syndrome has also been associated with other chromosomal abnormalities including an interstitial deletion of chromosome 6(q22.2q23) (316), a terminal deletion of chromosome 4[46,XX,del(4)(q33)] (317), as well as chromosomal translocations (318,319). However, the definitive basis remains unknown (315). In most cases, fluorescent in situ hybridization (FISH) using a probe for *ELN* is diagnostic (320,321).

The offspring of rabbits with vitamin D intoxication show similar skeletal features (mandibular hypoplasia and characteristic dental abnormalities) (322). It is uncertain, at this point, whether the pathogenesis of the Williams syndrome involves a defect in calciferol metabolism or not.

### 98.8.6 Management

The most important aspect of the treatment of hypercalcemia is a low-calcium diet with elimination of vitamin D. In newborns and infants, the daily calcium intake should be kept below 100 mg and preferably as low as 25–35 mg; Calcilo XD is a suitable low-calcium infant formula that lacks vitamin D. It is necessary not only to eliminate vitamin D intake but also to protect the infant from exposure to direct sunlight. It has been recommended that the low-calcium, low-vitamin-D diet be maintained for at least 9 months after the serum calcium has become normal. Corticosteroids may be a useful adjunct in the acute stage to induce rapid lowering of the plasma calcium level and can usually be discontinued after a few days when the diet has produced its effects. In general, hypercalcemia resolves without specific treatment by the end of the first year of life. Intravenous bisphosphonates can also be used to treat the hypercalcemia (323). Otherwise, there is no specific treatment available other than specific treatment for skeletal deformities.

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**Dr Streeten** is an endocrinologist, clinical geneticist and Associate Professor of Medicine and Pediatrics at the University of Maryland School of Medicine. She has active practices of both metabolic bone disease and clinical genetics and teaches medical students, residents and fellows. Much of Dr Streeten's research has focused on the study of bone health in the Old Order Amish, including participating in meta-analyses of genomewide association studies of bone density and bone geometry. She has also been studying osteoporosis–pseudoglioma syndrome since 1998, focusing on evaluating bone quality (with peripheral CT) and developing new treatments to reduce fracture risk. Other research interests include hypercalcemia related to *CYP24A1* mutation and the effects of vitamin D repletion on cardiovascular risk factors.



# CHAPTER

# 99

## Inherited Porphyrias

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### 99.1 INTRODUCTION

The inherited porphyrias are a diverse group of inborn errors of metabolism, with each resulting from the deficient activity of a specific enzyme in the heme biosynthetic pathway (Tables 99-1 and 99-2). The resultant accumulation of porphyrin precursors and/or porphyrins causes the major clinical manifestations, neurologic symptoms, and/or cutaneous photosensitivity. These seven disorders are classified metabolically as hepatic or erythropoietic, depending on the primary source of their accumulated heme biosynthetic intermediates. They also are classified clinically as acute or cutaneous. Of the five hepatic porphyrias, four are characterized by life-threatening acute attacks of neurologic manifestations that occur in association with excess amounts of the porphyrin precursors, 5-aminolevulinic acid (ALA), and porphobilinogen (PBG), and they are classified as acute porphyrias. Three porphyrias have primarily cutaneous manifestations, including the two erythropoietic porphyrias and porphyria cutanea tarda (PCT). Two other hepatic porphyrias, hereditary coproporphyria (HCP) and variegate porphyria (VP), may cause acute neurologic attacks and cutaneous manifestations. The skin damage results from photoactivation of the accumulated porphyrins by long-wave ultraviolet (UV) light.

From a genetic point of view, the porphyrias are unique as five of the seven disorders are autosomal dominant enzymopathies. Of note, only a minority of heterozygotes becomes symptomatic. The onset and severity of the hepatic porphyrias are greatly influenced by environmental and metabolic factors, such as hormones, drugs, and nutrition. In addition, modifying genes presumably play an important role in the clinical expression of these disorders.

Here, we describe the clinical, metabolic, and genetic features of the seven porphyrias; mutations in the first enzyme in the heme biosynthetic pathway cause X-linked sideroblastic anemia (1). Optimal methods for their diagnosis and treatment are presented, and the current understanding of the genetic basis and disease pathogenesis in these acute and cutaneous disorders are discussed. Recent reviews on the inherited porphyrias are available (1–5). For lists of mutations causing each porphyria, please see the Human Gene Mutation Database ([www.hgmd.org](http://www.hgmd.org)) (6). Informative and up-to-date Web sites are sponsored by the American Porphyria Foundation ([www.porphyrifoundation.com](http://www.porphyrifoundation.com)) and the European Porphyria Initiative ([www.porphyrria-europe.org](http://www.porphyrria-europe.org)).

### 99.2 THE HEME BIOSYNTHETIC PATHWAY

#### 99.2.1 The Heme Biosynthetic Enzymes

The heme biosynthetic pathway is shown in Figure 99-1 and Table 99-1. Nine nuclear heme biosynthetic genes, including separate genes for the housekeeping and erythroid-specific isozymes of ALA-synthase, encode the enzymes that catalyze the eight steps in the conversion of glycine and succinyl-CoA to heme (1). The first and the last three enzymes are in the mitochondrion, and the other four function in the cytosol. The characteristics of the genes, their respective enzymes, and their chromosomal locations are summarized in Table 99-1. The heme biosynthetic pathway is responsible for the production of heme for hemoproteins, including the superfamily of cytochrome P450 enzymes, which are most abundant in the liver, and hemoglobin in erythrocytes.

**TABLE 99-1 Human Heme Biosynthesis Enzymes and Genes<sup>a</sup>**

Enzyme	Gene Symbol	Chromosomal Location	cDNA (bp)	Protein (aa)	Gene	
					Size (kb)	Exons <sup>b</sup>
5-Aminolevulinate synthase: (ALA-synthase)						
Housekeeping	ALAS1	3p21.1	2,199	640	17	11
Erythroid-specific	ALAS2	Xp11.2	1,937	587	22	11
5-Aminolevulinate dehydratase: (ALA-dehydratase)						
Housekeeping	ALAD	9q32	1,149	330	15.9	12 (1A + 2–12)
Erythroid-specific	ALAD	9q32	1,154	330	15.9	12 (1B + 2–12)
Hydroxymethylbilane synthase: (HMB-synthase)						
Housekeeping	HMBS	11q23.3	1,086	361	11	15 (1 + 3–15)
Erythroid-specific	HMBS	11q23.3	1,035	344	11	15 (2–15)
Uroporphyrinogen III synthase: (URO-synthase)						
Housekeeping	UROS	10q26.2	1,296	265	34	10 (1 + 2B-10)
Erythroid-specific	UROS	10q26.2	1,216	265	34	(2A + 2B-10)
Uroporphyrinogen decarboxylase	UROD	1p34.1	1,104	367	3	10(URO-decarboxylase)
Coproporphyrinogen oxidase	CPO	3q12.1	1,062	354	14	7(COPRO-oxidase)
Protoporphyrinogen oxidase	PPOX	1q23.3	1,431	477	5.5	13(PROTO-oxidase)
Ferrochelatase	FECH	18q21.31	1,269	423	45	11

<sup>a</sup>References in Anderson KE, Sassa S, Bishop DF, et al. (2001) Disorders of heme biosynthesis: X-linked sideroblastic anemias and the porphyrias. In Scriver CR, Beaudet AL, Sly WS, Valle D (eds): The Metabolic and Molecular Basis of Inherited Disease, 8th ed. McGraw-Hill, New York, p 2991.

<sup>b</sup>Number of exons and (in parentheses) those encoding separate housekeeping and erythroid-specific forms.

Different regulatory controls have evolved for hepatic and erythroid-specific heme synthesis, including negative feedback repression by heme in the liver, and separate erythroid-specific genes or promoters in the first four genes in the pathway (Figure 99-2). Each of the enzymatic steps in the pathway is briefly described in this chapter.

**99.2.1.1 5-Aminolevulinate Synthase.** The first enzyme in the pathway, 5-aminolevulinate synthase (ALA-synthase; also known as D-aminolevulinate synthase; E.C. 2.3.1.37), catalyzes the condensation of glycine (activated by pyridoxal phosphate) and succinyl coenzyme A to form ALA. Distinct human housekeeping and erythroid-specific ALA-synthase isozymes are encoded by separate genes: the ~17-kb housekeeping gene (ALAS1), located at chromosome 3p21.1, is expressed in all tissues, while the ~22-kb erythroid-specific gene (ALAS2), located at chromosome Xp11.21, is expressed only in erythroid cells to supply the large amounts of heme required for hemoglobin (see Figure 99-2). These findings provide a basis for the tissue-specific regulation of this pathway (for a review see Reference (1)). Of note, expression of the housekeeping gene ALAS1 in the liver is under negative feedback repression by the cellular heme concentration and functions to modulate the supply of heme for the hepatic cytochrome P450 enzymes and other hepatic hemoproteins (7). In acute porphyrias, the depletion of hepatic heme by various drugs, hormones, and glucose restriction, the increased synthesis of the housekeeping ALAS1 isozyme, and the generation of

the large amount of the porphyrin precursors, ALA and PBG, are the biochemical hallmarks of acute neurologic attacks (2).

Mutations in the X-linked ALAS2 gene and the resultant deficient activity of the erythroid-specific isozyme cause X-linked sideroblastic anemia (1,8). Over 35 mutations in the erythroid-specific ALA-synthase gene causing X-linked sideroblastic anemia are listed in the Human Gene Mutation Database ([www.hgmd.org](http://www.hgmd.org)) (6). Except for a mutation in the promoter region of the ALAS2 gene (9) and one nonsense mutation, all of the reported lesions have been missense mutations in the ALAS2 catalytic core encoded by exons 5 to 11, with the majority occurring in exons 5 and 9. Most mutations were pyridoxine responsive in vivo and when expressed in *Escherichia coli*. Molecular modeling of the ALAS2 isozyme, based on the crystal structure of a bacterial ALA-synthase, suggested the molecular basis for the pyridoxine responsiveness of certain mutations (10). Recently, gain of function mutations in exon 11 of ALAS2 that increase its activity have been shown to cause an X-linked form of erythropoietic protoporphyria (EPP), known as X-linked protoporphyria (XLP). To date, only two gain of function mutations in ALAS2 have been described. No deficiencies of the ALAS1 isozyme have been described; presumably, the enzymatic deficiency would be lethal.

**99.2.1.2 5-Aminolevulinic Acid Dehydratase.** The second enzyme in the pathway is 5-aminolevulinic acid dehydratase (ALA-dehydratase; also known as PBG synthase; E.C. 4.2.1.24). This enzyme catalyzes the

**TABLE 99-2 Classification of the Human Porphyrrias including Major Clinical and Biochemical Features**

						Biochemical Findings <sup>a</sup>
	Symptomatology Porphyria	Enzyme	H or E	Principal Inheritance	Deficient NV or CP	Classification Erythrocyte
5-ALA dehydratase-porphyria (ADP)	5-ALA-dehydratase	H <sup>b</sup>	AR	NV	Zn-Protoporphyrin	ALA, Coproporphyrin III
Acute intermittent (AIP)	HMB-synthase	H	AD	NV	—	ALA, PBG, Uroporphyrin
Congenital erythropoietic porphyria (CEP)	URO-synthase	E	AR	CP	Uroporphyrin I Coproporphyrin I	Uroporphyrin I Coproporphyrin I
Porphyria cutanea tarda (PCT)	URO-decarboxylase	H	AD <sup>c</sup>	CP	—	Uroporphyrin, 7-carboxylate porphyrin
Hepatoerythropoietic porphyria (HEP)	URO- decarboxylase	H	AR	CP	Zn-Protoporphyrin	Uroporphyrin, 7-carboxylate porphyrin
Hereditary coproporphyria (HCP)	COPRO-oxidase	H	AD	NV & CP (uncommon)	—	ALA, PBG, Coproporphyrin III
Variegate porphyria (VP)	PROTO-oxidase	H	AD	NV & CP	—	ALA, PBG, Coproporphyrin III
Erythropoietic protoporphyria (EPP)	Ferrochelatase	E	AD <sup>d</sup>	CP	Protoporphyrin	—

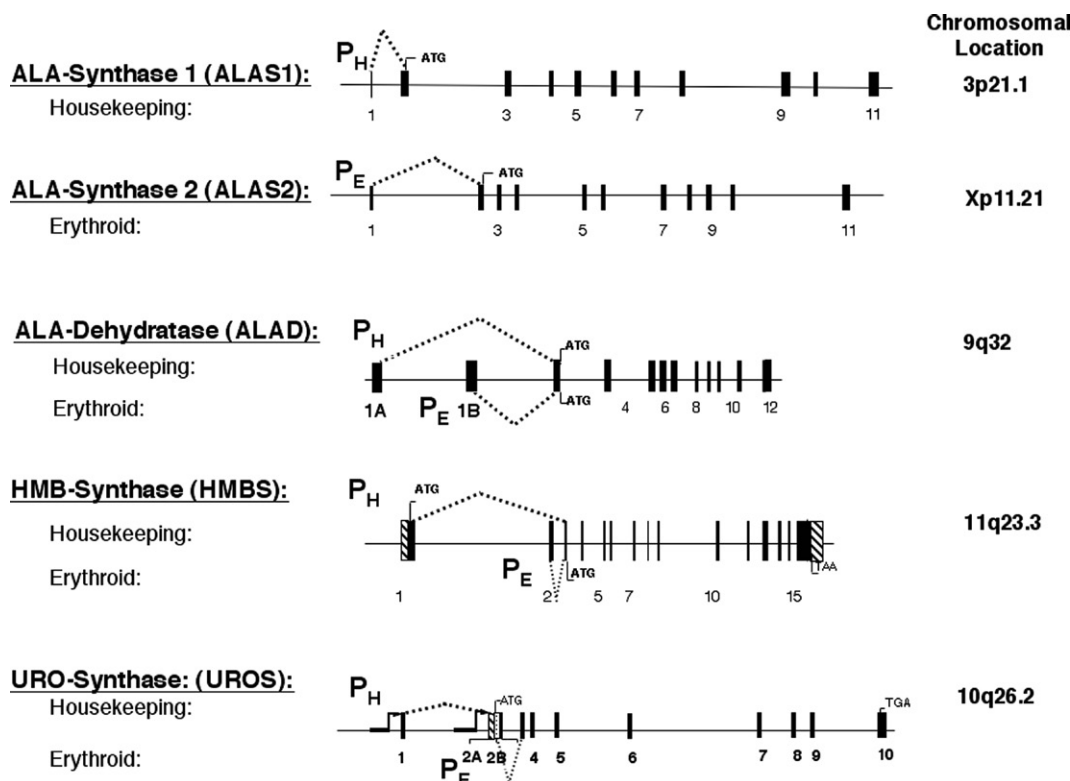
H, hepatic; E, erythropoietic; AR, autosomal recessive; AD, autosomal dominant; Type I isomers: ALA = 5-aminolevulinic acid; PBG = porphyrinogen; NV, neurovisceral; CP, cutaneous.

<sup>a</sup>Increases that may be important for diagnosis.

<sup>b</sup>These porphyrias also have erythropoietic features including increased erythrocyte porphyrins.

<sup>c</sup>Inherited deficiency of UROD is partially responsible for familial (Type II) PCT.

<sup>d</sup>Polymorphism in intron 3 of wild-type allele affects level of enzyme activity and clinical expression.



**FIGURE 99-1** The human heme biosynthetic pathway. Ac, acetyl; Pr, propionyl.

condensation of two molecules of ALA to form the cyclic pyrrole, PBG (see Figure 99-1). Human ALA-dehydratase is composed of eight identical 31-kDa subunits and eight atoms of zinc, which are required for both enzyme stability and catalytic activity. The zinc atoms are bound to each subunit by a typical zinc finger domain consisting of four cysteine and two histidine residues (1). The zinc atoms protect essential sulfhydryl groups in the enzyme and can be displaced by lead or other heavy metals. In fact, the measurement of erythrocyte ALA-dehydratase activity is a highly sensitive index of lead exposure.

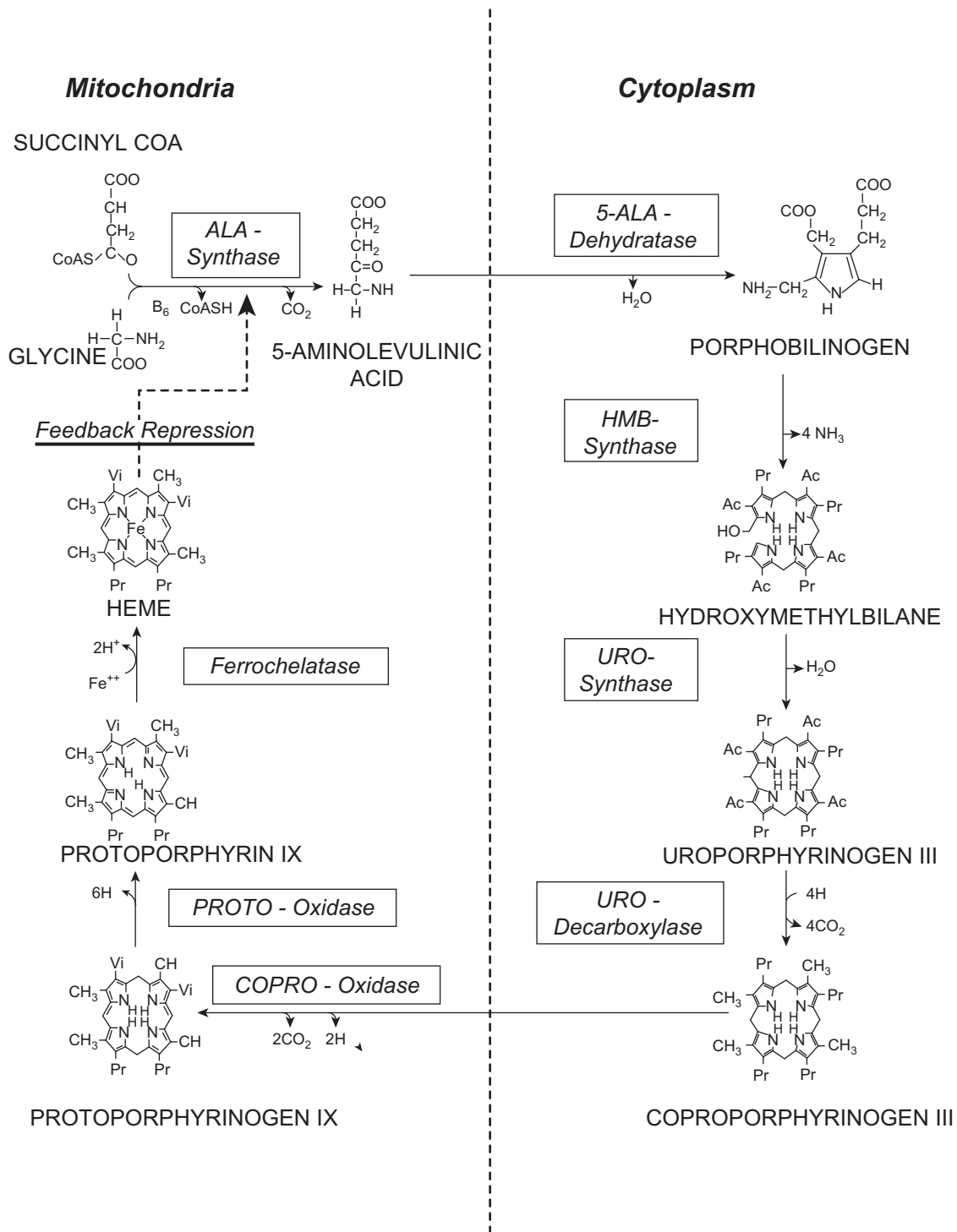
The 16-kb human ALA-dehydratase gene encodes housekeeping and erythroid-specific transcripts by alternative splicing (11); see Figure 99-2. Both transcripts encode the same amino acid sequence, as translation begins in exon 2. The housekeeping promoter region is upstream of exon 1A, while the erythroid-specific promoter is upstream of exon 1B. Mutations in the ALA-dehydratase gene result in the deficiency of ALA-dehydratase, causing a rare, recessively inherited acute hepatic porphyria, ALA-dehydratase-deficient porphyria (ADP) (6,12,13). Only six cases of ADP with 13 different ALA-dehydratase mutations have been reported ([www.hgmd.org](http://www.hgmd.org)) (6,14).

Of note, there are two common alleles at the ALA-dehydratase locus, ALAD1 and ALAD2, which are responsible for three electrophoretically distinguishable enzyme forms designated 1-1, 1-2, and 2-2. The frequencies of the corresponding phenotypes in white populations are about 80%, 18%, and 1%, respectively,

giving gene frequencies of 0.9 and 0.1 for the ALAD1 and ALAD2 alleles, respectively. Gene frequency of the ALAD2 allele is lower in Hispanics, Asians, and African-Americans (15), and in a Liberian population, the ALAD2 allele was not detected. The ALAD2 allele has normal ALA-dehydratase activity but may bind zinc more effectively. Several epidemiologic studies have demonstrated an association between the ALAD2 allele and high lead levels (16–20). Although blood and serum lead levels were 5%–10% greater in individuals with the ALAD2 allele than in individuals with the other alleles, bone lead was not increased (17).

**99.2.1.3 Hydroxymethylbilane Synthase.** Hydroxymethylbilane synthase (HMB-synthase; formerly known as PBG-deaminase or uroporphyrinogen I synthase; E.C. 4.3.1.8), the third enzyme in the pathway, catalyzes the head-to-tail condensation of four molecules of PBG by a series of deaminations to form the linear tetrapyrrole, hydroxymethylbilane (HMB) (see Figure 99-1). HMB can cyclize nonenzymatically to form uroporphyrinogen I, a nonphysiological and phototoxic compound. Because HMB-synthase activity is almost as low as ALA-synthase activity in the liver, it may become rate-limiting when the enzyme is partially deficient. The ~10-kb human HMB-synthase gene encodes erythroid-specific and housekeeping isozymes by alternative splicing (see Figure 99-2). The housekeeping and erythroid isozymes are monomeric proteins of 361 and 344 amino acid residues, respectively. The housekeeping promoter functions in all cell types, whereas the





a-helices and a discrete hydrophobic core. Because the human and *E. coli* HMB-synthase amino acid sequences have about 35% homology and greater than 70% similarity, it was possible to infer the structure–function relationships for certain human HMB-synthase mutations from the bacterial enzyme (24,25).

**99.2.1.4 Uroporphyrinogen III Synthase.** Uroporphyrinogen III synthase (URO-synthase; E.C. 4.2.1.75) catalyzes the rearrangement of HMB by inversion of the pyrrole D ring and ring closure to form the asymmetric uroporphyrinogen type III isomer (see Figure 99-1). In the absence of URO-synthase, HMB nonenzymatically cyclizes to form the uroporphyrinogen I isomer. This nonphysiologic compound can be metabolized to coproporphyrinogen I, but further metabolism cannot proceed as the next enzyme, coproporphyrinogen oxidase, is stereospecific for the III isomer.

The ~34-kb human URO-synthase gene has alternative promoters that generate housekeeping and erythroid-specific transcripts, which encode the same 265 amino acid polypeptide (see Figure 99-2) (26). The enzyme is active as a 29.5-kDa monomer. The human enzyme has been crystallized at a resolution of 1.85 Å (27). The protein folds into two alpha/beta domains connected by a beta-ladder, with the active site between the domains.

Mutations in the URO-synthase gene results in deficient but not absent, URO-synthase enzyme activity, causing congenital erythropoietic porphyria (CEP), an autosomal recessive erythropoietic porphyria (6,28). Over 35 URO-synthase mutations are listed in the Human Gene Mutation Database ([www.hgmd.org](http://www.hgmd.org)) (6).

**99.2.1.5 Uroporphyrinogen Decarboxylase.** Uroporphyrinogen-decarboxylase (E.C. 4.1.1.37), the fifth enzyme in the pathway, catalyzes the sequential removal of the four carboxyl groups from the acetic-acid side chains of uroporphyrinogen III (clockwise, starting with ring D) to form the four methyl groups of coproporphyrinogen III, a tetracarboxyl porphyrinogen (see Figure 99-1). The enzyme has no coenzyme or metal requirements, and iron does not appear to directly affect URO-decarboxylase activity in vitro.

The ~3-kb human URO-decarboxylase gene has a single-mRNA species, which expresses a 367-residue polypeptide in all tissues, where it is active as a homodimer (29,30). Recombinant human URO-decarboxylase has been crystallized at 1.60-Å resolution, and its reaction mechanism has been studied (30–33).

This enzyme is deficient in the liver in PCT, the most common porphyria. The majority (~80%) of PCT patients have no URO-decarboxylase mutations and are termed type I if the disease is sporadic or type III, if (rarely) more than one family member is affected. Heterozygous mutations and half-normal enzyme activities are found in all tissues (e.g. erythrocytes) in familial (type II) PCT (~20% of all PCT patients). In overt PCT of all types, hepatic URO-decarboxylase activity is always

reduced by additional factors to well below 50% of normal, which is consistent with an acquired tissue-specific inhibition of hepatic URO-decarboxylase. Hepatoerythropoietic porphyria (HEP) is the homozygous form of familial (type II) PCT and, generally, has a more severe phenotype (1,34). Over 100 URO-decarboxylase mutations identified in PCT and HEP are listed in the Human Gene Mutation Database ([www.hgmd.org](http://www.hgmd.org)) (6).

**99.2.1.6 Coproporphyrinogen Oxidase.** The sixth enzyme in the pathway, coproporphyrinogen oxidase (COPRO-oxidase; E.C. 1.3.3.3), catalyzes the decarboxylation of two of the four propionic acid groups of coproporphyrinogen III (on rings A and B) to form the two vinyl groups of protoporphyrinogen IX, a dicarboxyl porphyrinogen (see Figure 99-1). COPRO-oxidase is located between the mitochondrial inner and outer membranes, requires molecular oxygen for its activity, and contains no metals (1,35). An intermediate in the two-step decarboxylation is a 3-carboxyl porphyrinogen (termed harderoporphyrinogen, because this porphyrin in its oxidized form (harderoporphyrin) was first isolated from the rodent harderian gland). Coproporphyrinogen I, which is formed by decarboxylation of uroporphyrinogen I, is not a substrate for this enzyme and therefore is not metabolized to heme.

The ~14-kb human COPRO-oxidase gene encodes a single transcript, which expresses a 474-residue polypeptide including an N-terminal mitochondrial, targeting signal peptide of 120 residues (36–38). Human COPRO-oxidase has been crystallized to a resolution of 1.58 Å (39). Studies of the crystal structure confirmed that COPRO-oxidase functions as a dimer and identified the residues in the enzyme's active site (39).

Mutations in the COPRO-oxidase gene result in deficient enzymatic activity, causing HCP, an autosomal dominant disorder (40). Over 40 COPRO-oxidase mutations are listed in the Human Gene Mutation Database ([www.hgmd.org](http://www.hgmd.org)) (6). Mutation K404E in the COPRO-oxidase gene, when present in either the homozygous or compound heterozygous states, causes a biochemical variant, termed harderoporphyrin (39,41). Cases of homozygous dominant HCP have also been described (6,42,43).

**99.2.1.7 Protoporphyrinogen Oxidase.** The seventh enzyme in the pathway, protoporphyrinogen oxidase (PROTO-oxidase; E.C. 1.3.3.4), catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX by the removal of six hydrogen atoms (see Figure 99-1). The product of the reaction is a porphyrin (oxidized form), in contrast to the preceding several products, which are porphyrinogens (reduced forms). This oxidation occurs readily in vitro under aerobic conditions in the absence of the enzyme. PROTO-oxidase is an integral protein of the mitochondrial inner membrane spacing and appears to be active as a dimer. PROTO-oxidase is inhibited by bilirubin, perhaps accounting for the decreased levels of the enzyme activity in Gilbert disease.

The ~5.5-kb human PROTO-oxidase gene encodes a single ~1.8-kb mRNA in all tissues, which expresses a mitochondrial-targeted polypeptide of 477 amino acids (~51 kDa) (44–46). PROTO-oxidase lacks a typical mitochondrial targeting leader sequence but is effectively targeted by its 17 N-terminal residues (36).

Mutations in the PROTO-oxidase gene result in 50% of normal enzymatic activity, causing VP, a dominantly inherited hepatic porphyria (47). Over 150 PROTO-oxidase mutations are listed in the Human Gene Mutation Database ([www.hgmd.org](http://www.hgmd.org)) (6). Several cases of homozygous VP have also been described (48,49).

**99.2.1.8 Ferrochelatase.** The final step in heme biosynthesis is the insertion of ferrous iron into protoporphyrin IX to form heme. This reaction is catalyzed by ferrochelatase (heme synthetase or protoheme ferrolyase; E.C. 4.99.1.1), which is associated with the inner side of the inner mitochondrial membrane. The enzyme is specific for the reduced form of iron ( $\text{Fe}^{2+}$ ) but can use other metals (e.g.  $\text{Zn}^{2+}$  and  $\text{Co}^{2+}$ ) and other 2-carboxyl porphyrins. The enzyme appears to function as a dimer in mitochondria (50), and there is suggestive evidence that the membrane domains of PROTO-oxidase dock onto the dimeric structure of ferrochelatase (51).

The ~45-kb human ferrochelatase gene encodes a 423-amino acid polypeptide including a 54-residue leader sequence (36). An iron–sulfur cluster [ $2\text{Fe}-2\text{S}$ ] has been identified in recombinant human and mouse ferrochelatase (52,53) and is thought to be essential for enzyme activity (54). The putative iron–sulfur binding site is at the C-terminus in a 30-amino acid region that contains four cysteines. Recombinant human ferrochelatase has been crystallized and diffracted to about 2 Å (55,56).

Coding region mutations in the ferrochelatase gene result in a decreased enzymatic activity, causing erythropoietic protoporphyria (EPP) (57). Over 100 ferrochelatase mutations are listed in the Human Gene Mutation Database ([www.hgmd.org](http://www.hgmd.org)) (6). Clinical expression of this porphyria occurs when a disabling ferrochelatase mutation is heteroallelic with a polymorphism in intron 3 of the wild-type ferrochelatase gene that reduces expression of normal enzyme (58) or, less commonly, in individuals who inherit two mutations that impair enzyme function (e.g. coding region or splice-site mutations).

### 99.3 REGULATION OF HEME BIOSYNTHESIS

In humans, about 85% of heme is synthesized in erythroid cells to provide heme for hemoglobin, while most of the remaining heme is produced in the liver, where it is used primarily as the prosthetic group in cytochrome P450 enzymes and other hemoproteins. In the liver, the heme biosynthetic pathway is under a negative feedback control at the level of the first enzyme in the pathway, ALAS1 (the housekeeping form), by the concentration

of “free” heme (7). Heme represses the transcription and translation of liver ALAS1 mRNA, reduces ALAS1 mRNA stability (7), and interferes with the transport of the enzyme into mitochondria. High concentrations of free heme can also induce heme oxygenase and therefore stimulate heme catabolism (59). ALAS1 is inducible by many of the same chemicals that induce the hepatic cytochrome P450 enzymes. Because most of the heme synthesized in the liver is used for the synthesis of the cytochrome P450 enzymes, the induction of hepatic ALA-synthase and the cytochrome P450s occurs in a coordinated fashion. Recently, two sequence elements in the distal 5′-flanking region of the human ALAS1 gene were identified, which mediate direct transcriptional activation in response to drugs metabolized by the cytochrome P450s (60). When the regulatory “free” heme pool becomes depleted (which may occur, for example, when more heme is required for the synthesis of hemoproteins), the synthesis of ALAS1 is increased. Conversely, repression of ALAS1 synthesis results from augmentation of the regulatory heme pool. The evidence that ALA-synthase1 functions as a rate-controlling enzyme, at least in the liver, includes its relatively low  $V_{\text{max}}$  value (compared with most other enzymes in the pathway), its inducibility and short half-life, and its great sensitivity to repression by cellular heme (at concentrations below  $10^{-6}\text{M}$ ). In addition, ALAS1 mRNA is markedly increased under conditions when more heme is required by cells while expression of the other enzymes in the pathway do not change significantly (7). The low affinity of the enzyme for glycine suggests that the intracellular glycine concentration also determines the rate of ALA formation.

In erythroid cells, there are novel regulatory mechanisms for the production of the very large amounts of heme needed for hemoglobin synthesis. As noted previously, there is a separate erythroid-specific ALA-synthase gene (ALAS2) (1) and unique erythroid-specific promoters in ALA-dehydratase (11), HMB-synthase (23), and URO-synthase (61), the first four enzymes in the heme biosynthetic pathway (see Figure 99-2). The erythroid-specific gene ALAS2 on the X chromosome is expressed at high levels during erythroid differentiation. Synthesis of ALAS2, unlike ALAS1, is not repressed by hemin treatment and therefore is not regulated by the heme feedback repression. Transcriptional control of ALAS2 is exerted by erythroid-specific promoter elements in the 5′-flanking region of the gene. Translational control results from an iron-responsive element in the 5′-untranslated region of the mRNA. Transcription of the housekeeping ALAS1 gene may be downregulated during erythroid differentiation. In addition, heme regulates the rate of its synthesis in erythroid cells by controlling the transport of iron (required for ferrochelatase) into reticulocytes. Thus, the rate of iron acquisition from transferrin may be an important regulator of the erythroid heme biosynthesis (62).

These enzymes function in all cells to make heme for cytochromes and other hemoproteins. However, regulation of the heme biosynthesis in many tissues has not been the subject of intensive investigation, and some studies suggest that it may be different in tissues other than the liver and bone marrow (1).

## 99.4 CLASSIFICATION AND DIAGNOSIS OF THE PORPHYRIAS

As mentioned previously, the porphyrias can be classified as either hepatic or erythropoietic, depending on whether the heme biosynthetic intermediates that accumulate arise initially from the liver or developing erythrocytes, or as acute or cutaneous, based on their clinical manifestations. Table 99-2 lists the porphyrias, their symptoms, major biochemical abnormalities, and inheritance patterns. Further details on the deficient enzymes are given in Table 99-3. Of the five hepatic porphyrias, four of them, acute intermittent porphyria (AIP), HCP, VP, and ALA-dehydratase porphyria (ADP), are present with acute attacks of neurologic manifestations and elevated levels of one or both of the porphyrin precursors, ALA and PBG, and are thus classified as acute porphyrias. Symptoms of neuropathic abdominal pain, peripheral neuropathy, and mental disturbances develop during adult life and are more common in women than in men (1,2,4). By contrast, PCT, while classified as a hepatic porphyria, presents with blistering skin lesions and not acute attacks. HCP and VP may cause cutaneous manifestations similar to PCT, in addition to acute neurological symptoms. The erythropoietic porphyrias, CEP, and EPP, are characterized by elevations of porphyrins in bone marrow and erythrocytes and present with

cutaneous photosensitivity. X-linked protoporphyria, a variant form of EPP, has a clinical presentation identical to classic EPP. Lesions in CEP resemble PCT but are usually much more severe, whereas EPP causes a more immediate, painful, and nonblistering type of photosensitivity. Homozygous dominant forms of AIP, HCP, VP, and familial (type II) PCT (known as HEP) and the autosomal recessive ADP also have erythropoietic features (e.g. increased erythrocyte porphyrins). Rare patients who have mutations in two different heme biosynthetic genes have also been described (see “Dual Porphyrias” section).

### 99.4.1 Diagnosis

When porphyria is suspected clinically, proper laboratory testing is important to confirm or exclude the diagnosis for appropriate medical management and genetic counseling (2). Accuracy and speed are especially important in the diagnosis of an acute porphyric attack, so treatment can begin to prevent neurologic damage and even death. Tests for porphyria may be difficult to interpret because some abnormal results are seen in disorders other than the porphyrias; in particular, minimally elevated levels of urinary porphyrins may have little or no diagnostic significance.

Table 99-3 summarizes the major metabolites that accumulate in each porphyria. However, for initial diagnosis of porphyrias, it is unwise to measure all of these intermediates routinely or attempt to identify a diagnostic profile. For initial screening, we recommend relying on a limited number of tests that are sensitive, specific, and cost effective; additional testing should be done only if a screening test is positive. Testing for elevated urinary

**TABLE 99-3 Human Porphyrias Associated with Deficiencies of Specific Enzymes of the Heme Biosynthetic Pathway**

Inheritance	Deficient Enzyme	Subcellular Localization	Enzyme Activity % of Normal	Known Mutations n <sup>d</sup>	Porphyria OMIM Number <sup>a</sup>
5-ALA dehydratase-deficient	125270	AR	ALA-dehydratase	C	~5 9 porphyria (ADP)
Acute intermittent	176000	AD	HMB-synthase	C	~50244 porphyria (AIP)
Congenital erythropoietic	263700	AR	URO-synthase	C	1–5 36 porphyria (CEP)
Porphyria cutanea tarda	176100	AD	URO-decarboxylase	C	~50 <sup>b</sup> 55 (PCT, type II)
Hepatoerythropoietic	176100	AR	URO-decarboxylase	C	1–5 10 porphyria (HEP)
Hereditary coproporphyria	121300	AD	COPRO-oxidase	M	~50 37 (HCP)
Variegate porphyria (VP)	176200	AD	PROTO-oxidase	M	~50 129
Erythropoietic	177000	AD <sup>c</sup>	Ferrochelatase	M	~20–3088 protoporphyria (EPP)

AR, autosomal recessive; AD, autosomal dominant; C, cytosolic; M, mitochondrial.

<sup>a</sup>OMIM, Online Mendelian Inheritance in Man ([www.ncbi.nlm.nih.gov/entrez/query.fcgi?OMIM](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?OMIM)).

<sup>b</sup>Clinical expression occurs when an iron-mediated inhibitor of URO-decarboxylase is generated and enzyme activity is further reduced (see text for details).

<sup>c</sup>Polymorphism in intron 3 of wild-type allele affects level of enzyme activity and clinical expression.

<sup>d</sup>Number of known mutations from Human Gene Mutation Database ([www.hgmd.org](http://www.hgmd.org)) as of March 29, 2006.



PBG is especially important in screening for acute porphyrias. Active cutaneous porphyrias are readily detected by measuring total plasma porphyrin levels, although, if EPP is suspected, measurement of erythrocyte protoporphyrin is more sensitive. If a screening test result is abnormal, additional measurements are essential for establishing the specific type of porphyria (2). Urinary ALA and PBG are easily quantified by chemical methods (63), and the individual porphyrins in urine and feces can be separated and quantified by high-performance liquid chromatography. Assays are described in the literature for each of the eight heme biosynthetic enzymes, using erythrocytes, lymphocytes, cultured lymphoblasts, or cultured fibroblasts, although most are not widely available for diagnostic purposes (63). However, erythrocyte HMB-synthase activity is commonly measured to confirm the diagnosis of AIP and detect asymptomatic gene carriers.

Establishing the definitive diagnosis of a particular porphyria should include identification of the causative gene mutation(s). This is the preferred method for detecting asymptomatic relatives who carry the mutation identified in an index case (2).

### 99.4.2 5-Aminolevulinic Acid Dehydratase-Deficient Porphyria (ADP)

ALA-dehydratase-deficient porphyria (ADP) is a rare autosomal recessive acute hepatic porphyria caused by the severe deficiency of ALA-dehydratase activity (1,2). To date, only six cases have been reported with documentation by molecular methods (1,12–14). These affected homozygotes had less than 10% of normal ALA-dehydratase activity in erythrocytes, but their clinically asymptomatic parents and other heterozygous relatives had about half-normal levels of activity and did not excrete increased levels of ALA. The frequency of ADP is unknown, but the frequency of heterozygous individuals with less than 50% of normal ALA-dehydratase activity was ~2% in a screening study, in Sweden. Because there are multiple causes for deficient ALA-dehydratase activity, it is important to confirm the diagnosis of ADP by mutation analysis.

**99.4.2.1 Biochemical Aspects.** ALA-dehydratase-deficient porphyria is characterized by the markedly increased urinary excretion of ALA and coproporphyrin III and increased erythrocyte protoporphyrin (complexed with zinc). The markedly reduced erythrocyte ALA-dehydratase activity in these patients is not restored to normal by the *in vitro* addition of sulfhydryl reagents such as dithiothreitol. Immunologic studies in several cases revealed the presence of nonfunctional enzyme protein, which crossreacted with anti-ALA-dehydratase antibody.

**99.4.2.2 Molecular Aspects.** Molecular studies of ADP patients have identified nine-point mutations, two splice-site mutations, a two-base deletion, and

two different base changes at position -11 bp upstream of the exon 3 start site in the ALA-dehydratase gene (Human Gene Mutation Database; [www.hgmd.org](http://www.hgmd.org)) (6,12,14,64). The parents in each case were not consanguineous, and the index cases had inherited a different ALA-dehydratase mutation from each parent. In addition, a point mutation, F12L, was identified in an asymptomatic Swedish girl who had 12% of normal erythrocyte ALA-dehydratase activity (64,65).

The molecular basis of the ALAD2 polymorphism is a substitution of a lysine by an asparagine at residue 95 (K95N). To date, ADP has not been diagnosed prenatally, but this should be possible by determination of the ALA-dehydratase specific molecular lesions in cultured chorionic villi or amniocytes.

**99.4.2.3 Clinical Manifestations.** The onset, severity, and clinical presentations of ADP are variable, presumably depending on the amount of residual ALA-dehydratase activity. All patients had significantly elevated levels of plasma and urinary ALA, with little increase in PBG concentrations and ALA-dehydratase activities of 10% or less of normal. Four reported patients were male adolescents with symptoms resembling those of AIP, including abdominal pain and neuropathy (12–14). The third patient was an infant with more severe disease, including failure to thrive beginning at birth. The earlier age of onset and more severe manifestations in this patient reflect a more significant deficiency of ALA-dehydratase activity (64). Another patient was essentially normal until age 63, when he developed an acute motor polyneuropathy that was associated with a myeloproliferative disorder. This patient was heterozygous for an ALA-dehydratase mutation that presumably was present in erythroblasts that underwent clonal expansion due to the bone marrow malignancy (66).

**99.4.2.4 Differential Diagnosis.** Lead, styrene, and succinylacetone (which is structurally similar to ALA and accumulates in hereditary tyrosinemia type 1 due to fumarylacetoacetase deficiency) inhibit ALA-dehydratase, causing increased urinary excretion of ALA and coproporphyrin, and clinical manifestations that resemble those of the acute porphyrias. Idiopathic acquired ALA-dehydratase deficiency has also been reported. Therefore, these known causes of ALA-dehydratase deficiency should be considered in the differential diagnosis of ADP and the diagnosis of ADP confirmed by demonstrating the underlying ALA-dehydratase mutations.

**99.4.2.5 Treatment.** Because of the small number of ADP patients, there is a limited experience in treatment. Glucose has shown little benefit. Hemin therapy has helped the clinical symptoms in patients with adolescent-onset ADP (12,14,67) and was beneficial for preventing symptoms in one patient (12). A severely affected ADP child did not improve significantly with glucose, hemin, or liver transplant (13,64).

### 99.4.3 Acute Intermittent Porphyria

Acute intermittent porphyria (AIP) is an autosomal dominant acute hepatic porphyria resulting from half-normal levels of HMB-synthase activity. The disease occurs in all ethnic groups with an estimated frequency of individuals with acute attacks of 1 to 2 per 100,000 in most European countries, making it the most common acute porphyria (3,23). It is estimated that less than 10% of individuals with an HMB-synthase mutation have acute attacks (3). A survey of 3350 healthy French blood donors identified two with HMB-synthase gene mutations for a frequency of 1 in 1750, indicating that clinical expression is low (3). The highly variable symptoms and signs include visceral, autonomic, peripheral, and central nervous system manifestations. Activation of the disease is clearly related to ecogenic factors, as its expression is usually triggered by hormonal, metabolic, dietary, or environmental factors, which can precipitate acute attacks. Symptomatic patients always have increased urinary excretion of the porphyrin precursors ALA and PBG. However, the great majority of heterozygotes with HMB-synthase deficiency remains clinically asymptomatic (“latent” or presymptomatic) and may never have increased urinary ALA and PBG excretions.

**99.4.3.1 Biochemical Aspects.** The metabolic defect in AIP is the half-normal activity of HMB-synthase. For most HMB-synthase mutations, the enzyme activity is half-normal in all tissues. However, about 5% of patients have normal HMB-synthase activity in their erythrocytes (23) because they have mutations in or near exon 1 (see “Molecular Aspects” section). Moreover, the range of erythrocyte HMB-synthase activities in patients with classic AIP overlaps the range for normal individuals, as discussed later. Hepatic HMB-synthase activity in patients with active and latent AIP has not been compared.

**99.4.3.2 Molecular Aspects.** To date, over 300 mutations have been identified, most being either point or splice-site mutations (Human Gene Mutation Database ([www.hgmd.org](http://www.hgmd.org)) (6). Most are private, occurring in only one or a few unrelated families. Exceptions include W198X and R116W, which are common in the Swedish and Dutch populations, respectively and G111R, which was found in Argentinean AIP patients (68). A study of 143 Russian and Finnish AIP patients identified genotype-phenotype correlations (69). Based on the crystal structure of *E. coli* HMB-synthase, effects of specific AIP mutations on the human enzyme structure and function have been predicted (24,25).

In a variant form of AIP, molecular studies have identified mutations that impair splicing of exon 1 to exon 3, thereby preventing the formation of the housekeeping but not the erythroid-specific transcript (1,6,23). Other exon 1 mutations alter the initiation of the translation codon, thereby precluding the translation of the housekeeping transcript (70). These tissue-specific

splicing and initiation of translation mutations provide an explanation for the deficient activity in the liver and other nonerythroid tissues in these cases, while the levels of HMB-synthase activity in erythrocytes remain normal. This variant form of AIP can be suspected in a patient with increased PBG, normal erythrocyte HMB-synthase, and laboratory findings that exclude HCP and VP, and mutation analysis is required for confirmation.

Homozygous dominant AIP is a rare form of AIP in which patients have mutations in both of their HMB-synthase alleles and, therefore, very low (<2%) enzyme activity. The disease has been described in a Dutch girl, two young British siblings, and a Spanish boy (71,72). In these homozygous-affected patients, the disease presented in infancy with failure to thrive, developmental delay, bilateral cataracts, and/or hepatosplenomegaly. Interestingly, all these patients’ mutations (R167W, R167Q, and R172Q) were in exon 10 within 5 bases of each other.

**99.4.3.3 Clinical Manifestations.** Acute intermittent porphyria is characterized by neurovisceral disturbances that develop after puberty in a minority of heterozygotes with HMB-synthase deficiency. Symptoms and signs are nonspecific and require a high index of suspicion to suggest the proper diagnosis (2). Abdominal pain, which is the most common symptom, is usually steady and poorly localized, but may be cramping, and is accompanied by nausea and vomiting. Constipation and signs of ileus, including abdominal distension and decreased bowel sounds, are common. However, increased bowel sounds and diarrhea may also occur. These abdominal manifestations are neurologic rather than inflammatory, and therefore tenderness, fever, and leukocytosis are generally absent or mild. Tachycardia, hypertension, restlessness, fine tremors, and excess sweating may be explained by sympathetic overactivity. Dysuria and bladder dysfunction are common, and urinary retention may require catheterization. Chronic hypertension and impaired renal function may develop over a long term. AIP is also commonly associated with mild abnormalities in liver function and the risk of more advanced liver disease and hepatocellular carcinoma is increased.

Peripheral neuropathy in AIP is primarily motor and appears to result from axonal degeneration rather than demyelination. However, paresis does not develop in all patients who suffer from acute attacks, even when abdominal symptoms are severe. Muscle weakness most commonly begins proximally, more often in the arms than in the legs. Tendon reflexes may be normal or hyperactive in early disease stages but are usually decreased or absent with advanced neuropathy. Paresis can be asymmetric and focal. Cranial nerves, most commonly the tenth and seventh, can be affected. Rarely, involvement of the optic nerves or occipital lobes may produce blindness. Extremity pain and paresthesia and areas of loss of sensation are indications of sensory involvement. Muscle weakness can progress to respiratory and bulbar

paralysis and death, but this seldom occurs unless the porphyria is not recognized, harmful drugs are not discontinued, or appropriate treatment is not instituted. Sudden death, presumably due to cardiac arrhythmia, also may occur. Complete recovery even from severe neuropathy over a period of a year or longer is possible.

Central nervous system involvement during acute attacks may be manifested as anxiety, insomnia, depression, disorientation, hallucinations, and paranoia and may suggest a primary mental disorder. Some patients have been mistakenly regarded as hysterical. Depression and other mental symptoms may be chronic in AIP patients. However, it has not been proved that the prevalence of AIP is higher in psychiatric patients than in the general population. Seizures may occur as part of the acute neurologic manifestations of AIP or as a result of hyponatremia, which have been observed and may result from a variety of causes, including inappropriate antidiuretic hormone (ADH) secretion, gastrointestinal losses secondary to vomiting and diarrhea, poor intake, or excess renal sodium loss. Antiseizure drugs are problematic because almost all have at least some potential for exacerbating AIP, with clonazepam being less likely to do so than phenytoin or barbiturates. Improved overall morbidity and mortality in acute porphyrias in the past 20 to 30 years is attributable to earlier detection, less use of barbiturates and sulfonamides in clinical practice, and better treatment of acute attacks (4).

**99.4.3.4 Etiology and Pathogenesis.** Most of the factors known to precipitate acute porphyric attacks have the potential to induce the synthesis of ALAS1 in the liver, thereby increasing the accumulation of ALA, PBG, and other heme pathway intermediates. Normally, a half-normal amount of hepatic HMB-synthase activity is sufficient to avoid any accumulation of PBG. However, when certain environmental, metabolic, and hormonal factors increase the flux of ALA, PBG, and porphyrinogens through the pathway, the partially deficient activity of HMB-synthase may be insufficient to metabolize the increased amounts of PBG.

The etiology of the neurologic manifestations in AIP is not established (73). The possibility that ALA or PBG might be neurotoxic is favored by the increased production of porphyrin precursors during acute porphyric attacks. ALA is taken up by most tissues more readily than PBG, which appears to more readily cross the blood-brain barrier. These intermediates may be converted in vivo to other substances, including porphyrins, which may have neurotoxic potential. The fact that AIP, HCP, VP, ADP, plumbism, and hereditary tyrosinemia are all associated with increased ALA and similar neurologic manifestations favors a neuropathic role for ALA. Moreover, ALA is structurally analogous to  $\gamma$ -aminobutyric acid (GABA) and can interact with GABA receptors (73).

Alternatively, deficient HMB-synthase activity could lead to a functional heme deficiency in the nervous

system, or predispose to unsaturation of hepatic tryptophan pyrrolase, thus leading to altered tryptophan delivery to nervous tissue. Experimental observations regarding these and other possible mechanisms for neurologic dysfunction in the acute porphyrias are reviewed in more detail, elsewhere (2,72,73).

A mouse model in which HMB-synthase deficiency was introduced by gene targeting has been developed (74,75). These animals, when treated with a barbiturate, have impaired motor function, ataxia, increased levels of ALA in brain and plasma, and decreased heme saturation of liver tryptophan pyrrolase. Motor neuropathy can develop in these mice with normal or only slightly increased plasma or urinary ALA, suggesting a role for heme deficiency in nervous tissue (73,74). Studies of the brain MRIs of children with homozygous AIP have suggested damage primarily in white matter that was myelinated postnatally, while tracks that myelinated prenatally were normal (72). These findings suggest that a postnatal toxin such as elevated ALA or PBG rather than heme deficiency caused nervous tissue damage since prenatally elevated ALA and PBG would cross the placenta and be excreted in the mother's urine. Also, the recent finding that a hepatic transplant cured a woman with AIP who had 37 acute attacks in 29 months pre-transplant supports the notion that the acute attacks result from the excess porphyrin precursors produced in the liver (76).

**99.4.3.5 Precipitating Factors.** Certain clinical features of AIP suggest that endogenous steroid hormones are important precipitating factors (1). These include (i) the rarity of symptoms and excess porphyrin precursor excretion before puberty; (ii) more frequent clinical expression in women than in men; (iii) premenstrual attacks of the disease in some women and their prevention by gonadotropin-releasing hormone (GnRH) analogues; (iv) exacerbation of AIP due to exogenous steroids, such as oral contraceptive preparations; and (v) the presence of more subtle abnormalities in steroid hormone metabolism, such as a deficiency of hepatic steroid 5 $\alpha$ -reductase activity. The latter can predispose to the excess production of steroid hormone metabolites that are inducers of hepatic ALAS1.

**99.4.3.5.1 Pregnancy Is Usually Well Tolerated.** However, some women with AIP do experience an increased frequency of attacks during pregnancy. Earlier reports that worsening symptoms during pregnancy are more common may have been due in part to the use of barbiturates and perhaps to reduced caloric intake. Thus, pregnancy is not contraindicated in most women with AIP if harmful drugs are avoided and attention is given to proper nutrition.

Drugs are an important cause of AIP attacks, and the avoidance of harmful drugs can favorably impact the disease course. The major drugs known or strongly suspected by most observers to be harmful in the acute porphyrias, as well as drugs that are known to be safe, are listed in Table 99-4 and include most anticonvulsants,

**TABLE 99-4** Some Major Drugs Considered Unsafe and Safe in Acute Porphyrrias<sup>a</sup>

Unsafe	Safe
Alcohol	Acetaminophen
Barbiturates <sup>b</sup>	Aspirin
Carbamazepine <sup>b</sup>	Atropine
Carisoprodol <sup>b</sup>	Bromides
Clonazepam (high doses)	
Danazol <sup>b</sup>	Erythropoietin <sup>b,c</sup>
Diclofenac and possibly other	Gabapentin NSAIDs <sup>b</sup>
Ergots	Glucocorticoids
Estrogens <sup>b,d</sup>	Insulin
Ethchlorvynol <sup>b</sup>	Narcotic analgesics
Glutethimide <sup>b</sup>	Penicillin and derivatives
Griseofulvin <sup>b</sup>	
Mephenytoin	Ranitidine <sup>b,c</sup>
Meprobamate <sup>b</sup> (also mebutamate <sup>b</sup> ,	Streptomycin tybutamate <sup>b</sup> )
Methyprylon	
Metoclopramide <sup>b</sup>	
Phenytoin <sup>b</sup>	
Primidone <sup>b</sup>	
Progesterone and synthetic progestins <sup>b</sup>	
Pyrazinamide <sup>b</sup>	
Pyrazolones (aminopyrine, antipyrine)	
Rifampin <sup>b</sup>	
Succinimides (ethosuximide, methsuximide)	
Sulfonamide antibiotics <sup>b</sup>	
Valproic acid <sup>b</sup>	

NSAIDs, nonsteroidal anti-inflammatory drugs.

<sup>a</sup>More extensive list of drugs and their status are available in Anderson KE, Sassa S, Bishop DF, et al. (2001) Disorders of heme biosynthesis: X-linked sideroblastic anemias and the porphyrias. In Scriver CR, Beaudet AL, Sly WS, Valle D (eds): The Metabolic and Molecular Basis of Inherited Disease, 8th ed. McGraw-Hill, New York, p 2991; also see Web sites ([www.porphyrifoundation.com](http://www.porphyrifoundation.com); [www.porphyrria-europe.com](http://www.porphyrria-europe.com)).

<sup>b</sup>Porphyria is listed as a contraindication, warning, precaution, or adverse effect in the U.S. labeling for these drugs. For drugs listed as unsafe, absence of such cautionary statements in the U.S. labeling does not imply lower risk.

<sup>c</sup>Although porphyria is listed as a precaution in the U.S. labeling, these drugs are regarded as safe by other sources.

<sup>d</sup>Estrogens have been regarded as harmful, mostly from experience with estrogen–progestin combinations and because they can exacerbate porphyria cutanea tarda (PCT). Although the evidence that they exacerbate acute porphyrias is weak, they should be used with caution. Low doses of estrogen (e.g. transdermal) have been used safely to prevent side effects of GnRH analogues in women with cyclic attacks.

barbiturates, sulfonamide antibiotics, and metoclopramide. Other reviews and more extensive lists of drugs that are harmful or safe are published (1,2) or available through the American Porphyria Foundation Web site ([www.porphyrifoundation.com](http://www.porphyrifoundation.com)) and the European Porphyria Initiative Web site ([www.porphyrria-europe.org](http://www.porphyrria-europe.org)). Most porphyrogenic drugs (e.g. barbiturates) exert their action by induction of hepatic ALAS1, cytochromes P450, and heme synthesis in the liver. Smoking

results in exposure to chemicals that induce cytochrome P450 enzymes and heme synthesis in the liver, and may increase the risk of attacks.

A large retrospective study of risk from anesthetic use in AIP concluded that barbiturates or other inducing drugs are quite frequently detrimental in patients who have already displayed porphyric symptoms, but that they seldom exacerbate latent disease (77). Drugs are only rarely reported to cause acute symptoms in children, who have naturally low levels of endogenous hormones. Such observations indicate that attacks are likely to be due to the additive effects of more than one precipitating factor.

Attacks can also be provoked by intercurrent infections and other illnesses and by major surgery. The mechanisms are not understood but may involve metabolic stress, impaired nutrition, and the increased production of steroid hormones and their ALA-synthase-inducing metabolites. A low caloric intake, usually instituted in an effort to lose weight, is a common contributing cause of acute attacks. Caloric or carbohydrate restriction can precipitate acute symptoms of AIP and increase porphyrin precursor excretion. Recent findings indicate that hepatic ALAS1 is regulated by the peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ), which may represent an important link between nutritional status and acute porphyrias (78).

**99.4.3.6 Laboratory Evaluations.** Urinary excretion of porphyrin precursors is markedly increased during acute attacks of AIP. Fecal porphyrins are usually normal or minimally increased in AIP, which helps to distinguish this disorder from HCP and VP. Because ALA and PBG are colorless, the reddish urine observed in AIP is due to increased porphyrins, which can form nonenzymatically from PBG. Brownish discoloration may be due to porphobilin, a degradation product of PBG, or dipyrromethenes. A normal result of a quantitative test for urinary PBG during a symptomatic period virtually excludes acute porphyria as a cause for concurrent symptoms. An exception is ADP, in which there is an increase in ALA, but not PBG (2).

It is useful to follow ALA and PBG excretion in a symptomatic patient because the concentrations of these compounds generally decrease with clinical improvement. Such decreases are particularly dramatic after heme infusions. But, it is unusual for excretion of ALA and PBG to decrease to normal levels and remain normal unless the disease becomes clinically latent for a prolonged period. In contrast, ALA and PBG levels are often less elevated and may decrease to normal soon after acute attacks of HCP and VP.

The diagnosis of AIP heterozygotes can be confirmed by the finding of half-normal levels of erythrocyte HMB-synthase. However, normal erythrocyte HMB-synthase activity does not exclude AIP, as some mutations in the HMB-synthase gene lead to a deficiency of the enzyme in the liver and other organs, but not in erythrocytes



(1,78,79). A definitive diagnosis may also be precluded because of the following: (i) the normal range for erythrocyte HMB-synthase activity is wide (up to threefold) and low normal and high carrier values overlap; (ii) the enzyme activity is much higher in younger than older erythrocytes and therefore increases when erythropoiesis is stimulated; and (iii) improper processing, storing, and shipping of blood samples can decrease enzyme activity (2). The specific molecular defect in the HMB-synthase gene should be identified in each family in order to provide accurate diagnosis of presymptomatic AIP heterozygotes (2). Most HMB-synthase mutations are family-specific, with a few notable exceptions, where particular mutations have been transmitted over generations from single founders (1). Patients with AIP should have genetic counseling and be encouraged to inform family members about the disease and its genetics. Knowledge of genetic status enables family members to make informed decisions about lifestyle and to know the potential risks of certain drugs, preferably before the development of an acute illness. However, latent porphyria should not be construed as a health risk that limits health or life insurance. Prenatal diagnosis of AIP has been performed by enzymatic assay but is seldom indicated because the outlook for most carriers is favorable.

#### 99.4.3.7 Treatment.

**99.4.3.7.1 Supportive and Symptomatic Treatment.** Hospitalization may be required: for evaluation and treatment of severe pain, nausea, and vomiting; for administration of intravenous fluids, electrolytes, glucose, and hemin; and for close observation for electrolyte derangements and neurologic complications. Medications taken by the patient should be reviewed immediately and those identified as harmful stopped, if at all possible. Narcotic analgesics are usually required for abdominal pain, and small to moderate doses of a phenothiazine are indicated for nausea, vomiting, anxiety, and restlessness.

Carbohydrate loading provides nutritional replacement, has some repressive effect on hepatic ALA synthase, but is less effective than hemin. It may suffice for mild attacks in patients with low narcotic requirements and without hyponatremia or paresis. Sucrose, glucose polymers, or carbohydrate-rich foods may be given to patients without abdominal distention and/or ileus and who can tolerate oral treatment. The standard intravenous regimen is 10% glucose for a total of at least 300–500 g daily. However, large volumes of 10% glucose may increase the risk of hyponatremia. Severe or prolonged attacks should be treated with hemin and may also require more nearly complete nutritional support.

Tachycardia and systemic arterial hypertension may be treated cautiously with  $\beta$ -adrenergic blocking agents, but they may be hazardous in patients with hypovolemia, in whom increased catecholamine secretion may be an important compensatory mechanism (2). Seizures

are difficult to treat because almost all antiseizure drugs can exacerbate an attack. Gabapentin, and probably vigabatrin, can be given safely and benzodiazepines are relatively safe. Careful correction of hyponatremia and hypomagnesemia is important, particularly when associated with seizures.

**99.4.3.7.2 Hemin Therapy.** Intravenous hemin addresses the underlying pathophysiology by repressing hepatic ALAS1, hence decreasing the overproduction of ALA and PBG. Hemin given intravenously at moderate dose (3–4 mg/kg day for 4 days) is mostly taken up in the liver, and can at least transiently replenish the depleted heme pool that regulates the synthesis of ALAS1. It cannot be given orally because it is catabolized by heme oxygenase during intestinal absorption.

Hemin therapy should be started early (2). Although product labeling recommends an initial trial of intravenous glucose, hemin is the preferred therapy (80–82). The standard regimen is 3–4 mg of hemin per kilogram of body weight, infused intravenously once daily for 4 days. Hemin (Panhematin®), Lundbeck Pharmaceuticals, is available in the United States as lyophilized hydroxyheme (hematin) for reconstitution with sterile water just before infusion, and it is approved by the FDA for amelioration of acute porphyric attacks. Degradation products form rapidly in vitro when this product is reconstituted with sterile water, as recommended in product labeling, and these adhere to endothelial cells, platelets, and coagulation factors and cause a transient anticoagulant effect and often a phlebitis at the site of infusion. With repeated administration, phlebitis can compromise venous access. It is recommended that lyophilized hemin be reconstituted with human albumin to enhance stability (2). Another hemin preparation, heme arginate, is more stable in solution but is not available in the United States.

Reconstitution of lyophilized hydroxyheme with albumin enhances stability of lyophilized hemin, decreases the incidence of phlebitis, and may enhance efficacy. Other uncommon reported side effects of hemin include fever, aching, malaise, hemolysis, a case of circulatory collapse that resulted in full recovery after subsequent hemin infusions, and one case of transitory renal failure after a dosage of 1000 mg. Experience indicates that hemin can be administered safely during pregnancy.

Patients should be monitored closely during management of acute attacks for complications and signs of progression of acute porphyria such as electrolyte imbalance, acute psychiatric manifestations, muscle weakness, bladder retention, and ileus (2). Spirometry is sometimes indicated daily to detect respiratory impairment at least until the attack begins to resolve. Since patients with respiratory impairment can deteriorate rapidly, it is recommended they be placed in intensive care. ALA and PBG usually fall to normal whether therapy is started early or late, but this does not necessarily predict a clinical response.

Clinical improvement may occur within 1 to 2 days if hemin is started early in an attack. Patients can sometimes be discharged from the hospital within several days, although we recommend completion of the standard 4-day treatment course in the outpatient clinic. If initiated late, efficacy of hemin may not be immediately apparent because neuronal damage may already be advanced and slow to recover. In such cases, treatment for longer than 4 days should be considered, although the evidence that this improves the outcome is lacking. Hemin is seldom effective for chronic symptoms. Hemin therapy can be given in outpatient settings or in the home, if this facilitates prompt therapy and reduces medical care costs in patients with frequent attacks.

Chronic renal failure has developed in some AIP patients and required renal transplantation. This may be caused by the development of chronic hypertension and prevented by control of blood pressure. AIP also increases the risk of chronic liver disease and especially hepatocellular carcinoma. These tumors seldom increase serum  $\alpha$ -fetoprotein levels. Therefore, periodic screening by ultrasound or another hepatic imaging technique is recommended.

**99.4.3.7.3 Transplantation.** An allogeneic liver transplant was performed on a 19-year-old female AIP heterozygote who had 37 acute attacks in the 29 months prior to transplantation. Posttransplantation, her elevated urinary ALA and PBG levels returned to normal in 24 hours, and she did not experience acute neurologic attacks for more than 18 months posttransplant (76). Two AIP patients had combined liver and kidney transplants secondary to uncontrolled acute porphyria attacks, chronic peripheral neuropathy, and renal failure, requiring dialysis. Both patients had marked improvement with no attacks and normal urinary PBG levels posttransplantation, as well as improvement of their neuropathic manifestations (83). It should be noted that liver transplantation is a high-risk procedure and should not be considered as an established treatment for acute porphyrias. Recently, liver-directed gene therapy has been proven successful in the prevention of drug-induced biochemical attacks in a murine model of human AIP (84).

**99.4.3.8 Prevention of Acute Attacks and Later Complications.** Prevention of future attacks requires identifying precipitating factors. Educating the patient to avoid alcohol, smoking, and drugs that can induce exacerbations (see Table 99-4) is important, as in maintaining adequate nutrition. Lists of safe and harmful drugs are available (see previous discussion for references) but these are not infallible. Medical alert bracelets and wallet cards can help notify emergency medical personnel and ensure that unsafe drugs are not given to patients in emergencies. Some patients have frequent attacks even after exacerbating factors are removed, possibly because of unidentified modifier genes or environmental or endogenous precipitating factors. These patients should

be evaluated by a nutritionist and follow a well-balanced diet with sufficient calories to maintain weight.

Gonadotropin-releasing hormone (GnRH) analogues can be highly effective for women with frequent cyclic attacks when symptoms are confined to the luteal phase of the menstrual cycle (1). The low-dose estrogen patch has been successful in reducing side effects when treatment beyond six months is contemplated. Gynecological examinations and bone density determinations are advised every 6 months during treatment. Continued need can be assessed every 1 to 2 years by stopping the treatment.

Pregnancy increases levels of progesterone, a potent inducer of heme biosynthesis in the liver but nevertheless is well tolerated in most women with acute porphyria. For example, in a large series of women with AIP or VP who had 176 deliveries, porphyric symptoms were absent in 92% of their pregnancies (85). Because some women experience more frequent attacks during pregnancy, counseling women who wish to become pregnant must be individualized.

Recurrent noncyclic attacks are sometimes prevented by weekly or biweekly infusions of single doses of hemin (3–4 mg/kg). Frequent treatment with hemin has a theoretic risk of iron overload (100 mg of hemin contains 8 mg of iron); therefore, serum ferritin levels should be monitored. In selected rare instances of severe, unremitting symptomatic disease, consideration might be given to orthotopic liver transplantation (76). Transplantation of hepatocytes or specific gene replacement therapy is a possible future therapeutic strategy.

## 99.4.4 Congenital Erythropoietic Porphyrria

Congenital erythropoietic porphyria (CEP), also known as Günther disease, is an autosomal recessive disorder due to the markedly deficient activity of URO-synthase, the fourth enzyme in the heme biosynthetic pathway. CEP is panethnic, and as of 2000, about 160 cases were reported (28,86).

**99.4.4.1 Biochemical Aspects.** The deficient activity of URO-synthase is the enzymatic defect in CEP. Affected homozygotes have markedly deficient, but not absent, URO-synthase activity, as sufficient enzyme is required to produce uroporphyrinogen III for normal (or even increased) rates of heme production. Most CEP patients have less than 10% of normal erythrocyte URO-synthase activity. The deficient URO-synthase activity leads to the accumulation of the substrate, hydroxymethylbilane (HMB), most of which is converted nonenzymatically to uroporphyrinogen I. Although uroporphyrinogen I can undergo decarboxylation by URO-decarboxylase to form hepta-, hexa- and pentacarboxyl porphyrinogen I and finally coproporphyrinogen I, further metabolism cannot proceed because the next enzyme in the pathway, COPRO-oxidase, is stereospecific for the III isomer.

Therefore, the isomer I porphyrins are nonphysiologic, in that they cannot be metabolized to heme, and are pathogenic when they accumulate in large amounts and undergo auto-oxidization to their corresponding porphyrins. In patients with CEP, the large amounts of isomer I porphyrinogens that accumulate in bone marrow erythroid precursors (especially normoblasts and reticulocytes) and erythrocytes undergo auto-oxidation to the corresponding porphyrins, which damage erythrocytes, cause cutaneous photosensitivity, are deposited in tissues and bones, and are excreted in large amounts in the urine and feces.

**99.4.4.2 Molecular Aspects.** The isolation and characterization of the URO-synthase cDNA and genomic sequences have permitted the identification of mutations in CEP patients (26,28,87). Over 39 mutations have been detected in unrelated CEP families including missense and nonsense mutations, large and small deletions and insertions, splicing defects, intronic branch point mutations, and erythroid-specific promoter mutations (6). Most mutations have been detected in only one or a few unrelated families, except for C73R, which has been found in about 33% of the alleles studied, L4F in 7%, and T228M in 6% (28). The recent discovery of alternative housekeeping and erythroid-specific promoters in the human URO-synthase gene facilitated the identification of four-point mutations within a 20-bp region of the erythroid-specific promoter in six unrelated CEP probands (26,88). These mutations included a -70T to C transition altering a GATA-1 binding element, a -76 G to A transition, a -86C to A transversion in three unrelated patients, and a -90C to A transversion that altered a putative CP2 binding element. These four pathogenic erythroid promoter mutations impaired erythroid-specific transcription, caused CEP, and identified functionally important GATA1 and CP2 transcriptional binding elements for erythroid-specific heme biosynthesis. To date, these are the only known promoter mutations in the erythropoietic porphyrias. For a review of URO-synthase mutations causing CEP, see (6,28).

Genotype-phenotype correlations are possible in CEP once a patient's mutations are known. Prokaryotic expression and gene promoter-reporter systems have been used to determine the *in vitro* levels of enzymatic activity expressed by the missense mutations and the promoter function of mutations in the erythroid-specific promoter. The prokaryotic expression of URO-synthase constructs containing missense mutations resulted in levels of enzymatic activity that ranged from essentially nondetectable to about 35% of the mean activity expressed by the wild-type allele in *E. coli*. The effect of the four promoter mutations on transcription also was assessed *in vitro* by determining the luciferase activity of each lesion using promoter-reporter gene constructs in uninduced and induced (with hemin) K562 erythroleukemia cells (88).

For genotype-phenotype correlations, a series of CEP patients were classified as very mild to severely affected, based on age, degree of hemolytic anemia, organomegaly, osteopenia, and cutaneous involvement (28). Homoallelism for the most common allele, C73R, was correlated with the most severe phenotype, nonimmune hydrops fetalis and/or transfusion dependency from birth. Consistent with the severe phenotype of C73R/C73R homozygotes, expression of the C73R allele in *E. coli* resulted in the detection of less than 1% of the activity expressed by the wild-type allele. The fact that the C73R/C73R homozygotes are viable and do not die early in fetal life indicates that the mutant enzyme retains a very small amount of residual activity that is sufficient to produce enough heme for the biosynthesis of hemoglobin and other essential hemoproteins. Alternatively, if the C73R mutation produced only nonfunctional or barely functional enzyme, then the fact that affected fetuses survive suggests the possibility of another gene that is responsible for URO-synthase activity during development. However, knockout mice homozygous for a null mutation in the URO-synthase gene died early in embryogenesis, indicating that the total deficiency of URO-synthase activity was an embryonic lethal (89).

Patients heteroallelic for C73R and another mutation that expressed little residual activity, such as P53L, also resulted in a severe or moderately severe phenotype. Patients heteroallelic for mutations that expressed more residual activity such as A104V (7.7% of normal activity), A66V (14.5% of normal activity), and V82F (35% of normal activity) had milder forms of CEP, even if the other allele was C73R or another mutation that did not express detectable activity (e.g. nonsense and frameshift mutations). For example, a teenage boy whose genotype was C73R/A66V had only mild cutaneous involvement. Genotype-phenotype correlations for CEP probands with erythroid promoter mutations also have been made (88). For example, a proband heterozygous for a promoter mutation with low activity (-70C) and for C73R had the severe nonimmune hydrops fetalis phenotype, while a proband with the C73R mutation in one allele but with a promoter mutation with more activity (-76A) in the other allele had a mild cutaneous disease phenotype (88). As additional mutations are identified and expressed, more information will become available to evaluate genotype/phenotype correlations.

Affected fetuses can be detected *in utero* by determining the uroporphyrin I levels in amniotic fluid, the URO-synthase activity in cultured amniotic fluid cells or chorionic villi, and/or by molecular analysis in families where the URO-synthase mutation(s) has been identified (90), or by a combination of these methods (91).

**99.4.4.3 Clinical Manifestations.** The age at onset and clinical severity of CEP are highly variable, ranging from nonimmune hydrops fetalis due to severe hemolytic anemia *in utero* to milder, later-onset forms that have only cutaneous lesions in adult life (28,87). At least some

of the late onset cases have been associated with myeloproliferative disorders, in which a clone of erythroid cells carries expresses URO-synthase deficiency (66). A number of factors are responsible for the phenotypic variability including (i) the amount of residual URO-synthase activity, (ii) the degree of hemolysis and consequent stimulation of erythropoiesis, and (iii) exposure to UV light. Therefore, as in other porphyrias, an interplay of environmental factors with the deficient enzyme activity determines the clinical expression of disease. Life expectancy may be diminished in more severely affected patients owing to the hematologic complications and the increased risk of infection (28).

The major debilitating clinical features of CEP are photosensitivity and anemia. Severe cutaneous photosensitivity begins in early infancy and is manifested by increased friability and blistering of the epidermis on the hands and face and other sun-exposed areas. Skin manifestations resemble those of PCT, but with the much higher levels of porphyrins in plasma are usually much more severe. Bullae and vesicles contain serous fluid and are prone to rupture and infection. The skin may be thickened, with areas of hypo- and hyperpigmentation. Hypertrichosis of the face and extremities is often prominent. Recurrent vesicles and secondary infection can lead to cutaneous scarring and deformities, as well as to loss of digits and facial features such as eyelids, nose, and ears. Corneal scarring can lead to blindness. Porphyrins deposited in the teeth produce red fluorescence on exposure to long-wavelength UV light and a reddish-brown color in natural light, termed erythrodontia. Bone demineralization may result from expansion of the hyperplastic bone marrow and associated porphyrin deposition in bone (1,92).

Hemolysis is accompanied by anisocytosis, poikilocytosis, polychromasia, basophilic stippling, reticulocytosis, increased nucleated red cells, decreased serum haptoglobin, increased unconjugated bilirubin, increased fecal urobilinogen, and increased plasma iron turnover, and probably results from the accumulated porphyrins in erythrocytes. Development of secondary splenomegaly may contribute further to the anemia and may also result in leukopenia and thrombocytopenia. The latter is sometimes associated with significant bleeding, and in such cases, splenectomy may be beneficial. Hemolytic anemia is especially severe if the bone marrow does not compensate, and some patients are transfusion dependent. For example, CEP-genotype C73R/C73R usually presents in utero with hemolysis and nonimmune hydrops, which if recognized can be treated by intrauterine transfusions.

To better understand the pathophysiology and for studying treatment modalities, mouse models of CEP using knock-in techniques have been developed in which the mice have low URO-synthase activity and clinical symptoms, including erythrodontia, characteristic light-induced cutaneous involvement, hepatosplenomegaly, and hemolytic anemia (93,94).

**99.4.4.4 Laboratory Evaluation.** CEP should be suspected as a cause of nonimmune hydrops and in infants or young children with severe photosensitivity and markedly increased urinary and plasma porphyrins. Reddish urine in the diaper shortly after birth is often the first suggestion of this disease. Milder cases of CEP may be developed later in life in the presence of a myeloproliferative disorder and resemble PCT. Accumulation of isomer I porphyrins, especially uroporphyrin I and coproporphyrin I in bone marrow, erythrocytes, plasma, and urine is the biochemical hallmark of the disease. Urinary porphyrins are primarily uroporphyrin I and coproporphyrin I, the intermediate 7-, 6-, and 5-carboxyl porphyrins being excreted in excess as well. Although there is a great predominance of type I isomers, type III isomers are also increased. Protoporphyrin IX is sometimes the predominant porphyrin in erythrocytes in CEP, as in other autosomal recessive porphyrias. Urinary ALA and PBG are not increased. Fecal porphyrins are markedly increased, with a predominance of coproporphyrin I.

URO-synthase activity can be measured in erythrocytes and cultured cells using either direct or coupled enzyme assays (95). CEP should be differentiated from other porphyrias with cutaneous photosensitivity. For example, HEP often mimics CEP clinically but is the homozygous dominant form of URO-decarboxylase deficiency. HEP is distinguishable from CEP by porphyrin patterns resembling PCT, including high levels of isocoproporphyrin in feces and urine and markedly decreased URO-decarboxylase activity in erythrocytes. Very rare homozygous forms of VP and HCP also may be characterized by photosensitivity in childhood and increased erythrocyte porphyrins.

**99.4.4.5 Treatment.** Skin Protection. Protection of the skin from sunlight and minor trauma is essential. Sunscreen lotions and b-carotene are sometimes beneficial. Bacterial infections that complicate cutaneous blisters require timely treatment in an effort to prevent scarring and mutilation. Severe infections such as cellulitis and bacteremia may require intravenous antibiotics.

**99.4.4.5.1 Marrow Suppression.** Frequent blood transfusions are sometimes essential for severe anemia. Transfusions repeated frequently enough to suppress erythropoiesis, and thereby decrease porphyrin production, can greatly reduce porphyrin levels and photosensitivity. Such therapy is likely to be successful if the hematocrit remains above 35% and deferoxamine is administered to reduce the resulting iron overload. Treatment with hydroxyurea to reduce the bone marrow porphyrin synthesis may be considered, especially after puberty when porphyrin production may increase (96). Splenectomy has substantially reduced transfusion requirements in some patients. Oral charcoal has increased fecal loss of porphyrins with milder disease, but seems less successful in more severe cases (97). Hemin therapy, which is effective for the treatment of the acute hepatic porphyrias, may be somewhat effective in CEP



but has not been extensively studied. Chloroquine has not been beneficial.

**99.4.4.5.2 Bone Marrow Transplantation.** Bone marrow transplantation (BMT) has proved curative for patients with CEP. To date, nine transplanted patients have been reported, and when successful, BMT has resulted in marked reduction in porphyrin levels and photosensitivity (for reviews, see (98,99)). The source of the hematopoietic stem cells has included bone marrow or umbilical cord blood from histocompatible sibs as well as from unrelated HLA-matched marrow.

**99.4.4.5.3 Experimental Gene Therapy.** The success of BMT provides the rationale for hematopoietic stem cell gene therapy. The stable transduction of the patient's own stem cells with vectors containing the URO-synthase cDNA would abrogate the need for HLA-identical donors and the risk of rejection. Various retroviral and lentiviral vectors expressing human URO-synthase have been used to transduce a variety of cell types including mononuclear cells derived from the bone marrow of normal and CEP subjects (100–106). Better transduction efficiencies were obtained with the lentiviral vectors than the retroviral vectors and all transduced cell types had increased URO-synthase activity and suppression of porphyrin accumulation. These studies are encouraging; however, in vivo efficacy of individual vector constructs may not be predictable from these in vitro experiments, and animal studies are needed. Such in vivo experiments could determine whether transduction of hematopoietic stem cells can be efficient enough to minimize the proportion of nontransduced progenitors capable of producing toxic quantities of porphyrins in their descendants.

## 99.4.5 Porphyrria Cutanea Tarda

Porphyrria cutanea tarda (PCT) is caused by reduced activity of URO-decarboxylase, the fifth enzyme in the heme biosynthetic pathway. PCT is unique among the porphyrias as this cutaneous and biochemical phenotype results from an acquired, liver-specific enzyme inhibition in both sporadic (type I) and familial (types II and III) forms (1,34,107). PCT is the most common porphyria and has an estimated frequency of 1 per 25,000, of which ~80% have type I disease (34,107). This porphyria can also result from exposure to certain polyhalogenated aromatic hydrocarbons. The most notable occurrence of environmentally induced PCT was an outbreak in Turkey in the 1950s, caused by the ingestion of wheat treated with the fungicide hexachlorobenzene (HCB).

### 99.4.5.1 Biochemical Aspects.

**99.4.5.1.1 Types I–III PCT Are Clinically Very Similar.** The enzymatic activity of hepatic URO-decarboxylase must be decreased by ~75% before porphyrins accumulate and clinical symptoms occur (34,107). However, the amount of hepatic URO-decarboxylase enzyme protein is not decreased below

its genetically determined level but is inhibited by a substance, not yet characterized, derived from a heme pathway intermediate (107). Production of the URO-decarboxylase inhibitor is iron-dependent. The multiple factors that can precipitate types I–III PCT act mostly be increasing hepatic iron content or oxidative stress (see later discussion). The genetically determined level of URO-decarboxylase in type II PCT is half-normal in all tissues, and the disease becomes manifested only when the hepatic enzyme becomes further reduced.

**99.4.5.1.2 Type I Porphyrria Cutanea Tarda.** In type I PCT, URO-decarboxylase activity is deficient in the liver and is normal in all other tissues such as erythrocytes. No URO-decarboxylase mutations have been identified in type I PCT. Moreover, there are no tissue-specific isoenzymes of URO-decarboxylase, and therefore, a mutation of the gene for this enzyme is unlikely to lead to a tissue-specific enzymatic deficiency. The tissue-specific enzymatic deficiency in type I PCT appears to be acquired because the amount of hepatic URO-decarboxylase protein, as measured immunochemically, is normal, suggesting that the enzyme has been inhibited, and with phlebotomy the enzyme activity gradually increases and both catalytic and specific activity may return to normal (108). URO-decarboxylase activity is not directly inhibited by iron. Considerable evidence suggests that type I PCT is caused by inhibition or inactivation of structurally normal URO-decarboxylase by a liver-specific, iron-dependent process that promotes the oxidation of uroporphyrinogen to uroporphyrin and a product that inhibits URO-decarboxylase. Efforts to isolate and characterize this inhibitor are currently in progress using laboratory models (107).

**99.4.5.1.3 Type II Porphyrria Cutanea Tarda.** In type II or familial PCT, the genetically determined level of URO-decarboxylase activity in all tissues is half-normal due to the autosomal dominant inheritance of a URO-decarboxylase mutation. The half-normal amount of enzymatic activity is the product of the normal URO-decarboxylase allele. Half-normal enzyme protein and activity is demonstrated in nonhepatic tissues such as erythrocytes and cultured skin fibroblasts in clinically affected individuals and in family members with latent disease. However, manifest type II PCT develops only when hepatic URO-decarboxylase becomes reduced considerably below the inherited enzyme level, and to levels of activity corresponding to type I PCT (107). The amount of hepatic enzyme protein remains half-normal in type II PCT.

URO-decarboxylase is not a rate-limiting enzyme for heme biosynthesis, and therefore most type II heterozygotes do not develop PCT unless precipitating factors are present (see later discussion) (107,109).

**99.4.5.1.4 Type III Porphyrria Cutanea Tarda.** Type III PCT is rare and is presumed to be inherited because more than one family member is affected. However, it resembles type I in that URO-decarboxylase

activity is normal in extrahepatic tissues such as erythrocytes and URO-decarboxylase mutations have not been identified (108). Therefore, the genetic basis for this type of PCT is not yet understood, and it is not readily differentiated from type I.

#### 99.4.5.1.5 *Hepatoerythropoietic Porphyrria.*

Hepatoerythropoietic porphyria (HEP) is the homozygous dominant form of type II PCT. This disease is rare, as only about 30 cases from 24 families have been reported (110). The URO-decarboxylase mutations causing HEP often do not lead to complete loss of the enzyme activity, and some are CRIM-positive (29,111). The URO-decarboxylase activity in HEP patients has ranged from 3% to 28% of normal (29).

**99.4.5.2 Genetic and Molecular Aspects.** URO-decarboxylase gene mutations are found only in type II PCT, as well as HEP. However, in types I and III PCT, other genetic as well as environmental factors (see later discussion) appear to contribute to the reduction of hepatic URO-decarboxylase in liver that is necessary for development of the clinically manifested disease.

The half-normal activity of URO-decarboxylase in type II PCT is clearly inherited as an autosomal dominant trait. Over 100 mutations in the URO-decarboxylase gene have been identified, including over 50 mutations underlying type II PCT, and ten different missense mutations causing HEP (Human Gene Mutation Database; [www.hgmd.org](http://www.hgmd.org)) (6). The ten mutations in the URO-decarboxylase gene identified in HEP include five such as G281E (112) that also have been found in type II PCT (29,31,110,113–116). HEP patients are either homoallelic or heteroallelic for the URO-decarboxylase mutations.

Of the URO-decarboxylase mutations listed in the Human Gene Mutation Database (6), 57.4% are missense, 0.7% nonsense, and 13.1% are splice-site mutations. Most URO-decarboxylase mutations have been identified in only one or two families. Exceptions include mutations g10insA (found in six Argentinean families) (111), G281E (found in 14 unrelated Spanish families) (112), and IVS6+1 (found in 6 unrelated families).

Type III PCT has been studied in four Spanish families, in which at least two relatives had clinical manifestations of PCT with decreased URO-decarboxylase activity in liver but normal levels in erythrocytes and other tissues. To date, no mutations of the URO-decarboxylase locus have been detected in type III PCT.

**99.4.5.3 Precipitating Factors.** A number of inherited and environmental factors contribute to the profound inhibition of hepatic URO-decarboxylase activity and the development of clinically manifested PCT. In type II PCT, the inheritance of a heterozygous URO-decarboxylase mutation predisposes to the disease because the amount of enzyme is half-normal initially, and less inhibitor is required to develop overt PCT (107). Other inherited susceptibility factors include HFE mutations (C282Y and H63D) that increase iron retention, and possibly

polymorphisms of cytochrome P450 enzymes (117). Environmental and infectious factors include excess alcohol consumption, hepatitis C virus infection, HIV infection, estrogen use, smoking, decreased levels of antioxidants such as vitamins E and C, and carotenoids (3,107,108,118). These appear to contribute to development of PCT by increasing oxidative stress in hepatocytes and the production of an inhibitor of URO-decarboxylase. Multiple factors are present and may act in an additive fashion in the individual PCT patient (119). A study of 84 Swedish patients with PCT found that 23% of the patients had Type II PCT and 57% had hemochromatosis mutations, 14% of whom were homozygous for the C282Y mutation. Other risk factors included alcohol abuse (38% of males), estrogen treatment (55% of females), and antihepatitis C virus (29% of males) (120).

Mutations in the hemochromatosis (HFE) gene may result in elevated tissue iron levels and therefore predispose to PCT. There are two common mutations causing hemochromatosis: the C282Y mutation causes hemochromatosis in homozygotes in northern Europeans and their descendants, and the H63D mutation, which is more common in southern Europe. One in ten normal individuals is a carrier of a HFE gene mutation and an increased frequency occurs in both sporadic and familial PCT patients (107,108,121). In American PCT patients, 63% to 73% had HFE mutations (119,122,123); in one series, 17 of 87 (19%) PCT patients were homozygous for C282Y compared with zero for the 56 controls (123). Among English PCT patients, 17% were found to be homozygous for the C282Y mutation and 20% were heterozygous (124). A review of eight studies (121) found a mutant hemochromatosis allele in 17% to 47% of patients with sporadic or familial PCT suggesting that the HFE gene is an important predispositional modifier gene for the clinical expression of PCT (121).

**99.4.5.4 Porphyrria Cutanea Tarda due to Halogenated Hydrocarbons.** In 1955 to 1958, an extensive outbreak of PCT occurred in eastern Turkey as a result of the ingestion of wheat treated with the fungicide HCB (1,108). Feeding of HCB to animals was subsequently shown to decrease hepatic URO-decarboxylase activity and to produce a porphyria biochemically similar to human PCT. Smaller case clusters have also been reported after exposure to other chemicals, including di- and trichlorophenols and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) (108).

**99.4.5.5 Clinical Manifestations.** Skin lesions consist of fluid-filled vesicles and bullae on sun-exposed areas such as the dorsa of the hands and feet, the face, forearms, and legs. These may rupture, form crusted lesions, and heal slowly with residual scarring. Secondary infection may occur. Sun-exposed skin becomes friable, and minor trauma may precede the formation of bullae or may cause denudation of the skin. Small white plaques, termed milia, may precede or follow vesicle formation. Other cutaneous manifestations include hypertrichosis

and hyperpigmentation, especially of the face, which can present in the absence of vesicles. Thickening, scarring, and calcification of affected areas of skin are sometimes striking and has been termed pseudoscleroderma because it resembles the cutaneous changes of systemic sclerosis. The skin lesions in PCT are generally indistinguishable from those in VP and HCP.

PCT is commonly associated with evidence of chronic liver disease and sometimes with cirrhosis. Patients with PCT are also at risk to develop hepatocellular carcinoma; in several series, the incidence has ranged from 4% to 47%. These tumors appear as a complication of PCT and do not themselves contain or produce porphyrins in large amounts. They may result from longstanding liver damage in PCT, hemosiderosis, coexistent chronic hepatitis C infection, exposure to halogenated chemicals that are also carcinogenic, or to the effects of porphyrin deposition in the liver. PCT associated with advanced renal disease is often more severe and intractable than PCT occurring in its absence (1).

HEP resembles CEP clinically and usually presents in infancy or childhood with onset of blistering skin lesions, hypertrichosis, scarring, and red urine. HEP is genetically heterogeneous, and unusually mild cases have been described (125). Two mild cases of HEP with only minor scarring have recently been described in two unrelated patients, one in a 5-year-old boy and the other in a 38-year-old man (110,126). Interestingly, both patients were homoallelic for the same mutation F46L, which resulted in less than 10% of normal URO-decarboxylase activity in these patients.

**99.4.5.6 Laboratory Evaluation.** PCT is strongly suggested by the characteristic skin lesions in sun-exposed areas, especially on the backs of the hands. However, similar skin blistering is seen with other cutaneous porphyrias, with the exception of EPP. The most useful initial diagnostic test is a total plasma porphyrin determination. A normal plasma porphyrin level excludes PCT, whereas a high level with a fluorescence emission maximum at neutral pH near 619 nm excludes VP and is highly suggestive of PCT (108). Measurements of urinary and fecal porphyrins provide confirmation of PCT. URO-decarboxylase activity can be measured in erythrocytes and lymphoblasts (89) and can be used to distinguish Types I and II since Type 1 PCT patients will have normal levels of erythrocyte URO-decarboxylase, while Type II PCT patients will have ~50% of normal levels (15). Molecular analysis more reliably diagnoses type II patients by identifying the underlying URO-decarboxylase gene mutations (127).

Porphyrins are increased in the liver, plasma, urine, and stool in PCT (63). A slight increase in ALA is noted in some patients, but PBG excretion is normal. Urinary porphyrins consist mostly of uroporphyrin and 7-carboxyl porphyrin, with lesser amounts of coproporphyrin and 5- and 6-carboxyl porphyrins. The excess urinary uroporphyrin in PCT is predominantly

isomer I; 7- and 6-carboxyl porphyrins are mostly isomer III; and 5-carboxyl porphyrin and coproporphyrin are approximately equal mixtures of isomers I and III.

The finding of increased isocoproporphyrins, most readily demonstrated in feces, is diagnostic for a deficiency of URO-decarboxylase (34). These unusual 4-carboxyl porphyrins are produced when hepatic URO-decarboxylase is deficient, because 5-carboxyl porphyrinogen III, which accumulates in PCT, can be metabolized by COPRO-oxidase to yield dehydroisocoproporphyrinogen. This porphyrinogen is excreted in bile and undergoes auto-oxidation and side-chain modification by bacterial enzymes in the intestine to give isocoproporphyrin and deethylisocoproporphyrin, the major representatives of the isocoproporphyrin series in the feces of PCT patients. Increased liver porphyrins are composed mostly of uroporphyrin and 7-carboxyl porphyrin.

The biochemical findings in HEP are similar to those in other forms of PCT. In addition, the concentration of protoporphyrin in erythrocytes is increased and is predominantly zinc protoporphyrin.

**99.4.5.7 Therapy.** The diagnosis of PCT should be firmly established by biochemical investigations before treatment is initiated, because VP and HCP can produce similar cutaneous lesions but are unresponsive to measures that are highly effective in PCT. Imaging studies are advisable to exclude complicating hepatocellular carcinoma and to serve as a baseline for follow-up. Testing for known precipitating factors is recommended to include hepatitis C and HIV infections. Patients should abstain from alcohol, estrogens, iron supplements, or other exogenous agents that may exacerbate the disease. Drugs such as barbiturates, phenytoin, and sulfonamides that are harmful to patients with acute porphyrias are seldom reported to contribute to the clinical expression of PCT, but should be avoided as a precaution.

Standard therapy consists of repeated phlebotomy, which can produce remission in almost all patients (1). The aim is to gradually reduce excess hepatic iron by removing about 450 mL of blood at intervals of 1 to 2 weeks until the serum ferritin reaches the lower limits of normal (108). Plasma (or serum) porphyrin levels decrease in parallel and become normal within 1 to 2 months after the target ferritin concentration is achieved. Hemoglobin or hematocrit levels should be followed closely to prevent the development of symptomatic anemia. Continued phlebotomies are seldom needed even if ferritin levels later return to normal. However, it is advisable to follow porphyrin levels and reinstitute phlebotomies if porphyrin levels begin to rise. Even cutaneous scarring and pseudoscleroderma can improve with phlebotomy and serum markers for the liver cell function to normalize (108). Recombinant erythropoietin is an effective treatment and can support phlebotomy therapy when PCT is associated with end-stage renal disease (1).

Small doses of chloroquine (125 mg twice weekly) or hydroxychloroquine (100 mg twice weekly) are also

effective in producing remissions of PCT (127). These drugs promote excretion of accumulated porphyrins in the liver in PCT, possibly by complexing with porphyrins. However, the standard antimalarial doses of these drugs may induce acute hepatic damage, nausea, vomiting and fever, and elevated urinary and plasma porphyrin levels due to the release of large amounts of stored porphyrins from the liver (1). There may be a transient increase in photosensitivity. Such side effects are minimal or absent with much lower doses. This treatment is sometimes combined with repeated phlebotomy (108). Prospective study comparing these two forms of therapy is lacking.

### 99.4.6 Hereditary Coproporphyria

Hereditary coproporphyria (HCP) is an acute hepatic porphyria and results from the deficient activity of COPRO-oxidase, the sixth enzyme in the heme biosynthetic pathway. HCP is inherited as an autosomal dominant trait due to COPRO-oxidase mutations, whose clinical expression is influenced by ecogenic and metabolic factors. This condition is less frequent than AIP or VP (40). Clinical symptoms rarely occur before puberty and are very similar to AIP with the exception of photosensitivity, which can develop in HCP patients but much more rarely than in VP (1). Certain mutations in the COPRO-oxidase gene, such as K404E, when present in either the homozygous or compound heterozygous states produce a biochemical variant called harderoporphyria, characterized by increased fecal levels of a tricarboxylic porphyrin, harderoporphyrin (41,129). Cases of homozygous HCP have also been reported (42,43).

**99.4.6.1 Biochemical Aspects.** The activity of the mitochondrial enzyme COPRO-oxidase is about 50% of normal in cultured fibroblasts, circulating lymphocytes, and leukocytes from HCP heterozygotes. In one case of homozygous HCP, the residual COPRO-oxidase activity (approximately 2% of normal) had a normal  $K_m$  value. By contrast, in three cases in a family with harderoporphyria, the mutant enzyme exhibited increased thermostability and reduced affinity for both harderoporphyrinogen and coproporphyrinogen III, which is consistent with a structurally altered enzyme.

**99.4.6.2 Molecular Aspects.** Over 40 mutations in the COPRO-oxidase gene are listed in the Human Gene Mutation Database, including missense, nonsense, splice-site, and small insertion or deletion mutations (Human Gene Mutation Database; [www.hgmd.org](http://www.hgmd.org)) (6,130). Most have been identified in only one family (40). In a study of 17 unrelated British HCP patients, only one mutation, 1277G→A, which causes exon 6 skipping was found in more than one patient (131). No genotype–phenotype correlations have been observed in HCP (131). Five single nucleotide polymorphisms were identified in the COPRO-oxidase gene, but none appear to play a major role in clinical expression of HCP (132).

Studies of the human COPRO-oxidase crystal structure and a hydrophobic cluster analysis method indicated that only missense mutations in amino acids positions 400–404 in exon 6 resulted in harderoporphyria. In fact, mutation K404E is found either homoallelic or heteroallelic in all patients with harderoporphyria studied, to date. The amino acids in this region appear to be involved in retaining harderoporphyrinogen for the second decarboxylation step whereas mutations in these amino acids lead to the release of the porphyrinogen intermediate (39,41).

**99.4.6.3 Clinical Manifestations.** The neurovisceral symptoms of HCP are identical to those of AIP; however, the disease is probably less severe than AIP, and only a few patients have been reported to die from respiratory paralysis. Photosensitivity similar to that in PCT and VP sometimes occurs. In one series of 50 patients, the most common clinical manifestations were abdominal pain (80%), vomiting (34%), skin lesions (29%), neuropathic involvement (23%), psychiatric symptoms (23%), and constipation (20%) (40). In a study of 53 German HCP patients during acute manifestations, 89% had abdominal pain, 33% had neurologic symptoms, 28% had psychiatric symptoms, and 25% had cardiovascular symptoms, while only skin photosensitivity was observed in only 5% (114). In 69% of these patients, the abdominal pain occurred without other neurologic manifestations, while 27% of the patients had both abdominal and other neurologic symptoms; 4% had only neurologic symptoms (114). HCP can be exacerbated by many of the same factors that cause attacks in AIP, including drugs such as barbiturates and endogenous or exogenous steroid hormones. The disease is latent before puberty, and symptoms are more common in adult women than in men. Hepatitis and other superimposed liver diseases in an HCP patient can increase porphyrin retention and photosensitivity.

Patients with homozygous HCP, including some with harderoporphyria, developed symptoms in early childhood. Manifestations have included jaundice, severe hemolytic anemia with splenomegaly, and compensatory hyperactive bone marrow (43,133).

**99.4.6.4 Laboratory Evaluation.** A common and characteristic biochemical change in HCP is a large, isolated increase in fecal coproporphyrin, predominantly isomer III (40). Feces of symptomatic heterozygotes have a 10-fold to 200-fold increase in coproporphyrins and little or no increase in protoporphyrin. An increase in the ratio of coproporphyrin III to coproporphyrin I in feces is useful for diagnosis of both active and latent HCP. Increased urinary excretion of ALA, PBG, and total porphyrins (mostly uroporphyrin III and coproporphyrin III) is observed during acute attacks (114).

With resolution of symptoms, ALA and PBG levels revert to normal more readily in HCP (and VP) than in AIP, and HCP patients in nonacute stages may show only increases of urinary and fecal porphyrins. For



example, in a series of 53 German HCP patients, the average total urinary porphyrins during acute phases was 29,905 nmol/24 hr (6 patients) and around 1100 nmol/24 hr during subclinical and latent phases (average of 47 patients); normal levels were <224 nmol/24 hr (113). Total fecal porphyrins were 5508 nmol/g dry weight at active stages of HCP, compared with 1730 and 694 nmol/g dry weight (normal <224 nmol/g dry weight) in subclinical and latent phases, respectively. However, the percentage of the fecal coproporphyrin III isomer was still elevated in the patients in the subclinical and latent phases (114).

Porphyrin excretion patterns in homozygous HCP resemble those observed in heterozygotes but reflect a more profound enzymatic deficiency. Harderoporphyria is characterized by a marked increase in fecal excretion of harderoporphyrin as well as coproporphyrin. In one patient with harderoporphyria, 90% of the porphyrins in his feces were in the form of harderoporphyrin (129).

COPRO-oxidase activity can be measured in mononuclear cells (134). Individuals with a mutation in one of their COPRO-oxidase alleles have about 50% of normal activity in mononuclear cells. For screening family members, measurement of fecal porphyrins may be useful, especially if the ratio of coproporphyrin isomers I and III is determined; however, asymptomatic adults and children with the enzymatic deficiency may not excrete excess porphyrins.

**99.4.6.5 Therapy.** Acute attacks of HCP are treated in the same manner as in AIP (2). Hemin (lyophilized hydroxyheme or heme arginate) therapy is helpful in treating acute attacks of HCP (40). Cholestyramine may be of some value for photosensitivity occurring with liver dysfunction, but phlebotomy and chloroquine are not effective.

## 99.4.7 Variegate Porphyrria

VP is an autosomal dominant hepatic porphyria resulting from the deficient activity of PROTO-oxidase, the seventh enzyme in the heme biosynthetic pathway. The disorder is described as variegate because it can present with neurologic manifestations, photosensitivity, or both. The clinical penetrance of VP in adults is estimated at ~40% (135–137). In most countries, VP is less common than AIP, with the notable exception of South Africa, where 3 of every 1000 white persons have inherited VP. This high prevalence is due to a founder effect from a Dutch couple who immigrated to South Africa in 1688, one of whom carried a specific PROTO-oxidase mutation (133). A number of cases of homozygous dominant VP have also been described (49,50,138–141).

**99.4.7.1 Biochemical Aspects.** PROTO-oxidase activity is approximately half-normal in cultured skin fibroblasts and lymphocytes from VP patients. Because PROTO-oxidase is a mitochondrial enzyme, it is not present in mature erythrocytes. Assays for

PROTO-oxidase in lymphocytes or cultured cells are difficult and are not widely available for diagnosis and family screening (136).

Protoporphyrinogen IX, the substrate for PROTO-oxidase, accumulates in patients with VP and undergoes auto-oxidation to protoporphyrin IX, which is characteristically increased in VP. A close functional association between PROTO-oxidase in the inner mitochondrial membrane and COPRO-oxidase in the intermembrane space may relate to the excess excretion of both protoporphyrin IX and coproporphyrin III in this disease.

**99.4.7.2 Molecular Aspects.** Around 150 mutations in the PROTO-oxidase gene have been identified in patients with VP including missense, nonsense, splice site, and small deletions and insertions (Human Gene Mutation Database; [www.hgmd.org](http://www.hgmd.org)) (6,136). The missense mutation, R59W, is the common mutation in most South Africans with VP of Dutch descent (133). In 108 unrelated English and French VP patients, 66 mutations were identified; most were found in only one or two unrelated families, but five (L15F, E198X, L295P, 1082insC, and Q435X) had frequencies of 7% to 12% (142). No genotype/phenotype correlations were identified. In 21 Finnish VP families, the common missense mutation R152C was identified in 11 (52%), while the missense mutation I12T was found in two large families (9.5%) (137). Of interest, none of the patients with the I12T mutation had photosensitivity, only one had an acute attack, and all had lower levels of porphyrins than patients with mutation R152C (137). Mutations have also been identified in patients with the rarer homozygous dominant form of VP (48,49,138–141). Most have some residual enzymatic activity, allowing for synthesis of heme in amounts sufficient for many essential hemoproteins.

**99.4.7.3 Clinical Manifestations.** Clinical expression of VP before puberty is rare; the disease may even present late in life (143). VP can present with skin photosensitivity, acute neurovisceral crises, or both (47). In two large studies of VP patients from Europe and South Africa, 59% had only skin lesions, 20% had only acute attacks, and 22% had both (142). Among Finnish VP patients, the frequency of skin symptoms was 40% and acute attacks 27% (137). Interestingly, the proportion of Finnish patients with acute attacks decreased from 38% to 14% among patients diagnosed before and after 1980, while the proportion with skin symptoms remained similar. The reasons for the decline in symptoms are unclear (137). A similar decline in the frequency of acute attacks, from 38% before 1980 to 4% in 2004, was observed among South African VP patients, presumably due to increased detection and counseling of heterozygotes to avoid use of acute attack-inducing drugs, steroids, and dieting (135).

The neurovisceral manifestations of abdominal pain, vomiting, constipation, hypertension and tachycardia, and peripheral neuropathy are indistinguishable from those of AIP and HCP. Skin manifestations are very

similar to those of PCT and HCP, are usually of longer duration, and may occur apart from the neurovisceral symptoms. Photosensitivity is more common than in HCP. Drugs, steroids, and nutritional factors that are detrimental in AIP can also provoke exacerbations of VP (1,2). Photosensitivity may be less commonly associated with VP in more northern countries, where sunlight is less intense (136).

Patients with homozygous dominant VP have the early onset of symptoms, severe photosensitivity, absence of acute attacks, and elevated erythrocyte zinc protoporphyrin levels (49). In some cases, neurologic symptoms and developmental disturbances, including growth retardation, were noted in infancy or childhood. One homozygous VP patient had severe photosensitivity and mild sensory neuropathy and over a 20-year period developed IgA nephropathy (48). The heterozygous parents of these patients had approximately half-normal enzyme activity as expected.

**99.4.7.4 Laboratory Evaluation.** Fecal protoporphyrin and coproporphyrin and urinary coproporphyrin are markedly increased in clinically expressed VP. Urinary and fecal coproporphyrin is mostly type III. Urinary ALA, PBG, and uroporphyrins are increased during acute attacks but may be normal or only slightly increased during remission. ALA and PBG may be less elevated and return to normal more rapidly in VP and HCP than in AIP. Plasma porphyrins, consisting in part of a dicarboxylate porphyrin tightly bound to plasma proteins, are increased in VP particularly when photosensitivity or other symptoms are present. The fluorescence emission spectrum of plasma porphyrins at neutral pH in VP is characteristic and can distinguish this disease from other types of porphyria, especially PCT: the emission maxima occurs at 626 nm in VP; 619 nm in PCT, CEP, HCP, and AIP; and 634 nm in EPP (63). This method of measuring plasma porphyrins is perhaps the most sensitive method of detecting latent cases of VP, other than measuring PROTO-oxidase in lymphocytes or detecting a specific mutation by molecular methods. VP can be distinguished from HCP by fecal and plasma porphyrin analyses.

Assays for PROTO-oxidase are difficult, since the enzyme is not present in erythrocytes and are not widely available for diagnostic and family screening (63). Mutation analysis is preferred for confirming the diagnosis and detecting heterozygous relatives.

**99.4.7.5 Therapy.** Glucose, hemin, and other measures employed in AIP are recommended for the treatment of acute attacks of VP (2,47,136). Other therapies such as propranolol, D-penicillamine, hemodialysis, alkalization of urine, and b-carotene are of little or no benefit. Repeated venesections and chloroquine are not effective for skin manifestations in VP, even though these appear identical to those of PCT. Measures to protect the skin from sunlight with appropriate clothing and opaque sunscreen preparations are useful. Exposure to short-wavelength UV light, which does not excite

porphyrins, may provide some protection by increasing skin pigmentation.

### 99.4.8 Erythropoietic Protoporphyria

Erythropoietic protoporphyria (EPP) is due to the partially deficient activity of ferrochelatase, the last enzyme in the heme biosynthetic pathway (1,57). EPP also has been termed erythrohepatic protoporphyria and protoporphyria. EPP is the most common erythropoietic porphyria and, after PCT and AIP, is the third most common porphyria. EPP is an autosomal dominant disease in most affected families. However, many EPP patients had only 10% to 30% of normal ferrochelatase activity instead of the expected 50% of normal activity. Recently, it was shown that an intronic polymorphism in the wild-type allele reduces its expression, accounting for the lower level of enzymatic activity. EPP is therefore an autosomal dominant porphyria in which a low-expression polymorphism predisposes to clinical expression (penetrance) (134). The presence of the intronic polymorphism and the nature of the other mutation (e.g. null vs. missense) may be responsible in part for the penetrance and variable expressivity of this disease. In some families, the pattern of inheritance is autosomal recessive, with affected individuals inheriting a coding region mutation from each parent (144). Although the disease is most common in whites, it does occur in persons of other races, including blacks. The low expression polymorphism is found in ~10% of the normal white population and is also common in Asians but is rare in Africans (58). Recently, deletions in exon 11 of the ALAS2 gene have been described, which cause an X-linked protoporphyria (XLP) which is clinically indistinguishable from EPP. The deletion of the c-terminal amino acids of ALAS2 results in increased ALAS2 activity and the accumulation of protoporphyrin (145). XLP accounts for approximately 2% of cases with the EPP phenotype (146).

**99.4.8.1 Biochemical Aspects.** Partially deficient ferrochelatase has been documented in bone marrow, reticulocytes, liver, cultured fibroblasts, and blood or leukocytes from patients with EPP (1). The deficient enzyme activity becomes rate-limiting for protoporphyrin conversion to heme primarily in bone marrow reticulocytes. Ferrochelatase activity in tissue lysates of EPP patients has been reported to be as low as 10% to 30% of normal, which is much less than the 50% of normal activity that would be expected if EPP were inherited as an autosomal dominant enzymopathy. In a study of French and Swiss EPP patients, ferrochelatase activity ranged from 15% to 50% of normal (147). In a recently described variant form of EPP, ferrochelatase activity was normal, and an abnormality in iron delivery to the normal enzyme was postulated (148).

**99.4.8.2 Molecular Aspects.** To date, over 100 mutations in the ferrochelatase gene have been identified that cause EPP (Human Gene Mutation Database;

[www.hgmd.org](http://www.hgmd.org)) (6,147) including nonsense, missense, splice-site mutations, nonsense, and insertions/deletions or rearrangement mutations. Based on the type of mutation, about 75% of lesions result in an unstable or absent protein (null alleles). It has been suggested that there are genotype–phenotype correlations between mutations that result in unstable proteins (null) and liver complications in EPP patients. A study of 112 EPP patients with known ferrochelatase mutations found that all 18 EPP patients with severe liver complications had “null” alleles, while none of the 20 patients who had missense mutations developed liver complications (149). Another study of 31 EPP patients found that all 15 EPP patients with liver disease had null mutations (splicing, nonsense, or frameshift mutations), while 50% of the 16 patients who had only photosensitivity had missense mutations (150,151).

Mutation analysis of 105 English EPP patients identified three typical EPP patients with homozygous EPP, having two different mutations and one who was homoallelic for a missense mutation (152). The phenotypes of these homozygous EPP patients were very similar to EPP patients with one disabling mutation and the low expression intron 3 polymorphism, but their low ferrochelatase activity increased their risk for liver disease (152). In XLP, the erythrocyte protoporphyrin levels appear to be higher than other forms of EPP, and the proportions of free and zinc protoporphyrins are approximately equal.

**99.4.8.3 Genetic Aspects.** As noted previously, many EPP patients have about 20% to 30% of normal ferrochelatase activity rather than the 50% expected if the EPP was inherited as a simple autosomal dominant trait. Recent studies have shown that the presence of a polymorphism in intron 3 of the normal ferrochelatase allele affects the level of ferrochelatase activity and the severity of EPP. When the normal allele has a C at position -48 of intron 3, a cryptic acceptor splice site is activated, resulting in the insertion of 63 bp of the intron into the coding sequence (134). The IVS3-48C mRNA transcript contains a new stop codon and is rapidly degraded. The IVS3-48C polymorphism was present in ~10% of normal European individuals, 43% of normal Japanese, 31% of normal southeast Asians, and <1% of normal black West Africans (58,134,152). Transfection studies showed that the IVS3-48C construct produced ~40% aberrantly spliced mRNAs, while the common IVS3-48T construct produced only 20% aberrantly spliced mRNAs (134).

In studies of 40 unrelated European and 31 American symptomatic EPP patients, 95% (38 of 40) and 94% (29 of 31), respectively, had the IVS3-48C allele (132,150). Of note, none of 12 asymptomatic individuals with ferrochelatase mutations had the IVS3-48C allele (151). Additional studies of 113 overt French EPP patients and 61 asymptomatic carriers showed that none of the asymptomatic carriers had the IVS3-48C allele while 93 of 95 overt patients did (58). The EPP patients with the

IVS3-48C allele had lower levels of ferrochelatase activity than individuals with the IVS3-48T allele. These studies and others (150,153–155) indicate that symptomatic disease in most EPP patients is the result of a mutation in one ferrochelatase allele that alters markedly the structure, stability, and activity of the enzyme protein and a low expressing normal ferrochelatase allele, which is caused by the presence of the IVS3-48C polymorphism. Therefore, it appears that in most families the presence or absence of the IVS3-48C allele determines which individuals will be symptomatic.

In patients who do not have the IVS-48C polymorphism, it is possible that their reduced ferrochelatase activities result from the fact that the enzyme is a homodimer, and the dimerization of mutant and wild-type polypeptides may reduce the total activity. Recent studies of recombinant human wild-type and mutant ferrochelatase, expressed in *E. coli*, confirmed that ferrochelatase functions as a dimer and indicated that, for some mutations, the dimeric enzymes containing only mutant or mutant and wild-type heterodimers may be unstable or have markedly reduced activity (50,156).

Mouse models of EPP (157,158) may help further clarify the lower than expected ferrochelatase activity in EPP patients. In these models, the different genetic backgrounds or the absence of the heme-regulated eIF2a kinase (HRI) can effect the clinical severity of EPP (157,159). In XLP, to date, only two ALAS2 mutations, all deletions of 1 to 4 bases, have been described, which markedly increase ALAS2 activity (145).

**99.4.8.4 Etiology and Pathogenesis.** Porphyrins absorb light maximally at wavelengths near 400 nm (the Soret band) and enter an excited energy state that is manifested by fluorescence and, in the presence of molecular oxygen, by the formation of singlet oxygen and other oxygen species that can produce tissue damage. As might be expected, the skin is maximally sensitive to 400-nm light in EPP. Light-induced tissue damage may be accompanied by lipid peroxidation, oxidation of amino acids, and crosslinking of proteins in cell membranes. Histologic changes, predominantly in the upper dermis, may include amorphous material deposited around blood vessels and may resemble the findings in PCT. Immediate light-induced damage to capillary endothelial cells in the upper dermis has been described in EPP.

Circulating erythrocytes are an insufficient source for the excess protoporphyrin produced and excreted in EPP, and the presence of brightly fluorescent immature erythroid cells in the bone marrow of EPP patients presumably is a major source of the excess protoporphyrin (57). The liver also may be a source for some of the accumulated protoporphyrin (108); however, the relative contribution of hepatic and erythroid sources of the excess protoporphyrins is unclear. Studies in an EPP mouse model showed that bone marrow cells from a wild-type animal when transplanted into an irradiated EPP animal corrected the photosensitivity and fatal liver

disease, suggesting that the bone marrow was the major site for the excess protoporphyrins (160). In the reverse experiment, transplanting EPP bone marrow cells into irradiated normal mice resulted in protoporphyria (38% of erythrocytes contained fluorescent protoporphyrins), but not in liver disease, suggesting that the absence of ferrochelatase activity in the liver is a necessary component for development of EPP-associated liver disease (161). The normal recipients of EPP bone marrow cells also showed minimal skin photosensitivity despite high levels of plasma and erythrocyte protoporphyrins, suggesting that normal ferrochelatase activity in skin prevented photosensitivity (161).

Free protoporphyrin in EPP binds less readily to hemoglobin than does zinc protoporphyrin and diffuses more rapidly into the plasma. Moreover, UV light may cause free protoporphyrin to photodamage its hemoglobin-binding site and thus be released from the erythrocytes, even without disruption of the cell membrane. Protoporphyrin may then diffuse into the plasma, where it is bound to albumin. This light-mediated mechanism for the release of free protoporphyrin from hemoglobin in EPP may be important because binding of excess free protoporphyrin to hemoglobin is usually greater than binding to plasma proteins. Most of the protoporphyrin in erythrocytes is found in a small percentage of cells, and the rate of protoporphyrin leakage from these cells is proportional to their protoporphyrin concentration. The capacity of the liver to take up and excrete protoporphyrin into bile may also influence the flux of protoporphyrin from erythroid cells to the plasma.

In uncomplicated cases, hemolysis is uncommon or very mild. However, mild anemia with hypochromia and microcytosis or mild anemia with reticulocytosis is sometimes noted (144). Depletion of iron stores may be relatively common even in the absence of iron-deficiency anemia in EPP. Iron accumulation in erythroblasts and ring sideroblasts occur in some EPP patients.

Patients with EPP have high concentrations of protoporphyrin in bile and seem predisposed to develop gallstones that are fluorescent and composed at least in part of protoporphyrin. Protoporphyrin is cholestatic when infused intravenously in rodents. The potentially life-threatening hepatic complications of EPP are often preceded by increasing levels of erythrocyte and plasma protoporphyrin, abnormal liver function tests, and marked deposition of protoporphyrin in liver cells and bile canaliculi.

**99.4.8.5 Clinical Manifestations.** A major clinical feature of EPP is cutaneous photosensitivity, which usually begins in childhood. Photosensitivity is associated with substantial elevations in erythrocyte protoporphyrin and occurs only in patients with a genotype that results in ferrochelatase activity below ~35% of normal (3,108). Protoporphyrin levels remain quite constant over time, although cutaneous symptoms are generally more troublesome in the spring and summer when

sunlight exposure is greatest. Burning, itching, erythema, and swelling are the most common symptoms and can occur within minutes of sun exposure. Even brief exposure to the sun may result in intense skin pain lasting for hours, which has been described like “having a lighted match held against the skin or hot needles stuck into it” (108). Edema of the skin may be diffuse and resemble angioneurotic edema. Burning, itching, and intense pain can occur without obvious skin damage. Vesicles and bullae are absent or sparse. Epidermal intracellular vacuoles and interstitial edema are seen in fresh lesions, accompanied by acute inflammatory changes and extravasated red cells (57). Some residual scarring from vesicles or severe swelling may occur, but this is rarely severe or deforming. Pigment changes, friability, and hirsutism also are not characteristic of EPP. Thus, the cutaneous features of this disease are distinct from those of other cutaneous porphyrias. Also, in contrast to CEP, there is no discoloration or fluorescence of the teeth. It is also notable that neuropathic manifestations are not found in EPP, except rarely with advanced liver failure.

Although this is an erythropoietic porphyria, the hepatic complications that develop in a small percentage of patients (probably less than 5%) are most life threatening. Liver function is usually normal in this disease, but up to 20% of patients may have minor abnormalities of liver function. In XLP, about 17% of patients had overt liver disease, suggesting that the risk of liver disease may be higher with XLP than classic EPP (145). Rapidly progressive liver disease appears to be related to the cholestatic effects of protoporphyrin and is associated with increasing protoporphyrin levels due to impaired hepatobiliary excretion and increased photosensitivity (144). Splenic enlargement and hemolysis may be accompanying features. Upper abdominal pain may suggest biliary obstruction (57). Concurrent factors impairing liver function or the metabolism of protoporphyrin to heme, such as viral hepatitis, alcohol, iron deficiency, and fasting or oral contraceptive steroids, have played a role in some patients. An enterohepatic circulation of protoporphyrin may favor its retention in the liver, especially when liver function is impaired. Liver biopsies of EPP patients with severe liver disease contain dark brown pigment with a typical birefringence under polarized light. This pigment is protoporphyrin, which has been deposited in hepatocytes, macrophages, bile canaliculi, and small bile ducts. For a detailed discussion of the hepatic complications of EPP, see Cox (144) and Thunell and coworkers (162).

**99.4.8.6 Clinical and Laboratory Evaluation.** EPP should be suspected in individuals with intense skin pain after short exposure to the sun without blistering skin lesions (144). A lack of severe cutaneous signs distinguishes this disease from all other cutaneous porphyrias. Protoporphyrin concentrations are increased in the bone marrow, circulating erythrocytes, plasma, bile, and feces of EPP patients.



A substantial increase in erythrocyte protoporphyrin concentration, which is a readily obtained measurement, is essential for diagnosis of EPP. Erythrocytes also exhibit red fluorescence when studied by fluorescence microscopy at 620nm (163). However, an increased erythrocyte protoporphyrin concentration is not specific to EPP. Erythrocyte protoporphyrin concentrations are increased in other conditions such as lead poisoning, iron deficiency, anemia of chronic disease, and various hemolytic disorders, and also in all homozygous forms of porphyria and sometimes in acute porphyrias. However, the increased protoporphyrin in conditions other than EPP is in the form of zinc protoporphyrin, whereas in EPP it is free protoporphyrin (not complexed with zinc). Many assays for erythrocyte protoporphyrin or “free erythrocyte protoporphyrin” measure both the zinc-chelated and the free protoporphyrin. Free protoporphyrin is distinguished from zinc protoporphyrin by ethanol extraction or HPLC (144). In XLP, both free and zinc protoporphyrins are increased in equal proportions.

Plasma porphyrins may be less increased in EPP than in other porphyrias with cutaneous manifestations (1). Other heme pathway intermediates do not accumulate in EPP. Thus, urinary porphyrin and porphyrin precursor concentrations are generally normal. Fecal total porphyrins may be normal or somewhat elevated. Life-threatening hepatic complications of EPP are commonly preceded by increased photosensitivity and by increasing erythrocyte and plasma protoporphyrin levels. The ratio of erythrocyte to fecal protoporphyrin and the ratio of biliary protoporphyrin to biliary bile acids may also be observed to increase as liver failure develops. Ferrochelatase is a mitochondrial enzyme and can be measured in lymphocytes isolated from peripheral blood. As mentioned previously, EPP patients may have only around 20% to 30% of normal activity. Mutation analysis can be used to identify mutations and polymorphisms in the ferrochelatase gene.

A high prevalence of Vitamin D deficiency has been observed in EPP patients (164) and measurement of Vitamin D 25 OH levels would be recommended for monitoring these patients.

**99.4.8.7 Treatment.** Photosensitivity is managed by avoiding excessive sunlight and long-wave UV light. b-carotene was developed as a drug for treating EPP (162) and may improve tolerance to sunlight. Doses of 120 to 180mg daily in adults are usually required to maintain serum carotene levels in the recommended range of 600–800mg/dL. Improvement is noted 1–3 months after the initiation of treatment. With pure preparations of b-carotene, no side effects other than a mild and dose-related skin discoloration due to carotenemia have been noted. The mechanism of action may involve quenching of singlet oxygen or free radicals. The drug appears less effective in other forms of porphyria associated with photosensitivity, such as CEP and PCT. Dihydroxyacetone and lawsone (naphthoquinone),

which darken the skin when applied topically, partially block exposure of the dermis to light and are of some benefit in EPP. Cholestyramine, which may interrupt the enterohepatic circulation of protoporphyrin and promote its fecal excretion, has been reported to reduce liver protoporphyrin and improve cutaneous symptoms in some EPP patients. Increasing skin pigmentation by exposure to short-wave UV light may also offer some protection. Caloric restriction, drugs and hormone preparations that exacerbate acute porphyrias are often avoided in EPP, and iron deficiency should be corrected if present. Vitamin D should be supplemented if deficient.

Treatment of hepatic complications is difficult and must be individualized. Cholestyramine and other porphyrin absorbents, such as activated charcoal, should be considered. Oral bile acid supplementation has shown benefit in some animal models but has been little studied in EPP patients. Resolution of hepatic complications may also occur spontaneously, especially if another reversible cause of liver dysfunction, such as viral hepatitis or alcohol, is contributing. Splenectomy may be beneficial when EPP is complicated by hemolysis and splenomegaly. Other therapeutic options include transfusions and intravenous hemin to suppress erythroid and hepatic protoporphyrin production, as well as liver transplantation. However, liver disease may recur after transplantation (165).

### 99.4.9 Dual Porphyrrias

Patients with deficiencies of more than one heme biosynthetic enzyme are classified as having dual porphyria. For example, kindreds with individuals having both VP and familial PCT have been described. Patients with deficiencies of both HMB-synthase and URO-decarboxylase may develop symptoms of AIP, PCT, or both. COPRO-oxidase deficiency inherited from one parent and URO-synthase deficiency from both parents was found to cause severe porphyria in an infant. Coexistence of URO-synthase and URO-decarboxylase deficiencies has been described in a patient with features of an erythropoietic porphyria (166). Mutation analysis was not performed on these patients to confirm that the patient actually has mutations in two different genes. This is important, as shown by studies of a patient initially thought to have both VP and AIP but who was found to have a PROTO-oxidase mutation but no HMB-synthase mutation (167). A patient with both sporadic PCT and HCP due to an inherited COPRO-oxidase mutation was identified based on the urinary porphyrin pattern (168). Recently, ALA-dehydratase and COPRO-oxidase gene mutations were confirmed in one patient (169), and HMB-synthase and URO-decarboxylase gene mutations in another (170), as predicted by biochemical findings. These are the first two cases of dual porphyria where mutations in two different genes have been identified.

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## CHAPTERS TO CROSS-REFERENCE

2. Medicine in a Genetic Context; 3. Nature and Frequency of Genetic Disease; 7. Mutations in Human Disease: Nature and Consequences; 8. Mendelian Inheritance; 14. Pathogenetics of Disease; 23. Diagnostic Molecular Genetics; 24. Heterozygote Testing and Carrier Screening; 29. Gene Therapy.

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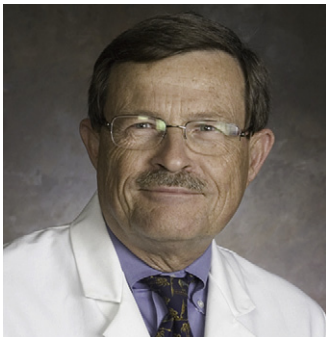
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# CHAPTER

# 100

## Inherited Disorders of Human Copper Metabolism

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### 100.1 INTRODUCTION

Copper is an essential dietary component, required in small amounts (1.5–3.0 mg/day) and distributed throughout the body (1–5). While the highest concentrations are in the liver, brain, heart, and kidneys, about 50% of total body copper is harbored in the large mass of muscle and bone. In humans, 30–60% of ingested copper is absorbed in the stomach and small intestine. The metal is then transported from the intestine to the blood, where copper binds to albumin at a specific site or to amino acids and is delivered to all tissues including liver. In the liver, copper is incorporated into the iron oxidase, ceruloplasmin, which is synthesized and metallated in the liver, and exported into plasma. Also in the liver, copper is excreted into the bile as amino acid and bile acid complexes, by way of a high-capacity excretory pathway in hepatocytes. This final sequence represents the major excretory pathway for copper, and the metal so processed is not available for resabsorption.

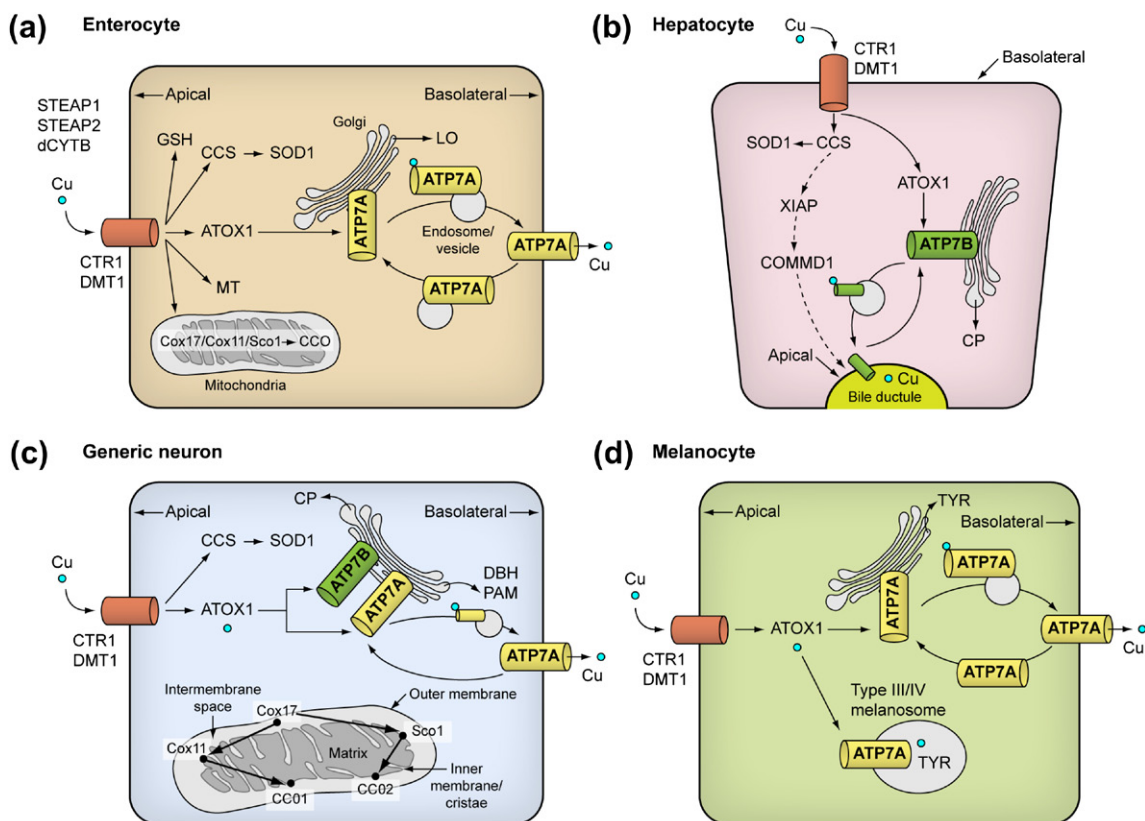
Copper transport and utilization in mammalian cells can be divided into three distinct and interrelated steps: copper uptake, copper excretion, and intracellular copper distribution and utilization (6,7). Intestinal absorption of copper across the apical membranes of enterocytes is mediated by the copper uptake proteins copper transporter 1 (CTR1) and divalent metal transporter 1 (DMT1) possibly in concert with the metalloredutases STEAP1, STEAP2, and dCYTB (Figure 100-1a). The roles of CTR2 (not shown) and DMT1 in this process are less certain. Within enterocytes, glutathione (GSH) and small proteins called metallothioneins (MTs) function in copper sequestration and storage. The copper chaperones CCS, ATOX1, Cox17, Cox11, and Sco1 ferry copper to specific proteins or organelles. As intracellular copper levels increase, ATP7A,

a copper-transporting ATPase, trafficks from the trans-Golgi network to the basolateral cell membrane and pumps copper into the bloodstream, entering the portal circulation.

Upon reaching the liver, the major organ of copper homeostasis, copper entering hepatocytes is directed toward one of several destinations (Figure 100-1b). Copper may be stored within the hepatocytes (bound to MTs), secreted back into the circulation (bound to ceruloplasmin), or excreted into the bile. The latter process is mediated by ATP7B, a copper transporter closely related to ATP7A, and may require interaction with the protein COMMD1. In neurons (Figure 100-1c) and other specialized cells (e.g. melanocytes, Figure 100-1d), other cuproenzymes (DBH, PAM, TYR) are efficiently metallated by the copper ATPase.

Since 90–95% of total plasma copper is bound to ceruloplasmin, and copper does not readily dissociate from ceruloplasmin, it was once hypothesized that copper entered peripheral cells following a ceruloplasmin-receptor interaction at cell surfaces. Since plasma copper also binds to albumin, alpha-2-macroglobulin, and amino acids such as histidine, these complexes were considered to be additional mediators of copper delivery to peripheral cells (8,9). However, individuals with the genetic disorders aceruloplasminemia (10), and analbuminemia (11), have normal copper status, indicating that neither ceruloplasmin nor albumin is essential for delivery of copper to mammalian cells. Table 100-1 provides detailed descriptions of proteins involved in human copper metabolism.

In all polarized cells, ATP7A and ATP7B reside in the *trans*-Golgi network and transport cytoplasmic copper to this compartment for incorporation into copper enzymes but relocate toward the plasma membrane to



**FIGURE 100-1** Cellular copper metabolism. (a) In enterocytes, copper (Cu<sup>+</sup>) uptake is mediated by CTR1, possibly in concert with the metalloregulators STEAP1, STEAP2 and dCYTB. The roles of CTR2 (not shown) and DMT1 in this process are less certain. Within enterocytes, GSH and MT function in copper sequestration and storage. The chaperones CCS, ATOX1, Cox17, Cox11 and Sco1 ferry copper to specific proteins or organelles. With an increase in copper levels, ATP7A traffics to the basolateral surface and pumps copper into the blood. (b) In hepatocytes, ATOX1 provides copper to ATP7B for metallation of CP and traffics to the apical membrane to pump copper into the bile, the body's major mechanism for copper removal. CCS has been proposed to deliver copper to XIAP, which may interact with COMMD1, a protein mutated in hepatic copper toxicosis of Bedlington terriers and which may modulate ATP7B activity. This putative pathway is denoted by dashed lines. (c) In neuronal cells, both ATP7A and ATP7B are expressed and required for maturation of CP, DBH and PAM. Details of CCO metallation are illustrated here. (d) In melanocytes, TYR acquires copper within melanosomes via ATP7A, which also localizes to the trans-Golgi. Abbreviations: ATOX1, copper transport protein ATOX1; ATP7A, copper-transporting ATPase 1; ATP7B, copper-transporting ATPase 2; CCS, copper chaperone for SOD1; CCO, cytochrome c oxidase; COMMD1, COMM domain-containing protein 1; COX11, cytochrome c oxidase assembly protein Cox11; Cox17, cytochrome c oxidase copper chaperone; CP, ceruloplasmin; CTR, copper transporter; DBH, dopamine-β-hydroxylase; dCYTB, cytochrome b reductase 1; DMT1, divalent metal transporter 1; GSH, glutathione; LO, lysyl oxidase; MT, metallothionein; PAM, peptidylglycine α-amidating monooxygenase; Sco1, protein SCO1 homolog; SOD1, superoxide dismutase; STEAP, six-transmembrane epithelial antigen of prostate; TYR, tyrosinase; XIAP, X-linked inhibitor of apoptosis.

mediate an exodus of copper from the cell in response to an increase in the intracellular concentration of this metal (12). ATP7A typically traffics to the basolateral membrane, while ATP7B moves toward the apical surface (13–16). The molecular domains responsible for the intracellular relocation and trafficking of ATP7A, along with their effects, are summarized in Table 100-2.

One might predict that mutations causing deficient function of copper chaperones would lead to mitochondrialopathies or to neurodevelopmental abnormalities with clinical and chemical evidence of copper deficiency. Mutations in the copper chaperone genes have not been associated with heritable disorders in humans, except for a single patient of consanguineous parentage homozygous for a loss-of-function missense mutation in CCS, associated with a complex clinical and biochemical phenotype (17).

Enzymes that utilize copper as a cofactor in functional catalytic reactions include the following: cytochrome c

oxidase (mitochondrial respiratory chain); superoxide dismutase-1 (free radical eradication); lysyl oxidase (rate-limiting collagen and elastin crosslinking reaction steps); tyrosinase (melanin formation); ceruloplasmin (iron oxidase, iron homeostasis); dopamine-β-hydroxylase (synthesis of catecholamines, neurologic development); and peptidylglycine α-amidating monooxygenase, responsible for chemical modifications of a significant fraction of neuropeptides (Table 100-1).

It should be clear from the foregoing that copper is an essential nutritional requirement for growth and development. However, at both organismic and cellular levels, extensive regulatory mechanisms exist to maintain a delicate balance (18) between necessity and toxicity. In a large measure, these homeostatic regulatory mechanisms are needed because the oxidative properties of copper are utilized in copper-dependent functions. Failure to maintain such a delicate balance can occur in

**TABLE 100-1 Proteins Important in Human Copper Metabolism**

Category	Name	Description
Copper import	CTR1	High-affinity copper uptake; localization at apical and basolateral plasma membranes and intracellular vesicles
	CTR2	Proposed low-affinity copper transporter; lacks methionine-rich domains present in CTR1
	DMT1	Facilitates divalent metal ( $\text{Fe}^{2+}$ , $\text{Cd}^{2+}$ , $\text{Co}^{2+}$ , $\text{Cu}^{2+}$ , $\text{Ni}^{2+}$ , $\text{Mn}^{2+}$ , $\text{Pb}^{2+}$ , $\text{Zn}^{2+}$ ) uptake via proton exchange
	STEAP	Reduces cupric ( $\text{Cu}^{2+}$ ) and ferric ( $\text{Fe}^{3+}$ ) ions in conjunction with cellular metal uptake
Copper export	ATP7A	P-type copper ATPase; mutated in Menkes disease, OHS and a distal motor neuropathy
	ATP7B	P-type copper ATPase highly expressed in hepatocytes; mutated in Wilson disease
	COMMD1	Required for hepatic copper excretion (is mutated in Bedlington terrier copper toxicosis); physically interacts with ATP7B
Copper chaperone	ATOX1	Cytosolic copper chaperone that ferries $\text{Cu}^+$ to ATP7A and ATP7B
	CCS	Cytosolic copper chaperone that ferries $\text{Cu}^+$ to SOD1 and XIAP
	COX17	Mitochondrial copper chaperone responsible for supplying copper ions, through the assistance of SCO1 or SCO2 and COX11, to cytochrome c oxidase, the terminal enzyme of the mitochondrial energy producing respiratory chain
	COX11	Accessory factor required to mediate copper transfer from COX17 to the $\text{Cu}_\text{B}$ site of cytochrome c oxidase subunit 1
	SCO1	Receives copper in transfer from COX17 en route to metallation of the $\text{Cu}_\text{A}$ site of CCO subunit 2
	SCO2	Partners with SCO1 in mammalian cells for delivery of copper to CCO
Storage and transfer	ALB	Albumin; a protein abundant in serum and cerebrospinal fluid; binds copper via a four-amino acid (DAHK) motif in amino-terminal region
	A2M	$\alpha_2$ -Macroglobulin or transcuprein; a major serum macroglobulin that also binds copper
	GSH	Glutathione; a sulfhydryl-containing tripeptide—abundant in the reducing environment of the cytosol—that binds and retains copper in the $1^+$ valence state
	MT	Metallothionein; a cysteine-rich metal-binding protein that sequesters excess intracellular copper
Copper enzyme	CP	Ceruloplasmin; a copper-containing serum ferroxidase needed to load ferric iron into transferrin
	HEPH	Hephaestin; a copper-containing transmembrane ferroxidase that exports iron from cells
Copper enzyme	SOD1	Superoxide dismutase; cytosolic antioxidant enzyme that catalyzes the conversion of superoxide to oxygen and hydrogen peroxide
	CCO	Cytochrome c oxidase; terminal enzyme of mitochondrial energy-producing respiratory chain
	DBH	Dopamine- $\beta$ -hydroxylase; key enzyme in catecholamine biosynthetic pathway; active in noradrenergic neurons
	PAM	Peptidylglycine- $\alpha$ -amidating monooxygenase; critical for amidation of numerous neuropeptides, which can enhance their bioactivity by several orders of magnitude.
	LO	Lysyl oxidase; oxidative deamination of lysine residues in collagen and elastin precursors needed for maturation of connective tissue
	ABP1	Diamine oxidase; a cuproenzyme required for degradation of histidine
	TYR	Tyrosinase; key enzyme in melanin biosynthetic pathway. Metallated in the Golgi compartment as well as in melanosomes
Regulator	XIAP	X-linked inhibitor of apoptosis; receives copper from CCS and, in turn, can regulate the metallochaperone via ubiquitination; also interacts with COMMD1; when not metallated, XIAP mediates degradation of COMMD1, reducing copper export

nutritional copper deficiency, caused by malnutrition, prolonged diarrhea in infants and children, and long-term parenteral alimentation without copper supplementation. The anemia, neutropenia, blood vessel fragility, skeletal defects, central nervous system dysfunction, and hypopigmentation of nutritional copper deficiency may be explained by functional deficiencies in the activities of the multiple cuproenzymes noted previously (19,20). If copper deficiency persists, patients may show decreased bone density and, in some cases, a peripheral neuropathy (21–28). In contrast, acquired acute copper poisoning manifests as nausea, vomiting, and diarrhea (29). Pathology includes ulceration of the intestinal mucosa, hepatic cell necrosis, jaundice, and hemoglobinuria. Toxicity of

copper excess may be ascribed to the inhibition of multiple enzyme systems, the mediation of free radical production, and the direct oxidation of cellular components (30,31).

Importantly, the symptoms of copper deficiency overlap with those of the genetic disorder, Menkes disease, in which cells and organs show a functional copper insufficiency, deficient copper utilization, or both. In contrast, the symptoms of copper poisoning mirror the manifestations of the copper overload observed in the genetic disorder, Wilson disease. Accordingly, the elucidation of the pathogenesis of Menkes disease and Wilson disease has directly impacted our understanding of the biology of mammalian copper transport and utilization.

**TABLE 100-2 ATP7A Molecular Traffic Signals and Effects**

1.	38 amino acid segment in 3rd transmembrane domain – Movement from endoplasmic reticulum to <i>trans</i> -Golgi
2.	N-terminal Cu-binding motifs – Movement from <i>trans</i> -Golgi network to plasma membrane
3.	C-terminal di-leucine motif at positions 1487–1488 – Endosomal retrieval of ATP7A from the plasma membrane
4.	C-terminal PDZ motif (DTAL) at positions 1497–1500 – Basolateral (instead of apical) localization in polarized cells
5.	CPC motif in TM6 at positions 1000–1002 – Cu-induced relocalization from post-Golgi vesicles to PM
6.	Phosphorylation motif (DKTG) at positions 1044–1047 – Cu-induced relocalization from post-Golgi vesicles to PM
7.	Phosphatase motif (LITGEA) at positions 873–878 – Endosomal retrieval of ATP7A from the plasma membrane

In turn, the resultant expansion in our knowledge of copper biology has led to an improved understanding of disease mechanisms and given investigators the tools to uncover other human genetic disorders of copper transport. In Menkes disease and Wilson disease, we have truly proceeded “from patients to gene,” and back to patients (32).

## 100.2 MENKES DISEASE

Menkes disease is an X-linked recessive neurodegenerative and connective tissue disorder, in which defective cellular copper export causes trapping of copper in some tissues (notably intestinal mucosa and kidney), leading to failure of copper delivery to other tissues (such as the central nervous system), and resultant systemic copper insufficiency. The disease was described by John Menkes nearly 50 years ago (33), and the X-linkage and syndromic nature of the disorder were noted in that early work. The disorder was subsequently shown by Danks and coworkers to involve defective copper homeostasis (34), with a copper deficiency phenotype due to failure of copper absorption from the small intestine. The disorder was later shown to be due to mutations in a gene encoding a copper-transporting P-type ATPase, ATP7A, or MNK (35–37). Subsequent experimental work showed that the MNK transporter functions in intracellular translocation and cellular efflux of copper (38–40). The identification of the X-chromosomal Menkes gene quickly led to the identification and cloning of a homologous autosomal gene, ATP7B, which was shown to be the gene for Wilson disease (41–43). Finally, the isolation of the Menkes- and Wilson disease genes led to the confirmation that mutations in homologs of the human genes are the proximate causes of the phenotypes of animal models for these disorders: the mottled mouse series for Menkes disease, and the toxic milk mouse, and LEC rat for Wilson disease (44). Experiments in these true animal analogs have considerably improved our understanding of the physiologic

role of the transporters (40,45) and the pathophysiology of the human diseases (46).

### 100.2.1 Clinical Features

Classical and lethal Menkes disease, with an estimated incidence of 1 in 90,000 to 1 in 254,000 live births (47), presents in the newborn period or in early infancy with nonspecific neurologic manifestations. Hypothermia, lethargy, poor feeding and failure to thrive, and myoclonic seizures are common (16,48). There are various degrees of spasticity, with limited spontaneous movement. At least two severe cases have been described with neonatal cutis laxa (49,50). Approximately 50% of patients show severe ocular manifestations, including poor visual acuity, myopia, strabismus, and peripheral retinal hypopigmentation (51). Development is generally severely delayed, and death occurs in early childhood.

There is a facial resemblance among patients, due in part to the distinct craniofacial configuration, and in part to the hair abnormalities. The face is described as “pudgy” or “cherubic” (Figure 100-2). Based on the steely, depigmented hair, the disorder was used to be known as “kinky hair disease” or “steely hair disease.” The hair is fragile and frequently broken; pili torti is seen on microscopic examination (Figure 100-3). Seborrheic dermatitis can be a persistent skin manifestation.

Central nervous system features include demyelination, reactive gliosis, and neuron loss in the cerebral hemispheres, the cerebellum, and the spinocerebellar tract (52). However, central nervous system manifestations in Menkes disease include not only neurodegenerative changes of a diffuse and unspecified nature but also selective defects that may be of developmental origin. These include abnormal dendritic arborization of pyramidal neurons; primary cellular degeneration in the thalamus; and reduced number and abnormal dendritic arborization of Purkinje cells (52). The developmental nature of these defects and early onset of neurological disease suggest that Menkes disease could have prenatal effects.

Radiographic studies are helpful in the diagnosis of Menkes disease and in characterization of aspects of the phenotype, especially those related to connective tissue defects. Skeletal changes include wormian bones in the lambdoidal and sagittal sutures, anterior rib flaring or cupping, and lateral or medial spur formation on the proximal and distal femoral and humeral metaphyses (53). Decreased bone density and osteoporosis can be seen after an age of 6 months. Arteriography shows tortuosity, narrowing, and dilatation of cerebral, visceral, and limb arteries, relatable to the intimal hyperplasia, and the fragmentation and beading of elastic tissue (54). Radiographic studies also reveal lobular bladder diverticuli in some patients. Gastric polyps have been noted (55), and the clinical course in at least one patient was complicated by a progressive sliding hiatal hernia, a manifestation





**FIGURE 100-2** Facial features in a child with Menkes disease. Note the jowly facial appearance and abnormal hair.

that should be considered in patients with recurrent gastrointestinal or respiratory symptoms (56). MRI of the brain shows intracranial vascular tortuosity, as well as the progressive postnatal decrease in cortical mass, ventricular dilatation, and white matter atrophy.

Patients with milder or atypical manifestations have been reported (57–60). A number of such children were, in fact, severely affected but with a longer life span than “classical” Menkes disease. Such longevity may reflect improvement in supportive care, rather than a distinct clinical variant. However, many such patients have shown a milder phenotype in general, with later onset of symptoms and a less severe clinical course. As such, Menkes disease must be considered a disorder with a spectrum of severity (55), with implications for clinical evaluative considerations in children with psychomotor retardation of unknown etiology.

A specific variant of Menkes disease, at the mildest end of the spectrum of severity, is occipital horn syndrome (OHS), previously called X-linked cutis laxa. While originally proposed as a form of Ehlers–Danlos syndrome (type IX) due to lysyl oxidase deficiency (61), subsequent documentation of abnormal copper metabolism (62) and definitive molecular genetic analyses (59,63–67) confirmed that the OHS is indeed an allelic variant of Menkes disease. The OHS is therefore a connective tissue disorder caused by a secondary deficiency of the cuproenzyme, lysyl oxidase. Hyperelastic and “bruisable” skin, bladder diverticulae, varicosities, hypermobile joints, and skeletal abnormalities including short, broad clavicles, fused carpal bones, thoracic malformations, and bony exostoses of the occiput characterize the syndrome. The OHS is sometimes accompanied by mild neurologic impairment, in contrast to the severe neurologic degeneration of Menkes disease.



**FIGURE 100-3** Phase contrast illumination showing a hair shaft (left) from a Menkes disease patient with 180-degree twisting (*pili torti*) compared with two normal hair shafts (right).

### 100.2.2 Heterozygosity for X-Linked Menkes Disease

While the natural history and variability of clinical expression in affected hemizygotes has been well studied and well documented, little is known about the phenotypic effects in heterozygotes. As with other X-linked recessive disorders, genetic counseling is often experientially based on a presumption of the benignity of heterozygosity. The emphases of the clinical geneticists are therefore placed on the prenatal or postnatal diagnosis and treatment of affected males. While some females with Menkes disease have had X chromosome abnormalities (68,69), others have not (70). It is therefore reasonable to consider that a fraction of heterozygotes may exhibit connective tissue or central nervous system manifestations in varying degrees—based on severity of mutation, skewing of lyonization (71), or other disease mechanisms. As in, for example, ornithine transcarbamylase deficiency heterozygosity, such patients may proceed through diverse medical interventions without a diagnosis or with an incorrect diagnosis. Another study of six female heterozygote relatives of three unrelated hemizygotes with different severe mutations reinforced the admonition mentioned previously (72). Importantly, all heterozygotes showed some measure of mild impairment on cognitive testing or neurologic examination, or both. Integumentary findings were observed on physical examination, and the majority of women showed subtle skeletal abnormalities on bone radiographs. Three women showed blood vessel tortuosity, or premature volume loss, or both, on brain MRI studies. Given the small numbers of well-studied heterozygotes, risk figures for clinically significant manifestations are not calculable. However, it is fair to conclude that there is a finite risk of

clinically significant manifestations in carriers of severe Menkes disease mutations. Further, while some manifestations (e.g. patchy skin findings) may be ascribed to differential lyonization, others (e.g. a spectrum of nonspecific neurologic features) may reflect the more generalized effect of a threshold- and age-based central nervous system copper deficiency (72). Such possibilities should be considered in diagnostic protocols, in genetic counseling, and in making decisions on whether treatment may be indicated for some heterozygotes.

### 100.2.3 Biochemical Pathology

In the patient with Menkes disease, oral copper is poorly absorbed, and there is abnormal distribution of body copper: low levels are found in, for example, plasma, liver, and brain, while excessive accumulation has been documented in other tissues, such as intestinal mucosa, kidney, and placenta. Measurements of cuproenzyme activities in tissues of Menkes disease patients have confirmed reduced specific activities, and it is therefore reasonable to ascribe many of the clinical manifestations to deficiencies of cuproenzymes in multiple tissues. However, studies in cell culture systems have revealed that the pathophysiology is somewhat more complex. Defective copper export is the basic cellular defect observed in cultured cells; with the exception of hepatocytes, all tested Menkes cells exhibit excessive copper accumulation (7). Kinetic studies revealed a specific defect in copper efflux, with normal uptake, and with normal transport of other trace metals in mutant cells under experimental conditions (73–75). These observations led to the hypothesis that defective copper efflux was the consequence of an abnormality in intracellular translocation of copper. This notion was later learned to be consonant with the basic genetic defect and with the cell biology of the Menkes disease transporter ATP7A. Under this construction, copper would be taken up by intestinal mucosal (or renal tubular) cells but not released across the serosal membranes, thereby causing systemic copper insufficiency, reduced cuproenzyme activities, and Menkes disease. Theoretically, treatment might be effected by the bypassing of the intestinal absorptive block. However, additional complexities may be of significance. In particular, a sufficiency of copper availability to a mutant cell can correct some cuproenzyme activities—superoxide dismutase-1 (74), tyrosinase, and cytochrome c oxidase (76)—but not others, such as lysyl oxidase (77). In the case of lysyl oxidase in Menkes mutant cells, holoenzyme formation may be reduced because of inhibition of transcription or reduction in stability of lysyl oxidase mRNA (78). While preliminary and suggestive data are in place for lysyl oxidase, similar considerations may obtain for other, heretofore uncharacterized, copper-dependent functions and may be of importance in the eventual design of effective therapy.

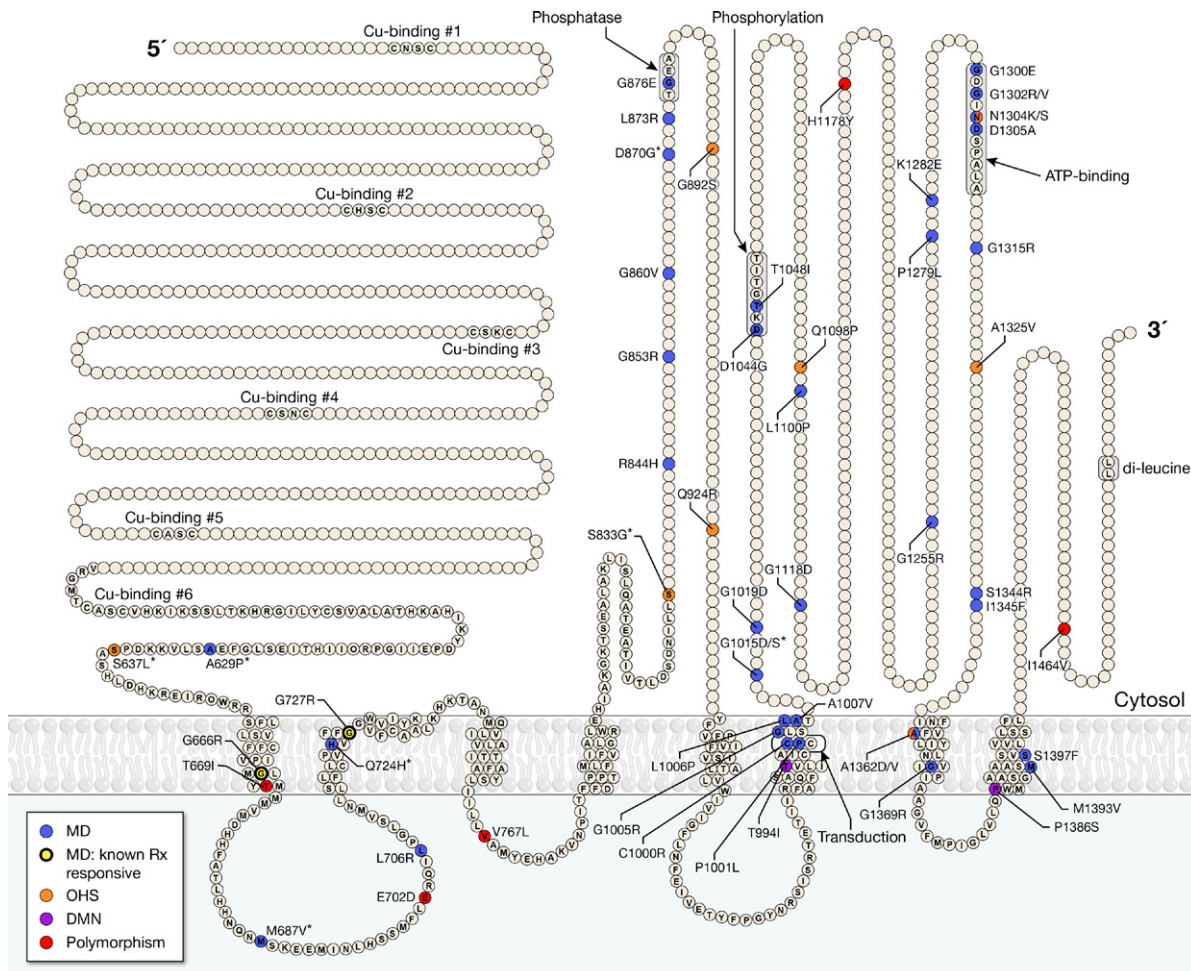
The mechanisms resulting in the developmental cerebral and cerebellar abnormalities are unknown and

under investigation. In an important heuristic approach to this question, expression profiles in Menkes brain tissue were studied using microarray analysis, leading to the observation of significant downregulation of multiple genes, involved in energy metabolism, myelination, and ribosomal function (79). An additional area of investigation is focusing on copper-dependent impairments in energy metabolism, with consequent mitochondrial damage, increased levels of cytochrome C release from mitochondria, and enhanced apoptosis leading to neurodegeneration (80,81).

### 100.2.4 Molecular Pathology

Following the cloning of the gene responsible for Menkes disease, the complete genomic structure was elucidated (82,83), identifying a gene with 23 exons over a genomic region of approximately 150 kb. The Menkes gene, designated as MNK or ATP7A, is expressed in all tissues except liver, a pattern consistent with a housekeeping role in cell copper homeostasis and with the Menkes disease phenotype. Sequence analysis predicts a 1500-amino acid protein, with the structure of a copper-transporting P-type ATPase (37) (Figure 100-4). The N-terminal domains of the protein and of the homologous Wilson disease transporter, ATP7B, have been shown to specifically bind copper (84) and may function as sensors of intracellular copper concentration (85). The MNK protein is localized in the membrane of the trans-Golgi network (39,86–88) and cycles between the trans-Golgi and plasma membrane localizations in response to basal conditions or conditions of copper excess, respectively (38,89). Structural and experimental studies have suggested that the MNK protein contains specific motifs that govern trafficking of the protein to the trans-Golgi network, and retrieval from the plasma membrane (88,89). Based on the aggregate studies, it is apparent that copper dyshomeostasis and disease can result not only from mutations that abolish the transport function of MNK but also from mutations leading to incorrect intracellular localization (40,90,91).

Diverse mutations of the MNK gene have been described in severe Menkes disease, and all such mutations would be predicted to result in severe impairment of the expression, structure, or function of the protein. Approximately, 15% of patients with severe Menkes disease have partial nonoverlapping deletions (92). Additional categories of mutations are different in each family and include nonsense, missense, and splice-site mutations, and small deletions or insertions (93,94). Analyses in patients with milder disease, and especially with the OHS, have revealed splice-site mutations in splice donor or acceptor sites that are not in the invariant dinucleotide sequences at the intron boundaries. In such instances, expression of some fraction of correctly spliced full sequence mRNA would presumably result in the synthesis of a small amount of normal MNK protein



**FIGURE 100-4** Model of ATP7A with functional domains indicated, as well as known amino acid substitutions and their phenotypic correlations. ATP7A has eight transmembrane segments and six copper-binding domains. The protein also has phosphatase, phosphorylation, transduction and ATP-binding domains. The majority of ATP7A missense mutations affect the carboxyl-terminal half of the protein. Early diagnosis and treatment is currently rare in Menkes disease; thus, little is known about the treatment responsiveness of the missense mutations associated with this phenotype. Of the mutations displayed, only two (Gly666Arg and Gly727Arg) have been evaluated in terms of early intervention, and each proved responsive to early treatment. Thus, newborn screening for Menkes disease that would detect affected infants in the first week of life is urgently needed. The locations of missense mutations that cause OHS and isolated distal motor neuropathy are also noted.

and result in the milder occipital horn cell phenotype (59,66,95–97). Cases of the OHS have also been shown to have mutations affecting mRNA splicing, in regulatory regions of the gene (64), and mutations affecting cellular localization (65,98); correlations with the milder occipital horn phenotype are more complex in these cases.

### 100.2.5 Diagnosis and Genetic Counseling

Once the diagnosis is confirmed in a proband by biochemical or molecular studies, genetic counseling proceeds as with other X-linked disorders. In the instance of a singleton case of severe Menkes disease and absent definitive data on maternal heterozygosity, conventional Bayesian calculations may be employed as for other X-linked recessive disorders with male lethality. Diagnosis in the proband may proceed on the basis of clinical, biochemical, and molecular studies, as outlined later in this chapter. Diagnosis in a proband can be confirmed

biochemically, while mutation identification is usually required for definitive documentation of heterozygosity. Rapid and reliable molecular diagnostic protocols have been developed, using sequential stepwise approaches that include multiplex PCR techniques, heteroduplex analyses, RT-PCR, and direct sequencing (99,100). These approaches are being increasingly employed in clinical molecular diagnostic laboratories, enabling physicians caring for Menkes patients and their families to provide accurate diagnosis, heterozygote detection, and prenatal diagnosis.

Serum copper and ceruloplasmin concentrations are decreased when measured beyond the first 6 weeks of life, but these may be indistinguishable from normal when measured in cord blood or during the first weeks of life. The hepatic copper content is markedly decreased, even in the immediate perinatal period (see Table 100-3). Daily urine copper excretion may be increased during the first year of life. Finally, the measurement of plasma and



**TABLE 100-3** Effects Seen in Nutritional Copper Deficiency in Humans, Sheep, Rats, Pigs and in Menkes Syndrome

Effect	Man	Sheep	Rat	Pig	Menkes Syndrome
Anemia	++	++	++	++	–
Neutropenia	+	+	+	+	–
Abnormal hair structure	±	++	+	+	++
Depigmentation	±	+	+	+	+
Arterial rupture	?	–	+	++	++
Myocardial fibrosis	?	–	+	+	–
Osteoporosis	+	±	+	++	+
Emphysema	?	–	+	–	+
Cerebellar ataxia	–	+ <sup>a</sup>	+ <sup>a</sup>	+ <sup>a</sup>	+
Other brain damage	–	+ <sup>a</sup>	+ <sup>a</sup>	+	++

<sup>a</sup>Seen only after fetal copper deficiency.

cerebrospinal fluid catecholamine levels is finding increasing utility in the diagnosis of Menkes disease. In particular, there can be found elevations of dihydroxyphenylalanine (DOPA) and dihydroxyphenylacetic acid (DOPAC), with decreased levels of specific norepinephrine metabolites, or of norepinephrine, or epinephrine themselves (101–104). Elevations in the ratios of DOPA or DOPAC to norepinephrine or derivatives thereof likely reflect dopamine-beta-hydroxylase deficiency, in the face of reduced availability of copper to the central nervous system. Dopamine-beta-hydroxylase deficiency also leads to increased urine ratios (over 4.0) of homovanillic acid/vanillylmandelic acid (HVA/VMA), and this clinical chemistry can also be used to screen patients for Menkes disease (105).

Menkes disease should be considered in any male infant with unexplained seizures, mental retardation, and hypothermia. The hair changes and radiologic findings are especially helpful differential features and might even be used in initial clinical evaluations for possible heterozygosity in female patients with unexplained neurodevelopmental presentations.

Menkes disease and OHS are expressed in cultured skin fibroblasts, and an elevated copper content can be detected by atomic absorption spectrophotometry or as the accumulation of radioactive copper (106). Most characteristically, kinetic studies reveal a markedly reduced efflux of copper in mutant fibroblasts (74). Such studies should be performed by laboratories experienced in these analyses. While the analyses may be employed in the initial assessment of heterozygosity in the presence of a positive family history, there may be as high as a 50% rate of false negativity, and the results must be interpreted with caution.

Second trimester prenatal diagnosis of male fetuses at risk has been performed by copper accumulation and kinetic studies in cultured amniotic fluid cells (106,107). First trimester prenatal diagnosis has been accomplished by measurements of copper content in chorionic villus samples (108). Because of specific sources of ambiguity in both kinds of prenatal measurements, prenatal diagnosis should be performed by a laboratory with extensive experience in tissue copper biochemistry.

Prenatal diagnosis and heterozygote detection have also been accomplished by mutation detection, in instances in which the mutation in a given family was already known or identified as part of the clinical diagnostic sequence (109,110). With respect to the genetic counseling of heterozygotes, it should be noted that there have been instances of recurrence in women who are not carriers of mutations, suggesting that germline mosaicism cannot be excluded as a mechanism in the transmission of the Menkes disease mutations (111). Finally, in practice, clinical molecular diagnosis and the availability of mutation identification may be problematic, since most ATP7A mutations appear to be unique to each family. If, in a given family, the mutation is not already known, the identification of a mutation may take some time, and clinical geneticists should be prepared to avail themselves of accurate biochemical testing protocols for genetic management.

### 100.2.6 Treatment

Postnatal postsymptomatic treatment is predicated on the bypassing of the intestinal absorptive block, by the parenteral administration of copper, usually as copper histidine, as early in the course of the disease as possible. The results of earlier treatment attempts were not encouraging. However, there have been recent reports of therapeutic amelioration of symptoms, in milder or atypical patients, or when treatment has begun very early in life (102,104,112–114). In some instances, significant improvements in neurologic status have occurred, but patients have developed some of the severe connective tissue manifestations of the OHS (112). It has been hypothesized that treatment outcome is primarily predicated on the existence of residual functioning MNK protein (102). A different hypothesis emphasizes that positive effects can be achieved, even in the face of severe mutations, if treatment is to begin very early, perhaps in a baby intentionally delivered prematurely. This latter hypothesis is consonant with the notion that some of the pathology begins in utero (115) and that parenteral copper is more likely to enter the brain prior to the maturation of exclusionary brain transport mechanisms. Given all of these uncertainties—both empiric and theoretical—it must be emphasized that treatment of Menkes disease, at this writing, is investigational; patients should therefore be referred for treatment to centers with ongoing systematic treatment protocols in place.



A combination of gene therapy with adeno-associated virus serotype 5 (AAV5), harboring a truncated (owing to AAV vector packaging limits) but functional version of ATP7A plus copper chloride, both delivered by intracerebroventricular injection, enhanced the survival of *mottled brindled* male mice in comparison with no treatment (116). *Mottled brindled* is a mouse model of Menkes disease based on mutation of *atp7a*, the murine homolog of ATP7A (see mouse models below). AAV5 transduces the choroid plexuses of the cerebral ventricles (117), and these specialized polarized epithelia appear crucial for copper transport to the CNS (118).

Finally, the medical geneticist should ensure that a patient with Menkes disease is treated by an experienced multidisciplinary medical team, including neurologists, nutritionists and gastroenterologists, pulmonologists, urologists, and social service personnel.

### 100.2.7 A Novel ATP7A-Related Phenotype

A third clinical phenotype—distal motor neuropathy without overt copper metabolic abnormalities—was recently found to be associated with mutations in ATP7A in two large families with multiple affected males (119). Distal hereditary motor neuropathies (HMNs) comprise a clinically and genetically heterogeneous group of disorders that predominantly affect motor neurons in the *peripheral nervous system* (PNS) (120). Distal HMNs have been classified into seven main subtypes on the basis of mode of inheritance, age of onset, distribution of muscle weakness, and clinical progression (121). Ten genes associated with distal HMNs have been identified to date (122–124). These genes encode a functionally diverse array of proteins, including two cation transporters (ATP7A and transient receptor potential cation channel subfamily V member 4), a transfer RNA synthetase, two heat shock proteins, and a microtubule motor protein involved in axonal transport.

ATP7A-related distal motor neuropathy is associated with unique missense mutations in ATP7A affecting amino acids within or near transmembrane segments of the protein (Figure 100-4). The resulting amino acid substitutions—Thr994Ile in transmembrane domain 6 and Pro1386Ser in the short extracellular loop between transmembrane domains 7 and 8—may contribute to the abnormal intracellular trafficking phenotype observed with these defects and may prove relevant to the underlying mechanism of this form of motor neuron disease.

### 100.2.8 Clinical Features

As in CMT, type 2 (125), the newly described ATP7A-related phenotype features progressive distal motor neuropathy with less prominent sensory loss (Table 100-4).

Symptoms begin with distal muscle weakness and atrophy of the lower extremities, followed by involvement of the upper limbs, reductions in tactile and vibratory sensation, and loss of deep tendon reflexes. Foot and hand deformities such as pes cavus (Figure 100-2c), hammer toes, and curled fingers are typical (126). The age of onset varies from the first to sixth decade of life, with the majority of cases presenting between 10–35 years of age (122). Nerve conduction studies show reductions in compound motor amplitudes with generally normal ( $\geq 40$ s) conduction velocities (122,126,127), indicative of an axonopathy rather than a demyelinating process. The phenomenon of “dying-back neuropathy,” in which degeneration begins in the distal portions of axons and slowly advances back toward the motor neuron cell body, is usually a result of a metabolic disturbance or toxin. The delayed-onset (often in adulthood) character of ATP7A-related distal motor neuropathy implies that the mutations associated with this disease have subtle effects that require years to provoke pathological consequences.

### 100.2.9 A Novel Mechanism of Disease?

The mechanism underlying ATP7A-related distal motor neuropathy seems to be distinct from the pathophysiology of Menkes disease and OHS. Individuals with ATP7A-related distal motor neuropathy have no neurological problems other than motor neuron disease, and no clinical or biochemical findings similar to those observed in patients with Menkes or OHS (Table 100-5). Specifically, no patients with ATP7A-related distal motor neuropathy, examined to date, have shown hair, skin, or joint abnormalities, low serum copper, abnormal plasma catecholamine levels, or renal tubular dysfunction (122), all of which are considered to be hallmarks of mutations at the ATP7A locus. Conversely, three patients with Menkes disease and four individuals with OHS examined recently showed no clinical or electrophysiological evidence of motor neuron dysfunction (S. G. Kaler, unpublished work). The individuals with OHS included the first patient in whom the condition was molecularly defined (56), who is now 32 years old, and an unrelated individual with OHS, who is now 19 years old (67). At these ages, one would have expected ATP7A-related distal motor neuropathy to be clinically manifest, if a common pathogenetic mechanism were involved. Delineation of the underlying mechanism in this form of motor neuropathy will help elucidate the normal role of ATP7A in peripheral nerve biology. Copper deficiency from various causes is known to induce transient sensory or motor neuropathy (22–28), and both ATP7A and ATP7B are expressed in mouse spinal cord neurons (128); however, the identification of ATP7A-related distal motor neuropathy is the first evidence that ATP7A has a direct role in motor neuron function.

TABLE 100-4 Synopsis of Inherited Copper Transport Diseases							
Condition/ Inheritance Pattern	Age of Onset (yrs)	Neurological Signs	Other Clinical Findings	Clinical Biochemical Findings	Molecular Defects	Treatment Options	Prognosis
Menkes Disease/ X-linked recessive	0–1	Hypotonia; seizures; developmental delay	Coarse hair; jowly facies; lax skin and joints; decreased bone density; bladder diver- ticula; gastric polyps; vascular tortuosity and distension	Low-serum copper and ceruloplas- min; Abnormal plasma and CSF neuro- chemicals; Increased urine $\beta$ 2-microglob- ulin.	Diverse mutations in ATP7A; 0–15% residual function	Early copper replacement	Difficult, u early o treatm (withi of birt
OHS/X-linked recessive	3–10	Dysautonomia; <sup>a</sup> slightly reduced muscle strength	Coarse hair; occipital exostoses; hammer- shaped clavicular heads; lax skin and joints; bladder diverticula; vascular tortuosity and distension	Low-normal serum copper and ceruloplasmin; Abnormal plasma and CSF neurochemicals	Leaky splice junction or hypofunctional ATP7A missense muta- tions; 20–30% residual function	Copper replacement; L-dihydroxy- phenylserine (L-DOPS) for dysautonomia	Fair; long- natural not kn may b for vas proble
ATP7A-related distal motor neuropathy/ X-linked recessive	5–50	Atrophy and weakness of distal muscles; foot drop; decreased or absent deep tendon reflexes; abnormal nerve conduction studies <sup>b</sup>	Pes cavus foot deformity; No other specific clinical abnormalities	No specific lab abnormalities	Missense mutations near luminal loops in carboxyl half of ATP7A (Figure 100-3); 60–70% residual function	None currently recommended (due to incomplete understanding of disease mechanism)	Uncertain natural unkno slow d progre likely
Wilson disease/ Autosomal recessive	3–50	Tremor, spastic rigidity, dystonia, dysgraphia, dysarthria, depression, schizophrenia	K–F corneal rings, jaundice, hepato-megaly, osteoporosis, amenorrhea	Low serum copper and ceruloplasmin; Elevated LFTs; Increased hepatic copper	Diverse mutations in ATP7B; 50% missense. Large deletions rare	Penicillamine Trien Zinc Tetrathio- molybdate	Excellen good c compl
Idiopathic Copper Toxicosis/ Autosomal recessive (presumed)	0–6	Irritability	Jaundice, hepatomegaly, ascites, aminoaciduria	Elevated serum & urine copper; Normal or high serum ceruloplasmin; High hepatic copper	Unknown	Penicillamine	Poor with ages treatm

<sup>a</sup>Syncope, dizziness, orthostatic hypotension, abnormal sinoatrial conduction, nocturnal bradycardia, and bowel or bladder dysfunction.

<sup>b</sup>Decreased peroneal and median muscle amplitudes with normal conduction velocities.

### 100.2.10 Mouse Models of Menkes Disease

The mottled mouse provides an excellent mammalian model for Menkes disease (129,130). The mottled (Atp7a) and human ATP7A loci are located in homologous regions of their respective X chromosomes. The Mouse Genome Informatics database of the Jackson Laboratory lists 88 Atp7a alleles, including 38 spontaneous or induced (through radiation and/or chemical mutagenesis) mutants, as well as 48 gene-trapped and two gene-targeted alleles (131). The molecular bases for 14 Atp7a allelic variants have been characterized (67,131–139). One of the best-studied mouse mutants, the mottled brindled male hemizygote shows tremor, a decrease in coat pigmentation, general inactivity, death at  $\approx 14$  days of age, an increase in intestinal copper levels with low levels of the metal in the liver and in the brain, and reductions in copper enzyme activities (129). Of interest has been the observation that normal viability can be restored in these mutant animals if a single copper injection is provided during the first 7–10 days of life, a response also characteristic of the mottled macular mouse, a biochemically similar model of Menkes disease discovered in Japan (76,139). Nevertheless, at least in *mottled brindled* animals, this treatment response seems to be dependent on modifier genes, as no response to therapy is shown in mottled brindled mice on a homogeneous C57BL/6J background, a strain that often maximizes expression of mutant phenotypes. In contrast to *mottled brindled* animals, mice harboring the *mottled blotchy* mutant allele are viable, even without treatment, although these latter mice show more pronounced connective tissue abnormalities than the former. Cultured fibroblasts from all *atp7a* mutant mice tested have demonstrated increased copper accumulation compared with similar cells from wild-type animals (140).

Investigation of the biochemical phenotype in *mottled brindled* and *mottled blotchy* mutant lines has been extensive (129,131,141–144), while recent cell biological studies have provided new insights into the effects of specific mottled (Atp7a) mutations (145,146). The *mottled brindled* and *mottled blotchy* mutant proteins do not traffic efficiently from the *trans*-Golgi to the plasma membrane in response to elevations in copper (145). The *mottled viable brindled* and *mottled macular* defects each shift the steady-state equilibrium of Atp7a to one where the protein is mainly localized to the plasma membrane under basal copper conditions, whereas mottled 11H, an embryonic lethal allele, encodes an isoform of Atp7a that is unable to exit the endoplasmic reticulum (146). In conjunction with the earlier work (147), the findings in *mottled viable brindled* and *mottled macular* imply that Atp7a trafficking is delicately controlled by phosphorylation and dephosphorylation. Specifically, these mutations seem

to stabilize Atp7a in a distinct conformation that leads to an increase in phosphorylation and alters intracellular localization (146,147).

Embryonic gene replacement accomplished by crossing mottled female heterozygotes with male transgenic strains corrected lethality in *mottled tohm* (a large intragenic deletion of Atp7a) and *mottled brindled* mutant mice (138,148).

The *mottled* mouse mutants will continue to serve as useful agents for the study of Menkes disease and other ATP7A-related diseases, and for evaluation of potential new therapies.

## 100.3 WILSON DISEASE

Wilson disease is an autosomal recessive disorder characterized by increased copper deposition in the liver, brain, and cornea. The clinical features have been described over the past 90 years (149), and the involvement of copper became central to considerations of pathogenesis during the past 50 years (150). In contrast to Menkes disease, Wilson disease is a disorder of copper toxicity (Table 100-5). The basic defect had been broadly understood to reside in impaired intracellular transport of copper into a hepatocyte pool or compartment essential for biliary excretion of copper, and for incorporation of copper into ceruloplasmin. The cloning of the Wilson disease gene, located on chromosome 13q, showed it to be a second P-type ATPase (ATP7B), with 55% amino acid homology to the Menkes disease gene (41–43). As with Menkes disease, the identification of the Wilson disease gene, and the characterization of its function, has greatly advanced our understanding of Wilson disease and its pathogenesis.

### 100.3.1 Clinical Features

Wilson disease has an estimated incidence of 1 per 30,000 (151), can present with quite variable clinical manifestations, and shows a range of age of onset of from 3 to over 50 years of age (151–153). Classically, the disease is characterized by the coexistence of specific neurologic findings that tend to be progressive—chronic liver disease with cirrhosis, renal tubular dysfunction, and pigmented corneal rings. However, the variability of the disease is such that it should be considered in any patient with unexplained neurologic or psychiatric dysfunction, hepatitis, hemolytic anemia, certain skeletal lesions, renal Fanconi syndrome, or hematuria. An earlier age of onset in a given patient is, in general, associated with hepatic disease, often without neurologic manifestations.

Liver involvement in Wilson disease (154,155) may resemble subacute viral hepatitis, chronic active hepatitis, juvenile cirrhosis, posthepatic cirrhosis, and cryptogenic cirrhosis. The disorder can present as chronic or as fulminant liver disease, with liver failure. Early signs of hepatic involvement may be hepatomegaly and jaundice; a patient may also present with the rapid onset of

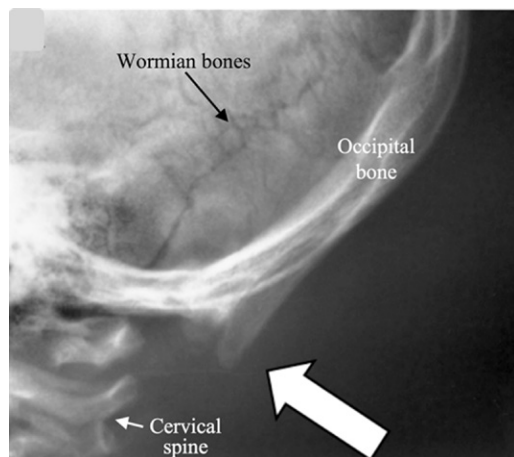
edema, ascites, or GI hemorrhage. Histologic findings are not specific and range from fatty cellular degeneration to macronodular cirrhosis.

Wilson disease is also a disorder of movement (153,156), and neurologic symptoms generally occur after the second and third decades. Specific manifestations include behavior disturbances, tremors, spasticity, rigidity, dysarthria, clumsiness, abnormalities in handwriting or other fine movements, and deterioration in performance in structured situations, such as school settings. Dystonia may include difficulties with facial muscles and drooling, and patients may appear parkinsonian in presentation.

The symptoms reflect the particular involvement of the basal ganglia in the disorder; pathologically, there is extensive neuronal loss, astrogliosis, and cavitation in the basal ganglia. Psychiatric symptoms—suggesting depression or schizophrenia—may be the signal presentation in as many as 20% of patients (157), and can precede the appearance of movement abnormalities (156). A strictly neurologic presentation is rare in children, and when present, latent liver disease can often be found in such patients.

In psychiatric presentations, changes in personality (irritability, anger, poor self-control), depression, and anxiety are common symptoms. Patients presenting in this fashion are typically in their late teens or early 20s, a period during which substance abuse is also a diagnostic consideration. Wilson's disease should be formally excluded in all teenagers and young adults with new-onset psychiatric signs.

The Kayser–Fleischer corneal ring (Figure 100-5) is the most important ophthalmologic finding in Wilson disease.



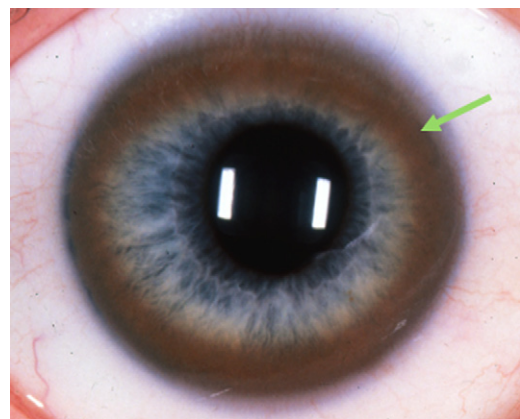
**FIGURE 100-5** The occipital “horns” of OHS. Occipital horns (large arrow) are calcified tendons of the trapezius and sternocleidomastoid muscles at their attachment to the occipital bone. The precise basis for these calcifications is not known, but they are not found in patients with classical Menkes disease or ATP7A-related distal motor neuropathy. The formation of occipital horns could represent an inflammatory response to chronic injury of the involved tendons caused by a combination of reduced connective tissue strength from lysyl oxidase deficiency and the capacity of patients with OHS to use these muscles frequently. Wormian bones (black arrow) are extra bone pieces that occur within a suture line in the skull and are not specific for OHS versus Menkes disease.

The ring is a brown-to-green discoloration in the limbic region of the cornea and consists of layers of copper granules in the Descemet membrane. Slit-lamp biomicroscopy is required to conclusively document the presence or absence of the rings. Approximately 95% of patients with neurologic signs manifest the Kayser–Fleischer ring compared with approximately 65% of those with hepatic presentations (158). Copper chelation therapy causes fading and even disappearance of corneal copper over time. While pigmented corneal rings can be seen in other liver disorders, these can be distinguished from Kayser–Fleischer rings by expert examination. Sunflower cataracts (159), which do not obstruct vision, may also be observed (in up to 13% of adult patients) on slit-lamp examination (Figure 100-6).

Age influences the specific presentation in Wilson's disease. Nearly all individuals who present with liver disease are younger than 30 years, whereas those presenting with neurologic or psychiatric signs may range in age from the first to the fifth decade. This reflects the sequence of events in the pathogenesis of this disease (see earlier discussion). However, regardless of clinical presentation, some degree of liver disease is invariably present. In one series of 400 adult patients with Wilson's disease, approximately 50% presented with neurologic and psychiatric symptoms, 20% with neurologic and hepatic symptoms, and 20% with purely hepatic symptoms (160).

Other clinical findings include acute hemolytic anemia with jaundice, which can occur prior to hepatic and neurologic symptoms. Hemolysis results from the direct toxic effects of copper on red blood cell membranes. When it occurs, this is usually associated with release of massive quantities of hepatic copper into the circulation, a phenomenon that can be sudden and catastrophic.

Renal involvement (161) can occur in approximately 50% of patients and includes hematuria and the renal Fanconi syndrome, the latter directly related to the



**FIGURE 100-6** Kayser–Fleischer ring in Wilson disease, representing copper deposition in Descemet's membrane of the cornea. (Image courtesy of T.U. Hoogenraad, MD, University Department of Neurology, University Hospital, Utrecht, The Netherlands.)



toxic effects of copper deposition. The renal tubular dysfunction can have secondary consequences, including phosphaturia and uricosuria. The former can lead to hypophosphatemia, with resultant renal rickets, osteoporosis, bone fractures, and pseudofractures. The uricosuria can result in a low serum urate, a relatively unique clinical chemistry, and a helpful diagnostic clue for Wilson disease. Renal function must be delineated in every patient prior to treatment, because certain therapeutic agents can be nephrotoxic.

Some untreated women may show amenorrhea and impaired fertility. Further, in some adults with longstanding Wilson disease, hepatocellular carcinoma has been observed. Additionally, some patients may have cardiac involvement, including arrhythmias and cardiomyopathy.

### 100.3.2 Molecular Pathology

The Wilson disease gene on the long arm of chromosome 13 (162) was identified by positional cloning strategies, based on the hypothesis that the Wilson disease protein might bear similarities to the Menkes disease transporter (37). The gene, termed WND or ATP7B, is expressed in liver, kidney, and placenta, in contrast to the widespread expression of the Menkes gene. The WND gene encodes a 1411-amino acid P-type copper-transporting ATPase. In the liver, the ATPase is localized to *trans*-Golgi vesicles in the pericanalicular area, with small amounts of the protein in the canalicular membrane (163). In the presence of elevated intracellular copper concentrations, the ATPase is distributed close to the bile canaliculi (164). It is possible that the Wilson disease ATPase interacts with the gene product of the *COMMD1* 1 gene (implicated in a copper toxicosis syndrome in the Bedlington terrier (165)) in the mediation of biliary excretion of copper (166). Such localization, translocations, and possible interactions are consistent with two functions of the WND protein: the transporting of copper into the hepatocyte secretory pathways for incorporation into ceruloplasmin and the excretion of copper into bile (167). Impaired function of the WND protein results in copper accumulation in the hepatocyte cytoplasm and, ultimately, liver cell necrosis, spillover of copper into plasma, and toxicity to extrahepatic organs, including brain (168).

In contrast to Menkes disease, large gene deletions do not occur in Wilson disease patients, and over 50% of the (over 300) WND mutations are missense mutations (169,170). A few mutations in the Wilson disease gene account for a significant fraction of cases in specific populations (41,169,171,172). For example, the H1069Q mutation accounts for 30% of all mutations in European populations, while the R778L missense mutation is common in Asian populations. In addition, Wilson disease mutations are in general associated with specific chromosomal haplotypes in a given population (173). Both

kinds of genetic epidemiologic correlations are applicable to clinical molecular diagnosis.

A Wilson disease mutation database contains over 300 different mutations reported at the ATP7B locus (<http://www.wilsondisease.med.ualberta.ca/database.asp>).

For families in which the mutant alleles have been determined, molecular diagnosis is highly reliable. While there is great variability, and even intrafamilial variability, some broad genotype–phenotype correlations have been described (172,174). For example, mutations such as nonsense mutations, deletions, and duplications, which would be predicted to result in a truncated or absent gene product, tend to be associated with early-onset liver disease. In contrast, the H1069Q missense mutation, in a homozygote or compound heterozygote, generally results in either neurologic or liver disease symptoms in adolescence (151). Even in the case of the H1069Q mutation, studies have revealed no significant differences between H1069Q homozygotes as compared with H1069Q compound heterozygotes in terms of age of onset and clinical and biochemical manifestations (172).

### 100.3.3 Diagnosis and Genetic Counseling

Once a diagnosis has been established in a given patient, genetic counseling can proceed as appropriate for an autosomal recessive disorder. To make the diagnosis of Wilson disease, one can perform a staged evaluation, as outlined later in this chapter. If mutations at both alleles are identified in the proband, then an accurate molecular diagnosis of Wilson disease can be made in presymptomatic or future siblings. The availability of mutation data also enables the accurate assignment of heterozygote status to relatives of a given proband. The problem often faced in clinical practice is the absence of knowledge of the actual mutation(s) in a patient with clinically documented Wilson disease. In such circumstances, and in some families and populations, it may be possible to use haplotype linkage analysis to trace the segregation of mutant genes through a pedigree.

The diverse manifestations of Wilson disease make the diagnosis quite difficult, and, in some systematic surveys, diagnostic errors—and consequent delay in treatment—are frequent (175). In this context, awareness of the multiple presentations of Wilson disease is, indeed, essential so that the diagnosis can be considered in a broad spectrum of patients. Formal recommendations and scoring systems may be helpful as guidelines (169,176). Wilson disease should be included in the differential diagnosis of patients with unexplained hepatic, neurologic, renal, or bone disease, or hemolytic anemia. Kayser–Fleischer rings are an especially important clinical finding on examination, but their absence, especially in the instance of hepatic presentation or in a patient with hemolytic anemia, does not exclude the diagnosis.

Clinical laboratory investigations include measurements of serum copper, urinary copper excretion, serum ceruloplasmin, tissue copper levels, and radiocopper kinetic studies (152–154,177,178). Ceruloplasmin levels (and serum copper concentrations) are decreased in most patients with Wilson disease. However, 4% to 28% of patients show serum ceruloplasmin concentrations in the normal range, and these individuals may show a normal serum copper concentration. Mostly, such patients are children or adolescents with liver disease. Conversely, ceruloplasmin levels may also be low in other conditions, including chronic active hepatitis with or without liver failure. The distinction between Wilson disease and chronic active hepatitis can, in fact, be quite difficult because of the similarity of clinical course, ceruloplasmin levels, and urine copper excretion. Absence of a molecular diagnosis, measurements of hepatic copper concentration, and even radiocopper kinetic studies may be required, to make this distinction.

Urine copper excretion at baseline, and after a dose of D-penicillamine, can be a useful diagnostic parameter. Urine copper excretion in patients is 100 mg/24 h to 1000 mg/24 h, in comparison with the normal excretion of <40 mg/24 h. After a dose of 1 g of D-penicillamine, normal adults will excrete 600–800 mg copper/24 h, while patients with Wilson disease will excrete well over 1000 mg/24 h. Because the excretion of copper reflects excess body stores, presymptomatic patients, or patients diagnosed very early, may have normal excretion values. Conversely, hypercupriuria can be observed with prolonged or severe cholestasis for reasons other than Wilson disease; elevations in urine copper are therefore not specific for Wilson disease.

The copper content of liver, brain, kidney, and cornea is increased in Wilson disease, and hepatic content can serve as a diagnostic discriminator. Hepatic copper content in symptomatic and presymptomatic patients ranges from 2 to 90 times normal, as measured in mg/g of dry weight. For comparison, hepatic copper content in chronic active hepatitis is generally 7–15 times normal.

Patients with intermediate or otherwise indeterminate hepatic copper contents require additional diagnostic testing. For such patients, a specific diagnostic test is the kinetic analysis of plasma and ceruloplasmin radioactive copper concentration after an intravenous dose of  $^{64}\text{Cu}$ . Patients with Wilson disease do not have a normal secondary rise in plasma radioactivity or incorporation of  $^{64}\text{Cu}$  into ceruloplasmin, even in those patients with normal ceruloplasmin levels. (Note that patients with Menkes disease do have a normal secondary response.) In heterozygotes, the rate of incorporation of  $^{64}\text{Cu}$  into ceruloplasmin is decreased compared with normals but is still measurable. This kind of testing should be performed at a center with experience in such analytic protocols.

Finally, mutation analysis may be employed to confirm a diagnosis suspected on the basis of clinical and biochemical parameters. This approach is particularly

important in the absence of typical symptoms, ambiguity of biochemical markers, or in asymptomatic siblings. A stepwise approach to molecular analysis has recently been outlined, beginning with rapid screening for a highly prevalent mutation in a given population or ethnic group (e.g. the H1069Q mutation in European populations). Screening for a panel of additional mutations present in significant frequency can ensue, if necessary, followed by analysis of exons (and intron boundaries). Such systematic molecular testing is becoming increasingly available and has, in fact, been put forth as an additional diagnostic criterion (172,179) for a disorder that requires early and, in some circumstances, rapid intervention.

### 100.3.4 Treatment

The prognosis in Wilson's disease is generally favorable. Current therapeutic approaches can prevent or reverse most of the significant clinical signs and symptoms, including Kayser–Fleischer rings. However, if treatment is stopped, potentially fatal liver damage inevitably occurs. The objective of treatment is to prevent copper from accumulating in tissues. While intake of copper can be decreased by restricting foods high in copper, the mainstay of medical therapy has been the use of the copper-binding agent, D-penicillamine (b,b-dimethylcysteine) (180), in doses of 1 g/d in adults and older children, and 20 mg/kg/d for children under 10 years. Pyridoxine supplementation is required, as D-penicillamine is a pyridoxine antagonist. Therapy is monitored with periodic slit-lamp examinations: there is reduction in pretreatment deposits, but total clearing may require several years (181). Treatment is also monitored by periodic measurements of urine copper excretion: after an initial maximum, excretion should stabilize at 500–800 mg/d during maintenance therapy.

If therapy is begun in presymptomatic patients, the development of symptoms can be prevented. If treatment is begun early in the course of the disease, neurologic and hepatic function can be normalized. However, patients with psychiatric disease may improve, but be left with residual psychiatric symptoms (182). Further, advanced disease may not be reversible, and some patients show neurologic worsening with initial treatment (183–185). D-penicillamine treatment has been shown to exacerbate clinical manifestations in about 50% of patients with neurologic symptoms, and half of those who worsen fail to regain baseline function (21,156). Finally, side effects of D-penicillamine can be serious, ranging from early hypersensitivity reactions (in 25% of patients) to more severe later reactions, including immune complex nephritis, drug-induced lupus erythematosus, Goodpasture syndrome, oral ulcerations, and agranulocytosis. If the drug must be withdrawn in the face of side effects or severe complications, attempts can be made to reinstitute therapy, beginning with very low doses, until the therapeutic range is again achieved.

Since such reinstitution of therapy is not always successful, a number of additional agents have been tested in the treatment of Wilson disease (183). One is the alternative chelating agent, triethylene tetramine HCl (trientine). Trientine is an effective chelating agent, but experience is limited, and the full spectrum of toxicity is not known. A second alternative drug, ammonium tetrathiomolybdate (183,186,187), has been used primarily as a rapidly acting initial treatment for patients with acute neurologic symptoms. In the intestine, this agent complexes copper and food protein, preventing absorption; in the blood, it complexes copper and albumin, making the copper unavailable for cellular uptake (21). In a randomized, controlled, double-blind clinical trial comparing tetrathiomolybdate plus zinc to trientine plus zinc for Wilson disease patients with neurological presentation, a statistically significant decreased occurrence of neurological relapse was found in the tetrathiomolybdate plus zinc treatment group (187).

An increasing number of patients are being treated with oral zinc sulfate or zinc acetate, at 75–100 mg of elemental zinc per day. After a lag of 1–3 weeks, the zinc blocks copper absorption, producing a negative copper balance. The effect is thought to be mediated by induction of intestinal cell MT, which binds copper with high affinity, preventing the transfer to copper into the blood. Treatment with zinc is nontoxic, does not produce initial neurologic worsening, and prevents hepatic reaccumulation of copper. Treatment with oral zinc has been shown to be effective, with clinical amelioration reported in multiple studies (181,184,187–189). Oral zinc may be especially useful for maintenance, following D-penicillamine initiation, or in conjunction with another agent (183,188,189). In pregnant women being treated with D-penicillamine, the teratogenic effects of the drug must be considered. There are reports of connective tissue defects of variable severity following pregnancies, marked by maternal penicillamine use (190). The magnitude of the risk may be low as numerous normal babies have been born to mothers treated with D-penicillamine. Accordingly, a balancing of the risks would suggest that drug treatment should be continued during pregnancy, at 1 g/d or lower doses, with the dose lowered even further near delivery, if a cesarian section is contemplated. Maintenance of oral zinc therapy may prove to be an important nonteratogenic adjunct during pregnancy.

Finally, improvements in the morbidity and mortality of orthotopic liver transplantation have made liver transplant the therapy of significant choice in Wilson disease patients with fulminant hepatitis, or with progressive, unremitting hepatic insufficiency. In general, transplantation results in removal of excess body copper, neurologic improvement, and disappearance of Kayser–Fleischer rings (191–193). Posttransplant patients do not require chelation therapy, and no recurrence of Wilson disease in a transplanted liver

has been reported. This latter observation was, in particular, made in recipients of grafts from living related heterozygotes for Wilson disease (191). Of additional importance is a report of quality of life data in patients who had received orthotopic liver transplants, showing improvement in overall quality of life in this cohort of Wilson disease patients (194).

### 100.3.5 Future Directions

Gene therapy for Wilson's disease is a possibility. Because the Wilson's copper transporter is expressed most prominently and functions most critically in the liver, this organ could be specifically targeted by the use of adenoviral, adeno-associated viral, or replication-deficient lentiviral vectors. Hepatocyte transfer, an alternative to gene therapy, may also be applicable to the treatment of liver-specific metabolic disorders through therapeutic liver repopulation (195). The Long-Evans-Cinnamon (LEC) rat, toxic milk mouse (tx), and Jackson toxic milk mouse (tx<sup>1</sup>) are naturally occurring rodent models of Wilson disease for which specific *Atp7b* defects have been demonstrated (196). A complete gene knock-out (*Atp7b*<sup>-/-</sup>) has also been generated (197). These models should be useful for preclinical explorations of novel therapeutic approaches.

### 100.3.6 Idiopathic Hepatic Copper Toxicosis

A disease variously termed Indian childhood cirrhosis, non-Indian childhood cirrhosis, copper-associated childhood cirrhosis, and, more recently, idiopathic hepatic copper toxicosis may represent a group of similar disorders, or a single entity with a variable spectrum of severity, and a multifactorial environmental and genetic etiology. The disease was first recognized in eastern India in 1887 and, subsequently, was noted in various other regions of that country. Most cases occurred in male infants from middle class Hindu families residing in rural communities. Indian childhood cirrhosis involved massive accumulation of copper in the liver, and development of early cirrhosis and liver failure. Serum ceruloplasmin levels were normal, and the histopathology of Indian childhood cirrhosis, characterized by hepatic necrosis with ballooning, Mallory's hyaline and absence of fatty change, was easily distinguishable from that of Wilson disease. Segregation analyses of multiple families in one study suggested an autosomal recessive inheritance pattern (198).

Heating and storage of foods, including cow or buffalo milk, in copper-containing (brass) vessels and pots in Indian households were implicated as causes of excessive copper ingestion and an environmental etiology for this disorder (199). The hypothesis was supported by a significant decline in the incidence of Indian childhood cirrhosis when this environmental



factor was addressed (200). Further, children who survived treatment with D-penicillamine showed no subsequent evidence of liver disease (115).

However, it since has become apparent that cases of a very similar form of infantile or early childhood cirrhosis may exist in other countries and even in the absence of overt environmental exposure to sources of excess copper (115). In one study of a Northern Germany population isolate, documentation of parental consanguinity implied autosomal recessive inheritance of a predisposition to development of infantile cirrhosis (201).

Studies in fibroblast cell lines from patients with typical Indian childhood cirrhosis excluded abnormal MT induction in response to metals as a molecular mechanism of disease (202). A reverse argument for a genetic factor was derived from studies showing that elevated copper content in a water supply, per se, resulted in no cases of idiopathic copper toxicosis in a region of Massachusetts over a 23-year period (115,203).

In sum, the aggregate data support the notion that the pathogenesis of idiopathic copper toxicosis—a reasonably distinct clinical and histologic entity—does indeed involve the interaction of genetic and environmental factors. In some patients or settings, either of these factors may play the role of a determinant of major effect. Careful study of the pathology, and of copper trafficking and utilization in tissues and cells from cases in population isolates or familial clusters, and from patients who develop disease in the absence of exposure to excess copper, should be of particular value in elucidating the mechanism of this disorder.

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# CHAPTER

# 101

## Iron Metabolism and Related Disorders

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### 101.1 INTRODUCTION

Iron is one of the most abundant elements on earth and a vital component of the cellular processes of most living things. It is used in many enzymatic reactions, including multiple steps of the electron transport chain and the tricarboxylic acid cycle, as well as in reactions catalyzed by microsomal cytochromes involved in detoxification of drugs and other foreign substances. The majority of iron functions as oxygen carrier in the heme groups of hemoglobin and myoglobin. In large quantities and when unbound, iron can be highly toxic to cells. Much of iron metabolism involves maintaining a delicate balance between sequestered and free ion, absorption, elimination, and reutilization. Over time, seemingly small changes in this balance can cause disease. Recent advances in understanding the molecular causes of common and rare disorders of iron metabolism have revealed regulatory pathways previously unappreciated, which suggest novel therapeutic strategies.

### 101.2 IRON BALANCE AND THE IRON CYCLE

Under normal circumstances, the total body iron content is maintained within narrow limits. In men, the average

concentration is 50 mg/kg but in women it is only 35 mg/kg, reflecting the high incidence of iron deficiency (1). Most of the body's iron is found in the form of heme, which serves as a prosthetic group on hemoglobin and myoglobin, for the enzymes required for oxidative phosphorylation, for the enzymes involved in splitting hydrogen peroxide, and on other enzymes required for detoxification of chemical agents or drugs. About 30% of iron is stored as a complex formed with ferritin. A small amount is present within the plasma bound to transferrin, the major plasma iron transport protein. There is no significant mechanism for iron excretion other than through cell loss. Daily losses are generally fixed at 1–2 mg/day due to normal epithelial sloughing of the skin and gastrointestinal (GI) tract, menstruation, and stool and urinary losses. With an adequate diet, this amount is absorbed daily through the small intestine. Under the conditions of iron deficiency, the amount of iron absorbed from the diet can be increased to as much as 4–5 mg/day. Thus iron balance is regulated at the level of absorption. In addition to total body iron, the rate of erythropoiesis also influences intestinal iron absorption (2).

Much of the iron contained in red blood cells is recovered and reutilized (3). This cycling process can be viewed as a series of compartments through which iron is passed. Central to the flow of iron within this cycle is the plasma

compartment, where the majority of iron is bound to the carrier protein, transferrin. As much as 90% of the iron bound to transferrin is targeted to developing red cells in the bone marrow, and ~90% of this is eventually incorporated into heme (3). At the end of their 120-day life cycle, red blood cells are phagocytosed by macrophages in the spleen and the iron is recovered. Depending on requirements, a fraction of the recovered iron is stored in macrophages. Under equilibrium conditions or under conditions of increased erythropoiesis, iron is not stored but passed quickly through the macrophages and is released into the plasma compartment. Other tissues also absorb iron for their own use. The liver is the most prominent site, receiving about 5% of plasma iron (3). Iron is also deposited in the liver in pathologic states, especially under the conditions of high transferrin saturation (TS).

### 101.2.1 Dietary Absorption of Iron

Iron is ingested in two principal forms: complexed in heme or as elemental iron. In both cases, the primary site of dietary iron absorption is within the villous tips of

the duodenum. Although heme iron typically constitutes only one-third of dietary iron, it has much greater bioavailability and can account for as much as two-thirds of iron stores. Much of this may be related to heme being more soluble in the alkaline environment of the duodenum. Surprisingly, heme iron absorption does not appear to be as readily responsive to regulation as non-heme iron. Although nonheme iron absorption can be increased by dietary contents such as ascorbic acid, chelators and phytates can bind iron and inhibit absorption (4–6). The inverse relationship between total body iron stores and iron absorption has been known for decades and with recent discoveries of iron transporters and their regulators, a clear picture of these mechanisms has developed. These findings have also led to links between inflammation and iron deficiency. The role of dietary iron absorption is also influenced by the rate of erythropoiesis. Interestingly, iron absorption is increased in anemias characterized by ineffective erythropoiesis where erythroid cells are destroyed within the bone marrow, suggesting that signals from the bone marrow compartment also influence mucosal iron absorption.

**TABLE 101-1 Hereditary Disorders of Iron Metabolism**

	<b>HFE-Related Adult-Onset Hemochromatosis</b>		<b>JH</b>	<b>TFR2-Related Hemochromatosis</b>	<b>FPN1-Related Iron Overload</b>
OMIM Classification	Type 1	Type 2A	Type 2B	Type 3	Type 4
Gene	HFE	HJV	HAMP	TFR2	SCL40A1
Aliases	HLA-H	JH, HFE2A, RGMC	HEPC, LEAP-1		MTP1, IREG1, FPN1, HFE4
Location	6p21.3	1q21	19q13.1	7q22	2q32
Gene Product	HFE	Hemojuvelin	Hepcidin	Transferrin receptor 2	FPN1
Function	Regulation of hepcidin expression through interaction with TFR1 and TFR2	Modulation of hepcidin expression acting as co-receptor for BMPR	Inhibition of iron export from intestine and macrophages by FPN	Uptake of iron by hepatocytes and regulation of hepcidin expression	Export of iron from enterocyte macrophages, placental cells, and hepatocytes
Inheritance	Autosomal recessive	Autosomal recessive	Autosomal recessive	Autosomal recessive	Autosomal dominant
Tissue Iron Accumulation	Liver, endocrine glands, heart	Liver, endocrine glands, heart	Liver, endocrine glands, heart	Liver, endocrine glands, heart	Liver, spleen
Cellular Iron Accumulation	Parenchymal	Parenchymal	Parenchymal	Parenchymal	Reticuloendothelial
Organ Damage	Variable	High	High	Variable	Low
Anemia	No	No	No	No	Possibly menstruating women, post phlebotomy
Treatment	Phlebotomy-excellent response	Phlebotomy-excellent response	Phlebotomy-excellent response	Phlebotomy-excellent response	Phlebotomy-fair response
Decade Onset	4th or 5th	2nd or 3rd	2nd or 3rd	4th or 5th	4th or 5th

Source: Modified from Pietrangelo (92).

## 101.2.2 Molecular Biology of Iron Metabolism

The molecular details of iron storage, transport, regulation, and absorption are described in the following. Table 101-1 lists the known molecules of iron metabolism involved in inherited disorders.

**101.2.2.1 Iron Storage.** Ferritin is a ubiquitous protein found in all cells, where its principal function appears to be storage of iron. Ferritin concentrates cellular iron and protects cells from its toxic effects. Storage iron is sequestered in a hollow sphere composed of 24 ferritin subunits (7). There are two distinct subunits of ferritin: the H subunit of 21,000 MW and the L subunit of 19,000 MW (8). The primary sequences for H and L subunits share 55% identity but adopt similar secondary and tertiary conformations. Multiple isoforms of ferritin have been isolated from different tissues that differ in the number of H and L subunits (9,10). Acidic isoforms contain a greater number of H chains and are characteristically found in cardiac tissue. Basic isoforms are found in the liver, spleen, and plasma. The active gene for the H

subunit is located on chromosome 11, whereas the gene encoding the L subunit is located on chromosome 19 (11,12). Multiple pseudogenes for ferritin subunits that lack regulatory sequences have been described on other chromosomes (13).

**101.2.2.2 Iron Transport.** Iron is transported in the plasma bound to transferrin. Transferrin is a protein of ~75,000 MW, capable of binding two atoms of ferric iron, and is synthesized primarily in the liver (14). In contrast, many cells, especially those that proliferate, rapidly express transferrin receptors on their surfaces. The transferrin receptor 1 (TFR1) exists as a dimer of a transmembrane glycoprotein of 90,000 MW (15,16). Both the transferrin gene (*TF*) and the *TFR1* map to the long arm of chromosome 3 (17,18). Transferrin protein, with two iron molecules (also known as holotransferrin or diferric transferrin), binds with high affinity to transferrin receptors at neutral pH; apotransferrin (lacking iron) does not. The holotransferrin–transferrin receptor complexes are internalized by receptor-mediated endocytosis (19–21). Acidification of the endosome results in the release of iron from holotransferrin, generating

Rare Disorders of Local or Cellular Iron Overload

Atransferrinemia	PKAN, HARP	Aceruloplasminemia	Neuroferritinopathy	Anemia, sideroblastic, spinocerebellar ataxia	Friedreich's Ataxia
TF	PANK2	CP	FTL	ABCB7	FRDA
3q21	20p13-p12.3	3q23-q24	19q13.3-q13.4	Xq13.1-q13.3	9q13
Transferrin	Pantothenate kinase 2	Ceruloplasmin	Ferritin light chain	ATP-binding cassette, subfamily B, member 7	Frataxin
Circulating iron transport molecule	Brain specific enzyme; defect results in cysteine accumulation which may chelate iron	Oxidation of ferrous to ferric iron required for release of cellular iron to transferrin	Intracellular iron storage	Transport of FeS clusters from mitochondria to cytosol	Mitochondrial iron metabolism; fes cluster assembly
Autosomal recessive	Autosomal recessive; some dominant alleles	Autosomal recessive	Autosomal dominant	X-linked recessive	Autosomal recessive
Liver, heart	Brain: basal ganglia	Liver, pancreas, brain	Brain: basal ganglia	Brain, erythroid cells	Brain, heart
Reticulo-endothelial	Parenchymal	Parenchymal	Parenchymal	Mitochondrial	Mitochondrial
Variable	High	High	High	High	High
Yes	No	No	No	Yes	No
Human transferrin; transfusion-phlebotomy	Pantothenate?	Desferoxamine			Antioxidant therapy?
1st–2nd	1st–2nd	5th–6th	5th–6th	1st	1st–2nd



apotransferrin (22–24). Apotransferrin is returned to the cell surface, where it dissociates from the transferrin receptor and can be used again as an iron carrier within the plasma.

The biosynthesis of transferrin, transferrin receptor 1, and ferritin is influenced by the amount of iron in the cell. Regulation of transferrin expression occurs at the level of mRNA synthesis, which is inversely correlated with the amount of cellular iron (25). Transferrin receptor 1 synthesis is largely regulated by the stability of its mRNA, whereas ferritin synthesis is regulated at the level of protein translation. Both ferritin and transferrin receptor 1 mRNAs contain stem-loop sequences, known as iron-responsive elements (IREs), which can bind to iron regulatory proteins (IRPs) with high affinity (26).

The binding of IRP to IREs is enhanced by low cellular concentrations of iron. A single IRE is located within the 5′ untranslated region of the ferritin mRNA. In contrast, transferrin receptor 1 mRNA contains multiple IREs within the 3′ untranslated region. This differential placement results in opposite effects on expression: Under the conditions of low cellular iron concentration, IRPs have high affinity for IREs, resulting in binding to the 5′ end of ferritin mRNA and the inhibition of translation. IRP binding to the 3′ IREs increases transferrin receptor mRNA by increasing message stability. The situation is reversed under the conditions of high iron concentration. High iron decreases IRP binding to IREs, allowing efficient translation of ferritin mRNA, but also renders transferrin receptor 1 mRNA less stable and subject to degradation.

Two distinct IRPs have been cloned. The gene encoding *IRP1* maps to chromosome 9, and a second highly homologous gene, *IRP2*, maps to chromosome 15 (27). *IRP1* is a protein of 90,000 MW and has strong homology to mitochondrial aconitase, including all active site residues and three cysteines that serve as ligands for a 4Fe–4S cluster (28,29). Further studies suggest that *IRP1* may function as a cytosolic aconitase (29).

The binding of iron in the 4Fe–4S complex of aconitase is unstable (28,29). This suggests that *IRP1* affinity for IREs is influenced by the stability of the iron–sulfur complex, which can be related to cellular iron concentration (30). If iron is abundant in the cell, the iron–sulfur complex of *IRP1* is stable and the molecule functions as a cytoplasmic aconitase but binds IREs with low affinity; under iron-poor conditions the iron–sulfur complex is destabilized and the molecule loses aconitase activity but binds IREs with high affinity. In fact, the IRE-binding site and the aconitase-active sites overlap, indicating that enzymatic activity and IRE binding are mutually exclusive (31,32).

*IRP2* binds to IREs with affinity similar to *IRP1* (33–35). However, *IRP2* lacks aconitase activity and appears unable to form an iron–sulfur complex (33,36). In addition, unlike *IRP1*, *IRP2* is substantially degraded in the

presence of iron (33,35). Absence of *IRP2* in mice results in misregulation of iron in the intestinal epithelium and neurons of the CNS causing neurodegeneration and iron accumulation in the brain (37). Factors other than iron have been identified that also regulate IRP binding. These include hypoxia, phosphorylation, and certain cytokines.

**101.2.2.3 Iron Absorption.** Nonheme iron is mostly absorbed in the duodenum where ferric iron ( $\text{Fe}^{3+}$ ) in food is reduced to  $\text{Fe}^{2+}$  by the cytochrome reductase *DcytB* and then transported across the apical membrane into the enterocyte by divalent metal transporter 1 (DMT1), also known as divalent cation transporter 1 and natural resistance-associated macrophage protein 2 (Nramp 2), a proton-coupled, divalent metal ion transporter (38). Located on endosomal membranes as well, (39) DMT1 is responsible for the transport of nonheme iron into the intestinal epithelial cell from the intestinal lumen (38). In addition, DMT1 is involved in the transport of iron from the endosome to the cytoplasm. DMT1 is mutated in the microcytic anemia (*mk*) mouse (40), a strain exhibiting a recessive disorder characterized by microcytic hypochromic anemia due to defects in intestinal iron absorption and erythroid iron utilization (41). An orthologous mutation is found in the Belgrade (*b*) rat, which exhibits a similar phenotype (42). Thus DMT1, the iron transporter of intestinal villi, also plays an important role in the transferrin cycle.

Ferroportin (FPN1) functions to export iron out of the intestinal epithelial cells. In yeast, high-affinity iron uptake is mediated by the iron transporter, *FTR1*. The vertebrate equivalent of *FTR1* was identified through positional cloning of the zebrafish mutant, *weissherbst* (43). This mutant develops a hypochromic anemia due to a deficiency in FPN1, which is responsible for the transport of iron from the maternally derived yolk sac stores to the embryonic circulation. Human FPN1 localizes to the basal surface of placental syncytiotrophoblasts, where it transports iron from mother to embryo. It is also highly expressed in the liver and the duodenum, specifically localizing to the basolateral membrane of intestinal epithelial cells and to Kupffer cells of the liver. In addition to placental transport, FPN1 mediates transfer of iron from intestinal epithelial cells into the circulation and the release of iron from macrophages.

Just as the apical flux of iron into the cell is associated with a reductase, the export of iron requires the ferroxidase activity of hephaestin, presumably to promote iron binding to apotransferrin. Hephastin was first identified as the causative gene mutated in the sex-linked anemia (*sla*) mouse, which exhibits a microcytic anemia due to defective intestinal iron absorption. However, unlike the *mk* mouse, the *sla* mouse is capable of normal iron uptake into the intestinal mucosal epithelium but is unable to effectively transfer iron to the circulation (44). Cloning of the gene responsible for *sla* led to the identification of hephaestin, a transmembrane-bound ceruloplasmin homolog, which putatively functions as

a ferroxidase required for the transfer of iron from the enterocyte into the plasma (45).

Although heme iron absorption accounts for a greater percentage of dietary iron absorption, our understanding of its mechanisms remains poor. Two possible mechanisms have been proposed (46). The first theory based on binding and transport studies of heme suggests an active receptor-mediated endocytic pathway. Multiple studies have shown specific binding and energy-dependent uptake of heme by intestinal epithelial cells. Presumably, heme-bound iron is either released by heme oxygenase 2 and transported out of the endosome by DMT1 into the cytoplasmic iron pool following a pathway similar to nonheme iron, or transported intact by the heme-responsive gene-1 (HRG-1) (47). Notably, however, a heme-binding receptor has not been identified to date.

The second possible pathway for heme iron absorption involves three recently identified heme transporters: heme carrier protein 1 (HCP1), also known as proton-coupled folate transporter (PCFT) and SLC46A1, feline leukemia virus subgroup C cellular receptor 1 (FLVCR), and HRG-1 (SLC48A1). HCP1/PCFT was originally isolated by a subtractive hybridization technique in hypotransferrinemic mice and was shown to be expressed on the apical membrane of duodenal epithelial cells and mediate heme transport (48). Interestingly, HCP1/PCFT has independently been shown to function as a proton/folate symporter and several mutations in HCP1/PCFT have been associated with hereditary folate malabsorption (OMIM 229050) (49–53). Sequence analysis of *HCP1/PCFT* in a multiethnic cohort found mutations to be rare and not associated with iron overload (54).

Within the intestinal epithelial cell, iron may be released from heme by endoplasmic reticulum bound heme oxygenase 1 and follow the nonheme iron-bound pathway. Alternatively, FLVCR and/or HRG-1 may be involved in its transport across the basolateral membrane where it is bound to hemopexin (55). FLVCR serves as a heme exporter in erythrocytes and has a critical role in maintaining heme homeostasis within these cells (56). In addition, FLVCR is expressed in the intestinal cell line called Caco-2. However, further studies are needed to elucidate its role in intestinal iron absorption. HRG-1 was initially identified in a gene array study of heme responsive genes of *Caenorhabditis elegans*, which are heme autotrophs and thus completely dependent on heme transport (47). Functional studies have demonstrated that HRG-1 does transport heme and is localized not only to endosomes but also to the basolateral membrane of polarized epithelial cells (57).

### 101.2.3 Regulation of Intestinal Iron Absorption and Body Iron Trafficking

The primary method of regulating iron stores is by modifying dietary absorption through the mucosal villi of the duodenum. Normal iron homeostasis depends on

dietary iron absorption to match the 1–2 mg of normal daily losses, with the capacity for increasing iron absorption under conditions of iron deficiency to 5 mg/day. In concert with the regulation of intestinal iron absorption, body iron stores are also regulated so that iron stores are released in states of iron deficiency. While evidence supporting the role of local iron concentration in the regulation of iron homeostasis has been reported, a more complex regulation involving multiple input signals has become clearer over the past decade.

Under certain conditions such as acute iron deficiency, intestinal iron absorption can be induced by direct intestinal factors including the upregulation of intestinal *DMT1* and *Dcytb* through hypoxia-inducible factor (*HIF*) 2 $\alpha$ -mediated signaling (58). In addition, some *DMT1* transcripts contain an IRE in the 3'-UTR and are stabilized by IRP binding while an IRP in the 5'-UTR of *FPN1* inhibits translation.

More relevant to the long-term maintenance of iron stores is hepcidin, the key regulator of iron homeostasis. Hepcidin is a circulating hormone similar to defensins. It is synthesized by hepatocytes, circulates in the blood, and inhibits the export of iron from intestinal epithelial cells by FPN. Hepcidin binds directly to FPN inducing its internalization and degradation in a Jak2-dependent manner (59,60). Presumably, hepcidin, via its effect on *FPN1* in reticuloendothelial cells, would similarly inhibit iron egress from these cells as well. In nearly all genetic conditions of iron overload, there is a deficiency of hepcidin or defect preventing its interaction with FPN. In contrast, inflammatory states are characterized by excessive hepcidin levels driven by IL-6 and resulting in hypoferremia.

Expression of hepcidin can be regulated by several signal pathways, but the precise nature by which iron is sensed remains unclear. A link with the bone-morphogenic protein signaling pathway was an unexpected observation of mice genetically engineered to lack *Smad4* in hepatocytes which were found to develop severe hepatic iron overload (61). The mutant animals also had 100-fold lower levels of hepatic hepcidin mRNA. In addition, the stimulation of the upstream signaling pathway of SMAD with several of the bone morphogenic proteins (BMPs) induced hepcidin in control hepatocytes suggesting that SMAD4 positively regulates hepcidin expression. Subsequently, BMP6 has been found to be the key ligand in this pathway (62,63).

The full activation of BMP requires a BMP receptor (BMPR) as well as hemojuvelin (HJV), a glycosylphosphatidylinositol-linked membrane-bound protein that can be regulated by matriptase-2 (also known as transmembrane protease, serine 6 (TMPRSS6)), a cell surface serine protease. (64) When activated, matriptase-2 cleaves HJV preventing it from functioning as a BMPR coreceptor (65). HJV can also be secreted in a soluble form, which acts in an opposite manner to inhibit hepcidin expression by binding BMP (66).

The regulation of hepcidin by iron is not well understood, but appears to be regulated through the interaction between TFR1 or transferrin receptor 2 (TFR2) with the hemochromatosis gene product, HFE (67). Unlike TFR1 which is ubiquitously expressed, TFR2 expression is restricted to the liver (68). Both receptors bind to HFE but at different domains (69,70). HFE can compete with transferrin for TFR1 binding, which is believed to sequester HFE from the cell surface and prevent its association with TFR2 (71). In contrast, the binding of TFR2 by HFE and holotransferrin results in the induction of hepcidin expression possibly through ERK1/2 signaling.

This chapter describes clinical disorders that arise from derangements in molecules that are part of the iron metabolic/regulatory pathway. In most cases, the clinical consequences of these disorders result from iron deficiency, iron overload, or anemia. Particular attention is given to classic hereditary hemochromatosis (HH), which is the most common and best studied of genetic disorders of iron metabolism. While many questions concerning iron regulation remain unanswered, many subtypes of iron overload syndrome, types 1, 2a, 2b, 3, and 4, and several molecular mediators have been identified (Table 101-1). Further details, where known, concerning structure–function relationships of these molecules, are discussed below in reference to individual genotypes and phenotypes.

### 101.3 SYNDROMES OF IRON OVERLOAD

Several clinical syndromes characterized by increased body iron stores have been identified (Table 101-2). Typically, these are classified as primary or secondary disorders of iron overload. Various classification schemes have been proposed for the primary disorder, but no consensus has been reached. Nevertheless, five disorders leading to iron overload can be classified as primary disorders and are characterized by clinical, biochemical, and genetic criteria. Whether they should all be classified as “hemochromatosis” is a matter of semantics. The most extensively studied of these disorders is the classic HH (MIM #235200), which affects adults in their later life and is caused by mutations in the *HFE* gene. Juvenile hemochromatosis (JH or HFE2, MIM #602390) is a syndrome similar to HH, but presents with massive iron loading in the second or third decade. Cardiac involvement is usually a prominent feature. Two genes have been implicated in this form of iron overload. Rare cases have been reported with mutations in the *HAMP* gene, which encodes hepcidin. However, most cases show a pattern of recessive inheritance that maps to chromosome 1q (72–74). Mutations in the hemojuvelin (*HJV*) gene suggest that this is the causative gene (75,76). A variety of mutations in the *TfR2* gene have been characterized as a rare cause of iron overload in families without typical mutations in *HFE* and has been classified as HFE3 (77–80). Mutations in *SLC11A3* (FPN1) produce iron overload in an

**TABLE 101-2 Disorders of Iron Metabolism**

#### Syndromes of Iron Overload

HH (HFE1, MIM #235200)  
 C282Y homozygosity  
 C282Y/H63D compound heterozygosity  
 JH (HFE2, MIM #602390)  
 TFR2-related hemochromatosis (HFE3, MIM #604250)  
 NH (HFE4, MIM #231100)  
 Atransferrinemia (MIM #209300)

#### Secondary Iron Overload Syndromes

Iron-loading anemias  
 Thalassemia major  
 Sideroblastic anemia  
 Chronic hemolytic anemias  
 Iron overload associated with chronic liver disease  
 Chronic viral hepatitis (B and C)  
 Alcoholic liver disease  
 NASH

#### Other Disorders Resulting in Derangements of Iron Handling

IRIDA (MIM #206200)  
 Hypochromic microcytic anemia with iron overload (MIM #206100)  
 African iron overload (MIM #601195)

#### Primary Defects of Iron Handling as a Cause of Neurological Disease

Friedreich ataxia (MIM #229300)  
 XLSA/A (MIM #300751)  
 Aceruloplasminemia (MIM #604290)  
 Neuroferritinopathy (MIM #606159)  
 HHCS (MIM #600886)

#### Other Neurological Disorders Associated with Abnormal Iron Handling

PKAN (MIM #234200)  
 HARP syndrome (MIM #607236)  
 INAD (NBIA2A, MIM #256600)  
 Atypical neuroaxonal dystrophy (NBIA2B, MIM #610217)

autosomal dominant pattern and have been classified as HFE4 (81–85).

The genetic basis of other primary disorders of iron overload including neonatal hemochromatosis (NH), African iron overload, and a disorder in a population in the Solomon Islands, has not been identified. NH (MIM #231100) is characterized by prenatal onset of liver disease and extrahepatic iron deposition that spans the reticuloendothelial system. NH is not genetically linked to chromosome 6p or mutations of the *HFE* gene. Recently, it has been proposed that NH is the result of a maternal alloimmune reaction and that iron loading is only secondary to the hepatitis (86). African iron overload (MIM #601195) was first described in the Bantu tribe of Africa, and was thought to be due to excessive dietary iron found in ale brewed in cast-iron drums. Recent studies, however, suggest a strong genetic influence on the development of this condition (see below). Dominant inheritance of an iron-loading syndrome has been identified in a population on the Solomon Islands. Eason et al. described a 96-member Melanesian kindred with 31 cases of iron overload (87). Liver biopsies from

19 of these patients showed features similar to those of the HFE-linked autosomal recessive HH in Caucasians. However, this family showed a pattern of autosomal dominant inheritance, with the involvement of three and possibly four consecutive generations, with a high frequency of transmission from parents to children and equal gender distribution. Linkage and segregation analysis support autosomal dominant inheritance, with no demonstrable HLA linkage.

Iron overload may also occur as a result of multiple transfusions such as those in sickle cell disease or from disrupted iron metabolism due to an underlying disease. Clinically, the iron overload in these conditions may be indistinguishable from primary disorders of iron overload. Generally, iron loading may occur as a result of ineffective erythropoiesis or chronic liver disease. The former includes sideroblastic anemia and thalassemia while the latter is frequently seen in alcoholic liver disease, chronic hepatitis C and B, and nonalcoholic steatohepatitis (NASH).

### 101.3.1 Hereditary Hemochromatosis

The French internist Armand Trousseau is credited with the first case report of hemochromatosis in 1865 (88). The term “Hemochromatosis” was later coined by the German pathologist Von Recklinghausen (1899) as a clinical condition characterized by the classic triad of hepatomegaly, diabetes, and bronzing of the skin (89). The disease was thought to be caused by a “chromogenic” substance carried in the heme, hence its name. Sheldon later suggested that the disease was an inherited process and established the link with abnormal iron metabolism (90). Simon et al. established the recessive nature of inheritance and linked the syndrome to the major histocompatibility complex (MHC) on chromosome 6 (91). In 1996, Feder et al. identified the *HFE* gene and delineated the mutations therein responsible for the clinical condition. These mutations may lead to excessive iron loading, evidenced by biochemical markers of iron stores and subsequently, organ damage and the clinical manifestations of HH. Although many still consider HH a rare disease, population-based testing has demonstrated a prevalence of C282Y homozygotes of five per 1000 among the white populations in the United States, Europe, and Australia, making it the most common inherited disease of the liver (92,93).

The cause of iron overload in HH is misregulation of dietary iron absorption in the duodenum, the site of highest iron absorption in humans. Normally, homeostasis of body iron levels is maintained by regulating intestinal iron absorption. When iron stores are adequate, liver hepcidin expression is induced. Hepcidin circulates to the duodenum where it binds FPN, which is then degraded preventing the export of iron into circulation. In the periphery, downregulation of FPN increases stored iron in macrophages by inhibiting the release of iron. In HH, there is a failure to downregulate intestinal

iron absorption despite adequate body iron stores. This causes an insidious accumulation of iron in the body with no compensatory mechanism of elimination. In addition, the failure to inhibit iron release from macrophages leads to increased levels of circulating iron and preferential accumulation in parenchymal cells. Over time, excess iron accumulates in vulnerable tissues, such as the liver, causing cirrhosis and possibly hepatocellular carcinoma; in the heart, causing congestive failure and dysrhythmias; in the pancreas, causing diabetes; in the skin, causing melanoderma; and in organs of the endocrine system, causing amenorrhea or loss of libido. Although it appears the symptoms of HH are due to iron-related injury, iron depletion does not reverse all of the clinical features, particularly arthropathy. It has been suggested that the degree of symptoms depend on the degree of overload, age, and sex, along with other genetic modifiers and comorbidities. Generally, older patients present with vague complaints of malaise, abdominal pain, or arthritis. Younger patients generally present with amenorrhea or cardiac symptoms.

The diagnosis of HH is made by a combination of clinical suspicion and biochemical and genetic testing. At the least, elevated iron stores in the body without a clear cause for secondary iron overload should lead the clinician toward a diagnosis. TS is almost universally increased in patients with hemochromatosis with a rise in serum ferritin levels later, indicating the accumulation of iron in tissues. Although serum ferritin measurements are a good reflection of body iron stores, they can often be elevated secondarily to chronic inflammation, alcohol abuse, steatohepatitis, and obesity, which may confound the diagnosis of HH. While fasting, TS values exceed 50% and 60% for women and men, respectively, the sensitivity is 0.92 with a specificity of 0.93. At this cutoff value, the positive predictive value is ~86%. Lowering the cutoff of transferrin to 45% predictably increases sensitivity, but will likely include HH heterozygotes and diseases of relatively minor iron overload. Instead, the pairing of serum ferritin with TS has a sensitivity of up to 94%, better than either test alone (94,95). Liver biopsy is no longer necessary for diagnosis in most cases and is often considered optional, with a shifting focus toward prognostication and evaluation of concomitant diseases. Prussian blue stain classically shows the accumulation of iron in parenchymal cells and the advanced disease will likely show nodular cirrhosis.

Phlebotomy remains the treatment of choice but must be started before irreversible tissue damage has occurred. In early studies, 27% of untreated patients developed advanced cirrhosis and hepatocellular carcinoma. Later studies show that phlebotomy leads to some improvement in the severity of hepatic fibrosis, the management of varices, and some reversal of hypogonadism (in young men) and cardiac dysfunction (92,96–98). Phlebotomy is inexpensive and is widely available. Early recognition of signs and symptoms, and aggressive screening in families



of newly described probands are the cornerstones of successful management.

**101.3.1.1 Clinical Features.** Intracellular iron overload carries a toxic burden that affects the heart, pancreas, endocrine system, joints, and most consistently, the liver (95). Iron-related oxyradicals promote the peroxidation of lipid membranes and subsequently, organelle disruption and cellular toxicity. If left unchecked, this process can contribute to hepatocellular necrosis, stellate cell activation, fibrosis, and eventual cirrhosis. Excessive iron accumulation occurs when the total body burden of iron reaches ~8g. In males, this typically occurs in the fourth decade of life. In females, menstruation delays iron accumulation for about a decade and symptoms usually begin after menopause. This may explain why, in adults, HH is found 2–10 times more frequently in males than in females (99–101).

The earliest studies of HH were criticized for lacking control groups and the notable exception of patients with milder disease. The signs and clinical symptoms at the time of diagnosis of HH have changed over time as awareness and screening have increased. Older studies of HH cohorts involved the most severely affected while screening studies of large populations suggest that most individuals with HH have no significant disease. In a cohort of German patients, cirrhosis was present in nearly 80% of those diagnosed between 1947 and 1969, compared to only 44% in those diagnosed after 1970 (102). Similarly, an Italian study of a prospectively collected cohort of patients with hemochromatosis, diagnosed between 1976 and 2007, indicated that patients diagnosed in recent years are more likely to have disease characterized by milder iron overload and lower prevalences of cirrhosis and extrahepatic manifestations of iron loading (103). Of note however, was that the risk of developing hepatocellular carcinoma in cirrhotic patients did not change over the years.

Common symptoms of HH include weakness, abdominal pain, arthralgia, loss of libido, and amenorrhea (102). Some degree of weight loss, fatigue, and lethargy are almost invariable complaints at the time of presentation, with extreme fatigue in 46% (104–106). Abnormalities in liver-associated enzymes are found in over 70% of patients at the time of diagnosis. Common physical findings include hepatomegaly, increased skin pigmentation, loss of body hair, and splenomegaly (102). Arthralgias and fatigue are the most common early symptoms, occurring in over 20% of precirrhotic HH individuals (101,107,108).

Just as the liver has tremendous capacity for regeneration, it also has an impressive ability to store iron. This capacity to accumulate iron within hepatocytes is the main reason it requires years before the development of symptoms. Hepatomegaly is a frequently observed sign of liver disease and is associated with the presence of cirrhosis, although it may often be lacking in the young asymptomatic homozygous patient (95,109). Up

to 93% of symptomatic adult patients have a palpable liver (100,110). Serum bilirubin and transaminases may be normal or slightly elevated, but usually not greater than twice normal (111).

As with many other causes of cirrhosis, HH is often associated with diabetes mellitus but apparently at a higher frequency. In addition, the glucose intolerance described in HH patients is often more severe than in other forms of cirrhosis (111,112). The abnormal glucose tolerance of HH is due to both impaired insulin secretion by pancreatic islet cells and peripheral insulin resistance (113,114). Recent research has found reduced insulin sensitivity in non-HH subjects with greater iron stores, raising the possibility that even moderate degrees of iron excess may result in increasing degrees of glucose intolerance, as with type 2 diabetes (97,115). Diabetes had been previously seen in up to 83% of adult patients with HH, including nearly 20% of HH patients without cirrhosis (90,101,102,104).

The characteristic skin hyperpigmentation (melanoderma) is seen only in very severe forms of HH. When pronounced, it is often gray but sometimes brownish bronze, diffuse in distribution, and mainly affects the sun-exposed areas of the skin. The pigmentation is not iron but rather melanin induced by iron's effects on melanocytes in the setting of epidermal thinning (116,117). Hemosiderin is also present in varying quantities in the dermal basement membranes of eccrine sweat glands or in the connective tissue stroma surrounding these cells. Mucosal pigmentation may also be seen, with slate-gray patches in the mouth. About half of the patients show cutaneous atrophy, ichthyosiform changes, and flattening of the nails or true spoon nail (118). Loss of body hair is also a common skin finding in these patients without clear improvement after treatment.

Arthritis is an early and common symptom of HH in approximately one-third to one-half of the patients (100,111,119–122). Joint involvement can often be severe, especially in patients with substantially elevated ferritin and the damage can require replacement of joints (122,123). The first, second, and third metacarpophalangeal and proximal interphalangeal joints are typically involved, with the next likely being the wrist and knee joints. Radiographic studies frequently show subchondral cyst formation, and bone demineralization is a common finding (124). Degenerative joint disease, osteophyte formation, and joint-space narrowing are present in 64% of patients (121). Unfortunately, phlebotomy does not prevent the onset or progression of arthritis and may even worsen these symptoms (111,119).

Heart disease is present in 15–20% of adult patients, usually as congestive heart failure and arrhythmias, such as atrial fibrillation (125). Electrocardiographic abnormalities consist of a decrease in QRS amplitude and T-wave flattening or inversion. Echocardiography typically shows ventricular dilatation and global dysfunction (126), but restrictive features have also been described

(127). The cardiac implications of HH are still evolving, but recent evidence suggests a correlation with essential hypertension (96,98). The extent to which iron loading causes cardiac symptoms is also unclear, as it is possible that oxidative stress in asymptomatic patients may be related to left ventricular diastolic dysfunction (128).

**101.3.1.2 Pathophysiology of HH.** A similar model to physiological iron regulation can be found in normal glucose regulation; whereas the secretion of insulin by the pancreas regulates glucose levels, and the production of the protein hepcidin by the liver regulates the absorption and mobilization of iron (129). As detailed earlier in this chapter, hepcidin, a protein encoded by *HAMP*, binds to the cellular iron exporter, FPN1 and induces its degradation. Through a signaling pathway that involves HFE as well as TFR2, HJV, and BMPR, excessive iron leads to an upregulation of hepcidin expression (129,130). Inflammatory agents such as interleukin 6 also upregulate hepcidin production. In cases of low iron, the synthesis of hepcidin is downregulated, leading to increased intestinal absorption and mobilization of iron. The core problem in HH is a genetic defect in HFE that impairs the signaling pathway leading to decreased synthesis of hepcidin. This, in turn, results in a failure to adequately reduce intestinal iron absorption and an inappropriate two- to fourfold increase in dietary iron absorption (131–133). In both HH and normal individuals, the percentage of iron absorption is inversely related to body iron stores (131–136). However, when body iron stores are replete or elevated, HH patients fail to downregulate both heme and nonheme iron absorption. This problem is compounded because iron excretion is a nonregulated process; that is, there is no physiological mechanism for excreting excess iron. Over the progression of years, this results in the insidious accumulation of toxic levels of iron in vulnerable tissues. The precise mechanism through which reactive iron induces cellular oxidant damage involves lipid peroxidation, DNA modifications, and protein damage (137). While the majority of iron is bound to ferritin or transferrin and is not available for redox reactions, a component of the nontransferrin-bound iron, the labile plasma iron pool, is available to catalyze the formation of reactive oxygen species as a Fenton agent, catalyzing Haber–Weiss reactions and it is this fraction that is notably elevated in HH (138). Iron and hydrogen peroxide ( $H_2O_2$ ) produce reactive oxygen species that cause tissue damage via oxidation of lipids, polyunsaturated fatty acids, and proteins along with nucleic acids (139).

As discussed previously, the progressive accumulation of iron in the liver may lead to fibrosis and eventually cirrhosis. The initial deposition of iron is primarily in the pericanalicular hepatocytes, with greatest concentration in the periportal hepatocytes. When an unclear threshold of iron accumulation is reached, cirrhosis ultimately develops. Although there is perhaps a role for inflammatory mediators, HH is relatively unique

among progressive liver diseases for a lack of inflammation prior to the development of serious disease. It is primarily thought that sideronecrosis activates macrophages, which along with activated Kupffer cells, release profibrotic cytokines (140,141). Stellate cells are also a critical part of the progression toward cirrhosis in HH, although their exact role is yet to be determined.

The frequency of *p53* mutations in the liver tissue of HH subjects is suggestive of oxyradical damage, although the exact mechanism by which *p53* mutations occur in hemochromatosis is not known. The direct mutagenic properties of iron also likely contribute to the development of hepatocellular carcinoma in these patients. Although hepatocellular carcinoma is a known complication of all causes of cirrhosis, other liver diseases rarely have such high rates of *p53* mutations (142). Evaluating the DNA from 170 cases of hepatocellular carcinoma, Vautier et al. found that 29% of all cases incurred *p53* mutations (143). *p53* mutational clustering was seen in 10 of 14 (71%) hepatocellular carcinomas from HH patients with A:T to G:C transitions at codon 220 in five cases. This mutation would be predicted to change a tyrosine residue to cysteine within the core domain of *p53*, the region that interacts directly with DNA. However, A:T to G:C transversions are not consistent with iron-induced oxidation of guanine to 8-hydroxy-2-deoxyguanosine, a frequent measure of oxidant DNA damage. In a separate study by Hussain et al., higher frequencies of G:C to T:A transitions were seen at codon 249 and C:G to T:A at codon 250 (144). This was also seen with the in vitro treatment of normal human fibroblasts with  $H_2O_2$  and  $FeCl_3$ , resulting in G:C to T:A transversions at codon 249 of the *p53* protein (145). Carmichael and colleagues did not find an increase in 8-hydroxy-2-deoxyguanosine in liver DNA from HH patients, but they did observe enhanced levels of bulky DNA lesions (146). Subsequently, they found a fourfold higher level of etheno-DNA adducts in HH livers compared to controls (147). These findings suggest that lipid peroxidation and subsequent modification of DNA bases may be an important mechanism in the formation of hepatocellular carcinoma in HH. Of interest, the aforementioned Hussain study also demonstrated that 28% of HH cases demonstrated higher levels of inducible nitric oxide synthase in the liver. Although more commonly associated with Wilson's disease (60% of cases), this could potentially implicate nitric oxide as a source of increased oxidative stress (144).

**101.3.1.3 HFE Gene.** Up to 90% of HH patients of northern European ancestry are homozygous for the same 845 G-to-A mutation of the *HFE* gene, resulting in a substitution of the cysteine to tyrosine at codon 282 (C282Y mutation) (148). HFE is highly expressed in hepatocytes and its importance in iron regulation has been confirmed by hepatocyte-specific knockouts of the *HFE* gene in mice. Feder et al. first identified the *HFE* gene by a positional cloning approach genotyping a cohort of

101 HH patients with 45 CA-dinucleotide markers saturating the distal MHC region on chromosome 6 (149). A number of genes were found in close proximity to markers D6S2240 and D6S2233, which described a peak of genetic association spanning 600,000 base pairs. One candidate gene, eventually named *HFE*, encoded a novel MHC class I-like protein whose relationship with iron metabolism was initially unclear. Later, a second mutation was identified in individuals heterozygous for the C282Y mutation but still with clinical evidence of HH. These patients carried a C-to-G transversion at position 347 in exon 2, resulting in a change from histidine to aspartic acid at residue 63 (H63D). Sequence analysis of the translated product initially suggested that the C282Y mutant protein fails to associate with  $\beta_2$ -microglobulin ( $\beta_2m$ ), which is required for surface expression of MHC class I protein. Consistent with these findings was the prior description of the  $\beta_2$ -m deficient mouse recapitulating HH (150–152). Subsequent biochemical analysis of the HFE protein confirmed that the C282Y mutation significantly reduces cell surface expression of the HFE- $\beta_2$ -m complex in transfected cells, while the H63D mutation has little to no effect on  $\beta_2$ -m binding (150,151). Thus, the C282Y mutation has a much more pronounced effect on HFE protein function than H63D.

Genetic knockout studies have since shown that mice lacking HFE develop iron overload and other features similar to HH, including a high TS and accumulation of iron in hepatocytes (152). Of note, these mice develop more severe iron overload than “knock-in” mice homozygous for the orthologous C282Y mutation of HFE (153).

**101.3.1.4 HFE Protein.** The HFE protein is expressed in all tissues except for the brain (154), with a prominence in duodenal crypt cells. A possible mechanism for the involvement of HFE in iron metabolism was first suggested by studies showing that HFE associates physically with the TfR (151,155,156). Biochemical studies show that HFE can form a ternary complex with TfR and transferrin (157). The interaction between wild-type HFE and TFR is pH dependent, with high-affinity binding observed at the basic pH of the cell surface and no binding at the acidic pH of the intracellular vesicles. Transfection studies of HFE in a variety of cell types have led to conflicting conclusions regarding the consequences of this binding. Transfection of the human duodenal adenocarcinoma cell line HuTu-80 with wild-type HFE leads to decreased ferritin and increased TFR. This is consistent with inhibition of transferrin-bound iron uptake by intestinal epithelial cells (158). This contradicts the *in vivo* studies of *Hfe*-deficient mice, however, which demonstrate the impaired uptake of plasma transferrin by the duodenum, suggesting that HFE promotes uptake of transferrin-bound iron by intestinal epithelial cells (159). Nonetheless, most studies in which HFE is overexpressed have reported reduced uptake of transferrin-bound iron (155,160).

Following the identification of TfR2 and hepcidin, it became clear that the primary role of HFE is to regulate

hepcidin expression through its interaction with TfR2 on hepatocytes. HFE has been shown to bind to both TfR1 and TfR2, competing with transferrin in the former and noncompetitively in the latter (161). In contrast to the binding to TfR1, which involves the  $\alpha$ -1 and  $\alpha$ -2 domains of HFE (69), binding with TfR2 occurs via the  $\alpha$ -3 domain of HFE (70). In addition, the binding sites for holotransferrin and HFE overlap on TFR1 but not on TFR2 (69,162). Ultimately, the interaction between HFE and TfR1 and TfR2 results in changes in hepcidin expression. For example, mice with a mutation that prevented the interaction of Hfe and TfR1 displayed high levels of hepcidin (71). In contrast, mice carrying mutations favoring constitutive binding of Hfe to TfR1 exhibited low levels of hepcidin suggesting that TfR1 serves to sequester HFE, preventing its interaction with TfR2, thereby decreasing signaling for hepcidin expression. Accordingly, holotransferrin competes with HFE for TfR1 binding and allows HFE to interact with TFR2.

The C282Y mutation disrupts a disulfide bond of the alpha-3 domain that is critical for correct folding and association with  $\beta_2m$ . MHC class I proteins require  $\beta_2m$  for normal trafficking from the Golgi to the cell surface. The outcome of transfection studies with *HFE* mutants and transferrin receptor would therefore be predicted by the amino acid sequence. The H63D substitution is in a loop within the  $\alpha$ -1 domain, where the histidine participates in a salt bridge. The H63D mutation disrupts this salt bridge and may thereby cause a local change in the tertiary structure of the protein that could affect the function of the HFE protein. The C282Y mutant is not expressed on the cell surface, and therefore does not bind transferrin receptor and does not inhibit cellular iron accumulation. In contrast, the H63D mutant is expressed on the cell surface of transfected cells, but has little effect on cellular iron accumulation. Neither of these mutations has been found to alter the affinity of HFE for TfR1.

The importance of hepcidin in the development of HH has been well established. Decreased levels of hepcidin are associated with higher levels of serum and tissue iron, and liver hepcidin mRNA is inappropriately low in patients with *HFE*-associated HH (163,164). Moreover, forced expression of hepcidin prevents iron loading in *Hfe*-deficient mice (165). In the C282Y mutation, the HFE protein fails to interact with TfR1 and/or TfR2, causing a decrease in the necessary inhibitory pathway.

**101.3.1.5 Genetics.** Sheldon first proposed that hemochromatosis was caused by a generalized disturbance of cellular iron metabolism that prevented iron from exiting the cell (90). Simon and colleagues (91) demonstrated that the inheritance of the hemochromatosis gene is associated with inheritance of certain HLA alleles, and Kravitz and coworkers (166) established formal genetic linkage with certain HLA alleles, thereby fixing the gene location nearby the MHC region on chromosome 6p21.3.

Based on the population distribution of HH, Simon and colleagues postulated that the HH allele originated in a Celtic population (167). Analysis of the C282Y mutation has lent further support to this hypothesis. The Celts first appeared in Europe, north of the Alps around 1000 BC. Although never a united empire, the Celtic culture spread throughout central, southwestern, and south-central Europe. Present-day “Celts” include the Irish, Scots, Welsh, and Bretons. These populations have an increased C282Y allele frequency of 0.069 compared to Anglo-Saxon (0.059), southern European (0.025), and Russian (0.018) populations (168,169). The C282Y allele frequency of 0.065 in Nordic populations of Denmark, Sweden, Finland, and Norway is similar to that found in Celtic populations. Two hypotheses might explain the high frequency of C282Y in the Nordic population. The first contradicts Simon’s original hypothesis that the allele originated in a Celtic population. It states that the C282Y mutation originated in a Nordic population from which the Celts were derived. Alternatively, the C282Y mutation may have been brought to Nordic populations during invasions of Celtic populations, around 900 AD. Based on the linkage disequilibrium surrounding the C282Y mutation and recombination frequencies in this region, Ajioka and colleagues (170) estimated that the C282Y mutation originated 60–70 generations ago. Assuming an average generation time of 20 years, the C282Y mutation is estimated to have occurred around 600–800 AD. This is most consistent with the latter hypothesis and a Celtic origin of HH. However, other estimates place the origin of the C282Y mutation as early as 4000 BC (171–173).

Several authors have commented on the possible selective advantages the HH phenotype might offer. Cazzola and coworkers (174) proposed that the HH allele has been maintained in the population as a result of a heterozygote advantage. The proposed advantage is the slightly increased iron absorption in the heterozygote, but to a degree that is unlikely to produce iron overload. In times of war and famine, when iron in the diet is scarce, the heterozygote may have an advantage. Ritter and colleagues (175) speculated that the HH phenotype could be beneficial in conditions of iron deficiency: homozygous males would not lose reproductive capacity from testicular effects; and females, homozygotes, and perhaps heterozygotes as well, would be better prepared to meet the increased iron demands of pregnancy. In this regard, the phenotype could favor multiparity in that it might help to avoid iron deficiency in the pregnant mother. In fact, females heterozygous for the C282Y mutation have a lower incidence of iron deficiency than controls (176). Rotter and Diamond (177) offer an evolutionary biologist’s perspective of HH stating “That the hemochromatosis gene is considered deleterious at all reflects a male-chauvinist evolutionary view: increased iron absorption is good for most women. From an unbiased

evolutionary view, the deaths of some post-reproductive males are a small price to pay for protecting many women in the same population against anemia.”

More recently, resistance to intracellular pathogens has been proposed as a potential advantage of the C282Y mutation (178). Iron deficiency has long been and continues to be the most common single nutrient deficiency worldwide and the *HFE* gene is conserved throughout vertebrates. Therefore, a unique selective pressure may have been operative during the expansion of the C282Y mutation. In Europe, this may have included epidemics of *Yersinia pestis* (Black Plague) and *Mycobacterium tuberculosis*. Both of these pathogens are intracellular organisms that can evade immune defenses of macrophages and are more virulent in the presence of iron. Although this may seem antithetical, if iron overload did not occur until later in life, if ever, the relative iron-deficient state of macrophages may have added additional protection for these infections.

**101.3.1.6 Penetrance and Modifiers.** HH is a disease of variable penetrance. Biochemical penetrance defined as elevated iron indices is incomplete, with 75% of men and 50% of women homozygous for C282Y showing evidence of iron loading. Although previous estimates ranged as high as 50%, newer data suggest the frequency of clinical sequelae of iron overload is particularly low in women and ~25% in men (179). Complicating the study of the natural history of HH is the latent period, in which iron is deposited in tissues over years of clinically silent disease. The factors that contribute to the varying expression of disease manifestations may include factors that potentiate iron loading or modify the disease phenotype (e.g., accelerating the progression to cirrhosis) and may include both genetic factors that influence iron metabolism, inflammation, or fibrosis. Additional non-genetic risk factors include age, diet, insulin resistance, and alcohol intake (Table 101-3) (148,180).

Evidence of strong concordance between iron overload and disease severity among related individuals has been identified in several studies, but is consistent with shared genetic or environmental factors (181–185). However, a study of monozygotic and dizygotic twin pairs showed that the pattern of residual variation in serum iron indices, after adjusting for the effect of the C282Y mutation, was consistent with additive effects of multiple genes (186). After correcting for age and body mass index, the proportion of variance explained by additive genetic factors was estimated to be 23% and 31% for iron, 66% and 49% for transferrin, 33% and 47% for TS, and 47% and 47% for ferritin for men and women, respectively. In addition to these studies in C282Y homozygotes, a genome-wide association study of healthy adolescent twins and siblings on serum markers of iron status identified not only the *HFE* C282Y association, but also associations between a *TM6RS6* SNP and serum iron and multiple *TF* SNPs and serum transferrin (187). Specifically, the variants in transferrin



**TABLE 101-3 Potential Modifiers of Hereditary Hemochromatosis Expression**

Modifier	Effect on Iron Loading	Effect on Liver Cirrhosis	Reference
<i>Environmental Factors</i>			
Gastric acid suppression	Reduced venesection requirements in those using proton pump inhibitors	<i>Unknown</i>	(219)
Dietary tannins	No significant effect on venesection requirements	<i>Unknown</i>	(217)
Dietary iron intake	Positive correlation between dietary heme iron intake and serum ferritin in postmenopausal women	<i>Unknown</i>	(134)
Blood donation	No significant difference in venesection requirements	<i>Unknown</i>	(487)
Alcohol consumption	Decreases hepcidin and increases iron loading in mouse model of HH	9-fold greater incidence of cirrhosis in those drinking more than 60g/day	(214)
Viral hepatitis	<i>Unknown</i>	Infection with hepatitis C virus was associated with cirrhosis at a younger age and with lower hepatic iron concentration	(215)
Hepatic steatosis	<i>Unknown</i>	Steatosis on biopsy correlates with fibrosis prior to venesection	(220)
<i>Genetic Factors</i>			
Hepcidin ( <i>HAMP</i> )	Rare alleles p.[Arg56X], p.[Arg59Gly], and c.[−153 C>T] have been associated with severe iron loading in <i>HFE</i> C282Y homozygotes		(196,197)
Hemojuvelin ( <i>HJV</i> )	Rare alleles p.[Lys101Pro] and p.[Gly320Val] have been associated with severe iron loading in <i>HFE</i> C282Y homozygotes		(488)
Haptoglobin ( <i>HP</i> )	Conflicting results on effect of Hp2-2 polymorphism on serum ferritin levels and venesection requirements		(198–201)
Tumor necrosis factor ( <i>TNF</i> )	Conflicting results on effect of c.[−238G>A] and c.[−308G>A] on various measures of iron loading	Conflicting results on effect of c.[−238G>A] and c.[−308G>A] on cirrhosis	(202,203,489)
Transforming growth factor-β ( <i>TGFB1</i> )		Single study associated p.[Arg25Pro] with cirrhosis	(204)
Myeloperoxidase ( <i>MPO</i> )	c.[−463G>A] was not associated with elevated serum ferritin and hepatic iron index	c.[−463G>A] was associated with cirrhosis in the same study	(205)
Mitochondrial DNA (mtDNA)	mtDNA [16189 T>C] was associated with biochemical iron overload in <i>HFE</i> C282Y homozygotes		(206)
Bone morphogenic protein 2 ( <i>BMP2</i> )	dbSNP rs235756 was associated with higher serum ferritin and venesection requirements but has not been replicated		(207–209)
Cytochrome b reductase ( <i>CYBRD1</i> )	dbSNP rs884409 was with lower serum ferritin		(209)
Matriptase-2 ( <i>TMPRSS6</i> )	dbSNP rs4820268 was associated with lower TS adjusting for <i>HFE</i> genotype		(187,209)

(*TF*) along with the C282Y mutation at *HFE* accounted for ~40% of variations of serum transferrin levels.

The earliest studies searching for genetic modifiers reported more severe phenotypes in individuals carrying the ancestral HLA-A3/B7 haplotype (188,189) or the HLA-A1/B8 haplotype (190,191) suggesting genetic modifiers within the MHC region. However, these associations have not been consistently observed in more recent studies (190,192–194).

Rare alleles found in genes associated with other forms of iron overload syndromes have been identified in some severe cases of HH. There have been severely affected C282Y patients found to have rare *HAMP* and *HJV* mutations, accounting for a very small proportion of disease variants (195). Island et al. also showed massive iron overload in a C282Y homozygote with a C to T transition in the *HAMP* promoter sequence (196). This particular mutation was found within a BMP-responsive element,

decreasing the transcriptional activity of the *HAMP* promoter and the ability to bind to the SMAD protein complex (196). Further, variants in the *HAMP* gene have been associated with severe disease phenotype (197).

More common alleles in candidate genes have also been associated with both iron loading and liver disease severity. However, in most cases, including *HP*, *TNF*, *TGFB1*, and *MPO*, replication has been inconsistent or limited to a single study (198–206). The most recent example was a study of 592 C282Y homozygotes from France in which multiple SNPs spanning genes linked to non-*HFE* hemochromatosis (*TFR2*, *HAMP*, and *SLC40A1*) and to hepcidin expression (*BMP2*, *BMP4*, *HJV*, *SMAD1*, *SMAD4*, *SMAD5*, and *IL-6*) were genotyped and a significant association was detected between serum ferritin level and a common SNP in *BMP2* (rs235756) ( $P=4.42 \times 10^{-5}$ ) (207). However, a subsequent study by the same investigators failed to replicate the association with serum ferritin, though they did find an association with the amount of iron removed by phlebotomy (208). Indeed, another study also failed to replicate the association between this *BMP2* SNP and serum ferritin (209). This same study involving 863 subjects stratified by *HFE* genotype found that an SNP in the promoter of *CYBRD1* (rs884409) was associated with significantly lower serum ferritin levels in C282Y homozygotes and accounted for 11% of the variance in serum ferritin levels in C282Y homozygotes (209). Interestingly, in heterologous expression assays this polymorphism resulted in a 30% decrease in basal promoter activity consistent with its protective effects against iron loading.

Regardless of whether or not insulin resistance begets iron overload, or the reverse, the resultant oxidative stress likely contributes to the progression of steatosis. Recently, Hatunic et al. studied insulin secretion and insulin sensitivity in patients with HH, showing that a significant proportion (28%) of recently diagnosed HH subjects had abnormal glucose tolerance with insulin resistance (97). Furthermore, this study suggested that HH is an independent risk factor for abnormal glucose tolerance and possibly implementing glucose tolerance testing of individuals with hemochromatosis. Of interest, phlebotomy has been suggested to improve insulin sensitivity in patients with NASH, along with a decrease in alanine transaminase (210). The concept of glucose intolerance as an intrinsic risk factor of HH is still being studied. The normalization of ferritin and transferrin by phlebotomy in HH has also been shown to improve glucose tolerance in some patients (those with impaired glucose tolerance) but has little effect on insulin secretion (97). Regardless, interventions to normalize iron levels in subjects with HH and abnormal glucose metabolism should be started early to prevent progression to diabetes and the development of diabetes-related comorbidities.

In addition to the genetic factors associated with disease expression, several nongenetic factors clearly play a role. Age has a significant effect on the disease burden, as

iron accumulates over time. Cirrhosis and other complications of HH do not generally occur until serum ferritin levels rise above 1000 µg/L (211,212), which typically occurs in men at 50 years of age, or older (179,213). Female sex is generally thought to be protective due to iron losses from menstruation and pregnancy. Alcohol intake and chronic viral hepatitis C are risk factors for cirrhosis by themselves and may accelerate liver fibrosis progression directly or through enhanced iron accumulation (214,215). Hepcidin concentrations had previously been shown to be significantly lower in alcoholic liver disease subjects compared to healthy controls. In mice, hepcidin is downregulated by alcohol and accompanied with an increase of DMT1 and FPN1 (216).

The effects of dietary factors such as dietary iron, vitamin C that promotes iron absorption, and tea which contains tannins that inhibit iron absorption, have been suggested to modify the expression of iron loading in C282Y homozygotes but the evidence is limited (134,217). Interestingly, low serum copper levels have been associated with higher serum and hepatic iron levels, perhaps acting through ferroxidase activity of hephaestin and ceruloplasmin (218). Use of proton pump inhibitors that can cause iron deficiency due to altered gut acidity may also suppress the absorption of dietary iron in HH (219).

Obesity and nonalcoholic steatohepatitis are also important modifiers of HH. This is particularly important in the United States, where there has been a dramatic increase in obesity rates. The CDC estimates that by 2009, only Colorado and the District of Columbia had a prevalence of obesity less than 20%. Of this number, at least 20% of the obese population has significant steatosis. NASH is defined by histopathological changes on liver biopsy (macrovesicular steatosis, mixed inflammatory infiltrate, hyaline bodies, etc.) that are similar to those of alcoholic liver disease, in the absence of significant alcohol use. Insulin resistance is an important step in the progression of liver disease in patients with underlying diabetes and obesity. An association between liver steatosis and fibrosis in HH has been reported in one study (220).

**101.3.1.7 Prevalence of HH.** The prevalence of HH based on genetic and biochemical criteria has been well established in several large studies of various populations throughout the world. Before the identification of *HFE* as the hemochromatosis gene, biochemical studies estimating the prevalence of HH—defined by elevated TS and/or ferritin—ranged from 0.002 to 0.005 in the white populations of Australia, France, Scotland, Sweden, and the United States (221–226). In 1988, Edwards and colleagues (227) published the results of a screening study involving 11,065 presumably healthy blood donors in the state of Utah (227). Subjects with a TS of more than 62% underwent HLA typing, serum ferritin measurements, and liver biopsy. The HLA-identical siblings of probands also underwent liver biopsy. A TS of more

than 62% in an HLA-identical sibling was accepted as evidence of an HLA-linked abnormality of iron metabolism. This study demonstrated homozygosity for HH in men at a frequency of 0.0045. These studies clearly show that HH is the most common genetic disease affecting the liver, and is one of the most common recessive metabolic disorders in white populations (228). It is most common among individuals of northern European descent, and particularly those of Nordic or Celtic origin (229).

Cross-sectional studies of the frequency and distribution of the two *HFE* mutations in different populations confirm this association (168,230). The frequency of the C282Y mutation is highest in individuals of northwestern European origin, particularly in Ireland and the west coast of Brittany where allele frequencies of 0.094–0.142 have been reported (231–233). Allele frequencies ranging from 0.075 to 0.085 have been reported from parts of the United Kingdom, including Aberdeen, East Anglia, and Jersey as well as Oslo, Norway, and Umea, Sweden (234–237). A gradient of decreasing frequency of the C282Y mutation occurs from northwest to southeast with C282Y allele frequencies less than 0.01 in parts of Italy, Portugal, Spain, and Greece (238–243). Outside of white populations, the C282Y allele is rarely found in the indigenous populations of Africa, Central and South America, Eastern Asia, and the Pacific Islands. Interestingly, the H63D mutation has a global distribution that is somewhat similar to that of C282Y, and is most frequent in Europe, Asia Minor, and the Indian subcontinent and rare in Africa and among indigenous populations in Central and South America. The H63D mutation is more common than the C282Y mutation, found in 15–40% of European populations.

The prevalence of clinical HH varies depending on the criteria used for diagnosis and the geographic region studied. However, in general, studies of patients with HH agree with the findings on the geographic variation

in *HFE* mutations. Homozygosity for C282Y is present in 60–100% (average 85%) of white patients with clinically diagnosed HH (Table 101-4) (108,149,244–251). Highest rates of C282Y homozygosity are reported among patients who are of northern European extraction. Lower rates are found in patients from southern Europe (230,249,252), and few instances of homozygosity for C282Y have been reported among African-Americans with iron overload (253–255).

Although the C282Y mutation has a relatively high frequency in many populations and homozygosity is found in the majority of clinical hemochromatosis, questions remain regarding the disease burden of HH. Estimates of clinical penetrance as discussed above, suggest 25–50% of C282Y homozygotes develop iron overload and 10–25% develop some form of HH-related morbidity (256). Several large screening studies have previously been undertaken to assess the prevalence of biochemical iron overload and disease due to iron overload in HH. Olynyk and colleagues (213) screened 3011 unrelated adults of Bussleton, Australia, and identified 16 (0.5%) individuals who were homozygous for C282Y. Twelve underwent liver biopsy, which revealed fibrosis in three individuals and cirrhosis in one who also had a history of alcohol abuse. Symptoms that the authors attributed to HH were present in eight of the 16 C282Y homozygotes. However, a similarly sized study from a primary care clinic in Rochester, New York, identified 12 individuals as homozygous for C282Y, with none having evidence of cirrhosis or cardiac failure (257).

One of the largest of these studies involved the screening of 41,038 individuals at a health appraisal clinic in San Diego, California (258). All patients answered a health survey and were examined by a physician; 152 C282Y homozygotes were identified and compared to controls from the same population. Although C282Y homozygotes were more likely to report “liver problems” and to

**TABLE 101-4 Genotype Frequencies for *HFE* Mutations in Patients with Hemochromatosis**

Country	Number of Subjects	Genotype Frequency (%)					
		HHCC	HDCC	DDCC	HHCY	HDCY	HHYY
Czech Republic (490)	12	0.0	0.0	0.0	0.0	0.0	100.0
Australia (250,491)	184	1.1	0.5	0.0	2.7	0.0	95.7
Germany (492,493)	149	0.0	2.0	0.0	1.3	4.0	92.6
UK (237,494)	133	3.	0.0	0.8	0.8	2.3	92.5
Ireland (232,233)	90	3.3	0.0	1.1	2.2	1.1	92.2
Sweden (248)	87	1.1	1.1	1.1	1.1	3.4	92.0
Scotland (236)	54	3.7	0.0	0.0	0.0	5.6	90.7
Spain (495,496)	53	7.5	1.9	1.9	0.0	3.8	84.9
Portugal (497)	25	0.0	4.0	0.0	8.0	4.0	84.0
France (247,498–500)	496	4.4	3.6	2.6	1.0	6.0	82.5
Brittany (501,502)	843	4.2	3.4	1.2	4.0	5.1	82.1
US (149,246,503)	399	9.3	2.5	1.5	3.5	5.0	78.2
Austria (504)	40	10.0	2.5	2.5	0.0	7.5	77.5
Italy (249,505)	263	17.1	7.2	1.5	4.9	5.7	63.5
<b>Total</b>	<b>2,828</b>	<b>5.7</b>	<b>3.0</b>	<b>1.3</b>	<b>2.8</b>	<b>4.6</b>	<b>82.7</b>

have an elevated serum aspartate amino transferase level, there was no greater prevalence of diabetes, arthralgias, or heart disease. Liver biopsies were not performed, so little can be concluded about the degree of liver injury. Similarly, a Norwegian study of 65,238 individuals using biochemical markers, followed by genetic testing, identified 269 individuals with HH, but only four, all men, had advanced liver disease (99). In African- and Asian-American populations, HH and the C282Y mutation are uncommon (230,254,255,259–262). In Beutler and colleague's study of 9650 individuals, allele frequencies of C282Y by ethnicity were reported to be 0.063 for Whites, 0.027 for Hispanics, 0.002 for Asians, and 0.051 for Blacks (263). In their larger study of the same population, the prevalence of C282Y homozygotes was 0.0046 in Whites but only 0.0025 in Hispanics and 0.0023 in those reporting a mixed race or race other than those listed (258). No C282Y homozygotes were identified in over 1400 African Americans. The low frequency of iron overload and the C282Y allele in African-Americans is supported by the Rochester study where 16,031 primary care patients were screened, including more than 2000 African-Americans; only 25 patients—all White—were confirmed to have HH by liver biopsy (255). Of an additional 20 patients with TS consistently greater than 45%, serum ferritin greater than 200 mg/L, and no obvious cause of secondary iron overload, only one was African-American. Thus, the prevalence of clinically diagnosed HH based on the 45 patients was 0.0056 among Whites and 0.0009 among African-Americans.

In the Hemochromatosis and Iron Overload Screening (HEIRS) study, almost 100,000 participants from ethnically diverse populations were recruited and tested for TS, serum ferritin, and C282Y and H63D mutations of *HFE* (130). The estimated prevalence of C282Y homozygotes was higher in non-Hispanic whites (0.0044) than in Native Americans (0.0011), Hispanics (0.00027), Blacks (0.00014), Pacific Islanders (0.00012), or Asians (0.00000039). However, despite the lowest prevalence of C282Y, Pacific Islanders and Asians had the highest geometric mean levels of serum ferritin and mean TS, and of the 364 participants in whom iron overload had not been diagnosed and who had a serum ferritin level greater than 1000 µg/L, only 29 were C282Y homozygotes. These findings illustrate that iron overload syndromes in non-White populations may be even more common than *HFE*-related hemochromatosis. At this time, the genetic and pathophysiological basis of these common conditions is largely unknown.

#### 101.3.1.8 Diagnosis and Differential Diagnosis.

Diagnosis of HH occurs in three general settings: (1) when an isolated proband presents with suggestive clinical features, laboratory evidence of excessive iron stores, and a liver biopsy showing the characteristic features of iron deposition with fibrosis or cirrhosis; (2) in the investigation of relatives of a diagnosed proband; (3) in the setting of population screening or serendipitous

screening, as may occur with routine blood work as part of a regular health maintenance program. Because each setting reflects different relative risks of having HH, the diagnostic workup differs. Even within a given setting, additional factors such as age, sex, and history of chronic blood loss must be considered.

Physicians and healthcare workers must be aware of the early minimal signs, such as a slightly enlarged liver or metacarpophalangeal arthropathy, and of their value for early diagnosis. Appropriate laboratory tests and possibly liver biopsy should follow. Prenatal diagnosis is not recommended since penetrance is highly variable, symptoms due to fully penetrant HH are preventable, and a full and productive life can be expected with pre-symptomatic diagnosis and treatment. General population screening is currently not recommended (256).

Biochemical testing for iron overload is useful for both screening and management of HH. The most useful biochemical tests are TS (serum iron/total iron-binding capacity  $\times$  100) and serum ferritin. In adult HH patients, serum iron levels are commonly greater than 170 mg/dL (30 mmol/L). However, because serum iron alone correlates poorly with body iron stores, it is not a satisfactory test for screening.

TS is an early and sensitive indicator of iron overload. In a study of 537 subjects from 18 pedigrees, a TS above 62% correctly identified 92% of homozygous siblings of known HH patients (223). In the same study, the serum ferritin concentration identified only 72%. The TS is the most reliable single laboratory test for screening in children and adults (222,264–269). As a screening tool, TS has been established as the preferred biochemical test. The sensitivity and specificity depends on the cutoff value used. TS  $>45\%$  has a sensitivity of 98% and identifies 6.3% of the population, whereas a cutoff of  $>60\%$  has a sensitivity of 86%, with 1.1% of the population called positive (270–272). Sensitivity is also affected by sex, with the same cutoff being more sensitive in males than in females. For universal screening purposes, the increased sensitivity of a lower cutoff must be weighed against an increase in false positives. Assuming a prevalence of 0.45% in the population, a cutoff of 45% would have a positive predictive value of 7%. Alternatively, a cutoff of 60% would have a positive predictive value of 35%.

Serum ferritin is a practical test for estimating total body iron stores and the severity of iron overload in otherwise healthy individuals. Ferritin is an acute-phase reactant and is elevated in states of inflammation. C-reactive protein and erythrocyte sedimentation rate can help distinguish between an elevated ferritin due to increased iron stores and that due to inflammation. Normal values for serum ferritin range between 20 and 350 mg/L and are age and sex dependent. In normal subjects, 1 mg/L of ferritin correlates with 140 mg of iron storage per kilogram of body weight (273).

A combination of serum ferritin and TS seems to be more sensitive than the individual measurement of each,



with a sensitivity of 94%, specificity of 86%, positive predictive value of 73%, and a negative predictive value of 97% (265,266,274–276). Serum ferritin has previously been demonstrated to correlate well with mobilized body iron in healthy control subjects and in patients with advanced HH (224,277,278). Bassett (266) showed an excellent correlation of serum ferritin with body iron stores, even in young homozygous subjects with early iron overload. In contrast, serum iron concentration has a low sensitivity and specificity and poor predictive value for detecting homozygotes. Unbound iron concentration (UBIC) has been proposed as a more cost-effective screening tool. Unlike the two-step TS, UBIC is a single-step test. The validity of UBIC has been confirmed in large screening studies, but has not gained widespread acceptance (279–281).

The high frequency of the C282Y mutation in HH has made the testing of this mutation invaluable for the diagnosis of HH. In the setting of increased TS, homozygosity for C282Y confirms the diagnosis of HH without the need for further testing. Confusion often arises in the interpretation of C282Y/H63D compound heterozygotes and H63D homozygotes. Both of these genotypes are common in the normal population and carry only a small relative risk of developing clinical manifestations of HH (282). Depending on the clinical situation, liver biopsy may be indicated to establish the diagnosis in these situations. Other sequence variants have been identified but their contribution to HH has not been established. S65C is a single base-pair substitution of A by T at nucleotide 193, which leads to a serine to cysteine missense. This is in the region implicated in transferrin receptor binding (283). After correcting for the presence of C282Y, Arya and associates found no difference in the frequency of S65C in voluntary blood donors with TSs greater than 45%, compared to those less than 45% (0.017 vs. 0.022, respectively) (284). Parametric analysis by others, however, shows a significant increase in mean TS when S65C is present compared to the wild-type allele (285). In addition, the frequency of S65C is increased on non-C282Y/non-H63D chromosomes in HH compared to controls (0.16 vs. 0.02, respectively) (286). A novel mutation was identified in an individual heterozygous for C282Y and with typical features of HH. A G to T substitution at position +1 of intron 3 was identified and shown to result in loss of exon 3 in the mature transcript (287).

Prior to the identification of the *HFE* gene, liver biopsy and hepatic iron index (HII) were generally required for the diagnosis of HH (288,289). Early in the course, liver biopsy shows stainable iron in the periportal areas. In the later stages, it is widely distributed throughout the hepatic lobules. The extent of stainable parenchymal iron in the liver is graded from 0 to 4, with grades 0 to 1 being normal. Grades 2–4 indicate increased parenchymal iron stores. Grade 4 stainable iron is usually seen with advanced disease. In HH, unlike in secondary iron

overload syndromes, hepatocytes rather than Kupffer cells are preferentially loaded with iron.

The hepatic iron concentration is the most objective means of assessing body iron stores (288). The range of normal values is 5–40 mmol/g (mean 15 mmol/g). In advanced disease, the hepatic iron concentration frequently exceeds 150 mmol/g. Because iron normally accumulates in tissues with age, the HII (hepatic iron index = hepatic iron concentration/age) corrects for this normal process (290). Originally developed to discriminate heterozygous from homozygous HH, a HII >1.9 was later shown to differentiate HH from alcoholic liver disease (288,289,291). However, a HII >1.9 has been reported in many different forms of cirrhosis. In fact, of 106 liver transplant patients nearly one-third had evidence of HH by serum iron indices. Four of these patients had a HII >1.9, but none were homozygous for C282Y (292).

Nevertheless, liver biopsy may still be a valuable tool in certain individuals. The identification of established fibrosis portends an increased risk of hepatocellular carcinoma even after iron depletion. Factors that have been associated with liver fibrosis include age greater than 40 years and serum ferritin greater than 1000 mg/L. If any of these conditions is present, liver biopsy should be strongly considered (212,293).

Noninvasive methods for measuring tissue iron content, by computed tomography (CT) (109,275,294) or superconducting quantum interference device (SQUID) biomagnetic liver susceptometry, have been reported to be helpful in the diagnosis of hepatic iron overload (295,296), but their low diagnostic accuracy has limited their use in the past. Magnetic resonance imaging (MRI) has more recently been accepted as a noninvasive method for quantification of hepatic iron (297,298).

CT evaluation of the liver in HH demonstrates increased attenuation and correlates with serum biochemical studies, but not with HII (299–301). In addition, imaging studies cannot differentiate between parenchymal cell iron deposition consistent with HH and Kupffer cell iron deposition seen in other iron overload syndromes. Initial studies on the use of MRI did not prove to be reliable in assessing iron stores (302,303). Recent modifications have been used to establish a method of MRI imaging that correlates hepatic iron concentration and the natural logarithm of the ratio of the signal intensity of liver to the standard deviation of background noise ( $r = -0.94$ ). SQUID biomagnetic liver susceptometry measures also correlate with liver iron stores, but its availability is limited (304–306). Recently, its use in the imaging of fetal liver iron overload has been suggested, possibly prompting ante- or neonatal treatment in severe cases (307).

Historically, the distinction between advanced HH and secondary causes of iron overload, particularly alcoholic cirrhosis, has been difficult, as the pathologic findings are often similar (288). HH patients who use alcohol

to excess have compounded the confusion in analyzing clinical studies. The use of the HII had resolved much of the confusion by delineating the lesser hepatic iron content typical of alcoholic liver disease, when corrected for age. LeSage and coworkers (308) demonstrated that the hepatic iron content in alcoholic HH patients was the same or even less than for age-matched nonalcoholic HH patients, but well within the hepatic iron levels diagnostic for HH. The iron overload observed in patients with alcoholic liver disease has been suggested to be caused by the heterozygous state for the HH gene. However, they are clearly unrelated with no significant increase in C282Y prevalence among patients with alcoholic liver disease (309).

In addition to chronic liver diseases, iron overload consistent with HH has been reported in patients with beta-thalassemia trait (310), hereditary spherocytosis (311–313), refractory sideroblastic anemia (314,315), and congenital dyserythropoietic anemias (316). The presumed etiology for iron overload in these patients is increased iron absorption caused by ineffective erythropoiesis. A study of 40 patients with acquired sideroblastic anemia demonstrated that significant iron loading occurred in the absence of either the C282Y or H63D mutation (317). On the other hand, a significantly higher frequency of coinheritance of the C282Y allele was found in 18 unrelated X-linked sideroblastic anemia hemizygotes (318).

Porphyria cutanea tarda (PCT) has also been associated with increased iron due to alcohol, (see Chapter 99) chronic hepatitis C, and HH (319–322). Clinical improvement with phlebotomy supports the role of iron in the pathogenesis of PCT. Alcohol is an important factor in certain regions and is involved much more often than any other, including the *HFE* gene. In other regions where alcohol intake is lower, the expression of PCT could depend on other factors, including the *HFE* gene. Mutational analysis of *HFE* in several populations, including North America, Australia, Britain, and Italy, have clearly demonstrated that both the C282Y and the H63D mutations are cofactors in the expression of PCT (323–326).

Patients on hemodialysis also frequently have elevated iron indices, but C282Y does not appear to increase the risk of iron overload in these patients (327). Individuals who receive more than 60–100 blood transfusions for chronic anemia (aplastic anemia, chronic renal failure, and refractory anemias) are at high risk of developing parenchymal iron deposition and associated organ toxicity (328,329). Early clinical signs of iron overload, including hepatomegaly, carbohydrate intolerance, and cardiac arrhythmias, are noted with the accumulation of 10g of iron. Congestive heart failure is noted with 75–100g of iron (329,330). Reports of prolonged oral iron therapy causing iron overload syndrome are infrequent, although there is a tendency among some physicians to treat microcytic anemias empirically with iron (331).

**101.3.1.9 Population Screening.** Population screening for HH and *HFE* mutations specifically is not recommended (256,332,333). Not all C282Y homozygotes will develop clinical features of hemochromatosis, and some homozygotes will never present with iron levels high enough to warrant phlebotomy. Several investigators suggested implementing screening for HH in different populations even prior to the identification of the *HFE* gene (227,265,276,334,335) and the American College of Pathologists and other experts advocated screening by serum iron indices (332,336,337), but because of concerns over cost effectiveness and genotype–phenotype correlation, others argued against screening (338–340). The World Health Organization (WHO) and the US Preventive Services Task Force have developed general criteria for population screening of genetic diseases (341,342), and HH meets many of these. The WHO criteria as specifically applied to HH:

1. First and perhaps the most compelling criterion promulgated by the WHO for population screening is that the condition should be an important health problem. Although HH is the most common inborn error of metabolism in people of European descent, the age distribution of C282Y homozygotes was no different than controls in Beutler and colleagues' study suggesting that there were no significant differences in mortality (258). This is supported by a cohort of 23 Danish C282Y homozygotes who did not develop manifestations of disease after 25 years of follow-up (343).
2. A latent period should exist to permit the opportunity for screening and treatment before the development of morbidity and mortality. In addition, an acceptable treatment should be available and effective to those identified with the condition. Iron depletion by phlebotomy has been the standard of care for several decades. Although no randomized trials exist to support its use and ethical considerations will prevent any such studies in the future, phlebotomy appears to be an effective treatment when instituted early in the disease process. In particular, liver fibrosis appears to halt and in some cases improve (102).
3. A suitable screening test must be available for the condition. TS has been advocated as the most suitable test for HH screening. As discussed above, it has a reasonable sensitivity and can identify individuals prior to the onset of significant iron overload. An added benefit is the identification of iron deficiency and other disorders, such as chronic liver disease. The positive predictive value of genetic screening remains uncertain particularly for the H63D mutation, which confers only a small risk toward the development of iron overload.
4. The cost of screening should be reasonable. Analysis of cost effectiveness for serum iron indices indicates a very favorable cost compared to screening programs

for other conditions (344–349). Even though the cost of high-throughput genotyping has plummeted in the past few years, the cost of population-wide genetic testing would still be expensive (345). The combination of serum iron studies followed by genetic testing has been suggested as the most cost-effective approach (336).

5. The test must also be acceptable to the target population. Serum TS has been widely accepted in screening trials. Genetic testing may encounter significant resistance owing to legal, financial, and ethical concerns. However, genetic screening for HH appears to be well accepted (350–352).

Uncertainty surrounding screening for HH primarily concerns the natural history of HH and whom to treat. A systematic review for the US Preventive Services Task Force examined the burden of suffering and the potential of a preventative intervention (256). The conclusion of this analysis was that there remains insufficient evidence to confidently project the impact of, or estimate the benefit from, widespread or high-risk genetic screening for HH.

**101.3.1.10 Clinical Management.** The concept of repeat blood letting for treatment of HH was first studied in the 1950s (353). Treatment of HH with established hepatic iron overload involves iron depletion, nearly always by whole-blood phlebotomy (276,334). The importance of early treatment cannot be overemphasized. Treatment in the precirrhotic stage of HH can yield a normal life expectancy (101,102).

Every 1 mL of packed red blood cells contains ~1 mg of iron so that a 500-mL phlebotomy contains between 200 and 250 mg of iron (354). In adults, the initial phase of treatment involves rapid-sequence phlebotomy designed to eliminate excessive iron stores and minimize organ injury. Adults with severe iron loading usually tolerate the removal of 500 mL of blood once or twice each week until iron-limited erythropoiesis occurs (334). Iron depletion can be confirmed by the presence of slight anemia, decreased mean red cell volume (usually <75 fL), and a markedly decreased TS and serum ferritin concentration (<15% and <20 mg/L, respectively). The serum ferritin concentration usually normalizes before the TS (334,355). After initial iron depletion is accomplished by rapid-sequence phlebotomy, lifelong maintenance phlebotomy therapy is begun. Most patients require phlebotomy therapy at intervals of 2–6 months. The serum ferritin concentration can be measured each year to estimate body iron stores and to adjust the frequency of phlebotomies (334).

The results of vigorous phlebotomy therapy have only been studied on a large scale in older patients with HH (101,102,104). Symptoms due to congestive heart failure (356), diabetes mellitus (357), hypogonadism (358), and general malaise improve with phlebotomy treatments. Patients who are diagnosed and treated in

the precirrhotic stage have been shown to have a normal life expectancy. Cirrhotic patients have a shortened life expectancy and a high risk of liver cancer, even when complete iron deficiency is achieved (101,102). The effect of phlebotomy on lipid metabolism has shown no effect on blood glucose and total cholesterol, whereas triglyceride lowering is seen when ferritin and TSs are dropped (359). Unfortunately, as discussed above, some complications of HH have not shown significant improvement with phlebotomy, including arthropathy. These patients may develop osteoarthritis, severe enough to warrant surgical repair. Total ankle arthroplasty, for example, has been shown to be associated with a low risk of complication in HH with significant pain relief (360). Oral or intravenous iron chelation therapy is not generally recommended, but may be considered in cases in which phlebotomy cannot be tolerated.

A question frequently asked by pediatricians is what infant formula to recommend to families in which a proband has been identified with HH. Iron-fortified formula significantly protects against iron deficiency and its sequelae in child development. As the presentation of HH is relatively uncommon in children and because iron deficiency is such a prevalent problem with serious sequelae, an iron-fortified formula is recommended in such families when not breastfeeding.

Olsson and colleagues were unable to show any negative effects of generalized iron fortification of food on populations with a high prevalence of HH (361). It is generally recommended that some foods and iron supplements be avoided by patients with iron overload, such as iron-rich foods and vitamin C, which reportedly increase dietary iron absorption (160,362). At present, there are no data on the use of low-iron diets in the prevention or treatment of HH, and such diets may be impractical.

### 101.3.2 Juvenile Hemochromatosis

After the identification of the *HFE* gene, it was discovered that some causes of hemochromatosis were due to non-*HFE* mutations. JH or type 2 HH is an autosomal recessive disorder of early onset of iron overload resulting in cardiomyopathy, hypogonadism, and diabetes, presenting in the teens and early 20s (75,363,364). While once characterized as an early manifestation of classic HH (type 1), the clinical presentation, genetic linkage studies, and the most recent molecular discoveries show that JH is a distinct disease. JH is uncommon, yet the recent discoveries of hepcidin and hemojuvelin, the protein protagonists that cause hemochromatosis type 2b (HFE2b) and type 2a (HFE2a), respectively, have completely changed our understanding of iron homeostasis, HH, and related disorders.

**101.3.2.1 Clinical Features.** Clinical and pathologic descriptions of what is now known as JH, in contrast to classical type 1 adult-onset disease, date back well before molecular diagnoses became available. JH usually

presents before the age of 30. The pattern of organ involvement is similar in both HH and JH, except that JH patients have a more fulminant clinical course, especially as a result of hypogonadism and cardiomyopathy, which are more prominent and severe (174). In contrast to adults with HH, females and males are equally affected in JH (365).

Symptoms of hypogonadism are frequently chief complaints in JH (131,174,365,366), whereas they usually have less clinical relevance in HH (367). Hypogonadism, as manifested by secondary amenorrhea, diminished libido, loss of sexual hair, or impotence, is observed in 64% of patients under 30 years, compared to 30% of older patients (365). Autopsy studies in young patients demonstrate iron deposition in the pituitary, adrenal cortex, gonads, thyroid, and parathyroid glands (90,366,368–370). Compared to iron deposition in other tissues, gonadal iron accumulation in young patients is relatively small and is present predominantly in the blood vessel endothelium (366). The pathogenesis of hypogonadism appears to be identical in both age groups and is caused by the specific distribution pattern of excess iron accompanied by functional impairment of affected parenchymal cells (174,365,371). However, for reasons that remain unclear, younger patients are more frequently affected (365).

Cardiac dysfunction is the usual cause of death in untreated JH (131,174,366). The cardiac disorder has been characterized as a restrictive cardiomyopathy in appropriately examined younger patients (126,365,372). However, the frequency of cardiomyopathy in the younger group (58%) is greater than in the older group (35%) (365). Heart failure is extremely common in younger subjects, and symptoms may develop suddenly with rapid progression to death (100,366). Because there is a direct correlation between the severity of myocardial siderosis and cardiac dysfunction (373), young patients probably accumulate large amounts of iron in the myocardium (174). This is supported by autopsy studies where a substantial deposition of iron was found in the heart of a 21-year-old man with cardiac complications (374), and in a report where severe myocardial siderosis was found in German twins who died at ages 19 and 20 (174).

Hepatic involvement is present in 83% of young patients reported in the literature, and is significantly less than the 93% frequency reported for the over-30 age group (365). A postmortem examination of four young patients aged 19–28 showed heavy iron deposition in parenchymal liver cells and varying amounts of portal fibrosis (366). A nodular hepatic architecture was present in other young patients (366,375), and portal hypertension has also been described (376).

Both diabetes and arthritis are less prevalent in younger patients. The frequency of diabetes mellitus or glucose intolerance is only 34% in patients younger than 30 (365). There are reports of arthritis in young patients

(377,378), but in Lamon and colleagues' review of 52 young patients dating back to 1895, only 5 had symptoms of arthritis (365).

**101.3.2.2 Molecular Biology of JH.** The mouse models of hepcidin deficiency and overexpression inspired the search for mutations of a human gene that could cause or contribute to hemochromatosis. Two families with severe JH were reported by Roetto and colleagues (379). One family had a homozygous frameshift mutation and the other a homozygous premature stop (nonsense) mutation; both in the coding region of hepcidin. Since a genetic locus for JH was previously mapped to chromosome 1q (HFE2a), rare mutations of *HAMP* encoding hepcidin on 19q13.1 were assigned to the “HFE2b locus.”

The first genetic mapping of a JH locus was to chromosome 1q (originally called the “HFE2 locus” and later “HFE2a” after mutations of the hepcidin gene were found) preceding the discovery of the first JH gene, *HAMP* on 19q13.1, by several years (72,380). Thus *HAMP* was designated the “HFE2b locus” because it was discovered after the 1q locus. Discovery of the *HJV* gene (HFE2a) quickly followed *HAMP* (HFE2b). Papanikolaou and associates reported the positional cloning of the 1q locus associated with JH and the identification of a gene, *HJV*, crucial to iron metabolism, which they called hemojuvelin (75). Studies in Greek, Canadian, and French families indicated that a G-to-T variant at position 1284 in the *HJV* gene, resulting in a glycine320 to valine missense, was present in all three populations and accounted for two-thirds of the mutations. The hemojuvelin protein is also called Repulsive Guidance Molecule c (RGMc), a member of a three-gene family of RGMs. The other members of this family include RGMa and RGMb, proteins found in the nervous system. RGMc/*HJV* is expressed in skeletal muscle, cardiac myocytes, and hepatocytes. Initially, RGMc/*HJV* was identified as a novel transcript that was expressed during the differentiation of skeletal muscle (381). Subsequently, it has been shown to be involved in the regulation of hepcidin expression through its actions as a BMP coreceptor and that it can be regulated itself by maltriaptase (TMPRSS6).

**101.3.2.3 Management of JH.** Treatment in children is accomplished by initially removing 5–7 mL/kg of blood every 7–10 days (275). Phlebotomy is continued at this frequency until the iron overload is relieved and equilibrium reached, in which iron loss by phlebotomy matches intestinal absorption. Attainment of equilibrium status is signified by normal values for serum iron, ferritin, and TS. Once the goal of removing excess iron has been achieved, the frequency of phlebotomy can be reduced to once every 1–3 months, maintaining serum ferritin and TS within the low reference range for age. Hemoglobin levels and mean corpuscular volume (MCV) should also be monitored regularly because excessive phlebotomy results in anemia (276).



### 101.3.3 Neonatal Hemochromatosis

Neonatal hemochromatosis (MIM #231100) is a rare gestational condition characterized by iron accumulation in the liver and extrahepatic sites of the fetus, and sparing the cells of the reticuloendothelial system (reviewed in Knisely et al., 2003) (382). This pattern of iron deposition resembles that seen in classic adult-onset type 1 *HFE*-associated hemochromatosis (HH). Liver damage is extensive and frequently the dominant clinical feature. NH often results in late gestation fetal loss, or death within a few days of birth despite aggressive support. It is the most frequent cause of liver failure in newborns. The recurrence rate in sibships is greater than that predicted for simple Mendelian autosomal inheritance, but the etiology, inherited or acquired or both, remains unclear. The diagnosis is frequently overlooked until autopsy. Treatment remains inadequate.

**101.3.3.1 Clinical Features.** Clinical manifestations of NH begin prenatally, usually in the last trimester of pregnancy. Fetal losses in late gestation are common. Placental edema, fetal hydrops, edema, ascites, intrauterine growth retardation, and oligohydramnios are frequent complications, although polyhydramnios has been reported as well (383). Birth frequently occurs before term.

The dominant presenting feature in live born infants is that of acute liver failure. Liver disease is usually apparent within hours of birth, though in rare cases may manifest days to weeks after birth (384–386). Prominent features typical of acute liver failure include hypoglycemia, marked coagulopathy, hypoalbuminemia, and edema with or without ascites, and oliguria. Initial treatment almost always is for presumed sepsis or septic shock. Disseminated intravascular coagulation with increased fibrin split products may play a role in coagulopathy. Placental edema may be due to decreased oncotic pressure. Neonatal or fetal oliguria may be the result of hepato-renal syndrome, or rarely renal tubular dysgenesis (387). Mixed hyperbilirubinemia with elements of both conjugated and nonconjugated bilirubin results in jaundice during the first few postnatal days. This may be because of an increased bilirubin load from intravascular hemolysis and extravasated blood, or impaired hepatic bilirubin conjugation and excretion. Decreased serum alpha-1-antitrypsin, ceruloplasmin, and transferrin have been reported (383). While abnormal serum bile acid profiles have been reported, it is unclear if this represents a primary defect of bile acid synthesis, or a sequelae of severe hepatocellular disease (388,389). Serum amino transferase levels are disproportionately low for the degree of hepatic dysfunction, presumably due to the loss of functional hepatocyte mass. Serum alpha-fetoprotein levels are characteristically highly elevated, in the range of 100,000–600,000 ng/mL. Levels of this magnitude are almost exclusively seen in NH or hereditary tyrosinemia, and are much higher than those typically observed in viral or toxic hepatitis of the newborn.

**101.3.3.2 Etiology.** There are two main hypotheses concerning the etiology of NH: (1) fetal liver injury causes abnormal iron handling, and (2) abnormal iron handling by the placenta leads to hyperaccumulation of iron and to liver injury. Unlike classic adult-onset or JH, no genetic mutations have been associated with NH, and no locus has been identified by genetic linkage. The *HFE*-associated locus on 6p21.3 has been excluded by linkage analysis (390). Consequently, both hypotheses concerning the etiology of NH could be valid depending on the circumstances. For example, NH or iron-overload features have been reported with hereditary tyrosinemia (391,392), Down syndrome (393), tricho-hepato-entero syndrome (394), GRACILE syndrome (395), congenital cytomegalovirus (CMV) infection (396), and non-A non-B hepatitis (382). All of these cause liver injury, which presumably can impair iron handling (hypothesis 1 above), though they rarely are associated with iron overload and generally do not present as acute liver failure in the newborn.

Almost all of the molecular constituents of iron absorption and regulation that are active in the intestinal enterocyte have been described in the placental trophoblast. These include the transferrin receptor, FPN1, *HFE*, and hepcidin. The potential roles that abnormal iron handling within the placenta (hypothesis 2), abnormal fetoplacental, or maternoplacental interaction, may play in NH has not yet been determined, though there are some interesting data from animal studies. Mouse pups that constitutively overexpress hepcidin are born iron deficient suppressing transplacental iron flux into the fetus (397). In contrast, hepcidin knockout mouse pups develop iron overload several months after birth (398). While these models show that hepcidin is important in regulating placental iron flux, human NH is significantly different in that it begins sometime during gestation. Furthermore, while hepcidin mutations cause JH, no mutations of hepcidin or hemojuvelin (shown to regulate expression of hepcidin), identified in a neonate or mother, have been associated with NH; and cases of NH have not been described in classic HH or JH kindreds.

Interestingly, the observed recurrence rate of NH in sibships exceeds 75% (399). This is high for recessive or dominant inheritance. While no man has been reported to have fathered infants with NH with different mothers, several kindreds have been described in which a woman has borne infants with NH to different fathers (400,401). This suggests that some cases of NH may be caused by gonadal mosaicism for new and dominant mutations lethal in spermatogenesis but not oogenesis, or mitochondrial disease inherited through the mother, or maternal transmission of an imprinted gene.

The high recurrence rate in sibships is also compatible with an acquired and persistent maternal factor reminiscent of gestational disease such as hydrops fetalis caused by Rh incompatibility and alloimmune thrombocytopenia. Such a factor in NH could be an antibody directed against a fetal or placental antigen involved in iron

handling (382,402). Fetal liver injury secondary to an alloimmune process, as opposed to a primary derangement of iron metabolism, has become increasingly favored as the primary cause of NH due to the recent reports of successful treatment of at-risk pregnancies (see treatment below) as well as the emergence of evidence in NH cases supporting an immune-based mechanism of hepatic injury (403,404).

**101.3.3.3 Treatment and Outcome.** A number of management strategies and treatments have been devised for NH, but regardless the overall prognosis remains poor (382,405). These include preterm delivery, a cocktail of antioxidants and iron chelators, orthotopic liver transplantation, and maternal intravenous gamma globulin (IVIG) infusions. IVIG treatment was devised on the theory that NH is an alloimmune gestational disorder. Whittington and Kelly reported results based on 48 women who previously had children with NH (406). The gestational histories of these women were remarkable in that of the 44 healthy babies delivered, 42 occurred prior to the index case. Following the index cases, there were 21 gestations by 13 women of which 19 resulted in NH, consistent with previous reports of high NH recurrence. Fifty-five subsequent gestations were treated with weekly IVIG infusions beginning at the 18th week of gestation. Of these, 53 pregnancies resulted in “good outcomes,” which is a marked improvement compared to the gestational histories of these mothers. Of the 41 infants who had measurements of serum ferritin and alfa fetoprotein (AFP), 29 babies showed elevations suggesting involvement of NH, but none had evidence of liver disease. Nine infants were treated with chelation/antioxidants. Additional case reports have suggested success with this approach in at-risk pregnancies as well as for the use of exchange transfusion for treatment of NH (407–409).

### 101.3.4 Atransferrinemia

Atransferrinemia (MIM #209300) is an extremely rare recessive disorder caused by the absence—or, in the case of hypotransferrinemia, severe deficiency—of the circulating iron transport molecule transferrin. To date, only 12 cases have been reported in the literature. (410–412). Transferrin is a glycoprotein with a molecular weight of 75,000–80,000; ~6% is carbohydrate (413). Transferrin is the major iron transport molecule of the circulation and the transferrin gene is part of a larger family whose members include p97, a membrane protein expressed in human melanoma cells and lactoferrin, which is the major iron-binding protein in milk. Interestingly, the genes for all three of these products are located on chromosome 3. Because 80% and 90% of transferrin traffic is directed to normoblasts for hemoglobin synthesis (3), the most striking clinical consequences of transferrin deficiency are due to the failure of hemoglobin synthesis and anemia.

**101.3.4.1 Clinical Features.** In 1961, Heilmeyer and colleagues described the first reported case in a girl who presented with severe hypochromic anemia at the age of four months (414). Since then, 11 other cases have been reported, with age at diagnosis ranging from birth to 20 years. In several cases, severe hypochromic microcytic anemia was found, with hemoglobin concentrations as low as 3.2 mg/dL (415). Evidence of iron overload in the liver and reticuloendothelial system was detected at autopsy or by liver biopsy in at least four of these cases, and elevated ferritin levels have been reported in others. In the case described by Heilmeyer et al., the patient died from cardiac failure, presumably due to severe hemosiderosis of the heart, which was found at autopsy (414). The patient reported by Goya and coworkers presented late, at the age of 7 following an acute febrile illness that was treated by chloramphenicol (416). In addition to anemia, this patient also had developmental and growth delay that persisted for a period of 5 years after presentation. Subsequently, he had significant catch-up growth (417). This recovery correlated with a rise in transferrin levels from below 10 mg/dL to around 20 mg/dL suggesting that symptoms are only apparent at transferrin concentrations below 10 mg/dL.

**101.3.4.2 Genetics.** In all cases, the mode of inheritance was presumed to be autosomal recessive. Hayashi and colleagues (417) restudied the family reported by Goya and colleagues (416) and found a small amount of a transferrin variant detected by isoelectric focusing gels in the proband and two of his siblings, although only the proband was symptomatic. The authors suggested that this patient represented a compound heterozygote consisting of a variant allele transmitted by the father and shared by two siblings, and a null allele inherited from the mother.

In some cases, affected individuals have been suspected of being heterozygous for a null allele and a hypomorphic variant accounting for a relatively mild course and/or a later onset of disease. For example, small amounts of transferrin were detectable by isoelectric focusing and anemia was first noted at age 7 in the patient studied by Hayashi and colleagues (417). Genetic characterization suggested that this individual carried both a missense allele (G1180A resulting in E394K) and a second undetected mutation located outside of exons or near splice sites (418). Genetic mutations have been reported in four other cases. Compound heterozygosity (G1429C resulting in A477P and a frameshift in exon 5 resulting in a nonsense mutation) was reported in a patient with onset of manifestations at age 20 (410). Cell lines established from the second known case of atransferrinemia, in which the onset of symptoms occurred during infancy, were homozygous for the missense mutation G229A (resulting in D77N) (419). Aslan et al. reported a C137Y mutation in a 4-month-old girl from Turkey (411). In the case reported by Chen et al., a G502C mutation was detected by sequencing in a 10-year-old boy (412). In

addition, a large number of transferrin alleles have been described that do not result in symptoms and which therefore represent polymorphic variants (420).

**101.3.4.3 Diagnosis and Differential Diagnosis.** The diagnosis is based on the assay of transferrin by nephelometry or immunoassay. Normal serum values range between 2.0 and 4.0 g/L (421). Congenital hypotransferrinemia must be distinguished from acquired hypotransferrinemia, which can be found in the setting of liver disease, nephropathies, enteropathies, inflammatory diseases, and malignancies (416). Atransferrinemia is exceedingly rare except in the genetic disorder.

**101.3.4.4 Management.** Goya and others (416,422) reported treatment with purified iron-free human transferrin, although its availability and cost may be prohibitive. The patient reported by Goya (an 8-year-old male) received 2 g of apotransferrin (9.8% protein in normal saline) in three doses over 16 days, resulting in an increase of hemoglobin concentration from 6.4 g/dL to more than 10 g/dL. The patient required an additional 1 g infusion of transferrin after 3 months and a third dose after 6 months, although the response was less marked than after the first infusion. At age 10, his infusion dosage was increased to 2 g, which he received approximately every 6 months. At this dose, his hemoglobin concentration remained near 10 mg/dL (417). Fresh frozen plasma transfusion has also been performed, although the quantity of transferrin infused is difficult to assess in these cases. Because iron overload has been reported for all surviving patients with atransferrinemia, whole blood or red cell transfusions should be avoided and iron chelators should be considered for patients demonstrating signs of iron overload.

## 101.4 OTHER DISORDERS RESULTING IN DERANGEMENTS OF IRON HANDLING

### 101.4.1 Connection between Disorders of Iron Utilization and Iron Absorption

Many diseases result in abnormalities of iron handling or deposition, although not all are due to a primary derangement of molecules normally involved with iron metabolism. The largest category of such disorders is anemia due to a variety of defects of hemoglobin synthesis or red blood cell architecture. These disorders are associated with secondary iron overload because they cause an accelerated rate of hematopoiesis. This leads to increased intestinal iron absorption that may be compounded by the need for transfusion therapy. Other causes of secondary iron overload include chronic liver disease due to a variety of causes, portacaval shunting, and chronic hemodialysis. However, at least two disorders—iron-refractory iron-deficient anemia and hypochromic microcytic anemia with hepatic iron overload—demonstrate the molecular network connecting the iron needs for red blood cell production and systemic iron absorption. Moreover,

conditions such as African iron overload suggest that there are genetic influences on the control of iron metabolism that remain to be identified.

### 101.4.2 Iron-Refractory Iron-Deficient Anemia

In 1981, Buchanan and Sheehan suggested that a form of microcytic anemia was due to a primary derangement of iron metabolism (423). Three siblings were demonstrated to have malabsorption of medicinal iron and responded partially to intramuscular iron dextran. Finberg et al. referred to this phenotype as iron-refractory iron-deficiency anemia (IRIDA, MIM #206200) (424). IRIDA is characterized by congenital hypochromic, microcytic anemia with low mean corpuscular erythrocyte volume, low TS, and abnormal iron absorption that is unresponsive to oral iron and partially responsive to parenteral iron. Several authors have reported cases with these characteristics. Finberg et al. noted that whereas urinary levels of hepcidin (MIM #606464) are typically undetectable in individuals with iron deficiency, hepcidin-creatinine ratios were within or above the normal range in IRIDA cases. Mutations in the *TMPRSS6* gene have been found in kindreds with IRIDA (424,425). *TMPRSS6* encodes a type II transmembrane serine protease expressed primarily in liver and orthologous mutations in the mouse result in anemia as a result of defective dietary iron uptake. It has been suggested that *TMPRSS6* is a negative regulator of hepcidin transcription.

### 101.4.3 Hypochromic Microcytic Anemia with Iron Overload

In 1981, Bannerman suggested that some human iron handling disorders may be analogous to that of the *mk* mouse, which exhibits iron deficiency and severe microcytic anemia as a result of defects in the *Slc11A2* gene. *Slc11A2* encodes a divalent metal ion transporter responsible for absorption of ferrous iron in the duodenum (426). It is also expressed in the erythroblast where it may facilitate absorption of transferrin-bound iron into the cytoplasm. A patient with a mutation within the human homolog of this gene, *SLC11A2* (*DMT1*), has been described (427). The patient had a homozygous mutation in the ultimate nucleotide of exon 12 (G1285C) resulting in a preferential skipping of exon 12 during processing newly transcribed RNA as well as an E399D missense mutation. Clinically, this patient presented in infancy with microcytic anemia and subsequently developed liver dysfunction with iron overload characterized by increased iron deposition in both Kupffer cells and hepatocytes demonstrated on liver biopsy at the age of 19 (Hypochromic microcytic anemia with iron overload, MIM #206100). Additional reports of patients with mutations in the *SLC11A2* gene (428) presenting with hypochromic, microcytic anemia and severe hepatic

iron overload suggest that unlike the rodent model, the *SLC11A2* mutations in humans result in defective iron utilization in the reticulocyte but does not eliminate iron absorption from the gut.

#### 101.4.4 African Iron Overload

Other diseases have been reported that may result from primary abnormalities of iron metabolism. The prevalence of iron overload in certain populations of Africa (African iron overload, formerly Bantu siderosis, MIM #601195) has been felt to reflect the consumption of large quantities of home-brewed beer that is extremely high in iron content (429). These patients demonstrate panlobar iron deposition in the liver, with marked deposition in the Kupffer cells. This distribution is in contrast to patients with HH, who predominantly exhibit a periportal pattern of iron deposition that affects mainly hepatocytes, with sparing of the Kupffer cells. A genetic predisposition for development of systemic iron overload when exposed to high dietary iron intake in Africans has been proposed (430,431) although linkage with HFE1 on chromosome 6 has been excluded (432). There have been recent reports of associations between iron overload in Africans and polymorphic variants in other genes involved in iron metabolism including *SLC11A3* (FPN1 or HFE4) and *CYBRD1* (encoding a putative plasma membrane diheme protein, expressed on the apical duodenal membrane, with ferric reductase activity) (433,434). However, the relationship between polymorphisms in these genes and development of iron overload is complex and the risk to carriers of these variants for developing disease from iron overloading is uncertain.

#### 101.4.5 Primary Defects of Iron Handling as a Cause of Neurological Disease

In addition to the five genes for which variants have been determined to cause iron overload syndromes (Table 101-1), a number of genes whose roles include support of systemic, cellular, or intracellular iron homeostasis have been described in recent years. Variants of these genes have been described in a number of degenerative neurological disorders suggesting a role of abnormal iron metabolism or handling in their pathogenesis, consistent with observations in diseases of iron abnormalities but often local in certain cells or tissues. These include Friedreich ataxia (MIM #229300), and X-linked sideroblastic anemia and spinocerebellar ataxia (MIM #301310), aceruloplasminemia (MIM #604290), and neuroferritinopathy, a form of neurodegeneration with brain iron accumulation (NBIA) due to mutations in L-ferritin.

#### 101.4.6 Friedreich Ataxia

Friedreich ataxia is a rare autosomal recessive inherited neurological ataxia, most often caused by a GAA-repeat

expansion in the *FRDA* gene, with a few cases (2%) caused by *FRDA* point mutations (435), which encodes frataxin (MIM #229300) (see Chapter 118). Of interest to iron metabolism is that frataxin is a mitochondrial protein with high homology to YFH1, a yeast protein involved in iron homeostasis and respiratory function (264). Deficiency of YFH1 in yeast results in mitochondrial iron accumulation, whereas *FRDA* mutations in humans also result in increased mitochondrial iron (436,437). Frataxin deficiency also results in increased sensitivity to oxidative stress, decreased activities of mitochondrial iron-sulfur proteins, decreased mitochondrial DNA content, and impaired cellular respiration (438,439). Potential functional roles of frataxin include iron-sulfur cluster assembly (440), maturation of proteins containing such clusters (441), and mitochondrial iron storage (442). These findings suggest that frataxin is required for mitochondrial iron metabolism and that frataxin deficiency leads to iron accumulation, oxidative stress, and mitochondrial dysfunction. Idebenone, coenzyme Q<sub>10</sub>, and other antioxidants have been suggested as treatments (362,443). Treatment with deferiprone, a “reversed” siderophore, has been suggested as mechanism for iron redistribution and an open-label trial has suggested some benefit (444,445).

#### 101.4.7 X-Linked Sideroblastic Anemia with Ataxia

X-linked sideroblastic anemia with ataxia (XLSA/A, MIM #300751) is a rare disorder characterized by a mild X-linked sideroblastic anemia, elevated free erythrocyte protoporphyrin levels, mitochondrial iron accumulation, and nonprogressive cerebellar ataxia with onset early in childhood. Allikmets et al. demonstrated a sequence variant (T1200G) in the *ABCB7* gene resulting in the missense mutation, I400M in a three generational pedigree with four affected males (446). The *ABCB7* gene encodes an ATP-binding cassette transporter localized to the inner mitochondrial membrane and is believed to participate in iron-sulfur protein maturation by supplying iron-sulfur clusters to the cytosol; disruption of this process in XLSA/A leads to mitochondrial iron loading and mitochondrial dysfunction (447).

#### 101.4.8 Aceruloplasminemia

Ceruloplasmin is a copper-dependent ferroxidase involved in the oxidation of ferrous iron to ferric iron, and its deficiency is thought to interfere with the release of iron from cells into the plasma as transferrin (448). Several mutations have been described in the ceruloplasmin gene in aceruloplasminemia (MIM #604290), all of which lead to truncation of the carboxyl terminal portion of the protein, which is known to be essential for the coordination of a trinuclear copper cluster (reviewed by Harris et al., 1998) (449). The clinical features of the



disease are marked by iron overload of the liver parenchyma, pancreatic islet cells and brain, especially the retina and basal ganglia, resulting in diabetes and neurological symptoms (450–452). Treatment with desferoxamine has been advocated (453).

### 101.4.9 Neuroferritinopathy

Intracellular storage of iron occurs mainly in the form of iron–protein complexes. Ferritin is the major intracellular iron storage protein in all organisms. Ferritin consists of H and L subunits that form a 24 subunit shell enclosing variable amounts of ferric hydroxide phosphate complexes. The synthesis of ferritin subunits is regulated at the translational level by iron through interaction with the cytoplasmic IRP or IRE-binding protein. As discussed above, the IRE is a conserved nucleotide motif present in the 5′ noncoding region of all ferritin mRNAs. During periods of low cellular iron, the IRP binds the IRE preventing translation of ferritin subunits.

A point mutation (A49U) located in the IRE motif of H-ferritin mRNA was found in members of a Japanese family with iron overload inherited as an autosomal dominant trait (454). This mutation caused an increase in affinity of IRP for the IRE of H-ferritin mRNA containing the A49U variant, as demonstrated *in vitro* by Kato et al., which presumably resulted in decreased amounts of H-subunit synthesis and a decrease of intracellular iron storage capacity as ferritin complexes leading to pathological iron deposition in tissues (454).

A mutation in the L-ferritin subunit gene (*ins460A*) was described by Curtis et al. in patients with dominantly inherited extrapyramidal dysfunction and degenerative changes of the basal ganglia with onset in the fifth to sixth decade of life (neuroferritinopathy, a form of NBIA, MIM #606159) (127). Accumulation of iron was demonstrated in the neurons of affected individuals especially in the globus pallidus, as well as in the forebrain and cerebellum. The L-ferritin mutation *ins460A* results in disruption of the C-terminal 22 amino acids of the protein, which the authors speculate may destabilize the L-ferritin subunit altering the properties of the ferritin complex causing release of free iron into the cytosol. A second family was reported by Chinnery et al. (455).

### 101.4.10 Hereditary Hyperferritinemia–Cataract Syndrome

Other mutations in the ferritin light-chain IRE have been found to cause the hereditary hyperferritinemia–cataract syndrome (HHCS, MIM #600886). This autosomal dominant disorder is characterized by elevated serum ferritin and nuclear cataract (456,457). Most mutations described to date have been point mutations (457–461), although a deletion has also been reported (462). The pathophysiology of this disorder involves the dysregulation of L-ferritin expression, resulting from the

destabilization of the IRE or the disruption of IRP–IRE recognition (463). Although the pathophysiology for this disorder involves regulatory control of a molecule important in iron metabolism, patients with HHCS are neither iron overloaded nor deficient.

### 101.4.11 Other Neurological Disorders Associated with Abnormal Iron Handling

Local iron overload, deposition, or deficiency has also been a finding in the context of certain Mendelian disorders as well as other complex genetic diseases involving the central nervous system. It is less clear in these cases whether abnormal iron handling is a primary etiologic event or a secondary phenomenon due to cellular injury from other mechanisms (464). Pantothenate kinase-associated neurodegeneration (PKAN, MIM #234200) and disorders associated with PLA2G6 mutations may fall into this category. Iron overload in particular brain regions has also been reported in Parkinson’s disease (465,466) and Alzheimer’s disease (467), while brain iron deficiency has been associated with restless legs syndrome (RLS) (468) and attention-deficit hyperactivity disorder (469).

### 101.4.12 Pantothenate Kinase-Associated Neurodegeneration

PKAN is a disorder associated with iron deposits in the basal ganglia (470–472), characterized by progressive dystonia, dysarthria, and rigidity beginning in childhood. Differential diagnosis includes other NBIA disorders such as PLA2G6-associated neurodegeneration, neuroferritinemia, and aceruloplasminemia. Diagnosis can be made with the aid of MRI, which often shows decreased signal intensity in T2-weighted images, compatible with iron deposition, and with a small central area of hyperintensity (“eye-of-the-tiger” sign) (473). Zhou et al., using positional cloning methodology based on classical linkage analysis, identified mutations in the coding sequence of a gene called *PANK2* that has homology to murine pantothenate kinase-1 (474). *PANK2* is one of four pantothenate kinases, but it is the only one localized to mitochondria (475). A description of classic disease (characterized by early onset with rapid progression) versus atypical disease (later onset with slow progression) was made (476). In their study of 98 families, Haflick et al. identified mutations in *PANK2* in all families with classic disease (49/49) but in only 35% of families with atypical disease (17/49) (476). Null mutations (alleles predicting protein truncation) were prevalent in individuals with classical disease (36/92 alleles) but rare among those with atypical disease (2/31 alleles). This suggests that residual enzyme activity is preserved in some *PANK2* variants arising from missense mutations possibly accounting for a less aggressive clinical presentation. Patients with atypical disease who had *PANK2*

mutations were also more likely to have speech-related and psychiatric symptoms than patients with classic disease or mutation-negative patients with atypical disease. All patients whose clinical picture included the eye-of-the-tiger sign were found to carry *PANK2* mutations, while conversely, those patients without this sign were all mutation negative. These authors report that the G1231A mutation (resulting in G411R) was common in their patient series and may represent a founder mutation of European ancestry; interestingly this mutation may be semidominant. Mutations in *PANK2* have also been reported in individuals with HARP (Hypoprebetalipoproteinemia, acanthocytosis, and retinitis pigmentosa; MIM #607236) syndrome (477,478). This syndrome also presents with the eye-of-the-tiger sign. Treatment is primarily aimed at palliation and is often temporally limited in terms of clinical benefit (479). Supplemental pantothenate (vitamin B<sub>5</sub>) has been proposed as a potential therapy for patients carrying *PANK2* mutations retaining partial enzyme activity, as has the chelating agent deferiprone.

#### 101.4.13 *PLA2G6*-Associated Neurodegeneration and Other Non-PKAN Forms of NBIA

The presentation of disorders caused by mutations in the *PLA2G6* gene is clinically heterogeneous and includes a spectrum of progressive psychomotor deficits, mostly pyramidal but partially overlapping those of PKAN, which range from neonatal infantile neuroaxonal dystrophy (INAD, NBIA2A, MIM #256600) to mid-childhood (atypical neuroaxonal dystrophy, NBIA2B, MIM #610217) in onset. Cerebral atrophy and brain iron accumulation, usually in the globus pallidus, are typical findings, although not all affected individuals exhibit accumulations of brain iron. Mutations in *PLA2G6* have been described in a variety of kindreds within this spectrum (480,481). *PLA2G6* encodes a cytosolic calcium-independent phospholipase A2. The iPLA2 enzymes catalyze the release of arachidonic acid from membrane phospholipids and are critical in cell membrane homeostasis. However, as molecular testing has increased, cases have been reported of patients with later onset and slower progression without high brain iron (482,483). Mutations in addition to those described above have been described in at least three other genes for various cases of NBIA including *fatty acid-2 hydroxylase* (484), *lysosomal type 5 P-type ATPase* (485), and *C2orf37* (479,486).

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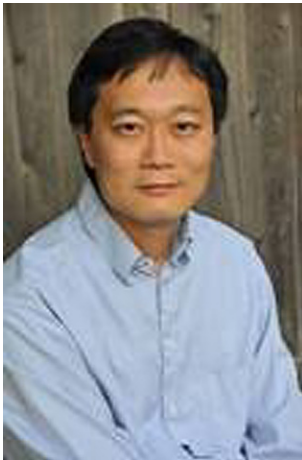


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## Mucopolysaccharidoses

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## 102.1 GENERAL ASPECTS

### 102.1.1 Definition

The mucopolysaccharidoses (MPSs) are a family of disorders due to inherited defects in the catabolism of sulfated components of connective tissue known as glycosaminoglycans (GAGs). GAGs are long-chain complex carbohydrates consisting of a variety of uronic acids, amino sugars, and neutral sugars. They are usually linked to proteins to form proteoglycans. Proteoglycans are the major constituent of the ground substance of connective tissue and they are also present in mitochondria and nuclear and cell membranes. The major GAGs are chondroitin-4-sulfate (C4S), chondroitin-6-sulfate (C6S), heparan sulfate (HS), dermatan sulfate (DS), keratan sulfate (KS), and hyaluronic acid (HA).

### 102.1.2 Etiology

The genes for all MPSs have been cloned ([Table 102-1](#)), and numerous, mostly private, mutations have been identified. Inheritance is autosomal recessive in all MPSs, except MPS type II (MPS II), which is X-linked.

### 102.1.3 Pathogenesis

The MPSs are degradation defects of GAGs. GAGs are broken down by the sequential action of lysosomal enzymes leading to a stepwise shortening of the GAG chain (for example see [Figure 102-1](#) and Reference (1)). The absent activity of a lysosomal enzyme results in the gradual accumulation of partially degraded GAG molecules in lysosomes. Distended lysosomes accumulate in the cell and by an unknown mechanism interfere with normal cell function.

Different MPSs are caused by different enzyme deficiencies leading to the accumulation of biochemically different GAG degradation products. As a general rule, the impaired degradation of HS is more closely associated

with mental deficiency and the impaired degradation of DS, chondroitin sulfates, and KS with mesenchymal abnormalities. Some of the salient features of the MPSs are summarized in [Table 102-1](#).

Some clinical manifestations of the MPS, such as coarse facial features, thick skin, corneal clouding, and upper airway obstruction, can be regarded as the direct expression of GAG accumulation in tissue. Others, such as mental retardation, growth deficiency, and skeletal dysplasia, are the result of defective cell function. Joint contractures and hernias point to an interference of accumulated GAGs with other metabolic substances such as collagen or fibronectin. Cardiac disease may be the result of the impaired elastogenesis which has been demonstrated in MPS I (Hurler disease). It appears that the accumulation of the GAG DS (but not HS) is linked to impaired elastic fiber assembly (2) and upregulation of elastases (demonstrated in animal models) secondary to cytokine activation may be important (3). The Toll-like receptor 4 pathway may have an important role in the evolution of bone and joint disease in MPSs and therefore treatments that inhibit the production of inflammatory cytokines may have a role to play in management (4,5).

### 102.1.4 Incidence

The crude cumulative rate for all types of MPSs is around 3.5 in 100,000 live births (6). There are striking variations in the frequency of various forms of MPSs in different populations. For example in the Irish Travelling community, Hurler syndrome is exceptionally common with a birth incidence of one in 371 and a carrier frequency of one in 10 (7).

### 102.1.5 Diagnosis

Although technically possible (8,9) and supported by patients and families (10) newborn screening for MPS disease is not yet available at a population level. Initial diagnosis depends on clinical acumen.

TABLE 102-1 MPS Enzyme Deficiency, Genetics and Clinical Features						
Disease	Enzyme Deficiency	Storage Material	Chromosome Location	Gene (OMIM Id)	Gene Mutations	Main Clinical Features
MPS						
MPS I (Hurler, Scheie, Hurler/Scheie)	Iduronidase (EC 3.2.1.176)	DS, HS	4p16.3	<i>IDUA</i> (252800)	W402X, Q70X plus many others	HSM, CNS, SD, DYS, OPH, CAR
MPS II (Hunter)	Iduronate-2-sulfatase (EC 3.1.6.13)	DS, HS	Xq27–28	<i>IDS</i> (300823)	No common mutations	HSM, CNS, SD, DYS, OPH, CAR, SK
MPS III (Sanfilippo)						
IIIA	Heparan-N-sulfatase (EC 3.10.1.1)	HS	17q25.3	<i>HSS</i> (605270)	R245H, R74C and many others	CNS, SD (+/–), DYS (+/–)
IIIB	N-acetyl-glucosaminidase (EC 3.2.1.50)	HS	17q21.1	<i>NAGLU</i> (609701)	No common mutations	CNS, SD (+/–), DYS (+/–)
IIIC	Acetyl CoA glucosamine N-acetyl transferase (EC 2.3.1.3)	HS	8p11.1	<i>HGSNAT</i> (610453)	No common mutations	CNS, SD (+/–) DYS (+/–)
IIID	N-acetyl-glucosamine-6-sulfatase (EC 3.1.6.14)	HS	12q14	<i>GNS</i> (607664)	Very few patients studied	CNS, SD(+/–), DYS (+/–)
MPS IV (Morquio)						
IVA	Galactose-6-sulfatase (EC 3.1.6.4)	KS	16q24	<i>GALNS</i> (612222)	I113F (UK and Ireland)	SD, CAR, OPH (+/–)
IVB	β-Galactosidase (EC 3.2.1.23)	KS	3p21-pter	<i>GLB1</i> (611458)	No common mutations	SD, CAR
MPS V	No longer used formerly Scheie disease					
MPS VI (Maroteaux–Lamy)	Galactosamine-4-sulfatase (EC 3.1.6.12)	DS	5q13–q14	<i>ARSB</i> (611542)	No common mutations	HSM, SD, DYS, OPH, CAR
MPS VII (Sly)	β-Glucuronidase (EC 3.2.1.31)	HS, DS	7q21.1–q22	<i>GUSB</i> (611499)	Very few patients studied	HF, HSM, CNS, SD, OPH, CAR
MPS VIII	No longer used					
MPS IX	Hyaluronidase (EC 3.2.1.35)	HA	3p21.3	<i>HYAL1</i> (607071)	Very few patients studied	U/K

Abbreviations: CAR, cardiac disease; CNS, regression; DS, dermatan sulfate; HS, heparan sulfate; KS, keratan sulfate; HA, hyaluronic acid; SA, sialic acid; DYS, dysmorphic appearance; HSM, hepatosplenomegaly; OPH, eye signs—corneal clouding; SD, dysostosis multiplex; SK, dermatological signs; WBC, white blood cell. (+/–), sign not always present or mild.

1. Low activity in CVB—caution re: contamination with maternal decidua.
2. Always do fetal sexing as some unaffected female fetuses will have very low enzyme results.
3. Difficult because of cross-reactivity from other sulfatases.



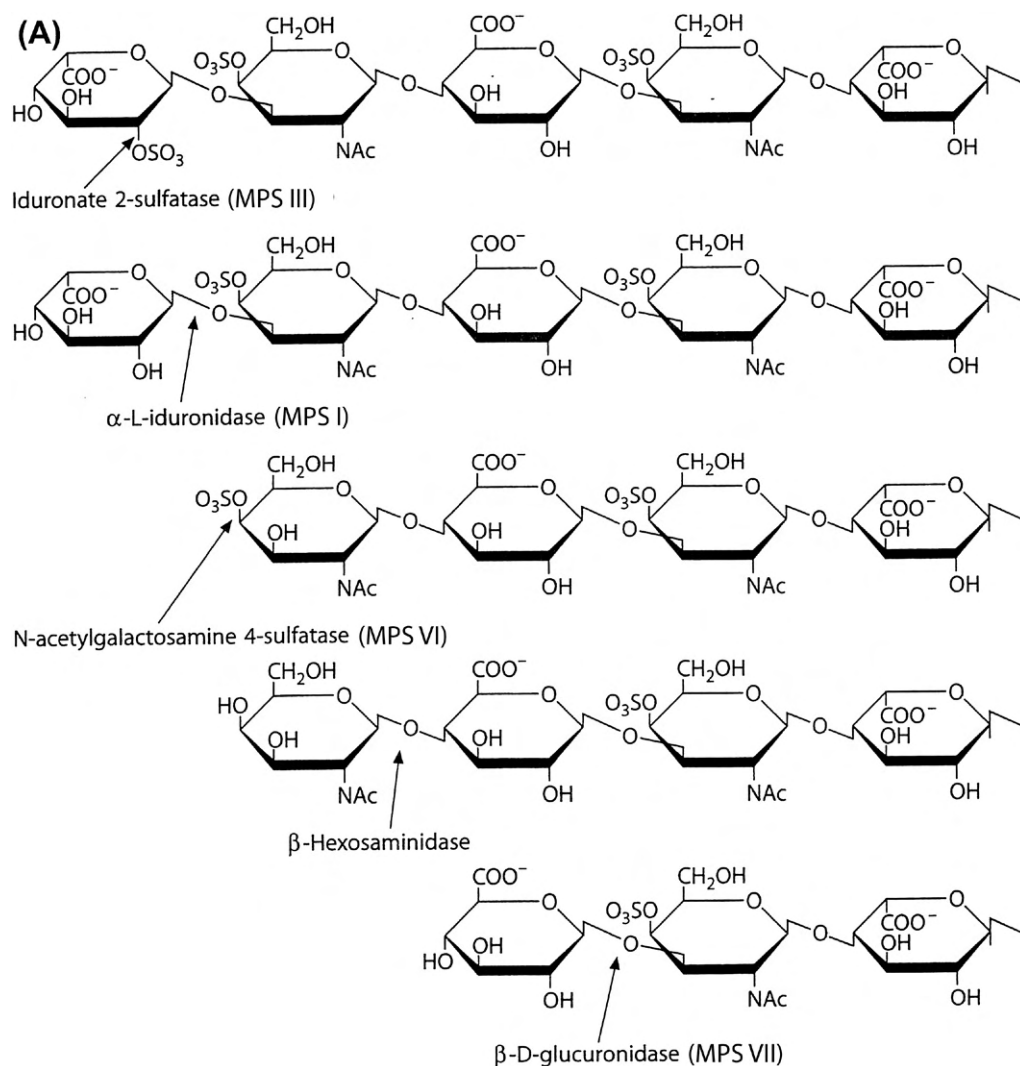
The MPS disorders tend to present in one of three ways:

- (1) As a dysmorphic syndrome (MPS IH, MPS II, MPS VI) often with early onset middle ear disease, deafness or upper airways obstruction
- (2) With learning difficulties, behavioral disturbance and dementia and mild somatic abnormalities (MPS III)
- (3) As a severe bone dysplasia (MPS IV)

Although initial medical contact is likely to be with the family physician or primary care practitioner it is unlikely that the diagnosis of these rare disorders will be suspected by these colleagues. Most will have never seen an MPS patient before in their clinical practice. Most diagnoses are made by metabolic physicians, clinical geneticists or general pediatricians. Other colleagues often have an opportunity to make an early clinical diagnosis of some MPS subtypes. This includes orthopedic surgeons (11) and otolaryngologists (12) in severe MPS I, II, IV or VI. In more attenuated variants (e.g. MPS I,

Scheie disease) rheumatologists may play a critical role in early diagnosis (13).

The first step in laboratory diagnosis is the analysis of urinary GAGs. A number of different methods exist for this screening test ranging from simple “spot” tests (that tend to be inaccurate and prone to false negative results (14)) to sophisticated analytical methods involving mass spectrometry (which are not universally available (15,16)). The most commonly available, reliable, method combines a quantitative measurement of total urinary uronic acid following precipitation with a dye (commonly dimethyl methylene blue) (17) with qualitative analysis of the species of GAG in the extracted sample by either one- or two-dimensional electrophoresis (18). It is important to stress that neither of these methods can diagnose a specific MPS disorder but an abnormal test does indicate that an MPS disorder is likely to be responsible for the patient’s symptoms. The pattern of GAG excretion seen on electrophoresis guides the clinician toward the



**FIGURE 102-1** The catabolism of dermatan, heparan, and keratan sulfate.

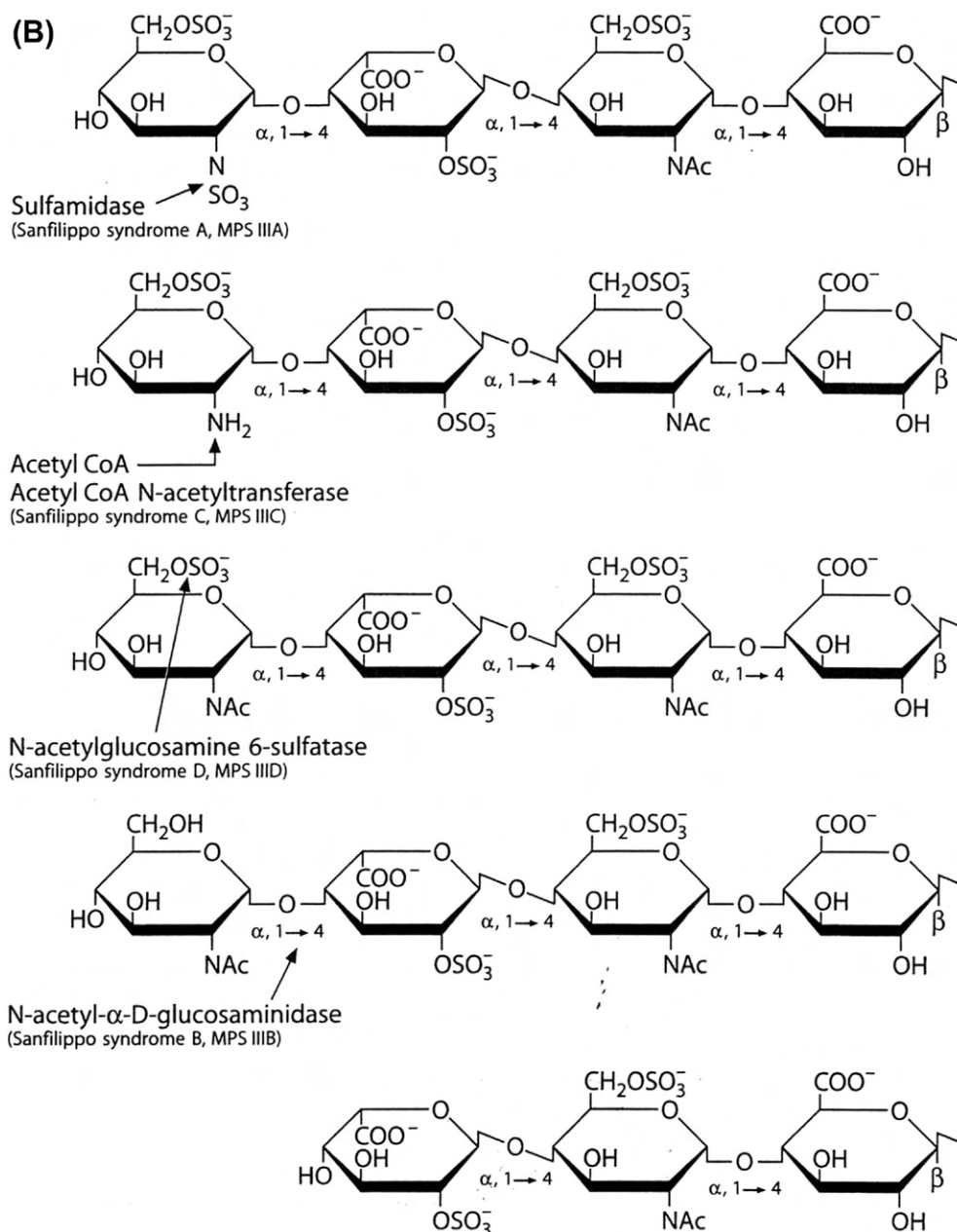


FIGURE 102-1—cont'd

appropriate enzyme analyses necessary to confirm the diagnosis (Table 102-1).

In positive cases, an enzyme diagnosis can be followed by specific mutation analysis in all of the MPS disorders.

Prenatal diagnosis is also possible for all MPS disorders. This is still often done by enzyme assay on uncultured chorionic villus biopsy (CVB) material but it is essential to combine this with fetal sexing in MPS II (Hunter syndrome) where some female carriers may have very low enzyme activities (19). Where the DNA mutation in an index case or family is known, prenatal testing can be performed by directly looking for the disease causing mutation in DNA extracted from a CVB sample.

For some families preimplantation genetic diagnosis is possible, although this is not universally available (20,21).

### 102.1.6 Dyostosis Multiplex

The skeletal abnormalities associated with MPSs are known as dysostosis multiplex (DM). These are progressive changes seen most floridly in the severe forms of MPS I (Hurler syndrome) and MPS VI (Maroteaux-Lamy syndrome). Patients with MPS II usually have less severe skeletal involvement although in most patients it remains significant whilst patients with MPS III (Sanfilippo syndrome) usually have very mild skeletal involvement.

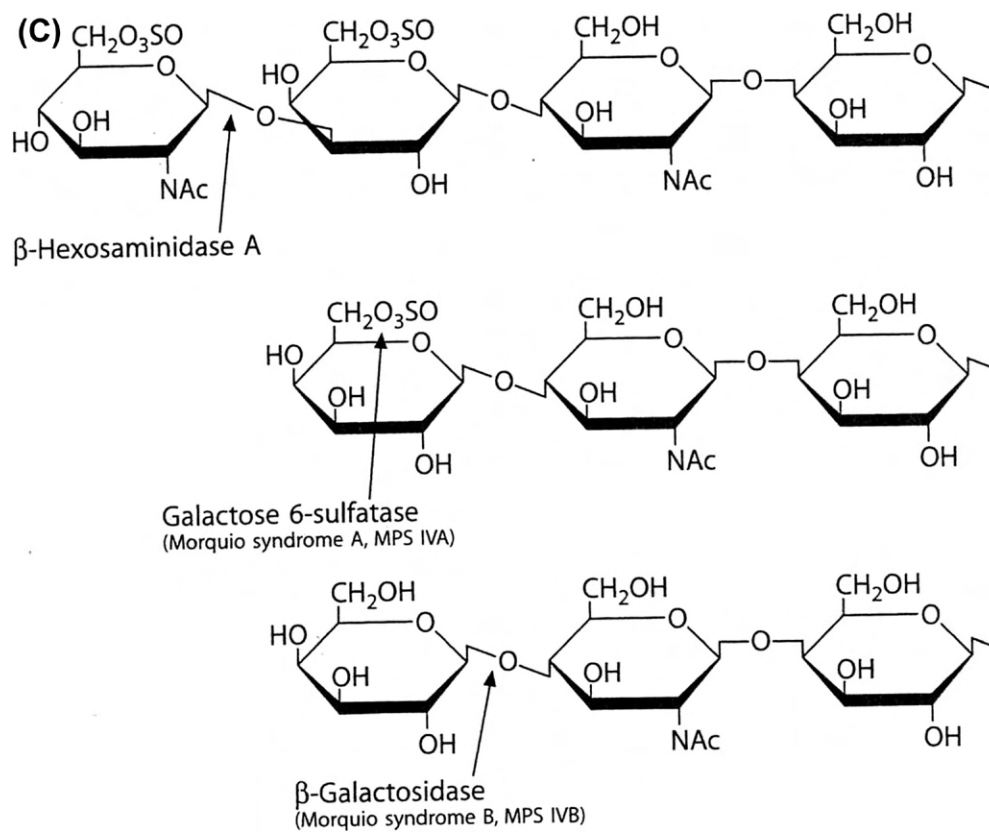


FIGURE 102-1—cont'd

Patients with MPS IV (Morquio syndrome) have a very distinct skeletal dysplasia characterized by platyspondyly and this will be discussed further in [Section 102.8](#).

The major features of DM are a large skull with a deep, elongated, J-shaped sella, oar-like ribs, deformed, hook-shaped lower thoracic and upper lumbar vertebrae, pelvic dysplasia, shortened tubular bones with expanded diaphyses, and dysplastic epiphyses ([Figures 102-2 to 102-9](#)).

For a comprehensive review of DM the reader is guided to [Reference \(22\)](#).

## 102.2 MUCOPOLYSACCHARIDOSIS I (IH HURLER, IS SCHEIE AND IH/S HURLER-SCHEIE DISEASE)

MPS I is caused by a deficiency of the lysosomal hydrolase  $\alpha$ -L-iduronidase, an enzyme that removes terminal iduronic acid residues during the sequential degradation of dermatan (DS) and heparan sulfate (HS). As a consequence, DS and HS accumulate and interfere with cellular function (by an unknown mechanism) leading to a chronic and progressive multisystem disease. In addition, DS and HS are excreted into the urine in excess ([Table 102-1](#)).

In the United Kingdom, the birth prevalence for MPS I is 1.07/100,000 births with a prevalence of the subtypes of MPS I calculated as 0.76/100,000 (Hurler), 0.24/100,000 (Hurler/Scheie) and 0.07/100,000 (Scheie) ([23](#)).

### 102.2.1 Mucopolysaccharidosis IH (Hurler Disease)

MPS IH serves as a model for all of the other MPS disorders as affected children exhibit almost the full range of effects that can occur from abnormal GAG storage. Affected infants are normal at birth and appear to develop normally for the first few months of life although there does appear to be an increased incidence of talipes equinovarus foot deformity ([24](#)). In the first 6 months of life, spinal gibbus deformity may be noted by the parents ([11](#)) and often children have a history of frequent ear, nose and throat infections. A persistent nasal discharge is commonly present as well as conductive hearing loss due to middle ear effusions. The pediatric otolaryngologist has an important role in both early diagnosis and management of affected patients ([12](#)).

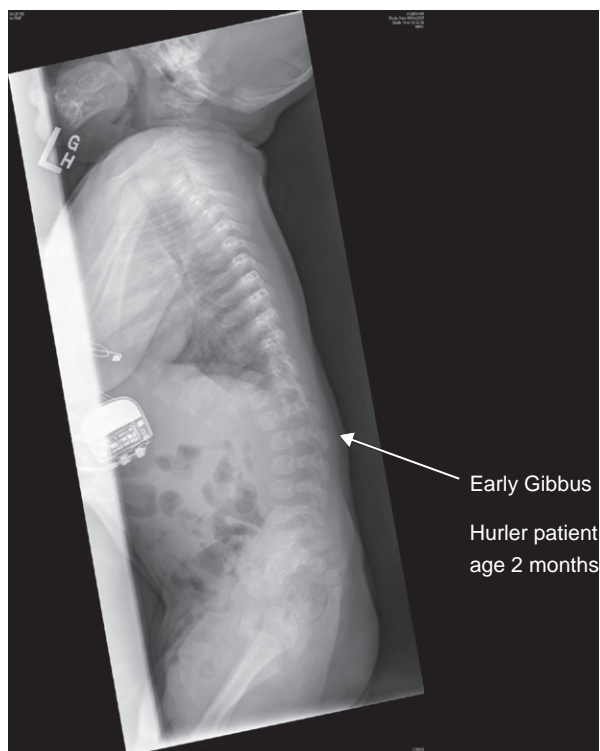
Between the ages of 6–12 months the facial features begin to change and are commonly described as “coarse,” being characterized by an increasing head circumference with frontal bossing, a wide nasal bridge and flattened mid face combined with an enlarged tongue. The skull becomes increasingly scaphiocephalic and by 18 months to 2 years the facial features are fully developed and the clinical condition becomes instantly recognizable to the majority of clinical geneticists and pediatricians ([Figures 102-10 and 102-11](#)).

In the first year of life, upper respiratory tract obstruction is often present and this can present with signs of obstructive sleep apnea which can be severe enough to precipitate right heart failure (25). The airway is generally narrowed, and there may be GAG deposition in the pharyngeal wall, tonsils, adenoids and soft tissues

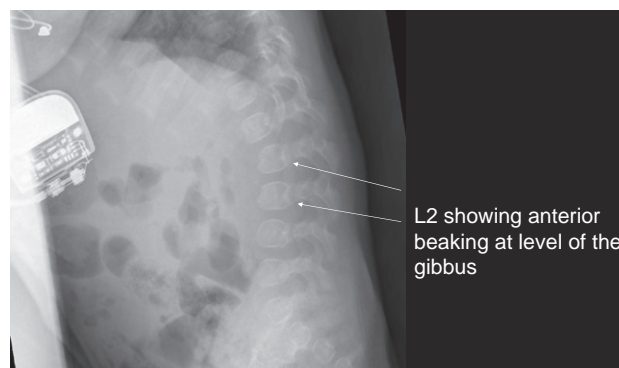
around the larynx causing the glottis to narrow significantly during respiration. This can cause problems in some patients with anesthesia which should always be undertaken with great caution in MPS patients (26). The upper airway disease also contributes to Eustachian tube dysfunction which along with dysostosis of the ossicles and scarring from frequent episodes of otitis media contributes to the hearing loss that is very commonly found in these patients.

Other early features of MPS IH include umbilical and inguinal herniae, which may recur following surgical repair as well as enlargement of the liver and spleen. Development starts to slow down from the age of two years. The head circumference can enlarge rapidly as high-pressure communicating hydrocephalus may occur (Figure 102-12).

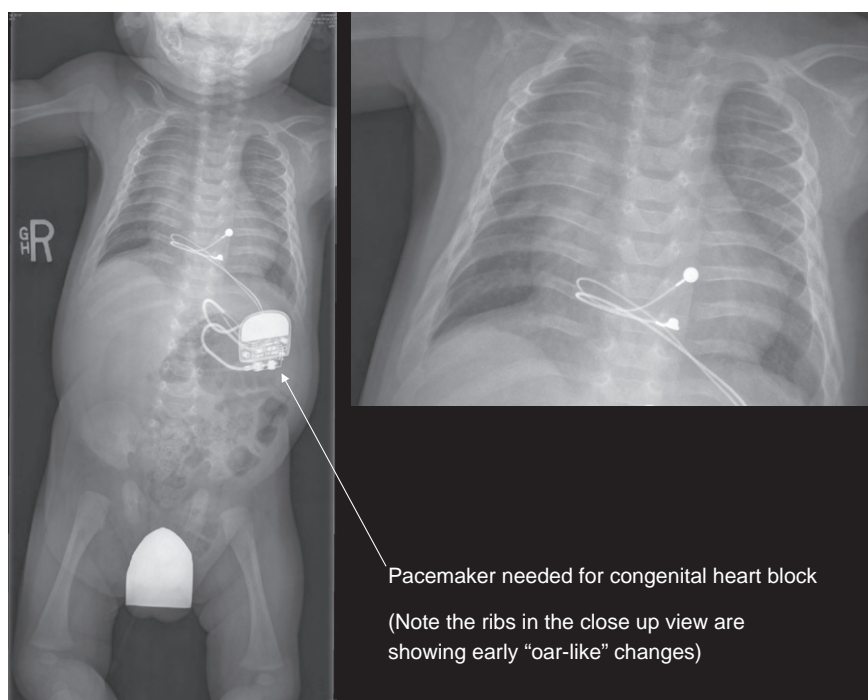
Growth also slows and affected patients develop contractures and joint stiffness. Corneal clouding starts to



**FIGURE 102-2** Hurler syndrome patient age 2 months.



**FIGURE 102-3** Hurler syndrome patient age 2 months.



**FIGURE 102-4** Hurler syndrome patient aged 2 months.



appear in the second year of life and becomes increasingly dense (Figure 102-13).

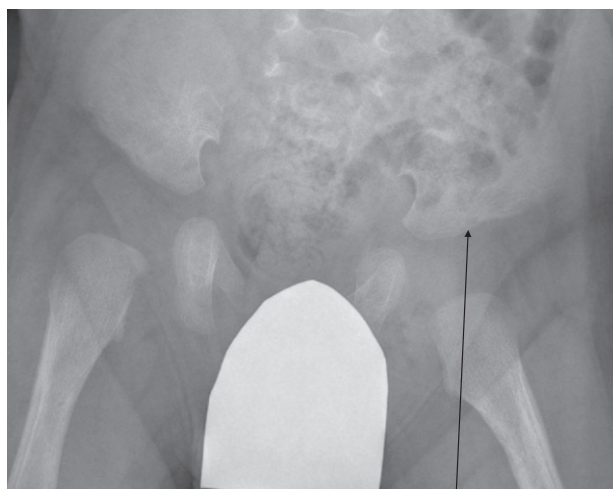
Cardiac disease is a significant complication and can present early with cardiac failure secondary to cardiomyopathy or later with valvular dysfunction (27,28). Progressive deposition of GAG in the myointima of the coronary arteries may be under recognized as a potential cause of cardiac complications, such as arrhythmia or ischemia (29,30).

DM progresses and affected children are short and most have a significant gibbus. Of more concern is that some patients have a hypoplastic or dysplastic odontoid and are unstable at the craniocervical junction. Flexion and extension X-rays should be performed on all patients with MPS IH to look for evidence of abnormal movement of C1 on C2 (Figure 102-14).

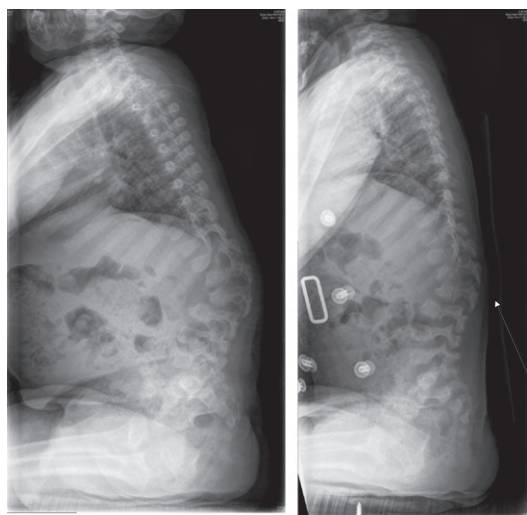
Throughout early childhood, the clinical picture continues to deteriorate and in the terminal stages of

the illness a combination of severe obstructive airway disease, neurological regression and cardiac involvement lead to death in most patients before the age of 10 years (23).

**102.2.1.1 Treatment of MPS IH.** For the past 30 years the standard treatment of choice for patients with MPS IH has been hematopoietic stem cell transplantation (HSCT), initially using bone marrow transplant (BMT) as the cell source but more recently and increasingly utilizing umbilical cord blood transplant (UCBT) or mobilized peripheral blood stem cells as the source. The initial publication by Hobbs et al. outlined the good short-term results of BMT with a reduction of urine GAG, resolution of hepatosplenomegaly and apparent arrest in developmental deterioration (31). It quickly became apparent, however, that even in the very best centers there was significant graft rejection, morbidity from graft versus host disease (GVHD) and mortality from various complications associated with the procedure (32,33). A number of complications that occur in MPS IH make transplantation more hazardous in this group of patients when compared with patients of a similar age undergoing transplantation for other



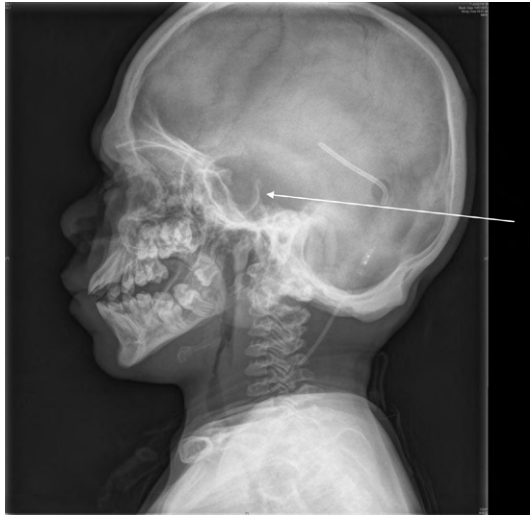
**FIGURE 102-5** Hurler patient age 2 months—under developed iliac bones (arrow).



**FIGURE 102-6** Lateral spine Hurler patient age 22 months some correction by brace (arrow).



**FIGURE 102-7** Same patient as Figure 102-6 X-ray shows uncovering of femoral heads due to acetabular dysplasia and genu valgum deformity.

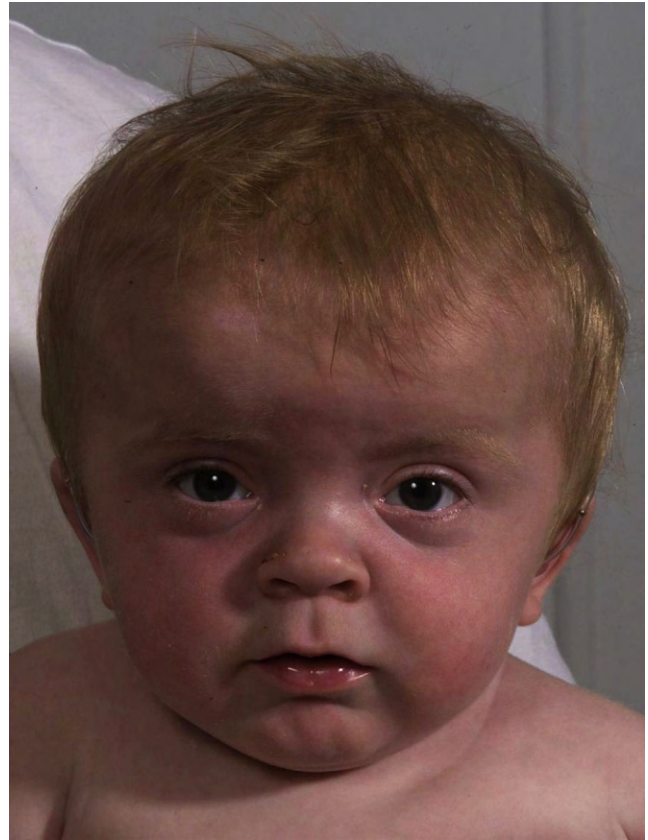


**FIGURE 102-8** MPS II patient with enlarged sella turcica ventriculo-peritoneal shunt in situ for communicating hydrocephalus (arrow).

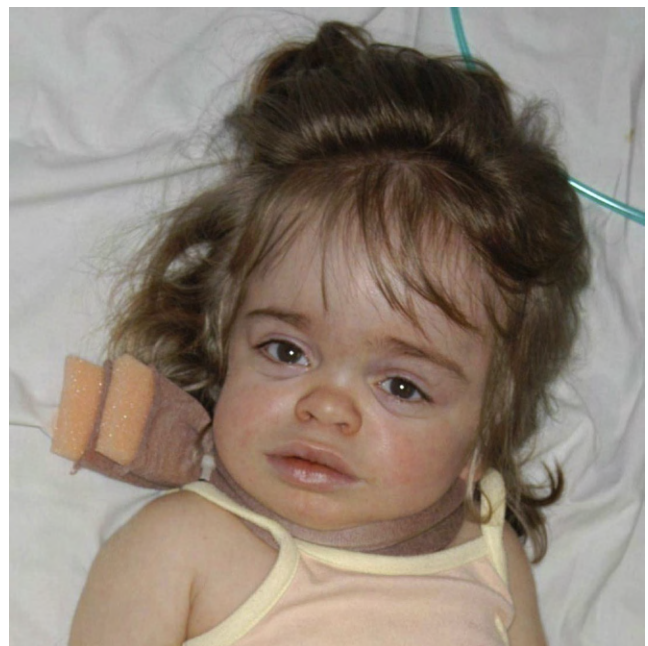


**FIGURE 102-9** Right hand of MPS II patient age 13 years. Note soft tissue flexion deformity of the interphalangeal joints. Metacarpals short, broad with proximal tapering. Abnormal ends of radius and ulna.

genetic disorders, such as hemoglobinopathies. In particular, the presence of lower airway disease, a common complication in MPS IH, appears to be particularly significant (34). In terms of graft survival, better matching and busulfan targeting protect against rejection (35) and these measures plus the advances in pediatric intensive



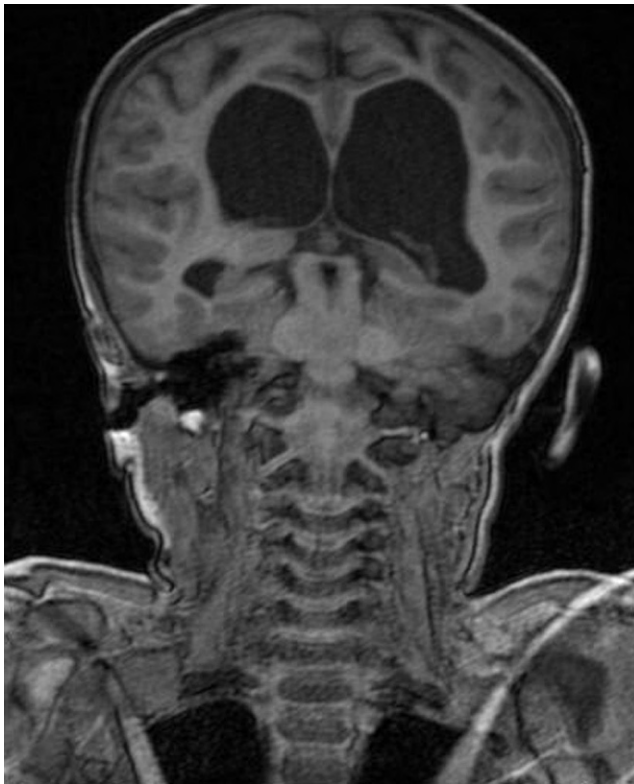
**FIGURE 102-10** MPS IH facial features at diagnosis aged 8 months.



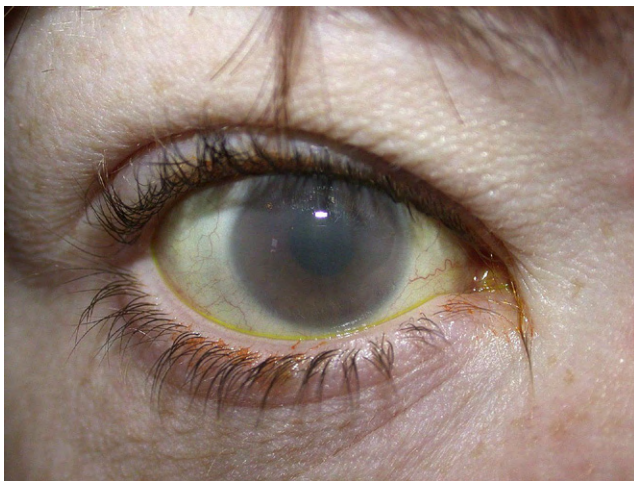
**FIGURE 102-11** MPS IH facial features at 27 months.

care, the increasing use of umbilical cord blood as a cell source and the use of enzyme replacement therapy (ERT) in the pretransplant period have all played a role in the improved graft and patient survival seen in the last decade (36–38).





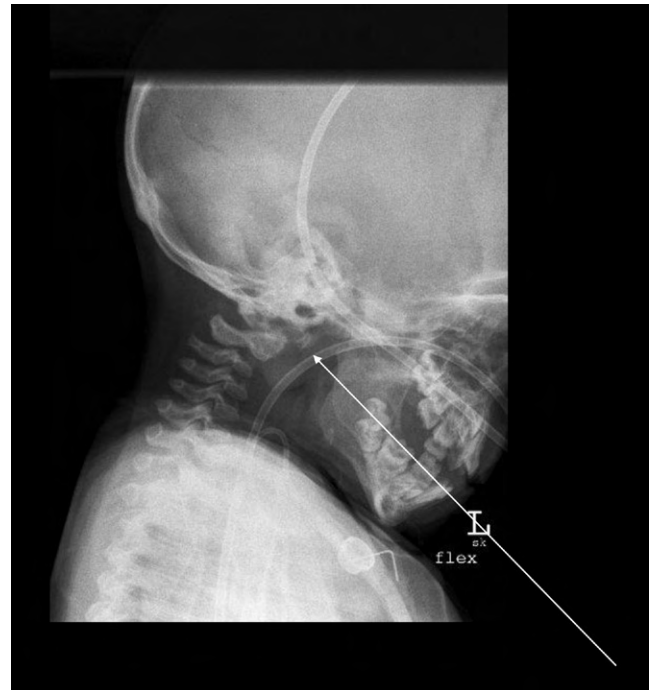
**FIGURE 102-12** Communicating hydrocephalus MPS IH.



**FIGURE 102-13** MPS I corneal clouding MPS IH age 2 years 6 months.

Despite the use of ERT and improvements in graft survival and patient morbidity and mortality, the results of successful HSCT remain variable and there is often a considerable residual disease burden. It is usually impossible to predict the outcome of a successful transplant in an individual patient. Generally, the earlier the transplant performed, the higher the enzyme level achieved; and the more favorable the chimerism, the better the outcome (18), although there are exceptions to this (39).

In favorable cases, there is rapid resolution of hepatosplenomegaly, decline in urine GAG excretion and improvement in respiratory function including resolution



**FIGURE 102-14** MPS IH flexion X-ray of C spine with anterior slip of C1 on C2 (arrow).

of upper airways obstruction (40). In the heart, cardiomyopathy improves (41), coronary arteries are patent (42) but valve disease often progresses (43). The coarse hair and facial features soften and become more normal (Figures 102-15 to 102-17).

Corneal clouding clears, but not completely and most patients develop slowly progressive retinopathy with attenuation of the electroretinogram (ERG) (44). Sensorineural hearing loss does not improve post transplant and many children require amplification to prevent delay in language acquisition and speech and language therapy has an important role in trying to maximize the beneficial effects of transplantation on development (45). To complete the findings in the head and neck post transplant, complex dental abnormalities persist and require specialist input for management (46).

Untreated MPS IH is associated with rapid cognitive decline and HSCT can stabilize this but only if performed before significant cognitive impairment has occurred. Most transplanted patients continue to gain new skills but acquisition of these is often slower than normal and most patients whilst capable of being educated within a normal school do need extra help and some have significant special educational needs. Certainly, treatable factors that may impair developmental progress should be sought and managed. This would include sensory loss such as poor hearing or vision as well potentially more serious complications such as communicating hydrocephalus.

The long-term cognitive outcome following HSCT has been reported in a number of publications (33,47–52) and for a comprehensive review see Reference (53).

In addition, to the cognitive issues there is additional evidence to suggest that transplanted children have significant psychosocial impairment (54).

By far, the most disappointing result of transplantation is its failure to improve the DM which continues to progress, despite very good enzyme levels being achieved in many patients. Although odontoid dysplasia may improve post-HSCT (55), cervical instability, spinal deformity, hip dysplasia and genu valgum deformities all deteriorate, greatly impairing functional abilities and often requiring very invasive orthopedic procedures to try and improve skeletal outcome (56–61) (Figures 102-18 to 102-26).

Despite early orthopedic intervention, growth is often poor post-HSCT; and in addition, a number of endocrine abnormalities have been detected that may contribute to this. Growth hormone therapy may have a role in carefully selected patients (62,63). Some long-term female survivors remain fertile (64,65).

In addition to the major orthopedic complications, more minor problems such as carpal tunnel syndrome or trigger digits are also common and the hands are often short and stubby in post-transplant patients (66) (Figure 102-27).

It is unclear whether or not additional enzyme replacement therapy post-HSCT will lead to an improvement in

some of the residual disease burden (67). It is important that these patients are assessed, treated and followed up by multidisciplinary teams experienced in the management of MPS IH.

Enzyme replacement therapy alone has been used in patients with MPS IH (68). It was shown to be safe and associated with a reduction in urine GAG level and size of liver. Left ventricular hypertrophy decreased and sleep studies either showed improvement or stabilization in the majority of patients. As the drug cannot cross the blood–brain barrier, no improvement in developmental delay can be expected with ERT and therapeutic goals with ERT in this patient group should be limited to symptom control as part of a wider package of palliative care (69). Not all patients respond favorably to ERT as in some skeletal and visceral response is poor (70,71). Because the therapy is invasive, ERT should be prescribed for a limited period and only continued if measured, positive benefits can be seen. The details of enzyme replacement therapy are considered in more detail in the next section.



FIGURE 102-15 MPS IH BMT at age 10 years.



FIGURE 102-16 MPS IH BMT at age 10 years.





**FIGURE 102-17** MPS IH BMT at age 10 years.

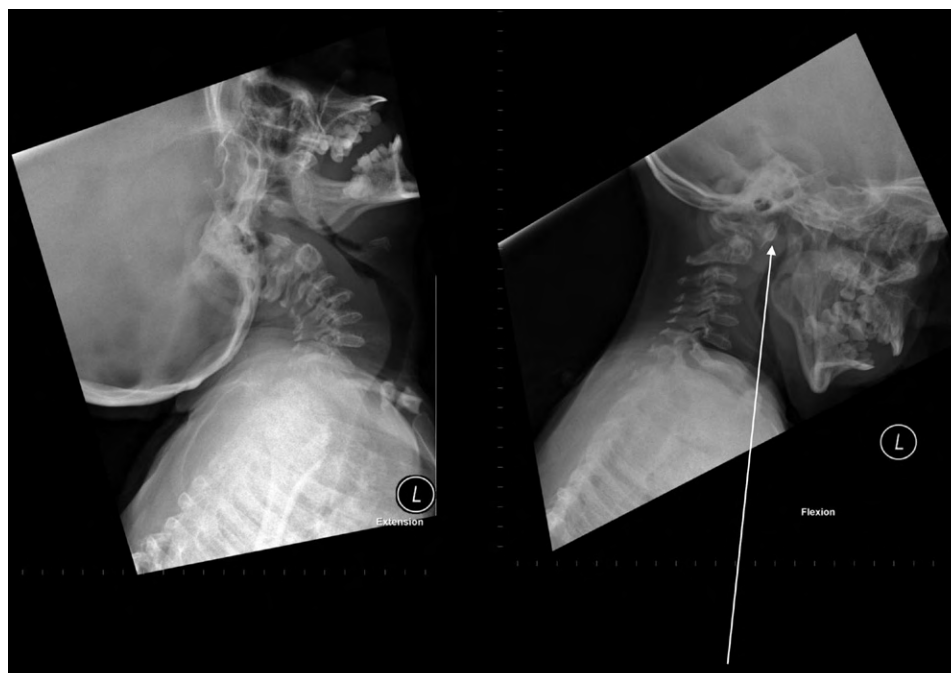
### 102.2.2 Mucopolysaccharidosis IH/S (Hurler/Scheie Disease)

Patients with the attenuated forms of MPS I have an extremely heterogeneous disorder and there is considerable overlap between the types labeled as Hurler/Scheie or Scheie. Both have in common little or no cognitive involvement but the skeletal and visceral manifestations can vary widely from patient to patient. The differences cannot be distinguished by either enzyme deficiency or GAG excretion and mutation analysis is of limited value except when two nonsense mutations are found, as this is invariably associated with a severe phenotype (72).

In our own patients with Hurler/Scheie disease, mean age of onset of symptoms was 2.0 years (median 1.4 years; range 4 months–6 years) whilst the mean age of diagnosis was 6.5 years (median 4 years; range 1.3–32 years) indicating significant diagnostic delay in this group of patients a point of pivotal importance when considering the benefits of ERT (73).

The commonest clinical presentation in this group of patients is joint stiffness (40% of patients), often misdiagnosed as juvenile rheumatoid arthritis. Other features present at diagnosis in some patients include corneal clouding (13%), recurrent ear, nose and throat infections (27%), deafness (20%), umbilical hernia (27%), abnormal facial features (13%), hepatomegaly (13%) and short stature (13%) (73). Joint contractures in the absence of inflammatory changes have been flagged as an indication to test for attenuated forms of MPS disease (13,74).

With time the condition progresses and the facial appearance changes. The mandible is often very underdeveloped, limiting mouth opening and contributing the anesthetic difficulties seen in these patients (75–77) (Figures 102-28 and 102-29).



**FIGURE 102-18** MPS IH post-HSCT flexion and extension views of C spine demonstrating anterior atlantoaxial subluxation (arrow).

Corneal clouding worsens and is often associated with optic nerve swelling or a retinopathy (78,79).

Restrictive respiratory disease increases, cardiac valve lesions are common and may require surgery (80). The bone and joint disease limits mobility and dexterity with additional problems including an exaggerated lumbar lordosis with an underlying spondylolisthesis and also thickening of the dura in the cervical region causing compression on the spinal cord in this area, leading to cervical myelopathy (73). The surgical burden borne by the patients is significant (81) (Figures 102-30 to 102-32).

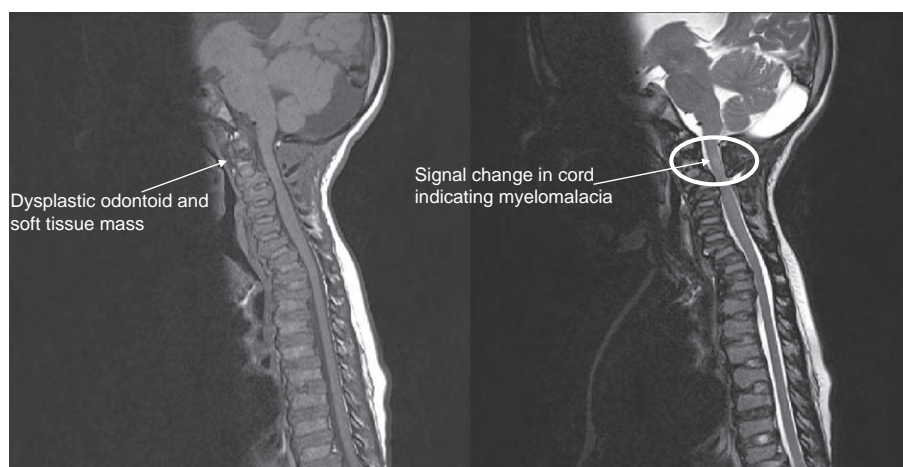
Some patients with MPS IH/S have significant cognitive impairment, but many are normal and most can cope with normal school with extra help for their physical and sensory disabilities.

Before enzyme replacement therapy became available, most patients with Hurler–Scheie disease died in their 20s

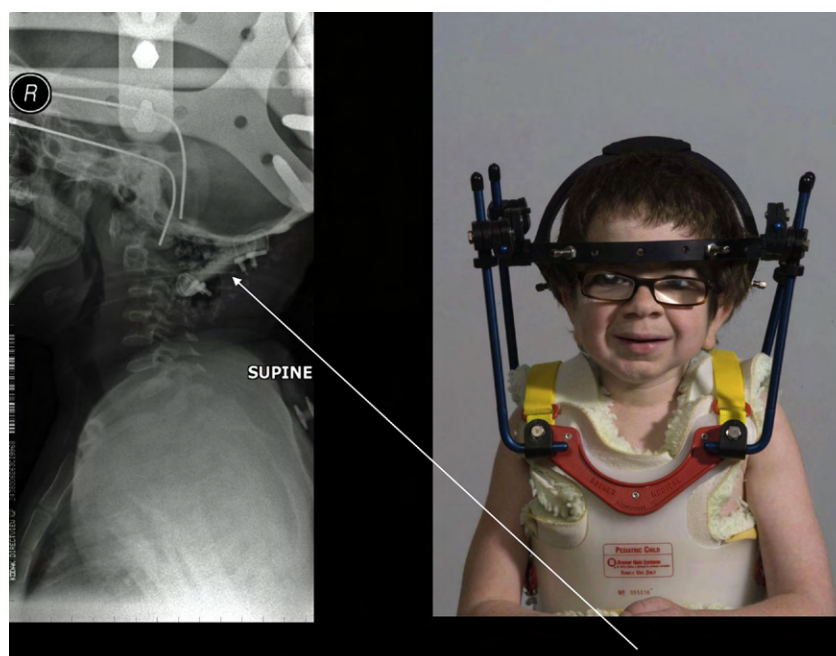
(mean 21.6 years, range 19.3–24.0 years, (23)) from the cardiorespiratory disease. It is too early to say whether or not this will be significantly prolonged by ERT.

### 102.2.3 Mucopolysaccharidosis IS (Scheie Disease)

The most attenuated form of MPS I is known as Scheie syndrome after the ophthalmologist who first described the condition in 1962 (82). Most patients are diagnosed as teenagers (mean age of diagnosis 13.3 years, median 7 years, range 2.5–40 years (73)). Patients often have considerable symptoms in childhood and most by the age of 12 years have a combination of, hepatomegaly, joint contractures, cardiac valve abnormalities and corneal clouding (83). Later complications include carpal tunnel syndrome (84) and cervical myelopathy secondary to



**FIGURE 102-19** T1 and T2 weighted images of the C spine (same patient as Figure 102-20).



**FIGURE 102-20** X-ray of C spine with bone graft in situ. Patient wearing halo fixation device.

dural thickening (85). Cognitive impairment is not usually a feature of Scheie disease and prolonged survival is usual although considerable disability may be present and most patients have undergone a significant number of surgical procedures (81).

**102.2.3.1 Treatment of MPS IH/S and MPS IS.** ERT using recombinant human  $\alpha$ -L-iduronidase, laronidase, (Aldurazyme, BioMarin Pharmaceutical Inc., Novato, California and Genzyme Corp., Cambridge, MA) is now part of the standard therapy for MPS IH/S and MPS IS. In a double-blind, placebo-controlled clinical trial in 45 patients with attenuated MPS I, laronidase was shown to reduce urine GAG excretion and to improve respiratory function and physical endurance (86). Laronidase is a life-long therapy and is given by weekly intravenous infusion over 3 to 4 hours. It has generally proven to be safe even when given to severely affected patients that one would expect to have null mutations (68).

A limited number of prolonged follow-up studies of MPS I patients treated with laronidase suggest that improvements are maintained in many areas (87,88), but like the results of HSCT this can be variable. Assuming no problems with the initial infusions,

the treatment can be administered in the patient's home (89).

The biochemical effect of ERT is possibly less than that achieved by HSCT (90) but it is difficult to directly compare the two populations of patients treated by these different modalities and both have been demonstrated to restore dermal fibroblast morphology (91).

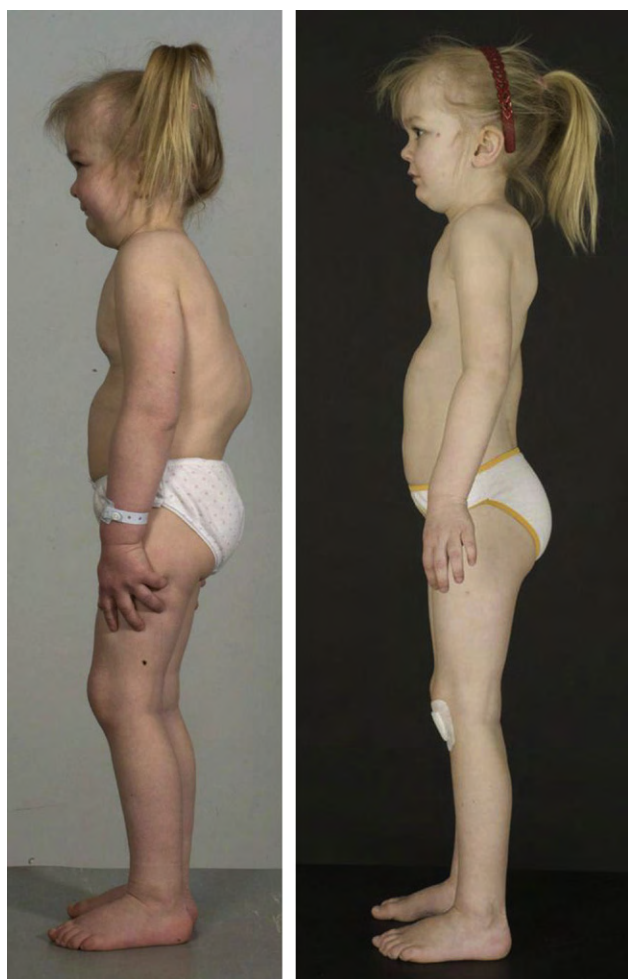
It has been clearly demonstrated both in the MPS I dog and MPS I human that early treatment gives the best chance of a successful outcome with treatment (92,93).

The limited reports of the results of long-term ERT in MPS IH/S and MPS IS suggest that over a 6-year period respiratory function tests remained stable and there is a small increase in distance walked in a 6 min walk test (88). Joint mobility may improve but this may take place gradually over a prolonged period of time (94,95). Dural thickening is not improved by intravenous ERT, but intrathecal ERT may be effective in this situation in carefully selected patients (96). Cardiac valve lesions do not improve with ERT and the majority of patients with significant corneal or optic disc changes continue to deteriorate.

The vast majority of patients develop antibodies to laronidase (93%, (88)). The significance of these remains uncertain but in the animal model, at least, the induction of immune tolerance has a significant impact on the efficacy of therapy and may have some relevance to the human situation (97). About half of all patients treated will have an infusion associated reaction (53%, (88)), but fortunately the majority of these will be mild and very easily managed with premedication with an antihistamine or slowing down the rate of infusion (98). In most patients, the frequency and intensity of reactions decreases over time. Anaphylactic reactions have been reported in the product safety information ([http://www.aldurazyme.com/hc/abt/az\\_us\\_hc\\_safety.asp](http://www.aldurazyme.com/hc/abt/az_us_hc_safety.asp)) but in practice these must be very rare.

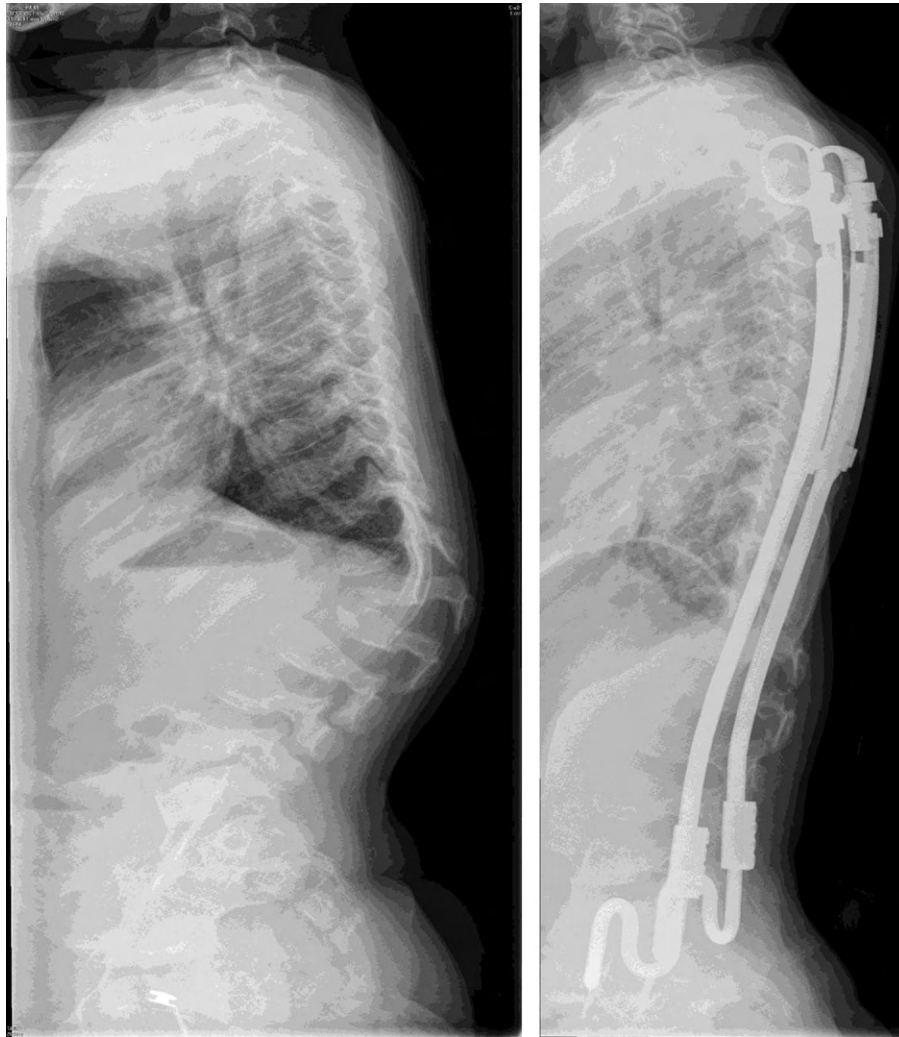
### 102.3 MUCOPOLYSACCHARIDOSIS II (HUNTER SYNDROME)

MPS II (Hunter syndrome) is an X-linked recessive disease caused by the deficiency of the lysosomal enzyme iduronate-2-sulfatase (I2S). I2S functions to cleave O-linked sulfate moieties from both DS and HS; therefore, due to the deficiency of I2S, these molecules progressively accumulate in MPS II. Apart from in the Far East, MPS II is not as prevalent as MPS I, and occurs an estimated incidence of one in 162,000 live births (99). Like MPS I, the clinical phenotype is secondary to GAG accumulation in almost all cell types, tissues and organs of the body. Although the condition is X-linked, a number of affected females have been reported. Most have a severe clinical phenotype and preferential X chromosome inactivation of the nonmutant allele resulting in low I2S enzyme activity (100). Affected female patients should be managed in the same way as affected males.

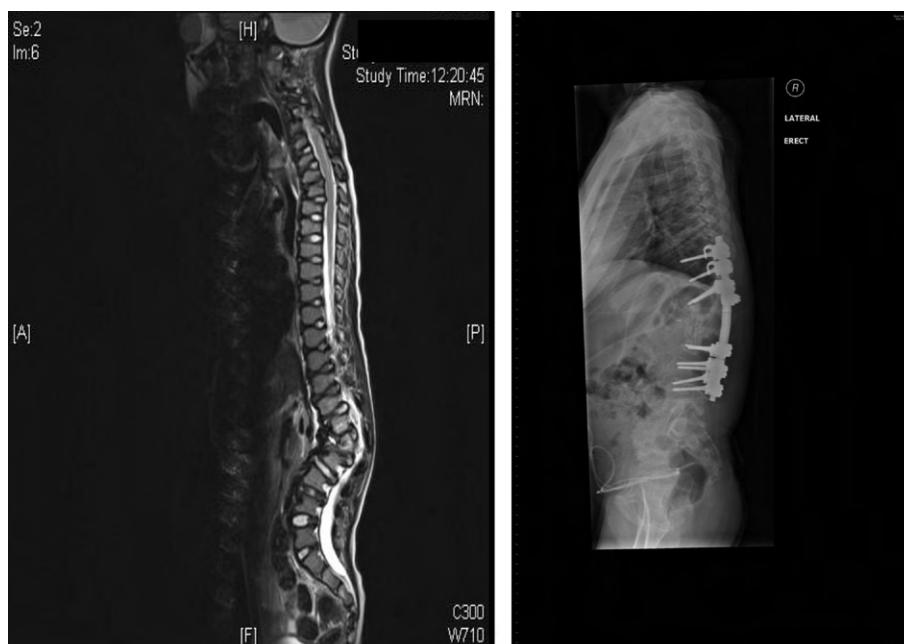


**FIGURE 102-21** MPS I post-HSCT pre- and post-op kyphosis surgery.





**FIGURE 102-22** MPS I post-HSCT pre- and post-op kyphosis surgery.



**FIGURE 102-23** MPS I post-HSCT pre- and post-op kyphosis surgery.





**FIGURE 102-24** Genu valgum deformity post-HSCT.

MPS II is a very heterogeneous disorder, both in terms of age at onset of symptoms and disease severity. The approach to recognition and diagnosis has been reviewed recently (101) and will be summarized here.

The median age of onset of symptoms and diagnosis of Hunter syndrome is 1.5 and 3.5 years, respectively (102). Many patients have already undergone surgical intervention before diagnosis and repeated early surgical interventions especially for hernias are common. Such a surgical history should raise suspicion of the possibility of an underlying MPS disorder (103). Most affected male patients are normal at birth but during the first 3 years of life grow rapidly and are often large infants. Growth velocity usually slows by the end of the third year and ultimately most patients are small (104).

It is usual to divide the patients into two groups, “severe” or “mild” based on survival and the presence or absence of neurodegeneration and cognitive impairment. This is an oversimplification as the disease, like MPS I, is a spectrum and it is often difficult at presentation to

predict neurological outcome. Retrospective studies of patients with MPS II have shown that patients with neurological involvement have an earlier age of onset of symptoms (2.47 years) compared with those with no cognitive impairment (4.3 years) and in addition have a much lower mean age at death (11.7 years in severe patients and 21.7 years in mild or attenuated patients (105)). There is some evidence that brain imaging may help to determine at an early stage patients that are destined to be significantly cognitively impaired but many more patients need to be studied to confirm this (106,107).

The most common presentation is a history of recurrent upper airway infections combined with a coarsening of the facial features with an enlarged head circumference. There is flattening of the nasal bridge and prominent supraorbital ridges and X-ray examination reveals evidence of DM (101) (Figure 102-33).

When compared with MPS IH at a similar age, it is clear that patients with MPS II have less severe skeletal involvement at a young age and are generally less



**FIGURE 102-25** Genu valgum corrected by medial epiphyseal fusion using eight plates.



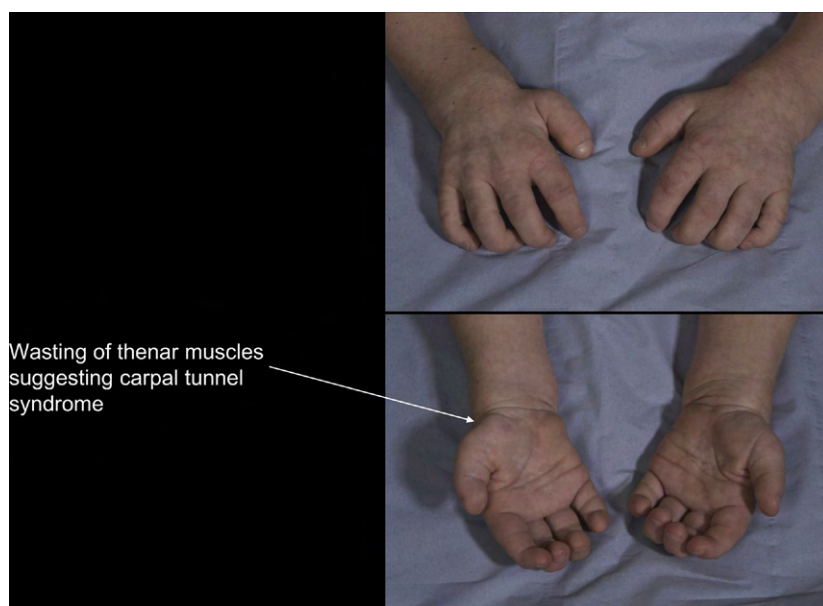
**FIGURE 102-26** X-ray and MRI of hips in 5-year-old MPS IH patient post BMT. Shallow acetabula shown on MRI to be due to failure of ossification of lateral border of the acetabulum. Coxa valga also present (arrow).

dysmorphic, explaining the difference in mean ages at diagnosis (9 months MPS IH compared with over 2 years for patients with MPS II). [Figure 102-34](#) illustrates this point.

Organ involvement is broadly the same as in MPS I. Frequent upper respiratory tract infections, mixed conductive

and sensory hearing loss ([108](#)) and obstructive sleep apnea are common ([109](#)).

Progressive airway obstruction is common and is combined with restriction due to the immobility of the thoracic cage secondary to skeletal involvement. Tracheobronchomalacia is common and the end result may



**FIGURE 102-27** 13-year-old MPAS IH patient post-HSCT. Short stubby hands.

be progressive respiratory failure and noninvasive ventilation is often required at night to maintain airway patency (110,111). Video 102-1 is a video of a laryngoscopy on a teenage boy with MPS II. Abundant soft tissue around the larynx is seen to prolapse into the orifice to cause obstruction during inspiration. Later in the video, the trachea is seen as narrowed and partially collapsed due to tracheomalacia.

In older patients, tracheal stenosis may be an additional problem (112).

The ocular findings in MPS II differ from those seen in MPS I. Corneal clouding is not a feature and raised intraocular pressure is very rare. The sclera is usually thicker than normal (113) and optic disc edema can mimic papilledema (114). Figure 102-35 shows the disc appearance of a teenager with MPS II. Despite this appearance, visual acuity was normal. Progressive retinopathy associated with attenuation of the ERG and thinning of the retina can lead to progressive visual loss (115).

Another finding that seems to be specific to MPS II is a distinctive papular skin lesion found around the scapulae and upper thighs of affected boys (Figure 102-36). The papules are ivory colored and range in size from 0.2 to 0.5 cm in diameter and often coalesce as seen in Figure 102-37. Biopsy of the lesions reveals interstitial mucin deposited throughout the reticular dermis (116).

Almost all affected boys develop cardiac valve lesions and cardiac disease is a common cause of death in this cohort (117).

Central nervous system involvement is variable. In severely affected patients it manifests with delay in development, behavioral disturbance and then progressive loss of skills between the ages of 5 and 10 years. Epileptic seizures are common and death usually occurs in the first or second decade of life. At the other end of the

clinical spectrum, patients with attenuated disease may have normal or near-normal cognitive abilities despite significant somatic disease.

Despite normal cognition, magnetic resonance imaging (MRI) scans of the brain in attenuated patients are often very abnormal (118). Cervical myelopathy due to hyperplasia of the transverse ligament and dural thickening is a common complication from teenage years onwards. This usually presents with a gradual loss of endurance coupled with difficulty with rising from a chair. If untreated, it progresses to a slowly ascending paralysis with sphincter involvement. Prompt cervical decompression can prevent this progression (Figure 102-38).

Carpal tunnel syndrome is common in affected males but is often asymptomatic. It should be screened for from a young age and surgical decompression formed early to preserve nerve function (119).

### 102.3.1 Treatment of MPS II

Multidisciplinary management is essential for affected patients with MPS II. Patients require access to ENT surgery, pulmonologists, orthopedists, neurosurgeons, cardiologists, ophthalmologists and audiologists among others. Skilled anesthesiology is a prerequisite for any planned or emergency surgical procedure. In severe neurologically impaired patients, management may be similar to that described for patients with MPS III and may include anticonvulsants, gastrostomy feeding and behavior modifying medications.

HSCT has not been reported as being successful in preventing the neurological deterioration that occurs in severe MPS II and is therefore not routinely offered as a form of therapy for these patients (120,121). It should be noted however that the numbers treated are small and the



patients have been offered treatment at a relatively late stage in their illness in the majority of cases. No systematic clinical trials of HSCT in MPS II have been performed and so these results should be interpreted with some caution.

Idursulfase (Elaprase, Shire Human Genetic Therapies Inc., Cambridge, MA, USA) is a recombinant form of I2S produced in a continuous human cell line and is used as ERT in MPS II. A large clinical trial demonstrated clinical benefit from weekly Idursulfase in a dosage of 0.5 mg/kg/week by intravenous infusion. Ninety-six patients were studied and the primary efficacy end point used was a composite of distance walked in 6 min and

the percentage of predicted forced vital capacity based on the sum of the ranks of change from baseline (122). There have been very few follow-up studies reported but the drug has been evaluated in children below the age of 5 years (123) and in adults with attenuated disease (124). A positive effect on growth has been reported especially in patients treated from under the age of 10 years (125). Although a number of patients have had infusion associated reactions (98) and some develop antibodies to idursulfase, the treatment is generally safe and can be administered in the patient's home (126).

Like laronidase, elaprase is unable to cross the blood-brain barrier and therefore, intravenous therapy cannot prevent or correct the neurological deficits seen in severe MPS II. A clinical trial of intrathecal ERT has commenced in these patients: (<http://www.clinicaltrials.gov/ct2/show/NCT00920647?term=mucopolysaccharidosis+type+II&rank=7>).

#### 102.4 MUCOPOLYSACCHARIDOSIS IIIA (SANFILIPPO SYNDROME, MPS IIIA)

MPS III is the most common of the MPS disorders with an estimated incidence of all types together of around

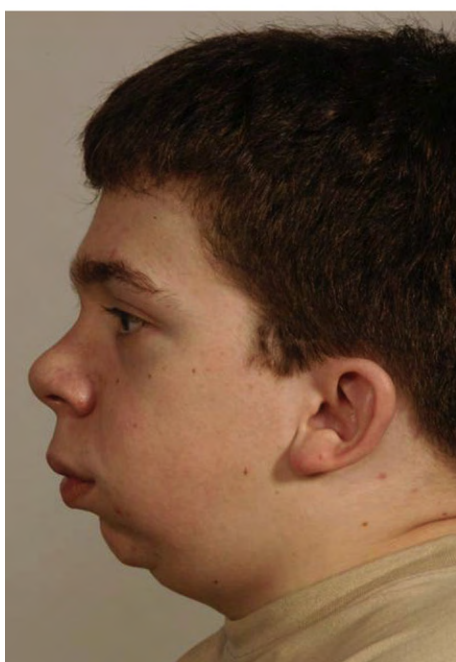
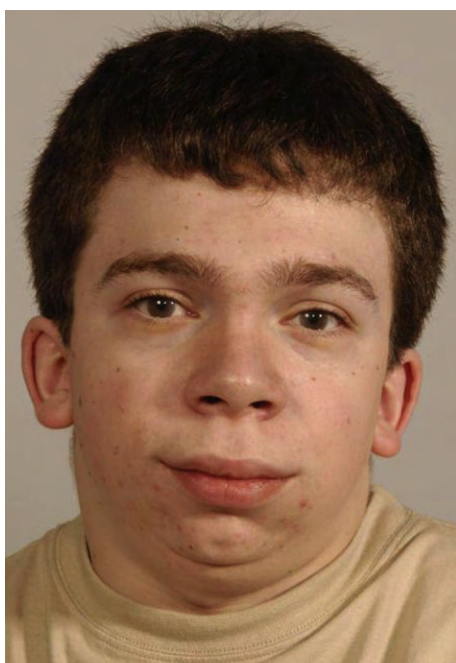


FIGURE 102-28 Teenager with MPS IIH/S—note micrognathia.

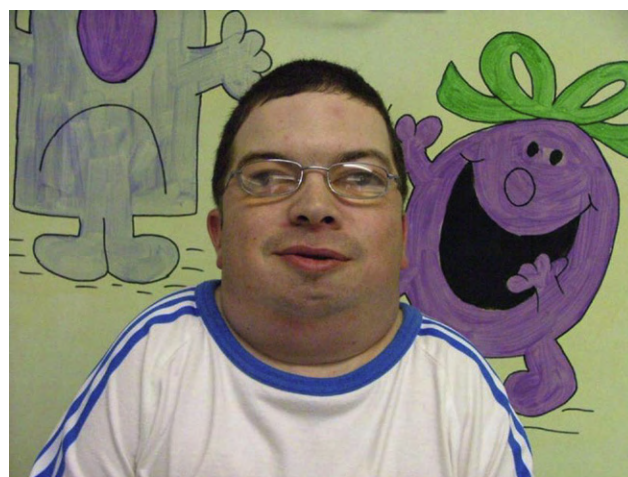


FIGURE 102-29 Teenager with MPS IIH/S—note micrognathia.



1:60,000–70,000 births (99). All of the disorders result from a failure to catabolize the GAG heparan sulfate. The enzyme deficiencies behind each of the subtypes of MPS III are documented in Table 102-1. MPS IIIA, the commonest of the MPS III subtypes in Western Europe is associated with a deficiency of the enzyme heparan-N-sulfatase (HNS). In our own patients, MPS IIIA was the clinical subtype in 47 out of the first 62 patients seen and assessed in our own unit (127).

MPS IIIA, like all of the MPS conditions, is a heterogeneous disorder. In our patients, the mean age at diagnosis was 4.9 years (range 0.8–16 years) and this seems consistent with other, more recent, studies (128,129).

Although diagnosis is usually made after the age of 4 years, the patients often have symptoms from the first year of life. Speech delay, recurrent ear, nose and throat infections, diarrhea and hearing loss are common presenting features. The absence of significant somatic disease usually means that a diagnosis of MPS is not

considered by the nonspecialist pediatrician as a potential cause for what are very nonspecific clinical symptoms. Enlargement of the liver and spleen and cardiac lesions are rare and the facial features are much “softer” than MPS I or II (Figure 102-39).

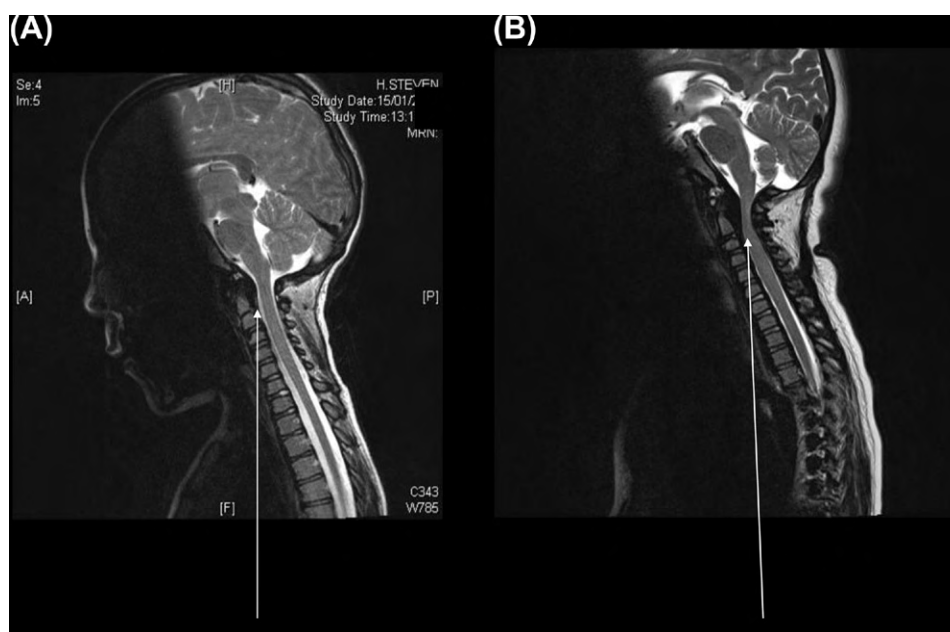
In a typical patient, the phenotype evolves over three overlapping phases (127). The first phase (usually before diagnosis) consists of developmental delay alone. This is most often delay in speech development and initially this is often ascribed to coexisting middle ear disease but when grommets are inserted there is no improvement in speech acquisition. In addition, many patients have



**FIGURE 102-30** Fixed flexion deformities of the fingers in a patient with MPS IH/S.



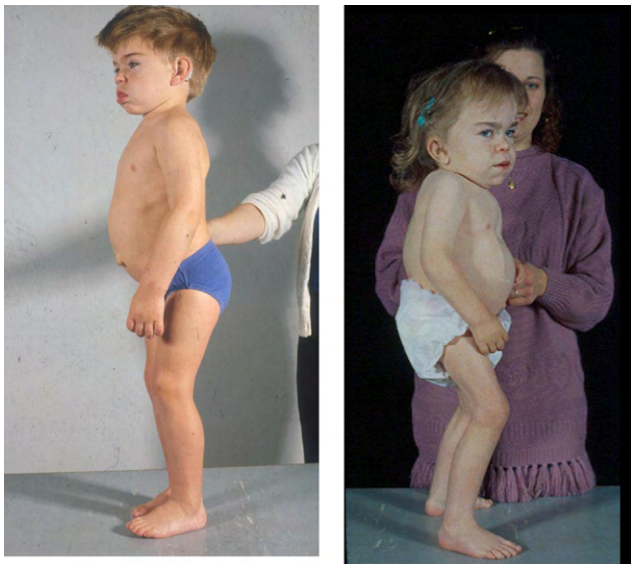
**FIGURE 102-31** MPS IH/S spondylolysis (arrow).



**FIGURE 102-32** (A) MRI scan of cranial–cervical junction clear rim of cerebrospinal fluid seen around the upper cord. (B) MRI scan CCJ of MPS IH/S patient with dural thickening (no rim of CSF) and early signal change in cord (arrow).



**FIGURE 102-33** MPS II facial features age 8 years.



**MPS II**

**MPS I**

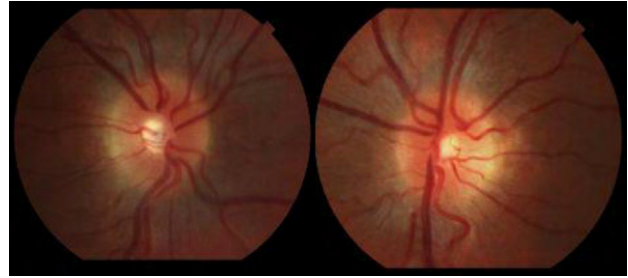
**FIGURE 102-34** Two 4-year-old children with MPS II and I.

gastrointestinal problems with chronic diarrhea being particularly prevalent (130).

The second phase of the illness usually begins around the age of 3 or 4 years and is characterized by severe behavioral disturbance. The behavior is challenging and characterized by increasingly frequent and severe temper tantrums. Hyperactivity is usual and is often combined with a severe sleep disturbance. There may be panic attacks and as the children are normally grown they can be very difficult to manage in social situations.

At the end of the first decade, the illness changes again as children enter the third and final stage of the illness. The patients become quieter, fall more frequently and develop neurological dysphagia. Gastrostomy feeds become necessary, the children become wheelchair bound and a significant number of patients develop seizures.

The radiological changes of DM are generally mild but patients are prone to vascular necrosis of the femoral head which may be unilateral or bilateral (127) (Figure 102-40).



**FIGURE 102-35** MPS II full optic discs both eyes.



**FIGURE 102-36** MPS II papular rash around scapula.



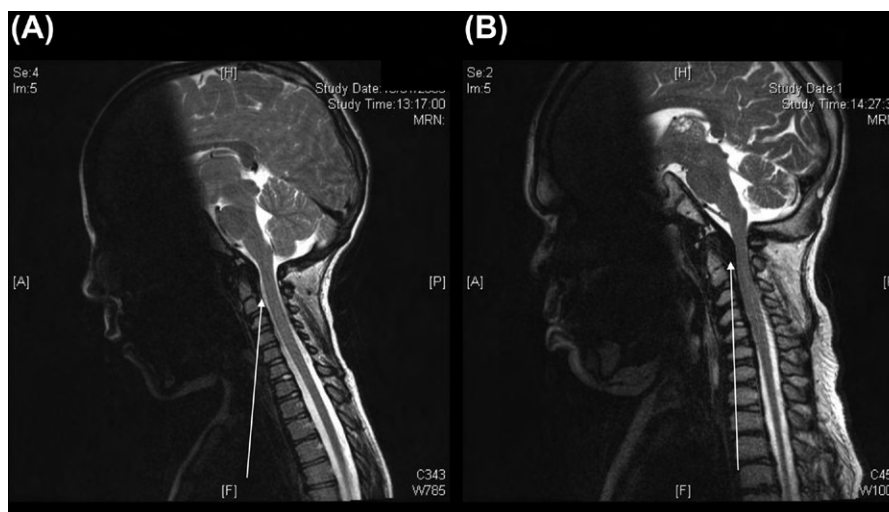
**FIGURE 102-37** MPS II patient with normal cognitive development.

In typical, severely affected patients, death usually occurs in the second or early in third decade of life. The usual cause of death is respiratory infection in a profoundly handicapped child or young adult.

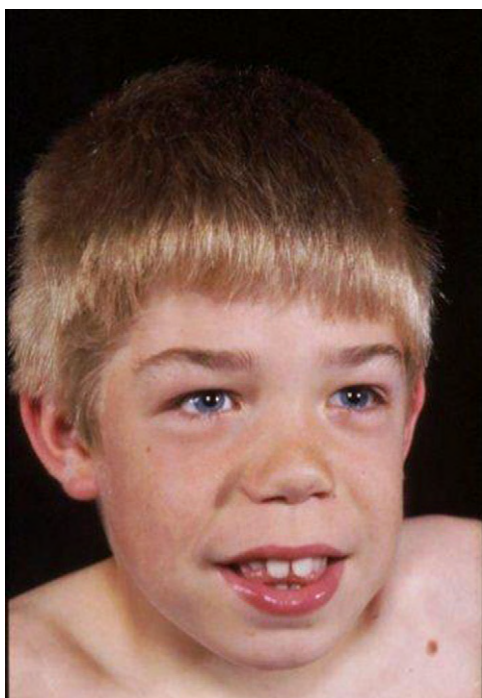
In keeping with other CNS disorders, precocious puberty has been reported in MPS III (131). Like all the MPS disorders, there are attenuated patients and a late onset visceral presentation without neurological disease has been reported in MPS IIIA (132).

Numerous mutations have been identified in the *HNS* gene and in western Europe the missense mutation p.R245H is prevalent (35% of alleles in Germany, 57% of alleles in The Netherlands) (133). Establishing a good genotype–phenotype correlation in MPS IIIA is complicated by the presence of a number of polymorphisms of





**FIGURE 102-38** MPS II normal MRI scan—clear rim of CSF around cord in C region MPS II with cervical myelopathy no CSF visible around cord in C region (arrow).

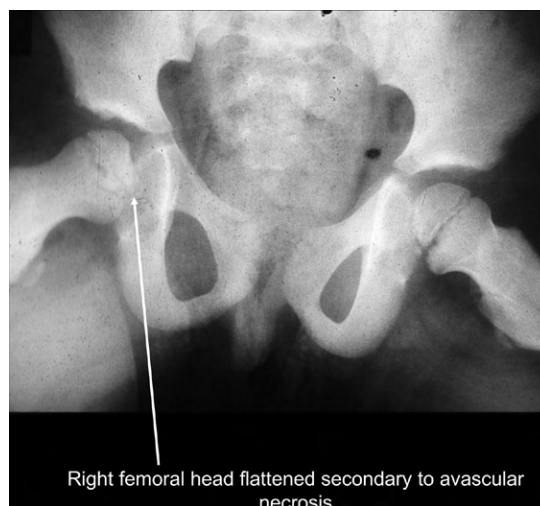


**FIGURE 102-39** MPS III facial features.

unknown significance but can be done (129). One mutation (p.Ser298Pro) is associated with a slowly progressive phenotype and survival well into adult life (134). These patients can be very difficult to diagnose as the very slow progression can be missed and the condition misdiagnosed as a static encephalopathy.

### 102.5 MUCOPOLYSACCHARIDOSIS IIIB (SANFILIPPO SYNDROME, MPS IIIB)

MPS IIIB is caused by a deficiency of the enzyme *N*-acetyl- $\alpha$ -D-glucosaminidase (NAGLU) which, like a deficiency of HNS, leads to an accumulation of HS and a Sanfilippo



**FIGURE 102-40** MPS III unilateral hip dysplasia.

phenotype. In the United Kingdom, the clinical phenotype and disease progression is exactly the same as that described for MPS IIIA, but the disease is less prevalent (127). In The Netherlands, however, MPS IIIB presents, predominantly, as an indolent, slowly progressive disorder compatible with prolonged survival and a median age at death of 43.5 years (135,136).

### 102.6 MUCOPOLYSACCHARIDOSIS IIIC (SANFILIPPO SYNDROME, MPS IIIC)

The gene responsible for MPS IIIC, *TMEM76* (the transmembrane protein 76 gene), is the most recently identified MPS gene (137) and it encodes for an enzyme (acetyl-coenzyme A: $\alpha$ -glucosaminide *N*-acetyltransferase, HGSNAT) that is active in the lysosomal membrane. HGSNAT catalyzes acetylation of the terminal glucosamine residues of the GAG heparan sulfate prior to its hydrolysis by  $\alpha$ -N-acetyl glucosaminidase (138).

A deficiency in HGSNAT leads to accumulation of heparan sulfate and a Sanfilippo phenotype. Most of the missense mutations identified affect folding of the mutant protein raising the possibility of pharmaceutical chaperones as a potential therapy in the future (139). MPS IIIC is a rare disease with an estimated prevalence of 0.07 per 100,000 live births (99) and generally results in an attenuated MPS III phenotype. In a study of 29 patients from The Netherlands, the mean age of death was 34 years (range 25–48 years) (140).

## 102.7 MUCOPOLYSACCHARIDOSIS IIID (SANFILIPPO SYNDROME, MPS IIID)

MPS IIID is caused by a deficiency of the lysosomal hydrolase  $\alpha$ -N-acetylglucosamine-6-sulfate sulfatase (GNS). GNS cleaves the sulfate at the 6-hydroxyl position moiety of N-acetylglucosamine from heparan sulfate leading to accumulation of this metabolite and a Sanfilippo phenotype (141).

The disorder is very rare (1:1,000,000 live births) (99) and the largest case series in the literature describe 12 patients (142). The patients reported have clinical features characteristic of MPS III A, B and C and no significant differences between the phenotypes is apparent.

### 102.7.1 Treatment of MPS III

The treatment of MPS III is symptomatic and palliative. The condition does not respond to HSCT (143) and ERT has not yet been developed.

Sleep disturbance can have a profound effect on the health of carers and needs to be tackled aggressively. Melatonin has the highest chance of success but in some patients sleep disturbance remains resistant to all forms of therapy (144). A clear disturbance of circadian rhythm has been demonstrated in the mouse model of MPS III and presumably similar defects in the human contribute to the sleep disturbance (145).

The challenging behavior during the day can also be difficult to manage. The hyperactivity responds poorly to behavior therapy and psychostimulants such as methylphenidate. Usually, major tranquilizers are needed and the only one that has been examined in a randomized trial in MPS III patients is risperidone (0.125–2 mg/d) (146). In this study, most improvement was seen in hyperactivity and defiance with less effect noted on conduct disorder. In the mothers of the treated children, there was a significant reduction in anxiety and depression scores. In this study, risperidone appeared to be both effective and safe but in other patients extrapyramidal side effects have been reported, especially in patients on relatively high dose (>4 mg/d) (147).

Toward the end of the first decade of life, neurological dysphagia becomes problematical for a significant number of patients. Weight loss, increasing time taken to feed and chest infection herald the need for gastrostomy

placement to prevent aspiration. Inhibition of saliva production can be successfully achieved by either scopolamine patches or glycopyrrolate. Both have been subjected to controlled trials in disabled patients and found to be effective but both have a significant incidence of side effects, in particular, agitation (148,149).

Over the age of 8 years, seizures become more common. These can be a mixture of different types and no specific anticonvulsant is better than another in management. Other nonepileptic paroxysmal behaviors also occur particularly at night. The etiology of these is often unclear but parents often ascribe them to pain as during the episode the patients often seem very distressed. Management of these episodes can be very difficult and one usually has to rely on sedatives running the risk of over sedation in particularly difficult patients. Video 102-10 is a short clip of a 15-year-old MPS III girl demonstrating the characteristic “distressed” behavior often seen in MPS III.

More experimental forms of therapy are in development. As this is a primary CNS disorder, a more inventive method of therapy will be required as intravenous ERT cannot be effective in such disorders as the enzymes are unable to cross the blood–brain barrier. Three approaches are in development and potentially more could be applicable in the future:

- (1) Intrathecal enzyme replacement therapy is in human clinical trial (December 2010) for MPS IIIA (<http://clinicaltrials.gov/ct2/show/NCT01155778?term=mucopolysaccharidosis+type+III&rank=1>)

Studies in both mice and dogs suggest that this approach may reduce primary substrate storage (150,151). In MPS IIIA mice, ERT from birth (when the blood–brain barrier is still open) delays the onset of neurological and behavioral disturbance seen in the untreated animal (152). Clearly, there are a number of additional safety issues to be considered with this therapy in humans. The nature of the delivery device, the necessary frequency of infusion and the possibility of infection or immunological problems are just a few of the variables that need to be carefully considered with this method of therapy.

- (2) Genistein, a naturally occurring isoflavone, has been proposed as a potential treatment for MPS III (and other MPS disorders) as it can inhibit the synthesis of GAGs (153). This approach to therapy is known as substrate reduction therapy (SRT) and is an alternative approach to enzyme enhancement (by ERT, HSCT or gene transfer). To be successful, there must be a small amount of residual enzyme activity to catabolize substrate already stored whilst the SRT drug hopefully prevents accumulation of further substrate thus restoring metabolic balance.

Genistein is known to occur in many different health food supplements, but not all contain sufficient amounts to inhibit GAG synthesis in vitro (154). In mouse models



of MPS III, the best results are obtained with high dosages of chemically synthesized, pure genistein aglycone (155,156). A placebo-controlled study is required to assess the potential of genistein therapy in humans.

Rhodamine B, another inhibitor of GAG synthesis has also been used in murine MPS IIIA with promising results but has not yet been tried in humans (157).

(3) Gene transfer using a variety of vectors has been attempted and successfully completed in murine models of both MPS IIIA and IIIB (158,159). In affected MPS IIIB dogs, immunosuppression is necessary to prevent elimination of transduced cells by neuroinflammation, but subsequent results suggest that this approach may be applicable for use in affected humans (160).

### 102.8 MUCOPOLYSACCHARIDOSIS IVA AND IVB (MORQUIO SYNDROME, MPS IVA, MPS IVB)

MPS IVA is very different from the other MPS disorders. A deficiency of *N*-acetylgalactosamine-6-sulfatase (GALNS) leads to a failure to degrade and therefore an accumulation of KS and C6S resulting in a severe skeletal dysplasia (161). MPS IVB is a rarer, distinct and different genetic disorder caused by a deficiency of the enzyme  $\beta$ -galactosidase due to defects in the  $\beta$ -galactosidase gene (*GLB1*) (162). The major substrates for  $\beta$ -galactosidase are KS, galactose-containing oligosaccharides as well as GM1 ganglioside, GA1, and lactosylceramides. The primary disorder associated with defects in the *GLB1* gene is GM1 gangliosidosis. The mutations in *GLB1* that underlie Morquio type B cause the enzyme to lose specificity for KS but retain the ability to degrade GM1 ganglioside. Several missense mutations in *GLB1* have been identified as causing Morquio type B but not all mutations in *GLB1* are predictive of phenotype (163). From a

clinical point of view, the skeletal dysplasia in Morquio B disease is generally milder than that seen in classical MPS IVA and the patients usually attain greater height. The condition is usually distinguished from juvenile GM1 gangliosidosis by the absence of cognitive impairment but there can be significant overlap. The approach to management follows that of patients with MPS IVA.

MPS IVA is rare with a reported prevalence of approximately 1:200,000 live births (99). Like the other MPS disorders, it is heterogeneous but the most common presentation is with a very severe skeletal dysplasia associated with profound growth failure (final adult height 95–110 cm). Numerous mutations and polymorphisms have been identified in the *GALNS* gene in MPS IVA but there is only limited genotype:phenotype correlation (164). There is surprisingly little reported on the histological changes in bone and connective tissues in affected patients. The cartilage cells in the epiphyseal plate are disorganized and vacuolated and there is loss of the normal columnar architecture. Calcification in the plate is reduced (165). The extracellular matrix of cartilage is also disorganized resulting in a cartilage more prone to early degeneration (166). The end result is growth failure and a tendency to early onset arthritic changes in affected joints.

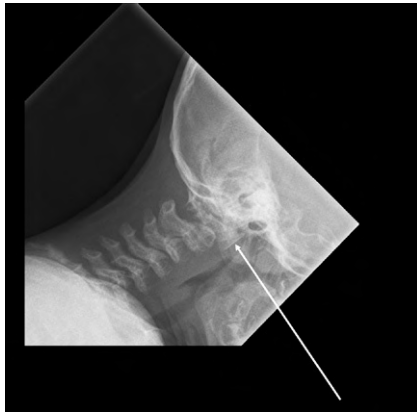
There have been recent reviews of the clinical manifestations and disease course in MPS IVA and in addition growth charts for affected patients have been produced (167,168). In our own patients, predominantly affected by severe MPS IVA, the diagnosis is usually made clinically toward the end of the first year of life (169). The parents of affected children most often notice a lower thoracic-upper lumbar vertebral gibbus or a pectus carinatum deformity of the chest (Figure 102-41).

The radiological abnormalities are distinct from the DM seen in other MPS disorders. In MPS IV there is platyspondyly and the odontoid is always dysplastic (170). In severely affected patients the odontoid is

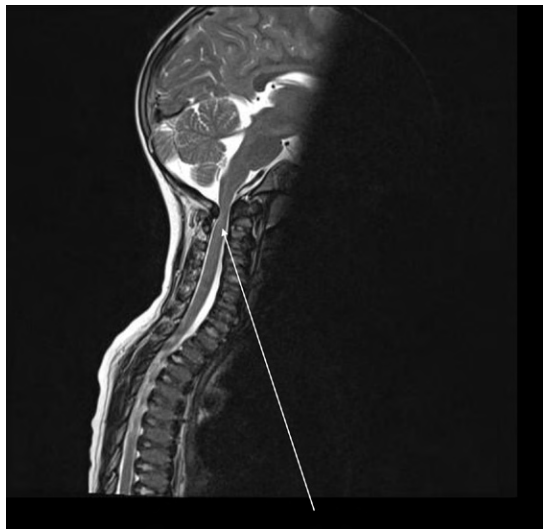


MPS IV patient at age 3 years

FIGURE 102-41 MPS IV patient at age 3 years.



**FIGURE 102-42** MPS IV hypoplastic odontoid and anterior subluxation of C1 (arrow).



**FIGURE 102-43** Same patient as Figure 102-42 MRI scan showing compression at craniocervical junction with signal change within the cord (arrow).

virtually absent and instability at the craniocervical junction is a major cause of morbidity and potential mortality. In addition, the hips are dysplastic and there is genu valgum deformity at the knees (Figures 102-42 to 102-44).

Although MPS IVA is a multisystem disease most time is spent analyzing the patient's craniocervical junction and assessing the presence or absence of instability and the potential risk of cervical cord damage (cervical myelopathy). MRI combined with plain radiology flexion and extension studies are a requirement for every patient with MPS IV. Figure 102-45 shows a typical MRI appearance of the C spine of a young MPS IV patient in whom plain radiology suggested instability at the craniocervical junction. The MRI scan shows an anterior soft tissue mass indenting the cord and causing compression in addition to the instability. Histological examination of this tissue shows that it has the appearance of nonspecific immature fibrous and fibrocartilaginous tissue (171).

The clinical presentation of cervical myelopathy can be acute with sudden death due to upper cord/brain stem



**FIGURE 102-44** Lateral spine—platyspondyly and gibbus. Lower limbs dysplastic hips and genu valgum deformity.

contusion (2 out of 37 patients in our original series (169)), but is more commonly insidious with loss of exercise tolerance often the first sign. If left untreated, there is ascending paralysis with initially hyper reflexia and clonus in the lower limbs followed by weakness and hyper reflexia in the upper limbs.

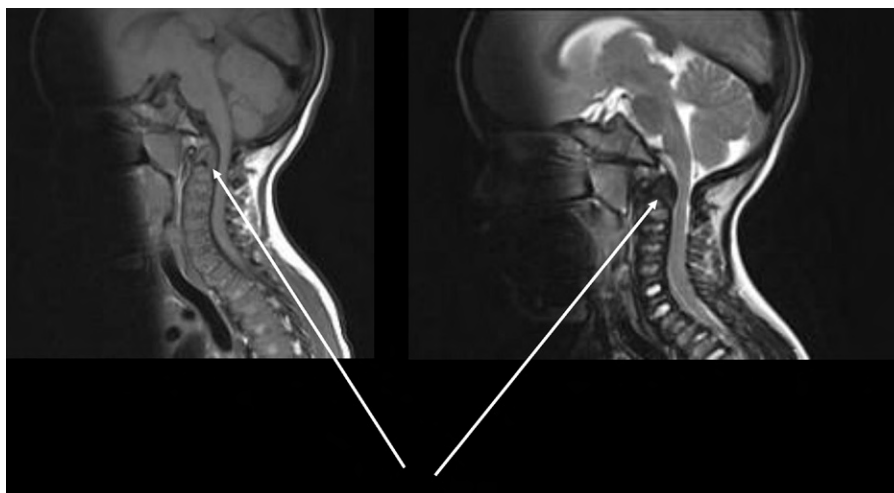
Other skeletal involvement includes hip dysplasia and genu valgum and both of these abnormalities can limit mobility and as a consequence many teenagers and young adults with MPS IVA prefer to use powered wheelchairs rather than relying on their own motor skills.

Delayed ossification of the carpal bones and ligamentous laxity at the wrists leads to very poor hand function. Grip is often very weak causing difficulties with dressing, personal hygiene and writing.

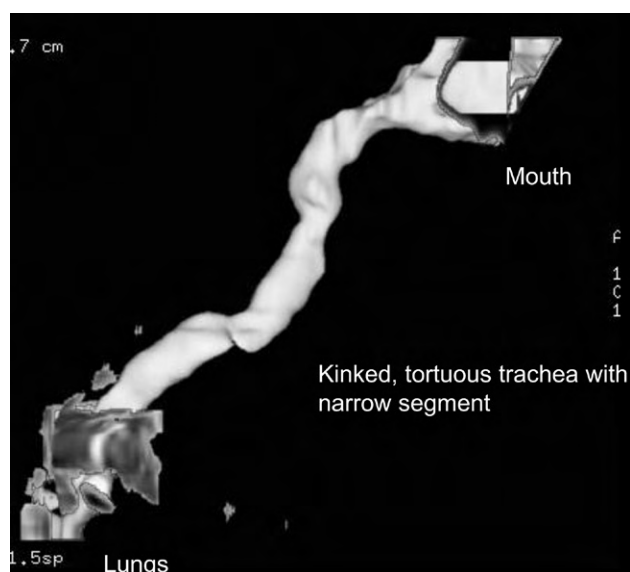
Aches and pains in various joints plus the onset of arthritic changes early in life compound the misery and difficulties caused by the skeletal dysplasia.

Outside of the skeleton, the major effects of the disease are on hearing, breathing, vision and cardiac function. Hearing impairment due to a combination of conductive and sensorineural deafness is common. Upper airway obstruction due to tonsillar and adenoidal hypertrophy is exacerbated by tracheomalacia and by the tendency of the trachea of affected patients to “kink” when the neck is flexed. Many children and young adults hold their necks in an extended posture to keep their airway open. Figure 102-46 illustrates the tracheal abnormalities in a young teenager with MPS IVA. The trachea is “kinked,” tortuous and narrower than normal.

As the disease progresses, a restrictive respiratory defect becomes more obvious and whilst formal respiratory function tests are difficult to interpret due to the short size of the patients it is clear that the thoracic organs are severely compressed by the small bony thorax and respiratory failure is a common cause of death in early adult life in severely affected MPS IVA patients.



**FIGURE 102-45** MPS IVA C spine MRI (T1 and T2 images) anterior soft tissue compression at tip of dysplastic odontoid process (arrow).



**FIGURE 102-46** MPS IVA CT reconstruction of trachea.

In the heart, early onset coronary artery disease has been reported in MPS IV but in practice this is not common (172). Valve abnormalities are much more prevalent primarily affecting the left side of the heart (173). The most common ophthalmological abnormality is fine corneal stromal clouding this is rarely severe enough to require any specific therapy. Both glaucoma and retinopathy have been reported in isolated cases but neither has been seen in our patient group. Dental abnormalities are universal in severely affected patients (174). Pointed cusps, thin enamel, spade shaped incisors and pitting are common and the patients are prone to extensive dental carries unless very careful dental follow-up is carried out.

Attenuated patients have a very different presentation. Common mutations causing a mild phenotype have been reported in both Finnish (175) and Northern Irish (176) patients. Typically affected patients are taller but are prone to premature osteoarthritis. Bilateral hip

and knee replacement surgery is commonly required to maintain pain-free mobility. Figure 102-47 illustrates a typical young teenager with attenuated MPS IVA (photographs courtesy of Dr Fiona Stewart, Consultant Clinical Geneticist, Belfast, Northern Ireland).

### 102.8.1 Treatment of MPS IVA

A clinical trial of ERT in MPS IV has commenced (December 2010, <http://clinicaltrials.gov/ct2/show/NCT00884949?term=mucopolysaccharidosis+type+IV&rank=3>). An improvement in growth and respiratory function in affected patients treated by ERT will be a prerequisite for a successful study. Preclinical studies in a murine model of MPS IVA have demonstrated clearance of KS from the blood and tissues of affected animals. Unfortunately, affected mice have very little skeletal disease and so these results are difficult to extrapolate to the human situation (177).

Surgical treatment remains a very important part of management. The timing of intervention of cervical decompression and fusion is of critical importance. The risks of anesthesia must be weighed up carefully against the risk of leaving a potentially unstable patient untreated. A prophylactic approach to surgery has long been advocated (178) and there have been few recent advances to alter this general advice. It has been our practice to perform posterior cervical decompression with occipitocervical fusion using a skull graft as the strut as illustrated in Figure 102-50. Immobility is maintained in the postoperative period by use of a halo vest fixation device for 34 months (Figures 102-22 and 102-48).

Complications of the halo vest include pin site infection and loosening, but these are usually easily managed. Long term, some patients develop further abnormal movement in the spine below the fused segment and the occasional patient will require repeat surgery. In our experience failure to fuse is rare. As with all operations on or near to the spinal cord there is a risk of damage to



the cord at surgery, either directly or by interfering with the blood supply to a segment of cord.

Lower limb and pelvic surgery is also often performed in affected patients in an attempt to maintain mobility and to prevent later osteoarthritic changes. There is no consensus as to what surgery should be performed and at what age. Surgery to correct genu valgum is often undertaken. Temporary hemiepiphysiodesis cannot be successful in severe MPS IVA as the patient's growth velocity is minimal from the age of 3 years onwards and in most MPS IVA patients satisfactory leg alignment can only be achieved by wedge osteotomy.

Other more invasive surgical treatments include both hip and knee replacements (179,180). The main indication for replacement surgery would be to effectively manage pain from the joint deformity and arthritic changes that are commonly present in the abnormal joints.

Like other MPS disorders ear, nose and throat surgery including grommets, tonsillectomy and adenoidectomy are commonly performed. In contrast to some other MPS disorders, heart valve surgery (181) and corneal transplant are rarely required (182).

Management of the respiratory disease is particularly challenging. The etiology is multifactorial with obstruction (in some cases positional, (183)), restriction due to thoracic deformity (184) and malacic changes in the trachea and bronchi (185). Patients commonly present with hypoxemia during sleep but eventually they become breathless and increasingly distressed during the day whilst awake. The lack of any significant respiratory reserve makes any superadded respiratory infection potentially life threatening. Gradually, hypercapnia supervenes and noninvasive ventilation is often suggested at this stage and whilst this can keep

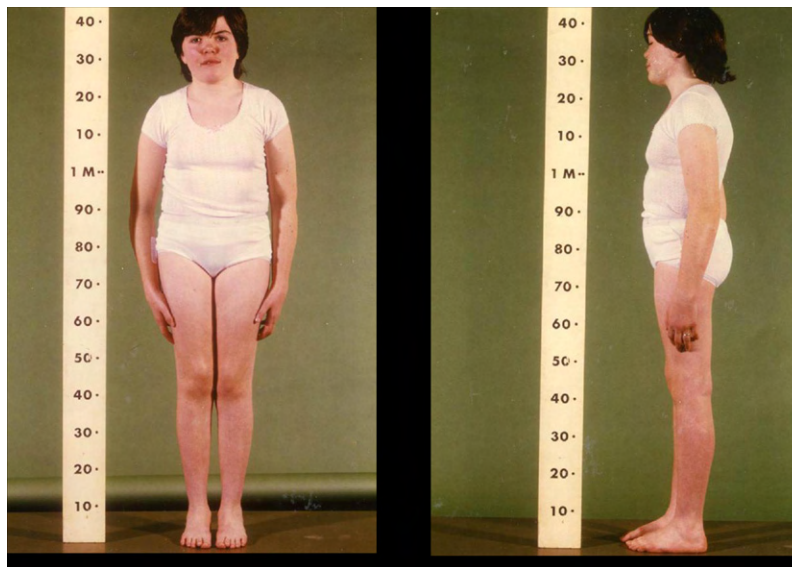


FIGURE 102-47 Attenuated MPS IVA age 12 years.

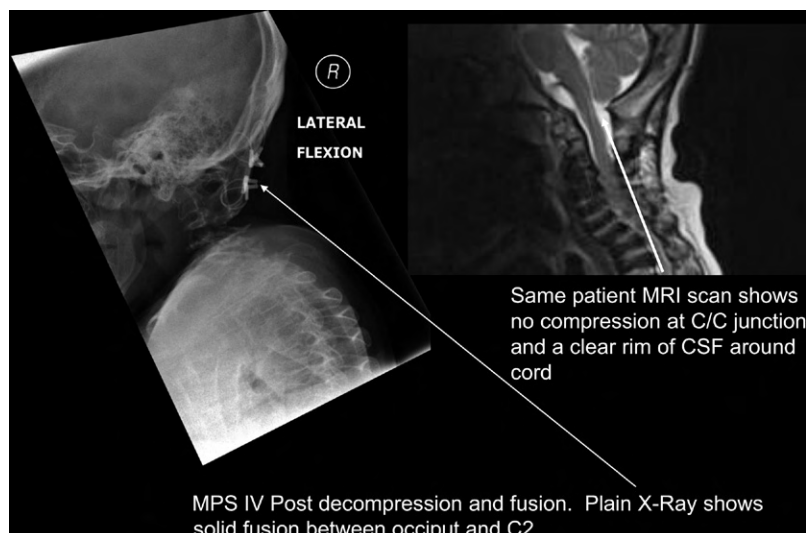


FIGURE 102-48 MPS IV post-decompression and fusion.



a malacic airway patent during sleep, it is less effective in treating the restrictive element of the respiratory disease. Most families, in our experience, have opted out of tracheotomy and invasive ventilation as they perceive the quality of life that this allows to be very poor and at this stage a palliative care program would be instituted.

### 102.9 MUCOPOLYSACCHARIDOSIS V (SCHEIE SYNDROME, MPS V)

Before being recognized as an  $\alpha$ -iduronidase defect, Scheie disease was classified as MPS V. The entry is retained for historical purposes only.

### 102.10 MUCOPOLYSACCHARIDOSIS VI (MAROTEAUX-LAMY SYNDROME, MPS VI)

MPS VI is caused by a deficiency of lysosomal enzyme N-acetylgalactosamine-4-sulfatase secondary to defects in the aryl sulfatase B (*ARSB*) gene which is located on chromosome 5 (5q13–5q14 (186)). The enzyme deficiency results in the accumulation of partially degraded DS which appears to be responsible for the disease manifestations. Another GAG, C4S is also broken down by the same enzyme but accumulation of this substrate and its derivative products does not appear to cause intracellular problems.

MPS VI is a rare MPS disorder with a reported prevalence of approximately 1:250,000 live births (99). Like other MPS disorders, it is heterogeneous. Unlike other MPS disorders that are usually divided into severe and attenuated phenotypes, the spectrum presented by MPS VI is usually divided into “rapidly progressing” disease or “slowly progressing” disease, correlating with the severe and mild ends of the clinical spectrum, respectively.

Patients with forms of the disease between these two extremes are also seen.

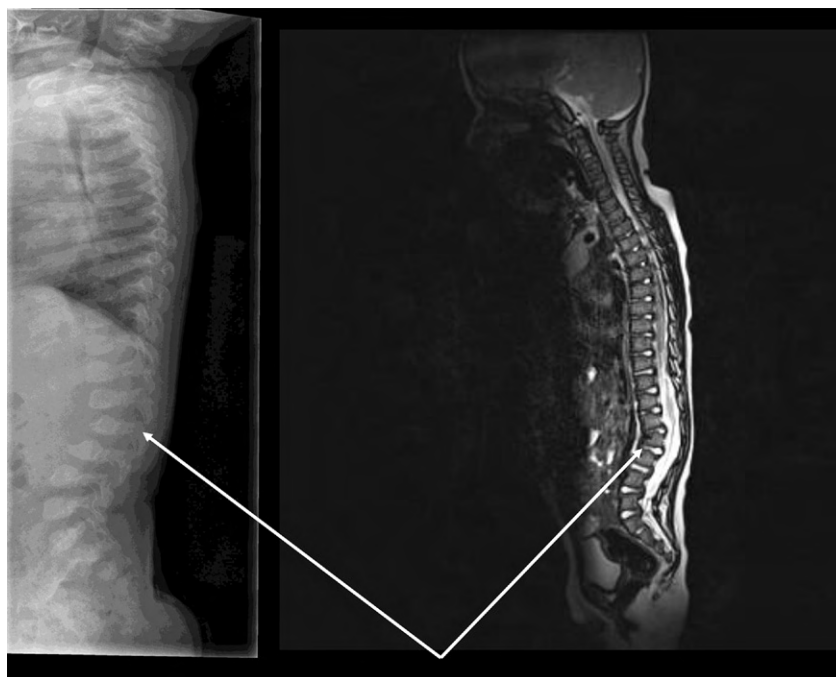
A cross-sectional natural history study of 121 MPS VI patients has provided insights into the relationship between disease severity, rapidity of progression and urine GAG excretion (187). In this study, high urinary GAG values (>200 mg/mg creatinine) were associated with rapidly progressive disease, impaired endurance, poor respiratory function, greater joint restriction and short stature. An attempt to correlate disease severity with *ARSB* mutation identified a clear correlation between genotype and urinary GAG and confirmed that urinary GAG values could be used to predict likely disease severity, confirming the findings of the natural history study (188).

In patients with rapidly progressing MPS VI, the onset of symptoms is usually before the age of 3 years and a number of patients are diagnosed in the first year (189,190). Affected patients have a phenotype rather like MPS IH with the exception that cognitive impairment is not present. The DM is severe and growth consequently severely restricted. In rapidly progressing patients, it is unusual for the patients to achieve a final adult height of greater than 120 cm (187). Most other organ systems are affected. The facial features are characteristic and again similar to MPS IH. The mid face is underdeveloped, the gums are thick and the tongue is large and protruding. Dental eruption is often considerably delayed (191). Hernias are common and the parents may notice a spinal deformity (Figure 102-49).

Upper airway obstruction and a persistent nasal discharge are universal. Many patients have obstructive sleep apnea and most require ear, nose and throat surgery (192). A combination of conductive and sensorineural hearing loss is usual. Anesthesia is hazardous and must only be performed by anesthetists experienced with the disorder.



**FIGURE 102-49** MPS VI patient aged 10 months. Parents were concerned about pectus deformity of chest. X-rays suggested an MPS disorder (Figure 102-53).



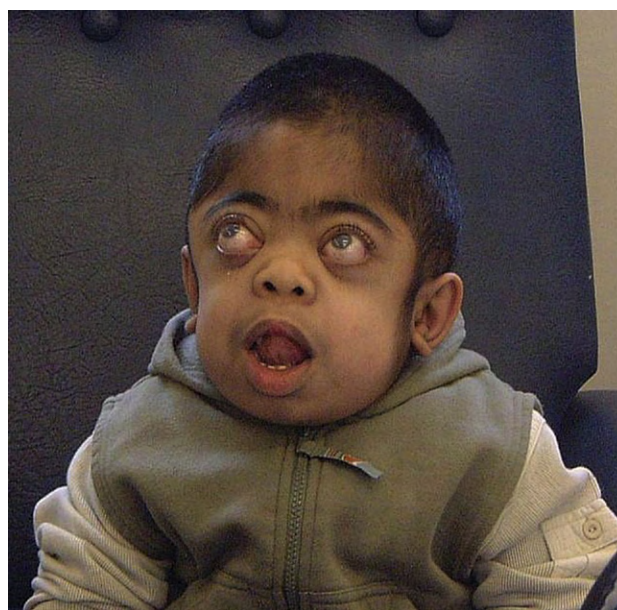
**FIGURE 102-50** Same patient as Figure 102-42 hypoplastic, beaked lumbar vertebrae (arrow).

The eye can be severely affected in MPS VI and visual impairment is unfortunately not uncommon. The orbit is shallow and as a result proptosis is common. Corneal clouding can be severe and raised intraocular pressure can occur. Retinopathy is rare but the optic nerve is vulnerable to both raised intracranial pressure as well as thickening of the dura and sclera. Sudden loss of vision can occur and the patients require careful ophthalmological follow-up (193) (Figure 102-51).

Lower respiratory tract involvement becomes progressively worse over time. Restrictive respiratory disease and airway obstruction are a significant cause of morbidity and mortality in rapidly progressing patients from the age of approximately 10 years onwards. Pulmonary hypertension secondary to chronic hypoxemia can occur and a number of patients will require noninvasive ventilation and some tracheostomy to maintain oxygen levels (192).

Cardiac disease ranges from cardiomyopathy in early infancy to chronic and progressive valve lesions. Valve replacement may be necessary but is technically challenging in rapidly progressing patients (194–196).

Orthopedic issues include spinal deformity with kyphosis and scoliosis, large joint contractures, claw-hand deformity, carpal tunnel syndrome and finally spinal cord compression secondary to dural hyperplasia. Studies in animal models have shown that accumulation of the GAG DS can have a profound effect on articular chondrocytes with a high rate of apoptosis and the release of inflammatory cytokines into the joint space (197). A combination of structural abnormality or deformity plus inflammation appears to be central to the development of the severe skeletal dysplasia seen

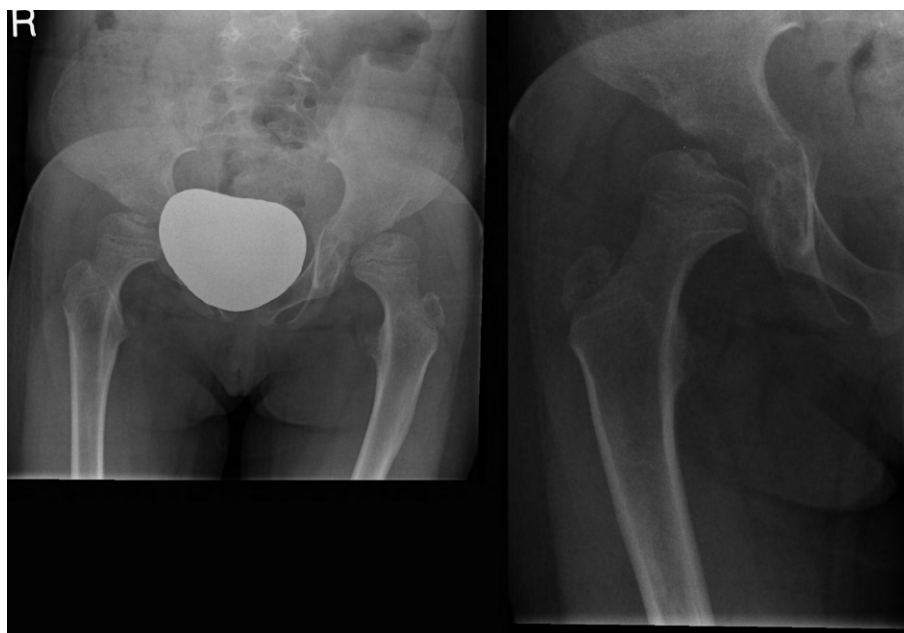


**FIGURE 102-51** Rapidly progressing MPS VI patient age 10 years. Proptosis, corneal clouding, large tongue.

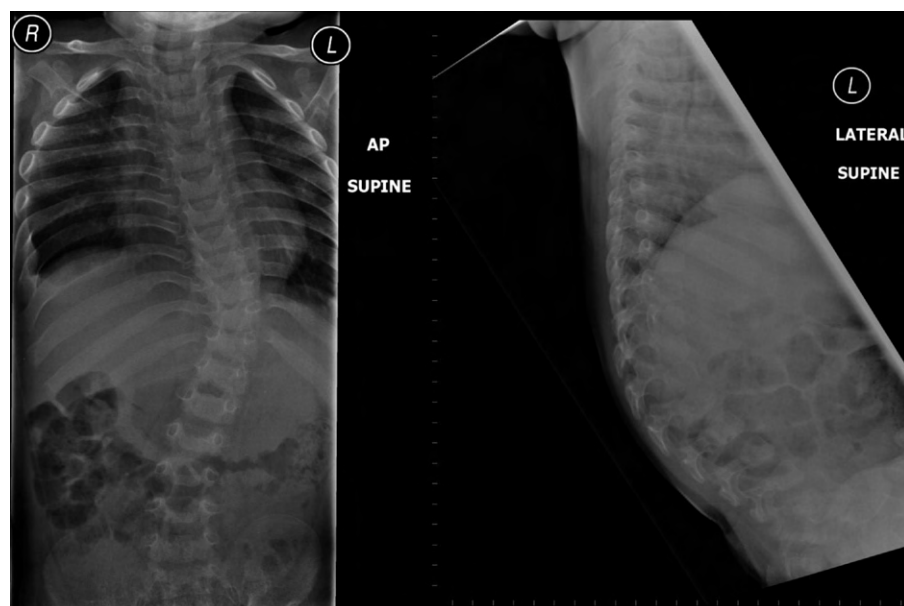
in patients with rapidly progressing disease. This can be seen clearly in the pelvis and hip-joint X-rays where dysplastic acetabulae are associated with fragmented flattened and fragmented femoral heads as illustrated in Figures 102-52 and 102-54.

As well as kyphosis, scoliosis may occur and this can require major spinal surgery to correct (Figure 102-53).

Virtually, all patients with rapidly progressing disease will go on to develop spinal cord compression if they survive long enough. This is due mainly to hyperplasia of the dura and ligamentum flavum and most commonly



**FIGURE 102-52** MPS VI pelvis and hips: underdeveloped acetabula, flattened femoral heads.



**FIGURE 102-53** MPS VI patient aged 12 months. X-rays at presentation illustrate the kyphoscoliosis that can occur in this disorder.

occurs in the cervical region (198) although it can occur at multiple sites along the spinal canal (199). In some patients, the odontoid will be dysplastic and decompression will need to be followed by occipitocervical fusion. Figure 102-56 illustrates the typical cervical MRI scan findings in MPS VI.

A 15-year-old patient with rapidly progressing MPS VI is illustrated in Video 102-12. The patient is blind from previous raised intracranial pressure, has a tracheostomy for upper airway obstruction and illustrates the severe joint stiffness seen in the disorder.

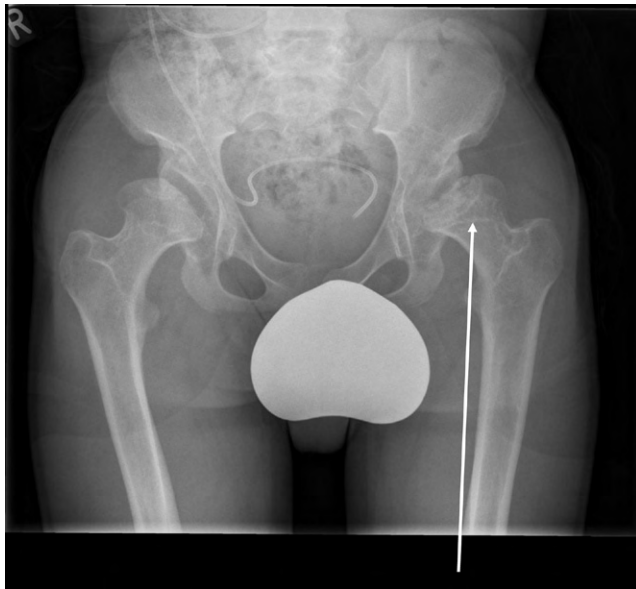
A number of mutations are known to be associated with slowly progressive MPS VI (188). Some of

the reported patients can be exceptionally mild and a number would presumably never be correctly diagnosed (200). In general, these patients have slowly progressive bone and joint disease, most eventually develop cardiac valve lesions and some have significant visual problems later in life. Urinary GAG excretion (below 100  $\mu\text{g}/\text{mg}$  creatinine) is much lower than what is seen in rapidly progressing patients and patients can survive into their 50s and occasionally beyond (187). The patient illustrated in Figure 102-55 presented at the age of 14 years to the growth clinic as she had fallen across the height centile chart. Minor radiological abnormalities on skeletal survey prompted investigation for an MPS

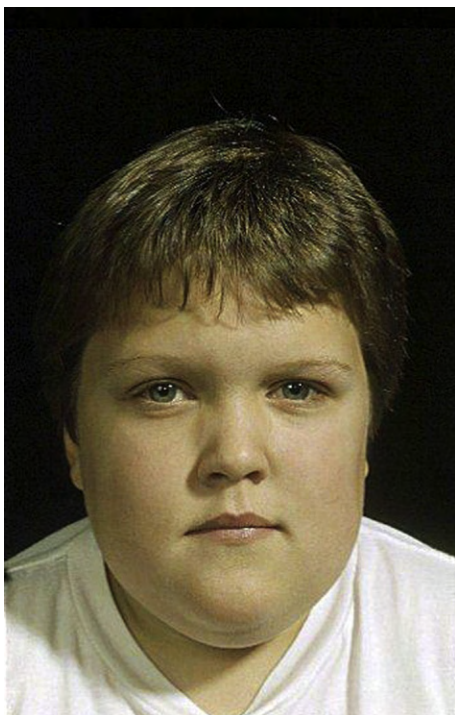


disorder and MPS was diagnosed. [Video 102-13](#) shows the same patient carrying out a range of tasks. Apart from her stature, there is little to suggest an underlying MPS disorder.

Patients with an intermediate phenotype usually develop the signs and symptoms seen in rapidly progressing patients but at a later age. In addition, as the skeletal dysplasia is less severe there is never the same degree of restrictive respiratory disease, allowing for much longer survival.



**FIGURE 102-54** MPS VI fragmentation of left acetabular head (arrow).



**FIGURE 102-55** Slowly progressive MPS VI patient aged 14 years.

### 102.10.1 Treatment of MPS VI

Historically, the treatment of MPS VI was palliative with surgical treatment for upper airway obstruction, spinal cord compression, corneal clouding and nerve entrapments. Like other MPS disorders, this is best provided in major centers with full access to the multidisciplinary care necessary for successful management.

HSCT was introduced as a treatment for rapidly progressing MPS VI in 1984 ([201](#)) and led to widespread clinical improvements in the treated patient who went on to survive at least 20 years following the transplant with a good quality of life ([202](#)). A review of HSCT in MPS VI reported mainly on safety of the procedure and not clinical outcome ([203](#)). In 45 MPS VI patients identified in the study, the probability of survival was 78% (65–89%) at 100 days and 66% (52–79%) at 1 and 3 years. The main causes of death were infection (44%) and organ failure (31%). Many of the transplants were performed over 10 years ago (latest 2007) and it is uncertain how relevant the safety data would be when compared with HSCT performed from 2010 onwards.

In terms of clinical outcome, long-term follow-up of UK patients documented improvements or stabilization of cardiac function but progression of skeletal disease ([204](#)).



**FIGURE 102-56** Intermediate MPS VI in a male child aged 6 years.



Since the introduction of ERT, HSCT is no longer recommended as the therapy of choice by the majority of treating centers.

The development of ERT for MPS VI has been greatly helped by studies on a cat model of the disease (205). These have demonstrated the importance of early treatment (206) and also the possibility of using enzyme therapy to treat both joint disease (207) and dural hyperplasia (208).

Human recombinant *N*-acetylgalactosamine 4-sulfatase (rh4S, Galsulfase, [Naglazyme™, BioMarin Pharmaceutical Inc., Novato, CA]) is licensed for the treatment of all forms of MPS VI. In an initial Phase I/II clinical trial, there were no drug-related serious adverse events or infusion associated reactions. The treatment was well tolerated and there was a rapid fall in urine GAG as well as an improvement in a 6-min walk test and shoulder range of movement (209). Additional improvements in endurance, mobility and joint function after 48 weeks of treatment were observed in the same group of patients (210).

A subsequent Phase III, randomized, double-blind and placebo-controlled study in 39 MPS VI patients confirmed efficacy with significant improvements in endurance (211). Subsequent long-term follow-up of this cohort of patients over a 5-year period confirmed sustained improvements over time (212). Subsequent data analysis on the same group of patients has reported improvements in long-term pulmonary function (213) and growth (214).

Like other MPS disorders, the best results from ERT in MPS VI are likely to be seen in patients treated from a very young age (215). Intrathecal ERT has also been used, unsuccessfully, in a patient with pachymeningitis cervicalis (216).

Naglazyme™ is given by weekly intravenous infusion (1 mg/kg) and can safely be given in the patient's home (126).

### 102.11 MUCOPOLYSACCHARIDOSIS VII (SLY SYNDROME, MPS VII)

Mutations in the *GUSB* gene on the long arm of chromosome 7 (7q21.11) lead to a deficiency of the enzyme  $\beta$ -glucuronidase and an accumulation of the GAGs DS, HS and chondroitin-4,6-sulfate. Accumulation of these partially degraded GAGs results in the very rare MPS disorder, MPS VII or Sly syndrome. The disorder is very rare (<1:1,000,000 live births) and less than 50 case reports exist in the literature. The prevalence may be underestimated as the commonest presentation is an antenatal form that presents as nonimmune hydrops fetalis and the diagnosis in these patients may be missed. Certainly, the diagnosis should be considered in families where there is a history of recurrent fetal hydrops (217).

In patients who survive the prenatal period, the disorder is very heterogeneous ranging from a severe neonatal presentation with coarse faces, macroglossia, hepatosplenomegaly and cholestatic jaundice (218) to

an attenuated disorder with survival well into adult life (219). Numerous mutations have been identified in the *GUSB* gene and are presumably responsible for the heterogeneity seen in the affected patients (220).

Cognitive impairment is a feature of all variants of MPS VII and radiological investigations reveal typical changes of DM. Craniovertebral instability with spinal cord compression has also been described (221).

The diagnosis is suggested by demonstrating abnormal GAG excretion in the urine followed by  $\beta$ -glucuronidase assay in white blood cells.

Treatment is generally supportive although HSCT would be a potential treatment for early diagnosed patients. BMT in a 12-year-old patient (diagnosed at 1 month of age with a severe neonatal presentation) led to motor improvements and reduced respiratory infections but no improvement in cognitive abilities (222).

Animal models of MPS VII have been used extensively to assess the possibility of gene transfer as a therapy for MPS disorders (223).

### 102.12 MUCOPOLYSACCHARIDOSIS VIII

The designation was given to a single patient with a presumed deficiency of *N*-acetylglucosamine-6-sulfate sulfatase. The disorder has not been confirmed, and the entry is left open (OMIM # 253230, (224)).

### 102.13 MUCOPOLYSACCHARIDOSIS IX (NATOWICZ SYNDROME, MPS IX)

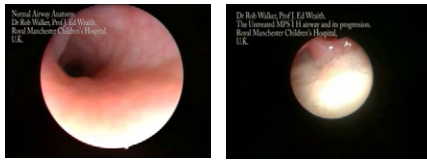
The disorder has been reported in two families and is caused by the deficiency of hyaluronidase 1, one of two hyaluronidases degrading HA (hyaluronan). In the first patient, clinically the defect led to the development of multiple periarticular masses, which were painful during exertion or febrile illness. Other manifestations in the patient included a flattened nasal bridge, submucous cleft palate, and short stature appearing in later childhood. Radiographs showed intra-articular soft-tissue masses, and acetabular erosions in the pelvis, but no signs of DM (225). In the second family the presentation was compatible with a diagnosis of juvenile idiopathic arthritis (229).

Urinary GAG and oligosaccharide excretion is normal in MPS IX, serum HA is increased and there is a deficiency of hyaluronidase activity in plasma. Histology reveals intralysosomal storage of undegraded hyaluronan in synovial histiocytes and skin fibroblasts.

Subsequent studies in the patients demonstrated mutations in *HYAL 1* one of three genes (*HYAL 1*, *HYAL 2*, *HYAL 3*) that play a role in HA metabolism (226). A mouse model of MPS IX (*HYAL 1*) exhibits osteoarthritis (227) and *HYAL 2*-deficient mice have skeletal dysplasia and thrombocytopenia (228). The human equivalent of *HYAL 2* deficiency has not yet been reported and presumably *HYAL 3* deficiency is likely to have a similar phenotype.

## APPENDIX A SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/B978-0-12-383834-6.00107-5>.



**VIDEO 102-1:** (a) demonstrates a normal airway and (b) the typical progressive changes seen with age airway in an MPS IH patient as seen down a rigid and fiberoptic bronchoscope.



**VIDEOS 102-2 AND 102-3:** These videos show a 4-year 4-month-old MPS IH patient interacting with her physical therapist. The typical facial features, abdominal distension and joint contractures are all clearly demonstrated.



**VIDEO 102-4:** This video illustrates a 15-year-old MPS IH patient treated by BMT at the age of 18 months. He is short in stature, has residual joint stiffness and has problems with dexterity secondary to his hand abnormalities. Despite these difficulties, he is able to cope well with most tasks and attends a normal school.



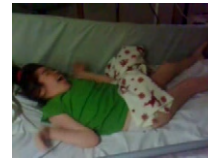
**VIDEOS 102-5 AND 170-6:** These are two short videos of two patients with MPS IH/S demonstrating the difficulties with dexterity. In Video 102-6 note the exaggerated lordosis.



**VIDEOS 102-7 AND 102-8:** These videos illustrate two 17-year-old Scheie patients performing the same tasks as the MPS IH/S in Videos 102-5 and 102-6.



**VIDEO 102-9:** This video illustrates the characteristic breathing during sleep in a young patient with MPS II.



**VIDEO 102-10** Severe nocturnal agitation in an MPS III patient. The cause of such a presentation is often unclear.



**VIDEO 102-11:** This video illustrates the typical gait of a young teenager with MPS IVA.



**VIDEO 102-12** A patient aged 12 years with rapidly progressing MPS VI.



**VIDEO 102-13** A 16-year-old girl with slowly progressing MPS IV.

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## RELEVANT WEB SITES

<http://www.mppsociety.org/>  
<http://emedicine.medscape.com/article/1115193-overview>  
<http://www.ninds.nih.gov/disorders/mucopolysaccharidoses/mucopolysaccharidoses.htm>  
[http://www.alduzyme.com/global/az\\_us\\_home.asp](http://www.alduzyme.com/global/az_us_home.asp)  
<http://www.elapraxe.com/>  
<http://www.naglazyme.com/>

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He was born in the northeast of England, graduated from Sheffield University in 1977 and received his postgraduate qualification in pediatrics (MRCP) in 1980. In 1993, he became a Fellow of the Royal College of Physicians (FRCP) and in 1997 a Fellow of the Royal College of Pediatrics and Child Health.

He has served on the Editorial Board of the Archives of Disease in Children and is an editor of The Journal of Inherited Metabolic Disease. Additionally, he is a medical advisor to the UK Mucopolysaccharide Society and the Niemann–Pick Disease group.

Dr Wraith is currently researching several projects, including enzyme replacement therapy for the treatment of a number of lysosomal storage diseases and the use of substrate reduction therapy in patients with neurodegenerative disease.

He has written over 200 articles, abstracts, and book chapters on topics related to inborn errors of metabolism in neonates, children and adults.

# Oligosaccharidoses: Disorders Allied to the Oligosaccharidoses

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## 103.1 OLIGOSACCHARIDOSES

Oligosaccharidoses are a group of inborn errors that adversely affect the catabolism of the glycan parts in glycoproteins and glycolipids. The several inborn errors due to deficient synthesis of these glycans, called congenital disorders of glycosylation (CDGs), are the subject of a separate chapter. The multisystemic clinical features, the progressive natural course, and the cellular and chemopathology findings prompted the concept of lysosomal storage disorder (LSD) as an initial characterization of the common pathogenesis. Not all oligosaccharidoses are true LSDs although excessive oligosacchariduria is the common hallmark. The ever more impressive physiologic importance of N- as well as O-glycosylation in intermediary metabolism explains the diseases' multisystemic nature that includes the central nervous system (CNS).

The term oligosaccharidosis (OS) has been aptly substituted for the initial name mucopolidosis (ML) that stressed the clinically challenging relationship between the oligosaccharidoses on the one hand and the mucopolysaccharidoses (glycosaminoglycan storage disorders) and lipidoses on the other hand.

Because of their adverse effect on glycoprotein catabolism due to either primary or secondary deficiency of lysosomal glycosidases or proteases, the term glycoproteinoses would be an adequate alternate to this chapter's title.

Table 103-1 lists the disorders discussed in this chapter that more or less fit the definition of OS. In all, the specific enzyme defect is known. In all, the mutant gene has been sufficiently characterized in order to make specific mutation screening the optimal method for formal confirmation of the clinical diagnosis.

Section 103.2 treats a heterogeneous group of metabolic disorders, once rightly or wrongly considered allied with oligosaccharidoses. The results of the more recent investigations show more clearly that the pathogenesis is

at least indirectly a lysosomal one. See the listing in Table 103-2 pertaining to Section 103.2.

Each one of the oligosaccharidoses is inherited as an autosomal recessive trait with the recurrence risk of one in four for full sibs of probands. As causal or truly effective treatment is still lacking, management of the disorders remains in the realm of preventive medicine. Paradoxically, this prevention finds its retrospective foundation in early diagnosis in the proband and requires genetic counseling and prenatal diagnosis as measures of practical strategy. Fetal monitoring by either relevant enzyme assay or mutation screening in chorionic villi or in cultured amniotic fluid cells is available and is reliable. Elective abortion remains one of the options to parents within the flow sheet of preventive measures.

### 103.1.1 Sialidoses

The term sialidosis (MIM # 256550) should be reserved for the clinical conditions due to the primary deficiency of lysosomal glycoprotein sialidase (MIM # 608272). Four nosological entities had been discerned clinically under the common name sialidosis (Table 103-1) before the elucidation of the enzyme defect and the molecular characterization of *NEU1*, the gene encoding acid sialidase, also known as neuraminidase 1 (NEU 1) (MIM # 608272). The entities delineated still serve as points of reference in what is gradually emerging as a continuous phenotypic spectrum by the reports of more patients and the characterization of the *NEU1* mutations causing their illness.

#### 103.1.1.1 Clinically Delineated Phenotypes.

**103.1.1.1.1 Childhood Dysmorphic Sialidosis or Sialidosis Type II.** Originally called lipomucopolysaccharidosis on its clinical delineation, childhood dysmorphic sialidosis (CDS) was subsequently named ML I. In many reviews, this sialidosis with gradually coarsening facial features from early childhood has been called Sialidosis type II, whereas in these sources the

TABLE 103-1 Oligosaccharidoses

Phenotype		Gene			Enzyme		
Group Entity (Synonym)	MIM#	Gene	Genome Location	MIM#	Defect	Symbol	EC#
Sialidoses (S) Childhood dysmorphic S; (ML I) Juvenile normosomatic S; (CRSM) <sup>a</sup> Perinatal onset S; (Hydropic; Infantile)	256550	NEU1	6p21.3	608272	Acid sialidase (neuraminidase)	SIAL1, NEU1	3.2.1.18
β-D-Galactosidase deficiency disorders <sup>b</sup>	230500	GLB1	3p21.33	611458	β-D-galactosidase	GLB1	3.2.1.23
Galactosialidoses (GS) Congenital GS Late infantile (D) GS Juvenile (J) GS	256540	CTSA	20q13.1	613111	Protective protein cathepsin A (carboxypeptidase)	CTSA (PPGB)	3.4.16.5
GlcNAc-PT deficiency disorders					GlcNAc-1-phosphotransferase		
ML II; (I-cell disease, ICD)	252500	GNPTAB	12q23.3	607840	α2β2 subunits	GNPTAB	2.7.8.17
ML III αβ; (pseudo-Hurler polydystrophy)	252600	GNPTAB	12q23.3	607840	α2β2 subunits	GNPTAB	2.7.8.17
ML IIIγ; (pseudo-Hurler polydystrophy)	252605	GNPTG	16p13.3	607838	γ2 subunits	GNPTAG	2.7.8.17
Mannosidoses							
α-Mannosidosis	248500	MAN2B1	19cen-q12	609458	α-D-mannosidase	LAMAN (MAMB)	3.2.1.24
β-Mannosidosis	248510	MANBA	4q22-q25	609489	β-D-mannosidase	MANBA	3.2.1.25
Fucosidosis	230000	FUCA1	1p34	612280	α-L-fucosidase	FUCA	3.2.1.52
Aspartylglucosaminuria	208400	AGA	4q32-q33	613228	N-aspartyl-β-glucosaminidase	NAGA	3.5.1.26
Kanzaki disease Schindler disease	608241 608242	GALB	22q11	104170	α-N-acetylgalactosaminidase	GALB	3.2.1.49

<sup>a</sup>CRSM: Cherry-red spot myoclonus syndrome.<sup>b</sup>Group of disorders discussed in the chapter on lipidoses.

disorder with adolescent onset is known as Sialidosis type I (1).

The onset in early childhood and the slowly progressive natural course set CDS apart from either the congenital and early infantile subgroup of patients with more aggressive disease or the juvenile normosomatic sialidosis that are discussed below. Coarse facial features, slow psychomotor development, and thoracolumbar kyphosis are noticed in early childhood. Inguinal herniae may be present before other findings prompt metabolic studies. Also, sensorineural deafness is a feature from early childhood. Facial puffiness, low nasal bridge, bulbous nose with anteverted nostrils, mildly hypertrophic gums, and broad maxilla are reminiscent of what is seen in the patient with Hurler disease (Mucopolysaccharidosis IH: MPS IH; MIM # 607014). The teeth are widely spaced and the tongue is enlarged. Intellectual development and statural growth are slower than normal. Height is below the third centile from 3 to 5 years of age. The thoracic cage is barrel shaped with pectus excavatum and some asymmetry due to mild or moderate kyphosis. Neuromuscular signs becoming more apparent after the age of 10 years include progressive ataxia, nystagmus,

strabismus, muscle wasting, and worsening weakness. The motion range in the small joints of the hands stays intact and the limitation of large joint movements is only mild. By then, ophthalmologic examination for complaints of decreasing vision reveals a macular cherry-red spot and inconsistent cataract and some corneal opacity. CDS patients become chair bound by or even before adolescence. The appearance of a coarse tremor and bouts of myoclonic jerks compound the neurologic syndrome. Peripheral neuropathy is documented by finding decreased nerve conduction velocity. Intellectual disability is a consistent feature, but true intellectual deterioration has not been reported. Fatal outcome in adolescence or early adulthood relates to adverse complications of the neurologic syndrome, pneumonia, and/or cardiomyopathy often insufficiently studied in the older CDS patient. α-Mannosidosis is an alternate differential diagnosis in the older CDS patient, especially when retinal changes and peripheral nervous system disease are absent.

Radiographically, the skeletal manifestations in CDS remain subtle and progress to only mild dysostosis multiplex (1).



**103.1.1.1.2 Juvenile (Adult) Normosomatic Sialidosis; Cherry-Red Spot Myoclonus Syndrome; Sialidosis Type I.** Almost simultaneously with the discovery of the primary metabolic defect in CDS, the specific activity of the same glycoprotein acid sialidase was found to be deficient also in the cherry-red spot myoclonus (CRSM) syndrome that accordingly was named sialidosis type I.

The initial complaints in adolescent or young adults concern gait difficulties and/or declining visual acuity. The designation CRSM syndrome adequately refers to the main clinical features in the fully developed disorder: slowly progressive reduction of visual acuity, first evident in some instances before 10 years of age, and a crippling, often generalized action myoclonus sometimes appearing from as early as the onset of the second decade of life. Demonstration of the macular cherry-red spot is rarely concomitant with the initial complaints about the vision deficit. A long time lag may exist between the onset of reduced vision and that of myoclonus. Seizures not associated with the loss of consciousness are shown to be repetitive bursts of generalized severe myoclonus, but not always promptly recognized as such. Nystagmus, dysarthria, and mild ataxia may become additional components in the syndrome. Muscle weakness, atrophy, and also neuropathy have been documented in juvenile (adult) normosomatic sialidosis (JNS), thereby providing also a physiopathologic link between JNS (CRSM syndrome) and CDS. It needs pointing out that intellectual disability and skeletal dysplasia changes are absent in JNS. For the issue of clinical differentiation from the late-onset type of galactosialidosis, the reader is referred to [Section 103.1.7](#). For references to the early reports on sialidosis, the reviews by Leroy (1) and by Thomas (2) may be consulted.

#### **103.1.1.1.3 Early-Onset Sialidoses.**

**103.1.1.1.3.1 Congenital Nonimmune Hydrops; Perinatal Sialidosis.** Acid sialidase deficiency has been shown to result also in hydrops fetalis in the prematurely born or the at-term neonate. The infant is small for date, but clearly hydropic with extensive edema, massive ascites, and hepatosplenomegaly. There may be pleural effusion and signs of life-threatening cardiorespiratory failure. Supportive measures in the neonatal intensive care unit may be only transiently effective. Fatal outcome often occurs within the first days or weeks of life. Usually, there is evidence that the hydrops had an in utero onset (3,4).

Neonatal nonimmune hydrops is far from pathognomonic for early-onset type sialidosis. It may result from the deficiency of almost any one of the lysosomal glycosidases (5). Therefore, samples for metabolic and enzymatic diagnostic work-up should be taken. Postmortem examination is strongly advisable. The generalized cytoplasmic vacuolization and more detailed EM findings in any parenchymatous organs and connective tissue cells including cultured fibroblasts support the diagnosis of any lysosomal enzyme deficiency. Study of sialyloligosaccharides (SOSs) or other macro compounds in urine or other body fluids using advanced analytical methods should orientate or

complement the assay of lysosomal enzymes in cultured fibroblasts.

**103.1.1.1.3.2 Infantile Sialidosis and Nephrosialidosis.** Sialidosis has also been diagnosed in young infants with only minor or transient edema or without any sign of postnatal hydrops. The patients are physically normal at birth except for mildly coarse facial features that may remind the examiner of features in the early-onset mucopolysaccharidoses. Visceromegaly is present and dysostosis multiplex is gradually evident in skeletal radiographs. Growth rate remains subnormal. There is no macrocephaly and joint mobility is only minimally affected. Only in some of these patients, a rapidly evolving glomerular nephropathy has been documented. Such observation in 1978 was the basis for Maroteaux terming the disorder in these infants nephrosialidosis (MIM # 256150). The rather fulminant kidney disease is soon fatal. Without the adverse renal component, the further clinical course of infantile sialidosis (IS) is indistinguishable from that of CDS.

**103.1.1.2 Cyto- and Chemopathology.** Peripheral lymphocytes contain large vacuoles and the histiocytes in bone marrow smears have a foamy cytoplasm. These findings are less consistent in JNS. Electron micrographs of these cells show enlarged, membrane-bound vesicular, lysosome-like organelles enclosing the reticulogranular material that fills the major portions of the cytoplasm. Similar histopathologic changes are found in Küpffer cells and hepatocytes as well as in neurons and mesenchymal cells.

Urinary excretion of several SOSs and sialylglycopeptides is excessive. The sialyl moieties occupy the terminal nonreducing position. Quantitatively, the excessive excretion of SOSs is more extreme in the infantile types of sialidosis and less pronounced in JNS. As the enzyme-based or mutation screening-based diagnosis may be delayed until adulthood in the latter type, oligosacchariduria may no longer be detectable. When studied by routine analytical methods, urinary excretion of SOSs is qualitatively similar to or indistinguishable from that observed in patients with galactosialidosis or with GlcNAc-1-phosphotransferase deficiency disorders. By more advanced and not routinely available analytic and spectroscopic techniques, qualitative differences between the SOSs excessively excreted in the urine can be demonstrated (6,7).

Free sialic acid is near or completely normal in the urine of the sialidosis patients, where it is bound to partly degraded glycoproteins and glycolipids. This negative result pleads against the diagnosis of a sialic acid storage disorder (SASD) or sialuria (see [Section 103.2](#)). Storage products in CDS brain include lipid-(mainly ganglioside-) bound and protein-bound sialic acid. Accumulation of free and bound sialic acid in sialidosis fibroblast strains matches the one in I-Cells and in galactosialidosis fibroblasts (see subsequent sections).

Since the advent of mutation screening adequately oriented by the clinical diagnosis, chemopathology data

have become ancillary at best in formally establishing the diagnosis.

**103.1.1.3 Lysosomal Acid Sialidase: Primary Enzyme Defect and the Role in Diagnosis.** Somatic cell hybrids between CDS and JNS or early-onset sialidosis fibroblast strains constructed in the 1980s failed to metabolically complement one another providing in vitro evidence that all types of sialidosis are due to functionally allelic mutations. The biochemistry-based diagnosis of sialidosis involves not only the demonstration of a significantly deficient glycoprotein sialidase in fresh leukocytes, cultured fibroblasts, amniocytes, or postmortem tissues. It requires, in addition, establishing in these cells or tissues that  $\beta$ -D-galactosidase is normally active (see further sections in this chapter). Sialidosis has been diagnosed prenatally by enzyme assay (4). In parents of the patients, the expected intermediate sialidase activity has been found in fresh leukocytes or cultured fibroblasts. Acid sialidase activity is destroyed by freezing.

Sialidase 1 (EC 3.2.1.18) or neuraminidase 1 (NEU1; molecular mass: 76kDa; 425 amino acids) belongs to the small family of NEUs widely distributed in nature. Besides its involvement in the cellular immune response, the enzyme catalyzes the intralysosomal hydrolysis of ketosidically linked sialic acid residues in glycoconjugates (Figure 103-1). NEU1 is the most widely and intensely expressed mammalian sialidase in all cell types. Hence, its absence generates the multisystemic disease sialidosis in man and in NEU1<sup>-/-</sup> mouse models (see next paragraphs).

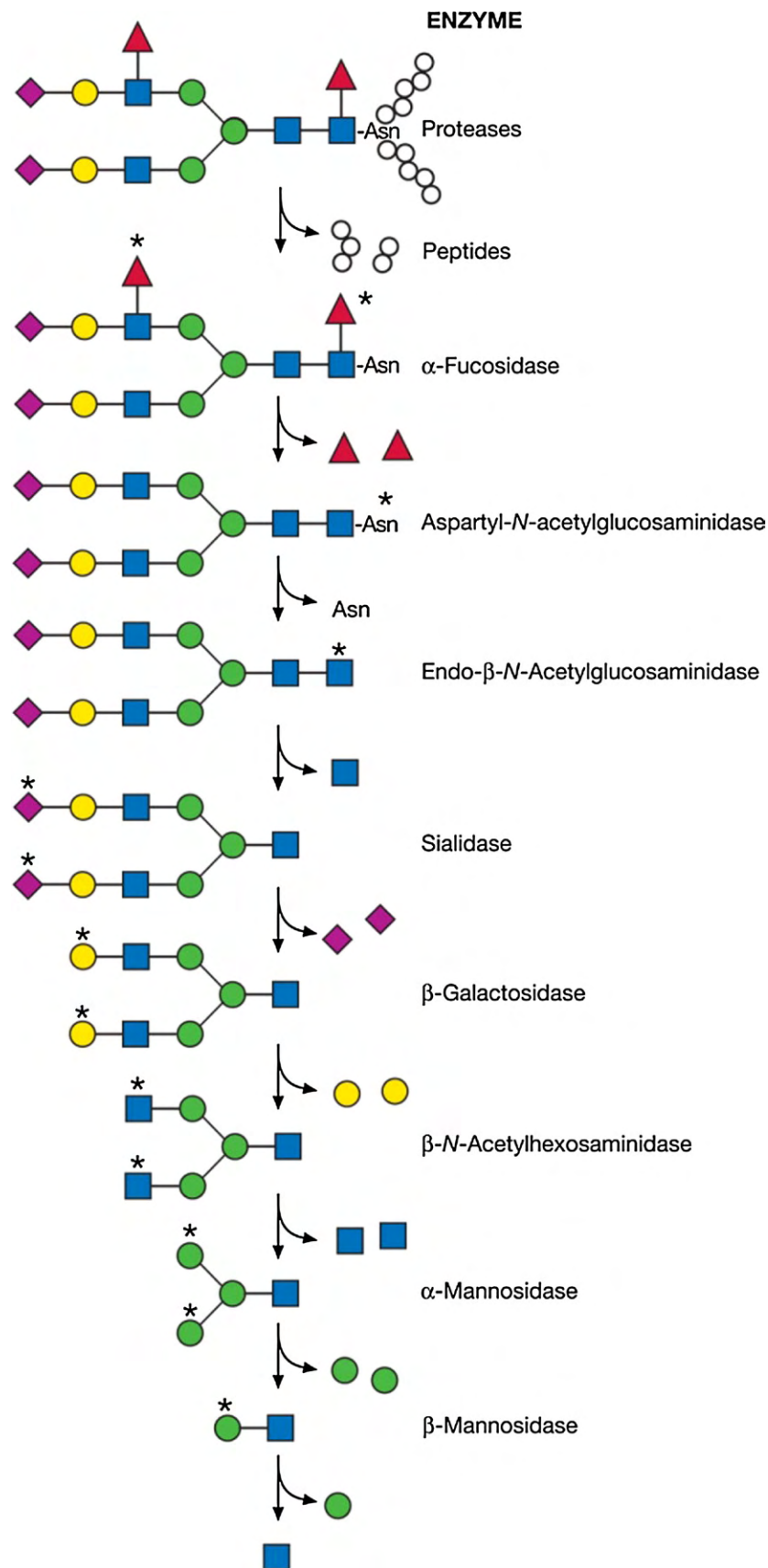
Already, the original purification procedure provided proof that the enzyme occurs in a macromolecular complex of proteins that also encompasses a protective protein with its own cathepsin A (CA) activity (CTSA; OMIM # 613111) and  $\beta$ -D-galactosidase (GLB1; OMIM # 230800). Soon after biosynthesis, nascent sialidase forms a heterodimeric structure with the protective protein in the larger protein complex and thus ensures its effective trafficking to the lysosomal compartment and intralysosomal stability and activation (8). Heterodimerization with the chaperone-type CTSA prevents premature oligomerization and degradation (9–11).

**103.1.1.4 Studying Mouse Models Relevant to Pathogenesis.** The disease that develops in NEU1<sup>-/-</sup> mice is also multisystemic and similar to severe human sialidosis. The affected animals develop progressive splenomegaly with extramedullary hematopoiesis. Yogalingam et al. have recently demonstrated that NEU1 controls sialylation in the hematopoietic cells and the concomitantly normal turnover of lysosomal-membrane associated protein 1 (LAMP1). Hyper-sialylation of LAMP1 at the lysosomal membrane prompts preferential docking of lysosomal organelles onto the plasma membrane (PM) and their exocytosis. Excessive exocytosis of lysosomal enzymes such as serine proteases leads to the premature degradation of vascular cell adhesion molecule 1 and loss of bone marrow cells (10).

Abnormally intense exocytosis is likely to have additional effects in sialidosis. Oversialylation, increased expression of LAMP1 and other LAMPs, and their apical localization in the marginal cells of the stria vascularis in the mouse cochlea suggest that there is excessive exocytosis into the endolymph with failure to adequately transduce sound through the sensory hair cells (12). Moreover, increased exocytosis from fibroblasts may represent at least one of the mechanisms underlying progressively abnormal remodeling of the extracellular matrix (ECM) and the overproliferation of the connective tissue (13) relevant to the coarsening of facial features and possibly to the bone dysplasia in early-onset and severe sialidosis. The expanding connective tissue around and within muscle fibers leading to their dysfunction and ultimate loss is likely to have a similar basis. Muscle weakness and atrophy are known to become prominent in long surviving patients. Thus, the mouse model studies reveal that sialidosis is an inborn error of metabolism in which lysosomal exocytosis is exacerbated (8,10).

**103.1.1.5 NEU1 Encoding Sialidase and Formal Diagnosis by Mutation Screening.** The gene encoding lysosomal sialidase, called NEU1 (OMIM # 608272) is located on the short arm of human chromosome 6 at 6p21.3 and hence within the large genomic site harboring the major histocompatibility gene loci. NEU1 contains six exons and spans about 3.8kb of genomic DNA. The gene and its encoded protein, the most abundant and most widely expressed mammalian sialidase, have considerable homology with other NEUs, including homology with the enzyme from the most divergent sources in nature such as some well-characterized bacterial sialidases (8).

Mutation screening has become the preferred method for formal confirmation of the diagnosis since the molecular characterization of NEU1. Over 40 different mutations have been identified in either homozygous or compound heterozygous genotypes in patients with any of the various types of sialidosis. Studying the correlation between the phenotype and the mutant genotype has yielded the expected result in most patients with severe diseases, where often at least one nonsense mutation or one mutation that impacts the reading frame (Frame Shift: FrSh) is revealed. The phenotypes are associated with complete absence of sialidase activity and the latter mutations are termed phenotypically “null” or “amorph.” The report of the amorph effect of a homozygous large 11kb interstitial deletion that had removed the entire NEU1 coding region is a case in point (14). Early-onset severe disease was also the clinical effect of the homozygous splice-site mutation that deleted the exon 5 completely (15) or of homozygous or compound heterozygous nonsense mutations with the effects of protein truncation and the complete loss of enzyme activity (16). If such amorph mutation is accompanied by a NEU1



**FIGURE 103-1** “Wild-type” lysosomal degradation pathway of complex *N*-glycans in glycoproteins, including lysosomal hydrolases, proceeds simultaneously on both the protein and the glycan components. *N*-glycans are degraded sequentially by the lysosomal hydrolases (mainly exoglycosidases). Deficiency of one hydrolase causes accumulation of the substrate involved and adverse effects on other catabolic steps. (Figure reproduced from Freeze, H. H. *Genetic Disorders of Glycan Degradation*. Chapter 41, pp. 567–583, Figure 41.1, In: *Essentials of Glycobiology*, 2nd ed.; Varki, A. et al., Eds.; CSHL Press: Woodbury, NY, 2009, with gratefully acknowledged permission by the editors and the publisher.)

missense type, called hypomorph, considerable residual sialidase may be detectable in the patient with late-onset, mild JNS.

In addition to the molecular type, also the location of mutations within *NEU1* is a challenging factor for predicting the phenotype associated with missense (MS) mutations located in the periphery of the tridimensional protein. Some of the latter adversely affect the binding of sialidase to its protective chaperone, PPCA (protective protein CA), and within the larger quaternary ( $\beta$ -D-galactosidase, CA, sialidase (GCS)) protein complex (16,17).

Sequence analysis and homology alignment methodology with other mammalian sialidoses have contributed to the tridimensional model building of the wild-type enzyme (18,19). It has turned out to be helpful in predicting the impact of MS mutations also on the enzyme's normal subcellular trafficking and location and on the concomitant biochemical maturation as studied by immunohistochemical methods (17). Among others, Seyrantepe et al. (16) have proposed to subdivide sialidase MS mutations into three groups: (1) mutations substituting amino acids within the enzyme's catalytic site and having a severe effect and an early onset; (2) mutations in the periphery of the enzyme without any major effect on the tertiary structure with the mild late-onset type clinical disease; (3) MS mutations located at the periphery of sialidase, but located within peptides necessary for binding into the quaternary GCS complex. The latter consistently result in early onset and severe disease.

### 103.1.2 Disorders due to the Deficiency of $\beta$ -D-Galactosidase

The disorders due to the acid  $\beta$ -D-galactosidase deficiency known as the various types of  $G_{M1}$ -gangliosidosis are discussed in the chapter on lipidoses. The term gangliosidosis implies only defective ganglioside metabolism and lysosomal storage of gangliosides with progressive CNS disease mainly due to neuronal cell death. It is hardly applicable to patients with Morquio type B disease with a rather specific spondyloepiphyseal dysplasia as the main or sole clinical consequence. See Chapter 102.

Patients with these disorders have excessive urinary excretion of OSs with  $\beta$ -galactose at the terminal non-reducing end. The diagnosis is confirmed by finding a severely deficient acid  $\beta$ -D-galactosidase (EC 3.2.1.23) (Figure 103-1) in leukocytes or cultured fibroblasts and a normal specific activity of acid sialidase. Adequate function of  $\beta$ -D-galactosidase is dependent on the intact structure and function of other proteins, notably their protecting and activating factors (next section in this chapter). The gene *GLB1* encoding  $\beta$ -D-galactosidase (OMIM # 611458) has been mapped to chromosome location 3p21.33. A steadily increasing number of mutations, single nucleotide substitutions, and larger structural

rearrangements have been identified. The clinical effect of progressive brain disease is most often severe and is evident from early infancy, occasionally expressed from late infancy or toddler age, and rarely first apparent from young adulthood. *GLB1* has at least two mRNA transcripts due to alternative splicing. Besides the encoding of  $\beta$ -D-galactosidase, it also yields an mRNA species with the genetic code for the catalytically inactive elastin binding protein (EBP) with a role in elastic fiber production.

### 103.1.3 Galactosialidoses

The term “galactosialidosis” is applied to a group of allelic lysosomal disorders due to mutations in the gene encoding the lysosomal protective protein cathepsin A (CTSA) resulting in the combined deficiency of both  $\beta$ -D-galactosidase and acid sialidase as the main metabolic consequence. Clinically and biochemically, the disorders resemble the age of the onset of the matched types in sialidosis (Section 103.1.1) and  $G_{M1}$ -gangliosidosis (Section 103.1.2 and Chapter 104). Moreover, the early-onset type of galactosialidosis represents a diagnostic challenge in discerning it clinically from ML II (see Section 103.1.4).

The juvenile (adult) type of galactosialidose (GS) mostly reported from Japan is the most frequent entity worldwide, but also late infantile GS (IGS) and even non-immune perinatal hydrops due to congenital GS (CGS) have been reported. In the large majority of cases, galactosialidosis is a severe disease most often with protracted natural course. Based on the increasing number of clinical reports, GS represents overall a continuum of phenotypes with the frequency distribution skewed toward late childhood or adolescent age of onset and slow rate of progression.

#### 103.1.3.1 Phenotypes Delineated.

**103.1.3.1.1 Congenital Galactosialidosis.** Congenital galactosialidosis presents as non-immune “hydrops fetalis,” which requires immediate intensive neonatal care that may be unable to prevent fatal outcome. The hydrops already recognized ultrasonographically in the third trimester of pregnancy (20) are often associated with polyhydramnios and hydropic placenta. The often prematurely born neonate is heavy for dates, but short and may have congenital inguinal herniae. In addition to the pronounced and extensive edema, there is hepatosplenomegaly, ascites, and inconsistent pleural effusion. The corneae are cloudy. The macula may be grayish or may already show a small central red spot. There is often proteinuria, anemia, and thrombocytopenia. Cytoplasmic vacuolization is a consistent finding in peripheral lymphocytes. In longer survivors, failure to thrive, refractory to management, and poor neuromotor developments are common. Cardiorespiratory failure compounded by sepsis is often the cause of death in the neonatal period or somewhat later in infancy (20,21).



Fetal loss late in pregnancy has also occurred in families with hydropic CGS. The pathogenesis in this and other genetic types of nonimmune hydrops remains poorly understood since some infants with proven CGS do not show significant edema at birth, but instead start a slowly progressive disease course indistinguishable from that in children with the late infantile type of GS.

The longer surviving patients initially show discrete signs of dysostosis multiplex. This type of skeletal dysplasia is consistently less pronounced than in patients of similar age with either infantile  $G_{M1}$ -gangliosidosis type I or ML II (see next section). The clinical syndrome in a surviving CGS patient is soon indistinguishable from the IGS phenotype.

**103.1.3.1.2 Late Infantile Galactosialidosis.** Only a few patients with clinical onset of GS during or before the second year of life have been reported. Clearly, in at least some of them, the true clinical onset in early infancy had not been recognized. Hence, CGS and late-onset IGS only represent points of clinical reference within the continuously variable, early, but severe GS. The face is slowly coarsening. Statural growth velocity gradually decreases. Head size remains proportional to body size. Failure to thrive is a common complaint. There is corneal clouding at least by slit-lamp examination. Macular cherry-red spot is at first inconsistent. Hepatomegaly is moderate, splenomegaly mild. There is developmental delay and neuromotor progress is slower than normal. In long survivors, cognitive deficiency resembles that in patients with adolescent or adult-onset GS as is true also for the skin changes. Thickening of the cardiac valves and later mild cardiomegaly and cardiomyopathy are consistent features. The rate of clinical progression is remarkably heterogeneous in IGS. Respiratory infections recur often, become increasingly more morbid, and ultimately result in very variably timed fatal outcome of this severe disease (22).

**103.1.3.1.3 Juvenile Galactosialidosis: Goldberg Syndrome.** Juvenile or adult galactosialidosis (JGS) (OMIM #256540), the most frequent form of GS with high prevalence reported from Japan may have its clinical onset from mid-to-late childhood until the fourth decade of life. This interfamilial variability of the age of onset is however larger than the intrafamilial one. See references to original reports in (1,22,23). The first patients described with the Goldberg syndrome have been examples of protracted JGS. In the first survey of Japanese patients, JGS had been called galactosialidosis type II. Parental consanguinity has often been recorded. The composite clinical picture resulting from an astute comparison of the fully developed JGS with the similar but milder JNS, known as CRSM syndrome (see Section 103.1.1.1.2), is helpful for both clinical diagnosis and differential diagnosis. It includes (1) slowly progressive axial and appendicular ataxia without nystagmus, tremor, severely handicapping action myoclonus, hyperactive deep tendon reflexes, and peripheral

neuropathy (24); (2) decreasing visual acuity and macular red spot, corneal clouding often detectable by slit-lamp examination, and infrequently lens opacities; (3) moderate short stature, coarsening facial features, and platyspondyly in the thoracolumbar spinal column as the main expression of otherwise mild dysostosis multiplex; and (4) initially mild, but gradually moderate with a clear mental impairment. The latter two among the four component groups are consistently absent in JNS. Therefore, the designation “cherry-red spot myoclonus syndrome plus” may be an alternate term for JGS provided the following remarks are taken into account: Any one of the clinical components may either be of primordial and/or early concern or lacking altogether; in older patients with diagnosis late in the clinical course, several signs and symptoms may be found retrospectively: Angiokeratoma is frequently an example; hepatomegaly is equivocal at worst, and splenomegaly is not observed in JGS patients. As is the case in the early-onset types of GS, cardiac involvement, especially valvular dysfunction and/or cardiomyopathy, combined with intervening infection fatally compound the ever more dreary neurologic syndrome of JGS (22,23,25,26).

**103.1.3.2 Histo- and Chemopathology: Biochemical Diagnosis.** Patients with any type of GS have vacuolated lymphocytes in the peripheral blood smears aligning them not only with sialidosis but also with many other LSDs. Follow-up reports on the neuro-radiology aspects of GS remain unavailable. Reports on postmortem neuropathology have not been added to the literature in the past decade. Marked atrophy of the brain has been described to be associated with severe neuronal loss, fibrillary gliosis, and histochemically heterogeneous storage in various parts of the brain, brain stem, spinal cord, and in Schwann cells surrounding atrophic peripheral nerves. Careful studies in mutant mice, although phenotypically not completely congruent to the corresponding human diseases, show various lesions in the brain and the cerebellum that are more extensively affected in the sialidosis than in the galactosialidosis model (27). Ultrastructurally, a variety of single membrane-bound cytoplasmic inclusions, some with granular and others with concentric lamellar shape, is found. Vacuoles are readily apparent also in the cytoplasm of hepatocytes, Küpffer cells, and in epithelial cells of the glomeruli and renal tubuli. Nephropathy has been documented in the final years of chronically evolving late IGS (28). Neither the inclusions seen in cultured fibroblasts from IGS patients nor the ones found in parenchymous organs are distinguishable from the ones encountered in subjects with sialidosis or several other lysosomal enzyme deficiency disorders. In hydropic IGS, there is extensive cytoplasmic vacuolization of the trophoblastic cells in the placental tissue (21), indistinguishable, however, from the findings in the perinatally expressed nonimmune

hydrops due to any other inborn lysosomal disorder. The same applies to the pathology features in parenchymatous organs even in the longer surviving CGS patients (29). The storage adversely affects endothelia especially in the capillaries within angiokeratoma lesions (26). See also Reference (1) for original literature reports.

The storage material in the brain tissue resembles histochemically as well as biochemically the macro compounds accumulated in the CNS and other organs in  $G_{M1}$ -gangliosidosis. However,  $G_{M1}$ - and other ganglioside species are less abundant in GS. The OSs in the accumulated glycoproteins and glycolipids are of the *N*-acetylglucosamine type with the mannose- $\beta$ -1-4 GlcNAc sequence preserved at the reducing ends (Figure 103-1). The structures of the OSs excessively excreted in the urine of GS patients are very variable but similar to the ones found in other OS patients. The combination of several types of chemical analysis (30,31) or advanced analytic methodology is required in order to identify disease-specific features in the body fluids (6,7).

Demonstrating the combined deficiency of  $\beta$ -D-galactosidase and glycoprotein sialidase in fresh leukocytes or cultured fibroblasts provides confirmation of the diagnosis of GS. It needs pointing out that the deficiency of the former enzyme is not found in all tissues and not in plasma. Where detectable, this deficiency is consistently less pronounced than in patients with  $G_{M1}$ -gangliosidosis. In obligate heterozygotes, glycoprotein sialidase activity is intermediate in fresh cells, but  $\beta$ -D-galactosidase is normal. The specific activity of CA by the lysosomal protective protein (PPCA) is at present preferentially assayed and found to be largely deficient in leukocytes and fibroblasts (21). Average values for CA activity are half of normal in obligate heterozygotes. This carboxypeptidase (EC #3.4.16.5) has amply sufficient normal activity in chorionic villi and in amniocytes in order to recommend its assay in a prenatal diagnosis setting. Because of the possibility of a mutation that interferes substantially more with the protective function than with its CA activity, it is advisable to ascertain the coexisting secondary deficiencies of  $\beta$ -D-galactosidase and acid sialidase for confirming the diagnosis, especially in circumstances when or where gene mutation screening is not available.

**103.1.3.3 Molecular Data on CTSA and Its Gene and Formal Diagnosis by Mutation Screening.** Because fibroblast strains from any two clinical types of GS did not show mutual in vitro complementation, the causal mutations were recognized in the 1970s as being functionally allelic before being identified. Immunoprecipitation used in the isolation procedure of the mutant enzymes revealed the existence of a “corrective factor” inactive in GS fibroblasts and tissues, but apparently essential for the adequate catalytic function of

both  $\beta$ -D-galactosidase and acid sialidase. The initially unknown polypeptides of 54, 32, and 20 kDa that coprecipitated with the anti- $\beta$ -galactosidase antibodies were soon identified as molecular species representing the “corrective factor,” because in vitro uptake of the 54 kDa protein promptly normalized the molecular processing of nascent  $\beta$ -D-galactosidase in GS cells. Thus, the primary metabolic defect in GS was elucidated to be the absence or complete deficiency of the 32-/20 kDa two-polypeptide mature protective protein (PP) and/or of its 54 kDa precursor. GS mutant fibroblasts are also deficient in protective protein-specific mRNA. Transient in vitro expression of the wild-type cDNA in COS-1 cells has resulted in the synthesis of the 54 kDa precursor protein, in its conversion into a 32/20 kDa two-polypeptide containing mature enzyme, and its normal routing to lysosomes. Subsequent coprecipitation studies have identified a quaternary multiprotein complex into which the “PP” must enter in order to adequately fulfill its enzyme protective and catalytic role. Not only  $\beta$ -D-galactosidase, but also nascent lysosomal acid sialidase becomes a constituent of this heteromeric protein (GCS) complex while still in the endoplasmic reticulum (ER) in order to avoid premature degradation and to achieve effective intracellular trafficking to and function in the lysosomal compartment (8).

In addition to its protein-protective chaperone-like function, the lysosomal PP has been identified as CA with carboxypeptidase activity at acid pH. Hence, the multifunctional protein is often symbolized by PPCA. At neutral pH, the enzyme can also function as a deaminase/esterase. For routing toward the lysosomal compartment and catalytic function, acid sialidase is totally dependent on integration into the macromolecular protein complex and PPCA integrity, whereas  $\beta$ -D-galactosidase in dynamic equilibrium of its monomeric, homodimeric, and homomultimeric conformations can exist and somehow function independently. The latter applies to the cathepsin A (CTSA) activity of PPCA as well (20).

The multienzyme complex in which PPCA is a protagonist protein contains still other components such as the lysosomal enzyme GalNAc-6-sulfate sulfatase (MIM # 612222), catalyst in the initial step of keratan sulfate degradation and known to be deficient in Morquio disease type A (MPS IVA; MIM # 253000). The urinary excretion of keratosulfate is excessive in GS and even comparable to that in MPS IVA patients. In GS patients, GalNAc-6-sulfatase activity is significantly lower than normal (32).

PPCA also plays an important role in another protein complex called the elastin binding protein receptor (EBPR) located and functional at the PM instead of the lysosomes. It is composed of three subunits, including EBP, PPCA (most often called CTSA), and the *NEU1* gene product, acid sialidase. Also, in this macromolecular

protein complex, PPCA protects the molecular integrity of both EBP and acid sialidase that apparently favors the attachment of the EBPR complex to the PM surface and has also a pivotal role in the secretion and the assembly of tropoelastin monomers into elastic fibers (33).

Beyond its effect in elastogenesis, cathepsin A (PPCA or CTSA), within the PM-bound EBPR complex, is also of regulatory importance in vasoconstriction, blood pressure regulation, and when mutant, a factor favoring the development of the cardiomyopathy in GS that is hardly directly explainable by lysosomal dysfunction. In *Ctsa* gene-targeted mice homozygous for the Ser190Ala MS mutation, located within the active site of the serine carboxypeptidase,  $\beta$ -D-galactosidase and acid sialidase had normal catalytic activity, but cathepsin A was clearly deficient. Further results confirmed that CTSA failed to hydrolyze some regulatory peptides including angiotensin (ang)2 and endothelin1 (ET-1) a peptide of 21 amino acids, most abundantly detectable in endothelia, where CTSA is most strongly expressed. Reduced degradation of ET-1 has been shown spectrophotometrically in the blood and tissue of the mutant mice. The affected animals had a significantly raised arterial pressure. In addition, the role of elastogenesis was confirmed immunohistochemically by finding a decreased amount of elastin containing microfibrils in skin, arterial walls, and lung alveoli (33,34). Paradoxically, elastic fiber formation and EBP mRNA expression studied by immunofluorescence with antitropoelastin antibody and by RT-PCR selective for EBP, respectively, was not significantly different from control values in mutant fibroblasts from either Morquio B patients (*GLB1* gene mutations), galactosialidosis (*PPGB* gene mutations), or sialidosis patients (*NEU1* gene mutations) (35). Either the physical difference between the in vitro ECM in cell cultures and that in tridimensional tissue, or the failing stability of the elastic fibers rather than the formation of the microfibrils containing them may preclude effective comparison of the results reported.

Mutations that adversely affect the posttranslational processing of the CTSA protein have been extensively reviewed by d'Azzo et al. (22). Nascent human CTSA is synthesized as a 480-amino acid precursor protein (54kDa), where Asn 117 and Asn 305 serve as N-glycosylation anchors. The protein contains nine cysteines and accommodates a hydrophobic signal peptide (SP) of 28 amino acids cleaved off upon ER entry, where CTSA is promptly N-glycosylated and dimerizes. While passing through the cis Golgi cisterns only the Asn 117 oligosaccharide acquires the mannose-6-phosphate (M6P) marker, required for proper routing of the dimer to the lysosomal compartment. Following the conclusion of this voyage, the precursor undergoes endoproteolytic cleavage into the 34- and 20-kDa inactive intermediates and a 2-kDa “excision” peptide is subsequently removed from the C-terminus of the larger protein that finally becomes the mature 32-kDa subunit. However, several

disulfide bridges hold the two polypeptides together as a single unit. By the removal of the “excision” peptide, the catalytically active site of the CTSA protein composed of the triad of amino acids serine (S) 150, histidine (H) 429, and aspartic acid (D), becomes exposed. Effective catalysis at the active site depends also on the tridimensional conformation of CTSA and on the integrity of the multiprotein complex in which it plays its multifunctional role (22).

The gene encoding the protective protein CA has received various names abbreviated as *PPCA*, *PPGB*, or *CTSA*, the latter becoming the more official one. It has been mapped to chromosome 20q13.12 and spans about 7.5kb of genomic DNA. It has 15 exons that, except for a short 5' untranslated region (UTR), represent the coding region. The latter is followed by the 3' UTR and the polyadenylation sequence. Human *CTSA* and mouse *ctsa* are very similar including the 5' promotor regions. The protein coding *CTSA* transcript of 2.1kb (one of the six protein-encoding transcripts) is expressed ubiquitously. The protein encoded is predicted to have 498 amino acids.

The clinical heterogeneity of GS is largely surpassed by the number of mutations in the *CTSA* gene with morbid effect in homozygous and compound heterozygous mutant genotypes.

Among the juvenile-onset patients (JGS) reported from Japan, homozygous deletion of the complete exon 7 is frequently found. It is due to the splice-site mutation IVS7, c.A>G, +3→Ex7del. Its late-onset, slow course, and rather mild features remain incompletely understood. The Ex7del mutation is leaky because in the homozygote a small amount of normally spliced transcript is detectable. When this Ex7del mutation is combined either with the MS mutation Trp65Arg (exon 3), the Ser90Leu (exon 3), or the Tyr249Asn (exon 8) (MS mutations), the clinical result is the more severe late IGS. Interestingly, the MS mutation Tyr395Cys associated with the Ex7del mutation in a compound heterozygous genotype yields a GS of intermediate severity. The homozygous Tyr395Cys genotype was found to result in early-onset IGS, where residual enzymatic activity was absent. Lack of phosphorylation of the CTSA protein precludes proper routing to the lysosomes (see next section) and in homozygotes is the primary metabolic consequence of the mutations Val104Met, Leu208Pro, or Gly411Ser. Each of these mutant genotypes results in early and severe IGS. The same clinical effect has been observed in Lys453Glu homozygotes. The latter MS mutation is located at the interface of the PPCA dimer (36).

As expected, some deletions or additions of nucleotides result in the shift of the mRNA reading frame and in the creation of abnormally located stop codons. In the homozygotes with the CTSA protein being at least truncated and often quickly degraded, non-immune hydrops CGS is the consistent clinical outcome. The clinical effect of the single nucleotide C899 insertion in exon 10 in two

unrelated Dutch patients with nonimmune fetal hydrops due to early lethal CGS is an example. The mutation was homozygous in one patient. It was associated with the MS transition c.169G>A in exon 3 (G57S: substitution of a nonconserved glycine) in the other patient. In both infants, the amount of CTSA mRNA was considerably reduced. The insertion of the single cytosine with frameshift effect caused premature termination of PPCA precursor synthesis and the predicted lack of the 20-kDa subunit. Moreover, the remaining mutant protein is being misfolded, fails being incorporated into the multienzyme complex, and cannot be routed to the lysosomes. Both the catalytic activity and the protecting and activating roles cannot be played: hence, the severe clinical consequences (21). In an Italian family with multiplex occurrence of CGS and nonimmune hydrops, two previously unreported CTSA mutant alleles were detected: c.60DelG and IVS2+1G>T. In this genotype, either mutation with different frameshift effect contributed to the complete absence of CTSA and of EBP translated from the alternate mRNA transcribed off the *GLB1* gene. This observation has provided proof of the role played by CTSA in the EBPR complex at the PM, where it coregulates elastic fiber synthesis and suppresses fibronectin production. This study also found interesting genetic polymorphisms in the CTSA (*PPBG*) gene (20). CGS in a prematurely born female with clear signs of fetal hydrops, who died at over 200 days of age due to renal failure, was due to homozygosity for the c.146A>G→p.Gln49Arg MS mutation in exon 1 of the CTSA gene. The mutation had been reported earlier in a compound heterozygous patient with JGS who also carried the DelEx7 splice-site mutation. Given its rare incidence, still too few reports are available describing this severe, most often chronic metabolic disorder. It is unfortunate that often-adequate clinical detail is lacking in the reports most informative regarding the molecular biology of GS. In contrast, in several clinically adequate to excellent papers, no information on the mutant genotype is provided. Future improvement of the genotype-phenotype correlation in GS also awaits more results of thorough studies on its complex pathology.

### 103.1.4 N-Acetylglucosamine Phosphotransferase Deficiency Disorders

#### 103.1.4.1 Clinically Delineated Syndromes.

**103.1.4.1.1 ML II or I-Cell Disease.** ML II is a slowly progressive disorder with clinical onset at birth and fatal outcome in childhood. It may be suspected prenatally when ultrasonographic examination in the last trimester of pregnancy finds punctate extraosseous calcifications around the ankles, wrists, spine, or pelvis in a small-for-date fetus. The neonate with I-Cell disease has on average a low birth weight, a plump and somewhat edematous, flat facies, and a skin that is thick and stiff in particular about the earlobes. Congenital inguinal herniae are

consistently present in males. One or more orthopedic abnormalities are common: clubfoot, dislocation of the hip(s), thoracic deformity, with or without kyphosis. Despite generalized hypotonia, the range of motion in the shoulders is limited. In the more severely affected neonates, crying is weak, respiration may temporarily need support, and alertness is below normal. Head size is proportional to stature. Growth failure is always severe and the final height of 80 cm is rarely exceeded. Unlike in the patient with Hurler disease (MPS IH: MIM # 607014), there is no temporary acceleration of statural growth around the age of 1 year. Instead, growth decelerates within 6 months from birth and ceases often before 15 months of age. Stiffening of all joints, large and small, occurs from the first year of life. Neurodevelopmental delay is severe in some, but more moderate in others. Only few patients come to unaided ambulation. Social interaction is better than the patient's hoarse speech that rarely reaches the stage of two-word sentences. Upper respiratory infection and otitis recur frequently. Hearing deficit that worsens over time is often evident. Breathing is noisy from the start and remains so even in the absence of bronchitis or lung infection. Because of the narrow airway, the gradually decreasing mobility of the neck compounded by hypertrophic gums, both intubation and detubation are difficult assignments for the anesthesiologist. The face of the ML II patients is reminiscent from that in MPS IH, but shows consistent differences including small orbits, hypoplastic supraorbital ridges, mildly proptotic eyes, a tortuous pattern of periorbital veins, telangiectatic capillaries over midface and cheeks, impressive gingival hyperplasia precluding adequate eruption of deciduous teeth, and a prominent mouth (Figure 103-2). The corneae are often considered clear clinically, but slit-lamp examination consistently shows haziness. Facial features are gradually coarsening in the longer-surviving patients and wrists, hands, and fingers broaden, become more immobile, and adopt a clawlike configuration in radial deviation. Cardiac murmurs increase gradually with slowly worsening insufficiency of several cardiac valves. The abdomen is protuberant and an umbilical hernia often noticed. Hepatosplenomegaly is moderate or equivocal. Bronchopneumonia and congestive heart failure are the usual causes of fatal outcome, usually before the age of 5 years. See original reference in Reference (1) and/or in other extensive reviews (37,38).

Affected siblings are clinically more similar than patients in different families.

Qualitatively, the radiological abnormalities in ML II are indistinguishable from the ones in MPS IH (OMIM #) and in G<sub>M1</sub>-type I GS (OMIM # 230500). ML II shares with the latter the most severe form of "Dysostosis multiplex," the osteochondrodysplasia (OCD) common to most oligo- and mucopolysaccharidoses and expertly described by Spranger et al. (39). At this stage, attention is due to some transient radiographic and related phenomena observed in or from the perinatal stage of





**FIGURE 103-2** Clinical manifestations in ML II. (a–c) Patient 1 at 5 years 10 months; (d, e) patient 2 at the ages of 3 months and 20 months; (f, g) patient 3 at 13 months and 3 years; (h) patient 4 and (i) patient 5 both at 3 years; (j, k) patient 6 at 13 months and 3 years. Coarsening of facial features, apparent from infancy, evolves in subsequent few years of life. Common features include flat face with depressed nasal bridge, prominent mouth and gingival hypertrophy, shallow orbits, thick skin, full cheeks, deep infraorbital creases, prominent cutaneous veins, and capillaries on the face. Metopic prominence, which creates the impression of craniosynostosis, is depicted in (b), (h), and (i). Short, broad “claw”-like hands and contractures in knees and ankles are seen in (c). (Figure reproduced from Cathey, S. S.; et al. *J. Med. Genet.* 2010, 47, 38–48, Figure 1, with gratefully acknowledged permission from authors and BJM Publishing Group Ltd, London, UK.)

ML II that include: (1) punctate or dot-like extra-osseous calcifications mainly in hands and feet, but also over the pelvic region and about the spinal column. These puncta, when detectable by prenatal ultrasonography, were initially considered to be the more typical feature in “Pacman” dysplasia (40,41), an OCD that was thought of as sharing features with ML II, but with perinatal fatal outcome. Meanwhile proof has been provided that most instances of Pacman type OCD represent the prenatal expression of I-Cell disease (ML II) (41,42); (2) Excessive periosteal new bone formation alongside the diaphyses of the large tubular bones, a phenomenon also called “periosteal cloaking,” the term used to describe the double outline of the bone cortex in the large tubular bones. The phenomenon is no longer visible from the age of 18 months when the calcified outline seems to coalesce with the diaphyseal cortex (39); (3) Severe diaphyseal

widening in the small tubular hand bones concomitantly rendering the metaphyseal ends very narrow, a process that may last until early childhood (43); (4) Perinatal hyperparathyroidism supported by relevant biochemical findings in plasma (42,44). It is unknown at present whether hyperparathyroidism represents the true cause of the transient bony abnormalities or the consequence of an as-yet unknown effect of the mutant genotype set in motion at or before birth. Also, the transient nature of the described set of phenomena awaits pathogenetic explanation.

In patients with ML II, the urinary excretion of SOSs is excessive and that of glycosaminoglycans (GAGs) is normal. This finding is nonspecific as it is hardly distinguishable from the results in urine from patients with sialidosis (Section 103.1.1) or galactosialidosis (Section 103.1.3), when studied by routine laboratory methodology. More

advanced technology, not commonly available, is able to improve chemical differentiation (6,45,46). At present, the latter methodology has interesting potential in more effective newborn screening, but has become of secondary importance in the field of diagnosis since the introduction of mutation screening.

**103.1.4.1.2 ML III $\alpha$ / $\beta$ ; Pseudo-Hurler Polydystrophy.** Delineated and named Pseudo-Hurler polydystrophy (PHP) by Maroteaux in 1966, it was called ML III by Spranger and Wiedemann in 1970 before its alignment with ML II was proved. Since the elucidation of the enzyme defect and of its oligomeric structure, it has been shown that two nonallelic types of PHP exist. The nomenclature has been adapted accordingly (47). In addition to ML III $\alpha$ / $\beta$  at hand, there is ML III $\gamma$ , the subject of the next paragraph.

Complaints of physical slowness, worsening stiffness in one or more of the large joints, and sometimes slow growth around the average age of 3 years mark the clinical onset. Slow cognitive development is rarely among the initial parental concerns. ML III $\alpha$ / $\beta$  patients retain family facial characteristics, but the facies is rather flat and coarsens gradually throughout the long and slowly progressive clinical course. Motion range, particularly in shoulders and hips, is reduced from the early stages of the disorder. The slowly progressive flexion contractures in hips and knees are evident early in the standing patient viewed from the side. Raising the arms above the head is difficult and soon impossible. Birth weight and length are normal in ML III, but below average and gradually decreasing statural growth rate is evident in childhood. The head circumference remains proportional to stature (Figure 103-3). The corneae are clear by clinical inspection, but show opacities on slit-lamp examination. Liver and spleen are not usually enlarged. Intellectual deficiency is mild and nonprogressive or absent. Speech and language development are normal. In the patient with later clinical onset of the disorder, the diagnosis may be postponed because of earlier referral to and follow-up in the rheumatology clinic. The early radiographs may show only mild dysostosis multiplex and are sometimes read as normal. However, skeletal age is found to be considerably delayed and moderate osteopenia already noticed. Contrary to the severe OCD in the hands of ML II patients, the hand bones themselves remain normal or near normal, but the progressive hardening of the soft connective tissue around the small joints results in worsening flexion contractures in the fingers. Already from preadolescence, ML III $\alpha$ / $\beta$  patients consistently have moderate but often severe and very painful hip disease. It is an almost consistent reason why the patients cease walking on average from the early teens. Unfortunately, the pain is not effectively dealt with even in the more immobile wheelchair-bound patient, because it results not only from the destructive hip dysplasia, but also from worsening osteopenia throughout the skeleton. In the older patients, the deficient function of some of the

heart valves apparently due to connective tissue hardening, becomes of increasing concern. Throughout the clinical course, respiratory infections are not significantly more frequent than in the patients' peers, unlike their morbid role in ML II. ML III patients surviving beyond the fifth decade of life have been reported, but comprehensive data on life expectancy and on specific causes of fatal outcome remain unavailable.

The distinct dividing line between ML II and ML III $\alpha$ / $\beta$  has been documented in a recent large genotype–phenotype correlation study supported by the results of mutation screening (43). Significant clinical differences include growth rate and final statural height, hand abnormality, qualitative differences of dysostosis multiplex, neuromotor and speech and language development, intellectual functioning, rate of clinical progression, and life expectancy. The rather rare phenotypically intermediate patients have specific mutant genotypes. See subsequent paragraphs on molecular genetics.

**103.1.4.1.3 ML III $\gamma$  (PHP).** Because the first report on the molecular basis of an ML III $\gamma$  has preceded any comparable result on either ML II or ML III $\alpha$ / $\beta$  (48), the clinical phenotype of this disorder deserves separate attention. From the few reports with useful clinical information already available, the ML III $\gamma$  phenotype is probably not distinguishable from ML III $\alpha$ / $\beta$ , but as the latter quite distinct from ML II. The age of onset, natural course, and the major sites of morbidity as well as life expectancy appear to be rather congruent to what has been recorded for ML III $\alpha$ / $\beta$ . Remarkably, the majority of observations reported so far concerns families in various parts of the Middle East. Parental consanguinity is noticed in most of these families. Nevertheless, the intrafamilial clinical variability among them may be larger than in ML III $\alpha$ / $\beta$ . Slowly, progressive stiffening of the shoulder joints, of the hands with claw-type deformity, slow growth, and short stature are among the first parental concerns. Intellectual disability is mild or more often absent. Radiographic evidence of mild dysostosis multiplex is initially often overlooked unless osteochondrodystrophy of the hip joints is already present. Some young patients have been observed with “isolated” hip involvement. From adolescence or even earlier, painful hip joints at walking and pain in the lower limbs also when immobile, become the patients' major handicap. Facial coarsening is late and possibly milder than in ML III $\alpha$ / $\beta$ . Thickening of some cardiac valves slowly leading to dysfunction is often recorded and probably consistent in the older patient (48–51).

**103.1.4.2 Histo- and Chemopathology in ML II and ML III $\alpha$ / $\beta$ .** The single most characteristic feature is the presence of large numbers of cytoplasmic, unit-membrane-bound vacuoles abundant in connective tissue cells in skin, gingivae, heart valves, and in the areas of endochondral and membranous bone formation. Because of the many empty-looking vacuoles





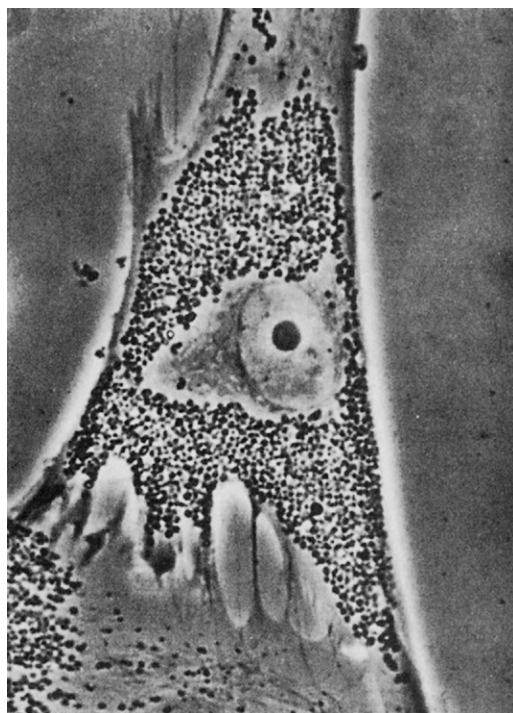
**FIGURE 103-3** Manifestations in ML III  $\alpha/\beta$ . (a–d) Patient 1 (female) at 17 years; (e–h) Patient 2 (male) at 9 years. Facial coarsening is mild and gradual in ML III and more apparent in profile, with full cheeks, low nasal bridge and prominent mouth; Stiffness in all large and small joints is slowly progressive, but disabling hip pain from late childhood is the major contributor to morbidity. Contractures of the hips and knees cause the squatting standing posture. Limited range of motion in the shoulders is an early consistent feature. Hand configuration is near normal although soft-tissue hardening and contractures are progressively evident. (Figure reproduced from Cathey, S. S., et al. *J. Med. Genet.* 2010, 47, 38–48, Figure 2, with gratefully acknowledged permission from authors and BJM Publishing Group Ltd, London, UK.)

in the cytoplasm, the swollen cells are called foam cells. Pericytes of capillaries, adventitial cells, and Schwann and perineural cells are similarly affected. In the renal glomeruli, there is foamy transformation mainly in the visceral and barely in the parietal cells of Bowman's capsule. Pathologic changes in neurons and glial cells are minimal, inconsistent, and may be of a secondary nature. Unfortunately, recent postmortem reports are as few in number (52) as the ones in former decades referred to in Reference (1). Histopathology findings are similar in ML II and ML III. In either type of mutant fibroblast strain, the many enlarged cytoplasmic inclusions are filled with pleomorphic material. Contrary to the in vitro observation of I-cells instead of normal fibroblasts in the phase-contrast microscope (Figure 103-4), the in vivo inclusions in mesenchymal cells are either empty or have only sparse granulofibrillar

contents. This paradox also applies to the chemo-pathology data obtained in patient tissues. There is neither histochemical nor biochemical evidence of significant accumulation of lipids, GAGs, glycogen, or lipid- and protein-bound sialic acid in postmortem brain and in visceral organs. In contrast, I-cells accumulate in vitro macro compounds, including GAGs, lipids, and sialylated glycoproteins in addition to free sialic acid. This paradox raises the question of whether the in vitro cultured fibroblasts are unable to achieve the clearance of macromolecular metabolites as they are physically separated from the in vivo neighboring parenchymous tissues. The specific activity of all lysosomal enzymes known to be deficient in I-cells and much increased in the corresponding culture media, are normally active in postmortem liver, spleen, kidney, and also in brain. Only  $\beta$ -D-galactosidase and acid

sialidase have a reduced specific activity in the organs mentioned. The activity of most lysosomal enzymes is much enhanced in the patients' plasma and other body fluids. This finding indicates that at least in connective tissues of the ML patient, the lysosomal hydrolases are unable to reach their intracellular site of function, and enter the ECM and the body fluids. The hyperactivity in plasma is thus an important indication of the diagnosis of either ML II or ML III. That this hyperactivity of plasma lysosomal hydrolases is more pronounced in ML II than in ML III can however not differentiate between the two clinical entities.

The secondarily impaired degradation of glycoproteins is sufficiently extensive in the ML II and ML III patients in order to result in excessive urinary excretion of several SOSs. With  $^1\text{H}$ -NMR spectroscopy-supported analytical technology, the qualitative pattern of the SOSs excreted in ML II or ML III urine samples can be differentiated from that in other oligosaccharidoses (6,7). Methods used in the routine lab for biochemical diagnosis can hardly make such diagnostic distinction. However, the excessive urinary excretion of SOSs continues to have a value of orientation toward the diagnosis. In



**FIGURE 103-4** Living inclusion (I)-cell type fibroblast observed in culture by phase-contrast microscopy (original magnification:  $\times 1000$ ). All cells in fibroblast strains derived from ML II or ML III skin biopsy specimens have a similar in vitro appearance with a juxtanuclear zone that encloses the ER and Golgi apparatus, free of dense granular inclusions. In cultures from obligate heterozygotes, clusters of “I-cells” are found among the normal-appearing fibroblasts (1). (Figure reproduced from Leroy, J. G. (2007) *Oligosaccharidoses, Disorders Allied to the Oligosaccharidoses*, Chapter 108, pp. 2413–2448, Figure 108-2, with gratefully acknowledged permission from the Publisher, Churchill Livingstone, Elsevier, Philadelphia, PA, USA.)

early infancy, interference by the smaller OSs present in any type of milk must be excluded.

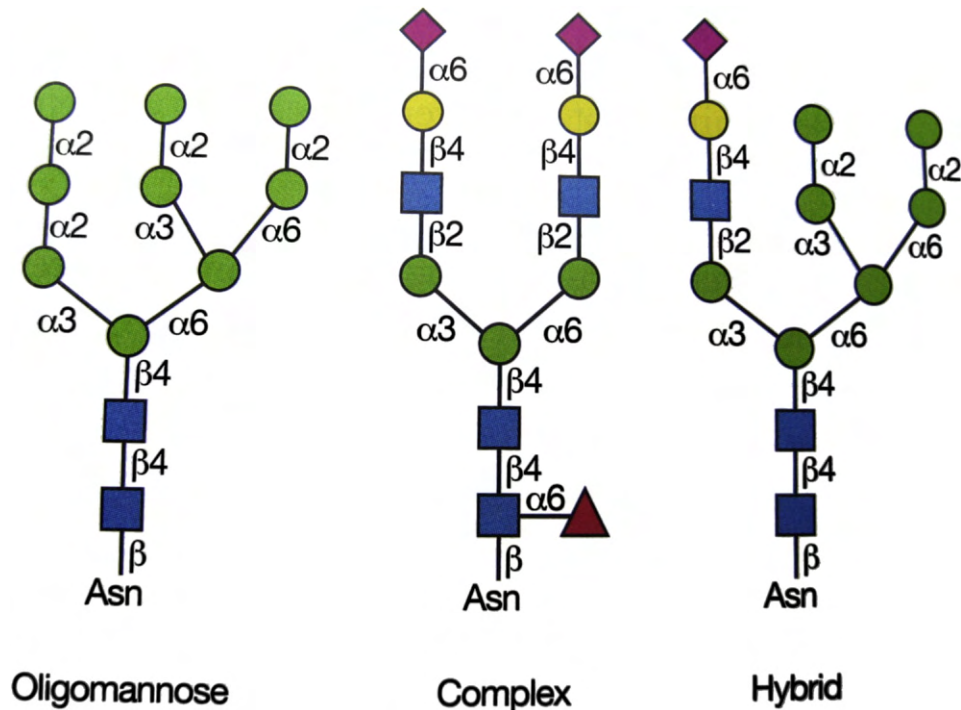
**103.1.4.3 From Lysosomal Enzymes to the Primary Metabolic Defect.** Finding the same primary enzyme deficiency in either disorder, albeit less complete in ML III patients, was the formal confirmation that pathogenesis and genetic cause of PHP and I-cell disease are similar and probably allelic. The “I-cell” phenomenon was found to be the consequence of faulty intracellular location and tissue distribution affecting many lysosomal hydrolases and to point to a defect of intracellular trafficking in both disorders. The lysosomal enzymes in plasma affected by the metabolic error and most often used in the laboratory of diagnostic biochemistry in support of the clinical diagnosis include N-acetyl- $\beta$ -D-glucosaminidase (EC 3.2.1.50),  $\beta$ -D-galactosidase (EC 3.2.1.23),  $\beta$ -D-glucuronidase (EC 3.2.31), arylsulfatase A (ASA) (EC 3.1.6.8),  $\alpha$ -D-mannosidase (EC 3.2.1.), and  $\alpha$ -L-fucosidase (EC 3.2.1.51). Other glycoproteins are known to be physically associated with the lysosomal and PM, among them  $\beta$ -D-glucosidase (EC 3.2.1.21) and acid phosphatase (EC 3.1.3.2), are barely if at all affected. Even the more moderately increased activity in plasma of either ML II or ML III obligate heterozygotes, relates to the underlying metabolic defect. The mutant enzymes egress from the cells into the ECM and cannot reenter, contrary to normal mature hydrolases that as phosphoglycoproteins (high-uptake type lysosomal enzymes) easily reenter by pinocytosis into any type of cultured fibroblast, including I-cells. Lysosomal enzymes in I-cells lack a common recognition marker essential for effective intravesicular routing to the lysosomal compartment and for uptake from the ECM. The carbohydrate nature of this recognition marker initially and the functional importance of its phosphate content subsequently were established as of crucial importance. In I-cells, phosphorylation of the oligomannosyl-type glycan in the hydrolases does not occur (ML II) or is much deficient (ML III). Lacking the phosphate-containing recognition marker is the reason that nascent I-cell hydrolases although catalytically active, cannot reach the intracellular lysosomal compartment nor enter cells from the ECM. I-cell lysosomal enzymes isolated from plasma or culture media are so-called low-uptake forms. Recent reviews on the progress in understanding the cause of ML II and ML III contain most references to the original reports (1,38).

Understanding the biosynthesis of the common recognition marker requires knowledge about N-glycosylation of lysosomal hydrolases that are initiated from the moment of their cotranslational insertion into the ER. The glycan precursor is first assembled on a dolicholpyrophosphoryl ER membrane-bound lipid carrier. Mutations that adversely affect the catalysis of any one of these many assembly steps each result in one of the morbid type I congenital N-glycosylation disorders (53). On completion in the ER, the OSs are transferred “en bloc” from the dolichol carrier onto some of the asparagine



(Asn) moieties (glycosylation sites) in the nascent proenzymes. At this stage of completed assembly, the glycan side chains on the immature hydrolases contain no less than 14 monosaccharide residues (Glc3Man9GlcNAc2-Asn). From this point on, the glycoproteins traverse the Golgi cisterns where sequential enzymatic modification takes place along two different pathways. One of the pathways results in N-glycan side chains of the complex type that are not phosphorylated and are unaffected by the ML mutations. The small amounts of normal hydrolases structurally matured along this pathway are either secreted into the ECM or have an incompletely known role within the PM. The larger part of the glycans in precursor enzymes matures along the alternate pathway into oligomannosyl-type OS side chains (Figure 103-5). Phosphorylation occurs as a two-step process in the cis Golgi stacks and involves first the transfer of GlcNAc-1-P to C6 in some of the terminal mannose residues producing initially *N*-acetylglucosamine (GlcNAc)-covered mannose-6-phosphate (M6P). This first step is catalyzed by the UDP-GlcNAc: lysosomal hydrolase GlcNAc-1-phosphotransferase (EC 2.7.8.17) for which the trivial name GlcNAc-1-phosphotransferase (GNPT) is adopted henceforth. This GNPT is completely inactive in ML II and significantly deficient in ML III. The second step that completes the synthesis of the M6P recognition

marker hydrolyzes the blocking GlcNAc and exposes the M6P marker. It is catalyzed by the microsomal, specific glucosidase named GlcNAc-1-phosphodiester  $\alpha$ -*N*-glucosaminidase (EC # 3.1.4.45). It is normal in ML II and in ML III. Mutations affecting this phosphodiester-glucosidase would probably result in either the ML II or ML III phenotype, but have not yet been recorded. This enzyme exerts its activity mainly in the trans-Golgi network (TGN) and it cycles between this TGN compartment and the PM. The uncovering of M6P occurs almost simultaneously with binding of the enzymes to the M6P receptors (MPRs) known to be located in the TGN membranes but also in the intracellular transport vesicles, the prelysosomal organelles, and in the PM. The binding into ligand-receptor complexes ensures the intravesicular transport of hydrolases to the prelysosomal compartment of the cell, where they dissociate due to the acid pH. The ligand hydrolases are delivered to lysosomes, while the MPRs recycle to the TGN membranes and do not enter lysosomes. In patients with I-cell disease or PHP, lysosomal enzymes lack the recognition marker, ligand-receptor complexes are not or barely formed and the segregation into lysosomes is not achieved. Instead in these patients, the abnormal glycoproteins leave the cells and appear in high quantity in body fluids or culture media.



**FIGURE 103-5** Symbolic representation of the most common N-glycans covalently attached to polypeptides at Asparagine (Asn) residues within Asn-X-Ser/Thr sequons, by  $\beta$  N-glycosidic bond. After enzymatic removal of some peripheral mannose residues, the oligomannose type N-glycans (left in figure) are phosphorylated (see Section 103.1.4) in order to acquire the mannose-6-phosphate (M6P) recognition marker of crucial importance for binding of lysosomal enzymes to the M-6-P receptors (MPRs) and ensuring entry into the lysosomal compartment. Unphosphorylated hydrolases lose more mannose residues and are substrates for stepwise synthesis of the complex (middle) and the hybrid type N-glycans (right side of figure). (Figure reproduced from Stanley, P.; Schachter, H.; Taniguchi, N. *N-Glycans* (Chapter 8, Fig 8.1). In *Essentials of Glycobiology*, 2nd ed.; Varki, A., et al, Eds.; CSHL Press: Woodbury, NY, 2009; pp 101–114, with gratefully acknowledged permission by editors and publisher.)

It should be pointed out that both pathways of OSs processing are not as completely distinct as presented above for didactic purposes. Hybrid forms of N-glycans are found on lysosomal hydrolases. However, their binding onto MPRs is considerably less effective than the adequately phosphorylated oligomannosyl-type OSs. Phosphorylation is actually blocking the Golgi-based  $\alpha$ -mannosidases. It has become clear also that the M6P marker has been found also on other glycoproteins that are not targeted to lysosomes. Hence, the M6P structure has other functions as yet incompletely understood (54).

**103.1.4.4 M6P Receptors.** Two distinct M6P receptors (MPRs) have been identified. Once purified, the structural characterization has been helped by cloning the corresponding cDNAs. Both receptors are type I integral membrane glycoproteins composed of an amino-terminal extracytoplasmic domain, a unique transmembrane region, and a cytoplasmic carboxy-terminal domain phosphorylated and palmitoylated at specific sites. One of the MPRs is a large, about 275-kDa protein mainly because of its large extracytoplasmic domain that comprises 15 contiguous repeats of about 147 amino acids each. The repeats have a similar tertiary structure. The other MPR has a molecular size of only 46 kDa and has in its much smaller extracytoplasmic domain. Only a single domain of 159 amino acids is homologous to the repeats in the large receptor. The small MPR requires the presence of cations for optimal ligand binding and is mainly present in its homodimeric conformation. It is called the cation-dependent M6P receptor (CD-MPR). The large receptor, also the most effective one, is the cation-independent receptor (CI-MPR) because it binds M6P marked glycoproteins effectively irrespective of the presence of cations. It adopts the homodimeric form for effective binding of M6P-bearing ligands. The latter occurs more specifically at the repeat segments 3 and 9 in each monomer. The CD-MPR has but one binding site per monomer. Either receptor complements the other one only partially. The absence of both is barely compatible with postnatal survival in MPR knockout mice (38,54,55). There is some specificity to ligand binding onto either MPR. The CI-MPR is a multifunctional molecule. It also binds insulin growth factor II (IGF-II) that does not carry M6P markers. Binding occurs at a single site, repeat N°13 in the monomeric form, and thus different from the anchoring sites for M6P-bearing glycoproteins. It is not clear at present whether or not binding of one type of ligand interferes in any way with the binding efficiency of another. The CI-MPR may have a role in regulating the amount of IGF-II available. Also, retinoic acid is a ligand to the CI-MPR at a site different from both the binding locations of M6P-marked glycoproteins or of IGF-II. One of the less well-defined functions of the large MPR is its role as a liver “tumor suppressor.”

M6P markers are also found on several nonlysosomal proteins, transforming growth factor beta precursor being one among them. The marker is lost from its

mature molecular form, but may target the precursor to the CI-MPR for activation. The function of several other of the M6P-marked proteins remains unknown at present (54).

### 103.1.4.5 Molecular Biology of GlcNAc-1-Phosphotransferase and of Its Genes.

**103.1.4.5.1 ML III $\gamma$ .** An important milestone toward the identification and characterization of the gene(s) encoding the GNPT enzyme has been reached by the successful purification of the bovine GlcNAc-1-phosphotransferase from lactating mammary glands late last century by the group of Canfield in Oklahoma City. The enzyme was found to be an oligoheteromeric protein complex composed of three different polypeptides, each represented in duplicate and represented symbolically as  $\alpha^2\beta^2\gamma^2$ . Original references are available in Reference (1). This result prompted success in identifying the orthologous gene(s) in the human species first with proving that mutations in the *GNPTG* gene (OMIM # 608838), mapped to human chromosome 16p13.3, are the cause of the variant ML IIIC (48), henceforth called ML III $\gamma$  (47), observed in three unrelated families of Druze and Muslim origin. The patients in all three families had consanguineous parents. All were homozygous for a cytosine insertion at nucleotide 500 in exon 7 that causes a shift of the DNA reading frame (FrSh) and the introduction of a premature stop codon (c.500InsC-exon 7→p. 167FrShX91). By the same study, the complete 1219 bp cDNA nucleotide sequence was made available. It predicted a mature protein of 305 amino acids comprising only two glycosylation sites. The gene contains 11 exons and spans about 11.4 kb of genomic DNA. A single 1.2 kb transcript (1221 bp) is detectable in all tissues but the lungs, where a transcript as large as 7.5 kb is found apparently due to alternate splicing and incomplete processing.

Identifying the most probable candidate gene causing the disorder in the families mentioned depended on the results of in vitro complementation studies in heterokaryons formed by pairwise fusion of two different ML III “I-cell” strains. The complementation of either strain resulted in the restoration of the intracellular activity of lysosomal acid hydrolases and of GlcNAc-1-phosphotransferase. Such studies had identified two complementation groups among the ML III patients. One of the groups did not show complementation with ML II-derived cells. The donors were initially called ML IIIA patients. The second group, considered “variant ML III,” was called ML IIIC. Fibroblasts from the latter group were found to be deficient in GNPT activity only when assayed with lysosomal enzymes as phosphate-acceptor natural substrates, but not when the  $\alpha$ -methylmannoside acceptor was used. Complementation group A turned out to be very deficient in GNPT activity irrespective of the type of substrate used. The original reports of results on this subject obtained in the 1970s and 1980s are referred to in the previous edition (1).

In the most recent years, approximately two-dozen different mutations have been characterized in the *GNPTG* gene. The initial impression of a higher prevalence of ML III $\gamma$  in people of Middle East origin is probably due to the inherent ascertainment bias by the first reports (48,49), as most recently Caucasian patients from various European countries have been reported (50,51,56–59). Attempts at comparing the prevalence of ML III $\gamma$  to that of ML III $\alpha/\beta$  are premature at this time. Either disorder is a rare inborn error of metabolism. As parental consanguinity is more often observed in reports on ML III $\gamma$ , it may indeed be the rarer entity of the two. It is of interest that all *GNPTG* mutations so far detected have occurred between the exons 4 and 9 and include among others single nucleotide insertions or deletions and larger molecular deletions, all resulting in the shift of the nucleotide reading frame and in a premature stop signal further downstream. MS mutations and several interesting splice-site mutations have been reported as well. Some of the latter result in exon skipping and/or retaining the introns. Some mutations barely affect the normal level of *GNPTG* mRNA, while others expose the mRNA to nonsense-induced degradation. Details on the ML III clinical syndrome are rarely provided in papers concentrating on the results of *GNPTG* mutation screening. Nevertheless, it is clear that not a single patient with ML II has been described to be associated with a mutant *GNPTG* genotype. Even patients with a so-called intermediate ML syndrome have not been observed. ML III $\gamma$  appears to be a clinically similar, on average probably a somewhat milder disorder than ML III $\alpha/\beta$ . There is a wide clinical spectrum for either. The examples of intra-familial variability in some ML III $\gamma$  families are most striking (49). Contrary to what will be concluded from the *GNPTAB* genotype–phenotype correlation studies (next paragraphs), the phenotypic consequence of any homozygous or compound heterozygous *GNPTG* genotype appears to be of the later onset, milder morbidity type ML III irrespective of mutation nonsense or MS type. This conclusion is in support of the view that the  $\gamma$  subunits in the GlcNAc-1-phosphotransferase protein complex play an incompletely known role, secondary to the catalytic role assigned to the  $\alpha$  and  $\beta$  subunits. One can but wonder whether reports on mutations nearer the 5' or the 3' end of the gene will require adapting the current conclusion about ML III $\gamma$ .

**103.1.4.5.2 ML II and ML III $\alpha/\beta$ : Allelic Disorders.** Both the 5597bp cDNA, the reverse transcribed product of a 6.2kb  $\alpha\beta$  transcript, and the genomic DNA encoding the  $\alpha\beta$  gene were cloned in 2005 by the Canfield group (60) and independently in the same year by Tiede et al. (61). The human gene mapped to chromosome 12q23.3 was shown to contain 21 exons and to span 85kb of gDNA. The prediction that it encodes the precursor of both  $\alpha$  and  $\beta$  subunits in the GlcNAc-1-phosphotransferase protein complex, a protein of 1256 amino acids, was confirmed. Following its translation

into an  $\alpha\beta$  precursor polypeptide of 144kDa with two transmembrane domains, the  $\alpha$  and  $\beta$  subunits are generated by proteolytic cleavage at the lysine 928-asparagine 929 peptide bond. The larger N-terminal  $\alpha$  subunit contains 928 amino acids and the shorter  $\beta$  subunit represents the C-terminal part of the precursor and has 328 amino acids. There are 17 potential N-glycosylation sites in the  $\alpha$  subunit and 3 in the  $\beta$  subunit. In addition to arguments from comparative protein biochemistry, the finding that in vitro transfection experiments with  $\alpha\beta$  precursor cDNA with or without the  $\gamma$ -subunit precursor cDNA resulted in a 17- or 3-fold increased expression of GNPT activity, made it highly likely that the  $\alpha\beta$  precursor cDNA encodes the important catalytic domain of the transferase complex.

Since 2005, the mutant *GNPTAB* genotype in well over 150 patients with either ML II or ML III $\alpha/\beta$  has been reported. All expected types of mutations have been detected by ever more effective mutation screening molecular methodology. They may be subdivided into two groups according to the biological implications. The first group included nonsense (NS) mutations with generation of a stop codon, and microdeletions (del), microinsertions (ins), or duplications (dup) involving a single or only few nucleotides, all resulting in the shift of the nucleotide-reading frame and bringing about a premature stop codon downstream of the mutation site. The members of this mutation group are often called “null” or “amorph” because there is no residual function of the gene products encoded. The second group of mutations comprises MS mutations and the intervening sequence splice-site mutations at or near the intron-exon boundaries. The effect of these mutations is more variable and quite dependent on the precise location and the physicochemical nature or the degree of phylogenetic conservation of the amino acid in question. The effect and the mutations are called “hypomorph” or “leaky” as some residual wild-type function may still be maintained (43,56,59,61–70). In several of the reports cited, the challenging differentiation between MS mutations with morbid effect and the large number of nucleotide polymorphisms has been adequately addressed.

Only occasionally, a larger or more extreme structural alteration of the *GNPTAB* gene has been observed. In five patients in the cohort reported from Japan, the duplication of exon 2 also with frameshift effect has been found in five unrelated patients (68). One structurally aneuploid allele has been explained by the insertion of an ALU sequence, obviously with “null” effect (71). The large structural rearrangement in one allele in a compound heterozygous ML II patient is the sole incompletely resolved defect in the “core” group of the cohort of 61 ML II and ML III $\alpha/\beta$  probands reported by Cathey et al. (43).

Some general conclusions may have already been drawn from the reports on *GNPTAB* mutation screening that represents the major parts of the world. They

confirm that both ML II and ML III $\alpha/\beta$  occur worldwide as rare disorders with a combined prevalence of well below  $15 \times 10^{-6}$  (72) (references in Reference (37)). A most interesting exception is the prevalence of 1/6184 births (carrier rate estimate: 1/39) in the Saguenay–Lac St. Jean region of Quebec. The two-nucleotide deletion 3503\_3504 delTC, known to represent the most frequent mutant *GNPTAB* allele in patients of European descent, is the sole cause in all MLII patients diagnosed in that part of Canada during the last decades. The authors document evidence in support of the founder effect of past centuries and of the population structure with high-distant inbreeding coefficient that explains the extraordinary prevalence figure (67).

In over 95% of either the ML II or the ML III $\alpha/\beta$  patients reported, both *GNPTAB* mutations have been identified. In none of the few bonafide exceptions where no mutation could be revealed, *GNPTG* mutation screening did not find any mutation either. Not a single instance among many dozens of ML II patients has been associated with any *GNPTG* mutant genotype. Hence, ML II $\alpha/\beta$  may be too redundant a term (47) and ML II may remain the more appropriate designation of “I-Cell” disease.

Clearly, the clinically hardly distinguishable entities ML III $\alpha/\beta$  and ML II  $\gamma$  are nonallelic. It is possible, though rather unlikely that the latter would be more prevalent than the former in the countries of the Middle East. More data from everywhere are awaited in order to settle this issue.

Mutation screening is the most effective way to reach formal confirmation of the diagnosis. The abnormal ratio between the specific activity of many lysosomal hydrolases in plasma and cultured fibroblasts does not differentiate ML II from either ML III $\alpha/\beta$  or ML III $\gamma$ , but strongly orients the clinician toward anyone of these disorders. Assay of GNPT requires special laboratory expertise and methods and is not routinely available in the laboratories of clinical biology.

Mutations in the *GNPTAB* gene have been detected in nearly all exons and in many exon-intron borders. The mutation spectra reported from various locations in the world are congruent with only a few exceptions. The mutation c.3503\_3504del CT is by far the most often detected. The frequency reached between 40 and almost 50% of all mutant *GNPTAB* mutant alleles. The report from Japan shows that that country of the Far East the NS c.3565C>T→p.R1184X mutation was found in over 40% of morbid mutant alleles detected in 40 patients. It was found in 8/25 ML II subjects (68). The phenotype of the patients, compound heterozygous with this mutation, depends on the type of the accompanying mutant allele. Its high frequency in Japan may be due to a founder effect. The c.3565C>T NS mutation has been found in much lower frequency in studies representing the countries of the United States, South Korea, Israel, and western Europe respectively (43,64,66).

All results show that the type of *GNPTAB* mutation more than its intragenic location determines the phenotypic implication. This is at variance with the conclusion drawn in the previous section: Neither type of mutation nor intragenic location seems to matter in the *GNPTG* gene. ML III $\gamma$  is the consistent clinical result. However, the intrafamily variability in ML III $\gamma$  remains unexplained.

The large *GNPTAB* genotype–phenotype correlation study by Cathey et al. (43) confirms the consistent findings in previous studies with less clinical details on the cohorts of patients studied (56,57,61,62,64,66,68,70). The rather homogeneous phenotype ML II is directly and strongly correlated with *GNPTAB*-mutant genotypes homozygous or compound heterozygous for two alleles expected to produce no or nearly no *GNPTAB* precursor polypeptide: nonsense and reading frameshift inducing mutations. Premature truncation of either  $\alpha$ - or  $\beta$  subunit in the GBNPT complex is likely to abolish all encoded enzyme activity. Absence or near inactivity of GlcNAc-1-phosphotransferase has been documented in patients with mutant genotypes composed exclusively of “null” or “amorph” mutations (62,64).

The presence of at least one hypomorph allele, MS, or mRNA-splicing mutation, appears to protect against the ML II outcome and consistently results in ML III  $\alpha/\beta$ . The hypomorph allele yields either poorly effective enzyme or insufficient amounts of it and hence much reduced activity (usually between 1 and 10% of normal GNPT) in the ML III $\alpha/\beta$  patients (43,62). Patients with ML III $\alpha/\beta$  associated with two hypomorph *GNPTAB* mutant alleles do not consistently have a milder clinical syndrome than the ones with only a single MS or splice-site mutation in the compound heterozygous genotype. Unfortunately, it is most often in the former type of patient that the clinical onset and the diagnosis may be delayed at least until adolescence. The morbidity, especially the painful hip disease, although sometimes somewhat postponed, becomes equally severe.

The several recent ML studies on large cohorts of patients support the view that the phenotypic spectrum of the GlcNAc-1-phosphotransferase deficiency disorders is at least dichotomous rather than continuously variable (43,56,62,66,68,70). There is still the need for more corroborating data of the contention that the complete lack of GNPT is associated with ML II and some residual activity with ML III $\alpha/\beta$  as the morbid result.

Admittedly, the clinical features in a small minority of ML patients have an intermediate phenotype because they tend to straddle some of the clinical and radiographic criteria set for delineation of the reference ML types, while taking the known variability into account. This small group is likely to be still genetically heterogeneous. In order to discern them, not only the specific mutant genotype but also much detailed information on the clinical features is required (43). The existence



of intermediate phenotypes supports the direct correlation of genotype–phenotype in ML and strengthens the idea of the discontinuous phenotypic spectrum instead of weakening it.

**103.1.4.6 Treatment/Management in ML II and ML III; Animal Models.** Attempts in the last two decades at treating LSDs by allogeneic hematopoietic stem cell transplantation (HSCT) (initially bone marrow transplantation (BMT); more recently cord-blood-derived stem cells) from HL-A compatible donors have met with variable and partial success. Best-known examples of the latter include Fabry disease, Gaucher disease type 1, and Pompe type glycogenosis type 2, and were followed by interesting results in the milder variants of MPS I and MPS VI. Enzyme replacement therapy (ERT) that does not cross the blood–brain barrier, has also brought impressive results to the former group of LSDs, but met with more limited successes in the MPS storage disorders. Few patients with ML II and probably less with ML III $\alpha$ / $\beta$  or ML III $\gamma$  have been subjected to HSCT, still fewer if any to ERT. Even in the few ML II patients who had HSCT in the course of infancy, the treatment known to be challenging to the affected, the hospital staff, and the family, has failed to show any beneficial effect either to the symptoms or the clinical course. The known aspects of the disorder’s pathogenesis most probably preclude any effect by substituting lysosomal hydrolases. Moreover, the likely secondary damage inflicted on the ECM in hard (osteopenia) as well as soft connective tissue (contractures), manifest most early in ML II, and initially less evident but inexorably progressive in the longer-surviving ML III $\alpha$ / $\beta$  or ML III $\gamma$  patients, awaits and requires molecular elucidation before any effective therapeutic approach will become feasible.

Treatment of manifestation issues and medical and routine surgical management are presented in detail elsewhere (37,73). ML II in particular and also the older patients with either ML III $\alpha$ / $\beta$  or ML III $\gamma$  represent serious challenges to the anesthesiologist as both intubations for surgical intervention and detubation are difficult and hazardous. Therefore, any type of surgery should be avoided as much as possible. The airway is smaller and because of mucosal thickening, more marrow than that in age-matched control children. The tracheal wall is stiffer than in normal subjects. Moreover, the swollen mucosae of mouth and pharynx, the enlarged tongue, and the limited extension and flexion in the cervical spine are additional difficulties. Clinical examination of the patient by the anesthesiologist and personal contact with the parents before the procedure is mandatory. The initial diagnosis in some patients with ML II was craniosynostosis and some of them have undergone corrective surgery as treatment. Craniosynostosis is not a feature of ML II.

The osteochondrodystrophy responsible for early growth failure, dysmorphic changes, and much impaired mobility in ML II is milder and more slowly progressive

in ML III. However, in ML III, locomotion becomes increasingly difficult and painful from late childhood because of serious hip disease. Not only joint pain, but also bone pain associated with worsening osteopenia in the progressively more wheelchair-bound ML III adolescent is a consistent complication. Cyclic intravenous (IV) bisphosphonate treatment may reduce the pain and osteopenia caused by excessive bone resorption. This symptomatic treatment has its beneficial effect after a few monthly injection cycles, but must be at least initiated in special treatment centers, where doctors may adequately assess limitations and potential complications of the treatment. The latter is hardly relevant in ML II patients (37,73).

The available animal models are ideally suited to study new therapeutic approaches prospectively. However, until now and in the near future, in vivo and in vitro studies of animal models have been and will be of more immediate importance for improving understanding of the pathogenesis in the mucopolidoses. Heuristic examples with interesting potential include the natural feline ML II model (74) and the knockout mutant mouse models with abolished function of either the *GNPTAB* or the *GNPTG* gene (75,76). The cats with ML II have both clinical picture and course that are congruent to human ML II, including its complications and early, though somewhat variable, timing of fatal outcome. Birth weight in the kittens is within normal limits but growth and weight gain are deficient. The in vitro “I-Cell” phenomenon including both the morphologic and the enzymatic features is congruent to that in human “I-Cell” cultures. Frank blindness is a rare sign in human ML II, except occasionally in the longer-surviving patient. In the absence of signs of decreasing eyesight, the ophthalmologic examination in ML II does not include advanced electrophysiological studies. The affected animals have poor muscle tone as have the affected infants. Progressive ataxia in the cat model has not been reported in the human ML II patient, where it may however help explain that very few children with ML II achieve unaided ambulation. The interesting radiographic features of osteochondrodystrophy should kindle prospective research on the possibly transient skeletal features and hyperparathyroidism in neonatal and young affected kittens. These features were described in the clinical paragraphs of this section (42,44). Proof that the feline model involves indeed the orthologous *GNPTAB* gene has not yet been provided (74).

The “knockout” *gnptab* mouse was obtained by the treatment of mouse embryonic stem (ES) cells with the gene-trapping retroviral vector that had inserted itself in intron 1 of the gene with the effect of truncation of the  $\alpha\beta$  precursor protein behind amino acid 39, out of the normal 1256. The directed gene deletion approach eliminated exons 4–11 of *gnptab* with consequently a much truncated polypeptide from amino acid 59. The ES cell strains were then used to generate mice heterozygous

for the structural ablation of the *gnptab* gene. The latter were inbred in order to generate homozygous offspring.

The specific phenotypic consequences of mice lacking *gnptab* gene function include small size at birth and slower growth rate, severe retinal degeneration with numerous depigmentation spots and attenuated retinal vessels, and markedly hypertrophic tracheal chondrocytes with abundant microvacuoles. Apparently, retinal development is normal in *gnptab*<sup>-/-</sup> mice, but sustaining histology and function of photoreceptor cells is soon deficient. Cytoplasmic inclusions were not seen in fibrocytes and fibroblasts in all tissues examined. However, in secretory cells of several exocrine glands, pancreas and parotid glands among them, tissular disarray and cytoplasmic vacuolization were found in acinar cells. Disrupting the *gnptab* gene completely abolishes phosphorylation of high mannose OSs in acid hydrolases, whereas the knockout of the *gnptg* gene does not affect the size at birth and does not produce animals that have blindness by 5 months of age. Phosphorylation of OSs in lysosomal enzymes is only partial. The  $\alpha$  and  $\beta$  polypeptide subunits in the murine GlcNAc-phosphotransferase enzyme must have adequate power to recognize the lysosomal hydrolases as specific substrates. The  $\gamma$  subunit enhances and/or maintains the transient substrate binding (75–77). Recent reports about in vitro studies of human mutant fibroblasts point at the balance that appears to be held between  $\alpha$  and  $\beta$  subunits on the one and the  $\gamma$  subunit on the other hand. Mutations in either one enhance the presence of the unaffected enzyme component(s) (59,78,79).

Recently, a zebrafish ML II model has been developed by inhibiting *GNPT* expression with translation blocking or splice blocking specific antisense morpholino oligonucleotides. Morphant zebrafish embryos manifest craniofacial defects, impaired motility, abnormal otolith, and pectoral fin development. Altered timing and localization of both type II collagen and Sox9 expression in the craniofacial region of the embryos suggest accelerated chondrocyte differentiation. Excessive accumulation of collagen II is also noticed in the misshaped cartilage tissues. Interpreting the findings and comparison with the slowly progressive features in mammals is hardly possible. The model may point at the protective power exerted by the maternal heterozygous organism during pregnancy against possible adverse effects of human *BNPTAB* mutant genotypes in early stages of embryonic and/or fetal development in mammals (80).

### 103.1.5 $\alpha$ -Mannosidosis

Alpha-Mannosidosis due to deficient lysosomal  $\alpha$ -D-mannosidase (EC 3.2.1.24) among the more slowly evolving oligosaccharidoses has been described first in 1967 by the Swedish physician, Öckerman, in a 4-year-old boy with dysmorphic facial features reminiscent of the ones in Hurler disease, already better-known at

the time. The phenotypic differences among patients, sometimes even within a single sibship, have resulted in controversy about the terminology and the need for delineation of various clinical types within the rather continuously variable spectrum.

$\alpha$ -Mannosidosis is a rare inborn error of metabolism with worldwide prevalence below  $3 \times 10^{-6}$  and with most often a very slowly progressive course and moderately reduced life expectancy. The elevated urinary excretion of mannose-rich OSs and normal excretion of GAGs, only of orientation value to the diagnosis, warrants the disorder's spot among the oligosaccharidoses.

**103.1.5.1 Clinical Phenotype(s).** Controversy about phenotypic delineation relates more to differences in the age of onset and clinical course than to the component symptoms in any fully developed, rather consistent phenotype. Here, the early childhood-onset  $\alpha$ -mannosidosis is termed type I. The clinical course, either short in number of years or quite prolonged, defines the subdivision into types IA and IB, respectively. The late-childhood or adolescent-onset disease is called type II.

The neonatal and infancy period is uneventful in  $\alpha$ -mannosidosis, although a small minority of patients may have congenital equinovarus deformation of the feet and/or may develop hydrocephaly during infancy (81). Instead, neuromotor development is often within normal limits throughout infancy. On average, the milestone of unaided walking is reached with moderate delay. Facial features are coarsening gradually but never reach the severe characteristics known in Hurler disease (MPS IH). The head circumference is large, the forehead prominent, the eyebrows more rounded than normal, and the nasal bridge flattened. Over the years, the enlarging tongue, the wide spacing between the teeth, and some prognathism complete the set of gradually coarsening facial features. The corneae are clear. The delay of speech development, more often than the mildly deficient psychomotor development, is the initial reason for parental concern. Mild hypotonia is a common finding. Joint mobility is only minimally impaired. Fine motor skills remain subnormal and render the patients rather clumsy. Statural growth is most often within normal limits. The clinical description of type IA  $\alpha$ -mannosidosis is thus complete, except for the fact that patients of any subgroup are very prone to respiratory infection and otitis (82). Some immunodeficiency, as yet insufficiently defined, has been documented in  $\alpha$ -mannosidosis (see Section 103.1.5.2). Patients of type IA have been labeled type III in a recent excellent survey (81). Patients grouped here under IB (type II in Reference (81)) may have a less clearly defined, yet early childhood onset. Their overall metabolic syndrome hardly differs from that in subjects belonging to group IA. However, as they do not succumb to the recurrent infections, they run a long clinical course and thus allow the observer to record more late-onset symptoms as is the case of type II patients.

OCD of the skeleton of the dysostosis multiplex type may be mild to moderate. Therefore, it is often not recognized early in the clinical course of the patient without deficiency of statural growth unless clinical kyphoscoliosis prompts the need for radiographic evaluation. The skeletal survey in later childhood shows the thickening of the calvarium, some vertebral dysplasia, narrowing of the basilar parts of the ilia, and slightly widened diaphyses of the short tubular bones in hands and feet (39). Genu valgum deformation, sometimes requiring epiphyseal arthrodesis, is common also in the young  $\alpha$ -mannosidosis patient. Patellar dislocation, often bilateral, is another regularly occurring complication in the knee regions that often requires surgical intervention (83). In either subtype, especially in type IB as well as in the late-onset  $\alpha$ -mannosidosis type II, neurosensory hearing deficit or frank deafness is formally diagnosed although it is not consistently the initial parental complaint except if recurrent bouts of otitis receive professional attention and treatment early in the clinical course (81,84).

Type II  $\alpha$ -mannosidosis has its observable clinical onset beyond the age of 10 years and is often named the clinically mild form. It is called type I in the recent survey earlier referred to in (81).

Intellectual impairment is common to all types of  $\alpha$ -mannosidosis and can be confirmed as soon as psychometric testing becomes feasible. The cognitive deficit is usually mild, only occasionally pronounced. Affected sibs can be much discordant regarding intellectual ability. In all long survivors including the “mild” type II patients, the deficit worsens very slowly over the next decades. Deficient speech remains a consistent and prominent problem as it is resolved only partially by sustained speech therapy. Moreover, declining receptive speech is noticed in the longer survivors. Physical mobility decreases due to persistent or increasing muscle weakness, arthritic and even destructive polyarthropathy (85,86), and the ataxia, apparently associated with generalized cerebral and cerebellar atrophy, features probably not directly related to demyelination (87). In the older patients especially, retinal dystrophy may be shown to be the reason of declining vision (88).

Attention needs to be drawn to the psychiatric signs and symptoms that are significant in a quarter of the adult patients with  $\alpha$ -mannosidosis. Because they are intellectually disabled, recognizing the early stages of this complication is difficult. They can already appear in adolescent patients. The behavioral changes, signs of confusion, depression, and hallucinations tend to occur in episodes of several weeks and are followed by periods of hypersomnia. The psychiatric problems may precede the decline or in some instances the temporary loss of acquired skills (84,89).

The more important differential diagnoses relevant to  $\alpha$ -mannosidosis in the longer surviving patients include CDS and even JNS (see Section 103.1.1), the early onset but long-surviving patients with galactosialidosis (see

Section 103.1.3), ML III $\alpha$ / $\beta$  and ML III $\gamma$  (see Sections 103.1.4.1.2 and 103.1.4.1.3), and obviously the patients with one of the more dysmorphic GAGs storage disorders such as MPS I HS (MIM # 607015) and I S type (MIM # 607016) and also the mild MPS VI (MIM # 253200).

**103.1.5.2 Cyto- and Histopathology.** The vacuolization in peripheral lymphocytes is prominent and more easily demonstrable in  $\alpha$ -mannosidosis than in the other LSDs, but qualitatively not different in the light microscope. In liver tissue, numerous clear vacuoles are seen in hepatocytes and in K  pffer cells. Such cytoplasmic inclusions are found also in bone marrow histiocytes, in endothelial cells of sinusoid vessels, and in lymphocytes in spleen and lymph nodes. The cytoplasm of neurons is distended throughout the CNS. It is packed abundantly with single membrane-bound vacuoles. The latter appear rather clear by EM as they contain but sparsely dispersed reticulogranular material. The severe morphological changes in hepatocytes and in neurons are due to accumulation of abnormal amounts of mannose-rich OSs (see original references in Reference (1)). The accumulation in lysosomes of the specific storage material itself cannot fully explain the pathogenesis of the disorder. Recurrent infection in the first decennium of life contributes significantly to the disorder’s morbidity. Its fatal outcome in childhood is the main point of difference between the clinical Types IA and IB. Decades ago, impaired leukocyte chemotaxis and decreased phagocytosis were documented in a type IA patient. Studying the matter of probable immunodeficiency in  $\alpha$ -mannosidosis is hard, given the paucity of patients and the dependence of subjects at a different age and stage of disorder. In one study of humoral and cellular immunity in six patients, postimmunization levels of antibody were lower in patients than in age- and sex-matched controls. Moreover, a serum factor with phagocytosis-inhibiting effect was detected only in the plasma of the patients (90). Plasma oligosaccharides comprising five and six mannose residues bind to the receptors of interleukin-2 (IL-2) and thus interfere adversely with IL-2-dependent immune responses, such as activation of T-, B-, and NK cells. The blocking of this receptor is probably contributing to the immunodeficiency in patients with  $\alpha$ -mannosidosis (81,90). Autoimmune diseases are apparently more prevalent in mannosidosis patients than in the unaffected population (81,91). In a mouse model, reduction of the degree of N-glycan branching in the complex type glycans induces an autoimmune disorder that resembles human systemic lupus erythematosus (SLE) with the induction of anti-nuclear antibodies (92).

**103.1.5.3 Chemopathology, Enzyme Deficiency, and Diagnosis.**  $\alpha$ -Mannosidosis is an OS because of the excessive urinary excretion of mannose-containing OSs of small molecular size such as Man( $\alpha$ 1 $\rightarrow$ 3)Man( $\beta$ 1 $\rightarrow$ 4)GlcNAc; Man( $\alpha$ 1 $\rightarrow$ 2)Man( $\alpha$ 1 $\rightarrow$ 3)Man( $\beta$ 1 $\rightarrow$ 4)GlcNAc, and Man( $\alpha$ 1 $\rightarrow$ 2)Man( $\alpha$ 1 $\rightarrow$ 2)Man( $\alpha$ 1 $\rightarrow$ 3)Man( $\beta$ 1 $\rightarrow$ 4)

GlcNAc. Other OSs are less abundantly present (see [Figures 103-1 and 103-5](#)). All compounds have GlcNAc at the reducing end (see original references in Reference [\(81\)](#)). These findings are the consequence of the deficient exoglycosidase function of lysosomal  $\alpha$ -D-mannosidase that normally catalyzes the hydrolysis of the mannosidic linkages  $\alpha(1\rightarrow2)$ ,  $\alpha(1\rightarrow3)$  and  $\alpha(1\rightarrow6)$ , present in both the high mannose and the hybrid-type glycan side chains in lysosomal proteins. More refined analytical chemistry of the excreted compounds allows relating the mannose-rich metabolites still more specifically to the metabolic defect at hand [\(6,93\)](#).

The diagnosis of  $\alpha$ -mannosidosis is based on the demonstration in tissues or body fluids of the severe deficiency of lysosomal acid  $\alpha$ -D-mannosidase (LAMAN) with optimal specific activity near pH 4.5. Precautions against  $\alpha$ -mannosidases active at more neutral pH but still interfering with the assay are relevant when artificial enzyme substrates are used. In human plasma, the lysosomal acid enzyme is much less abundant than an “intermediate” also called “neutral” form of  $\alpha$ -D-mannosidase. The former is thermostable and the latter, most active at pH 5.5–6.0, is thermolabile at 56°C. Hence, plasma lysosomal  $\alpha$ -mannosidase can be assayed at its pH optimum of 4.6 most reliably only after thermoinactivation of samples following dialysis against phosphate-buffered saline. In leukocytes or cultured fibroblasts, the enzyme activity is preferably assayed at pH 4.2. Under this condition, the interference by neutral isoenzymes is negligible. Prenatal diagnosis of  $\alpha$ -mannosidosis has been successfully made by showing severe deficiency of acid  $\alpha$ -D-mannosidase activity in cultured amniotic cells or in uncultured chorionic villi (see original references in [\(1\)](#)).

**103.1.5.4 Molecular Biology of  $\alpha$ -D-Mannosidase and Its Gene: Animal Models.** The gene *MAN2B1* (OMIM # [609458](#)) encoding LAMAN (EC 3.2.1.21) has been mapped to chromosome 19 (19pcen-p13.1), spans 21.5 kb of genomic DNA, and is composed of 24 exons. The *MAN2B1* mRNA of about 3.5 kb has slightly different lengths as at least two transcription initiation sites have been identified. Gene expression is ubiquitous, but most prominent in lung, kidney, pancreas, and leukocytes in peripheral blood (see original references in References [\(1,81\)](#)).

LAMAN has been purified and its amino acid sequence completely characterized. The nascent enzyme is synthesized as a single precursor protein. It comprises a 48 amino acid-containing signal peptide at the N-terminal end. On arrival in the lysosomal compartment, the pre-enzyme, a single polypeptide of 962 amino acids (molecular mass: 108.6 kDa), is split enzymatically into three glycopeptides, called A, D, and E, starting from the N-terminal, of sizes 70, 42, and 15 kDa, respectively. The largest among them representing the precursor's N-terminal part, is itself proteolyzed further into three peptides, named A, B, and C, that remain joined by disulfide bridges. The

amino acid sequence of human LAMAN is completely known as it was deduced from PCR-generated cDNA (3030 bp open reading frame) from lung tissue and from cultured fibroblasts. A high degree of similarity has since been confirmed between the human LAMAN amino acid sequence and the orthologous gene product in cattle, cat, and guinea pig. The mature enzyme, in fact a complex of five interacting polypeptides, has undergone extensive molecular folding in the ER, thus rather early in the course of its intravesicular trafficking toward the prelysosomes. Each one of the A, B, and C polypeptides contains one N-glycosylation site occupied by an oligomannosyl-type OS that already has acquired the M6P before leaving the Golgi apparatus. Both the D- and the E-peptide comprise several Asn-glycosylation sites for potential occupation by a complex type of OS.

Over 40 morbid mutations have been reported in the human *MAN2B1* gene, more than half of them being the MS or NS type. At present, nine small deletions and insertions with shift of reading frame as molecular effect have also been detected. Only one large deletion, but no gross rearrangements of the gene have been found. Eight different splice-site mutations are also known [\(94–101\)](#) (consult also earlier original references in References [\(1,81\)](#)).

In disorders with autosomal recessive inheritance, the homozygous or compound heterozygous mutant genotype is expected to be the major determinant of the clinical phenotype rather than the separate effect of either mutant allele. Correlating mutant genotype and resulting  $\alpha$ -mannosidosis phenotype remains challenging for several reasons. Firstly, defining and delineating different phenotypes within a probably continuous clinical spectrum of variation is a difficult assignment, irrespective of the terminology adopted or adhering to. The labels “severe” and “mild” are inadequate, if only the age of onset is taken into account. Clinical complications often determine the patient's fatal outcome. There are few, if any, follow-up reports on patients in adulthood. Secondly, often insufficiently detailed information on the clinical phenotype accompanies reports on causal mutations. Thirdly, the phenotypes in several animal models that continue to be very helpful in understanding pathology and molecular biology aspects show heterogeneous phenotypic expression that is consistently severe in cattle and in cats, and much milder in the guinea pig model. Finally, the clinical difference among affected sibs remains unexplained and may show that environmental and/or genetic factors outside *MAN2B1* mutations also contribute.

The mutations with morbid effect are scattered over the entire *MAN2B1* gene. Except for the MS Arg750Trp (p.R750W) mutation so far most frequently found, most mutations occur in only a few families or are entirely “private.” In homozygous patients, neither the type nor location of the mutations within the gene correlates directly with the clinical phenotype. Even when the amount of residual acid LAMAN activity in leukocytes



or cultured fibroblasts is taken into account, attempts to correlate the severity of the patient's disorder directly with the mutation's location within the linear sequence of the wild-type enzyme fail. However, more recent work supports the conclusion that taking into account the quaternary molecular structure of LAMAN itself, the result of complex molecular folding processes during its transient stay in the ER, is helpful in predicting the residual catalytic function of the mutant enzyme and ultimately the severity of the resulting phenotype especially in homozygous mutant subjects. Comparison of the stereochemical role of the wild-type amino acids to that of the mutant ones encoded by MS mutations in various component peptides identifies mutations that destroy the catalytic site of LAMAN and discerns them from substitutions well outside the active site. The efficacy of the intracellular trafficking of any mutant enzyme is another criterion on which the genotype–phenotype correlation may be built. By various in vitro site-directed mutagenesis experiments, Hansen et al. (97) have introduced 11 MS mutations into human LAMAN cDNA. The wild-type human enzyme synthesized as a 120-kDa precursor was both the target and the reference in these experiments. The resulting mutant proteins were expressed in COS cells (some of them in Chinese hamster ovary cells as well). The enzymes encoded by the mutants p.T355P (exon 8, polypeptide B), p.P356R (ibidem), p.W714R (exon 17, D), p.R750W (exon 18, D), p.L809P (exon 20, D), or by two deletion mutants, respectively, were misfolded, arrested in the ER, and functionally inactive. The LAMANs produced by six other mutations were transported to the prelysosomes and had either less than 5% (H72L; D196E; D196N; R220H) or more than 30% of the normal specific activity: E402K (exon 9, polypeptide B); F320L (bovine exon 3, polypeptide A). Modeling the mutations into the three-dimensional reference structure showed that the mutants with much reduced enzyme activity had substituted amino acids that directly partake in the LAMAN active site as Zn<sup>++</sup>-coordinating amino acids (His72, Asp 196), stabilizing the active-site nucleophile (Arg220) or positioning the active-site residue (Asp319, Phe320) (97). Most MS mutations resulted in misfolding and ER retention. Hence, more amino acids are involved in “wild-type” protein folding in the ER than in direct participation in the catalytic site of LAMAN. Predicting the clinical phenotype is to be more reliable while dealing with genotypes composed of mutations with better biochemical and molecular characterization.

The recent, still more comprehensive homology modeling study of the wild-type and inherited mutations of LAMAN in four species including man, cow, cat, and guinea pig reveals a significant correlation between the mutant genotype and the  $\alpha$ -mannosidosis phenotype (96). The study was based on the premise that the overall protein structure is better preserved during evolution than its linear primary amino acid sequence. This applies

even more to oligomeric proteins, in which ER folding insures that several component peptides contribute one or more amino acids to the enzyme's active site and that several other residues at different sequence locations in the linear precursor protein are determinants of the “wild-type” outcome by the folding process itself. Clearly, homology modeling involves generating three-dimensional structural models of complete mature wild-type bovine LAMAN to serve as a reference model and aligning any target sequence with that in the template or reference structure. Mutations, although scattered over the entire *MAN2B1* gene, seem to be clustered in five mutational hotspots including from the 5' to the 3' end, the following nt sequences: 157–323; 562–679; 961–1204; 1383–1815; and 2124–2746. Each of these regions harbors indeed most of the mutations described in the reports on human  $\alpha$ -mannosidosis in the reports referred to earlier. This approach of comparative collocating the mutations in orthologous positions in different mammals and including the proximity criterion to the enzyme's active site provides better molecular understanding of phenotypic variation and improves predicting the probability of morbid effect in the human species.

**103.1.5.5 Enzyme Therapy; Relevance of Animal Models.** There is still a paucity of well-documented reports on LAMAN treatment, either by allogeneic HSCT or by IV-ERT, in humans with  $\alpha$ -mannosidosis. In particular, more long-term follow-up data about the treatment effects in patients reported previously are required. One report on HSCT applied in four patients with early-onset  $\alpha$ -mannosidosis, in whom slowing neurocognitive development and sensorineural hearing loss had been of major concern, included an evaluation at 1–6 years after the transplant (102). Normalization of leukocyte LAMAN activity, stabilization of cognitive function, and improvement of verbal memory and adaptive skills were the apparent results. The dysostosis multiplex, most often evolving very slowly also in untreated patients, had not worsened since the time of treatment. An objective report on the clinical course of the patients' residual disease after six more years of follow-up would be of great interest.

The feline model of in utero intraperitoneal (IP) transplantation of monocytic cells derived from normal marrow, into fetuses with  $\alpha$ -mannosidosis is potentially valuable not only because it involves a larger animal, but also as a potential way of preventing in utero morbidity before definitive enzyme substitution can be instituted postnatally. The donor monocytic cells engraft and persist for at most 125 days in the recipient's liver, spleen, and even brain, but in numbers below what would be needed in order to halt progression of the inborn error (103). The significant decrease of stored mannose-rich OS in brain is an interesting result. Although termed safe and feasible by the investigators, the need for repetitive IP injections into the fetus render the procedure most

invasive. Follow-up reports on possibly persistent favorable effects are awaited.

The mouse model of  $\alpha$ -mannosidosis has been used several times to study IV ERT and its effects. Following IV administration of LAMAN from bovine kidney, human (h) recombinant (r), or mouse rLAMAN, correction of OS accumulation in affected mice was transient and time as well as tissue and dose dependent. Remarkable but as yet unexplained are the facts in this report that both the human (most effective) and the bovine enzyme (least effective) were barely phosphorylated and that mannose-containing OS in mouse brain was significantly reduced (104). In a more recent study of the IV mouse model, the administration of different doses of hrLAMAN and the clearance of stored OS from visceral tissues was accomplished by low doses, but significant decrease of accumulated OS in the CNS and in peripheral neurons had required much higher doses. Successful transfer of enzyme across the blood–brain barrier was proved by the finding of hrLAMAN in hippocampal neurons. The uptake by the latter cells appears to be independent of the MPRs in the mouse (105).

### 103.1.6 $\beta$ -Mannosidosis

First recognized in goats with a severe affliction of the central as well as the peripheral nervous system and rapidly fetal outcome,  $\beta$ -mannosidosis (OMIM # 248510) was not recognized in humans until 1986 (see original references in Reference (1)). Until the present time, less than two-dozen patients have been reported, unfortunately often at different stages of the disorder's rather severe and often long clinical course. The disease is slowly progressive but with less aggressive morbidity than documented in the caprine species. A disease-free interval in infancy is seen in the majority of patients, but at least one instance of rapidly evolving CNS disease with status epilepticus by 1 year of age and fatal outcome by 15 months is on record. This young victim had angiokeratoma, a symptom more regularly observed in adolescent and adult patients (106–108). Intellectual deficiency often with delayed and poor speech development and also perceptive hearing loss is consistently observed. Recurrent respiratory infections throughout childhood, also before the diagnosis is made, are often reasons for medical attention. Behavioral problems such as hyperactivity, attention deficit in childhood, inappropriate impulsivity, and even aggressive and destructive actions from adolescent age are component features. The clinical diagnostic challenge is enhanced by the fact that physical signs, facial coarsening, and organomegaly are equivocal. Statural growth is not significantly impaired. In some patients, peripheral neuropathy is progressively evident and in others, spinocerebellar ataxia (109), seizures, features of Gilles de la Tourette syndrome, and/or increasing signs of dementia (110) become the sources of growing concern. The neurological features in the  $\beta$ -mannosidosis patient appear often late in the disease course. There are

few if any data on life expectancy. At present, also the age of onset of this rare metabolic disorder is still ill defined, as several of the initial complaints may be too aspecific in order to be interpreted as true, first clinical signs. Follow-up reports on patients already presented to the professional readership are much awaited.

Generalized cerebral atrophy appears to be the hallmark neuroimaging feature in  $\beta$ -mannosidosis patients. The brain stem and cerebellar vermis appear more severely touched by this CNS atrophy (109). Recently, the brain disorder in a patient homozygous for a MS mutation was clinically and neuroradiologically read as a severe leukoencephalopathy (111).

Because routine urinary screening of OS excretion may fail to detect excessive oligosacchariduria, biochemical confirmation of the diagnosis is not an easy assignment. Quantitative assay of the small OSs species, most specifically of the disaccharide Man( $\beta$ 1–4)GlcNAc, is required. Lysosomal  $\beta$ -mannosidase (EC 3.2.1.25) is the exoglycosidase that catalyzes the hydrolysis of the penultimate  $\beta$ -type chemical bond in the glycan N-linked to the glycoprotein (see Figure 103-1). It is specific for the  $\beta$ -mannosidosis activity because all other mannose moieties located more peripherally in the glycan tree have  $\alpha$ -linkages between them.

The clinical diagnosis is confirmed by the demonstration of the completely deficient or significantly decreased specific activity of lysosomal  $\beta$ -mannosidase in either leukocytes or cultured fibroblasts. This enzyme assay is not routinely included in the set of acid hydrolases associated with better-known LSDs.

Molecular studies of the caprine gene *manba* have greatly contributed to the molecular investigation of the human orthologous *MAMBA* gene (OMIM # 609489). It spans 12.9 kb of genomic DNA and has been mapped to 4q22–q25. It is composed of 17 exons separated by rather large introns and is transcribed into a 3293 bp mRNA encoding a single polypeptide of 879 amino acids (see original references in Reference (1)). More than a dozen mutations that are spread over the entire gene, including eight MS and NS, four splicing altering mutations, and two small deletions have been detected in patients and their families (106–114). Finding any meaningful genotype–phenotype correlation between, for example, “null” mutations and severe type of disease must await more clinical detail and follow-up information, more molecular biology–based results, more uniform reporting, and hopefully more worldwide cooperation in studying this rare lysosomal disorder.

In recent years, interesting and relevant molecular biology results have been obtained in mouse models with  $\beta$ -mannosidosis (115,116).

### 103.1.7 Fucosidosis

Originally described as an atypical MPS without urinary excretion of GAGs, fucosidosis is a rare inborn error

of metabolism worldwide. Following the demonstration that the vacuoles in peripheral lymphocytes and Kupffer cells are abnormally swollen lysosomes, Van Hoof and Hers' colorimetric assay of several lysosomal hydrolases with artificial substrates, newly available in the late 1960s, demonstrated the profound deficiency of  $\alpha$ -L-fucosidase in the patient. The label fucosidosis was applied to this disorder. The conclusion of the very low incidence of fucosidosis is supported by the observation of parental consanguinity in more than one-third of the families (1).

**103.1.7.1 Clinical Phenotype.** In probably more than half of the patients, an initial, symptom-free interval of between 6 and 12 months is followed by recurrent upper respiratory infections at the time when a delay in neuro- and psychomotor development also becomes overt. Physical examination at this stage notices slightly coarse facies, thickened lips, and tongue, vaguely reminiscent of the Hurler phenotype, tight skin, and generalized hypotonia with depressed deep tendon reflexes. A sizable proportion of the affected never walk unaided. The initial developmental delay soon alters into decline of neurological skills and severe mental deficiency. Abundant sweating with increased salinity of the sweat has been observed in some of these young patients. Slowing of linear growth becomes apparent before the third birthday. Hepatosplenomegaly, inconsistent in early childhood, is most often absent in older patients. Mild thoracolumbar kyphosis is often confirmed radiographically. Cardiomegaly is initially radiographic rather than a clinical sign. The corneae remain clear. Ophthalmologic examination reveals some tortuosity and caliber irregularity in the retinal vessels. From as early as 3 years of age, the clinical course is compounded by progressive loss of motor skills, hypertonia, and spasticity with hyperreflexia, and sometimes by seizures. Cognitive deficiency progresses inexorably. Patients with this fast-evolving encephalopathy quickly reach the stage of dementia and decerebrate rigidity. They succumb in childhood due to the complications of the CNS disease (117–122). In the past, the disease with this dramatic clinical course had been termed fucosidosis type I.

About half of the patients reported also had severe intellectual handicap with less well-defined clinical onset and almost nonprogressive disease. Angiokeratoma and/or telangiectasias, a dermatological phenomenon indistinguishable from the ones known in patients with Fabry disease (MIM # 301500), were nearly consistent findings in adolescent or adult patients. The observation of this slowly evolving, though clinically severe, disorder had initially prompted the term fucosidosis type II. The longer surviving patients have recurrent, but potentially fatal episodes of dehydration apparently due to the inability to control body temperature (1).

As more clinical reports became available, the contention of two distinct clinical phenotypes could not be upheld. Vascular markings and pinhead-sized skin

lesions similar to angiokeratomata in Fabry disease are not only seen in older patients with slowly evolving disease. Extensive telangiectasia may be present also in the patients with early-onset fucosidosis, particularly in the thenar and hypothenar regions, on the lateral side of the feet as well as in the genital region (119,122,123). Moreover, patients with either type of disease have been observed in a single large pedigree and also among sibs. In somatic cell hybridization experiments with fibroblast cultures derived from either type of fucosidosis, mutual complementation has never been observed. Identical mutant genotypes are detected in patients of either type.

Radiographic skeletal changes are mild in all young patients, but skeletal age lags consistently behind the chronologic age. There is definite dysostosis multiplex in fucosidosis. The mild-to-moderate spondyloepiphyseal dysplasia and the more severe hip dysplasia are usually more pronounced than the diaphyseal widening of long bones (39).

Current magnetic resonance imaging (MRI) illustrates and confirms in vivo the progressive encephalopathy in all patients with fucosidosis as it does in several other hypomyelination disorders, other LSDs among them. In the latter, including fucosidosis, symmetrical, extensive, and confluent signal hyperintensity on T2-weighted images of cerebral periventricular, cerebellar, lobar, and subcortical white matter are consistently found. Following the evaluation of magnetic resonance images by clustering analysis and multivariate testing, Steenweg et al. (124) concluded that T2 hypointensity of the globus pallidus was the most specific and discerning feature in the fucosidosis brain. Signal abnormalities in the basal ganglia and thalamus have been reported in a number of LSDs (125), but only in fucosidosis T2 hypointensity of the globus pallidus has been previously reported (117,120,123).

**103.1.7.2 Histo- and Chemical Pathology.** Weakly periodic acid Schiff (PAS)-positive vacuoles are found in peripheral lymphocytes. By electron microscopy, two types of unit-membrane-bound cytoplasmic vacuoles can be discerned in hepatocytes and Kupffer cells: One type has very light, loosely structured contents; the other is filled with rounded osmiophilic lamellar structures. Also, histiocytes, glomerular endothelium cells, epithelial and other cells in conjunctiva and skin (irrespective of the presence of angiokeratoma), and endothelial cells in bronchial and rectal mucosa are swollen by the presence of similar lysosome-like inclusions. Neuronal and myelin loss in brain is considerable. There are large units, membrane-bound vacuoles in the surviving neurons.

Fucose-containing glycolipids, glycoproteins, and also glycosylceramides accumulate in liver and brain. There is excessive urinary excretion of fucose-containing OS. Thin layer chromatography can distinguish the pattern of OS excretion in fucosidosis from that of other oligosaccharidoses provided the appropriate control samples are simultaneously studied. The consistent biochemical feature

among the many fucosylglycoasparagines, isolated, separated chromatographically, and characterized from urine of patients with fucosidosis, is the disaccharide-containing fucose  $\alpha(1-6)$  linked to GlcNAc (see Figure 103-1). In case of defective or absent  $\alpha$ -L-fucosidase, this bond can barely or not at all be hydrolyzed from the nonreducing end in many types of glycoproteins and glycolipids. One of the glycolipids accumulating in the CNS and in other tissues is the ABO blood group H-antigen, the glycolipid Fuc ( $\alpha 1-2$ )Gal ( $\beta 1-4$ )GlcNAc- $\alpha$ -Gal-ceramide. The implication of fucosidosis on the quantity and quality of the fucosyltransferase(s)-dependent blood group antigens (H, Le<sup>a</sup>, and Le<sup>b</sup>) remains incompletely resolved.

#### 103.1.7.3 Enzyme Defect: Formal Diagnosis.

Assay of  $\alpha$ -L-fucosidase in leukocytes using the fluorescence-generating substrate—4-methylumbelliferyl- $\alpha$ -fucopyranoside—is the routine method for establishing or excluding the diagnosis of fucosidosis (see Figure 103-1). The enzyme deficiency in leukocytes, cultured fibroblasts, amniocytes, or various tissues such as liver, brain, or kidney, is equally profound in patients with the more protracted course as in patients with rapidly advancing disease.

It must be stressed that assaying  $\alpha$ -L-fucosidase in serum or plasma is of no value in the diagnosis of fucosidosis because in about 12% of normal people the average serum or plasma activity is only 5% of that in the rest of the population. In this minority group of normals,  $\alpha$ -L-fucosidase activity in leukocytes is within normal limits.

**103.1.7.4 Molecular Biology of  $\alpha$ -L-Fucosidase and Its Gene, *FUCA1*.** The enzyme has been purified from a variety of tissues. It occurs predominantly in the homotetrameric form but also exists in multiple other combinations of the single, mature 50–60 kDa polypeptide. Glycosylation of the enzyme is important as the size of the nascent monomeric precursor polypeptide is only 51 kDa. In some patients with fucosidosis, the precursor polypeptide remains unprocessed. The gene encoding  $\alpha$ -L-fucosidase has been mapped to chromosome 1p34 and named *FUCA1*. By fluorescence in situ hybridization (FISH), a pseudogene labeled *FUCA1P* has been mapped to 2q31-q32.

*FUCA1* spans 23 kb of genomic DNA and comprises eight exons. The cDNA consists of 2047 bp and includes a 1383 bp open reading frame that encodes a signal peptide of 22 and a mature protein of 439 amino acids. The nucleotide sequence in the pseudogene *FUCA1P* is 80% identical to that in *FUCA1* cDNA, but lacks an open reading frame. By Northern blot analysis, the size of *FUCA1* mRNA was shown to be 2.3 kb in length. This molecular characterization is the prerequisite to detecting a variety of mutations, the spectrum of which has been reviewed in 1999 (126). All mutations, mostly NS, small deletions or insertions, and only a few MS mutations are spread throughout *FUCA1*'s open reading frame. Most of them are encountered in homozygous genotypes, not surprisingly given the high proportion of consanguinity in the families affected. Only the NS Q351X mutation

has been observed in several families. Most of the others are single occurrences and therefore private for the time being. All mutations result in nearly absent enzyme activity and barely detectable cross-reacting material. Apparently, the clinical variability of the disease course is not correlated with either intragenic location or the type of mutations in the mutant *FUCA1* genotypes. Unknown genetic or environmental factors may play a role here. Willems review includes data on genetic polymorphisms within the *FUCA1* gene that explain neither the conclusions regarding the variability of the clinical course nor the variant enzyme activity in human serum (126).

### 103.1.8 Aspartylglucosaminuria

Aspartylglucosaminuria (AGU) (MIM # 208400), an OS with particularly high incidence of 1/18,500 in Finland, is worldwide only occasionally observed. Hence, no wonder that clinical description and delineation, and also metabolic and molecular elucidation have been mainly contributed by Finnish clinicians and scientists.

**103.1.8.1 Clinical Phenotype.** The clinical delineation has been contributed by the original M. Arvio studies (127–130). Umbilical and inguinal herniae and the unusual susceptibility to respiratory tract and ear infections are the most prominent parental concerns and complaints in patients at toddler age or in early childhood. In some, weak suck and general hypotonia could be recorded retrospectively. Infections recur less frequently after the age of 6 years. In 33 cases, the diagnosis was made between the ages of 10 months and 11 years. Intellectual disability, a consistent feature in later childhood, was not established in any patient before the diagnosis of AGU. The milestones of gross motor development are reached at the appropriate age. AGU is not an easy diagnosis before the patient's fifth birthday. Parental concern related most commonly to delay in speech development and short attention span often associated with motor clumsiness and restless behavior that only in a minority of patients was labeled hyperactive, destructive, or violent. The phenotype in early childhood is that of gradually apparent speech and cognitive developmental delay associated with only mildly coarsening facial features. Recently, systematic attention has been paid also to the slowly progressive facial dysmorphism in AGU patients (131). Following the period of slow but still slightly positive neuromotor and mental development in childhood, gradual loss of skills is observed from adolescence and on an average, a more rapid decline of cognition and the ability to interact socially starts after the age of 25 years. Regarding adaptive skills, school-aged patients remain superior, but the middle-aged affected become inferior to individuals with moderately intellectual disability. Behavior problems are encountered in about one-quarter of the adult patients. Seizures and sleep disturbance have been observed and may affect the patients' behavior (132–134). The disorder is slowly progressive and



hence interferes unfavorably with life expectancy (135). Remarkable is the fact that in older patients, apparently true reduction of head size has been carefully documented (136). AGU, clinically a rather homogeneous but rare inborn error, is probably underdiagnosed.

**103.1.8.2 Enzyme Defect: Formal Diagnosis.** This OS is the clinical consequence of the deficiency of aspartyl- $\beta$ -glucosaminidase (AGA) (EC 3.5.1.26), the lysosomal amidase that catalyzes the hydrolysis of the amide bond between asparagine of the N-glycosylation site in glycoproteins and the core GlcNAc residue in any N-linked OS side chain. In the AGU patient, aspartylglucosamine (2-acetamido-1-L- $\beta$ -aspartamido-1,2-dideoxy- $\beta$ -D-glucose) accumulates in tissues and is excessively excreted in the urine (see Figure 103-1). This abnormal metabolite is easily discerned by high-voltage electrophoresis/ninhydrin technology for routine diagnostic purposes (130,137). More sensitive methods for detection of the metabolite in urine and in lymphocytes have been developed.

#### 103.1.8.3 Molecular Biology of Aspartyl- $\beta$ -Glucosaminidase and of AGA, the Gene Encoding It.

The AGA gene has been mapped to 4q32-q33 by deletion mapping and FISH. Isolation and cloning of cDNA were the steps preceding its complete nucleotide sequencing. The human AGA covers 13 kb of genomic DNA and has its open reading frame distributed over nine exons. AGA mRNA is translated as a single pro-precursor polypeptide of 346 amino acids. The N-terminal signal sequence of 23 amino acids is cotranslationally cleaved off in the ER, where major folding of the nascent precursor (49 kDa) and homodimerization occurs. Subsequently, this dimeric complex is autocatalytically activated by proteolysis of the peptide bond between aspartic acid 205 and threonine 206 in each monomer, thus exposing the latter residue for an important role in the enzyme's catalytic site and yielding a tetrameric protein composed of two  $\alpha$  and two  $\beta$  subunits ((138) and references therein). In the Golgi cisterns, glycosylation and the M6P-targeting signal are added for routing of the enzyme to the cell's lysosomal compartment, where still a small reduction in size of the component polypeptides occurs. X-ray crystallographic studies have revealed the three-dimensional structure of the AGA enzyme and the specific roles of the amino acids within the active site (138). This report provides in vitro evidence for the adverse effect of some AGU mutations on the intracellular trafficking of the AGA protein complex. Several steps in the AGA enzyme maturation and macromolecular conformation have been confirmed by studying mutations in AGU patients and even by studies about in vitro mutagenesis. These findings have been extended by Saito et al. (139). At least as effective and useful has been the study of the "founder-type Finnish MS mutation" located in exon 4 that results in the Cys163Ser substitution concomitantly eliminating the all-important disulfide bond with misfolding, failing intracellular routing,

and catalytic deficiency as a result (140,141). The rather complete compilation of AGA mutations up to 2001 (138) and subsequent observations (142) have offered an excellent opportunity to explain in detail the molecular pathogenesis of AGU patients. Most MS mutations that substitute the "wild-type" amino acid for a bulkier one distort the enzyme's macromolecular conformation significantly. Mutations at the dimer interface preclude dimerization in the ER. Active-site mutations not only eliminate catalytic activity but also may adversely affect maturation and hence routing of the precursor protein to the lysosomes.

Mouse models have been studied (137,143,144) and demonstrated the impressive molecular and genomic similarities of the clinical pathogenic effect of mutations in the orthologous *aga* gene in addition to similar cyto- and histopathology. These data are potentially useful for future therapeutic trials. ERT has not been reported in AGU studies. Few results of BMT have led to the conclusion that this type of therapy cannot be recommended in patients after infancy in a disorder that is hard to formally diagnose before the age of 3 years (145). Unfortunately, this conclusion may still stand even following the report of a 5-year follow-up of the apparently stable clinical, biochemical, and neuro-imaging results in two BMT sibs during later childhood (146).

#### 103.1.9 NAGA Deficiency: Schindler Disease and Kanzaki Disease

This severe type of metabolic encephalopathy was first observed in 1989 by Schindler in two German sibs of consanguineous parents. Clinical onset had been in late infancy when the cessation of psychomotor development associated with hyperacusis or exaggerated startle response to noise was noticed. The disorder was soon characterized by deterioration of the CNS function expressed as axial and appendicular hypotonia, grand mal type seizures, poor motor coordination, strabismus, nystagmus, and visual impairment. Joint contractures and the appearance of submucous nodules—the ones in the vocal cords probably explaining the progressive hoarseness—compounded the clinical picture. By 4 years of age, this impressive CNS disorder resulted in severe mental deficit, cortical blindness, rigidity with flexion contractures, myoclonus, and decorticate posturing. In this "vegetative state," the patients had only minimal contact with the immediate environment. Neuropathology studies have shown that Schindler disease is nearly exclusively a disorder of the CNS and the peripheral nervous system without primary involvement of other organ systems (147,148). The clinical course and axonal ultrastructural findings are reminiscent of either Seitelberger-type infantile neuroaxonal dystrophy (MIM # 256600) or of Hallervorden-Spatz disease (MIM # 234200). Some of the patients reported previously with one or the other

neurodegenerative disorder in whom no enzyme studies were performed, may have been examples of Schindler disease.

More recently, two Dutch sibs have been reported: a girl with a neurological disease significantly milder than Schindler disease and the younger brother initially considered to be still presymptomatic because in both the same compound heterozygous mutant genotype was detected (149). Also in 1989, Kanzaki et al. reported on an adult mildly retarded female with disseminated angiokeratoma and glycopeptiduria and with large numbers of vacuoles in skin and kidney cells. The patient had consanguineous parents, but also two unaffected children. Telangiectatic lesions were found also in the conjunctiva, the eye fundus, and the gastric mucosa as endoscopically demonstrated. The disorder in this patient has been called Kanzaki disease (150). Similar patients have since been reported and shown to differ from any other LSD (151).

The abnormal thin-layer chromatographic urinary OS profile pointed to the metabolic nature of the disorder in Schindler's patients. The blood group A trisaccharide, GalNAc- $\alpha$ (1-3)Gal $\alpha$ (2-1)Fuc was a major compound among other glycoconjugates present in excess. All had terminal  $\alpha$ -NAc-galactosaminyl moieties as are found in both the N- and the O-linked glycopeptides, glycoproteins, and glycosphingolipids. Moreover, keratan sulfate had accumulated in various tissue biopsy specimens and in cultured fibroblasts. Therefore, the specific deficiency of lysosomal N-acetyl  $\alpha$ -galactosaminidase (NAGA), predicted in Schindler disease, was confirmed and demonstrated in Kanzaki disease as well. The residual activity was well below 2% of normal in the former and less than 5% of normal in the latter (152). Immunoblotting with monospecific rabbit antihuman enzyme antibody failed to show cross-reacting material in the patients.

The causal mutation in Schindler disease did not interfere neither with the synthesis of the 52 kDa precursor protein, nor with the generation of the mature enzyme (48 kDa) that was however less stable in the German patients than in the Japanese woman.

The *GALB* gene that encodes  $\alpha$ -NAc-Galactosaminidase is located at 22q13. The full-length 2.2 kb cDNA and the complete genomic DNA have been isolated, sequenced, and expressed in COS-1 cells (153,154). The gene has 9 exons and an open reading frame of 1236 bp encoding a 411 amino acid-containing protein. The German sibs were homozygous for the 973G>A transition resulting in the MS mutation that substitutes lysine for glutamic acid (Glu325Lys). In the cDNA of the Kanzaki proband, the 985C>T transition led to the Arg329Trp MS mutation (155). The Chabas Kanzaki-type patients were shown to be homozygous for the NS mutation Glu193X in exon 5. Their disease was apparently due to the complete loss of the NAGA protein (156). The latter authors have shown also that the Dutch siblings were compound heterozygous for the mutant alleles Glu325Lys and Ser160Cys.

All patients reported up to 2001 have been reviewed by Desnick and Schindler (148) and by Bakker et al. (157). The large phenotypic difference between the two types of patients with either Schindler or Kanzaki type of disease, some among them with substitutions in NAGA only three amino acids apart, remains unexplained. Unknown, genetic and/or environmental factors must play a role in the remarkable clinical heterogeneity. That the neuroaxonal dystrophy component may have been coincidental is improbable, but cannot be ruled out as an alternate hypothesis (157).

## 103.2 DISORDERS ALLIED TO OLIGOSACCHARIDOSES

By strictly biochemical criteria, the inborn errors of metabolism to be discussed in this section of the chapter are not oligosaccharidoses. The hereditary disorders associated with excessive urinary excretion and accumulation in tissues of free sialic acid as common hallmark differ pathologically more than clinically from the true oligosaccharidoses (Table 103-2). In one among them, the genetic defect adversely affects the egress of this and other monosaccharides out of the lysosomes; in the other, a rare condition without direct lysosomal involvement, there is constitutive overproduction of free sialic acid. The latter has, contrary to the autosomal recessive mode of inheritance in all other disease entities, been recognized as an autosomal dominant trait. The entities, multiple sulfatase deficiency (MSD) and ML IV were initially included among the mucopolysaccharidoses because the clinical and radiographic phenotypes were considered rather congruent. ML IV has in fact no pathogenetic features connecting it directly with either ML II or ML III $\alpha$ / $\beta$  or even ML I. However, it stresses the role of the transporter proteins in the lysosomal membrane. Mucosulfatidosis has more in common with the GAG storage than with the OSs storage disorders. The causal defect is not lysosomal, but renders the catalytic effect of sulfatases within the lysosomal compartment much deficient. Pyknodysostosis (PKD), a well-known OCD, results from a functionally deficient cathepsin K, a true lysosomal hydrolase. This second part of the chapter closes with the succinct discussion of nodulosis–arthropathyosteolysis (NAO) syndrome, the composite of at least three osteochondrodystrophies originally delineated as separate entities but at present known to be allelic because of a common molecular and biochemical defect.

### 103.2.1 Sialic Acid Storage Disease(s)

**103.2.1.1 Phenotypes Delineated.** Initially, two phenotypes have been discerned that still adequately fulfill the role of clinical reference type. They include (1) infantile free sialic acid storage disease (ISSD) (MIM # 269920) with perinatal or neonatal onset, multisystemic

TABLE 103-2 Disorders Allied to the Oligosaccharidoses

Phenotype		Gene		Enzyme (Protein)			
Group Entity (Synonym)	MIM#	Gene	Genome Location	MIM#	Defect	Symbol	EC#
Free sialic acid storage disorders (SASD)							
ISSD <sup>c</sup>	259920	<i>SLC17A5</i>	6q14–q15	604322	Solute carrier family 17 member 5	Sialin	–
SALLA disease (late ISSD)	604369						
Sialura <sup>a</sup>	269921	<i>GNE</i>	9p13.3	603824	UDP-GlcNAc-2-epimerase/ManNAc-kinase	GNE	5.1.3.14 2.7.1.60
Mucosulfatidosis, MSD <sup>d</sup>	272200	<i>SUMF1</i>	3p26	607939	Sulfatase modifying factor 1, (formylglycine generating enzyme)	SUMF1 (FGE)	1.8.99
Mucopolipidosis IV (Berman disease)	252650	<i>MCOLN1</i>	19p23–p13.2	605248	Mucolipin 1	TRPML1 (MCOLN1)	
Pyknodysostosis (PKD)	265800	<i>CTSK</i>	1q21	601105	Cathepsin K	CTSK	3.4.22.38
NAO syndrome <sup>b</sup>	259600	<i>MMP2</i>	16q13	120360 605156	Matrix metalloproteinase 1	MMP2	3.4.24.24

<sup>a</sup>See text for hereditary inclusion body myopathy (HIBM).

<sup>b</sup>NAO syndrome: nodulosis–arthropathy–osteolysis syndrome.

<sup>c</sup>Infantile sialic acid storage.

<sup>d</sup>MSD: multiple sulfatase deficiency.

but predominantly CNS morbidity, and fatal outcome before the third year of life; (2) the late infantile-onset type, a slowly progressive brain, and a peripheral nervous system disease with significantly less adverse effect on life expectancy. This type known as Salla disease (MIM # 604369) has a high incidence in the Salla region of northeastern Finland. From the time of delineation, ISSD and Salla disease have been viewed as allelic disorders because of common histopathologic and metabolic findings. Proof of this thesis was provided by the finding of mutations causing either disease in the *SLC17A5* gene (158), which encodes an important lysosomal membrane transporter (159). Not unexpectedly also intermediate phenotypes have been recorded prompting the hypothesis of a continuously variable phenotypic spectrum.

ISSD may present as an obstetric and neonatologic emergency as it figures among the most frequent causes of metabolic non-immune hydrops fetalis. Either disorder represents a diagnostic challenge because screening of urinary excretion of GAGs or OSs yields normal results. Unfortunately, the assay of free sialic acid is too often omitted from the panel of diagnostic tests applied to infants and children with probable LSD.

#### 103.2.1.1.1 Infantile Sialic Acid Storage Disorder.

ISSD, an inborn error of metabolism without ethnic predilection, was first delineated in Finland by Aula et al. in 1979 (160) and subsequently reported from various clinical centers (161) (see more original references in Reference (1)). Ultrasonographic studies during pregnancy have revealed that in a large proportion of cases, disease onset is prenatal and manifests as either frank hydrops fetalis or ascites and/or prominent fetal edema. Concomitantly, intrauterine growth retardation

is recorded (162,163). Premature birth had already been noticed frequently in ISSD (160). In some of these infants, nephrotic syndrome has been formally documented (162,164).

The infant patient is axially hypotonic, has a fair-to-pale complexion, and has an expressionless and sometimes already coarse face. He or she soon presents with often-refractory feeding difficulty and weak cry, and inconsistently with hepatomegaly, cardiomegaly, and/or pericardial effusion. The corneae remain clear, but the retinas are often described as albinoid. Failure to thrive and severe neurodevelopmental delay are soon apparent. At least some facial coarsening with flat nasal bridge and anteverted nostrils becomes consistently evident in later infancy. There is no restriction of motion range in the joints. Inguinal herniae are frequent in males. Neuromental development is minimal. Deep tendon reflexes may become hyperactive. Convulsions are noticed. Recurrent respiratory infections become progressively more threatening and often compounding the failing CNS and other organ functions that cause fatal outcome often around the first birthday.

The radiographic survey finds the skeleton to be osteopenic. There is hip dysplasia, sometimes thoracolumbar kyphosis, and often calcified stippling in soft tissues around the tarsal bones, the hips, or spine, where the vertebrae retain an ovoid configuration. However, the dysostosis multiplex type OCD is usually mild in ISSD (39).

**103.2.1.1.2 Salla Disease.** Patients with Salla disease, first described by Aula et al. in 1979 (165), are normal at birth. The first parental concerns arise by the time the infant reaches the age of 6–12 months. They

relate to slow neuromotor development and generalized hypotonia. Clinical examination confirms the symptoms and may reveal also incipient hyperreflexia and pyramidal spasticity in the legs, truncal ataxia, and nystagmus. Organomegaly is not usually present in patients with Salla disease. The early neuromotor milestones are reached with moderate delay and only about one-third of the patients achieve unaided ambulation. Speech development is more deficient. It has often a dysarthric character and is usually limited to single words. The language acquired in early childhood regresses considerably in subsequent years. Statural growth is only moderately impaired and pubertal development appears to be clinically normal. Facial coarsening is rather mild. At variable ages, tonic/clonic seizures and/or “absence”-like epileptic crises, often refractory to treatment, compound the neurologic syndrome. Interactive non-verbal communication remains remarkably effective whereas motor performance and visually constructive actions and assignments are performed poorly (166,167). The patients may still make some progress in understanding speech and in interacting with the immediate environment (168), while the disorder seems to become more stationary or shows slowing of regression. They are usually sociable and good-humored despite spasticity and athetoid spurious movements. From adolescence, they are often wheelchair-bound and bedridden later on. Motor scales always yield lower results than the mental ones, both being obviously limited. Visual deficiency and retinal changes have not been observed in Salla disease. Motor conduction velocity is slowed and abnormal somatosensory evoked potentials provide indications that also the peripheral nervous system is affected in Salla disease. Average life expectancy is clearly reduced, but patients have been reported who had reached over 60 years of age. Although an inborn error with high incidence in Finland, it has been observed and reported from various places in the world (162,163,169–174) (see also older references in Reference (1)).

The radiographic changes of the skeleton remain mild in Salla disease and at worst approach from a distance, the ones in LSDs with fully expressed dysostosis multiplex (39). MRI type neuroimaging documents hypomyelination of cerebral and cerebellar white matter, already unequivocal in the young patient. Abnormal density changes are found in the basal ganglia. Hypoplasia of the corpus callosum is consistent. Later in the course general cerebral atrophy is manifest due to be further demyelination (175–177).

No wonder that in this predominantly neurologic disorder, only occasional results of more multisystemic examinations are reported. In a Japanese patient, serious renal disease was associated with Salla disease, probably an indication of its allelic nature to ISSD (158). Deficiency of growth hormone and gonadotropins has been detected in an Italian patient (169), a probable indication of damage inflicted on pituitary secretion.

Clearly, colleagues with considerable experience are able to recognize patients with distinctly more severe Salla disease and to correctly predict mutant genotypes, different from the so-called classic Finnish phenotype (168,178,179). See the next section on gene mutation and comments on phenotypic variability in the free sialic acid storage diseases. The phenomenon of intrafamilial clinical variability still remains enigmatic.

**103.2.1.3 Pathology, Biochemical Findings, and Formal Diagnosis.** Peripheral blood smears of patients with either disorder show vacuolization in some of the lymphocytes. Phase-contrast microscopy reveals numerous “empty” cytoplasmic vacuoles in mutant-cultured fibroblasts. Similar vacuolar structures are seen in skin or in conjunctiva biopsy specimens. In parenchymous organs, light and electron microscopy reveal the same abnormalities and demonstrate once more the lysosomal nature of the free SASDs. See older references in Reference (1).

Few reports of classic neuropathology in either ISSD or Salla disease are available in the literature. Postmortem examination of the CNS in two unrelated Salla disease subjects aged over 40 years showed severely reduced white matter and more specifically, marked loss of axons and myelin sheaths accompanied by pronounced astrocytic proliferation. Interesting but probably less specific are the findings of abnormal lipofuscin in cortical nerve cells and neurofibrillar tangles only in neocortical cells and in some basal nuclei (180). An *in vitro* assay that characterized human sialin function (181) revealed that mutant sialin encoded by mutations associated with ISSD completely abolished the transport of sialic acid, whereas the well-known R39C Salla disease-causing mutation slowed down but did not halt the transport. Relevant to the disorders’ pathogenesis are also the results of an astute *in vitro* procedure for structural and functional analysis of transporter molecules of any species using the expression in insect cells (182,183). Glutamate is an excitatory neurotransmitter molecule. The neurotransmitting role of aspartate usually co-stored in synaptic vesicles of hippocampal neurons was controversial because the location and the role of vesicular storage of aspartate were hitherto unknown. The experiments referred to have established that sialin possesses the dual physiological functions of vesicular aspartate/glutamate transporter in addition to its H<sup>+</sup>/sialic acid cotransporter role. Sialin mutations known to be associated with ISSD lose the latter function completely, but partially maintain the former. People with Salla disease lose aspartergic and glutaminergic neurotransmission, but have the sialic acid transport partially preserved. Results recently obtained in the sialin<sup>-/-</sup> mouse model (184) are equally relevant to the pathogenesis of the disorders at hand. These mice are smaller than the control animals, have uncoordinated behavior, and die prematurely. Myelination of the optic nerve, the main site of study, is delayed and deficient in the mutant animals and



the apoptotic death of cells in the oligodendrocytic lineage is enhanced. The authors also found that down-regulation by the heavily sialylated cell-surface adhesion protein (PSA-NCAM), a known myelination inhibitor is concomitantly delayed.

Urinary excretion of free sialic acid is 5–20 times normal in the free SASDs. This initial finding prompted studies of the metabolic turnover of sialic acid and the demonstration that all enzymes catalytically involved in the synthesis or metabolism of sialic acid function normally in these patients. Subsequently, specific intralysosomal retention of sialic acid was recognized and explained by the finding of its negligible rate of egress in patients. In a normal individual this function is dependent on the intralysosomal sialic acid load and correlated with the ambient temperature suggesting a protein-carrier-mediated mode of transport, which also applies to the transport of glucuronide, other acidic monosaccharides, cystine, and apparently also aspartic acid (160,182,185,186). The mean transport rate of sialic acid and glucuronic acid from lysosomal membrane-bound vesicles in obligate heterozygotes was approximately half the average in control cell strains (1,187). Interesting progress has been made regarding the more specific biochemical effects of various *SLC17A5* mutations by the recent studies of Wreden et al. (188). At present, liquid chromatography-tandem mass spectrometry assay, a method not usually available in the routine clinical biochemistry laboratory can quantify free as well as total sialic acids in body fluids including cerebrospinal fluid. This is a major step forward for the diagnosis and even more important for better comprehension of the pathogenesis of the many disorders directly or indirectly related to gene mutations adversely affecting sialic acid metabolism (189).

Prenatal diagnosis of any free SASD must depend on prior identification of the disease-causing mutation in the family. Assay of free sialic acid (SA) in amniotic fluid is an unreliable method for this purpose. Cultured amniocytes (approach not reliable in case of suspected Salla disease) or tissues obtained by CVS can be used for measuring intracellular free SA in the event of suspected ISSD. Demonstration of intralysosomal SA accumulation most reliably provides or excludes the diagnosis (190,191).

**103.2.3.1 The SA Transporter, Sialin: Molecular Biology of Its Gene *SLC17A5*.** The gene causing either free SASD has been mapped to chromosome 6q14–q15 and has been identified, characterized, and sequenced by Verheijen et al. (158). Originally also termed “anionic sugar transporter,” it has been recognized as a member of the SLC17 anion transporter family of phylogenetically related genes and labeled *SLC17A5* (MIM # 604322). It contains 11 exons, all of them coding exons. A cDNA clone of 2.5 kb was shown to contain an open reading frame of 1485 bp, predicting a protein sequence of 495 amino acids.

The protein encoded by *SLC17A5* has been purified, characterized, and termed Sialin (158,192). The amino acid sequence in the SA transporter is extensively analogous to that in other human and mammalian symport proteins, except for the N- and C-terminal regions. Sialin contains no less than 12 transmembrane domains. Northern blot studies revealed the ubiquitous gene expression including the CNS. Various transcripts—the 3.5 kb and 4.5 kb ones being the major ones—have been identified in several human tissues (158,160). The initial RT-PCR analysis of *SLC17A5* cDNA found the homozygous c.115C>T transition resulting in the MS substitution p.R39C in five unrelated Finnish patients with Salla disease. The substitution of the highly conserved arginine by cysteine was confirmed in genomic DNA. This p.R39C MS mutation is a founder mutation in the Finnish population, where the homozygous mutant genotype is present in well over 90% of “classic” Salla disease patients. Verheijen et al. (158) found several different mutations in the *SLC17A5* gene, in either homozygous or compound heterozygous genotypes in different ISSD patients from other countries. Over two dozen pathogenic mutations have meanwhile been identified (163,168,170–173,178,193) and also some apparently nonpathogenic variants of which p.A43T is an example (194).

Some consistency in genotype–phenotype correlations has also become clear: (1) The homozygous MS mutant genotype p.Arg39Cys MS, the single Finnish founder mutation leads to typical Salla disease; (2) the compound heterozygous patients for the p.Arg39Cys and for another *SLC17A5* mutation leads to the more severe, also called intermediate, Salla disease. The same is true for the homozygotes for the p.Lys136Glu MS mutation (195); (3) Compound heterozygosity for other mutations leads invariably to the severe, early-onset type ISSD with more overt multisystemic involvement (194).

## 103.2.2 Sialuria

This inborn error, more interesting from a scientific than from a clinical vantage point, was initially called “French type sialuria” based on the facts that it was first described and biochemically characterized in 1968 by Fontaine et al. in a French patient and that it took almost 20 years before a second patient was reported from New Zealand by Wilcken et al. (see original reference in Reference (1)). The term was created in order to differentiate the disorder from Salla disease that was called “Finnish type Sialuria” at the time. Only the finding of excessive urinary excretion of free SA makes this “disorder” a differential diagnostic challenge for establishing or excluding the diagnosis of a free SASD.

**103.2.2.1 Clinical Aspects.** Drawing a composite picture of the salient features may still be preliminary because of the paucity of patient reports available (165,196,197) and because of the remarkably mild

phenotype. Information on the long-term outcome and prognosis of this inborn error is still lacking.

Five of the six patients observed in different countries and diagnosed in childhood may have been simplex occurrences in the nonconsanguineous families (165,198–200). The mother, a normally functioning homemaker, of the sixth nonconsanguineous patient was also found to be affected (196). This observation supports the autosomal dominant mode of inheritance of sialuria.

The prenatal and neonatal histories of all patients were unremarkable. Birth weights were in the low normal range and so were the infants' length and occipitofrontal circumference, where available. Some of the infants (four males; two females) had mildly coarse facial features. Hypotonia, hepatomegaly, prolonged neonatal jaundice, hypochromic anemia, episodes of diarrhea with threatening dehydration, transient failure to thrive, and hoarseness were inconsistently recorded. In the first 2 years, frequently recurring respiratory infections were compounding the clinical picture. The milestones of neuromotor development were reached with some delay. From the second year of life, facial coarsening and delay in language and cognitive development were additional parental concerns in some instances. Physical growth remained normal in all. General health improved gradually from the second year of life. Except for some mild hypotonia, the neurologic examination did not show any deficiency. In two instances, however a mild seizure disorder persisted, as did mild hepatomegaly in the majority of patients. In all these instances, the disorder appeared to be nonprogressive. Some patients have attended regular-grade school. Others made good progress in special education classes.

The only adult with documented sialuria so far, the mother of one of the probands, had attended regular-grade school classes only unlike all of her unaffected sibs, who had at least successfully completed high school. She is of normal stature and has no facial coarsening but remarkably edematous eyelids. Sialuria has been ruled out in the father and the two older sibs, all also physically and mentally normal. In the proband, urinary excretion of free SA has repeatedly been between 100 and 200 times normal. It was about 80 times normal in the mother (196).

**103.2.2.2 Pathology Studies, Diagnosis, and Molecular Biology.** There is no histologic evidence of lysosomal storage in either peripheral blood lymphocytes or cultured fibroblasts. Abnormalities detected electron microscopically in the mitochondria have been inconsistent and are probably secondary phenomena. Formal diagnosis of sialuria and differentiation from the SASDs is not achieved by the finding of the extraordinary increase of free SA in the patient's urine or its 100-fold excess detectable in sialuria fibroblasts. It must be stressed that expertise is required in selecting the appropriate methodology for the assay of free and total SA (189,197,201,202). The diagnosis may depend

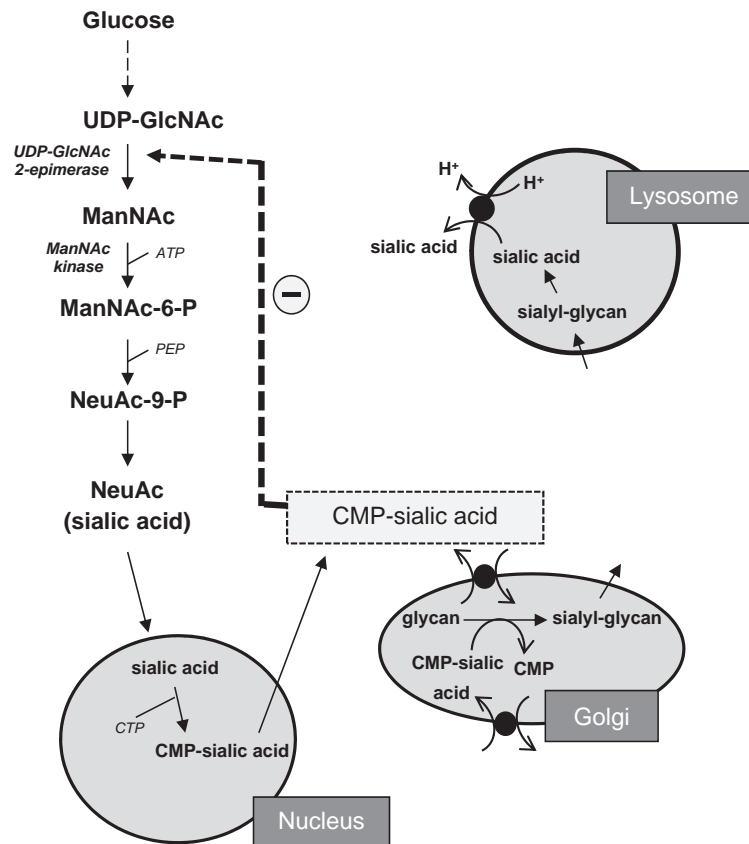
on comparative fibroblast cell fractionation studies: In sialuria, ~90% of free SA is found in the cytosolic cell fraction; that is, 50% in controls and only 10% in SASD fibroblasts, where the storage of free sialic acid is in the lysosomal fraction (165,196) as shown in the previous section.

The assay of the specific activity of the bifunctional enzyme (see further) that catalyzes the first rate-limiting and also the second step in the biosynthesis of SA, yields slightly elevated or near-normal results and is by itself almost irrelevant to the diagnosis of sialuria. Instead of demonstrating a nearly complete lack of feedback inhibition of the first biochemical reaction by CMP-Neu5Ac (CMP-sialic acid), the physiologic end product of SA synthesis, is causally associated with sialuria (Figure 103-6). This lack of allosteric inhibition, a major physiologic control mechanism, causes constitutive overproduction of free SA and was identified as the metabolic defect in sialuria (165,196,197,199). It results in highly excessive production of SA and in very elevated concentrations in the cellular cytoplasm, interstitial tissues, and body fluids such as urine. Based on data obtained by purification and characterization of the rat enzyme, Seppala et al. have cloned and sequenced the human cDNA encoding the corresponding bifunctional enzyme (Figure 103-7) (199).

The gene, termed *GNE*, has been mapped to human 9p13.3. It covers about 100 kb of genomic DNA and consists of 14 exons. Exon 1A is 20 kb upstream from the others all located closely together. Four different splice variants are transcribed from the *GNE* gene. The isoform hGNE is ubiquitously expressed (203). Two of the splice variants encode the hGNE1 protein of 722 amino acids, also called GNE/MNK. The latter symbolic term stands for uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) 2-epimerase (GNE) (EC 5.1.3.14)/*N*-acetylmannosamine(ManNAc) kinase (MNK)(EC 2.7.1.60) (165,199,204). The epimerase domain is in the amino-terminal portion of the protein (amino acids 1–378). The kinase domain is in the carboxy-terminal half (amino acids ~410–722) (Figure 103-7) (199,205).

The allosteric site resides in exon 5 within the epimerase domain. Two observations in sialuria patients are remarkable. First, all sialuria mutations are found in the heterozygous state. This prompts the conclusion that this inborn error is an autosomal dominant trait and confirms the findings in one family pedigree (196). Second, all patients so far reported have one of three MS mutations clustered very closely together: Arg266Trp, Arg266Gln, and Arg263Leu. The short stretch of nucleotides has thus been recognized as the putative allosteric site. Although the boundaries remain undefined, the allosteric site must comprise the codons 263 and 266.

Whether the sialuria in the remaining patients is due to a new mutation or inherited from one of the parents has not been explored. Because of the mild phenotype, sialuria



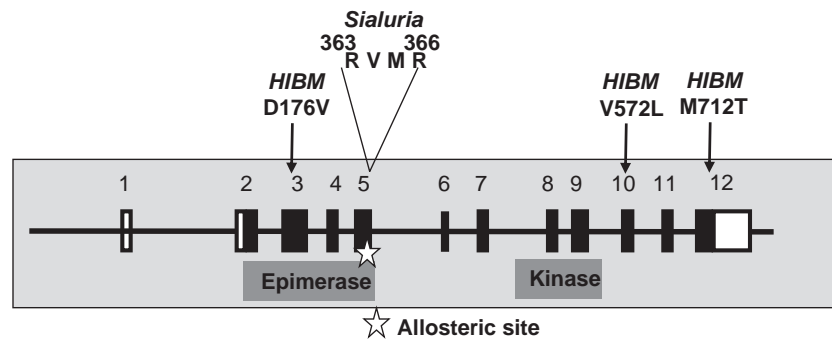
**FIGURE 103-6** Overproduction of free sialic acid in Sialuria results from failing allosteric inhibition of the rate-limiting, bifunctional GNE (UDPGlcNAc 2-epimerase and manNAc kinase) by CMP-sialic acid that cannot bind to the mutant allosteric site. This is an autosomal dominant trait. CMP-sialic acid is formed in the nucleus by CMP-sialic acid synthase. Its transport to the Golgi apparatus is assisted by a specific Golgi membrane protein. In this location it is a substrate for various sialyltransferases that sialylate complex and hybrid-type glycans among other acceptors. In sialidosis and galactosialidosis (see previous sections in this Chapter) lysosomal catabolism of these macrocompounds is impaired. Free sialic acid leaves lysosomes with the pivotal help of the transporter Sialin that if deficient by mutations blocks or impairs egress with ISSd or Salla disease as clinical result (See previous sections in this Chapter). Also the autosomal recessive hereditary inclusion body myopathy (HIBM) results from mutations in the GNE gene and deficient synthesis of sialic acid. (Figure with minor modifications by M. Huizing, reproduced from Huizing, M.; Krasnewich, D. M. *Hereditary Inclusion Body Myopathy: A Decade of Progress*. Biochim. Biophys. Acta. **2009**, 1792 (9), 881–887, with gratefully acknowledged permission from authors and publisher.)

may not be as rare a disorder as its infrequent reporting makes one assume.

The mutations generating the allosteric metabolic defect are of considerable importance in the study of the physiologic role of free SA and of sialylation in tissues. The metabolic trait is even important for the production of biologic molecules with therapeutic potential and in testing the feasibility of silencing effects by siRNA. Overexpression of GNE in CHO cells containing the allosteric mutation generating p.Arg263Leu resulted in the overproduction of SA and in significant increase of polysialic acid bound to neural cell adhesion molecules (NCAM). Persons with the rather mild entity, sialuria, may be at risk for harmful consequences on the maintenance of cerebral and/or peripheral neurologic functions (206). The GNE sialuria mutation in the model CHO-cell expression system produces recombinant human erythropoietin (EPO), a cytokine for erythrocyte precursors, and expresses homogeneous, highly sialylated EPO of

the desired therapeutic quality instead of the incompletely and heterogeneously sialylated product (207). In vitro silencing of a GNE mutation by synthetic small interfering RNAs in primary sialuria fibroblasts resulted in significantly decreased levels of free SA and thus the restoration of feedback inhibition by CMPneu5Ac (208).

**103.2.2.3 The GNE Gene and Hereditary Inclusion Body Myopathy.** Remarkably the importance of detecting the GNE gene as the cause of the probably mild phenotype sialuria is much enhanced by the finding that mutant genotypes either homozygous or compound heterozygous located anywhere outside the putative allosteric site, cause hereditary inclusion body myopathy (HIBM) (MIM # 600737), first described in 1984 by Argov in Jewish patients of Persian descent. The disorder has its onset in adulthood and is slowly progressive with distal and later also proximal muscular weakness. The lower limbs are more prominently affected, but in many patients the M. Quadriceps is more or less spared. Hence, the



**FIGURE 103-7** Diagram of the GNE protein (derived from GNE transcript isoform 1, also called transcript variant 2) (Ensemble database) (Genbank NM\_005476) with GlcNAc 2-epimerase (left) and manNAc kinase (right) domains. Star points at the location of allosteric site, defined by mutations in either amino acid R363 or R366 in sialuria patients and probably enclosing V364 and M365 as well. The intragenic location of three mutations most commonly causing hereditary inclusion body myopathy in descending order of prevalence include M712T (Persian-Jewish), V572L (Japanese/Asian), and D176V (Japanese) as indicated. (Figure reproduced with gratefully acknowledged permission from designer Dr M Huizing, NIH, Bethesda, MD, USA.)

alternate name of quadriceps sparing myopathy (see earlier references in Reference (1)). Recently, some observations regarding myopathic cardiac involvement in some HIBM patients have been reported (209,210).

Meanwhile, the mutant gene causing HIBM had been mapped to 9p12–p13 and was found to be *GNE* (211,212). The disorder called Nonaka myopathy originally described in Japan (213) has also been etiologically aligned as allelic with HIBM. None of the patients with the myopathy have sialuria. Unlike the heterozygous sialuria patient with a dominant so-called gain-of-function mutation in the allosteric site, patients with HIBM have a haploinsufficiency loss-of-function genotype. In fact, the metabolic effect of the mutations involved is the reduction of GNE-epimerase and/or MNK-kinase specific activity to 30–60% of control values in several in vitro cell types and in cell-free transcription-translation studies (214). As a monomer, GNE/MNK has no catalytic activity and requires di- or even multimerization of the nascent polypeptides in the ER in order to become the mature catalytically active protein. All mutant genotypes wherever located in *GNE* adversely affect the catalytic activity of either protein domain (203). The allelic heterogeneity in HIBM has currently been expanded beyond the 62 different mutations listed in 2009 (204,215). Three among them are found more frequently and appear to be associated with more or less defined ethnic groups: p.Met172Thr is predominantly in patients of Persian-Jewish extraction (216). p.Val572Leu and p.Asp176Val are most often identified in Japanese people (217–219) (see Figure 103-7). It is of interest that NS or frameshift effect mutations with “amorph” or “null” phenotypic consequence represent only one-sixth of the total listed and that homozygotes for mutations with “null” effect have not yet been detected among HIBM patients. Complete absence of GNE/MNK activity is probably lethal as soon observed after the embryonic stage of development in the *Gne* knockout mouse models (204).

Because of its rate-limiting role in the biosynthesis of SA, GNE/MNK is a major determinant of cell-surface glycoconjugate sialylation and a critical regulator of the function of some cell-surface adhesion molecules. Bound SA is widely distributed in normal tissues, in the consistent terminal sugar residue of N-linked complex-type glycan side chains in glycoproteins, and is an important component in gangliosides. However, the expected decrease of intra- and pericellular SA content in HIBM-derived muscle and other tissues has not yet been documented consistently. Hence, the question of an adverse role of hyposialylation of specific glycosylated proteins such as  $\alpha$ -dystroglycan and NCAM in muscle degeneration observed in this myopathy remains unresolved (220–223). Results in mouse models have however indicated that HIBM or Nonaka disease is a potentially treatable entity. In transgenic mice with the Asp176Val mutant genotype and ensuing myopathy, oral administration of ManNAc, an SA metabolite, can prevent and/or postpone muscle weakness and atrophy as well as the histopathologic features usually found in HIBM (224). In another transgenic mouse model (225), oral ManNAc treatment was able to rescue pups homozygous for the Met712Thr variant from the severe glomerular and concomitant proteinuria they had developed instead of signs of myopathy. In the much longer surviving treated mice, sialylation of podocalyxin, the major podocyte sialoprotein, had much increased. Besides the obvious potential as a preclinical treatment in humans, the ManNAc-related results are useful also in the evaluation of hypotheses regarding muscle loss in HIBM and the role of apoptosis (226). Hyposialylation of neprilysin, a metallopeptidase that normally cleaves amyloid- $\beta$ , is known to accumulate in HIBM muscle and promotes muscle degeneration (227). Animal models may be useful in solving questions about the interaction of GNE/MNK with proteins that regulate development (203) and about its probable role in mitochondrial processes (228). The recently published proteomic profile of HIBM has identified more specific



pathways affected by the mutant *GNE* genotype, including most prominent proteins involved in cytoskeleton and sarcomere organization.

Based on the view that adequate sialylation is important at least for maintaining physiologic functioning of several organs, more specifically of muscles, several interesting, careful, and scientific evidence-based efforts at *GNE* gene treatment have been reported. Expression systems have included well-defined in vitro cell strains (229) and the mouse animal model (230). The results have been expertly reviewed by Malicdan (224). It may suffice to point out the significant durable improvement in locoregional muscle function in a human subject #001 with advanced HIBM and without other therapeutic options, who, treated per compassionate investigational new drug, had received four intramuscular injections of wild-type, CMV-driven *GNE* gene plasmid vector encapsulated in cationic liposomes (*GNE* Lipoplex). The local clinical result obtained was in correlation with *GNE* transgene upregulation and local SA induction (231). Transient low-grade fever and pain at the injection site were the sole adverse effects, but proof of principle was obtained for manufacturing “clinical”-grade safe and active “*GNE* Lipoplex” and for opening the road to phase I clinical trials in patients with less-advanced HIBM.

### 103.2.3 MSD (Mucosulfatidosis)

**103.2.3.1 Challenging Clinical Phenotype.** This severe metabolic disorder with an incidence of less than  $1 \times 10^{-6}$  live births in Brazil (232), was first described by Austin in 1965, who subsequently detected the deficiency of several sulfatases, microsomal as well as lysosomal. From the metabolic vantage point, the effects of secondarily affected sulfatases place this disorder among the LSDs. The complete phenotype may be viewed as the composite of features of some of the mucopolysaccharidoses, metachromatic leukodystrophy (MLD) (MIM # 250100) and X-linked ichthyosis (MIM # 308100). The more or less complete phenotype is only observed in the rare patients with true neonatal onset (233) (see earlier references in Reference (1)). Early diagnosis is difficult because in the more “classic” patients with onset in later infancy, only a single or few aspecific symptoms or signs become the first reason of parental concern (232,234–236). They include frequent respiratory infections, hepatomegaly, inguinal herniae in males, microcephaly (233), developmental delay, mildly coarse facial features, limited mobility in hips and knees, broad thumbs and great toes (237), and moderate dysostosis multiplex detected in the radiographic skeletal survey. Even urinary excretion of GAGs and/or sulfatides may not be elevated. The disorder is inexorably progressive. Craniofacial coarsening, a consistent feature in early childhood, is less prominent than in MPS IH (MIM # 607014), but the face in these patients with increasing intellectual disability

becomes expressionless as the patient withdraws from his immediate surroundings. By the third birthday, the metabolic syndrome becomes more completely manifest and the appearance of dermal scaling or frank ichthyosis may point directly at the clinical diagnosis (234). Loss of the few acquired motor skills is unequivocal. The more advanced neurologic picture includes gradual quadriplegia, ataxia, convulsions, nystagmus, and a serious decrease of vision and hearing. The corneae remain clear on clinical inspection. The bedridden MSD patient evolves toward an average fatal outcome in mid-childhood. At present, there is still a need for more detailed clinical reporting, especially in papers that concentrate attention on the causal mutations. Variant phenotypes with truly late childhood onset have been reported (234). MSD in the fetus has been recognized by Steinmann as a rare cause of low urinary estriol during pregnancy (1,236).

**103.2.3.2 Many Sulfatases Affected and Biochemical Diagnosis.** Quantitative assay of GAGs and sulfatides in the patient’s urine may be useful, but in some variant phenotypes unexpectedly useful regarding the orientation toward the diagnosis. Formal confirmation of the diagnosis can be based on demonstrating the concomitant deficiency of several lysosomal sulfatases in peripheral leucocytes or cultured fibroblasts, more particularly of these sulfatases known to be causally related to disorders representing some differential diagnoses under consideration. These lysosomal sulfatases include arylsulfatase A (ASA) or cerebroside sulfatase (EC 3.1.6.8), arylsulfatase B (ASB) or GalNAc-4-sulfate sulfatase (EC 3.1.6.12), L-iduronosulfate sulfatase (EC 3.1.6.13), heparan-N-sulfate sulfatase (EC 3.10.1.1.), GlcNAc-6-sulfate sulfatase (EC 3.1.6.14), and GalNAc-6-sulfate sulfatase (EC 3.1.6.4). These enzymes are known to be specifically deficient in metachromatic leukodystrophy (MLD) (MIM # 250100), Maroteaux–Lamy disease (MPS VI) (MIM # 253200), Hunter disease (MPS II) (MIM # 309900), Sanfilippo A disease (MPS IIIA) (MIM # 252900), Sanfilippo D disease (MPS IIID) (MIM # 252940), and Morquio disease type A (MPS IV) (MIM # 253000), respectively. Assay of the microsomal sulfatase, Arylsulfatase C, using the natural  $3\beta$ -hydroxysteroid sulfate as substrate, an enzyme known to be deficient in X-linked ichthyosis (MIM # 308100) (EC 3.1.6.2), is also recommended as a support of the diagnosis of MSD. A total of 17 sulfatases have been identified in the human genome, 13 of which are already characterized biochemically (238,239).

**103.2.3.3 Deficient Activation of Sulfatases and Primary Genetic Defect.** More than two decades ago, in vitro complementation tests had already ruled out that any gene encoding anyone of the various sulfatases mentioned above would be the cause of MSD. Moreover, in vitro expression of “wild-type” sulfatase cDNAs in MSD-mutant cells have revealed normal synthesis of the various sulfatase polypeptides, but deficient catalytic activity and in some instances decreased stability of these

polypeptides. Expression of the same cDNAs in normal non-MSD cells yields quite active sulfatase components instead. Hence, the sulfatasases require the contribution by the product of a second gene in order to reach normal, full activity. Purified ASA subjected to trypsin digestion yields tryptic peptides separable by reverse-phase high performance liquid chromatography. Amino acid sequencing and determination of molecular mass showed that the peptide containing the amino acids 59–73 had a lower molecular mass than that predicted from the ASA-cDNA nucleotide sequence. Molecular characterization of this peptide revealed the presence at position 69, of 2-amino-3-oxopropanoic acid (C $\alpha$ -formylglycine or FGly) instead of the cysteine predicted for that particular spot. FGly turned out to be the substitute for that specific cysteine in all known eukaryotic sulfatasases. In MSD fibroblasts, the nearly inactive ASA and ASB proteins were shown to have retained the cysteine predicted by the corresponding cDNA codon. Older references to the important results of the independent research groups led by von Figura (Germany) and by Ballabio (Italy) are available in Reference (1).

Chemical modification and activation of nascent sulfatasases by the soluble formylglycine generating enzyme (FGE), also called sulfatase modifying factor 1 (SUMF1) occurs in the ER before their molecular folding and oligomerization. Purification and molecular characterization of SUMF1 have been crucial to the identification of the *SUMF1* gene (MIM # 607939) (240). Cosma et al. have identified this gene independently by microcell-mediated chromosome transfer methodology and in vitro complementation (241). The gene has been mapped to human chromosome 3p26. It spans 101 kb of genomic DNA and has nine exons. Northern blotting detected a 2.1 kb transcript in fibroblasts and in several human organs, thus providing evidence for ubiquitous expression. SUMF1-nascent protein has a molecular mass of 42 kDa. It has 374 amino acids, a number that includes a signal sequence. There is only one glycosylation site in the protein.

The results of several studies have recently shown that *SUMF1* codelivered with arylsulfatase cDNA per adeno-associated virus or lentivirus delivery into cells from patients with one of the several LSDs clearly enhances the sulfatase activity implicated. Moreover, the enhanced effect improved clearance of the intracellular GAG and sulfatide accumulation. Similar results were obtained in vivo in mouse models indicating that this type of combined treatment while providing proof of the sulfatase activating role by SUMF1 may generate beneficial effect in patients (242).

Also, useful data have become available on the intracellular location and trafficking of SUMF1, on its fine regulation, on binding to the sulfatase substrates, and on its more general at least indirect role in metabolism and cellular maintenance.

Although the canonical amino acid ER protein retention sequence KDEL is absent in SUMF1, a substantial

fraction is retained in the ER where it exerts catalytic activation on nascent sulfatasases. A part of the protein is secreted to be taken up by distant cells. To this end, that part of SUMF1 travels to the Golgi, where it is processed into a N-terminally truncated form that appears in the medium apparently for the purpose of endocytosis by other cells (1,243,244). Dynamic interaction with other proteins, such as protein disulfide isomerase (PDI), Erp44, and ERGIC 53, seems to ensure SUMF1's presence in the ER. PDI couples activation and ER retention. With Erp44, the protein is a member of the thioredox protein family and resides in the early secretory pathway. Normal SUMF1 shuttles between the ER and the Golgi as it is protected from proteasomal degradation by their physical association with ERGIC 53. The latter and Erp44 favor SUMF1's export from and retrieval by the ER, respectively (245,246).

All MS mutations encountered so far have affected strictly conserved amino acid residues. Translation of the corresponding mutant mRNAs has not really decreased. Yet, all normal amino acids appear to have a pivotal role in the stability and binding capacity of the "wild-type" protein. Mapping the substituent amino acids to the available crystal structure of "wild-type" SUMF1 (247) showed that they did not directly affect the substrate-binding groove and neither the oxygen-binding pocket nor the catalytic site in the vicinity of the redox-active cysteines. Hence, precise prediction of the pathogenic effect remains difficult. Maintaining the ER location is an important prerequisite for secretion of SUMF1, but clearly not as strict a condition for stability (236,248). Also, the active site in the various sulfatasases is their most evolutionary constrained region making them most probably the likely sole targets of their modifying factors. Gene interaction studies suggest the existence of at least two other cysteine-to-formylglycine converting mechanisms without the involvement of SUMF1 (238). By finding extensive sequence similarity, an SUMF1 paralog protein, called either SUMF2 (249) or pFGE (250), was detected that colocalizes with the active SUMF1 (FGE), has the same tridimensional structure and forms heterodimers with the latter. Obviously, either protein forms homodimers as well. pFGE lacks catalytic activity but can associate with sulfatasases. Overexpression of pFGE (SUMF2) has an inhibitory effect upon the catalysis by the active enzyme. This competitive type of binding to nascent sulfatasases has a fine regulatory effect upon the activation of sulfatasases by SUMF1.

Few if any new reports are available on the neuropathology of MSD in humans. Serial MRI of the CNS in a patient admitted to the hospital at 9 months, showed abnormally high signals in the deep and subcortical white matter. At the time of diagnosis at the age of 29 months, prompted by the appearance of ichthyosis, the patient's neurologic syndrome had much worsened including the earlier recorded MRI findings, in addition to the abnormalities in the peripheral nerves.

Fatal outcome was at the age of 4 years (251). Because of the lack of human data, pathology results in animal models are of special interest. In a mouse model generated with null mutations and complete inactivity of sulfatases, congenital growth retardation, skeletal abnormalities, significant neurologic deficits, and early mortality were recorded. There was progressive cytoplasmic vacuolization and storage of GAGs. In addition, the generalized inflammatory response in the affected animals was much enhanced as indicated by the massive presence of highly vacuolated macrophages near the sites of lysosomal storage. Microglial cells in the cerebellum and the cerebral cortex were activated and accompanied by astroglial and neuronal loss. At the age between 4 and 6 months, a significant increase of inflammatory cytokines and apoptosis marker molecules was found in the CNS and the liver (252). Lysosomal storage impairs autophagosomal delivery of bulk cytosolic content to lysosomes. Mutant mice with diseases corresponding to the human MSD or MPS IIIA had the expected severe neurodegenerative consequences, but showed also the accumulation of autophagosomes apparently because of seriously impaired autophagosome-lysosome fusion. In addition, the massive accumulation of polyubiquitinated proteins and dysfunctional mitochondria and the putative mediators of cell death were demonstrated. Hence the heuristic hypothesis is generated that LSDs are also disorders of autophagy either from the clinical onset or from some time in the later natural course (253).

An interesting effect of the biological role of SUMF1 in some wider metabolic and probably developmental fields of interest is illustrated by the results reported by Buono et al. in the mouse model (254). The concerted activities of the fibroblast growth factor (FGF), Wnt, and Notch pathways are known to balance self-renewal and differentiation processes in hematopoietic stem cells (HSCs). More adequate fine-tuning of the participating protein activities is accomplished by enzyme-mediated remodeling of the large heparan sulfate proteoglycans. SUMF1 activates Sulf 1 (MIM # 610012) (itself probably causally involved in the autosomal dominant mesomelia synostosis (MIM # 600383) in humans (255)) and Sulf2, both being sulfatases that remodel the HSPGs. The authors show that FGF signaling in mice is constitutively activated in *Sumf1*<sup>-/-</sup> HSCs and in hematologic progenitor cells that show upregulation of some specific kinases that promote  $\beta$ -catenin accumulation. This activation by FGF signaling blocks erythroid and B lymphocyte differentiation at specific stages. The animals can be rescued from this adverse effect by blocking the *Sumf1*<sup>-/-</sup> mutation.

The first carefully documented FGE genotype-phenotype correlation study in 10 MSD patients shows that a patient compound heterozygous for two “null” mutations has indeed been born alive, but as expected, had a neonatal onset and early fatal outcome after a short clinical course more severe than the one in four MS mutant homozygotes. Both residual FGE catalytic activity and protein stability

are determining the clinical phenotype but are not consistently predictable in the more classic MSD patients (236).

### 103.2.4 ML IV and Berman Disease

The name mucopolipidosis dates from the time prior to the discovery of the genetic defect, and is a misnomer because the disorder is neither clinically nor pathogenetically directly related to ML I (Sialidosis) or the GlcNAc-1-phosphotransferase deficiency disorders, ML II and ML III, all true oligosaccharidoses. However, the pathogenesis of ML IV being based physically in the membranes of the late endocytic vesicles involved in intracellular trafficking and in the PM, the disorder clearly belongs to the LSDs.

**103.2.4.1 Clinical Phenotype.** ML IV (MIM # 252650) was first observed and described by Berman et al. in 1974. In its classic, unfortunately severe form, the disorder is clinically rather homogeneous and has a high incidence in the Ashkenazi Jewish (AJ) population, but has occasionally occurred in most other ethnic groups. With onset either at birth, infancy, or early childhood, the presenting symptoms include developmental delay, hypotonia that later on may be substituted for some spasticity in the lower limbs, strabismus, corneal opacities, and visual deficit (see further). Unaided walking and effective language are achieved only in a minority of “classic” patients (256). Hand usage is significantly impaired. Growth and physical development are only moderately deficient. The level of social contact and cognition only reach the average expected in a 2-year-old child, and in most instances tend to not change significantly rather than showing signs of further deterioration. This clinical picture remains stable throughout childhood and adolescence and into adulthood. Yet, in some patients, further clinical deterioration may be observed. Since the detection of the genetic defect and the elucidation of some of its clinical effects, it is crucial for the diagnosis that the patient’s constitutive achlorhydria is being documented and that plasma gastrin levels are found to be much elevated. Biopsy specimens of the stomach mucosa consistently show selective vacuolization of the parietal cells. The ML IV patient remains axially hypotonic. There is severely impaired oromotor function and swallowing difficulty (256). The patient may show only brisk tendon reflexes or pyramidal spasticity in the lower limbs. Average life expectancy is decreased as recurrent infections and/or neurological complications represent the most probable dangers to fatal outcome. A few “negatives” need being pointed out: facial coarsening, macrocephaly, and organomegaly are not the features of ML IV.

Radiographically there are no detectable skeletal abnormalities. MRI and MRS of the brain reveal consistently a hypoplastic corpus callosum, demyelination of white matter, and increased ferritin deposited in the basal ganglia including the thalamus as reported by Frei (see

reference in Reference (1)). The degree of prominence of these findings has recently been correlated with the patient's mutant genotype (256–258).

All patients in a series of 22 studied by Smith et al. had some degree of corneal haze (sometimes lesions of erosion), optic nerve pallor, and vascular attenuation and characteristic pigment epithelial changes in the retina. The ophthalmological changes are clearly progressive. In biopsy specimens of the conjunctiva lysosomal histopathology characteristics of ML IV are readily detectable (259).

The number of patients reported with the significantly milder type of ML IV is small. Most examples are encountered in non-Jewish patients in whom growth and physical maturation are normal and neuromotor development only moderately delayed. Easy fatiguing, unsteady gait, and clumsy motor coordination may represent the main clinical complaints. Increased briskness of the deep tendon reflexes is often the only neurological finding. In some patients, a pyramidal encephalopathy is clinically more apparent (256–258). Some patients attend special education classes. However, also here the visual handicap represents the more important threat. With a somewhat more slowly progressive course, all ocular signs that supplement the classic type of ML IV, are detectable also in this variant ML IV (259) (earlier references in Reference (1)).

**103.2.4.2 Relevant Pathology Findings.** Only a few reports on postmortem findings are available (references in References (1,256)). They have shown the presence of histochemically different storage materials in different organs. In conjunctival or skin biopsy specimens, and also in cultured mutant fibroblasts as well as amniocytes, EM study revealed grossly swollen lysosomes with lamellar heterogeneous inclusions. The abnormalities were similar in cell strains derived from either the severe or the variant clinical type of ML IV, and similar also to the heterogeneous storage of gangliosides, phospholipids, and water-soluble macro compounds such as GAGs and glycoproteins, in cell strains from other LSDs with known enzyme defect (260). The in vitro results pointed at a possible defect in the normal function in the membranes of vesicles engaged in endocytotic intracellular trafficking. Meanwhile, mutual complementation tests had supported the conclusion that all ML IV patients have mutations in the same gene.

**103.2.4.3 Molecular and Metabolic Defects.** Genetic linkage studies aiming at mapping the mutation causing ML IV in large Ashkenazy–Jewish families, resulted in locating the causal gene to chromosome 19p13.2–p13.3 and to its cloning and molecular characterization (261–263). The gene termed *MCOLN1* contains 14 exons and spans over 13kb of gDNA. The cDNA sequence has a 1740bp open reading frame predicting an encoded protein of 580 amino acids. Northern blotting in human tissue showed that *MCOLN1* mRNA is ubiquitously expressed in adult and fetal tissues.

It was soon evident that over 95% of AJ patients, all with severe ML IV, have only two mutations in *MCOLN1* considered founder mutations (264) in this ethnic group. The affected are either homozygous for one or compound heterozygous for either. The most frequent mutation, also called the “major” mutation in AJ people, is the 486-2A>C transition in the acceptor site of the third intron. It results in skipping entirely exon 4 in the transcript and the creation of a stop codon further downstream. Premature termination of the translation yields a much truncated and inactive protein. The “minor” mutation, second in frequency among AJ-affected families, is a large genomic deletion of over 6.4 kb. It begins upstream of *MCOLN* and runs as far as into exon 7. It has been designated also as the “c.511del6434” mutation and results in complete loss of the protein encoded by the normal gene. These two “founder” mutations were detected in 73% and 23% of chromosomes in AJ patients (262). Effective and reliable short-cut methodology has been set up in order to screen AJ populations (265,266). Dozens of other *MCOLN1* mutations, most of them in non-AJ patients have been reported (256–258,262,267).

**103.2.4.4 Mucolipins as “Cation Channels” and ML IV Pathogenesis.** The protein encoded by *MCOLN1* called mucolipin 1 shows much structural similarity to proteins in the transient receptor potential (TRP) superfamily of cation channels, mostly Ca<sup>2+</sup> channels. As has been established for this group of proteins, mucolipin1 has six transmembrane domains. Both the N- and the C-terminus reside in the cytoplasm. Therefore, according to convention, mucolipin1 is a multiple pass type III integral membrane protein (molecular model in Reference (256)). A functional stretch of amino acids, located between transmembrane regions 5 and 6 in the TRP cation channel, has been identified as the channel pore domain. The functions of other domains in mucolipin1, at present appropriately called TRP mucolipin 1 (TRPML1), such as a serine lipase domain remain largely unknown (268). In vitro studies of mutant ML IV fibroblasts with documented heterogeneous storage in the lysosomal cell compartment and the accumulation of autophagosomes and fragmented mitochondria indicate that complete and/or partial loss of the several physiologic functions of TRPML1 causes the severe, chronic LSD, ML IV, without the direct involvement of any lysosomal hydrolase. The key question of why the dysfunction of this complex integral membrane protein more specifically causes the impressively serious encephalopathy and ophthalmopathy, is at present receiving its first answers.

The identification of *MCOLN1* soon led to the detection of two closely related genes, both located on chromosome 1 (261). All three encode largely similar integral membrane proteins and are known as TRP mucolipins (TRPMLs) 1, 2, and 3, respectively. TRPML1 is exclusively located in late endosome/lysosome vesicular membranes (269). Its main but not



exclusive function is that of a  $\text{Ca}^{2+}$  channel as shown by electrophysiology studies. It is modulated by intracellular changes in  $\text{Ca}^{2+}$  concentration and by the local low pH. It plays a key role in the biogenesis of lysosomes, a function that is strongly conserved throughout nature (270) (see also references in Reference (268)). Calcium signaling is disturbed in ML IV fibroblasts apparently because TRPML1 channels are unable to release  $\text{Ca}^{2+}$  ions sufficiently with impaired fusion of late endosomes and lysosomes as well-documented consequence (271–273). The TRP channels are required also for efficient fusion of autophagosomes with lysosomes. In addition to the accumulation of autophagosomes, increased amounts and even aggregates of p62 are measured in ML IV cells. This fact suggests abnormal accumulation of ubiquitinated protein inclusions (269). TRPML1 can function also as a  $\text{Fe}^{2+}$  channel. Its failing as such may explain the insufficient plasma  $\text{Fe}^{2+}$  in a sizable minority and the frank microcytic iron deficiency anemia in a smaller proportion of ML IV patients (see Reference (256) and references in Reference (268)). TRPML2 and TRPML3 (mutations in *Trpml3* cause deafness in varitint-waddler mice) both located in the PM and primarily in vesicles along the endocytic pathway, are bound to share functions with TRPML1. Because the three closely related channel-forming proteins form hetero- and homomeric complexes that are crucial regulators of cell viability and of in vitro starvation-induced autophagy (274), all TRPML channel proteins are somehow involved in ML IV's pathogenesis. RNA interference with *MCOLN1* closely mimics the in vitro ML IV phenotype in HEK-293 cells, where chelatable zinc is stored in the accumulated membranous vacuoles. This has been found also in ML IV fibroblasts, but not in controls. The zinc-66 isotope is known to be markedly elevated in brain tissue of TRPML<sup>-/-</sup> mice, not in that of healthy mice. Loss of TRPML1 function also generates dyshomeostasis of intracellular chelatable zinc and is likely to contribute to the ML IV type encephalopathy (275). Also, chaperone-mediated autophagy (CMA) is deficient in ML IV fibroblasts, where the amount of LAMP-2A (lysosome-associated membrane protein 2A) is significantly reduced. TRPML1 may be a docking site for intralysosomal chaperones allowing them to pull substrates for CMA more efficiently (276). As has been noticed also in the previous section on MSD, lysosomal storage itself impairs fusion of autophagosomes with lysosomes (253).

Recent molecular and cell biology studies in cells and animal models representing more than one delineated entity point to the common component of impaired autophagy as of significant importance in the pathogenesis of the clinically most vulnerable CNS phenotype in many of the LSDs. Hopefully improved scientific insight will lead to the design of more rational and effective causal treatment.

### 103.2.5 Pyknodysostosis

As a rare osteosclerotic OCD apparent from early childhood, first described and delineated in 1962 by Maroteaux and Lamy, PKD (MIM # 265800) is characterized by the following physical features: short-limb type, short stature, disproportionally large skull with frontal and occipital bossing, late or never closing fontanelles, rather proptotic eyes, small slightly beaked nose, and receding chin. Often a midline-grooved ridge is present in the palate. Tooth eruption is delayed. A double row of teeth may be present. The enamel is hypoplastic. Early and extensive caries are often noticed. Dental extraction predisposes to recurrent and refractory osteomyelitis of the mandibula that requires skillful surgical intervention. The shortened fingers have a drumstick appearance. Nearly all patients have a history of multiple bone fractures.

At first, the skeletal radiographs reveal only mild and atypical bone density changes that in later childhood become more sclerotic. Many Wormian bones are detectable in the calvarium in addition to open cranial sutures and still wide fontanelles. The mandibular angle is obtuse, the facial bones proportionally small, and the metaphyses of tubular bones undermodeled. The patients have coxa valga. The acromial ends of the clavicles are underossified. Such type defects may also be found in some vertebrae. The terminal phalanges of fingers and toes may also have signs of hypoplasia, but paradoxically in this sclerotic OCD, osteolytic bony lesions as well. Bone density increases with age, but often-radiographic surveys of patients reveal signs of old bone fractures (39). The latter recur frequently in PKD even without or following minor trauma (277–282) (see also earlier references in Reference (1)). Despite the rather unique phenotypic features, the diagnosis has come late in some patients.

Osteoclasts (OCs) that appear normal under light microscopy have an enlarged pericellular zone of demineralization. When studied by EM, these cells contain large, abnormal cytoplasmic vacuoles that contain collagen type I fibrils. Mutant OCs apparently contribute normally to the demineralization process, but do not effectively degrade the organic matrix of bone. The study of bone biopsy specimens (282) from two unrelated PKD patients with severely osteosclerotic skeleton showed multinucleated osteoblasts next to areas of demineralized bone matrix consisting of undigested collagen. Bone trabecular structure was highly disorganized and the decrease of bone remodeling was readily apparent. Apparently normal cathepsin K exerts balancing power over bone turnover. Its deficiency results in profound deterioration of bone quality.

The high proportion of consanguineous families among the PKD probands points to the rarity of the disorder (incidence estimated to be around  $1/75 \times 10^5$ ) and supports the autosomal recessive mode of inheritance. Several families with multiplex patient involvement are

known. The gene has been mapped to 1q21 (references in Reference (283)) characterized subsequently, termed the cathepsin K gene, and labeled *CTSK* (MIM # 601105). It covers 12.12 kb of genomic DNA and contains eight exons, the first one of which is not transcribed. Its expression is most intense in bone (especially osteoclasts: OCs), cartilage, and in skeletal muscle. Human *CTSK* cDNA produces a 1692 b transcript predicting a polypeptide of 329 amino acids (37kDa: molecular mass). The nascent pre-propeptide comprises, as is typical for the cysteine proteinases, a 15-amino acid N-terminal signal peptide, a larger pro-polypeptide with 99 amino acids with a single putative glycosylation site (see earlier references in Reference (1)). The monomeric proenzyme is targeted to the lysosomal compartment via its sole M6P glycan recognition marker and its binding to the MPR receptors (see Section 103.1.4.4). It is surprising that the proenzyme undergoes full maturation and activation only at its target location. The pH-activity profile of cathepsin K, a lysosomal cysteine proteinase most intensely expressed in OCs, is wide with an optimum at pH 6.1. It is a strongly collagenolytic protease with also strong elastase and gelatinase activities. However, the catalytic functions on these important proteins with role mainly in the ECM do not adequately explain the finding of bone sclerotic aspects next to the osteolytic ones in PKD.

Recent work with a the new 129/Sv *ctsk*<sup>-/-</sup> knock-out mouse model that truly imitates the human disorder instead of producing osteopetrosis only has been most revealing in support of explaining both osteolysis and osteopetrosis in the same organism. The findings are most probably relevant for the human PKD as well (284). Mutant *ctsk*<sup>-/-</sup> OCs lacked normal apoptosis and senescence and exhibited both in vivo and in vitro overgrowth. These phenomena were obviously associated with an unusually high number of OCs. The different effects of cathepsin K activity on the skeleton is due to site-specific variation of bone homeostasis, such as osteopetrosis in the tibiae in constant bone turnover during growth on the one hand and osteolysis in calvaria and terminal phalanges, where actual growth comes to a complete halt much earlier. Cathepsin K plays a key role in OC apoptosis and senescence. This work brings the first evidence of the importance of OC senescence in bone homeostasis, of its relevance to the full pathogenic mechanism, and points to the potential as treatment target in PKD (284).

This section ends by referring to the interesting report of promising long-term results in three unrelated PKD patients obtained by a long-term growth hormone treatment program longitudinally monitored by both growth rate measurement and circulating IGF-I assay. All patients have reached near normal stature and skeletal proportions after 12, 6.5, and 5 years, respectively, of personalized treatment. They were homozygous for different *CTSK* MS mutations (285). The treatment

protocol has followed recommendations listed in the consensus statement on diagnosis and the treatment of children with idiopathic short stature (286).

### 103.2.6 NAO Syndrome

The three separately delineated multicentric osteolysis syndromes, Winchester syndrome (MIM # 277950), Torg syndrome (MIM # 259600), and the more recently delineated nodulosis–arthropathy–osteolysis (NAO) syndrome (MIM # 605156) (287,288) have multicentric osteolysis with cutaneous nodule formation and arthropathy as hallmark clinical features. The disorders present in childhood with osteolytic lesions in peripheral parts of the skeleton and nodule formation in the dermal skin layer mainly of the foot soles. The increasing physical disability results from the skeletal changes, but at least as much by progressive contraction and deformity of the small and large joints and by arthralgia. There are some reports of coarsening facial features. In familial consanguineous patients reported from Saudi Arabia with what was called NAO syndrome (MIM # 605156) (287–289), causal involvement of mutations in the matrix metalloproteinase 2 (*MMP2*) gene, located at 16q13, was formally proved. This proteinase, also called gelatinase, is composed of 610 amino acids. Subsequently, also in patients with multicentric osteolysis and with what had been called either Winchester, Torg syndrome, or NAO in several other places in the world, MS, NS, and/or other molecular mutations in the *MMP2* gene (MIM # 120360) were detected (290–297). The NAO syndrome had already deserved a place as Winchester–Torg syndrome in the *Atlas on Bone Dysplasias* by Spranger et al. (39). Detailed radiographic and clinical data on 14 patients in 9 families were offered by Al-Otaibi (298). The NAO syndrome was also presented as differential diagnosis in a large study of familial arthropathy in Saudi Arabian children (289).

The reports on cyclic IV treatment with pamidronate render inconsistent conclusions so far. In seven children with NAO syndrome, aged between 5 and 14 years treated every 3 months during 1 year, conventional radiographs showed increased skeletal mineralization. Bone mineral density in the lumbar spine had increased and the excretion of cross-linked *N*-telopeptide type I collagen had gradually decreased. Limb and joint pain had decreased, but improvement of functional ability was not statistically significant. Also, plasma alkaline phosphatase and osteocalcin levels remained unchanged. The period of treatment was relatively short (299). Phadke et al. (300) have treated two siblings with NAO syndrome for 3 years. There was no clinical improvement although bone mineral density had improved in the axial skeleton. Osteoporosis and osteolysis continued to worsen in the appendicular skeleton.

Clearly, there are several other forms of more or less isolated or syndromic multicentric osteolysis that are not

caused by *MMP2* mutations (301). Discussion of the differential diagnosis falls beyond the scope of this chapter. There is currently an *Mmp2*<sup>-/-</sup> mouse model available enabling effective research on the molecular pathogenesis of NOA (302).

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## RELEVANT WEBPAGES

OMIM (Online Mendelian Inheritance in Man): <http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim>, Including Mapviewer: <http://www.ncbi.nlm.nih.gov/mapview/>.  
 UCSC Genome Browser: <http://genome.ucsc.edu>.  
 GeneTests; GeneReviews: <http://www.ncbi.nlm.nih.gov/sites/GeneTests/review?db=Genetests>.

HGMD (Human Gene Mutation Database): <http://www.hgmd.cf.uk/ac/index.php>.  
 E! Ensemble Genome Browser: <http://www.ensembl.org/index.html>.  
 Orphanet Journal of Rare Diseases: <http://www.ajrd.com/>.  
 Gene Cards Version 3: <http://www.genecards.org/>.  
 Enzyme Nomenclature: <http://www.chem.qmul.ac.uk/iubmb/enzyme>.

## Biography



Jules G Leroy MD, PhD, a native and citizen of Belgium, graduated from Ghent University Medical School, Ghent, Belgium, in 1959 and obtained his MS degree in Biochemistry in 1961 from the Ghent University Graduate School.

He trained as a fellow in Pediatrics at Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts, from 1962 till 1964 and in 1968. As a Helen Hay Whitney Foundation fellow, he was a graduate student in Genetics (major) at the Department of Genetics, University of Wisconsin, Madison, Wisconsin, and in Pediatrics (minor) (Department of Pediatrics) (1964–1967). He obtained his PhD degree in Genetics in 1967. Dr Leroy's thesis was on "Studies on the genetics of the Hurler syndrome and related disorders," which contained the discovery and initial delineation of Mucopolidosis II (I-cell disease).

As an associate professor at the University of Antwerp, Belgium (1967–1983), he was the founder and initial director of the Division of Genetics and subsequently promoted to professor and chairman of the Department of Medical Genetics at Antwerp (1973–1983).

Dr Leroy returned to the University of Ghent as professor and chairman of Pediatrics at the University Children's Hospital (1983–1999) and also headed the Department of Medical Genetics between 1984 and 1995.

Obligatory retirement according to Belgian law came in 1999. Since 2000, he has spent periods of four to six months a year as a visiting professor at the Department of Molecular and Human Genetics, Baylor School of Medicine, Houston, Texas (2001–2006). He is still a "visiting senior scholar" during annual periods of two months at the Greenwood Genetic Center, Greenwood, South Carolina. At both locations, he was and is involved in clinical research, teaching, and writing.

Dr Leroy's main professional and scientific interests include inborn errors of metabolism, especially "lysosomal storage disorders"; studies on intellectual disability and pediatric neurology; congenital malformation syndromes and skeletal dysplasias; and studies on genotype–phenotype correlation in monogenic disorders.

Dr Leroy has contributed to well over 200 original publications and review articles in peer reviewed scientific journals or in books.

# CHAPTER

# 104

## Sphingolipid Disorders and the Neuronal Ceroid Lipofuscinoses or Batten Disease (Wolman Disease, Cholesteryl Ester Storage Disease, and Cerebrotendinous Xanthomatosis)

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### GLOSSARY

**Ceramide** – ceramide is the fundamental structural unit common to all sphingolipids. It is a fatty acid chain attached through an amide linkage to sphingosine

**Chaperone therapy** – this therapy consists of enzyme inhibitors at suboptimal concentrations that are able to shuttle the mutant enzymes through the secretory pathway, protecting them from degradation

**Enzyme replacement therapy** – intravenous, intracerebral, or intraventricular delivery of intact recombinant enzyme protein as a form of treatment for a storage disorder due to a mutant enzyme defect

**Glycosphingolipids** – these are ceramides with one or more sugar residues joined in a  $\beta$ -glycosidic linkage at the 1-hydroxyl position

**Sphingolipids** – a class of lipids derived from the aliphatic amino alcohol sphingosine attached to a fatty acid and an R headgroup

**Sphingomyelin** – sphingomyelin has a phosphocholine or phosphoethanolamine molecule with an ester linkage to the 1-hydroxy group of a ceramide

**Substrate reduction therapy** – oral therapy using inhibitors of sphingolipid synthetic enzymes to compensate for the absent-to-low degradative enzyme with the goal of reducing the storage of accumulating sphingolipids in the peripheral and the central nervous systems

### 104.1 INTRODUCTION

Tremendous advances have been made in sphingolipidoses and the various forms of Batten disease over the past

decade. In sphingolipid diseases, of note is the description of the first “loss-of-function” mutation of a key synthetic enzyme for gangliosides, GM3-synthase, in humans (1). We presume that many more defects in synthetic enzymes will be described in the coming years.

A large number of Batten disease genes have been identified, with others on the verge of discovery. Most exciting is the improved understanding of the pathobiology and cell biology of these genetic diseases, which at last is providing real opportunities for therapy. Enzyme replacement therapy (ERT) for Gaucher disease (GD) and Fabry disease (FD) is now a common occurrence. Substrate reduction therapy (SRT) has been approved for Gaucher and may also prove beneficial for the gangliosidoses and FD. Enzyme inhibitors at suboptimal concentrations are able to shuttle the mutant enzymes through the secretory pathway, protecting these enzymes from degradation (chaperone therapy), hence raising residual enzyme activities, and being evaluated for specific gene mutations. Early, presymptomatic bone marrow (BM) replacement is of proven benefit for asymptomatic Krabbe disease and has helped patients with juvenile or later onset forms of metachromatic leukodystrophy (MLD). Rational therapies based on underlying biochemical processes, such as the use of phosphocysteamine (Cystagon®) to overcome the abnormal thio-ester linkage in the infantile form of Batten disease (infantile neuronal ceroid lipofuscinosis (INCL)), are under study. Antiapoptotic therapies such as the drug flupirtine maleate (Katadolon®) are being considered for the treatment of the various forms of Batten disease. Virally mediated gene therapies have given encouraging results in animal models, but much work must be done before use in humans becomes a reality. Stem cell therapies, in spite of the ongoing controversies, are being proposed for use in the late infantile form of Batten disease and other diseases as well. In spite of the positive and hopeful mood of this introduction, let me add that these novel therapeutic interventions are far from perfect and real-life medicine is messy (2). Frequently, children with these diseases are often not ascertained until considerable and non-reversible neurological impairment has occurred. Offering or denying these therapies to families both pose challenging ethical and human problems beyond the scope of this chapter. Avoiding these diseases through genetic screening programs in populations at risk, newborn screening for treatable disorders, and providing genetic counseling and prenatal diagnosis for pregnancies at risk remain the best remedies that we have to lessen the impact of these diseases on families and society.

## 104.2 GM1-GANGLIOSIDOSIS (β-GALACTOSIDOSIS)

### 104.2.1 Introduction

Decreased or absent activity of the lysosomal hydrolase, acid β-galactosidase, can result in one of the two diseases: GM1-gangliosidosis or Morquio disease type B. We discuss GM1-gangliosidosis primarily in this chapter. The

GM1-gangliosidoses are a group of autosomal recessive lysosomal storage diseases, characterized by accumulation of GM1-ganglioside, oligosaccharides, and keratan sulfate (3). Morquio disease, type B, which will be discussed elsewhere, is due to allelic mutations in the acid β-galactosidase gene. Patients have bony disease and corneal clouding, but no involvement of the central nervous system (CNS).

### 104.2.2 Clinical Presentation

There are three clinical types of GM1-gangliosidosis according to the age of disease onset. The classic infantile form (type I) is characterized by onset before 6 months, and death by 2 years of age. This type combines the features of neurolipidosis (neuronal death and macular cherry-red spots) and features of mucopolysaccharidosis (MPS) (hepatosplenomegaly and cardiomegaly, mild dysostosis multiplex). Initial presentation is the failure to thrive and decreased feeding. These children develop gum hypertrophy, macroglossia, corneal clouding, a protuberant abdomen (4), flexion contractures, kyphoscoliosis, frontal bossing, coarse facies, and hypertelorism. Neurologically, these patients are retarded, hypotonic, have an exaggerated startle response to noise, and suffer from generalized seizures. Death ensues at about 2 years due to pneumonia and/or cardiopulmonary failure (Figure 104-1). The juvenile form of GM1-gangliosidosis (type II) presents, usually before the second year of life, as a progressive psychomotor retardation. A triad of ataxia, dystonia, and spasticity of increasing severity develops and death ensues by 20 years of age. They have a lower incidence of organomegaly, skeletal diseases, and cherry-red spots (5). The adult form of GM1-gangliosidosis (type III) is characterized by a later onset during childhood or adolescence with symptoms of dementia, Parkinsonism and dystonia; visceral, macular, and bony involvements are rare. Age at death is variable (6). Few cases of GM1-gangliosidosis presenting with nonimmune hydrops fetalis (NIHF) are reported, with the onset of symptoms as early as 23 weeks of gestation. GM1-gangliosidosis rarely has a prenatal presentation, which usually is fetal or neonatal ascite. Storage



**FIGURE 104-1** Infant with GM1 gangliosidosis and hepatosplenomegaly.

material in Kupffer cells, sinusoidal obstruction and subsequent portal hypertension or hypoproteinemia due to hepatocellular dysfunction, are hypothesized as possible explanations. Other presentations include intrauterine growth restriction (IUGR) and, rarely, pleural effusion and renal tubular dysfunction (7). Severe glycolipid deposition in lymphoreticular organs can induce various complications, as reported in a 5-year-old patient with late infantile GM1-gangliosidosis who developed rare respiratory and intestinal complications. These included common bile duct dilatation due to compression by lymph node swelling of the head of the pancreas and ileus. Adenoidal hypertrophy can lead to obstructive apnea and bronchial compression can result from hilar lymph node swelling (8). More than a dozen cases of extensive dermal melanocytosis in association with GM1-gangliosidosis have been reported. Findings of large, ectopic, and extensive Mongolian spots in newborns may lead to early detection and treatment. Before irreversible organ damage occurs, identification of at-risk families and prevention of complications are needed (9,10).

### 104.2.3 Diagnosis/Testing

Measuring  $\beta$ -galactosidase enzyme activity in leukocytes is diagnostic for affected patients, but is not a good screening method for carriers (11). Quantitative or qPCR assays offer a sensitive, rapid, and reproducible technique for allelic discrimination. These assays lead to rapid diagnosis of gangliosidoses in cats and Shiba dogs and provides a means for large-scale screening for the carrier state, accelerating the eradication of these diseases from feline and canine populations (12,13). This is applicable to humans where there is a prevalence of common alleles. Based on dog studies, amnion and placenta are not useful enzyme sources in GM1-gangliosidosis because of the risk of misdiagnosis as canine placenta contains maternal cells (14). DNA from amniocytes, on the other hand, is a good template for genotyping. Vacuolation of lymphocytes and histiocytes is present as with other lipid storage diseases. Histiocytosis is common in the liver and spleen. Lipid accumulation is noted in BM cells, in neurons, and rectal ganglion cells from rectal biopsies. Radiographs show changes characteristic of dysostosis multiplex. Vertebral deformities are remarkable for hypoplasia and anterior beaking of vertebral bodies in the thoracolumbar region. Bone age is delayed. Computed tomography (CT) and magnetic resonance imaging (MRI) show prominent atrophy, white matter demyelination with or without basal ganglia changes, and enlargement of the ventricular system. There may be increased density of the thalami and reduced intensity of basal ganglia on CT. MRI shows hyperintensity of thalami and basal ganglia on T2-weighted images and hypointensity on T1-weighted sections suggesting a delay in myelination. Cranial imaging findings are quite similar in GM1-gangliosidosis types I and II (15). In the adult form, the putamen may show increased intensity on T2-weighted scans. Accumulation

of GM1-gangliosides in neurons can increase tissue viscosity. This can also sometimes lead to hypointensity of thalami on T2-weighted images (16). A peculiar MRI appearance was recently described in one patient with GM1-gangliosidosis and correlated with postmortem pathology. It consisted of radially oriented stripes of normal signal intensity within diffusely abnormal cerebral white matter. Histopathologic studies confirmed that the stripes represented relative sparing of myelin in the perivenular regions (17).

Proton magnetic resonance spectroscopy (MRS) shows decreased *N*-acetyl aspartate to creatine (NAA/Cr) and increased choline to creatine (Cho/Cr) ratios reflecting relative paucity of axons and neurons, and increased levels of myoinositol, suggestive of gliotic white matter changes (18,19). Cerebral fluorine-18 labeled 2-fluoro-2-deoxyglucose positron emission tomography (PET) shows decreased uptake (20). Electroretinography (ERG) is normal. Visual evoked responses (VERs) peak latencies are delayed. Electroencephalography (EEG) shows nonspecific slowing with epileptogenic foci.

### 104.2.4 Pathology

All patients with GM1-gangliosidosis store GM1-ganglioside in neurons, but only type I patients display coarse facial features, hepatosplenomegaly, prominent skeletal abnormalities (21), and cardiomyopathy. Neuronal cell death and the development of connective tissue and skeletal abnormalities are largely attributable to the intracellular and extracellular storage of galactose-terminal oligosaccharides. There is diffuse brain atrophy in patients with early-onset GM1-gangliosidosis. Neurons are filled with numerous membranous cytoplasmic bodies. As for demyelination, myelin deficit in type I seems to be due to abnormal axoplasmic transport, perhaps consequent to massive neuronal storage of GM1 (22). It also appears that CNS inflammation may play a role in the pathogenesis of GM1- and GM2-gangliosidosis with evidence for microglia/macrophage activation (23). Histiocytes with distended cytoplasm, filled with a fine granular material, are observed in the following organs: liver, spleen, lymph nodes, and thymus (8,24). There is impaired elastic fiber assembly by fibroblasts from type I GM1-gangliosidosis patients, which may explain connective tissue abnormalities (25). Corneal opacities have been described in Shiba dogs with GM1-gangliosidosis and are likely to be the sequel of abnormal accumulation of neutral carbohydrates in the lysosomes of corneal keratocytes. This accumulation induces keratocyte dysfunction and swelling, finally resulting in the irregular arrangement of collagen fibrils in the corneal proper substance (26).

### 104.2.5 Biochemistry

All the three forms of GM1-gangliosidosis are caused by a deficiency in  $\beta$ -galactosidase activity. The latter is a glycoprotein containing 7.5–9% carbohydrate. The terminal



galactose in most sphingolipids is cleaved by  $\beta$ -galactosidase, except for galactosylceramide (GalCer) and galactosyl sphinganine. Acid  $\beta$ -galactosidase is a lysosomal hydrolase that normally removes terminal beta-linked galactose from glycoconjugates, like GM1-ganglioside, generating GM2-ganglioside (Figure 104-2). These patients accumulate GM1, keratan sulfate, asialo-GM1, lyso-GM1, and oligosaccharides in their tissues.  $\beta$ -Galactosidase is also deficient in Morquio disease, type B.  $\beta$ -Galactosidase could be found as a monomer, dimer, and/or in a multiprotein complex with neuraminidase (sialidase) and a protective protein. The protective protein is a glycoprotein that is associated with  $\beta$ -galactosidase and neuraminidase in the lysosome, stabilizing the former and activating the latter (27). Differences in synthesis, posttranslational modification, and degradation of  $\beta$ -galactosidase can give rise to different clinical phenotypes of  $\beta$ -galactosidase deficiency (Morquio syndrome, type B, or GM1-gangliosidosis). GM1-ganglioside is four times higher than normal in the gray matter of diseased patients. Asialo GM1 exceeds normal values by up to 20 times. In type III GM1-gangliosidosis, lipid accumulation is limited to the caudate and putamen. In the liver, only a small amount of GM1-ganglioside accumulates, whereas the levels of keratan sulfate are 50 times higher than what is expected.

One theory about neurodegeneration in GM1-gangliosidosis implicates progressive depletion of iron as demonstrated in mouse brain tissue. Key regulators of iron homeostasis, hepcidin and IL-6, are increased in

gangliosidosis mice. Mouse brain transferrin was reduced, accompanied by a progressive inability of the brain to acquire iron from the circulation. Expression of the transferrin receptor was upregulated reciprocally. Iron administration prolonged survival in the diseased mice by 38%, with delayed disease onset and preserved motor function (28).

Abnormal activation of autophagy accompanied with mitochondrial alterations in a murine model of GM1-gangliosidosis has also been implicated. Modulation of autophagy and restoration of mitochondrial functions may be of therapeutic benefit in this disease (29).

The  $\beta$ -galactosidase gene can give rise to two transcripts: GLB1 and elastin-binding protein (EBP). Also, the accumulation of keratan sulfate in the setting of primary GLB1 mutations or defects can give rise to secondary defects in elastogenesis. Impaired elastogenesis, due to a secondary deficiency of EBP and keratan sulfate storage, was demonstrated in fibroblasts from a juvenile GM1-gangliosidosis patient (30).

### 104.2.6 Genetics

The gene responsible for GM1 gangliosidosis has been identified and is located on chromosome 3p21.33. The mode of inheritance is autosomal recessive. Molecular genetic analysis revealed heterogeneous gene mutations in all clinical forms of GM1-gangliosidosis with no clear genotype–phenotype correlation evident. Neither the

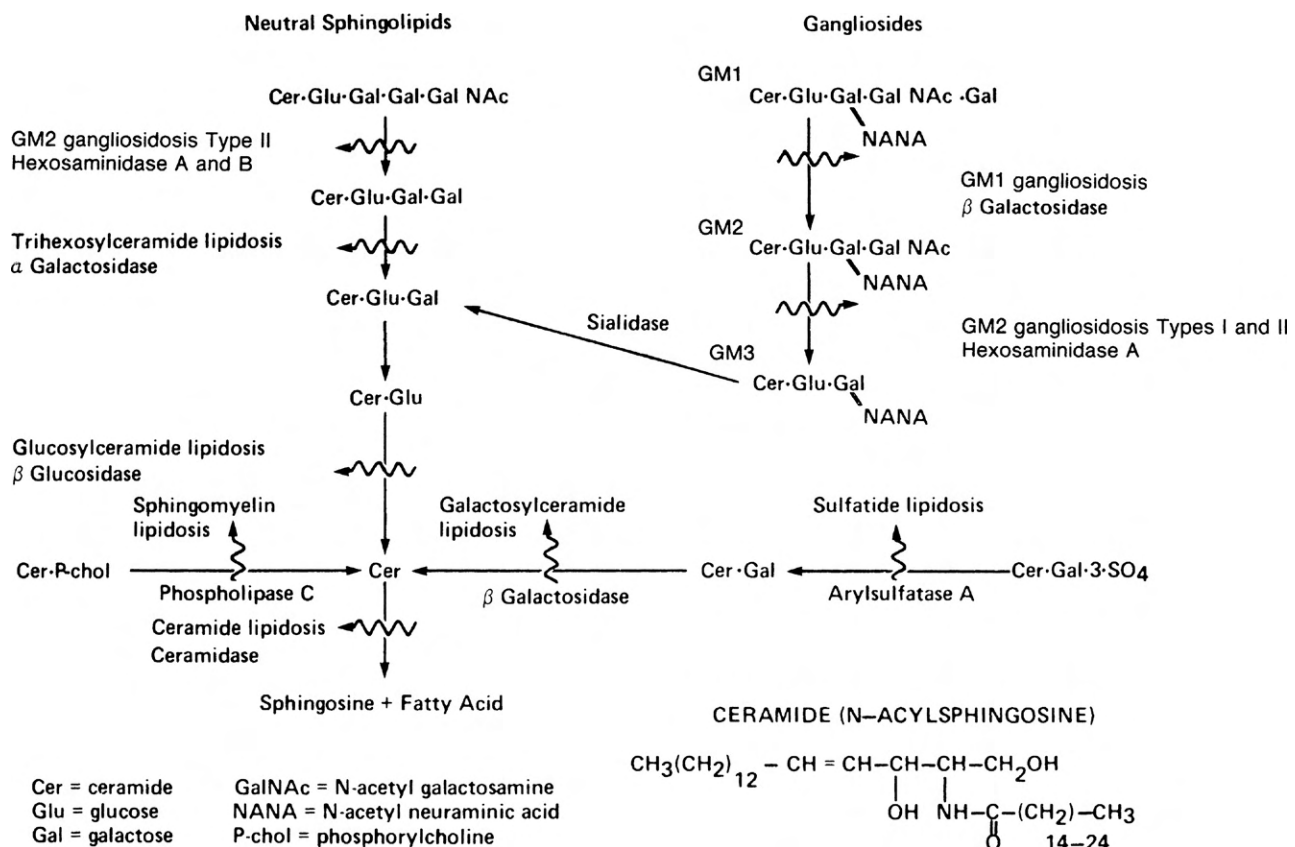


FIGURE 104-2 Sphingolipid and ganglioside degradation defects.

type nor the location of the mutation in the gene correlates with the clinical picture. Cardiac abnormalities have been correlated with specific mutations. Patients with cardiac involvement are homozygous for certain mutations. Several mutations have been described, many in Japanese patients. A common mutation in Japan, R201C, results in a juvenile or adult variant, and the molecular defect affects interaction with the protective protein (6). Other mutations exist in Brazilian patients. An Italian mutation affects synthesis (R482H), and one in North American and Puerto Rican cases (R208C) also affects synthesis (31). The p.Arg148Cys and the p.Pro549Leu mutations are found in a homozygous state in patients with the infantile form of the disease and are correlated with a severe phenotype. The p.ArgR201His mutation is predicted to be responsible for the juvenile phenotype (32). In one case presenting as NIHF, the molecular analysis of the  $\beta$ -galactosidase gene (*GLB1*) revealed a previously unreported splicing mutation (IVS1+2 insT) in a homozygous state (33). Three novel genetic mutations in the *GLB1* coding region identified in Chinese patients with GM1 were correlated with disease severity (34). Four mutations correlated to a distinct GM1-gangliosidosis phenotype due to their occurrence in homozygous patients. The mutations directly related to infantile (c.245+1G>A, p.I181K, and p.C230R) and juvenile GM1 (p.C127Y), respectively. The patient homozygous for the mutation (p.C230R) had early and severe neurological symptoms (severe spasticity, cherry-red spots) and poor cognition, lack of cardiac dysfunction, absence of hepatosplenomegaly, and minor bony disease. Also, certain missense and nonsense mutations led to mislocalization of the mutant protein (35). The Arg482His mutation in the  $\beta$ -galactosidase gene is responsible for a high frequency of GM1-gangliosidosis carriers in a Cypriot village (36). Two novel mutations were identified in four patients of Arab descent. The splice-site mutation in the first two siblings was associated with the severe infantile type I disease, whereas the missense mutation in patient 3 was associated with the juvenile type II form of GM1-gangliosidosis. The mutation analysis of the human  $\beta$ -galactosidase gene (*GLB1*) in both the siblings revealed a homozygous mutation in intron 8; the normal nucleotide sequence of CTTgtgagt was replaced by CTTgtgggt (IVS8+4A>G). This mutation causes a splicing defect. A silent polymorphism was also found in exon 1, as well as homozygous T→C transition at nucleotide 29 (29T>C), which results in Leu→Pro substitution at amino acid 10 (L10P). A missense mutation of the human *GLB1* gene due to a homozygous G→T transversion at nucleotide 451 in exon 4 (451G>T) results in Asp→Tyr substitution at amino acid 151 (D151Y) (37). R59H is a founder *GLB1* mutation leading to infantile GM1-gangliosidosis in the Gypsy population. The R59H carrier rate is more than 2% in the general Gypsy population and greater than 10% in the Rudari subisolate. Haplotype analysis suggests that the Gypsy diaspora may

have contributed to the spread of this mutation to South America (38). Mutational analysis was performed in 19 patients from South America, mainly from Argentina. Two were of Gypsy origin. Of the 22 mutations found, 14 mutations were novel, including five deletions. Four were small, while one was a large deletion that included exon 5. All but four unrelated patients were classified as types II, III, and II/III (two cases). The two type II/III patients bore the p.R201H mutation, while the adult patient bore the new p.L155R. The juvenile patient bore two novel mutations: p.S434L and p.G554E. The two Gypsy patients were homozygous for the p.R59H mutation, previously described in other Gypsies. Analysis of another 19 unrelated South American patients uncovered 38 alleles. Fourteen mutations were novel, seven missense (p.G134V, p.L155R, p.L162S, p.S434L, p.D491Y, p.P549L, and p.G554E), one a nonsense mutation (p.W576X), one a donor splice-site mutation (c.106811G.T), and five were deletions (c.435\_440delTCT, c.845\_846delC, c.1131\_1145del15, c.1706\_1707delC, and c.458-401\_55211033del1529) (39). Mutation analysis on 30 GM1-gangliosidosis and five Morquio B patients of Spanish origin was performed and 30 different causative mutations identified, 21 of which were novel. The novel mutations p.T420K and p.L264S were associated with the adult and the juvenile forms, respectively. In addition, the novel mutation p.Y83C was associated with Morquio B disease. Of these 30 patients, six were of Gypsy origin (Roma). Moreover, those six Gypsy patients shared the same mutation (p.R59H) indicating a founder effect. Screening of the p.R59H mutation first is therefore appropriate in suspected GM1-gangliosidosis patients of Gypsy origin (40). The p.Arg595Trp mutation, frequent in the Basques population (3.2%), partially reduces  $\beta$ -galactosidase activity (41). Four other (S54N, C230Y, T329A, R442Q) and two known (R59C, R201H) mutations in the *GLB1* gene of three GM1-gangliosidosis patients represent a clinical range of the disorder that include infantile, juvenile, and adult phenotypes. In spite of mutations in *GLB1*, the infantile patients have low EBP due to defective assembly, hence considered a primary EBP defect. In the juvenile variant, EBP levels were low due to the accumulation of keratan sulfate. In summary, *GLB1* and EBP proteins, altered in function and/or distribution, contribute differently to the specific clinical manifestations of patients with mutations in the *GLB1* gene. The missense mutations detected in these patients confirm the genetic heterogeneity of GM1-gangliosidosis (30). The lack of EBP of primary and secondary origin has been linked to impaired elastogenesis in GM1-gangliosidosis patients. A reduction in EBP is linked to cardiac involvement in infantile GM1-gangliosidosis patients. *GLB1* and EBP mRNA levels were both reduced in three patients carrying splicing defects (32,36). Cardiac involvement is also reported in patients without mutations affecting the EBP protein. This may be explained by glycosphingolipid (GSL)

storage, with the inhibition of secondarily involved lysosomal enzymes and the presence of undegraded products which accumulate, such as keratin sulfate (KS). As observed in patients affected by MPS IV A (Morquio syndrome), the presence of elevated urinary glycosaminoglycan, KS in particular, could explain the patients' specific cardiac abnormalities (32). The assessment of EBP levels may prove helpful in early prognostic assessment of and decisions regarding therapy. An updated catalog of mutations of the *GLB1* gene is found in a recent review (42).

### 104.2.7 Animal Models

Naturally occurring animal models of GM1-gangliosidosis have been identified in cats, dogs (43), sheep (11), and calves. A GM1-gangliosidosis mouse model has been created through the targeted disruption of the  $\beta$ -galactosidase gene (44). Animal models show CNS manifestations. Animal models are a versatile system to test gene therapeutic approaches for GM1-gangliosidosis (45).

### 104.2.8 Therapy

Therapy is supportive. Competitive inhibitors of  $\beta$ -galactosidase in vitro can serve as stabilizers of mutant proteins in the cell serving as a chemical chaperone: *N*-octyl-4-epi-beta-valienamine and *N*-octyl beta-valienamine (NOEV) lead to a remarkable correction of enzymatic activities in cultured fibroblasts derived from some patients with GM1-gangliosidosis (46–48). NOEV, given orally to a mouse model for juvenile GM1-gangliosidosis, traversed the blood–brain barrier (BBB), increased  $\beta$ -galactosidase activity, reduced the storage of GM1 and GA1-ganglioside, and prevented neurological deterioration within a few months (49,50).

Galactose was also proposed as a potential therapeutic agent for this disease in combination with GSL synthesis inhibitors or other chemical chaperones. Galactose may be utilized to synthesize chemical derivatives with chaperone properties, as reported for the NOEV chemical chaperone (51).

Anti-inflammatory drugs may prove to be effective or helpful in the treatment of GM1 in conjunction with other treatment modalities as inflammation appears to contribute to this disease (23,52,53).

SRT is being evaluated for a number of sphingolipidoses. The goal is to decrease the rate of GSL biosynthesis to counterbalance the impaired rate of catabolism. The imino sugar *N*-butyl-deoxygalactonojirimycin (NB-DGJ), a competitive inhibitor of ceramide glucosyltransferase, reduces neonatal brain ganglioside content in a mouse model of GM1-gangliosidosis (54). This suggests that NB-DNJ may prove to be an effective early intervention in GM1-gangliosidosis. Ganglioside reductions had no adverse effects on behavior or CNS development (38). The imino sugar drugs NB-DNJ and NB-DGJ both inhibit glucosylceramide synthase (GlcCerS) (21,55) the enzyme that

catalyzes the first step in GSL biosynthesis. These drugs therefore have the potential to be used to treat all lysosomal storage diseases resulting from the storage of glucosylceramide or GluCer-based GSLs (20). These imino sugars can cross the BBB (56) and, therefore, may be used to treat GSL storage diseases with CNS manifestations. A limitation is that non-GSL substrates will continue to accumulate. NB-DGJ was better tolerated than NB-DNJ, due to the intrinsic gastrointestinal tract dysfunction that was exacerbated by NB-DNJ. Functional improvement was greatest with NB-DNJ treatment because of its additional anti-inflammatory properties (52).

Intracerebral injection of adeno-associated virus (AAV) in mice resulted in the elevation of enzyme activity throughout the CNS and normalization of GSL storage. Survival was significantly longer in AAV-treated GM1 mice than in untreated mice. The motor performance of AAV-treated GM1 mice, however, declined over time at a rate similar to that observed in untreated GM1 mice (57,58)  $\beta$ -Gal-expressing BM-derived cells selectively migrate to the CNS under a gradient of chemokines and can become a source of enzyme to neurons in patients with GM1-gangliosidosis (59). Bone marrow transplantation (BMT) was attempted unsuccessfully in juvenile GM1-gangliosidosis (60). Fetal brain cells (FBCs), BM-derived mesenchymal stem cells (MSCs), and mixed FBCs and MSCs were injected into the ventricle of newborn  $\beta$ -galactosidase knockout mouse brain with no engraftment detected at 6 months (61).

Certain cerebrospinal fluid (CSF) biomarkers could be utilized to evaluate the efficacy of novel therapies proposed for this disease (62). Periodic studies demonstrated that GM1-ganglioside concentration, activities of aspartate aminotransferase and lactate dehydrogenase, and concentrations of neuron-specific enolase (NSE) and myelin basic protein in CSF were significantly higher in dogs with GM1-gangliosidosis than those in control dogs, and correlated with age and clinical course.

## 104.3 GM2-GANGLIOSIDOSIS

### 104.3.1 Introduction

GM2-gangliosidoses are a group of lipid storage diseases caused by mutations in one of the three recessive genes, HEXA, HEXB, and the gene for GM2-activator protein (GM2A), that lead to the accumulation of GM2-ganglioside. Three different gene products are necessary for the degradation of GM2-ganglioside. These genes code for the HEXA ( $\alpha$  subunit of  $\beta$ -Hexosaminidase A on chromosome 15), HEXB ( $\beta$  subunit of  $\beta$ -Hexosaminidase A on chromosome 5), and the GM2A on chromosome 5. A defect in any one of these proteins can lead to the accumulation of GM2-ganglioside and clinical disease (63).

### 104.3.2 Clinical Presentation

**104.3.2.1 Infantile Acute GM2-Gangliosidosis.** Clinical phenotypes of the acute infantile forms of the



GM2-gangliosidosis are essentially indistinguishable. These are infantile Tay–Sachs disease (TSD) due to defects in the *HEXA* gene, Sandhoff disease due to defects in the *HEXB* gene, and the ABO variant due to defects in the *GM2A* gene. Affected infants appear normal at birth. The earliest sign of the disease is mild motor weakness, beginning at about 2 months of age. There is nonspecific slow down of growth and dullness of responses to outside stimuli. An exaggerated startle response to noise or hyperacusis is observed at an early stage. Regression and loss of already acquired psychomotor skills becomes obvious. There is progressive weakness and hypotonia with loss of gross motor skills such as sitting and crawling before 10 months of age. Seizures occur several months after the onset. Ophthalmoscopic examination often reveals macular cherry-red spots (Figure 104-3). Progression of the disease is rapid as the infant becomes less responsive to parents and surroundings. Hyperacusis, vision deterioration, and seizures are common by the end of the first year of life (64,65). The electroencephalogram shows rapidly progressive deterioration until death (66). Visually evoked potentials are abnormal, whereas the electroretinogram (ERG) is consistently normal. Macrocephaly, due to reactive cerebral gliosis, begins by 18 months of age. Further deterioration leads to decerebrate rigidity, difficulty swallowing, increasing seizure activity, and eventually a vegetative state. Death is often caused by aspiration pneumonia by the age of 4 years.

Neuroimaging studies are characterized by low density in the thalami, basal ganglia, and cerebellum on CT (67), and high signal intensity on T2-weighted images by MRI. Enlargement of the caudate nucleus is observed as the disease progresses. Brain atrophy is present in advanced disease (68). A decrease in T2 signal intensity in the thalami has also been described (16). MRI imaging modality diffusion-weighted imaging indicates that the apparent diffusion coefficient decreases in the *HEXB*–/– mouse several months before the appearance of the symptoms and could potentially be useful in assessing the severity of presymptomatic Sandhoff's disease (69). Fluorine-18 fluorodeoxyglucose-positron emission tomography (FDG-PET) scan may be a useful tool in detecting the extent of the brain lesion as it shows decreased uptake



**FIGURE 104-3** Tay–Sachs disease: fundus photograph of cherry red macula.

in affected brain regions (70). Proton MRS also is a valuable tool in monitoring disease progress and response to therapy. Results have documented a progressive and significant elevation in myo-inositol/creatine (mI/Cr) and choline/creatine ratios. A decrease in the *N*-acetyl aspartate + *N*-acetyl aspartate glutamate/creatine (NAA/Cr) ratios and decrease in the *N*-acetyl aspartate/creatine ratio have also been reported. Metabolic alterations were supportive of demyelination, inflammation, gliosis, and neuronal loss in the neuropathological process. Elevation in mI is more sensitive than other metabolite alterations (71,72). *N*-acetylhexosamine is a brain metabolite and a specific marker for Sandhoff's disease indicating accumulation of hexosamine-containing oligosaccharides (73). This interpretation is supported by a recent in vitro MRS study of a Sandhoff mouse model (74).

**104.3.2.2 Late-Onset Forms.** Disease onset can be at any time from the late infantile period to adult age. There is involvement of the deeper brain structures and less of cortex in comparison to the overwhelming gray matter involvement in the infantile form. Manifestations include dystonia, ataxia, spinocerebellar degeneration, and lower motor neuron disease. In the adult-onset form of TSD, an amyotrophic lateral sclerosis-like or ALS-like motor neuron involvement and psychosis develop (75–78). Rarely, it may mimic primary lateral sclerosis (79). Late-onset forms of GM2-gangliosidosis can occur as variant forms of TSD or Sandhoff disease. GM2-activator deficiency only results in infantile GM2-gangliosidosis, indistinguishable from infantile TSD or Sandhoff disease (80). Some medications, particularly haloperidol, risperidone, and chlorpromazine, have been reported to result in neurologic worsening in late-onset forms of the disease (81). In most cases, the later onset forms of expression result from the presence of at least one allele (usually the G269S mutation) associated with residual enzyme ( $\beta$ -hexosaminidase A) activity. A positive family history is a valuable clue, enabling early diagnosis. Nonspecific cerebellar atrophy on brain imaging is another important finding. This entity should be considered in patients with speech, gait, and balance problems, and with psychiatric disorders even when focal neurologic deficiencies are absent (82). Isolated stutter is sometimes the sole initial manifestation of late-onset TSD years before developing other manifestations (83). Changes in metabolites on MRS closely reflect clinical features. Follow-up examination by MRS is useful for evaluation of neuronal changes, even prior to major anatomical changes associated with late-stage clinical disease (74). MRS might be a sensitive method for detecting and quantifying neuroaxonal injury and monitoring response to emerging treatments even in the absence of radiologic abnormalities on conventional MR imaging (84).

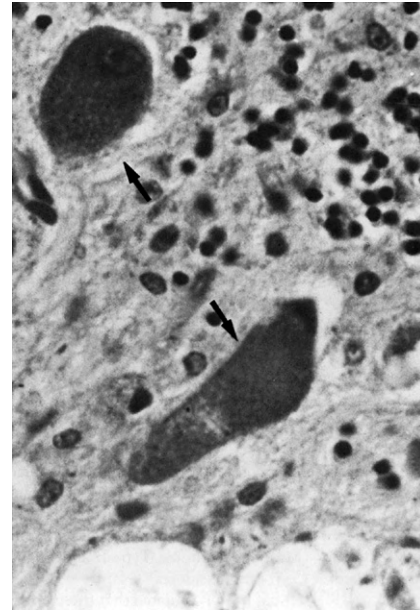
**104.3.2.3 Subacute GM2-Gangliosidosis.** Generally, ataxia develops between 2 and 10 years of age. Developmental regression and dementia are leading features of this variant. Progressive psychomotor deterioration, increasing spasticity, and seizures develop by the end of the first decade of life (80). Optic atrophy and retinitis



pigmentosa may be seen late in the course of this condition, and are the main reasons for visual loss. A vegetative state with decerebrate rigidity develops by 10–15 years of age, followed by death, usually precipitated by aspiration pneumonia. In some cases, the disease takes on a particularly aggressive course, with death occurring between 2 and 4 years of age (85). This subacute phenotype has been described in patients with HEXA or HEXB mutations.

**104.3.2.4 Chronic GM2-Gangliosidosis.** Clinical onset is anywhere from childhood to adulthood (75). Variations in clinical manifestations and clinical course are extreme. TSD variants are more common than Sandhoff disease variants. Dystonia and extrapyramidal signs are common. The clinical phenotype is similar in some cases to that seen in Friedreich's ataxia. Cerebellar signs can be the major symptoms (78,86). In some cases, prominence of cerebellar symptoms, along with spasticity and muscle wasting, suggest an atypical form of Friedreich's ataxia with normal or increased deep tendon reflexes (87). A number of older patients have a pattern of muscle wasting and weakness with fasciculation and secondary skeletal abnormalities indistinguishable from late-onset spinal muscular atrophy or Kugelberg–Wielander disease (88). In others, the condition resembles ALS. In some cases of chronic Sandhoff disease, autonomic nervous system involvement is pronounced and may include achalasia (89). Most patients with late-onset GM2-gangliosidosis have upper rather than lower motor neuron abnormalities. Electromyography (EMG) findings are consistent with chronic active denervation with re-nerivation. Nerve conduction velocities are usually normal, and muscle biopsy shows denervation atrophy. EEG is generally unremarkable, and rectal biopsy shows the membranous cytoplasmic bodies (MCBs) seen in the severe clinical variants of GM2-gangliosidosis. Neuroimaging studies demonstrate severe cerebellar atrophy with some cerebral atrophy (88). Psychiatric abnormalities are relatively common, affecting as much as 40% of patients with the disease. This may be the most notable feature of the condition, and can range anywhere from depression to schizophrenia.

**104.3.2.5 Pathology.** Due to gliosis, brain weight and volume increase during the second year of life. Cystic degeneration of the cerebral white matter, along with atrophy of the cerebellar hemispheres, is frequently present. In the brain, neuronal number is diminished, and remaining neurons appear ballooned due to the stored material (Figure 104-4). Glial cells are also engorged. Spinal anterior horn cells are affected. This explains the hypotonicity, hyporeflexia, and weakness. Lipid-laden macrophages are present in liver, spleen and lung. Electron microscopy reveals lipid-laden lysosomes located throughout the cytoplasm of neurons. In late-onset GM2-gangliosidosis, storage is preferentially localized to the thalamus, substantia nigra, cerebellum and brainstem nuclei. This is a progressive neurodegenerative disease caused by GM2-ganglioside accumulation in lysosomes giving rise to membranous cytoplasmic bodies or MCBs,



**FIGURE 104-4** GM2 gangliosidosis brain with ballooned neuron.

which also contain cholesterol and sphingolipid. There is apoptosis of vulnerable neurons. Phagocytic clearance leads to chronic inflammation involving elevated cytokine production and reactive oxygen/nitrogen species. A compromised BBB amplifies inflammation even further and promotes secondary oxidative stress, leading to apoptosis and neurodegeneration. Jeyakumar et al. demonstrated that microglial/macrophage activation plays a key role in the pathological process in mouse models for GM1-/GM2-gangliosidoses (23).

Both late endosomes and lysosomes, not early endosomes, have a higher density in Sandhoff fibroblasts. These have an intracellular distribution of terminal endocytic organelles different from the perinuclear punctate pattern observed in normal fibroblasts and endocytic vesicles are larger. This alteration in the terminal endocytic organelles of Sandhoff fibroblasts suggests the involvement of this compartment in the disruption of cell metabolic and signaling pathways leading to the pathological state (90). Autopsy studies of late-onset GM2-gangliosidosis are sparse with one adult case on record. The autopsy findings show severe accumulation of lipid and regressive changes in the anterior horns of the spinal cord. Less-severe storage was found in other spinal cord neurons, brainstem and selected basal ganglia. Cerebral cortex was virtually spared from storage but was the site of excessive lipofuscin, also present in many other neurons in the CNS. The involvement of sensory neurons was prominent and potentially related to allodynia. Marked storage and ganglionic loss were also found in the dorsal root ganglia, and the fasciculus gracilis was severely depleted of myelinated fibers. Electron microscopy showed homogeneously accumulated gangliosides in the form of single and coalescing zebra bodies, contrasting with heterogeneity of neuronal inclusions found

in other chronic cases. In Sandhoff mice, neuronal cells and pigment epithelial cells are vulnerable to excess ganglioside more than other retinal cells, as demonstrated by cytoplasmic inclusions and vacuolations (91).

**104.3.2.6 Biochemistry.** The defect in GM2-gangliosidosis is caused by the failure of  $\alpha$ -hexosaminidase to hydrolyze the terminal amino sugar from GM2-ganglioside. Disease severity correlates inversely with the amount of residual enzyme activity. The enzyme exists as two isoenzymes,  $\beta$ -hexosaminidase A (HEXA) and  $\beta$ -hexosaminidase B (HEXB). HEXA ( $\alpha\beta$ ) is a thermolabile protein composed of a dimeric structure of  $\alpha$  and  $\beta$  subunits, whereas HEXB ( $\beta\beta$ ) is a heat-stable dimeric structure composed of two  $\beta$  chains. A GM2A is essential for the hydrolysis of GM2-ganglioside. HEXA and HEXB hydrolyze *N*-acetylhexosamine from glycoproteins, glycolipids, glycosaminoglycans and oligosaccharides. HEXA metabolizes GM2-ganglioside. HEXB metabolizes globoside as well as the compounds listed previously. In humans, GM2 is degraded in the lysosome by  $\beta$ -hexosaminidase A, which removes the *N*-acetyl-galactosaminyl group. Yet, the water-soluble enzyme cannot hydrolyze the membrane-bound GM2 due to steric hindrance by the membrane surface. GM2-A is necessary to present the GM2-ganglioside lipid to the water soluble enzyme for terminal sugar hydrolysis. The hexosaminidases and GM2-A are glycoproteins that are synthesized in the endoplasmic reticulum (ER) lumen and processed in the Golgi. They are transported via the mannose-6-phosphate receptor to the lysosome. Both  $\alpha$  and  $\beta$  subunits possess an active site, yet dimer formation, as HEXA ( $\alpha\beta$ ), HEXB ( $\beta\beta$ ), and HEX S ( $\alpha\alpha$ ) are required for catalytic activity. The  $\alpha$  and  $\beta$  subunits exhibit different substrate specificities. The  $\beta$  subunit preferentially hydrolyzes neutral water-soluble substrates. The  $\alpha$  subunit also hydrolyzes negatively charged substrates such as GM2-ganglioside, dermatan sulfate, and chondroitin sulfate. The primary amino acid sequences of  $\alpha$  and  $\beta$  chains share a great degree of primary structure homology. Also, the gene structures of HEXA and HEXB are similar with respect to the number and placement of intron/exon junctions (64,92). Twelve of the 13 introns interrupt the genes at corresponding positions. The GM2 activator gene is 16 kb long and contains four exons.  $\alpha$ -Subunit defects result in HEXA deficiency (TSD and juvenile and adult-onset GM2-gangliosidosis);  $\beta$  subunit defects affect HEXA and HEXB (Sandhoff's disease), and defects in the GM2-A results in normal HEXA and HEXB activities with accumulation of GM2-ganglioside (the ABO variant). In the B1 variant, described mainly in Portuguese patients, there is a defect in the  $\beta$  catalytic site. Enzyme activity loss in the latter variant can only be demonstrated when a sulfated artificial substrate is used to assay enzyme activity. Loading studies of fibroblasts with radiolabeled gangliosides can help identify all these variants, but is particularly useful for identifying the B1

and ABO variants (93). In GM2-gangliosidosis, GM2 is markedly increased in the nervous system, representing ~90% of neutral tissue gangliosides. GM2 is also stored in the liver and spleen. Asialo and Lyso forms of GM2 are also increased in brain tissue of GM2-gangliosidosis patients. Lyso compounds are cytotoxic and are also present in the brains of patients affected by MLD, Krabbe disease and Gaucher disease. The acute forms of GM2-gangliosidosis are associated with complete HEXA deficiency and early death. Enzyme activity levels of ~5% are present in the juvenile and adult forms. Some unaffected individuals have only 10% enzyme activity compared with normal controls. Sandhoff and Conzelmann have estimated that 5–10% of normal HEXA activity levels represent a “critical threshold” below which disease exists (94).

Astrocytes were isolated from the neonatal brain of Sandhoff disease model mice in which the *N*-acetyl- $\beta$ -hexosaminidase  $\beta$  subunit gene is genetically disrupted (ASD). Results indicated that the upregulation of extracellular signal-regulated kinase (ERK) phosphorylation and the increase in proliferation of ASD astrocytes were dependent on GM2/GA2 accumulation. The phosphorylation of ERK in ASD cells was not dependent on extracellular growth factors. Treatment of ASD astrocytes with recombinant *N*-acetyl- $\beta$ -hexosaminidase A resulted in a decrease of their growth rate and ERK phosphorylation. These findings may represent a mechanism linking nerve cell death and reactive gliosis in Sandhoff disease (95).

Another theory about neurodegeneration in GM2-gangliosidosis implicates altered iron homeostasis. Progressive depletion of iron in brain demonstrated in mice seems to correlate with neurodegeneration. Key regulators of iron homeostasis, hepcidin and IL-6, are increased in gangliosidoses mice. In the brain, the principal iron transport and delivery protein transferrin is reduced, as is the ability of the brain to acquire iron from the circulation. Expression of the transferrin receptor was upregulated reciprocally. Administration of iron prolonged survival in the diseased mice by up to 38%, with onset of disease delayed and motor function was preserved (28).

There is a functional role for Sphingosine-1-phosphate (S1P) synthesis and receptor expression in astrocyte proliferation leading to astrogliosis during the terminal stages of neurodegeneration in Sandhoff disease mice. When sphingosine kinase 1 (Sphk1) is deleted in Sandhoff disease mice, a milder disease course occurred, with decreased proliferation of glial cells and less-pronounced astrogliosis. Astrocyte responses are involved in many types of neurodegeneration, and the sphingosine kinase 1/sphingosine-1-phosphate (Sphk1/S1P) receptor signaling axis may be important during the pathogenesis of neurodegenerative diseases (96).

There is accumulation of  $\alpha$ -synuclein in brain tissue of some lysosomal storage diseases, in both neurons and glial cells.  $\beta$ -Synuclein was detectable only in the pons of Sandhoff disease cases. This differential accumulation of

$\alpha$ - and  $\beta$ -synucleins in human lipidoses may be related to functional differences between these two proteins (97).

**104.3.2.7 Genetics.** The majority of mutations cause the severe infantile onset disease. Several mutations are responsible for certain subtypes of HEXA deficiency. The B1 subtype includes HEXA mutations in the catalytic  $\beta$ -subunit domain. They do not interfere with the synthesis and the activity of the HEXA heterodimer, but the enzyme is inactive toward the sulfated artificial fluorescent substrate used to determine enzyme activity, 4MUGS, as well as the natural substrate, GM2-ganglioside. Mutations causing the chronic disease phenotypes result in an unstable protein that fails to associate with the  $\alpha$  subunit or is otherwise incompletely processed. HEXA pseudodeficiency is due to a point mutation that leaves HEXA with lower activity toward the artificial substrate, but with sufficient GM2-ganglioside hydrolyzing activity to escape disease.

TSD occurs more commonly in Ashkenazi Jews, French Canadians from eastern Quebec, Cajuns from Louisiana, and Pennsylvania Dutch. About 1 in 3600 Ashkenazi Jews are at risk for TSD. However, TSD is not only restricted to these groups, but occurs in all ethnic groups. Juvenile TSD has no ethnic predominance. Inheritance is autosomal recessive. TSD is genetically heterogeneous, and multiple allelic mutations have been described. Even in single ethnic groups, multiple mutations exist. More than 75 mutations of the gene for the  $\alpha$  subunit are known. These mutations can result in wide variations in residual enzyme activity, extent and distribution of GM2-ganglioside accumulation in the brain and spinal cord, and great diversity of clinical phenotypes. An online database enumerating alleles, mutations, phenotypes, and authors is available. Only four mutations have been documented in the gene for the GM2A.

Gene sequencing detected four novel mutations, three of which are predicted to be disease causing [118.delT, 965A3T (D322V) and 775A3G (T259A)]. Six novel mutations are also described: one large deletion of 2406 nt (c.299+1471\_408del2406), one frameshift mutation c.965delT (p.I322fsX32), one nonsense c.1372C>T (p.Q458X), and three splicing mutations (c.299G>T, c.300-2A>G and c.512-1G>T) (98). A homozygous missense HEXB mutation (p. D459A) was discovered in six patients with a rare juvenile variant disrupting a salt bridge between aspartate D459 and arginine 505 at the subunit interface. R505 mutations are reported in late-onset Sandhoff disease (99).

A novel c.1556A>G transition in exon 12 of the *HEXB* gene associated with chronic Sandhoff's disease changes a conserved aspartic acid to glycine at position 494 of the Hex  $\beta$ -subunit. This A>G transition affects both *HEXB* mRNA processing and biochemical properties of the  $\beta$  subunit (100).

Of 31 Italian patients, 22 with the infantile, acute form of TSD and 9 with the subacute juvenile form, biochemically classified as B1 variant, are reported: Of the 29 different alleles identified, 14 are due to 15 novel mutations, two being *in-cis* on a new complex allele (101).

In patients with the Tay-Sachs variant, the presence of R178H and R499H mutations was predictive of an early onset and rapidly progressive course. The presence of either G269S or W474C mutations was associated with a later onset of symptoms along with a more slowly progressive disease course (102).

A 2-year-old black child with juvenile-onset disease, who presented with abnormal eye movements and cherry-red spots of the maculae, was a compound heterozygote (M1V/Y37N) of the *HEXA* gene. The M1V mutation was previously described in an African-American child with acute infantile GM2-gangliosidosis. The Y37N mutation is novel. This combination of mutations is consistent with the juvenile-onset disease, and provides further evidence for the association of the M1V mutation with individuals of black ancestry. The presence of oculomotor abnormalities is an unusual finding in this form of GM2-gangliosidosis, and adds to the phenotypic spectrum (103).

Gene sequencing is useful in identifying rare mutations in patients with TSD and their families, in evaluating spouses of known carriers for TSD who have indeterminate enzyme analysis and are negative for common mutation analysis, and in resolving ambiguous enzyme testing results (104).

### 104.3.3 Diagnosis and Carrier Detection

With the development of specific enzymatic and molecular diagnostic methods, ascertainment of carriers and affected patients can be achieved with readily available tissues or body fluids, such as serum, tears, chorionic villi, skin fibroblasts, leukocytes and buccal mucosa cells. In a given family, with a known mutation, specific prenatal and postnatal diagnosis of the heterozygous, homozygous, or compound heterozygous state, can be made by direct DNA analysis. The availability of rapid and inexpensive methods for identification of carriers has made it possible to develop programs for screening of populations at risk. Furthermore, coupling this with DNA-based diagnostics allows distinction between infantile, subacute, and chronic disease mutations, as well as pseudodeficiency alleles that do not result in disease. Carrier frequencies are 0.006 for *HEXA* mutations and 0.0036 for *HEXB* mutations. Among Ashkenazi Jews, the carrier frequency for *HEXA* mutations is as high as 0.033. Successful screening programs targeting Ashkenazi Jewish groups have resulted in a drastic reduction in the incidence of this fatal disease in this population by almost 90%. The adult-onset form, which presents with lower motor neuron symptoms and signs of psychosis, has primarily been described in those of Ashkenazi Jewish descent. They carry the common Ashkenazi mutation on one allele, and a milder mutation on the other allele. It is this second allele that defines the less severe phenotype. Other groups at high risk for TSD include Pennsylvania Dutch, Louisiana Cajun, and French Canadian populations. Epidemiologic studies have found a high carrier rate up to 1/52 in Irish Americans (105). Current



guidelines limit testing to selected high-risk groups and may miss potential carriers, despite the availability of an inexpensive enzyme screen. More than 100 mutations in the hexosaminidase A gene have been identified, some associated with later onset or chronic forms. HEXA enzyme analysis, HEXA DNA common mutation assay, and *HEXA* gene sequencing improve the sensitivity for carrier detection in non-Ashkenazi individuals. Sensitivity of TSD carrier detection is 91% for gene sequencing compared with 91% for the enzyme assay and 52% for the common DNA assay. Gene sequencing combined with enzyme testing had the highest sensitivity (100%) for carrier detection. This has important implications if TSD population screening is expanded to non-Ashkenazi Jewish populations (104).

Molecular analysis yields a false negative rate of 11.4% with a sensitivity of 88.6%. Due to changing demographics of the Ashkenazi Jewish population, molecular testing alone is not sufficient and biochemical analysis should be the assay of choice. Platelet *HEXA* enzyme assay is associated with a very high detection rate (1 in 29; 3.4%) and a very low inconclusive rate (0.4%). Platelet assay is superior to serum with an inconclusive rate that is 40-fold lower (106).

An accurate and fast isotope dilution tandem mass spectrometry method for the simultaneous measurements of GM1 and GM2 in human CSF samples measures ganglioside content in the CSF of patients with TSD, including presymptomatic cases. This assay is important for evaluating therapeutic effects of ganglioside elimination (107).

Prenatal diagnosis of TSD disease is accomplished within 24–48 h from sampling. The preferred strategy is to simultaneously carry out enzymatic analysis in the amniotic fluid (AF) supernatant or chorionic villi and molecular DNA-based testing of an AF cell-pellet or chorionic villus sample (CVS) (108). If the affected alleles are known from previous affected siblings or pregnancies, qPCR assays offer a sensitive and rapid technique for allelic discrimination (13).

Based on the preceding information, the Committee on Genetics makes the following recommendations:

- (1) Screening for TSD should be offered before pregnancy if both members of a couple are of Ashkenazi Jewish, French-Canadian, or Cajun descent. Those with a family history consistent with TSD also should be offered screening.
- (2) When one member of a couple is at high risk (of Ashkenazi Jewish, French-Canadian, or Cajun descent or has a family history consistent with TSD) but the other partner is not, the high-risk partner should be offered screening. This is particularly important if there is uncertainty. If the woman is already pregnant, it may be necessary to offer screening to both partners simultaneously to ensure that results are obtained promptly and that all options are available to the couple.

- (3) Biochemical analysis should be used for individuals in low-risk populations.
- (4) If TSD biochemical screening is performed in women who are pregnant or taking oral contraceptives, leukocyte testing must be used.
- (5) Ambiguous screening test results or positive screening test results in individuals should be confirmed by biochemical and DNA analysis for the most common mutations. This will detect patients who carry genes associated with mild disease or pseudodeficiency states. Referral to a geneticist may be helpful in these cases.
- (6) If both partners are determined to be carriers of TSD, genetic counseling and prenatal diagnosis should be offered (109).

Education and counseling are clearly important components of screening, and issues of privacy and confidentiality must be addressed, as well as insurability and employment. Conflict of interest by entrepreneurial scientists, clinicians, institutions and other advocates of widespread screening are also potential concerns (110).

### 104.3.4 Animal Models

GM2-gangliosidosis has been reported in dogs (111), cats, mice (112), and pigs. They all exhibited neuronal storage, but visceral storage was only found in the feline model.

Feline GM2-gangliosidosis is unique among large animal models of disease in the number and variety of mutations that have been reported (113). GM2-activator deficiency has been described in humans, dogs, and mice (114).

An animal model (American Flamingo, *Phoenicopterus ruber*) of TSD with HexA deficiency occurs spontaneously in nature. The spontaneous appearance of this animal model of TSD with features similar to those of the human form of this disorder could be a possible research tool for further studies of pathogenesis and the treatment of TSD (70).

### 104.3.5 Therapy

Therapy is primarily supportive. Several approaches to therapy are being investigated in animal and cell culture models. Most gene therapy experiments have involved the production of a recombinant virus, either retrovirus or adenovirus containing the *HEXA* cDNA. Substrate deprivation therapy has been tested by oral administration of NB-DNJ in Tay–Sachs mice, resulting in a 50% reduction in GM2-ganglioside accumulation (21,54,115). Other approaches still under investigation and research are bone marrow (BM) and neural progenitor cell transplantation. The attractiveness of SRT is that it is oral therapy and that the drug crosses the BBB and can act centrally. Also, risks and complications are relatively minor. Enzyme replacement is much less effective in diseases that primarily affect the CNS because of the difficulty of large molecules to cross the BBB. Theoretically, BM or cord blood transplantation should provide some enzyme-producing glial cells to the



CNS, but the high morbidity and mortality and the doubtful effectiveness of this approach make it undesirable, particularly in light of the low chance of efficacy. Unfortunately, these therapies have less than optimal chances at making a difference, as in most cases, once the diagnosis is made, considerable CNS damage has already occurred.

Recombinant human  $\beta$ -hexosaminidase A is a potentially therapeutic enzyme for GM2-gangliosidosis. Recombinant HEXA produced from certain yeasts and lentiviral (LV) vectors is effectively incorporated into cultured fibroblasts and neural cells, and rapidly degrades GM2-ganglioside in vitro (116–118). Gene transfer strategies using nonreplicating Herpes simplex vector encoding for the HexA  $\alpha$  subunit succeeded in replacing the missing enzyme in Tay–Sachs animal models when injected in the internal capsule of the affected mice without adverse effects (119). Similarly, hippocampal neurons and microglia of Sandhoff disease animal models were transduced with lentiviral and adeno-associated viral vectors encoding the Hex  $\beta$  subunit. This resulted in the elimination of the intracellularly accumulated GM2 in the brain and spinal cord, reduction of inflammation, preservation of motor power, and increased survival (120–122). Neonatal intraperitoneal administration led to reduction in neuroinflammation, attenuation of GM2 storage and amelioration of neurodegeneration and motor behavioral deterioration (118). SRT aims to decrease the rate of GSL biosynthesis to compensate for slowed catabolism. The imino sugar, NB-DGJ inhibits the first step in GSL biosynthesis. NB-DGJ treatment, administered from postnatal day 2–5 (600 mg/kg/day), significantly reduced total brain ganglioside and GM2 content in Sandhoff disease (Hexb $^{-/-}$ ) mice. NB-DGJ is effective in reducing total brain ganglioside and GM2 content at early neonatal ages (123).

In a multicenter, open-label, 12-month study involving patients aged 18 years or older randomized to miglustat (Zavesca) (200 mg TID) or “no miglustat treatment,” miglustat treatment did not lead to measurable benefits in late-onset TSD (124). In another open-label study over a 24-month period, five juvenile GM2 patients on oral miglustat at doses of 100–200 mg t.i.d. did not improve from a motor standpoint, but had stabilization of brain MRI lesions and some aspects of cognitive function over 24 months (125–127). Miglustat is a safe drug. Major adverse events in infantile patients include abdominal discomfort and flatulence, and in the juvenile group, diarrhea and weight loss (128).

One promising therapy for GM2-gangliosidosis involves the use of beta-*N*-acetylhexosaminidase inhibitors as chemical chaperones to enhance the enzyme activity above subcritical levels. Pharmacological chaperones (PC) are small molecules that can stabilize the conformation of a mutant protein, allowing it to pass the quality control system of the ER. They are used as enzyme enhancement therapy agents that are competitive inhibitors of the target enzyme beta-*N*-acetylhexosaminidase and may be useful as therapeutic agents for treating adult TSD and Sandhoff disease. The L-iminosugar LABNAc

and its derivatives are potent noncompetitive inhibitors of some beta-*N*-acetylhexosaminidases, while the D-iminosugar DABNAc and its derivatives were found to be weaker competitive inhibitors (129).

Pyrimethamine, a potent inhibitor, functions as a mutation-specific PC, enhancing residual lysosomal HEXA levels in late-onset GM2 patient cells (130).

Intracranial injection of neural stem cells (NSCs) into the brain of adult symptomatic Sandhoff (Hexb2/2) mice, results in cell migration far from the injection site and integration into the host cytoarchitecture, restoring  $\beta$ -hexosaminidase enzyme activity and promoting neuropathologic and behavioral improvement. Mouse life span increased, neurological function improved, and disease progression slowed. Clinical benefits correlated with neuropathological correction at the cellular and molecular levels, reflecting enzyme cross-correction, cell replacement, tropic support, and direct anti-inflammatory action. Pathotropism (i.e. migration and homing of NSCs to pathological sites) could be imaged in real time by MRI. Differentially expressed chemokines might play a role in directing the migration of transplanted stem cells to sites of pathology. Significantly, the therapeutic impact of NSCs implanted in even a single location was surprisingly widespread due to both cell migration and enzyme diffusion. Because many of the beneficial actions of NSCs observed in newborn brains were recapitulated in adult brains to the benefit of Sandhoff recipients, NSC-based interventions may also be useful in symptomatic subjects with established disease (125). In addition to neuronal replacement, NSCs also increased brain  $\beta$ -hexosaminidase levels, reduced ganglioside storage, and diminished activated microgliosis. When oral GSL biosynthesis inhibitors ( $\beta$ -hexosaminidase substrate inhibitors) were combined with NSC transplantation, substantial synergy resulted. Efficacy extended to human NSCs, both to those isolated directly from the CNS and to those derived secondarily from embryonic stem (ES) cells (131).

Trials of bone marrow transplantation (BMT) failed to ameliorate the natural course of the disease (132). Immunomodulation by inhibition of infiltrating peripheral blood mononuclear cells (PBMC) infiltration in the HexB $^{-/-}$  mouse model of Sandhoff disease brain retards disease progression and neurodegeneration in a mouse model of GM2-gangliosidosis. Ablation of the chemokine receptor CCR2 in the Hexb $^{-/-}$  mouse resulted in significant inhibition of PBMC infiltration into the brain, decrease in TNF- $\alpha$  and MHC-II mRNA abundance, and retardation in clinical disease development (133).

Caloric restriction may be effective in improving motor coordination and extending longevity in Hexb $^{-/-}$  mice during the symptomatic phase of SD, when extensive inflammatory processes are active. Furthermore, this improvement was observed without changes in brain GSL composition or cytoplasmic neuronal vacuoles and was not associated with observable adverse effects (134).

## 104.4 LOSS-OF-FUNCTION MUTATION OF GM3-SYNTASE

### 104.4.1 Introduction

Most disorders discussed under sphingolipidoses have involved hydrolysis enzyme defects. Although the existence of a sphingolipid class synthesis disorder has been postulated for a long time, this is the first report in humans of a documented molecular defect. The importance of this first description of a GM3-synthase disorder in humans suggests that other enzymes involved in the synthesis of gangliosides or other sphingolipids or glycolipids might be products of candidate genes at the root of other epilepsy syndromes (1,135).

### 104.4.2 Clinical Description

The first symptoms appear between 2 weeks of age and 3 months and consist of irritability, poor feeding, and failure to thrive. Generalized tonic-clonic seizure activity begins before 1 year of age. Other seizure types, resistant to therapy, including startle myoclonus, set in. Once seizures begin, complete developmental stagnation and regression are the norm. Affected children are mute, nonambulatory, and do not reach for objects, but have nonpurposeful choreoathetoid movements. They are cortically blind and some develop optic atrophy. EEG is slow with superimposed epileptiform discharges. MRI demonstrates progressive cortical atrophy.

### 104.4.3 Biochemistry

The protein catalyzes the formation of GM3-synthase from lactosylceramide, and is the initial and most crucial step in the formation of complex a- and b-series gangliosides. Affected children completely lack GM3 and its downstream derivatives in their serum, but accumulate the precursor, lactosylceramide, and show evidence of increased flux through the globoside and paragloboside alternate pathways. It is not known whether this severe neurological impairment is due to the lack of GM3 and its derivatives, or results from accumulation of precursors. In some studies, pharmacological depletion of cellular gangliosides did not impede stimulated neurite outgrowth (136) or neuronal differentiation of cultured cells suggesting yet other explanations for the neurological deficits (137).

### 104.4.4 Genetics

Simpson and associates (1) reported GM3 synthase deficiency in a large, older Amish pedigree. The defect is inherited in an autosomal recessive manner. All cases have a nonsense mutation in the *SIAT9* gene (R232X), which codes for the GM3 synthase enzyme.

### 104.4.5 Animal Models

A line of mutant mice with the same defect have a mild phenotype with increased insulin but no seizures, in contrast to the severe phenotype in humans. Mice lacking ganglioside GM3 synthase exhibit complete hearing loss due to selective degeneration of the organ of Corti (138). Mice unable to synthesize ganglioside GM3 are viable and do not have major abnormalities (139), but mice carrying double null mutations in both GM2 and GM3 synthases develop severe neurodegenerative diseases that result in death (140). Histopathological examination revealed striking vacuolar pathology in the white matter regions of the CNS with axonal degeneration and perturbed axon–glia interactions.

### 104.4.6 Treatment

Treatment is largely supportive at this point and encompasses nutritional management and seizure control. Seizures are notoriously difficult to control necessitating polypharmacy and vagal nerve stimulators. Most children have required feeding tubes.

## 104.5 NIEMANN–PICK DISEASE

### 104.5.1 Introduction

Niemann–Pick disease (NPD) constitutes a heterogeneous group of lysosomal storage diseases with an autosomal recessive mode of inheritance. Four clinical forms are described, which were initially grouped together because of pathologic similarities of the cases and the appearance of sea-blue histiocytes in the reticuloendothelial system. Types A and B caused by low acid sphingomyelinase (ASM) activity are characterized by the accumulation of sphingomyelin and arise because of defects in the same gene (141). Type C1, which includes an allelic variant from a genetic isolate from Nova Scotia, formerly delineated as Niemann–Pick, type D, accounts for 95% of Niemann–Pick, type C, cases. More recently, a second gene, NP-C2, has been identified. Niemann–Pick, types C1 and C2 defects, result in a distinctive lipid trafficking perturbation of low density lipoprotein (LDL)-derived cholesterol that remains trapped in the lysosomal compartment. Sphingomyelin, as well as other lipids, are increased secondary to abnormal cholesterol sequestration in lysosomes (142).

## 104.6 NIEMANN–PICK DISEASE, TYPES A AND B

### 104.6.1 Clinical Presentation

**104.6.1.1 Niemann–Pick Disease, Type A (Infantile or Acute Form).** Typically, hepatosplenomegaly develops in the first few months of life along with

moderate lymphadenopathy. There is a preponderance of NP-A Ashkenazi Jewish cases. The abdomen becomes protuberant. A moderate microcytic anemia, which may be responsive to iron, and a decrease in platelet counts occur later. Neurologic findings include hypotonia and muscular weakness (feeding difficulties). Recurrent vomiting and constipation are main complaints. About 50% of patients have macular cherry-red spots. ERG responses are diminished and EEG shows nonspecific slowing and voltage attenuation. Auditory-evoked brainstem responses may be abnormal, and nerve conduction velocities may be reduced. Death ensues at 4 years after a relentless neurodegenerative course. Early laboratory markers of disease include elevated levels of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), total cholesterol, and triglycerides (TGs) and low HDL-C (143).

**104.6.1.2 Niemann–Pick Disease, Type B (Chronic, Non-Neuronopathic Forms).** Type B is known as the chronic, non-neuronopathic form because of prolonged survival and the absence of neurologic involvement. Hepatosplenomegaly is particularly prominent in childhood. In most type B patients, decreased pulmonary diffusion due to alveolar infiltration becomes evident in childhood and progresses with age. Patients may experience significant pulmonary compromise by 15 years of age. This is usually manifested with low oxygen tensions in the blood, dyspnea on exertion, bronchopneumonias that may be life threatening, and cor pulmonale. Severely affected patients may have liver involvement and eventually develop cirrhosis. Rarely, joint and bone pain may be the presenting symptoms (144). In general, type B patients do not have neurologic involvement and are intellectually intact. Some NP-B patients have cherry-red maculae or a gray granular pigmentation around the fovea that appears like a halo (145). Two adults with psychiatric and pulmonary disease and low ASM activity have been described (146). The oldest patient at the time of diagnosis was a 68-year old woman (147). Fifty-nine demographically diverse patients demonstrate multisystem involvement and clinical variability of Niemann–Pick B disease. Most of them initially present with splenomegaly (78%) or hepatomegaly (73%). Frequent symptoms include bleeding (49%), pulmonary infections and shortness of breath (42% each), and joint/limb pain (39%). Growth is markedly delayed during adolescence. Patients commonly have low levels of platelets and HDL, elevated levels of LDL, Very low density lipoprotein (VLDL), TG, leukocyte sphingomyelin, and serum chitotriosidase, and abnormal liver function test results (148). NPD type B also can affect the cardiovascular system due to the abnormal lipid profiles. In addition to abnormalities in LDL and TG, patients have elevated cholesterol and low HDL, which lead to accelerated atherosclerotic heart disease, with coronary artery calcification in both children and adults (149). Massive

pulmonary involvement in early life is extremely rare. An infant presenting with recurrent respiratory tract infections has been reported (150). Patients commonly show restrictive lung disease physiology with impaired pulmonary gas exchange and decreased maximal exercise tolerance (148). Radiographically, pulmonary involvement manifests as a reticulonodular pattern, interlobular septal thickening, ground-glass density, and subcentimeter, sometimes calcified nodules. On occasion, the combination of these findings will result in the “crazy-paving” sign seen on CT in a variety of diseases. The imaging findings generally begin in the bases and progress cranially. Rarely, pulmonary nodules and cysts are described (151). The aurora sign, a sonographic sign found on the sagittal and transverse view, referring to multiple bands of ring-down artifacts posterior to the right hemidiaphragm has been reported in a patient with NPD type B (152). Imaging features do not necessarily correlate with the degree of pulmonary function impairment (153).

Cardiac dysfunction is not a usual complication in this disease, and is usually secondary to cor pulmonale. Recently, myocardial dysfunction without pulmonary disease was reported in two sisters in their third decade (genotype: S436R/S436R). The cardiac involvement was refractory to treatment in one, resulting in death (154).

Abdominal manifestations include hepatosplenomegaly, but adrenals, kidneys, and retroperitoneal lymph nodes can be involved (149). Splenomegaly is a prominent clinical manifestation of NPD type B, which occurs because of the accumulation of lipid-laden macrophages in the reticuloendothelial system (RES). Splenic masses are typically echogenic on ultrasound and low density on CT, and can have variable signal characteristics on MRI, with the most common being isointensity on T1-weighted images and high signal on T2-weighted images (149). The degree of splenomegaly correlates with most aspects of disease, including hepatomegaly, growth, lipid profile, hematologic parameters, and pulmonary function. Spleen volume correlates with disease severity, and may be a useful surrogate end point in treatment trials (148).

Hepatomegaly is also a prominent clinical finding in patients with NPD type B. The liver enlarges because of the accumulation of lipid-laden macrophages in the RES of the liver. Abnormal liver function tests and in rare cases, cirrhosis and hepatic failure can occur. The liver can be imaged with ultrasound, CT, or MRI. Repeated imaging studies are necessary to monitor disease and MRI is the modality of choice. CT, however, is better for detection of small foci of calcium in the liver and other organs (149). Niemann–Pick cells infiltrate the bone marrow (BM), leading to leukopenia and thrombocytopenia with normal hemoglobin levels. Adolescent patients with NPD type B have delayed skeletal maturation and growth restriction. Patients may have osteopenia or osteoporosis, detectable radiographically. MRI shows low T1 signal in the BM. Adrenal glands, kidneys, and lymph nodes can be involved in NPD type B and

have intermediate signal on T1- and T2-weighted imaging by MRI (149,155).

### 104.6.2 Pathology

The hallmark of this disease is the histochemically characteristic lipid-laden cells known as foam cells. These appear as sea-blue histiocytes in the BM. They are also called Niemann–Pick disease cells or NPD cells. These histiocytic cells are about 30–70  $\mu\text{M}$  in diameter and the cytoplasm is chock full with lipid droplets that have a mulberry-like appearance. NP-A brains are firm, with a reduced weight and a shrunken cerebellum and to a lesser extent, the cerebrum. Glial cells and foam cells are laden with lipid and are common around blood vessels. Neurons, when found, are swollen and there is a drastic loss of myelin. The spinal cord and deep nuclei may show similar changes. Peripheral nerves can be severely affected with the loss of both myelin and inclusion bodies in Schwann cells. Visceral involvement is severe in the BM and then the spleen, which can be enlarged up to 10 times its original size. Lymph nodes, liver, and kidneys are often affected. Lung pathology is prominent, particularly in NP-B cases. They are increased in size and foam cells invade vessels, interlobular septae, pleura, and even accumulate within the alveolar spaces (149,154,156). In the liver, the sinusoids are filled with numerous Kupffer cells or macrophages with abundant clear and vacuolar cytoplasm (Niemann–Pick cells). Hepatocytes are swollen with pale cytoplasm, suggesting an accumulation of lipid substances. The liver also shows varying degrees of fibrosis with focal regenerative proliferation of hepatocytes. In the spleen, the red pulps are infiltrated with numerous Niemann–Pick cells and the white pulps are atrophic. In a case of myocardial dysfunction leading to death, autopsy revealed a flabby, enlarged heart with marked dilatation of the left ventricle due to atherosclerosis. In the atherosclerotic lesions, large numbers of foam cells accumulating great amounts of cholesteryl ester were present (154).

### 104.6.3 Biochemistry

ASM degrades sphingomyelin to ceramide and phosphorylcholine. NP-A and NP-B are characterized by abnormal accumulation of sphingomyelin in most tissues, secondary to ASM deficiency (157). The types and amounts of lipids stored in the RES and visceral organs are similar. The profile of lipids in the brain differs. In one study, there was an increase in sphingomyelin in extraneural tissues of both types. Lyso-sphingomyelin was elevated in the brain of two type A patients and was normal in the brain of one type B patient (4). These lyso-sphingolipids seem to be toxic metabolites in the brain of patients with sphingolipid storage diseases, and have been implicated in the pathogenesis of Niemann–Pick as well as other diseases such as Krabbe disease. Type A has extensive

accumulation of sphingomyelin up to 50-fold of normal values, representing 2–5% of total body weight (158). Bis-mono-acylglycero-phosphate increases up to 100-fold, and the level of cholesterol is also somewhat elevated. There are reports of accumulation of GSLs and glycolipids. These include glucocerebroside, GM2- and GM3-gangliosides. In addition, lesser accumulation of lactocylceramide, globotriaosylceramide, and globotetraocylceramide are reported in liver and spleen (159).

Patients with Niemann–Pick, type A, have ASM activity levels ranging from nondetectable to 5% of normal, when determined using cultured fibroblasts/leukocytes as the enzyme source (157,160,161). Similar findings have been reported in tissues, including liver and brain. ASM activity in cells or tissues from type B patients is more elevated than that from Niemann–Pick type A patients. When determined in cultured cells, residual enzyme activities range between 2 and 10% in Niemann–Pick, type B. It is also possible to measure sphingomyelin hydrolysis using cell-loading assays and fluorescently labeled C12-sphingomyelin (BODIPY-SM, Molecular Probes, Inc., Eugene, OR) as substrate. These cell-loading assays reveal a higher rate of sphingomyelin hydrolysis in cultured cells from type B patients. The enzyme assay is not helpful in ascertaining carriers due to the overlap in enzyme activities between normals and carriers. For a family with known mutations, it is best to screen for carriers using molecular DNA analysis. Prenatal diagnosis using measurement of ASM activity, amniocyte cell-loading studies, and/or DNA analysis is available and reliable.

Plasma chitotriosidase and the chemokine CCL18 may also serve as markers for the formation of pathological lipid-laden macrophages in type B NPD. In situations where the differential diagnosis is not obvious clinically, these markers may help narrow the differential diagnosis and are of assistance in identifying the most probable specific confirmatory enzyme assay in comparable populations of children with organomegaly (162,163).

In NPD, sphingosylphosphocholine (SPC, a sphingomyelin metabolite) accumulates in various tissues, including the brain, where it might act as a toxic stimulus, contributing to the development of neurological symptoms. Acute administration of SPC to astrocytes in culture promotes  $\text{Ca}^{++}$  responses and the release of glutamate that leads to cytosolic  $[\text{Ca}^{++}]$  elevation in neurons. Chronic stimulation by SPC leads astrocytes to proliferate, transforming them into an activated state contributing to the inflammatory response. Acute SPC stimulation causes activated astrocytes to release more glutamate. In conclusion, chronic and acute exposure to SPC constitute harmful signals that may have a role in the events leading to neurodegeneration. These new insights into the interplay between astrocytes and neurons offer new opportunities for therapeutic approaches in Niemann–Pick type A disease as well as in other neurodegenerative diseases (164).



### 104.6.4 Genetics

The human ASM (*hASM*) gene has been mapped to chromosome 11p15.1–15.4. Types A and B are allelic. Carrier frequency for NP-A in Ashkenazi Jewish is high at 1:80 individuals. More than 50 mutations are known for this disease of which three, R496I, I302 P, FSP330 (stop codon), account for 92% of all mutant alleles in Ashkenazi Jews. NP-A has been described in non-Jewish populations. NP-B is rare in the Ashkenazim. The R496I mutation has been found in some type B Ashkenazi Jewish patients on one of the alleles, with a different mutation on the other allele. One of these mutations, p.R608del, is found in NP type B patients of both Ashkenazi Jewish and north African background. In fact, this mutation accounts for 87% of alleles in NP-B patients from the Maghreb countries (Algeria, Morocco, and Tunisia). Cases with a form of protracted neuronopathic disease have been due to combinations of mutations that produce a greater deficiency in ASM than typical type B patients. NP phenotype–genotype correlations hold true for the common Ashkenazi Jewish mutations, yet often the identical combination of mutations in other populations does not predict a similar clinical phenotype or course. Frameshift mutations due to splicing, small and large insertions and deletions, and splicing defects with little or no residual ASM activity are called type A alleles. Missense and in-frame codon deletions and splicing mutations retaining significant residual activity (>5% of in vitro-expressed wild-type activity) are neuroprotective and are called type B alleles (55). Inheritance of two type A alleles predicts a type A phenotype with a neurodegenerative disease course. Inheritance of one type B allele is neuroprotective and predictive for a type B phenotype, even if the other allele is an A allele (165). The measurement of ASM activity in peripheral leukocytes or cultured skin fibroblasts is commonly used for the diagnosis of ASM-deficient NPD; however, falsely normal enzyme activity has been reported in some patients (11). Thus, DNA diagnosis plays an important role in accurate diagnosis. More than 100 mutations within the *SMPD1* gene causing ASM-deficient NPD are known (human gene mutation database: <http://www.hgmd.org/>). Functional changes in the protein resulting from some mutations have been characterized (105). According to demographic data, most reported cases of type A NPD occur in Ashkenazi Jews. Three common mutations comprise >90% of the mutant alleles in this ethnic population (166). In contrast, type B NPD is panethnic and genetically heterogeneous (55,167). Few mutations have been found with a relatively high frequency in specific populations. Mutation c.1823\_1825delGCC (p.R608del) is always associated with the type B phenotype. This mutation has a low frequency in Europe: 9.4% in Italy, the Czech Republic, and Slovakia. Reported frequencies of mutation p.R608del in type B NP-D patients were 9.4% in Italy, 12% in 324 patients

of multiethnic origin, 86.6% in northern Africa, and 100% in four patients from the Canary Islands. Mutations p.Y67C and p.A482E were associated with type A. The p.R608del-type B association is confirmed (168). Eight new mutations causing types A and B NPD in six unrelated patients were identified: c.631t>c (p.W211R), c.757g>c (p.D253H), c.1729A>G (p.H577R), c.940G>A (p.V314M), c.1280A>g (p.H427R), c.1564A>G (p.N522S), c.1657delACCGCCT (F5253), and c.1575G>C (p.Q525H (169). A novel allele c.1352C>A was recently reported in a Taiwanese (167). An exhaustive molecular analysis of 19 Spanish NMA/B patients and two from Maghreb was conducted. Ten of the detected mutations were novel. The most frequent mutations in the 21 NPD patients were c.1823\_1825delGCC (p.R608del) (38%) and c.1445C>A (p.A482E) (9%). The mutations p.W168X, p.Y313X, p.F390del, p.Y467S, p.A482E and p.T592del found in the homozygous state were associated with type A disease. The same correlation was established for mutations p.G245S, p.Y367C and p.H421R, found in compound heterozygotes. The p.R608del accounted for 61.5% of mutant alleles in the type B subgroup of patients (168). The mutations, L137P, SSP189, and I549P comprise more than 70% of all mutations in Turkish patients with NPD type B. A novel mutation R505G has been recently described in a patient presenting with epigastric pain and hepatosplenomegaly (170).

### 104.6.5 Animal Models

Two mouse models of NP disease types A and B have been constructed by gene-targeting strategies. The NP disease “knockouts” were normal until about 3 months of age when progressive ataxia developed and death was around 7 months (171–173).

### 104.6.6 Treatment

BMT has failed for NP-A. There is, however, room for its use in NP-B patients. Similar results were obtained with preclinical testing of enzyme replacement in mouse models. Again, enzyme replacement is unlikely to work for NP-A due to the impenetrability of the BBB. Hematopoietic stem cell-mediated gene therapy also holds promise for treatment of NP-B, but not NP-A. Studies in mouse models have shown little or no improvement in the CNS.

Cloned mouse NSCs or human NSCs were injected into CNS sites in high concentrations into neonatal ASM knockout mice, and into 10 wild-type mice. Noninjected ASM controls had developed widespread neuronal and glial vacuolation and lysosomal accumulation of sphingomyelin and cholesterol when examined histologically at 16 weeks of age. At 16 weeks, NSC-injected mice showed a dramatic decrease in neuronal and glial vacuolation and in cholesterol accumulation throughout the cerebral neocortex, hippocampal formation, striatum,

and cerebellum, with lesser but clear improvement throughout the brainstem. Loss of Purkinje neurons and decline in rotarod performance were still present in injected ASM mice. Stem cells may not distribute widely enough within the CNS to produce optimal therapeutic results in the large human brain, even at the high-injection dosage of stem cells used. Also, long-term effects are unknown, mouse experiments being limited to months rather than to years (174).

Gene therapy has been intensively investigated with AAV being the most widely accepted among several vehicles for *in vivo* targeting of nondividing cells in the CNS. This approach has been effective in the ASM mouse model (174).

Gene therapy with a retroviral vector encoding for hASM in deficient mice has been explored (175). Miranda et al. transplanted newborn ASM-deficient mice and found higher levels of ASM activity in the white blood cells compared with untreated litter mates. In addition, levels of ASM activity in the spleen, liver, and lung were higher than untreated animals; however, levels in the brain remained low, similar to those not receiving a BM transplant. These levels correlated with decreases of sphingomyelin deposition in the spleen, liver, and lung. Animals undergoing transplant survived longer than those not treated, but developed ataxia, weight loss, and eventually died. None of these therapies are recommended for NP (166,176,177).

Intracerebroventricular (ICV) delivery of recombinant hASM into ASM mice is effective. ICV delivery of enzyme leads to distribution of the hydrolase throughout the CNS. A significant reduction in lysosomal accumulation of sphingomyelin is observed throughout the brain, spinal cord and viscera. Repeated ICV infusions of ASM are effective at improving the disease phenotype in the ASM mouse as indicated by reduced SPM accumulation within the CNS and partial alleviation of motor abnormalities (178).

Direct intraparenchymal brain injections of purified recombinant hASM corrects the storage pathology in a mouse model of NPD-A (ASMKO). Immunohistochemical analysis after 1 week indicated that animals treated with greater than 1 µg hASM/site showed detectable levels of enzyme around the injected regions. Localized clearance of sphingomyelin and cholesterol storage was observed in animals that administered lower doses of enzyme, starting at 100 ng hASM/site. Areas of correction were noted at distal sites such as in the contralateral hemispheres. Indications of storage reaccumulation were seen after 2 weeks postinjection. Injections of hASM did not cause any significant cell infiltration, astrogliosis, or microglial activation. Intraparenchymal injection of hASM is associated with minimal toxicity and can lead to regional reductions in storage pathology in the ASMKO mouse. They were safe and effective at correcting the lysosomal storage pathology. The extent of correction achieved in the vicinity of the injection site was

substantial for mouse brain, but needs to be significantly improved in order to treat a larger brain. Repeated administration every 2–3 weeks would be necessary to maintain the correction of lysosomal storage pathology. Achieving global distribution of the enzyme and long-term reversal of storage pathology in a larger brain will require further optimization of the route of delivery and methods for repeated infusions into the brain (179).

A new delivery vehicle for ERT of type B NPD consists of polystyrene and poly(lactic-coglycolic) acid polymer nanocarriers targeted to intercellular adhesion molecule (ICAM)-1, an endothelial surface protein upregulated in type B NPD. ICAM-1-targeted nanocarriers enhance the ERT for type B NPD. ICAM-1 targeting provides a means to enhance the delivery of recombinant ASM to vascular endothelium. Anti-ICAM particle formulations achieved improved pulmonary uptake compared with the naked enzyme, providing a means to enhance enzyme delivery to this critical type B NPD target organ (180).

In one 18-month-old with NP type B who underwent two allogeneic hematopoietic progenitor cell (HPC) transplants from her HLA-identical sister, engraftment was durable, with the normalization of sphingomyelinase. Normal BM, hepatic and pulmonary functions were regained. At 13 years of age, the patient was able to perform activities of daily living without assistance and attend school full time, in spite of extensive graft versus host disease (GVHD) and extremity contractures as transplant complications. Two others had less clear cut benefits with continued neurological deterioration in one case transplanted at age 3.5 years, and another with the resolution of pulmonary symptoms but continued hepatomegaly and neurocognitive decline (22,171,177,181).

A prenatally diagnosed girl with NPD type A underwent cord blood stem cell transplantation at 3 months of age. She was neurologically intact at the time of transplant. There were severe neurologic and visceral complications several months following transplant (182). Transplantation may be considered for those with severe disease, especially pulmonary symptoms. Successful hematopoietic stem cell transplantation (HSCT) for NPD type B is reported to improve the patient's pulmonary symptoms, with the caveat of graft versus host disease and other complications (183).

In general, patients with NP type B disease have not received transplant as definitive therapy because of the lower incidences of neurologic sequelae. HPC transplantation for patients with NP type B disease is feasible and potentially beneficial when performed prior to the onset of severe pulmonary and liver diseases. Given the serious potential sequelae of NP type B disease, HPC transplant should be considered in patients with severe visceral complications (177). Future directions should be directed toward ERT or gene therapy, which would solve the problem of unsuitable donors, thus reducing the complications of transplant.

## 104.7 NIEMANN–PICK DISEASE, TYPES C AND D

### 104.7.1 Clinical Presentation

Age of presentation is variable from the perinatal period to adulthood. Initial manifestations can be neurologic, hepatic, or psychiatric. The courses of systemic and neurologic disease are independent. Liver involvement is often present in the first month of life. Hepatosplenomegaly is a common finding, but may be absent in 15% of patients. The most frequent form begins in childhood with ataxia, vertical supranuclear palsy, and psychomotor regression. Dysarthria, dystonia, cataplexy, tremors, Parkinsonism seizures, jaundice, and hepatosplenomegaly can also be present. There is variability in clinical presentation, with patients developing clinical signs in their second, third, fifth, or sixth decades. The subacute form of NP-C1 (previously known as NP-D) described in French Canadians of Acadian descent seems to have a more homogeneous phenotype. It is presumed that all these patients were traced to a small set of common ancestors who immigrated to Nova Scotia from France and have a similar genetic defect. Other variants include an acute form with hydrops, an early form with neonatal hepatitis, and a slow chronic form with progressive neurologic deterioration with patients surviving into adulthood. An early lethal form of NP-C has been reported in three patients with severe pulmonary involvement (184,185). Gingival enlargement has been reported in a patient with NPD (186).

Antenatal presentation of NPC disease is most often correlated with a severe and rapidly fatal form of the disease. Sonographic signs consist of fetal ascites with hypoferritinemia, with or without hepatosplenomegaly. Prenatal diagnosis is possible using AF and/or ascites fluid samples, preceded by fetal blood screening for high chitotriosidase levels. Poor prognosis during the perinatal period correlates with hepatocellular insufficiency. In siblings with NP-C disease, the infant may develop a prenatal or neonatal form of variable severity and evolution, independent of the type of mutation, highlighting the complexity of genetic counseling for this disorder (187). Prenatal onset may be detected antenatally by congenital thrombocytopenia, congenital anemia, petechial rash, cholestasis and hepatomegaly immediately after birth (188).

Psychiatric signs and cognitive troubles constitute the most frequent symptoms in adults and may appear after 50 years. Main clinical features include vertical supranuclear gaze palsy, cerebellar ataxia, dysarthria, cognitive decline and movement disorders. Recognizing mild vertical supranuclear gaze palsy is a valuable clue to the diagnosis as it is a constant and specific feature (189). Early nonspecific signs result in an average delay in diagnosis of 6.2 years (181). This is particularly true for psychiatric symptoms (190). A patient with bipolar disorder in adolescence was recently reported (191). Whilst postictal

psychosis is rarely reported prior to 16 years, NPC may increase the risk for postictal psychosis in the pediatric population (192). Cataplexy and cortical myoclonus rarely do occur as the main symptoms of NPC and severely impair motor performance (193). NPC cases with cataplexy demonstrate low levels of CSF hypocretin-1 (194). Very rarely, NPD type C may present with purely visceral disease without neurological symptoms. Therefore, NPD type C should be considered in differential diagnosis of isolated hepatosplenomegaly with foam cells in adulthood (195). The rare type C2 almost uniformly presents with respiratory distress in early infancy. Pulmonary alveolar proteinosis due to NP-C2 disease should be considered in cases of infants with respiratory symptoms of bronchiolitis or pneumonia not responding to common treatments (196).

### 104.7.2 Diagnosis

Diagnosis of NPC requires a high index of suspicion and should be considered in a patient with hepatosplenomegaly with or without neurodevelopmental signs. The initial presentation in a pediatric patient may be restricted to isolated hepatomegaly, splenomegaly, or both. When neurological symptoms are absent, one may confuse this entity with NPD type B or GD type II. Decreased activity of acid  $\beta$ -glucosidase in leukocytes and elevated serum chitotriosidase are present; however, fibroblast  $\beta$ -glucosidase will be normal. Cholesterol esterification assays in cultured skin fibroblasts and NPC gene analysis always leads to the correct diagnosis of NPC. Therefore, diagnostic delay may occur in NPC due to false positive testing for Gaucher disease (GD) (197). Denaturing high-performance liquid chromatography is a rapid, low-cost, highly accurate, and efficient technique for the detection of NPC genetic variants and mutations (198). BM aspirates are not reliable as a screening test in the diagnosis of NPC presenting with hepatomegaly. While a positive result is helpful, a negative result cannot exclude the diagnosis. Skin biopsy for fibroblast culture enables definitive tests to be performed (199). Voxel-based morphometry of T1-weighted images and tract-based spatial statistics of diffusion tensor images demonstrate reduction in gray matter and white matter tracts due to impaired myelination and altered axonal structure. Volumetric analysis of gray matter and diffusion tensor imaging (DTI) may be useful modalities for indexing illness stage and monitoring the response to emerging treatment (200). Ocular motor measures provide an index of disease severity and may be a useful adjunct for monitoring illness progress and medication response (201). Differing aspects of tremor, Parkinsonism, ataxia, and dystonia are quantifiable in NPC patients through spiral analysis, which is a computerized method of analyzing upper limb motor physiology. This may serve as ancillary measures of NP-C pathophysiology, allowing for the objective evaluation of new therapies (202).



### 104.7.3 Pathology

While the precise cause of neurodegeneration in NP-C remains unclear, it is clear that defects in either NP-C1 or NP-C2 protein cause a “traffic jam” of lipids in the late endosome (203,204).

Foam cells are found in many tissues. Sea-blue histiocytes are seen in the BM with Giemsa–Wright stains. Such cells are not specific for NP-C and may be absent in cases without enlarged organs. Characteristic inclusions may be identified in skin. Neuronal storage, swollen perikarya, and cytoplasmic ballooning with pleomorphic, electron-dense inclusions are found in neurons and other cells throughout the nervous system. Apoptosis of neurons and Purkinje cells in the cerebellum are prominent. Meganeurites reminiscent of the ones seen in GM2-gangliosidosis are present. Axonal spheroids in the thalamus may be present.

In NPD C2, lung involvement is universal. Histology shows typical signs of pulmonary alveolar lipoproteinosis with an abnormal intra-alveolar accumulation of surfactant rich in cholesterol, invasion of macrophages, and hyperplasia of the alveolar cells (196,205).

### 104.7.4 Biochemistry

The metabolic basis of NPD, types C (1 and 2) and D was not fully elucidated until fairly recently. In fact, NP-D and NP-C1 are allelic variants due to defects in the same gene (see “Genetics” section). They have been reclassified as cellular lipid trafficking disorders, following several studies by Pentchev in the early 1980s (206,207). Type D refers to a genetic isolate from Nova Scotia (208). ASM is elevated or normal in leukocytes and tissues of NP-C patients (209,210). Cultured fibroblasts, however, show a decrease in exogenous sphingomyelin degradation (157,211,212) and ASM activity. The decrease in ASM activity in NP-C fibroblasts has been attributed to excessive sequestration of cholesterol. GM2-ganglioside accumulates in lysosomes of cultured cells from patients, even though hexosaminidase A activity is normal (213). The reason for this may be impaired cellular transport of GM2-ganglioside (214,215). There is also a many-fold elevation of free sphingoid long-chain bases in tissues and cultured cells (216). The primary biochemical defect in NP-C is aberrant intracellular transport of endocytosed cholesterol (203,217). The internalization, transport to endocytic vesicles, and hydrolysis of LDL is normal, yet further transport of unesterified cholesterol is impaired. Hence, there is the accumulation of unesterified cholesterol in the late endosome/lysosomal compartments with subsequent failure to induce all LDL-mediated homeostatic responses (218). In NP-C1 cells, LDL cholesterol traffics directly to lysosomes through endosomes, bypassing the plasma membrane and becoming entrapped. The function of NP-C1 protein is presumably the transport of LDL to PM (219). NP-C1 protein is necessary for the distribution of PM-derived cholesterol (220).

Recent studies have established that NPC is associated with an induction of autophagy in human fibroblasts and in mice. Autophagy is a regulated and evolutionarily conserved process by which cytoplasmic proteins are sequestered within autophagosomes and targeted for degradation. This pathway enables recycling of damaged macromolecules to promote cell survival and in other instances, autophagy leads to cell stress and programmed cell death. Autophagy is evidenced by the presence of autophagic vacuole-like structures, and increases in autophagic activity are associated with alteration in lysosomal function and protein ubiquitination (221,222). Cholesterol accumulation can have a detrimental effect on phagosome maturation by impairing the activation of Rab7 leading to failure of the Niemann–Pick type C phagosomes to fuse with lysosomes (223).

Autophagy induction and flux are increased in NP-C by signaling through the class III PI3K/Beclin-1 complex, which is critical for the formation of autophagosomes (224,225). Determining the role of autophagy in NP-C pathogenesis is an important task since targeting this pathway is a potential therapeutic strategy for treating this incurable disease. Hyperphosphorylation and the aggregation of the microtubule-binding protein tau characterize a diverse array of neurodegenerative disorders (226). NPC1/tau double-null mouse mutants exhibit an exacerbated NP-C phenotype, including severe systemic manifestations, and die significantly earlier than NP-C1 single-null mutants. Acute reductions of tau in NP-C1-deficient fibroblasts significantly decrease autophagic induction and flux, while having no effect on the autophagic pathway in control cells. Tau’s normal function is critical to the induction of autophagy in NP-C1 deficiency (227). Increased oxidative stress may be a contributing factor to the pathology of NP-C (228).

NP-C1 deficiency leads to cell autonomous, selective neurodegeneration. Ataxic symptoms of NP-C disease may arise from Purkinje cell death rather than cellular dysfunction (229). Neurodegeneration of *Npc1*<sup>−/−</sup> mice is greatly affected by the loss/dysfunction of fibrillary astrocytes (230). Estradiol treatment may be useful in ameliorating the progression of the disease (231).

Wild-type RID- $\alpha$  rescues lipid-sorting defects in cells from patients with this disease by a mechanism involving a class III phosphatidylinositol-3-kinase. In contrast to NP-C disease gene products that are localized to late endosomes/lysosomes, RID- $\alpha$  induces the accumulation of autophagy-like vesicles with a unique molecular composition. Ectopic RID- $\alpha$  regulates intracellular cholesterol trafficking at two distinct levels: the egress from endosomes and transport to the ER necessary for homeostatic gene regulation. RID- $\alpha$  also induces a novel cellular phenotype, suggesting that it activates an autonomous cholesterol regulatory mechanism distinct from NPC disease gene products (232). The presence of neurofibrillary tangles (NFT) in the hippocampus of a 4-year-old NP-C patient suggests that NFTs are not



aging dependent. Activation of cdc2/cyclin B kinase and downstream mitotic indices are associated with NFT formation, signifying the contribution of abortive cell cycle to neurodegeneration. Cdc2 inhibitors may be therapeutically used for the early intervention of neurodegeneration and NFT formation in NPC (233).

A cholesterol-binding protein, AnxA6, is an important molecule regulating the organization of lysosome-like structures responsible for the abnormal accumulation of cholesterol and GSLs in NP-C fibroblasts. Annexin A6 may participate in the formation of cholesterol-rich lipid raft (LR) platforms in late endosomes contributing to the pathology of NP-C disease (234). Defects in synaptic transmission at glutamatergic and GABAergic synapses may result in part from the impairment of synaptic vesicle trafficking contributing to progressive neurological impairments in NP-C disease (235,236). Enhanced excitatory synaptic transmission and decreased synaptic plasticity due to decreased adenosine release may contribute to seizures, neurodegeneration, and dementia in NP-C (237).

Sphingosine storage in NP-C1 disease pathogenesis leads to altered calcium homeostasis and the secondary storage of sphingolipids and cholesterol. This may explain the block in late endosome-to-lysosome transport in NP-C disease, resulting in downstream storage of cholesterol and GSLs (238). The presence of lysosomal phospholipid lyso-bisphosphatidic acid (LBPA) in the lysosome may be important for NP-C2-membrane interactions, suggesting an important role for LBPA in NP-C2-mediated cholesterol trafficking (58).

Decreased activity of acid  $\beta$ -glucosidase, elevated serum chitotriosidase, and tartrate-resistant acid phosphatase led to an initial diagnosis of GD in a patient. The failure to respond to ERT after 1 year put the diagnosis in question. Cholesterol esterification assays in cultured skin fibroblasts and gene analysis led to the correct diagnosis of NPC. The patient had markedly reduced cholesterol esterification and was a compound heterozygote for known and novel mutations in the *NPC* gene (395delC and 2068insTCCC), which predict a truncated protein. Acid  $\beta$ -glucosidase activity was in the normal range in skin fibroblasts. Therefore, diagnostic delay may occur in NP-C due to false positive testing for GD (197).

Loss of NP-C1 function alters TG metabolism in murine hepatocytes by increasing the metabolic flux of carbons into cholesterol synthesis. There is evidence that the *NPC1* genotype contributes to serum TG levels in humans, which is consistent with the observation that TG levels are increased in a subset of patients with NP-C1. In a genome-wide association study, the *NPC1* locus was detected as a risk locus for morbid obesity. Based on the recent literature (75,141,176), the *NPC1* gene is emerging as a player in TG metabolism and obesity (239).

Early endocytic defects impact NP-C disease and such heterogeneity in NP-C disease results in diverse responses to therapeutic interventions aimed at modulating the trafficking of lipids. The nucleotide cycling

or cellular knockdown of the small GTP-binding protein, ARF6, markedly impacts cholesterol homeostasis. Unregulated ARF6 activation attenuates the NPC phenotype by decreasing cholesterol accumulation and restoring normal sphingolipid trafficking (240). Cell death in the brain of NP-C disease occurs through apoptosis and is mediated by the TNF receptor superfamily pathway (241). Degeneration of cultured NPC1-like cortical neurons can be attenuated by the inhibition of cathepsin B or D enzyme activity. This suggests that the modulation of activity and subcellular distribution of cathepsins may impact neuronal vulnerability in *Npc1*<sup>-/-</sup> brains. Their inhibitors may have therapeutic potential in attenuating NP-C pathology (242). Apoptosis via activation of the c-Abl/p73 pathway is an early process in cerebellar neuronal death in NP-C mice. Inhibition of c-Abl treats the neurodegeneration of Purkinje neurons and ameliorates neurological symptoms in NP-C mice (243).

Studies have demonstrated that a lack of synthesis of the neurosteroid allopregnanolone in the early neonatal period may contribute to neuropathology in NP-C mice. Treatment studies suggest that allopregnanolone may function in the early postnatal period in the brain of mice, and may be related to cellular development, proliferation, and migration. Injection of allopregnanolone in *Npc1*<sup>-/-</sup> mice reduces cholesterol accumulation, improves autophagic and lysosomal function, enhances myelination, and reduces inflammation (244). The mechanism(s) through which allopregnanolone functions in NP-C is unknown. Allopregnanolone can mediate beneficial actions in cultured Purkinje neurons, which are mediated through GABAA-receptors (245).

NPC1 human skin fibroblasts overexpressing endosomal Rab proteins (Rab7 or Rab9) show a correction in the storage disease phenotype. Recombinant human Rab9 fused with herpes simplex virus VP22 protein fragment was overexpressed, purified, and added to the culture medium to induce protein transduction. VP22-Rab9 transduction into NPC1 fibroblasts led to significant reduction in cellular free cholesterol levels with no cytotoxicity. The reduction in cellular free cholesterol levels was associated with the correction of abnormal intracellular trafficking of BODIPY-lactosylceramide and an increase of sterols in the culture media. The clearance of lysosomal-free cholesterol was also associated with a decrease in LDL-receptor levels. In addition, the reduction of intracellular cholesterol by VP22-Rab9 transduction in NP-C2 fibroblasts and in NP-C1 neurons of cultured mouse was demonstrated, as well as the correction of membrane traffic suggesting this could be a novel therapeutic approach (246).

### 104.7.5 Genetics

NPD type C is panethnic. Approximately 95% of patients have mutations in the *NP-C1* gene mapped at 18q11 (56kb, 25 exons). The remainder have mutations in the

*NP-C2* gene, which maps to 14q24.3 (13.5kb, 5 exons). *NP-C1* encodes a 1278 amino acid integral membrane protein with 13 transmembrane domains, one cytoplasmic loop, three large luminal hydrophilic loops, a luminal amino terminus, and a cytoplasmic tail with a dileucine motif (247). More than 243 different loss-of-function mutations of *NPC1* have been reported (75), in addition to 60 different non-disease-causing polymorphisms (105,248). These mutations are scattered through the *NP-C1* gene and affect all functional domains with the exception of the leucine zipper motif (luminal amino terminus). Three frequent mutations have been described. Meaningful genotype and clinical phenotype associations are difficult, primarily due to the fact that most *NP-C1* patients are compound heterozygous for different mutations, yet some associations have been established. For *NP-C1* patients with homozygous mutations, the relatively common I1061T mutation, present in ~20% of all known mutations for *NPC1* and prominent among individuals of western European descent, predisposes patients to the classic *NP-C1* clinical phenotype (249–251). It is highly prevalent in a Spanish American isolate in southern Colorado and New Mexico (37). P1007A is the second most frequent mutation in Europe. G992W is typical of Nova Scotian patients and rare in other populations. Some mutations are prevalent only in Japanese (R518Q) (185) or in Italian patients (P474L) (252). Molecular analysis of the *NP-C1* gene is problematic because of the size of the gene, the large number of private mutations, and the occurrence of numerous polymorphisms. Report of the National Niemann–Pick Type C1 Disease Database found (1) a significantly decreased concentration of plasma LDL cholesterol and increased plasma TGs in the majority of *NP C1* disease patients, (2) a significantly decreased concentration of HDL cholesterol for all patient groups that were not associated with the concentration of plasma TGs, (3) a significant inverse association between the concentration of HDL cholesterol and biochemical phenotype and HDL cholesterol and the age of death for *NPC1* patients, and (4) five previously unreported disease-causing mutations in *NPC1* (Y628C, P887L, I923V, A1151T, and 3741\_3744delACTC). The results indicate the presence of a unique *NP-C1*-related dyslipidemia characterized by hypoalphalipoproteinemia, which may serve as a valuable and convenient biomarker of the biochemical severity of the disease, in addition to monitoring the potential benefit of therapies used for the eventual treatment of *NPC* disease. The following are severe novel mutations (c.2792\_3delAG and c.1437\_42delCACCAT, and c.1241\_2delTC), as are previously reported mutations c.2279\_81delTCT, p.T1205K, and p.L1213V that correlate with a severe phenotype (188). Two novel sequence variants, c.1997G>A (S666N) and c.2882A>G (N961S), in the *NPC1* gene account for a purely visceral form of *NP-C* in adults. *NP-C1* deficiency results in abnormal expression of genes involved in steroid synthesis and cholesterol transport and cell adhesion,

which could be responsible for neuroinflammation and hypomyelination. Dysregulated genes from microarray data, some validated, represent genes belonging to classic immune pathways (e.g. B cell receptor signaling pathway) in cerebellar pathogenesis in *Npc1*<sup>–/–</sup> mice. Based on these results, treatments targeting cell adhesion and/or immune responses combined with the facilitation of cholesterol egress from late endosomes/lysosomes have been proposed (253).

The *HE1* or *NP-C2* gene was identified using a proteomic approach to characterize soluble lysosomal proteins (254). The 130-aa *NP-C2* protein was previously characterized (255) as a major secretory protein present in mammalian epididymis and functions as a cholesterol-transfer protein. Porcine *NP-C2* binds cholesterol with 1:1 stoichiometry. Potential physiological functions of *NP-C2* include preventing inappropriate sterol intercalation into the lysosomal membrane, presentation of sterol to a membrane-bound transporter such as *NP-C1*, or some other regulatory or enzymatic activity. It was found mutated in *NP-C2* patients. Only a few cases are known to date. Nearly all have nonsense or frameshift mutations. The E20X mutation accounts for nearly half of the mutant alleles published. Only 13 different disease-causing mutations have been described for *NP-C2*. Functional characterization of mutant proteins shows good genotype–phenotype correlation for some of the mutations described (264–266). These mutations are c.133C4T, c.141C4A, and c.295T4C, a mutant carrying two in cis mutations, (p.E1188fsX54, and p.T1205NfsX53). An intronic c.464-2A>C change at the 3' acceptor splice site of intron 4 affected *NP-C1* messenger RNA processing, as well as a *NP-C2* mutant caused by a change of the first codon (p.M1L) have been described (265).

Six novel mutations were recently reported in Taiwanese/Chinese patients (N968S, G1015V, G1034R, V1212L, S738Stop, and I635fs) (266). A novel point mutation c.1554-1009G>A has also been recently reported in a Spanish patient (13). Specific diagnosis of *NP-C* is best achieved by filipin staining of cultured fibroblasts grown in the presence of LDL. Filipin binds to the excess unesterified cholesterol and appears as dense birefringent granules in the cytoplasm. Estimation of the degree of impaired LDL-induced cholesterol esterification identifies 80% of patients. Filipin staining is the more sensitive, cheaper, and easier test.

### 104.7.6 Animal Models

Pentchev et al. (142) described a strain of BALB/C mice with many of the characteristics of *NP* disease. These mice exhibited reduced ASM activity and elevated levels of sphingomyelin and cholesterol. An independent strain of C57BLKS/J mice with similar biochemical and clinical findings of ataxia and premature death was described (255). The ASM gene was sequenced from both strains of mice, and no molecular abnormalities were identified

(172). These are naturally occurring mouse models for NP-C1 disease.

Current mouse models of NP-C are not well suited for studying liver disease due to rapidly progressing neurological disease. To facilitate the study of NP-C-associated liver dysfunction, novel mouse models have been developed using antisense oligonucleotides to ablate NP-C1 expression primarily in the liver. NP-C1 knockdown leads to a liver disease phenotype similar to that of patients with NP-C and the NP-Cnih mouse model (267).

NP-C knockout models in the nematode *Caenorhabditis elegans* are available to study human disease (268). Also, an NP-C disease model has been established through the mutation of *Drosophila* NP-C1a (*dnp1a*). Loss of dNP-C1a caused profound changes in cholesterol distribution and thus disrupted LRs resulting in disease mimicking the human form (269).

In *Saccharomyces cerevisiae*, a homolog of the human NP-C1 protein encoded by the *NCR1* gene is Ncr1p. It localizes to the vacuole, the yeast equivalent of the mammalian endosome-lysosome system. Deletion of *NCR1* from the yeast genome results in resistance to the ether lipid drug, edelfosine. Results indicate that edelfosine has a cytotoxic, rather than cytostatic, effect on wild-type yeast cells. One amino acid change that severely compromises Ncr1p function as assessed using the edelfosine resistance assay established *S. cerevisiae* as a model system that can be exploited to analyze the molecular consequences of patient mutations in NPC1 (270).

The yeast homolog of human NP-C2, *S. cerevisiae* Npc2p, is evolutionarily related to mammalian NP-C2 proteins. Through colocalization, subcellular fractionation, and secretion analyses, yeast Npc2p is similar to human NP-C2 when expressed in mammalian cells. It can efficiently correct unesterified cholesterol and GM1 accumulation in *hNPC2*<sup>-/-</sup> fibroblasts demonstrating it is a functional homolog of human NP-C2. The yeast genome encodes functional homolog of hNP-C1 (11,248) and hNP-C2 (271).

### 104.7.7 Treatment

Treatment is primarily supportive and includes anticonvulsants for the treatment of seizures and protriptyline in the treatment of cataplexy. SSRIs are effective for cataplexy, but at higher doses than those usually used for depression (75,141,262,263,272,273). Mood stabilizers in some of the chronic later onset patients are beneficial. A multidisciplinary approach involving physical therapy, speech therapy, neurology, and nutritionists has been useful. Cholesterol-lowering drugs have been tried, and although tissue stores of cholesterol go down with therapy, there was no impact on the neurological deterioration or progression of this neurodegenerative disorder. Liver and BMT have failed to alter the course of this

disease as well. Several therapies have shown efficacy in the feline and mouse models of NP-C including miglustat, antioxidants, and pregnane X receptor (PXR) activators (member of the nuclear receptor superfamily of proteins that modulate the expression of genes involved in the clearance of structurally diverse endogenous and exogenous compounds) cyclodextrin and allopregnanolone alone or in combination with a drug that activates PXR.

Experts agree that the stabilization of neurological disease is the best attainable therapeutic goal in patients diagnosed with NP-C due to the fact that the irreversible damage or loss of neurons will likely already have occurred by the time the diagnosis of NP-C is made (274). With the recent approval of miglustat (275) and the possible development of additional disease-specific therapies (22,276), treatment can be aimed toward stabilizing neurological disease (277).

### 104.7.8 Approved Therapies

SRT represents a potential strategy for treatment. Very recently, SRT using miglustat has been approved in Europe for treating NPC (278). Miglustat, a small iminosugar, is a reversible inhibitor of the enzyme glucosylceramide synthase, which catalyzes the first step in the biosynthesis of most GSLs. Miglustat has pharmacokinetic properties that allow it to cross the BBB, making it a potential therapeutic agent for treating neurological symptoms in NPC patients.

The efficacy, safety, and tolerability of miglustat in juvenile, adult, and pediatric patients with NP-C have been demonstrated in clinical trials (181,273,279,280) and observational studies, (88,281–283), supported by findings from off-label use (284–286), preclinical studies (24,287,288), case reports (31,68,145,261,273,274,289–292), and the miglustat postauthorization surveillance program (274,293). Miglustat stabilized neurological disease progression in pediatric patients with comparable safety and tolerability to that observed in adults and juveniles. Age at diagnosis influenced response to treatment, and clinical benefit or slowing of disease progression was also seen in the earliest childhood forms (294). Findings demonstrate clinically beneficial effects of miglustat on neurological disease progression in adult, juvenile, and pediatric patients, particularly in those diagnosed in late childhood (6–11 years) and juveniles and adults 12 years and older, compared with those younger than 6 years (22,262,263,274,295). The safety/tolerability of miglustat in children was consistent with that observed in adult and juvenile NP-C patients (259). Tremors, diarrhea, flatulence, abdominal pain, and weight loss were frequently reported adverse events (181,273,291,296).

Decisions to treat NP-C patients with miglustat should be guided by a scheme included in consensus recommendations. Patient disease course and response to therapy should be followed up regularly (every 6–12 months)



based on investigations and the NP-C functional disability scale considered a useful measure. There are no reliable, validated biochemical markers of neurological disease progression. Imaging techniques have potential, but further evaluation is required (80,203,204,277,297).

Cyclodextrins (CDs) can sequester lipids in the hydrophobic core of their cup-like structure, enabling them to bind and traffic cholesterol away from cell bodies (22,175,298). In vitro studies have demonstrated that methyl- $\beta$ -cyclodextrin is more potent than hydroxypropyl- $\beta$ -cyclodextrin in reducing cholesterol and bis(monoacylglycerol) phosphate accumulation in NP-C-mutant fibroblasts. Brief treatment of cells with CDs causes an increase in cholesterol esterification by acyl CoA: cholesterol acyl transferase indicating increased cholesterol delivery to the ER. These findings suggest that cyclodextrin-mediated enhanced cholesterol transport from the endocytic system can reduce cholesterol accumulation in cells with defects in either NP-C1 or NP-C2 (299,300).

A derivative of  $\beta$ -cyclodextrin, 2-hydroxypropyl- $\beta$ -cyclodextrin (HP- $\beta$ -CD) is a promising experimental therapy for NPD type C. Several studies in mice have shown that it is capable of overcoming the block in cholesterol delivery to the ER allowing the sequestered sterol to be excreted from the body as bile acid. Liberated cholesterol flows into the cytosolic ester pool, suppresses sterol synthesis, downregulates SREBP2 and its target genes, and reduces the expression of macrophage-associated inflammatory genes (175,301,302). Treatment with CD delayed clinical disease onset, reduced intraneuronal storage and secondary markers of neurodegeneration, and significantly increased the life span of both Npc12/2 and Npc22/2 mice (303). Studies of the efficacy of HP- $\beta$ -CD in feline model of NP-C disease recognized a dose-dependent increase in hearing threshold associated with therapy as determined by brainstem auditory evoked response (BAER) testing (304). HP- $\beta$ -CD has been approved by the Food and Drug Administration (FDA) for the treatment of NP-C disease (176,298).

### 104.7.9 Experimental Therapeutics

Targeting inflammation in the brain represents a potential clinical intervention strategy that aims to slow the rate of disease progression and improve the quality of life. Non-steroidal anti-inflammatory drugs (NSAIDs) significantly prolonged the life span of NP-C1 mice and slowed the onset of clinical signs. Combining NSAID therapy with SRT resulted in additive benefit in murine models, whereas the antioxidant therapy was of no value (278).

ASM activity defects in fibroblasts from NP-C1 patients were corrected by sphingomyelin phosphodiesterase-1 (SMPD1) transfection or sphingomyelinase enzyme replacement. Both treatments resulted in a dramatic reduction in lysosomal cholesterol in fibroblasts (305).

In vitro studies have shown that the overexpression of certain Rab GTPases corrects defective membrane trafficking and reduces lipid storage in cultured NP-C fibroblasts. Rab9 overexpression has the potential to reduce stored lipids and prolong life span in vivo using a murine model of NP-C. Histological studies and lipid analysis of brain sections indicate that NP-C mice carrying the Rab9 transgene had dramatically reduced the storage of GM2- and GM3-gangliosides relative to NP-C animals lacking the transgene (306).

Currently, there are no proper evidence-based trials showing efficacy in humans using stem cell therapy in NP-C disease. Studies in mice suggest that neural stem cell NSC transplantation may have therapeutic effects. It protects Purkinje neurons without significant immunological response (307). Bone marrow-derived stem cells (BMDSCs) are able to promote neuronal network formation with functional synaptic transmission after transplantation into NP-C mouse cerebellum. The transplantation of BMDSCs reduces the inflammatory response by suppressing astroglial and microglial activation in a murine model of NP-C (308,309). Adipose tissue-derived stem cells (ADSCs) can rescue imperiled Purkinje neurons and alleviate the inflammatory response in NP-C disease mice (310).

## 104.8 FARBER'S DISEASE

### 104.8.1 Introduction

Farber's disease is a rare disorder caused by defects in acid ceramidase (aCDase), the lysosomal enzyme that breaks down sphingomyelin-derived ceramide, with excess ceramide storage in lysosomes. It is characterized by the triad of subcutaneous nodules over extensor joints, painful arthritic joints, and hoarseness due to laryngeal involvement (Figure 104-5). The nodules may involve the eyelids, lips, and gums. Less than 100 cases are reported since the first cases in 1957. Due to the extremely small patient population, the natural history and underlying pathophysiology of this disease are poorly described (311).



**FIGURE 104-5** Farber's disease: subcutaneous nodules over extensor joints.



### 104.8.2 Clinical Description

Up to seven clinical variants are described. An extremely rare and fatal neonatal form presenting with hydrops fetalis is reported. Neonates may present during the first week of life (312). A rare presentation in the neonatal period is cholestatic jaundice and rapidly evolving liver failure (313). The classic variant is the most common with presentation between 4 months and 4 years. All cases manifest the triad. A variable number have lung infiltrates, cherry-red spots, organomegaly, progressive neurological deterioration with the CNS, anterior horn cell, and peripheral nerve involvement. Most die within 1–2 years of onset. Mild and intermediate forms exist with mild or no neurologic involvement. A neurologically progressive form exists with milder visceral disease with little if any organomegaly and no lung infiltration. One unusual case with simultaneous Farber's disease and Sandhoff disease has been described (314). Prosaposin deficiency, with resultant SAP A, B, C, and D deficiencies, causes symptoms characteristic of Farber's disease and Gaucher disease (GD) type II. These patients develop seizures, organomegaly, and respiratory and cardiac failure (315). Patients may present with nodular swellings around the joints, leading to misdiagnosis as juvenile idiopathic arthritis, or the angle of the mouth and conjunctiva. This disease is usually lethal within the first two decades of life (11,316).

Diagnosis of Farber's disease is confirmed by the demonstration of deficient aCDase activity and subsequent storage of ceramide. Existing methods for diagnosis include complex assays with radiolabeled compounds. A new fluorogenic method offers a rapid and accurate determination of aCDase activity and, consequently, the diagnosis of Farber's disease (317). Prenatal diagnosis is achieved by measuring ceramidase activity in CVSs (316). FD is diagnosed by demonstration of reduced aCDase activity and/or abnormally high ceramide levels in cultured cells, biopsy samples or urine, and the presence of "Farber bodies," comma-shaped curvilinear tubular structures, by transmission electron microscopy.

### 104.8.3 Pathology

There are prominent macrophages and histiocytes, as well as foam cells. These sometimes form multinucleated cells around central foam cells. Skin, joints, and larynx have granulomatous regions. The lungs, including alveoli, are infiltrated by many macrophages. Aortic and mitral valves occasionally are thickened with nodules. The brain shows storage material in neuronal cells. Anterior horn cells, brainstem nuclei, cerebellum, basal ganglia, and cortical and retinal ganglion cells can be involved. Ultrastructural studies demonstrate tubular structures in the cytoplasm representing ceramide and zebra bodies consistent with the storage of gangliosides.

### 104.8.4 Biochemistry

In severely affected cases, ceramide content of subcutaneous nodules, liver, kidney, and brain can be quite high. Mildly affected cases may have normal levels. Other glycolipids, particularly gangliosides, accumulate. PM and mitochondrial membrane ceramides have important biomodulatory roles in cell growth, apoptosis, and in regulating other cell-stress responses. The lysosomal ceramide that accumulates in Farber's disease does not cause increased apoptosis of cultured fibroblasts. There are other neutral and alkaline ceramidases active in different compartments of the cell that are distinct from aCDase. ACDase is a heterodimer made up of a longer glycosylated  $\alpha$ -subunit and a nonglycosylated  $\beta$ -subunit. ACDase (*N*-acylsphingosine deacylase, EC 3.5.1.23) is the lipid hydrolase responsible for the degradation of ceramide into sphingosine and free fatty acids within lysosomes. ACDase not only hydrolyzes ceramide into sphingosine, but also synthesizes ceramide from sphingosine and free fatty acids in vitro and in situ. This "reverse" enzymatic activity occurs at a distinct pH from hydrolysis ("forward") reaction (6.0 vs. 4.5, respectively), suggesting that the enzyme may have diverse functions within cells dependent on its subcellular location and pH. ACDase plays a central role in sphingolipid metabolism, controlling levels of ceramide and sphingosine that regulate many vital cellular processes like proliferation, growth, and apoptosis. Animal studies revealed that aCDase knockout (*Asah1*<sup>−/−</sup>) mice had a lethal phenotype (318). Saposins (sphingolipid activator proteins) are soluble glycoproteins necessary for the presentation of hydrophobic lipids like ceramide to lysosomal hydrolases for degradation. SAP C and SAP D present neutral ceramide for degradation in the lysosome into sphingosine and fatty acid by aCDase. The epidermal water permeability barrier or the intercellular lipid barrier of the stratum corneum is made up of ceramide, cholesterol, and free fatty acids, with the ceramide covalently bound to proteins on the surface of the skin (319). Activation of the ceramide pathway leads to sustained activation of ERK-1/2, redistribution of c-Fos and SOX9, and downregulation of type II collagen. This defines a new mechanism for cartilage degeneration seen in Farber's disease (320).

### 104.8.5 Genetics

Farber's disease is rare. Most cases are offspring of consanguineous marriages. The disease is inherited as an autosomal recessive trait, and mutations in the *N*-acylsphingosine amidohydrolase (*ASAH1*) gene, which codes for the aCDase enzyme, cause the disease. There is a notable absence of Jewish cases. Over a dozen point mutations and exon skipping mutations are known. Most affected cases have less than 5% aCDase activity in leukocytes, cultured fibroblasts, and tissues. Carrier diagnosis is possible using enzyme assays and DNA

analysis. Prenatal diagnosis is possible by measuring aCDase activity or performing lipid cell loading studies in cultured amniocytes or CVSs.

A novel mutation in the eighth exon of the *ASAHI* gene located in the  $\alpha$ -subunit of the aCDase protein was described in two Indian siblings. It is a missense mutation causing replacement of valine by leucine at codon p.L182V 182 (321). The c.502G>T substitution is a novel mutation in a Croatian boy leading to the replacement of glycine by tryptophan at position 168 of the aCDase  $\beta$ -subunit (322).

### 104.8.6 Treatment

BMT has been performed and has resulted in regression of the nodules and the organomegaly. Neurologic deterioration, however, was not impacted. If the selection of cases without neurologic involvement were possible, this would be considered a successful form of therapy. This, however, is not always possible as neurologic deterioration can set in as late as 4 years of age. Experimental therapeutic work in cell model systems with viral gene delivery systems is ongoing.

Current treatment focuses on pain management, physical treatment, surgical correction of contractures and treatment with anti-inflammatory drugs. Considerable and progressive joint involvement may result in the inability to walk and in repeated surgical procedures to improve mobility. Patients usually die in the second decade as a result of respiratory insufficiency.

HSCT may correct the abnormal inflammation and be an option in mild and intermediate forms of Farber's disease without CNS involvement. So far, three patients from Germany were transplanted. All showed improvement in mobility, had less pain and considerable gain of function. Despite this limited experience of HSCT in patients with Farber's disease with CNS sparing, the results are encouraging (323).

Gene therapy using oncoretroviral vectors (RV) could restore enzyme activity in Farber patient cells. Novel RV and LV vectors with the coexpression of aCDase and a cell-surface-marking transgene product human CD25 (huCD25) were used to transduce Farber patient fibroblasts and B cells resulting in the overexpression of aCDase and leading to a 90% and 50% reduction in the accumulation of ceramide, respectively. Vectors were also evaluated in human hematopoietic stem/progenitor cells (HSPCs) and by direct in vivo delivery to mouse models. In a xenotransplantation model using NOD/SCID mice, transduced CD34<sup>+</sup> cells repopulated irradiated recipient animals, as measured by CD25 expression. When virus was injected intravenously into mice, soluble CD25 was detected in the plasma and increased aCDase activity was present in the liver 14 weeks postinjection, suggesting that vector and transgene expression can persist long term. The results of these animal studies represent significant progress

toward the development of gene therapy strategies for Farber's disease (324).

## 104.9 ACID LIPASE DEFICIENCY (WOLMAN DISEASE AND CHOLESTERYL ESTER STORAGE DISEASE)

Wolman disease (WD) and cholesteryl ester storage disease (CESD) disease result from defects in acid lipase, which causes the accumulation of cholesteryl esters and TGs in the spleen, liver, BM, intestine, adrenal glands, and lymph nodes. WD manifests after 2 weeks and before 1 year of age with abdominal distension due to a large liver and spleen, steatorrhea, failure to thrive, and adrenal calcification, which is the hallmark of this disease (Figure 104-6). Most patients die by 6 months of age (171).

Although worldwide incidence is estimated at 1/350,000 newborns, WD occurs at higher than expected frequency in the Iranian Jewish community of the Los Angeles area. In the Iranian Jewish population, a heterozygous frequency is 3.086%. Prevalence for couples at risk is estimated to be as high as 1 in 4200 newborns. Additional studies are required to determine if populations of Middle Eastern descent are at a higher risk for WD (325).

CESD has a prevalence of <1/2000. It is more benign and is seldom detected before adulthood. Hyperbetalipoproteinemia and a large liver are the presenting symptoms. Patients may be completely asymptomatic at diagnosis (326). CESD usually presents during the first or second decade of life with males and females equally affected. The phenotype of CESD is highly variable. Abdominal pain is common and is often accompanied by growth failure and chronic diarrhea (105). Hepatomegaly, splenomegaly, and malabsorption are usual. Gall bladder dysfunction (GD) has also been reported (327). Long-term complications include premature atherosclerosis and stroke (55), BM suppression, and testicular storage (21). Patients with CESD may develop progressive liver failure necessitating liver transplantation. Sometimes, CESD is associated with mesenteric lipodystrophy. One case of cholangiocarcinoma has been reported in an adult patient with CESD (166).



FIGURE 104-6 Wolman's disease: adrenal calcification by X-ray.

Hepatocellular carcinoma (HCC) complicating CESD was serendipitously found during liver transplantation in an 11-year-old patient with the disease (328).

Human lysosomal acid lipase/cholesteryl ester hydrolase plays a crucial role in lysosomal hydrolysis of TG and cholesterol esters. Deficient hydrolysis results in lysosomal accumulation of nonhydrolyzed cholesterol esters and TGs and the upregulation of endogenous cholesterol and LDL synthesis. Biochemical evaluation commonly reveals liver dysfunction, increased total cholesterol and LDL cholesterol, decreased HDL cholesterol, and slightly elevated TGs. Pathologic atherosclerosis may potentially be significant, but most patients do not present with cardiovascular disease. Neurologic symptoms are typically absent from both. Diagnosis is made by demonstrating low acid lipase activity in leukocytes or cultured skin fibroblasts. Human lysosomal acid lipase is encoded for by a gene on the long arm of chromosome 10 (gene locus 10q24–25). Currently, more than 50 mutations have been described (262). Both WD and CESD are allelic, autosomal recessive variants. The former is due to the total absence of enzyme activity and results from a number of mutations. CESD patients, on the other hand, usually have 5% or more acid lipase activity. The (934G→A) allele, when present, is predictive of CESD. Compound heterozygosity is the rule (11) in CESD with the majority being carriers of a splice junction mutation (E8SJM). The prevalence has recently been calculated to be as high as 25/106 (20,327). Recently reported mutations include a novel nonsense mutation c.652 C>T (p.R218X) and a complex insertion/deletion leading to a premature termination codon at position 82 (329). No obvious genotype–phenotype correlation has been firmly established (272,326). Carrier diagnosis and prenatal diagnosis by measuring enzyme levels in fibroblasts, amniocytes or chorionic villi, respectively, have been achieved.

Pathologically, lipid-laden foam cells are seen in the digestive tract and aorta, liver, and other organs as well. The BM may show lipid-laden foam cells; for example, sea-blue histiocytes (20,141) and storage macrophages, which can be differentiated from Gaucher or pseudo-Gaucher cells only by an experienced pathologist. Micro- and macro-vesicular steatosis can be seen. Portal and periportal fibrosis may be marked, and there may even be frank cirrhosis. CESD can be distinguished from other lysosomal storage disorders by the examination of frozen liver sections under polarized light. In CESD, liver tissue reveals birefringence that disappears on heating to 50–60°C and reappears on cooling. Heat-sensitive birefringence of liver tissue is highly suggestive for CESD and WD and is not observed in other lysosomal disorders (166). Ultrastructurally, lucent lipid droplets with an electron-dense rim are apparent. In rare cases of WD, sudanophilic material is seen in neurons, glia, endothelial cells, and Schwann cells in peripheral nerves. The adrenals have a yellowish appearance and are difficult

to cut because of a gritty consistency. They are increased in weight due to the deposition of cholesteryl esters and TGs.

Treatment is nonspecific. Suppression of cholesterol synthesis and apolipoprotein B production with HMG-CoA reductase inhibitors is recommended, and has led to improvement in hepatosplenomegaly, HDL levels, and adrenal dysfunction in CESD. Whereas WD is usually lethal, current therapy of CESD consists of a low-fat diet and lipid-lowering drugs such as lovastatin and cholestyramine. The youngest reported patient in the literature treated with HMG-CoA reductase inhibitor is a 2-year-old boy (330). Single reports show increased efficacy by adding ezetimibe to the statin (331). Therapy with intravenous alimentation or dietary supplementation has failed to improve the prognosis of WD. Patients with decompensated liver cirrhosis may require transplantation.

BMT has only had a minor therapeutic impact, owing to the need for matched donors and cachexia of affected individuals (55,332). Krivit et al. (333) reported successful BMT in a 6.5-month-old child with WD (334). Tolar et al. reported long-term survival in four Wolman patients, two of whom are the longest survivors to date (4 and 11 years). Results showed resolution of diarrhea within weeks after engraftment, normalized hepatic function, improved hepatosplenomegaly, and in one patient normal adrenal function. The older patient had normal adaptive functions but mild to moderate neurocognitive deficiencies. The younger patient had age-appropriate neurodevelopment and adaptive abilities (335). Despite this report, WD remains fatal almost universally.

Curing this disease with stem cell transplantation is extremely challenging, with the majority of attempts resulting in death because of liver failure from sinusoidal obstruction syndrome, as well as other transplant-related complications including infection, GVHD, and the failure to engraft (336). Unrelated umbilical cord blood transplantation (UCBT) to treat WD may be more promising (21,332). One paper reported the successful use of umbilical cord blood or UCB-derived stem cells from unrelated HLA-mismatched UCB-derived stem cells in an infant with WD. The patient underwent UCBT at 3 months of age, and her psychomotor development at age 4 years seems to be normal, although laboratory results are suggestive of gonadal failure. The transplantation may result in the restoration of normal acid lipase levels before the onset of permanent end-organ damage and, if performed early, can potentially cure the disease (334).

Plant-produced recombinant protein is active in vitro and in vivo. Intraperitoneally administered active enzyme into deficient mice produced normalization of hepatic color, decreases in hepatic cholesterol and TG contents, and diminished foamy macrophages in liver, spleen, and intestinal villi (332). Transgenic acid lipase-deficient mice injected intravenously with adenoviral vectors containing human acid lipase cDNA (Ad-hLAL)

had increased hepatic enzyme activity, decreased hepatomegaly, and a partial normalization of histopathology, compared with saline-injected controls. Mice showed TG reductions in liver, spleen, and small intestine of 68, 54, and 50%, respectively, and cholesterol reductions of 55, 52, and 34%, respectively, at 20 days postinjection (263,331).

Studies show specific cellular targeting and physiologic effects of differentially oligosaccharide-modified human lysosomal acid lipases and that lysosomal targeting of mannose-terminated glycoproteins occurs and storage can be eliminated effectively (337).

### 104.9.1 27-Hydroxylase Deficiency or Cerebrotendinous Xanthomatosis

Cerebrotendinous xanthomatosis (CTX) is a relatively rare disorder described in Jews of north African descent, a group of Japanese cases, and Arab Druze communities, Italy, The Netherlands, and Pakistan. The defect arises because of deficiency in 27-hydroxylase (215,257) which leads to a block in bile synthesis, and accumulation of substrates for this enzyme, including cholesterol, resulting in an increase in the conversion of cholesterol to cholestanol. There is widespread tissue deposition of cholestanol and cholesterol, resulting in tendinous xanthomas, juvenile cataracts, progressive neurological defects, and premature death from arteriosclerosis (338). Plasma cholesterol is often normal, but cholestanol levels are always elevated. Patients can present with cataracts, ataxia, mental retardation or dementia, seizures, a peripheral neuropathy, and xanthomas. Osteoporosis has been described. Spasticity and spastic paraparesis are the most common and relevant clinical hallmarks of motor system involvement in CTX. Cerebellar signs are frequent in CTX phenotype, but contribute less to disability (339). Occasionally, psychiatric disease may precede neurologic involvement. Behavioral and psychiatric manifestations may include attention deficit hyperactivity disorder (ADHD) and oppositional defiant disorder (340). Some manifest before the age of 15 years or in childhood; others do not become symptomatic until later. Parkinsonism and oromandibular dystonia rarely occur (341). Achilles tendon xanthomas are common. These tendon xanthomas can develop later in the third or fourth decade of life. Cardiac manifestations are less frequent and may include coronary obstructive disease (342). Liver involvement is described in patients with neonatal cholestasis (105). Prolonged jaundice occurs more often in adult CTX patients than in the general population (55,343).

### 104.9.2 Diagnosis

CT and MRI are notable for atrophy and focal lesions in the basal ganglia and cerebellum. The diagnosis should be suspected in any one with tendon xanthomas and normal

or elevated cholesterol and in cataracts with a juvenile onset. Diagnosis is usually made by measuring cholestanol levels in plasma. Elevated cholesterol precursors other than cholestanol, including 7-dehydrocholesterol (7DHC), 8-dehydrocholesterol (8DHC), lathosterol, lanosterol, and sitosterol, can be a hallmark for CTX (344). Carriers can be distinguished from normal by an abnormally high excretion of bile alcohols in the urine after a cholestyramine challenge. The assay for the enzyme is available, but tedious (176). Treatment effects can be noted radiologically with brain single-photon emission computed tomography (SPECT). Cortical volume is diffusely decreased in CTX patients and correlates closely with the patient's clinical status. Magnetic resonance-based quantitative metrics allow accurate and objective estimation in lesioned and normal appearing brain (345). Proton MR spectroscopy showed significant signal increases in lactate and decreases in NAA above the lateral ventricles and in the cerebellar hemispheres. Response to treatment can be documented by observing decrease in lactate within cerebellar lesions (109). Transcranial magnetic stimulation (TMS) represents a sensitive indicator of corticospinal tract dysfunction and subclinical improvements in pyramidal function after chenodeoxycholic acid (CDCA) therapy (339).

### 104.9.3 Pathology

Cholestanol is found in tendon xanthomas. Yellow xanthomas are sometimes seen in the cerebellum. There is atrophy of the cerebellar folia with Purkinje and granule cell loss and demyelination. Areas of demyelination are characterized by needle-shaped clefts. These often contain multinucleated giant cells with a foamy cytoplasm. The gray matter is usually not affected. Granulomatous lesions have rarely been documented in the vertebrae, femur, and the lung. In the peripheral nervous system (PNS), there is cholestanol accumulation within Schwann cells, with severe loss of myelinated fibers, predominantly larger diameter myelinated fibers. Axonal degeneration may be secondary to the loss of myelin sheath in CTX. Lipid granules in cytoplasm of Schwann cells with apoptotic nuclei are seen. Fibrous tissue is arranged in parallel on the surface of the tendinous xanthoma (346).

### 104.9.4 Genetics

The gene was discovered in 1991 (276). The CYP27 gene is located on chromosome 2q35-qter. About 50 different mutations are identified in the CYP27A1 gene. DNA diagnosis is possible in a family with a known gene defect. A recent report in Italian patients described four novel mutations located in exons 2–5 of the CYP27A1 gene. The first one is a nonsense mutation in exon 4 (Ser251X). The second mutation, Gly145Arg, is located outside functional domains. The glycine at highly conserved position 145 most likely has a key role in enzyme



function (22). The third mutation (c.647-1 G>T) occurs in the AG consensus acceptor site, a highly conserved site affecting normal splicing, leads to an aberrant protein. The fourth mutation (c.863 delA in exon 5) results in a frameshift from glutamic acid at position 288 (Glu288fs) causing alterations in protein function (347). A novel mutation in exon 1 (11\_12insTGGGCT-GCGC) was described in two Argentinian siblings who developed mental retardation and cataracts without tendon xanthomas (348). Recently described mutations include 1017G>C (exon 5) (349) and a nucleotide deletion (c.1330–1333delTTCC) that results in a frameshift and the occurrence of a premature stop codon leading to a truncated protein of 448 amino acids (350). Another novel (R104Q) mutation was reported in a Japanese patient (351). Other novel mutations include base substitutions in exon 8, C478A, and C479A, which may affect the heme-binding domain of the enzyme (352) and a novel (c.11\_20dup) mutation in a compound heterozygous form. This latter mutation causes an early frameshift and consequent amino acid sequence alterations leading to a premature stop at the 175th amino acid position of CYP27A1 (p.Arg8fsX175) (353).

### 104.9.5 Treatment

It is most important to make the diagnosis of CTX as early as possible. Treatment with CDCA and HMG-CoA reductase inhibitors, if instituted during childhood, will prevent neurologic deterioration and may even lead to improvement (176). If diagnosed neonatally, prompt treatment may prevent the onset of neurological problems (354). In a 14-year study, prompt preclinical administration of CDCA completely prevented the CTX phenotype in two sisters. Prevention is particularly significant in light of the availability of early genetic diagnosis of CTX and the devastating effects of this illness when not treated. Three essential steps can prevent irreversible multiorgan damage in patients with CTX: (1) recognition of early symptoms, especially chronic diarrhea and juvenile cataracts, (2) definitive diagnosis of CTX biochemically and by mutation analysis, and (3) prompt treatment with CDCA to prevent the CTX phenotype (355). Treatment with farnesoid X receptor ligands can promote liver regeneration of patients with low bile acid levels. This could be developed clinically to promote liver regeneration in CTX (169).

Oxidative stress may be a critical component to the neurodegenerative process in CTX. In one study, CML adducts within foamy histiocytes were found in CTX cerebellar tissue, consistent with the presence of reactive oxygen species (ROS). This is supported by the fact that CDCA is important in absorbing fat-soluble vitamins, so untreated patients might be relatively deficient in antioxidant defenses (356). This may provide a rationale for the combined treatment of chenodeoxycholate with antioxidants.

A young man with CTX was on high-dose steroids for a misdiagnosed chronic inflammatory demyelinating polyneuropathy. He had a normal level of serum cholestanol, and when steroids were discontinued, markedly elevated serum cholestanol was measured concomitant with marked clinical worsening. This observation may imply that steroids can lower plasma cholestanol, possibly by directly inducing residual CYP27A1 activity, or alternative pathways for cholestanol elimination (357).

## 104.10 GAUCHER DISEASE

### 104.10.1 Introduction

GD is the most common lysosomal storage disorder. It arises because of the deficiency in the lysosomal hydrolase, acid  $\beta$ -glucosidase (263,358). It results in the accumulation of glucosylceramide, mainly in mononuclear phagocytes, which is responsible for the hepatosplenomegaly seen, as well as many of the other symptoms of the disease (359). Three types of GD are described: non-neuronopathic or GD type I, acute neuronopathic or GD type II, and subacute neuronopathic or GD type III.

### 104.10.2 Clinical Presentation

**104.10.2.1 Gaucher Disease, Type I.** This non-neuronopathic form of GD encompasses the majority of patients with GD in North America and Europe. Although this disease is panethnic, the prevalence is high in Ashkenazi Jews (1 per 850) compared to other populations (1 per 40,000). Age of presentation can be anywhere from infancy to the eighth decade. Initial presentation may be at birth. Most patients present with hepatosplenomegaly, BM abnormalities leading to anemia and thrombocytopenia, and bony changes due to the expansion of the marrow space. Mildly symptomatic patients are diagnosed later in life. Visceral disease is best understood in terms of distribution of storage cells or “Gaucher cells” in the RES. These are macrophages filled with insoluble glycolipids and derived from senescent WBCs and RBCs. Fatigability is a common complaint due to anemia and increased proinflammatory cytokine secretion. Splenomegaly is present in 90% of patients and splenic infarction is one of the more serious complications of this disease. Liver failure, cirrhosis, and portal hypertension occur in less than 10% of cases (360). At the beginning, thrombocytopenia is explained by splenic sequestration of platelets, and responds to splenectomy. In splenectomized patients, it is due to marrow failure as a result of invasion by Gaucher cells. Lymphoproliferative disorders, malignant proliferations, amyloidoses, and gammopathies occur with a greater frequency in GD-I patients than in the overall population. Skeletal involvement is frequent and ranges from osteopenia to severe destructive lesions. Medullary infarction, evidenced by 99m-technetium bone scintigraphy,

is not uncommon. Degenerative joint disease due to aseptic osteonecrotic lesions (particularly of the hips), thinning of cortical bone, and sacroiliac osteosclerosis are frequently described. Pulmonary involvement is rare, but may occur due to the infiltration of alveolar spaces, perivascular, peribronchial, and septal region by Gaucher cells. A restrictive lung syndrome may also occur due to hepatosplenomegaly or kyphoscoliosis (361). Pulmonary hypertension is a complication of GD. The clinical course varies widely. Patients homozygous for the c.1226A→G mutation demonstrate minimal progression during adulthood, whereas clinical manifestations appear to be progressive in the majority of adult and pediatric patients who are compound heterozygotes. Oral findings in the absence of clinical symptoms may lead to early diagnosis of GD. These are yellow pigmentation of the oral mucosa and petechiae, delayed eruption of permanent teeth (56% of young patients <20 years of age). Jaw involvement is often asymptomatic and can be detected as an incidental finding on routine dental radiographs including generalized osteopenia, loss of trabecular structure, effacement of lamina dura, and displacement of the mandibular canal, pseudocystic radiolucent lesions, and apical root resorption of teeth adjacent to the lesions, all of which appear in the mandible (362).

Although type I GD spares the CNS, Parkinson's disease (PD) has been reported as a late feature in GD-I patients. Furthermore, GD heterozygote state is now the strongest genetic risk factor for PD (4–5% of sporadic PD). About 60 patients with GD-I and adult onset PD have been reported. Pathology shows brainstem Lewy bodies and perivascular Gaucher cells characteristic of GD (11,20,21,55,56,105). GD-I may be asymptomatic or cause mild cytopenia and visceromegaly, and may be worth screening in patients from endemic ethnic groups presenting with PD. Clinicians should be aware of this association between atypical Parkinsonism and GD, which may be included in the list of etiologies of corticobasal syndrome (363). Relative to the general population, Parkinsonism in GD patients was more frequent, occurred at an earlier age, responded less well to levodopa, and was more frequently associated with signs of cortical dysfunction. ERT and SRT were ineffective in GD-associated Parkinsonism, suggesting that Parkinsonism itself is not an indication for ERT or SRT in this setting (364). Another rare but fatal complication of Type I GD is pulmonary hypertension that typically occurs in asplenic patients, and may arise via direct involvement of endothelial cells in the disease process (365,366). Other examples of unusual complications include myocardial involvement (367) and peripheral neuropathy (368,369). The principal manifestations of type I GD (increased risk of bleeding, anemia, splenomegaly, hepatomegaly, and bone disease) affect females during reproductive events such as menarche and menstruation; fertility, pregnancy, parity, delivery and lactation; and menopause. Enzyme therapy with alglucerase and/or imiglucerase may reduce

menorrhagia, spontaneous abortions and complications associated with delivery, and the postpartum period. Menarche may be delayed in girls with GD. Menorrhagia is common in GD and is ameliorated by alglucerase/imiglucerase. There is no evidence of decreased fertility in GD. Pregnancy in GD may be complicated by hematological disease, organomegaly, and bone involvement. GD is often diagnosed in pregnancy. There is a reduced risk of spontaneous abortion in women treated with alglucerase/imiglucerase; reduced risk of GD-related complications during delivery, and a reduced risk of GD-related complications during the postpartum period. There are no adverse effects of alglucerase/imiglucerase on the fetus or infants breast fed by mothers receiving alglucerase/imiglucerase. The impact of GD on menopause requires further study in relation to bone pathology (370).

**104.10.2.1.1 Neuronopathic Variants: Types II and III.** These variants manifest CNS involvement with many distinguishing features. Accumulation of glucosylceramide in CNS neurons occurs, but neuronal cell death not storage explains the symptomatology.

**104.10.2.1.2 GD, Type II.** GD, Type II, is the most severe form and is characterized by early onset and rapid disease progression. Formerly known as the acute infantile form, GD II accounts for less than 1 case per 500,000 births. The onset is declared by bilateral fixed strabismus and/or bulbar signs, progressive spasticity, and choreo-athetosis within the first 3–6 months of life, followed by neurological deterioration. Convulsions are observed in some patients. Death ensues during the first 2 years of life, due to psychomotor deterioration. Gaucher cell infiltration of the lung, liver, and spleen is the rule. The neurological presentation of type II GD is homogeneous and characterized by precocious, severe, and rapidly progressive brainstem degeneration. Frequent initial signs are hyperextension of the neck, swallowing impairment, and strabismus. Provoked asphyxial episodes and prolonged spontaneous apneas are main features. Epilepsy, myoclonic epilepsy/myoclonus, trismus, stridor, and progressive microcephaly are less characteristic. Psychomotor regression may occur, but is not a typical feature of the disease onset. Chronic or subacute pulmonary disease predominates in visceral involvement. Hepatosplenomegaly, failure to thrive, thrombocytopenia, and anemia are other remarkable, nonspecific, features. The inflammatory component of GD is underlined by unexplained fever (371).

**104.10.2.1.3 Gaucher disease, Type III.** Gaucher disease, Type III, has a later onset and a more protracted course. It accounts for less than 1 case per 100,000 births. A number of cases have been described from the northern Swedish region of Norrbotten. A functional differentiation between GD types II and III is based on the presence of bulbar signs in type II and their absence early on in type III as well as age of onset (372). Visceral involvement is moderate in GD III, but disease course is severe here with dementia and ataxia. GD III has been

further subdivided into three entities. Type IIIA is the Norbotten form. Type IIIB develops in early childhood with visceral disease manifestations resulting in death due to pulmonary and/or portal hypertension. Type IIIC (mutation c.1342G→C(D409H)) has few visceral manifestations, but has early-onset oculomotor paralysis. Individuals homozygous for this mutation may have mitral and aortic valve involvement, nonatherosclerotic coronary artery disease, hydrocephalus (166,369), and myoclonic epilepsy (55,94,289,366,369,373,374).

There is increased risk for malignancy in patients with GD with a relative risk estimated at 3.6 in one report. The most consistent association of GD and cancers is that with hematological malignancies like lymphoma, myeloma, and leukemia (relative risk 14.7). There is an association between GD and multiple myeloma. The association of GD and multiple myeloma in a population of mixed ethnicity is not fortuitous, although the link between the two has still to be defined. From the clinical point of view, the association remains infrequent and may not drastically change the management of patients with GD. Clinicians must keep in mind the sixfold increase of multiple myeloma incidence in GD patients, and thus pay particular attention to proteinuria, uncommon bone lesions, or clinical evolutions in patients older than 50 years (375). Solid tumors in patients with type I GD are documented in lung, bone, spleen, breast, prostate, colon, brain, and liver (21,22,56,171,175,176,181,272,301,376). All observed carcinomas or tumors develop in systems or organs that harbor GD cells. It is hypothesized that chronic stimulation of the immune system by the accumulation of glucocerebroside may lead to hematological carcinogenesis, whereas damage to the immunosurveillance system of T lymphocyte function may contribute to the development of solid tumors (166,377). Injured macrophages may release secretory products such as ferritin that promote carcinogenesis. There is no solid evidence of a causative relationship between GD and tumorigenesis. The association may just be coincidental (209). Two cases of HCC associated with GD are reported (55,105). Other conditions coexistent with GD may play a role in hepatocarcinogenesis. These are hepatitis B infection and the use of HCG-contaminated analogs of human  $\beta$ -glucocerebrosidase (378).

A strict division in three different forms has been the subject of debate. In a study of a large Dutch cohort of type I GD patients, presence of neurological disease occurred 34 times in 75 patients. Forty-five patients reported at least one neurological symptom during the median follow-up time of 11 years. There are 86 studies in which type I GD patients or carriers of a glucocerebrosidase mutation were described in association with the neurological disease. Non-neuronopathic GD may be an inappropriate characterization of type I GD. The neurological signs and symptoms in type I GD are different and much less severe than the signs and symptoms associated with types II and III disease. Type I disease

should therefore be classified as a separate phenotype. Given the many neurological symptoms and diseases in the Dutch cohort and the many reports on central and peripheral neurological involvement in the literature, the word nonneuronopathic is somewhat problematic. As the neurological changes in type I GD are of a different kind and, of much less severity in comparison with the changes associated with type II and III disease, the presence of neurological changes in type I disease should not lead to the concept of a continuum of symptoms and signs in the three types of GD (379).

At the extreme end of the phenotypic continuum is the perinatal lethal variant, typically presenting in utero or the neonatal period as hydrops and/or congenital ichthyosis, with severe and progressive neurological involvement. Insights from the null-allele Gaucher mouse model significantly contributed to the appreciation of this phenotype by unraveling the critical role of glucocerebrosidase in fetal development. While multiple mutations are encountered, many affected infants are homozygous for recombinant alleles. The diagnosis is often missed due to early lethality and the failure to recognize the association between lysosomal disorders and hydrops fetalis. The incidence of severe perinatal GD may prove more common than previously appreciated. The differential diagnosis of hydrops fetalis and/or congenital ichthyosis should always include lysosomal storage disorders, particularly GD. Diagnosis will enable accurate genetic counseling and facilitate prenatal diagnosis for families affected with this disorder (380).

### 104.10.3 Pathology

This disease is characterized by gliosis in the brain and fibrosis in the visceral organs. In the viscera and bones, infarction, necrosis, and scarring occur. In the brain, the most obvious manifestation is neuronal loss. Unlike other lysosomal storage diseases, most of the storage material in Gaucher cells in viscera is derived from phagocytosis of cells, cell membranes, and cell debris, and are not from cell-specific glycolipid synthesis. Brain neurons are the only cells where endogenous synthesis plays a role in pathogenesis. Little is known about the mechanism leading from lipid accumulation to disease, particularly in the neuronopathic forms of GD. The general hypothesis is that cell death and injury in GSL storage diseases may be mediated by the inhibition of protein kinase C by lysosphingolipids (381). A number of studies have suggested different causes leading to neuronal cell death among which are downregulation of the neuroprotective protein, BCL-2 (382), and defective calcium homeostasis.

### 104.10.4 Biochemistry

The human acid  $\beta$ -glucosidase is a homomeric glycoprotein. Some studies (292) suggest that the normal enzyme may be dimeric in cells, whereas other data (383) are

consistent with the enzyme being a monomer. Several investigators have suggested the existence of groups of mutant enzymes in GD sources, based on different phenomenologic responses to various modifiers of acid  $\beta$ -glucosidase activity (65,166,280) and altered processing/stability. The two classes of mutant enzymes are (1) those with decreased stability, normal interaction with inhibitors, and severely decreased catalytic rate constants; this group of mutant enzymes has been found mostly in non-Jewish type I, II, and III patients and (2) those with essentially normal stability, decreased affinity for active site-directed inhibitors, and moderately decreased catalytic rate constants; this group of mutant enzymes has been mainly found in Jewish patients.

Occasionally, GD is associated with manifestations without an obvious connection to the enzymatic defect and storage of glucosylceramide. This underscores our poor understanding of the biology of glucocerebrosidase gene or GBA1 mutations and the consequences of glucosylceramide accumulation in macrophages and effects on other cell types. Severe Parkinsonian syndrome described in some patients with type I GD has challenged its non-neuronopathic designation (94,384). Parkinsonian syndrome in type I GD is believed to arise from synuclein aggregation within dopaminergic neurons induced by gain-of-function mutations in GBA1 that lead to protein misfolding (i.e. N370S is such a mutation) or the accumulation of lipids (385,386). The increased risk of multiple myeloma and gammopathies in type I GD is attributed to chronic immune stimulation via activated macrophages (276). In type III GD, a distinct phenotype includes cardiac calcification, nonatherosclerotic coronary artery disease, hydrocephalus (166), and myoclonic epilepsy (94). Colloidion skin is the most severe manifestation of type II disease, a phenotype that is recapitulated in germ line GBA1 null mice (372). Taken together, these observations have challenged the macrophage-centric view of GD. Insights into the pathophysiology of GD have revealed important roles of cell types other than phagocytes in the development of GD (359,369).

### 104.10.5 Diagnosis

Measuring acid  $\beta$ -glucosidase activity in leucocytes delivers the diagnosis. Cultured skin fibroblasts also show decreased enzyme activity and may also be used. Enzymatic activity does not differentiate between the three types of GD since there is no correlation between residual enzyme activity and clinical phenotype. Ancillary diagnostic markers consist of increased activities of the following enzymes in GD patients: substrate-resistant acid phosphatase, ACE, and chitotriosidase. Chitotriosidase is produced and secreted by the pathological storage macrophages (Gaucher cells). Plasma chitotriosidase levels are elevated on average 1000-fold in symptomatic patients with GD and reflect the body burden on storage cells. Chitotriosidase is a member of the chitinase

family of enzymes in mammals. Amongst these is acidic mammalian chitinase, implicated in the pathogenesis of asthma. Chitinases are omnipresent throughout nature and are also produced by vertebrates and play important roles in defense against chitin-containing pathogens and in food processing (387). Changes in plasma chitotriosidase reflect changes in clinical symptoms. Monitoring of plasma chitotriosidase levels is commonly used in decision making regarding the initiation and optimization of costly therapeutic interventions (ERT or SRT). A novel substrate has been developed that further facilitates the measurement of chitotriosidase in plasma samples. It has also been shown that the chemokine CCL18 is 30-fold elevated in plasma of GD patients. Therapeutic efficacy can be assessed by monitoring levels of the enzymes mentioned previously, and demonstrating a lowering of CCL18 plasma levels (259), particularly in patients lacking chitotriosidase due to a genetic mutation (388).

In Ashkenazi Jewish cases of GD I, a small number of mutations are involved, and the diagnosis may be made by the analysis of five common alleles. In the overall population, however, the existence of numerous rare alleles restricts DNA diagnosis to the study of certain cases with an established molecular defect or defects in the gene. Prenatal diagnosis is best carried out by the determination of acid  $\beta$ -glucosidase activity in CVSs at week 10 of pregnancy, or in cultured amniotic cells at week 14 of pregnancy if the fetus is at risk for GD types II and/or III.

Population screening is not recommended, according to the NIH Technology Assessment Panel on GD, as the disease does not meet the population screening criteria. The debate regarding prenatal Gaucher screening is complex. Many healthcare centers in Israel offer prenatal panel screening. Controversy exists over the inclusion of GD in the panel screening, especially since GD screening lacks prognostic reliability. Most screening participants do not discriminate between the specific tests in the panel and are unable to discern between severe, life-threatening diseases and those that are less severe and even treatable. By including screening for GD in the panel screening program, there is risk of a “panel effect,” leading to the termination of a pregnancy positive for GD, without sufficient knowledge of the disease. Increasing medical/public awareness of the disease, its prognosis, and treatment options may reduce the rate of underinformed abortions associated with prenatal screening for GD. For the couple with a genetic susceptibility for GD, especially those at high risk for mutations with severe presentations, prenatal screening should be offered. It is important to explain that the genotype–phenotype correlation is imperfect and the prognosis is variable. Screening must include education about GD and its treatment, genetic counseling, and prenatal options so that the couple may make an informed decision regarding the future of their fetus. Healthcare systems should permit flexibility in screening according to individual preference (389).



Once a diagnosis of GD is confirmed, the focus should be on comprehensive evaluation of all disease domains to establish baseline disease characteristics, to determine candidacy for ERT, and to develop personalized therapeutic goals and monitoring strategy. Quality of life measures and a severity score calculation using one of the novel instruments applicable in the clinic are of value in making treatment decisions. Accurate skeletal assessment is important as it is the site of the most disabling, irreversible complications (369).

All Gaucher patients should receive a comprehensive initial radiologic evaluation for bone disease and ongoing radiological monitoring at least once in every 2 years. Plain X-rays are helpful in assessing bone disease, but MRI remains the most sensitive method for monitoring BM infiltration by Gaucher cells (390).

### 104.10.6 Genetics

Almost 300 unique mutations have been reported with a distribution that spans the *GBA* gene. These include 203 missense mutations, 18 nonsense mutations, 36 small insertions or deletions that lead to frameshifts or in-frame alterations, 14 splice-junction mutations, and 13 complex alleles carrying two or more mutations in cis. The mutations 84insG (84G>GC), c.1448T→C (L444P), and c.1226A→G (N370S) account for 8, 20, and 55% of the patient population with GD I, respectively. The (N370S) mutation is present in 6% of the Ashkenazi Jewish population. Investigation of the previously listed three mutations in Ashkenazi Jewish patients allows diagnosis of 93% of the alleles, and 95.5%, if the IVS2+1G→A allele is included. The three mutations account for only 70% of alleles in non-Jewish populations. The (N370S) mutation is described in patients from Portugal, Spain, Germany, and The Netherlands (158,259,289). In contrast, the most prevalent disease genotype worldwide across many ethnicities is L444P, the homozygous state being associated with severe childhood onset and a high risk of CNS involvement (166,175,181,369). Many deleterious alleles, also known as complex alleles (RecNciI and RecTL), occur in GD patients. These genes arise because of a gene-pseudogene crossing over, conversion, duplication, or fusion event. Their presence correlates with disease severity. Identification of novel mutations continues (373,374,391). Two cases of GD are described with no mutations in the  $\beta$ -glucosidase gene, but defects in the gene for sphingolipid activator, saposin C. The clinical phenotype varies from an asymptomatic course to cases of extreme severity in patients with same *GBA1* genotype and in affected sib pairs (160,369). Genotype–phenotype correlations are weak. The best genotype–phenotype correlation includes the presence of an N370S allele, which predicts the absence of primary CNS involvement due to GD. Yet, carrying one or two copies of the N370S allele, although protective for neuronopathic GD, predisposes individuals to early-onset

PD that is refractory to therapy. Study of sporadic Parkinson patients revealed that 14% of the cohort had mutations in the  $\beta$ -glucosidase gene.

Phenotype is influenced only in part by *GBA1* gene mutations and the important role of genetic/epigenetic modifiers has been invoked but awaits delineation (293,369). Despite advances in knowledge regarding the spectrum of *GBA* mutations, the ability to make prognostic predictions from genotypic data is quite limited. While it is possible to enumerate individual mutant alleles encountered in patients with type I, II, and III GD, this approach has limited utility, since it is the combination of mutations on both alleles that is important in defining the phenotype. Most *GBA* mutations are found in patients with more than one type of GD.

The vast majority of mutations identified have not been found in the homozygous form, and the “severity” of a given mutation is hard to predict, especially those found in only a few individuals. It is becoming increasingly difficult to categorize patients into one of the three classic types. Patients who present in infancy or childhood without neurological signs, in some instances, have been reported to be type I in the original description of the causative alleles. However, the development of neurological signs later in life has subsequently resulted in the reclassification of these individuals as type III. There are patients who seem to fall in an intermediate range between the classic type II phenotype and type III. Ultimately, it may be more accurate to envision the associated phenotypes as a continuum, with the major distinction being the presence and degree of neurological involvement. Clinicians have noted that patients with similar phenotypes may have many different genotypes, even in unique subgroups of patients. Conversely, individuals sharing the same genotype can present with and exhibit different disease manifestations, clinical courses, and responses to therapy. Differences are even observed among siblings and twins. Subjects with genotype [c.1226A4G]+[c.1226A4G] (N370S/N370S) can vary from asymptomatic adults to children with significant organomegaly, growth delay, or bone disease. A review of 35 patients with genotype [c.1448T4C]+[c.1448T4C] (L444P/L444P), each confirmed not to have a recombinant allele, demonstrated phenotypes ranging from death in early childhood to autism to being successful college students. One of the few identified genotype–phenotype correlations involves mutation c.1226A4G (N370S), which is encountered solely in patients with type I disease. Since the observed frequency of c.1226A4G homozygotes is considerably less than expected when calculated from the allele frequency in the Ashkenazi Jewish population, the majority of individuals with this genotype are probably asymptomatic or do not reach medical attention. Although the degree and severity of systemic involvement can vary, for the purpose of genetic counseling, this observation can be reassuring to couples seeking to clarify the phenotypic spectrum that may be associated with

an affected pregnancy. The distribution of mutations differs significantly in patients with the neuronopathic forms of GD. Among patients with type II GD, recombinant and rare alleles are especially prevalent. In patients with type III GD, those primarily manifesting with visceral manifestations often carry c.1448T4C (L444P) and/or c.1504C4T (R463C), while c.680A4G (N188S), c.1246G4A (G377S), and c.1297G4T (V394L) are more commonly seen in patients with myoclonic epilepsy, often together with a null or recombinant allele. One atypical phenotype, which includes calcification or fibrosis of the cardiac valves, corneal opacities, hydrocephalus, and dysmorphic features, is associated with mutation c.1342G4C (D409 H). Not all individuals homozygous for this mutation develop this unique phenotype, and when c.1342G4C is in cis with c.882T4G (H255Q), it presents as a type II phenotype. With patients harboring rare alleles or alleles associated with more than one phenotype, caution must be exercised in prediction of disease severity. One approach to making genotypic predictions is to focus on phenotypes encountered in individuals with homozygous genotypes. Approximately 30 different homozygous genotypes have been reported in the literature. There is clearly a dosage effect with some alleles, such as c.354G4C (K79N), c.680A4G (N188S), or c.1246G4A (G377S), where homozygosity for the mutation results in type I disease, while compound heterozygosity with a null allele leads to a type III phenotype. Likewise, individuals homozygous for c.754T4A (F213I) or c.1448T4C (L444P) usually develop chronic neuronopathic GD, but either mutation together with a null allele is more likely to have a type II phenotype. Mutation c.1504C4T (R463C) is particularly confusing, as homozygotes have been described with type I disease, yet when inherited with a null allele, either type I or type III ensues. While different groups have expressed specific mutant alleles *in vitro*, the results have been variable and difficult to compare. The residual amount of enzymatic activity is not associated with the patient phenotype. As seen in other Mendelian disorders, many factors, including complex alleles, alternate substrates, contiguous genes, environmental/infectious exposures, and many potential genetic modifiers, contribute to all the phenotypes in GD (392).

Type I GD is common in the Jewish Ashkenazi population, both other types are not. Phenotypically, GD-II in Ashkenazi Jews is similar to that in other ethnic groups. Homozygosity for certain Ashkenazi alleles might be lethal, leading to a lower frequency of GD2 explaining why it is so rare in the Jewish Ashkenazi population. No case of homozygous L444P has ever been described in Ashkenazi Jews (393). In view of the high carrier rate (1:17) among Ashkenazi Jews (377), it is intriguing that GDII is so rare among Ashkenazi Jewish children. No Ashkenazi Jewish GD-II cases or L444P homozygous patients has ever been reported in the international Gaucher registry. One Jewish non-Ashkenazi type III patient with L444P/L444P genotype is known, the parents being

first cousins from Kurdistan. The common N370S allele, not associated with neuronopathic disease, accounts for about 75% of mutant alleles in Ashkenazi Jews. Also two of the more common Ashkenazi alleles, 84insG and IVS2+1, are thought to be null alleles. Homozygosity would likely result in early lethality and missed diagnoses. The lack of the expected L444P/L444P patients may be another factor responsible for the relative rarity of this condition in Ashkenazi Jews. An awareness that type II GD can occur, albeit rarely, among Ashkenazi families, is of great value while performing genetic counseling in this population (393).

The majority of Chinese GD patients have an early age of onset, severe hematological and skeletal complications, and neurological involvement, resulting in early childhood death. In 29 cases of GD, 13 were diagnosed as type I, 10 as type II, and 6 as type III. A novel mutation, del 205–209ACCTT, was identified in the heterozygous form with mutation R353W (c.1174C>T) by DNA sequence analysis in five type I patients. Other common mutations in that population include L444P and F213I. The majority of the Gaucher mutations identified in Chinese patients were rare or absent in other populations. With the exception of N370S and R353W found only in the type I form, the majority of these mutations are severe resulting in poor prognosis and types II and III GD (394). In a study including 193 unrelated patients from the Spanish GD Registry, 9 novel mutations (4 missense R395C, R463H, W312R, and V398I, 1 nonsense R359X, 4 frameshift c.708delC, c.1214–1215delGC, c.1439–1445del7, and c.42–65del24) were reported. The prevalence of N370S mutation in Spanish GD patients is one of the highest in Europe, and that of L444P one of the lowest worldwide. Considered together, they account for 68.7% of mutated GD alleles in Spain. The latter contrasts with the findings in closed populations, such as Ashkenazi or Norrbottnian, in which the “founder” effect is evident. In these, the number of mutations giving rise to GD is small, facilitating rapid genetic diagnosis. A wide phenotypic difference was observed within each genotypic group and 9% of diagnosed type I patients developed neurological involvement including Parkinsonism, tremor, hypoacusia, and eye movement abnormalities. Findings indicate a significant genotypic heterogeneity explaining the huge phenotypic variation among Spanish GD patients (395).

### 104.10.7 Animal Models

A glucocerebrosidase-deficient mouse has been produced using the techniques of targeted disruption. Homozygotes died within 34 hours of birth and manifested extensive lysosomal storage of glucosylceramide.

### 104.10.8 Treatment

ERT using Cerezyme® is central to GD management (359,372). The aim of ERT is to reverse the symptomatic

clinical manifestations and prevent fibrosis of viscera. Patients generally respond well to ERT, irrespective of disease severity. Patients who are severely affected respond just as well as mild cases. There is reduction of existing hepatomegaly during the first 6 months of treatment. Hemoglobin levels normalize 1 year after treatment in anemic patients (396) and sooner in splenectomized patients. Bone and lung involvement do not respond as well due to poor enzyme delivery. The inability of the enzyme to cross the BBB limits effectiveness in neuronopathic forms of the disease. The main adverse event is the development of neutralizing antibodies that diminish the response to ERT over time and that manifests as worsening after initial improvement. A disadvantage is the cost and the need for intravenous infusions every 2 weeks (365). ERT was introduced as treatment for non-neuronopathic GD more than 15 years ago. Alglucerase, extracted from human placenta, was the first enzyme treatment modality for GD patients to provide specific treatment. Major limitations of this product were its low degree of reproducibility (because of low number of available placentas), risk of blood-borne infections, and short half-life (about 4.7 min in blood and 2 h in visceral organs including liver and spleen) (281,360). The limitations led to the development of a recombinant enzyme; imiglucerase. Imiglucerase (Cerezyme), a recombinant formulation of human glucocerebrosidase, is generated from transduced Chinese hamster ovary cells, and modified by sequential deglycosylation of its carbohydrate side chains to expose alpha-mannosyl residues that mediate the uptake of the intravenously infused enzyme. This enzyme has a longer half life and lack of blood-borne infection risk. It ameliorates systemic manifestations of hepatosplenomegaly, anemia, thrombocytopenia, and skeletal abnormalities in patients with type I and type III (chronic neuronopathic) GD and enhances health-related quality of life. In a randomized controlled trial involving 29 patients with imiglucerase (287), the primary end point was the improvement in bone mineral density of the lumbar spine. ERT significantly improved hemoglobin, platelet counts, and liver volume but did not repair the bone composition in splenectomized adult Gaucher patients. One randomized trial found no significant difference between imiglucerase and alglucerase. A second randomized controlled trial study (397) showed that low frequency administration of ERT in adult Gaucher type I patients maintains stable disease in most patients. The number of randomized controlled trials in ERT is small, but this treatment option has been administered to ~4000 patients worldwide (398). There is a dose-response relationship for imiglucerase therapy (314). For patients in whom immediate disease control is a priority, or those with life-threatening complications such as hepatopulmonary syndrome and pulmonary hypertension, the initial dose of imiglucerase is 120 U/kg body weight over a 4-week period. Patients may transition to lower-maintenance dosing regimens of 30 and 60 U/kg per month after initial disease control. For adult patients

with less severe disease, initial dosing of 60 U/kg body suffices (369). In the younger patients, there is no clear benefit of high-dose ERT (399). The treatment is well tolerated, and infusion-related adverse events such as pruritus and hives, observed primarily in patients who have developed antibodies to the enzyme, are manageable. ERT is the standard of care for patients with GD type I. Imiglucerase is also given to a majority of GD III patients, as a means of alleviating their systemic problems. ERT, however, does not eliminate the neurologic problems in GD III (400).

Velaglucerase- $\alpha$  is a gene-activated human recombinant glucocerebrosidase being developed by Shire Human Genetic Therapies Inc as an ERT for GD I. In vitro, velaglucerase- $\alpha$  was internalized by human macrophages more rapidly than imiglucerase. Clinical trials in patients with GD demonstrate that the safety and efficacy of velaglucerase- $\alpha$  is comparable with historical imiglucerase data, but head-to-head data are unavailable (401). Although effective in improving organ volume, anemia, thrombocytopenia, bone markers, and biomarkers in patients with GD, some patient needs remain unmet because of the limited availability of ERT, inaccessibility of certain disease sites, and emerging disease manifestations. Cases of type 2 diabetes and metabolic syndrome, as well as hematological, lymphoreticular, and immune system malignancies have been observed in patients with GD, but mechanisms underlying the development of these are not fully understood. Animal studies suggest that these complications may be related to the reduction of glucocerebroside levels by the enzyme administered. Glucosylceramide has an immunomodulatory effect through the promotion of dendritic cells, natural killer T cells, and regulatory T cells. The breakdown of glucosylceramide to ceramide can explain part of these findings (402). Splenectomy for treatment of thrombocytopenia is not recommended as pulmonary (361) and bone involvement worsen in splenectomized patients. BMT and its associated morbidity and mortality are not indicated for GD type I.

SRT aims at reducing tissue GSLs by decreasing production of glucosylceramide. One such drug is the *N*-alkylated imino sugar analog, *N*-butyldeoxynojirimycin or Zavesca®. This drug is now approved for use in patients for whom ERT is not suitable (360). This includes rare cases of allergy/hypersensitivity to imiglucerase, or those in whom venous access is not possible. NB-DNJ or miglustat is used in GD to reduce the formation of glucosylceramide by inhibiting glucosylceramide synthase. Various studies address the efficacy of SRT, which leads to improved blood counts, decreased liver and spleen volumes, and increased quality of life (288,403). A recent study gave comparable results to ERT (24). Major drawbacks of SRT are safety issues. Theoretically, the long-term suppression of GSL synthesis may produce detrimental results, but no major adverse events are reported to date. In patients who received previous ERT, the switch to SRT is well

tolerated (282). SRT is currently limited only to adult GD I patients who cannot tolerate ERT (398). A systematic review of studies examining the effects of ERT and SRT on bony complications of GD I concludes that ERT is very effective in ameliorating BM involvement. The effects of SRT on improving BM disease Z-scores of lumbar spine and femoral neck look very promising and seem to take a shorter period of time compared to ERT (404). Miglustat reduces the incidence of patients reporting bone pain and improves bone mineral density seen within the first 24 months of therapy (405). Two advantages are that it is an oral drug and that it crosses the BBB. Abdominal pain, weight loss, and diarrhea are problematic for patients, particularly during the initiation of treatment. Tremor and a reversible peripheral neuropathy are of concern as well. All patients receiving this treatment should undergo baseline and repeat neurological evaluations at ~6-month intervals. Males may experience infertility due to abnormal spermatogenesis. Downstream effects on vital GSLs beyond glucosylceramide may have deleterious effects on the peripheral and central nervous systems and therefore SRT should not be used in children under 18 or adults over 65, in accordance with FDA-approved guidelines. Because responses to miglustat are slower and less robust than those observed with ERT, and because miglustat is associated with significant side effects, clinicians who care for patients with GD should become familiar with the limited indications for its use and the circumstances when it may be prescribed appropriately.

The available ERT and SRT options are expensive as the annual cost of ERT is around 1 billion US dollars (289), and individual responses for these treatments are highly variable so that some patients may report no improvement. Decision for treatment must be individualized. Severe organomegaly, high degree of cytopenia, minor bleeding due to thrombocytopenia, bone disease, liver enzyme elevations with severe organomegaly, and the presence of any organ involvement other than the liver–spleen–bone triad are indications for treatment. The aims of treatment are reversal of organomegaly, prevention of complications, and improvement in the quality of life. Some patients may not require disease-specific treatment because they have very mild manifestations. Such patients are usually of the N370S homozygous genotype. These patients should be monitored regularly to detect signs of disease progression so that treatment can be initiated before new symptoms/irreversible damage occurs. Maximal therapeutic gains can be achieved by preemptive therapy.

Pharmacological chaperones that can enhance trafficking of certain mutant forms of  $\beta$ -glucosidase (N370S) through the secretory pathway, that are otherwise retained in the ER and degraded, can enhance enzyme activity. These agents penetrate the PM and the ER, and by binding to the folded mutant enzyme population in the ER, shift the equilibrium toward folding allowing mutant enzyme to be trafficked to the Golgi and on

to the lysosome more efficiently, where the high substrate concentration and low pH environment stabilize the  $\beta$ -glucosidase fold enabling it to degrade glucosylceramide. The resulting increase in mutant lysosomal enzyme concentration and cellular activity is beneficial. Eliglustat tartrate (Genz-112638), currently under development by Genzyme Corp, is a glucosylceramide synthase inhibitor for the treatment of GD. In vitro studies demonstrate that, following exposure to eliglustat tartrate, the abundance of GM1- and GM3-gangliosides in cultured human erythroleukemia cells and murine melanoma cells were decreased. In vivo, eliglustat tartrate administered to Asp409Val/null mice lowered the concentrations of glucocerebroside in the liver, lung, and spleen and reduced the number of Gaucher cells in the liver. In a phase Ib clinical trial in healthy volunteers, plasma glucocerebroside concentrations were decreased after dosing with eliglustat tartrate, and in phase II clinical trials in patients with type I GD, spleen and liver volumes were diminished. Patients also reported improved bone mineral density, correction of abnormal BM signal by MRI, and the normalization of glucocerebroside and ganglioside GM3 levels (406). These agents have the potential to be useful for treating patients with type II and III GD, for which there are currently no therapeutic options (407). Use of the  $\beta$ -glucosidase inhibitor, *N*-nonyl-deoxynojirimycin, at suboptimal concentrations in cultured cells has resulted in an increase in enzyme activity. Perhaps a combination of therapies can be tailored for patients. Control of secondary inflammatory changes may also alter disease progression. Further work on pharmacologic chaperones and the development of more efficient ERT or small molecule therapies may lead to improved treatment options (46). Stem cell transplantation is a high-risk procedure with possible long-term benefits in the regression of skeletal and neurological changes in people with GD. There are no clinical trials that have assessed the safety and efficacy of stem-cell transplantation in comparison to other conservative measures (408).

### 104.11 GALACTOSYLCERAMIDE LIPIDOSIS, GLOBOID CELL LEUKODYSTROPHY, OR KRABBE DISEASE

#### 104.11.1 Introduction and Historical Aspects

Krabbe disease (KD) arises because of defects in the galactosylceramidase (galactocerebroside  $\beta$ -galactosidase or GALC) protein that normally degrades the GSL, galactosylceramide. Krabbe's report of two infants who died of a familial and acute form of diffuse brain sclerosis was published in 1916. There were, however, two previous reports consistent with KD by Bullard et al. and Beneke that appeared in 1906 and 1908, respectively (248). Collier and Greenfield introduced the term globoid cells to describe the multinucleated, hematogenously derived



macrophages that are chock full of galactosylceramide (280). These are found in the central and peripheral nervous systems of patients. The symptomatology is entirely neurologic. The most common variant is the infantile form, which presents between 3 and 6 months of age with irritability, extreme spasticity, opisthotonic posturing, and an inability to be consoled by holding. Patients become blind and are severely impaired by the time they die during the second year of life. Late infantile, juvenile, and adult forms exist and are characterized by varying degrees of spasticity, peripheral neuropathy, dementia, and blindness.

### 104.11.2 Clinical Presentation

The most common initial symptoms between ages 0 and 12 months are crying and irritability, stiffness, and seizures. Older children are more likely to present with gait disturbances or loss of milestones. Survival differs depending on the age of onset. Children with the early infantile type (onset 0–6 months) have significantly worse survivals than those with a later onset (409).

**104.11.2.1 Infantile Form.** After a period of normal development, these infants manifest marked irritability, inconsolable crying, and opisthotonous posturing, with hands held in a claw-like position. Touching, stroking, or holding these infants does not have a soothing effect as one expects, but may provoke more crying, perhaps because of hyperesthesias. Psychomotor decline ensues. Although the head may be large, microcephaly is more common. Optic atrophy appears a few months into the illness. One single case has been reported with cherry-red spots. Peripheral neuropathy is often, but not always present. Reflexes may initially be hyperactive, only to disappear later. Seizures are quite common, particularly late in the course. These children seldom live past their second birthday. More recently, a Palestinian Muslim female presented with progressive neurologic deterioration at 3 months of age with rapid decline and death at 8 months of age. She had a defect in sphingolipid activator protein A or saposin A (SAP A).

**104.11.2.2 Late Infantile Form.** The age of onset is from 6 months to 3 years. Manifestations include psychomotor decline, irritability, ataxia, spasticity, loss of vision, and may include peripheral neuropathy and seizures. Children usually die 2 years after the initial diagnosis.

Acquired obstructive hydrocephalus is reported in patients with late infantile and with early infantile forms. CSF protein elevation may cause obstructive hydrocephalus through an effect on CSF drainage. Elevation of CSF protein increases viscosity, slows its circulation, and reabsorption. Elevated CSF protein may produce an aseptic arachnoiditis at the basal cisterns, with irreversible blockage. Functional obstruction at the aqueduct of Sylvius may also occur (410). Seizures may

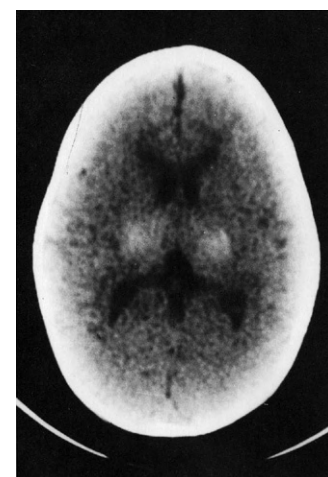
develop months to years after onset. Rarely do they signal the onset. Myoclonic seizures are an infrequent early symptom (411).

**104.11.2.3 Juvenile Form.** This variant becomes apparent between the ages of 3 and 8 years. Spasticity, ataxia, hemiparesis, and peripheral neuropathy are less common. Ultimately, cognitive and motor declines ensue. In spite of an initial rapid decline, the course levels off over a number of years with some patients surviving into the second decade (412,413).

**104.11.2.4 Adult Form.** These cases are the most difficult to diagnose. Individuals may be neurologically intact during childhood, or they may have some fixed neurologic deficit such as club feet, ataxia, tremor, or rigidity only to deteriorate later in life. In some cases, a late-onset cognitive decline or dementia can set in late in the course. Peripheral neuropathy has been described in some cases, but does not need to be present. A common clinical presentation for the adult form is one of familial spastic paraparesis (377,414,415).

**104.11.2.5 Laboratory Findings.** *Routine Blood Work:* Laboratory values are normal with two exceptions. CSF protein is almost always elevated in infantile and late infantile forms. There have been reports of elevated lactate levels in KD that may falsely point to a mitochondrial problem. Another leukodystrophy in which lactate may be elevated is Alexander's disease.

*Neuroimaging:* MRI and CT can greatly assist in narrowing down the diagnosis to a demyelinating disorder (Figure 104-7). High-signal, T2-weighted changes in the thalami, corona radiata, cerebellum, internal capsule, and periventricular white matter have been described in MRIs from these patients. Rarely, the enhancement of cranial nerves or lumbosacral nerve roots is observed early in the course of infantile cases. The detection of optic nerve enlargement and enhancement on MRI scans, not found in other types of leukodystrophies, is an auxiliary tool in the differential diagnosis of infantile KD (84,416–418). Rarer imaging characteristics of



**FIGURE 104-7** Krabbe disease: demyelinating lesions by CT scan.

early infantile KD include cystic lesions adjacent to the ventricles (419). Enhancement can be seen in the late form between the abnormal white matter and the spared subcortical U-fibers of white matter. Symmetric abnormalities were observed in the pyramidal tracts and optic radiations of an adult patient by fluid-attenuated inversion recovery (FLAIR) MRI. Cases of KD disease with selective involvement of corticospinal tract have been reported only in adults. Case reports of children with predominant involvement of corticospinal tracts are very few, but may show involvement of corticospinal tracts on MRI as the initial feature without other characteristic features of KD (420). Thinning of the spinal cord has also been reported in adult KD (421). Proton MRS has shown increased peaks of choline and myo-inositol with a decrease in *N*-acetyl aspartic acid; the first two findings are characteristic of demyelination, and the last suggests neuroaxonal destruction. The MRS abnormalities are milder with increasing age of onset. Also, lactate peaks by MRS have been reported. Diffusion-weighted MR imaging reveals high apparent diffusion coefficients (ADC) and low apparent anisotropy values before demyelination becomes apparent by routine MRI. These findings distinguish KD from other leukodystrophies such as MLD, Canavan's disease, and Pelizaeus–Merzbacher disease. Radial stripes by MRI have been studied pathologically in KD and correspond to perivenular clusters of globoid cells filled with galactosylceramide. DTI with quantitative tractography may detect significant differences in the corticospinal tracts of asymptomatic neonates who have the early-onset form of KD. DTI has the potential for use as a marker of disease progression in neonates diagnosed through screening programs in addition to monitoring response as future therapeutic modalities emerge (422). Infantile KD is reported to have MRI findings of diffuse cranial nerve/cauda equina nerve roots enhancement. Such findings may exist with or precede typical white matter abnormalities and may facilitate earlier diagnosis. These findings provide a rationale for use of contrast agents and craniospinal MR imaging in a child with a history of psychomotor regression and evidence of peripheral nerve involvement (423).

**Neurophysiological Tests:** Although EEGs may be normal initially, almost all infantile and late infantile cases have notable abnormalities later in the course. Multifocal epileptiform discharges, a slow and disorganized background, as well as asymmetry have been described. Nerve conduction velocities are almost always slowed, especially in the early forms, and often enough in the juvenile and adult forms. Peripheral neuropathy occurs very early in KD and affects the nerves uniformly. Marked nerve conduction abnormalities were found in 1-day-old and 3-week-old neonates, the youngest patients reported to date. The severity of the demyelination on nerve conduction correlates well with the clinical severity of the disease (424). EMG can also be abnormal. Evoked potentials have been closely studied in

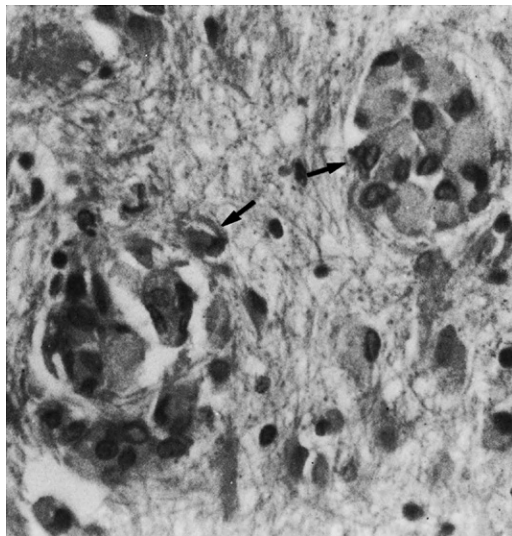
the early Krabbe form. BAERs are abnormal in 88% of cases, and flash VERs in 53% of early cases. For the later onset cases, BAERs were abnormal in 40% and VERs were normal in all cases. The severity of the abnormality correlated with more extensive disease by MRI. In the early-onset group, all symptomatic cases had abnormal BAERs, and in the asymptomatic early group 50% had brainstem auditory evoked potential (BAEP) abnormalities. For VERs, 67% of symptomatic cases had abnormalities, but none of the presymptomatic children did (175,425,426).

Diagnosis is confirmed by measuring galactocerebrosidase (GALC) activity in leukocytes or fibroblasts, and occasionally using DNA molecular diagnostics in specific families or ethnic groups (see “Biochemistry” and “Genetics” sections). A novel assay measures  $\beta$ -GALC activity following complete inhibition of  $\beta$ -galactosidase activity. This method unequivocally discriminates cells from healthy subjects and Krabbe patients and is therefore suitable for diagnostic applications. The procedure is straightforward, rapid, and reproducible, and the assay method is ideal for the diagnosis of KD (427). Prenatal diagnosis has been successfully performed on AF cells and CVSs from pregnancies at risk. Carrier detection is possible, though there is an overlap of enzyme values between noncarriers and carriers. It may be helpful to simultaneously assay parents together with those members of the family seeking clarification of their carrier status. When clinical course and neuroimaging studies are supportive of a diagnosis of KD in the setting of normal to minimally low enzyme activity and normal gene sequence for GALC, a defect in the activator saposin A must be suspected. The diagnosis can be made using natural substrate metabolism studies in cultured fibroblasts or DNA analysis of the *SAP A* gene. It is highly recommended that the biochemical assays only be performed in laboratories with experience in the diagnosis of the sphingolipidoses.

**104.11.2.6 Pathology. Central Nervous System:** Pathologic changes are largely limited to the nervous system. The brain is shrunken with large sulci and large ventricles. There is devastation of white matter with relative preservation of gray matter (Figure 104-8). Histologically, there are three striking features: loss of myelin and loss of oligodendroglia, the presence of multinucleated globoid cells, and astrocytic gliosis. Demyelination is most severe in the phylogenetically more recently myelinated structures such as the centrum semiovale and the cerebellum. There is secondary axonal degeneration. Mononucleated and multinucleated giant globoid cells are phagocytic in origin and tend to cluster around blood vessels (Figure 104-9). They are periodic acid Schiff (PAS) positive and stain intensely with acid phosphatase. Experimentally, globoid cells have been produced in murine brain by injection of solid galactosylceramide. Golgi preparations of pyramidal neurons reveal intact dendritic processes. At the end, relatively



**FIGURE 104-8** Krabbe disease: shrunken brain with large sulci and ventricles and relative preservation of white matter.



**FIGURE 104-9** Krabbe disease: giant globoid cells in the brain cluster around blood vessels (arrows).

little myelin is present and there is intense astrocytic gliosis (428–430).

**Peripheral Nervous System:** Peripheral nerves are thickened and rubbery in texture. There is segmental demyelination with variable distances between nodes of Ranvier. There is endoneural fibrosis, fibroblast proliferation, and perivascular presence of PAS-positive macrophages. Axonal degeneration is often present. Schwann cells often contain the typical needle-like tubular inclusions that are also seen in globoid cells in the brain by electron microscopy (426,431,432).

### 104.11.3 Late-Onset Krabbe Disease

Degeneration of the optic radiation and of the frontoparietal white matter with secondary degeneration of the corticospinal tracts has been described in two siblings. Peripheral nerves can be affected and Schwann cells with the typical needle-like inclusions have also been seen in sural nerve biopsies from older patients.

In fetal KD, the spinal cord begins to show abnormalities at about 20 weeks gestation: globoid cells are often observed in the dorsal columns and contain the typical tubular inclusions characteristic for this disease. The pons may be involved. There is no delay in myelination.

### 104.11.4 Biochemistry and Pathobiology

Galactosylceramide and its sulfated form, sulfatide, are major constituents of healthy myelin. The dilemma in KD is that although the hydrolytic GALC enzyme is missing, galactosylceramide does not accumulate in the brains of Krabbe patients or animal models. Multiple explanations have been put forth for this peculiarity. There is substrate specificity overlap between GALC and  $\beta$ -galactosidase, the enzyme deficient in GM1-gangliosidosis. The latter enzyme also can degrade galactosylceramide. Another substrate for GALC, psychosine, which is made up of galactose and sphingosine, or galactosylceramide minus the fatty acid, is not a substrate for  $\beta$ -galactosidase, and accumulates up to 100-fold in preserved myelin. The Krabbe–Psychosine hypothesis correctly claims that psychosine, a potent killer of oligodendrocytes by apoptosis, accumulates. Ultimately, dwindling numbers of oligodendrocytes prevent normal formation and turnover of myelin, which explains the lack of increase in galactosylceramide. Globoid cells, however, are induced by galactosylceramide as has been shown by injecting rat brains with galactosylceramide. Moreover, the needle-like and tubular inclusions seen in Schwann cells and globoid cells are quite similar to galactosylceramide aggregates. GALC was discovered in 1970. Determination of enzyme activity remains the best and quickest test for diagnosis. As stressed previously, the enzyme assay for GALC and the interpretation of results may not be entirely straightforward, and are best carried out in specialized laboratories.

The lysosomal GALC enzyme is involved in the maintenance of a functional HSPC niche by contributing to the control of the intracellular content of key sphingolipids. Both insufficient and supraphysiologic GALC activities,



by inherited genetic deficiency or forced gene expression in patient cells and in the disease model, induce alterations of the intracellular content of the bioactive GALC downstream products, ceramide and sphingosine, and thus affect HSPC survival and function, as well as the functionality of this stem cell niche. GALC and other enzymes may control the concentration of these sphingolipids within HSPCs (433).

Psychosine accumulation in LRs has functional consequences that range from interference with cell signaling to deregulation of raft-mediated endocytosis. Clearance of psychosine from LRs and the recovery of endocytosis are achievable with GALC replacement in vitro (434). The mutation of GALC and the resultant LR-targeted accumulation of psychosine disrupt the normal architecture of rafts. This drives a cascade of events in which there is an interference of protein–protein interactions that normally occurs in rafts, resulting in aberrant cell signaling, which then could lead to a decrease in normal overall cellular function and survival. This series of events should be considered, at the least, as a potential contributing factor in the overall decline in health of those suffering from KD (435). Psychosine accumulation induces an inflammatory process in nervous tissue and likely leads to impaired peroxisomal structure and function. This leads to downregulation of peroxisomes and the loss of peroxisomes, in turn, upregulating inflammatory disease (436). A psychosine-induced inflammatory process also occurs in liver and possibly other organs. The expression of cytokines and their effects on peroxisomal structure/function in Twitcher mouse liver indicate that Twitcher mouse pathobiology extends to the liver, where the induction of TNF- $\alpha$  and IL-6 compromise peroxisomal structure and function (437).

In rhesus macaques, globoid cells and parenchymal microglia express HLA-DR, indicating immune activation. Increased expression of iNOS, TNF- $\alpha$ , and IL-1 $\beta$  were observed in affected white matter, colocalizing with globoid cells, activated microglia, and astrocytes. This data suggest that the dysregulation of monocyte/macrophage/microglia and the upregulation of certain cytokines may contribute to the pathogenesis of KD (438).

The role of inflammatory cells and mediators in KD pathogenesis is suggested by increased production of TNF- $\alpha$  in peripheral blood mononuclear cells and the potentiating effect of psychosine in stimulated cells. Inflammation may be secondary to the damage induced by psychosine accumulation but it partly has a primary role in determining pathogenesis (439). Monocytes from Krabbe patients have a proinflammatory pattern that is amplified by psychosine. Atypical cytokine production suggests the involvement of inflammation in immune peripheral cells (440). Prostaglandin (PG) D<sub>2</sub> is a mediator of inflammation. Hematopoietic PGD synthase (HPGDS) is responsible for the production of PGD<sub>2</sub> involved in inflammatory responses. Microglial activation and astrogliosis occur during neuroinflammation,

particularly demyelination. In the Twitcher mouse, a model for human KD, activated microglia express HPGDS and activated astrocytes express the DP1 receptor for PGD<sub>2</sub> in the brain of these mice. Cultured microglia actively produced PGD<sub>2</sub> by the action of HPGDS. Cultured astrocytes expressed two types of receptors, DP1 and DP2, and enhanced GFAP production after stimulation of receptors with their respective agonist. The blockade of the HPGDS/PGD<sub>2</sub>/DP signaling pathway using HPGDS- or DP1-null *twitcher* mice, and *twitcher* mice treated with an HPGDS inhibitor, HQL-79 (4-benzhydryloxy-1-[3-(1H-tetrazol-5-yl)-propyl]piperidine), resulted in the suppression of astrogliosis and demyelination, and a reduction in twitching and spasticity. Furthermore, the degree of oligodendroglial apoptosis was reduced in HPGDS-null and HQL-79-treated *twitcher* mice. This suggests that PGD<sub>2</sub> is the key neuroinflammatory molecule mediating the pathological response to demyelination in *twitcher* mice (441).

In the *Twitcher* mouse model, neurological symptoms parallel severe lymphopenia. Although lymphopoiesis is normal before disease onset, primary and secondary lymphoid organs progressively degenerate. Hematopoietic cell replacement experiments support the existence of an epigenetic factor in mutant mice reconcilable with a progressive loss of autonomic axons that hamper thymic functionality and loss of immune competence. This finding suggests that KD patients are at risk of immune-related pathologies, and identifies a novel target for therapeutic interventions (442). Saposin A deficient mice demonstrate clinical, biochemical, and pathological phenotypes of a chronic form of globoid cell leukodystrophy (GLD) establishing that saposin A is essential for in vivo degradation of galactosylceramide. Saposin D deficient mice accumulate ceramides containing  $\alpha$ -hydroxy fatty acids (HFA/d18:1) in the brain and kidney and suffer from renal tubular degeneration and cerebellar Purkinje cell loss (443).

Psychosine induces the generation of lysophosphatidylcholine (LPC) and the release of arachidonic acid (AA) via the activation of sPLA<sub>2</sub> activity. Activation of PLA<sub>2</sub> generates ROS. Psychosine-mediated effects are nullified by the sPLA<sub>2</sub> inhibitor 7,7-dimethyleicosadienoic acid, which blocks the activation of mitogen-activated protein kinase (MAPK) and the generation of ROS by psychosine in oligodendrocytes. N-acetylcysteine (NAC) also attenuates LPC accumulation, AA release, ROS generation, and cell death induced by psychosine (4), suggesting involvement of oxidative stress in psychosine-mediated effects in oligodendrocytes. Generation of LPC has been implicated in demyelination (256,257) by the activation of cell death in mature oligodendrocytes and their progenitors (258,259). Metabolites of AA cause oxidative stress, regulating redox sensitive transcription factors (75,141,263) and cell death (261,262). Psychosine-mediated oligodendrocyte cell death is mediated via the sPLA<sub>2</sub> signaling pathway. Inhibitors of sPLA<sub>2</sub> may have



therapeutic potential protecting against oligodendrocyte cell death and demyelination in KD (444).

There is progressive loss of peroxisomal proteins/functions and induction of inflammatory TNF- $\alpha$  in Twitcher brain. The decrease in peroxisomal proteins was accompanied by lowered peroxisome proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ), a transcription factor required for expression of peroxisomal genes. Psychosine-induced downregulation of PPAR activity and cell death were attenuated by sPLA2 inhibitors. This suggests that the dysfunction of peroxisomes may play a role in the pathogenesis of KD and other lysosomal disorders. Peroxisomes and their function are compromised by psychosine accumulation. This compromised function may be responsible for oligodendrocyte loss and demyelination observed in Twitcher and Krabbe brains. This represents lipid-mediated downregulation of peroxisomal transcriptional activity and peroxisomal dysfunction in a storage disorder with psychosine-mediated pathobiology. Restoration of PPAR- $\alpha$  activity and the inhibition of cell death by sPLA2 inhibitors may provide novel therapies for KD (445). Psychosine accumulates in bones of mice. It may act as a factor or in conjunction with decreased plasma IGF-1 levels, impairing osteoclastic function, stunting bone growth. Treatment regimens may consider supplementation with paracrine factors (446).

### 104.11.5 Genetics

The human gene for GALC was identified in 1993. It consists of 17 exons and 16 introns. It codes for a 669 amino acid protein. The precursor protein is ~80 kDa and is processed to a 50–52 kDa and 30 kDa subunit. Both are important for enzyme activity. About 80 mutations have been reported as a cause of KD [<http://www.hgmd.org>]. A common mutation is the 502T/del, which accounts for 75% of Swedish cases and more than 50% of all European cases, suggesting a Swedish founder effect. There are two other mutations with high-carrier frequency. One occurs in Palestinian Druze (T1748G), and the other in a Palestinian Muslim group from a village close to Jerusalem (G1582A) (297,447–449). The mutation consists of a 30-kb deletion starting with a 502 C-to-T polymorphism beginning in the middle of intron 10 and extending to the end of the gene. This deletion eliminates the entire 30-kDa protein and 15% of the 50–52-kDa protein. All patients homozygous for this deletion have the infantile form of the disease. Recently, a prevalent GALC missense substitution p.G553R (G537R) was described in the Italian population with a high frequency and appears to be due to a common founder haplotype background in patients originating from Naples (450). The founder mutation c.169G>A (121G>A) is responsible for the other known KD “hotspot” in Catania (Sicily), associated with the late-onset form of the disease (451). There are a number of common polymorphisms (C502T, G694A, A865 G, and T1637C) that can occur together, or with disease-causing

mutations on the same allele. Presence of these polymorphisms is thought to contribute to the severity of the phenotypic disease expression. In a recent study of 30 European unrelated patients affected by KD, 15 previously unreported mutant alleles were described. These included four novel missense mutations that replaced evolutionarily highly conserved residues (p.P318R, p.G323R, p.I384T, p.Y490N), most of the newly described lesions altered mRNA processing, seven frameshift mutations (c.61delG, c.408delA, c.521delA, c.1171\_1175delCATTTCinsA, c.1405\_1407delCTCinsT, c.302\_308dupAAATAGG, c.1819\_1826dupGTTACAGG), and three nonsense mutations (p.R69X, p.K88X, p.R127X) one of which (p.K88X) led to skipping of exon 2, and a splicing mutation (c.1489+1G>A) that induced the partial skipping of exon 13. In addition, six previously unreported GALC polymorphisms were identified (450). A recent study in 17 Japanese patients with KD six mutations were novel, including two nonsense mutations, W115X and R204X, two missense mutations, S257F and L364R, a small deletion, 393delT, and a small insertion, 1719-1720insT. The two most frequent mutations 12Del3Ins and I66M+I289V account for 37% of all mutant alleles. With two additional mutations, G270D and T652P, these account for up to 57% of genetic mutations in Japanese patients (452). It is difficult to discern a trend in terms of a genotype–phenotype relationship. In addition to the mutational heterogeneity, the highly variable polymorphic background plays a role in modulating the genotype–phenotype relationship. Clinical phenotypes differ between individuals with the later onset forms of the disease, including siblings. The GALC mutational profile differs between European and Japanese KD patients. The most common large deletion, c.1161+6532\_polyA+9kdel (IVS10del30kb) plus three other mutations [c.1586C>T, c.1700C>T, c.1472delA (1538C>T, 1652A>C, 1424delA)] account for 60% of alleles in European ancestry KD with the classic infantile form (413,453). In the Japanese, these lesions are absent. By contrast, ~30% of Japanese GALC alleles associated with the infantile form of the disease possess c.683\_694del12insCTC or c.2002A>C (635\_646del12insCTC and 1954A>C) (452).

The common 30 kb deletion (c.1161+6532\_polyA+9kdel) is frequently found in patients with the infantile form of the disease (but sometimes with the juvenile form, depending on combination with other mutant alleles). The p.G286D (G270D) mutation might be associated with the juvenile/mild forms of KD. In vitro expression studies indicate that GALC enzymatic activity is absent in association with p.G553R (G537R), and is consequently associated with severe infantile KD in homozygosity or compound heterozygosity. The mutation p.R79H (R63H) is reported in association with the late-onset form of the disease. The p.E130K (E114K) mutation predicts a severe infantile form phenotype (450). The p.Gly41Ser mutation is described in the late-onset form and is associated with longer survival

(454). A high proportion of patients with late-onset forms of KD are observed in a region north of Catania in Sicily in which the founder mutation c.169G>A (121G>A) is described (451). In the Japanese population, I66M+I289M, G270D, and L618S contribute to a mild phenotype (452). Of particular interest is a patient with white matter disease restricted to one hemisphere and no symptoms. The mutation on one allele and the three polymorphisms associated with reduced expression on the other allele were present with enzymatic activity similar to that in heterozygotes without symptoms, or with mild phenotypes. A second somatic mutation in the *GALC* gene or another unidentified modifier gene may be present (455).

### 104.11.6 Animal Models

There are numerous naturally occurring animal models for KD (448,456). The most famous and useful has been the Twitcher mouse. A variety of naturally occurring canine models also exist (West Highland White terriers and others). Much of the work done in the murine model for the disease has provided novel insights into disease severity and progression and has resulted in ideas to approach therapy. There is a fair contribution of inflammatory processes to disease severity in this leukodystrophy. Crossing immunocompromised mice with the Twitcher model has resulted in progeny with attenuated disease severity. Also, apparently estrogens abundant during the pregnancy of some of the mice resulted in milder forms of the illness. Crossing the *GALC*-deficient mouse with a mouse heterozygous for galactosylceramide synthase or transferase gene (*GTC*) resulted in litter with milder disease, suggesting that there is some rationale to substrate reduction as well. The pioneering work on the beneficial effects of BMT KD was also first performed in the Twitcher mouse.

### 104.11.7 Treatment

Beneficial effects of BMT or cord blood transplants have been documented when the procedure was carried out in presymptomatic infants who had older siblings with the disease or in later onset cases (282). In utero BMT has not been successful in the few cases attempted. There are numerous words of caution that must be stated (181,249,333,457–459). Apparently, L-cycloserine is a potent inhibitor of psychosine production and its use as part of the treatment protocol in hematogenously derived stem cell transplantation may be responsible for many of the beneficial effects observed, suggesting its own potential as therapy. BMT or cord blood transplants may not be as helpful in already symptomatic infants and children, presumably because of advanced destruction of oligodendrocytes and the inability of these individuals to ever remyelinate normally. It becomes difficult to deny this extreme form of therapy in some but not all patients.

Although as physicians, we may understand the rationale and ponder the extravagance of expensive and futile drastic treatments, parents are more difficult to convince of their usefulness in some but not all affected children. Numerous other avenues for therapy are being explored in animal models: retroviral and adenoviral transduction of a variety of cells, direct introduction of *GALC* in viruses or liposomes into the brain, direct introduction of controversial stem cells directly in the brain, anti-inflammatory and antiapoptotic oral drugs, and the development of substrate reduction approaches, or combinations of any of the aforementioned approaches. Studies in Twitcher mice using gene therapy with intracerebrally administered genetically modified neural progenitor cells led to increased enzyme activity in oligodendrocytes, correction of astrocytic gliosis, and remyelination (280). As inflammatory mechanisms are involved in KD, anti-inflammatory treatment strategies are being investigated in GLD mice, in combination with HSCT (94,460). Universal newborn screening for KD in New York State and the screening in Illinois as of 2010, dictate the knowing outcome of available treatments. Expert consensus states that presymptomatic children with early infantile KD who are transplanted in the first 3 weeks of life survive longer than those without intervention. The vast majority eventually develop progressive neurologic/somatic growth impairment (461).

**104.11.7.1 Hematopoietic Stem Cell Transplantation.** The only available treatment for infants with early infantile KD is hematopoietic cell transplantation (HCT), typically using umbilical cord blood. HSCT in the Twitcher mouse and other animal models for KD significantly extends life span, normalizes psychosine levels, and improves central and peripheral neuropathological findings and motor ability. That being said, all transplanted animals have ultimately died of KD. In infants diagnosed with KD, HSCT significantly increases life span and ameliorates neurological outcome when performed before disease onset (282). In most cases, there is only a mitigation of disease severity. Despite improvement in white matter myelination after HSCT, children with KD still develop motor difficulty due to peripheral nerve involvement, as well as persistent cognitive deficits. Eighty percent do not walk independently at the age of 2 years (462). When the patient is already symptomatic, HSCT does not significantly modify the course of the disease (282,463). The efficacy of transplantation in later onset GLD remains less well delineated. Patients with later onset disease are likely to benefit from transplantation if undertaken early in the course of their disease, although no long-term data are available (464). HSCT leads to improvement in peripheral nerve conduction abnormalities in patients, suggesting remyelination of nerves occurs, particularly if performed early in the course (465). Allogenic HSCT has been beneficial; however, no transplanted patient achieved complete normalization of peripheral nerve

function, despite well-documented remyelination of the CNS and PNS (466). HSCT should be viewed as a treatment that attenuates the clinical course and improves survival, but is not curative. The majority of presymptomatic children transplanted for KD have developed motor and language deterioration, the cause and extent of which is unknown (461). Animal studies in Twitcher mouse suggest a possible role for IL-6 in the pathogenesis of GVHD in transplanted patients with GLD (467). The exposure of neonatal brain to irradiation performed prior to BMT, can lead to long-lasting alterations of postnatal neurogenesis and myelination, which might contribute to the progression of disease in myelin mutants and reduction in the success of BMT (468). In addition, inflammation, neurotoxic effects of preparative drugs for transplantation, and other factors may contribute to neurological deterioration. HSC-based gene therapy is being explored for GLD. Forced GALC expression is toxic to HSCs and early progenitors, highlighting the need for improved regulation of vector expression. By incorporating microRNA target sequences into a GALC-expressing vector, GALC expression can be suppressed while maintaining robust expression in mature hematopoietic cells. This approach protects HSCs from GALC toxicity and allows successful treatment of the mouse GLD model (469).

**104.11.7.2 Enzyme Replacement Therapy.** Intravenous administration of the deficient enzyme results in increased brain enzyme activity in the Twitcher mouse and prolonged survival times, weight gain, and improved gait (470). Enzyme replacement via direct injection into the brain parenchyma, the ventricles, or the lumbar subarachnoid space has been attempted (463). Peripheral injection of recombinant GALC, administered every other day, results in substantial improvement in early clinical phenotype of the Twitcher mouse. Following ICV administration of GALC, a 16.5% reduction in psychosine is noted. Recombinant GALC reached periventricular regions and sites distant to injection in the cerebral cortex and cerebellum. Animals receiving a single ICV dose at postnatal day 20 survived up to 51 days, compared to control Twitcher mice, which normally live to postnatal day 40–42. So, a single ICV administration of recombinant enzyme can have a significant clinical impact and suggests that lysosomal storage disorders with CNS involvement may similarly benefit from single-dose ICV administration of GALC, improving survival (471).

**104.11.7.3 Gene Therapy.** There is a growing literature on gene therapy in the brain of small and larger animal models with lysosomal storage diseases, although getting the viral vector into the brain remains problematic. The recombinant vector is injected directly into the nervous system. This approach has been successful in models of KD using AAV. A spontaneously transformed progenitor cell line was isolated from an astrocyte-enriched fraction of normal mice, partially characterized and transduced

with a retrovirus-containing mouse GALC cDNA to produce increased GALC activity (20- to 30-fold above baseline). These cells were injected into brains of newborn Twitcher mice. While there was only a modest increase in life span and body weight, there was clear evidence for the correction of astrocytic gliosis, normal-appearing oligodendrocytes, and evidence for remyelination. This treatment resulted in a 5-day delay in the onset of tremors, but only a modest increase in life spans and body weight. This treatment did result in the betterment of brain pathology. This includes an improvement in the appearance of oligodendrocytes with donor cells expressing GALC activity, and a reduction in staining for GFAP, indicating much less astrocytic gliosis (472). In vivo brain viral gene transfer of GALC has only shown modest impact on disease development in Twitcher mice, in spite of GALC LV vectors transduced neurons, oligodendrocytes, and astrocytes with efficiencies above 75% conferring high levels of enzyme activity. GALC accumulated in lysosomes of transduced cells and was also secreted to the extracellular medium. Conditioned GALC medium was able to correct the enzyme deficiency when added to nontransduced Twitcher glial cultures. Mice that received intraventricular injections of GALC vector showed accumulation of GALC in ependymal cells, but no diffusion of the enzyme from the ependymal ventricular tree into the cerebral parenchyma. Significant expression of GALC was detected in neuroglioblasts when GALC LV vectors were injected in the subventricular zone of Twitcher mice. Life span and motor conduction, however, were not significantly ameliorated (473).

**104.11.7.4 Combination Therapies.** Combined therapies will be needed to completely treat GLD. One treatment may help prevent early pathological events such as inflammation and apoptosis, while another may provide long-term supply of GALC activity to oligodendrocytes or replace dead/dying oligodendrocytes. Treatment of the PNS may require an entirely different approach. The combination of BMT and CNS-directed gene therapy may have a synergistic effect in ameliorating certain aspects of GLD. A combination of BMT and AAV2/5 results in improved brain pathology, mean life span, and behavior in mice (474). In the Twitcher mouse, combining L-cycloserine, an inhibitor of 3-ketodihydrosphingosine synthase, the synthetic enzyme of the toxic substance psychosine, and HSCT enhanced life span synergistically (461).

**104.11.7.5 Anti-inflammatory modalities.** Psychosine mediates inactivation of AMP-activated protein kinase (AMPK), which causes lipid alteration in astrocytes and oligodendrocytes. AMPK activator does not rescue oligodendrocytes from psychosine-mediated cell death, but downregulates psychosine-mediated production of NO and the expression of inflammatory cytokines/mediators (iNOS and Cox-2) in primary astrocytes. Loss of oligodendrocytes and the induction of inflammatory disease in KD/Twitcher brain suggest

that the inhibitors of sPLA2 and the inhibitors of inflammatory responses may be potentially therapeutic for KD (475). Mutations in *GALC* can cause GLD by impairing protein processing and/or folding. Pharmacological chaperones, therefore, may be potential therapeutic agents for patients carrying certain mutations (476). There is poor correlation between neuroimaging, neurophysiologic studies, and clinical course. Improvements identified in the white matter on MRI/DTI post-transplant are reported, despite evidence of continued neurologic dysfunction. Similarly, nerve conduction studies improve posttransplant, but clinical deterioration occurs. More sensitive techniques are, therefore, needed to monitor response (461). KD patients who underwent stem cell transplantation within the first month of life showed substantially smaller decreases in anisotropy ratios in four white matter regions than those treated later in the first year of life. These findings correlate well with global assessments of disease progression by neurodevelopmental evaluations and conventional MR imaging. Diffusion-tensor MR imaging offers a quantitative, reproducible method for evaluating white matter abnormalities (458).

## 104.12 METACHROMATIC LEUKODYSTROPHY

### 104.12.1 Introduction and History

MLD arises because of the failure of breakdown of sulfatide and other sulfated lipids primarily in the white matter of the peripheral and central nervous systems. This autosomally inherited illness occurs because of a deficiency in the arylsulfatase A (ASA) enzyme that normally desulfates 3-O-sulfogalactosyl-containing lipids. The accumulated sulfated glycolipids appear brown in tissue sections stained with cresyl violet, hence the name. The pathologic hallmark in humans is extensive demyelination in both the CNS and the PNS. Apparently, the first case of MLD described was by Alzheimer in 1906, followed by other reports from Witte, Scholz, and others (105). Jatzkewitz and Austin described excess sulfatides in tissues from patients. The ASA enzyme deficiency was discovered in 1965. Mehl and Jatzkewitz also confirmed this finding and later also discovered a heat-stable factor that increases enzyme activity several-fold, which we now know is the activator saposin B (385,477,478). Defects in saposin B account for a rare form of MLD with normal ASA activity (479). In 1965, yet another form of ASA deficiency with features of a leukodystrophy, ichthyosis, and a MPS was described, also known as multiple sulfatase deficiency, as seven different sulfatases are deficient (480,481). There are also numerous references to normal individuals with absent or very low ASA levels reported from MLD families. These individuals possess the “pseudo-deficiency” allele (384,482).

### 104.12.2 Clinical Presentation

**104.12.2.1 Late Infantile Variant.** The most well-known type of MLD is the late infantile variant. Additionally, there are juvenile- and adult-onset variants. The late infantile variant presents between 18 and 24 months. Children begin walking, but never outgrow their waddling gait stage. Typically, they have absent ankle jerks due to a peripheral neuropathy, Babinski signs, and the evidence of demyelination on neuroimaging and a peripheral neuropathy. They have optic atrophy, develop cognitive decline, and speech disturbances, only to become quadriparetic and generally do not survive the first decade. Mean age at death is 4.2 years and 5-year survival from onset of symptoms is 52% (483). Many develop seizures late in the course of the illness. They have elevated CSF protein, which is not uncommon in white matter diseases. An unusual occurrence in MLD may be papillomatosis of the gall bladder, which can present as acute cholecystitis or an abdominal mass due to the deposition of sulfatide in the gall bladder (22,484). Very rarely, hemobilia with massive intestinal bleeding occurs, necessitating cholecystectomy (485).

**104.12.2.2 Juvenile Variant.** This type can be early with presentation before 4–6 years, or late juvenile with age of onset between the ages of 6 and 16 years. Symptoms include difficulties with walking, ataxia, progressive spasticity, a peripheral neuropathy more prominent with earlier onset cases, and slow cognitive decline. Spinal fluid protein is also elevated, and optic atrophy may or may not be present. The older onset cases also may have psychiatric issues. Multiple polypoid masses in the stomach and duodenum can result in vomiting, gastrointestinal bleeding, and intussusception, as described in a 5-and-a-half-year-old girl with juvenile MLD (486).

**104.12.2.3 Adult Variant.** Quite frequently, the presenting symptoms may be psychiatric with slow, barely perceptible mental decline. Overt psychiatric illness is rare, but has been reported in two patients presenting with disorganized schizophrenia-like symptoms, and one patient presenting with postpartum depression (487). Peripheral neuropathy may or may not be present. CSF protein can be normal or mildly elevated. A myelopathy with spasticity is also observed in rare cases. Occasionally, ataxia, dystonia, bulbar signs, and tremor can be present, as well as optic atrophy. The course can be as short as a few years and may last for a number of decades. Terminally, patients are quadriparetic and spastic with increased deep tendon reflexes and some do develop seizures (46,250,488,489).

**104.12.2.4 Saposin B Deficiency.** Very few cases have been described. The clinical picture resembles that of ASA deficiency, but enzyme activity levels are normal. The age of onset is quite variable. Sphingolipid activator protein B deficiency is more common than ASA-deficient MLD in Arabs, a notion that has potential diagnostic and preventive implications (490). A high index of suspicion in



the setting of a degenerative demyelinating process and normal ASA enzyme levels leads to diagnosis, which can then be confirmed by the measurement of urine sulfatides, the assessment of sulfatide metabolism in cultured fibroblasts, or by direct sequencing of the gene (491).

**104.12.2.5 Multiple Sulfatase Deficiency.** A clinical picture similar to late infantile MLD with white matter changes on neuroimaging, and a Hurler-like MPS appearance with coarse facial features, deafness, dysostosis multiplex, and organomegaly, are consistent with the diagnosis of multiple sulfatase deficiency (MSD). The most common form is an infantile variant with mild MPS features, clear corneas, ichthyosis, optic atrophy, and retinal degeneration with rare cherry-red maculae. There is a neonatal severe form with prominent MPS features, an early infantile variant described in children from Saudi Arabia less than 1 year of age with dwarfism, severe MPS changes of dysostosis, craniosynostosis, hydrocephalus, and cervical cord strangulation, but the absence of ichthyosis or retinitis. MSD is due to the deficiency of seven different sulfatases, ASA being one of them. The defects arise because of a change in posttranslational modification affecting all sulfatases (see “Genetics” and “Biochemistry” sections later in this chapter) (480,481).

**104.12.2.6 Laboratory Tests.** *Routine Tests:* These are normal with the exception of CSF protein, which is elevated in the late infantile and early juvenile forms. The diagnosis is usually made based on ASA enzyme activity, urine sulfatide measurement, sulfatide turnover in cultured fibroblasts, and in some instances molecular DNA analysis.

*Neuroimaging:* Progressive loss of myelin in MLD has characteristic appearances by CT and MRI. The centrum semiovale is almost always involved with low signal on T1-weighted images and bright signal on T2-weighted sections. Arcuate fibers are almost always spared. There is prominent involvement of both frontal and parieto-occipital white matter with occipital regions being severely involved. The cerebellum is involved only in advanced stages of disease (492). A tigroid striped quality to the white matter is also sometimes seen. Proton MRS is abnormal with elevated myo-inositol values and

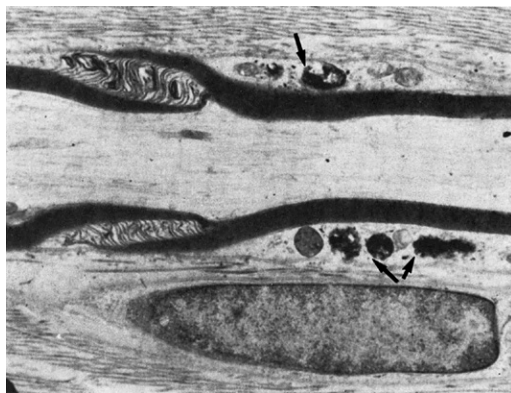
marked reduction in the *N*-acetyl aspartate peak, which suggests neuronal loss. There is elevation of the lactate peak in the white matter. MRS may provide a valuable tool for measuring the effects of treatment interventions. PET scans have demonstrated low metabolic rates in the thalami (46,489). MLD should be in the differential diagnosis of acute/subacute demyelinating polyneuropathy in children younger than 2 years, even with normal initial brain MRI scans (493). Late infantile MLD can present with marked contrast enhancement of nerve roots at the level of the cauda equina or isolated cranial nerve enhancement without brain white matter involvement at the time of diagnosis, in spite of the presence of clinical signs (494,495).

*Neurophysiology:* The EEG, although normal initially, becomes slow and abnormal. Some patients have epileptiform activity and many patients develop seizures late in the course of the illness (258). Nerve conduction velocities are slowed, particularly in the late infantile and early juvenile types, but can be involved in late juvenile and adult variants. Abnormalities in visual-, brainstem-, and somatosensory-evoked responses have been reported for all types, but are more common in the severe early-onset types. There can be prolongation of interpeak latencies loss of wave components (496).

**104.12.2.7 Pathology.** The pathologic hallmarks of MLD in the CNS are massive demyelination with sparing of U fibers within deep gray structures and metachromatic granules that stain brown with acidified cresyl violet (Figure 104-10). These granules are also observed in peripheral nerves and visceral organs. They appear as spherical structures with a diameter of 15  $\mu$ m. The ultrastructure is quite characteristic and appears as prismatic inclusions that have a herring-bone lattice, which appears cross-sectionally to have a honeycomb pattern. Another type of inclusion body described as Tuffstone-like may also be seen and has been reported in the thalami of adult MLD cases. In the late infantile variant, demyelination of the brainstem and spinal cord are prominent. There is a reduction in the number of oligodendrocytes and a reactive gliosis. The cerebellum is shrunken with the loss of both Purkinje and granule cells. Remaining Purkinje cells have torpedo-like swellings. The peripheral nerves,



**FIGURE 104-10** Metachromatic leukodystrophy: CNS demyelination with sparing of U fibers and metachromatic granules (arrows).



**FIGURE 104-11** Metachromatic leukodystrophy: peripheral nerve with segmental demyelination and metachromatic granules in macrophages and Schwann cells.

particularly in the early-onset severe forms of MLD, show segmental demyelination and metachromatic granules in macrophages and Schwann cells (Figure 104-11). Muscle histology may be distinctive for fiber-type disproportion with the atrophy of type I fibers. There is marked reduction in the thickness of myelin sheaths. Visceral organs, especially those with an excretory function such as the kidneys, the gall bladder, and the testis, accumulate sulfated lipids in adult MLD cases. Liver intrahepatic bile duct cells also contain metachromatic granules.

**104.12.2.8 Biochemistry.** Sulfatide and deacylated sulfatide of white matter are increased in late infantile MLD. It was also increased in cerebellum, brainstem, and spinal cord of a 24-week fetus. In adult MLD, white matter sulfatide is not as high, but gray matter sulfatide is more elevated than it is in late infantile MLD. They are also increased in gall bladder, liver, and kidney. Other sulfated glycolipids such as lactosylceramide 3-sulfate and seminolipid also accumulate, the latter only in testis. Sulfatide is a sulfated ester of galactosylcerebroside with the sulfate joined by an ester linkage to the C-3 hydroxyl of galactose. Formation of sulfatide is maximal during periods of myelination. Galactosylceramide, from which sulfatide is derived, and sulfatide maintain insulator function, are able to undergo hydrophilic and hydrophobic reactions, are active in Na transport, and are involved in binding of GABA, opiates, gp120 of HIV, prionic protein, and  $\beta$ -amyloid to the cell surface. The primary enzyme defect in all forms is in ASA, which is deficient in all tissues and cells from these patients. It is thought that excess sulfatide leads to myelin breakdown because it lowers the critical temperature for myelin. Late-onset forms of MLD presumably have residual enzyme activity such that sulfatide accumulates at a lower rate than early forms of MLD (260,497). Lysosulfatide is a cytotoxic compound in cell culture and may be involved in the pathology of MLD. ASA-null mutant mice show increased brain lysosphingolipid. Furthermore, a developmental increase of lysosulfatide is observed. Lysosulfatide is considered to be a possible pathogenic agent

in MLD contributing to severe demyelination occurring in patients. The mechanism of involvement is not known (498).

Transgenic ASA-deficient [ASA(-/-)] mice overexpressing the sulfatide synthesizing enzymes UDP-galactose:ceramide galactosyltransferase (CGT) and cerebroside sulfotransferase (CST) in neurons have been generated to provoke neuronal lipid storage. CGT-transgenic ASA(-/-) [CGT/ASA(-/-)] mice accumulate C18:0 fatty acid-containing sulfated GalCer in the brain. Histochemically, an increase in sulfolipid storage could be detected in central and peripheral neurons of both CGT/ASA(-/-) and CST/ASA(-/-) mice compared with ASA(-/-) mice. CGT/ASA(-/-) mice developed severe neuromotor coordination deficits and weakness of hindlimbs and forelimbs. Light and electron microscopic analyses demonstrated nerve fiber degeneration in the spinal cord of CGT/ASA(-/-) mice. CGT/ASA(-/-) and, to a lesser extent, young ASA(-/-) mice exhibited cortical hyperexcitability, with recurrent spontaneous cortical EEG discharges lasting 5–15s. These observations suggest that sulfatide accumulation in neurons contributes to disease phenotype (499).

Concentrations of cholesterol and its metabolites were determined in ASA-deficient [ASA(-/-)] mice, an animal model of MLD. A significant reduction in cholesterol content in the brain of adult ASA(-/-) mice compared to wild-type controls was observed. This was not due to the loss of myelin because ASA(-/-) mice do not demyelinate. Other cholesterol metabolites were not changed significantly in ASA(-/-) mice, except for an increase in lathosterol. Moreover, reduced cholesterol levels were found in tissue samples from two juvenile MLD cases. Since high cholesterol levels are important for myelination and various cellular processes, such as vesicular trafficking and signal transduction, reduced cholesterol might be an important factor in the molecular pathology of MLD. Arylsulfatase A and B proteins exist on the cell surface in mammalian tissues, including sinusoidal endothelial cells, hepatocytes, and sinusoidal macrophages (Kupffer cells), as well as the lysosome. They also colocalize with heparan sulfate proteoglycan. The extracellular localization of ASA may provide new insights for MLD (500).

### 104.12.3 Genetics

All MLD variants, with the exception of cases due to saposin B defects and cases with MSD, are inherited as autosomal recessive traits, and are due to allelic mutations affecting ASA. The actual defect in MSD remains unknown but affects the serine at position 69 in ASA, which should become formylglycine that is necessary for catalytic function of all sulfatases. In Sweden, the incidence of the disease is 1 per 40,000. It is lower than that in other European countries. Habbani Jews originating from Yemen have an incidence of 1 in 75 cases due to a high carrier rate and common consanguinity (501).

Arabs from an area north of Nazareth have an incidence of 1 in 8000. Two groups with high incidence are Eskimos (1/2500) and Navajo Indians (1/6400). The latter two groups carry the same genetic defect (502).

It is important to understand the genetics of ASA pseudodeficiency since the correct interpretation of ASA activity test results is crucial to correct diagnosis. This is a misnomer as the pseudo allele codes for a low-enzyme activity variant (5–10% of normal) without clinical consequences. This pseudo allele often is present in families that also carry a true MLD allele. The frequency of the pseudo allele in Europe can be as high as 10–20%. The incidence for heterozygotes carrying one pseudo and one ASA allele is 1 per 1500 individuals. A small proportion of these have neurological complaints are not related to MLD. The pseudo allele has two polymorphisms: (1) the first causes loss of one of three glycosylation signals resulting in a slightly shorter protein (N350S), and (2) the second polymorphism affects the polyadenylation signal, which precludes generation of the long ASA transcript. The N-glycosylation defect results in loss of 50% of ASA activity. Presence of the second polymorphism causing loss of the polyadenylation signal results in ASA activity that is 10% of normal. More than 68 mutations have been described in MLD. Three alleles account for most European cases. One of these alleles, c.459+1A→G, which results in loss of a splice-acceptor site, is found among Arabs. Seven of the MLD-causing mutations occur in the background of a pseudo allele. It is really the amount of residual activity determined with a sulfatide loading test that correlates best with disease severity. Patients with one pseudo and one MLD allele have 5–10% enzyme activity and are normal. Adult MLD patients have 2–5% enzyme activity, and patients with late infantile MLD have even lower activity from 0 to 2% determined in this manner.

In a multicenter European study with different forms of the disease, c.459+1G→A was found in 25%, whereas p.P426L was identified in 18.6%. Mutation c.459+1G→A was most frequent in late infantile MLD patients (40%), while p.P426L was most frequent in adults (42.5%). The mutation p.P426L was also found in a few late infantile patients (0.9%), and c.459+1G→A was present in some adults (9%). Mutation c.459+1G→A is more frequent in countries situated at the western edges of Europe. Mutation p.P426L is most prevalent in The Netherlands, Germany, and Austria. In other Central European countries, the frequency of both c.459+1G→A and p.P426L is 8–37.5% (503). P426L homozygotes present with progressive gait disturbance caused by spastic paraparesis or cerebellar ataxia. Mental disturbance was insignificant at the onset but became more apparent as the disease evolved. Reduced peripheral nerve conduction velocities and higher residual ASA activity are common (504). The I179S allele predisposes to psychiatric symptoms. A novel mutation, T279I, correlates with schizophrenia-like symptoms, neurological signs, and cognitive impairment

early in the course and fast progression toward dementia (505). Similarly, compound heterozygous missense mutations (p.G99D/p.T409I) may correlate with frontal lobe dysfunction manifesting as disinhibition and impaired executive function (506).

Two sisters with adult-type MLD developed psychiatric symptomatology, but differed in the expression of psychotic and depressive symptoms. Association studies had indicated that polymorphisms in genes encoding serotonin/dopamine transporters and receptors are related to symptoms of schizophrenia/depression, hence both sisters were genotyped for these candidate genes. The sisters shared dopamine receptor D2 (DRD2) c.1047GG (p.311Ser/Ser) and c.-141Cins/ins polymorphisms, which are also associated with schizophrenia, but differed in the serotonin transporter gene-linked polymorphic region and serotonin. A large sample is needed to have sufficient statistical power to develop a meaningful hypothesis. This is the first report genotyping candidate genes in MLD patients for psychiatric disorders, although MLD had been proposed as a model for schizophrenia (507). The sulfatide loading test also helps in identification of saposin B-deficient patients. Urine sulfatide excretion can also be used to differentiate these forms from each other. In determining carrier status, it is always best to be aware of the mutations carried by obligate carriers in that family. In prenatal diagnosis also, it is important to determine if the parents carry a pseudo allele as well. Sulfatide loading tests in this instance are extremely helpful (482). The ability to use dried blood spots to measure ASA protein and activity will simplify procedures for collection, handling, and storage of patient samples. The ease of transporting dried blood spots, combined with the diagnostic sensitivity and specificity of these assays, provide a powerful approach to the diagnosis of MLD. After further investigation and validation, these assays may be used for newborn screening for MLD and other lysosomal storage disorders (55,508). Presymptomatic detection of affected individuals may be possible with the introduction of newborn screening programs. The ability to accurately predict clinical phenotype and the rate of disease progression in asymptomatic individuals is essential to assist the selection of appropriate treatment strategies. Incorporating the determination of residual enzyme protein/activity using immune-based assays and metabolite profiling using electrospray ionization-tandem mass spectrometry were performed on urine and cultured skin fibroblasts from patients representing the clinical spectrum of MLD and unaffected controls. Residual enzyme activity in fibroblasts differentiated unaffected controls, ASA pseudo-deficient individuals, pseudo-deficient compound heterozygotes, and affected patients. MLD phenotypes were distinguished by quantification of sulfatide and other altered lipids in urine and skin fibroblasts. This enabled differentiation of the late infantile form of the disorder from the juvenile/adult forms. Prediction of the rate of disease progression for MLD requires a



combination of information on genotype, residual ASA protein/activity, and the measurement of sulfatide and other lipids in urine and cultured skin fibroblasts. A combined approach using genotype determination, where appropriate, followed by urine analysis combined with ASA and lipid measurements in cultured skin fibroblasts will provide good prediction of the age of onset and the rate of progression (509). Patients homozygous for the null allele suffer from the late infantile form. Heterozygosity for a null allele and a non-null allele are associated with the juvenile form, and homozygosity for non-null alleles is more frequent in attenuated adult-onset form. Genotype and mutation studies are valuable tools for accurate selection of patients for new therapies as well as for precise prognostication in presymptomatic cases (510).

A patient with adult-onset MLD, heterozygous for a novel nonsense mutation (C38X) (TGC/TGA) and a known missense mutation (T409I) (ACT/ATT), showed severely decreased nerve conduction velocities without obvious external clinical signs of neuropathy (511). On the other hand, a novel mutation (F219V) associated with an adult-onset case of MLD presented with progressive psychocognitive impairment without clinical or electrophysiologic signs and only minor morphologic signs of peripheral nerve involvement (512). A recent report described a Korean male with a novel heterozygous splicing mutation (c.1101+1G>T) in intron 6 and a heterozygous missense mutation in exon 2 (c.296G→A; Gly99Asp). The patient's elder brother who died of MLD is believed to have had the same mutation, which may be correlated with a rapidly deteriorating and lethal clinical course (513). Recently reported novel mutations in the Indian population include c.752\_753insT (p.Leu251fs) and c.98ANC (p.Tyr33Ser) that cause a late infantile form; c.508GNA (p.Gly170Ser) and c.1288GNT (p. Asp430Tyr) that result in an early-juvenile form; and c.1300GNA (p.Asp434Asn) causing a late-juvenile form (317). A first mutation of the ASA gene has been recently described in the Tunisian population. This was a novel missense mutation resulting in the substitution of Trp with Gly p.W124G and causing a late infantile form of MLD (514). In a study involving unrelated Italian patients, 11 new ASA alleles (c.53C>A; c.88G>C; c.372G>A; c.409\_411delCCC; c.634G>C; [c.650G>A; c.1108C>T]; c.845A>G; c.906G>C; c.919G>T; c.1102-3C>G; and c.1126T>A) were discovered (515). Other reports from Italy identified three novel mutations: Ser406Gly, Glu329Tr associated with late infantile MLD, and Leu52Pro with juvenile MLD (516). In addition, eight newly identified ASA mutations in the same population were identified and characterized through LV vector-based expression studies in cell lines and ASA-defective murine fibroblasts. The novel mutations c.155T>C, c.504C>A, c.634C>G, c.911C>T, c.919G>A, 1216A>G, c.1222\_1223delAC, and c.412C>G have resulted in null or very low residual

activities, whereas c.412C>G showed residual activity (517). In a study of Polish patients with different types of MLD, six novel mutations were identified: one nonsense (p.R114X), three missense (p.G122C, p.G293C, p.C493F), and two frameshift mutations (g.445\_446dupG and g.2590\_2591dupC). Substitutions p.G293C and p.C493F and duplication g.445\_446dupG caused a severe reduction of enzyme activity in transient transfection experiments in mammalian cells (less than 1% of wild-type (WT) ASA activity). Duplication 2590\_2591dupC preserved low-residual ASA activity (10% of WT ASA) (518). Molecular characterization of a cohort of 26 MLD patients allowed the identification of 18 mutations, 10 of which are rare and 8 novel: 1222–23delAC Asp407fs, 155T.C Leu52Pro, 504C.A Cys168STOP, 634G.C Ala212Pro, 911C.T Thr304Met, 1216A.G Ser406Gly, 412C.G His138Asp, 919G.A Glu307Lys (519). Two novel frameshift mutations (c.179\_180dupCA and c.1338dupC) were recently described in Chinese patients (520).

Juvenile MLD may rarely present with atypical, sometimes episodic, symptoms and findings. One patient demonstrated acute intermittent encephalopathic episodes for 1 year after having received the diagnosis of MLD at the age of 6 years. He was a compound heterozygote for the c.1277C>T (p.Pro426Leu) MLD mutation, and had a novel complex deletion/insertion mutation (c.1268\_1278delATGAGCCCC CGinsCCCCCCCC). Another patient homozygous for the mutation (c.1071A>G) presented at the age of 5 years with acute hemiparesis, which resolved in 3 weeks and developed progressive neurological deterioration after 2 years of remission. The episodic manifestations in both patients were associated with acute, resolving cerebral lesions on MRI accompanying or preceding the classical demyelinating lesions of MLD (521). A novel mutation at codon 1204 consisting of one base insertion or c. 1204+1G > results in a complete loss of enzymatic activity of ASA and subsequently a late infantile type MLD patient. MRI of the affected patient demonstrated the “tigroid” or “leopard-skin” demyelination pattern (522).

A patient with prosaposin precursor gene defects with saposin B deficiency presented with a MLD-like disorder, but had normal arylsulfatase activity. The SapB-deficient patient was a compound heterozygote. A 2-bp deletion (c.828–829delGA) in exon 8 on one allele resulted in the complete absence of the transcript, most likely due to nonsense-mediated decay, so no prosaposins or saposins could be generated from this allele. On the other allele, the c.577-2A>G splicing mutation led to the formation of two alternative transcripts, both of which carry an in-frame deletion of a portion of the SapB domain. This mutation exclusively affects the SapB domain and did not result in the deficiency of other saposins. In keeping with this finding, the biochemical and clinical phenotype in this patient was consistent with an isolated absence of SapB (321). Prosaposin activator gene analysis in



four unrelated Saudi families uncovered a homozygous mutation, a 722G>C transversion resulting in C241S change. This suggests that sphingolipid activator protein B deficiency is likely to be a common cause of MLD in this population. C241S is a prevalent mutation in Arab patients with MLD, and can be used for rapid diagnosis and preventive interventions (490).

A deletion on one chromosome and on a mutant allele on the other may cause an autosomal recessive disease. Patients with mental retardation, dysmorphic features, and low catalytic activity of ASA are described. One patient had a pathogenic mutation in ASA and succumbed to MLD. The other patient had a pseudo allele, which does not lead to MLD. The presenting clinical features and low ASA activity were explained in each patient, by a deletion of 22q13 and, thereby, of one allele of ASA (523). More than 100 cases of the 22q13 deletion syndrome (21,176,301) and ~90 cases of ring chromosome 22 [r(22)] have been reported (272,376). The ring formation of chromosome 22 is assumed to be associated with concomitant loss of telomeric material. Gene dosage studies have shown partial deficiency of ASA in patients with deletion of 22q13 (209). The patient reported (377) had a leukocyte activity of ASA at 47% of the normal. Whether the progressive peripheral neuropathy in their patient was related to this low ASA activity and MLD remained unanswered. Mental and physical regression in two of five patients with r(22) is described (272). One of the patients also had a diagnosis of bipolar affective disorder at the age of 12. The activity of ASA was not measured. The other patient had low ASA level, but molecular investigation for MLD was not performed. In 1995, a patient was reported with MLD and a r(22) deleted for ASA (524). ASA on the other allele carried a pathogenic mutation. The concurrence of MLD and the deletion of 22q13 may not be rare. It is suggested that ASA be measured in patients with 22q13 deletion syndrome for proper counseling, prognosis, and treatment (523).

#### 104.12.4 Animal Models

No naturally occurring animal models exist, but a transgenic mouse has been created that demonstrates sulfatide storage in the brain, kidney, and other organs. The neuropathologic lesion is similar to that in humans with more sulfatide deposited in the white matter than in the gray, loss of Purkinje cells, and a peripheral neuropathy. There is astrogliosis in the white matter but normal glia in mice of 1 year of age. By the age of 2, they demonstrate glial activation. There is a marked difference from human disease, however, in that the mice do not show CNS demyelination. They have a milder phenotype than one expects. Gene therapy experiments have shown variable positive results with decreased storage and mild clinical improvement (386). A second mouse model of MLD was optimized by transgenic expression of an inactive hASA mutant on the ASA-/- background.

Transgene expression does not alter the MLD-like phenotype, but confers absolute immune tolerance to wild-type hASA. The novel mouse strain represents an MLD variant with residual enzyme levels, which account for the vast majority of MLD cases. In contrast to conventional ASA-/- mice, the new strain allows for long-term ERT in the absence of immunological side effects (525).

#### 104.12.5 Treatment

Currently, no available treatment can reverse the fatal outcome of this devastating disease, and therapy is only supportive. Physical therapy, muscle relaxants such as vigabatrin and baclofen reduce spasticity. The former drug and other antiepileptics reduce seizures when present. BMT may have beneficial effects in the later onset forms of the disease but has shown no benefit for early-onset, late infantile MLD (256). Only a minority of transplanted MLD cases have been repeatedly reported in small series or as single-case reports. The posttransplant follow-up is short and does not allow proper interpretation of the clinical outcome. HSCT proved ineffective in patients with overt neuropsychological and/or neurological signs or in those with early-onset/aggressive infantile forms. Late infantile MLD cannot be effectively treated by HSCT of any type, cord blood or BM (20,56,141). The claim that late infantile MLD patients transplanted when asymptomatic or at least 1 year before the age of onset, will remain asymptomatic, has never been proven. Some case reports have suggested that BMT can stop the progress of juvenile MLD and may be indicated early in the disease as shown by the improvement in clinical, electrophysiological, and neuroradiological data obtained over long-term periods (526). Despite full engraftment and normalcy of enzymatic activity in juvenile/adult forms, the only benefit might be prolonged survival in selected cases, but without improved or stabilized quality of life (262,263,527,528).

In few patients, UCBT has been beneficial in stopping the progression of the disease clinically, neurophysiologically, and pathologically. Whether these are temporary or permanent effects will require long-term monitoring (529). The response to unrelated UCBT appears to depend on the stage of disease in each child at the time of transplant. A relationship between an individual's genetic makeup and his/her response to UCBT cannot be ruled out, so caution is advocated when recommending this modality to other families with MLD (530). Surprisingly, intravenous ASA enzyme replacement trials in the transgenic mouse model, given once a week for 4 weeks, result in reduced sulfatide storage in viscera, spinal cord, and brain. Efficacy in humans has not yet been tested, although this approach seems quite promising (531). Currently, a phase I/II trial is ongoing to evaluate the safety, efficacy, and pharmacokinetics of rhASA administration in patients with late infantile MLD. Efficacy data are still unavailable. There

are significant limitations such as the BBB, which limits access of the recombinant product to the nervous tissues. Furthermore, recurring intraparenteral administration of an exogenous protein carries the risk of inducing an immune response against the enzyme, thus reducing the therapeutic impact of the treatment (528). Therapeutic trials in knockout mice showed that ERT was tolerated without side effects and improved disease manifestations in a dose-dependent manner (532).

Injection of recombinant human ASA (hASA) in ASA-deficient mice reduced sulfatide storage in peripheral nerves and was effective in reducing sulfatide storage in the brain and spinal cord of treated mice. PNS symptoms prevail in infantile forms of MLD, and are nearly always present in other forms of MLD. ERT improved neuro-motor coordination capabilities and normalized peripheral compound motor action (531). ERT might have important clinical benefits if given in combination with other therapeutic approaches targeting the brain, but also in patients who have undergone allogeneic HCT. One may anticipate that early ERT treatment might have long-term clinical benefits in MLD patients, particularly if used to treat/prevent the motor disability due to the involvement of the spinal cord and PNS, not amenable to cell or gene therapy (533). ICV ERT at low dose reverses previously intractable ataxic symptoms of MLD mouse models and results in marked histological improvement, without adverse immunological effects, and may be an option for future treatment of MLD (534).

**104.12.5.1 Gene Therapy Strategies.** Intracerebral injection of adenovirus, AAV, and LV vector encoding hASA corrects the biochemical, neuropathological, and behavioral abnormalities in ASA-deficient mice without adverse effects, when AAV-ASA injections were performed at an early stage of the disorder. Injection at a later symptomatic stage prevented sulfatide storage and neuropathological abnormalities; however, neuromotor disability was unchanged (535–538).

**104.12.5.2 Cell-Based Therapy within the Brain.** An alternative approach to gene therapy is the direct engraftment of oligodendroglial progenitors or neuronal progenitors and of ES cells into the CNS of MLD patients, taking advantage of the regenerative capacity and cross-corrective activity of these cells. Mouse oligodendrocyte progenitors can be grafted into neonatal MLD brain, and ~10% of these cells survive in adult-treated mice. Transplanted ASA-deficient mice display reduced sulfatide accumulation in the CNS. This results in the prevention of motor deficits and electrophysiological abnormalities that characterize ASA-deficient mice. Migration and targeting of oligodendroglial progenitors into white matter was significantly greater when these cells were intravenicularly transplanted in neonate MLD mice. Transplanted cells seemed to tolerate the toxic accumulation of sulfatides, although this is minimal at birth in ASA-deficient mice. The distribution of these cells was more restricted when transplantation was performed 2 months

after birth (539). Neural progenitor cells (neurospheres) prepared from the striatum of E14 embryo ASA-deficient transgenic mice were transduced with a LV vector carrying ASA cDNA, and injected into the brain parenchyma of adult ASA-deficient mice (540). Transduced neurospheres retained their potential for differentiating into neurons, astrocytes, and oligodendrocytes in vitro. One month after injection, transduced cells were detectable in the brain, and partial reduction of sulfatide was observed. ASA enzyme was present in both transduced and nontransduced cells, suggesting that genetically engineered neural progenitor cells (neurospheres) could potentially be used for ex vivo therapy in MLD. Transplanting engineered murine ES cells to overexpress the hASA in neonatal ASA-deficient mice resulted in a reduction in sulfatide accumulation up to 175  $\mu$ m from the donor cell, and in vivo differentiation of the grafted cells into astrocytes and oligodendrocytes (541). These experiments demonstrate that ES cells may constitute a potential donor source for cell-mediated ASA delivery. Human MSCs have already been infused into patients following HCT for MLD. MSCs were isolated and expanded from a BM aspirate from the original donor for HCT. Four patients showed significant improvements in nerve conduction velocities after MSC infusion (533).

In vitro treatment with encapsulated cells overexpressing ASA was able to correct ASA deficiency in MLD skin fibroblasts. The implant of microcapsules within the brain could be a potential new therapeutic approach along with ERT. Cell encapsulation would target the CNS whereas ERT would reach the PNS. Continued studies are needed for perfecting timing, routes, and methods of delivery of gene/proteins to their targets in the brain and the PNS. PNS symptoms prevail in infantile forms of MLD, and are nearly always present in other forms of MLD (542).

## 104.13 FABRY DISEASE

### 104.13.1 Introduction

Fabry disease is an X-linked recessive lysosomal storage disease associated with systemic deposition of neutral GSLs, due to defects in the enzyme  $\alpha$ -galactosidase A (543). It was first described by Anderson from England and Fabry from Germany in the 1800s.

### 104.13.2 Clinical Presentation

Clinical onset typically starts during childhood or adolescence, but often is delayed until the third decade. The majority of FD complications are nonspecific and include left ventricular hypertrophy, conduction abnormalities, vascular spasms, proteinuria, and renal insufficiency, which are the reasons behind FD remaining largely underdiagnosed. Female carriers are at risk of developing disease, but this tends to be milder and more slowly

progressive than in males, although some women can display the entire range of clinical manifestations (544). Excruciating pain is the most incapacitating symptom of the disease, and can be episodic or constant. The pain begins in childhood in parallel with disease onset, and is a burning pain starting at the palms and soles of the feet radiating to other parts of the body and is termed acroparesthesias. Fever, exercise, fatigue, and stress trigger painful crises. Patients also suffer from hypohidrosis or anhidrosis and fevers. Another hallmark is the development over time, typically just before or around puberty, of angiokeratomas or angiokeratoma corporis diffusum in a bathing trunk distribution often involving the buttocks and scrotum, and sometimes the hips, thighs, umbilicus, and mucosal surfaces (524). Cardiac involvement is common and affects valves (mitral insufficiency), ventricles (left ventricular enlargement), and the conduction system (376). EKG changes are not due to necrosis, but due to GSL deposition in the myocardium. Patients may present with angina pectoris, dyspnea, palpitations, or syncope. Angina is often reported, but the incidence of epicardial coronary stenosis is not a dominant feature. Small vessel disease can occur. With the progression of the disease, patients die due to heart failure. Some patients manifest the milder “cardiac variant” in which cardiovascular symptoms exist in the absence of acroparesthesias or skin manifestations. This implies that some patients with unexplained myocardial hypertrophy may have undiagnosed FD. Cardiologists should play a key role in suspecting and diagnosing FD. Cerebrovascular disease results from multifocal small vessel involvement, and may end in thrombosis, transient ischemic attacks, basilar artery aneurysm, seizures, aphasia, hemiplegia, and hemianesthesia. MRI and proton MRS are useful in evaluating cerebrovascular disease (216,545,546). Conventional MRI shows micro- and macroangiopathic changes such as severe and progressive white matter lesions at an early age on T2- and FLAIR-weighted images, increased signal intensity in the pulvinar on T1-weighted MRI, as well as tortuosity and dilatation of the larger vessels (dolicho-ectasia). DTI shows brain tissue alterations predominantly in the periventricular white matter. DTI is more sensitive in detecting changes than conventional MRI. DTI measurements could provide appropriate surrogate parameters with which to monitor the natural history of structural brain involvement and potential effects of therapy such as enzyme replacement (547). GSL deposition in the kidney results in proteinuria, with progressive deterioration in renal function and the development of azotemia by middle age. Recently, kidney biopsy data have shown early renal histologic changes in pediatric patients, and kidney dysfunction, primarily proteinuria, is more common in girls. Renal disease with presentation of an ischemic kidney with no albuminuria has been reported (548). Currently, the recommendation is to evaluate systematically all children in registries and to measure albumin excretion

rates regularly and precisely. Renal biopsy could be a method to follow disease progression and responsiveness to treatment. No adequate data on the timing of ERT and the benefits of renal biopsies exists. Currently, it is recommended to perform follow-up noninvasive renal function tests such as three consecutive early morning urine samples for microalbuminuria, a marker of early renal disease (549). Birefringent lipid globules with characteristic “maltese crosses” are observed in the urine in desquamated cells by polarization microscopy. MRI reveals loss of corticomedullary differentiation. Death results from uremia. Gastrointestinal disease due to GSL deposition in intestinal small vessels and in autonomic intestinal ganglia leads to symptoms of diarrhea, abdominal pain, flank pain, nausea, and vomiting. Airflow obstruction, reduced diffusing capacity, and a reduction in the  $V_{\max}$  values may lead to chronic bronchitis, wheezing, and dyspnea. Minor changes are seen on the EEG and EMG. The cornea, lens, conjunctiva, and retina are affected. Spoke-like opacities in the corneas and characteristic lenticular inclusions (290,550) are evident by slit-lamp examination. Conjunctival vessels are tortuous and prominent (Figure 104-7) (551). Retinal infarcts are often present. Decreased erythrocyte survival often leads to anemia. Musculoskeletal involvement is common. There can be damage to the distal interphalangeal joints with limitations of that joint, avascular necrosis of the head of femur or talus, and the involvement of the metacarpal, metatarsal, and temporomandibular joints (48). Osteonecrosis is rarely reported in patients with FD (552). Hearing impairment and tinnitus may be present. Priapism has been reported in ~20 males from the Fabry registry. Derangement in the nitric oxide (NO) pathway may be responsible for this (553). FD is probably underdiagnosed, and the identification of undiagnosed Fabry patients represents a major challenge because heterogeneous phenotypes exist in male and female patients. Females with FD who present with pain are often ignored because the disorder is X-linked. Patients must be identified and treated to improve the quality of life and to protect from organ damage with ERT (554).

### 104.13.3 Pathology

Deposition of crystalline GSLs in endothelial cells of all areas of the body occurs in FD. These lipids show birefringence with characteristic “maltese crosses” under polarization microscopy. Deposition occurs in lysosomes of endothelial, perithelial, and smooth muscle cells of blood vessels, and less so in reticular and histiocytic cells. In the kidneys, GSLs accumulate in the epithelial cells of the glomerulus and of the distal tubules. Lipid-laden tubular epithelial cells desquamate and are detected in the urinary sediment. Renal blood vessels are extensively involved and arterial fibrinoid deposits are very common. GSL deposition occurs in the liver sinus epithelial cells and Kupffer cells, but not in hepatocytes. In the

heart, deposition occurs in myocardial cells and valvular fibrocytes, resulting in cardiac disease (283). Endomyocardial biopsy shows optically empty myocytes on light microscopy and dense osmiophilic bodies constituted of globotriaosylceramide on electron microscopy. Vascular ischemia and lipid deposition in the perineurium of peripheral nerves lead to a painful peripheral neuropathy with documented conduction abnormalities (555). Involvement of sweat glands due to lipid deposition and an inability to sweat raises core temperatures of patients during heat and exercise, exacerbating their painful small myelinated fiber and unmyelinated fiber neuropathy. Immunohistochemical studies using a sensitive anti-globotriaosylceramide monoclonal antibody revealed a selective pattern of neuronal involvement with deposition in the spinal cord and ganglia, brainstem, amygdala, hypothalamus, and entorhinal cortex, with sparing of adjacent areas. Many other organs, including the eyes, adrenals, gastrointestinal tract, prostate, testes, urinary bladder, and thyroid, demonstrate the involvement of blood vessels, smooth muscles, ganglia, and nerves.

#### 104.13.4 Biochemistry

Low activity  $\alpha$ -galactosidase A leads to the accumulation of neutral GSLs with terminal  $\alpha$ -galactosyl residues. There is widespread accumulation of globotriaosylceramide or ceramide trihexoside (CTH) in the lysosomes of vascular endothelial and smooth muscle cells, and in the epithelial and perithelial cells of most organs. CTH levels in hemizygote males may be 30–300 times that in normals (434,556). Galabiosylceramide also reaches high levels in FD patients, but in a tissue-specific manner. Affected tissues include kidney, pancreas, right heart, lung, dehiscent renal tubule cells, and spinal and sympathetic ganglia (557). Blood groups B and B1 GSLs that inhibit blood group B agglutination accumulate in patients with blood groups B and AB. GSLs are degraded in a stepwise fashion by a family of specific exoglycosidases found predominantly in lysosomes. These exoglycosidases are glycoproteins with optimal catalytic activity at acidic pH.  $\alpha$ -Galactosidase A deficiency causes FD, whereas  $\alpha$ -galactosaminidase B deficiency causes a type of neuroaxonal dystrophy called Schindler disease (397,558).  $\alpha$ -Galactosidase A is a protein of ~101 kDa. Affected males have normal plasma  $\alpha$ -galactosidase A activities, but deficient peripheral leukocyte  $\alpha$ -galactosidase A. Plasma GSLs are synthesized in the liver and incorporated into lipoproteins in the systemic circulation. Hepatic enlargement/dysfunction is not a feature of FD, although storage occurs in hepatocytes. Twenty-five percent of the plasma GSL pool is newly synthesized each day, and a portion is derived from the turnover of senescent red blood cells. The rate of GSL exchange between plasma and that found in cell membrane has not been determined. This has to be taken into account while measuring changes in plasma CTH

and while trying to correlate it to clinical response to therapy and/or disease progression. It has been suggested that the changes in urinary CTH may be a more sensitive and specific measure of tissue and body CTH burden (559).

The mechanism by which accumulating GSLs cause multiorgan disease is not completely understood. It cannot be explained by pure substrate storage (560). The mechanical deposition of storage material in blood vessels was believed to lead to decreased blood supply with consequent organ dysfunction. Many secondary biochemical processes may be involved in the pathogenesis of FD. Compromised energy metabolism occurs in vitro and in vivo, and altered lipid composition of membranes can lead to abnormalities in the trafficking and sorting of raft-associated proteins, leading to cellular and organ dysfunctions (561). In the brain, the pathogenesis of FD vasculopathy may be associated with endothelial dysfunction, cerebral hyper-perfusion, and a prothrombotic state with increased production of ROS. These abnormalities are further modified by genetic and possibly other vascular risk factors.

#### 104.13.5 Genetics

FD is panethnic. The  $\alpha$ -galactosidase A protein is encoded by a 12-kb gene mapped to the long arm of the X chromosome (Xq22.1). The gene is composed of seven exons. FD is caused by a wide variety of molecular defects: 57% of disease alleles are missense mutations, 18% partial gene deletions, 11% nonsense mutations, 6% insertions, and 6% RNA processing defects due to faulty splicing. Mutations have been found in all seven exons (56). Most enzyme mutations are private (i.e. confined to a single Fabry pedigree), but several occur in multiple, unrelated families (559). Many of the latter occur at CpG islands that are mutation hotspots because of the deamination of methylcytosine to thymidine. N215S, a common mutation in atypical asymptomatic or mildly affected patients with only cardiac involvement does not occur at these CpG dinucleotide hotspots. Efforts to establish genotype-phenotype correlations are limited, since the majority of the mutations are private and because the clinical phenotype, age of onset, and course of FD are very variable, even within the same family. Attempts to predict the phenotype require more extensive clinical information from unrelated patients with the same genotype. In addition, attempts to predict the clinical phenotype on the basis of the type or location of a molecular lesion are premature. For example, several mutations, N215S, Q279E, M296V, and R301Q, located in exons 5 and 6 lead to atypical or mildly affected cases. Nearby missense mutations, however, such as S297F (adjacent to M296V) result in a severe form of the disease. Patients from unrelated families with the G328R, R301Q, and R112H have presented with the classic disease phenotype in one family and mild disease in another (56).



The diagnosis of FD should prompt screening of family members. Although the disease presents in hemizygous males, the condition can be expressed in obligate female carriers because of nonrandom, random X chromosome inactivation.

### 104.13.6 Diagnosis/Testing

The diagnosis is made once low  $\alpha$ -galactosidase A activity is established in leukocytes or cultured skin fibroblasts. Enzyme activity is not a reliable way to distinguish affected hemizygous males from unaffected obligate carrier females. Genetic analysis is required for the diagnosis of female heterozygotes with FD. With a positive family history, carrier diagnosis is best confirmed by molecular testing in cases in which the defect is known or by linkage analysis when the specific gene defect is unknown. Carrier detection has been established in the past by the analysis of urinary sediments, which show elevations in the total glycolipid fraction, and elevated CTH and digalactosylceramide levels. Measurement of CTH may be used as a diagnostic tool in women with suspected FD. In men, urine CTH could be used to screen for FD. In men with suspected FD, diagnosis should be made by measuring enzyme activity (562). FD can be detected prenatally by the analysis of enzyme activity and the determination of the sex of the fetus, or by DNA analysis of CVSs or cultured amniocytes. Recognized MRI abnormalities in the brains of patients with FD include the consequences of infarction and hemorrhage, nonspecific white and gray matter lesions, vascular anomalies, in particular dolicho-ectasia, and a characteristic appearance of the posterior thalamus. Analysis of MRI findings in patients indicates that most patients have abnormal scans, most commonly cerebral white matter lesions. The number of the lesions increases with the patient's age and decreases in response to ERT (563).

### 104.13.7 Animal Models

A mouse model for FD was generated by the disruption of the  $\alpha$ -galactosidase A gene in mouse ES cells. Tissues from these mice had no detectable  $\alpha$ -galactosidase A activity (564) and elevated levels of globotriaosylceramide (GB3). They, however, were not affected clinically. They do, however, provide an excellent model to test the efficacy of different therapies.

### 104.13.8 Treatment

The management of patients with FD requires a multidisciplinary approach, because of the multiorgan nature of the disease. Historically, the treatment of FD has been symptomatic and palliative, and was based on the use of antihypertensives, phenytoin, gabapentin, and carbamazepine for the treatment of chronic neuropathic pain and ticlopidine for the prothrombotic state

that characterizes the disease. In the 1980s, a trial with  $\alpha$ -galactosidase A enzyme purified from human plasma demonstrated that this enzyme was effective in depleting the circulating GB3, but clinical application was limited due to the difficulty in producing large amounts of the enzyme. More than 20 years after purified enzyme infusion, a patient with the cardiac variant of FD was treated with galactose infusions, based on the principle of chaperon-mediated therapy. After 3 months of therapy, there was an improvement in cardiac contractility and a reduction in left ventricular mass (LVM), but the long-term benefits remain to be determined. Recently, ERT with recombinant human  $\alpha$ -galactosidase A was proposed as a specific treatment for FD. Early trials demonstrated the safety of ERT, as well as the efficacy in reducing GB3 storage in several organs and tissues, and in improving the signs and symptoms of the disease and the quality of life. The response of the neuropathic pain has been questionable. Longer follow-up is necessary to fully evaluate these treatments. Treatment with ERT should start as soon as clinical signs and symptoms appear. Some suggest that ERT should be given to all hemizygous male patients to prevent organ damage or to slow down the progression of preexisting severe organ damage (565). Kidney transplantation and dialysis have been necessary for some patients. To date, ERT is the only available FDA-approved therapy for FD. Two different forms of  $\alpha$ -galactosidase A ERT are available for treatment of FD: one genetically engineered in human cell lines (agalsidase  $\alpha$ , Replagal, Shire) and the other produced in a Chinese hamster ovary cell line (agalsidase  $\beta$ , Fabrazyme, Genzyme) (566). Studies with both preparations have described a reduction in plasma, urinary sediment, and tissue levels of GB3. Some reports support clinical benefit from ERT with a decrease in the frequency of pain crises and a reduction in left ventricular mass, and the improvement or stabilization of renal function, particularly when treatment is started at an early stage before irreversible tissue damage has taken place (567). There is considerable variation in the response to ERT due to heterogeneous phenotypic expression in patients of all ages and the lack of clear guidelines about when and which patients to treat. Five randomized controlled studies comparing agalsidase  $\alpha$  and  $\beta$  with both preparations administered as infusions every fortnight, concluded that aggregate results and differences were nonsignificant (568). ERT is entirely safe. In the first 3 months of treatment, headaches, hot flushes, and elevated temperature may be experienced as side effects. Patients may develop nausea and vomiting, flushing, and chills. The therapy has to be continued for a patient's entire lifetime. Costs are substantial, amounting to \$350,000/patient/year. Initial clinical trials showed great promise, but long-term effects are not as robust as initially anticipated (569). Information about long-term treatment with ERT is culled from cohort studies derived from patient registries, or from open extension studies of phase III trials.

Patients benefit to a variable extent from ERT. Whether treatment prevents organ manifestations in early symptomatic/presymptomatic patients and reduces mortality is still debatable (544). Controlled trials are needed to test whether treating children early will improve the neurological outcome. Studies with symptomatic female carriers are needed as are investigations of optimal time for early initiation of ERT to prevent irreversible organ damage (570). Large studies are needed to demonstrate the benefits of using concomitant angiotensin-converting enzyme inhibitors for their renoprotective properties or antiplatelet aggregating agents for primary and secondary stroke prevention in affected individuals, whether receiving ERT or not (568). There is no evidence that statins are effective in ameliorating vasculopathic changes in FD (571). ERT does not normalize the function of the PNS although neuropathic pain, sweating, and heat intolerance are somewhat improved (572). The available evidence supporting ERT in patients with renal disease (573,574) is not compelling with regard to outcomes, course of cardiomyopathy, or survival. That being said, ERT can be considered on an individual basis, particularly if the treatment is instituted early in the course of renal disease (575). Management of cardiovascular symptoms and the prevention of complications still rely on conventional pharmacologic and device-based therapies. Advanced cardiac disease may require permanent pacemakers and cardiac transplant. There is improvement of left ventricular mass and systolic function in patients with FD after 12 months of ERT. Many of the patients studied, however, are relatively young and have mild cardiac abnormalities (576). There are reports of cardiac damage present in older patients with FD, despite the use of ERT. The role and timing of ERT are still controversial in the prevention and amelioration of cardiac complications of FD (577). Beneficial effects of ERT on gastrointestinal symptoms in FD disease have been reported (578).

Galactose at subinhibitory doses to the enzyme in the presence of some mutations may prove to be beneficial as well. Currently, there are also investigations into other therapeutic approaches, such as SRT and enzyme enhancement with pharmacologic chaperones, which may have therapeutic value (568). 1-Deoxygalactonojirimycin is a potent inhibitor of  $\alpha$ -galactosidase A. It is an effective active site-specific chaperone and increases the residual enzyme activity in cultured fibroblasts and lymphoblasts established from Fabry patients with a variety of missense mutations. Oral administration of DGJ to transgenic mice expressing a mutant form of human  $\alpha$ -galactosidase A yielded higher  $\alpha$ -galactosidase A activity in major tissues. DGJ may be effective for patients who have missense mutations that primarily lead to misfolding of the mutant protein. DGJ could also be useful as an adjunct therapy with ERT for patients whose residual enzyme activity cannot be increased by DGJ alone to a level that reverses disease development. This

would provide undeniable advantages of convenience, cost savings, and ease of accessibility by the drug to tissues, including the CNS (579).

## 104.14 NEURONAL CEROID LIPOFUSCINOSIS OR BATTEN DISEASE

### 104.14.1 Introduction

The neuronal ceroid lipofuscinosis (NCL) or Batten disease are a group of clinically and genetically heterogeneous neurodegenerative diseases affecting predominantly the pediatric population with a worldwide incidence of 1 in 12,500 live births. Clinical features are progressive, which include visual loss, cognitive and motor deterioration, seizures, and early death (367). The NCLs were thought to comprise 10 variants termed CLN1-10, until recently when CLN9 was found to be a juvenile variant of CLN5 (580,581). A genetic origin with autosomal recessive inheritance is attributed to all of these variants except for the adult variant previously classified as CLN4, but now we know that autosomal recessive mutations in the *CLN6* gene accounts for some type A Kufs patients, and that mutations in cathepsin F or CLN13 account for some adult Kufs, B variants. CLN4/Kufs disease has both autosomal dominant and recessive patterns of inheritance. Also, two other recently described variants include mutations in progranulin or CLN11 accounting for some adult cases, and ATP13A2 or CLN12. Variants are grouped by the gene that bears the mutation and the age of onset of disease symptoms. Eleven genes have been identified and account for the many pediatric and adult clinical phenotypes: CLN1/protein palmitoyl thioesterase or PPT1, CLN2/tripeptidyl peptidase or TTP1, CLN3/battenin, CLN5, CLN6, CLN7, CLN8/EPMR, and CLN10, CLN11, CLN12, and CLN13. The clinical types are (1) congenital (CLN10), (2) classical infantile (INCL/CLN1), (3) classical late infantile (LINCL (late infantile neuronal ceroid lipofuscinosis)/CLN2), (4) variant late infantile/early juvenile Finnish (CLN5), (5) variant late infantile, Costa Rican or Portuguese, Pakistani, and others (CLN6), (6) classical juvenile (JNCL/CLN3), (7) epilepsy with mental retardation/LINCL (EPMR/CLN8), (8) Turkish variant late infantile (tLINCL/CLN7), CLN12 (juvenile Kufor-Raheb/CLN12 or ATP13A2), and (9) adult-onset variants recessively inherited (261,582). Recently, some cases of autosomal recessive Kufs type A disease were found to be caused by mutations in the *CLN6* gene (583) and adult Kufs B were found to be caused by CLN13 or Cathepsin F. Turkish vLINCL was previously believed to represent a distinct clinical and genetic entity, CLN7 (584), but is now known to be genetically heterogeneous with at least three underlying genes. These include *CLN6* (585), *CLN8* (586), and the *CLN7/MFSD8* gene, the identification of which occurred in Turkish patients (587). Autofluorescent

storage material accumulation is a feature common to all NCLs, and is generally a combination of proteins, proteolipids, and metals (588). The main type of storage material is subunit C of mitochondrial adenosine triphosphate (ATP) synthase which accumulates in CLN2, CLN3, CLN5, CLN6, CLN7, and CLN8 disease (440). In CLN1 disease and congenital NCL (CLN10 disease), there is the accumulation of sphingolipid-activating proteins (saposins A and D) (589,590).

The majority of variants manifest neuronal and photoreceptor-programmed cell death. Neuronal loss is viewed by CT and MRI as cerebral and cerebellar cortical atrophy (Figure 104-12A–B). Apoptosis of photoreceptor cells is documented as low amplitude a and b waves by ERG. Ultrastructural features by electron microscopy include vacuolated lymphocytes, granular osmiophilic deposits (GRODS) in INCL, curvilinear bodies in LINCL, curvilinear and/or fingerprint-like inclusions in juvenile neuronal ceroid lipofuscinosis (JNCL), and combinations of them in the others. These inclusions are present in multiple cell types including liver, muscle, conjunctiva, and of course, neurons.

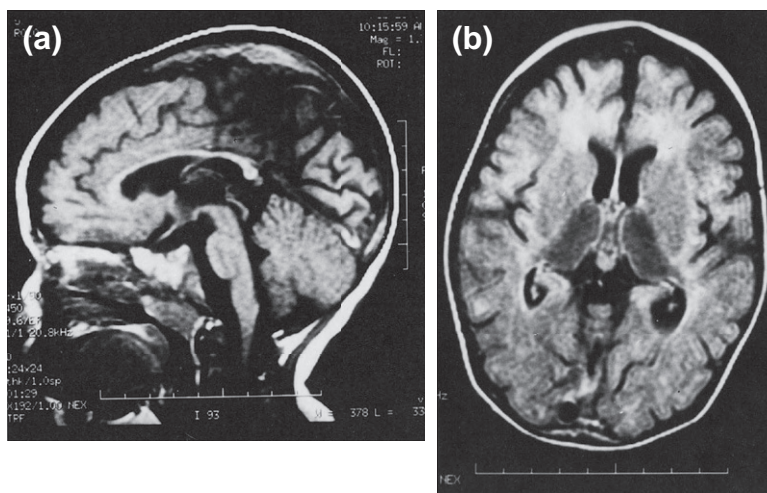
A deluge of new information has materialized over the past decade regarding the genetics, molecular and cell biology, and biochemistry of these diseases. PPT1, TPP1, and cathepsin D are soluble lysosomal enzymes with known function, although these variants are not typical lysosomal storage diseases.

CLN6 and CLN8 are ER-resident proteins, and CLN3 is a protein that traffics between Golgi, early recycling endosomes, and LRs at the cell surface. CLN3, CLN6, and CLN8 are hydrophobic membrane proteins. CLN5 protein localization has been debated but the current consensus is of a predominantly soluble protein that is trafficked to the lysosome (591). CLN7/MFSD8 is a member of the major facilitator superfamily of proteins that act as transporters of small solutes by chemiosmotic gradients. CLN7 protein localizes to the lysosome

and is thought to act as a novel lysosomal transporter (587,592).

Both naturally occurring and engineered animal models are described in mice, dogs, cats, sheep, cattle, pigs, and birds. The *nclf* and *mnd* mice are naturally occurring CLN6 and CLN8 variants, respectively. The New Zealand South Hampshire sheep are excellent models for CLN6. Transgenic mouse models for CLN1, 2, 3, 5, 6, 7, 8, and 10 variants are also available.

Diagnosis is based on clinical course and supplemented by tell-tale neuroradiologic and electrophysiologic studies and established by the corresponding enzymatic (PPT1, TPP1, or cathepsin D activity in CLN1/CLN2 and CLN10, respectively) or DNA-based laboratory tests. Skin biopsy electron microscopy remains as a mainstay of our diagnostic tools, especially when it comes to the delineation of new clinical variants not caused by any of the known gene defects. Current studies also highlight the role of gene mapping as a useful diagnostic tool especially in atypical NCL cases (593). As with most genetic diseases, prenatal diagnosis is possible through chorionic villus sampling, followed by enzyme testing, mutation analysis, or identification of inclusions by electron microscopy. For families at risk with a known predetermined mutation, preimplantation diagnosis is theoretically possible on day 4–5 after conception, but the authors are unaware of this having ever taken place. Treatment options no longer include just supportive antiepileptic, nutritional, and physical measures, but now comprise targeted approaches in light of newly delineated biochemical and cell biological processes. Examples include cysteamine or Cystagon in INCL, and Flupirtine in INCL, LINCL, JNCL, and CLN6 variants (288). Immunosuppression with mycophenolate was shown to be beneficial in mice models of juvenile NCL (594) and a trial is being currently planned for JNCL patients. Preclinical trials in ERT (595,596), gene therapy (597,598), and stem cell therapy (599) are



**FIGURE 104-12** CLN13 disease, juvenile variant MRI. (a) Sagittal section showing gaping sulci and cerebral and cerebellar atrophy. (b) Coronal section showing altered T2 signal of thalami and basal ganglia.



ongoing for CLN1, CLN2, and CLN10 diseases. Stem cell replacement is actively being pursued as potential therapy for CLN1 and CLN2 diseases.

### 104.14.2 History and Terminology

In 1826, Stengel described the first JNCL cases, followed by clinical and pathological descriptions by Batten, Mayou, Spielmeyer, Vogt, and Sjögren (301,600–602). The *CLN3* gene responsible for JNCL was identified in 1995 (603). First reports of the late infantile form (LINCL) were made by Jansky and Bielchowski in 1908 and 1913 (275). LINCL and JNCL were previously known as Batten disease. Batten disease now refers to all variants. The adult form or Kufs disease, an early-onset dementia with seizures and absence of visual findings, was described in 1925 (403). There are sporadic and familial reports of Adult Neuronal Ceroid Lipofuscinosis (ANCL) reported, with some families suggesting a dominant mode of inheritance. The *CLN4* genes responsible for the adult disease have not been identified. The term neuronal ceroid lipofuscinosis was introduced by Zeman and Dyken in 1969 to describe the autofluorescent, waxy, and dusky lipid deposits seen in neuronal endosomes (604). The infantile form or INCL was first described by Hagberg, followed by Haltia and Santavuori in 1973 (484,605,606). The *CLN3* gene was mapped in 1990 (607), followed by the *CLN1* gene (608,609), and the *CLN2* gene responsible for LINCL (610,611). Various other variant late infantile forms and early juvenile forms with defects in the *CLN5*, *CLN6*, *CLN7*, and *CLN8* genes are now recognized (207,293,612–614). The terminology is confusing. The terms INCL, LINCL, JNCL, and ANCL were chosen to classify these diseases according to the age of onset. This no longer holds true for all the types described. Discovery of the more recent genes and better ascertainment of atypical cases with a variable age of onset muddled the story somewhat. Different defects in one gene can manifest with variable clinical course and more than one age of onset. A genetic reference to the gene defect may be best for the moment (CLN1-, CLN2-, CLN3-, CLN5-, CLN6-, CLN7-, CLN8- and CLN-10).

Naming the genes *CLN* genes as opposed to *NCL* genes is regrettable, because yeast cyclin genes are also called *CLN* genes in the scientific literature. Changing matters at this juncture will make things even more difficult. In the United States, all forms are referred to as Batten disease. Although historically incorrect, it is a practical choice easy for all to remember and pronounce. The term Batten disease is universally recognized and accepted by families, physicians, private foundations, and government agencies in the United States. Some of our European colleagues on the Continent disagree. In view of the current genetic, allelic, and phenotypic heterogeneity of NCL, an axial nomenclature system, similar to that used for the epilepsies and

mental disorders, has been proposed that is acceptable to all involved:

Axis 1: Affected gene—*CLN1*, *CLN2*, *CLN3*, etc.

Axis 2: Mutation diagnosis

Axis 3: Biochemical phenotype—PPT1/TPP1/CTSD deficiency

Axis 4: Clinical phenotype—Congenital, infantile, late infantile, juvenile, adult onset, etc.

Axis 5: Ultrastructural features—For example, GROD, should include the specimen type studied (e.g. skin, conjunctiva)

Axis 6: Functionality—describes the current level of functioning using a universal scoring system, (Hamburg scales and the Unified Batten Disease Rating Scale)

Axis 7: Other remarks—relevant to the clinical condition; for example, stressors that may affect disease progression

### 104.14.3 Major Batten or Neuronal Ceroid Lipofuscinosis Syndromes

**104.14.3.1 INCL (CLN1 Disease, Infantile Variant, INCL, Haltia–Santavuori Variant, Palmitoyl-Protein Thioesterase or PPT1-Deficient).** INCL is caused by a deficiency in palmitoyl-protein thioesterase or PPT1, whose function is to remove fatty acids attached in thioester linkages to cysteine residues in proteins (615). The first description of this disease was by Hagberg (484). A more comprehensive clinical and pathological description of this autosomal recessive disorder was supplied by Haltia and Santavuori (606,616,617).

**104.14.3.1.1 Clinical Description.** Most cases of CLN1 disease in the Finnish population have an infantile onset. Only 50% of the CLN1 cases have an infantile onset in the United States. The other cases have late infantile, juvenile, or adult onset. Typical onset is in infancy but can be delayed until adulthood. Development is normal until 10–18 months of age, with slowed head growth beginning at 5 months. Developmental stagnation occurs and fine motor skills are impaired and hypotonia and ataxia follow. Patients become microcephalic. Visual impairment is present at 1 year of age and leads to blindness at the age of 2 years. Optic atrophy, thinned retinal vessels, and a discolored brownish macula are present. Myoclonic jerks begin after year one, with many developing generalized seizures. Rett-like hand knitting movements are observed early on but vanish by 2 years. At 3 years, children are nonambulatory, spastic, hypotonic, and irritable. Severe flexion contractures, acne, hirsutism, and precocious puberty often develop. Death ensues between the ages of 7 and 13 years. Atypical cases may have a delayed age of onset to 4 years. An adolescent-onset form reminiscent of the classical juvenile variant occurs in patients of Scottish descent (618). A single report describes two families with adult onset and mild course.



**104.14.3.1.2 Clinical Diagnostic Tests.** Measurement of PPT1 enzyme activity in leukocytes delivers the diagnosis (287). Enzyme activity can be measured from a dried blood spot on filter paper, or from cultured fibroblasts. Enzyme activity less than 5% of normal is diagnostic for INCL. DNA diagnostics is offered by specialized laboratories. Electron microscopic examination of skin shows characteristic membrane-bound GRODS seen in endothelial or perithelial cells and nerve cells of the submucosal myenteric nerve plexus. The EEG can be normal, and then gradually worsens. Lack of sleep spindles and the absence of the attenuation in amplitude, usually seen with eye opening, occurs by 16–24 months. The EEG becomes isoelectric by age 3 years. ERG, VEPs, and SEPs are abnormal, but seldom resorted to. The ERG is abnormal with cones affected before rods. CT and MRI findings include signal loss in the thalami, cerebral atrophy, and thinned periventricular rims early on. Postmortem MRI T2-weighted images reveal hypointensity of gray with respect to white matter. Prenatal diagnosis has been accomplished using CVSs at 11 weeks, and amniocytes at 16–18 weeks: EM or ultrastructural studies show GRODS and PPT1 enzyme assay and/or DNA analysis confirm the result. EM remains incredibly useful, when enzymatic diagnosis is not available, or equivocal, or knowledge of the family mutation is missing. All three diagnostic modalities should be used. The diagnosis of a normal or carrier fetus should be confirmed at birth by analysis of cord blood.

**104.14.3.1.3 Pathology.** Initial pathological events include progressive thalamic neuron loss and localized astrogliosis. Cortical neuronal loss follows thalamic neuron loss (618). Subtotal loss of neurons occurs by 4 years with significant decrease in brain weight. Betz cells and neurons of the hippocampal CA1 and CA4 sectors are preserved and reactive astrocytes become progressively more prominent. GRODS in neurons and macrophages are seen at 8 weeks of gestation. There is extensive gliosis. The brainstem and basal ganglia are also involved. The anterior horn cells demonstrate storage. Storage granules are seen in all tissues yet tissue destruction only occurs in the brain and retina (368,619). In addition to the involvement of the forebrain, there is significant hindbrain pathology manifested by widespread degeneration of cerebellar afferents and efferents and the death of Purkinje cells progressing from anterior to posterior cerebellar lobes, followed by granular cell layer degeneration (620). There is also prominent cerebellar astrocyte activation, gliosis Purkinje cell deficit, and GRODS accumulation (621).

**104.14.3.1.4 Biochemistry, Cell Biology and Pathophysiology.** Loss of function of PPT1, which removes long-chain fatty acids attached in thioester linkage to proteins, is the biochemical basis for this disorder. The severity of CLN1 disease correlates with residual PPT1 activity (622–624). The fatty acylated-cysteine-residue containing proteins found at the inner PM leaflet are

normally subjected to acylation and deacylation. Loss of this may impact protein–protein and protein–lipid membrane interactions. S-acylated protein degradation in lysosomes can also be impaired in CLN1 disease, which, like most of the NCLs, differs from other storage diseases because what accumulates in the cell has no apparent link to the actual defect, and may be a secondary occurrence. PPT1 is taken up via mannose-6-phosphate receptors into lysosomes, but is also activated at neutral and basic pH, and hence must function elsewhere in the cell. PPT1 also colocalizes with synaptophysin to presynaptic vesicles in neurons. Sphingolipid activator proteins A and D accumulate in storage cytosomes (218). Brain sphingomyelin and other phospholipids are decreased in INCL brain. Rates of apoptosis are increased in lymphocytes, cultured lymphoblasts, fibroblasts, and PPT1-deficient neurons (527). Increased apoptosis is a common theme in neurodegenerative disorders. The defect in deacylation of S-acylated proteins and the increased apoptosis of PPT1-deficient cells and neurons have led to specific therapeutic approaches (see “Management and Treatment” section).

**104.14.3.1.5 Genetics.** To date, there are 48 or more known mutations in the *CLN1* gene reported in the United States, Europe, China, and the Middle East. These are not limited to exons, as they affect introns, the promoter, and the 3′ untranslated region. (<http://www.ucl.ac.uk/ncl/cln1.shtml>). Pathogenic mutations include insertions, deletions, aberrant splicing, and frameshift and initiation defects.

**104.14.3.1.6 Management and Treatment.** Supportive therapies are important. Chloral hydrate is used for sleep disturbances. Baclofen and benzodiazepine treat irritability, sleep disturbances, spasticity, and rigidity. Topiramate valproic acid, piracetam, and benzodiazepine derivatives are used for seizures and pain. Physical therapy delays the onset of contractures. Virally mediated *CLN1* gene delivery clears storage and symptoms of a neurologically impaired mouse model. A recent and important finding is that very early (pre-symptomatic) therapy provides better results. Enzyme replacement is also a theoretical possibility and may be achieved via direct injection into the brain or CSF shunt. Stem cell therapy trials in a mouse model for *CLN1* are underway. Attempts at BM transplants have not been successful even when performed at preclinical stages of the disease. Drugs that allow read-through of mutations that introduce premature stops in PPT1 DNA are being considered. Drugs that help the mutated protein to fold correctly and regain activity, or escape degradation by increasing the amount of chaperones, such as Hsp70, are also under investigation. This approach is likely to be most suitable for proteins containing a missense mutation. Clinical trials using cystagon (cysteamine), a drug that breaks down the storage material in combination with Mucormyst, which may decrease apoptosis, are ongoing. Flupirtine is an oral antiapoptotic drug with

analgesic, muscle relaxant, and weak anticonvulsant properties that protect PPT1-deficient cells from apoptosis. It is an approved analgesic and antispasmodic in Europe. Its clinical efficacy in INCL is unknown, but anecdotal reports suggest a positive effect on EEG, spasticity, and seizure control.

#### 104.14.4 LINCL or Late Infantile Batten Disease (CLN2 Disease, Late Infantile Variant, Jansky–Bielchowski, Tripeptidyl Peptidase, or TPP1-Deficient)

LINCL is caused by defects in the gene for a lysosomal tripeptidyl peptidase (TPP1; CLN2) (611). First reports were by Jansky in 1908, then Bielchowski in 1913. It occurs in European, Middle-Eastern, Chinese, Pakistani, and Indian patients. It is the second most common form of Batten disease in the United States, although the total number of cases at any point in time is less than 500 making it an orphan disease according to FDA guidelines. A large number of cases originate from Europe. Atypical cases with juvenile onset and a protracted course have been reported (i.e. CLN2 disease, Juvenile variant).

**104.14.4.1 Clinical Description.** Late infantile phenotype: The disease presents with seizures and ataxia between the ages of 2.5 and 3.5 years. After 6 months, vision, motor, and cognitive skills deteriorate. Patients become blind by age 4 years due to tapeto-retinal degeneration, and are bedridden and mute by 5 years, requiring tube feeding. Myoclonic jerks prominent in the face but also involving trunk and extremities set in. Hypotonia is replaced by spasticity with flexion contractures as the disease progresses. Mottled, cold hands and feet are common. Hypothalamic involvement leads to hyperthermia and hypothermia. The former leads to negative fever workups. Excessive secretions and weak breathing effort result in pneumonia. Infections and refractory seizures are the cause of demise during the second decade of life (261,625).

**104.14.4.2 Juvenile Phenotype.** Onset is delayed to ages 6–8 years. Initial progressive cognitive decline is followed by seizures, ataxia and motor dysfunction. Vision loss is variable and survival up to the fourth decade is possible.

**104.14.4.3 Clinical Diagnostic Tests.** CLN2: Tripeptidyl peptidase 1 (TTP1) enzyme activity can be measured in leukocytes, cultured fibroblasts, amniocytes, dried blood spots, and saliva. Fibroblast TTP1 activity is approximately 17,000 micromoles produced per hour per mg of protein. The TTP1 activity in CLN2 disease typically is lower than 5% of normal activity. DNA-based diagnosis is available, but less practical, particularly if the involved mutation or mutations are unknown. Usually, the most commonly reported mutations are ruled out first. Ultrastructural examination of a skin biopsy provides diagnostic information as well. Curvilinear bodies enclosed within unilamellar endosomes in

endothelial cells, pericytes, and Schwann cells are diagnostic. Sometimes, fingerprint profiles also appear. EM remains a valuable diagnostic tool when other diagnostic measures are unavailable, and in unusual or atypical cases. The ERG shows reduced amplitudes early, before thinned vessels and pale discs become apparent. It is extinguished after a few months. Large and characteristic giant occipital polyspike discharges are activated in response to a single flash of light or to low-frequency repetitive stimulation by EEG, and is nothing but the early appearance of an exaggerated VER. VER and somatosensory-evoked response wave amplitudes are high, but these tests are not particularly useful. An initial CT or MRI may be normal, but within 6 months of onset, cerebral and especially infratentorial atrophy are prominent. Children with CLN2 disease, late infantile type, begin to differ from controls at age 5 years where a 40% loss in cerebellar volume and marked dilatation of the ventricular system are observed. Caudate and thalamic volumes are markedly reduced compared to age-matched controls with the relative preservation of brainstem volume early in the course of the disease (262,626).

**104.14.4.4 Pathology.** Brain weight is markedly diminished and is anywhere from 250 to 700 g. The calvarium is thickened. Sulci are wide in the occipital regions and elsewhere. Cerebellar folia are prominent and ventricles are wide. There is laminar necrosis and massive neuronal loss with some preservation of layer III. The preserved neurons represent meganeurites. Purkinje and granule cells are wiped out from the cerebellum. Putamen and subthalamic and brainstem nuclei also demonstrate neuronal loss. The white matter is pale and there is reactive astrocytosis with activation of microglia, yet monocyte-derived macrophages evident in chronic and acute inflammation are conspicuously absent. This strongly implies that the initial event in LINCL is neuronal loss with a secondary, reactive gliosis. The few neurons remaining have swollen cell bodies, and a granular cytoplasm that reacts positively with PAS, Luxol fast blue, and Sudan black B. The white matter appears intact, negating a primary inflammatory component in LINCL. Condensed chromatin by EM, upregulation of Bcl-2 protein, and positive terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) stains provide substantiation for apoptosis (627). There is a strong reactivity with an antibody to subunit C of mitochondrial ATP synthase (628). The cause for this remains obscure, but may represent a form of apoptosis common to neurodegenerative disease known as mitophagy. Neurons and other cells contain curvilinear inclusions enclosed within a single membrane, sometimes admixed with fingerprint profiles. This is seen in smooth muscle cells, eccrine sweat glands, endothelial cells, and pericytes outside the brain. A pathological feature that distinguishes CLN2 disease from other vLINCL forms is the invariant presence of curvilinear bodies, which occurs only occasionally in other vLINCL forms. Prenatal diagnosis has been

achieved by analyzing the ultrastructure of amniocytes at 16–17 weeks gestation, measuring enzyme activities and *CLN2* gene sequences.

For clinical staging, the Weill Cornell LINCL scale and the modified Hamburg LINCL scale are used. Both correlate with age and time since the onset of initial clinical manifestations, but the former also utilizes imaging measures (629).

**104.14.4.5 Biochemistry.** Pepstatin-insensitive lysosomal tripeptidyl peptidase is a 46-kDa protein that normally removes tripeptides from the N-terminus of proteins. TPP1 activity is lost in the brain, liver, heart, kidney, and the intestine of LINCL patients. TPP1 expression is developmentally controlled, reaching its peak at the same age of symptom onset in LINCL (630). Loss of TPP1 activity contributes to the accumulation of mitochondrial subunit C in the lysosome. Residual activity of mutated TPP1 can ameliorate the LINCL phenotype (631). It is not known how subunit C accumulation relates to TPP1 deficiency. NCL disorders with normal TPP1 activity (*CLN3*, *CLN4*, *CLN6*, and *CLN8*) also accumulate subunit C. It more than likely represents a secondary process and may be related to the increase in apoptosis observed in *CLN2*-, *CLN3*-, *CLN6*-, and *CLN8*-deficient cells. Measurement of TPP1 enzyme activity is the most practical diagnostic tool available for diagnosis of LINCL.

**104.14.4.6 Genetics.** LINCL is a panethnic, autosomal recessive disorder with cases diagnosed from countries from all continents. It is the second most common form of NCL disease in the United States and accounts for one-third of cases. Over 72 mutations have been described so far. Two common mutations account for 65% of diagnosed cases in the United States. One is a nonsense mutation, Arg208X, and the other affects a splice-junction site, IVS5-1G>C. Some cases with a milder course and later onset bear one of these two mutations on one allele, and an Arg447His on the other. Dog and mouse models for this disease are now available (632,633).

**104.14.4.7 Management and Treatment.** Treatment is supportive. Most problematic are recalcitrant seizures requiring multiple anticonvulsants. Valproic acid, clonazepam, and clorazepate have benefited a number of cases. Phenobarbital, Zonigran, and Keppra are helpful in others. Tube feeding becomes necessary in most cases after 5–7 years of age. Frequent aspiration pneumonias reflect difficulty swallowing. Contractures must be minimized with diligent physical therapy. BMT has failed. Replication deficient AAV gene transfer vector (AAV2-mediated *CLN2* gene transfer) has been studied in mice, rats, and nonhuman primates with *CLN2*. A safety trial for therapy in 10 children with mutations in *CLN2* using delivery by brain surgery is completed without any benefit. Enzyme replacement trials in a mouse model for *CLN2* disease show that it is possible to deliver TPPI enzyme to the brain using the CSF. Stem cell therapy is currently being developed. It has been proposed that use of

the antiapoptotic drug, flupirtine, may slow progression of this disease. The safety of this drug and its analgesic, antispasmodic, and weak anticonvulsant effects make it particularly attractive (288). The efficacy of this drug in LINCL is not determined.

#### **104.14.5 Variant Late Infantile Forms (vLINCL; Finnish Type: *CLN5*-Deficient; Costa Rican/Portuguese/Lake-Cavanaugh Variant: *CLN6*-Deficient; Northern Epilepsy or Epilepsy with Mental Retardation or EPMR, Turkish vLINCL: *CLN8*-Deficient and Turkish vLINCL: *CLN7/MFSD8*)**

A series of variant late infantile types with ages of onset between 5 and 8 years and a clinical profile reminiscent of the late infantile type, but with a protracted course have been described. So far, four different genes are identified. The *CLN5* gene, identified in Finland and believed to be exclusively Finnish, was reported later in patients of various ethnic backgrounds and in cases with early juvenile onset (634,635). Four patients of Serbian and German descent with juvenile NCL, previously described as *CLN9*, are now reclassified to the *CLN5* variant. This has brought new cytological and biochemical data to this *CLN5* variant with emergence of new clues to the function of the *CLN5* protein (580,581). The *CLN8* gene defective in Northern Epilepsy was identified in the northeast of Finland. Some but not all Turkish cases with variant LINCL are due to mutations in the *CLN8* gene. The variant LINCL type, also known as Costa Rican/Portuguese or *CLN6*-deficient type, affects patients with Venezuelan, Pakistani, and Indian descent and from the United States. The first reports were called “early juvenile” and are referred to as the Lake-Cavanaugh variant (293,414,636). In 2007, a novel gene named *MFSD8* was found affected in a subset of Turkish families with variant LINCL (587) and later identified as a common cause of vLINCL in different populations (612,637,638).

##### **104.14.5.1 Clinical Description.**

**104.14.5.1.1 Finnish Variant LINCL (*CLN5*-Deficient).** Late infantile phenotype: Initial symptoms may be clumsiness and/or difficulties concentrating noticed at age 4.5 years. Cognitive decline at the age of 6 and generalized and myoclonic epilepsy at the age of 8 follow. Children are blind by the age of 8, lose the ability to walk by the age of 10, and die between the ages of 14 and 34 years. Mutations in the *CLN5* gene can also cause milder disease.

**104.14.5.1.2 Early Juvenile Phenotype (*CLN5*-Deficient).** The clinical course is similar to JNCL disease with decreased vision at the age of 4 years, cognitive decline at the age of 6 years, and ataxia and rigidity by the age of 9 years. Dysarthria, scanning speech, and mutism may set in by the age of 12 years. Hallucinations and behavior problems are common and intractable seizures may develop during the early teens. Retinitis with pigmentary changes is



documented. Behavior disturbances/mental deterioration can be the dominant manifestations at onset (635).

**104.14.5.1.3 Northern Epilepsy or Epilepsy with Mental Retardation (CLN8-Deficient).** The disease manifests with frequent but short generalized tonic-clonic convulsions and complex partial seizures, as well as cognitive decline to a low average level after the age of 5 and before puberty. After puberty, noted are slow movements and a plateau in the rate of cognitive decline. In the final stage, seizures diminish and mental dullness/decline result in mental retardation by the age of 40 years. At the end, patients are clumsy, ataxic, and have impaired vision. Patients die at the age of 17 years to late middle age.

**104.14.5.1.4 Turkish vLINCL or tLINCL (CLN8-Deficient).** The clinical phenotype is more severe than that of EPMR. Patients present between the ages of 2 and 5 with severe seizures, intellectual decline, and blindness. Behavioral problems develop, and are prominent by the age of 8–9 years. Most patients are wheelchair-bound by 10 years of age.

Costa Rican/Portuguese vLINCL/Lake-Cavanaugh variant (CLN6-Deficient) (639). The onset is between the age of 18 months and 8 years with ataxia and speech difficulties following normal development. Visual failure due to retinitis pigmentosa, myoclonic jerks, and other seizures and intellectual decline follow. Loss of motor skills occurs between 4 and 10 years. Patients succumb to the disease in the early to mid teens. Most mutations in the *CLN6* gene cause a variant of late infantile onset neuronal ceroid lipofuscinosis. Recently, mutations in *CLN6* have shown to be a major cause of recessive Kufs type A disease, an adult form of NCL (see below).

**104.14.5.1.5 Turkish Variant Late Infantile NCL (CLN7/MFSD8-Deficient).** Disease onset/progression is similar to CLN6 disease. Mutations in the *CLN7* gene can cause milder disease. The occurrence of seizures and motor difficulties before visual failure is peculiar to CLN6 and CLN7 diseases.

**104.14.5.2 Clinical Diagnostic Tests.** JNCL with atypical features or symptoms suggestive of classical LINCL, normal PPT1/TPP1 enzyme activities, absence of vacuolated lymphocytes, and a normal *CLN3* gene suggest one of the variant LINCL types. Skin/rectal biopsy ultrastructure, ethnic background, and subtle features for one of these subtypes may raise suspicion in favor of one of the variant LINCL types. Fingerprint and rectilinear structures suggest the CLN5 variant. A combination of curvilinear and fingerprint bodies favors the Costa Rican/Portuguese variant. Finnish CLN8 EM findings include loose curvilinear-like structures, and the Turkish CLN8 variant is characterized by dense fingerprint profiles and dark amorphous material. MRI of CLN6 and CLN5-deficient vLINCL demonstrates severe cerebral and cerebellar cortical atrophy, low densities in the thalami and basal ganglia, and hyperintensities of the white matter. MRI of tLINCL/CLN8 is characterized by cerebellar and cerebral atrophy as well as atrophy of the brainstem. The

EEG shows giant amplitude occipital spikes in response to low-frequency photic stimulation in all the variants. In CLN7 disease, brain MRI demonstrates global atrophy and periventricular leukoencephalopathy. In view of the common clinical and radiological findings in the vLINCL group, molecular genetics methods are the mainstay for diagnosis. In the case of CLN, sequencing of the *MFSD8* gene remains the most practical alternative for diagnostics, as the majority of patients have private family mutations.

#### 104.14.5.3 Pathology.

**104.14.5.3.1 Finnish Variant LINCL (CLN5-Deficient).** The brain weighs about 500g. Severe cerebellar atrophy is documented. Findings are otherwise very similar to classical LINCL. Strong immunoreactivity to subunit C of mitochondrial ATP synthase and weak immunoreactivity to saposins is reported. EM is conspicuous for the presence of rectilinear profiles, curvilinear, and fingerprint bodies (367,368,640). Neuronal loss occurs both in the cerebellum and the neocortex. Cortical neuronal loss affects laminae III and V most severely. In the cerebellum, there is almost the complete loss of Purkinje and granule cells. Data from *Cln5*-deficient mice reveal that neuron loss starts in the cortex and subsequently involves the thalamus, as opposed to CLN2, CLN3 and CLN10, where the loss of thalamic relay neurons occurs before the onset of neuron loss within the corresponding cortical region (641).

**104.14.5.3.2 Costa Rican/Portuguese vLINCL/Lake-Cavanaugh Variant (CLN6-Deficient).** The brain weighs between 600 and 900g. Neuronal loss is pervasive and very prominent in neocortex layer V. Granule cells in the cerebellum are wiped out completely, but a few Purkinje cells remain. There is strong immunoreactivity with subunit C in neuronal tissues, which is absent in peripheral organs. By EM, curvilinear, fingerprint bodies and rectilinear profiles are documented in the brain. Rectilinear, fingerprint, and curvilinear bodies are present in viscera.

**104.14.5.3.3 Northern Epilepsy or Epilepsy with Mental Retardation (CLN8-Deficient).** The brain weight at autopsy is between 1000 and 1600g. The brain may appear normal or mildly atrophic. Storage material and meganeurites are prominent in layer III of cortex. Neuronal loss is conspicuous in cortex layer V, with minimal accumulation of subunit C in substantia nigra, cerebellar Purkinje cells, and locus ceruleus, and strong reactivity with antibodies to  $\alpha$ -amyloid and saposin D. EM of storage bodies is noted for curvilinear and granular material.

**104.14.5.3.4 Turkish vLINCL or tLINCL (CLN8-Deficient).** EM of skin is noted for curvilinear, rectilinear, and fingerprint profiles (640).

**104.14.5.3.5 Turkish Variant Late Infantile NCL (CLN7/MFSD8-Deficient).** The ultrastructural pattern of the storage material in CLN7 patients, consisting of rectilinear/fingerprint complexes occasionally associated with curvilinear inclusions, closely resembles EM findings in other vLINCL forms.



**104.14.5.3 Genetics.**

**104.14.5.3.1 Finnish Variant LINCL (CLN5-Deficient).** The *CLN5* gene localizes to chromosome 13q21.1–q32. There are 27 disease-causing mutations reported pan-ethnically. A 2-bp deletion in exon 4 (c.1175delAT) causes 94% of Finnish cases. Most result in the classical LINCL phenotype. A one base pair insertion in exon 1 (c.291dupC) responsible for early onset (4 months) NCL was reported in Argentina, and late onset (age 17) missense mutations were also reported in exons 2 (c.335G>A, c.377G>A) and 4 (c.1121A>G).

**104.14.5.3.2 Costa Rican/Portuguese vLINCL/Lake-Cavanaugh Variant (CLN6-Deficient, CLN6 disease).** The *CLN6* gene localizes to 15q21–q23. The nonsense mutation reported in 20 Costa Rican families, c.214G>T is the most common. Fifty-three mutations are recognized. This variant has a worldwide prevalence with cases reported from Turkey, Pakistan, Costa Rica, Portugal, Saudi Arabia, and Gypsy families from the Czech Republic. A 2-bp deletion in exon 4 (c.395\_396delCT) is described from India, Italy and Saudi Arabia. The *nclf* mouse is a naturally occurring model for *CLN6*-deficient vLINCL (582).

**104.14.5.3.3 Northern Epilepsy or Epilepsy with Mental Retardation (CLN8-Deficient) and Variant Turkish tLINCL.** The *CLN8* gene localizes to chromosome 8p23.3. A single-point mutation in exon 2 (c.70C>G) accounts for all Northern Epilepsy patients. The 13 other mutations cause the clinically more severe phenotype. These occur in Turkey, Pakistan, Germany, Finland, Italy and Israel. The *mnd* mouse is a naturally occurring animal model for *CLN8*-deficient variants (586).

**104.14.5.3.4 Turkish vLINCL CLN7/MFSD8.** There are 23 disease-causing mutations in the *MFSD8* gene localized on 4q28.2. The most common mutation is a missense mutation affecting exon 10 (c.881C>A), reported from Slovakia, Spain, Italy, and Turkey.

**104.14.5.4 Biochemistry and Cell Biology.** *CLN6* and *CLN8* proteins (*CLN6p*, *CLN8p*) are transmembrane proteins that reside in the ER. Evidence shows that *CLN6p* is required for degradation pathways via lysosomes through an unknown mechanism (642). When screened against a human fetal brain library, *CLN6* peptide fragments interacted with collapsin response mediator protein-2 (CRMP-2), a protein that guides axonal growth (643). This may have implications in the maturation and integrity of axons in vLINCL patients.

*CLN8p* is predominantly localized in the ER but colocalizing experiments showed *CLN8* staining beyond the ER, toward the neuronal periphery (644). Further, mouse experiments corroborated the existence of *CLN8p* outside the ER indicating that *CLN8p* has an unidentified vesicular localization in neurons. *CLN8p* belongs to the TLC (TRAM Lag1 *CLN8*) family of proteins, which includes ceramide Synthase enzymes (CerS1-6), and *CLN8p* functions suggested have included lipid

synthesis, trafficking, and as a sensor (645). Examination of lipid extracts from EPMR patients (646), and *mnd* mice (581) revealed reduced levels of ceramide and its downstream metabolites. Taken together, these results support a role for *CLN8p* in sphingolipid homeostasis through an undetermined mechanism. The *CLN5* protein is a lysosomal glycoprotein. *CLN5*-deficient fibroblasts derived from German and American Serbian patients have a distinctive morphology and biochemical phenotype. They are small, rounded, and adhere poorly to the culture dish. They grow rapidly, but with an increased sensitivity to apoptosis. A number of genes involved in cell adhesion and apoptosis are dysregulated by gene profiling and are posted at ([www.dbsr.duke.edu/pub/cln9/](http://www.dbsr.duke.edu/pub/cln9/)). Gene expression of cyclins A2, B1, C, E2, G1, and T2 were increased, and expression of cyclin D1, a proto-oncogene involved in malignant transformation of breast tissue was decreased, as was member 1A of the tumor necrosis factor superfamily. Subunits of cytochrome c oxidases and glutathione S-transferase were also increased. Levels of sphingolipids downstream of dihydroceramide synthase (CerS) in the de novo sphingolipid synthesis pathway: Ceramide sphingomyelin, lactosylceramide, CTH, and globoside were decreased 60–100%. The key regulating enzyme in the ceramide de novo synthetic pathway, serine palmitoyl transferase, was three- to four-fold upregulated. Recent work showed that in the setting of a defective *CLN5* protein,  $\gamma$ -actin fails to bind to CerS1 on one hand and to vimentin and histone proteins on the other hand (581).  $\gamma$ -Actin is the major cellular intermediate filament and is involved in cellular morphology, division and intracellular movements. Vimentin plays a crucial role in cellular shape, resilience and intracellular transport of globoside. Histones are known to regulate the cell cycle. In view of the function of the above proteins, it is likely that this defective protein interaction brought about by defects in *CLN5p* could explain the *CLN5*-deficient phenotype. Recent work showed molecular connections of *CLN5p* to five other NCL proteins: *CLN1/PPT1*, *CLN2/TPP1*, *CLN3*, *CLN6* and *CLN8* proteins suggesting a central role for *CLN5p* in the NCL network. This implies that these proteins may be functionally related. A close interaction between *CLN5* and *CLN8* proteins is further supported by the fact that *CLN8p* is the only NCL protein to correct the *CLN5*-deficient cellular phenotype in these cells and both *CLN5* and *CLN8* are implicated in ceramide synthesis, particularly impacting the step catalyzed by dihydroceramide synthase. This can have useful implications for therapy. Fenretinide, an activator of CerS, improved the *CLN5* phenotype in vitro. This could also apply to *CLN8*-deficient cells.

**104.14.5.5 Management and Therapy.** Supportive therapies for these variants are very similar to those outlined for classic LINCL and JNCL. Northern Epilepsy responds very well to monotherapy with clonazepam, with some patients remaining seizure free for many years

on this drug. Seizure frequency in this variant tends to decrease over time.

#### 104.14.6 Juvenile Neuronal Ceroid Lipofuscinosis or Juvenile Batten Disease (CLN3 Disease, JNCL, Spielmeyer–Vogt–Batten–Mayou, CLN3-Defective/Deficient)

There is a preponderance of JNCL cases with Northern European ancestry (Finland, Iceland, Norway, Sweden, Denmark, Germany, and Holland), and notable absence of African or Jewish cases. Japanese, Portuguese, Polish British, Turkish, Moroccan, Lebanese cases, and others from many countries have been described. It is the most prevalent type of NCL in the United States (261). The first Batten variant to be recognized was JNCL, and the gene responsible for it, CLN3, was first to be cloned (603). Description of the first juvenile cases is credited to a Danish physician, Otto Christian Stengel, in 1826 (601). The four affected siblings of a Norwegian family established the genetic nature of the illness.

**104.14.6.1 Clinical Description.** Early development is unremarkable and the first symptom is decreased central vision due to retinitis pigmentosa at 4–6 years of age. Patients become completely blind at variable ages from 10 to 14 years. Complete blindness and a disturbed sleep–wake cycle and insomnia are common. Some affected children reported difficult behavior between the ages of 7 and 9 years. By the age of 10 years, cognitive decline is apparent with diagnosis first suspected by teachers for the blind who are familiar with this condition in the pediatric visually impaired population. Seizures appear at the age of 12 years or sooner, but may not declare themselves until the age of 14 years. Early-onset seizures, difficult to control, often foretell a more rapidly declining course. Repetitive echolalic speech is universal to all cases and perseveration of motor actions becomes common. A cogwheel rigidity of the limbs and a stooped, shuffling gait is reminiscent of patients with Parkinson’s disease. Intention tremor may be present and can be of variable severity. Patients stabilize for a few years in their mid teens. Psychiatric symptoms occur in 74% of patients. Many become depressed and agitated, some become aggressive and psychotic. These patients often have a positive family history for unipolar or bipolar illness. Treatment is not always necessary, as it does aggravate extrapyramidal signs and symptoms. Hallucinations are common, but are often of a pleasant and repetitive nature. Some patients have constant imaginary friends. Growth and physical maturity are normal, and sexual development may become a problem, particularly for teenage girls. Contraceptive measures are often sought and given for affected teenage girls. Drooling, difficulty swallowing, and weight loss become problems late in the course, and are managed by gavage feeding. Temperature instability with hypothermia (93F) alternating with

hyperthermia is caused by hypothalamic disease. Seizures increase to 150–200 per day in spite of numerous medications and become impossible to control. Some patients develop a cardiomyopathy or sick sinus syndrome with bradycardia, requiring pacemakers. Patients succumb in their early to mid-twenties due to uncontrollable seizures or cardiopulmonary arrest. A small number of patients survive into the fourth decade.

**104.14.6.2 Clinical Diagnostic Tests.** DNA-based CLN3 gene tests confirm the diagnosis. EEG is abnormal from the age of 9 years onwards with large amplitude spike and slow wave complexes. CT and MRI, though initially normal, ultimately reveal cerebral atrophy with gaping sulci and large ventricles (Figure 104-12). Cerebellar atrophy is prominent usually after the age of 15. Morphometric MRI measurements document loss of hemisphere, caudate, thalamic, and lenticular volumes (262). A voxel-based morphometric study (647) showed marked reduction in the gray matter volume of the dorsomedial thalami and decreased white matter volume of the corona radiata. A low signal is observed in the white matter in T2-weighted images. PET shows decreased glucose utilization in the calcarine area, which progresses to involve all gray structures. The latter two techniques are not routinely offered, but were carried out to better understand disease progression. ERG is abnormal before decreased vision becomes apparent. Visual-evoked potentials show reduced amplitude potentials, and somatosensory-evoked potentials are enhanced. EM of a skin biopsy is often helpful, particularly if the mutations are not known. Schwann cells, endothelial cells, pericytes, neurons, macrophages, and eccrine sweat glands contain typical inclusions. Fingerprint-like inclusions enclosed by a unit membrane are common, often in association with curvilinear inclusions. Vacuolated lymphocytes are a hallmark of JNCL, but blood must be processed swiftly and correctly; otherwise false positives become problematic. Few diagnostic laboratories can evaluate this. Skin EM is more robust and reliable. On neuropsychological testing, children with CLN3 disease had significant impairment in auditory attention, memory, verbal intellectual function, and fluency. Neuropsychological impairment was progressive over time and correlated with disease duration and motor function (648).

**104.14.6.3 Pathology.** The brain at death weighs between 450g and 1100g. There is thinning of the cortical mantle with moderate neuronal loss, gliosis, and accumulation of autofluorescent material that is Sudan Black B and PAS positive. There is selective necrosis of stellate cells in cortical layers II and III and the loss of pyramidal cells in layer V. Meganeurites are seen in the basolateral amygdaloid complex and in cortical layer V. Purkinje cell and granule cell loss is dramatic. Apoptotic neurons with dark, shrunken, and fragmented chromatin are seen in the cerebral cortex by EM. A number of neurons are TUNEL-stain positive, confirming the

existence of apoptotic neurons. Surviving neurons are immunoreactive with antibodies to Bcl-2, a neuroprotective protein, and subunit C. Lipopigment accumulates in anterior horn cells of the spinal cord and receptor cells of the organ of Corti. In neuronal cells, fingerprint profiles and in non-neuronal cells curvilinear inclusions prevail. Also, blood lymphocytes contain cytoplasmic vacuoles.

**104.14.6.4 Biochemistry, Cell Biology, and Pathophysiology.** Despite a decade of research into the function of CLN3 protein, the role of this protein remains elusive. CLN3 has been reported to be present in multiple cellular compartments including nucleus, Golgi, mitochondria, PM, LRs, endosomes and lysosomes, suggesting that this protein dynamically traffics between cellular compartments and may function in multiple regions of the cell. The motif consisting of the amino acids valine, tyrosine, phenylalanine, alanine, and glutamic acid or VYFAE, embedded within the CLN3 protein is part of a larger galactosylceramide lipid, raft-binding domain. In CLN3-deficient cells, both mutant CLN3 protein and galactosylceramide (GalCer) remain in the Golgi, never reaching their final destination or LRs. This is reversed after restoring CLN3 to deficient cells suggesting that CLN3 may function as a GalCer transporter from Golgi to LRs. The increase in apoptosis is initiated from LRs, and the revved up production of sphingolipids is an attempt to correct the GalCer deficiency in LRs. Ceramide, the proapoptotic lipid second messenger, was elevated in JNCL brains (649). This correlated with the identification of apoptosis in JNCL brain and antiapoptotic amino acid stretches within the CLN3 protein (142). In addition, other GSLs and sphingomyelin were elevated, pointing to sphingolipid overproduction (650). The CLN3 protein was upregulated in human and mouse cancer cell lines, and solid colon cancer specimens (651). In the setting of a deficient CLN3 protein, cathepsin D transport and processing are altered. CLN3 was also reported to interact with many proteins including the cytoskeletal protein  $\beta$ -fodrin, the  $\text{Na}^+$ - $\text{K}^+$  ATPase complex (652), and with the Notch and JNK signaling pathways (653). Together, these results imply a role for CLN3 protein in cytoskeleton dynamics, cell migration, and adhesion, and the modulation of ionic flux and lysosomal homeostasis.

**104.14.6.5 Genetics.** The CLN3 gene localizes to chromosome 16p12.1. JNCL is the most common form of NCL in the United States. The prevalence of this autosomal recessive disease is 7 per 100,000 live births in Iceland, and 0.71 per 100,000 live births in Germany. The prevalence drops the further away one is from Scandinavian countries. The prevalence in the United States is less. This classifies JNCL as an orphan disease, according to FDA guidelines. The gene has 15 exons, and the protein is 438 amino acids long and is a hydrophobic protein with 5–7 potential transmembrane domains. It is highly conserved across species. There are over 50 disease-causing mutations. A 1.02-kb deletion accounts for 85% of cases while the remaining

15% result from point or frameshift mutations. DNA-based carrier testing is available, provided the family mutation is known. Prenatal diagnosis has been achieved using EM-based and DNA diagnostics at 11 weeks gestation. It is best to confirm diagnosis at birth in cord blood.

**104.14.6.6 Management and Treatment.** Clearly, JNCL is the most difficult of the clinical types to manage. Initial seizure control is easily achieved with one drug, but late in the course, patients progress to having over 100 seizures per day, in spite of numerous antiepileptic medications. The emotional and psychiatric aspects of JNCL pose neurologists with a therapeutic conundrum. Antipsychotics and mood stabilizers are necessary but they lower seizure threshold and aggravate Parkinsonian symptoms. Insomnia must be addressed with benzodiazepines and other drugs. Weight loss in the final years necessitates gastrostomy tubes for adequate caloric/liquid and medication intake. Anecdotal reports of the antiapoptotic medication, flupirtine, suggest improved seizure control and sleep patterns. Some patients develop bradycardia, requiring pacemaker placement. Death in the early mid-twenties is common. Some patients die early at 13 years of age, and some survive to the age of 40 years. The average survival has increased with the use of feeding tubes and better anticonvulsants and antibiotics. Patients do not suffer and continue to enjoy human contact and music late into the course. CLN3p is a membrane protein and, therefore, protein and/or gene replacement are not actively sought. The immune system seems to be affected in JNCL, albeit in a secondary manner. Immunodeficient and mycophenolate-treated JNCL mice have a milder clinical phenotype than immunocompetent JNCL mice. A clinical trial using mycophenolate in JNCL patients is planned. Prednisolone given to juvenile patients in Finland reduces antibodies to GAD65 without any effect on disease course. Stem cell approaches may some day have a role in therapy. Recently, EGIS-8332, a drug which targets  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor or AMPA receptors, improved the performance of a JNCL mouse model. Trials in mice on talampanel (LY300164), which targets the same receptors used to treat seizures and Parkinson disease, are underway. The hope for lessening the impact of JNCL must await better understanding of the cell biology and biochemistry of this disorder.

## 104.15 Kufs Disease or Adult NCL

### 104.15.1 Clinical Description

Kufs disease represents 1.3% of the NCL. Symptoms start at the age of 30 years, but can present at an earlier age. Both autosomal recessive and dominant inheritance patterns have been described. Type A is the predominant form and is characterized by progressive myoclonic epilepsy followed years later by dementia and ataxia.



Pyramidal and extrapyramidal symptoms occur as well. Mutations in the *CLN6* gene were found to be responsible for a number of cases of Kufs type A disease. In type B, onset may be delayed to after the age of 50 years. Behavioral abnormality and dementia occur early and are closely followed by progressive motor dysfunction, ataxia, and extrapyramidal and suprabulbar symptoms. Facial dyskinesias are common. More recently, mutations in cathepsin F have accounted for some of the Kufs, type B patients described (261). In both, there is absence of retinal pathology and no visual impairment, although marked photosensitivity is observed in some patients with the type A clinical phenotype.

### 104.15.2 Clinical Diagnostic Approach

A skin biopsy is the least invasive diagnostic approach. The presence of mixed (membrane-bound GRODS, rectilinear, and curvilinear) profiles in association with fingerprint-bearing cytosomes without membrane-bound vacuoles is diagnostic. In rare instances, enzyme testing on leukocytes or fibroblasts shows lysosomal PPT1 activity and gene analysis reveals *CLN1* mutations (654). EEG findings may assist in the diagnosis of Kufs disease. In type A, there are generalized atypical spike and slow wave complexes and photoparoxysmal responses at low flash frequencies. In type B, there is generalized slowing (655).

### 104.15.3 Pathology

Brain weight is 900–1245 g. Most dominant ANCL cases show mild to severe global cerebral and cerebellar atrophy with marked depigmentation of the substantia nigra (656–658). Neuronal loss is variable. The stellate cells of cortical layers II and III are severely affected with mild neuronal loss in layer Vb. Purkinje cells of the cerebellum are also considerably reduced in number.

### 104.15.4 Genetics

The genetic defects underlying most cases of ANCL are unknown. Exceptions are those that carry mutations in *NCL* genes with delayed onset (late teenage years or adulthood). These include *CLN1*, *CLN5*, and notably *CLN6* gene defects with cases reported in the United States, Australia, Italy, and Ireland suggesting that *CLN6* mutations are largely responsible for adult NCL (583). In addition, single mutations of chloride channel 6, the *CLCN6* gene, are described in two late-onset NCL patients. More recently, mutations in cathepsin F or *CLN13* have accounted for Kufs disease, type B cases.

### 104.15.5 Management and Treatment

Supportive and palliative measures are the mainstay of treatment.

## 104.16 CONGENITAL NCL/CNCL-CLN10/CATHEPSIN D OR CTSD DEFICIENCY

### 104.16.1 Clinical Description

CNCL is a rare congenital disorder that was first described in 1941 (659). It represents the earliest onset and the most aggressive form of NCL. Clinical features include congenital microcephalus, overriding sutures, receding forehead, and low-set ears. Deceleration of head growth begins prenatally during the third trimester and jerky fetal movements may be interpreted as seizures. Spasticity is congenital and status epilepticus and respiratory insufficiency occur soon after birth. Death occurs within few hours, days, or weeks after birth. A more protracted form of the disease with a course resembling vLINCL has been described (660).

### 104.16.2 Clinical Diagnostic Test

CLN10 disease should be suspected when an infant presents with intractable seizures and microcephaly. Survival is limited to a few days. The later onset, protracted form initially presents with visual disturbances and ataxia. The presence of granular storage material in skin Schwann cells is indicative of CLN10/CTSD deficiency, provided *CLN1*/PPT1 mutations have been ruled out. Diagnosis is confirmed by the measurement of cathepsin D activity or DNA analysis of CTSD.

### 104.16.3 Pathology

The storage material in CNCL stains positively for sphingolipid activator proteins also found in INCL patients.

Postmortem examination reveals extreme brain atrophy with disorganization of neurons in cerebral cortex, and loss of Purkinje and inner granule cells in cerebellum. The white matter lacks myelin (661–664). Most cells within the CNS are loaded with GRODs. Cathepsin D staining is absent in paraffin-embedded brain specimens.

### 104.16.4 Biochemistry, Cell Biology and Pathophysiology

Mature cathepsin D is a ubiquitously expressed, lysosomal protease belonging to the pepsin family (665). It consists of two polypeptides encoded by the *CTSD* gene. Both polypeptides contain an aspartic acid residue essential for enzymatic activity of the mature protein (666,667). The mature protease is 31 kDa in size. There are several proteins described to function as substrates of CTSD in vitro but the in vivo substrates are still unknown. Aspartyl proteinases consist of two domains, each of which contains an aspartate residue. The residues come together and link to a water molecule at the active site where the substrate peptide bond is hydrolyzed.



Hence, mutation of the aspartate residues that may be present at distant regions of the protein results in the elimination of enzymatic activity of cathepsin D, without affecting its processing (668,669).

### 104.16.5 Genetics

The *CTSD* gene consists of nine exons and is located on chromosome 11p15.5. There are a total of four disease-causing mutations in humans. P.Tyr255X results in truncation of the CTSD protein by 158 amino acids causing complete lack of enzymatic activity and CNCL. Other missense mutations result in effects ranging from complete enzyme inactivation to residual enzymatic activity, suggesting that partial enzyme inactivation leads to later onset CTSD. CTSD knockout mice exhibit the earliest onset and most severe phenotype, while American bulldogs carrying a missense mutation (p.Met199Ile) with 36% residual CTSD activity, start developing symptoms relatively late (between 1 and 3.5 years).

### 104.16.6 Management and Treatment

Currently, only symptomatic treatment is available especially in patients with later onset CTSD deficiency where the main purpose is to ameliorate motor and behavioral complications as well as feeding difficulties.

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### Biographies



**Dr Boustany** completed her Medical degree and Pediatric training at the American University of Beirut in 1980. She subsequently specialized in Pediatric Neurology at the Massachusetts General Hospital (MGH), Harvard Medical School, followed by postdoctoral training in Neurogenetics/Neurochemistry. In 1985, she joined the Neurology faculty at MGH, and stayed for four and a half years. During that time, she was the Co-Director of the Lysosomal Storage Disease Laboratory at the Shriver Center for Mental Retardation. She moved to Duke University in 1989, where she was on the faculty in Pediatrics and Medicine and completed a fellowship in Molecular Biology at the University of North Carolina at Chapel Hill. She became full Professor in Pediatrics and Neurobiology in 2003. She established her research laboratory at Duke in 1992, which remained operational until December, 2007. In 2006, she moved to head the Abuhaidar Neuroscience Institute at the American University of Beirut, a position she held until July 2009. She currently directs the Neurogenetics Program and heads the Division of Pediatric Neurology at the American University of Beirut Medical Center, where she directs basic research and academic clinical programs. Her research interests are Batten disease, lysosomal storage diseases, sphingolipids and their role in cell death.

**Dr Ibraheem Al-Sharreef** completed training in Pediatrics at the American University of Beirut and has just finished his training in Pediatric Neurology at the same institution.

**Dr Sariah El-Haddad** went to Medical School at the American University of Beirut after which she spent three years in the laboratory of Rose-Mary Boustany as a post-doctoral researcher working on the molecular biology and biochemistry of various forms of the neuronal ceroid lipofuscinoses. She is presently in her second year of Psychiatry training in the Department of Psychiatry at the University of Virginia Health Systems.

## Peroxisomal Disorders

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**ABBREVIATIONS**

ACALD – adolescent cerebral ALD  
 ACOX – acyl-CoA oxidase  
 ADHAPS – alkyl-DHAP synthase  
 AGT – alanine glyoxylate aminotransferase  
 AMACR – 2-methyl-acyl-CoA racemase  
 AMN – adrenomyeloneuropathy  
 BAAT – bile acid-CoA: amino acid N-acyltransferase  
 CCALD – childhood cerebral ALD  
 CDCA – chenodeoxycholic acid  
 CG – complementation group  
 CNS – central nervous system  
 DBP – D-bifunctional protein  
 DHAPAT – dihydroxyacetonephosphate-acyltransferase  
 DLP1 – dynamin-like-protein-1  
 ER – endoplasmic reticulum  
 ESRD – end-stage renal disease  
 FA – fatty acid  
 HCT – allogeneic hematopoietic stem cell transplantation  
 HSC – human stem cell  
 IRD – infantile Refsum disease  
 LBP – L-Bifunctional protein  
 MFE – multifunctional enzyme  
 MFP – multifunctional protein  
 NALD – neonatal adrenoleukodystrophy  
 PBD – peroxisome biogenesis disorder  
 PBE – peroxisomal bifunctional enzyme  
 PD – peroxisomal disorder  
 PH – primary hyperoxaluria  
 PLP – pyridoxal-5<sup>1</sup>-phosphate  
 PMP – peroxisomal membrane protein  
 PTS – peroxisomal targeting signal  
 RCDP – rhizomelic chondrodysplasia punctata  
 RD – Refsum disease  
 RP – retinitis pigmentosa  
 SKL – serine-lysine-leucine  
 SCP X – peroxisomal sterol-carrier protein X  
 UDCA – ursodeoxycholic acid

VLCFA – very long-chain FA  
 X-ALD – X-linked adrenoleukodystrophy  
 ZS – Zellweger syndrome  
 ZSD – Zellweger spectrum disorder

**105.1 INTRODUCTION**

The peroxisomal disorders (PDs) constitute a diverse group of inherited diseases in man, either caused by (1) the deficient activity of a specific peroxisomal enzyme or metabolite transporter or (2) the functional loss of a specific component of the peroxisome biogenesis system. At present >15 different peroxisomal disorders have been identified all of which are autosomal recessive with the exception of X-linked adrenoleukodystrophy. The prototype of the group of PDs is the cerebro-hepato-renal syndrome, better known as Zellweger syndrome (ZS), as first described by Bowen et al. (1) who described a familial syndrome of multiple congenital defects in two pairs of siblings. This report was soon followed by a series of papers describing additional cases. In 1973, Goldfischer and coworkers (2) published their—in retrospect—seminal findings documenting the absence of morphologically distinguishable peroxisomes in hepatocytes and kidney cortex cells of ZS patients. Until then virtually nothing was known about peroxisomes and this observation was left unnoticed. In fact, much more attention was paid to the mitochondrial abnormalities described in the same paper as is clear from the title “Peroxisomal and mitochondrial defects in the cerebro-hepato-renal syndrome.” Indeed, Goldfischer et al. (2) documented clear mitochondrial abnormalities characterized by a markedly reduced rate of oxygen uptake of mitochondria isolated from a brain biopsy of a ZS patient and a liver biopsy from another

ZS patient with malate (plus glutamate) as substrate but not with ascorbate plus TMPD as substrate. These findings led the authors to conclude that “the cytochrome portion of the electron transport chain is intact but that there is a defect in electron transport prior to the cytochromes” (2). Based on these results ZS was considered to be a mitochondrial disorder. Subsequent studies by other investigators, however, revealed that the mitochondrial abnormalities at the level of the respiratory chain were remarkably variable among patients ranging from near normal to grossly impaired, which already argued against ZS as a mitochondrial disorder. Another argument against ZS as a mitochondrial disorder was the observation that ZS patients usually have no lactic acidemia.

Two key observations in the early 1980s clearly established that ZS is indeed a PD and not a mitochondrial disorder. The first was the finding by Brown et al. reported in 1982 (3) that the plasma levels of very long-chain fatty acids (VLCFAs) in ZS patients were markedly elevated in contrast to the levels of the long-chain fatty acids which were normal. A few years earlier Lazarow and DeDuve (4) had described the presence of a fatty acid (FA) beta-oxidation system in peroxisomes. The significance of such a second beta-oxidation system next to that in mitochondria, had remained unclear, but the findings by Brown et al. (3) immediately suggested that the peroxisomal and mitochondrial beta-oxidation systems might serve different physiological purposes, catalyzing the oxidation of different sets of substrates. This view turned out to be correct as outlined below.

The second key observation was published 1 year later in 1983 by Heymans et al. (5) who reported the marked deficiency of plasmalogens—a specific type of phospholipids—in tissues from ZS patients. Taken together, these observations clearly identified ZS as a disorder of the peroxisome. In subsequent years a number of additional abnormalities has been reported in ZS patients including the accumulation of phytanic acid and pristanic acid, the deficiency of polyunsaturated FAs and the increased urinary excretion of oxalate and glycolate, all of which can be explained on the basis of the metabolic functions of peroxisomes as we know them now. Earlier work had already led to the identification of other abnormalities in ZS patients including the accumulation of pipelicolic acid and the bile acid intermediates di- and trihydroxycholestanoic acid, which were originally thought to result from the mitochondrial abnormalities in ZS patients. Thorough reinvestigation of these abnormalities revealed that they were due to the peroxisomal rather than mitochondrial abnormalities in ZS patients. These detailed studies on ZS have been extremely important since they have led to the development of a set of peroxisomal biomarkers, i.e. metabolites, which can be measured in a single blood specimen. With

this “peroxisomal biomarker panel” at hand, many diseases in which a peroxisomal dysfunction was suspected on clinical grounds could be investigated which has brought us to where we are now: a group of inherited PDs now made up of >15 different diseases (Table 105-1). This peroxisomal biomarker panel continues to be of great relevance up until now and has contributed to the identification of patients with unique aberrant phenotypes hitherto not considered to have a peroxisomal origin (6,7).

## 105.2 PHYSIOLOGICAL ROLE OF PEROXISOMES

In contrast to mitochondria, which contain >1000 different proteins either encoded by the nuclear or mitochondrial genome, peroxisomes are relatively simple organelles containing far less proteins (current estimate: 50–100 different proteins) (8) all encoded by the nuclear genome. One reason for this apparent simplicity is that peroxisomes lack a respiratory chain (note that complex I of the respiratory chain alone already has >50 different protein components) and that the transport properties of peroxisomes are also relatively simple with most low molecular weight substrates and products (i.e. metabolites) going in and out of peroxisomes, not via specific carriers as in mitochondria, but via one or more porins. In this respect, the peroxisomal membrane resembles that of the mitochondrial outer membrane much more than the mitochondrial inner membrane.

Below we will briefly describe the metabolic pathways catalyzed by peroxisomes and the enzymes involved as far as relevant for human diseases.

### 105.2.1 Peroxisomal FA Beta-Oxidation

The peroxisomal FA beta-oxidation pathway resembles mitochondria in many respects and catalyzes the step-wise shortening of FAs by chopping off an acetyl-CoA unit upon each cycle of beta-oxidation. Each cycle involves four subsequent enzyme reactions, which are all required for the removal of an acetyl-CoA unit from a particular FA. The enzyme proteins catalyzing these four reactions are different from their mitochondrial counterparts although there is marked sequence homology between the mitochondrial and peroxisomal beta-oxidation enzyme proteins. Needless to say that different genes encode them.

From a physiological point of view, the most conspicuous difference between the mitochondrial and peroxisomal beta-oxidation systems is the fact that they oxidize different fatty acids. Indeed, the mitochondrial system catalyzes the oxidation of the bulk of FAs derived from our daily diet and converts the acetyl-CoA units as produced upon beta-oxidation into CO<sub>2</sub> and H<sub>2</sub>O via the citric acid cycle to produce ATP. At least in the liver and



TABLE 105-1 The Peroxisomal Disorders

Disorder	Abbreviation	MIM	Defective Mutant		
			Defective Protein	Mutant Gene	Locus
<b>Disorders of peroxisome biogenesis</b>	PBD				
• PBD-group A:					
Zellweger spectrum disorders	ZSD				
(1) Zellweger syndrome	ZS	214100	PEX1	<i>PEX1</i>	7q21.2
(2) Neonatal adrenoleukodystrophy	NALD	214110	PEX2	<i>PEX2</i>	8q21.1
(3) Infantile Refsum disease	IRD	202370	PEX3 PEX5 PEX6 PEX10 PEX12 PEX13 PEX14 PEX16 PEX19 PEX26	<i>PEX3</i> <i>PEX5</i> <i>PEX6</i> <i>PEX10</i> <i>PEX12</i> <i>PEX13</i> <i>PEX14</i> <i>PEX16</i> <i>PEX19</i> <i>PEX26</i>	6q24.2 12p13.3 6p21.1 1p36.32 17q12 2p14–p16 1p36.22 11p11.2 1q22 22q11.21
• PBD-group B:					
(4) Rhizomelic chondrodysplasia type 1	RCDP-1	215100	PEX7p	<i>PEX7</i>	6q21–q22.2
<b>Disorders of peroxisome function</b>	PFD				
• Fatty acid beta-oxidation					
(5) X-linked adrenoleukodystrophy	X-ALD	300100	ALDP	<i>ABCD1</i>	Xq28
(6) Acyl-CoA oxidase deficiency	ACOX-deficiency	264470	ACOX1	<i>ACOX1</i>	17q25.1
(7) $\alpha$ -Bifunctional protein deficiency	DBP deficiency	261515	DBP/MFP2/MFEII	<i>HSD17B4</i>	5q2
(8) Sterol-carrier-protein X deficiency	SCPx-deficiency	–	SCPx	<i>SCP2</i>	1p32
(9) 2-Methylacyl-CoA racemase deficiency	AMACR-deficiency	604489	AMACR	<i>AMACR</i>	5p13.2–q11.1
• Etherphospholipid biosynthesis					
(10) Rhizomelic chondrodysplasia punctata type 2	RCDP-2	222765	DHAPAT	<i>GNPAT</i>	1q42.1–42.3
(11) Rhizomelic chondrodysplasia punctata type 3	RCDP-3	600121	ADHAPS	<i>AGPS</i>	2q33
• Fatty acid alpha-oxidation					
(12) Refsum disease	ARD/CRD	266500	PHYH/PAHX	<i>PHYH/PAHX</i>	10p15–p14
• Glyoxylate metabolism					
(13) Hyperoxaluria type 1	PH-1	259900	AGT	<i>AGTX</i>	2q37.3
• Bile acid synthesis (conjugation)					
(14) Bile acid-CoA: amino acid N-acyltransferase deficiency	BAAT-deficiency		BAAT	<i>BAAT</i>	
• $H_2O_2$ -metabolism					
(15) Acatalasemia		115500	Catalase	<i>CAT</i>	11p13

kidneys, the acetyl-CoA units may also be converted into the ketone bodies acetoacetate and 3-hydroxybutyrate, which can be used as source of energy in most other tissues.

Peroxisomes contribute little to the oxidation of FAs derived from our daily diet, which renders peroxisomal beta-oxidation unimportant for energy purposes. However, peroxisomes are important for the oxidation of a range of FAs that cannot be oxidized in mitochondria as described in detail below:

It should be noted that peroxisomes are unable to degrade the acetyl-CoA units to completion, i.e. to  $CO_2$  and  $H_2O$ , or convert them into ketone bodies. Instead, the acetyl-CoA units produced in peroxisomes are transported out of the peroxisome into the cytosol, either as acetylcarnitine or acetic acid followed by subsequent metabolism in mitochondria (9). The different physiological roles of the mitochondrial and peroxisomal beta-oxidation systems are also reflected in different clinical phenotypes associated with deficiencies in either of the two systems.

The FAs that are oxidized in peroxisomes include:

- **Very long-chain FAs** like C24:0 and C26:0 are in part derived from our daily diet but are predominantly synthesized endogenously from shorter-chain fatty acids via chain elongation. Current knowledge holds that VLCFAs are not fully degraded into acetyl-CoA units in peroxisomes but instead are only partially degraded to produce acetyl-CoA units plus medium-chain acyl-CoAs which are then exported to the mitochondria as carnitine esters or as free acids (9) (Figure 105-1A).
- **Pristanic acid** (2,6,10,14-tetramethylpentadecanoic acid) is a branched-chain FA which is not biosynthesized endogenously but is solely derived from dietary sources either directly as pristanic acid itself or indirectly from alpha-oxidation of phytanic acid (Figure 105-1B). Phytanic acid is also derived from dietary sources only. Pristanic acid is known to undergo three cycles of beta-oxidation in peroxisomes with acetyl-CoA, propionyl-CoA and 4,8-dimethylnonanoyl-CoA as end products, which are all transported out of the peroxisomes either as carnitine ester or as free acid (9).
- **Di- and trihydroxycholestanoic acid** (DHCA and THCA) are intermediates in the biosynthesis of the primary bile acids chenodeoxycholic acid and cholic

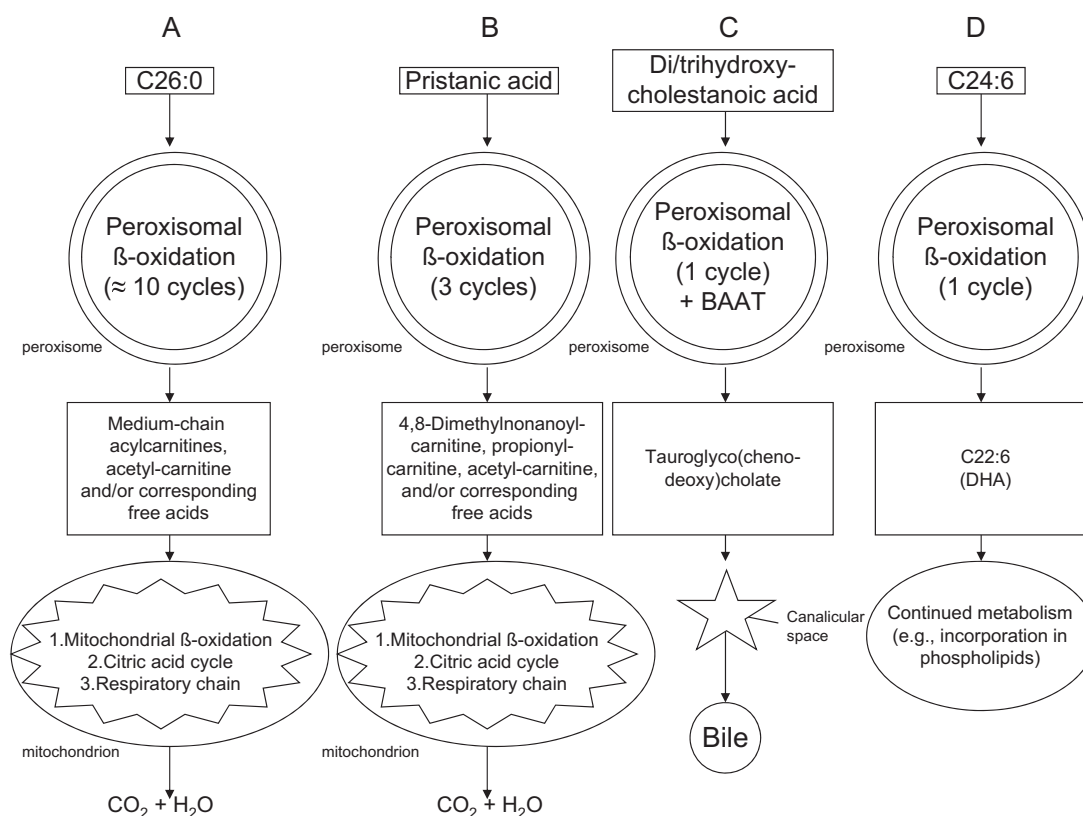
acid respectively and are produced from cholesterol in the liver (Figure 105-1C). After formation of the coenzyme A esters of DHCA and THCA, one cycle of beta-oxidation in peroxisomes produces propionyl-CoA plus the CoA esters of chenodeoxycholic acid and cholic acid which are then converted into the corresponding glycine/taurine conjugates in peroxisomes (9).

- **Tetracosahexaenoic acid** is synthesized from linolenic acid (C18:3n-3) via subsequent steps of elongation and desaturation to produce tetracosanoic acid (C24:3n-3) which undergoes one cycle of beta-oxidation in peroxisomes to yield docosahexaenoic acid (C22:6n-3) (Figure 105-1D).

### 105.2.2 The Peroxisomal FA Beta-Oxidation Enzymes

Peroxisomes contain the full enzymatic machinery to oxidize different FA substrates listed above. These enzymes include:

- **Acyl-CoA oxidases:** Human peroxisomes contain two acyl-CoA oxidases catalyzing the first step of beta-oxidation. The first enzyme (ACOX1) is specific for saturated acyl-CoAs and is nonreactive with 2-methyl-branched-chain acyl-CoA like pristanoyl-CoA



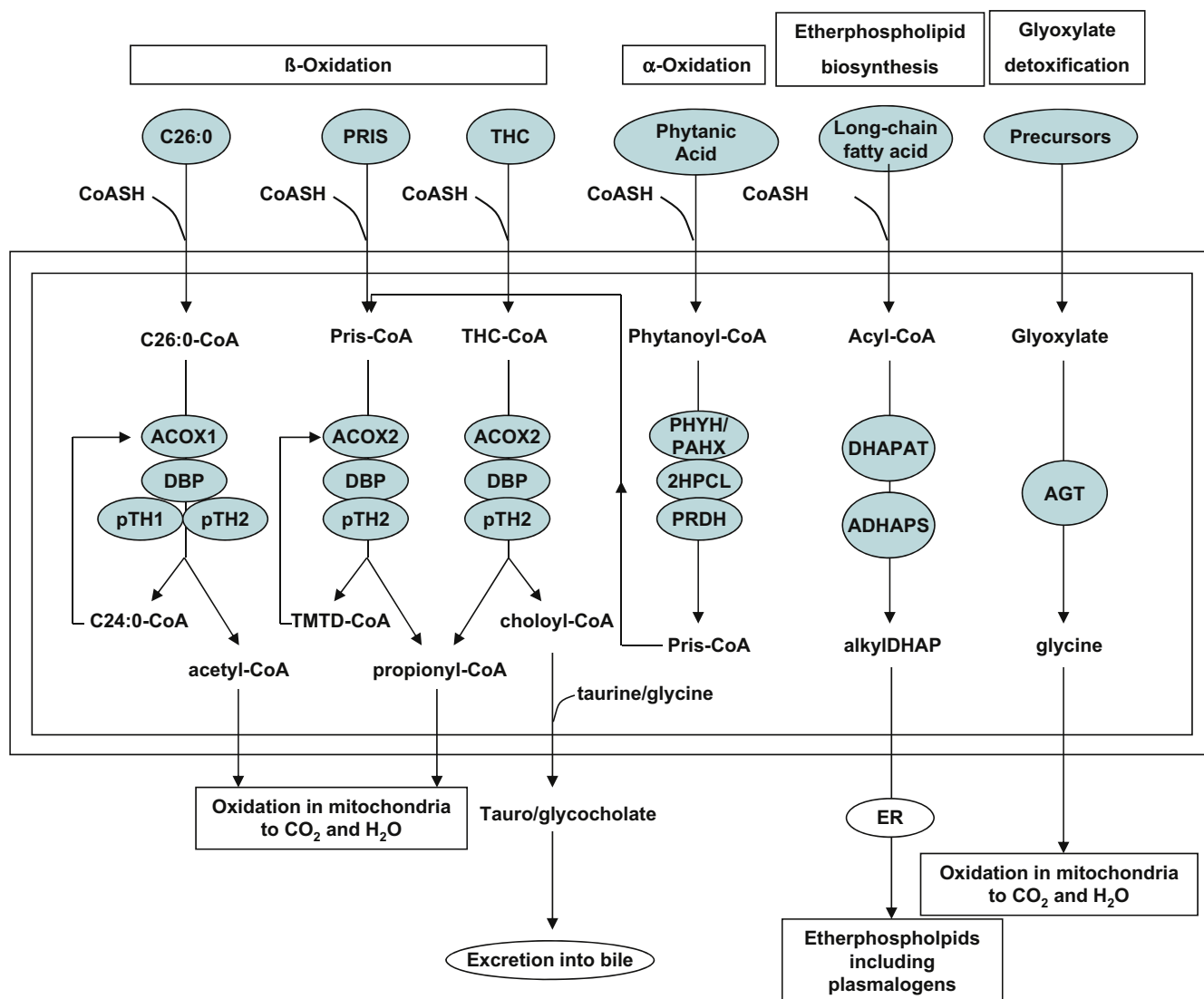
**FIGURE 105-1** Schematic diagram depicting the fate of the main FA substrates oxidized in peroxisomes, which include (1) the VLCFA C26:0 (hexacosanoic acid); (2) the branched-chain fatty acid pristanic acid (2,6,10,14-tetramethylpentadecanoic acid); (3) the bile acid synthesis intermediates DHCA and THCA, and (4) the polyunsaturated fatty acid tetracosahexaenoic acid (C24:6n-3). See text for further details.

and di- and trihydroxycholestanoyl-CoA while the second enzyme (ACOX2) preferentially reacts with pristanoyl-CoA and di- and trihydroxycholestanoyl-CoA. ACOX1 is the dominant enzyme in the oxidation of very long-chain FAs, whereas ACOX2 is indispensable for the oxidation of pristanic acid and di- and trihydroxycholestanic acid. Only human ACOX1 deficiency has been described so far as described in detail below.

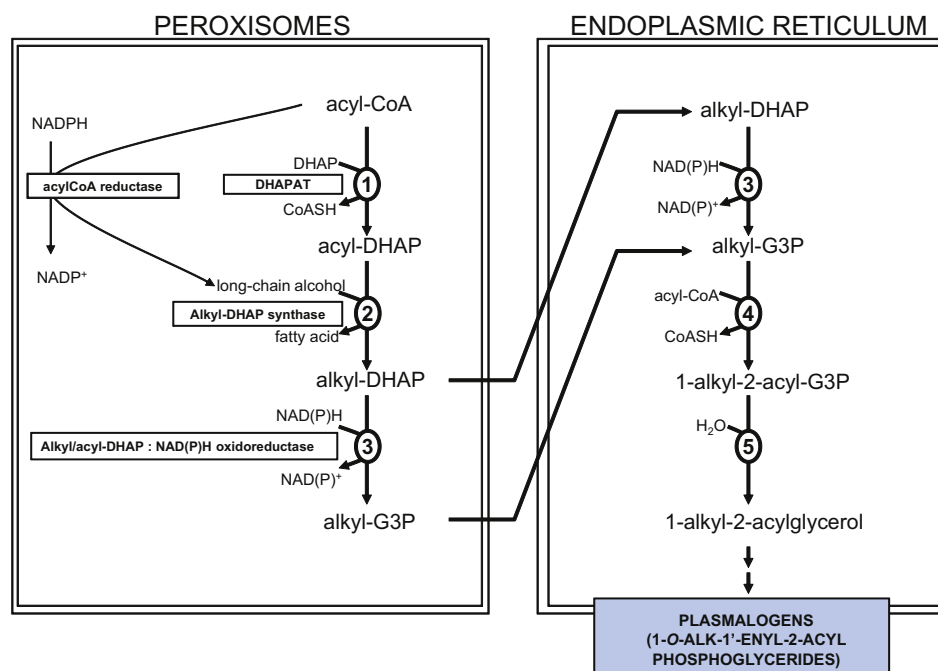
- **Combined enoyl-CoA hydratases/3-hydroxyacyl-CoA dehydrogenase enzymes:** Human peroxisomes contain two different the so-called bifunctional proteins which harbor both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities that allow these proteins to catalyze the second and third step of beta-oxidation in peroxisomes. The enzymes are alternatively named as multifunctional enzyme (MFE) I/II, multifunctional protein (MFP)

1/2, peroxisomal bifunctional enzyme 1/2 and L- and D-bifunctional protein (LBP/DBP), respectively. DBP is the central enzyme involved in the oxidation of virtually all beta-oxidation substrates including C26:0, pristanic acid and di- and trihydroxycholestanic acid (Figure 105-2). The role of LBP in FA oxidation has remained obscure although oxidation of some long-chain dicarboxylic acids appears to involve LBP rather than DBP. Only DBP deficiency has been described in humans as described in detail below.

- **Peroxisomal thiolases:** Human peroxisomes contain two different thiolases named peroxisomal thiolase 1 and 2. The latter thiolase named pTH2, better known as SCPx, is reactive with the 3-oxoacyl-CoA esters of branched as well as unbranched FAs whereas the first thiolase (pTH1) only reacts with the 3-oxoacyl-CoA esters of straight-chain FAs. Only a deficiency of the second thiolase has been described so far (10).



**FIGURE 105-2** Schematic diagram showing the four major metabolic functions of peroxisomes that include: (1) fatty acid beta-oxidation; (2) fatty acid alpha-oxidation; (3) etherphospholipid biosynthesis, and (4) glyoxylate detoxification plus the individual peroxisomal enzymes involved and the end products of each of the four pathways in peroxisomes. See text for further details.



**FIGURE 105-3** Enzymology of the etherphospholipid biosynthesis pathway. Etherphospholipid biosynthesis is fully dependent on peroxisomes since the first two enzymes of the pathway, i.e. DHAPAT and ADAPS are strictly peroxisomal whereas the next enzyme in the pathway is shared between peroxisomes and the ER. All subsequent enzymes are localized in the ER.

In addition to the enzymes described above, peroxisomes contain a number of additional enzymes involved in peroxisomal beta-oxidation. These include: (1) two different acyltransferases including carnitine acetyltransferase and carnitine octanoyltransferase for the conversion of the products of peroxisomal beta-oxidation i.e. acetyl-CoA, propionyl-CoA, and other chain-shortened acyl-CoAs into the corresponding carnitine esters, (2) different thioesterases catalyzing the cleavage of acyl-CoA esters into the corresponding free acids plus coenzyme A, (3) an enoyl-CoA isomerase and dienoyl-CoA reductase enzyme, and (4) the enzyme 2-methylacyl-CoA racemase. The latter enzyme abbreviated as AMACR, is required for the conversion of the (2R)-methyl group—as present in for instance pristanic acid—into the (2S)-configuration (Figure 105-4). The AMACR enzyme plays an indispensable role in the oxidation of (2R)-methyl-branched-chain FAs like pristanic acid, as well as di- and trihydroxycholestanic acid simply because the branched-chain acyl-CoA oxidase (ACOX2) only reacts with (2S)-acyl-CoA esters. Racemase deficiency has been identified in humans as described below (11).

### 105.2.3 Etherphospholipid Biosynthesis

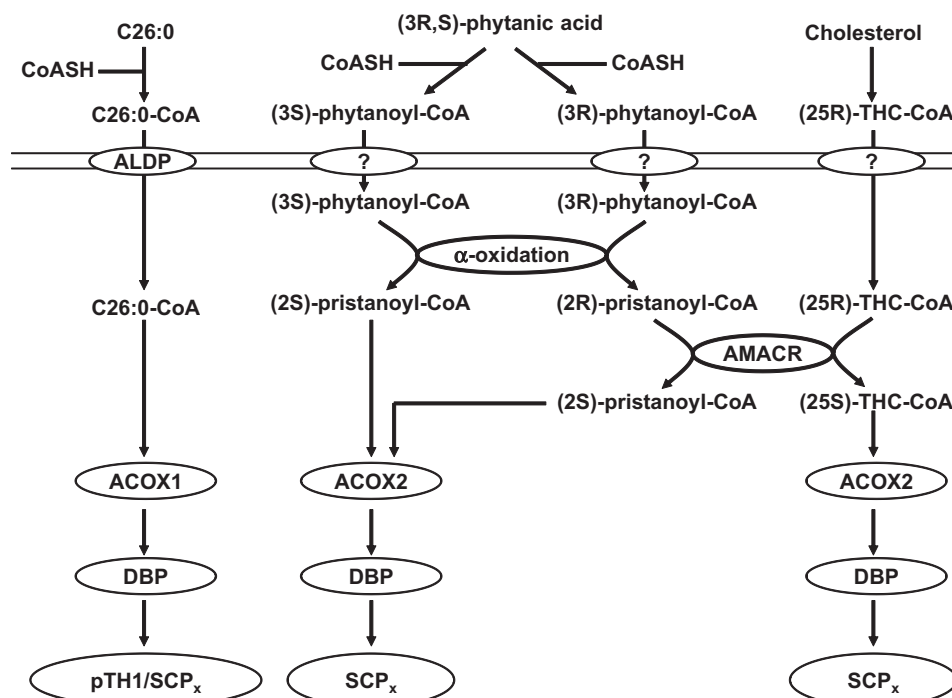
Etherphospholipids are a special class of phospholipids, which differ from the regular diacylphospholipids in the sense that an ether-linkage rather than an ester-linkage is present at the *sn*-1 position of the glycerol backbone. The ether group at *sn*-1 can have two different configurations, which explains why two groups of etherphospholipids can be distinguished either with a

1-O-alkyl or 1-O-alk-1'-enyl linkage. The latter phospholipids (1-O-alk-1'-enyl-2-acyl-phosphoglycerides) with an alpha-, beta-unsaturated ether-bond are also known as plasmalogens. Platelet-activating factor (1-O-alkyl-2-acetyl-glycerophosphocholine) is the best-known etherphospholipid. Figure 105-3 depicts the enzymology of the etherphospholipid biosynthetic pathway with part of the enzymes localized in peroxisomes and another part in the endoplasmic reticulum. Biosynthesis of etherphospholipids starts in the peroxisome with production of acyl-DHAP as catalyzed by the peroxisomal enzyme dihydroxyacetonephosphate-acyltransferase (DHAPAT), followed by the introduction of the typical ether-bond by the enzyme alkyl-DHAP synthase (ADHAPS). Both enzymes are strictly peroxisomal. The third step is catalyzed by the enzyme alkyl/acyl-DHAP: NAD(P)H oxidoreductase, which has a bimodal distribution in both peroxisomes and endoplasmic reticulum (Figure 105-3). The product alkylglycerol-3-phosphate (alkyl-G-3P) undergoes subsequent conversion into plasmalogens. Genetic deficiencies at the level of DHAPAT and alkyl-DHAP synthase have been described in literature as described below. Both of these deficiencies give rise to rhizomelic chondrodysplasia punctata (RCDP).

### 105.2.4 FA Alpha-Oxidation

3-Methyl-branched FAs cannot be beta-oxidized directly due to the presence of a methyl group at 3-position. Instead, 3-methyl FAs first undergo alpha-oxidation, which allows removal of the terminal





**FIGURE 105-4** Detailed description of the peroxisomal alpha- and beta-oxidation systems showing (1) the close interaction between the two pathways with respect to the oxidation of the branched-chain fatty acid phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) and (2) the importance of the enzyme AMACR in the oxidation of phytanic acid, pristanic acid and the bile acid synthesis intermediate THCA. See text for further details.

carboxyl group as  $\text{CO}_2$ . One of the most important 3-methyl-branched FAs is phytanic acid, long known to accumulate in Refsum disease (RD) as well as in other PDs as discussed below. The mechanism involved in the oxidative decarboxylation of 3-methyl FAs has been resolved in recent years. Figure 105-2 depicts the structure and enzymology of the peroxisomal fatty acid alpha-oxidation system. Phytanic acid first undergoes activation to phytanoyl-CoA and then passes the peroxisomal membrane probably as phytanoyl-CoA via a carrier protein. Once inside peroxisomes, phytanoyl-CoA undergoes hydroxylation, followed by cleavage to pristanal and formyl-CoA. Subsequently, pristanal is converted into pristanic acid, which is then activated to pristanoyl-CoA.

As discussed above, pristanoyl-CoA undergoes three cycles of beta-oxidation in peroxisomes after which the products are shuttled to mitochondria for full oxidation to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . Most enzymes involved in peroxisomal fatty acid alpha-oxidation and their encoding genes have been identified with the exception of pristanal dehydrogenase (see (9) for review). Interestingly, the alpha-oxidation system can handle both stereoisomers of phytanic acid including (3S)-phytanic acid and (3R)-phytanic acid with (2S)-pristanoyl-CoA and (2R)-pristanoyl-CoA as products, respectively. Since the peroxisomal beta-oxidation system only handles (2S)-FAs, (2R)-pristanoyl-CoA first needs to be converted into (2S)-pristanoyl-CoA, which requires the enzyme AMACR (see Figure 105-4).

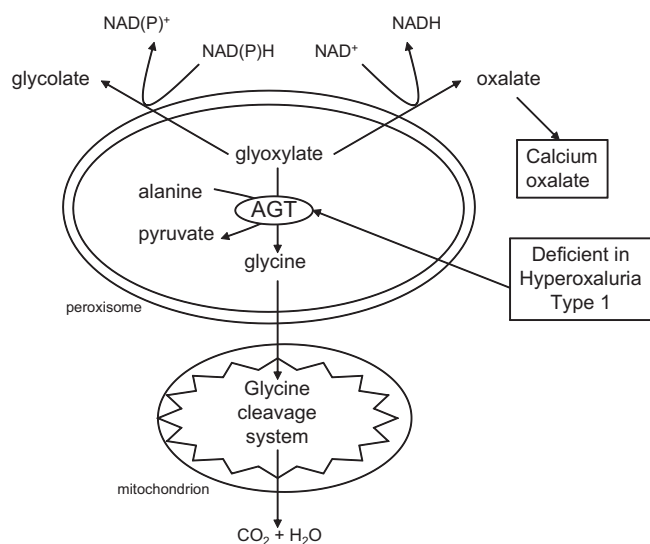
### 105.2.5 Glyoxylate Detoxification

Glyoxylate is a toxic metabolite. Under normal conditions, glyoxylate is rapidly converted into glycine via the peroxisomal enzyme alanine glyoxylate aminotransferase (AGT). In hyperoxaluria type 1 this enzyme is deficient, and glyoxylate accumulates, followed by oxidation to oxalate or reduction to glycolate (Figure 105-5). Oxalate rapidly precipitates as calcium oxalate with severe consequences for the tissues involved.

## 105.3 ADDITIONAL PEROXISOMAL FUNCTIONS

It should be pointed out that peroxisomes catalyze a number of additional metabolic functions. One such function involves the oxidation of L-pipecolic acid, which is a product of L-lysine. Although the precise metabolic route from L-lysine to L-pipecolic acid and onward has not been fully resolved yet, it is clear that L-pipecolic acid is degraded via the peroxisomal enzyme L-pipecolate oxidase.

Recent studies have also revealed that the enzyme bile acid-CoA: amino acid N-acyltransferase (BAAT) is exclusively localized in peroxisomes (12) (Figure 105-1) which implies that formation of taurocholate, glycocholate, taurochenodeoxycholate, and glycochenodeoxycholate is strictly peroxisomal, so that BAAT-deficiency is actually a peroxisomal disease (see below).



**FIGURE 105-5** Peroxisomes and the detoxification of glyoxylate to glycine as mediated by the peroxisomal enzyme AGT and the subsequent breakdown of glycine in mitochondria. In case of a deficiency of AGT, glyoxylate accumulates and is either reduced to glycolate which can be readily excreted via the urine, or oxidized to oxalate which precipitates in the presence of calcium (calcium oxalate stones). See text for further details.

Until recently it was generally assumed that peroxisomes play a major role in the biosynthesis of isoprenoids including cholesterol, since enzymes like mevalonate kinase, phosphomevalonate kinase, and mevalonate pyrophosphate decarboxylase were claimed to be localized in peroxisomes. Recent studies, however, have indicated that all these enzymes are in fact localized in the cytosol (see (9) for review). Based on these results we conclude that isoprenoid biosynthesis does not involve one or more peroxisomal enzymes. Accordingly, mevalonate kinase deficiency can no longer be considered a PD.

## 105.4 BIOGENESIS OF PEROXISOMES

The essential features of peroxisome biogenesis in humans have been nicely worked out in recent years, thanks to the work on lower eukaryotes, including bakers yeast (*Saccharomyces cerevisiae*). Since peroxisomes lack their own DNA, like mitochondria do, the genetic code for both the peroxisomal membrane proteins (PMPs) and peroxisomal matrix proteins is contained in nuclear DNA. The generally accepted idea is that peroxisomes start their life in the endoplasmic reticulum (ER) where an initial pre-peroxisomal structure is formed which buds off and develops into a full grown functional peroxisome. Large collections of proteins, which play a crucial role in the formation of peroxisomes, have been identified in recent years and are named “peroxins.” The corresponding genes are PEX genes. In humans at least 12 different PEX genes and the corresponding peroxins have been identified whereas in different yeasts quite a few more have been found (13). Although the

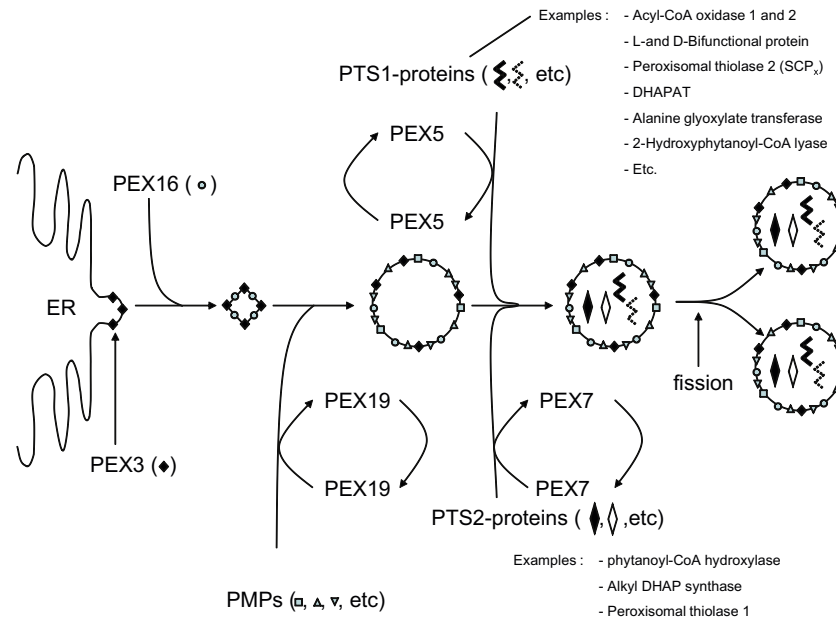
fine details of peroxisome biogenesis still need to be resolved, the general notion is that PEX3 plays an essential role in the initial phase of peroxisome biogenesis, i.e. the formation of the preperoxisomal vesicle in the ER (Figure 105-6). Once this initial vesicle is formed, different PMPs are inserted into the membrane of this vesicle, a process mediated by PEX19, which has been shown to bind multiple PMPs. Binding between PEX19 and different PMPs is supposed to happen in the cytosol after which these PMPs are specifically targeted to the membrane of the preperoxisomal vesicle with PEX3 acting as the docking partner for PEX19 thus ensuring specific targeting of PMPs to the preperoxisomal vesicle. Once this is done, the vesicle is ready to be filled up with various matrix proteins the majority of which are actually enzymes required for the execution of the different metabolic functions of peroxisomes. In order to target these proteins specifically to peroxisomes, peroxisomal matrix proteins contain one of two different peroxisomal targeting signals (PTS). The *first* one (PTS1) is made up of a set of three amino acids located at the carboxy-terminal end of the protein. These amino acids are: serine, lysine, and leucine at position 3, 2 and 1 of the carboxy-terminal end of the protein respectively. Some variants of this SKL theme are permitted.

The *second* signal (PTS2) involves a stretch of nine amino acids located at the amino-terminal end of peroxisomal matrix proteins. Most proteins possess a PTS1 signal. The PTS1 and PTS2 signals are recognized by soluble receptors, i.e. PEX5 and PEX7, respectively, in the cytosol (Figure 105-6). The two receptors loaded with “cargo” are recognized by the PEX13/PEX14 complex located in the peroxisomal membrane (Figure 105-6) and then release their cargo, i.e. a PTS1—or PTS2—protein followed by their extrusion into the peroxisome interior. This complicated process requires the active participation of a number of peroxins, including PEX2, PEX10 and PEX12 as well as PEX1 and PEX6.

After releasing their cargo PEX5 and PEX7 cycle back into the cytosol where they can bind another PTS1 or PTS2 protein. In addition to the peroxins described above, additional peroxins are involved in other aspects of peroxisome biogenesis including peroxisome division (PEX11-alpha, PEX11 beta, PEX11-gamma and DLP1). Defects at the level of most of these different peroxins have been identified as described in detail below.

### 105.4.1 Peroxisomal Disorders

The different PDs identified thus far are listed in Table 105-1 and are usually classified into two distinct groups including: (1) the peroxisome biogenesis disorders (PBDs) and (2) the disorders of peroxisome function. The latter group comprises of single peroxisomal enzyme deficiencies although in X-linked adrenoleukodystrophy (X-ALD) it is not an enzyme which is functionally defective but a transport protein as discussed in detail below.



**FIGURE 105-6** Biogenesis of peroxisomes in humans. Peroxisomes start their life in the ER but may also form from preexisting peroxisomes. The peroxin PEX3 is essential for this initial phase of peroxisome biogenesis in which a pre-peroxisome is generated. The peroxins PEX16 also plays a role—albeit it unresolved yet—in this initial phase of peroxisome biogenesis. Subsequently, the PMPs are inserted into this pre-peroxisomal vesicle with a key role for the cytosolic cycling receptor PEX19. The next phase is the import of matrix proteins mediated by PEX5 for PTS1 proteins and PEX7 for PTS2 proteins, respectively. Next, peroxisomes undergo proliferation and division. The division of peroxisomes involves three distinct sequential steps including elongation of peroxisomes, membrane constriction, and finally fission of peroxisomes. The proteins DLP1, mammalian fission 1 and mammalian mitochondrial fission factor are involved in this process. Remarkably, the latter three proteins do not only play a role in the fission of peroxisomes but also in the fission of mitochondria. With respect to the elongation of peroxisomes different PEX11 proteins are involved. See text for further details.

(1) The PBD group is heterogeneous in itself and is subclassified into two distinct subgroups A and B. The underlying basis for the subclassification is the fact that in the group A disorders both the PTS1- and PTS2-import pathways are disturbed (see [Figure 105-6](#)), whereas in PBD-group B disorders only the PTS2-pathway is affected. Importantly, the clinical signs and symptoms of patients with a PBD-group A disorder are widely different from those of patients affected by RCDP Type 1 which is so far the only PBD-group B disorder. Until recently, PBD-group A was comprised of three distinct PDs including ZS, neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD). The discovery, however, of phenotypes in between ZS and NALD, and NALD and IRD, respectively, plus the identification of additional phenotypes different from ZS, NALD, and IRD has prompted institution of a new name, i.e. “Zellweger spectrum disorders” (ZSDs). An additional benefit of this new nomenclature is that names like NALD and IRD will disappear which is good since they continue to generate confusion since they suggest that there is a link with X-ALD and RD respectively.

**105.4.1.1 Zellweger Syndrome (MIM 214100).** ZS was first described as cerebro–hepato–renal syndrome in 1964 by Bowen, Lee, Zellweger and Lindenberg (1) in two pairs of siblings. The clinical presentation of ZS is dominated by: (1) typical cranial facial dysmorphism and (2) severe neurological abnormalities. Facial

features include a large anterior fontanel with widely spaced sutures (96%), a broad full forehead (97%), micrognathia (100%), external ear deformity (97%), low and broad nasal bridge (100%), shallow orbital ridges (100%), and redundant skin folds of the neck (100%). The neurological picture is dominated by a profound truncal and peripheral hypotonia (99%), with poor sucking (97%) requiring gavage feeding, absence of visual and auditory responses, and seizures usually after the second week of birth and depressed neonatal and deep tendon reflexes. Other neurological abnormalities include an abnormal Moro response (100%), hypo/areflexia (98%) and epileptic seizures (92%). Ocular abnormalities include corneal clouding, congenital cataracts and glaucoma. Brushfield spots and retinitis pigmentosa (RP) with extinguished electroretinograms are frequently found. Hepatomegaly and cardiac defects are also present. Calcific stippling of the epiphyses visible on X-ray examination and small renal cysts that cannot be detected by echography have been reported. Brain abnormalities in ZS include a very specific cortical dysplasia with polymicrogyria and pachygyria in the perisylvian region, neuronal heterotopia, migration defects of Purkinje cells and discontinuations in the principle nucleus of the inferior olivary complex. There is dysmyelination rather than demyelination of the cerebral white matter. All these abnormalities can be diagnosed by MRI. More than 90% of ZS patients show postnatal growth failure.

Patients with ZS do not develop any psychomotor skills and usually die before 12 months of age.

**105.4.1.2 Neonatal Adrenoleukodystrophy (MIM 202370).** Patients with NALD have a somewhat less severe phenotype than ZS patients. They might be present at birth with severe hypotonia, absence of visual and auditory responses and seizures but the typical cranial facial dysmorphism observed in ZS is absent. RP and hepatomegaly are frequently found, however, brain abnormalities may include polymicrogyria, but without the severe cortical dysplasia seen in ZS. When NALD patients survive beyond 3 years of age, brain MRI may show progressive demyelination in addition to preexisting dysmyelination and is associated with rapid neurological deterioration. Occasionally NALD patients may develop some psychomotor achievements during the first year of life, but they remain severely retarded. Age of death is usually between 2–3 years of age. Needless to say that NALD and X-ALD are completely different diseases which only share a few abnormalities including demyelination of the central nervous system (CNS) white matter, atrophy of the adrenal cortex, ballooned adrenocortical cells, and splinter-like lamellar elements composed of electron-dense leaflets separated by a clear space which initially prompted the name NALD.

**105.4.1.3 Infantile Refsum Disease (MIM 266510).** Patients with IRD may have external features reminiscent of ZS but do not show disordered neuronal migration and no progressive white matter disease. The patients originally described as IRD had persistent jaundice and liver disease in the first 3–6 months of life. Later it became clear that there was also psychomotor retardation and RP. Remarkably, the liver disease recovered over the years, whereas the other abnormalities remained, including severe mental retardation, major behavioral problems, progressive blindness due to retinopathy, and peripheral deafness. In general, survival of IRD is variable. Most patients with IRD, however, do reach adolescence, and several even have reached adulthood. The clinical severity is remarkably variable. Some patients may have moderate learning disabilities with deafness and visual impairment that has sometimes led to the diagnosis of Usher syndrome (14) while others have severe and more global neurological handicaps.

**105.4.1.4 Other Phenotypes.** In the last few years a number of patients have been described with phenotypes different from ZS, NALD and IRD (6,7,14–18). Several of these patients have shown mild neurological symptoms, notably cerebellar ataxia, and peripheral neuropathy since adolescence with relative intactness of cognitive functions. From the second decade onward most of these patients end up developing obvious neurologic deterioration characterized by tetraparesis, dystonia, ataxia, and cognitive decline. Brain MRI frequently shows signs of cerebral demyelination mostly of the periventricular white matter. Initially progression of the neurological symptoms may be slow, but patients then aggravate

rapidly to a vegetative state or death. Remarkably, in recent years several patients with isolated cerebellar ataxia have been described who turned out to have a peroxisome biogenesis defect (6,7).

**105.4.1.5 Rhizomelic Chondrodysplasia Punctata Type I (MIM 215100).** RCDP Type I is a PD completely different from the ZSDs described above and is characterized by a disproportionally short stature primarily affecting the proximal parts of the extremities, typical facial appearance including a broad nasal bridge, epicanthus, high arched palate, dysplastic external ears and micrognathia, congenital contractures, characteristic ocular involvement, dwarfism and severe mental retardation with spasticities. Roentgenological studies have shown a series of abnormalities including symmetrical shortening of femur and humerus with irregular and broad metaphyses, calcific stippling mainly of the epiphyses, absent capital femur epiphysis, coronal clefts of vertebrae, increased intravertebral disc spaces, cupping of dorsal ribs, and a barrel-formed thorax. Neurological impairments include truncal hypertonia, spastic tetraplegia and epilepsy. Patients usually develop profound mental retardation and disproportionate growth failure before 3 years of age. In contrast to earlier views patients usually survive beyond 1 year of age. A subset of patients with RCDP has a milder clinical phenotype with less severe neurological impairment involving moderate to severe mental retardation with motor problems and nearly normal skeletal findings. No abnormalities on MRI are seen in the patients with milder phenotypes whereas delayed myelination, ventricular enlargement, and increased subarachnoid spaces, supratentorial myelin abnormalities, and progressive cerebellar atrophy are observed in patients with a severe phenotype of RCDP Type I (19).

## 105.4.2 Biochemistry and Molecular Basis of the ZSDs

In ZSD patients essentially all metabolic functions of peroxisomes are impaired due to the fact that proper peroxisome formation is disturbed as discussed in more detail below. This implies: (1) deficient beta-oxidation of FAs, normally handled by peroxisomes including VLCFAs like C26:0, pristanic acid, di- and trihydroxycholestanic acid, and tetracosahexaenoic acid (C24:6( $\omega$ -3)), (2) deficient alpha-oxidation of phytanic acid, (3) impaired synthesis of etherphospholipids including plasmalogens, (4) impaired degradation of glyoxylate, and (5) deficient oxidation of L-pipecolic acid. In general, all these abnormalities at the cellular level, are reflected in clear abnormalities in blood either in plasma (VLCFAs, pristanic acid, phytanic acid, di- and trihydroxycholestanic acid, pipecolic acid, plus C24:6 and C22:6), or in blood cells, notably erythrocytes (plasmalogens, C24:6 and C22:6). Also in urine abnormalities are usually found (oxalate, glycolate). In general, the abnormalities observed in plasma and/or erythrocytes reflect the extent of the



defect in peroxisome biogenesis especially with respect to (1) the VLCFAs, notably C26:0 in plasma and (2) plasmalogens in erythrocytes. Indeed, plasma C26:0 levels are in general highest in classical ZS patients and much lower in ZSD patients at the mild end of the spectrum whereas the reverse is true for the erythrocyte plasmalogens which may be virtually fully deficient in classical ZS but normal in milder ZSD patients. Phytanic acid and pristanic acid are solely derived from exogenous sources and may vary from normal to hugely elevated depending upon dietary intake. Di- and trihydroxycholestanic acid synthesized from cholesterol in the liver are in general higher in ZS patients but there are large variations between patients independent of phenotype (20). Finally, L-pipecolic acid is a useful marker of peroxisome dysfunction although again levels in individual patients vary widely independent from the phenotype (21).

Fibroblasts from patients have become the material of choice for diagnostic purposes since all metabolic functions of peroxisomes except bile acid synthesis and L-pipecolic acid oxidation are expressed in these cells. Furthermore, the morphology of peroxisomes can be studied easily using immunofluorescence microscopy analysis using antibodies against peroxisomal matrix proteins like catalase and PMPs like PMP70. Both antibodies are commercially available. Liver biopsies have long remained the golden standard for the diagnosis of patients but biopsies are no longer required nowadays although in some cases morphological analysis of peroxisomes in liver biopsy specimens has remained important. Lymphoblasts can also be used for diagnostic purposes.

In recent years, much progress has been made in the elucidation of the genetic basis of the different peroxisome biogenesis disorders. Earlier studies had already shown that the genetic basis of the ZSDs is very heterogeneous. This was concluded from the so-called complementation studies, which involves fusion of fibroblasts of two different patients with the same abnormality such as a defect in peroxisome biogenesis so that hybrid cells are generated containing nuclei from the two patients' fibroblasts. These cells are called heterokaryons. If the defective genes in the two patients' cell lines are different, one would expect restoration of peroxisome formation whereas in the other case when the mutant genes are identical, no complementation would occur. This is most easily done using catalase immunofluorescence. Collaborative studies involving laboratories in Japan (Gifu), USA (Baltimore) and The Netherlands (Amsterdam) have led to the identification of a total number of 12 different complementation groups (CGs) (22).

With the recent identification of *PEX26* as the defective gene in complementation group 8 (CG8) by Matsumoto et al. (23,24), the *PEX* genes underlying each of the CGs have all been identified now (Table 105-2). Most CGs include only a few patients. One exception to this rule is CG1 with *PEX1* as the defective gene, which is by

**TABLE 105-2** Frequency Distribution of *PEX* Gene Defects among 613 Patients Diagnosed with a ZSD

<i>PEX</i> Gene	Number of Patients Cell Lines Analyzed	Frequency (%)
<i>PEX1</i>	358	58
<i>PEX2</i>	23	4
<i>PEX3</i>	3	<1
<i>PEX5</i>	13	2
<i>PEX6</i>	97	16
<i>PEX10</i>	19	3
<i>PEX12</i>	54	9
<i>PEX13</i>	10	1
<i>PEX14</i>	3	<1
<i>PEX16</i>	8	1
<i>PEX19</i>	4	<1
<i>PEX26</i>	21	3

Data taken from Ebberink et al. *Hum. Mutat.* 2011 32, 59–69.

far the largest CG containing more than half of all Zellweger spectrum patients. In our own series of >600 Zellweger spectrum patients thereby excluding RCDP, 358 patients (58%) were found to belong to CG1 (*PEX1*) followed by 97 (16%) in CG4 (*PEX6*), and 54 (9%) in CG3 (*PEX12*) (25).

If all mutant *PEX* alleles are taken together, >100 mutations have been so far described in literature (25). In many cases mutations are private being restricted to single families only. Most mutations have been described in the *PEX1* gene (>80). Among these mutations a few common mutations have been identified. Most common is the missense mutation in exon 15 (c.2528G > A) leading to the substitution of the glycine at position 843 of *PEX1* by an asparagine (p.G843D) in the second ATP-binding domain. Patients homozygous for this mutation show the mild Zellweger spectrum phenotype (NALD/IRD). The frequency of the c.2528G > A (p.G843D) allele ranges from 0.25 to 0.37 in different cohorts. In our own cohort of *PEX1* mutated patients we found an allele frequency of 0.41 out of which 12% of the patients were homozygous and 17% were compound heterozygous for the c.2528G > A allele. The second most common mutation is a T insertion in exon 13 (c.2097\_2098insT), first described by Maxwell et al. (26) and Collins and Gould (27), which results in a frame shift and low steady state *PEX1* mRNA levels, presumably caused by nonsense mediated RNA decay. At the protein level it leads to truncation of the *PEX1* protein within the second AAA domain, abolishing *PEX1* function completely. In its homozygous form the mutation results in the severe Zellweger phenotype. Together, these two mutations account for around 50–60% of all mutant *PEX1* alleles. Interestingly, the mutation c.2528G > A encodes a *PEX1* mutant protein, which allows residual import for peroxisomal matrix proteins. The mutation seems to result in a misfolded

protein, which is more stable at lower than at higher temperatures, which explains the mosaic catalase immunofluorescence pattern at 37°C whereas a virtually normal pattern is observed at 30°C (28,29). The G843D amino acid substitution most likely disrupts the interaction between PEX1 and PEX6 (30,31).

### 105.4.3 Biochemistry and Molecular Basis of RCDP

RCDP type 1 is characterized by the deficiency of plasmalogens in all tissues including erythrocytes (32). Furthermore, plasma phytanic acid levels are increased in age and diet dependent manner. These data immediately suggested that at least two peroxisomal functions were impaired in RCDP type 1, including (1) plasmalogen biosynthesis and (2) phytanic acid alpha-oxidation. Detailed studies have shown that several peroxisomal enzymes are indeed deficient in RCDP type 1, including (1) DHAPAT, (2) alkyl-DHAP synthase, (3) phytanoyl-CoA hydroxylase, and (4) peroxisomal thiolase. The underlying basis for this surprising combination of defects in a single genetic disease became clear when the gene defective in RCDP type 1 was discovered. The gene involved is the *PEX7* gene, which codes for the PTS2 receptor. Alkyl-DHAP synthase, phytanoyl-CoA hydroxylase, and peroxisomal thiolase are all PTS2 proteins, which immediately explains their deficiency in RCDP type 1. The peculiar finding that DHAPAT is also deficient in RCDP type 1 has been resolved by De Vet et al., who found that alkyl-DHAP synthase and DHAPAT form a complex within peroxisomes so that DHAPAT is unstable in the absence of alkyl-DHAP synthase (33).

Two large studies have appeared on the molecular basis of RCDP type 1, which show many different mutations, although a single mutation (c.875T > A (p.L292X)) accounts for about half of the mutant *PEX7* alleles in RCDP patients (34,35). Haplotype analysis revealed that this mutation is on the same background, strongly suggesting a founder effect in Northern Europeans.

### 105.4.4 Treatment

At present there is no curative treatment for both the ZSDs and RCDP type 1. Treatment with docosahexaenoic acid (C22:6(ω-3)) has been claimed to be beneficial to patients (36), but studies in larger series of patients have shown no relevant clinical improvement (37). Inspired by the fact that the bile acid intermediates dihydroxycholestanic acid are considered very toxic to liver cells and may very well be responsible for the cholestasis frequently observed in ZSD patients, oral bile acid therapy has been tried in a few patients only with some therapeutic benefit (38,39). Indeed, Setchell et al. (39) were the first to report on such a bile acid treatment in a 6-months-old Zellweger patient. This patient received

the primary bile acids cholic acid and chenodeoxycholic acid (100 mg each/day) orally. A significant improvement in biochemical indices of liver function occurred with a normalization of the serum bilirubin and liver enzymes and a histological improvement in the extent of inflammation and bile duct proliferation and disappearance of canicular plugs. Steatorrhea improved as well and was accompanied by improvement in growth. Importantly, plasma and urinary C<sub>27</sub>-bile acid intermediates decreased with treatment. Despite the observed improvements the patient died at 1 year of age. Maeda et al. (38) have reported beneficial effects of ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA) treatment in two Japanese patients with Zellweger syndrome with the combination of UDCA and CDCA showing the best results. At present we are conducting a larger oral bile acid therapy study in our cohort of mildly affected ZSD patients (18).

In RCDP type 1 no curative treatment has been devised as well. Interestingly, Brites et al. (40) has found that in the *PEX7* knockout mouse model, which is a mouse model for RCDP type 1, a diet enriched with 1-O-octadecyl-*rac*-glycerol can restore plasmalogen levels in the peripheral tissues of the RCDP mouse but not in the CNS (40,41). This is most likely due to the inability of AG to cross the blood-brain barrier. When supplied prior to the occurrence of pathological changes, i.e. during pregnancy, the AG diet was found to ameliorate the peripheral pathology observed in the RCDP type 1-knockout mice, which is related to the extent to which plasmalogen levels are restored. This diet is currently being evaluated in patients with RCDP type 1 (Poll-The et al. work in progress).

## 105.5 SINGLE PEROXISOMAL ENZYME DEFICIENCIES

### 105.5.1 X-linked Adrenoleukodystrophy (MIM 300100)

X-ALD is a collective term, which includes a range of different phenotypes all due to mutations in *ABCD1* that codes for a protein named ALDP. The abnormalities involved in X-ALD primarily affect the CNS, adrenal cortex and Leydig cells of the testes. X-ALD comes in two main phenotypes including the cerebral demyelinating form of X-ALD and adrenomyeloneuropathy (AMN). AMN and cerebral ALD may occur in the same family, and there is no apparent correlation between the X-ALD phenotype and the mutations in the *ABCD1* gene. Cerebral ALD is characterized by a rapidly progressive inflammatory demyelination within the brain resulting in severe cognitive and neurologic disability and a vegetative state within 2–5 years after onset of symptoms and death thereafter. This phenotype is most common during childhood (childhood cerebral ALD: CCALD) and adolescence (adolescent cerebral ALD: ACALD).

**105.5.1.1 Cerebral ALD.** Cerebral ALD is the most severe type of X-ALD. Indeed, male newborns with bonafide mutations in *ABCD1* have 35–40% risk to develop childhood cerebral ALD between 5 and 12 years of age. Furthermore, 20% of patients initially showing the AMN phenotype develop cerebral ALD later in life so that the lifetime risk to develop cerebral ALD amounts to 55–60%. The typical presentation of CCALD is that of a boy presenting with learning disabilities, and behavioral problems, often diagnosed as attention deficit hyperactivity disorder may actually respond to stimulant medication (42). This is followed by neurological deterioration with cognitive decline, behavioral problems, impaired central auditory discrimination, cortical blindness, often seizures and quadriparesis. Patients may be completely normal until they develop cerebral demyelination visible on brain MRI (abnormal hypersignal of white matter on T2 and FLAIR sequences, hyposignal of white matter on T1 sequences). Usually, the initial lesions involve either the splenium of the corpus callosum and then extend into the adjacent white matter of the parieto-occipital loops or alternatively the genu of the corpus callosum and then extend into the white matter of the frontal loops. Initial demyelinating lesions may also occur in the pyramidal tracts, within the brain stem or internal capsule and then extend into the centrum semiovale (frontal white matter). The cerebral demyelinating lesions can be asymmetric, particularly when previous head trauma or even stroke seems to have initiated the onset of cerebral ALD. Initially, demyelinating lesions are not inflammatory and progress slowly. The patients then have virtually no neurological symptoms except mild cognitive dysfunction. Subsequently, when the disease becomes inflammatory rapid progression sets in and the patients enter into an active phase of devastating neurologic and cognitive deterioration that include emotional lability, hyper active behavior, school failure, and either visuo-spatial impairment or frontal syndrome according to the sites of the demyelinating lesions. This ends in the rapid development of motor symptoms (hemiplegia or quadriparesis), impaired central auditory discrimination, quadrantsopia and then loss of vision, and often seizures, which is subsequently followed by a vegetative state within 2–4 years, and to death at varying intervals thereafter.

**105.5.1.2 Adrenomyeloneuropathy.** The first symptoms of AMN usually appear when patients are between 20 and 30 years of age. AMN males develop progressive stiffness and weakness of the legs, impaired vibration sense in the lower limbs, sphincter disturbances, and impotence. Approximately two third of AMN patients also show clinical signs of a peripheral neuropathy which is usually not prominent and all males with AMN frequently have typical scanty scalp hair that often develops during adolescence. This hair abnormality can also be found in heterozygotes. The evolution of AMN in adult

males is quite variable. In two third of AMN patients the neurological disability progresses slowly. Within 10–15 years motor disability becomes more severe and requires the use of a cane or a wheelchair. However, 35% of patients with AMN have a marked progression of their myelopathy within the first 3 years after the onset of clinical symptoms. Adrenal insufficiency (Addison's disease) is present in approximately two third of males with AMN and in <1% of heterozygous women.

**105.5.1.3 Addison's Disease Only.** Adrenal insufficiency may be the only sign of X-ALD in approximately 10% of affected individuals (43). The signs include unexplained vomiting, weakness or coma. Some individuals have hyper-pigmented skin, due to increased ACTH secretion. Biochemical evidence of an adrenal insufficiency can be present for up to 2 years before the development of clinical signs. Some males with bonafide *ABCD1* gene mutations have been reported without clinical features of adrenal insufficiency. Most individuals, however, who present with isolated adrenal insufficiency, develop some form of X-ALD. In boys presenting with Addison's disease prompt evaluation for X-ALD is important because early diagnosis is likely to improve outcomes from therapy including hematopoietic stem cell transplantation (see below).

## 105.5.2 Female Carriers

Female carriers may be symptomatic depending upon the pattern of X-chromosome inactivation (44). Affected individuals typically present with mild spastic paraparesis in mid-adulthood. AMN-like symptoms affect up to 50% of female heterozygotes in middle age or later although the development of cerebral form of X-ALD is uncommon. Adrenal insufficiency is rare in females (1%).

## 105.5.3 Biochemistry and Molecular Basis of X-ALD

Plasma VLCFA analysis has proven to be an exceptionally robust initial biomarker for X-ALD, especially when the algorithm, as developed by Moser and coworkers (45) is used which is based on the analysis of three parameters including C26:0, C24:0/C22:0 and C26:0/C22:0. Whereas in males diagnosis on the basis of plasma VLCFAs is unambiguous, this is not true for heterozygotes. Indeed, in approximately 20% of obligate heterozygotes plasma VLCFAs are entirely normal, which immediately suggests that additional work has to be done to obtain a conclusive answer. Mutation analysis is the most reliable method for the identification of heterozygotes at least when the mutation in the family has been defined in an affected male or obligate heterozygote relative. If the female in question, however, has no family history of X-ALD, mutation analysis plus detailed studies in fibroblasts, notably immunofluorescence microscopy analysis of ALDP may be needed to obtain a definitive answer.

The X-ALD gene covers 19.9kb, contains 10 exons, and codes for a 745 amino acid peroxisomal transmembrane protein with the general structure of an ATP-binding cassette transporter. Mutation analysis of the *ABCD* gene in X-ALD patients has so far revealed 581 different mutations (<http://www.X-ALD.nl>). Of these mutations, 295 (51%) are missense mutations, 166 (29%) frame shift mutations, 67 (12%) nonsense mutations, 35 (6%) amino acid insertion/deletions, and 18 (3%) larger deletions of one or more exons. Importantly, 78% of all nonrecurrent *ABCD1* mutations resulted in the absence of ALDP (<http://www.X-ALD.nl>). As stated above there is no apparent genotype/phenotype correlation for X-ALD as exemplified by the presence of five different phenotypes in six male patients of a family with a destabilizing missense mutations (p.Pro484Arg) (46).

### 105.5.4 Treatment

It is crucially important to provide adrenal steroid hormone therapy for every ALD patient with adrenal cortical insufficiency. Almost all affected boys, and 60% of men with AMN have impaired adrenal reserve (47). Consequently, all patients diagnosed should undergo an ACTH stimulation test to detect frank adrenal failure or subclinical decreased adrenal cortical reserve. If left untreated, these patients may succumb to adrenal crises. However, adrenal steroid hormone therapy does not appear to alter the course of neurological deterioration. Based on the success of dietary restriction of phytanic acid in RD, a diet low in C26:0 was tried in X-ALD patients without success. Subsequently, a dietary therapy, based on supplementation with oleic acid and erucic acid (GTO/GTE) diet was tried, again without success. It should be noted that the GTO/GTE oil, also called Lorenzo's oil, is able to normalize the plasma levels of C26:0 after 3–5 weeks. This effect is most likely mediated through the competition of C18:1 and C22:1 with C18:0 and C22:0 for chain elongation. This pharmacological effect occurs in peripheral tissues in particular in the liver but apparently C18:1 and C22:1 are unable to cross the blood–brain barrier so that chain elongation of long-chain fatty acids to VLCFAs is not inhibited by C18:1 and C22:1 in the brain. The diet might have a preventive effect—albeit very limited—on the development of CCALD when given to boys before the age of 5 years. In any case this diet does not prevent the onset of CCALD and boys treated with Lorenzo's oil must have regular brain MRI examination, at least up to the age of 12 years to detect the early-onset of cerebral demyelination.

Allogeneic hematopoietic stem cell transplantation (HCT) (also known as bone marrow transplantation) is the only treatment that can arrest or even reverse cerebral demyelination provided the procedure is performed at an early stage of the disease and this point is absolutely

crucial (48–51). When patients have already obvious neurologic signs and extensive demyelinating lesions (Loes score >10) HCT has no effect and patients may even aggravate following the HCT procedure. The beneficial effect of HCT is mediated through the replacement of brain microglia in the patient by bone marrow derived myelo-monocytic cells from the donor that cross the blood–brain barrier and differentiate into microglia with normal ALDP function. HCT requires the availability of a HLA-matched donor who can be a nonaffected brother or sister, an unrelated donor or a cord-blood donor. In children the mortality risk of HCT with an unrelated or a cord-blood donor is approximately 15%. The mortality risk of HCT under the same conditions is 40% in adults. Although more than 200 X-ALD patients have now been transplanted successfully (52) and a 20-years follow-up of treated patients has confirmed the beneficial effect of allogeneic HCT in X-ALD definitively, the procedure remains associated with serious limitations in particular with respect to the availability of donor(s) and the mortality and morbidity risks of the procedure. Human stem cell (HSC) gene therapy will certainly be an alternative to allogeneic HCT in the near future. HSC gene therapy relies upon the reinfusion of the HSCs of the patient, which have been corrected ex vivo. Two X-ALD patients have recently been successfully treated by HSC gene therapy (53). The efficacy of the procedure in arresting the cerebral demyelination is identical to allogeneic HCT. In the study by Cartier et al. the HSCs were corrected ex vivo by a HIV-1 derived lentiviral vector expressing the *ABCD1* gene.

### 105.5.5 Acyl-CoA Oxidase Deficiency (MIM 264470)

Acyl-CoA oxidase (ACOX) deficiency first described in 1988 has so far been described in about 30 patients in literature. The clinical, biochemical and mutational spectrum of a cohort of 22 patients, affected by ACOX-deficiency has recently been published by us (54). Before the enzyme and molecular defect was identified, ACOX deficiency was referred to as pseudo-neonatal adrenoleukodystrophy since the clinical picture of the first patients described by Poll-Thé et al. (55) resembled that of patients affected by neonatal adrenoleukodystrophy as published by Kelley et al. (56) in 1986.

All patients identified so far had psychomotor retardation, but did acquire limited skills, including the ability to sit and stand up unsupported, voluntary control of hand function, and limited speech. In most patients (83%), however, there was loss of motor achievements with a mean age of regression of 28 months. Brain imaging (MRI and/or CT) revealed cerebral and/or cerebellar white matter abnormalities in all patients investigated (12/12). Three of these patients showed neocortical dysplasia. Other abnormalities include hypotonia (97%), seizures (91%), visual system failure (78%), impaired



hearing (77%), facial dysmorphism (50%), hepatomegaly (50%) and failure to thrive (37%).

Interestingly, two adult patients affected by ACOX1 deficiency have recently been described (57). Early developmental milestones were normal in these patients. At age 10, one patient was noted to have scoliosis and a clumsy-like hand. His gait showed progressive unsteadiness until the age of 28 when he became wheelchair bound. He had some urinary and fecal urgency. At the time of diagnosis (52 years of age), he had mildly impaired cognitive function. His memory was poor, but he was well oriented and could give an adequate description of his present problems. The visual acuity in the right eye was restricted to hand movements. He had small lens opacities. Retinitis pigmentosa was observed in both fundi. There was no optic disc pallor. Gaze evoked nystagmus on upgaze, downgaze and lateral gaze was present. He had a slurring dysarthric speech but near normal tongue movements. He had a jerky head tremor. There was some dystonic posturing of his arms and ataxia was seen on finger–nose testing. There was spastic paraparesis in the lower limbs. Sensation was normal with all modalities. Brain MRI showed profound atrophy of the brain stem and cerebellum, particularly evident in the pons, and modest cerebral atrophy. No other abnormality was seen. Nerve conduction studies were normal. Electroretinogram showed a virtually absent response from either eye. His sister was similarly affected at 55 years of age with onset of ataxia at 8 years.

**105.5.5.1 Biochemical and Molecular Basis of ACOX Deficiency.** Determination of the peroxisomal biomarker panel in plasma of ACOX-deficient patients has revealed that the abnormalities are restricted to the accumulation of VLCFAs that follow logically from the fact that the enzyme ACOX1 handles the CoA esters of VLCFAs but not of other peroxisomal substrates including pristanic acid and di- and trihydroxycholestanoic acid.

Elevated plasma VLCFA-levels have been found in all patients described in literature with proven ACOX1 deficiency except for one. Indeed, Rosewich et al. (58) described a case of ACOX1 deficiency with normal plasma VLCFAs. Based on the characteristic MRI findings a full study in fibroblasts was performed which revealed clear VLCFA abnormalities in fibroblasts and in addition a marked deficiency of the enzyme ACOX and clear-cut mutations in the ACOX1 gene. This case underlines once more that care should be taken in the interpretation of plasma metabolite results as detailed below.

The molecular basis of ACOX1 deficiency has been worked out by Ferdinandusse et al. (54), which revealed marked genetic heterogeneity with 20 different mutations. Two additional mutations have been published by Suzuki et al. (59).

No treatment options have been described for ACOX1 deficient patients.

## 105.5.6 DBP Deficiency (MIM 261515)

The first patient affected by DBP deficiency was described by Watkins et al. in 1989 (60). Interestingly, at that time it was believed that this patient had a deficiency of the other peroxisomal bifunctional protein, i.e. LBP. It took until 1999 before the true defect in this patient was identified at the level of DBP (61). The patient described by Watkins et al. (60) was born hypotonic and macrocephalic, had made no developmental progress at 6 weeks, continued to seize, remained hypotonic and macrocephalic, and was poorly responsive to stimuli. Fontanels were large and the metopic suture split. There was no cranial facial dysmorphism. Visual and brain stem auditory evoked responses were grossly abnormal. The skeletal survey and renal ultrasound were normal. A brain biopsy at 6 weeks revealed polymicrogyria. Post mortem examination revealed the cause of death to be bronchopneumonia and enteritis with patchy necrosis of the small bowel. Examination of the brain revealed polymicrogyria, demyelination of the white matter in the cerebrum, focal areas of cortical heterotopia, a few foamy macrophages in the perivascular spaces, and cystic degeneration of the periventricular zones. The cerebellum appeared normal. Since then many patients with DBP deficiency have been described. In 2006, Ferdinandusse et al. (62) reported on the clinical and biochemical spectrum of DBP deficiency in 126 patients. The clinical presentation of DBP deficiency is dominated by neonatal hypotonia (98%) and seizures (93%) within the first months of life. Nine patients (15%) were reported to have had infantile spasms at any time. Failure to thrive was observed in 43% of the patients. Visual system failure, including nystagmus, strabismus, or failure to fixate objects at 2 months was reported in 39 patients (54%). In addition, a progressive loss of vision and hearing was noted in 35% and 46% of the patients respectively. Almost none of the patients acquired any psychomotor developments and a few patients who did achieve some limited skills, showed progressive loss of motor achievements. The best achievements scored were head control when held at shoulders in nine patients (15%), sitting without support for more than 5 s in two patients (3%), voluntary reaching for objects and control of hand function in five (8%) and four (7%) patients respectively, and talking over to loose words with comprehension of their meaning in one patient only (2%). External dysmorphism was present in 52 patients (58%) and resembled that of patients with ZS. Frequently described were high forehead, high arched palate, and large fontanel, long philtrum, epicanthal folds, hypertelorism, macrocephaly, shallow super orbital ridges, retrognathia, and low-set ears. A hypoplastic/abnormally shaped thorax was reported in five patients and external genitalia abnormalities were reported in two

additional patients. Brain imaging (either MRI, CT, or ultrasound) showed growth dilatation of the ventricular system in 29% of the patients and neocortical dysplasia in 27%. Delayed maturation of the white matter before 1 year was reported in 16 of 47 patients and after 1 year in five of 14 patients. Demyelination of the cerebral hemispheres occurred in 17% of the patients whereas the occurrence of demyelination of the cerebellar hemispheres was 7%. Cerebellar atrophy was found in one patient and germinolytic cysts were found in 10 patients (20%). The following findings were described in 11 autopsy reports: generalized hyperplasia/atrophy (40%), (perisylvian) polymicrogyria (64%), atrophic/hypoplastic corpus callosum (55%), cerebellar hypoplasia (27%), heterotopic neurons in the white matter (35%) and gliosis (27%). DBP is subdivided into three different types depending on whether it is the complete DBP, which is missing (type I) or only the hydratase (type II) or 3-hydroxyacyl-CoA dehydrogenase component (type III), which is deficient. Kaplan–Meier survival analyses revealed that all type I deficient patients died within the first 14 months of life with 6.9 months as mean age of death. Four type II deficient patients survived beyond the age of 2 years. Two were still alive at the time of the study (62) including the oldest patient within the cohort (162 months). The mean age of death for type II deficiency was 10.7 months. The oldest type III deficient patient was 146 months at the time of the study and from this subgroup 8 patients survived beyond the age of 2 years. The mean age of death was 17.6 months. The immediate cause of death was pneumonia for almost all patients.

**105.5.6.1 Biochemistry and Molecular Basis of DBP Deficiency.** Since DBP plays a key role in the oxidation of multiple FAs including very long-chain fatty acids, pristanic acid, DHCA, and THCA, one would expect accumulation of all these metabolites in plasma from DBP-deficient patients except from pristanic acid and phytanic acid that are derived from exogenous dietary sources only. In the cohort of patients studied by Ferdinandusse et al. (62) VLCFAs were indeed abnormal in virtually all patients except three. In the latter patients phytanic and pristanic acid levels also were normal and the bile acid intermediates DHCA and THCA were undetectable in two of these patients. Phytanic and pristanic acid levels varied considerably in the cohort of DBP-deficient patients. Indeed, phytanic acid levels were increased in 60% of the patients and pristanic acid increased in 44%. In 12 patients no accumulation of DHCA and THCA was found. Detailed studies in fibroblasts from all these patients which includes enzyme activity measurements combined with immunoblot analysis revealed that 27% of the patients had DBP type I deficiency, 28% type II deficiency and 45% type III deficiency. Molecular analysis of the *HSD17B4* gene has revealed a large number of often-private mutations with

only one mutation being more frequent (c.46G → A); allele frequency: 24%). The latter mutation causes glycine to serine amino acid substitution (63), which effectively prevents binding of NAD<sup>+</sup> because of a steric clash (64).

No realistic therapeutic measures for DBP-deficient patients have been described.

### 105.5.7 Peroxisomal Sterol-Carrier Protein X Deficiency

This defect has only been described in a single patient so far (10). Although the name of the deficiency suggests otherwise, the primary abnormality in these patients is the inability to oxidize branched-chain fatty acids like pristanic acid and DHCA and THCA, which indeed accumulate in the patient's plasma. This is explained by the fact that the protein involved sterol-carrier protein X(SCPx) harbors two functional protein domains of which one has a high affinity for sterols whereas the other part actually has 3-ketothiolase activity with preference for the 3-ketoacyl-CoAs of branched-chain fatty acids (see Reference (9) for review).

The patient involved is a 45-years old white man with 28-years history of dystonic head tremor and spasmodic torticollis. He had noticed the start of symptoms for the first time at 7 years of age. At 17 years of age he developed spasmodic torticollis to the left side with dystonic head tremor in stressful situations. On fertility check-up at 29 years of age hypergonadotrophic hypogonadism and azoospermia were diagnosed. One of his two brothers was reported to have similar neurologic complains. Brain MRI showed bilateral hyper intense signals in the thalamus, butterfly-like lesions in the pons, and some lesion in the occipital region. Neurologic examination revealed hyposmia, pathologic saccadic eye movements, and a slight hypoacusis. Deep tendon reflexes were brisk in the arms, but diminished in the lower extremities. There were slight cerebellar signs with level-sided intention tremor and rebound phenomenon.

**105.5.7.1 Biochemistry and Molecular Basis of SCPx-Deficiency.** Analysis of the peroxisomal metabolites in plasma of the patient revealed no abnormalities except for trace amounts of the bile acid intermediates DHCA and THCA, which prompted additional analyses. Interestingly, marked abnormalities were found in the patient's urine, including large amounts of bile alcohols, probably produced via the alternative pathway for bile acid biosynthesis, i.e. the microsomal 25-hydroxylase pathway. The molecular basis of SCPx deficiency has also been determined (10).

### 105.5.8 2-Methyl-Acyl-CoA Racemase Deficiency (MIM 604489)

AMACR deficiency was first described in 2000 (11) in two patients whose clinical presentation was dominated

by the late-onset sensory motor neuropathy. Analysis of the peroxisomal metabolite profile in plasma revealed elevated levels of pristanic acid, DHCA and THCA and phytanic acid, but normal VLCFAs. This suggested a specific defect in the oxidation of branched-chain FAs with branched-chain acyl-CoA oxidase, SCPx, and AMACR as likely candidates (see Figure 105-2). Enzymatic and molecular studies revealed a deficient AMACR activity and mutations in the *AMACR* gene. Interestingly, Setchell et al. (65) described a completely different phenotype of SCPx deficiency dominated by severe liver abnormalities early in life. Recently, Kapina et al. (66) described yet another clinical presentation of SCPx deficiency, characterized by stroke-like episodes and recurrent rhabdomyolysis.

**105.5.8.1 Biochemistry and Molecular Basis of AMACR Deficiency.** The patients identified so far have shown clear abnormalities in plasma, characterized by elevated pristanic acid, DHCA and THCA, but normal VLCFAs. In line with the nature of the enzyme defect (see Figure 105-3) there is virtually exclusive accumulation of the (25R)-stereoisomers of DHCA and THCA, which discriminates AMACR deficiency from all other defects in which DHCA and THCA accumulate. Furthermore, there are clear bile acid abnormalities in urine from patients including the presence of many polyhydroxylated C27-bile acids like in ZSD patients.

## 105.5.9 BAAT Deficiency

Setchell et al. (67) were the first to identify defective bile acid conjugation in humans. This was based on the finding of absent glycine and taurine bile acid conjugates in all biological fluids characterized by the predominance of unconjugated cholic acid and lesser amounts of glucuronide and sulfate conjugates. The patient, an infant of Laotian ethnicity had conjugated hyperbilirubinaemia, mainly sulfate and glucuronide conjugates, fat-soluble vitamin malabsorption, early growth failure and a probably unrelated disorder, i.e. alpha thalassemia that required multiple blood transfusions. Two other patients, a 5-year-old Saudi Arabian boy and his 8-year-old sister, born from a consanguineous marriage, were also reported and had variable degrees of cholestasis. The boy had undergone a Kasai procedure in infancy for wrongly diagnosed biliary atresia whereas the girl was asymptomatic at the time of diagnosis. Liver function tests were either normal or mildly elevated at the time of diagnosis. In 2003, Carlton et al. (68) described the identification of patients from the Amish community with bonafide mutations in the *BAAT* gene. Patients were homozygous for a c.226A > G mutations (M76V) and showed increased serum bile acids which were virtually fully unconjugated. Clinical features of these patients included fat malabsorption, failure to thrive, coagulopathy, pruritus, and chronic upper respiratory infection. They did not have jaundice and had normal serum

gamma-GT concentrations. In 2007, Heubi et al. (69) reported six patients with mutations in the *BAAT* gene. The clinical phenotype included growth delay (3/6), neonatal cholestasis (3/6), including one with fulminant liver failure, and 5/6 with fat-soluble vitamin deficiencies, i.e. vitamin A, E or D. At diagnosis (5 months–19 years) all but one had normal bilirubin and normal or minimally elevated transaminases.

## 105.5.10 Primary Hyperoxaluria Type I (MIM 604285)

The primary hyperoxalurias (PH) are a group of rare inborn errors of glyoxylate metabolism that result in enhanced production of oxalate, mainly by the liver. Oxalate is an end product of metabolism that cannot be further degraded but can only be filtered at the glomerulus and excreted by renal tubules and to some extent the bowel. In case of a defect in glyoxylate breakdown, glyoxylate accumulates and is either oxidized to oxalate or reduced to glycolate. Glycolate is not toxic and can ratherly be excreted into the urine whereas oxalate due to its propensity to participate as calcium oxalate, is toxic to cells, especially in the kidneys which undergo progressive deterioration followed by urolithiasis, nephrocalcinosis, and end-stage renal disease (ESRD). The endogenous overproduction of oxalate with normal dietary oxalate absorption sets these Mendelian disorders apart from the secondary hyperoxalurias, which results from the increased oxalate absorption in the gut. At present, three distinct genetic defects have been identified which cause PH. These include: alanine glyoxylate aminotransferase deficiency (PH-I; gene: *AGXT*; MIM 602485), glyoxylate reductase/hydroxypyruvate reductase deficiency (PH-II; gene: *GRHPR*; MIM 604296) and 4-hydroxy-2-oxoglutarate aldolase deficiency (PH-III; gene: *HOGA1*; MIM 604296). Around 80% of PH patients suffer from PH-I, which is in fact the most severe PH type, often leading to ESRD. Built-up oxalate in the body may lead to oxalate deposition in multiple organs including bone, heart, skin, and retina. In case of a severe AGT deficiency death may occur. The phenotypic variability of PH-I is large, ranging from severe, early-onset oxalosis with early death to adult presentations that resembles idiopathic kidney stone disease. PH-I often goes undetected for years until severe, irreversible kidney damage has occurred. In general, PH-I especially in its severe form has a bad prognosis unless liver (plus kidney) transplantation is performed. PH-I should be considered in all cases of familial stone disease and renal failure of unknown cause.

**105.5.10.1 Biochemistry and Molecular Basis of AGT Deficiency.** AGT catalyzes the alanine-dependent transformation of glyoxylate into glycine with pyruvate as the other product of the AGT enzyme reaction, is localized exclusively in peroxisomes, and has pyridoxal-5-phosphate as cofactor. PH-I is caused by

either deficient or other mistargeted AGT. At present more than 150 different mutations in the *AGXT* gene have been identified.

**105.5.10.2 Laboratory Diagnosis.** Urinary oxalate analysis is the method of choice as initial method to check for PH. In PH-I patients overproduction of oxalate in the liver results in very high urinary oxalate excretion, typically amounting to  $>1 \text{ mmol}/1.73 \text{ m}^2/\text{day}$ . A raised urinary glycolate level is strongly suggestive for PH-I but glycolate may also be entirely normal in PH-I patients. Elevated L-glycerate on the other hand is characteristic for PH-II. Although measurement of AGT enzyme activity in a liver biopsy specimen has long been the golden standard for the diagnosis of PH-I, direct molecular analysis of the *AGXT* gene is rapidly becoming the first choice for diagnosis.

This is due to the fact that in a significant proportion of PH-I patients, AGT enzyme activity is not deficient, at least not in vitro in a liver homogenate prepared after biopsying the patient.

**105.5.10.3 Treatment.** Overall the management of PH-I greatly depends on the degree of renal function. Treatment is directed toward (1) decreasing oxalate production by inhibiting oxalate synthesis and (2) increasing oxalate solubility at a given urinary concentration of oxalate. Most of the efforts have concentrated on ways to increase the solubility of calcium oxalate. High-fluid intake and alkalinization of the urine remains the mainstay of this approach. Indeed, excessive volume is necessary to help excrete the enormous amounts of endogenously produced oxalate. Other helpful therapies may include the use of magnesium oxide (650–1300 mg/day). Furthermore, hemodialysis can remove large quantities of oxalate and its precursors. Attempts in trying to reduce the production of oxalate with succinimide, allopurinol, calcium carbimide, and isocarbazide have been unsuccessful.

Importantly, pyridoxine should be tried in every patient. One third of patients with PH-I respond to pharmacological doses of pyridoxine. Indeed, pyridoxine at the usual daily dose of  $1000 \text{ mg}/\text{m}^2$  body surface area can bring about a substantial reduction in the production and excretion of oxalate except in patients with a pyridoxine-resistant form of the disease. The efficacy of pyridoxine is probably directly related to the extent to which AGT is deficient. Indeed, if there is some residual enzyme activity high levels of pyridoxal-phosphate, which is the obligatory cofactor in the AGT enzyme reaction, may allow residual enzyme activity to operate optimally. In this way, flux through AGT may be considerably stimulated, leading to a reduced production of oxalate. Recent studies have shown a clear association between homozygosity for the two mistargeting mutations Gly170Arg and Phe152Ile, and pyridoxine responsiveness, the reason being that pyridoxine is able to interfere with the mistargeting of AGT to the mitochondrion thereby increasing the residual activity of AGT in peroxisomes from 5 to 10%. In our experience, all patients homozygous for the

Gly170Arg substitution, who had a preserved renal function at the time of diagnosis, were able to preserve renal function throughout the follow-up period when treated accordingly with pyridoxine, a high-fluid intake, and potassium citrate (70).

In cases in which AGT is fully deficient, pharmacological doses of pyridoxine show no effect. These patients will usually develop renal failure, requiring renal transplantation. The overall success rate of this treatment is however low, because the biochemical defect resides in the liver and not in the kidney. As a consequence, a renal transplantation gives only temporary relief, the new organ inevitably becoming obstructed by further deposition of calcium oxalate. Definite correction of the metabolic lesion requires liver transplantation. Mutation analysis may also be useful in taking decision whether to perform combined liver–kidney or isolated kidney transplantation in PH-I patients with ESRD. We believe that even in those PH-I patients, who already have developed ESRD, *AGXT*-mutation analysis can be very useful. It would seem advisable to perform only isolated kidney transplantation in patients homozygous for Gly170Arg or Phe152Ile. On the other hand, the adverse outcome of pyridoxine negative mutations would give a strong argument for combined liver–kidney transplantation or a timely planned liver transplantation in such patients.

## 105.6 RARE PEROXISOMAL DISORDERS

Recently, two new peroxisomal defects have been described both affecting the fission of peroxisomes. Defects affecting mitochondrial fission have already been described in literature. Indeed, mutations in the *OPA1* gene which codes for a mitochondrial dynamin-related guanosine triphosphatase have been associated with an autosomal dominant form of optic atrophy resulting in early-onset blindness (71,72). The first peroxisomal fission defect was described by Waterham et al. (73) and is an apparently autosomal dominant disease resulting from mutations in the *DLP1*-gene, which codes for dynamin-like-protein-1 (DLP1). The single patient identified with this defect so far, had microcephaly, abnormal brain gyration, optic atrophy, hypotonia, absence of any developmental milestones, and persistent lactic acidemia, which suggested a mitochondrial defect. However, plasma VLCFAs were also abnormal albeit mildly. Immunocytochemical studies in fibroblasts from the patient revealed clear abnormalities in the morphology of both mitochondria and peroxisomes, which ultimately led to the identification of DLP1 as the dysfunctional protein. The fact that DLP1 is known to be involved in both mitochondrial and peroxisomal fission explains the combination of mitochondrial (elevated lactate) and peroxisomal (elevated VLCFAs) abnormalities.

The other patient is a 25-year-old mentally retarded male who showed normal early development with walking at the age of 18 months, except from bilateral



congenital cataracts, which prompted cataract extraction at 4 months of age. At the age of 5 years a hearing problem was noted and this progressed to severe bilateral perceptive hearing loss. From the age of 10 years, he started to complain of painful muscles and showed a gradual loss of muscle strength. At the age of 12 years he was referred for extensive clinical and laboratory evaluation. Main features on neurological examination were nystagmus with a rotary component, normal muscle strength, low reflexes and normal sensibility in the arms, but low muscle strength, absent reflexes and disturbed sensibility in the legs. From the age of 15 years he had regular attacks of migraines and convulsions, was encephalopathic, complained of nausea and headache, had trouble with his vision and had a numb-feeling in his mouth and hands. These attacks often followed upon physical or mental exertion. The fact that VLCFAs had been abnormal although only once, prompted a detailed study in fibroblasts, which revealed clear abnormalities, showing elongated peroxisomes arranged in rows, indicative of a defect in peroxisome division. Subsequent studies ultimately led to the discovery of *PEX11 beta* as the mutated gene. *PEX11 beta* is known to be involved in peroxisome fission. Molecular analysis revealed homozygosity for a nonsense mutation (c.64C → T (p.Q22X)) in the *PEX11 beta* gene (74).

The identification of these two patients with atypical phenotypes resulting from defects in peroxisome fission may point to the existence of a new class of peroxisomal patients yet to be identified in detail.

In summary, much has been learned about the group of PDs in recent years. This includes the development of sensitive laboratory methods allowing the measurement of the panel of peroxisomal metabolites and follow-up enzymatic methods to pinpoint the underlying defect. Furthermore, the molecular basis of most—if not all—of the peroxisomal diseases identified so far has been resolved with obvious consequences for diagnosis, including prenatal diagnosis. The availability of these sophisticated and sensitive methods together with the rapid development of genetic techniques including whole exome and genome sequencing, has allowed the identification of atypical phenotypes including isolated cerebellar ataxia, a.o., which holds great promises for the future in terms of patient recognition. This is especially important since therapeutic measures will surely become available in the next years to come, surely for the milder variants.

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### Biography



**Ronald J A Wanders** studied chemistry (subspecialization: Clinical chemistry and Biochemistry) and performed his PhD in the section Medical Enzymology and Metabolism, E C Slater Institute for Biochemical Research (supervisor: Prof Joseph Tager) and studied the flux control of metabolic pathways with emphasis on mitochondrial oxidation phosphorylation, ammonia genesis and ammonia utilization (urea cycle). In 1983, he joined the Laboratory Genetic Metabolic Diseases, Departments of Paediatrics and Clinical Chemistry, Medical Faculty, University of Amsterdam as postdoctorate with the specific task to (1) setup a laboratory for the enzymatic diagnosis of patients suspected to suffer from certain inborn errors of metabolism (IEM) and (2) start a research project on peroxisomes aimed to develop post- and prenatal diagnostic laboratory methods for ZS and identify new PDs. He was appointed as a professor in 1996, and Head of the laboratory in 2003, which is a world-renowned expert center for the laboratory diagnosis of PDs and other IEMs. The author has also set up diagnostic and research activities in the field of mitochondrial FA oxidation, cholesterol biosynthesis, cardiolipin metabolism, amino acid degradation, a.o. The author has published >965 peer-reviewed papers, including book chapters and has received multiple awards including the International Federation of Clinical Chemistry (IFCC) Distinguished Award for Laboratory Medicine and Patient Care 2011.



# CHAPTER

# 106

## The Genetics of Personality

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### 106.1 THE NATURE OF PERSONALITY

One of the most salient features of human behavior is its diversity. Some individuals are shy and reserved while others are outgoing and loquacious. Some relish the adrenaline rush that accompanies jumping out of airplanes while others are content to experience their thrills vicariously by watching reality TV. Personality is one of psychology's core constructs, posited to account for both the marked differences in behavior that exist among individuals as well as the coherence of any given individual's behavior over time and context. In short, personality psychologists believe that personality is one of the reasons we behave consistently differently from one another.

Although there are many alternative theoretical conceptualizations of personality (1), most of the empirical, and indeed virtually all of the genetic, work in this area has been based on a trait conceptualization of personality. The existence of literally thousands of trait descriptors in the English language (happy, sullen, vibrant, impulsive, withdrawn, aggressive) appears to make trait approaches unwieldy, even if it is an indication that the general public has found trait terms to be a useful way to describe human behavior (2). There is, however, a growing consensus among personality psychologists that a large portion, although certainly not all, of the variation in human behavior reflected by these trait terms can be captured by a relatively small number of core dimensions. Although there remains debate over the exact number of core personality dimensions (e.g. 3, 5, 7 or 9), currently a model that posits five is in ascendance. The Five Factor Model (FFM) of personality emphasizes five major dimensions of personality that appear to be cross-cultural universals (3): extraversion (the tendency to be positively and actively engaged in one's social and work environments), neuroticism (the tendency to experience negative moods states and psychological distress), agreeableness (the tendency to be friendly, warm and cooperative),

conscientiousness (the tendency to be planful and conform to social norms), and openness (the tendency to welcome diverse intellectual, cultural, and artistic experience) (Table 106-1). Importantly, the ability to reliably and validly assess these dimensions through self-report questionnaire has made large-scale behavioral genetic studies of personality feasible.

Much of the latter half of the twentieth century saw a decline in personality research within psychology, largely over concern personality had limited utility for helping to explain differences in our behavior. Within the past 20 years, however, there has been a surge of research implicating personality in a broad array of important life outcomes. Differences in personality predict differences in physical health (4) and how long we live (5). It is an important predictor of academic and social achievement (6) as well as performance on the job (7). It helps explain differences in the quality of our relationships with romantic partners, relatives and friends (8) and even the likelihood we get married (9) or divorced (10). Indeed, personality is as strong a predictor of important life outcomes as other major behavioral or social predictors including cognitive ability and social class (11).

Nothing has done more to revive interest in personality within psychology and psychiatry than the demonstration of its link to mental health. Personality factors are associated with risk of mental disorder ranging from depression and anxiety (12) to attention deficit hyperactivity disorder (ADHD) (13) and substance abuse (14). Significantly, these associations appear to be established early in life as personality ratings of toddlers have been shown to be predictive of mental health problems in adulthood (15). Moreover, personality may be an essential component, rather than merely a predictor, of those mental health problems that represent the extreme of quantitative variation rather than a discrete clinical entity. For example, depression has been hypothesized to be the consequence of high levels of neuroticism coupled with low levels of

extraversion (16), while many acknowledge that ADHD likely represents the extreme of a continuum of impulsivity, inattention, and overactivity (17). Resolving the genetic basis of personality will be key to resolving the genetic basis of some mental disorders.

## 106.2 TWIN AND ADOPTION STUDIES

### 106.2.1 Twin Studies

The major evidence in support of a heritable basis to personality comes from the comparison of the similarity of reared-together monozygotic (MZ) and dizygotic (DZ) twins. Analysis of twin data is based on a biometric model first introduced by the population geneticist Falconer (18). If we assume that: (1) MZ twins are no more likely than DZ twins to share trait-relevant environmental influences, (2) there is no assortative mating for personality, and (3) all relevant genetic effects are additive, then the expected correlation for reared-together MZ ( $r_{\text{MZT}}$ ) and DZ ( $r_{\text{DZT}}$ ) twins along with the total trait variance (normed to 1.0) can be expressed as:

$$\begin{aligned} r_{\text{MZT}} &= a^2 + c^2 \\ r_{\text{DZT}} &= 1/2a^2 + c^2 \\ 1.0 &= a^2 + c^2 + e^2 \end{aligned}$$

where  $a^2$  is the proportion of trait variance associated with additive genetic factors (i.e. the additive heritability),  $c^2$  is the proportion associated with shared environmental factors (i.e. those environmental factors that are shared by reared-together relatives and thus contribute to their phenotypic similarity), and  $e^2$  is the proportion associated with non-shared environmental factors (i.e. those environmental factors that are not shared by reared-together relatives and thus contribute to their phenotypic dissimilarity). Simple algebraic manipulation of these equations produces the familiar Falconer estimators of the three variance components (variously called the Falconer or ACE model):

$$a^2 = 2(r_{\text{MZT}} - r_{\text{DZT}})$$

$$c^2 = 2r_{\text{DZT}} - r_{\text{MZT}}$$

$$e^2 = 1.0 - r_{\text{MZT}}$$

Table 106-2 summarizes a meta-analysis of twin studies of the Big Five personality factors published by Bouchard (19). Three general observations, all of which have been confirmed in virtually every large-scale study of reared-together twins, can be drawn from the data presented in the table. First, additive genetic factors are estimated to account for roughly 40–60% of the variance in personality. Second, shared environmental influences on personality appear to be negligible. Finally, non-shared environmental effects are substantial and account for approximately 50% of the variance in personality. Importantly, research subsequent to Bouchard's meta-analysis confirms the robust nature of his findings across country (20), assessment modality (20b,21), and age (22).

There is thus little doubt that reared-together MZ twins are more similar in personality than reared-together DZ twins. But, does this consistent observation necessarily implicate the existence of genetic influences on individual differences in personality? The validity of the MZ–DZ comparison rests on two fundamental assumptions: (1) that the personalities of twins do not differ from those of non-twins and (2) that MZ twins are no more likely than DZ twins to share trait-relevant environmental influences. While the psychological uniqueness of being a twin has been a subject of much speculation, the empirical evidence on this issue is overwhelmingly consistent.

**TABLE 106-1 The Big Five Model of Personality**

Personality Characteristics	Characteristics of	
	High Scores	Low Scores
Extraversion	Assertive, energetic, sociable	Quiet, shy, withdrawn
Neuroticism	Anxious, emotional, moody	Calm, at ease, unemotional
Conscientiousness	Dependable, organized, practical	Careless, irresponsible, frivolous
Agreeableness	Cooperative, generous, warm	Cold, quarrelsome, selfish
Openness	Curious, imaginative, insightful	Shallow and simple

**TABLE 106-2 Mean Twin Correlations and Falconer Estimates from a Meta-Analysis of Twin Studies of the Big Five Personality Traits**

Personality Dimension	No. of Studies	$r_{\text{MZT}}$	$r_{\text{DZT}}$	Falconer Estimates		
				$a^2$	$c^2$	$e^2$
Extraversion	30	0.52	0.25	0.54	−0.02	0.48
Neuroticism	23	0.51	0.22	0.58	−0.07	0.49
Conscientiousness	12	0.44	0.24	0.40	0.04	0.60
Agreeableness	6	0.49	0.23	0.52	−0.03	0.51
Openness	7	0.43	0.17	0.52	−0.09	0.57

Note:  $r_{\text{MZT}}$  is mean correlation for reared-together MZ twins,  $r_{\text{DZT}}$  is mean for reared-together DZ twins,  $a^2$  is proportion of variance attributable to additive genetic effects,  $c^2$  is the proportion of variance attributable to shared environmental effects, and  $e^2$  is the proportion of variance attributable to nonshared environmental effects. Table is adopted from material given in Bouchard (19).

There is no evidence that the personalities of twins differ systematically from that of singletons (23), supporting the generalizability of findings from twin studies to the larger non-twin population. The second, or so-called equal environmental similarity, assumption is the basis for the inference that if MZ twins are phenotypically more similar than DZ twins it is because of their greater genetic, and not environmental, similarity. This latter assumption has received substantial attention in the empirical literature, with the consistent finding that greater MZ than DZ similarity in personality cannot be attributed to differential treatment (e.g. by parents) or exposure (e.g. rearing neighborhood) (24).

The strongest evidence against the hypothesis that differential environmental similarity can account for reared-together MZ–DZ differences in personality similarity is studies on reared-apart twins. Although few in number, studies of reared-apart twins support the three general findings from studies of reared-together twins. Table 106-3 provides a summary of the four studies that have compared the similarity of reared-together and reared-apart MZ twins on self-report measures of extraversion and neuroticism (25). MZ twins who are reared apart nonetheless show substantial similarity in personality, a finding that underscores the importance of genetic factors given that these correlations cannot be accounted for by placement effects (26). Except for the Swedish study, reared-apart MZ twins are about as similar in personality as reared-together MZ twins, further suggesting that common rearing does not have a profound impact on personality similarity. Finally, the consistent observation of MZ correlations that are substantially less than unity indicates that non-shared environmental factors are a major source of individual differences in personality.

### 106.2.2 Adoption Studies, Family Studies and Nonadditive Genetic Influences

Adoption studies have consistently found little or no personality similarity among adoptive relatives, confirming that growing up in the same home has little impact on personality similarity. Using data reported in Loehlin (27), we computed the weighted-average adoptive sibling (i.e. nonbiologically related siblings who are reared together) correlation for the five basic dimensions of personality. These average correlations are 0.07 for

measures of extraversion (three studies, total  $N$  of 258), 0.11 for measures of neuroticism (three studies, total  $N$  of 258), 0.06 for measures of agreeableness (two studies, total  $N$  of 250), 0.02 for measures of conscientiousness (two studies, total  $N$  of 245), and 0.06 for measures of openness (two studies, total  $N$  of 241). Similarly, there is little personality resemblance between adoptive parents and the nongenetically related offspring they rear.

It is informative to compare the personality resemblance of adoptive relatives not with that for twins but rather with that for first-degree biological relatives. In a longitudinal study of over 200 adoptive and 200 non-adoptive families, for example, Plomin et al. (28) reported an average, across four personality measures obtained at five different ages, adoptive sibling correlation of only 0.01. The comparable correlation in a separate sample of biological siblings was only 0.05. Moreover, the average adoptive parent-adopted child personality correlation of 0.03 actually exceeded, albeit by a trivial amount, the average biological parent-adopted child personality correlation of 0.01 in this study. Thus, first-degree biological relatives appear to show little more similarity in personality than adoptive relatives.

The consistent observation of low sibling and parent-offspring personality resemblance necessarily implies that growing up in the same home has little impact on measured personality; it suggests that the genetic factors shared by parents with their children or among siblings may also have little influence on personality similarity. This finding appears to be at odds with the consistent observation of strong heritable effects in twin studies of personality. That is, an estimated narrow-sense heritability of 50% from twin studies would imply a correlation of 0.25 among first-degree biological relatives. In fact, as suggested by the Plomin study, personality correlations among first-degree biological relatives are characteristically much lower than 0.25. For example, in a large twin family study of neuroticism, Finkel and McGue (29) reported biological sibling correlations of 0.07–0.14 and parent-offspring correlations of 0.12–0.19, less than half of the corresponding MZ correlations of 0.42–0.49. Furthermore, in a study of neuroticism that included over 45,000 individuals (30), the average biological same-sex sibling correlation of 0.14 ( $N = 10,400$ ) was less than half the corresponding same-sex MZ twin correlation of 0.40 ( $N = 4628$  pairs). Family studies thus suggest

**TABLE 106-3** Reared-Together (MZT) and Reared-Apart (MZA) Monozygotic Twin Correlations for Extraversion and Neuroticism

Study	Country	Extraversion		Neuroticism	
		$r_{\text{MZT}}$ ( $N$ )	$r_{\text{MZA}}$ ( $N$ )	$r_{\text{MZT}}$ ( $N$ )	$r_{\text{MZA}}$ ( $N$ )
Shields (25c)	Great Britain	0.42 (43)	0.61 (42)	0.38 (43)	0.53 (42)
Langinvainio (25a)	Finland	0.33 (47)	0.38 (30)	0.32 (47)	0.25 (30)
Pedersen (25b)	Sweden	0.54 (150)	0.30 (95)	0.41 (151)	0.25 (95)
Tellegen (25d)	USA	0.63 (217)	0.34 (44)	0.54 (217)	0.61 (44)

a narrow-sense heritability of personality on the order of 20–30% (i.e. twice the first-degree relative correlation) rather than the 40–60% typically reported in twin studies.

Although there are several possible explanations for the discrepancy in heritability estimates from studies of twins and nuclear families, including biases in the twin method or age moderation of personality similarity (i.e. twins share an age while other biological relative pairings do not), the explanation that has the greatest empirical support is the existence of nonadditive genetic effects. Specifically, because MZ twins share all their genetic material, interactions between alleles at a single locus (genetic dominance) or among alleles at multiple loci (epistasis) will contribute to their phenotypic similarity. Because first-degree relatives, however, are not likely to share all the components in an interactive genetic system identical by descent, nonadditive genetic effects likely contribute minimally to their phenotypic similarity.

Combined biometric analysis of twin and non-twin family data support this conclusion. Finkel and McGue (29) reported that nonadditive genetic effects accounted for from 7% to 24% of the variance in measures of extraversion, from 3% to 36% of the variance in measures of neuroticism, and from 13% to 45% of the variance in measures of conscientiousness. Interactive genetic effects were also estimated to account for from 10% to 13% of the variance in neuroticism in Lake et al.'s (30) massive twin family study, from 5% to 45% of the variance in various personality measures in a unique study of biological and adoptive families (31), and for 12% of the variance in impulsivity based on a recent meta-analysis of twin, family and adoption studies (32). The existence of nonadditive genetic effects has implications far beyond accounting for discrepancies in heritability estimates. Most investigations aimed at identifying the specific genes affecting personality are based on a single-locus paradigm. That is, the effect of each genetic locus is assessed singly, independent of genetic variation at other loci. If there are significant nonadditive genetic effects, then the effect of one locus will depend on genotype status at one or more other loci. Researchers may risk failing to identify important genetic effects on personality unless they take a multi-locus approach.

### 106.2.3 Twin and Adoption Studies of Personality and the Nature of Environmental Influence

One of the most remarkable findings to emerge from twin and family studies of personality concerns the nature of environmental rather than genetic influence. Both studies of personality similarity among adoptive relatives and the comparison of personality similarity among reared-together versus reared-apart twins suggest that growing up in the same home has very little effect on personality. These findings stand in stark contrast

to models of personality development that dominated within psychology throughout most of the twentieth century and that emphasized the primary role of parents in shaping their children's personalities (33). But while behavioral genetic research has led to a re-evaluation of the nature of environmental influence, it has not led to a rejection of the existence of those influences. The personalities of MZ twins correlate only about 0.50, implying that fully half of the variance in personality can be attributed to environmental factors, albeit environmental factors that create differences rather than similarities among reared-together relatives. The unexpected nature of environmental influence implied by behavioral genetic research has resulted in a large research effort aimed at identifying specific non-shared environmental influences on personality (34) and a debate over whether these influences owe to idiosyncratic stochastic processes (35), epigenetic phenomenon (36), or systematic psychological processes (37). It remains to be seen whether this new perspective will finally allow researchers to determine the circumstances by which some people end up gregarious, adventurous, or compliant.

### 106.2.4 Summary of Twin and Adoption Studies of Personality

Twin and adoption studies have consistently implicated the importance of both genetic and environmental factors in the etiology of individual differences in normal-range variation in personality. Heritability estimates from studies of reared-together twins suggest that approximately 50% of the variance in personality is associated with genetic factors, with some significant portion of these heritable effects likely to be due to non-additive gene action. The heritable basis of personality provides justification for molecular genetic studies aimed at identifying the specific genes influencing personality. However, the complexity of the personality phenotype suggests that multiple genes are involved, and they will be difficult to identify if they interact in their effect on personality as is suggested by twin and adoption studies. Behavioral genetic research also suggests that the major source of environmental influence on personality may be due to factors that create differences rather than similarities among reared-together relatives. What these non-shared environmental factors are and how they combine with genetic factors to influence personality remain to be determined.

## 106.3 THE SEARCH FOR GENES THAT INFLUENCE PERSONALITY

### 106.3.1 Candidate Gene Approaches

Throughout the last decade of the twentieth and the early part of the twenty-first century, research aimed at identifying the specific genetic factors that underlie



the heritability of personality was dominated by the candidate gene approach. Several factors contributed to this focus. First, the biological basis of differences in personality had been hypothesized to be due to differences in brain neurotransmission systems, with different systems thought to underlie the different dimensions of personality. So, for example, the neurotransmitter dopamine had been implicated in brain reward and approach systems (38), making polymorphisms in the dopaminergic system a convenient target for study of personality traits such as impulsivity and sensation seeking. Alternatively, the serotonin system is known to be involved in emotion regulation (39) and pharmacologic interventions for depression and anxiety target the reuptake of serotonin into the presynaptic neuron by the serotonin transporter (5-HTT). Consequently, genetic variation in the serotonin system has been the target of candidate gene studies of neuroticism and anxiety-related personality characteristics. Second, relatively early on geneticists had identified markers, many of which were functional or in coding regions, for several key genes in the relevant neurotransmitter systems. Thus, a variable number of tandem repeat (VNTR) marker in the third exon of the gene that codes for the dopamine D4 receptor (*DRD4*) (40) has been the focus of a large number of behavioral studies as has a functional insertion/deletion polymorphism in the promoter region of the gene that codes for 5-HTT (gene label is *SLC6A4*, polymorphism is 5-HTTLPR) (41).

The year 1996 was a watershed for research on the genetics of personality. Over the span of just a few months, two of the most prestigious scientific journals in the world, *Nature Genetics* and *Science*, published separate research studies that each purported to have found a major gene variant associated with normal-range differences in personality. The January issue of *Nature Genetics* provided two independent reports of an association between the *DRD4* VNTR polymorphism and personality measures of novelty seeking (42), providing what appeared to be compelling confirmation of an influential hypothesis advanced by Cloninger et al. (43) that genetic variation in the dopamine system would be relevant to novelty seeking. In November of that same year, *Science* featured a report by Lesch et al. (44) associating a polymorphism in *SLC6A4* with neuroticism in two

independent samples. Particularly impressive was the amount of variance accounted for by these variants. The *DRD4* variant was estimated to account for 5% of the phenotypic and 10% of the genetic variance in novelty seeking; the 5-HTT polymorphism accounted for 3–4% of the phenotypic and 7–9% of the genetic variation in neuroticism. With specific genetic effects of this magnitude, there was considerable optimism that the genetic architecture of key personality dimensions would soon be resolved (45).

These initial publications were followed by a perplexing pattern of successful and failed attempts at replication. The failure to observe consistent replication was not, however, specific to personality. Failure to replicate initial genetic association results was characteristic of candidate gene association studies with a broad range of behavioral and nonbehavioral phenotypes (46). The factors that contributed to this poor record of replication are now fairly well understood. Initial candidate gene studies was substantially underpowered, designed to detect effects that were an order of magnitude larger than the magnitude we now know to be typical of the effects of specific genetic variants on complex phenotypes. An underpowered study is more likely to produce a false-positive finding with an overestimated effect size than a true-positive finding, a phenomenon geneticists have called the “winner’s curse” (47). The problem of candidate gene studies was further exacerbated by their focus on single genetic variants in each of a small number of relevant genes (i.e. the “usual suspects”), whose selection was driven as much by what polymorphisms could be genotyped as by strong theory as to which genetic systems were relevant.

In an attempt to circumvent the problems inherent to small sample studies, geneticists have increasingly turned to meta-analysis (48). Table 106-4 provides a summary of major meta-analyses of candidate gene studies of personality-related phenotypes published since 2008. Several features of the table warrant comment. First, several of the genetic variants that have been the focus of considerable personality research do not show significant meta-analytic effects. This is most notable for the *DRD4* VNTR polymorphism, which has been extensively investigated but for which there is little

**TABLE 106-4 Findings from Major Meta-analyses of Candidate Gene Studies of Personality-Related Phenotypes Published Since 2008**

Study	Gene	Polymorphism	No. of Samples	Personality Characteristics	<i>d</i> ( <i>p</i> -value)
Munafo (82)	<i>DRD4</i>	Exon 3 VNTR	36	Approach-related scales	0.04 ( <i>p</i> = 0.14)
		C521T	11	Approach-related scales	0.25 ( <i>p</i> < 0.001)
Munafo (83)	<i>SLC6A4</i>	5-HTTLPR	28	Harm avoidance	0.02 ( <i>p</i> = 0.37)
			28	Neuroticism	0.07 ( <i>p</i> = 0.001)
Munafo (60)	<i>SLC6A4</i>	5-HTTLPR	14	Amygdala activation	0.63 ( <i>p</i> < 0.001)
Frustaci (84)	<i>BDNF</i>	Val66Met	4	Neuroticism	0.24 ( <i>p</i> < 0.05)
			5	Harm avoidance	0.11 ( <i>p</i> > 0.05)

*d* gives the meta-analyzed standardized mean personality difference between the genotype.

evidence of an association with approach-related traits such as impulsivity and sensation seeking. Second, even in cases where the pooled effect is significant, the magnitude of the effect is modest. This is most notable for the 5-HTTLPR polymorphism, which is significantly associated with neuroticism but with a pooled effect size of less than 0.1 standard deviations. Finally, for amygdala activation in response to the presentation of emotional stimuli the effect size is sizable ( $d=0.63$ ), suggesting that nearly 10% of the variation in this phenotype is accounted for by the polymorphism. The phenotype in this case is, however, a measure of brain function underlying personality rather than a self-report measure of personality.

### 106.3.2 Genome-Wide Strategies

The limited success of candidate gene studies of personality has motivated alternative approaches that do not depend on strong hypotheses about the probable locus of genetic effects. There have been eight published genome-wide linkage studies of personality-related traits (49). Most of these studies involve neuroticism because it is the personality factor most strongly implicated as a risk factor for mental health problems. While these studies have reported suggestive linkage findings for several chromosomal regions, none of these regions has received consistent support and no confirmed associations between variants in these regions and personality have emerged as a result of the linkage findings. As is well known, one of the limitations of the linkage approach is that it is underpowered to detect variants other than those that have relatively large phenotypic effects (50). The failure of linkage to identify genetic variants associated with personality is likely a consequence of the small effects associated with these variants.

The recent development of efficient methods for high-throughput genotyping has made feasible what geneticists had only speculated about previously—the ability to comprehensively evaluate the association of complex phenotypes with common genetic variation throughout the whole genome. A genome-wide association study (GWAS) involves genotyping large numbers of individuals on several hundreds of thousands or even millions of genetic markers and then determining whether variation in each of these markers is associated with the target phenotype. Each marker is a known and common (greater than 5% of a population has the rarer allele) single-nucleotide polymorphism (SNP or single base pair change) selected so that in aggregate the entire genome is densely covered (a GWAS typically includes at least 500,000 SNPs). Association is estimated with a linear regression of the phenotype on each SNP (along with various covariates), with the  $p$ -value threshold set to  $p < 5 \times 10^{-8}$  to account for the multiple testing burden. Because of the density of coverage across the genome, this method can find important variants with much

smaller effects than those that can be detected with linkage analysis. Due to linkage disequilibrium (the probability that two SNPs that are located physically near on another will be inherited together), the interpretation of a significant association is not that the marker itself is causal but rather that it is close to the causal variant.

Only recently have personality researchers turned to GWAS. The first personality GWAS examined only neuroticism (51) but there soon followed a study of all Big Five traits (52) as well as a study of temperament (53). The most highly associated SNPs in these studies failed to meet the stringent significance threshold of  $p < 5 \times 10^{-8}$ , but several were marginally significant. Of these nominally significant SNPs, several showed some evidence of replication in the replication samples included in the original GWAS. Furthermore, many reside in or near genes involved somehow in the central nervous system and may be of interest in further studies. For example, a SNP in the gene *SNP25* on chromosome 20, associated with neuroticism traits (52), is known to be important in neurotransmitter release, axon growth, and synaptic plasticity. It is important to note, however, that over half of all genes are expressed in the brain, so these genes may still be dead ends.

Findings with other phenotypes, including those that are non-behavioral as well as behavioral, suggest the reason why GWAS have failed to provide conclusive evidence of SNP associations with personality. Specifically the magnitude of phenotypic effect associated with any specific genetic variant is likely to be small, accounting for less, and likely much less, than 0.5% of phenotypic variance. Because the initial round of personality GWAS had sample sizes around a few thousand individuals, however, they were substantially underpowered to detect associations of this magnitude. For example, a sample of 4000 unrelated individuals would have power of less than 20% to detect effects as large as 0.5% of variance. To remedy this problem, a meta-analysis of GWAS personality data from several research groups was recently carried out (54). After combining several datasets from Caucasian populations worldwide, researchers ended up with a sample of over 17,000 individuals. With this number of participants, power exceeds 80% to detect effect sizes of 0.25% or more. This meta-analysis provided evidence for genetic loci associated with two personality dimensions: a region near the gene *RASA1* with openness to experience and the *KATNAL2* gene and conscientiousness. Each locus explained about 0.25% of variance in the associated trait. *RASA1* produces a protein involved in intracellular signaling and cellular proliferation and differentiation and is expressed moderately in the brain. *KATNAL2* produces a protein that may be involved in neuronal migration, axonal growth, and dendritic pruning.

Although these findings are exciting, we cannot yet have confidence that they are true associations because neither finding remained significant in the analysis of the replication sample. Again, researchers are left to wonder if effect sizes are even smaller than a quarter of a percent,

requiring even larger sample sizes to detect them, or if rare SNPs or other kinds of genetic variation (e.g. large deletions) are responsible for personality variation. If the latter is true, genome sequencing may be a promising approach to finding important variants. Alternatively, it may be, as twin and adoption research suggests, that genetic effects on personality are nonadditive, in which case the standard one SNP at a time approach to analyzing GWAS data is known to be inefficient.

## 106.4 STRATEGIES TO INCREASE THE SENSITIVITY OF GENETIC STUDIES OF PERSONALITY

### 106.4.1 Genetic Scores

Given the small effect associated with any specific individual genetic variant, one strategy to increase the sensitivity of genetic analysis involves creating genetic scores that capture the aggregate effect of multiple variants. For example, in a sample of approximately 4000 adults, McCrae et al. (55) created aggregate genetic scores for each of the Big Five factors by summing SNPs that were associated with each factor at different  $p$ -value thresholds. The cross-validated correlations of these scores in an independent replication sample ranged from a nonsignificant 0.009 for extraversion to a significant 0.125 for openness. Although the cross-validated correlations are modest, these results suggest that there are individual signals being genotyped that should, in principle, be detectable in sufficiently large samples. Similarly, Chen et al. (56) showed that an aggregate score reflecting genetic variation in the dopamine system was significantly associated with a measure of highly sensitive personality. The development and validation of genetic scores hold great potential for better understanding the molecular basis of personality.

### 106.4.2 The Endophenotype Approach

Personality characteristics are clearly far removed from the primary function of DNA, so that any genetic effects on personality must be mediated by the multiple biological, physiological, and neurological systems that are intermediate between primary gene product and observed behavior. Gottesman and Gould (57) have argued that resolving the genetics of complex phenotypes such as personality may best be achieved indirectly, by first resolving the genetics of relevant intermediate or endophenotypes. For example, it may be conjectured that the effect of specific genetic variants may be stronger, and thus easier to identify, for endophenotypes than for observed behavior because the former are more proximal to the primary gene product than the latter. Consistent with this hypothesis, Ducci and Goldman (58) have shown that the magnitude of the effects of candidate polymorphisms on various endophenotypes is substantially greater than for phenotypic disease.

Although only at the initial stages of inquiry, promising findings are already emerging from endophenotype research on personality. Developments in imaging technology have allowed neuroscientists to learn much about the neurological basis of individual differences in personality (59), providing a rich source of potential endophenotypes. As noted above, genetic variation in 5-HTT appears to be relatively strongly associated with the processing of emotionally laden stimuli in the amygdala (60), while a recent meta-analysis reported a strong effect of a functional genetic variant in the gene coding for the enzyme catechol-O-methyltransferase (COMT, involved in the degradation of catecholamines) on activation of the prefrontal cortex (61). Similarly, functional polymorphisms in other dopamine or serotonin system genes have been associated with measures of brain function that have been implicated in trait anxiety and impulsivity (59b). The combination of genetic and neuroimaging approaches holds great potential for unraveling the genetics of complex behavioral systems like personality (62).

### 106.4.3 Animal Models

As any pet owner will know, animals, like people, have personalities. Indeed, there is considerable evidence that the five factors of personality seen in humans also emerge in behavioral ratings of individuals representing a wide range of different species (63). As with human personality, much of the individual differences in animal personality factors appears to be moderately to strongly heritable (64), suggesting that genetic variants affecting personality in humans might effectively be identified using animal models. Consistent with this proposition, genetic variation in DRD4, which we previously showed was related to approach-related personality factors in humans, has been associated with exploratory behavior in an avian species (65).

One of the most promising animal models for a complex behavioral system is the open-field model of emotionality in the mouse. When placed in a novel, brightly illuminated open field, mice display various levels of fear, which can be objectively quantified in terms of motor activity, wall-seeking behavior, and defecation. These behavioral indicators have been pharmacologically validated as measures of anxiety and are substantially heritable and evidence the nonadditive genetic effects characteristic of human personality (66). Flint et al. (67) reported a quantitative trait locus (QTL) analysis of emotionality in the F2 intercross progeny from parental lines selected for high or low open-field activity. Three QTL (murine chromosomes 1, 12 and 15) gave consistent evidence of linkage across four separate measures of emotionality. Each QTL accounted for from 3% to 9% of the trait variance. In a study of open-field behavior in the F2 progeny of A/J and C57BL/6J mice, Gershenfeld et al. (68) independently replicated the finding of QTL for emotionality on chromosomes 1 and 15.

Moreover, genetic analyses of intercrosses from C3H/HeJ by C57BL/6J (69) and C57BL/6J by DBA/2J (70) revealed a QTL on chromosome 1 that influenced fear conditioning in the mouse.

Taken in aggregate, the strongest evidence for a QTL affecting murine emotionality is on chromosome 1, although additional regions have emerged in subsequent research (71). High resolution mapping of the relevant region of chromosome 1 suggests, however, that there are likely multiple loci in this region affecting different aspects of open-field emotionality (72). Importantly, these regions are syntenic to regions on human chromosome 1q that have been implicated in linkage studies of neuroticism (73). Although no known candidate genes have as yet been identified in the replicated QTL regions, these findings clearly provide encouraging support for the existence of genes affecting emotionality in man and mouse.

### 106.5 GENE-ENVIRONMENT INTERPLAY AND THE DEVELOPMENT OF PERSONALITY

In evaluating genetic research on human personality, it is essential to recognize the fundamental differences that exist between human behavior and other heritable human phenotypes. First, environments are not distributed at random but rather are a reflection in part of an individual's own behavior. An easygoing, affable individual will experience a much different social environment than a tense, surly individual. It is highly unlikely that these experiential differences, which emerge early and can endure throughout development, are without psychological effect. Consistent with this perspective, research on twins indicates that individuals high in neuroticism are more likely to experience psychological adversity than individuals low in this personality trait (74), while individuals low in conscientiousness are more likely to place themselves in high-risk situations (75). The resulting "genetic" correlations between neuroticism and life stress or conscientiousness and risk taking raise the possibility that some of what is labeled as heritable effects on personality may actually reflect a complex interplay between genetic factors and the psychological environments our personalities help us create.

Second, genetic effects on personality are almost certain to depend on environmental exposure. The notion that gene-environment interactions play a fundamental role in the development of behavior gained widespread acceptance within psychology following two highly influential publications. The first was the finding that the 5-HTTLPR short allele increased risk of depression only when the individual is exposed to a stressful environment (76); the second was the observation that a functional polymorphism in the MAO-A gene is associated with increased risk of aggressive behavior only when a

child has a history of abuse (77). It would be hard to overstate the impact these two studies have had within psychology; the rate of publication of papers on gene-environment interaction and behavior went from being irregular to more than one per day (78). Despite the popularity of the approach, there is growing concern that gene-environment interaction research suffers from the same difficulty with replication that has plagued candidate gene association studies in psychology. The original interaction involving the 5-HTTLPR polymorphism has not held up in rigorous meta-analysis of relevant studies (79), and it appears that most interaction studies are substantially underpowered and thus likely to produce false-positive results (80). While there is little doubt that gene-environment interaction effects on behavior and personality are pervasive, reliable detection of these effects will likely require methodologically stronger research (81).

### 106.6 CONCLUSION

Genetic research on personality is at a transition. Early twin and adoption studies documented the importance of genetic influences and showed that the major source of environmental influence lies outside the family. The major goal of genetic analysis is now to identify the specific genes that influence individual differences in personality and determine how the effects of those genes interact with and are modulated by experience. As with any complex phenotype, we can expect that progress in mapping genes for personality will be slow and subject to false leads. Nonetheless, research in this area holds great promise in not only bringing about a better understanding of mental health but also in illuminating the biological basis of human nature.

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Multifactorial Inheritance and Complex Diseases; Twins and Twinning; Attention-Deficit/Hyperactivity Disorder; Schizophrenia and Affective Disorders; Addictive Disorders.

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# CHAPTER

# 107

## Fragile X Syndrome and X-linked Intellectual Disability

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### 107.1 OVERVIEW

Intellectual disability (ID) is a broad diagnostic category with a variety of underlying etiologies that include both environmental and genetic factors. “Intellectual disability” is replacing “mental retardation” as the preferred term, although they refer to the same impairment (1). A plethora of genes participates in the development of the human brain, and disruption of many of these processes can impede normal intellectual development. With the development of comparative genomic hybridization arrays, which fostered our ability to detect submicroscopic changes to chromosomes, structural chromosome aberrations have been observed in an increasing number of individuals with ID that had previously gone without an underlying cause (2). Although these defects have been found across the genome, the fact remains that the X chromosome has an overabundance of known ID genes. This is at least partly responsible for the finding by various epidemiological studies that there is ~1.4-fold excess of males affected with ID compared to females (3–5). Because males have a single X chromosome, they are more sensitive to mutations that reduce the expression or activity of genes encoded therein. Females, on the other hand, have a second X chromosome that, for many genes, can provide compensatory activity for the mutated gene/genes. However, with the advent of next generation DNA sequencing, autosomal recessive loci resulting in ID will be uncovered. While each locus is likely to be individually rare, the aggregate of autosomal loci will likely dwarf the number X-linked causes in the future.

X-linked ID (XLID) is estimated to account for 10–12% of ID cases in males (6). The most common form of XLID, and the first group that could be split from the heterogeneous nonsyndromic XLID (NS-XLID)

category, was fragile X syndrome (FXS). Mutations in many different X-linked genes have since been identified in individuals with both syndromic and nonsyndromic forms of XLID, although, individually, each of these genes accounts for a small fraction of ID overall. Despite the advances made in XLID research, the underlying etiology remains unclear in a significant proportion of families with probable XLID (6,7), and NS-XLID remains a viable diagnostic category. In this chapter, we will summarize what is broadly known about the genes that contribute to XLID with particular emphasis on the FXS.

### 107.2 FRAGILE X SYNDROME

With an estimated 1 in 4000 males affected, FXS is the most common inherited form of ID (8). ID and behavioral abnormalities are central to the phenotype. Approximately 85% males and 25–30% females with FXS will have an intelligence quotient (IQ) below 70, which falls in the ID range. The associated cognitive deficits also impact executive function, visuospatial-processing abilities, attention, and working memory. Although originally considered to be a nonsyndromic form of ID because its associated physical characteristics are relatively subtle, a more distinct syndromic picture is now apparent (Figure 107-1). Affected males tend to have a long narrow face, prominent ears, and joint hypermobility; macroorchidism is present from puberty (9). Individuals with FXS generally do not have significant medical issues. Recurrent otitis media and recurrent sinusitis may be present during childhood (10). Gastroesophageal reflux is also common during infancy and early childhood, and symptoms may include irritability and colic. The frequency of seizures in FXS is ~13–18 % in boys and ~5% in girls. The risk of seizures will be



**FIGURE 107-1** Two males affected with FXS. The boy on the left is 10 years of age, and the man on the right is 26 years old. One of the few recognizable facial features associated with FXS is prominent ears, which are notable on this child but less so on the older individual.

higher in childhood, with peak incidence between 6 months and 4 years, with mean age of onset of 2 years (11,12). Different types of seizures have been reported in FXS, with the most common type being complex partial seizures.

Behavioral and psychological issues are prominent and wide-ranging in individuals with FXS. The behavioral phenotype includes shyness, social avoidance, anxiety, hyperarousal to sensory stimuli, autistic behavior (e.g. hand flapping, poor eye contact, perseverative-behavior or speech), hyperactivity, impulsivity, inattention, self-injurious behavior, aggression, and mood instability (13–16). These features are typically grouped into the following symptom clusters: (1) Attention Deficit/Hyperactivity Disorder (ADHD)-like symptoms of hyperactivity, distractibility, impulsivity, and overarousal; (2) anxiety-related symptoms, including obsessive-compulsive disorder (OCD)-like and perseverative-behaviors; (3) emotional lability; and (4) aggressive and self-aggressive behavior (17).

FXS is the most common single gene cause of autism, resulting in 2–6% of all cases of autism (18). Based on the Autism Diagnostic Observation Scale and the Autism Diagnostic Interview, ~30% of males with FXS have autism spectrum disorder (ASD), and another 20–30% have pervasive developmental disorder, not otherwise specified (15,19). Even those children with FXS who do not meet the criteria for ASD diagnosis usually have one or more autistic characteristics, such as hand flapping, poor eye contact, repetitive behaviors and language, and tactile defensiveness. Individuals with FXS and comorbid ASD are more severely impacted by social impairment and lower cognitive functioning and adaptive behavior compared to those with FXS without ASD (20,21).

Females with FXS typically have milder behavioral and cognitive problems than males with FXS because they have one normal copy of the relevant gene. Approximately 25% of females with FXS have

an IQ <70; the remaining have a high propensity for learning and/or behavioral issues including depression, withdrawal, social anxiety, and executive dysfunction (22).

### 107.2.1 Diagnosis

FXS is typically not suspected in infants unless there is a family history of FXS and/or ID. The syndrome includes no distinct physical features at birth to prompt the diagnosis, and the physical features that do develop are often subtle prior to puberty and may become more apparent with increasing age (10). Macroorchidism generally begins to present after 8 years of age with testicles being 2–3 times normal in mid-adolescence (9). Typically, the presenting concerns for a child with FXS are developmental delays and/or behavioral problems. These delays can be evident in gross motor, fine motor, and/or language skills (23). The behavioral problems include autistic-like behaviors such as hand flapping, poor eye contact, and social deficits. Due to the overlap of FXS with more common forms of ID and autism and to the prevalence of FXS, it is recommended that genetic testing for FXS be offered for all children with developmental delay and/or autism.

A 2003 study conducted with 274 FXS families indicated that the typical diagnosis of FXS occurred at approximately 32 months of age, an average of 18 months after the family first identified concerns (24). Despite efforts to increase FXS awareness in the medical community through patient advocacy, professional recommendations regarding prompt diagnosis, and increased exposure to information about FXS in the pediatric literature, no change was detected in the age of diagnosis of FXS when this study was repeated in 2009 with 1250 additional families (25). The average age of FXS diagnosis remained relatively stable across the 7-year period at ~35–37 months.

### 107.2.2 Molecular Genetics

In the vast majority of cases, FXS is caused by a CGG trinucleotide expansion in the 5' untranslated region of *FMR1*. This leads to hypermethylation and transcriptional silencing of the gene, which normally encodes the fragile X mental retardation protein, FMRP. Expanded *FMR1* alleles arise from meiotic instability of the so-called premutation alleles, which have higher number of repeated units than those found in the general population. In other words, premutation alleles can change in size when they are passed from one generation to the next and when the allele expands greatly in size, the *FMR1* gene is shut off. The mechanism of this repeat instability is unclear.

There are four allelic classes for the *FMR1* trinucleotide repeats, and these categories are based on the observations of allele instability in affected families (Table 107-1). They are normal alleles, which range in size up to 40 copies; intermediate alleles, which range in size from 45–54 repeats and may exhibit some slight instability but are otherwise considered normal alleles; premutation alleles, which can expand to a full mutation and range from 55–200 repeats; and full mutations, which are >200 repeats in length and are hypermethylated (26,27). Although premutations can change to full mutations in a multistep (i.e. multigeneration) process, this allele class was defined by the smallest premutation allele that has been observed to expand to a full mutation in a single generation, which was 59 repeat units in size (28). The 55 repeat unit cutoff for the definition of premutation alleles reflects the fact that there is some laboratory-to-laboratory-based variability in allele sizing (26).

Intermediate-sized *FMR1* alleles are significantly less stable than are the normal alleles; in a large screen of mother–child pairs in the French Canadian population, 7% of the intermediate-sized alleles changed in size when transmitted from the mother to child, compared to 1% of normal transmitted alleles (27). Three out of four intermediate alleles that did change between generations increased or decreased by a single repeat unit (27). However, it is important to note that expansions of intermediate alleles to full mutations within two generations have been reported (29,30). A similar study by

Nolin et al. (31) found that 9% of maternal alleles sized 45–49 repeats were unstable when transmitted, but this fraction increased to 26% in larger intermediate-sized alleles of 50–54 repeats (Table 107-2). Of the unstable transmissions of intermediate alleles in the Nolin et al. study, 50% increased by one repeat unit, and 89% changed by ≤4 repeat units. In one case, an increase of 31 repeat units (from 54 to 85 units) was observed, and this resulted in the expansion of an intermediate allele into the premutation class.

Premutation alleles of *FMR1* are unmethylated alleles that are transcribed and translated. Relative to normal alleles, they produce increased *FMR1* transcript levels, and in general, slightly lower levels of FMRP (32,33). Alleles of this size can expand incrementally during meiosis to produce a slightly larger premutation allele. When passed from females, they can also undergo massive expansion into the full mutation range in a single generation. This expansion is restricted to females because full mutations cannot be maintained during spermatogenesis (34). In fact, full mutations are only rarely found in the sperm of FXS males; premutations are present instead (35). No differences are found in the stability of premutations based on the sex of the offspring (27,31).

The likelihood that a premutation will expand to a full mutation is governed by the size of the premutation allele, as illustrated in Figure 107-2 and Table 107-2, and by the presence of AGG interruptions to the CGG-repeat sequence. The longer the length of the 3' pure CGG repeat tract, the less stable the premutation allele (36). Conflicting data exist on the likelihood of premutation expansion based on family history of FXS. Levesque et al. (27) did not observe differences in premutation instability between families with a history of FXS compared to premutation carriers from the general population. On the other hand, Nolin et al. found that the likelihood of a premutation expanding to a full mutation was correlated with prior family history and suggested that this may be due to interfamilial differences in the pattern of AGG interruptions in the transmitted allele (31).

Although the length of the CGG repeat in *FMR1* determines its stability, the size of a full mutation does not determine the severity of FXS (37). In females with a full mutation in *FMR1*, the biggest determinant of the severity of the phenotype is X inactivation. In these females, the phenotype can range from normal intellectual abilities to full FXS (37), and the larger the active fraction of full mutation-carrying chromosomes in these females, the more severe will be the phenotype. In males with a full mutation, the methylation status of the mutation can influence the severity of the phenotype. Although complete methylation of the full mutation is the norm, some individuals exhibit methylation mosaicism, meaning that the full mutation is not methylated in all cells. Another type of mosaicism that can be observed in males with FXS is mutational mosaicism. In this situation, some

**TABLE 107-1** The Allele Classes of *FMR1* and Their Phenotypic Consequences

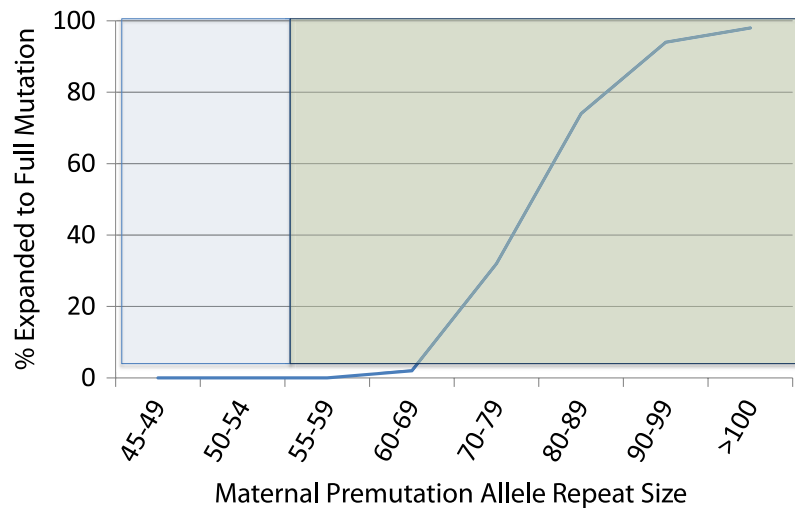
Category	Repeat Size	Consequence
Normal	≤45	—
Intermediate	45–54	No established clinical significance
Premutation	55–200	Risk of FXS in next generation(s) Primary ovarian insufficiency Fragile X Tremor–Ataxia syndrome
Full mutation	>200	Fragile X syndrome

Based on recommendations from: Kronquist et al., *Genetics in Medicine* 2008, 10, 845–847.

**TABLE 107-2 The Instability of Maternal Repeats Increases with Size**

Maternal Repeat Size	Fetal Outcome			
	Smaller than Full Mutation			Full Mutation (%)
	Unstable Transmissions (%)	Average Increase in Repeat Size	Average Decrease in Repeat Size	
45–49	5/55 (9)	1.0	0	0
50–54	13/51 (26)	5.1	8.7	0
55–59	37/86 (43)	5.9	2	0
60–69	60/79 (76)	13.2	12.3	2/81 (2)
70–79	32/33 (97)	22.4	20.5	15/47 (32)
80–89	16/16 (100)	29.3	18.2	45/61 (74)
90–99	0/1 (0)			31/33 (94)
100–200	2/2 (100)		68.0	93/95 (98)

Data derived from Nolin et al. *Prenatal Diagnosis* 2011, 10, 925–931. Fragile X Analysis of 1112 Prenatal Samples from 1991 to 2010.



**FIGURE 107-2** Risk of full mutation in offspring increases with maternal repeat size. Maternally transmitted intermediate-sized alleles (blue shading) do not increase to full mutations in one generation. The likelihood of transmitting a full mutation dramatically increases with the size of a maternal premutation (green shading).

cells in the body contain a premutation and others a full mutation allele that is hypermethylated and silenced. Both forms of mosaicism are correlated with increased IQ scores, presumably due to the fact that some FMRP is expressed in these individuals (38,39).

### 107.2.3 Cytogenetics

The term FXS describes the X-chromosome fragile site that is detectable cytogenetically when a full mutation is present in *FMR1*. The underlying mutational mechanism was not understood when this was first described, so observations of the fragile X chromosome were the first diagnostic test used for FXS. Peripheral lymphocytes must be grown under conditions of thymidylate stress for the fragile site to be observed, and it is seen only in a fraction of cells from an affected individual, so this was a less-than-optimal diagnostic approach. The cytogenetic approach also does not detect premutation alleles, which prevented accurate risk assessment in affected families.

It should be noted that the fragile site that can be documented in individuals with FXS is not the sole fragile site on the X chromosome. The FXS fragile site has been dubbed as FRAXA and is located at Xq27.3. Nearby at Xq27.2 is FRAXD, which is a common fragile site and has been confused with FRAXA in cytogenetic analyses, although it has no known pathologic significance (40). There are two more rare fragile sites at Xq28 that each result from expansion of trinucleotide repeat tracts. The FRAXF fragile site contains a gene called *FAM11A*, but has not been definitively correlated with a particular phenotype (40). Within the FRAXE fragile site, on the other hand, is the *FMR2* gene (also known as *AFF2*), which contains a CCG triplet repeat that expands in a fashion similar to that of the *FMR1* CGG repeat. This expansion has been described in individuals with a form of NS-XLID (41) but it is much more rare than FXS (42). Similar to FMRP, the product of *FMR2* is an RNA-binding protein that binds to G-quartet structures. It is believed to participate in the



regulation of RNA transcription and alternative RNA splicing (43–45).

### 107.2.4 Inheritance

FXS is inherited as an X-linked dominant trait with reduced penetrance, but the mutational mechanism for this disorder gives rise to a characteristic inheritance pattern in families. Typically, intellectually normal males pass the relevant allele on the X chromosome to an unaffected daughter, who then passes the allele to an affected son. Due to the instability of premutation alleles as they are passed from generation to generation, and to the fact that larger premutation alleles are more likely to expand to a full mutation (28), the likelihood that FXS will be penetrant in a family increases down through the generations. This provides the resolution for the so-called Sherman paradox, which was the finding that, in FXS families, the mothers of transmitting sons were less likely to have sons with FXS than were daughters of transmitting males.

### 107.2.5 Premutation Phenotypes

Individuals who carry a premutation allele at *FMR1* do not have FXS, but they are at risk of having other *FMR1*-related disorders. The best documented of these are fragile X-associated tremor/ataxia syndrome (FXTAS) and fragile X-related primary ovarian insufficiency/premature ovarian failure (FXPOI). Premutation carrier females may also be at increased risk for depression and anxiety, and late-onset neurological issues including hypothyroidism, fibromyalgia, and neuropathy (46,47), but these associations are not always confirmed once covariates are taken into account (48,49). While FXS is due to the lack of FMRP, the premutation-associated disorders result from the accumulation of CGG-repeat containing *FMR1* transcripts (50). This idea is supported by the fact that no RNA or protein is produced from *FMR1* full mutations, but there is an excess of *FMR1* transcript and a deficiency of FMRP associated with premutations (32,33). Further, individuals with FXS do not exhibit features of FXTAS nor FXPOI, suggesting they occur by a different mechanism.

FXTAS includes intention tremors, gait ataxia, cognitive decline and dementia, balance problems, and peripheral neuropathy (51). These symptoms may progressively worsen over time (52). Women with FXPOI have decreased ovarian reserve, irregular menstrual periods, hormone fluctuations, and increased risk for premature ovarian failure (menopause before the age of 40) (53). Both phenotypes exhibit age-dependent penetrance, and overall, the penetrance and severity of symptoms is correlated with the size of the premutation allele (53,54). This correlation is not linear for FXPOI; instead the highest risk of premature ovarian failure is found in the middle of the premutation

range and could be due to the excess FMRP production associated with alleles of this size (55). This hypothesis is supported by the identification of a family that carries a duplication encompassing *FMR1* in which all carrier females experienced premature ovarian failure (56).

The mean age of onset for FXTAS is in the early 60s (57), and nearly 50% of 70–79-year-old men who carry a premutation allele have features of FXTAS (58). Fewer female premutation carriers have FXTAS. In a study by Rodriguez-Revenga et al. (59), 16.5% of female premutation allele carriers had signs of FXTAS by age 50, compared with 45.5% of males. Although a larger fraction of women might have features of FXPOI, the penetrance of fragile X-related premature ovarian failure—which is the most extreme end of FXPOI—is 1% by age 18 and 15–24% by age 40 (53).

### 107.2.6 Genetic Testing

Genetic testing for FXS and for *FMR1* premutations involves an assessment of the size and methylation status of the CGG repeat tract in the 5' UTR of *FMR1*. A polymerase chain reaction with primers that flank the repeat tract is used to size the repeat, and the product is visualized by capillary gel electrophoresis. A Southern blot is used to determine the methylation status of the repeat, as well as to estimate the size of full mutations that are too long to be amplified by PCR. This approach will detect the vast majority of cases of FXS. It is believed that approximately 1% of cases of FXS are due to point mutations or full/partial deletions in *FMR1* (60), but a more accurate assessment of this prevalence is unavailable because *FMR1* sequencing is rarely performed. Although a limited number of *FMR1* mutations have been reported in boys who presumed to have FXS, it is not clear whether, in general, point mutations in *FMR1* will yield the same phenotype. Sequence analysis of *FMR1* in 50 boys with an FXS-like phenotype yielded one deletion in *FMR1*, but no other pathogenic sequence changes (61). A larger study of 963 boys with developmental delay identified one potentially pathogenic missense change in *FMR1* (62). Despite this low frequency of sequence mutations, *FMR1* sequence analysis can be considered in males with a clinical picture consistent with FXS in whom the *FMR1* CGG repeat tract is normal.

Due to the fairly high prevalence of FXS and the subtlety of the syndromic features, it is recommended that genetic testing for FXS be included as a first-line genetic test for all individuals with developmental delays, learning issues, ID and/or ASD (60). This is estimated to have a diagnostic yield of ~1–2% (63). Females with a history of infertility should also be screened for premutations in *FMR1*. A family history of movement disorders, early menopause, learning disabilities, ID, or autism should increase the suspicion for FXS (64).

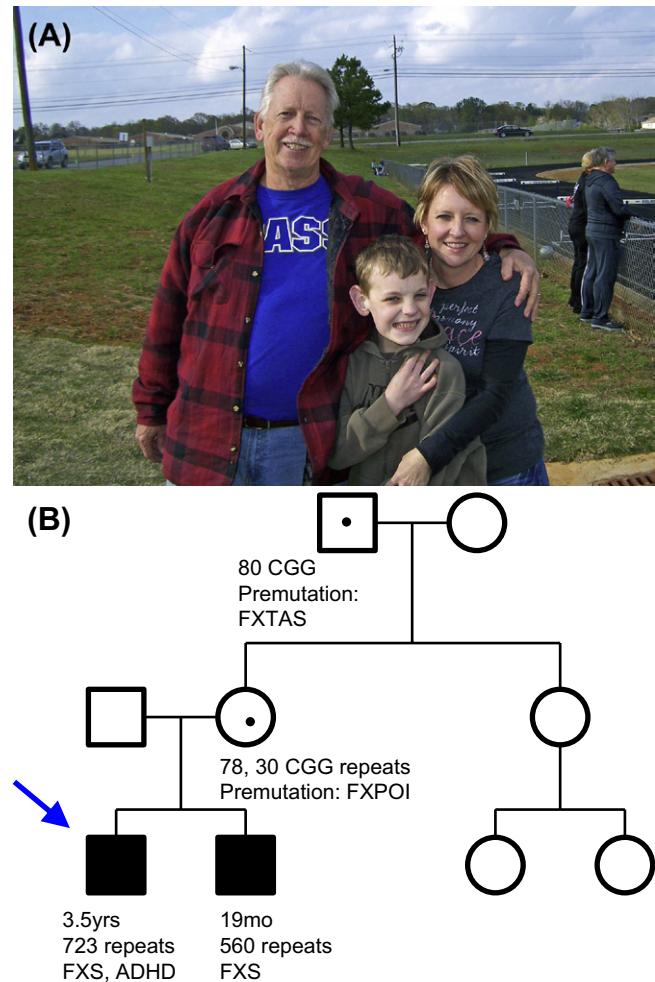
### 107.2.7 Genetic Counseling

Genetic counseling and cascade testing should be recommended upon the identification of FXS in a family with the aim of identifying both premutation carriers and individuals with FXS (64). FXS is often termed “a family of disorders” because there is a wide spectrum of involvement, both medical and psychiatric, in a single family (9), as illustrated in Figure 107-3. This means that early diagnosis has important implications not only for the child with FXS, but also for the immediate and extended family members.

Premutation carriers should be counseled as to their risks of having a child with FXS and to their own risks of developing FXTAS and/or FXPOI. As the cascade testing strategy is planned, particular attention should be paid to certain aspects of the extended family history, including whether and which individuals have ID, behavioral or learning issues, infertility or premature menopause, tremor, ataxia, or other neurological disorders, and psychiatric issues, such as cognitive decline, depression, dementia, or anxiety (64). This is particularly important due to recent evidence that the risk of passing a full mutation to a child is lower for premutation carriers with no family history of FXS (31).

One of the difficult issues that arise in counseling families for *FMR1*-related disorders is the identification of a CGG repeat tract that falls in the intermediate class of 45–54 repeats. Although no alleles in this size range have been reported to expand directly to a full mutation, and these alleles are generally considered to be stable, meiotic instability is significantly higher than is observed for alleles in the normal range (27), particularly in the 50–54 repeat range (31), so careful counseling of these families is warranted (37). Intermediate alleles have been observed to expand to full mutations in two generations (29,30).

Other than use of repeat size, it is difficult to predict which premutation carriers will suffer from FXPOI or FXTAS. There are limited data that hint that magnetic resonance imaging (MRI) studies may detect changes in prefrontal activity and reductions in gray matter in the anterior subregion of the cerebellar vermis in premutation carriers, even when they have not been diagnosed with FXTAS (65,66). It remains to be seen whether this turns out to be a predictor of FXTAS in premutation carriers. Predictions of ovarian dysfunction in premutation carriers have focused on hormone levels. Increased levels of follicle-stimulating hormone and decreased levels of inhibin B, inhibin A, and progesterone at certain times of the menstrual cycle have been recorded in female premutation carriers, even when they have regular menstrual cycles, and this is believed to be indicative of early ovarian aging (53,67,68). Anti-Müllerian hormone is reduced in premutation carriers with repeat sizes  $\geq 70$ , relative to those with shorter premutation alleles, and may better



**FIGURE 107-3** The family of Fragile X disorders. (A) A three-generation family affected by *FMR1*-related disorders. The boy on the right has FXS and an *FMR1* full mutation, which he inherited from his mother, who has FXPOI due to an *FMR1* premutation; her father has FXTAS due to the premutation. (B) Pedigree of the family at identification. The proband was a 3 ½-year-old boy with FXS. His 19-month-old brother also has FXS. The diagnoses and *FMR1* repeat allele sizes are indicated underneath each relevant individual.

predict early ovarian decline than the other hormones (69), but definitive assays for predicting FXPOI have not yet been developed.

The potential for preimplantation genetic diagnosis for FXS should be a component of the discussion with premutation carriers who are interested in having children. The effects of the premutation on reproduction, as described above, are relevant in this discussion. The potential for premature ovarian insufficiency may shorten the reproductive window in these women. In addition, ovarian stimulation for oocyte retrieval is more difficult in *FMR1* premutation carriers, and they may need more gonadotrophins for this stimulation than women without a premutation allele (70,71). The failure rate for ovarian stimulation is higher for women with a premutation allele (13%) than those without (~1%), but, of note, the majority of premutation carriers who

are completely refractory to ovarian stimulation have regular menstrual cycles (71).

### 107.2.8 Epidemiology

Approximately 1:5000–1:4000 males have FXS, and this frequency is lower in females, due to the reduced penetrance of the full FXS phenotype (72–74). A large, anonymous screen of samples for *FMR1* premutations (of which 99% were females) estimated that 1:178 females and 1:400 males in the general population is a premutation carrier (75). Based on previous penetrance estimates, these data were used to calculate that the prevalence for FXTAS in males is 1:4848 and for FXPOI in females is 1:3560 (75).

### 107.2.9 Screening

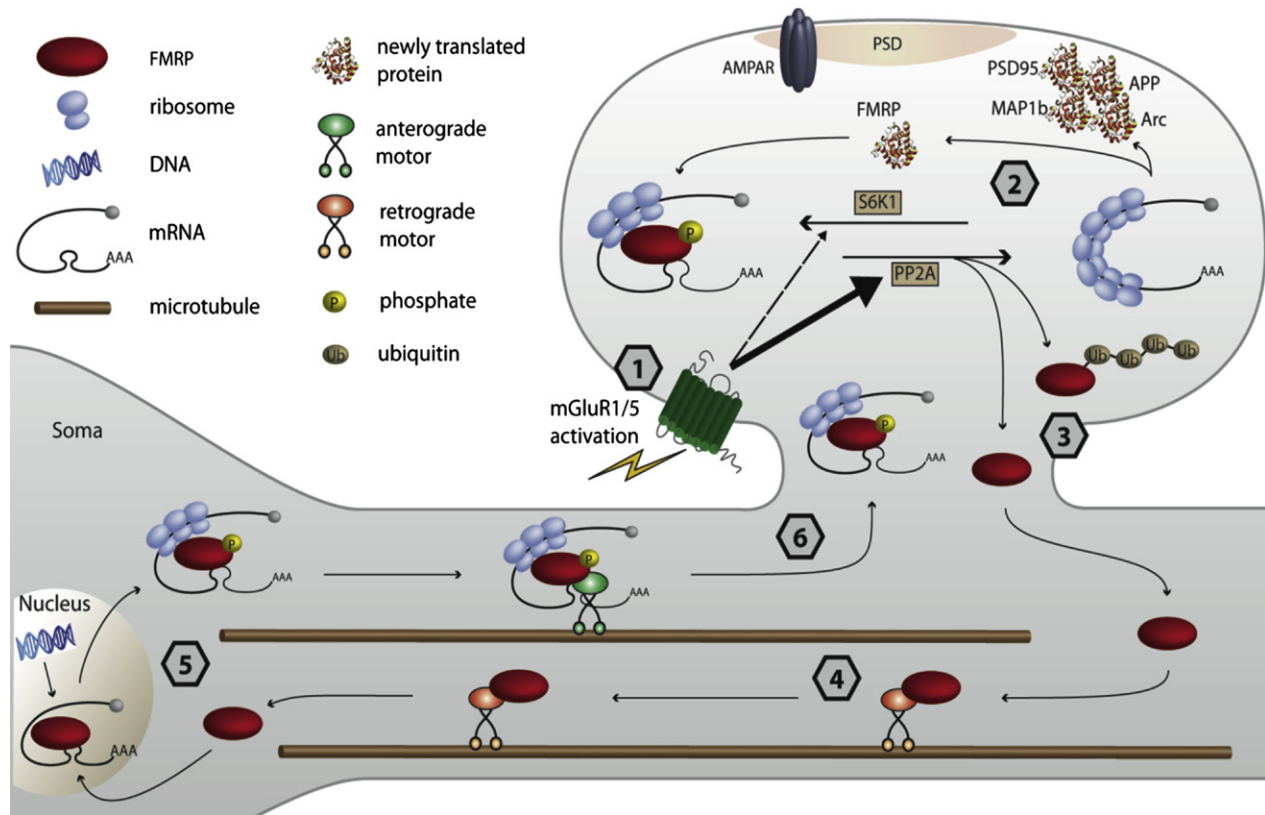
Traditional population screening includes conditions (e.g. phenylketonuria) for which early treatment can prevent severe morbidity and mortality. More recently, newborn screening programs have expanded to include conditions that may not meet all the traditional screening criteria. No states currently screen for FXS in newborns, but it has been considered for possible inclusion in these programs. The under-recognition of FXS despite its prevalence and the increased potential of targeted pharmacological intervention provide arguments for this type of screening, but it was not until recently that this type of screening was feasible on a large scale due to technological advances in our ability to detect *FMR1* mutations.

The techniques that are used to diagnose FXS are too labor-intensive and costly to incorporate into large-scale screening programs. Several PCR-based screening techniques have been developed to get around these problems. These include methods to facilitate the amplification of long GC rich tracts (76,77), and methylation-specific PCR methods that detect methylated full mutations of *FMR1* (72). It should be noted that the *FMR1* allele classes that can be detected via a screen are determined by the technique used; some techniques can detect premutations, whereas the methylation-based methods detect only full mutations. Further, the methylation-based techniques do not detect approximately 20% of females with a full mutation, due to the presence of the second X chromosome (72). Although these techniques make population screening for *FMR1*-related disorders possible, this idea is not without controversy (78,79), including the fact that some premutation carriers might not want to know from early in life that they are at risk of an *FMR1*-related disorder in adulthood and the fact that girls with a full mutation might be stigmatized by the screen even though they may not have ID. General population screening for FXS is not currently recommended by the American College of Medical Genetics (60) or the American College of Obstetricians and Gynecologists (80).

Population screening for FXS does not necessarily have to occur in newborns; preconception screening of *FMR1* in adult women has also been debated (78,79). Preconception screening (screening for premutation carriers) assesses a woman's risk for having a child with FXS and determines the woman's risk for FXPOI. It may be less stressful for women as compared to waiting for the screening test result during the pregnancy or after the birth of a child. Additionally, women can elect alternative reproductive options such as adoption, embryo donation, egg donor, or preimplantation genetic diagnosis. With preconception screening, women will have more time to make informed decisions. Access to genetic counseling, education, and resources will be important for the successful implementation of preconception screening. It should be kept in mind that premutation carriers identified through general population screens may not have as high a risk of having a child with FXS as those identified through cascade screening of affected families (31).

It often takes a long time for FXS to be recognized and diagnosed, and families may give birth to a second child before a diagnosis is made and are thus not counseled as to relevant reproductive options for their family (24). Furthermore, this can delay diagnosis of the premutation-associated disorders, FXPOI and FXTAS, in older members of the family. Thus, research has explored the attitudes of affected families toward FXS screening. In a survey of 442 parents of children with FXS, parents indicated their support for voluntary screening for FXS, especially carrier and newborn screening (81). Approximately 93% of the surveyed parents indicated that they believe that FXS genetic testing should be offered to parents prior to conception, and 83% felt this testing should be available for newborns. Parents made arguments in support of early testing, such as the potential to obtain services for their child earlier, increased understanding of their child's needs, and the ability to make informed reproductive decisions. These parents also remarked on the potential negative outcomes of screening, which could include increased worry about their child's development and future, increased stress, insurance discrimination, and strained relations with extended family members (81). Pediatricians also support early screening of high-risk infants for cystic fibrosis, Duchenne muscular dystrophy, and FXS (82). These physicians preferred the idea of infant screening over newborn screening for FXS, and they suggested that screening be voluntary and require informed consent. Screening in later infancy allows parents make an informed decision about the test and allows time for the parents to bond with their child before a diagnosis is made. Given that most states do not require parental consent for newborn screening, but not all parents want to know this diagnosis at the birth of their child, informed consent is an issue for FXS newborn screening.





**FIGURE 107-4** The function of FMRP in the neuron and its regulation by mGluR. FMRP regulates local translation of mRNAs near synapses. mGluR stimulation at synapses has several effects on FMRP. These include: (1) Rapid activation of PP2A, which dephosphorylates FMRP, resulting in rapid depression of target mRNAs and their subsequent translation. (2) This local translation of proteins such as PSD-95, MAP1b, APP, and Arc, leads to AMPA receptor internalization. Next, and with slower kinetics after mGluR stimulation, FMRP is rephosphorylated by S6K1, bringing it back to a state of translational repression. (3) mGluR stimulation leads to FMRP ubiquitination and its proteasome-dependent degradation, which is necessary for mGluR long-term depression. (4) A retrograde signal leads to transport of new FMRP-associated mRNAs from the soma. (5) FMRP can shuttle into the nucleus. (6) We speculate that it may pick up new target RNAs there and bring them back to the synapse. (Reprinted from *Neuron* 60(2) Bassell; Warren. *Fragile X Syndrome: Loss of Local mRNA Regulation Alters Synaptic Development and Function*, 2008; pp 201–214, with permission from Elsevier.)

### 107.2.10 Mechanisms

FMRP is an messenger RNA (mRNA)-binding protein (83) that is abundantly expressed in the brain, where it is involved in RNA trafficking (84) and the regulation of local protein synthesis in dendrites (85) (see Figure 107-4). FMRP is a negative regulator of translation, and—in its phosphorylated form—is associated with stalled polyribosomes (86). In the absence of FMRP, as in FXS, translation of specific mRNAs is dysregulated, and this is associated with altered synaptic function. FMRP has been shown to bind several specific mRNAs and influence their expression (Table 107-3). Intimately tied to the function of FMRP is signaling through the group 1 metabotropic glutamate receptors, mGluR1 and mGluR5. In hippocampal neurons, activation of mGluR can trigger long-term depression, which is a long-lasting change in synaptic strength. This process requires localized translation of mRNAs that are found in postsynaptic dendrites (87) and is accompanied by long-term changes to the rate of endocytosis of AMPA receptors (88). mGluR stimulation triggers the movement of FMRP and its bound mRNAs to dendrites and the release of the FMRP-mediated

brake on mRNA translation (89). This, in turn, stimulates endocytosis of AMPA receptors. In the absence of FMRP, the lack of feedback inhibition in this system gives rise to excess basal translation of FMRP-regulated mRNAs and a lack of mGluR-stimulated local translation (90), which lead to increased AMPA receptor internalization (91) and excessive hippocampal and cerebellar long-term depression (LTD) (e.g. weakening of synaptic connections) (92). This presumably underlies the abnormal dendritic morphology, which includes an overabundance of dendritic spines with an immature morphology, and the subsequent weakening of neural connections and synaptic plasticity that are evident in humans with FXS and in *FMR1* knockout mice (90). The definition of these molecular defects in FXS has led to the development of new therapies for FXS that are currently in trial, as will be described later in this chapter.

### 107.2.11 Treatment

The current psychopharmacology treatment in FXS is symptom-based combined with supportive strategies,



TABLE 107-3 FMRP Target mRNAs		
mRNA	Effect of mGluR Stimulation	References
App	Translation	{Westmark, 2007 #139}
Arc	mRNA transport; translation	{Park, 2008 #142; Steward, 2001 #140}
CamKII $\alpha$	mRNA transport; translation	{Hou, 2006 #145; Muddashetty, 2007 #144; Zalfa, 2003 #102} {Dictenberg, 2008 #101}
eEF1A	mRNA transport; translation	{Huang, 2005 #147; Sung, 2003 #148}
Fmr1	mRNA transport; translation; protein degradation	{Antar, 2004 #150; Schaeffer, 2001 #151; Weiler, 1997 #149} {Hou, 2006 #145}
GluR1/2	Translation	{Muddashetty, 2007 #144}
Map1b	mRNA transport; translation	{Antar, 2005 #154; Brown, 2001 #152; Darnell, 2001 #153; Davidkova, 2007 #155; Hou, 2006 #145; Menon, 2008 #156} {Dictenberg, 2008 #101}
Psd-95	mRNA stability; translation	{Muddashetty, 2007 #144; Todd, 2003 #157; Zalfa, 2007 #158}
Sapap3/4	mRNA transport; translation	{Brown, 2001 #152; Dictenberg, 2008 #101; Narayanan, 2007 #160}
Rgs5	mRNA transport	{Dictenberg, 2008 #101}
Gaba-A $\delta$	mRNA transport	{Dictenberg, 2008 #101}

Adapted from Bassell and Warren (2008) *Neuron*, 60, 201–214.

TABLE 107-4 Current Psychopharmacology Treatment in FXS		
Behavioral Problems	Medication Class	Medications
Hyperactivity, Inattention, Impulsivity	Stimulants or Non-stimulants	Stimulants: Methylphenidate and Amphetamine Non-stimulants: Strattera, Intuniv
Overarousal and hypersensitivity	Alpha-agonists	Clonidine and Guafacine
Anxiety, obsessive-compulsive disorder, perseverative	Selective Serotonin Reuptake Inhibitor (SSRIs)	Fluoxetine, Fluvoxamine, Sertraline
Aggression/self-injurious	Atypical Antipsychotics	Risperidone, Aripiprazole, Olanzapine, Quetiapine, Ziprasidone, and Aripiprazole

which include speech therapy, occupational therapy, behavioral interventions, and a specialized educational program. As behavior in FXS can greatly impact academic and social functioning, medications may be necessary to decrease maladaptive behavior and/or prevent dangerous consequences of the behavior. Additionally, medications may optimize the individual's functioning and potential, and allow for integration in the educational and social environment. Medications are always combined with intervention therapies (e.g. speech therapy), behavioral strategies, and educational support and resources. An overview of the pharmacological treatments currently used to manage FXS can be found in Table 107-4 and will be detailed below.

**107.2.11.1 ADHD Treatment in FXS.** The prevalence of ADHD symptoms is more common in children with FXS than it is in individuals with other genetic conditions or individuals with nonspecific ID (93). ADHD symptoms are the most prevalent problem behavior in individuals with FXS and in male premutation carriers (16,94). Overall, 54–59% of boys with FXS meet the diagnostic behavioral criteria for either ADHD-inattentive type only, ADHD-hyperactive type only, or ADHD-combined type based on parent or teacher report (16). In addition to behavioral intervention and individualized therapies, medications must be

considered to improve symptoms of hyperactivity, inattention, and impulsivity.

Stimulants are the most frequently used class of medication in boys with FXS (17). However, data on the efficacy of stimulants in children with FXS are limited. Stimulants continue to be the first-line of treatment for intellectually typical children with ADHD, but their efficacy in children with cognitive deficits has been questionable. Stimulants are frequently not effective in children with severe ID, and an IQ <45 is associated with poor response (95,96). For FXS, stimulants improve the hyperactivity and inattention symptoms of approximately 67–75% of affected children (17,97). In a double-blind crossover trial of methylphenidate and dextroamphetamine compared to placebo, 10 of 15 (67%) prepubertal boys with FXS were noted to have improvements in attention span and socialization skills. Response rate to stimulants may be relatively lower in adult males with FXS because of higher propensity toward anxiety and decreased activity level (17). Overall, our current knowledge about the benefits of stimulants for people with FXS continues to be positive, and these medications can improve distractibility, attention span, and impulsivity in individuals with FXS.

The most commonly used stimulants for treatment of ADHD in FXS are methylphenidate (Ritalin) and

amphetamine (Dexedrine and Adderall). Short-acting stimulants may require frequent dosing, as the duration of activity is typically 2–6 h. The long-acting stimulants first became available in 2000 and are the preferred treatment for most children with FXS. They are equally effective but with fewer side effects, such as the irritability and rebound in the late afternoon that are often seen with short-acting stimulants (97). Short-acting stimulants may be used in conjunction with long-acting stimulants to decrease symptoms of ADHD at the beginning or end of the day or to manage adverse effects as medication wears off. Examples of long-acting methylphenidates are Concerta, Metadate CD, Ritalin LA, and Focalin. Daytrana is the first methylphenidate transdermal patch, which offers flexible duration of action because it is applied once daily and the medication lasts approximately 3 h after the patch is removed. Examples of long-acting amphetamines include Vyvanse, Adderall XR, and Dexedrine Spansule. Pemoline is a stimulant with associated risk of hepatotoxicity that was discontinued in 2005.

Despite being the most prevalent medication used to manage FXS, it should be kept in mind that the efficacy and side effect profiles of stimulants in individuals with FXS vary considerably. The common side effects of stimulants include decreased appetite (with or without weight loss), abdominal pain, headache, insomnia, and growth retardation. Occasionally stuttering, decreased talking, or increased seizures may be seen (17). It is important to be cautious when increasing the dose because children with FXS are sensitive to stimulants and may have exacerbated outbursts and mood lability at higher doses. The cardiovascular system is also stimulated, resulting in an increase in heart rate and blood pressure. If motor or vocal tics develop, non-stimulants such as atomoxetine and guanfacine should be considered. Children taking stimulants should be monitored for weight, height, heart rate, and blood pressure before and during treatment. Non-stimulants may be preferred in children <5 years of age, as stimulants may induce irritability and other behavioral issues in young children. These behavioral problems may not be present when stimulants are reintroduced at an older age.

The first non-stimulant approved for the treatment of ADHD is Strattera (Atomoxetine), a norepinephrine reuptake inhibitor. Strattera is an alternative option for individuals with FXS who cannot tolerate stimulants. Anecdotal reports and our experience suggest that Strattera can be helpful for females with premutations who have ADHD and anxiety. The most common side effects of Strattera are decreased appetite, abdominal pain, nausea and vomiting, somnolence, and fatigue. Intuniv is a new non-stimulant for treatment of ADHD, and studies on its use in FXS have not been published.

Clonidine (Catapres) and guanfacine (Tenex) are alpha-adrenergic agonists that were initially developed to treat hypertension in adults but have been used for

years without Food and Drug Administration (FDA) approval to treat a variety of behavioral concerns in children, including those in children with FXS. In a survey of clonidine use in 35 children with FXS, 63% of parents reported that clonidine was helpful for their child, while 20% found it a little helpful, 11% indicated behavior was worse, and 6% reported that there was no effect (98). Clonidine is prescribed to treat the hyperactivity, overstimulation, and attention/concentration problems associated with FXS. Clonidine has also been used to treat sleep disorders, a common issue in children with FXS. At low doses, clonidine can increase rapid eye movement (REM) sleep and decrease non-REM sleep, whereas at higher doses, the effect is reversed in the second 1/3 of the night (99). The most common side effect of clonidine is sedation, which is often transient and manageable by dose reduction. Blood pressure should be monitored and abrupt withdrawal avoided, due to the risk of rebound hypertension and withdrawal arrhythmias.

Guanfacine can help with ADHD symptoms, including hyperactivity and frustration tolerance (100). Guanfacine is used commonly in FXS, as it is generally less sedating than clonidine and has a longer half-life. For example, a 0.1 mg dose of clonidine is equivalent to a 1 mg dose of guanfacine. As with clonidine, abrupt withdrawal should be avoided (101).

**107.2.11.2 Anxiety Treatment in FXS.** Selective serotonin reuptake inhibitors (SSRIs) are the treatment of choice for anxiety, depression, and mood symptoms (102). In the FXS population, antidepressants, particularly SSRIs are helpful more than 50% of the time in helping with anxiety and behavioral/emotional symptoms (17,103,104). Fluoxetine (Prozac) can be effective in treating selective mutism and extreme shyness in females with FXS (105). In addition, based on a self-reported survey, fluoxetine improves anxiety, tantrums, or aggression in 70% of adults with FXS.

Pediatric research is limited on SSRIs, as not all SSRIs have been approved by the FDA for use in children. Approved SSRIs are fluoxetine (Prozac; approved for depression and OCD), fluvoxamine (Luvox; approved for OCD), and sertraline (Zoloft; approved for OCD) (102). An unblinded prospective study in 12 children with FXS revealed that sertraline showed improvement in emotional and behavioral problems based on parental questionnaires; however, response in language fluency and pragmatics were not noted (17). SSRIs are beneficial in targeting obsessive-compulsive behavior, irritability, inability to tolerate changes and transitions, and social anxiety in individuals with FXS. Higher doses of SSRIs can lead to behavioral activation, which causes increased hyperactivity and disinhibition, changes in appetite, insomnia, nausea, and decreased sexual drive or impotence (17). SSRIs cause activation in ~20% of individuals with FXS, as evidenced by symptoms of restlessness, mood changes, and disinhibited behavior (e.g. aggression) (103). Of this class, fluoxetine is associated with

more activation and may be suitable for individuals with shyness, social anxiety, or withdrawal, whereas those who tend toward hyperactivity and impulsive behavior may respond better with the less activating SSRIs. Individuals who are over-sensitive to the activating effects of SSRIs may tolerate venlafaxine (Effexor) or tricyclic antidepressants, which decrease reuptake of both serotonin and norepinephrine, thereby targeting both anxiety and attention problems (17). Careful monitoring of EKGs and anticholinergic effects (e.g. dry mouth, dry nose, blurry vision, constipation, urinary retention) must be considered when using tricyclic antidepressants. Although it has not specifically been reported in individuals with FXS, SSRIs can increase suicidal ideation in depressed patients; careful monitoring of patients for mood changes is important. Currently, the FDA has not reported any completed suicides in youths with or without FXS on SSRIs (102).

**107.2.11.3 Treatment of Aggression and Mood Instability in FXS.** Atypical antipsychotics are generally helpful for individuals with FXS who exhibit severe behaviors such as aggression, irritability, mood instability, and perseverative behavior. Despite the finding that approximately 80% of individuals with FXS respond to at least one antipsychotic medication, these drugs are less widely used in individuals with FXS than are the stimulants and SSRIs (17,106). Risperidone (Risperdal) has been the most frequently used antipsychotic medication and showed high response rates for aggressive behavior and other aberrant behaviors in older males with FXS and in young boys with FXS and autistic traits. This is consistent with the double-blind, placebo-controlled trial in individuals with autism (but not FXS), which reported that risperidone is safe and effective for aggressive and aberrant behavior (107).

Aripiprazole (Abilify) is the second most frequently used atypical antipsychotic in FXS, with response rates of ~70% (108,109). Aripiprazole targets distractibility, anxiety, mood instability, aggression, and aberrant social deficits (110). Some individuals cannot tolerate aripiprazole because its side effects are similar to stimulants and can include agitation or aggravation of aggressive, irritable, and perseverative-behaviors (108).

In general, the side effects of atypical antipsychotics include sedation, nausea, constipation, extrapyramidal reactions, impaired coordination, gynecomastia, and weight gain (17). Tardive dyskinesia appears to be extremely rare in individuals with FXS on prolonged treatment with antipsychotics. Risks of diabetes, fatty liver, or metabolic syndrome may be increased in general populations treated with these drugs (17). There is no information available on these risks in the FXS population, although monitoring for these conditions with periodic glucose in fasting condition and liver function studies are necessary, especially for individuals experiencing rapid weight gain. The newer atypical antipsychotics (risperidone, olanzapine, quetiapine, ziprasidone,

and aripiprazole) are less sedating than older medications (haloperidol and thioridazine). Certain atypical antipsychotics (quetiapine, ziprasidone, aripiprazole) have less effect on weight and were helpful for aggressive behavior in some patients with excessive weight gain on risperidone or olanzapine (108).

#### **107.2.11.4 Future Pharmacotherapies for FXS.**

Although current therapies for FXS are aimed at symptom management, the future of psychopharmacology treatment in FXS is promising, as it is specifically directed at preventing and/or improving some of the cognitive and behavioral features in FXS. The enhanced mGluR5-dependent hippocampal and cerebellar LTD, exaggerated synaptic weakening, and increased internalization of AMPA receptors associated with loss of FMRP, as described above, are believed to underlie the cognitive deficits in FXS (89). Thus, therapeutic strategies that incorporate mGluR5 antagonists might reduce excess mGluR5-mediated dendritic translation and its downstream effects and thus be useful in treating some of the symptoms of FXS (111). Selective mGluR5 antagonists, including the prototype of the class, 2-methyl-6-(phenylethynyl)pyridine (MPEP) has been studied in animal models of FXS (112). It rescues hyperactivity and audiogenic seizures in knockout mice, and cognitive and neuroanatomical phenotypes in a *Drosophila* fruit fly model of FXS (113).

Clinical trials in individuals with FXS are currently being conducted with several compounds that target the mGluR5 pathway. Fenobam, a high potency and selective mGluR5 antagonist, is comparable to MPEP (114). Fenobam was previously investigated as an anxiolytic in several phase II studies in the early 1980s with reported central nervous system-related side effects including hallucinations, vertigo, paraesthesias, and insomnia, especially in higher doses (115). In a pilot open-label, single-dose trial of Fenobam in 12 adults with FXS, no significant adverse events were identified (116). Decreases in hyperactivity and anxiety were reported, and improvement of the prepulse inhibition was seen in 50% of the subjects. Phase I and II clinical studies in FXS are presently underway for several other mGluR5 antagonists: R04917523 (Roche, Basel, Switzerland), AFQ056 (Novartis, Basel, Switzerland), and STX107 (Seaside Therapeutics, Boston, MA). In a double-blind, crossover study of AFQ056 in 30 males with FXS, aged 18–35 years, adverse events were reported by 80% of the subjects, mostly fatigue and headache. No significant effects were noted between AFQ056 and placebo groups in the Aberrant Behavior Checklist scores. However, the study data suggested that the response in AFQ056 treatment might be predicted by the methylation status of the *FMR1* promoter. Seven subjects with full methylation showed significant effects of AFQ056 treatment in the ABC and Repetitive Behavior Scale, whereas, subjects with partial methylation did not show any significant improvements with AFQ056 treatment when compared to placebo (117).

Lithium is an alternative strategy to target the excessive signaling through the mGluR pathway and may potentially correct the overactive dendritic protein synthesis in FXS (118). Lithium reduces mGluR-activated translation and reverses phenotypes in the *Drosophila* FXS fly model and *FMR1* knockout mouse. In an open-label trial of lithium in 15 males with FXS (6–23 years of age), improvements in behavioral functioning, adaptive skills, and verbal memory were reported (119). Results were not as positive for the Ampakine compound CX516. This class of compounds facilitates the activity of AMPA receptors, thereby targeting a downstream effect of excess mGluR activity. A Phase II double-blind, placebo-controlled clinical trial evaluating the safety and efficacy of CX516 in individuals with FXS found no significant improvement in memory, language, attention/executive function, behavior, and overall functioning in CX516-treated subjects compared to placebo (120).

Targeting the GABA system may provide an alternative treatment strategy in FXS. GABA is a key inhibitory neurotransmitter system in the brain and works in opposition to the excitatory glutamate pathways. The expression of various subunits of the GABA-A receptor is reduced in knockout FXS mice and in *Drosophila* *dFMR1* knockout flies (121). This appears to render these organisms more sensitive to excess glutamate signaling and may be responsible for the observation that high levels of glutamate in food are lethal to *dFMR1*-deficient flies (122). This lethality provided the impetus for a screen of 2000 compounds to identify those that rescue this glutamate-induced lethality and lead to the identification of three that implicate the GABA inhibitory pathway as a counter-effect to the lethality. One of these was the GABA-B agonist baclofen. Baclofen has since been found to ameliorate the abnormal phenotypes in several animal models of FXS, including audiogenic seizures and hyperactivity in the FXS mice (123). In both human and animal studies, arbaclofen, which is the active isomer of racemic baclofen, appears better-tolerated and more efficacious than racemic baclofen. Studies are now being conducted to explore the efficacy, safety, and tolerability of arbaclofen, STX209, (Seaside Therapeutics, Boston, MA) in adolescents and adults with FXS.

Minocycline, a tetracycline antibiotic, inhibits the activity of matrix metallo-proteinase-9 (MMP-9), which is elevated in the hippocampus of *FMR1* knockout mice and may be partially responsible for the immature dendritic spine profile of hippocampal neurons (124). In an open-label, add-on trial of 20 adults with FXS (13–32 years of age), behavioral improvements were noted (125). Two subjects sero-converted to a positive antinuclear antibody test, which is suggestive of a lupus-like reaction likely induced by the drug. Additionally, minocycline can cause graying of teeth in children younger than 7 years of age if their permanent teeth have not erupted. Of note, with long-term use minocycline can cause graying of other tissues and increased sensitivity to sunlight.

It is hoped that the targeting of therapeutics to ameliorate the underlying pathophysiologic mechanism of FXS will result in improved outcomes for affected individuals. Many FXS clinical trials are currently underway and we await the results with eagerness. These medications could perhaps also be effective in the treatment of more common forms of autism or other neurodevelopmental disorders, which may overlap FXS in terms of the underlying molecular mechanisms.

### 107.3 OTHER FORMS OF XLID

Because ID is a heterogeneous grouping of disorders, the underlying etiology can be difficult to uncover. Several factors have made the X chromosome a research focus for genetic causes of ID, including the significant excess of males affected with ID, the relative simplicity of genetic analysis in males because they have a single X chromosome, and the fact that many of the genes encoded on the X chromosome are expressed in the brain. Nearly 100 of the more than 800 annotated protein-coding genes on the X chromosome have been implicated in XLID (Table 107-5) and there are estimated to be as many as yet-unidentified putative XLID genes. These XLID genes have been compiled by the Greenwood Genetics Center (<http://www.ggc.org/xlmr.htm>) and at the International Workshop on Fragile X and X-linked mental retardation (<http://xlmr.interfree.it/home.htm>). Other than *FMR1*, which is estimated to account for approximately 25–40% of XLID (126,127), and *ARX*, which was found to be mutated in 7.5% of XLID families (6), each accounts for only a small fraction of cases of XLID, meaning that there are many target genes when genetic testing for XLID is considered.

#### 107.3.1 Diagnosis

It is estimated that approximately 10% of ID in males is due to genetic defects on the X chromosome (127), including the FXS. A thorough evaluation must be undertaken before XLID is considered as a likely diagnosis, and a diagnosis of XLID is typically made in males once other causes of ID have been excluded (42). Consensus guidelines from the American Academy of Pediatrics, the American College of Medical Genetics, the American Academy of Neurology, and others suggest that the evaluation of a child with developmental delay or ID should include: a three-generation pedigree, a thorough clinical history, including pre- and perinatal history, and physical and neurological examinations that include particular examinations for minor anomalies or neurologic signs that may suggest a particular diagnosis (128–130). Environmental contributions to ID, particularly lead and prenatal alcohol exposure, must also be considered. A recommended first tier of laboratory testing for developmental delay includes fragile X testing and chromosomal microarray analysis (whole genome array-comparative genomic hybridization—array CGH), which has



TABLE 107-5 X-Linked Intellectual Disability Genes			
Gene	ID-Related Phenotype	MIM	Cytogenetic Location
<i>ABCD1/ALDP</i>	X-linked adrenoleukodystrophy	300371	Xq28
<i>ACSL4/FACL4</i>	NS-XLID	300157	Xq22.3
<i>AGTR2</i>	XLID with optic atrophy NS-XLID	300034	Xq23
<i>ARHGEF6/αPIX</i>	NS-XLID	300267	Xq26.3
<i>AP1S2</i>	Fried syndrome NS-XLID	300629	Xp22.2
<i>ARHGEF9</i>	XLID and sensory hyperarousal	300429	Xq22.1
<i>ARX</i>	NS-XLID Hydranencephaly with abnormal genitalia Agenesis of the corpus callosum with abnormal genitalia X-linked lissencephaly with abnormal genitalia West syndrome/infantile spasms X-linked syndrome X-linked myoclonic epilepsy with spasticity and intellectual disability Partington syndrome	300382	Xp22.13
<i>ATP6AP2</i>	XLID with epilepsy	300556	Xp11.4
<i>ATP7A</i>	Menkes disease Occipital horn syndrome	300011	Xq12–q13
<i>ATRX/XNP/XH2</i>	X-linked alpha–thalassemia–ID syndrome XLID-hypotonic facies syndrome	300032	Xq13
<i>BCOR</i>	Syndromic microphthalmia	300485	Xp11.4
<i>BRWD3</i>	XLID with macrocephaly	300553	Xq13
<i>CASK</i>	XLID with microcephaly and cerebellar hypoplasia XLID with nystagmus	300172	Xp11.4
<i>CDKL5/STK9</i>	Early infantile epileptic encephalopathy Atypical Rett syndrome	300203	Xp22
<i>CUL4B</i>	XLID with macrocephaly, short stature, seizures, aggression Cabezas syndrome	300304	Xq23
<i>DCX</i>	Lissencephaly Subcortical band heterotopia	300121	Xq22.3–q23
<i>DKC1</i>	Dyskeratosis congenita Hoyeraal–Hreidarsson syndrome	300126	Xq28
<i>DLG3</i>	NS-XLID	300189	Xq13.1
<i>DMD</i>	Duchenne muscular dystrophy	300377	Xp21.2
<i>FANCB</i>	Fanconi anemia X-linked VACTERL hydrocephaly	300515	Xp22.3
<i>FGD1/FGDY</i>	Aarskog–Scott syndrome NS-XLID	300546	Xp11.21
<i>FLNA/FLN1</i>	X-linked dominant periventricular heterotopia Otopalatodigital syndrome Frontometaphyseal dysplasia Melnick–Needles syndrome X-linked cardiac valvular dysplasia Terminal osseous dysplasia	300017	Xq28
<i>FMR1</i>	Fragile X syndrome	309550	Xq27.3
<i>FMR2</i>	NS-XLID	300806	Xq28
<i>FTSJ1</i>	NS-XLID	300499	Xp11.23
<i>GDI1</i>	NS-XLID	300104	Xq28
<i>GK</i>	Glycerol kinase deficiency	300474	Xp21.3–p21.2
<i>GPC3</i>	Simpson–Golabi–Behmel syndrome	300037	Xq26
<i>GRIA3</i>	NS-XLID	305915	Xq25–q26
<i>HCCS</i>	Microphthalmia with linear skin defects syndrome	300056	Xp22
<i>HPRT1</i>	Lesch–Nyhan syndrome	308000	Xq26–q27.2
<i>HSD17B10/HADH2</i>	17-β-hydroxysteroid dehydrogenase deficiency XLID with choreoathetosis	300256	Xp11.2
<i>HUWE1</i>	XLID with macrocephaly	300697	Xp11.2
<i>IDS</i>	Mucopolysaccharidosis type II	300823	Xq28
<i>IGBP1</i>	Agenesis of the corpus callosum with ID, ocular coloboma, and micrognathia	300139	Xq13.1–q13.3

Continued

TABLE 107-5 X-Linked Intellectual Disability Genes—Cont'd

Gene	ID-Related Phenotype	MIM	Cytogenetic Location
<i>IKBKKG/NEMO</i>	Incontinentia pigmenti	300248	Xq28
<i>IL1RAPL1</i>	NS-XLID	300206	Xp22.1–p21.3
	Autism spectrum disorder		
<i>IQSEC2</i>	NS-XLID	300522	Xp11.22
<i>JARID1C/KDM5C</i>	NS-XLID	314690	Xp11.22–p11.21
	XLID with short stature		
<i>KIAA1202/SHROOM4</i>	Stocco dos Santos XLID syndrome	300579	Xp11.2
<i>KIAA2022</i>	XLID with progressive quadriparezia	300524	Xq13.2
<i>KLF8/ZNF741</i>	NS-XLID	300286	Xp11.21
<i>LAMP2</i>	Danon disease	309060	Xq24
<i>L1CAM</i>	X-linked hydrocephalus	308840	Xq28
	X-linked spastic paraplegia/ MASA syndrome		
<i>MAGT1/IAP</i>	NS-XLID	300715	Xq13.1–q13.2
<i>MAOA</i>	Brunner syndrome	309850	Xp11.23
<i>MBTPS2</i>	Ichthyosis follicularis, atrichia, and photophobia (IFAP) syndrome	300294	Xp22.12–p22.11
<i>MECP2</i>	Rett syndrome	300005	Xq28
	Severe neonatal encephalopathy		
	XLID with progressive neurological involvement		
	NS-XLID		
<i>MED12/HOPA/TRAP230</i>	FG syndrome	300188	Xq13.1
	Lujan–Fryns syndrome		
<i>MID1</i>	Opitz syndrome	300552	Xp22
<i>MTM1</i>	X-linked myotubular myopathy	300415	Xq28
<i>NDP</i>	Norrie disease	300658	Xp11.4
<i>NDUFA1</i>	Mitochondrial complex I deficiency	300078	Xq24
<i>NHS</i>	Nance–Horan syndrome	300457	Xp22.13
<i>NLGN3</i>	XLID and autism	300336	Xq13
<i>NLGN4/KIAA1260</i>	XLID and autism	300427	Xp22.33
<i>NSDHL</i>	CK syndrome	300275	Xq28
<i>NXF5</i>	XLID	300319	Xq22.1
<i>OCRL1</i>	Lowe oculocerebrorenal syndrome	300535	Xq26.1
	Dent disease		
<i>OFD1/CXORF5</i>	Orofaciodigital syndrome I	300170	Xp22.3–p22.2
	Simpson–Golabi–Behmel syndrome, type 2		
	X-linked recessive Joubert syndrome-10		
<i>OPHN1</i>	XLID with cerebellar hypoplasia	300127	Xq12
	NS-XLID		
<i>OTC</i>	Ornithine transcarbamylase deficiency	300461	Xp21.1
<i>PAK3</i>	NS-XLID	300142	Xq21.3–q24
	XLID with microcephaly, speech defects, behavioral abnormalities		
<i>PCDH19</i>	Early infantile epileptic encephalopathy-9/Female-restricted epilepsy and ID	300460	Xp22
<i>PDHA1</i>	Pyruvate decarboxylase deficiency	300502	Xp22.2–p22.1
<i>PGK1</i>	Phosphoglycerate kinase 1 deficiency	311800	Xq13
<i>PHF6</i>	Borjeson–Forssman–Lehmann syndrome	300414	Xq26.3
<i>PHF8</i>	Siderius XLID syndrome/XLID with cleft lip and palate	300560	Xp11.2
<i>PLP</i>	Pelizaeus–Merzbacher disease	300401	Xq22
	Spastic paraplegia-2		
<i>PORCN</i>	Focal dermal hypoplasia/Goltz–Gorlin syndrome	300651	Xp11.23
<i>PQBP1</i>	Renpenning syndrome	300463	Xp11.23
	NS-XLID		
	ID with short stature		
<i>PRPS1</i>	Phosphoribosylpyrophosphate synthetase superactivity	311850	Xq22–q24
	Arts syndrome		
<i>PTCHD1</i>	XLID and autism	300828	Xp22.11
<i>RAB39B</i>	XLID and autism	300774	Xq28
<i>RPL10</i>	Autism spectrum disorder	312173	Xq28
<i>RSK2</i>	Coffin–Lowry syndrome	300075	Xp22.2–p22.1
	NS-XLID		

**TABLE 107-5 X-Linked Intellectual Disability Genes—Cont'd**

Gene	ID-Related Phenotype	MIM	Cytogenetic Location
<i>SLC6A8</i>	Creatine deficiency syndrome	300036	Xq28
<i>SLC9A6</i>	XLID with epilepsy and ataxia	300231	Xq26.3
<i>SLC16A2/MCT8</i>	Allan–Herndon–Dudley syndrome	300095	Xq13.2
<i>SMC1L1/SMC1A</i>	Cornelia de Lange syndrome	300040	Xp11.22–p11.21
<i>SMS</i>	Snyder–Robinson ID syndrome	300105	Xp22.1
<i>SOX3</i>	XLID with isolated growth hormone deficiency	313430	Xq26.3
<i>SRPX2</i>	Rolandic epilepsy with ID and speech dyspraxia	300642	Xq21.33–q23
<i>SYN1</i>	Epilepsy with learning disabilities	313440	Xp11.4–p11.2
<i>SYP</i>	NS-XLID	313475	Xp11.23–p11.22
<i>TIMM8A/DPP</i>	Mohr–Tranebjaerg syndrome	300356	Xq22
<i>TM4SF2/TSPAN7</i>	NS-XLID	300096	Xp11.4
<i>UBE2A</i>	ID with seizures, absent speech, and urogenital anomalies	312180	Xq24–q25
<i>UPF3B</i>	NS-XLID	300298	Xq25–q26
	Syndromic XLID		
<i>ZCCHC12/SIZN1</i>	NS-XLID	300701	Xq24
<i>ZDHHC9</i>	XLID with marfanoid habitus	300646	Xq26.1
<i>ZDHHC15</i>	NS-XLID	300576	Xq13.3
<i>ZNF41</i>	NS-XLID	314995	Xp22.1–cen
<i>ZNF81</i>	NS-XLID	314998	Xp22.1–p11
<i>ZNF674</i>	NS-XLID	300573	Xp11.3
<i>ZNF711</i>	NS-XLID	314990	Xq21.1–q21.3

This information was compiled from OMIM, the Greenwood Genetics Center, and the International Workshop on Fragile X and X-linked Mental Retardation.

supplanted karyotyping in the cytogenetic analysis of individuals with developmental delay due to its increased diagnostic yield (2). Provided no diagnosis is achieved following this evaluation, the family history must be evaluated, with particular attention to individuals with learning problems, autism, psychiatric problems, or ID itself because they could share an underlying genetic etiology and could suggest a diagnosis. Although in classic XLID pedigrees, one would expect multiple affected males who are related through unaffected females, it may not be possible to document an extensive family history in every family, nor are the families always large enough to observe this pattern. Thus, XLID should be considered in males with ID of unknown etiology, and it should not be ruled out in females with ID.

Traditionally, XLID has been broken into two categories: syndromic and nonsyndromic. NS-XLID includes those families in which ID is the only consistent clinical feature, although it is clear that syndromic XLID is under-recognized by clinicians, in part because the additional phenotypic features can be subtle and because they may not be readily apparent at young age. As described below, the lines between these categories often blur as the genes for XLID are defined and as groups of patients with ID due to mutations in the same gene are compared. For the most part, we will approach this discussion from a gene-based standpoint because of the problems with categorization. However, although these categories are somewhat artificial, careful examination for the physical features that might suggest one of the syndromic forms of XLID is the only way to systematically direct molecular

diagnosis of XLID. In the absence of such clinical clues, the XLID genes can be ordered for molecular diagnostics based on what is known about the frequency of mutation in a population. However, as sequencing technologies have improved and as the costs of large-scale sequencing have gone down, there is now the possibility of sequencing all, or at least many, potential XLID genes in a diagnostic setting at one time.

### 107.3.2 XLID in Females

The focus tends to be on affected boys when we think of XLID, but XLID can also manifest in females. For some conditions, such as FXS, males are more likely to be affected than females, and the phenotype tends to be less severe in females that are affected, due to the presence of a wild-type copy of the ID gene on the second X chromosome in females. For other conditions, such as Rett syndrome, the majority of affected individuals are female. It was once believed that mutations in *MECP2* were not compatible with life in males. A variety of mutations in *MECP2* have now been described in males. The most common phenotype in males is a severe phenotype of neonatal-onset encephalopathy with microcephaly, but they can also have nonsyndromic ID (131). The classic Rett phenotype is still largely restricted to females (132).

### 107.3.3 Nomenclature

NS-XLID families are given sequential MRX numbers that are coordinated through the Human Genome

Organization Nomenclature Committee (<http://www.genenames.org/>). Genetic linkage to the X chromosome must be established before an MRX number is assigned. These numbers are withdrawn as the relevant mutations are identified in these families, and the families are reassigned a relevant gene name. Because these numbers are family-based, sometimes multiple families share an underlying mutated gene.

### 107.3.4 Gene Discovery

Large consortia have been at the forefront of gene discovery in XLID. Two key consortia in this endeavor are the International Genetics of Learning Disabilities Consortium (GOLD; <http://goldstudy.cimr.cam.ac.uk/>) and the EuroMRX cohort consortium ([www.euomrx.com](http://www.euomrx.com)). Both have collected large samples of families with probable XLID that were prescreened for FXS and cytogenetic abnormalities. An inheritance pattern consistent with XLID is a key aspect of the inclusion criteria for both consortia, necessitating multiple affected males in each family. Each group has performed extensive molecular and cytogenetic analyses in an attempt to find the underlying genetic abnormality in each family. In the EuroMRX cohort, putative X chromosome mutations have been identified in 12% of the families, and this fraction increases to over 40% in families with stronger evidence of X-linkage (6). Members of the GOLD consortium sequenced the coding exons of 718 X-chromosome genes in probands from each of 208 families with probable XLID (7). The sequence data generated from this analysis has revealed several new XLID genes.

### 107.3.5 Copy Number Variation

Sequence variation is not the only pathogenic variation responsible for ID; pathogenic copy number variants (CNVs) on the X chromosome are also quite common. Comparison of GOLD probands indicates that, in general, the ID is more severe in cases with pathogenic structural variation compared to those with pathogenic sequence variation or to those with an unknown cause (133). There is an excess of pathogenic duplications on the X chromosome, perhaps because deletions are lethal in males, who are hemizygous for the X chromosome (134). This excess could also be partially due to the relative ease of array CGH-based detection of X-chromosome duplications in males, compared to the detection of duplications on autosomes (134).

Using high-resolution array CGH, pathogenic CNVs on the X chromosome are found in approximately 5% of cases of ID in males (134,135). Pathogenic CNVs have so far been detected in 10% of the GOLD consortium families that have been analyzed. This includes 3.6% with *MECP2* duplications and 1.6% with duplications that include *HUWE1* and *HSD1710B* (133). However, it should be noted that these numbers could be inflated

because some of the GOLD consortium samples were specifically included in the Whibley et al. study due to the fact that they were already known to have a known pathogenic CNV. However, *HUWE1-HSD17B10* duplications have been reported by others and appear to occur in patients with nonsyndromic ID (136). Other studies have confirmed that—in males with ID—duplications at Xq28 that include *MECP2* are one of the most common sources of pathogenic CNV on the X chromosome. These are estimated to occur in ~1% of cases of unexplained ID in males (137). The *MECP2* duplication phenotype will be described further below.

### 107.3.6 Genetic Testing

CNV on the X chromosome should be detected in whole genome array CGH, which is part of the recommended first tier of testing in individuals with ID. The likelihood of finding a causative CNV on the X chromosome is increased when the affected male comes from a large family with demonstrated XLID, although these changes are also found in sporadic cases (135). They are also more common in syndromic ID compared to nonsyndromic ID (135).

Due to the large number of XLID genes and the lack of a defined phenotype associated with many of them, sequence-based testing for XLID has not been used widely in the genetic evaluation of ID, but this is likely to be increasingly common as we gain a more complete understanding of the XLID genes and as DNA sequencing costs decrease. For example, Emory Genetics Lab offers sequencing of more than 90 genes in a panel-based format designed for clinical testing of XLID. In a clinical diagnostic setting, one must keep in mind that the likelihood of finding a pathogenic sequence change or CNV will be lower than described by the GOLD and MRX consortia, because the inclusion criteria for these consortia increase the likelihood of a causative genetic change on the X chromosome. A broader group of idiopathic ID patients, as would be expected in a clinical setting, would include both X-linked and autosomal causes of ID, as well as more complex etiologies. Although additional clinical features can be used to direct the diagnostic flowchart of tests ordered and this can improve the success rate of finding mutations (6), at this time, the causative mutation will go undetected in a significant fraction of patients even when whole genome array CGH, Fragile X testing, and extensive sequence analysis of the X chromosome are performed.

Also of note for clinical diagnostic testing is the fact that XLID is a clinically heterogeneous condition, making it difficult to judge whether a rare sequence variant or CNV causes disease (138,139). Clinical interpretation of rare genetic variation is further complicated when parental samples, extended pedigrees, and/or large, ethnically matched control samples are unavailable (138). Indeed, disease-causing variants in the X-linked genes make up



only a fraction of the rare, nonsynonymous variants present on the chromosome (7). The high prevalence of nonrecurring, private genetic variation on the X chromosome that is observed in families with ID makes this a large challenge for diagnostics, even when the variation is truncating or de novo (140). In fact, nonpathogenic, truncating sequence variants were found in up to 1% of X genes in the GOLD consortium study (141). These were judged to be nonpathogenic, based on additional analysis of family members and controls (7). Additional studies designed to look at rare variation are needed in order to understand the sequence variation that each X-linked gene can tolerate in an intellectually normal individual.

As with genetic variation in the rest of the genome, interpretation of sequence variation and CNV on the X chromosome can be informed through examination of segregation of the variant through a family, searches of databases of known pathogenic and polymorphic variation, such as the Database of Genomic Variants (<http://projects.tcag.ca/variation/>), DECIPHER (<http://decipher.sanger.ac.uk/>), dbVar (<http://www.ncbi.nlm.nih.gov/dbvar/>) and dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>). Unique to the interpretation of X-chromosome variation is the use of analysis of X-inactivation patterns in carrier females. Skewed X-inactivation patterns in carrier females are common with XLID mutations (142), so the observation of skewed X inactivation in a female carrier of a genetic variant on the X chromosome can be used to support its role in ID.

Although we are still likely to come up empty-handed, determining the underlying genetic explanation of XLID is an important goal to pursue in the clinical setting. A molecular diagnosis can allow for more specific prognosis and medical management, it can put an end to unnecessary testing and evaluation, and—for the family—it allows for more accurate risk assessment and for referral to specific support groups. Even without a molecular diagnosis, the recognition that the ID in a family is likely to be X-linked changes the calculated reproductive risk for siblings of an affected male from 1–2 to 10% for sisters of two affected brothers (42). With a defined molecular diagnosis, this reproductive risk can increase to 50% following carrier testing in sisters of affected males with a known X-linked mutation or CNV.

### 107.3.7 XLID Genes

An overview of the genes that have been implicated in XLID can be found in Table 107-5. Because a full description of each of these genes is beyond the scope of this chapter, we restrict the following discussion to those genes that are believed to be the most commonly mutated or for which the underlying pathogenic mechanism is at least partially understood.

**107.3.7.1 ARX.** Mutations in ARX are one of the most common causes of XLID. In the EuroMRX sample, ARX

mutations accounted for 7.5% of families with an obligate carrier female and 1.5% of affected brother pairs (6). ARX encodes a homeobox-containing transcription factor, and mutations in the gene have been associated with a range of phenotypes, all of which share ID as a consistent feature (143). At the milder end of the spectrum are cases with nonsyndromic ID, but ARX mutations can also cause brain malformations, such as lissencephaly, hydranencephaly, or agenesis of the corpus callosum. ARX mutations have been implicated in various syndromes, including Proud syndrome (agenesis of the corpus callosum with abnormal genitalia; MIM 300004), hydranencephaly with abnormal genitalia (MIM 300215), X-linked lissencephaly, Partington syndrome (XLID with dystonic hand movements; MIM 309510), and X-linked West syndrome (early infantile epileptic encephalopathy; MIM 308350). In general, the more severe the mutation, the more severe the phenotype, but one must be aware that missense mutations in key residues can cause a phenotype at the more severe end of the spectrum (143). In families in which affected males have a non-malformation phenotype, carrier females are generally unaffected. In those families with a more severe phenotype due to ARX, carrier females can be affected, albeit more mildly (143).

By far, the most common pathogenic mutation in ARX is a recurrent 24-bp duplication in exon 2 that expands a polyalanine tract. This duplication mutation is most often associated with NS-XLID, but it can also be found in patients with other phenotypes on the milder end of the spectrum, such as West or Partington syndrome (144). Although the relatively large contribution of ARX mutations to XLID overall suggests it is valid to screen ARX in cases of idiopathic ID, the frequency of ARX mutations in sporadic cases of ID is thought to be quite low (145,146). The likelihood of finding an ARX mutation is increased in patients in whom epilepsy or dystonia accompanies their ID (147).

**107.3.7.2 MECP2.** Mutations in MECP2 were originally reported in girls with Rett syndrome, and it was believed that, in boys, mutations in the same gene were not compatible with life (148). Subsequent research indicated that MECP2 mutations could also be found in boys, most often manifesting as a severe neonatal encephalopathy, but also as nonsyndromic ID (149). Early population-based studies suggested this might be as common a cause of XLID as FXS (150), but other groups have found that MECP2 mutations are not a common cause of NS-XLID and that interpretation of MECP2 sequence results is complicated by the existence of rare sequence variation in MECP2 that can be ruled out as pathogenic through careful segregation analysis in affected families (151). Some have suggested that MECP2 sequencing is not indicated in boys with ID unless there is unexplained familial XLID, if the boy had severe congenital encephalopathy, or if there is also a girl in the family with Rett syndrome (152). Others advise

*MECP2* sequence analysis in males with ID if there are progressive neurological manifestations (153). *MECP2* mutations have also been found in individuals with an Angelman syndrome-like phenotype and are believed to account for perhaps 3% of individuals referred for Angelman syndrome testing who did not have a methylation defect at chromosome 15q11–q13 (154).

Submicroscopic duplications at Xq28 that range in size from 0.3–4Mb and encompass the complete *MECP2* gene have been reported in males with ID (155). These individuals typically have moderate to severe ID and infantile hypotonia, as well as ataxia or an ataxic gait (137). Other features can include autistic features, recurrent infections, epilepsy, progressive spasticity, and sometimes developmental regression (155). As mentioned above, *MECP2* duplications account for ~1% of XLID, but this fraction may approach 15% in samples of males with ID and additional features of *MECP2* duplication syndrome, such as progressive spasticity (155). The duplication is also found in ~2% of males who were referred for *MECP2* mutation analysis but were negative for a mutation (137).

Although there is variable expressivity, the *MECP2* duplication syndrome is 100% penetrant in carrier males (155). The majority of affected males inherit the duplication from carrier females, who exhibit extreme to completely skewed X inactivation (156). Although carrier females do not have the same syndrome as their affected sons, they are likely to have psychiatric symptoms that can include anxiety, depression, and compulsive behaviors (156).

**107.3.7.3 SLC6A8.** The *SLC6A8* gene at Xq28 encodes a creatine transporter. Mutations in this gene cause one of the three cerebral creatine deficiency syndromes, which all share ID, speech/language delay, and seizures as common features. Males with *SLC6A8* mutations have ID that can range from mild to severe; the phenotypic spectrum is—not surprisingly—broader in carrier females, some of whom exhibit no obvious phenotype, whereas an estimated 50% have learning and behavioral problems that can range from mild to severe (157,158). Studies of families with XLID, or at least affected brother pairs, suggest that mutations in *SLC6A8* are one of the more frequent causes of XLID, accounting for an estimated 1–2% of XLID (159–162). Mutations in *SLC6A8* are associated with an increased creatine:creatinine ratio in urine, so it has been suggested that urine screens be used in males with ID to determine those that are a good candidate for *SLC6A8* sequence analysis (159). The creatine signal in the brain can also be assessed using proton magnetic resonance spectroscopy, and a low or absent signal can be used to guide molecular testing in boys with developmental delay, as well (163). Although creatine supplementation is used to treat the autosomal recessive forms of creatine deficiency, this is not of value in patients with the creatine transporter defect (157).

**107.3.7.4 Additional Genes.** Efforts to sequence large proportions of the X chromosome in large samples of XLID families have yielded several new XLID genes. Mutations in some of these genes are estimated to account for a significant proportion of XLID families, although, for many of the genes, it remains to be seen whether these estimates hold true when additional less-selected samples are tested. For virtually all of the genes, it is not known how frequently mutations will be found in sporadic cases of ID. These genes include: *CASK*, *PQBP1*, *IQSEC2*, *OPHN1*, and *JARID1C*. As XLID sequencing panels are developed and used in a more random sample of people with ID, we will have further insight into the relative frequency of mutations in each of these genes and into the clinical spectrum associated with mutations in each gene. Deletions and duplications have also been reported that include some of these genes, but, again, the frequency of these copy number changes in individuals with ID is unknown. As is indicated below, certain aspects of the clinical picture can be used to direct molecular testing for XLID, but until we collect a significant number of patients with mutations in any particular gene, it is hard to clearly define the phenotypic spectrum associated with mutations in any one gene, so, to begin, we must cast a fairly wide net in looking for mutations, although this will generally result in a low yield (164).

*CASK* encodes calcium-calmodulin-dependent serine protein kinase. Mutations in *CASK* were originally described in girls with severe ID, microcephaly, and cerebellar hypoplasia, and a boy who died at 2 weeks of age, leading to the idea that *CASK* mutations are dominant with reduced viability in boys (165). Subsequent research indicates that this may be true for severe, loss-of-function mutations in *CASK*, but that reduced function mutations are found in boys with ID. Tarpey et al. (7) found mutations in boys from four of 358 XLID families (1.1%) in the GOLD consortium sample. Congenital nystagmus was present in affected individuals from two of the original families, which led the researchers to subsequently look for mutations in *CASK* in 45 additional probands with ID and either nystagmus or microcephaly; 2 of the 45 probands (4.4%) had mutations in *CASK* (166). Thus, the presence of nystagmus in a boy with ID may be considered an indicator for *CASK* sequence analysis.

The GOLD consortium has also provided evidence that *CUL4B* could be a significant cause of XLID (167). They identified mutations in 8 out of 250 XLID families (3%). *CUL4B* encodes a ubiquitin E3 ligase. In addition to ID, boys with mutations tend to have macrocephaly, short stature, seizures, and aggressive outbursts (167).

The *IQSEC2* gene is another gene identified by extensive sequencing in the GOLD families (168). Four missense mutations were identified in 208 XLID families (1.9%). Mutations appear to be associated with language delay, seizures, and autistic features.

Mutations in *OPHN1* were first found to cause ID in the EuroMRX sample. *OPHN1* encodes oligophrenin 1,

a Rho-GTPase-activating protein. Mutations have been found in probands from families considered to have NS-XLID, but MRIs of affected individuals have documented cerebellar hypoplasia and dysplasia of the vermis (169). This led Zanni et al. to suggest that neuroradiologic investigation of individuals with ID be used to detect subtle brain abnormalities that could be used to direct molecular diagnostics (169). In the EuroMRX set, three of 63 families with obligate female carriers (4.8%) have mutations in *OPHN1*, and a similar fraction of families with affected brother pairs in their sample (3 of 65; 4.6%) have mutations in this gene (6).

Mutations in *PQBP1* have been found in both syndromic and nonsyndromic forms of XLID. This includes Renpenning syndrome, an XLID syndrome that includes microcephaly, small testes, and short stature (170). There is a wide clinical spectrum associated with mutations in *PQBP1*, and this can also include congenital heart defects and craniofacial abnormalities (171) and microphthalmia (172). Of 111 XLID families with obligate carrier females in the EuroMRX sample, de Brouwer et al. found five with mutations in *PQBP1* (4.5%); on the other hand, no mutations were found in 73 families with affected brother pairs or in 465 sporadic male cases of ID (6). A study by Jensen et al. again found a lower mutation frequency (0.7%) in families with fewer affected boys; one of 135 families with no more than two affected brothers was found to have a *PQBP1* mutation (160).

Mutations in *JARID1C* were originally found in 5 of 179 families from the EuroMRX sample (2.8%) (173). Further analysis of 144 NS-XLID families also collected through EuroMRX identified five *JARID1C* mutations, a frequency of 3.5% (174). This frequency did not hold up in all samples; Abidi et al. found one mutation out of 172 general XLID probands (0.6%) (175). Use of short stature as a selection criterion might increase this frequency; 1 of 92 probands with ID and short stature (1.6%) was found to have a *JARID1C* mutation (175). Finally, 2 of 208 XLID families (1%) in the GOLD sample had a mutation in *JARID1C* (176). Pooling data from all published individuals with a mutation in *JARID1C* indicates they have ID that ranges from mild to severe, as well as a clinical picture that can include short stature, seizures, hyperreflexia, and microcephaly (176). Carrier females can be mildly affected.

### 107.3.8 Disease Mechanisms

**107.3.8.1 ATRX.** Mutations in *ATRX* cause a spectrum of ID phenotypes with the classic presentation being the alpha-thalassemia XLID (*ATRX*) syndrome. *ATRX* syndrome can be recognized by characteristic facial features in the presence of (typically) severe ID and an unusual form of alpha-thalassemia. The facial features include midface hypoplasia, a tented upper lip and full, everted lower lip, upswept frontal hair, and a protruding tongue (177). The alpha-thalassemia is unusual in

that peripheral red blood cells contain hemoglobin H inclusions that can be used in the diagnosis of the syndrome (178). Mild skeletal abnormalities can also be present, such as clinodactyly, brachydactyly, tapered fingers, pes planus, clubfoot, and scoliosis (177). Postnatal microcephaly, sensorineural deafness, and seizures may also occur (178). Although affected individuals have a 46,XY karyotype, genital abnormalities are common and range from hypospadias to external female genitalia (pseudohermaphroditism). The range of syndromes associated with *ATRX* mutations encompasses: Carpenter–Waziri syndrome, Holmes–Gang syndrome, Chudley–Lowry syndrome, and XLID with spastic paraplegia, and these can be considered part of the same spectrum (179). Female carriers are intellectually normal and do not have consistent physical manifestations, due to marked skewing of X inactivation (177).

The *ATRX* protein is a member of the SWI/SNF family of DNA helicases that remodel chromatin. *ATRX* plays roles in sister chromatid cohesion during mitosis and in regulation of gene expression (180). The MECP2 protein recruits *ATRX* to pericentromeric heterochromatin (181), and these proteins seem to work in conjunction with cohesin to regulate postnatal expression of certain imprinted genes in the brain (180). The majority of mutations associated with *ATRX* syndrome are point mutations in the exons encoding the helicases and zinc-finger domains, although mutations do exist in other parts of the gene (179). Duplications and deletions of the gene have been reported less frequently.

The severity of the alpha-thalassemia component of the *ATRX* syndrome can be quite variable, even within a single family, and is due to decreased expression of the  $\alpha$ -globin locus on chromosome 16. This variability was puzzling given that individuals within a family would share the defect in *ATRX*. *ATRX* binds to a variety of sites across the human genome, including many gene promoters associated with CpG islands (182). One of these is a region approximately 1 kb upstream of the  $\alpha$ -globin gene *HBM* within a variable nucleotide tandem repeat (VNTR). The length of this VNTR is quite variable within the human population, and it is this second site variability that determines the severity of alpha-thalassemia in individuals with the *ATRX* syndrome. In the absence of *ATRX* expression, the VNTR exerts a repressive effect on transcription of *HBM*, and the longer the VNTR, the greater the reduction in  $\alpha$ -globin expression (182).

**107.3.8.2 XLID Pathways.** Many of the XLID genes encode proteins involved in basic cellular processes, including transcription regulation, metabolism, RNA processing, DNA metabolism, telomere maintenance, and the ubiquitin cycle (183). Several examples exist in which multiple ID genes work together in the same pathway, providing a potential common underlying mechanism for the development of ID. As mentioned above, MECP2 recruits *ATRX* to certain heterochromatic regions and works with the cohesin complex, a component of which



is encoded by *SMC1L1*, to regulate gene expression (180). Another example involves the XLID genes *PHF8*, *ZNF711* and *JARID1C*. *PHF8* is a demethylase that acts on di- and monomethylated lysine 9 of histone H3 that binds near transcription start sites of active or poised genes (184). Chromatin immunoprecipitation followed by DNA sequencing indicates that *PHF8* binds to a number of XLID genes, including *JARID1C*, *ZNF41*, and *ZNF81*. *PHF8* is recruited to some of these sites by the zinc-finger protein *ZNF711*, which is encoded by another XLID gene. The two proteins, together, bind at *JARID1C* and influence its expression (184). The linking of XLID genes in the same cellular pathway adds to our understanding of the processes that lead to ID and hint that other components of the same pathways may also be candidate ID genes.

## 107.4 CONCLUSION

The development of array CGH and the increase in our DNA sequence capabilities has led to an explosion in the number of genes and genomic regions that have been implicated in XLID. Right now, we are at a point where we have greater ability to identify CNVs and sequence variants than to interpret them, leaving us at a disadvantage in clinical diagnostics for XLID. In order for us to fully understand the tolerance for genetic variation on the X chromosome, it is crucial that sequence and copy number analysis could be performed in additional large samples of individuals with ID, particularly sporadic cases, and this could be linked to our understanding of variation in the normal human genome, which is coming from projects such as the Thousand Genomes Project, so that we can fully understand the tolerance of the X chromosome for variation. However, we should not downplay the fact that we can uncover the relevant genetic change in an increasing number of cases of ID, both inherited and sporadic, and that sequence analysis of a large number of XLID genes is available on clinical testing panels.

## CROSS REFERENCES

Human Gene Mutation in Inherited Disease; Molecular Mechanics and Clinical Consequences; Genetic Counseling and Clinical Risk Assessment; The Genetic Basis of Female Infertility; Abnormal Mental Development; Attention Deficity Hyperactivity Disorder and Other Behavioral Disorders; Autism and Autism Spectrum.

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## RELEVANT WEBPAGES

- Online Mendelian Inheritance in Man (OMIM): <http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim>.
- GeneReviews: <http://www.ncbi.nlm.nih.gov/sites/GeneTests/review?db=genetests>.
- XLID Update from Greenwood Genetics Center: <http://www.ggc.org/xlrm.htm>
- International Genetics of Learning Disabilities Consortium: <http://goldstudy.cimr.cam.ac.uk/>.
- The European Mental Retardation (EuroMRX) Consortium: <http://www.euomrx.com/>.
- Database of Genomic Variants: <http://projects.tcag.ca/variation/>.
- DECIPHER Database of Submicroscopic Chromosomal Imbalances: <http://decipher.sanger.ac.uk/>.
- dbVar Database of Genomic Structural Variation: <http://www.ncbi.nlm.nih.gov/dbvar/>.
- dbSNP Database of Single Nucleotide Polymorphisms: <http://www.ncbi.nlm.nih.gov/SNP/>.

## FURTHER READING

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## Biographies



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# CHAPTER

# 108

## Dyslexia and Related Communication Disorders

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### ABBREVIATIONS

SRD – specific reading disability  
SSD – speech sound disorder  
SLI – specific language impairment

### 108.1 INTRODUCTION

Nonsyndromic language/learning disorders are heterogeneous in presentation or symptomatology, life course, and etiology. Diagnostic criteria may vary depending on the examiner and the reason for the examination. Criteria that qualify a child for special education for learning disabilities may be quite different from the criteria for research into the underlying causes. Furthermore, there are no biologic tests for these types of disorders to date. Nevertheless, standards for diagnosis (e.g. the Diagnostic and Statistical Manual of Mental Disorders, 4th edition text revision (DSM-IV-TR)) do exist, and there is convincing evidence for organic and genetic etiologies. This chapter deals with the most common nonsyndromic learning disorder that is likely to confront physicians and geneticists: specific reading disability (SRD or dyslexia). Dyslexia is the most intensively studied childhood learning disorder from a genetic standpoint and appears to be related to other language and learning disorders, thus providing a model for further research into their causation and etiology.

### 108.2 DEFINITION

Specific reading disability is an unexpected difficulty learning to read (and/or spell) despite adequate intelligence and

opportunity and without demonstrable sensory, psychiatric, or neurologic factors that could explain the disorder. This definition, like that for many learning disorders, is exclusionary in that other causes of reading problems have been ruled out (e.g. emotional problems or environmental deprivation). It is noteworthy that there is a push in the field to start to define SRD more in terms of inclusionary symptoms, although a finite or gold-standard set of inclusionary criteria has yet to be accepted and there are arguments to support the idea that at least some exclusionary criteria may always be needed (45).

The diagnosis of SRD usually requires a measured discrepancy between reading or spelling ability and the level of the skills that would be expected based on age or intelligence. For example, the US federal definition of learning disabilities specifies a discrepancy of 1 standard deviation (SD) in achievement (e.g. reading) and ability (e.g. a standard measure of intelligence) (181); these are often used as criteria to qualify a child for additional educational services. Other definitions have required varying deficits in reading achievement, such as 2 SD below the mean for age or 2 years' delay for age or grade (116). Of course, this variation in phenotypic definition will affect estimates of prevalence.

A task force of investigators representing the International Dyslexia Association and the National Institutes of Health adopted the following version of a research definition of dyslexia in 2002.

Dyslexia is a specific learning disability that is neurological in origin. It is characterized by difficulties with accurate and/or fluent word recognition and by poor



spelling and decoding abilities. These difficulties typically result from a deficit in the phonological component of language that is often unexpected in relation to other cognitive abilities and the provision of effective classroom instruction. Secondary consequences may include problems in reading comprehension and reduced reading experience that can impede growth of vocabulary and background knowledge (99).

SRD is essentially the same as dyslexia, although the term dyslexia has been avoided by some because of additional connotations that have been attached to it in the past, such as right-left confusion, left-handedness, and reversals of letters and words. More recent studies have shown that these associated problems are certainly not pathognomonic of dyslexia and may not be greatly increased over the general population (9,12,116). Alternate terms are “specific dyslexia,” which emphasizes the lack of deficit in other areas of cognitive functioning; “developmental dyslexia,” to differentiate it from acquired causes such as trauma or stroke; and “reading retardation.” In contrast, “reading backward” or “garden-variety poor reading,” has been used when the reading level is consistent with generalized cognitive delays (44,116,142).

Initially, there was debate as to whether dyslexia actually exists as a separate learning disorder (44,116,141,157). It was argued that the poor reading in children with below-average intelligence quotient (IQ), or in whom there is no significant IQ-achievement discrepancy, is not qualitatively different from the poor reading seen in dyslexia (157). Some researchers maintained that, if dyslexia is etiologically distinct, it should produce a “hump” in the lower end of the otherwise continuous distribution of reading scores in children (169,192), but several studies did not detect such a hump (137,157).

As will be reviewed later in this chapter, genetic and linkage studies hold promise toward defining the phenotype more precisely; for example, the heritability of dyslexia is greater in a population selected for higher IQ (185), suggesting that broader definitions of SRD may include a number of factors that may depress cognitive functioning overall that are not specific to SRD. Moreover, at least for a locus on chromosome 6p22, linkage is more significant in populations with more severe dyslexia (49,55) or higher IQ (85).

### 108.2.1 Prevalence

Estimates of the prevalence of SRD depend on the definition used, the age of the population, and the language or culture examined. For example, more children with reading problems will be identified in first grade because of different rates of maturation; however, by third grade, many of those children will have caught up with their peers in reading ability. This catch-up phenomenon was demonstrated by Shaywitz and colleagues (157) and cited as evidence that SRD is an unstable trait. Huston (76) argued in response to this study that those children

who improved between first and third grade did not have dyslexia; those with persistent problems were the true dyslexics. Moreover, test-retest unreliability, which was not taken into account, could make a substantial contribution to the instability of the dyslexia diagnosis over time. Overall, typical estimates of prevalence of dyslexia in school children in third grade or beyond have been 5–10% (5,83). Similar estimates have been found for logographic languages such as Chinese (86). If a broader definition of poor reading is used that does not require a significant discrepancy with nonreading abilities, the prevalence may be as high as 20–30% (6,34); in contrast, in populations in which the written language is more phonetically consistent, such as Italian, the frequency of diagnosis of dyslexia is lower (115,181).

### 108.2.2 Sex Ratio

Clinically referred populations generally show male:female ratios from 3:1 to 6:1, but this appears to be due mainly to biases in referral favoring males, or to the effects of increased severity in males. In systematically ascertained populations of boys and girls, the male-to-female differences have not been as pronounced. For example, Shaywitz and associates (156) diagnosed dyslexia in 8.7% of the boys and 6.9% of the girls in a second-grade epidemiological sample, while their teachers identified 13.6% of the boys and 3.2% of the girls as disabled readers. Similarly, Wadsworth and colleagues (184) found equal proportions of affected boys and girls when the probands were ascertained by research criteria, rather than being part of a clinically referred population. In affected members of families ascertained through an SRD proband, the sex ratios are typically around 1.3–1.7:1, once ascertainment bias is corrected by elimination of probands. Although the trend toward excess males may remain, typically the sex ratios in well-defined research samples are not significantly different from 1:1 (24,133).

### 108.2.3 Natural History and Life Course

Although generally considered to be a school-age disorder, there is evidence that underlying language-based problems can be noted in the first few years of life, and signs and symptoms of dyslexia persist into adulthood. In a unique prospective study Scarborough (145) ascertained a sample of 34 children aged 2 years with a positive family history of reading disability, and matched them to 44 children with a negative family history. They were given a series of language tests over a 6-year span, and reading ability was assessed in second grade. As would be expected from the familial nature of SRD (see following paragraphs), 22 of the 34 children with a positive family history became poor readers, while only 2 of the 44 children of control families were poor readers. Moreover, the 22 poor reading children with a positive family history of SRD showed early language problems

that were not seen in the other 12 high-risk children who became normal readers. In fact, the early language abilities of the children who later became normal readers were indistinguishable from the controls. At 30 months of age, the children who became dyslexic had deficits in syntax that were correlated with later phonologic problems. At 42 months, they had poorer vocabulary scores, and at 5 years of age, they had deficits in vocabulary and letter/sound correspondence. By contrast, receptive phonologic skills were normal. Perhaps the most interesting finding of this study is that the dyslexic children showed a marked disinterest in books even before reading instruction was introduced to them. Differences in how often parents read to them were not large enough to account for this difference; rather, the children over 36 months of age were much less likely to seek out books themselves (146). The extent to which this lack of interest in reading is genetic is unclear, however. It is possible that this represents active genotype/environmental correlation (147); that is, a child with genetic susceptibility to SRD may tend to select nonreading activities. This has been referred to as “niche-picking” (61,126).

Prior to school age it is not uncommon to observe oral language problems in children who develop reading disability, and some have estimated that approximately 50% of SRD children had language problems before attempting to learn to read (17). The primary reading-related symptoms of SRD are noted in the first few school grades. These symptoms are particularly evident in oral reading and in learning to spell phonetically, and the children may produce unusual reading and spelling errors. Specifically, they produce a higher proportion of phonologically inaccurate errors than non-SRD children. In reading, they will substitute words that begin with the same letter as the target word, but otherwise are pronounced differently. In spelling, they will produce a misspelling in which consonants are missing, added, or substituted. Reading comprehension is closer to normal, particularly when the child is not pressured to read quickly. As reviewed by Pennington (116) and Lefly and Pennington (88), the correlated features of dyslexia, related to the underlying language disability, are articulation problems, problems with naming, and verbal memory deficits. Naming and verbal memory deficits may be secondary to the reading and phonological problems, since these skills are not deficient when compared to younger reading-age-matched controls. Letter and number reversal, usually thought of by lay people as important signs of dyslexia, are actually secondary to the phonologic confusion between the sounds the letters represent and are, in fact, developmentally normal early on as the child acquires reading skills. In contrast to the language difficulties, ability in arithmetic is often much better, as long as reading-related skills are not required.

The reading and spelling problems seen in childhood generally persist in the later grades. In a longitudinal study in which children with SRD were periodically

tested into their late teen years, reading ability stayed below that of controls. The gap between the two groups actually widened for symbol processing speed, perhaps because of lack of remediation in this particular skill (86). Similarly, a longitudinal study by Shaywitz and associates (155) documented that twelfth graders who had reading disability since elementary school still showed reading delays. These were related to phonological deficits (that is, the auditory, “phonetic” aspects of language, discussed in detail later in this chapter), and reading rate remained slow. Interestingly, reading comprehension was not important in discriminating the poor readers from average or superior readers. Although the high school seniors with reading disability had poorer academic achievement, there was no indication of behavioral problems. Two other studies of morbidity of behavior problems and reading disability also showed that the two were independent, and that often the behavior problems could be noted to predate reading disability (37,142).

Finally, SRD has been documented to have an effect on adult outcome. Finucci (40) reviewed a number of follow-up studies, most of which indicated a detrimental effect of SRD on educational attainment and occupation; however, several studies, including her own, indicated that severity of the dyslexia, IQ, and socioeconomic status also had significant influence on outcome. For example, individuals with dyslexia could succeed in college, but they generally took longer to complete the requirement for a degree and chose occupations with less demand on reading.

It has been questioned whether dyslexia represents a “developmental lag,” which would mean that children with SRD actually read like younger normal children and eventually attain more age-appropriate reading ability at a later age, or whether it represents a deficit that is qualitatively different and that is persistent into adulthood. The studies previously cited by Finucci (40), LaBuda and DeFries (86), and Shaywitz and associates (155) demonstrate that the effects of SRD do persist into adulthood, with a plateauing of reading ability. A number of other studies have also reported continued reading problems or have found deficits in underlying skills in adults with histories of SRD, including phonologic processing, selective auditory attention, naming, and short-term memory similar to those found in children with SRD (10,11,110,143,154). Others have noted that adults may exhibit a variety of symptoms related to their learning disability but not obviously or commonly associated with, say, a reading problem. These “subtle” clinical markers that may indicate a referral for a learning assessment include frequent tardiness, difficulty with mental math, avoidance of reading, poor sense of direction, and forgetfulness, among others (see Appendix 5 of Reference (135)).

Some adults do fully compensate for their childhood reading problems; that is, they attain expected reading

levels and are no longer diagnosable as dyslexic via standardized tests of reading/spelling-related errors. Several studies have reported compensation rates of approximately 22%, with slightly more females compensating than males (61,88,119,143,144). While the reading level that SRD children attain as adults may be related to IQ, education, and SES (36,59,63,143,144), underlying problems can still persist independent of these factors. For example, Lefly and Pennington (88) found that compensated adults who had reading levels similar to those of control adults had deficits in reading speed that were comparable to those in adults who were still dyslexic.

Most studies of adult outcome and compensation have relied on a self-reported diagnosis of dyslexia. While self-report is fairly reliable (63), the degree and specificity of the childhood reading problems often cannot be documented. The follow-up studies of Wood and Felton and their colleagues at Bowman-Gray School of Medicine are unique in that childhood test results are available on a group of adults diagnosed as dyslexic in childhood between 1957 and 1972. This has allowed the researchers to relate adult outcome to the severity of the SRD in childhood. Felton and colleagues (36) found that the severity of the reading problems in childhood predicted severity as an adult, while more mildly affected children were more likely to become compensated adults. Adults who were still dyslexic performed poorly on several neuropsychological tests compared to controls, but they were also found to have somewhat lower IQ, socioeconomic status, and educational achievement. When these factors were controlled, only phonologic tasks and rapid verbal retrieval were still significantly impaired.

One caveat in accepting outcome and rates of compensation from older studies is that the methods of remediation may have been less effective than the phonologically based methods currently used. In fact, Shaywitz and colleagues (155) noted that the remediation that had been available to their twelfth grade students when they were in elementary school in the early 1980s was sporadic and inconsistent in approach, which may have contributed to some of the persistence of reading problems.

### 108.3 PRIMARY COGNITIVE DEFICIT OF SPECIFIC READING DISABILITY

In the history of research in reading disability, it has generally been assumed that SRD is etiologically heterogeneous, and that identification of subtypes would aid in the development of appropriate remediation strategies for each type. Classification systems were developed based on clinical features or theoretical models of the reading process. Initially, the subtypes generally related to whether the predominant errors in reading and spelling seemed to reflect phonological problems (“auditory dyslexia,” “dysphonetic dyslexia,” “deep dyslexia”) or problems with visualization of words (“visual dyslexia,” “dyseidetic dyslexia,” “orthographic

dyslexia,” “surface dyslexia”). Phonologic problems can be conceptualized as difficulty attaching sounds to letters, resulting in particular problems sounding out unfamiliar words. This can be assessed by the ability to read “nonsense” words, which are phonetically regular and pronounceable, but are not real words, so that visual pattern recognition of the word is not involved in reading it. Conversely, orthographic problems are characterized by difficulty reading words based on their appearance, so that irregularly spelled words are not recognized, but words can be sounded out. This is tested by the ability to read “irregular” words in which the pronunciation does not match the phonetics, such as the word “yacht.” These words must be recognized by their appearance, since use of a phonological approach will be misleading. (Note that orthographic disability will be a problem primarily with languages with irregular spelling patterns, such as English.) These contrasts between phonological and orthographic abilities are also seen in acquired alexia, which is an inability to perceive/read written words, related to specific lesions of the brain (15). However, when subtypes of dyslexia were defined and children were categorized by whether they showed phonological or orthographic errors, it was found that the assignment was not always consistent within families or even in the same child over time, leading Snowling (165) to conclude that subtype classifications appeared to have more descriptive rather than etiologic utility. The contrast between phonological and orthographic processes has remained, however, in the efforts to determine if these represent two independent pathways to reading and if one or the other is more basic and more heritable.

Pennington and colleagues (121) have reviewed the theories that have related phonology and orthography. Initially, theories of learning to read took a hierarchical approach; normal readers were thought to rely first on phonologic skills, through slow and methodical “sounding out.” This process would reach a plateau and would be superseded by reliance on faster orthographic processes to facilitate word recognition. Dyslexics, it was postulated, could be blocked at either the phonologic or orthographic stage. This led to methods of remediation that concentrated on enhancing the orthographic stage, by teaching whole-word recognition. The results were dismal, and further research demonstrated that even skilled, mature readers utilized phonology for reading. In fact, the “dual process” theories of reading further declined when it appeared that phonological skills were deficient almost universally in reading-disabled children and adults, even when compared to younger controls reading at the same level as the dyslexics. Consensus built that phoneme awareness, the ability to understand and manipulate the basic sounds of language, was likely to be the primary deficit in reading. When matched on word reading and spelling accuracy, dyslexics do make more orthographically plausible errors in their spelling

attempts than younger controls whose errors tend to be more phonologic in nature, suggesting that orthographic knowledge is influenced by greater experience with text (48). The predominance of phoneme awareness as an underlying deficit in reading disability did not mean that orthographic skills were normal, however. In the majority of disabled readers, both skills were delayed (15).

In an effort to resolve the relationship between these two processes, Pennington and colleagues (118) and others have drawn upon the connectionist theory of Seidenberg and McClelland (153). This is a type of neural network design in which the input is the visual image of the word and the output is the recognition and pronunciation of the word. A three-layer network is hypothesized that contains both phonological and orthographic layers, and through the learning process, the network develops the interconnections to create a balance between both processes in deciphering a word. If any of the interconnections are faulty, the input is degraded, or there is too much reliance on one process at the expense of another, reading problems result (101). More detailed cognitive models are reviewed by Ramus (130).

It is important to mention two other aspects of reading disability that may constitute etiological subtypes. Wolff and colleagues (summarized in Reference (189)) have noted that about 50% of familial dyslexics show impairment in precise coordinated motor action in both manual fine-motor tasks and in speech, and that this constitutes a subtype that is consistent within families. In addition, Stein and colleagues (summarized in (168)) have described a small subset of dyslexics who report distinctive visual perceptual problems, such as seeing letters move on the page. These individuals show problems with vergence, and patching one eye appears to improve their vergence and reading ability. These reports, and others, suggest that reading or phonological awareness as the “pure phenotype” of a dyslexic individual may be complicated by other symptoms reflecting a complex neurological difference in these individuals (56,62). These data are examined further in the following sections.

In sum, the proper models for reading acquisition and the underlying cognitive deficits in dyslexia have not been fully determined, although phonological processing does appear to be the predominant deficit. The contributions and interactions of auditory and visual systems are now being assessed at neurological levels, and genetic studies are demonstrating that a single genetic locus can affect both orthographic and phonologic phenotypes at once (43,54–56,62).

## 108.4 INHERITANCE

The familial nature of reading disability was noted around the turn of the century, and numerous reports of multiplex families followed (e.g. (71); see reviews by Fisher and DeFries (42), DeFries and Gillis (28), DeFries and Alarcón (24), and Schulte-Korne (149). Systematic

family and twin studies have demonstrated genetic influences underlying the familial transmission.

### 108.4.1 Twin Studies

Although many twin studies of reading disability have been reported, a number of early studies suffered from poor measurements, small sample size, and/or biases in the ascertainment of twins (see Harris (71) and DeFries and Gillis (28) for reviews). The Colorado Twin Study of Specific Reading Disability, which was initiated in 1982, has avoided these problems, and has reported proband-wise concordance rates for SRD of 68% and 38% for monozygotic (MZ) and dizygotic (DZ) twins, respectively (24). This significant ( $p < 0.001$ ) difference confirms earlier reports of genetic influence in SRD.

DeFries and Fulker (25,26) have presented a method using regression analysis rather than concordance rates to examine quantitative differences between MZ and DZ twin pairs. An extension of this method can assess the genetic influence on extreme phenotypes, such as deficit in a quantitative phenotype. This method, which can be used for many complex phenotypes, involves the selection of a sample of MZ and DZ twins such that at least one member of each pair (i.e. the probands) exceeds a predetermined extreme on the phenotype. The extent to which the extreme performance of the probands is heritable ( $h^2_g$ ) can be measured by the degree to which their MZ or DZ co-twins regress toward the unselected population mean. Specifically, if deficits in performance are to some extent heritable, MZ co-twins should regress toward the mean less than their DZ counterparts. Using this methodology with a composite discriminant score of reading ability, the Colorado Twin Study reported an  $h^2_g$  value of approximately 0.56 (SE = 0.09) (24,28).

Twin studies have also been used to help resolve the phenotype of SRD. Using data from the Colorado twins, Pennington and colleagues (119) demonstrated that the requirement for a significant discrepancy between reading and intelligence may not define a genetically unique disorder, at least in children with normal intelligence. The twins had been selected such that at least one member of each pair was reading disabled according to school records, and then additional testing was done. All the twins had an IQ greater than 90. Two diagnostic criteria were applied, one based on age discrepancy with reading achievement (“backward readers”) and the other on IQ discrepancy (“true dyslexics”). It was found that both definitions of reading disability were moderately heritable, with  $h^2_g$  quotients of approximately 0.47. Furthermore, reading ability was highly correlated between both types of SRD ( $r = 0.93$ ), and this correlation was primarily due to shared genes. (Ninety-two percent of the covariance in reading ability between the two diagnoses was explained by a common genetic origin.) This suggested that the age and IQ diagnostic phenotypes both detected the same, etiologically related reading disorder in this



population. Thus, the argument for an etiologic distinction between backward and specific dyslexics may not be as tenable as was once proposed, at least in individuals with normal intelligence (192). Similar conclusions have been reached in the psychometric literature (44).

Olson and associates (112) have also used this sample of twins to examine the heritabilities of orthographic and phonologic reading and spelling skills. In a sample of 296 MZ and DZ twins, it was found that both skills were again equally heritable. The estimate of  $h^2_g$  for orthographic coding was 0.56 (SE 0.13), quite comparable to 0.59 (0.12) for phonologic coding. Using a bivariate analysis (selection of a proband based on deficit on one skill, and assessing heritability based on measures of the other skill), it was demonstrated that deficits in both skills were influenced primarily by the same genetic effects (53). Furthermore, both skills were related to phonologic awareness, with  $h^2_g = 0.60$  (0.17), and appeared to be the skill least influenced by shared family environment (i.e. family influence that will tend to make relatives alike, or  $c^2$ ). Enlarging the sample to 440 pairs of twins confirmed that phonologic and orthographic coding skills had shared as well as independent genetic influences (54). Thus, it would appear that these are not totally separate, modular skills, a conclusion that supports the neurological evidence of independent “dual route” as well as shared “connectionist” pathways.

Additional studies of the same twin sample (although expanded in size) examined whether subgroups classified as “phonologic” or “surface” (orthographic) had different heritabilities (16). If the processes were indeed independent, it was anticipated that the heritabilities would be different. Subjects were selected from the extremes of deficit in the two skills, nonsense word reading and exception word reading. Phonological dyslexics showed high heritability, with  $h^2_g = 0.67$  (SE = 0.14),  $p < 0.001$ . There was also evidence for a shared environment effect,  $c^2_g = 0.27$  (SE = 0.13),  $p < 0.05$ . Thus, genetic effects appeared to be the most important for this group. In contrast, for the surface dyslexics,  $h^2_g = 0.31$  (SE = 0.13),  $p < 0.05$ , and  $c^2_g = 0.63$  (SE = 0.13),  $p < 0.001$ , indicating that, while there was a significant heritable component for surface phenotypes, shared environment made a stronger contribution than in the case of phonologic phenotypes. This could lend support to the hypothesis that phonological skills are primary and that orthographic abilities are more sensitive to variation in environmental input, such as teaching methods or exposure to reading.

#### 108.4.2 Family Studies and Segregation Analysis

As mentioned previously, there have been many reports of families with histories of SRD. Hallgren (71) conducted the first formal study of dyslexic families and noted the high degree of familial occurrence. He used segregation analysis (as done in the day) to determine

whether the pattern of inheritance was consistent with the predictions of genetic transmission and found a fit with autosomal dominant inheritance; that is, when one parent was dyslexic, approximately half the children were also affected; however, the relatives of the proband were not actually tested to confirm the diagnosis.

One of the first family studies to carefully examine the first-degree relatives of SRD probands was done by Finucci and colleagues (41). Although multiple family members were found to be affected, the families were too few and too small to use statistical methods to establish a definite mode of inheritance. Similarly, Høien and associates (74) studied the families of 19 very carefully defined dyslexics. A positive family history of dyslexia was found in the nuclear families of 13 probands and in the extended families of 3 more. All the probands with a positive family history showed phonologic deficits when compared to matched controls, but orthographic word recognition was not delayed. By contrast, the phonologic deficits in the remaining 3 probands with negative family histories were not as severe as in the other 16. This was also a small study, and the mode of inheritance could not be determined, but all but one of the multiplex families had an affected parent.

The first segregation analysis of SRD that based the diagnosis in first-degree relatives on actual testing was done by Lewitter and colleagues (94). They analyzed the data on 133 nuclear families using a discriminant score as a continuous phenotype. When all the families were considered together, major gene inheritance could not be supported, but when the families of female probands were considered separately, autosomal recessive inheritance could not be rejected. Multifactorial inheritance was possible, but a multifactorial/threshold model (with higher threshold in females) was rejected. It was concluded that genetic heterogeneity was likely in this population, which could have masked a major gene occurring in a subset of families.

Since these early studies, more sophisticated forms of segregation analysis were developed that can consider a combination of major gene and polygenic influences (i.e. “mixed models”) simultaneously as well as correct for various statistical assumptions and sampling biases. Pennington and colleagues (120) used such a program, POINTER, and examined four sets of families from studies based in Colorado, Washington State, and Iowa, as well as a Linkage kindred sample collected specifically for genetic linkage analysis. This is the largest study of its kind to date. Evidence for a major dominant or additive gene was found for the Colorado, Washington, and the Linkage kindred samples. Segregation in the Iowa sample was more consistent with polygenic influence. This may have been due to a decrease in the accuracy of the diagnosis in more remote relatives, resulting in lower rates of affection that would mimic a polygenic model. For the dominant major gene model, penetrance in the heterozygote in the study with the most unbiased sample

(Colorado) was 1.0 for males and 0.56 for females. The Washington sample was biased toward large multiplex families and the Linkage kindred sample was selected for an autosomal dominant pattern; in those samples, the penetrance for males was still 1.0, but penetrance for females was 0.70–0.89. Estimates of the number of sporadic cases (i.e. cases of SRD without the putative dominant allele) were low; penetrance for the homozygous unaffected genotype ranged from 0.001 to 0.039 in males and was consistently 0.000 in females. While intuitively it seems unlikely that a single locus accounts for such a high proportion of SRD in the population, the results indicate that it is likely that genetic influences affect a significant number of cases.

Segregation analysis has also been performed on a quantitative measure of reading ability derived from standardized tests (59). This analysis was based on the 125 control families (i.e. selected through normal reading probands) of the Colorado Family Reading Study (94). The results were somewhat surprising: there was evidence for a fairly common major gene acting to produce normal reading variability. It was estimated that this gene accounted for 54% of the variance in normal reading ability. As in the previous segregation analysis, the authors cautioned that the analysis may have produced an overly simplistic picture. A combination of several “susceptibility” loci could mimic a single gene pattern with greater effect, and the sample size may not have been large enough to detect an additional multifactorial contribution.

More recently, segregation analyses have been used to estimate the number of genes that may contribute to dyslexia phenotypes and to determine the most likely mode of inheritance. Studies of two measures of phonological skills, phonologic coding (nonword repetition) and phonologic memory (digit span) indicated genetic influences for both. Monte Carlo Markov Chain (MCMC) analysis estimated that two to three genes contributed to nonword repetition, with a codominant mode of inheritance favored by complex segregation analysis, while one to two genes were estimated to contribute to digit span with a dominant mode of inheritance. When the digit span phenotype was adjusted by covariance by nonword repetition, the evidence for genetic influence disappeared. Adjusting the nonword repetition phenotype for digit span had little effect on the results, suggesting that the genetic influences on digit span are primarily due to the skills it has in common with nonword repetition, which are likely to involve language processes such as phonemic representation and retrieval (187). In further studies of phonologic skills, this same group has examined phonologic decoding (word attack) and phonologic decoding efficiency (a timed work attack skill). Again, both showed evidence of genetic contribution with genetic heterogeneity. Both phenotypes appeared to be influenced by one to two genes, although decoding efficiency showed greater evidence

for more than one gene in the MCMC analysis. Complex segregation analysis supported a dominant mode of inheritance with polygenic background for decoding efficiency, with polygenic inheritance for word attack. This could be due to the greater difficulty of the timed test, particularly for compensated adults in the study, and indicates that the timed task may be a better phenotype for linkage analyses geared toward detecting the major loci (19).

### 108.4.3 Linkage Analysis

As we begin this section it is worth noting that there has been a surge in the focus on finding the genes that are relevant to SRD. This research emphasis has met with some success as we will detail later. On the other hand, finding the specific shared or nonshared environmental factors that contribute to the risk for SRD has proved elusive and complex. Although several specific factors have been suggested, such as toxins, intrauterine hormones or viruses, educational opportunities, parental modeling, and others, no one factor has been shown to consistently predict SRD across samples. A better understanding of the role of the environment and its interactions with genes will be an important contribution to future work (9b 146,141,181)

Identification of a linkage of SRD with a known gene or marker would be conclusive proof of a genetic effect. Two methods of linkage analysis have been used in studies of SRD; the family study, LOD score approach (108) and studies of allele sharing between siblings, based on the sib-pair analysis of Haseman and Elston (72). In addition, definitions of phenotype have varied widely, from dichotomous affected/unaffected diagnoses to quantitative “summary” variables, to measures of phonologic and orthographic phenotypes. The results of these linkage studies have confirmed the prediction of genetic heterogeneity.

The majority of studies have focused on the first loci that were reported to show linkage, 15q and 6p. These localizations have been refined to the regions 15q21 and 6p22.2, and linkage has been confirmed by separate, independent studies. Additional studies, including several full genome screens, have suggested linkage for at least a dozen loci (Table 108-1), and five of these loci have also been replicated in independent samples (e.g. 1q36, 2p16-15, 3p12-q13, 18p11, and possibly Xq27.3).

Gene designations have been given to the nine loci for which linkage has been replicated: DYX1, 15q21; DYX2, 6p22.2; DYX3, 2p16-p15; DYX5, 3p12-q13; DYX6, 18p11.2; DYX8, 1p36; and DYX9, Xp27.3. DYX4 and DYX7 had been assigned to separate loci on chromosome 15, but currently these are subsumed under DYX1. DYX4 has also been applied unofficially to the 6q13-q16.2 locus. The current status of the major loci and the candidate genes that have been screened are summarized in the following paragraphs.

**TABLE 108-1 Gene Localization Studies with Reading Disability**

Supportive Results			Negative Results	
Region	Population	Reference	Population	Reference
1p36-p34 (DYX8)	9 extended families (US)	Rabin et al., 1993 (128)	8 extended families (US)	Grigorenko et al., 2001 (68)
	100 families (Canada)	Tzenova et al., 2004 (180)		
	291 families (Canada)	Couto et al., 2008 (206)		
2p16-p15 (DYX3)	1 extended family (Norway)	Fagerheim et al., 1999 (35)	119 nuclear families (US)	Francks et al., 2002 (46)
	111 nuclear families (US)	Chapman et al., 2004 (18)		
	89 nuclear families (UK)	Fisher et al., 2002 (42)		
2p12-q13 (DYX3)	96 families (Canada)	Petryshen et al., 2002 (123)	11 families (Finland)	Peyrard-Janvid et al., 2004 (125)
	11 families (Finland)	Anthoni et al., 2007 (3b)		
	251 families (Germany)	Anthoni et al., 2007 (3b)		
2p11	11 families (Finland)	Kaminen et al., 2003 (82)	11 families (Finland)	Peyrard-Janvid et al., 2004 (125)
3p12-q13 (DYX5)	1 extended family (Finland)	Nopola-Hemmi et al., 2001 (109)	89 nuclear families (UK),	Fisher et al., 2002 (46)
	119 nuclear families (US)			
6p22.2 (DYX2)	18 extended families (US)	Smith et al., 1991 (162)	8 families (US)	Grigorenko et al., 2000 (67)
	79 families (Canada)	Field and Kaplan, 1998 (38)		
	18 families; 41 sib-pairs (US)	Cardon et al., 1994, 1995 (13, 14)		
	7 families (Germany)	Nöthen et al., 1999 (111)		
	6 families (US)	Grigorenko et al., 1997 (66)		
	79 families (Canada)	Petryshen et al., 2000 (124)		
	82 families (UK)	Fisher et al., 1999 (43)		
	111 nuclear families (US)	Chapman et al., 2004 (18)		
	79 nuclear families (US)	Gayán et al., 1999 (80)		
	89 nuclear families (UK),	Fisher et al., 2002 (46)		
	104 nuclear families (US)	Kaplan et al., 2002 (82)		
	89 families (UK)	Marlow et al., 2003 (103)		
	264 nuclear families (UK)	Francks et al., 2004 (47)		
	96 families (Canada)	Petryshen et al., 2001 (122)		
	11 families (Finland)	Kaminen et al., 2003 (82)		
6q13-q16.2 (DYX4)	100 families (Canada)	Hsiung et al., 2004 (75)	178 parent/proband trios (Wales)	Turic et al., 2003 (178)
7q32	200 children with SRD (US)	Roeske et al., 2009 (136)		
11p15.5 (DYX7)	186 children with SRD (US)			
12p13.3	5 families. Specific language impairment (Canada)	Bartlett et al., 2002 (4)		
13q21	9 extended families (US)	Smith et al., 1983 (161)		
	9 extended families (US)	Rabin et al., 1993 (128)		
5q21 (DYX1C1)		(includes Smith et al., 1983 (161))		
			19 extended families	Smith et al., 1991 (162)
			19 extended families	Fulker et al., 1991 (49)
			6 extended families (US)	Grigorenko et al., 1997 (66)
	7 extended families (Germany)	Nöthen et al., 1999 (111)	>100 parent/proband trios (UK)	Morris et al., 2000 (106)
	2 families (Finland)	Nopola-Hemmi et al., 2000 (109)	1 family with translocation	Taipale et al., 2003 (171)
	23 families and 33 singletons		111 nuclear families (US)	Chapman et al., 2004 (18)
	121 nuclear families (Italy)	Marino et al., 2004 (103)	148 nuclear families (Canada)	Wigg et al., 2004 (186)
	89 nuclear families (UK)	Fisher et al., 2002 (42)	84 nuclear families (UK)	
	111 nuclear families (US)	Chapman et al., 2004 (18)		
	119 nuclear families (US)			
Xq27.3 (DYX9)	89 nuclear families (UK)	Fisher et al., 2002 (42)	119 nuclear families (US)	
	3 extended families, 67 male	de Kovel et al., 2004 (22)		

#### 108.4.4 DYX1, 15q21

Linkage near the centromeric region of chromosome 15q was first reported in 1983 (161), but was not supported by subsequent analysis by Rabin and associates (128), which included one of the families that contributed significantly to the linkage results in the Smith and colleagues study. Additional studies of more telomeric markers were positive, however (49,162), and this linkage has subsequently been replicated in families from the United States (18,66), Canada (186), Germany (111), Italy (103), and the United Kingdom (106). The exact location of the linkage peak is in doubt, however, and there may be two separate peaks, one near the candidate gene described in the following paragraph (18,171,186), and the other about 15 Mb more centromeric (66,103,105).

**108.4.4.1 Candidate Gene Studies.** This locus is the first for which a candidate gene has been named. In 2003, Taipale and coworkers (171) characterized a translocation breakpoint and determined that it disrupted a gene of unknown function they named DYX1C1 (DYX1 candidate 1), later determined to be homologous to mouse Ekn1, a ubiquitin-protein ligase involved in degradation of misfolded proteins (73). Single nucleotide polymorphisms (SNPs) were discovered through gene sequencing and typed in families of dyslexics in Finland. Two SNPs were found to be significantly associated with dyslexia, but subsequent studies of these two SNPs in populations from the United Kingdom and Italy did not replicate these findings (102,148); however, this could be due to separate founder effects in different populations. This supposition has been strengthened by the findings of Wigg and colleagues (186) who typed the original two SNPs as well as additional SNPs in their Canadian families. Linkage disequilibrium was found for an intronic SNP, as well as for the original SNPs; however, in the case of the original SNPs, the opposite alleles were associated with dyslexia, indicating that those particular alleles were neither causal nor in linkage disequilibrium with dyslexia in their population. The mutation in this region could be either in a regulatory region of EKN1 that has not been surveyed or in an adjacent gene. Mutation studies of two phospholipase genes located centromeric to EKN1, phospholipase C beta 2 (PLCB2) and phospholipase A2, group IVB (cytosolic; PLA2G4B), were both negative (105).

#### 108.4.5 DYX2, 6

The strongest and most consistent evidence for linkage with dyslexia has been found for chromosome 6p22.2. First reported in 1991 in a set of extended families selected for apparent autosomal dominant inheritance (162), significant linkage was confirmed in an independent set of nuclear families (13,14). This has been replicated in an independent set of families from the same research group (55) as well as by independent researchers on different samples (39,66,67,178). In those studies, linkage was

seen with all the component processes of single-word reading, phoneme awareness, phonologic coding, and orthographic coding (43,55). Conversely, linkage was not replicated in a Canadian population that assessed phonological coding (38,124) and in a German study that examined a spelling phenotype (111). These differences may be due to population differences or to differences in phenotype definition. In particular, the severity of the phenotype may be a factor in detection of linkage to this locus, since population selection for severity has consistently produced stronger linkage results (13,42,55).

**108.4.5.1 Candidate Gene Studies.** Recent studies in the United States and United Kingdom have narrowed the candidate region to 77–575 kb containing three to five candidate genes (23,47). These include vesicular membrane protein (VMP), doublecortin 2 (DCDC2), TRAF- and TNF receptor-associated protein (TTRAP), KIAA0319, and thioesterase superfamily member 2 (THEM2). Mutations have not been reported in any of these genes, however. Most activity recently has been with two of these genes, KIAA0319 and DCDC2. Significant associations were found with a haplotype of SNPs in the candidate region, with two SNPs in KIAA0319 largely responsible for the disequilibrium. One of these was a cSNP, changing an amino acid in exon 4 (20a). An independent study identified a separate haplotype of three SNPs spanning parts of KIAA0319, TTRAP, and THEM2 with association with dyslexia (47), and subsequent studies by the same group demonstrated that expression of KIAA0319 was reduced in cell lines heterozygous for that haplotype, while TTRAP and THEM2 did not show altered expression (113). There is also modest evidence that KIAA0319L, which is a homolog of KIAA0319, is a candidate gene for SRD. Like KIAA0319, this gene demonstrates expression in the brain, has a transmembrane protein domain, and potential function in neuronal migration (20b). Further, knockdown of KIAA0319 in developing rat brain interfered with neuronal migration to the cortex. Otherwise, the function of the gene is unknown. Similarly, SNPs in and around DCDC2 have been associated with dyslexia, as well as deletions and coding variations in intron 2 (103). The subjects in this study were part of the same US (Colorado) sample in which the initial SNP associations to the region were found (23). The intronic deletions included putative transcription binding sites that could affect expression level. A similar knockdown of DCDC2 function resulted in reduced neuronal migration, as well. A related gene, DCX, is on the X chromosome and produces lissencephaly in hemizygous males and subcortical heterotopia in heterozygous females (OMIM 300121). The latter is reminiscent of the heterotopia seen in autopsied brains of dyslexic individuals (50). Association of SNPs in this gene was also noted in an independent German population (152).

Other genes that have been screened for mutations with negative results include GABA B receptor 1 (GABBR1) (65), myelin oligodendrocyte glycoprotein



(MOG) (160), synaptic RAS-GTPase-activating protein 1 (SYNGAP1) (23a), and secretagogin (SCGN) (Deffenbacher, unpublished thesis).

#### 108.4.6 DYX8, 1p36-p36

Although first suggested by linkage results by Rabin and colleagues (128) that reported an LOD score of 2.33 in the region of the Rh gene, replication was not published until 2001 by Grigorenko and associates (68), who confirmed the linkage in a two-locus analysis, weighting the analysis by the identity by descent for a chromosome 6 haplotype. Recently, the linkage has also been replicated by Tzenova and coworkers (180), without digenic inheritance. Several other genome searches did not identify linkage in this region (35,42,109). Studies with positive linkage results tend to show two peaks of linkage depending on the phenotype and method of analysis, suggesting that there may be two loci spanning a region of about 20 cM. If so, this heterogeneity may have masked linkage in other studies.

**108.4.6.1 Candidate Gene Studies.** Anthoni and colleagues (3b) have refined the 2p12 candidate region in a Finnish and a German population sample, providing evidence for two candidate susceptibility genes, MRPL19 (mitochondrial ribosomal protein 19) and C2ORF3 (chromosome 2 open reading frame 3), for DYX3 locus. These genes are coexpressed in the adult human brain, and are in strong linkage disequilibrium. Moreover, their expression is correlated with DYX1C1, ROBO1, DCDC2 and KIAA0319 genes for SRD, such that individuals with this risk haplotype demonstrate attenuated expression of MRPL19 and C2ORF3 when compared to controls.

#### 108.4.7 DYX3, 2p16-p15

This locus was first identified by Fagerheim and associates (35) in a genome screen of a large Norwegian kindred, and linkage has been replicated in populations from the United Kingdom and Canada (42,123) and two populations from the United States (18,42,46). The linkage region is about 7 cM in size. Linkage to a more centromeric locus, 2p11, has also been identified in families in Finland (82,125), but a large study covering both loci would be needed to be certain that these are separate loci.

**108.4.7.1 Candidate Gene Studies.** Four genes have been screened for mutations with negative results: semaphorin 4F (SEMA4F) and orthodenticle 1 (OTX1) (46), calcineurin B (PPP3R1) (35), and the tachykinin receptor 1 (TACR1) (125). A translocation interrupting the ROBO1 gene in this region was found in one individual with dyslexia, which led the authors to examine the gene in a previously reported Finnish family showing linkage to DYX5 (109). They identified a unique SNP haplotype in ROBO1 with strong association to the dyslexia in that family. Expression of the gene was reduced in the mRNA of individuals who were heterozygous for the associated

haplotype. The haplotype was not found in an independent sample of 96 dyslexic individuals, however (71). The *Drosophila* homolog of this gene, robo, and the mouse homolog, Robo1, are both involved with midline neuronal migration (3a).

#### 108.4.8 DYX5, 3p12-q13

This linkage has been reported in three separate populations (42,109), but the critical region is quite large, spanning almost 25 cM.

**108.4.8.1 Candidate Gene Studies.** Two genes have been screened with negative results: the 5-hydroxytryptamine receptor 1F (HHT1F) and the dopamine receptor D3 (DRD3) (109).

#### 108.4.9 DYX6, 18p11.2

In genome screens of three independent populations (one from the United States and two from the United Kingdom), this locus provided the strongest evidence for linkage in all three (42). Linkage was not replicated by Chapman and colleagues (18), however. The linkage was reevaluated in one of the UK populations using a multivariate linkage analysis method (103), with a resulting decrease in the level of significance from  $p = 0.00001$  for single-word reading to  $p = 0.0011$  for all phenotypes simultaneously. This suggests that the significance level obtained in the original genome screen may be an overestimate.

**108.4.9.1 Candidate Gene Studies.** No gene mutation studies have been reported.

#### 108.4.10 DYX9, Xq27.3

Linkage to Xq27.3 has been reported in one large family in Holland (22), but was not replicated in a small set of sibling pairs that they analyzed. Linkage to this region is also mentioned in the report of genome screens by Fisher and colleagues (42).

**108.4.10.1 Candidate Gene Studies.** Four genes were screened in the linked family reported by de Kovel and colleagues (22): fragile X mental retardation 1 (FMR1), Cxorf1, DKFZp574M2010, and KIAA1854, with no mutations detected in any of the genes.

Linkage studies have also addressed phenotypic issues by comparing different phenotypic measures to see if there were differences in the linkage results. Grigorenko and associates (67) examined phonological and orthographic measures, as well as more global single-word reading measures. Linkage to chromosome 6p was most significant for a measure of phonemic awareness, although other phonologic measures were also significant. Conversely, linkage to chromosome 15q was significant for the single-word reading measure, leading them to conclude that the two genes influenced different reading processes. However, studies by Fisher and colleagues (43) and Gayán and associates (55) did not find similar

results for the chromosome 6p linkage; while phonologic awareness measures were important, significant results were also found for the other phonologic measures as well as orthographic coding measures, suggesting that the locus had a more general influence on reading ability. Subsequent analysis by Grigorenko and coworkers (67) with a larger sample confirmed the linkage to 6p, but the phenotype was expanded to include other phonologic measures and vocabulary. Thus, linkage to chromosome 6 does not support the assignment of a particular cognitive process to a specific gene, nor does it support dissociation between phonologic and orthographic abilities. The same pattern has been seen for other loci (42,46). It may be that, at this level of phenotypic analysis, these highly correlated phenotypes should not be expected to have separate, modular genetic influences (117). Refinements of the phenotypes along with larger sample sizes may indicate that some loci do have greater effects on one type of measure than another (187).

#### 108.4.11 New Candidate Loci

Using an event-related potential correlate of speech perception that has been shown to be impaired in dyslexic individuals, Roeske and colleagues (136) found a significant association with SLC2A3 on 12p13.3. They replicated this finding in a second independent sample of children with SRD. SLC2A3 facilitates glucose transport in neurons and is thought to lead to glucose deficits in children with SRD.

#### 108.4.12 Missing Heritability

Although it is clear that there is a strong genetic component underlying SRD, the proportion of variance accounted for by candidate loci does not add up to heritability estimates for SRD. This “missing heritability” problem, of course, is not specific to this disorder and is common to all studies of complex behavioral disorders (101). There are a number of hypothesis that suggest why this effect exists. Most notably, prominent scientists, such as Eric Lander, have suggested that epistasis (suppression of a gene by another gene) plays a major role when considered alongside current methodologies such as genome-wide association studies, which are underpowered to find gene–gene interactions.

DNA methylation, which is the addition of a methyl group to a stretch of DNA that often results in the silencing of the portion of DNA sequence, is the most stable form of epistasis in mammalian genomes. Recent studies of DNA methylation have shown that it is a common occurrence in the human genome (for a review see Reference (103)). The effects can be allele specific and quantitative or heterogeneous. In other words, the effects of methylation may not be evenly distributed across both alleles; they may differ across tissues and individuals, and 10% may be non-cis (acting from a different molecule) such as parental

origin. These methylation effects, which demonstrate that noncoding sequences can have functional effects, then reduce the observed relationships between genes and behavior when not accounted for directly. Another reason why some of the genetic variance may be unaccounted for could be shared prenatal effects or the “maternal effect” that can be transmitted to siblings and appear genetic when in fact, it is environmental in origin (183).

#### 108.4.13 Summary of Genetic Studies

The evidence so far supports the idea that both normal reading and SRD can be influenced by genetic factors. Segregation analyses support the hypothesis that a limited number of loci are involved, with at least some having a major gene effect, and linkage analyses have identified at least seven candidate loci. Some of the ambiguity in results or the lack of findings may reflect the fact that both segregation and linkage analysis are dependent on the definition of the phenotype. As the phenotypes are more closely defined and new techniques for analysis of quantitative trait loci are developed, the mode of inheritance and the role of individual loci should become clearer.

### 108.5 COMORBIDITY

SRD is frequently comorbid with other language disabilities, including specific language impairment (SLI), and speech sound disorder (SSD) (phonologic disorder). Comorbidity for two or more disorders may have a number of causes: they may be two separate but common disorders with different causes and independent segregation, complications of one disorder may produce a phenocopy of the other disorder, or the two disorders may be pleiotropic effects of the same underlying gene. There is evidence supporting all three of these possibilities.

#### 108.5.1 Speech Sound Disorder and Specific Reading Disability

SSD is a developmental disorder of articulation, specifically the production of developmentally inappropriate errors in pronunciation that interfere with the ability to produce understandable speech. SSD includes problems with articulatory output (deficits in phonetic structure due to poor motor skills) and phonological processes (failure to learn the pattern of sounds in a particular language) ([www.ASHA.org](http://www.ASHA.org)). While the etiology of some SSDs are known such as hearing loss, it is diagnosed in young children, and both prospective and retrospective studies have indicated that many of these children later develop reading disability (see Raitano and colleagues (129)). The prevalence of SSD at 3 years of age has been shown to be 16%; however, only 3.8% of children continue to present a speech delay at 6 years of age (10). This estimate does not include children who have speech/language problems secondary to other conditions.

Susceptibility to SSD has been shown to have a strong genetic influence (6a). For example, familial segregation has been demonstrated in 20–40% of first-degree relatives of children with SSD (93). Moreover, segregation analyses have also confirmed familial aggregation of SSD and supported both a major gene and polygenic modes of transmission of the disorder (6a,86,148). Stein and associates (167) ascertained families with children with SSD and found linkage with markers in the centromeric region of chromosome 3, within the region of linkage of SRD. Similarly, Smith and colleagues (163) presented evidence for linkage of SSD with the SRD regions of 1p36, 6p22.2, and 15q21. These results suggest that shared developmental deficits in phonological representation or retrieval affect both disorders.

### 108.5.2 Specific Language Impairment and Specific Reading Disability

Like SRD, SLI is primarily defined by exclusionary rather than inclusionary criteria. In general, a person with SLI exhibits unexplained deficits in receptive and/or expressive language skills, without any evidence of deficits in nonverbal IQ, neurologic impairment, or environmental or emotional problems that could explain the language delays. These deficits can be seen in some or all five global language domains: phonology (analysis of the sounds of language; articulation), morphology (e.g. word formation and grammar; prefixes, suffixes, tense marking), syntax (word order), semantics (word meaning), and pragmatics (the practical use of language to convey purpose and emotion) (92,172,176). Thus, children with this disorder have trouble with the formation and learning of the rules of language and their appropriate application more than with articulation and phonology (113). Given the wide range of SLI phenotypes, research in this area is difficult to consolidate.

There are several major hypotheses regarding the causes of SLI, and some show overlap with the hypothesized causes of SRD. For example, difficulty in auditory processing (e.g. References (174,175)) or in defective cognitive/representational abilities (80,81) has been proposed. Studies of auditory perception have focused on language stimuli, with apparent problems in the perception of artificially generated, very brief auditory signals (174,175). The deficits were corrected when the signal was prolonged. The work of Tallal and colleagues (e.g. Reference (134)) also found deficits in discriminating brief sounds, similar in length to stop consonants, along with poorer perception of degraded speech and difficulty processing rapidly presented auditory stimuli. These findings were replicated by Wright and associates (191) with psychophysical tests that showed perceptual deficits for brief tones. These theories have attracted a great deal of attention lately, in part due to their application to treatment/remediation techniques (see Reference (104) and later in this chapter).

Although these results are intriguing and provide some parallels to the visual and auditory evoked response studies reviewed previously for SRD, others have argued that the described deficits in auditory perception are too limited and are not sufficient to account for the full range of linguistic problems typically found in SLI children (8,64,91,92,185). In addition, Bishop and colleagues (7) used a twin design and determined that the auditory perception test used did not show significant heritability, whereas phonological measures did show high estimates of heritability. A shared environment appeared to be more significant in accounting for variation in performance on the auditory perception tests. It is still possible, however, that the auditory perception deficits are an important clue to an underlying neurologic abnormality.

There are also suggestions of similarity in pathogenesis between SLI and SRD. The deficit in the processing of rapid auditory stimuli proposed by Tallal and Stark (174) and Tallal and colleagues (175) could result from the same mechanism underlying the deficits in fast transient visual stimuli studied in dyslexics, perhaps also mediated by magnocells. Accordingly, Tallal and associates (173) expanded their hypothesis by proposing that problems in perception of any rapidly presented stimuli result in a cascading disruption of intersensory integration processes, including phonologic processing. Liberman (95) cautioned about taking the parallels between reading and speech disorders too far, particularly in attributing speech problems solely to difficulties in phonologic perception or awareness. He maintained that speech perception is not the same as reading in that speech deals with more than just phonology; one does not need to learn the underlying segmentation to understand speech, and oral motor feedback is also an important consideration. Thus, rather than being the primary deficit for SLI (or SRD), the difficulty in processing rapid stimuli may be correlated with the underlying deficit for SLI, which may be closely related to, but not exactly the same as, the underlying deficit in SRD.

While these differences would suggest that SLI and SRD show limited etiological similarity despite their comorbidity, linkage studies suggest that the etiologic overlap that exists may be small. For example, a genome screen of SLI phenotypes did not show localization to any known SRD loci (158), but further studies of a candidate locus on 16q that was identified in this screen showed that SRD phenotypes also linked to this region (159). In another study of families selected for SLI, Bartlett and colleagues (4) found linkage of SRD phenotypes (but not SLI phenotypes) to chromosome 13q21. Neither of these regions was identified in genome screens of SRD (42).

### 108.6 RECURRENCE RISKS

Certainly, there is great value in identifying children at high risk for SRD so that effective remediation is not delayed. However, since it is very likely that there is

etiologic heterogeneity in reading disability, it is impossible to give precise recurrence risks in individual cases. In some families, the inheritance pattern may suggest a dominant gene with high penetrance, while other children with SRD may be sporadic cases with low recurrence risks (see Reference (63) for illustration of risk estimate fluctuation as a function of major gene or polygenic influence on SRD). While a strongly positive family history would indicate a higher recurrence risk, regardless of the mode of inheritance, the exact magnitude of the risk can only be estimated. Empirical risk figures can be provided to the family, however, and the results of the segregation analysis by Pennington and associates (120) would suggest close to a 50% recurrence risk for male siblings, and 28% for female siblings of a dyslexic child.

Other types of studies have produced somewhat similar figures. Gilger and colleagues (63) used the data from three family studies to calculate the chance that an affected parent would have an affected child. Using their most unbiased data set and assuming 3.5:1 sex ratio for SRD, sons of an affected father had a 39–58% probability of being affected, and daughters had a 19–32% risk. Sons of an affected mother had a 30–48% probability of being affected, slightly less than if the father was affected, but her daughters had a comparable risk of 18–32%. (The two risk figures reflect estimated population base rates of 5 or 10%, respectively.) If a 1:1 sex ratio was assumed, the risks for daughters went up to 35–50% regardless of which parent was affected, and risks for sons went down to 29–45% with an affected father and 20–36% with an affected mother. Whether these sex differences are genetic or environmental is unclear; however, while these data may appear consistent with a multifactorial/threshold trait, the segregation analysis and linkage analysis results would suggest that the number of loci involved cannot be great, and that modification of a major QTL must also be considered.

It has also been suggested that the risk to offspring varies according to whether the parent has compensated for their childhood SRD (60). For example, if one parent had SRD that began in childhood and their spouse was unaffected, 60% of the children were SRD, but if the parent had compensated (i.e. was affected with SRD in childhood but not in adulthood), only 30% of the children were affected, and if both parents had never had signs of SRD, 20% of the children were affected. The latter figure of 20% is somewhat greater than the generally accepted population frequency of 5–10% as one might have expected, but this may be due to the truncated selection of the families in the study; that is, all the families had at least one affected child, the proband, at the outset and therefore were more heavily loaded for SRD than the population at large. This type of family study did not permit separation of genetic and environmental effects, so the mechanism of the compensation effect on recurrence could not be determined. In addition, it is possible that this effect is confounded somewhat by the sex effect

observed by Gilger and coworkers (63), since compensation is more common in females (see also Pennington and associates (120)). In sum, the studies we have reviewed indicate some fluctuation in the recurrence risk based on characteristics of the affected parent. These findings must be replicated and investigated further to determine whether sex and/or severity of the affected parent have an effect on transmission. If so, the mechanism of such an effect may reveal important aspects of the mode of inheritance of SRD.

## 108.7 DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

The diagnosis of dyslexia requires a convergence of data from three domains: the child's developmental and school histories, qualitative aspects of the child's reading and spelling performance, and quantitative test scores. These are all covered in greater detail elsewhere by Pennington (116) and Fletcher and associates (45). Briefly, the preschool developmental history is often essentially normal, although there may be mild speech delays and articulation difficulty. Problems with reading and spelling are usually noticed by first or second grade, with phonetic errors in reading and spelling. To confirm the diagnosis, psychometric testing needs to be done by a well-trained child clinical or educational psychologist who is familiar with dyslexia. At a minimum, this testing should include an IQ test, such as the WISC III, and tests of academic achievement, such as the Woodcock–Johnson, Revised, the Gray Oral Reading Test-Revised (GORT-R), and the spelling subtest of the Wide Range Achievement Test-Revised (WRAT-R). It is important that the academic battery include measures of spelling, nonword reading, and reading speed and fluency for paragraphs (e.g. the GORT-R), since these areas are particularly difficult for dyslexic individuals. Some dyslexics, especially older or brighter ones, may perform normally on untimed measures of single-word reading accuracy or reading comprehension, which can lead to a false-negative diagnosis if these other, more sensitive measures are not used. Of course, a variety of different tests are available for the assessment of the essential reading-related domains and an interdisciplinary diagnostic team is suggested and becoming common practice. Nonetheless, research is coalescing toward relatively reliable measures of early identification and formal and simple instruments have been developed that are becoming handy tools in this endeavor (69,166). (See also the Web site <http://dibels.uoregon.edu/>.)

## 108.8 TREATMENT

Treatment of dyslexia can be viewed from two perspectives: remediation and compensation. Remediation involves intensive training of the weak phonologic skills, since it is impossible to completely bypass phonics



in learning to read, particularly in English, but the best method to treat reading disability is debated.

There are several therapies for which scientific support is lacking or further research is required (Silver (157); Creaghead (21) and accompanying articles). Among these are tinted lenses, visual training, patterning, auditory processing training, fast-for-word (104), and various nutritional additives or adjustments. At this time, multisensory approaches such as Orton-Gillingham and systematic training on letter-to-sound correspondences are favored by the International Dyslexia Association (IDA) and many other experts (see IDA address at end of chapter and Hall and Moats (69)).

Compensation for SRD involves strategies that both the child and the educators can use to work around some of the problems posed by the disorder. For instance, reduced time pressure, marking but not penalizing for spelling errors, not being asked to read aloud in class, and possible waiver of foreign language requirements, are all things that the school environment can provide to help dyslexic children perform to their greatest potential. Things the child can do themselves include use of spell checkers (including handheld models), calculators to check computation, and tape recorders or other aids for note taking. Dyslexia can easily lead to secondary emotional problems, and these must be monitored carefully by both teachers and parents.

There is growing recognition that many of the common practices in the field of education today are based more on opinion or tradition than they are on scientifically sound evaluations of their efficacy (No Child Left Behind, Reading First, PL 107-110, 2002; (71,164). This is particularly true in the field of academic remediation for learning disabilities, where the types of help available to children are typically limited, conventional, and applied in a one-size-fits-all manner (71,177). The mantra of scientifically based treatments or interventions (SBI) is being echoed across the country, in part due to recent federal mandates (e.g. PL 107-110, 2002; IDEA, 2004). Public and private schools, clinics, and the like, are being asked to “step up to the plate” and make responsible efforts toward validating their treatment programs and/or developing treatment programs that have data-based validity.

The essentials of scientifically based interventions (SBI) for dyslexia can be found in several documents (see [www.whatworks.ed.gov](http://www.whatworks.ed.gov); Institute for Educational Sciences (IES), US Department of Education). Among the key requirements for evidence of efficacy to be considered “strong” and scientifically valid are that the intervention be tested using randomized (or other appropriate) types of controlled trials and that the intervention be shown to be effective in more than one setting and by more than one independent investigator. It is noteworthy that basic pre- and post tests and studies do not provide “strong” evidence of efficacy or even “likely” evidence and that such studies can produce misleading results (IES).

Literacy is one of the best studied domains of academic abilities. Reading teaching practices and reading remediation for the learning disabled or dyslexic individual have benefited from a great deal of basic research, although there is still much to be learned (135,170). Research has in fact identified the basic cognitive component areas or processes needed for the development of reading skills (30,132,164). These processes or abilities include phonemic awareness, phonics, fluency, vocabulary, and comprehension strategies, each of which is essential for the accurate and fluent identification of words and the ultimate construction of meaning (comprehension).

Researchers are beginning to use this general model of component skills as a framework for assessing the adequacy and potential effectiveness of specific reading teaching programs. This is particularly true in the case of programs for reading remediation where at the first step of validation, programs are examined for their adherence to training and development of the several componential skills identified previously. There are a number of reading remediation programs available, none of which meet the criteria to be an SBI, although many programs are now being studied with proper controls and replications as required by IES.

In summary, there is both a moral and legal obligation to develop and provide SBIs. There is a plethora of reading programs for dyslexics and nondyslexics alike that make far-reaching claims that have little or no support using objective data. Many of these programs have face validity, but upon closer scrutiny they do not train in any coherent way the several basic componential skills essential to reading development. Some programs, however, do appear to have support from a theoretical view in that they train in areas related to the componential skills and some programs do have at least preliminary data supportive of their efficacy. Among such programs are the several varieties of reading remediation based on the Orton-Gillingham multisensory approach (1,73). Sometimes referred to as multisensory-structured language (MSL) methodologies, this family of programs are typically intense, incremental, cumulative, explicit, and systematic, and address in some way the essential componential skills identified in the National Reading Panel report (30,132,164).

Significant support for families and dyslexic individuals can come from organizations such as the International Dyslexia Association (formerly the Orton Dyslexia Society), which has branches in many states. The national headquarters can be reached at

IDA

Chester Building, Suite 382

8600 LaSalle Road Baltimore, MD 21204-6020

800-222-3123

410-296-0232

[www.interdys.org](http://www.interdys.org)

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## Biographies



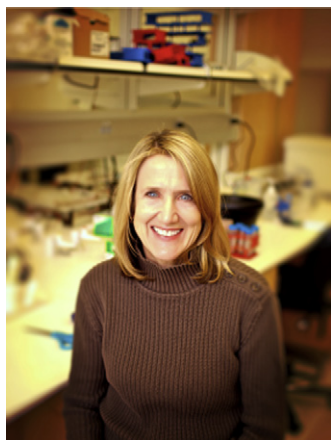
**Name:** Angela Friend

**Position/Title:** Research Associate

**Affiliation/Institution/Location:** University of Denver

**Discipline(s):** Cognitive Psychology, Behavioral Genetics

**Background-Training-Experience:** Angela Friend's background includes an MA and PhD in Cognitive Psychology with a specialty in Behavioral Genetics. Her training includes genetic methodology on twin and family data, structural equation modeling, multivariate statistics, item response theory, bioinformatics and genomics, and quantitative trait loci analysis. Her research has focused on understanding the etiology of component processes related to reading disability and, more recently, on gene by environment (GxE) interactions at the extreme tails of the distribution of reading ability through the application of quantitative behavioral genetic methodology in data from twins. Her current projects include studying the extent to which language impairment and other cognitive risk factors mediate the relationship between speech sound disorder and dyslexia at the phenotypic and genetic levels of analysis, specifically, how language impairment may mediate the relationship between speech sound disorder and dyslexia.



**Name:** Shelley D Smith

**Position/Title:** Professor of Pediatrics

**Affiliation/Institution/Location:** University of Nebraska

**Discipline(s):** Board Certified Medical Geneticist

**Background-Training-Experience:** Shelley Smith is a medical geneticist who conducted the first genetic linkage analysis of reading disability for her doctoral dissertation in 1978. In 1986, she edited a research monograph entitled Genetics and Learning Disabilities. Her collaboration with CLDRC investigators led to the identification of the region on chromosome 6 containing the candidate genes DCDC2 and KIAA0319, the most replicated of all the reading disability candidate genes. She is Professor of Pediatrics, Departmental Section Chief of Molecular Genetics, and Chair of the Developmental Neuroscience Department in the Munroe Meyer Institute for Genetics and Rehabilitative Medicine at the University of Nebraska Medical Center. She has been involved in gene identification studies for deafness disorders and learning disabilities, including separate R01s in collaboration with Dr Pennington, which study families with phonological disorder, and with Dr Mabel Rice at the University of Kansas focusing on specific language impairment. She is currently PI of an NIH/NCRR Center for Biomedical Research Excellence (COBRE) on the Molecular Biology of Neurosensory Systems, which supports three research core facilities and four research projects in studies of the development of auditory and visual systems. She is certified by the American Board of Medical Genetics as a PhD Medical Geneticist and provides clinical services in the neurosensory genetics clinic at Boys Town National Research Hospital in Omaha.





Name: **Jeffrey W Gilger**

Position/Title: Professor

Affiliation/Institution/Location: Purdue University

Discipline(s): Developmental Psychology, Behavioral Genetics

**Background-Training-Experience:** Jeff Gilger's background includes an MS and certification in Clinical Child/School Psychology, and an MA and PhD in Developmental Psychology with a specialty in Behavioral Genetics. His scholarship is inherently multidisciplinary spanning the clinical, educational and neuroscience disciplines. Dr Gilger's teaching and research has tended to focus on normal and abnormal neuropsychological development, genetics, and the etiology of learning-language disorders, especially dyslexia. Current research projects include the neurology/genetics of the gifted-learning disabled individual and the effects of subclinical brain damage in adolescents, among others.



Name: **Bruce F Pennington**

Position/Title: John Evans Professor of Psychology

Affiliation/Institution/Location: University of Denver

Discipline(s): Developmental Psychology

**Background-Training-Experience:** Bruce F Pennington, PhD, is a developmental neuropsychologist who has earned an international reputation for his research on dyslexia, ADHD, and autism. He is particularly interested in using genetic and neuropsychological methods to understand comorbidity among disorders, such as the comorbidity between dyslexia and ADHD. He has been the primary research mentor for 35 doctoral and postdoctoral students, many of whom are now pursuing their own research on developmental disabilities. He heads the Developmental Cognitive Neuroscience program. His honors include Research Scientist, MERIT, and Fogarty awards from the National Institutes of Health; the Samuel T Orton Award from the International Dyslexia Association; and the Emanuel Miller Lecture from the British Child Psychology and Psychiatric Association. He is also a Fellow of AAAS.

He recently published *The Development of Psychopathology: Nature and Nurture*. (Guilford Press, 2002). His first book, *Diagnosing Learning Disorders* (Guilford Press, 1991), has now been revised and the second edition was published in 2008. It emphasizes a close relationship between research and practice. In addition to being a researcher and research mentor, he is also a child clinical neuropsychologist, and has been active in clinical practice and training throughout his career.

# CHAPTER

# 109

## Attention-Deficit/Hyperactivity Disorder

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ADHD is a childhood-onset behavioral disorder characterized by excessive inattention and/or hyperactivity and impulsivity. It is common in populations across the globe. A recent paper reported a worldwide, pooled prevalence of approximately 5.3% (1); however, estimates are often as high as 10% (2). Although a subsample of affected individuals remit, the majority of patients continue to exhibit impairing symptoms into adulthood, and there is a strong association between the disorder and academic and career underachievement in addition to conduct problems and substance abuse (3,4). Thus, given its prevalence, the impact of this condition on society is vast in terms of financial cost, stress to families, and disruption in schools and the workplace.

The disorder was first described in detail in the late 1950s as “hyperkinetic or overactive behavior,” and subsequent labels such as “minimal brain dysfunction” and “hyperactive child syndrome” continued to emphasize hyperactivity as the essential component of the disorder. With the advent of DSM-III came the distinction between attention deficit disorder with (ADHD) and without hyperactivity (ADD). Owing to debates about whether inattention without hyperactivity was a clinically valid syndrome, DSM-III-R combined the two subtypes into a single entity. The publication of DSM-IV in 1994, however, marked a return to subtypes—including a primarily inattentive subtype, a primarily hyperactive/impulsive subtype and a third that mixes both symptom dimensions. At the time of this writing, changes are proposed to DSM-V, including the possible return to a single category ([www.dsmV.org](http://www.dsmV.org)), because of the instability of the different subtypes over time and the lack of strong neurobiological evidence for their distinction.

Since the mid 1960s, researchers have used behavioral genetic designs to examine the etiology of this condition. Despite a changing nosology, estimates of familiarity and heritability have been quite consistent over time (5) and, together, family twin and adoption studies indicate definitively that the development of ADHD is influenced to a large extent by genes. Nonetheless, these studies also support heterogeneity of the phenotype, and the more recent molecular genetics literature suggests that ADHD is complex and multifactorial.

### 109.1 BEHAVIORAL GENETICS

#### 109.1.1 Family Studies

There is substantial evidence that ADHD runs in families. Early studies of hyperactivity and subsequent studies of each recent variation of the DSM ADHD diagnosis agree that the parents of ADHD children have a two- to eightfold increase in the risk for disorder (5). In addition to confirming the transmission of ADHD, family data provided the initial evidence for the validity of the diagnosis in adults as well as particularly high rates of ADHD in their relatives, e.g. Faraone et al. (6), suggesting a higher genetic loading in persistent cases.

Our group confirmed and extended these family study findings using more definitive double-blind, case-control studies of large samples of boys (7) and girls (8) and adults (6) with DSM-III-R and DSM-IV ADHD. In addition, both male and female probands and their relatives had higher rates of comorbid conditions such as conduct, mood and anxiety disorders than respective controls. Further attention to these other psychiatric disorders in

family studies suggests genetic heterogeneity and shared risk with some but not all of the disorder's common comorbidities. Overall, independent samples suggest that (1) ADHD and major depression share common familial vulnerabilities (9); (2) ADHD children with conduct (10) and bipolar (11) disorders might be a distinct familial subtype of ADHD; and (3) ADHD is familially independent from anxiety disorders (12). Thus, stratification by conduct and bipolar disorders may cleave the universe of ADHD children into more familially homogeneous subgroups. In contrast, major depression may be a nonspecific manifestation of different ADHD forms.

### 109.1.2 Adoption Studies

Whereas parents can confer a disease risk to their biological children via both biological and environmental pathways, parents of adoptive children only confer risk via an environmental pathway. Thus, examining both the adoptive and the biological relatives of ADHD probands helps to disentangle genetic and environmental sources of familial transmission. Two early studies found that the adoptive relatives of ADHD children were less likely to have ADHD or associated disorders than the biological relatives of ADHD children (13,14). Biological relatives of ADHD children also performed more poorly on standardized measures of attention than did adoptive relatives of ADHD children (15). Consistent with these earlier studies, a more recent adoption study that examined DSM-III-R ADHD found higher rates of the disorder in the biological parents of ADHD children compared with adoptive parents (16). Conclusions from these studies must be considered in light of the limitation that the adoptive and biological relatives of the same children were not compared. Nonetheless, these data support the twin data, described below, to highlight the role of genes in the etiology of the disorder.

### 109.1.3 Twin Studies

Twin studies rely on the assumption that reared-together identical (or monozygotic [MZ]) and fraternal (or dizygotic [DZ]) twins share equal amounts of environmental influences but differ in their genetic similarity. While MZ twins share all of their genes, DZ twins share no more genes that siblings would, on average 50%. According to the logic of the twin design, the finding that MZ twin pairs are more likely to have the same disorder than are DZ twin pairs shows that genes play a causal role in the disorder.

When examined independently and together, twin studies of ADHD suggest that the heritability of the disorder is quite high. Our group estimated a heritability of 0.76 across studies in the literature (5), which places ADHD among the most heritable of psychiatric conditions. Yet these data also suggest that approximately a fourth of the variance in the population is due to

nongenetic factors, particularly factors that relate to the unique environment of individuals rather than the environment shared among family members (17). Specific nongenetic influences that have shown repeated association with ADHD in the literature include low birth weight (18) as well as maternal smoking and alcohol use during pregnancy (19). Recent data suggests that exposure to lead and environmental toxins including PCBs should be considered as well (17,20). How these environmental factors may interact with genetic vulnerability is an important topic for future studies.

Twin studies have also examined the genetic overlap between the two ADHD symptom dimensions, inattention and hyperactivity-impulsivity. On the basis of 288 male twin pairs, data from the Minnesota Twin Family Study (21) found that common genes contribute to both symptom dimensions, but the bivariate heritability was substantially larger when based on mothers' (0.86) as opposed to teachers' reports (0.33). Data from larger, recent studies (22,23) continue to support substantial genetic overlap of the dimensions as well; however, data from these projects further clarify the existence of a significant proportion of nonoverlapping genetic influences on each dimension.

Twin designs additionally provide a means of examining the genetic contribution to the comorbidity of ADHD and other disorders. Although it has been suggested that ADHD and reading disability were genetically independent (24), several recent reports (23,25,26) support mutual genetic influences on measures of reading and inattention symptoms, but low to negligible overlap between reading and hyperactive/impulsive symptoms. Overlap between conduct problems and ADHD has also been explored extensively. Nadder et al. (27) found that 50% of the correlation between the ADHD and CD was due to shared genes. These findings are consistent with those from our own family work (10) showing that ADHD and conduct disorder tend to cosegregate in relatives of ADHD probands. More recent twin studies have extended this investigation beyond DSM categories to document a more general, heritable liability for externalizing psychopathology factor that cuts across diagnoses of ADHD, CD and ODD and may in part account for their comorbidity (28,29). Finally, twin studies have documented genetic overlap between ADHD and the autism spectrum, in terms of specific diagnostic categories autistic traits that have been examined in nonclinical samples (30).

## 109.2 MOLECULAR GENETICS

### 109.2.1 Candidate Gene Studies

Biological candidate genes for ADHD have predominantly been related to the catecholamine system. Our 2005 meta-analysis (5) showed small but statistically significant pooled odds ratios, ranging from 1.1 to 1.5,

for five genes related to dopamine (DA) and/or its neurotransmission, including the dopamine (DA) transporter (*SLC6A3*), the DA 4 receptor gene (*DRD4*), synaptosomal associated protein 25 (*SNAP-25*), the DA 5 receptor gene (*DRD5*), and the gene coding for dopamine beta-hydroxylase (*DBH*). An updated meta-analysis (31) confirmed the association between these genes, with similar effect sizes, and also found association between the serotonin transporter (*5HTT*) and the serotonin receptor 1B (*HTR1B*). Owing to space constraints, we refer readers to these meta-analyses for further details; however, we briefly discuss three of what are arguably the most compelling candidate genes to be investigated.

*SLC6A3* was the first candidate gene to be examined for ADHD (32), and recent studies strongly support its involvement in the pathophysiology of the condition. The solute carrier transporter protein coded by the gene is integral to the reuptake of synaptic DA, and methylphenidate achieves at least part of its therapeutic effects by blocking its action (33). Increased transporter density in the striatum has been documented in several studies of adults with ADHD versus controls (34). *SLC6A3* also lies in a region on 5p13 that has been highlighted in genome-wide linkage studies of ADHD (described below) (35). A variable number tandem repeat (VNTR) in the 3' untranslated region (UTR) of the gene has been the primary focus of molecular genetic studies because of some evidence of functionality (36). Our 2005 meta-analysis (5) of 14 studies showed a small but statistically significant pooled odds ratio for this polymorphism (1.13; 95% CI 1.03–1.24) with the 10-repeat (10R) allele conferring increased risk for the disorder. An updated meta-analysis found a comparable, modestly but significantly increased risk for the disorder with the 10R allele (31) as well as evidence for heterogeneity of the effect size across studies. Recent reviews (31,37) agree that more attention to other markers in this gene is warranted. One other VNTR in intron 8 and three additional SNPs (in exon 8 and the 3' UTR region) have been examined with enough frequency to be included in Gizer et al.'s recent meta-analysis (31), with only the other VNTR showing significant association (OR = 1.19, CI = 1.05–1.34). Other recent investigations also suggest that environmental risk factors including maternal alcohol use and smoking during pregnancy may represent mediators of the association with *SLC6A3* variants and the disorder (38,39).

*DRD4* codes for a G-protein-coupled catecholamine receptor that is prevalent in frontal-subcortical networks implicated in ADHD by neuroimaging and neuropsychological studies (40). Authors of a *DRD4* knockout mouse study concluded that the gene was implicated in a variety of relevant phenotypes including activity level, sensitivity to methylphenidate and nigrostriatal DA neuronal activity (41). Researchers have focused on a polymorphic 48-bp repeat in exon III because in vitro studies have shown that the 7-repeat allele produces a blunted response to dopamine (42,43). The significant

association of the 7R allele with the disorder was demonstrated in our 2005 meta-analysis (5), with an odds ratio for case-control studies of 1.9 (95% CI 1.4–2.2), and for family-based studies of 1.4 (95% CI 1.1–1.6). Gizer et al.'s (31) update found a comparable effect (OR = 1.3, CI = 1.16–1.39) as well as significant heterogeneity across studies. Similar to *SLC6A3*, more work is needed to investigate other markers in the gene. Only an insertion/deletion in the promoter region and an SNP thought to influence promoter activity (rs1800955), have been examined with frequency, and Gizer et al. found a significant, though modest, association with its T allele (OR = 1.21, CI = 1.04–1.41).

*SNAP-25* encodes a plasma membrane protein involved in neurotransmitter release, axon growth and synaptic plasticity (44). The *Coloboma* mouse, considered an animal model of ADHD, is characterized by a hemizygous, 2-cM deletion on chromosome 2q that includes this gene. Such mice display spontaneous hyperactivity, delays in complex motor abilities, deficits in hippocampal physiology, and deficits in Ca<sup>2+</sup>-dependent striatal dopamine release (45). Previous studies have tested several markers within *SNAP-25*. Our prior meta-analysis showed a modest but significant effect (1.19 [CI 1.03–1.38]) in family-based studies for the T allele of rs3746544, located in the 3' UTR. Updated meta-analyses (31,46) examined this as well as a handful of other markers in the gene that have been explored in multiple studies. Both found the same allele in rs3746544 to be associated with ADHD (both OR = 1.15 [CI 1.01–1.31]). Because this SNP is not functional, more work is needed to determine its role in risk for the disorder or whether it is in linkage disequilibrium (LD) with a different risk variant.

In their recent review, Barr and Misener (37) note the strong support for the involvement of these and other DA-related genes in ADHD, but highlight gaps that must be filled by the next generation of studies. First, true risk alleles remain unclear because a few studies have examined allelic variation across entire genes. Second, the mechanism by which specific variants confer risk for the disorder is yet to be determined. Thus there is considerable interest in examining the association of ADHD-related candidate genes with more direct indices of the putative neurobiology of the disorder. Early work examining the association of candidate genes including *DRD4* and *SLC6A3* with neuroimaging and neurocognitive phenotypes has begun to reveal a relationship with prefrontal striatal neural networks relevant to ADHD (47).

## 109.2.2 Genome-Wide Linkage

Genome-wide linkage scans have aimed to identify chromosomal regions that harbor genes for ADHD. This approach examines many DNA markers across the genome to determine if particular markers are co-inherited with the disorder of interest. Although the markers in question may not themselves be functional, excess



sharing of alleles in affected family members suggests that the marker is in close enough proximity to true risk variant as not to be disrupted by meiosis. Regions identified in these studies can then be examined in more detail using additional markers.

The majority of genome-wide linkage studies of ADHD have targeted affected youth. The first of these, which examined 126 affected sib-pairs from the United States, found three regions showing some evidence of linkage (LOD scores  $>1.5$ ): 5p12, 10q26, 12q23, and 16p13 (48). An expanded sample of 203 families found stronger evidence for the 16p13 region, previously implicated in autism, with a maximum LOD score of 4 (49). A study of 164 Dutch affected sib-pairs also identified a peak previously noted in autism, at 15q15, with a peak LOD score of 3.5 (50). Two other peaks, at 7p13 and 9q33, yielded LOD scores of 3.0 and 2.1, respectively. Pooled analyses of these two studies (49,50) suggest that the lack of consistent findings is a reflection of between-population heterogeneity (51) and revealed a region on 5p13 that may reflect a common risk locus. A study of 155 sib-pairs from Germany (52) also reported a maximum LOD score of 2.59 for this distal arm of 5p at 17cM, providing further support for a potential ADHD locus in this region. They also reported nominal evidence for linkage to chromosomes 6q, 7p, 9q, 11q, 12q and 17p, which had been identified in previous scans. A genome-wide scan of families from an isolated community in Columbia implicated 8q12, 11q23, 4q13, 17p11, 12q23, and 8p23 (53).

More recent linkage studies have used an SNP-based panel of markers with arguably finer resolution than previous studies. With such a platform, our group at Massachusetts General Hospital found no regions of significant or suggestive linkage in 217 families when targeting the ADHD diagnosis and quantitative symptom (54); however, in this same sample, we identified a potential QTL at the suggestive level on 3q13 using a multivariate linkage strategy combining neurocognitive traits and ADHD symptoms (55). Specifically, all of the neurocognitive traits and inattentive but not hyperactive/impulsive symptoms showed linkage to this region, which has been implicated in autism (ref) and in dyslexia, which, as reviewed above, shares genetic risk with ADHD inattention symptoms. On the basis of the ADHD phenotype alone, Asherson et al. (56) and the International Multi Centre ADHD Genetics (IMAGE) Project identify two regions of suggestive linkage (16q23 and 9q22) that overlapped with prior studies. Converging regions also continue to be identified by linkage studies of dense families, e.g. 5q13 (57) and 16q23 (58), as well as of adults with the disorder, e.g. 16q23 (59). A recent genome-wide linkage study in adults also revealed significant novel loci of interest (18q21 and 2p25; (60)).

Although the linkage literature provides no replicated genome-wide significant findings using strict

criteria (61), regions highlighted by multiple studies (e.g. 5p13, 17p11, 16q23) are of considerable interest for further investigation given that these studies individually had low power to detect linkage to genes of small effects. To further delineate regions of interest among studies in this literature, Zhou et al. (62) conducted a genome scan meta-analysis of these data. They reported genome-wide significant linkage ( $P_{SR} = 0.00034$ ,  $P_{OR} = 0.04$ ) for at 16q23.1 that appeared to result from a contribution, though in some cases modest, from the majority of the seven studies examined. Additionally, results of individual and combined analyses suggest that many genes of moderately large effect are unlikely to exist in ADHD and that association methods will be more fruitful in the search for ADHD susceptibility genes.

### 109.2.3 Genome-Wide Association

Individual genome-wide association studies from the Children's Hospital of Philadelphia, an academic-industry collaboration between UCLA, Massachusetts General Hospital, University of Washington and Pfizer Pharmaceuticals (PUWMA), and two studies from the IMAGE consortium have not achieved genome-wide significant results ( $p < 5 \times 10^{-8}$ ). A meta-analysis combining these studies (63) has resulted in some promising findings, though again no results surpassed genome-wide criteria for significance. The top hit from the study (rs146807;  $p = 1.10E-06$ ) resides in a gene-poor region on chromosome 7. The next result most likely to be valid was found on chromosome 8 in a gene-rich area. The strongest signal was for rs7463256, a marker proximal to charged multivesicular protein 7 (CHMP7), involved in endosomal sorting. That study also implicated TNFRSF10D and TNFRSF10D, which are involved in mechanisms relevant to apoptosis, and LOXL2, which aids in the generation of chondrocyte, or connective tissue. As the authors note, this meta-analysis is the largest ADHD sample analyzed to date and these regions should be the bases of further study; yet, given sample sizes needed to achieve genome-wide significance for other complex psychiatric disorders, including schizophrenia and bipolar disorder, the sample lacks power to detect variants of the full range of expected effect sizes. Current efforts are now underway to amass even larger samples and are thus likely to drive power into the range where replicable genome-wide significant associations can be detected.

Exploratory analyses within GWAS data sets are also noteworthy because they highlight biological processes with a potential role in ADHD. Among the most interesting are those relevant to cellular adhesion and synaptic plasticity. For example, cadherin 13 (CDH13), which resides in the 16p23 region highlighted in linkage studies discussed above, was implicated in a pooled GWAS analyses of 343 ADHD adults and 250 controls (59) and

trait-specific analyses in the IMAGE project (64,65). Additionally, a bioinformatics pathway analysis of the five GWAS data sets has implicated proteins relevant to directed neurite outgrowth (66).

#### 109.2.4 Copy Number Variation

Finally, one of the most exciting findings to emerge in the recent literature is the presence of large (>500 kb) rare structural deletions or duplications of DNA that substantially increase risk for the psychiatric and neurodevelopmental disorders (e.g. autism (67) and schizophrenia (68)) in which they are found.

In the first investigation of this kind in ADHD, Elia et al. (69) extracted a gene set associated with copy number variants (CNVs) in their ADHD patients and parents. Compared to a large, control sample, they found no excess duplications or deletions; however, CNV-related genes were enriched for those previously identified for psychiatric disorders, learning and behavior domains and relevant neurodevelopmental processes such as synaptic transmission and central nervous system development. CNVs in the genes for protein tyrosine phosphatase (*PTPRD*) and metabotropic glutamate receptor 5 (*GRM5*) were highlighted as particularly notable because of the pattern of findings in the sample and the respectively uniform phenotypic features of patients.

Williams et al. (70) did identify an excess of rare deletions and duplications >500 kb in length in their ADHD sample (14%) versus controls (7%). Although ADHD youth with intellectual disability (ID) had a particularly high rate of these rare variants, an excess of large CNVs was still found in ADHD youth without ID compared to controls. Within this restricted group, CNVs in ADHD youth were more likely than those in controls to overlap with autism-related loci from the literature, and an excess of a particular schizophrenia-related duplication (16p13.11) was found as well. This 16p13.11 variant was also found to be a greater degree in ADHD Icelandic patients versus controls, and the replication sample showed further evidence of CNV occurrence at schizophrenia-related but not autism-related loci. There are multiple genes within this 16p13.11 region, with nuclear distribution gene E homolog 1 (*NDE1*) being of particular prior interest for schizophrenia because of its role in neurodevelopment and binding with the disrupted in schizophrenia 1 (*DISC1*) gene.

#### 109.2.5 Refined Phenotypes

Given the complexity of the ADHD phenotype, future studies will benefit from the use of large samples as well as efforts to target more homogenous and/or familial forms of the phenotype. The substantial evidence from twin studies suggesting partial rather than complete overlap for inattention and hyperactivity/impulsivity

symptoms suggests that in large samples, it will be fruitful to examine these symptom dimensions separately as well as in a combined manner. Previously, we have shown that the risk ratios in relatives of ADHD probands are increased when probands exhibit comorbidity with CD or BPD and when probands are shown to have ADHD that persists into adolescence (see Faraone et al. (71) for details). The statistical power of linkage and association studies increases with the magnitude of risk ratios. Additionally, phenotypes associated with the underlying pathophysiology of ADHD that lie in the pathway from genes to behavior are of interest to ADHD researchers. Such “endophenotypes” provide a way of targeting a phenotype that may be closer to gene products and less complex than the disorder as a whole. Thus far, endophenotypes based on neuropsychological impairments (72–74) and structural neuroimaging findings (75) appear promising in that they are identified in unaffected relatives of ADHD youth; however, considerably more work is needed to clarify their utility.

### 109.3 CONCLUSIONS

The evidence that genes influence susceptibility to ADHD continues to grow, and the field of ADHD genetics is poised for considerable advances in the coming years. In light of the changing ADHD nosology, the consistency of findings across behavioral genetic studies is striking. A growing number of candidate gene and agnostic genome-wide explorations have now been conducted. As with other neuropsychiatric disorders, the majority of genetic variance in the disorder remains unexplained. Yet promising leads exist. Meta-analyses of biological candidate genes have produced substantial evidence implicating *DRD4*, *DRD5*, *SLC6A3*, *DBH*, *SLC6A4*, *HTR1B* and *SNAP-25*; however, pooled odds ratios for these associations range from 1.16 to 1.45. Genome-wide linkage scans are not conclusive but highlight 5p13, 17p11, and 16q23 as regions likely to harbor additional susceptibility genes. Such studies also help to rule out genes of large effect in the etiology of the disorder. Although the first wave of GWAS studies has similar unyielded genome-wide significant results, they have identified the SNPs that require further exploration, and larger, better-powered studies are in the planning stages. Genome-wide data have additionally highlighted novel genes of interest, most notably *CDH13*. CNV studies have demonstrated an excess of rare duplications and deletions have been found in ADHD, and these variations overlap with other neuropsychiatric disorders, including autism and schizophrenia. In particular, a replicated duplication of 16p13.11 requires aggressive pursuit. Meta-analyses, collaborative studies with large sample sizes and examination of refined phenotypes that may reduce heterogeneity are certain to play a role in advances in the coming years. Studies of gene–gene interactions, and gene–environment interactions, will

be necessary to further clarify the mechanisms by which these genes interact with each other and with nongenetic factors. Such strategies, along with innovations in the technology and statistical methods, and opportunities for sequencing to further understand the role of rare variants, leave no doubt that further progress in understanding genetic risk for ADHD specifically, as well as risk that overlaps with other neuropsychiatric disorders, is on the horizon.

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<http://www.nimh.nih.gov/health/topics/attention-deficit-hyperactivity-disorder-adhd/index.shtml>  
<http://www.ncbi.nlm.nih.gov/omim/143465>

### Biographies



**Stephen Faraone** is a Professor of Psychiatry and of Neuroscience and Physiology at SUNY Upstate Medical University. He is also a Senior Scientific Advisor to the Research Program Pediatric Psychopharmacology at Massachusetts General Hospital. An author on over 700 publications, he was the eighth highest producer of High Impact Papers in Psychiatry from 1990 to 1999 according to the Institute for Scientific Information. In 2005, ISI determined him to be the second highest cited author in the area of Attention Deficit Hyperactivity Disorder and in 2007, he was the third most highly cited researcher in psychiatry for the preceding decade.

Dr Faraone is an Editor for the *Neuropsychiatric Genetics* and is a Deputy Editor for the *Journal of the American Academy of Child and Adolescent Psychiatry*, *Biostatistical and Methodology*, Editor for the *Journal of Attention Disorders*. In 2002, he was inducted into the CHADD Hall of Fame in recognition of his research on attention disorders. In 2008, he received the SUNY Upstate President's Award for Excellence and Leadership in Research. In 2009, he was awarded Alumni Fellow status at the University of Iowa. In 2010, he received the Chancellor's Award for Excellence in Scholarship and Creative Activities from the State University of New York.



**Dr Alysa Doyle** is an Assistant Professor of Psychiatry at Harvard Medical School (HMS) and on faculty of the Department of Psychiatry and the Center for Human Genetics Research at Massachusetts General Hospital (MGH). Within Psychiatry, she is a Scientific Director of Neuropsychology in the Pediatric Psychopharmacology Unit. She is also a consulting editor for the APA journal *Neuropsychology* and a licensed child and adult clinical psychologist.

Dr Doyle earned her BA in English and Psychology from Williams College. She received training in child and adult clinical psychology, pediatric neuropsychology and behavioral genetics at the University of Minnesota, where she earned her PhD. She completed her predoctoral internship and postdoctoral fellowship at MGH/HMS.

Dr Doyle's research focuses on the relationship between genes, neurocognition and risk for psychiatric disorders including ADHD, bipolar disorder, schizophrenia and autism. She also studies evidence-based clinical neuropsychological assessment in child psychiatric populations. Her work has been funded by the National Institute of Mental Health, Harvard Medical School and the Stanley Center for Psychiatric Research. She has received several awards including election to Sigma Xi by Williams College, a Dissertation Research Award from the American Psychological Association and a fiftieth Anniversary Scholars Award from Harvard Medical School.

# CHAPTER

# 110

## Autism Spectrum Disorders

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### 110.1 BACKGROUND

Autism Spectrum Disorders (ASDs) encompass a range of illnesses that share deficits in three core domains: social interaction, restricted interests/repetitive behaviors, and language (1). In the current clinical conception, there are three major categories of ASD, autistic disorder, Asperger syndrome and pervasive developmental disorder, not otherwise specified (PDD NOS). Autism or autistic disorder describes individuals with deficits in all three domains. Asperger's disorder describes individuals with deficits in social interaction and restricted interests/repetitive behaviors, but with normal language, but is likely to be removed from Diagnostic and Statistical Manual of Mental Disorders (DSM-V), which has lumped this category into the broad concept of ASD (2,3). PDD NOS describes individuals with one or more of these core deficits who do not meet the full diagnostic criteria for autism or Asperger's disorder as defined by the DSM-IV. Also, contained within the umbrella of autism spectrum disorders in DSM-IVR are childhood disintegrative disorder and Rett's syndrome, both of which are characterized by a period of normal development followed by a loss of motor, social, or language skills. Although there is a minimum set of symptoms that a patient must have to be diagnosed with an ASD the severity of various deficits must only be enough to cause "functional impairment" to be considered as symptoms. In reality, the severity of symptoms of ASD patients lies on a broad continuum. Thus, there is a great deal of clinical heterogeneity within any group of patients with an ASD. The diagnosis of autism, much like the diagnosis of epilepsy, gives a clinician only the broadest sense of what difficulties a given patient is dealing with and says very little about the severity, etiology, or prognosis of the problem.

Thus, in addition to significant genetic heterogeneity, as we will discuss later in this chapter, the genetic study of ASDs is challenged by what many consider extreme clinical heterogeneity. Before discussing the genetics of ASDs in detail, we first provide a brief overview of ASD diagnosis, followed by a discussion of medical comorbidities. Subsequently, we explore the evidence supporting a genetic etiology for ASDs, highlight the main findings from studies of linkage, association and chromosomal structural variation, and how these exciting findings inform our understanding of ASD pathophysiology, and its relationship to other neuropsychiatric and neurodevelopmental conditions.

### 110.2 DIAGNOSIS AND CLINICAL WORK UP

Technically, any physician taking a detailed history who asks about each of the criteria set forth in the DSM-IV can make a diagnosis of an ASD. Due to the variability of people interpreting those criteria, in the last 20 years, two psychological instruments were developed that are now the gold standard in evaluations for ASDs. The first, the ADI-R, is a clinician-administered questionnaire that asks parents or caregivers about a child's development (4). The second, the ADOS, is a semi-structured battery of social interactions between a clinician and the child being evaluated that is scored in a standardized manner (5). Although the ADOS and ADI-R have been validated as being sensitive and specific in children as young as 2 years, the average age of diagnosis for children with ASDs in the United States is about 5 years (6), suggesting that systemic barriers to timely diagnosis still exist.

Current estimates about the prevalence of ASDs vary from 0.6–0.9%, but there is growing consensus that at least part of the increase in prevalence during the last four decades is due to a standardization of criteria and increased referral to specialized evaluators on the part of families, schools, and primary care physicians (7,8). Once a diagnosis of an ASD is made, further evaluations such as a neurological evaluation, speech/language testing, audiology testing, IQ testing, and physical/occupational therapy evaluations are warranted to identify targets for treatment (9). In order to help distinguish between syndromic and idiopathic forms of ASDs, a detailed physical examination looking for dysmorphic features as well as Woods' lamp examination of the skin are recommended by the American Academy of Pediatrics (10). The development of array based comparative genomic hybridization and cytogenetic microarray technology has made genetic evaluation valuable in ASDs as well. Recent studies have determined that between 10 and 20% of patients with ASDs will have duplications or deletions that are identified by these tests, results observed in the American College of Medical Genetics recommend them as first-tier tests for patients with ASDs (11).

### 110.3 MEDICAL COMORBIDITIES

In addition to behavioral symptoms, individuals with ASDs can have variable impairments in sensory, motor, and medical domains (see Reference (12) for review). Classically, it was thought that 60–70% of individuals with autistic disorder have intellectual disability (ID) (13,14), but more recent estimates range between 34 and 55% (15–17). Epilepsy is highly comorbid with ASDs, though estimates of epilepsy prevalence in the ASD population vary from 6 to 60%. The higher estimates are due to inclusion of syndromic forms of ASD with a high occurrence of seizures, such as Tuberous Sclerosis Complex (TSC), and the prevalence of epilepsy in idiopathic ASD likely ranges between 5 and 10%. The loss of developmental milestones or skills after a period of normal development (developmental regression) is sometimes seen in patients with ASDs as well as certain syndromic forms of epilepsy (e.g. Dravet syndrome) (18). Although the definition of regression was not constant across studies, recent work has estimated the prevalence of developmental regression in ASDs to be between 22–53%, with the median age at the start of regression being between 20 and 30 months (19–21). Patients with developmental regression and autism may represent a subtype of ASDs with their own etiology, however they do not appear to have differences in prognosis or eventual levels of functioning compared to ASD patients without developmental regression (22).

Several studies have demonstrated that the mean head circumference in individuals with autism is larger than average; the rate of macrocephaly in ASD is between 17 and 20% (compared to 3% in the general population) (23,24). Courchesne et al. have presented evidence that

this may be due to brain overgrowth during the first year of life (25,26). Taken together, these findings indicate that the ASDs are caused by problems in early neural development and emphasize the importance of understanding developmental trajectories in addition to single epochs over time.

### 110.4 ETIOLOGY: GENES AND THE ENVIRONMENT

The purpose of this chapter is to review the literature concerning the genetic basis for ASDs, however there is an ongoing debate about what fraction of an individual's risk for having an ASD is due to genetic factors and what fraction is environmental. Rather than predicting the outcome of this debate, we take the position that the genetic data from genome sequencing and other efforts will help to settle this issue.

The first evidence implicating genetic factors was the discovery of chromosomal rearrangements in cases of autism, as well as the recognition that patients with certain rare genetic syndromes had deficits in the three core domains of ASDs. Some of the most important evidence supporting a major role for genes in ASDs come from twin studies (reviewed in Reference (27)). Early twin studies, using a more stringent definition of autism, found a high concordance among monozygotic (MZ) twins and almost no concordance among dizygotic (DZ) twins; these relatively small studies estimated that genetic factors comprised about 90% of the risk (28–30). More recently, larger twin studies have shown lower rates of concordance compared to the earlier studies among MZ twins (39–58%), and modestly increased rates of concordance among DZ twins (24–31%) (31,32). This suggests that the contribution of genetic factors to risk for ASDs is still significant and consistent with a heritability of 0.7. Although this heritability estimate is relatively large, it is on the lower end of previous estimates.

Previous studies have estimated that the sibling recurrence risk for ASDs (the odds that a future sibling of a child with an ASD will also have an ASD) to be between 3 and 10% (16,33,34). The most recent large scale, multisite prospective study of recurrence risk found it to be about 20% for autism (35), similar to the risk observed in DZ twins. This figure does not leave much room for sharing in utero environmental factors in ASD etiology. Other evidence for genetic factors playing a role in ASDs includes the heritability of subthreshold “autistic traits,” such as increased head circumference, and social dysfunction, as well as psychometric properties of language (14,24,32), which will be discussed later in the section on endophenotypes.

There is also evidence that environment can play a role in the occurrence of ASDs. In the 1960s an association was found between maternal viral infection during pregnancy (particularly rubella) and autism (36,37). Prenatal exposure to the antiepileptic drug sodium

valproate also significantly increased the risk of developing an ASD (38). If we assume the general population prevalence for ASDs to be about 0.8%, we can calculate the rough odds ratios (OR) for prenatal rubella infection or valproate exposure leading to ASDs as at least 9.9 and 6.0, respectively, which is higher than common genetic risk factors. Another environmental risk factor is increased parental age. Several studies have now replicated the finding that increased maternal and paternal age are associated with increased odds for ASDs in offspring, ranging from 1.3–1.6 for a single older parent to 3.1 if both parents are >40 years old at conception (39–41). A recent study by Volk et al. showed that the distance of maternal address during pregnancy from freeways was inversely correlated with risk for ASDs (42). The study showed the greatest OR of 2.2 for developing ASDs when mothers lived near a freeway during the third trimester. This suggests that airborne pollutants may play a role as well. Given the fact that all the subjects in the study were from a single geographic area (California), the study needs to be replicated in other parts of the world. Additionally, no actual airborne pollutants were measured in the study; proximity was taken as a surrogate measure of exposure. Many confounders are possibly explanatory and further work in this area is clearly needed.

Given the clear evidence for both genetic and environmental factors in ASD etiology it is not really that productive to argue which is more important. Common conditions are caused by a combination of genes and the environment, not one or the other. The benefit of a genetic approach is that it is a practical and efficient means to identify the etiologies of ASDs. One important issue that is often not discussed is that heritability estimates cannot take into account relatively invariant or pervasive environmental factors, which may be acting in concert with genetic factors. However, once genetic risks are identified, they provide a window through which environmental factors can be explored in an efficient manner.

### 110.5 PATHOPHYSIOLOGY

As ASDs are pediatric brain disorders and those with ASD do not have a shortened lifespan, obtaining post-mortem tissue for histopathological analysis has been challenging. Consequently, most pathological studies have had very small sample sizes and it has been difficult to compare across conditions and methodologies. Despite these limitations, two groups have made the observation that there appears to be an increase in the number of cortical mini columns in the brains of individuals with autism (43–45). These mini columns appear smaller in size, but contain a normal number of neurons, consistent with higher neuronal packing density overall. There has also been one report of an increase in cortical dendritic spine density in specific brain regions of

**TABLE 110-1** Syndromes with ASD as a Component

ASD Related Syndrome	Rough Percent of Cases with an ASD (%)	References
1q21 duplication	50	(67,68)
3p deletion/duplication	<50	(69–71)
15q11–q13 duplication (maternal)	>50	(72,73)
15q13 deletion	<50	(74,75)
16p11 deletion	55	(58,76–78)
22q11 deletion (DiGeorge syndrome/VCFs)	15–50	(79–81)
22q13 deletion	>50	(82,83)
15q13 maternal (Angelman)	40–80 <sup>a</sup>	(84,85)
11p15 (Beckwith Weidemann)	~7	(86)
7q35–36 (cortical dysplasia focal epilepsy)	70	(87–89)
10q23 (Cowden/BRRS)	20	(90,91)
Trisomy chr. 21 (Down)	6–18	(92,93)
Xq27 (fragile X)	25% of males, 6% of females	(94)
17p11 (Potocki–Lupski)	66–90	(95,96)
11q13 (Smith–Lemli–Opitz)	50	(97)
15q11–q13 paternal (Prader–Willi)	20–25	(98)
12p13 (Timothy)	60–80	(99–102)

<sup>a</sup>There exist some controversy whether or not the stereotypies and social deficits seen in Angelman syndrome are more related to ID than ASDs (see Reference (103) for review).

individuals with autism (46). These data, combined with fMRI and EEG studies (47–50), have led to the hypothesis that there is an overabundance of local neuronal connections within certain brain regions, at the expense of long-range (interhemispheric) connections (reviewed in Reference (51)). Another hypothesis about the pathophysiology of autism states that there is an imbalance between excitatory and inhibitory inputs (E–I imbalance) to neurons in various brain regions (52,53). Evidence for this theory comes from the observation of the comorbidity of epilepsy with ASDs (Table 110-1) as well as direct measurements in animal models of syndromic forms of autism (reviewed in Reference (54)). A third hypothesis, called the extreme male brain theory, posits that individuals with ASDs have brains in which exaggerated sex differences in neuro-circuitry result in differences in how they respond to social and other stimuli (55). Proponents of this theory argue that it helps explain the 4:1 male to female ratio seen in ASD cases and point to studies in which the levels of testosterone that a fetus was exposed to were later correlated with behaviors associated with ASDs (56,57). None of these theories is mutually exclusive and given the clinical heterogeneity of individuals with ASDs, all of them may help explain a proportion of cases. Our view is that the genetic data provides a solid and unbiased platform for understanding ASD



pathophysiology. Below, we first review chromosomal structural variation, followed by linkage and association.

## 110.6 SYNDROMIC FORMS OF ASD AND STRUCTURAL CHROMOSOMAL VARIATION

It has been estimated that 6–7% of individuals with ASD have large structural chromosomal variations visible with standard cytogenetic methods (58). Although analysis of common genetic variation based on SNP microarrays has not yielded strong genome wide association signals (see later in this chapter), these same data have been successfully used to identify structural chromosomal variation (59–61). Since the first studies showed that de novo nonrecurrent copy number variation (CNV) caused about 5–10% of ASDs (58,62), array technology has been steadily improving the resolution of rearrangements that can be identified (reviewed in Reference (63)). This led to the identification of several of the recurrent events discussed in Table 110-1, such as 16p del/dup. Two recent CNV studies in ASDs have identified the candidate genes *BZRAP1*, *MGDA2*(64), *SHANK2*, *SYNGAP1*, *DLGAP2*, and *PTCHD1*(60). These studies used either multiplex (more than one affected family member) or a combination of simplex (only one affected child) and multiplex families. Two more recent CNV studies have been on a collection of exclusively simplex families collected to help identify de novo events that contribute to ASDs (65). Sanders et al. found significant association between CNVs at 7q11.23(duplication), 16p13.2, and 15q11–13.1 in the Simons simplex collection (61). A parallel study by Levy et al. used a different platform and statistical tools on the same sample, and found consistent results, including an increased frequency of CNVs in girls; neither study identified an excess of transmitted CNVs in ASD, consistent with the model that the major risk conferred by CNV in ASDs is due to de novo events (59). Interestingly, an accompanying pathway analysis of the genes identified in the Levy et al. paper confirmed many of the candidate genes identified by a previous study (60) and implicated pathways involved in synapse formation and dendritic morphogenesis (66). But, despite a more than 10-fold increase in resolution over the last 5 years, the proportion of ASD that can be explained by large (>250kb) de novo CNV remains between 5 and 10%. It is also important to emphasize that although none of these variations individually account for more than 1% of total cases of ASDs, several of these CNVs and other syndromic forms of ASDs show a very high penetrance for ASD (see Table 110-1). These mutations with large effect sizes, including CNV spanning many genes, as well as syndromic forms of ID, have provided the first clear candidate genes for ASDs.

The identification of these syndromic forms of ASD is important because certain syndromes have other features relevant to treatment associated with them. For example,

sudden cardiac death is observed with increased frequency in 15q11–13, while astrocytomas and renal cell tumors are associated with TSC. As many of the newer syndromes become better clinically characterized, differences in phenotypes and trajectories are likely to be identified. A salient example is the difference in head circumference in 16p deletion versus 16p duplication patients (78,104), and the much lower penetrance for ASD and broader phenotypes observed in those with 16p duplication versus those carrying the deletion (76–78,105). Because of these issues, a molecular diagnosis is likely to be quite important from a clinical standpoint, especially as our characterization of some of the more recently identified CNV associated with ASD are studied in larger populations.

## 110.7 LINKAGE AND ENDOPHENOTYPES

### 110.7.1 Linkage Studies

Over the past decade, large linkage studies have identified numerous regions of interest that have met genome wide significance (3q25–27(106), 2q, 7q(107), 17q11–q21(108), and 20p13(109)), but only two (7q(110) and 17q11–q21(111)) have been replicated. Within these replicated linkage regions and others, where it has been assessed, no single common variant can account for the linkage signals (112,113). The lack of replication of large linkage studies is thought to be due to the heterogeneity present in ASDs. The lack of common variant association that can explain linkage under replicated peaks suggests that rare genetic variants play a more important role than previously realized.

### 110.7.2 Endophenotypes

One way to tackle heterogeneity is to use clinical features to identify what one hopes to be a more homogeneous subset of patients. For example, those with epilepsy, large heads, specific psychiatric comorbidities, ID, language delay, or even sex, may have distinct genetic risk factors. Multiple groups have devised strategies that perform linkage analyses for ASDs after stratification by phenotype with varying success. Stone et al. found a male specific linkage peak at 17q11 that reached genome wide significance (114). Molloy et al. (also using the Autism Genetic Resource Exchange (AGRE) sample) found suggestive peaks at 7q and 21q in ASD cases with regression (115). Salyakina et al. stratified for language by only analyzing cases with Asperger's disorder and found suggestive linkage peaks at 5q21 and 15q22 (116). When stratifying by IQ in the AGP dataset, Vieland et al. did not find any genome wide significant peaks, but did find that high and low IQ cases had distinct, mutually exclusive patterns of linkage (117). As with any such genetic finding, replication is critical, and the only finding from

stratification that has been formally replicated is the male-related locus on chromosome 17 (111).

Another approach uses the power of continuous quantitative traits of ASD features to find chromosomal regions of interest (reviewed in Reference (118)). Such quantitative endophenotypes should be observed in those with the disease and heritability (observed in those with the disorder and first degree relatives more than in the general population) (119). In theory, this QTL approach is more powerful than stratification, as it allows one to use all subjects, affected and unaffected, and does not require creating smaller subgroups of patients with a particular feature, which can reduce power by reducing sample size.

Several groups have adopted the idea that the overlapping genetic risk across ASDs, other psychiatric disorders, and the general population (120–123) represent a continuum of neurodevelopmental vulnerabilities and function. The existence of a “broad autism phenotype,” or subclinical features of ASDs found in family members of patients also argues that ASDs are an amalgam of traits (124) and the clinical heterogeneity of ASDs comes from the particular mix of traits observed in a given individual. Considerable progress has been made in defining those traits, determining their heritability, and developing psychometric instruments to quantitatively measure them in the past decade (reviewed in Reference (125)). Sung et al. found that traits for social motivation and range of interest/cognitive flexibility had the highest heritability (126). Wheelwright et al. developed an Autism Spectrum Quotient questionnaire that may be useful stratifying the patients with ASDs, but needs to be validated (127). Recent genome-wide scans have used older measures such as IQ (128), ADI-R subscales (129), and language delay (130) to identify loci of interest, but none have reached genome-wide significance.

As new measures and traits are identified, a comprehensive approach to identify the best endophenotypes for genetic study of ASD is needed. Genome-wide scans based on these traits are more likely to be successful than previous studies, which relied on targets of convenience, such as age of first word on ADI or IQ testing. Large-scale studies of the segregation of specific cognitive and behavioral features are needed in ASDs and across allied psychiatric diseases (see later in this chapter on cross disorder mutations).

## 110.8 GENOME WIDE ASSOCIATION STUDIES

Several genome wide association studies (GWAS) in ASD have been performed, but they have not revealed any loci with major effect sizes. Wang et al. published a high density SNP GWAS involving 2503 affected individuals and 6491 control subjects, starting with 1299 individuals from multiplex AGRE families. They found six SNPs in an intergenic region between the cell adhesion genes *CDH9* and *CDH10* as having replicated, genome wide

significance (131). Weiss et al. studied 1553 affected individuals, including a set of approximately 1175 individuals from AGRE that largely overlaps with the Wang et al. study. Weiss et al. found one highly suggestive SNP between the axon guidance factor *SEMA5A* and the taste receptor gene *TAS2R1* (109). These two studies, using different platforms, but a largely overlapping group of AGRE families, did not replicate each other’s findings. One possible explanation for this fact is that the two platforms had different areas of coverage (i.e. the platform used in Weiss et al. had poor coverage in the region identified by Wang et al. and vice versa). In light of a third study that failed to replicate either group’s findings (132), one has to consider the alternative that these initial findings are not generalizable. This is in contrast to the CNV studies mentioned earlier that have largely been consistent in their findings.

## 110.9 CANDIDATE GENES

To date, the list of genes that have been preliminarily associated with ASD (affected by CNVs or mutations) has over 100 members, representing a wide range in the level of supporting evidence (see Reference (63) for review). As multiple groups embark on whole genome and exome sequencing projects on patients with ASDs, this list is likely to change. Making sense of this growing list of genes presents a conceptual and bioinformatic challenge. One popular approach is to use gene ontology databases to order the genes on the list into common biological processes, and then construct networks between the genes based on different models of interaction (60, 132). There are several caveats to recognize with this approach. The first is that the candidate genes for ASDs are so disparate, that most biological processes undertaken by neurons are implicated. The second problem, which is related to the first, is that the approach is dependent on the quality and specificity of the gene ontology assignments, which vary greatly among genes. A more useful approach might be to categorize candidate genes based on their presence in biochemical complexes or pathways. The downside of this approach is that a greater number of the genes would remain uncategorized due to a lack of understanding of their biology. Table 110-2 outlines some of the biochemical pathways containing multiple ASD candidate genes.

In addition to the pathways mentioned in Table 110-2, there are several categories of proteins that contain members linked to ASDs that are intriguing as possibilities for future research. These include Actin regulators (*SYNGAP1*, *CYFIP1*, *ARHGEF6*, *NHS*), microtubule regulators (*LIS1*, *YWHAE*, *DCX*), ion channels and their regulators (*SCN1A*, *PRSS12*, *CACNA1c*, *CACNA1f*, *SLC9A6*, and *GRIA3*), transcription factors (*MEF2C*, *HOXA1*, *SATB2*, *FOXP1*, *FOXP1*, *FOXP1*, *RAI1*, *ARX*, *NFIX*, *TBX1*, *ZNF81*, *ZNF674*), and proteins involved in vesicle transport (*OCRL*, *AHI1*, *VPS13B*, *SYN1*, *RAB39B*).

**TABLE 110-2 ASD Linked Genes that Fall into Common Biochemical Pathways or Complexes**

Biochemical Pathway or Complex	ASD Linked Genes <sup>a</sup>	References
mTOR signaling pathway	( <i>TSC1</i> , <i>TSC2</i> ); <i>PTEN</i> ; <i>NF1</i> ; <i>MID1</i>	Reviewed in (133, 134)
MAPK/MEK/ERK signaling pathway	<i>BRAF</i> ; <i>HRAS</i> ; <i>KRAS</i> ; <i>MEK1</i> ; <i>CREBBP</i>	(135, 136)
Synapse assembly/maintenance complex	( <i>NRXN1</i> , <i>NLGN3</i> ); <i>NLGN4</i> ; <i>IQSEC2</i> ; <i>SHANK2</i> ; <i>SHANK3</i> ; <i>CASK</i> ; <i>DMD</i>	(137–142)
Splicing Factors/posttranscriptional regulators	<i>AFF2</i> ; <i>A2BP1</i> ; <i>CDKL5</i> ; <i>UPF3b</i> ; <i>PQBP1</i> ; ( <i>FMRP</i> , <i>CYPFIP1</i> )	(143–148)
DNA methylation/chromatin remodeling factors; Mediator complex	<i>NSD1</i> ; <i>JARID1c</i> ; <i>PHF8</i> ; <i>CHD7</i> ; <i>EHMT1</i> ; ( <i>MECP2</i> ; <i>ATRX</i> ; <i>SMC1A</i> ; <i>NIPBL</i> ; <i>MED5</i> , <i>MED12</i> )	(149–155)

<sup>a</sup>For references showing that these genes are linked to ASDs, see Reference (63). Genes within parentheses are known to form complexes.

A major unanswered question is whether these pathways converge on common molecular or biological processes (156,157). Recent gene expression studies in ASD brain suggest that despite the disparate processes implicated by mutations, there may be a large degree of convergence on final common pathways (158). This exciting finding awaits broader replication.

### 110.10 VARIABLE EXPRESSIVITY AND VARIABLE PENETRANCE—PSYCHIATRIC COMORBIDITY AND DISEASE BOUNDARY CONFUSION

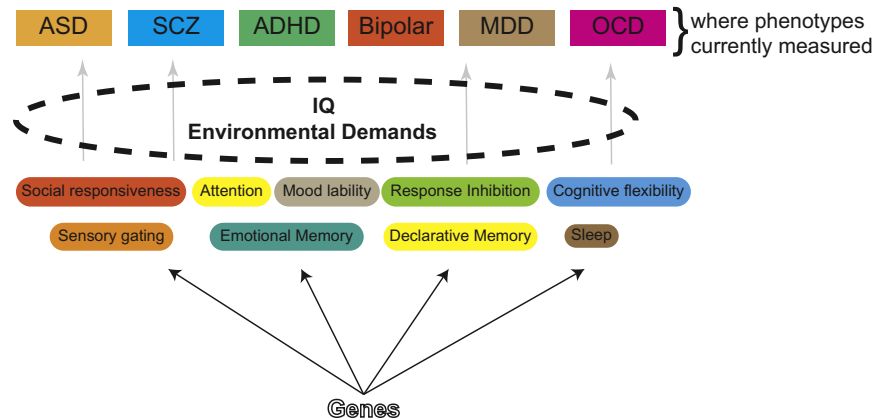
As our genetic knowledge advances, the classic boundaries that separate psychiatric diseases are being called into question; multiple genes and chromosomal regions implicated in ASDs have also been implicated in other psychiatric disorders such as schizophrenia, ADHD, and OCD (159). This raises the questions of variable penetrance and expressivity, or why mutations in a given gene can produce such different outcomes in different people? Another fundamental problem in the genetics of neuropsychiatric disorders is that the phenotypes used to assess penetrance, linkage, or association, are clinical assessments based on functional impairment that are far from the actual genetic derangement.

Figure 110-1 illustrates the concept that genes are likely to impact basic behavioral capabilities or parameters rather than specific disorders. Deficiencies in these capabilities can then interact with an individual's environment to form the constellations of symptoms we recognize as neuropsychiatric disorders. As a corollary, certain environments such as those found in wars, natural disasters, or extreme poverty, can overwhelm most people's functional reserve and produce symptoms in the absence of obvious genetic risk; however, the clinical manifestation of a mutation may not be observed until the individual's capacity to compensate is exceeded. From this perspective, resiliency or functional reserve may mask the effects of specific causes. Therefore, in order to understand the roles of specific genes in neuropsychiatric disorders, it may be more productive to ignore issues of penetrance and instead focus on understanding variable expressivity.

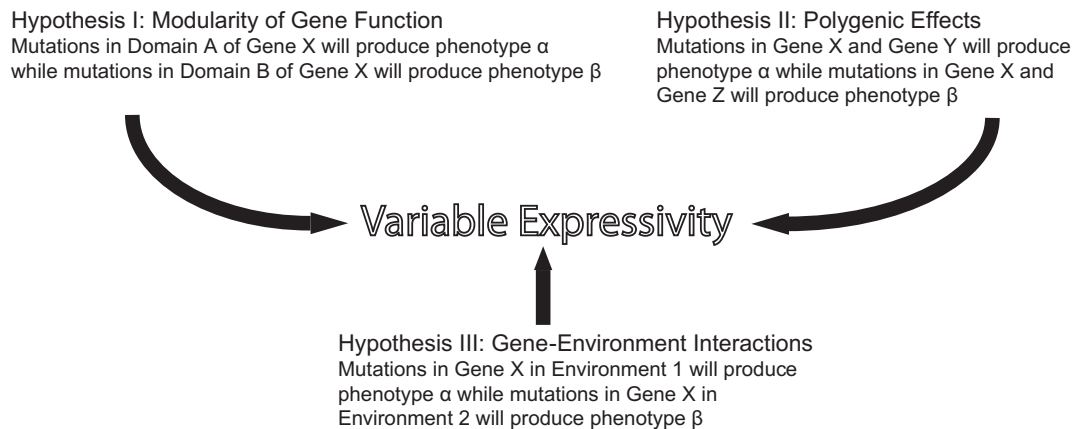
Understanding the mechanisms of variable expressivity in ASD candidate genes is important because they may help explain why there is such a great deal of clinical heterogeneity in ASDs. Additionally, being cognizant of these mechanisms is important because they form implicit assumptions that influence the design and analysis of genetic experiments. In many cases, studies are designed to minimize sources of variable expressivity. For example, researchers who believed that random X inactivation was a major source of variable expressivity may choose to study a disorder only in males or choose to focus on candidate genes on the X chromosome. Researchers who believed that an environmental factor, such as exposure to power lines, caused variable expressivity would choose to study their disorder in more rural populations.

Figure 110-2 illustrates three broad hypotheses about mechanisms of variable expressivity in ASDs. None of the hypotheses are mutually exclusive and, as we will now discuss, all have some evidence that they are relevant to ASDs.

The most obvious examples of Hypothesis I would be instances of pleiotropy, or one gene having multiple functions. However, mutations in regulatory regions, splice sites or imprinted genes would also be examples where the location of the mutation determines what disorder it produces. Recent re-sequencing efforts and deleterious SNPs found in a few ASD candidate genes offer the opportunity to see if Hypothesis I can be supported. *NRXN1* is a gene with considerable evidence linking it to ASDs, schizophrenia, and ID (reviewed in Reference (160)). Gauthier et al. re-sequenced the coding exons of *NRXN1* in individuals with ASDs, schizophrenia, non-syndromic Intellectual Disability (NSID), and controls, identifying rare variants that were more strongly associated with ASDs or schizophrenia (161). Looking at their data, one finds the interesting pattern that none of the missense mutations in *NRXN1* associated with just ASDs, or just schizophrenia and not with NSID, or controls, fall within a known domain (according to the NCBI Conserved Domain Database). In contrast, all of the missense mutations associated with NSID fall within one of the Laminin G domains of the protein. Re-sequencing of a related ASD candidate gene, *CNTNAP2* revealed rare



**FIGURE 110-1** Phenotypes in neuropsychiatric disease. Many neuropsychiatric genetic studies look for association or genetic effect at the level of neuropsychiatric disorders. However, these disease categories are predicated on functional impairment caused by a combination of environmental demands exceeding individual capabilities and compensatory mechanisms (which can often be influenced by IQ). ASDs provide an example of this where early-identified cases all had cognitive impairments and only after a seismic shift in awareness on the part of parents, educators, and clinicians were higher functioning cases identified. Individual genes are far more likely to impact basic behavioral traits such as the ones described. (Abbreviations: ASD: Autism Spectrum Disorder, SCZ: Schizophrenia, ADHD: Attention Deficit Hyperactivity Disorder, Bipolar: Bipolar Disorder, MDD: Major Depressive Disorder, OCD: Obsessive Compulsive Disorder.)



**FIGURE 110-2** Mechanisms of variables expressivity. Three plausible mechanisms for how variable expressivity can be observed when mutations in a hypothetical gene “X” are present. In this scenario, phenotype  $\alpha$  could be the disease state most often associated with Gene X, while phenotype  $\beta$  could be either another illness, or no illness (healthy individual). It is important to note that none of these hypotheses are mutually exclusive and they each may be true for a given gene in different biological contexts.

variants associated with ASD scattered throughout the protein (162), while studies looking at common variants associated with specific language impairment or selective mutism in the general population found significant SNPs clustering between exons 13–15 of the gene (163,164). As more sequencing efforts are published, we are likely to find more examples of different mutations in a given gene producing different neurobehavioral phenotypes, motivating cross disorder study of genetic risk factors that rely on careful quantitative measurement of behavior, cognition, and brain anatomy and function, rather than categorical disease diagnoses.

It is clear from genetic studies that ASDs are unlikely to be single gene disorders. Hypothesis II, which states that different combinations of mutations result in different phenotypes, provides a plausible explanation for how a polygenic, or complex genetic disease could have

significant clinical heterogeneity. The first evidence that Hypothesis II was applicable to ASDs was the observation that large deletions of candidate genes (presumably null mutations) were not fully penetrant in families (see Table 110-1, (165)). Early exome sequencing efforts of patients with ASDs have also identified rare variants that appear be associated with ASD when found together, but not in isolation (166). Gregor et al. re-sequenced two candidate genes (*NRXN1* and *CNTNAP2*) whose homozygous deletion is strongly associated with ASDs and ID in a population with ID and found heterozygous deleterious mutations in the two genes in a significant number of cases (167). This suggests that other genes are likely interacting with these two to produce ID. Levy et al. provided evidence for the idea that that ASDs are rarely caused by single gene mutations, but more often by CNVs that affected multiple genes, particularly in girls



(59). Again, further exome and whole genome sequencing will give us a clearer picture about how much of the clinical heterogeneity of ASDs is explained by Hypothesis II.

The previous two hypotheses assume that ASDs are result of aberrant neuro-developmental programs caused by one or multiple mutations. There is an alternate view that considers ASDs to be the result of an event, possibly a developmental insult. As reviewed earlier, several lines of evidence point to something happening either prenatally or during the first 6 months of life that results in a child displaying features of an ASD. Hypothesis III argues that the clinical heterogeneity of ASDs is due to a combination of the timing or nature of the insult and the patient's genetic background, which would confer varying levels of susceptibility to the insult. While we currently only have faint clues about what that insult might be (maternal infection, environmental contaminants, etc.), support for Hypothesis III exists in the findings that there appear to be haplotypes in the EN2 (168,169) and HLA DRB1 (reviewed in Reference (170)) loci that are protective for ASDs. Hypothesis III also offers a framework for considering epigenetic risk factors for ASDs. Hypothesis III is concordant with the idea that synaptic dysfunction is at the root of all psychiatric disorders in the same way that aberrant blood-flow is at the root of all strokes. In this model, the behavioral differences between schizophrenia, major depressive disorder, and ASDs result from different circuits, or populations of neurons being affected. If this were the case, one would expect to see a greater frequency of other psychiatric disorders in the families of patients with ASDs. Several small studies have suggested an increased prevalence of mood and anxiety disorders in the first degree relatives of patients with ASDs (171–173), but a large systematic investigation into the neuropsychiatric disease burden in families with ASDs needs to be undertaken.

It should be clear from the previous discussion that the genetic findings to date have not simplified our notion of psychiatric disease. From a clinical perspective, the issue of psychiatric comorbidity in ASDs is one that the field is still struggling with. Several of the diagnostic features or commonly associated symptoms of ASDs overlap with other disorders, such as sleep problems (overlapping with bipolar disorder), cognitive rigidity and repetitive behavior (overlapping with Obsessive Compulsive and Tic disorders), and social difficulties (often seen in ID or Social Anxiety Disorder). The DSM IV-TR and ICD-10 explicitly exclude diagnosing a patient with both ADHD and ASD, despite the fact that patients with ASDs frequently have symptoms of hyperactivity and difficulty paying attention (174). Parsing a patient's symptoms into subtly different categories often hinges on the patient's subjective experience of the symptoms, which can be difficult to elicit or correctly interpret in the case of patients with

ASDs due to their language and cognitive deficits. Furthermore, internal states such as sadness or anxiety may manifest through vastly different behaviors in patients with ASDs compared to neurotypical children. This has made quantifying the rates of comorbid psychiatric disorders with ASDs problematic. Our position is that researchers should essentially ignore these relatively arbitrary disease boundary issues, which are primarily useful for clinical reasons.

## 110.11 CONCLUSION

The clinical and genetic heterogeneity of ASDs have led to the suggestion that ASDs encompass hundreds of unique “autisms” (12), but this may be true of many neuropsychiatric disorders. The genetic data outlined in this chapter as well as what will be published in the near future promise to help resolve these clinical issues. As the many genes implicated in ASDs are organized and validated in pathways, biomarkers may be found for their dysfunction and specific therapeutic targets can be identified. Given the issues of variable expressivity we have highlighted (many ASD candidate genes are also implicated in other neuropsychiatric disorders), which specific quantitative cognitive, behavioral, biochemical or anatomical traits are influenced by particular genetic risk variants is a critical area of work in the near future. It is certainly plausible that such etiological information will lead to major revisions in diagnostic categories. A robust understanding of the genetics will finally give us improved diagnostic clarity, improved family and genetic counseling, as well as help guide treatment and prognostication. In the end, such information as to disease mechanism will serve to augment practice by providing individualized care, thus supplementing wise clinical practice and enabling us to truly treat the patient rather than the disorder.

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### Biographies



**Daniel Geschwind** is the Gordon and Virginia MacDonald Distinguished Professor of Neurology, Psychiatry and Human Genetics, and Director of the Neurogenetics Program and Center for Autism Research and Treatment in the Semel Institute at UCLA.

Geschwind obtained an AB degree in psychology and chemistry at Dartmouth College and his MD and PhD. degrees at Yale University School of Medicine. He completed his neurology residency at UCLA in 1995, where he has remained following training, joining the faculty in 1997. Dr Geschwind's research is in the area of neurogenetics and systems biology of human neuropsychiatric diseases.

His laboratory integrates basic neurobiology, genetics and genomics with translational studies of human diseases, ranging from autism to neurodegenerative diseases. Dr Geschwind has also put considerable effort into fostering large-scale collaborative patient resources for genetic research and data sharing. He played a major role in the founding and has provided scientific oversight for the Autism Genetic Resource Exchange (AGRE). He has published over 500 on numerous scientific advisory boards, including the NIH Council of Councils, the NIMH scientific advisory council, and the Faculty of 1000 Medicine. He received the Derek Denny-Brown Neurological Scholar Award from the American Neurological Association in 2004 and the Scientific Service Award from Autism Speaks in 2007.



**Sunil Mehta** is currently a Child and Adolescent Psychiatry fellow in the Semel Institute at UCLA. He received his BA in Biology and Philosophy of Science from the University of Pennsylvania and his MD and PhD degrees from Baylor College of Medicine. He completed his adult psychiatry residency at UT Southwestern Medical Center in 2009. Dr Mehta's research interests focus on understanding the biological functions of ASD candidate genes. He is currently using the visual system of *Drosophila melanogaster* to study the function of several ASD candidate genes.

# CHAPTER

# 111

## Genetics of Alzheimer Disease

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### 111.1 BACKGROUND AND HISTORY

Alzheimer disease (AD) is characterized by an insidious onset and progressive deterioration of memory and at least one other cognitive domain (language, praxis, recognition, or executive functioning). It is the leading cause of dementia in the elderly, affecting more than 5.4 million people in the United States (1,2). Prevalence increases with age, from 0.3–0.5% at age 60 to 11–15% at age 80 (3–5). There is substantial variability in these estimates, with some reports finding the prevalence to be as high as 47% after age 84 (6). Half the beds in long-term care facilities are already devoted to patients with dementia, and a majority of those patients have AD (7). As the population continues to age, the prevalence is expected to increase almost threefold by 2050 (1), making AD a growing public health and economic crisis.

#### 111.1.1 Alzheimer Disease History

In 1906, Bavarian psychiatrist Alois Alzheimer presented the first case of dementia characterized by histopathological signs of senile (neuritic) plaques and neurofibrillary tangles (NFTs). At the time, Alzheimer thought the disease was a rare cause of senility (8). In the 1960s, Blessed, Tomlinson, and Roth performed a series of autopsies on hundreds of brains affected with “normal senility” (9), and found the majority were affected by the plaque and tangle lesions characteristic of AD. In subsequent decades, it became increasingly clear that most dementias result from a specific pathological process, and are not simply the result of aging.

Despite advancements in clinical assessment, definitive diagnosis of a symptomatic person still requires either neuropathologic examination of brain tissues obtained postmortem, or pathogenic mutation in a causal AD gene (10–12). Further complicating the diagnosis for clinicians, 6–14% of autopsy confirmed cases have an atypical disease presentation (10,13–15).

Clinically, AD remains a diagnosis of exclusion, generally beginning with slowly progressive memory loss, and advancing to deficits in higher intellectual functions and cognitive abilities (16). Even an apparently clear clinical diagnosis of AD may be overturned later by histopathological investigation, revealing different or additional etiologic processes contributing to cognitive impairment (17). Standard criteria for the clinical diagnosis of AD were established by the National Institute for Neurological and Communicative Diseases and Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA criteria) in 1984 (18) (Table 111-1). In tertiary care facilities, clinical diagnosis is accurate in 85–90% of putative AD cases (19–21). Clinical diagnosis of AD in individuals aged 60 years and older is often complicated by numerous factors including cerebrovascular insults (22) and common, co-occurring neuropsychiatric conditions (23). In a study by Carney et al. (24), 9 of 101 clinically diagnosed cases of AD from a family research study had a different etiology for their dementia, including two within the same multiplex family. Another 18 cases had unrecognized contributing comorbidities in addition to AD pathology. Most of the cases with disparate clinical and histopathological diagnoses had significant Lewy body pathology, but Pick disease and progressive supranuclear palsy also contributed.

**TABLE 111-1 Criteria for Clinical Diagnosis of Alzheimer Disease**

- I. The criteria for the clinical diagnosis of probable Alzheimer disease include
  - Dementia established by clinical examination, documented by the Mini-Mental Test, Blessed Dementia Scale, or some similar examination, and confirmed by neuropsychological tests
  - Deficits in two or more areas of cognition
  - Progressive worsening of memory and other cognitive functions
  - No disturbance of consciousness
  - Onset between ages 40 and 90, most often after age 65
  - Absence of systemic disorders or other brain diseases that in and of themselves could account for the progressive deficits in memory and cognition
- II. The diagnosis of probable Alzheimer disease is supported by
  - Progressive deterioration of specific cognitive functions, such as language (aphasia), motor skills (apraxia), and perception (agnosia)
  - Impaired activities of daily living and altered patterns of behavior
  - Family history of similar disorders, particularly if confirmed neuropathologically
 Laboratory results of
  - Normal lumbar puncture as evaluated by standard techniques
  - Normal pattern or nonspecific changes in electroencephalograph such as increased slow-wave activity
  - Evidence of cerebral atrophy on computed tomography, with progression documented by serial observations
- III. Other clinical features consistent with the diagnosis of probable Alzheimer disease, after exclusion of causes of dementia other than Alzheimer disease, include
  - Plateaus in the course of progression of the illness
  - Associated symptoms of depression, insomnia, incontinence, delusions, illusions, hallucinations, catastrophic verbal/emotional/physical outbursts, sexual disorders, and weight loss
  - Other neurologic abnormalities in some patients, especially with more advanced disease and including motor signs, such as increased muscle tone, myoclonus, or gait disorder
  - Seizures in advanced disease
  - Computed tomography normal for age
- IV. Features that make the diagnosis of probable Alzheimer disease uncertain or unlikely include
  - Sudden, apoplectic onset
  - Focal neurologic findings such as hemiparesis, sensory loss, visual-field deficits, and incoordination early in the course of the illness
  - Seizures or gait disturbances at the onset or very early in the course of the illness
- V. Clinical diagnosis of possible Alzheimer disease
  - May be made on the basis of the dementia syndrome, in the absence of other neurologic, psychiatric, or systemic disorders sufficient to cause dementia, and in the presence of variations in the onset, in the presentation, or in the clinical course
  - May be made in the presence of a second systemic or brain disorder sufficient to produce dementia, which is not considered to be the cause of the dementia
  - Should be used in research studies when a single, gradually progressive severe cognitive deficit is identified in the absence of other identifiable causes
- VI. Criteria for a diagnosis of definite Alzheimer disease are
  - The clinical criteria for probable Alzheimer disease
  - Histopathologic evidence obtained from a biopsy or autopsy
- VII. Classification of Alzheimer disease for research purposes should specify features that may differentiate subtypes of the disorder, such as
  - Familial occurrence
  - Onset before age of 65
  - Presence of trisomy 21
  - Coexistence of other relevant conditions, such as Parkinson disease

### 111.1.2 Heritability and Segregation within Families

Several lines of evidence support heritable components of AD. Familial aggregation studies demonstrate the disease clustering within families (25–27), and in large pedigrees with early-onset Alzheimer disease (EOAD), the disease exhibits an autosomal dominant pattern of inheritance (28). Family studies of AD have found that the age at onset (AAO) of AD within families has a bimodal distribution. EOAD families were determined empirically to have a mean AAO less than 58 years (29). EOAD is generally defined as an onset of symptoms prior

to age 60 or 65 (30–32). Twin studies have also demonstrated a genetic component to AD. By their nature, twin studies of the elderly tend to be small, and early studies utilized varying diagnostic criteria; however, each published study found the concordance rate among monozygotic twins (22–83%), who share all their genes, to be higher than the concordance rate among dizygotic twins (0–50%), who share only half their genes on average (33–36). While the higher concordance rate in monozygotic twins supports the genetic influence on the disease, environmental factors are also suspected, since concordance was not 100%.



Segregation analysis in families affected by AD further supports the influence of genes on AD risk. Segregation analysis follows the inheritance of a disease through a family and attempts to fit a genetic model to the observed transmission pattern. Models assuming only sporadic AD with no major genes and models limited only to Mendelian inheritance patterns were rejected (37). Using only EOAD families, the pattern of inheritance is often consistent with classic Mendelian autosomal dominant inheritance, while inheritance patterns in late-onset families appear more complex. Late-onset Alzheimer disease (LOAD) follows a pattern of multifactorial inheritance involving a combination of genetic and nongenetic factors (38–40).

In summary, these studies have provided powerful evidence for a genetic contribution to AD, even before any specific genetic variants were identified. In the very rare early-onset autosomal dominant pedigrees, the evidence clearly points to Mendelian genes. Additional risk-conferring genetic variants inherited in more complex combinations were believed to contribute to AD in the majority of the population.

### 111.1.3 Environmental Risk Factors for AD

As noted earlier, the lack of perfect concordance in AD status among identical twins, who share 100% of their genes, strongly implies that not all risk for AD is attributable to genetic effects. In fact, a variety of environmental factors have been implicated in AD risk including socioeconomic features such as level of education, a multitude of risk factors for cardiovascular disease, and still others such as history of head injury and female gender. Immune pathways have also been implicated in AD.

Environmental risk factors of AD have been reviewed in considerable detail elsewhere (41). Major socioeconomic factors implicated in increases of AD risk include lower level of income, lower level of completed education, and lower occupational status (42). These factors are correlated with poor quality of and limited availability of resources needed during childhood development that tend to be associated with poor growth and physical development (42). These limitations contribute to developmental markers like low birth weight (LBW) and short stature, which have been shown to be associated with AD (43). LBW is often a marker of developmental deficiency and often correlates with limitations in cognitive ability that is present in childhood and may contribute to reduced education level. One study observed that limited linguistic ability in young women increased the likelihood of meeting neuropathologic criteria for AD in old age (44). Developmental deficiencies may result in reduced brain development with diminished reserve capacity, which may modify lifelong patterns of growth and risk of AD (42,44).

Early development correlates of LBW include the rapid gain of weight after birth, increased central adiposity, and metabolic irregularities such as insulin resistance. As these are also important early risk factors for

type 2 diabetes mellitus, it is not surprising that connections between features of diabetes and AD pathology have been drawn. Rat models have shown experimentally that killing insulin-producing cells in the brain in early life have produced AD-like pathological features. Furthermore, in work using transgenic murine models of AD, increased intake of water supplemented with sucrose advanced amyloidosis and the appearance of cognitive deficits, while caloric restriction was found to be protective (45).

Knowledge is limited about connections between environmental exposures such as infectious and chemical agents and AD. The relationship between environmental exposures and cognitive decline has been explored in some detail, especially the role of lead exposure in reduced cognitive function. One study (46) of blood levels in 172 children showed that even blood lead concentrations below 10 µg/dL are inversely associated with children's IQ scores at three and five years of age, with declines in IQ greater at smaller than at higher concentrations, suggesting a stronger effect of environmental exposure to lead in childhood than previously estimated. Cognitive decline from lead exposure has been found to continue through development and into adulthood, as seen through consistently lower IQ levels and behavioral changes (47). Furthermore, occupational lead exposure among adults correlates with lower neurobehavioral test scores and with deficits in several cognitive spheres, including measures of executive function and verbal intelligence and memory (48). Evidence for gene–environment interactions in cognitive decline is evidenced by findings that persons with at least one *APOE* ε4 allele may be more susceptible to the effects of prolonged lead exposure on the central nervous system (49). While a direct relationship between cognitive decline from lead exposure and AD development has not been shown in humans, a primate model of early life exposure to lead demonstrated a development of AD-like pathological abnormalities in the brains of infant monkeys; however, monkeys exposed later on in adulthood did not show similar pathology (50).

Head trauma is also associated with AD, but whether trauma occurring earlier or later in life increases risk is unknown. It should be noted that adult head circumference has been observed to correlate with cognitive performance; likewise, traumatic brain injury during childhood is associated with reduced head size (42,51), suggesting that head size may mediate the effect of brain injury on AD risk.

## 111.2 GENETICS OF EOAD

Linkage analysis, followed by positional cloning of candidate genes, was used to identify genetic variants of the three genes known to cause autosomal dominant EOAD. The three known AD-causative genes are amyloid precursor protein (*APP*) (52), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) (53–55).

### 111.2.1 Discovery of the *APP* Gene

The first AD-causing mutation found was in the *APP* gene. *APP* was initially cloned and localized to chromosome 21 in 1987 (56,57). Chromosome 21 was an intriguing location, since those with trisomy 21 (Down syndrome) develop the pathologic signs (neuritic plaques and NFTs) and symptoms of AD at an early age (58). Interestingly, an individual who was affected by translocation Down syndrome, but did not develop the characteristic Alzheimer dementing syndrome, was identified. On autopsy, the brain was free of the characteristic AD plaques and tangles. The individual's DNA revealed a duplication of the obligate telomeric Down syndrome region of chromosome 21, but the *APP* gene was not within this duplication (59). Within families affected by *APP* mutations, functional analyses have demonstrated the likely pathophysiologic processes causing AD. The identified missense mutations lie outside of the beta-amyloid region of the *APP* gene, and as *APP* can be cleaved into one of two amino acid sequences, these mutations appear to cause shifts in the proteolytic cleavage of *APP* toward the production of beta-amyloid ( $A\beta$ ) products. In neuroblastoma cell lines one mutation favors the production of a 42 amino acid residue-length  $A\beta$ .  $A\beta$  is a major component in senile plaques. This 42-residue beta-amyloid ( $A\beta_{42}$ ) is less soluble than the alternative 40-residue isoform ( $A\beta_{40}$ ) (60).  $A\beta_{42}$  is also associated with increased aggregation and neurotoxicity (Hilbich et al., 1991). *APP* mutations consistently show increased production and/or deposition of the pathogenic long- $A\beta$  product (60,61). The long- $A\beta$  isoforms are less soluble than the shorter  $A\beta_{40}$  isoform, and these  $A\beta$  deposits are the principal component of amyloid within the characteristic plaques of AD-affected brains (62). Transgenic mice expressing the human *APP* mutation develop senile plaques, but not the tangles, characteristic of AD (63). A second characterized mutation in *APP* causes double mutation-transfected neuroblastoma cells to secrete five times the  $A\beta_{42}$ , compared to control cells with wild-type *APP* (64). The neurotoxicity of  $A\beta$  has been well demonstrated in cell culture (65,66).  $A\beta$  also confers increased vulnerability to excitotoxic damage of hippocampal neurons in vitro (67). Furthermore, when  $A\beta$  is added to neuronal cell cultures, there is a distinct loss of synapses, analogous to the early pathologic processes of AD (68). Image analysis of  $A\beta$  load in AD patients and controls demonstrates a positive correlation between the amount of  $A\beta$  and severity of cognitive deficit.

While it was clear that some early-onset families were affected by *APP* mutations and showed linkage to chromosome 21 (69), many others were found not be affected by *APP* mutations (70,71). Worldwide, only about two dozen families have been described as carrying mutations of the *APP* gene. Among these families, at least 15 different pathogenic mutations have been characterized (Alzheimer Disease Mutation Database) (72). Therefore,

*APP* mutations that cause AD display allelic heterogeneity. Since the majority of early-onset familial AD is not caused by mutations in the *APP* gene, there is clearly locus heterogeneity for EOAD (73,74).

### 111.2.2 Discovery of the *PSEN1/PSEN2* Genes

Subsequent studies in additional families with autosomal dominant inheritance demonstrated genetic linkage to chromosome 14 (75–77). In 1995, the first AD-causing mutation in the *PSEN1* gene was identified (55). Since then, more than 150 different *PSEN1* mutations scattered throughout the gene have been described (78,79). Interestingly, the majority of the large autosomal dominant AD families appear to carry unique *PSEN1* missense mutations (Clark et al., 1995). While several families with the same *PSEN1* mutation have been identified, these families appear to be unrelated, providing evidence that the same mutation may have arisen more than once (80,81). Phenotypically, *PSEN1* familial AD is the most aggressive form of AD, and affected individuals generally have disease onset in their fourth or fifth decade of life (82). The characteristic amyloid plaques in brains of individuals affected with AD and a *PSEN1* mutation show a relative abundance of  $A\beta_{42}$  compared to the plaques in individuals with sporadic AD. Quantitative image analysis using  $A\beta_{42}$  and  $A\beta_{40}$  antibodies demonstrate a 1.5- to 3-fold relative increase in plaques containing  $A\beta_{42}$  peptides (58,83).

After characterization of *APP* and *PSEN1*, there remained EOAD-burdened families under study that did not show segregation of known mutations, and that did not link to either chromosome 21 (*APP*) or 14 (*PSEN1*). These included families originating from the Volga River basin of Russia (84) who showed linkage to chromosome 1 (53). Serendipitously, with the cloning of the *PSEN1* gene, a larger 7.5-kilobasepair (kb) alternative polyadenylation message was identified. The product represented a gene homologous to *PSEN1*. The new gene was mapped to a known linkage region on chromosome 1, and was identified as *PSEN2*. Mutations in *PSEN2* are rare, but have been described in the Volga River basin families and in one Italian pedigree (53,54). Individuals affected by *PSEN2* mutations have a variable time to disease onset, ranging from the fourth through the eighth decade of life.

### 111.2.3 Pathophysiologic Roles of *APP* and the Presenilins

The physiologic role of the presenilins remains unclear. However, the presenilin mutations do appear to have a pathophysiologic relationship with *APP*. Assays of  $A\beta_{40}$  and  $A\beta_{42}$  in the plasma and the cultured skin fibroblast media of individuals with presenilin mutations revealed a twofold elevation of  $A\beta_{42}$  levels, despite no mutations of

the *APP* gene (85). In addition, transgenic mice expressing *PSEN1* and *APP* mutations demonstrate A $\beta$ <sub>42</sub> plaques and an accelerated AD-like phenotype (86). These observations pointed to an interaction between *APP* and the presenilins. Whether the presenilins interact directly with *APP* through their putative  $\gamma$ -secretase activity, or act as cofactor for another  $\gamma$ -secretase, remains unclear; however, it is clear that, like *APP* mutations, the *PSEN1* and *PSEN2* mutations all lead to increased levels of A $\beta$ <sub>42</sub>. The precise role of A $\beta$  accumulation in the pathogenesis of AD remains under investigation. A $\beta$  accumulation may be the final common neurotoxic pathway, an additional toxic by-product of the primary neurotoxic pathway, or less likely, an inert by-product (79).

Variants in these three genes (*APP*, *PSEN1*, and *PSEN2*) account for between 30% and 50% of early-onset familial AD (87), yet together they account for less than 2% of all cases of AD (88–91). In addition, while the genetics underlying autosomal-dominant or familial EOAD may appear to be relatively straightforward, the variants in genes responsible for AD in many families have yet to be characterized. Furthermore, the relationship between identified causal mutations and AD phenotype is not necessarily simple. For example, *PSEN1* mutations associated with familial EOAD have been identified in individuals with frontotemporal dementia who have no evidence of the A $\beta$  accumulation characteristic of AD (92,93). Nevertheless, each gene discovered has contributed to the elucidation of the pathophysiologic mechanisms underlying AD.

### 111.3 GENETICS OF LOAD

LOAD cases comprise the majority (90–95%) of individuals afflicted with AD. The prevalence of LOAD increases exponentially with advancing age (5). LOAD also increases the risk for other major common disorders (e.g. cardiovascular and cerebrovascular diseases), which reduce life expectancy (94,95). Thus it is clear that LOAD has a significant negative impact on the quality of late life and will represent a major financial and emotional burden to society until effective treatments or preventions are available.

There is compelling evidence that the etiology of LOAD, like EOAD, is also strongly genetic. Family studies (96,97) and twin studies (36,98) have suggested that LOAD may aggregate within families. While the majority of LOAD cases are sporadic, 15–25% are familial (99). Segregation analyses suggest the inheritance of LOAD is consistent with Mendelian inheritance, with incomplete penetrance and an additional multifactorial component (38,40).

Despite strong evidence that LOAD is a genetic disease, the complex etiology of LOAD still poses a great challenge for the identification of susceptibility genes. The presence of co-occurring psychiatric disorders, such as depression, varies from case to case, suggesting the

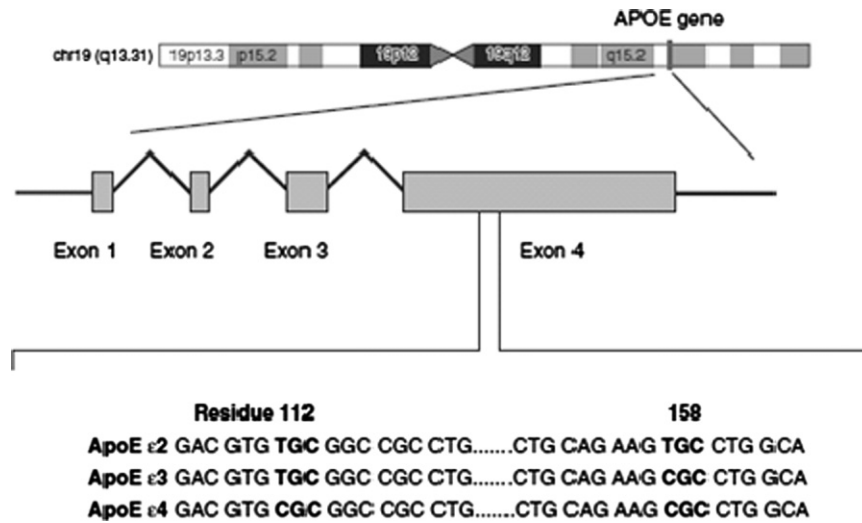
possibility of heterogeneity in disease etiology. Whether environmental exposures like lead and socioeconomic factors like access to education that factor into risk of disease interact with genetic causes of LOAD remains unclear, and gene–environment interactions may factor into the complex portrait of genetic susceptibility to LOAD.

#### 111.3.1 Discovery of the Apolipoprotein E Gene

The Apolipoprotein E (*APOE*) gene is the first and most widely accepted susceptibility gene for LOAD. It was initially discovered using affected relative pair linkage analysis in a subset of individuals diagnosed with AD at age 60 or older (100), and the signal was further refined by follow-up association analysis (101). The *APOE* gene lies on the long arm of chromosome 19 (19q13.2) (102,103) adjacent to another apolipoprotein gene (*APOC1*), and is also linked to the *APOC4*, *APOC2*, and *TOMM40* genes (104,105). *APOE* comprises four exons and three introns, spanning approximately 3.7 kb (102,106). It encodes the serum protein apolipoprotein E (ApoE), which is involved in the transport, storage, and metabolism of lipids and is synthesized in the central nervous system by astrocytes (107). There are three isoforms for ApoE protein: ApoE2, ApoE3, and ApoE4. These three isoforms result from single amino acid substitutions at residues 112 and 158 (108,109). The ApoE2 isoform is produced when the amino acid sequence contains cysteine at both residues 112 and 158. ApoE3, the most common isoform, contains cysteine at residue 112 and arginine at 158. The ApoE4 isoform is seen when the sequence contains arginine at both these two residues. These three isoforms are, therefore, produced by three different combinations of codons at the two loci: TGC (for cysteine) and CGC (for arginine), which result in three different alleles:  $\epsilon$ 2,  $\epsilon$ 3, and  $\epsilon$ 4 alleles (Figure 111-1). The frequencies of these three alleles are estimated to be 8, 78, and 14%, respectively, in most Caucasian populations (110). Other *APOE* alleles (such as  $\epsilon$ 1,  $\epsilon$ 5, and  $\epsilon$ 7), and therefore the ApoE isoforms they encode, are extremely rare in most populations. The *APOE*  $\epsilon$ 4 allele carries the strongest risk of any genetic variation for a complex disorder, and acts in a dose-dependent fashion (111). As such, the *APOE*  $\epsilon$ 4/ $\epsilon$ 4 genotype has a very high penetrance. Furthermore, the *APOE*  $\epsilon$ 2 allele appears protective for LOAD (112,113).

##### 111.3.1.1 Epidemiologic Features of the *APOE* Gene.

**111.3.1.1.1 The *APOE* Polymorphism Is Associated with Risk and AAO of LOAD.** While the role of *APOE* in cardiovascular diseases has been well characterized (114,115), its role in neurodegenerative disease has been the focus of investigation for over two decades. The  $\epsilon$ 4 allele has consistently been associated with risk for both sporadic and familial LOAD (111). In populations of European ancestry, the relative risks of LOAD for  $\epsilon$ 4



**FIGURE 111-1** Structure of the *APOE* gene and three ApoE isoforms.

homozygotes and for  $\epsilon 3/\epsilon 4$  heterozygotes are approximately 15 and 3, respectively, compared to  $\epsilon 3$  homozygotes (116). The  $\epsilon 4$  allele has the greatest impact on risk for AD among individuals who are between the ages of 60 and 79 years. Its effect is gradually attenuated as an individual surpasses age 80 (116,117). In contrast to the  $\epsilon 4$  allele, the  $\epsilon 2$  allele is protective for risk of LOAD with a reduction in risk of up to 20% (112,113,118).

The *APOE* polymorphisms also modulate the AAO for both familial (111,119–122) and sporadic forms of AD (123–125). The *APOE*  $\epsilon 4$  allele has a dosage effect on AAO. In one family-based study, the estimated mean AAO for subsets consisting of individuals carrying no  $\epsilon 4$  allele, one copy of  $\epsilon 4$  allele, and two copies of  $\epsilon 4$  alleles, were 84.3, 75.5, and 68.8 years, respectively (111). In addition, the effect of  $\epsilon 4$  allele on risk for LOAD is also age dependent. Although the *APOE*  $\epsilon 4$  allele appears to determine when an individual develops the first dementia-related symptoms, it remains arguable whether the  $\epsilon 4$  allele hastens disease progression. Two population-based studies reported that the *APOE*  $\epsilon 4$  allele was associated with a higher rate of memory deterioration in nondemented individuals (126,127). However, there is equivocal evidence regarding whether the *APOE*  $\epsilon 4$  allele speeds up (128,129) or slows (130,131) down disease progression in LOAD patients. Other studies report that the *APOE*  $\epsilon 4$  allele has little effect on disease progression (132,133).

**111.3.1.1.2 The Effect of *APOE* Polymorphism May Vary by Race/Ethnicity.** Although the *APOE*  $\epsilon 4$  allele is a risk factor for LOAD in nearly every population, the magnitude of association between the  $\epsilon 4$  allele and LOAD may vary by ethnicity. A weaker effect of the *APOE*  $\epsilon 4$  allele on risk of LOAD in African-Americans, compared with Caucasians, has been reported in several studies (134–136). A meta-analysis of 40 studies by Farrer and colleagues (116) found that the *APOE*  $\epsilon 4$  allele has less influence on risk among African-American and

Hispanic populations, compared with the Caucasian population, while the effect of *APOE*  $\epsilon 4$  allele was greater within the Japanese population. In addition, heterogeneity in *APOE*  $\epsilon 4$  allele effect size has been noted across studies of African-Americans (116) and Hispanics (137), suggesting that the effect of *APOE* on LOAD risk may depend on additional population-specific characteristics.

Variation in the magnitude of association between the *APOE* polymorphism and risk of LOAD may also reflect the variation in distributions of *APOE*  $\epsilon 4$  alleles in different racial/ethnic groups. For example, the *APOE*  $\epsilon 4$  allele frequency in cognitively normal individuals was significantly greater in the indigenous Australian population than in the Caucasian population (138). Another example from a study of three distinct ethnic groups in Malaysia showed that the *APOE*  $\epsilon 4$  allele frequency in the Indian population was higher compared to the Chinese and Malay populations (139). The “thrifty gene hypothesis,” initially proposed to study genetic mechanisms of type 2 diabetes (140), may also explain why high *APOE*  $\epsilon 4$  allele frequency does not lead to increased  $\epsilon 4$  allele-associated LOAD risk in some populations. High *APOE*  $\epsilon 4$  allele frequency in some indigenous ethnic groups may result from selection favoring the  $\epsilon 4$  allele because of its association with enhanced cholesterol uptake in environments where food supplies are more limited (141). Such a high *APOE*  $\epsilon 4$  allele frequency may not result in an increased risk of AD in these populations until more individuals start to consume a high cholesterol diet (142). The hypothesis may be supported, at least in part, by the findings of no association between the *APOE*  $\epsilon 4$  allele and LOAD in eastern Africa (143,144), while the *APOE*  $\epsilon 4$  allele is a well-established risk factor for LOAD in African-Americans (116,134–136).

**111.3.1.1.3 The Effect of *APOE* Polymorphism May Vary by Gender.** Female individuals have a 1.5- to 3-fold higher risk of LOAD compared with male individuals (reviewed in (145)). The gender effect on LOAD risk



appears to be most marked among individuals carrying two copies of the *APOE*  $\epsilon 4$  allele (116). Since estrogen is a putative neuroprotectant (reviewed in (146)), some have speculated that the higher risk of LOAD in women may be attributed to “estrogen deprivation” after the age of menopause; however, studies examining the protective effect of estrogen replacement therapy on cognitive function have produced inconsistent and sometimes contradictory findings. Inconsistencies may be due to the different study designs (e.g. observational studies versus prospective randomized clinical trials), and may relate to issues of treatment, such as timing of initiation of the therapy and type of therapy (147). The *APOE* polymorphism may also modulate the effect of estrogen on risk of LOAD. Decline in cognitive function is slowed by estrogen replacement therapy, particularly in women carrying no *APOE*  $\epsilon 4$  alleles (148). These lines of evidence suggest interactive effects between *APOE*  $\epsilon 4$  allele and gender and/or estrogen on development of LOAD.

**111.3.1.2 The Role of *APOE* on the Neuropathology of LOAD.** The definite diagnosis of AD is established based on neuropathological findings, including amyloid plaque, intracellular NFTs, and neuronal loss (149). In addition, cerebral amyloid angiopathy (CAA), defined by the accumulation of fibrillar  $A\beta$  in cerebral blood vessels, is also commonly seen in AD patients (150,151). The *APOE* polymorphisms are thought to influence LOAD risk through these amyloid-related neuropathological processes.

**111.3.1.2.1 Amyloid Plaque and NFTs.** Several studies report that individuals carrying two copies of *APOE*  $\epsilon 4$  alleles have a higher amyloid plaque density than individuals carrying one or no *APOE*  $\epsilon 4$  alleles (152–157). Two other studies did not confirm this finding (158,159). Similarly, there have been conflicting findings regarding whether the *APOE*  $\epsilon 4$  allele is associated with the density of NFTs. Several studies have reported an association between the *APOE*  $\epsilon 4$  allele and NFT density (153,156,160,161), but these have been refuted by other studies (154,157–159,162).

**111.3.1.2.2 Cerebral Amyloid Angiopathy.** CAA is present in more than 90% of individuals affected with LOAD. There is an association of CAA and the *APOE*  $\epsilon 4$  allele (156,163–165), and there is a dose-response relationship between the *APOE*  $\epsilon 4$  allele and the extent of CAA (163,166); however, a recent study has shown that *APOE* polymorphisms have no effect on the severity of CAA (Tian et al., 2004). Interestingly, although CAA is a risk factor for lobar cerebral hemorrhage, *APOE*  $\epsilon 2$  allele carriers have a higher rate of such hemorrhage related to CAA, compared with individuals carrying no *APOE*  $\epsilon 2$  alleles (167,168). This counterintuitive finding is likely due to the association of the *APOE*  $\epsilon 2$  allele and microangiopathic changes (e.g. fibrinoid necrosis and concentric splitting) occurring at the vessel wall (169).

**111.3.1.3 The Relationship of the *APOE* Gene with Anatomic Findings in LOAD.** The typical anatomic pathological change in brains of LOAD individuals is

whole brain atrophy. The medial temporal lobe, and in particular the hippocampus, manifests some of the earliest and most marked atrophy. Mild cognitive impairment (MCI) is generally understood to be an intermediate stage between the cognitive changes of normal aging and the serious cognitive declines related to AD. MCI is more likely to progress to AD in the presence of hippocampal atrophy (170). Several studies have shown that hippocampal volume is inversely associated with the number of *APOE*  $\epsilon 4$  alleles (171–173), but this finding was not supported by a different study (174). Other research has shown that the *APOE*  $\epsilon 4$  allele hastens hippocampal atrophy in nondemented individuals (175). In addition, the *APOE*  $\epsilon 4$  allele appears to exert a greater effect on hippocampal atrophy in women than in men (176).

The mechanism by which *APOE* polymorphisms influence risk and AAO in LOAD is yet to be uncovered. Several hypotheses have been posited, mostly centered on the relationship between the *APOE* polymorphisms and  $A\beta$ . One widely tested hypothesis proposes that the *APOE* polymorphisms may have an impact on the production, distribution, and clearance of  $A\beta$ . Supporting this hypothesis, the *APOE* polymorphisms influence AAO, particularly in individuals carrying the  $\beta$ -amyloid precursor protein ( $\beta$ APP) Val717Ile mutation, suggesting a possible interaction between the *APOE* gene and the *APP* gene (122). The density of  $A\beta$  peptide plaque is greater in individuals carrying at least one copy of the *APOE*  $\epsilon 4$  allele, compared to individuals carrying no copies of the *APOE*  $\epsilon 4$  allele (156). In addition, the hippocampal atrophy resulting from neuronal loss may, at least in part, stem from an impaired ability to clear extracellular  $A\beta$  due to the *APOE*  $\epsilon 4$  allele (177).

An alternative hypothesis proposes that the *APOE* polymorphisms influence risk of LOAD by altered cholesterol metabolism. Intracellular cholesterol availability impacts transport and storage of  $\beta$ APP, and therefore the reduction in intracellular cholesterol may lead to decreased  $A\beta$  formation (178). The cholesterol hypothesis is supported by observations that taking statin drugs to treat hypercholesterolemia may decrease risk of LOAD (179–183); however, a longitudinal community-based study refutes this finding (184).

## 111.3.2 Additional Candidate Susceptibility Loci for Late-Onset AD

**111.3.2.1 Overview.** While *APOE* is critically important, it does not account for all the genetic variations observed in AD. More than a third of AD cases do not have a single *APOE*  $\epsilon 4$  allele. The sibling relative risk ( $\lambda$ ) for the *APOE* locus is estimated to be about two, suggesting that *APOE* accounts for, at most, 50% of the total genetic effect in AD (116,123,185). To date, efforts to identify the remaining AD loci have taken several forms: regional and whole-genome scans for linkage in multiplex families, association tests of

candidate genes, the combining of findings across multiple studies through linkage and association meta-analyses, genome-wide association studies (GWAS), and the emerging technology of deep resequencing.

**111.3.2.2 Linkage Analysis.** Genome-wide linkage scans (GWLS) for LOAD have been published on many independent datasets since 1997 (137,186–195). Many of the scans (186–191) have focused on Caucasian families from the general population, while other scans have focused on special populations (137,189,190,193) or very large single inbred families (192,194,195). All these studies have used microsatellite markers with, at best, an average spacing of 8 cM. Some chromosomal regions have been studied extensively (most notably chromosomes 9, 10, and 12), although no consistently replicated AD gene has yet been identified within these regions. It should also be noted that while the majority of linkage

follow-up studies have focused on these chromosomes, there are numerous other linkage regions identified in more than one study that have been virtually ignored (Table 111-2).

For several reasons, reliance solely on linkage data has produced limited results and problematic inconsistencies across studies (196). One reason is that linkage analysis is more powerful than association analysis for identifying rare, high-risk disease alleles, but association analysis is more powerful for detecting common disease alleles conferring modest disease risk (197). Yet another explanation is that criteria for suggestive linkage may allow for too many false-positive regions; because of multiple hypothesis testing considerations, it is suggested that fine-mapping suggestive linkage peaks is only sensible when there is clear evidence for an excess of suggestive linkage across the genome (198).

**TABLE 111-2 Genetic Classification of Alzheimer Dementias**

Type	Chromosome	Gene
<b>I. The Alzheimer diseases</b>		
A. Early-onset familial, autosomal dominant mutations (AD1)	21	<i>APP</i>
B. Late-onset familial and sporadic associated, susceptibility gene (AD2)	19	<i>APOE</i>
C. Early-onset familial, autosomal dominant (AD3)	14	<i>PSEN1</i>
D. Early-onset familial, autosomal dominant (AD4)	1	<i>PSEN2</i>
E. Late-onset familial (AD5)	12	?
F. Late-onset familial (AD6)	10	?
G. Alzheimer disease, Lewy body variant	19, other?	<i>APOE</i> , other?
H. Other confirmed late-onset susceptibility genes (sporadic AD)	1	<i>CR1</i>
	2	<i>BIN1</i>
	6	<i>CD2AP</i>
	7	<i>EPHA1</i>
	8	<i>CLU</i>
	11	<i>MS4A</i> gene cluster
	11	<i>PICALM</i>
	11	<i>SORL1</i>
	19	<i>ABCA7</i>
	19	<i>CD33</i>
<b>II. Other causes of presenile dementias</b>	17	<i>MAPT</i>
A. Frontotemporal dementia with parkinsonism (FTDP-17) (Picks disease, nonspecific dementia, familial subcortical gliosis, frontal lobe degeneration)	4	<i>HD</i>
B. Huntington disease	21	<i>APP</i>
C. Hereditary cerebral hemorrhage with amyloidosis, Dutch	20	<i>CST3</i>
D. Hereditary cerebral hemorrhage with amyloidosis, Icelandic	19	<i>NOTCH3</i>
E. Hereditary multi-infarct type dementia (CADASIL)	13	<i>ITMB2</i>
F. Familial dementia, British (FDB) and Danish (FDD)	3	?
G. Familial nonspecific dementia	4	<i>SNCA</i>
H. Familial Parkinson disease, type 1	?	?
I. Adult-onset Parkinson disease, familial and sporadic		
<b>III. Mutations at the prion locus</b>		
A. Gerstmann–Straussler–Schenker (GSS)	20	<i>PRNP</i>
B. Creutzfeldt–Jakob, hereditary (CJD)	20	<i>PRNP</i>
C. Creutzfeldt–Jakob (transmitted)	20	<i>PRNP</i>
<b>IV. Nongenetic dementias</b>		
A. Infectious (e.g., AIDS, syphilis)		
B. Vasculitis		
C. Metabolic/nutritional (e.g., thyroid, B12 deficiency)		
D. Vascular (e.g., multi-infarct)		
E. Drug toxicity		
F. Tumors		

In linkage studies of AD, initial dataset sizes were quite small and had inadequate power to detect all relevant loci. While the datasets have subsequently increased, the microsatellite marker sets have recovered less than 50% of the potential linkage information in the families (199). Thus, many regions of true linkage may remain undetected, and false-positive results may have been inflated. Even under the best of circumstances, the regions identified through GWLS still contain hundreds of locational candidate genes. Thus, while GWLS data have been, and will continue to be, useful, they do not represent a definitive, comprehensive, nor sufficiently detailed examination of the whole genome. Despite these caveats, considerable analyses have been done following up GWLS regions without confirmation of new genes identified.

### 111.3.2.3 Candidate Gene Association Analyses.

The candidate gene approach for identifying susceptibility genes focuses on specific candidate genes selected because of their known (or more often, hypothesized) biological function relevant to AD. Hundreds of genes have been individually tested for association with LOAD, and nearly 150 genes have been reported to be associated, but few of these have gained wide acceptance (200,201). There are several reasons for this. First, our knowledge of gene function is still very limited, and it has been difficult to make direct observations of altered gene function or expression in Alzheimer tissues. Second, the sample sizes and single-stage study designs have generally been too small for the small-to-moderate effect sizes and locus heterogeneity that we now suspect underlie AD. Third, the use of clinical details and clinical subtypes to define clinical (and by proxy, genetic) heterogeneity has only recently been recognized, and only sporadically implemented. Fourth, the level of genomic detail that could be interrogated was low. All these issues conspire to make replication of any true effect difficult, and false-positive results rampant. Thus, while some of these reported associations could be and are real, it is not surprising that the evidence for these loci has been mixed. Meta-analyses and GWAS are two association approaches that have been used to overcome the limitations of the candidate gene approach for identifying genes, the first by combining statistical power to detect effects across multiples datasets, and the second by interrogating most of the genome agnostically with high-density coverage.

**111.3.2.4 Candidate Gene Meta-Analyses.** Given the limitation that sample sizes in candidate gene study designs have generally been too small to detect small-to-moderate effect sizes, one approach that benefits from the availability of genetic data in multiple samples is meta-analysis. A meta-analysis by Bertram et al. (201) served not only to catalog association results across many candidate gene association studies in the AlzGene database, as described before, but also to identify associations with AD by systematically combining data on genetic variants that had been genotyped commonly across multiple studies. This study performed meta-analyses systematically

on variants genotyped in three or more samples and identified variants with small-to-modest effect sizes in 13 candidate genes for further investigation (including *ACE*, *CHRNA2*, *CST3*, *R1*, *GAPDHS*, *IDE*, *MTHFR*, *NCSTN*, *PRNP*, *PSEN1*, *TF*, *TFAM* and *TNF*).

*PSEN1* is one particular gene known to be involved in familial, EOAD, suggesting that this approach may have identified valuable candidates for further examination. The authors noted a major limitation of this approach: candidate gene studies tend to examine genes with hypothetical functional roles in disease or proximity to strong linkage signals, and this has the potential to introduce bias from several sources into meta-analytic examinations. This limitation is partially addressed by the introduction of GWAS, which deal with several potential biases by examining high-density genotyping of markers capturing most common variations across the genome, reducing overrepresentation of variant data from particular genomic regions. The application of meta-analytic approaches to GWAS data represents an additional improvement upon meta-analysis in candidate genes by improving sample sizes and allowing for small-to-modest effects to be observed with fewer potential biases inherent, provided appropriate adjustment is made for differences between high-density genotyping platforms and for relevant issues of study design.

### 111.3.2.5 Genome-Wide Association Analyses.

More than 13 studies have tested association with LOAD on high-density, genome-spanning panels of SNPs. Grupe et al. (202) pooled samples and tested association with more than 17,000 gene-based putative functional SNPs across the genome, finding a signal at *APOE* that reached study-wide statistical significance, with multiple weaker associations observed elsewhere, many occurring in regions of known linkage. Coon et al. (203) reported results of association with half-million SNPs across the genome genotyped on over 1000 histopathologically verified AD cases and controls, identifying only *APOE* as a major susceptibility gene. A follow-up study (204) stratifying cases by *APOE* genotype detected strong associations with *GAB2* SNPs, and in follow-up work observed altered *GAB2* transcript levels in vulnerable neurons, and an effect of *GAB2* levels on tau phosphorylation; replication studies have observed mixed results (205–209). Abraham et al. (210) genotyped approximately 550,000 SNPs in more than 1000 pooled cases and 1200 pooled controls, and in testing genetic associations, observed genome-wide statistical significance only for SNPs in or near *APOE*. Following up on the strongest signals that did not attain statistical significance with more individual genotyping identified the gene *LRAT* (MIM:604863), which is involved in the vitamin A (retinoid) cascade, a system previously implicated in AD. Bertram et al. (211) analyzed 500,000 SNPs in 410 families, reporting an SNP associated with AD AAO on chromosome 14q31, and providing additional evidence of associations near *APOE* and near *GAB2*.

Beecham et al. (212) reported a GWAS on approximately 550,000 SNPs in nearly 500 AD cases and 500 cognitive controls. This study confirmed associations at *APOE* and identified an SNP on chromosome 12q13 meeting a genome-wide statistical significance threshold corresponding to a false-discovery rate (FDR) < 0.20. The strongest signals with less than genome-wide statistical significance in this study were identified in regions with prior linkage evidence, suggesting that some of these are likely to underlie true associations (213). A GWAS (214), genotyping 314,000 SNPs on 844 cases and 1255 controls, once again verified *APOE* associations, and in second-stage replication analysis, identified a novel signal on the X chromosome (combined  $P = 3.9 \times 10^{-12}$ ) in the gene *PCDH11X* (MIM:300246), encoding a protocadherin, a cell-cell adhesion molecule expressed in the brain. Finally, a study (215) using the original set of cases and controls from Beecham et al. (212) and additional newly identified cases and controls with high-density genotyping examined associations in a genome-wide set of markers (483,399 SNPs) and identified an association with LOAD of genome-wide significance ( $P = 4.70 \times 10^{-8}$ ) at 151.2 Mb of chromosome 6q25.1 in the gene *MTHFD1L*, which encodes the methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like protein, a gene involved in the pathway synthesizing methionine from homocysteine, and thus may influence homocysteine levels, known risk factors for AD.

While many of the aforementioned non-*APOE* associations attained genome-wide significance and were found to be associated in replication datasets, associations of these variants have not been found to be statistically significant in other independent studies, and for this reason, it remains unclear whether these genetic variations are truly associated with risk. A notable limitation of these studies is the lack of statistical power to detect small or even modest associations (odds ratio (OR) < 1.5) with samples sizes of ~1000 cases and ~1000 controls, or less. A second generation of GWAS with greatly increased sample sizes were able to detect the first set of associations of variants with small effects on LOAD and that replicated in multiple studies. The first of these by the European Alzheimer Disease Initiative (EADI) (216) examined associations in a total of 6010 LOAD cases and 8625 controls, and observed highly significant associations in *CLU*, a chromosome 8 p21.1 gene that encodes clusterin or apolipoprotein J, and in *CR1*, a chromosome 1q32.2 gene encoding the complement component (3b/4b) receptor 1. Effect sizes for associations of the minor alleles at these loci were either slightly deleterious (for *CR1*, OR = 1.21) or slightly protective (for *CLU*, OR = 0.86). The Genetic and Environmental Risk in Alzheimer Disease (GERAD) consortium (217) also observed genome-wide statistical significance for associations of non-*APOE* genomic variants in their independent dataset including a total of 5964 cases and 10,188 controls. These included strong associations in *CLU* (OR = 0.86),

one of the genes with significantly associated variants in the Lambert et al. study, and novel associations in the *PICALM* gene (OR = 0.86). A third study by the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium (218), which incorporated data from both the EADI and GERAD consortia to investigate an overall 9511 cases and 28,174 controls, identified associations with genome-wide statistical significance adjacent to two sets of previously unreported loci, near *BIN1* on chromosome 2q14.3 (OR = 1.13) and near *EXOC3L2/BLOC1S3/MARK4* on chromosome 19q13.3 (OR = 1.18). *BIN1* encodes the bridging integrator 1 protein, which is a nucleocytoplasmic adaptor protein that is heavily expressed in brain and muscle. Of the three genes proximal to the significant associations on chromosome 19q13.3 (signals that are independent of and not in linkage disequilibrium with associations in the *APOE* region), only two are potential biological candidates involved in AD-related pathways: *BLOC1S3*, which encodes subunit 3 of the biogenesis of lysosomal organelles complex-1 protein, influences endosomal to lysosomal routing, and is expressed in the brain; and *MARK4*, which encodes the MAP (mitogen activated protein)/microtubule affinity-regulating kinase 4 protein, is expressed exclusively in the brain, and participates in neuronal differentiation.

Most recently, two even larger GWAS, one from the Alzheimer Disease Genetics Consortium (ADGC) (219) and another from a joint GERAD/EADI/CHARGE analysis (GERAD+) (220), confirmed the genetic associations at *CLU*, *CR1*, *PICALM*, and *BIN1*, and further identified and confirmed several additional AD susceptibility loci. The ADGC examined data on nearly 2.5 million genotyped and imputed SNPs from 11,840 cases and 10,931 controls in 15 datasets using a three-stage design, all on samples from individuals of European ancestry. These novel SNP associations with LOAD at *MS4A4A*, *CD2AP*, *EPHA1*, *CD33*, and *ABCA7* bring the total of consistently replicated loci associated with LOAD risk to 10 (*APOE*, *CR1*, *CLU*, *PICALM*, *BIN1*, *EPHA1*, *MS4A*, *CD33*, *CD2AP*, and *ABCA7*), confirming the emerging consensus that common genetic variation plays a significant role in the etiology of LOAD. These two studies estimated that, with the inclusion of genetic effects contributed by the nine non-*APOE* loci, as much as 50% of the genetic effect of LOAD may now be explained.

The next generation of genetic studies will likely take several forms. Some will combine and meta-analyze findings from large GWAS consortia, and with the increase in sizes of these datasets to many tens of thousands of LOAD cases and controls, much greater power will be available to identify and replicate newfound genetic associations with LOAD, and to uncover the biological mechanisms underlying disease pathology. Others will integrate annotation data on biochemical pathways to perform associations of variants at multiple related loci



through pathway analyses, while still others will integrate data on important biomarkers like A $\beta$  in order to be able to capture genes more closely related to specific pathological features of disease.

### 111.3.3 Clinical Implications of Genetic Discoveries for AD

**111.3.3.1 Genetic Counseling and Testing.** Increased media attention and access to genetic testing through clinical laboratories and direct-to-consumer testing have enhanced public awareness of the role genetic factors play in the etiology of AD, but the personal and clinical utility of such information has thus far been unclear. It remains controversial whether genetic testing for AD is appropriate, particularly since effective treatments and preventions for AD are still lacking (88,221,222). In light of these facts, the National Society of Genetic Counselors and the American College of Medical Genetics published practice guidelines for clinicians to assess their patients' risk for AD, to determine when genetic testing is appropriate, and to counsel their patients accordingly (223).

Using information collected from a three-generation pedigree, risk should be assessed based on average AAO in the family (early or late onset) and pattern of inheritance observed (sporadic, familial, or autosomal dominant). Genetic counseling should include information about the patient's risk relative to the population and the current status of treatment and prevention options. For families in which an autosomal dominant form of AD is a possibility, it is recommended that genetic testing be offered for causative forms of dementia using the *Guidelines for Genetic Testing for Huntington's Disease (HD)* set forth by The Huntington's Disease Society of America (224). In particular, pretest genetic counseling should include a discussion about the likelihood of identifying a mutation in the family and, if so, risk to the patient and offspring, motivations and considerations for pursuing testing, permanence of the information, and the potential impact of insurance, psyche, life plans, and relationships, among others. Genetic testing for causative mutations in a referral-based series of AD cases was proved to be cost-effective (225), and when accompanied by appropriate genetic counseling, resulted in effective coping skills and absence of untoward psychological responses (e.g. severe depression, anxiety, or suicidal ideation) (226–230).

On the other hand, while guidelines recommend assessing and counseling about risk, genetic testing for those with familial or sporadic LOAD is not recommended in most instances. In addition to a lack of clinical utility for those who carry APOE  $\epsilon$ 4 allele(s), APOE testing has low sensitivity and specificity, and it is difficult to convey probabilistic risk based on the presence or absence of an  $\epsilon$ 4 allele (231). Based on data from the REVEAL study, which suggest that APOE testing

on a self-selected, highly motivated, and educated group does not result in significant short-term psychological distress, guidelines further state that testing may be considered at the clinician's discretion if the patient wishes to pursue testing despite genetic counseling and recommendations to the contrary (232). In such instances, the same testing protocol used for causative genes should be used.

**111.3.3.2 Pharmacogenomics.** Genetic information may help illuminate disease mechanisms and facilitate developing targeted treatments. Diverse genetic influences in AD may drive biochemical pathways that result in the clinical heterogeneity observed in AD. Therefore, individuals affected with different subtypes of AD or other dementias may be characterized by different disease courses, prognosis, and responses to treatments. Pharmacogenomics research may shed some light on heterogeneity in individuals' response to pharmaceutical compounds.

Donepezil, galantamine, rivastigmine, and tacrine are cholinesterase inhibitors (ChEIs) used to alleviate AD symptoms, with evidence of weak-to-moderate efficacy (233–235). Response to ChEIs may differ by APOE genotype (236). Specifically, carriers of at least one copy of the APOE  $\epsilon$ 4 polymorphism have been shown to be the less responsive to the ChEI tacrine (237).

The ChEIs are metabolized primarily by the cytochrome P450 drug metabolism proteins (238). Their baseline metabolic activity varies greatly from individual to individual, largely because of polymorphisms in the genes encoding them. One such polymorphism in the CYP2D6 gene has been found to contribute to 15% of cases of efficacy and/or adverse drug response in AD (239). There are ethnic and geographic differences in the frequency of these polymorphisms. For example, the CYP2A<sub>del</sub> mutation is present in 15% of Asians, but only 1% of Caucasians, and produces a protein product with no metabolic activity (240).

The clinical significance of P450 gene polymorphisms as related to response to ChEIs has not been thoroughly elucidated. Similarly, pharmacogenomic approaches to developing drug targets in AD have not been extensively utilized in clinical practice (241).

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### Biographies



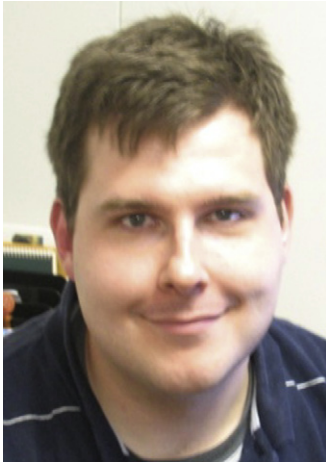
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# CHAPTER

# 112

## Schizophrenia and Affective Disorders

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### GLOSSARY

**Psychiatric disorder** – A state in which an individual's behavioral actions and state are not within the norms of their society.

**Schizophrenia** – A psychotic disorder with symptoms including hallucinations, delusions, thought processing defects, mood effects and social withdrawal.

**Affective disorder** – A disorder of emotional state that results in excessively negative and/or positive mood states, which may not be congruent to the individual's situation and which are protracted. This disorder may include swings from one state to the other and include psychotic elements.

### 112.1 INTRODUCTION

Psychiatric disorders exert a unique and profoundly negative effect on individuals, their families and society in general. Although not isolated to our species, they are often thought of as uniquely human because of their impact on the individual's personality and challenge our perceptions of what it is that constitutes a human individual (1).

Psychosis in particular has elicited contemplation, with etiological theories, and management recommendations recorded in some of the earliest written records from the Mesopotamian region (2). Indeed, extensive and diverse descriptions of psychopathology were offered throughout the ancient world (3). This is likely a result of the florid effects of this group of disorders and the fact that most members of society are, in one way or another, impacted by them. Interest not only was of an intellectual nature but also reflected the need to deal with the effects of the psychopathology, for the individual and/or society's sake.

This chapter, by focusing on classic schizophrenia, bipolar disorder 1 (BPD1) and major depressive disorder (MDD) will review current understanding of schizophrenia and the affective disorders from a genetic perspective.

The milder variants, e.g. bipolar disorder 2 (BPD2), schizophreniform disorder and other subclassifications as well as intermediate states such as schizoaffective disorder (which has elements of each of the primary disorders), are no less valid; however, understanding of these disorders is yet too tenuous to do more than complicate a field already challenged by diagnostic complexities and conflicting data. Therefore, except in so far as these diagnoses inform the classic disorders, they will not be discussed.

#### 112.1.1 Incidence of the Disorders

Almost half of all Americans are at risk for having a major psychiatric disorder at some point in their life. The affective, or mood, disorders affect one in five people in their lifetime. The prevalence of MDD accounts for 17%, and BPD (type 1 or 2) for 4%, of the prevalence, respectively. Affective disorders are the leading cause of disability in young adults (under 45 years of age) (4). Schizophrenia is reported to occur around the world with a prevalence ranging from 1.4 to 4.6 per 1000 of the population in most groups (5). There are some notable exceptions, including the significantly elevated incidence in some immigrant groups (6) as well as the significantly decreased rates found among the Hutterites of both Dakota and Manitoba (7).

The high incidence of these disorders therefore has significant implications on health, well-being and the economy; although the costs to individuals and society can be difficult to fully measure (8). It is estimated that BPD1 and schizophrenia each have annual public health costs in the tens of billions of dollars in the United States alone (9). The costs to a degree reflect the fact that management remains problematic. The reason for limited management capabilities, in part at least, is a consequence of our limited understanding of these disorders.

The human genome project may offer the first novel approach to unraveling the pathoetiology of these

disorders since ancient times and as a result has generated tremendous excitement and hope (10). In order to realize the potential of genetic research, however, it is crucial to be aware of the basic assumptions that underlie these disorders and psychiatry in general, in order to avoid simplification and misinterpretation of the data.

## 112.2 HISTORY AND DEFINITIONS OF THE DISORDERS

The primary problem facing psychiatric research and management is knowing what we are dealing with.

In order to understand what psychiatric illnesses are, it is crucial to view them from a historical context, as current understanding remains deeply rooted to previous concepts. This is evident in society's ongoing separation of the physical illnesses from the psychiatric ones, with the implication that there is something other than "physical" that underlies them. Furthermore, even within the field of psychopathology, conditions managed by psychiatrists and psychologists are defined as "disorders" or "behaviors," with the former considered to have a biological etiology, whereas the latter are considered "volitional," as, for example, cannabis use would be considered. Despite the recognition that there are underlying components to the behavior nonetheless, the supposition is that the behavior is secondary and, hence, once removed from the biology. Heritability estimates from modern twin studies, however, provide no evidence to support a differentiation of involuntary (disease) or voluntary (behavior), suggesting that mainstream psychiatric concepts remain detached from biology (11).

With respect to the etiological theories for psychiatric disorders, it appears that hypotheses are proposed that largely fit into the cultural views of the societies then extant. Thus it has been suggested that trephining holes found in the skulls of stone age people represent treatments designed to allow an escape portal for evil spirits (12). It is perhaps not surprising that preoperational, animistic and polytheistic societies develop such views. Interestingly, however, the theme of behavioral disorders occurring as a consequence of a spiritual vector re-emerged in late medieval Christian Europe. This is well described by Christian Brandt in his *Das Narrenschiff*, or "ship of fools," in which the passengers, as a consequence of their sins, were led to acts of "folly" or madness. Despite running counter to the professed free will beliefs of the society, the intelligentsia yet rationalized previous ideological systems into the framework of beliefs then current. There is no reason to believe that our society is less susceptible or that our current hypothesized etiological constructs for these disorders is less wedded to beliefs *au courant* than those of the past. This may be exemplified by the commonly accepted hypothesis that psychiatric disorders are largely multifactorial in origin; however, it has also been proposed that the fit is more semantic than data driven (13).

Regardless of potential cultural influences, observational descriptions of the psychopathology provide an additional layer of complexity. Focusing for the moment on schizophrenia, the current definition, according to the Diagnostic and Statistical Manual of Mental disorders 4th edition revised (DSM-IV-TR™) (14), includes the presence of at least two category A symptoms of delusions, hallucinations, disorganized speech, disorganized behavior and negative symptomatology, which includes flattened affect, *alogia* (poverty of speech) or *avolition* for at least 1 month coupled with a failure to maintain function in at least one domain of work, school, self-care or interpersonal relationships. Overall symptoms should be present for at least 6 months, although this time frame may include the presence of "prodromal" symptoms, e.g. odd beliefs or unusual perceptions. Fulfillment of these criteria defines one as having schizophrenia, whereas failure to do so may result in an alternate diagnosis. The subclassifications of paranoid, disorganized (hebephrenic), catatonic, undifferentiated and residual types of schizophrenia themselves have stated symptomatology required for diagnosis. Similarly, mood disorders involve characterized symptomatology that is required for a "scientifically" valid diagnosis.

A major mood disorder is classified by DSM-IV as having an at least 2-week period of depression that is not a part of bipolar disease, an underlying medical disorder or substance abuse. Depression is characterized by anhedonia or dysphoria and symptoms that may include changes in sleep, appetite, loss of sense of self-worth, guilt and suicidal ideations. BPD may or may not have a history of a mood disorder but includes a period of mania, as demonstrated by a period with expansive, persistently elevated or irritable mood, and may include increased self-esteem, grandiosity, talkativeness, distractibility, goal-oriented activity, disinhibited potentially reckless activities and decreased need for sleep. Psychotic symptomatology may be present in both disorders. Notably, the diagnosis of bipolar disease requires a diagnosis of schizophrenia to be excluded and the diagnosis of a major depression additionally requires bipolar disease to be excluded, reflecting a close relationship between the three disorders.

These diagnostic criteria are descriptive and arbitrary definitions lacking a pathological basis upon which to ground them. The field of psychiatry has been cognizant of this since the inception of these definitions and seeks to improve upon them with the DSM-V (currently anticipated for release in May of 2013). This has been in development since 1999 and is charged with expanding the scientific basis for psychiatric diagnosis. As a result, the definitions for many disorders will be changing. Schizophrenia, for example, will no longer include subtype classification. This will result in a broader overall definition of the disorder. This change in subject definition will have a knock-on effect for research, albeit the nature of this effect being unknown.



From a research perspective, however, psychiatric diagnosis, including schizophrenia and the affective disorders, have another layer of complexity. In addition to the DSM conceptualization (which is the product of the American Psychiatric Association), there coexists the International Classification of Diseases (ICD), which is supported by the World Health Organization. The ICD is a system ratified by the majority of countries in the world. While the two systems have much in common, there are differences. The ICD provides alternate diagnostic criteria for psychiatric disorders that are probably of etiological significance. For example, within the schizophrenia spectrum of disorders, DSM10 does not include schizotypal disorder like DSM, but rather places it within a personality disorder classification. In addition, individuals with medical and substance abuse specified precipitants are also not included in the schizophrenia group of disorders. At this time, it remains unclear whether these changes increase the diagnostic stringency and therefore the likelihood of finding discrete genetic factors, or exclude important groups of subjects, significantly reducing the power of population studies.

The classification issue becomes further complicated by cultural attributes, which may lend differing emphasis to elements of the diagnostic criteria as applied to different populations. This is well exemplified by the differences in categorizing catatonia depending on if one is in Wales or in India (15).

Regardless of this and of which criteria are used, the definitions of schizophrenia and the affective disorders remain mutable, and this will not change with the introduction of DSM-V or ICD 11, as each reflects the most recent iteration of consensus opinion rather than concrete objective diagnostic standards. This should not be viewed as a fault, given that we do not have sufficient understanding of these disorders to be both specific and sensitive. As far as currently possible, DSM-V is attempting to classify on an etiological basis (16). Lacking measurable criteria, these diseases must be described phenomenologically, but with awareness that what is defined may not represent discontinuous disorders with well-defined boundaries. As a consequence, research into psychiatric disorders, genetic or otherwise, is based on studies of perceived symptom clusterings and not necessarily discrete disorders.

One way to get around the difficulties involved in comparing individuals with these arbitrary and quite likely heterogeneously derived disorders is to identify endophenotypes to use as a proxy. Endophenotypes are quantifiable traits that may be chemical, anatomical or physiological and that are reliably present in affected individuals. They are considered to be biological intermediates between the underlying pathology and symptomatology, and as such, may provide greater sensitivity for the purported underlying disorder. The applicability of the endophenotype concept in psychiatry appears legitimate, although optimal candidates are lacking (17).

There are limitations because of sensitivity and specificity issues with currently identified endophenotypes, but they may still aid in second-level studies of candidate genes. For example, although the term neuroticism is used in some cases, MDD still lacks a well-agreed endophenotype (18). The options appear to be better for schizophrenia. Prepulse inhibition (PPI) of startle is recognized to be deficient in schizophrenics and their first-degree relatives (19). It is, however, also deficient in other situations, including drug usage, stress and fragile X syndrome (20). This lack of sensitivity limits its use diagnostically. PPI provides a measurable outcome for an experimental stimulus, e.g. the effect of candidate gene activity, particularly in animal models, and therefore can be very useful (21). Endophenotypes with higher sensitivity and specificity are being sought to improve experimental studies (22) and also to aid in improving diagnostic acumen. Interestingly, gene expression levels are themselves being postulated as endophenotypic measures in neurological research (23), although insufficient understanding means this is not yet an option with the neuropsychiatric disorders.

## 112.3 BIOLOGY OF THE DISORDERS

### 112.3.1 The Evolution of a Biological Perspective

Drugs heralded the approach to understanding schizophrenia and the affective disorders from a biochemical perspective. The first modern pharmacological agent proposed for these disorders was the use of lithium as a treatment for BPD1. William Hammond of New York pioneered the use of lithium specifically for mania in 1871. This likely followed both the work of Silas Mitchell, who prescribed lithium bromide for a variety of neuropsychiatric conditions from 1870, and from observations of specific European mineral wells and American “crazy waters” used therapeutically, which include lithium in their composition (24). This role for lithium continued to be explored and used, particularly among the Danish and French, but did not start to receive mainstream attention until Cade published his case series in 1949 in Australia (25). It was not until the late 1960s, however, that American psychiatrists, well behind many others, really started using the drug. Despite this long history, our understanding of how lithium exerts this effect remains unclear. Lithium inhibits the second messenger inositol pathway involved in the release of intracellular calcium in the frontal cortex and hippocampus, among other roles (26). In addition, lithium also regulates a number of other molecules, including protein kinase 3 and glycogen synthase kinase 3 (GSK3). Both are involved in signal transduction pathways in the brain. Providing an alternate pathway and possibly reducing the relevance of the inositol pathway to bipolar disease (27). Some genetic support for an inositol role is

suggested by the identification of the lithium-regulated protein, inositol monophosphate, in both the amoeba *Dictyostelium* and human cells (28). This finding may further be supported by the reported association of this gene with BPD in some families (28b,29) as well as for schizophrenia in the Japanese population (30).

Despite the early pharmacological beginnings in BPD, research into the biochemical processes underlying schizophrenia and psychiatric disorders generally received their most significant stimulus with the identification of chlorpromazine as a treatment for schizophrenia. This was recognized in Europe in 1951 but not until 1954 in the United States. The lag reflected the resistance of the psychoanalytic community, which was the arbiter of American psychiatric practice and theory at that time (31); also in 1954, iproniazid, which was under trial for tuberculosis, was found to elevate mood (32). This was confirmed in follow-up studies focusing specifically on its antidepressant role (33), although the drug's pharmacological mechanism of monoamine oxidase inhibition (MAOI) was not identified until 1963 (34). Interestingly, one of the most widely prescribed classes of drugs for depression, the tricyclic antidepressants also evolved in 1954 from the identification of the drug imipramine, a congener of chlorpromazine. Notably, all these drugs function by modulating monoamine neurotransmission. Thus, for the first time a biochemical direction from which to develop etiological clues to pathogenesis had become available for both schizophrenia and the affective disorders (35).

From these beginnings, other pharmacological agents notably mood stabilizers, selective serotonin reuptake inhibitors, atypical antipsychotic agents and others have evolved. These drugs both informed and developed out of the biochemical theories underlying these disorders. Despite this evolution, our understanding of these disorders remains limited and the biological role of these drugs is in some cases questionable (36). This is in part because of the above-discussed difficulty in providing defined measurable indices of the disorders and in part because of our limited understanding of neuropharmacological processes. The biochemical pathways implicated by drugs have different effects in different parts of the brain. These brain regions are not functionally independent, but operate within an organ system in which multiple components are involved in variably integrated ways. Furthermore, while neurons themselves are stable, their connections to other neurons are not. The brain as an organ is perhaps unique in its functional plasticity, allowing it to remodel in response to its environment. Thus, there are multiple variables co-occurring and the different factors are influenced by genetic and environmental effects (GXE) (37) to a variable degree, depending on the stage of development (38).

With respect to developmental influences, genetic control is generally greater early in development and it has been hypothesized that the very early *in utero*

environmental sensitivity may even provide an evolutionary advantage via brain response sensitizing the fetus preparatory to life *ex utero* (39). This fetal GXE interplay itself appears to have psychopathological effects. Stressors result in altered hypothalamic–pituitary–adrenal (HPA) axis functioning, and depending on the subsequent postnatal environment, may predispose toward depression, which is associated with disturbances of this axis (40). This itself may have positive competitive evolutionary advantages. Depressive elements such as fatigue would reduce energy usage in winter and times of deprivation and sadness could evoke support from the group in settings where real benefit to these emotions occurred, unlike in modern society where these are counterproductive (41). A potential confounder is, of course, a genetic predisposition to stress; however, in an elegant assay, the effects of maternal stress on *in vitro* fertilization (IVF) pregnancies, in which the implanted embryo was either related or not to the mother, demonstrated that maternal stress was associated with depression and anxiety, whereas ADHD was present only in the biologically related offspring, suggesting that the former had a less heritable component than the latter (42).

Biochemical motifs associated with schizophrenia may also have environment-dependent benefits. The level of dopamine activity in the prefrontal cortex is closely linked to schizophrenia, as discussed later. Metabolism of dopamine occurs via catechol-o-methyl transferase (COMT). The COMT gene has different polymorphisms affecting activity. Comparison of the met158Val polymorphism appears to show that those with the Val gene have better working memory under stress but poorer at baseline (43). This may indicate that there could be a competitive advantage for carriage of putative schizophrenia risk genes under specific circumstances. Extrapolating the carrier advantage concept could, at least in part, explain the enduring incidence of the disorder in the population.

### 112.3.2 Biology of Schizophrenia

It is important to recognize that schizophrenia is a consequence of antecedent factors that prime the individual for the disorder and the pathology necessary to precipitate and possibly maintain the disorder itself. The genetic and environmental factors that predispose an individual to develop schizophrenia are therefore not necessarily all the same as those that are involved in expressing the disorder.

From an etiological perspective, there is a large body of evidence pointing to the origins of schizophrenia as a neurodevelopmental disorder (44). Many of the risk factors implicated in schizophrenia appear to relate to environmental deficits in prenatal factors. These factors were originally posited as a consequence of the WWII Dutch hunger famine. Approximately 20 years after the Nazi blockade, there was a spike in schizophrenia among

individuals who were mid-late trimester fetuses to malnourished mothers at the time (45). Investigations including the Chinese great leap forward famine and others (46) have supported this initial finding. Micronutrients have been implicated to varying degrees (47). Most evidence points to low folate in combination with elevated maternal homocysteine levels in the third trimester being associated with an increase in risk of developing schizophrenia (48). Biologically this may support the glutamate hypothesis of schizophrenia wherein hypofunction of the N-methyl-D-aspartate receptor (NMDAR) is centrally involved in schizophrenia, (49) as discussed later. Hyperhomocystinemia, which can be a consequence of folate deficiency, appears to regulate NMDAR activity, which itself regulates neural crest formation in utero (50).

Iron deficiency, a very common finding in pregnancy, has also been implicated (51). This is perhaps not surprising, as some of the same nutritional deficits involved in folate deficiency would also predispose to iron deficiency. Iron is a cofactor for dopamine formation (52) as well as myelin and oligodendrocytes (53), both of which are abnormal in schizophrenia (54) and therefore there is biological plausibility. In addition, a fourfold increase in schizophrenia was seen in the offspring of Hb-deficient mothers (51) and a national Danish cohort demonstrated a dose-response of hemoglobin level to risk of schizophrenia (55). Although the cause for the anemia was not specified, iron deficiency is the most common recognized cause in pregnant women (56).

There is also a large body of evidence supporting the role of prenatal infections, inflammation and immune dysfunction predisposing an individual to neural stress, culminating in schizophrenia with immunological and brain maturation (57).

The relative role and possible interrelationships of each of these components remains unresolved. It is likely that our understanding will improve as the pathways continue to be worked out, although it is clear each of the hypothesized risk factors involves a GXE model and is neither genetically predetermined nor wholly a reflection of the environmental milieu.

Considering the disorder itself, schizophrenia appears to involve multiple disrupted neurotransmitter circuits (58) (particularly those involving dopaminergic and glutamatergic pathways). The role of dopamine in schizophrenia is supported by multiple different approaches; thus pharmacological, neurochemical, pathological, imaging and molecular studies all support a role for dopamine dysfunction (59). Striatal D2 overactivity, secondary to presynaptic stimuli and release of excess dopamine, appears to reflect the positive symptoms of schizophrenia, specifically the psychotic elements (60); indeed, this striatal dopamine excess is not present in other disorders such as bipolar disease and major depression in the absence of psychosis. In addition, prefrontal dopamine D1 levels are decreased and may be involved in the negative symptoms, including a possible learned

anhedonia secondary to an aberrant dopamine-related reward system, whereby received signals are miscued, resulting in blunted affect (61). Postmortem and genetic studies have yet to provide strong support for the dopamine hypothesis. Furthermore, extensive use of dopamine D2 targeted therapies only provide limited benefit to the majority of individuals for whom schizophrenia remains a chronic disabling condition. This implies a role for alternate pathways (62). Hypofunction of GABAergic interneurons in the prefrontal cortex and hippocampus of postmortem samples, as well as NMDA receptor hypofunction, particularly in the corticolimbic GABAergic tracts, is well characterized in schizophrenia and is supported by electrophysiological and pharmacological studies. Furthermore, the dissociative anesthetics ketamine and phencyclidine (PCP) can induce a psychosis indistinguishable from that seen in schizophrenia (63). Both drugs act by binding to and antagonizing the NMDAR, both in healthy volunteers and in schizophrenics in remission who experienced their previous unique positive symptomatology (64). NMDAR hypofunction appears to result in reduced GABAergic function in the regions seen in schizophrenia. Whether these are primary to the underlying pathology or supportive remains unclear, although a causative role may be implied via studies with clozapine. This atypical antipsychotic demonstrates unique efficacy in a subset of schizophrenics. Prescribing glycine and D-cycloserine with clozapine resulted in a worsening of effect. It appears that clozapine acts at the glycine modulatory NMDAR agonist site, causing these other coagonist agents to become, in effect, antagonistic in behavior, further implicating a direct role for the NMDAR in the schizophrenia phenotype (65).

Whether the dopaminergic or glutamatergic pathways are primary may be irrelevant, as both appear to be disrupted in schizophrenia. Therapeutic targets and candidate genes have been found in both pathways, as will be discussed later.

### 112.3.3 Biology of Affective Disorders

As mentioned earlier, disruption of the HPA axis may be a risk factor predisposing to depression. In general, however, major depression appears to be the result of a confluence of environmental and intrinsic factors interacting dynamically with altered receptive pathways and subsequent memory retrieval in response to stimuli (66). Low neuropeptide Y levels have been associated with depression, possibly via a role in decreasing activity of hypocretin, a peptide involved in arousal and appetite in the hypothalamus (67).

Being female is a well-described risk factor for depression (68); while there may also be a sexual disparity in schizophrenia with a slight preponderance toward males it is less pronounced (69) and also does not appear to be as relevant to BPD (70). The explanation for this sexual disparity is thought to be an admixture of psychosocial

environmental causes coupled with hormonal and immunological differences, possibly interfacing with the HPA axis (71). Medical disorders such as hypothyroidism, Cushing syndrome, cancers, Parkinson disease and dopamine deficiency are risk factors for depression (72). Some of these disorders clearly interact with the HPA, which appears to be dysfunctional in depression (73). This is not the only region affected by depression, however. Reductions in gray matter in the hippocampus and cortex are seen in postmortem samples, as are deep white matter hyperintensities (74), whereas activation in the amygdala is associated with dysphoria (75). Connections between forebrain regions are modulated by monoamines, the compounds targeted by the early drugs. Depletion of monoamines is insufficient to induce depression and the rapid rise secondary to treatment does not remit symptoms (76). While depression appears to have less of a genetic component than schizophrenia and less of a relationship than BPD, there are overlapping links; thus use of ketamine, an NMDAR antagonist, can reverse depressive symptoms (77). In addition, the role of peripheral molecules such as grehlin, neuropeptide Y, melanocyte stimulating hormone, and other compounds associated with arousal and activity states such as appetite remain unclear (78).

### 112.3.4 Biology of Bipolar Disorder

A comprehensive pathoetiological model for BPD is still lacking, despite treatments that are often effective. Excess dopamine appears to play a central role in symptomatology (79). It is recognized that psychostimulants such as amphetamine elevate dopamine and lead to effects very similar to mania, even in normal subjects with elevations in mood, with increased drive and less need for sleep via increasing synaptic dopamine (80), although in bipolar patients it appears that postsynaptic sensitivity, possibly via increased dopamine transporter levels, is increased rather than dopamine levels themselves (81). Reduction in dopamine production via tyrosine-free diets attenuates this effect and improves the symptoms in manic patients (82). Dopamine's action at the mesocorticol pathway appears to increase impulsivity and reward-seeking behavior, possibly regulated by the DRD2 gene (83). As mentioned earlier, dopamine may also play a role in the biology of depression (dopamine may be central to the depression of Parkinson disease and dopamine agonists can improve the depression in these patients) (84). Alleviation of depression in BPD2 patients with dopamine agonist pramipexole appears to be via dopaminergic agonist activity in the prefrontal cortex (85). Indeed, it has been suggested that the primary difference between BPD1 and schizophrenia is not that they are different disorders but rather that schizophrenia reflects additional developmental disorders (86). Both share many of the same risk factors and significant overlap in symptomatology, along with MDD, as well

as coaggregation between these disorders within families (87) that is suggested most likely to reflect shared genetics (88).

## 112.4 EVIDENCE SUPPORTING A GENETIC COMPONENT

### 112.4.1 Heritability

Perhaps the first to quantify heritability in psychopathology was Jenny Koller, who in 1895 compared psychiatric symptoms in family members of affected individuals and unaffected controls and noted that the hereditary loading was highest for psychosis (5). Comparing family members for pathological traits is, not surprisingly, a pursuit that has taken place throughout civilization. Twin studies in particular have occurred since at least the time of Hippocrates. It was not until the first decade of the twentieth century that the potential of monozygotic and dizygotic twins to separate out environmental from genetic factors was realized by Wilhelm Weinberg (89).

As a consequence of numerous twin and family studies, the heritability of these disorders has resolved, demonstrating a differential between the disorders. Depression appears to have a heritability around 37–50%, with a two- to four fold increased risk of developing MDD overall in first-degree relatives compared to the general population. This figure rises to up to an eight-fold increase for recurrent early-onset depression (90). Nonetheless, depression appears to be about half as heritable as bipolar disease, with heritability rates for BPD reported to range from 60 to 93% (88b,91), with concordance in monozygotic twins for BPD estimated at 0.67 as compared to 0.1 for dizygotic twins and first-degree relatives (9a,90c). Schizophrenia, similarly to BPD, appears to have a heritability of over 80% (92).

Overall it is clear that there is a significant heritable component for each of these disorders, but particularly BPD1 and schizophrenia. This has raised hopes that specific genes could be identified. It has proved to be more of a challenge than was anticipated, however, and the early linkage and population studies have evolved into more sophisticated and powerful genome-wide association studies (GWAS).

### 112.4.2 Molecular Studies

**112.4.2.1 Affective Disorders.** Linkage for MDD has been suggested for various chromosomal regions, including 15q25-26, 17p12 and 8p22-21.3 (93). Linkage studies led to a possible association for the NTRK3 gene in the 15q25-26 region (94). In addition, the 2q33-35 locus was raised as a possible candidate region for women with severe depression, possibly associated with the CREB gene, which interacts with estrogen receptors (95). Most recently, regions within chromosomes 2 and 17, as well as further support for the chromosome 8



locus, have been raised as having a possible association (96). Unfortunately, replication of these results has been challenging, with little support for any specific region(s) (97). The disappointment has, however, been tempered by the advent of GWAS, offering a variety of approaches to provide denser coverage of the genome. As a consequence, an increasing number of candidate genes and loci of interest have been identified. The initial meta-analysis of GWAS studies for depression identified the strongest association for APOE (apolipoprotein E), with lesser associations for variants in GNB3 (guanine nucleotide-binding protein, beta 3), MTHFR (methylene tetrahydrofolate reductase), and SLC6A4 (serotonin transporter) (98). GWAS findings also raised an association with GRM7 (metabotropic glutamate receptor 7) as well as associations with intronic markers suggested for ATP6V1B2, GRM7, and SP4. The ATP6V1B2 locus is also potentially associated with BPD (99), which is also associated with ATP6V1G1, a gene that contributes to the same molecular complex. A further GWAS association with mood disorder and GRM7 (18) noted earlier is interesting, as GRM7 is a group III metabotropic glutamatergic receptor, possibly interacting with antidepressants (100). A number of studies have suggested an association of this molecule with BPD as well as MDD (101). In addition to the GWAS polymorphism association, there also appears to be a link involving GRM7 and BPD within copy number variants (CNVs) (102).

CNVs are deletions or gains (from duplications to multiple repeats of a given sequence) that are currently testable to a resolution as small as 1 kilobase pair (kb) in size. They may include one or more genes potentially altering gene dosage. In addition, they may disrupt gene function directly by bisecting a gene, creating novel fusion products and/or affecting regulatory elements.

In depression, duplication at 5q35.1 is the first GWAS CNV variant identified for MDD. This locus includes three genes, including SLIT3, a potentially promising functional candidate, as it is involved in axon development (103). Other CNV regions of interest in affective disorders include the 3p21.1 locus. This has emerged in a number of studies to be associated with mood disorders including BPD (104,105).

With respect to BPD an increased frequency of the 3q13.3 duplication CNV has been reported. This appears to disrupt the 3' coding region of GSK3 $\beta$  (106), a bipolar candidate gene involved in the Wnt signaling pathway and sensitive to lithium, although, as is typical in the field, studies conflict on the significance of any association (107).

Linkage analysis for BPD, like MDD, has raised candidates that are not consistently replicated. Furthermore, the data are often confounded by the variable inclusion of the broader spectrum of affective disorders of BPD1, BPD2, and sometimes MDD altogether. Nonetheless, meta-analysis of 11 linkage studies implicated susceptibility loci on 6q and 8q for BPD (108).

GWAS studies have also implicated a greater number of genes for BPD than for MDD. Diacylglycerol kinase, a protein involved in the phosphatidyl inositol pathway that is targeted by lithium, has been associated (109). Similarly, the CACNA1C gene has been implicated in BPD (99,110), as have C15Orf53, ANK3 and SLITRK2. GWAS studies appear to have demonstrated a differential between Americans of European or African ancestry (111). The ANK finding as well as other candidate regions has also been demonstrated in GWAS SNP analysis of both Europeans and Han Chinese (104b,112). While these studies have limitations, they offer the possibility of identifying potential candidate genes. For example, ANK3 is a protein responsible for regulating sodium channel-dependent action potentials and may impact cognitive attentional processing, which in turn may have relevance to BPD (113). Subsequent candidate ANK3 SNP analysis for polymorphic susceptibility to BPD is further supported by studies in different European and Asian populations (114). Similarly, CACNA1A gene polymorphisms were found to be associated with anatomical and functional brain changes congruent with BPD, as well as in schizophrenic patients, and appeared to segregate to BPD patients and schizophrenics in further studies (115).

Even when there appears to be biological plausibility and other investigations such as animal studies coupled with GWAS SNP assays are suggestive of a relationship, there remains lack of clarity. For example, GRM7, Homer1 and the 2p16.1-15 region have been proposed as relevant candidates for MDD but meta-analyses have not supported the data (18,116).

In an attempt to better assess candidate genes, those strongly associated with mood disorders (BDNF, NTRK2, SLC6A4, TPH2, P2RX7, DAOA, COMT, DISC1, and MAOA) as identified from multiple studies (117) were sequenced from three separate population cohorts to determine if variants cosegregated with mood disorders. Only P2RX7 appeared to associate with both BPD and MDD, although the common alleles of SLC6A4 (also known as HTPPLR, the serotonin transporter linked polymorphic region) appeared to have an association with mood disorders (117). Further data are required to validate this study and the many others implicating these and other genes.

**112.4.2.2 Schizophrenia.** Over 4000 articles have been written on the role of genes in schizophrenia. This is twice as many as for BPD and perhaps reflects the intense interest and high expectation from this field. Indeed, the field is so active that a gene database on the Schizophrenia Research Forum (<http://www.schizophreniaforum.org>) provides systematic and regularly updated meta-analysis of genetic association studies as well as candidate genes to assist interested parties in keeping abreast of the studies.

Candidates for schizophrenia appear to have been less elusive, although work is still required to determine

if there are unifying biochemical pathways involved in all cases. Individuals with schizophrenia appear to have an increased number of CNVs relative to both the general population and bipolar patients (118,119). Many CNVs appear to be *de novo* and occur at a six fold higher frequency compared to unaffected individuals (120). Deletions and duplications in various regions have been reported from large-scale GWAS and implicated in numerous studies across ethnicities, including 1q21.1, 15q13.3, 15q11.2, 22q11.21 as well as exon-disrupting deletions in the *NRXN1* gene. In addition, duplications in 16p11.2, dup16p13.1 and 17q12 have also been reported (121). Many of these changes have also been associated with other neurodevelopmental disorders. Apart from *NRXN1*, these regions contain numerous genes, including potential candidates, such as *BCL9* in the 1q21.1 region (122), but further studies are required to conclusively determine relevance. In addition, although the odds ratios were relatively high and the data have been replicated in a number of studies, the majority of individuals with schizophrenia did not possess these changes. Characterized CNVs are present in only 3% of individuals (123). This raises the possibility that a relatively small number of genes may carry higher risk for schizophrenia in a subset of affected individuals, supportive of the “common disease, rare variant” model. Alternatively, a proportion of these CNVs may not exert a profound role. Determining the significance of a CNV involves assessing the biological plausibility of the CNV against the clinical phenotype (124). One small study of 3 monozygotic twins discordant for schizophrenia did not identify CNV variation between them (125). Although this is not surprising, given both the very small scale of the study and the monozygosity, it does raise an interesting perspective on analyzing very early somatic mutations as a source for variability (126). This applies to CNVs, which can differ in monozygotic twins with phenotypic variability (127), and, together with genomic sequencing techniques, offers the potential benefit of identifying candidate gene differences in otherwise relatively homogenous backgrounds. This approach could potentially return monozygotic twin studies to the center of genetic research.

**112.4.2.3 Candidate Schizophrenia Genes.** As implied earlier, multiple gene candidates are proposed and over 1000 genes have been studied in connection with schizophrenia-related biology; however, evidence remains limited and only a proportion of these genes are currently of high interest. Among the many genes under active investigation a small number deserve mention on the basis of possessing both biological plausibility and genetic survey candidacy.

COMT, also a BPD candidate gene (128), is of particular interest in schizophrenia given its central role in the enzymatic degradation of catecholamines including dopamine (129), a key hypothesized molecule in

the etiology and symptoms of schizophrenia. COMT, together with proline dehydrogenase (PRODH), is haploinsufficient in 22q11.2 deletion syndrome (also known as velocardiofacial syndrome (VCFS)), a genetic disorder with a 25% incidence of schizophrenia (130). Furthermore, genetic polymorphisms in COMT and PRODH are associated with morphological changes found in schizophrenic brains (131), as well as in affected individuals (132). Conflicting results of relevance with hypo-functioning alleles, specifically the COMT Val158 Met alleles (133), have confounded interpretation. More recent analysis, however, suggests that the effect of the COMT allele is epistatically regulated, and thus the genetic milieu may ameliorate potential effects (134). With respect to PRODH, this gene itself also merits serious consideration. PRODH polymorphism has been linked to schizophrenics independently (135). Key to the metabolism of proline to glutamate, haploinsufficiency potentially impacts NMDAR activation. Polymorphisms, as well as haploinsufficiency, have been associated with schizophrenics (136).

Neuregulin (NRG1) and its receptor ErbB4 are candidate susceptibility genes for schizophrenia and appear to stimulate synaptic connectivity of GABAergic interneurons (137). Furthermore, another major candidate gene for schizophrenia appears to be intimately involved with both NRG1 and ErbB4. This protein, the NR2 subunit of NMDAR is centrally involved in the development of negative as well as positive symptoms of schizophrenia. The NR2 subunit appears to share a common anchoring domain with ErbB4, with perturbations of NRG1 and the subunit potentially impacting the function of each other (138).

The identification of a chromosomal translocation that segregated with schizophrenia and related disorders in 2000 (139) created enormous excitement. This was particularly because the translocation disrupted a gene subsequently named *DISC1*, disrupted in schizophrenia 1. In the intervening time, further evidence of an association has evolved (140), although with it, the usual negative associations (141). It has also become clear that *DISC1* is not specific to this group of disorders but rather appears to be involved in neurodevelopmental processes through inhibition of GSK3 $\beta$  activity of the Wnt1 pathway involved in cortical neurogenesis (142). It would appear that *DISC1* may be involved in the developmental predisposition, rather than concurrent pathology of schizophrenia.

### 112.4.3 Overview of the Genetic Data

Although numerous candidate genes of interest have been identified for all three disorders, of the 50–90% heritability identified for these psychiatric disorders, less than 2% appears to be attributable to the identified candidate genes. It has been suggested that epigenetic and post-transcriptional modifications may account for some of this unexpected discrepancy (143), but to what degree

remains to be determined. The data available nonetheless provides useful directions for research but is of limited applicability to practice.

## 112.5 ROLE OF GENETICS IN CLINICAL PRACTICE

### 112.5.1 Genetic Counseling

Affected individuals and family members are concerned about the risk of inheriting or passing on affective disorders or schizophrenia. Perceptions about just how likely this is vary widely across the population. Studies suggest that people both overestimate and underestimate risk (144). While providing definitive risk figures is difficult, there is benefit in tackling family perceptions on the disease etiology, as they are often misinformed. Although people consider both genetic and environmental factors to be involved, there is often a significant sense of guilt and shame as well as blame. It appears that there is not generally strong conviction in the held views. Indeed, the counseling is of interest particularly with respect to obtaining an etiological understanding more than for the recurrence risk itself. The exception appears to be if the clients have children themselves, and even in this instance, this appears to be in hopes of being able to identify possible ameliorating factors (144). Counseling not only allows for a clarified perception but itself can provide a therapeutic effect for patient and family (145). Just as psychiatric illness carries stigma so too does genetic illness. Concern that a genetic discussion implies that the disorder is inevitable and untreatable, and may result in discrimination to the individual and their family, may need to be addressed before the counseling can take place (146). It is essential to provide accurate information, and while genetic counselors, who are the experts in providing counseling, are generally unfamiliar and uncomfortable with psychiatric issues, psychiatrists, to date generally appear to be unprepared with sufficient knowledge of genetics to provide this service (147).

Risk figures are themselves challenging to provide, as these depend on the individual circumstances. Empiric data are available (148), and provides a starting point from which specific risk figures can be extrapolated. These figures are influenced by the number of family members who are affected, age of onset of affected family members, relative number of females or males who are affected, and presence of a disorder such as deletion 22q11 syndrome in the family (148b). A careful individualized risk determination taking the various factors into account may give quite different risk to the patients/clients seeking this information. Given the potential disparity in risk figures, it is recommended that this be provided through a genetic counselor determining risk from the starting point of the a priori risk and working from there (149).

Another area where genetic counseling/analysis may be helpful is in pharmacogenetics. The identification and characterization of drug metabolizing alleles provides an opportunity to better determine appropriate dosing and indeed choice of drug(s) for an individual, based on their specific situation and their predicted response. Numerous CLIA (Clinical Laboratory Improvement Amendments) certified laboratories are offering this testing on a limited basis. The most experience right now for relevant pharmacogenetic testing is the cytochrome P450 enzyme system, which is central to the metabolism of many antidepressants (150). The role for pharmacogenetics is rapidly expanding and it is likely that care will improve significantly for both the affective disorders and schizophrenia as the relevance and utility of pharmacogenomic screening is realized.

## 112.6 SUMMARY

Family and twin studies have demonstrated a genetic basis for schizophrenia and the affective disorders. Application of the human genome project has generally been disappointing, perhaps more so for these disorders (and the rest of the psychiatric field) than any other area in medicine. While this may support the notion that psychiatric disease and possibly with it, the psyche, is unique and not subject to the same rules as the rest of biology, it is too early to make such pronouncements. Limited data appear to be emerging. Evidence for candidate genes is increasing, raising the possibility that, while no one gene or group of genes will likely dominate the risk for schizophrenia or the affective disorders, the roles of the many candidates will clarify the pathways involved in these disorders. The ability to determine the steps necessary to develop the disorders will provide further therapeutic targets that may be of direct relevance to individuals for whom the genetic evidence is scant but who nonetheless may be predicted to have unidentified perturbations in pathways, or in whom consolidation of pathways may help counter undefined defects. Thus, it may not be necessary to have a comprehensive explanation for a disorder that potentially has as many paths to symptomology as there are individuals affected by the disorders. The identification of themes rather than specifics may be sufficient to improve treatment.

The inability to develop a comprehensive genetic overview also forces us to question our understanding of the relationship of the genome to cellular function in the brain. This is already starting to evolve into a search for new etiological frameworks that may ultimately help us to understand brain function at a new level. It may be that the current challenges in preventing a simplified mechanistic theory from evolving is ultimately fortunate.

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Medicine in a Genetic Context; Twins and Twinning; Pharmacogenetics and Pharmacogenomics; Genetics Counseling and Clinical Risk Assessment.



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### Biography



Jonathan Picker is a geneticist focused on the interface between genetics and psychiatry, particularly from a developmental perspective. Following medical school in Aberdeen, Scotland he pursued behaviorally oriented pediatrics training in Newcastle upon Tyne, England. While at Newcastle University he also carried out Masters in Biochemistry and Genetics followed by a PhD in the Faculty of Agriculture investigating molecular regulatory mechanisms. This was followed by further training in clinical genetics and child and adolescent psychiatry at the Boston Children's Hospital (BCH), where he has remained since. At BCH his clinical work has specialized in the genetics underlying behavioral problems, primarily in children. As a part of this interest he founded and currently directs the BCH Fragile X Program. He is currently developing a detailed phenotyping registry with a view toward integrating this with translational genetic research endeavors. In addition, from a basic science perspective, he has been working with Joseph Coyle at McLean Hospital investigating epigenetic developmental risk factors for schizophrenia.

# CHAPTER

# 113

## Addictive Disorders

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### 113.1 INTRODUCTION

Etiologically, addictions are multistep pathologies that, by definition, require exposure to an agent, which is often a psychoactive substance. The probability of initial use as well as the probability of progression toward a pathological pattern of use (substance use disorder) is influenced by characteristics specific to the individual (e.g. genotype, sex, age, cohort and psychopathology), environment (e.g. drug availability, social support, stress and socioeconomic status) and addictive agent (e.g. addictive liability, mode of use or administration, physiological response and secondary pathology). Genetic and other influences may differ for each transitional step in the pathway to addiction.

Once use is initiated, repetitive exposure to the agent, and cycles of intoxication and withdrawal, induce neuroadaptive changes that further promote drug-seeking behaviors and ultimately lead to the maladaptive, persistent, and uncontrolled patterns of use that characterize addictions. These neuroadaptive changes are the bases for the establishment of tolerance, craving, withdrawal, and persistent affective disturbances that appear after consumption ceases (1). Together with failure to address and manage preexisting vulnerability factors, the self-maintaining and progressive neurobiology of addictions makes these disorders chronic and relapsing in nature. The wide variety of addictive agents includes foods, sex, video gaming and gambling. Any of these agents may lead to an “addicted state” through neurobiological pathways partially overlapping with those involved in addiction to psychoactive substances (2). As will be discussed, addictive agents vary substantially in addictive liability and there may be a relationship between this

critical property of addictive agents and the inheritance of liability to addictive diseases.

#### 113.1.1 Epidemiology

Worldwide, drug use is both widespread and common. According to the World Health Organization (WHO), there are 2 billion people who consume alcoholic beverages and 76.3 million with diagnosable alcohol use disorders, 1.3 billion tobacco users, and 185 million users of illicit drugs ([http://www.who.int/substance\\_abuse/facts/global\\_burden/en/](http://www.who.int/substance_abuse/facts/global_burden/en/)). In the United States, according to the 2009 National Survey on Drug Use and Health (NSDUH), 130.6 million people aged ≥12 years (51.9% of the American population within this age range) are current drinkers. According to the same survey, approximately 21.8 million people older than 12 years (8.7% of the population) had in the last month used an illicit drug including marijuana/hashish, cocaine, heroin, hallucinogens, inhalants, or prescription-type psychotherapeutics used without a medical prescription. Prevalence of current illicit drug use among youth aged 12–17 years has declined from 11.6% in 2002 to 9.3% in 2008, although rates trended higher in 2009 (10.0%). Similar trends have been observed for young adults (age 18–25 years). Changes in use of illicit drugs are mainly driven by consumption of marijuana, the most commonly used illicit drug. The worldwide large-scale consumption of nicotine with accompanying epidemics of smoking-related cancers and cardiovascular diseases is a twentieth century phenomenon initiated by the packaging of bright-leaf tobacco into the cigarette (3). Warning requirements, advertising and sales restrictions, and public education

efforts have had a positive effect. According to the Monitoring the Future Survey (MFS), nicotine use in American youth began to decrease in 1997, and continues to do so (4).

Most people are able to use addictive agents in moderation and only a minority develop pathological patterns of use (5). However, the contribution of psychoactive substances to the global burden of illness, disability, and death is high, and from a public health perspective this disease burden is directly related to use. In the United States, the Prohibition era, which began at midnight on 16 January 1920, as a result of the 18th Amendment and was proceeded by a vigorous Temperance Movement, was marked by a dramatic reduction in alcohol use and in cirrhosis deaths (which declined to about half former rates). Following the end of Prohibition in 1933 as a result of the 21st Amendment, and a process of gradual repeal of state-wide prohibitions that lasted until 1966, there was a 56% increase in per capita alcohol consumption, with an accompanying return of cirrhosis deaths to their former level. During the 1950s, there were slight changes in alcohol intake. The 1960s and 1970s saw large increases. Since 1980 there has been an overall steady decline.

Most nicotine and approximately half of alcohol are consumed by individuals who have a specific addiction diagnosis. Nicotine-dependent and psychiatrically ill individuals consume about 70% of all cigarettes smoked in the United States (6). Alcohol-dependent individuals are responsible for about 50% of the social, legal, and interpersonal alcohol-related problems in society (7).

Gambling is an example of a nonpharmacologic addictive agent. Pathological gambling is currently classified as an impulse control disorder rather than an addiction. However, monetary rewards in gambling tasks activate the brain in a similar fashion as addictive drugs (8) with hypersensitivity to these rewards in problem gamblers (9). Gambling is common, and has become increasingly so in the past several decades with new access to government sponsored lotteries in most states and some form of legalized gambling in almost all, and general access to casinos, slot machines, and Internet gambling. Some 80% of Americans have gambled, and many become pathological gamblers. Estimated rates of problem gambling, which combines problem and pathological gambling, range as high as several percentage. However, the National Comorbidity Survey, which used the Composite International Diagnostic Interview (CIDI) and DSM-IV criteria, estimated that the rate of pathological gambling was 0.6% in an epidemiologically ascertained sample of 9282 individuals (10). The British Gambling Prevalence Survey (<http://www.gamblingcommission.gov.uk>) has tracked the prevalence of gambling and problem gambling in the United Kingdom, where the prevalence of problem gambling is also 0.6%.

A variable used to estimate impact of diseases on public health is Disability Adjusted Life Years (DALYs), which is years of life lost because of premature mortality or disability. Worldwide, alcohol subtracts 69.4 million DALYs, which represents 4.5% of all DALYs ([http://www.who.int/substance\\_abuse/facts/alcohol/en/index.html](http://www.who.int/substance_abuse/facts/alcohol/en/index.html)); tobacco 59.1 million (4.1%); and illicit drugs 12.2 million, representing 0.8% ([http://www.who.int/substance\\_abuse/facts/global\\_burden/en/](http://www.who.int/substance_abuse/facts/global_burden/en/)). The addiction disease burden is also unequally distributed across countries, gender, and age groups. Psychoactive substances have a higher social impact in the United States and Europe than in developing countries, where life expectancies are shorter and most common causes of premature death are infectious diseases rather than addictions. Drug use is more common in men than in women. However, male-to-female ratios for use of psychoactive substances have narrowed, consistent with the changing social roles of women. The use of alcohol and other drugs by teenage girls is now similar to rates of consumption by boys in both the United States and Australia (11,12).

Illicit drug use inflicts its largest effects on mortality in adolescence and young adulthood, alcohol mainly before age 60, and 70% of tobacco-related deaths occur after the age of 60 ([http://www.who.int/substance\\_abuse/facts/global\\_burden/en/](http://www.who.int/substance_abuse/facts/global_burden/en/)). From an economic perspective, the cost of addiction in the United States is approximately \$484 billion/year (\$138 for smoking, \$161 for illicit drugs, and \$185 for alcohol). The societal cost of addiction is comparable to the worst chronic diseases, including diabetes (\$131.7 billion/year) and cancer (\$171.6 billion/year) ([www.drugabuse.gov/about/welcome/aboutdrugabuse/magnitude](http://www.drugabuse.gov/about/welcome/aboutdrugabuse/magnitude)).

The majority of users of addictive agents do not progress to dependence and the conditional prevalence of dependence among users is different for various agents. According to the National Comorbidity Survey (NCS) (5) the highest risk of dependence among users is for nicotine, 31.9%, double that of cocaine. Risk factors for the development of substance use disorders include preexisting psychopathology (especially a preexisting substance use disorder) and family history of a substance use disorder (13,14). Age at first use is a powerful predictor. Odds of lifetime substance dependence among users are reduced by 4% for illicit drugs and 9% for alcohol for each additional year drug use onset is delayed (15,16). Studies on offspring of addicted patients also reveal that different risk factors may be involved at different stages of the development of addiction. Peer influences are most important for exposure and initial pattern of use, whereas familiarity and psychopathology play a more salient role in the transition to problematic use (14). Addiction occurs more often within a matrix of social and economic disadvantage. The poor and the deprived have higher rates of mental disorders in general, including substance use disorders (WHO, 2002; available at <http://www.who.int/whr/2001/chapter1/en/>



[index2.html](#)). Substance use/misuse is strongly related to determinants of overall level of population health including education, social support network, income, social status, and living conditions including housing, nutrition, water and environmental quality, employment, and access to health services. Addictions partially mediate the impact of these determinants on health. The relationship between mental illness and poverty is complex, reciprocal, and self-sustaining.

### 113.1.2 Definitions of Disease

There are currently two widely used systems for diagnosing substance use disorders: the Diagnostic and Statistical Manual of Mental Disorder (DSM) of the American Psychiatric Association (APA) and the International Classification of Disease (ICD) of the World Health Organization (WHO).

Both systems recognize two types of substance use disorders: substance dependence and substance abuse (DSM)/harmful use (ICD). DSM-IV criteria for dependence (Table 113-1) are similar to ICD-10 criteria (Table 113-2), whereas DSM-IV Abuse and ICD Harmful Use differ somewhat (17). The ICD-10 differs in excluding Harmful Use among patients with current Dependence, but not in patients who have ever had Dependence.

The development of valid and reliable criteria for addictive disorders represented a fundamental contribution to communication between physicians as well as for research. DSM and ICD criteria provide a unifying framework for epidemiology, treatment, and genetic research worldwide (17). However, current nosology

of addiction has important limits for treatment and research. The diagnostic categories are syndromic (based on clusters of symptoms and clinical course) and not etiologically based (18). The diagnoses are categorical, assuming a cutoff between normal and abnormal variation, when in fact many of the problems associated with addiction are found in people who fall below the threshold for diagnosis (7). Pathological forms of use may be underdiagnosed using the categorical DSM approach. Binge drinking is a pattern of alcohol use characterized by intense bouts of episodic drinking (binge) during which a large quantity of alcohol is consumed. Between these episodes, the binge drinker may refrain from drinking. In some American Indian Tribes, binge drinking is a common pattern of alcohol use that is generally, but not always, present in the context of alcohol dependence but that is itself a strong independent predictor of problems in all four DSM addiction major symptom areas: social, work, physical, and violence/lawlessness (19). Furthermore, there is a need to understand, and integrate, etiologic factors that act across diagnostic boundaries. Twin studies detect evidence of etiological factors shared between addictions and other psychiatric diseases (20) and linking normal (personality) and abnormal variations (psychopathology) (21,22). Thus, addiction disease categories are etiologically connected to other psychiatric diseases and to “normality.” Future versions of the DSM may incorporate dimensional indices such as age at onset, years of drug use, frequency of use, and quantity of use. However, it is improbable that the nosology of addiction will advance until neurobiological indicators, including genotypes, are integrated.

**TABLE 113-1 Diagnostic Criteria for Substance Dependence and Abuse According to the DSM-IV-TR**

Criteria for substance dependence, according to DSM-IV-TR

≥3 of the following occurring at any time in the same 12-month period

A maladaptive pattern of substance use, leading to clinically significant impairment or distress

(1) Tolerance, defined by either

- (a) A need for markedly increased amounts of the substance to achieve intoxication or desired effect
- (b) Diminished effect with continued use of the same amount of substance

(2) Withdrawal, defined by either

- (a) The characteristic withdrawal syndrome for the substance
- (b) The same (or similar) substance is taken to relieve or avoid withdrawal symptoms

(3) The substance is often taken in larger amounts or over a longer period than was intended

(4) There is a persistent desire or unsuccessful efforts to cut down or control substance use

(5) A great deal of time is spent in activities necessary to obtain the substance, use the substance or recover from its effects

(6) Important social, occupational or recreational activities are given up or reduced because of substance use

(7) The substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance

Criteria for substance abuse, according to DSM-IV-TR

≥1 of the following occurring at any time in the same 12-month period

(1) A maladaptive pattern of substance use, leading to clinically significant impairment or distress

- (a) Recurrent substance use resulting in a failure to fulfill major role obligations at work, school, or home
- (b) Recurrent substance use in situations in which it is physically hazardous
- (c) Continued substance use despite having social or interpersonal problems caused or exacerbated by the effect of the substance
- (d) Recurrent substance-related legal problems

(2) The symptoms have never met the criteria for substance dependence for this class of substance

**TABLE 113-2 Diagnostic Criteria for Substance Dependence and Harmful Use According to the ICD-10**

Criteria for substance dependence, according to ICD-10

≥3 of the following present together at some time during the previous year

- (1) A strong desire or sense of compulsion to take the substance;
- (2) Difficulties in controlling substance-taking behavior in terms of its onset, termination, or levels of use;
- (3) A physiological withdrawal state when substance use has ceased or been reduced, as evidenced by the characteristic withdrawal syndrome for the substance or use of the same (or a closely related) substance with the intention of relieving or avoiding withdrawal symptoms;
- (4) Evidence of tolerance, such that increased doses of the psychoactive substances are required in order to achieve effects originally produced by lower doses
- (5) Progressive neglect of alternative pleasures or interests because of psychoactive substance use, increased amount of time necessary to obtain or take the substance or to recover from its effects;
- (6) Persisting with substance use despite clear evidence of overtly harmful consequences, such as harm to the liver through excessive drinking, depressive mood states consequent to periods of heavy substance use, or drug-related impairment of cognitive functioning; efforts should be made to determine that the user was actually, or could be expected to be, aware of the nature and extent of the harm.

Criteria for harmful use, according to ICD-10

The diagnosis requires that actual damage should have been caused to the mental or physical health of the user.

Harmful patterns of use are often criticized by others and frequently associated with adverse social consequences of various kinds.

The fact that a pattern of use or a particular substance is disapproved of by another person or by the culture, or may have led to socially negative consequences such as arrest or marital arguments is not in itself evidence of harmful use.

Acute intoxication or "hangover" is not itself sufficient evidence of the damage to health required for coding harmful use.

Harmful use should not be diagnosed if dependence syndrome, a psychotic disorder, or another specific form of drug- or alcohol-related disorder is present.

### 113.1.3 Subtypes of Addiction

The general recognition of the heterogeneous and multifactorial nature of addiction has led to the classification of addicted patients into more clinical homogeneous subtypes in which etiologic factors, including genes, tend to be shared, and represents a first systematic effort to individualize treatment and prevention. Characteristics used to subclassify alcoholism include age at onset (23), personality, comorbidity with other psychopathological conditions, familiarity and mode of inheritance, severity, clinical course, and response to treatment. Most of the typologies proposed recognize the existence of two basic types. Type A (e.g. Cloninger type I) comprises approximately two-thirds of alcoholics and is characterized by late onset, slower course, and better prognosis. Type B (e.g. Cloninger type II) is characterized by stronger familiarity, antisocial behavior, earlier onset, rapid course, and poorer prognosis. Type I alcoholism thus falls more in the category of internalizing disorders and type II in the externalizing disorder domain. The A/B distinction, first applied to alcoholism, appears to be applicable to other addictions (24). Typological systems identifying more than two categories also identify what appear to be similar categories, for example, negative-affect alcoholism, characterized by high-level depression (25,26). Physicians individualize therapy for addicted patients in many ways, for example, by addressing specific risk factors, barriers to abstinence, family and community supports, and medical consequences, but to this point formal diagnostic subclassification has mainly been a tool in research. Ondansetron was found to be more effective in early-onset alcoholics (27). Serotonin-specific reuptake inhibitors (SSRIs) seem to be effective in type A but not in type B alcoholics (28). Central serotonin dysfunction may

be specifically linked to type B alcoholism (29,30). Some genetic variants (e.g. the serotonin transporter (31) and the 5HT1B receptor (32)) have been shown to confer risk mainly in specific subgroups of alcoholics.

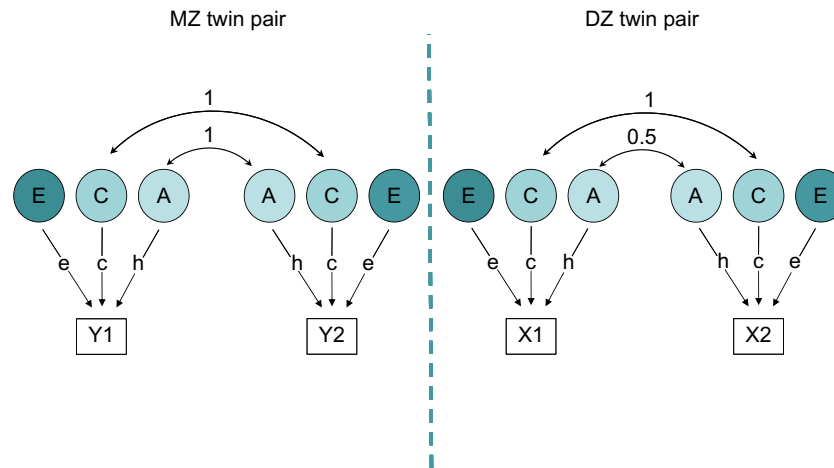
### 113.2 GENETIC ASPECTS OF ADDICTION

Evidence from family, adoption, and twin studies converge on the relevance of genetic factors in the development of addictions (13,33–38). The increased risk among relatives of addicted probands in the same family might result from either environmental or genetic influences. Twin studies and studies on adoptees can disentangle those contributions. In the classical twin study design the phenotypic resemblance of monozygotic (MZ) twins (who share 100% of their genes) is compared to that of dizygotic (DZ) twins (who share approximately 50% of their genes) to estimate three main sources of variation (Figure 113-1):

- (1) Heritability (A): Proportion of variance due to additive (A) genetic factors.
- (2) Shared (or common) environment (C): Proportion of the variance due to environmental factors shared by members of the same family. C can also be directly measured in the context of adoption.
- (3) Unique environment (E): Proportion of the variance due to environmental factors unique to the individual. This term also includes variance attributable to measurement error.

Both A and C are sometimes collectively called familial influences because they contribute to similarities between members of the same family.

It has already been noted that the diagnostic assessment of addictions is imperfect. The usual effect of



**FIGURE 113-1** Twin studies: heritability path diagram: the phenotypic resemblance of monozygotic (MZ) twins (who share 100% of their genes) is compared to that of dizygotic (DZ) twins (who share approximately 50% of their genes) to estimate three main sources of variation: 1. Heritability=proportion of variance due to additive (A) genetic factors; 2. Shared (or common) environment (C)=proportion of the variance due to environmental factors shared by members of the same family; 3. Unique environment (E): proportion of the variance due to environmental factors unique to the individual.

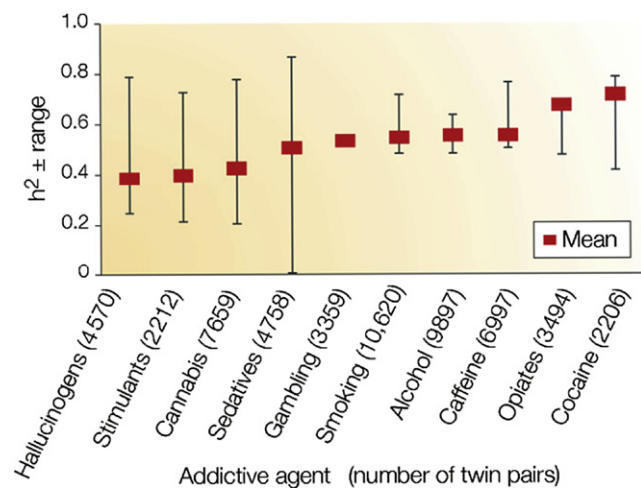
measurement error is to limit the maximum heritability (A) and to increase the proportion of variance attributable to E. In the same vein, it is important to stress that A, C and E are latent (unmeasured) influences that should not be interpreted as absolute values. They provide broad estimates of sources of variation in the particular context in which they are evaluated, and do not, positively or negatively, predict effects of intervention to prevent or treat addictions. Twin studies failed to take into account gene by environment interaction. Furthermore, they do not inform us of sources of variation at the individual level. Nor are they informative of the specific genes and environmental factors involved.

Several questions that can be addressed by twin studies of addictions include the following: Are addictions heritable? Do genetic and environmental risk factors contributing to drug initiation, the same contributing to drug addiction? What is the nature of inheritance? Are the genetic and environmental risk factors agent specific or nonspecific? Are genetic and environmental factors independent?

### 113.2.1 Heritability of Addiction

Weighted mean heritabilities for substance use disorders (substance dependence, substance abuse or dependence, or persistent use) computed from several studies of large, carefully phenotyped cohorts of twins are shown in Figure 113-2 (39). Addictions include several of the most heritable psychiatric diseases, as well as disorders that are not highly heritable. Heritability is lowest for hallucinogens (0.39) and highest for cocaine (0.72).

Heritability estimates are usually higher for dependence than abuse and for abuse than use; however, “no pathological drug use” and “initiation of use” are also heritable characteristics (35,37,40).

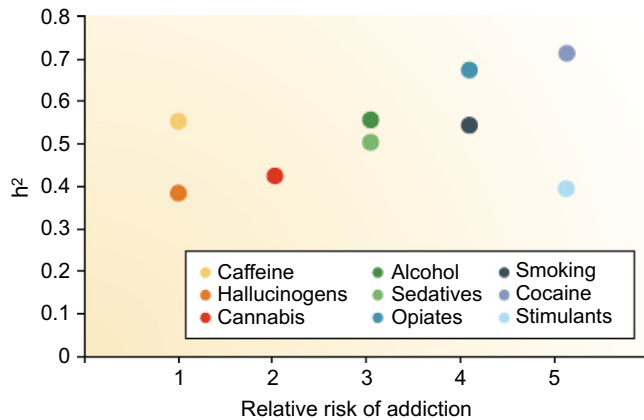


**FIGURE 113-2** Heritability (weighted means and ranges) of 10 addictive disorders: hallucinogens, cannabis, stimulants, sedatives, opiates, cocaine dependence or abuse, alcohol dependence, smoking, caffeine consumption or heavy use, and pathological gambling. Weighted heritability ( $h^2$ ) means were computed using data from large national surveys of adult twins. (Adapted from Goldman et al. (39).)

### 113.2.2 Addiction Liability and Heritability

Drugs and other agents differ in addiction liability (e.g. potential of an agent to lead to addiction). Although addiction liability is difficult to quantify, Goldstein and Kalant (41) ranked the relative risk of addiction of different psychoactive substances. Their rankings were based on animal behavioral models in which the addictiveness of a substance is measured by the pattern of self-administration of a drug (e.g. how many times an animal will press a lever to obtain the injection of a drug or the rapidity of reinstatement of high levels of consumption after a period of enforced abstinence). As shown in Figure 113-3, addiction liability is nominally correlated

with heritability. Cocaine and opiates, which are the drugs with the highest addictive properties, are also the most heritable. On the other hand, hallucinogens are among the least addictive, and least heritable. This positive correlation between addictive liability and heritability predicts that part of what is inherited is variation in the core neurobiology of addiction, potentially including



**FIGURE 113-3** Heritability ( $h^2$ ) versus approximate ranking for relative risk of addiction. Heritability estimates for addiction correlate linearly with the addictiveness of the agent. Relative risk of addiction is expressed on a five-point scale, one indicates the lowest risk and five denotes the highest risk. (Adapted from Goldman et al. (39).)

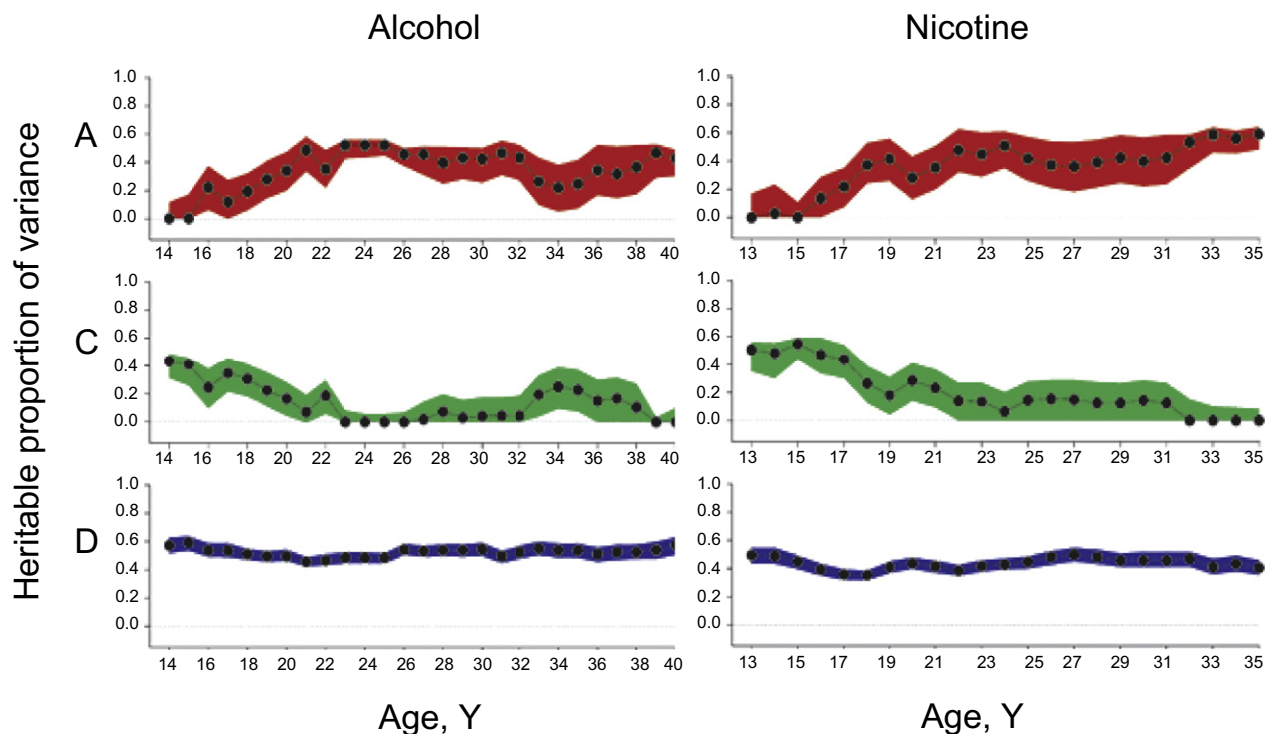
reward, behavioral control, obsessionality/compulsivity, and stress/anxiety response.

### 113.2.3 Developmental Dependence of Genetic and Environmental Risk

Genetic and environmental influences modulating risk of addiction, as well as other complex phenotypes, change during the lifespan. These developmental differences are thought to be both quantitative (i.e. the relative contribution of genetic and environment factors change over time) and qualitative (i.e. the specific genes and environmental factors involved in addiction during a certain developmental period are different from those involved in different periods). Longitudinal twin/family studies allow the exploration of developmental differences in gene/environment effects.

**113.2.3.1 Quantitative Differences.** Longitudinal studies reveal that the impact of genetic and environmental factors on addictions changes during development and across the lifespan. Kendler et al. (42) found that the effect of shared environment (C) declines moving from adolescence into adulthood and disappears by age 35 for nicotine and by age 40 for alcohol (Figure 113-4). In contrast, gene effects (A) are undetectable in early adolescence but their relative importance gradually grows.

A possible explanation for these results is that during the progression to adulthood, the individual has



**FIGURE 113-4** Age dependency of genetic and environmental contributions to variation in liability to alcohol (left) and nicotine (right) use. Additive genetic effects (A), familial environmental factors (C) and individual-specific environment (E) are represented by age in years (Y). The actual parameter estimates for A, C, and E are depicted by the black lines. Colored regions represent the possible range of estimates  $\pm 1$  standard error. For both nicotine and alcohol use, genetic influences increase and shared family environmental influences decrease, moving from adolescence to adulthood (see text). (Adapted with modification from Kendler et al. (42).)



increasing latitude to shape his/her own choices and social environment, thus increasing the relative role of genotype (43) (see also Section 113.2.6.1). Another possible explanation is that the action of some genetic factors appear only after repetitive exposure to the addictive agent (see also gene  $\times$  environment interaction), or by genes that act only after the brain has fully developed.

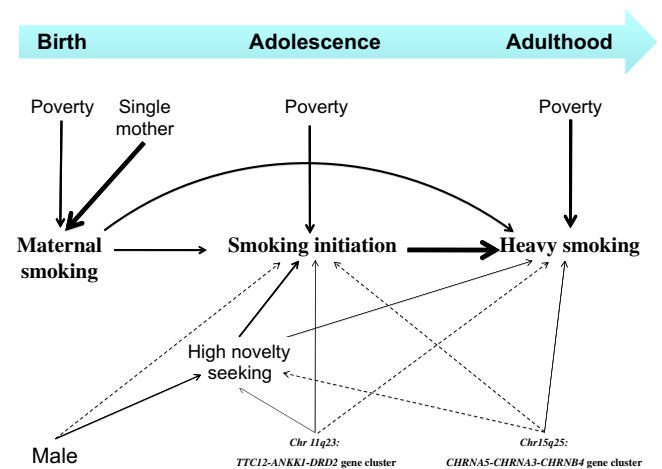
**113.2.3.2 Qualitative Differences.** Addiction is a multistep process from initial exposure to the agent, often occurring in adolescence, toward the development of maladaptive patterns of use and psychical/psychological dependence. In this view, it is not surprising that genes involved in initiation might be different from genes that are involved in persistence and dependence. Indeed, twin studies have shown that genetic risk factors for initiation only partially overlap with genetic determinants for developing substance use disorders. In a large twin study, genetic risk factors for initiation of illicit drug use were responsible for about half of heritability of abuse/dependence (40). This is consistent with the hypothesis that some genes influence addiction by increasing the probability of initiation (e.g. genes influencing temperament traits such as sensation/novelty seeking that promote drug experimentation) and other genes modify response to the drug and the probability of persistent use (e.g. genetic variants modulating the pharmacodynamics/pharmacokinetic of substance). For example, genetic variation within the chromosome 11q23 region where the gene encoding for the dopamine receptor 2 (DRD2) is located, has been associated with smoking initiation in adolescents and its effect on smoking appears to be partially mediated by its effect on high novelty seeking, a personality characteristic that is associated with drug-seeking behavior (22). Similarly, genetic variation within the monoamine oxidase A (MAOA) gene is associated with increased impulsive/antisocial behavior that usually predates alcohol and illicit substance use (44). In contrast, genetic variation within the *CHRNA5-CHRNA3-CHRNA4* gene cluster on chromosome 15q25 appears to be involved in the development of a heavy pattern of use in adulthood (22) and in smoking persistence (Figure 113-5). Among environmental risk factors, low socioeconomic status appears to increase risk of nicotine abuse across all life.

### 113.2.4 Shared and Unshared Inheritance of Different Addictive Disorders

Comorbidity (e.g. co-occurrence of different disorders in the same individual or an excess of disease co-occurrence in a population) between different addictive disorders is frequent (45–47). Studies on genetically informative samples (e.g. adoptive or twin studies) can measure the relative contribution of genes and environment to comorbidity by evaluating the cross-transmission of different traits. In this way, shared risk factors common to different

types of drugs or between addiction and other disorders can be differentiated from specific risk factors contributing to vulnerability for a particular addiction but not to the others. Based on results from these types of studies, inheritance of addiction vulnerability is in part substance specific but a large portion of vulnerability to addiction is attributable to shared factors that act across different addictive agents, including agents from seemingly different categories (48).

**113.2.4.1 Cross-Inheritance of Addictions.** Large twin studies reveal an overlap between genes for alcoholism and illicit drug use disorders (20,49). In a sample of male and female adolescents, a common vulnerability factor to alcohol, tobacco, and a combined class of illicit drugs had a heritability of 23% (50). Kendler et al. (51) explored the genetic overlap between several licit and illicit substances, including alcohol, caffeine, nicotine, cannabis, and cocaine, in a portion of a Virginia twin sample consisting of almost 5000 twins. In this study genetic risk factors for dependence on different psychoactive substances could not be explained by a single factor acting across all substances. Rather, two shared genetic factors were found: an illicit agent genetic factor mainly explained vulnerability to cannabis and cocaine dependence and a licit agent genetic factor mainly explained vulnerability to alcohol, caffeine, and nicotine. These



**FIGURE 113-5** Impact of genetic and psychosocial factors on smoking behavior moving from adolescence to midadulthood. Genes are the *TTC12-ANKK1-DRD2* gene cluster on chromosome 11q23 and the *CHRNA5-CHRNA3-CHRNA4* gene cluster on chromosome 15q25. Psychosocial risk factors include socioeconomic status, maternal smoking, and the personality characteristic of novelty seeking. The thicknesses of path lines are proportional to the strength of the association between the two variables connected by the path. Nonsignificant paths are shown with dashed lines. The effect of *TTC12-ANKK1-DRD2* gene cluster on smoking in adulthood is mainly indirect and mediated via its effect on smoking in adolescence and novelty seeking. In contrast, the effect of the *CHRNA5-CHRNA3-CHRNA4* gene cluster on smoking in adulthood is mainly direct and not mediated by smoking in adolescence or novelty seeking. Poverty and social disadvantage are associated with increased risk of smoking both in adolescence and midadulthood. (Adapted with modification from Ducci et al., (22).)

two factors were not independent but highly correlated. Large agent-specific genetic factors were found mainly for nicotine and caffeine (Figure 113-6).

**113.2.4.2 Cross-Inheritance of Addictions and Other Psychiatric Disorders.** Addictions frequently coexist with other psychiatric diseases, including both internalizing disorders (disorders marked by anxiety or problems of mood) and externalizing disorders (disorders marked by problems of impulse control) (46,47). Twin studies consistently reveal the existence of shared genetic influences between addiction and externalizing disorders (20,21,49). Longitudinal studies have shown that externalizing disorders of childhood such as conduct disorder (CD) and attention deficit hyperactivity disorder (ADHD) are important risk factors for the subsequent development of alcoholism (52). Evidence from twin studies for shared genetic influences between alcoholism and internalizing disorders is more controversial (53,54). However, longitudinal studies have shown that anxiety disorders such as panic disorder and social phobia predict subsequent alcohol problems in adolescents and young adults (55).

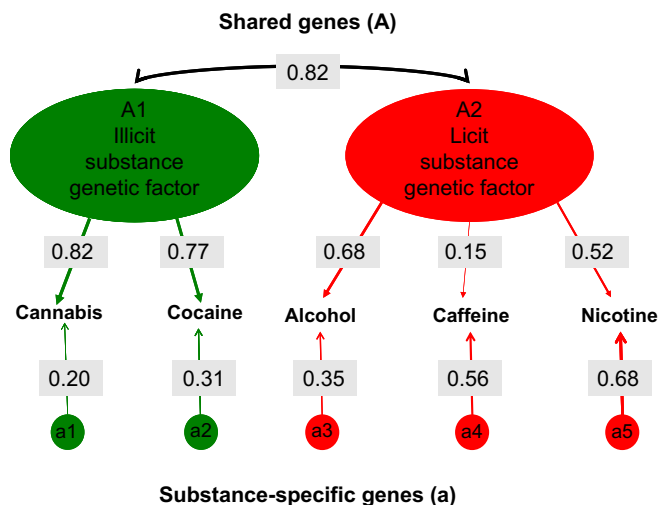
Overall, twin studies predict that genes involved in vulnerability to addiction include both agent-specific genes and genes that act on common pathways

involved in addiction to different agents. Agent-specific genes include genes involved in pharmacokinetic or pharmacodynamic processes specific to a particular drug. For example, genetic variation in alcohol metabolizing genes moderate risk to develop alcoholism. On the other hand, variation within genes involved in the pharmacodynamics of nicotine dependence moderate risk for nicotine addictions but not other types of addictions. Nonspecific genes include genes affecting neurobiological networks involved in vulnerability to different types of addictions as well as genes predisposing to addictions and other psychiatric diseases such as genes involved in reward, stress resiliency, behavioral control, and personality. For example, the dopamine system is fundamental for the reward effects of all addictive agents (56), and genetic variations in the gene encoding the dopamine two receptor (*DRD2*) have been linked to different types of addictions, although with some inconsistencies (57). As will be discussed, other genes such as monoamine oxidase A (*MAOA*), the serotonin transporter (*SLC6A4*), and catechol-O-methyltransferase (*COMT*) have been implicated in the shared genetic liability between addictions and other psychiatric diseases.

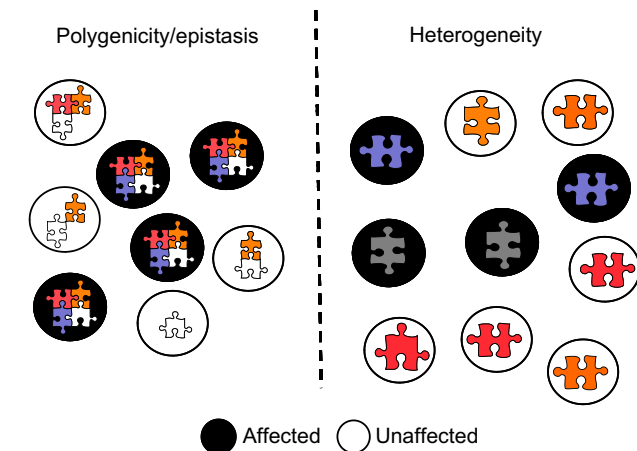
### 113.2.5 Mode of Inheritance

Genes influence addiction liability through a matrix of neural systems, molecular networks, and environmental interactions, providing a challenge for reductionism. Family studies do not reveal a simple Mendelian model of inheritance for any addiction. The genetic complexity of addictions can be expressed in a variety of ways: incomplete penetrance, variable expressivity, gene–environment interactions, and genetic heterogeneity. Two other sources of genetic complexity are polygenicity (the action of multiple variant alleles) and epistasis (nonadditive effects of combinations of genes), and for addictions these are strongly inter-related mechanisms because of the threshold nature of the trait. Vulnerability to addiction may be a continuous trait, but addictions are clinically defined as threshold traits, which is to some extent justified by the changes in brain functions that occur in addicted individuals.

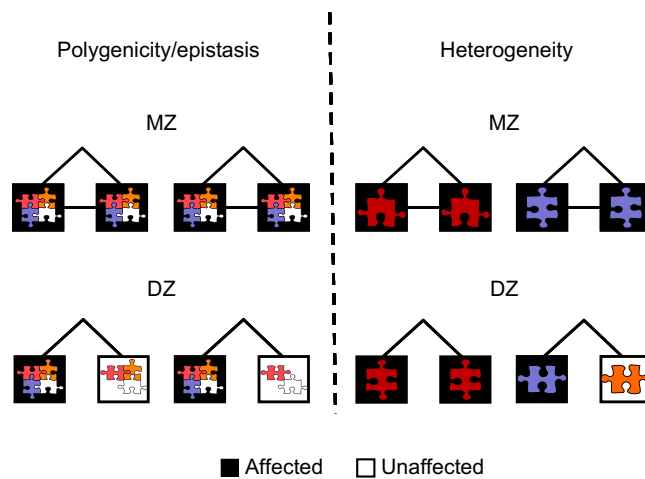
**113.2.5.1 Polygenicity vs Genetic Heterogeneity.** Frequently it is posited that psychiatric diseases are likely to be polygenic because the function of the brain depends on the action of thousands of interacting genes, many of which have functional variants. As just mentioned, for a threshold trait such as addictions a requirement for the action of multiple variants would also lead to epistasis. However, the molecular complexity of the brain and multiple processes underlying addiction are likely to lead to genetic heterogeneity. As shown in Figure 113-7 (39), under the polygenic/epistatic model the presence of combinations of genetic variants (each shown in Figure 113-7 as a piece in a small puzzle) is required to express a certain phenotype.



**FIGURE 113-6** Shared and substance-specific additive genetic influences acting on symptom counts for cannabis, cocaine, alcohol, caffeine, and nicotine abuse or dependence. Two common genetic factors are identified, one for illicit substances (A1) and one for licit substances (A2). Substance-specific genetic influences (a1–a5) are represented at the bottom of the figure. Path values are standardized loadings and thus need to be squared to reflect the proportion of variation in liability in the observed variable accounted for by the factor. For example, the illicit substance genetic factor (A1) accounts for 0.67 ( $0.82^2$ ) of the total variance in cannabis symptoms in this population. The cannabis-specific genetic factor (a1) accounts for 0.04 ( $0.20^2$ ) of the variance in this trait. Therefore, the total variance of cannabis symptoms determined by genetic factors—heritability—is 0.71 ( $0.67 + 0.04$ ). The remaining variance ( $1 - 0.71 = 0.29$ ) is explained by environmental influences that are not represented here. The double-headed arrow connecting the illicit and licit substance genetic factors represents the genetic correlation between these factors (see text). (Adapted with modification from Kendler et al. (51).)



**FIGURE 113-7** Genetic complexity in unrelated individuals: polygenicity vs heterogeneity. Each risk allele is represented as a puzzle piece of different color or shape. Black circles indicate affected individuals and empty circles denote unaffected individuals. (Adapted from Goldman et al. (39).)



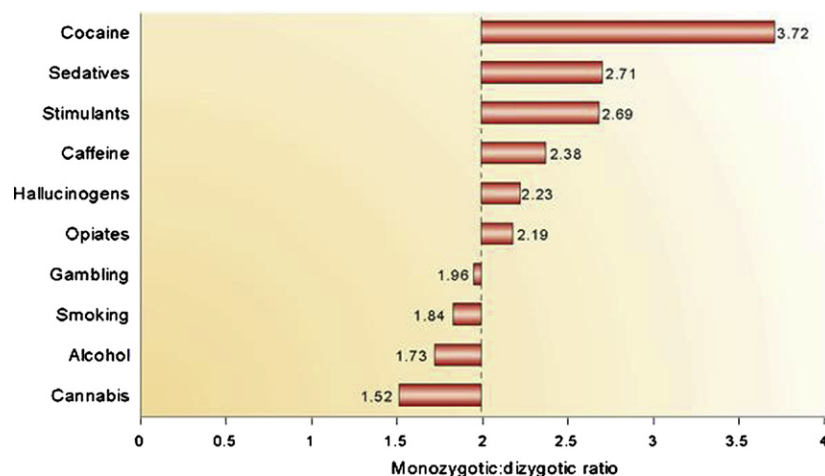
**FIGURE 113-8** Genetic complexity and twin concordance. Each risk allele is represented as a puzzle piece of different color or shape. Members of twin pairs are represented by squares. Black squares indicate affected individuals and empty squares denote unaffected. (Adapted from Goldman et al. (39).)

Under the genetic heterogeneity model different genetic variants produce the same phenotype in different individuals, but a single genetic variant can suffice to confer risk in an individual. Twin studies represent an important tool to clarify the relative importance of polygenicity/epistasis and heterogeneity in the inheritance of a trait, because polygenicity and heterogeneity generally have different effects on MZ:DZ concordance ratios, as shown in Figure 113-8.

Since MZ twins share all alleles and DZ twins only on average half, a polygenic model predicts a high discrepancy between the MZ and DZ concordance rates (high MZ/DZ ratio). DZ twins are unlikely to inherit exactly the same alleles at multiple loci, but the odds are 1/2 that a co-twin will inherit an individual risk allele. Certain neuropsychiatric disorders have high MZ:DZ ratios. For example, the MZ:DZ ratio for autism appears to be as high as 50:1. The MZ:DZ twin concordance ratios for several drugs (Figure 113-9) converge to 2:1, consistent with alleles of individual effect and with the genetic heterogeneity model.

In other words, systems of multiple loci are more likely to confer risk for addiction additively (e.g. one or at most a few major alleles lead to risk independently from the others). Nonadditive genetic influences that result from interaction between genetic mechanisms at a single locus (e.g. recessive action of alleles) or at different loci (e.g. epistasis) might contribute to the inheritance of cocaine, which has the highest MZ/DZ ratio (3.7:1).

Gene  $\times$  gene interaction studies in the addictions are sparse. In alcoholism, the protective effects of missense variants in *ADH1B* (Arg48) and *ALDH2* (Lys487) are additive (58), although these might have been thought to be synergistic because of their action in consecutive steps of the alcohol metabolic pathway. In nicotine addiction, two variants previously associated with smoking also appeared to act additively (22). The genetic risk variants involved are in the *CHRNA5-CHRNA3-CHRNA4* nicotinic acetylcholine receptor subunit cluster and in the *TTC12-ANKK1-DRD2*



**FIGURE 113-9** MZ/DZ twin concordance ratios for 10 addictions. MZ/DZ ratios tend to converge on two, consistent with the heterogeneity model (see text). (Adapted from Goldman et al. (39).)

cluster, which includes *DRD2*, a dopamine receptor important in nicotine reward. Adolescent carriers of three to four risk alleles (20% of the population) at these two loci had a 3-fold increase in odds of smoking regularly and 2.5-fold increase in odds of smoking occasionally as compared to noncarriers, who constitute 9% of the population. Carriers of one to two risk alleles were at intermediate risk. A similar stepwise increase in risk with allele dosage was observed in adulthood, and again consistent with additivity (Figure 113-10).

The mode of inheritance of a disease has implications for the manner in which genetic predictors may eventually be used in clinical treatment and in genetic counseling. Indeed, as more genetic risk variants for addiction (as well as other complex diseases) are discovered and extensive personalized genotyping and sequencing become widespread, there will be increased efforts to use multilocus genetic risk scores to predict specific diseases (59).

### 113.2.6 Interplay between Genetic and Environmental Factors

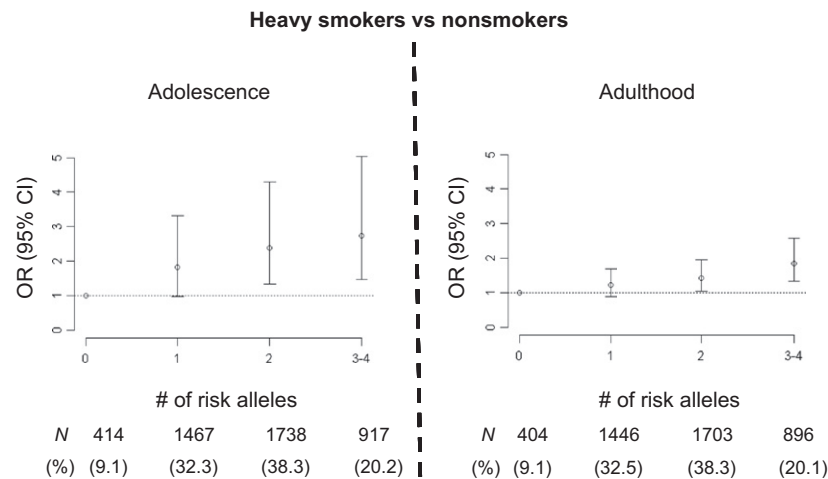
The simplest, and oldest, model for the etiology of behavioral differences and psychiatric diseases regarded genetic and environmental factors as separate and independent entities. This model polarized discussion, leading to the gene (*nature*) vs environment (*nurture*) debate, a false dichotomy.

The inheritance of addictions was a concern of the eugenics movement, which in the United States was led by Charles Davenport at the Cold Spring Harbor Laboratory. Social Darwinism led to the classification of people who were psychiatrically ill, cognitively deficient (for example, illiterate), or poor as genetically defective. In the first half of the twentieth century these ideas were promulgated in genetics textbooks. For example, the Kallikak family was used to illustrate the effects of

“bad breeding.” The founding father in the pedigree was a Revolutionary War soldier who sired offspring both by a dissolute barmaid and a Puritan wife. The barmaid’s side of the family tree had drunkards and criminals, with toadstools growing at the dark and twisted roots. The other branch of the family tree was supposedly replete with fine and moral citizens. Consequences of eugenics included the compulsory sterilization of many thousands of vulnerable individuals. *Buck vs Bell* was heard before the United States Supreme Court in 1927, Carrie Buck being a ward of the Virginia State Colony for Epileptics and Feeble-minded. Although Buck later became an avid reader, she had been proposed for sterilization because she was “feeble minded” and incorrigibly promiscuous (having given birth to a child after being raped). The clinching piece of evidence was that her mother had been a prostitute. Writing for the 8-1 majority, Chief Justice Oliver Wendell Holmes, Jr. stated,

*It is better for all the world, if instead of waiting to execute degenerate offspring for crime, or to let them starve for their imbecility, society can prevent those who are manifestly unfit from continuing their kind. The principle that sustains compulsory vaccination is broad enough to cover cutting the Fallopian tubes.*

Holmes then memorably concluded: “Three generations of imbeciles are enough.” Some 65,000 individuals were compulsorily sterilized in the United States, and there were similar programs in other countries, including Japan, Canada, and Sweden, where in that one nation 21,000 people were forcibly sterilized. The Eugenics era reached its nadir in Nazi Germany where >400,000 people were sterilized and >6 million institutionalized individuals, homosexuals, gypsies, and Jews were murdered on the false premise of their genetic inferiority.



**FIGURE 113-10** Additive effects of the *TTC12-ANKK1-DRD2* and *CHRNA5-CHRNA3-CHRNA4* gene clusters on smoking behavior in adolescence and adulthood. Risk of smoking heavily increases linearly with the number of risk alleles at the two loci. Odds ratios (OR) and 95% confidence intervals (CI). (Adapted from Ducci et al. (22).)



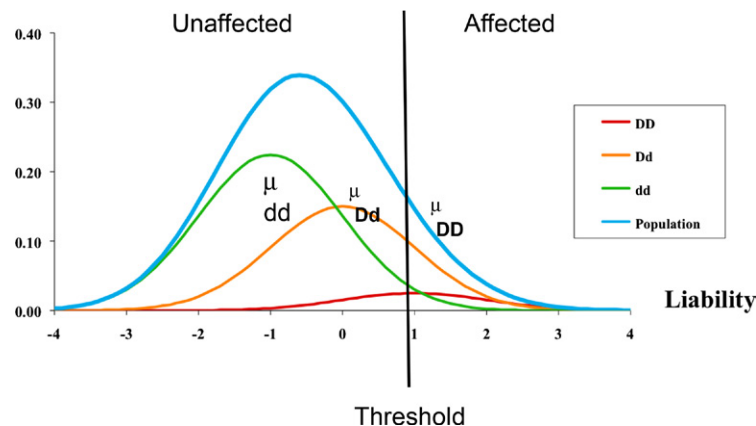
Although it is important to be aware that modern genetic studies on addictions and other psychiatric diseases have emerged from a desire to understand behavior and to prevent and treat these diseases, it remains imperative to be alert to misuse of genetic information.

Another ideologically tinged view of addictions and other psychiatric diseases is that these emerge only through upbringing, experience, and choice. In addition, in a confusion that we may charitably say is created by the difficulty of defining exact boundaries between what is and is not disease and because addictions involve choice and environmental exposure, it is sometimes alleged that these conditions are not diseases. However, addictions display the classic features of disease process, and are prevented and treated in the same fashion as other complex diseases, most of which also have volitional and environmental exposure components. The environmentalist viewpoint was also to some extent co-opted for political purposes, for example, by people who believe in equality of outcome rather than equality of opportunity. Regardless of whether their origins are genetic or environmental, behaviors can be used to stigmatize. American Indians have suffered from the “fire-water myth,” although particular American Indian communities have low rates of alcoholism and within tribes there is differential vulnerability owing to genetic variation. The Irish are frequently characterized as alcoholic, although rates of alcoholism are not higher than several other European countries. Differential vulnerability to alcoholism is again genetically transmitted within the Irish. Modern neuroscience has revealed that the potential for addiction is probably latent in all people, but to different extents. Destigmatization should flow from improved prevention and treatment and from a more sophisticated understanding of the role of natural processes in addiction, and the ways that these can be co-opted to lead to disease, and

because of the complexity of vulnerability such that all of us probably carry vulnerability alleles for one disease or another.

Genotype appears to determine reaction range—the range of possible responses to environment, and more importantly the probability of different reactions. Because people share the neurocircuitry of reward, it is likely that most or all are at some risk for addiction and could become addicted under certain circumstances. However, shifts in the level and type of environmental exposure can unveil new parts of the overall risk density distribution, as shown in Figure 113-11.

An important environmental risk factor is drug availability. Tobacco consumption and tobacco-related diseases in the United States have decreased dramatically in the last 50 years because of changes in public policy beginning with the Surgeon General’s warning in 1960, restrictions on indoor smoking and advertising, increased taxes on tobacco products, warnings placed on packaging and in advertisements, counter-advertising, and well-publicized lawsuits. As a consequence, different birth cohorts in the United States have faced different levels and qualities of exposure to tobacco. A large meta-analysis including more than 60,000 individuals has explored how the genetic impact of a synonymous variant in the nicotinic acetylcholine receptor subunit alpha three gene (*CHRNA3*) on risk for smoking changes across birth cohorts in the United States (Hartz et al., unpublished data). This variant is highly correlated with a functional missense polymorphism Asp398Asn located within the *CHRNA5* (see Section 113.2.6.1) and is therefore a proxy for Asp398Asn among Caucasians. The increasing social restriction may have strengthened the impact of this locus on smoking. The odds ratio (OR) effect of this locus was 1.6 for the 1970 birth cohort as compared to 1.18 for the 1950 birth cohort, who passed through adolescence



**FIGURE 113-11** Genotype-specific liability and disease threshold. Liability or predisposition to a disease is simplified here as a single continuous dimension. Once liability passes a threshold the discrete phenotype emerges (affected versus unaffected). Given a disease-linked locus with two different alleles (D and d), carriers of the three different genotypes (DD, Dd, dd) have three different liability curves that together compose the overall liability distribution for that population (assuming that this is the only disease-linked locus in this population). Factors that change the threshold will therefore change the number of affected and unaffected individuals within each genotype group as well as in the overall population.

and young adulthood when the cultural climate for smoking was more permissive. Such findings also suggest that social restrictions that are effective in reducing smoking behavior at the population level may be less effective for people with a stronger genetic propensity such as carriers of the asparagine allele.

In addition to environmental influences that are specific to the addictive agent, such as drug price, availability and advertising campaigns, many nonspecific environmental influences powerfully moderate risk of addictions. These nonspecific environmental factors include exposure to maltreatment during childhood (44), low socioeconomic status (60), adverse life events, low social support, poor parenting, religiosity (61), peer influences (62), and parental monitoring.

Having identified gene effects and environmental effects it is important to identify the two main types of violations of gene–environment independence. These are gene by environment interaction (Figure 113-12B–D) and the less well-recognized gene by environment correlation (Figure 113-12A).

**113.2.6.1 Gene by Environment Correlation.** A shown in Figure 113-12A, gene  $\times$  environment correlation (rGE) occurs when genotype correlates ( $r$ ) with the probability of exposure to an environmental risk factor. There are three main types of rGE: active rGE, evocative rGE, and passive rGE.

Active rGE occurs when an individual's genotype shapes her choice of environment. For example, children with CD, a precursor to antisocial personality disorder (APD), tend to seek out antisocial peers. Exposure to antisocial peers increases risk of developing antisocial behavior and addiction. An example of active rGE is the effect of *CHRNA5* Asn398 to increase risk of lung cancer. As already discussed, this functional allele is associated with heavy smoking. It thereby leads to the exposure of cells of the lung, and elsewhere in the body,

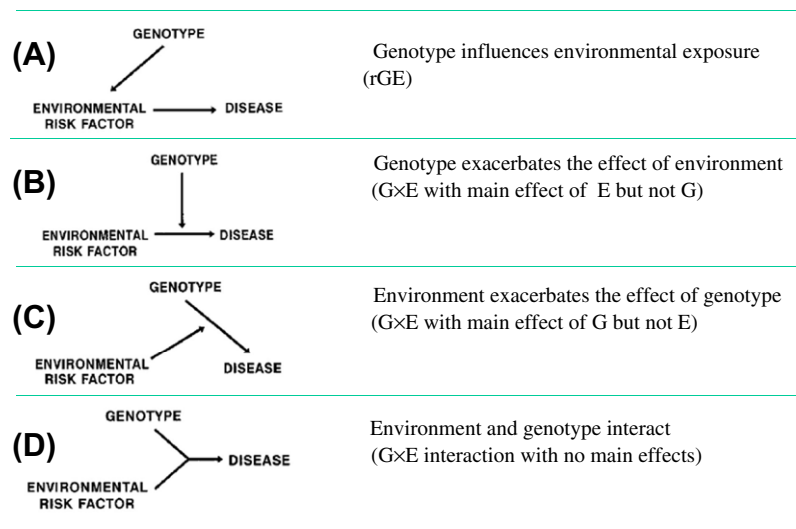
to carcinogens, and to cancer. A misinterpretation of these results would be that asparagine allele influences sensitivity to the carcinogenic effect of smoking; this is the  $G \times E$  model. The rGE model is of course consistent with a wealth of information on the carcinogenic effects of molecules in cigarette smoke.

In evocative rGE, an individual indirectly shapes his/her environment. For example, a child with CD may evoke harsher discipline from her parents, in turn promoting the risk of addictions and other pathologies.

In passive rGE, alleles conferring risk in a child also alter the behavior of the parent transmitting the allele. Thus, the children of an addicted parent are at enhanced risk both via transmission of risk alleles and via family environment and teratogenic effects of the drug. For example, maternal smoking is associated with increased risk of smoking in the offspring because mothers smoking heavily are at increased risk of carrying, and transmitting, the risk allele within the *CHRNA5* gene (63). In addition, mothers who smoke expose their offspring to a tobacco-enriched environment that is likely to promote smoking behavior.

Twin studies can address the existence of rGE by measuring the “genetics of the environment.” Although it may appear paradoxical, if behavior alters environmental exposures and if the relevant aspect of the behavior is subject to genetic influences, then the environmental factor will be heritable. Inheritance of environmental exposures has been observed. Kendler and Backer (64) reported modest to moderate heritabilities (ranging from 7 to 39%) for several categories of environmental factors important or potentially important in addiction vulnerability: stressful life events, parenting, family environment, social support, peer interactions, and marital quality.

An important example of rGE is age at first use of the psychoactive substance, which influences risk of becoming dependent. Early initiation of addiction use is associated with an increased risk of developing addiction. The



**FIGURE 113-12** Different patterns of gene (G)–environment (E) interplay: gene by environment correlation (A) and gene by environment interaction (B–D).

National Epidemiologic Survey on Alcohol and Related Conditions (NESARC) revealed that odds of lifetime substance dependence among users were reduced by 4% for illicit drugs and 9% for alcohol for each additional year that onset of drug use was delayed (15,16). This association between age at initiation and risk can arise from different mechanisms. It can result from a direct causal effect (early onset directly increases risk) or might be mediated by genetic/familial influences (early onset and addiction result from a broad shared liability). Results from the Virginia Twin Registry study are more consistent with the second hypothesis. Prescott and Kendler (65) showed that co-twins with late onset of alcohol use had the same risk of becoming alcoholics as their sibling twins with early onset of alcohol use. This result indicates that the association between early exposure and alcoholism may result from a shared genetic liability and that early exposure does not independently influence risk of developing alcoholism. This is important, because prevention efforts at delaying drinking are likely to be useful for preventing alcohol-related accidents and injuries, but might not necessarily reduce risk of developing alcoholism in adulthood. The impact of early exposures to alcohol, and other addictive agents, remains a critically important issue: few would risk unnecessary early exposures in children with developing brains and imperfectly developed impulse control.

### 113.2.6.2 Gene by Environment Interaction.

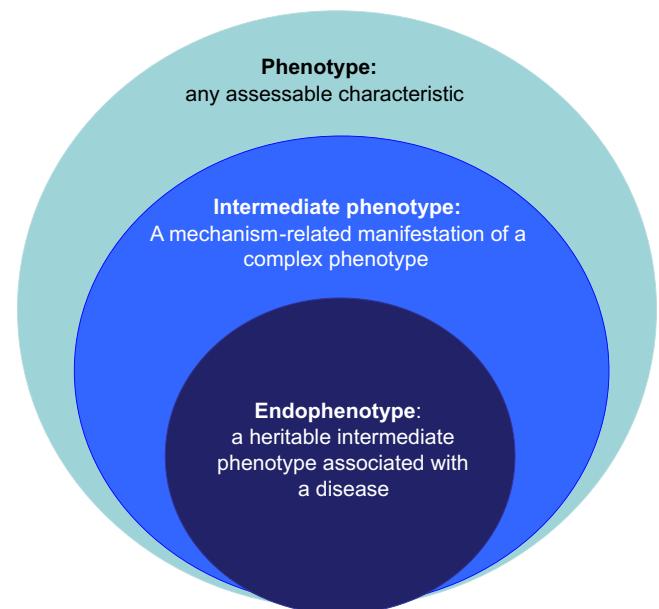
Gene  $\times$  environment interaction ( $G \times E$ ) occurs when the effect of the environmental exposure on a certain outcome is strongly influenced or contingent upon genotype and vice versa (gene effect on the outcome is contingent on exposure), see Figure 113-12B–D (for review see Reference (66)).

$G \times E$  has been observed for several functional alleles identified as having roles in addictions and other psychiatric disorders. By comparison with other complex diseases, including cancer, diabetes, cardiovascular disease, infectious diseases, and hematologic diseases, where genetic variation moderates resiliency and vulnerability to chemicals, pathogens, nutrients, caloric load, oxidants, and ionizing radiation, in psychiatric genetics much of the progress has been through discovery of alleles that influence stress resiliency. Severe childhood stress and neglect both increase vulnerability to addiction and multiple addiction-related psychiatric diseases, including APD, CD, anxiety disorders, and depression, with the risks of these common diseases being elevated several-fold in the stress exposed. However, not all people who are exposed to early life stress develop addiction or other psychiatric diseases, indicating wide variation in stress resiliency. Functional loci that have been shown to partially account for interindividual differences in stress resiliency include MAOA (44), the serotonin transporter (SLC6A4) (67), COMT (68), the corticotrophin-releasing hormone receptor 1 gene, neuropeptide Y (NPY) (69), and FKBP5 (70).

### 113.2.7 Progress through Intermediate Phenotypes

One strategy to discover gene effects in addictions and other etiologically complex diseases is the deconstruction of complex phenotypes into elements that are etiologically more homogeneous. Intermediate phenotypes access mediating mechanisms of genes and environmental effects on behavior. Heritable intermediate phenotypes that are disease associated have been termed “endophenotypes” (71) (Figure 113-13).

Several intermediate phenotypes have been specifically associated with addiction. These include alcohol-induced flushing, which is a protective endophenotype, and low response to the effects of alcohol, which is an endophenotype predictive of risk of alcoholism. In humans, the level of response to alcohol is believed to reflect mainly pharmacodynamic variation in response (72) rather than variation in metabolism. A low response to alcohol predicts increased risk of developing alcohol use disorders (73–75) and has been associated with genetic variation in the serotonin transporter gene (SLC6A4) and in the gene encoding the subunit  $\alpha 6$  of the gamma-aminobutyric acid receptor A (GABRA6) (76). Other intermediate phenotypes assist in the exploration of genetic vulnerability to addictions (as well as other psychiatric diseases), and these addictions-relevant intermediate phenotypes include electrophysiologic, neuropsychologic, neuroendocrinologic and, more recently, neuroimaging measures. Neuroimaging provides access to the neuronal mechanisms underlying emotion, reward, and craving and therefore represents an extraordinary tool to link genes to the neuronal pathways that produce behaviors (for review see (77)). For example, amygdala activation after exposure to



**FIGURE 113-13** Definitions of phenotype, intermediate phenotype, and endophenotype.

stressful stimuli predicts anxiety and captures interindividual differences in emotional response and stress resilience (78). On the other hand, activation of the prefrontal cortex during working memory performance is used to evaluate prefrontal cognitive function that is impaired in several psychiatric diseases including addictions.

As compared to complex phenotypes such as clinical diagnosis, intermediate phenotypes offer two main advantages:

- (1) They can help in identifying the neurobiological processes, or “missing links,” that translate gene effects into complex behavior.
- (2) They are less complex in their causal origins and therefore likely to be influenced by variations at fewer genes, thereby increasing the power to detect risk genetic variants (79,80). Indeed, effect sizes (i.e. the strength of the association) of genetic variations acting on intermediate phenotypes that are more proximal to gene action appear to be larger than effects on complex disease phenotypes such as clinically defined disease (79), and as illustrated, for example, by the dilution of action of a variant of NPY, an anxiolytic neuropeptide, from the molecular level (RNA and peptide level) to intermediate phenotypes (brain imaging) to anxiety (69).

### 113.3 GENE IDENTIFICATION

Two main strategies have been used and are increasingly integrated to identify genetic variations influencing addiction: (1) candidate gene and (2) genome-wide approaches. In the former, genes known to influence processes involved in the pathogenesis or treatment of addiction are selected. In the latter, the whole genome is interrogated simultaneously in a hypothesis-free way. A point of integration between the methods is the study of candidate genes located in chromosome regions implicated by genome-wide scans.

#### 113.3.1 Candidate Genes

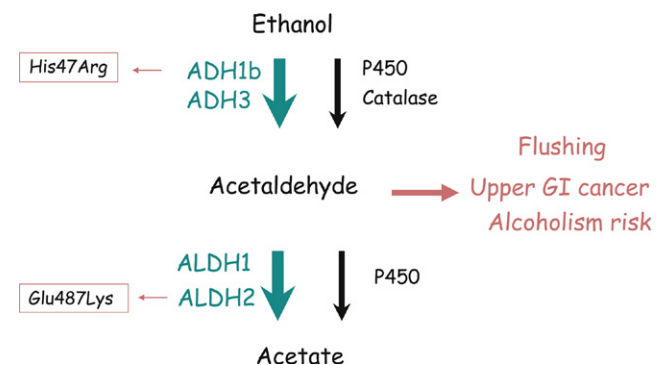
**113.3.1.1 Alcohol Metabolizing Genes: *ADH1B* and *ALDH2*.** The alcohol dehydrogenase IB (*ADH1B*) and aldehyde dehydrogenase 2 (*ALDH2*) genes encode enzymes catalyzing consecutive steps in alcohol metabolism. In adults, these enzymes play an important role, although there are also several other enzymes that can carry out both these metabolic steps, including catalase, cytochrome P450, and additional enzymes in the *ADH* and *ALDH* gene families. In the liver, three *ADH* genes are expressed at high levels and to some extent at different times of development, and these three enzymes are primarily expressed in hepatocytes. *ALDH2* is encoded in the nuclear genome but the enzyme is translocated to the mitochondrion, where it plays a critical role in the ability of hepatocytes, and other cells throughout the body,

to metabolize acetaldehyde. The metabolic functions that have maintained these genes and this enzyme pathway through at least 80 million years of mammalian evolution are in fact somewhat obscure. A possible explanation is that the body has to utilize alcohols that are the product of bacterial fermentation in the gut. These enzymes also act on other important substrates including retinoids and neurotransmitters.

The product of *ADH* is acetaldehyde, a toxic intermediate that may react with a variety of biomolecules. Indeed, acetaldehyde adducts with DNA and both acetaldehyde and alcohol are formally recognized as mutagens. Acetaldehyde is a potent releaser of histamine, and can trigger the aversive flushing reaction. Symptoms include headache, nausea, palpitations, and flushing of the skin. Ordinarily, acetaldehyde is rapidly converted to acetate, and levels of acetaldehyde remain very low—in the nanomolar range. However, if aldehyde dehydrogenase is blocked by disulfiram (which is a medication used to help alcoholics maintain abstinence) or certain drugs used to treat protozoal infections (e.g. metronidazole) then the flushing reaction is observed after the ingestion of only small quantities of alcohol. If acetaldehyde accumulates the individual is at substantially increased risk of upper gastrointestinal (GI) cancer, and this can occur because of either pharmacologic blockade of aldehyde dehydrogenase or natural genetic variation, a factor that physicians may wish to consider in counseling individuals who drink despite carrying the genetic variations that will next be described (81).

Nature has provided two common natural examples of genetic predisposition to alcohol-induced flushing, and it is not surprising that the enzyme variants that lead to flushing are protective against the development of alcohol use disorder (Figures 113-14 and 113-15).

The most important functional loci at *ADH1B* and *ALDH2* are the *ADH1B* His48Arg missense polymorphism, in which Arg48 is a hyperactive allele acting in codominant fashion, and *ALDH2* Glu487Lys, in which the Lys487 allele inactivates *ALDH2* dominantly



**FIGURE 113-14** Functional polymorphisms in ethanol metabolism: *ADH1B* His48Arg and *ALDH2* Glu487Lys. Higher activity of *ADH1B*, conferred by Arg48, or lower activity of *ALDH2*, conferred by Lys487, leads to accumulation of acetaldehyde following alcohol consumption and the flushing reaction.



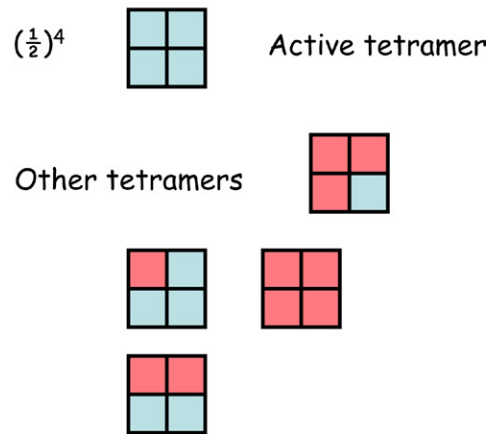
(a manifestation of the tetrameric structure of ALDH2). Higher activity of ADH1B, conferred by Arg48, or lower activity of ALDH2, conferred by Lys487, leads to accumulation of acetaldehyde following alcohol consumption and the flushing reaction. In east Asia populations (e.g. China and Japan), where both His48 and Lys487 are highly abundant, and in Jewish populations where His48 is abundant, many individuals carry genotypes protective against the development of alcoholism. The protective effect seems to vary across environments (82) and shows genotype–genotype additivity (58). Following up the connection of acetaldehyde to mutation,

both polymorphisms have also been associated with an enhanced risk of cancers of the oropharynx and esophagus (81) (Figures 113-16 and 113-17). As seen in the figure, rates of upper GI cancer tend to be higher in parts of the world where the ALDH2 Lys487 allele is abundant.

Both the ADH1B and ALDH2 polymorphisms appear to be ancient in human populations, occurring on characteristic and highly diverged haplotypes. On that basis it is unlikely that either the Arg48 or Lys487 were selected to high frequencies in east Asian populations as protective alleles against alcoholism. One possibility, still speculative, is that these polymorphisms alter susceptibility



### Structure leads to dominant action of Lys487



**FIGURE 113-15** ALDH2: A tetrameric oxidoreductase. ALDH2 is a tetrameric oxidoreductase, accounting for the dominant action of the Lys487 allele. His487/Lys487 heterozygotes have <5% of ALDH2 activity of His487/His487 homozygotes.



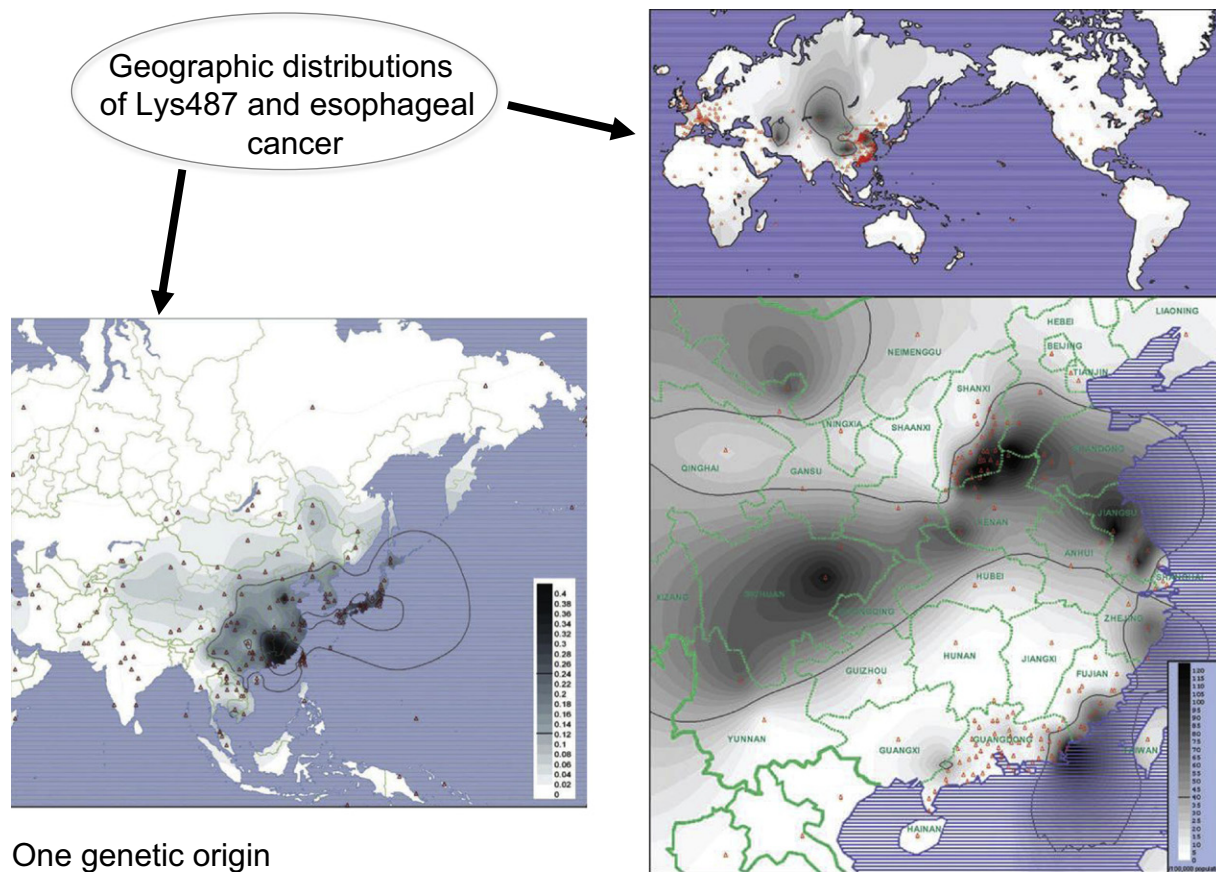
**FIGURE 113-16** Alcohol-induced flushing and cancer. The ADH1B His47Arg and ALDH2 Glu487Lys influence risk of developing cancers of the oropharynx and esophagus after alcohol consumption because of the toxic effect of acetaldehyde.

to protozoal infections of the gut, including amebiasis, because an action of metronidazole (an antiprotozoal drug of unknown mechanism) is to inhibit ALDH (83). However, regardless of the forces responsible for their high frequencies, the pervasive environmental exposure to alcohol that occurs in modern societies has added other dimensions to their effects.

**113.3.1.2 Genes Moderating Monoamines.** Monoamines including serotonin (5-HT), norepinephrine (NE), and dopamine (DA) are fundamental modulators of emotionality, cognition, reward, and behavioral response to stimuli. Therefore, it is unsurprising that genes regulating monoamines levels such as catechol-O-methyltransferase (COMT), MAOA, and the serotonin transporter (SLC6A4) have been implicated in vulnerability to several psychiatric diseases, including addiction, APD, depression and anxiety. In line with these ideas, drugs increasing monoamines in the synaptic cleft or drugs that target receptors of monoamine neurotransmitters are used in the treatment of several psychiatric diseases.

COMT metabolizes dopamine, NE, and other catecholamine neurotransmitters. COMT plays an important role in the regulation of dopamine in the prefrontal cortex because it is a region where dopamine transporter is less

expressed (84,85). COMT knockout mice have increased levels of dopamine in this brain region (86,87). In mammals, the COMT enzyme occurs in two forms: a soluble, cytoplasmic, protein (S-COMT) and a membrane-bound form (MB-COMT), which, in humans, has 50 additional amino acid residues at the N-terminus. S-COMT predominates in most tissues, accounting for 95% of total COMT activity (88). However, in the brain, the amount of MB-COMT activity is much higher (89). Val158Met is a common functional single nucleotide substitution of COMT (90), replacing methionine for valine at codon 158 of MB-COMT and at codon 108 of S-COMT. Via its effect on enzyme stability (91,92) the Met158 allele is three- to four-fold less active than Val158 (93), and the alleles act codominantly. Because of its higher activity and the importance of COMT for dopamine metabolism in the frontal cortex, the Val158 allele was predicted to lower dopamine level in that region. In line with this hypothesis, the Val158 allele has been associated with inefficient frontal lobe function evaluated with different psychological and neuroimaging methodologies (94–96). In addition, in a pharmacogenetic study, the COMT inhibitor tolcapone improved executive function in val/val homozygotes, but not in individuals homozygous for the met allele, a finding consistent with



**FIGURE 113-17** Geographic distributions of Lys487 and esophageal cancer (158). The Lys487 allele is highly abundant in southeast Asia, and the frequency falls off clinally. Lys487 is virtually absent in Europeans, Africans and Amerindian populations. Southeast Asia is also an epidemiologic hot spot for esophageal cancer, consistent with genetic epidemiological studies that have connected risk of esophageal cancer to moderate consumption of alcohol in carriers of the Lys487 allele. Acetaldehyde is a carcinogen (81).

the higher levels of cortical dopamine already expected in individuals with this genotype (97). On the other hand, the Met158 allele, although associated with better cognitive performance, is associated with decreased stress resilience and increased anxiety. This allele has been associated with increased anxiety among women populations (98), increased pain–stress response and a lower pain threshold (68,99), and increased amygdala reactivity to unpleasant stimuli (100). In certain addicted populations, e.g. polysubstance abusers (101), both the Val158 and Met158 alleles have been associated with addictions. The Val158 allele was found to be excess among methamphetamine, nicotine, and polysubstance addicts (101). On the other hand, in addicted populations with high frequencies of internalizing disorders, such as late-onset alcoholics in Finland (102) and Finnish social drinkers (103), increased risk appears to be conferred by the Met158 allele.

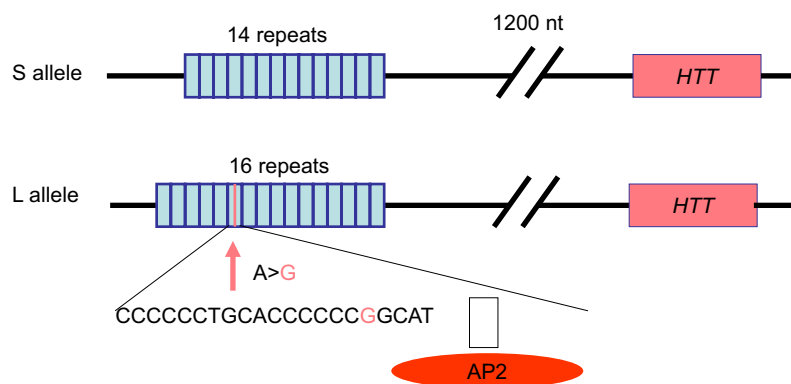
This story of multilevel association of a functional locus of the COMT underlines that a gene can influence different pathways to addictions, sometimes working in opposite directions. Frustratingly for researchers, substance use disorders are not etiologically defined and people become addicted for different reasons. An eventual use of genetic markers is to improve the nosology of these common diseases, but at present the disease diagnosis or some other relatively crude indicator (for example, the number of cigarettes or alcoholic drinks consumed each week) is often the primary phenotype being used in genetic analysis. From this perspective it is unsurprising that gene effects vary across different populations of patients (for example, addicted patients with different level of severity, different pattern of comorbidity, different age at onset, and different exposure to stress).

Addictions genetics is problematic if primarily conducted at the statistical level—it may be difficult to statistically replicate association to the crude addiction phenotype. Replication is an important scientific standard to establish validity, but it is not the only way to validate gene/phenotype relationships, and often does not further the goal of genetic studies, which is

to identify functional loci and mechanisms. The initial observation of genetic association, in which the statistical genetic study is treated as the first phase of a process, can lead to a more productive focus on intermediate phenotypes, clinical subgroups, individuals with particular histories of environmental exposure, and animal models. The goal is to connect gene to behavior through a rationally understood chain of causality, and as just described for COMT and the alcohol metabolic enzymes. In this regard, genes such as COMT and ALDH2 have validated roles in behavior, but the association findings to disease phenotypes, even now and years after discovery of these loci, do not measure up to the statistical standard required in the genome-wide association study (GWAS), which is approximately  $10^{-8}$ . However, as will be seen, that stringent standard also reflects the multiple testing that is performed as part and parcel of a GWAS.

The serotonin transporter (*SLC6A4*) is another gene that like COMT influences addiction and that is also not specific to a particular addictive agent, or to addiction. The serotonin transporter regulates the level of serotonin in the synapse, and this neurotransmitter has many functions in mood, behavior, appetite and impulse control. Reflecting these diverse actions, the most commonly prescribed category of medications for mental illness, namely, the SSRIs, is used to treat depression, anxiety, and chronic pain and their effect is mediated by blockade of the serotonin transporter. The serotonin transporter gene *SLC6A4* has a common variable-number tandem repeat (VNTR) in its promoter region (HTTLPR) (Figure 113-18).

The major alleles within this VNTR differ in the number of copies of a 20–23-bp imperfect repeated sequence. The L allele, which leads to increased transcription efficiency, has 16 copies of the repeat and the S allele has 14 copies (104). Furthermore, there is a relatively common, functional A>G single nucleotide polymorphism (SNP) within the L allele (105). The low-transcribing S allele has been inconsistently associated with trait anxiety, depression, and alcoholism. However, the effect of this allele on behavior appears stronger if stress exposure is taken into



**FIGURE 113-18** The serotonin-transporter-linked polymorphic region (HTTLPR). The human serotonin transporter gene has a common VNTR in its promoter region termed HTTLPR. The major alleles within this VNTR, namely, the L (long) and S (short) alleles, differ in the number of copies of a 20–23-bp imperfect repeated sequence. The L allele, which leads to increased transcription efficiency, has 16 copies of the repeat and the S allele has 14 copies. Furthermore, there is a relatively common, functional A>G SNP within the L allele (104, 105).



account. *HTTLPR* moderates the impact of stressful life events on risk of depression and suicide (106). Carriers of the low-transcribing *S* allele exhibit more depression and suicidality following stressful life events than *L* individuals with two copies of the allele (106).

Furthermore, *HTTLPR* has been shown to influence the activity of the amygdala, a brain region that regulates emotional response to environmental changes and is involved in the pathogenesis of depression and anxiety. Carriers of the low-activity allele display increased amygdala reactivity to fearful stimuli (67), reduced amygdala volume (107), and enhanced functional coupling between the amygdala and the ventromedial prefrontal cortex (108), a brain region that ordinarily represses amygdala activation. Closer to the molecular action of *HTTLPR*, an effect of the genotype on transporter expression in brain *in vivo* has been reported in post-mortem brain (109) and in life by neuroimaging (110), although not in all studies (111). The rhesus macaque has an orthologous polymorphism (*rs-5HTTLPR*) in the promoter region of its gene. Consistent with findings in humans, the macaque *rs-5HTTLPR* polymorphism influenced alcohol consumption and stress response, depending on rearing conditions. Carriers of the low-expression serotonin transporter genotype that were separated from their mothers at an early age (an animal model for childhood trauma) displayed higher stress reactivity and ethanol preference (112). The combined effect of *rh-HTTLPR* and environment on stress reactivity suggests that the influence of *HTTLPR* on behavior might be traced to altered regulation of the hypothalamic–pituitary–adrenal (HPA) axis.

Another key regulator of monoamine activity is MAOA that will be discussed in Section 113.3.3.

### 113.3.2 Genetic Mapping

Genetic mapping is the localization within the genome of genes underlying a disease on the basis of correlation with DNA variation, without the need for prior hypotheses as opposite to the candidate gene approach. For the addictions, genome-wide scans, including whole-genome linkage and whole-genome association (WGA), have implicated several chromosome regions.

**113.3.2.1 Whole Genome Linkage.** In whole-genome linkage a panel of polymorphisms is tested for meiotic linkage to a disease in family-based samples. This is done by identifying chromosome regions that are shared more often among phenotypically concordant relatives than among phenotypically discordant family members. The implicated chromosomal regions are usually very large, for example, greater than 10 Mb, and include therefore multiple genes. Therefore, a more refined search for the disease-associated gene within the disease-linked region is subsequently conducted via association or sequencing.

To perform whole-genome linkage analysis for alcoholism and other addictions, several large family-based

samples have been collected. These include the Collaborative Study on the Genetics of Alcoholism (COGA) (113); the Roscommon Study of Irish families (114); a sample of multiplex families from the Pittsburgh area (115); and samples collected from relatively isolated populations, including Native Americans (116,117) and Finns (32). Such isolated populations, and large families within them, are likely to confer the advantage of reduced genetic heterogeneity. A nonexhaustive list of convergent findings across family linkage studies includes a region on chromosome 4q that contains the alcohol dehydrogenase (*ADH*) gene cluster (113,114,116,117) and a chromosome 4p region containing a gamma-aminobutyric acid (*GABA*) receptor (*GABA<sub>A</sub>*) gene cluster (113,117). In the COGA sample there was also evidence for linkage to chromosomes 1 and 7, and to chromosome 2 at the location of an opioid receptor gene (115). A region on chromosome 1 was linked to alcoholism and depression in the COGA data set (118), further supporting the existence of a genetic overlap between alcoholism and internalizing disorders. A region on chromosome 7 was linked to alcoholism and/or illicit drug disorders in a subset of COGA families with high density for childhood and adult antisocial behaviors (119). Linkage analyses have also been conducted with intermediate phenotypes for alcoholism, including low response to alcohol (118), neurophysiological endophenotypes such as P300 (120), and reduced alpha power (121,122), implicating the corticotropin releasing factor-binding protein (*CRHBP*), another stress-related gene (122). Recently, multiple genome-wide significant loci for resting electroencephalography (EEG) were identified by GWAS (123), illustrating the power of combining GWAS with the endophenotype strategy. Chromosome regions identified by these studies overlap partially with those reported for alcoholism.

**113.3.2.1.1 *GABA<sub>A</sub>* Receptors.** Gamma-aminobutyric acid (*GABA*) is the primary inhibitory neurotransmitter in the central nervous system. *GABA<sub>A</sub>* receptor-mediated chloride currents into neurons are modulated by various drugs including ethanol, benzodiazepines, and barbiturates. *GABA* mediates many effects of alcohol including tolerance, dependence and cross-tolerance to benzodiazepines and barbiturates. A series of mouse ethanol-related behaviors, including preference, withdrawal severity, and sedation sensitivity, map to quantitative trait loci (QTL) regions where *GABA<sub>A</sub>* receptor gene clusters are located (124,125). In the rat, an Arg100Gln missense variant located in the *GABA<sub>A</sub>*  $\alpha 6$  subunit gene (*GABRA6*) was associated with variation in ethanol and benzodiazepine sensitivity (125). In humans, alcohol use disorder has been linked to both the chromosome 4 (121,126) and chromosome 5 *GABA* clusters. Linkage signals appear to derive from *GABRA6* on chromosome 5 (127) and *GABRA2* (121,126) and *GABRG1* on chromosome 4. In the COGA sample, the association between *GABRA2* and alcoholism was mainly driven by alcoholics who also abused illicit



substances, indicating that this gene might contribute to the shared liability to a different class of addictive disorders (128). A human variant of GABRA6 (Pro385Ser), which is located in the chromosome 5 cluster, was associated with sensitivity to alcohol (76) and benzodiazepines (129).

**113.3.2.2 Genome-Wide Association (GWAS).** Large-scale genotyping techniques have recently enabled genome-wide association analyses. These GWASs have the advantage of increased power for detecting effects of relatively common alleles (minor allele frequency (MAF) >5%) and more refined localization of signals to smaller chromosome regions as compared to family-based linkage analyses, which have a reciprocal advantage of being powerful for detecting effects of rare and uncommon alleles that are present in only a small proportion of probands and their families. An advantage of GWAS is that the same genotypes are obtained in different samples facilitating the combination of results from different studies in meta-analyses. This is a crucial aspect because extremely large study samples are necessary to be able to detect the small effects of many common variants on complex diseases. Of note in GWAS, up to 5 million SNPs can be simultaneously tested, raising the issue of false positive due to multiple testing. Therefore, genome-wide significance threshold is generally set at the  $p$  value level of  $10^{-8}$  rather than 0.05 or 0.01, which would be the equivalent  $p$  value for a single locus test.

GWASs have been particularly useful to identify genetic variation relevant to nicotine addiction. Results have been to some extent more disappointing for alcoholism; however, the datasets that have been analyzed have been substantially smaller.

**113.3.2.2.1 The *CHRNA5-CHRNA3-CHRNA4* Gene Cluster.** Although a large number of genetic loci potentially moderating smoking have been tested, only a few have been convincingly replicated. So far, the strongest evidence of association is for the *CHRNA5-CHRNA3-CHRNA4* gene cluster on chromosome 15q25 (130–135) (Figure 113-19). Gene clusters such as this one are thought to reflect ancient gene duplications, and can enable cis-regulatory effects on the expression of genes in the cluster.

Nicotinic acetylcholine receptors, nAChRs, are pentameric cholinergic receptors that form ligand-gated ion channels. They are key mediators of the effect of nicotine on the central nervous system. Neuronal subtypes of nAChRs include various homomeric or heteromeric combinations of 12 different nicotinic receptor subunits:  $\alpha 2$  through  $\alpha 10$  and  $\beta 2$  through  $\beta 4$ . The *CHRNA5-CHRNA3-CHRNA4* gene cluster encodes for the alpha five, alpha three, and beta four subunits. Association of genetic variation within this region to smoking behavior was initially discovered using a candidate gene approach (131,132) but was subsequently replicated by GWASs. GWASs detect a highly significant peak on chromosome

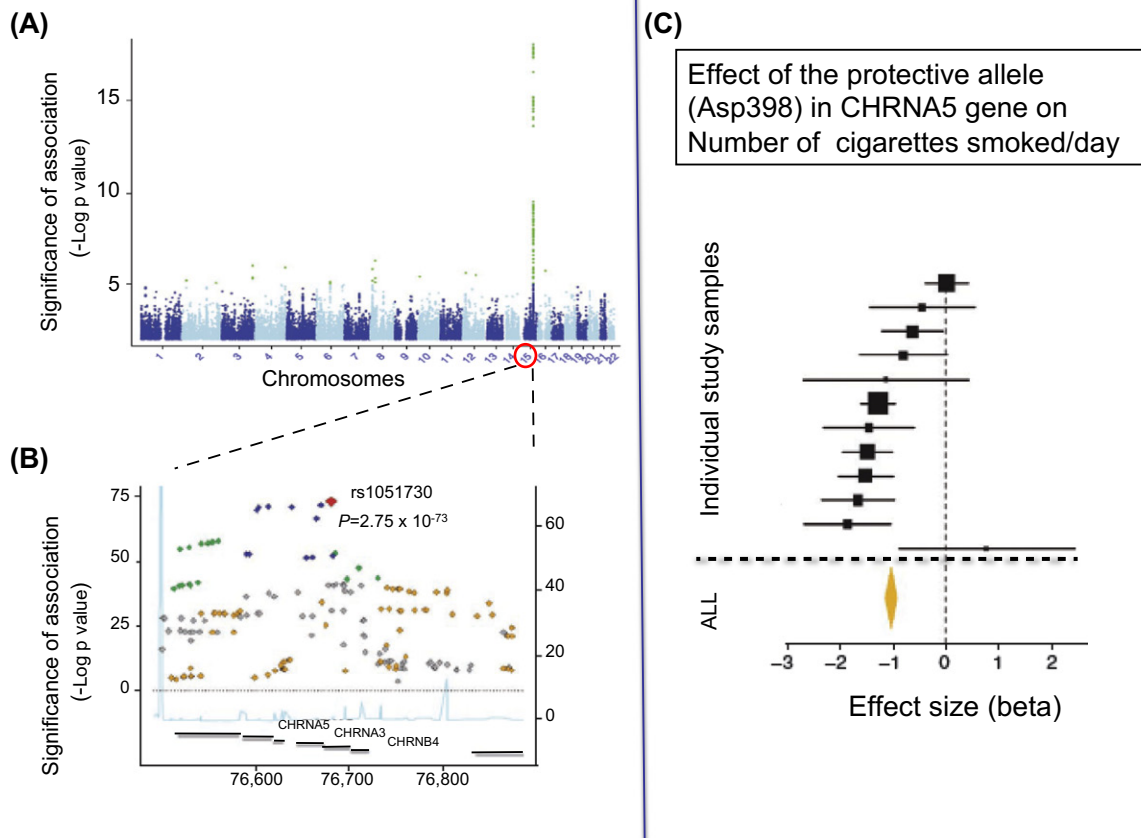
15q25 corresponding to the region where these three genes are located (Figure 113-19A,B).

Two SNPs in this region that have been repeatedly associated with smoking are the nonsynonymous (aspartic acid, Asp (D), to asparagine, Asn (N); rs16969968) SNP at codon 398 of *CHRNA5* and a synonymous SNP (rs1051730) in *CHRNA3*. These SNPs are in strong linkage disequilibrium with each other. The *CHRNA5* asparagine allele (Asn398) and the A allele at rs1051730 have been associated with nicotine dependence/heavy smoking (131,132), pleasurable response to smoking (133), decreased response to nicotine agonists in vitro (136), smoking quantity (134), smoking persistence and increased susceptibility to develop lung cancer and vascular disease among smokers (134,137,138). According to a recent meta-analysis, each copy of the A allele at rs1051730 accounts only for ~0.5% of the variance in number of cigarettes smoked per day. Nevertheless, the overall  $p$  value for this large meta-analysis including >74,000 participants was  $\sim 10^{-73}$ , making this finding one of the most consistent across the entire panorama of genetic-association studies on human behavior (139) (Figure 113-20C). Potentially explaining the mechanism by which the Asp398Asn locus alters propensity to nicotine addiction, the asparagine allele was found to predict the strength of a brain circuit connecting the anterior cingulate to the ventral striatum (140). The anterior cingulate is a component of the limbic system involved in emotional modulation, and the ventral striatum is a principal reward region of the brain. Strength of this circuit itself was associated with nicotine dependence, and this genotype predicted the circuit strength in both smokers and nonsmokers.

For alcoholism, results from GWASs have been less convincing. None of the alcohol dependence GWAS has yet yielded a finding that reached genome-wide significance (141–144). Larger studies and meta-analytic approaches are likely to change this picture. In addition, GWASs performed on alcoholism-relevant intermediate phenotypes are underway. For example, a GWAS on variation in the electroencephalogram yielded genome-wide significant loci (123).

### 113.3.3 Rare and Common Variants

The focus of genetic studies of addiction, as well as other common disorders, have been common genetic variation, namely, variants with a MAF >1%. The idea behind these studies is the common disease/common variant (CDCV) hypothesis according to which common alleles of ancient origin and with small to moderate effect lead to susceptibility to common disorders. The success of GWASs in explaining the genetic variance of common diseases is to some extent tied to the validity of this hypothesis. Consistent with the hypothesis, the effect sizes of loci detected by GWASs have generally



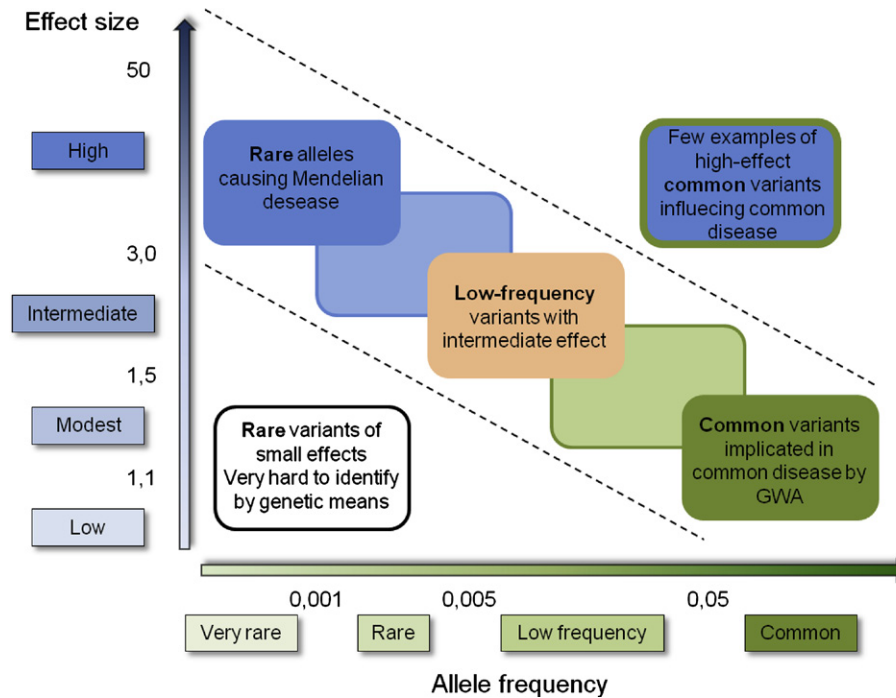
**FIGURE 113-19** Association between smoking behavior (number of cigarettes smoked per day, CPD) and genetic variation within the *CHRNA5-CHRNA3-CHRNA4* gene cluster on chromosome 15q25. (A) In the Manhattan plot, level of significance (–log  $p$  value) of association to SNPs covering 22 autosomal chromosomes is shown. SNPs reaching genome-wide significance ( $p < 10^{-8}$ ) are in green. Results show a strong signal of association within chromosome 15. (B) The chromosome 15 region contains the *CHRNA5-CHRNA3-CHRNA4* gene cluster. (C) The most significant SNP within this region is rs1051730, which is highly correlated with a nonsynonymous (aspartic acid (Asp) to asparagine (Asn)) SNP (rs16969968) at codon 398 of the *CHRNA5* gene. The asparagine allele at this locus is associated with increased risk of becoming a heavy smoker. In contrast, the aspartic acid allele is protective, as shown by the negative effect size (beta). (Adapted with modification from (159,160)).

been small, with a mean genotype relative risk (GRR) of 1.25 for all complex disorders (145). However, the total genetic variance accounted for has been less than half of what is estimated to exist, and usually less than 10%, the amount depending on the disease and the intensity with which it has been studied. It is perhaps dangerous to extrapolate, but this may indicate that common variants may not explain most of the heritability of common diseases. Recent evidence suggests that rare variants with larger GRR are likely to contribute to genetic vulnerability to common diseases (for review see Reference (146). Indeed associations with multiple rare variants with moderate-to-large effect sizes (GRR of approximately 10) have been recently reported for schizophrenia and autism (147,148). A rare stop codon was detected in the serotonin receptor 2B gene (*HTR2B*) that can lead to severe impulsivity (149). Some of these variants appear to be associated with severe forms of disease and are thought to be of recent origin and to some extent de novo. Consistent with that, the *HTR2B* stop codon was restricted to the Finnish population and is in that sense a “founder mutation,” and an *MAOA* stop codon that

also influences impulsivity, was restricted to only one Dutch family.

The contribution of rare variants in common diseases is still largely unknown. However, recent advances in sequencing technologies enable the sequencing of genome, exome (the expressed portion of the genome) or targeted gene panels at low cost, and have opened the way for extensive searches for rare variants. The probability of discovery of variants influencing vulnerability to addiction is dependent on their effect size and frequency, and the number of individuals studied (Figure 113-20).

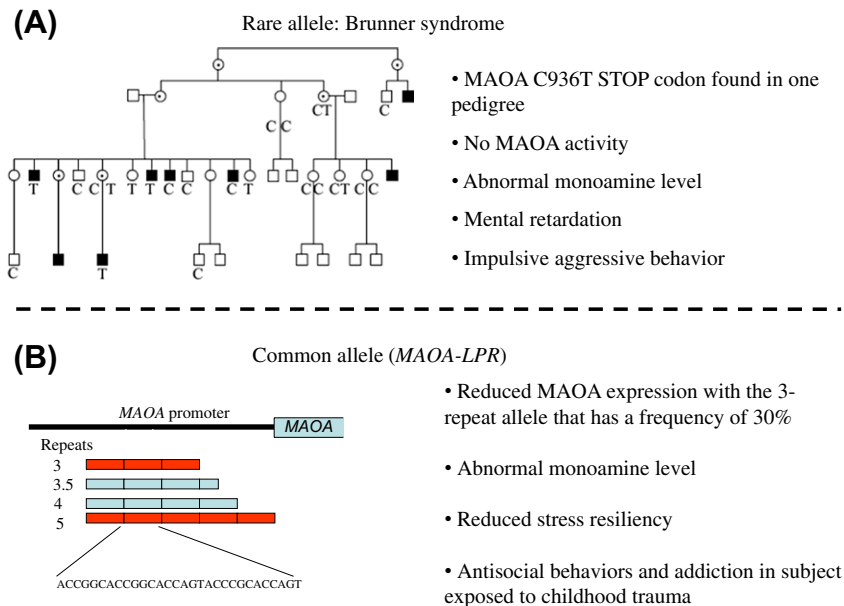
However, as illustrated by the discovery of the *MAOA* and *HTR2B* stop codons in impulsivity, the sequencing-based discovery of rare variants can be augmented by the study of genetically related individuals in families and founder populations and by the sequencing of individuals who are phenotypically extreme. It is also likely that statistical strategies will be further developed to pool effects of multiple rare variants at the same gene. Rare and uncommon functional variants may coexist at the same gene. The *MAOA* gene is an interesting example highly relevant to the addictions (Figure 113-21).



**FIGURE 113-20** Feasibility of identifying genetic variants by risk allele frequency and strength of genetic effect (odds ratio (OR)) in case/control samples. Effects of common variants (MAF > 5%) with low effect sizes (ORs approximately below 1.5) can be detected in very large samples. Effects of rare variants of large effect size may also be detected. Alternative strategies for rare variants include families, founder populations and “extreme” phenotypes. Of note, effects of rare variants with small effect size will be very difficult to identify. (*Adapted with modification from Manolio et al. (161)*).

MAOA is an X-linked gene encoding MAOA, a mitochondrial enzyme that metabolizes monoamine neurotransmitters including NE, DA and serotonin. MAOA knockout mice have higher levels of these neurotransmitters and manifest increased aggressive behavior and stress reactivity (150). In the human, different MAOA genetic variants impair MAOA activity to different degrees, and the reduction in enzyme activity appears to parallel the effect on behavior. In 1993, Brunner et al. (151) reported a Dutch family in which eight males were affected by a syndrome characterized by borderline mental retardation and impulsive behavior including impulsive aggression, arson, attempted rape, fighting, and exhibitionism (Figure 113-21A). The cause was a stop-codon variant in the eighth exon leading to complete and selective deficiency of MAOA activity, with an X-linked pattern of transmission. Discovery of this mutation led to attempts to identify it in other individuals with behavioral dyscontrol, including individuals accused of serious crimes. However, and despite intensive effort, the stop-codon variant was not found in other individuals, and thus represents an example of a rare, private allele. More recently, a common MAOA polymorphism influencing MAOA transcription was discovered (152). This locus, termed the MAOA-linked polymorphic region (MAOA-LPR), is a VNTR located approximately 1.2kb upstream from the MAOA start codon and within the gene’s transcriptional control region (152)

(Figure 113-21B). Alleles at this VNTR have a different number of tandem copies of a 30-bp sequence, with the three- and four-repeat alleles being by far the most common. Alleles with four repeats are transcribed more efficiently than alleles with three copies of the repeat, and therefore lead to higher MAOA enzyme activity (152). In a longitudinally studied cohort of boys, Caspi et al. (153) found that MAOA-LPR moderated the effect of childhood maltreatment on vulnerability to develop antisocial behavior. In this study maltreated boys with the low-activity genotype were more likely to develop antisocial problems later in life than boys with the high-activity genotype. Meta-analysis of several studies that represent attempts at replication revealed a significant pooled  $G \times E$  effect for MAOA and stress. A similar MAOA  $\times$  stress interaction appears to occur in women, although of course a much smaller percentage of women are homozygous for the low-expression allele (males being hemizygous for MAOA). In a sample of Native American women, the effect of childhood sexual abuse (frequent among women in this and other populations) on risk of developing alcoholism and APD was contingent upon MAOA-LPR genotype (44). Sexually abused women homozygous for the low-activity MAOA-LPR allele had high rates of both disorders, and heterozygous women displayed an intermediate risk pattern. However, in the absence of childhood sexual abuse, there was no relationship between MAOA genotype and these disorders.



**FIGURE 113-21** Rare and common variants within MAOA. (A) Pedigree of a Dutch family with eight males affected by Brunner syndrome, X-linked behavioral dyscontrol caused by a stop codon (C936T) in monoamine oxidase A that cause complete lack of MAOA activity (151). (B) The MAOA-linked polymorphic region (MAOA-LPR) is a common variable-number tandem repeat (VNTR) located approximately 1.2 kb upstream from the MAOA start codon and within the gene's transcriptional control region. Alleles at this VNTR have a different number of tandem copies of a 30-bp sequence, with the three- and four-repeat alleles being by far the most common. Alleles with three repeats are transcribed less efficiently, leading to lower MAOA enzyme activity.

MAOA G×E has also been studied in animal models. These are useful because of the ability to control stress exposures and many other environmental variables. In the Rhesus macaque (*Macaca mulatta*) early life stress exposure, particularly early separation from the mother, leads to dyscontrolled behavior and enhanced stress response later in life. The behaviors observed in the stressed animals include increased alcohol consumption, higher impulsive aggression, incompetent social behavior and serotonin dysfunction, and increased behavioral and endocrine responsivity to stress (for review see Reference (154)). Remarkably, an orthologous (same evolutionary origin and same function) VNTR polymorphism is also found in the promoter region of MAOA in the rhesus macaque. Also similar to the human, the lower activity allele predicts aggressive behavior in these animals, and the association is dependent on whether the monkey had been separated from its mother (155).

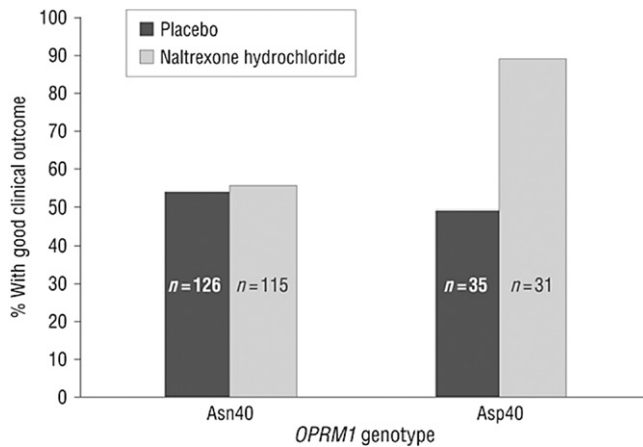
Impulsivity, “action without foresight,” is an important contributor to addictions of all types, and apparently there are many more rare and uncommon variants contributing to impulsivity that remain to be discovered. An example of a genetic variant leading to impulsivity, even severe impulsivity associated with criminal violence, is a stop codon in the HTR2B serotonin receptor (149). This stop codon, Q20\*, has an allele frequency of 1.2% in Finns (and >100,000 carry it), but is population specific. In that regard it can be regarded as relatively common, or rare, depending on whether one is taking a population-specific or world view. The variant was discovered by sequencing phenotypically extreme Finns. All

the sequenced probands were alcoholic violent offenders. Carriers of the stop codon who had committed violent crimes did so while inebriated with alcohol, and there were other risk factors—the stop codon alone was not sufficient. Mice with the *htr2b* gene knocked out are more novelty seeking and impulsive. For example, in a delay discounting task these *htr2b*–/– mice were less able to tolerate delay in order to receive a large reward. Just as genes such as NPY that alter resiliency play a role in addictions, the HTR2B stop codon and the variants at MAOA may be only the precursors of a larger pattern of genes influencing impulsivity.

### 113.4 TREATMENT OF ADDICTIONS

Treatment of addiction is enormously beneficial, in the same sense as it is also worthwhile to treat other diseases such as cancer, where success rates are substantially less than perfect, where there may be relapse, and where volition and lifestyle choices also play a powerful role in etiology and outcome. The maintenance of abstinence for multiyear periods has enormous benefits to the individual, family, and community. The complex nature of addictions makes their etiology difficult to comprehend, but paradoxically creates a broad range of opportunities for interventions that can be used often in combination. Effective interventions extend from the spiritual and religious to drug therapies that ease withdrawal, block the action of an addictive drug (antagonist therapies), substitute for the addictive agent (agonist therapies), or reduce symptoms such as anxiety and depression





**FIGURE 113-22** Genetic variation within the *OPRM1* gene and treatment response to naltrexone. The *OPRM1* Asp40 allele predicted good clinical outcome in patients treated with naltrexone (an antagonist of the mu-opioid receptor), shown in gray, as compared to patients treated with placebo (156).

that accompany long-term withdrawal and can trigger relapse. Lastly, the identification of genes altering the liability to addiction and ability to recover are a major focus for genetic studies, because these could provide new therapeutic targets and an ability to individualize treatment (so-called personalized medicine). One of the first examples of pharmacogenetic prediction of treatment response in the addictions is a common functional missense variant of the mu-opioid receptor (*OPRM1* Asn40Asp) (Figure 113-22)

In several studies, naltrexone, a mu-opioid receptor antagonist, was observed to augment abstinence and good therapeutic outcome in recovering alcoholics. Carriers of the Asp40 allele appear to be highly likely to show clinical improvement when treated with this drug (Figure 113-22), encouraging the idea that the treatment of this large clinical population can be better targeted (156,157).

### 113.5 CONCLUSION

Substance use disorders are common, chronic, and relapsing diseases that develop through a multiple-step process. The impact of addictions on morbidity and mortality is high worldwide. Twin studies have shown that addictions are among the most heritable of psychiatric diseases, with heritabilities ranging from 0.39 (hallucinogens) to 0.72 (cocaine). Twin studies indicate that genes influence each stage of psychoactive substance use, from initiation to addiction, although the genetic determinants may differ for each stage. Addictions are by definition the result of the gene  $\times$  environment interaction. These disorders, which are in part volitional, in part inborn, and in part determined by environmental experience, pose the full range of medical, genetic, policy, and moral challenges. Gene discovery is being facilitated by a variety of powerful approaches, but is

in its infancy. It is not surprising that the genes discovered so far act in a variety of ways: via altered metabolism of the drug (the alcohol metabolic gene variants for alcohol), altered function of the receptor of the drug (the nicotinic receptor for nicotine), and general mechanisms of addiction (genes such as MAOA and the serotonin transporter that modulate stress response, emotion and behavioral control).

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## Biographies

**Dr David Goldman** received his BS from Yale University in 1974. He received his MD degree in 1978 and completed residency training in psychiatry in 1979, both at the University of Texas Medical Branch. Dr Goldman joined the NIAAA in 1979 and has been Chief of the Laboratory of Neurogenetics since 1991. Throughout his career, Dr Goldman has focused on the identification of genetic factors responsible for inherited differences in behavior, and he has authored over 300 papers. This laboratory has recently completed projects including functional genomics studies identifying genetic variants that alter in vitro and in vivo response. These include multilevel studies on NPY, GCH1 and the serotonin transporter. His laboratory is currently exploring the genetics of alcoholism and related psychiatric diseases, and is well-known for his work identifying effects of functional genetic variants on intermediate phenotypes for complex behavioral diseases. Awards that Dr Goldman has received include NIMH Director's Honor Award for genetic factors that may alter susceptibility to schizophrenia, James Isaacson Research Award, International Society for Biological Research on Alcoholism, and the NIH Director's Award for the years 2002 and 2010. Dr Goldman is author of, "Our Genes, Our Choices."

**Dr Francesca Ducci** received her MD degree in 2001 and completed her residency training in 2004, both at the University of Pisa, Italy. Dr Ducci worked as postdoctoral visiting fellow from 2004 to 2008 at the Laboratory of Neurogenetics of NIAAA. In 2008 she moved to London where she is working as academic clinical lecturer at the Institute of Psychiatry, Kings College and at the Department of Epidemiology and Biostatistics of Imperial College. Her research interest is focused on the study of genetic and environmental factors that promote the early initiation of addictive substance use and the later development of Substance Use Disorders. Dr Ducci has authored more than 30 papers. These include studies on the effects of genes encoding for nicotine receptors, MAOA, and dopamine receptors on vulnerability to alcoholism and nicotine dependence. Awards that Dr Ducci has received include the American college of Neuropsychopharmacology travel award, the International Behavioral and Neural Genetics Society Outstanding Junior Faculty Travel Award and the Early Career Investigator award at the World Congress of Psychiatric Genetics. Dr Ducci is also working as specialist registrar in general adult psychiatry at the South West London and St George's Mental health trust.

**Dr Paola Landi** received her MD degree from the University of Pavia, Italy in 2008. She is currently attending her final year of residency training in psychiatry at the University of Pisa, Italy. She is also attending her last year at the school of Cognitive-Behavioral Psychotherapy in Milan. Her main research interest is to explore emotional processing in psychosis.

# CHAPTER

# 114

## Neural Tube Defects

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### 114.1 EMBRYOLOGY

Neurulation is the critical morphogenetic event occurring during the fourth week of human gestation, converting the previously developed neural plate into the ectoderm-covered neural tube that will eventually differentiate into the brain and spinal cord. Genetic regulation of the events involved in mammalian neural tube morphogenesis is a complicated process that involves a multitude of genes. These genes have vital functions in a range of biological activities that are thought to include signaling molecules, transcription proteins and factors, cytoskeletal and gap junction proteins, growth factors, and tumor suppressor genes (1–4). For decades, the process of neural tube closure has been oversimplified by authors who suggested that the neural plate is merely a sheet of cells that rolls into a hollow tube that closes continuously in a “zipperlike,” manner. This description leaves out the intricacies of cell migration and alterations in cellular morphology that underlie successful development and closure of the neural tube (5).

At the earliest stages of neural tube development the epithelia of the two-layered embryo is induced by signaling molecules to thicken and form a neural plate that is anchored over the notochord. The neural plate subsequently bends at the midline or medial hinge point (6). In a general sense, the lateral portions of the neural plate elevate, and the rostral regions of the neural folds bend inward at the dorsolateral hinge points (7–10). In addition to the bending motions, the movement of the neural folds is aided by the narrowing and lengthening of the cells of the neural plate. Two additional processes are fundamental to the creation and fusion of the neural tube. The first is

apical constriction, whereby columnar epithelial cells are converted into wedge-shaped cells by cytoskeletal elements (11–13). The second is convergent extension, whereby the flat sheet of epithelial cells is remodeled, via intercalation, into a longer, narrower configuration (14,15).

The neural tube, formed by the cell shape changes and movements described above, ultimately fuses at several discrete points. In the mouse, the neural folds begin to fuse at the extreme caudal portion of the hindbrain (actually in the midcervical portion of the embryo) at gestational day embryonic day (E) 7.0 (closure I). A second site at the forebrain–midbrain boundary begins to close at E8.0 (closure II). At E9.0, the neural plate at the rostralmost position of the developing embryo fuses to form the anterior forebrain (closure III), and by E9.5, the neural tube fuses in the midbrain–hindbrain junction (closure IV). Fusion from closure sites II and IV proceeds bidirectionally, until the two sites meet and complete the formation of the cranial neural tube. By E10.0, the neural tube is closed with the exception of the caudal end where the posterior neuropore becomes increasingly smaller and ultimately disappears.

The process of neural tube closure in humans is similar to that of the mouse. It has been proposed that human embryos have two successive sites of fusion of the neural folds, one in the rhombencephalic region and one in the prosencephalic region, adjacent to the chiasmatic plate (16). Fusion of the neural tube is initiated from site 1 at the level of somites 2 and 3, when 4–6 somitic pairs are present, and extends bidirectionally to involve the rhombencephalic and spinal levels, and ultimately the cervical, thoracic, mesencephalic, and prosencephalic levels (16). Fusion from site 2 is unidirectional (caudad



only). Accessory loci of fusion may also be present in older, Carnegie stage 10 embryos. However, the presence and location of such loci are inconsistent and their importance in neural tube closure remains speculative (16).

In humans, fusion of the neural tube initially involves surface ectoderm, followed by the neural ectoderm, and subsequently the interposition of mesenchyme, which is derived from the primitive streak/node and is responsible for elevation of the neural folds. The presence of mesenchyme in the crest at Stages 9 and 10 is likely to be a requirement for normal neurulation, and thus impairment of the primitive node and streak could lead to neural tube defects (NTDs). The observed fusions terminate in two neuropores, one rostral and one caudal. In humans, primary neural tube closure is thought to be complete when approximately 19–20 somite pairs have appeared (Carnegie stage 12) (16). The secondary neural tube develops between Carnegie stages 13–20, by a process of differentiation and canalization with the primary neural tube (17).

## 114.2 DEFINITION

Abnormalities of neural tube closure may result from alterations in any of the processes that are involved in the formation of the primary neural tube (e.g. elevation and bending, apical constriction, convergent extension). As these processes are under the control of a multitude of genes, the molecular basis of neural tube closure defects is likely to be quite heterogeneous. Indeed, in the mouse, defects of neural tube closure have been shown to result from genetic mutations affecting cell proliferation and apoptosis, convergent extension, elevation/apposition of the neural folds, and neural tube fusion (9,18–20).

Despite the complexity of the processes that are required for normal neural tube closure, the gross phenotypic consequences of abnormalities in these processes are relatively homogeneous. In general, failure of neural tube closure is associated with defects in the overlying bony structures (i.e. cranial vault and neural arches) such that the underlying neural tissue is exposed to the body surface. Consequently, defects of primary neural tube closure are often referred to as “open” NTDs. Further classifications of the open NTDs are based on the location and extent of the defect.

NTDs that are restricted to the cranial region of the neural tube are referred to as anencephaly. This condition is characterized by the absence of the cranial vault and absent or markedly diminished cerebral hemispheres. In addition, the cerebellum is usually absent and the brain stem may be hypoplastic. Anatomically, anencephaly is classified as holocrania if the defect involves the foramen magnum and merocrania if the foramen magnum is not involved. Most fetuses with anencephaly are aborted or stillborn; however, a small proportion of anencephalic infants is live born and can survive for short periods without significant medical support.

Neural tube closure defects that are restricted to the caudal portion of the neural tube are referred to generally as spina bifida or specifically as meningocele. This condition is associated with bony defects in the overlying neural arches, through which the meninges and spinal cord tissue are exposed to the body surface. Clinically, meningocele may be further characterized by the anatomic level and the extent of the lesion. The majority of fetuses with meningocele are live born and, with proper treatment, survival into the adult years is common. Failure of neural tube closure over the entire body axis, or craniorachischisis, is a lethal condition.

In addition to the open NTDs, there are also a number of “closed” or skin-covered conditions that involve the neural tube, including encephalocele, iniencephaly and lesions often referred to as occult spinal dysraphisms such as meningocele (that may be partially skin covered), spinal lipomas (lipomyelomeningocele or lipomeningocele), myelocystocele, split cord malformations, and various forms of sacral agenesis. In general, these conditions are not thought to result from defects in primary neural tube closure but may result from defects in secondary neural tube development. Nonetheless, there is evidence that open and closed NTDs may have some common etiological underpinnings. For example, families segregating both open and closed NTDs have been reported (21,22) and declines in the prevalence of encephalocele, as well as anencephaly and spina bifida, have been reported following folic acid fortification of the food supply in Chile (23–25).

## 114.3 PREVALENCE

As a group, the open NTDs (anencephaly, meningocele or spina bifida, and craniorachischisis) are relatively common malformations. However, the true incidence of these conditions is unknown, since only a proportion of affected conceptuses survive to a stage where their NTD is likely to be recognized. The prevalence of these conditions at birth is also difficult to estimate accurately, since some affected fetuses are identified and electively terminated in the prenatal period (26–31) and others are stillborn. Hence, estimates of the birth prevalence for these conditions may be influenced by the ascertainment methods (e.g. active vs passive surveillance), source documents (e.g. birth certificates vs chart review), and inclusion criteria (e.g. live born, stillborn, elective terminations) (32–35).

Despite differences between studies that make it difficult to directly compare estimates, several characteristic features of the prevalence of NTDs have been identified. In general, the prevalence of spina bifida is higher than that of anencephaly and the prevalence of craniorachischisis is much less than that of either spina bifida or anencephaly. In addition, the prevalence of NTDs is higher in females than in males (36) and varies by race/ethnicity (37), across regions, and over time (38,39). Moreover,

data from countries that have implemented mandatory folic acid food fortification programs indicate that the prevalence of NTDs is reduced by approximately 50% following such fortification (40).

## 114.4 RISK FACTORS

The open NTDs are recognized as being etiologically heterogeneous. A small proportion of affected individuals have an associated chromosomal or Mendelian malformation syndrome (41) and there are rare families in which NTDs segregate in patterns consistent with X-linked or autosomal recessive inheritance. In addition, a small proportion of cases can be linked to an established risk factor. The most notable of these factors are maternal pregestational insulin-dependent diabetes and maternal use of medications, in particular, specific anti-convulsant drugs.

### 114.4.1 Maternal, Insulin-Dependent Diabetes

The teratogenic potential of maternal pregestational diabetes is well established and includes a 2- to 10-fold increase in the risk of central nervous system malformations (including NTDs) among the offspring of affected women, relative to the general population (42). Moreover, the level of maternal periconceptional glycemic control has been associated with the risk of NTDs in the offspring of diabetic women (42–44).

As human embryos lack pancreatic function until after the seventh week of gestation, the teratogenic effect of maternal diabetes may be due to embryonic exposure to elevated glucose concentrations. In rat embryos, elevated glucose concentrations are known to induce oxidative stress and deplete inositol stores (45) and inositol supplementation has been found to be protective against posterior NTDs in a murine model system (46). In addition, inositol metabolism is impaired in three NTD mouse mutants (47). Although it remains to be seen whether inositol can confer protection against NTDs in humans, an initial investigation provided no strong evidence of an association between maternal myoinositol intake and risk of NTDs in humans (48).

Animal models have suggested that the association between maternal diabetes and NTDs may be mediated by the paired box (Pax) family of transcription factors (49,50). This family of genes plays critical roles in development. In humans, mutations in PAX3, which is expressed in the neuroepithelium, neural crest, and somatic mesoderm, are associated with Waardenburg syndrome, a condition that includes an increased risk of NTDs. Moreover, mouse strains carrying null mutations for the Pax-3 gene develop NTDs similar to those that occur in the embryos of diabetic mice, and diabetic mice heterozygous for a Pax-3 mutation demonstrate elevated rates of NTD-affected pregnancies compared to diabetic

mice lacking this mutation (51). Furthermore, when hyperglycemia is artificially induced in dams of inbred strains, Pax-3 expression is reduced, leading to neuroepithelial apoptosis and ultimately NTDs. Similar results are obtained in vitro through treatment of explants with glucose (49,52–54). Although an association between PAX3 and nonsyndromic NTDs has not been found in human studies (55–59), this association has not been fully investigated.

Rappaport and Smith (60) recently attributed 70–90% of the risks for chronic disease including adverse pregnancy outcomes to environmental factors. They brought to light the importance of understanding that “toxic effects are mediated through chemicals that alter critical molecules, cells, and physiological processes inside the body.” This would include the production of inflammatory molecules, reactive oxidative compounds, lipid peroxidation molecules and others (60). Environmental exposures including small particle air pollution and heavy metals such as arsenic and cadmium pose real threats to human health via their direct absorption into systemic circulation and their linkage to the pathogenesis of metabolic diseases, including diabetes (61–63). Xu and coworkers (64) demonstrated in genetically sensitive P47Phox mice that chronic exposure to PM2.5 resulted in heightened inflammatory responses and the formation of reactive oxidative species. Environmental arsenate exposure has been shown to directly affect both the maternal and embryonic glucose levels in an experimental mouse model system, paralleling the situation in maternal type 2 diabetes (39). The maternally administered arsenate crossed the placenta and produced a well-described pattern of fetal malformations that are primarily characterized by NTDs (38,65). Work by Salbaum and Kappen (66) further demonstrated that maternal diabetes alters the regulation of critical embryonic genes. This dysregulation of embryonic genes may include epigenomic changes via deregulated expression of chromatin-modifying factors. Using a mouse model of streptozotocin-induced diabetes, the prevalence of NTDs was tripled when the diabetic mice were maintained on a low-folate diet (67).

### 114.4.2 Maternal Use of Medications

The anticonvulsant drug valproic acid (VPA; Depakene, Abbott Laboratories) has been directly implicated as a potent neural tube teratogen. Among women taking VPA (alone or in combination with other anticonvulsant medications) the risk of having a pregnancy affected with spina bifida may be as high as 1–2%, whereas the risk of anencephaly does not appear to be increased (68–72). In humans, in utero VPA exposure has also been associated with craniofacial, cardiovascular, and skeletal defects (73–77). However, only a small percentage of VPA-exposed fetuses present with spina bifida or other malformations, suggesting that there may be genetically mediated differences in sensitivity to VPA teratogenicity.

Studies in murine model systems have revealed strain differences in NTD susceptibility to single maternal intraperitoneal (IP) injections of 600 mg/kg VPA on E8.5 (78). To identify the chromosomal locus associated with sensitivity to VPA-induced NTDs, a genome-wide linkage analysis was performed using SWV and C57 fetuses. The result of these analyses suggested that an autosomal recessive locus, plus a gender-related effect or an overall X-chromosome effect, determine sensitivity to VPA-induced exencephaly. Subsequent analyses identified a major locus for VPA-induced exencephaly linked to D7Mit285 ( $p < 2 \times 10^{-6}$ ) (79). This marker showed the most significant linkage in the secondary screen ( $p < 0.005$ ); therefore, additional BC1 samples were genotyped. This analysis of the BC1 panel (131 NTD pups total) yielded a probability of  $p < 2 \times 10^{-6}$ , greatly exceeding the threshold for highly significant linkage. A total of 13 additional markers flanking D7Mit285 were genotyped, which confirmed this observation and further refined the mapping of the sensitivity trait. Examination of recombination events showed that this gene is located between D7Mit285 and D7Mit101, which are 3.3 Mb apart. Within this region is the Acyl-CoA synthetase medium-chain (ACSM) gene family, which plays a role in conjugating VPA with coenzyme A before the molecule enters  $\beta$ -oxidation. Given this important biochemical role and the strong linkage data, the ACSM genes are leading candidates for a significant role regulating susceptibility to VPA-induced NTDs.

There are several other possible theories to explain a genetically regulated mechanism for susceptibility to VPA-induced NTDs, including the inhibition of folate metabolism by VPA (80,81). Specifically, VPA interferes with selected steps in the folate pathway, which may decrease the rate of methylation of essential, developmentally regulated genes during critical periods of embryogenesis. This could significantly enhance the sensitivity of the embryos to specific malformations. However, definitive interactions between folate metabolism, VPA therapy, and gene regulation have yet to be demonstrated.

Valproic acid may also influence neural tube development via its action as a potent inhibitor of histone deacetylases (HDACs) (82–85). Class I and II HDACs are involved in histone modification and therefore play a role in the regulation of gene expression. Specifically, histone acetylation is often related to transcriptional activation, whereas HDACs are capable of down regulating gene expression (86). The inhibition of HDACs by VPA has been associated with teratogenicity in a *Xenopus* model system (87). Thus, it is possible that post-translational gene modification is compromised by VPA exposure, which inhibits HDACs and limits the availability of folate molecules (87) and thereby increases the risk of NTDs. VPA inhibits HDAC activity most likely by binding to the catalytic center and blocking substrate access and causing hyperacetylation of the N-terminal

tails of histones H3 and H4 in vitro and in vivo. VPA serves to relieve the repression of transcription factors such as PPAR $\delta$ , which recruit HDACs. The inhibitory effect of VPA on HDACs was first discovered in HeLa cells (87). Because HDACs play important roles in transcriptional regulation and pathogenesis of cancer, VPA as an HDAC inhibitor, is considered a candidate drug for cancer therapeutics (82,87,88). In several cell lines, a therapeutic dose of VPA reduces proliferation, morphological changes, and marker gene expression, similar to the HDAC inhibitor TSA (5).

The literature concerning the teratogenic potential of the frontline antiepileptic drug carbamazepine (CBZ) is significantly more limited than the literature that exists for VPA (70,89–91). Nonetheless, it is clear that the risk for NTDs in infants exposed in utero to CBZ approaches that of VPA. Consistent with earlier reports, Hernandez-Diaz and colleagues (92) observed a seven-fold increased risk of NTDs in the offspring of women who used CBZ during pregnancy, compared with the offspring of women who did not. Moreover, based on a meta-analysis of the existing literature there was evidence (based on 1255 prospectively ascertained gestations exposed to CBZ) of a significantly increased risk of congenital malformations, primarily NTDs, amongst CBZ-exposed infants. No additional increase in risk was observed among the offspring of women who took CBZ and one additional anticonvulsant medication. However, when CBZ exposure occurred in the presence of two or more additional anticonvulsants, the risk to the exposed infant was significantly elevated relative to that of infants exposed to CBZ alone (58).

Similar to VPA, CBZ and its major metabolite, CBZ-10,11-epoxide, are both able to inhibit HDACs (86). Thus, it is possible that in utero exposure to CBZ inhibits embryonic HDACs, resulting in the repression of critical genes involved in neural tube closure. It has also been suggested that arene oxide intermediates are responsible for CBZ's teratogenicity. It is possible that the binding of a CBZ electrophile to a critical, developmentally regulated protein could compromise normal embryogenesis (93). There is an exhaustive literature suggesting that an inherited defect in epoxide hydrolase activity can place selected mother-offspring pairs at increased risk for antiepileptic drug-induced birth defects (94). Unfortunately, efforts to positively associate epoxide hydrolase activity with adverse CBZ pregnancy outcomes have not been reported to date.

It is also possible that, like VPA, CBZ influences the risk of NTDs via its role as a folic acid antagonist. Other folic acid antagonists, including aminopterin and methotrexate, have also been associated with an increased risk of NTDs in prenatally exposed infants. Moreover, relative to unexposed infants, infants who were prenatally exposed to any member of the group of folic acid antagonists including CBZ, phenobarbital, phenytoin, primidone, sulfasalazine, triamterene, and trimethoprim

were found to be at increased risk of NTDs, with the highest risks being observed for infants exposed to CBZ (OR = 6.9, 95% CI 1.9, 25.7) and trimethoprim (OR = 4.8, 95% CI 1.5, 16.1) (92). Furthermore, both dihydrofolate reductase inhibitors and drugs that influence folate concentrations through pharmacokinetic mechanisms appear to be associated with increased risk of NTDs (92,95).

Maternal use of other drugs/categories of drugs including opiod analgesics (96), selective serotonin reuptake inhibitors (97), sulfonamides (98) and nitrosatable drugs (99) has also been associated with the risk of NTDs in offspring. However, the reported associations tend to be weaker than those reported for VPA and CBZ and either many have not been the subject of replication or replication studies have provided conflicting results.

Although NTDs can occur as part of chromosomal or single gene disorders or as a result of teratogenic exposures, a specific causative agent cannot be identified for the majority of affected individuals. NTDs that cannot be linked to a specific cause are often referred to as being nonsyndromic and are thought to result from the cumulative effects of multiple genetic/environmental risk factors. Epidemiological investigations have implicated a relatively large number of variables as potential risk factors for nonsyndromic NTDs. Although many of the reported associations have been weak and difficult to replicate, there are several variables for which there is relatively strong evidence of an association with NTDs. In general, it appears that anencephaly and spina bifida share some, but not all, of these risk factors (100).

### 114.4.3 Family History

A family history of NTDs is one of the strongest risk factors for these conditions. The risk of NTDs in the siblings of affected individuals ranges from 3% to 8% and is consistently higher than that of the general population. An increase in the risk of NTDs has also been reported for second-degree and third-degree relatives of affected individuals.

### 114.4.4 Nutrition

Maternal intake of natural folate, or its synthetic form, folic acid, is the most widely recognized nutritional determinant of the risk of NTDs in offspring. Case-control studies, randomized clinical trials, and community-based interventions of vitamin supplements have demonstrated that failure to consume adequate amounts of folic acid or folic-acid-containing multivitamins increases the risk of having an affected child two to eightfold. Further evidence that maternal intake of folate/folic acid is causally related to NTD risk is provided by studies that demonstrate a dose-response relationship between the risk of having a pregnancy affected by an NTD and maternal folate intake/status

(101–103) and by the observed decrease in the prevalence of NTDs following mandatory folic acid food fortification programs in several countries (24,104–116). In the United States, estimates of the birth prevalence of spina bifida and anencephaly (including affected fetuses identified in the prenatal period) following mandatory folic acid food fortification (1999–2000) were 4.1 per 10,000 and 3.5 per 10,000, respectively (113). These are likely to represent some of the lowest NTD prevalence estimates for this period worldwide. At the other end of the spectrum, estimates of NTD prevalence (anencephaly and spina bifida combined) as high as 20 per 1000 births (117) have been reported in some regions of China.

The mechanism underlying the association between NTDs and folate has not been established. However, folate participates in two metabolic pathways that, if disrupted, could have an adverse effect on the development of the embryo. One of these pathways is important for nucleic acid synthesis, and the other for a vast range of methylation reactions. Disruptions in folate metabolism can also result in elevated homocysteine levels, which are teratogenic to the neural tube in some species (118).

There is also evidence that the risk of NTDs in offspring may be related to other aspects of maternal diet including vitamin B12 (119–123), choline (124,125) and zinc (126,127) levels as well as maternal glycemic index (44,101,128,129).

### 114.4.5 Obesity

The risk of having a child with an NTD increases with increasing maternal body mass index (BMI). Women in the highest BMI categories (usually defined as a prepregnancy BMI greater than 29 kg/m<sup>2</sup>) have a 1.5-fold–3.5-fold higher risk of having an NTD-affected child than women who have lower BMIs (130–138). It has been suggested that the increased risk of NTDs in the offspring of obese women may be attributable to hyperinsulinemia (130). This could provide a common explanation for the associations between NTD risk and maternal obesity and maternal pregestational diabetes, since hyperinsulinemia may coexist with both conditions.

The majority of studies linking NTD risk to maternal BMI have been based on data that were largely derived from Caucasian women of European ancestry. It is therefore of note that in a relatively large study conducted in China (*N*=511 NTD cases), the risk of NTDs was not increased in the offspring of obese women and was significantly lower in the offspring of overweight women as compared to the offspring of normal weight (139). As this study was conducted in a region of China that has a very high prevalence of NTDs, these findings could reflect differences in the underlying etiology of NTDs in this high-risk population (as compared to lower risk populations) or differences in the characteristics of obese women (e.g. nutritional status) across populations.



### 114.4.6 Hyperthermia

Clinical and epidemiological studies have shown an increased risk for NTDs, in particular anencephaly, in infants whose mothers were exposed to hyperthermia during the first trimester of pregnancy (37,140–146). As many as 10% of NTD-affected live births, and 14% of NTD-affected abortuses, may have been exposed to teratogenic levels of maternal hyperthermia during early pregnancy (144). In a study of 23,419 women, it was found that early hyperthermic exposure (i.e. sauna, hot tub, fever, or electric blanket) roughly doubled the risk of an NTD-complicated pregnancy, while exposure to more than one of these factors increased this risk by six-fold (143). Interestingly, among women who reported first trimester infection and fever in the National Birth Defects Prevention Study, the risk of anencephaly or craniorachischisis was significantly lower among the offspring of women who took acetaminophen as compared to those who did not (147).

In humans, the greatest hyperthermic risk is generally considered to be from maternal febrile illness. In a large, retrospective, population-based case-control study, maternal fever or febrile illness in the first trimester was associated with a twofold increased risk for an NTD pregnancy (148). However, the possibility that the increased risk was due to reporting bias cannot be completely discounted. Although the effects of hyperthermia are difficult to disentangle from those of the underlying illness (142,145,146), data from experimental animals support a direct relationship between maternal hyperthermia and NTD risk (149–152).

Evidence from animal model studies suggests that induction of NTDs through elevation of the maternal core temperature is an embryonic, not a maternal effect. This has been investigated by completely removing the rat embryos from maternal influence and exposing the explants to mild hyperthermic insult during the onset of neural tube closure. Embryos thus exposed displayed head and somite malformations, with nearly half of the exposed embryos being microcephalic (153). It appears that temperature and duration of exposure act together in a dose-response manner to induce NTDs in experimental animals (154). This provides an explanation for the failure of saunas to induce elevated rates of NTDs in Finnish women, who regularly indulge in saunas while pregnant (155). Consistent with this explanation, subjects in another study could not tolerate hot tub exposure long enough to elevate their core temperature to 38.9°C (156).

Interestingly, there are apparent similarities between VPA-induced and hyperthermia-induced exencephaly in animal models. Phenotypically, both teratogens are primarily associated with exencephaly, an anterior NTD, but may induce other forms of birth defects and homeotic transformations when the treatment regimen is slightly altered (117,150). In addition, the inbred mouse strain SWV/Fnn is highly susceptible to both

VPA-induced exencephaly and NTDs induced by maternal hyperthermia (11,19,157,158). Moreover, with both these teratogens, the embryos affected with NTDs have an insufficient proliferation of cells in the neural tube due to an embryonic cell cycle arrest (159), which may be accompanied by cell death (or apoptosis). These similarities suggest the possibility of a shared mechanism(s) underlying the teratogenic effects of VPA and hyperthermia. It is not known whether such mechanisms involve a common stress response reaction or a specific molecular pathway. However, given the dissimilarity of the physical nature between hyperthermia and VPA, the involvement of a general stress response reaction appears to be plausible.

Cells and tissues of essentially all eukaryotes respond the same way to a variety of stressful situations. Owing to the activation of heat-shock proteins following several types of environmental insult (including hyperthermia), an embryonic stress hypothesis has emerged (160). The hypothesis maintains that the induction of the heat-shock response in the mammalian embryo during the critical period of organogenesis can alter the established program of activation and inactivation of genes essential for normal fetal development, thus leading to anatomic malformations. However, direct genetic evidence for such a highly speculative mechanism of teratogenesis is lacking (79).

### 114.4.7 Reproductive Technologies

An association between maternal use of fertility drugs, in particular clomiphene citrate, and an increase in the risk of NTDs in offspring has been suggested. However, based on a pooled analysis of 10 epidemiological studies, the summary prevalence ratio for the association between NTD risk and clomiphene citrate was 1.08 (95% CI 0.76, 1.51) indicating that clomiphene citrate either does not elevate or only very modestly elevates the risk of NTD in the offspring of women who use this drug (161). More recently, it has been suggested that the risk of NTDs may only be increased in women who develop follicular cysts following treatment with clomiphene (162). However, this association remains to be confirmed in independent investigations.

It has also been suggested that there is an association between assisted reproductive technologies (i.e. in vitro fertilization/intracytoplasmic sperm injection) and the risk of congenital malformations, including NTDs (163,164). Furthermore, data from Sweden indicate that the risk of NTDs is three- to fourfold high in offspring conceived following in vitro fertilization as compared to the general population (165,166). However, a study of both in vitro fertilization and intracytoplasmic sperm injection found no evidence of an increased risk of NTDs (167). It is possible that at least some of the differences observed between studies are due to the inclusion of different assisted reproductive technologies that may

be associated with different risks for malformations in offspring (168).

#### 114.4.8 Autoantibodies to Folate Receptors

Evidence has emerged that maternal immunological responses can have substantive impact on embryonic development. When antibodies to rat placenta, kidney, heart, and other tissues are generated and administered to pregnant rats, they bind to the yolk sac and embryonic tissue and are believed to contribute to congenital abnormalities and embryonic death (169,170). It has been postulated that the antibodies binding to the yolk sac impair the delivery of nutrients to the embryo (170, 171). Further studies show that monospecific antibodies to folate receptors, also known as folate binding proteins, can bind in vivo to the membrane-bound receptors on embryonic and extraembryonic tissues, inducing resorptions and developmental defects by blocking cellular uptake of folate (172,173). Additionally, large doses of antiserum may cause immune-mediated damage to embryonic tissues (173).

Rothenberg and coworkers (171) reported the presence of autoantibodies to the folate receptor in 75% (9/12) of mothers who had given birth to NTD-affected infants, but in only 10% of mothers of nonmalformed infants. This study suggests that maternal autoantibodies, which bind to the folate receptor, may block the intracellular uptake of folate by target epithelial cells and cause NTDs. This observation could explain the beneficial effect of periconceptional folate supplementation. This finding was subsequently replicated in larger studies using midgestational serum samples (29 mothers of NTD cases and 76 control mothers) in California (174) and Norway (175), but was not replicated in the largest study (approximately 100 case and control women) that has been conducted (121). Clearly, there is still much to be learned about the role of immune factors in susceptibility to NTDs, including the role of maternal autoantibodies that block folate transport during development.

#### 114.4.9 Other Maternal Risk Factors

Other maternal factors of interest, which require additional research in order to establish their association with NTD risk, include diarrhea (176), dieting behavior (177), caffeine consumption (166,178), emotional stress (179), physical activity (180), social networks (181) and education (182).

#### 114.4.10 Environmental Exposures

While many environmental agents are suspected of possessing a teratogenic potential based on data from studies in animals, relatively few have been confirmed as human teratogens. This may be due to several factors, including

differences in dose: laboratory studies performed on mice generally utilize doses that are much higher than encountered by humans; exposure assessment: environmental exposures are more difficult to quantify in studies involving humans than in mice kept under laboratory conditions; and degree of experimental control: genetic and environmental differences that may influence disease susceptibility are more easily controlled in studies of laboratory animals than in studies of human populations. However, several environmental exposures have emerged as potential risk factors for NTDs.

Fumonisin is a family of mycotoxins produced by *Fusarium verticilloides*, which is commonly found as a contaminant of corn. The major isoform, fumonisin B1 (FB1), causes liver and kidney cancer in rodents (158) and may be a human carcinogen (183). In addition, FB1 appears to be teratogenic in laboratory animals (184–188) and is a suspected risk factor for human NTDs in populations consuming large amounts of fumonisin-contaminated corn products (189–191). Data from a study conducted along the Texas–Mexico border indicated that the risk of NTDs was increased in the offspring of women whose consumption of corn tortillas was moderate (301–400 tortillas during the first trimester) as compared to the offspring of women with low consumption (<100 tortillas) during the first trimester (OR=2.4, 95% CI 1.1, 5.3). Furthermore, in this same population, the maternal postpartum sphinganine:spingosine ratio (a biomarker of fumonisin exposure) was directly related to the risk of NTDs in offspring (192,193).

It has been proposed that FB1 may induce NTDs as a result of its ability to interfere with folate transport (189). Specifically, receptor-mediated folate uptake was found to be reduced by up to 50% in Caco-2 cells pretreated with fumonisin (194). Fumonisin disrupts sphingolipid metabolism by inhibiting ceramide synthases, which are critical enzymes involved in the de novo synthesis of ceramide from fatty acids and sphingoid bases (192,195,196). As this pathway is required for glycosphosphatidylinositol (GPI)-anchoring, fumonisin disrupts GPI proteins such as the folate receptors. This in turn disrupts the receptor's endocytic recycling (197) and the amount of the receptor available for substrate transport (189). Fumonisin's role in the disruption of folate transport and lipid rafts is further supported by rescue of fumonisin-exposed dams with gangliosides (10mg/kg) the day before, on, and the day following exposure (189). Thus, the disruption of sphingolipid-dependent processes appears to mediate fumonisin's wide range of biological functions, which may contribute to an increased risk for NTDs (185,188,195,198).

In several animal models, maternal oral fumonisin exposures have been largely unsuccessful in producing NTDs in offspring (33,199,200). However, fumonisin does produce NTDs and significant growth retardation in developing murine explants (36). In explants, the risk of exencephaly increases with increasing dose of fumonisin,

but the effects of fumonisin can be partially mitigated by the addition of folate to the growth medium (187). Murine studies also demonstrate that maternal IP dosing with fumonisin during neurulation induces exencephaly in LM/Bc/Fnn mice and that this effect of fumonisin can also be reduced by folate supplementation (185,186).

Exposures to metals may also influence the risk of NTDs. Periconceptional exposure to cadmium (201) and lithium carbonate (202) has been associated with NTDs in animal models. However, human studies have shown no increased NTD risk from exposure to tap water contaminated with a variety of metals including lead, calcium, magnesium, copper, lithium, zinc, nickel, selenium, mercury, chromium, silver, cobalt, cadmium, and molybdenum (203). Furthermore, studies of the association between zinc and NTDs in humans have provided conflicting results (24,79,126,127,204–210).

There are also data indicating a relationship between folate and iron. Specifically, iron can modulate the availability of folate, leading to symptoms of folate deficiency despite adequate folate intake and extracellular folate concentrations (211). Investigations have shed light on this relationship by demonstrating that the homocysteine remethylation pathway is regulated in part by serine hydroxymethyltransferase, the product of the SHMT gene that is regulated by the heavy-chain subunit of ferritin (212). It has also been suggested that lead may induce NTDs by reducing bioavailability of dietary zinc (181,207,210,213).

Inorganic arsenic (Asi) is a natural element found in the environment (drinking water, air, food) as arsenate (pentavalent, As (V)) or arsenite (trivalent, As (III)). Arsenic is used as a pesticide, herbicide, and even as a medication. An association between human exposure to Asi and various forms of cancer is well established (214), and it has been suspected that Asi is also a human teratogen (215). However, a conclusive association between Asi exposure and the occurrence of human malformations has not been established (216,217). Numerous animal studies have examined the role of arsenic-induced teratogenicity in various species using different chemical forms, routes of administration, and experimental designs. Although Asi was previously found to be teratogenic only when administered by injection and not as a result of oral or inhalation exposure, this is no longer the case. Hill and colleagues (38) orally intubated pregnant mice on embryonic day (E) 7.5 and E8.5 with 4.8, 9.6, or 14.4 mg/kg sodium arsenate. This treatment regimen produced a specific pattern of malformations, including neural tube and craniofacial defects. In addition, these effects were observed in multiple species with a typical dose response, indicating that Asi has a direct teratogenic effect (215,218,219).

Other environmental exposures of interest as potential NTD risk factors include organic solvents (220–222), air pollutants and in particular benzene (190,223), chlorination disinfection by-products in drinking water

(176,224–226), electromagnetic fields (227,228), proximity to hazardous waste sites (229,230) and pesticides (231–233).

#### 114.4.11 Occupational Exposures

There is considerable interest in the relationship between parental (maternal and paternal) occupational exposures and the risk of congenital malformations in offspring. Although paternal exposures are less commonly evaluated than maternal exposures, paternal exposures could influence the risk of birth defects in offspring via a direct effect on sperm DNA (mutations or chromosomal alterations), indirectly via transmission of agents in seminal fluid, or maternal exposure to agents brought home by father.

Paternal exposure to the herbicide Agent Orange has been associated with an increased risk of NTD in offspring, but this association is based on a very small number of affected offspring (234). A meta-analysis of relevant studies published between 1966 and 2008 indicates that, compared to the offspring of unexposed men, the risk of NTDs in the offspring of men exposed to Agent Orange is increased by approximately twofold (relative risk = 2.02, 95% CI: 1.48, 2.74) (235).

Other paternal occupations that have been associated with an increased risk of NTDs in offspring include technical, sales and administration; farming, forestry and fishing; and fabricators and laborers (risk was assessed relative to paternal managerial and professional occupations) (236) as well as occupations involving exposure to organic solvents (218,237). Additional studies are required to confirm these associations and determine the specific exposures that are associated with increased risk of NTDs in offspring.

Maternal occupation exposure to solvents has also been associated with an increase risk of NTD in the offspring of Mexican-American women (221,237). In addition, there is evidence that the risk of NTDs and other birth defects may be increased in women with occupational exposure to glycol esters (221), although several negative studies have also been published (7,238). Other maternal occupations that have been associated with an increased risk of NTDs in offspring include, but are not limited to, health care and cleaning (221).

#### 114.4.12 Genetics

Studies of the recurrence patterns in families ascertained through an individual affected with anencephaly or spina bifida indicate that these two conditions tend to cosegregate within families and are therefore, likely to share a common etiology. In addition, family studies have demonstrated that the relatives of an affected individual are at increased risk of having an NTD compared with the general population. The relative risk ratio (i.e. risk to relative vs. risk in general population) for the sib of an affected

individual is 30–50. This is much lower than the relative risks exhibited by Mendelian conditions and higher than expected for conditions that are determined solely by environmental factors (147). Hence, it is commonly believed that the risk of NTDs is determined by multiple risk factors, including both genes and environmental agents.

Epidemiological studies of potential environmental risk factors for NTDs have a relatively long history and have identified several factors (reviewed previously) that appear to be related to NTD risk. In contrast, the study of genetic risk factors for NTDs has a relative short history. Although traditional genetic linkage approaches (e.g. LOD score and affected relative pair analyses) have been applied to the study of other conditions since the 1980s, the data required by such approaches (i.e. DNA from multiple affected individuals within families) are largely unavailable for NTDs. Consequently, studies designed to identify genes that influence the risk of NTDs did not become feasible until the 1990s, when the Human Genome Project began to provide new information regarding the variability within the human genome. Such advances in our understanding of the human genome have provided new opportunities for studying the genetic contribution to conditions such as NTDs (e.g. case-control and family-based genetic association studies of non-Mendelian conditions).

The majority of the published genetic association studies of NTDs have focused on one or a few candidate genes that are involved in folic acid transport and metabolism, since maternal intake of folate/folic acid in the periconceptional period is significantly related to the risk of NTDs in offspring. Variants of several folate-related genes, including 5,10 methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), and methionine synthase reductase (MTRR), have been found to be associated with the risk of NTDs in one or more studies. These studies have provided evidence that both the maternal and embryonic genotypes are involved in determining NTD risk. However, most of the reported associations have been weak and have not been consistently replicated (239).

Three studies have undertaken more comprehensive evaluations of the association between NTDs and variants of folate-related genes (124,240,241). These studies differed both in the genes/SNPs that were included as well as the number of SNPs evaluated per gene, including between 46 and 118 SNPs in 11–45 genes. However, in two studies there was evidence for associations between NTDs and variants of CBS, DHFR, MTHFR and TYMS (Shaw, Martinez), which adds to the evidence suggesting that these genes are involved in the etiology of NTDs. Moreover, the study by Franke et al. implicated CUBN as a potential NTD risk factor and provided some evidence for an association of NTDs with SLC19A1, which has been implicated as an NTD risk factor in other studies (242).

Other groups of candidate genes have also been considered as potential risk factors for NTDs. Candidate

genes have been selected based on known risk factors for NTDs; for example, genes that predispose to the development of maternal conditions (e.g. diabetes, obesity) that are associated with an increased risk of NTDs (48,243), genes that are critical during embryogenesis (e.g. DNA repair genes) (244,245), genes involved in the metabolism of known teratogens (e.g. valproic acid), and genes involved in the metabolism of compounds (e.g. cholesterol) that are required for embryonic development (246). The human orthologs of genes, which when mutated or knocked out in the mouse are associated with NTDs, are also good candidate genes for NTDs in humans (247,248). Although candidate gene studies of non-folate-related candidate genes also have not identified any genes or genetic variants that are consistently associated with NTD risk, none of the genes that have been evaluated as NTD risk factors (including the folate-related candidate genes) have been studied in sufficient detail to warrant their exclusion as candidates.

In addition to candidate gene association studies, a genome-wide linkage analysis of NTDs has been completed (21). Through a collaborative effort, 44 families with two or more individuals affected with a defect of the neural tube were assembled for the purpose of a genome-wide linkage analysis. Owing to the rarity of such families, affected individuals had a broad spectrum of conditions including anencephaly, myelomeningocele, lipomyelomeningocele, encephalocele, and craniorachischisis. When a narrow phenotypic definition was applied (i.e. lumbosacral myelomeningocele), the sample was reduced to 17 families. A microsatellite-based genome screen in these families identified regions of interest on chromosomes 7 (attributable to a single large pedigree) and 10. The results of these analyses provide positional data for the prioritization of candidate genes in future studies.

The relative lack of success in identifying genetic variants that are related to the risk of NTDs in humans is somewhat surprising given the large number of mouse models that provide strong evidence of the genetic contribution of NTD. To date, over 240 gene mutations have been associated with NTD in mouse lines (47), yet none of the 155 genes implicated by these models has been identified as a determinant of NTD risk in humans. In general, the lack of success in past studies in humans is likely to reflect a number of factors including issues of sample size and power, etiologic heterogeneity and failure to adequately capture the etiological complexities that are likely to underlie human NTDs. These complexities are likely to include gene-gene and gene-environment interactions, maternal and the embryonic genotypic effects, the involvement of both rare and common variants, and consideration of pathways (as opposed to individual genes).

As an example of the need to consider pathways and rare variants, consider the following grouping of genes, which is suggested by mouse models of NTDs: Cobl, Vangl, Celsr, Scrb, Ptk7, Fuz, Fzd, and Dvl. These genes



are mechanistically connected through the planar cell polarity pathway (PCP) of noncanonical Wnt signaling and include both receptors and downstream effector molecules that play critical roles in determining cell shape and position during neural tube closure (248). In mouse embryos, defective PCP genes are associated with abnormal convergent extension (14,84,249). In humans, sequencing of PCP genes has led to the identification of rare mutations in *VANGL* and *VANGL1* in individuals with NTDs (250–252). These findings demonstrate that DNA resequencing of a reasonable number of subjects can identify important novel SNPs that are highly relevant to disease risk and that the interrogation of all variants in the protein coding regions of candidate genes in order to establish a clinically useful panel of predictive markers is important.

### 114.5 DIAGNOSIS, TREATMENT, AND OUTCOME

Screening to identify pregnant women who are at an increased risk of carrying an NTD-affected fetus can be achieved by the evaluation of maternal serum alpha-fetoprotein levels and/or ultrasound imaging (253–255). Follow-up studies for women with a positive result on either screening test include amniocentesis and/or detailed ultrasound evaluation. When amniocentesis is performed, evaluation of amniotic fluid alpha-fetoprotein and acetylcholinesterase levels can be used to confirm the presence of an open fetal malformation and differentiate between open ventral wall and open NTDs. In addition, the fetal karyotype can be evaluated to rule out chromosomal anomalies. Detailed ultrasound can also be used to differentiate between open defects of the ventral wall and neural tube, and to identify the presence of other associated anomalies (256). When a diagnosis of spina bifida is confirmed, ultrasound and prenatal magnetic resonance imaging (using ultrafast T2-weighted sequences) can be used to identify spontaneous leg and foot motion, leg and spine deformities, and the presence of a Chiari II malformation (257).

Although anencephaly and craniorachischisis are lethal conditions, individuals with spina bifida can survive with appropriate medical treatment. Mode of delivery (cesarean vs vaginal) does not appear to influence the outcome of children with spina bifida (258). However, cesarean section may be appropriate for infants with large spina bifida lesions, to reduce the risk of trauma (258), and is performed following in utero treatment of spina bifida because of the risk of dehiscence during labor.

The majority of individuals with spina bifida have their spinal lesion closed postnatally, usually within 72 h of birth (180,259). However, a small proportion of fetuses have had their lesion closed in utero. A randomized clinical trial designed to compare the outcome of infants treated postnatally with those treated in utero was initiated in 2003 and stopped in December 2010 based on

the evidence for efficacy of prenatal surgery (260). Based on data from 158 patients who underwent a 12-month evaluation, the primary outcome (death or need for cerebrospinal fluid shunt by 12 months) was significantly less common in infants undergoing prenatal as compared to postnatal surgery (relative risk 0.70; 97.7% CI 0.58, 0.84). The prenatal surgery group also had lower rates of hindbrain herniation, brain-stem kinking and syringomyelia and, at 30 months, the difference between the functional and anatomical level of the lesion was significantly better as compared to the postnatal surgery group.

Whether treated in utero or postnatally, individuals with spina bifida are at risk for associated malformations of the nervous system including hydrocephalus and Chiari II malformations. Lower extremity weakness and paralysis, sensory loss, and bowel and bladder dysfunction are also common. The most important determinant of neurological function is the upper level of the spinal lesion. Individuals with spina bifida are also at risk for a range of orthopedic abnormalities including clubfoot, contractures, hip dislocation, scoliosis, and kyphosis. Moreover, while most individuals with spina bifida have normal intelligence, specific cognitive and language difficulties are common (261). Given the range of physical and developmental problems associated with spina bifida, the care of individuals with this condition is best provided by a multidisciplinary team (259).

The most recent population-based data indicate that among individuals with spina bifida 1-year survival is approximately 87%, and approximately 78% survive to the age of 17 years (262). Among survivors there is significant morbidity. In a cohort of 54 adults with spina bifida (mean age 35 years), all of whom had their lesion closed with 48 h of birth, only 30% could walk at least 50 m without aid, 41% lived independently, 54% had passed a driving test, and 24% were in open employment. Relatively little is known about the specific health problems encountered by adults with spina bifida. However, it appears that excess morbidity and mortality do continue throughout the adult years (259,263). In a cohort of 117 individuals with spina bifida who were born in the United Kingdom between 1963 and 1971, the mortality rate from age 5–40 years was 10 times that of the national average. Approximately 50% (16/31) of the deaths that occurred in this age group were considered to be unexpected. The most common causes of unexpected death were epileptic seizure, pulmonary embolism, renal sepsis and acute hydrocephalus ( $N = 3$  each) (264).

### 114.6 PREVENTION

Like many other birth defects and genetic disorders, NTDs can be identified in the prenatal period and are, therefore, amenable to secondary prevention through elective termination of affected pregnancies. However, NTDs stand as one of the few birth defects for which primary prevention strategies are also available. Research

spanning several decades, including randomized (265) and community-based (266) clinical trials demonstrate that maternal periconceptional supplementation with folic acid alone, or multivitamins containing folic acid can reduce the risk of NTDs in offspring by 50–70%.

Numerous studies have demonstrated that women who take a daily folic-acid-containing supplement are at significantly reduced risk of having a child with an NTD, compared with women who do not take such supplements (204,265,267). Consequently, health agencies in several countries have established policies concerning the use of folic acid supplements to prevent NTDs (268,269). However, translating such policies into practice has proven to be extremely difficult. Despite numerous public health campaigns, a substantial proportion of reproductive age women are unaware of, or do not follow, recommendations regarding the need to take folic acid before and during pregnancy for the prevention of NTDs (270–276). Moreover, such policies do not appear to reduce the prevalence of NTDs at the population level (277,278).

Because of the difficulties inherent to public health efforts aimed at widespread changes in behavior, several countries have initiated mandatory folic acid food fortification programs. Cordero et al. (157) noted that 53 countries have established regulations for mandatory fortification of wheat flour with folic acid. Once implemented, such fortification programs can reduce the prevalence of NTDs by approximately 50% (40) and are associated with significant cost savings (110,279). Unfortunately, since folic acid fortification has not been universally implemented and those programs that have been implemented may not be fortifying at optimal levels, it is estimated that only 9% of the folate-preventable NTDs are currently being prevented (280). Indeed, the US Preventive Services Task Force has reiterated that “All women capable of pregnancy should take a daily vitamin supplement that contains 0.4–0.8 mg of folic acid” and noted that “It is not known whether women can get enough folic acid to prevent NTDs simply by eating folic acid-fortified foods” (281).

### 114.7 GENETIC COUNSELING

NTDs can be divided into those that are associated with a chromosomal abnormality, single gene disorder, or teratogenic exposure, and those for which a single causative agent cannot be identified. Therefore, accurate genetic counseling for affected individuals and their relatives requires a thorough evaluation of the underlying cause of NTDs in all affected family members. This includes evaluation of family, medical, and reproductive histories and, when possible, karyotype analysis and clinical or postmortem examination of the affected family members (41).

When an underlying cause of an NTD is identified (e.g. chromosomal aneuploidy, maternal diabetes), recurrence

risk counseling and guidance regarding prenatal care, screening, and testing should be tailored to the specific underlying cause. However, it is unclear whether NTDs that occur in association with an underlying condition or exposure are solely the result of that condition or whether the underlying condition or exposure increases the vulnerability (i.e. shifts the threshold) of the embryo to the development of an NTD (282). If the latter is true, then families that have had a child with an NTD that occurred in association with an underlying condition or exposure may be at increased risk (relative to the general population) of having a child with a nonsyndromic NTD (283).

Although NTDs can occur as part of chromosomal or single gene disorders or as a result of teratogenic exposures, a specific causative agent cannot be identified for the majority of affected individuals. NTDs that cannot be linked to a specific cause are thought to result from the cumulative effects of multiple genetic/environmental risk factors. Recurrence risk counseling for this group of NTDs is based on empirical risk estimates. In general, the recurrence risk for a sibling of an affected individual is reported to be 3–5%. Increased risks (relative to the general population) are also generally reported for second-degree and third-degree relatives, but these risks are less precisely defined than those for siblings. It is of note that the vast majority of studies that assessed recurrence risks for NTDs were conducted before the 1990s and the widespread recognition of the NTD protective effect of folic acid. Hence, it is unclear to what extent these estimates apply at the present time.

In the United States and many other areas of the world, it is recommended that all women of childbearing age consume a daily multivitamin supplement containing at least 0.4 mg of folic acid in order to reduce their risk of having a child with an NTD. Women who have had a child affected with an NTD may be counseled to take higher doses of folic acid as per the initial recommendations from the Centers for Disease Control (232). Specific recommendations for other categories of high-risk women (e.g. sisters of women who have had an affected pregnancy, women who take antiepileptic drugs such as valproic acid, and diabetics) are not available.

As folic acid supplementation does not prevent 100% of all NTDs, women who are at increased risk for having an affected child (whether or not they are taking folic acid supplements) should be informed of the availability of MSAFP screening/ultrasound/amniocentesis for the prenatal identification of affected fetuses. At this time, there are no recommended genetic screening or testing procedures for nonsyndromic NTDs. Evaluation of genetic variants such as the MTHFR C677T polymorphism, is not currently recommended because of the lack of consistent associations across studies, the relatively low proportion of cases that are likely to be attributable to any single genetic factor, and the fact that it would not change preconceptional advice regarding the utility of vitamin supplements or pregnancy management (284).

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Genetic Disorders of Cerebral  
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**115.1 INTRODUCTION**

The widespread clinical use of MRI, with its ability to image the human brain noninvasively, has revolutionized our understanding of cerebral cortical malformations. Although some dramatic cortical malformations can be associated with remarkably preserved intellect, most brain malformations cause severe neurological disability. Genetic disorders of cortical development include sporadic noninherited anomalies, as well as a growing variety of malformations with distinct inheritance patterns. Consequently, accurate diagnosis and genetic counseling requires precise definition of the anatomical abnormalities and the clinical spectrum of each disorder. Since malformations of the cerebral cortex have their origins during the dynamic process of cortical development, understanding normal cortical development is essential when considering these disorders. Therefore, the process of normal development of the human cerebral cortex will be reviewed briefly before discussing individual cortical malformations.

The neurons of the cerebral cortex are formed in a pseudostratified columnar neuroepithelium of the ventricular zone and then migrate over considerable distances to reach their final destination in the cortex. Neural progenitor cells in the ventricular zone are called radial glia cells (1). They give rise to postmitotic neurons, which migrate toward the surface, and also to a secondary population of progenitor cells in the subventricular zone (the region immediately superficial to the ventricular zone) (2). The

subventricular zone contains two major types of progenitors. Basal progenitors appear to be short-lived intermediate progenitors that produce individual cell types such as neuron or glia (2–5). In addition, a recently described population of multipotential progenitors with radial glia-like morphology in the outer subventricular zone is especially common in the brains of humans and other mammals with a large cerebral cortex (6–8).

The first group of postmitotic neurons produces a pioneer layer called the primordial plexiform layer (9,10). This layer, also called the “preplate” is later split into an outer layer (“marginal zone”) and a deeper layer (“subplate”) by the later-arriving neurons that form the cortical plate. Neurons add to the cortical plate in an inside out manner, so that newer-arriving neurons always migrate past the older cortical plate neurons until they arrest next to the marginal zone (11,12). The marginal zone, which contains specialized neurons called Cajal-Retzius cells, seems to be instrumental in establishing the inside out laminar gradient of the cortical plate. In the human cortex, postmitotic neurons start to migrate out of the ventricular zone between the sixth and seventh week of gestation to form the primordial plexiform layer (10,13). Migration of neurons appears to peak between the 11th and 15th week of gestation (11). Although it is not entirely clear when migration is finally completed in the cerebral cortex, the majority of neurons have entered the cortex by around the 24th week of gestation (10,14).

The migration of cortical plate neurons is guided by long radially aligned fibers of radial glial cells (12,15,16). The outermost processes of these radial glial cells form the outer limiting membrane of the brain, consisting of glial endfeet apposed to a basement membrane contributed both by neural and extra-central nervous system (CNS) cells [reviewed in (17)]. Nonradial (tangential) neuronal migration is also common in the developing cortex (18), with a large fraction of cerebral cortical neurons, particularly inhibitory interneurons, originating outside of the cortex itself, in other brain regions that give rise to the basal ganglia (19–21). A vast diversity of molecules has been implicated in the development of the cortex through animal studies but will be reviewed here only if they specifically relate to known human genetic disorders.

Although a standard and universally applied classification scheme for malformations of cortical development is yet to be developed, a system proposed by Barkovich et al. (22,23) serves as a useful framework (Table 115-1). The Barkovich et al. scheme is primarily organized according to a triad of embryology, genetics, and neuroimaging. Our presentation of cortical disorders will generally follow this outline.

## 115.2 MALFORMATIONS DUE TO ABNORMAL NEURONAL AND GLIAL PROLIFERATION OR APOPTOSIS

### 115.2.1 Microcephaly: An Overview

Microcephaly is a condition in which the cranial vault, defined by the occipitofrontal head circumference (OFC), is significantly smaller than expected for an individual's age and sex (>2 standard deviations smaller than the mean; or more conservatively, >3 standard deviations) (24,25). The etiology of microcephaly is extremely diverse, posing significant challenges to clinicians dealing with this condition, but can be broadly divided into environmental and genetic causes (26). Common environmental causes include congenital infection, intrauterine exposure to teratogenic agents, and hypoxic-ischemic injury. The clinical history usually provides important diagnostic clues in these cases. On the other hand, genetic causes of microcephaly are also quite diverse. Inherited metabolic disorders usually cause postnatal onset of microcephaly (acquired microcephaly) and are not discussed here.

Genetic causes of microcephaly that are associated with the presence of microcephaly at birth (congenital microcephaly) usually represent developmental malformations of the cerebral cortex. For example, microcephaly is frequently seen in association with a variety of chromosomal abnormalities and other well-defined genetic syndromes (e.g. Smith-Lemli-Opitz syndrome; OMIM 270400) that are discussed elsewhere in this book. In these cases, the characteristic patterns of involvement of other organ systems and/or the presence

of specific dysmorphic features often help make the diagnosis. Other well-characterized CNS disorders such as neuronal migration disorders (e.g. lissencephaly) can be associated with microcephaly without other organ involvement, and these conditions are described later in the chapter. Finally, there are an increasing number of syndromes and genetic loci (e.g. microcephaly vera) in which the CNS is typically the only affected organ system and in which the brain is characteristically quite small. These disorders are discussed below.

**TABLE 115-1 Classification of Cortical Malformations**

I. Malformations due to abnormal neuronal and glial proliferation or apoptosis
A. Decrease proliferation/increased apoptosis (microcephaly)
1. Microcephaly with normal or mildly simplified gyri
a. Microcephaly vera (primary autosomal recessive microcephaly)
b. Microcephaly, seizures and developmental delay (early infantile epileptic encephalopathy 10)
c. Microcephaly with proportionate short stature
d. Others
2. Microlissencephaly (severe microcephaly with significant simplification of gyri)
3. Microcephaly with other brain malformations (e.g. polymicrogyria, pontocerebellar hypoplasia)
B. Increased proliferation/decreased apoptosis (megalencephaly)
C. Abnormal proliferation and differentiation (nonneoplastic)
1. Cortical tubers of tuberous sclerosis
2. Focal cortical dysplasia
3. Hemimegalencephaly
II. Malformations due to abnormal neuronal migration
A. Classical lissencephaly/subcortical band heterotopia
1. LIS1-associated lissencephaly
2. X-linked lissencephaly 1
3. X-linked lissencephaly 2
4. TUBA1A-associated lissencephaly
B. Cobblestone dysplasia
C. Gray matter heterotopia
1. X-linked periventricular heterotopia
2. Autosomal recessive periventricular heterotopia with microcephaly
3. Heterotopia due to chromosomal aberration
III. Malformations due to abnormal cortical organization (including later stages of neuronal migration)
A. Polymicrogyria
1. Bilateral frontoparietal polymicrogyria
2. Bilateral perisylvian polymicrogyria
3. Bilateral occipital polymicrogyria
4. Bilateral generalized polymicrogyria
5. Other localized polymicrogyria syndromes
6. Tubulin-associated polymicrogyria
7. Polymicrogyria due to chromosomal aberration
B. Schizencephaly
IV. Malformations of cortical development, not otherwise classified
A. Malformations secondary to inborn errors of metabolism
B. Others

Modified from (22,23).

### 115.2.2 Microcephaly Vera

This term, meaning “true” microcephaly, was coined by Giacomini in 1885 to denote a condition in which no gross pathological abnormality other than the small size of the brain was observed (27). Clinically, this term has been used to describe a group of patients characterized by the presence of microcephaly at birth, relatively normal early motor milestones, and intellectual disability of variable severity. Microcephaly is usually profound, ranging between  $-4$  and  $-12$  standard deviations below the mean for the age (28,29). Usually there are few dysmorphic features other than those that arise as a consequence of the microcephaly, including a narrow sloping forehead and relative prominence of ears. Seizures are relatively uncommon in this group of patients, although reported in some cases (30). When early-onset seizures are a prominent feature but the rest of clinical characteristics resemble microcephaly vera, patients may fall into the category of the recently characterized syndrome of microcephaly, seizures, and developmental delay (see below).

**115.2.2.1 Genetics and Biology.** Microcephaly vera is inherited as an autosomal recessive trait, and it is genetically highly heterogeneous. To date, seven genes in seven loci (termed MCPH1 through 7) have been identified to be associated with microcephaly vera: *MCPH1* (also known as *microcephalin*; the MCPH1 locus; OMIM 251200) (31), *WDR62* (MCPH2; OMIM 604317) (32–34), *CDK5RAP2* (MCPH3; OMIM 604804) (35), *CEP152* (MCPH4; OMIM 604321) (36), *ASPM* (MCPH5; OMIM 608716) (37), *CENPJ* (MCPH6; OMIM 608393) (35) and *STIL* (MCPH7; OMIM 612703) (38). Among these genes, *ASPM* mutations appear to be the most common and may account for approximately 40% of patients who fit the clinical description of microcephaly vera (39,40).

Upon brain MRI, patients with microcephaly vera typically show a normal or mildly simplified gyral pattern. Cortical thickness appears normal. In patients with *ASPM* mutations, associated abnormalities such as agenesis of corpus callosum, enlarged lateral ventricles (particularly of the occipital horn), and focal cortical malformations (e.g. unilateral polymicrogyria or focal cortical dysplasia) are sometimes seen (41). *WDR62* mutations are associated with the more frequent presence of associated structural abnormalities, which include severe simplification of gyri, polymicrogyria hypoplasia or partial agenesis of the corpus callosum and schizencephaly (32–34). Missense *WDR62* mutations appear to be associated with less severe structural abnormalities compared to truncating mutations (33).

The proteins encoded by the genes associated with microcephaly vera localize, at least in part, to the mitotic centrosomes and are involved in the regulation of cell cycle and cell division during early cortical development (28). Thus these proteins are thought to regulate

proliferation of neural progenitor cells in the developing cerebral cortex. Although the exact mechanisms by which these proteins regulate cell cycle and cell division are not entirely clear, *ASPM* has been shown to maintain symmetrical cell division (in which neural progenitors generate two progenitors as daughter cells, as opposed to generating one progenitor and one postmitotic neuron) (42). This function likely prevents premature depletion of neural progenitors. *MCPH1* contains three BRCT (BRCA1 C-terminal) domains. Many proteins with this domain are known to function in DNA repair, and *MCPH1* has also been implicated in DNA damage response (43). In addition, it is implicated in chromosome condensation during mitosis (44) and chromatin remodeling (45).

Patients with congenital microcephaly who also have intractable seizures starting during infancy have been found to have recessive mutations in the DNA repair protein gene *PNKP* (46). This entity is variably called microcephaly, seizures, and developmental delay or early infantile epileptic encephalopathy 10 (OMIM 613402). These patients typically show head circumference of  $-2$  to  $-3$  SD at birth and  $-4$  to  $-7$  SD during childhood and have severe intellectual disability. However, milder mutations lead to milder microcephaly ( $-2$  to  $-3$  SD below the mean during childhood) and less severe seizures and intellectual disability. Unlike other disorders that affect DNA repair (e.g. ataxia telangiectasia), microcephaly due to mutations in *PNKP* has not been associated with immunodeficiency or cancer, although no long-term follow-up is available for patients with *PNKP* mutations.

Patients with microcephaly vera have severe microcephaly, but their linear growth is much less affected. On the other hand, there are patients with severe congenital microcephaly with proportionate growth retardation. Clinical entities associated with this type of presentation include Seckel syndrome (OMIM 210600 for the *SCKL1* locus) and microcephalic osteodysplastic primordial dwarfism types I (MOPD I; OMIM 210710) and II (MOPD II; OMIM 210720). Some patients with Seckel syndrome have been found to have recessive mutations in *ATR* (47), which is closely related functionally to *ATM*, the gene mutated in ataxia telangiectasia, and is also involved in DNA damage response (48). Recessive mutations in one of the genes associated with microcephaly vera, *CEP152*, have also been found in patients with a diagnosis of Seckel syndrome (49), suggesting overlap between these two conditions. MOPD II has been associated with recessive mutations in *PCNT2*, whose protein product localizes to centrosome (50,51) but may also be involved in DNA damage response like *ATR* and *MCPH1* (50). Thus these microcephaly syndromes with short stature and microcephaly vera may represent defects in overlapping biological pathways.

An autosomal dominant form of microcephaly (OMIM 156580) has been described and is perhaps

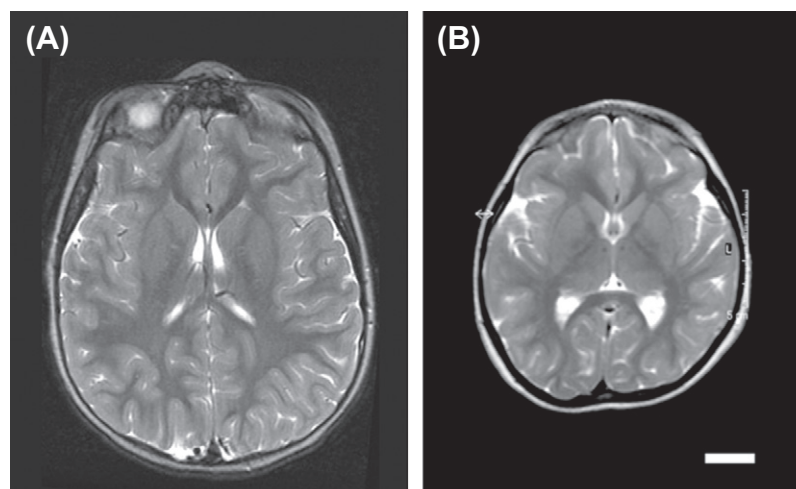
relatively common among individuals with mild ( $-2$  to  $-3$  SD) microcephaly, but little is known about its cause. This form of microcephaly is often associated with normal intelligence and may not come to clinicians' attention very often (52). The assessment of recurrence risk of microcephaly is therefore often challenging because of the heterogeneous nature of this condition. Unless the mode of inheritance is evident from family history, examination and laboratory investigations, accurate assessment may be difficult. In one population-based study in British Columbia, Canada, the recurrence risk of intellectual disability in the siblings of microcephalic individuals was estimated to be 5.9%, one-third of whom also had microcephaly (53). Another study estimated the recurrence risk of microcephaly in siblings of microcephalic individuals to be 19% (54), more suggestive of an autosomal recessive trait. This large difference may be due to differences in the percentage of autosomal recessive forms of microcephaly in each population studied.

In patients with microcephaly vera, the gyral pattern is relatively well preserved despite the often strikingly small size of the brain (Figure 115-1). Pathological studies of the brain are few, but they thus far reveal no microscopic abnormality in cortical laminar formation (55,56). In some cases, a reduced number of neurons in cortical layers II and III, which are superficial layers (57), or neuronal heterotopias (55) have been observed. Although a decreased number of neurons in the cerebral cortex is considered to be primarily responsible for the small size of the brain in microcephaly vera, there are many potential ways in which the number of cortical neurons can be subnormal. For example, decreased proliferation of neuronal progenitors, decreased production of mature neurons by each neuronal progenitor, or excessive cell death of neuronal progenitors or of mature neurons all lead to decreased numbers of neurons in animal models (58,59).

### 115.2.3 Microlissencephaly

It has been long known that some patients with congenital microcephaly present with more severe neurological signs, such as spasticity, severe developmental delay, and seizures. Some of those patients have striking simplification of cortical gyral pattern (more significant than what is seen in patients with microcephaly vera), and the term microlissencephaly (microcephaly + lissencephaly) may be applied to those cases (60–62). This is clearly a highly heterogeneous group. Survival varies significantly, with some patients dying within days or weeks (63) but others living for years. Associated neuroimaging findings may include pontocerebellar hypoplasia, enlarged extra-axial spaces, and polymicrogyria (64). This clinical and radiological variability may reflect their distinct pathogeneses.

**115.2.3.1 Genetics and Biology.** Recently, recessive mutations in the *NDE1* gene were identified in patients with severe congenital microcephaly, severe simplification of gyral pattern, and the variable presence of spasticity and seizures (65,66). The homolog of NDE1 in *Aspergillus nidulans*, NudE, is an essential regulator of nuclear migration pathway (thus the name Nuclear distribution E) and interacts with the homologs of LIS1 (whose mutations are associated with classical lissencephaly discussed below) (67). This interaction is also preserved in mammals (58,68,69). In addition, similar to other genes associated with microcephaly vera, NDE1 localizes to the mitotic centrosome, and its defects lead to failure in mitotic progression (65,66). Thus, the clinical characteristics of severe microcephaly associated with lissencephaly may be explained by the essential roles of NDE1 in both neurogenesis and neuronal migration. The genetic basis of other types of microlissencephaly has not been elucidated.



**FIGURE 115-1** T2-weighted axial MRI images of a normal 4-year-old girl (A) and a 3-year-old girl with microcephaly vera (B) are shown. The gyral pattern is mildly simplified in the patient, but there are no other structural abnormalities, except for the striking smallness of the brain. The patient was found to have a mutation of the *ASPM* gene. Scale bar = 2 cm.



### 115.2.4 Other Microcephaly Syndromes

Several other novel microcephaly syndromes have emerged in recent years. Intellectual disability and variable postnatal microcephaly (ranging from normal to almost –6 SD below mean; MRT13; OMIM 613192) has been associated with mutations in *TRAPPC9* (70–72). Brain MRI of these patients may reveal paucity and signal abnormalities of the cerebral white matter (70–72). The *CASK* gene on the X chromosome was found to be mutated in patients with congenital or postnatal microcephaly with disproportionate brainstem and cerebellar hypoplasia (OMIM 300749) (73).

### 115.2.5 Tuberous Sclerosis Complex

Tuberous sclerosis complex (TSC; OMIM 191100 for *TSC1* and 613254 for *TSC2*) is an autosomal dominant multiorgan disorder that commonly affects the brain, eyes, skin, kidneys and heart. TSC is discussed in this section as its fundamental defects include cell proliferation, fate determination and differentiation. Neurological manifestations, which are usually the major clinical problems, include epilepsy, intellectual disability and autistic symptoms. In the brain, the characteristic lesions of TSC are cortical tubers, subependymal nodules, and subependymal giant cell astrocytomas (SEGAs). Cortical tubers are circumscribed areas of dysplastic cortex, which appear pale and have firmer consistency compared to the normal cortex (74). Within the tubers, the normal laminar structure is lost and enlarged dysmorphic neurons as well as “giant cells” (or “balloon cells”) with abundant eosinophilic cytoplasm are seen. These giant cells variably express neuronal and glial markers (75). In TSC, subependymal nodules project into the third and lateral ventricles as smooth firm nodules, forming classic “candle gutterings” (74). They may continue to grow and form SEGAs, but the molecular mechanisms of this transformation remain to be understood. Many of the histological features that are observed in cortical tubers are also seen in subependymal nodules and SEGAs, but the cellular packing density of these latter lesions is much higher than that of cortical tubers.

**115.2.5.1 Genetics and Biology.** TSC is caused by mutations in one of the two genes, *TSC1* and *TSC2*. In *de novo* cases, *TSC2* mutations have been found 2–10 times more often than *TSC1* mutations (76–81). However, about half of the familial cases are due to mutations in *TSC1* and the rest are due to mutations in *TSC2* (81,82). *TSC2* mutations are associated with a higher frequency of cystlike tubers, which may predispose to a more aggressive seizure phenotype (83). The *TSC2* gene in chromosome 16p13 encodes a protein called tuberin, which encodes a GTPase activating protein (GAP)-related domain (84). Tuberin has been shown to have GAP activity for Rap1 and Rab5 (85,86). The *TSC1* gene in chromosome 9q34 encodes a protein named hamartin (87).

Tuberin and hamartin interact directly (88–90) and act as critical negative regulators of the mTOR (mammalian target of rapamycin) signaling pathway, an important pathway regulating cell growth in response to growth factors and other metabolic stimuli (91–93). Thus TSC lesions show abnormal mTOR activation, with enlarged and dysplastic-appearing cells.

The focal nature of TSC lesions appears to be explained by the “two-hit” hypothesis, originally developed by Knudson for the retinoblastoma gene (94). In the “two-hit” model, all somatic cells harbor a mutation in one allele, and a subsequent random “second hit” or spontaneous mutation in the other “healthy” allele is required for a dividing cell and the daughter cell of that cell to develop an abnormal phenotype. This hypothesis is supported by the fact that loss of heterozygosity (which indicates that the second hit has occurred) for *TSC1* or *TSC2* locus has been detected in some TSC lesions (95,96). In CNS lesions, loss of heterozygosity has been demonstrated to occur rarely if at all (97,98); whether this reflects a pathogenesis of CNS lesions different from other TSC lesions is unclear.

The central involvement of the mTOR pathway in the pathogenesis of TSC has led to the hypothesis that some manifestations of TSC may be amenable to treatment with rapamycin and its derivatives (99–102). The role of rapamycin vs neurosurgical intervention for the treatment of SEGAs is still to be determined at the time of the writing of this chapter (103), and clinical trials to evaluate the effects of rapamycin on neurocognitive outcomes in children with TSC are still in progress. TSC is an exemplar of a neurodevelopmental condition with potential disease-specific treatments that may follow from the study of the genetics of the disorder.

### 115.2.6 Focal Cortical Dysplasia

Focal cortical dysplasias (FCDs; OMIM 607341) are dysplastic zones of cortex, usually occurring as solitary lesions that interrupt the otherwise morphologically normal cortex. FCDs share some radiographic and histological similarities with the cortical tubers of TSC (104–106). Histologically, FCDs contain large bizarre neurons in all but the first cortical molecular layer, with a loss of cortical lamination. Grotesque, glia-like cells are present in the depth of the affected cortical region and in the underlying demyelinated white matter (105). “Balloon cells” with glassy eosinophilic cytoplasm and pleomorphic eccentric nuclei have been demonstrated in cases of FCD (105) and were found to express neuronal markers, glial markers, or both (107,108). FCD is a well-known cause of intractable epilepsy (109,110), but it is sometimes not identified in the initial MRI scan unless there is a high degree of suspicion for a focal abnormality at a specific location; appropriate signal sequences and reconstructions may need to be performed to optimize detection of small or relatively subtle FCD.

The most common MRI picture of FCD takes the form of a thickened bumpy cortex with shallow and wide sulci. Deep infoldings of thickened cortex may also be found (104).

The neuropathological findings of FCD, which are the basis of the classification of FCD into subtypes (110), have led to the hypothesis that FCDs may represent focal somatic mutations that have occurred early in cortical development, perhaps in the *TSC* genes or in related genes, but this has not been definitively demonstrated. The histological features of FCD are strikingly similar to that of TSC, and it has been debated whether sporadic FCD, in the absence of the other stigmata of TSC, represents a “forme fruste” of TS (111). Studies have shown different patterns of gene expression in these two disorders, and this may suggest distinct pathogenetic mechanisms at work (112,113). On the other hand, loss of heterozygosity at the *TSC1* locus has been identified in some of microdissected tissue samples of FCD, possibly suggesting a pathogenetic relationship (114,115).

FCD usually occurs in a sporadic, noninherited form. A pedigree was reported several years ago with apparent familial cortical malformations including FCD among other malformations; on careful inspection images from an affected individual with FCD appear more typical of asymmetric perisylvian polymicrogyria than the typical FCD (116). More recently, there has been one demonstration of a genetic etiology for focal cortical dysplasia in an Amish pedigree with an autosomal recessive mutation in the gene *CNTNAP2*; the affected individuals had medically intractable focal epilepsy, intellectual disability, and aggressive behavior (117). *CNTNAP2* has also been reported to be associated with autism (118–121) and Pitt-Hopkins-like syndrome 1 (OMIM 610042) (122).

### 115.2.7 Hemimegalencephaly

Hemimegalencephaly is characterized by unilateral enlargement of only one cerebral hemisphere. The entire cerebral hemisphere may be enlarged, but sometimes only half or part of the affected hemisphere is involved. The enlarged hemisphere usually shows gyral abnormalities in the form of agyria/pachygyria or polymicrogyria, and the lateral ventricle on the abnormal side is often enlarged (123). Boundaries of gray and white matter may be blurred, and gray matter heterotopia may be found. Microscopically, cortical laminar disorganization, neuronal cytomegaly, and heterotopic neurons in the white matter are seen (124,125). The diagnostic criteria for hemimegalencephaly are not precise, so the term is often applied to virtually any cortical malformation that involves most of a single hemisphere.

A typical clinical presentation includes contralateral hemiparesis, epilepsy, and intellectual disability (123). Seizures often start during the first days of life and are typically intractable to medical management. In fact, early resistance to antiepileptic drugs is common (126). Partial seizures with or without secondary

generalization and infantile spasms are common (127,128). Hemispherectomy can be an effective treatment for seizure control (126). Cognitive development may vary widely. Patients with normal intellectual development have been reported (129), but this is uncommon.

Hemimegalencephaly can be seen as an isolated malformation or in association with various neurocutaneous syndromes such as hypomelanosis of Ito (OMIM 300337) (130,131), Shimmelpenning-Feuerstein-Mims syndrome (also known as linear sebaceous nevus syndrome or epidermal nevus syndrome; OMIM163200) (132,133), Proteus syndrome (OMIM 176920) (134), and Klippel-Trénaunay-Weber syndrome (OMIM 149000) (135). In addition, rare association with neurofibromatosis type I (OMIM 162200) (136) and TSC (OMIM 19100 and 613254) (137) have been reported. Thus, a careful skin examination is mandatory in patients with this condition. Hemimegalencephaly is typically encountered as a sporadic, noninherited condition. Therefore, the risk of recurrence is considered small when there is no association with a known inherited syndrome.

Histological characteristics, such as neuronal cytomegaly and heterotopic neurons, closely resemble TSC and FCD. In some cases, “balloon cells” similar to those seen in TSC and FCD are also observed. In contrast, a comparison between “balloon cells” seen in hemimegalencephaly vs TSC has shown different immunohistochemical and ultrastructural characteristics of the abnormal neurons in each condition (138). It is conceivable that somatic mosaicism may play a role in the pathogenesis of hemimegalencephaly, but definitive evidence is lacking.

## 115.3 MALFORMATIONS DUE TO ABNORMAL NEURONAL MIGRATION

### 115.3.1 Classical Lissencephaly: An Overview

Lissencephaly represents a smooth brain with a lack or severe paucity of normal gyri. Classical (or Type I) lissencephaly is characterized by a severely thickened cerebral cortex with three or four abnormal layers instead of the normal six cortical layers. On the other hand, “cobblestone” (or Type II) lissencephaly is a completely different entity histologically (see Section 115.3.7). Several clinical entities are associated with classical lissencephaly, including isolated lissencephaly sequence (ILS), Miller-Dieker syndrome (MDS; OMIM 247200), X-linked lissencephaly 1 (OMIM 300067), and X-linked lissencephaly 2 (OMIM300215).

### 115.3.2 Isolated Lissencephaly Sequence and the Miller-Dieker Syndrome

The most common cause of classical lissencephaly is a mutation of an autosomal gene on chromosome 17p13

known as *LIS1*. Deletion or mutation in the *LIS1* gene is the cause of lissencephaly both in MDS and in ILS (139). Additional clinical findings in MDS include an abnormal facies with microcephaly, bitemporal hollowing with narrowing at the temples, tall and prominent forehead with vertical furrowing, hypertelorism with upward slanting palpebral fissures and ptosis, short nose with upturned nares, low-set ears with minor flattening of the helices, prominent philtrum with thin vermilion border of the upper lip, and small mandible (140–142). Digital abnormalities, such as syndactyly, congenital heart disease, and other visceral abnormalities may be seen in patients with MDS (142–144). Almost all children with classical lissencephaly have profound developmental delay and intellectual disability; many of them die during infancy or childhood. The majority of the patients have seizures, typically starting during the first 6 months of life (143,145).

On imaging studies, the cerebral hemispheres show agyria (lack of gyri) or pachygyria (broadening of gyri), and often these two features coexist. The frontal and temporal opercula (the parts of the brain that cover insular cortices) are not developed, leading to a characteristic “figure-eight” shape appearance of the brain on axial images and bitemporal hollowing. Agenesis or hypogenesis of the corpus callosum may be seen, and small midline calcifications in the region of the septum pellucidum may be observed in patients with MDS (140). The cerebellum is notably normal in MDS (146).

**115.3.2.1 Genetics and Biology.** Identification of patients with MDS and monosomy 17p led to a suggestion that this locus may harbor the causative gene (141). This eventually led to the identification of *LIS1*, a gene that is deleted in MDS and deleted or mutated in ILS (139). Subsequent studies showed visible cytogenetic or submicroscopic deletions of 17p13.3 in more than 90% of MDS patients and in approximately 40% of patients with ILS (140). Point mutations as well as intragenic deletions and duplications of *LIS1* have also been identified in patients with ILS (147–150). What distinguishes the group of patients with *LIS1*-associated lissencephaly is a posterior predominance of the gyral abnormality, such that the posterior cortex is essentially agyric while there is typically a pachygyria pattern anteriorly. In ILS, the submicroscopic deletion is smaller than its counterpart in MDS (151,152), and so it has been suggested that MDS may represent a contiguous gene syndrome, with deletion of additional gene(s) being responsible for the dysmorphic features of MDS (140). There are eight genes that are consistently deleted in patients with MDS; deletion of the genes *CRK* and *YWHAE* appears to be associated with more severe lissencephaly (153). Animal studies have also shown that the 14-3-3 epsilon protein encoded by *YWHAE* is important for neuronal migration (154).

Virtually all known *LIS1* mutations are de novo; thus if a deletion or a mutation of the *LIS1* gene is identified

in a patient, the recurrence risk is very low (155). There are rare cases in which one of the parents harbors a balanced translocation in the area involving the *LIS1* gene. In such cases, the risk is significantly higher, and up to 33% of the offspring having an abnormal genotype (i.e. deletion or duplication of 17p) and/or phenotype (156). Originally both Miller (157) and Dieker (158) reported familial cases of MDS, and later studies revealed translocations in both the families they described (159).

The normal cellular function of the *LIS1* protein is thought to be related to regulation of microtubules. Microtubules, which are major components of the cytoskeleton, play a critical role in essential cellular processes, such as cell division and migration. *LIS1* has been known to interact directly with microtubules and stabilize them. *LIS1* also interacts with motor protein dynein (160) and regulates the microtubule-based molecular motor (161–163).

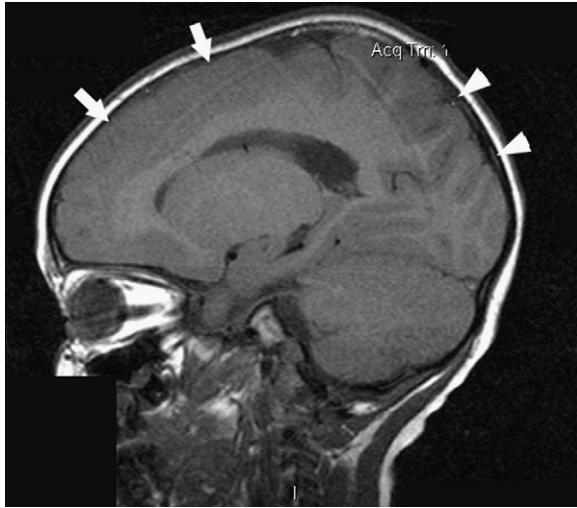
### 115.3.3 X-Linked Lissencephaly 1/ Subcortical Band Heterotopia

A familial, X-linked form of lissencephaly (*LISX1*; OMIM 300067) has also been identified and is associated with a higher recurrence risk than lissencephaly due to *LIS1* mutations. *LISX1* manifests in affected hemizygous males as a disorder quite similar to MDS/ILS, with severe intellectual disability, intractable seizures, classical lissencephaly with pachygyria/agyria, agenesis of corpus callosum, and death during infancy (Figure 115-2) (164). This disorder manifests as a much milder phenotype in heterozygous females, who present with subcortical band heterotopia (SBH; also known as “double cortex” syndrome). SBH is characterized by symmetric stretches of gray matter found in the central white matter between the cortex and the ventricular surface, associated with intellectual disability and epilepsy. There is a wide range of phenotypic severity—both in terms of cognitive outcome and epilepsy—associated with SBH (165).

Although the MRI findings of the brain of *LISX1* patients are generally quite similar to that of MDS/ILS, there is a notable difference in the pattern of malformation. The malformation is more severe posteriorly in patients with *LIS1* mutations and more severe anteriorly in patients with *LISX1* (Figure 115-2) (148,166). In addition, a neuropathological study of two fetuses, one with a *LIS1* mutation and the other with a *DCX* mutation, showed different patterns of cortical lamination in these two conditions, suggesting possibly dissimilar pathogenetic mechanisms in MDS/ILS and *LISX1* (167). Unlike MDS, there is no outward syndromic manifestation of *LISX1* or SBH.

**115.3.3.1 Genetics and Biology.** Linkage analysis of pedigrees with *LISX1*/SBH localized the gene to Xq22 (168,169). The gene responsible for *LISX1*/SBH was identified and named *doublecortin* (*DCX*, OMIM 300121) (170,171). In one study, mutations of





**FIGURE 115-2** T1-weighted sagittal MRI image of a patient with X-linked lissencephaly 1 (*LIS1*) is shown. The anterior cortex almost completely lacks gyri (arrows), but the gyral pattern of the posterior cortex is relatively intact (arrowheads). This is typical of lissencephaly due to *DCX* mutations. In lissencephaly due to *LIS1* mutations, this anterior–posterior gradient is reversed (see text).

*DCX* were identified in all eight familial cases and in 18 of 47 (38%) sporadic cases of SBH (172). Another study found mutations of *DCX* in all 11 familial cases of SBH, and in 22 of 26 (85%) sporadic SBH cases (173). Although most of the reported *DCX* mutations are point mutations, intragenic deletions have been reported (149). Unlike *LIS1* mutations, in which the risk of a second affected child is low, recurrence of either *LIS1* or SBH will be 50% if the mother is affected with SBH due to a *DCX* mutation. Therefore, in males with lissencephaly who do not show deletion or mutation of *LIS1*, mutation analysis of *DCX* should be considered. MRI examination of the mother may reveal SBH, particularly if the mother has epilepsy or cognitive difficulties. Maternal germline mosaic mutations in *DCX* have also been reported (173,174). Together, *LIS1* and *DCX* mutations have been estimated to cause the most cases (approximately 76%) of classical lissencephaly in the United States (148). There are likely to be other lissencephaly loci responsible in the rest of the patients. The presence of an autosomal recessive locus for lissencephaly has been suggested (i.e. Norman-Roberts syndrome; OMIM 257320) (175). It should also be noted that both male and female patients with SBH and somatic mosaic *DCX* mutations have been reported (165,174,176,177). Rare patients with mosaic mutations of *LIS1* presenting with SBH have also been reported (178). In more recent years, mutations in *DCX* have also been associated with a milder range of phenotypes, including nonsyndromic intellectual disability with normal MRI in females (179) and anterior-predominant pachygyria in males (180).

When compared to cases with *LIS1* mutations, cases with *DCX* mutations display variable

neuropathological patterns, some with a six-layer cortex and others with an ill-defined four-layer cortex. In individuals with mutations in *LIS1*, linear disruptions of the gray–white matter junction have been observed; in contrast, individuals with mutations in *DCX* displayed more irregular disruptions of the gray matter–white matter junction as well as nodular heterotopias (181).

*DCX* is expressed in postmitotic neurons throughout the developing nervous system. The *DCX* protein has been shown to interact with and stabilize microtubules (182–184). Note that *LIS1* and *DCX*, two genes that lead to the phenotype of classical lissencephaly, are both involved in the regulation of microtubules. The structure of *DCX* includes two microtubule binding domains in the protein (185). Thus, regulation of microtubules appears to be essential for migration of cortical neurons.

### 115.3.4 Lissencephaly Associated with *TUBA1A* Mutations

Mutations in *TUBA1A*, which encodes the microtubule-related protein  $\alpha$ -tubulin, have been associated with a wide range of cerebral malformations including lissencephaly (OMIM 611603) (186,187,337). The prototype of *TUBA1A*-associated lissencephaly includes additional abnormalities of the hippocampi, corpus callosum, brainstem, and cerebellum (187). Abnormalities in the basal ganglia have been seen as well in some cases (188). Histological findings in severe cases with mutations in *TUBA1A* include abnormal lamination of the cerebral cortex and hippocampi as well as heterotopic and misoriented neurons in the cortex (188). After several cases with varying degrees and patterns of lissencephaly were screened for mutations in *TUBA1A*, the spectrum of *TUBA1A*-associated lissencephaly has come to include a mild form with perisylvian pachygyria as well as a more severe form consisting of posteriorly predominant pachygyria (with a gradient similar to that seen in association with *LIS1* and *ARX*), microcephaly, cerebellar hypoplasia, and abnormalities of the anterior limb of the internal capsule (186,189). The associated phenotype is reported to include intellectual disability; the proportion of patients with mutations in *TUBA1A* with epilepsy is not yet reported but is expected to be very high.

While *TUBA1A* is relatively new to the lissencephaly genetic landscape, it is really not surprising, given the microtubule-associated roles of *LIS1* and *DCX*, that a gene encoding  $\alpha$ -tubulin would be associated with a defect in migration resulting in lissencephaly. Interestingly, similar to what has been observed for *ARX*, there have been abnormalities in interneuron migration suggested by the observation of decreased numbers of interneurons in a fetus with a known mutation in *TUBA1A* (188).



### 115.3.5 X-Linked Lissencephaly 2 (X-Linked Lissencephaly with Abnormal Genitalia)

There is another form of X-linked lissencephaly (X-linked lissencephaly 2, also known as X-linked lissencephaly with abnormal genitalia; LISX2; OMIM 300215). In a typical patient with LISX2, lissencephaly is associated with agenesis of the corpus callosum and ambiguous or underdeveloped genitalia (190–192). The causative gene for this syndrome was identified as the Aristaless-related homeobox transcription factor gene, *ARX* (193). *ARX* has been shown to regulate tangential migration of interneurons (193–196). It is also implicated in regulation of neuronal proliferation (193,195). In addition to LISX2, mutations in the *ARX* gene can cause a wide variety of syndromes, ranging from severe brain malformations, such as hydranencephaly, to neurological disorders with apparently normal brain structure, such as West syndrome, nonsyndromic intellectual disability or autism (197–199). There seems to be some degree of genotype–phenotype correlation in that mutations that cause premature termination are more likely to be seen in patients with overt malformations and mutations that cause polyalanine expansion are commonly seen in patients without malformations (197).

While the pattern of lissencephaly associated with *ARX* mutations is a posterior-predominant agyria, with a gradient similar to what is seen in the setting of *LIS1* mutations, there are several features that may distinguish the two: (1) a cell-sparse layer is observed with *LIS1*, but brains from patients with mutations in *ARX* have only three cortical layers and lack a cell-sparse zone (181); (2) agenesis of corpus callosum is commonly seen with *ARX* mutations (197); and (3) the basal ganglia are noted to be small in patients with lissencephaly due to *ARX* mutations (198).

### 115.3.6 Lissencephaly with Cerebellar Hypoplasia

A variety of types of lissencephaly with cerebellar hypoplasia have been defined (200), and one of them, an autosomal recessive form of lissencephaly with severe hypoplasia of the cerebellum, has been characterized genetically (201–203). Since no postmortem studies on this condition have been published, it is unknown whether it resembles type I lissencephaly microscopically or whether it shows a unique histological pattern. Mutations in a gene called *reelin* (*RELN*) have been identified in two families with this condition (204). The *RELN* gene had previously been identified in mouse as causing the *reeler* mutation, which is characterized by disorganized cortical lamination and severe hypoplasia of the cerebellum (205). Therefore, it is likely that the histological pattern in humans reflect similar defects. Reelin is a protein secreted by Cajal-Retzius cells, which are the specialized neurons that reside in the uppermost

layer of the cerebral cortex. Although there is evidence that Reelin acts as a “stop” signal for migrating neurons, the precise mechanism through which Reelin regulates neuronal migration is yet to be understood (206). Very-low-density lipoprotein receptor (VLDLR) and low-density lipoprotein receptor-related protein 8 (LRP8; also known as apolipoprotein E receptor -2) have been shown to act as receptors for Reelin (207), and mutations in the *VLDLR* gene have been associated with cerebellar hypoplasia similar to that seen with *RELN* mutations (208,209). Simplification of gyral pattern appears less severe in patients with *VLDLR* mutations.

### 115.3.7 Cobblestone Dysplasia

Cobblestone dysplasia is characterized by disorganized cortical layers, overmigration of neurons onto the outside of the brain through breaches in the pial surface, and gliovascular proliferation (210,211). The term “cobblestone” is applied because the ectopic neurons with gliovascular proliferation near the surface of the cortex give a bumpy cobblestone-like appearance. It has previously been called “type II lissencephaly” or “cobblestone lissencephaly.” The gyral pattern seen on imaging studies varies widely, including polymicrogyria, pachygyria, and agyria, therefore, the term “cobblestone dysplasia” appears to be more appropriate.

Cobblestone dysplasia is the characteristic brain malformation observed in a group of disorders, sometimes referred to as “dystroglycanopathies,” which includes three prototypic autosomal recessive disorders: Fukuyama congenital muscular dystrophy (FCMD), Walker-Warburg syndrome (WWS), and muscle-eye-brain disease (MEB). All three syndromes associated with cobblestone dysplasia affect the brain, muscle, and eye. As the genetic studies of these disorders progressed, it became clear that there was significant clinical and genetic overlap among these conditions. Since the fundamental biological defects in these disorders appear to be defects in glycosylation of  $\alpha$ -dystroglycan, the term dystroglycanopathy has been coined.

FCMD is most prevalent in Japan, although rare cases have been reported from other countries (212–214). It presents with hypotonia during infancy, generalized weakness, intellectual disability, and occasionally seizures (215). WWS has been reported worldwide and has generally a much more severe phenotype. Patients with WWS often present with severe hypotonia and lethargy during the neonatal period. Median survival in one study was 9 months (216). Various forms of eye abnormalities are seen, including retinal nonattachment/detachment, retinal dysplasia, cataract, persistent hyperplastic primary vitreous, microphthalmia, and coloboma (216–218). MEB is prevalent in Finland, although it has been reported in many countries. Patients with MEB often present with neonatal hypotonia and weakness, which develops into spasticity and contractures (219). Severe intellectual disability is the

rule, and eye abnormalities include severe visual failure and myopia (219).

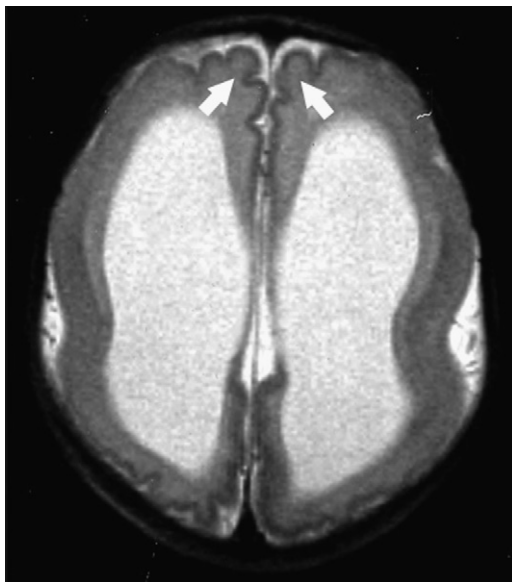
On imaging studies, regions of cobblestone dysplasia usually appear as agyric or pachygyric areas. In typical FCMD, the frontal lobes show polymicrogyria, and cobblestone dysplasia is limited to the temporo-occipital area (220,221). WWS presents more dramatically, with diffuse agyric or pachygyric areas (representing cobblestone dysplasia), enlarged ventricles, hypoplasia of the pons and cerebellar vermis, fusion of the superior and inferior colliculi, and a diffuse abnormality of the cerebral white matter (Figure 115-3) (221,222). Imaging findings of MEB are similar to that of WWS, but abnormalities are usually less extensive (221–224). Cerebellar polymicrogyria with or without small cysts can be seen in any of these disorders.

**115.3.7.1 Genetics and Biology.** Linkage mapping assigned the FCMD gene to 9q31 (225,226); subsequently the causative gene termed *FKTN* (*fukutin*) was cloned (227) and appears to encode a glycosyltransferase. Most (87%) FCMD-bearing chromosomes have been derived from a single mutation consisting of a retrotransposon insertion in the 3' untranslated region, suggesting an ancestral founder mutation in the Japanese population (227). Patients who are compound heterozygotes, carrying this founder mutation and a point mutation, have been found to be more likely to have severe phenotypes, including a WWS-like phenotype (228). Patients who are homozygous for nonfounder mutation have been reported, and the phenotype resembles WWS (229–231). These data suggest that the Japanese founder mutation may represent a partial loss-of-function allele. Subsequently, mutations in a gene with sequence similarity to *FKTN*, *FKRP*

(fukutin-related protein), have been identified in patients with WWS- and MEB-like phenotypes (232).

The first reported gene for the MEB phenotype was linked to 1p34-p32 (233). Subsequently, the responsible gene in this locus was identified as *POMGNT1* (protein O-mannose beta-1, 2-N-acetylglucosaminyltransferase) (234). The identification of an MEB gene as a glycosyltransferase made other glycosyltransferases potential candidate genes for this group of disorders. Indeed, another glycosyltransferase, *POMT1* (protein O-mannosyltransferase), was found to be mutated in some patients with WWS (235). However, *POMT1* mutations seem to account for only a minority of cases of WWS, possibly as low as 7% (231,236). Subsequently, mutations in several other genes have been associated with WWS and MEB-like phenotypes. These include *FKRP* (fukutin-related protein) (232,237), *POMT2* (238–240) and *LARGE* (241–243). In one series, nine of 27 European and American patients with WWS were accounted for by mutations in *POMT1*, *POMT2*, *FKTN* and *FKRP*, and five of those cases were due to *FKTN* mutations (231).

*POMT1*, *POMT2* and *POMGNT1* are glycosyltransferases, and although the biochemical functions of *FKTN*, *FKRP* and *LARGE* are not completely elucidated, they appear to play essential roles in  $\alpha$ -dystroglycan glycosylation. Animal studies have suggested that functional disruption of dystroglycan may be central to the CNS pathogenesis of these disorders (244). Hypoglycosylation of  $\alpha$ -dystroglycan has been shown in patients with FCMD and MEB, and this abolishes binding activity of dystroglycan for ligands such as laminin, neurexin, and agrin (245). Thus, it may be speculated that this defect in binding leads to loss of integrity of the pial surface and that subsequent overmigration of neurons through these breaches leads to the development of cobblestone dysplasia.



**FIGURE 115-3** T2-weighted axial image of a patient with Walker-Warburg syndrome is shown. Enlarged lateral ventricles are evident. The gyral pattern seen here is mostly agyria (absence of gyri), but some areas show pachygyria (broadened gyri; arrows).

### 115.3.8 X-Linked Periventricular Heterotopia

Gray matter heterotopias are masses of well-differentiated neurons in abnormal locations, reflecting arrested radial neuronal migration. Periventricular heterotopia (PH) can be encountered as a sporadic condition, but there are several genetic syndromes in which heterotopias are a cardinal feature. One such syndrome is X-linked PH (OMIM 300049), for which many familial cases are known (246–249). In these pedigrees, typically only females were affected and there was a high rate of miscarriages among the affected females. These observations led to the suggestion that the condition was an X-linked disorder with prenatal lethality in males (247).

Females affected with X-linked PH typically present with epilepsy, commonly generalized tonic-clonic or complex partial seizures. Typical age of onset is before the mid-twenties, and the average age is around 15 years.

Intelligence is usually normal, although some patients have borderline intellectual disability, and dyslexia is remarkably common (250,251). An increased incidence of patent ductus arteriosus and stroke at young ages has been noted (252). Abnormalities in cardiac valve development have also been reported in some cases (252,253), and an increasing number of affected patients have vascular manifestations of Ehlers-Danlos syndrome (254).

Brain MRI of affected females typically shows bilateral PH nodules, which show the typical signal characteristics of normal gray matter. Pathologically, brains of females with X-linked PH show continuous bands or discontinuous nodules of gray matter along the periventricular region, consisting of well-differentiated cortical neurons (255). The heterotopic subependymal nodules of X-linked PH may initially be misdiagnosed as TSC nodules (256), but classical lesions of X-linked PH appear as roughly symmetric nodules as opposed to the less-confluent and not necessarily symmetric nodules of TSC.

**115.3.8.1 Genetics and Biology.** X-linked PH was mapped to distal Xq28 (255), and subsequently mutations in the *FLNA* (*filamin A*) gene were identified to be the cause (252). Mutations in *FLNA* have been identified in almost 100% of familial cases of X-linked PH (257,258) and 26% of sporadic patients with classical bilateral nodular PH (257). In rare instances, male patients with PH have *FLNA* mutations, but the majority of male patients are negative for *FLNA* mutations (257,258). *FLNA* mutations have also been identified in patients with PH and coexisting Ehlers-Danlos syndrome (OMIM300537) (254) and X-linked chronic idiopathic intestinal pseudo-obstruction with CNS involvement (OMIM300048) (259). In recent years, the clinical spectrum of *FLNA* mutations has expanded to include conditions in which non-CNS involvement predominates or in which no CNS involvement is noted. For example, *FLNA* mutations that preserve the reading frame have been shown to be associated with otopalatodigital syndrome types 1 (OMIM311300) (260) and 2 (OMIM304120) (260), frontometaphysial dysplasia (OMIM305620) (260), Melnick-Needles syndrome (OMIM309350) (260), FG syndrome-2 (OMIM300321) (261), terminal osseous dysplasia (OMIM300244) (262), and cardiac valvular dysplasia (OMIM314400) (263). This suggests that *FLNA* is involved in a broad range of organogenesis involving the nervous, skeletal, and cardiovascular systems. It has been speculated that the mutations associated with some of these syndrome may be gain-of-function mutations affecting specific protein interactions (260,264), unlike mutations associated with PH, which are probably loss-of-function mutations.

*FLNA* encodes a large cytoplasmic actin-binding protein that was originally identified in macrophages as a protein that precipitated actin (265). It had been shown to be essential for migration in non-neuronal cell types (266,267), but its role in neuronal migration had

not been known prior to identification of mutation in patients with X-linked PH. *FLNA* is expressed by the cortical neurons during migration (252,268) and possibly regulates the actin cytoskeleton in response to the extracellular signals during neuronal migration (269).

### 115.3.9 Other Genetic Heterotopia Syndromes

An autosomal recessive syndrome with heterotopia associated with microcephaly (OMIM 608097) was found to be caused by mutation in the ADP-ribosylation factor guanine nucleotide-exchange factor-2 gene (*ARFGEF2*) (270). This gene functions in vesicle and membrane trafficking from the trans-Golgi network (TGN) and appears to be essential for proliferation of neuroblasts and migration of postmitotic neurons (270). PHs have been seen in association with various chromosomal anomalies. These include chromosome 5p anomalies (271), 5q deletion (272), 7q11.23 deletion (273), and 1p36 deletion (273,274).

## 115.4 MALFORMATIONS DUE TO ABNORMAL CORTICAL ORGANIZATION

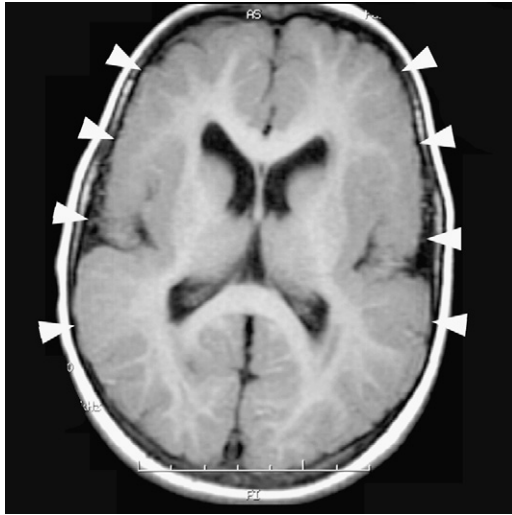
### 115.4.1 Polymicrogyria

Polymicrogyria refers to a cortical malformation characterized by numerous small gyri. Clinical presentations of polymicrogyria depend on the extent and location of the abnormal cortex. When the abnormality is diffuse, severe developmental delay is the rule, but when it is focal, developmental delay is less severe (104). Some individuals with small regions of focal polymicrogyria have normal intelligence. Seizures are common in both groups. On MRI, small meandering gyri of polymicrogyric cortex may appear as thickened cortex, and it may be difficult to distinguish from “pachygyria” (thickened cortex). Irregularity of the junction between the cortex and white matter is usually evident with high-resolution imaging (275,276).

In recent years, several distinctive syndromes of polymicrogyria have emerged (277). These syndromes, which are mainly distinguished by characteristic distributions of polymicrogyric cortex, include bilateral frontoparietal polymicrogyria (BFPP; OMIM 606854) (278), congenital bilateral perisylvian syndrome (CBPS; OMIM 300388; also known as bilateral perisylvian polymicrogyria (BPP)) (279), bilateral generalized polymicrogyria (BGP) (280), and bilateral occipital polymicrogyria (OMIM 612691) (281).

BFPP is associated with symmetrical distribution of polymicrogyria in the frontal and parietal cortex (Figure 115-4). The clinical presentation includes moderate to severe developmental delay, seizures (usual onset after 4–5 years), bilateral pyramidal and cerebellar signs, and dysconjugate gaze (278). CBPS is characterized by





**FIGURE 115-4** T1-weighted axial MRI image of a patient with bilateral frontoparietal polymicrogyria is shown. The frontoparietal cortex shows an abnormal gyral pattern (arrowheads), which represents clusters of abnormal small gyri. The occipital cortex appears typically spared in this condition, and appears relatively normal in this image. Mutations in *GPR56* are responsible for the condition.

diplegia of the facial, pharyngeal, and masticatory muscles, which can be explained by the location of abnormal cortex (282). Intellectual disability is common, but the severity ranges from mild to severe. Epilepsy is also common, and typically has its onset between 4 and 12 years of age (283). BGP is characterized by motor and cognitive delay, and seizures are frequent. Dysconjugate gaze, which is commonly seen in BFPP, or pseudobulbar palsy, which is a hallmark of CBPS, is not typically seen in BGP (280). A comprehensive review of the clinical features of 328 cases of polymicrogyria revealed that seizures occurred in 78%, global developmental delay in 70% and microcephaly in 50%; generally, the more severely affected children had more widespread polymicrogyria and earlier age at presentation with epilepsy or other neurological symptoms (277).

**115.4.1.1 Genetics and Biology.** Polymicrogyria is usually encountered as a sporadic condition; however, it has become evident that there are distinct genetic syndromes with polymicrogyria. Polymicrogyria in association with chromosome 22q11 deletions have been well documented (284,285,338), although only a minority of the patients with 22q11 deletion have polymicrogyria. Furthermore, recurrent copy number variations at 1p36.3, 2p16.1-p23.1, 4q21.21-q22.1, 6q26-q27, and 21q2 have been reported in patients with polymicrogyria (286), suggesting that genes in these regions may be involved in brain development, particularly proper cortical organization. It is important to note that the majority of individuals with polymicrogyria associated with these copy number variants displayed dysmorphic features that today would prompt evaluation with a chromosomal microarray to assess for deletions or duplications.

BFPP, originally described under several different monikers, is often seen in consanguineous pedigrees, suggesting

autosomal recessive inheritance (202,287–291). BFPP was mapped to 16q12.2-21 (292), and subsequently the causative gene was identified (293). The gene mutated in BFPP, *GPR56*, encodes a G-protein coupled receptor, whose ligand is not identified yet. *GPR56* appears to be essential for maintaining the integrity of pial basement membrane (294), and therefore the pathogenesis of BFPP appears to overlap with that of cobblestone dysplasia (see Section 115.3.7). BFPP is increasingly recognized as sharing radiographic and pathologic features with cobblestone dysplasia (295).

Recently, mutations in *LAMC3*, encoding a laminin protein that is characteristic of the pial basement membrane, has also been discovered in association with polymicrogyria in the occipital region (296). This adds to the evidence suggesting that disruption of the pial basement membrane is a common cause of polymicrogyria.

There have been many reports of familial cases of CBPS (279). Many pedigrees were found to be consistent with an X-linked mode of inheritance or autosomal dominant inheritance with incomplete penetrance, and genetic loci for CBPS have been identified at Xq28 (297) and Xq27 (298); however, other genetic loci may exist for this syndrome. Some patients with BGP are siblings and/or born to consanguineous parents, suggestive of autosomal recessive inheritance (280). In familial BGP cases, linkage to the BFPP locus on 16q has been ruled out, but the locus for BGP has not been identified (280).

There is also evidence that prenatal insults, such as hypoxic-ischemic injury, can lead to polymicrogyria. Polymicrogyria in some of these cases has been suggested to result from postmitotic encephaloclastic lesion (299,300), but animal studies have indicated that cortical injuries resulted in polymicrogyria only when they occurred during the course of migration (301–303). Other types of intrauterine insults (e.g. cytomegalovirus infection) and metabolic disorders (see below) have been associated with polymicrogyria as well, but genetic forms of polymicrogyria always need to be considered. For example, bandlike calcification of the brain with simplified gyral pattern and polymicrogyria (OMIM 251290), which resembles TORCH (Toxoplasmosis, Other, Rubella, Cytomegalovirus, Herpes simplex virus) infection and sometimes referred to as pseudo-TORCH syndrome, is associated with mutations in a tight-junction protein gene, *OCN* (304). Interestingly, a mutation in another tight-junction protein gene *JAM3* has been reported to cause calcification of the brain (OMIM 613730) (305). Patients with a mutation in *JAM3* often had severe perinatal intracranial hemorrhage, but no obvious polymicrogyria was noted.

Two families have been reported with perisylvian polymicrogyria and mutations in the sushi-repeat-containing gene *SRPX2*, located at Xq22; one mutation (N327S) was found in a French family presenting with rolandic seizures, oral and speech dyspraxia, and variable degrees of intellectual disability, and a second mutation (Y72S)



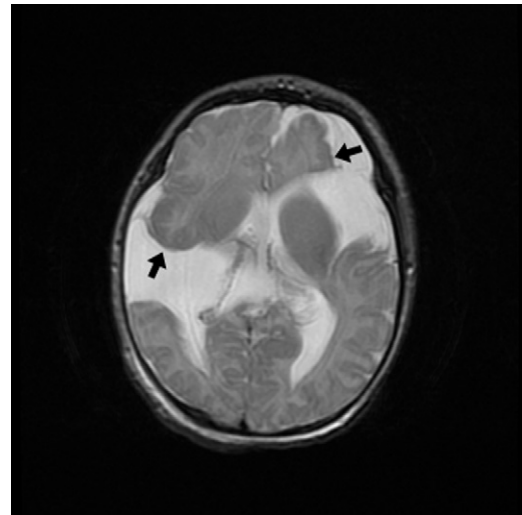
was reported in a male patient with seizures of the rolandic area and BPP whose female relatives with the same mutation had only mild intellectual disability without polymicrogyria (306).

### 115.4.2 “Tubulinopathies” and Polymicrogyria

As mentioned above, *TUBA1A* is a gene encoding a microtubule-related protein that is associated with brain malformations, typically lissencephaly with other features. Recent work suggests that mutations in *TUBA1A* are also responsible for some cases of BPP (187,189). Although it is not a common cause of BPP, the association between this gene—known to be involved in cortical migration—and a defect such as polymicrogyria that is thought to reflect a disorder of later cortical organization speaks to the idea that many of the genes in brain development are important through multiple stages of brain development. Along the same lines, mutations in the gene encoding  $\beta$ -tubulin, *TUBB2B*, have been reported in five cases of asymmetrical polymicrogyria (OMIM 610031) (307). Mutations in *TUBA8* have been implicated in a form of polymicrogyria associated with optic nerve hypoplasia (OMIM 613180) (308). Additionally, mutations in the gene *TUBB3* have been seen in cases of frontally predominant polymicrogyria associated with basal ganglia dysmorphism, corpus callosum abnormalities, and mild brainstem hypoplasia (309) without the feature of congenital fibrosis of the extraocular muscles reported in the original description of *TUBB3* mutations (310).

### 115.4.3 Schizencephaly

The term “schizencephaly” (OMIM 269160) was coined by Yakovlev and Wadsworth in 1946 and refers to a brain malformation characterized by full-thickness cleft of the cerebral mantle (311,312). The walls of the clefts are usually lined by polymicrogyric cortex. As Yakovlev and Wadsworth proposed, schizencephaly can be divided into two subtypes, namely closed-lip schizencephaly and open-lip schizencephaly. In closed-lip schizencephaly, two walls are in apposition and form a so-called “pial-ependymal seam.” On the other hand, in open-lip schizencephaly the two walls are apart and the space between the two walls is filled with cerebrospinal fluid (CSF) (Figure 115-5). The clinical presentation varies depending on the extent and location of anatomical abnormalities. Closed-lip schizencephaly often presents with hemiparesis or motor delay, and open-lip schizencephaly may present with the same features as well as seizures (313). Generally, the severity of motor and/or cognitive impairment is related to the extent of anatomic malformation; however, the presence or severity of epilepsy may not be predicted from the extent of the anatomical abnormality. Although schizencephaly is usually seen as sporadic cases, rare familial cases have been reported (314–318).



**FIGURE 115-5** T2-weighted axial MRI image of a patient with bilateral open-lip schizencephaly is shown. The walls of the full-thickness clefts are lined by abnormal cortices (arrows), which often prove to be polymicrogyria histologically.

The presence of *de novo* heterozygous mutations in the *EMX2* gene has been reported in sporadic and familial cases of schizencephaly (319–321); however, subsequent sequencing effort failed to identify any pathogenic *EMX2* mutation in a total of more than 100 patients with schizencephaly (322,323). Thus the role that *EMX2* plays in pathogenesis of schizencephaly is not entirely clear. Recently, a few patients with *WDR62* mutations were found to have schizencephaly as well as microcephaly (32,34), but the proportion of cases of schizencephaly caused by *WDR62* mutation is not known. Schizencephaly can also definitely be caused by nongenetic etiology, such as the death of monozygotic cotwin (324), and is associated with environmental risk factors, such as in utero exposure to warfarin, alcohol and cocaine and young maternal age (324), to a much greater extent than any other cortical malformation, suggesting a substantial role for nongenetic factors as well.

## 115.5 MALFORMATIONS OF CORTICAL DEVELOPMENT, NOT OTHERWISE CLASSIFIED

### 115.5.1 Malformations Secondary to Inborn Errors of Metabolism

Typically, inborn errors of metabolism cause degenerative cerebral lesions; however, several metabolic disorders are associated with developmental malformations of the cerebral cortex. For example, neuronal migration abnormalities, particularly perisylvian pachygyria-polymicrogyria, are a prominent feature of Zellweger syndrome (325–328). Pathological studies have shown abnormal pleomorphic cytosomes, presumably the results of excessive very-long-chain fatty acids, in astrocytes, neuroblasts, immature neurons and radial glia,

suggesting linkage between the underlying biochemical abnormality and migrational disturbances (326). Studies of an animal model of Zellweger syndrome have suggested that peroxisomal function in both brain and extraneuronal tissues is important to the normal neuronal migration (329).

Other inborn errors of metabolism associated with developmental disorders of the cerebral cortex include multiple acyl-CoA dehydrogenase deficiency (glutaric aciduria type II; OMIM 231680) (330) as well as mitochondrial disorders and disorders of pyruvate metabolism (331–334). The pathogenesis of cortical dysgenesis in these disorders is not well understood. Amish lethal microcephaly (OMIM 607196) is a metabolic disorder reported among the Old Order Amish of Lancaster County, Pennsylvania. The patients have severe congenital microcephaly and 2-ketoglutaric aciduria; they typically die within the first year of life. This is one of the few known metabolic disorders that are associated with congenital, rather than postnatal, microcephaly. The causative gene is *SLC25A19* (335), which may be involved in mitochondrial thiamine pyrophosphate transport and affects the function of the  $\alpha$ -ketoglutarate complex (336). Other metabolic causes of congenital microcephaly include maternal phenylketonuria and phosphoglycerate dehydrogenase deficiency (24).

## 115.6 CONCLUSIONS

Recent years have seen dramatic progress in the area of genetic malformations of the cerebral cortex. As more and more genes for brain malformations are identified, clinical syndromes are being redefined based on their underlying genetic basis. Although some of the disorders presented here are rare, they collectively account for a large number of patients with neurological disabilities. Identification of the causative genes has already led us toward the development of genetic testing and better genetic counseling for the patients and their families. In addition, studying the functions of these genes continues to lead to a better understanding of biological mechanisms of the human brain development. There still remains a stunning diversity of genetic disorders of cortical development that are yet to be characterized and studied, and each year brings further progress in understanding the genetic basis of many of those disorders. With the advent of next-generation sequencing, we expect to see an ever-increasing number of genes involved in brain development identified and characterized through the study of human cerebral malformation syndromes.

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- GeneTests, <http://www.ncbi.nlm.nih.gov/sites/GeneTests/>.
- Christopher, A. Walsh Laboratory, <http://www.walshlab.org/>.

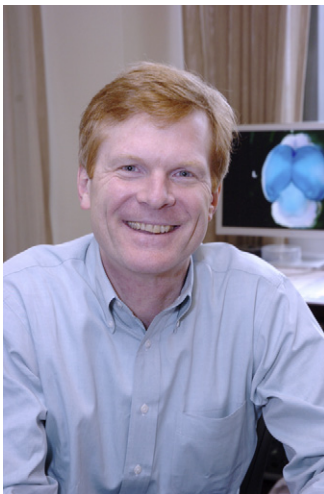
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**Dr Christopher A Walsh, MD, PhD** is Bullard Professor of Pediatrics and Neurology at Harvard Medical School, Chief of the Division of Genetics at Boston Children's Hospital, and an Investigator of the Howard Hughes Medical Institute. He completed his MD and PhD degrees at the University of Chicago. After a neurology residency and chief residency at Massachusetts General Hospital, he completed a fellowship in genetics at Harvard Medical School. He joined the faculty at Harvard Medical School in 1993 and has held the Bullard Professorship since 1999. Dr Walsh's research has focused on the development, evolution, and function of the cerebral cortex. He has pioneered the analysis of human genetic diseases that disrupt the cerebral cortex, including autism, intellectual disability, seizures, and cerebral palsy, identifying genetic causes for more than a dozen brain diseases of children. Among his awards are a Jacob Javits Neuroscience Investigator Award (National Institute of Neurological Disorders and Stroke), the Dreifuss-Penry Award Epilepsy Research Award (American Academy of Neurology), the Derek Denny-Brown Award and the Jacoby Award (American Neurological Association), the Research Award (American Epilepsy Society), and the Wilder Penfield Award (Middle Eastern Medical Assembly). He is an elected member of the American Neurological Association, the American Association of Physicians, and an elected fellow of the American Association for the Advancement of Sciences.

# CHAPTER

# 116

## Genetic Aspects of Human Epilepsy

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### ABBREVIATIONS

ACH – acetylcholine  
 AD – autosomal dominant  
 ADNFLE – autosomal dominant nocturnal frontal lobe epilepsy  
 ADPEAF – autosomal dominant partial epilepsy with auditory features  
 AED – antiepileptic drug  
 AGAT – arginine glycine amidino transferase  
 ANT1 – adenine nucleotide translocator 1  
 AR – autosomal recessive  
 ARFGEF2 – ADP-ribosylation factor guanine nucleotide-exchange factor-2  
 ARX – aristaless-related homeobox  
 AS – angelman syndrome  
 BECTS – benign epilepsy with central temporal spikes  
 BFIC – benign familial infantile convulsions  
 BFNC – benign familial neonatal convulsions  
 BFNIS – benign familial neonatal-infantile seizures  
 BTD – biotinidase  
 CACN1H – calcium channel  
 CAE – childhood absence epilepsy  
 CGH – comparative genomic hybridization  
 CHRNA4 – nicotinic cholinergic receptor alpha 4 subunit  
 CLCN – chloride channel  
 CNS – central nervous system  
 CPS – complex partial seizures  
 CSTB – cystatin B  
 DCX – doublecortin gene  
 DRPLA – dentatorubro-pallidoluysian atrophy  
 DS – Down syndrome  
 DZ – dizygotic twin  
 EEG – electroencephalogram  
 EPM1, EPM2 – epilepsy, progressive myoclonic, type 1/2  
 EPTP – epitempin  
 FEB1-4 – febrile seizure gene loci  
 FLNA – filamin A

FS – febrile seizures  
 GABAA – gamma aminobutyric acid receptor type A  
 GABAB – gamma aminobutyric acid receptor type B  
 GABARA1 – gamma aminobutyric acid receptor alpha 1 subunit  
 GABARG2 – gamma aminobutyric acid receptor gamma 2 subunit  
 GAMT – guanidino acetate methyl transferase  
 GEFSP – generalized epilepsy with febrile seizures plus  
 GLUT1 – glucose transporter 1  
 GTC – generalized tonic clonic seizures  
 hMASS1 – human ortholog, monogenic audiogenic seizure susceptibility gene  
 IGE – idiopathic generalized epilepsy  
 ILAE – International League Against Epilepsy  
 JAE – juvenile absence epilepsy  
 JME – juvenile myoclonic epilepsy  
 KCNQ2 – potassium channel  
 LGI1 – leucine rich glioma inactivated gene 1  
 M CURRENTS – muscarinic currents  
 MECP2 – methyl CPG-binding protein  
 MELAS – mitochondrial encephalopathy, lactic acidosis and stroke  
 MERRF – myoclonic epilepsy with ragged red fibers  
 MIM – Mendelian inheritance in man  
 MLPA – multiplex ligation-dependent probe amplification  
 MTHFR – methylenetetrahydrofolate reductase  
 MZ – monozygotic twin  
 NCL – neuronal ceroid lipofuscinosis  
 NFLE – nocturnal frontal lobe epilepsy  
 NH – nodular heterotopia  
 NHLRC1 – NHL repeat containing gene 1  
 NKH – nonketotic hyperglycinemia  
 PAS – periodic acid-Schiff  
 PH – periventricular heterotopia  
 SBH – subcortical band heterotopia  
 SCN2A – sodium channel  
 SLC2A – solute Carrier Family 2, Member 1

SMEI – severe myoclonic epilepsy of infancy  
 SNPs – single nucleotide polymorphisms  
 SPS – simple partial seizures  
 TNEP1/C21LRP – chromosome 21 leucine rich protein  
 TSC – tuberous sclerosis  
 TWINKLE – novel mitochondrial protein helicase  
 UBE3A – ubiquitin protein ligase E3A  
 VLGR1 – very large G protein-coupled receptor 1  
 VPA – Valproic acid  
 WHS – Wolf–Hirschhorn syndrome  
 XLIS – X linked lissencephaly

## 116.1 INTRODUCTION

Epilepsy is common, and affects 1% of the world population. The term “epilepsy” is drawn from the Greek word “*Epilambanein*”—that means to seize upon. In medical writings as early as 400 B.C., epilepsy was accorded the status of “*a sacred disease*”. The disorder is far reaching in its impact as fear, social stigma, and isolation taint affected individuals and families even today (1).

Human epilepsy is heterogeneous in origin; acquired causes (trauma, tumor, infection, vascular insufficiency, metabolic and drug induced encephalopathies) account for a third of all cases, in the remaining two thirds, a specific etiology is obscure (2). In the Rochester epidemiology project, the cause of epilepsy was presumed to be “idiopathic” in 68% of the incidence cases (1935–1984) signifying that familial/genetic factors were considered as contributory (3). The first human epilepsy gene (mutation involving a nicotinic cholinergic receptor subunit) associated with an idiopathic partial epilepsy syndrome (autosomal dominant nocturnal frontal lobe epilepsy) was identified in 1995 (4). With the convergence of bioinformatics, and high throughput technologies, rapid progress has been made possible in the identification of gene defects for many disorders including epilepsy. The use of microarray technology, next generation rapid sequencing technologies and exome sequencing will further enhance our knowledge about pathogenic gene mutations, and mechanisms underlying gene expression, and regulation of the level and timing of expression (5).

In this chapter, we will highlight the advances in our understanding of the genetic basis of epilepsy, its relevance in the evaluation and management of patients with epilepsy in the clinical setting.

## 116.2 EPILEPSY: DEFINITION

“Epilepsy” is recognized as a disorder in which recurrent seizures occur, accompanied by a variety of clinical phenomena involving motor, sensory, cognitive, psychic and autonomic manifestations, with or without loss of consciousness. The occurrence of at least two unprovoked seizures are necessary to fulfill generally accepted criterion to establish the diagnosis of epilepsy.

## 116.3 EPILEPSY: NEUROPHYSIOLOGICAL BASIS

The central nervous system (CNS) functions as a complex interconnected neuronal network. The clinical manifestations of epilepsy result from hypersynchronous firing of neurons within such cortical networks. Regions of hyperexcitability capable of spontaneous and aberrant behavior can be identified within epileptogenic foci. Intracellular recordings from neurons in such foci demonstrate the occurrence of spontaneous synchronized depolarization of large amplitude (paroxysmal depolarization shift), which is considered the electrophysiological equivalent of the epileptic spike seen on the electroencephalogram (EEG) (6).

Normally, this regional expression of hyperexcitability is limited and constrained by counterbalancing inhibitory processes within the brain. Under pathological conditions, simultaneously occurring increments in excitatory influences accompanied by decrements in inhibitory effects could potentially lead to an unstable electrical state, promoting cortical hyperexcitability and regional spread of excitation. Ultimately, the recruitment of increasing number of neurons and propagation of aberrant excitability through neuronal networks to other regions underlie the diverse seizure semiology encountered in the form of partial and/or complex partial seizures.

The anatomic substrate of a generalized spike wave discharge seen on the EEG in generalized epilepsies involves synchronization between neocortical pyramidal neurons, thalamic relay neurons, and GABAergic neurons of the nucleus reticularis thalami. Current electrophysiological and imaging studies in humans and animal models suggest that the entire brain is not involved homogeneously and the generation of generalized spike wave discharges do not involve a single common pathway or mechanism. Selective thalamocortical network loops are involved in the generation of the typical spike wave discharges in certain forms of generalized epilepsies, while focal or regional aberrations within cortical networks may be sufficient to provoke a transition to generalized spike waves from normal rhythms within thalamocortical networks (7).

Thus, the epileptic seizure represents an endpoint of the interactions between anatomical, biochemical, molecular and electrophysiological changes leading to the generation of cortical hyperexcitability within neuronal networks and spread of excitation along different pathways.

## 116.4 EEG IN EPILEPSY

The EEG records at the scalp surface are the patterns generated by electrical potentials within the brain. These potentials are amplified several thousand times and can be visually analyzed using analog (old, paper



based) and digital instruments (current). The presence of epileptiform patterns on the EEG record, pattern and morphology (sharps, spikes and spike and wave complexes), location/distribution, provide diagnostic clues that help distinguish focal from generalized seizures. We now associate specific epileptiform patterns with certain epilepsy syndromes, given the right clinical context. Some common examples are the finding of a “generalized 3 Hz spike wave pattern” as a marker for childhood absence and other idiopathic generalized epilepsies, “generalized 4–6 Hz polyspike wave/fast spike wave variant” in the context of juvenile myoclonic epilepsy (JME), “central temporal spikes” with benign epilepsy of childhood with central temporal spasms or West syndrome. It was recognized in early studies conducted on family members of probands with epilepsy that pure epileptiform abnormalities on the EEG could be inherited as genetic traits. Thus, the EEG plays an indispensable role in the investigation of epilepsy, by providing critical information and aiding in defining a “seizure type” and “syndromic” approach to epilepsy.

## 116.5 SEIZURE TYPES, EPILEPSY SYNDROMES

The International League Against Epilepsy (ILAE) has spearheaded efforts to provide a classification of seizure types and epilepsy syndromes (Table 116-1). The previously adopted classification is in the process of being revised. In the present chapter, we have used the proposed new terminology to try and use current nomenclature. Seizure types are broadly divided into (self-limited and continuous seizures) and further subdivided into focal/partial and generalized seizures. Details of seizure types and different epilepsy syndromes are available through the 2006 report of the ILAE classification core group (8).

Further attempts have been made to evolve and adapt current knowledge into the organization of epilepsies into electroclinical syndromes. Current recommendations use concepts of “genetic,” “structural–metabolic” and “unknown” to replace the terms previously used, i.e. “idiopathic,” “symptomatic,” and “cryptogenic.” Further, epilepsies are organized into distinct electroclinical syndromes, constellations (used to denote those entities that do not fulfill the concept of a syndrome, but

**TABLE 116-1 Proposed Seizure Classification (Task Force on Classification and Terminology)**

Comments	
<b>Generalized Seizures</b>	Generalized seizures are characterized by the appearance of a generalized bisynchronous epileptiform patterns (spike wave and/or sharp wave complexes) at a variable rate on the EEG, the clinical manifestations vary from negative phenomena such as motor arrest and unresponsiveness, to positive motor phenomena (tonic, clonic, tonic clonic, spasms, myoclonus)
Tonic clonic	
(in any combination)	
Absence	
* Typical	
* Atypical	
* Absence with special features	
Myoclonic absence	
Eyelid myoclonia	
Myoclonic	
* Myoclonic	
* Myoclonic atonic	
* Myoclonic tonic	
Clonic	
Tonic	
Atonic	
<b>Focal Seizures</b>	Focal or partial seizures exhibit a focal or regional epileptiform patterns of the EEG, with clinical manifestations dictated by the region of the brain that is the source, and the subsequent spread and activation of other brain areas that lie in the path of discharge.
	<b>Descriptors for focal seizures dependent on degree of impairment of consciousness</b>
	*Without impairment of consciousness/responsiveness
	+With observable motor or autonomic components (roughly corresponds to the concept of simple partial seizure)
	*Involving subjective sensory or psychic phenomena only (corresponds to the concept of aura)
	*With impairment of consciousness/responsiveness (roughly corresponds to the concept of complex partial seizure)
	*Evolving to a bilateral, convulsive seizure (involving tonic, clonic or tonic and clonic components; replaces the term secondarily generalized seizure)
<b>May be focal, generalized, or unclear</b>	
Epileptic spasms	

nonetheless are distinctive enough to be diagnostically meaningful and/or carry implications for treatment such as hypothalamic hamartoma associated with gelastic seizures, and Rasmussen's encephalitis), epilepsy with structural-metabolic causes, and epilepsy with unknown cause. These concepts are elaborated in detail in a special report of the ILAE Commission on Classification and Terminology 2005–2009; Table 116-2 (9).

## 116.6 GENETIC STUDIES IN HUMAN EPILEPSY

Support for the heritable basis to epilepsy is currently based on findings from (1) aggregation studies in families and twins, (2) linkage analysis and positional cloning of susceptibility genes, (3) association studies, and (4) study of single gene defects associated with human epilepsy, single gene defects in animal models of epilepsy (10).

A considerable volume of evidence comes from family- and population-based studies and has been extensively reviewed (10,11). There is a two- to four-fold increase in risk for epilepsy in patient families with a first-degree relative with epilepsy (12). Twins share similar environmental influences superimposed on either a monozygotic (MZ) or dizygotic (DZ) twin background. In childhood absence epilepsy (CAE) the high concordance rates in population-based studies of twins with epilepsy (for MZ versus DZ twins) provide strong supportive evidence for a genetic contribution (13). The application of contemporary ILAE classification schema to historical seizure data on twins collected by Lennox also provide evidence for a high concordance rate for idiopathic generalized epilepsies (MZ to DZ ratio (0.80 versus 0.00)) (14) (Table 116-3). Twin studies enable differentiation between the effects of shared genetic background and shared environmental influences. While population-based twin studies do offer investigators the ability to carefully phenotype individuals, twins may not necessarily be representative of the entire population. Such limitations involve problems of bias due to an excess of female MZ twins within volunteer registries.

Family-based studies of “multiplex families” are considered when several individuals within a single pedigree appear to be affected by the same epilepsy syndrome. Advances in molecular genetics have permitted the identification of mutations that account for specific epilepsy syndromes using carefully selected families. In the last two decades investigator groups have been successful in identifying several “epilepsy genes.” Using families with large numbers of affected individuals, using linkage analysis and positional cloning, gene loci were mapped and mutations affecting genes coding for different channel proteins identified. A closer look reveals that these are mostly “monogenic” epilepsies, i.e. these involve families showing a clear pattern of segregation consistent with Mendelian laws of inheritance. The concept of inherited epilepsy as a “channelopathy” took shape on the basis of these studies (15).

### 116.6.1 Monogenic Epilepsy

The majority of the causative mutations identified seem to affect ion- or ligand-gated channels with few exceptions. Mutations involving different gene loci may be expressed through a single phenotype (locus heterogeneity), while mutations involving the same gene may be expressed through completely different phenotypes at an individual level (variable expressivity). For example, the syndrome of generalized epilepsy with febrile seizures plus (GEFSP) is known to be extremely variable in its clinical expression, with multiple seizure type combinations associated with and without fever described in individuals within the same family. In GEFSP, mutations in 3 different genes are described; two sodium channel genes, *SCN1B* (16) and *SCN1A* (17), and a GABA receptor gene, *GABRG2* (18). In addition, at least 3 additional gene loci (2p24, 21q22, 8p23-p21) linked to this epilepsy phenotype have also been identified (19).

Mutations of *SCN1A* are also known to be causally associated with severe myoclonic epilepsy of infancy (SMEI). These are mostly de novo and truncating mutations (20), while the mutations leading to GEFS+ tend to be missense in type (21). Mutations of the *SCN1A* gene have also been identified in an expanding spectrum of epilepsy, including infantile epileptic encephalopathies (22) as well as cryptogenic generalized and focal epilepsy (23).

Mutations identified in the leucine rich, glioma inactivated 1 gene (*LGII*) have been found in 50% of families with two or more affected individuals with autosomal dominant partial epilepsy with auditory features (ADPEAF), however, in sporadic patients with the same clinical features, none were shown to carry an inherited mutation (24). Thus, the syndrome is dominant in some families; in others a “complex inheritance” is considered likely. These issues highlight the difficulties in fitting genetics of epilepsy into simpler schemes of Mendelian inheritance.

In addition to monogenic epilepsies above, nontraditional inheritance models also account for disorders with an inherited basis, where epilepsy is a prominent feature of the overall phenotype. These include imprinting disorders (differential expression of genes based on a parent of origin effect) (e.g. Angelman syndrome, mitochondrial inheritance) (e.g. myoclonic epilepsy with ragged red fibers, MERRF) and triplet repeat expansion (Dentatorubropallidoluysian atrophy, DRPLA) and dodecamer repeats (progressive myoclonic epilepsy). These have been extensively reviewed and discussed later (25).

### 116.6.2 Epilepsy with Complex Genetics

For geneticists, the former “idiopathic” epilepsy represents “epilepsy with complex genetics or complex epilepsy” i.e. multifactorial disorder with polygenic inheritance influenced by environmental factors as well as the internal milieu of the individual.

**TABLE 116-2 Proposed ILAE Classification of Epilepsy syndromes**

<b>Electroclinical Syndromes and Other Epilepsies</b>
Electroclinical Syndromes Arranged by Age at Onset*
Neonatal period
Benign familial neonatal seizures (BFNS)
Early myoclonic encephalopathy (EME)
Ohtahara syndrome
Infancy
Migrating partial seizures of infancy
West syndrome
Myoclonic epilepsy in infancy (MEI)
Benign infantile seizures
Benign familial infantile seizures
Dravet syndrome
Myoclonic encephalopathy in nonprogressive disorders
Childhood
Febrile seizures plus (FS+) (can start in infancy)
Early-onset benign childhood occipital epilepsy (Panayiotopoulos type)
Epilepsy with myoclonic atonic (previously astatic) seizures
Benign epilepsy with centrotemporal spikes (BECTS)
Autosomal-dominant nocturnal frontal lobe epilepsy (ADNFLE)
Late-onset childhood occipital epilepsy (Gastaut type)
Epilepsy with myoclonic absences
Lennox–Gastaut syndrome
Epileptic encephalopathy with continuous spike and wave during sleep (CSWS) including:
Landau–Kleffner syndrome (LKS)
Childhood absence epilepsy
Adolescence–Adult
Juvenile absence epilepsy (JAE)
Juvenile myoclonic epilepsy (JME)
Epilepsy with generalized tonic-clonic seizures alone
Progressive myoclonus epilepsies (PME)
Autosomal dominant partial epilepsy with auditory features (ADPEAF)
Other familial temporal lobe epilepsies
Less Specific Age Relationship*
Familial focal epilepsy with variable foci (childhood to adult)
Reflex epilepsies
<b>Distinctive constellations</b>
Mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE with HS)
Rasmussen syndrome
Gelastics seizures with hypothalamic hamartoma
<b>Epilepsies attributed to and organized by structural-metabolic causes</b>
Malformations of cortical development (hemimegalencephaly, heterotopias, etc.)
Neurocutaneous syndromes (Tuberous sclerosis complex, Sturge–Weber, etc.)
Tumor
Infection
Trauma
Angioma
Peri-natal insults
Stroke
Etc.
<b>Epilepsies of unknown cause</b>
<b>Conditions with epileptic seizures that are traditionally not diagnosed as a form of epilepsy per se</b>
Benign neonatal seizures (BNS)
Febrile seizures (FS)

Online source: <http://www.ilae-epilepsy.org/Visitors/Documents/ClassificationSummaryReportwebAug2009.pdf>

**TABLE 116-3** Concordance Rate for Epilepsy among MZ and DZ Twin Pairs

Study Authors	Nature of Epilepsy in Proband	Concordance Rates in Twins	
		Monozgotic <i>N</i> (%)	Dizygotic <i>N</i> (%)
Berkovic et al., 1998	Major epilepsy syndromes	108(62)	145(18)
Corey et al., 1991	Febrile seizures	95(19)	157(6)
Sillanpaa et al., 1991	Epilepsy	79(12)	201(3)

In “complex epilepsy” multiple susceptibility genes, and a triggering effect from the environment may be involved. There are no clear segregation patterns, and the phenomena of epistasis (intragenic interactions) influencing the gene expression are possible. Pleiotropic effects and epigenetic modification of expression also influence the final phenotype. The resulting phenotypic variability is such that stratification may be a problem.

Any discussion of epilepsy with complex genetics is linked to the nature of genetic variation and the role of natural selection in the occurrence of complex traits. A precise understanding of the nature of genetic variation in causation of epilepsy remains to be elucidated. Genetic variants linked to epilepsy could be common (allele frequencies >1%) or rare (allele frequencies <1%). The number and frequency of the susceptibility alleles are influenced by mutation, genetic drift and natural selection in the population. The common disease common variant model assumes that disease-causing variants have resisted natural selection to achieve a significant threshold within the population. This model has not found much support in terms of the identification of many common variants underlying human epilepsy, as association studies have failed to deliver consistent results (26). Another model, the common disease rare variant hypothesis (also called the multiple rare variant common disease model (MRVCE)) has been considered to be more applicable in the context of epilepsy. This model for complex epilepsy seems to involve causative variants in susceptibility genes drawn from a population of genes (with rare and low frequency polymorphic alleles) where none of the variants are sufficient in themselves to give rise to an epilepsy phenotype independently. The susceptibility alleles may give rise to the phenotype in different combinations, with each combination leading to a different endophenotype. As several single gene defects underlying monogenic epilepsy seem to code for proteins that make up ion- or ligand-gated channels, it would be natural to assume that variants in the genes coding for ion channels and their receptors would also be involved in complex epilepsy. Initial studies of variant polymorphisms with functional consequences—calcium channel subunits CACNA1H (CAE),  $\gamma$ -aminobutyric acid receptor subunits GABARD (GEFS+), and GABARB3 (idiopathic generalized epilepsy; IGE), potassium channel subunits KCND2 (TLE)—show themselves as carrying weak effects as far as their contribution to susceptibility to epilepsy is concerned. None of these variants have

been individually shown to have a causal association with genetic epilepsy (26,27).

From the foregoing discussion, both rare and common variant models for epilepsy individually are not broadly applicable to common epilepsy. To get around this problem a blended approach involving both the MRVCE (discussed above) and an ancestral common variant common epilepsy model has been suggested. In this polygenic heterogeneity model, the final expression of a disorder is determined by permutations and combinations of both common and low frequency polymorphic variants (26,28). With this background, it can be stated (at the risk of some oversimplification) that current thinking about genetic influences on the risk of epilepsy conceptually consist of the net effects resulting from a mix of “susceptibility genes with large effects” and those “with smaller effects,” the former producing Mendelian inheritance patterns, while the latter produce complex inheritance patterns. The detection and the construction of a composite of all genes with different contributions to determining susceptibility to epilepsy is the challenge for investigators. With the advent of high throughput technologies to study stable alterations in gene expression, contributions from structural variants in the genome (insertions, deletions duplications, inversions, and copy number variations) and nucleotide polymorphisms (SNP's) are seen to assume increasing importance in complex epilepsy.

### 116.6.3 Copy Number Variation in Epilepsy

Although a significant part of the human genome is conserved through evolution, even within these conserved regions, there are variations involving insertions and deletions of DNA ranging from 1 kb to several megabases constituting up to 12% of the human genome. Most of these variations termed copy number variants (CNV) are usually of no clinical significance (29); some, however, are considered as a source of mutations. CNV's can be detected by array specific comparative genomic hybridization (arrayCGH) and by SNP genotyping arrays (30). These technologies can be used to scan the entire human genome without any prior knowledge of the location of the “lesion.” CNVs can also be detected in targeted regions of the genome by the multiplex ligation-dependent probe amplification (MLPA) method. Previous studies have shown that CNV's are associated with many disorders with complex inheritance patterns



(intellectual disability, schizophrenia, and autism), and now have come under the scanner for complex genetically determined epilepsies (31). CNV's can be recurrent due to nonallelic homologous recombination in regions of segmental duplication. At least three microdeletions (15q11.2, 16p13.11 and 15q13.3) are now known to be associated with epilepsy; their combined frequency in populations with genetically generalized epilepsy is about 3%. The 15q13.3 microdeletion appears to be more specific for generalized epilepsy syndromes, whereas the other two have been described with both generalized and focal epilepsy syndromes (32). These larger recurrent CNVs appear to occur in rearrangement prone regions of the genome, hence detected more easily. The likelihood that rare nonrecurrent CNVs are also associated with epilepsy is a finding confirmed in recent studies (33). It has been suggested that the recurrent CNVs described above are providing an outline that encompasses a group of different electroclinical phenotypes within a "constellation" of genetic generalized epilepsies (childhood absence epilepsy, juvenile absence epilepsy, juvenile myoclonic epilepsy) syndromes.

## 116.7 MECHANISTIC DIVERSITY IN GENETIC EPILEPSY

The regulation of membrane excitability is a function of many types of gating channels. An ionic concentration gradient is maintained by all cells, with potassium being predominantly intracellular in its distribution, while sodium, chloride, and calcium are maintained at a higher extracellular concentration. These ionic gradients are maintained through the activity of ion channels that act as transmembrane pores, permitting rapid ionic fluxes to occur along electrochemical gradients. The ionic channels are capable of undergoing conformational changes, between an open and a closed state, enabling selective permeability to different ions, and to the direction of their flow under changing conditions. These processes are critical to electrical signal generation and transmission within the nervous system.

Ion channels are categorized as voltage-gated (their opening and closing depends on the electrical state of the neuronal membrane), or ligand-gated (the channel function is regulated by a ligand such as a neurotransmitter molecule, e.g. Ach or GABA, second messengers and metabolites). The location of these channels along the neuron, dendrites, and axons, presynaptic and postsynaptic processes is highly specialized. Regional differences in the distribution of ion channels, ligand-gated channels and signaling proteins, permits regional specialization of function. Structurally, these channels (Table 116-4) are formed by homomeric or heteromeric protein subunits coded by many different genes (34). Each ion- or ligand-gated channel is dependent on different families of subunits encoded by a multigene complex. The functional consequences of mutations in genes encoding

channel subunits can be variable. There may be a simple straightforward effect of either the loss of function or at other times a gain of function resulting in more complex effects on channel function and resultant seizure susceptibility. It is, however, important to point out that not all idiopathic epilepsies are channelopathies. A partial epilepsy syndrome (autosomal dominant lateral temporal epilepsy) is linked to a gene *LGII*, where the mutation involves a novel repeat motif coding for a leucine rich repeat protein, although the precise mechanism of how this links to epileptogenesis remains unknown. There are other examples for instance; mutations involving the *SLC2A1* gene that codes for a glucose transporter (GLUT1), that underlie early onset absence epilepsy, and the *EFHC1* gene mutations identified in juvenile myoclonic epilepsy point to the mechanistic diversity leading to seizure susceptibility.

### 116.7.1 Voltage-Gated Ion Channels and Epilepsy

**116.7.1.1 Loss of Function Mutations.** The identification of K channel mutations (KCNQ2, KCNQ3) associated with benign familial neonatal convulsions (BFNC) (35) preceded the recognition of Na channel (SCN2A) defects in the syndrome of benign familial neonatal-infantile seizures (BFNIS) (36). These findings provide irrefutable evidence linking ion channel dysfunction to epilepsy syndromes due to the loss of function effects of the pathogenic mutation. Voltage-gated potassium channels play a crucial role in neuronal physiology, involving post excitatory membrane repolarization by influencing M currents (K currents produced by activation of muscarinic receptors). When expressed in *Xenopus* oocytes, wild type KCNQ2 channels facilitate the development of M currents, while mutant channels show no such currents (37). By impairing the restoration of ionic balance after neuronal firing, potassium channel dysfunction likely leads to repetitive neuronal firing and a state of heightened excitability.

Sodium channel mutations also lead to a wide variety of neurological phenotypes, particularly within epilepsy syndromes including BFNIS, GEFS, and severe myoclonic epilepsy of infancy (Dravet syndrome). Sodium channels consist of an  $\alpha$  subunit (9 isoforms Nav1.1–Nav1.9) associated with two of four auxiliary  $\beta$ 1,  $\beta$ 2,  $\beta$ 3 and  $\beta$ 4 subunits in the CNS in a complementary fashion. The  $\alpha$  subunit contains the pore, whereas the associated  $\beta$  subunit determines the voltage dependency and other aspects of channel kinetics. There are four homologous domains (I–IV) and six transmembrane segments (S1–S6) forming  $\alpha$  subunits. A hairpin like P loop connects S5 and S6 forming a pore, while the intracellular loop connecting the domains III and IV forms the channel-inactivating gate (38). In the described mutations affecting BFNIS families, changes have been identified in the

**TABLE 116-4    Ion Channels: Structure and Subunit Composition**

<b>Ion Channel</b>	<b>Type</b>	<b>Associated Epilepsy</b>	<b>Composition</b>	<b>Mutations</b>	<b>Effect on Channel</b>
Sodium channel SCN1A/SCN2A SCN1B	Voltage-gated	GEFSP BFNIS SMEI	Each sodium channel is formed by 1 pore forming $\alpha$ -subunit and two $\beta$ -subunits that serve to modulate channel-gating kinetics. Each $\alpha$ -subunit is made from four tandem domains, each of which contains six transmembrane domains	SCN1B $\beta$ 1C121W SCN2A $\alpha$ 2L1330F $\alpha$ 2L1563V SCN1A Mutations are many and often occur de novo in the gene coding for this channel	Loss of function Slower
Potassium channel KCNQ2/KCNQ3	Voltage-gated	BFNC BFNC with myokymia	Each K channel is a tetramer made of four homologous subunits that contain six transmembrane domains. The fourth transmembrane domain carries amino acids that are positively charged which effects conformational change during depolarization. The pore-forming loop connects domains five and six	Multiple mutations described for KCNQ2	Loss of function Reduced current Both KCNQ2 and KCNQ3 are heteromultimers
Chloride channel	Voltage-gated	IGE	Chloride channels have two subunits each with its own pore	Three different mutations M200fsX231 IVS2-14del11, del 74-117 G715E	The first identified mutation in the $\text{ClC-1}$ gene
Nicotinic acetylcholine receptors CHRNA4/CHRNB2	Ligand-gated	NFLE	Pentamers with each subunit containing four transmembrane domains. In the human brain there are 10 subunits that can be expressed and may combine in heteromeric or homomeric combinations to form receptors. Relatively non selective channel	CHRNA4 $\alpha$ 4S248F $\alpha$ 4L776ins3 $\alpha$ 4S252L CHRNB2 B2V287L $\beta$ 2V287M	Gain of function Resulting in a nonfunctional receptor Mutations in the $\text{CHRNA4}$ gene
$\gamma$ -aminobutyric acid, subtype A GABRG2/GABRA1	Ligand-gated	GEFSP ADJME	Also shares a pentameric structure with four transmembrane domains Channel shows selective permeability to small anions and allows both chloride and bicarbonate	GABRG2 $\gamma$ 2K289M GABRA1 $\alpha$ 1A322D	Loss of function Mutations in the $\text{GABRG2}$ gene

$\alpha 2$  subunit of the sodium channel (SCN2A). Both the known mutations (L1330F, and L1563V) affect the highly conserved leucine, which results in an increased sodium current, which in turn reduces the rate of channel inactivation. SCN1A mutations have been described in association with GEFSP, Dravet syndrome and severe idiopathic generalized epilepsy of infancy (39). SCN1B mutations have been associated with GEFSP as well as a purely partial temporal lobe epilepsy phenotype (16,40).

**116.7.1.2 Gain of Function Mutations.** Activating mutations in inward rectifying potassium channels (KCNJ11) where a gain of function effect leads to a syndrome of developmental delay, neonatal diabetes, and epilepsy (41). Here the effects of heterozygous mutations in the *KCNJ11* gene result in the reduction of K(ATP) channel ATP sensitivity, increasing the K(ATP) current, which in turn inhibits beta-cell electrical activity and insulin secretion leading to diabetes, and in the brain paradoxically leads to increased excitability and epilepsy in the neonatal period. The condition can be ameliorated by the administration of a sulfonylurea that closes the potassium channel through an independent mechanism, particularly if the condition is recognized early (42).

The T type calcium channel is an obvious target channel as it plays a critical role in the thalamocortical oscillatory network. Missense mutations in a heterozygous state were identified in a gene coding for a calcium channel (CACN1H) in the 16p13 region in a small group of individuals with absence epilepsy, suggesting a role for calcium channels in the genetic susceptibility to absence epilepsy (43). The described mutations affect highly conserved regions in the T type calcium channel, functional studies using site-directed mutagenesis have shown a gain of function effect on calcium influx during physiological activation leaving the neuron vulnerable to repetitive firing (44).

**116.7.1.3 Ligand-Gated Ion Channels (Nicotinic Cholinergic Receptors, GABA Receptors).** The first evidence linking epilepsy to ion channel dysfunction came with the identification of a missense mutation ( $\alpha 4$ S248F) in the  $\alpha 4$  subunit of the nicotinic acetylcholine receptor (CHRNA4) in all 21 members of an Australian family affected by autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) (4). Different mutations in the *CHRNA4* gene (45), as well as in the gene coding for the *CHRNA2* ( $\beta 2$  subunit of the cholinergic receptor) have been identified in other families (46). The cholinergic receptor is a pentameric structure that is nonselective in its permeability to Na, K, and Ca. It appears that mutations directly affecting the channel pore can result in the epilepsy phenotype, as all of the known mutations appear to affect transmembrane domains that form the wall of the ion channel. Differences in the electrophysiological and pharmacological profiles among the different receptor mutations have been shown, such as increased cholinergic sensitivity, and reduced calcium permeability. It has been suggested that these differences

in mutant receptor function may account for additional specific phenotypic features such as the occurrence of neuropsychiatric symptoms (seen in individuals carrying the  $\alpha 4$ 776ins3 mutation) (47). In a family with individuals affected only with JME, a novel gene mutation (Ala322Asp) affecting the gene encoding for GABARA1 ( $\alpha$  subunit of the  $\gamma$ -aminobutyric acid receptor subtype A) has been identified. Voltage clamp recordings from HEK293 whole cell lines transfected with mutant and wild type GABA receptors show a reduction in GABA activated currents in the former. This observed effect is attributed to altered GABA receptor function, and not to reduced expression (48).

GABA receptor dysfunction can also be caused by mutations affecting other channels. Three different heterozygous mutations resulting in (1) a premature stop codon (M200fsX231), (2) an atypical splicing site (del74–117), and (3) an amino acid substitution (G715E), have been identified in the voltage-gated chloride channel gene (*CLCN2*) that co-segregate with other IGE traits in three families of 46 individuals selected for the study (49). Whole cell patch clamp recordings done after transfecting cell lines (tsA201 cells) with both mutant and wild type genes suggest an alteration of human *CLCN2* channel function, affected through different mechanisms. The end result is the induction of a hyperexcitable state at GABAergic synapses at a postsynaptic level. Altered GABA-related inhibitory influences underlie the IGE phenotype, despite the differing molecular targets of gene mutations.

In the above discussion, the results of pathogenic single gene mutations on seizure susceptibility are illustrated. A logical extension of this discussion is whether pathogenic variations in ion channel genes are enough to influence channel function in different ways serving to explain genetic risk of seizure susceptibility in complex epilepsy. In experimental work on a digenic animal model, which was generated carrying two mutations one in shaker K channel (*kcna1*), and another in calcium channel subunit (*cacn1a*) gene respectively had shown that while the mutations occurring singly served to enhance seizure susceptibility, the effect of carrying both mutations in the same animal model, however, resulted in a masking effect on the absence epilepsy associated with the calcium channel mutation (50). Such interactions between the effects of pathogenic genomic variants could potentially explain phenotypic variation seen within families in genetically complex epilepsy.

In summary, the genetic aspects of epileptogenesis seem to be characterized by involvement of multitude of genes, and other epigenetic processes involved in the regulation of gene expression. Ultimately, there is as a consequence of a change in the balance between excitability and inhibition in the nervous system and a defect in synaptic transmission. There is firm evidence for mechanistic diversity in the pathogenesis of idiopathic epilepsies. The pathways involve “channelopathy genes” as well as

other nonion channel genes. Several themes appear to emerge from the above discussion; firstly, that mutations in different genes may encode for subunits of the same channel, resulting in an epilepsy phenotype that is essentially related to the channel dysfunction in a nonspecific manner; secondly, mutations affecting different channels may result in the same phenotype, by net effects converging on a common pathway (convergent effect); and finally a situation where mutations affecting subunits of the same channel that may produce two entirely different epilepsy phenotypes (divergent effect).

## 116.7.2 Other Mechanisms (Channel Independent)

**116.7.2.1 Secretin Receptor Genes and Epilepsy Associated Repeats.** The identification of mutation in a G protein-coupled receptor underlying a murine audiogenic epilepsy phenotype (Frings and Frings mouse) led to the search for mutations in the human ortholog (*hMASS1*) (51). The gene was mapped to the 5q34 locus that exhibits linkage to febrile seizures (FEB4). Screening for mutations in the *hMASS1* gene in a family with this febrile seizure phenotype led to the identification of a nonsense mutation. Although the above mutation did not co-segregate with the febrile seizure phenotype in all families, it was suggested that a loss of function in this gene could lead to febrile and afebrile seizures (52). The genes *hMass1* and *VLGR1* appear to be two halves of a single gene now called MASSive G protein-coupled receptor 1 gene (*MGR1*) that belongs to a family of genes coding for secretin-coupled receptors (53).

In the rare ADPEAF, a gene mutation in the *LGII* gene was identified; this mutation also involves nonion channel genes. The structural aspects of the proteins coded by this and other genes (*VLGR1* on 5q14.1) and *TNEP1*(*C21LR* gene on 21q22.3) share a seven-fold repeated motif in the C terminal region that appears to define a superfamily of leucine-rich proteins termed EPTP which share seven bladed  $\beta$  propeller fold. The mechanisms through which these gene products influence epileptogenesis remain unclear at this stage (54).

JME is a genetic epilepsy syndrome where mutations in both ion channels and nonion channel genes have been found to play a causal role in some families. While GABARA1 mutation is described above, mutation in the gene *EFHC1* has been described in some Hispanic, Japanese and Italian families with JME. The gene codes for a conserved protein that is located in the choroid plexus is found in flagella, and motile cilia, mitotic spindles and appears to play a regulatory role in cell division. The deficiency of the same protein ortholog in mouse models is associated with a higher incidence of myoclonus and seizure susceptibility (55).

**116.7.2.2 Glucose Transporter Deficiency (Cerebral Energy Deficit).** A haploinsufficiency of the glucose transporter (*GLUT1*) caused by mutations in the gene

belonging to the solute carrier family (*SLC2A1*) underlies diverse group of neurological phenotypes from infantile epileptic encephalopathy, early-onset absence epilepsy to genetic generalized epilepsies, and movement disorders (56). The cerebral energy deficit within the nervous system characterized by a hypoglycorrachia leads to alteration in seizure threshold. The epileptic phenotype is often accompanied by generalized 2.5–4 Hz spike and wave complexes on the EEG (57).

**116.7.2.3 Somatic Mutations as a Cause of Sporadic Epilepsy.** Mutations occurring in somatic cell DNA acquired during brain development, or even in germ line DNA could potentially provide a pathogenic mechanism for certain kinds of sporadic epilepsies. An influence on tissue patterning may lead to the occurrence of two populations of cells; that either possess (or lack) the mutation. These mutational alterations may express themselves either through structural changes, or changes in receptor subunits within regions critical to epileptogenesis, eventually leading to epilepsy. This appears to be the case in some families with X-linked mental retardation associated with epilepsy (58). In a recent report of two Italian families with two sibs affected with SMEI, their parents were unaffected or had only experienced febrile seizures during childhood (59). In one family, DNA assays on parental lymphocytes demonstrated evidence of somatic mosaicism and in the other family there was evidence for germ line mosaicism in the mother.

## 116.7.3 Epigenetic Modification and Phenotypic Variability in Epilepsy

The biological explanation for the diversity of cellular proteins and their function lies in understanding the process of modulation and regulation of gene expression during cell development and proliferation. The rapidly expanding field of epigenetics (originally a term introduced by C. H. Waddington) (60) sheds new light on our understanding of phenotypic variability. Epigenetic processes in the form of DNA methylation, changes in nucleosome architecture and its regulation, regulatory non coding RNA's regulation and RNA editing, modifications to the histone proteins do not directly affect the genetic code; however, they modulate gene expression through changes in chromatin accessibility. DNA methylation and demethylation events are stable and are passed on during mitotic cell division. DNA methylation occurs at cytosines that reside as dinucleotide CpG-rich islands at the 5' end of regulatory regions. Through the addition of a methyl group to these cytosines, the gene is effectively silenced. Thus, methylation and demethylation processes effectively function as off-on switches for gene expression. Rett syndrome, a neurodegenerative disorder with significant epileptic seizures results from heterozygous mutations in the X-linked methyl CpG-binding protein 2 (*MeCP2*) gene. The *MeCP2* gene



product functions as a transcriptional repressor by binding to methylated DNA. The genetic deregulation that follows the occurrence of mutations in the X linked *MeCP2* genes, has been shown to affect the expression of genes (UBE-3 ligase and GABRB3) at other locations e.g. 15q11–q13 (Angelman syndrome) through possible histone modifications (61). Both Angelman and Rett syndrome share significant features of their clinical phenotype particularly in the occurrence of epileptic seizures. In addition to methylation, reversible posttranslational modifications of histone proteins are also involved in regulating gene expression. Histone modifying enzymes (histone deacetylases HDAC or histone acetylases HATs) as well as histone methyltransferases (HMTs or DNMTs) are involved in dynamic interactions with chromatin in determining whether the genes remain accessible for transcription (activation) or in a closed inaccessible state (silenced). Mutations in the *ATR-X* gene are responsible for  $\alpha$ -thalassemia mental retardation syndrome, a condition where 30% of patients are affected with epilepsy. These patients present with altered DNA methylation profiles and changes in chromatin architecture. *ATR-X* gene codes for a protein that interacts with a number of other epigenetic factors, including *MeCP2*, and is involved in diverse functions including transcriptional regulation, heterochromatin formation (62). *ATR-X* may mediate epileptogenesis through its activity in the maintenance, survival and differentiation of inhibitory interneurons thus altering the balance between excitation and inhibition (63).

Cellular methylation patterns are affected by changes in diet (availability of folate), hormone levels as well as inherited genetic polymorphisms (64). These aspects of epigenetics open up a Pandora's box of potential issues pertaining to the effects of dietary modifications. Inherited genetic polymorphisms (such as the C677T polymorphism) have been linked to elevated homocysteine levels following antiepileptic drug (AED) therapy as well as an increased risk of epilepsy in offspring born to Scottish women homozygous to the C677T susceptibility allele (65). The direct or indirect effects of the antiepileptic drugs on methylation patterns and epigenetic regulation remain to be better understood. Valproic acid (VPA) used commonly in long term epilepsy treatment has been of particular interest, as it actively enhances replication independent demethylation of DNA. VPA has been shown to block seizure-induced neurogenesis through its effect of HDACs (66). Its effectiveness thus may be linked to the prevention of aberrant neurogenesis that has been associated with the pathological consequences of status epilepticus. VPA and several other antiepileptic drugs are also folate antagonists and their effects of folate deficiency on methylation patterns remains to be fully understood. Nevertheless, one could speculate that these effects may have something to do with the teratogenic effects on fetal development as a consequence of pregnancy-related exposure. The reader desiring to learn

more about epigenetic processes that influence epileptogenesis is referred to this elegant review (67).

### 116.7.4 Modifier Genes

A role for modifier genes is in the final expression of an epilepsy phenotype that could contribute to the observed variability in phenotypic expression within a defined epilepsy syndrome as observed in the GEFSP and in SMEI (68).

In a mouse model of GEFSP carrying the *scn1a* mutation, mutant alleles carrying coding and noncoding variants in sodium (*scn1a*, *scn2a*) and potassium channels (*kcnq2*) have been shown to have a deleterious effect on seizure severity, while the presence of a mutant (*scn8a*) allele restores normal seizure threshold, as well as prolonging survival of these GEFSP mutant mice (68). Evidence from studies of mouse models carrying ion channel mutation strongly suggest that the explanation underlying phenotypic variability encountered within families carrying for instance a *SCN1A* mutation may be influenced by genetic modifiers, stochastic events during development, epigenetic and environmental influences. So in the future, genetic assessments of risk for epilepsy as well as for guiding treatments, molecular work up of patients will need to focus on a whole panel of ion channel genes than a single affected gene, and will have to take into account the influence of variants in the coding and noncoding regions and other modifier genes.

The gene mutations so far described result in altered channel characteristics through loss or gain of function. Among the nonion channel genes, mutations appear to affect proteins that share structural similarities (seven-fold repeat motifs in the LGI family of proteins), leading to the speculation of a final common pathway that may or may not necessarily be linked to ion channels. The finding of involvement of a glucose transporter protein, and the *SLC2A* gene in association with the genetically generalized epilepsies, provides yet another example of the diverse mechanisms underlying genetic aspects of human epilepsy. The challenge of breaking out of the channelopathy paradigm is already bearing results given the expanding research interests into a genotype first approach using current high throughput technologies to study variation within the entire genome in populations with epilepsy. Such a genotype-first approach accompanied by a careful search for rare variants within the phenotype or constellation of genetic generalized epilepsies will lead to a knowledge base where one may begin to build a classification of epilepsies based on the recognition of the molecular genetic basis. Such diagnostic testing should be possible through the availability of next generation high throughput exome sequencing technologies. Combined with this will be an ever-greater need for powerful bioinformatics as there will be enormous data generated, to sift through and understand what is truly a significant result.

## 116.8 SELECTED GENETIC EPILEPSY SYNDROMES

For the purpose of discussion, we have grouped disorders on the basis of age of onset and recognized electroclinical syndromes. The symptomatic epilepsies with a genetic basis present a challenge to categorize, as there is too much variability in seizure phenotype. Details of clinical descriptions are considered in brief or provided in several tables. (Table 116-5) The following discussion focuses on the gene defects identified and implications for the epilepsy phenotype.

### 116.8.1 Genetic Generalized and Focal Epilepsy syndromes

**116.8.1.1 Neonatal Period and Infancy.** At least two distinct electroclinical syndromes with Mendelian inheritance pattern have been identified, with seizures that begin in the neonatal period and infancy. The syndromes are characterized by an age-dependent onset of epileptic seizures, a relatively benign outcome in the majority, and at a molecular level are related to a pathogenic mutation in an ion channel. The seizure semiology in these syndromes is described in Table 116-5.

**116.8.1.1.1 Benign Familial Neonatal Epilepsy.** Mutations involving genes coding for a family of voltage-gated potassium channels (KCNQ) that play a critical role in the repolarization of neurons underlie this form of epilepsy (35,69). The reason why a fixed genetic deficit in potassium channel structure should lead only to intermittent seizures that tend to remit over time in early life may be related to changing spatial and temporal patterns of expression of potassium channel subunits during brain development (70). The possibility that this effect could also be mediated by genetic modifiers is also entertained.

**116.8.1.1.2 Benign Familial Neonatal-Infantile Seizures.** The seizures in this condition are of later onset around 3 months of age and seizures almost always remit by age 12 months. A mutation in  $\alpha 2$  subunit of the voltage-gated sodium channel (SCN2A) has been identified in individuals in an Australian family and in a family previously described by Lewis et al. (36). The clinical seizure phenotype has been named BFNIS, adding another seizure susceptibility syndrome to epilepsies beginning in early life. The mutations described are all missense and predicted to be associated with a gain of function, however recently three of the described mutations were found to be associated with a loss of function (71).

**116.8.1.2 Febrile Seizures and Related Syndromes.** Febrile seizures and other idiopathic epilepsies strongly related to the febrile seizure phenotype at presentation are discussed together (Table 116-6).

**116.8.1.2.1 Febrile Seizures.** Febrile seizures (FS) are classified as “situation-related” (72); they affect 2–5% (Europe) and 6–9% (Japan) of children in between

the ages of about 6 months and 5 years. FS are associated with a low risk of subsequent epilepsy, although a few children with FS, especially with the complex type, will develop temporal lobe epilepsy later in life (73).

The genetic basis for FS is considered likely, based on their increased occurrence in families. In particular, twin studies have demonstrated a much higher risk in MZ than in DZ twins (40% versus 7%) (74). A complex polygenic inheritance pattern is currently favored. At least six susceptibility loci have been mapped, although the causative genes have not been identified in most patients: FEB1 (8q13–q21), FEB2 (19p13), FEB3 (2q23–q24), FEB4 (5q14–q15), FEB5 (6q22–q24), and FEB6 (18p11). The FEB4 locus was also confirmed in nuclear families and may account for 70% of commonly encountered febrile seizure susceptibility. The FEB3 locus at 2q23 was reported in large Utah family, and the seizure descriptions are considered to be close to GEFSP, so this may be considered more as a GEFSP locus (75).

#### 116.8.1.3 Epileptic Encephalopathy of the Neonate and Infant.

**116.8.1.3.1 Ohtahara Syndrome.** The condition is associated with a progressive epileptic encephalopathy beginning in the first 2 months of life, severe seizures, and a characteristic suppression burst pattern on the EEG. While most infants with this condition have structural brain abnormalities demonstrable on neuroimaging, presently the syndrome appears to be associated with both genetic and metabolic etiologies. Pathogenic mutations in the Aristaless homeobox gene (ARX) (X linked infantile spasms), syntaxin binding protein (STXBP1) (76), and mitochondrial respiratory chain defects have been found causative (77). A pathogenic mutation in a mitochondrial glutamate transport carrier SLC25A22 has also been described in association with features of Ohtahara syndrome in an Arab-Israeli family with multiple affected individuals (78). Many of these patients transit into West syndrome in an age-dependent manner. It should be noted that STXBP1 mutations are also reported in an early-onset epileptic encephalopathy that did not clearly meet the criteria for the diagnosis of neither Ohtahara syndrome nor West syndrome (79).

**116.8.1.3.2 Infantile Spasms/West syndrome.** The diagnosis of West syndrome is based on the occurrence of characteristic seizure type (axial epileptic spasms), EEG findings of hypsarrhythmia, developmental arrest eventually leading to psychomotor retardation. While in a substantial proportion of infants diagnosed with infantile spasms are attributable to structural metabolic causes with a genetic basis, in a small proportion of cases genetic mutations have been identified in the “idiopathic/cryptogenic” cases. These mutations involve the X linked ARX gene, cyclin dependent kinase like-5 (CDKL5/ serine-threonine kinase 9) and syntaxin binding protein (STXBP1) and phospholipase C beta1 deficiency (76a,80). Mutations in these genes are associated with not only West syndrome, but also with

**TABLE 116-5 Electroclinical Syndromes by Age of Onset**

Epilepsy Type	Type of Seizure	Clinical and EEG Characteristics	Chromosomal Locus and Mode of Inheritance	Molecular Defect
Benign familial neonatal epilepsy	Unilateral or bilateral clonic, tonic clonic, apneic, and tonic seizures	Interictal EEG is normal, seizures are brief, on day 2 or 3 of life, recurrent, 15% may develop epilepsy in later life	Autosomal dominant 20q	Mutation in voltage-gated K channel genes (KCNQ2,KCNQ3)
Benign familial neonatal–infantile seizures (BFN-IS) MIM #607745	Mixed seizures similar to BFNC	Age of onset between 2 and 7 months, remit in the first year	Autosomal dominant 2q	Mutations in the $\alpha 2$ sub-unit of Sodium channel (SCN2A) gene
Benign familial infantile convulsions (BFIC1) MIM %601764	SPS CPS	Age of onset 4–8 months Parieto–occipital foci Remit by age 3 years	Autosomal dominant 19q11–13 in Italian families is also associated with another gene locus 16p12–q12	Causative gene remains unknown
Early infantile epileptic encephalopathy (Ohtahara)	Intractable tonic spasms Progressive Encephalopathy	Seizure onset in the first month, suppression-burst pattern on EEG Evolution to West syndrome and Lennox–Gastaut syndrome Often structural brain abnormalities seen	Sporadic forms	Mutations in <i>STXBP1</i> (syntaxin binding protein 1 gene) and <i>ARX</i> gene have been identified in sporadic patients with Ohtahara syndrome
X linked infantile spasms in boys	Epileptic spasms Flexor, extensor, mixed Psychomotor retardation	EEG shows hypsarrhythmia Progressive MR Male patients with MR, intractable epilepsy Rett like features in females	Recessive and dominant X linked forms identified	Recessive forms <i>ARX</i> gene mutations Dominant <i>CDKL5</i>
Epilepsy with mental retardation X linked	Seizure types include GTCS, myoclonic and tonic seizures, absences, and focal seizures	Seizures start from the age of 6–36 months and may be precipitated by fever	X linked	Mutations in the Protocadherin ( <i>PCDH19</i> ) gene
Generalized epilepsy with febrile seizures plus (GEFSP)	Febrile Afebrile seizures GTCS FS + Absences, myoclonic seizures Atonic seizures	FS extend beyond age 6 and accompanied by other seizure types Good AED response Normal interictal EEG	Autosomal dominant 50–60% penetrance	Mutations in <i>SCN1A</i> (5-10%), <i>SCN1B</i> (4.1%) and <i>GABRG2</i> genes <1%) All GEFSP mutations are missense
Dravet syndrome	Febrile hemiconvulsions in the first year	Mixture of intractable generalized (myoclonic or atonic seizures, atypical absences) and focal seizures, normal early development, Subsequent psychomotor retardation, and normal brain imaging at onset Interictal EEGs are initially normal but progressively show generalized spike-and-waves, polyspike-and-waves, focal abnormalities, and photosensitivity	Sporadic Denovo	Mutations in the <i>SCN1A</i> gene in 32–100% of patients Truncation Missense

**TABLE 116-6 Idiopathic/Genetic Generalized Epilepsy Syndromes**

Epilepsy Type	Type of Seizure	Clinical and EEG Characteristics	Chromosomal Locus and Mode of Inheritance	Gene Testing (Availability, Accuracy, Utility)
Idiopathic Generalized Epilepsy (IGE) MIM 600669	GTCS Absence Myoclonic	Normal intellectual development Age of onset and seizure type differ in different syndromes Generalized 3 Hz or faster spike, polyspike and wave complexes	Complex Polygenic 3q26 SLC2A1 mutations in 10% of early onset absence	Limited only for (SLC2A1) Accurate Implications for treatment and counseling
Childhood Absence Juvenile Absence Juvenile Myoclonic Epilepsy with grand mal on awakening				
Familial juvenile myoclonic epilepsy	GTCS Absence Myoclonic	Only pure JME in all affected individuals Age of onset 8–25 years. Diffuse polyspike–multispikes waves (3.5–6 Hz)	Autosomal dominant 5q34	No Not useful
Benign familial infantile convulsions (BFIC2) %6 05751	SPS CPS	Similar seizures with dyskinesias at onset Associated with paroxysmal choreoathetosis in French kindred	16p in French kindred	
Familial febrile seizures (FS) FEB1 MIM %602476 FEB2 MIM %602477 FEB4 MIM %604352	Generalized seizures	6 months–5 years Typical febrile convulsions	Genetic heterogeneity 8q13 (FEB1) 19p13 (FEB2) 5q14 (FEB4)	NA

other early onset epileptic encephalopathies as discussed above (76a,80,81). Of these three genes, *ARX* gene mutations exhibit remarkable phenotypic variability and pleiotropy: protein truncation mutations are associated with malformation phenotypes while missense mutations are associated with nonmalformation phenotypes. X-linked infantile spasms, are associated with missense mutations outside of the homeobox and expansion of the polyA tracts (82). Pathogenic mutations in the gene *SPTAN1* coding for  $\alpha$ -II spectrin has been reported in association with early-onset West syndrome associated with cerebral hypomyelination, visual symptoms, and developmental delay (83), adding to the panel of genes that will need to be screened for in cases of West syndrome where a genetic basis is suspected.

#### 116.8.1.4 Childhood Onset Epilepsy Syndromes.

**116.8.1.4.1 Generalized Epilepsy Febrile Seizures Plus.** The seizure phenotype in febrile seizures plus includes the persistence of febrile seizures beyond age 6 years or association with occurrence of afebrile seizures. Very often these seizures are generalized (tonic-clonic, myoclonic, atonic, or absence) seizures, however, focal seizures of frontal, temporal origin are also recognized (17b,84). The mode of inheritance in GEFS plus remains a matter of debate; in some a dominant pattern, in others an oligogenic effect is thought to account for the wide variation in clinical phenotype. So far the gene defects identified appear to involve subunits of voltage-gated sodium channels (17a), and subunits of the ligand-gated GABAergic receptors (18,85). Both types of mutations appear to affect the functional properties of the respective channels,

and may serve to enhance seizure susceptibility in a non-specific manner. The familial *SCN1A* mutations identified to date have all turned out to be missense in type (86).

**116.8.1.4.2 Severe Myoclonic Epilepsy (Dravet syndrome) and Other Epileptic Encephalopathies.** The phenotype in SMEI represents the severe end of the spectrum of the epilepsies encountered with *SCN1A* mutations. Prolonged unilateral clonic or hemiconvulsive febrile seizures at onset in the first year of life in developmentally normal children, progress to intractable afebrile seizures (atypical absence, myoclonic and partial seizures). Intractable epilepsy is accompanied by ataxia and the development of pyramidal signs. The interictal EEG shows bihemispheric generalized spike waved discharges, which can be asymmetrically expressed. Imaging studies fail to show structural abnormalities in the brain. There is often developmental regression and a long-term unfavorable outcome. About 400 mutations considered to be pathogenic have now been identified and described in the sodium channel gene *SCN1A*. The majority appears to be arising de novo. Missense (40%) and truncating mutations (50%) in the *SCN1A* gene (coding for the  $\alpha$  1 subunit for the sodium channel) constitute a majority of the known mutations associated with SMEI (20b,87). There is evidence to suggest that such de novo mutations may be of paternal origin, as the mutational events have a greater chance during mitoses occurring during spermatogenesis (88). There is considerable overlap with GEFS, where similar mutations have been described (89). The phenotypic variability of epilepsy phenotypes associated with sodium channel mutation has expanded



considerably with the description of patients with borderline severe myoclonic epilepsy (SMEB), with intractable epilepsy lacking specific features of SMEI, as well as reports of intractable childhood epilepsy with generalized tonic clonic seizures, infantile spasms, Lennox–Gastaut syndrome (22), atypical Panayiotopoulos syndrome (90), and Rasmussen’s encephalitis (91). An added mutational mechanism in SMEI has been the finding on novel copy number variants as duplications/deletions in SCN1A detected by multiplex ligation-dependent probe amplification (MLPA) and characterized further by arrayCGH in patients where initially no mutations were identified (92). Finally the discovery of SCN1A mutations in a substantial number of patients with vaccine associated encephalopathy adds yet another neurological phenotype, unmasked by administration of vaccine.

**116.8.1.4.3 Glucose Transporter 1 Deficiency Syndrome.** An infantile seizure disorder associated with hypoglycorrhachia was attributed to a defective glucose transport carrier across the blood–brain barrier was first reported in 1991 (93). Haploinsufficiency or hemizygosity of the GLUT1 transport carrier was identified as the cause (56a), with both sporadic de novo occurrence and autosomal dominant inheritance patterns identified (94). The condition can also be transmitted as an autosomal recessive in rare instances (95). The phenotype of the epilepsy syndrome associated with this condition has rapidly expanded to include both a severe form of an epileptic encephalopathy presenting with infantile seizures to milder intermittent symptoms that can range from paroxysmal movement disorders, intermittent seizures (56c). All seizure types (generalized tonic or clonic, myoclonic, atypical absence, atonic, and unclassified) are reported. Mutational hotspots in the gene coding for a solute transporter (*SLC2A1* gene) have been identified leading to a defective GLUT1 transport protein. The recognition of this condition is particularly important as the severity of the condition ameliorated and symptoms controlled by the administration of the ketogenic diet. An added discovery that up to 10% of patients with early-onset absence epilepsy as well as other types of genetic generalized epilepsies carry milder mutations in the *SLC2A* gene assumes great clinical importance as these cases are indistinguishable from other absence epilepsies. Further GLUT1 deficiency may be more amenable to treatment with the ketogenic diet in addition to the implications for genetic counseling.

#### **116.8.1.4.4 IGE Syndromes.**

**116.8.1.4.4.1 Childhood and Juvenile Absence Epilepsy, Juvenile Myoclonic Epilepsy, Epilepsy with Grand Mal on Awakening.** The clinical and EEG features of IGEs show considerable overlap. Seizure types include absence, tonic, tonic–clonic, clonic, or myoclonic seizures, the interictal EEG shows bursts of synchronous generalized epileptiform patterns over both cerebral hemispheres and a normal background. In each syndrome, there is an age-dependent onset of a particular

seizure type. Affected individuals are of normal intelligence, the neurological examination is usually normal. (Table 116-6).

Mutations hitherto identified in molecular studies appear to affect different targets that include a GABA receptor subunit (GABARA1) in JME (48), a chloride channel (CLCN2) in IGE (49), and a calcium channel (CACNA1H) in CAE (43). The mutations appear to carry a net effect on altered GABAergic function and altered patterns of synchronized burst firing within thalamocortical circuits (48,49).

**116.8.1.4.5 Idiopathic Partial Epilepsies.** Most partial or focal epilepsies were assumed not to have a genetic basis. Relatives of probands with partial epilepsy have an increased risk of epilepsy compared to the general population, suggesting a genetic influence in at least some forms of partial epilepsy. In order for partial seizures to be caused by a gene defect, the defective gene either has to cause localized cortical hyperexcitability, or has to be expressed only in certain neuronal regions, or its effects are modified by local factors (96). The partial epilepsy syndromes and the chromosomal loci to which they are mapped are summarized in Table 116-7.

**116.8.1.4.5.1 Nocturnal Frontal Lobe Epilepsy.** ADNFLE is transmitted in an autosomal dominant fashion with incomplete penetrance, thus displaying intra-familial variability in clinical expression. Cholinergic receptor mutations have been shown to underlie this epilepsy phenotype and have been discussed at length earlier.

**116.8.1.4.5.2 Familial Temporal Lobe Epilepsy and Other Partial Epilepsies.** Ottman et al. described a single family of 11 affected individuals who presented with partial seizures beginning during adolescence, and nonspecific auditory disturbances (97). The condition is now recognized by the term ADPEAF or autosomal dominant lateral temporal lobe epilepsy. Sequencing all the 28 genes in the implicated 10q24 locus, mutations were identified in the leucine rich glioma inactivated 1 gene (*LGI1*) (24a).

Other autosomal dominant inheritance patterns for partial epilepsies have been identified in clinical studies of large families: autosomal dominant partial epilepsy with variable foci (98), autosomal dominant rolandic epilepsy with speech dyspraxia (99) and benign familial infantile convulsions (BFIC) (100). While linkage studies have suggested associated loci, however so far, a mutation has not been identified.

**116.8.1.4.5.3 Benign Epilepsy of Childhood with Central-Temporal Spikes.** A strong genetic influence in benign rolandic epilepsy has been an early consideration (101). The epilepsy syndrome appears to be inherited as autosomal dominant with age dependent penetrance and the EEG trait is inherited similarly (102). There is an increased incidence of both focal and generalized EEG abnormalities among first-degree relatives. (Interestingly, in relatives generalized abnormalities are even

**TABLE 116-7 Genetic partial epilepsy syndromes**

Epilepsy Type	Type of Seizure	Clinical and EEG Characteristics	Chromosomal Locus and Mode of Inheritance	Gene Testing Availability Accuracy Utility
Nocturnal frontal lobe epilepsy (ENFL1, 2, 3) MIM #600513	SPS	Age of onset 8 y Nocturnal seizures, occur in clusters with aura Frontal lobe origin Tonic, hyperkinetic movements often mistaken for sleep disturbance	Autosomal dominant 20q13.2–13.3 15q24 1q	Yes Accurate Useful Establishes etiology Genetic counseling implications
Familial lateral temporal lobe epilepsy with auditory features (ADPEAF) MIM#600512	SPS	Childhood or adolescent onset Partial seizures Acoustic and visual hallucinations Aphasia Seizure precipitation by speech activation Vertigo	Autosomal dominant Genetic heterogeneity 10q22	Yes Accurate Not very useful
Familial Partial Epilepsy with variable foci MIM %604364	SP, CPS and SGTC	Age of onset 8–10 years Migrating spike foci Frontal, temporal Normal intelligence	Autosomal dominant 22q11–q12	
Benign epilepsy of childhood with central temporal spikes MIM % 117100	SPS CPS SGTC	Onset 3–13 years Partial seizures with speech arrest Hemifacial Somaotensory symptoms	Autosomal dominant with incomplete penetrance (EEG trait) 15q13	

more common than focal ones). Linkage to 15q13 locus has been demonstrated in family studies (103).

### 116.8.2 Progressive Myoclonic Epilepsy Syndromes

Progressive myoclonic epilepsies share in common the phenotypic features of myoclonic and generalized seizures, and progressive cognitive decline and ataxia. (Table 116-8). The differential diagnosis is wide and include many genetic conditions such as phakomatoses, inborn errors of metabolism, storage disorders, chromosomal abnormalities, mitochondrial disorders, neurodegenerative disorders (ceroid lipofuscinoses), Unverricht Lundborg (Baltic myoclonus) syndrome (cystatin B gene), Action myoclonus renal failure syndrome (SCARB2). Newer genes are being identified, where pathogenic mutations are associated with progressive myoclonic epilepsy (SCARB2 mutations in PME without renal failure, PRICKLE1 mutation in recessive PME with ataxia) (104). These disorders involve both Mendelian and non-Mendelian inheritance patterns and provide a paradigm for a common phenotypic expression for multiple defects at a molecular level resulting in myoclonic seizures. Selected conditions are discussed later, and the problem of progressive myoclonic epilepsies is reviewed in greater details elsewhere (105).

**116.8.2.1 Unverricht Lundborg syndrome.** EPM1 has been mostly reported in a genetically homogeneous population. The gene CST6 at the 21q22.3 locus codes for a protein called cystatin B (CSTB), a cystine protease

inhibitor. Cystatin B mRNA was found to be markedly reduced in EPM1 patients. Point mutations have also been described in the CST6 gene, accounting for 10% of EPM1 alleles, while in the most common EPM1 Baltic/Finnish or Mediterranean haplotypes, no point mutations were found. In the latter, a polymorphic unstable dodecamer repeat expansion (CCCCGCCCGCG) *n* located 174 nucleotides upstream of the initiation ATG codon of CSTB in the promoter region of the CST6 gene, was demonstrated (106). The condition may be under diagnosed in patients with drug resistant myoclonic epilepsy (107).

The process by which changes in the promoter region result in reduction in CSTB transcription remains unknown. The expansion is different from the triplet repeat expansion, but several lines of evidence suggest moderate intergenerational instability particularly with male transmission (106d). There is no relationship between the size of the repeat expansion and the age of onset of the disease. As a ubiquitous protease inhibitor, CSTB is speculated to play a critical role in the maintenance of neuronal integrity. Neuronal degeneration particularly of the cerebellar granule cells is noted in the knock out mouse models, with epilepsy being a secondary effect (108). In a human postmortem study evidence of neuronal degeneration, neuronal cytoplasmic inclusions containing the lysosomal proteins, Cathepsin-B and CD68, as well as immunoreactive intranuclear inclusions were prominent suggesting that neuronal degeneration in at least is in part mediated by deficient cystatin mediated neuroprotection (109).

**TABLE 116-8 Progressive Myoclonic Epilepsies (EPM)**

Epilepsy Type	Type of Seizure	Clinical and EEG Characteristics	Chromosomal Locus and Mode of Inheritance	Molecular Testing
Progressive myoclonic epilepsy of Unverricht Lundborg (EPM1) #MIM 254800	GTCS Myoclonic	Seizures begin early, with mental deterioration, ataxia a late feature Photosensitive myoclonus Spike wave discharges precede seizures	Autosomal recessive 21q22.3	Targeted mutation analysis Sequence Analysis Clinical testing available for mutations in cystatin B gene
Dentatorubro pallidoluysian atrophy (DRPLA) *MIM 125370	Myoclonic	Myoclonic epilepsy Dementia Ataxia Choreoathetosis	Autosomal dominant 12p13.31	Targeted mutation analysis for CAG expansion in the <i>ATN1</i> gene Clinical testing available
Myoclonic epilepsy of Lafora EPM1 EPM2 *MIM 254780	GTCS Myoclonic	Age of onset 15 years Rapid mental deterioration Psychosis Blindness PAS + bodies in myoepithelial cells of eccrine glands	Autosomal recessive 6q23.q25	Sequence and Deletion Analysis for mutations in the <i>EPM2A</i> , <i>NHLRC1</i> genes Clinical testing available
Myoclonic epilepsy with ragged red fibers (MERRF) #MIM 545000	Myoclonic	Myopathy with ragged red fibers Sensorineural hearing loss Myopathy Peripheral neuropathy Dementia Short stature Exercise intolerance Optic atrophy	Mitochondrial tRNA mutations affecting tRNA(lys)	Targeted mutation analysis for the <i>MT-TK</i> , <i>MT-TF</i> and <i>MT-TP</i> genes, as well as scanning for sequence variants Clinical testing available
Sialidoses I *MIM 256540	Myoclonic	Age of onset 8–15 years Vertex positive spikes Stimulus sensitive myoclonic seizures, Ataxia Visual failure with cherry red spots in the macula	Autosomal recessive 6p21.3	Targeted mutation analysis in the neuraminidase gene Clinical testing available
Ceroid Lipofuscinoses Neuronal (CLN2) (CLN5) Finnish *MIM 204500	Myoclonic, GTCS, Atonic, Atypical absence	Age of onset 2.5–4 years Often mistaken for Lennox Gastaut syndrome Photoparoxysmal response at <3 Hz stimulation	Autosomal recessive 11p15 15q21-23 (Finnish variant)	Targeted mutation analysis is available for <i>PPT1</i> ( <i>CLN1</i> ), <i>TPP1</i> ( <i>CLN2</i> ), <i>CLN3</i> , <i>CLN5</i> , <i>CLN6</i> , <i>CLN7/IMFSD</i> , <i>CLN8</i> , and <i>CTSD</i> genes Clinical testing available
Ceroid Lipofuscinoses Neuronal (CLN3) *MIM 204200	Atypical absence Myoclonic GTCS	Age of onset 4–10 years Visual loss Ataxia Dementia	Autosomal recessive 16p12.1	

Gene testing and relevant information on molecular diagnostic test, and laboratories performing such testing is accessible through the NCBI website. <http://www.ncbi.nlm.nih.gov/sites/GeneTests/lab?db=GeneTests>

### 116.8.2.2 Dentatorubral–Pallidoluysian Atrophy.

DRPLA is a disorder caused by expansion of trinucleotide repeats (CAG, >49 repeats) within a gene of unknown function (110). DRPLA exhibits genetic anticipation, variable penetrance, and heterogeneity. The gene codes for polyglutamine tracts and the defective protein has been termed “atrophin.” Attention has focused on the interaction of these polyglutamine tracts with other

intranuclear proteins that function as transcription factors (TATA, CREB and CREB binding proteins) (111). Progressive neuronal impairment is thought to result from irregularities in transcription induced by abnormal protein–protein interactions (112).

### 116.8.2.3 Myoclonic Epilepsy of Lafora/Lafora disease.

Progressive myoclonus epilepsy of the Lafora type (EPM2/MELF) presents during late childhood or

adolescence has a fatal outcome that is inevitable within a decade of the onset of initial symptoms. Periodic acid-Schiff (PAS) positive cytoplasmic inclusion bodies are identified intracellularly within neurons, skeletal muscle, hepatocytes, and sweat glands in the skin and are considered diagnostic. The inclusions (Lafora body) are composed of a starch-like substance termed polyglucosans and are identified within eccrine glands and in the myoepithelial cells surrounding apocrine glands of the skin (113). There is a cautionary note in that the cells of the apocrine gland itself may show PAS+ granules giving rise to a false positive result (114). Therefore, many centers prefer biopsy sites outside of the axilla where generally apocrine glands are absent. More recently it has been shown that skin biopsies can have a high negative rate even in classical form of Lafora disease (LD), where mutational analysis can confirm the presence of a pathogenic mutation.

The inheritance pattern for this disorder is autosomal recessive with genetic heterogeneity (2 loci *EPM2A*, *EPM2B*). The *EPM2A* gene codes for a gene product laforin, a dual function tyrosine phosphatase, which exists in two isoforms (cytoplasmic, ER, and nuclear) (115). Mutations affecting laforin appear to involve the cytoplasmic isoform. The *EPM2B* locus on DNA sequencing and screening leads to the identification of gene *NHLRC1* (NHL repeat containing gene 1) carrying a pathogenic mutation. Several mutations have now been identified in this gene in different families with EPM. The *NHLRC1* codes for malin, a 395 amino acid protein with a putative E3 ubiquitin ligase function that is predicted by the presence of zinc-binding RING finger domain (116). Both gene products appear to localize at the ER and may be involved in clearance of polyglucosans. It is suggested that the disorder arises through the accumulation of polyglucosans within neuronal dendrites and the resulting disturbance in synaptic function gives rise to increased seizure susceptibility in the nervous system (117). Further work in animal models has suggested that glycogen hyperphosphorylation is the principal biochemical abnormality in LD (118). Excess glycogen phosphate further leads to glycogen conformational change, unfolding, precipitation, and conversion to polyglucosan (119).

**116.8.2.4 Myoclonic Epilepsy with Ragged Red Fibers.** The disorder derives its name from the histopathological appearance of “ragged red muscle fibers” resulting from the subsarcolemmal accumulation of mitochondria, seen in muscle sections stained by the trichrome stain. Three mutations (A8344G, T8356C and G8363A), in the human mitochondrial DNA (mtDNA) affecting the tRNA (*Lys*) gene have been identified in the majority of patients with MERRF (120). The resulting defects in complex I and IV enzymes of the oxidative-phosphorylation system (121) are responsible for an energy deficit within affected cells carrying abnormal mitochondrial population above a certain threshold. The proportion of normal and mutant mitochondrial DNAs in affected tissues at a

particular age may be one of the factors determining the severity of symptoms. Currently, it is believed that the pathogenic tRNA mutations lack a posttranscriptional taurine-modification at the anticodon wobble uridine that leads to defective translation of either of its codons (AAA and AAG) and it is this inability that underlies the molecular pathogenesis of MERRF (122).

**116.8.2.5 Lysosomal Storage Disorders.** Sialidosis, neuronal ceroid lipofuscinosis (NCL) and GM2 gangliosidosis are autosomal recessive neurometabolic storage disorders in which characteristic inclusion bodies or storage material are identified within neurons, resulting in their degeneration. In these disorders, myoclonic seizures likely occur as a secondary phenomenon.

Sialidosis results from a mutation in the gene encoding glycoprotein specific  $\alpha$ -neuraminidase. Neuraminidase cleaves terminal sialic acid residues from sialoglycoconjugates, and deficiency of the enzyme results in accumulation of these conjugates within the cell (115a). The NCL present with a combination of seizures (with the exception of CLN1), psychomotor decline, and visual failure with varying age of presentation. The age of onset, clinical features, and the ultrastructural appearance of the intracellular lipopigment accumulations form the basis of division into subtypes. At least eight mutant genes (*CLN1*–*CLN8*) are suspected, while six gene loci have been mapped, with the exception of *CLN4* and *CLN7*. With the exception of the protein encoded by the gene *CLN1*, lysosomal accumulation of mitochondrial ATP synthase subunit “c” forms a substantial part of the inclusions in the remaining subtypes. The proteins defective in *CLN1* (123), and *CLN2* (124) are lysosomal enzymes, that are mannose 6-phosphate dependent for their trafficking and have a role in protein degradation. The remaining proteins associated with *CLN3* (125), *CLN5* (126) and *CLN6* are predicted to function as transmembrane proteins, while it is shown that *CLN8* protein is localized to the ER/Golgi apparatus.

Thus, impaired lysosomal degradation and trafficking of proteins may be the common theme underlying this group of disorders with overlapping clinical features. It is clear that CLN proteins are critical to the nervous system, as it bears the brunt of the damage in these disorders, even though the storage product is found in tissues outside the nervous system. It has been suggested the machinery for synaptic functioning bears similarities to the machinery involved in lysosomal protein trafficking. Impaired synaptic function may be the final common endpoint of enzymatic/genetic defects in the NCL group of disorders (127).

### 116.8.3 Genetic Epilepsy Associated with Structural Causes

Abnormal cortical development can result in a predilection for seizures, in addition to encephalopathy, developmental delay, and motor problems in early life. Disorders



of neuronal migration account for nearly 25% of recurrent childhood epilepsy. Studies of surgically resected specimens from patients with intractable seizures show a 30–50% incidence of developmental malformations, especially in children (128). Many single gene defects that lead to malformations of cortical development are now known that include tuberous sclerosis, lissencephaly, and periventricular nodular heterotopia (Table 116-9). These genes are involved in the regulation of intracellular signaling, as well as protein–protein interactions that control neuronal growth, differentiation, and migration.

**116.8.3.1 Tuberous Sclerosis.** The *TSC1* gene, on chromosome 9q34, encodes a widely expressed protein (hamartin) that is postulated to function as a tumor-suppressor gene (129). The *TSC2* gene, on chromosome 16p13.3, encodes a protein product (tuberin) (130). Tuberin and hamartin associate with each other physically in vivo and are jointly involved in protein–protein interactions that form intracellular signals that regulate cell size, protein synthesis and neoangiogenesis. The complex roles enacted by the gene products serve to explain the diverse clinical features of tuberous sclerosis, but the specific interactions underlying epileptogenesis remains to be elucidated (131).

**116.8.3.2 Lissencephaly and Subcortical Band Heterotopia.** Lissencephaly occurs as an isolated lissencephaly sequence as well as part of a dysmorphic

syndrome (Miller–Dieker syndrome). At least two genes have been identified within the chromosome 17 locus, *LIS1* encoding for platelet-activating factor acetyl hydrolase (PAFAH1B1) (132), and another gene *YWHAE* that encodes for 14-3-3ε(epsilon), a phosphoserine/threonine-binding protein (133). Mutations involving either of the genes can cause lissencephaly; both appear to influence neuronal migration by participating in a highly conserved evolutionary pathway that regulates cytoplasmic dynein function (134).

An X linked form of lissencephaly is recognized in hemizygous males (carrying the abnormal X chromosome), while heterozygous females manifest with a different, distinct neuronal migration defect termed subcortical band heterotopia (SBH) or double cortex syndrome. Here, bands of gray matter isointense with cortical ribbon are distributed within the white matter, separated by a thin layer of white matter representing an entire layer of neurons arrested in their course of migration. Boys with X linked lissencephaly resemble patients with classic lissencephaly phenotype, whereas females with SBH have milder mental retardation, fewer behavioral problems and milder epilepsy. SBH and X-linked lissencephaly are caused by mutation of the doublecortin (*DCX*) gene, with complete absence of the gene product in hemizygous males, while the milder SBH phenotype in heterozygous females results from X-inactivation

**TABLE 116-9 Epilepsy Associated with Malformations of Cortical Development**

Epilepsy Type	Type of Seizure	Clinical and EEG Characteristics	Chromosomal Locus and Mode of Inheritance
Tuberous sclerosis (TSC1) MIM 191100	Infantile spasms	Seizures Mental retardation Hydrocephalus secondary to cerebral glial nodules Hamartomas	Autosomal dominant 9q34
Tuberous sclerosis (TSC2) MIM191092	infantile spasms	Mental and motor retardation Intractable seizures Facial angiofibroma, skin changes Renal, cardiac and neural tumors	Autosomal dominant 16p13.3
Lissencephaly I Miller–Dieker syndrome MIM 247200	Infantile spasms	Characteristic facies Motor and mental retardation, neuronal migrational defect Deficient opercularisation	Autosomal recessive 17p13.3
X linked Lissencephaly MIM 300067	Intractable seizures Infantile spasms in males Mixed seizures in females	Lissencephaly in hemizygous males Subcortical band heterotopia in heterozygous females Mental retardation	X linked dominant Xq22.3–q23
X linked infantile spasms syndrome	Infantile spasms	Mental retardation Epilepsy Absence of cortical dysplasia	Missense/ insertion mutations in ARX
Bilateral Periventricular Nodular heterotopia (BPNH) MIM *300049	Heterogeneous types of epilepsy GTCS, TLE SGTC	Variable age No specific cognitive or neurological markers Heterotopias on imaging	Genetic heterogeneity X linked dominant Xq28 AR forms

Gene testing and molecular diagnostic tests, and laboratories performing such testing is accessible through the NCBI website. <http://www.ncbi.nlm.nih.gov/sites/GeneTests/lab?db=GeneTests>

(lyonization) (135). The *DCX* gene encodes a protein widely expressed in the developing brain (136), which likely plays a critical role in signal transduction in the developing cortex, involving the non-receptor tyrosine kinase cascade (137).

An unusual phenotype of X linked lissencephaly with ambiguous genitalia has also been described caused by a mutation in a highly conserved homeobox gene *ARX* (138). Mutations in the *ARX* gene have now been linked to a wide list of conditions characterized by abnormalities in cerebral development, neurological dysfunction (epilepsy, spasticity and dystonia), autism and mental retardation. Truncating mutations in the *ARX* gene lead to X linked forms of lissencephaly, while missense/insertion mutations lead to other epilepsy phenotypes without cortical dysplasia (139).

Several subtypes of lissencephaly are recognized based on the MRI imaging patterns of the severity and distribution of the agyric–pachygyric cortex (anterior–posterior gradients), the presence or absence of a double cortex, agenesis of the corpus callosum, and other associated malformations of the posterior fossa. The genotype–phenotype relationships and the molecular genetics of lissencephaly and SBH are discussed in greater depth in this paper (140). Furthermore, there is evidence that copy number variations seen with intragenic deletions and duplications also account for a number of cases of lissencephaly and SBH where no abnormalities were identified by mutation analysis (141).

**116.8.3.3 Periventricular Heterotopia.** Isolated periventricular heterotopia (PH) consists of well-differentiated cortical neurons and glia forming nodules in the subependymal zone along the ventricular surface. Individuals with PH are at high risk for epilepsy, although they may have no other neurological signs or external stigmata (142). Seizures starting in infancy have also been reported (143). Both X-linked dominant, as well recessive patterns of inheritance are known. In the majority of patients affected with the X linked form, mutations in the *FLNA* gene on Xq28 are described. The gene encodes a protein filamin A, considered essential for neuronal migration (144). The recessively inherited form is caused by mutations in the gene *ARFGEF2* (ADP-ribosylation factor guanine nucleotide-exchange factor-2) mapping to chromosome 20. The gene product is implicated in membrane and vesicle trafficking within the Golgi network (145,146).

## 116.8.4 Epilepsy Associated with Chromosomal Aberrations

The incidence of epilepsy associated with chromosomal imbalances is difficult to estimate together, and depends on individual conditions (147). Of 400 chromosomal imbalances that were systematically searched, eight disorders with a strong association with epilepsy were identified. These include; terminal deletions of 1q and

1p, Angelman syndrome, inversion duplication of chromosome 15, Wolf–Hirschhorn (4p-) syndrome, Miller–Dieker syndrome, and ring chromosome 14 and 20 (148). In addition to these, specific electroclinical associations have been noted in the epilepsy literature in the Fragile X syndrome, trisomy 12, and Rett syndrome. Trisomy 12p is associated with seizures of the generalized tonic clonic seizures (GTC) or myoclonic types. EEG findings in Trisomy 12p consisted of generalized 3Hz spike wave discharges. Mutations affecting genes (K channels and *GABARAPL1*) localized to 12p region, may lead to increased seizure susceptibility (149). Children with fragile X are reported to have a characteristic EEG pattern with centrottemporal spikes similar to those seen in benign rolandic epilepsy (150–152).

The conditions shown in Table 116-10 merit special attention due to the nature of seizures in the clinical phenotype, electroclinical correlates, and implications for epileptogenesis. In these situations the seizure phenotype is not simply related to gene dosage, but likely influenced by the combined effect on genes in and outside the affected region, as well as environmental influences.

**116.8.4.1 Angelman Syndrome.** More than 90% of children with Angelman syndrome (AS) develop seizures, usually by the third year of life. The seizures display age-dependent features; with febrile seizures or infantile spasms in infancy, evolving into mixed seizures in later life including atypical absences, myoclonic, astatic, and atonic seizures (153). Myoclonic status epilepticus is especially common (154). Seizures in AS often remit after puberty, providing additional support for their age-dependent nature. Highly characteristic EEG patterns are reported (154a,b,155).

A *UBE3A* mutation, uniparental disomy or an imprinting methylation abnormality are sufficient to produce the AS phenotype, but seizures and EEG abnormalities seem to be more severe if the 15q11–13 region is deleted (153b). Genotype–phenotype correlation studies suggest that AS patients with deletions have the highest incidence of seizures followed by those with a mutation in the *UBE3A* ligase gene (156). A study of GABA receptor subunit expression in the cortex of four subjects with AS in a postmortem study has provided useful insights. The key findings suggest; significantly decreased ratios of  $\beta(3)/\beta(2)$  and  $\alpha(5)/\alpha(1)$  subunit protein expression in AS cortex when compared with controls, further using *Xenopus* oocytes to incorporate membrane fractions from AS cortical cells, voltage clamp techniques were applied and receptor pharmacology was found to be altered. These alterations were found to be associated with an impaired extrasynaptic GABA mediated inhibition, but unchanged synaptic GABA mediated inhibition, and a predictable response of epileptic seizures to benzodiazepines (157).

**116.8.4.2 Inversion Duplication of Chromosome 15 (Inv Dup 15 syndrome).** Supernumerary marker chromosomes are small, extra pieces of a chromosomal material attached anywhere along a chromosome. Inversion

**TABLE 116-10 Selected Autosomal Chromosomal Aberrations Associated with Epilepsy**

Epilepsy Type	Type of Seizure	Clinical and EEG Characteristics	Chromosomal Locus and Mode of Inheritance
Angelman syndrome #MIM 105830	GTCS NCSE	Characteristic electroclinical features described (see text)	Deletion Imprinting Uniparental disomy 15q11–q12
Inverted duplication 15 MIM not assigned	Atypical absences Tonic Atonic Tonic-clonic	Characteristic facies, moderate to severe mental retardation, autistic features, hyperactivity EEG Diffuse and multifocal abnormalities, slow spike/polyspike wave abnormalities	Interstitial Dup 15q Inv dup (15)(pter-q12~13;q12~13-pter 4p- deletion *4p16.3
Wolff-Hirschhorn syndrome #MIM 194190	Myoclonic generalized or unilateral Atypical absences	Multiple anomalies "Greek Helmet" facies Characteristic electroclinical features described (see text)	4p- deletion *4p16.3
Pitt Hopkins syndrome	Mixed seizures	Typical Facial dysmorphic features, Hyperventilation episodic Intractable epilepsy	Mutation in Transcription factor (TCF4)
Ring chromosome 20 epilepsy syndrome MIM not assigned	Nonconvulsive status	Characteristic electroclinical features described	Locus of fusion p13q13, p13q13.3 p13q13.33
Trisomy 12p MIM not assigned	GTCS Myoclonic	Characteristic facies Mental retardation, hypotonia, cerebral malformations 3Hz Spike-wave complexes on EEG	Trisomy due to a breakpoint distal to 12p12
Rett syndrome MIM#312750	Partial complex seizures	Acquired microcephaly Autistic features Epilepsy "hand wringing" stereotypes	Xq28 Mutations in the <i>MECP2</i> gene
Fragile X *MIM 309550	GTCS SPS	Characteristic electroclinical features described that resemble the Rolandic trait	Triplet expansion Xq28
Down syndrome #MIM 190685	Infantile spasms	Phenotypic features often permits clinical diagnosis	Trisomy 21 21q22.3

duplication of chromosome 15 (inv dup 15 results in tetrasomy 15p and partial tetrasomy 15q), is associated with severe developmental delay, hyperactivity, aggressiveness, and intractable seizures (158). The predilection for seizures in the inv dup 15 syndrome could also be related to altered GABA receptor function, as three genes coding for GABAA receptor subunits are located on this chromosome (158a).

**116.8.4.3 Wolf–Hirschhorn Syndrome.** The Wolf–Hirschhorn (4p-) syndrome (WHS) is a contiguous gene syndrome. Seizures are reported in 50–100% of cases, with onset usually in the first year. There is a stereotyped electrographic and clinical picture, characterized by mixed seizures (GTC, atypical absences and myoclonic seizures) with characteristic EEG changes described (159). A second critical region WHSCR2 characterized recently, includes a Leucine zipper/EF hand containing transmembrane protein (*LETM1*) gene, that encodes for proteins belonging to the EF hand family of calcium signaling proteins, has been suggested as the gene responsible for the epilepsy phenotype (160).

**116.8.4.4 Rett Syndrome.** The classical form of Rett syndrome is a pervasive developmental disorder affecting primarily girls, with a defined genetic cause (161). The majority of patients with Rett syndrome will experience

epileptic seizures at a median age of 4 years and more than 50% experience a period of active epilepsy of >5 years duration. Distinctive features in the EEG consist of loss of normal background rhythms, frontal central rhythmic theta, and the presence of focal, and multifocal epileptiform patterns (162). Rett syndrome is caused by mutations affecting the *MECP2* gene located at Xq28 (163). The *MECP2* gene appears to be a housekeeping gene working as a transcriptional repressor. The gene appears to have an important role in synaptogenesis and dendritic arborization, in keeping with the pathological findings of reduced numbers of synapses due to either reduced formation or excess formation and pruning (164). Patients with Rett syndrome often have symptoms relating to autonomic dysregulation resulting in a number of behavioral spells often mistaken to be epileptic seizures, in other situations epilepsy may be under diagnosed as true epileptic seizures are overlooked owing to subtle signs and symptoms. Seizures are expressed in an age-dependent manner, and tend to be worse in patients with significant development delay and restricted mobility, and mutation type (p.R294X, p.R255X mutations and C terminal mutations are associated with lower seizure frequency; early truncating mutations are associated with a severe phenotype) (165).

**116.8.4.5 Pitt–Hopkins Syndrome.** This condition carries a typical facial gestalt, and is associated with bouts of hyperventilation, movement stereotypies, severe intellectual disability and intractable epilepsy. Some of the features usually prompt clinicians to investigate these patients for Angelman and Rett syndromes. The facial features are fairly characteristic; deep set eyes, strabismus, thin eyebrows in their midline portion, a large nose, high nasal bridge and flared nostril, protruding philtrum; M-shaped cupid’s bow; fleshy lips and wide mouth with shallow and broad palate; and widely spaced teeth. The helices of the ears are abnormal, dysplastic and thick, the jaw becomes protuberant in later stages. A pathogenic mutation in a gene coding for transcription factor (TCF4) is now shown to be associated with this phenotype (166). The gene encodes a protein that belongs to the “E” protein family that is a downstream target for WNT/ $\beta$ -catenin/TCF pathway. Epilepsy is reported more often in association with missense mutations on genotype–phenotype correlation studies (167).

**116.8.4.6 Ring Chromosome 20 Associated Epilepsy.** There is characteristic electroclinical phenotype associated with a ring chromosome 20 (r20). Though seizures are variable in onset from infancy to adolescence, they become intractable and treatment resistant. Characteristic nocturnal events that may be associated with hallucinations, motor tonic seizures and episodes of nonconvulsive status associated with a fugue-like state and regression are documented. EEG findings are typical during these spells, consisting of long runs of bifrontally dominant slow waves with or without spikes superimposed (168). As dysmorphic features are quite mild in this condition, specific testing for this chromosomal abnormality is indicated in all cases of intractable epilepsy with episodes of confusion, and the characteristic EEG findings.

**116.8.4.7 Down Syndrome.** Seizures occur in 5–10% of persons with DS, in particular infantile spasms (169). The high frequency of seizures in DS was previously attributed to a combination of inherent structural anomalies (brain dysgenesis) and common medical complications of the syndrome (hypoxia ischemia caused by cardiac defects, immune dysfunction with frequent infections, etc.) (169a). Recently, it has been suggested that the association may be more specific (170), with the recognition of the *TNEP1* (C21LR) gene belonging to the superfamily of genes coding for proteins with EPTP repeats, a novel candidate gene as it is localized near the DS critical region (54a).

## 116.8.5 Epilepsy Associated with Inborn Errors of Metabolism

Epilepsy associated with inborn errors of metabolism are characterized by the following clinical features: (1) frequent presentation in the neonatal period, infancy or early childhood years, (2) persistent neurological and

functional impairment in all developmental domains, coinciding or associated with the occurrence of frequent clinical and/or subclinical seizures, (3) resistance to conventional antiepileptic therapy, and (4) adverse effects on cognition, and long-term developmental outcomes. The most striking associations occur in the critical pathways of energy metabolism, neurotransmitter biosynthesis and breakdown. Substrate deficiency (glucose transporter defect, serine and creatine deficiency), as well as accumulation of toxic intermediates (Sulfite oxidase deficiency, nonketotic hyperglycinemia (NKH)) are also involved. Several conditions such as pyridoxine dependency (antiquitin deficiency), pyridoxal phosphate responsive, and folinic acid responsive seizures, creatine deficiency are potentially treatable. Selected conditions are presented in Table 116-11. Success at diagnosis involves evaluation in consultation with an expert in biochemical genetics, and appropriate investigations of blood, urine and cerebrospinal fluid (171). It is important to understand that as a few of these conditions are amenable to treatment, and an early diagnosis can be lifesaving.

Disorders such as, GABA transaminase and succinic semialdehyde dehydrogenase deficiencies involve defects in GABA metabolism. Reduced GABA levels in the brain cause a hyperexcitable state favoring an epileptic predisposition. Pyridoxine-dependent seizures, as well as aromatic acid decarboxylase deficiency, respond to either pyridoxine or pyridoxal phosphate. Pyridoxine functions as a cofactor in the function of the enzyme glutamic acid decarboxylase (involved in GABA synthesis) thus increased cerebral GABA mediated inhibition may be facilitated. Biotinidase deficiency (BTD) is a rare autosomal recessive disorder (172). The seizures in BTD are usually GTC, and less frequently infantile spasms or myoclonic seizures. The EEG shows a burst suppression pattern or multifocal paroxysmal abnormalities. Seizures respond well to administration of biotin. In NKH, there is accumulation of glycine in blood, brain and CSF due to defects in the cleavage system for glycine (173). Glycine functions as an inhibitory neurotransmitter in the brain, and triggers intractable myoclonic seizures and apnea in the neonate (174).

Sulfite oxidase and molybdenum cofactor deficiency are disorders affecting the xanthine metabolism. The presentation is often with very severe and intractable seizures in the neonatal period, often mistaken for a presentation akin to perinatal hypoxic ischemic encephalopathy. Uric acid levels are low in molybdenum cofactor deficiency, and elevated urinary sulfocysteine can be detected in both molybdenum cofactor deficiency and isolated sulfite oxidase deficiency. Imaging studies often demonstrate extensive cerebral atrophy and multicystic white matter change (175).

Cerebral serine deficiency occurs in 3-phosphoglycerate dehydrogenase deficiencies, and a low level of serine in the CSF is considered a marker. Seizures appear to respond to serine supplementation (176). CSF hypoglycorrhachia (low CSF/plasma glucose ratios) is a feature of



**TABLE 116-11 Inborn Errors of Metabolism and Epilepsy**

Inborn Error of Metabolism	Seizure Types	Clinical Features	Mode of Inheritance	Biochemical Abnormalities
Pyridoxine responsive seizures %266100	Multiple seizure types Intrauterine seizures	Prenatal and neonatal onset seizures refractory to treatment with conventional AEDs Responsive to pharmacological doses of Pyridoxine	AR 5q31.2	No specific markers Putative disorder of GABA metabolism
Folinic acid responsive seizures	Seizures tend to be generalized	Intractable seizures in the first week of life Refractory to conventional AEDs Seizures respond to small dose of Folinic Acid	AR	CSF analysis (HPLC) for neurotransmitter metabolites shows an abnormal peak of a hitherto unidentified compound
Succinic semialdehyde dehydrogenase deficiency +271980	Generalized tonic clonic Atypical absence, myoclonic	Psychomotor retardation Hypotonia, Ataxia seizures in 50%	AR 6p22	Increased 4 hydroxybutyric acid in urine, plasma and CSF (Organic acid analysis)
GABA transaminase deficiency +137150	Clonic tonic	Seizures begin in the neonatal period, psychomotor retardation Hypotonia	AR 16p	Rare disorder, increased free GABA levels in the plasma and CSF as well as elevated $\beta$ alanine levels
Non-ketotic hyperglycinemia #605899	Myoclonic encephalopathy	Intractable seizures in the neonatal period Hypotonia Apnea	AR 16q 9p 3p 2p	Plasma and CSF glycine levels are elevated Glycine cleavage system enzymes can be assayed in the liver
3-Phosphoglycerate Dehydrogenase deficiency #601815	Mixed	Microcephaly Psychomotor retardation Intractable seizures Seizures respond to serine supplementation	AR 1q	Cranial MRI shows marked attenuation of signals in the white matter Low serine, and glycine levels in the CSF, Plasma
Pyridoxine resistant Pyridoxal phosphate sensitive seizures	Mixed	Neonatal epileptic encephalopathy Suppression burst pattern on EEG Seizures respond to Pyridoxal phosphate	AR	CSF analysis reduced HVA, 5HIAA Increased 3 methoxytyrosine, threonine and glycine Increased urinary vanilactic acid
Glucose transporter (GLUT1) deficiency #606777	Mixed	Intractable seizures in early life Acquired microcephaly Psychomotor retardation Oscillatory eye movements Treatment response to ketogenic diet, Thioctic acid	AR 1p35	Persistent CSF hypoglycorrachia

GLUT1, which is accompanied by an intractable epilepsy phenotype. The condition responds to the administration of a ketogenic diet (56b). Deficiency of three enzymes; arginine glycine amidino transferase (AGAT), guanidino acetate methyltransferase (GAMT) and creatine transporter result in a clinical syndrome of mental retardation and epilepsy, with creatine deficiency as a common denominator. Measurement of guanidinoacetate in body fluids and demonstration of brain creatine deficiency using in vivo proton magnetic resonance spectroscopy are helpful in establishing a diagnosis. GAMT and AGAT deficiency can be treated by oral creatine supplementation (177). It is of interest that an X linked phenotype of epilepsy and mental retardation has been associated with a mutation in the gene encoding for a creatine transporter gene (SLC6A8) and mapped to the Xq28 locus (178).

#### 116.8.5.1 Mitochondrial DNA Depletion Syndromes.

Another group of disorders involving mitochondrial targeted nuclear DNA genes are the mitochondrial DNA depletion syndromes, which have come to be associated with early-onset epileptic encephalopathies. Germ line mutations in the genes involved in mitochondrial DNA duplication (particularly  $\gamma$  polymerase POLG) and recessive mutations in the nuclear genes (*TWINKLE* and *ANT1*) have been associated with the Alpers as well as other intractable epilepsy phenotypes (179). Children with intractable epilepsy should be screened for POLG mutations, as these individuals would be at risk of developing progressive hepatic failure (180). Valproate should particularly be avoided in scenarios where mitochondrial DNA depletion syndromes are being suspected.

A detailed discussion of this group of epilepsy syndromes is beyond the scope of the present review. Readers are referred to a review article written by the principal author addressing the causes, the clinical presentation, the approach to diagnosis and treatment of epileptic encephalopathies associated with inborn errors of metabolism (181).

## 116.9 CLINICAL AND LABORATORY EVALUATION

The clinical history should focus on elucidating information on the age of onset, seizure type, triggers or precipitating factors, prior history of CNS insult, and existence of comorbid neurological problems (mental retardation, developmental delay, risk factors of stroke, tumor, trauma, CNS infection). In the evaluation of the epilepsy phenotype, the assistance of a pediatric neurologist/epileptologist is essential. Seizure semiology and the electroencephalogram are critical in helping narrow an electroclinical syndrome. The age of onset of seizures, seizure type at onset, evolution and change in semiology must be documented. For instance, onset with prolonged febrile seizures that are hemiconvulsive in the first year of life, followed by the development of myoclonic jerks and other seizure types with cognitive decline would be very suggestive of severe myoclonic epilepsy of infancy. Thus, collecting clinical data, the nature of the epilepsy, progression, presence of associative features are all essential steps in identifying an epilepsy phenotype. In the family history, information on all affected individuals should be collected along the same lines as for the proband. The ethnic background could alert physicians to a founder effect (“Baltic myoclonus” in Finnish population), certain pathogenic mutations and phenotypes may have been described in certain ethnic groups. A family history of consanguinity will suggest autosomal recessive inheritance. Although a matrilineal inheritance pattern points toward mitochondrial cytopathies, these disorders can also show autosomal recessive or dominant inheritance patterns. Other valuable features include a positive family history for seizures/any history of developmental delay/developmental regression/other associated symptoms such as organomegaly/vision and hearing involvement.

The physical examination of the affected individual is directed toward assessing cognitive and developmental abilities, and the presence or absence of focal neurological deficits, and lateralizing signs (preferably conducted by a neurologist in consultation). Most of the patients with idiopathic/cryptogenic epilepsies show little or minimal cognitive or developmental dysfunction, no lateralizing signs, or focal neurological deficits on examination; however if the etiology is symptomatic, then the examination will provide clues to etiology. Anthropometry with special reference to head circumference can provide clues to certain genetic/metabolic syndromes (e.g. macrocephaly in Fragile X syndrome and microcephaly in Rett

syndrome, AS and other inborn errors of metabolism). A careful search for dysmorphic features is warranted, as they may suggest association with specific chromosomal rearrangements.

Examination of the eyes may disclose specific abnormalities of the fundus (cherry red spots, macular changes and pigmentary retinopathy storage disorders, degenerative diseases, and intrauterine infection), cataracts (metabolic disorders). A careful evaluation of the skin for hypopigmented macules (tuberous sclerosis), hyperpigmentation (café-au-lait spots, whorl-like pigmentation), and the presence of port wine nevi on the face may suggest neurocutaneous syndromes (NF1, incontinentia pigmenti, Sturge–Weber syndrome). The presence of visceromegaly, abnormal odors in sweat and urine are additional pointers toward metabolic disease. Examination of genitalia in the case of X-linked infantile spasms would be important to identify the presence or absence of ambiguous genitalia. The neurological examination may help in localizing the structural abnormality to a particular hemisphere or region. Clinical findings such as spasticity, dystonia, and ataxia suggest involvement of specific pathways (pyramidal and extrapyramidal tracts), and regions (subcortical white matter, basal ganglia, cerebellum and connections) within the nervous system.

The neurological history and examination must be supplemented by EEG data to characterize the seizure type and electroclinical epilepsy syndrome. The investigative work up should be individualized for each patient. The seizure types can be classified by the age of onset, type, and with/without fever, frequency, time of occurrence, and association with sleep. In the idiopathic generalized epilepsies, an imaging study is not usually considered as necessary. Video EEG studies may be considered to characterize the seizures and provide electroclinical correlation. The need for other investigations such as imaging arises in the case of a focal- or localization-related epilepsy to rule out a structural lesion or when a symptomatic etiology is suspected. If it is deemed that the clinical profile is not consistent with the well-established clinical phenotypes of genetic generalized epilepsies, other investigations may be selected on a case-by-case basis. Specialized genetic testing may become necessary when the clinical context and imaging studies suggest specific structural abnormalities for which a genetic basis is established (periventricular nodular heterotopia, lissencephaly, and so on). Genetic testing may also become necessary in cases of co-occurrence of epilepsy and syncope, and epilepsy and SUDEP (sudden death in epilepsy) as these conditions have been linked to channelopathies, particularly mutations affecting the potassium channel subunit (KCNQ1) (182).

### 116.9.1 Genetic Investigations and Gene Testing

The role of genetic testing takes on new urgency with the availability now to access such testing on a commercial

scale direct to consumers. Clearly high throughput technologies such as arrayCGH and exome sequencing are set to overtake traditional karyotyping and first generation technologies. The initial work up for every child referred for genetic evaluation should include arrayCGH to detect deletions/duplications, targeted mutation analysis by sequence analysis or detecting methylation status of the *FMR1* gene if indicated. Specific testing for Fragile X, Rett syndrome, Pitt–Hopkins, AS, and ring chromosome 20 become important in the panel of tests to be considered based on the clinical neurological phenotype. In the context of a specific genetic epilepsy syndrome, the age of presentation, the presence or absence of consanguinity, the seizure semiology, and the EEG findings are critically important in making a syndromic diagnosis. For an early epileptic encephalopathy, the targeted mutation analysis could include some of the following genes depending on the abovementioned factors in history and examination (*ARX*, *STXBP1*, *CDKL5*, *SLC25A22*). Similarly, if the phenotype directs the clinician to myoclonic epilepsy or a related “borderland” syndrome, mutation testing for sodium channel mutations would be relevant. In such cases there is a high clinical utility, as a positive test for a mutation could be useful in counseling and in directing treatment choices.

Direct to consumer testing poses many problems while advocates favor the autonomy it brings to patients, critics argue and worry about quality standards, issues pertaining to interpretation of results and the lack of follow-up genetic counseling in the commercial setting. For the geneticist, the ILAE commission on genetic testing has brought out a detailed special report looking at the clinical contexts of genetic testing (diagnostic, predictive, prenatal and carrier), the molecular methods (sequencing, mutation scanning, FISH, targeted mutation analysis, arrayCGH, SNP arrays, and MPLA), and evaluating potential benefits and harms. The special report specifically looks at the clinical validity and utility of diagnostic testing in individuals affected with epilepsy, and in unaffected relatives, as well as the ethical, social and legal implications of such testing. At present, the key message is that despite the availability of gene testing technologies, and many genes being identified, few have current clinical utility in the context of the common genetic epilepsies. The report summarizes the current status of such testing and it is likely that with time and advances in the field, recommendations may change. The reader is referred to this timely and detailed and useful document as the topic merits a separate discussion.<sup>(183)</sup> Links are also provided to web sites of the CDC that give information on the evaluation of genomic testing. <http://www.cdc.gov/genomics/gtesting/ACCE/>. In the data summarized in Tables 116-5–116-7, we have also included information on the availability, accuracy and utility value of available gene tests for the genetic generalized and partial epilepsy syndrome constellations. Gene tests for disorders leading to the progressive myoclonic

epilepsies, epilepsy associated with structural brain malformations, metabolic disorders leading to epilepsy are likely to be discussed in details elsewhere in the textbook.

### 116.9.2 Cranial Imaging

In order to define structural pathology within the nervous system, a cranial MRI should be performed using appropriate coils and protocols to provide high resolution imaging, when evaluating malformations of cortical development and disorders of neuronal migration. Patterns of imaging abnormalities provide critical diagnostic clues for static encephalopathies, degenerative and storage disorders. The use of other techniques such as magnetic resonance spectroscopy can be helpful for measurement of specific metabolites, e.g. a lactate peak (mitochondrial disorders), and the absence of creatine (creatine transporter deficiency).

### 116.9.3 Metabolic Investigations

A consultation with genetics and metabolic specialist is recommended. Ammonia level should be obtained in all encephalopathic individuals or with seizures to rule out urea cycle disorders. Abnormal patterns of plasma amino acids (quantitative) may suggest specific conditions for instance; high alanine levels (mitochondrial disorders), high phenylalanine (untreated PKU should be rare after introduction of newborn screening), low cystine in (isolated sulfite oxidase deficiency), low serine in plasma and CSF (serine deficiency). Urine organic acids should be assayed to rule out any organic acidemias. Low serum uric acid is seen in molybdenum cofactor deficiency, another cause of severe intractable seizures in childhood. Urine dipsticks will be positive for elevated sulfites in sulfite oxidase deficiency. Assays for specific lysosomal enzymes depend on the index of suspicion and clinical profile. DNA-based analysis is used often for mitochondrial point mutations such as in the MELAS, and MERRF syndromes.

*Analysis of cerebrospinal fluid* may provide useful diagnostic markers; elevated CSF lactate (mitochondrial disorders), an elevated CSF/plasma glycine ratio of more than 0.8 (NKH), low serine (serine deficiency) and persistently low CSF glucose to serum glucose ratios (GLUT1 deficiency).

### 116.9.4 Skin, Nerve and Muscle Biopsies

Electron microscopic examination of a skin/nerve biopsy for inclusions is of benefit in the evaluation of lysosomal storage disorders, neuronal ceroid lipofuscinoses, and Lafora body disease <sup>(184)</sup>. Skin should be stored for fibroblast studies, if needed. A muscle biopsy although invasive is the gold standard for diagnosing some mitochondrial disorders. Histopathology and electron microscopy are useful in defining integrity of mitochondrial

structure. Use of special stains such as succinate dehydrogenase and Gomori Trichrome stains can detect red ragged fibers, and cytochrome oxidase negative fibers. All these can suggest mitochondrial pathology; respiratory chain analysis on fresh muscle will also help with diagnosis of respiratory chain defects. An algorithm for the work up of genetic metabolic etiologies presenting with epileptic encephalopathies is suggested (181).

### 116.10 GENETIC COUNSELING ISSUES IN EPILEPSY

Genetic counseling has become a central component of the comprehensive care of the family with a child who has epilepsy. By providing the family with accurate genetic risks for specific epilepsy syndromes, and empirical risks for complex polygenic epilepsies, the physician enables the family to make informed life decisions. The central issues relating to genetic counseling involve the heritability of epilepsy syndrome, the risk of epilepsy in family members and offspring, and the value of genetic tests in epilepsy (10).

The seizure type, supplemented by the history, physical examination and EEG and relevant laboratory investigations will determine recognition of a specific electroclinical epilepsy syndrome, or a clinical phenotype associated with a known single gene defect. The next step in the process is the family history, where a detailed pedigree should be drawn to address key questions (Box 116-1).

The information obtained in combination with clinical data will suggest possible modes of inheritance. For disorders with classical Mendelian inheritance patterns, counseling is straightforward. For instance, in EPM1, and autosomal recessive disorder, with a penetrance of 100%, the sibling risk is 25% (one in four). In benign familial neonatal convulsions, an autosomal dominant inheritance pattern with incomplete penetrance (85%) translates into a 40–45% sibling risk. Matrilineal inheritance patterns suggest a mitochondrial disorder is likely. Counseling for mitochondrial disorders is complex and should take into account heteroplasmy. For disorders with single gene defects, the degree of penetrance and mode of inheritance determine risk.

For the non-Mendelian disorders (i.e. most common idiopathic epilepsies), estimates of risks are best expressed in terms of absolute risk (%) with a range of values as well as to provide comparison figures for the general population. The class of relative (in relationship to the probands) should be taken into account. Sibling and offspring risk estimates are based on the information from epidemiological studies. Risk estimates are influenced by a number of factors including seizure and syndrome type, type of EEG abnormality, age of onset, parental attributes (sex and affected status), and the number of individuals with epilepsy in the family. The risk of developing any form of idiopathic epilepsy by the age 20 years in a sib of an affected proband is around 3%, as

#### BOX 116-1 Key Issues in Genetic Counseling

- Is epilepsy inherited?
- Status of family members
  - Is the proband a twin born, monozygotic or dizygotic
  - Is the cotwin affected or unaffected
  - Number of sibs, affected or unaffected
  - Are parents affected? Mother or Father?
  - Does the proband have any children
  - Affected relatives, class of relatives
- Age of onset of epilepsy in affected individuals
- Seizure type and epilepsy syndrome in affected individuals
- Seizure etiology
- Role of genetic testing in epilepsy

compared to the general population risk of 1%. The risk estimates increase for instance with earlier age of onset, associated EEG patterns, and if one or both parents are additionally affected. Higher risks are associated with specific epilepsy syndrome type. In general, a 6% risk to offspring is considered as a reasonable estimate with an affected mother with idiopathic generalized epilepsy. Though risks in offspring do not seem to be influenced by the seizure type (generalized versus partial), they may be higher for certain specific epilepsy syndromes such as childhood absence epilepsy, and JME. Earlier onset of epilepsy appears to increase risk to offspring, but is not dependent on etiology of epilepsy (Idiopathic/cryptogenic versus symptomatic) Table 116-12 (10,185).

The role of genetic testing in the routine management of common idiopathic epilepsies is limited. The complex modes of inheritance and various confounders that can influence the reliability of a test result preclude routine use of genetic testing in this group. Affected families may opt to undergo genetic testing through participation in numerous research studies and programs such as the gene discovery project of the Epilepsy Foundation of America. For monogenic disorders where genes and common mutations are known and identified, direct mutation analysis is possible in disorders such as the progressive myoclonic epilepsies (EPM1, EPM2, MERRF, and the ceroid lipofuscinoses). These are serious disorders with considerable morbidity and mortality and genetic testing can be justified. Current information on the molecular diagnostic testing, details regarding the specific types of tests and a list of laboratories performing the tests are available through the NCBI web site, <http://www.ncbi.nlm.nih.gov/sites/GeneTests/lab?db=GeneTests>.

### 116.11 PHARMACOGENOMICS ISSUES IN EPILEPSY

The accelerated pace of identification of gene mutations, has opened up opportunities to understand the interactions of gene products, other proteins and drugs within the cell. We have entered into an era of understanding



**TABLE 116-12 Empirical Estimates of Risks of Epilepsy in First-Degree Relatives, and in Offspring**

Genetic Counseling Issues	Proband Attributes	Cumulative Risk Estimate in Terms of Percentage of Siblings by Age 20 Years (%)
<b>Sibling Risk to Age 20</b>		
	General population risk	1
Acute symptomatic	Febrile seizure	2.3
	Febrile seizure then epilepsy	6
Epilepsy age of onset	IGE Onset < age 15	4
	IGE Onset > age 25	2.6
	Overall	2
Epilepsy etiology	Postnatally acquired	1
	Epilepsy and MR/CP	3
Epilepsy seizure type	Partial epilepsy	2.5
EEG pattern	Epilepsy and GSW	6
	Epilepsy + (GSW + PPR) or GSW + multifocal spikes	8
Proband with epilepsy	Parent also affected	8
	Parent also affected either with GSW	12
	GSW in probands and sib	15
<b>Offspring risk for Epilepsy to age 20 where the proband is an adult<sup>a</sup></b>		
Proband attribute	Mother with epilepsy	6
	Father with epilepsy	2.4
Parent seizure type	IGE	3.3
	Pure absence epilepsy	9
	Partial epilepsy	4.6
Parent age of onset	Onset < 20 years	6
	Onset > 20 years	2.8
Parent seizure etiology	Idiopathic/cryptogenic	4.2
	Remote symptomatic etiology	
<b>Specific Epilepsy syndrome related risk to sibs and offspring<sup>b</sup></b>		
Risk type	Syndrome	Estimated Risk %
Sibling risk	Childhood absence	5–10
	Juvenile absence	5
	Juvenile myoclonic	5–7
	Myoclonic astatic	19–20
	Grand mal on awakening	No data
Offspring risk	Childhood absence	7
	Juvenile absence	5
	Juvenile myoclonic	7
	Myoclonic astatic	4
	Grand mal on awakening	5–7

<sup>a</sup>Data summarized from Reference (185).<sup>b</sup>The risks quoted in this section are slightly higher than in first section, the risk to sibling and offspring are similar.

of interaction between drugs and genotype. Variations in genotype will provide a rational basis for the understanding the differences in drug pharmacokinetics, as well as the effect of external factors that influence such differences (age, sex, race, etc.) (186). Proteomics and pharmacogenomics will help us better understand the mechanisms of effectiveness of antiepileptic drugs as well as drug resistance. It has been established for instance that genetic polymorphisms in the ATP-cassette binding member 1 (ABCB1) are associated with a drug resistant epilepsy phenotype (187). Thus, strategies that will block ABCB1, or drugs that will evade or be resistant to the effects of ABCB1, can be designed to improve effectiveness of AED therapy.

Aberrant effects of drugs can also be prevented through knowledge of specific interaction between drug and epilepsy phenotype; for example the use of carbamazepine in the treatment of patients with idiopathic generalized epilepsy presenting with absence seizures (precipitates nonconvulsive status or will induce appearance of other seizure types) (188). The mechanisms underlying this are not well understood but may be linked to the effects of gene mutations on thalamo-cortical networks. Similarly, the use of vigabatrin in the treatment of patients with AS and epilepsy leads to exacerbation of seizures (189). This clinical interaction is also likely linked to the genotype in patients with AS and the effect of vigabatrin in raising cerebral GABA

levels and altered inhibitory extrasynaptic effects on GABA-receptor subunits.

Another example of a pharmacogenomic interaction is the precipitation of hepatotoxicity in patients with mutations in *POLG*, an enzyme involved in mitochondrial DNA maintenance. Mutations in the gene coding for the mitochondrial  $\gamma$ -polymerase can be tested for, and used predictively to determine risk for valproic acid induced hepatotoxicity (180). In the case of *GLUT1* transporter deficiency, the selection of the ketogenic diet as treatment based on the understanding of biochemical aspects of the disorder has been rewarding (56b). An improved understanding of pharmacogenomic interactions in epilepsy will enable the physician in making correct choices of antiepileptic drugs in treatment of seizures, improvements in drug design, the prediction and avoidance of aberrant drug effects, and better strategies in dealing with drug resistance.

## 116.12 EPILEPSY AND PREGNANCY

Pregnancy in women with epilepsy increases the risk of fetal malformations. The risk of teratogenicity was highlighted several decades ago. Prospective studies were only carried out in the early 90s leading to the development of guidelines for care of pregnant women with epilepsy. Fetal malformations were thought to occur at 2–3 times higher rate (around 8–9%) in pregnant women with epilepsy in comparison to the general population (190). There is considerable variability in the reported incidence of birth defects in different studies, but a teratogenic effect attributable to the use of AED is noted, influenced in particular by the type of drug, number of drugs, and dosage during pregnancy (191).

Families with a higher incidence of birth defects, tend to report a higher incidence of malformations in pregnancies exposed to the teratogenic effects of AEDs. This suggests that a genetic susceptibility to malformations may be an added factor influencing the outcome in these pregnancies. Among the front line antiepileptic drugs, valproic acid appears to be the most teratogenic, with a well established dose-related effect, while drug combinations such as phenobarbital+ethosuximide, and primidone+valproic acid were associated with the highest rate of malformations in prospective studies conducted in Europe, and joint Japanese, Canadian, Italian studies respectively (191). Valproic acid exposure during pregnancy has been shown to be associated with a poorer cognitive outcome in children in comparison to carbamazepine, lamotrigine and even phenytoin (192).

Carbamazepine is also associated with the specific risk of spina bifida but to a lower extent than valproic acid (193). The formation of several International Collaborative Registries such as EURAP (European Registry of Antiepileptic Drugs and Pregnancy) as well as other national registries will provide meaningful data on the risks associated with individual antiepileptic drugs.

Women diagnosed with epilepsy and taking AEDs approaching childbearing years should be counseled regarding the risk of teratogenicity. A multidisciplinary consultation involving the geneticist, epileptologist, psychiatrist (if the indication for AED treatment also involves a comorbid psychiatric disorder) and a specialist in maternal-fetal medicine is advised in order to adjust and monitor treatment. A single AED in the lowest dose necessary to effectively control seizures should be chosen several months prior to conception. Valproic acid and polytherapy should be avoided in the first trimester. This will ensure that seizure control will be maintained during pregnancy. Periconceptional folic acid (4 mg/day) is advisable in mothers taking carbamazepine/valproic acid to reduce the risk of neural tube defects. Screening for neural tube defects, ultrasonography and fetal MRI can be helpful in the early detection of congenital anomalies. Additional supplementation of vitamin K (10 mg/day) is advised for 1 month prior to delivery to reduce the risk of hemorrhagic disease of the newborn (194).

### 116.12.1 Fetal Anticonvulsant Syndrome

The most common malformations associated with the use of AEDs include minor anomalies (mid facial hypoplasia, hypoplasia of nail and distal phalanges, and umbilical or inguinal herniae) and major defects (facial clefts, structural heart and neural tube defects, and urinary tract defects) (195). Delayed effects such as developmental delay and cognitive dysfunction are also known to be associated. The periconceptional use of folic acid as supplement, even in small doses as multivitamin supplements may help reduce the occurrence of these defects (196). The major fetal anomalies associated with common AEDs are summarized (Table 116-13).

### 116.12.2 Neural Tube Defects, and Use of AEDs

A 10–20-fold increase in the prevalence rates for spina bifida is noted following exposure to valproic acid in human fetuses. The mechanism by which this teratogenic effect is induced is not fully understood. One potential pathway involves folate metabolism, as folic acid has a known protective effect against neural tube defects in pregnancy, and valproic acid has a known inhibitory effect on folate metabolism.

A number of candidate genes affecting folate metabolism are known, and have been studied including folate receptor alpha (*FR $\alpha$* ), reduced folate carrier, 5,10-methylenetetrahydrofolate reductase (*MTHFR*), to name a few. *MTHFR* is involved in the regulation of intracellular concentration of homocysteine, and elevated homocysteine levels during pregnancy are associated with an increased risk of neural tube defects. This relationship has drawn particular attention as periconceptional administration of folic acid has been shown

**TABLE 116-13 Fetal Anticonvulsant Syndrome-Specific Drug-Related Effects**

Fetal Anticonvulsant Syndrome	Systemic Malformations
Phenytoin effects	Growth retardation Learning difficulties Frontal bossing, ocular hypertelorism, Nail and digital hypoplasia
Phenobarbital	Facial clefts Cardiac anomalies Neural tube defects Hypospadias Hernia Polydactyly
Valproic acid	High forehead Ridged metopic suture Medial deficiency of eyebrows Low set ears, posteriorly rotated Epicanthal folds Flat nasal bridge Thin vermilion border to upper lip Neural tube defects
Carbamazepine	Short nose Long philtrum Hypertelorism Nail hypoplasia Neural tube defects
Lamotrigine	No increase in risk reported in prospective studies

to reduce the risk of neural tube defects (197). Several studies have examined the relationship of MTHFR genotypes and risk of NTDs, as well as the interaction in women who use antiepileptic drugs during pregnancy. Homozygosity for the common 677C→T mutation appears to be associated with an increased risk of NTD in selected populations (198). No definite conclusions can be drawn regarding the interaction between this genotype and in utero exposure to specific AEDs, due to weaknesses in methodology (199).

Interference in methylation of developmentally regulated genes, involvement of genes regulating cell cycle and growth factors are potential mechanistic pathways. Critical perturbations in neurulation involving pathways that are folate sensitive provide an explanation of the influence of antifolate drugs such as valproic acid (200). Although it is now recognized that NTDs are also associated with the use of carbamazepine in pregnancy (201), insights into mechanisms underlying this interaction remain unexplored. Among the newer AEDs, the risk of major congenital malformations appears to be the same as for the general population with the use of lamotrigine as monotherapy (202), but is increased when evaluated for the combination regimen of lamotrigine+valproic acid, an effect similar to that observed with valproic acid monotherapy.

The AED Pregnancy Registry based at the Genetics and Teratology unit at the Massachusetts General Hospital

is a voluntary initiative to collect and analyze information of fetal risk following exposure in pregnancy (<http://www.aedpregnancyregistry.org/about.htm>).

## 116.14 SUMMARY

We have highlighted the diversity of causes, genetic mechanisms and the role of epilepsy genes influencing inherited susceptibility to epilepsy in humans (Figure 116-1). Our understanding of the function of these genes and the proteins that they code (203) for is in its infancy, but even these glimpses underscore the many pathways to epileptogenesis. From ion- and ligand-gated channels, signaling and regulatory molecules, chromosomal aberrations, and defects in intermediary metabolism, the heterogeneity of inherited epilepsy is truly breathtaking. New technologies hold the promise of opening many windows of understanding, and hopefully will lead to methods of early detection, counseling and designing effective therapies for affected individuals and their family dealing with this complex disorder.

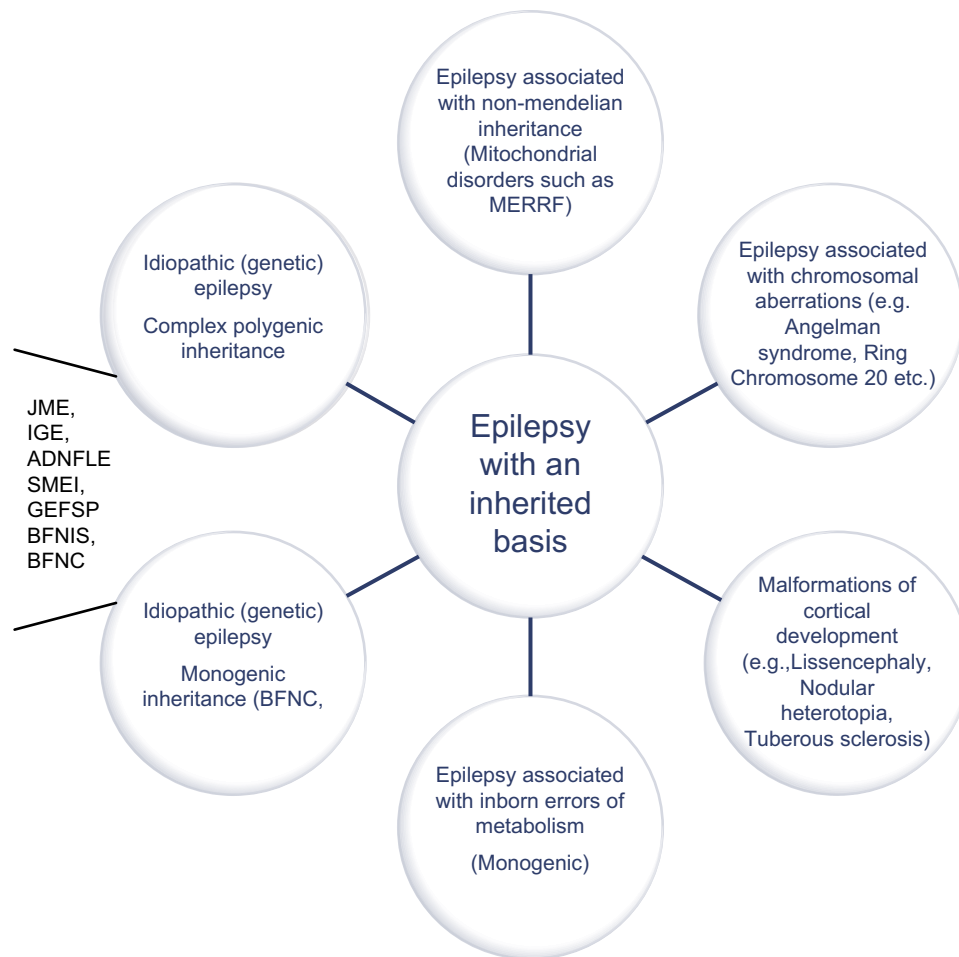
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**FIGURE 116-1** Genetic influences in human epilepsy. JME=juvenile myoclonic epilepsy, GE=idiopathic generalized epilepsy, ADNFLE=autosomal dominant nocturnal frontal lobe epilepsy, GEFSP=generalized epilepsy with febrile seizure plus, BFNIS=benign familial neonatal-infantile seizures, BFNC=benign familial neonatal convulsions.

and Other Structural Abnormalities of the Autosomes. Nancy B. Spinner; Amino Acid Metabolism. Raymond Y. Wang and William Wilcox; Disorders of Carbohydrate Metabolism. YT Chen and Priya Kishnani; Congenital disorders of protein glycosylation. Jaak Jaeken; Purine and Pyrimidine Metabolism. Naoyuki Kamatani; Organic Acidemias and Disorders of Fatty Acid Oxidation. Jerry Vockley; Copper Metabolism. Stephen G. Kaler; Iron Metabolism and Related Disorders. Jeffrey R. Gruen, Christopher Bowlus, Kaveh Hoda and Tom Chu; Mucopolysaccharidoses. James E. Wraith; Disorders of DNA Repair and Metabolism. Sharon E. Plon; Oligosaccharidoses and Allied Disorders. Juliaan G. Leroy; Gangliosidoses and Related Lipid Storage Diseases. Rose Mary N. Boustany, Ibrahim Al-Shareef and Sariah El-Haddad; Peroxisomal Disorders. RJA Wanders; Neural Tube Defects. Richard H. Finnell; Genetic Disorders of Cerebral Cortical Development. Christopher A. Walsh; Sleep Disorders. Allan Pack; The Phakomatoses. Susan Huson; Multiple Sclerosis and Other Demyelinating Disorders. AD Sadovnick.

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## RELEVANT WEBPAGES

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## Biographies



**Asuri Narayan Prasad, MBBS, MD, FRCPC, FRCPE** is an tenured associate professor in Pediatrics and Clinical Neurosciences at the Schulich School of Medicine and Dentistry at the Western University, London, Ontario, Canada. He is currently an academic pediatric neurologist on staff at Children's Hospital, and Director of the Pediatric Epilepsy Monitoring Service, London Health Sciences Center. He is also appointed as an associate scientist at Child Health Research Institute (CHRI) affiliated with the Children's Hospital. His research interests focus on pediatric epilepsy, the interface between genetics, metabolism and neurological disorders in childhood. In collaboration with Dr Chitra Prasad, a Neurogenetics and Neurometabolic (multidisciplinary) clinic service has been developed at the Children's Hospital since 2005. He has more than 100 peer-reviewed publications and abstracts to his credit in high impact journals including; "Epilepsia," "Brain," "Neurology," "Journal of Child Neurology," "Molecular Genetics and Metabolism," and the "Canadian Journal of Neurological Sciences." After serving as Secretary–Treasurer, he is currently Vice-President of the Canadian Association of Child Neurology and is a member of the Board of Directors of the Canadian Neurosciences Federation. He is committed to improving the lives of children with epilepsy and neurological disorders and their families.



**Dr Chitra Prasad MD, FRCPC, FCCMG** is an associate professor in the Department of Paediatrics, Section of Genetics and Metabolism, and Director of Metabolic Clinic at the Western University, London, Ontario since 2003. She is a certified pediatrician (Paediatrics from PGIMER (Post Graduate Institute of Medical Education and Research in India), Fellow of Royal College of Paediatrics Canada (FRCPC) and through American Boards in Paediatrics). She received postdoctoral training in medical genetics and inborn errors of metabolism at Children's Hospital, Harvard Medical School, and Boston. She has subspecialized in clinical genetics with certifications in both clinical genetics and biochemical genetics (American College of Medical Genetics and the Canadian College of Medical Genetics). Before coming to London, Dr Prasad held faculty positions in Medical Genetics at the Memorial University of Newfoundland, St. Johns, Newfoundland and University of Manitoba, Winnipeg. Dr Prasad has published many articles on a variety of genetic syndromes and their pathogenesis and metabolic disorders. Her clinical interests include dysmorphology, the pathogenesis of clinical genetic syndromes and inborn errors of metabolism. The main focus for her research has been establishing genotype–phenotype associations in genetic disorders and delineation of new syndromes as well as therapeutic aspects of metabolic syndromes.

# Basal Ganglia Disorders

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## 117.1 INTRODUCTION

The diagnosis and classification of basal ganglia disorders have traditionally rested on the clinical recognition of involuntary movement syndromes. The term “basal ganglia disorders” infers dysfunction of subcortical brain structures that include the neostriatum (caudate nucleus and putamen), globus pallidus (external and internal parts), subthalamic nucleus, and substantia nigra. These anatomic structures constitute the “extrapyramidal motor system,” a concept introduced by Kinnier Wilson in 1912 in his description of familial hepatolenticular degeneration, now called Wilson disease (1).

Clinically, extrapyramidal disorders can be divided into hypokinetic (associated with a paucity of movement) and hyperkinetic (associated with excessive movement) syndromes. The former for the most part is represented by forms of parkinsonism and is characterized by varying components of hypokinesia, bradykinesia, and rigidity, while the latter include dystonia, athetosis, chorea, tremor, and tics (see Glossary). In general, dystonia, chorea, and tremor constitute a phenomenologic continuum that ranges from sustained abnormal postures and very slow writhing gestures (dystonia) to more or less graceful continuous movements (chorea) to rapid and regular oscillating tremor (tremor). Tics are the most complex of hyperkinetic movements, often indistinguishable from voluntary movements only by their frequency and inappropriate timing.

Although the motor aspects are the signature feature of these disorders, and the one which usually brings them to clinical attention, almost all patients with extrapyramidal diseases manifest cognitive or behavioral abnormalities, although sometimes of a subtle nature, that reflect the important role of the basal ganglia in cognitive function (2). In addition, the disease processes that affect the basal ganglia may also involve structures outside these nuclei, leading to more prominent cognitive features that may include psychosis or dementia.

Extrapyramidal structural pathology is a well-established feature of many of the common basal ganglia disorders, including Parkinson, Huntington, and Wilson disease. In other disorders of this class, however, the structural nature has proved elusive: to date, there are no definitive structural changes associated with primary torsion dystonia or Tourette syndrome. The extrapyramidal nature of these diseases has been inferred from the similarities of the movement disorder to other disorders, the effects of pharmacologic treatment, and, recently, the identification of metabolic and neurochemical alterations by modern imaging methods such as positron emission tomography, single-photon emission computed tomography, magnetic resonance spectroscopy, and functional magnetic resonance imaging. The lack of reported pathology is perhaps best viewed as a weakness of the current methods for identifying such pathology.

Recently, the molecular basis of many of these diseases has been established, and the biologic consequences and pathways that are perturbed are now being investigated. This increasing knowledge has already allowed a more rational classification, as well as improved diagnostic accuracy. It is hoped and to be expected that improved and novel treatment modalities for these devastating diseases will follow.

## 117.2 DISEASES WITH PARKINSONISM

The term parkinsonism is used to describe syndromes characterized by hypokinesia, bradykinesia, rigidity, and sometimes tremor. Parkinsonism can be seen in a variety of settings, which include degenerative, metabolic, drug-induced, and vascular disorders. Parkinson disease (PD), a neurodegenerative condition, is by far the most common cause of parkinsonism in most clinical populations. In the case of a patient with parkinsonism, the major diagnostic considerations are PD or the associated conditions: dementia with Lewy bodies, drug-induced parkinsonism, progressive supranuclear palsy, multiple systems

atrophy, Wilson disease, and corticobasal ganglionic degeneration (CBGD). With the exception of Wilson disease (discussed elsewhere), the forms of parkinsonism that are commonly seen are sporadic in nature, lacking a clear pattern of inheritance. There are, however, hereditary forms of PD with classical Mendelian as well as genetic risk factors for PD, and these provide crucial insights into the pertinent mechanisms of neurodegeneration and neurotransmission.

### 117.3 PARKINSON DISEASE

In 1817, James Parkinson described a disease characterized by bradykinesia, hypokinesia, rigidity, and a resting tremor (3). PD is now recognized as the most common neurodegenerative movement disorder and a disease primarily of older people. Onset before age 40 is rare, but after this age incidence and prevalence rise steadily. Prevalence rates in white populations have been estimated to range between 100 and 350 per 100,000 in the general population, but over 600 in populations over age 75 (4). A 1991-European collaborative study yielded an overall prevalence in those over age 65 of 1600 per 100,000, with rates of 3600 per 100,000 reported in those over 85 years (5). Incidence rates are age-dependent, estimated at 114 per 100,000 in the age group 50–99 years but more than 300 over 80 years (6). There is a strong gender effect, with incidence in males which is nearly twice that of females at all ages (7). PD is more prevalent in whites, in whom the lifetime risk of developing the disease has been estimated at 2.4%. In contrast, in people of African or Asian descent, prevalence and risk rates may be considerably lower (8).

#### 117.3.1 Clinical Features of PD

The most commonly used criteria for the clinical diagnosis of PD are those promulgated by the UK Parkinson Disease Brain Bank (9,10). These require the presence of bradykinesia along with at least one additional feature: muscular rigidity, 4–6 Hz rest tremor, or postural instability. There are also several exclusionary criteria designed to screen for other disorders and positive supportive criteria, which include unilateral onset and response to levodopa therapy. The sensitivity and specificity of these criteria are good but not ideal. From a series of 100 autopsy cases that had received a clinical diagnosis of PD by experienced clinicians, only 76 were diagnosed correctly according to standard neuropathologic criteria (9). At least two of the triad of bradykinesia, rigidity, and tremor were present in 99% of the 76 cases during the course of the disease. In these 76 patients, resting tremor was an initial sign in 72%, while the disease started asymmetrically in 77%. In the course of the disease, 76% displayed tremor, while akinetic rigid disease without tremor was present in 24%. Experts in movement disorders have a higher degree of accuracy: in series of 143 autopsies from patients evaluated at the

National Hospital for Neurology and Neurosurgery in London, the positive predictive value of the clinical diagnosis of IPD was extremely high, at 98.6% (72 out of 73). This suggests that experts in the field may apply methods of pattern recognition that are more sophisticated than simple heuristic criteria (11).

The clinical diagnostic criteria emphasize the early features of the disease, when the diagnosis is most in doubt. Later in the course of the disease, balance problems and frequent falls often become problematic. Progressive dysarthria (loss of volume, monotony, and impaired articulation), dysphagia, and drooling are other common features. Depression is common, as is autonomic failure with orthostatic hypotension, and with longer follow up, many patients eventually develop dementia (12). The nosology of dementia associated with PD has undergone considerable evolution in recent years. As currently defined, dementia that develops in the context of established PD is termed “Parkinson disease with dementia” (PDD), while those patients who develop dementia followed by PD are described as “Dementia with Lewy bodies” (DLB) (13,14). The pathology is similar, and some in the field have argued that these should be viewed as different parts of the same spectrum of disease (15). Recent genetic findings strongly support this position as discussed in this chapter.

An interesting development in recent years has been the recognition that in addition to these late, nonmotor complications of PD, there is also a prodromal syndrome preceding the classic motor features (16). The earliest aspects of this may include hyposmia, as well as constipation (likely a consequence of involvement of the enteric nervous system). Indeed, the long-term Honolulu Heart Study showed that frequency of bowel movements was a strong predictor of the occurrence of PD at a follow up 30 years later (17,18). Another strong predictor of subsequent PD is sleep disturbance, particularly REM sleep behavior disorder. Follow up of men with this condition (it is an almost exclusively male disorder) suggests that as many as 50% will develop PD within 5 years of the identification of the sleep disorder (19,20). Together, these observations suggest that there may be a “premotor” state of PD that is potentially identifiable, and may provide an opportunity for intervention with neuroprotective therapies (21).

The natural course of the disease has dramatically changed since the introduction of levodopa therapy in the early 1960s. Prior to the advent of levodopa therapy, widely divergent clinical courses with deterioration within a few years, or survival for 25 years without losing much independence, were reported (22). With medication, the quality of life of PD patients has markedly improved, but age-specific mortality rates of those with PD or other causes of parkinsonism are still twice or more as high as in controls (23). Much of the late morbidity can be attributed to the nonmotor aspects, such as dementia and autonomic failure, which are often



refractory to dopaminergic medications. The development of hallucinations and cognitive impairment is one of the strongest predictors of death in PD (24).

### 117.3.2 Structural and Functional Neuropathology of PD

The best-known neuropathologic features of PD are neuronal loss in the substantia nigra pars compacta, with accompanying occurrence of local free melanin and glial proliferation. The presence of inclusions, termed Lewy bodies, in the remaining nigral neurons is required for pathological confirmation of the disease. Lewy bodies were first described in 1912 by F. H. Lewy, a neuropathologist from the German city of Breslau (currently Wroclaw, Poland) (25). They are typically round intracytoplasmic inclusions with a dense eosinophilic core and a nonstaining surrounding halo, and are often found together with elongated intraneuritic inclusions (“Lewy neurites”) (26). Lewy bodies can be stained by antibodies against ubiquitin and various neurofilaments.  $\alpha$ -Synuclein staining, a major feature of Lewy body immunohistochemistry, was discovered after  $\alpha$ -synuclein mutations were found to be associated with familial PD (see later). Although the presence of Lewy bodies in remaining dopaminergic neurons in the substantia nigra is the signature feature of PD, Lewy bodies and neurites may be found in various other brain areas. In some patients with dementia, cortical Lewy bodies are so prominent, with or without brainstem inclusions, and with or without clinical signs of parkinsonism, that the term diffuse Lewy body disease has been used. In a large cross-sectional study, Braak and colleagues mapped the distribution of Lewy bodies and neurites in more than 700 postmortem brains. Based on these observations, they proposed a sequential evolution of PD, with the earliest manifestations in the dorsal glossopharyngeal and vagal motor nucleus, the raphe nuclei and caeruleus complex, the midbrain (including the substantia nigra), the temporal mesocortex and allocortex, and finally the neocortex (27,28).

The defining neurochemical feature of PD, originally described by Hornykeiwicz and colleagues, is the depletion of dopamine from the striatum (29). This in turn drives the main motor features of the disease—bradykinesia, hypokinesia, and rigidity—and explains the excellent response to pharmacologic dopamine replacement or agonist treatment. There are also a variety of other neurochemical alterations involving other catecholamines and nonaminergic systems, but the specific relationships between these abnormalities and clinical symptoms are less clearly established. For example, due to the profound degeneration of noradrenergic neurons in the locus coeruleus, a mood disorder may arise that accompanies motor dysfunction (30).

The major issue in understanding the neuropathology of PD has always been to find the cause of the progressive

neurodegeneration. Why do substantia nigra neurons—and other neuronal populations—die? In the early 1980s, evidence in favor of environmental causes of PD seemed particularly strong. It was firmly supported by studies in humans and animals that were exposed, accidentally or intentionally, to methylphenyltetrahydropyridine (MPTP). This substance appeared as an accidental contaminant of illicit heroin analog synthesis and turned out to be a highly selective neurotoxin that primarily damages melanin-containing and dopamine-synthesizing neurons (31). MPTP is broken down by monoamine oxidase B into MPP<sup>+</sup>, which significantly interferes with the function of the mitochondrial respiratory chain and results in death of the dopaminergic neurons (32). The discovery of MPTP-induced parkinsonism has always been a major argument in favor of environmental toxins as causes for dopaminergic degeneration in PD, and has supported the notion of mitochondrial abnormalities as risk factors. But, as the 1980s gave birth to the notion of environmental toxins, the 1990s saw the rise of the genetic understanding of PD.

### 117.3.3 Genetics of Parkinson Disease

PD was long considered to have little or no significant genetic contribution to disease susceptibility. While families that clearly inherited disease from one generation to the next were described by prominent neurologists of the nineteenth and twentieth centuries, the debate centered on whether the familial variations of the disease shared enough overlap as parkinsonian disorders to be considered part of the usual spectrum of PD. Early twin studies were interpreted as providing evidence against the existence of important genetic susceptibilities (33–35), but the small sample sizes of these initial studies, reduced penetrance of the disease, and the inability to detect subclinical PD may have been confounding factors. Subsequently, the studies in the late nineteen-nineties yielded the pivotal discovery of  $\alpha$ -synuclein in PD, which uniquely links together lesions that define PD on a pathological level (i.e. Lewy bodies) with rare genetic variation of large effect. Thus, there is no longer a debate over whether there are important genetic susceptibilities in PD: there are now clear and important genetic factors involved, and these will be expanded upon in this chapter; however, it remains unclear the degree to which genetic (or epigenetic) variation ultimately influences disease susceptibility, how many genetic factors are yet to be discovered, and how genetic discoveries will impact clinical diagnosis and treatment.

### 117.3.4 Twin Studies and Familial PD

Initial twin studies were fairly unambiguous about the lack of a genetic involvement in PD. A more recent twin study in a large cohort from the National Academy of Sciences/National Research Council World War

II Veteran Twin Registry advocated the notion that—at least in those in whom PD starts before the sixth decade—genetic factors may play a role (36). Integration of modern imaging techniques identified preclinical loss of dopaminergic input into the striatum in monozygotic twins, initially classified as unaffected with an affected sibling, that are indicative of subclinical PD, demonstrating that twin studies likely underestimate genetic susceptibilities (37). Other recent studies have more closely examined familial aggregation of PD. The odds for any individual to develop PD are increased two to four times in the presence of a first-degree relative with PD (38–40). These odds further increase with increasing numbers of affected relatives and with younger affected relatives. Odds are higher in Caucasians than in African Americans or Hispanics, and they are twice as high for affected male relatives compared with females (39). In general, familial studies and twin studies have supported a much stronger role for genetic factors in individuals with the onset prior to age 50 (e.g. the early-onset PD), but genetic factors could not easily be ruled out in the late-onset disease.

### 117.3.5 Monogenetic Forms of Parkinsonism and Related Disorders

Most patients with PD do not report a family history of disease, and the number of families with evidence of Mendelian transmission (i.e. monogenic) makes up a small percentage of cases, likely 5% or less. The proportion of monogenic PD can be quite difficult to determine, and clinic and referral bias and ethnicity play variable modifying roles. In the families that are identified where PD segregates in a Mendelian fashion, disease often manifests with atypical clinical and neuropathologic features and often starts significantly earlier than idiopathic typical PD. This is congruent with a larger genetic contribution to the early-onset PD versus late-onset typical PD. Of the familial loci that are described in detail, the only known exception to the ‘early-onset’ rule in monogenic PD is the PARK8 locus, encoding the leucine-rich repeat kinase 2 (LRRK2) gene. Although monogenic PD is usually atypical in clinical presentation (save most LRRK2 families), the major rationale for focusing on monogenic PD is that the specific mutation may perturb more severely a pathway that is important in a sporadic typical PD that is of a later onset. Elucidating the associated pathways would also help identify targets for therapeutics and potentially enhance disease diagnosis.

Loci positively identified through linkage analysis in large PD families are usually designated as PARK loci, and these are distributed throughout the genome. However, some PARK loci are susceptibility genes and not associated with monogenic PD. Currently, there are 18 PARK loci defined, although there are presumably many other loci yet to be discovered in smaller monogenic (particularly recessive) families. Generally, loci are definitively

associated with disease when familial segregation of a specific mutation supports pathogenicity within a reasonable doubt. Other loci remain highly tentative and should be understood with caution until future studies can confirm the association. Of note, it is likely not possible to definitively rule out the existence of a particular locus, so the list of PARK loci will always expand but this does not always correlate with identification of clear genetic susceptibilities of disease. For example, type I errors are common in current GWAS studies and some PARK loci derive from GWAS studies. Both definitely associated and speculative genetic factors are described below:

### 117.3.6 Definitively Associated Autosomal Dominant Loci

Currently, there exist two genes where mutations underlie autosomal dominant PD. These genes are associated with the PARK1/4 and PARK8 loci.

**PARK1/4 ( $\alpha$ -Synuclein):** The first family in which extensive clinical and genetic studies led to the identification of a causative mutated gene was described by Golbe and colleagues (41). In this large family of Italian descent, autosomal dominant PD developed at an early age (average 45.6 years), ran a rapid course, and responded well to levodopa. Neuropathologic examination of affected persons revealed striking cortical Lewy bodies consistent with DLB and neuronal loss in the substantia nigra and the Locus coeruleus. In this family, linkage was found at chromosome 4q21–q23, where subsequently a causative base transition (G209A, leading to an A53T substitution at the protein level) in the  $\alpha$ -synuclein gene was found (42). This same mutation, A53T, causes considerable endophenotypic variation, exemplified by the range of ages of onset of symptoms (18 to 80 years) within the same family. The interpretation is that additional strong modifiers of disease must exist (e.g. stochastic probability, environmental effects, genetic modifiers), but the A53T mutation on its own is of very high penetrance. Only a few other families with this mutation have been identified, five of them of Greek descent (43). The A53T mutation is notable since a threonine residue at position 53 in rodent  $\alpha$ -synuclein is the wild-type sequence. Thus, A53T provides a striking exception to the usual rule in genetics that disease-associated mutations tend to occur in evolutionarily conserved residues. In addition to A53T, a German family with an  $\alpha$ -synuclein C88G/A30P substitution and a Spanish family with a G188A/E46K substitution are known (44,45). The Spanish family displays a DLB phenotype as well.

A second type of mutation that involves the  $\alpha$ -synuclein gene has also been identified and originally attributed to the PARK4 locus in the “Iowa kindred.” Similar to the Contursi kindred, autosomal dominant levodopa-responsive parkinsonism occurs prior to age 50, with memory loss and frontal lobe dysfunction,

in conjunction with extensive neuronal loss and Lewy bodies in both subcortical and cortical regions (46,47). Haplotype errors suggested locus copy number variation, and subsequent quantitative of  $\alpha$ -synuclein by polymerase chain reactions demonstrated triplication of the whole region, in which 17 genes are located (48). The triplication is thought to lead to four functional copies of the  $\alpha$ -synuclein gene in the Iowa kindred, and thus to a pathogenic dosage effect, akin to what has been described for trisomy 21 and early-onset Alzheimer disease. Further support for this notion comes from a Swedish-American family with a triplication (49), as well as from a family with autosomal dominant parkinsonism, without cognitive decline or dementia. In this family, a duplication of the  $\alpha$ -synuclein gene was found (50). Disease severity may be directly correlated to dosage of the  $\alpha$ -synuclein gene, where more copies are associated with more severe and earlier onset forms. Thus, simple overexpression of the wild-type protein leads to early-onset PD with dementia as potentially or more potent than the missense mutations originally identified.

Families with mutations in  $\alpha$ -synuclein are exceedingly rare but screening  $\alpha$ -synuclein for mutations is required in evaluating dominant-transmitting monogenic PD families.  $\alpha$ -Synuclein is a very small protein that is expressed in neuronal presynaptic membranes throughout many neurons of the central nervous system but is also expressed in many tissues outside of the brain. Notable protein interactions include the synphilin (synuclein loving) protein and the 14-3-3 class of proteins, both of which can sequester  $\alpha$ -synuclein and modify toxicity (51,52). The  $\alpha$ -synuclein protein is natively unfolded, lipophilic, and can become phosphorylated by Caesin Kinase II in vivo (53,54).  $\alpha$ -Synuclein is also known to interact with metals such as iron and magnesium, but is not considered a canonical metalloprotein. The highest levels of  $\alpha$ -synuclein in the brain are found in various cortical areas and the basal ganglia, particularly in the dopaminergic neurons of the substantia nigra (55). General consensus suggests that  $\alpha$ -synuclein plays a supportive role of vesicular neurotransmitter release at the presynaptic complex. In disease, the protein forms a major component of Lewy bodies and Lewy neurites in the brains of patients with PD (56).  $\alpha$ -Synuclein is not well conserved with evolution as orthologs are difficult to resolve in invertebrate or lower organisms. Knock-down of  $\alpha$ -Synuclein in rodents is well tolerated with subtle effects on dopaminergic transmission (57). Perhaps most striking is that  $\alpha$ -Synuclein knockout mice are resistant to MPTP intoxication, suggesting an integral role for cell death processes in MPTP susceptible neurons (58). Mice that overexpress  $\alpha$ -Synuclein are not further susceptible to MPTP damage, making this conclusion possibly premature (59).

Despite prominent neuronal localization of  $\alpha$ -synuclein, there is also prominent  $\alpha$ -synuclein aggregation in oligodendroglial and neuronal thread-like

inclusions in multiple systems atrophy (60,61). As a noncanonical secreted protein that is readily detectable in human CSF, it is probable that secreted  $\alpha$ -synuclein accumulates in a pathogenic manner in cells that do not endogenously express the protein.  $\alpha$ -Synuclein is a member of a family of three related synuclein genes. No mutations have been found in  $\beta$ - or  $\gamma$ -synuclein that could be related to PD, or any other neurodegenerative disease. The missense mutations that have been described (i.e. A53T) may enhance the rate of spontaneous self-aggregation that has been observed in the wild-type form of  $\alpha$ -synuclein, while multiplication of the  $\alpha$ -synuclein gene may exert increased aggregation propensity through mass action effects (62). How and why  $\alpha$ -synuclein aggregation leads to Lewy body formation and regional degeneration is currently still unclear and not well modeled in animal systems.

**PARK8 (LRRK2):** Families from Japan, the Basque region in Spain, the United Kingdom, Germany, and Nebraska have been reported with autosomal dominant PD linked to chromosome 12p11.2. Onset was later than other familial PD forms, and response to levodopa was uniformly excellent. In late 2004, missense mutations in the leucine-rich repeat kinase 2 (LRRK2) gene were identified (63,64). Soon after the discovery of LRRK2 mutations as a cause of late-onset familial PD, many additional and independent mutations in families from all over the world were identified, suggesting that PARK8 is indeed not a rare cause of familial PD (65–67). The most prevalent known mutation, G2019S, is responsible for about 5% of all autosomal dominant PD, and initial estimates suggested 1.5% of the so-called sporadic cases in European populations. The G2019S mutation, which probably accounts for the vast majority of LRRK2 linked PD, shows high variation in frequency depending on ethnicity. For example, the G2019S mutation can be found in up to 30% of PD occurring in Ashkenazi Jews and North African Arab Berbers (68,69). Of note, in these populations, mutations are nearly as prevalent in patients that report a family history as those that do not, despite clearly sharing the conserved and ancient disease-linked allele. A combination of incomplete penetrance and the aforementioned difficulties (mentioned in twin studies in PD section) in diagnosis are thought to contribute to this phenomenon. LRRK2 mutations clearly illustrate the problems with classification of familial versus sporadic PD in patient populations, and it is thought that many past studies underestimate the contribution of genetics in PD.

All pathogenic LRRK2 mutations thus far identified are missense mutations that alter conserved residues in functional domains. Unlike  $\alpha$ -synuclein, LRRK2 is part of an evolutionarily conserved protein family that can be traceable back to single-cell organisms. LRRK1 and LRRK2 comprise a subfamily of ROCO (Ras-of complex) proteins but in invertebrates such as drosophila, only one member of this family (dLRRK) is detectable, and

this protein is more closely associated with LRRK1. No mutations or linkage of PD with LRRK1 has been found. Thus, studies of LRRK2 function in lower organisms may be viewed with caution. As a mammalian specific protein, the function of LRRK2 in the brain and in other tissue is not clear. Very high levels of LRRK2 protein are detected in kidney and peripheral blood cells, with only moderate expression in the brain (70). LRRK2 is a protein kinase and the most common mutation G2019S in the kinase domain increases kinase activity in vitro 2–3 fold (71), although a substrate for LRRK2 activity has not been verified. Knockout of LRRK2 in mice is well tolerated with only modest descriptions of effects on neurite outgrowth in cultured neurons ex vivo. Other subtle effects on neurotransmitter and dopamine release have been suggested (72).

LRRK2 is a unique (save LRRK1) protein in the human proteome in that both a kinase and GTPase domain are encoded in the same peptide. GTPases are well-known to modify downstream protein kinases, and it is thought that the two enzymatic domains in LRRK2 serve as an intrinsic modifying pathway where kinase activity (or GTPase activity) represent outputs (73). Indeed, PD-causative mutations such as R1441C localize to the GTPase domain and not the kinase domain. Also similar to  $\alpha$ -synuclein, LRRK2 is expressed in many different neurons throughout the brain. Some of these neurons are susceptible to PD and some are not, making an immediate understanding of neuronal vulnerability in PD not forthcoming. The vast majority of individuals with PD that have the G2019S mutation have typical Lewy body formation consistent with sporadic late-onset PD. Thus, some relationship should exist between LRRK2 and  $\alpha$ -synuclein in late-onset PD, potentially analogous to APP and presenilin's in Alzheimer's disease. However, LRRK2 is not known to process  $\alpha$ -synuclein or directly interact despite extensive experimentation and it is thought that the two proteins function in the same pathway but do not directly interact. It is generally assumed that LRRK2 should lie in a biochemical pathway upstream of  $\alpha$ -synuclein as suggested by experimental crosses of double transgenic animals (74).

### 117.3.7 Definitively Associated Autosomal Recessive Loci

**PARK2:** Autosomal-recessive juvenile Parkinsonism (AR-JP) patients from Japan were described with the onset of motor symptoms in their 20s, such as foot dystonia, leg hyperreflexia, amelioration of signs and symptoms after sleep, and a good sustained response to levodopa, but with early occurrence of drug response fluctuations (75). Disease course is overall comparatively benign, and dementia and cognitive decline rare. On neuropathologic examination, loss of pigmented neurons in the substantia nigra and the locus coeruleus can be found, but, contrary to idiopathic PD, no Lewy

bodies were detectable. The gene for this condition was mapped to 6q25.2–q27, and subsequently homozygous deletions within the parkin gene were identified (76). Although the initial reports suggested deletions as the predominant mutational mechanism, subsequent studies showed a wide variety of mutations in many families (77). Moreover, the clinical phenotype extends well beyond Japanese AR-JP: for example, the age at onset in the fifth decade of life has been well-described. Parkin mutations seem to account for at least one third of all families in Europe with autosomal recessive early-onset (<45 years of age) PD (78). In a genome-wide linkage scan of early-onset PD, the parkin gene is highly associated suggesting genetic variability at this site is the most important contributor to the early-onset disease susceptibility (79). Recent evidence suggests that parkin functions together with PINK1 in mediating mitochondrial dynamics (PARK6, described below).

Many cases positive for early-onset- or late-onset PD have been described with only one detectable heterozygous mutation, such as a large deletion or putative pathogenic missense mutation, and another mutation cannot be detected despite exhaustive search (80). The debate over whether these mutations are coincidental to disease or causative, or some susceptibility in between, has not been fully resolved. There is some evidence to suggest that heterozygous deleterious mutations in parkin lead to increased susceptibility to the development of PD. However, parkin spans one of the most common fragile sites in the genome (FRA6E), and large copy number variations comprising deletions within this region are well described in databases that track chromosomal variants in “control” individuals (dGV, database for genetic variation). Thus, it is clear that two separate inactivating mutations each on separate alleles is linked to and probably the major genetic cause of early-onset PD via a haploinsufficiency model. However, it is not yet clear whether heterozygous mutations lead to increased susceptibility to PD.

Most discovered missense mutations in parkin are very difficult to attribute to disease susceptibility, as many benign alterations are likely to exist. However, functional studies suggest that, for at least the confirmed pathogenic mutations, parkin function is compromised in a number of ways by the presence of associated mutations (81). Parkin encodes an unusual E3 ubiquitin ligase together with an ubiquitin-like domain not usually found on this class of enzyme. The C-terminal sequence of parkin has two RING domains with an in-between RING domain, and RING domains are thought to facilitate transfer ubiquitin to target proteins through both K63 or K48 linkages (82). Parkin mutations have been described in all functional domains, particularly in the RING motifs (78). This links parkin to the ubiquitin-proteasome protein degradation machinery and suggests that parkin mutations impair breakdown of specific proteins. But parkin's E3 ligase-specific substrates have not



yet been established. Immunohistochemistry and in situ hybridization studies showed protein-containing and gene-expressing neurons widely distributed in the substantia nigra, the raphe nuclei, and the basal ganglia, but also in laminar structures such as the cortex and the hippocampus. In patients with parkin mutations who lack Lewy bodies, the protein is completely undetectable; in idiopathic sporadic PD, the protein seems present in normal amounts.

**PARK6 (PINK1):** In a large Sicilian family with recessive disease in four affected individuals, a genome-wide homozygosity screen revealed a locus at the short arm of chromosome 1. Phenotypically, affected patients showed the early-onset (range 32 to 48 years) parkinsonism, with slow progression and sustained response to levodopa (83,84). These clinical features were expanded in additional families from Europe and Asia, broadening the possible age-at-onset range from 18 to 68 years, with or without foot dystonia, with or without sleep benefit.

Valente and colleagues then identified two different homozygous mutations affecting the kinase domain of PINK1 (PTEN-induced kinase 1) in three consanguineous PARK6 families (83). Additional mutations have been described, both affecting the kinase domain and outside this important catalytic site. PINK1 is mitochondrially located and may exert a protective effect on the cell that is abrogated by the mutations, resulting in increased susceptibility to cellular stress. These data provide a direct molecular link between mitochondria and the pathogenesis of PD. Similar to parkin, individuals with a PD phenotype have been identified who are heterozygous for single pathogenic mutation. In these cases, heterozygous loss of PINK1 function may constitute a risk factor for PD, but as with parkin, further studies are required to assess risk and delineate pathogenic versus benign alterations.

PINK1 encodes a serine threonine kinase together with a mitochondrial localization signal (MLS). PINK1 has been demonstrated to provide protection from mitochondrial dysfunction and apoptosis, and removal of PINK1 is suggested to induce defective mitochondrial membrane potential and mitochondrial morphology (85–87). These defects are efficiently rescued by parkin, suggesting PINK1 functions upstream of Parkin in mediating mitochondrial function. Recent data from *Drosophila* and other model systems suggest that PINK1 and parkin act in a selective autophagic pathway to remove depolarized and damaged mitochondria from neurons (88).

**PARK7 (DJ1):** In a genetically isolated population in the Netherlands, Van Duijn and coworkers diagnosed four related patients with the early-onset parkinsonism at or before age 40 and a good response to levodopa (89). Homozygosity mapping identified a locus at 1p26. In an independent set of four consanguineous families, these linkage results were confirmed in at least three families. The phenotype was similar to the Dutch family, with

levodopa-induced dyskinesias occurring after treatment. In the original Dutch family, a homozygous deletion that contained DJ1 only cosegregated with the disease phenotype, while a homozygous L166P substitution in one Italian family confirmed DJ1 mutations as causative of recessive early-onset PD. Deletions and nonsense DJ1 mutations have been described, but these are an exceedingly rare cause of early-onset PD, and postmortem descriptions have not been possible, and it is unknown whether  $\alpha$ -synuclein aggregates are present. A number of missense mutations have also been described that are suggested to destabilize the protein and lead to reduced expression.

DJ-1 is expressed in most tissues throughout mammals and is a very evolutionarily conserved gene with orthologues detectable in single-celled organisms. DJ-1 may function as a redox-sensor protein with antioxidative activity in both neuronal and glial cells. Although knockout of DJ-1 in mice is well tolerated and only subtle defects described (e.g. hypersensitivity to MPTP exposure), DJ-1 removal may result in mitochondrial phenotypes that include reduced membrane potential, increased fragmentation, and accumulation of autophagic markers (90). It has been suggested that DJ-1 may act to maintain mitochondrial function in the context of oxidative stress. Thus, the direct interaction between the parkin/PINK1 pathway and DJ-1 is not clear, but there appears to be overlap in terms of the overall maintenance of mitochondrial dynamics in susceptible neurons.

**PARK9 (ATP13A2):** In a Jordanian consanguineous family, five affected sibs suffered from early-onset parkinsonism, with onset of symptoms between ages 12 and 16 years. Spasticity, supranuclear upgaze paresis, and dementia were additional nonparkinsonian features. Treatment with levodopa resulted in incomplete improvement of parkinsonian features. Magnetic resonance imaging (MRI) of the brain showed globus pallidus atrophy and later generalized brain atrophy (91). The gene was later mapped to 1p36 (92). But although this disorder, called Kufor–Rakeb disease by the original authors, has been assigned a PARK locus number, the condition does not meet criteria for a PD-like disorder. Postmortem descriptions have not been available and it is unknown whether  $\alpha$ -synuclein deposits are present in affected areas.

Mutations were identified in the ATP13A2 gene and the encoded protein has been described as a lysosomal membrane protein with encoded ATPase function that may traffic inorganic cations and other substrates in lysosomes (93). Pathogenic mutations in ATP13A2 are suggested to affect localization and stability of the protein via disruption of the transmembrane domain. Overexpression of ATP13A2 has been described to protect from  $\alpha$ -synuclein in some model systems (94). Although PARK9 may not represent a parkinsonian disorder, the function of APT13A2 in disease may reveal novel aspects of  $\alpha$ -synuclein biology relevant to PD.

*PARK14 (PLA2G6)* and other recessive parkinsonism diseases with complex phenotypes. Mutations in the *PLA2G6* gene were identified in a consanguineous Pakistani family with Neurodegeneration with Brain Iron Accumulation 2B (NBIA2B) (95), and as with *PARK9*, *PARK14*-associated disease does not meet criteria for a PD-like disorder. The clinical and pathological phenotypes associated with NBIA2B can be considered similar to those of *PANK2* mutations in NBIA1 syndrome (Hallervorden–Spatz disease). As with NBIA2, NBIA1 patients can develop a parkinsonism dystonia like phenotype. Neuropathological examinations reveal pronounced Lewy bodies in NBIA1. Mutations in the *NPC1* gene cause Niemann–Pick Type C, and neuropathological examination also reveals Lewy bodies in some cases. These genes and their encoded proteins may provide insight in  $\alpha$ -synuclein function in disease in sporadic PD.

*PARK15 (FBX07)*: Homozygous mutations in the *FBX07* gene were first identified in an Iranian family with a parkinsonian-pyramidal syndrome, and additional mutations identified in *PARK15* linked families of Italian and Dutch descent (96). Although no pathology has been reported in cases with mutations, the phenotype and pallidal syndrome features suggest strong overlap to *Park9* and *Park14* and divergence from typical PD linked phenotypes. F-box proteins like *FBX07* are cofactors for a class of E3 ubiquitin ligases proteins called SCFs that function in phosphorylation-dependent ubiquitination (97).

### 117.3.8 Putative (Unconfirmed) Monogenic loci

*PARK3*: Families with autosomal dominant PD have been described in which a locus at 2p13 (*PARK3*) is involved in the development of parkinsonism that closely resembles sporadic disease, both clinically and neuropathologically (98). Onset age in these families was 59 years, similar to sporadic PD. In two German and Danish *PARK3* families, with an assumed but unproven common founder, the penetrance of the mutation is below 40%. Particular haplotypes at this locus have repeatedly been found to be associated with the onset age in samples of PD patients (99). The *PARK3* locus includes several dozen genes and but no overt gene mutations have been identified despite exhaustive investigations. It is possible that a monogenic cause of PD does not localize to this region but rather a modifier of age of disease onset. A notable candidate is the sepiapterin reductase gene, which encodes a protein critical for dopamine biosynthesis.

*PARK5*: Ubiquitin Carboxyl-Terminal Hydrolase L1 (*UCHL1*). In two patients from one family, a putative mutation in the ubiquitin carboxyterminal hydrolase-L1 gene (*UCHL1*) has been described from a candidate-screening approach (100). This C277G transversion

results in an I93M amino acid substitution in a highly conserved region. No other families with mutations in this gene have been identified, and *UCHL1* linkage to PD should be considered putative.

*PARK10*: A susceptibility locus for late-onset idiopathic PD (not a monogenic PD-associated locus) was assigned as *PARK10* from studies in Icelandic PD patients (101). This locus has not replicated in GWAS studies or populations with strong genetic overlap with Icelandic populations such as Norwegians. In addition, the locus includes hundreds of genes and has not been refined or reconfirmed in the Icelandic population.

*PARK11*: A 39.5cM region on the long arm of chromosome has been described as linked to disease in North American PD families under an autosomal dominant model of inheritance (102). However, linkage to this region has not been observed in other populations. Missense mutations in the *GIGYF2* have been nominated as pathogenic in small PD families. However, other studies have demonstrated that these putative mutations are not pathogenic for PD, and the assignment of *GIGYF2* as a PD-associated gene is currently tentative (103). Other candidate genes for *PARK11* have not been nominated.

*PARK12*: Pankratz et al. employed a sib pair-based genome wide approach and identified a large region of Xq21–q25 associated with PD (genomewide  $p=0.04$ ) (102). As yet, no mutations have been identified in these patients, and the region has not replicated as associated in other studies.

*PARK13 (Omi/Htra2)*: Loss of function mutations in the gene encoding *Omi/Htra2* have been proposed in a candidate gene approach in German PD patients (104), but other studies suggest these rare alterations are not causative for PD but benign variants.

### 117.3.9 Important Genetic Susceptibilities in PD

*MAPT (PARK locus not assigned)*: The microtubule-associated protein Tau (*MAPT*) is primarily expressed in neurons in the brain and is thought to regulate the organization of microtubules in the cytoskeleton architecture. Tau coassembles together with tubulin and is enriched in axons. Tau positive inclusions are the pathological hallmarks of a number of neurodegenerative diseases that include Alzheimer's disease, corticobasal degeneration, PSP, and frontotemporal dementia with parkinsonism linked to chromosome 17. Although tau pathology is not common in PD and those PD cases described with tauopathy may be the result of a coincidental but separate disease, genetic variation in the *MAPT* gene associated with PD is arguably the most reproducible finding in PD genetics. *MAPT* resides in a large block of strong linkage disequilibrium, and the major haplotype named H1 is confirmed as a PD risk factor in every major GWAS

study in PD that involves European subjects (105). Interestingly, tau haplotypes are not associated with susceptibility to AD despite clear tau pathology in disease. Emerging evidence suggests that  $\alpha$ -synuclein may modify and compromise the ability of Tau to regulate cytoskeleton dynamics.

**PARK1/4 ( $\alpha$ -synuclein):** While extremely rare gene copy number alterations and missense mutations lead to early-onset PD with dementia, common genetic variants have been reproducibly demonstrated to modify susceptibility to PD. Some evidence suggests that  $\alpha$ -synuclein alleles that are associated with PD lead to increased transcription and expression (62). It is thought that the enhanced expression is not as high as that caused by a gene duplication, but that the mild elevation predisposes to disease in a dose-dependent manner. A complex repeat polymorphism located 10kbp upstream of the translation start site in the  $\alpha$ -synuclein promoter named REP1 is associated with PD, in addition to SNPs in the 3' end of the gene. Other SNPs within the  $\alpha$ -synuclein gene captured in GWAS studies reproducibly highlight  $\alpha$ -synuclein as a consistently associated gene that modifies PD susceptibility.

**PARK8 (LRRK2):** While missense mutations can potentially cause PD in European populations, these mutations are extremely rare in the Far-East Asian populations. However, in these Asian populations, several common nonsynonymous variants G2385R and R1628P potentially modify PD susceptibility (106,107). These variants are not common in European populations. A recent meta-analysis of the G2385R variant suggests a striking combined odds ratio for PD of 2.55 (95% CI, 2.1–3.1).

**GBA (PARK locus not assigned):** Recessive mutations in the glucocerebrosidase gene (GBA) lead to Gaucher's disease, which is considered a lysosomal storage disorder. Sidransky and colleagues found that parents and second-degree relatives of Gaucher's disease that presumably carry a heterozygous mutation in GBA often have PD (108). GBA mutations are more common in Jewish populations and occur at much higher frequencies in PD cases (>25%) versus PD-negative controls.

**PARK16/17:** Recent GWAS analyzes have nominated loci on 1q32 (PARK16) and on chromosome 4 (rs11248060, PARK17) (109). The underlying genes and their functions are not yet clear, or the reproducibility of the associations.

**HLA-DRB5 (PARK18):** One GWAS study identified a HLA haplotype associated with sporadic and late-onset disease. The association peak at SNP rs3129882 is a non-coding variant in HLA-DRA and is thought to segregate with modifiers of expression of HLA-DR and HLA-DQ (110). Some evidence suggests that PD-affected brains show an upregulation of DR antigens and DR-positive reactive microglia, linking the immune system on both a genetic and pathologic level to PD.

### 117.3.10 The Future of Genetic Research in PD

The identification of specific genetic causes of PD has revolutionized research in neurodegeneration in many ways. It is fitting that the first gene identified in familial PD,  $\alpha$ -synuclein, is likely the principal modifier and most important protein for disease. Genetic studies coupled with functional approaches have strongly suggested that the main genetic contribution relevant to  $\alpha$ -synuclein and PD centers on the regulation of  $\alpha$ -synuclein on a transcriptional level. The ideas that mutations and genetic variability should underlie proteins that comprise the majority of the pathological aggregate in the disease have been a common theme and confirmed in dozens of disorders. Thus, genetics guides pathology and pathology guides genetics in a feedback loop, with the overall goal of improving diagnosis and treatment.

$\alpha$ -Synuclein genetics and pathology extends beyond PD into many other neurodegenerative disorders that are disparate from PD on both clinical and pathological levels. The discovery of causative mutations in LRRK2 uniquely provides direct insight into pathological mechanisms specific to typical late-onset PD. Studies into LRRK2 shed light onto genetics of PD as a whole. First, dominant mutations of high penetrance were found at rates in many populations that were not predicted by earlier genetic studies and acutely illustrates that GWAS studies are not likely to be powered to detect rare variants of strong effect without tens of thousands (perhaps hundreds of thousands) patients included in the study. Second, LRRK2 studies demonstrate the lack of apparent utility of patient-derived family histories in deciding whether an individual or family has monogenic PD of dominant transmission, since many PD cases of reported sporadic origin harbor pathogenic mutations that are not de novo in origin and yet highly penetrant for disease. Third, the discovery of LRRK2 through genetic studies provides a potent example of how new pathways and targets for therapeutics can be identified for complex disease.

The autosomal-recessive forms of parkinsonism have unquestionably provided insights into biochemical pathways important for the health of neurons in the basal ganglia in neurodegeneration. However, their importance in terms of late-onset typical PD susceptibility has not been clear. Divergent clinical phenotypes and diverse cellular pathways have been implicated, with arguably the most excitement generated by the 'mitochondrialopathies' associated with PINK1, Parkin, and DJ1. Through identification of the biochemical pathways associated with loss of function mutations in these pathways, it is hoped that new therapeutic targets are resolved that can modify neurodegeneration in both early- and late-onset typical disease.

However, while the identification of LRRK2,  $\alpha$ -synuclein, and the plethora of genes associated with

recessive parkinsonism have been instrumental in shaping research objectives, there are limited numbers of families of sufficient size worldwide to detect new monogenic forms of PD. There is a large focus on GWAS studies that are powered to detect common variation of moderate to weak effects. It is likely that in the very near future, studies involving greater than ten thousand patients will identify new genetic susceptibilities. Exome resequencing has not been described in PD to any large extent but will likely help identify rare mutations of large effect in new genes that may help resolve common biochemical pathways in PD. Finally, the integration of gene expression data in affected brain tissue together with coding and noncoding DNA variation (i.e. epigenetic variation) will likely contribute to understanding PD on a genetic level. If the past is the best predictor of the future, we have only scratched the surface of understanding the genetics of PD.

## 117.4 PARKINSON PLUS SYNDROMES

Progressive supranuclear palsy (PSP, or Steele–Richardson–Olszewski disease) (111), multiple system atrophy (MSA) (112), and corticobasal degeneration (113) fall into the category of Parkinson plus syndromes. These are chronic diseases that in some ways may resemble PD. All three were initially considered sporadic diseases, but there is now substantial evidence that genetic factors, particularly related to the *MAPT* tau gene, play an important role in PSP and corticobasal degeneration. MSA, still considered a sporadic disease, may clinically resemble autosomal dominant cerebellar ataxia and therefore may present a diagnostic problem.

### 117.4.1 Progressive Supranuclear Palsy (OMIM 601104)

After PD, PSP may be the most prevalent cause of degenerative parkinsonism, with an estimated prevalence rate ranging between 2.5 and 10.5 per 100,000 (114). The mean annual incidence rate for those over age 50 may be about 5.3 per 100,000 (115). The disease is typically one of aging people, rarely starting before age 40 and rising in incidence over the decades up to 14.7 for those between 80 and 99 years old. It runs a relentlessly progressive course, with median survival of about 5.5 years. Clinically, the core features of the disease are early-gait and balance problems: vertical (also horizontal) saccadic slowing that develops into gaze paralysis, particularly on downward gaze; parkinsonism; axial dystonia; and cognitive decline (“subcortical dementia”) (116,117). A response to levodopa is temporary and slight, at best. Although PSP is generally considered to be a sporadic disease, familial cases consistent with autosomal dominant transmission have been described, not all of them typically in terms of clinical presentation (118).

Neuropathologically, neuronal degeneration and loss is primarily found in the striatum, the globus pallidus, the subthalamic nucleus, and various brainstem areas, among which is the substantia nigra (119). Characteristic fibrillary depositions (straight filaments) that immunostain with antibodies against the tau protein have been found in these structures. Abnormal accumulation of tau protein is also present in astrocytes. Therefore, PSP is considered a “tauopathy,” like frontotemporal dementia or Alzheimer disease. Interestingly, among the tau species deposited in PSP brains, the four-repeat tau (one of its six isoforms) seems enriched (120).

The link with tau is further demonstrated by a repeatedly confirmed association with a specific tau haplotype, often called H1, that seems to confer an increased susceptibility to PSP (121). Tau mutations have never been identified in the familial cases that have been described, apart from rare atypical cases with exon 10 mutations that may be more like frontotemporal dementia. A recent GWAS study involving more than 2000 cases of PSP has demonstrated association with several genes including *STX6*, *EIF2AK3*, and *MOBP*, encoding proteins for vesicle-membrane fusion at the Golgi-endosomal interface, for the endoplasmic reticulum unfolded protein response, and for a myelin structural component. This study also confirmed two independent variants in *MAPT* affecting risk for PSP, one of which influences *MAPT* brain expression (122).

### 117.4.2 Corticobasal Ganglionic Degeneration/Corticobasal Degeneration

The rare disease corticobasal ganglionic degeneration (CBGD) resembles PSP and may be actually confounded with it. The classical diagnostic feature of CBGD is unilateral asymmetrical dystonia or ideomotor apraxia (“the alien limb syndrome”), often associated with cortical sensory abnormalities. Cognitive decline, particularly manifesting as a subcortical dementia, is prominent in this disease. Like PSP, neuronal tau inclusions are a feature of CBGD type 1, including the enrichment of four-repeat tau (123). And like PSP, association with the tau-containing H1 haplotype has been found (124). Indeed, although pathological criteria for CBGD have been established (125), there are patients where the features overlap with those of PSP (126). These close similarities have led some to suggest that the two disorders be merged into a single entity, but in their classical forms, the clinical features are so distinct that most practitioners regard them as separate disorders. Although “familial CBGD” has not been described, this may turn out to represent semantic confounding. Those families with parkinsonism, subcortical decline, and asymmetrical dystonia or limb apraxia will probably be reported as having frontotemporal dementia with parkinsonism.



### 117.4.3 Multiple Systems Atrophy

In our current state of knowledge, this prevalent and highly incapacitating disease must be considered as a true sporadic disease. MSA is almost as prevalent as PSP (115). Survival is somewhat longer, with a median of up to 9 years. Clinical features are a mix of parkinsonism, cerebellar ataxia, autonomic dysfunction (orthostatic hypotension, bladder disturbances, erectile dysfunction), and pyramidal tract dysfunction, particularly of the lower legs (127). Another distinctive feature is the lack of dementia in these patients (128). Neuropathologically, silver-staining fibrillary inclusions (“argentophilic inclusions”) are found in oligodendroglia and neurons in affected brain areas (129). These inclusions are strongly immunoreactive for  $\alpha$ -synuclein, thereby establishing MSA as an “ $\alpha$ -synucleopathy,” similar to PD and diffuse Lewy body disease (130).

### 117.4.4 Wilson Disease

Rarely, Wilson disease may present as a parkinsonian syndrome, particularly in young patients. Although rare, it should be part of the differential diagnosis, as it can be easily diagnosed and is amenable to treatment. This condition is discussed elsewhere.

### 117.4.5 Dystonias

Dystonia is a clinical syndrome, identified by its characteristic features: sustained muscle contractions, twisting, and abnormal postures (131). Dystonia may be the main, or only, clinical symptom, or it may be seen in association with a variety of other disorders, such as stroke, PD, or other degenerative disorders of the brain. When dystonia is the primary symptom and there is no identifiable other disorder that is the cause, it is termed “primary” dystonia, with dystonias related to other diseases classified as “secondary dystonia” (132). Comprehensive lists of possible causes of secondary dystonia Table 117-1 can be found in the literature (133).

Most of the cases of primary dystonia encountered by clinicians are sporadic in nature, and of uncertain etiology. As in PD, however, in recent years genetic etiologies for primary dystonia have been identified, and these have begun to shed some light on the fundamental mechanisms responsible for these disorders.

## 117.5 GENERAL CLINICAL FEATURES OF THE DYSTONIAS

Dystonia is a movement disorder characterized by sustained muscle contractions, frequently causing twisting and repetitive movements or abnormal postures (131,134). Physiologically, it is characterized by concurrent contractions of agonist and antagonist muscles. Apart from dystonia, some additional features of disordered movement

**TABLE 117-1 Possible Causes of Secondary Dystonia**

#### Hereditary Neurodegenerative Diseases

Ataxia telangiectasia
Dystonia with striatal basal ganglia calcifications
Dystonic lipidosis
Glutaric acidemia type I
GM1 gangliosidosis
GM2 gangliosidosis
Hallervorden–Spatz disease
Hartnup disease
HGPRT deficiency
Homocystinuria
Huntington disease
Leigh disease
Metachromatic leukodystrophy
Neuroacanthocytosis CT lucencies
Neuronal ceroid lipofuscinosis
Neuronal intranuclear inclusion disease
Niemann–Pick disease, type A/B
Niemann–Pick disease, type C
Pallidal atrophies (including DRPLA)
Pelizaeus–Merzbacher disease
Spinocerebellar ataxias
Wilson disease

#### Sporadic Degenerative and Nondegenerative Diseases

Basal ganglia infarction,
Drugs (acute intoxications; dyskinesia)
Encephalitis
Head trauma
Intoxications
Multiple sclerosis
Vascular malformations and tumor
Drugs (acute intoxications: dystonia; chronic: tardive dyskinesia)
Perinatal damage
Spinal cord disease
(Manganese, CO, CS <sub>2</sub> )

#### Disorders Simulating Dystonia

Congenital	Muscular; postural; Klippel–Feil syndrome
Neurologic	Posterior fossa tumor; syringomyelia; Arnold–Chiari syndrome
Ocular	IVth nerve palsy
Psychiatric	Atlantoaxial subluxation;
orthopedic	ligamentous cervical problems; bone disease
Others	Hiatus hernia; Sandifer–Kinsbourne syndrome

CT, computed tomography; DRPLA, dentatorubral-pallidoluysian atrophy; HGPRT, hypoxanthine guanine phosphoribosyltransferase.

Adapted from Calne, D. B.; Lang, A. E. Secondary dystonia. *Adv. Neurol.* 1988, 50, 9–33.

are commonly encountered in conjunction with dystonia. There is often dystonic tremor; clinically, this is distinguished from the more common forms, such as essential tremor, by the coarse nature of the movement, the irregular frequency, and the association with fixed dystonia. Characteristically, the so-called sensory tricks (geste antagoniste) are able to correct the abnormal posturing.

For example, the gentle touch of the index finger may correct the twisted position of the head in torticollis.

Dystonic movements can be classified according to their distribution: focal (a single body part affected), segmental (spreading to a contiguous body part), multifocal, hemidystonia (left or right), or generalized. Age of onset is also important. In dystonia, it is customary to distinguish between early- (prior to age 28) and late-onset diseases (135). The rationale for selecting this particular age cutoff is based on the biology of DYT1 dystonia, as discussed below. Childhood-onset cases often start with focal or segmental dystonia that eventually spreads to affect multiple parts of the body. Marsden and Harrison, in a review of 42 patients, found that 80% of the cases with onset before age 11 had progression to severe generalized dystonia (136). Patients with generalized dystonia usually had onset in childhood or adolescence, and almost always by age 40. In contrast, about 70% of those with focal distribution tended to have onset after age 40 (137). In fact, focal or segmental dystonia could even start over age 70. In a series of 560 patients, Fahn and associates found a median age of onset of 8 years for generalized dystonia, with a peak between 6 and 10 years. Segmental dystonia had a median onset of 40 years but a bimodal distribution, with a small curve at a young age and a broader curve later in life. Focal dystonia has its mean onset at age 45 (137). Thus, the earlier the age of onset, the more likely there will be an evolution to generalized disease. These are of course generalizations, and the outcome will depend to a great extent on the underlying etiology.

The most common types of dystonia encountered in a general neurology practice are late-onset focal dystonias, including cervical dystonia (torticollis), oromandibular dystonia and blepharospasm (Meige syndrome), occupational dystonias (e.g. writer's cramp), isolated limb dystonia, and spasmodic dysphonia. Axial dystonia, involving the paraspinal musculature and twisting the body in grossly abnormal postures, is often part of generalized dystonia but is uncommon as an isolated symptom. In patients with late-onset focal or segmental dystonia, the movement disorder rarely generalizes.

### 117.5.1 The Neurobiology of Dystonia

The neurobiological mechanisms of dystonia are one of the most important unsolved questions in basal ganglia disorders (134). One of the key barriers to progress has been the lack of a clear association between the clinical symptoms of dystonia and corresponding pathological changes in the brain (138). It is clear that lesions in the striatum (caudate and putamen), globus pallidus, and thalamus may cause secondary dystonia, but these represent only a small minority of the cases of dystonia. Only a handful of cases of primary dystonia (either focal or generalized)

have been studied pathologically, and these have revealed no clear evidence for neurodegeneration or other structural changes. Clinical imaging methods such as CT and MRI usually do not reveal any abnormalities, although these studies may be helpful in excluding secondary dystonia, particularly in the case of focal or hemidystonia.

Recent work with functional and neurophysiological methods has begun to shed some light on brain abnormalities in dystonia. PET studies of dystonia patients have provided evidence for cerebral functional networks that are dysregulated in dystonia (139,140). Interestingly, some of these networks are shared by dystonias with different genetic etiologies (141). There is also recent evidence for changes in structural connectivity of the brain in dystonia, as revealed by diffusion tensor imaging. Interestingly, changes can be seen in individuals who carry genetic mutations for dystonia but do not manifest the symptoms (nonmanifesting carriers (142,143). From a physiological perspective, alterations in somatosensory processing, both at the level of the cortex and below, have been described consistently (144–147). The previously mentioned effects of “sensory tricks” support the role of sensory dysregulation as a core feature of dystonia (148).

Although the bulk of the evidence points to involvement of the basal ganglia in dystonia, there is also evidence for participation of other neural structures, particularly the cerebellum (142,149). In several animal models of dystonia, the movements have been traced directly to cerebellar abnormalities. Imaging in patients consistently reveals abnormalities in the activity of the cerebellum in dystonia. LeDoux and Brady reported a case series and extensive review of the literature of secondary forms of cervical dystonia (150). They observed that the majority of the cases had lesions in the cerebellum or associated with brainstem afferents to the cerebellum. In contrast, lesions isolated to the basal ganglia were uncommon in this series.

On the whole, the current level of understanding of the neurobiology of dystonia is unsatisfactory. It is clear that many forms involve abnormal function of the basal ganglia, but there is also participation of the premotor and motor regions of the cortex, sensory systems, the thalamus, and the cerebellum. In some cases, peripheral sensory feedback seems to be a contributor. Disentangling the relative roles of these components will require further improvements in methodology for studying the function of the human brain, as well as a clearer understanding of the proximate causes of dystonia.

### 117.5.2 Clinical Genetics: The Various Forms of Primary Dystonia

In the past decade, mapping and cloning efforts have resulted in a consistent classification of primary dystonias. From a clinical genetic perspective, a subdivision into autosomal dominant, recessive, X-linked, and sporadic forms remains a useful first approach to classification and counseling. Accurate assessment of the mode

of inheritance can sometimes be difficult because of the low penetrance of the various disorders in most families. The application of a few simple clinical guidelines can be helpful in making a correct diagnosis and in the selection of informative laboratory investigations.

**117.5.2.1 Autosomal Dominant Idiopathic (or Primary) Early-Onset Torsion Dystonia: DYT1 (OMIM 128100)** This is one of the most common forms of primary torsion dystonia, occurring with increased frequency in people of Ashkenazi Jewish descent. Onset is almost always in childhood or adolescence, with focal limb onset that over the years spreads to the other limbs and the axial musculature. Cranial and laryngeal involvement is rare. The disease is transmitted as an autosomal dominant disorder with incomplete penetrance of about 30% (135,151–153). Linkage data from both non-Jewish and Jewish families established the DYT1 locus to the 9q32–q34 region, although linkage to this locus could be excluded in some early-onset families. A common haplotype of 9q alleles was found among affected Ashkenazim, and it was concluded that the vast majority of early-onset cases in the Ashkenazi Jewish population are due to a single mutation that has been estimated to have occurred approximately 350 years ago. This mutation may have arisen from the northern part of the historic Jewish Pale of settlement (Lithuania and Byelorussia) (154).

The gene for DYT1 was cloned in 1997 by Ozelius and colleagues (151). They identified as the pathogenic mutation a GAG trinucleotide deletion in a conserved region of a novel ATP-binding protein called torsinA. The gene was renamed TOR1A. This deletion results in loss of one of a pair of glutamic acid residues. The mutation was found in all affected and obligate carrier individuals with chromosome 9-linked dystonia, including four non-Jewish families. A second mutation in TOR1A, an 18-bp in-frame deletion near the C-terminal part of the protein (Phe323–Tyr328del), was found in two patients with early-onset dystonia and myoclonic features (155). Whether this last alteration constitutes a pathogenic mutation is unclear: in both patients, a Leu196Arg mutation was also identified in exon 5 of the  $\epsilon$ -sarcoglycan gene. Mutations in this gene are commonly associated with myoclonic dystonia (see discussion of DYT11 later).

The DYT1/TOR1A gene has high homology to three additional vertebrate genes as well as to nematode and *Drosophila* genes (134,153). The TOR1A gene is located next to one of its homologs, TOR1B; both genes belong to the HSP/Clp and AAA+ ATPase superfamily of chaperones. Northern blot analysis of TOR1A revealed two ubiquitously expressed transcripts of 1.8 kb and 2.2 kb and an additional low-abundance transcript of 5 kb that was detected in the fetal brain, lung, and kidney, as well as in the adult brain, heart, and pancreas (151). Widespread expression was found in many brain areas, including the dopaminergic neurons of the substantia nigra (156). The deduced protein, torsinA, is a 332-amino acid, approximately 37-kDa protein that contains an

ATP-binding domain and a putative N-terminal leader sequence, as well as a membrane-spanning region that may be necessary for membrane translocation and targeting. Its function is uncertain, although most evidence suggests that it is endoplasmic reticulum resident protein involved in secretion and trafficking (157). Mouse models constructed either by introducing the human mutation into *dyl1*, the mouse homolog of TOR1A, or by transgenic expression of the human protein has not produced animals with obvious dystonia (158). Many of these animals do, however, have subtle motor phenotypes, and it has been suggested that they may model the “nonmanifesting carrier” state of DYT1 dystonia (159).

The GAG deletion results in the loss of one of a pair of glutamic acid residues in a conserved region near the carboxyl terminus of the protein. Mutational analysis of most of the coding regions and splice junctions of TOR1A and TOR1B failed to reveal additional mutations in typical early-onset cases of patients who lack the GAG deletion, in dystonic individuals with apparent homozygosity in the 9q34 region, or in an individual with late-onset dystonia who did share the common 9q34 haplotype (153).

Mutational analysis has also made possible a better appreciation of the clinical features of DYT1. The most common form is indeed an early-onset (prior to age 20 to 30) limb dystonia that gradually may generalize but typically spares craniocervical muscles. However, cases of isolated focal or segmental dystonia have been described (160–162). In patients with nonfamilial dystonia, particularly in those without generalized dystonia, with late onset, or with cranial dystonia, the number of individuals with a DYT1 mutation is very low. Brassat and colleagues found five sporadic patients with the mutation out of a group of 100 sporadic individuals (163). Four of these five had early-onset generalized dystonia. Among 300 cases with late-onset focal/segmental dystonia, only three tested positive for the GAG deletion (161).

**117.5.2.2 Autosomal Dominant Primary Dystonias: DYT6** In two Mennonite Amish families, dystonia started between 6 and 38 (mean, 18.9) years, with cranial or cervical dystonia in about half of those affected. Most of the later disability was caused by cranial or cervical manifestations. The locus was mapped to 8p21–q22 (DYT6); an identical haplotype in the region linked the two families to a recent common ancestor (164). DYT6 dystonia has recently been found to be caused by mutations in the transcription factor THAP1 (165). This has a large number of potential downstream targets, but interestingly one of the genes regulated by THAP1 appears to be TOR1A (166). Identification of the DYT6 gene has enabled molecular screening, and has revealed that THAP1 mutation may be a cause of apparently sporadic dystonia (167). Despite the interest in this disorder, there are so far no pathological studies of the condition.

**117.5.2.3 Autosomal Dominant Primary Dystonias: Other Forms (DYT7, DYT4, DYT13)** Apart from the DYT1 and DYT6 families that have been identified, many families in the world have been described in whom dystonia segregates as an autosomal dominant trait, not linked to chromosomes 8 or 9. Several different loci have been assigned, and more are expected to exist. Leube and coworkers identified a family with seven persons definitely affected by late-onset (28 to 70 years) focal dystonia (torticollis, dysphonia, writer's cramp) and six possibly affected (hypertrophic neck muscles, postural hand tremor). Linkage to 18p was demonstrated (168). In one Australian family with at least five generations of affected family members, in whom onset ranged from 13 to 37 years, a linkage to 9q could be excluded, and the locus in this family has been designated DYT4 (169). In a large, non-Jewish, three-generation Italian family, autosomal dominant ITD started at an average age of 15 years. Manifestations started in the cervical or craniocervical region or upper limbs and slow progressed to other body regions over decades. The gene was subsequently mapped to 1p36.32–p36.13 and the locus called DYT13 (170). In all of these autosomal dominant families, the same general clinical principle seems to hold true as in the DYT1 families. Early-onset dystonia is often limb onset and tends to generalize; late-onset dystonia often presents as a focal or segmental disorder and tends to remain localized.

**117.5.2.4 Autosomal Dominant Hereditary Progressive Dystonia with Marked Diurnal Fluctuations, or Dopa-Responsive Dystonia: DYT5 (OMIM 128230)** Hereditary progressive dystonia with marked diurnal fluctuations (HPD), or dopa-responsive dystonia, is a rare condition first described in 1976 by Segawa in Japan (171). However, despite its low prevalence, the biochemical and genetic basis for this disorder is currently well known, and it provides clues as to the neurobiology of dystonia in general. Several cases have been described, but many more cases may go unreported or indeed undiagnosed. The true prevalence of this disease, therefore, is unknown, but it seems to be more common in Japan than in North America or Europe.

Initial descriptions of HPD emphasized onset in the first decade, initially with asymmetrical dystonic foot posturing and walking impairment, with progression and gradual involvement of other extremities (172,173). The disease progresses over the first two decades but stabilizes from the third decade onward. Marked diurnal fluctuations are present, with dystonia being partially alleviated in the morning and aggravated during the course of the day. Particularly impressive is the effect of L-dopa, which gives marked and sustained relief in relatively low doses without the long-term side effects that occur in the treatment of

PD. Nygaard and colleagues, calling the disease dopa-responsive dystonia (DRD), later described additional aspects of the phenotype, such as early-developmental and motor delay-resembling cerebral palsy, later-life parkinsonism, late-onset parkinsonism in previously unaffected family members, and lack of fluctuations in 25% of patients.

HPD/DRD is an autosomal dominant disorder with low, sex-related penetrance and a female preponderance that may be as high as 4:1. The HPD/DRD gene was initially mapped to a 20-cM region on chromosome 14q. Using a candidate gene approach, the Japanese group then found that one of the tetrahydrobiopterin-synthesizing enzymes, GTP cyclohydrolase I, mapped to this same 14q region (174). Four variations in four families were identified: three single-base changes that predicted nonconservative amino acid substitutions in highly conserved regions of the GTP cyclohydrolase I gene (GCH1), and a 2-base insertion frameshift mutation. In one family, no mutation was found. In all families, however, GTP cyclohydrolase I activities in phytohemagglutinin-stimulated mononuclear blood cells of affected members was reduced from 2% to 20% of control values. Mutant fusion proteins, expressed in *Escherichia coli*, showed no detectable enzymatic activities for two of the base substitutions. In cases of severe loss of activity of GTP cyclohydrolase 1, presumably under 2% and presumably associated with mutations on both alleles, the affected infant displays mental retardation, epilepsy, disturbances of tone and posture, abnormal movements, hypersalivation, and swallowing difficulties (175).

Many mutations—missense, nonsense, and frameshift mutations—in the coding or noncoding regions or in the exon–intron junctions of GCH1 have been reported in patients with dominant HPD/DRD all over the world; most of these are new mutations (176). In about 40% of cases with a typical phenotype and low GTP cyclohydrolase 1, no mutations can be found. In such cases, mutations in the GCH1 promotor, enhancer, or transcriptional regulatory domains, as well as large genomic deletions, have been implicated (177).

GTP cyclohydrolase I is the rate-limiting enzyme for the biosynthesis of tetrahydrobiopterin, the cofactor for tyrosine hydroxylase, which is the first and rate-limiting enzyme of DNA synthesis. Apparently, mutations in GTP cyclohydrolase I lead to dopamine synthesis impairment that can be bypassed by administration of the dopamine precursor L-dopa. In a large series of 66 cases, the effects of L-dopa therapy were immediate and dramatic, with daily doses as small as 50mg being effective (178). Treatment duration of up to 23 years has been reported, and response fluctuations developed only rarely, leading to speculation that these latter cases might represent juvenile-onset PD rather than DRD. Thus L-dopa is the treatment of choice, as well as a diagnostic tool.



A markedly decreased striatal dopamine concentration and a mild decrease of levels of the dopamine metabolite homovanillic acid could be demonstrated in a postmortem brain (179). Homovanillic acid concentration decrease can also be detected in cerebrospinal fluid. But, contrary to PD, the striatal dopaminergic deficit is not caused by nigrostriatal neuronal loss, as the number of substantia nigra neurons is normal, and a normal pattern of tyrosine hydroxylase immunoreactive fibers is present in the striatum. Furthermore,  $^{18}\text{F}$ -dopa uptake on PET, which provides a measure of the functional integrity of the nigrostriatal dopaminergic projection, is normal (180,181). This integrity could very well explain the prolonged success of low-dose L-dopa therapy.

**117.5.2.5 Myoclonic Dystonia: DYT11 (OMIM 159900) and DYT15 (OMIM 607488)** This relatively mild form of dystonia is increasingly recognized in various parts of the world (182). In most cases, onset is before age 20, with presentation in the face, neck, and arms. Both dystonia and myoclonic jerks, often subtle, occur in patients, with different predominance in different patients, even within one family. In some patients or families, myoclonus, rather than dystonia, may be the most prominent sign. Quite characteristically, alcohol alleviates the movement disorder (183) and alcoholism may develop as a consequence. Clonazepam may be a long-term treatment alternative. Obsessive-compulsive phenomena may segregate with the movement disorder (184). Epilepsy may also be part of the phenotypic spectrum (185).

After the initial finding of linkage to 7q21 was confirmed in phenotypically similar families, mutations in the  $\epsilon$ -sarcoglycan gene (SGCE) were identified in most families (186).  $\epsilon$ -Sarcoglycan is a component of the dystrophin-sarcoglycan complex, and it is widely expressed in both nonneuronal and brain tissues. Although a variety of loss-of-function mutations have been described, all of them seem to result in parental origin-dependent penetrance; that is, the disease is more likely when the gene is inherited from the father than when originating from the mother. Maternal imprinting of SGCE is the most likely explanation, and this has indeed been observed (187). A second locus at 18p11—DYT15—has been identified in a Canadian family with myoclonic dystonia (188). Thus, the phenotype may be genetically heterogeneous.

**117.5.2.6 Autosomal Dominant Rapid-Onset Dystonia with Parkinsonism: DYT12 (OMIM 128235)** Various families with rapid-onset dystonia with parkinsonism have been described that share the following unique features: onset within a few hours to days to weeks, although a more protracted course has been described; severe bulbar manifestations, including dysarthria; and parkinsonism. Onset in these families varied from early to late adulthood. Penetrance

seemed incomplete, but male-to-male transmission has been described (189,190). The finding of linkage to chromosome 19 led to the subsequent identification of six mutations in the Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ 3 subunit gene (ATP1A3) in seven unrelated families with this unusual form of dystonia (191). The connection between the genetic disorder in energy metabolism and the abrupt and permanent nature of the symptoms has raised speculation that the defect may predispose the brain to an energy crisis under stress and subsequent neurodegeneration, but there is so far no clear evidence for a structural abnormality in DYT12. In a mouse model system, interactions between the basal ganglia, cerebellum, and thalamus appear important for generation of the phenotype (192).

**117.5.2.7 Recessive Forms of Dystonia: DYT2 (OMIM 224500)** It has always been assumed that cases of recessive primary dystonia could be identified, and the finding of affected offspring from consanguineous marriages has been used to support this possibility. In four Spanish Gypsy families, with three of them consanguineous, recessive inheritance seemed likely (193). Low penetrance of dystonia in families with an autosomal dominant form of the disease may simulate recessive disease, which has made identification of some of these genes difficult.

The best documented examples of recessive dystonias are those due to tyrosine hydroxylase deficiency, that resemble early- (childhood-) onset L-dopa-responsive dystonia (194–196). Much rarer genotypes, which often present with a much more severe phenotype, are 6-pyruvoyl-tetrahydropterin synthase deficiency (197), sepiapterin reductase deficiency (198), and aromatic L-amino acid decarboxylase deficiency (199).

**117.5.2.8 X-Linked Dystonia (“Lubag”): DYT3 (OMIM 314250)** An X-linked form of generalized dystonia with parkinsonism (XPD) has been identified in the Philippines, where this distinct syndrome reaches endemic proportions on the island of Panay (200–202). The disease is locally known by the name of lubag (twisting) and may have its onset between the ages of 12 and 48 years (mean,  $38 \pm 8.0$  years). In an assessment of 42 cases, the initial manifestations have been variably found in the lower or upper extremities, the trunk, or the face, in order of decreasing frequency (200). Major manifestations include gait abnormalities (90% of patients), leg dystonia (79%), oromandibular dystonia (64%), neck dystonia (57%), blepharospasm (57%), and truncal dystonia (52%). Dystonia tends to generalize within 1 to 11 years. Characteristically, 35% of the cases displayed parkinsonian signs: bradykinesia, rigidity, tremor, or loss of postural reflexes. Those with parkinsonism had a significantly later age of onset (40.5 years) than those without (32.4 years). Medications that may be useful in other forms of dystonia, such as trihexyphenidyl or L-dopa, are not effective in lubag.

XDP is genetically homogeneous and has its origins on the Panay Island of the Philippines. The locus for XDP has been mapped to the proximal long arm of the X chromosome at Xq12–q13.1. One gene encoded in this region is TAF1, a transcription factor expressed in the nuclei of neurons in the striatum and other regions of the brain (203), but there are several other candidates within the region, all of which exhibit strong linkage disequilibrium.

## 117.6 MANAGEMENT AND TREATMENT

The symptomatic treatment of ITD is a process of trial and error. It has been recommended to start with increasing doses of l-dopa, as some of these cases may not represent ITD but instead may be l-dopa-responsive dystonia. If this fails, anticholinergic medications (e.g. trihexyphenidyl) constitute the drugs of choice. The dose should be gradually increased until adverse effects (drowsiness, xerostoma, blurred vision, impotence, urinary retention, and obstipation) become limiting. In general, only high doses yield adequate effects, and these doses are best tolerated by young patients. After this, baclofen, carbamazepine, or benzodiazepines may be tried, although their efficacies are less than that of anticholinergics. Local injections of botulinum toxin in dystonic muscle groups is currently the treatment of choice for focal dystonias. This is a safe and very effective treatment but it must be repeated frequently. Both the patient and the clinician should be aware that, in general, treatment effects will be partial at best. A completely new and novel approach to treatment is the application of deep brain stimulation in patients with generalized dystonia. Preliminary results with this approach are encouraging (204,205).

## 117.7 CHOREIC DISORDERS

### 117.7.1 Huntington Disease

**117.7.1.1 Prevalence.** Multiple epidemiological surveys of Huntington disease (HD) have been presented from different parts of the world, including northwest Europe, the United Kingdom, Scandinavia, North America, Australia, Japan, and South Africa. While recorded rates differ, there is general agreement that the frequency of HD in populations of Western European descent is between 3 and 7 affected individuals per 100,000 of the population. However a few areas, such as certain regions of Venezuela or Scotland, show particularly high or low prevalence of HD. The disease is less frequent in Japan, China, Finland and in African blacks.

**117.7.1.2 Natural History.** A very broad definition of “age of onset” is the first time any neurologic or psychiatric symptoms appear that represent a permanent change from the normal state. The age at which signs of symptoms for HD occur has a mean of around 40

years and a standard deviation of approximately 10 years (206). The earliest age of onset has been approximately 2 years (207,208) (juvenile-onset), and patients with onset of the disorder in their 80s and even early 90s have been noted.

HD does not have a specific single presenting sign or symptom. Patients may exhibit early subtle changes in executing motor programs, with minor changes in coordination and involuntary movements. In addition, an insidious and slow deterioration of intellectual functions as well as mild personality change has been reported (209). The duration of HD is about 15 years, with no differences between the sexes (206). Survival with HD has not significantly changed over the past 50 years, which reflects the failure of medical therapy to delay disease progression. Even though the mean duration in many different studies has been constant, there are marked individual variations, extending up to as much as 40 years from time of onset on rare occasions. It would appear that, in some families, HD follows a milder course with longer survival.

#### 117.7.1.3 Clinical Manifestations and Diagnosis.

Most individuals affected by HD will initially display minor motor abnormalities. These include general restlessness, abnormal eye movements or abnormal optokinetic nystagmus, hyperreflexia, impaired finger tapping or rapid alternating hand movements, and excessive and inappropriate movements of the fingers, hands, or toes during emotional stress (209,210), as well as mild dysarthria. Minor motor abnormalities usually precede the obvious signs of extrapyramidal dysfunction by at least 3 years. Gene carriers with a completely normal neurologic examination have a 3% chance of being diagnosed within the next 3 years (209).

A definitive diagnosis of HD can be made when the following features are present: (i) demonstration of the pathognomonic mutation, a trinucleotide CAG repeat expansion is the only mutation observed; (ii) progressive motor disability involving both involuntary and voluntary movement; and (iii) mental disturbances, including cognitive decline, affective disturbances, and/or changes in personality. These features indicate that the disease is already manifested. The diagnosis of HD depends on a clinical examination. In the absence of clinically defined signs and symptoms but the presence of the mutation, the patient is presymptomatic. Demonstration of atrophy of the caudate nucleus and the putamen by CT or MRI provides additional support for the diagnosis. PET may reveal a decrease in the uptake and metabolism of glucose in the caudate nucleus before structural tissue loss becomes evident.

#### 117.7.1.4 Presentation of HD: Early- and Late-Onsets.

**Juvenile Onset.** Approximately 10% of all patients with HD have onset before age 20. In contrast to adult cases, bradykinesia and rigidity are conspicuous early, dominating the neurologic findings in about 50% of juvenile cases (206,211). Chorea is present in almost all cases

but is often of short duration and is superseded by rigidity (206). Frequent falls, dysarthria, clumsiness, hyperreflexia, and oculomotor disturbances are frequent in children with HD and occur early. Although difficult to assess in HD, cerebellar dysfunction is more prominent than in adult patients (210).

Mental deterioration in juvenile HD is first manifest by declining school performance. Over the years, a severe progressive dementia develops. Epileptic seizures, which are no more frequent in adult HD patients than in the general adult population (1%), are more common in early-onset cases with an estimated 30% to 50% of juvenile patients affected (206,211). Partial or generalized, tonic-clonic or absence seizures may all appear. Seizures should be differentiated from myoclonic jerks, which also rarely occur in adult cases. The epilepsy of juvenile HD patients is often difficult to control.

**Late-Onset.** In contrast, the manifestations in late-onset disease are less severe. Approximately 25% of all cases will display first signs and symptoms after age 50, and in these patients, the disease will follow a slower progression than usual (212). Chorea is the presenting motor disorder, and gait disturbances and dysphagia are common, though not severe. Cognitive impairment, although invariably present, may be less debilitating than in younger patients (212). A later onset is associated with a slower disease progression, as measured by functional disability. Even prior to clinical diagnosis, persons carrying the mutation can demonstrate cognitive dysfunction. Specifically, presymptomatic HD carriers have deficits in shifting strategy, psychomotor speed, recognition memory, planning, and verbal fluency (213–215). At this point, the impairments are subtle as many studies were unable to detect any difference in cognitive function prior to motor onset (216,217).

**117.7.1.4.1 Motor Dysfunction. Chorea.** Chorea is the major motor sign of the disease. These involuntary movements are continuously present during waking hours, cannot be voluntarily suppressed by the patient, and worsen during stress. Choreic movements of the face are common and present as pouting of the lips, irregular grimacing, twitching of the cheeks, and alternate lifting of the eyebrows and frowning. The neck is often involved, causing forward or backward bending of the head, or rotation. Breathing may become irregular. In the limbs, there is frequent flexion and extension of the fingers. The legs may be alternately crossed and uncrossed and the toes flexed and extended. Chorea is a feature of HD in over 90% of patients, increasing during the first phase (~10 years) of the patients' illness. With advancing duration, features of bradykinesia, rigidity, and dystonia become more evident. Chorea is seen less frequently in patients with juvenile onset (206,218). These cases often represent the so-called rigid Westphal variant (219,220).

**Bradykinesia and Rigidity.** Bradykinesia and rigidity are infrequent in the early phases of adult-onset HD.

However, they gradually appear until they often dominate the final stages of the illness, in which the patient will become severely rigid and grossly akinetic (210,219). Early in the illness, bradykinesia alone may contribute to an impairment in voluntary motor performance (221). In both juvenile and adult rigid cases, a coarse-resting tremor, distinct from a parkinsonian tremor, may complement the clinical picture (220).

**Dystonia.** Dystonia, characterized by slow abnormal movements and abnormal posturing, is infrequent in the early-symptomatic period but worsens and becomes a prominent feature toward the later stages of the illness (210).

**Oculomotor Dysfunction.** Oculomotor disturbances, apart from being among the earliest signs in the transitional phase, are present in the vast majority of affected patients. Slowing of saccades may be seen in up to 75% of symptomatic individuals, especially in early-onset cases, more particularly affecting vertical rather than horizontal movements (222,223).

**Voluntary Motor Dysfunction.** An early sign is impairment of voluntary motor function (221,224). Patients and their family describe clumsiness in common daily activities. Disturbances in motor speed, fine motor control, and gait correlate with disease progression and appear to be better measures of duration of illness than chorea. Clumsiness may increase with deterioration of functional capacity (210).

**Reflexes.** Hyperactive reflexes occur early in up to 90% of patients, while clonus and extensor plantar responses occur late and are less frequent (206,210). Again, these latter phenomena are predominant in juvenile and advanced adult cases (210). Frontal release reflexes like snouting, sucking, or grasping typically accompany significant cognitive decline.

**Gait.** Gait disturbances ultimately result in severe disability (210). Subtle changes in gait may be observed early in the illness, including difficulty with tandem walking, sudden stopping on command, and turning (225). With more advanced disease, walking difficulties are more pronounced. As a consequence, patients experience frequent falls with significant associated morbidity and often ultimate confinement to a wheelchair.

**Speech.** Most patients display speech abnormalities (206,218), which are present early in the illness (226,227). Initially mild disturbance of clarity appears, which is aggravated by changes in rate and rhythm of speech as the disease progresses.

**Dysphagia.** Disturbances in swallowing generally occur later with progression of the illness. Initially, this may primarily affect intake of fluids but later will also affect intake of solids. Choking with aspiration secondary to dysphagia is a common cause of morbidity.

**117.7.1.4.2 Cognitive Disturbances. Subcortical Dementia.** A global decline in cognitive capabilities is ultimately present in all HD patients. While global measurements of cognitive function may be preserved (228),

a typical pattern of decline becomes apparent very early in the disease, including slowness of thought, altered personality, affective changes, and impaired ability to integrate new knowledge.

**Memory.** Memory impairment is common early in the disease and is often one of the patient's presenting symptoms (229). Visuospatial memory is particularly affected, involving visual retention (228), while verbal memory remains fairly preserved until late. For example, patients have difficulty reproducing geometric designs but may remember facts, words, or stories. Retrieval of information is impaired (230), but verbal cues, priming, and sufficient time may lead to partial or correct recall (231,232). Recall of recent and remote events is equally impaired (233). The learning and acquiring of new motor skills (procedural memory) is also affected in HD. In contrast to other amnesic syndromes, orientation to both time and place remains intact until late in the illness (234).

**Attention and Concentration.** Attention and concentration are affected early (228), resulting in easy distractibility by interfering stimuli. Difficulty performing sustained simple motor tasks such as gazing laterally, sticking out the tongue, or tightly closing the eyelids may be a manifestation of this distractibility, rather than motor disturbance. Problems with organizing, sequencing, and planning; inability to coordinate and initiate complex actions; and inability to maintain a mental set or organize cognitive strategies constitute other early impairments (229).

**Language and Related Functions.** Dysarthria, slowness, and lack of initiative interfere significantly with fluency and spontaneous speech (235), but semantic and syntactic structure, word finding, and speech comprehension remain intact until the final stages of the disease (226). Difficulties in writing and recognition of objects have been ascribed to defective nonlinguistic modalities, such as visuoperceptual analysis, attention and concentration, and overall cognition (235). In contrast, simple naming of daily objects, as tested in the Mini Mental State Examination, may remain intact until the latest stages of the disease (234). Expression and perception of the musical, tonal, rhythmic, nonlinguistic aspects of language (prosody), which are a function of nondominant hemisphere structures (analogous to those mediating language in the dominant hemisphere), are impaired in affected HD patients (236).

**Visuospatial Functions.** Specialized neuropsychological tests reveal impaired visuospatial abilities that may be quite prominent in the later stages of the disease. However, patients are oriented to time and place and able to dress themselves, and have no obvious spatial neglect until late in the illness. Clearly, in comparison to Alzheimer disease, visuospatial disturbances are relatively preserved in HD (220). Similarly, a patient's insight into his or her deteriorating cognitive abilities, which is absent in Alzheimer disease, remains intact.

**Psychiatric Disturbances:** Although psychiatric disturbances are as characteristic of the disease as motor and cognitive abnormalities, these appear less consistently and are not necessarily related to the severity of chorea or dementia (237).

**Mood and Affect.** Changes in mood and affect are common, ranging from anxiety and ill-defined irritability to prolonged periods of depression. Suicide is more common in patients than in the unaffected population (238). Manic or hypomanic episodes also occur with increased frequency. Affective syndromes may precede the first signs of motor impairment by many years and do not usually manifest for the first time until late in the illness (220).

**Behavioral Disturbances.** Apathy, aggressive behavior, sexual disinhibition, and alcohol abuse are other symptoms seen in HD patients. They may be either a manifestation of the progressive cognitive decline, or, alternatively, manifestations of the mood disturbances, especially if they are reversible and related to the premorbid personality.

**Delusions and Hallucinations.** Delusions are frequent, occurring in up to 50% of the patients with advanced disease. They may be seen in depressive or manic episodes or they may be isolated, and they are frequently paranoid in nature. In contrast, hallucinations are less common (206).

**117.7.1.4.3 Other Manifestations. Weight Loss.** Weight loss is one of the features of late HD. Clinical follow-up (239) as well as anthropometric studies with dietary assessment (240), all show that the vast majority of HD patients lose weight in the course of the disease.

**Sleep.** Sleep may be disturbed in advanced disease, with frequent nocturnal sleeplessness and reversal of the day-night pattern of sleep (241). Choreic movements disappear during sleep.

**Incontinence.** Approximately 20% of all patients are incontinent of urine and feces in the terminal phases of the illness, but early incontinence is rare (206). Choreatic contractions have been electromyographically recorded from perineal musculature in affected patients (242).

**117.7.1.5 Neuropathology of HD.** Atrophy of the caudate nucleus and the putamen (the neostriatum) is the most characteristic pathologic feature of the disease (243–245), yet the severity of neostriatal abnormalities is highly variable. In a series of 163 clinically diagnosed cases, 13 lacked macroscopically visible atrophy, while in 18 cases the caudate was extremely shrunken and the putamen markedly atrophic (243). Microscopically, the neostriatal atrophy is characterized by neuronal loss and gliosis (243), which again may be highly variable. Full appreciation of neuronal loss, however, can only be obtained by cell counting. In these instances, even in the absence of caudate abnormalities macroscopically, regional loss of up to 40% of the normal neurons may be found (246). Medium- and small-sized neurons, those expressing enkephalin and Substance P, which are the



most abundant classes in human striatum, disappear, while larger interneurons appear relatively preserved.

Macroscopically, the whole brain often appears atrophic, with narrowed gyri, widened sulci, and a reduction of brain weight, sometimes by as much as 400 g (247). Cortical atrophy with changes in neocortical architecture has been frequently reported (247,248). The normal layered architecture is preserved, but alterations occur in layers III, V, and VI (236,248), with IV possibly also involved. The thalamus, similar to most structures, may be reduced in proportion to the rest of the brain (247,249). In contrast to variable changes in other extrastriatal structures, the hypothalamic lateral nucleus is atrophied while other brainstem nuclei are generally intact (250). Gross cerebellar atrophy is rare (251).

Ultrastructural studies of the caudate may reveal abnormalities in the nucleus and nucleolus, the endoplasmic reticulum, ribosomes, the Golgi apparatus, mitochondria, and lysosomes. In affected neurons, both degenerative and regenerative changes can be observed. In Golgi stains of medium-sized spiny neurons, abnormal dendritic branching, elongation of distal dendrites, and alterations in spine densities were visualized (252). Gliosis particularly manifested by astrogliosis may also be prominent. The caudate is more affected than the putamen (243).

**117.7.1.6 Neurochemistry.** The earliest and most extensively affected neurons are the medium-sized spiny striatal neurons that express  $\gamma$ -aminobutyric acid (GABA) and enkephalin (Enk) or GABA and substance P (243,253,254). A decreased number of fibers immunoreactive for Enk and substance P was already observable in the brain of an asymptomatic individual at high risk for having inherited the HD gene (255). Medium-size and large-size neurons containing the enzyme NADPH diaphorase (recently identified as a nitric oxide synthase) remain intact (256). These neurons contain somatostatin and neuropeptide Y as their neurotransmitters and are localized in the striatal matrix, probably serving as interneurons. Another relatively spared group are large aspiny, acetylcholinesterase-containing, and locally arborizing interneurons.

**117.7.1.7 Neuroimaging. CT and MRI.** CT and MRI scanning can demonstrate striatal atrophy in affected patients, but appreciable atrophy was thought to usually appear after the appearance of chorea (454); however, more recent work has clearly demonstrated significant striatal atrophy prior to the onset of overt motor deficits in persons at risk for HD (257–259). In the absence of genetic confirmation, a scan showing caudate atrophy supports a diagnosis, but a normal scan does not exclude the diagnosis. Extrastriatal atrophy has also been found in HD patients. There is reduced cortical thickness in early to moderate HD patients (260–262). Other subcortical structures including the globus pallidus, accumbens, and amygdala have also been found to be atrophied (249). In addition to gray matter, white-matter atrophy

is also present in HD patients and can be seen very early in the disease process for known gene carriers (258). While all of the MRI data are intriguing, methodological differences must be considered when reviewing this data. The data concerning striatal (caudate and putamen) and white-matter atrophy are highly reproducible; however, some of the data concerning the cortical gray matter and subcortical structures are not consistent. These differences make it difficult to generalize between studies; therefore, further refinement and standardization are needed.

**Positron Emission Tomography.** PET using  $^{18}\text{F}$ -deoxyglucose demonstrates decreased glucose metabolism in the caudate nucleus that appears normal on CT or MRI scan, both in affected persons and in some persons at risk (263–265). In addition, PET studies using radioactive tracers to examine dopaminergic receptor binding (indicative of neuronal loss in the striatum) in HD patients reveal changes in binding that is sometimes associated with clinical manifestations of disease (266). PET scanning may be a sensitive method to detect early HD-related changes, particularly during the transitional clinical phase when a definitive diagnosis cannot yet be made. Several PET measures in patients in this transitional period (pre-HD) are abnormal, including dopamine receptor binding and glucose metabolism (267,268). The finding of caudate hypometabolism lacks specificity since significantly decreased caudate glucose metabolism has been demonstrated in other diseases of the nervous system including (269).

**117.7.1.8 Genetics of HD.** HD is inherited as an autosomal dominant trait. The mutation is found in a gene encoding the protein huntingtin. A summary of normal and mutant HD allele sizes is presented in Table 117–2. The gene is transmitted by parents of either sex to children of either sex. Each child of an affected parent has an even chance of inheriting the gene. In fact, HD is an archetypical dominant disorder. Clinical differences are apparent between heterozygotes and homozygotes, where homozygosity is associated with a more severe clinical course (270).

In the past, it has been repeatedly stated that HD is completely penetrant. However, it is now evident that penetrance depends on the severity of the genetic abnormality, and only a proportion of patients with between 36 and 41 CAG repeats may manifest within their expected lifespan (271). The exact frequency of nonpenetrance is uncertain, but, if the number of CAG repeats is closer to 36, a greater proportion of persons would not be expected to manifest signs of the disease in their lifetimes.

**117.7.1.9 The HD Gene.** A novel gene containing a trinucleotide CAG repeat that is expanded on chromosome 4 was described (272). This highly polymorphic CAG repeat, located in the 5' region of the huntingtin gene, has been shown to range from 10 to 35 in the general population, while it is expanded beyond 36 repeats in

**TABLE 117-2 Normal and Mutant HD Allele Sizes**

Allele Type	CAG Repeat Size	Comments
Normal	≤26	
Intermediate	27 to 35	<p>Individual is not at risk of developing symptoms of HD but may be at risk of having a child with an allele in the abnormal range (284).</p> <ul style="list-style-type: none"> <li>The risk of a child inheriting a “mutant allele” from a parent with an intermediate allele is dependant on a variety of factors, including the sex of the transmitting parent (the conversion of an intermediate allele to a mutant allele as a result of CAG expansion has only been observed when the parent contributing the allele is male), the DNA sequence surrounding the CAG expansion, and the size of the allele.</li> </ul>
Mutant	≥36	<p>Alleles with 36 or more CAG repeats are observed in individuals with HD.</p> <ul style="list-style-type: none"> <li>Reduced-penetrance alleles: 36–40 CAG repeats. An individual with an allele in this range may or may not develop symptoms of HD in his/her lifetime (271, 281, 284).</li> <li>Full-penetrance alleles: 41 or more CAG repeats. Alleles of this size are associated with development of HD in a normal lifetime.</li> </ul>

HD patients. The HD gene is located on chromosome 4p16.3, encompasses 67 exons, and spans over 200 kb (272). The HD gene lacks homology to any previously characterized gene and encodes a protein of 3144 amino acids with a predicted molecular mass of 348 kDa. Multiple homologs of the HD gene have been cloned, including the rodent homolog (273,274). The human and the mouse genes are 90% homologous, with a high degree of sequence identity in the 5′- and 3′ untranslated regions.

The huntingtin protein is expressed in many different cells throughout the central nervous system (CNS) as well as in cell lines from different somatic tissues (275–277). There is no particular enrichment of the protein product in the basal ganglia. Furthermore, there is no altered expression of the protein with the expanded repeat in HD patients (276,277). Thus, the pattern of expression of both the messenger RNA and the protein does not provide clues to the causes for selective neuronal death in HD.

Gene targeting strategies resulting in homozygous deletion of this gene from mice reveal that offsprings are not viable. Timed pregnancy studies revealed that these

mice die before 8.5 days of age, showing that this protein is crucial for normal development and particularly for normal gastrulation (278–280). Studies of animals with heterozygous deletion of this gene have revealed that they have abnormal behavior characterized by increased locomotor activity as well as some changes in cognitive ability. Furthermore, detailed morphometric studies have revealed that these animals have significant reduction of neurons in the subthalamic nucleus (279). These studies, as well as others, have demonstrated the HD gene is also important for maintaining normal neuronal integrity in the basal ganglia.

**117.7.1.10 Relationship between Trinucleotide CAG Repeat Length and Clinical Features of HD.** A significant correlation between the number of CAG repeats and the age of onset of HD has been demonstrated (281,282). This association was present irrespective of the mode of clinical presentation at time of onset. The number of trinucleotide repeats in the upper allele account for up to 70% of the variation in the age of onset (283). Repeat length, however, is not indicative of any other particular clinical phenotype as there is no independent association between any particular clinical feature of the illness and the number of repeats.

Is it possible to predict the age at which, in an individual, the signs and symptoms of HD will start? In the initial studies that examined genotype–phenotype correlations, the scatter around the regression curve was too large to make meaningful predictions for individuals (281,282). Many of these studies were, however, confounded by not including carefully ascertained at-risk persons with CAG expansion who are asymptomatic. One study including such persons showed that it is possible to use CAG size to predict with narrow confidence limits expected age of onset (283).

**117.7.1.11 Molecular Analysis of New Mutations.** New mutations causing HD have been proposed to be exceedingly rare, with the mutation rate estimated as the lowest for any human genetic disease (218). This may reflect that proof of a new mutation in HD is difficult, as prior criteria for identification of a new mutation have stipulated that parents of the sporadic case must have lived beyond the expected age of onset of HD without any manifestations of the disease, paternity of the sporadic case must be confirmed, and the disease should be transmitted to the offspring of the sporadic case.

The CAG repeat lengths in sporadic cases of HD and their families were assessed in order to learn more about the molecular events underlying new mutations for HD. In these families, the sporadic HD patient had a number of CAG repeats in the upper allele that was in the size range seen in patients with this disorder. In all families in which DNA was available from parents, the repeat length on one parental allele was found to be significantly greater than that seen in the general population (more than 29) but below the range seen in patients with HD (an intermediate allele [IA]) (284). The sex of origin of

the IA was determined by examining DNA from parents. In all families (16 of 16), preferential origin of the new mutation from the paternally derived allele was demonstrated. This would suggest that the paternal allele in the IA range is more likely to undergo significant expansion to a repeat length in the range seen in patients affected with HD (284).

#### 117.7.1.12 Insights into Pathophysiology of HD.

Several reports have highlighted a characteristic morphologic feature in many of the polyglutamine expansion disorders. Intracellular aggregates have been found in neurons of affected patients (285) and in murine (286) and in vitro models of HD (287–289). The specific compositions of these aggregates are unknown, but recent evidence showed that truncated polyglutamine fragments are associated with an increased frequency of aggregate formation. This raised the possibility that aggregates are in some way associated with cell death. Findings in human studies showed that aggregates are present in numerous cells in which cell death does not occur or occurs less frequently. This includes regions outside the cerebral cortex and basal ganglia in patients with HD, such as the cerebellum, and also regions outside the CNS, such as peripheral tissues including muscle and the pancreas. Similar observations were reported in animal studies in which mice express full-length huntingtin (290). These studies suggested that the aggregates are markers for toxicity and are not directly causative of neurotoxicity. In one study, Arrasate and coworkers monitored inclusion formation and cell death in vitro in individual neurons that had been transfected with mutant huntingtin and observed that neurons with inclusions had an increased likelihood of survival compared to neurons without inclusions. This supported the hypothesis that huntingtin inclusions are not pathogenic and may be neuroprotective (291).

An accumulating body of evidence implicates proteolytic cleavage of mutant huntingtin in the pathogenesis of HD. Several studies have shown that the N-terminal region of huntingtin is cleaved by different families of proteases, including caspases, calpains, and aspartyl endopeptidases (292–294). Findings from human and murine studies also demonstrate intracellular accumulation of N-terminal huntingtin fragments (295,296). Several in vitro studies have demonstrated that expanded N-terminal fragments of huntingtin cause enhanced cytotoxicity compared with normal N-terminal huntingtin fragments (289), and transgenic animals expressing truncated mutant huntingtin (exon 1) demonstrate widespread neuronal degeneration in the brain (286,297,298). Transgenic animals expressing an expanded N-terminal fragment of huntingtin containing exons 1 and 2 show no neurodegeneration, indicating that expression of an expanded N-terminal fragment of huntingtin is in itself insufficient to cause neurodegeneration and pointing to a differential effect of distinct N-terminal fragments on neurotoxicity. Additional studies are needed to further

elucidate the role of huntingtin cleavage and proteolysis in the pathogenesis of HD.

Excitotoxicity is a process by which excessive activity of the glutamate signaling pathway, particularly through the N-methyl-D-aspartate (NMDA) type of glutamate receptor, leads to abnormal intracellular calcium levels resulting in cell death. The involvement of excitotoxicity in the pathogenesis of HD was first suggested by rodent studies in which intrastriatal injections of kainic acid or quinolinic acid produced lesions that mimicked many of the neurochemical and histopathologic features of HD (299–301) and were associated with HD-like behavioral deficits (302,303). Subsequent human studies demonstrated enhanced expression of members of the glutamate signaling pathway in striatal medium-sized spiny neurons selectively lost in HD patients compared to the spared interneurons. These observations provided a possible explanation for the selectivity in neuronal loss and further implicated aberrant glutamate signaling in the pathogenesis of HD (304–306). Enhanced activities of NR1/NR2B-type NMDA receptors and mGluR5 receptors have been identified as a key contributor to HD pathology (307–309). Along with human and animal studies showing abnormal mitochondrial calcium handling, these observations give rise to a coherent, multifactorial model of mutant huntingtin-mediated disruption of calcium homeostasis as a cause of excitotoxicity and neuronal degeneration in HD (310).

**117.7.1.13 Predictive Testing.** Predictive testing for HD has been offered in different parts of the world for several years. Prior to the introduction of these programs, research protocols were developed to evaluate the psychological impact of receiving either an increased or decreased risk result (311,312). There was major concern that an increased risk result would precipitate catastrophic reactions such as emotional breakdown or suicide.

Short-term follow-up of participants in the Canadian Predictive Testing Program have revealed that predictive testing for HD may maintain or even improve the psychological well-being of at-risk individuals (312). Most individuals who receive a decreased risk result have shown improvement in psychological health, while the individuals who have received an increased risk result have not responded to predictive testing in the negative manner feared when predictive testing programs for HD were first developed. Despite the fact that both groups as a whole responded well to predictive testing programs, several individuals did have difficulties in adjusting to their new status. For those who received an increased risk result, there has been a new focus on physical symptoms with request for physical examination and the need for continued support and reassurance that DNA testing is not synonymous with diagnosis of illness (313). Though it was expected that some individuals might have difficulty coping with an increased risk result, a similar frequency of problems was not expected among

those receiving a decreased risk result. About 10% of the decreased risk group have had serious difficulties adapting to their new status. The major hurdle for these individuals appears to be the realization that they are facing an unplanned future.

The demand for predictive testing has been lower than expected in studies conducted prior to the advent of predictive testing (314). In addition, prenatal testing for HD is not a frequently chosen option (315).

Cloning of the gene for HD has had impact on the demand for and attitudes of at-risk individuals toward both predictive and prenatal testing. The demand for predictive testing has increased significantly. Hope for an effective therapy that did arise as a result of the cloning of the gene for HD is likely to further reduce the demand for prenatal testing (316). The long-term effects of predictive testing for HD are not known, and there is a continued need for longitudinal investment to examine the psychological and social effects of testing and to collect data that best predict responses to change in risk status.

**117.7.1.14 Management of HD.** Drug therapy for the management of HD is currently still limited to symptomatic treatment. The dopamine depleting drug tetra-benazine has recently been approved in the United States for treatment of the chorea of HD (317,318). Choreic movements can be partially suppressed by neuroleptics, while hypokinesia and rigidity may be ameliorated by antiparkinsonian agents. Psychiatric disturbances may react well to psychotropic drugs. Cognitive impairment is not amenable to drug treatment. Successful symptomatic treatment, however, does not always lead to a significant improvement of functional capacity. Therefore, an important part of management is to help the patient and the family to cope with the disease. Effective therapy to retard disease progression has not yet been achieved, although recent pathophysiologic insights suggest novel approaches.

It should be stressed that the contribution of nonpharmacologic approaches to the general well-being of HD patients is more important than medication. For those patients still living in the community, the close attention of family and friends, the family doctor, the social worker, or a public health nurse will be increasingly required. While there are obvious declines in functional capacity, patients with HD can in many instances remain in partial employment and fulfill domestic responsibilities until far into the illness. Premature withdrawal from these activities may further exacerbate the feelings of inadequacy and loss of power associated with the illness.

Attention should also be paid to the well-being of family members. Apart from possibly being at risk for HD, they also suffer from their daily burden of providing adequate care for the patient. Intermittent, temporary relief from this burden through respite care may encourage them to continue. A crisis at home is the most common reason for forced institutionalization of HD patients.

Many of these options will depend on the society in which the patients live. The advice, help, and expertise of the national lay societies, therefore, are indispensable in accumulating knowledge about the specific problems and opportunities for people with HD in their own country.

## 117.8 DIFFERENTIAL DIAGNOSIS: OTHER CHOREIC DISORDERS

### 117.8.1 Benign Hereditary Chorea (OMIM 118700)

Benign hereditary chorea (BHC) (also known as chronic juvenile hereditary chorea) is much rarer than Huntington disease. The earliest descriptions date from 1966 and 1967 but few series of patients and families have been described (319,320). BHC starts before the age of 5 in the vast majority of cases, although onset shortly after birth or around age 10 may occur. Males and females seem equally affected. The severity of the choreic movements may reach its maximum between ages 10 and 20 but then tends to decrease. Apart from choreic movements that may affect the limbs (both proximally and distally), the neck, and the face, not many additional features occur. Dysarthria is rare, while tremor has been described in a few families. Motor development may be somewhat slow, and some patients manifest clumsiness. The disease does not lead to dementia and, in adulthood, is non-progressive. Although some individuals were reported to have intellectual disability, the majority seems to be of normal intelligence and cognitive development. Similarly, psychiatric manifestations are not part of the usual clinical course. Other findings, such as hypospadias and minor digital abnormalities, have been found in single families and may be chance occurrences. Neuropathologic examination of the brain of one affected individual did not yield any specific abnormalities (321).

BHC is inherited as an autosomal dominant trait. A locus at 14q was found in 2000 and later confirmed (322), but genetic heterogeneity is likely (323). The gene at the 14q locus turned out to be the gene for TITF-1, a homeodomain-containing transcription factor essential for the organogenesis of the lung, thyroid, and the basal ganglia (324). This gene was part of a 1.2-Mb deletion in one family. As a second mutation, a single nucleotide substitution of intron 2 of the TITF-1 gene has been described (321).

### 117.8.2 Dentatorubral-Pallidoluysian Atrophy (OMIM 125370)

DRPLA is a disorder that received its name from its neuropathologic characteristics. The initial reports from 1946 described sporadic cases, but in 1982, Naito and Oyanagi described five Japanese families with multiple affected members and an autosomal dominant pattern of inheritance (325). The disease is more common (although



still very rare) in Japan than in other countries. In fact, only a few families have been described from outside Japan. Three clinical phenotypes are evident. The first is dominated by initial ataxia and subsequent choreoathetosis. The second was called the pseudo-Huntington type, with choreic movements and dementia, and only mild cerebellar ataxia. The third consists of progressive myoclonic epilepsy: initial myoclonic and generalized seizures with progressive mental deterioration. Different phenotypes may occur in one family, but cerebellar ataxia could be elicited to some extent in almost all cases. Onset ranges from 6 to 69 years, and death occurs between ages 20 and 75. Disease duration is approximately 10 years. Anticipation was noted in some families, with early-onset, associated with myoclonic epilepsy, and later-onset, with ataxia, dementia, and choreoathetosis.

In the course of the disease, multiple CNS neuronal systems degenerate, consistently including the globus pallidus (especially its lateral part), the subthalamic nucleus or nucleus of Luys, the cerebellar dentate nucleus, and the red nucleus (326). In addition, several other structures in the brainstem and spinal cord may be affected. Similar to HD, neuronal loss and gliosis constitute the main microscopic findings, with secondary demyelination of affected tracts.

DRPLA is caused by CAG expansion in a novel gene on chromosome 12 (327,328). CAG expansion is strongly correlated with age of onset. In one African American family, a disorder clinically similar to typical DRPLA but lacking myoclonic epilepsy and basal ganglia degeneration (Haw River syndrome) has been shown to be caused by similar CAG expansion in the same gene (329). Antibodies directed against the C-terminus of the DRPLA gene product detect a protein of approximately 190 kD (330). Consistent with what is known from other CAG repeat disorders (see earlier section on Huntington Disease), small, round immunoreactive intranuclear inclusions in both neurons and glial cells in various brain regions have been found. Electron microscopy showed that such inclusions are composed of granular and filamentous structures that consist of polyglutamine-containing aggregated protein fragments (326).

### 117.8.3 Neuroacanthocytosis (OMIM 200150)

The presence of acanthocytes in a peripheral blood smear may be associated with neurologic disease. For clinical purposes, two groups of patients may be distinguished. The first may be additionally characterized by low serum  $\beta$ -lipoproteins and includes Bassen Kornzweig disease (autosomal recessive abetalipoproteinemia), a form of hypobetalipoproteinemia (331), and the HARP syndrome (hypoprebetalipoproteinemia, acanthocytosis, retinitis pigmentosa, and pallidal degeneration) (332). A second group comprises those with normal serum  $\beta$ -lipoproteins and, apart from a few patients with Neurodegeneration

with Brain Iron Accumulation (NBIA, formerly Hallervorden-Spatz disease) (333), includes patients with neuromuscular and basal ganglia disease with or without the McLeod blood group phenotype. In this latter group, the disorder is called neuroacanthocytosis, chorea-acanthocytosis, familial amyotrophic chorea with acanthocytosis, or the Levine-Critchley syndrome, after the authors of the two original papers that first described the disease.

Neuroacanthocytosis is a slowly progressive neurodegenerative disorder affecting basal ganglia, peripheral nerves, and muscle. In a review of 19 sporadic and familial British cases, as well as 26 cases from the literature, Hardie and coworkers (334) found onset varying between 8 and 62 years, with a mean of about 32.5 years. Of these 45 patients, 8 were reported to have died between 7 and 24 years after onset of the disease (mean, 13.9 years).

The clinical manifestations may resemble HD. Mild cognitive impairment and psychiatric symptoms may manifest early in the disease. Dementia will develop in most patients, but early in the disease, frontal lobe dysfunction dominates. Personality changes, impulsive and distractable behavior, mood disorder, paranoid delusions, and obsessive-compulsive features may necessitate psychiatric help (335). Contrary to adult patients with HD, epilepsy occurs much more frequently than in the general population, with about half of the reported cases affected.

Choreic movements of the limbs and face are present in almost all cases; they usually start in the legs. Orofacial dyskinesia may lead to tongue and lip biting, offering a strong clue to the diagnosis; it may cause severe problems with speaking and swallowing. Apart from chorea, dystonia, parkinsonism, and tics may occur in the course of the disease. Vocalizations and, rarely, coprolalia may resemble that seen in Tourette syndrome. CT or MRI studies of the brain may reveal atrophy of the caudate nucleus. In addition, in a number of cases focal symmetrical abnormalities can be demonstrated in various parts of the basal ganglia that consist of hyperintense lesions in T2-weighted MRI sequences. These lesions may be found in the caudate, putamen, or globus pallidus, where they may resemble the “eye-of-the tiger” sign in NBIA.

The clinical feature that distinguishes neuroacanthocytosis from HD is the presence of neuromuscular abnormalities consisting of hypo- or areflexia of tendon reflexes and distal amyotrophy. Serum creatine kinase activity is elevated in the majority of cases, while nerve conduction studies will reveal reduced sensory action potentials in about 50% of cases. In contrast, motor nerve conduction studies generally will be uninformative, and electromyography will reveal denervation in only a minority of cases (334).

The neuropathology of neuroacanthocytosis is distinct. In the CNS, the caudate nucleus, the putamen, and the globus pallidus consistently show atrophy with marked neuronal loss and gliosis. The substantia nigra, the thalamus, and the motor neurons in the spinal cord may

atrophy (336,337). In peripheral nerves, axonal loss primarily affects the large diameter-myelinated fibers distally, with signs of regeneration after axonal degeneration, but unmyelinated axons may not escape destruction (334).

Neuroacanthocytosis shows a recessive pattern of inheritance in most cases. A genome-wide scan in 11 families from geographically different areas revealed linkage in all families to a 6-cM region at 9q21 (399). In these families, 16 different mutations were identified in a gene called CHAC (338). CHAC encodes an evolutionarily conserved protein, called chorein by Ueno and associates, that may be involved in protein sorting (338,339). Various groups have confirmed mutations in this gene as the cause of neuroacanthocytosis, thus establishing CHAC as the major gene associated with neuroacanthocytosis. It is important to realize, however, that X-linked neuroacanthocytosis associated with the McLeod phenotype may present a clinical phenotype that may resemble CHAC-associated neuroacanthocytosis very closely (340).

#### 117.8.4 Paroxysmal Dystonic and Choreic Movement Disorders: DYT8 (OMIM 118800), DYT9 (OMIM 601042), and DYT10 (OMIM 128200)

Although the dystonia and chorea of the different disorders as described in the previous paragraphs may wax and wane with emotion, fatigue, and other exogenous factors, these involuntary movements are still considered to present a more or less static movement disorder. In contrast, the so-called paroxysmal hyperkinesias or dyskinesias present short-lasting (minutes to hours) episodes of dystonia or chorea, often with an identifiable precipitating event, without any signs of movement disorder in between.

The term paroxysmal dyskinesia encompasses various disorders, such as “familial paroxysmal choreoathetosis,” “familial paroxysmal dystonic choreoathetosis,” “paroxysmal kinesigenic choreoathetosis,” and others (341). A clinically useful classification has been proposed by Demirkiran and Jankovic, who used as classifying factors the duration of the attacks, the inducing factor, and whether the attacks are primary (familial or sporadic) or secondary (342). The most important classifying criterion, in their opinion, is whether or not attacks are induced by voluntary movements. The resulting categories are kinesigenic, nonkinesigenic, and exertion-induced paroxysmal involuntary movements. A secondary subdivision can be made to take into account the duration of the attacks—lasting longer or shorter than 5 minutes.

DYT8 is associated with the classical phenotype as described by Mount and Reback in 1940 as paroxysmal nonkinesigenic choreoathetosis (343). Attacks are typically brief, lasting a few minutes to hours only, and are precipitated by coffee, fatigue, emotional stress, alcohol, or tobacco. The movement disorder starts within the first few years of life. The locus was ultimately mapped to

2q33–q33 (135,143,385). In affected persons from two unrelated families, missense mutations in the myofibrillogenesis regulator gene (MR1) were then identified. These mutations were absent in control subjects and caused Ala7Val and Ala9Val substitutions. These disturb interspecies-conserved residues and are predicted to alter the amino-terminal  $\alpha$ -helix of MR1. The MR1 exon containing these mutations (exon 1) was expressed only in the brain, a finding that explains the brain-specific symptoms of subjects with these mutations (344).

DYT9, at 1p, is an autosomal dominant form of paroxysmal choreoathetosis with episodic ataxia (345). As in DYT8, episodes are typically brief, and precipitating factors were also similar to those in DYT8. The gene responsible has not been identified.

DYT10 is familial dominant paroxysmal kinesigenic choreoathetosis. Attacks are precipitated by sudden movements, onset is later in life, duration of attacks is seconds to minutes, there is daily occurrence, and patients show good response to anticonvulsants. A locus was mapped to 16p11.2–q12.1 (346), but the gene is still unknown.

It has been suggested that, in all instances, ion channels are excellent candidate genes, as ion channel mutations play a role in many other paroxysmal neurologic disorders, such as hypo- and hyperkalemic periodic paralysis, episodic ataxias, familial hemiplegic migraine, and various forms of familial epilepsy syndromes.

#### 117.8.5 Huntington Disease–like Illnesses: HD-like 1 and HD-like 2

Several genetic disorders have been identified which closely resemble Huntington disease. A 192-nucleotide insertion in the region of the prion protein gene (PRNP) encoding an octapeptide repeat in the prion protein on chromosome 20 is responsible for HD-like 1 (347), an inherited disorder with chorea and dementia. HD-like 2 (HDL2) also bears striking resemblance to HD. The genetic mutation associated with HDL2 is a CTG/CAG trinucleotide repeat expansion within the junctophilin-3 gene (JPH3) on chromosome 16q24.3 (348). In the normal population, the repeat length ranges from 6 to 27 CTG/CAG triplets, whereas affected individuals have 41 to 58 triplets. HDL2 manifests in the third or fourth decade with characteristic features of chorea, dystonia, or parkinsonism and progressive cognitive deficits over approximately 20 years. Early signs include behavioral and personality changes with deterioration of social interaction. Intracellular inclusions immunoreactive for expanded polyglutamine repeats are seen in the brains of patients with HDL2 (349).

#### 117.8.6 Neuroferritinopathy

This disorder typically presents with involuntary movements in people 40 to 55 years of age. Symptoms of

extrapyramidal dysfunction, including choreoathetosis, dystonia, spasticity, and rigidity, sometimes showing acute progression but not associated with significant cognitive decline or cerebellar involvement, are present. MRI showed cavitation of the basal ganglia confirmed by brain pathology (350,351). The disorder was mapped by linkage analysis to 19q13.3, which contains the gene for ferritin light polypeptide (FTL). An adenine insertion at position 460–461 that is predicted to alter carboxy-terminal residues of the gene product was found (352). Brain histochemistry disclosed abnormal aggregates of ferritin and iron. Low serum ferritin levels also are seen. Brain histopathology shows widespread reddish discoloration of the basal ganglia. The globus pallidus contains abundant iron-positive, roughly spherical inclusions most of which are also positive for ferritin. Many iron- and ferritin-positive profiles are present throughout the forebrain and cerebellum. The number and overall size of iron/ferritin elements greatly exceeded those found in elderly.

### 117.8.7 Familial Tic Disorders: Gilles de la Tourette Syndrome (Oimim 137580)

A tic is a sudden, rapid, recurrent, nonrhythmic, stereotyped motor movement or vocalization. Tics have long been considered the major manifestation of Gilles de la Tourette syndrome (TS), but the recognition of a range of behavioral abnormalities, particularly obsessive-compulsive disorder (OCD) and attention-deficit disorder (ADD), as part of the disease spectrum has greatly advanced our understanding of the pathophysiology and genetics of the syndrome (353,354). The diagnostic criteria for TS as formulated in the revised fourth edition of the DSM (DSM-IV-R) are as follows:

1. Both multiple motor and one or more vocal tics have been present at some time during the illness, although not necessarily concurrently.
2. The tics occur frequently throughout a period of more than 1 year, and during this period there is no tic-free period of more than 3 consecutive months.
3. Onset is before age 21.
4. Other causes of tics have been ruled out.

TS is considered to be a lifelong disorder with onset at a young age, but in a retrospective cohort study, 50% of TS patients were asymptomatic by age 18 (355). Although formerly considered to be rare, it has become clear that the disease is more prevalent than expected, and that the apparent rates may depend on the extent of surveillance and access to care. A community-based study of pupils enrolled in schools in Monroe County, New York, yielded an estimated prevalence of 3 per 10,000, with males being nine times as often affected as females (356). A study using a large insurance claims database found that in Medicaid-insured children, the rate of diagnosis of TS per 1,000 was 0.53 (95% confidence interval [CI]

0.51–0.55), and 0.50 (95% CI 0.49–0.52) in privately insured children (357).

### 117.9 CLINICAL FEATURES

Usually tics are perceived as involuntary motor acts, but many patients indicate that they feel a tension or “urge” building up that can be relieved by submitting to the tic. Therefore, tics may be voluntarily suppressed for some time (seconds to hours).

Tics may be divided into simple and complex motor tics, and phonic or vocal tics. Simple motor tics involve simple motor acts such as eye blinking, head nodding, or shoulder shrugging. These movements are characteristically brief and jerky. In contrast, complex tics involve an array of movements that are always characteristic and reproducible for one individual over a limited period of time. For example, standing up and striking one’s upper leg, jumping, or patting objects within reach may constitute the phenomena of complex tics. Similarly, vocal tics may be simple and complex. Sniffing, grunting, or coughing are examples of the former, while coprolalia, echolalia, or the stereotypic utterance of words and sentences are examples of the latter. Although too many coprolalia may be the most remarkable and characteristic feature of the condition, its frequency is probably less than 10% (358).

Characteristically, the frequency and severity of tics will wax and wane over time. Over the course of a day, they can be aggravated by anxiety, stress, and fatigue, while relaxation or concentration on an enjoyable task may lead to amelioration, but fluctuations also may occur over a timespan of months. In fact, during certain periods, tics may be minimal. In addition, their form and content may change over time, with a tendency for simple forms to be replaced by complex ones.

In its severity, TS stands at the extreme of a spectrum of tic disorders (353). *Chronic tic disorder* displays either motor or vocal tics, but not both. *Transient tic disorder* lasts from 2 weeks to 12 months. Benign tics of childhood may be recognized for a brief period in childhood, typically lasting less than 1 year. Finally, isolated, mostly simple tics, may develop later in life. Many of these individuals with non-TS tics may nevertheless represent milder manifestations of the same disease, as the prevalence of non-TS tic disorder in relatives of TS patients is increased.

Obsessive-compulsive symptoms were identified by Gilles de la Tourette, and since then they have been recognized as part of the disease. Estimates of their prevalence have ranged from 30% to 90%, depending on the assessment instruments used and whether it was OCD or obsessive-compulsive symptoms that were looked for. OCD has a negative impact on quality of life in individuals with TS (359). One survey revealed that about 20% of the first-degree relatives of TS patients displayed obsessive-compulsive manifestations without tics; this

was particularly the case among women (360). Similarly, in twin studies, high concordance rates for TS and obsessive-compulsive symptoms have been reported, higher in monozygotic than in dizygotic twins (361). It was concluded that obsessive-compulsive symptoms form part of the disease spectrum of TS.

Although TS is considered a basal ganglia disorder, there is only indirect evidence that the disease is caused by pathology in the basal ganglia. Simple and complex tics (“Tourettism”) have been observed after organic brain disease (e.g. encephalitis lethargica), trauma, stroke, tumors, carbon monoxide poisoning, herpes encephalitis, syphilis, multiple sclerosis, or Huntington, Alzheimer, or Creutzfeldt–Jakob disease (362). Tardive induction of TS-like symptoms by neuroleptic medication (363), aggravation of symptoms by dopamine agonists, but particularly the suppression of signs and symptoms by neuroleptics (see later) link TS to disturbances in dopaminergic transmission. Up to now, however, PET studies with metabolites and ligands involved in dopaminergic transmission have failed to yield abnormalities in TS patients. As with tics, obsessive-compulsive manifestations may be part of basal ganglia disorders such as Sydenham chorea or Huntington disease. In general, obsessive-compulsive signs are partially responsive to drugs such as fluoxetine and fluvoxamine—selective serotonin reuptake inhibitors that imply a role for this indolamine in the pathophysiology of the disease, in addition to dopamine.

Not unexpectedly, the complex behavioral disturbances in TS may be associated with altered activities in areas outside the basal ganglia. A fluorodeoxyglucose PET study of the brains of TS patients showed relatively decreased metabolic rates in paralimbic and ventral prefrontal cortices (particularly in orbitofrontal, inferior insular, and parahippocampal regions), as well as in subcortical regions that included the ventral striatum (nucleus accumbens/ventromedial caudate) and the mid-brain. These changes were more robust and occurred with greater frequency in the left hemisphere. They were associated with concomitant bilateral increases in metabolic activity in the supplementary motor, lateral premotor, and Rolandic cortices (364,365).

Postmortem studies of TS patients are rare. In one case, calcifications around the third ventricle and the mesencephalic periaqueductal gray matter were found, consistent with the localization of postencephalitic lesions that may be associated with Tourettism (366). Another study found immunohistochemical evidence for a decrease in dynorphin staining throughout the brain. The absence of dynorphin-containing striatal fibers projecting to the globus pallidus particularly was noted, while fibers that contained substance P and Enk were intact. These results suggest that dysfunction of multiple structures and systems may be associated with TS (367,368). In a recent set of 5 cases examined using quantitative methods, a

higher total neuron number was found in the globus pallidus pars interna, with a lower neuron number and density observed in the globus pallidus pars externa and in the caudate nucleus (369). A more striking deficit was revealed by examination of interneuron populations: TS patients demonstrated a 50%–60% decrease of both parvalbumin and cholinergic interneurons in the caudate as well as the putamen (370). Both of these observations suggest abnormalities in neurogenesis or neural migration.

### 117.9.1 Genetics

Strong initial support for a genetic contribution to the etiology of TS came from twin studies as well as from family studies, but the study of the genetics of TS has always been confounded by variations in clinical presentations and incomplete penetrance. Dominant inheritance with incomplete penetrance has been often proposed as the mode of inheritance, but linkage analysis of single- and multiple multigenerational pedigrees has yielded disappointing results.

A recent review discusses in detail the current state of the genetics of TS (371). Although several mechanistically based candidate genes have been examined, none yet has withstood the test of replication. Genome-wide scans have been limited in scope, with the largest published so far including only 111 individuals. The most informative studies have been in families with high penetrance. In a pedigree with a large number of familial TS cases, L-histidine decarboxylase (HDC), an enzyme central to histaminergic neurotransmission, was identified (372). Mice with deletion of HDC exhibit increased sensitivity to stereotypic behaviors after treatment with dopamine agonists (373), providing what may be a useful model for examining the disorder. It is likely, however, that the genetic basis of TS is polymorphic, and larger numbers of familial and sporadic cases will be required to establish a complete picture of the genetics underlying the disorder.

### 117.9.2 Treatment and Management

Counseling, psychological support, and education of affected and nonaffected family members, teachers, and coworkers constitute major goals in helping the patient to cope with the condition. A child psychiatrist's expertise is often required in handling issues related to the child's psychological development and maturation.

TS is characterized by episodic alterations in intensity of the tics over the years. Sometimes, in periods of mild tics, medication can be avoided. Should drugs be required, a gradually escalating approach is preferred. Often, partial relief may be obtained with the alpha-adrenergic agent clonidine, or the benzodiazepine clonazepam. Neuroleptics are more effective at suppressing tics but carry a much greater burden of potential side effects.



Traditionally, haloperidol has been the first choice for treatment since its efficacy in treating TS was demonstrated in 1961. Adverse reactions to haloperidol—and, indeed, to every neuroleptic drug—include drowsiness and sedation, mood alterations, parkinsonian signs, akathisia, acute dystonic reactions, and tardive dyskinesia. It has been estimated that only one third of all TS patients who start on haloperidol will eventually benefit from it for sustained periods. Pimozide also been studied extensively, and although effective against tics it also carries a risk of cardiac arrhythmia. In recent practice, the use of the “atypical” neuroleptics, such as risperidone, has become more common but long-term follow up data on these is lacking.

Apart from attempts to control tics, one may want to modify obsessive-compulsive symptoms. For this purpose fluoxetine, fluvoxamine, and clomipramine may be beneficial. Amphetamine-like substances such as methylphenidate, and again clonidine, have been employed to suppress hyperactivity and ADD in children. Care should be taken with methylphenidate, as this drug may exacerbate tics.

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The text refers to Wilson disease, which should be cross-referenced.

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### Biographies



**Dr West** graduated from Alma College in Michigan in 1999 and received a PhD in Molecular Neuroscience from the Mayo Clinic School of Medicine in the laboratories of Dr John Hardy and Matthew Farrer in 2003. He went on to complete a Postdoctoral Fellowships at the UCLA Neuropsychiatric Institute in Los Angeles in the laboratory of Nigel Maidment and in the laboratory of Ted and Valina Dawson at Johns Hopkins School of Medicine. He joined the Department of Neurology at the University of Alabama at Birmingham School of Medicine in 2007 as the John A. & Ruth R. Jurenko Research Scholar in Parkinson disease research. Dr West's laboratory in the Center for Neurodegeneration and Experimental Therapeutics focuses on discovering the biochemical and genetic basis of Parkinson's disease.



**Dr Gray** graduated from Alabama State University in 1997. She then received her PhD. in Molecular, Cellular, and Developmental Biology from Ohio State University with a focus on Developmental Neurobiology. Her post-doctoral training was obtained in the Brain Research Institute in the Semel Institute for Neuroscience and Human Behavior at the University of California, Los Angeles. She moved to the University of Alabama at Birmingham in 2008, and joined the Department of Neurology as an Instructor and the Dixon Scholar in Neuroscience. She is currently an Assistant Professor in the Department of Neurology and a member of the Center for Neurodegeneration and Experimental Therapeutics. Her work focuses on elucidating the contribution of astrocytes to Huntington Disease pathogenesis.



**Dr Standaert** graduated from Harvard College in 1982. He received his M.D. and Ph.D. degrees from Washington University in St. Louis. He completed a one-year internship in Medicine followed by a three-year Neurology residency at the University of Pennsylvania. He was appointed a Howard Hughes Medical Institute Physician Research Fellow, and completed a three-year research and clinical fellowship in Neurology (Movement Disorders) at Harvard Medical School and Massachusetts General Hospital in 1995, and subsequently joined the faculty at MGH. Dr Standaert is presently at the University of Alabama at Birmingham faculty as the John N Whitaker Chair and Professor of Neurology. He serves as Director of the Division of Movement Disorders, the Director of the APDA Advanced Center for Parkinson Research at UAB, and is the Director of the Center for Neurodegeneration and Experimental Therapeutics. He sees patients in a weekly clinic and oversees many clinical trials for new treatments of Parkinson's disease. He is a member of the Scientific Advisory Boards of the Michael J. Fox Foundation for Parkinson Research, the American Parkinson Disease Association, and the Bachmann-Strauss Dystonia & Parkinson Foundation.

# CHAPTER

# 118

## The Hereditary Ataxias

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### 118.1 RECESSIVE ATAXIAS CAUSED BY CATALYTIC DEFICIENCIES

A large number of hereditary ataxias appear to be caused by “inborn errors of metabolism”—a term used by Garrod in the early 1900s (1). Although the genes responsible for these diseases are only now being discovered, their genetic basis and relevant biochemical abnormalities have been appreciated for quite some time. Indeed, the dissection of metabolic pathways in the early half of the twentieth century has played an important role in suggesting candidate enzymes in gene-hunting strategies. This is in contrast to the degenerative ataxias, for which positional cloning, a more time-consuming process, was needed to identify novel genes encoding for the most part novel proteins whose functions are still to be unveiled.

Like most disorders resulting from catalytic deficiencies, the metabolic ataxias tend to be recessive, since half the complement of enzymatic activity in heterozygotes is sufficient for most metabolic pathways to proceed. Moreover, these pathways lie at the root of several housekeeping functions, so the resulting ataxia is just one component of a complex clinical phenotype. It is this clinical picture that usually betrays the underlying metabolic defect. The metabolic ataxias can be divided, yet again, into one of the two groups. The first group encompasses those ataxias that present intermittently when the biochemical abnormalities are at their worst; the second category is characterized by a more chronic and progressive ataxia induced by specific enzyme deficiencies (Table 118-1).

### 118.2 INTERMITTENT ATAXIAS

Of the group that present with an intermittent ataxia, the most common are the aminoacidurias resulting from deficits of urea cycle enzymes, and disorders of pyruvate and lactate metabolism. In all of these conditions,

affected infants usually display mental retardation and developmental delay. It is, however, important to distinguish between these subtypes since supportive measures differ.

Deficiency of any of the enzymes in the Krebs–Henseleit urea pathway is the most common metabolic cause of hyperammonemia and intermittent ataxia. Affected infants present with clumsiness, vomiting, and headaches. At the peaks of hyperammonemia, usually induced by stress or ingestion of proteins, infants display seizures, involuntary movements, and ptosis. Unfortunately, there is no treatment to reverse the underlying enzyme deficiency. Supportive therapy takes the form of hydration and dietary restriction of proteins. Inheritance of urea cycle deficits is autosomal recessive, with the notable exception of the X-linked and relatively common *ornithine transcarbamylase deficiency* (OTC) (Xp21.1). It is important to note that occasionally female carriers with OTC present with symptoms that are typically triggered by a protein-rich diet (2). In these symptomatic females, the attacks can come even in adulthood.

Ataxias seen in the aminoacidurias, such as *intermittent branched-chain ketoaciduria* and *isovaleric acidemia*, are usually diagnosed by the characteristic odor of the urine of these infants. *Hartnup* syndrome is another aminoaciduria that results in ataxia; this disorder results from a defect in renal and intestinal transport of amino acids (in particular, the neutral amino acids) rather than a defect of a metabolic pathway. Because of the associated niacin deficiency, features of pellagra (e.g. rash and mental confusion) are often seen. In contrast to the hyperammonemias resulting from urea cycle deficits, treatment of the aminoacidurias consists of a high protein diet along with nicotinamide supplementation. In 1997, a mouse mutant displaying several features of Hartnup disease was generated in an N-ethyl-N-nitrosourea (ENU) mutagenesis screen. This gene maps to mouse chromosome 7 (3). The first gene to unambiguously cause a Hartnup

**TABLE 118-1 Genetics of Inherited Ataxias with Known Enzyme Defects**

Disease	Locus	Enzyme/Protein Deficiency
<b>I. Intermittent ataxias</b>		
<i>Hyperammonemias and aminoacidurias</i>		
OTC deficiency	Xp21.1	Ornithine transcarbamylase
Citrullinemia	9q34	Arginosuccinate synthetase
Arginase deficiency	6q23a	Arginase
Arginosuccinaciduria	7cen-q11.2	Arginosuccinate lyase
Hyperornithemia-hyperammonemia-homocitrullinuria	13q14	Mitochondrial ornithine transporter syndrome
Hartnup disease	11q13	—
Isovaleric acidemia	15q14-q15	Isovaleric Acid CoA dehydrogenase
<i>Disorders of pyruvate and lactate metabolism</i>		
Pyruvate dehydrogenase complex	Xp22.2–p22.1 (most common)	E1- $\alpha$ subunit of PDH complex (most common)
Pyruvate carboxylase deficiency	11q13.4-q13.5	Holocarboxylase synthetase
<b>II. Progressive ataxias from metabolic insufficiency</b>		
Hexosaminidase deficiency-GM2 gangliosidosis	Genetically heterogenous	Hexosaminidase A, B or S isozymes (Tay–Sachs disease)
Niemann Pick	11p15.4-15.1	Acid sphingomyelinase
Niemann Pick type C	18q11-q12	NPC1
Cerebrotendinous xanthomatosis	2q33-qter	Mitochondrial sterol 27 hydroxylase
Metachromatic leukodystrophy	22q13.31-qter	Arylsulfatase A
Adrenoleukodystrophy	Xq28	Adrenoleukodystrophy protein
Abetalipoproteinemia	4q22-q24	Microsomal triglyceride transfer protein
Hypobetalipoproteinemia	2p24	ApoB
Ataxia with vitamin E deficiency	8q13.1-q13.3	Alpha-tocopherol transfer protein
Lesch–Nyhan syndrome	Xq26-q27.2	Hypoxanthine-guanine phosphoribosyl transferase
Wilson disease	13q14.3-q21.1	ATP7B protein
Ceroid lipofuscinosis	Several variants	Multiple gene products
X-linked ataxia, ichthyosis and tapetoretinal dystrophy	Xpter-p22.32	Arylsulfatase C
<b>III. Progressive ataxias associated with defective DNA repair mechanisms</b>		
Ataxia telangiectasia	11q2.3	ATM kinase
Xeroderma pigmentosa	Several variants	Multiple
Cockayne syndrome	Several variants	Multiple

syndrome in humans is SLC6A19 (mapping to 5p15), a gene that encodes a sodium dependent and chloride independent neutral amino acid transporter (4,5).

*Pyruvate dehydrogenase deficiency*, a less common cause of metabolic intermittent ataxia, is most often caused by a mutation in the E1- $\alpha$  subunit of the pyruvate dehydrogenase enzyme (Xp22.2–p22.1) (6). This condition is characterized by lactic acidosis, seizures, mental retardation, and spasticity. Recently, a zebrafish model of this enzymatic defect has been generated, and it should help in understanding how best to treat the metabolic sequelae of this syndrome (7). *Biotin-responsive multiple carboxylase deficiency* is another autosomal recessive infantile ataxia caused by a variety of mutations in the holocarboxylase synthetase (*HLCS*) gene at locus 21q22 and characterized by seizures, myoclonus, and nystagmus (8–10). A late-onset (juvenile) ataxia can also result from a defect in the biotinidase gene located

on chromosome 3p25 (11). Both the infantile and juvenile ataxic syndromes are responsive to high-dose biotin therapy.

Metabolic ataxias are usually diagnosed by screening biochemical tests when the neurological abnormalities are first noticed. Based on a positive family history, it is even possible to diagnose OTC deficiency (and several of the hyperammonemias) from blood samples taken in utero. Pyruvate dehydrogenase deficiency can, in addition, be corroborated by a relatively simple biochemical assay on cultured fibroblasts. Despite the identification of individual gene defects, genetic testing, at least at present, is impractical because of the large number of mutations in the relevant genes.

Apart from the supportive therapy of hydration and dietary restriction for many of the metabolic ataxias, there is yet no treatment for the underlying enzyme deficiency. If the liver is the site of enzyme synthesis, then

liver transplantation may be an option. Gene therapy for these diseases is still in its infancy (12–14).

### 118.3 PROGRESSIVE ATAXIAS FROM METABOLIC INSUFFICIENCY

A variety of etiologies underlie progressive ataxias induced by metabolic insufficiency. Unlike the intermittent ataxias, these diseases usually present in later childhood or adolescence, most likely because cumulative damage must reach a threshold before the clinical signs appear. The persistence of the biochemical abnormalities explains the progressive nature of the symptoms. Storage disorders predominate in this group of ataxias (comprising sphingomyelin lipidosis, metachromatic leukodystrophy, Krabbe disease, Niemann–Pick disease type C, X-linked adrenoleukomyeloneuropathy, and the genetically heterogeneous ceroid lipofuscinosis and hexosaminidase deficiencies (e.g. Tay–Sachs disease, Sandhoff disease—see Table 118-1 for a more comprehensive list, along with the relevant genetic defects). In each case, the clinical picture in conjunction with the histopathological findings or biochemical tests is telling: for instance, in the case of Niemann–Pick, one often sees foamy storage cells in bone marrow, and when accompanied by the typical supranuclear gaze palsy, the diagnosis is virtually certain. *Adrenoleukodystrophy* is often suspected when the X-linked inheritance is confirmed. Adrenoleukodystrophy is caused by defective beta-oxidation of fatty acids in peroxisomes. This leads to elevated levels of very long-chain fatty acids in the blood (a finding used for diagnosis) and accumulation of cholesterol esters of fatty acids and gangliosides in membranes of cells particularly in the brain and adrenal cortex; hence, the frequent accompaniment of adrenal insufficiency. Adrenoleukodystrophy is caused by a mutation in the adrenoleukodystrophy protein (ALDP) on Xq28 that belongs to the ABC family of transporter proteins (15,16). It is important to remember that because of variability in X chromosome inactivation patterns, a small percentage of females will show some of the symptoms in X-linked conditions. Unfortunately, there is no cure for the ataxias induced by storage defects, but there is now a growing list of ataxias for which specific measures can be taken to at least halt progression (Table 118-2). *Wilson disease* or *hepatolenticular degeneration* is probably the best known and is characterized by copper overload. Although the disease was described by Kinnier Wilson in 1911, the relevant gene *ATP7B*, encoding a copper transporting ATPase, was discovered relatively recently (17). The culprit gene maps to 13q14.3-q21.1. Because there are at least 25 different disease-causing mutations in a gene that occupies 80kb of genomic DNA, genetic testing is currently not possible and diagnosis rests on clinical tests, including the demonstration of Keyser–Fleischer corneal rings in slit-lamp examination and liver biopsy findings (18). Because Wilson's disease

is an eminently treatable ataxic syndrome, it should be suspected in any young patient (typically less than 40 years of age) with ataxia, especially when the ataxia is accompanied by other well characterized features of this syndrome. Treatment in Wilson's has a three-pronged strategy: 1. Reduction of accumulated tissue copper by the administration of copper chelators (such as penicillamine and trientine). 2. Reduction of copper absorption from the gut by oral zinc. Zinc induces metallothionein in enterocytes that helps chelate ingested copper that eventually is excreted in the stools as enterocytes turn-over. 3. Reduction of copper intake by avoiding foods rich in copper (for instance, shellfish, nuts, chocolates). With adherence to the treatment plan, patients generally respond well.

Ataxia with isolated vitamin E deficiency (AVED) is the name given to a group of autosomal recessive ataxic syndromes. This syndrome typically presents as a slowly progressive ataxia syndrome with neuropathy, sometimes with a phenotype virtually indistinguishable from FRDA (19,20). Some patients also develop retinitis pigmentosa (21,22). The most common genetic lesion is in the alpha tocopherol transfer protein TTPA encoded by the tocopherol transfer protein gene *TTP1* (8q13.1-q13.3) (AVED-MIM 277460) that is involved in the secretion of vitamin E from cells (23).

*Hypovitaminosis E* can also occur as part of abetalipoproteinemia, also known as *Bassen–Kornzweig disease* or *acanthocytosis* because of the presence of acanthocytes in peripheral blood smear evaluations. Characterized by hypocholesterolemia, hypolipidemia, and reduction of fat-soluble vitamins A, D and K, this ataxia is inherited in an autosomal recessive fashion. Patients often suffer from pigmentary retinopathy from the coexisting deficiency of vitamin A. The genetic defect is caused by mutations in the microsomal triglyceride transfer protein (MTP), resulting in an inability to form the apoB peptide of LDL, and VLDL (24). Recently, genetic defects that result in a similar ataxic syndrome have been identified in the apoB gene itself. In all these diseases, oral supplementation with vitamin E in high doses is effective in slowing down the progression. Therefore, it is important to test vitamin E levels in any chronic ataxic syndrome. Recently a mouse model for AVED has been engineered by deleting the gene encoding the mouse version of the alpha tocopherol transfer protein (25). This mouse model, like the human patient, responds to vitamin E therapy and should help in elucidating the cellular pathogenesis of this syndrome.

*Cholestenolosis* or *cerebrotendinous xanthomatosis* (CTX) is a relatively rare cause of progressive ataxia. Caused by any one of a variety of defects (missense, nonsense and frameshift) in the mitochondrial 27 hydroxylase gene (locus 2q33-qter), CTX is characterized by autosomal recessive ataxia, neuropathy, cataracts, and achilles tendon xanthomas (26). MRI reveals cerebral atrophy and evidence for demyelination. The diagnosis



**TABLE 118-2 Potentially Treatable Hereditary Ataxias**

Disorder	Metabolic Abnormality	Clinical Features	Treatment
Bassen–Kornzweig syndrome	Abetalipoproteinemia	Acanthocytosis, retinitis pigmentosa, fat malabsorption	Vitamin E
Ataxia with isolated vitamin E	Deficiency of alpha-tocopherol transfer protein	Progressive ataxic syndrome	Vitamin E deficiency (AVED)
Hartnup disease	Tryptophan malabsorption	Pellagra rash, intermittent ataxia	Niacin
Mitochondrial complex defects	Complexes I, III, IV	Encephalomyelopathy	Riboflavin, CoQ10, dichloroacetate
Multiple carboxylase deficiency	Biotinidase deficiency	Alopecia, recurrent infections, variable organic aciduria	Biotin
Pyruvate dehydrogenase deficiency	Block in energy metabolism	Lactic acidosis, ataxia	Ketogenic diet, chloroacetate
Refsum disease	Phytanic acid, alpha hydroxylase	Retinitis pigmentosa, cardiomyopathy, hypertrophic neuropathy, ichthyosis	Dietary restriction of phytanic acid
Urea cycle defects	Urea cycle enzymes	Hyperammonemia	Protein restriction, arginine benzoate, alpha ketoacids

is confirmed by elevated serum cholestanol in the face of normal cholesterol. Treatment with chenodeoxycholic acid appears to halt progression of the disease. In addition, statins help in delaying atherosclerosis in these patients (27,28).

*Refsum disease* is yet another treatable ataxia caused by an enzyme deficiency, in this case a deficiency of phytanoyl-CoA hydroxylase with the culprit gene mapping to locus 10pter-p11.2. Refsum disease should be suspected when autosomal recessive ataxia presents with the triad of ichthyosis, retinitis pigmentosa and neuropathy (29,30). Patients with this disease cannot degrade phytanic acid due to a deficient activity of phytanoyl-CoA hydroxylase (PhyH) (with mutations in the gene encoding this protein), a peroxisomal enzyme catalyzing the first step of phytanic acid alpha-oxidation (31). Since toxicity in this disease is a result of the elevation of phytanic acid, the disease is preventable by avoiding the food containing this substance or its precursors.

There are a set of ataxias that are caused by enzymes that involve in DNA repair mechanisms. Because they do not result in metabolic ataxias, we have discussed them in a separate section below.

## 118.4 PROGRESSIVE ATAXIAS THAT ARE NOT BECAUSE OF METABOLIC DEFECTS

### 118.4.1 Friedreich Ataxia (FRDA, Spinocerebellar Ataxia-MIM 229300)

Friedreich ataxia (FRDA) is the most common autosomal recessive spinocerebellar ataxia, with a prevalence rate of 1 to 2 per 100,000. In the typical case, symptoms begin in late childhood or adolescence. The neurological phenotype is characterized by the cerebellar features of ataxia, scanning speech and nystagmus. In addition, eye movements tend to be sluggish, the gait is disorganized

and lurching, and patients demonstrate signs of motor and sensory neuropathy. Because of motor neuropathy, especially in the later stages of the illness, the distal extremities become weak and wasted while sensory neuropathy causes loss of vibration sense and proprioception. Early evidence for sensorimotor neuropathy can be elicited clinically by absent ankle reflexes and impairments on electrophysiological tests. These constitute the core features of the diagnosis, formalized in Harding's essential criteria with the additional proviso of onset before the age of 25 with most patients wheelchair bound around 15 years after the onset of symptoms (32). Other noteworthy neurological features include bladder incontinence and deficits of the special senses that can further decrease the quality of life. Vision is often mildly affected by optic atrophy, which typically causes impaired color vision. Deafness is a relatively rare accompaniment (it occurs in less than 10% of patients) and results from degeneration of cochlear neurons. Despite widespread neurological involvement, intellectual deficits are not all that common. Among the pyramidal features, extensor plantar responses are common, and sometimes spasticity can be observed. Rarely, extrapyramidal involvement in the form of choreiform movements may be seen. The most common pathological findings include degeneration of the posterior columns, spinocerebellar tracts and large sensory neurons of the dorsal root ganglia.

Besides involvement of the nervous system, patients with FRDA often have musculoskeletal abnormalities. Most patients display some degree of scoliosis or kyphoscoliosis and pes cavus. Cardiac pathology is another hallmark and usually results from muscular subaortic stenosis and hypertrophic cardiomyopathy. In the early stages of the disease, however, a wide range of abnormal EKG findings have been observed: T-wave abnormalities, deep Q waves, low QRS complexes, and the more obvious heart-block. In the later stages, a dilated form of cardiomyopathy with wall motion abnormalities replaces

the earlier hypertrophic picture. At a pathological level, there is cardiomyocyte hypertrophy, fatty degeneration associated with interstitial fibrosis, and eosinophilic and lymphocytic infiltrates. Although there is variability in the progression of the disease, the mean age of death is in the fourth decade (33). Death occurs because of cardiac complications or bulbar dysfunction that results in an inability to protect the airway. As in AT, approximately one fourth of the patients with FRDA develop glucose intolerance and diabetes that increases morbidity.

Since discovery of the gene and the availability of genetic testing, we are beginning to learn more about the less typical FRDA phenotypes. For instance, FRDA can present as early as infancy and should be suspected when delay in achieving motor milestones is associated with skeletal abnormalities. This early-onset phenotype tends to be associated with a worse prognosis than typical FRDA. Surprisingly, FRDA can also present much later in life, even beyond the age of 40, in a so called “very late-onset FRDA” (34,35). Some of these atypical features are found in shorter repeats (36) or in compound heterozygotes where one allele has a pathogenic GAA allele, while the other has a point mutation (missense, nonsense, or intronic) (37–40). Since several of the cardinal features of FRDA are milder in this instance, with a preservation of reflexes, these patients were bracketed into a separate category in the pregenetic era: “autosomal recessive ataxia with preserved reflexes.”

In the past, the diagnosis of FRDA was primarily a clinical one, resting on the aforementioned signs and symptoms and supported by only a few ancillary tests. Electrophysiological testing provides additional evidence for motor and sensory axonal neuropathy, whereas EKG findings present supportive evidence relating to the cardiac manifestations of the disease. MRI scans in FRDA often show evidence for spinal cord and cerebellar (particularly vermal) atrophy. In the spinal cord, atrophy affects the posterior columns, the spinocerebellar tracts and dorsal root ganglion (41). Today, the only diagnostic test is, of course, genetic, and testing should be performed in all recessive and chronic ataxic syndromes, based on what we know of their broad phenotypic spectra.

FRDA is caused by a GAA repeat expansion in the first intron of the gene called *frataxin/FXN* or X25. This gene is located on chromosome 9q13 (42). Most normal individuals carry alleles ranging from 7 to 34 GAA repeats, while patients with the disease have alleles with a tract of over 100 repeats. Tract sizes that vary between 34 and 100 do not result in disease but are further divided, based on whether or not they are interrupted by non-GAA repeats. If interrupted, they are considered nonpathological, because the interruption stabilizes the repeat against expansions in subsequent generations. On the other hand, uninterrupted repeat tracts with 34 to 100 GAAs are considered to be premutations, since without the stabilizing influence of the interruption, they can expand to over 300 repeats in just a single generation (43).

The unstable repeats tend to expand in paternal transmission and can expand or contract in maternal transmission. As mentioned earlier, because of the recessive nature of the disease, with a requirement for two expanded alleles, there is no anticipation. Nonetheless, disease severity and onset of disease correlate with repeat length. This is not just confined to the neurological phenotype, but also holds true for the glucose intolerance and the skeletal and cardiac abnormalities (44,45).

As might be expected from its recessive genetics, FRDA is caused by a loss of function of the gene product, frataxin. The intronic repeat expansion results in a reduced expression of the messenger RNA encoding this 210 amino acid protein. The precise mechanism is still unknown. Nevertheless, it has been speculated that the expanded repeats result in a higher order DNA structure that stymies transcription (46). That FRDA, caused by a loss of function mechanism, is supported by the observation that a Friedreich phenotype can also be seen in patients carrying only one expanded allele of frataxin, provided that their second allele is inactive due to a point mutation (14). This situation might be more common than currently recognized, since most current genetic tests search for expansions and not for other types of mutations.

Recent research has focused on delineating the function of frataxin and explaining why its loss should result in such widespread and profound pathology. It appears now that frataxin is a mitochondrial protein, expressed at particularly high levels in tissues undergoing degeneration such as the brain, heart, and pancreas (35,47,48). From studying the yeast frataxin homologue (YFH1), it appears that this protein is involved in the normal efflux of iron from mitochondria (49). Iron accumulates in the mitochondria, which in turn might disrupt mitochondrial respiratory chain function and cause susceptibility to oxidative stress (50). Frataxin has also been demonstrated to function as an iron chaperone protein for aconitase, an enzyme essential in the citric acid cycle (51). In humans, iron deposits have been found in the myocardium of FA patients (52). This raises the intriguing possibility that we may be able to employ chelating agents and antioxidants to reverse pathology (53).

Mouse models of FRDA are providing clues to pathogenesis (reviewed in (54)). Frataxin knockout mice are embryonic lethal, while heterozygosity for the frataxin null allele shows no pathological phenotype. These results suggest that frataxin expression associated with expansion mutation is critical for survival and haplo-insufficient levels of frataxin can completely prevent deleterious consequence (55). Conditional animal models have been generated to reproduce certain morphological and biochemical features observed in patients with FRDA, including cardiac hypertrophy without skeletal muscle involvement, large sensory neuron dysfunction without alteration of small sensory and motor neurons, and deficient activities of complexes I through

III of the respiratory chain and of the aconitases, an enzyme essential in the citric acid cycle (reviewed in (54)). Another useful mouse model is a humanized GAA repeat expansion mouse model that was generated by combining the constitutive knockout model with the transgenic expression of a yeast artificial chromosome (YAC) carrying the human locus. These repeat expansion mice show oxidative stress and progressive neuronal and cardiac pathology (56).

A phenocopy disease of Friedreich's ataxia without mutations in the *FXN* gene has been described in a few familial cases (57,58). In one family, this disease has been linked to chromosome 9p23-p11 and has been called FRDA 2 (MIM 601992).

Current therapy aims at reducing oxidative stress that can be followed by markers of oxidative injury (59,60). These medications include antioxidants such as coenzyme Q10 and vitamin E. The the best predictor of a positive clinical response to CoQ(10)/vitamin E therapy appears to be pretreatment decreased serum CoQ10 levels often seen in FRDA. Studies to date suggest that both low- and high-dose CoQ10/vitamin E therapies were equally effective in improving ICARS scores (61).

Another promising agent appears to be the free radical scavenger, idebenone, a short-chain analog of coenzyme Q10 (Reviewed in Reference (62)). In initial studies, idebenone at 5mg/kg and 10mg/kg/day demonstrated improved cardiac function with no significant benefit on the progression of ataxia (62). It appears that higher doses of idebenone (up to 60 mg/kg/day) are well tolerated and provide some neurological benefit with an improvement in parameters that contribute to activity of daily life (ADL) (63).

Recent experiments suggests that expansion of intronic GAA repeats causes *FXN* silencing by causing chromatin changes in particular histone hypoacetylation that correlates with transcriptional repression (64). Treatment with a histone deacetylase (HDAC) inhibitors in mouse models appears to restore frataxin levels in the nervous system and heart (65). Whether these agents will be successful in human patients is clearly the next step that will depend on exploring this possibility in well-designed clinical trials.

Iron chelation has typically not been used because despite some evidence for iron accumulation in mitochondria in some brain regions, there is normal or even mildly reduced levels of plasma iron. There are some promising results with using the chelator deferiprone (20 to 30mg/kg per day in two divided doses) in combination with idebenone. Deferiprone unlike idebenone has the potential of significant side effects (has been reported to cause fatal agranulocytosis), and its use is confined to clinical trials (53).

Unfortunately, none of these treatments have a major impact on disease progression. Yet much can be done for each individual patient, and when treatment is instituted by a team of medical personnel, neurologists,

occupational and physical therapists are in the best position to address issues relating to the symptomatic treatment for spasticity, surgical correction of scoliosis, exercises to help with loss of balance, and poor motor control (along with recommending devices to assist in activities of daily living); cardiologists and endocrinologists can help with the management of cardiomyopathy and diabetes; finally, like any of the other chronic neurodegenerative syndromes, most patients require genetic and psychological counseling.

## 118.5 PROGRESSIVE ATAXIAS ASSOCIATED WITH DEFECTIVE DNA REPAIR MECHANISMS

Although caused by known enzymatic defects, these autosomal recessive disorders are caused not by derangements in intermediary metabolism but by a specific inability to repair damaged DNA. As might be expected from such a threat to the genome, all of these closely related conditions—ataxia telangiectasia, xeroderma pigmentosum, and Cockayne syndrome—are characterized by wide-ranging deficits.

*Ataxia telangiectasia* (AT; Louis Bar Syndrome-MIM 208900) is the most common of the three. The disease manifests in early childhood as growth retardation, hypotonia, and diminished reflexes. As the disease progresses, children develop ataxia along with oculomotor apraxia, choreoathetosis, nystagmus, and peripheral neuropathy (66). The hallmark telangiectases are most often seen in the conjunctiva of the eyes and in exposed areas of the skin (typically the nose, ears, and neck), and usually develop by the age of seven. Sometimes, other skin lesions such as vitiligo or hypopigmented spots can also be seen. In the absence of telangiectases, either early in the course of the disease or in atypical cases, AT can be difficult to distinguish clinically from other chronic ataxic syndromes (67). If ataxia develops early, it may be misdiagnosed as an ataxic variety of cerebral palsy, particularly since mental retardation is not a feature; when the onset is delayed, it is most often mistaken for FRDA. Dystonia and choreoathetosis occur in the majority of patients especially as they grow older. Patients often have an element of facial weakness that leads to poor facial expression and drooling, while the peripheral neuropathy leads to decreased vibration and position sense and poor stretch reflexes (68). Another characteristic feature is oculomotor apraxia that is best elicited by asking the patient to initiate saccades. Patients have problems with this task, and to compensate, they tend to thrust their head or blink excessively while initiating saccades (69).

Apart from neurodegeneration, growth retardation and gonadal dysfunction, AT is also characterized by a predisposition to cancer and immune function defects. The malignancies typically affect the lymphoreticular system (lymphomas, leukemias, and Hodgkin disease), but there is also increased risk of cancers of the breast,

skin, and stomach. Infections resulting from deficiency in both cellular and humoral immunity typically affect the airway and are often life-threatening. Other features of AT that contribute to morbidity include glucose intolerance because of peripheral insulin resistance and the associated complications of diabetes.

Of diagnostic importance are the low levels of IgA and IgE, a testimony to the defects in humoral immunity. Other humoral deficits that have been observed include elevation of immunoglobulins M, G1, and G3 and deficiency of immunoglobulins G2 and G4 concentrations.

In addition, deficient cellular immunity is reflected in anergy to intradermal injections of test antigens. Another helpful diagnostic feature seen in more than 90% of patients is the elevation of serum alpha-fetoprotein levels and carcinoembryonic antigen (CEA). Identification of the *AT* gene has now led to genetic testing, but diagnostic features clearly aid in pointing physicians to the right diagnosis. Moreover, since the cloning of the *AT* gene, it is becoming apparent that abnormalities in even one copy of the gene predispose heterozygotes to an increased incidence of cancer (particularly of the breast). Thus, although ataxia is a recessive trait, heterozygotes are clearly not normal, at least from the standpoint of predisposition to malignancies.

Both the central and peripheral nervous systems are involved in AT. The central nervous system pathology is more severe and is characterized by cerebellar atrophy, in particular, loss of Purkinje cells and granule cells with some loss of basket cells. In addition, patients sometimes show degeneration of the dentate and olivary nuclei, the substantia nigra and neurons of the oculomotor complex, pretectal nuclei, and hypothalamus. Pituitary cells often have enlarged or dysplastic nuclei. The spinal cord also demonstrates abnormalities, typically denervation of the posterior columns of the spinal cord, degeneration of the anterior horn cells, and decreased numbers of satellite cells, whereas the peripheral nervous system involvement is characterized by malformed nuclei in Schwann cells (70). In line with deficits in the immune system, the thymus is characteristically hypoplastic, with fewer lymphocytes and absent Hassall corpuscles.

AT is a difficult disease to treat and has an especially poor prognosis because of its multisystem involvement. There is no specific treatment for the ataxic syndrome or the progressive cerebellar neurodegeneration. For the ataxic symptoms, therapeutic agents like amantadine, fluoxetine, and buspirone can be tried. The tremors may respond to gabapentin, clonazepam, or propranolol. L-DOPA and anticholinergics may be tried to improve any associated extrapyramidal dysfunction (71). Infections should be aggressively treated with antibiotics and simple maneuvers such as postural drainage of lung abscesses. Diagnostic tests involving X-rays and ionizing radiation should be kept to a minimum, to avoid causing iatrogenic somatic mutations and malignancies. Treatment of malignancies, when they almost invariably occur,

is complicated by the fact that these patients cannot tolerate conventional doses of radiation therapy. Despite the best supportive measures, most patients die in their third decade. More recently, less severe variant forms of AT have been described, where neurological features of AT may occur with minimal immunodeficiency, telangiectasias, cancer, and sinopulmonary infections (72,73). These individuals have decreased chromosomal instability and cellular radiosensitivity that translates into a later onset and slower progression. In these variants, some ATM activity is preserved, which explains the less robust phenotype.

The *AT* gene was mapped to chromosome 11q22.3 in 1988, and the gene was finally cloned in 1995 (74,75). The gene product, ATM or ataxia telangiectasia mutant, is a 370-kDa protein (76) ubiquitously expressed in all tissues of the body. This protein belongs to a family of important protein kinases called inositol-3 kinases. The ATM kinase appears to play a key role in stalling cells at cell cycle checkpoints in response to DNA damage. This supervisory function of ATM offers the cell an opportunity to repair the damage rather than bequeath inappropriate genetic information to daughter cells. In the absence of this checkpoint, cells build up somatic mutations and thus increase the risk of cancer. The major mechanistic steps in ATM pathogenesis are only now being unraveled. It appears that in response to double-stranded DNA damage, the ATM kinase directly interacts with and phosphorylates the tumor suppressor protein p53 (77). Phosphorylated p53 serves as a transcriptional activator of genes that cause cell cycle arrest or apoptosis. In the absence of ATM, p53 is no longer phosphorylated and therefore cannot prevent the cell from moving into the next phase of the cell cycle. More recently, ATM has been shown to phosphorylate another tumor suppressor, Bcr1, one of the genes implicated in breast cancer (78). This interaction may explain how ATM predisposes patients and heterozygotes to breast cancer and why the body is so sensitive to ionizing radiation and radiomimetic chemicals. It also explains why the immune system is affected since breakage of double-stranded DNA is the mechanism by which diversity of antigen receptors is produced by lymphocytes. None of these molecular explanations explain why the nervous system, specifically the cerebellum, is affected in this syndrome. It is possible that ATM is crucial for surveying the genome for DNA damage in neurons that suffer from significant oxidative stress (79). Recent engineered mouse models of the disease promise to increase our understanding of the molecular mechanisms of pathogenesis. Disruption of the mouse version of the gene recreates the neurological phenotype, growth retardation, and defects in the immune system, apart from the sensitivity to radiation (80). This is an exciting area of scientific research at the confluence of tumor biology, cell cycle research, and neurodegeneration (81,82). It is also possible that deficiency of ATM contributes to neurodegeneration in pathways



distinct from its role in DNA damage. For instance, there is evidence to suggest that cytoplasmic ATM is involved in vesicular/protein transport (83).

The most immediate benefit of cloning the gene has led to diagnostic testing and describing unusual clinical variants as alluded to earlier. The *ATM* gene is itself very large, with 66 exons spanning 150kb of genomic DNA (84). Using a combination of techniques, it is estimated that more than 95% of affected individuals can be identified by genetic testing (85). Developing diagnostic test has been a challenge, given the variety of mutations found: it is estimated that truncating mutations account for about 85% of mutations, while point mutations are responsible for the rest (32). Furthermore, the majority of patients are compound heterozygotes.

A disease very similar to AT, known as the Aicardi variant, was in the past separated from AT by the lack of telangiectasia and severe limitation in vertical eye movements. This disease is now known to share the same genetics as AT. There are a few disorders that closely mirror AT but result from defects in genes other than *ATM*. The disease that resembles AT the closest is called ataxia telangiectasia-like-disorder (ATLD-MIM 604391). ATLD is caused by a mutation in a gene (*hMRE11*) encoding a protein involved in the DNA repair complex of which AT is another component (86). Patients with ATLD have a slowly progressive ataxia and ocular apraxia, but do not develop tumors. Furthermore, they did not have telangiectasia, raised alpha-fetoprotein, or reduced immunoglobulin levels (87).

Xeroderma Pigmentosa and Cockayne syndrome are two other syndromes that have deficiencies in DNA repair pathways and can be associated with ataxia.

*Xeroderma pigmentosum* (XP) has an incidence of approximately one in a million. This disease is characterized by a wide variety of cutaneous lesions from mild freckling to xerosis, erythema, bullae, telangiectasia, actinic keratosis, and skin malignancies. Most of these lesions occur around the eyes and eyelids. The eyes also show an increased incidence of keratitis, opacification of the cornea, iritis with synechia formation, and malignant melanoma of the choroid. The neurological features of xeroderma pigmentosa consist of progressive cognitive decline, sensorineural deafness, peripheral neuropathy and choreoathetosis. As with AT, XP is characterized by an increased risk of neoplasms (88). Oral administration of retinoic acid has been shown to be helpful in preventing skin cancer (89).

At a molecular level, XP constitutes a genetically heterogeneous group of disorders characterized by defects resulting in excision repair abnormalities (90). There are several complementation groups based on functional studies using fibroblasts derived from human patients. Patients in the same complementation group have a similar constellation of symptoms with variable neurological involvement that can range from severe to none at all. In addition, ultraviolet-sensitive Chinese hamster cells have

been instrumental in identifying genes involved in excision repair. Unfortunately, because some of these genes were named by both of these methods, there is some redundancy in the nomenclature.

*Cockayne syndrome* (CS) is a rare disorder (91). Although the clinical phenotype shows some overlap with XP with deafness and ataxia, patients with CS also show retinal degeneration and early ageing without displaying a tendency to develop cancer. Pathologically, there is evidence for demyelination in the central nervous system, along with basal ganglia calcification, both of which are detected in imaging studies. Like XP, CS is genetically heterogeneous, with several complementation groups. In fact, there are a few patients with a mixed XP-CS phenotype. The key defect in most cases appears to be insufficiency of transcription, or transcription-translation coupled repair, a phenomenon that pertains to excision repair of DNA that must precede the transcription of active genes (92). Mouse models of XP and CS have been developed; importantly, when crossed, they have a synthetic phenotype, suggesting yet again that these proteins are in the same genetic pathway (93).

Another set of diseases that mimic AT are the ataxia-ocular apraxia syndromes of which there are at least two: AOA1 and AOA2. AOA2 has also been called spinocerebellar ataxia of the recessive type 1. Other rarer ataxias that are being identified are being termed SCAR with numbers given in the order of locus identification (reminiscent of the autosomal dominant spinocerebellar ataxias described later in the chapter).

Ataxia-ocular apraxia type 1 (AO1; Early-Onset Ataxia with Ocular Motor Apraxia and Hypoalbuminemia-MIM 208920) (mapping to 9p 13.3 (94,95) is an autosomal recessive ataxia caused by mutation in the aprataxin (*APTX*) gene causing. Initially described in Japanese families, AOA1 presents with gait imbalance and dysarthria. These patients then progress over a few years to develop pronounced ataxia and ocular apraxia. Areflexia, dystonia, choreoathetosis, sensory and motor neuropathy, mental retardation, and retinal/macular lesions (picked up by fundoscopic examination) can all accompany the core clinical picture of ataxia and ocular apraxia. In addition, patients often develop hypoalbuminemia and hypercholesterolemia. AO1 is caused by a mutation in a histidine triad/zinc finger protein, called aprataxin, a protein involved in DNA single-strand break repair (96). Several mutations have been described including frameshift, nonsense mutations, and missense mutations with many patients being compound heterozygotes (96). Coenzyme CoQ10 has been found to be decreased in muscles of some patients with AO1, with some improvement with oral supplementation (97,98).

Ataxia-Ocular apraxia type 2 (AO2; mapping to locus 9q34) is another recessive ataxia characterized by unsteady gait, cerebellar atrophy, ocular apraxia, and a sensory-motor neuropathy. AO2 has more recently been called Spinocerebellar Ataxia, autosomal Recessive Type 1

(SCAR1-MIM 606002). But there are a few distinguishing features: this disease typically has a later age of onset, a less common ocular apraxia (only 50% of AO2 patients display ocular apraxia) and high levels of serum alpha-fetoprotein, creatine kinase, and gamma globulin with normal serum albumin and cholesterol (99). Patients may also show evidence of executive dysfunction on neuropsychological testing (100). At a molecular level, AOA2 is caused by mutations in the *SETX* gene, which maps to chromosome 9q34 (101). This gene encodes the protein, senataxin, which is a member of the helicase family of enzymes involved in RNA maturation and termination (102), although more recently, there is evidence to suggest that senataxin modulates transcription (103). Surprisingly, some mutations in the same gene can cause a juvenile form of ALS that is inherited in an autosomal dominant manner (104,105).

## 118.6 OTHER RECESSIVE PROGRESSIVE ATAXIAS

As more genetic recessive ataxias are being linked to specific loci, they are being classified as SCARs (Spinocerebellar Ataxia, Autosomal recessive). As mentioned above, Ataxia-Ocular apraxia type 2 (AO2) is also called SCAR1. In this section, we will briefly describe the other SCARs/recessive ataxias.

Spinocerebellar Ataxia, Autosomal Recessive 2 (SCAR2—MIM 213200); Cerebellar hypoplasia, nonprogressive Norman type. SCAR2 was described by (106) as an early-onset cerebellar ataxia with mental deficiency that can be associated with microcephaly, cataracts, and exaggerated tendon reflexes (106). It is not certain if SCAR2 is distinct from the disorder called cerebellar hypoplasia (MIM 213000). This disease is associated with cerebellar granule cell loss. The disease locus has been assigned to chromosome 9q34-qter (107).

Spinocerebellar Ataxia, autosomal Recessive 3 (SCAR3—MIM 271250); Spinocerebellar Ataxia with Blindness and Deafness (SCABD). This spinocerebellar ataxia was first described as an early-onset ataxia in an Israeli family. It is associated with retinal and cochlear degeneration causing blindness and deafness (108,109) and has been linked to the chromosome 6p23-p21 (110).

Spinocerebellar Ataxia, Autosomal Recessive 4 (SCAR4—MIM 607317); Spinocerebellar Ataxia with Saccadic Intrusions (SCASI). SCAR4 has been described in a family of Slovenian descent. It is a progressive ataxia with gait, trunk, and limb ataxia, associated with pyramidal signs. Most patients require walking aids by the sixth decade. This ataxia is associated with eye movement abnormalities, myoclonic jerks, fasciculations, cerebellar dysarthria, and pes cavus. There are large- and small-fiber sensory losses, and nerve condition studies show axonal sensorineural neuropathy. Neuroimaging shows cerebellar atrophy that primarily involves the dorsal vermis (111).

Spinocerebellar Ataxia, Autosomal Recessive 5 (SCAR 5—MIM 606937); Nonprogressive Congenital Cerebellar Ataxia with Mental Retardation, Optic Atrophy, and Skin Abnormalities (CAMOS). SCAR5 has been described in a large inbred Lebanese Druze family as a nonprogressive autosomal recessive ataxia that starts in early childhood and is associated with optic atrophy, mental retardation, short stature, speech defect, and cerebellar atrophy (112). This disease is associated with abnormal osmiophilic pattern of skin vessels that has been speculated to prevent normal exchange between the blood and surrounding tissues. This might also help underlie abnormalities in developmental processes involved in neurigenesis and neuronal migration. The disease locus has been mapped to a 3.6-cM interval on chromosome 15q24-q26 (113).

Spinocerebellar Ataxia, Autosomal Recessive 6 (SCAR6—MIM 608029); Norwegian Nonprogressive Infantile Cerebellar Ataxia (CLA3). SCAR6 was first described in a large inbred Norwegian family, ranged in age from 4 years to 59 years. All patients showed a nonprogressive cerebellar ataxia manifesting in early childhood associated with short stature and flat feet. The phenotype of this disorder includes defects in motor coordination, slow speech, poor eye movement coordination, slight spasticity, and normal intelligence (114). The disease has been chromosomally mapped to a 19.5-cM interval on chromosome 20q11-q13 (115).

Spinocerebellar Ataxia, Autosomal Recessive 7 (SCAR7—MIM 609270). SCAR7 has been described in a nonconsanguineous Dutch family. SCAR7 presents with slowly progressive spinocerebellar ataxia, dysarthria, nystagmus, saccadic pursuit eye movements, a postural tremor, hyperreflexia, extensor plantar responses, and decreased vibration sense (116). The disease severity is variable ranging from mild ataxia to wheelchair bound. The gene for SCAR7 has been tentatively mapped to a 5.9-cM interval on chromosome 11p15 (116).

Spinocerebellar Ataxia, Autosomal Recessive 8 (SCAR8—MIM 610743); Cerebellar ataxia, autosomal recessive, type 1 (ARCA1); Recessive ataxia of Beauce. SCAR8 was identified in 26 French Canadian families mostly from the Beauce and Bas-St-Laurent regions of the province of Quebec in Canada. This autosomal recessive disease starts at 17 to 46 years of age with a relatively pure cerebellar ataxia although patients often display mild oculomotor abnormalities, occasional exaggerated tendon reflexes in the lower extremities. Neuroimaging shows diffuse cerebellar atrophy (117).

SCAR8 has been mapped to chromosome 11p15 with various mutations in the gene *SYNE1* (118). *SYNE1* encodes a very large protein with 8797 amino acid residues. *SYNE1* is a part of the spectrin family, a group of structural proteins that share a common function of linking the plasma membrane to the actin cytoskeleton, a protein that is abundantly expressed in Purkinje cells and neuromuscular junctions (118). A splice-site

mutation was the most common mutation, occurring at a frequency of 50.8% among 124 patients (117). SCAR8 shows slow progression and causes moderate disability.

Spinocerebellar Ataxia, Autosomal Recessive 9 (SCAR9—MIM 612016); Cerebellar ataxia, autosomal recessive, type 2 (ARCA2). SCAR9 has been described in a consanguineous Algerian family and in three other sporadic patients with slowly progressive childhood-onset gait ataxia and cerebellar atrophy. SCAR9 is often associated with pes cavus, exaggerated tendon reflexes, mild psychomotor retardation, and mild axonal degeneration of the sural nerve. An unusual feature in some patients is exercise intolerance that is associated with elevated serum lactate. SCAR9 is caused by a homozygous splice-site mutation in the *aarF*-domain-containing kinase 3 gene (*ADCK3*) (chromosome 1q41). *ADCK3* is a mitochondrial protein homologous to the yeast CoQ8 and the bacterial UbiB proteins required for Muscle coenzyme Q10 (CoQ10 or ubiquinone) biosynthesis (119). Most of the SCAR9 patients showed decreased level of CoQ10 (119). Intriguingly, CoQ10 deficiency had been reported low in some patients with childhood-onset cerebellar ataxia (120). It is likely that CoQ10 supplementation will be beneficial for SCAR9 patients, as has been suggested for other mitochondrial respiratory chain dysfunction-related ataxias.

Cerebellar Ataxia, Cayman Type (*ATCAY*—MIM 601238). *ATCAY* is a recessive nonprogressive ataxia described in an isolated population from the Grand Cayman Islands. Ataxia is associated with hypotonia from birth, psychomotor retardation, nystagmus, intention tremor, and dysarthria. Neuroimaging shows isolated cerebellar atrophy. The *ATCAY* gene has been identified and maps to chromosome 19q13.3 (121,122,123). The *ATCAY* gene is expressed only in neurons in the brain and in components of the spinal cord and peripheral nervous system. Two mutations identified include nucleotide substitutions, one in exon 9 resulting in a serine to arginine substitution, and one in intron 9 that interferes with splicing (124). *ATCAY* encodes the caytaxin protein, a protein that shares a conserved domain with *TTPA* (see Vitamin E deficiency syndromes, above). However, it does not appear that caytaxin binds vitamin E, and its role is still unclear.

Infantile-Onset Spinocerebellar Ataxia (*IOSCA*—MIM 271245). *IOSCA* was first described in Finland, with a population carrier frequency of more than 1:230. It maps to 10q24 (125). As its name suggests, dysfunction starts in infancy (9–18 months of age) with muscle hypotonia, athetosis, ataxia, ophthalmoplegia, sensorineural hearing deficit, sensory axonal neuropathy, epileptic encephalopathy, and female hypogonadism (126,127). Other features include sensory axonal neuropathy and progressive atrophy of the cerebellum, brain stem, and the spinal cord (127). As children, most patients develop migraine-like headaches and seizures progressive to a severe epileptic encephalopathy (128). Some patients

have psychiatric problems including disorders of mood or even psychosis. Brain imaging and postmortem pathology show widespread brain edema and necrosis.

*IOSCA* results from a recessive mutation in *C10orf2* encoding Twinkle (Y508C), a mitochondrial deoxyribonucleic acid (mtDNA)-specific helicase (125). Different mutations in this same gene cause autosomal-dominant progressive external ophthalmoplegia (adPEO) with multiple mtDNA deletions (MIM 606075), a neuromuscular disorder sharing a spectrum of symptoms with *IOSCA* (125). The depletion of mtDNA has been shown in *IOSCA* patients' brains and places *IOSCA* in the family of mtDNA depletion syndromes (129).

One such disorder is *Autosomal Recessive Spastic Ataxia of Charlevoix-Saguenay* named after the region in Quebec, where it was first described. The gene, mapping to chromosome 13q11, has recently been cloned (130). It encodes a large protein with a predicted molecular weight of 437kDa that shares some homology to molecular chaperones, proteins speculated to play a role in protein misfolding. This is intriguing, since protein misfolding appears to be central to the polyglutamine induced autosomal dominant ataxias, as will be discussed later in this chapter.

*Wolfram syndrome* is a rare ataxia characterized by diabetes insipidus, diabetes mellitus, optic atrophy, and deafness (*DIDMOAD*) (4p16), can also present with ataxia. It is now known to be caused by a mutation in a transmembrane protein expressed predominantly in the pancreas and the brain (131).

Among the cerebellar ataxias yet to be genetically characterized are *Marinesco-Sjögren syndrome*, wherein cerebellar ataxia coexists with congenital cataracts, impaired physical growth, mental deficiency, myopathy and skeletal abnormalities, and *Mathews-Rundle syndrome* in which ataxia is combined with hypogonadism.

## 118.7 THE AUTOSOMAL-DOMINANT HEREDITARY ATAXIAS

The autosomal-dominant cerebellar ataxias have an interesting history. They were first recognized by Marie in 1893 as a syndrome distinct from *FRDA*, based on their clinical features (132). Gordon Holmes criticized Marie for lumping together clinically and pathologically heterogeneous group of ataxias, with only some being clearly documented as familial (133). Nonetheless, we now know that there are indeed numerous adult-onset autosomal dominant ataxias. Several classifications have been proposed to sort individual members of the group by clinical spectrum. The most recent was proposed by Anita Harding in 1984. She divided these ataxias into three distinct groups called autosomal-dominant cerebellar ataxia types I, II and III: where type I syndromes are associated with ophthalmoplegia, optic atrophy, dementia, and extrapyramidal features; type II with pigmented maculopathy (with or without ophthalmoplegia or



extapyramidal features); while type III syndromes are pure ataxic syndromes.

Over the last several years, however, a new classification based on the genetic loci of the spinocerebellar ataxias (SCA) has gained wide acceptance. These loci have been numbered, based on their order of identification: SCA1 through 31 (and the number is bound to grow with advancements in positional cloning). In previous editions, we have tried to correlate the older Harding classification with this genetic nomenclature; however, because of the broad overlap of clinical features and phenotypic variations within each disorder, such a correspondence is difficult to achieve. Therefore, we now favor a purely genetic classification based on the genetic loci of the SCAs. Several of the SCAs are nucleotide repeat disorders and, therefore, present with the common findings of anticipation and increased severity correlating to the size of the nucleotide expansions within the relevant gene (134); Table 118-3.

### 118.7.1 Spinocerebellar Ataxia Type 1 (SCA1—MIM 164400)

Spinocerebellar ataxia type 1 was the first dominantly inherited ataxia gene to be linked to a locus (6p22-23). It is characterized by progressive cerebellar ataxia, dysarthria, oculomotor deficits, and relentless bulbar dysfunction including dysphagia and respiratory failure that results in death. Other accompaniments include hyperreflexia, increased tone, and extensor plantar responses. Occasionally the peripheral nervous system involvement can obscure these upper motor neuron findings and can result in wasting of the extremities and generalized fasciculations. The disease most often presents in the third decade of life, but there is tremendous variability in onset and clinical severity (as early as 4 years of age and as late as 60) (135). In fact, it was this clinical feature that led to a targeted search for trinucleotide repeat expansions, known to be the defect in causing anticipation in other neurodegenerative conditions. Pathologically, the primary sites of neurodegeneration in SCA1 include cerebellar Purkinje cells, brain stem cranial nerve nuclei, and the inferior olive and spinocerebellar tracts (135).

SCA1 is caused by a CAG repeat expansion in the coding region of a novel gene *SCA1* that was mapped to chromosome 6p22-23 and the mutation identified using a positional cloning approach (136,137). Even in the general “normal” population, the number of CAG repeats is polymorphic and ranges from 6 to 44. In patients, the CAG repeats range from 39 to 82 on SCA1 chromosomes (138,139). The distinction between disease-causing and “normal” alleles in the range of overlap is made on the basis of the fact that the repeat tract on normal chromosomes is interrupted by 1 to 4 CAT trinucleotides in alleles with 20 or more repeats, whereas SCA1 chromosomes contain a “pure,” uninterrupted CAG tract (140).

There is an inverse correlation between repeat size and age of onset. Patients with 70 or more CAG repeats have childhood-onset, a rapidly deteriorating course, and death within 4–5 years, whereas adult-onset patients have fewer than 70 repeats and typically live 10–20 years after symptoms appear (135). A prominent feature of SCA1, like other polyglutamine disorders, is *genetic anticipation* or the tendency of affected individuals in each successive generation of a kindred to show earlier and earlier disease onset (often with worsening severity). The mechanism involves progressive expansion of the polyglutamine repeats in disease alleles, in particular those that are transmitted through the father (137).

In SCA1, the protein carrying the CAG repeats is ataxin, a protein encoding 792–830 amino acids, depending on the size of the repeat (136). Ataxin-1 appears to be a nucleoplasmic shuttling protein that is predominantly nuclear in neurons and cytoplasmic in peripheral cells (138,139). A 120-residue region of the protein shares significant sequence similarity with the HMG-box transcription factor HBP1. This domain is thought to play a role in protein–protein interactions and possibly in protein–RNA interactions (104,105).

The cellular functions of ataxin-1 are still unclear. Loss of ataxin-1 function alone, however, cannot explain SCA1 pathogenesis, given that mice lacking ataxin-1 do not display ataxia or neuronal degeneration—although they do have spatial and motor learning deficiencies as well as impairment of short-term plasticity (141). On the other hand, there is considerable support for a gain-of-function pathogenic mechanism. The first evidence came from mice overexpressing a mutant *SCA1* cDNA with 82 glutamines (82Q) specifically in Purkinje cells. These mice developed progressive ataxia and Purkinje cell degeneration (142). The mutant ataxin-1 accumulates in nuclear inclusions (NIs) along with components of the proteasome, suggesting that mutant ataxin-1 might misfold and resist degradation (143). Similarly, fruit flies that overexpress human ataxin-1 (82Q) also developed progressive neuronal degeneration and ataxin-1-containing NIs that colocalized with chaperones and ubiquitin. Moreover, NIs have also been noted in other glutamine repeat diseases as well. These inclusions not only contain ataxin-1 but they also stain positive for chaperones and components of the ubiquitin-proteasomal pathway, both instrumental in protein clearance. Recent work suggests a scenario where ataxin-1 tends to accumulate in SCA1, probably because of an altered conformation induced by the polyglutamine tract. Ubiquitination and proteasomal degradation probably represents an appropriate response since interventions to prevent these processes in SCA1 and other polyglutamine diseases only serve to increase neuronal toxicity (144–147). Based on these findings, strategies aimed at improving protein folding or clearance are gaining center stage (148).

To characterize the effects of the SCA1 mutation on all ataxin-1-expressing neurons, an expanded CAG tract of



**TABLE 118-3 Genetics of Chronic Progressive Hereditary Ataxias**

Disease	Gene	Locus	Protein	Genetic Basis	Repeat Size	
					Normal	Disease
Friedreich ataxia	X25	9q13.1-21.1	Frataxin	GAA repeat expansion in intron 1 of the gene	7–34	>100 <sup>a</sup>
Spinocerebellar ataxia type 1	SCA1	6p23	Ataxin-1	CAG repeat in coding region of gene	6–44 <sup>b</sup>	36–121
Spinocerebellar ataxia type 2	SCA2	12q24.1	Ataxin-2	CAG repeat in coding region of gene	15–31	36–63
Spinocerebellar ataxia type 3 (Machado–Joseph disease)	SCA3 (MJD1)	14q32.1	Ataxin-3	CAG repeats in coding region of gene	12–40	55–84
Spinocerebellar ataxia type 4	SCA4	16q22.1	–	–	–	–
Spinocerebellar ataxia type 5	SCA5	11p11-11q11	–	–	–	–
Spinocerebellar ataxia type 6	SCA6	19p13	Alpha-1A voltage-dependent calcium channel subunit	CAG repeat in coding region of gene	4–18	21–33
Spinocerebellar ataxia type 7	SCA7	3p12-13	Ataxin-7	CAG repeat in coding region of gene	4–35	37–306
Spinocerebellar ataxia type 8	SCA8	13q21	None	CTG repeat in the 3' terminal exon (antisense)	16–37	110–<250?
Spinocerebellar ataxia type 9	No category assigned			–	–	–
Spinocerebellar ataxia type 10	SCA10	22q13ter	–Ataxin-10	–Caused by pentanucleotide (ATTCT) repeat expansion in intron 9 of the ATXN10 gene	–10–29	400–4500
Spinocerebellar ataxia type 11	SCA11	15q14-q21	–Ataxin-11	–Caused by mutations in TTBK2 gene that encodes tau tubulin kinase-2	–	–
Spinocerebellar ataxia type 12	SCA12	5q31-33	Protein phosphatase 2A	CAG repeat in 5' UTR	7–28	66–78
Spinocerebellar ataxia type 13		19q13	KCN3	Two missense mutations identified		
Spinocerebellar ataxia type 14		19q13 Protein kinase C gamma (PRKCG)	Protein Kinase C-gamma	Missense mutation in exon 4		
Spinocerebellar ataxia type 15		3p24.2-3pter	Type 1 inositol 1,4,5-triphosphate receptor (ITPR1)	Haploinsufficiency or missense		
Spinocerebellar ataxia type 16		Same disorder as SCA15				

**TABLE 118-3 Genetics of Chronic Progressive Hereditary Ataxias—cont'd**

Disease	Gene	Locus	Protein	Genetic Basis	Repeat Size	
					Normal	Disease
Spinocerebellar ataxia type 17		6q27	Tata-Binding Protein (TBP)	CAG repeat	25–44	47–63
Spinocerebellar ataxia type 18		7q22-q32	Interferon-related developmental regulator gene 1 (IFRD1)			
Spinocerebellar ataxia type 19		1p21-q21				
Spinocerebellar ataxia type 20		Chromosome 11				
Spinocerebellar ataxia type 21		7p21.3-p15.1				
Spinocerebellar ataxia type 22		Likely identical to SCA19				
Spinocerebellar ataxia type 23		20p13-p12.3				
Spinocerebellar ataxia type 24 (redesignated SCAR4)						
Spinocerebellar ataxia type 25		Chromosome 2				
Spinocerebellar ataxia type 26		19p13.3				
Spinocerebellar ataxia type 28	AFG3L2	18p11.22q11.2	Multiple missense mutations described			
Spinocerebellar ataxia type 29		3p26				
Spinocerebellar ataxia type 30		4q34.3-q35.1				
Spinocerebellar Ataxia type 31		16q22.1	Repeats in introns of the TK2 gene and the BEAN gene (on opposite strands and transcribed in opposite directions)	Insertion containing pentanucleotide repeats including a long (TGGA)n sequence		
DRPLA	DRPLA	12q	Atrophin-1	CAG repeat in coding region of gene	6–35	49–88

<sup>a</sup>34–100 can be premutations (see text).

<sup>b</sup>Alleles with 21 or more repeats are interrupted by 1–3 (CAT) units; disease alleles contain pure CAG tracts.

154 repeats was targeted in place of the typical 2 CAGs present in the endogenous mouse locus. *Sca1*<sup>154Q/+</sup> mice reproduced several key features of human SCA1 including ataxia, brain stem dysfunction, Purkinje cell degeneration, and premature death (149).

Several insights into SCA1 pathogenesis have come from studying the cellular properties of ataxin-1. For instance, phosphorylation of ataxin-1 at serine 776 (S776) appears to be important for ataxin-1 accumulation.

Moreover, mice that express ataxin-1 (82Q) that cannot be phosphorylated at this site (with an alanine at position 776) do not develop ataxia or pathology in spite of the long glutamine tract. These data show the following: (1) toxicity is mediated by the protein and not RNA; (2) the protein context is very important given that a single amino acid change abrogated accumulation and toxicity; and (3) a long polyglutamine tract can be efficiently handled by the cell's degradation machinery (150).

Furthermore, there is evidence to suggest that toxicity of ataxin-1 depends on its native but not novel, protein interactions (151). Lim et al. proposed a two-pronged model of SCA1 neurodegeneration in which increased function of a particular stable endogenous protein complex is combined with a simultaneous loss of function of other, stable endogenous protein complexes (152). Although the precise functions of ATXN1 remain elusive, several findings implicate ATXN1 in gene regulation by interacting with chromatin-modulating proteins and transcription factors (153–159).

No treatment is currently available for SCA1. Patients experience progressive limitations in their activities, lose the ability to walk, and eventually become bedridden. So far, based on studies in SCA1 animal models, it appears that lithium and neural stem cell transplantation in the cerebellum could be viable therapeutic approaches for SCA1 patients (160,161).

SCA2 (SCA2-MIM18309) was described as a form of dominantly inherited spinocerebellar ataxia that occurs at an estimated frequency of 41 per 100,000 in the province of Holguine, Cuba (162). It has since been described in several regions of the world (163). SCA2 is characterized by ataxia, dysarthria, tremor, and extremely slow saccades. Hyporeflexia of the upper limbs and ophthalmoparesis are seen in over half the patients. Dementia occurs in a significant minority of patients. Genetically, it was set apart from SCA1 when its locus (12q23-24) was identified (164–166).

Age of onset varies (2–65 years); when SCA2 alleles contain more than 200 repeats, the disease can present as early as infancy with hypotonia, developmental delay, dysphagia, and retinitis pigmentosa (167). The genetic background may be relevant, since atypical features are seen in geographically distinct families: mental deterioration in an Italian family (167,168), for example, and chorea and dystonia in families from Tunis and Martinique (169,170). At a pathological level, there is severe loss of Purkinje cells and of neurons in the substantia nigra and basis pontis. Mild to moderate neuronal loss is also seen in the inferior and accessory olives; dentate, arcuate, gracile, and accessory cuneate nuclei; internal granule cell layer of the cerebellum; and in the anterior horns of the spinal cord. Axonal loss from the dorsal roots and posterior columns, the dorsal spinocerebellar tract, and often from the anterior spinal roots is also found. Reactive gliosis can be seen in the globus pallidus, thalamus, subthalamus, and periaqueductal regions (171,172).

The CAG trinucleotide repeat lies within the coding region of a novel protein termed ataxin-2 that was identified by positional cloning (173–175). Normal alleles vary between 15 to 24 CAG repeats (with 22 repeats found 94% of the time), while disease alleles have 35 to 59 repeats. The range of pathogenic alleles in SCA2 is shifted toward shorter repeats compared with other SCAs, suggesting that the expanded repeat within

ataxin-2 is more deleterious to neurons. The CAG repeat in the SCA2 gene is interrupted by 1 to 3 CAA units only on normal alleles suggesting that, as in SCA1, interruption of CAG repeats confers stability. Finally, there is a strong inverse correlation between the size of the repeat and the age of symptom onset.

The SCA2 gene contains 25 exons and two alternately spliced forms of mRNAs are generated, one of which results in a protein that is truncated by 70 amino acids (176). Ataxin-2 mRNA is found in multiple tissues and in all regions of the CNS (173–175) where it is present predominantly in neurons and is highly expressed in Purkinje cells (171).

The SCA2 gene encodes a protein called ataxin-2 that contains structural elements that appear to be important in RNA splicing. Ataxin-2 regulates the intracellular concentration of its interaction partner, the poly(A)-binding protein, a stress granule component and a key factor for translational control (177). Ataxin-2 is associated with rough ER (178). Ataxin-2 associates with polyribosomes under normal conditions and is recruited to stress granules upon environmental stress. Mutant ataxin-2 sensitizes Purkinje cells to glutamate-induced apoptosis. Glutamate-induced cell death of 58Q PC cultures was attenuated by dantrolene, a clinically relevant ryanodine receptor (an intracellular calcium channel) inhibitor and Ca<sup>2+</sup> stabilizer. Thus, neuronal Ca<sup>2+</sup> signaling may play an important role in SCA2 pathology, and the ryanodine receptor could be a potential therapeutic target for treatment of SCA2 patients (179). Intriguingly, a recent study implicates this gene as a modifier of amyotrophic lateral sclerosis (180).

SCA3 or Machado-Joseph disease (MJD; MIM 109150) is probably the most common of the autosomal dominant spinocerebellar ataxias and also has a broad clinical spectrum. In fact, the disease initially linked to the spinocerebellar ataxia 3 locus was not recognized as an MJD variant, since the initial SCA3 patients did not exhibit the extrapyramidal syndrome which was then thought to be characteristic of the MJD syndrome. Only when linkage analysis assigned both of these ataxic syndromes to the same genetic locus on the long arm of chromosome 14 (14q24.3-q32) did it become clear that they were one and the same polyglutamine disease (181). SCA3 encodes a protein referred to as ataxin-3, a 42 kDa predominantly cytoplasmic protein. MJD is also a CAG repeat disorder. Normal MJD1 alleles contain from 13 up to 47 CAG repeats, while expanded alleles contain 45–84 repeats (182). Much like SCA1, the mutant protein forms nuclear inclusions (183,184).

Besides ataxia, clinical features in SCA3 include slow saccades and saccadic pursuit. Other features include lid retraction that gives the impression of a persistent stare, and signs of brain stem dysfunction including dysarthria, difficulty in swallowing, poor cough, and tongue fasciculations. Cognitive impairments such as deficits in verbal

and visual memory, poor verbal fluency, and visuospatial impairments and autonomic dysfunction have also been described (185,186). Because of peripheral nervous system involvement, the neurological examination usually shows a mix of upper motor neuron and lower motor neuron findings. Thus tone can range from hypotonia to significant rigidity, and reflexes can be exaggerated or absent. The plantar response tends to be extensor. The common extrapyramidal features include rigidity and dystonia. In the past, MJD had been subclassified as type I when spasticity was the major sign without significant ataxia; type II when ataxia was alone; and type III when ataxia was accompanied by peripheral neuropathy. Beyond formalizing the phenotypic variability that we now know is most likely contributed by genetic background and polyglutamine length, this classification adds little to the diagnosis.

Ataxin-3 is the smallest of the polyglutamine proteins with a ubiquitin interaction motif (UIM) situated near the polyglutamine domain. Several isoforms have been described. Ataxin-3 interacts with Rad23 and valosin-containing protein (VCP) (187). Rad23, a member of proteasomal protein degradation machinery, in turn, interacts with the proteasome subunit S5a, suggesting a role of the protein complex in “protein shuttling” for translocating proteins to the proteasome for degradation. VCP, also called CDC48, is an ATPase associated with various activities and has been implicated in multiple cellular functions ranging from organelle biogenesis to ubiquitin-dependent protein degradation (reviewed in Reference (182)). Mutation in VCP causes inclusion body myopathy with Paget disease and frontotemporal dementia (IBMPFD MIM 167320). Interestingly, Parkin, the E3 ubiquitin ligase, which is frequently mutated in early-onset autosomal recessive Parkinson’s disease, promotes ubiquitination and degradation of ataxin-3 (188).

The pathogenesis of MJD/SCA3 is still poorly understood. The introduction of ataxin-3 with an expanded polyglutamine tract into cultured cells induces apoptosis, suggesting that the mutant protein is either directly or indirectly involved with a cellular suicide pathway (183). Other studies suggest that protein misfolding, presumably initiated by the expanded polyglutamine tract, leads to ubiquitination and subsequent formation of intranuclear inclusions (144,145). This is a common pathway in polyglutamine expansion diseases. How this leads to cell dysfunction and death and why the clinical timecourse takes so long to evolve remain unknown.

SCA4 (MIM 600223) has no unique features that distinguish it from the other ataxias, except perhaps for an exaggerated sensory axonal neuropathy and extensor plantar reflexes (189,190). The gene, which maps to 16q22.1, has yet to be identified.

SCA5 (MIM 600224) and 6 (SCA6—MIM 183086) are both characterized by an almost pure cerebellar

syndrome with global cerebellar atrophy. SCA5, in particular, is a relatively mild syndrome with a slow progression. The disease is caused by mutations in the beta III spectrin gene (SPTBN2) (191) located on the centromeric region of chromosome 11 (192). As an interesting historical aside, SCA5 was described initially in descendants of President Abraham Lincoln’s family.

SCA6 is more common than SCA5. Indeed in some studies it accounts for 15–17% of dominant cerebellar ataxic syndromes (193–195), and see review in Reference (196). The mutation is occasionally found in sporadic cases as well. In SCA6, there is occasional horizontal and vertical nystagmus and abnormal vestibulo-ocular reflex (197). Both diseases begin in the third decade, and MRI examinations on affected patients show cerebellar atrophy. SCA6 is known to be a polyglutamine disorder (198). The CAG tract, however, is small compared with that of other polyglutamine diseases—21 to 33 repeats in the disease state (normal less than 18). The genetic locus was mapped to chromosome 19q13, where a CAG repeat expansion in exon 47 at the 3’ region of the *CACNA1A* gene was identified as the causative mutation (198). *CACNA1A* codes for the brain-specific, voltage-sensitive  $\alpha_{1A}$  ( $Ca_v2.1$ ) subunit of the P/Q-type calcium channel, which is highly expressed in Purkinje cells (199). Alternative splicing leads to six isoforms, three of which contain the polyglutamine sequence. Repeat expansions are the smallest of the triplet repeat diseases, with 21 or more repeats being pathogenic. Individuals who are homozygous for expanded repeats show no phenotypic or age of onset differences (200). Phenotypic severity is inversely correlated with the number of CAG repeats, as is the age of onset (201). Clinical anticipation has been observed, but does not always appear to stem from CAG repeat instability. No CAA interruptions are present in the repeats. Proteins with the expanded repeat aggregate specifically in the cytoplasm of Purkinje cells (199). These cytoplasmic inclusions are not ubiquitinated and lack several other components found in the inclusions of other CAG repeat disorders.

Possible mechanisms of neuronal cell degeneration include increased calcium channel entry and cytoplasmic aggregation of channel protein (199,202). Intriguingly, this gene has been implicated in episodic ataxia type 2 and familial hemiplegic migraine, although the mutational mechanisms are distinct (203). Nonetheless, compromise of the function of the calcium channel might be central to all three, since there is some overlap of symptoms. Like episodic ataxia, SCA6 can present with intermittent ataxia in the early stages, and all three conditions (SCA6, EA2, and familial hemiplegic migraine) often involve cerebellar atrophy. Nakamura et al. in a 4-week trial on 11 SCA6 patients showed that treatment with Gabapentin, which interacts with  $\alpha_{2\delta}$  subunit of the P/Q-type Voltage-dependent calcium channel, might be beneficial for SCA6 patients (204).



Pathology specimens demonstrate loss of Purkinje and granule cells, with proliferation of Bergmann glia, that is more prominent in the vermis than in the hemispheres (197,201). Loss of inferior olive neurons also occurs, but may be secondary to the cerebellar changes.

Families with significant phenotypic overlap have been identified, leading some to postulate that the three disorders represent a spectrum of disease rather than truly separate entities. The identification of small triplet expansions (20 and 23 repeats) in two kindreds whose members presented with EA2, and the identification of a point mutation leading to early hemiplegic migraine, and later progressive ataxia, is supportive of this interpretation (205,206).

Among the spinocerebellar ataxias, SCA7 (SCA7—MIM 164500) has perhaps the most variable expression. When the onset is in childhood, seizures and myoclonus along with cardiac involvement are seen alongside the usual features of ataxia. (207,208). It can be rapidly debilitating, culminating in death by age three or earlier. The infantile-onset form is also associated with cardiac abnormalities, particularly patent ductus arteriosus (207,209,210). Childhood-onset SCA7 is less aggressive than the infantile-onset form but more progressive than the adult-onset form.

In adults, ataxia is accompanied by oculomotor abnormalities. Other features include pyramidal tract signs, supranuclear ophthalmoplegia, and dysarthria/dysphagia; occasionally dementia and deafness are seen as well (207). In both childhood and adult cases, visual loss from pigmentary macular degeneration is almost invariable and can precede the ataxic syndrome. Since this sets it apart from the other autosomal dominant ataxias, defects in color vision and electroretinogram abnormalities might suggest the diagnosis in early cases (211) (reviewed in Reference (212)). The retinal changes in SCA7 start in a central location and move peripherally as the disease progresses, thus distinguishing the eye findings from those in retinitis pigmentosa.

Brain imaging in all cases typically demonstrates cerebellar atrophy; ventricular dilatation and delayed myelination can be found in infantile cases. Brain pathological specimens show atrophy of the cerebellum with loss of dentate neurons and Purkinje cells, and loss of inferior olivary neurons in the brain stem; posterior column, dorsal and ventral spinocerebellar tract loss in the spinal cord; and macular degeneration with loss of photoreceptive cells in the retina.

SCA7 is a glutamine repeat disorder mapping to chromosome 3p12-p21.1 (211,213,214). SCA7 accounts for roughly 5 percent of dominant spinocerebellar ataxias (193,195). SCA7 encodes a novel protein, ataxin-7, with a polyglutamine tract at the amino-terminus (215). The SCA7 CAG repeat ranges in size from 4 to 35 repeats in normal alleles, and from 37 to 306 repeats in expanded alleles. Another notable feature of SCA7 is the

remarkable intergenerational instability, with expansion particularly likely upon paternal transmission (215,216). An inverse correlation exists between repeat length and age of onset, and repeat expansions >59 repeats tend to develop visual impairment prior to ataxia (209). Alleles ranging in size from 34 to 36 repeats have been observed in individuals who are at risk but asymptomatic at the time of evaluation (207,209,214,215).

Like SCA2, it is the extreme expansion in SCA7 that contributes to the severe infantile phenotype (207). Mice expressing mutant ataxin-7 have features similar to those seen in the human disease with neurodegeneration affecting the cerebellum and retina (217,218).

Expression of the expanded ataxin-7 protein in transgenic mice leads to the development of intranuclear inclusions and the degeneration of rod photoreceptors and Purkinje cells, findings that are consistent with the human phenotype (219). In retinal photoreceptor cells, the expression of expanded ataxin-7 is associated with alterations in gene expression that predate photoreceptor degeneration (217,220). Another interesting finding is that the speed of ataxin-7 accumulation varies in different neuronal populations, suggesting that intrinsic differences in the ability to clear mutant protein may explain the variable susceptibility of different cell types in this and other polyglutamine diseases (217). Cellular dysfunction predates the appearance of neuronal inclusions, suggesting that, while they are part of the pathogenesis, inclusions in and of themselves are not the primary cause of dysfunction (217).

Recently ataxin-7 has been shown to be a core component of a transcription coactivator complex called STAGA (reviewed in Reference (221)), the mammalian equivalent of a yeast transcription coactivator named SAGA. This complex contains histone acetyltransferase (HAT) activity, mediated by the Gcn5 enzyme. Sgf73, the yeast homologue of ataxin-7, is a component of the SAGA complex with essential function in yeast (222). Two independent groups have shown that ataxin-7 is indeed a core component of STAGA complex in mammals (223,224). It is still unclear why, in spite of ubiquitous expression of the STAGA complex, neurodegeneration happens only in specific group of neurons. The answer might underlie identification of STAGA-dependent transcription factors whose functional impairment underlies the production of the SCA7 phenotype.

SCA8 (SCA8—MIM 608768) is difficult to distinguish clinically from the other SCAs. Pathologically, there appears to be degeneration of Purkinje, inferior olivary, and nigral cells, accompanied by periaqueductal stenosis, with MRI of the brain demonstrating cerebellar atrophy (225) (SCA8 was mapped to chromosome 13q21, and the vast majority of disease alleles in individuals of European descent share a common haplotype (226).

Genetically, SCA8 is the only SCA with a possibly novel mechanism of pathogenesis for triplet repeat disorders that involves a CTG expansion in the noncoding

region of the relevant gene. There is a high variability in the repeat length of wild-type alleles: more than 99% of unaffected individuals have repeat lengths less than 74, but a few unaffected individuals have 800 repeats, by far the largest number of normal repeats of any SCA. This remarkable overlap of repeat size in pathogenic and nonpathogenic alleles is unique among the SCAs. Furthermore, homozygosity for an expanded allele does not appear to exacerbate the disease phenotype, as it does in SCA1 and DRPLA. The CTG expansion shows significant genetic instability. Repeat lengths contract with paternal transmission and expand with maternal transmission, another unusual finding of this disease (227,228).

Initial studies suggested that the SCA8 gene might encode an antisense RNA that regulates the levels of a neighboring messenger RNA that encodes a putative actin-binding protein (229). More recent data suggest that the CTG expansion is transcribed in both the forward and reverse directions to yield an RNA with a CUG expansion and a peptide with CAGs encoding polyglutamines (230). It is possible that both the RNA and the protein product produce toxic effects. The RNA gain of function might be similar to that suggested for myotonic dystrophy types 1 and 2 (DM1: MIM 160900; DM2: MIM 602668)—repeat disorders where the repeat does not occur in the protein coding region of the gene. Recently, Daughters et al. have presented some evidence that SCA8 CUG<sup>exp</sup> transcripts form hallmark ribonuclear inclusions that colocalize with MBNL1 in humans and mice and that genetic loss of Mbnl1 enhances motor coordination deficits in SCA8 BAC-EXP mice. Furthermore, ATXN8OS CUG<sup>exp</sup> transcripts dysregulate MBNL1-CUGBP1 pathways in the CNS and trigger downstream molecular changes in GABA-A transporter 4 (*Gabt4*) regulation through an RNA gain-of-function mechanism (231). Unlike DMPK and ZNF9 (the genes responsible for DM1 and DM2, respectively), which have broad expression patterns, SCA8 is almost exclusively expressed in the CNS; the spatial and temporal expression differences between SCA8, DMPK, and ZNF9 could explain the phenotypic differences between these three diseases (reviewed in Reference (232)).

Thus, SCA8 appears to be a very complicated syndrome with many questions unanswered. Further confusing the picture is the coexistence of SCA8 expansions with those of SCA1, SCA3, or SCA6 in some kindreds (233,234). SCA8 expansions have also been found in patients with Alzheimer's disease, Parkinson's disease, and an individual with vitamin E deficiency heterozygous for the TTPA mutation (233,235,236). How these factors influence the pathogenesis of the disease is unknown. Although a relationship between repeat size and clinical signs was demonstrated in the initial report, other families with this disorder have shown greater variability in penetrance (226). This has led some authors in the past to question the pathogenesis of the expansion itself (237–239). It appears that sequence interruptions

of the CTG expansion and the size of the CTA tract that precedes the CTG expansion are among the multiple factors that may influence SCA8 disease penetrance.

SCA9 has not been assigned to a clinical disorder. In SCA10 (MIM 603516) seizures coexist with spinocerebellar ataxia. Mood disorders and polyneuropathy diagnosed by nerve conduction studies can also be seen, and neuroimaging shows cerebellar atrophy. SCA10 was identified in a single four-generation Mexican kindred (239) and is the second most common form of dominantly-inherited ataxia after SCA2 in people of Mexican descent and MJD in southern Brazil. This observation, along with the rarity of expanded alleles in other populations, has led to the hypothesis that the mutation arose in the New World (240). SCA10 is caused by a pentanucleotide (ATTCT) expansion in an intron of the affected gene (chromosome 22q13). Like trinucleotide expansions, pentanucleotide expansions are also unstable when transmitted paternally, leading to the clinical phenomenon of anticipation in this syndrome as well (241,242) (maternal transmission results in more stable repeats). Instability of the repeat region can lead to as many as 4500 repeats (242).

The ataxin-10 protein is widely expressed throughout the brain and in several other tissues (243). It is localized to the cytosol and perinuclear region in neurons. Whether the disease phenotype stems from genomic disruption, RNA gain-of-function, or ataxin-10 loss of function is unknown; however, decreased ataxin-10 RNA levels in primary cerebellar and cortical neuronal culture result in increased apoptosis—suggesting that loss of function may be the pathogenetic mechanism (243).

The remaining SCAs are rare. SCA11 (SCA11—MIM 604432) is a relatively mild, pure cerebellar ataxia with brisk reflexes. Mean age of onset was about 25 years. Genetic anticipation does not appear to be present. It is caused by mutations in the *TTBK2* gene that encode tau tubulin kinase-2 (locus at 15q14-q21.3) (244,245). Neuropathological examination does not reveal neuronal inclusions using hematoxylin and eosin, p62, ubiquitin or 1C2 immunohistochemistry, but neurofibrillary tangles, neuropil threads and tau-positive neuritis are visible in the medullary tegmentum, nigra, midbrain tegmentum and tectum, and putamen. The cerebellum showed cell loss but surprisingly no other visible pathology, perhaps due to the high resistance of the cerebellum to forming tangles (244).

SCA12 (SCA12—MIM 604326) is a trinucleotide repeat disorder caused by a CAG trinucleotide expansion in the 5' UTR of a gene encoding a brain-specific regulatory subunit of protein phosphatase 2A (genetic locus 5q31-q33) (246). This ataxia is often complicated by tremor, parkinsonian features, and dementia in the later stages of disease progression. In fact, action tremor is the most distinguishing clinical feature and is typically the initial symptom. Other features include hyperreflexia and abnormal eye movements. Two patients in the initial

kindred were reported with childhood-onset symptoms: one had nystagmus from birth, while the other had lower extremity dystonia that developed in childhood (247). MRI of the brain reveals both cortical and cerebellar atrophy. Pathology is available only on a single brain and revealed diffuse atrophy of cerebral and cerebellar cortices and specifically loss of Purkinje cells. SCA12 is rare except in India, where it is the third most common SCA (248,249).

Genetic analysis revealed a triplet CAG expansion on chromosome 5q31-q33. No apparent relationship exists between repeat size and age of onset. Unlike other SCAs with CAG expansions, the expanded allele does not lead to a polyglutamine tract. Rather, the CAG expansion is found in 133 nucleotides upstream of the transcription start site for PPP2R2B. The SCA12 gene product is a brain-specific regulatory subunit of protein phosphatase 2A and regulates neuronal survival through the mitochondrial fission/fusion balance (250).

SCA13 (SCA13—MIM 605259) is characterized by cerebellar ataxia and mental retardation described in French and Filipino pedigrees (251,252). It is caused by mutations in the potassium channel gene *KCN3* on chromosome 19q13 (253). Two distinct mutations have been identified in distinct Filipino and French pedigrees. Both mutations alter *KCN3* function in a *Xenopus laevis* oocyte expression system. An F448L mutation is expected to be more severe because it alters key gating properties of *KCN3* channels. In contrast, an R420H would be expected to reduce channel activity without changing the functional properties of the residual current (253). This speculation is consistent with the childhood-onset with concurrent mental retardation (IQ = 62–76) and seizures in members of the French pedigree and their absence in the Filipino patients. These mutations are expected to change the output characteristics of fast-spiking cerebellar neurons, where *KCN3* channels confer capacity for high-frequency repetitive firing.

SCA14 (SCA14—MIM 605361) is caused by missense mutations in exon 4 of the protein kinase C gamma gene, a member of a serine/threonine kinase family that is involved in cell proliferation and signal transduction processes (254–256). This gene maps to 19q13.4 (255,257,258).

The phenotype of SCA14 is quite variable. Patients who present before 27 years of age manifest axial myoclonus prior to the development of ataxia, while those with onset after age 39 present with a pure cerebellar ataxia. Gait ataxia, dysarthria, horizontal gaze nystagmus, abnormal smooth pursuit movements, hyper- and hyporeflexia, and peripheral neuropathy were described in all of the families (255,257,258). Imaging reveals atrophy of the cerebellar vermis either alone or in conjunction with the cerebellar hemispheres. Lifespan is not significantly affected, and ataxia displays a slowly progressive course. Genetic anticipation occurs. Limited pathologic specimens suggest a primary Purkinje cell defect (257).

SCA15 (MIM 606658) is a slowly progressive cerebellar ataxia that may be associated with a mild postural or action tremor and titubation. Its onset is variable from childhood to middle age (259–261). Neuroimaging typically reveals atrophy of the cerebellum, particularly the vermis. The locus for SCA15 was first mapped to 3p24.2-3pter in an Australian family (262). Hara et al. mapped two Japanese families presenting with ataxia and postural tremor of the head, arm, or trunk (263). The first family had partial deletions involving both the type 1 inositol 1,4,5-triphosphate receptor (*ITPR1*) and sulfatase modifying factor 1 (*SUMF1*) genes, and the second family had only a point mutation in *ITPR1* (P1059L). The proline was highly conserved, and the mutation was absent in 234 chromosomes in Japanese controls (263). These results suggest that *ITPR1* but not *SUMF1* is the causative gene for SCA15. On the other hand, Iwaki et al. showed that haploinsufficiency in *ITPR1* alone is the cause of what had been thought to be a different disease, SCA16 (MIM 606364) (264). Since these discoveries, SCA16 is thought to be same disorder as SCA15.

SCA17 (MIM 607136) is yet another polyglutamine disease with the expansion occurring in the general transcription factor TATA-binding protein (*TBP*) on chromosome 6q27 (265). The ataxic syndrome is associated with dementia, extrapyramidal features, and even psychiatric symptoms such as depression and hallucinations (265,266). SCA17 can also mirror Huntington disease by causing generalized chorea (267). Marked anticipation can occur as observed in Italian kindred, with the youngest family member in the fourth generation presenting with dysarthria and ataxia at age three (268). Repeat sizes of 42–48-repeats show variable penetrance. Like other expansion diseases, an inverse correlation exists between repeat size and age of onset.

Neuroimaging typically demonstrates cortical and cerebellar atrophy. SCA17 mouse models have been generated by expressing *TBP* under the prion promoter that also demonstrate a neurodegenerative syndrome. There is evidence to suggest that the expansion of the polyglutamine protein enhances the interaction of *TBP* with the general transcription factor *IIB* causing transcriptional misregulation (269). Pathologic specimens reveal neuronal loss in many brain areas, including loss of Purkinje cells (265,270). Immunostaining for ubiquitin, *TBP*, and polyglutamine tracts demonstrates their presence in intranuclear inclusions in several neuronal cell types, including Purkinje cells (265,270).

SCA18 (Sensory/Motor Neuropathy with Ataxia—MIM 607458) is an ataxic syndrome associated with pyramidal tract signs, muscle weakness, sensory axonal neuropathy, and mild cerebellar atrophy. Distal muscle weakness and atrophy along with pes cavus are occasionally seen. It has been described in an American family of Irish ancestry (257). Haplotype analysis links SCA18 to 7q22-q32. Neuroimaging may show



evidence of mild cerebellar atrophy. EMG/NCV shows evidence of denervation and axonal sensory neuropathy. Thus, the overall picture is one of a mixed cerebellar degeneration and peripheral neuropathy. There is evidence to suggest that the interferon-related developmental regulator gene 1 (IFRD1) is mutated in this disease (271).

SCA19 (MIM 607346) was identified in a single Dutch family and manifests as a relatively mild cerebellar ataxia with cognitive impairment, myoclonus, and tremor. Imaging shows atrophy of the cerebellar hemispheres or vermis (272). SCA19 links to chromosome 1p21-q21 (273), a region that has also been linked to SCA22. Thus, SCA19 and SCA22 are probably allelic forms of the same gene ((274) #4503).

SCA20 (MIM 608687) is an ataxic syndrome described in a family of Anglo-Celtic descent that maps to chromosome 11 (275). Age of onset in 14 family members was 19–64 years (mean 46.5). In this disease, dysarthria is usually the presenting feature with subsequent limb ataxia, palatal tremor, and dysphonia. This disease is also characterized by calcification of the dentate nucleus visualized by brain imaging with CT or MRI. The genetic locus overlaps with that of SCA5, so the separate identity of this SCA remains to be established.

SCA21 was identified in a French family as gait ataxia and akinesia, with variable features of dysarthria, hyporeflexia, and mild cognitive impairment. Brain imaging shows marked atrophy of the cerebellum. Eye movements are typically preserved, and disease progression is slow. The phenomenon of anticipation has been suggested; however, the responsible gene has not yet been identified. SCA21 maps to chromosome 7p21.3-p15.1 (276,277).

SCA22 was identified in a Taiwanese family mapping to chromosome 1p21-q23 (278). Clinically, this is a slowly progressive ataxia with variable dysarthria and hyporeflexia, with neuroimaging showing cerebellar degeneration (46). As mentioned earlier, it is likely that SCA19 and SCA22 are allelic forms of the same gene (272,274,279).

SCA23 is a late-onset (age > 40 years) ataxia described in a Dutch family mapped to chromosome region 20p13-p12.3. 2 (280). It is associated with variable dysarthria, ocular dysmetria, and distal sensory deficits (280). Neuroimaging demonstrates severe cerebellar atrophy. Pathological examination in a single patient who died at 80 years of age showed loss of Purkinje cells, neurons in the dentate nuclei and inferior olives and thinning of the cerebellopontine tracts. Also observed was demyelination of the posterior and lateral columns in the spinal cord—and ubiquitin-positive, polyglutamine-negative intranuclear inclusions in nigral neurons that resembled Marinesco bodies.

SCA24, unlike the other SCAs that are autosomal dominant, is a recessively inherited ataxia. It has therefore been redesignated as SCAR4 (see below).

SCA25 (MIM 608703) was described in a French family as a cerebellar ataxia with sensory neuropathy (281). The phenotype itself is variable with regard to age of onset, severity, and clinical manifestations. Thus, the disease can range from infantile-onset cerebellar ataxia with pure sensory neuropathy, suggestive of Friedreich's ataxia, to a form with mild cerebellar ataxia and prominent sensory neuropathy suggestive of Charcot-Marie-Tooth SCA25 maps to chromosome 2.

SCA26 (MIM 609306) was described in a Norwegian family. It is characterized by a pure slowly progressive cerebellar ataxia with autosomal dominant inheritance (282). SCA26 maps to chromosome 19p13.3 that is located close to the locus responsible for SCA6 (282). Genetic analysis, however, has excluded mutations in CACNA1A gene (the gene mutated in SCA6).

SCA28 (MIM 610246) was first described in an Italian family. It is characterized by a juvenile-onset (mean age 19.5 years), slowly progressive cerebellar ataxia with eye movement abnormalities and, in some cases, pyramidal tract signs. Associated features include dysarthria and hyperreflexia (283,284). SCA28 is caused by missense mutations in the mitochondrial protease gene AFG3L2, a gene that maps to chromosome region 18p11.22-q11.2 (285). AFG3L2 protein is a homolog of paraplegin, a protein mutated in one form of hereditary spastic paraplegia (SPG7). AFG3L2 and paraplegin are nuclear-encoded mitochondrial proteins that form a hetero-oligomer. Both of these proteins belong to the family of m-AAA proteases that are involved in protein quality control in the inner mitochondrial membrane. Mutations in paraplegin are responsible for a recessive form of hereditary spastic paraplegia (SPG7—MIM 607259), whereas mutant forms of AFG3L2 cause dominantly inherited SCA28. Haploinsufficient mice for AFG3L2 recapitulate important pathophysiological features of the human disease. Respiratory chain dysfunction and increased production of the reactive oxygen species might explain the pathophysiology of SCA28 (286).

SCA29 (MIM 117360) is characterized by an early-onset, and nonprogressive ataxia is characterized by a variable atrophy of the cerebellar vermis. It maps to chromosome 3p26 and may be an allelic variant of SCA15 described above (287).

SCA30 is an ataxia with a mid- to late-life onset associated with hypermetric saccades and minor pyramidal signs described in an Australian family of Anglo-Celtic origin (288). Brain MRI shows atrophy of the cerebellar vermis and cerebellar hemisphere. SCA30 maps to chromosome 4q34.3-q35.1. The patients did not have nystagmus or neuropathy.

SCA31 (MIM 117210) is characterized by late-onset cerebellar ataxia and reduced muscle tone that may be associated with sensorineural hearing loss (289–291). Notably, there are no signs of pyramidal tract involvement



(190). It maps to chromosome 16q22.1, close to SCA4. SCA31 is caused by an insertion containing pentanucleotide repeats including a long (TGGAA)<sub>n</sub> sequence within introns of the TK2 gene and the BEAN gene (on opposite strands and transcribed in opposite directions) (292).

Based on this summary of the known autosomal dominant spinocerebellar diseases and their phenotypes, it should not be misconstrued that all genes contributing to dominant spinocerebellar ataxia have been identified. Indeed, only 60–70% of patients have mutations in the known loci. This situation is likely to change and web-based resources such as the Online Mendelian Inheritance of Man ([www.OMIM.org](http://www.OMIM.org)) provide an excellent means for keeping track of advances in this area.

Besides the numbered spinocerebellar ataxias, other autosomal dominant conditions that result in ataxias include dentatorubropallidoluysian atrophy (DRPLA). This ataxia is relatively common in Japan. It should be suspected when ataxia and rigidity are accompanied by choreoathetosis, myoclonic epilepsy, and dementia. Other features include hyperreflexia and slowing of saccades. Caused by an expanded CAG repeat, DRPLA presents with anticipation, especially when transmission is via the father. The relevant protein encoded by the *DRPLA* gene (locus 12p) is called atrophin-1. This is a cytoplasmic protein with a molecular weight of 125 kDa (293,294). Calcification of the basal ganglia and leukodystrophic changes are often seen on imaging studies. Haw–River syndrome is a variant of DRPLA, without the characteristic myoclonic epilepsy, seen in a few families of African American descent in North Carolina.

Another ataxia that should be mentioned in the differential of spinocerebellar ataxic conditions is the Fragile X-associated tremor ataxia syndrome (FXTAS). This disease is seen in some older men (approximately a third) who carry a permutation in the fragile X mental retardation 1 gene (*FMR1*) (a gene on the X chromosome) (295–297). Fragile X syndrome, a common cause of mental retardation in males, is caused when the CGG expansion extends typically beyond 200 and is not associated with ataxia or tremor. The premutation consists of a trinucleotide CGG repeat length ranging from 50 to 200. It is also associated with dementia, parkinsonism, and autonomic dysfunction. Since the prevalence of the premutation carrier state is as high as 1 in 813 males, this syndrome could prove to be a common genetic cause of ataxia. Increased age and length of the CGG expansion are associated with worsening neurological features. FXTAS was thought to only affect men carrying the permutation. Women have been identified with this syndrome likely because of X inactivation of the normal allele. Premutation carriers in women can present with premature ovarian failure. The molecular mechanism causing FXTAS differs from that causing the fragile X syndrome. In fragile X syndrome, methylation of DNA sequences in the promoter where the

expanded CGG resides causes silencing of the *FMR1* gene and complete loss of *FMR1* protein. In FXTAS, fragile X RNA is produced but it is poorly translated into protein. This translational deficit is thought to cause a compensatory increase in the levels of *FMR1* transcripts. Unfortunately, the RNA bearing the expansion now causes a toxic gain-of-function effect triggering a downstream protein accumulation and neurodegeneration (298). Experimental mouse and *Drosophila* models have highlighted the similarity between FXTA and polyglutamine induced ataxias (299,300). This is an active area of research, with implications for some of the other repeat disorders in untranslated regions of RNA, including SCAs 8, 10 and 12.

Another class of diseases that may resemble the spinocerebellar ataxias are the prion disorders with mutations in the prion or *PRNP* gene. Of these, *Gerstmann–Straussler–Scheinker disease* (GSS), particularly the ataxic variant, is the most likely to mimic a spinocerebellar syndrome. GSS is diagnosed when autosomally dominant ataxia coexists with cognitive and motor decline alongside pathological findings of prion protein-containing amyloid plaques. Unlike those afflicted with the other SCAs, GSS patients often complain of altered sensation or even occasional leg pain; moreover, they go on to rather rapidly develop cognitive decline and EEG changes, similar to the more common variants of prion diseases such as CJD. GSS represents a group of at least six mutations in the *PRNP* gene, with the ataxic variant caused by a proline to leucine missense at codon 102 (301–303).

### 118.7.2 Diagnostic Studies in the Autosomal Dominant Spinocerebellar Ataxias

In all autosomal dominant ataxias, neuroimaging studies by Magnetic Resonance Imaging (MRI) and Computerized Axial Tomography (CAT) mirror the clinical and pathological phenotypes of each of the spinocerebellar ataxias. Cerebellar atrophy is the most commonly reported finding, along with enlargement of the fourth ventricle. The relative amounts of degeneration vary, with SCA2 patients showing the most atrophy and those with SCA5 and 6 the least. Surprisingly, brain stem atrophy, which one would expect to be almost universal, can be minimal in SCA3 and DRPLA, and is rare in SCA6. On the other hand, it is quite characteristic of SCA1, 2 and 7. Cerebral atrophy and compensatory enlargement of the lateral ventricles can be seen in DRPLA, SCA2, and the infantile variant of SCA7.

More expensive imaging studies such as magnetic resonance spectroscopy and positron emission tomography (PET) scanning are sensitive markers for deterioration and show abnormalities beyond the structural changes revealed by MRI scans. Their role, however, is controversial, and at the moment, these modalities should be reserved for research rather than diagnosis.

Most spinocerebellar ataxias cause defects in nerve conduction, especially SCA4. The predominant axonal neuropathy affects mainly sensory neurons, and sural nerve action potentials are commonly absent. Nerve conduction abnormalities, however, are rarely helpful in determining the genetic subclass of the ataxia. On the other hand, visual evoked potentials can be helpful to suggest SCA7, while interictal electroencephalograph (EEG) abnormalities might suggest DRPLA. Although seizures are common in SCA10, interictal EEGs are typically normal.

Genetic testing is the only definitive diagnostic test. A few differentiating features (Table 118-4) may help in suggesting the likely candidates in a stepwise screen. More often, blood is sent out to test for mutations in a battery of genes. So far, this list includes SCA1, 2, 3, 6, 7, 8, 10, 17 and DRPLA, but the number is likely to increase. Online resources e.g. [www.geneclinics.org](http://www.geneclinics.org), are extremely informative.

**TABLE 118-4** A Few Differentiating Features of the Autosomal Dominant Spinocerebellar Ataxias

Distinguishing Features of the Spinocerebellar Ataxias	
SCA1	Relatively nondescript spinocerebellar ataxia with neuropathy and pyramidal signs
SCA2	Slow saccades, myoclonus, areflexia
SCA3	Bulging eyes, fasciolingual fasciculations, extrapyramidal signs
SCA4	Sensory neuropathy
SCA5	Slow course despite early onset
SCA6	Very late-onset, mild, apparently sporadic onset
SCA7	Macular degeneration
SCA8	Mild
SCA9	Unassigned
SCA10	Generalized or complex partial seizures
SCA11	Mild
SCA12	Tremor, dementia
SCA13	Mental retardation
SCA14	Often associated with myoclonus
SCA15	Mild
SCA16	Head tremor
SCA17	Bradykinesia and dementia
SCA18	Mixed sensory and motor neuropathy
SCA19	Dementia, myoclonus and tremor
SCA21	Dementia
SCA22	Dysarthria
SCA25	Sensory neuropathy
SCA26	Slow progression
SCA28	Juvenile-onset with eye movement abnormalities, in some cases pyramidal tract signs, dysarthria, hyperreflexia
SCA29	Early-onset, nonprogressive
SCA30	Hypermetric saccades and minor pyramidal signs
SCA31	Reduced muscle tone, possible associated with sensorineural hearing loss
DRPLA	Chorea, seizures, myoclonus

No effective treatment is available for the SCAs although we generally treat our patients with antioxidants such as vitamin E. Like Friedreich's ataxia a team approach involving physical therapists, occupational therapists, and genetic counselors help advise each individual patient.

## 118.8 EPISODIC ATAXIAS

There are seven varieties of dominantly inherited episodic ataxias called episodic ataxia type 1 (EA1) to episodic ataxia type 7 (EA7) (304). EA1 and EA2 are the most common. Both of these diseases begin in late childhood or adolescence and respond to treatment with acetazolamide.

Patients with EA1 show short episodes of ataxia rarely lasting beyond a few minutes. Ataxic spells sometimes are induced by exercise and may be accompanied by myokymia in the hands and face. Pathology is minimal with mild atrophy of anterior cerebellar vermis. The genetic basis for EA1 is the presence of point mutations in a voltage-gated potassium channel gene, *KCNA1*, located on chromosome 12p13 (305,306). Recently, a mouse model of EA1 has been engineered that should lead to important insights into pathogenesis and possibly even treatment options (307).

In EA2, the attacks are longer, lasting usually for a few hours or even days. Moreover, these episodes appear to result in cumulative damage as patients often go on to develop cerebellar symptoms and atrophy. Thus, subtle cerebellar signs such as nystagmus and mild clumsiness should tilt the diagnosis toward EA2 rather than EA1. As mentioned in the context of SCA6, EA2 is caused typically by mutations in the  $\alpha_{1A}$  subunit of the calcium channel gene, the gene involved in SCA6 and also familial hemiplegic migraine (203,308–310). There is at least one family with a mutation in the gene for the beta-4 subunit of this channel (311). It is possible that mutations in the P/Q-type calcium channel reduce calcium-dependent neurotransmitter release that then triggers ataxia (312). Recently the potassium channel blocker 4-aminopyridine has been shown to reduce the frequency of attacks, presumably by first increasing the excitability of cerebellar Purkinje cells followed by a subsequent release of the inhibitory neurotransmitter GABA (313).

Patients with episodic ataxia type 3 have brief attacks of ataxia, vertigo, tinnitus, and myokymia that have a variable age of onset (314). It appears to be linked to chromosome 1q42 (315), and the attacks are responsive to acetazolamide.

Patients with episodic ataxia type 4 have attacks of vertigo, tinnitus, diplopia, and ataxia beginning in early adulthood (316). Patients often display poor smooth pursuit with respect to their eye movements. They also display gaze-evoked nystagmus (317). EA4 is typically unresponsive to acetazolamide.

Patients with episodic ataxia type 5 display ataxia reminiscent of EA2 with attacks lasting hours (318). EA5 is caused by a mutation in the CACNB4 gene on chromosome 2q22-23 (311).

EA6 is caused by a heterozygous mutation in the SLC1A3 gene, a member of the solute carrier family that encodes the excitatory amino acid transporter 1 (EAAT1) (319). Episodic ataxia can also be associated with seizures, migraine, and alternating hemiplegia.

EA7 has been identified in a single family with clinical features of EA2 except that they do not display persistent cerebellar symptoms with time (320). This gene has been linked to chromosome 19q13.

### 118.9 X-LINKED ATAXIAS

Besides adrenomyeloneuropathy (described earlier), which can present as an ataxic syndrome, there are a few X-linked progressive ataxias. These can be almost purely cerebellar syndromes (321,322) or associated with a wider spectrum of signs such as anemia, deafness, spasticity, or dementia (323–326). In most instances, the genetic loci are still unknown. One X-linked ataxia where the gene has been identified is X-linked sideroblastic anemia with ataxia (ABC7 transporter gene at Xq13) (327). How this defect leads to ataxia is still unclear. Another X-linked ataxia that has been mapped to a locus is the early-onset X-linked ataxia that is characterized by deafness and loss of vision (links to locus Xq21.2–q24) (328). The Fragile X-associated tremor ataxia syndrome (FXTAS) has been described above in the differential diagnosis of the autosomal dominant SCAs.

### 118.10 MITOCHONDRIAL ATAXIAS

Mitochondrial deficits can present with a progressive or intermittent ataxia. For instance, *Leigh syndrome*, a genetically heterogeneous syndrome caused by mutations in several genes affecting mitochondrial function, often presents with an intermittent ataxia and atypical necrotizing encephalitis not unlike that seen in thiamine deficiency. An MRI scan is informative in revealing characteristic subcortical necrotic lesions. Leigh syndrome phenotype has been associated with autosomal dominant, X-linked, or with mitochondrial DNA (maternal inheritance) mutations. Mutations in SURF1, a gene located on chromosome 9q34, have been identified in several COX-deficient patients with Leigh syndrome (329,330).

Leigh syndrome is also often accompanied by cardiomyopathy, presumably because of mitochondrial energy failure in cardiac muscle. Ataxia and myoclonus can also be seen in other defects of the mitochondrial genome. Both larger deletions and duplications characteristic of Kearns–Sayre syndrome and maternally-inherited point mutations in mitochondrial genes encoding tRNA, the MELAS (mitochondrial encephalopathy, lactic acidosis,

and stroke-like episodes), and MERRF (myoclonic epilepsy with ragged red fibers) have been associated with the ataxic phenotype. Historically, these diseases contributed to the Ramsay Hunt syndrome, an ataxic syndrome characterized by myoclonus. Other etiologies of this phenotype include some of the storage disorders described above, in particular sialidosis, but this constellation of symptoms is also seen in *Baltic myoclonus of Unverricht-Lundberg*, an autosomal recessive ataxia, where the underlying defect is in the gene encoding the protease inhibitor cystatin B. This disease is prevalent in Scandinavia and maps to locus 21q22.3 (331). Surprisingly, in most instances, this is also a repeat disorder, but the repeat is a dodecamer repeat in the gene's promoter region. With better genetic delineation, the term “Ramsay Hunt syndrome” will probably become relegated to history, since it does not really throw light on the etiology of a variety of disparate conditions.

### 118.11 CONCLUSIONS

A decade ago, it would have been difficult to predict the rapid advances in our understanding of the genetics of hereditary ataxias. This progress has not been confined to the identification of the genetic basis of individual diseases; we have begun to sort out candidate pathways in pathogenesis, using a plethora of tools from the growing armamentarium offered by present day molecular biology and genomics. The development of animal models, in particular, is now recognized as a notable first step in hypothesis testing and, more importantly, as an approach for testing possible therapies. The various animal models—the mouse, *Drosophila*, *Caenorhabditis elegans* and yeast—offer powerful, complementary tools for elucidating complex pathways at the level of the whole organism.

An excellent example that validates such an approach has been the dissection of the chaperone and ubiquitin-proteasomal pathway in polyglutamine disease. Initial work on this pathway in transfection experiments has found confirmation in genetic screens in the fly, where overexpression of heat shock proteins can rescue a polyglutamine-induced phenotype (332–334). Another example has been the pivotal role of yeast in elucidating the role of the oxidative stress induced by the mitochondrial accumulation of iron (49). In the prevailing climate of combinatorial chemistry and rational drug design, we anticipate high-throughput screens for chemical modifiers using simple animal models. Using such techniques, pharmaceutical companies could potentially identify candidate agents that can then be tested as therapeutic agents first in animal models and then in the human patient. Currently, there are clinical trials testing the role of agents that modulate synaptic activity (riluzole and varenicline), antioxidants such as coenzyme Q10, and lithium for ataxia treatment. Other strategies being tested include RNA interference or micro-RNA-based



mechanisms to shut down gene expression of mutant proteins. Thus, for the patient, there is reason for hope, even though the desired outcome of actual therapies remains to be seen. For the present, genomics and web-based dissemination of information have already wrought changes in the way patients view their chronic diseases. They are better informed about the genetics of transmission and disease prognosis and are therefore in a better position to plan their lives. In return, they are often more willing to provide blood samples for DNA analyses and enroll in variety of clinical trials that are crucial for clinical progress. The National Ataxia Foundation ([www.ataxia.org](http://www.ataxia.org)) and nonprofit organizations such as [www.wemove.org](http://www.wemove.org) are playing important roles in educating patients and the larger public.

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## LIST OF RELEVANT WEB PAGES

- <http://www.ncbi.nlm.nih.gov/omim>: Online Mendelian Inheritance in Man. This is the premiere web site for genetic information and references to original scientific publications across the spectrum of human genetic diseases.
- <http://www.ncbi.nlm.nih.gov/sites/GeneTests/>: The GeneTests Website is a publicly funded medical genetics information resource developed for physicians, other healthcare providers, and researchers.
- <http://www.uptodate.com/index>: A subscription-based website that serves as an on-line text book. This is written by experts and is constantly updated.
- <http://clinicaltrials.gov/>: This website provides a registry of federally and privately supported clinical trials conducted in the United States and around the world.
- <http://www.ataxia.org/>: This is the official website of the National Ataxia Foundation. This is an excellent site to refer patients and families suffering from rare ataxias to learn more about the disease.



### Biographies



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# CHAPTER

# 119

## Hereditary Spastic Paraplegia

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### 119.1 INTRODUCTION

The hereditary spastic paraplegias (HSPs) are a clinically and genetically diverse group of disorders in which the predominant symptom is lower extremity weakness and spasticity that usually, though not always, progresses insidiously. HSP is also referred to as familial spastic paraplegia (FSP), hereditary (or familial) spastic paraparesis, and Strümpell–Lorrain syndrome (1).

This chapter summarizes clinical features including diagnosis, classification, differential diagnosis, treatment and counseling, HSP-related disorders, complicated HSP syndromes, and advances in our understanding of the molecular genetic basis of HSP. For previous review of HSP and early descriptions see (2).

#### 119.1.1 History

The earliest reports of HSP were those of Seeligmüller in 1876 (3), who described a family with progressive spastic weakness of the lower extremities. Subsequently, Strumpell in 1880 and Lorraine in 1898 (4) described families with hereditary progressive lower extremity spastic weakness in the absence of other neurologic abnormalities (1,5,6).

#### 119.1.2 Ethnicity

HSP kindreds have been described from diverse geographic and ethnic backgrounds including European (Belgian, Dutch, English, Icelandic, Irish, Italian, French, German, Greek, Norwegian, Portuguese, Romanian, Spanish, Swedish, Swiss, Turkish), South American (Brazilian and Columbian), Asian (Chinese, Japanese, Korean, Tibetan), Middle Eastern (Egyptian, Jordanian, Iraqi, Israeli), Australian, and Indian ancestry. Although there are relatively few reports of HSP kindreds of African descent (7), HSP has been described in many Tunisian kindreds, in a South African Zulu family (8), and in a family from Mali (9).

#### 119.1.3 Prevalence

The prevalence of HSP has been estimated to be 1.27 in Ireland (10), 2.7 per 100,000 in Italy (11), 5.75 per

100,000 in Tunisia (Sfax district) (12), 7.4 per 100,000 in Norway (13), and 9.6 per 100,000 in Spain (14). In Norway, the prevalence of autosomal dominant HSP (5.5 per 100,000) exceeded that of autosomal recessive HSP (0.6 per 100,000) and isolated cases of spastic paraplegia (1.3 per 100,000) (13).

#### 119.1.4 Genetic Classification

HSP is classified genetically according to the mode of inheritance (autosomal dominant, autosomal recessive, and X-linked HSP). Genetic loci for HSP are designated “SPG” and numbered one through 48 in order of their discovery (Table 119-1). In addition, several genetic types of HSP have been reported, which do not have SPG loci designations (e.g., SPOAN syndrome and mitochondrial ATP6 gene mutation, Table 119-1).

#### 119.1.5 Clinical Classification

Historically, HSP clinical syndromes have been classified (56) as “uncomplicated” (also referred to as “pure” or “non-syndromic” HSP) if neurologic deficits are limited to progressive lower extremity spastic weakness, hypertonic urinary bladder disturbance (57), and often, but not always, mild diminution of lower extremity vibration sensation. HSP is classified as “complicated” (“syndromic”) if, in addition to the impairments present in “uncomplicated” HSP, the *heritable syndrome includes other neurologic or systemic abnormalities that are not attributable to alternative or coexisting disorders*. Thus, “complicated HSP” is characterized by insidiously progressive lower extremity weakness and spasticity, accompanied by such neurologic or systemic impairments as seizures, mental retardation, dementia, cataracts, amyotrophy, optica atrophy, ataxia, extrapyramidal disturbance, cutaneous abnormalities, or peripheral neuropathy.

As noted below, there is imperfect correlation of HSP clinical syndromes with specific genetic types of HSP. Increasingly, it is recognized that a given genetic type of HSP (including the most common dominant and

TABLE 119-1 Genetic Types of HSP

Spastic Gait (SPG) Locus	Clinical Syndrome	Protein	Gene Testing	References
<b>Autosomal dominant HSP</b>				
SPG3A (14q11-q21)	Uncomplicated HSP: symptoms usually begin in childhood (and may be nonprogressive); symptoms may also begin in adolescence or adulthood and worsen insidiously. Genetic nonpenetrance reported. <i>De novo</i> mutation reported. Presenting as spastic diplegic cerebral palsy	Atlastin	ADL	(15–17)
SPG4 (chr.2p22)	Uncomplicated HSP, symptom onset in infancy through senescence, single most common cause of autosomal dominant HSP (~40%); some subjects have late-onset cognitive impairment	Spastin	ADL <sup>a</sup>	(18–22)
SPG6 (15q11.1)	Uncomplicated HSP: prototypical late-adolescent, early-adult-onset, slowly progressive	"Not imprinted in Prader Willi/Angelman 1" (NIPA1)	ADL	(23–26)
SPG8 (8q23-q24)	Uncomplicated HSP	KIAA0196/Strumpellin	ADL	(27–31)
SPG9 (10q23.3-q24.2)	Complicated: spastic paraplegia associated with cataracts, gastroesophageal reflux, and motor neuronopathy	Unknown	No	(32,33)
SPG10 (12q13)	Uncomplicated HSP or complicated by distal muscle atrophy	Kinesin heavy chain (KIF5A)	Lab	(34,35)
SPG12 (19q13)	Uncomplicated HSP	Unknown		(28)
SPG13 (2q24-34)	Uncomplicated HSP: adolescent and adult-onset	Chaperonin 60 (also known as heat shock protein 60, HSP60)	Lab	(36–38)
SPG17 (11q12-q14)	Complicated: spastic Paraplegia associated with amyotrophy of hand muscles (Silver syndrome)	BSCL2/seipin	Lab	(39–41)
SPG19 (9q33-q34)	Uncomplicated HSP	Unknown	No	(42)
SPG29 (1p31.1–21.1)	Complicated: spastic paraplegia associated with hearing impairment; persistent vomiting due to hiatal hernia inherited	Unknown	No	(43)
SPG31 (2p12)	Uncomplicated HSP or occasionally associated with peripheral neuropathy	Receptor expression enhancing protein 1 (REEP1)	ADL	(44–46)
SPG33 (10q24.2)	Uncomplicated HSP	ZFYVE27/protrudin}	Lab	(47)
SPG36 (12q23-24)	Onset age 14–28 years, associated with motor sensory neuropathy	Unknown	No	(48)
SPG37 (8p21.1-q13.3)	Uncomplicated HSP	Unknown	No	(49)
SPG38 (4p16-p15)	One family, five affected subjects, onset age 16–21 years. Subjects had atrophy of intrinsic hand muscles (severe in one subject at age 58)	Unknown	No	(50)
SPG40 (locus unknown)	Uncomplicated spastic paraplegia, onset after age 35, known autosomal dominant HSP loci excluded	Unknown	No	(51)
SPG41 (11p14.1-p11.2)	Single Chinese family with adolescent-onset, spastic paraplegia associated with mild weakness of intrinsic hand muscles	Unknown	No	(52)
SPG42 (3q24-q26)	Uncomplicated spastic paraplegia reported in single kindred, onset age 4–40 years, possibly one instance of incomplete penetrance	Acetyl CoA transporter (SLC33A1)	No	(53–55)
<b>Autosomal recessive HSP</b>				
SPG5 (8p)	Uncomplicated or complicated by axonal neuropathy, distal or generalized muscle atrophy, and white matter abnormalities on MRI	CYP7B1	Lab	(123,418,419, 473, 497–500)
SPG7 (16q)	Uncomplicated or complicated: variably associated with mitochondrial abnormalities on skeletal muscle biopsy and dysarthria, dysphagia, optic disc pallor, axonal neuropathy, and evidence of "vascular lesions," cerebellar atrophy, or cerebral atrophy on cranial MRI	Paraplegin	ADL et al	(155,239)

**TABLE 119-1 Genetic Types of HSP—cont'd**

SPG11 (15q)	Uncomplicated or complicated: spastic paraplegia variably associated with thin corpus callosum, mental retardation, upper extremity weakness, dysarthria, and nystagmus; may have "Kjellin syndrome": childhood-onset, progressive spastic paraplegia accompanied by pigmentary retinopathy, mental retardation, dysarthria, dementia, and distal muscle atrophy; juvenile, slowly progressive ALS reported in subjects with SPG11 HSP; 50% of autosomal recessive HSP is considered to be SPG11	Spatacsin (KIAA1840)	Lab	(169,501,502)
SPG14 (3q27-28)	Single consanguineous Italian family, three affected subjects, onset age ~30 years. Complicated spastic paraplegia with mental retardation and distal motor neuropathy (sural nerve biopsy was normal)	Unknown	No	(267)
SPG15 (14q)	Complicated: spastic paraplegia variably associated with associated with pigmented maculopathy, distal amyotrophy, dysarthria, mental retardation, and further intellectual deterioration (Kjellin syndrome)	Spastizin/ZFYVE26	Lab	(258,259)
SPG18 (8p12-p11.21)	Two families described with spastic paraplegia complicated by mental retardation and thin corpus callosum	Unknown	No	(503)
SPG20 (13q)	Complicated: spastic paraplegia associated with distal muscle wasting (Troyer syndrome)	Spartin	Lab	(264,266,275, 428,471)
SPG21 (15q21-q22)	Complicated: spastic paraplegia associated with dementia, cerebellar and extrapyramidal signs, thin corpus callosum, and white matter abnormalities (Mast syndrome)	Maspardin	Lab	(162)
SPG23 (1q24-q32)	Complicated: childhood-onset HSP associated with skin pigment abnormality (vitiligo), premature graying, characteristic facies; Lison syndrome	Unknown	No	(263)
SPG24 (13q14)	Complicated: childhood-onset HSP variably complicated by spastic dysarthria and pseudobulbar signs	Unknown	No	(504)
SPG25 (6q23-q24.1)	Consanguineous Italian family, four subjects with adult (30–46 years) onset back and neck pain related to disk herniation and spastic paraplegia; surgical correction of disk herniation ameliorated pain and reduced spastic paraplegia. Peripheral neuropathy also present	Unknown	No	(505)
SPG26 (12p11.1–12q14)	Single consanguineous Bedouin family with five affected subjects. Complicated: childhood onset (between 7 and 8 years), progressive spastic paraparesis with dysarthria and distal amyotrophy in both upper and lower limbs, nerve conduction studies were normal; mild intellectual impairment, normal brain MRI	Unknown	No	(271)
SPG27 (10q22.1-q24.1)	Complicated or uncomplicated HSP. Two families described. In one family (seven affected subjects) uncomplicated spastic paraplegia began between ages 25 and 45 years. In the second family (three subjects described) the disorder began in childhood and included spastic paraplegia, ataxia, dysarthria; mental retardation, sensorimotor polyneuropathy, facial dysmorphism and short stature	Unknown	No	(32,256)
SPG28 (14q21.3-q22.3)	Uncomplicated: childhood-onset progressive spastic gait	Unknown	No	(506)
SPG29 (14q)	Uncomplicated HSP, childhood onset	Unknown	No	(507)

Continued



**TABLE 119-1 Genetic Types of HSP—cont'd**

SPG30 (2q37.3)	Complicated: spastic paraplegia, distal wasting, saccadic ocular pursuit, peripheral neuropathy, mild cerebellar signs	Unknown	No	(508)
SPG32 (14q12-q21)	Mild mental retardation, brainstem dysraphia, clinically asymptomatic cerebellar atrophy	Unknown	No	(509)
SPG35 (16q21-q23)	Childhood onset (6–11 years), spastic paraplegia with extrapyramidal features, progressive dysarthria, dementia, seizures. Brain white matter abnormalities and brain iron accumulation; an Omani and a Pakistani kindred reported	Fatty acid 2-hydroxylase (FA2H)	Lab	(160,161,510)
SPG39 (19p13)	Complicated: spastic paraplegia associated with wasting of distal upper and lower extremity muscles	Neuropathy target esterase (NTE)	Lab	(273)
SPG43 (19p13.11-q12)	Two sisters from Mali, symptom onset 7 and 12 years, progressive spastic paraplegia with atrophy of intrinsic hand muscles and dysarthria (one sister)	Unknown	No	(9)
SPG44 (1q41)	Allelic with (PMLD, early-onset dysmyelinating disorder with nystagmus, psychomotor delay, progressive spasticity, ataxia). GJA/GJC2 mutation I33 M causes a milder phenotype: late-onset (first and second decades), cognitive impairment, slowly progressive, spastic paraplegia, dysarthria, and upper extremity involvement. MRI and MR spectroscopy imaging consistent with a hypomyelinating leukoencephalopathy	Gap junction protein GJA12/GJC2, also known as connexin47 (Cx47)		(290)
SPG45 (10q24.3-q25.1)	Single consanguineous kindred from Turkey, five subjects described: affected subjects had mental retardation, infantile onset lower extremity spasticity and contractures, one subject with optic atrophy, two subjects with pendular nystagmus; MRI in one subject was normal	Unknown	No	(511)
SPG46 (9p21.2-q21.12)	Dementia, congenital cataract, ataxia, thin corpus callosum	Unknown	No	(512)
SPG47 (1p13.2-1p12)	Two affected siblings from consanguineous Arabic family with early childhood-onset slowly progressive spastic paraparesis, mental retardation, and seizures; one subject had ventriculomegaly, the other subject had thin corpus callosum and periventricular white matter abnormalities	Unknown	No	(513)
SPG48 (7p22.1)	Analysis of KIAA0415 gene in 166 unrelated spastic paraplegia subjects (38 recessive, 64 dominant, 64 “apparently sporadic”) and control subjects revealed homozygous mutation in two siblings with late-onset (6th decade) uncomplicated spastic paraplegia; and heterozygous mutation in one subject with apparently sporadic spastic paraplegia	KIAA0415	Lab	(316)
“SPOAN” syndrome (11q23)	Complicated: Spastic paraplegia, optic atrophy, neuropathy (SPOAN)	Unknown	No	(118)
5p15.31-14.1 No SPG designation	Complicated spastic paraplegia associated with mutilating sensory neuropathy	Epsilon subunit of the cytosolic chaperonin-containing t-complex peptide-1 (Cct5)	Lab	(119,450)
<b>X-linked HSP</b>				
SPG1 (Xq28)	Complicated: associated with mental retardation, and variably, hydrocephalus, aphasia, and adducted thumbs	L1 cell adhesion molecule (L1CAM)	Lab	(151)
SPG2 (Xq28)	Complicated: variably associated with MRI evidence of CNS white matter abnormality; may have peripheral neuropathy	Proteolipid protein (PLP)	Several labs <sup>b</sup>	(514–517)

**TABLE 119-1 Genetic Types of HSP—cont'd**

SPG16 (Xq11.2-q23)	Uncomplicated; or complicated: associated with motor aphasia, reduced vision, nystagmus, mild mental retardation, and dysfunction of the bowel and bladder	Unknown	No	(277,278)
SPG22 (Xq21)	Complicated (Allan–Herndon–Dudley syndrome): congenital onset, neck muscle hypotonia in infancy, mental retardation, dysarthria, ataxia, spastic paraplegia, abnormal facies	Monocarboxylate transport 8 (MCT8)	Lab	(518–520)
SPG34	Uncomplicated, onset 12–25 years			(521)
Maternal (mitochondrial) inheritance HSP				
No SPG designation	Adult-onset, progressive spastic paraplegia, mild to severe symptoms, variably associated with axonal neuropathy, late-onset dementia, cardiomyopathy.	Mitochondrial ATP6 gene	Lab	(212)

<sup>a</sup>ADL: Athena Diagnostics Laboratory, Boston.

<sup>b</sup>Several laboratories including Dupont Nemours Clinic and Baylor University; Lab = research laboratory testing.

Source: Updated from (522).

recessive forms) may manifest as both “complicated” and “uncomplicated” HSP syndromes.

Previously, Harding (56) proposed subclassifying autosomal dominant, uncomplicated HSP according to the age-of-symptom onset, the relative degrees of spasticity and weakness, and the rate of progression. According to this scheme, individuals with autosomal dominant uncomplicated HSP Type I had symptom onset before the age of 35, greater lower extremity spasticity than weakness, and slow progression. Individuals with type II uncomplicated HSP had symptom onset after age 35, significant lower extremity weakness in addition to spasticity, mild distal sensory loss, urinary bladder disturbance, and faster progression (56,58). Classification based on age-of-symptom onset is problematic because the age-of-symptom onset may be extremely variable even within a given family. We (59) and others (60,61) have observed HSP kindreds in which some family members developed symptoms in childhood and others in late adulthood, consistent with both Type I and Type II occurring within the same family. Furthermore, although *average* age-of-symptom onset in some forms of HSP is earlier (e.g., SPG3A, SPG10, SPG12) than others (SPG4, SPG6, SPG8, and SPG13), there is significant overlap in the *range of ages* at which symptoms first appear between “early” and “later” onset HSP (Table 119-2) (62). For these reasons, HSP is not classified by age-of-symptom onset alone.

*Complicated HSP syndromes* are those in which the heritable syndrome is predominated by insidiously progressive, bilaterally symmetric, lower extremity spastic weakness and accompanied by additional systemic or neurologic abnormalities (which are part of the heritable syndrome and not due to coexisting neurologic disorders). Spastic weakness in the legs of mild to severe degree is a feature of many unrelated neurologic syndromes that are not considered “complicated HSP.” For example, Machado–Joseph disease, early-onset familial Alzheimer’s disease due to presenilin 1 gene mutation, (63) and amyotrophic lateral sclerosis (ALS) may all have lower extremity spastic weakness, yet none are

considered “complicated HSP.” Diagnosis of uncomplicated or complicated HSP requires progressive lower extremity spastic weakness to be *the predominant clinical feature*; and the *exclusion (by clinical evaluation, laboratory studies, electromyography, nerve conduction testing, and neuroimaging) of alternative disorders*.

The “complicated spastic paraplegia” classification includes many diverse syndromes (Table 119-1) that undoubtedly represent many etiologically distinct disorders. In general, genetic analysis of complicated HSP syndromes has been hampered by the small size of individual families and by the likelihood of genetic heterogeneity. In addition to the syndromes listed (Table 119-1), there are many case reports of HSP associated with other systemic disorders (64–68), neurologic syndromes (e.g., restless leg syndrome (69), Parkinson’s (70), and ALS (71)). For individual kindreds of limited size, it is often uncertain whether two unrelated disorders simply coexist in the family such as HSP and epilepsy (72) or HSP and cardiac abnormalities (73); or whether the extra-spinal abnormalities are variant manifestations of HSP.

**119.1.5.1 Imperfect Correlation between Clinical and Genetic Classifications.** Classifying the clinical syndrome in an individual subject or family as “complicated HSP” or “uncomplicated HSP” is useful in terms of diagnostic recognition of the HSP syndrome, prognosis, and genetic counseling. Nonetheless, it is important to recognize that there is imperfect agreement between genetic and clinical classifications. For example, while some subjects with SPG7 autosomal recessive HSP (due to paraplegin gene mutation) and SPG10 HSP (due to kinesin heavy chain mutation) have uncomplicated HSP, other subjects with these types of HSP have complicated HSP syndromes (compare, e.g., descriptions of “uncomplicated” (74) and “complicated” (75)) SPG10 HSP due to KIF5A mutation; and complicated and uncomplicated forms of SPG7 HSP due to paraplegin mutation (76–79).

For an individual subject and their affected family members, the certainty of determining that an HSP syndrome is “uncomplicated” or “complicated” often

depends on the nature and extent of diagnostic evaluations (e.g., were nerve conduction studies and formal neuropsychometric studies performed in every subject?); and the extent of long-term, serial evaluation to observe the natural history of the disorder. Similarly, determining that an individual genetic type of HSP is always (e.g., 100%), usually (e.g., 50–100%), sometimes (e.g., 25–50%), occasionally (e.g., 10–25%), rarely (<10%), or never (0%) associated with either “uncomplicated” or “complicated” syndromes depends on long-term, serial evaluation of many individuals from ethnically diverse, unrelated families using detailed and standardized methods. With the exceptions of SPG1, SPG2, SPG3A, SPG4, SPG7, and SPG11, descriptions of most HSP types have been limited to one or several kindreds. Therefore, the phenotypic spectrum of most types of HSP must be considered incompletely known.

Rather than rigidly classifying a given type of HSP as “complicated” or “uncomplicated”, it is increasingly evident that various genetic forms of HSP are more *reliably* classified as *typically associated with uncomplicated HSP*, *variably associated with complicated HSP* (i.e., being associated with either uncomplicated or complicated HSP), *and typically associated with complicated HSP*. As this knowledge is unfolding, the certainty that a given type of HSP is “typically uncomplicated,” “variably complicated,” or “typically complicated” should consider the published number, size, and extent of evaluation of individuals and families with this type of HSP. Particular caution must be exercised (1) in too readily ascribing a variant neurologic or systemic symptom as representing a “complicated” feature of HSP (rather than investigating the symptom directly for alternative causes); (2) assuming that

**TABLE 119-2    Complicated HSP Syndromes<sup>a</sup>**

<b>Spastic Paraplegia and Peripheral Neuropathy</b>	
HSP types SPG2, 3A, 5, 7, 10, 25, 27, 30, 31, SPOAN, Cct5 (epsilon subunit), mutation, mitochondrial ATP6 gene mutation	See Table 119-1 and text for references
Spastic paraplegia, neuropathy, poikiloderma; autosomal dominant	(523)
Spastic paraplegia and peripheral neuropathy; autosomal dominant	(524)
Spastic paraplegia, early-onset painless perforating and mutilating ulcers of the hands and feet. Loss of pain and of the sensations of touch, heat, and cold over the feet. Shooting pains in the legs, and occasionally in arms. Probably autosomal recessive	(122,525)
Spastic paraplegia and sensory neuropathy with mutilating ulcerations of the hands and feet. Possibly autosomal dominant	(526)
Early onset of paraparesis (first decade), peripheral neuropathy, depigmented areas of skin and hair. Probably autosomal recessive	(527)
Spastic paraplegia, mental retardation, distal motor neuropathy. Autosomal recessive	(267)
<b>Spastic paraplegia and distal amyotrophy</b>	
HSP types SPG3A (rare feature of), 4 (rare feature of), 5, 10, 14, 15, 17, 20, 26, 30, 38, 39, 41, 43	See Table 119-1 and text for references
Spastic paraplegia, onset in the second decade with slight progression, normal life span, amyotrophy of the hands; autosomal dominant	(525)
Spastic paraparesis with distal muscle wasting, microcephaly, mental retardation, arachnodactyly, tremor; autosomal recessive	(528)
Spastic paraplegia with peroneal amyotrophy and fever-induced episodes of pain, dysethesia, tetraparesis; autosomal recessive	(529)
Spastic paraplegia (nonprogressive, infantile onset), distal muscle wasting, mental retardation, short stature; probably autosomal recessive	(530)
Spastic paraplegia, mental retardation, distal motor neuropathy; autosomal recessive	(267)
<b>Spastic paraplegia and mental retardation</b>	
HSP types SPG 1, 11, 14, 16, 18, 20, 22, 26, 27, 32, 44, 45, 47	See Table 119-1 and text for references
Spastic paraplegia with glaucoma and mental retardation; probably autosomal recessive	(531)
Spastic paraplegia, dysarthria, ataxia, mental retardation, pigmentary macular degeneration; probably autosomal recessive	(532)
Mental deficiency and spastic paraplegia; autosomal recessive	(533)
Spastic paraplegia (nonprogressive, infantile onset), distal muscle wasting, mental retardation, short stature; probably autosomal recessive	(530)
Spastic paraparesis (childhood onset), dysarthria, mental retardation, cerebellar ataxia, tremor, and periventricular white matter abnormalities; probably X-linked	(534)
Spastic paraplegia, mental retardation, precocious puberty; autosomal recessive	(535)
Spastic paraplegia, mental retardation, distal motor neuropathy	(267)
<b>Spastic paraplegia and dementia</b>	
SPG4, 15, 21, 35, 46, and Mitochondrial ATP6 gene mutation	
Spastic paraplegia, dementia, cardiac abnormalities; autosomal recessive	(73)
Adult-onset-HSP dementia and thalamic degeneration; autosomal dominant	(536)

**TABLE 119-2    Complicated HSP Syndromes<sup>a</sup>—cont'd**

<b>Spastic paraplegia and blindness</b>	
HSP types SPG15, 16, 45, 46, and SPOAN syndrome	See Table 119-1 and text for references
Spastic paraplegia with retinal degeneration; autosomal recessive	(537)
Spastic paraplegia, optic atrophy, dementia; autosomal dominant	(538)
Bilateral hypoplasia of the optic nerve. Pupillary light-near dissociation, peripheral neuropathy, fasciculations, cardiomyopathy	(539)
Spastic paraplegia, dysarthria, ataxia, mental retardation, pigmentary macular degeneration; probably autosomal recessive	(532)
Spastic paraplegia, mental retardation, amyotrophy, pigmentary retinal degeneration	(257)
Macular degeneration and mild mental retardation; autosomal recessive	(525,540–543)
Spastic paraplegia, short stature, seizures, retinal degeneration, ichthyosiform skin changes; autosomal recessive	(544,545)
<b>Spastic paraplegia and deafness</b>	
SPG29	
Spastic paraplegia, bilateral sensorineural deafness, and intellectual retardation associated with a progressive nephropathy; autosomal dominant	(546)
Spastic paraparesis with short stature, hypogonadism, lens opacities, and deafness	(547)
<b>Spastic paraplegia and skeletal abnormalities</b>	
SPG25	
Skeletal anomalies of the hands and feet (brachydactyly, cone-shaped epiphyses), and dysarthria	(548)
Spastic paraparesis, macrocephaly, distinct craniofacial appearance; autosomal recessive	(549)
<b>Spastic paraplegia and extrapyramidal movement disorder</b>	
HSP types SPG21, 35	
Rhythmic myoclonus of palate, pharynx, larynx, and face; truncal ataxia; probably autosomal dominant	(550)
Spastic paraparesis and extrapyramidal movement disorder; probably autosomal dominant	(525), Dick and Stevenson (1953)
Spastic paraplegia onset second-third decade: basal ganglion manifestations such as dysarthria, and athetosis <sup>a</sup> ; autosomal recessive	(525)
Spastic paraplegia (nonprogressive, infantile onset), distal muscle wasting, mental retardation, short stature; probably autosomal recessive	(530)
<b>Spastic paraplegia and epilepsy</b>	
HSP types SPG35, SPG47	
Spastic paraplegia with epilepsy	(72)
Spastic paraplegia, short stature, seizures, retinal degeneration, ichthyosiform skin changes (Sjogren–Larsson syndrome); autosomal recessive	(544,545)
<b>Spastic paraplegia and dysarthria</b>	
HSP types SPG7, 15, 22, 24, 27, 35, 43, 44,	
Spastic paraplegia and ataxia	See Table 119-1 and text for references
HSP types SPG7, 21, 22, 27, 30, 32, 46	
Spastic paraplegia (early onset) with ataxia, dysarthria, nystagmus, mitral valve prolapse, muscular atrophy; autosomal recessive	(525)
Spastic paraplegia with thin corpus callosum, dementia, ataxia, peripheral neuropathy; autosomal recessive	(551)
<b>Spastic paraplegia and hematologic abnormality</b>	
Spastic paraplegia and May–Hegglin anomaly: cytoplasmic inclusions in leukocytes, giant platelets, and thrombocytopenia; autosomal dominant	(552)
Spastic paraparesis and Evan’s syndrome (Coombs-positive hemolytic anemia and immune thrombocytopenia without a known underlying etiology); probably autosomal recessive	(553)
<b>Spastic paraplegia and MRI brain abnormalities</b>	
HSP types SPG1, 2, 5, 7 (variably abnormal), 11, 15, 18, 21, 32, 35, 44, 46, 47	
<b>Spastic paraplegia and skin abnormalities</b>	
SPG23	
Short stature: seizures, retinal degeneration, ichthyosiform skin changes (Sjogren–Larsson syndrome); autosomal recessive	(544,545)
SPG23 HSP: Childhood-onset spastic paraplegia associated with skin pigment abnormality; autosomal recessive	(263)
Early onset of paraparesis (first decade), peripheral neuropathy, depigmented areas of skin and hair; probably autosomal recessive	(527), Abdallat et al. (1867)
Spastic paraparesis with skin manifestations: vitiligo, hyperpigmentation of exposed areas, multiple lentigines, premature graying of hair, café-au-lait spots; probably autosomal recessive	(554)
Spastic paraplegia, neuropathy, poikiloderma; autosomal dominant	(523)
<b>Spastic paraplegia and endocrine disturbance</b>	
Spastic paraplegia and Kallman syndrome (hypogonadotrophic hypogonadism and anosmia)	(555)

<sup>a</sup>Note that the “complicating feature” may be frequently or infrequently associated with a specific genetic type of HSP.



additional neurologic impairments do not sometimes occur in forms of “uncomplicated” HSP; and (3) assuming (and so counseling subjects and families) that all subjects with a “typically complicated” type of HSP will experience the “complicating” feature (such as mental retardation, epilepsy, or dementia).

**119.1.5.2 Practical Syndrome Recognition: Six HSP Syndromes.** Forms of HSP for which genetic loci have been identified are listed in [Table 119-1](#). On a practical, clinical basis, it is useful to approach the numerous clinical and genetic types of HSP by recognizing six HSP syndromes: (1) uncomplicated HSP; (2) spastic paraplegia associated with peripheral motor neuropathy and/or distal wasting; (3) spastic paraplegia associated with cognitive impairment; (4) spastic paraplegia associated with ataxia; (5) spastic paraplegia associated with neuroimaging abnormality; and (6) spastic paraplegia associated with additional neurologic and systemic abnormalities.

### 119.1.6 Clinical Features of Uncomplicated HSP

Subjects with uncomplicated HSP experience insidiously progressive weakness and increased muscle tone in their legs. These symptoms may begin at any age from early childhood, for example [\(80\)](#), until late adulthood. Gait becomes progressively impaired over many years and subjects often require canes, walkers, or wheelchairs. Urinary urgency and lower extremity paresthesia often occur. In uncomplicated HSP, weakness and paresthesia involve the legs only. In subjects with uncomplicated HSP, there is no weakness, increased muscle tone, slowing of movements, or loss of dexterity or coordination in the upper extremities. Dysphagia and dysarthria are similarly absent in uncomplicated HSP (see [\(24,81–83\)](#) for examples of typical clinical features of uncomplicated HSP).

There is an important exception to the generalization that HSP inevitably causes progressive motor impairment. Many subjects with very early-onset HSP experience very little worsening even over several decades. The disorder in these individuals may resemble that of spastic diplegic cerebral palsy. This is particularly common in SPG3A HSP but also has been described in several other types of HSP [\(60,84\)](#).

*Neurologic examination of uncomplicated HSP subjects* demonstrates bilateral lower extremity weakness and increased muscle tone that is maximal in iliopsoas, hamstrings, and tibialis anterior muscles and which is approximately symmetric; bilateral lower extremity hyperreflexia and bilateral extensor plantar responses. Mildly diminished vibration sensation in distal lower extremities is frequently observed in HSP although, as noted by Brugman et al. [\(85\)](#), may also be present in subjects with primary lateral sclerosis (PLS).

Although upper extremity deep tendon reflexes are often brisk (grades two to three on zero to four scale), it is extremely rare for subjects with uncomplicated HSP to

develop weakness, increased muscle tone, or decreased dexterity of upper extremities. Significant upper extremity weakness is more indicative of motor neuron disorders or PLS than uncomplicated HSP. Similarly, dysphagia, dysarthria, and cranial nerve abnormalities are not features of uncomplicated HSP.

It is not uncommon for subjects with uncomplicated HSP to have decreased muscle mass in distal lower extremities (particularly the shins). While this may reflect disuse atrophy, it may also reflect involvement of anterior horn cells or peripheral motor axons. Slight loss of anterior horn cells has been observed at postmortem examination of uncomplicated HSP (discussed below). Nonetheless, other features of motor neuron disease (muscle cramps, fasciculations, and prominent amyotrophy) are not features of uncomplicated HSP.

*Pes cavus* is often but not invariably present in uncomplicated HSP. *Pes cavus* and hammer toes (hallux flexus) may be prominent in older affected subjects, or conversely, may be absent even in severely affected subjects.

Slight terminal dysmetria on finger-to-nose testing may be present in elderly subjects with otherwise uncomplicated HSP. Tedeschi et al. [\(86\)](#) noted abnormal saccadic eye movements in subjects with otherwise uncomplicated HSP. The occurrence of functionally significant cerebellar disturbance, such as wide-based gait, dysarthria, or oculomotor disturbance, co-inherited with spastic paraplegia would be inconsistent with classification as “uncomplicated” HSP and could represent an olivopontocerebellar atrophy, spinocerebellar ataxia (SCA), or a complicated form of HSP (such as SPG7 HSP due to paraplegin gene mutation).

Subclinical cognitive disturbance and dementia have been reported in some subjects with otherwise uncomplicated HSP. Tedeschi et al. [\(86\)](#) observed cognitive disturbance in five of seven uncomplicated HSP patients, and Lizcano-Gil et al. [\(87\)](#) reported two kindreds with late-onset spastic paraparesis and dementia in whom identification of the genetic locus was not performed. Cognitive disturbance and dementia have been described in uncomplicated autosomal dominant HSP kindreds linked to the SPG4 locus as discussed below [\(88–92\)](#). In general, clinical features of dementia in HSP have included both memory impairment and patterns of behavioral disturbance suggestive of frontotemporal dementia [\(93,94\)](#). Frontotemporal dementia [reviewed in [\(95\)](#)] frequently accompanies ALS, a motor neuron disorder whose neuropathology overlaps that of HSP. Nonetheless, it is not known if dementia in some subjects with HSP is an extra-spinal manifestation of HSP; or represents the occurrence of a separate disorder (such as senile dementia of the Alzheimer’s type) co-segregating with HSP in these kindreds.

**119.1.6.1 Atypical Presentations for “Uncomplicated” HSP.** Additional neurologic impairments may occur in genetic types of HSP typically associated with uncomplicated spastic paraplegia. This phenotype

variation may occur within a family in which all affected individuals share the same HSP gene mutation. The full range and frequency of such atypical presentations and whether each is uniquely associated with a specific mutation is not known. Because SPG3A HSP and SPG4 HSP typically manifest as “uncomplicated” HSP syndromes, the occurrence of additional neurologic abnormalities is particularly noteworthy with these forms of HSP.

**119.1.6.1.1 Cognitive Disturbance in SPG4 HSP.** Most often, subjects with SPG4-linked HSP exhibit uncomplicated spastic paraplegia that begins at any age from childhood to senescence (average age of onset 26–35) (21,59,62,89,92,96–98). Nonetheless, subclinical cognitive disturbance, mental retardation, and dementia have been reported in some SPG4 kindreds (88,89,92,99,100). Scheur et al. (101) reported decreased regional (frontotemporal cortex) cerebral blood flow in SPG4 HSP patients whose neuropsychometric impairment was confined to recognition of faces. Ribai et al. (102) reported 13 patients from three SPG4 HSP families who had cognitive impairment. Among these, one had mental retardation, two had isolated psychomotor delay, and ten had social dependence (five of whom required institutional care). The occurrence of cognitive impairment in some of these SPG4 HSP subjects may be related to a modifying gene because their SPG4 mutations (p.Arg459Thr or p.Arg499Cys) had been previously reported in uncomplicated HSP subjects. It is not known whether nonprogressive cognitive impairment and dementia are unique to SPG4 HSP are more commonly associated with SPG4 HSP compared to other types of “typically uncomplicated” HSP.

**119.1.6.1.2 Unusual Manifestations of SPG4 HSP.** Ataxia, ALS, Thin Corpus Callosum, Muscle Atrophy, Peripheral Sensory-Motor Neuropathy, and Congenital Arachnoid Cysts. As noted above, the single most common cause of autosomal dominant HSP, SPG4 HSP due to SPG4/spastin gene mutation is usually associated with an uncomplicated HSP syndrome. Nonetheless, ataxia, cerebellar atrophy, and posterior fossa developmental abnormality have been reported in SPG4 HSP. For example, Nielsen et al. (103) reported a novel SPG4/spastin mutation (GLN490Stop) in subjects with autosomal dominant HSP associated with cerebellar ataxia. Scuderi et al. (104) reported a kindred with SPG4 due to five base pair deletion in exon 9 (1215-1219delTATAA) in which seven of nine affected subjects had large retrocerebellar fluid collection that was accompanied in three subjects by defect of the posterior part of the tentorium and occipital bone modifications. This report included an additional, unrelated subject with *de novo* SPG4 mutation (p.R581X), who had hypoplasia of the cerebellar vermis.

Alber et al. (105) reported dysplasia of the corpus callosum in one subject with autosomal dominant HSP who had SPG4/spastin mutation C1120A. Other spastic paraplegia subjects in this family did not have corpus

callosum dysplasia, however. Orlacchio et al. (106) also reported congenital arachnoid cysts in HSP subjects with SPG4/spastin T614I mutation.

**119.1.6.1.3 SPG4 HSP Associated with Lower Motor Neuron Involvement Including Peripheral Neuropathy and Muscle Atrophy.** Orlacchio et al. (107) reported peripheral sensory-motor neuropathy and cognitive impairment in subjects with SPG4 906delT frameshift mutation. They also reported (50) a kindred with SPG4 mutation (frameshift mutation p.Asp321GlyfsX6) in which affected subjects exhibited muscle wasting in the hands, cognitive impairment, and temporal lobe epilepsy. The authors noted that distal muscle wasting associated with this SPG4 mutation conformed to “Silver Syndrome” (dominantly inherited spastic paraplegia and distal muscle wasting) (39–41). In light of previous description of distal muscle wasting in subjects with SPG17/BSCL2 mutations (41), this finding indicates that Silver syndrome is genetically heterogeneous.

Meyer et al. (71) described an individual with early-onset, slowly progressive ALS who had spastin gene mutation (c.304\_309dupGCCTCG). See below for further description of the relationship between HSP and ALS.

#### **119.1.6.1.4 Unusual Manifestations of SPG3A HSP.**

**119.1.6.1.4.1 Motor-Sensory Axonal Neuropathy, Distal Wasting, Thin Corpus Callosum, Hereditary Sensory Neuropathy.** Motor-sensory axonal neuropathy, ranging from subclinical involvement evident only on electromyography and nerve conduction studies (which is not uncommon) to severe distal wasting (very rare) is recognized increasingly in SPG3A HSP. Ivanova et al. (108) reported that motor-sensory axonal neuropathy was relatively common (17% of affected subjects) in SPG3A HSP and associated with a number of different SPG3A/atlastin mutations (F151S, Q191R, M408 T, G469A, R495 W). Motor sensory neuropathy was particularly severe in one subject [with M408 T SPG3A mutation (109)] who had severe motor impairment, delayed motor milestones, a combination of global hypotonia and hyperreflexia, marked muscle distal atrophy in all limbs, impaired upper extremity dexterity, dysphagia, and dysarthria. Interestingly, a similar phenotype (although less severe) including impairment of fine hand movements, distal atrophy in the upper extremities, and chronic denervation pattern on electromyography was associated with a separate mutation disturbing this same amino acid (M408 V) (110). Axonal neuropathy has also been reported with SPG3A mutations M347T, R495 W, and S398F (111–113).

Recently, Guelly et al. (114) reported that SPG3A/atlastin mutation p.Asn355Lys was associated with the syndrome autosomal dominant hereditary sensory neuropathy type 1 (HSN 1) rather than with the syndrome of HSP. HSN 1 is an autosomal dominant disorder characterized by marked, early sensory loss (which may be

associated with painless injuries, impaired healing, and osteomyelitis that necessitate amputations). Distal motor involvement may occur in advanced cases (114). The report of Guelly et al. (114) significantly expands the phenotypic spectrum that may be associated with SPG3A/atlastin mutations. These phenotypes include childhood-onset, relatively nonprogressive, uncomplicated spastic paraplegia that resembles spastic diplegic cerebral palsy (the most common SPG3A HSP syndrome); adult-onset, progressive uncomplicated spastic paraplegia (115); spastic paraplegia associated with motor-sensory axonal neuropathy (111); and dominantly inherited sensory neuropathy without spastic paraplegia (114).

The finding that mutation in SPG3A can manifest as sensory neuropathy highlights three other associations between spastic paraplegia and sensory neuropathy. First, subclinical sensory neuropathy has been described in otherwise uncomplicated HSP (116,117). Second, sensory neuropathy, which may be severe, is a feature of SPOAN (spastic paraplegia, optic atrophy, neuropathy) syndrome, linked to chromosome 11q23 (118). And finally, mutations in the *Cct5* gene cause “mutilating sensory neuropathy with spastic paraplegia” due to *Cct5* gene mutation (119–122).

Thin corpus callosum and mental retardation are common features of SPG11 HSP due to Spatacsin mutation (see Table 119-1). Orlacchio et al. (8) reported thin corpus callosum in a South African Zulu family with SPG3A mutation (R416C). In addition to SPG3A and SPG11 HSP, thin corpus callosum has also been described in many types of HSP including SPG4, SPG7, SPG15, SPG21, SPG32, and SPG47 (see Table 119-1).

### 119.1.7 Neurophysiology

*Electromyography and nerve conduction studies.* Although results of these studies are quite variable (both between genetic types of HSP and often within individual families), a number of generalizations can be made. Nerve conduction studies in uncomplicated HSP are usually normal (123–125). Schady and Scheard (116) and Schady and Smith (117), however, found subclinical sensory neuropathy in otherwise uncomplicated HSP. Tredici and Minoli (126) observed degenerative changes in the large myelinated fibers, onion bulb formations, and clusters of small myelinated fibers in a sural nerve biopsy in a 16-year-old subject with autosomal dominant HSP, who had borderline slowing of motor and sensory nerve conduction velocities.

There is increasing awareness that peripheral neuropathy (clinically evident or subclinical) is a feature of many types of HSP, including some of those previously considered to be “uncomplicated.” For example, Ivanova et al. (108) reported motor-sensory axonal neuropathy in 17% of SPG3A HSP subjects. At this stage, the presence of clinically symptomatic peripheral neuropathy would warrant classification as a complicated HSP syndrome

(if neuropathy were part of the inherited syndrome and not due to a coexisting disorder such as diabetes). Nonetheless, it should be noted that nerve conduction studies have not been performed in most subjects with uncomplicated HSP from the various genetic types. Therefore, the true incidence of subclinical peripheral sensory and/or motor nerve involvement in otherwise uncomplicated HSP is not known.

*Somatosensory evoked potentials* (SSEPs) from the lower extremities often, but not always, show conduction delay in dorsal column fibers (123,127–130). In contrast, SSEPs from the upper extremities are usually normal.

*Cortical evoked potentials* [reviewed in (131)], are used to measure the sensitivity of cortical motor neurons and the neurotransmission of corticospinal tracts. These studies have shown greatly reduced conduction velocity and amplitude of evoked potential when stimulating corticospinal tracts subserving the legs (129,131–135). Often, there is no cortical evoked potential elicited in muscles innervated by lumbar spinal segments. These findings indicate that there are decreased numbers of corticospinal tract axons reaching the lumbar spinal cord and that the remaining axons have reduced conduction velocity.

In general, cortical evoked potentials of the arms are usually normal or show only mildly reduced conduction velocity (136). This generalization may not apply to all types of HSP. Recently, for example, Manganelli et al. (137) reported abnormal cortical evoked potentials in both upper and lower extremities (as well as somatosensory, visual, and brainstem evoked potentials) in subjects with SPG5 HSP.

Jorgensen et al. (138) compared motor-evoked potentials in 29 subjects with multiple sclerosis and nine subjects with SPG4 HSP. Using various stimulus intensities, there was no difference in recruitment of motor-evoked potentials between multiple sclerosis and HSP subjects, indicating that this method could not be used to distinguish between these disorders.

Several investigators have reported the peroneal H reflex to be a marker of upper motor neuron involvement in HSP (81,125,134).

Following transcranial magnetic stimulation-induced muscle contraction, there is a period in which voluntary muscle contraction is suppressed. This period, evident by the absence of EMG activity, is referred to as the cortical silent period (CSP) (139) and considered to reflect inhibitory cortical mechanisms (see (139) for methods and discussion of CSP). Sartucci et al. (134) studied 12 subjects with SPG4 HSP and observed that following transcranial magnetic stimulation, the CSP was significantly reduced when measured in the legs and that this correlated with clinical assessments of spasticity. CSP measurements from the hands were normal. Sartucci et al. (134) concluded that shortened CSP reflected impairment of supraspinal or intracortical inhibitory pathways, together with increased segmental motor neuron excitability, and that CSP may be a useful physiologic marker of spasticity.

**119.1.7.1 Central Motor Reorganization in HSP.** As stated by Konrad (140), “plasticity as an intrinsic property of the central nervous system can also be expected in degenerative motor neuron diseases.” Functional reorganization of cortical and subcortical motor networks has been demonstrated in ALS (141,142), and recently in HSP. Using positron emission tomography to study 13 subjects with SPG4 HSP, Scheuer et al. (143) demonstrated that increased activation of cerebral blood flow (in bilateral primary motor cortices, supplementary motor areas, and the right premotor cortex compared to controls) only occur during movement of affected ankles, but not during movements of the unaffected shoulders. These results suggest that upper motor neuron degeneration in SPG4 HSP (and presumably other types of HSP) is sufficient to induce cortical reorganization. Whether the process of cortical reorganization itself is influenced by HSP gene mutation and the extent to which cortical reorganization contributes to clinical manifestations of HSP (such as influencing the apparent rate of progression or response to therapy) are unknown.

### 119.1.8 Neuroimaging

Brain and spinal cord MRI scan are important in the clinical evaluation of subjects suspected as having HSP as part of the exclusion of alternative or coexisting disorders (see Table 119-3).

**119.1.8.1 Spinal Cord MRI.** The primary neuropathology of uncomplicated HSP is axonal degeneration that is maximal in the ends of the lateral corticospinal tracts in the thoracic spinal cord; and the ends of *fasciculus gracilis* fibers in the cervical-medullary region. This axonal degeneration may be severe enough to affect the cross-sectional area of the spinal cord on magnetic resonance imaging (MRI). Spinal cord MRI in HSP subjects has been normal (144); or has shown mild to marked atrophy of cervical and thoracic segments (31,145–148). For example, Hedera et al. (146) reported significantly reduced cervical (C2) and thoracic (T9) spinal cord cross-sectional areas in subjects with four different types of autosomal dominant HSP (SPG3A, SPG4, SPG6, and SPG8). Spinal cord atrophy was more prominent in patients with SPG6 and SPG8 than in subjects with SPG3 and SPG4.

Vassilopoulos et al. (149) reported decreased sagittal and transverse diameter of cervical and thoracic spinal canal in HSP subjects compared to normal control subjects. They concluded that this indicated that the patterns of growth of spinal cord (which may be atrophic in HSP) and the spinal canal are parallel.

**119.1.8.2 Brain MRI.** Although conventional brain MRI is usually normal in uncomplicated HSP, detailed measurements (using brain parenchymal fractions calculated from three-dimension MRI data) have shown reduced brain volumes in both uncomplicated and complicated

**TABLE 119-3 Differential Diagnosis of HSP**

Category	Disorder	Clinical Features that Differentiate from HSP	Diagnostic Evaluation
Structural spinal cord abnormality	Arnold–Chiari malformations	Ataxia	Magnetic resonance imaging (MRI) of posterior fossa
	Cervical or lumbar spondylosis	Involvement of upper extremities; radiculopathy	Spine X-rays and MRI
	Tethered cord syndrome		MRI of conus medullaris
	Neoplasm involving spinal cord	Pain, loss of sensation, asymmetric involvement	MRI of spinal cord
	Spinal cord arteriovenous malformation	Saltatory progression; spinal sensory level	MRI, spinal arteriography
	Granuloma (e.g., Tuberculosis) involving vertebrae with spinal	Back pain, subacute course	Spine X-rays and MRI
Degenerative diseases	Multiple sclerosis	Exacerbations, remissions, ataxia, optic neuritis, Lhermitte’s sign	MRI, cerebrospinal fluid examination, visual evoked potentials
	Amyotrophic lateral sclerosis	Fasciculations, amyotrophy	Electromyography and nerve conduction testing
	Primary lateral sclerosis	PLS is typically sporadic. HSP may have dorsal column disturbance which is not a feature of PLS.	Family history, dorsal column impairment (evident clinically or with somatosensory evoked potentials)
	SCA including Machado–Joseph disease	Ataxia, dysarthria	Prominent ataxia; Machado–Joseph disease also has extrapyramidal involvement
	Friedreich’s ataxia	Peripheral neuropathy	FRDA gene testing

*Continued*



TABLE 119-3 Differential Diagnosis of HSP—cont'd

Category	Disorder	Clinical Features that Differentiate from HSP	Diagnostic Evaluation
Metabolic disorders <sup>a</sup>	Adrenoleukodystrophy (ALD), adrenomyeloneuropathy (AMN)	ALD and AMN are X-linked. In childhood-onset a/d progressive cognitive impairment and peripheral neuropathy accompany corticospinal tract signs. In adolescent/adult-onset AMN, cognition is normal peripheral neuropathy is variable.	Serum very long chain fatty acid analysis
	Metachromatic leukodystrophy (MLD)	MLD (autosomal recessive) in children is typically associated with psychomotor regression and peripheral neuropathy.	MRI (brain), arylsulfatase
	Krabbe (globoid cell) leukodystrophy	Krabbe disease is an autosomal recessive disorder. Peripheral neuropathy, a feature of childhood-onset Krabbe disease, may be absent in adolescent/adult-onset Krabbe disease.	MRI (brain), galactocerebrosidase
	Subacute combined degeneration	Peripheral neuropathy, marked dorsal column involvement	Serum vitamin B12 concentration
	Mitochondrial encephalomyopathy	Short stature, retinitis pigmentosa, and multiple stroke-like episodes may help distinguish this diverse group of disorders from HSP	MRI (brain), serum lactate and pyruvate
	Abetalipoproteinemia (Bassen–Kornzweig disease)	Peripheral neuropathy	Lipoprotein electrophoresis
	Methylenetetrahydrofolate reductase deficiency	Psychiatric disturbance, spastic paraplegia, neuropathy; thromboemboli	Hyperhomocysteinemia, hypomethioninemia, low folic acid
	Cobalamin C defect	Psychiatric disturbance, peripheral neuropathy, dorsal column impairment, retinitis pigmentosa	Hyperhomocysteinemia, hyomethioninemia, methylmalonic aciduria
	Arginase deficiency and	Episodic nausea, vomiting, headache, confusion triggered by high protein intake	Hyperargininemia, (variable hyperammonemia
	Hyperornithinemia-hyperammonemia-hypercitrullinemia)	Episodic nausea, vomiting, triggered by high protein intake; ataxia, mental retardation	Hyperammonemia
	Biotinidase deficiency	Optic atrophy, deafness, neuropathy, dermatitis, alopecia	Serum biotinidase activity
	Phenylketonuria	Extrapyramidal disturbance, dementia, mental retardation	Serum phenylalanine
	Nonketotic hyperglycinemia	Mental retardation, ataxia, neuropathy, optic atrophy	Serum glycine
	Central folate deficiency	Extrapyramidal disturbance, deafness, ataxia, atrophy	Low 5-methyltetrahydrofolate concentration in CSF
	Cerebrotendinous xanthomatosis	Juvenile cataracts, tendon xanthoma, peripheral neuropathy, leukodystrophy	Serum cholestanol
	Vitamin E deficiency	Peripheral neuropathy	Serum vitamin E concentration
Infectious diseases	Tertiary syphilis (hypertrophic pachymeningitis)		VDRL, FTA
	Tropical spastic paraparesis	Subacute course	HTLV-I antibodies
	Acquired immunodeficiency syndrome (AIDS)	Subacute course	HIV antibodies
Miscellaneous	DOPA-responsive dystonia	Diurnal fluctuation; response to low-dose levodopa-carbidopa	Therapeutic trial of low-dose levodopa-carbidopa

<sup>a</sup>See Reference (219).  
Modified from (59).

HSP subjects (150). Brain MRI in HSP subjects may also disclose syndrome-specific abnormalities including hydrocephalus [typical of X-linked SPG1 HSP due to L1CAM mutation (151,152)], thin corpus callosum [discussed below, common in SPG11 but also reported in SPG3A, SPG4, SPG7, SPG15, SPG21, SPG32, SPG47, and the syndrome “HSP with thin corpus callosum and epilepsy” (8,153,154)], and cerebellar atrophy (which is frequent in SPG7 HSP (155) and uncommon in SPG4 HSP (103)).

*Diffusion Tensor Imaging (DTI)* in the brain of HSP subjects has shown evidence of corticospinal tract degeneration. Duning et al. (100) performed DTI in six uncomplicated SPG4 HSP subjects and observed widespread white matter disturbance primarily affecting corticospinal tracts. Interestingly, these white matter abnormalities were present in subtly affected subjects (with isolated hyperreflexia) who had pathogenic SPG4 mutations. Similarly, Unrath (156) performed DTI in 24 HSP subjects (six with SPG4/spastin mutations) and reported predominant deterioration of corticospinal tracts in addition to widespread white matter abnormalities.

DTI has shown more widespread involvement in complicated HSP due to SPG11/spatacsin mutation. Chen et al. (157) performed DTI in two SPG11 HSP subjects with thin corpus callosum and observed increased mean diffusivity and fractional anisotropy to be much more widespread (brainstem, internal capsule, cingulum and subcortical white matter including superior longitudinal fascicle and inferior longitudinal fascicle) than previously recognized (corpus callosum, cerebellum, and thalamus) or evident on conventional MRI.

**119.1.8.3 Magnetic Resonance Spectroscopy.** Erichsen et al. (158) reported abnormal brain magnetic resonance spectroscopy in a study of eight subjects with SPG4 HSP and reported abnormal brain. They observed that the choline-to-creatine ratio was significantly reduced in motor cortex of HSP subjects (compared to control subjects); and that reduced choline-to-creatine ratio was associated with reduced verbal learning and memory in the SPG4 HSP subjects.

Dreha-Kulaczewski et al. performed serial magnetic resonance spectroscopy and DTI in a 25-year-old subject with adolescent-onset spastic paraplegia, dementia, and dysarthria. Genetic type of HSP was not determined (159). Magnetic resonance spectroscopy of white matter revealed metabolic abnormalities (reduced concentrations of N-acetylaspartate and N-acetylaspartyl-glutamate, creatine and phosphocreatine, and choline-containing compounds and elevated levels of myo-inositol) that showed progression over 5 years. DTI showed increased diffusivity and reduced fractional anisotropy in periventricular white matter. These findings were interpreted as indicating axonal degeneration and astrocyte proliferation in white matter.

**119.1.8.4 Variable Degree of White Matter Disturbance Occurs in Some Types of HSP.** It is noteworthy that spinal cord and brain MRI in uncomplicated HSP usually do not show evidence of demyelination (despite neuropathologic evidence of myelin loss commensurate with axonal degeneration in the spinal cord). On the other hand, white matter abnormalities have been observed in many forms of complicated HSP (e.g., due SPG5/CYPB7, SPG7/Paraplegin, SPG21/Masparidin, and SPG35/FA2H gene mutations, see Table 119-1) (78,155,160–163).

In humans, heterozygous mutation of the spastin gene causes distal axon degeneration in SPG4 HSP, the single most common form of dominantly inherited HSP. Mild demyelination of corticospinal tracts is also noted and is considered commensurate with, and secondary to, axon degeneration. In contrast, in congenital bovine dysmyelination, homozygous mutation of the bovine spastin gene causes severe dysmyelination (164). It is not clear why homozygous spastin mutation in cattle causes predominant dysmyelination while heterozygous spastin mutation in humans causes primarily axonal degeneration. Our SPG4/spastin mutant mice did not show extensive demyelination in homozygous mutant animals (J. K. Fink, et al. unpublished studies). If this can be extrapolated to cattle, it is therefore unlikely that dysmyelination is due to homozygosity of the spastin mutation alone.

Although white matter abnormalities may be evident in brain MRI in some forms of HSP, evidence of significant, generalized demyelination should prompt further diagnostic evaluation for leukodystrophies unrelated to HSP. For example, extensive white matter disturbance in subjects with X-linked spastic paraplegia would raise the possibility of adrenomyeloneuropathy or proteolipid protein (PLP) gene mutation (causes allelic disorders X-linked, SPG2 HSP, and the dysmyelinating disorder, Pelizaeus–Merzbacher, discussed below).

As noted above, the primary pathology of uncomplicated (and many forms of complicated) HSP is axon degeneration (maximal at the distal ends of corticospinal tracts and dorsal column fibers). Nonetheless, for most types of HSP, it is not known whether axon degeneration is “cell autonomous” (due to intrinsic disturbances only in neurons) or “non-cell autonomous” (axon degeneration depends in part on interaction with nonneuronal cells). Some HSP genes are primarily expressed in oligodendroglia and not neurons (e.g., PLP and GJA12/Connexin 47, mutations in which cause SPG2 and SPG42, respectively). Proteins encoded by these genes are important factors in myelin formation. These examples indicate that axon degeneration in these types of HSP is non-cell autonomous; and furthermore, underscore the concept that primary disturbance of myelin can result in axon abnormalities.

*Thin corpus callosum* (particularly its anterior portion) initially was identified as a very frequent (but not constant) feature of SPG11 HSP (e.g., (159,165–169)).

Subsequently, thin corpus callosum has been reported in many forms of HSP, including “typically uncomplicated” types (SPG3A and SPG4) (8,105); and “typically complicated” types of HSP (SPG7, SPG15, SPG21, SPG32, and the syndrome “HSP with thin corpus callosum and epilepsy” (153,154)). Whereas thin corpus callosum in SPG11 HSP is usually associated with cognitive impairment (studied in detail in two subjects by Siri et al. (170)), thin corpus callosum in other types of HSP is not always associated with cognitive impairment. For example, Alber et al. (105) reported thin corpus callosum in one of two affected subjects with SPG4 HSP (due to Spastin mutation C1120A) that was not associated with cognitive impairment.

Rather than being simply a coexisting feature accompanying some forms of HSP, corpus callosal abnormalities may contribute to spastic gait. As noted by van Swigchem et al. (171), damage to the corpus callosum is frequent in spastic diplegic cerebral palsy. These authors found that transcallosal inhibition (suppression of voluntary electromyographic activity in ipsilateral muscles after focal transcranial magnetic stimulation) in the upper extremities was abnormal in cerebral palsy patients with upper extremity involvement, but was normal in subjects with HSP who did not have upper extremity involvement or thin corpus callosum. It is tempting to speculate that transcallosal inhibition of the lower extremities contributes to spasticity in subjects with HSP who have thin corpus callosum.

### 119.1.9 Neuropathology

Schwarz (172), Schwarz and Liu (173), Deluca et al. (174), and White et al. (175) reported postmortem findings of uncomplicated HSP subjects as showing the following: (1) demyelination and destruction of axis cylinders in lateral corticospinal tracts, primarily in the thoracic spinal cord with less severe involvement in the cervical spinal cord; (2) less severe demyelination of ventral corticospinal tracts (in four of seven subjects); (3) demyelination of spinocerebellar tracts; (4) demyelination of fasciculus gracilis, consistently more severe in the cervical spinal cord; (5) diminished number of neurons in Clarke’s columns (in two of seven subjects); (6) decreased number of anterior horn cells (in one of seven subjects); (7) minor changes in cerebellum, basal ganglia, and rubrospinal tract (in one of seven subjects); (8) changes in the internal capsule, cerebral peduncles, pons or medulla (in one of seven subjects); atrophy or decreased numbers of Betz cells (in three of seven subjects). Behan and Maia (176), Harding (58), Sack et al. (177), and Buge et al. (178) confirmed the primary neuropathology of uncomplicated HSP as demonstrating primary axonal degeneration that was maximal in distal aspects of the corticospinal tracts in the thoracic spinal cord, and to a lesser extent, the distal ends of dorsal column fibers (especially fasciculus gracilis fibers) in the cervicomedullary region. Mild loss

of anterior horn cells may be observed. Demyelination, if present, is generally consistent with the degree of axonal degeneration (although, as discussed above, many forms of complicated HSP have significant white matter abnormalities).

There are very few reports of postmortem studies in which the genetic type of HSP is known. Wharton et al. (179) reported postmortem examination in three SPG4/spastin mutation HSP subjects. Typical of previous neuropathologic findings, they identified distal degeneration of long tracts in the spinal cord accompanied by a microglial reaction. In addition, they reported cytopathologic findings of anterior horn cells (novel hyaline inclusions), alteration in immunostaining for cytoskeletal proteins (nonphosphorylated neurofilament protein and  $\beta$ -tubulin), and abnormal mitochondrial distribution as well as evidence of tau pathology outside the motor system. In addition to confirming distal degeneration of long axons, these findings highlight involvement (albeit usually subclinical) of anterior horn cells. These findings also indicate that neurodegeneration is widespread in SPG4 HSP, the single most common form of dominantly inherited HSP. Moreover, findings indicative of cytoskeletal protein disturbance may be consistent with spastin’s microtubule severing properties and effect of spastin mutations on axonal transport (180–182).

White et al. (175) reported neuropathologic findings in a subject with HSP due to SPG4/spastin mutation (R424G) who had dementia, decreased facial expressions, and mild tremor. Their findings were consistent with previous descriptions of axonopathy in HSP including corticospinal tract degeneration that was maximal in thoracic and lumbar cord, associated myelin pallor, preservation of corticospinal tracts in cerebral peduncles, and no evidence of loss of anterior horn cells or Betz cells. Regarding dementia, the hippocampus showed loss of neurons with remaining neurons displaying frequent neurofibrillary tangles that were immunoreactive for tau. There was severe neuronal loss and neurofibrillary tangle formation in the entorhinal cortex. Senile plaques were not observed. Ballooned neurons with granulovacuolar degeneration, and stained strongly for tau and  $\alpha$ - $\beta$ -crystallin, were especially prominent in limbic areas. Consistent with the subject’s decreased facial expressions and mild tremor, the substantia nigra showed moderate loss of pigmented dopaminergic neurons and frequent Lewy bodies and pale bodies.

Kuru et al. (183) reported postmortem findings in an individual with HSP and thin corpus callosum. The genetic type of HSP was not determined. In addition to adolescent-onset progressive spastic weakness in both legs, this subject manifested upper extremity involvement (spastic tetraplegia), dementia, neuropathy, and muscle wasting. Autopsy revealed a marked brain atrophy, extremely thin corpus callosum and widespread neurodegeneration involving corticospinal tracts, thalamus, cerebral white matter, and substantia nigra.

Degenerative changes involving anterior horns and dorsal columns were also observed. Significant accumulation of lipofuscin and eosinophilic granules was noted in remaining neurons. The authors commented that the occurrence of severe gliosis in the cerebral white matter and substantia nigra indicated that neurodegeneration likely followed substantially normal neurodevelopment, and therefore the primary process was neurodegenerative rather than developmental abnormality.

It is difficult to make generalizations from individual postmortem descriptions of isolated subjects with spastic paraplegia syndromes, particularly when the genetic mutation is not known. As noted above, progressive spastic paraplegia may be a feature of numerous disorders, not all of which are properly classified as HSP. Indeed, given the fact that uncomplicated HSP usually does not shorten the life span, there may be ascertainment bias toward greater number of postmortem studies in subjects with unusual, complicated, and severe spastic paraplegia syndromes. (See, e.g., the description by Nomura et al. (184) of postmortem findings in an individual with a likely autosomal recessive spastic paraplegia syndrome that was complicated by upper extremity involvement, dysarthria, and dementia, becoming bedridden at age 40 and leading to death at age 50 from aspiration pneumonia.)

### 119.1.10 Diagnostic Criteria of Uncomplicated HSP

HSP is diagnosed *clinically* by the following criteria (59): (1) *characteristic clinical symptoms* of bilaterally symmetric, lower extremity spastic weakness that progresses insidiously (when symptoms begin after childhood) or essentially nonprogressive (when symptoms begin in early childhood); (2) *neurologic examination* demonstrates approximately symmetric deficits involving corticospinal tracts subserving the bilateral lower extremities [lower extremity spastic weakness, hyperreflexia, and typically extensor plantar responses (occasionally, plantar responses are flexor in obviously affected subjects) (36)], often accompanied by mildly impaired vibration sensation in the distal lower extremities, hypertonic urinary bladder, and pes cavus; (3) *family history* consistent with inheritance of an autosomal dominant, autosomal recessive or X-linked disorder; (4) *and most importantly, exclusion of alternate and coexisting diagnoses* (Table 119-3). The role of HSP gene analysis in confirming the clinical diagnosis of HSP is discussed below.

*Laboratory testing for HSP gene mutations* is increasingly available. Mutations in 22 genes have been discovered, which are associated with various HSP syndromes. In the United States, commercial analysis is available for X-linked, SPG2/PLP gene (tested at several laboratories, including A.I. duPont Nemours Hospital, Wilmington, Delaware), SPG3A/Atlastin, SPG4/spastin, SPG6/NIPA1, SPG7/Paraplegin, SPG8/Strumpellin,

SPG11/Spatacsin, SPG17/BSCL2, and SPG31/REEP1 available through Athena Diagnostics (Boston, MA). Together, this gene analysis will confirm the diagnosis in ~75% of autosomal dominant HSP. In addition, depending on the extent of genetic testing, ~10–20% of subjects with no family history of HSP (“apparently sporadic spastic paraplegia,” discussed later in the chapter) can be shown to have a potentially pathogenic mutation in an HSP gene for which testing is clinically available (185,186).

HSP gene testing is used most reliably to confirm the clinical diagnosis of HSP. When an HSP gene mutation is identified in an affected subject with clinically definite HSP, this information can be applied to prenatal genetic testing (187–189).

**119.1.10.1 Limits to HSP Gene Analysis and Interpreting the Relevance of HSP Gene Variations of “Unknown Significance”.** HSP gene testing must be interpreted in light of the clinical syndrome. It is very difficult to know the significance (including implications for genetic counseling) of a novel HSP gene variation in a subject who does not meet clinical diagnostic criteria for HSP.

In the case of SPG4/spastin, more than 300 mutations have been reported, the majority of which are “private,” being uniquely present in the family in which they are discovered. In this situation, it is common for an affected subject to have a novel SPG4/spastin mutation that is of uncertain significance. Often, clinicians must decide whether the mutation is likely to be pathogenic and the proper explanation for the patient’s symptoms. Although molecular abnormalities (such as microtubule severing function), in vitro models, and in vivo models associated with SPG4/spastin mutation are being studied, these have not yet been translated into validated biochemical or functional assays that can be used to evaluate the significance of novel SPG4/spastin mutations (or mutations in other HSP genes). Whether the subject actually has HSP depends more on the constellation of clinical features, family history, and exclusion of alternate disorders than on the results of HSP gene analysis alone. Even with the emerging availability of laboratory-based testing to confirm the diagnosis of HSP, it is still important that subjects with progressive spastic paraparesis undergo evaluation, including neuroimaging as indicated, to exclude alternative or coexistent disorders.

There are important limits to HSP gene testing. Presently, gene testing is available commercially for only a subset ( $N = 9$ ) of identified HSP genes ( $N = 22$ ), which in turn represent less than half of the known genetic types of HSP ( $N = 50$ ). Gene testing for other discovered HSP genes is available only on a research basis. For genes whose analysis is commercially available, only the coding sequences and intron-exon splice junctions are examined. In the case of SPG4/spastin, exon deletion is also assessed (responsible for ~10% of SPG4 HSP). For SPG2/PLP, gene copy number is also evaluated. Sequence



abnormalities in gene promoter and other gene regulatory elements are not assessed. Therefore, while identification of an HSP gene mutation of known or likely pathogenic significance can confirm the clinical diagnosis of HSP, the absence of a detectable mutation among currently available HSP genes does not exclude the diagnosis.

### 119.1.11 Differential Diagnosis

It is important to consider HSP a diagnosis of exclusion and to use appropriate laboratory and neuroimaging studies to rigorously exclude other disorders. Consideration of these alternative disorders (see Table 119-3 and (190)) is most relevant when examining patients with atypical features and those with no family history of a similar disorder. Clearly, the presence of neurologic or systemic abnormalities, in addition to spastic paraplegia, does not exclude the diagnosis of HSP but rather may indicate a form of “complicated” HSP. Documenting associated deficits (such as ataxia, retinitis pigmentosa, peripheral neuropathy, amyotrophy, and extrapyramidal signs) is the basis for classifying HSP patients as uncomplicated or complicated and helps distinguish uncomplicated HSP from other neurologic disorders.

The differential diagnosis of *uncomplicated HSP* includes *structural abnormalities* involving the brain or spinal cord, such as tethered cord syndrome and spinal cord compression (191); *other motor neuron disorders* such as slowly progressive ALS (which may present at an early age with insidiously progressive lower extremity weakness, hyperreflexia, and distal amyotrophy) (192–195); PLS (discussed below); distal hereditary motor neuropathy (196); *leukodystrophies* such as steadily progressive multiple sclerosis (197) and Pelizaeus–Merzbacher disease (PMD) (X-linked, allelic with SPG2 HSP), Pelizaeus–Merzbacher-like disease (PMLD) (allelic with SPG44 HSP), and those due to metabolic abnormalities such as B12 deficiency, Krabbe disease (autosomal recessive), metachromatic leukodystrophy (autosomal recessive), and adrenomyeloneuropathy (which, though X-linked, has mimicked autosomal dominant HSP) (198–201); SCAs, such as Machado–Joseph disease SCA 3, which may include spasticity (202–205), or other SCAs including SCA type 17 (206) and SCA 28 (207); Friedreich’s ataxia [which may present with spasticity instead of hyporeflexia, see, e.g., (208)]; olivopontocerebellar atrophy (209), autosomal recessive spastic ataxia of Charlevoix–Saguenay (210), and spastic ataxia due to mitochondrial mRNA maturation (211). Regarding *mitochondrial disturbance*, it is notable that abnormalities involving mitochondrial proteins underlie at least four forms of HSP: SPG7 (paraplegin gene), SPG13 (heat shock protein 27 gene), SPG31 (REEP1 gene), and the mitochondrial ATP6 gene (212). These examples of autosomal recessive (SPG7/Paraplegin, SPG13/heat shock protein 27), autosomal dominant (SPG31/REEP1), and maternal (mitochondrial ATP6 gene) inheritance of

mitochondria-related HSP highlight the importance of considering mitochondrial disorders whose wide variety of clinical presentations may include spastic paraplegia (213,214).

The differential diagnosis of progressive spastic paraplegia includes viral infection with human immunodeficiency virus (HIV/AIDS) as well as tropical spastic paraplegia [also known as human T-cell leukemia virus 1 (HTLV1)-associated myelopathy] (203). Familial clusters of tropical spastic paraplegia have been reported (215–218).

Although pediatricians, geneticists, and neurologists are well aware of the numerous metabolic disorders associated with lower extremity spastic weakness in childhood, there is much less awareness that many of these disorders also may begin later in adolescence and adulthood and manifest as either “uncomplicated” progressive spastic paraplegia or “complicated” spastic paraplegia syndromes. Sedel et al. (219) reviewed metabolic disorders in which adult-onset spastic paraparesis is reported to be a presenting sign or an important feature. It is very important to note that treatment approaches exist for many of these disorders (reviewed in Sedel et al. (219), see Table 119-3).

In addition to adrenomyeloneuropathy, Krabbe disease, and metachromatic leukodystrophy, discussed above, these disorders include homocysteine remethylation defects due to methylene tetrahydrofolate reductase deficiency and cobalamin C disease, urea cycle defects (in particular, arginase deficiency and hyperornithinemia–hyperammonemia–homocitrullinuria syndrome), biotinidase deficiency, phenylketonuria, nonketotic hyperglycinemia, cerebral folate deficiency, homocarnosinosis, cerebrotendinous xanthomatosis (220), Sjögren–Larsson syndrome [caused by deficiency of fatty aldehyde dehydrogenase (FALDH)], polyglucosan body disease, and nucleoside phosphorylase deficiency (221). For review of these disorders including a summary of their clinical presentation, diagnostic testing, and treatment, see Sedel et al. (219).

Insofar as some forms of HSP are associated with dementia (discussed above), the differential diagnosis in appropriate individuals may extend to frontotemporal dementia (95) as well as dementia related to presenilin 1 gene mutation whose presentation may include lower extremity spasticity (222).

Dopa-responsive dystonia should be considered in all subjects, particularly children with progressive gait disturbance. We often recommend a 2-week trial of levodopa-carbidopa, increasing to 25/100 B.I.D. Although rare, dopa-responsive dystonia is treatable with low-dose levodopa-carbidopa (223,224).

Note that the differential diagnosis of HSP includes familial clustering of nongenetic disorders (tropical spastic paraparesis) (215–218), disorders for which specific treatments are available (e.g., B12 deficiency, DOPA-responsive dystonia, cervical spondylosis), and those

whose prognosis differs significantly from HSP (e.g., familial ALS and PLS).

### 119.1.12 Genetic Types of HSP: Autosomal Dominant, Autosomal Recessive, X-linked, and Maternal Inheritance

Each of these types of HSP is genetically heterogeneous: mutations in separate genes cause disorders that share the feature of lower extremity spastic weakness. Some forms of HSP are indistinguishable clinically while others are recognized by syndrome-specific constellations of neurologic (and sometimes systemic) features. Genetic loci for HSP are designated “spastic gait” (SPG) loci 1 through 48, in order of their discovery. In addition, there are several forms of HSP that do not have SPG loci designations (Table 119-1).

**119.1.12.1 Autosomal Dominant HSP Shows Extreme Genetic Heterogeneity.** The first locus for autosomal dominant HSP was identified by Hazan et al. (15) on chromosome 14 and designated SPG3 (X-linked HSP loci SPG1 and SPG2 had previously been described). Hazan et al. (15) and others (225) demonstrated that autosomal dominant HSP was genetically heterogeneous by identifying other autosomal dominant HSP kindreds for which SPG3 locus was excluded. To date, 19 genetic loci for autosomal dominant HSP have been identified (Table 119-1). The existence of one additional locus (SPG40) was suggested by exclusion of the then known HSP loci (51).

SPG4 HSP, due to mutations in the *SPG4*/spastin gene (discussed below), is the single most common type of dominantly inherited HSP, representing ~40% of autosomal dominant uncomplicated HSP kindreds (61,82). Next in frequency is SPG3A (due to mutations in the *SPG3A*/atlastin gene) and SPG6 (due to mutations in the *SPG6*/NIPA1 gene), which represent ~15% and ~5% of dominantly inherited HSP, respectively. Other types of dominantly inherited HSP are much less common, each having been reported in only one family or at most in several families.

**119.1.12.2 Genotype-Phenotype Correlation in Autosomal Dominant HSP.** The phenotype spectrum and therefore genotype–phenotype correlations are poorly understood for most types of HSP. This is largely because clinical descriptions for many genetic types of HSP are based on only one or several families, and detailed, longitudinal analyses to document the natural history are rare. With this proviso, a number of generalizations can be made.

“Complicated” forms of HSP (summarized in Table 119-1) are distinguished by the presence in the *majority of subjects (but perhaps not all)* of clinical features in addition to lower extremity spastic weakness. Clearly, some subjects with some types of HSP considered to be “complicated” (e.g., SPG10 due to KIF5A mutation) may have “uncomplicated” HSP syndromes (34). Conversely,

additional neurologic impairments (and therefore “complicated HSP syndromes”) may occur in some subjects with types of HSP generally considered to be “uncomplicated”. For example, although SPG3A and SPG4 HSP are usually “uncomplicated”, SPG3A HSP may be associated with peripheral axonal neuropathy and muscle atrophy; and SPG4 HSP may be associated with dementia, peripheral axonal neuropathy, thin corpus callosum, and muscle atrophy. In general, it is not possible to attribute these “variant” presentations to specific gene mutations.

For subjects manifesting the syndrome of autosomal dominant, uncomplicated progressive spastic paraplegia, it is generally not possible to distinguish reliably the genetic type of HSP using clinical parameters alone (59,62,82,96,226,227). This is due, in part, to the narrow diagnostic criteria of uncomplicated HSP in which the inherited syndrome is limited to insidiously progressive, symmetric, spastic weakness of the lower extremities, frequently accompanied by urinary bladder disturbance and mild impairment of vibration sensation in distal lower extremities.

There is variability, however, in the age at which symptoms first appear and the extent of functional disability. These features are variable both within a given kindred, between kindreds linked to the same locus, and between different genetic types of HSP. In general, symptoms begin earlier (less than age 11 years, on average) in SPG3A, SPG10, and SPG12 than for SPG4, SPG6, SPG8, and SPG13 (in which symptoms begin after age 20, on average) (62). Although various types of dominantly inherited HSP differ in the *average* age-of-symptom onset, there is considerable overlap in the *range* of ages when symptoms first appear between various types of HSP. This finding limits the ability to predict the HSP locus from the age-of-symptom onset *alone*.

*Autosomal dominant*, “typically complicated” and “variably complicated” HSP includes those syndromes in which spastic paraplegia is *usually or often* associated with additional neurologic abnormalities. These include SPG17 HSP (Silver syndrome, due to *BSCL2*/seipin gene mutation discussed below) in which dominantly inherited spastic paraplegia is associated with distal muscle atrophy (41); SPG9 HSP in which dominantly inherited spastic paraplegia is associated with cataracts, gastroesophageal reflux, and motor neuropathy (33); and SPG29 HSP in which spastic paraplegia associated with hearing impairment and persistent vomiting and hiatal hernia (228).

In some forms of dominantly inherited HSP, the “complicating features” are *variably present*. For example, SPG10 HSP (due to mutations in the *KIF5A* gene discussed below) is a dominantly inherited HSP syndrome in which affected subjects may have either uncomplicated spastic paraplegia; or spastic paraplegia complicated by distal muscle atrophy (229). In addition, although SPG3A HSP (due to *SPG3A*/atlastin gene

mutation) typically is “uncomplicated,” peripheral axonal neuropathy has been reported in ~17% of subjects with (108).

**119.1.12.3 Genetic Anticipation in Autosomal Dominant HSP.** Some autosomal dominant HSP kindreds exhibit progressively younger age-of-symptom onset or increased disease severity in succeeding generations. Although genetic anticipation is not typical of all dominantly inherited types of HSP (59), it has been reported in SPG4 HSP (98,230–233) and observed in SPG3A HSP. Despite these observations, tandem repeat expansions, the molecular mechanism responsible for genetic anticipation in more than two dozen inherited neurologic disorders, have not been identified in HSP with anticipation for which HSP gene mutations have been identified. In contrast, the HSP gene mutations in such families have been point mutations, deletions, and aberrant splicing. For example, we identified an SPG3A HSP family in which the 73-year-old grandfather was neurologically normal, his son had mild spastic gait beginning in his 20s and his grandson had marked spastic gait beginning at age 7 (234,235). Each of these individuals was heterozygous for SPG3A/atlastin mutation V253I.

The absence of trinucleotide (or other) repeat expansion in families with apparent genetic anticipation could be explained by the presence of an expanded repeat in an HSP modifying gene (236); a molecular mechanism for anticipation other than tandem repeat expansions; or ascertainment bias, in which variable age-of-symptom onset in small families is interpreted as an intergenerational trend toward earlier symptom onset.

### 119.1.13 Autosomal Recessive HSP

In Norway, the prevalence of autosomal recessive HSP was determined to be 0.6 per 100,000, being less prevalent than autosomal dominant HSP (5.5 per 100,000) and isolated cases of spastic paraplegia (1.3 per 100,000) (13). Individuals with autosomal recessive HSP (like those with other autosomal recessive disorders) often do not have family history of similarly affected relatives (because parents, children, and, on average, 75% of siblings of individuals with recessive disorders are unaffected). Therefore, we may underrecognize the genetic basis for the disorder in many individuals with “apparently sporadic” spastic paraplegia (ASSP). Indeed, analyses of ASSP subjects consistently identify gene mutations in both autosomal recessive and autosomal dominant types HSP. For example, Brugman et al. (185) reported that 6.7% of subjects (7 of 105) with apparently sporadic upper motor neuron syndromes had potentially pathogenic SPG4/spastin mutations. In addition, 11% (7 of 65) spastic paraplegia subjects had homozygous or compound heterozygous SPG7/paraplegin mutations (185,237).

Hentati et al. (238) studied Tunisian HSP kindreds and identified the first locus for autosomal recessive HSP

(SPG5) on chromosome 8. Subsequently, DeMichele et al. (155), Garner et al. (239), and Martinez-Murillo et al. (240) identified autosomal recessive HSP loci on chromosomes 16 (SPG7) and 15 (SPG9), respectively. Presently, 26 loci for autosomal recessive HSP have been identified. SPG11 is the single most common genetic type of autosomal recessive HSP, initially estimated to be responsible for 50% of autosomal recessive HSP (240). SPG11 HSP often (not always) presents as a “complicated” spastic paraplegia syndrome, typically (though not always) associated with thin corpus callosum and mental retardation (and sometimes associated with distal wasting and retinal pigmentary abnormality, conforming to Kjellin syndrome, discussed below). The proportion of SPG11 among autosomal recessive forms of HSP varies in part on whether subjects were selected on the basis of corpus callosum thinning. For example, Shibasaki et al. (241) showed that 10 of 13 (77%) Japanese families with autosomal recessive HSP, mental retardation, and thin corpus callosum were linked to this locus. In contrast, Boukhris et al. (12) studied Tunisian autosomal recessive HSP subjects (not selected for corpus callosum thinning) and found linkage to the SPG11 locus in seven of 38 families (18.4%). Similarly, Stevanin et al. (242) analyzed the SPG11/spatacsin gene coding sequence in 43 autosomal recessive families and 33 isolated patients and identified mutations in 41% of subjects with spastic paraplegia and thin corpus callosum, but only in 4.5% of subjects with spastic paraplegia and cognitive impairment who did not have thin corpus callosum.

**119.1.13.1 “Typically Uncomplicated” Autosomal Recessive HSP.** Whereas 12 of 20 genetic types of autosomal dominant HSP are “typically uncomplicated” (Table 119-1), only five (SPG5, SPG28, SPG29, and SPG48) of 26 genetic types of autosomal recessive HSP are “typically uncomplicated” (Table 119-1), with the caveat that since very few kindreds with each of these HSP have been described, the full phenotypic spectrum may not be completely known.

**119.1.13.2 “Variably Complicated” Autosomal Recessive HSP: SPG7, SPG11, SPG27.** These autosomal recessive types of HSP are “variably complicated” insofar as the “complicating” features are *variably present* in some but not all affected individuals. The fact that some subjects with these types of HSP have “uncomplicated” spastic paraplegia (even in families in which other individuals have “complicated” spastic paraplegia syndromes) must be considered when providing genetic counseling and prognosis.

Subjects with SPG11 HSP may demonstrate either mild or severe syndromes (243) of uncomplicated HSP; HSP with mental retardation but normal corpus callosum (244), or more frequently, HSP associated with thin corpus callosum, mental retardation, upper extremity weakness, dysarthria, and nystagmus (240,241). Some SPG11 subjects have distal muscle atrophy and retinal pigmentary abnormality consistent with “Kjellin syndrome,”



discussed in the following section (245). For additional descriptions of SPG11 HSP, see (153,170,242–251). Variant presentations of SPG11 mutation as juvenile parkinsonism (252) and juvenile ALS (253) are discussed in the following.

Similarly, some subjects with autosomal recessive SPG7 HSP (due to paraplegin gene mutations, discussed in Section 119.5.2) have uncomplicated HSP and others have additional neurologic abnormalities (155,239) including dysarthria, dysphagia, optic disc pallor, axonal neuropathy, and evidence of “vascular lesions”, cerebellar atrophy, or cerebral atrophy on cranial MRI (254).

Subjects with SPG27 HSP exhibit either uncomplicated, adult-onset spastic paraplegia or spastic paraplegia associated with dysarthria (32). For additional descriptions of SPG7 HSP see (76–79).

Only two families with autosomal recessive SPG27 HSP have been described. The SPG27 locus was identified by Lossos et al. (255) who reported seven affected subjects in French–Canadian family in which uncomplicated spastic paraplegia began between ages 25 and 45 years. In contrast, the Tunisian family linked to the SPG27 locus reported by Ribai et al. (256) had a childhood-onset disorder in which spastic paraplegia was accompanied (to variable extent) by ataxia, dysarthria, mental retardation, sensorimotor polyneuropathy, facial dysmorphism, and short stature.

**119.1.13.3 “Typically Complicated” Autosomal Recessive HSP.** Among 19 types of “typically complicated” HSP (see Table 119-1), the most common additional neurologic impairments are (1) peripheral motor-sensory neuropathy, distal muscle wasting, or sensory neuropathy (occurring in 13 types); (2) mental retardation, dementia (or both mental retardation and dementia, occurring in 12 types); (3) dysarthria (occurring in seven types); (4) ataxia and/or cerebellar atrophy (occurring in five types); (5) symptomatic upper extremity involvement (occurring in five types); (6) optic atrophy, maculopathy, or cataract (occurring in four types); and (7) thin corpus callosum (occurring in three types) and brain white matter abnormalities (occurring in three types). Some of these types of HSP conform to named syndromes.

*Mast syndrome* refers to SPG21 HSP (due to maspardin gene mutations) in which spastic paraplegia is associated with dementia, cerebellar and extrapyramidal deficits, and white matter abnormalities (162).

*Kjellin syndrome.* Kjellin (257) described an autosomal recessive disorder characterized by childhood-onset, progressive spastic paraplegia that is accompanied by pigmentary retinopathy, mental retardation, dysarthria, dementia, and distal muscle atrophy. Huges et al. (258) studied two families with Kjellin syndrome and identified linkage to a then novel locus on chromosome 14q, designated SPG15. Recently, SPG15 HSP has been shown to be due to Spatzin gene mutation (259). Subsequently, Kjellin syndrome was identified in subjects with SPG11

HSP (245,260), recently shown to be due to Spatacsin gene mutation (251). It is noteworthy that the full manifestations of Kjellin syndrome are not constant features of either SPG11/spatacsin or SPG15/spatzin gene mutations but instead are variably present in subjects with these types of HSP (258,259).

*Spastic Paraplegia as Part of a Neurocutaneous Syndrome.* Sjögren–Larsson syndrome (261,262) is an autosomal recessive disorder characterized by congenital ichthyosis, mental retardation, and spastic diplegia. Sjögren–Larsson syndrome is due to mutations in the SALDH3A2 gene that encodes FALDH. SPG23 HSP is characterized by childhood-onset spastic paraplegia and cutaneous pigment abnormality (263).

*SPOAN syndrome* is characterized by infantile spastic paraplegia associated with progressive optic atrophy, neuropathy, dysarthria, and progressive joint and spine deformities (118).

*HSP with thin corpus callosum* was initially recognized as a frequent (but not constant) feature of SPG11 (159,165–169). Subsequently, it was recognized that thin corpus callosum is not unique to SPG11 but is also a frequent or variable feature of additional types of autosomal recessive HSP [SPG7, SPG15, SPG21, SPG32, SPG47, and the syndrome “HSP with thin corpus callosum and epilepsy” (153,154)]. Thin corpus callosum has also been reported in autosomal dominant SPG3A and SPG4 HSP, both of which are typically “uncomplicated.” Thin corpus callosum in SPG11 HSP is frequently (but not always) associated with cognitive impairment (characterized in two subjects by Siri et al. (170)). Similarly, thin corpus callosum in other types of HSP is not always associated with cognitive impairment (105).

*Autosomal Recessive HSP with Distal Muscle Atrophy (“Troyer Syndrome”) and/or Peripheral Motor Neuropathy.* Autosomal recessive HSP associated with distal muscle atrophy was initially described in Amish families (264). Designated “Troyer syndrome”, this progressive spastic paraplegia syndrome was associated with not only distal muscle atrophy, but also short stature, mental retardation, and dysarthria (265). The disorder in these families was subsequently linked to the SPG20 locus and causative mutations in a novel gene (designated “Spartin” for spastin-related, autosomal recessive troyer syndrome) was discovered (266).

Subsequently, distal muscle wasting or motor neuropathy in at least some subjects has been reported with five other genetic types of autosomal recessive HSP: SPG11, SPG14, SPG15, SPG26, SPG39, and SPG43. SPG11 has a variety of presentations, most commonly associated with thin corpus callosum, although it may also exhibit pigmentary retinopathy, mental retardation, dysarthria, dementia, and distal muscle atrophy and conform to Kjellin syndrome (discussed above). SPG14 HSP, described in three subjects in an Italian family, was associated with mental retardation and motor neuropathy (distal atrophy per se was not mentioned) (267). SPG15



has been described in 13 families of Irish, Arabic, and Tunisian descent (258,259,268,269). Some of these subjects exhibited distal muscle atrophy, pigmented retinopathy, mental retardation, and dementia (258,259,270) and conform to “Kjellin syndrome,” discussed above. Recently, ZFYVE26 gene mutations have been identified in SPG15 HSP subjects (259). SPG26, in five siblings from a single, consanguineous Bedouin family, is described as childhood-onset progressive spastic paraplegia associated with distal amyotrophy in both upper and lower limbs (with normal nerve conduction studies), dysarthria, and variable intellectual impairment (271). Meilieur et al. (9) described two sisters from Mali, symptom onset seven and 12 years, progressive spastic paraplegia with atrophy of intrinsic hand muscles and dysarthria (one sister). Genetic analysis identified a linkage to novel HSP locus (19p13.11-q12) designated SPG43.

Distal muscle atrophy is a characteristic of SPG39 due to neuropathy target esterase (NTE) gene mutation (272,273). SPG39 HSP was described in six subjects from two unrelated families and characterized by childhood onset, slowly progressive spastic paraplegia that becomes associated in adolescence with progressive, ultimately severe wasting of intrinsic hand and distal lower extremity muscles. Peripheral motor neuropathy with normal sensory nerve conduction studies was demonstrated in SPG39 HSP (also known as NTE-related motor neuron disease) (272,274). SPG39 patients do not have additional features of short stature, mental retardation, and dysarthria, which are present in many SPG20 HSP subjects (272,275).

SPG14, SPG26, and SPG43 HSP have each been described in single families.

*X-linked HSP* is also genetically heterogeneous. Like autosomal dominant HSP and autosomal recessive HSP, X-linked HSP exists as both “uncomplicated” (276) and “complicated” spastic paraplegia syndromes. SPG16 HSP begins in childhood and may manifest either as uncomplicated HSP or as spastic paraplegia associated with aphasia, visual impairment, and mental retardation (277,278).

**119.1.13.4 L1CAM Mutations: X-linked Hydrocephalus, MASA Syndrome, and X-linked Spastic Paraplegia.** SPG1 HSP is an Xq28-linked HSP associated with mental retardation and adducted thumbs and is due to mutations in the neuronal cell adhesion molecule L1CAM. In addition to X-linked spastic paraplegia with mental retardation, and adducted thumbs, mutations in the L1CAM gene also cause X-linked hydrocephalus, MASA syndrome (mental retardation, aphasia, shuffling gait, adducted thumbs), and CRASH syndrome (corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraparesis, and hydrocephalus) (151,279,280).

**119.1.13.5 PMD Is Allelic with SPG2 HSP; PMLD Is Allelic with SPG44 HSP.** SPG2 HSP is due to mutations in the PLP gene. The PLP gene encodes an intrinsic

myelin protein (281). In addition to SPG2 HSP, mutations in the PLP gene cause PMD, an X-linked, infantile-onset, severe neurologic disorder due to abnormal myelin formation. Often, subjects with Xq21-22-linked HSP have MRI evidence of central white matter disturbance (144,282,283). It is noteworthy that the underlying pathology for slowly progressive spastic paraplegia in SPG2 is considered to represent axonal degeneration rather than demyelination despite the fact that SPG2 is due to abnormality in an intrinsic myelin protein (284). This is supported by murine models of SPG2 HSP that show axonal degeneration as the consequence of oligodendroglial/myelin abnormality (285,286).

The basis for clinicopathologic differences between PLP mutations that result in PMD and X-linked HSP is not completely established. In some patients, phenotype variations correlate with specific PLP gene mutations associated with each disorder (287). In other families, both early-onset severe Pelizaeus–Merzbacher leukodystrophy and later-onset insidiously progressive spastic paraplegia have occurred in individuals with precisely the same PLP mutation.

Despite thorough investigation, some subjects linked to Xq22 do not appear to have PLP gene mutations. This raises the possibility of an additional gene at this locus for X-linked spastic paraplegia (277).

As noted above, PMD is an X-linked, hypomyelinating, congenital leukodystrophy due to PLP gene mutation. Recently, mutations in two other genes have been shown to cause autosomal recessive (rather than X-linked) forms of this syndrome. Specifically, PMLD are associated with mutation in the ARS-interacting multifunctional protein 1 (AIMP1) gene (identified in consanguineous Israeli Bedouin family) (288) as well as mutations in the GJA12 gene, which encodes gap junction protein alpha 12 (also known as connexin 47) (289). Like PLP, GJA12/connexin 47 is an important factor in central and peripheral myelin formation.

Just as PMD is allelic with a more slowly progressive disorder (SPG2 HSP), so too does PMLD due to GJA12/connexin 47 mutation. Recently, Orthmann-Murphy, et al. (290) identified homozygous GJA12 mutations in three subjects from a possibly consanguineous, Italian kindred. Affected subjects had symptom onset in the first or second decade, very slow progression, mild to severe gait disturbance, and variable involvement of upper extremity, speech, cerebellum, and cognition. EMG and nerve conduction studies were normal. Brain MRI was consistent with hypomyelination. This syndrome is designated SPG42 HSP.

The findings that PMD is allelic with SPG2 HSP, and that PMLD is allelic with SPG42 have three important implications. First, they indicate that infantile-onset leukodystrophies may manifest as later-onset, milder phenotypes. (The concept that infantile-onset metabolic disorders may manifest in adulthood as relatively more slowly progressive disorders is well known and illustrated

by other notable examples: adrenoleukodystrophy/adrenomyeloneuropathy, Tay–Sachs disease/partial hexosaminidase deficiency, and infantile Krabbe/late-onset Krabbe disease.) Second, these disorders underscore the concept that primary myelin abnormalities may cause axonopathy (291). In this regard, mouse models of SPG2 HSP exhibit either axonal degeneration (consistent with SPG2 HSP) or demyelination (consistent with PMD) depending in part on the nature of the PLP gene disturbance (284,292,293). And finally, these syndromes highlight the growing awareness that myelin abnormalities are present in a number of forms of HSP (discussed below). Note in particular that mutation of the bovine SPG4/spastin gene causes congenital dysmyelination.

*Allan–Herndon–Dudley (AHD) syndrome (SPG22)* is an X-linked disorder characterized by congenital onset, neck muscle hypotonia in infancy, mental retardation, dysarthria, ataxia, spastic paraplegia, and abnormal facies. Recently, Schwartz et al. (294) showed that AHD patients had mutations in the SLC16A2 gene that encodes monocarboxylate transporter-8 (MCT8). Previously, mutations in this gene were reported in males with hypotonia, involuntary movements, and mental retardation (295,296). MCT8 is a thyroid hormone transporter. MCT8 mutations may be associated with increased serum triiodothyronine (T3) (295).

## 119.2 MATERNAL (MITOCHONDRIAL GENOME) INHERITANCE

### 119.2.1 Mitochondrial ATP6 Gene

Verny et al. (212) reported a late-onset spastic paraplegia-like disorder that affected five subjects of a family (a mother, all three of her daughters, and two of her four sons). Although there was extreme variability in symptom severity and disability, lower extremity pain was a common feature. One individual had slowly progressive spastic paraplegia and lower extremity pain that began at age 30 leading to wheelchair dependence in his late 40s. This individual also had diabetes mellitus, hypertrophic cardiomyopathy, associated with supraventricular arrhythmia and ataxia (excess alcohol may have been a contributing factor). Two individuals had painful legs, mild sensory loss, but only minimal disability. One subject had axonal neuropathy and yet another had moderate spastic paraplegia, lower extremity pain, and reduced vibratory sensation.

The authors discovered mutation (m.9176 T>C) in the mitochondrial ATP6 gene and showed that the clinical severity of the disease correlated with the biochemical and assembly defects of the ATP synthase (212). This same mitochondrial ATP6 gene mutation had previously been reported in Leigh syndrome, thus expanding the phenotypic spectrum associated with this mutation and implicating the effect of modifying genes and/or environmental factors.

### 119.2.2 “Apparently Sporadic” Spastic Paraplegia

Many subjects who have all the signs and symptoms of uncomplicated HSP have no similarly affected relatives. Despite thorough neurodiagnostic analysis, alternative disorders are not identified. This is the syndrome of ASSP (297). In our database of more than 300 unrelated HSP syndrome subjects, ~25% conform to ASSP. The disorders among ASSP syndrome subjects are heterogeneous and may include both nongenetic disorders (e.g., copper-deficient myelopathy or paraneoplastic disorders), early stages of PLS and ALS, and those for whom the disorder is autosomal recessive HSP, X-linked HSP, or autosomal dominant HSP (with absent family history due to incomplete or inaccurate family information, late age-of-symptom onset, incomplete penetrance, *de novo* mutation, or mistaken paternity). Depending on the extent of genetic testing, ~5–18% of individuals with ASSP have been shown to have a mutation in an HSP gene (298–301) including SPG4/spastin mutations, which are generally associated with autosomal dominant HSP with high genetic penetrance (186,237,297). Undoubtedly, this figure will increase as additional HSP genes are available for evaluation in ASSP syndrome subjects. Identifying an HSP gene mutation in subjects with ASSP would change the diagnosis to HSP and have important implications for genetic counseling (discussed below).

Other than family history, there are no clinical features (such as age-of-symptom onset, rate of symptom progression, or extent of disability) that distinguish uncomplicated spastic paraplegia subjects with (autosomal dominant, recessive, or X-linked HSP) from those with uncomplicated ASSP. A cautiously optimistic, wait-and-see approach is advised regarding the prognosis of such individuals. After many years, a relative may become similarly affected and the disorder will be recognized as HSP. Some individuals, however, will continue to have slowly progressive spastic weakness that is confined to the lower extremities and that is clinically indistinguishable from uncomplicated HSP. Other individuals, however, will develop upper extremity and corticobulbar muscle weakness and be re-classified as PLS (discussed below) (85). For still other individuals, “apparently sporadic HSP” represents an early phase of ALS (302–306).

### 119.2.3 Penetrance in Autosomal Dominant HSP

The frequency with which obligate carriers of autosomal dominant HSP pathogenic gene mutations exhibit the disorder is age-dependent, very high, but incomplete. Incomplete genetic penetrance has been reported for SPG4 (61), SPG8 (31), and SPG3A HSP (307,308). Mitne-Neto et al. (309) described sex-dependent penetrance (earlier age-of-symptom onset in males) in subjects with SPG4 HSP due to tandem duplication of SPG4/spastin exon 10–12.

As yet, too little information exists to know whether reduced penetrance is more frequent for certain mutations in these genes than for other mutations. Fonknechten et al. (61) provide the best estimate of genetic penetrance for SPG4 HSP (due to spastin gene mutations, discussed below). They examined the frequency of clinical signs and symptoms among 238 obligate gene carriers (those with spastin gene mutations) from 44 autosomal dominant HSP kindreds. The age-of-symptom onset varied from 0 to 74 years (mean  $\pm$  standard deviation was  $29 \pm 17$  years). Among 238 subjects from SPG4-linked kindreds shown to have mutations, only 179 (75%) were aware of having symptoms (61). They observed clinical symptoms and signs of HSP in 85% individuals with spastin gene mutation who were older than 40 years (61).

In general, incomplete penetrance in HSP is observed more often in kindreds in whom age-of-symptom onset extends over a broad range into senescence, than in families with uniform symptom onset in early childhood. Careful neurologic examination of many asymptomatic obligate gene mutation carriers often demonstrates lower extremity corticospinal tract disturbance (hyperreflexia and often extensor plantar response). For example, Durr et al. (82) studied 12 SPG4-linked kindreds. Among 93 gene carriers, 32 (34%) were asymptomatic with very few exceptions; neurologic examination of asymptomatic gene carriers disclosed lower extremity corticospinal tract signs. Nonetheless, incomplete penetrance has also been reported for SPG3A HSP, which typically begins in early childhood (108,234,307,308).

For some asymptomatic obligate gene carriers, lower extremity deep tendon reflexes and the remainder of the neurologic examination are entirely normal. Depending on their age, and the age-of-symptom onset within the family, these individuals may be considered either still at risk of developing the disease or genuinely nonpenetrant. For example, Hedera et al. (31) reported a chromosome 8q-linked autosomal dominant HSP kindred in which age-of-symptom onset ranged from 22 to 60 years (mean  $37.3 \pm 12.2$  SD). One obligate gene carrier was entirely asymptomatic and had a normal neurologic examination at age 60. It is uncertain whether this individual is a rare example of nonpenetrance or whether she will ultimately develop HSP. Similarly, the extended kindred reported by Cooley et al. (310) contained one elderly asymptomatic carrier of autosomal dominant, uncomplicated HSP.

HSP's apparently high degree of genetic penetrance may include an ascertainment bias. Autosomal dominant HSP is more readily diagnosed in large kindreds for whom the disorder is highly penetrant. This could contribute to overrepresentation of highly penetrant HSP kindreds in published studies. Unless genetic testing is performed, a mutation in an HSP gene that is known or likely to be pathogenic is discovered, and unaffected family members tested, it is not possible to estimate

genetic penetrance in kindreds consisting of affected siblings (with unaffected parents) since this could represent an autosomal recessive disorder or an autosomal dominant disorder with incomplete penetrance, or in kindreds consisting of an affected child, an affected parent, and unaffected grandparents since this could represent either incomplete penetrance (in a grandparent) or a new mutation (in a parent). Caution must be observed when providing genetic counseling for spastic paraplegia subjects with no previous family history in whom a pathogenic mutation for dominantly inherited HSP is found (but other family members not tested). In addition to *de novo* mutation, mistaken paternity, and incompletely ascertained family members, absence of previous family history could represent incomplete genetic penetrance (or late-onset and mild symptoms in some subjects).

In general, there is very limited information about the genetic penetrance of autosomal recessive and X-linked forms of HSP.

#### 119.2.4 Modifying Factors

HSP's genetic penetrance is age-dependent, and although high, it is not complete (discussed above). Moreover, there is often marked phenotype variability between subjects with the same genetic type of HSP and even between affected subjects in the same family who share the precise HSP gene mutation. Incomplete penetrance and marked phenotype variation between subjects with the same mutation implicate modifying factors (environmental and genetic) in the development, severity, and age at onset of lower extremity spastic weakness. There is one report (311) of a two generation kindred with autosomal dominant, uncomplicated HSP in which one of two monozygotic twins developed HSP. (Monozygosity was confirmed by testing 24 informative genetic markers.) Observations of discordancy between monozygotic twins and extreme variability in age-of-symptom onset and degree of disability among individuals in the same family who have the same HSP gene mutation indicate the effect of modifying factors (both genetic and nongenetic factors in the development of spastic paraplegia in HSP).

One source of modifying genes is variation in HSP genes themselves. This includes mutation-related variation in HSP gene transcript abundance (312,313) and HSP coding sequence polymorphisms. Svenson et al. (314) analyzed *benign* SPG4/spastin polymorphisms (S44L and P45Q) and showed that L44 and Q45 are each associated with a striking decrease in age at onset in the presence of the *pathogenic* mutations in SPG4/spastin's AAA domain. Schickel et al. (315) showed that spastin polymorphisms S44L, E43Q, and P45Q enhanced the stability of alternative spastin isoforms. They propose that the relative abundance of alternative spastin isoforms could ultimately influence the

phenotype by increasing competition of various spastin isoforms for interacting proteins, substrates, or oligomerization partners.

**119.2.4.1 Emerging HSP “Protein Interactome”.** One source of modifying factors is the proteins that interact directly with HSP proteins. Presently, nine of the 22 discovered HSP proteins interact directly with another HSP protein or share common interaction with a third protein (see Table 119-4). Indeed, Slabicki et al. (316) and Mannan et al. (47) evaluated spastin-interacting proteins and identified mutations in ZFYVE27 and KIAA0415 that cause SPG33 and SPG48 HSP, respectively. Direct interaction between HSP proteins raises the possibility that the phenotype in subjects with pathogenic mutation HSP gene mutations could be influenced by otherwise benign variation (including coding sequence polymorphisms, gene regulation, and copy number variants) in other HSP genes.

**119.2.4.2 HSP Genotype–Phenotype Correlation.** International efforts are underway develop a database of HSP gene mutations that lists, for each mutation in each HSP gene, specific phenotype parameters (age-of-symptom onset, genetic penetrance, associated neurologic or systemic impairments, extent of functional disability) as well as family history, ethnicity, and comorbid conditions (317). Once established, this database will provide information regarding the range of symptoms and functional severity. This information will guide prognosis and genetic counseling. By combining clinical information with *in silico*, *in vitro*, and *in vivo* models, the relative pathogenicity of novel HSP gene mutations can be assessed.

In a broad sense, however, the clinical phenotype associated with a given HSP gene mutation is influenced by a myriad of factors beyond the discrete mutation itself. The clinical phenotype of HSP reflects the composite outcome of numerous factors including (1) genetic regulation (e.g., regulation of the HSP gene bearing the pathogenic mutation, regulation of other HSP genes, regulation of other genes influencing axon growth and maintenance, genetic regulation of inflammatory response to antigens liberated in neurodegeneration); (2) genetic polymorphisms (including copy number variants) in the HSP gene bearing the pathogenic mutation, other HSP genes, other genes influencing or compensating for axon degeneration; (3) epigenetic influences (potentially, age-, diet-, or environmentally related gene silencing of the aforementioned genes); (4) neuroplasticity (in turn affected by age, diet, exercise, and self-specific (idiotypic) combinations of genetic factors including those mentioned above); (5) environmental factors (such as exposure to neurotoxins as well as the influence of diet and exercise); (6) general health (including coexistent medical conditions, quality of sleep, pain, depression); and (7) age (including chronobiologic effects on neurologic and immunologic function).

### 119.3 ASSOCIATION OF HSP GENE MUTATIONS WITH SYNDROMES OTHER THAN SPASTIC PARAPARESIS

#### 119.3.1 BSCL2/Seipin Mutations Cause Autosomal Dominant HSP (SPG17), Charcot–Marie–Tooth Type 2, and Distal Hereditary Motor Neuropathy Type V

These syndromes vary in the degree of upper motor neuron involvement (i.e., there is significant spasticity and hyperreflexia in the SPG17 HSP syndrome), lower motor neuron (i.e., there is significant atrophy in the distal hereditary motor neuropathy syndrome), and peripheral axonal neuropathy [which is marked in Charcot–Marie–Tooth (CMT) type 2] (318).

*BSCL2/Seipin mutations cause congenital generalized lipodystrophy type 2 (CGL2)*, manifesting as severe lipoatrophy, insulin resistance, hypertriglyceridemia, and mental retardation (318,319). It is important to point out that whereas CGL2 is an autosomal recessive disorder due to homozygous or compound heterozygous BSCL2/Seipin gene mutation, SPG17 HSP is an autosomal dominant disorder due to heterozygous BSCL2/Seipin gene mutation (41).

#### 119.3.2 Juvenile Parkinsonism

Anheim et al. (252) described juvenile parkinsonism in two subjects from a consanguineous Turkish family who had a novel SPG11/Spatacin mutation (homozygous for c.704\_705delAT, p.H235RfsX12). In the first subject, parkinsonism was the initial feature including marked rest tremor, moderate akinesia, and rigidity. At the time of initial examination (age 12) the patient had brisk reflexes, bilateral extensor plantar response, and reduced vibration sensation. Despite initial benefit from levodopa, both extrapyramidal and pyramidal syndromes worsened progressively and the patient became wheelchair-dependent at age 22. At age 15, the second subject had spastic paraparesis, decreased vibration sensation, and dementia. This was followed by parkinsonism at age 16 that did not respond to levodopa. Both extrapyramidal and pyramidal syndromes worsened progressively. As it frequently occurs in SPG11 HSP, each subject was mentally retarded and had thin corpus callosum and axonal neuropathy.

Independently, Micheli et al. (320) described two Korean siblings with thin corpus callosum (a frequent feature of SPG11 and several other types of HSP), one of whom had primarily levodopa-responsive parkinsonism, and the other had progressive spastic paraplegia. Genetic testing was not performed. Extrapyramidal rigidity in the hands has also been described in one subject with SPG15 HSP (259), which also is associated with thin corpus callosum.



TABLE 119-4 HSP Proteins

HSP Gene/Protein	Functions	References
SPG1/L1CAM	Integral membrane glycoprotein, cell adhesion molecule in the immunoglobulin superfamily; mediates cell-to-cell and cell-to-matrix attachment; functions include guidance of neurite outgrowth during development, neuronal cell migration, and neuronal cell survival. <i>Interacts with Bone Morphogenic protein (affects L1CAM gene regulation)</i>	(451,454,455)
SPG2/PLP	Proteolipid protein (PLP) is an integral myelin protein expressed in oligodendroglia and Schwann cells but not in neurons	(461)
SPG3A/atlastin	Dynamin family GTPase, interacts with HSP proteins spastin, NIPA1, and REEP1; involved in membrane fusion and severing; contributes to endoplasmic reticulum morphology. <i>Interacts with SPG4/spastin, REEP1, NIPA1, DP1/Yop1 p and reticulon families of ER-shaping proteins, HPK/GCK-like kinase (HGK) (a protein kinase in the c-Jun N-terminal kinase signaling pathway; Inhibits BMP signaling in Zebrafish model)</i>	(375–381,382,556,557)
SPG4/spastin	Cytosolic (and possibly nuclear) protein with AAA domain; (AAA domain is also present in paraplegin); interacts with microtubules and has microtubule severing properties; interacts with atlastin and REEP1 and contributes to endoplasmic reticulum morphology; mutations affect axonal transport. <i>Interacts with: SPG3A/Atlastin, SPG31/REEP1, SPG33/ZFYVE27, ESCRT-II complex associated CHMP1B, Reticulon 1 (endoplasmic reticulum protein), Reticulon 3, CREL5, COP55, Tubulin; inhibits bone morphogenic protein signaling</i>	(47,379,390,400,557–559)
SPG5/CYP7B1	Cytochrome P450-7B1 (CYP7B1) provides primary metabolic route for cholesterol derivatives dehydroepiandrosterone (DHEA) and related hydroxysteroids via 7 $\alpha$ -hydroxylation; and that in the liver; and an alternative route for cholesterol metabolism.	(418)
SPG6/NIPA1	“Not imprinted in Prader Willi/Angelman 1” (NIPA1): nine alternating hydrophobic-hydrophilic domains predicts integral membrane localization; NIPA1 binds to BMP-II receptor to inhibit BMP signaling; NIPA1 transcription is induced by low extracellular Mg <sup>++</sup> ; NIPA1 expression causes inwardly direct Mg <sup>++</sup> conductance. <i>Interacts with Type II bone morphogenic protein (BMP) receptor 2 (BMPRII) to inhibit BMP signaling and SPG3A/atlastin</i>	(400)
SPG7/paraplegin	Mitochondrial metalloprotease, involved as protein chaperone in mitochondrial protein quality control. <i>Interacts with AFG3L2 (mutations cause SCA type 28)</i>	(407,423)
SPG8/Strumpellin (KIAA0196)	KIAA0196/Strumpellin, mutations may be pathogenic through protein aggregation: Strumpellin binds to valosin-containing protein (VCP; also known as p97, TER ATPase and Cdc48 p); VCP-positive inclusions occur in a wide variety of neurodegenerative disorders including Parkinson’s disease, Lewy body disease, Huntington’s disease, amyotrophic lateral sclerosis, and SCA type III (Machado–Joseph disease); Strumpellin may also regulate actin dynamics through its interaction with WASH; <i>interacts with Valosin-containing protein (VCP; also known as p97, TER ATPase and Cdc48 p); WASH, a Wiskott–Aldrich syndrome protein (WASP) family member, via SWIP (Strumpellin and WASH interacting protein (WASH localizes to endosomal subdomains and regulates endocytic vesicle scission in an Arp2/3-dependent manner).</i>	(560)
SPG10/KIF5A	Kinesin heavy chain (KIF5A) a molecular motor subunit that participates in the intracellular movement of organelles and macromolecules	(465)
SPG11/Spatacsin	Unknown function; spatacsin is a component of a protein complex that includes two other HSP proteins, SPG15/ZFYVE26 and SPG48/KIAA0415 (316), suggesting that these forms of HSP may share similar molecular pathogenesis. <i>Interacts with SPG48/KIAA0415, SPG15/ZfyVe26, C200RF29, DKFZp761E198</i>	(316)
SPG13/KIF5A	HSPD1 gene encodes Chaperonin 60 (also known as heat shock protein 60, HSP60), the large subunit of the mitochondrial Hsp60/Hsp10 chaperonin complex; functions in mitochondrial protein quality control (binding and sequestration, and refolding of unfolded proteins)	(414)
SPG15/Spastizin (ZFYVE26)	FYVE-domain proteins have diverse functions including membrane trafficking, signal transduction, regulating the cytoskeleton, and serving as phosphatidylinositol 3-phosphate (PtdIns3P) phosphatases. Spastizin colocalizes with ER and endosome markers and is a component of a protein complex that includes two other HSP proteins, SPG11/spatacsin and SPG48/KIAA0415. <i>Interacts with SPG11/Spatacsin and SPG48/KIAA0415</i>	(259,316,425)

TABLE 119-4 HSP Proteins—cont'd

HSP Gene/Protein	Functions	References
SPG17/BSCL2	BSCL2/seipin: Endoplasmic reticulum transmembrane protein; mutations appear pathogenic through induction of endoplasmic reticulum stress-mediated apoptosis	(318,415,416)
SPG20/spartin	Spartin: N-terminal region similar to spastin; homologous to proteins involved in the morphology and trafficking of endosomes; also localizes to mitochondria; interacts with Eps 15, a protein known to be involved in endocytosis and the control of cell proliferation, suggesting that spartin may be involved in endocytosis, vesicle trafficking, or mitogenic activity; spartin promoter hypermethylation is associated with cytokinesis arrest in colorectal carcinoma. <i>Interacts with GRP78, GRP75, ubiquitin, and the E3 ubiquitin-protein ligases AIP4/Itch and AIP5/WWP1, and; nucleolar protein "nucleolin"; inhibits BMP signaling</i>	(358,400,427,428,431,561,562)
SPG21/masparidin	Appears to be a cytosolic protein partitioned between the cytosol and vesicles of the endosomal/trans-Golgi network; may play a role in endosomal trafficking; may be similar to a noncatalytic $\alpha/\beta$ hydrolase protein NDRG1, mutations in which cause peripheral neuropathy with early axonal involvement. <i>Interacts with Aldehyde dehydrogenase ALDH16A1</i>	(162,432)
SPG22/MCT8	Monocarboxylate transport 8 (MCT8) is a thyroid hormone transporter, results in elevated serum triiodothyronine (T3) levels	(462)
SPG31/REEP1	Receptor expression enhancing protein 1 (REEP1), structurally related to the DP1/Yop1p family of endoplasmic reticulum-shaping proteins; required for ER network formation in vitro; forms complexes with atlastin and spastin within endoplasmic reticulum; also binds to microtubules and promotes ER alignment along the microtubule cytoskeleton in vitro. <i>Interacts with SPG3A/Atlastin and SPG4/spastin</i>	(379)
SPG33/ZFYVE27	ZFYVE27/protrudin has a role in Rab11-mediated membrane trafficking and promotes neurite outgrowth. <i>Interacts with SPG4/spastin and RAB11</i>	(47,74)
SPG35/FA2H	Fatty acid 2-hydroxylase (FA2H) in oligodendroglia catalyzes the 2-hydroxylation of myelin galactolipids, galactosylceramine, and sulfatide, and therefore helps determine myelin lipid content	(563)
SPG39/NTE	Neuropathy target esterase (NTE) is a phospholipase localized to ER membranes; may reduce cytotoxic lysophosphatidylcholine; implicated in toxic organophosphorus compound induced delayed neurodegeneration (OPIDN); may function to regulate cyclic AMP-dependent protein kinase. <i>Interacts with Cyclic AMP-dependent protein kinase (in Drosophila)</i>	(434–443,445,446)
SPG42/SLC33A1	SLC33A1 is an acetyl CoA transporter that moves acetyl-CoA into the Golgi apparatus where it may be transferred to sialyl residues of gangliosides and glycoproteins	(53)
SPG44/GJA12/GJC2	Gap junction protein (GJA12/GJC2) also known as connexin47 (Cx47); important factor in formation of specialized channels ("gap junctions") between cells that allow selective movement of ions and metabolites between adjacent intracellular compartments	(288,290,447)
SPG48/KIAA0415	KIAA0415 encodes a putative helicase involved in DNA repair; Interacts with SPG11/Spatacsin, SPG15/ZFYVE26, C20ORF29, DKFZp761E198	(316)
Cct5	Epsilon subunit of the cytosolic chaperonin-containing t-complex peptide-1 (Cct5); contributes to the protein folding and assembly of a wide variety of cytosolic proteins including actin and tubulin	(119)
Mitochondrial ATP6 gene	Synthesis of ATP (from ADP) in mitochondria is coupled to translocation of protons from the mitochondrial matrix to specific intermembrane sites. Mitochondrial ATP6 forms a channel through which protons flow back from intermembrane sites into the mitochondrial matrix; and is thus an important factor in mitochondrial ATP production	(212,564)

### 119.3.3 Sensory Neuropathy

As noted above, SPG3A/atlastin mutation p.Asn355Lys was associated with the syndrome autosomal dominant HSN 1 rather than with the syndrome of HSP (114). Sensory neuropathy occurs with spastic paraplegia in two additional syndromes, (SPOAN syndrome, linked to chromosome 11q23) (118) and “mutilating sensory neuropathy with spastic paraplegia” due to *Cct5* gene mutation (119–122).

### 119.3.4 HSP-Related Disorders

ALS is often an important consideration in the differential diagnosis of subjects with progressive spastic paraplegia, particularly when there is no clear family history or presence of a causative gene mutation to support the diagnosis of HSP. “Upper motor neuron predominant” ALS may begin with lower extremity spastic weakness, and particularly in young individuals, be slowly progressive (192,193,195).

For ~10% of subjects, ALS is a familial disorder. Mutations in a number of genes (321,322) have been identified in both familial and “apparently sporadic” ALS subjects. The discovery that ALS can have multiple separate causes (mutations in different genes) indicates that ALS, like HSP, is a clinicopathologic syndrome that can result from molecularly distinct inciting abnormalities.

Neuropathologic features of ALS (degeneration of corticospinal tract axons, loss of anterior horn cells, degeneration of peripheral motor axons, and degeneration of pyramidal neurons in the motor cortex) overlap those of HSP (degeneration of corticospinal tract axons with variable peripheral motor axon degeneration, variable and mild degeneration of anterior horn cells, and degeneration of *fasciculus gracilis* fibers). As previously noted, distal muscle atrophy and peripheral motor neuropathy are typical or variable features of many forms of HSP (see Table 119-1). Indeed, at times, lower motor neuron involvement in some subjects with HSP meet diagnostic criteria for ALS. For example, the combination of spastic paraplegia and distal muscle atrophy in some (though not all) subjects with SPG11 HSP meet criteria for juvenile ALS (253). Although a case of ALS occurring with SPG4/spastin mutation (c.304\_309dupGCCTCG in exon 1) has been described (71), it is difficult to know if this is an unusual form of ALS with prolonged survival (more than 40 years), or an unusual form of SPG4 HSP with bulbar muscle and marked upper extremity involvement and distal wasting (71). In addition, muscle wasting in subjects with SPG39/NTE mutation (272), and rarely with SPG3A/atlastin mutation (109), may meet ALS diagnostic criteria.

Rather than concluding that these HSP gene mutations cause ALS, such cases may reflect the inclusive nature of *El Escorial* criteria for diagnosing ALS. Summarized in the Amyotrophic Lateral Sclerosis Association website ([http://www.alsa.org/assets/pdfs/fyi/criteria\\_for\\_diagnosis.pdf](http://www.alsa.org/assets/pdfs/fyi/criteria_for_diagnosis.pdf)),

these criteria state that “Definite ALS is defined on clinical grounds alone by the presence of upper motor neuron (UMN) as well as lower motor neuron (LMN) signs in the bulbar region and at least two of the other spinal regions or the presence of UMN and LMN signs in three spinal regions. The important determinants of diagnosis of definite ALS in the absence of electrophysiological, neuroimaging and laboratory examinations are the presence of UMN and LMN signs together in multiple regions.” Many subjects with complicated HSP associated with distal muscle atrophy or motor neuropathy may fulfill these criteria. Nonetheless, the insidiously progressive course (over decades) of bilaterally symmetric, lower extremity spastic weakness usually distinguished HSP syndromes (uncomplicated or complicated) from typical, adult-onset ALS syndromes (familial or sporadic), which generally progress more rapidly (e.g., over 1–5 years,) and often exhibit markedly asymmetric, unilateral, or single-limb weakness. One caveat, however, are juvenile forms of ALS, in which more gradually progressive and symmetric weakness and spasticity may be similar to some complicated HSP syndromes.

Recent results of a study of genome copy number variants in ALS are intriguing from the standpoint of potential HSP–ALS relationship. This study (323) analyzed genome copy number variants in 1875 ALS subjects and 8731 control subjects. Although this study did not find evidence for a difference in global copy number variant between ALS and control subject cohorts, two loci met criteria for follow-up investigation: the DPP6 locus (which has already been implicated in ALS pathogenesis) and the 15q11.2 locus, containing NIPA1, mutations in which cause autosomal dominant SPG6 HSP. Clearly, NIPA1 and the molecular cascade with which it interacts warrant further investigation in ALS pathogenesis.

PLS, independently described by Erb and Charcot (324), originally referred to a clinicopathologic syndrome in which progressive spastic weakness involving the lower extremities, eventually the upper extremities and corticobulbar muscles (325,326), was associated with degeneration of corticospinal and corticobulbar tracts. More recently, it is recognized that rather than being a single etiologic entity, the PLS syndrome (insidiously progressive spastic weakness involving lower and upper extremities, speech, and swallowing) can be a feature of genetically heterogeneous disorders. Although for the vast majority of subjects, PLS occurs as an “apparently sporadic” disorder (without known family history), both an autosomal recessive form of juvenile PLS and a family with an autosomal dominant PLS syndrome have been described. Like HSP, neuropathologic analysis of PLS shows axonal degeneration of corticospinal tracts with general preservation of the anterior horn cells. Autopsy of some, but not all (326), PLS subjects revealed decreased number of Betz cells in layer 5 and decreased number of pyramidal tract neurons in layers 3 and 5 of the precentral gyri (325,327). As noted above, atrophy

or decreased number of Betz cells has been described in HSP (172,173). For recent reviews of PLS and HSP, see (85,304–306,328).

Classically, PLS is distinguished clinically from HSP on the basis of (1) inheritance: PLS is usually an “apparently sporadic” disorder (although, as noted above, an autosomal recessive juvenile form and a family with dominantly inherited PLS have been described (329,330)); and (2) symptomatic upper motor neuron deficits involving upper extremities, speech, and swallowing (whose presence makes PLS discernible from “uncomplicated,” “apparently sporadic” HSP but not necessarily separable from some complicated forms of HSP (Table 119-5)). On the other hand, early stages of PLS (prior to development of upper extremity weakness, dysarthria, and dysphagia) may not be distinguished from “apparently sporadic” HSP (discussed above). Previously (303), assessment of distal lower extremity dorsal column function (by vibratory sensation in the toes and SSEPs) was considered useful to distinguish HSP (considered to be a central nervous system, motor-sensory axonopathy often involving mild lower extremity dorsal column impairment) from PLS (considered to be a pure upper motor neuron degenerative process sparing dorsal column

fibers). Recently, however, Brugman et al. (85) showed that a subset of PLS subjects had lower extremity dorsal column impairment. Involvement of dorsal columns in a subset of PLS subjects both challenges the concept that this clinical parameter can distinguish HSP from PLS and may expand our understanding of the PLS syndrome to include mild, sensory (dorsal column) involvement.

**119.3.4.1 Juvenile PLS and Familial PLS.** We (J. K. Fink, unpublished observations) and others (329,331) have observed infantile and early childhood-onset lower extremity spastic weakness among siblings that for several years was consistent with autosomal recessive uncomplicated HSP; and which was followed later by progressive involvement of the upper extremities, speech, and swallowing, consistent with the diagnosis of PLS. Such families in which PLS is transmitted as an autosomal recessive syndrome could be classified as either familial PLS or as a complicated form of HSP with upper extremity and bulbar muscle involvement (332–335).

Yang et al. (335) and Hadano et al. (336) identified mutations in the ALS2 gene (protein: “ALSin”) in juvenile onset autosomal recessive PLS. Depending on the particular ALSin gene mutation, subjects exhibited either a progressive upper motor neuron syndrome (spastic

**TABLE 119-5 Animal Models of Hereditary Spastic Paraplegia**

Spastic Gait (SPG)	Protein Name	Mouse	<i>Drosophila</i>	<i>Zebrafish</i>	<i>Caenorhabditis elegans</i>	Other
SPG1	L1 cell adhesion molecule (L1CAM)	Cohen et al., 1998, Dahme et al., 1997 (456,487–489)			(449)	
SPG2	Proteolipid protein	(459,460,565) Al-Saktawi et al. (2003) (480–486)				
SPG3A	Atlastin	(476)	(495)	(556)		
SPG4	Spastin	(477,566)	(468,469,567,568) (490)	(569–571)	(572,573)	Cattle (164)
SPG5 (8 p)	CYP7B1	(574)				
SPG6	“Not imprinted in Prader Willi/Angelman 1” (NIPA1)		(401)		(163)	Rat (575)
SPG7 (16q)	Paraplegin	Ferreirinha et al. (2004) (424,472,576,577)				
SPG8	KIAA0196/Strumpellin		(29)	(404,570)		
SPG10	Kinesin heavy chain (KIF5A)	(465)				
SPG11 (15q)	Spatacsin (KIAA1840)			(578)		
SPG13	HSPD1 gene encodes Chaperonin 60 (also known as heat shock protein 60, HSP60)	(579)				
SPG21 (15q21-q22)	Maspardin	(580)				
SPG39 (19p13)	Neuropathy target esterase (NTE)	(479)	(492,493,581)			
SPG42 (3q24-q26)	Acetyl CoA transporter (SLC33A1)			(53,570)		



weakness involving the upper and lower extremities, spastic dysarthria, and dysphagia) or also had lower motor neuron signs (e.g., muscle atrophy) consistent with slowly progressive ALS (335). The absence of detectable ALSin mutations in some families suggests that autosomal recessive familial PLS/juvenile ALS is genetically heterogeneous. For additional clinical and molecular features and animal models of ALS2/Juvenile PLS, see (337–340).

Chow et al. (330) described a French–Canadian kindred with an adult-onset (age 30–60 years), PLS syndrome (progressive spastic paraparesis, followed closely by spastic weakness of the upper extremities and later associated with spastic dysarthria and dysphagia). Subsequently, a genetic locus (4ptel-4p16.1) segregating with this disorder was identified (341). The possibility that genetic factors contribute to PLS in some subjects may be supported by the recent observation of FIG4 mutations in two subjects described as having PLS (342).

**119.3.4.2 Infantile Onset HSP and Familial Cerebral Palsy.** Nonprogressive lower extremity spastic weakness dating to infancy and unaccompanied by mental retardation or other neurologic abnormalities (“spastic diplegic cerebral palsy”) often occurs in the absence of obvious risk factors (such as prematurity, small size for gestational age, and multiple pregnancy, or intrauterine hypoxia) (343,344). A study performed in England and Sweden estimated that 2% of all cerebral palsy cases are due to a genetic cause (345,346). Considering all types of cerebral palsy together, the risk of recurrence in sibships is low. However, bilaterally symmetric spastic cerebral palsy (as opposed to hemiplegic or monoplegic cerebral palsy) for which there is no identifiable cause has recurrence risk in sibs of approximately one in eight (345).

McHale et al. (347) identified a locus for autosomal recessive spastic cerebral palsy on chromosome 2q24-25 with a minimum locus interval of approximately 5cM between the markers D2S124 and D2S2284. It is important to note that this locus for autosomal recessive, bilaterally symmetric, spastic cerebral palsy overlaps the SPG13 locus for uncomplicated autosomal dominant HSP (36). It is intriguing to consider that both disorders are due to mutations in the same gene.

Clearly, subjects with some forms of HSP may be misdiagnosed as having spastic diplegic cerebral palsy (235,348–350). It is uncertain, however, whether some or all kindreds with autosomal recessive familial cerebral palsy (351) should be considered forms of autosomal recessive HSP with infantile onset symptoms. It is unknown, for example, whether autosomal recessive familial cerebral palsy linked to chromosome 2q24-25 (347) shares the same pathologic changes as HSP (distal axonal degeneration of corticospinal and dorsal column tracts in the spinal cord).

As a generalization, the primary clinical feature that distinguishes HSP from bilaterally symmetric spastic diplegic cerebral palsy is the *progressive* nature of lower

extremity weakness in HSP (348) (as opposed to *non-progressive* lower extremity spastic weakness in cerebral palsy). Dorsal column impairment, a feature of HSP but not spastic cerebral palsy, is less helpful because vibratory sensation is usually normal in children with HSP.

Nonetheless, there are important exceptions to the generalization that HSP is always a progressive disorder. Specifically, HSP beginning in infancy (or earlier) might not be evident as a progressive disorder. Rather, such children may present with delayed acquisition of walking milestones, lower extremity spastic weakness, and toe-walking in a manner that may not be distinguished from spastic diplegic cerebral palsy. Moreover, for some families with classic features of HSP, the degree of progression may be so insidious and so mild as to be clinically inapparent during childhood. We (350) and others have observed affected subjects with no previous family history of HSP who were diagnosed initially as having spastic diplegic cerebral palsy, only to have this diagnosis changed to autosomal dominant HSP following the appearance of the disorder in their child. In two unrelated families, we documented that this was due to *de novo* SPG3A/atlastin mutation in the proband (235,350). In addition to SPG3A/atlastin mutation, *de novo* mutation in SPG4 has also presented as familial cerebral palsy (352).

**119.3.4.3 Hereditary Motor Sensory Neuropathy (HSMN, also Known as CMT).** Previously, the HSPs were considered categorically distinct from HSMNs. Clinicians can readily distinguish the lower extremity hyperreflexia of HSPs from the lower extremity hypo- or areflexia of the HSMNs. There is growing awareness that these syndromes sometimes overlap. As noted above, many forms of HSP have peripheral neuropathy including forms of HSP that were usually considered “uncomplicated” (e.g., SPG3A and SPG4 HSP). Often, the neuropathy associated with HSP is “axonal” rather than “demyelinating.” This is consistent with the general concept that the lower extremity spasticity and hyperreflexia is due to corticospinal tract and dorsal column axon degeneration; and suggests that axon degeneration (in these forms of HSP) involves both central and peripheral motor and sensory fibers. On the other hand, some forms of HSP, such as X-linked SPG2, due to PLP gene mutation, may have demyelinating or mixed axonal-demyelinating peripheral neuropathy. Indeed, mutations in one gene (BSCL2/Seipin) can manifest as either a predominant peripheral neuropathy syndrome (consistent with a form of type 2, CMT) or a form of HSP associated with peripheral neuropathy (SPG17).

Furthermore, there is increasing awareness that some molecular mechanisms implicated in axon degeneration in HSP are similar to those implicated in axon degeneration in some forms of CMT. For example, whereas mutations in the kinesin heavy chain subunit KIF5A cause SPG10 HSP (353), mutations in a functionally related molecular motor subunit KIF1B- $\beta$  cause a form of CMT

(CMT2A1) (354). Similarly, whereas gap junction A12/connexin 47 gene mutations cause SPG42 HSP (as well as the demyelinating disorder, PMLD) (289), mutations in gap junction beta 1 cause an X-linked form of CMT (CMT1X) (355). In a related manner, FIG4 mutations are implicated in both CMT (CMT4J) (356), and have been identified in both PLS and ALS subjects (357). These examples indicate that the same or overlapping pathogenic mechanisms underlie many forms of these ostensibly clinically disparate syndromes (spastic paraplegias versus peripheral neuropathies).

**119.3.4.4 SPG20/spartin and Colorectal Carcinoma.** Lind et al. (358) reported that SPG20/spartin promoter is hypermethylated in colorectal carcinomas and adenomas, but was not hypermethylated in normal mucosa. They proposed that SPG20/spartin gene methylation status be used as a biomarker of colorectal tumors. In addition to being a biomarker, Lind et al. (358) noted that SPG20/spartin promoter hypermethylation could contribute to neoplasia. Specifically, they observed an association between SPG20/spartin promoter hypermethylation, transcription silencing of the SPG20/spartin gene, and cytokinesis arrest. They noted that cytokinesis arrest could lead to aneuploidy, a mechanism of neoplasia (358).

### 119.3.5 Treatment

There is no effective treatment to prevent, retard, or reverse the process of distal axonal degeneration that underlies HSP. Nonetheless, treatment approaches used for chronic paraplegia from other causes are useful. In particular, spasticity may be reduced with oral or intrathecal Lioresal (baclofen) or oral Dantrolene or Tizanidine and selective injection of botulinum toxin (Botox) (359–361). Oxybutynin is often effective in reducing urinary urgency. Physical therapy is recommended to subjects with HSP to improve range of motion, maintain and increase lower extremity strength, and increase cardiovascular conditioning, which may improve endurance and lessen fatigue. Toe-dragging may be reduced by ankle foot orthotics as well as by devices that provide gait phase-dependent, transcutaneous peroneal nerve stimulation (362).

Regarding placement of intrathecal baclofen pump, it is essential to establish, by one or more trial injections, the extent to which intrathecal baclofen provides improved walking ability. It is emphasized that the important outcome parameter for these trials is not the degree to which lower extremity spasticity is reduced, but rather, the degree to which walking is improved. In general, we have found that reduction of spasticity (e.g., either by oral or intrathecal baclofen) provides greatest functional benefit in HSP subjects who have relatively preserved lower extremity strength. Conversely, in our experience, subjects for whom gait disturbance is attributed to roughly equal contributions of weakness and spasticity benefit less from spasticity-reducing strategies.

**Genetic counseling** in HSP must be individualized for each individual and family. It is important to recognize that genetic penetrance in autosomal dominant HSP is age-dependent and may be as low as 70% (82). Moreover, the age at which symptoms begin and the ultimate degree of disability may vary widely within a given family, between families with the same genetic type of HSP, and between genetic types of HSP. For this reason, it is generally prudent to limit the premise upon which genetic counseling is based on the age-of-symptom onset, phenotypic variability, and genetic penetrance that are present in the patient's family (rather than rely primarily on data from published kindreds describing the same genetic type of HSP). Although the risk of inheriting the HSP disease gene and genetic penetrance can be estimated, it is generally not possible to predict accurately whether HSP symptoms will develop early (e.g., before age 10) or late (e.g., after age 40) or whether HSP will lead to mild or severe disability.

Extreme caution must be exercised when providing genetic counseling to a subject who has all the signs and symptoms of HSP but who has no other similarly affected relatives. While such “apparently sporadic, uncomplicated spastic paraplegia” subjects may have autosomal recessive HSP (and thus low risk of transmitting the disorder to progeny), it is also possible that the disorder is autosomal dominant (with absent family history due to incomplete genetic penetrance, the disorder being due to a *de novo* HSP gene mutation, or mistaken paternity). Five to ten percent of ASSP subjects have been shown to have mutations either in SPG4 or less commonly in SPG3A HSP genes (both of which are autosomal dominant and therefore imply up to 50% risk of disease reoccurrence in each first-degree relative).

**119.3.5.1 Genetic Counseling of Subjects with SPG7 HSP.** A number of subjects with ASSP (both uncomplicated and complicated syndromes) have been shown to have mutations in the SPG7/paraplegin gene (76,297,363). The majority of SPG7/paraplegin mutations discovered in spastic paraplegia subjects are in homozygous or compound heterozygous states. Recently, several groups (76,363,364) have described both “uncomplicated” and “complicated” HSP in subjects who are *heterozygous* for pathogenic SPG7 mutations. In some of the subjects with spastic paraplegia due to *heterozygous* SPG7/paraplegin mutations, muscle biopsies have shown evidence of mitochondrial disturbance including ragged red fibers and cytochrome C oxidase negative muscle fibers. There is incomplete information to determine reliably which SPG7 mutations are pathogenic when homozygous or in compound heterozygous states, which SPG7 mutations are pathogenic when heterozygous, and whether other mutations in regulatory and noncoding SPG7 regions coexist with SPG7 mutations associated with spastic paraplegia when heterozygous. Nonetheless, the finding that at least some SPG7 mutations cause spastic paraplegia when heterozygous has important implications for

genetic counseling. Specifically, subjects with SPG7 HSP who are homozygous for SPG7/paraplegin gene mutation should be informed that unlike “typical” autosomal recessive disorders, their progeny (obligate SPG7 mutation heterozygotes) have some risk of developing some symptoms of HSP which could range from subtle, *forme fruste* to full manifestations of SPG7 HSP.

Some HSP subjects request prenatal diagnosis. For identified HSP genes for which analysis is available (either in commercial laboratories or on a research basis, Table 119-1), prenatal genetic testing can be performed using chorionic villus samples, amniocytes, and blastocyst cells (for preimplantation genetic testing). Prenatal testing for SPG3A, SPG4, and SPG42 HSP (54,188,365) as well as detection of PLP mutations (mutated in SPG2 HSP and PMD) have been reported.

If the HSP locus is known in a given family (but the gene is yet to be identified), it is possible to perform prenatal risk assessment by analyzing fetal inheritance of haplotypes that segregate with “normal” or “HSP affected” alleles in the family (365). Such methods estimate the risk of fetal inheritance of the HSP gene mutation. Genetic counseling must consider not only this information but also estimates of penetrance, range of age-of-symptom onset, and variable disease severity that occur within the particular family.

### 119.3.6 HSP Genes and Encoded Protein Function

A comprehensive catalog of HSP gene mutations and their associated phenotypes and thorough presentation of the emerging molecular biology of HSP proteins is beyond the scope of this chapter. The following section is an overview of HSP protein function.

## 119.4 AUTOSOMAL DOMINANT HSP

### 119.4.1 SPG3A/atlastin (17)

SPG3A gene mutations are the most frequent cause of childhood onset, dominantly inherited HSP (308,366,367). Several dozen SPG3A/atlastin mutations have been identified. Often, mutations are “private,” being uniquely present in the families in which they are identified. The existence of SPG3A/atlastin mutation that disrupts a conserved GTPase motif (R271Q) indicates the likelihood that atlastin is a functional GTPase (368). SPG3A/atlastin mutations appear to be pathogenic through haploinsufficiency. This is supported by observations of SPG3A/atlastin insertion mutation that leads to a stop codon and premature protein truncation (369), and by the occurrence of gait impairment and neuropathologic changes in mice bearing targeted mutation of SPG3A/atlastin gene (J. K. Fink et al. unpublished observation).

SPG3A/atlastin is homologous to guanylate-binding protein 1 (GBP1), a member of the dynamin family of

large GTPases (17). Dynamins play essential roles in a wide variety of vesicle trafficking events during formation of clathrin-coated vesicles from the plasma membrane, receptor-mediated endocytosis, and endosome trafficking to the *trans*-Golgi network (370,371). Consistent with this role, Zhu et al. (372) showed that atlastin was present in *cis*-Golgi membranes of cortical motor neurons.

Atlastin binds to itself (homodimer) in a nucleotide-dependent manner (373). Luan et al. (374) reported that atlastin interacts *in vitro* with HPK/GCK-like kinase (HGK), a protein kinase in the c-Jun N-terminal kinase (JNK) pathway. JNK pathway signaling leads to diverse effects including differentiation and apoptosis.

Recently, atlastin has been demonstrated to interact *in vitro* with three other HSP proteins, SPG4/spastin, SPG6/NIPA1, and SPG31/REEP1, and to play a critical role in development of endoplasmic reticulum morphology, particularly in the formation of three-way tubular junctions (375–382).

Endoplasmic reticulum stress cascade (e.g., due to misfolded proteins) is a widely recognized factor in many neurodegenerative disorders (e.g., those due to polyglutamine repeat expansions) including a number of forms of HSP (notably, SPG8/Strumpellin and SPG17/BSC12 (seipin)). Demonstration that atlastin, spastin, REEP1, and NIPA1 interact and that atlastin, spastin, and REEP1 help determine endoplasmic reticulum morphology represent seminal advances in our knowledge of the molecular mechanisms of four types of HSP (SPG3A, SPG4, SPG6, and SPG31). Moreover, these discoveries emphasize the importance of endoplasmic reticulum function and morphology in the maintenance of axon structure in health and disease.

### 119.4.2 SPG4/Spastin Gene (383)

SPG4 is the single most common type of autosomal dominant HSP, representing ~40% of such patients. More than 300 SPG4/spastin mutations have been identified in HSP patients. These mutations have been of all types (missense, nonsense, deletion, insertion, whole exon deletion, and aberrant splicing) except tandem repeat expansions. Whereas nonsense, frameshift, and splice site mutations are distributed throughout the gene (except exon 4 which is not expressed in the central nervous system), missense mutations are located predominantly in spastin’s AAA domain (“ATPase associated with diverse cellular activities”) (383). Many SPG4/spastin mutations are predicted to cause loss of spastin function. Both haploinsufficiency and dominant gain function mechanisms have been proposed for spastin mutation pathogenicity (384).

Spastin is expressed widely; although within the spinal cord, spastin is present only in neurons and not glia (18). Spastin’s intracellular distribution has been the subject of controversy as both nuclear (18) and cytoplasmic (385–388) localizations have been reported using



various techniques. There is evidence that spastin has dual nuclear and cytoplasmic localization owing to alternate translation initiation that results in large (68 kDa) and small (60 kDa) spastin isoforms (389). Whereas both isoforms are imported into the nucleus, the larger (68 kDa) isoform contains two nuclear export signals and is efficiently exported to the cytoplasm. The larger (68 kDa) spastin isoform is more abundant in brain and the spinal cord compared to other tissues.

Reid et al. (390) showed that spastin interacts with CHMP1B, a protein associated with the endosomal sorting complex required for transport (ESCRT)-III complex. This suggests that spastin participates in intracellular membrane traffic events.

Azim et al. (391) showed that spastin interacts with tubulin in vitro (391). Studies of transfected cells expressing wild-type and mutant spastin suggest that HSP-specific *SPG4* mutations disturb spastin's interaction with microtubules (385–387). Spastin's interaction with microtubules appears to be mediated through spastin's N-terminal region and regulated through the ATPase activity of the AAA domain (385). Compared to wild-type spastin, studies in transfected cells showed that mutant spastin had aberrant cytoplasmic localization and diminished promotion of microtubule disassembly (386,387). Transfection studies also provide evidence that mutant spastin expression leads to altered distribution of organelles (386) including mitochondria and peroxisomes. McDermott et al. (386) concluded that mutant spastin interferes with anterograde transport of these organelles.

A portion of spastin shares homology with katanin, a microtubule severing enzyme (392). There is in vitro and in vivo evidence that spastin has microtubule severing properties (19,393,394). For example, overexpression of *Drosophila*'s spastin homolog (*Dspastin*) in *Drosophila* S2 cells produced nearly complete absence of cytoplasmic microtubules (393). Trotta et al. (395) used RNA interference to examine loss of spastin function in *Drosophila*. They observed that loss of spastin resulted in aberrant stabilization of microtubule cytoskeleton in neurons and defects in synaptic growth and neurotransmission.

Axonal cytoskeletal stability and axonal transport depend to a large extent on dynamic microtubule “remodeling” (assembly, disassembly, and movement of short microtubules into new patterns of organization (396)). The ability of neurons to mobilize microtubules appears to depend on microtubule severing enzymes, such as spastin and katanin, which cleave microtubules into shorter fragments that can be moved to new locations and subsequently elongated (396). Spastin mutations in *SPG4* HSP appear to reduce microtubule severing and thus compromise neuronal cytoskeletal remodeling and possibly interfere with axonal transport.

As noted above, spastin interacts with atlastin (378,379,381). Further, in addition to tubulin binding and microtubule severing properties, Spastin has been

shown to participate with atlastin and REEP1 in the coordination of microtubule interactions with tubular endoplasmic reticulum (379).

### 119.4.3 SPG6/NIPA1

SPG6 HSP is a prototypical example of slowly progressive, uncomplicated HSP that usually begins in adolescence or adulthood (see reviews by Fink et al. (24) and Fink and Hedera (62)). It is notable that only 4 *SPG6*/*NIPA1* amino acid substitutions have been identified among five unrelated SPG6 families. Rainier et al. (26) reported T45R mutation in an Iraqi family and an Irish family. G106R mutation has been reported in a British family, a Brazilian family, and in two unrelated Chinese families (23,397,398). It is interesting that in the case of the two Chinese SPG6 families, the G106R mutations arose by two different mutations in each family involving the same nucleotide: one family had mutation at nucleotide 316 G→C and one family had mutation at the same nucleotide but with 316 G→A. Each change led to the same amino acid substitution (G106R). These observations suggest that this *SPG6*/*NIPA1* region may be a mutational “hot spot” (23).

*NIPA1* is expressed predominantly in the central nervous system. The presence of nine alternating hydrophobic–hydrophilic domains suggests that *NIPA1* encodes a membrane protein. *NIPA1* mutation T45R appears to act through a “dominant negative” gain of function. This prediction is based on the observation that subjects who are missing one *NIPA1* gene entirely (e.g., subjects with Prader–Willi and Angelman syndromes who have deletions involving this region of chromosome 15q) do not develop HSP (26,399).

Recent insights into *NIPA1* function are emerging. There is evidence, for example, that *NIPA1* binds to bone morphogenic protein 2 (BMP-II) receptor to inhibit BMP signaling (400,401) (BMP signaling has also been shown to be inhibited by *SPG4*/spastin and *SPG20*/spartin (400)). In addition, Goytain et al. (376) showed that *NIPA1* transcription is enhanced by low extracellular Mg<sup>++</sup>, and that *NIPA1* expression causes inwardly direct Mg<sup>++</sup> conductance in vitro. Finally, as discussed above, *NIPA1* has been shown to interact with *SPG3A*/atlastin, which has been recently implicated in determining endoplasmic reticulum morphology. *NIPA1* accumulation in a *Caenorhabditis elegans* model was associated with evidence of endoplasmic reticulum stress response (163).

### 119.4.4 SPG8/KIAA0196/Strumpellin

Following discovery of the *SPG8* locus on chromosome 8q23–q24 (27), Valdmann et al. (29) identified KIAA0196 (“Strumpellin”) gene mutations in affected subjects in this kindred. To date, *SPG8*/*KIAA0196*/Strumpellin mutations have been reported in four unrelated families (27,29,402,403).



SPG8/KIAA0196/Strumpellin mutations may be pathogenic through protein aggregation. Clemen et al. (404) showed that Strumpellin binds to valosin-containing protein (VCP). VCP-positive inclusions occur in a wide variety of neurodegenerative disorders including Parkinson's disease, Lewy body disease, Huntington's disease, ALS, and SCA type III (Machado–Joseph disease) (405). Through induction of endoplasmic reticulum stress response, protein accumulation is increasingly recognized as a common pathologic mechanism in a wide variety of degenerative neurologic disorders (reviewed in (406)).

Ropers et al. (407) showed that Strumpellin is present in a protein complex that includes WASH, and which localizes to endosomal subdomains and regulates endocytic vesicle severing. WASH's action on endocytic vesicles depends on Arp/23 (407), an important factor in nucleation of actin from monomers (408,409). By extrapolation, this raises the possibility that Strumpellin mutations could be pathogenic by misregulation of actin dynamics on endosomes.

#### 119.4.5 Kinesin Heavy Chain (KIF5A) Mutations Cause SPG10 HSP

KIF5A is a molecular motor that participates in the intracellular movement of organelles and macromolecules. KIF5A gene mutation was recently identified in affected HSP subjects linked to the SPG10 locus (353). Subjects with KIF5A mutation exhibited either uncomplicated HSP, or HSP associated with distal muscle atrophy (353). Finding *KIF5A* mutations in SPG10 HSP suggests that degeneration of distal axons in this and possibly of other forms of HSP is related to disturbance of axonal transport (410).

It is noteworthy that mutations in another molecular motor component, kinesin light chain, cause axon degeneration limited to the *peripheral nervous system* (CMT type 2A1) (354). Together with the finding that dynactin mutations cause motor neuron disease (411), there is increasing evidence that abnormalities in axonal transport cause degeneration of both central nervous system axons (leading to an HSP phenotype), and peripheral axons (leading to a phenotype of peripheral neuropathy and lower motor neuron disease).

Wang and Brown (412) examined the consequences of SPG10/KIF5A mutation N256S on neurofilament transport in vitro. Expression of this mutation in cultured mouse cortical neurons decreased both anterograde and retrograde neurofilament transport flux by decreasing the frequency of anterograde and retrograde movements. Ebbing et al. (413) studied four different KIF5A mutations and observed that some mutations reduced KIF5A's affinity for microtubules, other mutations affected KIF5A's gliding velocity, and some mutations affected both microtubule binding and gliding velocity.

*Mutation in heat shock protein 60 (also known as chaperonin 60)*, a mitochondrial protein, cause SPG13 ADHSP, an autosomal dominant form of uncomplicated HSP mapped (37). The mechanism by which Hsp60 mutations cause HSP is not clear.

Hansen et al. (414) examined lymphoblastoid cell lines and fibroblasts from a subject with Hsp60 mutation p.Val98Ile. They studied mitochondrial membrane potential, cell viability, and sensitivity to oxidative stress and found no differences from control cells. Interestingly, they observed decreases in mitochondrial protein quality control proteases, Lon and ClpP, and proposed that this may be an adaptive mechanism in response to increased refolding attempts of Hsp60 substrate proteins. It is intriguing that paraplegin, mutations in which cause SPG7 HSP, is also a mitochondrial protein that may play a role in mitochondrial protein quality control.

#### 119.4.6 BSCL2 Mutations Cause Dominantly Inherited HSP Associated with Distal Wasting (Silver Syndrome) and Distal Hereditary Motor Neuropathy (41)

The BSCL2 gene encodes seipin, an integral endoplasmic reticulum membrane (ER) protein. Null mutations in BSCL2 cause congenital lipodystrophy REF. BSCL2 mutations (N88S and S90L) in Silver syndrome affect glycosylation of seipin and result in aggregate formation, leading to neurodegeneration (41,415). In this regard, seipinopathies are regarded as important examples of disorders due to abnormal protein conformation in which activation of the endoplasmic stress response evokes a molecular cascade resulting in neurodegeneration (318,415,416).

#### 119.4.7 SPG31/receptor Expression Enhancing Protein 1 (REEP1)

Beetz et al. (44) found 15 REEP1 mutations (in 16 subjects) among 535 unrelated individuals including subjects with HSP and subjects with apparently sporadic spastic paraplegia. Most REEP1 mutations are considered to be pathogenic through haploinsufficiency (44). Park et al. (379) showed that REEP1 is structurally related to the DP1/Yop1 p family of endoplasmic reticulum-shaping proteins that are required for endoplasmic reticulum network formation. They showed that REEP1 forms complexes with atlastin and spastin within endoplasmic reticulum; and furthermore that REEP1 binds to microtubules and promotes alignment of endoplasmic reticulum along microtubules in vitro (379).

#### 119.4.8 SPG33/ZFYVE (Protrudin)

ZFYVE27 encodes a protein with several transmembrane domains, including a Rab11-binding domain and a lipid-binding, FYVE finger domain REF. ZFYVE27/protrudin

has a role in Rab11-mediated membrane trafficking and promotes neurite outgrowth (74).

Mannan et al. (47) performed yeast two-hybrid analysis to identify spastin-interacting proteins and identified ZFYVE27 and four additional proteins. REF In their analysis of 43 German HSP families, they identified an autosomal dominant kindred in which ZFYVE27 mutation p.G191V was present in two affected siblings, absent in an unaffected siblings, and absent in 210 control chromosomes. Subsequently, Martignoni et al. (417) reported that pG191V was a benign polymorphism which, in their in vitro studies of neurite extension and Rab11 binding, functioned similar to wild-type ZFYVE27. Pending identification of additional HSP families with ZFYVE27 mutation and clear demonstration of mutation pathogenicity, assignment of ZFYVE27 as a cause of HSP is controversial.

#### 119.4.9 SPG42/SLC33A1

Genetic linkage analysis and positional candidate gene analysis led to identification of SLC33A1 gene mutation (resulting in p.S113R) in one Chinese, autosomal dominant HSP kindred (53). Analysis of 220 European, autosomal dominant HSP families did not identify SLC33A1 mutation (55). RNAi-mediated knockdown of SLC33A1 resulted in an abnormal tail phenotype in Zebrafish (53). This abnormal phenotype could be rescued by wild-type but not mutant human SLC33A1 mRNA (53).

SLC33A1 encodes an acetyl CoA transporter that moves acetyl-CoA into the Golgi apparatus where it may be transferred to sialyl residues of gangliosides and glycoproteins (see (53) for discussion and references).

### 119.5 AUTOSOMAL RECESSIVE HSP GENES

#### 119.5.1 SPG5/CYP7B1

Hentati et al. (1994) identified the SPG5 locus in five Tunisian families with uncomplicated HSP. Wilkinson et al. and Muglia et al. refined this locus and Tsaousidou et al. (418) identified mutations in the cytochrome P450-7B1 (CYP7B1) gene in five SPG5-linked families. Studies by Biancheri et al. (419) and Goizet et al. indicate that CYP7B1 mutations are a frequent cause of autosomal recessive HSP occurring in 7.3–8% of subjects as well as ~3% among subjects with apparently sporadic spastic paraplegia.

Tsaousidou et al. (418) point out that CYP7B1 provides the primary metabolic route for cholesterol derivatives dehydroepiandrosterone (DHEA) and related hydroxysteroids *via* 7 $\alpha$ -hydroxylation, and that in the liver, CYP7B1 provides an alternative route for cholesterol metabolism. Discovery that CYP7B1 mutations cause HSP represents the first example that altered cholesterol metabolism can result in motor neuron disease.

It is noteworthy that altered cholesterol metabolism from mutations in another cytochrome p450 protein (CYP27A) results in a neurodegenerative disorder (cerebrotendinous xanthomatosis) whose clinical presentation (including juvenile cataracts, neuropathy, spastic paraparesis, ataxia, and white matter abnormalities on MRI) may overlap some forms of complicated HSP.

#### 119.5.2 SPG7/Paraplegin

Casari et al. (254) discovered the paraplegin gene at the SPG7 locus and found disease-specific paraplegin gene mutations in affected subjects from two unrelated autosomal recessive HSP kindreds. Like spastin, paraplegin contains an AAA motif. Paraplegin is homologous to several yeast mitochondrial metalloproteases (AFG3, RCA1, and YME1), which have both proteolytic and chaperone activities (254,420). Casari et al. (254) demonstrated that paraplegin is localized exclusively to mitochondria and that some affected HSP affected subjects with paraplegin gene mutations have histologic evidence (ragged red fibers) and histochemical evidence (no reaction to cytochrome C oxidase) in skeletal muscle biopsy.

Atorino et al. (421) showed that paraplegin interacts with a homologous protein AFG3L2 to form a complex in the inner mitochondrial membrane. This paraplegin/AFG3L2 complex is reduced in fibroblasts from SPG7 HSP subjects who have homozygous paraplegin mutations. Such fibroblasts show reduced mitochondrial complex I activity and an increased sensitivity to oxidative stress, features that can be rescued by expression of wild-type paraplegin. Nolden et al. (422) studied the AAA-protease-dependent processing of the mitochondrial ribosomal protein MrpL32 in mice bearing targeted paraplegin gene mutation. They observed that maturation of MrpL32 and mitochondrial protein synthesis was impaired in paraplegin-deficient mice. This suggests that paraplegin mutations in SPG7 HSP may be pathogenic by impairing mitochondrial protein synthesis.

As noted above, paraplegin complexes with AFGL2 to form a heteromeric mitochondrial complex that contributes to mitochondrial protein quality control and provides chaperone-like activity on the respiratory chain complexes (423). While SPG7/paraplegin mutations cause corticospinal tract degeneration (often associated with cerebellar and peripheral nerve involvement), mutations in AFGL2 cause mitochondria-dependent, Purkinje cell degeneration ascertained clinically as SCA type 28 (SCA28). In mouse models, the SPG7/paraplegin knockout phenotype is made more severe in the presence of AFGL2 knockout (424). This raises the possibility that variation in AFGL2 function (such as through genetic polymorphism) may modify the SPG7 HSP phenotype (and conversely, that SPG7/paraplegin functional variation may modify the phenotype of SCA 28).

SPG11/*Spatacsin* function is not known. Spatacsin does not share significant sequence similarity with other

proteins (251). Nonetheless, spatacsin is a component of a protein complex that includes two other HSP proteins, SPG15/ZFYVE26 and SPG48/KIAA0415 (316), suggesting that these forms of HSP may share similar molecular pathogenesis.

*SPG15/Spastizin (ZFYVE26)* is one of more than 30 mammalian proteins that share a highly conserved, zinc-finger-binding domain characterized by eight conserved cysteine residues (summarized in (259)). FYVE-domain proteins have diverse functions including membrane trafficking, signal transduction, regulating the cytoskeleton, and serving as phosphatidylinositol 3-phosphate (PtdIns3P) phosphatases (reviewed in (425)). Spastizin colocalizes with endoplasmic reticulum and endosome markers, suggesting that it may play a role in endosomal trafficking (259). It is notable that ZFYVE26/Spastizin was demonstrated to be a component of a protein complex that includes two other HSP proteins, SPG11/spatacsin and SPG48/KIAA0415 (316). Moreover, there is evidence that another FYVE-containing protein (ZFYVE27) interacts with SPG4/spastin and may be implicated in SPG33 HSP REF. These observations suggest that these forms of HSP (SPG4, SPG11, SPG15, SPG48, and possibly SPG33) may share common molecular pathogenesis, and that disturbance of membrane trafficking may be an important mechanism (426).

### 119.5.3 SPG20/Spartin (266)

This gene is designated “Spartin” (SPastin-related Autosomal Recessive Troyer protein) because the amino-terminal region is similar to that of spastin’s mutations which cause SPG4 HSP. Spartin’s function is unclear and it is intracellular and may be complex. Bakowska et al. (427) observed that spartin was distributed to both cytosolic and membrane fractions. Whereas Lu et al. (428) observed Spartin localization to mitochondria, Robay et al. (429) noted that spartin’s location (in SH-SY5Y cells) changed with differentiation. In undifferentiated cells, spartin displayed diffuse nuclear and cytosolic localization. Following differentiation into neuroblasts, spartin colocalized with synaptic vesicle marker synaptotagmin and was enriched in synaptic-like structures. Robay et al. (429) showed that following epidermal growth factor (EGF) stimulation, spartin translocated from cytoplasm to the plasma membrane, and suggested that spartin may be involved in the intracellular trafficking of EGF receptor. Eastman et al. (430) showed that spartin associates with surface lipid droplets and can regulate their size and number. Milewska et al. (431) investigated spartin-interacting proteins, confirmed that spartin interacted with GRP78, GRP75, ubiquitin, and the E3 ubiquitin-protein ligases, AIP4/Itch and AIP5/WWP1, and established that spartin interacted with the nucleolar protein nucleolin. Their studies suggested that spartin is a multifunctional protein with potential functions in protein folding and protein turnover both in mitochondria and in endoplasmic

reticulum. The observation that spartin interacts with Eps 15, a protein known to be involved in endocytosis and the control of cell proliferation, suggests that spartin may be involved in endocytosis, vesicle trafficking, or mitogenic activity (427).

Recently, Lind et al. (358) reported that SPG20/spartin promoter is hypermethylated in colorectal carcinomas and adenomas, that hypermethylated spartin promoter leads to transcription silencing of the SPG20/spartin gene, and that reduced SPG20/spartin is associated with cytokinesis arrest. Cytokinesis’ arrest may lead to aneuploidy, a mechanism of neoplasia (358). The authors propose that SPG20/spartin gene methylation status be used as a biomarker of colorectal tumors.

*SPG21/Maspardin* mutations cause “Mast syndrome,” an autosomal recessive complicated form of HSP associated with slowly progressive ataxia and extrapyramidal signs, thin corpus callosum, white matter abnormalities, and dysphagia (162). The disorder progresses insidiously beginning in adolescence or adulthood and leads to akinetic mutism in the most severely affected subjects. Simpson et al. (162) mapped the disorder to a locus on chromosome 15q22.31 and identified disease-specific mutations in a novel gene (Maspardin) within this locus. Maspardin’s function is unclear, although there is evidence that it may function in endosomal trafficking. Maspardin appears to be a cytosolic protein partitioned between the cytosol and vesicles of the endosomal/trans-Golgi network (162,432). Protein homology comparisons suggest that maspardin is related to an  $\alpha/\beta$  hydrolase superfamily of diverse enzymes and may be similar to a noncatalytic  $\alpha/\beta$  hydrolase protein “N-myc downstream regulated” (NDRG1). Mutations in NDRG1 cause autosomal recessive peripheral neuropathy with early axonal involvement (162). Hanna et al. (432) showed that maspardin interacts with aldehyde dehydrogenase ALDH16A1. There are at least 19 other human aldehyde dehydrogenases. Although the specific functions of ALDH16A1 are not known (432), it is relevant that mutations in another aldehyde dehydrogenase ALDH3A cause spastic paraplegia, mental retardation, and congenital ichthyosis (Sjögren–Larsson syndrome) (432) (reviewed in (262)).

### 119.5.4 SPG35/Fatty Acid 2-Hydroxylase (FA2H)

Dick et al. (160) identified mutations in the fatty acid 2-hydroxylase (FA2H) gene in an Omani and a Pakistani family with complicated spastic paraplegia. Affected subjects had childhood onset of spastic paraplegia, progressive dysarthria, dementia, seizures, and variable thin corpus callosum, and brain white matter abnormalities.

FA2H is an important factor in myelin formation and is an important factor in determining myelin lipid content. FA2H is present in oligodendrocytes where it catalyzes the 2-hydroxylation of myelin galactolipids,

galactosylceramine, and sulfatide (160,433). Edvardson et al. (433) reported FA2H mutations in subjects with childhood-onset spasticity, dystonia, cognitive dysfunction, and periventricular white matter disease. The variable phenotype associated with FA2H mutation was expanded by Kruer et al. (161) to include childhood onset, spastic quadriparesis, ataxia, and dystonia and episodic neurological decline, and importantly, the appearance of iron accumulation in brain MRI.

### 119.5.5 SPG39/Neuropathy Target Esterase

Rainier et al. (274) analyzed two autosomal recessive kindreds with childhood-onset spastic paraplegia associated with progressive muscle atrophy in distal upper and lower extremities. The phenotype in these families conformed to that of Troyer syndrome. Homozygosity mapping in one consanguineous kindred suggested linkage to a locus on chromosome 19p13 that contained the NTE gene. NTE was considered a high priority candidate gene because of its involvement in the pathogenesis of OP-induced delayed neuropathy (OPIDN) syndromes in humans and laboratory animals (434–439). OPIDN syndromes include axon degeneration particularly involving long, central, and peripheral fibers, findings that were present in the HSP family with NTE mutation. Rainier et al. (274) showed that affected subjects in the consanguineous kindred were homozygous for M1012 V mutation that disrupted an interspecies conserved residue in NTE's esterase domain. Affected subjects in the nonconsanguineous kindred were compound heterozygotes for two NTE mutations: one allele had 2826A > G mutation which caused substitution of an interspecies conserved residue R890H in NTE's catalytic domain, the other allele had a four base pair insertion (NTE mRNA position 3104) that caused protein truncation after residue 1020. Observations of NTE mutation as the cause of corticospinal and peripheral motor axon degeneration in SPG39 HSP indicates the importance of the NTE pathway in maintaining axon integrity, and raises the possibility that NTE pathway disturbances (and possibly organophosphate exposure) could contribute to other motor neuron disorders including ALS.

NTE may play a role in membrane lipid homeostasis. NTE is a widely expressed, endoplasmic reticulum membrane-associated phospholipase A1 that deacylates intracellular membrane phosphatidylcholine to glycerophosphocholine (440–443). Phosphatidylcholine is a major component of animal cell membranes. High concentrations of lysophosphatidylcholine (LPC) are cytotoxic (444). Recent studies (445) suggest that NTE is involved in LPC homeostasis, protects cells from LPC cytotoxicity (445), and affects membrane fluidity. NTE's *Drosophila* homolog SWS binds to the C3 catalytic subunit of PKA, inhibit its activity, and tethers it to endoplasmic reticulum membranes (446). From their observations, Bettencourt da Cruz et al. (446) propose

that “SWS acts as a noncanonical subunit for PKA-C3, whereby the complex formation regulates the localization and kinase activity of PKA-C3, and that disruption of this regulation can induce neurodegeneration.”

### 119.5.6 SPG44/GJA12/GJC2 (Connexin 47, Cx47) (290)

Specialized channels (“gap junctions”) between cells allow selective movement of ions and metabolites between adjacent intracellular compartments. This is an important factor in the molecular and electrical functional integration of cells and tissues (see review by (288)). To a large extent, gap junctions are formed by connexin proteins (288). For review of the gap junction mutations in myelinating cells, see (447).

Mutations in the gap junction protein alpha 12 [GJA12/GJC2] gene (which encodes connexin 47) cause the autosomal recessive disorder, “PMLD” (289,448), an early-onset dysmyelinating disorder associated with nystagmus, psychomotor delay, progressive spasticity, ataxia. GJA12/GJC2 gene mutations also cause a relatively milder phenotype (designated SPG44) that begins in the first and second decades and is characterized by cognitive impairment, slowly progressive, spastic paraplegia, dysarthria, and upper extremity involvement. As with PMD and PMLD, MRI and MR spectroscopy imaging in SPG44 HSP is consistent with a hypomyelinating leukoencephalopathy (290).

In addition to GJA12/GJC2 mutations, Wang et al. (449) recently reported that mutations in the AIMP1 gene (which encodes ARS-interacting multifunctional protein 1) also cause autosomal recessive, infantile onset, PMLD disease. Although a late-onset, more mild phenotype (compatible with complicated HSP) has not been described, the examples that early-onset dysmyelinating disorders, PMD and PMLD, are allelic with later-onset, more slowly progressive spastic paraplegias (SPG2 and SPG44 HSP), make it likely that some AIMP1 gene mutations also cause later-onset, more slowly progressive disease consistent with a complicated form of HSP.

SPG48/KIAA0415 encodes a putative helicase involved in double-strand DNA repair (316). KIAA0415 interacts with SPG11/Spatacsin and SPG15/ZFYVE27 suggesting that common molecular mechanisms may be involved in these forms of HSP (316).

*Epsilon subunit of the cytosolic chaperonin-containing t-complex peptide-1 (Cct5) gene* causes mutilating sensory neuropathy associated with progressive spastic paraplegia (119,450). An “SPG” designation has not been made. As a component of two chaperone systems, Cct5 contributes to the protein folding and assembly of a wide variety of cytosolic proteins including actin and tubulin (119). This suggests that Cct5 mutations could be pathogenic through protein misfolding [a mechanism invoked for SPG8/Strumpellin, SPG13/Chaperonin 60,



and SPG17/BSCL2 (Seipin)] and/or through cytoskeletal abnormalities including axon transport disturbance.

## 119.6 X-LINKED HSP GENES

*Neuronal cell adhesion molecule L1 gene (L1CAM) mutations* cause several neurologic disorders including X-linked hydrocephalus, MASA syndrome, and complicated spastic paraplegia (451,452). Although some of these syndromes correlate with specific L1CAM mutations (e.g., see (453)), modifying factors are implicated because X-linked hydrocephalus, MASA, and X-linked spastic paraplegia have occurred in the same kindred in which affected individuals had the same L1CAM mutation (280).

L1CAM is an integral membrane glycoprotein cell adhesion molecule in the immunoglobulin superfamily. L1CAM is found primarily in the nervous system. Cell adhesion molecules mediate cell-to-cell and cell-to-matrix attachment. For example, L1 interacts with integrin to regulate attachment to proteins in the extracellular matrix (454). L1CAM functions include guidance of neurite outgrowth during development, neuronal cell migration, and neuronal cell survival (451,454,455). Mice bearing targeted L1CAM gene disruption (456) exhibited weak hind limbs and reduced size of corticospinal tracts. It is interesting that bone morphogenic protein (BMP) increases expression of L1CAM (457) in light of observations that SPG4/spastin, SPG6/NIPA1, and SPG20/Spartin appear to inhibit BMP signaling (400,401).

### 119.6.1 PLP Gene Mutations Cause Both PMD and X-Linked, Complicated SPG2 HSP (144,458)

PLP gene mutations cause PMD which is characterized by severe dysmyelination (such as in jimpy mice) (459,460). In stark contrast to the childhood leukodystrophy PMD, SPG2 HSP patients develop normally and experience slowly progressive spastic gait beginning in childhood and adolescence. SPG2 is considered a complicated form of HSP because affected subjects may have peripheral neuropathy and abnormal appearing white matter on brain MRI scans indicative of abnormal CNS myelin. PLP is an integral myelin protein expressed in oligodendroglia (which express an oligodendroglial-specific splice variant of PLP known as DM20 (461)), and Schwann cells but not in neurons. Murine models of SPG2 HSP exhibit either demyelination or axonal degeneration depending on the nature of the PLP mutation (discussed below). Axon degeneration in SPG2 HSP thus appears to be secondary to an intrinsic disturbance in oligodendroglial cells that disturbs axon–glial interaction (291), rather than from an intrinsic abnormality within neurons per se.

*SPG22/Monocarboxylate transport 8 (MCT8) gene* mutations cause AHD syndrome, characterized by neck

muscle hypotonia in infancy, mental retardation, dysarthria, ataxia, spastic paraplegia, and abnormal facies (462). MCT8 encodes a thyroid transporter, essential for triiodothyronine (thyroid hormone T3) uptake into neurons, where it is transported to the nucleus, interacts with nuclear receptors, and then degraded. MCT8 mutations block T3 uptake which prevents both its interaction with its intracellular receptors (therefore preventing its modulation of expression of a variety of genes), as well as its intracellular degradation (therefore resulting in elevated serum triiodothyronine (T3) levels) (462).

*Mitochondrial ATP6 (mATP6) gene mutations* cause a variety of clinical syndromes depending in part on heteroplasmy (relative abundance of mutant and normal mitochondria in each tissue). Genetic background, including both nuclear and mitochondrial genes (e.g., mitochondrial haplogroup) also appears to contribute to phenotypic variation. MATP6-associated syndromes include neuropathy ataxia and retinitis pigmentosa, neonatal Leigh syndrome, and recently, a syndrome of adult-onset, progressive, mild to severe spastic paraplegia variably associated with axonal neuropathy, late-onset dementia, and cardiomyopathy (212).

Verny et al. (212) showed that mATP6 gene mutation discovered in their spastic paraplegia subjects (m.9176 T > C) caused severe reduction in ATP synthesis. The finding that mATP6 mutations can present as progressive spastic paraplegia underscores observations that at least two other HSP genes encode mitochondrial proteins [SPG7/paraplegin and SPG13/chaperonin 60/heat shock protein, (37,155)]. In addition, discovery of mATP6 mutation-related progressive (complicated) spastic paraplegia is the first example of maternally inherited HSP.

## 119.7 EMERGING CONCEPTS OF HSP PATHOGENESIS

Analysis of the 22 HSP genes discovered to date indicates that HSP syndromes result from a variety of primary molecular abnormalities (181,410,463). Rather than reflecting a common biochemical disturbance, phenotypic similarity between different types of HSP appears to reflect the limited repertoire of neurologic symptoms that are caused by relatively selective degeneration of long CNS axons, as well as the limited sensitivity of neurodiagnostic studies to discriminate between these disorders.

Analysis of HSP genes discovered thus far suggests the following primary biochemical abnormalities in different types of HSP: (1) axonal transport abnormality; (2) disturbance in ER morphology; (3) mitochondrial abnormality; (4) primary myelin abnormality; (5) abnormal protein conformation leading to ER-stress response; and (6) disturbance in corticospinal tract development. Whether these biochemical abnormalities ultimately converge into one or more common biochemical pathways remains to be determined.

Axonal transport disturbance has long been suspected as an underlying molecular process in HSP in view of the relatively selective degeneration of the longest CNS axons, the fact that degeneration is maximal at the distal ends of these axons, and the importance of axon transport in axonal biology in general and long axons in particular (410,464). The clearest example of axonal transport disturbance in HSP is autosomal dominant SPG10 HSP due to kinesin heavy chain (KIF5A) mutation. KIF5A is a molecular motor involved in anterograde axonal transport of organelles and macromolecules including neurofilaments (465). SPG4 HSP may also be an example of HSP due to axonal transport disturbance. *SPG4*/spastin interacts with microtubules and appears to be involved in microtubule severing (19,22,179,300,364,386,466–469). Kasher et al. (470), using time-lapse microscopy, showed that neurons from spastin mutant mice had abnormal anterograde and retrograde transport (including the transport of mitochondria).

Two types of HSP, SPG3A and SPG17, may be due to Golgi abnormalities. Although the functions of these *SPG3A*/atlastin and *SPG17*/spartin are unknown, both atlastin and spartin have been shown to be localized to Golgi (372,471).

Three HSP genes encode integral mitochondrial proteins: chaperonin 60/heat shock protein 60, mutations in which cause autosomal dominant uncomplicated SPG13 HSP (37); and paraplegin, mutations in which cause autosomal recessive SPG7 HSP; and mitochondrial ATP6 (254,421,472,473). Some, but not all, SPG7 HSP subjects have morphologic and histochemical abnormalities of mitochondria in skeletal muscle biopsy (155). These findings indicate that mitochondrial disturbance underlies at least two types of HSP, an observation consistent with emerging evidence of mitochondrial abnormalities in other degenerative neurologic disorders (474,475).

Three HSP protein, *SPG3A*/Atlastin, *SPG4*/spastin, and REEP1, have been implicated in determining ER maturation of ER into a meshwork of interconnected tubules. It is noted that mitochondrial abnormality may involve specific oxidative phosphorylation impairment, mitochondrial protein quality control, or both.

Discovery that mutations in PLP, an intrinsic myelin protein, and GJA12/connexin 47 cause either a childhood-onset leukodystrophy (PMD and PLMD, respectively) or slowly progressive spastic paraplegia (SPG2 and SPG44) provides important insights into axonal biology and the causes of HSP. This underscores the concept that axon degeneration in at least two forms of HSP arises from glial abnormality rather than intrinsic neuron abnormality.

Several forms of HSP appear to be *developmental* disturbance rather than progressive neurodegeneration. These include AHD syndrome (SPG22) and SPG1 HSP, both of which are congenital. In the case of SPG1 HSP, the implicated gene (L1CAM) is involved in neuronal migration and corticospinal tract development. AHD

syndrome (SPG22) is a form of congenital insensitivity to thyroid hormone due to mutations in the thyroid transporter MCT8, essential for T3 uptake into neurons.

Abnormal protein accumulation and induction of ER stress cascade have been implicated in many forms of HSP including SPG6, SPG8 (163), and SPG17. In particular, SPG17 (due to BSCL2/seipin gene abnormalities) (318,415,416), SPG8 (due to Strumpell's mutation) (404), and "mutilating sensory neuropathy with spastic paraplegia" due to *CcT5* mutation (119) are important examples of spastic paraplegia protein misfolding and protein accumulation.

## 119.8 ANIMAL MODELS OF HSP

There are numerous animal models of various types HSP (476) including mouse, rat, *Drosophila*, zebrafish, *C. elegans*, and cattle (summarized in Table 119-6). In general, these models have proven useful in evaluating the functional significance of specific HSP mutations; and in exploring the molecular pathogenesis of various forms of HSP. As a generalization, mouse models of HSP (either spontaneous mutants such as "rump-shaker" model of SPG2 or gene knockout models), typically exhibit much more mild phenotypes than humans with the same or equivalent mutations. Nonetheless, these models have proven valuable in the investigation of pathogenic mechanisms underlying HSP. The occurrence of congenital bovine spinal dysmyelination in American Brown Swiss cattle as a consequence of bovine spastin gene mutation is discussed above.

### 119.8.1 SPG4/Spastin Mutation Mice

*SPG4*/spastin mutations are the single most common cause of autosomal dominant, uncomplicated HSP. Tarade et al. (477) reported studies of *SPG4*/spastin gene mutation mice. The Cre/LoxP gene targeting vector was used to create mice in which *SPG4*/spastin exons 5 through 7 were deleted and replaced by the inserting vector. By crossing mice that were heterozygous for this mutation (*Sp<sup>del/+</sup>*) with either CMV-Cre transgenic mice or with NSE-Cre transgenic animals, mice were created in which the deleted spastin gene was present in all tissues (*Sp<sup>del/+</sup>/CMV-Cre* mice) or only in neurons (*Sp<sup>del/+</sup>/NSE-Cre* mice). In preliminary analysis, some *Sp<sup>del/+</sup>* mice exhibited impaired rotarod performance compared to normal littermates. This phenotype was highly variable, however, indicating the importance of modifying genetic factors.

In our laboratory, we created mice in which the *SPG4*/spastin gene is mutated (J. K. Fink et al. unpublished observations) (476). We observed that mice bearing heterozygous deletion of *SPG4*/spastin mutant exon 8 exhibit age-dependent gait disturbance due to impaired hind limb locomotor ability. These deficits were observed in open field assessment and rotarod performance. *SPG4*

mutant mice older than 1 year tended to walk with hind limbs externally rotated and often demonstrate impaired trunk control, tail dragging, intermittent “scooting” (dragging the hindquarters instead of hind limb stepping), or “hopping” (instead of hind limb stepping).

These observations support the concept that *SPG4*/spastin gene mutations are pathogenic through haploinsufficiency. Although these observations are preliminary, they support “proof of concept” that mice bearing *SPG4*/spastin mutations exhibit a recognizable behavioral phenotype of impaired hind limb motor performance; and the usefulness of these animals to investigate *SPG4* pathogenesis and treatment.

### 119.8.2 *SPG3A*/Atlastin Mutation Mice (478)

Our laboratory also created mice in which the *SPG3A*/atlastin gene is mutated targeted deletion of all of *SPG3A* exon 8 which results in a frameshift and protein truncation after residue 242 (of atlastin’s 559 amino acids). These animals show mild age-dependent hind limb motor impairment, evident in open field evaluation, footprint analysis, and grid-walking and rotating rod assessments. Preliminary neuropathologic studies indicate axonal degeneration involving spinal cord axons, anterior horn cells, and peripheral nerve axons and changes in skeletal muscle consistent with chronic denervation. Lower motor neuron involvement has been reported in *SPG3A* HSP (110).

These preliminary observations support the concept that *SPG3A*/atlastin gene mutations are pathogenic through haploinsufficiency. Observations that mice bearing *SPG3A*/atlastin mutations develop age-dependent gait disturbance and neuropathologic changes similar to humans with *SPG3A*/atlastin mutation indicate that these animals are an important resource in which to explore *SPG3A*/atlastin function, the pathogenesis of *SPG3A*/atlastin mutations, and ultimately, treatments for HSP.

### 119.8.3 *SPG10*/KIF5A Knockout Mice (465)

KIF5A null mice die immediately after birth. Therefore, Xia et al. (465) used a synapsin-promoted Cre-recombinase transgene to create mice in which targeted disruption of the KIF5A gene occurred in neurons postnatally. The majority (75%) of mutant mice developed seizures and died at approximately 3 weeks. Older surviving animals had age-dependent hind limb paralysis, sensory neuron degeneration, accumulation of neurofilament in neuron cell bodies, reduction in axon number, and loss of large caliber axons.

### 119.8.4 *SPG7*/Paraplegin Knockout Mice

Homozygous mutations in *SPG7*/paraplegin cause a rare form of complicated autosomal recessive HSP. *SPG7*

encodes a mitochondrial metalloprotease designated “paraplegin.” Some, but not all, *SPG7* HSP patients have abnormal-appearing mitochondria (“ragged red” fibers and cytochrome C oxidase negative fibers) in biopsies of skeletal muscle.

Ferreirinha et al. (472) reported studies in homozygous *SPG7*/paraplegin knockout mice. These animals displayed impaired performance on rotarod apparatus starting at age 6 months and worsening with age. Spinal cord histology showed axonal swellings (through massive accumulation of organelles and neurofilaments), more prominently in the lateral columns of the lumbar spinal cord. This phenotype was slowly progressive, with signs of axonal degeneration becoming prominent at 12 months of age. Changes in mitochondrial morphology appeared to precede axonal changes in this system, suggesting that degeneration is due to impaired mitochondrial function. This study therefore indicated that *SPG7*/paraplegin gene disruption in mice recapitulates the histologic and, albeit to a lesser extent, the locomotor disturbance of humans with *SPG7* HSP.

### 119.8.5 *SPG39*/NTE Knockout Mice

Akassoglou et al. (479) used Cre-Lox recombination to create mice in which NTE was deleted in neurons. These animals showed prominent neuronal pathology in the hippocampus, thalamus, and cerebellum, disruption of endoplasmic reticulum, vacuolization of nerve cell bodies, and abnormal reticular aggregates.

### 119.8.6 *SPG2*/PLP Mutation Mice (459,460)

PLP is an intrinsic myelin protein. PLP gene mutations cause both PMD, an X-linked leukodystrophy, as well as X-linked HSP (*SPG2*). To some extent, the phenotype depends on the nature of the PLP gene mutation. Although severe disease (PMD leukodystrophy) is usually caused by increased expression of PLP due to gene duplication, a number of PLP point mutations have also been shown to cause PMD. There are naturally occurring mouse models containing PLP mutations (jimmy(jp), jimpy-4J, msd, and rumpshaker); as well as PLP-null mice in which the PLP gene has been disrupted by gene targeting. The phenotype of these animals often depends on the particular mutation and genetic background (480). Depending on the particular mutation, PLP missense mutations and aberrant mRNA splicing mutations result in severe hypomyelination and early death (481–485), or simply tremor and normal life span (486). In contrast, mice in which the PLP gene has been disrupted by gene targeting display length-dependent axonal degeneration that is similar to patients lacking PLP. These studies demonstrate that: (1) genetic background (e.g., the impact of modifying genes) influences the PLP gene mutation phenotype; (2) PLP gene mutations can cause either hypomyelination or primary axonal degeneration; and

(3) axonal degeneration in HSP may arise from a primary glial (oligodendroglia in this case) abnormality.

### 119.8.7 SPG1/L1CAM Knockout Mice

Mutations in L1CAM cause X-linked “CRASH” syndrome, “MASA” syndrome, and X-linked hydrocephalus associated with spastic paraplegia. Depending on the particular mutation introduced, L1CAM knockout mice have shown hind limb motor impairment and reduced size of the corticospinal tracts (456,487); and number of brain defects including reduced size of the corpus callosum and hippocampus, enlarged ventricles, septal abnormalities, and abnormal migration of mesencephalic dopamine neurons (487–489). These results provided evidence that L1CAM plays an important role in brain development, particularly that of corticospinal tracts and the migration of dopamine neuron. Furthermore, studies of L1CAM mutations in humans and mice illustrate that the HSP syndrome may be caused by developmental disturbance of corticospinal tract formation rather than corticospinal tract degeneration.

*Drosophila* models of HSP (SPG4, SPG3A, SPG39): Kammermeier et al. (490) identified the *Drosophila* homolog of spastin (*Dspastin*). Orso et al. (491) created *Drosophila* bearing *Dspastin* insufficiency (via RNA interference [RNAi]) and those overexpressing a neuronally targeted *Dspastin* mutant (*Dspastin* K467R mutant corresponding to pathologic mutation K388R in humans). They observed that loss of *Dspastin* produced adult-onset progressive locomotor impairment, neurodegeneration, and early death; within 15–20 days of age, flies stopped moving and had neurodegeneration in the brain with obvious neuronal apoptosis. Both *Dspastin* deficiency and mutant *Dspastin* expression resulted in excessive stabilization of microtubules in the neuromuscular junction (NMJ) synapse (observations consistent with other evidence of spastin’s microtubule-severing properties discussed above). Moreover, they observed that vinblastine, a microtubule destabilizing drug (but not a functionally related agent nocodazole), significantly attenuated disease-related abnormalities in vivo. At the cellular level, vinblastine treatment was associated with suppression of induced cytoskeletal abnormalities of the neuromuscular synapse. Sherwood et al. (468) generated *Drosophila* bearing *Dspastin* deletions and observed a similar, very severe behavioral phenotype (null mutants were flightless), striking morphological changes in NMJs, reduced synaptic vesicle release, and shorter lift span. Overexpression of *Dspastin* in muscle erased the muscle microtubule network, consistent with other evidence (described above) of spastin’s microtubule severing properties.

The *Drosophila* homolog of NTE is the Swiss Cheese protein (SWS) (492). *Drosophila* *sws* mutants are an important model of NTE mutation-induced neurodegeneration (492). Early death occurs in *sws* flies due to

neuronal vacuolization, apoptosis, and glial hyperwrapping. *sws* is localized to 7D1 on the X chromosome. Therefore, age-dependent neurodegeneration occurs in *hemizygous male and homozygous female sws* flies. *sws* mutations include missense mutations in the esterase/patatin domain and the regulatory domain and a non-sense mutation that yields a truncated protein missing the esterase/patatin domain (6). These are similar to the location of NTE mutations we identified in NTE-MND families. NTE esterase activity measured in the heads of *sws1* mutant flies is decreased as compared to controls (493).

Lee et al. (494) generated atlastin null *Drosophila* and observed age-dependent locomotor impairment (flies were paralyzed by mechanical shock such as being bumped) that was associated with degeneration of dopaminergic neurons. This phenotype could be rescued by targeted expression of wild-type atlastin in dopaminergic neurons or by levodopa administration. On this basis, Albin et al. performed DTBZ-PET scans in humans with SPG3A/atlastin mutation and observed normal nigrostriatal dopaminergic terminal density. This indicates important differences between molecular consequences of atlastin mutation in *Drosophila* and humans.

Subsequently, studies of atlastin null and atlastin-depleted (via RNA interference methods) *Drosophila* provided evidence that atlastin is required for normal growth of muscles and synapses at the NMJ (495), and clearly demonstrated atlastin’s function in endoplasmic membrane fusion (30,496).

## 119.9 CONCLUSIONS

Although the primary symptoms of HSP are insidiously progressive spastic weakness in the legs, it is important to remember that HSP, even when “uncomplicated” or “pure” is not simply a motor neuron disorder affecting corticospinal tracts. Dorsal column fibers are often involved (manifested as mild distal vibratory sense impairment) although typically later and more mildly. The majority of genetic forms of HSP involve peripheral neuropathy or other neurologic abnormalities in at least a subset of patients. The concept that HSP exclusively involves length-dependent distal axonal degeneration involving both motor and sensory fibers is being modified to include neurodegeneration involving shorter axons including those in the cerebellum and those traversing the corpus callosum.

Even in light of the tremendous advances in HSP gene discoveries, for most subjects, HSP is a diagnosis of exclusion. It is imperative that alternative disorders be considered carefully and evaluated by appropriate neurodiagnostic studies.

HSP exhibits extreme genetic heterogeneity. Genetic linkage analyses have identified 51 different HSP loci. Among these, 25 HSP genes have been identified. Discovery of HSP genes has permitted development of



gene-based testing to confirm a clinical diagnosis of HSP (presently available for ~70–80% of dominantly inherited HSP and two forms of X-linked HSP, and for genetic counseling and prenatal testing). Discovery of HSP genes has provided seminal insights into the molecular basis of this large class of motor neuron disorders and permitted development of important animal models. These advances permit direct exploration of HSP's pathophysiology and bring us closer to developing treatments for this group of disorders.

## CROSS-REFERENCES

Hereditary Muscle Channelopathies: Myotonias, Periodic Paralyzes, Malignant Hyperthermia, and Core Myopathies.

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# CHAPTER

# 120

## Autonomic and Sensory Disorders

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### 120.1 INTRODUCTION

The hereditary sensory and autonomic neuropathies (HSAN) are a group of rare disorders that illustrate the intimate relationship of the development and maintenance of sensory and autonomic neuronal populations. Both neuronal populations derive from the neural crest and develop into unmyelinated neurons by a complicated process of migration and differentiation that can be disrupted by various genetic mutations. Analgesia of varying degree and abnormal autonomic function are common to all the disorders. Because the autonomic nervous system (ANS) is pervasive and integrates multiple functions, symptoms can be widespread and extremely variable (1). Identification and classification of the HSAN disorders are ongoing. In addition to the numerical classification of four distinct forms of hereditary sensory neuropathy that was proposed by Dyck and Ohta (2), additional entities continue to be described (3).

Diagnosis of the various disorders depends primarily on clinical and biochemical evaluations, with pathologic examinations serving to further confirm differences. Some of the disorders have been localized to specific chromosomal regions and specific genetic mutations identified, allowing for definitive diagnoses. With the exception of HSAN type I, which is a dominant disorder presenting in the second decade, that affects serine palmitoyltransferase function and sphingolipid synthesis (4–6), the other HSAN are autosomal recessive disorders whose mutations impede normal neural crest migration and differentiation and result in decreased neuronal populations. Familial dysautonomia (FD) (Riley–Day syndrome or HSAN type III) (3,7,8) and congenital sensory neuropathy (HSAN type IV) (3,9,10) are two such disorders that have been particularly well described and categorized and for which genetic mutations have been identified.

Table 120-1 summarizes some of the other HSAN disorders in which sensory and autonomic functions are

also affected. Table 120-2 lists the specific genetic mutations that have been identified.

### 120.2 FAMILIAL DYSAUTONOMIA

#### 120.2.1 Clinical Features

FD has the most protean nervous system abnormalities of this group of disorders. Pain and temperature sensation are decreased and there is marked autonomic dysfunction (3,7,8). Signs of the disorder are present from birth and neurological function slowly deteriorates with age (3,7,11).

Autonomic dysfunction is pervasive and affects peripheral and central functions. Abnormalities of autonomic function include lack of overflow tears, supersensitivity of the pupil to autonomic drugs, postural hypotension, episodic hypertension, skin blotching, inappropriate hyperhidrosis, and episodic hyperadrenergic vomiting crises.

Earliest signs include feeding difficulties with a poor suck, uncoordinated swallowing, and frequent misdirection of ingested material into the trachea. Recurrent misdirection, especially with liquids, causes aspiration pneumonias and may lead to bronchiectatic disease, atelectasis, and even lung abscesses. Gastroesophageal reflux further increases the risk of aspiration in some patients. Cinesophagrams have demonstrated delay in cricopharyngeal relaxation, abnormal gastrointestinal motility, and reflux.

Vomiting crises associated with irritability, negativistic behavior, hypertension, tachycardia, blotchy erythema, and diaphoresis are observed in 40% of patients. Crises, which may occur as often as once a week, typically last for 1–3 days but can persist for up to 10 days. Dehydration and aspiration of vomitus may be fatal. The crises are associated with elevated circulating serum catecholamines (12).



TABLE 120-1    Affected Neurologic Functions and Genetics of Nine Congenital Sensory Disorders								
Neurologic Function	Congenital Sensory Familial Dysautonomia (HSAN Type III)	Congenital Insensitivity Neuropathy with Anhidrosis (HSAN Type IV)	Congenital to Pain without Anhidrosis (HSAN Type V)	Congenital Autonomic Sensory Neuropathy (HSAN Type II)	Sensory Neuropathy Dysfunction with Universal Pain Loss	Progressive with Skeletal Dysplasia	Hereditary Panneuropathy with Hypotonia	Radiating Neuropathy (HSAN Type I)
<b>Sensory</b>								
Pain	Reduced	Absent	Absent	Absent	Absent	Absent	Reduced	Distal
Temperature	Reduced	Reduced	Normal	Absent	Absent	Absent	Reduced	Distal
Touch	Normal	Reduced	Normal	Absent	Reduced	Reduced	Normal	Reduced
Position sense	Reduced to normal	Normal	Normal	Reduced	?	?	Reduced	Reduced
Visceral pain	Normal	Reduced	?	?	Absent	Reduced	Normal	?
Axon flare	Absent	Absent	Normal	Absent	Absent	Absent	Absent	Local
<b>Reflexes</b>								
Tendon	Hypoactive or absent	Hypoactive or normal	Normal	Hypoactive	Absent	Absent	Absent	Hypoactive or normal
Superficial	Hypoactive or normal	Hypoactive or normal	?	Hypoactive	Absent	Hypoactive	?	Normal
<b>Cranial nerves</b>								
Corneal (V)	Normal, diminished or absent	Absent	Variable	Diminished	Absent	Absent	Diminished	?
Gag (IX, X)	Diminished or normal	Normal	?	Diminished	Diminished	Diminished	Diminished	?
Pain (V)	Reduced	Reduced	?	Absent	Absent	Absent	Reduced	?
Taste (XII)	Reduced	Normal	Normal	Reduced	?	?	Reduced	?
<b>Autonomic</b>								
Sweating	Increased (with stress)	Absent	Normal	Reduced or normal	Increased (with stress)	Reduced	Increased	Reduced
Tear production	Decreased	Normal	Decreased	Normal	Decreased	Normal	Normal, then reduced	Normal
Postural hypotension	Present	May be present	Not present	Not present	Present	?	Present	Normal
G1 motility	Abnormal	Normal	Abnormal	Variably abnormal	Variably abnormal	Abnormal	Abnormal	?
<b>Intelligence</b>	Normal	Decreased	Decreased	Decreased	Decreased	Decreased	Normal	Mild or normal
<b>Genetics</b>	Autosomal recessive	Autosomal recessive	?Autosomal recessive	?Autosomal recessive	?Autosomal recessive	?	Autosomal recessive	Autosomal recessive

**TABLE 120-2 Genetic Mutations Identified for the Hereditary Sensory and Autonomic Neuropathies**

Gene	HSAN	Type	Chromosome
Familial dysautonomia	III9q31	9q31	<i>IKBKAP</i>
Congenital sensory neuropathy with anhidrosis	IV	1q21–22	TrkA/NGF-receptor
Congenital insensitivity to pain without anhidrosis	V	1p11.2–p13.2	NGF-beta chain –
Congenital sensory neuropathy	II	?	?
Congenital autonomic dysfunction with universal pain loss	—	?	?
Congenital sensory neuropathy with skeletal dysplasia	—	?	?
Progressive panneuropathy with hypotonia	—	?	?
Hereditary radicular neuropathy	I	9q22.1–22.3 3q13–22	<i>SPTLC1</i>

Abnormal responses to hypoxic or hypercarbic states compound the problem of aspiration pneumonias and chronic lung disease in FD (13,14). When oxygen tension is lowered (as at high elevations, during air travel or in tunnels) minute ventilation does not increase appropriately. The ensuing hypoxemia may lead to hypotension, bradyarrhythmia, and even syncope. Diving or underwater swimming can be hazardous. Agitated patients are able to hold their breath without discomfort long enough to produce cyanosis, syncope, decerebrate posturing, and seizures. Tachypnea does not accompany respiratory infections.

Consistent lack of overflow emotional tearing is pathognomonic of the disorder. As corneal hypesthesia or anesthesia is a component of the disease, reflex tearing in response to intraconjunctival foreign material is also absent. Although baseline tearing varies among affected individuals, hypesthesia results in a decreased blink frequency and causes rapid evaporation of baseline moisture. The relatively dry eye is then at risk for corneal de-epithelialization, ulceration, and scarring with opacification. The pupil responds appropriately to light and accommodation, suggesting intact parasympathetic pathways, yet is supersensitive to infused methacholine. Abnormal permeability of the damaged cornea might contribute to this sensitivity.

Blotchy erythema occurring during eating or emotional excitement is one indication of abnormal vasomotor control. Supersensitivity to circulating catecholamines, giving rise to hypertension during crises, has already been alluded to. In periods between crises there is supine hypertension, as well as postural hypotension without compensatory tachycardia; heart rate and blood pressure move in parallel, which demonstrates lack of afferent baroreflex feedback (12,15,16). Excitement can cause profuse sweating. The extremities may intermittently become cold, red, and mottled. Inappropriate temperature response to infection or environmental changes can result in either hypothermia or hyperthermia.

Sensory involvement is most prominent in temperature discrimination (11,17). Nevertheless, hypoalgesia is first noted, most often by the blunted response to trauma. Tongue sores on the ventral surface due to repetitive thrusting against newly erupted teeth are

common and there is decreased response to injections, venipuncture and fractures. Although bone and skin pain are poorly perceived, sensitivity to visceral pain is intact. Appropriate responses to pleural effusions, esophageal irritation, and menstrual cramping indicate that there is no central error in the interpretation of pain. These also suggest that not all sensory components of the peripheral nervous system are equally affected. Touch sensation is relatively well preserved and proprioceptive abnormalities appear in older patients (11).

Defective afferent conduction may account for the absence of deep tendon reflexes. Motor neuron involvement may underlie the infantile hypotonia, abnormal gait, and high incidence of juvenile scoliosis in FD. By 10 years of age, 85% of patients have scoliosis. There is a higher incidence of left thoracic curves than in idiopathic scoliosis (18,19).

As a group, patients have average intelligence, with a general tendency for better verbal than motor performance, but developmental milestones are commonly delayed. There is a subgroup of affected individuals with pervasive developmental delay; some of these individuals never develop normal expressive language. Memory is usually excellent but executive planning skills are frequently impaired and there is a tendency to concrete thinking with problems in extrapolation. Seizures with decerebrate posturing can follow breath holding, even in children with normal electroencephalograms (EEGs).

Somatic growth is poor; the average adult height being about 5ft. Growth hormone levels are usually normal but some patients have responded well to growth hormone administration, suggesting decreased responsiveness to physiological levels (20). Puberty is commonly delayed in both sexes. The average age at menarche is 15 years compared with a normal of 12.8 years. Nevertheless, primary and secondary sexual characteristics do eventually develop. Males are capable of erection and ejaculation. Fertility in both males and females has been proved and offspring of affected individuals have been normal.

Progressive diminution of renal function with age is a frequent observation (21,22). Moderate azotemia is an early sign. Creatinine clearance decreases, and many patients have subnormal renin excretion. Progression to

renal failure has led to dialysis for a number of patients as well as renal transplants (23).

### 120.2.2 Genetics

FD is an autosomal recessive disorder that appears to be largely confined to Ashkenazi Jews. In this population, the carrier rate is approximately 1 in 30 with a disease frequency of 1 in 3600 live births. In 1993, the gene for FD was localized to the long arm of chromosome 9 (24). Because one major haplotype was found for over 98% of the FD chromosomes, it was hypothesized that there was one founder mutation for this gene among the Ashkenazim (25).

In 2001, the gene was identified as *IKBKAP* (26,27). FD remains almost exclusive to individuals of Eastern European Jewish extraction. Greater than 99% of individuals with FD are homozygous for a mutation in intron 20: a T to C change located at base pair 6 of intron 20 (IVS20+6T>C) that results in tissue-specific mis-splicing, with variable decrease in expression of the protein product IKAP/ELP1 that is especially profound in neuronal tissue (27). Two other missense mutations have been reported. One mutation, in exon 19, was noted in four unrelated Jewish patients heterozygous for the major splice mutation; the second mutation, in exon 26, was reported in a single patient who was also heterozygous for the major splice mutation but inherited the new mutation from a non-Jewish parent (28).

FD is the only HSAN for which carrier testing of the general population has been recommended.

Now that the gene has been identified, its mode of action must be explored before we can consider that definitive therapies are being explored to either increase general production of IKAP or improve splicing efficiency (29).

### 120.2.3 Diagnosis

Although DNA diagnosis is now available, clinical suspicion is based on the recognition of sensory and autonomic dysfunction that starts at birth. The following criteria are used for clinical confirmation of the disorder. The criteria are listed in order of frequency of observation.

- (1) Signs of sensory dysfunction
  - (a) Absence of fungiform papillae on the tongue and decreased taste
  - (b) Absence of flare after intradermal histamine
  - (c) Decreased or absent deep tendon reflexes
  - (d) Decreased or absent corneal reflexes
  - (e) Decreased response to pain and temperature
- (2) Signs of autonomic dysfunction
  - (a) Absence of overflow tears
  - (b) Miosis following intraocular administration of dilute methacholine 2.5% or pilocarpine 0.0625%
  - (c) Postural hypotension

- (d) Blotching
- (e) Increased sweating
- (f) Episodic hypertension

Further supportive evidence includes feeding difficulties, repeated aspiration, episodes of hypothermia, breath holding spells, hypotonia and delayed motor development, repeated vomiting, abnormal cinesophagram, spinal curvature, poor somatic growth, and Ashkenazi Jewish parentage.

Because individuals affected with the other HSAN disorders will also fail to produce an axon flare after intradermal histamine, careful assessment of the other clinical signs and symptoms is necessary to distinguish between these disorders (3). Because there can be extreme variability in expression, clinical criteria are not always sufficient. In such cases molecular diagnosis is indicated.

### 120.2.4 Pathologic and Biochemical Findings

FD appears to represent a disorder in which there is developmental arrest of the sensory and autonomic systems, with sympathetic development more widely affected than parasympathetic.

Sympathetic ganglia are about one-third the normal size and have only about one-tenth the normal population of neurons (30). The number of neurons tends to be fewer in older patients, but degenerative changes are not prominent. Surviving neurons tend to be slightly larger than normal and to have markedly increased immunoreactive tyrosine hydroxylase (31). Sympathetic terminals are sparse or absent on the vessels of skin, nerves (17,32,33), or kidneys (22). Preganglionic sympathetic neurons in the spinal cord are reduced to about half the normal population (30).

Parasympathetic ganglia are variably affected (34). The ciliary ganglion is normal or marginally depleted of neurons. The sphenopalatine ganglion has less than one-tenth the normal population. Sensory ganglia are also small and have markedly reduced numbers of neurons (35).

Increasing residual nodules and decreasing neurons in older patients indicate a degenerative component. The sensory sural nerve is hypoplastic and contains markedly reduced populations of nonmyelinated and small myelinated fibers and there is lesser depletion of large-myelinated axons (36,37). Lissauer's tract is small in the spinal cord (30). The dorsal columns conducting vibration and proprioceptive and touch information are initially normal but diminish markedly in older patients.

Lingual sensory axons and submucosal neurons are diminished in number (38). Circumvallate papillae are hypoplastic and bear few taste buds. Fungiform papillae are absent and filiform papillae are rudimentary (38,39).

No consistent qualitative central nervous system (CNS) anomalies have been found as yet.

On measurement of urinary catecholamine metabolites, FD patients have been found to have elevated levels of homovanillic acid (HVA) and normal to low levels of vanillylmandelic acid (VMA), resulting in elevated HVA:VMA ratios (40). These findings are consistent with the neuropathological descriptions of a decreased sympathetic neuronal population. Although supine plasma levels of norepinephrine are normal or elevated, FD patients, like most other patients with neurogenic orthostatic hypotension, do not have an appropriate increase in plasma levels of norepinephrine and dopamine  $\beta$ -hydroxylase (D $\beta$ H) with standing (12,16,41). Circulating D $\beta$ H levels have been reported as diminished by Weinshilbaum and Axelrod (42), but this is not apparent when age-matched controls are used (43). In addition, FD patients appear to have a distinctive pattern of plasma levels of catechols (44). Regardless of posture, plasma levels of dopa are disproportionately high and plasma levels of dihydroxyphenylglycol (DHPG) are low, resulting in elevated plasma dopa:DHPG ratios, which are not seen in other disorders associated with neurogenic orthostatic hypotension. During emotional crises, dopamine and norepinephrine are markedly elevated (12).

Patients are also hypersensitive to sympathomimetics and parasympathomimetics. Small doses of methacholine cause profound falls in blood pressure in the absence of compensatory tachycardia. Doses of norepinephrine that would be below threshold in normal subjects cause hypertension; again, there is no cardiac compensation in the form of bradycardia. The pupil is supersensitive to constriction by methacholine but not to dilation by epinephrine.

### 120.2.5 Management

The disease process cannot be arrested. Supportive therapy is directed toward specific problems that vary considerably from patient to patient. Uncoordinated swallowing usually makes breast feeding impossible. A gastrostomy is often required to maintain adequate caloric intake to avoid dehydration and to prevent aspiration (3,7,45).

Pyrexia in response to dehydration, mucous plugs, environmental temperature, or stress is usually brief but associated with muscular spasms and extreme discomfort. Antipyretics may be ineffective and tepid sponging or a cooling mattress may be required. A muscle relaxant such as diazepam is often helpful in reducing spasms and anxiety during pyrexia.

A high index of suspicion for pneumonia should be maintained rather than attributing febrile episodes to the disorder itself. Radiographic examination is often necessary for diagnosis. Pathogens cultured from tracheal aspirations are often uncommon agents requiring use of broad-spectrum antibiotics (46). In individuals with chronic lung disease, postural drainage and inhalation

therapy may have to be used as a daily routine. For those who have sleep-disordered breathing with oxygen desaturations and hypercarbia, noninvasive nocturnal ventilation is suggested.

Breath holding episodes are often interpreted as seizures. They can be frightening but are self-limiting and, in our experience, have never been fatal in normal atmosphere. The cyanosis of breath holding must be differentiated from the cyanosis that can occur with mucous plugs. Either cause of hypoxia can produce seizurelike movements and decerebrate posturing. EEGs are not helpful. The frequency of hypoxic seizures is unaffected by anticonvulsant therapy. Appropriate instructions to parents are indicated regarding underwater swimming, air travel, and visits to high altitudes. Antiepileptic drugs are used in some patients who are thought to have autonomic seizures manifested by daily crises that appear to be provoked by arousal (47).

Corneal complications have been decreasing with regular use of artificial tear solutions containing methylcellulose and maintenance of normal body hydration. Artificial tears are instilled three to six times daily, depending on the child's baseline eye moisture and environmental conditions and on whether the child is febrile or dehydrated. Moisture chamber spectacle attachments help maintain eye moisture and protect the eye from wind and foreign bodies. Tarsorrhaphy of the medial or lateral part of the palpebral fissure has been reserved for unresponsive and chronic situations. Cautery of the tear ducts increases baseline moisture and is beneficial in promoting corneal healing.

Management of a "vomiting crisis" has five goals: (1) adequate hydration, (2) relief of gastric distention to reduce the risk of gastroesophageal reflux and aspiration, (3) suppression of vomiting, (4) relief of hypertension, and (5) induction of sleep (45). The latter seems essential for resolution of a crisis. Dehydration is characteristically isotonic and is best estimated on weight change, as tearing and blood pressure are unreliable indicators. Diazepam is the most effective antiemetic for the dysautonomic vomiting crisis. In addition to its antiemetic effect, diazepam normalizes blood pressure and decreases agitation. Clonidine works in a synergistic manner and aids management of associated diastolic hypertension. H<sub>2</sub> antagonists are useful adjuncts in reducing emesis volume. The crisis usually resolves abruptly and is marked by normalization of personality and return of appetite.

Postural hypotension between crises may respond to increased salt and fluid intake. Caffeinated beverages and waist-high elastic stockings have been helpful. Fludrocortisone and midodrine may be useful in severe cases (48,49).

Decreased response to pain and temperature requires that parents be alert to any unusual swellings or skin discolorations. Smoothing of newly erupted teeth prevents tongue ulcerations in young children. Self-mutilation is rare.



Annual examination of the spine will permit early diagnosis of scoliosis, as well as appropriate institution of brace and exercise therapy. Braces may ulcerate the insensitive skin. The abdominal breathing pattern of these patients may be inhibited and esophageal reflux induced if the brace has a high epigastric projection. Rapid progression and severe curvature at time of presentation are indications for spinal fusion (18,19).

### 120.2.6 Prognosis

With improved supportive measures increasing numbers of patients are reaching adulthood (50). Survival statistics prior to 1960 reveal that 50% of patients died before 5 years of age. Current survival statistics indicate that a newborn with FD has a 50% probability of reaching 40 years of age (50). Many FD adults have been able to achieve independent function. Both men and women with FD have married and reproduced. All offspring have been phenotypically normal despite their obligatory heterozygote state.

Causes of death are less often related to pulmonary complications, indicating that aggressive treatment of aspirations has been beneficial. Of recent concern have been the patients who have succumbed to unexplained deaths, which may have been the result of unopposed vagal stimulation or a sleep abnormality. A few adult patients have died of renal failure.

## 120.3 CONGENITAL SENSORY NEUROPATHY WITH ANHIDROSIS (HSAN TYPE IV)

### 120.3.1 Clinical Features

Congenital sensory neuropathy with anhidrosis (HSAN type IV) is characterized by decreased pain perception, anhidrosis and learning disabilities (9,51,52).

The earliest and most common sign of the disorder is hyperpyrexia. Episodic fevers with extreme hyperpyrexia can be induced by illness and overheating. The fevers can start in infancy and persist throughout the childhood years. Traditionally these febrile episodes have been explained by the inability to sweat. A general defect in thermoregulation, however, actually may be present, as extreme hypothermia has also been observed. With time, the skin may become thick and calloused with lichenification of palms, dystrophic nails, and areas of hypotrichosis on the scalp (3,9,53). As further evidence of autonomic dysfunction, patients exhibit miosis with dilute intraocular methacholine and have mild postural hypotension, but in contrast to patients with FD, there is compensatory tachycardia. Some patients may have a heightened sympathetic response, as erythematous blotching of the skin with excitement has been seen in younger patients. Acrocyanosis is notably lacking.

Other central and peripheral autonomic disturbances are infrequent. On occasion there may be a weak suck, but aspiration is unusual and respiratory problems are not characteristic. Weight gain may be poor. Vomiting is not a feature of the disease. Cyclical crises do not occur and tearing is normal.

Decrease in pain and temperature sensation is extensive and profound, and touch may also be affected (53). Self-inflicted injury is a more prominent feature than in FD (3,9). Biting of lips and fingers and traumatic tongue lesions are common. Injuries to soft tissues, bones, and joints may go unrecognized with subsequent severe secondary changes. Repeated fractures of the lower extremities and poor healing result in aseptic necrosis, Charcot joints, and even frequent osteomyelitis. Visceral sensation is also severely impaired, judged by diminished response to testicular compression and increased intra-esophageal pressure.

Hypotonia is seen in the early years. Although strength and tone normalize as the patient gets older, many patients overstretch their large joints as a means of self-stimulation.

From experience following a number of patients from early childhood to adulthood, it appears that some of the neurological features of the disorder are slowly progressive. Initially, both deep tendon and superficial reflexes are usually preserved. With time, the reflexes may decrease and vibration and joint position sense may become impaired. Cranial nerves are normal, with the exception of pain sensation on the face and corneal reflexes. Spinal curvature has not been described but has been noted in some of our patients. It may be secondary to leg length discrepancies due to repeated fractures of the lower extremities.

Patients tend to be irritable, hyperactive and emotionally labile. Hyperactivity and learning disabilities are prominent. EEGs are normal. Seizure activity and breath holding spells are not characteristic.

### 120.3.2 Genetics

HSAN type IV is caused by mutations of the neurotrophic tyrosine receptor kinase 1 gene (*NTRK1*). The human *NTRK1* gene, located on chromosome 1 (1q21–q22), encodes a receptor tyrosine kinase (RTK) for nerve growth factor (NGF) (9). Binding of NGF to RTK stimulates maturation and functioning of nociceptive sensory and sympathetic neurons. One half of reported cases of this autosomal recessive disorder have occurred in consanguineous marriages. In 1996 it was reported that some HSAN type IV patients from Japan and two brothers from Ecuador had mutations in a gene on chromosome 1 (9,10). Using a candidate gene strategy, the genetic basis for HSAN IV was identified when *NTRK1* loss-of-function mutations were found (10). Since this report numerous mutations have

been identified within the *NTRK1* domain (54). In some cases the mutations seem to correlate with phenotypic expression. For example the mildest patient was the one individual who was compound heterozygous for two different mutations (54).

### 120.3.3 Diagnosis

Consistent features of the disorder include anhidrosis and insensitivity to pain. Some authors believe that severe intellectual disability is also a constant feature, but we have seen a few patients with normal intelligence.

- (1) Signs of sensory dysfunction
  - (a) Absence of flare after intradermal histamine
  - (b) Profoundly decreased response to pain and temperature
  - (c) Decreased visceral sensation
  - (d) Absence of corneal reflexes
- (2) Signs of autonomic dysfunction
  - (a) Hypohidrosis or anhidrosis
  - (b) Episodic fevers
  - (c) Abnormal response to intraocular methacholine 2.5% or pilocarpine 0.0625%
  - (d) Blotching with excitement
  - (e) Mild postural hypotension
  - (f) Absent sympathetic skin response

Supportive evidence includes self-mutilation, hypotrichosis of scalp, and lichenification of skin, history of unrecognized fractures or osteomyelitis, joint injuries, poor somatic growth, hyperactivity, developmental delay, and emotional lability.

### 120.3.4 Pathologic and Biochemical Findings

Virtual absence of unmyelinated fibers has been noted in peripheral nerves and in Lissauer's tract from the spinal cord, and in dorsal root ganglia (55,56). The overall size of the sural nerve is markedly reduced. Skin biopsy morphology of HSAN IV patients reveals deficient C and Ad fibers in the epidermis and absent or hypoplastic dermal sweat glands without innervation (57,58).

Nerve growth factor radioimmunoassay was normal in the serum of four patients but decreased biological activity of fibroblast NGF was observed (59). This is consistent with eventual findings of abnormal receptors for NGF.

Involvement of the sympathetic nervous system terminals is indicated by an increased urinary ratio of homovanillic acid (HVA) to vanillylmandelic acid (VMA) with decreased excretion of VMA, 3-methoxy-4-hydroxyphenylglycol (MHPG), and normetanephrine (NM) (60,61). Intravenous infusion of norepinephrine produces a supersensitive response with hypertension and blotching (61).

Important differentiations from FD are normal tearing, normal deep tendon reflexes, normal taste and presence of fungiform papillae, no swallowing problems, and no vomiting.

### 120.3.5 Management

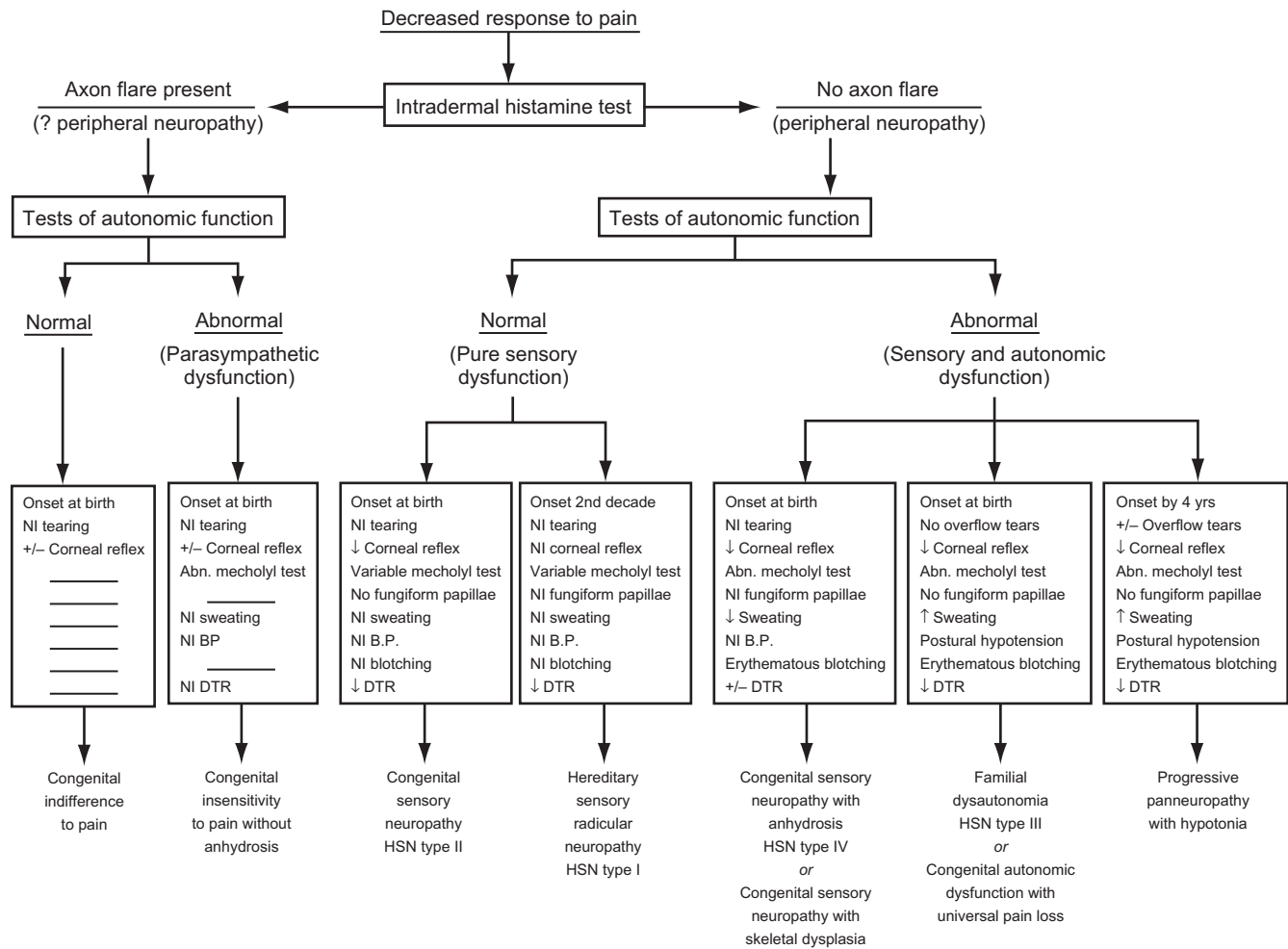
There is no specific treatment for congenital sensory neuropathy with anhidrosis. Extreme care must be taken to guard against inadvertent trauma. The ankles, knees, and fingers are particularly susceptible to injury. Early treatment of traumatic injuries is necessary to prevent secondary degenerative changes. Orthopedic procedures pose major challenges as patients can move within their casts and develop skin breakdown and even osteomyelitis.

Hyperpyrexia can be fatal. Aggressive treatment includes the use of cool baths and sponging and rubbing of the trunk and extremities. Overheating should be avoided and early use of antipyretics is advised. For extreme irritability, chlorpromazine (but not diazepam) has been beneficial. Treatment of the hyperactivity remains difficult, with poor response to stimulants.

### 120.3.6 Differential Diagnosis

Classification of the HSAN remains incomplete and has been complicated by lack of consistency in nomenclature. Each disorder has more than one name and some disorders share the same name. HSAN type II has been used to describe a disorder that is very similar to FD or HSAN type III (3,51) with decreased deep tendon reflexes, absent lingual papillae and decreased response to pain but normal tearing; HSAN type II has been used to describe a disorder characterized by distal neuropathy and trophic ulcers (52). For the former disorder no specific gene has yet been identified but for the latter disorder the causative gene has been reported to be *HSN2* (62), suggesting that term HSAN type II now includes a variety of genotypes.

As illustrated by the two HSAN disorders that are described in detail in this chapter, the HSAN disorders affect both autonomic and sensory function, but there are definite phenotypic differences (Table 120-3). The lack of an axon flare after intradermal histamine is common to all the HSAN disorders, not just FD (3,51,63). By refining clinical studies of the sensory and autonomic function, and with further information regarding neuropathology, distinctions between the other HSAN disorders should become clearer and permit accurate classification. Although the schematic outline in Figure 120-1 provides a helpful preliminary approach to differential diagnosis, it is anticipated that this will be modified as new entities are described. As more of the genetic mutations are identified, diagnosis can be more exact and facilitate genetic counseling.



**FIGURE 120-1** Evaluation of the patient with decreased response to pain. NI, normal; BP, blood pressure; DTR, deep tendon reflex.

Several conditions should be considered in the differential diagnosis of the HSANs. When sensory dysfunction is suspected, because of a history of self-mutilation or decreased responsiveness to pain, great care should be taken to document that there is sensory dysfunction rather than a behavioral or metabolic abnormality, as might occur with either cognitive impairment or Lesch–Nyhan syndrome. If sensory dysfunction is then confirmed, it should be determined which sensations are abnormal, the degree of the abnormality, and the somatic distribution. Loss of touch, pressure, vibration, and joint position senses occur in the autosomal recessive disorder Friedreich ataxia. Vibration and position sense in deep tissues are lost in Refsum disease,

but cutaneous sensation tends to remain intact. In dysraphia and syringomyelia pain pathways may be interrupted at a secondary neuronal level in the spinal cord.

When both autonomic and sensory functions are impaired, one must determine which sympathetic, parasympathetic, and enteric dysfunctions are present. Variable combinations of autonomic and sensory dysfunction may be observed in children with mitochondrial myopathies (64). Perturbations in autonomic and sensory function due to diabetes mellitus, Fabry disease, and amyloidosis is present in the adult years. Isolated autonomic dysfunction associated with developmental anomalies of neurons occur in hypertrophic pyloric stenosis and Hirschsprung disease.

**TABLE 120-3 The Eight Sensory Disorders with Peripheral Neuropathy and Their Pathologic Findings**

	<b>Familial Dysautonomia (HSAN Type III)</b>	<b>Congenital Sensory Neuropathy with Anhidrosis (HSAN Type IV)</b>	<b>Congenital Insensitivity to Pain without Anhidrosis (HSAN Type V)</b>	<b>Congenital Sensory Neuropathy (HSAN Type II)</b>	<b>Congenital Autonomic Dysfunction with Universal Pain Loss</b>	<b>Congenital Sensory Neuropathy with Skeletal Dysplasia</b>	<b>Progressive Panneuropathy with Hypo-</b>
Sural nerve	Decreased total size	Marked reduction in total size			Marked reduction in total size	Normal overall nerve size; faint myelin stain; ballooning of inner laminae	?
	Small myelinated fibers slightly decreased	Normal myelinated population	Large myelinated fibers are reduced	Severe depletion of myelinated axons	Marked decrease in myelinated fibers	Marked decrease in myelinated fibers	?
	Unmyelinated fibers markedly decreased (about 25% of normal)	Severe reduction in unmyelinated fibers (about 5–10% of normal)	Unmyelinated fibers normal	Moderate decrease in unmyelinated fibers	Only slight decrease in unmyelinated fibers	Unmyelinated fibers normal	?
Sensory ganglia	Decreased total size, decreased neuronal count, residual nodules; increase in number with age	Absence of thin myelinated fibers; absence of small neurons	?	?	?	?	Extremely small; only residual nodules remaining in ganglion
Sympathetic ganglia	1/3 of normal size 1/10 number of neurons; increased proportion of tyrosine hydroxylase	Normal	?	?	?	?	?



**TABLE 120-3 The Eight Sensory Disorders with Peripheral Neuropathy and Their Pathologic Findings—cont'd**

	<b>Familial Dysautonomia (HSAN Type III)</b>	<b>Congenital Sensory Neuropathy with Anhidrosis (HSAN Type IV)</b>	<b>Congenital Insensitivity to Pain without Anhidrosis (HSAN Type V)</b>	<b>Congenital Sensory Neuropathy (HSAN Type II)</b>	<b>Congenital Autonomic Dysfunction with Universal Pain Loss</b>	<b>Congenital Sensory Neuropathy with Skeletal Dysplasia</b>	<b>Progressive Panneuropathy with Hypo-</b>
Parasympathetic ganglia	Ciliary ganglion is normal, sphenopalatine ganglion is 1/10 of normal size	?	?	?	?	?	?
Spinal cord	Lissauer's tract is decreased in size; dorsal columns decrease in size with age	Absence of Lissauer's tract	?	?	?	?	Marked demyelination and atrophy of posterior columns; degeneration of intermediate lateral columns
Blood vessels (from skin and sural nerve)	No dense-core vesicle terminals	Complete absence of innervation ultrastructurally	?	Not innervated but many nerves present outside adventitial sheath; subcutaneous unmyelinated fibers with dense-core vesicles in skin biopsy	?	No dense-core vesicle terminals	?

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### Biography



**Dr Felicia B Axelrod** is Professor of Pediatrics and Neurology at the NYU School of Medicine. Her career has focused on understanding and treating rare hereditary sensory and autonomic disorders (HSAN). Her initial body of work dealt with familial dysautonomia (FD) but then she expanded her interest to other disorders affecting development of the autonomic and sensory nervous systems. With the support of the Dysautonomia Foundation and NYU Medical Center she founded the Dysautonomia Treatment and Evaluation Center in 1970. Dr Axelrod has centralized the care of patients with FD resulting in new treatments and dramatically lengthening of life expectancy. In collaboration with Harvard University, Dr Axelrod identified the responsible genetic mutation for FD. This has lead to prenatal testing and global population screening, which has successfully reduced the number of new cases to less than 5 per year worldwide.

Dr Axelrod was awarded the Carl Seaman Family Professor Chair for Dysautonomia Treatment and Research in 1992. NYU School of Medicine awarded her the Soloman A Berson Medical Alumni Achievement Award in Health Science in 1998 and the Samuel Leidesdorf Alumni Award in 2003. She was a past President of the American Autonomic Society and the David H Streeten Lecturer.



# CHAPTER

# 121

## The Phakomatoses

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### 121.1 INTRODUCTION

The term phakomatosis derives from phakos, the Greek word for birthmark. It was originally used by van der Hoeve in 1923 (1) to describe neurofibromatosis and tuberous sclerosis. von Hippel–Lindau (VHL) disease was added in 1932 (2) and Sturge–Weber syndrome (SWS) in 1936 (3). As other neurocutaneous diseases of a spotty or patchy nature have been recognized, they have been incorporated into the spectrum of the phakomatoses. A list of disorders that have been classified with the phakomatoses is presented in Table 121-1. In this chapter, we consider the original conditions included by van der Hoeve, as well as some of the more common ones listed in the table.

### 121.2 THE NEUROFIBROMATOSES

Until the late 1970s, many publications did not distinguish between the different forms of neurofibromatosis and used von Recklinghausen disease or multiple neurofibromatosis as all-encompassing terms. We now recognize three distinct forms of neurofibromatosis: neurofibromatosis type 1 (NF1), neurofibromatosis type 2 (NF2), and schwannomatosis (Table 121-1). Although each disorder is characterized by multiple nerve sheath tumors, the natural history for each disorder is different and each is due to mutations in a distinct gene.

#### 121.2.1 Neurofibromatosis Type 1 (NF1)

NF1 is the form of neurofibromatosis described by von Recklinghausen and was formerly referred to as von Recklinghausen disease or peripheral neurofibromatosis. It is the most common form of the disease and one of the most common single gene disorders. Most studies have found a disease prevalence of around 1 in 3000 (4,5). Evans et al. (6) estimated the disease prevalence of NF1 at 1 in 4560 and birth incidence at 1 in 2699.

**121.2.1.1 Clinical Features.** The clinical features of NF1 and the age of presentation are summarized in Table 121-2. The phenotype tends to evolve with age and is highly variable, both within and between families.

**121.2.1.1.1 Café-au-Lait Spots and Freckling.** Café-au-lait spots (Figure 121-1) are usually the first feature to appear; some may be present at birth, and they continue to develop and increase in size until puberty. In the diagnostic criteria, six or more café-au-lait spots are required because one to three spots are a relatively frequent finding in approximately 10–15% of the general population (7). The number of café-au-lait spots decreases with time, as they fade and disappear in adulthood (8). There is no correlation of the location of café-au-lait spots of average diameter less than 10 cm with the location of other NF1-associated lesions, such as neurofibromas, and there is no correlation of the overall number of café-au-lait spots with the severity of the disorder. Some plexiform neurofibromas have café-au-lait pigmentation overlying them, and this can be the first obvious change in early childhood (9). Therefore, particularly large areas of pigmentation need to be monitored for plexiform change. Such areas often have irregular edges. Café au lait spots vary in diameter, but are mainly less than 10 cm. They usually have smooth contours, but some, particularly the larger ones, have irregular outlines. Their color intensity varies with background skin pigmentation (10). Occasionally, in children with very pale complexions, café-au-lait spots may be difficult to see with the naked eye and are best assessed with a Wood's lamp.

The other characteristic pigmentation, which appears to be unique to NF1, is freckling in specific body areas not related to sun exposure (11). The classical area for freckling is the axilla, but it is also seen around the base of the neck, the groin, and the submammary region in women. These resemble café-au-lait spots (1–3 mm in

TABLE 121-1 The Phakomatoses

Disorder	Frequency	Gene	Major Features
NF1	1:3000	<i>NF1</i> ; GAP protein	Café-au-lait macules, neurofibromas, optic gliomas, malignant peripheral nerve sheath tumors, learning disabilities, skeletal dysplasias
NF2	1:33,000	<i>NF2</i> ; cytoskeletal protein	Vestibular schwannomas, meningiomas, ependymomas, cataracts
Schwannomatosis	Unknown	<i>SMARCB1</i> ; chromatin remodeling protein	Schwannomas of peripheral nerves, pain
Tuberous sclerosis complex	1:6000	<i>TSC1/TSC2</i> ; mTOR regulator	Hypomelanotic macules; angiofibroma; cortical tubers; seizures; intellectual disability; renal angiomyolipomas; cardiac rhabdomyomas
von Hippel–Lindau syndrome	1:40,000	<i>VHL</i> ; angiogenesis regulator	Hemangioblastomas of cerebellum, brainstem, spinal cord, retina; renal cell carcinoma; pheochromocytoma
PTEN hamartoma syndrome	Rare	<i>PTEN</i> ; cell signaling protein	
Proteus syndrome	Rare	<i>AKT1</i> ; cell signaling protein	Bony and cutaneous overgrowth; disfigurement

diameter) and develop after the café-au-lait spots appear, usually between 3 and 5 years of age (12). Some patients have small freckles all over the trunk.

**121.2.1.1.2 Peripheral Neurofibromas.** Nearly all adult patients with NF1 have neurofibromas (see Figure 121-1). Those within the epidermis are soft on palpation and may become papillomatous. Neurofibromas located deeper within the dermis often have an overlying violaceous color. Neurofibromas under the surface of the skin or deeper in the body have a nodular configuration and are firm in texture. The number of neurofibromas is only roughly proportional to age; severely and mildly affected cases can be seen in all age groups beginning in the teens (8,13). At present there is no means of predicting the number of neurofibromas someone will develop, with the exception that individuals with deletions of the entire *NF1* gene tend to develop large numbers of neurofibromas (14). Neurofibromas most commonly appear at times of hormonal change, including puberty and pregnancy (15). One study did not reveal an increase in the rate of growth of neurofibromas in women using oral contraceptives, except in two women receiving a high-dose depot form of progesterone (16).

Dermal lesions are rarely painful, although some patients complain of pruritis (9). Numerous lesions present a significant cosmetic burden (17). Dermal neurofibromas rarely, if ever, undergo sarcomatous change. Deeper nodular neurofibromas may give rise to neurologic symptoms due to nerve compression and are painful, particularly to pressure.

In addition to neurofibromas, glomus tumors have been identified in some individuals with NF1. These occur at the tips of the digits and present with pain that can be relieved with tumor resection (18).

**121.2.1.1.3 Plexiform Neurofibromas.** Plexiform neurofibromas involve multiple fascicles of a nerve, extending along the length of the nerve and involving multiple branches (19). In the Welsh study, 26.7% of patients had plexiform neurofibromas (13); 1.2% had

large facial plexiform neurofibromas of the head and neck (associated with major cosmetic problems) and 5.8% had large lesions of the trunk or limbs associated with significant skin hypertrophy or overgrowth of the underlying bone (Figure 121-2). Imaging studies reveal a higher frequency of plexiform neurofibromas than may be apparent clinically, with whole body magnetic resonance imaging (MRI) studies showing 50–100% of patients having plexiform tumors (20–22). Plexiform neurofibromas, particularly those with extensive internal involvement, may undergo malignant change.

Plexiform neurofibromas are clinically and pathologically distinct from dermal neurofibromas. There are two types of plexiform neurofibromas. The most common present as large subcutaneous swellings with ill-defined margins and vary from a few centimeters in diameter to those involving a whole area of the body. Their consistency is usually soft, although sometimes hypertrophic nerve trunks can be palpated within the mass. The skin overlying the lesion is often abnormal because of a combination of hypertrophy, hyperpigmentation, and hypertrichosis. Unless it is very obvious that the lesion is limited to the skin, an MRI scan should be considered to delineate the extent of internal involvement. Tumors that invade skin may have a diffuse appearance by MRI (23). Patterns of growth differ depending on whether the tumors are superficial or invasive (24), and lesions often undergo rapid growth in the early years of life, after which they remain relatively quiescent, growing only in proportion to body growth (25). Aside from disfigurement, some may impinge on critical structures such as the airway or urinary tract and thereby cause significant morbidity (26,27). Surgery is difficult because of the diffuse nature of the lesions and because they may be very vascular (28); recurrence after surgery is related to the degree of resection (29).

The other form of plexiform neurofibroma is the nodular variant. In these lesions, the nerve trunks develop multiple nodular neurofibromas that merge into one

**TABLE 121-2 NF1 Clinical Features and Age of Onset**

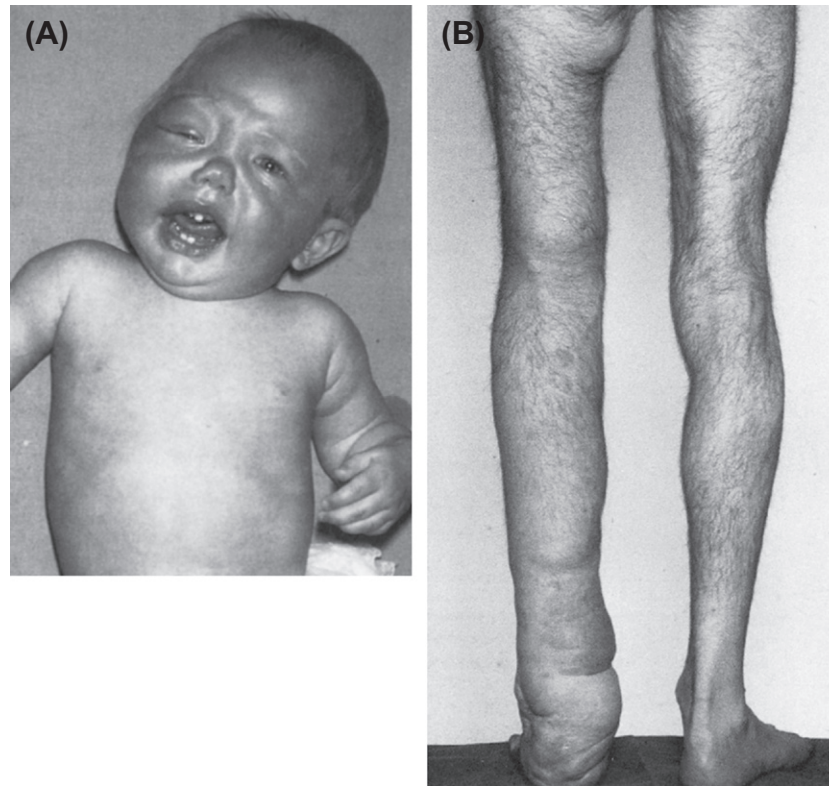
Disease Feature	Frequency (%) <sup>a</sup>	Age at Presentation
Café-au-lait spots	>99	0–2 y
Skinfold freckling	67	3–5 y
Peripheral neurofibromas	>99	≥7 y
Plexiform neurofibromas		
All lesions	30	0–18 y
Head and neck	1.2	0–3 y
Trunk/extremities with hypertrophy	5.8	0–5 y
Lisch nodules	90–95	≥3 y
Macrocephaly	45	Birth
Short stature	31.5	Birth
Intellectual disability		
Severe	0.8	0–5 y
Moderate	2.4	0–5 y
Minimal/learning difficulties	29.8	0–5 y
Attention deficit disorder ± hyperactivity	48 <sup>b</sup>	Childhood
Epilepsy		
No known cause	4.4	Lifelong
Secondary to disease complications	2.2	Lifelong
Hypsarrhythmia	1.5	0–5 y
CNS tumors		
Optic glioma	1.5 <sup>c</sup>	0–20 y
Other CNS tumors	1.5	Lifelong
Spinal neurofibromas	1.5	Lifelong
Aqueductal stenosis	1.5	Lifelong
Malignancy		
Malignant peripheral nerve sheath tumor	1.5 <sup>d</sup>	Lifelong
Rhabdomyosarcoma	1.5	0–5 y
Juvenile myelomonocytic leukemia	<1	0–18 y
Orthopedic		
Scoliosis, requiring surgery	4.4	0–18 y
Scoliosis, not requiring surgery	5.2	0–18 y
Long bone dysplasia	3.7	0–2 y
Vertebral scalloping	10 <sup>e</sup>	Lifelong
GI tumors (neurofibromas and GISTs) <sup>f</sup>	2.2	Lifelong
Renal artery stenosis	1.5	0–20 y
Pheochromocytoma	0.7	≥10 y
Duodenal carcinoid	1.5	≥10 y
Juvenile xanthogranuloma	0.7	0–1 y
Congenital glaucoma	0.7	0–1 y
Pulmonic stenosis	<1	Childhood
Sphenoid wing dysplasia	<1	Congenital
Lateral meningocele	<1	Lifelong
Cerebrovascular disease (moyamoya)	<1	Childhood
Glomus tumors of nailbeds	<1	Adulthood

<sup>a</sup>Data from Huson and colleagues (13).<sup>b</sup>Data from Mautner and colleagues (524).<sup>c</sup>Optic glioma is seen in 15% of children if imaging study is done (56).<sup>d</sup>Lifetime risk may be 5–10% (98).<sup>e</sup>Data from Riccardi and Eichner (525).<sup>f</sup>See Takazawa and colleagues (101).**FIGURE 121-1** The major defining features of NF1. Café-au-lait spots and cutaneous neurofibromas in an adult with NF1. (From Huson S. M. *Neurofibromatosis 1: A Clinical and Genetic Overview*. In Huson S. M.; Hughes R. A. C.; Eds.; *The Neurofibromatoses: A Pathogenetic and Clinical Overview*. Chapman and Hall, London, 1994, pp 176. Reproduced with permission of Edward Arnold.)

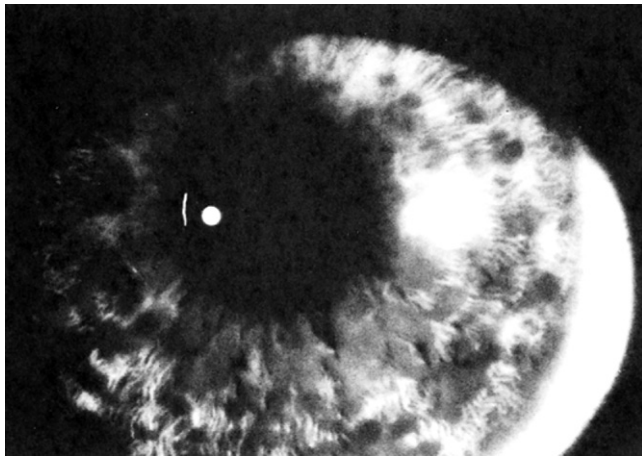
another. They may involve one nerve or a whole plexus of nerves. The patient usually presents because of pain related to the lesions. If they involve a relatively superficial nerve trunk, they present as smooth subcutaneous masses, which are often painful on palpation. When deeper nerve trunks are involved, the lesions are only detectable on imaging (30,31).

**121.2.1.1.4 Lisch Nodules.** Lisch nodules are asymptomatic iris hamartomas. They can occasionally be seen with the naked eye, but slit-lamp examination is preferable to distinguish them from common iris nevi (Figure 121-3). On slit-lamp examination, they have a smooth, dome-shaped appearance and are usually light brown, although some can be very pale (32). They develop during childhood, after the appearance of café-au-lait spots but before peripheral neurofibromas and so may be useful in confirming the diagnosis of NF1 in children with no family history and only multiple café-au-lait spots (12). They are highly specific for NF1 and are present in over 90% of affected adults (33,34).





**FIGURE 121-2** Plexiform neurofibromas. (A) Facial plexiform neurofibroma in a 2-month-old girl. (B) Plexiform neurofibroma of left leg, associated with limb overgrowth, in a 38-year-old man. He had required epiphyseal plating of the tibia to prevent further overgrowth as a teenager. (From Huson S. M. *Neurofibromatosis 1: A Clinical and Genetic Overview*. In Huson S.M.; Hughes R. A. C.; Eds.; *The Neurofibromatoses: A Pathogenetic and Clinical Overview*. Chapman and Hall, London, 1994, pp 177. Reproduced with permission of Edward Arnold.)



**FIGURE 121-3** Lisch nodules.

#### 121.2.1.1.5 Macrocephaly and Short Stature.

Macrocephaly and short stature are common in NF1 but are not specific to the condition and therefore cannot be used as diagnostic criteria. Macrocephaly was found in 45% of the Welsh population (13), and 31.5% of individuals were at or below the third centile for height. Growth curves have been produced for use in individuals affected with NF1 (35).

**121.2.1.1.6 Learning Difficulties.** All the population-based studies of NF1 have shown that at least 25–30% of patients require educational assistance because of learning difficulties (36). Hyman et al. (37) found academic difficulties in 52% of children with NF1, of which one-third had lowered IQ. Specific learning disabilities in those with normal IQ included problems with attention, planning, visuospatial skills, and language. Those without learning difficulties did have minor problems with sustained attention, planning, and visuospatial skills. Comorbid attention deficit disorder with hyperactivity was found in 38% of children. The situation is further compounded by coordination problems in NF1-affected children (38–41). Behavior problems and sleep disturbance also occur with increased frequency (42,43). Cognitive problems appear to begin early in life (44) and do not improve with age (45).

The pathogenesis of learning difficulties in NF1 patients is undetermined. Some authors have found a correlation between the number of enhanced T2-weighted signal abnormalities seen in NF1 on MRI (46,47) or their location in the thalamus (Moore 1996) but others have not found this correlation (48,49). Evidence for frontal cortical dysfunction has been obtained from functional MRI studies (50), and MRI morphometric studies have shown gyral anomalies (51) or increased size of the



corpus callosum (52,53) that may contribute to learning problems. Studies in a mouse model suggest that dysfunction of the Ras signaling pathway due to NF1 haploinsufficiency may be responsible for learning problems (54).

It is important to be vigilant for learning problems and neuropsychological assessment should be provided for those in whom learning problems are suspected. If children are found to have very severe learning problems or moderate to severe intellectual disability, then it is important to ensure that the child does not have a complication of NF1 such as hydrocephalus secondary to aqueduct stenosis. Children with deletions of the entire *NF1* gene also may display severe cognitive impairment (55). If these NF1-related causes of severe learning impairment are excluded, then other causes should be considered, such as fragile X syndrome.

**121.2.1.1.7 Optic Glioma.** Approximately 15% of children with NF1 display thickening of the optic nerve visualized by computed tomography (CT) or MRI (56–58). Although biopsy is rarely done, this is attributed to optic glioma; in cases in which biopsy has been done, the pathology is typically pilocytic astrocytoma. Optic glioma may involve the orbital portion of the optic nerves, the retro-orbital portion, the chiasm, or any combination of these components. Orbital optic glioma can produce proptosis, interference with extraocular movements, and loss of visual acuity or visual fields. Visual loss can also occur from retro-orbital or chiasmatic tumors, and the latter can involve the hypothalamus, usually presenting as precocious puberty (59,60), which may be accompanied by growth hormone excess (61).

Asymptomatic children in whom optic gliomas are diagnosed incidentally by MRI should be followed up clinically with repeated ophthalmologic assessment and MRI but is not treated unless visual impairment (loss of visual acuity or fields or proptosis) occurs (56). Spontaneous plateau of tumor growth is common, and regression has been seen even in untreated patients (62–64). In the past, symptomatic optic gliomas were treated with radiation, but cranial radiation in young children results in a high frequency of cognitive, endocrine, and vascular complications (65). Chemotherapy is more commonly used now, particularly vincristine and carboplatin (66–68), although many affected children continue to lose vision following treatment (69,70). Surgery is rarely indicated, since the diagnosis does not require biopsy and there is a high likelihood of visual impairment after surgery. Surgical treatment is reserved for orbital tumors that are causing pain or proptosis.

**121.2.1.1.8 Skeletal Dysplasia.** Long bone dysplasia, most commonly involving the tibia and sometimes also the fibula (71), occurs in approximately 3% of individuals with NF1 (72). Other long bones can be involved more rarely. Dysplastic bone includes cortical thickening (73) and medullary canal narrowing, and there may

be cystic changes (74,75). Usually there is no associated neurofibroma in the region. Long bone dysplasia occurs congenitally and usually is clinically evident during the first year of life as anterolateral bowing. Dysplasia of the greater wing of the sphenoid may occur, usually in association with orbital plexiform neurofibroma (76). This can lead to enophthalmos, or, if there is orbital tumor, exophthalmos. Dysplastic vertebrae may occur, causing a dystrophic scoliosis, most commonly involving the thoracic spine. Nondystrophic scoliosis also occurs at an increased frequency (77). There can also be bone cysts that can result in fracture, although most are asymptomatic. Individuals with NF1 have been reported to have decreased bone mineral density, reduced vitamin D levels, and increased risk of fracture (78–84). Osetoclast activity has also been reported to be higher in NF1-affected individuals (85) and osteoblast function is reduced (86).

**121.2.1.1.9 Cardiovascular Complications.** Cardiovascular complications are an important source of morbidity and even mortality. Arterial occlusions may occur due to nodular proliferation of spindle-shaped cells in the arterial wall and can cause renovascular hypertension (87–89), aneurysm, arterial dissection, and cerebrovascular occlusion and moyamoya disease (90,91). Congenital heart defects, particularly pulmonic stenosis, are also seen with increased frequency (92,93). NF1 patients with hypertension should also be evaluated for pheochromocytoma (94). Instances of pulmonary hypertension in adults with NF1 have also been reported (95–97).

**121.2.1.1.10 Malignant Tumors.** The lifetime risk of malignancy (excluding optic glioma) in NF1 has been estimated at approximately 8–13% (98); Walker et al. (99) estimated a risk of 20% by 50 years of age. Major tumor types include gliomas, malignant peripheral nerve sheath tumors (MPNST), leukemia, rhabdomyosarcoma, pheochromocytoma, and gastrointestinal stromal cell tumors (100,101). One study indicates a moderately increased risk of breast cancer in women with NF1 (102). Gliomas may occur anywhere in the central nervous system (CNS) and are seen in both adults and children. Most are low-grade pilocytic astrocytomas, but more aggressive tumors can occur (103–106). Care must be taken in distinguishing gliomas from benign foci of enhanced T2 signal intensity. MPNST are highly aggressive sarcomas that usually arise from preexisting plexiform neurofibromas or nodular neurofibromas. Although they are most common in young adults, children can be affected (107). Sudden growth or unexplained pain is a sign of malignancy, but diagnosis can be difficult since the malignant tumor often lies adjacent to a preexisting benign neurofibroma. MRI findings may be helpful in distinguishing benign from malignant tumors, with signs such as peripheral enhancement or edema and cystic appearance suggestive of malignant change (108). Positron emission tomography with [18F]-deoxyglucose may be useful,

revealing enhanced uptake in MPNST (109–114). Some MPNST have features of rhabdomyosarcoma. The relative risk of leukemia is increased in NF1, particularly nonlymphocytic leukemia (115). Some children with NF1 develop juvenile xanthogranuloma (JXG) (small yellowish nodules on the skin, seen especially near the hairline, in early childhood) (116,117). JXG can also be seen in individuals with myeloid leukemia, but it is not established that JXG in children with NF1 is predictive of leukemia.

**121.2.1.2 Diagnostic Criteria.** NF1 is diagnosed using clinical criteria set forth by the National Institutes of Health (NIH) Consensus Development Conference (118,119). An individual who fulfills two or more criteria can be diagnosed as having NF1. Many of the features are age dependent, however, so children who have a single feature, such as multiple café-au-lait macules, often must be followed for several years before a second feature is seen (120). The use of MRI to detect foci of enhanced T2 signal has been proposed as a diagnostic criterion, but the specificity of this finding is unclear (121). Genetic testing is now available to help resolve uncertain diagnoses (see below). The NIH Consensus Development Conference Statement diagnostic criteria for NF1 are met in an individual who has two or more of the following:

- (1) Six or more café-au-lait macules of over 5 mm in greatest diameter in prepubertal individuals and over 15 mm in greatest diameter in postpubertal individuals
- (2) Two or more neurofibromas of any type or one plexiform neurofibroma
- (3) Freckling in the axillary or inguinal regions
- (4) Optic glioma
- (5) Two or more Lisch nodules (iris hamartomas)
- (6) A distinctive osseous lesion such as sphenoid dysplasia or long bone dysplasia
- (7) A first-degree relative (parent, sibling, or offspring) with NF1 by the above criteria

These criteria have stood the test of time well. Individuals with Legius syndrome or homozygous mutation of mismatch repair genes may also fulfill NF1 diagnostic criteria. These entities are discussed in detail below.

**121.2.1.2.1 Segmental or Localized NF1.** The term segmental or localized NF1 is used to describe the patients with disease features limited to one or more body segment. Ruggieri and Huson (122) have reported their series of 124 patients with segmental NF1. They estimated a prevalence between 1 in 36,000 and 1 in 40,000 individuals in the general population. Most patients were asymptomatic and sought medical opinion because of the unusual appearance of the skin. In the majority, the area involved was unilateral and varied in size from a narrow strip to one quadrant and occasionally one half of the body. Some patients had more than one segment involved on both sides of the midline, either in a symmetrical or

an asymmetrical arrangement. Within the affected area, the patients either had NF1-related pigmentary changes, neurofibromas alone, or both. Eight of the 124 patients had a solitary plexiform neurofibroma as their only manifestation. Seven of the remaining 116 patients (6.9%) had specific NF1 complications, including learning difficulties, plexiform neurofibromas, optic pathway gliomas, and pseudarthrosis. Other NF1 complications were only identified in patients with segmental pigmentary changes. Mosaicism for NF1 mutation can be demonstrated in most cases, sometimes in blood but otherwise in Schwann cells from neurofibromas or melanocytes from café-au-lait macules (123). The importance of recognizing this group is for their different natural history and because they have much lower recurrence risks in offspring. There are, however, well-recorded examples of parents with segmental NF1 having children with full-blown NF1 (124).

**121.2.1.3 Differential Diagnosis.** NF2 should be considered in assessing children or teenagers with a few café-au-lait spots (usually six or less) in association with any other NF2 features. One should be particularly cautious in children with multiple nodular peripheral nerve lesions—the clinical appearance of peripheral schwannomas in NF2 is similar to that of peripheral nodular neurofibromas in NF1. A useful distinguishing feature of NF2 is the cutaneous NF2 plaque. Young children who have six or more café-au-lait spots and no family history should be followed up. They usually have NF1, and other disease features gradually appear (12). Café-au-lait spots can be seen in association with other syndromes, such as tuberous sclerosis complex (TSC), ataxia telangiectasia, and Noonan syndrome, although other features of these conditions should establish the correct diagnosis. Rare individuals with homozygous mutation of mismatch repair genes have presented with multiple café-au-lait spots and skin fold freckling, thereby fulfilling diagnostic criteria for NF1. Most of these individuals die at a young age of malignancies (125–133).

Brems et al. (134) reported five families with autosomal dominant transmission of multiple café-au-lait spots, with or without skin fold freckling, macrocephaly, and learning disabilities associated with mutation in the *SPRED1* gene. *SPRED1* is a negative regulator of Ras, which is the target of the product of the *NF1* gene. This condition has come to be known as “Legius syndrome.” Subsequent analysis has indicated that affected individuals do not appear to be at risk for tumors associated with NF1, such as neurofibromas, optic gliomas, or malignancies (135). Cognitive impairment is also not as significant as for patients with NF1 (136). Implications for genetic testing will be discussed below.

**121.2.1.4 Natural History.** NF1 is characterized by a wide range of phenotypic variability, both between and within families. The major morbidity and mortality in NF1 is caused by the occurrence of disease complications, many of which present in childhood or not at all (see Table 121-3). Individuals with NF1 face a decreased

**TABLE 121-3 NF2 Clinical Features**

Feature	Frequency (%)
<b>Tumors</b>	
Vestibular schwannoma (bilateral)	85
Vestibular schwannoma (unilateral)	6
Cranial meningioma <sup>a</sup>	45–58
Spinal tumors (extramedullary) <sup>b</sup>	63–90
Spinal tumors (intramedullary) <sup>c</sup>	18–53
Peripheral neuropathies	10 <sup>d</sup>
<b>Peripheral schwannomas</b>	
Overall	68
>10 tumors	10
NF2 plaques	48
Nodular schwannomas	43
NF1-like dermal neurofibromas	27
Plexiform schwannomas	<1
<b>Café-au-lait spots</b>	
1–2 spots	35
3–4 spots	7
6 spots	1
<b>Ophthalmologic features</b>	
Cataracts	60–81
Epiretinal membranes	12–40
Retinal hamartomas	6–22

<sup>a</sup>A higher frequency of tumors is found on cranial and spinal MRI, a significant proportion of which will never require treatment.

<sup>b</sup>Dorsal root schwannomas and, less frequently, meningiomas.

<sup>c</sup>Ependymomas, infrequently astrocytomas and schwannomas.

<sup>d</sup>Frequency of symptomatic neuropathies in one series (205), up to 66% have electrophysiological evidence of a neuropathy (227).

Data from Evans et al. (205) and Asthagiri et al. (526).

overall life expectancy (137–140). Major causes of death related to NF1 are malignancy and vascular complications such as hypertension and hemorrhage.

**121.2.1.5 Genetics.** NF1 is an autosomal dominant condition with variable expression but virtually 100% penetrance by the age of 5 years (5,13). Approximately 50% of cases represent new mutations, and the mutation rate is one of the highest recorded in humans. Crowe and colleagues (4) estimated a mutation rate on the order of  $1.4\text{--}2.6 \times 10^{-4}$ , although subsequent studies have shown a lower rate,  $3\text{--}6.6 \times 10^{-5}$  (5,141). The reasons for the extreme variability in NF1 are unknown. Studies of concordance in twin pairs (142,143) indicate that some features, such as number of café-au-lait spots and average number of tumors, are concordant, whereas others, such as presence of plexiform neurofibromas, are not concordant. This suggests that both genetic factors, including the *NF1* mutation and other modifying loci, as well as nongenetic or stochastic factors are involved in determining the specific NF1 phenotype.

**121.2.1.5.1 Genetic Testing.** Genetic testing is available for NF1 and can be used to assist in diagnosis of affected individuals and for prenatal testing. Given the large size of the gene (>350kb) and the large diversity of mutations, multiple approaches need to be used to achieve nearly complete ascertainment of mutations

(144). Most mutations lead to loss of function of the gene product, either due to deletion of the gene or due to premature termination of translation due to stop mutation, frameshift, or abnormal splicing. Only a few genotype–phenotype correlations have been reported. These include a severe phenotype (large tumor burden, developmental impairment, and dysmorphic features) in those with large deletions that encompass the entire *NF1* gene and several nearby genes (14,145), absence of neurofibromas in those with a specific three-base deletion in exon 17 (146), and presence of nodular, spinal, and extensive plexiform neurofibromas with minimal dermal involvement with missense or splicing mutations (147,148).

Given the relative paucity of genotype–phenotype correlations, *NF1* genetic testing is not useful in those where a definitive clinical diagnosis has been established, unless prenatal diagnosis is desired. It can be useful establishing an NF1 diagnosis in a young child who presents with a single NF1 feature, such as multiple café-au-lait spots. It may take several years before a child will develop additional signs of NF1. Although clinical management is currently limited to surveillance, establishing a definite diagnosis can be reassuring to some parents. Genetic testing can also distinguish between Legius syndrome and NF1, which can be important given the more benign course of Legius syndrome. Legius syndrome is much less common than NF1, so genetic testing for *NF1* mutation is likely to be more productive than *SPRED1* (135). The highest proportions of *SPRED1*-positive patients are postpubertal individuals with multiple café-au-lait spots, no NF1-related tumors, and a family history of similar manifestations.

**121.2.1.5.2 Genetic Counseling.** The 50% risk to offspring of an affected individual is straightforward; more difficult to share with families is the varied and unpredictable nature of the disease. If children at risk of NF1 have not developed multiple café-au-lait spots by the end of their second year of life, then it is very likely that they have not inherited the *NF1* gene. In the 50% of cases with no family history, recurrence risk for the parents is minimal. Examination of the skin and eyes of the parents is important, as children with NF1 have been born to parents with segmental neurofibromatosis (124) or Lisch nodules only. These parents represent gonosomal mosaics. Pure gonadal mosaicism is a rare event in NF1 (149).

**121.2.1.6 Pathogenesis.** *NF1* was identified in 1990 (150–153). The gene spans over 350kb of genomic DNA, consists of 60 exons, and encodes a 2818-amino-acid protein referred to as neurofibromin (154,155). There are three additional genes (*EV12A*, *EV12B*, and the oligodendrocyte myelin glycoprotein gene [*OMGP*]) located within intron 27B of *NF1*. The transcriptional orientation of the three intronic genes is reversed compared to that of *NF1*.

Neurofibromin has a domain homologous to the GTPase-activating protein (GAP) family (152,156,157).

This region is known as the GAP-related domain (NF1-GRD) and accounts for about 13% of the gene sequence. Neurofibromin is involved in the regulation of the signal transduction molecule Ras, stimulating conversion of Ras-GTP to Ras-GDP. The mechanism whereby increased Ras-GTP leads to neurofibroma formation remains unclear. Studies have shown hyperactivity of the mammalian target of rapamycin (mTOR) pathway in neurofibromin-deficient astrocytes, suggesting another potential target for development of therapeutics for NF1 (158–160).

Dominant inheritance of NF1 coupled with the occurrence of multiple tumors at multiple sites throughout life have suggested the possibility that NF1 might function as a tumor suppressor, analogous to hereditary retinoblastoma. This predicts that both copies of *NF1* would be mutated in a tumor such as a neurofibroma, with one mutation representing the inherited change and the other being acquired somatically. Loss of heterozygosity for NF1 or biallelic mutation has been demonstrated in MPNST (161), leukemias (162), bone dysplasias (163), melanocytes in café-au-lait macules (123), and Schwann cells in neurofibromas in NF1 patients (164–169). MPNST tend to accumulate additional genetic changes besides biallelic *NF1* mutation (170), as is common in malignant tumors.

The tumor suppressor hypothesis is also supported by animal models. Mice with an *Nf1* knockout mutation do not develop neurofibromas or other classical signs of NF1, but do get sarcomas and leukemias (171,172). Homozygous animals die in early development due to cardiovascular anomalies (173), but mice that are rendered chimeric for *Nf1*<sup>-/-</sup> cells on a wild-type background develop plexiform neurofibromas (171). Conditional knockout strategies have been used to generate models that develop plexiform neurofibromas (174), optic gliomas (175), dermal neurofibromas (176), vascular dysplasia (177), and skeletal dysplasia (178,179).

Although the tumor suppressor mechanism in Schwann cells appears to be the primary cellular event in neurofibroma formation, there is evidence for a role of haploinsufficiency of NF1 expression in other cells, particularly mast cells (180,181), being necessary for tumor formation (174). Studies of mast cells from *Nf1* mice (182–185) have revealed abnormal proliferation of these cells and hypersensitivity to growth factors. Furthermore, in animal studies, loss of NF1 function in Schwann cells does not result in neurofibroma formation if the gene is normal in all other cell types. Tumor formation only occurs with nullizygous Schwann cells on a heterozygous background. The origin of *-/-* cells in neurofibromas is also a matter of investigation. Dermal neurofibromas appear to arise from Schwann cell precursor cells in the skin (176,186); plexiform neurofibromas most likely arise from embryonic Schwann cells (187), which explains their typical congenital appearance.

**121.2.1.7 Management.** It is recommended that individuals with NF1 have an annual clinical review (119). Since the purpose of the clinical assessment is to detect complications, the age of presentation of the various complications (see Table 121-3) must be appreciated so that an appropriate assessment can be offered to patients of different ages. In the first 2 years of life, one is particularly monitoring for plexiform neurofibromas, sphenoid wing dysplasia, and tibial dysplasia. Neuropsychological assessments become important in preschool children. Children should have annual ophthalmological evaluations to screen for visual dysfunction; tests of visual fields may be difficult, but visual acuity should be possible to assess (188). Apart from learning difficulties and plexiform neurofibromas, most complications of NF1 present with symptoms. The exception to this is hypertension due to renal artery stenosis or pheochromocytoma, so the annual review for all ages must include measurement of blood pressure. There are no specific treatments for any of the disease features. Patients with cosmetic problems need the support of a plastic surgeon.

In our opinion, none of the complications warrants screening investigations, although this is a matter of debate, particularly with regard to the role of routine cranial MRI scans. Cranial MRI scans in children with NF1 will detect optic gliomas in 15%, the majority of which will remain asymptomatic, and in 60% enhanced signal intensity will be found on T2-weighted or fluid-attenuated inversion recovery (FLAIR) images (189). Occasionally, other asymptomatic lesions with mass effect are also observed, particularly in the brainstem; if an asymptomatic lesion or an optic glioma is detected, follow-up scans and clinical assessments are needed. In our opinion, the detection of these asymptomatic lesions does not alter clinical management, as they are treated only if symptomatic and their detection often only serves to increase parental anxiety, but other authors have advocated MRI screening for the early detection of optic glioma (190).

We advocate the value of the specialist neurofibromatosis clinic. In this setting, patients are offered coordinated care and follow-up by a group of health professionals, including a pediatrician, ophthalmologist, dermatologist, neurologist, neurosurgeon, orthopedic surgeon, otolaryngologist, plastic surgeon, geneticist, specialist genetic nurse/genetic counselor, social worker, and psychologist. Usually one of the group acts as the overall coordinator of patient care and involves the others at certain times in the patient's life or because of specific symptoms. The clinic is used for family support when NF1 is newly diagnosed, for specialist genetic counseling, and for assessment of unusual symptoms or complications.

Insights into pathogenesis and the availability of mouse models are providing an opportunity to perform drug screening. Previous trials of ketotifen (a mast cell stabilizer) (191), interferon alpha-2a, cis-retinoic acid,



thalidomide (192), pirfenidone (193), and tipifarnib (194) have not revealed definitive evidence of effectiveness. One case has been reported indicating shrinkage of a plexiform neurofibroma treated with imatinib (184); this finding is being followed up in a phase II clinical trial. Neurocognitive improvement has been noted in *Nf1*<sup>+/-</sup> mice treated with lovastatin. One trial in humans with simvastatin did not show definitive improvement (195), but additional studies with statins administered to children with documented neurocognitive impairment are underway (196).

## 121.2.2 Neurofibromatosis Type 2

Writing in the twenty-first century with easy availability of MRI scans and molecular genetic knowledge, it is hard to see how the different forms of neurofibromatosis were ever considered to be one disease (197–199). The overlap with NF1 occurs because some cases of NF2, particularly the severe form that presents in childhood, may initially present with cutaneous features that overlap with NF1. NF2 was not established as a distinct entity until 1970 (200). The other factor that caused confusion was the pathological nomenclature for different nerve tumors. The main lesions in NF2 are on the vestibular branch of the eighth nerve and are schwannomas, yet formerly they were always referred to as acoustic neuromas. Hence NF2 was formerly referred to as bilateral acoustic or central neurofibromatosis. The 1991 NIH Consensus Development Conference Statement on Acoustic Neuroma (201) recommended replacing the term acoustic neuroma with vestibular schwannoma.

NF2 is much less common than NF1, with a prevalence of around 1 in 60,000 and an estimated birth incidence of 1 in 33,000 (202).

**121.2.2.1 Clinical Features.** NF2 causes significantly more morbidity and mortality than other forms of neurofibromatosis largely due to vestibular schwannomas and other cranial or spinal tumors (197,202–204). The frequency of the different clinical features is shown in Table 121-3. MRI scans often show NF2 patients to have multiple lesions, a significant proportion of which will never need treatment (202–204).

**121.2.2.1.1 Vestibular Schwannomas.** Vestibular schwannomas are the most consistent disease feature and were present bilaterally (and symptomatic) in 85% and 92.1% of cases in two large series (203,205). A further 6% of cases in both studies had unilateral vestibular schwannomas. The presenting symptoms of NF2 are related to vestibular schwannomas in the majority of affected individuals (202–204). Early symptoms include hearing loss with tinnitus or vertigo related to pressure on the cochlear nerve. Vestibular schwannomas are usually diagnosed synchronously, but there may be a time interval in their development; in 10 of 58 cases with vestibular schwannomas, Parry and associates (203) report a mean interval of 7.5 years (range,

0.5–20 years) between diagnosis of the first and second tumor.

**121.2.2.1.2 Other Cranial Tumors.** Schwannomas can develop on other cranial nerves and were found on cranial MRI in 51% of 83 cases in one study (206). They can develop on any nerve except one and two, which are CNS derived. They are most frequent on the third, fifth and seventh cranial nerves. Seventh nerve tumors are often only identified at surgery for a vestibular schwannoma (197,206). Many of these never cause major problems and, if so, do not require treatment. Lesions of the lower cranial nerves can cause significant morbidity if symptomatic because of swallowing problems. Cranial meningiomas are found in approximately 50% of patients in cross-sectional studies (207), although some will never cause problems. Optic nerve meningiomas are seen in NF2 much more frequently than in the general population and are a particular problem as curative surgery is difficult (198,207).

**121.2.2.1.3 Spinal Tumors.** The most frequent spinal tumors in NF2 are dorsal root schwannomas. They usually originate within the vertebral canal and extend both within and out of the canal giving rise to a “dumbbell” appearance on scan. Spinal meningiomas are less common but do occur. NF2-related intramedullary tumors are usually ependymomas histologically, although both astrocytomas and intramedullary schwannomas also occur (197,208–211). Many spinal tumors seen on scan will never cause significant problems; for example, two series documented an overall frequency of spinal tumors of 75% and 89% of patients, but only 26% of these were symptomatic (203,204).

In assessing potential cases of NF2 one must be aware of frequent nosologic confusion between schwannomas and neurofibromas. Spinal schwannomas can appear identical with the spinal neurofibromas seen in NF1, both radiologically and at surgery. Although schwannomas and neurofibromas have distinctive histologic appearances, we have seen a significant number of NF2 patients in whom spinal and peripheral lesions were initially reported as neurofibromas, which were actually schwannomas.

**121.2.2.1.4 Other CNS Manifestations.** Imaging and postmortem studies have shown a number of other abnormalities that are usually not associated with symptoms. Benign intracranial calcifications on CT scans, similar to those seen in tuberous sclerosis, do occur (212). When present they are seen in the choroid plexus, cerebellum, and cerebral parenchyma.

Meningioangiomas is often only diagnosed on postmortem. It is a multifocal cortical plaque-like proliferation (213). Histologically there is a meningotheial and vascular component. The other lesion seen in postmortem studies is glial microhamatoma in the cortex and basal ganglia (214).

**121.2.2.1.5 Peripheral Nerve Tumors.** There are three main types of peripheral nerve tumor in NF2. These have different clinical features, but on histologic

examination are usually schwannomas. Rarely, histology will show mixed appearance of both schwannoma and neurofibroma or even just neurofibroma. The most common peripheral tumors, occurring in 48% of patients in the Evans et al. study (205), are referred to as NF2 plaques and have a distinctive appearance (Figure 121-4). They are discrete, well-circumscribed, slightly raised, cutaneous lesions, usually less than 2 cm in diameter. Their surface is roughened, they may be slightly pigmented, and the overlying skin is often hairy. The second type of peripheral nerve tumors are nodular, subcutaneous lesions on peripheral nerve and were present in 43% of patients in the Evans et al. study. These tumors are clinically indistinguishable from their NF1 counterpart, as are the least common type of peripheral tumors, which appear similar to the dermal lesions of NF1. These were found in 7% of patients in the Evans et al. study, but were fewer in number than would normally be seen in an adult with NF1. Rare patients, usually with severe NF2, have lesions that are clinically similar to diffuse plexiform neurofibromas. Again, on histology these usually have the features of plexiform schwannomas.

**121.2.2.1.6 Café-au-Lait Spots.** As can be seen in Table 121-4, more cases of NF2 than expected have a few café-au-lait spots, but only the occasional patient has six or more (205). The café-au-lait spots are not associated with other NF1 pigmentary abnormalities such as axillary freckling.

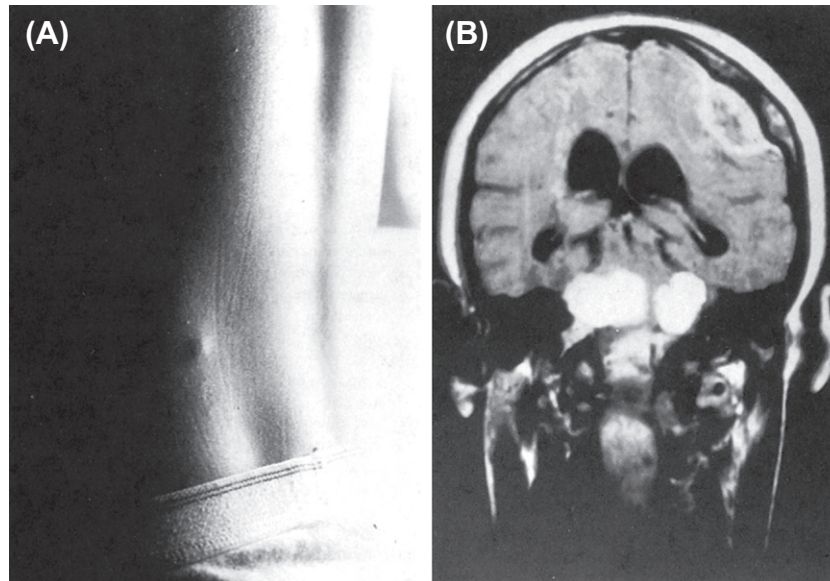
**121.2.2.1.7 Ophthalmologic Features.** Eye problems in NF2 fall in to two categories: (1) Primary NF2-related eye lesions such as cataracts and congenital retinal/disk problems, which usually present in childhood

and (2) problems occurring secondary to cranial nerve palsies caused either from an NF2-related tumor or operative nerve damage. In a condition where the patient will almost certainly lose all/some hearing, loss of vision can be a major issue.

The commonest eye findings are cataracts, which were present in 81% of patients in the series reported by Parry et al. (203); 72.4% had posterior capsular cataracts, 41.4% had cortical cataracts, and 32.8% had both types. Fortunately they only cause significant visual disturbance in a minority of cases (two of 47 individuals in the Parry et al. series). Other ocular features in NF2 are much less frequent (215–220) and include retinal hamartomas (either combined pigment epithelium and retinal hamartomas or astrocytic hamartomas) and epiretinal membranes. Whether these cause symptoms depends on position. Even in the absence of a causative lesion, more than the expected number of NF2 patients have no useful vision in one eye—34% (24/73) patients had only unocular vision in one series (220); there was also a high frequency of strabismus.

Secondary eye problems often relate to facial or trigeminal nerve damage at the time of vestibular schwannoma treatment (ptosis, nystagmus, reduced tear production, corneal insensitivity). Schwannomas on the relevant nerves can affect eye movement. Optic nerve meningiomas usually result in visual loss.

**121.2.2.1.8 Peripheral Neuropathies.** In addition to loss of nerve function related to tumors, NF2 patients are also at risk of two forms of peripheral neuropathy: an axonal neuropathy (221,222) and focal amyotrophy (223). The latter presents as a relatively sudden onset of



**FIGURE 121-4** NF2. (A) Cutaneous NF2 plaque. (B) Coronal T1-weighted gadolinium-enhanced MRI demonstrating severe NF2 in an 18-year-old. There are bilateral vestibular schwannomas, an intrinsic brainstem lesion, and a left convexity meningioma. (From Short P. M.; Martuza R. L.; Huson S. M. *Neurofibromatosis 2: Clinical Features, Genetic Counselling, and Management Issues*. In Huson S. M.; Hughes R. A. C., Eds.; *The Neurofibromatoses: A Pathogenetic and Clinical Overview*. Chapman and Hall, London, 1994, pp 423, 432. Reproduced with permission of Edward Arnold.)

**TABLE 121-4 Clinical Features of TSC**

Feature	Frequency (%)	Age of Onset	Comments
Seizures	78		
Infantile	54	0–1 y	Half infantile spasms
Childhood	21	1–18 y	
Adulthood	3	>18 y	
Neurocognitive			
Learning disability	53	0–5	Related to seizures autistic sleep disorders, hyperactivity, aggressive behavior
Behavior disorder	>50	Childhood	
CNS lesions			
Subependymal nodules	95	Birth	
Cortical tubers	93	Birth	
White matter abnormality	93	Birth	
Subependymal giant cell astrocytoma	6–7	1–31 y	
Cutaneous lesions			
Hypomelanotic macules	78–87	Birth	
Facial angiofibromas	80–90	5 y–puberty	
Shagreen patch	21–41	>10 y	
Forehead plaque	26	0–10 y	
Dental enamel pits	48	>6 y	
Ungual fibromas	17–47	>15 y	
Renal lesions			
Angiomyolipomas	37–67	Childhood	May become symptomatic in adulthood
Cysts	10	Childhood	
Polycystic kidney disease	1.5	Childhood	
Renal cell carcinoma	Rare	Adulthood	rarely symptomatic with PKD1 deletion
Retinal hamartoma	50	Childhood	
Cardiac rhabdomyoma	50	Prenatal	Regress early in childhood
Pulmonary lymphangiomyomatosis	0.8 (symptomatic)	>18 y	Mostly in females
Liver hamartomas	45	Childhood	Usually asymptomatic
Microhamartomatous rectal polyps	78	Childhood	Usually asymptomatic

See text for sources of data.

weakness and wasting of specific muscle groups causing, for example, facial weakness or foot drop. These neuropathies are thought to result from compression effects of multiple tumorlets, by local influences of the endoneurial pathological cells and/or the inability of these cells to properly adhere to the axon (221). In the clinical study of Evans et al. (205) 6% had a clinical peripheral neuropathy and 4% evidence of focal amyotrophy. When patients have detailed neurological and neurophysiological studies, two-thirds show electrophysiological evidence of peripheral neuropathy, the majority of who are asymptomatic (221).

**121.2.2.1.9 Other Manifestations.** The possibility of NF2 vascular involvement has been raised by cases reports of renal artery stenosis and cerebrovascular problems (224). Although mesothelioma occurs at an increased frequency in *Nf2* +/- mouse models, there have only been occasional reports in NF2 patients exposed to asbestos may have an increased risk of mesothelioma (225).

**121.2.2.2 Diagnostic Criteria.** The original NIH neurologic criteria (118) were too narrow for routine clinical use (205). The Manchester diagnostic criteria are now most widely used and have been found to improve sensitivity without affecting specificity (225). Using these criteria, NF2 is diagnosed in any individual with one of the following criteria:

Bilateral vestibular schwannomas

First-degree relative with NF2 and

–Unilateral vestibular schwannoma *or*

–Any two of meningioma, schwannoma, glioma, neurofibroma, posterior subcapsular lenticular opacities

Unilateral vestibular schwannoma *and* any two of meningioma schwannoma, glioma, neurofibroma, and posterior subcapsular lenticular opacities

Multiple meningioma (two or more) *plus* unilateral VS *or* any two of glioma, schwannoma, neurofibroma, and posterior subcapsular lenticular opacities

**121.2.2.3 Differential Diagnosis.** With improvements in neuroimaging, the diagnosis of NF2 is now usually straightforward on clinical grounds. The main time the diagnosis is missed is in children, with no family history, who present with marked cutaneous features and a diagnosis of NF1 is considered. The most helpful features to distinguish the two conditions are the much lower number of café-au-lait spots and the absence of axillary freckling in NF2 and the finding of NF2 plaques. Another problem is that NF2 is not considered in young people presenting with single vestibular or spinal schwannomas and meningiomas. After treatment, they are not followed up and re-present when other tumors become symptomatic. Diagnosing sporadic pediatric cases early is important, as they go on to develop severe NF2 with multiple neuraxis lesions (227,228). The vestibular lesions in such cases can reach a considerable size before causing

symptoms; detection at an earlier stage offers the chance of much improved treatment outcomes.

Multiple meningiomas not associated with other tumors is a recognized, although extremely rare, autosomal dominant trait (229,230). In one family in which meningiomas and ependymomas were segregating as dominant, Pulst et al. (231) excluded linkage to markers that flanked the NF2 locus. The underlying genetic mechanism has not yet been elucidated (232). The diagnosis of familial non-NF2 meningiomas can only be made with a clear family history. In sporadic cases, the causes include NF2 mosaicism, noncontiguous spread of a single sporadic tumor, or new mutation in the as yet unidentified gene(s) responsible for non-NF2 familial meningiomas.

Autosomal dominant inheritance of spinal and peripheral schwannomas without vestibular nerve lesions is seen in schwannomatosis, which is discussed later. From a practical viewpoint, isolated cases of multiple meningiomas or schwannomas should be assessed as if they have NF2, as it is probable they have mosaic NF2. Finally in sporadic cases with no peripheral manifestations, there have been reports of other causes of cerebellopontine angle lesions being initially diagnosed as bilateral vestibular schwannoma (233).

**121.2.2.4 Natural History.** First symptoms usually develop around the age of 20 years on average, but with a range varying from infancy to the sixth decade (203,205,234). These studies showed an average delay of 7 years to diagnosis; again with a considerable range—from 5 to 66 years in the Evans study (205). Fortunately, with increased awareness and routine use of cranial MRI in investigating hearing loss, such long delays are now rare in clinical practice. Our recent clinical experience suggests the cases sometimes still missed at an early stage are children with no family history who present with NF2-related eye or skin problems or an isolated schwannoma or meningiomas.

Nearly all patients eventually develop symptoms related to the vestibular schwannomas and typically progress to bilateral deafness, although this can take many years in mild cases (235). Large vestibular schwannomas may not cause hearing loss and the patient then presents with symptoms related to brain stem compression. The larger the tumor at the time of surgery, the higher the frequency of complications such as facial nerve palsy and bulbar problems.

The number of other neuraxis tumors makes a significant contribution to disease-related morbidity and mortality. Two studies have shown that the age of death is inversely proportional to the age of diagnosis (234,236). Children presenting with symptomatic NF2 have the worst prognosis, as they have multiple neuraxis tumors in addition to bilateral vestibular schwannomas. At the other end of the spectrum are adults with just bilateral vestibular schwannomas presenting in middle age.

In the large UK study reported in 1992 (205) the mean actuarial survival was 62 years, although over

40% would be expected to have died by 50 years. In a Japanese study of 74 cases (234), the overall 5-, 10- and 20-year survival rates were 80, 60 and 28% respectively. Analysis of patients presenting after 25 years gave survival rates of 100, 87 and 62%. It is likely that future studies will show significant improvement relating to modern imaging techniques, developments in microsurgical technique and increasing recognition of the need for treatment in a specialist center.

In the most severe cases, there are so many tumors and other disease-related morbidities, such as loss of vision and neuropathies, that, despite surgery, the patient has significant neurological disability in early adulthood. For these patients, the development of possible systemic treatments discussed below will be of major benefit.

The final factor affecting mortality is treatment center. Baser et al. (236) showed that the relative risk of mortality in patients treated in one of three UK specialist centers was 0.34 compared with those treated in nonspecialist centers.

#### 121.2.2.5 Genetics.

**121.2.2.5.1 Inheritance and Mutation Rate.** NF2 is inherited as an autosomal dominant condition with almost complete penetrance by the age of 60 years (237). Approximately half the cases are sporadic, and approximately one-third of this group is somatic mosaics (238,239). Depending on the timing of the somatic mutation, mosaic NF2 patients present with either mild, generalized or more localized disease, for example, a unilateral vestibular schwannoma with one or more ipsilateral NF2-related tumors. In contrast to NF1, all studies of the clinical course of the disease in NF2 have shown strong intrafamilial correlation but marked interfamilial variation. The exception to this is affected children of a mosaic parent. Earlier studies suggesting a maternal effect on disease severity have not been confirmed (203).

**121.2.2.5.2 The NF2 Gene.** Cytogenetic studies of meningiomas showing frequent loss of chromosome 22 provided the clue to the chromosomal localization of NF2. Seizinger et al. (240) studied loss of heterozygosity of DNA for chromosome 22 markers in vestibular schwannomas from both isolated and NF2 cases. Their positive findings gave strong circumstantial evidence to the location of NF2, which was subsequently confirmed by linkage studies (241). Further studies identified flanking markers and showed no suggestion of locus heterogeneity (242,243).

The identification of a number of germline deletions in NF2 patients in this area facilitated the cloning of the disease gene by two groups (244,245). The gene spans 110kb and has 16 constitutive exons and one alternatively spliced exon. The gene encodes for a 69kDa protein called merlin or Schwannomin (commonly known as merlin).

**121.2.2.5.3 Genotype–Phenotype Correlation.** Clinical studies prior to the molecular era, led to the suggestion



of at least two types of NF2 (203). They suggested there were mild families, which they named Gardner after the first author of the paper of an illustrative family, where the main problems related to the bilateral vestibular schwannomas with few other tumors, relatively late presentation and slow progression. At the other extreme were severe cases, named Wishart, with multiple neuraxis and peripheral NF2 tumors, with a young age of presentation often from other tumors than the vestibular schwannomas. In these cases disease course was rapid with severe disability or death at a young age. There were, however, some families that overlapped the two extremes. The clinical subtypes are now rarely referred to, as clinically useful genotype–phenotype correlation has emerged.

Genotype–phenotype correlation studies have provided a molecular explanation for the interfamilial heterogeneity:

Constitutional nonsense or frameshift mutations are associated with severe disease regardless of their position in the gene (246,247). In studies of spinal involvement, truncating mutations correlate with an increased number of spinal tumors (208,209).

Missense and in-frame or large deletions are associated with mild disease (246,248). Missense mutations are associated with the mildest disease, with a lower risk of mortality (236).

Splicing mutations have been associated with various levels of severity, although disease may be milder if in the 3' end of the gene (249,250).

Mutations in the 3' half of the gene, especially exons 14–16 are associated with a lower risk of meningioma (251).

**121.2.2.5.4 Ring Chromosome 22 and Other NF2-Associated Chromosome Abnormalities.** In a small proportion of cases, NF2 develops because of a constitutional chromosome abnormality (252). These include balanced reciprocal translocations with breakpoints through or near to the NF2 gene (252,253); therefore if no mutation is found on molecular analysis, cytogenetic studies should be done. Patients with ring chromosome 22 are at risk of developing NF2 (252,254). These patients usually present in early childhood with moderate–severe developmental delay, absent expressive speech, and variable facial dysmorphism. As the ring chromosome is prone to loss during mitotic cell division, carriers of the ring may become monosomic for chromosome 22 and lose one NF2 allele, thus they are effectively NF2 mosaics and a mutation in the second allele will give rise to an NF2-related tumor. Zirn et al. (254) review the clinical picture in 10 cases, and this ranges from full blown NF2 to just multiple meningiomas. The age of symptomatic tumor diagnosis ranged from puberty to 52 years. It is therefore important that regular assessments for NF2-related problems is undertaken and continued into adulthood.

**121.2.2.6 Pathogenesis.** The NF2 gene acts as a tumor suppressor in the pathogenesis of schwannomas and other NF2-associated tumors. Somatic inactivation of both alleles also happens in sporadic schwannomas (>90%), meningiomas (50%), and ependymomas (5%) (197,255). NF2 mutations are also seen frequently in sporadic mesotheliomas and less commonly in thyroid cancer and melanoma. Animal models have also demonstrated the tumor suppressor function (256–258). NF2<sup>−/−</sup> genotypes are embryonic lethal in both mice and *Drosophila*, suggesting a vital role in embryogenesis. NF2<sup>+/−</sup> mice develop metastatic osteosarcomas, fibrosarcomas, and hepatocellular carcinomas, but not NF2-related tumors. The best NF2 models result from selective inactivation of NF2 in Schwann cells or leptomeningeal cells, which produces schwannomas and meningiomas, respectively (259). Two major isoforms of merlin exist (1 and 11); only isoform 1 is able to undergo the protein folding that enables the tumor suppressor activity (260–262).

**121.2.2.7 NF2 Protein (Merlin).** The NF2 gene sequence shows strong homology to the highly conserved protein 4.1 family. The NF2-encoded protein is most similar to moezin, ezrin, and radixin and it was named merlin for moezin–ezrin–radixin-like protein (245). The primary role of the protein 4.1 family appears to be in mediating communication between the extracellular milieu and cytoskeleton (263–265). In normal cells, merlin is expressed diffusely across several cell types including Schwann, meningeal, mesothelial, and lens cells.

Merlin is a multifunctional protein involved in integrating and regulating the extracellular cues and intracellular signaling pathways that control cell fate, shape, proliferation, survival and motility (reviewed in (266–268)). The main merlin functions identified to date involve:

Regulating cell–cell and cell–matrix adhesions and functions of the cell surface and extracellular matrix receptors such as CD44.

Stabilizing the contact inhibition of proliferation and regulating activities of several receptor tyrosine kinases including the ErbB family, platelet-derived growth factor receptor  $\beta$ , insulin-like growth factor 1 receptor, and vascular endothelial growth factor (VEGF).

Through these different roles merlin affects downstream signaling of several signaling pathways that are critical for cell growth, protein translation and cellular proliferation including the phosphoinositide-3 (PI3K) pathway, the mitogen-activated protein kinase pathway (MAPK) and mTOR complex 1 (mTORC1) pathway.

**121.2.2.8 Candidate Drugs.** The identification of the signaling pathways affected by merlin dysregulation is now translating in to preclinical and clinical trials (269). Drugs targeting the key pathways such as lapatinib (ErbB1/2 inhibitor) and Platelet Derived Growth Factor

Receptor (PDGFR) inhibitors (nilotinib, sorafenib) are in early-phase human trials. The first drug that has been shown to be effective in growth control of NF2-related vestibular schwannomas is bevacizumab (270,271). Bevacizumab is a monoclonal antibody that binds all biologically active forms of human VEGF, and VEGFs are widely expressed in vestibular schwannoma (270). In a series of 10 NF2 patients at risk of complete hearing loss, Plotkin et al. (270) reported reduction in tumor volume  $\geq 20\%$  in six of 10 patients. In four of seven evaluable patients there was significantly improved hearing. Mautner et al. (271) reported similar response in two patients. Further trials are ongoing and confirming these response rates. Recent case reports suggest that when the drug is stopped the radiographic and clinical benefit is reversed (272). Bevacizumab has had little effect on meningiomas in these cases, and as it affects wound healing, surgery can only be scheduled after the drug has cleared the system (269).

**121.2.2.9 Management.** The successful management of NF2 patients involves coordination between several different specialties, principally neurosurgeons, otolaryngologists, neurologists, ophthalmologists, geneticists and increasingly neurooncologists. Ideally patients are seen in a multidisciplinary clinic (273). Patients also need ongoing support from other health professionals, such as teachers of communication to the deaf and social workers. Treatment in a specialist center has been shown to be associated with a decrease in disease-related mortality (225). This is probably related to two aspects of care: the improved outcome of vestibular schwannoma surgery in specialized centers (269,274,275) and the expert knowledge of disease natural history, recognizing that many tumors seen on scan in NF2 will never need treatment. It is therefore important that patients are referred to such centers as soon as the diagnosis has been made.

The overall aim of NF2 care is to maintain function whenever possible. Improved microsurgical techniques and the development of cochlear/auditory brainstem implants has made this increasingly possible (269,273). The mainstay of management is at least annual clinical, audiological and radiological monitoring of tumors. The only tumors that may be treated before causing symptoms are the vestibular schwannomas, as early surgery on small tumors provides the opportunity for hearing preservation surgery or at least cochlear nerve preservation. The latter allows the opportunity for a cochlear nerve implant, which provides more useful hearing than the auditory brain stem implant. To avoid the complications of surgery, stereotactic radiation therapy can be used for the treatment of vestibular schwannomas less than 3 cm in diameter; although as for surgical outcomes, the results of treatment in NF2 are not as good as for sporadic vestibular schwannomas (276,277). There are now reports of malignant change following radiation therapy, bringing its use into question, at least for younger patients (278).

**121.2.2.10 Genetic Counseling.** Although inheritance is autosomal dominant, genetic counseling is more complicated than usual because of the high frequency of mosaicism in sporadic cases (238,239). The risks given and tissues submitted for mutation analysis will vary according to whether the consultand is the first case in the family or not (279). Evans et al. (279) provide a guideline for genetic counseling and management of at-risk individuals in NF2. In established families the risk to children is 50% and there is relatively strong intrafamilial correlation of disease severity. Molecular analysis is now able to assist the counseling process in the majority of families, providing the options of preimplantation, and prenatal and presymptomatic diagnoses.

**121.2.2.10.1 Mutation Testing.** Using sequence analysis and multiplex ligation-dependent probe amplification (MLPA) the disease causing mutation is identified in around 93% of individuals in the second or subsequent generations of NF2 families (279). As a result of mosaicism, this falls to around 60% in sporadic patients with typical NF2 (Evans 2007) and is very low in patients with an atypical presentation that suggests mosaicism (e.g. a unilateral vestibular schwannoma and ipsilateral schwannomas/meningiomas). The detection rate is also age dependent in people presenting with bilateral vestibular schwannomas; it is 86% in sporadic cases presenting under 20 years of age but this falls to 39% in those presenting with bilateral vestibular schwannomas after 40 years (279). In rare families with no mutation, DNA marker studies can be used.

In sporadic patients with no mutation found in lymphocyte DNA, mutation analysis in two or more tumors usually identifies the causative mutation. If only one tumor is available but both mutations are found, then at-risk children can be tested for both. If only loss of heterozygosity is shown, then this is still useful for counseling at-risk children. If they inherit the allele lost in the tumor they are at very low risk, because this allele is unlikely to have a disease-causing mutation (239). Tumor analysis can either be on tumor tissue taken at the time of neurosurgery or on peripheral tumors specifically removed for genetic analysis.

**121.2.2.11 Genetic Risks for First-Degree Relatives of Nonmosaic Sporadic Cases.** Parents and siblings: If the relatives do not have any symptoms it is unlikely they are affected, but mutation testing can be offered for reassurance. There has been a single case of germline mosaicism reported (203).

Children: The children will have a 50% risk.

**121.2.2.12 Risks for First-Degree Relatives of Mosaic Sporadic Cases.** Parents and siblings: Can be completely reassured, as by implication the proband has had a somatic mutation.

Children: The risk is lower than 50% and is affected by several factors; the risk is increased if the mutation is present in detectable levels in lymphocyte DNA, the parent had a young age of onset and with bilateral rather than a unilateral vestibular schwannoma, or other

combination of NF2 features (239,279). Although the risk is lower, if the child does inherit the mutation the disease is likely to be more severe. The recent UK guideline (279) provides risk levels and management plans for various mosaic presentations at different ages.

**121.2.2.13 Counseling Individuals with Possible NF2.** With increasing awareness of NF2 presentations in childhood (228,280), families may be referred for genetic counseling when their child has presented with, for example, an isolated vestibular schwannoma or meningioma or a typical NF2 retinal hamartoma. These patients should have a full clinical NF2 evaluation and mutation testing. If the results are normal then there is still a risk of a missed mutation or mosaicism; Evans et al. (281) provide follow-up guidelines depending on the clinical scenario.

**121.2.2.14 Management of At-Risk Children.** As the disease can be symptomatic in early childhood and screening is recommended, parents should know that genetic testing is available from early childhood. In our experience, many families defer the genetic testing until around the age of 10, when the at-risk individual can take part in the counseling process. The timing of genetic testing in children is an individual choice for families.

For a child at risk, the first screening test is in infancy when an ophthalmic check is recommended to exclude NF2-related problems that will affect vision. After this, unless the family has particularly severe disease, there is no need for regular review or scans, unless the child develops symptoms or the parent has concerns, until the age of 10. Some families prefer annual review with audiology from infancy. Scans are indicated before the age of 10 only if there are clinical concerns. From the age of 10, annual review with audiological assessment and a cranial MRI is recommended. There should also be a baseline spinal MRI but this only needs repetition every 3 years or if new symptoms develop (199).

In rare families where no genetic testing is available, cranial and spinal MRI at age 10 years is recommended if no tumor is identified. Evans et al. (199) recommend semiannual cranial scans in the teens, reducing to every 3 years from 20–35 years. If the patient wants to be ultra-cautious then annual audiology should be carried out between scans. The spinal scans are repeated every 5–10 years. If no tumor is found by the age of 35 years then the patient can be reassured.

Once an asymptomatic gene carrier has been identified or NF2 is diagnosed in a child with no family history, it is recommended that he or she be cared for by a specialist NF2 clinic so the chances of hearing preservation can be optimized, either through surgery on small tumors or through optimal use of brain stem/cochlear implants.

### 121.2.3 Schwannomatosis

Although not recognized as a form of neurofibromatosis by the NIH Consensus Development Conference (118),

an entity referred to as schwannomatosis is now recognized as a distinct form of neurofibromatosis (282–284). The frequency of schwannomatosis is not known, but it is less common than NF2. A proportion of cases have been shown to have germline mutations in the *SMARCB1* gene (also known as *INI1*, *SNF5* and *BAF47*), which lies proximal to NF2 on chromosome 22 (285). Mutations in the same gene are also associated with malignant rhabdoid tumors (MRTs) (286,287); although MRTs were not originally recorded in schwannomatosis, since the finding of the shared pathogenesis, several families with both MRTs and schwannomas have been reported (286–289).

**121.2.3.1 Clinical Features.** Patients with schwannomatosis develop peripheral nerve and spinal root schwannomas almost exclusively, but with no dermal tumors. These tumors often cause persistent problems with pain. Meningiomas occur rarely. There is no eye involvement and ependymomas have not been seen (282–284,290,291). Cranial nerve involvement is less common than in NF2 and nearly always involves nerves other than the vestibular nerve (292). Malignant transformation of schwannomas in schwannomatosis has been reported, although it seems to be a very rare occurrence (289,293). It is therefore important that patients have access to rapid assessment for rapidly growing tumors.

**121.2.3.2 Overlap with Mosaic NF2 in Sporadic Cases.** In the absence of a clear family history, the clinical dilemma is distinguishing schwannomatosis from mosaic NF2 (284). The appearance of the tumors is clinically and radiologically the same as in NF2. The more severe pain seen in schwannomatosis nerve lesions is a helpful clinical clue. Peripheral nerve lesions in NF2 are often only painful on pressure. Clinical assessment to exclude any signs of NF2 is essential when a diagnosis of schwannomatosis is considered and should include a thorough cutaneous and eye examination for signs of NF2 and full MRI neuraxis imaging. Molecular analysis is also available now, as discussed below. After tumor analysis, Smith et al. (292) found four of nine (40%) of their sporadic cases with multiple schwannomas had mosaic NF2.

**121.2.3.3 Risk of Malignant Rhabdoid Tumors?** The identification of *SMARCB1* mutations in some schwannomatosis patients raised the question as to whether there was also a risk of MRTs. This is because germline *SMARCB1* mutations have also been found in the extremely rare families with a predisposition to these tumors (286,288). Rhabdoid tumors are aggressive pediatric malignancies with a poor prognosis that develop in infancy and early childhood. They most commonly occur in the brain (where they are called atypical teratoid/rhabdoid tumors), kidney, and soft tissue.

At the cellular level, the tumors result from biallelic *SMARCB1* inactivation, in the majority of cases due to somatic mutation. Familial cases have been reported with germline mutations. Eaton et al. (Eaton 2011)

found germline mutations in 35 of 100 cases with MRT. In 22 of 35 cases parental samples were available and in 7 of 22 of these cases one parent had the mutation. In a further two families with affected siblings, parental analysis was normal, demonstrating germline mosaicism. Two of the seven parents with mutations had one or more schwannomas and a positive family history of schwannomas; one of these families has been reported previously (Swensen 2009). A third reported family was ascertained through the mother who had sarcomatous change in a schwannoma (289). A son had died of cerebellar rhabdoid tumor 6 years earlier. During the mother's genetic counseling, a younger son developed a cerebellar rhabdoid tumor. In the Eaton et al. (286) series, three of seven parents with mutations had no MRT or schwannomas and one an ill-defined CNS lesion not suggestive of MRT. These findings demonstrate nonpenetrance for *SMARCB1* in MRT families.

The emerging literature suggests that *SMARCB1* immunohistochemistry may contribute in assessing cases. The majority of schwannomas in schwannomatosis and NF2 show a mosaic pattern of *SMARCB1* staining (294). In this study no correlation was available with germline *SMARCB1* status. In MRT families the tumors show complete loss of *SMARCB1* staining. In one of the three families with both schwannomas and MRT, Carter et al. (289) reported that both the malignant peripheral nerve tumor and schwannomas showed complete loss of *SMARCB1*. Furthermore, all but one of the schwannomas studied were neuroblastoma-like schwannomas, a very rare variant.

**121.2.3.4 Diagnostic Criteria.** MacCollin and colleagues (284) have proposed diagnostic criteria. Definite schwannomatosis would be diagnosed in an individual at least 30 years of age with two or more nonintra-dermal schwannomas (at least one histologically confirmed), no evidence of vestibular schwannoma by high-quality MRI, and no known NF2 mutation. The diagnosis would also be made on pathologic confirmation of a schwannoma in a first-degree relative of an individual known to be affected. Criteria were also proposed for possible diagnosis in individuals under age 30 who fulfill these criteria but may be too young to manifest vestibular schwannoma, as well as in older individuals who have no symptoms of vestibular tumor but have not had imaging studies. A segmental form was proposed in which the features are limited to one limb or five or fewer contiguous spinal segments.

Smith et al. (292) have recently reported two cases with a clinical diagnosis of schwannomatosis, both of whom had neither NF2 nor *SMARCB1* mutations on testing. They did, however, have unilateral vestibular schwannomas and in one case, mutation analysis on tumors was compatible with schwannomatosis rather than NF2 (see below). They also reported a family with an *SMARCB1* mutation, in whom the proband had previously been diagnosed with NF2 after presenting with

spinal schwannomas and was found to have a right cerebellopontine angle lesion that was thought to be a vestibular schwannoma. Radiological review suggested that the tumor was in fact on the jugular nerve. Smith et al. (292) concluded that vestibular schwannomas may rarely occur in schwannomatosis and highlight the importance of expert radiological review of cerebellopontine angle lesions to determine the cranial nerve on which the schwannoma(s) has arisen.

**121.2.3.5 Genetics.** The majority of cases of schwannomatosis are sporadic, with some patients having localized lesions suggestive of mosaicism. The risk to offspring of sporadic cases is much less than 50%; MacCollin et al. (284) suggest an empirical risk of less than 15%. In familial cases inheritance is dominant but expression is variable and incomplete penetrance is recorded (284). Germline mosaicism of an *SMARCB1* mutation in schwannomatosis has been reported (295).

**121.2.3.6 Pathogenesis.** The genetic mechanisms underlying schwannomatosis are gradually being elucidated. The gene has been localized to chromosome 22 proximal to NF2 and mutations in the *SMARCB1* tumor suppressor gene were first reported in 2007 (285). Combining their own and other published series, Smith et al. reported that in familial schwannomatosis cases 45% (22 of 49) have *SMARCB1* mutations but only 7% (14 of 190) of sporadic cases have these mutations (292,296–299). Tumor analysis has shown a complex mechanism of tumorigenesis in schwannomatosis, which requires somatic mutation in both copies of the NF2 gene as well as in *SMARCB1* (Hadfield 2008; Sestini 2008). The majority of reported cases/families with *SMARCB1* mutations have just had schwannomas. As discussed above, there are families with both schwannomas and MRTs reported. Two schwannomatosis families with meningiomas have also been shown to have mutations (290,291). *SMARCB1* encodes a core component of the SW1/hSNF chromatin remodeling complex, which regulates expression of around 5% of genes across the genome (reviewed by Reisman et al. (300)).

**121.2.3.7 Genotype–Phenotype Correlation.** The reasons that mutations in the same gene cause schwannomatosis in some cases but MRT in others, with little overlap, are emerging. Studies to date show that the majority of mutations in schwannomatosis-only families are nontruncating, which is in marked contrast to MRT, where the majority are truncating mutations or deletions or duplications (288,292). It has been proposed that there is an early developmental window in which a mutation carrier is predisposed to MRT, and those that survive may have a later predisposition to schwannomas. It seems likely that different mutations confer different risks of the two tumors. Many schwannomatosis mutations may be hypomorphic, conferring a very low risk of MRT but resulting in multiple schwannomas later in life.

**121.2.3.8 Genetic Counseling and Genetic Testing.** Our knowledge of schwannomatosis is increasing



rapidly. It is therefore essential that up to date information is used when counseling families. Although inheritance is dominant in familial cases, there is well-recorded nonpenetrance. With regard to molecular testing at the current time, if the clinical picture is very typical of schwannomatosis (e.g. family history and no vestibular schwannomas, or schwannomas and meningiomas or MRT in family members) direct *SMARCB1* testing is appropriate.

In sporadic cases, prior to genetic testing it is essential to check that a full workup to exclude NF2 has been done. If tumor tissue is available from two or more tumors it is then usually more helpful to proceed to tumor mutation analysis of *NF2* and *SMARCB1*. In mosaic NF2 each separate tumor will share one mutation in common, whereas in schwannomatosis the *NF2* mutations are different in each tumor. If no tumor is available then *NF2* and *SMARCB1* analysis should be done. This will be normal in the majority of cases, and the empirical risk to children is much reduced.

#### 121.2.4 Tuberous Sclerosis Complex

Tuberous sclerosis complex (TSC) is the now preferred name for the condition previously known as tuberous sclerosis. The addition of the term complex emphasizes the multisystem involvement and variable expression of the disease. In the older literature, it is often referred to as Bournville disease, giving eponymous credit to Bournville (301), who gave the first detailed report of the neurologic symptoms and gross cerebral pathology of TSC. Bournville coined the term tuberous sclerosis of the cerebral convolutions to describe the brain of one of his cases because its nodular appearance resembled tubers. Another old and unfortunate term for TSC, used mainly in the United Kingdom, was epiloia, reflecting the combination of epilepsy with anopia, or mindlessness.

TSC was previously considered to be relatively rare. As more mildly affected cases have been diagnosed, estimates of prevalence/incidence have increased. In a UK study, taking account of underascertainment, O'Callaghan and associates (302) reported a prevalence of 1 in 12,500, whereas a study by Devlin et al. calculated a prevalence in Ireland of 1 in 24,956 (303). The birth incidence may be as high as 1 in 6000 (304,305).

**121.2.4.1 Clinical Features.** The clinical features of TSC involve several body systems and develop at different ages (Table 121-4). This means that, as in NF1, the assessments should vary according to the age of the individual. For many years, it was believed that the triad of features identified by Vogt (306) of mental retardation, epilepsy, and adenoma sebaceum had to be present for the diagnosis of TSC; however, as this triad is only present in approximately 30% of cases, many patients were undiagnosed.

##### 121.2.4.2 Cutaneous Features.

**121.2.4.2.1 Hypomelanotic Macules.** Hypomelanotic macules are the first cutaneous feature to become

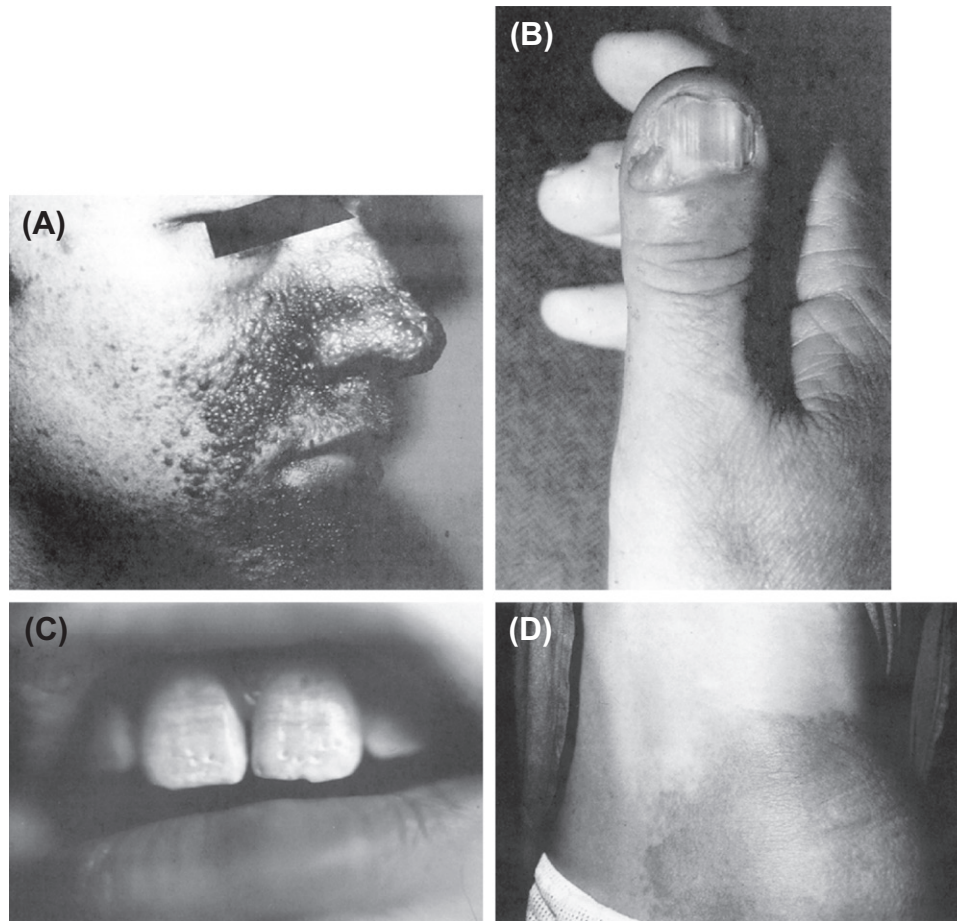
obvious; they are present from birth or develop during early childhood, and may increase in number during childhood (307). In the majority of patients, the macules are easily visualized under normal lighting. There may be between 1 and 100 hypopigmented patches, present in 80–90% of patients (308–310). They are usually easily seen in room light, but the Wood's lamp may help to visualize hypopigmented macules if they are not seen with room lighting. Fitzpatrick and colleagues (311) described three main types of hypopigmented lesions in TSC:

- (1) The type seen most frequently has a rounded shape like a thumbprint, 0.5–2 cm in diameter.
- (2) The second type has a lancinate-ovate shape, 1–12 cm in length, said to resemble the leaf of a mountain ash tree (and may be known as an “ash-leaf spot”).
- (3) The least frequent type consists of patches of skin containing multiple areas of small (1–3 mm) hypopigmented lesions (“confetti” lesions).

Hypopigmentation in TSC can also involve the hair of the scalp (poliosis), eyebrows, and eyelashes. Caution must be exercised when hypopigmented patches are found as the only feature in childhood, as 2 or 3 per 1000 normal neonates also have them (312,313).

**121.2.4.2.2 Facial Angiofibromas (“Adenoma Sebaceum”).** Facial angiofibromas (Figure 121-5) are present in 80–90% of older TSC patients (308,310). They begin to develop from around the age of 5 years to puberty, when they often become more numerous and prominent. The inaccurate name of adenoma sebaceum derives from the fact that there are a large number of sebaceous glands in the facial area where these lesions develop. Histologically, the angiofibroma is a hamartoma of dermal connective tissue and blood vessels. Typically red or pink nodular lesions appear distributed symmetrically on both sides of the face. They often involve the whole nose, spread onto the cheeks (particularly the nasolabial folds), and may also occur on the chin. These lesions can present a considerable cosmetic burden. A variety of options are available to remove these lesions, including shave excision, cryosurgery, desiccation, dermabrasion, and carbon and argon laser (308,309). The use of medications to treat angiofibroma and other skin lesions will be described later in this section.

**121.2.4.2.3 Shagreen Patch.** Shagreen patches (Figure 121-5) usually develop in later childhood. Webb and colleagues (308) found 41% of patients to have these patches and Chopra reported a frequency of 65% (310). A shagreen patch appears as a slightly elevated, roughened patch of skin, yellowish brown or pink in color, with the texture of pigskin or orange peel. They are firm or rubbery in consistency and vary in size from a few millimeters to 10 cm or more. They are usually found on the dorsal surface of the trunk, particularly in the thoracolumbar region, but may occur in the dorsal cervical area. They rarely cross the midline and are rarely seen on the ventral aspect of the trunk.



**FIGURE 121-5** Tuberous sclerosis complex. (A) Facial angiofibromas. (B) Periungual fibroma. (C) Dental pits. (D) Shagreen patch.

**121.2.4.2.4 Forehead Plaque.** Forehead plaques often develop in early childhood and may grow slowly before puberty or involute at an older age. They appear as raised soft patches of skin and can be red, yellow, or flesh-colored. They were present in 26% of cases in the Osborne et al. series (304).

**121.2.4.2.5 Ungual Fibromas.** Ungual fibromas usually develop in adult life (Figure 121-5), with a reported frequency up to 88% (308). The lesions may be peri- or subungual and appear as dull red or flesh-colored papules or nodules. The periungual lesion is a fibrous nodule arising from the unguinal groove of fingers or toes. It grows on the nail plane in the same direction as the nail and may be 1 mm or more in diameter. The subungual fibroma grows under the free edge of the nail as a slightly raised nodular tumor, which lifts the nail off the nail bed. Some lesions can lead to longitudinal splitting of the nail. Ungual fibromas are found more often on the toes than on the fingers and are more common in females.

**121.2.4.3 Oral Findings.** Pitted enamel hypoplasia (Figure 121-5) has been found in both adults and children with TSC (314), but is never symptomatic. Sampson's group found no pits in deciduous teeth, but in the permanent teeth of TSC patients they found pits easily visible to the naked eye in 48% of patients, compared

with 5 of 563 controls (0.88%) (314). Sparling et al. reported enamel pits in 97% of 56 patients (315). Some investigators have used techniques that allow the detection of microscopic pits; although microscopic pits are found in a higher proportion of TSC patients, they are also found in more of the general population. Sparling et al. (315) also noted fibromas in the oral cavity in 69% of their patients, not only on the gingiva but also on the tongue, palate, and buccal surfaces.

#### **121.2.4.4 Central Nervous System Involvement.**

**121.2.4.4.1 Pathology.** The two characteristic features of CNS involvement in TSC are the cortical tubers and subependymal nodules. The tubers are focal lesions that occur predominantly at the gray–white matter interface. Macroscopically they are paler and firmer than the rest of the brain, and microscopically they show loss of normal cortical cytoarchitecture with occurrence of abnormal neurons and glial cells. The neurons may be gigantic and the glial cells may also be enlarged and morphologically abnormal (316,317). The cortical tubers disrupt and displace normal cortical architecture, and their number or location roughly correlate with the severity of the seizures and mental retardation in TSC (318,319). They are present in the majority of patients, being found in 95% in one series (320), and are best seen by MRI

using the FLAIR sequence (321). White matter lesions, consisting of radial bands, are best visualized with T1 spin echo with magnetization transfer contrast medium pulse on resonance (T1-SE/MTC) (321).

The subependymal nodules are found lining the third ventricle. They are composed of large irregular cells that are more densely aggregated and more uniform in appearance than cortical tubers. In the Braffman et al. series (320), 95% of the patients had subependymal nodules on MRI. Some of the nodules grow larger than 3 cm in diameter and these larger lesions are often referred to as subependymal giant cell astrocytomas (SEGAs). O'Callaghan et al. (322) found SEGAs measuring at least 1 cm in 17% of 41 patients and symptomatic lesions in 10 of 149 patients. These lesions can grow (323) and obstruct the foramen of Monro, leading to hydrocephalus (324). The average age of presentation of these tumors is 13 (range, 1–31 years) (325).

**121.2.4.4.2 Epilepsy.** In the UK population-based survey (326), 78% of individuals (102 of 131) had seizures; 69% of those had seizures in the first year of life and in 4% the seizures began in adulthood. About 50% of patients presenting with infantile spasms, with the characteristic electroencephalographic (EEG) finding of hypsarrhythmia, will turn out to have TSC. In the Webb et al. survey (326), half the TSC patients presented with a variety of other forms of seizure (partial, tonic-clonic, myoclonic, and febrile seizures). Seizure control was poor in 25% of patients in this series (defined as daily or weekly seizures). Resistant seizures (defined as more than one per year despite treatment) were more common following infantile spasms and myoclonic seizures and when onset of seizures occurred under 2 years of age. Males were three times more likely to have weekly or daily seizures resistant to treatment. Chu-Shore et al. (327) studied the natural history of seizures in 291 patients. A history of seizure was found in 85.2% and infantile spasms in 37.8%; multiple seizure types were seen in 54.1%. Seizures began in the first year of life in 63.2% and 12% of adults had the first seizure in adulthood. Patients with infantile spasms have the highest risk of intellectual disability (328) and may benefit from treatment with vigabatrin (328–331).

**121.2.4.4.3 Learning Disability and Behavior Disorders.** The level of intellectual disability in TSC is much more severe than that seen in NF1; 53% of individuals in the Webb and colleagues survey (326) had learning disability, 59 of these 68 patients (87%) required at least some supervision of daily living, 65% had little or no language, and 63% needed daily help with feeding, dressing, or toileting. Winterkorn et al. (332) found normal IQs in 57% of 107 patients.

Various studies have shown that all TSC patients with intellectual involvement have had seizures at some stage (326,333). The converse is not true, and in the Webb series 31% of patients with epilepsy had a normal intellect (326). Prognosis for intellectual outcome is worse

with an earlier onset of seizures and the occurrence of infantile spasms (332) (see above). Intellectual disability is also associated with the type of seizure and the level of seizure control (331,334). Behavior disorders, including attention deficit disorder, are a significant problem in TSC; 20–50% of the children show autistic features (335–338). Adults have an increased frequency of psychiatric disorders, including interpersonal sensitivity, psychoticism, depression, and obsessive-compulsive symptoms (339), as well as sleep disturbance (340).

**121.2.4.5 Renal Involvement.** Two characteristic renal lesions are seen in TSC: angiomyolipomas and renal cysts. An individual patient may have one or both of these lesions or be unaffected (341,342). They are usually multiple and bilateral; most are asymptomatic. There may also be a slightly increased frequency of renal cell carcinoma (343,344).

Angiomyolipomas were found in 67% of reviewed autopsy cases when kidney examination was specifically mentioned (345) and in 49% of patients in a clinical study (341). They were found more frequently in older patients—only 8% of children aged 5 years or younger at autopsy had them, compared with 93% of those older than 10 years. In a population-based study of renal disease in the United Kingdom (346), 6 of 131 patients (4.6%) had symptomatic renal angiomyolipomas. They had presented because of abdominal pain with or without hematuria, and a renal mass was then found on examination. In the same study, a subpopulation of 21 patients had renal ultrasound; two of these patients were known to have symptomatic angiomyolipoma, and ultrasound scanning detected a further five lesions, giving an overall frequency of 33%. The lesions were more frequent in females (45%) than in males (20%). Similarly, most of the patients in the study by Cook et al. (341) were asymptomatic, with hematuria, abdominal pain, and urinary tract infection being the most common clinical problems in symptomatic individuals. Rakowski et al. (347) reported renal lesions in 96 of 167 patients, of which 85.4% were angiomyolipomas. Hemorrhage occurred in six patients. Conservative management and monitoring are appropriate for most patients (348); symptomatic tumors can be treated by embolization, radio-frequency ablation, or partial nephrectomy (349–354) and may also respond to medical therapy (see below).

Renal cysts in TSC can be few and asymptomatic or numerous and macroscopically similar to adult polycystic kidney disease (345). The basis for development of multiple renal cysts appears to be codeletion of the *TSC2* and *PKD1* genes on chromosome 16 (355). Cook and associates (341) found renal cysts in 32% of patients with TSC and Rakowski et al. (347) reported cysts in 44.8% of the patients who had renal lesions. In the UK population-based study (346), 2 of 131 patients (1.5%) had polycystic kidney disease; both presented in early childhood with abdominal masses, one child also had hematuria, and both were hypertensive. None of the



cases in the UK population study had renal carcinoma, whereas Rakowski et al. (347) reported renal cell carcinoma in 4 of 167 patients; the overall risk in the TSC population appears to be small. An increased relative risk is suggested, however, by the fact that reported cases of renal carcinoma in TSC have occurred at a young age and the cancers have been multiple and bilateral in several patients (343,345), although a meta-analysis by Tello and coworkers (344) did not reveal an increased risk of renal cell carcinoma in association with TSC.

**121.2.4.6 Cardiac Rhabdomyomas.** On echocardiography, 50% of children with TSC are found to have cardiac rhabdomyomas (356). These lesions tend to be multiple, and their size and number decrease with age (357). Most lesions are asymptomatic. Those that will cause problems tend to present in infancy with heart failure (either due to outflow obstruction or because of replacement of normal myocardium with hamartoma), or arrhythmias (typically preexcitation syndromes) (358). In the UK population-based study, Webb et al. (326) identified only 2 of 131 living patients (1.5%) who had had symptomatic rhabdomyomas in early childhood. Cardiac tumors have been used as a basis of prenatal diagnosis of TSC (359,360) and are often the first presenting sign in a newborn or a young child (361,362).

**121.2.4.7 Ophthalmic Features.** The ophthalmic features of TSC are usually asymptomatic. Approximately half (68 of 139) of the patients in the Mayo Clinic series (363) had retinal hamartomas. There were three types of lesions: (1) relatively flat with a smooth surface and round or oval with indistinct boundaries, salmon to salmon-gray color, present in 28% of patients; (2) classical, relatively easily seen retinal astrocytomas, elevated multinodular lesions that resemble mulberries and are calcified, present in 24% of patients; and (3) transitional in appearance between the other two kinds, present in 5.7% of patients. The retinal hamartomas in TSC rarely cause significant visual problems (364). In addition to the hamartomas, some patients also show pigmentary lesions of the retina; 13% of the patients in the Mayo Clinic series had pigmentary abnormalities, usually in the form of depigmented patches (363).

**121.2.4.8 Other Lesions.** TSC can affect virtually any part of the body except perhaps the skeletal muscle. Apart from the disease features already discussed, the only other lesions that caused symptoms in the UK population-based series (326) were pulmonary involvement and colonic angiodysplasia, both occurring in 1 of 131 cases (0.8%).

Pulmonary involvement in TSC is five times more common in girls and can present with respiratory failure, hemoptysis, and/or spontaneous pneumothorax. The characteristic pathology is lymphangioleiomyomatosis (LAM). Symptoms seldom occur before the third or fourth decade (365–367), but the prognosis is poor once severe pulmonary involvement has been demonstrated. LAM tends to occur in association with

renal angiomyolipoma and may represent a metastatic spread of the renal lesion (368). The tumors are estrogen sensitive, possibly explaining their female predilection. Subclinical LAM is found in a high proportion of women with TSC if they are imaged with high-resolution CT (369).

Microhamartomatous rectal polyps were found on rectal biopsy in 14 of the 18 patients (78%) examined by Gould (370). The patients studied were intellectually impaired but, as far as could be ascertained, had no gastrointestinal symptomatology.

Asymptomatic liver hamartomas were found in 23.5% of children examined by abdominal ultrasound, more often in girls than boys (ratio 5:1) (371). Their incidence increased with age, reaching 45% in children over the age of 10. The lesions are presumed to be angiomyolipomas. Black et al. (372) reported angiomyolipomas, cysts, and other lesions in 30% of 205 patients, but none had clinical symptoms because of their lesions.

**121.2.4.9 Diagnostic Criteria and Differential Diagnosis.** The diagnosis of TSC is straightforward when children present with epilepsy and are found to have characteristic signs on cutaneous examination and neuroimaging. Not all patients with TSC present in this manner, however, and difficulties arise because of the diverse phenotypic expression and variation in age of onset of disease features. Difficulties in diagnosis have led to the evolution of detailed diagnostic criteria over the past two decades (373,374). The clinical features of TSC are divided into major and minor features. Definite diagnosis requires either two major features or one major feature with two minor features. Probable TSC results when one major feature and one minor feature are present. The occurrence of either one major feature or two or more minor features raises suspicion of possible TSC, but does not establish the diagnosis.

- (1) Major features: facial angiofibromas or forehead plaque, nontraumatic ungual or periungual fibroma, hypomelanotic macules (at least 3), shagreen patch, multiple retinal nodular hamartomas, cortical tuber, subependymal nodule, SEGA, cardiac rhabdomyoma (single or multiple), lymphangioleiomyomatosis, renal angiomyolipoma.
- (2) Minor features: multiple pits in dental enamel, hamartomatous rectal polyps, bone cysts, cerebral white matter radial migration lines, gingival fibromas, non-renal hamartoma, retinal achromic patch, “confetti” skin lesions, multiple renal cysts.

If cortical dysplasia and white matter migration tracts occur together, they should be counted as only a single feature; if lymphangioleiomyomatosis and renal angiomyolipomas are both present, one other feature of TSC should be present before a diagnosis is established.

In terms of differential diagnosis, there is no disease that has close phenotypic overlap with TSC; we do, however, see patients with misdiagnosis of specific disease



features—for example, early facial angiofibromas are often misdiagnosed as acne. The most difficult issues in relation to the diagnosis of TSC are in the interpretation of the finding of isolated disease features in individual patients.

**121.2.4.10 Natural History.** TSC exhibits a wide range of variable expressivity. Diagnosis may be established at birth, or even prenatally, or may be delayed until well into adulthood. Some adults learn of their own diagnosis when the condition is diagnosed in a child. Morbidity and mortality in TSC are dictated by the presence or absence of the various features of the disease and their severity. In the UK population-based study, Webb and colleagues (326) found the major cause of morbidity to be epilepsy, as discussed earlier. Shepherd and Gomez (343) studied the causes of death in the Mayo Clinic cohort: 49 of a cohort of 355 patients had died; in 40 of 49 the cause was related to TSC. The most common cause of death was renal disease, occurring in 11 of 49 patients, all of whom were more than 10 years of age. The frequency of renal death increased with age. Seven patients died of renal failure, two of bleeding angiomyolipomas, and two of renal cell carcinoma. The next most frequent cause of death was SEGAs in 10 patients; death was due to the tumor in 6 cases and related to treatment in 4 cases. These tumors occurred most frequently in the 10- to 19-year-old group. Four patients died from lymphangio-myomatosis of the lung (three female, one male), all of whom were older than 40 years. A total of 13 patients with severe mental handicap died at different ages, with the cause of death listed as status epilepticus in nine and bronchopneumonia in four. The final two patients died of cardiovascular causes in early childhood, one of multiple cardiac rhabdomyomas at 3 days and another of rupture of thoracic aortic aneurysm at 3 years.

**121.2.4.11 Genetics.** TSC follows autosomal dominant inheritance with variable expression both within and between families. Between 50% and 75% of all cases are sporadic, and the mutation rate is in the about  $2.5 \times 10^{-5}$  per generation (304). Sampson et al. (375) found no significant parental age effect for the new mutations. Because of the disease-associated morbidity, large families with TSC are uncommon, but within those studied there has been no evidence of nonpenetrance. Germline mosaicism has been demonstrated, as has somatic mosaicism in instances in which a parent shows only minimal clinical signs of the disorder (376).

**121.2.4.12 Pathogenesis.** Before molecular genetic analysis of TSC, there had been no real insights into the pathogenesis of the disease. The nature of the lesions suggests that a tumor suppressor or “two-hit” model would be a reasonable assumption. Genetic linkage studies indicated the existence of two distinct TSC loci, one on chromosome 9 (377) designated *TSC1* and the other on chromosome 16 (378) designated *TSC2*. *TSC1* was cloned by van Sleight et al. (379). The gene is located at 9q34 and encodes a protein of 1164 amino acid residues referred to as hamartin. *TSC2* is located at

16p13 and encodes a protein of 1784 amino acid residues, referred to as tuberin (380). Tuberin and hamartin are both expressed in neurons and interact with one another (381,382), suggesting that they act together in the same cellular pathway. Tuberin shows a region of homology with a GAP domain and functions as a negative regulator of the Ras-family GTPase Rheb, which in turn is a positive regulator of mTOR (383,384). Loss of tuberin GAP activity leads to high levels of Rheb-GTP, activation of mTOR, and consequent inhibition of S6K and activation of 4EBP1, two proteins involved in the control of ribosome function (385) and protein biosynthesis. Tuberin thereby functions in the Akt/PI3 kinase pathway, which regulates cell growth in situations of nutrient deprivation.

Support for a tumor suppressor model comes from the study of loss of heterozygosity for the *TSC1* and *TSC2* regions in various hamartomas from TSC patients (386–391), and also by the finding that rat *TSC2* is mutated in the Ecker rat, which manifests hereditary renal cell carcinoma (392). Activation of mTOR has been shown in some tubers (393), compatible with effects of loss of Rheb inhibition by tuberin. Loss of heterozygosity or small point mutations have not been found in most tubers (390,394), but Crino et al. (395) did identify biallelic mutations in giant cells microdissected from tubers. This suggests that tubers arise from interactions between cells with biallelic mutations and surrounding haploinsufficient cells.

**121.2.4.13 Genotype–Phenotype Correlation.** Mutations are widely distributed across *TSC1* and *TSC2*, with most leading to lack of expression of the respective gene products (396,397), although some missense mutations have been identified (398). The detection rate in 325 patients in the study of Au et al. (397) was 72% in de novo cases and 77% in familial cases. Kozlowski et al. (399) found large deletions in 0.5% of patients with *TSC1* mutation and 6% of those with *TSC2* mutation. Somatic mosaicism accounts for some individuals with undetected mutations, but so far has not been detected in all (400). Although phenotypes may vary even within a family (401), it appears that phenotypic effects may on average be milder for patients with *TSC1* rather than *TSC2* mutations (397). Analysis of deletions of the *TSC2* locus has identified one specific correlation to date. As we have discussed, severe polycystic kidney disease, presenting in early childhood, is a rare manifestation of TSC. Brook-Carter et al. (402) studied six sporadic patients with this TSC phenotype and found that all had large deletions disrupting both *TSC2* and the nearby polycystic kidney disease gene *PKD1*. Disruption of *PKD1* was not found in other TSC patients with less-severe cystic involvement, indicating that renal cysts in TSC can arise by at least two different mechanisms.

#### **121.2.4.14 Management/Treatment.**

**121.2.4.14.1 Affected Individuals.** In the past, the management of affected individuals with TSC was dictated by the problems caused by the condition; many

patients were under the care of pediatric and adult neurologists because of their epilepsy. As there are many possible manifestations of the disease, it is advisable for all children with TSC to be reviewed at least annually, and there may be a role for multidisciplinary TSC clinics. Adults with ongoing problems will also require regular review.

Recommendations for screening were proposed by the NIH Consensus Development Conference (373), and evidence-based guidelines have also been proposed (403). Brain MRI was recommended by the NIH panel every 1–3 years in children, with a reduction in frequency of monitoring in adults. Renal imaging by ultrasound, CT, or MRI was recommended every 1–3 years in adults. Children should be monitored closely for development (404). Other studies, such as EEG, electrocardiography or echocardiography, dermatologic screening, and pulmonary CT would be performed as clinically indicated.

The discovery of the role of dysregulation of mTOR in the pathogenesis of lesions associated with TSC has suggested the possibility of treatment with drugs that inhibit mTOR activity. This has been borne out in preclinical studies (405,406) and early clinical studies showed regression of SEGAs (407) and renal angiomyolipomas (408). Bissler et al. (409) conducted an open-label trial in 25 patients with renal angiomyolipomas. After 12 months of therapy, the mean volume decreased to approximately 53% of baseline, but regrowth occurred to a mean volume of approximately 86% of baseline; 5 of 20 patients who were evaluated at 24 months had a 30% or greater persistent reduction in volume of angiomyolipomas. Patients with LAM experienced an improvement of pulmonary function while on treatment. Krueger et al. (410) conducted an open-label trial of the mTOR inhibitor everolimus in 28 children aged 3 years or older with SEGAs. At least a 30% reduction in size occurred in 21 patients by 6 months of therapy. An improvement in seizure control was also noted. Similar results were reported by Curran (411). Dabora et al. (412) noted regression of angiomyolipomas of the kidney or liver as well as SEGAs in adults treated with sirolimus in a phase 2 trial. Either oral or topical rapamycin has been reported to result in improvement in angiofibroma (413–416). Everolimus has now been approved by the Food and Drug Administration in the United States for treatment of symptomatic SEGAs and clinical trials are ongoing to assess the effectiveness in the treatment of other TSC lesions. Side effects of mTOR inhibitors include oral ulcers, increased susceptibility to infection, hypercholesterolemia and hyperlipidemia, and, in rare cases, pneumonitis.

**121.2.4.14.2 Investigation of Relatives of Apparently Isolated Cases.** The role of the clinical geneticist in TSC families is usually in the investigation of family members of a proband. Very occasionally it becomes obvious during the clinic assessment that one of the parents has mild undiagnosed TSC, but more often the proband will be an apparently isolated case. Clinical evaluation

of parents should include fundoscopic examination, brain MRI, renal imaging, and dermatologic screening as part of the evaluation of asymptomatic relatives. Even when both parents are apparently unaffected, there is a small but significant risk of germline mosaicism. Clinical studies have shown the risk to lie between 1% and 6% (375,417,418). Prenatal diagnosis can be achieved in families in which the mutation has been identified. It has also been accomplished using ultrasound to detect cardiac rhabdomyoma (419) or MRI to detect subependymal nodules (420,421).

**121.2.4.15 Molecular Genetic Testing.** Molecular genetic testing is possible, although complicated by the fact that two distinct genes must be tested, with a wide diversity of possible mutations in either gene (422,423). Testing may be helpful to corroborate the diagnosis in cases of uncertainty or for prenatal testing and may reveal the presence of a gene mutation in advance of clinical signs (424).

## 121.2.5 von Hippel–Lindau Disease

VHL disease is the third most frequent phakomatosis, with a disease prevalence in the range of 1 in 40,000–53,000 (425,426). Maher and colleagues (425) estimated the birth incidence to be 1 in 36,000 live births. VHL is different from the other phakomatoses in that there is no cutaneous involvement, but there is multiple system involvement of the eye, CNS, pancreas, adrenal gland, kidney, and epididymis. VHL's eponymous name originates from the contributions made by Eugene von Hippel (427) and Arvid Lindau (428). von Hippel studied the retinal lesions, concluded they were hemangioblastomas, and coined the term angiomatosis retinae. Lindau, in a study of cerebellar cysts, concluded that most are associated with an angio-blastic neural tumor often with hemangioblastomas elsewhere in the CNS or retina, or renal cysts. van der Hoeve (2) first used the term VHL disease to describe cases with both angiomatosis retinae and cerebellar hemangioblastomas in his Doyne Memorial lecture. We briefly discuss the different disease features of VHL, its age of presentation, and family management.

**121.2.5.1 Clinical Features.** The frequency and age of presentation of the disease features are shown in Table 121-5 (429,430). Approximately 8% of patients will have only one disease manifestation (426,430).

**121.2.5.2 Retinal Angiomas (Hemangioblastomas).** Although the retinal lesions in VHL are hemangioblastomas, they are referred to as retinal angiomas in the ophthalmology literature. All patients with multiple retinal hemangioblastomas have VHL disease by definition. Isolated lesions can occur in the general population. Retinal hemangioblastomas are the most common presenting feature of VHL disease and are multiple and bilateral in about one half of the cases (431). In the study by Maher et al. (430), they were present in 89 of 152 cases (59%), and in a large literature review (432), in

317 of 554 cases (57%). Retinal hemangioblastomas have rarely been reported in early childhood and in the ninth decade of life (433), but the usual age of presentation is in the early twenties (431). In the Maher series (430), 54 of 89 patients had symptomatic retinal lesions that presented at a mean age of 24.5 years (range, 4–46 years) and the remaining 35 were asymptomatic, with a mean age of detection of 27 years (range, 7–68 years).

The typical direct ophthalmoscopic appearance of a retinal hemangioblastoma is that of a dilated artery

leading from the disk to a peripheral tumor with an engorged vein. Indirect ophthalmoscopy is usually needed to detect smaller lesions at an early stage, when they appear similar to diabetic microaneurysms but lie peripherally between large arterial and venous trunks (431,434). Screening for the retinal manifestations of VHL consists of direct ophthalmoscopy through dilated pupils as a minimum; indirect ophthalmoscopy is more sensitive for smaller lesions.

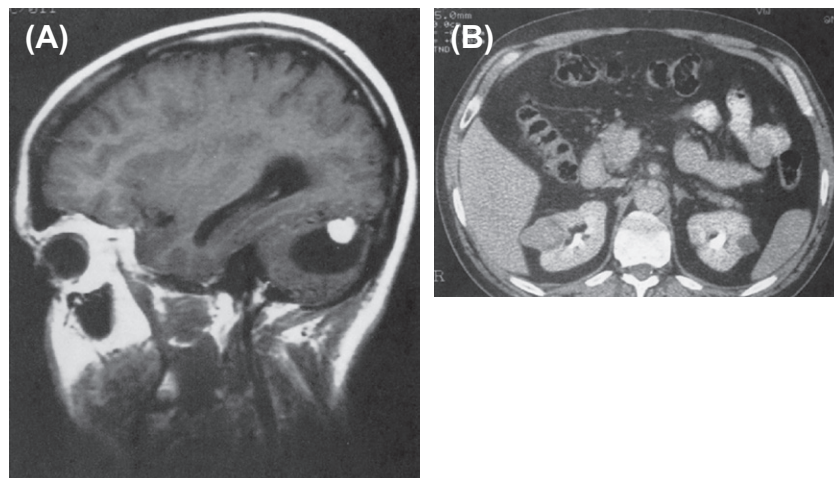
Small peripheral retinal lesions are usually asymptomatic, but if they enlarge or are located centrally, they can cause profound visual loss. Arteriovenous shunting and leaky capillaries lead to exudation of fluid, and hemorrhage can result in a number of complications, including retinal detachment, macular edema, and glaucoma. The cumulative risk of visual loss from retinal angiomas was estimated at 35% in all gene carriers and 55% in patients with retinal angiomas at age 50 years (434). Management is therefore directed toward detecting asymptomatic lesions to prevent complications. Most angiomas respond well to laser photocoagulation or cryotherapy. Optic disk lesions are difficult as there is a risk of nerve damage with treatment. They are therefore usually kept under surveillance unless there is evidence of progression. Antiangiogenic therapy may be an option for lesions where conventional therapy is contraindicated (435,436).

**121.2.5.3 Central Nervous System Hemangioblastomas.** CNS hemangioblastomas develop most commonly in the cerebellum (59% of VHL cases) (Figure 121-6), but are also found in the spinal cord (13%), brain stem (4%), or elsewhere in the CNS (less than 1% of cases) (429,437). All cases with multiple CNS hemangioblastomas have VHL disease. Huson et al. (438) found that 40% of a series of patients with cerebellar hemangioblastomas had VHL disease; they were distinguished from isolated lesions by a younger mean age of

<b>TABLE 121-5 von Hippel–Lindau Disease: Clinical Features and Age at Presentation</b>		
<b>Feature</b>	<b>Frequency (%)</b>	<b>Mean Age at Presentation</b>
<b>Hemangioblastoma</b>		
Retinal	59	24 (4–46)
Cerebellar	59	29 (1–36)
Spinal cord	13	33.9 (11–60)
Brain stem	4	NA
Elsewhere	<1	NA
<b>Renal lesions</b>		
Carcinoma	28	46.2 (20–69)
Cysts	37	34.6 (25–50)
<b>Pheochromocytoma</b>	7–19	20.2 (12–36)
<b>Pancreatic lesions</b>		
Cysts	40	NA*
Microcystic adenomas	4	NA*
Islet cell tumors	2	NA*
Carcinoma	0.7	NA
<b>Epididymal lesions</b>		
Cystadenomas	10–26	NA*
Cysts	NA	NA*

NA, data not available; NA\*, usually asymptomatic; Asymptomatic, detected through screening as opposed to presenting with symptoms.

Data from Maher and colleagues (430) and Choyke and coworkers (429).



**FIGURE 121-6** von Hippel–Lindau imaging. (A) MRI appearance of cerebellar haemangioblastoma in sagittal section. (B) Abdominal CT showing an asymptomatic renal carcinoma detected on initial screening of a 52-year-old man who had had three cerebellar hemangioblastomas removed.



presentation—the isolated cases presented at a mean age of 53 years (range 38–75), but in VHL they presented at 28 years (range 10–49 years). Spinal hemangioblastomas are associated with VHL in 80% of cases (429). The mean age of presentation of cerebellar hemangioblastoma in VHL in Maher and colleagues' series (430) was 29 years (range 13–61 years).

The signs and symptoms related to the CNS hemangioblastomas are related to their location. In addition to pressure-related symptoms, there is also a risk of hemorrhage. Spontaneous hemorrhage without prior symptomatology is unusual and occurred in only 1 of 20 of the lesions in the Cardiff series (438). Gadolinium-enhanced MRI is the most sensitive method for detecting these lesions. With screening MRI now being used in asymptomatic family members, small haemangioblastomas that remain static for some years are being detected. Removal of asymptomatic lesions is not usually indicated (439,440). The outcome of surgical resection is usually good in cerebellar lesions, but surgery can be more difficult elsewhere in the CNS and in cases with multiple or recurrent lesions (439,441). Stereotactic radiotherapy may be an alternative to conventional surgery in noncystic small lesions particularly in difficult locations, although complications can occur (439,441).

**121.2.5.4 Renal Involvement.** Patients with VHL can have both cysts and renal carcinoma. The cysts are usually multiple but are rarely symptomatic or affect renal function. Their lining epithelium may be dysplastic or show carcinoma in situ, which may then give rise to renal cell carcinoma. The carcinomas are clear cell in type. Examination of kidneys removed from VHL patients shows large numbers of microscopic tumor foci in apparently normal parenchyma; this explains the high risk of multiple and bilateral carcinoma in VHL (442).

Renal carcinoma is a major cause of death in VHL and screening programs are geared toward early detection and treatment of small tumors (435). The risk of renal carcinoma varies in different subtypes (see Section 121.2.5.11). In the most common forms (type 1 and 2B) the lifetime risk is approximately 70% (443). The mean age of symptomatic diagnosis is around 40 years. In screened patients asymptomatic lesions are detected much earlier, but rarely before age 16 years (435).

When renal carcinomas are detected presymptomatically treatment depends on size. Most small renal tumors enlarge slowly (<2 cm per year) (429), and once the growth rate of a given lesion is determined, it can be kept under radiographic surveillance. The cutoff for treatment is 3 cm diameter, and then nephron-sparing surgery or an alternative technique such as radiofrequency ablation is used (444,445). Repeated surgeries may eventually necessitate renal replacement therapy. Renal transplantation has been undertaken successfully (446).

**121.2.5.5 Pheochromocytoma.** Pheochromocytomas only occur in specific VHL subtypes (type two). Mean age

at diagnosis of pheochromocytoma in VHL is 30 years (435). The lesions are usually in the adrenal. Although extra-adrenal lesions can occur in VHL, the finding of an extra-adrenal lesion in a pheochromocytoma-only family makes a succinate dehydrogenase subunit mutation more likely. The symptomatology, diagnosis, and treatment of pheochromocytomas in VHL is the same as when these lesions occur in isolation (429).

**121.2.5.6 Pancreatic Lesions.** The most common pancreatic lesions in VHL are cysts; these are usually asymptomatic and found on screening (447). In one series, 21 of 52 patients (40%) who underwent screening with abdominal CT had pancreatic cysts (448). The earliest age they have been identified was 15 years; in screening programs they usually are picked up between 20 and 40 years. The other benign pancreatic lesion in VHL is the serous adenoma or microcystic adenoma, which was present in 4% of the Hough and colleagues series (448). Both cysts and microcystic adenomas are usually asymptomatic, but if they are large, or occur in a critical place (e.g. near the bile duct), they can be symptomatic. Occasionally the cysts are so numerous that pancreatic function is affected.

Solid pancreatic tumors in VHL are usually nonsecretory islet cell tumors (447,449–451). These can be malignant and surgery is recommended in tumors >3 cm in diameter.

**121.2.5.7 Other Lesions.** Papillary cystadenomas of the epididymis occur in up to 60% of men with VHL disease and are often bilateral (452). Unilateral papillary cystadenomas of the epididymis occur rarely in the general population. When they are bilateral, they are virtually pathognomonic of VHL disease. Epididymal cysts with no solid component are also commonly found in VHL patients; however, they are seen in 23% of the general population, making them an unreliable marker for VHL disease. These lesions rarely cause symptoms, and the cystadenomas are benign; surgery is therefore indicated only for the occasional patient who presents with symptoms. Women can develop papillary cystadenomas in the broad ligament, the embryonic counterpart of the epididymis. These lesions are exceedingly rare (429).

Endolymphatic sac tumors can be detected by MRI or CT imaging in up to 11% of VHL patients (453). Bilateral lesions are considered pathognomonic for VHL. The endolymphatic sac is located at the end of the lymphatic duct and lies within the dura of the posterior fossa. Sac tumors can grow outward into the cerebellum or cerebellopontine angle and thus mimic other tumors more commonly found at these sites. They can also erode the vestibular aqueduct to involve the inner ear structures. Symptoms reflect the direction of growth. These tumors are histologically similar to the papillary cystadenomas of the epididymis. They are locally invasive but not known to metastasize. The tumors can be treated by surgical resection (454).



**121.2.5.8 Natural History.** The introduction of screening programs for disease complications for VHL from the late 1980s onward has much improved the natural history. The study of Maher et al. (430) consisted largely of unscreened patients. The mean age at presentation of the symptomatic cases in the series was  $27 \pm 12.6$  years. As in the other phakomatoses, the natural history depends on which lesions develop; in unscreened populations the disease is associated with significant morbidity and mortality. In the Maher et al. series (430), 51 of 152 patients had died. Mean age at death was 41 years (range 13 to 67 years). Renal carcinoma was the most common cause of death (24 of 152 or 16%), followed by cerebellar hemangioblastoma (21 of 152, or 14%). Screening programs are improving the natural history of the disease. Wilding et al. (455) recently showed a significant increase in life expectancy before and after the introduction of a genetic register and routine surveillance of VHL families. The median life expectancy before introduction was 43.1 years (95% CI 39.2–47); this improved to 59.4 (95% CI 48.1–70.8). There remains, however, a cohort of patients for whom we have no satisfactory treatment. For example, patients with symptomatic optic disk angiomas usually lose vision, and those with multiple CNS hemangioblastomas may reach the stage at which further surgery is considered impractical and are left with severe neurologic deficits.

**121.2.5.9 Diagnostic Criteria and Differential Diagnosis.** Using the clinical diagnostic criteria for VHL (456), the diagnosis is made in the following:

- A simplex case (i.e. no known family history) presenting with two or more characteristic lesions (e.g. two hemangioblastomas or a hemangioblastoma and a visceral lesion).
- An individual with a positive family history and one or more of the following: retinal angioma, CNS hemangioblastoma, pheochromocytoma, multiple pancreatic cysts, epididymal or broad ligament cystadenoma, multiple renal cysts, or renal cell carcinoma before age 60 years.

The main problem in the diagnosis of VHL is that the condition is not considered in patients with isolated lesions that are part of the disease spectrum. Often the significance of other disease features in relatives is not appreciated, and the patient is discharged after treatment for the particular problem, only to present at a later stage with another disease feature.

Other causes of hereditary renal cancer such as hereditary leiomyomatosis and renal cell cancer and the Birt–Hogg–Dube syndrome have no other features that overlap with VHL (457).

**121.2.5.10 Genetics.** VHL disease is inherited as an autosomal dominant condition with almost complete penetrance by age 60 years. In their study, Maher's group (425) found that approximately 25% of cases were sporadic. The mutation rate was calculated to be in

the region of  $2.3\text{--}4.4 \times 10^{-6}$  per gamete per generation. There was no parental age effect for new mutations, and reproductive fitness was 0.89. As in the other phakomatoses, the disease in a proportion of the sporadic cases results from somatic mutation. Sgambati and colleagues (458) studied the apparently sporadic cases in the NIH VHL cohort. They showed two (4.8%) of the 42 to have parents mosaic for VHL. In VHL, when manifestations such as renal cancer do not present clinically until it is too late for satisfactory treatment, it is suggested that at least one round of screening be undertaken (and more depending on age) of apparently unaffected parents who are mutation negative on lymphocyte DNA.

**121.2.5.11 Heterogeneity and Genotype–Phenotype Correlation.** All families with VHL map to the same locus on chromosome 3 and in nonmosaic patients with classical VHL mutation detection is >95% (435). There is interfamily variation, with particular reference to pheochromocytomas and renal carcinoma. Families can be grouped into VHL subtypes as follows:

- Type 1: All the VHL features except pheochromocytoma occur. Truncating mutations or missense mutations that grossly disrupt the protein are associated with type 1 (459). A further subgroup of type 1 patients is sometimes referred to as 'type 1B'. These patients have deletion of all or part of the *VHL* gene and the *HSPC300* gene and have a very low risk of renal carcinoma (460–462).
- Type 2: This is VHL with pheochromocytoma and families usually have a missense mutation. Type 2 is further divided into three groups:
  - Type 1A: Low risk of renal carcinoma.
  - Type 2B: The most frequent type 2 subtype, with a high risk for renal carcinoma.
  - Type 2C: Pheochromocytoma is the only feature.

Maher et al. (435) feel that this classification is most useful in the research setting for correlating mutation with protein function; in the clinical setting, one has to be cautious not to use the classification too rigidly. They use the example of an index case with retinal angiomas and renal carcinoma who would be type 1, but the finding of a pheochromocytoma in a relative makes it a 2B kindred.

**121.2.5.12 Chuvash Polycythemia.** This is the only other phenotype associated with VHL mutations (463). It is autosomal recessive and is endemic in the Chuvashia region of Russia. In this region most people are homozygous for p.Arg200Trp mutation. Patients have elevated erythropoietin levels and thrombosis and/or hemorrhage can occur. No VHL disease features have been reported. Chuvash polycythemia is now recognized in many ethnic groups and with other mutations (464,465). VHL analysis is indicated in all patients with congenital erythrocytosis.

**121.2.5.13 Pathogenesis.** The *VHL* gene was localized to chromosome 3p by linkage (466–468), and tumors were found to display loss of heterozygosity for this region

(469,470), consistent with a tumor suppressor mechanism. The gene was cloned in 1993 by Latif and colleagues (467). The *VHL* gene has three exons and encodes for two VHL proteins, a full-length 213-amino-acid protein (pVHL<sub>30</sub>) and a smaller protein lacking the first 53 amino acids (pVHL<sub>19</sub>). Functional studies suggest that the two proteins have the same function (471). The VHL protein is widely expressed in fetal and adult tissues (472,473).

Hypoxia-inducible factor (HIF) is hydroxylated in the presence of oxygen, which targets it for ubiquitination and subsequent degradation. HIF is not hydroxylated in low oxygen conditions, allowing it to function as a transcription factor to induce genes involved in angiogenesis. Loss of VHL function prevents the ubiquitination of HIF and leads to localized hyperactivity of HIF, with resultant tumor formation. This mechanism suggests the possibility of use of inhibitors of angiogenesis in the treatment of VHL-associated tumors. VEGF levels rise in response to HIF activity. Understanding the disease pathogenesis has led to new therapeutic opportunities (435). Tyrosine kinase inhibitors that target the VEGF pathway have been shown to be effective in sporadic renal cancer (474). VHL-specific trials have shown good preliminary results in some tumors (475).

**121.2.5.14 Management.** The features of VHL disease that can cause significant morbidity and mortality are all potentially treatable if diagnosed early (429) (Figure 121-6). This has led to the development of screening protocols for the follow-up of affected individuals and their at-risk relatives. All individuals with VHL should be offered annual clinical review. Further clinical examinations and other screening techniques are added in at various ages for different disease manifestations. Additional examinations and investigations are instigated at different ages depending on the average age of presentation of the VHL manifestation being targeted. The following surveillance protocol is recommended (435).

*Screen for retinal angiomas:* Annual ophthalmic examinations (including both direct and indirect ophthalmoscopy) beginning in infancy or early childhood.

*Screen for CNS hemangioblastomas:* MRI scans of the head (and/or the spine) every 12–36 months beginning in adolescence.

*Screen for renal cell carcinoma and pancreatic tumors:* MRI or ultrasound examination of the abdomen annually from age 16 years. Although CT is the most sensitive method for detecting renal tumors, particularly in the presence of renal cysts, other methods are preferred for screening to avoid a large cumulative radiation dose.

*Screen for pheochromocytomas:* Annual blood pressure monitoring and biochemical screening for pheochromocytoma from early childhood. Measurement of plasma catecholamine metabolites is the most sensitive test and is replacing the traditional 24-hour urine collection in some centers. In families at high risk of pheochromocytoma the annual abdominal MRI should be introduced in childhood before 16 years.

Other investigations are driven by symptoms, for example, MRI of internal auditory canals if hearing loss, tinnitus, or vertigo is present. Although the screening protocols have been shown to decrease disease-related morbidity and mortality, patients with VHL may still have to face multiple treatment episodes to different body systems during their lifetime. This is compounded by the fact that the natural history cannot be predicted and that different family members can be affected in different ways. It is important when entering families into screening programs for the first time that the full implications of the screening program be discussed. This is particularly the case when the diagnosis is first made in adulthood and the first round of tests may identify multi-organ involvement, some of which will require treatment even though asymptomatic.

**121.2.5.15 Molecular Genetic Testing.** As the screening programs for VHL disease manifestation start in early childhood, VHL is one condition for which childhood genetic testing is justified. Even though molecular testing of children at risk of VHL in early childhood is justified clinically, the report of Levy and Richard (476) suggests that not all families will want to consider this. In our own practice, we give parents the option as to whether they want to consider having the children tested, or have the children enter the screening program until the time when the children can decide about gene testing on their own account. Mutations can be now identified in most patients, and this testing is routinely available in service laboratories. This enables only those shown to have inherited the mutation to proceed to the screening programs.

It is also recommended that patients presenting at a young age with any of the individual features of VHL be assessed for the disease (434,438,477). If screening for disease manifestations and mutation analysis is normal, it is unlikely that they have VHL; however, because of the possibility of mosaicism, as mutation detection is not 100% sensitive, and if they have VHL they would be at risk of life-threatening conditions such as renal carcinoma at an older age, long-term surveillance is recommended. In our own practice, if such patients reach the age of 50 with no manifestations, they are discharged from follow-up.

## 121.2.6 Other Phakomatoses

Several additional disorders display features of “spottiness,” with prominent effects on the nervous system that overlap with van der Hoeve’s original classification sufficient to be included among the “phakomatoses.” These include both inherited and sporadic disorders. The latter group was suspected on clinical grounds to be caused by genetic mosaicism (478) and this is now being confirmed at the molecular level with a series of mosaic overgrowth phenotypes resulting from mutations in the genes in the PI3Kinase-AKT

pathway. The clinical relevance of including them in this chapter is that their phenotypes can overlap with the classical phakomatoses, particularly NF1.

**121.2.6.1 PTEN Hamartoma Tumor Syndromes (PHTS).** *PTEN* hamartoma tumor syndromes (PHTS) is the collective name given to a group of overlapping disorders that predispose to hamartomas and certain malignancies and are caused by germline mutations in the *PTEN* gene on 10q23.3 (Phosphatase and TENsin homolog deleted on chromosome TEN (479–481)). One of the most consistent features of all the related phenotypes is macrocephaly. Inheritance is autosomal dominant.

**121.2.6.1.1 Cowden Syndrome (CS).** CS predisposes to a mixture of hamartomas and benign and malignant tumors of various organs. The most prevalent feature are the mucocutaneous manifestations, which are present in 99% of individuals by the third decade of life (481). The mucocutaneous manifestations include trichilemmomas (benign neoplasms derived from the outer root sheath of the hair follicle), papillomatous papules (benign epithelial neoplasms—on the skin and oral mucosa) and keratoses of peripheral parts of the body (acral), particularly the soles of the feet. In clinical practice, however, the significance of the skin features may not be appreciated until the patient presents with one of the benign or malignant tumor related to the disease or a relative is diagnosed with PHTS.

The tumor predisposition in CS gives an increased risk of benign and malignant disease in affected organs. For example, affected women have a risk of up to 67% of benign breast disease and an 85% lifetime risk of breast cancer with 50% penetrance by 50 years (481,482). The thyroid, uterus and bowel also show the mixed picture of benign and malignant lesions. Other cancers that occur at increased risk are renal papillary carcinoma and melanoma. When a rare CNS tumor, cerebellar dysplastic gangliocytoma (Lhermitte–Duclos disease), occurs in an adult it is pathognomic of CS.

**121.2.6.1.2 Bannayan–Riley–Ruvalcaba Syndrome (BRRS).** In contrast to CS, the BRRS phenotype is usually diagnosed in childhood in children presenting with macrocephaly often associated with frontal bossing and downslanting palpebral fissures. Birth weight is usually greater than 4000 g and length greater than the 97th centile (483). Postnatal growth decelerates and, other than the head circumference, the proportions of older patients are normal. Hypotonia, severe motor delay and subsequent moderate to severe learning problems are seen in up to 50% of cases, with seizures in up to 25% (483). Approximately 60% of cases have a proximal myopathy with a lipid storage picture on biopsy (484,485).

About 75% have lipomas, some of which can be aggressive and cause complications (486). Characteristic macular pigmentation is found on the penile glans and shaft in the majority of affected males; this may be subtle and needs to be sought for specifically. A few patients are reported as having café-au-lait spots.

Hamartomatous polyps cause symptoms more frequently than in CS, presenting with bleeding and intussusception (481). BRRS was not originally thought to be associated with an increased risk of malignancy, but as no clear genotype–phenotype correlation has emerged and in some families the BRRS and CS phenotypes coexist it is now recommended that patients with BRRS are followed up as patients with CS.

**121.2.6.1.3 PTEN-Related Mosaic Overgrowth/Type 2 Segmental CS.** A number of patients have been reported with germline *PTEN* mutations and a clinical picture dominated by a mosaic overgrowth pattern similar to that seen in Proteus syndrome (487–490). Some cases have been in classical CS families. Affected individuals have had a mixture of arteriovenous and lymphatic vascular malformations, lipomatous overgrowth and linear epidermal naevus. In cases where different tissues have been analyzed either a second somatic mutation or evidence of loss of heterozygosity has been found.

The nomenclature of these cases needs clarification. Eng (481) refers to Proteus syndrome and Proteus-like syndrome as part of Pitt–Hopkins syndrome (PTHS). However, no patient with classical PS has been found to have *PTEN* mutations and as discussed below has now been shown to result from somatic mutation in *AKT1* (491). Eng refers to this as “*AKT1*-related Proteus syndrome” and comments that as *PTEN* downregulates *AKT1* it can be viewed as a “*PTEN*-pathway-opathy.” Caux et al. (490) described two cases in CS families and suggested the acronym SOLAMEN for segmental overgrowth, lipomatosis, arteriovenous malformation and epidermal nevus. Although this describes the phenotype, it seems a retrograde step in the molecular era. Happle (492) proposed we use the term type 2 segmental Cowden disease, which fits with the classification he proposed for segmental manifestations of dominant skin disorders in 1997 (478).

**121.2.6.1.4 Other PTHS Phenotypes and Genes.** *PTEN* mutations have been found in 10–20% of patients ascertained through autistic spectrum disorders and macrocephaly (493,494).

Not all patients with CS clinically have *PTEN* mutations. Approximately 30% of such patients have a germline *KLLN* epimutation, which is associated with a greater frequency of breast and renal carcinoma (495). Germline variants in three of the succinate dehydrogenase genes (B, C and D) have also been reported in both *PTEN*-positive and *PTEN*-negative CS patients (496); in the former they appear to modify the risk of breast and thyroid cancer.

**121.2.6.1.5 Management of PTHS.** It is now recommended that all patients with *PTEN* mutations are followed using the surveillance recommendations originally developed for surveillance of patients with the CS phenotype (481). One child with a germline *PTEN* mutation and life-threatening respiratory dysfunction and malnutrition because of progressive hamartomas of



the chest, abdomen and pelvis showed dramatic response to the mTOR inhibitor, rapamycin (497).

**121.2.6.2 Proteus and Other Syndromes Caused by Constitutional or Somatic Mutation of Genes in the Phosphatidylinositol 3-Kinase (PI3K)-AKT Signaling Pathway.** This group of patients present with a combination of specific skin changes, dysregulated growth of one or more body parts or of a specific tissue type and vascular malformations. The majority of cases are sporadic and few, if any, convincing familial cases exist. In many cases the phenotype is very patchy or localized. These findings suggested new dominant mutations or somatic mutation of genes that would be lethal if germline. As systematic clinical research was applied it became clear that there were specific subgroups of patients. The importance of recognizing these subgroups was not just of academic interest; the natural history of the dysregulated growth and of specific types of vascular manifestation is different. Thus Biesecker and colleagues (498,499) developed very specific criteria for Proteus syndrome as it is the progressive overgrowth of parts of the body compared with the general growth rate that causes some of the major challenges for patients.

Oduber and colleagues (500) proposed a classification of entities combining vascular malformations and deregulated growth. However, this may well now be superseded by a molecular classification. The past year has seen a series of papers reporting germline and somatic mutations in genes in the PI3K-AKT pathway that have been identified as groups have applied improved sequencing technologies to search for mutations in different tissues from this group of patients (491,501–508). At an early stage it is clear that some phenotypes, such as Proteus, are due to a specific somatic mutation in one gene. Furthermore, in the three *AKT* genes the mutations are homologous (p.Glu17Lys) and the phenotypes reflect the tissues where the protein is most active during development.

### **121.2.6.3 Klippel–Trenauney–Weber Syndrome.**

**121.2.6.3.1 Clinical Features.** The main feature of Klippel–Trenauney–Weber syndrome (KTS) consists of a cutaneous vascular nevus over the trunk or limbs in an asymmetrical distribution (509). The nevus can be associated with varicosities, lymphatic anomalies, and asymmetrical hypertrophy of all or part of a limb—the nevus and hypertrophy need not necessarily overlap. The lesions are usually unilateral, but can also be bilateral. The hypertrophy of the limbs is due to hyperplasia of both bone and soft tissue, and arteriovenous anastomoses are found in occasional cases. The CNS is rarely involved (506,507). Pain control is an important component of clinical management (510,511).

**121.2.6.3.2 Genetics.** Most cases have been sporadic (512). Discordant monozygotic twins were reported by Hofer and coworkers (513). The vascular anomalies overlap with those seen in CLOVES. Kurek et al. reported *PIK3CA* missense mutations in lesional tissue from 3 of 15 individuals with KTS (501).

### **121.2.6.4 Encephalocraniocutaneous Lipomatosis.**

**121.2.6.4.1 Clinical Features.** Encephalocraniocutaneous lipomatosis (ECCL) is localized to the craniofacial area and some authors suggest it is a localized form of Proteus syndrome (514). Against this is the fact that the features are present at birth and are nonprogressive, with the exception of skeletal cyst and jaw tumors if present. The following are the main features:

- Cutaneous: Nonscarring alopecia,  $\pm$  underlying fatty tissue are the most frequent. The nevus psiloliparus is characteristic and is composed of a smooth, hairless fatty tissue nevus of the scalp.
- Ophthalmic: Choristomas,  $\pm$  other eye anomalies are present in most cases. They are benign ocular tumors and include epibulbar or limbal dermoids and lipodermoids.
- CNS anomalies: These include intracranial and spinal lipomas often in association with other abnormalities including porencephalic and arachnoid cysts. Intracranial calcification similar to that seen in SWS has also been described (504,505).
- Neurology: Patients exhibit a variable degree of developmental delay and epilepsy.
- Evidence of extracranial manifestations should be looked for (506–508).

**121.2.6.4.2 Genetics.** All cases of ECCL to date have been sporadic (515). ECCL has been reported in one patient in whom a germline *NF1* mutation has been demonstrated—the authors speculated that the ECCL may result from somatic mutation in the normal *NF1* allele or in another unrelated gene (516).

### **121.2.6.5 Sturge–Weber Syndrome/Encephalofacial Angiomatosis.**

**121.2.6.5.1 Clinical Features.** SWS is a congenital condition marked by capillary malformations of the facial skin in the distribution of the first division of the trigeminal nerve (port-wine stain/nevus). The other second and third areas of trigeminal supply may also be affected. The other features are cerebral venous malformations (leptomeningeal angiomatosis) and glaucoma with ocular venous vascular malformations. Only 8–20% of patients presenting with facial port-wine nevus develop neurological symptoms, and the diagnosis of SWS is only made with the combined brain involvement (517,518).

The cutaneous features are present at birth and are nonprogressive. The neurologic features of SWS seem to be a consequence of the leptomeningeal angiomata (519), although other cerebral vascular malformations may occur. The characteristic “tramline calcification” appearance results from gyriform calcification of the cerebral cortex and usually develops after 2 years of age. Seizures, mental retardation, and hemiparesis may occur. Developmental delay occurs in approximately 50% of affected individuals, although it is much less likely if there are no seizures and more likely if seizures began



in the first year of life (517,520). The seizures may be generalized or focal, affecting the contralateral side of the body. They may be difficult to control, and surgery should be considered if they remain refractory to medical treatment (521). The neurologic manifestations are often progressive.

SWS should be considered in any child with a facial capillary hemangioma, and radiologic studies are helpful in delineating the degree of meningeal and cerebral involvement. However, it should be noted that both CT and MRI studies can be normal in the newborn. Lo et al. (517) review developments in imaging techniques, and that of possible biomarkers, which will assist both in diagnosis and prognosis.

**121.2.6.5.2 Genetics.** All cases of SWS have been sporadic except for one reported case of an affected father and son (522). Monozygotic twins have been reported that were concordant (523,524) or discordant for the condition (525). The most likely explanation remains somatic mosaicism.

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## Biographies



**Susan M Huson, MD, FRCP.** Dr Sue Huson is a Consultant Clinical Geneticist in the department of Genetic Medicine at the Central Manchester University Hospitals NHS Foundation Trust and an honorary senior Lecturer in Clinical Genetics at the University of Manchester. She trained in medicine in Edinburgh. After general training in adult medicine and neurology, she was a research fellow in Cardiff with Professors Peter Harper and Alastair Compston. She did a population based study of NF1 and gene mapping studies of NF1. Her work made a major contribution to the mapping of the *NF1* gene to chromosome 17 in 1987. Her MD degree, based on this work, was awarded with distinction and gold medal. She then trained in Clinical Genetics in London with the late Professor Robin Winter. As a consultant, first in Oxford and now in Manchester she has focused her clinical research on defining the different neurofibromatosis phenotypes and developing models of neurofibromatosis care. She is the director of one of the two English centers nationally commissioned for complex NF1 care.



**Bruce R Korf, MD, PhD.** Dr Korf received his MD degree from Cornell University Medical College and his Ph.D. degree in genetics and cell biology from Rockefeller University. He then completed training in pediatrics, pediatric neurology, and genetics at Children's Hospital, Boston. He served as clinical director in the Division of Genetics at Children's Hospital from 1986 to 1999 and as the medical director of the Harvard-Partners Center for Genetics and Genomics from 1999 to 2002. Currently he is the Wayne H and Sara Crews Finley Chair in Medical Genetics, Professor and Chair, Department of Genetics and Director, Heflin Center for Genomic Sciences at University of Alabama at Birmingham. Dr Korf has completed terms as president of the Association of Professors of Human and Medical Genetics, President of the American College of Medical Genetics, member of the boards of directors of the American College of Medical Genetics and the American Society of Human Genetics, member of the Liaison Committee on Medical Education, and the National Cancer Institute Board of Scientific Counselors. He currently is President of the ACMG Foundation for Genetic and Genomic Medicine and serves on the Board of Scientific Counselors of the National Human Genome Research Institute.

# CHAPTER

# 122

## Multiple Sclerosis and Other Demyelinating Disorders

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### 122.1 INTRODUCTION

Demyelination occurs when normal functioning myelin sheaths—lipid-rich insulators that surround axons in the central nervous system (CNS) and the peripheral nervous system—are broken down. The most common inherited demyelinating disease is multiple sclerosis (MS), which affects approximately 0.1% of Caucasians of northern and central European ancestry. This chapter will focus on MS (<http://mssociety.ca>, <http://www.nationalmssociety.org>, <http://www.mscares.org>), although brief reference will be made to a few other inherited demyelinating diseases.

MS is an inflammatory disease of the CNS characterized by myelin loss, varying degrees of axonal pathology, and progressive neurological dysfunction. MS is the most common cause of nontraumatic neurological disability in young adults in Europe and North America. The clinical onset of MS may be monosymptomatic or polysymptomatic. Onset symptoms can include sensory disturbances (numbness, tingling), visual disturbances (optic neuritis, diplopia), motor weakness and ataxia. The diagnostic criteria for MS have evolved over time (1,2), most recently to include findings on magnetic resonance imaging in the definition of dissemination in time and space (3). The most frequent revisions to diagnostic criteria also take into account the observation that MS is now recognized increasingly in pediatric (4) and non-Caucasian populations (3) (see Figure 122-1 for geographic distribution of MS).

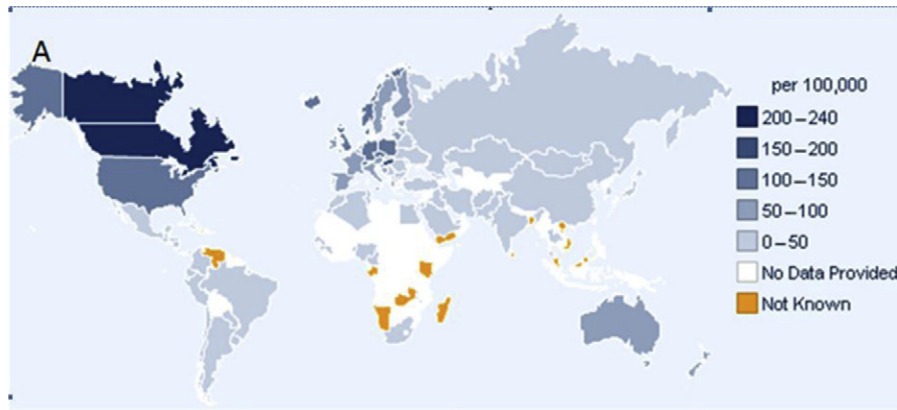
Prognosis cannot be accurately determined, but MS tends to be a progressive disorder with the majority of relapsing-remitting cases becoming progressive over time, as shown by several natural history cohorts (5–8). While not a fatal disease, a reduced life expectancy (about 10 years) has been reported (9).

MS disability is often assessed using the Extended Kurtzke Disability Scale or EDSS (10). The EDSS scale ranges from “0” to “10.” Zero refers to no disability, 6 is “needs a cane,” 8 is “bedridden” and 10 is “dead from MS.” Although this scale has definite limitations, it remains the most widely accepted disability and clinical measurement when evaluating the natural progression and outcome of clinical trials (e.g. beta-interferon-1b (INFB) 16-year follow-up) in MS (11).

Genes have important roles in MS susceptibility, with the human leukocyte antigen (HLA) class II locus exerting the strongest effect on MS risk (12). However, it is also very clear that broad, population-based environmental (nongenetic) factors are also very influential in determining disease susceptibility/protection (e.g. (13,14)). Thus, it is now accepted that genes, environment, and interactions between genes and the environment are important in the etiology of MS (e.g. (12–14)).

Current data provide evidence for the following statements (15–19):

- There is a true familial aggregation of MS;
- The familial aggregation is because of genetic material shared by individuals and not the shared familial microenvironment;
- Factors determining geographic distribution of MS are not operative in familial microenvironment but at a broad population level and exert a huge effect;
- The rate of MS is increasing but too rapidly to be due to genetic factors; environmental changes must be critical;
- There are probably different genetic and environmental triggers or agents involved in MS onset versus MS progression;
- MS natural history does not run true in families;
- Parental and gender effects are important in MS risk and allele transmission;



**FIGURE 122-1** Global MS prevalence. Maps courtesy of the Atlas of MS database (<http://www.atlasofms.org/index.aspx>).

- MS is not a “transmissible” disease in either childhood or adulthood, other than by genes;
- There is no evidence for a single deterministic gene/locus/allele in MS (not even HLA);
- The genetic interactions in MS are much more complex than initially believed;
- The “critical window of opportunity” to prevent MS may be much earlier than puberty;
- There are no biomarkers to definitively predict the clinical onset of MS;
- A maternal effect is well validated;
- Genetic factors have a role(s) in MS course as well as susceptibility;
- Epigenetic factors are increasingly being recognized;
- Evidence from studies of disease risk in relation to the month of birth implicates early-life environmental factors in MS susceptibility;
- Migration data together with variability in disease onset and relapse rates suggest that environmental factors might also act later in life influencing MS risk and disease course;
- Vitamin D is an attractive candidate risk/protective factor implicated by the association of MS risk with latitude and month of birth, and is likely to act early in life;
- Epstein–Barr virus (EBV) seems to act in adolescence or early adulthood to alter MS susceptibility;
- Smoking is associated with increased susceptibility to adult-onset MS when individuals are exposed later in life, and can alter the course of established MS;
- Strategies aimed at preventing or treating MS by manipulating environmental factors may provide a powerful way of reducing the prevalence.

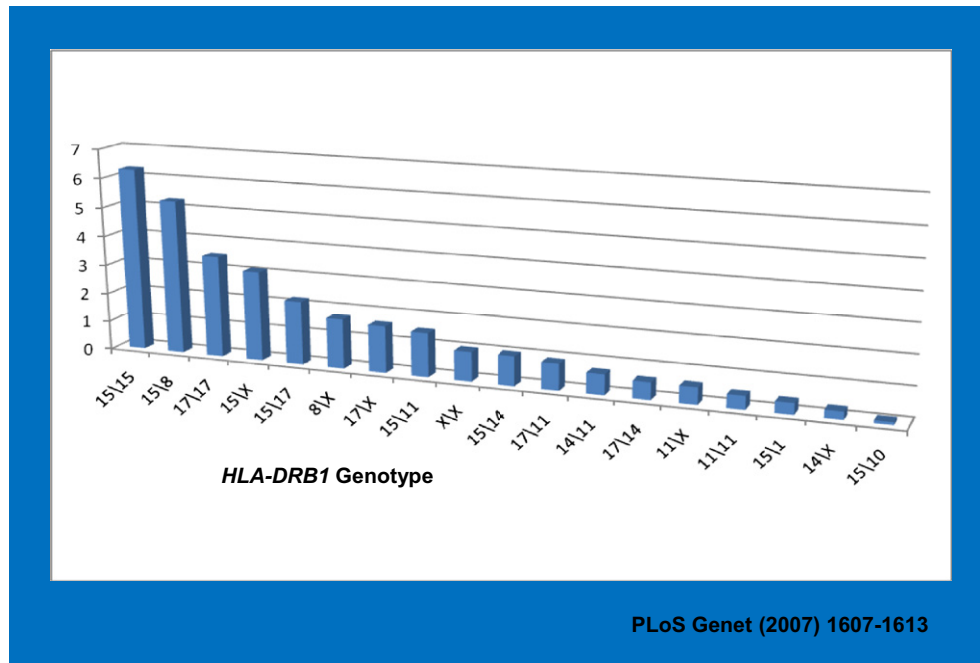
Genes have been implicated in MS since the first linkage reports in the 1970s, with HLA and familial cases of the disease being reported as early as 1933 (15). In the mid-1990s, the first generation of genomic screens for linkage in MS were performed using a variety of methodologies and family types. These implicated many distinct regions of interest,

although with little consensus. The one exception was the major histocompatibility complex (MHC) on chromosome 6p21, which was first noted to have linkage with MS in the 1970s (15,17–19). Genome-wide association studies (GWAS) in MS led to the identification of additional loci of interest (20–24) but HLA class II extended *HLA-DRB1\*15* haplotype (*HLA-DRB5\*0101*–*HLA-DRB1\*1501*–*HLA-DQA1\*0102*–*HLA-DQB1\*0602*) has remained the most consistent association (25).

Replication and meta-analyses following GWAS have suggested up to 20 genes affecting MS susceptibility including *CD6*, *CD25* (interleukin 2 receptor, alpha, *IL2RA*), *CD40*, *CD58*, *CD226*, C-type lectin domain family 16, member A (*CLEC16a*), ectopic viral integration site 5 (*EVI5*), glypican 5 (*GPC5*), regulator of G protein signaling 1 (*RGS1*), tyrosine kinase 2 (*TYK2*), tumor necrosis factor receptor superfamily member 1A (*TNFRSF1A*), interferon regulatory factor 8 (*IRF8*), ribosomal protein L5 (*RPL5*), kinesin family member 21B (*KIF21B*), membrane metalloendopeptidase-like 1 (*MMEL1*), methyltransferase like 1 (*METTL1*), but additional, independent replication is still necessary for several genes and none meet the level of the *HLA-DRB1* (17–19,26). Even with respect to HLA, the genetics of MS remain complex and topics continuing to be studied include epistatic effects and parent origin effects to name a few (see (27–30)). This is clearly shown in Figure 122-2 (31) as it is the HLA haplotype and not the specific allele that influences risk/susceptibility to MS (e.g. 15/15 versus 15/04 (Figure 122-2)).

Thus, despite progress, the genetics in MS remains a conundrum and work must continue along several avenues (see (17,19)).

A number of strategies have been employed to dissect the environmental from the genetic components (i.e. nature versus nurture) underlying MS susceptibility, e.g. half-sibling, adoptee, step-sib and conjugal MS studies; see review articles (17,32) for descriptions



**FIGURE 122-2** Genotype relative risk for DRB1.

of these studies. Taken together, these have clearly shown no evidence for environmental factors operative within the familial microenvironment, either in childhood or adulthood. Thus, DNA sharing is responsible for most, if not all, of the familial aggregation of MS.

Half-sibling data initially suggested a maternal effect for MS (recurrence risk for maternal half-sibs was 2.35% compared to 1.31% for paternal half-sibs (33)), despite the mothers not having MS. This maternal effect has since been replicated in a Dutch extended pedigree (34), a study of avuncular pairs (35) and an investigation of interracial matings (36). The mechanism of the increased risk conferred maternally remains to be elucidated but epigenetic (study of changes in gene expression that may or may not be heritable and do not stem from a change in the DNA sequence (37)) mechanisms are implicated (38). Epigenetic marks (mainly DNA methylation and histone modification) can be modulated by the environment, thus integrating the external environment and internal genetic systems (28,39,40). At present, there are three major environmental factors associated with the risk of developing MS (32,41): (i) latitude and sunshine exposure and the resulting vitamin D levels, which are closely related, (ii) EBV seropositivity, and (iii) smoking. For a review of MS observations with potential regulation through epigenetics, see (42). Research into epigenetic mechanisms of MS continues.

## 122.2 PEDIATRIC MS

MS is increasing being diagnosed in children (43), often within 3 years of an acquired demyelinating syndrome (ADS) (see (4)). Recent diagnostic criteria for MS have recognized pediatric MS (4). For an overview of pediatric MS, see Reference (44). It still remains unclear whether susceptibility risk factors for pediatric MS are the same as those in adult-onset MS. Work continues in this area. HLA-DRB15 is a risk factor in both groups (45), and the role of EBV remains unclear (e.g. risk factor, protective factor, etc. (4,46)), as does race/ethnicity (47). Recurrence risks are yet to be validated in pediatric MS but at present appear to be lower than for adult-onset MS. However, rates may be confounded by an increased number of “at risk” relatives in families of pediatric cases (see Reference (48)).

## 122.3 PRACTICAL APPLICATIONS OF GENETIC STUDIES

### 122.3.1 Presymptomatic Treatment of MS

Given the anticipated complexity associated with the genetics of MS, it is unlikely that predictive testing (genetic or biomarkers) will be an option in the very near future; however, work continues to identify biomarkers of MS in asymptomatic individuals. There are several international groups working on the identification of



reliable and replicable MS biomarkers. Even if a presymptomatic biomarker is identified, one cannot ignore issues including who would define the cutoff for “at risk” individual (e.g. physician, drug company, etc.) and at what age would any preventive therapy be initiated. There is increasing evidence that vitamin D may have a preventive role in childhood, adulthood and even as early as conception and gestation. There may also be interactive effects between vitamin D, EBV and genes (e.g. (14,49–53)). There is also increasing interest in prodromal pathways in MS, which, if identified and replicated, may indicate windows of opportunity to potentially reverse early disease processes before clinical MS becomes evident. This is important for both adult-onset (54) and pediatric MS (4).

### 122.3.2 Genetic Counseling

Genetic counseling for MS must take into consideration the known facts, the complexity of the disease susceptibility and the as yet unknown factors and stochastic events. As shown in Figure 122-3, up to 30% of MS patients have at least one biological relative with MS based on data from the longitudinal, population-based Canadian Collaborative Project on Genetic Susceptibility to MS (CCPGSMS) (e.g. (33,35,55)).

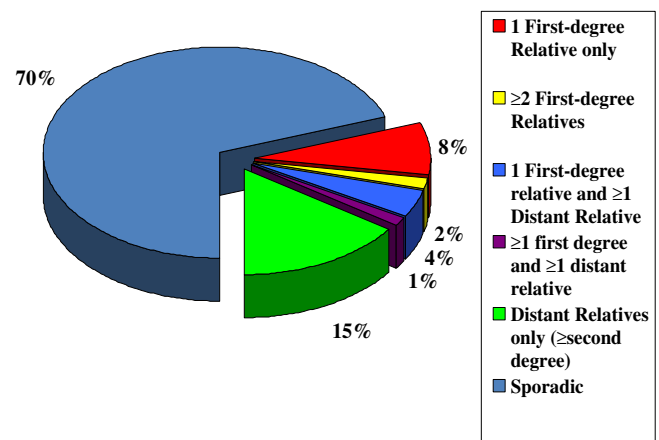
Genetic epidemiological data suggest that genetic counseling must take into account individual family structure, i.e. family-specific factors such as gender, age of MS onset for affected relatives, twin status and whether any (both) parent has MS.

A “liability” threshold model may be helpful when explaining to consultants that each individual, controlling for ethnicity, has a certain amount of “genetic” and “nongenetic” risk factors, which together represent the person’s “liability” (or overall risk). Once the total liability is sufficient to cross a threshold, MS is clinically manifested. This explanation makes no assumption about the exact nature of the individual liability (genetic/environmental and interactions thereof). Nevertheless, taking covariates of age of onset, gender and parental MS status into account, it is likely that genetic loading may vary especially in view of the “nongenetic” factors that affect the population in general (e.g. climate, hours of sunlight) rather than the family-specific environment, as has been discussed previously in this chapter.

Lifetime risks for biological relatives of persons with MS, controlling for the amount of genetic sharing, are given in Table 122-1.

Depending on the amount of genetic (DNA) sharing, the recurrence risks for MS can range from 2/1000, i.e. the general population lifetime risk for Caucasians (e.g. adopted sibs, step-sibs), to 340/1000 (female monozygotic co-twins of MS probands with whom they share 100% of their genetic material).

**Demographics of CCS participants:  
Distribution of Familial MS**



**FIGURE 122-3** Demographics of CCS participants distribution of familial MS.

When counseling full sibs of affected individuals, risks can be refined from those given in Figure 122-1 when information is available on gender of the MS proband and the sib (56).

During genetic counseling sessions, patients and family members often ask the following questions, which can now be answered as follows:

- (1) Can my family members also “inherit” MS if I have MS?

MS itself cannot be directly inherited as is the case for a single gene disorder (such as cystic fibrosis and Huntington’s disease). However, a genetic susceptibility (“risk”) to develop MS does exist. This is highlighted by the fact that the increased frequency of MS among family members only holds true for family members who share genetic material (DNA). Family members who grow up together in the same environment but do not share genetic material (e.g. adopted brothers and sisters, as well as step-siblings) have no increased risk to develop MS compared to the general population.

Conversely, if you do share genetic material, your risk does not change whether you were “raised together” or “raised apart” from the family member who eventually develops MS. The risk of MS among family members can be influenced by several factors including gender, ethnicity, country/location where one grows up, age and biological relationship (percentage of DNA sharing) to other family members with MS.

If you are concerned about your risk to develop MS because you have other family members with MS or you are worried about the risk of passing on MS because you and another family member have MS, please contact genetic counseling services in your area for more information (<http://www.nsgc.org>; [cam medical org](#)).

**TABLE 122-1** Recurrence Risk of Multiple Sclerosis by Relationship

Relationship	Percent
First-degree relative; index case only MS	3.0
Monozygotic female co-twin	34.0
Dizygotic co-twin	5.4
Adopted sib	0.2
Maternal 1/2 sib	2.2
Paternal 1/2 sib	1.2
Step-sib	0.2
Offspring of conjugal mating	30.5
Offspring of consanguineous mating	9.0
Sister of female MS patient with onset under 30 plus 1 affected parent	12.7

**TABLE 122-2** Topics to be Included in Reproductive Counseling for Individuals with Multiple Sclerosis

<i>Effects of MS on Pregnancy</i>
Contraception <sup>a</sup>
Conception <sup>a</sup>
Pregnancy management
Pregnancy outcome <sup>a</sup>
<i>Effects of Pregnancy on MS</i>
Short-term
Long-term
<i>Recurrence Risks<sup>a</sup></i>
Factors to include in determining recurrence risks include gender, ages, family structure, simplex or multiplex with respect to MS, ethnicity, consanguinity, conjugal mating, etc. (See Table 122-1).
<i>Teratogenicity of MS Treatments<sup>a</sup></i>
Symptom specific
Relapses
Disease modifying
<i>Psychosocial Issues<sup>a</sup></i>

<sup>a</sup>Important to discuss regardless of whether it is mother or father who has MS.

Data from Dwosh, E.; Guimond, C. G.; Sadovnick, A. D. Reproduction Counseling in MS: A Guide for Healthcare Professionals. *Int. MS J.* 2003, 10, 67.

- (2) Can my child “catch MS” if he or she hugs, kisses or shares a cookie with my relative (e.g. mother, mother-in-law, sister) who has MS?

MS is not a transmissible disease and cannot be caught by human contact either during childhood or adulthood. This is clearly shown by a variety of genetic epidemiological studies, which have repeatedly shown this finding in separate groups (e.g. adoption, step-sib and conjugal studies discussed earlier in this article).

- (3) If my relative also develops MS, will he or she have the same clinical course of the disease that I do?

The clinical course of MS does not appear to “run true” in families if more than one family member is

affected. Thus, if you develop MS, you cannot assume that you will have the same disease course as your mother, sister, or other relative with MS.

- (4) Can you predict, in advance of any signs or symptoms, who is destined to develop MS in the future?

There are no definitive biomarkers for MS. A biomarker can be “anatomic, physiologic, biochemical, or molecular parameters associated with the presence and severity of specific disease states” (<http://www.biomarkers.org/NewFiles/faqs/definition.html#Anchor-What-35882>). This means that if you study two large groups, for example, 1000 MS patients and 1000 unaffected controls, you may find “risk factors” occurring more often in the affected group than in the controls, but these risk factors will still exist in both groups. Thus, you cannot test an unaffected person for a specific biomarker (such as HLA genotype or low levels of vitamin D) and then state with any certainty whether a person with or without this factor will end up being affected or unaffected by MS in the future. Hence we use the term “susceptibility” with respect to MS risk rather than “causal.”

- (5) Can MS be prevented?

There is no way to prevent MS. No fault can be assigned if someone develops MS. There are no clear protective preventive measures that can be taken.

- (6) If I have MS, should I have or father a child?

Much is still to be known about reproduction and MS. If you have MS and are planning to get pregnant or to father a child, you may want to discuss various issues involved in the decision-making process with your health care professionals. There is no right or wrong answer. Each couple must make their own informed decision. Topics to consider include the risk of pregnancy on MS, the risk of MS on pregnancy, possible risks of MS therapy at the time of conception and/or gestation, psychosocial issues, and the long-term commitment to raising a child (e.g. see References (57,58)). Please note that while several factors should be considered in advance, many individuals with MS have been able to successfully raise children.

- (7) If I am from a region where MS is rare (e.g. Shanghai, China) and am Chinese, do I change my risk to develop MS when I move to Canada or the northern United States? Do my genes change?

Although your genes do not change when you move, your environment does. Thus, by moving from Shanghai to Canada/United States, you may have a higher risk of developing MS than if you stayed in Shanghai. This may be due to different environmental exposures as well as genetic and environmental interactions (59,60).

### 122.3.3 MS Treatment—Risk of Teratogenicity

The majority of MS patients have the disease onset during their reproductive years and it is well known that females develop the disease up to three times as often as males. It is therefore imperative to weigh the pros and cons of current immunotherapies and other treatments (61) against potential teratogenic effects on developing fetuses when young females with MS are contemplating pregnancy or males are planning to father a pregnancy. Data are insufficient to answer with any degree of certainty whether such treatments would have effects on the developing fetus and/or the child's immune system over the long term.

**122.3.3.1 Other Demyelinating Diseases of the CNS.** Unlike MS, for which multiple genes, gene–gene, gene–environment and even environment–environment interactions are implicated, the following examples are relatively rare and are single gene disorders. In addition, as does MS, these examples have both adult and childhood subtypes.

### 122.4 KRABBE DISEASE (GLOBOID CELL LEUKODYSTROPHY)

Krabbe disease (globoid cell leukodystrophy) is an autosomal recessive disorder (25% sibling recurrence, 67% chance that asymptomatic siblings are carriers, females are affected as often as males) resulting from the deficiency of galactocerebrosidase (GALC), leading to the accumulation of a cytotoxic metabolite (galactosylsphingosine or psychosine), which results in the apoptosis of myelin-forming cells (62,63). The estimated carrier frequency is 1/150 for the general population. Prenatal diagnosis is possible either by measurement of GALC enzyme activity or by molecular genetic testing if both disease-causing alleles in an affected family member are known (62).

Recent guidelines have been developed as an educational resource for confirmatory testing and subsequent clinical management of presymptomatic individuals suspected to have a lysosomal storage disease, including Krabbe disease as well as a research agenda for longitudinal studies (64).

### 122.5 METACHROMATIC LEUKODYSTROPHY

Common forms of arylsulfatase A deficiency (metachromatic leukodystrophy or MLD) are infantile, juvenile and adult (66). Although all forms of MLD follow the autosomal recessive mode of inheritance, each form appears to be a distinct genetic entity and the type seems to run true in families. Pseudo-aryl sulfatase A deficiency has been reported.

Genetic counseling is based on the autosomal recessive model but there are reports of cases in which one

copy of the *ARSA* gene has been deleted and a single disease-causing *ARSA*-MLD mutation on the remaining allele results in MLD. Therefore, mutation testing is recommended for both parents when counseling recurrence risk data for other family members (65). There is evidence for a genotype–phenotype correlation (66).

Recent guidelines have been developed as an educational resource for confirmatory testing and subsequent clinical management of presymptomatic individuals suspected to have a lysosomal storage disease, including MLD, as well as a research agenda for longitudinal studies (64).

### 122.6 X-LINKED ADRENOLEUKODYSTROPHY

Adrenoleukodystrophy shows a wide range of phenotypes, with the two most common being the childhood and adult forms. Intragenic deletions of the adrenoleukodystrophy gene and point mutations have been identified, although most families have unique mutations. About 93% of cases have inherited the *ABCD1* mutation from one parent; at most, 7% of individuals with X-ALD have a de novo mutation. Males who inherit the mutation will be affected (67,68). Mosaicism has recently been identified (68). About 20% of females who are carriers develop neurologic manifestations but have later onset (age = 35 years) and milder disease than do affected males (67).

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### Biography



**Dr Sadovnick** was born in Montreal and obtained degrees from McGill University (BSc, Honors Genetics; MSc, Human Genetics) and the University of British Columbia (PhD, Genetics). Dr Sadovnick is a Professor in the Department of Medical Genetics and the Faculty of Medicine, Division of Neurology, UBC. She is the director of the Western Pacific Regional Research and Training Center for Multiple Sclerosis (MS), established by the MS Society of Canada. Dr Sadovnick is the Principal Investigator of several multicenter Canadian and International collaborative research projects on multiple sclerosis, pediatric multiple sclerosis and dementia. Dr Sadovnick was one of the developers of the MSc Genetic Counseling Training Program at UBC and has served as codirector and is now on the advisory panel. She has published extensively (over 300 articles in peer-review journals) and serves as a reviewer for a wide variety of medical journals and grant review panels. She is often an invited speaker at Canadian, American and International (Europe, South America, Russia, Asia) scientific meetings and task forces. Dr Sadovnick is a member of several international advisory groups for both MS and dementia. She is currently involved in Canadian–Chinese initiatives in both MS and early onset familial Alzheimer’s disease (AD).

# CHAPTER

# 123

## Genetics of Stroke

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### 123.1 INTRODUCTION

Strokes result from a focal reduction of blood flow to the brain. Around 83% of strokes are due to arterial vascular occlusion (termed *ischemic stroke*) and around 17% are due to vascular rupture (termed *hemorrhagic stroke*). Arterial vascular rupture may occur into the brain parenchyma resulting in intracerebral hemorrhage (ICH) or into the subarachnoid space what results in subarachnoid hemorrhage (SAH). In about 1% of cases, strokes occur in the venous system (termed cerebral venous thrombosis). Strokes are a leading cause of morbidity and mortality in the US. There is a substantial additional burden of asymptomatic cerebrovascular disease.

Evidence for a genetic basis for stroke comes from twin and family studies. Furthermore, a number of monogenic disorders cause stroke, as either a primary manifestation or a secondary manifestation. The contribution of these factors has been increasingly recognized over the past 10–20 years with the description of monogenic disorders such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). Advances in genomics methods, including genome-wide association studies, are permitting possible new genetic risk markers to be explored.

In this chapter, we will review the genetics of stroke and cerebrovascular disease. The burden and prevalence of stroke, along with stroke classification systems, will first be described. Then, evidence from twin and family studies will be presented followed by monogenic disorders associated with stroke.

#### 123.1.1 Prevalence and Incidence

Stroke represents a serious burden to the worldwide population in terms of prevalence, incidence, long-term disability, and mortality, generating substantial costs across the globe. Stroke statistics differ by gender, age groups, and among the four main racial groups: Whites, Asians, Blacks and Hispanics. According to an estimate,

7,000,000 Americans of  $\geq 20$  years of age have had a stroke, setting the stroke prevalence at the level of 3.0%. Another projection is that by 2030, another 4 million will suffer from stroke, increasing the prevalence by 24.9% (1). Every year, 795,000 people experience a new (77%) or recurrent stroke (23%). Gender and racial differences are seen in both stroke prevalence and incidence, with higher overall incidence in women and the highest incidence and prevalence in non-Hispanic blacks (1). The distribution of stroke subtypes has not changed since 2011, with 87% of ischemic strokes, 10% of hemorrhagic strokes, and 3% of SAH strokes (2). Over the past 20–30 years, stroke incidence has declined significantly in whites, both in men and women (3). Differences in stroke prevalence and incidence between blacks and whites highlight the importance of race.

#### 123.1.2 Mortality

Stroke is now the 4th leading cause of death behind the causes of the diseases of the heart, cancer, and chronic lower respiratory diseases. In the US, a person suffers a stroke every 4 minutes and stroke causes 1 of 18 deaths (1). The 30-day mortality is 12.6% for all strokes, 8.1% for ischemic strokes, and 44.6% for hemorrhagic strokes (4).

#### 123.1.3 Common Stroke Risk Factors

Common stroke risk factors include the following: high blood pressure, atrial fibrillation (AF), smoking, diabetes mellitus (DM), transient ischemic attack (TIA), dyslipidemia, renal disease and sleep apnea, and physical inactivity. TIA substantially increases the short- and long-term stroke rates (1). High blood pressure nearly doubles the lifetime risk of stroke (5). Impaired glucose tolerance and DM respectively doubles and triples the risk of stroke (6). AF independently increases the risk of stroke by five fold throughout all ages (7). Smoking is the most important modifiable stroke risk factor that increases 2 to 4 times the risk of stroke among current smokers (8).

### 123.1.4 Poststroke Long-term Disability

Stroke is a leading cause of serious, long-term disability in the US (1). Neurological deficits, hemiparesis and cognitive deficits at six months poststroke, are present in almost half of the patients. Hemianopia, aphasia, and sensory deficits are present respectively at 19.6, 18.9 and 15.4% of patients. In addition, women were found to be at greater risk for disability and institutionalization (9).

Stroke imposes a substantial economic burden both for society and individual. In the US, the mean stroke expense was \$ 7657 per person in 2007, and the mean estimated stroke care lifetime cost is estimated at \$ 140 048 (1).

## 123.2 STROKE PHENOTYPES

An understanding of stroke classification is essential in reviewing stroke genetics. This is because the different monogenic disorders are specifically associated with certain stroke types and subtypes. According to the World Health Organization, stroke was defined in the 1970s as “neurological deficit of cerebrovascular cause that persists beyond 24 hours or is interrupted by death within 24 hours” (10). Stroke is a heterogeneous disease represented by five main types: ischemic stroke, hemorrhagic stroke, subarachnoid stroke, cerebral venous thrombosis and spinal cord stroke. Furthermore, ischemic stroke is subdivided into four etiological categories: atherothrombotic, small-vessel disease, cardioembolic, and other causes Table 123-1.

### 123.2.1 Ischemic Stroke—Subtypes, Pathomechanism

Ischemic stroke occurs when a blood vessel that supplies the brain is occluded by a clot. There are two kinds of clots: thrombus and embolus. Thrombus forms in an artery that is already narrow and causes thrombotic stroke. Embolus is a clot that forms in another place

in the blood vessels of the brain, or some other part of the body, and travels up to the brain to block a smaller artery causing an embolic stroke.

Ischemic stroke may be divided into three categories, based on the clot source. It may arise from the atherosclerotic large cerebral arteries (e.g. carotid, middle cerebral, or basilar arteries) or atherosclerotic small cerebral arteries (e.g. lenticulostriate, basilar penetrating, and medullary arteries); finally, ischemic stroke may also be cardioembolic in origin.

**123.2.1.1 Large-Vessel Disease.** Large-vessel disease (LVD) or artery-to-artery embolism accounts for 15 to 20% of all ischemic strokes (12). In the case of LVD, emboli may originate from the aorta, extracranial carotid, or vertebral arteries, or intracranial arteries. The embolic material is composed of clot, platelet aggregates, or plaque debris that usually breaks off from atherosclerotic plaques (13).

**123.2.1.2 Cardioembolic Stroke.** Cardioembolism accounts for 20 to 30% of all ischemic strokes (12). Embolus originating from the heart causes severe strokes, but it is also prone to early recurrence. Due to their large size, cardiac emboli flow to the intracranial vessels in most cases and cause massive, superficial, single large striatocapsular or multiple infarcts in the middle cerebral artery.

**123.2.1.3 Small-Vessel Disease.** Small-vessel disease (SVD) accounts for another approximately 20 to 30% of all ischemic strokes (12). It is strongly associated with hypertension and is characterized pathologically by lipohyalinosis, microatheroma, fibrinoid necrosis, and Charcot–Bouchard aneurysms.

**123.2.1.4 Other Causes.** This category includes rare causes of stroke. Patients assigned to this group should have clinical and imaging signs of a stroke. Common causes of stroke (SVD, LVD, and Cardioembolism) should be excluded (14).

**123.2.1.5 Intermediate Phenotypes.** Patients are assigned to this group if the cause of stroke cannot be determined. This applies to patients with two or more potential causes of stroke, for example SVD and LVD or LVD and Cardioembolism.

**TABLE 123-1 Annual Ischemic Stroke Incidence Rates per 100, 000 among Whites, Blacks, and Hispanics (11)**

Ischemic Stroke Subtype	Whites %	Hispanics %	Blacks %
Large-vessel disease (LVD)	9.09	14.77	16.75
Intracranial LVD	3.41	8.72	7.85
Extracranial LVD	5.68	6.04	8.90
Small-vessel disease	14.77	20.81	20.94
Cardioembolic	23.86	20.13	17.28
Cryptogenic	51.14	44.30	42.93
Other	1.14	0.67	1.05
Total			

### 123.2.2 Hemorrhagic Stroke—Subtypes and Pathomechanisms

Hemorrhagic stroke results from a weakened vessel that ruptures and bleeds into the surrounding brain. The blood accumulates and compresses the surrounding brain tissue. There are two distinct mechanisms for two main types of hemorrhagic strokes: bleeding directly into the brain parenchyma (ICH), or bleeding into the cerebrospinal fluid containing sulci, fissures, and cisterns (SAH). Other disorders involving bleeding inside the skull include epidural and subdural hematomas, which are usually caused by a head injury and are not considered strokes.

**123.2.2.1 Intracerebral Hemorrhage.** Primary spontaneous ICH occurs as a result of the spontaneous rupture of small blood vessels damaged by hypertension or amyloid angiopathy. This has an incidence of 7–17 per 100,000 and accounts for 78–85% of all cases (15). Secondary spontaneous ICH occurs in the presence of a preexisting lesion such as avascular or parenchymal abnormality, the most common cause being arteriovenous malformations. Other causes include arteriovenous malformations, cavernous hemangiomas, intracranial aneurysms, venous sinus thrombosis, hemorrhagic transformation of ischemic stroke, coagulopathy, intracranial tumors, or vasculitis.

**123.2.2.2 Subarachnoid Hemorrhage.** Rupture of a cerebral aneurysm is the most common cause and accounts for about 85% of SAH. Cerebral aneurysms are present in about 2% of asymptomatic adults. Intracranial aneurysms are found in 2–5% of all autopsies; however, the incidence of rupture is only 2–20/100,000 individuals/year (16). Nonaneurysmal perimesencephalic SAH accounts for 21–68% of angiography negative SAH (17). The presentation of this subgroup is typically indistinguishable from other types of SAH, but their prognosis is excellent.

### 123.2.3 Cerebral Venous Thrombosis

Cerebral venous thrombosis (CVT)—i.e. thrombosis of the intracranial veins and sinuses—is a rare type of cerebrovascular disease that affects about five people per million and accounts for 0.5% of all strokes (18). The risk factors for venous thrombosis in general are linked classically to the Virchow triad of stasis of the blood, changes in the vessel wall, and changes in the composition of the blood. Risk factors are usually divided into acquired risks and genetic risks (19).

### 123.2.4 Spinal Cord Stroke

Spinal cord infarction is uncommon and accounts for 1.2% of all strokes (20). It is a rare but often devastating disorder caused by a wide array of pathologic states. The onset of spinal cord infarction is typically abrupt, and the vascular territory involved largely defines neurologic presentation.

### 123.2.5 Stroke Classification

It is not always possible to identify a specific cause of stroke; in fact, most registers failed to do so in 25–39% of stroke patients (21). Number of classifications on stroke subtype have been published. Their purpose was to uniformly classify patients for clinical trials, genetic, or epidemiological studies and to help classify patients for therapeutic decision making in daily practice.

Stroke Data Bank Subtype Classification (22) recognized five major groups: brain hemorrhages; brain

infarctions; cardioembolic stroke; lacunar stroke; and stroke from rare causes or undetermined etiology.

The Oxfordshire Community Stroke Project (OCSP) was proposed to characterize this population-based epidemiological study. This classification was based on clinical findings only. OCSP classification recognized four types of stroke: lacunar infarct (LACI); total anterior circulation infarct (TACI); partial anterior circulation infarct (PACI); and posterior circulation infarcts (POCI).

The Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification is the most commonly used by clinical researchers (14). This classification is used to categorize ischemic stroke etiology into five groups: large-artery atherosclerosis; cardioembolism; small-vessel occlusion (lacunae); stroke of other determined cause; and stroke of undetermined cause.

## 123.3 HERITABILITY: TWIN AND FAMILY STUDIES

In twin studies, the concordance rates for stroke are 17.7% in monozygotic twins and 3.6% in dizygotic twins (23,24). The heritability of stroke has varied in family studies, but allowing for some methodological weaknesses, it appears that a family history of stroke increases a subject's stroke risk by 2- to 3-fold (25,26). In the Framingham study, using the information obtained across three generations, including the original and offspring cohorts, a parental history of stroke was associated with an approximately 2-fold increase in stroke risk: a paternal history of stroke was associated with a relative risk of 2.4 and a maternal history with a risk of 1.4 (25). In later studies, looking at the heritability of stroke subtypes, the large-artery atherosclerosis and small-vessel subtypes were found to be more commonly inherited than the cardioembolic subtype (27). Few studies have separated ischemic stroke risk from that of ICH, probably because of the smaller frequency of the ICH stroke type and the difficulty in retrospectively obtaining this information. It appears that ICH in a first-degree relative increases a subject's odds by as much as 2- to 6-fold (28). SAH has a distinct clinical profile, and a family history of SAH has been associated with an increased risk for SAH of about 4-fold (29); because of this association, screening of first-degree relatives of patients with SAH is currently recommended (30). Evidence of a gene-environment interaction with smoking, another risk factor for SAH, also may exist for aneurysmal SAH (31).

## 123.4 SINGLE-GENE DISORDERS CAUSING STROKE

Single-gene disorders is a group of diseases caused by mutation in a single gene. In contrast, polygenic disorders are due to interaction of multiple gene mutations with lifestyle and environmental factors.



### 123.4.1 Ischemic Stroke

Ischemic stroke was divided into etiological categories according to TOAST criteria (14).

### 123.4.2 Small-Vessel Disease

#### 123.4.3 CADASIL – Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (NOTCH3 gene)

Cerebral arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)—Online Mendelian Inheritance in Man (OMIM) #125310 (<http://www.ncbi.nlm.nih.gov/omim/125310>, Accessed June 20, 2011)—is an autosomal dominant disorder. It affects small arterial vessels due to mutation in the NOTCH3 gene mapped on 19p13.2-p13.1.

From 1955, symptoms were recognized as Binswanger disease without hypertension (32), until 1991 when the relevant gene was mapped on chromosome 19 (33). Product of the NOTCH3 gene large type-1 transmembrane receptor (NOTCH3—N3) is mainly expressed in vascular smooth muscle cells and pericytes. Mature N3 consists of two independent domains: intracellular N3-ICD present in nucleus and responsible for gene expression regulation and extracellular N3-ECD. In CADASIL, mutations are present in N3-ECD within one of the 34 epidermal growth factor (EGF)-like repeat domains, each including six cysteine residues. All mutations, missense, deletion, and splice site, lead to an odd number of cysteine residues. Odd number of cysteines due to CADASIL mutations has been linked to multimerization of N3-ECD (34). Deposits of multimerized N3-ECD are components of the granular osmiophilic material pathognomonic for CADASIL on electron microscopy.

**123.4.3.1 Clinical Presentation.** CADASIL starts at the mean age of 45 years and leads to death in 10 to 25 years (35). The disease is characterized by five main symptoms: migraine with aura, subcortical ischemic events, mood disturbances, apathy, and cognitive impairment.

**Migraine with aura** occurs in approximately 20–40% of CADASIL patients—five times more than in general population. Frequency of migraine without aura is the same as in general population (32). Migraine is also a first presenting symptom in 34–36% of patients—usually before the age of 40 years (35). Symptoms, frequency, and severity of migraines vary across patients. Most attacks involve visual and sensory aura lasting 20–30 minutes, followed by prolonged headache. Atypical attacks with basilar, hemiplegic, or prolonged aura may be difficult to differentiate from ischemic episodes. Frequency of migraines may increase until first stroke, thereafter it cease or decrease.

**Subcortical ischemic events** represented by TIA and ischemic strokes affect up to 84% of patients with

CADASIL. In 70% of patients, these are the first CADASIL symptoms (35), occurring at the mean age of 49 years (32). Ischemic events are almost always subcortical and lacunar, affecting white matter and basal ganglia. Patients experience recurrent strokes, leading to progressive disability and pseudobulbar palsy.

**Mood disturbances and apathy** are the most common psychiatric manifestations of CADASIL. Mood disorders are present in 20% of patients with moderate depression, being the most common symptom. Other manifestations include aggression, agitation, delusional states, dysthymia, emotional lability, mania, paranoia, and schizophrenia-like symptoms (36). Apathy is present in 40% of patients with CADASIL and is associated positively with the load of white matter MRI lesions and with a decrease in quality of life (37).

**Cognitive impairment** is the second most frequent symptom in patients with CADASIL, rarely present at the onset of the disease. Frequency varies from 30 to 90% depending on patient's age (36). Cognitive deficit is slowly progressing, worsening after recurrent strokes.

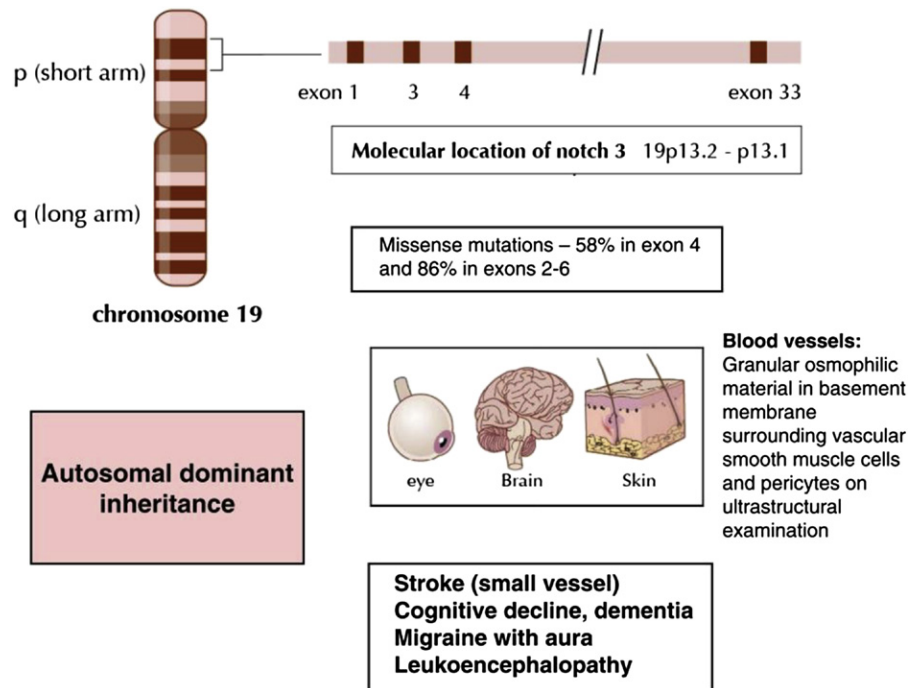
**Other clinical manifestations** include primary and secondary to stroke seizures 5–10% of patients, ocular symptoms, and ICHs (32) Figure 123-1.

**123.4.3.2 Diagnosis.** Genetic testing for the presence of EGF domain mutation in NOTCH3 gene is the only method to diagnose CADASIL. Due to the expensive and time-consuming procedure, patients should be preselected for genetic analysis based on diagnostic screening criteria and clinical presentation. MRI neuroimaging is the primary test that may be supported with the results from skin biopsy.

**123.4.3.3 Neuroimaging.** In most cases, MRI changes precede other common symptoms by 10–15 years appearing at the age of 30–35 years (32). Angiography is usually normal and is contraindicated due to increased risk of complications. MRI changes are present in T2- and T1-weighted images and fluid attenuated inversion recovery (FLAIR). Typically, symmetrical and diffuse MRI changes are present in periventricular areas and centrum semiovale. LACIs occur mostly in external capsule and anterior part of temporal lobes—location characteristic for CADASIL (32). Less frequently, changes may be present in the basal ganglia and thalamus, sporadically in the brainstem and corpus callosum (39). Focal microbleeds are commonly found in CADASIL patients (40).

**123.4.3.4 Skin biopsy.** Skin biopsies, based on two methods, were shown to be helpful in selecting patients for genetic testing. Samples stained with monoclonal antibodies specific for NOTCH3 showed high sensitivity (93%) and high specificity (100%) (41), whereas ultrastructural testing for extracellular deposits of granular osmiophilic material (GOM) in the tunica media was less sensitive (57%) and equally specific (100%) (42).

**123.4.3.5 Treatment.** So far, there is neither a specific nor a proven treatment for CADASIL. Separate treatment of CADASIL symptoms has also not been validated.



**FIGURE 123-1** Pathophysiology of CADASIL (38), by permission of Elsevier Inc. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), autosomal dominant disorder due to mutation in the Notch 3 gene. This gene is located on chromosome 19, on the p (short) arm, at 19p13.2 to p13.1. Notch 3 gene contains 33 exons; the majority of single-point or missense mutations occur on exons 2 to 6. There is an accumulation of osmophilic granules in smooth muscle of blood vessels throughout the vasculature, particularly in the brain, the retina, and the skin.

#### 123.4.4 CARASIL – Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (HTRA1)

Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL)—OMIM #600142 (<http://omim.org/entry/600142>; Accessed June 20, 2011)—is an autosomal recessive disorder. It was named after Bowler and Hachinski in 1994 (43). Disease was diagnosed approximately in 50 Asian cases; except for two Chinese patients, all come from Japan (44). So far, there was only one Caucasian case of CARASIL carrying HTRA1 gene nonsense mutation (44). Disease affects small arterial vessels due to mutations in the HTRA1 gene mapped on chromosome 10q25.3-q26.2 (45). HTRA1 gene codes for HtrA serine peptidase/protease 1 (HTRA1) that represses signaling by transforming growth factor (TGF)- $\beta$  family members (46). Genome-wide linkage analysis in six consanguineous Japanese families with CARASIL identified 45 mutations in HTRA1 gene. In the case of a mutation, protein fails to repress TGF- $\beta$ , and the amount of TGF- $\beta$  is increased in the media of cerebral small arteries (45). As a result, intense arteriosclerosis is present mainly in the small penetrating arteries of the basal ganglia and cerebral white matter. Different than CADASIL, there are no GOM deposits of amyloid in the tunica media (46).

**123.4.4.1 Clinical Presentation.** Cerebral onset of CARASIL is between 20 and 45 years of age, with the

mean age of 32 (46). Duration of life ranges from 10 to 20 years; however, patients become bed-ridden within 10 years of onset (45). The disease is characterized by four main symptoms: ischemic stroke or stepwise deterioration; cognitive deficits; orthopedic complications; and alopecia (premature baldness).

**123.4.4.2 Ischemic Stroke or Stepwise Deterioration.** Characteristic lacunar strokes localized in basal ganglia and brainstem are present in 50% of the patients. Remaining patients develop stepwise deterioration, pseudobulbar palsy, pyramidal and extrapyramidal signs and gait disturbances (46).

**123.4.4.3 Cognitive Deficits.** Cognitive deficits occur in almost all CARASIL patients developing dementia by the age of 30–40 years. Symptoms that may occur include: forgetfulness, dyscalculia, time disorientation, personality changes, emotional incontinence, severe memory dysfunction, and finally abulia and akinetic mutism (46). Focal signs like aphasia or apraxia and abnormal behavior were not seen (46).

**123.4.4.4 Orthopedic Complications.** 80% of CARASIL cases develop acute middle to lower back pain. Spondylosis deformans or disk degeneration occurs, based on magnetic resonance imaging (MRI) and X-ray imaging, in cervical and/or thoracolumbar spine (46). Other orthopedic complications include osseous structures like kyphosis, ossification of intraspinal canal ligaments, deformity of the elbows, and arthropathy of the knee joints (47).

**123.4.4.5 Alopecia.** Hair loss present in 90% of the patients starts as early as adolescence and is limited to the head (46). Alopecia is less prominent but also present in affected women (47).

**123.4.4.6 Diagnosis.** Patients presenting with CARASIL's characteristic symptoms, lacunar strokes and white matter changes and cognitive deficits, alopecia and spondylosis, should be tested for HTRA1 gene mutations to confirm the diagnosis. Differential diagnosis should include Binswanger disease, angiitis of the central nervous system (CNS), CADASIL, chronic progressive multiple sclerosis, and leukodystrophies with dermatologic or skeletal disorders (46).

**123.4.4.7 Treatment.** At present, there is neither specific treatment of CARASIL itself nor its symptoms.

### 123.4.5 RVCL – Retinal Vasculopathy with Cerebral Leukodystrophy (TRESX1)

Retinal vasculopathy with cerebral leukodystrophy—RVCL—OMIM#192315 (<http://omim.org/entry/192315>; Accessed June 20, 2011)—is an autosomal dominant disease caused by mutations in TRESX1 gene on chromosome 3p21. Since 2007, RVCL encompasses three diseases—cerebroretinal vasculopathy (CRV), hereditary vascular retinopathy (HVR), and hereditary endotheliopathy with retinopathy and stroke (HERNS). Although clinical symptoms differ in the pathomechanisms of those three diseases, genetic mutations are the same (48). The TRESX1 gene codes for 3' > 5' DNA exonuclease. Mutations are present in C-terminus of TRESX1 gene and cause frame shift. Gene product retains exonuclease activity but loses normal perinuclear localization (48).

**123.4.5.1 Clinical Presentation.** RVCL ranges from 30 to 50 years of age, and death occurs within 5–10 years from the symptoms onset (49). Features commonly noted in patients with RVCL include neurological and ophthalmological manifestations. Other organs may also be involved and appear as renal impairment, proteinuria, hematuria, micronodular cirrhosis, gastrointestinal bleeding, anemia, and Reynaud's phenomenon.

**123.4.5.1.1 Neurological Manifestations.** A neurological manifestation encompasses clinical, radiological, and pathological changes. Strokes are usually localized in deep white matter and often resemble tumors due to irregular shape, mass effect, and edema. Other manifestations include seizures, migraine-like headaches, motor/sensory/cerebellar deficits, and psychiatric disturbances (50). Localized necrotic changes in the walls of the vessels may resemble obliterative vasculopathy.

**123.4.5.1.2 Ophthalmological Manifestations.** Progressive visual loss is the main feature of RVCL. Predominantly changes occur around macula. Loss of vision is secondary to retinopathy, microaneurysms with telangiectasia, and capillary dropout.

**123.4.5.2 Diagnosis.** Genetic screening for TRESX1 mutation will be positive and negative for mutations in NOTCH3 and HTRA1 genes.

**123.4.5.3 Treatment.** There is no specific treatment for RVCL.

### 123.4.6 Large-artery disease

**123.4.6.1 EDS IV – Ehlers–Danlos Syndrome Type IV (COL3A1).** Ehlers–Danlos Syndrome type IV (EDS IV)—OMIM#130050 (<http://omim.org/entry/130050>; Accessed June 20, 2011)—is an autosomal dominant disease. EDS IV is the vascular type, one of the six types of Ehlers–Danlos Syndrome (51). Disease is caused by mutation in type III collagen (COL3A1) gene on chromosome 2q31 (52). Mutations in COL3A1 are responsible for the structural defects in the protein pro  $\alpha$  1(III) chain of collagen type III. Presence of at least two major symptoms is indicative of the diagnosis; however, laboratory tests are needed. Four major diagnostic criteria include 1) thin and translucent skin, 2) fragility or rupture of arteries, uterine or intestines, 3) extensive bruising, and 4) characteristic facial appearance (51).

**123.4.6.1.1 Clinical Presentation.** Major clinical manifestations are seen in most patients by the age of 40 years, and median life span reaches 48 years (52). Vascular type is characterized by the highest mortality among other EDS types, high pregnant-related complications, fragile vessel prone to rupture joint hypermobility dermatological manifestations, and gastrointestinal complications.

Dermatological manifestations are usually the earliest in EDS IV. Vascular complications are leading causes of death with cerebrovascular events represented by stroke in young age, carotid–cavernous fistula, dissection of extracranial and intracranial segments of the vertebral and carotid arteries and aneurysms (53). In EDS IV, due to high complication rate, arteriography is not recommended (53).

**123.4.6.1.2 Diagnosis.** In addition to the presence of at least two major diagnostic criteria, abnormal structure of COL3A1 protein or identification of COL3A1 gene mutations is required to confirm the diagnosis (52).

**123.4.6.1.3 Treatment.** In EDS IV, there is no specific treatment, and medical interventions are limited to symptomatic treatment.

### 123.4.7 PXE – Pseudoxanthoma Elasticum (ABCC6)

Pseudoxanthoma elasticum (PXE)—OMIM #264800 (<http://omim.org/entry/264800>; Accessed June 20, 2011)—is an autosomal recessive disease affecting connective tissue. Disease is an effect of the mutation in ABCC6 gene on chromosome 16p13.11. Polymorphisms in xylosyltransferase genes XYLT1 and XYLT2 may modify severity of PXE. The prevalence is estimated to be about one in



25,000 to 100,000, with high variability both in the age of onset and in the severity of organ involvement. The disease primarily affects the skin, retina, and cardiovascular system. PXE is characterized pathologically by high elastic fiber mineralization (elastorrhexia), resulting in increased rate of elastin synthesis and degradation (54).

**123.4.7.1 Clinical Presentation.** The skin is the first affected organ. Yellow papules of 1 to 5 mm are the primary skin lesions. They are typically located on the neck and in flexural surfaces. Mucosal, genital, and navel area lesions are found less frequently.

The eye contains thin layer of elastic tissue called Bruch's membrane, located between the retinal pigment epithelium and the choriocapillaris. Elastic fiber alterations result in angioid streaks, characteristic for PXE. Other ocular manifestations—less specific for PXE—include appearance of 'peau d'orange, drusen, and comet-like streaks.

**Cardiovascular system** complications occur due to the changes in elastic fiber-rich arterial wall. Slowly progressing segmental arterial narrowing affects small- and medium-size arteries. Cardiovascular manifestations of PXE may be further divided in to the occlusive arterial disease and mucosal bleeding. Most commonly observed are coronary artery disease, arterial hypertension, restrictive cardiomyopathy, sudden cardiac failure, and gastrointestinal hemorrhages (55).

**Neurological complications** in PXE are mainly expressed by increased incidence of ischemic strokes due to small-vessel disease (56). ICH has also been reported in few PXE patients (57). However, IA has not been found in any of 100 PXE patients studied by Berg and colleagues (56).

**123.4.7.2 Diagnosis and Treatment.** Presence of angioid streaks in eye examination and dermal elastorrhexia (with or without clinically visible skin changes) represent the minimum criteria to diagnose PXE. Occurrence of ABCC6 gene mutation in one of the 31 exons confirms the diagnosis (55).

There is no specific treatment; however, antiplatelet drugs, high blood pressure, and contact sports should be avoided. Symptomatic treatments include retinal laser treatment and photodynamic therapy and valvular surgery in selected cases (57).

## 123.4.8 Small-Vessel Disease and Large-Artery Disease

**123.4.8.1 FD – Fabry Disease (GLA).** Fabry Disease (FB)—OMIM# 301500 (<http://omim.org/entry/301500>; Accessed June 20, 2011)—is an X-linked congenital dysfunction of glycosphingolipid (GSL) catabolism subsequent to deficient or absent activity of the lysosomal enzyme alpha-galactosidase A ( $\alpha$ -gal A) coded by gene *GLA* located on chromosome Xq22.1.  $\alpha$ -gal A activity in FD patients is usually lower than 1%, whereas the symptoms of enzyme deficiency are present below the level of 5–10% (58). Defects in  $\alpha$ -gal A impair its ability to

degrade membrane GSL, especially globotriaosylceramide (Gb3), which accumulates in various tissues throughout the body (59). Gb3 is found in all parts of blood vessel wall, in Schwann cells, dorsal root ganglia and in CNS neurons, leading to organ dysfunction (60).

Polymorphisms in genes, coding for interleukin-6, endothelial nitric oxide synthase, factor V and protein Z, affect the phenotypic expression of single-gene disorder in FD probably due to protein interactions (60).

The incidence of FB is estimated at one in 55000 male births (58); however, results from a newborn screening study by Spada et al. estimate higher rate of FD incidence falling between one in 3,100 and one in 4,600 individuals (61).

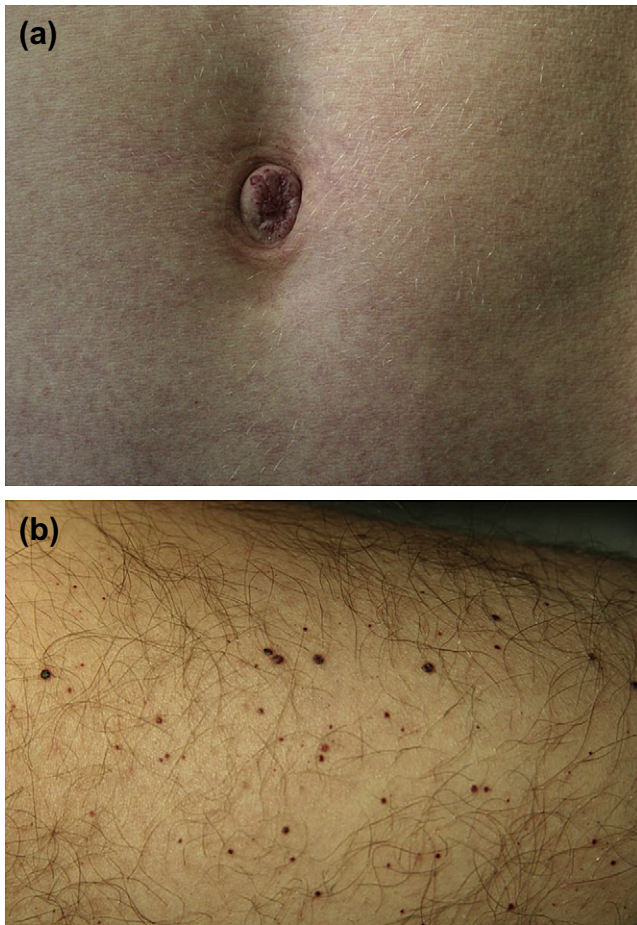
Some patients with residual  $\alpha$ -gal A activity develop a variant of the disease in which they develop only cardiac disease, specifically left ventricular hypertrophy, with or without renal failure, in the sixth decade of life (62). Although FB is an X-linked disease, it also affects females. Some woman may develop significant life-threatening conditions, requiring medical treatment and intervention (63).

**123.4.8.2 Clinical Presentation.** Major clinical manifestations develop from childhood until third decade in most cases. Median life span is decreased by 15–20 years (58). Clinical onset is characterized by painful burning sensations in the hands and feet (acroparesthesias), typical skin lesions (angiokeratomas), hypohidrosis, and corneal opacities. Further stages of the disease lead to the renal failure and vascular disease of the heart and the brain, with premature death in the fourth and fifth decades of life. Late-onset variants start from the sixth decade, and patients develop renal and/or cardiac disease without other neurologic symptoms. Based on results from Fabry Outcome Survey (64), neurologic symptoms were the most frequent, affecting 75% of males and 61% of females. Renal failure was present in 19% of males and 3% of females. Cardiac manifestations were recorded in 60% of males and in 50% of females. Cerebrovascular events occurred in 25% of males and 21% of females, and stroke was present in 5% of females and in 9% of males.

**123.4.8.2.1 Pain and Skin Lesions.** Neuropathic burning pain (acroparesthesia) usually involves hands and feet. It may be transient or may persist for several hours. The acroparesthesia is typically resistant to treatment with conventional analgesics and may require narcotic analgesics, phenytoin, amitriptyline, or gabapentin or combinations of these drugs (65). The pain is supposed to be caused by lysosomal accumulation of GSL in peripheral nerves, dorsal root ganglia, and the spinal cord and atrophy of the small, unmyelinated nerves involved in pain and temperature sensation (58).

FD is characteristic of angiokeratomas—vascular lesions characterized by thin-walled vessels beneath a hyperkeratotic epidermis. Skin lesions are usually located in periumbilical, scrotum, and penis areas (58) **Figure 123-2.**





**FIGURE 123-2** Umbilical (a) and thigh (b) angiokeratomas—vascular lesions characteristic for Fabry Disease. Image courtesy of M. Podolec-Rubis, MD and K. Podolec, MD (Department of Dermatology UJ Medical College).

**123.4.8.2.2 Impaired Renal Function and Cardiac Changes.** Renal failure occurs in midadulthood; the first indication is often isosthenuria, followed by proteinuria and gradual decline in glomerular filtration rate over time, leading to end-stage renal failure (66).

Cardiac complications of FD may include cardiac arrhythmias and conduction defects developed in the first two decades of life—progressive left ventricular hypertrophy, aggravated by arterial hypertension, which is followed by progressive impairment of diastolic filling that leads to decreased cardiac output and early death (58).

**123.4.8.2.3 Neurological Changes.** Population studies estimate that FD is responsible for 1.2% of cryptogenic strokes in patients younger than 55 years, and women are more likely to be affected than men (27% vs 12%) (67). Stroke may result from either cardiac or vascular factors and may cause both large- and small-vessel diseases. The large vessels dilate, resulting in dolichoectatic changes characteristic for FD. Subsequent flow stagnation increases the risk of artery-to-artery embolism and vessel thrombosis. These changes are found more frequently in the posterior circulation

(60). Stenosis in small arterial vessels is caused by gradual accumulation of GSL in endothelial and vascular smooth muscle cells (67).

**123.4.8.3 Diagnosis.** FD may be diagnosed based on the direct measurement of  $\alpha$ -gal activity in leukocytes or plasma in all except heterozygotic patients. In those subjects, gene sequencing and genetic linkage studies may be necessary (60).

**123.4.8.4 Treatment.** Treatment with  $\alpha$ -gal enzyme replacement therapy (ERT), approved by the US Food and Drug Administration in 2003, results in the reduction in the amount of vascular endothelial GSL deposits. ERT decreased the progression and severity of the changes observed in kidney, heart, skin, and liver. Unfortunately, ERT treatment did not change the incidence of stroke and other vasculopathic manifestations of the disease (67). Therefore, the use of antiplatelet and antihypertensive treatment is necessary to prevent secondary and primary strokes (60).

**123.4.8.5 Homocystinuria (CBS).** Homocystinuria indicates an increased urinary excretion of the oxidized form of homocysteine, homocystine. Classic homocystinuria—OMIM#236200 (<http://omim.org/entry/236200>; Accessed June 20, 2011)—is an autosomal recessive metabolic disorder caused by mutation in the gene encoding cystathionine beta-synthase (CBS) located on chromosome 21q22.3. The CBS gene has been both cloned and sequenced, revealing more than 140 mutations (68).

Homocystinuria is characterized by elevated levels of plasma. Usually, plasma homocysteine concentrations rises above 100 mmol/l, which is around 10-fold higher than normal (69). Homocysteine level elevation with a normal methionine level may be caused by metabolic errors that affect the conversion of homocysteine to methionine, such as methylene tetrahydrofolate reductase deficiency and disorders of cobalamin (vitamin B12) metabolism. The mechanism by which elevated homocysteine concentrations result in clinical manifestations remains unclear, although it has been shown to directly damage endothelium and promote smooth muscle cell proliferation (69).

The incidence of homocystinuria differs across countries and is estimated from 1:58,000 to 1:1,000,000, with overall rate of 1:344,000 (68). Clinically, two equally prevalent phenotypes have been described: a milder pyridoxal phosphate (vitamin B6), responsive form, and a more severe pyridoxal phosphate, nonresponsive form.

**123.4.8.6 Clinical Presentation.** The most common type homocystinuria type I is characterized by intellectual disability, lens dislocation, skeletal abnormalities, and thrombotic vascular disease due to a deficiency of the enzyme cystathionine synthase. Homocystinuria may also be due to defects in methyl cobalamin formation, which are specific for homocystinuria type II characterized by the triad of megaloblastic anemia, homocystinuria, and hypomethioninemia. Homocystinuria type III is a result of the deficiency of the enzyme methyltetrahydrofolate

reductase. Type III is characterized by homocystinuria and homocystinemia with low- or normal-blood methionine levels (70).

Homocystinuria can cause stroke through atherosclerosis, thromboembolism, small-vessel disease, and arterial dissection. Cerebrovascular events represent around 30% of thromboembolic complications (71).

**123.4.8.7 Diagnosis and Treatment.** The diagnosis of homocystinuria is based on clinical symptoms and laboratory studies. Plasma tests usually reveal hyperhomocystinemia, hypermethioninemia, and hypocystinemia, and the urinary excretion of methionine, homocysteine, and its oxidized form (homocystine) is elevated. Cultured fibroblasts, amniotic fluid, and chorionic villi cells are used to evaluate the activity of cystathionine synthase activity (72).

Treatment in hypocystinemia is aimed at lowering the plasma level of homocysteine—possibly to the normal values. Patients must adhere to a methionine-restricted diet. Roughly 50% of them respond to pyridoxine (vitamin B6). In addition, folate, betaine, and vitamin B12 are used to promote metabolism of homocysteine to methionine (72). Dietary supplementation with folic acid lowers plasma homocysteine concentrations by about 25%. Additional B-vitamin lowers homocysteine by about 10 to 15% (73).

## 123.4.9 Cardioembolic Stroke

**123.4.9.1 MS – Marfan Syndrome (FBN1).** Marfan Syndrome (MS)—OMIM#154700 (<http://omim.org/entry/154700>; Accessed June 20, 2011)—first described by Antoine-Bernard Marfan in 1986 is an autosomal dominant disorder of connective tissue caused by heterozygous mutation in fibrillin-1 gene (*FBN1*) located on chromosome 15q21.1. Main manifestations of MS involve cardiovascular system, eyes, skeleton, pulmonary system, skin, and dural sac. Family history is not conclusive in 27% of cases due to sporadic de novo *FBN1* gene mutations (74). Both hereditary and new mutations in *FBN1* gene lead to abnormal protein folding and enhanced proteolytic degradation. Mutant protein boosts matrix metalloproteinase (MMP) 2 and 9 activity and interferes with TGF  $\beta$  (TGF $\beta$ ) pathway. Recent evidences show that both abnormal TGF $\beta$  pathway and mutations in *TGFBR1* and *TGFBR2* genes may lead to Marfan-like phenotypes including Marfan syndrome II and Loeys-Dietz aortic aneurysm syndrome.

### 123.4.9.2 Clinical Presentation.

**123.4.9.2.1 General Complications.** Clinical characteristics of MS include tall stature, arm span higher than patient's height, reduced upper-to-lower body segment ratio, pectus carinatum or excavatum, ectopia lentis, scoliosis, mitral valve prolapse, aortic root dilatation, and aortic dissection (74).

**123.4.9.2.2 Neurological Complications.** In the largest retrospective study of MS patients by Wityk et al.

(75), neurovascular complications were found in 3.5% of patients. TIA was the most common complication, and cardiac source of embolism was found in 77% of all ischemic events. Neither cerebral artery dissection nor intracranial aneurysms (IA) were found in any of the studied patients. Another two studies by Conway and colleagues—firstly, found no statistical difference in the prevalence of IA between MS patients and general population (76); secondly, found no MS patients among 710 neurosurgical patients treated for IA (77).

**123.4.9.3 Diagnosis.** One of 500 so far discovered mutations in *FBN1* gene spanning 235 kb may occur in any of 65 exons, making the genetic testing costly and time consuming (78). Therefore, according to revised Ghent criteria, diagnosis is established primarily on clinical characteristics and family history (79). Personal or familial history of thoracic aortic aneurysm in tall, thin patients optionally with scoliosis, arachondactyly should always be suspected of MS.

## 123.4.10 Familial Cardiomyopathies and Familial Arrhythmias

Cardioembolic stroke and TIA are common complications of all types of cardiomyopathy, with AF being the main cause. Cardiomyopathies are classified according to their morphological characteristics as hypertrophic (HCM), dilated (DCM), arrhythmogenic right ventricular (ARVC), and restrictive cardiomyopathy (RCM). A genetic cause has been shown in significant number of patients: HCM (50%), DCM (35%), and ARVC (30%) (80). Cardiac dysrhythmias may be divided to supraventricular and ventricular. Dysrhythmias has been linked to number of genes and chromosomal loci. AF has been extensively studied and was mapped to four genes and nine chromosomal loci (81). In a largest study on 900 patients with hypertrophic cardiomyopathy by Maron et al. (82), 57 (6%) experienced thromboembolic event. Six of them were due to hemorrhagic stroke or brain tumor; Ischemic stroke occurred in 44 patients; 70% out of the remaining 51 with nonhemorrhagic thromboembolic event. Cardioembolic etiology of ischemic stroke was definite in eight cases and probable in 31, all of them had AF.

## 123.4.11 Other causes

**123.4.11.1 MELAS – Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke (A3243G).** MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes syndrome)—OMIM# 540000 (<http://omim.org/entry/540000>; Accessed June 20, 2011)—can be caused by mutation in several mitochondrial genes, including *MTTL1*, *MTTQ*, *MTTH*, *MTTK*, *MTTC*, *MTTS1*, *MTND1*, *MTND5*, *MTND6*, and *MTTS2*. Mitochondrial (mt) DNA is inherited exclusively from the mother (maternally

inheritance). The most common mtDNA mutation—A-to-G transition at nucleotide 3243—is present in 80% of MELAS patients (67). A3243G mutation is in the tRNA—leucine (UUR) gene, associated with respiratory chain complex I deficiency (67). The prevalence of the A3243G mutation varies across different population—it is estimated to be 7.59 per 100 000 persons in North East England, 16.3 per 100 000 in Northern Finland, and 236 per 100 000 in Australia (83).

Although the etiology of MELAS is not completely understood, there is likely a role for mitochondrial angiopathy, vascular dysfunction, and hyperemia, as well as mitochondrial-mediated cytopathic mechanisms, resulting in energy failure. Neuronal hyperexcitability may also play a role (84).

**123.4.11.1.1 Clinical Presentation.** Normal early development, followed by stroke-like episodes (before the age of 40 years), mitochondrial encephalomyopathy, and lactic acidosis, is typical for MELAS. Other clinical manifestations of MELAS may include exercise intolerance, short stature, central and peripheral nervous system involvement, hearing loss, and eye, heart, and gastrointestinal complications with accompanying DM (67).

CNS manifestations include stroke-like episodes, seizures, encephalopathy or dementia, headache, and elevated level of proteins in cerebrospinal fluid. Peripheral nervous system manifestations are represented by myopathy and peripheral neuropathy. The etiology of stroke-like lesions is not yet understood. The lesions have predilection to the posterior areas of the brain and may not follow arterial territory distribution (67).

**123.4.11.1.2 Diagnosis and Treatment.** Due to unequal load of mutated mtDNA, its content varies across different tissues, and genetic tests are not always conclusive. Sue et al. compared the detection rates for A3243G point mutation in muscle, blood, and hair follicles and found that in 50% mutation was absent in blood (85). Therefore, clinical symptoms, a key factor in the MELAS diagnosis, supported by laboratory tests, confirmed by biopsy and genetic studies are all essential in the diagnostic workup. Patients usually have increased lactate and pyruvate levels in serum and cerebrospinal fluid, and elevated lactate to pyruvate ratio. Muscle biopsy reveals typical ragged-red fibers (86).

Recently Janssen et al. presented a new method to diagnose MELAS—the mitochondrial energy-generating system (MEGS). MEGS as an indicator for the overall mitochondrial function related to energy production showed a great capacity for detection of subtle mitochondrial dysfunction (83). This method may be very useful in diagnosing patients with rare or new mitochondrial DNA mutations, and with low mutation loads.

Several treatment approaches have been tested to treat MELAS patients so far. Coenzyme Q10 and its synthetic analog Idebenone have been used to improve electron transfer in the mitochondrial respiratory chain. Their role in MELAS remains to be proven (67). Clinical

trial with another agent, dichloroacetate, was terminated earlier owing to frequent peripheral nerve toxicity (87). Finally, treatment with L-arginine (L-arg) shortly after the onset of stroke-like episodes improved patient's outcome and normalized the concentration of lactate and pyruvate (88).

### 123.4.12 NF 1 – Neurofibromatosis type I(NF-1)

Neurofibromatosis type I (NF1)—OMIM# 162200 (<http://omim.org/entry/162200>; Accessed June 20, 2011)—also known as von Recklinghausen disease is an autosomal dominant disorder caused by mutation in the neurofibromin gene (NF1) on chromosome 17q11.2. New mutations are very common, since 50% of patients are the first to be affected in the family (89). The NF1 gene codes for neurofibromin, a protein that is highly expressed in the nervous system. It functions as a tumor suppressor, and therefore, its loss leads to development of benign and malignant tumors (89). NF1 is approximately one per 2500 to 3000 individuals (90). NF1 decreases mean and median ages at death from 70.1 and 74 years to 54.4 and 59 years, respectively (91).

**123.4.12.1 Clinical Presentation.** Clinical features of NF1 encompasses café-au-lait spots, intertriginous freckling, and Lisch nodules, cutaneous, subcutaneous, and plexiform neurofibromas, macrocephaly, optic glioma, and other neoplasms (92). Based on the most common clinical features, the National Institutes of Health Consensus Development Conference formulated the diagnostic criteria for NF1 (93) (Table 123-2).

Reports on the incidence of neurological complication vary among authors, from 2.5% according to Roser et al. (94) to 20% and 26%, respectively; according to Griffiths et al. (95) and Hsieh et al. (96) Epilepsy (8.7%) and cerebral infarction (7.2%) are the most common neurological complications (96).

### 123.4.13 Ischemic and Hemorrhagic Stroke

**123.4.13.1 SCD – Sickle-Cell Disease (HBB).** Sickle-cell disease (SCD) refers to all the different genotypes that cause the characteristic clinical syndrome, whereas

**TABLE 123-2 NF1 Diagnostic Criteria (93)**

**At least two of the following criteria are required to diagnose NF1**

- Six or more café-au-lait macules (>0.5 cm in children or >1.5 cm in adults)
- Axillary or inguinal regions freckling
- At least 2 neurofibromas of any type or one plexiform fibroma
- Optic pathway glioma
- Two or more Lisch nodules (iris hamartomas)
- Osseous dysplasia
- A first-degree relative with NF1 diagnosed by these criteria



sickle-cell anemia—OMIM# 603903 (<http://omim.org/entry/6039030>; Accessed June 20, 2011)—represents the most common form of SCD, which is the result of the mutation in beta globin (*HBB*) gene located on chromosome 11p15.4. The most common type of sickle-cell disease is the homozygosity for the  $\beta$ S allele (HbS), and the remaining types include hemoglobin SC disease (HbSC disease) with coinheritance of the  $\beta$ S and  $\beta$ C alleles and HbS/ $\beta$ -thalassemia due to coinheritance of  $\beta$ S with a  $\beta$ -thalassemia allele (97).

HbS is caused by a T>A mutation in the  $\beta$ -globin gene in which the 17th codon is changed from thymine to adenine and the sixth amino acid in the  $\beta$ -globin chain becomes valine instead of glutamic acid (98). Mutant protein causes  $\beta$  globin chains to crystalize, resulting in a sickled appearance. Changes disrupt erythrocyte architecture and flexibility and promote cellular dehydration with physical and oxidative cellular stress (98). The disease severity is determinant by the rate and extent of HbS polymerization. Several mechanisms are involved in the pathophysiology of the SCD: vaso-occlusion with ischemia-reperfusion injury and hemolytic anemia causing hemoglobin and arginase-1 release into the circulation. Often triggered by inflammation, vaso-occlusion is caused by entrapment of erythrocytes and leucocytes in the microcirculation, causing vascular obstruction and tissue ischemia. Subsequent reperfusion of blood flow further promotes tissue injury (99). Hemolysis, also driven by HbS polymerization, causes anemia, fatigue, cholelithiasis, and, what was recently noticed, a progressive vasculopathy (97). Hemoglobin released from hemolyzed erythrocytes generates reactive oxygen species (ROS), such as the hydroxyl and superoxide radical. ROS are a potent scavenger of nitric oxide that disrupts endothelial cell function and induces the nitric oxide resistance (100), whereas free plasma arginase-1 transforms arginine—a nitric oxide substrate—into ornithine, decreasing bioavailability of nitric oxide in SCD patients (101).

In the largest autopsy study, Manici et al. (102) reviewed the cause of death in 306 SCD patients. The most common cause of death for all sickle variants and for all age groups was infection (33 to 48%) due to *Streptococcus pneumoniae* or *Haemophilus influenza*. Other causes of death included stroke (9.8%), complications of therapy (7%), splenic sequestration (6.6%), pulmonary emboli/thrombi (4.9%), renal failure (4.1%), pulmonary hypertension (2.9%), hepatic failure (0.8%), massive hemolysis/red cell aplasia (0.4%), and left ventricular failure (0.4%). In 40.8% of them, death was sudden and unexpected; in 28.4% occurred within 24 hours after presentation; and in 63.3% was associated with acute events (102).

**123.4.13.1.1 Clinical Presentation.** Sickle-cell disease phenotype is very complex, ranging from early childhood mortality to virtually no-symptom condition. Clinical features are a consequence of vaso-occlusion, hemolysis anemia, or infection.

Complications due to vaso-occlusion are complex and include painful episodes, stroke, acute chest pain, priapism, liver disease, splenic sequestration, spontaneous abortion, leg ulcers, osteonecrosis, and proliferative retinopathies renal insufficiency. Complications of hemolysis include anemia, cholelithiasis, and acute aplastic episodes. Infections are caused by *Streptococcus pneumoniae* in children and *Eserichia coli* in adults, causing sepsis, and both *Salmonella* and *Staphylococcus aureus* causing osteomyelitis (103).

Sickle-cell anemia is the most common cause of stroke in children. 11% of sickle-cell anemia patients suffer from stroke by 20 years and 24% by 45 years of age (104). Adams et al. (105) found a higher than previously expected (11 to 24% versus 6 to 8%) incidence of cerebral vascular events in the first 2 weeks of life of SCD patients. This finding reflects a large number of silent strokes. Ischemic stroke (54%) is the most common type of cerebrovascular accident, followed by hemorrhagic stroke (34%) and TIA (11%). The greatest risk of ischemic stroke is in the first two decades of life, whereas hemorrhagic stroke falls in the third decade (100).

**123.4.13.1.2 Diagnosis and Treatment.** All newborns are screened for sickle-cell disease in the US. Diagnosis is based on hemoglobin high-performance liquid chromatography (HPLC), electrophoresis, or isoelectric focusing. Transcranial Doppler ultrasound is a useful tool to screen for significantly narrowed vessels in high-risk children and adults. Genetic counseling requires genetic tests for globin gene mutations (106). Prenatal and antenatal tests are also available (103).

Hydroxycarbamide is a cytotoxic drug that increases the fetal hemoglobin (HbF) concentration, which inhibits HbS polymerization. Treatment decreases the frequency of painful episodes, acute chest syndrome, demand for blood transfusion, and admission of SCD patients to hospital. It may also protect against cerebrovascular events and decrease hypoxemia and proteinuria (97). Blood transfusion with ion chelators in case of chronically transfused patients with sickle-cell disease has an established role in SCD treatment. Repeated transfusions improve oxygen saturation and reduce red blood cell sickling (69). Introduced 30 years ago, bone marrow transplantation is the only potential cure for sickle-cell anemia; however, due to safety concerns, it is limited to HLA-compatible siblings (97).

## 123.4.14 MMD – Moyamoya Disease

Moyamoya disease (MMD) is an uncommon cerebrovascular disorder predominantly affecting East Asians. The disease was named after characteristic cerebral angiographic picture “moyamoya” what in Japanese means “something hazy like a puff of cigarette smoke, drifting in the air.” The pathogenesis of the disease remains to be discovered. Data from epidemiological studies indicate that infection in the head and neck might be implicated



**TABLE 123-3 Genetic Types of MMD (<http://omim.org/entry/252350>; Accessed June 20, 2011)**

Phenotype	Location	Phenotype MIM Number	Gene/Locus	Gene/Locus MIM Number
Moyamoya disease 1–MYMY1	3p26-p24.2	252350	Unknown	Unknown
Moyamoya disease 2–MYMY2	17q25.3	607151	<i>RNF213</i>	613768
Moyamoya disease 3–MYMY3	8q23	Unknown	Unknown	Unknown
Moyamoya disease 4–MYMY4	Xq28	300845	Unknown	Unknown
Moyamoya disease 5–MYMY5	10q23.31	614042	<i>ACTA2</i>	102620

in the development of MMD (107). Genetic factors also play an important role in MMD. Associations with loci on chromosomes 3, 8, 10, 17, and X have been described (Table 123-3). MMD inheritance pattern is polygenic or autosomal dominant with a low penetrance (108).

The incidence of MMD is highest in countries in East Asia; however, it is also present throughout the world in people of many ethnic groups. MMD onset peaks in two age groups: children who are approximately 5 years of age and adults in their mid-40s (109). There are nearly twice as many female patients as male patients (110).

MMD affects—usually bilaterally—terminal portions of the internal carotid vessels as well as cerebral vessels originating from the circle of Willis. In addition to the steno-occlusion, affected arteries show fibrocellular thickening of the intima, an irregular folding of the internal elastic lamina, and reduction of the media. The perforating arteries in subcortical areas are either dilated (in children) or stenotic with thick walls (in adults). These changes predispose to microaneurysmal formation and subsequent intracerebral and intraventricular hemorrhage (111).

**123.4.14.1 Clinical Presentation.** Symptoms may be divided in two main etiological categories: related to ischemia and related to hemorrhage. Ischemic symptoms are more common in children and comprise ischemic stroke and TIA. Ischemia may cause hemiparesis, dysarthria, aphasia, and cognitive impairment. Seizures, visual deficits, syncope, or personality changes are less common in ischemic etiology. Hemorrhage is common in adults (50%); however, it was also noted in children (112). It may be located in intraventricular, intraparenchymal, or subarachnoid space. Hemorrhage is either due to rupture of dilated, fragile affected vessels, or rupture of saccular aneurysms in the circle of Willis (107). Rare symptom include seizures, migraine-like headache, choreiform movements due to basal ganglia involvement and occasionally present ophthalmologic findings like “morning glory disk” (110).

**123.4.14.2 Diagnosis and Treatment.** Diagnosis of patients suspected of MMD is based on imaging studies. Research committee on Spontaneous Occlusions of the Circle of Willis of the Ministry of Health and Welfare of Japan published revised diagnostic criteria (Table 123-4). Additional useful diagnostic tests include electroencephalography (EEG) and cerebral blood-flow studies.

So far, there is no treatment that reverses the primary disease process; however, symptomatic treatment focused on improvement of cerebral blood flow reduces the frequency of MMD symptoms (107). Medical therapy—antiplatelet and less frequently anticoagulant—is used mainly in children due to very low percentage of hemorrhagic strokes, whereas surgical treatment is used both in young and adult patients. Since the external carotid artery is spared in MMD, direct, indirect, and combined surgical bypass operations are performed to improve the cerebral blood flow (110).

#### 123.4.15 Collagen Type IV (COL4A1 Gene and COL4A2 Gene)

The collagen IV molecule is a heterotrimer composed of two alpha-1 chains and one alpha-2 chain. *COL4A1* OMIM# 120130 and *COL4A2* OMIM# 120090 genes are coding for alpha-1 chain and alpha-2 chain of type IV collagen respectively. They are associated together structurally and functionally with each other (<http://omim.org>; Accessed June 20, 2011). Both genes are located on chromosome 13q34 and are universally expressed in basement membranes during early stages of development (113). A number of mutations have been described for *COL4A1* gene (114) and recently also for *COL4A2* gene (115,116). In addition to porencephaly, infantile hemiplegia and hemorrhagic stroke *COL4A1* gene mutations induce cerebral small-vessel disease (117). Hemorrhagic strokes are usually associated with physical activity, trauma and anticoagulant therapy, whereas SVD was expressed as leukoaraiosis (63.5%), microbleeds (52.9%), lacunar infarction (13.5%), and dilated perivascular spaces (19.2%) (118). Based on results by Janne et al. (115), mutations in *COL4A2* gene contribute to sporadic cases of ICH, due to intracellular accumulation of gene product.

#### 123.4.16 Hemorrhagic Stroke

**123.4.16.1 CCM 1 to 3-Cerebral Cavernous Malformations (KRIT1, Malcavernin, PDCD10).** Cerebral cavernous malformations (CCM) are relatively common lesions occurring incidentally or in autosomal dominant fashion. The autosomal dominantly inherited type is caused by mutations in one of at least three

**TABLE 123-4 Diagnostic Criteria for MMD (107,112)**

1. Cerebral angiography findings:
  - a. Stenosis or occlusion at the terminal portion of the ICA and/or at the proximal portion of the ACAs and/or the MCAs.
  - b. Abnormal vascular networks in the vicinity of the occlusive or stenotic lesions in the arterial phase.
  - c. "a" and "b" are present bilaterally.
2. MRI and MRA findings:
  - a. MRA showing stenosis or occlusion at the terminal portion of the ICA and at the proximal portion of the ACAs and MCAs.
  - b. MRA showing an abnormal vascular network in the basal ganglia. Abnormal vascular network can also be diagnosed when more than 2 apparent flow voids are observed in one side of the basal ganglia on MRI.
  - c. (1) and (2) are observed bilaterally.
3. Elimination of the following conditions: arteriosclerosis, autoimmune disease, meningitis, brain neoplasm, Down syndrome, Recklinghausen disease, head trauma, irradiation to the head and other conditions (sickle-cell disease, tuberous sclerosis).
4. Pathological findings:
  - a. Intimal thickening with resulting stenosis or occlusion of the lumen usually observed on both sides, both in and around the terminal portion of the ICA. Lipid deposits are infrequently noted in the proliferating intima.
  - b. Stenosis of various degrees or occlusion associated with fibrocellular thickening of the intima, a waving of the internal elastic lamina, and an attenuation of the media is characteristic for ACAs, MCAs, and posterior communicating arteries, constituting the circle of Willis.
  - c. Perforators and anastomotic branches forming small vascular channels are frequently observed around the circle of Willis.
  - d. Presence of reticular conglomerates of small vessels is common in the pia mater.

Autopsy cases without cerebral angiography should refer to 4.

1. **Definite case:** adults, either 1 and 3 or 2 and 3; children, either 1-a and 1-b or 2-a and 2-b with sever stenosis at the terminal portion of the ICA on the opposite side.

2. **Probable case:** fulfills 3 and either 1-a and 1-b or 2-a and 2-b.

ICA- internal carotid artery, ACA- anterior cerebral artery, MCA- middle cerebral artery, MRI- magnetic resonance imaging, MRA- magnetic resonance angiography.

**TABLE 123-5 Genetic Types of CCM (<http://omim.org/entry/116860>; Accessed June 20, 2011)**

Phenotype	Location	Phenotype MIM Number	Gene/Locus	Gene/Locus MIM Number
CCM-1; Cerebral cavernous malformations-1	7q21.2	116860	<i>CCM1</i>	604214
CCM-2; Cerebral cavernous malformations-2	7p13	603284	<i>C7orf22</i>	607929
CCM-3; Cerebral cavernous malformations-3	3q26.1	603285	<i>PDCD10</i>	609118

genes *CCM-1*, *CCM-2* and *CCM-3* (Table 123-5). The prevalence of CCM has been estimated from 0.17 to 0.55 per 100000 in general population (119).

CCMs are slow-flow, sinusoid blood vessels, lacking smooth muscle cells and elastic lamina. They are lined with endothelial cells that do not have tight junctions, which make them prone to intracranial hemorrhage (120). Blood at various stages of thrombosis usually fills CCM vessels forming mulberry-like shapes. Even in the absence of obvious hemorrhage, all lesions are surrounded by the deposits of hemosiderin (119).

More than half of CCMs are familial (121). Multi-locus linkage analysis revealed that *CCM1* is present in 40%, *CCM2* in 20%, and *CCM3* in 40% of inherited cases. *CCM* genes products, respectively *CCM1/Krit1*, *CCM2/macaverinin*, and *CCM3/PDCD10*, are specifically expressed in endothelium, neurons, and astrocytes (121). Based on immunohistochemical staining, Pagenstecher and colleagues showed loss of expression of

*CCM*-coded proteins, limited only to endothelial cell within cavernous malformation. Additionally, their results demonstrated endothelial cell mosaicism within cavernous tissue (122).

**123.4.16.1.1 Clinical Presentation.** If not symptomatic (47% of cases), CCM presents typically with epileptic seizures (25%), intracranial hemorrhage (12%), or focal neurologic deficits including headaches (15%) (120). The hemorrhage rate in patients with CCM has been estimated to be 0.7%–4.2% (123). The five-year annual rates of hemorrhage among patients presenting with hemorrhage, with symptoms not related to hemorrhage and an incidental finding, were respectively 6.19%, 2.18%, and 0.33% (124). Risk of recurrent hemorrhage decreases over time from 19.8% in the first year to 5% in the fifth year (120).

**123.4.16.1.2 Diagnosis and Treatment.** MR imaging with T2-weighted gradient-echo imaging is currently the gold standard imaging technique for detecting both

sporadic and familial CCMs (125). Currently, surgical removal is the only treatment option in CCM. After careful patient selection, surgery significantly improves patient's neurological condition, from 5.9 NIHSS score after the first episode to 1.7 NIHSS score after 40 months postoperative follow-up (126). Experimental results have shown that mutations in two CCM (KRIT1 and OSM) genes cause RhoA activation. Treatment aimed at blocking activated RhoA may potentially stop the disease (127).

**123.4.16.2 HHT – Hereditary Hemorrhagic Telangiectasia (ENG, ACVRL1 and SMAD4).** Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant vascular dysplasia. Rendu, Osler, and Weber independently gave the original description of HHT in the nineteenth century, hence the eponymous Rendu–Osler–Weber syndrome. So far, based on the affected gene/locus, five types of HHT have been described. (Table 123-6). Telangiectasias are characterized by the presence of multiple arteriovenous malformations (AVMs) of different sizes. AVMs lack intervening capillaries that result in direct connections between arteries and veins (128). They are usually found in the skin, mucosa, and viscera.

Although the prevalence of HHT is estimated from one per 5000 to one per 10,000, it may be underdiagnosed due to variable phenotypes and diagnostic limitations (129).

Each of the genes in HHT1, HHT2 and JHPT encodes a protein involved in TGF- $\beta$  superfamily signaling. The genes within chromosomal regions in HHT3 and HHT4 types remain to be discovered. Endoglin (ENG)—HHT1—is expressed predominantly on endothelial cells, syncytiotrophoblasts, activated monocytes, and tissue macrophages. ENG is a coreceptor from the TGF- $\beta$  family and binds TGFB1 and TGFB3 proteins (128). Activin A receptor type II-like 1 (ACVRL1)—HHT2—codes for the activin receptor-like kinase (ALK) 1, which is a type I receptor from the TGF $\beta$  superfamily ligands. ALK1 is expressed on endothelial, lung, and

placental cells (128). MADH4—JHPT—encodes the transcription factor Smad4. Mutations in Smad4 cause juvenile polyposis/HHT syndrome by disturbing TGF / BMP pathway (128).

**123.4.16.2.1 Clinical Presentation.** Main HHT symptoms include epistaxis, telangiectasia, gastrointestinal (GI) bleeding, pulmonary AVM (PAVM), cerebral system HHT complications, and hepatic vascular abnormalities. The age of AVM development or AVM symptom onset varies and is organ-specific. AVMs in the brain are usually present at birth, whereas those in liver and in lungs develop or grow over time (128).

Nasal bleeding is the major manifestation of mucous telangiectases and, in HHT, is the main reason to seek medical attention. On average, epistaxis starts at the age of 12 years but may range from infancy to adulthood. It affects more than 95% of patients (130).

Telangiectases of the face, oral cavity, or hands develop as often as epistaxis, but later in life. One-third of affected patients develop telangiectases before the age of 20 years, with remaining two-thirds developing symptoms before the age of 40 years (131). The color of the telangiectases may range from pink to red, and the size may vary from pinhead-size lesions to larger, sometimes raised purple lesions. Telangiectases may be distinguished from petechiae and angiomas by blanching upon pressure and recurrent immediate refill (128).

GI bleeding due to AVM affects 15–45% of patients with HHT and begins after the age of 50 years. If prolonged, this can lead to anemia and the need for transfusion. Telangiectases usually develop in upper GI tract; however, other localizations are also possible (128).

Although 74% of HHT patients develop hepatic vascular abnormalities, only 8% are symptomatic (128). Three different types of vascular malformations may be observed in the liver: hepatic artery to hepatic veins, hepatic artery to portal veins, and portal veins to hepatic veins (130).

**TABLE 123-6 Genetic Types of HHT (<http://omim.org/entry/187300>; Accessed June 20, 2011)**

Phenotype	Location	Phenotype MIM Number	Gene/Locus	Gene/Locus MIM Number
Telangiectasia, hereditary hemorrhagic, type 1- HHT1	9q34.11	187300	ENG	131195
Telangiectasia, hereditary hemorrhagic, type 2- HHT2	12q13.13	600376	ACVRL1	601284
Juvenile polyposis/hereditary hemorrhagic telangiectasia syndrome - JHPT	18q21.2	175050	MADH4	600993
Telangiectasia, hereditary hemorrhagic, type 3- HHT3	5q31.3-q32	601101	5:139,500,000–149,800,000 <sup>a</sup>	
Telangiectasia, hereditary hemorrhagic, type 4- HHT4	7p14	610655	7:28,800,000–43,300,000 <sup>a</sup>	

<sup>a</sup>The Genome Reference Consortium Human genome build 37 (GRCh37), from NCBI.

Prevalence of PAVMS ranges from 15 to 59%, depending on the method used for detection (128). Patients with ENG gene mutations seems to have higher incident of PAVMS compared with ACVRL1 mutation carriers (129). PAVMS frequently cause neurological complications. TIA, ischemic stroke or brain abscess were found in 30–40% of patients with PAVMs. Other pulmonary complications include massive hemoptysis or hemothorax and pulmonary hypertension (132).

AVMs may be found in the brain and less frequently in the spine; overall, CNS vessel malformation rate ranges from 10 to 23% in HHT patients (130). Based on a retrospective study, bleeding risk is approximately 0.5% per year (130). The wide spectrum of CNS symptoms includes headaches, acute or subacute hemorrhage, back pain, acute or progressive paraparesis/tetraparesis, sciatic pain, and sphincter disturbance.

**123.4.16.2.2 Diagnosis and Treatment.** The Curaçao criteria (Table 123-5) have been established for the HHT in 1999 (133) and are as follows: (1) spontaneous recurrent epistaxis; (2) multiple telangiectases at characteristic sites; (3) family history; and (4) visceral lesions. If at least three criteria are present, the diagnosis is definite. Diagnosis is possible or suspected if two criteria are present, and unlikely if less than two criteria are present. Mutations in *ACVRL1*, *ENG* and *MADH4* genes are found in 90% of definite HHT patients. Genetic tests are currently used to diagnose asymptomatic patients and to avoid complications (130).

Epistaxis may be treated surgically with steroids and with antifibrinolytic drugs. Cutaneous and labial telangiectases may be treated with laser therapy. GI treatments have not been successful, and transplantation is the only treatment option for hepatic involvement (130). Because lung and brain AVMs may cause serious complications, they should be treated before they become symptomatic. Screening for brain AVMs should first include contrast-enhanced MR imaging and, if necessary, should be evaluated with angiography. Surgery is the first brain AVM treatment option; however, due to difficult localization, this may not be possible. Therefore, in certain situation, surgery should be replaced by stereotactic radiosurgery or by embolization (130).

### 123.4.17 CAA – Cerebral Amyloid Angiopathies

Cerebral amyloid angiopathy (CAA) is a process of progressive pathological deposition of amyloid proteins in arterial and arteriole walls and less frequently in veins and capillaries of the CNS. Amyloid deposits damage the vessel wall and activate matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) (134). CAA may occur as sporadic, familial, or hereditary disease. Familial and hereditary forms of CAA are described in Table 123-7. Deposited pathologic proteins are products of large-protein proteolysis. The most common form of CAA is due to A $\beta$  deposition—product of amyloid precursor protein (APP) proteolysis. Other less common proteins include both ABri and ADan, which are products of amyloid Bri precursor protein (ABriPP) proteolysis, mutant cystatine C (Acys), mutated transthyretin (ATTR), mutated gelsolin (AGel) and disease-associated prion protein (PrP) (134). Based on A $\beta$  form of CAA, in initial stages, cerebrovascular amyloid appears around smooth muscle of tunica media and adventitia, then gradually infiltrates, and finally replaces the smooth muscle cells (135). CAA is generally assumed to be a risk factor for ICH, ischemic stroke, and white matter lesions. Nevertheless, in sporadic cases, CAA may not suffice to explain the incidence of hemorrhage (134).

### 123.5 CVT – CEREBRAL VENOUS THROMBOSIS (FVL, PROTHROMBIN)

Cerebral venous thrombosis (CVT) is a rare cause of stroke due to the thrombosis of the dural sinus and/or cerebral veins. CVT affects approximately 5 persons per million annually accounting for 0.5 to 1% of all strokes (19). CVT is more common (78%) in patients under 50 years of age (19). The acute phase of CVT-case fatality is around 4%, whereas overall death or dependency rate is around 15% (18).

There are multiple predisposing causes of CVT (Table 123-8). All are linked to the Virchow triad of alterations in blood flow, vascular endothelial injury, or alterations in the constitution of the blood. Other causes of CVT

**TABLE 123-7** Familial and Hereditary forms of Cerebral Amyloid Angiopathy (<http://omim.org>; Accessed June 20, 2011)

Location	Phenotype	Phenotype OMIM Number	Gene/Locus	Gene/Locus OMIM Number
21q21.3	Cerebral amyloid angiopathy, Dutch, Italian, Iowa, Flemish, Arctic variants	605714	<i>APP</i>	104760
20p11.21	Cerebral amyloid angiopathy, Icelandic type	105150	<i>CST3</i>	604312
13q14.2	Dementia, familial British	176500	<i>ITM2B</i>	603904
13q14.2	Dementia, familial Danish	117300	<i>ITM2B</i>	603904
18q12.1	Amyloidosis, hereditary, transthyretin-related	105210	<i>TTR</i>	176300
20p13	Prion protein-related cerebral amyloid angiopathy	176640	<i>PRNP</i>	



**TABLE 123-8 CVT Risk Factors (19)**

Condition	Prevalence, %	OR (95% CI; p-value)
Prothrombotic conditions	34.1	NA
Protein C deficiency	N/A	11.1 (1.87 to 66.05; P=0.009)
Protein S deficiency	N/A	12.5 (1.45 to 107.29; P=0.03)
Antiphospholipid and anticardiolipin antibodies	5.9	8.8 (1.3 to 57.4; NA)
Mutation G20210A of factor II	N/A	9.3 (5.9 to 14.07)
Resistance to activated protein C and factor V Leiden		3.4 (2.3 to 5.1)
Hyperhomocysteinemia	4.5	4.6 (1.6 to 12.0; NA)
Pregnancy and puerperium	21	NA
Oral contraceptives	54.3	5.6 (4.0 to 7.9; NA)

N/A – not available.

can be divided into acquired and genetic risks. Main (18) acquired causes comprise antithrombin III, protein C, and protein S deficiency; antiphospholipid and anticardiolipin antibodies; hyperhomocysteinemia; pregnancy and puerperium; oral contraceptives and cancer (18).

The prevalence of thrombophilic symptoms in children with CVT varies between 10 and 78% (136). Both mutations in factor V Leiden gene (R506Q) due to resistance to activated protein C and prothrombin gene mutation (G20210A) causing a slight elevation of prothrombin level are independent CVT risk factors. While on oral contraceptive treatment, odds ratio for CVT is dramatically increased for G20210A mutation to 149.3 (95% CI 31.0 to 711.0) (19).

### 123.5.1 Clinical Presentation

The wide range of presenting symptoms may be classified into two main categories: (1) related to increased cranial pressure due to impaired venous drainage; and (2) related to focal brain injury due to venous ischemia/infarction or hemorrhage, with a number of patients presenting both mechanisms (19). The most frequent symptoms are headache, seizures, focal neurological deficits, altered consciousness, and papilledema, (18) all of which may be present isolated or in association with other symptoms.

### 123.5.2 Diagnosis and Treatment

Diagnosis of patients suspected of CVT is based on clinical suspicion and imaging confirmation that may be supported with selected laboratory tests. Laboratory tests include routine blood work to reveal potential prothrombotic conditions and a level of D-dimers (137). Noninvasive imaging modalities include CT, MRI, and ultrasound. CT Venography (CTV) and magnetic resonance venography (MRV) are most useful. Invasive diagnostic angiographic procedures comprise cerebral angiography and direct cerebral venography (19).

Anticoagulant therapy has been successfully introduced in CVT treatment in order to prevent thrombus growth, facilitate recanalization, and to prevent DVT. Both LMWH and UFH are safe and effective, with a

possible advantage of LMWH (19). Direct intrasinus thrombolytic techniques and mechanical therapies may be considered in three cases—first, if despite the use of anticoagulation clinical deterioration is present; second, if mass effect from a venous infarction occurs; and third, if ICH that causes intracranial hypertension is resistant to standard therapies (19).

## 123.6 GENOME-WIDE ASSOCIATION STUDIES AND GENOMICS

Advances in genetics and genomics may permit new insights. In recent genome-wide association studies, a number of single-nucleotide polymorphisms have been associated with specific stroke subtypes and major stroke-risk factors such as diabetes and AF, but these have yet to be replicated. Studies of messenger RNA expression have also shown promise for the development of genomic signatures for stroke classification.

## 123.7 SUMMARY

At present, the contribution of genetic factors to stroke etiology and risk is small, involving familial predisposition, a small number of monogenic disorders such as CADASIL—the prototype genetic disorder associated with stroke—and polymorphisms associated with cerebral venous thrombosis. Possible new associations are being explored in genome-wide association studies but no markers have yet emerged.

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A Fellow of the Royal Australasian College of Physicians, Dr. Baird received her medical degree from the University of Melbourne, Australia, and a Master of Public Health from Harvard. After completing a Clinical Stroke Fellowship at Beth Israel Deaconess Medical Center and Harvard University in Boston, she joined the Neurology faculty at Harvard Medical School.

Dr. Baird lectures worldwide and has published widely. She has performed extensive clinical and translational research. She serves on the editorial boards of a number of medical journals. Dr. Baird is a Fellow of the American Heart Association/American Stroke Association, the Royal Australasian College of Physicians and a member of the American Academy of Neurology, the American Neurological Association, the New York State Neurological Society, and the Society for Neuroscience.

# CHAPTER

# 124

## Primary Tumors of the Nervous System

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### ABBREVIATIONS

ATM – Ataxia-telangiectasia mutated  
 EGFR – Epidermal growth factor receptor  
 NF-1 – Neurofibromatosis 1  
 NF- $\kappa$ B – Nuclear factor kappa-light-chain-enhancer of activated B cells  
 PDGF – Platelet-derived growth factor  
 PTCH1 – Protein patched homolog 1  
 PTEN – Phosphatase and tensin homolog  
 TP53 – Tumor protein 53  
 TSC – Tuberous sclerosis protein

### 124.1 INTRODUCTION

This chapter reviews the current knowledge about the molecular genetic alterations associated with the initiation and progression of the most common forms of primary nervous system tumors. These include glial tumors, primitive neuroectodermal tumors (PNETs), meningiomas, and schwannomas. We briefly review recent epidemiologic data and the present histopathologic classification of primary nervous system tumors. In addition, we discuss the hereditary syndromes predisposing to the development of tumors of the nervous system.

### 124.2 EPIDEMIOLOGY

Estimates of the incidences of the various tumors of the nervous system vary considerably depending on the source of data (1). Tumors of the central nervous system (CNS) are common and occur with an incidence of 6–16 per 100,000 (2). Consistent with this estimate, according to the Year 2000 Standard Statistical Report of the Central Brain Tumor Registry of the

United States (CBTRUS, [www.cbtrus.org](http://www.cbtrus.org)), the overall annual incidence rate for primary benign and malignant brain tumors in the United States is 14.1 cases per 100,000 (3). The relative incidence of CNS tumors is age dependent. Intracranial neoplasms represent the most common solid tumors in children younger than 15 years of age, among which primary tumors of the nervous system comprise nearly 20% of all cancers, making them the second most common form of childhood cancer next to leukemias (4). The incidence of primary brain tumors has apparently increased among the elderly population (2,5), even after correction for improved diagnostic methods (6).

### 124.3 CLASSIFICATION

There are multiple systems for the classification of brain tumors. Attempts are being made to revise classifications from a morphology-based approach identifying a “cell of origin” or “embryogenetic” phase to a more comprehensive approach incorporating molecular data (7). Applications of these approaches may help to validate some of the more recent classification concepts, such as the classification of embryonal tumors; however, many of the original terms are still in clinical use. For example, medulloblastoma was named after the presumed progenitor cell, the “medulloblast.” The newer classification systems should increase prognostic accuracy and may provide a basis for developing treatment strategies for specific tumor types.

Several different types of brain tumors can be distinguished based on classic morphologic criteria. Overall, the most common type of brain tumor is the glioma, which constitutes more than half of all brain tumors, followed by meningioma and schwannoma, which make up

20% and 10%, respectively. Of the rarer brain tumors only ependymoma and medulloblastoma are discussed.

The location and histologic type of brain tumors differ in children and in adults. In children, brain tumors occur most frequently in the posterior fossa. The most frequent tumor types are ependymoma, medulloblastoma, and astrocytic tumors, including spongioblastoma, cerebellar astrocytoma, and optic nerve glioma. In adults, the majority of tumors are supratentorial. Meningiomas and gliomas predominate.

The World Health Organization (WHO) has established a widely used grading system for classifying CNS tumor malignancy (8). This classification, based on histopathologic tumor typing, also has an optional WHO grading that ranges from WHO grade I (benign) to WHO grade IV (malignant). Tumors with minimal proliferative potential are classified as WHO grade I lesions. Such tumors include pilocytic astrocytomas, subependymomas, myxopapillary ependymomas of the cauda equina, a variety of neuronal and mixed neuronal/glia tumors, schwannomas, and most meningiomas. Tumors with lower mitotic activity and a tendency for recurrence are classified as WHO grade II. Grade II tumors include well-differentiated astrocytomas, oligodendrogliomas, mixed gliomas, and ependymomas. Neoplasms with histologic evidence of anaplasia, generally in the form of increased mitotic activity, increased cellularity, nuclear pleomorphism, and cellular anaplasia, are classified as WHO grade III. WHO grade IV is assigned to mitotically active and necrosis-prone highly malignant neoplasms. Typical examples include glioblastomas and PNETs (8).

The WHO classification presently is the standard classification system for CNS tumors. The general use of this classification provides a common morphologic baseline in neuro-oncology and greatly facilitates comparison of clinical and laboratory results from different institutions around the world. Gilles and colleagues (9) highlighted certain limitations of the WHO classification of childhood tumors for prognosis. They proposed a classification technique that simultaneously accounts for all reliably recognized histologic features.

A new classification system has emerged for glioblastomas based on extensive genomic analysis, revealing four distinct subtypes: classic, neural, proneural, and mesenchymal.

## 124.4 INHERITED TUMOR SYNDROMES PREDISPOSING TO CENTRAL NERVOUS SYSTEM TUMORS

Most brain tumors occur in a sporadic fashion. On rare occasions, however, brain tumors may occur as part of known inherited cancer syndromes (Table 124-1). Often, the germline mutations manifest themselves by the development of non-CNS malignancies. Occasionally, however, brain tumors may be the presenting tumor in such cancer-prone patients.

In addition to the familial occurrence of brain tumors as part of recognized inherited cancer syndromes, there are rare pedigrees in which several family members develop a specific histologic type of brain tumor consistent with autosomal dominant inheritance, but other signs of phakomatoses or non-CNS malignancies are absent. These pedigrees are described later under the respective tumor type.

### 124.4.1 Neurofibromatosis 1

Neurofibromatosis 1 (NF1) is transmitted in an autosomal dominant manner, with 50% of patients representing de novo mutations (reviewed in Reference (10)). The gene is located on chromosome 17q and encodes a protein with properties of a guanosine triphosphatase (GTPase) activating protein. NF1 affects approximately 1 in 3000 individuals around the world. Details about NF1 are presented in Chapter 121. Although the characteristic lesion of NF1 is the neurofibroma, a benign

TABLE 124-1

Inherited Cancer Syndromes and Tumors of the Nervous System

Syndrome	Gene	Location	Predominant Tumor Type
Neurofibromatosis 1 peripheral	NF1	17q11.2	Neurofibroma, malignant nerve sheath tumor, optic glioma
Neurofibromatosis 2 ependymoma	NF2	22q12	Schwannoma, meningioma
Von-Hippel–Lindau disease	VHL	3q25	Capillary hemangioblastoma, renal cell carcinoma, pheochromocytoma
Tuberous sclerosis astrocytoma	TSC1	9q34	Subependymal giant cell TSC2 16p13.3
Li–Fraumeni syndrome	P53	17p13.1	Soft-tissue and bone sarcomas, breast carcinoma, glioma, leukemia, PNET
Gorlin syndrome	PTCH	9p22	Basal cell carcinoma, PNET, meningioma
Ataxia–telangiectasia	ATM	11q22-23	Lymphoid tumors
Cowden syndrome	PTEN	10q23	Dysplastic gangliocytoma of the cerebellum, meningioma
Werner syndrome	WRN	8p12	Meningioma, astrocytoma
Turcot syndrome	APC	5q21	Colon carcinoma, glioblastoma
	hPMS2	7p22	PNET
	hMLH1	3p21.3-p23	

PNET, primitive neuroectodermal tumor.

tumor of the peripheral nerve, patients with NF1 may also develop gliomas (11). These lesions typically involve the optic nerves or optic chiasm and may occur in up to 15% of patients if detailed neuroimaging is used for detection. The great majority of these tumors is asymptomatic and shows little progression. The histology is typically that of a pilocytic astrocytoma. Gliomas may also occur less frequently in the brainstem and hypothalamus, and rarely in the cerebellum or spinal cord. Reports of meningiomas in NF1 most likely represent the chance association of a common brain tumor with a common genetic disorder.

### 124.4.2 Neurofibromatosis 2

Neurofibromatosis 2 (NF2) is another autosomal dominant condition, affecting approximately 1 in 40,000 individuals (12). NF2 is due to mutation in a gene on chromosome 22q that encodes a cytoskeletal protein (13,14). It is described in detail in Chapter 121. NF2 patients are characterized by bilateral vestibular schwannomas, a hallmark feature of the disease. Commonly, NF2 patients have other cranial and spinal schwannomas and meningiomas. Gliomas are also found in patients with NF2, most commonly in the spinal cord (15). Rarely, gliomas may occur in the posterior fossa or in a supratentorial location, and they may represent the sole manifestation of NF2 in the occasional patient (16). About 80% of gliomas in NF2 patients are intramedullary spinal or cauda equina tumors, and the vast majority of these are ependymomas (17,18).

### 124.4.3 Von Hippel–Lindau Disease

Von Hippel–Lindau (VHL) disease is transmitted as an autosomal dominant trait and is described in detail in Chapter 121. The responsible gene is located on chromosome 3p (19). Hemangioblastomas are found in the majority of patients with VHL disease (20) and may be a cause of death (21). The majority of hemangioblastomas in VHL disease occur in the cerebellum, followed by locations in the spinal cord and brainstem (20,21). Approximately half the tumors are asymptomatic (20). Capillary hemangioblastomas in VHL patients tend to manifest in younger patients than sporadic capillary hemangioblastomas and are more often multifocal (20).

### 124.4.4 Tuberous Sclerosis

Tuberous sclerosis (TS) is the second most frequent hereditary tumor syndrome of the nervous system after NF1 (22). Details are presented in Chapter 121. Two different genes have been linked to the onset of TS, TSC1 located at chromosome 9q34 and TSC2 located at 16p13.2 (23,24). Neuroimaging studies show CNS lesions in the great majority of patients with TS, including hamartomas such as cortical tubers and subependymal nodules (25).

Only approximately one-fourth of the lesions are tumorous and represent giant cell astrocytomas. Giant cell astrocytomas, in contrast to subependymal nodules, show marked enhancement.

### 124.4.5 Li–Fraumeni Syndrome

The Li–Fraumeni syndrome is a rare, dominantly inherited syndrome associated with germline mutations in the *TP53* gene (26). Although soft-tissue sarcomas and breast cancers predominate, approximately 13% of patients develop brain tumors that typically show the histology of astrocytic glioma, followed by PNETs (27). In addition to patients with the Li–Fraumeni syndrome, *TP53* germline mutations have been occasionally identified in patients with nonfamilial malignancies with early onset or multifocality. First-degree relatives of these patients are also at an increased risk of gliomas (28,29).

### 124.4.6 Gorlin Syndrome

Gorlin syndrome, also called nevoid basal cell carcinoma syndrome, is an autosomal dominant disorder leading to the development of multiple basal cell carcinomas of the skin as well as palmar and plantar pits, odontogenic keratocysts, and skeletal anomalies (30). Childhood medulloblastoma, meningioma, craniopharyngioma, and neurofibroma have been described in patients with Gorlin syndrome (31–33). Gorlin syndrome has been linked to mutations in the tumor suppressor gene *PTCH*, which is the human ortholog of *Drosophila* patched (34–36). Somatic mutations in *PTCH* have been detected in sporadic basal cell carcinomas, PNETs, medulloblastomas, and certain other types of sporadic tumors (37–39).

### 124.4.7 Ataxia-Telangiectasia

Ataxia-telangiectasia is a recessive trait mapped to the *ATM* gene on chromosome 11q. *ATM* is a PI3-kinase-related protein kinase whose function in cell cycle control is lost by truncating mutations that result in loss of the C-terminally located kinase domain or by point mutations (40,41). Lymphoid malignancies are frequently seen in patients with ataxia-telangiectasia. Although solid tumors occur, primary CNS tumors are infrequent (25a,42).

### 124.4.8 Cowden Syndrome

Cowden syndrome, also known as multiple hamartoma syndrome, is an autosomal dominant cancer syndrome that predisposes to a variety of hamartomas and neoplasms. The major CNS lesion associated with the disease is dysplastic gangliocytoma of the cerebellum (Lhermitte–Duclos disease) (43). Other associated CNS lesions include megalencephaly and gray matter heterotopias. Occasional cases of meningiomas in patients



with Cowden syndrome have also been documented (44). Peripheral manifestations include multiple trichilemmomas of the skin, cutaneous keratoses, oral papillomatosis, gastrointestinal polyps, hamartomas of soft tissues, thyroid tumors, and benign and malignant breast tumors (45,46). Germline mutations in the *PTEN* tumor suppressor gene at 10q23 have been linked to Cowden syndrome (47,48).

#### 124.4.9 Werner Syndrome

Werner syndrome is a recessive trait with clinical symptoms resembling premature aging. The responsible gene maps to the short arm of chromosome 8, and has been identified by positional cloning (49). In addition to premature aging, some individuals with Werner syndrome develop tumors, including CNS tumors such as meningiomas and, less frequently, astrocytomas (50–52).

#### 124.4.10 Turcot Syndrome

Turcot syndrome describes a rare heterogeneous disorder characterized by the association of colonic polyposis and malignant primary neuroepithelial tumors of the CNS. Colonic polyposis in patients with Turcot syndrome appears to be the result of mutations in genes encoding Wnt signaling pathway proteins (*APC* and *beta-catenin*). Some patients with Turcot syndrome have been shown to possess mutations in the gene for familial adenomatous polyposis coli (*APC*) (53,54). A patient with Turcot syndrome and congenital hypertrophy of the pigment epithelium, commonly seen in familial *APC*, has also been described (55). The incidence of glial tumors and medulloblastomas appears to be increased in patients with colonic polyposis (see Reference (56) and references therein). On the other hand, somatic *APC* mutations are not a major cause of brain tumors. Using RNase protection analysis Mori and associates (54) did not detect any *APC* gene mutations in 47 medulloblastomas, 8 glioblastomas, 22 astrocytomas, and 2 oligodendrogliomas. Furthermore, mutations in Wnt pathway proteins are less frequent in sporadic medulloblastomas: Huang and coworkers (57) detected mutations in the *APC* or *beta-catenin* genes in only six of 46 of such tumors.

In patients with Turcot syndrome, germline mutations have been identified in three different genes. Of 14 families with Turcot syndrome, 10 had germline mutations in the *APC* gene, and two had mutations in *hPMS2* or *hMLH1*-mismatch repair genes (45a). Mutations in *hPMS2* may predispose the patient to extreme DNA instability. One patient was studied who had an inherited *hPMS2* missense mutation. Genetic characterization of the patient's tumors, which included one astrocytoma, three colon carcinomas, and two colon adenomas, showed additional mutations in the *TGFbetaRII*, *E2F-4*, *hMSH3*, *hMSH6*, *APC*, or *TP53* genes (58).

### 124.5 RELATIVE RISK OF CANCER IN FIRST-DEGREE RELATIVES

In epidemiologic studies, only a small increased risk for brain tumors was detected for relatives of patients with brain tumors. Choi and associates (59) found a ninefold increase in the incidence of brain tumors among relatives of glioma patients compared with controls. Even this increased relative risk translated into the relatively small absolute risk of 0.6% in this study. In other studies, the increased relative risk was less significant (60,61). Gold and colleagues (62) compared several risk factors in 361 children with brain tumors to 1083 matched controls. Although a family history of tumors did not contribute to an increased risk of brain tumors in children, a modest increase in risk of childhood brain tumors was associated with a maternal family history of birth defects. Kuijten and coworkers (63) found a modestly increased risk of childhood cancers only in relatives of patients with PNETs, whereas for relatives of astrocytoma patients, this risk was not significantly increased.

### 124.6 GLIAL TUMORS

Gliomas are a heterogeneous group of mostly sporadic neoplasms derived from glial cells. They account for about 40–45% of all intracranial tumors and, thus, are the most common tumors among the primary CNS neoplasms (1). Depending on morphologic appearance and presumed histogenesis, gliomas are subdivided into several subgroups, the most important being astrocytic tumors (including the glioblastoma), oligodendroglial tumors, mixed gliomas (oligoastrocytomas), and ependymal tumors.

A genetic predisposition for the development of gliomas is seen in NF1 and NF2, Li-Fraumeni syndrome, TS, Gorlin syndrome, Turcot syndrome, and ataxia-telangiectasia. The specific type of glioma or its location may vary depending on the disorder. For example, pilocytic astrocytoma of the optic nerve is typical for NF1, whereas ependymoma of the spinal cord is characteristic for NF2. Of 282 children with astrocytoma examined by Kibirige and colleagues (64), 21 had a diagnosis of NF1 and four had TS. Familial glioma not associated with a specific genetic syndrome does occur but is infrequent, and it is exceedingly rare to see more than two first-degree relatives with glioma. Vieregge and associates (65) reviewed 39 reports of familial glioma and concluded that 60% involved affected siblings. Although gliomas are seen in familial *APC* and Turcot syndrome, somatic mutations in the *APC* gene in primary brain tumors are rare and were not detected in 91 neuroepithelial tumors including gliomas (54).

#### 124.6.1 Astrocytoma and Glioblastoma

Astrocytoma is a generic term applied to diffusely infiltrating tumors composed of well-differentiated neoplastic astrocytes (8). The astrocytomas, or astrocytic gliomas,

may be subdivided into two major groups: (1) the more common group of diffusely infiltrating tumors, comprising astrocytoma, anaplastic astrocytoma, and glioblastoma and (2) the less common group of tumors with more circumscribed growth consisting of pilocytic astrocytoma, pleomorphic xanthoastrocytoma (PXA), and subependymal giant cell astrocytoma of TS. Astrocytomas tend to infiltrate the surrounding brain. Therefore, despite their slow growth, even well-differentiated astrocytomas (corresponding to grade II histologically) tend to recur. Anaplastic (malignant) astrocytomas show diffuse anaplasia (e.g. increased cellularity, pleomorphism, nuclear atypia, and mitotic activity). Histologically, they correspond to grade III. Glioblastoma is an anaplastic, often cellular brain tumor composed of poorly differentiated, fusiform, round or pleomorphic cells and occasional multinucleated giant cells. The presence of prominent vascular proliferation or necrosis is essential for the histologic diagnosis. Histologically, glioblastomas correspond to grade IV. Astrocytomas and glioblastomas account for about 17% of primary brain tumors in adults, whereas in children they account for only 4%.

The most malignant type of glioma, the glioblastoma (WHO grade IV), is also the most common, making up close to 50% of all gliomas (66). The incidence of glioblastomas peaks between 45 and 60 years of age. In adulthood, glioblastomas share a preferential supratentorial location with the other diffuse astrocytomas. By computed tomography (CT) and magnetic resonance imaging (MRI), these tumors usually appear as a ring structure with a hypodense center (necrosis) surrounded by a ring of contrast-enhancing vital tumor tissue and edema. Macroscopically, typical glioblastomas are largely necrotic masses with a peripheral zone of fleshy gray tumor tissue. Intratumoral hemorrhage is a frequent finding. Histologically, glioblastomas are cellular tumors that may show a variety of tissue and cell differentiation patterns.

Although they are histologically indistinguishable, there are genetically distinct subtypes of primary and secondary glioblastoma. Primary glioblastomas account for the vast majority of cases in adults older than 50 years. After a short clinical history of usually fewer than 3 months, they manifest *de novo*, without clinical or histologic evidence of a less malignant precursor lesion. In contrast, secondary glioblastomas usually develop in patients younger than 45 years of age, with malignant progression from grade II or III astrocytoma. The interval of progression may be from less than 1 year to as long as 10 years, with a median interval of 4–5 years (67). The prognosis of glioblastoma patients is extremely poor, with a median postoperative survival time of only 12 months (68).

### 124.6.2 Molecular Genetics of Astrocytic Gliomas

Several molecular mechanisms have been implicated in the development of gliomas and their progression to more

malignant histologic grades on recurrence. These involve activation of dominantly acting oncogenes, as well as inactivation of recessive tumor suppressor genes. Among primary glioblastomas, 60% have overexpression of the epidermal growth factor receptor (EGFR) and 40% have EGFR amplification. EGFR VIII, a mutant EGFR receptor, is coexpressed in nearly 50% of glioblastomas with EGFR amplification (69). Other genetic alterations observed in primary glioblastomas include overexpression of the *MDM2* (murine double minute 2) gene and mutation loss of the tumor suppressor protein PTEN. Secondary glioblastomas typically have mutations of the *p53* tumor suppressor gene and overexpression of platelet-derived growth factor (PDGF) ligands and receptors (67). Genetic alterations on chromosomes 9p, 10q, 11p, 17p, 19q, and 22 have also been associated with events leading to gliomas.

As we observe these molecular genetic alterations of tumors, efforts are targeted at correlating these changes with clinical parameters including prognosis and response to treatment. As an example, we can look more closely at the *MGMT* gene located on chromosome 10q26, which encodes a DNA-repair protein that removes alkyl groups from the O6 position of guanine. High levels of *MGMT* activity diminish the therapeutic effects of alkylating agents. Loss of *MGMT* expression occurs by epigenetic silencing of the *MGMT* gene by promoter methylation. In a series of 206 glioblastomas, the *MGMT* promoter was methylated in 45%. This change correlated with a statistically significant increase in median overall survival of 18.2 months compared with 12.2 months in those without promoter methylation. Moreover, those patients with the *MGMT* promoter methylation status benefited from a combination of chemotherapy with an alkylating agent, temozolomide, and radiation, with a median survival of 21.7 months compared with treatment with radiation alone, with a median survival of 15.3 months (70).

Genetic alterations leading to glioma formation are described later in greater detail, and a simplified summary of events leading to glioma formation and progression is shown in Figure 124-1.

**124.6.2.1 Chromosome 2q and IDH1.** The isocitrate dehydrogenase 1 (*IDH1*) gene encodes for an enzyme in the citric acid cycle that converts NAD<sup>+</sup> to NADH and drives the oxidative decarboxylation of isocitrate. Genomic analysis uncovered somatic mutations in 12% of glioblastoma multiforme (GBM) tumors. Large-scale screening of gliomas showed that mutations were uncommon (5%) in primary adult GBMs, but they were present in 85% of secondary glioblastomas (71). Mutations in a functional equivalent region of *IDH2* (chromosome 15q) were also observed in low-grade gliomas, but at 1/20 the frequency (71). Pediatric gliomas, as well as primary adult GBMs, rarely contain mutations in *IDH1* (27,71–73). *IDH1* mutations are an early transformation event that occurs in tumors that acquire either *p53* mutations or 1p and 19q deletions (72,74). Tumors with *IDH1* mutations appear to occur in a genetically distinct



subtype given their common occurrence in secondary GBMs, and mutual exclusivity with other common genetic aberrations including PTEN deletions and EGFR copy-number amplification (16).

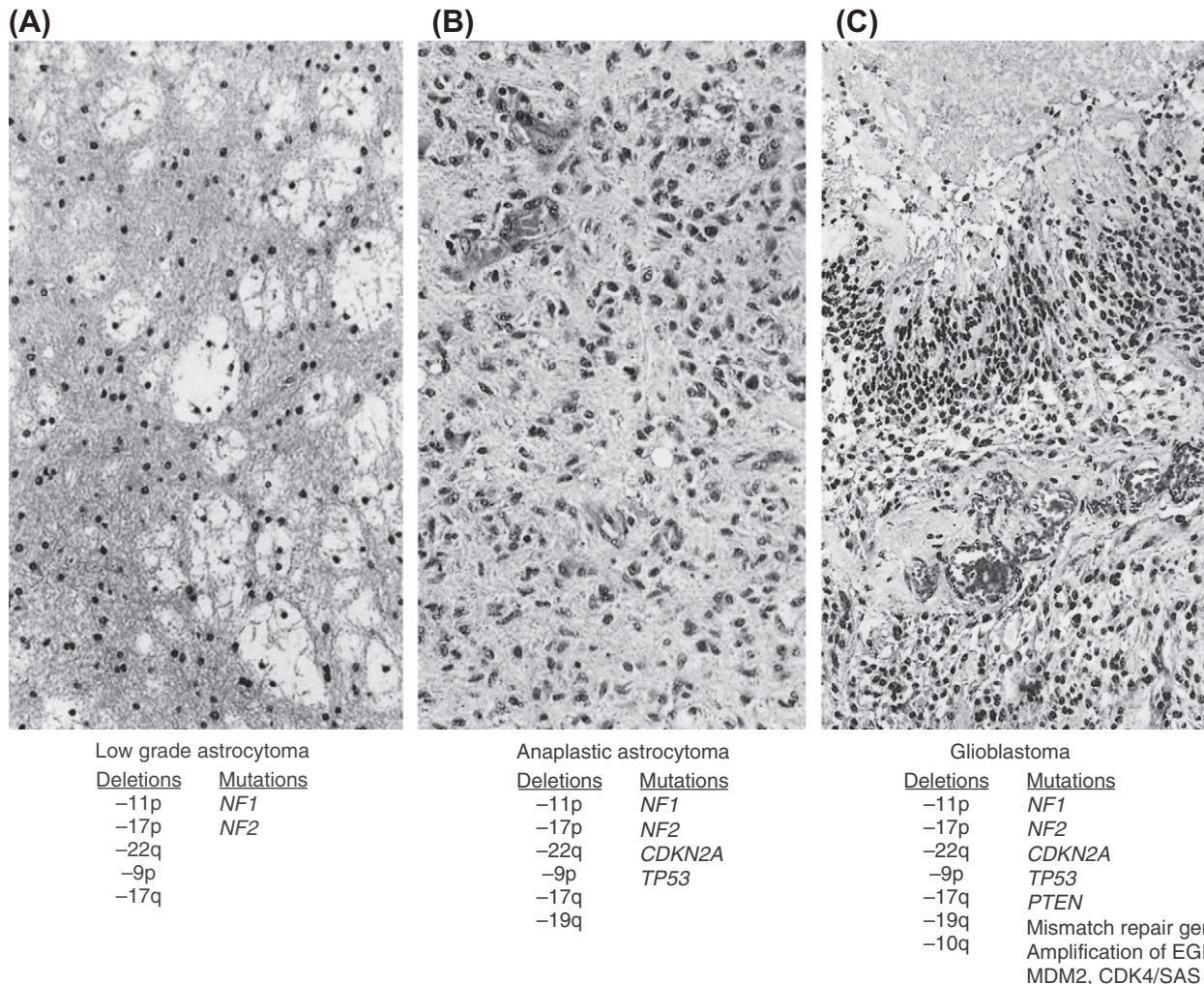
**124.6.2.2 Chromosome 4q and PDGFR $\alpha$ .** Activation of platelet-derived growth factor receptor alpha (PDGFR $\alpha$ ) initiates mitogenic signaling and is amplified in up to 17% of GBMs and is observed in all four subtypes, but alterations are more common in the proneural subtype (16,34).

**124.6.2.3 Chromosome 7p and EGFR.** Amplification of chromosome 7 is the most common alteration observed in glioblastomas, resulting in gains of EGFR copy number. EGFR amplifications are observed in 50–60% of GBM tumors and are correlated with increased expression. Although EGFR activation typically results from ligand stimulation, a common mutation in a quarter to two-thirds of GBM tumors with EGFR amplifications express an aberrantly active version

referred to as EGFRvIII. EGFRvIII is constitutively active, avoids degradation, and contributes to genomic instability (75). Alternatively, the majority of tumors that lack EGFR amplifications have little or no detectable levels of EGFR (60). Alterations in EGFR are observed in all four tumor subtypes, but are a hallmark of the classic subtype, with a mutually exclusive relationship between EGFR and TP53 mutations (7,16,68).

Cyclin dependent kinase 6 (CDK6) contributes to the progression through the G1 mitotic phase and is commonly upregulated in gliomas (76,77). Although chromosome 7 has common regions of amplification in gliomas, the 7q21 region containing CDK6 is not coamplified with MET or EGFR, suggesting a distinct selective pressure (3). Amplifications in the CDK4 gene occur in 18% of glioblastoma tumors (78), and tumors lacking CDK4 alterations are likely to contain mutations in RB1 (59).

The MET proto-oncogene, also referred to as c-MET, encodes for the hepatocyte growth factor receptor (HGFR)



**FIGURE 124-1** Simplified schematic representation of glioma pathogenesis and progression. (A) Fibrillary astrocytoma with low cellularity and microcyst formation; (B) anaplastic astrocytoma with nuclear atypia, and frequent mitoses; (C) glioblastoma with small, anaplastic tumor cells, vascular proliferation, and necrotic areas with pseudopalisading of tumor cells. Mut., mutation, amplif., amplification. Parentheses indicate a minor or less well-established role of a particular genetic change in the pathogenesis of gliomas. (From Kleihues, P.; Burger, P. C.; Scheithauer, B. W. *Histological Typing of Tumours of the Central Nervous System, 2nd ed.*; Springer-Verlag, Heidelberg, 1993.)

protein. The expression of MET correlates with tumor grade (79), and gene amplification appears to occur independent of the EGFR region (80). MET amplifications in gliomas are far less common than those of EGFR, but are a part of the upstream PI3K pathway (78). Inhibition of MET by antibodies reduces tumor growth in vivo (80), and higher expression is associated with a shorter time to tumor recurrence (79). MET signaling contributes to drug resistance (81), and synergistically regulates EGFR signaling, particularly with EGFRvIII (82–84). MET itself is regulated by the tumor suppressor PTEN (83,85). Interestingly, MET may be critical to cancer stem cells, since expression induces signaling network alterations producing a cancer stem cell-like phenotype (6).

**124.6.2.4 Chromosome 9p.** The critical gene on chromosome 9 is the *CDKN2A* (MTS1) tumor suppressor gene, which encodes p16 (p16INK4a), a negative regulator of cell cycle progression (86). *CDKN2A* mutations have been identified in melanoma, astrocytoma, and glioblastoma (87,88). The p16 protein normally binds the cyclin D-cyclin-dependent kinase 4 or 6 (Cdk4, Cdk6) complex, thereby inactivating the retinoblastoma protein Rb, resulting in cell cycle arrest (89).

Glioblastomas frequently show deletions of one or both copies of the *CDKN2A* tumor suppressor gene on 9p21. Schmidt and coworkers (90) found homozygous deletion of *CDKN2A* in 41% and hemizygous loss of *CDKN2A* in 28% of primary glioblastomas. In glioblastoma cell lines, the incidence of homozygous *CDKN2A* loss seems to be even higher, reaching 70% (42a). In addition, inactivation of *CDKN2A* either by point mutation or by 5' CpG island methylation has been found in some glioblastomas (83,91). Chromosome 9p deletions are not exclusive to malignant astrocytomas, and may occur in lower grade astrocytomas and oligodendrogliomas (92). Interestingly, there is a well-established mutually exclusive relationship between mutations in *CDKN2A* and *RB1*, whereas cells that lack mutations in *CDKN2A* typically have mutations in *RB1* (59,93). Molecular subtype analysis of tumors reveals that although deletion events in the *CDKN2A* gene are common (70%), they occur in 95% of tumors with the classic subtype (16a).

**124.6.2.5 Chromosome 10.** Monosomy 10 is a frequently detected karyotypic abnormality in gliomas and is typically associated with a more malignant histologic type. Using comparative genomic hybridization, Schrock and colleagues (94) demonstrated chromosome 10 loss in one of two astrocytomas and seven of seven glioblastomas. The incidence of chromosome 10 loss in glioblastomas varies between different studies, ranging from 60% to more than 90% of the cases (82). Loss of heterozygosity (LOH) in gliomas for chromosome 10 genetic markers often involves markers spanning the whole chromosome; however, partial deletions have recently been identified (95).

Three different regions on both arms of chromosome 10 have been implicated as potential sites of

glioblastoma-associated tumor suppressor genes (95). The region most frequently deleted is located at distal 10q and spans approximately 5 cM between the loci D10S587 and D10S216 (96). A candidate tumor suppressor gene designated *DMBT1* ("deleted in malignant brain tumors 1"), has been cloned and mapped to this region (97). Intragenic homozygous deletions in *DMBT1* were found in about 23% of glioblastomas (97). Another candidate gene from distal 10q is the *MXI1* gene, which codes for a negative regulator of the Myc oncoprotein. Mutations of *MXI1* have been detected in prostate cancer (98). Glioblastomas have not been studied in detail for *MXI1* mutations. In contrast, somatic mutations in the *PTEN* tumor suppressor gene at 10q23 have been detected in about one-third of glioblastomas (74,99,100). *PTEN* is also altered in some breast carcinomas, prostate carcinomas, and malignant melanomas (99,100).

Deletions in chromosome 10, including the region containing the tumor suppression gene *PTEN*, commonly occur in tumors containing *EGFR* amplifications (101,102). These results suggest that *PTEN* function may serve to modulate the effects of enhanced *EGFR* signaling in gliomas and has been implicated as a means for resistance to *EGFR* inhibitor therapy (98). Mutations are observed in 23% of GBM tumors, but deletions in the gene region are far more common and occur in 85% of tumors, although less frequent in the proneural subtype (16a). The 10q22–23 region that is often deleted also contains a novel tumor suppressor gene annexin 7 (*ANXA7*). Deletions in *ANXA7* occur in 77% of tumors and mutations are observed in 6%, resulting in lower *EGFR* degradation and a worse survival outcome (101).

**124.6.2.6 Chromosome 11p.** Eleven of 43 malignant astrocytomas showed LOH of markers in 11p (103). The *HRAS* gene that maps to this region was excluded as a candidate gene by single strand conformation polymorphism (SSCP) analysis. Loss is detected in low- as well as high-grade gliomas, suggesting that these events occur early in tumorigenesis.

**124.6.2.7 Chromosome 12.** Cyclin dependent kinase 4 (*CDK4*) facilitates cell cycle progression by regulating *RB1*. Amplifications of *CDK4*, like *CDK6*, are observed in glioblastomas and are often overexpressed (77,104). Activation of retinoblastoma signaling is predictive of the effects of *CDK4* inhibition (76), while tumors with wild-type *CDK4* are more likely to contain *RB1* mutations (59).

MDM2 is a negative regulator of tumor suppressor p53, preventing its transcriptional activity and targeting it for proteosomal degradation. Amplification of MDM2 occurs in 14% of glioblastomas (78) and is associated with worse survival (104).

**124.6.2.8 Chromosome 13 and *RB1*.** The retinoblastoma protein (*RB1*) is a critical tumor suppressor gene that blocks cell cycle progression by inhibiting E2F transcription factors when unphosphorylated. On phosphorylation by cyclin-dependent kinases, *RB1* is targeted for degradation and the E2F transcription factors



are allowed to be active. The RB signaling network is often disrupted in GBMs (50,69). Mutations in RB1 are observed in glioblastomas, particularly when tumors are wild type of cyclin dependent kinases (59,76,93). Chromosomal deletions in the region that correspond to the *RB* gene are a frequent occurrence in GBMs (44%), but are less common in the classic subtype (16a).

**124.6.2.9 Chromosome 14q and NFKBIA.** NF- $\kappa$ B signaling contributes to invasiveness and drug resistance. NFKBIA inhibits the nuclear factor of kappa light polypeptide gene enhancer in B cells (NF- $\kappa$ B) transcription factor by sequestering the transcription factor dimer in the cytoplasm. Following stimulation, NFKBIA is phosphorylated and targeted for proteosomal degradation, allowing NF- $\kappa$ B to translocate to the nucleus and transcribe target genes. Constitutive activation of NF- $\kappa$ B is commonly observed in GBMs and can be achieved through multiple signaling pathways. Monoallelic deletions in the chromosome 14q region corresponding to the *NFKBIA* gene are observed in approximately a quarter of GBM tumors (23,92). Deletions of NFKBIA allow for NF- $\kappa$ B signaling in tumors lacking EGFR amplification and are not typically observed in tumors with a classic phenotype (92).

**124.6.2.10 Chromosome 17 TP53 and NF1.** Loss of chromosome 17p is an early and frequent event in astrocytomas, and losses are frequently accompanied by mutation in the *TP53* gene, which is located in 17p13.1. This was not surprising because the occurrence of glial tumors in patients with the Li-Fraumeni syndrome pointed to the importance of the *TP53* gene in the formation of sporadic gliomas. In addition, Kyritsis and coworkers (28) identified *TP53* germline mutations in six of 19 patients with multifocal glioma, including two with a family history of cancer, one with another primary malignancy, and two with all three risk factors; one of four patients with unifocal glioma, another with primary malignancy, and a family history of cancer; and two of 15 patients with unifocal glioma and a family history of cancer but no second malignancy. In a family ascertained through the occurrence of childhood adrenocortical carcinoma a mutation of codon 307 in exon 8 was identified in the proband's tumor as well as in an astrocytoma from the proband's father (29). However, van Meyel and coworkers (105) did not identify germline mutations in 26 members of 16 families with glioma in exons 5 through 9 of the *TP53* gene.

The wild-type *TP53* gene product is a nuclear phosphoprotein that suppresses cell and tumor growth. In a study of 120 primary brain tumors, *TP53* mutations were detected only in 59 astrocytic tumors (106). Of these, six tumors with *TP53* mutations were either anaplastic astrocytomas or glioblastomas. Four of the six tumors had lost heterozygosity for 17p markers as well. Del Arco et al. (84) suggested a two-step model for the inactivation of the *TP53* gene in astrocytomas. A single *TP53* mutation seemed to occur in the initial stage of tumorigenesis,

because low-grade astrocytomas were heterozygous for the mutation; loss of the remaining wild-type allele was associated with a higher degree of malignancy. Sidransky et al. (107) proposed that histologic progression of astrocytomas was associated with a clonal expansion of cells that had previously acquired a mutation in the *TP53* gene. By studying low-grade tumors that had recurred as more malignant tumors they could show that a subpopulation of cells in the initial tumor that contained *TP53* mutations made up the majority of cells in the recurrent tumor, which had progressed to glioblastoma. Inactivation of *TP53* may not be an obligatory step because four of 13 glioma cell lines contained a nonmutated *TP53* gene with wild-type function in a functional assay using transcriptional elements that are induced by wild-type but not mutant *TP53* (108). However, these tumors may contain amplifications of the *MDM2* gene (see later).

Wild-type p53 has a short half-life, and is present in such small quantities in normal cells that it cannot be detected immunocytochemically. Mutations in the *TP53* gene are associated with a slower turnover and may result in abnormal expression leading to p53 accumulation in the cell nucleus so that staining can be detected by immunocytochemistry. Aberrant or increased expression of p53 has been observed in many astrocytic tumors (109). Haapasalo et al. (110) stained sections of 102 astrocytic tumors with two antibodies to wild-type and mutant *TP53*. None of the grade 1 astrocytomas were positive, but 29% of grade 2 tumors and 49% of grade 3–4 astrocytomas were positive. Some mutations in the conserved *TP53* exons may be missed by immunocytochemistry, and p53 accumulation may occur independent of mutations in these exons (111). Nonetheless, mutations in p53 are well established in GBMs, particularly those without EGFR amplifications and from a proneural subtype (7,16a,68).

The *NF1* gene encodes for the tumor suppressor protein neurofibromin 1 on chromosomal region 17q11.2 that negatively regulated the oncogenic Ras pathway. Although mutations in this gene are commonly associated with neurofibromas, mutations are found in 17% of GBMs. In fact, 53% of GBMs with the mesenchymal phenotype either contain deletions in the *NF1* gene or harbor sequence mutations (16a).

**124.6.2.11 Chromosome 19.** LOH on chromosome 19p is a common alteration found in astrocytomas (112,113). Ritland et al. (112) showed LOH at 19p13.2-pter in 17 of 23 studied astrocytomas, consistent with findings of the previous work by von Deimling et al. (113). Alterations on chromosome 19q are also less commonly linked to the development of astrocytomas, involving 19q13.2–q13.4 (114).

**124.6.2.12 Chromosome 22.** Using comparative genomic hybridization, Schrock et al. (21) identified loss and gain of chromosome 22 in malignant gliomas. Five of nine tumors had lost all or part of chromosome 22q. A novel amplification site was mapped to chromosome 22q12.

**124.6.2.13 Gene Amplifications.** The most commonly amplified gene in glioblastomas is the gene coding for the *EGFR* on chromosome 7, correlating with the frequent observation of trisomy 7 in glioblastomas. *EGFR* is a transmembrane protein with tyrosine kinase activity. Its extracellular domain binds EGF and transforming growth factor (TGF)- $\alpha$ . *EGFR* may interact with its ligands in an autocrine fashion, leading to an increase in cell proliferation.

*EGFR* abnormalities are specifically associated with GBM (115), and *EGFR* amplification is associated with a shorter median survival (116). In a study of 58 glioblastomas, von Deimling et al. (113) detected *EGFR* gene amplification only in tumors with loss of chromosome 10, suggesting that *EGFR* abnormalities follow chromosome 10 loss in the cascade of tumor progression.

Abnormalities of the *EGFR* gene in glioblastomas include most commonly not only amplification and overexpression but also rearrangements and deletions resulting in abnormal binding of ligands (104,115,117). Most frequently, rearrangements are deletions affecting the 5' end (coding for the extracellular domain) and, more rarely, the 3' end (coding for the intracellular domain) (81). The most common of these rearrangements, an in-frame deletion of 801bp resulting in the aberrant splicing of exon 1 to exon 8, causes the expression of a truncated receptor molecule lacking parts of the extracellular domain necessary for ligand binding. Functional characterization of this *EGFR* variant has revealed that it shows constitutive tyrosine kinase activity and may thereby confer enhanced tumorigenicity on human glioma cells (84).

In addition to *EGFR*, the cellular *MDM2* (murine double minute 2) gene on 12q is amplified in a large percentage of human sarcomas and in other human tumors. The gene product can complex with p53 and inhibit its function (118). Reifenger et al. (119) studied 157 primary brain tumors and found that the *MDM2* gene is amplified and overexpressed in 8–10% of anaplastic astrocytomas and glioblastomas. No *TP53* mutations or LOH for 17p were detected in these tumors, suggesting that *MDM2* amplification may be an alternative mechanism for abnormally regulated p53 growth control. Reifenger et al. (120) showed that 15% of astrocytomas and glioblastomas show amplification of 12q13–14 and identified some tumors that had amplicons not containing *MDM2*, but *CDK4* and *SAS*. More recently, another murine double minute gene, *MDM4*, has been shown to be amplified in gliomas lacking *TP53* mutations that lacked *MDM2* amplification (121). Other genes including *NMYC* and *MET* may be amplified as well, albeit at a much smaller frequency (72,122).

Pilocytic astrocytomas constitute a separate clinical and histopathologic entity, and are the most common astrocytic tumors in children. In contrast to adult astrocytomas, allelic losses on chromosomes 10, 17p, and 19q are not found in pilocytic astrocytomas, nor

are alterations in the *EGFR* gene. von Deimling et al. (123) detected loss of alleles on 17q in 4 of 20 tumors. One tumor contained an interstitial deletion encompassing the region of the *NF1* gene. Mutations of the *TP53* gene were not identified in 12 juvenile pilocytic astrocytomas (124).

### 124.6.3 Oligodendroglioma

Oligodendroglioma is a tumor composed predominantly of neoplastic oligodendrocytes (8). Oligodendrogliomas are typically slow growing and usually occur during adulthood. They are most commonly located in the cerebral white matter and deep gray structures. Oligodendrogliomas have a lesser tendency to malignant transformation than astrocytomas. Histologically, oligodendrogliomas most often correspond to WHO grade II, whereas the anaplastic oligodendrogliomas are WHO grade III.

Most oligodendrogliomas are sporadic neoplasms. Occasional cases of familial clustering have been reported by Parkinson and Hall (125) (oligodendroglioma in two brothers), Roosen et al. (126) (oligodendroglioma in a mother and her daughter), Roelvink et al. (127) (oligodendroglioma in twin sisters), and Ferraresi et al. (128) (oligodendroglioma in a father and his son). In addition, a family with polymorphous oligodendrogliomas in brother and sister has been shown, whose tumors were immunoreactive for p53, suggesting that each tumor was associated with a mutation in the *TP53* gene (129).

### 124.6.4 Molecular Genetics of Oligodendrogliomas

There are multiple molecular mechanisms that account for the development of oligodendrogliomas. Genetic alterations on chromosomes 1, 10, and 19 have been associated with events leading to oligodendrogliomas. Amplification of *EGFR* has also been observed in oligodendrogliomas. *EGFR* amplification is considerably more infrequent than in other glial tumor types, whereas *EGFR* overexpression is common. LOH on the chromosome arms 1p and 19q is frequent in oligodendrogliomas. LOH of chromosome 1p or combined loss involving chromosomes 1p and 19q is statistically significantly associated with both chemosensitivity and longer recurrence-free survival after chemotherapy (76). LOH on 1p and 19q in patients with anaplastic oligodendrogliomas treated with chemotherapy with or without radiation had a median time to progression of 86 months and a median overall survival of 91 months compared with those without LOH who had a median time to progression of 39 months and a median overall survival of 46 months (130).

**124.6.4.1 Chromosome 1.** Loss of alleles on the short arm of chromosome 1 is common in oligodendroglioma neoplasms. Reifenger et al. (120) showed LOH on

1p in 67% (14/21) of studied tumors. Bello et al. (101) found LOH on 1p in six of six oligodendrogliomas and five of six anaplastic oligodendrogliomas. Kraus et al. (131) detected LOH in three of nine oligodendrogliomas and three of six anaplastic oligodendrogliomas.

In anaplastic oligodendrogliomas, when chromosome 1p deletions are absent, chromosome 9p deletions often occur, and loss of *CDKN2A*, which encodes a cell cycle regulatory molecule (p16INK4A). *CDKN2A* has a close homolog, *CDKN2C*, located at chromosome 1p32. Tipped by these reciprocal 1p/9p deletions, Pohl et al. (132) demonstrated homozygous deletions of the *CDKN2C* gene on the short arm of chromosome 1 in a subset of oligodendrogliomas, suggesting that *CDKN2C* may be oncogenic in these tumors.

Concurrent deletions of chromosome regions 1p and 19q are commonly observed in oligodendrogliomas and may be an early transformation event (32), although somatic alterations in these regions are rarely observed (33). Patients with tumors containing these deletions have better survival outcomes and response to treatment (133,134). Deletions of 1p and 19q in pediatric oligodendrogliomas are rare (12). Interestingly, there is a high frequency of concurrence with 1p and 19q LOH and mutations in *IDH1* in oligodendrogliomas (71,74), but typically do not occur in tumors with p53 alterations (130).

**124.6.4.2 Chromosome 2q and *IDH1*.** Mutations in the isocitrate dehydrogenase 1 (*IDH1*) gene are common in oligodendroglial and oligoastrocytic tumors (27,71,73,74). Mutations in *IDH1* occur at amino acid R132 in 80% and 86% of grade 2 oligodendrogliomas and grade 3 anaplastic oligodendrogliomas, respectively (71). *IDH2*, located on 15q26, encodes for a functionally equivalent enzyme that can also be mutated in the analogous R172 residue in tumors, albeit at 1/10 to 1/20 the frequency (71). Pediatric oligodendroglial tumors and glioblastomas with oligodendroglial differentiation do not contain *IDH1* mutations (27).

**124.6.4.3 Chromosome 10.** Wu et al. (117) report a patient presenting with an oligodendroglioma that recurred with the histology of a glioblastoma 5 months later. DNA analysis of the initial tumor showed loss of alleles on chromosome 10, which is typically found in more malignant gliomas. The authors suggested that loss of chromosome 10 alleles may be predictive of malignant tumor growth even when morphologic criteria of aggressive growth are absent. Chromosome 10 deletions in low-grade oligodendrogliomas were more recently narrowed to 10q25–26 (135).

**124.6.4.4 Chromosome 19.** The most frequent genetic alteration in oligodendrogliomas is LOH on the long arm of chromosome 19 (113,114,136,137). Ritland et al. (112) examined region-specific LOH and its relation to the morphologic type of glioma. In astrocytomas, allelic loss was most commonly observed for 19p, whereas loss of alleles in 19q and retention of alleles in

19p were associated with oligodendrogliomas and mixed oligoastrocytomas (112).

Allelic loss on 19q showed a striking association with LOH on 1p, a finding suggesting a synergistic effect of both alterations in providing a selective growth advantage (78,120,131,138).

**124.6.4.5 Amplification of *EGFR*.** *EGFR* gene amplifications are the most common amplifications found in glioblastomas. *EGFR* amplifications are relatively uncommon in oligodendrogliomas, whereas overexpression of *EGFR* protein is a common feature of oligodendrogliomas. Reifenberger et al. (139) studied 13 grade II oligodendrogliomas and 20 grade III anaplastic oligodendrogliomas for *EGFR* gene amplification. They observed *EGFR* gene amplification in only one anaplastic tumor. However Reifenberger et al. (139) also observed that overexpression of *EGFR* mRNA is relatively common in both low- and high-grade oligodendrogliomas (six of 13 oligodendrogliomas, and 10 of 18 anaplastic oligodendrogliomas).

## 124.6.5 Ependymoma

Ependymoma is a tumor composed predominantly of neoplastic ependymal cells. Ependymomas are moderately cellular with low mitotic activity. They are thought to arise from the ependymal or subependymal cells surrounding the ventricles, the central canal, or within the filum terminale. Ependymomas usually present as a posterior fossa mass in children between the ages of 2 and 10 years but are also found in the spinal canal.

Ependymomas may occur in patients with NF2 and have been described in one individual who was part of a sibship with autosomal dominant meningiomas (140). Familial ependymoma occurred in a family of 11 siblings (141). Four siblings developed ependymomas or subependymomas, and one additional sibling had a brain tumor with unverified histology. Gilchrist and Savard (142) described ependymomas in two sisters and their maternal male cousin.

Because ependymomas are found in patients with NF2, the *NF2* gene was a likely candidate for an ependymoma gene; however, loss of chromosome 22 in sporadic ependymomas is rarely determined by cytogenetic or molecular studies (143–145). Rubio et al. (144) detected the loss of chromosome 22 alleles and mutation in the *NF2* gene in only one of eight ependymomas. Segregation analysis with chromosome 22 markers identified chromosome 22pter–22q11.2 as a region containing the ependymoma locus, and it clearly excluded the *NF2* gene locus (146). This is consistent with the finding in a single patient of an ependymoma-associated constitutional translocation, t(1;22) (p22;q11.2) (147). It should be noted that ependymomas in NF2 patients usually occur in the spine, whereas most sporadic tumors have an intracranial location.

Mutations in the *TP53* gene are also rare in ependymomas. In 15 ependymomas, only one contained a



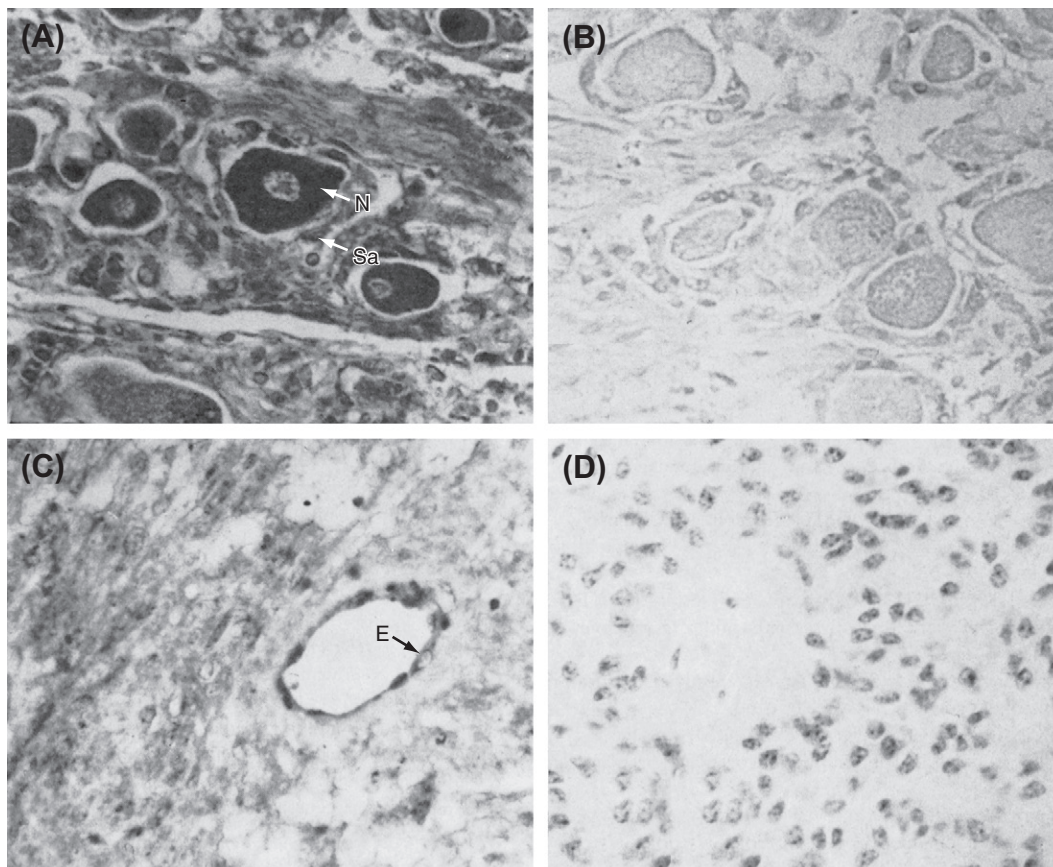
silent mutation in exon 6. Metzger et al. (148) detected a germline mutation in codon 242 of the *TP53* gene in a patient with a malignant ependymoma of the posterior fossa. Several of the relatives had died at a young age from a variety of cancers. Similarly, in a survey of relatives of 195 children younger than 15 years of age with soft-tissue sarcomas, only one sib died from an ependymoma (149).

Evidence for other ependymoma loci has come from several different approaches. In addition to chromosome 22 abnormalities, cytogenetic analyses of ependymomas have shown abnormalities of chromosomes 6, 11, 16, and 17 (150–152). Comparative genomic hybridization studies and cytogenetic analysis have shown that loss of chromosome 6p is common in the pediatric ependymoma, in addition to 17p and 22q abnormalities (153,154). By immunocytochemical investigation of ependymomas using antibodies directed against schwannomin, Huynh and colleagues (155) divided ependymomas into two groups: one group with lack of staining suggesting that loss or mutation of both *NF2* alleles had occurred, and a second group with normal staining suggesting that mutation in other genes caused ependymoma formation

(Figure 124-2). An ependymoma locus in addition to *NF2* was also suggested by genetic linkage analysis of a family with autosomal dominant meningiomas and ependymomas (149).

### 124.6.6 Molecular Predictors of Glioma Progression

In gliomas, more than in any other type of brain tumor, recurrence is associated with a more malignant histologic type, and response to treatment may vary greatly despite identical histologic classification (Figure 124-3). Patients with *EGFR* gene amplification in their gliomas were found to have a significantly shorter survival than those without amplification ( $P < 0.01$ ) (116). Analysis of tumor cells in a plaque assay allowed Sidransky et al. (107) to detect a subpopulation of tumor cells that contained mutations in the *TP53* gene. Progression to a more malignant histology in the recurring tumor was due to clonal expansion of those cells containing *TP53* mutations. In a long-term follow-up study of 52 patients with low-grade astrocytomas, a trend toward more aggressive growth was detected for patients with p53-positive



**FIGURE 124-2** Detection of schwannomin in normal human vestibular nerve and ependymomas. (A) Staining of a paraffinized section of normal human vestibular nerve with an antibody raised against schwannomin (155). White and black arrows indicate cytoplasm of a vestibular neuron (N) and a satellite cell (Sa), respectively. Schwannomin is present in the cytoplasm of both vestibular neurons and satellite cells. (B) Staining of the adjacent section with the same antibody after preabsorption. (C) Detection of schwannomin in an ependymoma. In this ependymoma, endothelial cells (E, at arrow) and tumor ependymal cells were stained. (D) Absence of schwannomin in another ependymoma, as indicated by negative antibody staining in tumor cell cytoplasm (note nuclear counterstain). Cell nuclei in all sections were counterstained with aqueous hematoxylin (132 $\times$ ).





tumors (19). Five years after diagnosis, the survival estimate with the Kaplan-Meier method was 21% for p53-positive patients but 46% for patients whose tumors lacked p53 immunoreactivity. Glioblastomas in patients with Turcot syndrome caused by mutations in mismatch repair genes may have a prolonged survival (45a).

The WHO classification defines PNETs as small cell malignant tumors of childhood with predominant location in the cerebellum and a noted capacity for divergent differentiation, including neuronal, astrocytic,

Genetic disorders associated with medulloblastoma include Gorlin syndrome (31,33,34), familial APC and Turcot syndrome (45a). One patient in a family with

VHL disease presented with a PNET with multipotent differentiation in the cerebellum (102).

Familial medulloblastoma is very rare, but it has been reported in two newborn sisters and identical twin girls (157). Another sib pair was recently reported by Hung et al. (158) including a review of the relevant literature. One study detected a germline *TP53* mutation in two siblings with cerebral PNETs (159).

### 124.7.1 Molecular Genetics of Primitive Neuroectodermal Tumors

Molecular abnormalities on chromosome 9, 11, and 17 have been linked to the development of PNETs. Other common genetic abnormalities in medulloblastomas are gains of portions of chromosome 1 and deletions of 1q, 6q, 11p, and 16q (5,129,134). Mutations in the genes encoding Wnt signaling pathway proteins APC and beta-catenin occur rarely in sporadic medulloblastomas (57,160). No mutations in the *APC* gene were detected in 91 neuroepithelial tumors including medulloblastomas (54); however, 12% of sporadic medulloblastomas (of 86 tumors plus 11 cell lines investigated) had *Axin1* gene deletions (83a). The *Axin1* gene is located at 16p13.3, and its gene product Axin is a negative regulator of Wnt signaling.

**124.7.1.1 Chromosome 9.** LOH of chromosome 9q involving the Gorlin syndrome gene locus at 9q22 (*PTCH*) has been found in a subset of medulloblastomas, including desmoplastic variants (161,162). In addition, somatic mutations in the *PTCH* gene have been found in about 10–15% of medulloblastomas (37,163).

**124.7.1.2 Chromosome 11.** Three of 11 PNETs showed LOH of markers in 11p (103). The *c-HRAS* gene that maps to this region was excluded as a candidate gene by SSCP analysis.

**124.7.1.3 Chromosome 17 and *TP53*.** Isochromosome 17q has been consistently observed in cytogenetic studies of PNETs, which has been detected in about 30–50% of the tumors (133,134). Isochromosome 17q results in the loss of one copy of 17p, and molecular studies have indeed confirmed LOH on 17p in a similar percentage of medulloblastomas (80,143,164). Loss of 17p in medulloblastomas is usually not associated with *TP53* mutation (71,80,81,165–167). Mutations in the *TP53* gene are relatively infrequent in medulloblastomas. Although four of 22 tumors had lost 17p, only two harbored mutations in the *TP53* gene, and the mutation was homozygous in only one of these tumors (16a). Intense overexpression of p53 by immunocytochemical analysis of tumor sections was associated with a significantly reduced survival (168). The infrequent mutation of *TP53*, together with the finding of LOH at distal 17p not including the *TP53* locus, suggest the existence of a second, not yet identified, tumor suppressor gene located at 17p13.3 (71,169). The *HIC-1* gene is a good candidate for this suspected tumor suppressor gene (170,171),

but further studies are needed to substantiate the role of this gene in PNETs.

### 124.8 SCHWANNOMA (NEURILEMMOMA, NEURINOMA)

Schwannomas are encapsulated and sometimes cystic tumors composed of spindle-shaped neoplastic Schwann cells (8). Tumors contain cellular areas with compact elongated cells, often with palisading (Antoni A areas) and less dense areas with cells containing lipid (Antoni B).

Schwannomas account for 8% of intracranial tumors and 29% of intraspinal tumors. Vestibular schwannomas (VSs) are also (somewhat erroneously) referred to as acoustic schwannomas or neuromas and occur commonly as single tumors on the vestibular branch of the eighth cranial nerve. They have an incidence of around 13 million per year (172). In patients with NF2, vestibular schwannomas are often bilateral and occur at a much earlier age than in patients with sporadic unilateral tumors. About 4% of vestibular schwannomas are bilateral, and virtually all these patients have NF2. Schwannomas also occur on other cranial and spinal nerves. When MRI imaging is used, spinal schwannomas are as common in NF2 as vestibular schwannomas (15,18). A proband presenting with multiple spinal schwannomas harboring a deletion in the *NF2* gene has been described (173). Two relatives carrying the same deletion were subsequently shown to have asymptomatic bilateral vestibular schwannomas. A second pedigree with autosomal dominant spinal tumors, but lacking vestibular schwannomas or other CNS tumors by autopsy or MRI, has also been described (174).

Frequent loss of alleles in 22q12 in sporadic vestibular schwannomas and NF2 tumors indicated a common pathogenetic mechanism for these tumors (175). These findings were further supported by establishing linkage of NF2 to genetic markers on chromosome 22 (176), leading to the subsequent cloning of the *NF2* gene in 1993 (13,14). The *NF2* gene product, designated schwannomin or merlin (see Reference (46)), is a 595 amino acid protein of the protein 4.1 superfamily of proteins, with highest similarity to the ERM protein family (including ezrin, radixin, and moesin), which links the cell membrane and the cytoskeleton.

The action of the *NF2* gene is that of a classic tumor suppressor. Inactivation or loss of the second allele and lack of the *NF2* gene product could be demonstrated in most schwannomas ((177), see later). Reduction of schwannomin synthesis in Schwann cells by use of anti-sense oligonucleotides leads to morphologic changes and loss of cell attachment (155). Schwannomin is known to directly interact with a variety of proteins including ezrin, moesin, radixin, betaII-spectrin, actin, HRS, eIF3c, CD44, paxillin, N-WASP, magacin, PIKE-L, and EBP50/NHE-RF (39,178–186).

In addition, schwannomin colocalizes and forms complexes that may or may not be direct with betaI-integrin (21a), and CD44 (152a).

The majority of *NF2* gene mutations identified in vestibular schwannomas are deletions leading to a premature stop codon (119,156,177,187–191). Although no clear hot spots for mutations have been identified, C to T transitions leading to change of an arginine codon to a stop codon occur more commonly than expected (192). With the exception of an increased frequency of C to T transitions (192) and mutations resulting in skipping of exon 4 (193), recurrent mutations are rare in the germline or in schwannomas (194).

Most *NF2* mutations are expected to result in loss of the majority of interactions between schwannomin and other proteins. *NF2* mutation also significantly decreases schwannomin stability. Sainz et al. (177) demonstrated absence of schwannomin staining in all of 30 immunohistochemically stained vestibular schwannomas. Two other studies demonstrated loss of schwannomin expression in 93% of stained schwannomas, 75% of meningiomas, and 33% of ependymomas (155,195). Thus, in contrast to meningiomas and ependymomas, mutations in the *NF2* gene appear to be the major if not exclusive molecular event leading to schwannoma formation.

Schwannomatosis describes a condition of multiple schwannomas and represents a unique class of NF that may or may not involve *NF2* gene mutations (196). Patients with schwannomatosis lack vestibular schwannomas, and some cases show multiple schwannomas localized to just the spine. Some patients with schwannomatosis have been characterized to possess no identifiable *NF2* gene mutations (75). In another study, pedigree analysis of 20 families with spinal schwannomatosis revealed truncating *NF2* gene mutations and LOH (197). Some patients proved to be sporadic mosaics for *NF2* gene mutations, whereas others had familial. This is reviewed in the phakomatosis chapter (21); schwannomatosis is associated with SMARCB1 mutations in the germline, and homozygous loss of SMARCB1 and *NF2* in tumors.

## 124.9 MENINGIOMA

Meningioma is a tumor composed of neoplastic meningeothelial (arachnoid) cells (8). Several histologic variants are recognized, such as meningothelial, fibrous (fibroblastic), transitional, and psammomatous meningioma.

Meningiomas are the most common benign brain tumors, and account for about 15% of all intracranial tumors and 25% of intraspinal tumors. The frequency of meningioma increases with advancing age, and meningiomas are more common in women. Although meningiomas are frequently attached to the dural membranes, they may occur in unusual sites, for example, within the ventricular space.

Meningiomas occur frequently in patients with *NF2* (12,177,198), and less frequently in Werner and Gorlin syndromes. Many reports of familial meningioma may represent patients with *NF2* who were inadequately evaluated for the presence of small vestibular or spinal schwannomas or lens opacities. For example, Delleman et al. (85) reported a family with four members in two generations with meningiomas. Other signs of *NF2* were missing. A fifth member of the pedigree, however, had multiple meningiomas and vestibular schwannomas. Because it is advisable to remove vestibular schwannomas before they lead to loss of hearing, signs of *NF2* should be carefully sought in all cases of familial meningioma, multiple meningiomas, or meningiomas in young patients. Dominantly inherited meningioma without other evidence of *NF2*, however, does occur (see References (73) and (199) and references therein).

### 124.9.1 Molecular Genetics of Meningiomas

**124.9.1.1 Chromosome 22.** Zang and Singer (49) reported the loss of chromosome 22 in short-term cultures of fresh meningiomas. Subsequently, Seizinger et al. (175) reported LOH of chromosome 22 DNA markers in 17 of 40 meningiomas. Molecular studies of sporadic meningiomas and meningiomas from *NF2* patients have confirmed these findings (93,185). Inactivating mutations in the *NF2* gene have been detected in 20–30% of sporadic meningiomas, often accompanied by mutations or loss in the second allele (189,190). Schwannomin staining is absent in more than half of sporadic meningiomas (see earlier) (155,195).

Additional genes may be involved in meningioma pathogenesis on chromosome 22. A translocation (16a,68) observed in a meningioma cell line recently led to the identification of a gene, dubbed *MN1*, which is also involved in translocations with the *tel* gene in the pathogenesis of leukemias (200). This gene is centromeric but in close proximity to the *NF2* gene. What role *MN1* plays in the formation or progression of most meningiomas is still unresolved. Other genes more recently linked to meningiomas pathogenesis include *DAL1* and protein 4.1R (201).

In a screen of 81 meningiomas for mutations in the *NF2* gene, LOH for chromosome 22 markers was detected only in those meningiomas that also had *NF2* gene mutations (202). Of the 81 meningiomas, 44 had LOH of 22q, 29 had LOH of 1p, and 23 had LOH of 14q (202). The authors concluded that the formation of aggressive meningiomas follows a multistep tumor progression model involving genes on 1p, 14q, and 22q. Immunocytochemical studies of meningiomas support this hypothesis. Schwannomin immunoreactivity was absent in more than half the meningiomas, whereas the remainder showed strong schwannomin staining suggesting that the *NF2* gene was not mutated (155,195).

Genetic linkage studies provide further evidence for the existence of a second meningioma locus. Analysis of a pedigree with autosomal dominant meningiomas and ependymomas excluded the mutation from the *NF2* region (199). The region excluded by linkage analysis encompassed the region centromeric to *NF2* that contains the *MNI* gene.

**124.9.1.2 Chromosome 1.** Cytogenetic studies had indicated that next to loss of chromosome 22, deletions of chromosome 1p are common in meningiomas. Bello et al. (101) studied 50 meningiomas and identified 13 meningiomas with loss of chromosome 1 alleles, of which 12 had also lost alleles on chromosome 22. Most of these tumors showed aggressive growth. Analysis of 16 anaplastic meningiomas detected loss of chromosome 1p alleles at almost the same frequency as the loss of chromosome 22 (203), confirming the role of genes in 1p in the progression of meningiomas. Comparative genomic hybridization has shown that a meningioma locus maps to a distal chromosome 1p region (204). Analysis of 157 blood/tumor pairs showed LOH for 1p in 34% of cases, and high-resolution deletion mapping defined a 1.5 cM region within 1p32 as a candidate meningioma locus (205). More recently, loss of expression of the alkaline phosphatase *ALPL* that maps to 1p36.1-p34 was shown to be involved in meningioma progression (206).

**124.9.1.3 Chromosome 14.** There is considerable evidence supporting the existence of a meningioma locus on chromosome 14q. Aberrations including LOH on 14q are common in low- and high-grade meningiomas (202,207,208). As for changes in chromosome 1p alleles, alterations of the chromosome 14q locus may not be causative but rather related to in tumor progression, because many tumors also displayed LOH of the *NF2* locus (202,207).

## CROSS REFERENCES

Epigenetics; The Molecular Biology of Cancer; Bioinformatics

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## RELEVANT WEBPAGES

- National Cancer Institute the Cancer Genome Atlas <http://cancergenome.nih.gov/>.
- National Cancer Institute REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) <https://caintegrator.nci.nih.gov/rembrandt/>.
- The Central Brain Tumor Registry of the United States (CBTRUS). <http://www.cbtrus.org/>.
- Welcome Trust Sanger Institute <http://www.sanger.ac.uk/>.

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# CHAPTER

# 125

## Muscular Dystrophies

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### GLOSSARY

**Becker muscular dystrophy** – an X-linked genetic condition, caused by mutations in the dystrophin (*DMD*) gene, leading to reduced production of the dystrophin protein within the muscle. Patients affected by Becker muscular dystrophy usually show first symptoms in their childhood or early teens, such as difficulties running or walking up stairs, frequent falls, and calf hypertrophy. Creatine kinase values are highly increased. Becker muscular dystrophy is a condition allelic to Duchenne muscular dystrophy.

**Muscle biopsy** – the removal of a sample of muscle tissue for histopathological examination or protein analysis in order to help with the diagnosis of a muscle condition.

**Calpain 3** – encoded by the *CAPN3* gene, calpain 3 is a calcium-dependent protease located both at the cytosol and the nucleus, with a possible role in regulating levels of muscle-specific transcription factors and muscle cell differentiation. Recessive mutations in the *CAPN3* gene are responsible for limb-girdle muscular dystrophy type 2A.

**Collagen type VI** – a microfibrillar protein forming a network that associates with other extracellular matrix proteins, with a role in stabilizing the myofibrils during contraction by anchoring the basement membrane to the interstitial extracellular matrix. Mutations in the genes encoding for three subunit chains of collagen VI are responsible for Ullrich congenital muscular dystrophy and for Bethlem myopathy.

**Congenital muscular dystrophy** – a muscle condition usually diagnosed at birth or shortly after, characterized by variable degrees of muscle weakness, increased creatine kinase values, and other associated features such as central nervous system involvement, eye abnormalities, and developmental delay. Congenital muscular dystrophies are caused by mutations in several different genes.

**Distal myopathy** – a muscle condition characterized by predominant distal involvement, i.e. of muscles of hands and feet.

**Duchenne muscular dystrophy** – an X-linked genetic condition caused by mutations in the dystrophin (*DMD*) gene, causing absence of the dystrophin protein within muscle. Patients affected by Duchenne muscular dystrophy usually show first symptoms in their childhood with difficulties running, walking up stairs, frequent falls and calf hypertrophy. Creatine kinase values are highly increased. Duchenne muscular dystrophy is a condition allelic to Becker muscular dystrophy.

**Dysferlin** – encoded by the *DYSF* gene, dysferlin is predominantly localized in the muscle membrane, playing an important role in muscle membrane maintenance and integrity, vesicle trafficking, phagocytosis, focal adhesion, and myogenic differentiation. Recessive mutations in the *DYSF* gene are responsible for limb-girdle muscular dystrophy type 2B and Miyoshi myopathy.

**Dystrophin** – a cytoskeletal protein encoded by the *DMD* gene, dystrophin is localized to the muscle cell membrane through a dystrophin-associated protein complex and acting as a mechanical link between the cytoskeletal actin and the extracellular matrix. Reduction or absence of this protein causes Becker or Duchenne muscular dystrophies.

**Dystrophin – glycoprotein complex** – a group of proteins forming a critical link between the inside and outside of muscle cells, essential for its structure and correct functioning. Mutations in genes encoding for members of this protein complex are responsible for different muscular dystrophies.

**Dystroglycanopathies** – a genetically heterogeneous group of muscle disorders, characterized by detection of hypoglycosylated  $\alpha$ -dystroglycan on muscle biopsy by immunolabeling and/or on western blot analysis. Dystroglycanopathies might clinically range from severe congenital muscular dystrophies to milder, later onset limb-girdle muscular dystrophies.

**Emerin** – encoded by the X-linked *EMD* gene, emerin is a nuclear membrane protein. Mutations in the *EMD* gene are responsible for the X-linked form of Emery–Dreifuss muscular dystrophy.



**Emery – Dreifuss muscular dystrophy** – a muscle condition mainly characterized by progressive muscle weakness, contractures, and cardiac defects, and caused by mutations in the *EMD*, *FHL1* (X-linked forms), and *LMNA* genes (autosomal dominant and recessive forms).

**Facioscapulohumeral muscular dystrophy** – muscle condition mainly characterized by weakness of the facial, shoulder, upper arm muscles. The conditions is slowly progressive and later may also involves other muscle groups and in particular the lower limbs. The condition is caused by a molecular defect on the short arm of chromosome 4.

**Gowers' maneuver** – a characteristic sign observed in presence of weakness of the pelvic girdle and proximal lower limb muscles. Patients use their arms and hands to push up on their legs to stand up from a lying position.

**Lamin A/C** – encoded by the *LMNA* gene, Lamin A/C is a major component of the nuclear lamina, a proteinaceous meshwork underlying the inner nuclear membrane. Lamin A/C is involved in several cellular and molecular processes of nuclear architecture, DNA synthesis, chromatin organization, gene transcription, cell cycle progression, cell differentiation, migration, and response to DNA damage. Dominant and rare recessive mutations in *LMNA* are responsible for different types of muscular dystrophies, cardiomyopathies, and other rare disorders also with no major cardiac or respiratory involvement.

**Limb-girdle muscular dystrophy** – muscle conditions characterized by weakness in the upper and lower girdle muscles. There are different types of LGMDs, classified according to their inheritance (dominant-LGMD1- and recessive-LGMD2-), and the underlying genetic defect.

**Merosin (laminin alpha 2)** – a protein encoded by the *LAMA2* gene and essential for maintaining the structure and function of muscles. Merosin protein is absent in biopsies from patients affected by congenital muscular dystrophy type 1A.

**Muscular dystrophy** – the term dystrophy derives from the greek words “dys” (“faulty”) and “trophe” (“nourishment”). Muscular dystrophy refers to progressive muscle conditions characterized by weakness and wasting of the voluntary muscles.

**Pseudohypertrophy** – typically observed in Duchenne muscular dystrophy, this term refers to prominence of muscles, in particular of calves, due to increased fat deposits rather than true hypertrophy of muscle tissue.

**Revertant fibers** – rare fibers observed in muscle biopsies from patients with Duchenne muscular dystrophy, showing positive labeling for dystrophin in presence of a mutation causing absent production of this protein. Revertant fibers are caused by second mutations in the *DMD* gene, giving rise to exon skipping and production of a shorter but functional dystrophin.

**Sarcoglycans** – encoded by the four sarcoglycan genes (*SGCA*, *SGCB*, *SGCD*, and *SGCG*), sarcoglycans are transmembrane proteins within the dystrophin-glycoprotein complex at the muscle cell membrane with possible role in stabilizing the membrane. Recessive mutations in the  $\gamma$ -sarcoglycan,  $\alpha$ -sarcoglycan,  $\beta$ -sarcoglycan and  $\delta$ -sarcoglycan genes are responsible for limb girdle muscular dystrophy types 2C, 2D, 2E, and 2F.

**Ullrich congenital muscular dystrophy** – a congenital muscular dystrophy caused by recessive mutations in the genes encoding for subunit chains of the collagen type VI protein. The condition is characterized by contractures of the proximal joints, marked distal laxity, hypotonia, and progressive scoliosis often associated with rigid spine. Maximum functional ability is variable.

**Walker-Warburg syndrome** – a form of congenital muscular dystrophy characterized by brain and eye abnormalities in addition to muscle weakness. Walker-Warburg syndrome is a highly genetically heterogenous disorder caused by recessive mutations in four different genes (*POMT1*, *POMT2*, *FKRP*, and *FCMD*).

## 125.1 INTRODUCTION

Muscular dystrophies are a group of inherited primary diseases of the muscle clinically characterized by muscle weakness and pathologically by abnormalities of muscle fibers. According to the most recent classifications (1) and publications, muscular dystrophies and their major differential diagnoses can be classified based on their inheritance and genetic defects, as shown in Table 125-1. A schematic representation of the proteins involved in the most common forms of muscular dystrophy is shown in Figure 125-1.

## 125.2 DYSTROPHINOPATHIES

### 125.2.1 Definition

Dystrophinopathies represent a wide spectrum of mild to severe progressive muscle diseases caused by mutations in the *DMD* gene. The well-recognized Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) represent the best-understood and most common diseases within this spectrum, affecting boys (and less frequently carrier females), and primarily causing skeletal muscles weakness. Isolated asymptomatic increase of creatine kinase (CK) values and cardiomyopathies represent the milder ends of the spectrum.

### 125.2.2 History

DMD is named after Guillaume Benjamin Amand Duchenne, a French physician who described this condition in 1861. However, an English physician, Edward Meryon, described the same disorder several years earlier. He noted that the disease affected males, segregated

**TABLE 125-1 Classification of Muscular Dystrophies**

Disease	Inheritance	Locus	Gene	Protein
DMD/BMD	X-R	Xq21.2	<i>DMD</i>	Dystrophin
FSMD type 1	AD	4q35	?	?
FSMD type 2	AD	4q35	?	?
EDMD X-linked type 1	X-R	Xq28	<i>EMD</i>	Emerin
EDMD X-linked type 2	X-R	Xq26.3	<i>FHL1</i>	Four-and-a-half LIM Domain 1
EDMD Autosomal Dominant	AD	1q21.2	<i>LMNA</i>	Lamin A/C
EDMD-AR	AR	1q21.2	<i>LMNA</i>	Lamin A/C
EDMD with Nesprin-1 defect	AD	6q25	<i>SYNE1</i>	Spectrin repeat containing, nuclear envelope 1 (Nesprin-1)
EDMD with Nesprin-2 defect	AD	4q23	<i>SYNE2</i>	Spectrin repeat containing, nuclear envelope 2 (Nesprin-2)
Muscular dystrophy with generalized lipodystrophy	AR	17q21–q23	<i>PTRF</i>	Polymerase I and transcript release factor (cavin-1)
LGMD type 1A	AD	5q31	<i>MYOT</i>	Myotilin
LGMD type 1B	AD	1q21.2	<i>LMNA</i>	Lamin A/C
LGMD type 1C	AD	3p25	<i>CAV3</i>	Caveolin-3
LGMD type 1D	AD	7q	<i>DNAJB6</i>	DNAJB6
LGMD type 1E	AD	6q23	?	?
LGMD type 1F	AD	7q32	?	?
LGMD type 1G	AD	4p21	?	?
LGMD type 1H	AD	3p23–p25	?	?
LGMD type 2A	AR	15q15.1	<i>CAPN3</i>	Calpain-3
LGMD type 2B	AR	2p13	<i>DYSF</i>	Dysferlin
LGMD type 2C	AR	13q12	<i>SGCG</i>	γ-sarcoglycan
LGMD type 2D	AR	17q12–q21.33	<i>SGCA</i>	α-sarcoglycan
LGMD type 2E	AR	4q12	<i>SGCB</i>	β-sarcoglycan
LGMD type 2F	AR	5q33	<i>SGCD</i>	δ-sarcoglycan
LGMD type 2G	AR	17q12	<i>TCAP</i>	Titin-cap (telethonin)
LGMD type 2H	AR	9q31–q34	<i>TRIM32</i>	Tripartite motif-containing 32 (ubiquitin ligase)
LGMD type 2I	AR	19q13.3	<i>FKRP</i>	Fukutin-related protein
LGMD type 2J	AR	2q31	<i>TTN</i>	Titin
LGMD type 2K	AR	9q34	<i>POMT1</i>	Protein-1-O-mannosyl-transferase 1
LGMD type 2L	AR	11p14.3	<i>ANO5</i>	Anoctamin 5
LGMD type 2M	AR	9q31	<i>FKTN</i>	Fukutin
LGMD type 2N	AR	14q24	<i>POMT2</i>	Protein-O-mannosyl-transferase 2
LGMD type 2O	AR	1p34	<i>POMGNT1</i>	Protein-O-linked mannose beta1,2-N-aminyltransferase 1
LGMD type 2Q	AR	8q24	<i>PLEC1</i>	Plectin 1
LGMD type 2?	AR	3p21	<i>DAG1</i>	Dystrophin-associated glycoprotein 1
CMD with merosin deficiency (MDC1A)	AR	6q2	<i>LAMA2</i>	Laminin alpha2 chain of merosin
CMD	AR	1q42	?	?
CMD and abnormal glycosylation of dystroglycan (MDC1C)	AR	19q13	<i>FKRP</i>	Fukutin-related protein
CMD and abnormal glycosylation of dystroglycan (MDC1D)	AR	22q12	<i>LARGE</i>	Like-glycosyl transferase
FCMD	AR	9q31–q33	<i>FCMD</i>	Fukutin
WWS	AR	9q31–q33	<i>FCMD</i>	Fukutin
		9q34	<i>POMT1</i>	Protein-1-O-mannosyl-transferase 1
		14q24	<i>POMT2</i>	Protein-O-mannosyl-transferase 2
		1p34	<i>POMGNT1</i>	Protein-O-linked mannose beta1,2-N-aminyltransferase 1
		19q13	<i>FKRP</i>	Fukutin-related protein

Continued

**TABLE 125-1 Classification of Muscular Dystrophies—cont'd**

Disease	Inheritance	Locus	Gene	Protein
MEB disease	AR	1p34	<i>POMGNT1</i>	Protein-O-linked mannose beta1,2-N-aminyltransferase 1
		19q13	<i>FKRP</i>	Fukutin-related protein
		14q24	<i>POMT2</i>	Protein-O-mannosyl-transferase 2
CMD due to glycosylation disorder	AR	9q34.1	<i>DPM2</i>	Dolichyl-phosphate mannosyl-transferase polypeptide 2
		1q21.3	<i>DPM3</i>	Dolichyl-phosphate mannosyl-transferase polypeptide 3
CMD with mitochondrial structural abnormalities	mtDNA	22q13	<i>CHKB</i>	Choline kinase
Rigid spine syndrome	AR	1p36	<i>SEPN1</i>	Selenoprotein N1
Ullrich syndrome	AR	21q22.3	<i>COL6A1</i>	Collagen type VI, subunit alpha 1
		21q22.3	<i>COL6A2</i>	Collagen type VI, subunit alpha 2
		2q37	<i>COL6A3</i>	Collagen type VI, subunit alpha 3
BM	AD	21q22.3	<i>COL6A1</i>	Collagen type VI, subunit alpha 1
		21q22.3	<i>COL6A2</i>	Collagen type VI, subunit alpha 2
		2q37	<i>COL6A3</i>	Collagen type VI, subunit alpha 3
	AR	2q37	<i>COL6A3</i>	Collagen type VI, subunit alpha 3
CMD with integrin $\alpha 7$ defect	AR	12q13	<i>ITGA7</i>	Integrin $\alpha 7$
CMD with integrin $\alpha 9$ defect	AR	3p21.3	<i>ITGA9</i>	Integrin $\alpha 7$
MFM, DES-related	AD	2q35	<i>DES</i>	Desmin
MFM, ZASP-related	AD	10q22	<i>LDB3/ZASP</i>	LIM domain binding-3/Z band alternatively spliced PDZ motif
Myofibrillar myopathy, MYOT-related	AD	5q31	<i>MYOT</i>	Myotilin
MFM, FLNC-related	AD	7q32	<i>FLNC</i>	Filamin C
MFM, BAG3-related	AD	10q25–q26	<i>BAG3</i>	BLC2-associated athanogene 3
Inclusion body myopathy associated with Paget disease of the bone and frontotemporal dementia	AD	9p13.3	<i>VCP</i>	Valosin-containing protein
Welander distal myopathy	AD	2p13	?	?
Tibial muscular dystrophy	AD	2q31	<i>TTN</i>	Titin
Laing distal myopathy	AD	14q11.2	<i>MYH7</i>	Myosin heavy chain 7, cardiac muscle, beta
Distal myopathy with rimmed vacuoles	AR	9p12–p11	<i>GNE</i>	Glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase
Nebulin distal myopathy	AR	2q23.3	<i>NEB</i>	Nebulin
Distal myopathy, Kelch-like homolog 9-related	AR	9p21.2-p22.3	<i>KLHL9</i>	Kelch-like homolog 9 protein
Distal myopathy, Matrilin 3-related	AD	5q31	<i>MATR3</i>	Matrilin 3
Distal myopathy, ZASP-related	AD	10q22	<i>LDB3/ZASP</i>	LIM domain binding-3/Z band alternatively spliced PDZ motif
Distal myopathy, VCP-related	AD	9p13.3	<i>VCP</i>	Valosin-containing protein
Distal myopathy, MYOT-related	AD	5q31	<i>MYOT</i>	Myotilin
Myoshi distal myopathy	AR	2p13	<i>DYSF</i>	Dysferlin

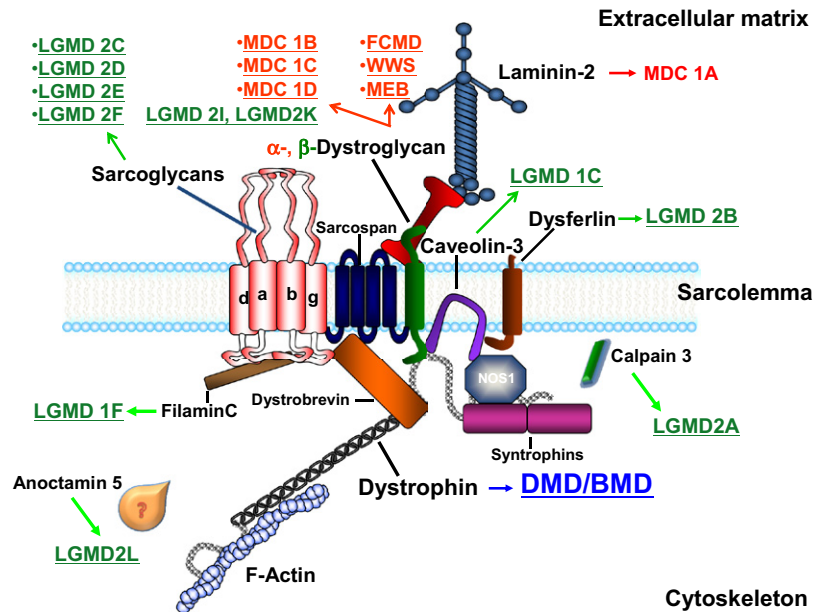
?: not known.

in families and essentially affected muscle, providing a detailed clinical, genetic, and pathologic study of the condition. In 1955, Peter Emil Becker delineated a milder form of X-linked muscular dystrophy, now referred to as BMD. In 1983, Davies et al. showed that the gene responsible for DMD was located on the short arm of the X chromosome, making prenatal testing available. The work from Harper et al. showed that BMD mapped to the same locus as DMD confirming these conditions were allelic. A few years later, cloning of the *DMD* gene

was accomplished by means of mapping of X chromosome translocations in manifesting female patients and use of deletion-detecting clones, and by 1988, specific DNA and protein-based diagnostics for dystrophinopathies was available.

### 125.2.3 Prevalence

DMD represents the most common inherited muscle disease of childhood, with an estimated point prevalence



**FIGURE 125-1** Schematic representation of the main proteins involved in muscular dystrophies, with their localization and reciprocal interactions. Disease names are indicated in abbreviations in underlined capital letters. Proteins are in lower case letters. *Reproduced with adaptation from Figure N. 1. of Straub and Bushby, 2006 (Straub and Bushby. The Childhood Limb-Girdle Muscular Dystrophies. Semin. Pediatr. Neurol. 13:104–114.)*

in northern England of 8.29/100.000 males (2), and birth prevalence of BMD of 1/18,450 live male births. It has been suggested that incidence rates of familial cases may be now declining as a result of prenatal testing (in countries where this service is offered to families with previous cases), leading in the long term to a relative increase of *de novo* cases. A high rate of new mutations maintains an essentially stable population frequency despite current practices of family counseling in identified families.

## 125.2.4 Duchenne Muscular Dystrophy

**125.2.4.1 Clinical Features.** DMD is mainly characterized by progressive, symmetrical muscle weakness affecting males, with an onset usually before the age of 5 years (3). A raised CK (typically up to  $>10$  the normal value) can be detected from birth. The most common first symptoms are walking delay ( $>18$  months of age) and general clumsiness compared to siblings and peers. Later, waddling gait, difficulties getting up stairs and from the floor (the so-called “Gower’s maneuver”), is caused by the presence of muscle weakness, affecting the pelvic girdle (Figure 125-2). The average age at diagnosis is 4–5 years (3). After this age, even in the context of a positive family history, a normal muscle examination greatly decreases the likelihood of a positive diagnosis, and this is highly unlikely above the age of 10. Some muscle groups and, in particular, the calves might appear enlarged (pseudohypertrophy), and muscle imbalances might cause contractures, in particular, shortening of the Achilles tendons. Flexion contractures of elbows, knees, hips and *talipes*

*equinovarus* can also develop after loss of ambulation. Muscle cramps and stiffness of the calf muscles are common, and myoglobinuria can also be observed. With no interventions, DMD patients are wheelchair bound before the age of 13 years. Respiratory muscle weakness and thoracic scoliosis contribute to respiratory insufficiency and together with cardiac involvement, in particular dilated cardiomyopathy and/or arrhythmias developing in the second decade, represent the most common causes of death. The intelligence coefficient is typically 1 SD below the general population, but cognitive impairment is not progressive and not correlated with weakness. Verbal intelligence and verbal skills appear more affected than performance. Smooth muscles can also be involved with occasional bladder paralysis, paralytic ileus, and gastric dilation. Delayed puberty and low bone mineral density is also observed (3–5).

**125.2.4.2 Natural History.** DMD shows a predictable clinical course in the absence of treatment. Independent ambulation is lost around the age of 9 years and, by definition, by the age of 13 years. Progression of muscle weakness to further muscle compartments and organs results in scoliosis, respiratory and cardiac impairment. The mean age at death in the absence of interventions is around age 19 years, 90% attributable to respiratory and 10% to cardiac causes.

## 125.2.5 Becker Muscular Dystrophy

BMD is also characterized by progressive symmetrical muscle weakness, often associated with calf hypertrophy, but onset is usually later than in DMD (mean age 11) (6),





**FIGURE 125-2** The sequence of the Gower's maneuver in a boy affected by Duchenne muscular dystrophy. (Figure by courtesy of Richard S. Finkel, Director of the Neuromuscular Program, The Children's Hospital of Philadelphia.)

and some patients show onset of mild symptoms only in late adulthood. Calf pain and cramps, in particular during and after exercise, is commonly observed and often represents the major symptom at onset. CK values are increased, usually up to  $>5$  times the normal values. Progression of weakness is slower than in DMD, and ambulation is longer preserved, and wheelchair dependency develops per definition after the age of 16 years and often very much later. Contractures of Achilles tendons are commonly observed. Cardiomyopathy is a common finding in BMD with cardiac symptoms also sometimes occurring before the onset of weakness, and it represents the cause of death in up to 50% of patients (7).

### 125.2.6 Manifesting Carriers of Dystrophinopathies

Heterozygous female carriers of *DMD* gene mutations are mostly asymptomatic; however, between 2.5 and 7.8% of carriers develop some symptoms of the condition (manifesting carriers—MCs) ranging from very mild late-onset muscle weakness to a rapidly progressive DMD-like phenotype (8). Asymmetry of weakness is a common finding and likely related to somatic mosaicism. Cardiomyopathy is also common, with positive echocardiographic evidence in up to about 36–38% of MCs. Decline in cardiac function can be acute, in particular, during pregnancy,

making monitoring of cardiac function pivotal for MCs. CK values are elevated up to 2–10 times the normal values in around 50% of females with a truncating *DMD* mutation and in around 30% of females with in frame changes.

### 125.2.7 *DMD* Gene-Associated Dilated Cardiomyopathy

Isolated dilated cardiomyopathy caused by *DMD* gene mutations is characterized by predominant involvement of cardiac muscles, with relative sparing of skeletal muscles, occurring in both males and females. Males, usually symptomatic between ages 20 and 40 years, show a more severe and progressive phenotype, and cardiac transplant is frequently recommended. CK values are elevated, and on muscle biopsy analysis myopathic changes can be observed.

### 125.2.8 Genetics and Pathogenesis

Dystrophinopathies are X-linked conditions caused by mutations in the dystrophin (*DMD*) gene.

**125.2.8.1 The Dystrophin Gene.** The *DMD* gene is located on chromosome Xq21.1 and spans 2.4Mb of genomic DNA, being the largest known human gene. The coding sequence of 11 kb represents only 0.6% of the entire gene, and it is divided in 79 exons with multiple promoters.

**125.2.8.2 The Dystrophin Protein.** The dystrophin protein has a molecular weight of 427kDa and consists of 3685 amino acids. Dystrophin is an N-terminal actin-binding protein, with 24 spectrin-like repeat units interspersed by four hinge regions, followed by a cysteine-rich domain and a C-terminal domain. The N-terminal domain has three actin-binding sites with a fourth one located within the 11–17th spectrin-like repeats. Dystrophin is a cytoskeletal protein, localized to the muscle cell membrane through a dystrophin-associated protein complex and acts as a mechanical link between the cytoskeletal actin and the extracellular matrix (Figure 125-1). Its C-terminal cysteine-rich domain binds to the  $\beta$ -dystroglycan, which via the transmembrane  $\alpha$ -dystroglycan is connected to the extracellular matrix protein laminin  $\alpha$ -2. The dystrophin protein is also a key mediator in the communication between the extracellular matrix and the cytoskeleton. There are different known isoforms of the protein, and each isoform is under the control of one of the different promoters, with shorter transcripts being generated from internal promoters. Full-length proteins are found in the muscle, brain and Purkinje cells. Shorter brain isoforms have been implicated in mental retardation, while mutations in the muscle promoter and first exons are related to the isolated forms of cardiomyopathies.

**125.2.8.3 *DMD* Gene Mutations.** A large spectrum of *DMD* gene mutations has been described so far, including

deletions of the entire gene, deletions/duplications of one or more exons, small deletions, insertions, or single base changes. Probably due to its extreme size, the mutation rate is higher than the estimated average mutation rate in humans ( $1.10^{-4}$  versus  $10^{-5}$ – $10^{-6}$  for human genes) with about 1/3 of all mutations being *de novo* changes with more than 4700 different mutations described so far (9). Mutation hot spots are recognized with partial deletions and duplication mutations clustering in two recombination hot spots, one proximal to the 5' end of the gene involving exons 2–20 ( $\approx 30\%$  of the mutations), and one more distal, involving exons 44–53 ( $\approx 70\%$  of mutations). Duplications cluster near the 5' end of the gene, with duplication of exon 2 being the most common duplication (9). The reading frame rule was first described by Monaco et al. in 1988, giving an explanation for the phenotypic difference between DMD and BMD. Mutations identified in DMD patients are shown to affect the translational open reading frame (ORF) of triplet codons, and each mutation is predicted to result in truncated abnormal products, while mutations identified in BMD patients maintain the translational ORF. An in-frame deletion will produce a shorter lower molecular weight and semifunctional protein giving rise to a milder clinical phenotype. The same ORF mechanism is also applicable for splice site mutations. About 10% of known mutations do not follow the reading frame rule, and this could be explained by mechanisms holding true the reading frame rule at the RNA level (9). In particular, missense and also in-frame deletion/duplication mutations can affect splicing and thus create out-of-frame transcripts, while large in-frame deletions may result in nonfunctional product, especially if they involve key domains such as the cysteine-rich domain. Conversely, nonsense mutations and out-of-frame deletions/duplications can also affect splicing, leading to in-frame transcripts and, therefore, BMD phenotypes. These events have been observed in about 2% of BMD patients (9). Finally, a number of mutations have been identified in both DMD and BMD patients, and again it has been suggested that this could be caused by alternative splicing of one of more flanking exons or use of alternative translation initiation codon, such as the three in-frame start codons in exon 8. mRNA analysis is currently not always feasible, and predictive softwares, such as ESEfinder, can help to identify exonic splicing-enhancer sequence motives to further define the effect of novel *DMD* mutations. Report of families segregating the same *DMD* mutations in family members showing different phenotypes indicate that other factors can also play a role and part of this clinical variability could possibly be explained by transacting polymorphisms, e.g. within muscle performance genes or splicing factors as well as cis-acting polymorphisms in the *DMD* locus itself.

**125.2.8.4 Pathogenesis.** The most likely sequence of events in the pathogenesis of DMD and BMD is that the deficiency of dystrophin (and the resulting reduction of all the dystrophin-associated proteins) causes a disruption

of the cell membrane and an efflux of muscle proteins (e.g. CK) and an influx of calcium. The latter leads to mitochondrial overload with ATP depletion, decreased oxidative phosphorylation, and cell death (muscle cell necrosis). An imbalance between muscle fiber necrosis and regeneration is considered the main cause of the clinical manifestations of dystrophinopathies, with the primary pathologic feature necrosis, and with decrease of regenerative capacity with age, leading to replacement of muscle fibers with connective and adipose tissue (10). Several aspects of the pathogenesis of dystrophinopathies are far from clear. In particular, the cause of the delay in onset and the thereafter-progressive nature of the diseases remain hard to explain.

Several different pathophysiological hypotheses have been described in more detail elsewhere (10). Currently, the mechanical and the impaired calcium homeostasis hypothesis represent the two main leading hypotheses. In addition, a contribution of inflammation, apoptosis, and regeneration has also been suggested. According to the mechanical hypothesis, absence of dystrophin affects the membrane structure. Concurrent delocalization of dystrophin-associated proteins from the membrane leads to membrane fragility and increased membrane permeability, all greatly affected by exercise. Structural weakness and tears in the sarcolemma and consequent fail in membrane repair cause influx of calcium-rich extracellular fluid. When the calcium-buffering capacities of the sarcoplasmic reticulum, the mitochondria and intracellular calcium-binding proteins are reached, calcium-dependent proteases are activated leading to digestion of cytoskeletal and myofibrillar proteins, together with activation of calcium-mediated phospholipases that further damage the membrane. Dystrophin deficient cells have an inherent defect in calcium regulation also in the presence of the intact plasma membrane, due to increased opening of calcium leak channels. An increased calcium level makes mitochondria form a large pore complex, leading to loss of matrix and intermembrane contents, and to mitochondrial swelling, rupture and then necrosis and apoptosis. Increased influx of extracellular fluid also causes intracellular activation of complement, leading to further damage of intracellular membranous organelles and recruitment of macrophages. Interestingly, glucocorticoid treatment in DMD has been shown to decrement the number of inflammatory cells, but the beneficial effect of the treatment is unlikely related to its antiinflammatory effect. Oxidative stress has also been implicated in DMD pathophysiology, as *mdx* mice muscles show increased levels of antioxidant enzymes even before onset of myopathology. Oxidative stress combined with additional insult to the cell homeostasis such as a physical damage could promote cell pathology. Sparing of extraocular muscles could be attributed to better calcium handling capability, higher utrophin levels, and smaller size of muscle fibers that can influence the tension exerted on the plasma membrane.

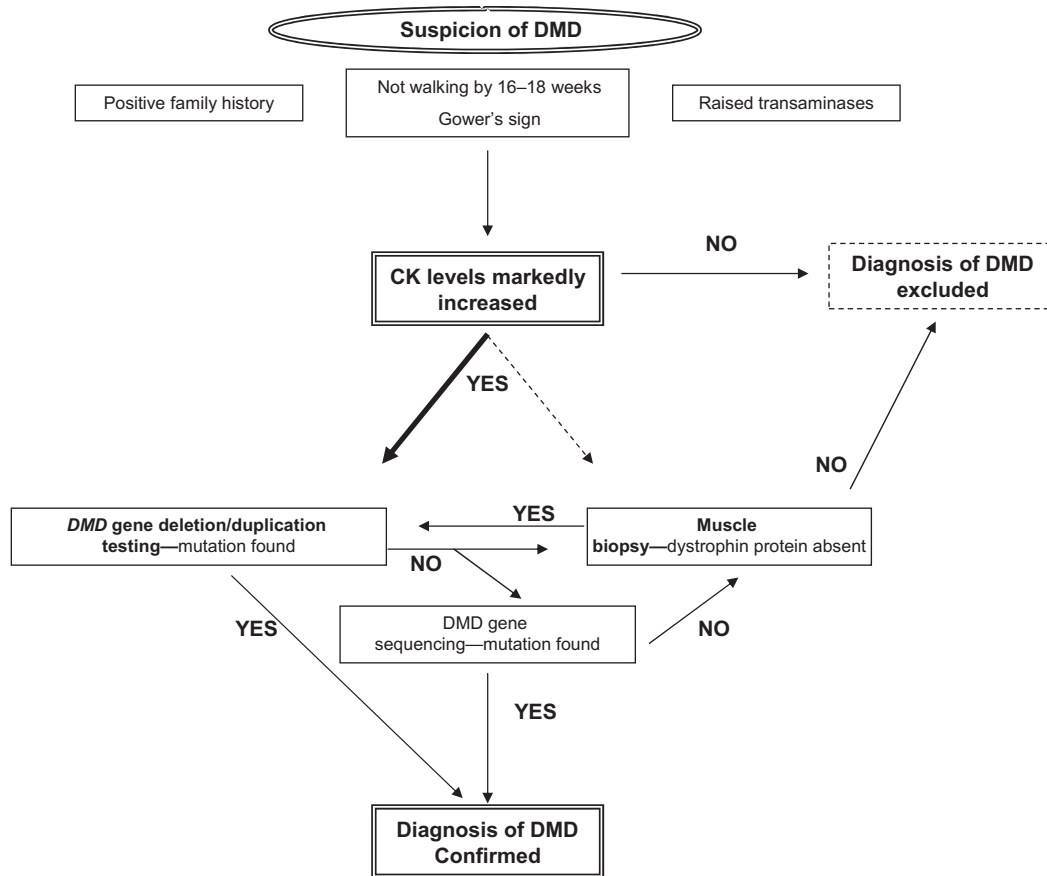
### 125.2.9 Diagnosis

Diagnosis of dystrophinopathies and in particular of DMD should be suspected in any child with a positive family history but, as new mutations are so commonly seen, also in the case of (A) a male child with abnormal muscle function such as delayed walking (>16–18 months of age), frequent falls, difficult running, and climbing stairs, Gower's maneuver, waddling gait; (B) increased CK values; and (C) increased transaminases. In the case of a positive family history, increased CK values would be indicative of a positive diagnosis. Speech and language delay also supports positive diagnosis (4,5).

Diagnostic algorithms include clinical and genetic investigations that should be done and interpreted together (Figure 125-3). In the case of abnormal muscle function in a male child, increased CK levels should prompt *DMD* gene analysis, with deletions/duplications analysis as first step, preferably by a technique that screens all exons such as multiplex ligation-dependent probe amplification (MLPA) (11). Presence of a mutation in the *DMD* gene confirms a diagnosis of dystrophinopathy. If no mutations are found, a muscle biopsy can confirm the absent dystrophin labeling on immunohistochemistry that should prompt direct sequencing of the gene. If the muscle biopsy analysis shows normal protein labeling, alternative diagnoses should be considered, although very rare cases of DMD and BMD probably do have normal localization of protein. Many other forms of muscular dystrophy (for example some forms of limb-girdle muscular dystrophies (LGMDs) or congenital muscular dystrophies) cause an elevated CK, so the finding of an elevated CK without confirmatory tests should never be considered diagnostic of dystrophinopathy. It is important to note that dystrophinopathy is much more common than other forms of muscular dystrophy, so the diagnosis should always be considered seriously (for example, in sporadic female cases, the diagnosis of MC is important to consider, and BMD is much more common than the different forms of LGMD).

The test most commonly used to identify deletions/duplications of the *DMD* gene mutations is MLPA; however, other techniques such as multiplex amplifiable probe hybridization and single condition amplification/internal primer are also available, and the latter also provides sequence data alongside deletions (11–14). If these tests are negative, full *DMD* gene sequencing should be performed in order to identify point mutations and small deletions/insertions (15). As none of these techniques is 100% sensitive and universally available, muscle biopsy can facilitate diagnosis and, in yielding a quicker diagnosis, minimize anxiety of the families (16). An open biopsy is necessary so that adequate tissue amount is available, in particular if alternative diagnoses need to be considered as well. Cocothome technique has the advantage of providing a larger sample compared with single-core needle biopsy and does not require open surgical procedure (17).





**FIGURE 125-3** Diagnostic approach in case of suspicion of Duchenne muscular dystrophy (DMD).

A muscle biopsy alone in absence of confirmed genetic result is not sufficient to confirm a diagnosis of dystrophinopathy and crucially is not sufficient to offer the carrier testing, which is then mandatory to suggest for the rest of the family. Noteworthy, in the presence of positive genetic testing, muscle biopsy is not required to confirm the diagnosis, although some centers may like to perform dystrophin quantification in conjunction with the genetic testing and clinical assessment.

**125.2.9.1 Other Clinical Investigations.** Recent recommendations for diagnosis and care indicate that electromyography (EMG) and nerve conduction studies are not necessary for the specific assessment of DMD (4,5). In fact, EMG analysis shows nonspecific findings observed in all myogenic disorders, and it is therefore of relative inutility in the diagnostic process of dystrophinopathies.

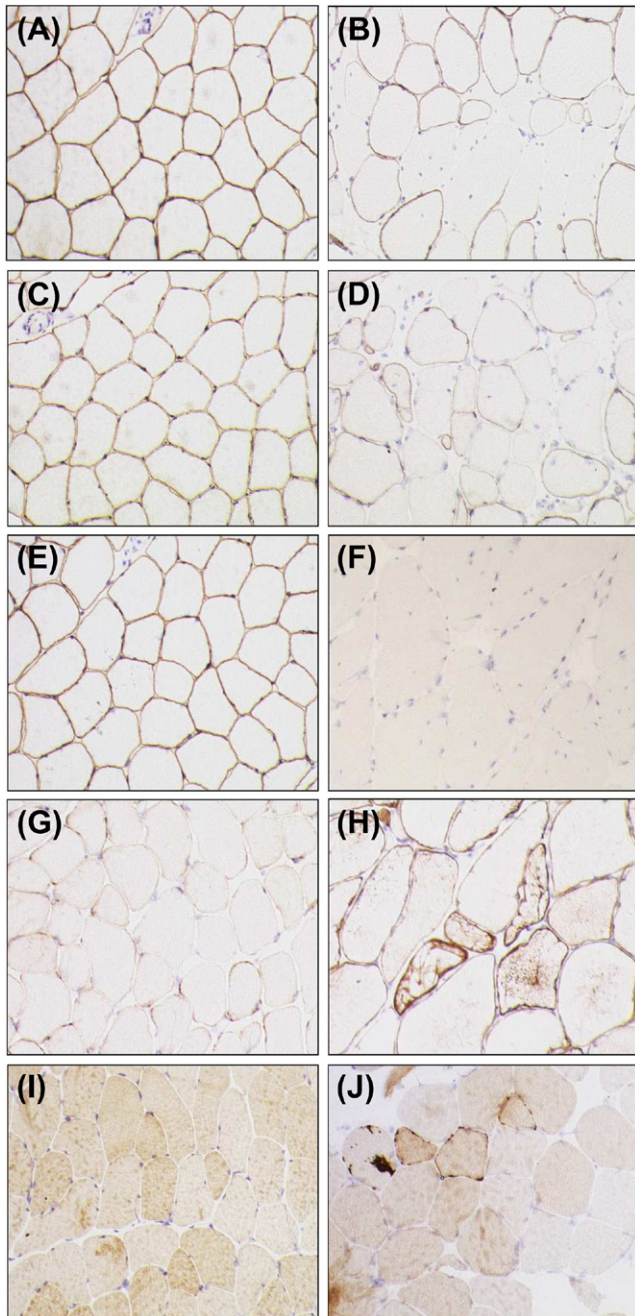
**125.2.9.2 Muscle Biopsy and Protein Analysis.** Muscle histology in the early stages of the disease shows nonspecific dystrophic changes such as variation in fiber size, foci of necrosis, and regeneration and hyalinization. In later stages, deposition of fat and connective tissue is evident. Necrotic fibers are surrounded by macrophages and CD4+ lymphocytes. Small internally nucleated fibers reflect muscle regeneration. Immunohistochemistry analysis shows complete or almost complete absence of dystrophin in DMD,

and dystrophin is typically not detectable on Western blot analysis (Figures 125-4 and 125-5) (4,5). In BMD, labeling of dystrophin can appear normal, patchy, or reduced on immunohistochemistry, while Western blot analysis could show bands of normal or abnormal weight and quantity (Figures 125-4 and 125-5). Dystrophin abundance could be studied by immunohistochemical techniques, and Western blot analysis is able to offer valuable semiquantitative data (16). Recently, a method has been developed to quantify relative levels of sarcolemma-associated proteins using digitally captured images of immunolabeled sections of skeletal muscle (18). This method appears useful not only to establish both the abundance and localization of dystrophin but also to assess the efficacy of experimental therapies leading to partial dystrophin restoration or upregulation (18).

Female carriers typically show a mosaic pattern of immunohistochemistry, with variable amounts of protein on Western blot, depending on the mutation and the amount of X-chromosome inactivation, although these changes may be subtle (Figure 125-5). Electron microscopy (EM) is not required for diagnosis of dystrophinopathies.

**125.2.9.3 DNA Analysis.** Molecular confirmation of dystrophinopathies is achieved when a clearly pathogenic *DMD* gene sequence variant is found in an affected





**FIGURE 125-4** Main muscle immunohistochemical findings in patients with muscular dystrophies. Control staining for Dystrophin,  $\alpha$ -dystroglycan, Caveolin 3, Desmin, and Myotilin are in panels A, C, E, G, and I respectively; (B) dystrophin staining in a female carrier for DMD; (D)  $\alpha$ -dystroglycan staining in a LGMD2I patient with *FKRP* gene mutations; (F) Caveolin-3 staining in a patient with LGMD1C; (H) Desmin staining in a patient with *DES* gene-related MFM; (J) Myotilin staining in a patient with *MYOT* gene-related MFM. (Figure courtesy of Dr Rita Barresi, NCG Diagnostic and Advisory Service for Rare Neuromuscular Diseases, Muscle Immunoanalysis Unit, Newcastle upon Tyne, UK.)

individual (19). The current recommended diagnostic workup of dystrophinopathies is shown in the algorithm in Figure 125-3. If a deletion/duplication is found, a diagnosis of BMD or DMD is made according to the reading frame and the overall clinical presentation.

Where duplication is detected, the prediction of the reading frame is not feasible with genomic analysis only, as the determination of the orientation of duplicated exons requires mRNA analysis.

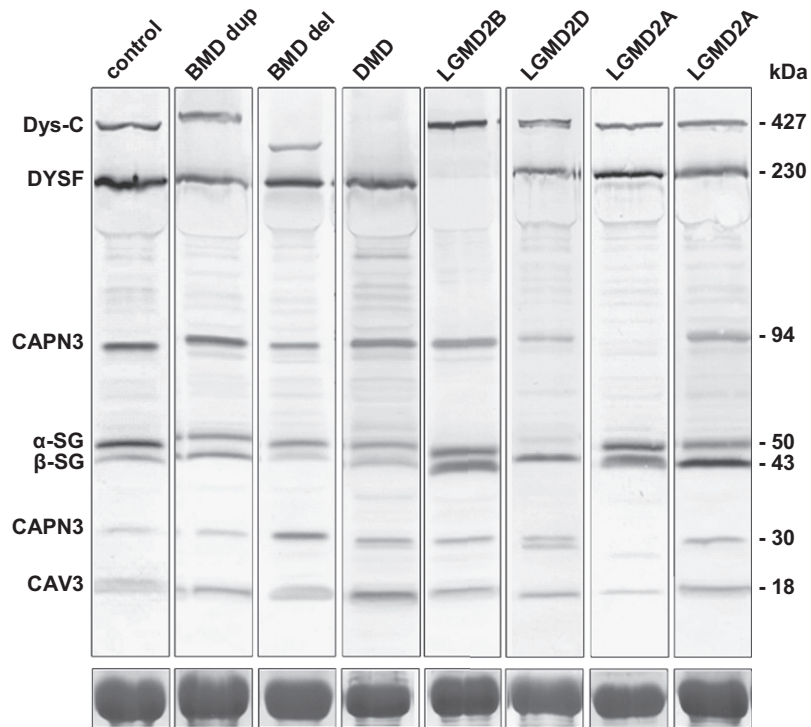
If no mutation is found, the likelihood of the diagnosis is reduced depending on the sensitivity of the screening technique. Since none of the current molecular tests is 100% sensitive, it is not possible to refute a diagnosis if no mutation is found. Indeed, recent reports show that about 4–7% of symptomatic individuals show no detectable mutation by genomic analysis, and analysis of mRNA or muscle biopsy analysis would be required to confirm the diagnosis in these patients (14).

### 125.2.10 Prevention and prenatal testing

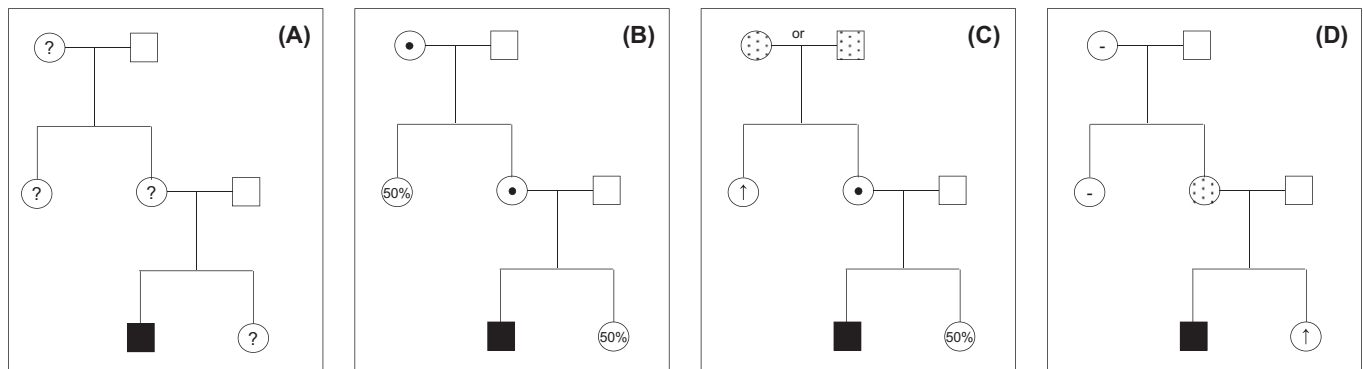
Prevention of dystrophinopathies is possible by means of counseling and prenatal diagnosis.

**125.2.10.1 Genetic Counseling.** Dystrophinopathies are X-linked conditions. Fathers of affected males are therefore not affected nor carry the mutation. Mothers and other relatives from the maternal line are at risk of being affected or being carriers for the disease (Figure 125-6). A woman with more than one affected son is either a carrier or has germ line mosaicism for the disease-causing mutation. If the proband is the only affected individual in the family, the mother is either a carrier or the mutation is occurred *de novo* in the egg or sperm at time of conception (germ line mutation) (20). The mother could also present mosaicism (somatic or germ line) for the mutation. If the mother is a carrier, she could have inherited this mutation from her mother (maternal grandmother of the affected individual) who could be either a carrier or a somatic mosaic, or have had germ line mosaicism or a *de novo* mutation (these last two options also valid for the maternal grandfather). The risk for the sibs of affected individuals depends on the carrier status of the mother, although also needs to take into account germ line mosaicism (Figure 125-7) (20). If the mother is a carrier, the risk of transmitting the disease mutation is 50% for each pregnancy. Males affected by dystrophinopathies have no risk of transmitting the disease to their male offspring, while their daughters will be obligate carriers.

**125.2.10.2 Carrier Testing.** About 2/3 of cases of dystrophinopathies are transmitted from carrier females, and testing of mothers and sisters is therefore fundamental not only because of counseling issues but also to identify potential MCs. Prior identification of the disease-causing mutations in the affected individuals is crucial in testing potential at risk relatives. When the mutation is known, screening of the disease-causing mutation can be performed on a DNA sample from the at-risk relative. About one-third of the potential carriers are still not tested and therefore missed, and discussion about the indication of a more active approach of at risk family members is in progress (21). If the affected male



**FIGURE 125-5** Schematic representation of the main muscle Immunoblot findings in patients with muscular dystrophies. Specific protein labeling is indicated on the left side of the image. Protein weights of each fragment are indicated in kDa on the right side of the image. The first lane represents the control muscle. The following lanes represent patients affected by (from left to right) BMD due to an in-frame duplication of the *DMD* gene; BMD due to an in-frame deletion of the *DMD* gene; DMD; LGMD2B, LGMD2D and LGMD2A. The last lane represents an example of a patient with confirmed diagnosis of LGMD2A, based on clinical and molecular data, with normal Calpain 3 labeling on immunoblot. (Figure courtesy of Dr Rita Barresi, NCG Diagnostic and Advisory Service for Rare Neuromuscular Diseases, Muscle Immunoanalysis Unit, Newcastle upon Tyne, UK.)

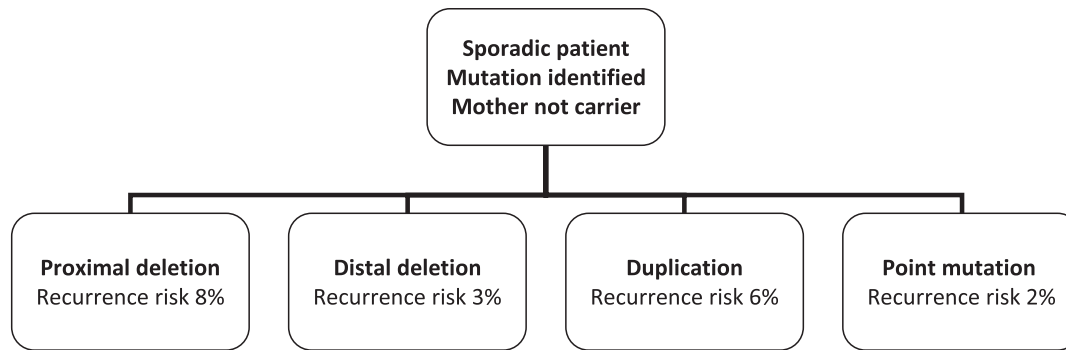


**FIGURE 125-6** Carrier status and germ line mosaicism in DMD. Dotted circles/squares represent males and females with germ line mosaicism. (A) In the case of a sporadic affected male, females through the maternal line are at risk of being somatic carriers. (B) In case the mother is a carrier, the sister of the affected has 50% risk of being carrier. If the maternal grandmother is also a carrier, the maternal aunt has a 50% risk of being a carrier. (C) If the grandmother is not a carrier, there is a low residual risk due to germ line mosaicism in the grandparents for the maternal aunt of being a carrier. (D) In case the mother is not a carrier, there is low residual risk due to germ line mosaicism for the sister of the affected of being a carrier.

relative is not available, at-risk females should be tested as discussed for affected individuals.

In the case of an unknown *DMD* mutation, the presence of signs and symptoms of the disease in at risk relatives could also help to estimate carrier status and genetic counseling. The Bayes' theorem has for a long time been a valuable tool to calculate carrier risk in case of a single child affected by dystrophinopathy with no mutation

found and depending on CK values. The Bayes' theorem is based on the prior, conditional, joint, and posterior probabilities of the mother being a carrier. The prior probability is based on the knowledge of the antecedents and sibs, and the conditional probability is the probability of being carrier or not depending on the CK values of the mother and the number of unaffected sons she might have. The product of the prior and the conditional



**FIGURE 125-7** Recurrence risk in case of a sporadic patient affected by Duchenne muscular dystrophy, with the mother not being a carrier. The indicated risks are for an affected son only and with no information about the haplotype. (Reproduced with adaptation from Figure N. 3 of Helderma-van den Enden et al. (20).)

probability is the joint probability, while the posterior probability of a woman to be carrier for dystrophinopathy is the joint probability of being carrier divided by this plus the joint probability of non being a carrier (3).

**125.2.10.3 Haplotype Analysis.** Where there are multiple affected individuals in the same family, and in the case of a clearly defined clinical diagnosis with no living relatives for direct carrier testing, haplotype analysis could be offered by some laboratories to trace the at-risk DMD haplotype for carrier testing and prenatal diagnosis. At least, three informative markers should be used, one of which is intragenic and one extragenic flanking the DMD gene on either side. Interpretation needs to take into account recombination risk, and therefore it is not in common use, now that direct methods of mutation detection have become more widespread.

**125.2.10.4 Germ Line Mosaicism.** About 1/3 of patients with dystrophinopathy show a *de novo* DMD gene mutation that occurred during cell division (mitosis or meiosis). As the general mutation rate is relatively low, the risk of a second independent mutational event in a family or individual is estimated to be low. Germ-line mosaicism was first described in DMD in the late 80s, and at that time, the recurrence risk for noncarrier females was estimated to be between 14 and 20%. Recent data suggest that the real estimate of recurrence risk in case of *de novo* DMD mutation is much lower, being 8.6% (4.8–12.2), if the risk haplotype is transmitted (20). In the current counseling practice where risk haplotype is not usually investigated, the recurrence risk is estimated to be 4.3%. This increased risk also applies for sisters of apparently *de novo* carrier females because of germ line mosaicism in the maternal grandmother or grandfather that could cause an increased risk of being carrier themselves and therefore of transmitting the disease. Remarkable differences have been noted if the mutation is a proximal (15.6%) or distal deletion (6.4%) (Figure 125-7) (20). Most mutations, and in particular deletions, originate on the X-chromosome of the maternal grandmother, whereas point mutations originate on the X-chromosome of the maternal grandfather. Germ line mosaicism should therefore be considered when

counseling families with *de novo* DMD mutations and prenatal diagnosis should be discussed in all pregnancies of mothers of isolated cases and carrier testing for their sisters (20).

**125.2.10.5 Prenatal Testing.** Prenatal testing is offered in case of male pregnancies to females who are known to carry a DMD gene mutation or who are at risk of germ line mosaicism for known DMD gene mutations. The procedure usually involves extraction of DNA from fetal cells (chorionic villi at 10–12 weeks' gestation or amniotic cells at 15–18 weeks' gestation). In the case of an unknown mutation, linkage analysis could also be offered; however, this should take into account the high risk of recombination. Currently, it is not possible to predict if a carrier female will manifest any signs or symptoms of the disease, and therefore, it is generally considered inappropriate to offer prenatal testing in case of female fetus. Examination of possible maternal cell contamination of the fetal DNA must be carried out as this, in particular with specific type of mutations such as deletions, could affect the interpretation of the test giving false negative results. Preimplantation genetic diagnosis (PGD) is offered in a limited number of centers throughout the world and special requirements apply (22).

**125.2.10.6 Neonatal Screening.** In the case of suspicion of dystrophinopathy at birth, such as grossly elevated CK values at birth or because of a positive family history, genetic testing can be performed at an early stage, allowing early institution of treatment and prompt genetic counseling. Currently, some countries are performing newborn CK screening programs, and this is currently considered also by other countries in view of possible future presymptomatic treatments (23). This sort of screening program holds several ethical and practical implications that need further discussion (24,25).

## 125.2.11 Management and Treatment

To date, there is no curative treatment for dystrophinopathies. Over recent years, advances in medical management and treatment of complications of dystrophinopathies have made a significant difference in terms



of natural history of the disease and also in terms of life expectancy, in particular, for DMD. The key interventions for DMD are use of steroids, surgical management of scoliosis, management of cardiac and respiratory complications as well as enhancement of quality of life, with timely supply of aids, adaptations, and access to independent living (26). All affected individuals should have access to the same clinical care, and nihilistic approach should not be accepted. In 2009, the international “DMD care considerations working group” evaluated assessments and interventions used in the management of DMD and developed care recommendations for this condition. These recommendations represent a framework for a coordinated multidisciplinary care, including rehabilitation, gastroenterology, nutrition, orthopedic, surgical, respiratory, and cardiac cares (4,5).

Benefits of steroids on DMD were first suggested in 1974, and glucocorticoids still represent the gold standard in DMD care, being the only currently available medication able to slow down the disease progression in terms of muscle strength and function. This also reduces risk of scoliosis, stabilizes respiratory function and improves cardiac function. Randomized control trials (RCTs) showed that treatment with 0.75 mg/kg of prednisone/prednisolone improves muscle strength, being daily regimes more effective than alternate (4,5,26–28). Deflazacort, a sodium-sparing glucocorticoid used in Europe but not approved in the US, has been shown to have a similar efficacy at a daily dose of 0.9 mg/kg and associates with lower risk of side effects (29). Long-term use of glucocorticoids prolongs ambulation and reduces speed of muscle function decline. Particular care need to be taken for steroid-related side effects and their prevention, and management has to be proactive. A multicenter, double-blind, parallel-group study, aiming to compare three steroid regimes (prednisone 0.75 mg/kg/day, prednisone 0.75 mg/kg/10 days on 10 days off, deflazacort 0.9 mg/kg/day) is currently underway in order to assess whether daily steroids are of greater benefit than intermittent steroids, and whether deflazacort does indeed associate with a better side effect profile than daily prednisone (30). At the moment, there are no evidence-based guidelines regarding when to start glucocorticoid treatment. Current consensus suggests that unless there are major preexisting risk factors for side effects, steroid treatment should be proposed when the child reaches the so-called *plateau* phase (age 4–8 years) when there is no progress in motor skills but prior to decline of muscle function, as evidenced by history and timed testing (4,5).

Anabolic steroids are neither necessary nor appropriate. There are no clear recommendations for use of creatine in DMD, and a RCT of creatine in DMD did not show clear benefits. Coenzyme Q10, amino acids, antiinflammatories, antioxidants, and potential disease modifying drugs, such as pentoxifylline and herbal and botanic agents, have been used or indicated for DMD treatment, but there are no supportive data to corroborate their use (4,5).

Due to the much less-progressive clinical course seen in most cases of BMD, steroids are much less frequently used. Cardiac and respiratory surveillance and timely intervention (including for some patients cardiac transplantation and ventilator support) are the mainstay of management along with physiotherapy and timely provision of aids and adaptations.

**125.2.11.1 Molecular Therapies.** Different molecular strategies, such as cell and gene therapy, mutation specific and cellular pathway approaches have been tested in cell lines, animal models, and clinical trials. At the current time, stem and progenitor cell therapies appear more distant from therapeutic applications (31–33). This field by definition is moving very rapidly, and the following is a summary of the state of the art at the time of writing—with the results of ongoing trials, there will be new findings in this area in the coming years.

**125.2.11.1.1 Gene Replacement Therapy.** The main obstacle for gene replacement is the size of the DMD gene and the difficulty of fitting it in gene transfer vehicles, such as adeno-associated virus. Mini or microdystrophins are able to transfer in adenovirus, and in *mdx* mouse, they give rise to robust gene expression correcting the muscle defect. However these approaches result more difficult in humans because of unpredicted obstacles such as immune response that requires further studies (34). Human artificial chromosomes containing the entire dystrophin genome with patient-derived and induced pluripotent stem cells and artificial zinc finger transcription factors targeting the utrophin gene promoter have been also proposed as alternative approaches, offering lower risk of immunorejection. Finally, utrophin upregulation has been modeled in various ways, and this approach could add beneficial effects if combined with other treatments.

**125.2.11.1.2 Mutation Suppression or Stop Codon Read Through.** Aminoglycoside antibiotics possess the unique property to read through stop codons in pro- and eukaryotes, introducing a nucleotide sequence at the mRNA level that creates a missense mutation instead of the stop codon that permits the translation of the full-length protein. Gentamicin-induced mutation suppression was observed in the *mdx* mouse, with expression of full-length protein and reduction of serum CK. This mutation suppression treatment would potentially be applicable only for patients with mutations leading to premature termination codons that in humans represent 13–15% of all DMD/BMD cases. Treatments with gentamicin showed that in order to achieve efficacy in restoring muscle function, doses would need to be increased with potential renal and auditory toxicity (35,36).

PTC124 (Ataluren, PTC therapeutics, South Plainfield, NJ, USA) is a 284-Da, 1,2,4-oxadiazole-linked furobenzene and benzoic rings drug that have been designed to readthrough stop codon mutations, and its therapeutic application are currently underway for cystic fibrosis and hemophilia. A phase IIb clinical trial with



Ataluren in DMD/BMD showed no statistically significant change in the primary endpoint of the trial (6-minute walking test) within the 48 weeks duration of the trial; however, the drug has been shown to be safe (<http://www.clinicaltrials.gov>). Discussion is currently in progress about the real efficacy of PTC124 to readthrough stop codons, and it has also been suggested that this agent could reactivate silent retroelements with potential negative consequences in future generations (37).

**125.2.11.1.3 Exon Skipping and Antisense Oligonucleotides.** The exon skipping approach is based on the observation of rare dystrophin-positive revertant fibers in muscle biopsies from DMD patients. This phenomenon is caused by spontaneous second mutations that cause exon skipping, giving rise to restoration of the reading frame and therefore production of shorter but functional dystrophin. This approach is based on direct manipulation of the dystrophin transcript, via antisense oligonucleotides (AONs), chemically synthesized single-strand DNAs designed to hybridize with a complementary sequence of mRNA, able to alter RNA process excluding one or more exons from the mRNA. When appropriately designed, AONs are able to restore the reading frame leading to a partially functional dystrophin protein (38–41). Feasibility of exon skipping has been demonstrated by local and systemic administration of AONs in mice, CXMD dogs, and utrophin/dystrophin double-ko mice. Study of proof of principle and safety of exon 51 skipping with 2 different backbones (2-O-methylAONs-PRO051 and morpholino AONs-AVI-4658) following intramuscular injections, demonstrated partial restoration (3–35% expression) and sarcolemmal localization of newly synthesized dystrophin (42). Phase I/II studies of intravenous infusions of AVI-4658 and subcutaneous administration of PRO051 have been recently performed to evaluate their safety profile first and then their ability to induce exon skipping and dystrophin expression. Both studies showed variable dose-dependent systemic restoration of dystrophin (43,44). Moreover, PRO051 administration also showed a modest anecdotal improvement in the 6-minute walk test. Preliminary results appear promising, and current molecular data indicate that up to 60% of DMD patients would potentially benefit from single-exon skipping approach. Single- and double-exon skipping would be applicable to 79% of deletions, 91% of small mutations, and 73% of duplications, amounting to 83% of all DMD mutations. Exon 51 skipping alone would be applicable up to 13% of all DMD patients (45). Multiexon skipping approaches would have the benefit of covering large number of mutations and patients with a single treatment. The open question is whether partial restoration of dystrophin expression is sufficient to induce clinical and functional improvement and different chemistries with improved delivery systems are currently in study. Worth mentioning, none of these therapies are currently able to target the heart in the *mdx* mouse at clinical doses, and ways to overcome this are currently explored.

## 125.3 FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY

Facioscapulohumeral muscular dystrophy (FSHD) is a dominantly inherited muscular dystrophy and represents the 3rd most common muscular dystrophy after dystrophinopathies and myotonic dystrophy, with an estimated prevalence of 1–15.00/20.000 (46,47).

### 125.3.1 Clinical Features

FSHD is characterized by typical distribution of muscle weakness that usually begins involving the facial muscles, and it is often mild and asymmetric. Subsequently, weakness involves the scapular, humeral, truncal, and lower extremity muscles, and it often keeps its prominent asymmetric pattern. Pain and/or fatigue are common complaints occurring in up to about 70% of the individuals (48). Abdominal muscle weakness is often observed. Clinically relevant respiratory dysfunction occurs in less than 1% of patients, while cardiac involvement, in particular atrial arrhythmias, is observed in about 5% of patients, although only a few require treatment. Mild high-frequency hearing loss occurs in up to 75% of patients, and it appears more profound in patients with infantile onset. Retinal vasculopathy is frequent but rarely leads to a symptomatic exudative retinopathy (Coats' syndrome), which can in turn result in significant visual loss (47).

FSHD shows some clinical variability between patients and also within families. The condition appears more severe in males than females and penetrance also shows sex difference (49). By age 30 years, penetrance is 95% for males and 69% for females. Somatic mosaicism is common (50) and can considerably affect clinical presentation and recurrence risks. Somatic mosaicism often associates with absence of symptoms, in particular in females, while mosaic male subjects can show a milder phenotype. Offspring of a mosaic individual will likely show a more severe phenotype than expected based on the clinical presentation of the mosaic parent, explaining cases of anticipation observed in some FSHD families (50).

Atypical presentations are well-recognized, in particular facial sparing forms and infantile onset forms, with severe rapidly progressing disease observed in about 4% of cases from a UK cohort (46,51). These patients also show mild to moderate cognitive impairment and epilepsy and higher occurrence rate of hearing loss and retinopathy. Interestingly, homozygosity for FSHD does not appear to correlate with a more severe phenotype (49).

### 125.3.2 Genetics

FSHD is inherited as an autosomal dominant condition, although up to 10–30% of cases are sporadic or due to germ line mosaicism. The large majority of patients with FSHD (>95%) have a partially deleted D4Z4 repeat

array in the subtelomeric region of chromosome 4q35 (FSHD1). This repeat normally consists of 11–100 D4Z4 units, each 3.3 kb in size and ordered head to tail. Patients with FSHD1 have a partial and internal deletion of the repeat array leaving 1–10 units on one chromosome 4. An almost identical repeat array is also present in the chromosome 10q subtelomere, but contractions on this chromosome are nonpathogenic. Each D4Z4 unit has several transcripts, none of which is stable likely because of absence of polyadenylation signal in internal D4Z4 units. The major transcript in each D4Z4 unit is the *DUX4* gene, a retrogene encoding a double homeo-box protein.

About 3% of FSHD patients have no D4Z4 contractions (FSHD2) but show changes in the chromatin structure of D4Z4 similar to what is observed in FSHD1 patients. Clinically, patients with FSHD2 are indistinguishable from patients with FSHD1, although FSHD2 patients are more frequently sporadic cases and show no gender difference in terms of disease severity (52).

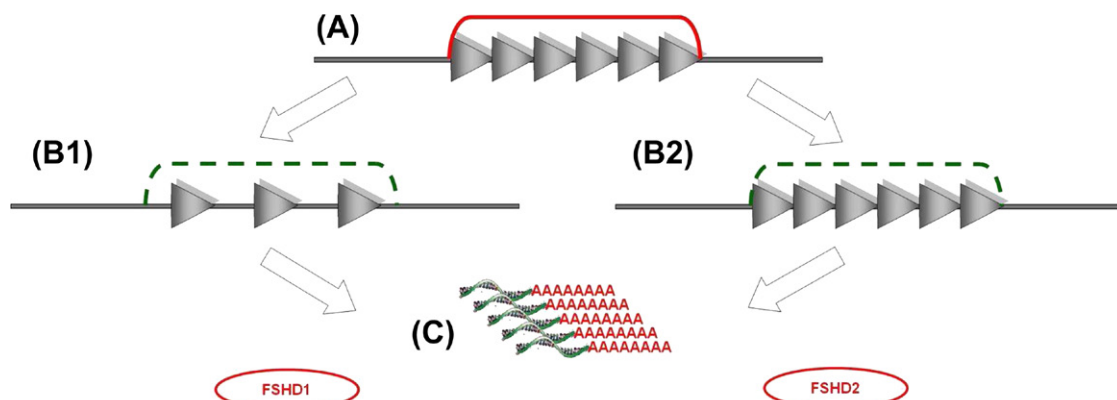
A correlation has been made between the size of the contraction and severity of the disease, in particular age at onset and rapidity of progression. Supporting this correlation is the finding of a more severe phenotype in patients with *de novo* changes, who usually show larger D4Z4 contractions.

### 125.3.3 Pathogenesis

To date, disease models have suggested that contraction of D4Z4 repeats causes chromatin remodeling and transcription deregulation of genes close to the D4Z4 units. Normally, D4Z4 repeats are in a relatively closed chromatin configuration. Repeat contraction causes partial loss of DNA methylation, which, together with loss of repressive histone modifications, opens the chromatin structure, potentially affecting transcript processing

(53,54). Upregulation of several genes located proximal to D4Z4 was reported in FSHD1 patients but has not been confirmed by subsequent studies. The 4q shows several different configurations (4qA and 4qB) and variants due to 4q polymorphisms, with a few specific sequence variants being casually related to FSHD1 (with the 4qA161 being the most common) (55,56).

It has been observed that at least one D4Z4 unit is required to develop FSHD, as monosomy of 4q does not lead to FSHD. Based on this observation, Lemmers and coworkers reasoned that the minimal pathogenic region might reside in the first or the last D4Z4 unit, which indeed has been shown to have a transcriptional profile different from what is observed for the internal units (56). Indeed, immediately distal to the last unit, there is a region called pLAM1 that has been shown to play a crucial role in the development of FSHD. The pLAM1 sequence contains a poly(A) signal that presumably stabilizes transcripts of the *DUX4* gene in the last D4Z4 unit. Patients with FSHD1 have been shown to have an identical sequence in the last D4Z4 unit and the immediately flanking pLAM1 sequence, and specific sequence variants unique to the permissive haplotypes confer pathogenicity to the repeat irrespective of its chromosomal localization (56). The distal pLAM1 sequence is preserved in FSHD1 patients, with deletion extending proximally to the D4Z4 repeat array, and also in FSHD2 patients, with a similar chromatin relaxation of a 4qA161 chromosome independent of D4Z4 repeat array contraction (56). This model by Lemmers and coworkers therefore suggests that two polymorphisms create a polyadenylation site for the distal *DUX4* transcript, located in the pLAM1 sequence, which, in combination with the chromatin relaxation of the repeat, leads to increased *DUX4* transcript levels. A toxic gain of function caused by the stabilized distal *DUX4* transcript could therefore give rise to the disease (Figure 125-8) (56).



**FIGURE 125-8** Schematic representation of a unifying mechanism for pathogenesis of facioscapulohumeral muscular dystrophy (FSHD). (A) Indicates the normal D4Z4 repeats (indicated with gray triangles) with closed chromatin configuration (solid line); (B1) In case of contraction of the D4Z4 repeats (FSHD1), the chromatin adopts a more open configuration (dotted line), leading to the expression of the *DUX4* mRNA as indicated in (C), that on permissive chromosomes is stabilized by the presence of the polyadenylation signal distal to the D4Z4 repeats. (B2) Rare patients may present no contraction of the D4Z4 repeats (FSHD2) but still show a more open configuration of the chromatin, leading to the expression of the *DUX4* mRNA as indicated in (C) in the case of FSHD1 patients.

### 125.3.4 Genetic Counseling

FSHD is inherited with autosomal dominant manner and most individuals (70–90%) with this condition have one parent showing some clinical symptoms of the condition. The rest of the cases are caused by a *de novo* event of mitotic origin or germ line mosaicism. In view of the extreme clinical variability of the condition and of reported somatic mosaicism that could affect recurrence risks (50), it is appropriate to offer appropriate clinical examination and genetic testing to parents in absence of positive family history.

If one of the parents is affected, the recurrence risk for the sibs of an affected individual is 50%, independent of sex. The incidence of germ line mosaicism is unknown, but recurrence risk in the case of absence of contraction in the parents is reported as low. Affected individuals have a reproductive risk of 50%. Following appropriate genetic counseling, molecular testing could be offered to asymptomatic at-risk family members aged above 18 years.

### 125.3.5 Genetic Diagnosis

Genetic confirmation of FSHD is currently performed by Southern blotting and hybridization of a set of probes able to establish the size of the D4Z4 repeat array and also to determine the genetic background of 4q35 (4qA or 4qB). Unaffected individuals have two 4q35 alleles of >40 kb on an EcoRI DNA digestion. Affected individuals will have one allele between 10–38 kb. Results of 40–50 kb are inconclusive. Long-range PCR and the newest molecular combing techniques have been recently developed to facilitate genetic diagnosis of FSHD (57). Diagnosis of FSHD2 is currently only made on a research basis, and there is no currently validated genetic test available for these patients.

### 125.3.6 Prenatal Testing

Prenatal testing is available in the case of at-risk pregnancies, given that the mutation of the affected parent is known. DNA of the fetus is extracted from cells obtained by sampling of chorionic villi at 12 weeks pregnancy or by amniocentesis (at 15–18 weeks of gestation). As conventional Southern blot is not suitable at the single-cell level, PGD cannot be offered at the moment. A PCR-based multiplex approach on single cells was developed to perform an indirect familial segregation study of pathogenic alleles; however, the relatively high recombination risk reduces the sensitivity of this technique in PGD (58).

### 125.3.7 Management and Treatment

To date, no disease-specific treatment for FSHD is available. Medical management includes physiotherapy, use of assistive devices and pain medication. Availability of

treatments of complications, such as hearing loss and retinopathy, justifies regular surveillance, in particular for patients with the early-onset forms. Respiratory and cardiac involvement should be monitored and managed accordingly. Scapular fixation has been shown to enhance arm mobility in patients with FSHD; however, no clear controlled studies are available.

Different pharmacological interventions, in particular corticosteroids and  $\beta$ -2 antagonists, have been tested, showing inconsistent but mainly ineffective results. A clinical trial testing the effect of myostatin block on muscle growth showed no benefits on muscle function or strength (59). Currently, other novel myostatin inhibitors are considered to have potential beneficial effect on FSHD as well as other muscular dystrophies but these remain to be tested in clinical trials.

## 125.4 EMERY–DREIFUSS MUSCULAR DYSTROPHIES AND OTHER CONTRACTURAL PHENOTYPES

### 125.4.1 Emery–Dreifuss Muscular Dystrophies

Emery–Dreifuss muscular dystrophies (EDMD) represent a clinically and genetically heterogeneous group of diseases. The clinical diagnosis of EDMD is based on the presence of (1) early contractures, in particular of elbow flexors, Achilles tendons, and spinal extensor muscles; (2) slowly progressive muscle wasting and weakness with scapulo–humero–peroneal distribution and (3) cardiac involvement, usually by the 3rd decade of life, in particular conduction defects and arrhythmias. CK levels are usually normal or moderately elevated (2–20 times the upper normal limit). Muscle histopathology shows non-specific myopathic or dystrophic findings.

EDMD is caused by mutations in three different genes. Two autosomally inherited forms are due to either dominant (AD-EDMD) or recessive (AR-EDMD) mutations in the *LMNA* gene (60), while mutations in the *EMD* and *FHL1* genes are responsible for the X-linked forms (X-EDMD) (61). AD-EDMD is the most common form, and it is often more severe than the *EMD* gene-related form. The AR and the *FHL1*-related forms are rare and reported so far only in a few patients. For the *FHL1* gene-related form, see paragraph “*FHL1* gene-related myopathy.” Nesprin mutations are also discussed later. An important differential diagnosis of a contractural phenotype is Bethlem myopathy, discussed in a separate paragraph.

The overall prevalence of EDMD is unknown. In the past, EDMD was estimated to be the third most common form of muscular dystrophy after DMD and BMD. In the northern English population, however, the X-linked form has a prevalence of 0.13/100,000, while combined prevalence of AD-EDMD and LGMD1B (also caused by *LMNA* gene mutation) is about 0.20/100,000 (2).

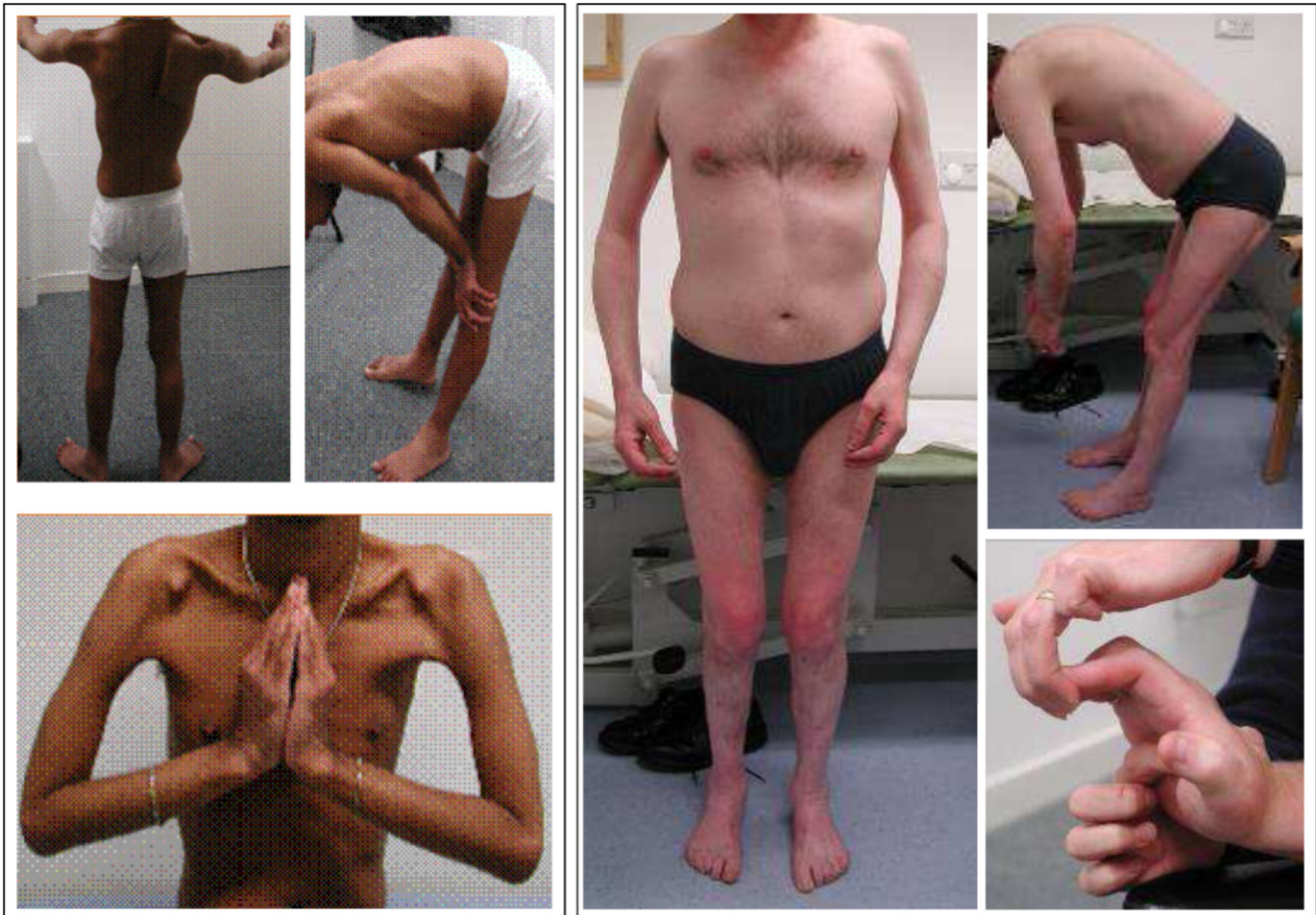


#### 125.4.1.1 Clinical Features.

**125.4.1.1.1 AD and X-EDMD.** AD-EDMD and X-EDMD show partly overlapping clinical phenotypes (Figure 125-9). AD-EDMD is characterized by early onset of muscle weakness with scapulo–humero–peroneal distribution that might precede joint contractures. Wasting of the upper arms and lower legs is common. In patients with X-EDMD, muscle involvement is usually preceded by joint contractures, and the muscle weakness and wasting shows a typical humeroperoneal distribution. In both conditions, the weakness is usually slowly progressive in the first decades, while in later stages, weakness spreads to the scapular and pelvic girdles. Loss of ambulation might also occur, in particular after the third decade in the AD-form, when the progression of the muscle wasting becomes more rapid. Interestingly, patients with *LMNA*-gene mutations, independent of the variety of clinically detectable skeletal muscle involvement, show the same pattern of leg muscle involvement on leg muscle imaging, in particular within the soleus and medial head of the gastrocnemius (62). Contractures usually involve elbows, ankles, cervical, and spinal muscles causing spinal rigidity. The progression is variable and not age-dependent. Severity of contractures of the

spine and lower limbs can greatly influence ambulation. Surgical release of Achilles tendon contractures as well as orthoses can improve ambulation.

Cardiac involvement usually arises by the 3rd decade, with palpitations, presyncope, syncope, poor exercise tolerance, and dyspnea being the first symptoms. Patients can show a wide range of cardiac involvements, such as supraventricular arrhythmias (atrial premature contractions, tachycardia or fibrillation, atypical atrial flutter and atrial paralysis), different degrees of atrio-ventricular block, ventricular arrhythmias (ventricular premature beats or unsustained ventricular tachycardia), nondilated, dilated, or restrictive cardiomyopathy, and sudden death that can occur despite pacemaker implantation (63,64). The risk of ventricular tachyarrhythmia and dilated cardiomyopathy with left ventricular dilatation and dysfunction is higher in AD-EDMD than in the X-linked form. Rather than the pacemaker, ICD implantation in patients with a *LMNA* mutation is more likely to be effective in treating possibly lethal tachyarrhythmias and should be considered in all EDMD patients with confirmed *LMNA* mutation (65). Patients can also develop respiratory impairment and cerebral emboli (64).



**FIGURE 125-9** Clinical images of patients with Emery–Dreifuss muscular dystrophy (EDMD). In the left panel, a patient with *LMNA* gene mutation (AD-EDMD). In the right panel, a patient with *EMD* gene mutation (X-EDMD).



Intrafamilial and interfamilial variability of phenotypes is common, in particular, in the AD-form, and incomplete penetrance or semidominant inheritance has also been reported (66,67). Female carriers of the X-linked form usually do not present muscle weakness but can be at risk of cardiac defects.

Different phenotypes of laminopathies (EDMD, LGMD1B or cardiomyopathy) have been observed in different members from the same family segregating the same mutation in the *LMNA* gene. More severe phenotypes have been observed in rare patients segregating mutations in both *EMD* and *DES* gene (68) or in *EMD* and *LMNA* (69), suggesting that digenism, in addition to environmental factors and modifier genes, may partially explain the extreme clinical variability observed in some rare EDMD families.

**125.4.1.1.2 AR-EDMD.** Autosomal recessive inheritance of EDMD has been described in a single patient (70). The patient was homozygote for the *LMNA* mutation C664T, causing the amino acid change in a conserved histidine in position 222 of the gene. The patient showed first symptoms at 14 months, with difficulties walking and then later difficulties standing because of contractures. At age 40, he showed severe and diffuse muscle wasting and was wheelchair bound. He did not show any cardiac involvement. Heterozygous parents were unaffected.

#### 125.4.1.2 Genetics.

**125.4.1.2.1 *LMNA* Gene.** Mutations in the *LMNA* gene are responsible for the AD as well as for the rare AR-EDMD form. The *LMNA* gene, located in 1q21.3, contains 12 exons and by alternative splicing encodes the two A-type lamins, Lamin A and Lamin C. In addition to EDMD, mutations in the *LMNA* gene are responsible for a wide and heterogeneous group of disorders called “laminopathies” and also responsible, in addition to the neuromuscular disorders affecting the striated muscles (AD-/AR-EDMD, LGMD1B, *LMNA*-related congenital muscular dystrophy and dilated cardiomyopathy with conduction system defects), for disorders of the peripheral nerve or fatty tissue, as well as disorders involving multiple tissues such as mandibuloacral dysplasia, Hutchinson–Gilford progeria (HGPS), atypical Werner syndrome, etc. (71). A strict genotype–phenotype correlation has not been yet identified, although HGPS tends to correlate with C-terminal splicing defects, while other conditions present specific mutation hot spots (72). EDMD-associated mutations are distributed throughout the gene although a few mutations recur (72,73). Marked clinical heterogeneity is observed even between patients with the same mutation, and some mutations appear to be partially penetrant.

Mutations in the *LMNA* gene are detected by direct sequencing in about 45% of cases of AD-EDMD (66). The majority of mutations are missense (>80%), but nonsense mutations as well as small deletions/insertion can also occur. The UMD-*LMNA* mutation database

included, to date, more than 400 mutations for all known laminopathies, and about 46% of these changes were detected in EDMD patients only (UMD-*LMNA* database at [www.umd.be/LMNA](http://www.umd.be/LMNA)). Large deletions/duplications of the *LMNA* gene have not yet been reported in patients with EDMD. Missense changes give rise to normal-size mutant proteins, and Western blot analysis of patients demonstrate normal levels of the protein. Conversely, nonsense mutations cause haploinsufficiency with 50% of normal protein expression (74).

Lamins A (72 kDa) and C (67 kDa) are type V intermediate filament proteins with a common nonhelical N-terminal domain, a central  $\alpha$ -helical coiled-coil rod domain, and a unique C-terminal tail domain. Lamins A, B, and C are the major components of the nuclear lamina, a proteinaceous meshwork underlying the inner nuclear membrane that provides a structural framework for the nuclear envelope and offer an anchoring site at the nuclear periphery for interphase chromosomes. Nuclear lamins also interact with integral proteins of the inner nuclear membrane and proteins of nuclear pore complexes, coordinating nuclear architecture, DNA synthesis, chromatin organization, gene transcription, cell cycle progression, cell differentiation, migration, and response to DNA damage.

**125.4.1.2.2 *EMD* Gene.** X-EDMD is mostly caused by mutations in the *EMD* gene, located in Xq28, encoding emerin, a small integral membrane protein that is located in the inner nuclear membrane and a member of the LEM domain proteins (75). Mutations in *EMD* also cause a rare form of X-linked LGMD (76,77) and an X-linked isolated cardiac disease with prominent sinus node disease and atrial fibrillation (69).

*EMD* has six exons, and sequencing analysis is able to detect almost 100% of mutations in patients, with established X-EDMD with no emerin on immunohistochemistry, that correspond to about 61% of all cases of X-EDMD (61). To date, the UMD database for *EMD* genes lists more than 96 different mutations spread throughout the gene with no mutation hot spots (UMD-*EMD* database at [www.umd.be/EMD](http://www.umd.be/EMD)).

Emerin is a 34-kDa protein with a hydrophobic C-terminus anchored in the nuclear membrane and an N-terminal tail projecting into the nucleoplasm. The majority of the mutations (95%) are null mutations, giving rise to absence of emerin on immunostaining of emerin at the nuclear membrane. A few missense or in-frame mutations result in aberrant targeting at the inner nuclear membrane and binding to lamins.

**125.4.1.3 Pathogenesis.** Emerin and Lamin A/C are thought to have a close functional relationship, and direct interaction between these proteins has been demonstrated (78). Currently, it is believed that two mechanisms could be involved in EDMD pathogenesis: (1) structural mechanisms caused by mechanical stress in skeletal and cardiac muscle, and (2) modification of gene expression relative to abnormal chromatin organization

associated with alteration of proliferation/differentiation of muscle cells (60,71,73). The basis of the extreme clinical variability observed in EDMD is still unclear. It has been shown that patients with frameshift *LMNA* mutations present with a late-onset phenotype caused by a loss-of-function mechanism secondary to haploinsufficiency, while dominant-negative or toxic gain-of-function of the protein may cause a more severe phenotype with early onset (79). The occurrence of extreme phenotypes associated with mutations in the same codon suggests the presence of genetic modifiers. Supporting this theory is the observation of patients with digenism (68). In particular, the occurrence of independent mutations in related interacting proteins (such as emerin and Lamin A/C) may synergistically contribute to disease severity of EDMD.

**125.4.1.4 Genetic Analysis and Counseling.** According to the mode of inheritance, testing of the different genes should be performed. In the case of sporadic male patients, emerin (and if available FHL1) immunoanalysis can help to distinguish if it is an X-linked or AD-form. Affected females are more likely to have the AD-form, prompting *LMNA* gene testing. Carrier testing of at risk relatives for the AR- and X-linked forms, as well as pre-natal, and PGD for at risk pregnancies requires prior identification of the mutations in the affected individual.

## 125.4.2 *SYNE1*- and *SYNE2*-Related EDMD-Like Myopathies

Mutations in the *SYNE1* and *SYNE2* genes, encoding nesprin 1 and 2 proteins, have been described in a few patients affected by myopathies with cardiac involvement and muscle involvement—overall reminiscent of EDMD but not totally typical of the EDMD phenotype (80). Nesprins are spectrin-repeat proteins that bind both emerin and lamins A/C and link the nucleoskeleton to the inner and outer nuclear membranes, membranous organelles, the sarcomere, and the actin cytoskeleton. Indeed, fibroblasts of patients with mutations in the *SYNE1* and *SYNE2* genes exhibited nuclear morphology defects and specific patterns of emerin mislocalization. Interestingly, a homozygous splice-site mutation of the *SYNE1* gene has also been reported in an autosomal recessive form of congenital muscular dystrophy segregating in a consanguineous Palestinian family (81). The phenotype was characterized by bilateral clubfoot, decreased fetal movements, hypotonia, delayed motor milestones, and progressive motor decline.

## 125.4.3 *FHL1* Gene-Related Myopathy

*FHL1*-related myopathies represent a recently recognized heterogeneous group of disorders, with X-linked myopathy with postural muscle atrophy and generalized hypertrophy (XMPMA) (82), and X-linked dominant scapuloperoneal myopathy (X-SM), being the first

associated with *FHL1* gene mutations (83). Subsequently, *FHL1* gene mutations were identified in a wide spectrum of partly overlapping conditions such as reducing body myopathy (RBM) (84), rigid spine syndrome (RSS) (85), EDMD (61), and in a single family with contractures, rigid spine, and cardiomyopathy (86). *FHL1*-related myopathies share some clinicopathological features with EDMD, such as the scapuloperoneal involvement, the asymmetric muscle involvement, and the rigid spine, but they differ for age at onset, distribution and severity of muscle weakness, progression rate, cardiac and respiratory involvement, and presence or absence of reducing bodies (RB) on Menadione- nitroblue tetrazolium (NBT) staining. *FHL1*-related disorders could be divided into two main subgroups, the “RB subgroup” including RBM, X-SM, and RSS, all characterized by RB at histological examination, and a second subgroup, including the XMPMA and EDMD, with later onset and absence of RB (61).

The human *four-and-a-half LIM domain 1* (*FHL1*) gene, located on Xq26.3, is a 32-kDa protein with four-and-a-half tandem repeat LIM domains cysteine-rich, tandem zinc finger protein interaction motifs first recognized in the Lin-11, Isl-1, and Mac-3 homeodomain transcription factors (87). LIM domain proteins are involved in the skeletal and cardiac structure and function (88,89). *FHL1* has at least three isoforms (*FHL1A*, *B*, and *C*) with different primary structures, expression patterns, binding partners, and subcellular localizations (90). All isoforms are highly expressed in skeletal muscle and to a lesser extent in heart, but *FHL1A* is the prevalent muscle isoform (91). *FHL1* localizes to the sarcomere and the sarcolemma and participates in muscle growth, differentiation and sarcomere assembly (92).

**125.4.3.1 Reducing Body Myopathy.** RBM is a rare and severe condition characterized by early age of onset (usually infancy or childhood), progressive proximal muscle weakness, and spinal rigidity, with rapid progression into respiratory failure and death. In cases of adult onset (third to fourth decade), cardiac involvement is also observed. Both sexes can be affected, but onset is usually earlier in males (average 5 years in males versus 7 years in females). CK levels can be found increased up to 10 times the normal levels, with usually higher increase in males than females (92).

RBM is histologically characterized by the presence of intracytoplasmic inclusions of RB in the muscle fibers. Because of their high sulphhydryl content, RBs reduce NBT and therefore strongly stain with menadione-NBT in the absence of the substrate  $\alpha$ -glycerophosphate (93). The *FHL1* protein has been shown to be a major component of RB (84). *FHL1* gene analysis in 16 unrelated RBM families led to the identification of 10 missense mutations and one in-frame deletion (84,92,94). All variants so far identified localize in the second LIM domain and mainly affect zinc-coordinating cysteine and histidine residues, leading to instability of the domain and protein

misfolding. A more severe phenotype is observed in association with mutations at residue 123 (92).

**125.4.3.2 Rigid Spine Syndrome.** Rigid spine is a common feature in FHL1-related myopathies, in particular in RBM, X-SPM, XMPMA, and EDMD. Interestingly, a unique *FHL1* gene mutation was identified in a single patient presenting with RSS and later muscle atrophy (85). Muscle analysis identified RB, suggesting that this case could indeed represent a milder form of RBM. To corroborate this hypothesis, the mutation identified in this patient (p.151-153delVTC) affects a cysteine residue in the second LIM domain, as for the RBM cases.

**125.4.3.3 X-linked Scapuloperoneal Myopathy.** X-SPM is characterized by proximal upper limb weakness with adult onset (second or third decade), scapular winging, early foot drop, rigid spine, and late contractures (83). Cardiac involvement has also been observed, while respiratory involvement appears uncommon. Men are usually more severely affected and may become wheelchair bound. RBs have been observed on muscle biopsy analysis with menadione-NBT analysis, and together with the observed clinical overlap with adult-onset RBM, it suggests that X-SPM and RBM could represent extremes of a unique clinical continuum. Two *FHL1* gene mutations, affecting residue tryptophan 122, have been described in the two unrelated families so far reported (83,95). Molecular dynamics simulation of the W122C mutation revealed no major distortions of the protein structure or disruption of zinc binding. An increase in the nonpolar, solvent-accessible surface area has been observed in one or both of two clusters of residues, suggesting that the mutant proteins have a variably increased propensity to aggregate (95).

**125.4.3.4 X-linked Myopathy with Postural Muscle Atrophy and Generalized Hypertrophy.** XMPMA is characterized, as indicated by the name, by late-onset postural muscle atrophy associated with generalized muscle hypertrophy (82). Onset is in the third decade, and patients show a typical pseudoathletic appearance, associated with moderate scapulo-axio-peroneal weakness, atrophy of postural muscles, and bent spine. Cardiac and respiratory failure is also observed and may be the cause of death. CK levels are normal or increased up to 10 times the normal levels. So far, *FHL1* mutations have been described in 7 families with XMPMA (82,96). Two missense mutations affect conserved cysteine or histidine residues in the fourth LIM domain (C224W and H246Y) (82,96). The third known missense change in exon 7 only affects isoform FHL1B, indicating that this also plays an important role in skeletal muscle function (V280M). Another *FHL1* mutation (127-I-128) results in the insertion of an isoleucine in the linker region between the two zinc fingers of the second LIM domain (82,97). This two-amino acid linker (C-X2-C) is typically invariant in length, and the insertion of a large aliphatic hydrophobic isoleucine between the

rigid aromatic phenylalanine-127 and the hydrophilic threonine-128 is supposed to alter the orientation and the spatial relationship between the tandem zinc fingers (98). This mutation has been identified in three apparently unrelated British families, with a broad phenotypic spectrum within members of the same families as well as between families. Females were variably but more mildly affected. Haplotype analysis of the *FHL1* region revealed that the families share a common haplotype suggesting a British founder effect (97). Finally, a splice site mutation in intron 4 (c.688+1G>A) has been detected in another family, leading to splicing from exon 5 to exon 8 and the expression of a C-terminal truncated protein identical to isoform FHL1C (96).

**125.4.3.5 Emery–Dreifuss Muscular Dystrophy.** *FHL1* gene mutations have been so far reported in seven families segregating X-EDMD (61). At onset, the phenotype is characterized by a myopathy with scapuloperoneal and/or axial distribution and joint contractures, with age at onset ranging from childhood to the fifth decade. All patients showed pelvic, peroneal or pelvi-peroneal weakness, with associated upper limb (mainly scapular) involvement in most of them. Additional axial and facial involvements were also observed. Limb joint (ankles, elbows, hips, knees, and rarely wrists, fingers, and shoulders) contractures, neck stiffness, and rigid spine were also common features. Patients invariably also showed cardiac involvement (conduction defects, arrhythmias, and hypertrophic cardiomyopathy) with onset ranging from early teens to adulthood, with symptoms of supraventricular or ventricular arrhythmias. Heterozygous female carriers were either asymptomatic or had cardiac disease and/or mild myopathy. Interestingly, four male relatives with *FHL1* gene mutation presented isolated cardiac disease, and an overt hypertrophic cardiomyopathy was present in two. CK levels were normal or moderately elevated. The *FHL1* mutations responsible for the EDMD phenotype are located in the most distal exons (exons 5–8), affecting all three FHL1 isoforms. One of these EDMD-causing mutations (C209R) has also been reported in a German family with male patients affected by hypertrophic cardiomyopathy and rigid spine, but no myopathy (86).

**125.4.3.6 Genetics and Pathogenesis.** More than 25 *FHL1* mutations have been reported to date, the majority of which affecting the second LIM domain. Mutations affecting the critical zinc-binding residues, such as in RBM patients, appear to affect the protein folding and structure. All LIM2 domain mutations disrupt tandem zinc-finger topology and the interface required for protein–protein interaction (87). Modeling studies have shown that the mutations do not cause protein misfolding per se, but the protein aggregation may be caused by the exposure of nonpolar surfaces (95). Aggregates increase over time in RBM, suggesting that toxic accumulation of protein aggregates might be responsible for the disease. Mutations observed in XMPMA and EDMD

patients do not give rise to RB, although desmin-positive aggregates are observed in the muscle from these patients (82). It has been hypothesized that this second group of mutations causes loss of normal protein function by reducing FHL1 protein expression and/or impairing protein partner binding. The milder phenotype associated in association with mutations affecting the most distal part of the gene is also due to the different effect of the mutations on the three FHL1 isoforms (98).

FHL1 plays an important role in the heart, connecting the muscle stretch sensor machinery interacting with titin and the downstream hypertrophic response, such as the members of the MAPK signaling pathway (99–101). Indeed, FHL1 knockout mice show reduced hypertrophy-dependent activation of ERK2, one of the members of MAPK pathway (99). It has also been shown that FHL1 has a role in the regulation of the functional expression of KCNA5, a component of the voltage-gated K<sup>+</sup> channel (Kv1.5), a fundamental protein in atrium repolarization, which also represents a therapeutic target in the treatment of atrial fibrillation (101–103).

**125.4.3.7 Genetic Counseling.** FHL1-myopathy is an X-linked condition differently affecting the two sexes, with variable mode of inheritance (recessive or dominant) according to the specific mutation. Females can be completely asymptomatic or show a variable degree of symptoms, ranging from a mild phenotype, with later onset, to the more severe early-onset RBM phenotype (92,97). Carrier testing of at-risk relative and eventual prenatal testing requires prior identification of the mutation in the affected subject.

## 125.4.4 Bethlem Myopathy

Bethlem myopathy (BM), allelic to the more severe form of Ullrich congenital muscular dystrophy (CMD) (see Section 125.7) is an important differential diagnosis for EDMD. The extracellular matrix protein collagen VI (ColVI) is composed of three chains,  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  that assemble in tetramers to form a microfibrillar network in many tissues, including skeletal muscle. The three ColVI peptides associate into collagen VI triple helical monomers, followed by assembly into disulfide bonded dimers that then align laterally to form tetramers, the secreted form of collagen VI, which associate end to end outside the cell to form the characteristic microfibrils. Despite its ubiquitous expression pattern, effects of collagen VI mutations in human or mouse show strikingly muscle specific phenotypes. Indeed, Collagen VI chains are encoded by the *COL 6A1*, -2 and -3 genes, and mutations in these genes cause ColVI myopathies, including Ullrich CMD, BM, and other intermediate phenotypes, constituting a continuous clinical spectrum. Because collagen VI is thought to play a role in mediating tissue morphogenesis as well as playing a potential role in cell cycle signaling and tissue homeostasis, it is possible that mutated collagen

VI leads to myopathy by disruption of one or both of these roles.

Classic BM is characterized by the combination of a relatively mild proximal myopathy and variable contractures. Ullrich CMD is the most severe end of the spectrum, presenting early in life with hypotonia, muscle weakness, motor delay, proximal contractures, and distal hyperlaxity (for more details see “Congenital muscular dystrophies”). Other phenotypes included in the spectrum are severe early onset forms of BM, milder forms of Ullrich CMD, or intermediate forms not classified as BM or Ullrich CMD (104). Moreover, rare forms of LGMDs (105) and a rare form of autosomal recessive myosclerosis myopathy (106) are also included in this group of diseases. Inheritance of BM was classically described as autosomal dominant, however, recent reports have shown different inheritance patterns, further increasing the clinical and genetic complexities of this disease. Prevalence of BM is low, with estimated point prevalence in northern England of 0.77/100.000 live births, respectively (2).

**125.4.4.1 Clinical Features.** BM may present at any age, and indeed, it may be recognizable from birth with affected children presenting sometimes with torticollis and contractures. Prenatal onset of muscle weakness has been suggested in some families with diminished fetal movements, and affected infants are often hypotonic with delayed motor milestones. Proximal muscle weakness from childhood onward tends to be only slowly progressive, and there may be long periods of time when the condition does not progress at all. Other patients may be asymptomatic in childhood and present with mild muscle weakness and contractures in adult life. Considerable disability may be seen in some patients in late adult life, with more than two-thirds of patients older than 50 years requiring aids for ambulation outdoors, and some with respiratory failure secondary to diaphragmatic paralysis. A hallmark of this condition is the development of contractures, especially of the fingers, wrists, elbows, and ankles, and these, in addition to weakness, contribute to disability. The contractures can be dynamic in nature during childhood, and hypermobility of distal interphalangeal joints can be present together with long finger flexion contractures. Skin features typically seen in connective tissue disorders, such as keloid formation and “cigarette paper” scarring, as well as follicular hyperkeratosis, can also be present in patients with BM. Clinically, the condition may show overlap with autosomal dominant forms of LGMD (105) because of the proximal muscle weakness, especially as the full spectrum of the disease may not be seen in every patient. The presence of the contractures may cause confusion with EDMD, particularly if a rigid spine is present. In BM, there is no evidence of any cardiac involvement.

**125.4.4.2 Diagnosis.** Clinical diagnosis of BM is suggested by the presence of proximal muscle weakness associated with variable contractures, and unusual skin



features. CK levels are normal or mildly elevated. Muscle biopsy shows myopathic or dystrophic changes. In contrast to Ulrich CMD, collagen VI immunolabeling of muscle is often normal. Immunofluorescent (IF) labeling of collagen VI in fibroblast cultures is useful to guide molecular genetic testing of BM in a cost-effective and timesaving manner. Indeed, fibroblast IF technique is highly predictive of the presence of a *COL6A* mutation, with a positive predictive value of 75%, a sensitivity and negative predictive value of 100%, and a specificity of 63% (107). Muscle MRI is an additional tool in guiding molecular diagnosis of BM and often requested prior to performing molecular analysis. *Vasti* muscles are the most frequently and markedly affected muscles in the thigh. A rim of abnormal signal at the periphery of each muscle, with relative sparing of the central part, is also observed. Characteristic is the peculiar involvement of the rectus femoris, with a central area of abnormal signal within the muscle (108). BM patient also shows a rim of abnormal signal at the periphery of soleus and gastrocnemii.

Confirmation of a diagnosis of BM relies on the detection of a mutation in one of the three collagen genes, *COL6A1*, -2, and -3. These genes have 107 coding exons and frequent polymorphisms, thus mutation analysis is not straightforward. Sequence analysis allows detection of *COL6A* gene mutation in 60% of classical or unusually severe BM phenotypes (109). The most common pathogenic mechanism is represented by single amino acid substitutions disrupting the Gly-Xaa-Yaa motif of the highly conserved triple helical domain of one of the *COL6A* genes, followed by splice-site mutations, in particular of exon 14 in *COL6A1*, causing small in-frame deletions or insertions or frameshifting and nonsense mRNA decay.

Severity of the phenotype depends on the ability of mutant chains to be incorporated in the multimeric structure of collagen VI (110). The majority of mutations causing BM are usually heterozygous, dominantly acting mutations, resulting in either haploinsufficiency or with a dominant negative effect, affecting secretion and deposition of structurally abnormal collagen VI or compromising its interactions in the extracellular matrix of muscle (109,111). Recently, recessive inheritance of BM was reported in homozygous or compound heterozygous patients with a functional null mutation and a missense *COL6A* mutation (112,113).

Genetic heterogeneity within BM was suggested by exclusion by linkage and/or direct sequencing of the *COL6* loci in patients with a typical BM phenotype, suggesting that mutations in other genes may underlie at least some of these cases (Hicks et al, unpublished data).

**125.4.4.3 Genetic Counseling.** Recurrence risk for siblings in BM due to heterozygous mutations will depend on the affected status of the parents, requiring careful examination and molecular genetic testing of parents. In case of confirmed *de novo* mutations, recurrence risk will be low and will relate to the possibility of gonadal

mosaicism. In case of recessive mutations, recurrence risk for siblings will be 25%.

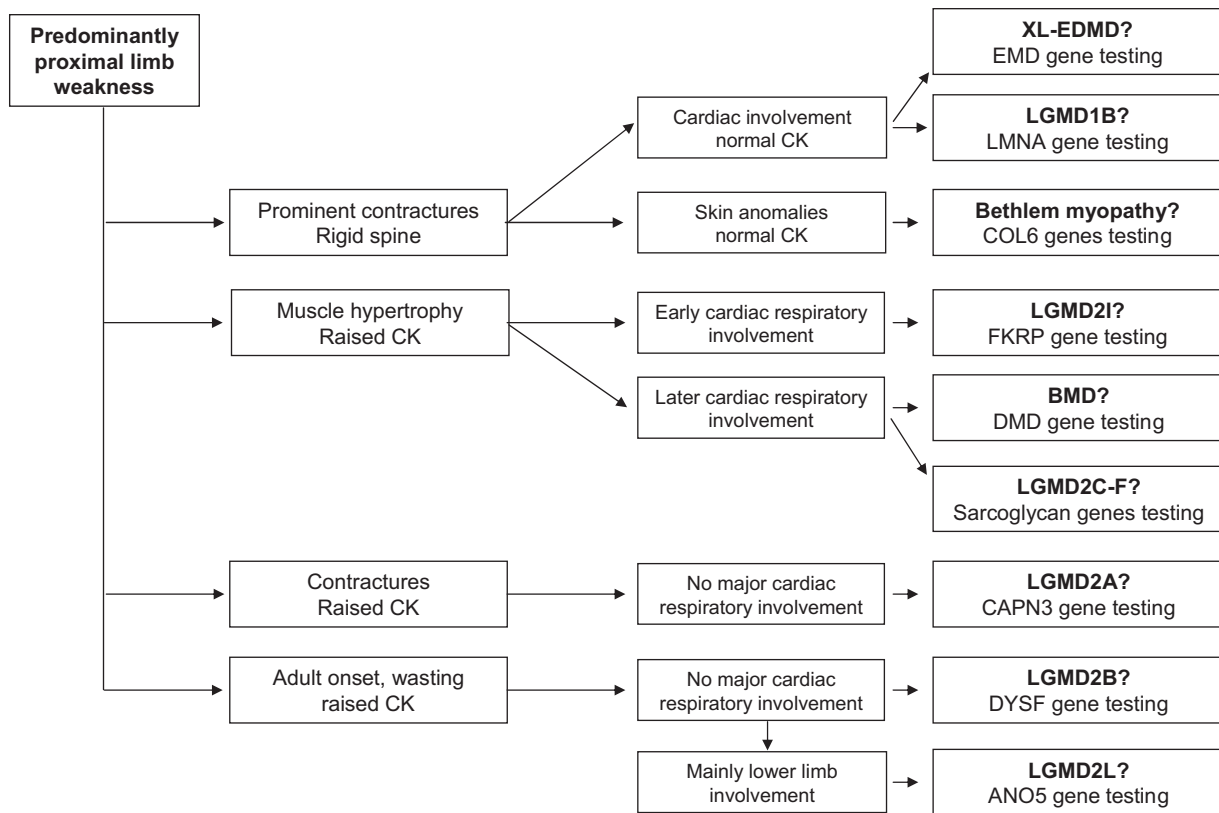
## 125.5 LIMB-GIRDLE MUSCULAR DYSTROPHIES

A highly genetically heterogeneous group of muscular dystrophies can be recognized as sharing the features of predominant involvement of the proximal musculature. This group is known as the LGMDs and is an area where great progress in diagnostic investigations has been made (114–116). The ability now to achieve a precise gene or protein-based diagnosis in many patients in this category means that the correct disease designation should include an indication of the molecular pathology whenever possible. Equally, the ability to distinguish the various subgroups within the LGMD category means that there is increasing knowledge about the various phenotypes seen in association with the genetically defined subtypes. The increasing awareness of the complications accompanying the various subtypes of LGMD is also crucial in management of patients and improving quality of life and longevity. Therefore, careful clinical characterization still has to take its place alongside the molecular diagnostic tools of gene and protein analysis.

In a patient presenting with suspected LGMD, mode of presentation and in particular the specific pattern of muscle involvement, additional clinical features, CK levels, and family history are key handles to reach a precise diagnosis (Figure 125-10). It is important to keep in mind that LGMDs are singularly rare and more common, and more likely diagnoses should be considered first, in particular dystrophinopathies. Muscle analysis is helpful in about 2/3 of the cases, but mutation analysis is the gold standard for reaching diagnosis. Currently, with appropriate testing, it is possible to reach a diagnosis in about 75% of cases of LGMD, but multidisciplinary approach for diagnosis is better achieved in specialized centers and is helpful for management, in particular, of cardiac and respiratory complications. Diagnostic precision is also pivotal for future genetic therapies.

### 125.5.1 Autosomal-Dominant LGMDs

Only a relatively small proportion of LGMD (possibly around 10%) show autosomal dominant inheritance (AD-LGMD) (114–116). The 2010 Gene Table lists eight genetic types of AD-LGMDs, including a number of disorders with clinically significant cardiac involvement (1). An important distinguishing feature in some of these disorders compared with autosomal recessive LGMD (AR-LGMD) is the lower CK, which may be in the normal range or elevated only 3–5 times. Individually, many of the AD-LGMDs are relatively rare and restricted to specific family or population groups, with the important exception of the *LMNA* gene-related form. Most of the AD forms of LGMD are caused by mutations in genes,



**FIGURE 125-10** Flow chart for diagnostic approach in case of a patient with suspicion of limb girdle muscular dystrophy.

which are also associated with other phenotypes. In assessing a family with possible LGMD and dominant history, important differential diagnoses are FSHD and BM. Facial muscle involvement in individual patients with FSHD may be relatively mild, and this diagnosis could be easily checked by DNA analysis.

**125.5.1.1 LGMD1A.** LGMD1A is a rare disease, first described in a large north American pedigree with a predominantly proximal muscular dystrophy associated in some cases with dysarthria and tight Achilles tendons. Linkage to chromosome 5q led to identification of mutations in the myotilin (*MYOT*) gene, which encodes a component of the muscle sarcomere (117). No patients had arm weakness without leg weakness, and distal weakness was a late feature. Age at onset ranged from 18 to 35 years, with some suggestion of anticipation. Progression of the disease was very slow, with very few patients confined to a wheelchair. CK values were usually only mildly elevated. *MYOT* mutations more commonly cause myofibrillar myopathy (MFM) (118). Following the first description of LGMD1A, further families segregating *MYOT* gene mutation have been described, giving rise to a clear-cut LGMD phenotype different from MFM. A novel *MYOT* mutation was recently reported in a LGMD1A family with patients presenting with a late onset and rapidly progressing weakness, leading to loss of ambulation and respiratory failure (119). The onset of weakness in proximal muscles and muscle MRI findings were clearly different

from those observed in MFM patients, and myofibrillar pathology was limited. Although cardiac and respiratory complications are less common in patients with *MYOT* mutation compared with other genetic forms of MFMs, a high level of suspicion for these complications is always necessary in patients with LGMD1A. The *MYOT* mutations described in LGMD1A families (R6H, S55F, T57I, and R405K) are localized in different exons of the protein, indicating no genotype–phenotype correlations.

**125.5.1.2 LGMD1B.** Autosomal dominant LGMD is one of the various clinical presentations observed in patients with mutations in the *LMNA* gene. Of all the disorders classified within the AD-LGMD group, laminopathy in its various manifestations would appear to be numerically the most significant, to date.

LGMD1B presents with a predominantly proximal muscular dystrophy with minimal or late contractures and a tendency to atrioventricular conduction defects that increased with age. Indeed, when there is skeletal muscle involvement with laminopathy, there is also a very high risk of cardiac disease, in particular, progressive atrial arrhythmias with a subsequent risk of cardiomyopathy and ventricular arrhythmias. Even with pacing, there is a high risk of sudden death and consideration of the use of implantable defibrillators in the presence of cardiac involvement is recommended (120).

The essentially invariable cardiac involvement in the muscle-related laminopathy phenotypes make imperative to make a clear diagnosis. Moreover, given the variety

of presentations, potentially not yet fully elucidated, genetic diagnosis of laminopathy should be considered in any families with muscular dystrophy with a dominant family history, especially if there is a history of cardiac disease or sudden death. Diagnosis relies upon the demonstration of a *LMNA* gene mutation, because the protein is not altered in muscle biopsy samples. The ability to study the *LMNA* gene directly has also revealed the presence of a large number of *de novo* mutations and a significant level of germ line mosaicism. Because of the high rate of new mutations in this condition, the diagnosis should be considered in other cases of muscular dystrophy without a clear cause from examination of the muscle biopsy.

The majority of reported LGMD1B mutations are confined to the first 10 exons of the gene, with rare patients with mutation in exon 11 showing remarkable clinical variability, with specific features not reported in other LGMD1B patients (121). In particular, one patient had congenital weakness and died in early childhood. In two other patients, severe cardiac problems arose early, and in one of these, cardiac signs preceded the onset of skeletal muscle weakness by many years. The fourth case had a mild and late-onset LGMD1B phenotype. A lethal phenotype has been observed in association with a homozygous *LMNA* nonsense mutation (Y259X), where, in the heterozygous state, the mutation caused a classic LGMD1B phenotype in the same family (122).

Among the myriad of diseases caused by mutations in the *LMNA* gene, as well as AD-EDMD (see Section 125.4), congenital muscle diseases have also been reported (for more details, see Section 125.7).

**125.5.1.3 LGMD1C.** LGMD1C is caused by mutation in the Caveolin 3 (*CAV3*) gene, a muscle-specific component of the caveolin membrane, which is probably involved in signal transduction. Caveolin 3 localizes to the sarcolemma, coinciding with the distribution of dystrophin, with which it can also be shown to associate by immunoprecipitation, although it is not believed to be an integral part of the dystrophin complex. Caveolin 3 also associates with dysferlin. In addition to LGMD1C, *CAV3* mutations are also responsible for a variety of phenotypes including a distal phenotype, rippling muscle disease, myalgia, cardiomyopathy, and hyperCKaemia (123,124). Phenotypes might evolve over time, and the diseases can show different manifestations at different ages. Moreover, the same mutation in the *CAV3* gene may cause different presentations in members of the same family.

Patients with *CAV3* mutations may show very good muscle power and athletic ability. The most common symptom and reason for referral is myalgia, but patients can also present with cramps or distal and proximal muscle weakness. Rippling can be detected in childhood, and it is more frequently observed than weakness. Rippling and percussion-induced repetitive contractions should therefore be looked for in suspected cases, because they

may be easy to miss. Indeed, patients may be unaware that these phenomena are related to any muscle problem. Myoglobinuria as well as hypoglycemia can also occur (125). Progression of the disease is usually gradual, but relatively few patients with *CAV3* mutations had long-term follow-up (125). Cardiac and respiratory involvement is usually not observed. LGMD1C is the exception to the general rule that patients with AD disease generally have a lower level of serum CK, because here CK levels may be very high (10 times the upper limit of normal), and indeed, caveolinopathy may present only as hyperCKaemia. Rare homozygous cases have a more severe phenotype (126). LGMD1C is a rare form of LGMD. Frequency in Italian LGMD population is about 1%, while it has not been reported in northern England (2,127,128).

Diagnosis of LGMD1C may be suggested by the finding of reduced or absent caveolin 3 labeling in muscle, and it may be confirmed by mutation analysis (Figure 125-4) (127). Differential diagnosis should include autoimmune-mediated rippling muscle disease, where a mosaic pattern of caveolin loss is observed on muscle biopsy in absence of *CAV3* gene mutations.

A subgroup of patients presenting with muscular hypertrophy, muscle mounding, mild metabolic complications, and raised CK levels, and muscle biopsies with reduction of caveolae structure (129), have been shown to have homozygous mutations in another gene, called polymerase I and transcript release factor (*PTRF*; or cavin) involved in formation of caveole and stabilization of caveolins. In addition to the muscular dystrophy and lipodystrophy described in these patients, cardiac abnormalities leading to sudden death (long-QT syndrome, bradycardia, supraventricular and ventricular tachycardias) as well as smooth-muscle hypertrophy, leading to impaired gastrointestinal motility and hypertrophic pyloric stenosis, were also reported (130).

**125.5.1.4 Other AD-LGMDs.** Other forms of AD-LGMDs, defined only by their genetic localization in single families, have no known genetic cause, to date (1). Careful examination of these families and exclusion of known genes are pivotal. As cardiac and respiratory involvement is typical for dominant LGMDs, screening of these genetically unknown families is appropriate.

Following the identification of the locus for LGMD1D on chromosome 7q36 in two families, this rare form of AD-LGMD has also been described in several Finnish families (131), and mutations in the *DNAJB6* genes have recently been identified in a few of these and other families with different geographic origins (132,133). The phenotype is characterized by slowly progressive distal and proximal weakness, normal or mildly raised CK values but no major cardiac or respiratory involvement. *DNAJB6* is a member of the HSP40/DNAJ molecular cochaperone family with role in protein aggregation, and indeed affected muscles showed abnormal



protein aggregation and rimmed vacuoles, similar to what observed in MFM.

LGMD1E has a locus on 6q23, and it was reported in a single large family with affected subject presenting with proximal muscle weakness, dilated cardiomyopathy, and cardiac conduction abnormalities. Men were more severely affected than women for both skeletal and cardiac muscle manifestations of the disease. Progression of muscle symptoms was slow, and CK levels were raised to 1½–3 times the normal levels. Conduction disturbances preceded congestive heart failure in cases under follow-up, while sudden death was reported in other family members.

Few clinical details are yet known for LGMD1F (7q32), LGMD1G (4p21), and LGMD1H (3p23–p25) (134–136).

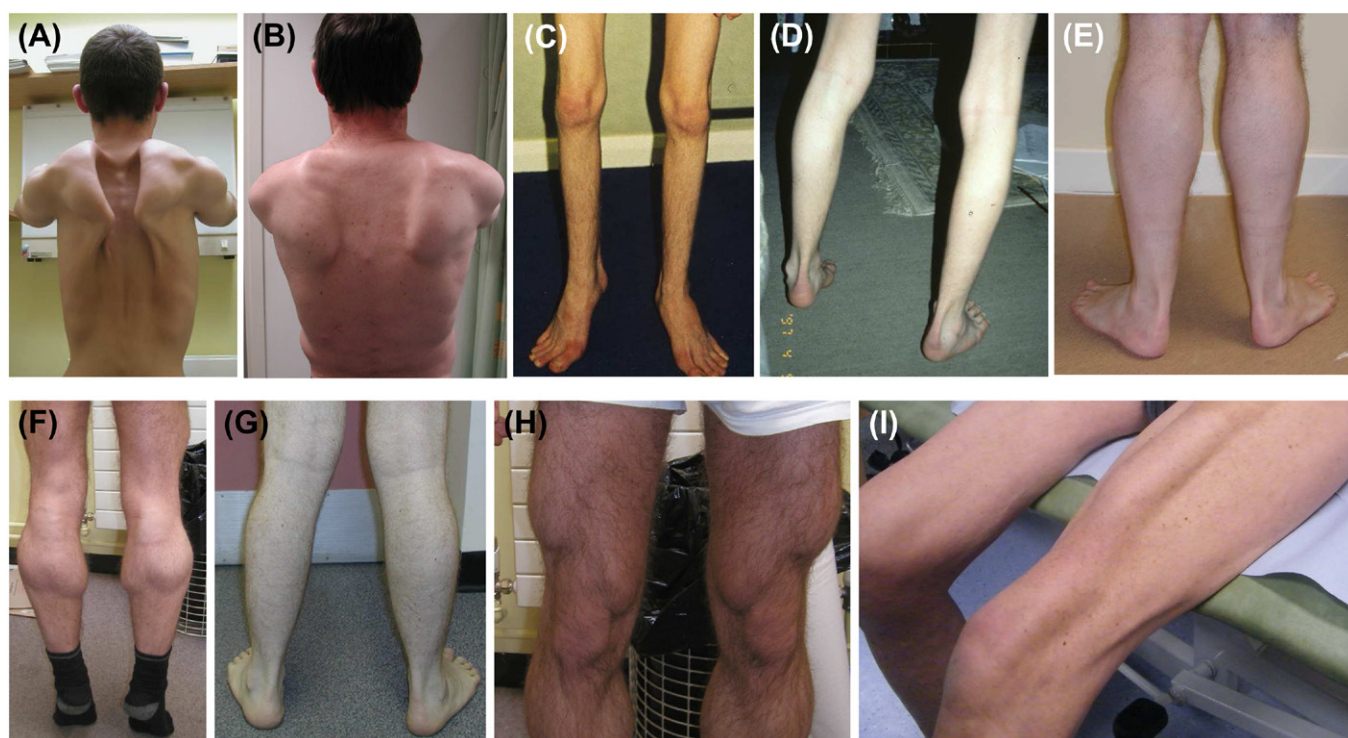
## 125.5.2 Autosomal Recessive Limb-Girdle Muscular Dystrophies

The autosomal recessive forms of LGMD (AR-LGMD) are more frequent than the dominant forms. Population-based studies show variation in frequencies of the different forms in different countries, reflecting the different gene frequencies in these population groups. LGMD2I is common in northern Europe, with a particularly frequent mutation (137–139), whereas in southern Europe, LGMD2A appears to be more prevalent (140).

**125.5.2.1 LGMD2A or calpainopathy.** LGMD2A has a fairly consistent and characteristic clinical

presentation (141–144). The weakness is typically proximal and affecting the posterior muscles of the lower limbs, associated with shoulder girdle involvement leading to scapular winging (Figure 125-11). The disease is predominantly symmetrical and atrophic, with prominent calves seen only in a minority of cases. Achilles tendon contractures may be an early sign, and contractures elsewhere, including of the spine and elbows, may also be prominent (141–143).

A large study by Saenz et al. showed a mean age at onset of <14 years, with first symptoms between 6 and 18 years of age in 71% of patients, with rare cases with onset in early childhood or adulthood (144). Pelvic girdle weakness is present and symptomatic from the onset, together with marked posterior thigh weakness and wasting, but often with very striking sparing of the hip abductors, even relatively late in the course of the disease. These features, combined with laxity of the abdominal muscles, lead to the development of a particularly characteristic stance in these patients (143). Scapular winging is usually present from the early stages, although it may be asymptomatic. A small minority of patients present muscle pain and can be misdiagnosed as polymyositis or metabolic myopathy; others are reported with hyperkalemia. Respiratory, and more frequently, cardiac function is usually preserved, and therefore a normal lifespan is expected. The disease shows high intrafamilial and interfamilial variability, in particular with regard to age at onset and rate of progression (145). Wheelchair dependency is typically observed after 10–20 years of



**FIGURE 125-11** Clinical images of patients with limb-girdle muscular dystrophy. In panel (A) LGMD2D; (B), (C), and (D) LGMD2A; (E) LGMD2I; (F) LGMD2D; (G) LGMD2L; (H) LGMD2D; (I) LGMD2L.



disease progression, with a mean age of 32.2 years (144). CK values are usually 10 times over the upper limit of normal.

Calpainopathy is a relatively common cause of LGMD. Norwood et al (2) recognized LGMD2A as the most common form of LGMD in the northern English population, with a prevalence of 0.60/100,000 (26.5% of the total LGMD group), similar to what was reported in an unselected LGMD population (39%) as well as in the Italian population (28.6%), with a carrier frequency in the north-eastern Italian population of ~1:160 (128,140). Founder effects are, in part, responsible for the higher prevalence observed in Turkey, Basque Country, and Russia, as well as in La Reunion, where the prevalence of calpainopathy is estimated to be 48/1,000,000.

Diagnosis of LGMD2A can be challenging. Immunoblot is currently the preferred laboratory test to assist the diagnosis (Figure 125-5). Protein analysis on muscle biopsy could be difficult to interpret, as the results of muscle immunoblotting are often complex and several calpain 3 bands could be detected, all susceptible to degradation, as the protein may have poor stability in experimental conditions. Immunohistochemistry with exon 1 monoclonal antibody can be useful for distinguishing the majority of genuine calpain 3 defects from secondary protein reduction (146). It has been suggested that, in cases with normal immunoblotting (Figure 125-5), further investigation of the autoproteolytic function of calpain 3 may offer an alternative route to diagnosis (147,148). In calpainopathy, examination of dystrophin, the sarcoglycans, and all other muscle proteins appear normal. It has to be noted, however, that secondary reduction in calpain 3 levels has been described in patients with a primary dysferlinopathy, reflecting an interaction between these proteins (149). The possibility of other, as yet unrecognized, secondary causes of reduction of the protein is another reason to confirm the diagnosis by mutation analysis.

The demonstration of mutations in the *CAPN3* gene is the gold standard to confirm calpainopathy, though this may be guided by linkage to chromosome 15 in families where the structure is suitable. Mutations in *CAPN3* may be missense or nonsense substitutions, small deletions or small insertions, spread widely over the gene (although missense mutations are relatively infrequent in the first protein domain and some exons remain free of mutations). A few recurrent mutations have been reported, with a mutation in exon 22 (c.2362\_2363delAGins TCATCT) accounting for over 30% of chromosomes analyzed by Saenz et al (144). One mutation does appear to be conserved with a founder haplotype in families from such diverse geographical origins as America, Brazil, and Reunion. Common mutations in some populations are due to founder effect. Unfortunately, around 23% of cases with LGMD2A only have one mutation found by commonly used mutation technologies. Sequencing of *CAPN3* transcripts present in muscle or peripheral blood

could be applied as a new approach for LGMD2A diagnosis, although lack of exon 15 in isoforms expressed in blood and presence of mRNA degradation would suggest the combined use of mRNA and DNA analyzes (150,151). Detection of the causative mutation is also necessary for carrier detection and prenatal diagnosis when required (144,148).

Genotype–phenotype correlations are difficult except in patients who are homozygous for a *CAPN3* mutation; as a general rule, patients with homozygous null mutations tend to have the most severe clinical course, in particular with regard to age at loss of ambulation. Although there are occasional reports of elevated CK in carriers especially, it has been suggested, in carriers of a particularly deleterious mutation, the general applicability of this has yet to be determined.

Calpain 3 is the muscle-specific member of a family of calcium-dependent proteases. It has four protein domains similar to those found in ubiquitous calpains, as well as three unique regions (NS, IS1, and IS2) possibly conferring its muscle specificity. It is still unclear how a *CAPN3* mutation causes muscular dystrophy, but various insights are emerging. Indeed, *CAPN3* mutations frequently lead to protein loss from skeletal muscle, and loss of proteolytic function is a common consequence of *CAPN3* mutations (147,148). Calpain 3 interacts with titin through one of the IS2 muscle-specific sequences, which also contains a nucleus translocation signal-like sequence (152). Calpain 3 is located in both the cytosol and the nucleus, suggesting that it may have a role in regulating levels of muscle-specific transcription factors and muscle cell differentiation. An apparent increase in apoptosis has been described in calpain 3-deficient muscle. A defect in sarcomere remodeling with loss of ubiquitination has also been described (152). Calpain 3 coexists in the dysferlin protein complex with AHNK, a protein involved in subsarcolemmal cytoarchitecture and membrane repair. AHNK is cleaved by calpain 3 and lost in cells expressing active calpain 3. Conversely, AHNK accumulates in muscles from patients with LGMD2A. The AHNK fragments cleaved by calpain 3 lose their affinity for dysferlin suggesting an interconnection between LGMD2A and LGMD2B (153).

#### 125.5.2.2 LGMD2B or Dysferlin Myopathies.

Dysferlinopathies are a heterogeneous group of recessive muscular dystrophies, ranging from a predominantly proximal disease (LGMD2B), a more posterior and distal phenotype predominantly affecting the gastrocnemius, the Miyoshi myopathy (MM), to rarer phenotypes such as a distal myopathy, affecting the anterior tibial muscles (154), or isolated hyperCKaemia. LGMD2B and MM are the most common presentations, observed in about half of patients with dysferlin (*DYSF*) mutations only, but unusual phenotypes are indeed common (155). No significant differences are observed in progression rate, functional prognosis, MRI pattern of muscle involvement, or *DYSF* mutations between patients with MM

and LGMD2B, favoring the merging of these diseases into a unique clinical continuum called dysferlin myopathy (156). Corroborating this conclusion is the observation of families with affected members presenting with different phenotypes.

Patients affected by dysferlin myopathies are often very active and sporty before the onset of symptoms. About 25% of patients have first symptoms before the age of 13 years with a window from 17–25 years (156), although rare congenital (157) as well as late onset in elderly has also been described (158). Independent of the initial mode of presentation, gastrocnemius appears to be the most severely affected muscle leading to an inability to stand on tiptoes, and lower limbs are affected more severely than upper limbs (159) (Figure 125-11). In contrast to LGMD2A, scapular involvement is minor and is not present at the onset. Conversely, a distinct “bulge” of the deltoid muscle is a striking feature of the disease. Calf hypertrophy as a transient feature early in the course of the disease has been reported in a minority of patients, and temporary painful swelling of the calf is another unusual presentation. Calf wasting is also commonly seen, while early contractures are rare. Progression is generally slow, and there is not a consistent relationship between mode of presentation and subsequent progression. Cardiac and respiratory functions are usually preserved. CK is usually massively increased up to 100 times the upper limit of normal.

Prevalence of dysferlinopathy varies among populations. The disease appears to be more prevalent in southern than northern Europe and Japan (160–163). In Italy, frequency of LGMD2B is indeed high (18.7%) being second only to LGMD2A (128). Conversely, in northern England dysferlinopathy is less common at 5.9% with a prevalence of 0.13/100,000 (2).

Muscle biopsies show generally nonspecific dystrophic changes, although inflammatory features are also often prominent, leading to polymyositis having been a frequently considered differential diagnosis. Demonstration of loss of dysferlin labeling in muscle biopsies is an extremely useful guide to which patients are to be analyzed for mutations (Figure 125-5). Dystrophin and sarcoglycan analysis in these patients is normal. In approximately half of the dysferlinopathy patients so far studied, calpain 3 levels on immunoblotting are reduced, and caveolin 3 immunolabeling may also be altered (164). Dysferlin can also be studied in monocytes (165).

The LGMD2B locus was first identified in families with a predominantly proximal muscular dystrophy with onset in the late teens (166,167). The gene for MM was shown to localize to the same genetic interval on chromosome 2 as LGMD2B, and subsequent analysis has confirmed the two disorders to be allelic. Not all cases of MM are associated with *DYSF* mutations, with a second locus tentatively assigned to chromosome 10.

Diagnosis of dysferlinopathy is based on detection of *DYSF* gene mutations. Linkage to chromosome 2p13 in

large families may be indicative of the diagnosis. The *DYSF* gene has 55 exons and three alternative exons (1, 5a and 40a), and encodes a 230-kDa ferlin-1 like protein. *DYSF* has a large mutational spectrum, but unfortunately, the detection rate is incomplete. Mutations in dysferlin are spread widely over the coding sequence of the gene, but alternative exons 1, exon 5a, and exon 40a show no disease-causing mutations (168). A particularly prevalent *DYSF* mutation is present in approximately 10% of the Libyan Jewish population. Founder mutations have also been recognized in Spain and Italy, in part responsible for the higher prevalence in these populations (160,162). The possibility of internally deleted functional dysferlin protein has been suggested by the description of mildly affected patients missing one or more *DYSF* exons (169,170). Thus, exon skipping might also be applicable for dysferlinopathy. It has been indeed shown that *DYSF* exon skipping could be as straightforward as DMD exon skipping, as AONs to induce efficient skipping of four *DYSF* exons were readily identified (171).

Carriers of a single *DYSF* gene mutation can rarely be symptomatic (172). No genotype–phenotype correlations are recognized. These findings, together with the wide clinical heterogeneity and the intrafamilial variability, suggest that additional factors, either genetic or nongenetic, may contribute to the pattern of the muscle involvement observed.

Dysferlin is a member of a novel mammalian gene family with homology to a nematode protein (FER-1) involved in spermatogenesis. Another FER-1-like gene (otoferlin) is involved in autosomal recessive deafness, whereas the third human member of this novel gene family, myoferlin or FER-1L3, which is highly homologous to dysferlin, is not yet associated with any human disease but may be upregulated in some forms of muscular dystrophy. The proteins in this group share the structural features of multiple C2 domains, with a C-terminus transmembrane domain. Dysferlin localizes predominantly to the muscle fiber membrane and shows weak interactions with caveolin 3, the annexins and calpain 3 (149,173–175). It is expressed from very early in human development and also in the placenta. Dysferlin is the first example of a protein containing multiple C2 domains, being involved in a muscular dystrophy phenotype, and defines a novel mechanism for the production of a muscular dystrophy via the disruption of the mechanisms for muscle membrane repair (176–180). Dysferlin also plays an important role for muscle membrane maintenance and integrity, and recent data support the role of dysferlin in vesicle trafficking, phagocytosis, focal adhesion, and myogenic differentiation (181). In view of the muscle inflammation often observed in LGMD2B patients, it has been hypothesized that a dysfunction of monocytes and macrophages might contribute to disease pathogenesis and progression. Indeed, it has been observed that dysferlin deficiency enhances monocyte

phagocytosis, suggesting that the myofiber damage might stimulate an inflammatory cascade that can exacerbate the underlying dystrophic process (182). In addition, dysferlin also plays a role in muscle regeneration and maintenance, as well as neutrophil response. In addition to the muscle membrane repair model, dysferlin appears also to be involved in the release of chemotactic agents. In fact, in dysferlinopathy-reduced neutrophil recruitment results in incomplete cycles of regeneration, which together with the membrane repair deficit ultimately triggers dystrophic pathology (183). A predominantly cytoplasmic localization of dysferlin can be observed in regenerating myofibers in dysferlinopathy patients, but the subcellular compartment responsible for this labeling pattern is not yet known. Dysferlin associates with the developing T-tubule system *in vitro* and *in vivo*, and dysferlin-deficient muscle revealed primary T-tubule anomalies, indicating a role of dysferlin in T-tubule formation (184).

**125.5.2.3 LGMD2C-D-E-F or Sarcoglycanopathies.** LGMD2C–F are caused by mutations in one of the genes, encoding for protein of the sarcoglycan complex. The sarcoglycans are transmembrane proteins within the dystrophin-glycoprotein complex at the muscle cell membrane whose functions may include its stabilization, which may play a role in cell signaling. Primary genetic defects in one of four ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ) sarcoglycans are now established as the cause for four subtypes of AR-LGMD (185,186). A fifth sarcoglycan ( $\epsilon$ -sarcoglycan), homologous to  $\alpha$ -sarcoglycan, appears to be maternally imprinted and is mutated in myoclonus-dystonia syndrome (187,188).  $\zeta$ -Sarcoglycan is not associated with any disease phenotype (189). Because loss or deficiency of any of at least four sarcoglycans causes muscular dystrophy, these proteins must play a critical role in the maintenance of membrane integrity through the maintenance of the link from the extracellular matrix to the proteins of the cytoskeleton and contractile apparatus. In  $\beta$ - and  $\delta$ -sarcoglycanopathies, there are also some indications that smooth vascular muscle disease may add to the dystrophic process, although this mechanism has not yet been fully established.

The clinical spectrum of sarcoglycanopathies overlaps with dystrophinopathies, with the distinction that learning difficulty is not seen in sarcoglycanopathies, and scapular winging is more frequent (Figure 125-11). Presentation may range from early childhood to late adulthood, and progression may be rapid or much more benign. Early presentation usually indicates more rapidly progressive disease, and childhood presentation predominates, being  $\alpha$ -sarcoglycan deficiency responsible for 22% of patients with a progressive muscular dystrophy beginning in childhood. Cases of LGMD2D ( $\alpha$ -sarcoglycan) with a late onset show indeed a milder course with respiratory insufficiency while patients are still ambulant (190). Up to 6% of adult-onset cases are  $\alpha$ -sarcoglycan deficient. Muscle hypertrophy is common,

and at presentation, patients show relatively great involvement of the scapular muscles and hamstrings. Cardiac and respiratory involvement is also reported alongside the progression of muscle weakness, similar to what seen in DMD. CK values are increased up to 10 times the upper limit of normal.

Prevalence of sarcoglycanopathy is variable. The combined frequency of sarcoglycanopathy in the northern England population is 11.7% with a prevalence of 0.27/100,000 (2), while frequencies in Italy were as follows: LGMD2C ( $\gamma$ -sarcoglycan) 4.5%; LGMD2D 8.4%; LGMD2E ( $\beta$ -sarcoglycan) 4.5%; LGMD2F ( $\delta$ -sarcoglycan) 0.7% (128). Prevalence of LGMD2D in Finland is higher (1:250,000) with a carrier frequency around 1:150 (191).

Muscle immunoanalysis using specific antibodies to the various sarcoglycans is the primary diagnostic tool. Use of antibodies against all four sarcoglycans for immunoanalysis of skeletal muscle sections is recommended, as residual sarcoglycan expression is highly variable and does not enable an accurate prediction of the genotype (192). Multiplex Western blotting may also be very helpful in trying to resolve the primary abnormality (Figure 125-5). A concomitant reduction of dystrophin and  $\beta$ -dystroglycan is also frequently observed and illustrates the important differential diagnosis of DMD and BMD for sarcoglycanopathies (193). The most worrying diagnostic confusion may be with isolated women with muscular dystrophy who, if a full range of antibodies is not used, may be misdiagnosed as manifesting carriers of DMD, with potentially disastrous results for genetic counseling (193). In addition, the issue of misdiagnosis in men (between BMD and sarcoglycanopathy) can also be important (Figure 125-10).

Mutation analysis is necessary in order to provide carrier testing or prenatal diagnosis. Mutations identified in the *SGCA* gene on chromosome 17q21, coding for the 50-kDa  $\alpha$ -sarcoglycan (previously called adhalin), cause the most commonly reported sarcoglycanopathy (LGMD2D). LGMD2E, LGMD2C, and LGMD2F are caused by mutations in the *SGCB*, *SGCG*, and *SGCD* genes on chromosomes 4q12, 13q12, and 5q33, respectively. A wide range of mutations has been described in the four sarcoglycan genes. Many mutations are missense, and most are found in the portion of the genes, encoding the large extracellular domains. A CpG site in exon 3 of the *SCGA* represent a mutation hot spot, being mutation R77C the most frequently reported. Various founder mutations are also described. Not all patients with absent or reduced sarcoglycan staining on a muscle biopsy can be shown to have a mutation in one of the sarcoglycan genes by standard techniques, in particular sequencing (193). Exclusion of large deletions may be useful in these cases. In particular, MLPA analysis is able to detect copy number changes in about 15% of tested cases, with *SGCG* gene accounting for 8% of cases, as



the size of the gene makes it vulnerable to large exonic deletions (193).

It has been suggested that missense mutations in these membrane proteins may lead to aberrant protein processing and degradation. From this model, missense mutations in a member of the complex might cause improper processing or assembly of the whole group, leading to rapid turnover of the mutated protein. In  $\beta$ - and  $\delta$ -sarcoglycanopathy, disruption of the smooth muscle sarcoglycan complex may contribute to the development of cardiomyopathy (185). Indeed, rare cases of hemizygous  $\delta$ -sarcoglycanopathy have also been reported with an isolated cardiomyopathy phenotype (194), and in particular, the S151A mutation has been reported to cause severe lethal form of dilated cardiomyopathy. The same mutation was also detected in a consanguineous family with LGMD also segregating a homozygous novel missense mutation in the same gene in exon 6 (A131P) (195). Compound heterozygous family members revealed no signs of cardiomyopathy, questioning thereby the role of the *SCGD* gene in general as a disease-causing gene for autosomal-dominant dilated cardiomyopathy.

Mendell et al. (196) have shown use of gene therapy as a potential treatment approach for LGMD2D. In fact, they attained long-lasting sustainable gene expression of  $\alpha$ -sarcoglycan in LGMD2D subjects mediated by adeno-associated virus gene transfer under control of a muscle-specific promoter.

**125.5.2.4 LGMD2I and 2K.** LGMD2I is one of the most frequent forms of LGMDs, and the protein involved is the fukutin-related protein (FKRP). Mutations in the *FKRP* gene cause a remarkable spectrum of disease severity, from a severe congenital muscular dystrophy to a mild form of LGMD (197,198). FKRP belongs to a growing family of proteins involved in producing muscular dystrophies that are involved in the O-glycosylation of  $\alpha$ -dystroglycan. Disruption of the link through the muscle fiber mutation through  $\alpha$ -dystroglycans is a novel and increasingly important mechanism for producing muscular dystrophy (199–201).

LGMD2I is the most common form of LGMD in northern Europe (138,139,202) with a common founder mutation (C826A) in this population, which is also present in the Hutterite population of Canada (203). Minimum prevalence in the Norwegian population is 1/54,000 with a carrier frequency of 1/116 (203). In northern England, LGMD2I represents 19.1% of the total LGMD group with a prevalence of 0.43/100,000 (2).

Presentation can be at any age, with a spectrum of disease severity similar to that seen in dystrophinopathy. The ability to identify LGMD2I has brought to light some misdiagnoses amongst the supposed DMD and BMD population, critical for genetic counseling, and highlighting the importance of full genetic diagnosis in all cases (138). Clinical clues for diagnosis are hypertrophy of the calf and sometimes other muscles (including the tongue),

as well as cardiomyopathy (reduced left ventricular ejection fraction) and diaphragm involvement that could cause particular problems with breathing when lying flat (Figure 125-10 and 125-11). Cardiac and respiratory involvement carries indeed important implications for medical surveillance and treatment, and patients often require respiratory support even when the patient is still ambulant (137,204). Oligosymptomatic patients and individuals with predominant cardiac involvement have also been described, with cardiomyopathy being the first sign of the disease (204). Gene mutation and the severity of the muscle disease are usually not predictive of cardiac involvement. Abnormal glycosylation of  $\alpha$ -dystroglycan in LGMD2I may interfere with brain development and cognitive performances, involving the frontal and posterior parietal regions, but does not result in specific brain MRI abnormalities (205).

LGMD2K also involves glycosylation defects and is caused by mutations in the *POMT1* gene. LGMD2K is allelic to a severe form of CMD (see “Congenital muscular dystrophies”). Patients appear to have mental retardation as well as muscular dystrophy. This disorder is common in Turkey (206).

Examination of the muscle biopsy in patients with LGMD2I and LGMD2K typically shows a secondary reduction in  $\alpha$ -dystroglycan (Figure 125-4), with a secondary reduction in laminin  $\alpha$ -2 on immunoblotting. The diagnosis of LGMD2I is through mutation analysis of the *FKRP* gene. The vast majority of LGMD2I cases (typically all cases in northern Europe) have at least one copy of the common C826A mutation, while *FKRP* mutations found in CMD are much more variable (for more details see “Congenital muscular dystrophies”). If the *FKRP* gene was normal in patients with LGMD and abnormal  $\alpha$ -dystroglycan, it would be appropriate to direct mutation detection to the other genes involved in glycosylation of  $\alpha$ -dystroglycan. It is likely that milder mutations in all of the genes also involved in CMD phenotypes will eventually be described in LGMD, as for LGMD2I and LGMD2K.

**125.5.2.5 LGMD2G.** LGMD2G is a rare form of LGMD representing about 3% of all LGMDs, and it has been so far observed mainly in patients from Brazil and Italy. The phenotype can be more variable than that initially suggested. Clinically, patients between ages 9 and 15 years present with difficulty climbing stairs and running, although foot drop is also an early feature. Proximal and distal lower limb weakness is present from the onset, whereas in the upper limbs, the proximal musculature is usually more severely affected. Confinement to a wheelchair usually occurs after the 4th decade. Females appear to be less severely affected than males. Cardiac involvement is frequent. CK levels are elevated to 3–30 times.

The locus for LGMD2G was identified in 17q11–12 through the study of large LGMD families in Brazil, and mutations in the telethonin gene, which encodes a sarcomeric protein, are now known to be responsible for



this form of LGMD (1). Examination of muscle biopsies shows a large number of rimmed vacuoles (a feature not commonly reported in AR-LGMD) and loss of telethonin labeling. Diagnosis needs to be confirmed by mutation analysis.

**125.5.2.6 LGMD2H.** This disorder was first described in 1976 in the Hutterite population in Manitoba, and it is caused by a founder homozygous mutation (D487N) in the E3 ubiquitin ligase *TRIM32* gene (207,208). One sib pair has been identified in a non-Hutterite family in Germany (the country of origin of the Hutterites). Affected individuals are described as presenting usually with proximal lower limb weakness with a variable age at onset (from early childhood to mid-20s). Facial weakness is observed as the disease progresses. Mild distal limb involvement is also seen, involving the brachioradialis and anterior peroneal muscles, but most patients remain ambulant late into adult life. CK values are typically elevated up to five times the normal values. Muscle biopsy analysis shows no pathognomonic changes, and diagnosis is based on identification of *TRIM32* gene mutations. Patients with sarcotubular myopathy, also of Hutterite origin, have now been identified to have *TRIM32* mutations, but this does appear to be a rare cause of muscle disease (209).

**125.5.2.7 LGMD2J.** LGMD2J is rare form of LGMD described only in Finland in very few cases, and it is caused by homozygous mutations in the titin *TTN* gene, representing the severe form of the milder tibial muscular dystrophy. LGMD2J is characterized by severe progressive proximal weakness, with onset ranging from the 1st to the 3rd decade. Weakness can also involve the distal compartments, and about half of the patients require the use of a wheelchair. Contractures and cardiac or respiratory involvement are not reported. CK values are massively raised. On immunohistochemistry, severe reduction or absence of calpain 3 is detected, as reflection of the interaction of titin with calpain 3. Diagnosis is based on *TTN* gene analysis, and almost all affected individuals have a homozygous 11-bp deletion/insertion in the last exon termed Finnish FINmaj, being very common in the Finnish. Recently, a novel mutation has been described in a French family (210).

**125.5.2.8 LGMD2L.** LGMD2L is caused by mutations in the *ANO5* gene, which encodes a putative calcium-activated chloride channel belonging to the Anoctamin family of proteins. Mutations in *ANO5* were first identified in families with LGMD2L and non-dysferlin MM (211). Screening of additional cohort of patients from England and Germany showed that LGMD2L is indeed is relatively common form of LGMD (212). In particular, a truncating mutation (c.191dupA) in exon 5 of *ANO5* has been identified in homozygous or combined heterozygous state in all patients from these cohorts, suggesting a founder effect for this mutation in the northern European population. Patients with LGMD2L show adult-onset (from early 20s to 50s) proximal lower limb weakness

with highly raised CK values (average 4500 IU/l). Distal presentation is much less common, but a milder degree of distal lower limb weakness is often observed. Muscle wasting mainly affects quadriceps, hamstrings, and the medial gastrocnemius (Figure 125-11). Knee hyperextension is a common and often causes progressive functional disability. Asymmetry of muscle weakness and atrophy is common (90% of patients) and represents, together with thigh muscle atrophy and later age at onset, a useful predictor to differentiate LGMD2L from LGMD2B. The weakness is generally slowly progressive, with most patients remaining ambulant for several decades. In later stages of the disease, mild weakness and wasting of the upper limbs, in particular of biceps brachii and brachioradialis, are also observed. Cardiac and respiratory function is normal. Intrafamilial variability in mode of presentation and disease progression is also observed, and CK measurement and molecular testing could be used to identify asymptomatic or mildly affected family members. Females represented a minority of cases of the mutation positive patients and appear to be less severely affected, suggesting a strong bias toward affected males in the LGMD2L population.

A predominantly distal phenotype is more often observed in association with other *ANO5* mutations, and one in particular appears common in the Finnish population. The phenotype observed in the Finnish patients is characterized by onset in the third decade, with first symptoms being burning sensation on the calves and, later on, calf tightness during running. Muscle weakness and wasting were asymmetric and early involving the calf muscles, later spreading to the thigh muscles. Involvement of biceps brachii was a later manifestation (213).

Although the same *ANO5* mutation can also lead to a variable clinical presentation at onset (LGMD or distal myopathy), with disease progression, the phenotypes largely overlap and merge into a more homogenous clinic entity.

The minimum prevalence of LGMD2L in the North of England is 0.27/100,000, being the 3rd most common form of LGMD. The apparent frequency of this condition in this group compared with some of the other forms of LGMD confirm the need to incorporate *ANO5* mutation screening (at least for the common mutation) at an early stage in the diagnostic algorithm for patients presenting with an LGMD phenotype.

*ANO5* encodes a member of the anoctamin family of proteins, which contain eight transmembrane domains. Dominant mutations in the *ANO5* gene are associated with the skeletal disorder called gnathodiaphyseal dysplasia (214). The role of *ANO5* is still unknown, but the homologous proteins *ANO1* and *ANO2* are known to be calcium-activated chloride channels (215–219). However, it has been suggested that *ANO5* may be important in the development and maintenance of skeletal muscle and the clinical overlap between LGMD2L and

LGMD2B further suggests that mutations in *ANO5* may be involved in membrane repair.

**125.5.2.9 Other Causes of Autosomal Recessive Limb-Girdle Muscular Dystrophy.** The list of AR-LGMD genes does not yet appear to be completely exhaustive, and other rare forms of LGMDs, allelic to CMD, are caused by mutations in the *FKTN* (LGMD2M), *POMT2* (LGMD2N), and *POMGNT1* gene (LGMD2O) (1). An additional form of AR-LGMD associated with cognitive impairment has been now linked to a mutation in the *DAG1* gene encoding dystroglycan (220). Moreover, mutations in the *PLEC1* gene, also responsible for epidermolysis bullosa simplex with muscular dystrophy, have been identified in a consanguineous Turkish family (221). This new form of LGMD has now been coded LGMD2Q (<http://omim.org/entry/613723>). In populations with a high rate of consanguinity, the possibility of further loci is suggested by the existence of families that are still unlinked to any of the known genes. Use of the full battery of investigative tools in all patients with a suggestive phenotype will no doubt reveal other presumably secondary protein deficiencies. The key role of the muscle biopsy in elucidating both primary and secondary effects in LGMD is very clear, and in all patients with suspected LGMD, this investigation should be recommended, with frozen tissue kept available for further analyzes as these become available.

## 125.6 MYOFIBRILLAR MYOPATHIES AND OTHER DISTAL PHENOTYPES

### 125.6.1 Myofibrillar Myopathies

**125.6.1.1 Clinical Features.** Myofibrillar myopathies (MFMs) represent a clinically and genetically heterogeneous group of conditions characterized by a similar distinctive myopathological phenotype of myofibrillar dissolution and myofibrillar degradation products, giving the name to these conditions. So far, mutations in eight different genes (*DES*, *MYOT*, *CRYAB*, *FLNC*, *ZASP*, *BAG3*, and *FHL1*) have been described in MFM or MFM-like conditions (118). FHL1-related myopathy is covered separately in view of its contractural phenotype. Inheritance of MFMs is autosomal dominant, although rare exceptions with autosomal recessive inheritance have been described (222). A significant number of patients with MFM are sporadic, and incomplete penetrance has also been observed.

The clinical phenotype of MFM is extremely variable and, in part, depends on the different molecular etiology, and several genotype–phenotype correlations have been drawn (118). In general, MFMs are characterized by progressive adult-onset muscle weakness involving more the distal than the proximal compartments. Onset can vary according to the underlying molecular defect. Classically, *DES* and *CRYAB* gene mutations associate with

an onset in early or mid adulthood, while *MYOT*, *ZASP* and *FLNC* gene mutations tend to manifest later in life, usually after the fourth or fifth decades. Congenital or childhood onset has been described in association with recessive *DES* and *CRYAB* gene mutations, as well as in families with *BAG3* gene mutations (222–225). Patterns of muscle weakness are variable. Distal onset in the lower limbs is the most common, followed by a more LGMD (with *MYOT* gene mutations), scapuloperoneal (with *DES* gene mutations), or a more general distribution of weakness. The weakness is progressive and later may involve facial, truncal and neck muscles and can also cause swallowing difficulties and dysarthria, in particular with *DES*, *CRYAB*, and *MYOT* gene mutations. Cardiac and respiratory involvement is common, as well as involvement of other organs and apparatus, such as skin, joints, and nervous system. Cardiac disease, mainly arrhythmias (AV nodal conduction blocks, supraventricular and ventricular ectopic beats, tachycardia) and/or cardiomyopathy (dilated, hypertrophic, or restrictive), is observed in up to 30% of individuals, most commonly in association with *DES*, *ZASP*, and *BAG3* gene mutations. *DES* and *BAG3* gene mutations have also been reported in patients affected by isolated cardiomyopathy (226,227). Treatments include pacemaker and implantable cardioverter defibrillator as well cardiac transplantation in case of life-threatening cardiomyopathy (223). Respiratory insufficiency, caused by weakness of respiratory muscles, is common in particular in patients with *FLNC* and *BAG3* gene mutations. Joint contractures and/or rigid spine are observed in patients with *BAG3*, *MYOT*, as well as in rare patients with *DES* or *CRYAB* gene mutations with congenital or early-childhood onset. Neuropathy has been reported in patients with *BAG3*, *ZASP*, or *FLNC* mutations. Cataract has been observed in three families with *CRYAB* gene mutations. CK levels are usually normal or moderately elevated, but marked increases were also observed in association with *BAG3* and *MYOT* gene mutations. EMG is usually myopathic although rare cases of mixed myopathic and neuropathic findings have also been observed.

Mutations in two further genes (*TTN* and *DNAJB6*) have recently been identified in families segregating an autosomal dominant myopathy and showing histopathological changes such as eosinophilic inclusions, rimmed vacuoles, and Z-disc abnormalities, suggestive of MFM (132,133,228,229). A single heterozygous mutation in the A-band of Titin was detected in a few dominant families. The phenotype is mainly characterized by predominant distal, proximal, and respiratory weaknesses, with variable age at onset, CK being normal or increased up to 4–5 times the normal values (228,229). As previously mentioned, the phenotype associated with *DNAJB6* mutations is also characterized by distal and proximal weakness, but no major respiratory or cardiac involvement was found in the few families described so far (132,133).

Diagnosis of MFM is greatly based on observation of myopathological findings. In addition to myopathic changes, usual findings are subsarcolemmal and/or sarcoplasmic protein aggregates, cytoplasmic bodies, rimmed and nonrimmed vacuoles, and core-like lesions (230,231). Specific findings can give an indication of the underlying molecular defect. Not all MFM biopsies show the above-mentioned findings, and unspecific and normal muscle biopsy analyses have also been observed in genetically confirmed MFM patients. Immunostaining using antibodies for desmin, myotilin and  $\alpha$ B-crystallin shows pathological protein aggregation and it is commonly used in diagnostic laboratories (Figure 125-4). Western blot analysis is usually nondiagnostic, except for rare cases of truncating, X-linked or recessive mutations. EM analysis shows pathological protein aggregation and myofibrillar degeneration.

**125.6.1.2 Genetics and Pathogenesis.** The genetic bases of MFM are only partially known. Mutations in the known genes are responsible for about 50% of MFM cases and being *MYOT* and *ZASP* gene mutations the most common (13 and 14%, respectively) followed by *DES* (8%) and then by *CRYAB*, *FLNC* and *BAG3* genes mutations, each responsible for 4% of cases (232). Prevalence of *TTN* and *DNAJB6* mutations in MFM patients is not known, to date.

Most of MFM patients are sporadic or with a dominant family history. Germ line mosaicism has been described in families with multiple affected siblings, harboring heterozygous *BAG3* gene mutations (226). Recessive mutations in the *DES* and *CRYAB* genes have been reported in a few families with atypical or more severe presentations (222,233), with heterozygous parents showing no clinical symptoms. Prenatal and preimplantation genetic testing could be offered in families with known mutations.

Molecular pathogenesis may vary according to the underlying genetic defect. In general, disease proteins are involved in maintaining the structural integrity of the Z-disk. Desmin is a type III intermediate filament protein present in striated and smooth muscle cells. Desmin has a tripartite structure with a central  $\alpha$ -helical coiled-coil rod domain, flanked by non- $\alpha$ -helical head and tail domains. The rod domain plays a key role in the assembly of desmin filaments and formation of extrasarcomeric cytoskeleton, forming a three-dimensional scaffolding structure of the Z-disks, interlinking myofibrils and connecting them to the nuclei, mitochondria, and sarcolemma. For its proper function, desmin interacts with other binding proteins such as plectin and  $\alpha$ B-crystallin. Myotilin, Filamin C and *ZASP* are key Z-disk proteins interacting with each other or with other Z-disk or sarcolemmal proteins. *BAG3* is a cochaperone for the Z-disk with antiapoptotic properties. *DNAJB6* interacts with this, and other proteins involved in chaperone-assisted selective autophagy and current data suggest that *BAG3* might indeed have a role in mediating the pathogenic effect of *DNAJB6*

mutations (133). Defects in MFM proteins therefore cause disintegration of the Z-disk, leading to myofibril degeneration. Metabolic abnormalities have also been reported, in particular based on the mitochondrial pathology, protein quality control mechanisms related to the ubiquitin–proteasome system, and the autophagolysosomal pathway.

## 125.6.2 Inclusion Body Myopathy Associated with Paget Disease of the Bone and Frontotemporal Dementia

**125.6.2.1 Clinical Features.** Inclusion body myopathy associated with Paget disease of the bone (PDB) and frontotemporal dementia (FTD) is a rare adult onset condition, characterized by proximal and distal muscle weakness, early onset PDB and premature frontotemporal dementia (IBMPFD) (234). Prevalence of IBMPFD is unknown, but it is probably an under-diagnosed condition. The myopathy is usually characterized by adult onset (20–40s) progressive weakness usually with proximal LGMD distribution. However, a distal onset involving hands and feet has also been described. Muscle weakness progresses to limb and respiratory muscles, and in later stages, the disease can lead to loss of ambulation. Cardiac failure due to cardiomyopathy is observed in later stages of the disease and together with respiratory failure is the prevalent cause of death, usually occurring in the sixth or seventh decade. About half of the affected individuals also show PDB, characterized by focal areas of increased bone turnover, leading to bone pain, localized painful bone enlargement and deformities of long bones, rare fractures, and deafness. PDB is often asymptomatic and is usually diagnosed based on increased alkaline phosphate concentration, with a mean age at diagnosis of 42 years. FTD is characterized by degeneration of the frontal and anterior lobes controlling reasoning, personality, movement, speech, social graces, and language, being memory unaffected. FTD affects about 1/3 of patients with IBMPFD with a mean age at onset of 55 years. A single family with prominent sphincter disturbance involving bladder, bowel, and erectile function in all affected individuals has also been reported (235), possibly caused by spinal cord and nerve pathology and consistent with pathological findings of spinal cord inclusion bodies (236).

Clinical diagnosis of IBMPFD is based on the combination of the major manifestation of the disease: myopathy, PDB, and FTD. However, only a minority of all affected individuals (12%) shows all the three major manifestations of the disease, while about 50% shows two, and about 30% of patients only show myopathy. CK values are normal or mildly elevated. EMG is myopathic. Muscle analysis shows degenerating fibers, rimmed vacuoles, and sarcoplasmic inclusions, containing ubiquitin and TAR DNA-binding protein 43.



**125.6.2.2 Genetics and Pathogenesis.** IBMPFTD is caused by heterozygous mutations in the VCP gene, encoding valosin-containing protein (VCP) (237). The VCP gene, localized on chromosome 9p, has 17 exons, and mutations (all missense) have been described mainly in the N-terminal CDC48 domain involved in ubiquitin binding. The majority of mutations are located in exon 5, in particular involving residue Arg155. Some genotype–phenotype correlations have been described (234,238). In particular, the Arg155Cys mutation has been associated with an earlier onset of IBM, while patients with the Arg155His mutation show a later onset of PDB (238). A more severe phenotype was observed in association with the Ala232Glu mutation in exon 6, involving a residue localized in the main catalytic D1 ATPase domain. Genetic heterogeneity has been shown, and several families with a clinical diagnosis of IBMPFTD (234) or overlapping phenotypes do not show linkage to 9p nor mutations in the VCP gene (239).

VCP is a 97-kD protein, and it is a member of the type II AAA ATPases that associate with a variety of activities, characterized by the AAA domains. VCP catalyzes ATP hydrolysis to generate energy and perform mechanical work in cells. VCP is involved in several cell cycle protein pathways, including ubiquitin-dependent protein degradation, and quality control process that eliminates aberrant proteins in the secretory pathway (240). VCP has been shown to participate in aggregate-prone proteins degradation, principally mediated by autophagy (241). Indeed, VCP mutations lead to accumulation of autophagic structures in patients and transgenic animal tissue, likely due to a defect in VCP-mediated autophagosome maturation (242).

### 125.6.3 Distal Myopathies

Distal myopathies (DM) are a heterogeneous group of muscle disorders characterized by weakness and atrophy starting from hands and feet, later involving lower legs and forearms. Although traditionally defined as myopathies, in view of the progressive nature of these diseases as well as of the typical histological changes, DMs are indeed muscular dystrophies. Genetic splitting and lumping has been observed, with mutations in the same gene responsible for different clinical phenotypes (243,244).

**125.6.3.1 Welander Distal Myopathy.** Welander distal myopathy (WDM) is a late-onset disease (40–60s), characterized by weakness first involving the index finger extensor. Initial lower leg involvement is observed in 1/3 of the patients, while proximal muscles are usually spared. Later, the disease can also involve the other finger extensors and flexors as well as the thenar and hand muscles. Ankle reflexes are lost in later stages of the disease. Asymmetry is commonly observed. MRI of lower legs shows fatty degeneration of the posterior and anterior compartments. CK levels are normal or increased up to 2–3 times the normal values. Muscle biopsy

analysis can show rimmed vacuoles but no inflammatory changes (245).

The inheritance of the disease is dominant, and it has been mainly reported in the Swedish and Finnish populations. A locus has been found in 2p13 with a unique founder haplotype in Scandinavian patients that could help for genetic counseling of families (246). Although extensive sequencing has been performed, no gene defect has been found, so far.

**125.6.3.2 Tibial Muscular Dystrophy.** Tibial muscular dystrophy (TMD) is a condition with a relatively high prevalence in Finland where it represents the most common muscle disease (10/100,000). TMD is a slow progressive disease, with onset in the late 30s. The first symptom is often an asymmetric weakness in ankle dorsiflexion, later associated with atrophy of the muscles of the anterior lower leg compartment. Typical sparing of extensor digitorum brevis as well as of hand muscles is a useful diagnostic handle. Late-onset proximal lower-limb weakness is observed, but loss of ambulation is a rare finding. MRI studies can show fatty degeneration of anterior tibial muscles, later also involving soleus and medial gastrocnemius. CK values are normal or mildly elevated. Muscle biopsy shows many rimmed vacuoles (243).

TMD is caused by heterozygous mutations in the titin (*TTN*) gene. Mutations typically involve the C-domain located in the M-line of the sarcomere. Finnish patients carry a common deletion–insertion in-frame mutation called FINmaj, while different point mutations were found in other populations. The mutated proteins are incorporated in the sarcomere, but they are likely lacking the whole C-terminus, as C-terminal antibodies do not recognize their epitopes. Dominant mutations therefore have a likely gain of function effect, different from what observed in the rare cases of homozygous *TTN* gene mutations observed in LGMD2J patients.

**125.6.3.3 Laing Distal Myopathy.** Laing distal myopathy (LDM) is a rare condition mainly characterized by mild foot drop and hanging of the big toe. The condition is clinically variable and onset may range from birth to the sixth decade (247). In addition to great toe/ankle dorsiflexors, weakness often interests the neck flexors, the finger extensors, as well as the facial muscles. Proximal and axial muscles are also affected clinically or by muscle imaging, with some patients showing scapuloperoneal or limb-girdle phenotypes from onset. Dilated cardiomyopathy, skeletal deformities, and myalgias have also been observed. The condition could be extremely mild with asymptomatic individuals or, more severe, with patients showing bone deformities, ankle contractures, and abnormal head posture, as well as loss of ambulation. CK is usually normal or moderately elevated (twice the normal limits). Most common pathological findings are predominance of atrophy/hypotrophy of type I fibers, core/minicore lesions, as well as mitochondrial abnormalities. MRI studies typically show involvement of the anterior compartment of the lower legs. EMG showed



frequent neurogenic features, in keeping with denervation–reinnervation activity, reflecting an abnormal motor unit remodeling process, and that could indeed lead to misdiagnosis (247).

Inheritance is autosomal dominant, and the condition is caused by mutations in the *MHY7* gene. The *MYH7* gene encodes for MyHC I or slow/ $\beta$ -cardiac myosin, and it is expressed in slow, type 1 muscle fibers as well as in the ventricles of the heart. To date, nine different mutations in the rod domain (between exon 32 and 37) have been described (244,247,248). Major clinical variability is observed in patients from the same family or with the same mutation.

**125.6.3.4 Distal Myopathy with Rimmed Vacuoles.** Distal myopathy with rimmed vacuoles (DMRV) or inclusion body myopathy type 2 (IBM2) is a rare condition characterized by distal lower limb weakness, with onset in late teens to early adulthood. Patients show gait disturbance with foot drop due to anterior tibialis muscle weakness. The weakness later involves proximal lower limb muscles and hands, but typically, but not constantly, spares quadriceps muscles. Shoulder muscles could be mildly affected, but triceps are spared. Neck flexors are affected. Rare patients with complete heart block have been reported. The condition is progressive, and after about 20 years of disease progression, patients usually become wheelchair bound. CK values are elevated up to 2–3 times the normal values. Muscle biopsy analysis shows rimmed vacuoles, with basophilic granular material on hematoxylin and eosin stain and purple-red with modified Gomori. It has been suggested that these represent autophagic vacuoles.

The condition has been described worldwide, but of the greatest majority of >200 patients so far described are from Iranian Jewish descendant or from Japan. Prevalence rate of the disease in Iranian Jews is estimated to be 1/500–1000. Inheritance is autosomal recessive and mutations of *GNE*, encoding for UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosaminase kinase, are the only known to be responsible for DMRV (249). In the middle-eastern Jewish population, 100% of patients carry a common missense change (M712T). Different mutations, including a large deletion, have been reported in different populations. No genotype–phenotype correlations have been described so far. Rare cases of lack of penetrance have been reported (250,251).

**125.6.3.5 Distal Nebulin Myopathy.** Distal nebulin myopathy is a rare condition, caused by homozygous mutations in the nebulin (*NEB*) gene (252). Affected individuals show childhood or adult-onset foot drop. Muscle weakness predominantly affected ankle dorsiflexors, finger extensors, and neck flexors, clinically resembling LDM. On muscle biopsy analysis, nemaline bodies are absent on routine light microscopy while inconspicuous or absent on EM. Rimmed vacuoles are also absent. The two reported missense mutations are localized in the C domain of nebulin and have been observed in

combination with more disruptive mutations in patients with nemaline myopathy.

**125.6.3.6 Kelch-Like Homolog 9 Gene-Related Distal Myopathy.** Cirak et al. described 10 affected members from a German kindred with a new form of DM (253). Affected individuals showed weakness and atrophy of the anterior tibial muscles leading to difficulty walking on heels and high stepping gait, with onset in late-childhood teens. Reduced sensation with stocking distribution in lower limbs has been observed in most of the patients. The weakness later spread to the intrinsic hand muscles and sensory symptoms in the hands have also been reported. Ankle joint contractures were also observed, likely secondary to the foot drop. CK levels ranged between 200 and 1400 IU/L. There was no evidence of a neuropathy on nerve conduction. MRI of lower limbs showed symmetric fatty atrophy of muscles and muscle biopsy showed myopathic findings. Genome wide analysis revealed a locus at 9p21.2–p22.3 that led to the identification of a heterozygous mutation in the Kelch-like homolog 9 gene, encoding for bric-a-brac Kelch protein.

**125.6.3.7 Matrilin 3-Related Distal Myopathy.** Mutations in *MATR3* gene, encoding matrilin 3, cause a new form of adult-onset, progressive autosomal-dominant DM, with dysphagia and dysphonia, also called vocal cord and pharyngeal weakness with distal myopathy (VCPDM). This condition has been first described and then mapped to chromosome 5q31 in a North American pedigree, and then fine mapping and sequencing of the critical interval allowed identification of mutations in the *MATR3* gene in two families (254). Matrilin 3, expressed in skeletal muscle, is a component of the nuclear matrix, a proteinaceous network extending throughout the nucleus. Mutations in *MATR3* do not appear as a common cause of muscular dystrophies (255).

**125.6.3.8 Other Forms of Distal Myopathies.** Mutations in genes responsible for muscular dystrophies with different distributions and phenotypes have also been associated with a more distal phenotype and could therefore be classified as pure DMs. Among these, the best examples are the genes responsible for MFMs and in particular *ZASP*, *VCP*, and *MYOT* genes (256–258) as well the *DYSF* gene, also responsible for the more distal Miyoshi myopathy phenotype (156).

## 125.7 CONGENITAL MUSCULAR DYSTROPHIES

### 125.7.1 Introduction

The congenital muscular dystrophies (CMD) are a clinically and genetically heterogeneous group of disorders, presenting at birth or within the first few months of life, with hypotonia, muscle weakness, contractures, and motor developmental delay. They are classically

described as having autosomal recessive inheritance; however, it has recently emerged that one form can also result from *de novo* dominant mutations. In the last decade, several new genes and proteins responsible for individual forms of CMD have been identified and the classification has expanded considerably.

### 125.7.2 Background

In 1993, an International Consortium on CMD proposed a classification based on clinical and imaging findings, defining four distinct phenotypes: (1) “classic” CMD; (2) Fukuyama congenital muscular dystrophy (FCMD); (3) muscle–eye–brain disease (MEB); and (4) Walker–Warburg (WWS). In contrast to the other three forms of CMD, “classical” CMD had no clinical evidence of central nervous system (CNS) or eye involvement. Also in 1993, the locus for FCMD was mapped to chromosome 9. The other forms of CMD with mental retardation and CNS or ocular involvement did not map to the FCMD locus. In 1994, an exciting discovery helped explain some of the clinical findings. A proportion of children with CMD were found to show an absence or marked deficiency in an extracellular matrix protein, merosin. This divided “classic” CMD into two groups: merosin deficient and merosin positive. Although the clinical spectrum associated with merosin-deficient CMD was relatively homogeneous, the merosin-positive group included several distinctive phenotypes, such as the Ullrich phenotype with distal laxity or the form with rigid spine, which in the last few years have been shown to be distinct genetic entities.

Another significant advance has recently come from the discovery that a number of forms of CMD with differing clinical phenotypes have in common a profound depletion of  $\alpha$ -dystroglycan, which can be demonstrated by immunolabeling. These forms, collectively categorized as dystroglycanopathies, have mutations in genes encoding known or putative glycosyltransferase enzymes, which among their substrates most likely include  $\alpha$ -dystroglycan.

To date, the genes responsible for more than 26 of those forms have been identified (Table 125-1). These forms can be classified into five main groups according to clinical, pathological, and genetic data:

1. CMD due to mutations in genes encoding structural proteins of the basal membrane or extracellular matrix of the skeletal muscle fibers. These include forms due to mutations in the genes encoding collagen VI, laminin  $\alpha$ -2 (merosin), and integrin  $\alpha$ -7 and integrin  $\alpha$ -9.
2. CMD due to mutations in genes encoding putative or demonstrated glycosyltransferases, which affect the glycosylation of  $\alpha$ -dystroglycan. These include FCMD, MEB, and WWS, and other forms with normal brain MRI, with and without mental retardation.

3. CMD with rigidity of the spine, due to mutations in the *SEPN1* gene, which encodes selenoprotein 1, an endoplasmic reticulum protein of unknown function.
4. CMD due to abnormalities of nuclear envelope proteins (Lamin A/C and nesprin).
5. CMD with mitochondrial structural abnormalities (CMDmt).

This classification includes only forms of CMD in which the primary genetic defect has been identified, but there are several other forms with distinctive phenotypes in which the underlying defect has not yet been identified. In this chapter, we describe in detail the forms of CMD most frequently found in clinical practice, such as merosin-deficient CMD and Ullrich CMD, followed by dystroglycanopathies and CMD with rigidity of the spine (RSMD1).

### 125.7.3 Merosin-Deficient Congenital Muscular Dystrophy

**125.7.3.1 Pathogenesis.** Merosin (laminin  $\alpha$ -2) is a subunit of laminin, a heterotrimeric extracellular matrix protein that links with dystrophin on the inner side of the muscle membrane through a group of dystrophin-associated glycoproteins (sarcoglycans and dystroglycans) that straddle the muscle membrane. Deficiency of merosin in this form of CMD results in loss of the critical linkage between the extracellular matrix and the intracellular actin-associated cytoskeleton. Merosin is expressed in the Schwann cell basement membrane in peripheral nerve, where it promotes neurite outgrowth and Schwann cell differentiation, migration, and adhesion (259). It is also expressed in the basement membrane of cerebral capillaries, and in neuronal processes of limbic brain regions and the cerebellum. Deficiency of merosin in these locations underlies the CNS and peripheral nervous system manifestations of this form of CMD.

#### 125.7.3.2 Clinical Features.

**125.7.3.2.1 Muscle Involvement.** Hypotonia is the most common presenting feature at birth or in the first months of life, followed by contractures and congenital dislocation of the hip. Neonatal respiratory and feeding problems may also occur. Motor development is delayed, and these children rarely achieve independent walking. Maximal motor ability is generally sitting unsupported and standing with support or, less commonly, walking with support. These children show respiratory problems as a result of diaphragmatic involvement and tend to develop nocturnal hypoventilation, sometimes associated with only moderately abnormal values of daytime oxygen saturation and forced vital capacity. Feeding problems and failure to thrive are also frequent. In our detailed study on 14 children (age range 2–14 years) with merosin-deficient CMD, 12 of the

14 children were below the third centile for weight. On videofluoroscopy only, the youngest child (2 years old) had a normal result, and five children required a gastrostomy.

The phenotype of the merosin-deficient group has expanded further with the recognition of other forms with atypical phenotypes but still with documented mutations in the *LAMA2* gene, such as a form with late onset.

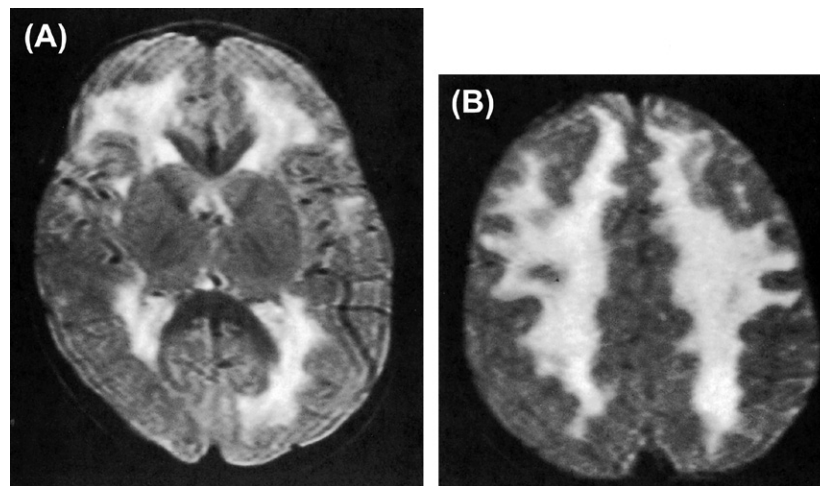
**125.7.3.2.2 Central Nervous System Involvement.** White matter changes are a consistent feature in patients with merosin-deficient CMD. The changes are diffuse, resembling a leukodystrophy, and affect both hemispheres (Figure 125-12) but spare the internal capsule, corpus callosum, basal ganglia, thalami, and cerebellum. This pattern becomes more evident after 6 months but can be visualized in the neonatal period using appropriate MR sequences (260). In a proportion of children with merosin-deficient CMD, the white matter changes can be associated with structural brain changes, such as cortical dysplasia or cerebellar hypoplasia. Children with isolated white matter changes usually have associated normal cognitive function, but they may have mild perceptuomotor difficulties, when compared with both normal controls and children with CMD and normal scans. In contrast, the patients with white matter changes and additional cerebellar hypoplasia often have lower scores on the performance scales on cognitive tests. Epilepsy is common and may occur in about 20% of the cases. Visual function is normal, but visual evoked responses are generally abnormal.

**125.7.3.2.3 Peripheral Nervous System Involvement.** Merosin is expressed in the basement membrane of Schwann cells, and a demyelinating neuropathy is a feature of merosin-negative CMD, which can be

demonstrated by reduced peripheral motor nerve conduction velocity (261).

**125.7.3.3 Muscle Pathology.** The muscle biopsy shows a wide variation in fiber size and an increase in endomysial connective tissue and adipose tissue. Immunofluorescence techniques can readily demonstrate the reduction or absence of laminin  $\alpha$ -2. In some cases, the merosin is totally absent; in others, traces are present, but it is still markedly reduced compared with normal controls. The reduction of the merosin chain can sometimes be demonstrated only by the combined use of antibodies to both the globular 80-kDa components, which contain the C-terminal end, and to the 300-kDa N-terminal end. Laminin  $\alpha$ -2 can be also demonstrated in the skin, and a skin biopsy can provide useful information when muscle is not available.

**125.7.3.4 Molecular Genetics.** Merosin-deficient CMD is autosomal recessively inherited. The 64-exon *LAMA2* gene is located on chromosome 6q22–23. Most mutations causing complete merosin deficiency are non-sense or frame-shifting mutations. These are predicted to encode truncated proteins lacking the C-terminal G domain that is involved in  $\alpha$ -dystroglycan and integrin binding, sometimes together with the preceding coiled-coil forming domains that are required for assembly of the laminin heterotrimer (262,263). Mutations are scattered throughout the length of the gene, and the only common mutation is a 2-bp deletion, 2098delAG (703X), which has been reported in several families of different nationalities (264). Recently, two siblings with strikingly different clinical phenotypes were described who were both homozygous for the same out-of-frame deletion in *LAMA2*. Both had complete merosin deficiency in muscle, but one had a classical merosin-deficient CMD phenotype, whereas the other had milder muscle weakness and was still ambulant aged 13 years (265). Modifying



**FIGURE 125-12** Merosin-deficient boy 7 years of age. T2-weighted SE 2500/80 sequences. A, Low ventricular level showing abnormal, increased signal intensity in a periventricular distribution. Note that the white matter of the basal ganglia is spared. B, Centrum semiovale, showing extensive white matter lesions. (From Philpot, J.; Topaloglu, H.; Pennock, J.; Dubowitz, V. *Familial Concordance of Brain Magnetic Resonance Imaging Changes in Congenital Muscular Dystrophy*. *Neuromuscul. Disord.* 1995, 5, 227–231).

genes might have affected the clinical phenotype in this family.

Patients with partial merosin deficiency have a variety of mutations including missense and non-sense mutations, splice-site mutations (resulting in exon skipping or altered splicing efficiency), or small, in-frame deletions (262,266,267). These are mostly predicted to result in expression of a partially functional protein or low levels of a normal protein, in contrast to the situation in complete merosin deficiency, in which mutations result in a truncated, nonfunctional protein.

A recent paper examining the relationship between degree of merosin expression, genotype, and clinical features in 51 patients with MDC1A has reported that *LAMA2* mutations were distributed throughout the gene in patients with absent merosin and more severe phenotype, with minor clusters in exon 27, 14, 25, and 26 (55% of mutations). Patients with residual merosin and milder phenotype often carried at least one splice-site mutation and less frequently frameshift mutations (268).

**125.7.3.5 Genetic Counseling.** A diagnosis of merosin-deficient CMD can be confirmed by *LAMA2* mutation analysis or by linkage analysis in suitable families, and these families can be offered antenatal diagnosis. Merosin is expressed in placental trophoblast, and antenatal diagnosis can be performed by analysis of merosin expression in chorionic villous samples. Because secondary merosin deficiency occurs in other forms of CMD, great care should be taken when considering this approach in families where no mutation in *LAMA2* has been found.

**125.7.3.6 Animal Model.** The *dy/dy* mouse was studied extensively during the 1960s as a possible model for DMD, but enthusiasm waned when it was shown to also have peripheral nervous system involvement. Merosin is markedly reduced in the dystrophic *dy/dy* mouse. The *dy* locus has been located close to the gene for merosin in the mouse, suggesting that the mouse *dy* mutation is in the merosin gene. The presumed mutation, however, is yet to be identified. A milder allelic mutant of the *dy* mouse (the *dy<sup>2J</sup>/dy<sup>2J</sup>*), whose muscle is merosin-positive, has been shown to have a splice-site mutation within the merosin gene.

#### 125.7.4 Congenital Muscular Dystrophy with Partial Merosin Deficiency

A number of patients with partial merosin deficiency and a documented mutation in the *LAMA2* gene have also been reported. The clinical phenotype is generally less severe than in the form with complete or near-complete merosin deficiency. Unlike the complete form, these patients show delayed walking but independent ambulation. These patients also show white matter changes on MRI, and in some of them, epilepsy has also been

reported. On muscle biopsy, it is the N-terminus of laminin  $\alpha 2$  that is more substantially decreased, whereas the C-terminus can be normal or only mildly decreased. A recent paper comparing patients with absent and partial reduction has reported that compared with the absent merosin group, patients with partial merosin deficiency had later presentation ( $>7$ days) ( $P=0.0073$ ), were less likely to lack independent ambulation ( $P=0.0215$ ), or require enteral feeding ( $P=0.0099$ ) and ventilatory support ( $P=0.035$ ) (268).

#### 125.7.5 Ullrich Congenital Muscular Dystrophy

This form is reported to be one of the most, if not the most common form of CMD, but its prevalence is not yet fully appreciated.

**125.7.5.1 Pathogenesis.** Ullrich CMD results from a defect in collagen type VI. The three collagen VI subunit chains assemble into microfibrils, which form a network that associates with other extracellular matrix proteins. It is thought that collagen VI stabilizes the myofibrils during contraction by anchoring the basement membrane to the interstitial extracellular matrix, and that this function is perturbed in Ullrich CMD (269,270).

**125.7.5.2 Clinical Features.** The typical clinical features are those reported by Ullrich in 1930, when he first described a form of “congenital atonic-sclerotic muscular dystrophy” characterized by contractures of the proximal joints, marked distal laxity, and normal intelligence. Contractures and hypotonia, which are often associated with torticollis and hip dysplasia, are usually present at birth. However, a proportion of children may present with delayed milestones. Maximum functional ability is variable, because some will only achieve assisted ambulation, whereas others will acquire the ability to walk independently. Patients with Ullrich CMD usually develop scoliosis often associated with rigidity of the spine irrespective of whether they have achieved independent ambulation. The laxity of hands and feet is always significant and often associated with proximal contractures that become more marked with time. Failure to thrive and early and marked respiratory involvement are generally obvious by the end of the first decade and become more severe in the second decade, often requiring treatment with noninvasive nocturnal ventilation between the ages of 10 and 15 years (271). On examination, there are associated foot deformities with protrusion of the calcaneum. There is also a tendency to develop skin manifestations such as follicular hyperkeratosis, and in some cases, hypertrophic scars and keloids. Serum CK is normal or only mildly elevated.

**125.7.5.3 Muscle Pathology.** Muscle biopsy can show a variable pathology ranging from myopathic to clearly dystrophic patterns. Collagen VI is generally deficient in muscle and skin on immunofluorescence. However,



although a deficiency of collagen VI is a clear marker of Ullrich CMD, normal collagen VI expression in muscle does not exclude a diagnosis of CMD, and collagen VI status should also be checked on fibroblast cultures. There is some correlation between the extent of reduction in collagen VI in muscle and clinical severity: Patients without collagen VI always have a severe phenotype, whereas patients with a mild collagen VI reduction have a more variable phenotype (272).

**125.7.5.4 Molecular Genetics.** Ullrich CMD can be caused by recessive mutations in the *COL6A1* or *COL6A2* genes on chromosome 21q22, or the *COL6A3* gene on chromosome 2q37 (109,273,274). Heterozygous mutations in the same genes cause the milder allelic condition BM. These genes have 107 cumulative coding exons and frequent polymorphisms, thus mutation analysis is not straightforward. A relatively small number of mutations have been reported, and their mechanisms are still being elucidated (275). Truncating mutations have been reported most commonly and include nonsense and splice-site mutations, as well as small deletions, insertions, or duplications (109,273,274,276,277). These may result in nonsense-mediated mRNA decay and a corresponding reduction in collagen VI or synthesis of abnormal monomers, which form a structurally abnormal microfibrillar network (278). In one patient with a homozygous nonsense mutation in *COL6A3*, the mild clinical phenotype may have been explained by alternative splicing (273). Other mutations in Ullrich CMD are predicted to result in in-frame exonic deletions, and these patients can have classic or mild phenotypes (269,273).

Genetic counseling in Ullrich CMD has been complicated by the recent identification of an increasing number of *de novo* dominant mutations in some patients (279,280). These mutations encode structurally abnormal monomers that have a dominant negative effect on collagen VI microfibrillar assembly. In addition, *de novo* dominant putative mutations in the collagen VI genes have been identified in 10 of a cohort of 26 Ullrich CMD patients, suggesting that *de novo* dominant mutations might account for a significant proportion of Ullrich CMD, although the pathogenicity of these putative mutations is yet unproved (109).

Heterozygous, dominantly acting, mutations in *COL6A1*, *COL6A2*, or *COL6A3* are known to cause BM (270), even though recessive mutations have also been reported (112,113). Some mutations result in haploinsufficiency, whereas others have a dominant negative effect on microfibrillar assembly. Genotype–phenotype correlations are becoming clearer, and with few exceptions, particular mutations are strictly associated with either Ullrich CMD or BM (273). The difference between Ullrich and BM, however, is not always straightforward and a number of phenotypes have clinical findings that are intermediate and not easily classifiable as Ullrich or BM, especially in young children (271,280).

There is definite genetic heterogeneity within Ullrich CMD, although the extent of this is not yet clear. cDNA sequencing excluding mutations in the *COL6* genes in several Ullrich CMD patients with classic clinical phenotypes (including reduced collagen VI immunolabeling in muscle) strongly suggest that mutations in other genes may underlie at least some cases of Ullrich CMD (269).

**125.7.5.5 Genetic Counseling.** Genetic counseling in Ullrich CMD is complicated by the possibility of recessive or *de novo* dominant mutations in Ullrich CMD, the clinical overlap with BM, and the difficulties in mutation analysis of the large *COL6* genes.

The sibling recurrence risk for recessive cases of Ullrich CMD is 25%. The recurrence risk in families in which the index case has a *de novo* dominant mutation will be low and will relate to the (still theoretical) possibility of gonadal mosaicism. Cases with mild Ullrich CMD overlap clinically and pathologically with severe BM cases. As BM is generally, but not always, dominantly inherited and shows variable expression, a parent may be minimally affected, and in this situation, there would be 50% sibling recurrence risk. Careful examination of parents and consideration of muscle biopsy or MRI can be helpful in this regard, but again, a definitive resolution may require molecular genetic testing.

Antenatal diagnosis can be performed by linkage analysis or direct mutation analysis, where there is a molecular diagnosis in the proband. Collagen VI is expressed in the placenta, and its deficiency can be detected by immunolabeling in chorionic villous samples (281). Antenatal diagnosis was reported in a family in which reduced collagen VI immunolabeling was documented in the proband, in combination with molecular testing. Antenatal diagnosis using collagen VI immunolabeling would be possible in families in which the proband has a definitive reduction in collagen VI in muscle. Careful consideration would be recommended before offering this approach without accompanying molecular analysis.

**125.7.5.6 Integrin Alpha 9.** A number of cases with Ullrich CMD-like phenotypes, but with normal collagen VI in muscle and absence of collagen VI subunit mutations have been reported (269,279), suggesting that other disorders may share clinical signs with Ullrich CMD. Mutations in the integrin  $\alpha$ -9 gene have been found in 14 cases from 11 French Canadian families with congenital hypotonia, weakness, contractures, distal joint hyperlaxity, scoliosis, normal intelligence, and delayed motor milestones but with normal collagen 6 on muscle biopsies and no mutations in the *COL6* genes. At variance with Ullrich CMD, they all acquire the ability to walk and do not develop respiratory failure. A genome-wide scan established linkage to a region on chromosome 3p23–21 (282). In the reported cases haplotype analysis defined a 1.6-cM candidate interval, suggesting that two common mutations accounted for 78% of carrier chromosomes and more recently mutations in the *ITGA9* were also reported (283).

**125.7.5.7 Integrin Alpha 7.** Deficiency of integrin  $\alpha 7$ , a basal lamina muscle-specific laminin receptor, has so far been reported in three male children. All three patients had delayed motor milestones and two of the three achieved independent ambulation while the third one had a more severe phenotype. A recent paper reported the progression of clinical signs in these patients with progressive weakness, scoliosis, and respiratory impairment, requiring noninvasive ventilation at the age of 12 years (284).

### 125.7.6 Congenital Muscular Dystrophy with *SEPN1* Mutations and Rigidity of the Spine

This form, characterized by rigid spine, early respiratory failure, and slowly progressive weakness, was first described as a subgroup of CMD. Following the identification of the gene on chromosome 1p35–36, the phenotype has been more clearly defined and expanded, and it has become obvious that this disorder is relatively frequent (285).

**125.7.6.1 Clinical Features.** Mild hypotonia and weakness may be present in the first months of life, but developmental milestones are often normal and patients with CMD with rigidity of the spine develop Achilles tendon contractures and rigid spine in the first years. On examination, there is weakness of the axial muscles and, to a lesser extent, the proximal muscles. Muscle weakness does not increase significantly with age, and patients affected by this form rarely lose the ability to walk independently although their motor abilities may decrease because of the tendency to develop progressive contractures. The spine becomes increasingly rigid with time, and the condition often develops into progressive and severe scoliosis. Progressive respiratory involvement is typically observed in the first decade, and respiratory failure requiring nocturnal ventilatory support is invariable in the second decade of life, when patients are often still ambulant. Serum CK is either normal or minimally elevated. Muscle MRI shows a typical pattern that helps in the differential diagnosis with the other forms of CMD with rigid spine (286).

**125.7.6.2 Muscle Pathology.** Muscle biopsy changes are variable, ranging from minimal myopathic changes to dystrophic (285). There are no specific antibodies to detect possible deficits of selenoprotein N, and immunocytochemistry does not provide additional information.

**125.7.6.3 Molecular Genetics.** CMD with rigidity of the spine results from recessive mutations in the *SEPN1* gene on chromosome 1p36, which encodes an endoplasmic reticulum protein, selenoprotein N (287), a member of the selenoprotein family, so called because the proteins incorporate selenium in the form of selenocysteine. A role for selenium in muscle was suggested a long time ago by the association of selenium deficiency and skeletal and cardiac muscle disease in livestock and the more

recent observation that selenium deficiency in humans is associated with cardiomyopathy. The pathogenesis of this form of CMD remains unclear.

Mutations in *SEPN1* were initially described in families with CMD with rigidity of the spine (287) but have since been identified in another two groups of patients: those with classic multimini core disease [2890] and those with desmin-related myopathy with Mallory body-like inclusions (288). Despite striking overlap in their clinical phenotypes, these patients had previously been considered to have separate disorders on the basis of distinctive pathologic abnormalities demonstrated on muscle biopsy. Now, it is apparent that different histologic changes can coexist in the same patient, if different muscles are examined (285).

The mutations found in *SEPN1*-related myopathies are scattered throughout the gene and can be nonsense, missense, or frameshifting. Mutations in exon 1, encoding a region implicated in ER localization of *SEPN1*, tend to result in more severe phenotypes, but there are no other genotype–phenotype correlations (285). The same deletion has been described in two patients with different pathologic abnormalities. One case was homozygous for the deletion and had typical Mallory bodies, whereas the other was a compound heterozygote, with the deletion on one allele and a second mutation on the other, and did not have Mallory bodies (288).

CMD with rigidity of the spine is also genetically heterogeneous. At least one third of patients with an indistinguishable clinical phenotype do not harbor mutations in *SEPN1* (285). Recently a form with axial muscular dystrophy and other features similar to those found associated with *SEPN1* mutations have been found in patients with a multisystem disorder and mutations in the selenocysteine (Sec) insertion sequence-binding protein 2 (also known as SBP2). These individuals have reduced synthesis of most of the 25 known human selenoproteins, resulting in a more complex phenotype including azoospermia, photosensitivity, and impaired T lymphocyte proliferation and abnormal mononuclear cell cytokine secretion, highlighting the role of selenoproteins in different biological processes (289).

### 125.7.7 Dystroglycanopathies

The term “dystroglycanopathies” has been used to describe a genetically heterogeneous group of muscle disorders, in which the diagnosis is based on the detection of hypoglycosylated  $\alpha$ -dystroglycan by immunolabeling and/or on Western blot on muscle biopsy. The spectrum of dystroglycanopathies ranges from CMD forms with structural brain changes and often eye abnormalities and mental retardation to mild cases of LGMDs with late onset and no brain or eye involvement. In this chapter, we will only report the cases with early onset labeled as CMD.

**125.7.7.1 Pathogenesis.** The dystroglycanopathies result from abnormal glycosylation of the sarcolemmal protein  $\alpha$ -dystroglycan, owing to mutations in genes encoding known or putative glycosyltransferase enzymes (199,201).  $\alpha$ -Dystroglycan is a peripheral membrane glycoprotein that associates tightly with transmembranous  $\beta$ -dystroglycan (290,291). Glycosylated epitopes on  $\alpha$ -dystroglycan bind extracellular matrix ligands, including laminin, perlecan, agrin, and the neuron-specific cell surface proteins neuexins. The cytoplasmic tail of  $\alpha$ -dystroglycan interacts with dystrophin, or its homolog utrophin, which, in turn, binds cytoskeletal actin, thus linking the extracellular matrix to the actin-associated cytoskeleton.

In the dystroglycanopathies, mutations in glycosyltransferase genes are known or proposed to result in reduced activity of the corresponding enzymes (292–294). As a result,  $\alpha$ -dystroglycan is hypoglycosylated and has reduced affinity for its ligands (295–301). In skeletal muscle, this is presumed to disrupt the critical linkage between the extracellular matrix and the intracellular cytoskeleton. Hypoglycosylation of  $\alpha$ -dystroglycan is presumed to play a major role in pathogenesis of the brain malformations in dystroglycanopathy patients, although hypoglycosylation of other proteins could also play a part. In the brain, dystroglycan is expressed at the glia limitans (302). The integrity of the glia limitans is necessary for correct organization of the radial glial cells, which are involved in guiding cortical neurons on their inside-out migration from the periventricular regions to the surface of the brain (303–306). Defects in the glia limitans are observed in patients with dystroglycanopathy and allow migration of postmitotic neurons and glial cells through the pia into the subarachnoid space, giving rise to the cobblestone appearance of the brain surface (300).

**125.7.7.2 Clinical Features.** Dystroglycanopathies are a very complex group of disorders, and while distinct phenotypes had been originally thought to be associated with the involvement of individual genes, the same phenotype has subsequently been reported in association with many of the known genes. Conversely, mutations in individual genes have been associated with different phenotypes. The CMD forms share a considerable clinical, pathologic, and imaging overlap (201,285,306,307), and the involvement of the CNS plays an important role in defining the different phenotypes.

The classification of these forms has changed over the years in order to take into account the heterogeneity of both of phenotypes and genetic defects. While the early studies following the identification of the first genes involved mainly focused on distinct known phenotypes, namely MEB, WW, and Fukuyama, or other phenotypes with normal brain MRI scans, more recent studies have reported that the spectrum of brain lesions also includes a significant number of posterior fossa lesions, such as cerebellar dysplasia or hypoplasia (307,308). A simplified clinical classification system was suggested in a

paper reporting a large series of all dystroglycanopathies, from the CMD, i.e. those with early onset discussed in this chapter, to the LGMDs that have later onset and often a milder phenotype than CMD (306). The CMD spectrum included five forms: WWS, MEB, CMD with cerebellar involvement (CMD CRB), CMD with normal MRI and mental retardation (CMD MR), and CMD with normal MRI and no mental retardation (CMD no MR). A simplified version of this nomenclature has been recently suggested following new OMIM entries. The group of muscular dystrophies with deficit of dystroglycan (MDDG) has been first subdivided into three broad phenotypic groups denoted types A, B and C, each including some of the seven categories proposed by Godfrey et al. (307).

- Type A represents the phenotypes with cortical involvement and the most severe phenotypes: WWS, WWS-like, MEB and Fukuyama congenital muscular dystrophy (FCMD)-like;
- Type B represents cases of CMD with posterior fossa abnormalities or normal MRI: CMD CRB, CMD MR, and CMD no MR,
- Type C represents both LGMD with MR and LGMD with no MR. These are rare causes of LGMD to be considered in patients with reduction of  $\alpha$ -dystroglycan and no mutations in *FKRP*.

#### **125.7.7.2.1 Phenotypes with Cortical Involvement.**

**125.7.7.2.1.1 Fukuyama Congenital Muscular Dystrophy.** FCMD is characterized by muscular dystrophy with mental retardation. It is the second most common form of childhood muscular dystrophy in Japan after DMD, with an incidence of seven to 12/100,000. Onset is extremely early, and many infants present with poor sucking during the neonatal period. Muscle weakness is generalized and involves the facial muscles, giving a typical appearance. Motor milestones are markedly delayed, but most achieve the ability to sit unaided and to crawl on the knees, with the maximal motor ability acquired between the ages of 2 and 8 years. A few patients achieve independent ambulation, but most are unable to walk. With time, however, motor function gradually deteriorates, and contractures become more evident. By the age of 10 years, most of these children become immobile, and the majority dies by their late teens. All cases of FCMD have a high CK, even in the early stages of the disease that tends to decline with age.

##### **125.7.7.2.1.1.1 Clinical Features.**

- CNS involvement

Intellectual retardation is usually severe, with more than one half unable to speak. However, the intelligence quotient varies from 20 to 90 so that, in some cases, intelligence is within the normal range. The patients with better mental function tend to be those with the least motor

disability. There is also a high association with seizures. MRI studies demonstrate abnormal neuronal migration during development, resulting in a distorted cerebral gyral pattern with areas of polymicrogyria, macrogyria, and agyria. White matter changes are also frequent, but sequential MRI studies have demonstrated that the abnormal signal in the white matter is most likely to be due to delayed myelination rather to dysmyelination, as it improves with age.

– Eye involvement

Eye abnormalities occur in approximately 60 to 70% of these patients and are rarely severe, with myopia being the most frequent abnormality. More severe ocular changes, such as optic nerve atrophy, cataract, and retinal detachment, have been reported but are rare.

**125.7.7.2.1.1.2 Muscle Pathology.** The muscle usually shows a marked dystrophic picture. Muscle immunocytochemistry reveals a severe depletion of  $\alpha$ -dystroglycan and a secondary reduction of merosin expression (297).

**125.7.7.2.1.1.3 Molecular Genetics.** FCMD results from recessive mutations in the 10 exon *FCMD* gene, which is located on chromosome 9q13 and encodes a putative glycosyltransferase, Fukutin. There is a founder mutation in the Japanese population, shared by more than 80% of FCMD chromosomes. This retrotransposal insertion of novel tandemly repeated sequences within the 3' untranslated region causes a profound reduction in mRNA levels in patient lymphoblasts, perhaps by altering mRNA stability. Most patients who are homozygous for the founder mutation are mildly or typically affected and have very low levels of Fukutin mRNA. Most compound heterozygotes for the founder mutation and a second missense or nonsense mutation are severely affected and more likely to have severe WWS-like manifestations such as hydrocephalus and microphthalmia. These patients have more moderately reduced mRNA levels. It is suggested that the founder mutation causes reduced levels of a normal Fukutin protein, whereas the other mutations have more severe effects by resulting in structurally abnormal Fukutin.

Two non-Japanese patients with novel, homozygous, nonfounder mutations in *FCMD* have been described (13–314). Both patients had severe phenotypes, indistinguishable from WWS. The mutations in both cases were truncating mutations that would be predicted to lead to complete loss of Fukutin activity, in contrast to mutations causing an FCMD phenotype, which are predicted to result in some residual enzyme activity. This expands the spectrum of phenotypes resulting from FCMD mutations and highlights the need to consider FCMD mutations in non-Japanese patients.

**125.7.7.2.1.2 MEB Disease.** The vast majority of reported patients come from Finland, although there have been reports of affected individuals from other

regions (314). Clinical manifestations include severe mental retardation, muscle weakness, and poor vision.

**125.7.7.2.1.2.1 Clinical Features.**

– Muscle involvement

Most patients with MEB have symptoms by 2 months of age, including severe hypotonia, sucking difficulties, and failure to thrive. Children often have a similar facial appearance, with a large head, prominent forehead, and wide fontanelle. The midface is flat, and the nose and philtrum are short. Patients are severely affected both mentally and physically, but a spectrum of severity is also found. They have a generalized weakness, involving the limbs and trunk. Motor development is extremely delayed, but a small minority eventually achieves ambulation with or without support. Over time, however, motor ability deteriorates, with the lower limbs becoming spastic, with increased reflexes and marked contractures. Overall, the disease appears to be progressive, with patients gradually losing their few acquired skills with time. Survival is variable, but many patients lived into adulthood, with some surviving into the 40s and 50s. The CK is usually grossly elevated but can be normal during the first year of life, with the highest values recorded between the ages of 5 and 15 years of age. With time, the CK levels appear to fall, with no abnormal levels found in patients older than 40 years of age.

– CNS involvement

All patients are mentally retarded, most of them severely, although some achieve limited speech. Epilepsy is a common associated feature, with the majority having generalized seizures, which are usually infrequent and associated with fever. Some patients had frequent seizures in the early childhood, with significant fits in the second decade. The electroencephalogram has always been found to be abnormal after 1 year of age. Hydrocephalus is common, and some patients require shunting. Although there is variability between patients, all basically exhibit features of cobblestone lissencephaly with pachygyria over the frontal, temporal, and parietal regions, and polymicrogyria over the occipital region. Enlarged ventricles, brainstem hypoplasia, and cerebellar hypoplasia are also present in most cases. Patchy white matter changes are occasionally seen, but in contrast to patients with WWS, the patches are not present in every case, nor are they as severe.

– Eye involvement

Ocular involvement is a characteristic feature MEB. The most common findings are severe myopia and retinal hypoplasia. Other abnormalities include congenital and infantile glaucoma, nystagmus, and cataract. Electrophysiologic studies in these patients are extremely interesting. In most cases, the electroretinogram is abnormally low, and the visual-evoked potentials abnormally



high. The combination of low electroretinogram and high visual-evoked potentials is exceptional and a striking feature of this disease.

**125.7.7.2.1.2.2 Muscle Pathology.** The muscle biopsy has a dystrophic picture, with variation in fiber size and an increase in connective tissue and adipose tissue, a reduction of merosin expression, and a severe depletion of  $\alpha$ -dystroglycan.

**125.7.7.2.1.2.3 Molecular Genetics.** MEB is autosomal recessively inherited. Mutations in the 22 exon *POMGnT1* gene, located on chromosome 1q32–34, have been identified in the classic Finnish MEB cases and in a proportion of patients with MEB from other geographical regions (294).

*POMGnT1* encodes the Golgi-resident glycosyltransferase, protein O-linked mannosyl  $\alpha$  1,2-*N*-acetylglucosaminyl transferase 1. *POMGnT1* transfers *N*-acetylglucosamine from UDP-GlcNAc to O-mannosyl glycoproteins. It is implicated in the second step in synthesis of a common tetrasaccharide structure on  $\alpha$ -dystroglycan (Sia  $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc $\beta$ 1-2Man-Ser/Thr) that has been directly implicated in laminin binding.

There is a founder mutation in the Finnish population, specifically a splice-site mutation in intron 17, which is present on 99% of Finnish MEB chromosomes, as well as in patients from other countries who may share a common ancestor (315). Other MEB-causing mutations are scattered through the *POMGnT1* gene. They are mostly either mutations predicted to result in a truncated protein or missense mutations within the catalytic domain (294,315). One study suggested that mutations at the 5' terminus of the *MEB* gene resulted in more severe brain abnormalities than mutations at the 3' terminus (316). However, this correlation was not supported by a subsequent study (315). Those mutations studied, to date, all virtually abolish *POMGnT1* enzyme activity, and this can be assayed in extracts from muscle biopsy samples (293,294,317).

MEB phenotypes have also been reported with mutations in *POMT1* and *POMT2*, *FKRP*, and *LARGE*. These cases were clinically indistinguishable from patients with *POMGnT1* mutations.

*POMT1* encodes an endoplasmic reticulum-resident glycosyltransferase, protein O-mannosyltransferase 1 (295,318). *POMT1* is proposed to catalyze transfer of the initial mannose residue to the tetrasaccharide structure, Sia  $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc $\beta$ 1-2Man-Ser/Thr, on  $\alpha$ -dystroglycan. The *POMT2* gene is homologous to *POMT1* and encodes another mannosyltransferase, *POMT2*. Neither *POMT1* nor *POMT2* is active when expressed alone, suggesting that formation of an active enzyme complex is required for glycosyltransferase activity (318). *FKRP* (Fukutin-related protein), a small gene with a single-coding exon was identified on the basis of its sequence homology with FCMD and, like FCMD, encodes a putative glycosyltransferase whose mode of action is not yet known.

**125.7.7.2.1.3 Walker–Warburg Syndrome.** WWS is a rare form of CMD, presenting with the triad of eye and brain malformations and muscular dystrophy. It has been reported in patients from many different nationalities and races.

**125.7.7.2.1.3.1 Clinical Features.** WWS has the most severe phenotype of the MEB syndromes, and the condition is usually associated with early death.

#### – Muscle involvement

In pregnancy, mothers usually notice decreased fetal movements, often complicated by polyhydramnios. As a result of marked hydrocephalus causing cephalopelvic disproportion, delivery is often by caesarean section. Commonly, resuscitation is required at birth, and a significant majority of them are still born or die in the perinatal period. On reviewing the survival data, median survival time for all liveborn infants was 18 weeks, although about 5–10% survived more than 5 years. The longer survivors usually were less severely mentally retarded. Of the survivors, most have profound mental and motor retardation, but as with the other syndromes, there is a spectrum of severity, and some of the less affected eventually acquire the ability to sit and roll. The CK level is usually elevated but actual values can vary tremendously between patients and in individual patients over time. Patients with normal values have been recorded, but they were either infants or children older than 10 years.

#### – CNS involvement

As suggested by the extremely severe phenotype, full expression of cobblestone lissencephaly is usually observed in this syndrome. Most affected children have severe gyral changes, with agyria and additional areas of macrogyria and polymicrogyria. The leptomeninges are thick and can obliterate the interhemispheric fissure and subarachnoid space. The cortex is abnormally thick; frequently, the corpus callosum and septum pellucidum are absent or hypoplastic. Microscopically, the cortex is severely disrupted with no recognizable neuronal layers. Unlike the other syndromes, the white matter is extensively involved with diffuse changes throughout the cortex. Ventricular dilation, with or without progressive hydrocephalus, is extremely common and, at one time, was a required diagnostic criterion. In addition, some patients have been found to have congenital microcephaly. Cerebellar malformation is seen in all patients. The cerebellum is hypoplastic, particularly the posterior vermis, and the gyral pattern is abnormal. EM shows that the neuronal layers are severely disrupted. In some patients, hypoplasia of the vermis is associated with enlargement of the fourth ventricle, the Dandy–Walker malformation. Seizures are common in these patients and can be difficult to control.

#### – Eye involvement

Patients with WWS show a wide spectrum of eye abnormalities, with abnormal differentiation of the

retina being also described. The spectrum of retinal involvement varies, ranging from severe to mild. Severe abnormalities include microphthalmia, colobomatous malformation, and retinal detachment secondary to retinal dysplasia. Milder changes include abnormal retinal vascularization, absent macular and optic disc hypoplasia. Anterior chamber malformations can also be found in some patients; common abnormalities including corneal clouding and narrowing of the iridocorneal angle, with or without glaucoma and cataract.

**125.7.7.2.1.3.2 Muscle Pathology.** Muscle biopsy is dystrophic, but there have been reports of normal muscle biopsies in affected infants during the first months of life, and it has been suggested that the biopsy becomes more dystrophic with time. Muscle involvement may not always be uniform, and multiple biopsies from individual patients have shown variability in the degree of dystrophic changes. Immunocytochemistry shows a reduction of merosin expression and a severe depletion of  $\alpha$ -dystroglycan (295,319).

**125.7.7.2.1.3.3 Molecular Genetics.** WWS is a highly genetically heterogeneous disorder. Autosomal recessive mutations in four different genes have been identified so far, which result in indistinguishable clinical phenotypes. These genes explain only a minority of cases (138), and genome wide linkage studies predict a minimum of 10 loci (285). The first gene found to cause WWS was *POMT1* (295,318), mutated in up to 20% of WWS cases (295,320). The mutations in *POMT1* reported in WWS are either nonsense mutations, single-base pair insertions, or missense mutations scattered throughout the gene. The nonsense mutations are predicted to encode nonfunctional proteins. The other reported mutations in *POMT1* have been demonstrated to abolish its mannosyltransferase activity, thus, presumably, resulting in hypoglycosylation of  $\alpha$ -dystroglycan (292). WWS phenotype has subsequently been also found in patients with mutations in *POMT2* (306,307,321). Two patients were homozygous for mutations predicted to result in truncated proteins, while the other patient was homozygous for a splice site mutation. WWS phenotype has also been found in association with mutations in the *FCMD* or *FKRP* genes (313,314,322), again highlighting the overlapping phenotypes that result from mutations in glycosyltransferase genes.

#### **125.7.7.2.2 Dystroglycanopathies with Posterior Fossa Abnormalities or Normal MRI.**

**125.7.7.2.2.1 Clinical and Imaging Findings.** A number of patients with CMD and  $\alpha$ -dystroglycan deficiency have been found with posterior fossa abnormalities. The cerebellum is more often involved, in isolation with signs of hypoplasia or dysplasia (cerebellar cysts) or in combination with brainstem and pons abnormalities. Cerebellar involvement is generally associated with mental retardation and often with microcephaly (307,308). Patients with normal brain MRI can also have microcephaly and mental retardation (306), but a proportion of

them will have normal head circumference and no mental retardation. This group was originally described by Brockington et al. (323) and has been labeled as MDC1C according to the old nomenclature. Patients affected by both forms with and without mental retardation generally show hypotonia and feeding difficulties at birth and markedly delayed motor milestones. They usually achieve the ability to sit but do not achieve independent ambulation. On examination, there is a generalized hypertrophy of the lower limb muscles with wasting of the shoulder girdle and a peculiar posture of the arms, with cubital pronation of the forearm and partial flexion of the fingers. Weakness is more marked in the arm than in the legs. Feeding difficulties may persist after the neonatal period, and gastrostomy may be required, especially if aspiration is detected on videofluoroscopy. They often develop respiratory failure and echocardiography evidence of left ventricular dilatation in the second decade of life.

**125.7.7.2.2.2 Molecular Genetics.** Cerebellar cysts are more often found in patients with *FKRP* and *POMGnT1* mutations but have also been reported in association with *POMT1* and *POMT2* mutations. Cerebellar hypoplasia and microcephaly in contrast are generally associated with *POMT1* and *POMT2*. Patients with normal brain MRI but with microcephaly and mental retardation are more often related to mutations. The form with normal brain MRI and no mental retardation generally results from autosomal recessive mutations in *FKRP*.

**125.7.7.3 Dystroglycanopathies: Genetic Overview.** The spectrum of phenotypes associated with mutations in the individual genes responsible for dystroglycanopathies expands beyond the CMD cases, and some of the known genes cover a broad range of phenotypes. *FKRP* is the best example of the wide heterogeneity of phenotypes. Shortly after the initial description of MDC1C, mutations in *FKRP* were found to underlie a common form of LGMD, LGMD2I (197). In MDC1C and LGMD2I, a correlation has been shown between the immunohistochemical reduction in  $\alpha$ -dystroglycan, the mutation, and the clinical phenotype (296). In MDC1C, there is severe depletion of  $\alpha$ -dystroglycan, and patients are either compound heterozygotes for one missense and one nonsense mutation, or homozygous for a missense mutation. Compound heterozygosity for two null alleles has not yet been reported in *FKRP*, suggesting that a total absence of enzyme activity may result in embryonic lethality. Patients with severe LGMD2I, who have a DMD-like course, show a significant, but lesser, depletion of  $\alpha$ -dystroglycan and are compound heterozygotes for a missense mutation, L276I and another, variable, second mutation. Patients at the milder end of the LGMD2I spectrum show a variable but subtler depletion of  $\alpha$ -dystroglycan and are either L276I heterozygotes or, more commonly, homozygotes. *FKRP* mutations have subsequently also been found in patients with cerebellar cysts, MEB, and in WWS patients (295,306,307). Mutations have been described scattered throughout the

coding sequence of *FKRP* although not yet in the transmembrane region (197,202,323,324). Those mutations causing brain involvement are not located in a different *FKRP* domain compared with other reported mutations (295,325). The cerebellar cyst and WWS patients were homozygous for novel, missense mutations. The most likely explanation for the brain involvement in some patients with *FKRP* mutations is that such mutations reduce enzyme activity below a certain threshold that is required for normal brain development. In support of such a hypothesis, the patients with cerebellar cysts and MEB, in whom skeletal muscle was available for study, showed a virtual absence of glycosylated  $\alpha$ -dystroglycan (Voit and Muntoni, personal communication) and references (321,326).

The spectrum of phenotypes resulting from mutations in *FKRP* is the broadest of all the glycosyltransferase genes to date, but *POMT1* and *POMT2* mutations have also been associated with a similar spectrum of phenotypes from WWS and MEB to milder LGMD phenotypes. *POMGnT1* in contrast is more often associated with MEB and cerebellar cysts phenotypes, and so far, less phenotypic heterogeneity has been reported. *LARGE* mutations are much less common and so far have been reported in a case with white matter changes and in WW phenotype.

Although it may be possible to prioritize testing based on clinical phenotype, the phenotypic overlap means that it is necessary to test all known genes in all patients with dystroglycanopathy. It is also important to highlight that approximately 50% of the patients with CMD and  $\alpha$ -dystroglycan deficiency do not show mutations in the known genes, suggesting further genetic heterogeneity.

### 125.7.8 Other Forms of CMD due to Glycosylation Disorders

While until recently it has been assumed that all the forms with muscle involvement were dystroglycanopathies due to O-glycosylation defects, in the last year there has been increasing evidence that muscle may also be involved in patients with N-glycosylation defects, well known to be responsible for congenital disorders of glycosylations. A recent paper has reported a homozygous missense mutation in *DPM3*, in a patient with mild muscular dystrophy, dilated cardiomyopathy and stroke-like episodes with no brain involvement, and a reduction in Dol-P-Man synthase activity on fibroblasts. Mutations in *DPM2* have also been identified in two siblings with muscular dystrophy, severe mental retardation, microcephaly, myoclonic epilepsy, and cerebellar hypoplasia on brain MRI (312,327).

### 125.7.9 Congenital Muscular Dystrophy Type 1B

This autosomal recessive form of muscular dystrophy, presenting with proximal girdle weakness, generalized muscle hypertrophy, rigidity of the spine, and

contractures of the Achilles tendon, with grossly elevated serum CK, was originally described in a consanguineous family from the United Arab Emirates. Early respiratory failure was a feature in these patients. There is secondary merosin deficiency and  $\alpha$ -dystroglycan deficiency in skeletal muscle (201). MDC1B has been mapped to chromosome 1q42.

### 125.7.10 Congenital Muscular Dystrophy Type 1D

Mutations in the 16-exon *LARGE* gene, located on chromosome 22q12.3-13.1, and encoding a putative glycosyltransferase, have been identified in one patient from the United Kingdom (328). This gene was analyzed because it is mutated in the *myd LARGE* mouse, a model of muscular dystrophy in which  $\alpha$ -dystroglycan is abnormally glycosylated (329). The patient was a compound heterozygote; she had a missense mutation affecting a highly conserved residue in a putative catalytic domain, together with a 1-bp insertion, predicted to encode a truncated protein lacking part of a putative catalytic domain. The patient muscle showed milder hypoglycosylation of  $\alpha$ -dystroglycan than the *myd LARGE* mouse. This may be explained by residual *LARGE* activity in the patient, in contrast with the *myd LARGE* mouse, which has a homozygous out-of-frame mutation predicted to encode a nonfunctional protein. Interestingly, the patient had a neuronal migration defect and electroretinographic abnormalities that were also similar to those seen in the *myd LARGE* mouse.

### 125.7.11 Other Dystroglycanopathies

**125.7.11.1 Microcephaly-Normal Structural Brain.** Another form of CMD with mental retardation but normal brain MRI was reported in two siblings who never achieved independent ambulation. Laminin  $\alpha$ -2 and  $\alpha$ -dystroglycan were reduced in the muscle biopsy.

**125.7.11.2 Microcephaly-Pachygyria-Peripheral Neuropathy.** Four siblings in one family have been described with generalized muscular wasting and weakness, calf pseudohypertrophy and joint contractures, microcephaly, and severely delayed psychomotor development. There was pontocerebellar hypoplasia, bilateral opercular abnormalities, and focal cortical dysplasia on brain MRI. One patient showed electrophysiologic evidence of demyelinating peripheral neuropathy. Laminin  $\alpha$ -2 expression was normal but  $\alpha$ -dystroglycan was virtually absent (319).

### 125.7.12 LMNA-Related Congenital Muscular Dystrophy and Infantile Onset Inflammatory Myopathy

*De novo* heterozygous mutations in the *LMNA* gene have recently been described in rare patients with infantile onset, showing a phenotype characterized by severe

muscle weaknesses in the first year of life (330). Most of the patients showed a classic “dropped-head” syndrome phenotype. The weakness appears to be selective, with wasting of the cervicoaxial muscles, associated with rigid spine and proximal involvement in upper limbs and distal in lower. Later onset of proximal limb contractures, ventilatory support, cardiac arrhythmias were also observed in many patients. Muscle appeared dystrophic with inflammatory markers. This new phenotype, now called LMNA-related CMD (L-CMD) and overlaps with the other striated muscle laminopathies, in particular EDMD, because of the humeroperoneal distribution of limb weakness, but the early onset, the head drop, absence of elbow contractures, and rapidly progressive respiratory insufficiency can help to distinguish L-CMD. The specific *LMNA* mutation found in these patients is currently considered as the major cause of the severity of this phenotype, although one of the mutations was also reported in association with a milder phenotype, suggesting, again, the possible involvement of other genetic modifying factors, as for EDMD and LGMD1B. Following this first report, *LMNA* mutations were also found in patients showing inflammatory myopathy with infantile onset, with presence of perivascular cuffing and/or endomysial/perimysial lymphocyte infiltration. Joint contractures and cardiac involvement developed later in life (331). These findings further broaden the phenotypic spectrum of laminopathies.

### 125.7.13 Other Forms of Congenital Muscular Dystrophy

Apart from the forms described, in which the primary genetic defect has been identified, there are a number of forms with distinctive phenotypes in which the underlying genetic or protein defect has not yet been identified:

**125.7.13.1 Congenital Muscular Dystrophy with Cataracts.** This form of muscular dystrophy is characterized by mild mental retardation, bilateral cataracts, and normal cranial magnetic resonance imaging

**125.7.13.2 Congenital Muscular Dystrophy with Short Stature, Mental Retardation, and Distal Laxity.** We recently described a form with distal laxity, early respiratory impairment, and a significant overlap with Ullrich CMD but associated with short stature and mental retardation. Linkage to the collagen VI genes was excluded (328).

### 125.7.14 Making a Diagnosis in Congenital Muscular Dystrophy: Practice and Pitfalls

Reaching a correct diagnosis in a patient with CMD requires a combination of careful history taking, clinical examination, selected investigations, and interpretation of the muscle biopsy. Only then can targeted mutation analysis be employed, to confirm the clinical diagnosis and allow genetic counseling.

A thorough clinical history is essential. Although CMD is defined as having an onset in the first 6 months of life, patients may present at a later age, and parents may not recall mild early signs, unless questioned specifically. A detailed family history should be taken, including questions about consanguinity, neonatal deaths, and similarly affected relatives. It is important to ask about mild symptoms in the parents, because several dominantly inherited disorders are included in the differential diagnosis of CMD. Clinical examination may give clues to a particular subtype. The skin changes and joint hypermobility in Ullrich CMD can lead to milder patients being misdiagnosed with benign hypermobility or Ehlers–Danlos syndrome if a systematic search for joint contractures and mild muscle weakness is not made. Patients with RSMD1 can have minimal limb weakness, and so it is important to assess axial muscle power, which is more affected in these patients.

Investigations should always include serum CK, which is a useful guide to likely differential diagnoses. The CK level allows particular disorders to be excluded; for example, patients with merosin deficiency or a dystroglycanopathy will rarely have a CK below 1000 IU/L. Nerve conduction should be measured because this is usually abnormal in merosin-deficient patients, and an alternative diagnosis should be considered in patients with normal nerve conduction. Brain MRI should be performed if merosin-deficient CMD is suspected, looking for the characteristic white matter changes. MRI should also be considered in suspected dystroglycanopathy patients, who may have structural brain abnormalities that may help direct genetic testing. Similarly, detailed ophthalmologic assessment should be performed in suspected dystroglycanopathy patients. Muscle MRI shows a characteristic pattern of muscle involvement in RSMD1 and Ullrich CMD. MRI may also be helpful when considering other differential diagnoses; for example, patients with some congenital myopathies show a characteristic pattern of muscle involvement.

Muscle histology usually shows dystrophic changes in CMD; however, muscle in RSMD1 may show only myopathic changes. Patients with a congenital myopathy due to mutations in the ryanodine receptor 1 gene (*RYR1*) are not infrequently misdiagnosed as having CMD, particularly Ullrich CMD, because both disorders involve distal laxity. A diagnosis of RSMD1 or *RYR1*-associated myopathy should be considered in patients with corelike areas on muscle biopsy. The presence of frequent internal nuclei is another pointer to an *RYR1*-associated myopathy. Another possible misdiagnosis is in patients with dystroglycanopathies, who can have an inflammatory infiltrate and upregulation of HLA in muscle, and may be wrongly diagnosed with an inflammatory myopathy. Immunolabeling of muscle allows the diagnosis of specific CMD subtypes; however, this analysis is not always straightforward. Immunohistochemical changes may be subtle in partial merosin deficiency, and antibodies to both the 80-kD and 300-kD fragments of



merosin should be used to avoid missing minor abnormalities. It should also be noted that secondary merosin deficiency could occur in the dystroglycanopathies. Although immunolabeling of collagen VI is reduced in Ullrich CMD, this reduction can be mild and difficult to appreciate, and the diagnosis could be missed in such cases.

In conclusion, the diagnosis of CMD requires substantial clinical expertise and specialized histopathologic techniques. Referral to a specialist neuromuscular center is helpful in reaching a diagnosis in difficult cases.

## 125.8 CONCLUSIONS

The muscular dystrophies represent a widely variable and heterogeneous group clinically (with onset from infancy to late adult life), genetically, and at the level of the underlying protein defect. Precise diagnosis can be sought in the majority of cases. Precise diagnosis can be used to help predict prognosis and plan supportive therapies, which are proven to influence longevity and quality of life. Current diagnostic strategies rely on a combination of review of clinical symptomatology, MRI studies, muscle biopsy analysis (especially immunolabeling techniques), and direct mutation testing. Current novel technologies will most likely elucidate new causes for muscular dystrophies, new diagnostic algorithms, and the hope is that they will also provide routes for specific therapies.

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## LIST OF RELEVANT RELEVANT WEB PAGES

- The Treat-NMD neuromuscular network: <http://www.treat-nmd.eu/>.
- Leiden Muscular Dystrophy pages: <http://www.dmd.nl/>.
- The ClinicalTrials.gov website: <http://www.clinicaltrials.gov>.
- The UMD central website: <http://www.umd.be/>.
- The UMD-LMNA mutations database: <http://www.umd.be/LMNA/>.
- The UMD-EMD mutations database: <http://www.umd.be/EMD/>.
- OMIM #613723 OMIM #613723, Muscular Dystrophy, Limb-Girdle, type 2Q; LGMD2Q: <http://omim.org/entry/613723>.

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### Biographies

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# CHAPTER

# 126

## Hereditary Motor and Sensory Neuropathies

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### 126.1 INTRODUCTION

Hereditary motor and sensory neuropathies (HMSNs) are a group of clinically and genetically heterogeneous disorders primarily affecting the PNS. The diagnosis is based on the presence of lower motor neuron signs and evidence of sensory involvement. If lower motor neuron signs are present with normal sensory system, the diagnosis of peripheral motor neuropathy or lower motor neuron disease is likely. Sensory neuropathies in the absence of lower motor neuron signs, and usually associated with autonomic dysfunction are the hereditary sensory and autonomic neuropathies (HSANs), discussed in another chapter. Peripheral neuropathy is frequently part of complex neurologic or multisystemic disorders. In these patients, there is striking evidence of other system involvement, and these disease entities are discussed in other chapters.

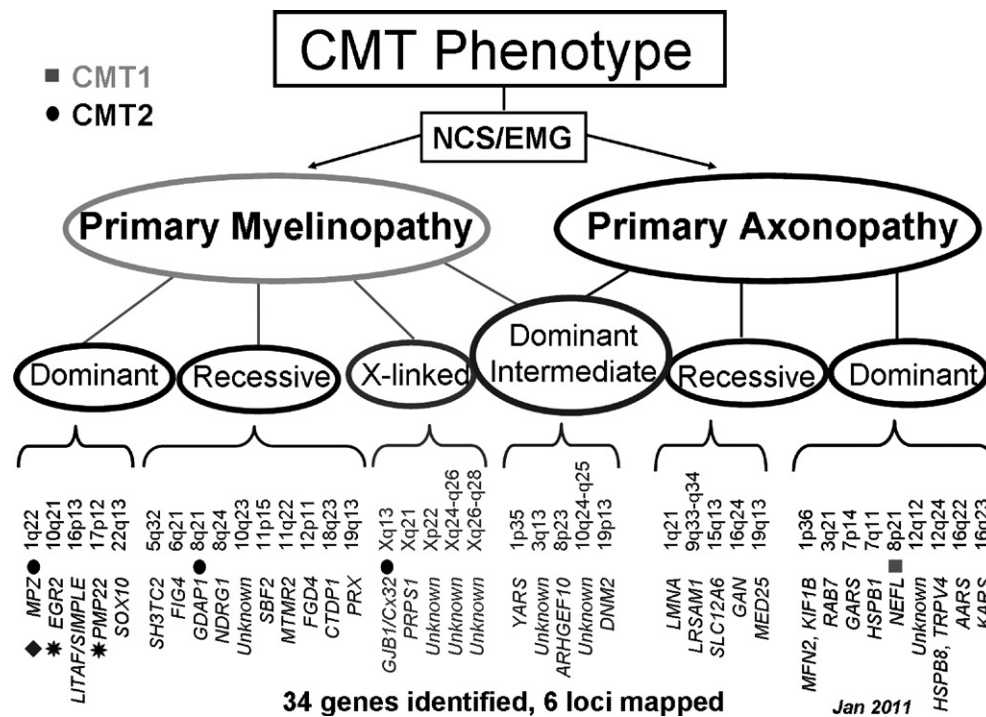
### 126.2 HEREDITARY MOTOR AND SENSORY NEUROPATHIES

HMSNs, or Charcot–Marie–Tooth (CMT) disease and related neuropathies, represent a heterogeneous group of hereditary disorders (1–6) of the PNS with an estimated frequency of 1 in 2500 individuals (7). CMT is characterized by slowly progressive, length-dependent neuropathy, manifesting as distal weakness of the legs progressing proximally, followed, in some cases, by hand involvement. Based on motor nerve conduction velocities (NCVs), two major types can be distinguished—type 1 or the demyelinating (CMT1) form (note: refers to all demyelinating forms independent of inheritance pattern), which is characterized by symmetrically slowed NCV

(usually <38 m/s; normal is >45 m/s), and type 2 or the axonal (CMT2) (all axonal independent of inheritance pattern) form, associated with normal or subnormal NCVs and reduced compound muscle action potential (8). Pathologic studies of patients with myelinopathies show segmental demyelination and remyelination, that is, the so-called onion bulb formation (9), whereas studies of patients with axonopathies show normal myelin but fewer nerve fibers. Both CMT1 and CMT2 can be inherited as either an autosomal dominant (AD), autosomal recessive (AR), or X-linked (XL) trait, but often present as sporadic neuropathy (Figure 126-1).

The disease spectrum of both CMT1 and CMT2 is a continuum, often in the same family extending from severe infantile-onset disease to mild adult-onset disease (2). Traditional clinical classifications also include Dejerine–Sottas neuropathy (DSN) and congenital hypomyelinating neuropathy (CHN) (10–14). By definition, the diagnosis of DSN requires the presence of developmental delay, indicating very early onset. The clinical definition of CHN overlaps with DSN, the terminology indicating congenital onset. However, symptoms are frequently recognized as developmental delay in this group of patients also. The distinction between DSN and CHN thus requires a pathologic diagnosis, the presence of onion bulbs in DSN indicating episodes of demyelination and remyelination (12,14–16).

During the previous two decades, an enormous amount of information regarding peripheral nerve function and dysfunction has been obtained through the identification of genes responsible for disease in patients, manifesting inherited peripheral neuropathies, generating a complex



**FIGURE 126-1** Genetics of CMT neuropathy. This figure depicts the delineation of the CMT phenotype into primarily myelin versus axon involvement, the modes of inheritance that can be observed, and the 40 different linked genetic loci. For 34 of these loci, specific genes have been identified and are shown below. Note that, at some of the loci, either dominant or recessive inheritance may be observed depending on the specific mutation. Also, mutations in *GJB1*, encoding the gap junction protein connexin-32, can present as either an axonal or demyelinating neuropathy, or have features of both. In other genes, specific mutant alleles can cause either a CMT1 or CMT2 phenotype.

classification based on the locus or gene involved (Table 126-1). In some instances, mutations in the same genes can cause both demyelinating and axonal neuropathy; the same mutations can have variable disease onset even in the same family, and the inheritance pattern can vary whether the same gene has a dominant negative or a loss of function mutation (17). Thus, this classification will need revision in the future. In this text, CMT1 and CMT2 refer to demyelinating and axonal CMT, respectively, independent of inheritance pattern. Some of these genes or mutations contribute to a significant fraction of inherited peripheral neuropathy cases and, thus, molecular analysis can play a substantial role in establishing a precise and accurate etiological diagnosis, whereas other genes may be involved in only a small minority of patients. This chapter expands upon and updates our previous one (1).

### 126.3 DISEASES PHENOTYPES

#### 126.3.1 Charcot–Marie–Tooth Disease (MIM 118200, 118220)

Clinical symptoms most frequently appear in the first or second decade of life, including tripping, followed by difficulties with heel walking. Weakness of the peroneal muscles impairs foot dorsiflexion and eversion during gait, for which patients compensate by flexing the hip and knee with each step, producing the steppage gait. To achieve better foot stroke patients flex their toes, which with time

becomes rigid (hammer toes). Foot deformities such as pes cavus and heel varus occur late. In most cases, weakness and wasting of the intrinsic hand muscles occurs late in the course of the disease but is not usually related to the degree of leg weakness or atrophy and is also not related to the age of the patient. The thumb lies flat in the plane of the hand instead of opposing the other fingers, giving the appearance of claw hand. Decreased or absent ankle reflexes are virtually universal, and most patients are areflexic. Mild sensory loss can be detected in approximately 70% of the cases. Patients rarely lose the ability to ambulate. Slow NCVs, as seen in the demyelinating form or CMT1, differentiate this form from the axonal or neuronal type or CMT2, in which the NCVs are normal or slightly slow, with reduced amplitudes. Approximately 60–70% of patients have CMT1, whereas about 20–40% are diagnosed with CMT2. Restless leg syndrome occurs in nearly 40% of CMT2 patients (18) but is rare in CMT1 cases. Pathologic studies of patients with myelinopathies show segmental demyelination and myelin sheath hypertrophy, that is, the so-called onion bulb formation, whereas studies of patients with axonopathies show normal myelin but fewer nerve fibers (9,19).

#### 126.3.2 Hereditary Neuropathy with Liability to Pressure Palsies (MIM 162500)

The clinical phenotype of hereditary neuropathy with liability to pressure palsies (HNPP) is characterized by

**TABLE 126-1 Genetic Classification of Charcot–Marie–Tooth Disease and Related Peripheral Neuropathies**

CMT	Locus	Gene	Product	OMIM
CMT1A	17p11.2	<i>PMP22</i>	Peripheral myelin protein 22	118220
CMT1B	1q22	<i>MPZ</i>	Myelin protein zero	118200
CMT1C	16p13.1-p12.3	<i>LITAF</i>	Lipopolysaccharide-induced TNF factor	601098
CMT1D	10q21.1-q22.1	<i>EGR2</i>	Early growth response protein 2	607678
CMT1E	17p11.2	<i>PMP22</i>	Peripheral myelin protein 22	118300
CMT1F	8p21	<i>NEFL</i>	Neurofilament triplet L protein	607734
CMT2A	1p36	<i>MFN2</i>	Mitofusin 2	118210
CMT2B	3q21	<i>RAB7</i>	RAS-related protein RAB-7	600882
CMT2B1	1q21.2	<i>LMNA</i>	Lamin A/C	605588
CMT2B2	19q13.3	<i>MED25</i>	Mediator complex subunit 25	605589
CMT2C	12q24	<i>TRPV4</i>	Transient receptor potential cation channel V	606071
CMT2D	7p15	<i>GARS</i>	Glycyl-tRNA synthetase	601472
CMT2E	8p21	<i>NEFL</i>	Neurofilament triplet L protein	607684
CMT2F	7q11.23	<i>HSPB1</i>	Heat-shock protein B 1	606595
CMT2G	12q12-q13	Unknown	Unknown	608591
CMT2H	8q21.11	<i>GDAP1</i>	Ganglioside-induced differentiation protein 1	607731
CMT2I	1q23.3	<i>MPZ</i>	Myelin protein zero	607677
CMT2J	1q23.3	<i>MPZ</i>	Myelin protein zero	607736
CMT2K	8q21.11	<i>GDAP1</i>	Ganglioside-induced differentiation protein 1	607831
CMT2L	12q24	<i>HSPB8</i>	Heat-shock 22 kDa protein 8	608673
CMT2N	16q22	<i>AARS</i>	Alanyl-tRNA synthetase	613287
CMT2O	14q32.31	<i>DYNC1H1</i>	Dynein, cytoplasmic 1, heavy chain 1	614228
CMT4A	8q13-q21.1	<i>GDAP1</i>	Ganglioside-induced differentiation protein 1	214400
CMT4B1	11q22	<i>MTMR2</i>	Myotubularin-related protein 2	601382
CMT4B2	11p15	<i>SBF2/MTMR13</i>	SET binding factor 2	604563
CMT4C	5q32	<i>SH3TC2</i>	SH3 domain and tetratricopeptide repeat domain 2	601696
CMT4D	8q24.3	<i>NDRG1</i>	NDRG1 protein	601455
CMT4E	10q21.1-q22.1	<i>EGR2</i>	Early growth response protein	607678
CMT4F	19q13.1-q13.2	<i>PRX</i>	Periaxin	145900
CMT4G	10q23	Unknown	Unknown	605285
CMT4H	12p11.21	<i>FGD4</i>	RhoG and PH domain-containing protein	611104
CMT4J	6q21	<i>FIG4</i>	Homolog of <i>S. cerevisiae</i> FIG4	611228
DI-CMTA	10q24.1q25.1	Unknown	Unknown	606483
DI-CMTB1	19p12-13.2	<i>DNM2</i>	Dyamin 2	606482
DI-CMTB2	16q22.2-q22.3	<i>KARS</i>	Lysyl-tRNA synthetase	613641
DI-CMTC	1p34	<i>YARS</i>	Tyrosyl-tRNA synthetase	608323
DI-CMTD	1q22	<i>MPZ</i>	Myelin protein zero	607791
CMTX1	Xq13.1	<i>GJB1</i>	Gap junction beta-1 protein, Connexin 32	302800
CMTX2	Xp22.2	Unknown	Unknown	302801
CMTX3	Xq26	Unknown	Unknown	302802
CMTX4	Xq24-q26.1	<i>NAMSD</i>	Neuropathy, axonal, motor sensory, deafness	310490
CMTX5	Xq21.32-q24	<i>PRPS1</i>	Phosphoribosyl pyrophosphate synthetase 1	311070

recurrent episodes of nerve palsies at compression sites (20,21). The symptoms are transient numbness or weakness lasting hours to weeks. Approximately 64–78% of patients present with acute painless mononeuropathy or brachial plexopathy (22). After multiple attacks, the recovery may be incomplete owing to permanent nerve damage. The repeated demyelination and remyelination result in sausage-like thickening of the myelin sheath (tomacula) (23). The neurologic impairment is usually milder than in CMT1 but can become similar after the fifth decade. Electrophysiologic findings include conduction blocks and mildly slow motor conduction velocities during exacerbations (23).

### 126.3.3 Dejerine–Sottas Neuropathy (MIM 145900)

DSN was originally described as an interstitial hypertrophic neuropathy of infancy. The patients present with motor developmental delay, hypotonia, and areflexia (24,25). Many patients walk eventually, although walking is often delayed beyond the second year. Probably because of proprioceptive deficits, all patients have ataxia and abnormal coordination. Virtually all patients are areflexic. Beginning with the distal musculature of the lower extremities, patients develop progressive muscular atrophy and weakness. By the second decade of life, nearly all



patients have developed proximal weakness in addition to distal weakness and moderate to severe sensory deficits. Although some deteriorate over the first two decades of life and may eventually die from respiratory complications, others have a relatively mild disease course except for progression of limb deformities and scoliosis (25,26). NCVs show marked slowing (<6 to 12 m/s) in a uniform pattern affecting all nerves and nerve segments (24,25). The hypertrophic superficial nerves may be visible and palpable. Patients with DSN have the neuropathologic features of both de/remyelination (onion bulb) and hypomyelination (thin myelin sheets) (15,25).

### 126.3.4 Congenital Hypomyelinating Neuropathy (MIM 605253)

CHN is the most severe form of the HMSNs. The clinical features are present at birth with marked hypotonia, areflexia, and distal muscle weakness (10–14). In some cases, decreased fetal movements and arthrogryposis multiplex congenita have been described (10,14). The NCVs are usually less than 6 to 12 m/s. Clinical distinction between CHN and DSN is often difficult, because frequently the first measurable clinical sign is motor development delay. The diagnosis of CHN is established by nerve biopsy showing hypomyelination, with only a few thin myelin lamellas left without active myelin breakdown products and early onion bulb formations.

### 126.3.5 Roussy–Lévy Syndrome (RLS, MIM 180800)

Roussy–Lévy syndrome (RLS) combines the CMT1 phenotype with marked tremor and sensory ataxia (6,27). Symptoms include early pes cavus, distal leg muscle weakness and atrophy, distal sensory loss, and gait ataxia. As the full phenotypic spectrum of CMT has been defined, however, tremor and sensory ataxia have been clearly identified as common although variable features of CMT (28,29). Therefore, although initially considered a *forme fruste* of Friedreich ataxia, RLS is a CMT variant. Genetic testing confirms this clinical impression. The original family described by Roussy and Lévy segregates an *MPZ* mutation (30), and RLS has also been associated with the *PMP22* duplication and point mutations in *GJB1* (31–33). RLS has never been associated with mutations in *FRDA*, the gene associated with Friedreich ataxia.

## 126.4 DISTAL SYMMETRIC POLYNEUROPATHY—EVIDENCE-BASED MEDICAL GUIDELINES

In 2009, the American Academy of Neurology, American Association of Neuromuscular and Electrodiagnostic Medicine and American Academy of Physical Medicine and Rehabilitation issued a joint practice parameter that provided evidence based recommendations for the role of

laboratory and genetic studies in the process of evaluation of distal symmetric polyneuropathy (DSP). The panel of specialists reviewed available literature devoted to evaluation of polyneuropathy published during the years 1980–2007 and recommended genetic testing to be considered in patients with DSP or an hereditary neuropathy phenotype. According to the guidelines, clinical presentation, mode of inheritance, and electrophysiological findings should be used to guide molecular testing (34,35).

## 126.5 MODES OF INHERITANCE

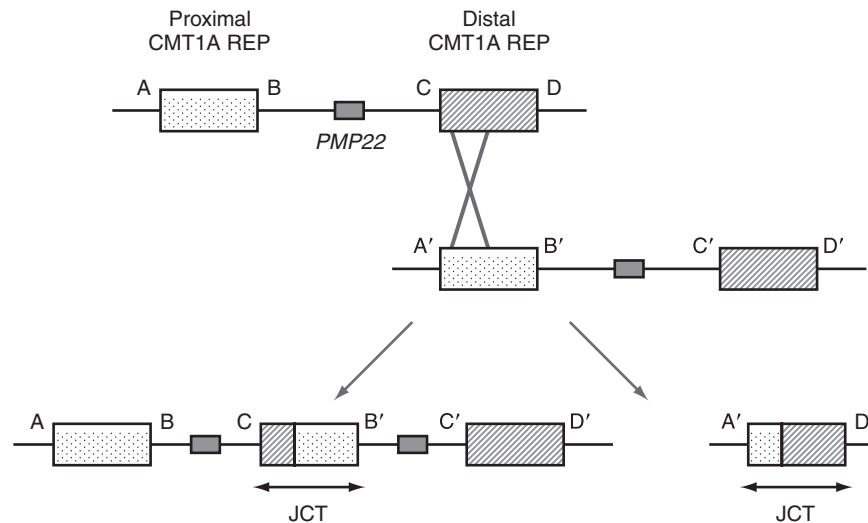
CMT and related neuropathies exhibit all forms of Mendelian inheritance—AD, AR, and XL. AD-CMT1 is the most frequent pattern observed (2). Forty linked loci (20 AD, 15 AR, and 5 XL; although rarely some mutations in a gene isolated as a dominant locus may behave as a recessive allele in a given family) and 34 CMT-associated genes have been identified (see Figure 126-1 and Table 126-1). HNPP and RLS show AD inheritance, whereas CHN is AR or sporadic. DSN shows both AD and AR forms. Sporadic disease is often a result of new mutation, and thus, the absence of a family history does not preclude molecular genetic testing.

## 126.6 GENETICS

Mutations in genes encoding proteins required for proper development, maintenance, or function of the peripheral nerve may result in neuropathy. Several of the disease genes initially identified encode (i) structural proteins that are important in myelination (e.g. *PMP22*, *MPZ*), (ii) proteins involved with radial transport through the myelin membrane sheath (e.g. *Cx32*), (iii) proteins involved with axonal transport (e.g. *NEFL*, *GAN1*), (iv) transcription factors associated with onset of myelination (*EGR2*, *SOX10*), (v) members of signal transduction pathways (e.g. *PRX*, *MTMR2*, *SBF2*, *NDGR1*), (vi) mitochondrial function-related proteins (e.g. *MFN2*, *GDAP1*), (vii) endosome-related proteins/endocytic recycling (*RAB7*, *SH3TC2*, *FIG4*, *SIMPLE*) and (viii) chaperones (*HSP22*, *HSP27*), (ix) a gene involved in DNA single-strand-break repair (*TDP1*), (x) protein synthesis (*GARS*, *KARS*, *AARS*, *HARS*, *YARS*), and (xi) other genes (e.g. *LMNA*, *KCC3*, *DNM2*) whose peripheral nerve specific functions are less clearly established.

### 126.6.1 Genes Associated with Peripheral Nerve Structure

**126.6.1.1 Peripheral Myelin Protein 22.** The first molecular event discovered, responsible for the majority of CMT, was the duplication of the chromosomal segment harboring *PMP22* (2,36). This discovery introduced a novel molecular mechanism in human mutagenesis, nonallelic homologous recombination (Figure 126-2) and defined a new group of disorders, the genomic disorders



**FIGURE 126-2** Reciprocal recombination resulting in duplication causing Charcot-Marie-Tooth disease type 1A (CMT1A) or deletion associated with hereditary neuropathy with liability to pressure palsies (HNPP). Proximal CMT1A-REP is depicted by a dotted box, distal CMT1A-REP is depicted by a hatched box, and the *PMP22* gene-encoding peripheral myelin protein depicted by a gray box. A, B, C, D, and A', B', C', D' represent unique sequences flanking the CMT1A-REP low-copy repeats on each of the two chromosome homologs, respectively. Gray lines depict the site of crossover and the resulting rearrangements are labeled ABCB'C'D' for the CMT1A duplication and A'D for the reciprocal HNPP deletion. The JCT refers to specific junction fragments detected by probes that are used in diagnostics (PFGE). Homologous meiotic recombination between nonallelic copies of CMT1A-REP's leads to either the CMT1A duplication or HNPP deletion. The CMT1A duplication results in three copies (two on the duplicated chromosome and one on the normal homolog) of the dosage-sensitive *PMP22* gene.

(37,38,230). The reciprocal molecular event, deletion of the same fragment, was predicted and found in HNPP (39,40). This molecular mechanism and the diseases provided substantial evidence for the presence of dosage-sensitive genes in the human genome. Peripheral myelin protein 22 (*PMP22*) is an integral membrane protein of 160 amino acids with four transmembrane domains. The function of *PMP22* is still unknown; however, recent evidence shows that *PMP22* and P0 are involved in both transhomophilic and transheterophilic interactions in cell culture-based assay systems using retrovirally transduced HeLa cells (41). An extra copy of *PMP22*, due to the CMT1A duplication, is associated with CMT1 (2,42,43), and this single molecular event accounts for 70% of families with dominant CMT1 (44,45) and 76–90% of sporadic CMT1 (44,46). The CMT1A duplication is also associated with neuropathy in patients manifesting wide variations in clinical phenotypes such as DSN, RLS, calf hypertrophy, and scapuloperoneal atrophy or Davidenkow syndrome (2). Deletion of *PMP22* leads to HNPP (40). In one study (47), 50% of patients diagnosed with a multifocal neuropathy had the 17p11.2 deletion associated with HNPP. Thus, *PMP22* is a dosage-sensitive gene that, when duplicated or deleted, gives rise to demyelinating neuropathies. Point mutations in *PMP22* have been seen in CMT1, DSN, and CHN phenotypes (43,48,49). As anticipated, loss of function mutations (50), including frameshift, nonsense, and splice-site mutant alleles, result in HNPP, because like the HNPP deletion, they effectively result in *PMP22* haploinsufficiency. Most of the missense mutations occur in the four predicted transmembrane domains.

Heterozygous *Pmp22* knockout mice display clinical manifestations comparable to HNPP (51). Transgenic mice and rats that overexpress *PMP22* mimic the human disorder (52,53). The finding that such mice also develop a pronounced distal axonopathy (54) led to a reappraisal of disease manifestation in CMT1A, and a careful examination of a cohort of such cases revealed that reduced compound motor and sensory nerve action potentials correlate with clinical disability, while motor NCV does not (55). Consistent with this finding is an earlier observation (56) that motor NCVs and clinical motor examination did not change significantly over a period of 22 years in eight CMT1A duplication cases.

*PMP22* is expressed predominantly in the PNS (42,57). Most of the newly synthesized *PMP22* is retained in the endoplasmic reticulum (ER), where it is degraded (54). Only a small percentage of *PMP22* is transported from the ER to the Golgi, where it undergoes complex glycosylation and becomes more stable. Axonal contact appears to stimulate the redistribution of *PMP22* to the Schwann cell plasma membrane as myelination occurs (58). The ultrastructural pathology of the HNPP phenotype, tomacula formation and reduced myelin compaction (59), suggests that *PMP22* plays a structural role in myelin formation or maintenance, or both.

Strategies aimed at normalizing *Pmp22* expression in transgenic mice have been encouraging (60). When overexpression of *Pmp22* is switched off in adult mice, correction begins within 1 week, and myelination is well advanced by 3 months (although the myelin sheaths are still thinner than normal), indicating that the Schwann cells are poised to start myelination. It has been suggested

that ascorbic acid ameliorates neuropathy in transgenic mice overexpressing *Pmp22* (61); however, extensive studies with human subjects with CMT1A disease did not confirm benefits of the ascorbic acid supplementation (62). The observations of steroid hormone effects on *Pmp22* expression in *in vitro* cultures (63) prompted studies where the effect of progesterone and its antagonist, onapristone, was investigated in a rat model overexpressing *Pmp22*. Interestingly, supplementation with progesterone resulted in increased expression of *Pmp22* mRNA, decreased number of axons on histopathological examination of sciatic nerve, and worsening of the neurological phenotype; conversely, animals supplemented with progesterone antagonist demonstrated opposite findings (64).

**126.6.1.2 Myelin Protein Zero.** The gene for myelin protein zero (MPZ), also known as  $P_0$ , maps to 1q22. MPZ protein contains a single membrane-spanning region, a large hydrophobic glycosylated immunoglobulin-like extracellular domain, and a smaller basic intracellular domain (65). MPZ is normally expressed exclusively by myelinating Schwann cells and accounts for 50% of the total PNS myelin protein (66). More than 120 different myelinopathy-associated MPZ mutations have been described (<http://molgen-www.uia.ac.be/CMTMutations/>). These are mostly associated with CMT1 and sometimes DSN and CMT2 phenotypes. A few cases of CHN have also been reported to be associated with MPZ mutations (67–69). Some evidence suggest that a milder course of CMT disease is associated with heterozygous loss of function MPZ alleles, while severe phenotypes are caused by gain-of-function mutations (70). *In vitro* functional studies were able to demonstrate that the MPZ-truncating mutations associated with a more severe form of peripheral neuropathy result in premature stop codons within the terminal or penultimate exons, and thus, they escape nonsense-mediated decay and are stably translated into mutant proteins (71). A subset of these mutations, despite escaping nonsense-mediated decay, results in a mild form of peripheral neuropathy. Further *in vitro* experiments have demonstrated that the severity of disease phenotype is a result of the retained function of the escaped mutant protein. If the mutant protein has altered function of the cytoplasmic domain and thus impairs adhesion, the mutation acts as a null allele. If the mutation disrupts function of the transmembrane domain, the mutant protein suffers ER retention and protein aggregation, and induces apoptosis (72).

Interestingly, one severe MPZ mutant allele (68) was identified in a patient who presented as a floppy infant and was originally diagnosed with a myopathy, suggesting that innervation may be necessary for proper muscle differentiation and development. The original Roussy-Levy family reported in 1926 has been shown to harbor a point mutation causing a missense amino acid substitution in the extracellular domain of MPZ (30). Thus, mutations in MPZ show a clinically diverse spectrum of CMT and related phenotypes. A recent observation elegantly

demonstrated how various truncating mutations in MPZ convey distinct neurologic phenotypes, depending on whether the truncated mRNA undergoes or escapes nonsense-mediated decay (71). It has been shown that some of MPZ-truncating proteins escape NMD, accumulate at the ER, and trigger apoptosis. Interestingly, supplementation with curcumin, a chemical compound present in the popular turmeric spice, may abrogate ER retention and aggregation-induced apoptosis (72).

Patient genotype–phenotype studies suggest two major types of disease associated mutations: one more severe with early onset and the other type with adult onset (73). Knockout mice heterozygous for *Mpz* mimic the human CMT1 phenotype caused by MPZ mutations (74). Homozygous *Mpz* knockout mice show severe hypomyelination with signs of noncompact myelin (75) and pronounced loss of distal axons (76). The cellular and molecular mechanism of MPZ mutations were studied extensively by Grandis et al. who demonstrated that sequence variants associated with late-onset disease were transported to the membrane and conveyed moderate effect on MPZ-mediated intracellular adhesions. Interestingly, MPZ mutations, causing severe CMT phenotypes, demonstrated multiple gain-of-function effects that resulted in reduction of cellular viability (73).

## 126.6.2 Gene Associated with Transport through Myelin

**126.6.2.1 Connexin 32.** The connexin 32 (*Cx32*) (Gap junction B1; *GJB1*) gene maps to Xq13.1. It consists of two exons and encodes a gap junction protein containing four transmembrane domains. A connexon (hemichannel) consists of six connexin subunits and two connexons, each from one of the two apposing membranes, which form a functional channel that allows rapid transport of ions and small molecules (77). *Cx32* is expressed in myelinating Schwann cells and is localized to noncompact myelin in the paranode and Schmidt-Lanterman incisures consistent with its role in providing a radial diffusion pathway between the adaxonal and perinuclear cytoplasm of the Schwann cell (78,79).

More than 300 different mutations have been described (<http://molgen-www.uia.ac.be/CMTMutations/>). These mutations occur throughout the entire *Cx32* protein and, unlike the *PMP22* and  $P_0$  mutations, are not concentrated in transmembrane or extracellular domains. Mutations in *Cx32* account for nearly 10% of all CMT cases and are the most frequent cause of CMT after *PMP22* duplication. The nature of the neuropathy in CMTX, whether primarily axonal, demyelinating, or a mixed neuropathy, has been debated (80–83). The recent comprehensive evaluation of 73 male patients with CMT1X and 28 *GJB1* mutations revealed that most mutations render a clinical phenotype similar to that observed in patients with a whole gene deletion. These data suggest that the neuropathy phenotype is caused by loss of function mechanism.

It has been proposed that *GJB1* mutations lead to axonal loss rather than demyelination (84).

Cx32-deficient mice mimic the human CMT1X phenotype (85). These mice develop a slowly progressing demyelinating neuropathy, with enlarged periaxonal collars, abnormal noncompacted myelin domains, and axonal sprouts (86). This suggests that reflexive gap junctions may be required for myelin compaction; or alternatively, Cx32 may play a structural role in myelin compaction. Mice lacking Cx32 show a distinct pattern of gene dysregulation in Schwann cells (87), indicating that Schwann cell homeostasis is critically dependent on the correct expression of Cx32.

### 126.6.3 Genes Associated with Axonal Transport

**126.6.3.1 Neurofilament Light Chain.** Neurofilament light chain (*NEFL*) encodes for one of the three subunits of neurofilaments, which are the major type of intermediate filaments found in neurons. Mutations in *NEFL* have been originally identified in two independent families associated with AD CMT2 (88,89). Recent studies, (90–92), have identified additional mutations in *NEFL* among CMT and DSN cases. In the earlier study (90), individuals harboring *NEFL* mutation had an early onset, severe CMT, or DSN phenotype with moderate to severely reduced NCVs. *In vitro* functional studies with mutated *NEFL* demonstrated defects in the assembly of intermediate filament networks, defective targeting of neurofilaments into processes, and altered intracellular distribution of mitochondria, suggesting defective axonal transport as an underlying pathomechanism (93). Yum et al. reported an autosomal recessive family with four siblings affected with severe, early-onset neuropathy (CMT2E) who were homozygous for p.E210X nonsense mutation. Affected individuals had features of axonal neuropathy as evidenced by neurophysiological tests and histopathological studies of the sural nerves. It has been postulated that p.E210X mutation prevented formation of intraaxonal filament network and exerted its pathogenic effect through a loss of function mechanism (94). *Nefl* knockout mice develop normally and show no overt phenotype (95). In another transgenic model, a point mutation (Leu394Pro) causes massive, selective degeneration of spinal motor neurons accompanied by abnormal accumulations of neurofilaments and severe neurogenic atrophy of skeletal muscles (96). Thus, both mouse mutants are not suitable models for human CMT2.

**126.6.3.2 Kinesin Family Member 1B.** Kinesin family member 1B (*KIF1B*), a member of the kinesin superfamily, encodes a molecular motor that transports specific organelles as cargo along microtubules. It has two isoforms—long (beta) and short (alpha). The alpha form is expressed in a variety of tissues and is responsible for the transport of mitochondria. The beta form is expressed specifically in the neurons. Knockout mice heterozygous for *KIF1B*

have a defect in transporting synaptic vesicle precursors and develop progressive weakness similar to that found in human neuropathies (97). A loss-of-function mutation in the motor domain of human *KIF1B* was identified in a pedigree with AD CMT2. Subsequently, an *MFN2* mutation was segregating with disease phenotype in the same family (98). Thus far, no other mutations of *KIF1B* have been found in human peripheral neuropathy.

### 126.6.4 Transcription Factors Associated with Myelination

**126.6.4.1 Early Growth Response 2.** Early growth response gene (*EGR2*), also known as *KROX20*, maps to 10q21 and consists of two exons that encode for a Cys<sub>2</sub>His<sub>2</sub> type zinc-finger-containing protein. Mouse *Egr2* is implicated in the establishment of myelination, and thereafter, its expression is restricted to myelinating Schwann cells (99,100). Homozygous knockout mice for *Egr2* show disruption in hindbrain segmentation (101,102), and the Schwann cells are blocked at an early stage of differentiation (103). Mutations in human *EGR2* are detected in patients with CMT1, DSN, and CHN and frequently associated with cranial nerve dysfunction and early respiratory compromise (104–107). Most mutations occur in the zinc-finger domain. Functional studies have shown that most *EGR2* mutations affect the DNA binding, and the amount of residual binding directly correlates with transcriptional activity and disease severity (17,107). Recently, it has been demonstrated that DNA binding sites for *Egr2* and *Sox10* are in close proximity, and disease-causing *EGR2* mutations affect *SOX10* binding to regulatory sequences and subsequently disturb myelination (108). Different pathogenic mechanisms are proposed for mutations in the R1 domain of *EGR2*, which is known to bind with NAB corepressors and prevent its interaction with NAB proteins, which leads to an increase in the transcriptional activity of *EGR2* (17). Thus, failure to activate or inactivate downstream genes or deregulation of *EGR2* activity could be a possible pathologic mechanism for the development of the disease phenotype.

**126.6.4.2 SRY-Related HMG-Box Containing Gene 10.** Although mutations in *SOX10* mostly cause a distinct phenotype with peripheral neuropathy, demyelinating leukodystrophy and Waardenburg–Hirschsprung disease (109–111), cases with a pure severe hypomyelinating neuropathy presentation have been reported (109). *In vitro* functional studies of *SOX10* mutations demonstrated clear genotype–phenotype correlations based on whether the mutation results in the escape of nonsense-mediated decay (71). PCWH (Peripheral demyelinating neuropathy, Central dysmyelination, Waardenburg syndrome, Hirschsprung disease, MIM#609136) is a novel clinical phenotype associated with gain-of-function mutations in *SOX10*. The *SOX10* activates Cx32, MPZ, and myelin basic protein (MBP), which play a significant role in Schwann cell differentiation; however, *SOX10* is unable



to regulate the expression of myelin genes independently but rather functions synergistically with EGR2 (108,112).

### 126.6.5 Genes Associated with Signaling

**126.6.5.1 Periaxin.** Human periaxin (*PRX*) maps to 19q13. It encodes a protein consisting of an N-terminal PDZ domain, followed by a basic domain, a nuclear localization signal domain, 57 imperfect tandem pentameric repeats that may have a tripeptide spacer and an acidic domain. Mutations in *PRX* are associated with AR DSN and CMT4F (113–115). *PRX* mutations cause an early-onset, but slowly progressive neuropathy with marked sensory component and often sensory ataxia, moderate to severe reduction of NCVs and demyelination with onion bulb formation (116,117). Alternative splicing results in two forms—L-*PRX* and S-*PRX* (118). S-*PRX* is restricted to the cytoplasm. L-*PRX* is initially seen in the nuclei of embryonic Schwann cells and subsequently in the plasma membrane of myelinating Schwann cells (119). It is expressed in the first uncompacted lamellae of the Schwann cell membrane that ensheathes the axon, and further synthesis of the protein in the rat sciatic nerve parallels the deposition of myelin (120). In the mature myelin, *PRX* is found in the cytoplasm-filled periaxonal regions of the sheath but is excluded from the compact myelin. Mice disrupted for *Prx* develop PNS compact myelin that degenerates as the animals age (121) consistent with the requirement of *PRX* in myelin stability. These mice are an important model for the study of neuropathic pain in late-onset demyelinating disease. To date, more than 10 mutations to *PRX* were reported. Interestingly, all mutations are loss of functions (nonsense and frameshift) and are predicted to result in truncated proteins (<http://molgen-www.uia.ac.be/CMTMutations/>).

**126.6.5.2 Myotubularin-Related Protein 2.** Myotubularin-related protein 2 (*MTMR2*) maps to 11q22 and encodes for a dual-specificity phosphatase. It also contains a GRAM domain, a SET-interacting domain, and a PDZ-binding domain. Mutations in *MTMR2* cause a type of AR CMT1 (CMT4B1) and CHN (122,123). CMT4B1 is characterized by focally folded myelin. The mutations are distributed throughout the open reading frame. *MTMR2* uses the lipid second messenger, phosphoinositol 3-phosphate, (PI[3]P), as a physiologic substrate. The known (124) disease-associated *MTMR2* mutations show reduced phosphatase activity (125), indicating that the phosphatase activity of *MTMR2* is crucial for its proper function in the PNS. A mouse model of CMT4B1 neuropathy demonstrates phenotypic findings observed in human subjects with *MTMR2* mutations, including myelin infoldings and outfoldings present in peripheral nerves (126).

**126.6.5.3 SET Binding Factor 2.** SET binding factor 2 (*SBF2*) maps to 11p15 and encodes for an *MTMR2*-related protein. *SBF2* is a member of the pseudophosphatase branch of myotubularins (it is also known as *MTMR13*). A homozygous inframe deletion encompassing exons 11

and 12 was detected in a consanguineous Turkish family (127). Recently, the Japanese family from one of the clinical reports of CMT and glaucoma (128) was found to have a nonsense mutation in *SBF2* that segregated with a phenotype of markedly decreased NCV, myelin folding, and juvenile-onset glaucoma (129). It is expressed in various tissues including spinal cord and peripheral nerve. The histopathologic hallmarks of the disease phenotype are focal outfoldings of myelin in nerve biopsies.

**126.6.5.4 N-Myc Downstream-Regulated Gene 1.** N-myc downstream-regulated gene (*NDRG1*) maps to 8q24 and encodes for a phosphatase. A homozygous C-to-T transition in exon 7 (R148X) was identified in 60 individuals affected with hereditary motor and sensory neuropathy, Lom type (HMSNL) (130). HMSNL is an AR CMT1 type of disorder with deafness and unusual neuropathologic features (131). *NDRG1* is ubiquitously expressed and appears to play a role in cell growth and differentiation.

### 126.6.6 Genes Associated with Endocytic Recycling

**126.6.6.1 RAB7.** Ras-related protein 7 (*RAB7*) encodes a GTP-binding protein that is a member of the RAB family of small GTPases, which are important regulators of vesicular transport and are located in specific intracellular compartments. *RAB7* is universally expressed and has been localized to the cytosolic side of some intracellular organelles. The protein has been shown to be important in membrane trafficking including the late endocytic pathway and retrograde axonal transport (132–134). Mutations in *RAB7* have been associated with CMT2B, an axonopathy (135). The disease-causing mutations in *RAB7* target highly conserved amino acid residues and result in an abnormal GTP hydrolysis rate that may influence axonal transport (134).

**126.6.6.2 SH3TC2.** Mutations in *SH3TC2* result in early onset, slowly progressive neuropathy with frequent spine and foot deformities, and they are identified in 10 to 26% of patients with a CMT4 phenotype making this gene a significant contributor to autosomal recessive CMT or CMT4 (127,136). The gene encodes a 144-D protein that is exclusively expressed in Schwann cells and involved in endocytic membrane trafficking. The protein is localized to the plasma membrane and perinuclear recycling compartment. *In vitro* studies demonstrated that *SH3TC2* interacts with Rab11, a small GTP-ase that regulates the recycling of membranes and receptors to the cell surface. Interestingly mutations identified in CMT4C patients abate protein–protein interactions between *SH3TC2* and *RAB7* that disturbs the endocytic and membrane recycling pathway (137,138).

Recently, the whole genome-sequencing approach was utilized to identify sequence variants in a proband with a CMT type I phenotype and resulted in the identification of two disease-causing mutations in the *SH3TC2*. This was one of the first studies documenting a successful

application of this powerful technique for identification of causative mutations. Interestingly, extended clinical and molecular analysis of the index family revealed that *SH3TC2* haploinsufficiency confers predisposition to a mild polyneuropathy with particular susceptibility to the carpal tunnel syndrome (139).

**126.6.6.3 FIG4.** FIG4 exhibits lipid phosphatase activity toward phosphoinositides and metabolizes phosphoinositol 3,5-bisphosphate, PI(3, 5)P<sub>2</sub>, which plays a role in intracellular signal transduction. The studies of fibroblasts obtained from FIG4-deficient patients demonstrated decreased cellular concentrations of PI(3, 4)P<sub>2</sub> and abnormal intracellular transport of organelles (140). The importance of phosphoinositides (PI) in the regulation of intracellular membrane trafficking has been known for years, and abnormal PI metabolism has been associated with other AR forms of CMT including those caused by mutations to *MTMR2* and *MTMR13* (123,141). Patients deficient for FIG4 present with atypical peripheral neuropathy with severe motor dysfunction and rapid progression. There is an animal model deficient for FIG4 activity, *plt*, pale tremor mouse, that presents symptoms of severe tremor and abnormal gait.

**126.6.6.3.1 LITAF (SIMPLE).** *LITAF* (lipopolysaccharide-induced tumor necrosis factor- $\alpha$  factor), also known as *SIMPLE* (small integral membrane protein of the lysosome/late endosome) and *PIG-7* (p53 inducible gene-7), encodes for an unglycosylated small integral membrane protein that localizes to lysosomes/late endosomes (142,143). The function of the protein remains unknown; however, it has been proposed to play a role in protein degradation. Bioinformatics analysis suggests that *LITAF* may be a member of RING-finger motif-containing subfamily of E3 ubiquitin ligases (144). Mutations in *LITAF* may cause both demyelinating and axonal neuropathy, and they are found in <1% of cases with CMT1 (145–147).

## 126.6.7 Mitochondrial Function Genes

**126.6.7.1 GDAP1.** Ganglioside-induced differentiation-associated protein (*GDAP1*) encodes a ganglioside-induced differentiation-associated protein originally isolated using a tetracycline-regulated expression system from differentiated Neuro2a cells (148). It is expressed at high levels in the brain and spinal cord, and at lower levels in human sural and mouse sciatic nerves (149). The *GDAP1* mutations affect both Schwann cells and neurons and elicit distinct phenotypes including (i) AR demyelinating CMT4A, (ii) AD axonal CMT2E, (iii) AR CMT2 with vocal cord paresis and hoarseness, and (iv) AR intermediate CMT (149–153). Mutations are among the most frequent cause of AR CMT and present in up to 25% of patients (154). The gene encodes a mitochondrial fission factor that plays an important role in mitochondrial division.

The functional studies of disease-causing sequence variants demonstrated that autosomal recessive phenotypes are caused by decreased fission capacity of mutant protein. Dominant mutations to *GDAP1* result in

mitochondrial damage that lead to increased production of reactive oxygen species and increased susceptibility to apoptotic stimuli (155–157).

**126.6.7.2 Mitofusin 2.** Mutations in mitofusin 2 (*MFN2*) are the most frequent cause of the axonal form of CMT neuropathy (98,158–160). Most patients have a moderately severe axonal neuropathy, with onset in childhood. *MFN2* is localized to the outer mitochondrial membrane, and it regulates mitochondrial network architecture by fusion of mitochondria. Mitochondria are dynamic organelles and highly motile with frequent fusion and fission. The potential role of *MFN2* in axonal transport was suggested upon demonstration of its interactions with the *Miro/Milton* complex that connects mitochondria to kinesin motors and plays a crucial role in mitochondrial motility and fusion–fission dynamics (155,161). Although *MFN2* is ubiquitously expressed in the peripheral nerve, the mitochondrial network has to be maintained for long distances from the cell body. This may explain the length-dependent axonal neuropathy developing in patients with *MFN2* mutations (160). Patients with *MFN2* mutations present with normal or slightly decreased NCVs and evidence of axonal degeneration with accumulation of abnormal mitochondria in the axonal periphery (155,162). The mouse model expressing p.T105M mutation replicates the clinical features of CMT2A neuropathy and shows typical histopathological findings observed in patients with *MFN2* mutations (163).

## 126.6.8 Chaperones

**126.6.8.1 Heat-Shock Protein 27 and Heat-Shock Protein 22.** Small heat-shock protein 27 (*HSP27*) mutations were found in distal motor neuropathy and in a family with CMT (164). In this case, distal motor neuropathy and CMT are allelic, suggesting that these two groups of disorders are intimately related. Although *HSP22* mutations were originally found in the distal motor neuropathy group (164), as anticipated, recently an *HSP22* mutation was identified in a family with CMT (165). The pathomechanism of the development of neuropathy is less clear. In vitro data suggest that neuronal cells transfected with mutant *HSP27* are less viable than cells transfected with the wild-type protein. When the mutant *HSP27* is cotransfected with NEFL, neurofilament assembly is altered. In a yeast two-hybrid system, *HSP22* and *HSP27* were found to interact (166).

## 126.6.9 Genes Associated With DNA Single-Strand Break Repair

**126.6.9.1 Tyrosyl DNA Phosphodiesterase 1.** Tyrosyl DNA phosphodiesterase 1 (*TDPI*) maps to 14q32 and codes for a DNA repair enzyme that repairs abortive single-strand breaks (SSBs) created by topo1 (167) and also repairs 3'-phosphoglycolated overhangs of DNA double-strand breaks (DSBs) (168). In the repair of SSBs,

TDP1 cleaves the covalent bond formed between the tyrosine moiety of TopoI and the 3' end of the DNA and thus generates a 3' end compatible with ligation (169). In the case of DSBs that leave a 3'-phosphoglycolate overhang, TDP1 removes the glycolate to leave a 3' phosphate, which forms the substrate for ligation. One familial homozygous *TDP1* mutation has been associated with AR spinocerebellar ataxia and axonal neuropathy (SCAN1) (170). *In vitro* functional studies revealed that mutations associated with the SCAN1 phenotype alter the sequestration of TDP1 into multiprotein single-strand break repair complexes, making these complexes catalytically inactive (171). Another set of *in vitro* functional studies showed that these mutations abolish the 3'-phosphoglycolate processing activity of the enzyme (172). The phenotype caused by mutations in *TDP1* does not quite classify as CMT, because central nervous system involvement is also present. Rather, it belongs to a new group of disorders affecting oculomotor praxis, the cerebellum, the spinal cord, and the peripheral nerves in various combinations. These disorders are caused by alteration of the DNA repair pathways and include the ataxia-oculomotor apraxias type 1 and 2 (AOA1 and AOA2) (173,174). AOA1 results from mutations in aprataxin (175), a protein shown to act in the single-strand DNA break/repair (SSBR) complex (176). AOA2 is due to mutations in senataxin (174), and other mutations of this protein have been associated with familial ALS (177).

### 126.6.10 Genes Associated with Other Peripheral Nervous System-Specific Functions

**126.6.10.1 Lamin A/C.** Lamin A/C (*LMNA*) maps to 1q21 and encodes a structural protein with similarity to cytoplasmic intermediate filament proteins. One familial mutation in *LMNA* is associated with AR CMT2 (CMT2B1) (178). Other mutations in *LMNA* are associated with several different disorders including Emery-Dreifuss muscular dystrophy (EDMD) (179), limb-girdle muscular dystrophy (180), dilated cardiomyopathy (181), familial partial lipodystrophy (182), and mandibuloacral dysplasia (183). Thus, mutations in a single gene can cause different diseases affecting diverse tissues and organs including neurons, muscles, cardiovascular and skeletal systems, and fat cells.

Lamins are the major structural proteins of the nuclear lamina underlying the nuclear membrane. They appear to play a role in DNA replication, chromatin organization, spatial arrangements of nuclear pore complexes, nuclear growth, and anchorage of nuclear envelope proteins (184). Mice, lacking *Lmna*, develop to term with no overt abnormalities (185). However, their postnatal growth is severely retarded and is characterized by the appearance of muscle weakness.

### 126.6.10.2 Potassium Chloride Cotransporter 3.

Potassium chloride cotransporter 3 (*KCC3*) maps to 15q13 and codes for a  $K^+$ - $Cl^-$  cotransporter. Mutations in *KCC3* are associated with AR peripheral neuropathy with agenesis of the corpus callosum (MIM 604878; 218000), also known as Andermann syndrome (186). Heterozygous mice transgenic for deletion of the mouse homolog *Slc12a6* are indistinguishable from the wild type in appearance and gross behavior; however, homozygous animals exhibited weakness of the rear limbs beginning at 2 weeks. Hypomyelination, myelin decompaction, demyelination, axonal swelling, and fiber degeneration were observed in the sciatic nerves of homozygous mice. *KCC3* protein is detected in the brain and the spinal cord and, at low levels, in the dorsal root ganglion (42,186).

**126.6.10.3 Protein Synthesis.** Mutations in four genes encoding aminoacyl-tRNA synthetases including glycyl- (*GARS*), tyrosyl- (*YARS*), alanyl- (*AARS*) and lysyl- (*KARS*) synthetases have been identified in patients with CMT type 2 axonal neuropathies. The genes encode aminoacyl tRNA synthetases involved in charging tRNAs with their cognate amino acids. For the vast majority of ARS, there are separate genes encoding cytosolic and mitochondrial enzymes. The mechanism leading to the pathology remains an enigma. It has been hypothesized that mutations in ARS. (i) change the structure of ARS and allow misincorporation of amino acids; (ii) decrease enzymatic activity that in turn affects protein synthesis; and (iii) result in mislocalization in cellular compartment (187–189). Mutations in glycyl tRNA synthetase (*GARS*) have been found in patients with AR CMT axonal neuropathy type 2, designated CMT2D (190). Distal spinal muscular atrophy type V (DSMAV) is an allelic disorder with a similar phenotype. The clinical picture of patients with *GARS* mutations was different from other axonal CMT2 types in that weakness and atrophy were more severe in the hands than in the feet, and that sensory impairment had the same prevalence as the motor involvement (191). Patients with mutations in *YARS* present with dominant intermediate neuropathy characterized by intermediate NCVs and histological evidence of both axonal and demyelinating features. The original patients described suffered from the distal leg and arm weakness, numbness, and prominent sensory defects (192). It has been hypothesized that disease-causing mutations may cause a dominant-negative effect on *YARS* in neuronal endings that leads to axonal loss and signs of peripheral neuropathy (193). Alanyl-tRNA synthetase mutations were originally described in two French pedigrees. Affected individuals presented with predominant axonal neuropathy leading to sensory-motor distal degeneration and demyelinations. Both families were found to have the p.Arg329His mutation that was shown to reduce tRNA-Ala aminoacylation in *E. coli* (194). Lysyl-tRNA synthetase mutations were reported in two families with CMT neuropathy. The phenotype of an affected patient with two disease-causing



*KARS* mutations presented with an intermediate form of autosomal recessive CMT disease accompanied by developmental delay and dysmorphic features. Both mutations represent loss of function alleles with severely depressed aminoacylation potential (195).

**126.6.10.4 Dynamin 2.** Mutations in dynamin (*DNM2*) on chromosome 19p12-13.2 were found in three unrelated families with CMT originating from Australia, Belgium, and North America. *DNM2* belongs to the family of large GTPases and is part of the cellular fusion–fission apparatus. *In vitro* experiments showed that mutations of *DNM2* substantially diminished binding of *DNM2* to membranes by altering the conformation of the beta3/beta4 loop of the pleckstrin homology domain. Additionally, two different mutations affecting the same amino acid, Lys558, segregated with CMT and neutropenia, which has not previously been associated with CMT neuropathies (196).

**126.6.10.5 Inverted Formin 2 (IFN2).** Mutations in the inverted formin 2 (*IFN2*) gene were recently identified in patients with CMT neuropathy associated with focal segmental glomerulosclerosis (FSGS). Patients with *IFN2* mutations present with early onset of renal diseases followed by end-stage renal disease (median age 18 years and 21 years, respectively). Their neurological problems including walking difficulties, muscle wasting, and areflexia are frequently seen as early as in the teenage years. These symptoms are accompanied by other CNS abnormalities, for example sensorineural hearing loss and white matter hyperintensities on brain MRI. *IFN2*-associated disease was classified as intermediate AD CMT based on moderate reduction of NCV and the combination of axonal and demyelinating features on nerve biopsy studies. The gene is expressed in both Schwann cells and podocytes. It encodes a formin protein that is involved in intracellular polymerization and depolymerization of actin and other elements of the cytoskeleton. It has been postulated that *IFN2* mutations disturb cytoskeletal networks and targeting of protein to plasma membrane in various types of cells. Interestingly, mutations associated with CMT + FSGS phenotype are localized exclusively in diaphanous inhibitory domain (DID) as opposed to more scattered distribution observed in patients with renal disease alone (197).

## 126.7 CHARCOT–MARIE–TOOTH “DISEASE GENES” REVEAL A CELLULAR AND MYELIN DEVELOPMENT/ MAINTENANCE NETWORK

Genes implicated in the pathogenesis of peripheral neuropathies encode proteins that form a multitiered network of structural proteins, enzymes, and transcription factors that are implicated in development and maintenance of the nervous system. This hierarchy is exemplified by the central position of *SOX10*, which orchestrates spatial and temporal expression of

downstream transcription factors (e.g. *EGR2*) that in turn control expression of myelin genes (*PMP22*, *MPZ*, and others) (Figure 126-4). Further elucidation of these relationships is crucial for better understanding of mechanisms leading to the disease (198).

### 126.7.1 Genetic Testing—Single Locus/Gene Approach

Recent advances in identifying specific mutations in various genes have led to extensive genotype–phenotype correlation studies, which have confirmed and elucidated further that genetic heterogeneity, age-dependent penetrance, and variable expressivity are key characteristics of the HMSN. Molecular tools have increased the possibility of establishing a specific molecular diagnosis. The expense associated with evaluating multiple genes for disease-causing mutations has also escalated. When deciding on genetic testing, one should consider multiple factors, including (i) availability of clinical testing, (ii) the yield of a specific molecular test, (iii) the aim of establishing a molecular diagnosis, and (iv) in sporadic cases, the frequency of *de novo* mutations.

Hereditary polyneuropathy is common, and powerful diagnostic tests are clinically available. Forty loci and 34 genes (see Figure 126-1 and Table 126-1) have been identified in CMT and related peripheral neuropathies, generating a complicated molecular classification and making it difficult to apply the vast amount of information in clinical practice. In order to establish evidence-based guidelines, we recently reviewed the medical literature and identified 10 population-based studies from various ethnic backgrounds (44,45,48,199–206). These studies reported results on five genes or genomic rearrangements: *PMP22* duplication/deletion; *MPZ*, *Cx32*, and *PMP22* point mutations. The mutation frequencies of the individual genes in the total population and in phenotypic subgroups are depicted in Table 126-2. Similar mutation frequencies were detected in the various studies, revealing a uniform distribution of pathogenic mutations. Applying a simple clinical classification (demyelinating versus axonal neuropathy) and considering the inheritance pattern (205) markedly improved the diagnostic yield.

In order to estimate the relative frequencies of the genes for which population-based studies are not available, we used our own cohort of 153 consecutive unrelated CMT cases collected before the availability of genetic testing in commercial laboratories (precommercial era). We have screened 14 genes and genomic rearrangements (*PMP22* dup/del, point mutations in *Cx32*, *MPZ*, *PMP22*, *EGR2*, *PRX*, *NEFL*, *SOX10*, *SIMPLE*, *GDAP1*, *LMNA*, *TDP1*, *MTMR2*) in this cohort to estimate the relative frequency of pathogenic mutations in these genes. The frequencies of the five mutations screened in the population-based studies were similar, suggesting that estimates from this cohort are adequate. Seemingly, mutations in the genes for which population studies are not available



may contribute to only a small minority (<1 to 2%) of patients with the CMT phenotype. Molecular testing in commercial laboratories confirmed the relative frequencies of the various genes harboring pathogenic mutations.

Duplication of a chromosomal segment harboring *PMP22* (i.e. the CMT1A duplication) (2) represents 43% of the total CMT cases, whereas the yield of duplication detection rises to 70% in CMT1. This group of patients represents the potential beneficiaries of the novel therapeutic interventions, and being the most common mutation, it is likely that drug toxicity studies will address this population initially, just like the data of idiosyncratic vincristine toxicity have emerged from patients with this mutation (207,208). Thus, the aim is to identify all subjects with the CMT1A duplication; therefore, the test should be used as a screening test.

The deletion of the same chromosomal segment results in HNPP (40). HNPP can mimic multifocal neuropathy (47), a frequently inflammatory disorder that requires immunosuppressant therapy. The individuals with HNPP among this group of patients need to be identified in order to do no harm. Although detection of deletion has a low yield in the CMT population, the clinical picture is distinctive, and the deletion is specific for HNPP. Thus, testing of this phenotype identifies deletion mutations in >90% of patients. Deletion testing should serve as a confirmatory test. Importantly, essentially all commercially available DNA diagnostic tests for the common CMT1A duplication and HNPP deletion rearrangements can detect either mutation using a single assay.

After the CMT1A duplication and HNPP deletion, *Cx32* mutations are the next most common culprits in inherited neuropathy. A dominant inheritance pattern and lack of male-to-male transmission point to this gene on the X chromosome. Identification of a *Cx32* mutation determines an XL-dominant inheritance pattern, enabling both genetic counseling and accurate estimation of recurrence risk. As the phenotype is intermediate, molecular testing for *Cx32* is appropriate in both CMT1 (after duplication testing) and in CMT2.

The population-based studies suggest that in patients with the CMT1 phenotype *MPZ* and *PMP22* mutations are the next most common after *PMP22* duplication and *Cx32* mutations. In the CMT2 group, recent data, although not population based, suggest that *MFN2* mutations are one of the most common causes of CMT2 present in up to 20% of patients, followed by *Cx32* and *MPZ* mutations in frequency (98,160). *GDAP1* and *SH3TC2* compound heterozygous and homozygous mutations account for a significant fraction of autosomal recessive CMT4.

Mutations in other genes are responsible for the CMT phenotype in only a small minority of patients; however, unique clinical features may point to a specific gene (e.g. *EGR2* external ophthalmoplegia, respiratory compromise; *SBF2* glaucoma or *IFN2* glomerulonephritis)

(Table 126-3). In the absence of a clinical clue, the likelihood of establishing a molecular diagnosis is low.

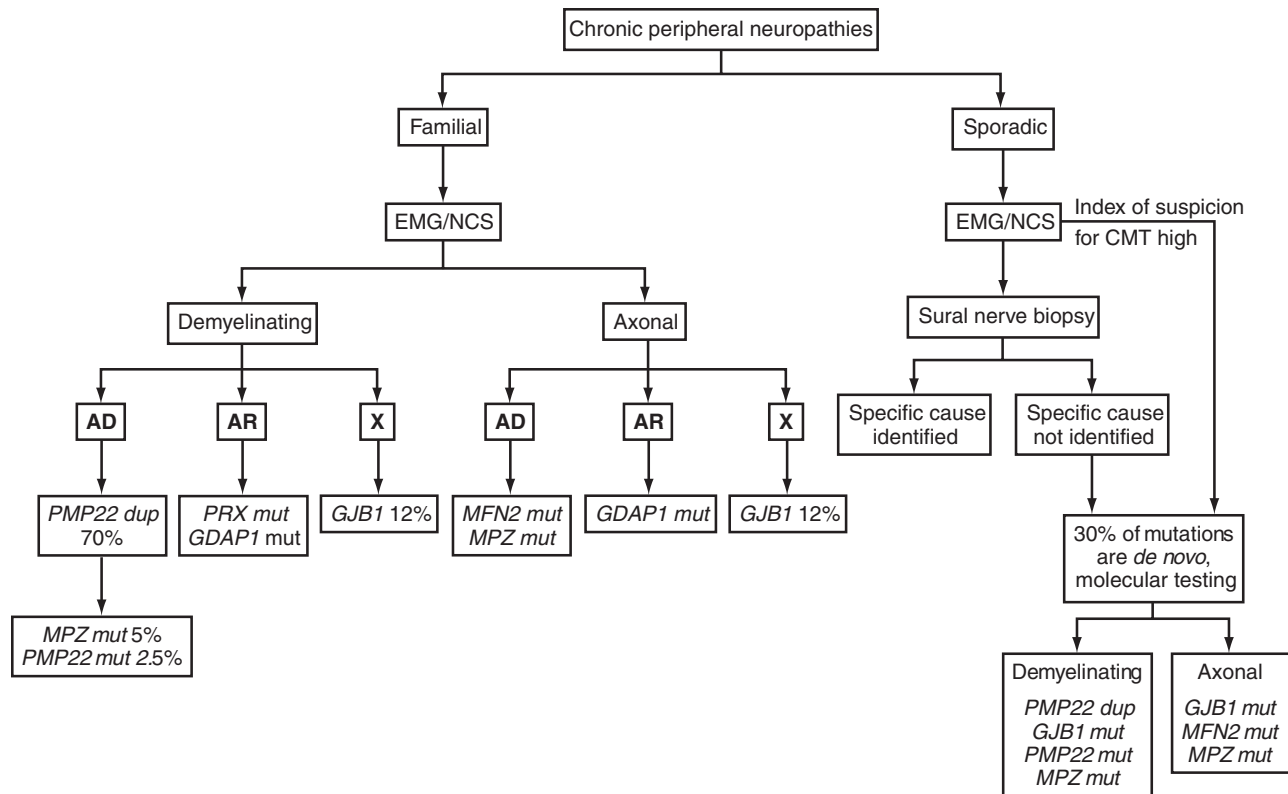
The high frequency of *de novo* mutations in duplication/deletion (37 to 90%) (44,46) illustrates that genetic disease is commonly sporadic in presentation, lacking a family history. Our data suggest that point mutations also occur frequently *de novo* (199). The frequently occurring *de novo* event necessitates having an index of suspicion for genetic disease, even in the absence of a family history. In fact, in a patient presenting with chronic polyneuropathy in the absence of other signs and symptoms, after the most common systemic and treatable causes such as diabetes, uremia, and nutritional deficiency, genetic causes are more common than autoimmune or paraneoplastic neuropathy. A rational diagnostic approach is presented as Figure 126-3.

Finally, when performing genetic testing, one must consider the specific question posed and the likelihood that the result alters medical management. In adults with the CMT phenotype, *PMP22* duplication and *Cx32* mutation analysis establish the molecular diagnosis in 65% of patients. The combination of *PMP22* duplication and *Cx32* mutation testing identifies the candidates for the clinical trials, and in the near future, those who may benefit from treatment with gene expression modifiers (selective progesterone antagonist (64), vitamin C (61)), identifies families whose members are at risk for idiosyncratic drug reactions, and determines the inheritance pattern, establishing grounds for accurate genetic counseling, and prenatal diagnosis. If patients with the demyelinating form are tested as a group, the diagnostic yield increases to over 80% by performing *PMP22* duplication and *Cx32* mutation testing.

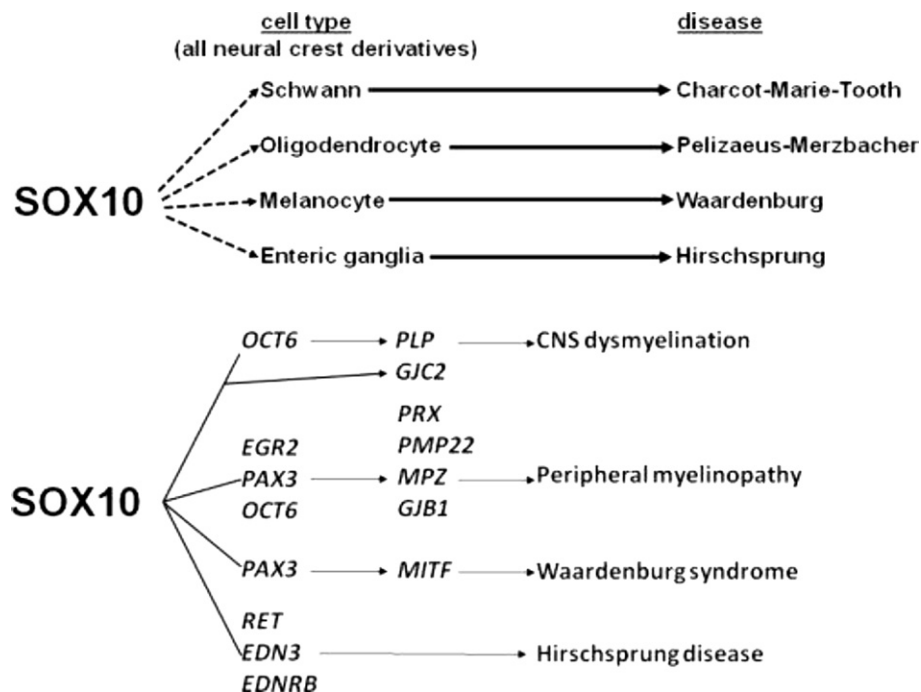
In the pediatric population, the aim of testing has a different emphasis. The parents frequently have a devastated child with severe weakness and normal intellect. The parent's major concerns are prognosis and recurrence risk. The answer will depend on an accurate molecular diagnosis. In children after testing for the common causes of peripheral neuropathy, *PMP22* duplication, and *Cx32* mutations, the physician should consider proceeding to panel testing for all the genes implicated in that phenotype or potentially consider a genome analysis.

### 126.7.2 Genetic Testing—Genome Analysis by Whole Genome/Exome Sequencing

We are now witnessing rapid developments in the field of genomic DNA analysis that is changing the landscape of clinical molecular genetic testing. The introduction of genomic methodology, based on array comparative genomic hybridization (aCGH), single-nucleotide polymorphism microarrays (SNP arrays), and next-generation whole genome or exome sequencing, has already proven its diagnostic and sometimes therapeutic potential (139,209). The application of aCGH and SNP arrays allows detection of disease-causing copy-number changes



**FIGURE 126-3** Flow diagram depicting a stepwise approach in the diagnosis of CMT and related peripheral neuropathies. In cases with a positive family history, electrophysiology establishes the objective diagnosis of demyelinating versus axonal disease. Taking into account the inheritance pattern helps limit the possible causative genes, then testing can proceed in the order from most common to least common. In sporadic cases of peripheral neuropathy, after exclusion of the most common causes of peripheral neuropathy, a sural nerve biopsy may indicate a specific nongenetic etiology or suggest genetic disorder. If the index of suspicion for CMT is high, genetic testing can follow the establishment of the demyelinating versus axonal phenotype by electrophysiology even preceding sural nerve biopsy.



**FIGURE 126-4** SOX10 in nervous system development and disease. (Adapted from Lupski (2010).)

**TABLE 126-2 Mutation Frequencies (%) for CMT and Related Neuropathies in 10 Population Studies**

Phenotype	CMT1A Duplication	HNPP Deletion	PMP22 Mutation	Cx32 Mutation	MPZ Mutation
Total	25–59	11–26	1–4	5–8	1–6
CMT1	54–81	ND	0–2	5–19	2–7
HNPP	ND	84–100	0–20	ND	ND
CMT2	ND	ND	ND	3–57	ND
CMT	3	ND	ND	18	2–40
DSN	ND	ND	14–50	ND	14–25

CMT, Charcot–Marie–Tooth disease; CMT1, Charcot–Marie–Tooth disease type 1; CMT2, Charcot–Marie–Tooth disease type 2; DSN, Dejerine–Sottas neuropathy; HNPP, hereditary neuropathy with liability to pressure palsies.

**TABLE 126-3 Clinical Pearls in CMT**

Gene	Special Features
<b>I. Peripheral neuropathy</b>	
<i>GJB1</i>	1. Early upper extremity involvement
<i>MPZ, NDRG1, PRX</i>	2. Marked sensory involvement
<i>LMNA</i>	3. Proximal weakness
<i>GJB1, MPZ, PMP22</i>	4. Intermittent symptoms
<b>II. Cranial nerve involvement</b>	
<i>PMP22, GJB1, NDRG1,</i>	1. Deafness <i>EGR2, PRX, SOX10</i>
	2. Pupillary abnormalities: MPZ a. Adie's pupil b. Argyl–Robertson pupil
<i>EGR2</i>	3. Ophthalmoplegia
<i>EGR2, MTMR2, PMP22</i>	4. Facial weakness
<i>GDAP1, MTMR2</i>	5. Vocal cord paralysis
<i>EGR2, MTMR2, MPZ</i>	6. Bulbar
<b>III. CNS involvement</b>	
<i>GJB1, SOX10</i>	1. Focal demyelination
<i>PMP22 dup</i>	2. Static tremor
<b>IV. Other organ system</b>	
	1. GI:
<i>MPZ</i>	a. Diarrhea, vomiting
<i>SOX10</i>	b. Pseudoobstruction
<i>NEFL</i>	2. Hyperkeratosis
<i>EGR2</i>	3. Respiratory compromise
<i>MTMR13/SBF1</i>	4. Juvenile glaucoma
<i>DNM2</i>	5. Neutropenia
<i>IFN2</i>	6. End-stage renal disease.
<b>V. Pathology</b>	
<i>MPZ, PRX, MTMR2</i>	1. Focally folded myelin sheaths
<i>NDRG1</i>	2. Intraaxonal curvilinear profiles
<i>PRX</i>	3. Detachment of terminal paranodal myelin loops from the axon and loss of septate-like junctions and transverse bands
<i>IFN2</i>	4. Focal segmental glomerulosclerosis (FSGS)

in patients with various clinical phenotypes. Identification of recurrent and often *de novo* copy-number variant (CNV), the CMT1A duplication (36,210), initiated the molecular genetic revolution in the study of peripheral neuropathy. CMT1A neuropathy represents a classical and one of the earliest examples of genomic disorders,

resulting from a pathogenic copy-number variation (211). Recent studies reveal rare nonrecurrent CNV of different sizes can cause CMT1A or HNPP (212). Furthermore, comprehensive analysis of *GJB1* in patients with a classical CMT1X phenotype but no detectable single nucleotide variants revealed the presence of disease-causing CNVs, disrupting the whole gene (213). We may anticipate that future studies will bring more examples documenting the role of pathogenic CNVs in the etiology of CMT.

Next generation sequencing has been already introduced to clinical laboratories and becoming a reliable and affordable tool to analyze DNA and genomes. Interestingly, one of the first patients ever diagnosed using whole-genome sequencing had CMT type I due to *SH3TC2* mutations (139). The advantages of the whole-genome approaches are especially apparent for disorders like CMT disease that is extremely heterogeneous at both the clinical and molecular levels. The genome-wide analysis with next generation sequencing allows testing all known CMT genes at once. It is expected that this broad approach may enable identification of genetic factors that are modulating the clinical phenotype (modifier genes) and further explain phenotypic heterogeneity. Whole-genome analysis brings also some ethical challenges that need to be addressed prior to its widespread implementation. These challenges are related to discovery and disclosure of unexpected findings generated by whole-genome studies, including carrier status of AR conditions, mutations in genes predisposing to late-onset diseases, or unexpected familial relations e.g. nonpaternity, incest, or consanguinity. Whole genome sequencing does not detect the CMT1A duplication; testing for CMT1A duplication/HNPP deletion should be performed prior to instituting whole genome sequencing.

### 126.7.3 Management

Treatment approaches to the HSMNs can be divided into preventive, symptomatic, and etiologic approaches. Treatment is directed to maintain function of joints and muscles. Because CMT is a slowly progressive neurodegenerative disease, these areas need to be assessed periodically. Physiotherapy and occupational therapy aid in maintaining range of motion and thus help in functioning (214,215). After a thorough evaluation, the patient and family are

**TABLE 126-4** Partial Listing of Neurotoxins Associated with Peripheral Neuropathy

Drugs	Heavy Metals	Organic Chemicals
Amiodarone	Arsenic	Ethanol
Amphotericin	Lead	Carbon monoxide
2',3'-dideoxycytidine	Mercury	Carbon disulfide
2',3'-dideoxyinosine	Thallium	Cyanate
2',3'-didehydro-3'-deoxythymidine		N-hexane
Cisplatin		Hydroxyquinolines
Ethambutol		Organophosphates
Ethionamide		Triorthocresyl phosphate
Isoniazid		
Lithium		
Metronidazole		
Nitrofurantoin		
Phenytoin		
Taxanes		
Thalidomide		
Vincristine		

instructed on an exercise program that can be carried out at home with periodic reassessments to modify the program, if needed, in the light of changing manifestations of the progressive disorder. The application of orthotic devices and assistive equipment can be made, if warranted. High top shoes, and with progression, ankle-foot orthoses can stabilize weak ankles and improve gait (216). Such intervention rarely requires prolonged care by the physician or occupational therapist. In some instances, surgical interventions for the hands and feet are necessary (217,218).

Symptomatic treatment may have a substantial impact on the patient's quality of life. Maintenance of normal weight prevents strain on weak muscles and joints, and maintains ambulation longer. Excess weight and the development of low back pain correlates with CMT, as well as in other neurologic disorders. Nonsteroidal antiinflammatory drugs may help to relieve lower back or leg pain. In cases in which neuropathic pain develops, antiepileptic drugs (gabapentin, topiramate) or tricyclic antidepressants (amitriptyline) can be used (219–221). The tremor may respond to beta blockers or primidone (222). Caffeine and nicotine can aggravate the fine intentional tremor, therefore avoidance of these substances is recommended. Neurotoxic drugs (Table 126-4) and excessive alcohol should be avoided. A list of such drugs is available with the Charcot–Marie–Tooth Association (<http://www.charcot-marie-tooth.org/>). Vincristine, a cancer chemotherapeutic agent, causes neuropathic side effects in a dose-limiting manner (223). A small dose of vincristine can produce a devastating effect in CMT patients (207). However, early detection of HMSN can avoid life-threatening vincristine neurotoxicity (224).

Although etiologic treatment is currently unavailable, recently, two potential small molecule therapeutic

approaches (61,64) have proved effective in the treatment of animal models for a specific molecular form of CMT neuropathy due to the CMT1A duplication. A selective progesterone antagonist slowed the progression of peripheral neuropathy in a transgenic rat model of CMT1A caused by overexpression of *Pmp22*, whereas vitamin C therapy slowed progression of neuropathy in a mouse model of CMT1A duplication; however clinical trials of vitamin C in patients with CMT1A duplication showed no demonstrable benefit. These observations will initiate clinical trials in patients with the CMT1A duplication and may prove as etiologic therapy for this specific molecular diagnosis.

## 126.8 GENETIC COUNSELING

Because CMT follows the principles of Mendelian inheritance, genetic counseling for recurrence of CMT1 and CMT2 is relatively straightforward if the family history for an affected individual is defined. Because of intrafamilial variability in disease expression, the definition of parental disease status requires either testing for a mutation defined in the proband or, if the mutation is not identifiable, a thorough neurologic examination with objective nerve conduction studies.

### 126.8.1 Positive Family History

An affected parent with AD or XL-dominant CMT1 or CMT2 has a 50% risk of having a child with the same mutation. The risk that a child with a mutation will be clinically affected sometime during his or her life is not known, however, because the clinical penetrance of CMT1 and CMT2 has not been determined for genetically well-defined patient populations. In general, though, few patients with AD CMT1 have substantial difficulty walking before age 50 years, although most, if not all, patients express some symptoms by the sixth decade of life (27). Similarly, only a small percentage of patients with AD CMT2 have substantial difficulty walking before age 50 years, and a small number may remain unaffected throughout life. For fathers with XL-dominant CMT, the risk of having an affected son is negligible, but the risk of having an affected daughter is 50%, whereas, for mothers with XL-dominant CMT, the risk of having an affected son or daughter is 50%.

The risk of developing AD CMT1 can be assessed by DNA testing if the causative mutation is known or by electrophysiology if the mutation cannot be identified. For AD CMT1, NCV slowing is detectable by age 2–5 years (225,226); therefore, if a young adult has normal NCVs, his or her risk of developing AD CMT1 is negligible, whereas if the NCVs are abnormal, the patient has at least a 90-% lifetime risk of developing symptoms.

In contrast to CMT1, DNA testing for CMT2 leads to a molecular diagnosis in a smaller percentage of patients, and the electrophysiologic changes associated with AD CMT2 develop with disease progression. Because of



these confounding factors, only about half of patients can be identified by age 20 years (29).

Most AR forms of CMT manifest in childhood or early adolescence. The risk of a young adult developing AR CMT is, therefore, low.

### 126.8.2 Negative Family History

For unaffected parents with a child affected with CMT1 or CMT2, four possibilities exist: a *de novo* dominant mutation in the affected child, AR inheritance, XL inheritance, or nonpaternity. Distinction between these possibilities requires either the identification of the causative mutations or identification of affected siblings. The identification of a *de novo* heterozygous presumed dominant mutation suggests a low recurrence risk for the parents; however, the risk is higher than that for the general population because of germline mosaicism (227,228). A proband with a heterozygous presumed dominant mutation has a 50-% risk of having affected children. For AR inheritance, the parental risk of an affected child is 25% because penetrance is nearly complete.

## 126.9 SUMMARY

CMT represents a clinically and genetically heterogeneous group of disorders, caused by aberration of the intimate relationship between the Schwann cell sheath and the neural axon. The clinical symptoms of weakness and muscle atrophy, and DSP, ultimately results from axonal death and muscular denervation, the final common pathway of nerve damage. A simple clinical classification of CMT (demyelinating versus axonal) improves the yield of genetic testing and determines which genes should be tested for. In the demyelinating form, the combination of CMT1A duplication and Cx32 mutation testing has a yield of approximately 80%. MPZ and PMP22 mutations are the next most common culprits. In cases of axonal CMT, MFN2 mutations are most prevalent and present in 20% of patients with CMT2 followed by mutations to MPZ and Cx32 in the population-based studies. For AR CMT, GDAP1 and SH3TC2 appear to be significant contributors to disease. Recent discoveries of potential small molecular treatments for a specific subtype of CMT shifted the emphasis in genetic testing: instead of a molecular diagnosis for the few patients referred to tertiary care centers, we need to find most patients with the potentially treatable molecular pathomechanism. Although the robust amount of new information taught us about peripheral nerve function and dysfunction, it also made genetic testing for all known genes impractical in clinical practice. However, the low cost of genome analysis may make this approach very pragmatic after PMP22 duplication/deletion analysis (229). Today, genetic testing should be used to address specific questions in a logical stepwise fashion based on evidence from population-based studies. However, the

introduction of powerful new genomic analysis tools may dramatically change clinical genetic testing and obviate the need for the disease gene panel testing in genetically heterogeneous conditions like CMT.

## FURTHER INFORMATION

CMT Mutation Database: <http://molgen-www.uia.ac.be/CMTMutations/>.

GeneClinics: <http://www.geneclinics.org>.

GeneTests <http://www.ncbi.nlm.nih.gov/sites/GeneTests/>.

CMT Association, 2700 Chestnut Street, Chester, PA 19013-4867, 610-499-9264 or 610-499-9265 1-800-606-CMTA (2682). Fax 610-499-9247, <http://www.charcot-marie-tooth.org>.

CMT International: 1 Springbank Dr. St. Catharines, Ontario, Canada L2S 2K1, (905) 687-3630 Fax (905) 687-8753 anytime <http://www.cmtint.org>.

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### Biographies

**Wojciech Wiszniewski** is Assistant Professor at the Department of Molecular and Human Genetics. He received his MD degree from Medical University of Warsaw and PhD from National Research Institute of Mother and Child in Warsaw, Poland. Dr Wiszniewski completed residency training in medical genetics in 2011 at Baylor College of Medicine, Houston, TX. He has studied the mechanisms of Charcot–Marie–Tooth (CMT) neuropathies in Dr Lupski’s laboratory.



**Kinga Szigeti** is Assistant Professor of Neurology. Dr Szigeti received her MD at the University of Pecs, Hungary in 1994. In 1997 she moved to the United States and completed a postdoctoral fellowship at Harvard University. She completed her clinical training in neurology (1998–2002) at the University at Buffalo and medical genetics (2002–2004) at Baylor College of Medicine. She studied Charcot–Marie–Tooth disease in Dr Jim Lupski’s lab. She was instrumental in developing evidence-based guidelines for genetic testing in CMT and related peripheral neuropathies. She is a neurogeneticist and neurologist with special interest in peripheral neuropathies and Alzheimer’s disease. She has coauthored 28 scientific publications and is a coinventor on two patents.



**Jim Lupski** is Cullen Professor and Vice Chair of Molecular and Human Genetics. Dr Lupski received his initial scientific training at the Cold Spring Harbor Laboratory as an Undergraduate Research Participant (URP) and at New York University completing the MD/PhD program in 1985. In 1986 he moved to Houston, Texas for clinical training in pediatrics (1986–1989) and medical genetics (1989–1992) and then established his own laboratory at Baylor College of Medicine where he remains, and as of 1995, as the Cullen Professor of Molecular and Human Genetics. Through studies of Charcot–Marie–Tooth peripheral neuropathy, a common autosomal dominant trait due to a submicroscopic 1.5-Mb duplication, and Smith–Magenis syndrome, a contiguous gene deletion syndrome, his laboratory has delineated the concept of “genomic disorders” and established the critical role of copy-number variation (CNV) and gene dosage in conveying human disease phenotypes. An increasing number of human diseases are recognized to result from recurrent DNA rearrangements (recent examples include obesity and both autism and schizophrenia), involving unstable genomic regions and have thus been classified as genomic disorders. Dr Lupski’s laboratory has also used chromosome engineering to develop mouse models for genomic disorders. Recently, the laboratory’s CMT studies in collaborations with Richard Gibbs and the Baylor Human Genome Sequencing Center resulted in the first personal genome sequence to identify a “disease gene” by whole genome sequencing (WGS) and demonstrated the utility of WGS for optimizing patient management. These latter investigations further elucidated the potential role of rare variants in complex traits such as carpal tunnel syndrome and fibromyalgia. For his work on human genomics and the elucidation of genomic disorders, Dr Lupski was awarded a Doctor of Science degree honoris causa in 2011 from the Watson School of Biological Science at the Cold Spring Harbor Laboratory. He has coauthored over 500 scientific publications, is a coinventor on more than 20 patents, and has delivered over 400 invited lectures in 32 countries.

# CHAPTER

# 127

## Congenital (Structural) Myopathies

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### 127.1 INTRODUCTION

The term “congenital myopathies” does not, as the name might imply, refer to just any inborn primary myopathy. Rather, it is used to designate a group of congenital muscle disorders defined on the basis of structural abnormalities of the muscle fibers, which are visible after staining of muscle biopsy sections by histochemical methods. This chapter addresses the most clearly defined entities within this group. A report by Goebel and Bönnemann describes some of the more unusual structural myopathies (1). The current use of the term congenital myopathies does not include the muscular dystrophies, metabolic myopathies, or mitochondrial disorders, although in individual cases there may be considerable clinical and histopathological overlap.

Congenital myopathies are rare. The incidence of all congenital myopathies taken together is estimated to be around 0.06 per 1000 live births, or one-fifth of the incidence of Duchenne muscular dystrophy and one-tenth of all cases of neuromuscular disorders (2). The prevalence of all congenital myopathies in the adult and pediatric population in northern Ireland, UK, and in the pediatric population of western Sweden is estimated to be 1:28,600 and 1:22,480, respectively (3,4); however, these data are likely to be an underestimate as patients often present with only subtle clinical and pathological manifestations.

### 127.2 DIAGNOSIS

#### 127.2.1 Clinical Features

The congenital myopathies are characterized by generalized muscle hypotonia and weakness of varying severity. They cannot be distinguished from each other or other congenital muscle disorders on clinical grounds alone; diagnosis is dependent on muscle biopsy findings. These, in turn,

require the use of histochemical staining methods applied to fresh-frozen sections of the sample. There are, however, some differences between the disorders in their predilection for, and distribution of, muscle weakness and in their associated features. These are discussed later separately for each disorder. A systematic diagnostic approach for the congenital myopathies outlined by an international group of experts has been published (5). Many of the causative genes implicated in the congenital myopathies have now been identified, a majority of them encoding sarcomeric proteins and proteins directly involved in calcium homeostasis and excitation–contraction (EC) coupling (Table 127-1).

Many patients present at birth with severe floppiness and muscle weakness, and have difficulties with respiration and feeding. Arthrogryposis is not a characteristic feature, with the exception of rare, very severe cases (44,45). The face is often elongated and expressionless, the mouth tent-shaped, and the palate high-arched (Fig. 127-1). Retrognathia and later jaw lock from masseter contractures may occur. In some patients, chest deformity is evident at birth. These features are likely to be secondary to muscle weakness, as are the commonly encountered foot deformities and hip dislocations. The dysmorphic features do not amount to, but can be reminiscent of, Marfan's syndrome (46–48) or 22q11.2 deletion syndrome. Most other patients present later in infancy or childhood with delayed attainment of motor milestones and a waddling gait, and some with a disturbance of speech articulation.

Clinical presentation later in life is infrequent, but occasionally mild congenital muscle weakness may go unnoticed and patients may thus present in adulthood, either with progressive weakness or with respiratory insufficiency. These patients usually lack the dysmorphic features and deformities that are thought to be secondary to congenital muscle weakness (49). In many adult-onset cases, it remains to be determined how frequently the

**TABLE 127-1 Genes for the Major Congenital Myopathies, Myofibrillar Myopathies and Myosin Storage Myopathies**

Disorder	Inheritance	Gene Location	Gene Symbol	Gene Product	MIM	Key References
Nemaline myopathy	AR	2q21.2–q22	<i>NEB</i>	Nebulin	256030	(6,7)
Nemaline myopathy	AD	1q42.1	<i>ACTA1</i>	$\alpha$ -Actin, skeletal	161800	(8)
Nemaline myopathy	AR	1q42.1	<i>ACTA1</i>	$\alpha$ -Actin, skeletal	256030	(8)
Actin myopathy	AD	1q42.1	<i>ACTA1</i>	$\alpha$ -Actin, skeletal	102610	(8)
Nemaline myopathy	AD	1q21–q23	<i>TPM3</i>	$\alpha$ -Tropomyosin	161800	(9,10)
Nemaline myopathy	AR	1q21–q23	<i>TPM3</i>	$\alpha$ -Tropomyosin	191030	(11)
Nemaline myopathy	AD	9p13.2–p13.1	<i>TPM2</i>	$\beta$ -Tropomyosin	190990	(12)
Nemaline myopathy	AR	19q13.4	<i>TNNT1</i>	Troponin T, slow	605355	(13)
Nemaline myopathy	AD	15q21–q23	<i>KBTBD13</i>	Kelch-repeat and BTB (POZ)	609273	(14)
Myotubular myopathy	X	Xq28	<i>MTM1</i>	Myotubularin	310400	(15,16)
Centronuclear myopathy	AD	19p13.2	<i>DNM2</i>	Dynamin 2	160150	(17)
Centronuclear myopathy	AR	7p14–p13	<i>BIN1</i>	Amphiphysin 2	255200	(18)
Centronuclear myopathy	AR	19q13.1	<i>RYR1</i>	Ryanodine receptor type 1	117000	(19)
Central core disease	AD	19q13.1	<i>RYR1</i>	Ryanodine receptor type 1	117000	(20,21)
Central core disease with rods	AD	19q13.1	<i>RYR1</i>	Ryanodine receptor type 1	180901	(22,23)
MmD	AR	1p36	<i>SEPN1</i>	Selenoprotein N	117000	(24)
MmD, with ophthalmoplegia	AR	19q13.1	<i>RYR1</i>	Ryanodine receptor type 1	255320	(25,26)
MFM	AD	2q35	<i>DES</i>	Desmin	601419	(27)
MFM	AR	2q35	<i>DES</i>	Desmin	601419	(27)
Myofibrillar myopathy	AD	11q22	<i>CRYAB</i>	$\alpha$ -B-Crystallin	608810	(28)
MFM	AR	11q22	<i>CRYAB</i>	$\alpha$ -B-Crystallin	608810	(29,30)
Myofibrillar myopathy	AD	5q31	<i>MYOT</i>	Myotilin	609200	(31)
MFM (Mallory body myopathy)	AR	1p36	<i>SEPN1</i>	Selenoprotein N	602771	(32)
MFM	AD	10q22.2–q23.3	<i>ZASP</i>	Z-band alternatively spliced PDZ-motif containing protein	609452	(33)
MFM	AD	7q32	<i>FLNC</i>	Filamin C	102565	(34)
MFM	AD	10q25.2–q26.2	<i>BAG3</i>	Bcl-2 associated athanogene 3	609524	(35)
Myosin storage myopathy	AD	14q12	<i>MYH7</i>	Myosin heavy chain, slow/b	608358	(36,37)
Congenital fiber type disproportion	AD	1q42.1	<i>ACTA1</i>	$\alpha$ -Actin, skeletal	255310	(38)
Congenital fiber type disproportion	AR	1p36	<i>SEPN1</i>	Selenoprotein N	606210	(39)
Congenital fiber type disproportion	AD	1q21–q23	<i>TPM3</i>	$\alpha$ -Tropomyosin	255310	(40)
Congenital fiber type disproportion	AR	19q13.1	<i>RYR1</i>	Ryanodine receptor type 1	180901	(41)
Congenital fiber type disproportion	AD	14q12	<i>MYH7</i>	Myosin heavy chain, slow/b	160760	(42)
Congenital fiber type disproportion	X				300580	(43)

AD, autosomal dominant; AR, autosomal recessive; X, X-linked; MmD, multi-minicore disease; MFM, myofibrillar myopathy.

histologic findings suggestive of a congenital myopathy are in fact due to non-hereditary disease processes, such as for example in sporadic late-onset nemaline myopathy (50), see also Section 127.5.

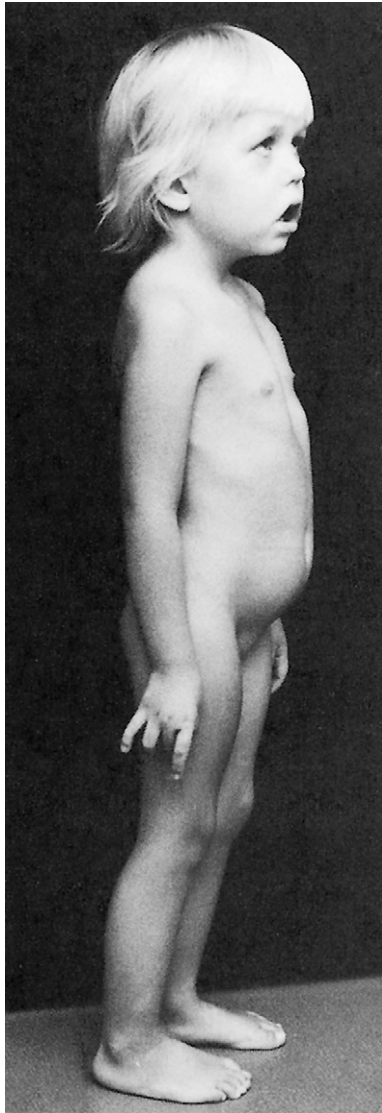
Some infants die from respiratory complications, but even patients with severe floppiness and lack of spontaneous respiration at birth have been known to survive, some of them with little residual disability (51–57). Others may experience deterioration during the prepubertal period of rapid growth, and some will require a wheelchair from this time. Otherwise, the course of the disease is often only very slowly progressive, and most patients will be able to lead an active life.

The main factors influencing prognosis seem to be respiratory capacity and the development of scoliosis (56,58,59). In many congenital myopathies the diaphragm along with the intercostal muscles and the accessory muscles of respiration are affected; this is of special

importance during sleep and when the patient is in the recumbent position. The monitoring of respiratory function and of the spine are essential elements in the ongoing care of these patients (60). Respiratory infections are common in the preschool years, but this susceptibility is often overcome with time (53,61).

The gait is usually waddling because of pelvic girdle weakness, and some patients have foot drop. The build is slender but muscle bulk is not necessarily small, especially not in young children. The spine is hyperlordotic or sometimes rigid (24,56,62–66). Tendon reflexes are weak or absent. Gross motor activity is slow, whereas fine motor activity is normal. Many patients have hypermobility of joints, and contractures and deformities of the joints commonly develop with time.

Intelligence is usually normal. Cardiac involvement is uncommon in association with most of the currently known genetic defects, but an initial evaluation to exclude



**FIGURE 127-1** A 3½-year-old girl with congenital nemaline myopathy. Note myopathic facies, chest deformity, and hyperlordotic posture.

structural cardiac abnormalities or a cardiomyopathy is indicated as in other neuromuscular conditions. Cardiac follow-up is necessary when hypoxia carries the risk of cor pulmonale (58), in genetically unresolved cases and probably also in patients with mutations in the skeletal  $\alpha$ -actin gene, because of the late expression of this gene in cardiac muscle (67) and rare reports of an associated cardiomyopathy (68).

Although a variety of abnormalities interpreted as neurogenic have been reported (69–71), most patients will have normal findings on examination of peripheral nerves, including normal conduction velocities. Autopsy findings in the central nervous system (CNS) and peripheral nerves have also been normal in most cases (72–74). Electromyography (EMG) may be normal in young patients and mild cases but usually shows polyphasic motor unit potentials with small amplitude, a full interference pattern during weak effort, and normal

fiber density (75,76). In addition to these “myopathic” features, EMG signs often interpreted as neurogenic, such as large motor unit potentials with discrete pattern on full effort, abnormal jitter, and increased fiber density, may develop with time, especially in distal muscles (75,77–80). In some patients, neurophysiological assessment may suggest an associated neuromuscular transmission defect (81). Severely affected neonates may even show spontaneous activity to the extent of mimicking spinal muscular atrophy (8,72,76,82–87).

Ultrasonography often shows abnormally high echogenicity in affected muscles and computed tomography (CT) shows low density of muscles with preservation of volume. Magnetic resonance imaging (MRI) commonly reveals fatty infiltration of the muscle tissue (88–91) and often identifies patterns of selective muscle involvement, which may aid the genetic diagnosis in individual cases (see paragraphs on specific conditions). For review of the role of muscle MRI in the diagnosis of the congenital myopathies and other neuromuscular conditions see references (92,93).

Serum concentrations of creatine kinase (SCK) are usually normal or only slightly elevated, particularly in those with myopathies due to dominant mutations in the skeletal muscle ryanodine receptor (*RYR1*) gene.

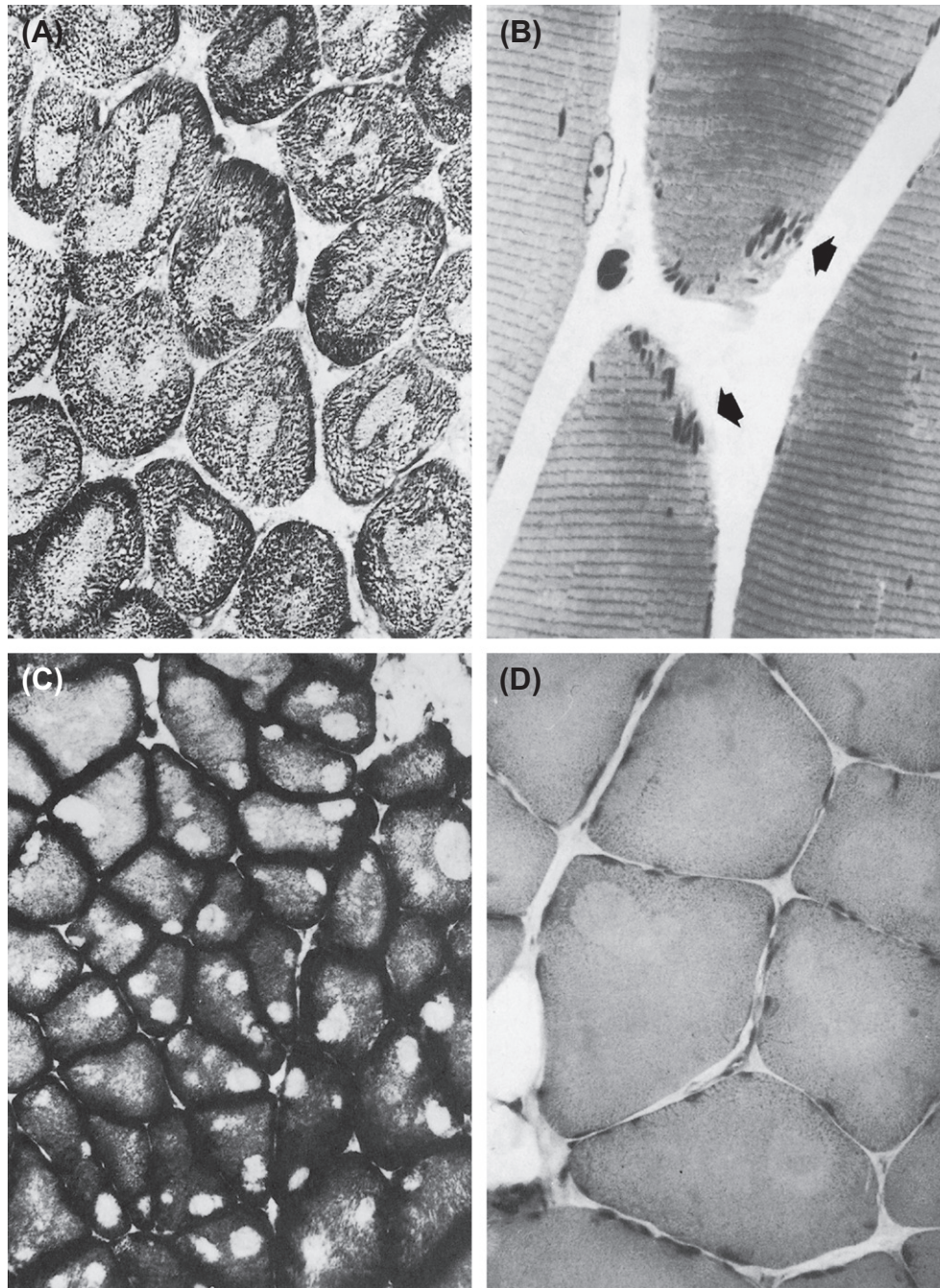
### 127.2.2 Muscle Biopsy

Routine hematoxylin and eosin (H&E) staining of muscle sections shows pathologic features common to most cases of congenital myopathy, but histochemical staining of fresh-frozen sections is necessary to reveal the more specific characteristics of each of the congenital myopathies (Fig. 127-2). Muscle fibers are often small, although, especially in older patients, there may be an additional population of large fibers. Replacement of muscle fibers by fat and fibrous tissue may be seen in advanced cases and occasionally at presentation, but necrotic and regenerating fibers are uncommon. Inflammation is not a typical feature. Internal nuclei may be numerous, and there may be occasional fiber splitting. Some patients with variable genetic backgrounds may show abnormal variability in fiber size, with both atrophy factors and hypertrophy factors abnormally high (94).

In addition to histologic changes, there are histochemical abnormalities that relate to the different fiber types. In a normal muscle, the histochemical differences between the two main fiber types are due to biochemical and electrophysiologic differences reflecting their functional properties. The initial distinction between type 1 (slow, oxidative) and type 2 (fast, glycolytic) fibers in congenital myopathies was drawn by Dubowitz and Pearse (95) and has served as the basis for a more detailed classification of fiber types.

The fibers of the two main types are usually of approximately equal size, but in the congenital myopathies, atrophy or hypotrophy of type 1 fibers is common (56,94,96–100). By definition, if the mean diameter of the



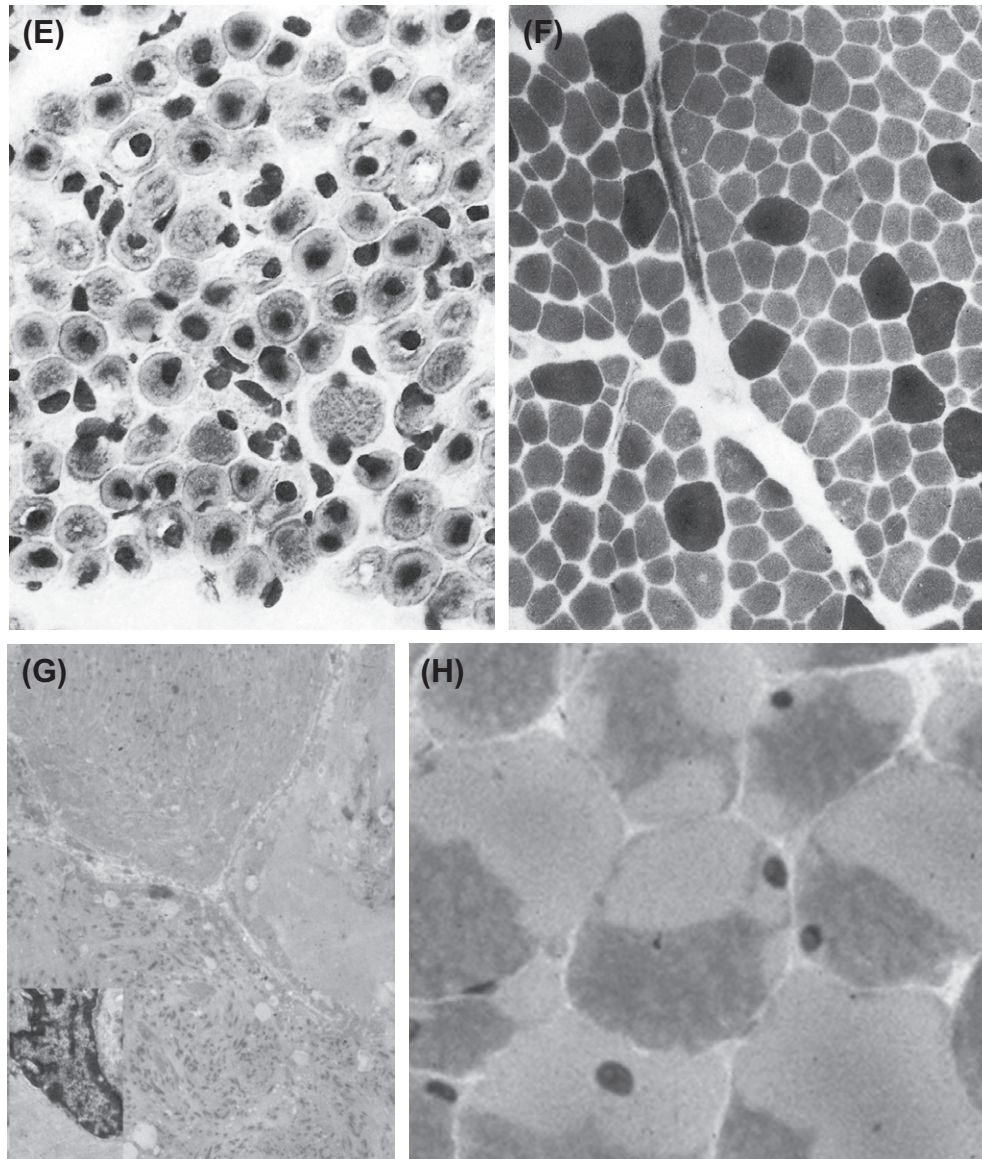


**FIGURE 127-2** Characteristic features of muscle biopsies in the various congenital myopathies. (A) Central core disease. (Frozen section stained with NADH,  $\times 500$ .) (B) Congenital nemaline myopathy. Nemaline bodies in subsarcolemmal location indicated by arrows. (Epoxy resin-embedded toluidine-stained section,  $\times 1200$ .) (C) Multi-minicore disease. (Frozen section stained with NADH,  $\times 300$ .) (D) Same biopsy as in (C) (Routine H&E stain with abnormality hardly detectable,  $\times 500$ .)

type 1 fibers is smaller than that of the type 2 fibers by more than 12%, (or 35–40%, as more recently proposed) (5), while the variability coefficient of the type 2 fibers (standard deviation of the fiber diameters divided by the mean of the fiber diameters) is normal (less than 0.25), selective atrophy or hypotrophy of type 1 fibers is recognized (96,101). In the absence of other diagnostic features, this is termed congenital fiber type disproportion (CFTD). It has been suggested that a predominance of type 1 fibers is also necessary for this diagnostic label to be applied,

although this may exclude some cases that are otherwise compatible with the criteria (102). The proportion of different fiber types varies in different muscles; predominance of type I fibers is defined as greater than 55% in the biceps brachii or vastus lateralis muscles (94). Predominance of type 1 fibers is a common feature of the congenital myopathies, and some patients show exclusively type 1 fibers (85,103–106). A selective smallness of type 2 fibers, on the other hand, has been reported in congenital myopathies in combination with type 1 fiber predominance (107–109),





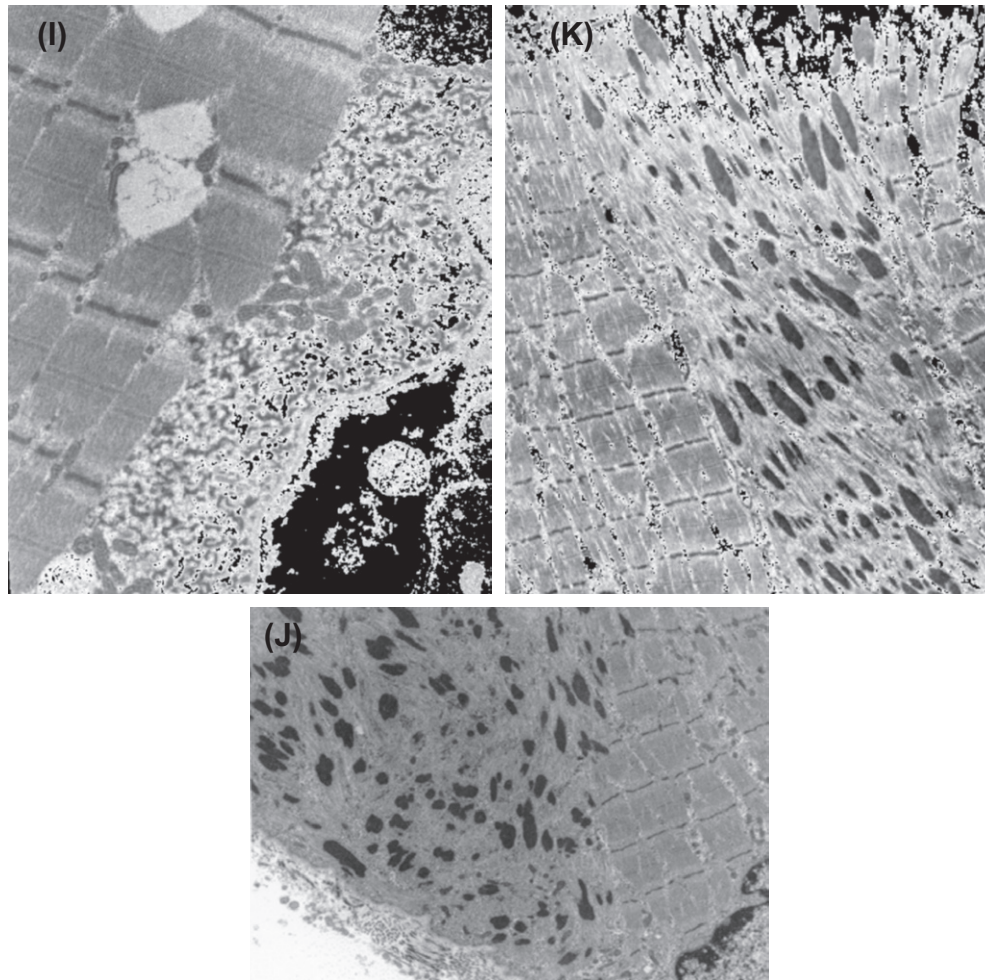
**FIGURE 127-2, cont'd** (E) X-linked myotubular myopathy. (Frozen section stained with routine H&E,  $\times 500$ .) (Photomicrograph by courtesy of Prof Juhani Rapola.) (F) Congenital fiber type disproportion. Numerous small, lightly stained fibers are of type 1, and darker, larger fibers are of type 2. (Frozen section stained with ATPase after preincubation at pH 9.4,  $\times 250$ .) (Photomicrograph by courtesy of Prof Juhani Rapola.) (G) Actin myopathy. Biopsy from a severely affected neonate with a mutation in the actin gene. Pale areas of accumulated actin filaments and dense rod-like structures in an adjacent fiber. The insert shows a high power magnification of the actin filaments. (Electron micrograph by courtesy of Prof Caroline Sewry.) (H) Myosin storage myopathy/hyaline body myopathy with large pale areas of granular material within muscle fibers. (Modified Gömöri trichrome stain.) (Photomicrograph by courtesy of Prof Hans H. Goebel.)

but is more often seen in other circumstances, such as for example, central hypotonia and simple disuse atrophy (94). Selective type 2 atrophy may also be a feature in congenital myasthenic syndromes (110). In the congenital myopathies, fiber types may not be well defined, but different patterns may be apparent depending on the analytic method used (111). This poor differentiation of fiber types is being explored through the study of developmental and alternative isoforms of the various proteins involved (112). Some patients with a clinical picture compatible with a congenital myopathy show only the nonspecific histologic abnormalities described earlier, with no additional more specific abnormality (113–115).

The characteristic histologic features upon which the definitions of each disorder are based are discussed under the respective subheadings. Electron microscopic studies of muscle ultrastructure are useful in confirming the diagnosis of the congenital myopathies, but immunohistochemical studies do not have the same role as in the diagnosis of the congenital muscular dystrophies.

### 127.2.3 Inheritance

Although there may be phenocopies, cases showing a typical clinical picture in combination with typical histologic features can reasonably be assumed to be caused



**FIGURE 127-2, cont'd** (I) Myofibrillar myopathy (desmin myopathy). Biopsy from a patient with a mutation in the desmin gene. Dense granulomatous material at the periphery of a fiber. (Electron micrograph by courtesy of Prof Caroline Sewry.) (J) Rod-core disease. Nemaline bodies (A) and core-like area (B) in a patient with rod-core disease caused by *KBTBD13* mutation. (Photomicrograph by courtesy of Dr Phillipa Lamont.) (K) Rod-core disease. Nemaline bodies in a core region that lacks mitochondria. (Electron micrograph by courtesy of Prof Caroline Sewry.)

**TABLE 127-2** Modes of Inheritance for Congenital Myopathies, Myofibrillar Myopathies and Myosin Storage Myopathies

Myopathy	Inheritance
Myotubular (centronuclear)	X-linked, AD, AR
Nemaline (rod) myopathy	Mainly AR, less frequently AD, new dominant mutations common (ACTA1)
Actin myopathy	Mostly new dominant mutations
CCD	Mainly AD, less frequently AR
MmD	Mainly AR, rarely AD
Congenital fiber type disproportion	Sporadic, AR and AD
Myosin storage myopathy	AD, AR, often sporadic
MFM	AD, AR, often sporadic

AD, autosomal dominant; AR, autosomal recessive.

genetically. More than one mode of inheritance is known for most of the congenital myopathies (Table 127-2). The proportion of new mutations and the incidence of germ line mosaicism are uncertain. A number of genes for congenital myopathies have been identified (see Table 127-1), most of them encoding sarcomeric proteins and proteins involved in calcium homeostasis and EC coupling. With a few exceptions such as the giant nebulin gene, for most of the genes identified in the congenital myopathies to date, molecular genetic testing is now available for diagnostic purposes (116–119); this is discussed under the specific subheadings for each congenital myopathy.

Where molecular genetic analysis is not yet routine, an attempt should be made to clarify the mode of inheritance in an individual family through clinical examination of both parents, to rule out any minor muscle weakness. Muscle biopsy of the parents was previously commonly performed (120–124), and in some families, both



clinically healthy parents have shown abnormalities on muscle biopsy, suggestive of heterozygote manifestations of a recessive gene (56,125,126). Some, but not all, carriers of X-linked myotubular myopathy have abnormal biopsy findings (57,127). EMG of either or both asymptomatic parents may also show abnormalities (128–131). The interpretation of slight abnormalities on clinical examination, EMG, or muscle biopsy remains difficult. Evaluating the mode of inheritance in sporadic cases, therefore, requires great care, and all families should be provided access to genetic counseling. After the discovery of many of the causative genes, molecular genetic studies are most useful in resolving these problems and are likely to replace alternative means of parental investigation.

### 127.2.4 Diagnosis and Differential Diagnosis

The diagnosis of a congenital myopathy can be made in the presence of a typical clinical picture and typical structural abnormalities of the muscle fibers visible in muscle biopsy sections stained by histochemical methods. For an increasing number of the congenital myopathies, molecular genetic confirmation of the diagnosis is possible (Table 127-1). In view of the rarity of the congenital myopathies, it is worth concentrating diagnostic investigations at centers that have sufficient experience with clinical diagnosis and muscle pathology of neuromuscular disorders (5). Because many of the characteristic histologic abnormalities are not specific but have been encountered in a number of probably unrelated conditions (94,132–140), the diagnosis of a congenital myopathy is not justified in the absence of a typical clinical picture.

Combinations of histologic abnormalities characteristic of different congenital myopathies have been encountered within families, sometimes even in the same patient (59,100,141–144), and could recently been attributed to different genetic backgrounds. For example, the combination of nemaline bodies and central cores (“core-rod myopathy”) has been noted in a number of families (22,23,123,145–156), and has been recently attributed to mutations in the ryanodine receptor type 1 gene (*RYR1*) on chromosome 19q13 (22,23), the skeletal muscle  $\alpha$ -actin (*ACTA1*) gene on 1q42.1 (157), the cofilin-2 (*CFL2*) gene on 14q11 (158), the nebulin gene (*NEB*) on 2q21.2-q22 (159) or the *KBTBD13* gene on 15q22.31 (14). Core formation has also been noted in centronuclear myopathy, and some of these cases may be due to *RYR1* mutations (19). Myosin storage myopathy due to mutations in the *MYH7* gene (160) may resemble the core myopathies, Central Core Disease (CCD) and Multi-minicore Disease (MmD). Selcen et al. (161) described a family with cardio-neuromyopathy, histologically characterized by central hyaline masses surrounded by nemaline bodies.

Many patients with one of the other congenital myopathies also fulfill the mathematical criteria of CFTD. For example, some patients with nemaline myopathy have

shown nemaline bodies only in the second muscle biopsy (83,85,104,106,162,163), whereas the fiber type disproportion was evident in both biopsies. Patients with biopsy findings characteristic of myotubular myopathy in one biopsy have shown only nonspecific abnormalities in another (164–166). Increasingly, in patients with CFTD and no other specific features, mutations are being identified in the known genes for congenital myopathies. After the first identification of mutations in the *ACTA1* gene (38), a greater number of patients have shown either dominant mutations in *TPM3* (40) or recessive mutations in *RYR1* (41), while recessive mutations in *SEPN1* appear to be less frequent (39).

Abnormalities of the CNS may cause floppiness and predominance of type 1 fibers (167). A central cause should therefore be excluded. In cases presenting as floppy infants, the differential diagnosis includes the Prader–Willi syndrome, which causes severe hypotonia and feeding difficulties. This diagnosis can be confirmed by molecular genetic studies. Because of the clinically and histologically similar picture, the congenital form of myotonic dystrophy (see Chapter 122) needs to be excluded in all new cases suspected of X-linked myotubular myopathy prior to performing a muscle biopsy. This can preferably be done using molecular genetic methods, or, if not available, by performing EMG to exclude myotonia in the mother. The severe form of spinal muscular atrophy and other anterior horn cell disorders should also be excluded, as should congenital muscular dystrophy and the motor neuropathies (see Chapters 116, 118 and 120).

Rarely, the muscle weakness in sporadic cases has shown a scapulohumeral distribution, with terracing of the shoulders resembling severe FSHD (168–171).

In addition to mutations in beta-tropomyosin (*TPM2*) gene causing nemaline myopathy, the mutations in this gene have also been identified in distal arthrogryposis (172).

The recent identification of two nemaline myopathy families presenting with distal weakness indicates that nemaline and distal myopathy form a continuum, and that nemaline myopathy needs to be taken into account in the differential diagnosis of distal myopathies (173).

For patients in whom the initial workup reveals no specific histologic features, and no genetic cause has been identified, a later reassessment is recommended. This includes MRI of muscles to identify any selective pattern of muscle involvement to guide mutation detection, and, if this is inconclusive, a second biopsy should be performed some years after the initial muscle biopsy (5).

## 127.3 MANAGEMENT

No curative treatment is currently available for the congenital myopathies. However, much can and should be done for the patient. Management is best entrusted to a multidisciplinary team familiar with the treatment of neuromuscular disorders in the appropriate age group (174). In view of the favorable outcome documented for



some patients, active treatment is indicated initially even in the severe congenital cases (51,52,55,56,175). Because no reliable prognostic indicators have been established, we believe that judgments of how actively an infant should be treated should not be based on diagnosis as such. Any decision to refrain from active treatment needs to be taken *in casu* using the same criteria as for children with other neonatally severe conditions.

An international group of experts have suggested strategies for the assessment and treatment of respiratory insufficiency in the congenital myopathies (60). Regular monitoring of respiratory capacity is most important, especially because respiratory compromise may be out of proportion to the patient's overall muscle weakness, and special attention should be paid to possible signs of nocturnal hypoventilation and hypoxia. Thus, even in ambulant patients, the need for intermittent or permanent noninvasive mechanical ventilation should be evaluated at an early stage, because of the risk of sudden respiratory failure (53,58–60,176–179). Continuous recording of overnight pulse oximetry during sleep should be performed annually when forced vital capacity (FVC) is less than 60%, and more often when FVC is less than 40% (60). The results of treatment of patients with congenital myopathies by noninvasive mechanical ventilation are often very satisfactory because of the absence of primary lung involvement. Respiratory infections should be treated actively. Follow-up care should include the assessment of cardiac status because of the risk of cor pulmonale (58,180).

Swallowing difficulties are common and may require intervention to prevent aspiration. Conservative treatment by a speech therapist may also help toward overcoming any feeding difficulties. Early speech therapy is recommended to support normal speech development, particularly for patients with dysarthria.

Should scoliosis develop, in patients with respiratory impairment early operation is preferable to bracing because of the tendency of bracing to restrict respiration still further. The timing of surgical treatment should be before the scoliosis has become too severe and while respiratory capacity is still sufficient for such a major operation (181). Surgical stabilization of the spine may also help maintain an adequate vital capacity.

Other deformities and contractures should be treated conservatively but actively in the first instance to avoid surgical interventions requiring immobilization. Orthopedic treatment should be undertaken very selectively and only at centers with extensive experience with neuromuscular diseases, and postoperative mobilization ought to be rapid. If immobilization for other reasons (e.g. trauma) cannot be avoided, its adverse effects in terms of irreversible muscle atrophy should be counteracted by early intensive physiotherapy.

The aim of regular physiotherapy includes the preservation of muscle power and function, with emphasis on maintenance of cardiorespiratory capacity, thoracic mobility, coughing, and drainage. It is uncertain whether

specific training of inspiratory muscle function is beneficial, whereas there is more evidence supporting the use of mechanical aids for assisted coughing exercises (60). The most useful exercises for improving cardiorespiratory function are those that promote endurance, such as swimming and horseback riding (182). Other goals are the prevention of scoliosis, back pain, and contractures, and the maintenance of mobility, head control, and independence in the activities of daily living.

CCD and probably MmD secondary to mutations in patients with skeletal muscle ryanodine receptor (*RYR1*) gene, are at a greater than normal anesthetic risk of malignant hyperthermia (MH) reactions during anesthesia (183). A higher risk is not clearly associated with the other congenital myopathies, but it is necessary for the anesthetist to be aware of the patient's diagnosis and to plan the anesthesia accordingly (184–188). Careful preoperative assessment is necessary (60). Wedel (189) reviewed the association between MH and this group of disorders, suggesting that succinylcholine should generally be avoided, even in the absence of a specific contraindication. Most myopathies lead to disturbances of calcium metabolism in muscle tissue, and this condition apparently confers an increased risk of complications when succinylcholine is used for relaxation or when the volatile anesthetics are employed. More recent reviews have focused on *RYR1*-related myopathies and the potentially associated MH risk (190).

Patients should be encouraged to choose an occupation fitting their intellectual capacity, and free from physical strain, high risk of infection, and exposure to tobacco smoke and other inhaled irritants.

Many patients have gone through pregnancy and delivery without significant problems (188,191–197). However, the pregnancies of affected women should be carefully supervised, with care coordinated cooperatively by the obstetrician and neurologist, taking into account the patient's respiratory capacity, muscle weakness, and any contractures potentially complicating delivery. Although involvement of smooth muscle is not usually a feature of the congenital myopathies, the process of labor in affected women must be carefully planned and managed individually.

## 127.4 GENETIC COUNSELING

Genetic counseling should be offered to all families in which a diagnosis of a congenital myopathy has been made. Care should be taken while interpreting the mode of inheritance, especially in families with only a single affected member. Primary (i.e. in the context of genetics, phenotypic) prevention of the disorders is currently not possible.

Molecular genetic confirmation of diagnosis, mode of inheritance, and carrier status is already possible in several of the congenital myopathies, and as other causative genes are being found, counseling can and should increasingly be based on molecular genetic testing.

## 127.5 NEMALINE (ROD) MYOPATHY

Nemaline myopathy was first described in 1963 in two independent reports (198,199). The autosomal dominant forms (MIM \*161800) and the more common autosomal recessive forms (MIM \*256030) may be histologically similar. Both display clinical and genetic heterogeneity; seven genes are known to cause nemaline myopathy when mutated: nebulin (*NEB*) (200), slow skeletal muscle actin (*ACTA1*) (8), alpha-tropomyosin (*TPM3*) (9), beta-tropomyosin (*TPM2*) (12), slow troponin T (*TNNT1*) (13), cofilin-2 (*CFL2*) (158) and Kelch-repeat and BTB (POZ) domain containing 13 (*KBTBD13*) (14) (Table 127-1). Of these, nebulin and actin are the two main causative genes. Genetic linkage studies indicate that at least one further gene exists (201). All the causative genes identified to date encode proteins of the thin filament, with the exception of the latest one to be found, *KBTBD13*, the function of which remains to be clarified.

There have been a number of suggestions for subclassification of congenital nemaline myopathy (202–204). The classification by the International Consortium on Nemaline Myopathy, mainly designated for gene identification, is based on what is recognized as the typical form of nemaline myopathy, and on how other types differ from this form (205). Studies of genotype–phenotype correlations in patients with known mutations may bring about a revised classification, but currently still, a clinical classification permitting accurate prognostication or the determination of the mode of inheritance in singleton cases awaits further molecular genetic studies and genotype–phenotype correlations. Comparisons of the clinical pictures in patients with nebulin and actin mutations indicate some differences in severity and distribution of muscle weakness (206). For clinical reviews, see North et al. (74), Ryan et al. (53), Agrawal et al. (207), and Wallgren-Pettersson et al. (206).

Isolated adult-onset cases (49,208–214) differ from the other forms in the absence of dysmorphic features; in these cases, the disease often follows a clearly progressive course. The presenting feature may be so-called dropped head (215–217). In this group of patients, sporadic late-onset nemaline myopathy has been recently defined as a clinical entity. This disease has been found to be autoimmune in origin, raising the possibility of therapy using stem cell transplantation (217–221).

### 127.5.1 Special Features of Muscle Pathology

The histologic definition of nemaline myopathy is based on the presence of thread- or rod-like bodies, the nemaline (rod) bodies in the muscle fibers of patients (Greek *nema*=thread) (see Fig. 127-2B). The nemaline bodies are most readily visualized after staining with the Gömöri trichrome method, applicable to paraffin as well as fresh-frozen sections (94,222), or in toluidine blue-stained semi-thin plastic sections. Electron microscopy confirms the lattice

structure of the nemaline bodies. These are derivatives of the Z disk and the thin filament,  $\alpha$ -actinin being a main constituent. Nemaline bodies are also labeled with antibodies against myotilin and telethonin and associated with them are also actin, tropomyosin, and desmin (223–228). There is often a deficiency of either or both subtypes of the fast, type 2 muscle fibers (106), and often the exact fiber types may be difficult to define histochemically (205). No histologic means of definitively distinguishing between the various forms of nemaline myopathy has yet been found, although specific histologic features, if present, may be helpful in some cases in directing molecular genetic studies (5,162,229–231). (For reviews, see References (5,74,206).)

Some cases have shown intranuclear nemaline bodies, with or without sarcoplasmic nemaline bodies (for review, see references (232) and (233)). A number of these patients have been found to have mutations in the skeletal muscle  $\alpha$ -actin gene (*ACTA1*) (8,234).

A pathogenetic relationship between nemaline myopathy and congenital myopathy with excess of thin filaments (233) was established through the findings of mutations in the actin gene in both groups of patients (234).

It is to be noted that in the typical autosomal recessive form of nemaline myopathy, even heterozygote carriers may show deficiency of type 2B muscle fibers (61).

Nemaline bodies are not pathognomonic for congenital nemaline myopathy but have been described in combination with features of other myopathies (see Section 127.2.4), and in a number of contexts probably unrelated to muscle disease. These include patients with acute psychosis (134), patients who have undergone radiotherapy for cancer (235–237), patients undergoing hemodialysis (136), and animal muscle after tenotomy (137,238,239). They also occur normally in extraocular muscles (240), myotendinous junctions (241), and in the muscles of aged persons (242). Therefore, it is not justified to make the diagnosis of hereditary nemaline myopathy without a typical clinical picture.

Nemaline bodies in patients with human immunodeficiency virus infection have usually been regarded as epiphenomena, but in some cases, they have been associated with myopathy (138,243–248). One patient treated for dermatomyositis was examined because of lacking response to treatment and was found to have nemaline bodies (249). Cases of nemaline myopathy have been reported with mitochondrial abnormalities (250–255).

Yet another pathological pattern is exemplified by a sib pair with cardioneuromyopathy showing histologically characteristic central hyaline masses surrounded by nemaline bodies (161).

### 127.5.2 Clinical Characteristics

In the typical autosomal recessive form, most often caused by nebulin mutations, the patients commonly have a nasal voice or even dysarthria, the palatal reflex is usually absent, and most patients are unable to lift

their heads in the supine position (56,201). The weakness is more pronounced in the axial muscles and the limb girdles than in the proximal limb muscles. A definite distal involvement, including foot drop, may be noted later in addition to the proximal muscle weakness; this may be confused clinically with the peroneal paresis of a peripheral neuropathy, or with the distal myopathies. In fact, recently, nemaline myopathy has been shown occasionally to present with distal weakness (173). The tongue often becomes relatively small and furrowed. The facial and bulbar muscles are weak, but the extraocular muscles are spared. A skew toward higher levels of intelligence has been noted in one small series of individuals with nemaline myopathy (56).

Respiratory problems are a common feature of congenital nemaline myopathy, not only in the neonatal period, but also throughout life, and may be the presenting feature (157). Respiratory infections are common, especially from birth to school age, and should be treated vigorously but may subside with time (53). Although they are symptom free, most patients, including ambulant ones, will show restriction of their respiratory capacity on testing. The patients run a great risk of insidious nocturnal hypoxia, even in the absence of morning symptoms, and several patients have experienced sudden respiratory failure (53,56,58,59,256–259). Continuous monitoring of respiratory capacity and early employment of mechanical ventilation at night and, if required, intermittently during the daytime is therefore recommended (60,260). Freedom from hypoxia is vital for the patient's quality of life and for avoiding complications such as cor pulmonale. Strategies have been outlined for ventilatory support in the congenital myopathies (60).

Cardiac contractility is usually normal in congenital nemaline myopathy (261). Although cardiac involvement has been reported in some cases showing nemaline bodies in skeletal or cardiac muscles (262–270), only two cases appear indistinguishable from congenital nemaline myopathy, and in addition, show childhood onset of histologically verified dilated cardiomyopathy with nemaline bodies (262,263). Furthermore, nemaline bodies have been observed in developing cardiac hypertrophy in patients with no clinical evidence of cardiomyopathy and with no disease of the skeletal muscles (271). Post-mortem investigations have in most cases not revealed nemaline bodies in heart muscle (69,72,73).

To our knowledge, there have been no reports of MH in congenital nemaline myopathy, although one report describes three children with nemaline myopathy in whom the heart rate decreased during induction of anesthesia for cardiac surgery, and body temperature increased during or after surgery (272). Thus, although patients with nemaline myopathy are not regarded as being at risk for MH, the anesthetist needs to be aware of the patient's muscle disorder and take precautions accordingly (185,187,188,273). This may be especially important in cases with unknown cause of the disease.

Fetal akinesia sequence has been postulated as an entity within the nemaline myopathies (44,274,275, 276), in addition to previous descriptions of arthrogryotic cases (277–279).

The International Consortium on Nemaline Myopathy has defined six clinical categories of nemaline myopathy, based on knowledge of the typical form and how some groups of patients differ from those with that form: (1) the severe congenital form, with patients lacking spontaneous movements or respiration at birth, or with fractures or severe contractures at birth; (2) the intermediate congenital form, with patients moving and breathing at birth but who are later unable to achieve ambulation or respiratory independence; (3) the typical congenital form, with typical distribution of muscle weakness, milestones delayed but reached, and slowly progressive or nonprogressive course; (4) mild nemaline myopathy with childhood onset; (5) adult-onset nemaline myopathy; and (6) other forms of nemaline myopathy with unusual associated features (205).

Adult-onset cases are heterogeneous and most are unlikely to be genetic in origin. The recently defined sporadic late-onset nemaline myopathy (SLONM) (217–221,280) with subacute presentation after the third decade may show gammopathy, degeneration and a relentlessly progressive course (209,281–285), while patients without gammopathy appear to have a better prognosis (217). Recently, successful treatment for SLONM has been reported using stem cell transplantation or immunoglobulin (217–221,280). Some cases present with respiratory failure without any noted preceding muscle weakness (286–288), while others present with “dropped head” (215,216,289) or camptocormia (290).

### 127.5.3 Modes of Inheritance and Genotype–Phenotype Correlations

In familial cases of nemaline myopathy, autosomal recessive inheritance is more common than autosomal dominant inheritance (61,201). Autosomal recessive nemaline myopathy is most commonly caused by mutations in the *nebulin* gene, *NEB* (6–8,200,206,291,292). Deletion of exon 55 is a founder mutation with worldwide occurrence in the Ashkenazi Jewish population, the reported carrier frequency being 1:108–1:168 (293). Rarer causes of recessively inherited nemaline myopathy include mutations in the slow skeletal alpha-actin gene, *ACTA1* (8,158,206,207,294), the genes for beta- or alpha-tropomyosin, *TPM2* and *TPM3*, troponin T1, *TNNT1*, or cofilin-2, *CFL2* (11,158,295). Recessive mutations identified in *ACTA1* are genetic or functional null mutations causing severe myopathy (296). These patients retain expression of cardiac actin in their skeletal muscles and the muscle fibers may also show zebra bodies. In *TPM3*, a homozygous deletion, a likely founder mutation has been identified in the Turkish population (297).



Autosomal dominant nemaline myopathy is often caused by mutations in the actin gene *ACTA1* (8,53,298,299) but may also be caused by mutations in *TPM2* or *TPM3* (9,10,12,300–302). In the first family to be described, an Australian family with a dominant *TPM3* mutation, weakness was most pronounced in the lower limbs (9,10), but subsequent descriptions have shown that this is not consistent (301,303). Fiber type distribution varies, but nemaline bodies may be present in type 1 fibers only. A small number of families have been described with dominant mutations in *TPM2* (12). Dominantly inherited nemaline myopathy caused by mutations in the *ACTA1* gene may be slightly more common, but even these cases fail to show consistent differences compared with the recessively caused cases and there is variability even within families (8,207,294,299,304). A peculiar form of muscle slowness, with inability to correct body position to avoid falls, has been described in two-thirds of families showing linkage to the chromosome region 15q, with a myopathy featuring onset of weakness in early childhood and the presence in the muscle fibers of both nemaline bodies and core-like formations (148,305–308). This form of dominantly inherited nemaline myopathy has recently been shown to be due to mutations in the gene *KBTBD13* (14).

Sporadic cases are often caused either by new dominant mutations in *ACTA1* (8,207,234,294,298,309,310) or by two recessive nebulin mutations (292). Thus, it is worth testing first for mutations in *ACTA1*, because this is a small gene of six coding exons, and testing is available as a service. Molecular genetic confirmation of the diagnosis in nemaline myopathy caused by mutations in the other major gene, nebulin, still poses a problem because of the size of the gene. It comprises 249kb of genomic sequence and 183 exons (311). Mutation analysis of the other small genes is thus recommended before embarking on nebulin testing.

In addition to the above-mentioned seven genes, at least an eighth gene is postulated for nemaline myopathy (201,312), and candidates are being explored among the genes for thin filament proteins and the Z disk (313).

Genotype–phenotype correlations among families in which mutations have been identified are slowly emerging, but appear complicated (205,206,292,314–318). A locus-specific mutational database has been established for mutations in *ACTA1* (<http://waimr.uwa.edu.au>), in which no missense polymorphisms have been reported. A similar database for *NEB* is under way ([http://www.dmd.nl/nmdb2/variants.php?action=search\\_unique](http://www.dmd.nl/nmdb2/variants.php?action=search_unique)). In addition to nemaline myopathy, mutations in *ACTA1* can cause intranuclear rod myopathy (8,234,319–321), actin filament aggregate myopathy (8), congenital fiber type disproportion without nemaline bodies (38), myopathy with core-like areas (322), and cap disease (323). *NEB* mutations may cause distal nebulin myopathy, core-rod myopathy and nemaline myopathy with a distal presentation (159,173,324).

A detailed study of 60 patients with mutation of one of the two most common causative genes, *ACTA1* and *NEB*, showed some differences in clinical severity and in the distribution of muscle weakness (206,314), corroborated by comparisons of muscle MRI results of patients with mutations in the same genes (90,91). Severe nemaline myopathy is more often caused by *ACTA1* mutations. In both groups of patients, muscle weakness was generalized, usually symmetric, and most pronounced in the neck flexors. In patients with *NEB* mutations, the ankle dorsiflexors were also very weak, whereas the extensors of the knees were well preserved in comparison with the knee flexors. In patients with *ACTA1* mutations, the knee extensors were weaker than the flexors, and there was better preservation of the ankle dorsiflexors.

In some cases, histologic findings may direct mutation detection. If the muscle biopsy shows uneven staining for nebulin, a nebulin mutation is probably more likely than an *ACTA1* mutation (200,229,230), and if there are intranuclear nemaline bodies, an excess of thin filaments, retained expression of cardiac actin in muscle or zebra bodies, an *ACTA1* mutation is likely (8,316). In patients with both fast and slow muscle fibers present, more severely affected type 1 fibers and normal-appearing type 2 fibers may be a clue to a mutation in *TPM3*, not expressed in fast fibers (297,301,325). Core-rod myopathy may be caused by mutations in *RYR1* (22,23), *ACTA1* (157), *CFL-2* or in *KBTBD13* (14,148,158,305).

The typical form of nemaline myopathy (56,205) is usually due to mutations in *NEB* (6,7,200,291), but sometimes to new dominant mutations in *ACTA1* or *TPM2* (206,294,298).

In families with severe nemaline myopathy, mutations have often been identified in *ACTA1* (8,207,294,298,326) and, more rarely, in *NEB* (327). In a small number of families with *ACTA1* mutations, one parent showed somatic mosaicism for the mutation, a phenomenon not hitherto encountered in families with mutations in *NEB*. This needs to be taken into account in genetic counseling. Somatic mosaicism has been observed in two out of nearly 80 families in total in which *ACTA1* mutations have been identified (294). Moreover, an instance of gonadal mosaicism has been observed (Professor Nigel G. Laing, personal communication). The intermediate form may be caused by mutations in *TPM3* (295), *ACTA1* (206,294,315) or *NEB* (206,292). Some of the patients with recessive mutations in *ACTA1* have a milder myopathy than those with heterozygous mutations (316).

Mild nemaline myopathy has been caused by mutations in *ACTA1*, *NEB*, *TPM2*, *TPM3* or *KBTBD13* (14,206). Many of the patients with mutations in *KBTBD13* show a distinct slowness of movements (14,148,305–308).

A homozygous nonsense mutation in the slow tropomyosin T gene (*TNNT1*) causes an autosomal recessive form of nemaline myopathy in children of the Old Order Amish



community (13,328). Special features of this nemaline myopathy are tremor and progressive contractures.

The two patients reported with homozygous mutations in *CFL-2* differed from patients with the typical congenital form of nemaline myopathy in lacking facial weakness and foot drop (158).

#### 127.5.4 Pathogenetic Considerations

The pathogenetic mechanisms leading from mutation to nemaline myopathy are becoming discernible through functional studies of the implicated genes and their sarcomeric proteins. Models under study include mouse models (275,329–337), spontaneously occurring myopathies in animals (338–340), *Drosophila* models (341–343), baculovirus expression systems (343), tissue culture models, and functional, physiological and direct visualization studies of normal and altered proteins (16,275,276,311,320,321,325,344–358).

Mutations in the actin gene causing nemaline myopathy are mostly missense mutations, and they are spread all across the six coding exons (294). They are usually dominant mutations arising *de novo*, with dominant-negative effects (344,345). No missense polymorphisms have been identified (296). A mouse model has been generated for dominant *ACTA1* nemaline myopathy, confirming the observations in patients that the mutant protein load determines disease severity (332). The muscle fibers of these mice have lower calcium sensitivity than normal mice.

Patients with recessive mutations in *ACTA1* usually have no functional skeletal actin, and the severity of their disease is related to the proportion of retained expression of cardiac actin in their skeletal muscles (207,316). Normally, the expression of cardiac actin, predominating in skeletal muscle in early development, is later replaced by skeletal actin, while cardiac actin is the predominating form of actin present in the adult human heart (359). This explains why mutations in *ACTA1* do not, as a rule, cause cardiac involvement.

Because of the spectrum of different disease mechanisms caused by *ACTA1* mutations (16,296,320,321,342,350–352), it has been suggested that future therapies should be targeted toward a method that would be independent of the function of skeletal muscle actin (315). A mouse model has been generated to test the hypothesis that upregulation of cardiac actin may alleviate the myopathy caused by mutations in *ACTA1* (8,207,360). The results are very promising, with the skeletal actin knockout mice living into adulthood with good muscle strength. This leads on to the question of how this could be utilized for therapy in human patients. Increasing the proportion of normal skeletal actin in heterozygous patients is another possibility to explore further (332).

Mutations in the nebulin gene causing nemaline myopathy have been identified along the length of this enormous gene (291,292,311). All mutations published

to date have been recessive (7,200,291). Despite the fact that most of the mutations are truncating, the patients surprisingly express the C-terminus of the protein (200,229,230), and severe cases are rare. This may be explained by differential expression of the numerous isoforms of nebulin (311). Nebulin plays roles in specifying thin filament length, stabilizing and activating the filaments, as well as in ensuring force generation and efficiency of contraction (354–356). Based on the few mutations that have been studied in detail in terms of their effects, pathogenetic mechanisms leading to muscle weakness include dysregulation of thin filament length and cross bridge kinetics (355,361–363). The recently generated *NEB* knockout mouse models showing muscle weakness, but not consistently forming nemaline bodies (335,337), are contributing to the understanding of nebulin's role in the muscle sarcomere. The knockout mice have shorter thin filament lengths than normal mice and indicate roles for nebulin in capping thin filaments, in maintaining contractility and force generation and in stabilizing sarcomere structure, including Z disk structure and myofibrillar alignment (333,334). Interestingly, studies of the diaphragm muscle of the mice, a muscle severely affected in nemaline myopathy patients, show that myofibrillar disorganization begins to appear when the mice start using this muscle for breathing (335). These mouse models, however, being devoid of nebulin, and showing early lethality, mirror the human disease only to a limited extent. Since no human patients with mutations causing total absence of nebulin have been reported, it has been thought that null mutations in *NEB* would be lethal. It is thus remarkable that the knockout mice do form sarcomeres despite the absence of nebulin, indicating that nebulin is not required for myofibrillogenesis.

The mouse models with mutations in the *TPM3* and *ACTA1* genes have muscle weakness and show nemaline bodies in their muscle fibers (329–332,360). The onset of muscle weakness in the *TPM3* mouse, caused at least partially by hypotrophy of type 1 fibers, appears to be delayed by compensatory hypertrophy of type 2 fibers (329). Previous observations of immobilization having adverse effects on muscle volume and strength in nemaline myopathy (56) are corroborated by studies of the mice, which appear to benefit from postimmobilization endurance exercise (330,331). The mechanisms of muscle repair differ from those in normal mice, not exhibiting the classic features of regeneration (330,364). This is in accordance with what is known from follow-up biopsies in humans with nemaline myopathy, indicating an active disease process with segmental degeneration, reinnervation and regeneration (56,80,106). The quantity of nemaline bodies in the mice correlated with weakness, but this has not been the case in studies of patients (69,106,125,162,298). The mice showed cytoplasmic bodies also, which can indeed sometimes be found in patients with nemaline myopathy (144,329).

Missense mutations in *TPM2* appear to affect contractility of muscle fibers in a mutation-dependent way (347,349). Studies on animal models are needed to explore the recent suggestion that calcium sensitizers might have beneficial effects on single muscle fibers expressing tropomyosin altered by *TPM2* mutations (349). Mutation of *TPM2* causing absence of  $\beta$ -tropomyosin was found to underlie Escobar syndrome associated with nemaline myopathy (365).

Mutations in *TPM3* have been thought to cause nemaline myopathy by altering the stoichiometry and interrelations of tropomyosin dimers and their affinity for binding partners (325,346). Recent studies of five patients with *TPM3* mutations indicate that the contractile dysfunction is due to impaired cross bridge cycling kinetics, partly compensated by enhanced calcium sensitivity of force generation (362).

The truncating mutation in *TNNT1* causing nemaline myopathy with early respiratory failure in the Old Order Amish leads to the absence of slow skeletal muscle troponin T, and it has been suggested that upregulation of cardiac or embryonic troponin might be a therapeutic possibility (366,367), similarly to cardiac actin compensating for the loss of normal slow skeletal actin in *ACTA1*-caused nemaline myopathy.

The literature on management and therapy is scarce (5,368–370). The apparently beneficial effects a limited clinical trial of dietary tyrosine supplementation (369) warrants further study.

### 127.5.5 Actin Myopathy, Actin-Related Myopathies, Core-Rod Myopathy, Cap Myopathy, and Other Myopathies Within the Nemaline Myopathy Spectrum Caused by Mutations in Known Nemaline Myopathy Genes

In some patients with nemaline myopathy, a histologic finding in addition to that of intranuclear or cytoplasmic nemaline bodies is accumulation of excess thin filaments, the main constituent of which is actin (MIM 102610) (233). Another group of patients shows actin filament aggregation only. Mutations have been identified in the  $\alpha$ -actin gene in both groups of patients (8) and, interestingly, the mutations causing actin myopathy without nemaline bodies (294) and those causing intranuclear rod myopathy (315) tend to be clustered in particular parts of the gene corresponding to a sterically defined region of the actin protein. A recent study indicated that in intranuclear rod myopathy, the rods are formed within the nucleus (320,321). These cases often, but not always (371) carry a bad prognosis. One patient with mutation of *ACTA1* had extramuscular involvement including osteosclerosis (372). Zebra bodies and retained expression of cardiac actin have been identified in patients with *ACTA1* null mutations causing severe nemaline myopathy (316).

Moreover, mutations in the actin gene can cause CFTD with no additional specific features (38,373), also caused by *TPM3* mutations (40), or a myopathy with cores only (322).

*NEB* mutations may cause core-rod myopathy (159) as well as distal nemalin myopathy, with only distal weakness and the absence or inconspicuous presence of nemaline bodies (324). The recent identification of two families with distal presentation and nemaline bodies in their muscle fibers indicates that nemaline and distal myopathy form a continuum (173).

Cap myopathy may overlap with nemaline myopathy both clinically and histologically, some cases showing caps only (374). Causative genes include *TPM2* and *TPM3* (302,375,376), with evidence for a dominant-negative effect of one of the *TPM3* mutations identified (377).

Mutation of *TPM2* has been reported to cause dominant distal arthrogryposis (172,378) while a homozygous null allele leading to the absence of  $\beta$ -tropomyosin caused Escobar syndrome associated with nemaline myopathy (365).

## 127.6 MYOTUBULAR (CENTRONUCLEAR) MYOPATHIES

Myotubular myopathy (MTM) (or centronuclear myopathy, CNM) first described in 1966 (379), exists in all three Mendelian forms. The X-linked form (XLMTM) is the best characterized (MIM \*310400) (380) (55,238,381,382), and has been attributed to mutations in the myotubularin (*MTM1*) gene on chromosome Xq28 (15,383). Diagnostic criteria for this disorder have been outlined by the International Consortium for Myotubular Myopathy (384). The autosomal-dominant (MIM \*160150) (380) form has been associated with mutations in the dynamin 2 (*DNM2*) gene (17) while mutations in the amphiphysin 2 (*BIN1*) gene (18) and the skeletal muscle ryanodine receptor (*RYR1*) gene (19) have been implicated in autosomal-recessive forms of CNM (MIM 255200) (380). For an overview of different forms of CNM see references (385,386).

### 127.6.1 Special Features of Muscle Pathology

Histologically, the three forms of myotubular myopathy share similar features, with small muscle fibers showing a central area devoid of myofibrils and mitochondrial aggregates around the centrally located, often large nuclei (see Fig. 127-2E). Histopathological appearance at presentation may be nonspecific (387). The name “myotubular myopathy” derives from the resemblance of these fibers to normal muscle myotubes during fetal development, but in contrast to earlier suggestions (112,384,388–392) fibers do appear to mature over time (112) and the term “centronuclear myopathy” is often preferred, particularly for autosomal forms. Although strictly centralized

nuclei are more common than multiple internalized nuclei in the *MTM1* and *DNM2*-related forms (393), the opposite applies to *RYR1*-related cases (19). A radial distribution of sarcoplasmic strands on NADH staining has been reported in autosomal cases (394) and seems to be a specific feature of the late-onset form related to mutations in the dynamin 2 (*DNM2*) gene (17); the latter may also exhibit almost dystrophic features (395). Cores in addition to the myotube-like fibers may evolve over time in the *RYR1*-related form (116,396) but have also been reported in *DNM2*-related cases (397) and mildly affected patients harboring *MTM1* mutations (398,399). “Necklace” fibers may be an additional feature in various forms of CNM (398,399).

### 127.6.2 Clinical Characteristics

In addition to the features typical of all the congenital myopathies, the myotubular myopathies frequently cause ophthalmoplegia. Clinical differences between the X-linked and autosomal forms of myotubular myopathy are quantitative rather than qualitative. The X-linked form XLMTM is the most severe and has the earliest onset, the autosomal recessive form is intermediate in both respects, and the autosomal dominant form mostly has a later onset and a milder course than the other two (394). (For review, see also References (385,386).) Cardiomyopathy has been described in two sporadic cases in which mutation analysis of the *MTM1* gene was not performed (400,401). Malignant hyperthermia has been reported in a patient with adult-onset, genetically unresolved CNM (402).

### 127.6.3 Genetic Background

Identification of mutations in the myotubularin (*MTM1*) gene (15) in XLMTM, the dynamin 2 (*DNM2*) (17) gene in autosomal-dominant CNM and the amphiphysin 2 (*BIN1*) (18) and the skeletal muscle ryanodine receptor (*RYR1*) gene (19), respectively, in autosomal-recessive CNM have permitted mutation detection and confirmation of the mode of inheritance (15,383). A proportion of cases remain currently genetically unresolved.

In XLMTM, some carriers will show histologic abnormalities, and some have muscle weakness on examination (119), but it is uncertain whether similar manifestations occur in heterozygotes for the autosomal recessive genes (394) or in subclinical cases of the autosomal dominant form. Furthermore, the proportion of sporadic cases caused by new mutations remains to be determined for the genes more recently implicated in CNM.

Because the clinical differences between the autosomal and the X-linked forms are quantitative rather than qualitative, in all new (and in particular, sporadic and male) cases of CNM, it is worth checking for mutations in the *MTM1* gene in the first instance, also considering the relatively small size of the gene. Female cases may

be due to a *MTM1* gene defect in combination with a chromosomal rearrangement or skewed X-inactivation (119,403–408). If a mutation cannot be detected in the *MTM1* gene, depending on clinical and pathological presentation, either unusual *MTM1* variants (409) or mutations in one of the autosomal genes associated with CNM ought to be considered. Myotonic dystrophy is an important differential diagnosis for all forms of CNM and should be considered early in the diagnostic process.

### 127.6.4 X-Linked Recessive Form

The X-linked form commonly has its onset in utero, and the pregnancy is often complicated by polyhydramnios, (55,57,114,127,132,381,382,384,410–414) a feature rarely seen in the other congenital myopathies. The family history often includes miscarriages and male neonatal deaths in the maternal line (415) but there may be marked phenotypical variability even within the same family (416). Many affected infants lack spontaneous antigravity movements, and some fail to establish spontaneous respiration at birth (417). Contractures of the hips and knees are commonly present. Affected boys are often long, with low birthweight (418) and large head, and many show ophthalmoplegia. EMG may be normal, or “myopathic,” or may show abnormal spontaneous activity (76). Neuromuscular junction abnormalities and/or an associated neuromuscular transmission defect have been reported in XLMTM and other forms of CNM (81,419). The disease usually, but not invariably, follows a fatal course over days or weeks. Other patients may be mildly affected from the neonatal period onward with little residual disability (51,55,57,417,420–422). However, treatment policies may influence survival as much as the underlying mutation: A study from the United States (423), where treatment policies are active, reported 26 of 35 male patients surviving for at least 1 year. However, about 60% of these patients aged 1–27 years were completely ventilator-dependent, and some showed associated medical problems related to organs other than muscle, such as pyloric stenosis, gallstones, and liver hemorrhage, although those features are not universally observed in adult survivors (51,420,422).

Some female carriers of the X-linked form have slight muscle weakness on examination (119,132,424), and a few have been reported as being overtly manifesting due to skewed X-inactivation (119,404–408) or abnormalities of the X chromosome (403). Manifestation in female carriers of XLMTM may be very late in life (425). Muscle biopsy studies of obligate female carriers indicate that about one-half of carriers may have specific abnormalities on muscle biopsy, but a normal biopsy does not exclude the risk of being a carrier (57,127). Carrier status is best determined by molecular genetic methods.

Muscle biopsies of boys with the X-linked form of myotubular myopathy have been reported to show

abnormal persistence of the proteins desmin and vimentin (391,392), but this is not a consistent feature (112,426) and does probably not distinguish XLMTM from autosomal forms of CNM (427,428).

Following initial studies linking XLMTM to proximal Xq28 (403,429–436) causative mutations in the myotubularin (*MTM1*) gene at this locus were subsequently identified (15,383,417,437,438). It is to be noted that the previous indication of genetic locus heterogeneity in X-linked myotubular myopathy (439,440) was later refuted (440); the failure to identify causative mutations in affected males with typical clinical features may be due to the presence of unusual *MTM1* mutations (409).

The *MTM1* gene encodes four active sites, and mutations have been identified in all of these (116). Although *MTM1* mutations are spread across the length of the gene, exons 12, 8, and 14 are more commonly mutated than the others, harboring about 50% of mutations, whereas seven mutations account for about 25% of cases (116,118,438,441–446). Approximately 30% are missense mutations affecting conserved residues (116,441). Some deep intronic mutations may not be detectable on routine sequencing (409). Molecular genetic confirmation of diagnosis, determination of carrier status, and prenatal diagnosis are available as a routine service (116–119). New mutations in affected male patients are not as common as previously thought (441); a majority of mothers of affected boys will be found to be carriers (116). At least five instances of mosaicism have been documented (441,447,448), so that, even in cases in which the mutation detected in an affected son has not been found in the mother's lymphocyte DNA, the parents might want to consider prenatal mutation testing of male fetuses in subsequent pregnancies.

No strong correlation appears to exist between clinical severity and the nature of the mutation; severity varies even within families. Two boys with myotubular myopathy and intersexual genitalia had a deletion extending beyond the myotubularin gene (449). An extensive study addressing genotype–phenotype correlations (417) indicated that some nontruncating mutations are associated with a milder clinical course (see also references (51,420,422)), whereas other nontruncating mutations and all known truncating ones are associated with the common, neonatally very severe form.

### 127.6.5 Autosomal Dominant Form

A substantial number of families compatible with autosomal-dominant inheritance have been reported in the premolecular era (57,168,394,427,450–462) and a proportion of cases have now been attributed to mutations in the dynamin 2 (*DNM2*) gene (17,393,463–466).

Typical *DNM2*-related CNM is a relatively mild condition but severity varies widely, with onset from birth to the fifth decade (463,467). Even the most severe cases with neonatal onset may show improvement over time

(463,468), provided respiratory complications are managed proactively as those may be fatal if left untreated (21). Facial weakness, ptosis and ophthalmoplegia are common. Muscle weakness is often predominantly proximal, but many patients show additional truncal and distal involvement (395,397). Marked muscle hypertrophy, particularly affecting the calves, has been reported in some patients (399). *DNM2*-related CNM is allelic to a form of dominant intermediate Charcot–Marie–Tooth disease (CMTDIB) (469) and occasional, subtle signs of peripheral or central nervous system involvement also reported in *DNM2*-related CNM suggest a continuum between the two entities in some patients (393,464,467). As in CMTDIB patients, non-muscular features such as neutropenia (399) or cataracts (21) may also be observed in a subgroup of patients with *DNM2*-related CNM. Muscle MRI shows a pattern of selective muscle involvement distinct from congenital myopathies with other genetic backgrounds (395,397,465).

In addition to the histopathological features shared by all forms of CNM, late-onset *DNM2*-related CNM typically features additional radial strands (17), and almost dystrophic features as well as core-like structures are not uncommon (395,397). “Necklace” fibers may be observed (399) but are not specific. In contrast to the *RYR1*-related form and in common with XLMTM, strictly centralized nuclei are more common than multiple and internalized nuclei (393).

Mutations affecting the *DNM2* middle domain are usually associated with milder presentations (17), whereas those concerning the pleckstrin homology domain give rise to more severe forms with earlier onset (21,395,463).

### 127.6.6 Autosomal Recessive Form

Autosomal recessive inheritance of CNM has been reported in the premolecular era in a number of families with suggestive clinical and pathological features (57,70,124,130,142,394,470–474) and has been recently attributed to mutations in the amphiphysin 2 (*BIN1*) (18) and the skeletal muscle ryanodine receptor (*RYR1*) (19) gene. In both forms age at onset is mostly in infancy or early childhood and ophthalmoplegia, ptosis, and facial weakness are common clinical features.

*BIN1*-related CNM has been reported only in few families to date (18,475–477) mainly associated with early onset and a severe phenotype, which may feature associated respiratory impairment and may result in loss of ambulation (477). Marked intrafamilial variability and associated mental retardation has been reported in one consanguineous family (475).

Mutations in the skeletal muscle ryanodine receptor (*RYR1*) gene, one of the more common causes of inherited neuromuscular disorders (see below), have been reported as another and probably relatively common cause of congenital myopathies with central nuclei (19,478).



Clinically, *RYR1*-related CNM is typically of early onset and moderate severity with improvement over time but may occasionally mimic the appearance of XLMTM in males. Ptosis and extraocular muscle involvement are common. Bulbar involvement requiring nasogastric tube feeds and respiratory impairment needing ventilatory support are often transient but a permanent ventilatory requirement has been reported in the most severe cases. On the histopathological level, multiple internalized nuclei in addition to typical centralized nuclei are common and core-like structures more suggestive of *RYR1* involvement may evolve over time (19,478). Associated congenital fiber type disproportion, another *RYR1*-associated histopathological feature (41), may be prominent. *RYR1*-related congenital myopathy with central nuclei is often associated with compound heterozygosity for *RYR1* missense mutations and *RYR1* mutations resulting in low abundance of functional RyR1 protein (19), corresponding to the situation in other recessively inherited *RYR1*-related myopathies (25,479). There appear to be no mutational hotspots. Some *RYR1* mutations may not be detectable on routine sequencing (480), probably explaining the substantial proportion of cases with presumably recessive inheritance but only one heterozygous missense mutation identified, either inherited from an asymptomatic parent or of de novo occurrence (396). *RYR1*-related CNM is common in the South African population due to the presence of multiple founder effects (19).

There is likely to be further heterogeneity. Tosch et al. reported two CNM patients with missense variations in the novel phosphoinositide phosphatase (hJUMPY), in one patient associated with a de novo mutation in the *DNM2* gene. The inheritance in these families is uncertain but could be either recessive with an undetected second allele, or digenic (481).

### 127.6.7 Pathogenetic Considerations

Most of the genes implicated in various forms of CNM to date, namely *MTM1*, *DNM2* and *BIN1*, encode proteins involved in various aspects of membrane trafficking (for review, (482)) and endocytosis. Alterations of skeletal muscle ryanodine receptor (RyR1) assembly and function have more recently emerged as an important pathogenetic mechanism in CNM, both primary and secondary, the latter mediated through indirect effects of *MTM1*, *DNM2* and *BIN1* mutations on the assembly of the EC machinery (483).

*Myotubularin* belongs to the large family of dual-specificity phosphatases that play a role in the epigenetic regulation of signaling pathways involved in growth and differentiation (for review see references (484,485)). There are homologs of myotubularin in many species, including mice and yeast, as well as many human homologs (15,441,486–489), some of them found to be mutated in distinct forms of Charcot–Marie–Tooth

disease (490–493). A specific function has been proposed for myotubularin in dephosphorylating phosphatidylinositol 3-phosphate (PtdIns3P), a lipid second messenger with a crucial role in membrane trafficking (383,494–505). In addition to the catalytic site, myotubularins contain lipid and protein binding sites and form homo- and heterodimers, in which active and inactive protein binding to each other may produce conformational changes affecting substrate affinity, hydrolysis, and binding to associated proteins (502,506–508). More recently, secondary effects have been reported of loss of myotubularin on T-tubule assembly (509), endosomes (510) as well as desmin intermediate filament architecture and mitochondrial dynamics (511,512). The deleterious effect of specific *MTM1* mutations may thus be due to destabilization of the three-dimensional structure, loss of enzymatic activity or disturbed protein–protein interactions (513). Findings in a myotubularin knockout mouse model (514) suggest that myotubularin is necessary for the maintenance of muscle fibers but not for myogenesis, and that the myotubularin-deficient phenotype may be correctable by delivery of the functional protein through viral vectors (515) or other therapeutic interventions (516).

Mutations in the *dynamamin 2* (*DNM2*) gene have been shown to disturb specific enzymatic functions (517), dimerization of the mutant protein (518) and endocytosis pathways (519). A knock-in mouse model of the common *DNM2* R465W mutation (520) exhibits impaired skeletal muscle structure and function, with specific evidence of increased intracellular calcium and dysferlin accumulation. Disturbed reticular assembly and secondary mitochondrial alterations are a feature in the mouse model and have also been reported in humans (521). (For review of dynamamin 2 physiology and pathophysiology see reference (522).)

Mutations in the *BIN1* gene encoding *amphiphysin 2* have been demonstrated to abolish interactions with dynamamin 2 and to disrupt the membrane tubulation properties of the mutant protein (18), corresponding to the important role of the functional protein in T-tubule biogenesis (523).

An autosomal form of CNM in Labrador retriever dogs has been attributed to dominant mutations in the *PTPLA* gene and may provide a model for currently genetically unresolved forms of the condition in humans.

## 127.7 THE “CORE MYOPATHIES”: CCD AND MMD

Core myopathies are a clinically and genetically heterogeneous group of congenital myopathies with the common defining histopathological feature of focally low oxidative activity on muscle biopsy (for review see references (524,525)). The variability of oxidative stain abnormalities is reflected in the wide range of descriptive terms (“central

cores, minicores, multicores, multi-minicores”) applied to these conditions and has provided the basis for the designation of histopathologically defined entities, namely central core disease (CCD) and multi-minicore disease (MmD). Recent years have seen major advances in the genetic resolution of the core myopathies, with mutations in genes encoding proteins involved in calcium homeostasis and EC coupling, namely the skeletal muscle ryanodine receptor (*RYR1*) and the selenoprotein N (*SEPN1*) gene, being the most common identifiable causes. Congenital myopathies with cores on muscle biopsy have been less frequently reported in genetically distinct forms, often associated with clinico-pathological features unusual in the context of the more common genetic backgrounds. The process from the histopathological diagnosis of a core myopathy to the identification of a specific genetic defect is complex and ought to be informed by a combined appraisal of histopathological, clinical and, increasingly, muscle MRI data in a multidisciplinary setting.

### 127.7.1 Central Core Disease

In 1956, Magee and Shy (526) reported a family where five individuals over three generations were affected by congenital, nonprogressive hypotonia and weakness, sometimes with prenatal onset. Muscle biopsy in the affected members of this family showed amorphous central areas within almost every muscle fiber stained with the modified Gömöri trichrome technique.

The term CCD (MIM \*117000) (380) was introduced later (527), and a characteristic absence of oxidative enzyme activity in the core area secondary to mitochondrial depletion was identified as the histopathologic hallmark of the condition (528).

#### 127.7.1.1 Special Features of Muscle Pathology.

Muscle biopsy demonstrates amorphous central areas in most muscle fibers. Histochemical staining shows the absence of oxidative and glycolytic enzymatic activity from these central core regions. Central cores may be single or multiple, or central or eccentric (95,528–530), but they commonly run along a significant extent of the longitudinal muscle fiber axis (531,532). Marked type 1 predominance or uniformity and hypotrophy are typical, whereas type 2 fibers may show hypertrophy, splitting or atrophy. Type 1 uniformity may be the only abnormal feature at presentation (530,533). Central cores are typically found in type 1 fibers but may occasionally also be observed in type 2 fibers; one report describes the predominance of type 2C fibers with cores in most of these fibers (534). The degree of histopathologic changes may be variable and, as with all pathological interpretation, sampling site and the age of the patient have to be taken into account. Substantial increases in fat and connective tissue but not more overtly dystrophic features such as necrotic or regenerating fibers have been described in some patients, probably reflecting a marked degree of selective involvement as demonstrated on muscle MRI (89,535).

Electron microscopy demonstrates that: (1) the cores lack mitochondria and sarcoplasmic reticulum; (2) there are variable degrees of myofibrillar disorganization; and (3) abnormal Z-band material has accumulated. The cores are usually sharply demarcated from the surrounding normal myofibrils (536). Some (“structured”) cores preserve a degree of myofibrillar organization and, therefore, retain some ATPase activity; other (“unstructured”) cores do not, and can be found in the same muscle biopsy (79,531). The sarcoplasmic reticulum and T-tubule systems are also affected, with an increase in the abundance of both organelle systems (537).

Immunohistochemical studies have demonstrated abnormal expression of various sarcomeric and intermediate filament proteins (112,538,539). Abnormal expression of slow myosin isoforms corresponding to the observed type 1 predominance on histochemical stains has been repeatedly described, but the organization of myosin and other sarcomere components is rarely disturbed (539). Abnormal distribution of desmin in and around the core areas is prominent (112,538). Central cores stain strongly positive with antibodies to the actin cross-linking protein filamin C, but this is a nonspecific finding and may be observed in other situations associated with core formation (540). More recently, distinct abnormalities of proteins involved in calcium handling and homeostasis have been suggested to differentiate between distinct specific genetic backgrounds associated with core myopathies (541).

Both the etiology and pathogenesis of central cores remain unresolved and are the topic of ongoing investigation in animal models of the condition (for a review, see Reference (542)). Core formation is not a primary pathologic process specific to CCD but can be observed in other contexts such as in several different clinical disorders (543), following tenotomy (137), secondary to denervation (“target-fibers”) (529), during reinnervation in experimental animals (544) or even in healthy probands following eccentric exercise (545). Biopsy features of CCD may be found in association with hypertrophic cardiomyopathy but, sometimes, with little or no clinical evidence of a skeletal myopathy (160,546). It is also important to emphasize that the presence of cores on muscle biopsy without associated weakness, as has been reported in some malignant hyperthermia susceptible individuals (547), is not sufficient to constitute a diagnosis of a core myopathy. Features of CCD and other congenital myopathies may be observed in the same biopsy, and the common occurrence of central cores, minicores, and nemaline bodies in the same patient has been repeatedly reported (123,154,155) and recently been attributed to a wide range of different genetic backgrounds (see Section 127.7.1.6). Therefore, care must be taken in the interpretation of a muscle biopsy with features of CCD in the presence of another disease entity or if the clinical presentation is atypical.

**127.7.1.2 Clinical Characteristics.** CCD is typically transmitted as an autosomal dominant trait and presents

in infancy with hypotonia or in early childhood with motor developmental delay (59,79,534,548–552); there is, however, marked clinical variability, and much milder (122,531,553–555) as well as more severe presentations within the range of the fetal akinesia sequence (45) have been reported, often in sporadic cases and associated with recessive inheritance. Variation in age of onset and severity may occur within the same family (536,556,557). Myalgia, muscle stiffness and exertional weakness with or without rhabdomyolysis are often associated and may be the only presenting feature (558–560). Weakness in most familial cases is pronounced in the hip girdle and axial muscle groups (79,534,548–552), and, in rare patients reported before the molecular resolution of the condition, may be associated with focal wasting (561). An adult-onset, almost exclusively axial myopathy has been recognized recently and may be a late myopathic manifestation of some MH mutations (555). Facial involvement is usually mild, and lack of complete eye closure may be the only finding. Extraocular muscle involvement is not a prominent feature in dominant CCD but is common in recessive *RYR1*-related myopathies. Bulbar involvement and associated chewing or swallowing difficulties are uncommon.

Orthopedic complications are common in CCD (562). Congenital dislocation of the hips, often resulting in persistent instability or hip contractures, is frequently associated and may be more likely when the child has been delivered through breech presentation (128,557,562,563). Scoliosis is a frequent feature and may be present from birth (553). Foot deformities include talipes equinovarus and pes planus (562). Contractures other than tendon Achilles tightness are rare, and many affected individuals have marked ligamentous laxity, occasionally associated with patellar instability (562,564).

Cardiac abnormalities other than mitral valve prolapse appear to be rare (560). An associated cardiomyopathy has not been reported in *RYR1*-related CCD but may occur in other myopathies with cores on muscle biopsy. For example, a group of patients with hypertrophic cardiomyopathy secondary to mutations in the *MYH7* gene encoding the slow/β myosin heavy chain may show central cores on muscle biopsy but typically do not have associated muscle weakness (see Section 127.9.1 and Section 127.7.1.6) (160). Respiratory involvement in typical dominantly inherited CCD is rare and has only been reported in a few isolated cases (565,566).

Malignant hyperthermia is a frequent complication (183). Many patients with CCD test positive for the malignant hyperthermia susceptibility (MHS) trait (560,567) whereas MH-susceptibility has been excluded in others. Considering that many of the more recently identified *RYR1* mutations have not yet been functionally characterized or formally assessed for their potential MH risk, our personal practice is to consider all patients with CCD at risk and to advise appropriate precautions

during general anesthesia unless proven otherwise. For the association between CCD and the potential MH risk see reference (190).

Apart from the most severe neonatal cases and some of those with congenital dislocation of the hips (45,126), most patients achieve the ability to walk independently. The course of CCD is static or only slowly progressive, even over prolonged periods of follow-up (568). Affected women may show intermittent deterioration of symptoms during or after pregnancy (192).

SCK activity is usually normal, but some patients have shown elevations up to 6–14 times normal (556,569,570).

Muscle ultrasound may be helpful in the assessment of individuals from CCD families, as it often shows a striking increase in echogenicity within the quadriceps even in paucisymptomatic individuals, with relative sparing of the rectus femoris compared with the vastus intermedius (88,571). A characteristic pattern of selective involvement on muscle MRI has been reported in patients with typical dominantly inherited CCD (89) and is distinct from that observed in core myopathies unlinked to the *RYR1* locus and from other congenital myopathies (89,535,572).

**127.7.1.3 Central Core Disease, Malignant Hyperthermia, and the King–Denborough Syndrome.** The relationship between CCD and MH is complex. Both conditions have been associated with mutations in the skeletal muscle ryanodine receptor gene (*RYR1*) but penetrance of the MH trait is low and there are likely to be additional modulators.

MH is a pharmacogenetic disorder of skeletal muscle characterized by an abnormal response to muscle relaxants such as succinylcholine and to volatile anesthetics (573). It is a severe and sometimes fatal reaction characterized by muscular rigidity, rhabdomyolysis, rapid increase in body temperature, and signs of generalized metabolic decompensation; survivors can suffer severe renal and neurologic damage (for review see references (574,575)). Males are more often affected than females at a ratio of 2:1. The estimated incidence of MH reactions lies between 1:15,000 in children and 1:50,000 in adults (576–578); however, the trait has low penetrance (576) and susceptibility may be much higher (579), as supported by studies indicating the frequency of the MHS trait as 1:2000–1:3000 in France (580) and the carrier frequency for heterozygous *RYR1* mutations in the Japanese population as 1:2000 (581).

Diagnosis of MHS is possible by applying the in vitro contracture test (IVCT), an invasive diagnostic test based on the mechanic response of muscle fibers to the triggering agents halothane and caffeine. The IVCT is interpreted according to two different protocols, the European MH group (EMHG) protocol (582) and the North American MH registry (NAMHR) (583) protocol. According to the EMHG protocol, individuals are classified as MH normal if they are not hypersensitive to either agent and as MHS if they are hypersensitive to both. In

contrast to the NAMHR protocol, individuals are classified as MH equivocal (MHE) if they are hypersensitive to either of the two agents.

MH was recognized as a familial, autosomal dominant trait by Denborough et al. (584) in Australia. Later studies of MHS families in Australia and New Zealand (585) and other countries identified two main groups, families in which MHS was clearly transmitted as an autosomal dominant trait with or without myopathic features, and sporadic or multiplex cases with negative family history and an associated combination of consistent dysmorphic findings.

In the first group of families with clear autosomal dominant inheritance, at least some individuals had evidence of a myopathy with clinical features similar to CCD; however, central cores on biopsy were absent in those patients (550) or not always typical of CCD (532). The link between MH and CCD was established in a family in which the proband had suffered an MH reaction and his aunt was found to suffer from a mild congenital myopathy characterized by central cores and type 1 atrophy on muscle biopsy; she also had an abnormal response to halothane (183). Other individuals with CCD were subsequently reported who developed MH under anesthesia or who were MHS on testing (550,560,563,569,586). Elevated SCK levels have often been found in those individuals with a myopathy or known to be MHS (550,563,585), but the predictive value of these findings for MHS, even in CCD families, is questionable (560,587).

The second group of patients described in the original Australian series (585) and subsequent reviews (588,589) were characterized by MHS, a mild myopathy, delayed motor development, and consistent dysmorphic features comprising ptosis, down-slanting palpebral fissures, neck webbing, scoliosis, pectus deformity, short stature, and cryptorchism (i.e. King–Denborough syndrome, KDS). Additional features in other families have also included vertebral fusion, eventration of the diaphragm and spinal cord tethering (590). The four cases in King and Denborough's original series were all sporadic and male, but subsequent reports have included a few women and siblings with unaffected parents and a severe phenotype with prenatal onset (591–593). A presumably recessive MH-associated phenotype with dysmorphic features identical to the KDS has been described in the Lumbee Indians of south central Carolina ("Native American myopathy, NAM") (OMIM 255995) (380), a specific ethnic group with probable consanguinity (594). Although the majority of parents of sporadic or multiplex cases with KDS and NAM were clinically normal, in other families, mild myopathic features such as ptosis, high SCK levels, MHS on IVCT (595), and increased fatigability (596) have been reported.

KDS shares many dysmorphic features with Noonan's syndrome, and the phenotypic overlap between the two conditions has been systematically evaluated (597). Despite

the striking overlap of facial features, kyphoscoliosis is less common in Noonan's syndrome and heart defects are not usually a feature of KDS. Moderate elevation of SCK and MHS, which are common in KDS, have been reported in only one patient each with apparent Noonan's syndrome (597,598). These syndromes are likely to be etiologically distinct, and assignment of the correct diagnosis in individual cases is important because of the association with MHS in KDS but not in Noonan's syndrome.

Susceptibility to MH or MH-like disorders has also been associated with a number of other muscle disorders, including carnitine palmitoyl transferase deficiency (599), hypokalemic periodic paralysis (600), and myotonia congenita (585). For a review of the relationship between MH and other neuromuscular disorders, see references (189,190).

**127.7.1.4 Genetics of Central Core Disease and Malignant Hyperthermia.** The homology of MH to the porcine stress syndrome, a naturally occurring animal model with almost identical features due to a founder mutation (601), led to the consideration of the skeletal muscle ryanodine receptor (*RYR1*) gene at human chromosome 19q13 as a likely candidate for this condition. Its function was shown to be abnormal in MH (602,603), and some families with MH showed linkage to this area (602,604–606), as did those with CCD (607–609). The identification of mutations in the *RYR1* gene, which segregate with the disease in families with MH and CCD, suggested that the two disorders could be caused by mutations in the same gene (20,610–612). An identical mutation (Arg163Cys) was found in a family with MH and in a family with CCD (612). In another family identified because the proband had CCD, several individuals were found by IVCT to be susceptible to MH but had no evidence of CCD on muscle biopsy (613). In such families, the mutation conferring susceptibility to MH may also predispose to CCD in the presence of other (probably genetic) triggering factors (612). This model of MH and CCD is supported by reports of *RYR1* mutations that are associated with MHS in most individuals but only occasionally with CCD, or with multi-minicore myopathy (547).

*RYR1* is a large gene with 106 exons (614), and it encodes a protein of 5037 amino acids, the skeletal muscle ryanodine receptor (RyR1), a  $\text{Ca}^{++}$ -release channel located in the sarcoplasmic reticulum terminal cisterna (615), playing a crucial role in EC coupling.  $\text{Ca}^{++}$ -release via the RyR1 is primarily triggered by the dihydropyridine (DHPR) receptor, which undergoes voltage-induced conformational change allowing it to directly interact with, and activate, the RyR1 (for review, see reference (616)). The predicted structure of the ryanodine receptor suggests that the  $\text{Ca}^{++}$ -release channel is located in the C-terminal part of the protein, whereas the remaining N-terminal portions face the myoplasm and constitute the visible foot structure that interacts with the DHPR receptor (617,618).



More than 100 mutations have been identified to date in the *RYR1* gene (for review, see references (619–621); also (22,581,622–629).) The majority of *RYR1* mutations associated with CCD and MHS identified to date were dominant missense mutations with only a few small deletions reported (22,581,623,624,626–633). Of note, missense mutations associated with MHS in the heterozygous state may give rise to congenital myopathies in the homozygous state (634).

The frequency of specific MH-related mutations depends on the study population, and there are indications that a few MH-causing *RYR1* mutations may account for a significant proportion of MH cases. A single mutation (Gly341Arg) accounted for 10% of MH-susceptible cases in one series (635), and the Arg-2433Gly mutation discussed earlier was present in eight probably unrelated families. Four adjacent, novel mutations were found to account for 11% of *RYR1* mutations in a collaborative European study (636). The Arg614Cys mutation is more common in German families (637), the Gly341Arg change is frequently observed in Irish/English families (636), and the Val2168Met substitution is prevalent in Swiss MH pedigrees (636,638). The Arg4861Cys substitution has been identified in three unrelated CCD families (622), but data regarding the frequency of other CCD-related mutations are currently still emerging.

The complex genotype–phenotype correlations associated with mutations in the *RYR1* gene may be partly explained by the degree of functional differentiation within this large protein. The first mutations identified predominantly gave rise to the MHS phenotype and affected two regions of the protein mainly: the cytoplasmic N-terminal domain (MHS/CCD region 1, amino acids 35–614) and the cytoplasmic central domain (MHS/CCD region 2, amino acids 2163 to 2458). Although mutations associated with a congenital myopathy phenotype have been identified in these regions (20,612,635,639), initially many families with CCD remained genetically unresolved despite typical clinical and histopathologic features. Following identification of an “unusual” *RYR1* C-terminal mutation in a large Mexican pedigree with a severe CCD phenotype (640), there has been increasing evidence that mutations affecting the C-terminal portion of the receptor molecule (MHS/CCD region 3, amino acids 4550 to 4940) are common in patients with CCD (22,23,622–624). It has now emerged that dominant *RYR1* mutations affecting the cytoplasmic N-terminal (MHS/CCD region 1, amino acids 35–614) and central (MHS/CCD region 2, amino acids 2163–2458) domains of the protein give predominantly rise to the MHS phenotype (641), whereas the CCD phenotype is closely associated with dominant *RYR1* C-terminal (MHS/CCD region 3, amino acids 4550–4940) mutations (23,622–624,640,642), with few reported families deviating from this rule (20,639,643,644). However, as some of the above studies were based on a limited screening of the *RYR1* gene, our understanding of genotype–phenotype correlations is

likely to evolve further now that sequencing of the entire *RYR1* coding sequence has become more common.

The majority of *RYR1* mutations associated with MHS or CCD described to date was dominantly inherited, but recent evidence suggests that dominant inheritance cannot always be assumed in cases with unusual features or unexpected severity when only part of the *RYR1* gene has been screened. Homozygosity or heterozygosity for *RYR1* mutations has been rarely described in association with MHS but is increasingly recognized in the context of congenital myopathy phenotypes, more commonly of the MmD rather than the CCD type (see below). The recognition of healthy (but MHS) individuals homozygous for certain *RYR1* mutations already suggested that some mutations are milder than others (645), although for some homozygotes, the sensitivity to caffeine on the IVCT may be enhanced (640). More recently, homozygosity and compound heterozygosity for *RYR1* mutations inherited from asymptomatic parents has been reported in mild cases with histopathologic features of MmD, (see below) and, less frequently, CCD (63,625), in some cases associated with a fetal akinesia syndrome with dysmorphic features and extraocular involvement (45). One affected infant in the latter series was a compound heterozygote for Arg614Cys, one of the most common MH-associated mutations previously not associated with a congenital myopathy phenotype, and a novel Gly215Glu mutation affecting the same domain of the protein. These findings are in keeping with observations that clinically silent MH mutations may give rise to a severe phenotype in the compound heterozygous or homozygous state, owing to a combined deleterious effect on the tetrameric RyR1 protein (634).

Considering the prominence of dysmorphic features and skeletal malformations associated with recessively inherited *RYR1* mutations, compound heterozygosity or homozygosity for *RYR1* mutations is also a probable mechanism in the KDS and other related syndromes. Most published cases of KDS were either sporadic or compatible with recessive inheritance, and subtle clinical or biochemical abnormalities in a parent in some of the reported families are compatible with the carrier state of a clinically not fully penetrant mutation. Indeed, Rueffert et al. (590) reported a 15-year-old boy with MH and clinical features of KDS who had inherited a novel *RYR1* mutation in exon 48 from his mother, who only had MH and central cores on muscle biopsy but no weakness, suggesting the presence of additional modifiers in the child. Another patient with KDS was found to carry a heterozygous *RYR1* missense mutation that had occurred de novo (646). A more recently published series indicates that KDS may be consistently due to compound heterozygosity for *RYR1* missense mutations, often associated with MH and running in the family, and a second *RYR1* mutation, easily missed on routine sequencing or if only partial *RYR1* screening was performed, resulting in low quantities of functional RyR1 protein (647).

The functional effects of specific *RYR1* mutations have been studied in response to the IVCT, in cultured myotubes from probands and in various homologous and heterologous expression systems. *RYR1* mutations are thought to alter excitability and calcium homeostasis within muscle cells, but the precise molecular mechanisms behind specific genotype–phenotype correlations are still emerging. Two models for mutation-induced receptor malfunction have been proposed: depletion of sarcoplasmic reticulum calcium stores with resulting increase in cytosolic calcium levels (“leaky channel” hypothesis) (648), and a specific disturbance of EC coupling (EC uncoupling hypothesis) (649). The pathogenetic consequences of individual *RYR1* mutations appear to depend on the specific effect on this structurally complex protein.

An earlier collaborative European study (636) already suggested an association between a specific mutation and the response to the IVCT; there were also some indications of a correlation between the particular *RYR1* gene mutation in a family and the severity of MH or liability to CCD (567,636,650). Further studies based on human myotubes demonstrated increased agonist sensitivity associated with *RYR1* mutations (648,651,652); however, agonist sensitivity appears to depend on the location and nature of the mutation and may even be reduced by mutations affecting certain portions of the protein (653). Studies of a mutation (I4898T) in the RyR1 C-terminal domain associated with a severe CCD phenotype demonstrated a severe reduction in agonist-induced calcium release and an increase in the resting calcium concentration (640,654); investigation of the same mutation also revealed a marked disturbance of EC coupling (655), which was also found for some of the other mutations affecting the RyR1 C-terminal domain (656). Additional studies of *RYR1* mutants expressed in myotubes of *RYR1* knockout (“dyspedic”) mice (657) indicate that MH-only mutations increase basal release channel activity insufficiently to alter net sarcoplasmic reticulum calcium content (“compensated leak”), whereas mixed MH/CCD mutations are associated with increased channel activity sufficient to deplete sarcoplasmic reticulum calcium stores, elevated intracellular calcium stores, probably mediated by decreased calcium sensitivity (658) and reduced maximum voltage-gated calcium release.

In addition to the more established tools for RyR1 functional studies, B-lymphocytes have recently emerged as a relatively easily accessible cellular system to study the pathogenesis of *RYR1* mutations in vitro (624,659–662). Expression of functional RyR1 channels in B-lymphocytes and dendritic cells leading to altered IL-6 secretion seen with some *RYR1* mutations also suggest a potential role of RyR1 in the immune system (654,663).

A number of animal models of *RYR1*-related MHS and CCD, namely the Y522S, the R163C and the I4895T knock-in mouse, have emerged over recent years, providing valuable insights into the pathophysiology of these

conditions and the evolution of associated histopathologic changes (542,664–671).

**127.7.1.5 Penetrance and Heterogeneity in Malignant Hyperthermia.** Mutations in the *RYR1* gene account for the majority of familial MH cases, with a prevalence of causative mutations indicated at 50–70% in different populations (626,628,629,632,641,644,672,673). However, a number of studies have shown discordance between the MH phenotype and the *RYR1* genotype (650,674–681); In a family with MHS, five mutation-negative individuals gave IVCT results as MHS, although they did not carry the G1021A mutation associated in seven other family members with MHS (682). Similarly, the MHS relatives of a girl with CCD showed recombination within the *RYR1* locus (613,683). Conversely, the MH-associated mutation C1840T has been found in individuals whose IVCT test results have been normal (645,675). These findings may reflect the presence of additional *RYR1* mutations in the same patient not detected if only a partial screen was performed (673), complex genomic rearrangements involving the *RYR1* gene not identifiable on routine sequencing (480), or variations in other, functionally related genes modifying the phenotype (684,685).

Limitations of the IVCT’s capacity to assign the MHS state correctly have also to be taken into account. The IVCT was designed as a clinical test to safely identify patients at risk for MH reactions, and thresholds for this testing procedure were chosen to avoid false-negative results with potentially grave clinical consequences. In both the North American and the European protocol, therefore, IVCT sensitivity is higher than specificity, but neither reaches 100%, with important implications for the study of phenotype–genotype correlations and linkage analysis. The occasionally reported discordance between *RYR1* genotype and MH phenotype (650,681) may therefore have been due to incorrect assignment of the MH-susceptibility state, resulting in obscured genetic linkage between MHS and *RYR1* and other loci (686).

Matters are complicated further by the possibility that the *RYR1*-related MH phenotype may be more common than previously thought, as suggested by the relatively high incidence of the MHS and MHE phenotypes (1% and 5%, respectively) in a low-risk European control population (687), frequent MHS in asymptomatic parents of MH probands (688), and the recent identification of MH-related *RYR1* mutations in compound heterozygotes with a congenital myopathy phenotype and unaffected parents (45). Particularly in families where only partial *RYR1* screening has been performed, two or more recombination events may therefore be explained by the presence of independent *RYR1* mutations running within a pedigree.

Although it now seems likely that *RYR1* mutations account for the majority of MH cases, it has been suggested that the MHS phenotype may reflect the compound influence of several genes rather than one major

gene defect (689). Locus heterogeneity has been indicated by variable degrees of linkage evidence for several loci and, less frequently, cosegregation of MHS with mutations in candidate genes. Because most of these loci have been implicated only in a limited number of families or certain ethnic groups, they are, however, unlikely to account for the large number of *RYR1*-recombinant families:

- Linkage to a locus on chromosome 17q has been suggested in five MH families (lod score 3.26) (690,691), but this could not subsequently be replicated in other families (692). A five-generation Canadian family with hyperkalemic periodic paralysis has been reported, in which three affected individuals have had definite episodes of MH and another four may have had milder MH-related events (693). The two conditions cosegregate with each other and with the adult skeletal muscle sodium channel (*SCN4A*) on chromosome 17q (lod score for MH < 3). A Gly1306Ala variation in the *SCN4A* gene at this locus was also found to be associated with masseter muscle rigidity, a minor MH variant, and a positive IVCT in a single family (694).
- In a single German family, there was some evidence of linkage (lod score < 3) to a marker on chromosome 7q, adjacent to *CACNL2A*, the gene for the  $\alpha$ 2-delta subunit of the 1,4-dihydropyridine-sensitive voltage-dependent calcium channel (VDCC) (695).
- In another single German family, MH linkage to chromosome 3q13.1 (MHS4) was demonstrated (lod score 3.22) (696), although the precise gene locus remains to be defined.
- In a single French family, MH showed linkage to the  $\alpha$ 1 subunit of the 1,4-dihydropyridine-sensitive VDCC on chromosome 1q, and a likely causative mutation in this gene (*CACLN1A3* or *CACNA1S*) has been recognized (697). Previously reported mutations in this gene have been implicated in hypokalemic periodic paralysis. Another mutation in this gene was identified in a North American MH pedigree (698) and an isolated Canadian patient (699) but *CACNA1S* mutations do not appear to play a significant role in other MH populations (700).
- A European collaboration has also suggested that another candidate gene for MH may be located on chromosome 5p (701), based on studies in a Belgian MH kindred.

Malignant hyperthermia susceptibility is a feature in NAM (702), a dysmorphic syndrome recently linked to 12q13.13–14.1 (703).

#### 127.7.1.6 Heterogeneity in Central Core Disease.

Although most studies have confirmed the association between CCD and mutations in *RYR1*, the penetrance of *RYR1* mutations associated with CCD is clearly incomplete. Although the classic phenotype of CCD appears tightly associated with *RYR1* mutations, there are reasons for not assuming that every case

with cores on muscle biopsy is caused by a mutation in *RYR1*:

- One family demonstrated recombination events between CCD and *RYR1*, although too little information is provided about the CCD phenotype in this family to be certain about the interpretation (704). Also, corresponding to MHS, recombinants in a family might be caused by separate *RYR1* mutations running in the same pedigree.
- Screening of the entire *RYR1* coding sequence failed to reveal a mutation in a severely affected neonate with histopathologic and clinical features of CCD (45), indicating probable genetic heterogeneity or failure to identify a more complex rearrangement by conventional sequencing techniques as demonstrated recently (480).
- Mutations in the *ACTA1* gene, previously associated with nemaline myopathy, have also been implicated in an autosomal dominant myopathy with cores (322). However, some patients in this pedigree had an associated cardiomyopathy, which is not a feature in *RYR1*-related CCD.
- Biopsy features of CCD have been found in 17 of 25 patients with hypertrophic cardiomyopathy associated with missense mutations in the slow/ $\beta$  myosin heavy chain, *MYH7* (160). Although only two of the patients in the original pedigree had muscle weakness, more recent reports suggest that *MYH7* mutations may be associated with more overt skeletal muscle myopathies, which, however, typically show more distal involvement (705) compared with *RYR1*-related CCD.
- Central cores were demonstrated in only one of identical twin boys with CCD and arthrogryposis (706); other phenocopies have been discussed earlier (see Section 127.7.1.1).
- A patient with a CCD-like presentation and muscle biopsy was found to have a specific deficiency of fructose 1,6-diphosphatase (707). Other “genocopies” may exist.
- In addition, the common occurrence of cores and other structural abnormalities such as nemaline bodies or centralized nuclei, recognized for a long time (123,154,155) has recently been attributed to mutations in the *RYR1* (22,23,157), *DNM2* (397), *KBTBD13* (14) and *NEB* (159) genes.

It remains a matter of controversy whether every patient with CCD should undergo IVCT or be assumed to be at risk of MH. Not all patients with CCD are at risk of MH (e.g. references (63) and (640)), but susceptibility should be assumed in the absence of firm evidence to the contrary. Although some authorities recommend that every individual with CCD should have an IVCT (708), it may be more appropriate to discuss the issue with affected individuals and their relatives, and to consider other approaches, such as deferring a decision until molecular genetic testing may be better able to provide



clinical guidance. For a recent review on the relation between core myopathies and the potentially associated MH risk see reference (190).

### 127.7.2 Multi-Minicore Disease

In 1971, Engel et al. (709) described two unrelated children with nonprogressive hypotonia and muscle weakness from infancy and a characteristic muscle biopsy appearance of multiple, small, well-circumscribed foci of myofibrillar degeneration with loss of histochemical staining for mitochondrial enzymes. Since the original description, at least 100 other cases with similar histopathologic features and a wide range of clinical phenotypes have been reported under the names of multicore disease, focal loss of cross-striations, minicore myopathy, myopathy with multiple minicores, or pleocore disease. The original name for this condition, “multicore” disease (MIM \*255320, 157550) (380), has now been largely replaced by the more helpful term MmD (642), reflecting the larger number and smaller size of characteristic lesions when compared with classic CCD.

**127.7.2.1 Special Features of Muscle Pathology.** Multifocal, well-circumscribed areas characterized by reduction of oxidative staining and low myofibrillar ATPase activity are the histopathologic hallmark of MmD (709,710). The cores are often not evident on routine histologic staining and may vary substantially in size; a presentation with focal loss of cross-striations in the muscle fibers has been described in some families (179,711). In contrast to central cores, minicores that affect both type 1 and type 2 fibers are typically unstructured and extend only for a short distance along the longitudinal axis of the muscle fiber (94); the latter feature distinguishes them more clearly from CCD than does their multiplicity. Predominance of hypotrophic type 1 fibers is commonly associated and may precede the appearance of more specific features (99). Distinction between CCD and MmD may be difficult in individual cases due to a shared genetic background, and in some, minicores may evolve into central cores over time (63,530).

An increase in internal nuclei and type 2 hypertrophy are commonly associated (712), whereas in some families, whorled fibers, an increase in fat and connective tissue, and more dystrophic changes have been observed (24,94), suggesting a potential overlap with the milder end of the congenital muscular dystrophy spectrum.

Minicores are evident on electron microscopy as areas of myofibrillar disruption and paucity of mitochondria, often with degeneration of the sarcomeres and swelling or proliferation of the sarcoplasmic reticulum and transverse tubules (94). Ultrastructural studies indicate different stages of minicore formation in the same biopsy, ranging from Z-line streaming with preserved myofibrillar structure to areas with complete loss of sarcomeric organization (709).

Immunohistochemical studies in minicore myopathy have been limited and suggest sarcoplasmic reticulum and desmin abnormalities corresponding to those observed in central core disease (112). As with central cores, minicores stain strongly positive with antibodies to the actin cross-linking protein filamin C (540). More recently, distinct abnormalities of proteins involved in calcium handling and homeostasis have been reported to differentiate between distinct specific genetic backgrounds in the core myopathies (541).

Minicores in muscle are found within a range of other clinical contexts (709) such as dystrophy, denervation, and inflammatory and endocrine myopathies. Minicore formation may be induced by eccentric contractions (i.e. lengthening during activity) in healthy humans (545) and has been described in association with emetine (713) or steroid administration in rats (714). Primarily metabolic conditions such as type III glycogenosis (135) or short-chain acyl-CoA dehydrogenase (SCAD) deficiency (715) may also feature minicores on muscle biopsy.

Minicores have also been reported as an additional feature in other congenital myopathies, and the association with myotubular (centronuclear) myopathy has been particularly well documented (142,143,404,716). The simultaneous occurrence of nemaline bodies and minicores has been described occasionally (123,155). More recently, minicore-like structures, with or without additional nemaline bodies, have been reported in association with dominant mutations in the *ACTA1* gene (157).

Because minicores are thus not specific for minicore disease, the diagnosis of MmD depends on the biopsy appearance within the specific clinical context of each individual case.

**127.7.2.2 Clinical Characteristics.** MmD usually presents in infancy or childhood with hypotonia or proximal weakness; prenatal onset is also well recognized (712,717). The weakness of some individuals improves with age (718–720), although the condition of others deteriorates slowly. Several individuals have been reported with onset of MmD in adult life; they have generally had a progressive weakness and some have suffered from progressive respiratory or cardiac failure (721–724). Clinical features associated with the histopathologic appearance of MmD are markedly heterogeneous depending on the genetic background and comprise at least four different subgroups.

The most instantly recognizable classic phenotype of MmD (24,712,717) has now been attributed to recessive mutations in the selenoprotein N (*SEPN1*) gene and is characterized by spinal rigidity, scoliosis, and early respiratory impairment. Patients with this form typically present in infancy, and occasionally associated are feeding difficulties with failure to thrive. Myopathic facial features are common, the voice is typically high pitched, and there may be an associated high-arched or cleft palate. Weakness is pronounced in the axial muscles, in particular the neck and trunk flexors, and proximally,



typically affecting the shoulder girdle more than the hip girdle. Failure to acquire head control is a constant early sign. There may be marked muscle wasting, mainly affecting axial groups, the shoulder girdle and the inner thigh (“bracket-like thighs”). Scoliosis, invariably present by the early teens, is usually progressive, cervico-dorsal, and associated with dorsal lordosis and lateral trunk deviation. Respiratory failure often develops after 10 years of age in the presence of severe scoliosis and can lead to secondary cardiac failure (178,712,725,726). Respiratory impairment is often out of proportion to the overall degree of weakness, and many patients on nocturnal ventilation remain independently mobile.

The other three subgroups of MmD have now all been associated with recessive *RYR1* mutations and despite some relatively distinct features there is clearly a clinical continuum, not surprising considering the shared genetic background. Extraocular muscle involvement (MmD with external ophthalmoplegia) (MIM 255320) (380) pronounced on abduction and upward gaze may be an additional feature in a subset of patients with an otherwise similar distribution of weakness and wasting (25,179,642,711,712,727). However, with the exception of the most severely affected neonatal cases (26), respiratory impairment is usually milder than in the classic form and may improve over long periods of follow-up. External ophthalmoplegia is not always observed at presentation and may evolve over time.

Another group of patients show a milder phenotype similar to CCD (moderate form of MmD with hand involvement) characterized by predominant hip girdle weakness with relative sparing of respiratory and bulbar muscles (63). A common complaint is exercise-induced myalgia. In males, cryptorchism may be an additional feature. Patients in this group may also show a pattern of selective muscle involvement on muscle imaging similar to that observed in classic CCD caused by mutations in the C-terminal end of the *RYR1* gene (63,89,625), and distinct from the selective muscle involvement described in *SEPN1*-related myopathies (728,729). In some patients, there is additional marked distal weakness and wasting, predominantly affecting the hands (63). The observation of extraocular muscle involvement evolving over time in this group suggests a clinical continuum between the latter groups rather than distinct clinical entities. Respiratory involvement is prominent in the classic form of MmD but is less pronounced, or absent, in other clinical subgroups.

A severe form with antenatal onset, generalized arthrogryposis, dysmorphic features and mild to moderate compromise of respiratory function has been described in a few patients (MIM 607552) (717,730).

A small group of patients have been reported with congenital cardiac defects (709), and mitral valve prolapse appears particularly common. Cardiac, mainly right ventricular impairment is usually secondary to marked respiratory involvement in the *SEPN1*-related

form, but a number of cases with different genetic backgrounds have shown primary cardiomyopathy in the absence of significant respiratory compromise. Cardiac involvement adversely affects the clinical course (723). Primary cardiomyopathies can be restrictive, hypertrophic, or dilated in type (722,723,731,732) and may be associated with additional accumulation of desmin in skeletal and cardiac muscle fibers (731). This indicates that the pathogenetic mechanisms may be distinct from those involved in MmD without primary cardiac involvement, as has indeed been demonstrated in some families recently (see below).

An episode of possible MH has been reported in one boy with minicore disease (733) and in an affected woman during pregnancy (194). Features of minicore myopathy have been noted in muscle biopsies from a few families with mutations in the *RYR1* gene and susceptibility to MH but no other clinical features of a congenital myopathy (547,734). As with the other congenital myopathies, the use of potentially MH-triggering agents should be avoided. For a review on the relationship between core myopathies and MH risk see reference (190).

Single cases of MmD have been reported in association with anhidrotic ectodermal dysplasia (727), type III glycogenosis (135), and SCAD (715). Although these associations are likely to be coincidental, the reported association of MmD with multiple pterygium syndrome is more likely to be of pathogenetic significance (735), considering that both conditions share the MHS trait as a potential complication (591). Chudley et al. (736) reported an adult brother and sister from a consanguineous family with MmD who also had short stature, severe mental retardation and dysmorphic features resembling those of the KDS, musculoskeletal and facial anomalies, and pituitary hypoplasia with hypogonadotrophic hypogonadism.

**127.7.2.3 Genetics of MmD.** MmD is mainly inherited as an autosomal recessive trait, as documented in several families, most with siblings of either sex affected in one generation (59,123,179,711,718,737). In addition, numerous sporadic cases have been reported, as well as one set of identical male twins (718,738–740).

Only few reports of MmD have described pedigrees compatible with autosomal dominant inheritance (123,720,723,741–743); dominant or X-linked inheritance was proposed in one family with affected male sibs (718). In some of these families, autosomal dominant inheritance was suggested because of a parental history of mild muscle weakness in childhood without any clinical signs of myopathy but with slightly abnormal muscle biopsy features (123,720,725); however, these findings may have reflected carrier status of a recessive mutation as can be observed in other congenital myopathies. When muscle biopsies from the parents of singleton patients have been studied, they have usually been normal (123,744). In other families with proposed autosomal dominant inheritance, the diagnosis was later

changed to a muscular dystrophy (745), or unusual clinical features such as electrocardiographic evidence of a cardiomyopathy suggested a distinct entity altogether.

The marked phenotypic variability of MmD is reflected in genetic heterogeneity, and recessive mutations in both the selenoprotein N (*SEPN1*) and the skeletal muscle ryanodine receptor (*RYR1*) gene have been recently identified in clinically distinct subgroups of the condition.

**127.7.2.4 MmD and the Selenoprotein N (*SEPN1*) Gene.** Investigation of the selenoprotein N (*SEPN1*) gene on chromosome 1p36 as a candidate for MmD was prompted by the considerable clinical and histopathologic overlap between the classic phenotype of MmD and congenital muscular dystrophy with rigidity of the spine (RSMD), previously attributed to *SEPN1* mutations (746). RSMD patients have a similar clinical phenotype with marked axial weakness, spinal rigidity, early scoliosis, and respiratory impairment, and histopathologic features may be rather more myopathic than dystrophic, usually with normal SCK.

*SEPN1* involvement in the classic phenotype of MmD was suggested both by linkage data and direct mutational analysis in around 50% of patients with these clinical features (24). More than 30 *SEPN1* mutations associated with a congenital myopathy phenotype have been identified to date. *SEPN1* mutations in RSMD and MmD predominantly result in a truncated protein; missense mutations are rarer and typically affect functionally important domains of the protein such as the SECIS (747) or the selenocysteine redefinition element (748). A multisystem disorder with associated myopathy has been reported associated with mutations in the gene for selenocysteine insertion sequence-binding protein 2 (SECISBP2) (749). In addition to compound heterozygosity, homozygous mutations are unexpectedly common, even in nonconsanguineous families, reflecting the presence of few founder mutations in different European populations.

Selenoprotein N, a glycoprotein localized in the endoplasmic reticulum, belongs to a family of at least 30 different proteins that mediate the effect of selenium and play a role in several metabolic pathways and various antioxidant defense systems. Selenium is essential for the normal function of many physiologic systems (750), and deficiency states have been associated with skeletal and cardiac muscle phenotypes both in animals (751) and humans (752–754).

Selenoprotein N is highly expressed from mid-gestation to immediately prenatally (755), suggesting an important part in embryogenesis. A more specific role in myogenesis is indicated by abundant expression in fetal muscle precursor cells and the observation of a specific disturbance of satellite cell function in the *sepn1*  $-/-$  knockout mouse (756). Although the precise function of selenoprotein N in muscle remains unclear, certain important aspects have been recently emerging. A role

in calcium homeostasis is suggested by a structural motif similar to those found in calcium-binding proteins (746) and the close functional and spatial relationship between selenoprotein N and RyR1 reported in normal zebrafish and the *sepn1*  $-/-$  morphant (757,758). The latter observation in conjunction with a proposed role of Selenoprotein N in redox-regulated calcium homeostasis may explain the many clinico-pathologic similarities between *RYR1*- and *SEPN1*-related core myopathies. In addition to its implications for redox-regulated calcium homeostasis, the specific involvement of selenoprotein N in antioxidant defense systems opens the prospect of *ex vivo* administration of antioxidants such as acetylcysteine as a possible therapeutic approach (759,760).

**127.7.2.5 MmD and the Skeletal Muscle Ryanodine Receptor (*RYR1*) Gene.** MmD and CCD secondary to dominant *RYR1* mutations have generally been regarded as separate entities, considering differences in core morphology, clinical phenotype, and mode of inheritance. However, recent genetic studies have implicated recessive homozygous and compound heterozygous *RYR1* mutations in clinically distinct subgroups of MmD.

The moderate form of MmD with hand involvement shares many features with CCD and linkage evidence suggested *RYR1* involvement in families with and without additional hand involvement (63,625). In two of these families, a consanguineous Algerian and a consanguineous British family, homozygous *RYR1* mutations were subsequently identified. Despite a distinct histopathologic appearance, the pattern of selective involvement on muscle MRI and CT was similar to that observed in association with heterozygous dominant *RYR1* mutations.

MmD with external ophthalmoplegia (MIM 255320) (380) in a severely affected patient, an isolated case from a consanguineous Tunisian family, was attributed to a homozygous *RYR1* mutation, introducing a cryptic splice site in intron 101 (26). Both parents were asymptomatic carriers. This mutation was the first out-of-frame mutation described in *RYR1* and resulted in a marked depletion of the normal RyR1 transcript, probably explaining the severe phenotype. *RYR1* involvement was also suggested by linkage evidence in four additional families with a similar phenotype (25), including the family previously reported by Swash and Schwartz (179).

The *RYR1* gene is also a likely candidate for the severe form of MmD with neonatal onset and arthrogryposis, considering the phenotypic overlap with the form of CCD with fetal akinesia sequence (45).

Identification of recessive *RYR1* mutations in MmD provided the molecular basis for a possible histopathologic and clinical continuum between MmD and CCD. Although the histopathologic appearance of MmD appears to be more closely associated with recessively inherited *RYR1* mutations, dominant *RYR1* mutations occasionally give rise to minicores on muscle biopsy (530) and may have accounted for a proportion of MmD pedigrees with autosomal dominant inheritance reported

in the premolecular area. Moreover, the histopathologic appearance of MmD due to recessive *RYR1* mutations may evolve into the classic picture of CCD over long periods of follow-up (63). The clinical and histopathologic spectrum associated with mutations in the *RYR1* gene is currently expanding and may account for a wider range of phenotypes than those included in the original descriptions of CCD and MmD. Recessively inherited mutations associated with MmD and related phenotypes are more widespread throughout the *RYR1* gene (25,63) and are often associated with very low presence of the functional RyR1 protein.

In contrast to the extensively characterized dominant *RYR1* mutations associated with MHS and CCD, functional effects of recessive *RYR1* mutations associated with CCD and MmD have only been characterized in few studies to date (761–764). Based on these limited studies, pathogenetic mechanisms underlying recessive *RYR1*-related myopathies appear to be more variable with loss of calcium conductance, probably mediated by marked RyR1 protein reduction, a relatively common observation.

There is currently no murine model for recessive *RYR1*-related myopathies, however, the zebrafish *relatively relaxed* mutant (765), a sporadic mutant with marked reduction of functional RyR1 protein, closely mimicks one of the probable molecular mechanisms underlying recessive core myopathies.

**127.7.2.6 Evidence for Genetic Heterogeneity in MmD.** Although a substantial proportion of MmD could be attributed to mutations in the *SEPN1* or *RYR1* gene, there is evidence for further genetic heterogeneity:

- Half of all cases with the classic phenotype of MmD are not associated with the *SEPN1* gene and may have mutations affecting a functionally related protein (24).
- Although a proportion of MmD pedigrees with autosomal dominant inheritance are likely to be due to dominant *RYR1* mutations, distinct clinical features such as a primary cardiomyopathy (723,731) suggest a different genetic background in other families with this unusual mode of transmission. Cardiac involvement secondary to respiratory impairment is common in *SEPN1*-related MmD, but primary cardiomyopathies have notably not been documented in patients with MmD owing to confirmed mutations in the *SEPN1* or *RYR1* genes. Dominantly inherited desmin myopathy (see below) may feature minicores in addition to more typical inclusions on muscle biopsy, and the marked desmin accumulation reported in some cases with MmD and a primary cardiomyopathy (731) suggest the desmin gene as a likely candidate for this subgroup. In general, although the histopathology is usually distinct, myofibrillar myopathies other than desmin myopathy may be confused with core myopathies on a more superficial histopathologic assessment. A cardiomyopathy associated with

multi-minicores on muscle biopsy has also been documented in severely affected siblings with homozygous truncating titin mutations (766). Dominant mutations in the *ACTA1* gene, previously implicated in nemaline myopathy, may feature cores with or without nemaline bodies on muscle biopsy and are associated with a hypertrophic cardiomyopathy (322).

## 127.8 CONGENITAL FIBER TYPE DISPROPORTION

Congenital fiber type (size) disproportion (CFTD) (MIM 255310) is a histopathologic diagnosis characterized by consistent smallness of type 1 fibers compared with type 2 fibers and the absence of more specific histopathologic features. CFTD has been reported in some children with the clinical picture of a congenital myopathy but also in many other neurologic conditions and is therefore not a precise diagnostic entity. Moreover, although in some patients the histopathologic appearance of CFTD persists even after prolonged follow-up (767), in others appearances may change or more specific features may evolve over time. Insofar as CFTD is indeed a diagnosis, it is one of exclusion. For a discussion of the controversy surrounding CFTD as a diagnostic entity see reference (768).

The few incidence figures that have been published are based on studies of archival biopsies and vary between 5:4000 (767) and 149:2212 (98).

### 127.8.1 Special Features of Muscle Pathology

The diagnosis of CFTD may be employed when a patient has typical clinical features of a congenital myopathy and when muscle pathology reveals predominance and selective smallness of type 1 fibers compared with type 2 fibers (see Fig. 127-2F) but no pathognomonic features of a distinct myopathy. The pathologic criteria for this diagnosis are given in Section 127.2.2 (see Reference (94) for a full discussion). The selective smallness of type 1 fibers may be accompanied by hypertrophy of type 2 fibers, and there are some cases reported with a preponderance of type 2 fibers (102).

The first report of the histology of CFTD was that of Farkas-Bargeton et al. (769), but the term CFTD was coined by Brooke, who defined the clinical presentation of 22 affected children as well as the histologic characteristics (96,770). The essentially pathologic nature of the diagnosis has been emphasized (771), and since the consistency even of this is doubtful (767), it is recommended that genetically unresolved patients with CFTD and no specific histologic features undergo a second biopsy some years after the initial one to see if more specific histopathologic features have evolved (5). In particular, Iannaccone et al. (772) reviewed the cases of 37 children with hypotonia and identified a wide range of eventual diagnoses

underlying the pathologic appearances of CFTD, including dystrophies, neuropathies, and spinal muscular atrophies, as well as myopathies. Distinct conditions in which selective smallness of type 1 fibers may be found include the other congenital myopathies (98), congenital myotonic dystrophy (773), and congenital muscular dystrophies (82). Some children have been reported where in addition to CFTD the biopsy also showed central nuclei and myofibril degeneration, suggesting a spectrum between CFTD and centronuclear myopathy (114,454,774,775). There are a number of additional reports in the literature suggesting that the biopsy features of CFTD are also found in a variety of other disorders, including metabolic diseases and other neuromuscular or neurologic conditions, including globoid cell leukodystrophy (776). Pompe's disease (glycogenosis II), other glycogen storage diseases (776), Krabbe's leukodystrophy (771,777), facioscapulohumeral dystrophy (101), hypertrophic neuropathy (778,779), spinal muscular atrophy (780), hypothyroidism (781), Lowe's syndrome (782), deletion of the short arm of chromosome 1 (1p36) (783), or fetal alcohol syndrome (776). Clarke and North (784) reviewed the literature on CFTD to this regard and emphasized that care should be taken to exclude other conditions with type 1 hypotrophy.

### 127.8.2 Clinical Characteristics

The clinical features reported in association with CFTD in the premolecular era were highly heterogeneous and are likely to reflect marked genetic heterogeneity. In the original series of Brooke and Engel, infants presented at or soon after birth with hypotonia and associated orthopedic complications were common. Facial appearance was myopathic with frequent extraocular muscle involvement and ptosis. The severity of the weakness varied considerably, but despite significant residual disability, evidence of progression was uncommon, particularly after the age of 1-years (101). Inconsistently associated features have included short stature, spinal rigidity (64,785), cerebellar hypoplasia (786–788), moderate learning difficulties (789), and seizures and aberrant skeletal maturation (790). Microstomia, campodactyly, and talipes equinovarus have been found in two patients with CFTD who were given a diagnosis of Freeman–Sheldon syndrome (791).

There have also been a number of more unusual associations, such as those of typical clinico-pathologic features of CFTD with cardiomyopathy and myofibrillar lysis inherited as an autosomal recessive trait in one family (51), and with insulin resistance in two brothers who were compound heterozygotes for mutations at the insulin receptor locus (792).

The clinical course in genetically unresolved cases with CFTD is highly variable, ranging from slow improvement (165,793) to progressive deterioration (784), the

latter often due to progressive respiratory failure requiring long-term tracheostomy (794).

The biopsy features of CFTD are also sometimes found in children or adults without a history of weakness or hypotonia in infancy, and in whom the clinical features have developed subsequently (62,795).

### 127.8.3 Genetic Background

Most cases reported in the premolecular era were sporadic but familial cases have also been described, suggestive of both autosomal-dominant (770,779,784,796–798) and autosomal-recessive inheritance (82,770,789,794,799). A number of causative genes have now been identified, some of them also implicated in other congenital myopathies such as nemaline myopathy, central core disease, multi-minicore disease and centronuclear myopathy, emphasizing the genetic and, possibly, clinico-pathologic continuum between CFTD and other congenital myopathies. Further genetic heterogeneity is expected.

Heterozygous missense mutations in the skeletal muscle  $\alpha$ -actin (*ACTA1*) gene were the first mutations identified in CFTD, in three Japanese patients with severe weakness and respiratory impairment but no extraocular muscle involvement (38).

Two sisters with CFTD, insulin resistance, severe scoliosis and respiratory muscle weakness were found to be homozygous for a G943A mutation in the selenoprotein N (*SEPN1*) gene (39), also implicated in a subgroup of MmD (24).

Heterozygous missense mutations in the  $\alpha$ -tropomyosin 3 (*TPM3*) gene, also implicated nemaline myopathy (9), were identified in five CFTD families, associated with proximal and distal weakness, respiratory impairment and ptosis but no extraocular muscle involvement (40). In a sixth family, the recurrent p.Arg168His mutation was associated with CFTD and nemaline myopathy in different family members.

Compound heterozygous mutations in the *RYR1* gene were identified in seven families with CFTD (41). Extraocular muscle involvement was a frequently associated feature and may help distinguishing distinguish *RYR1*-related CFTD from other genetic backgrounds. CFTD is also a common additional feature in *RYR1*-related congenital myopathies with central nuclei (19), suggesting a continuum between different recessively inherited *RYR1*-related myopathies.

Features of CFTD and a myosin storage myopathy were found in different members of a large dominant family harboring the same c.5807A>G mutation in the *MYH7* gene encoding the slow/ $\beta$ -cardiac myosin heavy chain (42).

Clarke et al. reported a form of CFTD compatible with X-linked inheritance in a large family with facial, bulbar and cardiorespiratory involvement and possible linkage to two loci on the long arm of the X chromosome (43).



## 127.9 MYOSIN STORAGE (HYALINE BODY) MYOPATHY

Myosin storage myopathy, previously called hyaline body myopathy (MIM 608358) (380), is clinically and probably also genetically heterogeneous. Histologically, it is characterized by subsarcolemmal accumulations of slow/ $\beta$  skeletal myosin heavy chain-positive material with a glassy (hyaline) appearance in type 1 muscle fibers. For review see reference (800).

### 127.9.1 Special Features of Muscle Pathology

The H&E and trichrome stains show subsarcolemmal or intersarcomeric accumulation of amorphous material, and often splitting of fibers, internal nuclei, fibrosis, and fatty infiltration. Fiber typing shows the affected fibers to be of type 1 (801), and in most cases, this is also the predominating fiber type. The hyaline regions are Congo red negative, devoid of oxidative enzyme activity, and rich in ATPase activity after preincubation at low pH values (802). Immunohistochemical labeling shows the regions to be occupied by slow myosin. There is no binding of antibodies against desmin (except in some at the rims of these regions), actin, tropomyosin,  $\alpha$ -B-crystallin, and several other proteins (36,802,803). Ultrastructural features include accumulation of granular, filamentous, or amorphous material without sharp demarcation toward regions of normal-looking myofibrils unconnected to the membrane (36,801–804)

### 127.9.2 Clinical Characteristics

In some of the sporadic cases and those consistent with recessive inheritance (801–803,805), onset of muscle weakness has been congenital, whereas most families with autosomal-dominant inheritance except one (806) have shown onset later in childhood or in adulthood (36,804,807). The clinical course in these families was highly variable, regardless of the age at onset. The distribution of weakness has been of limb-girdle type or scapuloperoneal. Some patients show neck flexor weakness, lumbar hyperlordosis, or calf hypertrophy. CK concentrations have been normal or slightly higher than normal, whereas electromyographic findings have been normal or myopathic (36,801–804,806). Cardiorespiratory involvement is variable but is an important cause of mortality.

### 127.9.3 Genetic Background

The first published report (801) describes an affected sib pair with healthy parents, whereas some cases have been sporadic (802,803,805) and yet another few families have shown autosomal-dominant inheritance (804,806,807). Two separate research groups were

guided by the histologic finding of accumulation of one myosin heavy chain subtype to identify the cause of this disorder, namely heterozygous missense mutations in the rod domain of the gene for slow/ $\beta$  myosin heavy chain, *MYH7* (36,808), and a genome-wide scan in a large family resulted in similar findings (37). To date, four different *MYH7* mutations have been identified in association with MSM, three of them (Leu1793Pro, Arg1845Trp and His1901Leu) associated with autosomal-dominant (36,37,809) and one (Glu1883Lys) associated with autosomal-recessive inheritance (810).

Mutations in the region of the same gene corresponding to the tail of the protein forming a coiled coil have been associated with distal myopathy type Laing (811) and other less well-defined myopathies (705). More recently, features of both CFTD and MSM have been reported in a large dominant pedigree harboring a novel heterozygous *MYH7* c.5807A>G mutation (42). In addition, *MYH7* mutations in the globular head and tail cause hypertrophic cardiomyopathy (160,812,813), occasionally associated with central cores on muscle biopsy (160).

Possible genetic heterogeneity in MSM is suggested by a family with two sibs affected by a myopathy with histologic similarity to MSM, where linkage was found to a locus on the short arm of chromosome 3 (814). However, the biopsies in this family had not been labeled with antibodies against myosin and the causative gene has not been identified yet.

## 127.10 MYOFIBRILLAR MYOPATHIES

Myofibrillar myopathy (MFM) (MIM 601419) (380) is a clinically and genetically heterogeneous disorder often occurring sporadically, with onset of muscle weakness mainly in adulthood (for review see references (815,816)). The term “myofibrillar myopathy” was introduced in the 1990s and reflects the shared pathologic features of myofibrillar dissolution with resulting accumulation of degradation products in patients with often-diverse clinical features and different genetic backgrounds. Familial cases have increasingly been reported, however, and in some of these cases, onset was in childhood. Because of the description of a few congenital cases, and in view of the structural aberrations characteristic of this disorder sometimes similar to those found in the congenital myopathies, we have included myofibrillar myopathy among the congenital muscle disorders in the current chapter.

The definition of a myofibrillar myopathy rests on the histologic findings of myofibrillar disruption and intracellular aggregation of a variety of proteins. A multitude of histologically descriptive terms, namely “desmin-storage myopathy” (817) or “desmin-related myopathy” (818) were coined because of an observed accumulation of desmin in the muscle fibers of patients (819,820), but are now used less frequently as the extended histopathologic

and genetic spectrum of these conditions is increasingly recognized (815,816,821,822).

### 127.10.1 Special Features of Muscle Pathology

The hallmark of the MFMs is disruption of myofibrils in combination with accumulation in the muscle fibers of proteins derived from the Z disk, intermediate filament or sarcolemma, or of chaperone proteins (823,824). Light microscopy shows unspecific myopathic changes such as fiber splitting and variability of fiber size, often but not invariably in combination with aggregates of granulofilamentous or amorphous congophilic material, and a variety of inclusion bodies best revealed by the trichrome method (825,826). In the regions of the aggregates, oxidative enzyme activity is low. There may be groups of small fibers, and sparse inflammatory infiltrates, but usually no necrosis or regeneration. Many of the childhood cases show predominance of type 1 fibers (827). Although histopathologic changes are nonspecific, those tend to be less pronounced with mutations in the desmin (*DES*) and  $\alpha$ B-crystalline (*CRYAB*) genes compared with other genetic backgrounds (see below). Abnormal nuclei are particularly common in *BAG3*- and *CRYAB*-related MFM.

Immunohistochemical labeling reveals an abundance of desmin (819,820), myotilin,  $\alpha$ -B-crystallin, and other muscle proteins (821,825,826,828,829). The lytic regions, in contrast, are devoid of proteins such as actin,  $\alpha$ -actinin and other sarcomeric proteins.

Electron microscopy shows disruption of the myofibrillar network, streaming of components from the Z disks, and dense structures composed of filamentous material and degrading organelles resembling hepatic Mallory bodies (820,822,826). There may be a few cytoplasmic or nemaline bodies, or autophagic vacuoles. The suggestion of distinct EM patterns associated with different genetic backgrounds has been controversial (815,830).

### 127.10.2 Clinical Characteristics

Muscle weakness is usually both proximal and distal, and symmetric and slowly progressive. Facial muscles are often spared and there is no extraocular muscle involvement. A subgroup of patients have presented with predominant involvement of respiratory muscles (32,831–833). Many patients have hypertrophic cardiomyopathy (824,826), which occasionally may be the presenting features. Some show peripheral neuropathy, including slow nerve conduction velocities. Electromyographic findings have been compatible with a myopathy in most patients but some show additional neurogenic findings and even myotonic discharges (817,826). Cataracts have been reported in some forms.

A small number of patients with congenital onset (29,32,827) have shown axial and neck flexor weakness

with hyperlordosis, scoliosis, and rigid spine. A few patients have had facial weakness. Electromyographic findings were normal or unspecifically abnormal, and SCK levels were normal or very slightly higher than normal. The remaining children in this group have had early and severe involvement of respiratory muscles. A phenotype with similar pathologic features has been reported under the name “hypertonic muscular dystrophy” in native Canadian Indians (834).

### 127.10.3 Genetic Background

A majority of patients have been sporadic cases. Although familial cases mostly show autosomal dominant inheritance (835–837), the mode of inheritance in a few families, including those in which onset has been congenital, has been autosomal recessive (27,29,32,827,834). MFM is genetically heterogeneous and mutations in genes other than those listed below are likely to be identified in future:

Mutations in the desmin (*DES*) gene were the first to be associated with MFM and have now been identified in a number of additional families (27,838–842). Onset varies from childhood to late adulthood. An associated cardiomyopathy is common and both respiratory and bulbar involvement may occur. Recessively inherited *DES* deletions have been associated with severe infantile-onset cardiomyopathy (843) or an Emery–Dreifuss muscular dystrophy-like phenotype (844).

Mutations in the  $\alpha$ -B-crystalline (*CRYAB*) gene on chromosome 11q22.3–q23.1 have been identified in a few families, associated with autosomal-dominant inheritance, late-onset, a slowly progressive course and, variably, cardiomyopathy, respiratory impairment, cataracts and peripheral nerve involvement (28,845,846). Recessive *CRYAB* mutations have been more recently identified in Canadian native Indians (30) and an isolated Caucasian case (29) with suggestive histopathologic features but an unusual clinical phenotype featuring profound truncal muscle stiffness.

Dominant mutations in the myotilin (*MYOT*) gene (31), previously reported in limb girdle muscular dystrophy LGMD1A (847,848), were described in six of 57 patients with MFM (825). *MYOT*-related MFM is associated with late-onset, frequent peripheral nerve and variable cardiac involvement.

Mutations in the *ZASP* gene encoding the Z-band alternatively spliced PDZ-motif containing protein were initially identified by Selcen and Engel (33) and subsequently in a large family with similar features previously reported by Markesbery et al. (849,850). *ZASP*-related MFM is characterized by distal weakness and variable degrees of peripheral nerve and cardiac involvement but may present with isolated high SCK.

A heterozygous Trp2710X mutation in the filamin C (*FLNC*) gene was identified in several German MFM kindreds (34), probably reflecting a founder effect, and associated with cardiorespiratory and peripheral nerve

involvement. Additional *FLNC* mutations associated with MFM were subsequently identified in different ethnic groups (851,852).

The de novo heterozygous Pro209Leu mutation in the *BAG3* gene encoding the Bcl-2 associated athanogene 3 has been identified in several sporadic cases with a particularly severe form of MFM (35,853), characterized by early-onset and a rapidly progressive course with severe cardiorespiratory involvement and an associated neuropathy.

#### 127.10.4 Pathogenetic Considerations

The pathogenesis of the MFMs has been most extensively studied for the *DES*-related forms and is currently only partially understood. Current hypotheses concerning pathogenetic mechanisms implicated in the MFMs include aborted or abnormal intermediate filament formation (854,855), disturbed interactions between desmin and its binding partners as well as downstream effects on mitochondrial positioning and function (856) or protein control mechanisms involving the ubiquitin-proteasome system and the autophagic lysosomal pathway (857). For review of molecular mechanisms in MFMs see reference (858).

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### Biographies



**Carina Wallgren-Pettersson** is a medical doctor specialized in Medical Genetics, working as a clinician and head of department at the Folkhälsan Department of Medical Genetics, and as a teacher and research group leader at the Department of Medical Genetics, Haartman Institute, University of Helsinki, and the Folkhälsan Institute of Genetics in Helsinki, Finland. Her research has concentrated on neuromuscular disorders, mainly on nemaline myopathy and myotubular (centronuclear) myopathy. Together with Prof Nigel G. Laing in Perth, Australia, in 1996, she initiated the forming of the International Consortium on Nemaline Myopathy. From 1993 until the beginning of the year 2000 she coordinated the research activities of another international research network, the International Consortium on Myotubular Myopathy. Carina Wallgren-Pettersson was a member the Governing Board of the EU-funded Network of Excellence Translational Research in Europe–Assessment and Treatment of Neuromuscular Diseases (TREAT-NMD), as representative for the University of Helsinki, and is now a member of the TREAT-NMD Alliance.



**Heinz Jungbluth** is a Pediatric Neurologist specialized in neuromuscular disorders, working as a clinician and head of the neuromuscular service at the Evelina Children's Hospital, St Thomas Hospital, London, and as a researcher at the Clinical Neuroscience Division, IoP, King's College, London, United Kingdom. His main research interest is in the genetics of neurological and neuromuscular disorders. His research to date has concentrated mainly on the congenital myopathies, in particular those associated with mutations in the skeletal muscle ryanodine receptor (*RYR1*) gene, one of the more common causes of inherited neuromuscular disease. He has organized and chaired several dedicated European Neuromuscular Centre (ENMC) workshops on Multi-minicore Disease, *RYR1*-related disorders (both jointly with Dr Ana Ferreiro, Paris and Prof Francesco Muntoni, London) and Centronuclear/Myotubular Myopathy (jointly with Dr Carina Wallgren-Pettersson, Helsinki, Finland, and Dr Jocelyn Laporte, Strasbourg). He is a member of the International Congenital Myopathy Standards of Care Consensus Group.



## Spinal Muscular Atrophies

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**128.1 INTRODUCTION: DEFINITION**

The term spinal muscular atrophy (SMA) comprises a clinically and genetically heterogeneous group of diseases characterized by degeneration and loss of the anterior horn cells in the spinal cord, and sometimes also in the brainstem nuclei, resulting in muscle weakness and atrophy (1). The sensory neurons are clinically spared, and there are generally no signs of upper motor neuron (pyramidal tract) involvement. With increasing identification of the underlying genetic defects, our understanding of the pathogenetic mechanisms, clinical spectrum, and possible therapeutic options has much improved in the past 20 years. Because no cure is yet available, genetic counseling and prognostic considerations are of great importance.

The subdivision of the SMAs into separate genetic and clinical entities (Table 128-1) is still controversial without distinct biochemical or molecular genetic criteria for each of the listed entities. The criteria used are age of onset, severity (progression and age of death), distribution of weakness, inclusion of additional features, and modes of inheritance. Gene defects have been identified by the end of 2010 in infantile SMA type I–III, X-linked SMA, SMA with primary respiratory distress type 1 (SMARD1), few autosomal dominant SMA types, different types of distal SMA (hereditary motor neuropathy (HMN)), and bulbospinal neuronopathy (Kennedy's syndrome).

Much semantic confusion exists in the description of the various SMAs, which is a result of the widespread use of synonyms. In this chapter, a rational approach is made to clarify the relevant types, synonyms, and eponyms under consideration of the current advances in molecular genetic research.

**128.2 PROXIMAL SPINAL MUSCULAR ATROPHY**

The proximal SMAs can be divided into autosomal recessive and autosomal dominant types. Clinically, there is no way of separating the dominant from the recessive forms. Infantile SMA is one of the most common inherited

diseases leading to death in early infancy. Because of the relative importance in neuropediatric departments and genetic counseling, the clinical features and genetics are reviewed in detail.

**128.2.1 Epidemiology**

Most cases of proximal SMA are inherited as autosomal recessive traits. Epidemiologic data of childhood-onset SMA have been summarized by Emery (2) and reviewed by Ogino and Wilson (3) and Jedrzejowska et al. (4).

Data for the most severe type I (Werdnig–Hoffmann) disease, in which affected children usually do not survive beyond the first few years, suggest that the birth incidence varies between 1:25,000 and about 1:10,000 (3). The incidence is much higher in certain inbred communities. Variants of early-onset SMA are very rare, accounting for only about 2% of infantile and childhood cases (5).

The more benign forms of the disease (type II and type III) have a prevalence among children as high as 1:25,000 and around 1:83,000 in the general population (3). Assuming an incidence of about 1:10 000 for all types of autosomal recessive SMAs, a heterozygosity frequency for purposes of genetic counseling would be 1 in 50; however, by molecular genetic carrier screening in the normal population it was found that the carrier frequency in the Caucasian population is much higher, i.e., 1 in 35 carries a heterozygous SMN1 gene deletion (4). This discrepancy is still a matter of debate and may be explained by a reduced penetrance or by atypical clinical pictures that escape diagnostic recognition.

It has been estimated that adult SMA accounts for 8% of all SMA cases, with a prevalence of 0.32 per 100,000 of the population (6).

Autosomal dominantly inherited SMA is extremely rare. According to a rough estimate, less than 2% of cases with an onset before 10 years of age show a parent-to-child transmission (7). Although vertical transmission of childhood-onset SMA is an exception, autosomal dominant transmission can be found in about two-thirds of the adult-onset proximal SMAs ((8), own series).

**TABLE 128-1 Classification of Spinal Muscular Atrophies**

1. Proximal SMA (80–90%)
  - 1.1 Autosomal recessive SMA
    - Infantile SMA (SMA I–III)
    - Adult SMA (SMA IV)
  - 1.2 Autosomal dominant SMA (juvenile and adult forms)
2. Variants of infantile SMA
  - 2.1 Diaphragmatic SMA (SMARD) (ar)
  - 2.2 SMA plus cerebellar hypoplasia (ar)
  - 2.3 SMA plus arthrogryposis and bone fractures (ar, XI)
  - 2.4 SMA plus myoclonic epilepsy (ar)
3. Nonproximal SMA
  - 3.1 Distal SMA (ad, ar, XI)
  - 3.2 Scapuloperoneal SMA (ad, ar)
4. Bulbar palsy
  - 4.1 Adult-onset bulbar palsy (ad)
  - 4.2 Progressive bulbar palsy of childhood type Fazio–Londe(ar)
  - 4.3 Bulbar palsy with deafness (Brown–Vialeto–van Laere syndrome) (mostly ar)
5. Spinobulbar muscular atrophy type, Kennedy’s disease (XI)

ar, autosomal recessive; ad, autosomal dominant; XI, X-linked.

## 128.2.2 History

In 1891, Guido Werdnig (1844–1919), an Austrian neurologist from the University of Graz, described two brothers with proximal SMA (9). Two years later (1893), Johann Hoffmann (1857–1919), a German neurologist from the University of Heidelberg, described four cases belonging to two families (10). Some of these children did not show the fatal course, which is usually meant by the term Werdnig–Hoffmann disease. The description of Oppenheim’s “myatonia congenita,” later changed to “amyotonia congenita,” led to confusion for several decades. It is believed that heterogeneous conditions have been summarized, among them many proximal SMAs. Kugelberg and Welander (11,12) described 12 patients belonging to eight sibships with an onset between 2 and 17 years. All were able to walk at least 8 to 9 years after the symptoms started.

Following the first descriptions, more than a century ago, many systematic studies about various aspects on SMA have been published. Most of the older descriptions are relevant and important even today. The first systematic monograph was published in 1951 by Sven Brandt, who collected and investigated all patients in Denmark (13). Irena Hausmanowa-Petrusewicz from Poland made essential contributions to nearly all aspects of childhood-onset SMA (14). John Pearn performed a systematic study about various aspects of childhood and adult SMA in northeastern England and, between 1977 and 1980, published numerous fundamental papers about SMA.

In 1990, the gene responsible for infantile and juvenile SMA was mapped to chromosome 5q13, and this entity was further denoted as SMA 5q. In 1995, homozygous deletions in the survival motor neuron (SMN1) gene were found by the group of Judith Melki, France, to be the leading cause of SMA 5q (15), whereas a smaller number of patients showed homozygous deletions in other genes of this highly complex region, such as the neuronal apoptosis inhibitory protein (NAIP) gene (16). The SMN1 gene proved to be the most important causative gene of SMA 5q and has further been studied on the protein level, in cell cultures, and in animal models.

With the foundation of the International SMA Consortium in 1990, supported by the European Neuromuscular Research Center and the American Muscular Dystrophy Association, diagnostic criteria have been proposed and research activities coordinated. Before understanding the genetic basis of infantile SMA, diagnostic criteria were defined in order to collect a homogeneous sample of patients for subsequent linkage studies (17). Following the SMN gene identification, the American self-support group “Families of SMA” initiated and financed international research activities with the aim to find a treatment for infantile SMA ([www.fsma.org](http://www.fsma.org)). In Europe TREAT-NMD (Translational Research in Europe for the Assessment and Treatment of Neuromuscular Diseases), a Network of Excellence funded within the sixth Framework Programme of the European Commission, was founded in 2007 ([www.treat-nmd.eu](http://www.treat-nmd.eu)). TREAT-NMD is a network for the neuromuscular field focused on the development of novel therapeutic approaches and establishing the best-practice care for neuromuscular patients worldwide. TREAT-NMD created a global registry for SMA combining standardized information from each of the national registries.

## 128.2.3 Clinical Picture

The clinical and laboratory criteria of proximal SMA were repeatedly defined by the International SMA Consortium (Table 128-2) (17,18). Meanwhile genetic screening of the SMN1 gene has become an important diagnostic tool and has generally replaced invasive procedures in SMA 5q. In other SMA forms, where the genetic basis is heterogeneous or largely unknown, clinical work-up still needs to be done in experienced hands.

As far as the clinical investigations are concerned, biochemical investigations of serum or cerebrospinal fluid show no specific abnormality. Muscle enzyme (creatine kinase (CK)) activity is normal or only slightly increased mostly in juvenile-onset cases (19). If the serum CK level is more than fivefold above the upper limit of normal, a myopathy is more likely.

Electromyographically, neurogenic changes can be seen with abnormal spontaneous activity (fibrillations, positive sharp waves, fasciculations) in progressive disease courses. Motor unit potentials elicited by

**TABLE 128-2** Diagnostic Criteria of Proximal Spinal Muscular Atrophy (18)

Inclusion Criteria	Exclusion Criteria
Symmetric muscle weakness of trunk and limbs	Involvement of extraocular muscles, diaphragm and myocardium*
Proximal muscles > distal	Marked facial weakness*
Lower limbs > upper limbs	CNS dysfunction
Fasciculations of tongue, tremor of hands CK usually <5 times the upper normal limit	Arthrogryposis (but rarely seen in severe SMA type I)
Neurogenic changes in EMG and muscle biopsy	
Homozygous absence/mutation of the SMN1 gene	Reduction of motor nerve conduction velocities <70% of lower normal limit or abnormal sensory nerve action potentials in SMA II–III (motor and sensory conduction can be markedly abnormal in SMA I)

CK, creatine kinase; CNS, central nervous system; EMG, electromyography; SMA, spinal muscular atrophy; SMN, survival motor neuron.

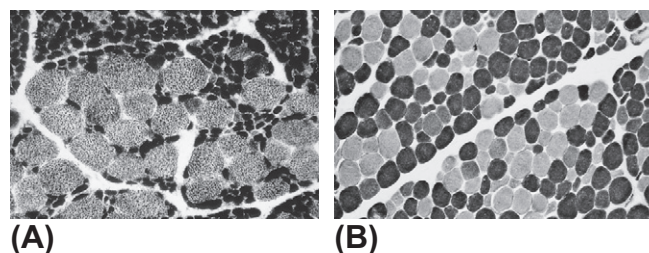
\*Exceptions are rare congenital onset cases with external ophthalmoplegia and facial weakness.

voluntary effort have an increased mean duration and amplitude, indicating reinnervation. The interference pattern on increasing effort is reduced because of loss of motor units.

Peripheral nerve conduction is generally normal. However, in severe cases of SMA type I, motor and sensory conduction velocities can be reduced owing to depletion of fast-conducting large-diameter nerve fibers.

Muscle biopsy specimens typically show large group atrophy in severe SMA (Figure 128-1), whereas the atrophic groups of both fiber types appear to be smaller in the chronic forms. Histochemical staining reveals fiber type grouping mainly in the more chronic cases reflecting reinnervation. In SMA I and II, hypertrophic fibers of type I are distributed among the atrophic fibers of either histochemical type. In SMA III and IV, there is prominent reinnervation with type grouping, but small angular fibers occurring in small groups or singly are also present and signal ongoing denervation.

In accordance with the diagnostic criteria, peripheral nerves are morphologically normal in the vast majority of SMA patients. When axonal degeneration is observed in motor nerves, it has been attributed to a dying-forward phenomenon as a consequence of motor neuron degeneration, but recent studies suggest a primary axonal dysfunction in SMA 5q. Corresponding to the electroneurographic findings, there can be significant axonal degeneration not only in motor but also in sensory nerves at least in SMA I (20).



**FIGURE 128-1** Muscle biopsy findings in severe (A) and mild (B) SMA. Note large group atrophy and type I fiber hypertrophy in severe SMA, whereas small group atrophies and fiber type grouping predominate in mild SMA (ATPase staining).

In the spinal cord (Figure 128-2), pathologic features are gliosis of anterior horns without areas of demyelination. Although the posterior roots appear to be normal, distinct demyelination can be seen in the anterior roots. The degenerated anterior horn cells become small and pycnotic until they completely disappear. Because in patients with severe SMA other parts of the central nervous system (CNS) also show degenerative processes (21), the full spectrum of pathologic abnormalities in SMA is yet to be clarified.

**128.2.3.1 Clinical Course and Classification.** The clinical picture of proximal SMA is highly variable, indicating a continuous spectrum with ages of onset from before birth to adulthood rather than to distinct disease subgroups. The currently used classification schemes take age of onset, life span, and motor development into account but vary to a large extent, so that it is difficult to compare the defined subgroups with each other. This problem was addressed by Dubowitz (22) and Russman (23) and resulted in the proposal of a common classification by the International SMA Consortium. The subgrouping of childhood-onset SMA was basically suggested as a guideline for inclusion of patients in DNA studies and later analysis of genotype–phenotype correlations after identification of the gene for autosomal recessive childhood SMA. A major problem with the existing classifications is that the prognosis often turns out to be better than that associated with a defined subtype. This means that a patient initially classified as severe type I SMA may have to be reassigned to intermediate type II or even type III when the patient's life span exceeds the predicted age of death. Therefore, it is better to define SMA types I–IV by achieved motor functions and by age of onset rather than by prognostic limitations (Table 128-3).

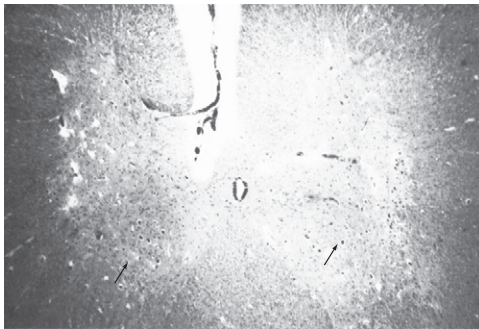
**128.2.3.2 SMA Type I.** The clinical signs of the most severe SMA type, which is also called Werdnig–Hoffmann disease, are evident at birth or soon thereafter, with a median age of onset of 1 month (24). Nearly all patients present by 6 months of age, and one-third have decreased fetal movements.

Symptoms are profound hypotonia and generalized weakness (Figure 128-3). The infants do not kick well and are never able to sit unaided. Weakness is proximally

**TABLE 128-3 Classification of Proximal Spinal Muscular Atrophy**

SMA Type	Principal Synonyms	Definition	Genetics
I	Werdnig–Hoffmann disease Acute infantile SMA	Sitting not achieved Onset usually within the first 6 months Death >90% by 10 years	Autosomal recessive
II	Chronic childhood SMA  Arrested Werdnig–Hoffmann disease	Unaided sitting possible, walking not achieved Onset usually in the first year of life Survival >90% by 10 years	Autosomal recessive
III	Kugelberg–Wielander disease Juvenile SMA	Walking without aids achieved – IIIa: Onset ≤ 3 years – IIIb: Onset >3 years Mild course, life span not markedly reduced	Autosomal recessive Excess of men
IV	Adult SMA	Onset >30 years Variable severity, normal life span	Mostly sporadic Autosomal dominant Autosomal recessive (extremely rare)

SMA, spinal muscular atrophy.



**FIGURE 128-2** Cross-section of gray matter in spinal cord of an infant who died from severe SMA. Motor neurons are grossly reduced in number, and surviving cells are pycnotic (arrows). (Courtesy of Professor John Pearn, Herston Qld, Australia.)

pronounced, and the legs are weaker than the arms. Tendon reflexes are absent. Tongue fasciculations and hand tremor are characteristic features of infantile SMA, whereas muscle wasting is often inconspicuous. The diaphragm and the extraocular muscles tend to be spared, which distinguishes classic SMA from the rare diaphragmatic SMA variant. A certain percentage of infants show mild joint limitations from birth (limited hip abduction or knee and elbow extension and ulnar deviation of the hands), but this is much less severe than in infants with arthrogryposis multiplex congenita. Typical ulnar deviation in the hands is shown in [Figure 128-4](#). Weakness affects both bulbar and respiratory muscles with a rapid progression in most cases; the median age at death of patients with diagnosis in the first 6 months was 6–7 months (25). Chronic forms exist ([Figure 128-5](#)), and some patients survive into adulthood (26). Because an increasing number of countries are offering mechanical respiratory support either through tracheostomy or noninvasive ventilation, the survival probabilities have changed dramatically (27–29). Although the question



**FIGURE 128-3** Girl with SMA type I at the age of 10 months, a few weeks before her death.

of quality of life is still a matter of debate in the totally paralyzed and ventilated SMA type I children, survival into the thirties has rarely been reported on a stable functional level. Moreover, there is concern about previously unknown complications from the cardiovascular side in long-term SMA type I, including bradycardia progression to a cardiac standstill (30) and other features suggesting autonomic nervous system defects.

**128.2.3.3 SMA Type II.** The clinical course of SMA type II or chronic childhood SMA, arrested Werdnig–Hoffmann disease, or intermediate-type SMA is marked by periods of apparent arrest in the clinical progression. The age of onset and presenting signs may be indistinguishable from SMA type I, although the median age of onset is generally later (8 months). The children fail to pass motor milestones because of proximal weakness and hypotonia within the first 18 months of life. There is a wide variability of clinical severity, ranging from children who have early difficulty sitting ([Figure 128-6](#)) or rolling over to patients who can crawl or walk with support. For practical purposes, this group is defined by the ability to sit independently, because the children never learn to stand or walk unaided. Pronounced weakness of trunk muscles in the nonambulatory patients results





**FIGURE 128-4** Ulnar deviation in the hands of a 2-month-old paralyzed infant with severe SMA type. (Courtesy of Professor John Pearn, Herston Qld, Australia, by kind permission of Dr John Wilson, The Hospital for Sick Children, London.)

in spine deformities and reduces lung capacity. Contractures develop early in all major joints owing to synergist-antagonist imbalance (Figure 128-7). Hand tremor and fasciculations are characteristic features. Most patients survive into adulthood (Table 128-3), and life span can be influenced by unrelated factors, such as body weight, growth spurts, and infections.

Because progression is slow and survival into adulthood is the rule rather than the exception, major emphasis must be placed on schooling and education. It is important to know that the cognitive functions are not affected in SMA. Children with chronic SMA often seem to be brighter than others of the same age, which may be related to early speech development. In a large German study, general intelligence measured in 96 SMA patients, who had mostly type II SMA, was not different from that of age-matched controls and their healthy sibs (31).

**128.2.3.4 SMA Type III.** A mild form of childhood and juvenile SMA type III is known as Kugelberg-Welander disease and shows a wide range of clinical onset from the first year of life until the third decade. Patients with SMA type III learn to walk without support, which distinguishes them from those with SMA type II. For prognostic reasons, this group has been separated into types IIIa and IIIb (Table 128-3).

In SMA type IIIa, onset is in the first 3 years of life, the children have early walking difficulties and often fail to pass further motor milestones, for example, rising from the floor or climbing steps (Figure 128-8). Because many patients are nonambulatory by school age, they are much more handicapped than those whose walking difficulties begin in youth or adulthood. In a German and Polish study, 50% of SMA type IIIa patients were confined to a wheelchair 14 years after onset (32). Similar results were obtained in a study undertaken in Hong Kong, where 50% of patients were chair-bound 20 years after onset (28). In SMA type IIIb, the first signs of weakness occur between 3 and 30 years and are mainly problems in running, climbing, and sports. As in SMA II, tremor of the outstretched hands and limb



**FIGURE 128-5** SMA type I patient with prolonged survival. Current age 7 years, deformed thorax; death at 9 years after numerous lung infections.

fasciculations are frequent findings. The progression can be very slow or even imperceptible. About 50% of SMA type IIIb patients are still ambulatory after having had the disease for 45 years (28,32). Depending on the degree of weakness, spine deformities and contractures are frequent complications, mainly in the chair-bound patients. Respiratory insufficiency requiring assisted ventilation can become a problem in older, immobile patients late in the disease; however, life expectancy is not much reduced.

**128.2.3.5 SMA Type IV.** Although there is a spectrum of manifestations in SMA types I–III, with overlapping features between the subgroups, SMA type IV or adult SMA is a more distinct and heterogeneous entity. Lower motor neuron disease is also seen in series of patients with motor neuron disease including amyotrophic lateral sclerosis (ALS), i.e., a distinction is not always possible and variable phenotypes can occur within a family. Attempts were made to classify subtypes of adult-onset SMA according to the distribution for weakness and progression (33,34). Because the underlying pathogenetic mechanisms of the different types remain to be identified, subgrouping and prediction of disease courses is difficult at least at the time of diagnosis.

Only exceptionally, SMA 5q starts after 30 years of age. These patients have a strictly proximal involvement, with marked weakness of the pelvic girdle muscles, particularly of the iliopsoas muscle. It is believed that these patients have a mild SMA 5q owing to a large number



**FIGURE 128-6** Twin brothers with early-onset SMA type II. A sitting position is maintained only for several minutes followed by a “pocket knife” phenomenon.

of SMN2 gene copies, but clinical variability cannot be explained by the SMN genes alone. Some patients have an age of onset between 20 and 32, and can thus be classified borderline between SMA III and IV. In our study, onset in adult SMA ranged from 30 to 60 years, with pronounced proximal weakness, particularly of the limb girdle and thigh muscles. The condition is relatively benign with slow clinical progression, and a normal life span can be expected (34,35). It must be stressed that in contrast to SMA I–III, recurrence of SMA IV within a sibship is exceptional. Since there is a marked predominance of males, hormonal or modifying genetic factors are assumed to influence the pathophysiology (34).

### 128.2.4 Genetics

The proximal SMAs are genetically heterogeneous; both autosomal recessive and autosomal dominant genes are known to cause childhood- or adult-onset SMA. The clinical picture does not allow the distinction of different modes of inheritance, thus leading to problems of genetic counseling in families in which only one subject is affected and in whom no gene defect is detected.

Except for Kennedy syndrome and the distinct entity of infantile SMA with congenital contractures and bone fractures (see section on Variants of Childhood Onset), there is no convincing evidence of an X-linked proximal SMA. There is an excess of male patients, however, best noted among the simplex families and in the milder forms of proximal SMA.

**128.2.4.1 Autosomal Dominant SMA.** As stated earlier, autosomal dominant proximal SMA is extremely rare, but was observed more frequently in late onset SMA IV than autosomal recessive inheritance in some European studies. The course is usually mild or only slowly progressive, and most patients remain ambulatory many years after onset.

Pearn (8) suggested that there are two dominant genes, one accounting for the childhood-onset form and one for the adult-onset form. Penetrance was thought to be nearly complete in both forms. There is marked intrafamilial variability of age of onset and



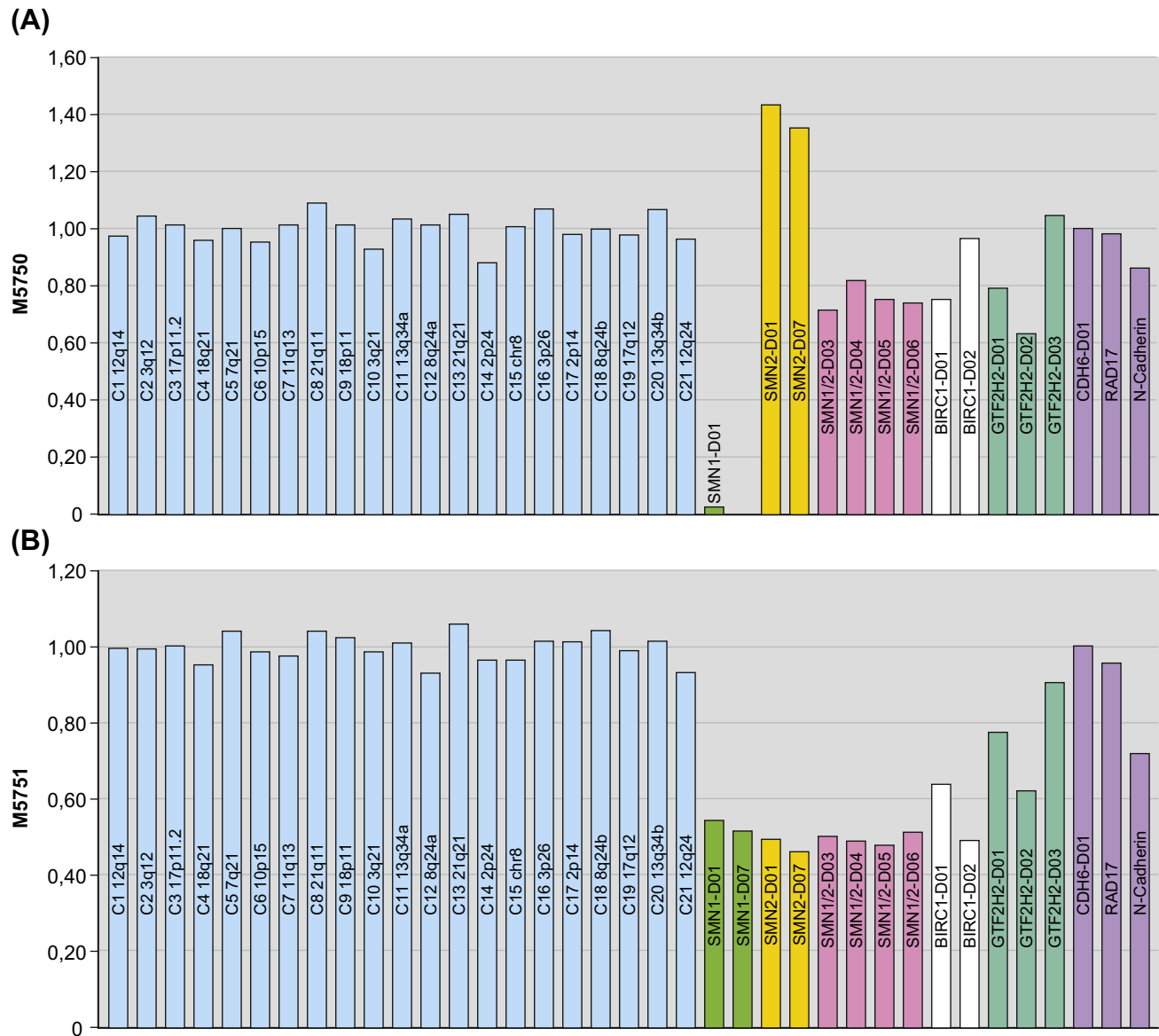
**FIGURE 128-7** Proximal childhood-onset SMA. An 11-year-old girl showing gross kyphoscoliosis. (Courtesy of Professor John Pearn, Herston Qld, Australia.)



**FIGURE 128-8** SMA type III in a 3-year-old boy. Rising with Gower's sign, proximal muscle wasting.

progression, which is not compatible with the subdivision into two clinically defined genetic entities (36). Pedigree analysis and molecular testing can be helpful to identify pseudodominant families in which one parent is homozygous and the other heterozygous for an autosomal recessive gene.

Large dominant pedigrees of late-onset SMA were reported from Brazil and designated SMA type Finkel (OMIM 182980) (37,38). Mean age of onset was 48 years; muscle cramps and respiratory insufficiency occurred early in the disease course. The gene defect has been recently identified on chromosome 20q13.3 (VABP gene) and gave evidence of a founder effect from a common Portuguese ancestor in these and other families (39). Mutations in the VABP gene were also detected in atypical and typical ALS families with marked interfamilial and intrafamilial variability, and some families showed overlapping features of SMA and ALS (39). In other populations, VABP gene mutations seem to be an exception (40).



**FIGURE 128-9** Multiplex ligation-dependent probe amplification (MLPA): detection of a homozygous (A) or heterozygous (B) SMN1 gene deletion.

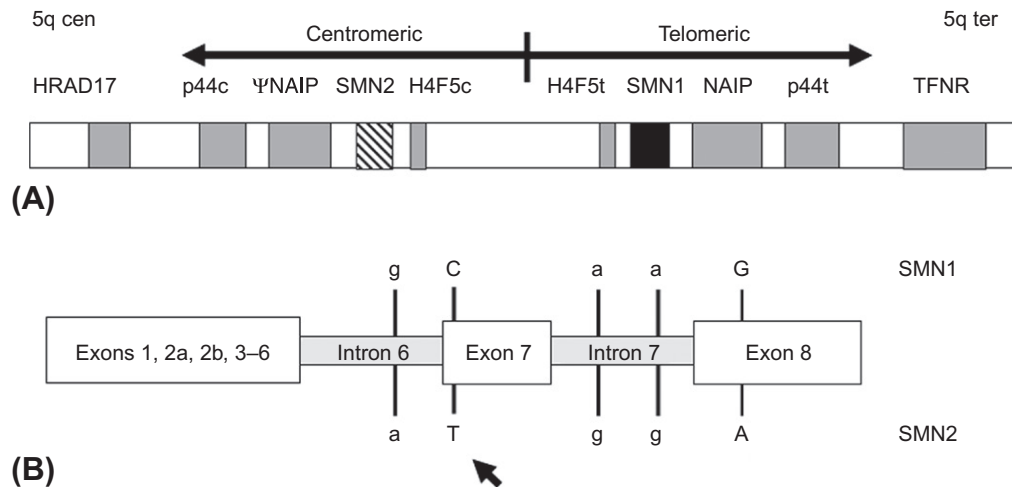
A further locus for an early-onset proximal SMA was demonstrated on 14q32 in a large North American family (41), but still many genetic defects and chromosomal loci are so far unknown in autosomal dominant SMA.

**128.2.4.2 Autosomal Recessive SMA.** The majority of cases with SMA are autosomal recessively inherited. Taking the acute and chronic forms of childhood SMA together, the incidence is at least 1:10,000 among whites.

**128.2.4.3 Molecular Biology.** The recessive gene responsible for most families with childhood SMA has been localized in 1990 to a small region of the long arm of chromosome 5 (5q11.2-q13.3). Different genes and microsatellite markers were identified in the 5q region that can be deleted in SMA patients. Deletions or mutations of the telomeric copy of survival motor neuron (SMN1) gene are disease specific and confirm the diagnosis (15). The SMN1 gene consists of eight exons spanning about

20 kb genomic region. It encodes a 38 kDa protein with 294 amino acids, the SMN protein. Homozygous deletions of the SMN1 gene are detected in more than 90% of SMA type I–III patients and only exceptionally in SMA IV. Homozygous deletions can be made visible by single-strand conformational analysis (SSCA) or by restriction digest of polymerase chain reaction (PCR) products (42). In the vast majority of patients, exons 7 and 8 are absent, followed by a 5–10% deletion rate of exon 7 only. Moreover, isolated deletion of exon 8 has been detected in rare instances of mild SMA (43). Quantitative methods allow a direct estimation of the number of SMN1 and SMN2 gene copies, e.g., by multiplex ligation-dependent probe amplification (MLPA) (Figure 128-9).

The centromeric copy of the SMN gene (SMN2) is nearly identical with the SMN1 gene except for five



**FIGURE 128-10** Schematic representation of the two SMA genes in the chromosomal region (5q13) (A), localization of the nucleotides by which SMN1 can be distinguished from SMN2 (B). The arrow indicates the C>T base exchange in exon 7 of SMN2 that causes alternative splicing.

nucleotide differences in exon 7 and 8 (Figure 128-10), yet their transcriptional products are not the same. Although SMN1 produces full-length transcripts, SMN2 primarily gives rise to truncated transcripts lacking exon 7 (60%), exon 5 or both. The protein product SMN $\Delta$ 7 is nonfunctional and is degraded rapidly. Only minor amounts of full-length SMN transcripts (10–20%) are produced by SMN2, but this has been in the focus of therapeutic research in the past 10 years (see section on Management). The critical difference between SMN1 and SMN2 is a C>T base change in exon 7 that was believed to disrupt a putative exonic splice enhancer (ESE) and cause alternative splicing of SMN2 exon 7 (44,45). In the past years it was shown that SMN splicing machinery is much more complex and includes positively and negatively acting regulatory elements (46). Homozygous deletions of SMN2 gene can be detected in about 5% of the normal population but are not associated with a disease phenotype if at least one SMN1 copy is retained.

Only in a small number of patients (approximately 3–4%), subtle mutations in the SMN1 gene have been identified (47,48) that mostly occur as compound heterozygotes in conjunction with a deletion. Compound heterozygotes can be detected after PCR-based densitometric assessment of SMN-gene copy number, by pulsed field gel electrophoresis or by fluorescence-based carrier testing, but in the past years quantitative analysis of SMN1 and SMN2 copy numbers largely replaced these laborious methods. In routine analysis, technology based on real-time fluorescence PCR using a LightCycler (49) and TaqMan technology (50), and increasingly MLPA are in use. These quantitative methods identify patients and relatives who retain only one copy of SMN1. The information obtained in this way can be used for further diagnostic work-up of patients for genetic risk calculation in affected families (see section on Genetic Counseling).

The SMN protein is expressed in all somatic tissues and is highly conserved from yeast to man. It is involved

in RNA processing and is localized in structures called “gems” in the nucleus. The SMN protein acts in concert with several other proteins in the regeneration of the small nuclear ribonucleoproteins (snRNPs), acting as an assemblyosome in the formation of diverse RNP particles (for a review see References (46,51)). It is still unclear why SMN protein deficiency results in selective motor neuron loss, because the gene is ubiquitously expressed. More recent studies of cell cultures and animal models have shown that the SMN protein is important for axonal growth and transport of motor neurons. Additional functions are ascribed to the skeletal muscle and to the integrity of the neuromuscular junction.

**128.2.4.4 Animal Models.** The SMN protein is transcribed by the two SMN genes that were generated by a duplication on chromosome 5q around a million years ago (52); therefore, no natural animal model for infantile SMA exists. Before the SMN gene was discovered, it was hoped to find a human homolog derived from the different mouse models of motor neuron degeneration (for a review see Reference (53)). This approach was eventually successful in identifying the human homolog of the nmd mouse (54), which turned out to be the IGHMBP2 gene responsible for the SMA variant with diaphragmatic palsy (SMARD1).

Several groups have attempted to model SMA in zebrafish, *Drosophila*, and *Caenorhabditis elegans* but it was difficult to reconcile conclusions generated in different species (for a review see Reference (53)). New and emerging insights were obtained from model mice. As regards murine *smn* and *naip* genes, the number of *naip* genes is increased, but there is only one *smn* copy. Replacement of murine *smn* by human SMN2 genes resulted in transgenic mice that show features of the human disease (55–57). These mice not only produced different SMN2 numbers and had varying phenotypes resembling human SMA but also had additional features like necroses of the tail, ear and digits (58). Moreover, it was shown that



there are early abnormalities of the neuromuscular junction while motor neuron loss was recorded later in the disease course of severely affected mice (59). Splice correction by antisense oligonucleotide (ASO) therapy into the CNS increased SMN protein levels and also corrected the tail and ear necroses, which therefore appeared to be related to an innervation deficit (60).

In the transgenic mouse model created by the French investigators, exon 7 of the murine *smn* gene was removed from both mouse chromosomes in a tissue specific manner by using the Cre/loxP recombination system. Although mice with SMN deficiency restricted to the spinal cord (neuronal mutant) developed an SMA-like phenotype (61), different disease phenotypes were induced in mice in which *smn* genes were deleted in the muscle (62) or in the liver (63). These data suggest a ubiquitous role of full-length SMN protein in various mammalian cell types. It appears that the residual amount of SMN protein produced by the SMN2 genes in humans allows a normal function of other organs except motor neurons.

Eventually, treatment studies of transgenic mice have supplied first proof of concept of the feasibility of modulating the SMN2 gene for therapeutic purposes.

**128.2.4.5 Genotype–Phenotype Studies.** The proportion of clinically typical SMA patients who do not show a homozygous SMN1 deletion does not exceed 5% in SMA type I and II, but increases to 10–20% in mild SMA type III (64). Although deletions of variable size occur in SMA I, in the milder SMA II and III, gene conversion of SMN1 to SMN2 is responsible for the reduced transcription of the SMN protein. Several studies have demonstrated chimeric SMN genes with SMN2 exon 7 fused to SMN1 exon 8, which increases the number of SMN2 copies to 3–4 in milder SMA types. Thus, SMA I is caused by physical deletions of SMN1, whereas the mutations in type II and III SMA consist of replacement of SMN1 by SMN2. The number of SMN2 copies correlates with SMA subtype, age of onset, and length of survival, that is, the clinical phenotype depends primarily on the level of functional SMN protein (25,49,65). However, it is not possible to predict the course of the disease in individual patients from the size of the observed deletion or the gene conversion event, because there is a wide overlap of molecular genetic findings in the different types of SMA.

**128.2.4.6 Abnormal Phenotypes Associated with SMN Gene Deletions.** Based on molecular genetic findings, the clinical spectrum of SMA 5q was extended to more severe and atypical presentations as well as to subclinical manifestations. It is now clear that a “congenital” type of infantile SMA exists, which includes severe neonatal onset with respiratory insufficiency (66) and congenital contractures (25,67,68) (Figure 128-11). In some of these patients, a severe SMN protein deficiency was seen by the presence of homozygous SMN1 gene deletions and only one SMN2 gene copy (25). Life span is short, with death occurring soon after birth



**FIGURE 128-11** Patient with congenital SMA presenting with arthrogryposis multiplex and respiratory distress from birth, fetal arrhythmia and polyhydramnios in pregnancy. Picture taken at age 10 days, death after 18 days. Molecular genetic analysis showed a homozygous SMN1 deletion and only one SMN2 gene copy (patient 3 from (25)).



**FIGURE 128-12** Sural nerve biopsy in a patient with severe SMA type I featuring signs of congenital hypomyelination. Severe axonal loss points toward primary involvement of the sensory nervous system (patient 2 from Reference (69)).

unless under mechanical ventilation. Rarely external ophthalmoplegia, and severe peripheral (sensory and motor) nerve involvement (Figure 128-12), clinically indistinguishable from congenital hypomyelination neuropathy, can be a consequence of severe SMN deficiency (69). In this context it has to be clarified whether the rarely observed vascular perfusion abnormalities resulting in digital necroses (70,71) are a consequence of an innervation deficit or of an independent mechanism in the blood vessels.

More recently it turned out that congenital heart disease is also a feature of severe SMA associated with a small number of SMN2 copies. In our series of patients with one SMN2 copy, three out of four had a congenital heart defect, mostly atrioventricular septal defects

(72). Following this observation in humans, recent studies of transgenic mouse models confirmed early-stage developmental defects of the heart, i.e., interventricular septum and left ventricular wall remodeling along with interstitial fibrosis and thinning of arterial walls (73). Other mouse models developed severe bradyarrhythmia and dilated cardiomyopathy (74,75). These studies shed a new light on SMN function in other tissues.

**128.2.4.7 Modifying Factors.** The presence of modifying factors had been studied soon after the discovery of the SMN1 gene, following the observation of families, primarily published as unlinked to chromosome 5q markers, in whom SMN1 gene deletions were found, not only in the patients but also in their unaffected sibs (Figure 128-13) (64,76–78).

Preliminary data indicate that a small fraction of patients with mild SMA have haploidentical sibs who also show deletions in the SMN1 gene, thus confirming the responsible gene locus on chromosome 5. This finding suggests that there are environmental or genetic mechanisms that can suppress the effect of

a nonfunctioning SMN1 gene and therefore reduces penetrance of the disease. The most important modifier is the SMN2 gene itself because a large number of SMN2 copies can suppress disease manifestation, but this has not explained the nonaffected relatives with homozygous SMN1 deletions. In addition, a positive modifier of SMN expression from the retained SMN2 genes is the c.859G>C variant in SMN2 first described by Prior et al. (79). A larger Spanish study showed that this variant is present in about 1–2% of the normal population but was detected in about half of the patients with two SMN2 copies and an SMA type II or III phenotype (80). The group of Wirth identified mutations in the X-linked plastin gene as an explanation for a reduced expression and a milder phenotype in females (81). Moreover, it was shown that ciliary neurotrophic factor (CNTF) deficiency results in a deterioration of SMA in a transgenic mouse model (82); comparable data in humans are not available as of February 2011.

**128.2.4.8 Sex Influence.** The question of a possible influence of gender has been a matter of controversial discussion. There was a predominance of men in the whole German study group (243:202), which was most pronounced in the mild form (type III) (82:52) (35). Only a small number of girls developed the disease after puberty. Very similar results have been obtained by Hausmanowa-Petrusewicz (83). It still needs to be clarified whether a “female-sparing factor” is responsible for a remarkable decrease in the number of affected girls in the late-onset group. Among children belonging to sibships with at least two affected siblings, no significant sex difference could be obtained in our data of 101 affected sibs out of 48 families (84).

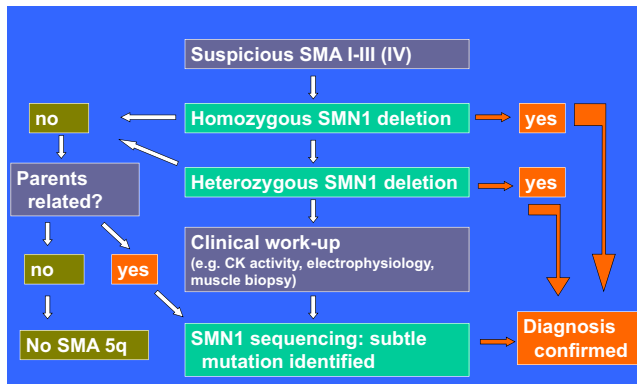
**128.2.4.9 Genetic Counseling.** Most cases of proximal SMA follow an autosomal recessive mode of inheritance. For genetic counseling, the estimated heterozygosity frequency for SMA 5q is about 1 in 50 in the Caucasian population (see section on **Epidemiology**).

The detection of homozygous deletions in the SMA region confirms the diagnosis and establishes autosomal recessive inheritance in SMA types I–III. Patients with adult-onset SMA (onset >30 years) are only exceptionally associated with alterations of the SMN1 gene. In clinically typical patients, a heterozygous SMN1 gene deletion is likely to act with a subtle mutation on the other chromosome (3–4% of patients), while in consanguineous families a homozygous subtle mutation has to be taken into account. Linkage analysis with genetic markers of the SMN region on 5q13 may point toward homozygosity and the correct diagnosis. The diagnostic algorithm for SMA 5q (Figure 128-14) has to consider the clinical picture and pedigree information. Mutation analysis of the SMN1 gene is hampered by the complex genomic region and not offered on a routine basis. It is established by cDNA analysis following RNA isolation (85) or by a long-range PCR method (86). The



**FIGURE 128-13** Family with homozygous deletions of SMN1 not only in the affected son with classical SMA type II but also in the healthy mother.





**FIGURE 128-14** Diagnostic algorithm of SMA 5q.

genetic basis of patients who are unlinked to chromosome 5q remains to be identified; in those patients other diagnoses have to be ruled out by thorough clinical examination.

Carrier testing of relatives or spouses is meanwhile an integral part of genetic counseling and has improved in sensitivity and specificity in the past years. The consequences for genetic risk calculation have been outlined in detail (3). The SMN1 copy number can be measured by quantitative methods and can reliably identify heterozygous carriers of SMN1 gene deletions. The test sensitivity does not exceed 95%, because two or more SMN1 copies are present on normal chromosomes in 3–4% of carriers. In addition, new SMN1 deletions occur in about 1% of SMA chromosomes, and subtle mutations cannot be identified by the quantitative test. Therefore, it is recommended to investigate the spouse, if the person at risk carries an SMN1 deletion or cannot be excluded as a gene carrier from previous investigations in the family.

In families in which the index patient had died before molecular studies were undertaken, the evidence of a heterozygous SMN1 gene deletion in the parents gives a clue toward the genetic defect and allows a correct risk estimation of further relatives.

Prenatal testing can be safely performed in families with proven homozygous SMN1 deletions/mutations. If there is only a heterozygous SMN1 deletion in the patient and the second mutation is unknown, prenatal diagnosis can be offered through analysis with chromosome 5q markers. A typical clinical picture in the patient is a prerequisite. Prenatal testing is not applicable in families without evidence of alterations in the SMA region on chromosome 5q.

If a spouse of a proven gene carrier has normal SMN values, this reduces the remaining risk to the offspring to a level where prenatal testing is no longer recommended. However, the detection rate through SMN1 gene deletion screening in fetal DNA has a higher sensitivity than the risk calculation based on carrier testing in the parents. If prenatal diagnosis is undertaken for unrelated reasons in a pregnancy at risk, SMN1 gene deletion screening can further reduce the fetal risk. Considering

the implications of carrier testing, it is mandatory to offer molecular genetic studies only within the context of genetic counseling.

### 128.2.5 Pregnancy

Women affected with childhood-onset SMA are unlikely to have their own children, but increasing reports of successful pregnancies even in severely handicapped SMA type II patients are encouraging. In long-standing diseases with stable lung function, the risk of major complications or deterioration seems to be small. In chair-bound patients, the preferred mode of delivery is elective caesarean section, which can be safely conducted under subarachnoid block (87). In SMA II the respiratory situation is essential for pregnancy planning. While successful pregnancies have been reported in women with vital capacities as low as 11% (88), the risk for miscarriages or stillbirths depending on the lung function is increased but yet ill-defined. In SMA II and SMA III there is an increased preterm birth rate and most pregnancies end these days via a planned caesarean section (89). There is still controversy about the appropriate options for anesthesia and analgesia management in SMA patients, as information is limited (88,90). No deleterious effects on fetal or maternal outcome were seen in a series of 13 patients with SMA type III (89). Exacerbation of muscle weakness after the second trimester was experienced by some of these patients, most striking and consistent in women with a presumed autosomal dominant inheritance of proximal SMA (89). Nonetheless, the majority of women emphasized the great personal values of raising children, who were born mostly against medical advice, especially in childhood-onset cases. Recommendations for affected women who plan a pregnancy have recently been established during a workshop organized by the European Neuromuscular Center (ENMC) at the end of 2010 (91).

### 128.2.6 Differential Diagnosis

The differential diagnosis of acute SMA type I comprises the whole spectrum of the floppy infant syndrome, including cerebral malformations, perinatal asphyxia, metabolic defects, Prader–Willi syndrome, Zellweger’s syndrome, mitochondrial disease, and other congenital neuromuscular disorders. A careful neuropsychiatric examination is required to exclude other diseases if SMN1 deletion screening is negative. If there is clear evidence of anterior horn cell disease, the so-called SMA plus variants have to be taken into account as separate genetic entities.

The clinical picture of the mild SMA type III is often indistinguishable from the muscular dystrophies even after neurologic investigations. The neurogenic changes in long-standing SMA courses become less prominent with increasing age, so that a definite diagnosis may be impossible. Families classified as X-linked proximal SMA turned out to have a deletion in the dystrophin gene, confirming X-linked muscular dystrophy. In many

patients with limb-girdle muscular dystrophy, it is now possible to classify the immunohistochemical defect in muscle. If cardiomyopathy is seen in patients with apparent SMA, this should prompt a search for a mutation in the lamin A/C gene and immunostain studies for emerin to exclude the autosomal dominant and X-linked forms of Emery–Dreifuss muscular dystrophy. Lamin A/C gene mutations were seen in 10% of our families with the diagnosis of autosomal dominant proximal SMA (92).

Hexosaminidase A deficiency may rarely produce a clinical picture resembling SMA type III, because signs of cerebellar dysfunction (mainly dysarthria) may appear years after clinical onset. In particular, if atypical features in juvenile-onset SMA are present, a hexosaminidase deficiency should be excluded by laboratory studies (93).

The most important differential diagnoses of SMA type IV are Kennedy syndrome, with an X-linked mode of inheritance (see later), and ALS, which is rarely familial (10–20%) and can be inherited as an autosomal dominant or recessive trait. ALS often starts with pure lower motor neuron signs, but progression of weakness is more rapid and bulbar symptoms usually appear in early stages of the disease. In most cases, there is evidence of upper motor neuron involvement soon after clinical presentation, and this problem should resolve diagnostic difficulties. In occasional cases, upper motor neuron signs are lacking, and a distinction might not be possible. This is underscored by the observation of families with an overlapping SMA/ALS phenotype and marked intrafamilial variability based on VAPB gene (39) or SETX gene mutations (94).

Interestingly, alterations of the SMN genes have been demonstrated in patients with sporadic ALS and with slowly progressive SMA, indicating that deficiency of the SMN protein can also modify the phenotype of adult motor neuron diseases (95–97).

Acid maltase deficiency can also mimic SMA type IV or the Kennedy syndrome, because glycogen accumulation is not always apparent in the biopsied muscle.

In dominant SMA families, proximal myopathic myopathy (PROMM) or myotonic dystrophy type 2 (DM2) has to be considered because changes suggesting neurogenic atrophy may appear in muscle. The presence of myotonia and cataracts, and molecular genetic screening of the expanded CTTG repeat in the ZNF9 gene on chromosome 3 should clarify the diagnosis.

Postpoliomyelitis muscular atrophy is supposed to be due to the lack of an aging motor neuron to nourish an abnormally large number of regenerated sprouts (98) and is a potential cause of diagnostic and genetic confusion.

### 128.2.7 Variants of Childhood Onset (“SMA Plus”)

The applicability of DNA studies in families with childhood SMA makes a separation of classical SMA from variants with atypical or additional features necessary. This issue has been addressed in an ENMC workshop 2001 (99).

**128.2.7.1 SMA with Respiratory Distress, SMARD (Diaphragmatic SMA).** One important differential diagnosis of classic infantile SMA is anterior horn cell disease with initial respiratory insufficiency due to diaphragmatic palsy (diaphragmatic SMA), first described in the 1980s (100). The presenting symptom is acute respiratory distress at birth or soon thereafter, requiring permanent ventilation. Generalized weakness and hypotonia are usually associated with the respiratory distress, whereas distally pronounced weakness becomes evident later in the disease course (Figure 128-15). The chest X-ray study shows eventration or abnormal movements of the diaphragm. Anterior horn cell loss and diaphragmatic atrophy have been confirmed at autopsy in some cases. Because the diaphragm is usually well preserved in proximal SMA, this feature is an important clinical criterion for differential diagnosis (101).

SMARD is a rare autosomal recessive disorder and is genetically heterogeneous. One type (SMARD1) is caused by mutations in the IGHMBP2 gene on chromosome 11q13 (102). After linkage studies excluded SMA 5q, a locus was discovered on chromosome 11q13-q21 that led to the identification of the IGHMBP2 gene, the human homolog of the murine *ighmbp2* gene, which causes the neuromuscular degeneration (*nmd*) phenotype in mice (54). SMARD1 patients of various ethnic backgrounds have been described with mutations in the IGHMBP2 gene, further defining the clinical spectrum of this entity (103–105). Meanwhile, long-term survivors into the second decade under permanent ventilation are reported (101); moreover, some patients do not develop diaphragmatic palsy even years after onset of weakness (106). There is no apparent genotype–phenotype relationship, and mutation screening is hampered by a large gene with numerous polymorphisms. The pathogenic pathway of IGHMBP2 deficiency is still unknown, but there may be a similar involvement in the RNA metabolism like in SMA 5q.

**128.2.7.2 SMA with (Olivo-)Ponto-Cerebellar Hypoplasia.** In patients with pontocerebellar hypoplasia or early



**FIGURE 128-15** Diaphragmatic SMA characterized by initial respiratory failure due to diaphragmatic palsy. Generalized muscular hypotonia and distally pronounced weakness.



atrophy and anterior horn cell involvement, a profound floppiness at birth can be seen, which is followed by mental retardation, multiple joint contractures, and cerebellar signs (vision impairment, nystagmus, ataxia). Life span does not exceed a few months in most instances (107). However, longer survival up to several years has been described (108). The early-onset form of the disease is denoted as pontocerebellar hypoplasia (PCH)-I (109) and is consistent with autosomal recessive inheritance. This rare disorder might be mistaken for severe infantile SMA, especially because electromyography (EMG) and muscle biopsy give evidence of neurogenic changes. Following clinical examinations and negative molecular genetic testing for infantile SMA, brain imaging methods might be helpful to identify cerebellar hypoplasia (Figure 128-16) and more widespread volume loss of pons and brainstem. Postmortem examinations show neuronal depletion in the cerebellar hemispheres and vermis, and may include the pons and olivary nuclei in addition to anterior horn cell degeneration. Neuronal loss in other parts of the spinal cord, basal ganglia, and brainstem suggest a more widespread neuronal degeneration, at least in a subgroup of patients. Linkage with chromosome 5q markers has been excluded in two families, and the results of genome scan approaches are awaited for families corresponding strictly to PCH-1. In 2012, mutations of the EXOSC3 gene were discovered as the major cause for PCH-1 of variable severity (110). EXOSC3 is a core component of the human RNA exosome complex. PCH-1 is the first disease found to be associated with a dysfunction of an RNA exosome core component. Further genotype-phenotype studies confirmed biallelic mutations in 10 of 27 (37%) families collected worldwide (111).

**128.2.7.3 SMA with Arthrogryposis Multiplex.** Arthrogryposis multiplex is a heterogeneous group of neuromuscular disorders. A neurogenic type is caused by anterior horn cell degeneration and leads to severe muscle weakness similar to SMA type I and congenital contractures. Although the majority of cases are believed to be nonprogressive and nonfamilial, there are patients with more

complex pathology that follows autosomal recessive or X-linked traits.

In some patients, the clinical picture of severe SMA is combined with contractures and congenital fractures of the long bones; this phenotype has been thoroughly described by Borochowitz and colleagues (112). Anterior horn cell involvement and neuronal degeneration in other parts of the CNS can be seen pathoanatomically. In extremely rare cases with congenital SMA 5q, fetal weakness can lead to congenital contractures with bone fractures and early lethality (see section on Genotype-Phenotype studies). The majority of patients with neurogenic arthrogryposis multiplex congenita have a different genetic background, as linkage analysis with chromosome 5q markers excluded the gene locus for childhood SMA in most families with autosomal recessive inheritance. The underlying gene defects are as yet unknown.

Because there is a preponderance of males, a significant proportion of cases might be X-linked. One convincing form of an X-linked infantile SMA is associated with arthrogryposis, genital abnormalities, and facial dysmorphism (113). This syndrome gene was mapped to Xp11.3-q11.2 and mutations were found in the UBE1 gene (114).

Autosomal dominant forms of congenital arthrogryposis in combination with lower motor neuron disease of milder course are also reported (115,116). The gene for one Dutch family was mapped to the long arm of chromosome 12 with evidence of heterogeneity (117), before mutations were found in the TRPV4 gene (118).

**128.2.7.4 SMA and Myoclonic Epilepsy.** The combination of infantile SMA and myoclonic epilepsy has rarely been reported. It was first described as an autosomal dominant disease with distally pronounced weakness (119), but subsequent cases showed a more proximal or generalized pattern of muscle atrophy and recessive inheritance (120). The patient described by Lance and Evans (121) had bilateral hearing loss associated with action myoclonus, ataxia, and SMA. Normal intelligence and neurogenic findings on EMG and muscle biopsy are cardinal diagnostic findings; the genetic basis remains to be identified. Mitochondrial disease, metabolic, and lysosomal disorders must be excluded.

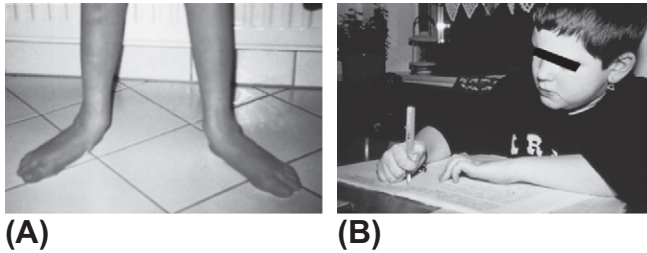


**FIGURE 128-16** SMA and pontocerebellar hypoplasia in a 9-month-old boy who was initially thought to have Werdnig–Hoffmann disease and finally showed biallelic mutations of the EXOSC3 gene.

## 128.3 NONPROXIMAL SPINAL MUSCULAR ATROPHY

### 128.3.1 Distal

Distal SMA or distal HMN comprises a group of genetically and clinically heterogeneous disorders. In a population survey in northeast England, distal SMA accounted for about 10% of all cases (122). Both autosomal dominant and recessive genes cause childhood- and adult-onset forms, which normally have a chronic and benign course. X-linked distal SMA is very rare, and only described in a large Brazilian family (123).



**FIGURE 128-17** Distal SMA. Denervation atrophy of the calves (A) and the forearm and hand musculature (B) in a 9-year-old boy.

**128.3.1.1 Clinical Picture.** The early-childhood form of distal SMA starts soon after birth with distal hypotonia and wasting, and leads to pes planus and sometimes scoliosis within the first years of life. Despite considerable handicap due to weakness of the hands (Figure 128-17), these patients' scholastic and occupational attainments are often better than expected. Juvenile- and adult-onset distal SMA is usually characterized by slowly progressive weakness and wasting of the extensors muscles of the toes and feet. Later on, weakness and wasting also involves the distal upper limb muscles. Progression can be variable; some patients become wheelchair bound, and some develop respiratory insufficiency. There are different distal SMA subtypes defined by additional findings, such as vocal cord palsy, spasticity, pyramidal tract signs (ALS-like features) and diaphragmatic palsy (Table 128-4).

**128.3.1.2 Differential Diagnosis.** The differential diagnosis of distal SMA includes hereditary motor and sensory neuropathy (HMSN) or Charcot–Marie–Tooth (CMT) disease and HMN, which are much more common than distal SMA. The clinical picture may be indistinguishable if overt sensory disturbances are absent. Therefore, it is important to exclude peripheral nerve involvement by electrophysiologic investigations or DNA studies before diagnosing distal SMA. Sensory disturbances, abnormal sensory nerve action potentials, and markedly reduced nerve conduction velocities indicate polyneuropathies and exclude the diagnosis of SMA. Because the list of genes causing variable phenotypes, including polyneuropathies, axonopathies, and complex motor neuron diseases, is still growing, a subdivision becomes increasingly difficult (124,125).

Distal SMA must also be distinguished from distal myopathy. Either the high serum CK level or muscle biopsy studies that include immunocytochemical and immunoblot analyses or, in some instances, DNA studies will help to exclude Miyoshi distal myopathy, dysferlinopathy, hereditary inclusion body myopathy, tibial muscular dystrophy, or Welander dystrophy. In nonfamilial cases of distal SMA, intraspinal pathology, for example, ependymoma or lumbar stenosis, must be excluded by magnetic resonance imaging (MRI).

**128.3.1.3 Genetics.** Following epidemiologic studies from northeast England, autosomal recessive inheritance of distal SMA is more frequent (~75% of cases) than autosomal dominant inheritance (122). A heterozygosity frequency of 1:100 has roughly been estimated for

a childhood- and an adult-onset genes (122). Whether these data are valid for all types of distal SMA or hereditary motor neuropathies must be clarified, because dominant new mutations play an important role at least in HMSN (CMT neuropathies).

In case of parental consanguinity or sibships with multiple affected siblings, autosomal recessive inheritance with 25% recurrence risk is most likely. For siblings of sporadic cases, a risk of 1:4 should be given, although it is probably significantly smaller. Children of affected subjects have a risk of about 1:8, because dominant inheritance cannot be excluded even in patients whose parents show no signs of distal SMA.

The 17 affected men of the rare X-linked form (123) had an age at onset in the first decade, and the first presenting symptom was foot deformity or gait instability, followed by distal lower limb weakness. Finally, the hands were affected. Disease progression was slow; all patients remained ambulant. The gene defect was assigned to Xq13.1-q21 and a few years later, mutations in the ATP7A gene were identified (126).

Similar to HMSN, distal SMA shows clinical and genetic heterogeneity (for review, see References (124,125)). With increasing knowledge of the genetic basis and overlapping phenotypes, the distinction between distal SMA, HMN, and CMT neuropathy becomes less clear and hampers classification approaches. The gene defects in most distal SMAs remain to be determined, although many genes have been identified until the end of 2010 (Table 128-4). The SMN1 gene does not play a role for pathogenesis. In a large patient cohort with distal HMN/SMA pathogenic mutations were found in 17 out of 112 index patients in four genes (BSCL2, HSPB8, HSPB1, SETX), resulting in an overall diagnostic yield of 15% and of 30% in the subgroup of families with autosomal dominant inheritance (125). In summary, genetic testing of BSCL2 is recommended in patients with distal HMN showing the only two known mutations in 5–10% of patients, in particular if a variable degree of upper motor neuron signs is present.

**128.3.1.4 Juvenile Distal-Type Hirayama.** Juvenile distal SMA is also known as “monomelic juvenile SMA type Hirayama,” “benign monomelic amyotrophy,” or “juvenile segmental SMA.” It has first been reported in Japanese literature (127) but was later found in many populations. Sporadic occurrences have been reported in more than 200 cases, with a large predominance of men. The onset is insidious and usually occurs usually between 15 and 25 years of age. The cardinal features are asymmetric wasting and weakness confined to a single upper limb (hand and forearm), which might spread to the contralateral or lower limb. The condition is very benign with an initial progressive phase coming to a halt within 2–4 years after onset in most cases. Bulbar, sensory, and pyramidal signs are lacking so that motor amyotrophy is likely. A postulated pathogenesis is circulatory insufficiency due to chronic compression of the spinal cord (128). Other authors are of the opinion that the focal spinal cord compression is flexion induced. MRI studies and spinal

**TABLE 128-4 Genetic Classification of Distal SMA (Hereditary Motor Neuropathy (HMN) and Its Variants) (2010)**

Mode of Inheritance	Type (OMIM)	Clinical Features	Gene Locus (Gene)
Autosomal dominant	Distal HMN I (182960)	Distal weakness starting in the lower limbs, onset in childhood and youth (median 10 years), involvement of the upper motor neuron described (juvenile ALS)	7q34-q36
	Distal HMN II/CMT2L/CMT2F (158590)	Distal weakness starting in the lower limbs, variable age at onset, rapid progression, sensory nerve involvement described (CMT2L/CMT2F)	HMNIIA: 12q24.3 (HSP8) HMNIIB: 7q11-21 (HSP1)
	Distal HMN V/CMT2D (600794)	Distal weakness starting in the hands, later spreading to the lower limbs, onset in youth or adolescence, sensory nerve involvement described (CMT2D)	7p15 (GARS)
	Distal HMN with spasticity/Silver syndrome (606158)	Variable clinical picture corresponding to HMN I, II, or V, mild to severe spastic pareses (may not be present), onset in youth or adolescence	11q12-14 (BSCL2)
	Distal HMN VII/HMN7B/SMA with vocal palsy (158580, 608465)	Distal weakness predominantly of upper limbs, onset from childhood to adulthood, hoarse voice, stridor, vocal cord changes, facial weakness described	HMN7A: 2q14 HMN7B: 2p13 (DCTN1)
	Distal HMN with pyramidal tract signs/ALS4 (608465)	Distal and proximal weakness starting in the lower limbs, onset mostly in youth or adolescence, brisk reflexes, positive Babinski sign	9q34 (SETX)
	Congenital distal SMA (600175)	Congenital distal muscle wasting and weakness of the lower limbs associated with contractures, scoliosis, no progression	12q23-34 (TRPV4)
Autosomal recessive	Distal HMN VI/SMARD I/DSMA1 (604320)	Starting with generalized muscular hypotonia, later distal weakness, onset mostly in the first months of life, sudden respiratory distress due to diaphragmatic palsy	11q13.2-13.4 (IGHMBP2)
	Distal HMN of Jerash type/DSMA2 (605726)	Distal weakness starting in the lower limbs, onset in youth to adulthood, brisk reflexes, positive Babinski sign	9p21.1-12
	Distal HMN III/HMN IV/DSMA3 (607088)	Distal weakness starting in the lower limbs, onset in childhood or youth, variable progression, diaphragmatic palsy may be present	11q13.3
	Distal HMN/distal SMA/DSMA4 (611067)	Distal weakness with rapid progression, onset in the first years of life, early immobilization and respiratory insufficiency	1p36 (PLEKHG5)
X-linked	Juvenile distal SMA/adult-onset distal SMA (300489)	Distal weakness starting in the lower limbs, onset variable in childhood to adolescence, slow progression	Xq13.1-q21 (ATP7A)

CMT, Charcot–Marie–Tooth disease; HMN, hereditary motor neuropathy; SMA, spinal muscular atrophy; ALS, amyotrophic lateral sclerosis.

angiography argue against this and support the view that Hirayama's disease is an intrinsic motor neuron disorder (129,130). If there is evidence of spinal cord compression, typical juvenile distal SMA has most likely to be regarded as nongenetic.

Some cases of segmental SMA are familial (131), and some have an asymmetrical but progressive weakness that can be either distal or proximal. To clarify the variants of the chronic monomelic amyotrophy syndrome, Serratrice (132) classified three subgroups, including

(i) a benign, nongenetic form due to focal amyotrophy, restricted to young men; (ii) a more progressive and sometimes familial form affecting either an upper or a lower limb; and (iii) patients with recurrent episodes of monomelic atrophy. Molecular genetic screening for SMN1 gene alterations showed no evidence of a modifying influence (133,134).

There are no distinctive findings in segmental SMA. If spinal pathology has been ruled out by imaging studies, only long-term observation over many years might point toward the correct diagnosis. The differential diagnosis includes, for example, distal SMA or HMN, multifocal motor neuropathy, inclusion body myositis, dysferlinopathy, and early myotonic dystrophy.

### 128.3.2 Scapuloperoneal SMA

Autosomal dominant familial SMA with scapuloperoneal involvement and adult onset was first described by Stark (135) and Kaeser (136), but the original family later turned out to be a desmin myopathy due to a heterozygous mutation in the DES gene (137). A rare autosomal recessive form also exists (1). The German eponym SMA type Vulpian-Bernhardt, which has been used to denote a heterogeneous group of disorders, should be avoided.

The clinical manifestations are variable. Weakness may first appear in the foot and toe extensors, and then spread to the shoulder girdle and the proximal muscles of lower limbs. Tendon reflexes are diminished, and fasciculations are present. The dominant form usually presents in adolescence or early adult life (138,139); the recessive form tends to present earlier and shows a more progressive course (1,140). Since the literature has become sparse on the subject in the past 20 years, the relevance of this entity among the SMAs remains doubtful.

Because facio-scapulo-humeral muscular dystrophy (FSHD) can be clinically indistinguishable from scapuloperoneal SMA and may show changes suggesting denervation in muscle, scapuloperoneal SMA can be diagnosed only after FSHD has been excluded by DNA analysis.

Some patients with scapuloperoneal weakness also have cardiac conduction defects. The clinical distinction from autosomal dominant or X-linked Emery-Dreifuss muscular dystrophy can be difficult; therefore, a thorough investigation and molecular studies are required before diagnosing SMA plus cardiomyopathy.

There is a rare and clinically highly variable subtype of autosomal dominant SMA, which has previously been classified as scapuloperoneal SMA (OMIM 181405) and is characterized by congenital absence of muscles, progressive scapuloperoneal atrophy, laryngeal palsy, and progressive distal weakness and atrophy (141). This distinct entity turned out to be allelic to a congenital benign form with joint contractures caused by mutations of the TRPV4 gene on chromosome 12q23-34 (OMIM 60015) (142,143).

### 128.3.3 Progressive Bulbar Palsy

Progressive bulbar palsy is extremely rare and generally categorized into childhood- and adult-onset forms. The eponym Fazio-Londe disease has been used for the autosomal recessive type of childhood onset, although the family reported by Fazio (144) followed an autosomal dominant pattern.

In the childhood form, first signs of facial weakness and dysarthria are present between 2 and 14 years of life. Subsequently, dysphagia, respiratory insufficiency and involvement of other cranial nerves develop with a rapid progression. Death occurs in most cases within a few years after onset. Autosomal recessive and dominant pedigrees have been described.

There are juvenile- and adult-onset forms that are very rare and follow an autosomal dominant mode of inheritance (145,146). Since the majority of patients with the diagnosis of progressive bulbar palsy progress to a clinical picture designated as ALS (147), the classification as a separate disease entity is difficult as long as the pathogenesis or the genetic basis is unknown.

The Brown-Vialetto-Van Laere syndrome is characterized by bulbopontine paralysis and bilateral nerve deafness (Figure 128-18). The age at onset ranges from birth to adulthood, with a median of 13 years. According to Sathasivam (148) progressive hearing loss is the presenting sign in the vast majority of cases. The weakness is in the distribution of motor cranial nerves III, and VII to XII; scapuloperoneal or distal muscle atrophy occurs



**FIGURE 128-18** Brown-Vialetto-van Laere syndrome in a 14-year-old boy a few months before he died. Note facial weakness with severe ptosis. Following bulbar dysfunction, he developed scapuloperoneal atrophy.



later in the disease course. Progression is variable, and life span is reduced because of respiratory complications caused by diaphragmatic palsy. About half of the cases are sporadic, and the majority of the familial cases are autosomal recessively inherited. Recently, mutations in the C20orf54 gene were detected in seven unrelated families (149), which can produce a metabolic profile mimicking mild multiple acyl-CoA dehydrogenation defect (MADD) (150). Another family was suggestive of an autosomal dominant pattern with variable expressivity (151) but this awaits further confirmation.

### 128.3.4 Spinobulbar Neuronopathy (Kennedy Syndrome)

An important differential diagnosis of late-onset SMA in association with bulbar palsy and gynecomastia is spinobulbar atrophy type Kennedy (152). The mean age at onset is 30–40 years (range 15–60 years); however, there is an average delay of the correct diagnosis of about 5 years (153). Clinically, there are pronounced muscle cramps and fasciculations accompanying predominantly proximal and symmetrical weakness that affects the legs more than the arms. Facial and perioral contraction fasciculations and postural tremor of the hands are frequently observed. There are often signs of partial androgen insensitivity, with gynecomastia, impotence, testicular atrophy, and reduced fertility. Androgen levels and strength are inversely correlated with age. As yet, there are no prospective hormonal intervention studies that gave conclusive results, despite the fact an antiandrogen treatment in animal models proved to be efficient (153).

Involvement of the sensory neurons is usually subclinical, but can be detected by electrophysiologic investigations. Dysarthria, dysphonia, and dysphagia result from progressive degeneration of spinal and bulbar motor neurons. The serum CK level is often markedly elevated (>35-fold above the upper normal limit). Life expectancy can be shortened by aspiration pneumonia. The disease is X-linked and has a prevalence of about 1:40,000. Female carriers may manifest with cramps, mild muscle weakness and fasciculations (154,155). Kennedy disease is caused by a CAG repeat expansion of the androgen-receptor gene (156). The diagnosis is confirmed when there are more than 40 CAG repeats, whereas there is only a weak inverse correlation between the number of repeats and the age of onset severity of the clinical picture (157,158).

## 128.4 MANAGEMENT

A curative treatment of SMA is not yet available, but the knowledge of the genetic defect in SMA 5q currently has a major impact on therapeutic strategies. Because an increased number of SMN2 copies might partly

compensate for or prevent the motor neuron loss, agents that enhance the production of the SMN protein might be a means of therapy. Strategies include (i) upregulation of SMN expression either by stimulation of promoter activity or by enhancement of exon 7 inclusion of the SMN2 gene, (ii) gene therapy to supply extra copies of a functional SMN gene or the SMN cDNA in motor neurons, or (iii) compensation of the functional consequences caused by SMN deficiency (for review see Reference (159)).

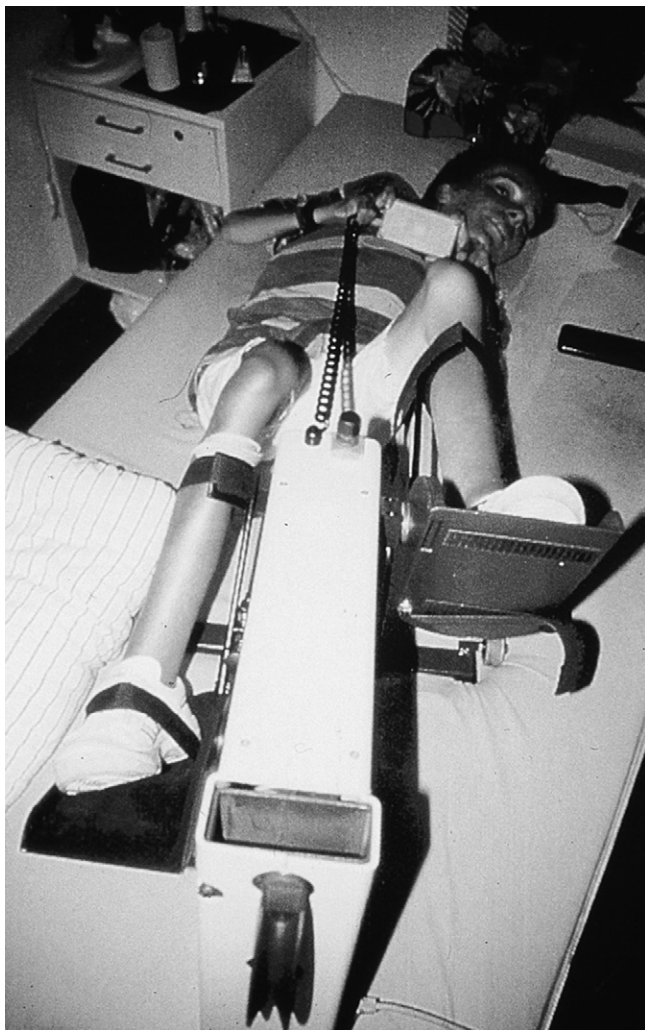
The upregulation of SMN2 gene transcription has been intensely studied for about a decade after identification of histone deacetylase inhibitor Htra-beta1 (160). Several splice enhancers have entered clinical trials, a summary of completed and ongoing clinical trials by 2008 was given by Lunn and Wang (51). By 2010, only few randomized, double-blind, and placebo-controlled trials were published and did not demonstrate a therapeutic effect of splice enhancers. This applies to phenylbutyrate in SMA type II (161) and to valproic acid and L-carnitine in a nonambulatory cohort of SMA patients (162). At the time of this writing (February 2011), no medication is known that has a proven positive influence on the course of the disease.

The development of ASO therapy in SMA has been reviewed by Burghes and McGovern (163). ASOs can be used to alter splicing of a gene and either restore or eliminate protein products. Since it was shown in a mouse model (60) that restoration of SMN was achieved by ASO blocking, it is hoped that this could be used for clinical trials in SMA; however, there is still limited information on the different chemistries of ASOs and their action in the nervous system. The same applies to stem cells approaches that await further studies before entering clinical practice (159).

The main therapeutic elements in SMA are physical and orthopedic therapy and ventilatory support (164). Goals of physiotherapy are prevention of contractures and, in the milder types, the maintenance of mobility and ambulation as far as possible. Contractures may be treated by passive mobilization and stretching (Figure 128-19). Preliminary results of studies using isometric strength training in mild SMA have at least given some evidence of a beneficial effect. At its present state, electrical stimulation cannot be recommended as a therapeutic tool.

Main orthopedic problems in early-onset SMA are inability to sit, contractures of lower limbs, and scoliosis. Orthopedic treatment includes conservative methods (orthotic devices and physiotherapy) as well as surgical procedures for the limbs and spine.

Respiratory failure is common in SMA patients—treatment is similar to that in other neuromuscular disorders, with a major role for assisted ventilation. Noninvasive methods of intermittent ventilation is recommended in chronic or late-onset forms of SMA (Figure 128-20) and increasingly offered to patients with severe SMA type I.



**FIGURE 128-19** SMA type II patient with severe contractures that are treated by passive mobilization and stretching.



**FIGURE 128-20** Intermittent noninvasive ventilation in a 32-year-old SMA type II patient. The ventilator is fixed at the back of the electric wheelchair to allow a maximum mobility.

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### Biography



**Sabine Rudnik-Schöneborn** is a senior lecturer in human genetics with a medical background holding a permanent position as a professor for clinical genetics at the Institute for Human Genetics, Medical faculty, RWTH Aachen, Germany. **Klaus Zerres** is professor and head of the Institute for Human Genetics, Medical Faculty, RWTH Aachen, since 1999. He is also a medical doctor trained in human genetics. Our group has been engaged in the clinical description and genetic characterization of spinal muscular atrophies (SMA) for more than two decades. In addition, we studied reproductive issues of women with neuromuscular disorders in a similar time frame. After identification of the gene defect (SMN1 gene) for classical proximal SMA in 1995, we early focused on the genetic characterization of SMA-plus types. Within the framework of the International SMA Consortium and the European Neuromuscular Centre (ENMC) we contributed to the definition of diagnostic criteria, the classification of disease variants and the identification of disease genes. We were involved in identifying the gene for SMA and respiratory distress (SMARD1) and performed genotype-phenotype studies thereafter. On the basis of a large collection of families with SMA and pontocerebellar hypoplasia (PCH-1), we recently identified the EXOSC3 gene as the major gene defect together with the group of Joanna Jen, University of California, Los Angeles, USA, and broadened the clinical spectrum. We were first to give pathoanatomical evidence of a sensory nerve involvement in SMA, and moreover we defined congenital heart disease and vascular dysfunction as a feature of severe SMA. We reviewed genotype-phenotype correlations in infantile SMA in German patients in order to provide reliable data on natural disease course and clinical variability as a basis for clinical trials.

# CHAPTER

# 129

## Hereditary Muscle Channelopathies

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### 129.1 INTRODUCTION

Defective ion channels may play a causal role in disease pathogenesis. This implication was first concluded from the observation of an abnormal ion conductance in muscle fibers biopsied from myotonic goats (1). In man, a similar conclusion was reached for patients with paramyotonia congenita and periodic paralyses (2,3). The term “ion channelopathies” was then coined in the 1990s (4), and defined for disorders that are caused by malfunction or altered regulation of ion channel proteins. Channelopathies can, therefore, be either hereditary or acquired (usually caused by autoantibodies).

Hereditary channelopathies can be categorized as those affecting the motor end plate (congenital myasthenic syndromes (CMS)), the sarcolemma (myotonias and periodic paralyses) and excitation–contraction coupling (malignant hyperthermia (MH), central core myopathy and multiminicore myopathy). The channel defects result in changes of excitability that one would expect to be constantly present. Fortunately, this is not the case. Clinical symptoms mainly appear episodically, provoked by an out-of-the-normal situation, a so-called trigger (5). Compensatory mechanisms such as the normalization of the serum potassium level often allow an episode to cross over to complete remission. In addition to the episodes, progressive manifestations with muscular degeneration are present in a substantial percentage of patients.

The total prevalence of monogenic muscle channelopathies is about 5:100,000, i.e. they are rare diseases. Pharmacogenetic influences on abnormal muscle channels, which may generate the susceptibility to MH, increase the clinical relevance of channelopathies considerably. As many disease mechanisms have been elucidated by functional characterization on the molecular level, the channelopathies are regarded as model disorders. Molecular genetics identify the underlying mutations and thus often enable one to predict the course of the diseases and to assign an individual therapy for the patient. They also allow one to calculate the patient’s risk of having affected offspring.

### 129.2 ELECTROPHYSIOLOGY OF CLINICAL WEAKNESS AND PARALYSIS

The excitability of a muscle fiber, and thus its ability to generate force and contraction, depends on a *high* resting potential across its sarcolemma (−80 mV inside with respect to outside). Such fibers are assuming the P1 state in the nomenclature of Jurkat-Rott et al. 2009 (6), and they make up the majority of the fibers in a healthy muscle. Owing to the complicated voltage dependence of the membrane conductance on the membrane potential, muscle fibers can also assume a *low* stable resting potential of about −60 mV. Such “depolarized” fibers are said to be in the P2 state and their major functional difference is that they cannot be excited and, therefore, are “paralyzed”. In a healthy muscle, fibers in the P2 state make up only a small minority. Mutations in the genes encoding the various ion channels responsible for the membrane potential may influence the electrical stability so that fibers can shift from the P1 state to the P2 state—not only for short time intervals but also for very long durations. The number of excitable fibers is then very much reduced.

### 129.3 HEREDITARY CHANNELOPATHIES AFFECTING THE END PLATE

#### 129.3.1 Congenital Myasthenic Syndromes

Of the myasthenic disorders, the CMS are the rarest, affecting the neuromuscular transmission of up to three individuals per million. Like the autoimmune disease myasthenia gravis, the myasthenic syndromes are characterized by defective excitation causing muscle fatigue (7,8). Although rare, they form a very heterogeneous group of inherited disorders. They arise from mutations affecting presynaptic, synaptic or postsynaptic proteins at the neuromuscular junction on which synaptic formation and function depend. The majority are recessively inherited. Detection of the genetic defect is important for the selection of the appropriate drug therapy (Table 129-1).



Although anticholinesterase medication is beneficial in most forms, it can be deleterious in others.

**129.3.1.1 Clinical Symptoms.** Weakness is usually evident at birth or within the first or second year of life, and results in feeding or breathing difficulties, ptosis, ophthalmoplegia, and delayed motor milestones. Strength sometimes improves during adolescence. In about 15% of the cases, the weakness does not become evident before adolescence or even adult life. A major group of patients show a limb girdle pattern of muscle weakness that can be mistaken for a myopathy (9). These muscles have small, simplified neuromuscular junctions but normal acetylcholine receptor and acetylcholinesterase function. Reflexes are usually brisk and muscle wasting does not occur. Congenital arthrogryposis multiplex may be present involving reduced fetal movement and multiple joint contractures in the neonate (10).

The slow-channel syndrome is the only disease of this group that presents in childhood, adolescence or particularly adult life, with upper limb predominance and contractures. Patients show an increased synaptic response to acetylcholine with characteristic repetitive discharges in response to a single supramaximal stimulus. It is aggravated by cholinesterase inhibitors and is progressive.

A CMS channelopathy so far identified only in a single family affects the skeletal muscle sodium channel Nav1.4 encoded by *SCN4A* (11). Another CMS also observed only in one family is caused by a mutation in *MUSK* on chromosome 9q31.3-q32 that encodes MuSK, a muscle-specific kinase.

A rapsyn defect causes either mild generalized muscle weakness of late onset, fluctuating ptosis and intermittent double vision or life-threatening episodes of early-onset

**TABLE 129-1 Overview of Hereditary Muscle Channelopathies**

Disease/Susceptibilities	Gene	Protein	Inheritance	Mutation	Therapy
Congenital myasthenic syndrome	<i>CHAT</i>	Ch-A-T	Recessive	Loss	AChE-I, DAP
	<i>COLQ</i>	AChE	Recessive		Avoid AChE-I
	<i>CHRNA-E</i>	nAChR	Dominant/recessive	Gain or loss	AChE-I, DAP <sup>a</sup>
	<i>RAPSN</i>	rapsyn	Recessive	Loss	AChE-I, DAP
	<i>MUSK</i>	MuSK	Recessive		
	<i>SCN4A</i>	Nav1.4	Recessive		
	<i>DOK7</i>	Dok-7	Recessive		Ephedrin, albuterol
	<i>GFPT1</i>	GFPT1	Recessive	Loss	
Thomsen myotonia	<i>CLCN1</i>	ClC1	Dominant	Loss	Propafenone,
Becker myotonia			Recessive	Loss	flecainide,
Sodium channel myotonia	<i>SCN4A</i>	Nav1.4	Dominant	Gain ( $\alpha$ -pore)	acetazolamide
Potassium-aggravated myotonia					
Paramyotonia congenita					
Hyperkalemic periodic paralysis					HCT, albuterol
Normokalemic periodic paralysis				Gain ( $\omega$ -pore)	(K), CAI, AA
Hypokalemic periodic paralysis 2				Gain ( $\omega$ -pore)	K, CAI, AA
Hypokalemic periodic paralysis 1	<i>CACNA1S</i>	Cav1.1	Dominant	Gain ( $\omega$ -pore)	K, CAI, AA
Thyrotoxic periodic paralysis <sup>b</sup>	<i>KCNJ18</i>	Kir2.18	Dominant	Loss	Symptomatic
Andersen-Tawil syndrome	<i>KCNJ2</i>	Kir2.1	Dominant	Loss	CAI
Malignant hyperthermia <sup>c</sup>	<i>CACNA1S</i>	Cav1.1	Dominant	Gain	Dantrolene (crisis)
	<i>RYR1</i>	RyR1	Dominant	Gain	Dantrolene (crisis)
Central core disease			Dominant/recessive	Gain or loss	Exercise
Multiminicore disease			Recessive	Loss	Exercise

AChE-I, acetylcholine esterase inhibitor; Ch-A-T, cholin–acetyl transferase; Nav, voltage-gated sodium channel; DAP, 3,4-diaminopyridine; HCT, hydrochlorothiazide; K, potassium; CAI, carbonic anhydrase inhibitor; AA, aldosterone antagonists.

<sup>a</sup>AChE-I and DAP to be avoided in the slow-channel syndrome.

<sup>b</sup>Susceptibility in Singapore individuals.

<sup>c</sup>Susceptibility in world population.



is haploinsufficient. The reduced availability of sodium channels decreases the safety factor of synaptic transmission and thereby causes myasthenic syndrome (11).

Other postsynaptic CMS are caused by a rapsyn defect due to loss-of-function *RAPSN* mutations (17). Rapsyn plays a key role in acetylcholine receptor clustering during development, and is another relatively frequent cause of CMS. Recessive Dok-7 mutations also result in a defective structure of the postsynaptic membrane. The p.N88K rapsyn mutation and the c.1124\_1127dupTGCC duplication in *DOK7* are frequent in Europe. Recently, mutations in *GFPT1* encoding the glutamine-fructose-6-phosphate transaminase 1 have been identified in this also recessive CMS type. Consistent with these data, downregulation of the *GFPT1* ortholog *gfpt1* in zebrafish embryos altered muscle fiber morphology and impaired neuromuscular junction development. *GFPT1* is the key enzyme of the hexosamine pathway yielding the amino sugar UDP-*N*-acetylglucosamine, an essential substrate for protein glycosylation (18).

## 129.4 HEREDITARY CHANNELOPATHIES AFFECTING THE PLASMALEMMA

### 129.4.1 Nondystrophic Myotonias

**129.4.1.1 Clinical Symptoms.** The major symptom of the myotonias is a generalized muscle stiffness that appears particularly following strong and/or sudden muscle contractions, e.g. when a patient gets scared. The stiffness is caused by series of involuntary action potentials that can occur in practically all skeletal muscle fibers. The muscles are often hypertrophied, in contrast to what is seen in most other muscle diseases. The involuntary action potentials are caused by one (or two in the case of recessive Becker myotonia) of a great number of mutations in genes encoding muscular ion channels, which all result in increased excitability of the sarcolemma. Depending on the type of channels affected, chloride and sodium channel myotonias are distinguished (19) (Table 129-1).

There are two forms of chloride channel myotonia, the rare dominant Thomsen myotonia and the more common recessive Becker myotonia. Both forms are characterized by generalized myotonia and the so-called warm-up phenomenon, a reduction of myotonia in the course of repeated contractions. A typical feature of myotonia would be the inability to let go after a hearty handshake. Such myotonia during closure of the fist is most pronounced when the muscles have remained relaxed for at least 10 minutes. Another typical sign is the “lid-lag” phenomenon, whereby after a brief look upward, upon a brisk look downward the eyelids do not immediately follow the eyeballs because of muscle stiffness. Gentle tapping on a relaxed muscle may elicit a “myotonic reaction”, a local muscle contraction called “percussion myotonia.” A distinct myotonia may be followed by a “transient weakness” of the same muscle. This weakness

hampers the patient more than the stiffness (20,21). Hypertrophy is more pronounced with the Becker than with the Thomsen form, whereby the glutei, the femurs and the calves are most affected. Paradoxically, the power produced by these muscles is less than expected. In severe cases the hyperactivity will result in shortened muscles leading to pes equinus with consequent lordosis and in a reduced extension of the elbow and wrist joints. Males are usually more affected than females. Females often develop myotonic symptoms only after hypothyreosis or gravidity. In Becker myotonia, myotonic stiffness is usually first detected within the rather long time span between first school enrollment and the third decade of life. During adulthood the severity of myotonia remains constant; life expectancy is normal. Becker patients have reduced manual skills; this should be given consideration when occupational choices are made.

The sodium channel myotonia is also called potassium-sensitive myotonia because the myotonic stiffness is increased after oral intake of potassium (c. 1 hour after 1–2 potassium tablets). Depending on the mutation, symptoms may occur in a wide range of severity. The most benign form is called myotonia fluctuans (22), and the most severe form myotonia permanens (23,24). With myotonia fluctuans work-induced stiffness does occur, however, usually with a delay of about an hour. It then lasts for about 1–2 hours. The typical warm-up phenomenon is often masked by this delay, but with repeated contractions and relaxations of the extremities it may be observed. Patients having myotonia permanens may be more or less immobile by the continual activity in many of their muscles. With these patients a diagnostic test applying oral potassium must not be performed because of the danger that stiff respiratory muscles lead to respiratory insufficiency (25). Patients with sodium channel myotonia tend to suffer from cramping when their muscles are being stressed (26).

For a clinical discrimination between chloride and sodium channel myotonia one may use the fact that chloride channel myotonia shows warm-up of the eyelid muscles, whereas sodium channel mutations cause paradoxical eyelid myotonia (25,27,28). Further specification is possible by investigating the reactions to cold and potassium. For instance, after cooling of an eye for 10 minutes with an ice bag, a forceful closure of the eye may lead to the inability to open for many seconds. Creatine kinase (CK) values of five times the norm may be observed in sodium channel myotonia, whereas in chloride channel myotonia they are at the most a little increased. Some patients, in particular those with a sodium channel myotonia, suffer from muscle pain that increases with the amount of muscle work.

**129.4.1.2 Clinical Neurophysiology.** Typical signs of all types of myotonia are bursts of discharges in the EMG that are elicited by the movement of the needle or by tapping on the muscle. In addition, sodium channel myotonias show long-lasting series of fibrillation

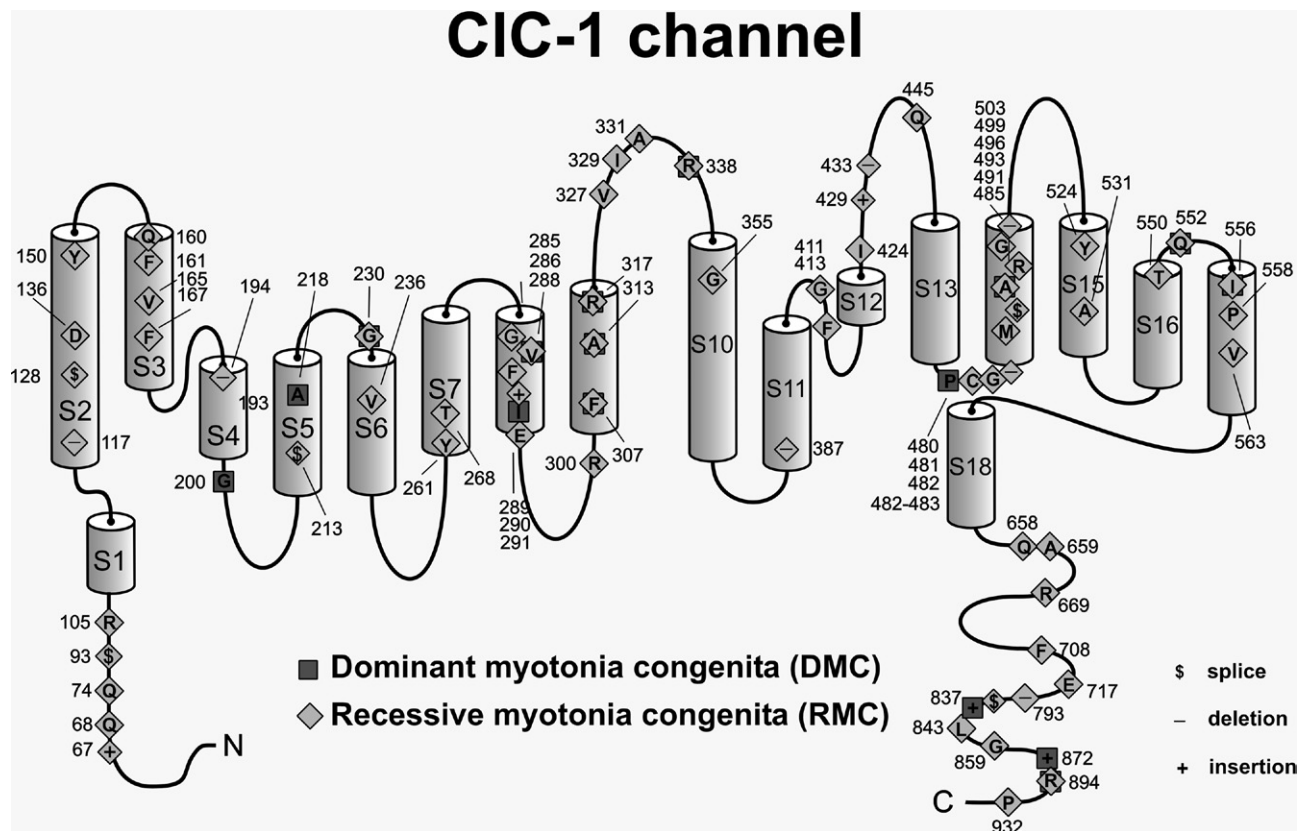
potentials. Like with myasthenia gravis at 3Hz, repetitive nerve stimulation at 10Hz yields a decrement in chloride channel myotonia, but in contrast to myasthenia, the decrement begins later, is more pronounced, not antagonized by tensilon, and runs parallel with the described transient weakness, i.e. the amplitude recovers with continuing stimulation (29). No decrement is found in sodium channel myotonia.

**129.4.1.3 Pathogenesis.** The mutations responsible for the myotonias are located in the genes *CLCN1* and *SCN4A* encoding the muscular chloride and sodium channels, respectively. Both chloride channel myotonias (Becker and Thomsen forms) have in common that the mutations lead to decreased activity of the chloride channels in the sarcolemma, which reduces the chloride conductance of the latter. Physiologically the chloride conductance of a muscle fiber comes up to 80% of the total membrane conductance at rest. This resting conductance is passive ("ohmic"), and its rather high value is very important for a stabilization of the muscle fiber's resting potential of the P1 state close to  $-80$  mV. When the chloride conductance is lower than 30% of the total membrane conductance, the muscle fibers still stay favorably in the P1 state at rest, but they become hyperexcitable. Clinically, this results in myotonia (30). During the state of transient weakness, an increased number of muscle fibers is believed to stay

in the P2 state at the end of a myotonic series of action potentials.

Mutations of the muscular chloride channel gene with dominant and recessive mode of inheritance are responsible for Becker and Thomsen type myotonias, respectively (31) (Figure 129-2). Functionally, the approximately 10 dominant mutations exert a dominant-negative effect on the homodimeric channel complex as shown by coexpression studies, meaning that mutant/mutant and mutant/wild-type complexes are malfunctional. The most common feature of the resulting chloride currents is a shift of the activation threshold toward less negative membrane potentials almost out of the physiological range (32,33). As a consequence, chloride conductance is drastically reduced in the vicinity of the resting membrane potential. Interestingly, both testosterone and progesterone rapidly and reversibly exert a similar effect on chloride conductance (34). The approximately 100 recessive mutations do not functionally hinder the associated subunit. This explains why two mutant alleles are required to reduce chloride conductance sufficiently for myotonia to develop clinically in Becker myotonia. Heterozygous carriers of a recessive mutation are healthy but may exhibit some myotonic runs in the EMG.

Dominant mutations in the gene encoding the muscular voltage-dependent sodium channel Nav1.4 are



**FIGURE 129-2** Membrane topology model of the CLC1 monomer. The functional channel is an antiparallel assembled homodimer. The channel is functional without any other subunits. Symbols are used for the mutations leading to either dominant or recessive myotonia congenita. The amino acids at which substitutions occur are indicated by one-letter abbreviations and numbered according to the protein sequence.



responsible for sodium channel myotonia (22,24,35) (Figure 129-3). The pathogenesis is based on the fact that mutant Nav1.4 channels have a reduced refractory time and this may generate series of action potentials as a consequence (24,36,37). Recently, aberrant splicing has been reported to cause sodium channel myotonia (38).

**129.4.1.4 Epidemiology.** The prevalence of Thomsen myotonia is now estimated at approximately 1:400,000 (25), i.e. much lower than the 1:23,000 of the premolecular era (39). This is owing to the fact that many families with dominant myotonia are now identified as having sodium channel mutations that result in a different disease with very similar symptomatology. Other families were found to have Becker myotonia with pseudodominant inheritance. Conversely, the prevalence of Becker myotonia is now thought to be 1:24,000 (25,40), much higher than Becker's original estimate of 1:50,000 (39). This suggests that the prevalence of a single recessive *CLCN1* mutation is 1:78.

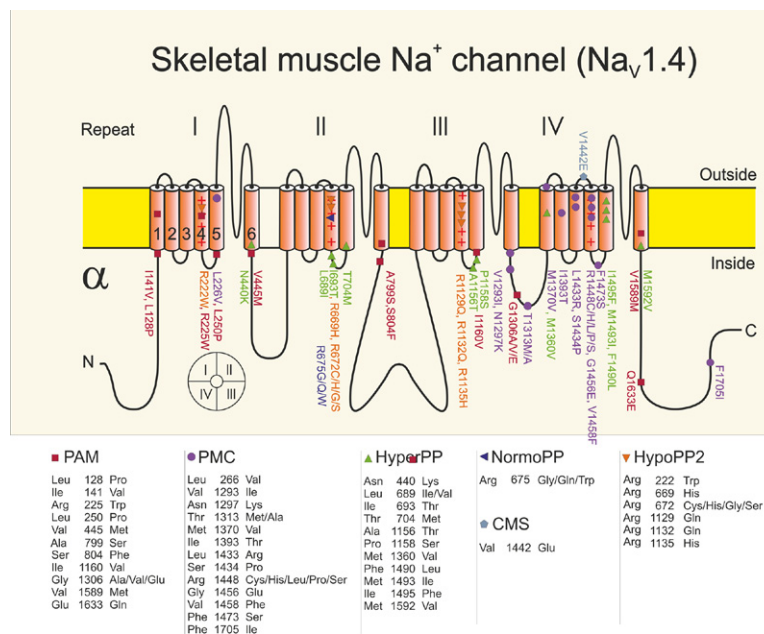
## 129.4.2 Paramyotonia Congenita

**129.4.2.1 Clinical Symptoms.** Paramyotonia is a sodium channel myotonia that, depending on the specific mutation, may or may not also show characteristics of hyperkalemic periodic paralysis (Table 129-1, Figure 129-3). Typically, the myotonic stiffness is negligible in warm muscles, but may assume very cumbersome degrees during exposure

of the muscles to cold. A slight cold draft may suffice to “freeze” the musculature. The warm-up phenomenon might be present in some muscles; however, repeated contractions usually rather increase the muscle stiffness (“paradoxical myotonia”). With extended duration of cooling, the stiffness tends to give way to weakness, which then can last for hours, even after warming the muscles. The mentioned periods of generalized paralysis experienced by patients with certain mutations may occur at rest and in a warm environment.

**129.4.2.2 Clinical Neurophysiology.** In patients with myotonia in a warm environment, there are myotonic bursts and myotonic after-activity reveals a warm-up phenomenon. In patients without myotonia in a warm environment, electrical discharges in the EMG may be absent at normal or increased temperature, but occasional myotonic runs do occur. On cooling, a fibrillation-like spontaneous EMG activity develops consistently, which is maximal at a muscle temperature of about 29°C. With a further drop in temperature, this spontaneous activity decreases and also voluntary activity almost disappears at 24°C when the muscle gets paralyzed. Consequently, compound action potential (CMAP) amplitudes decrease upon cooling, corresponding to the developing weakness.

**129.4.2.3 Pathogenesis.** Most paramyotonia mutations are situated in protein parts relevant for channel inactivation, in the inactivation gate itself (i.e. the intracellular loop connecting domains III and IV like



**FIGURE 129-3** Cartoon of the alpha subunit of the voltage-gated sodium channel Nav1.4 indicating all known mutations responsible for potassium-sensitive myotonia (PAM), paramyotonia (PMC) and hyper- (HyperPP), normo- (NormoPP), and hypokalemic periodic paralysis type 2 (HypoPP2). The alpha subunit consists of four almost homolog domains (repeats I–IV), formed by six transmembrane alpha-helical segments (1–6). Within the membrane, the four domains are so arranged that they form a central pore with segments 5 and 6 lining its wall. The segments 4 contain several positively charged amino acids (arginine and lysine), which are responsible for the voltage sensitivity of the channel. The intracellular loop between domains III and IV represents the channel's inactivation gate. Also domain IV is important for the inactivation process. All the HypoPP mutations replace the arginines within the voltage sensors by neutral amino acids (which are given in the three-letter code, e.g. Arg for arginine). Likewise, HypoPP1 is caused by neutralizing Arg substitutions within the voltage sensors of the similar voltage-gated calcium channel Cav1.1.

T1313M), in the outermost arginine of the voltage sensor in domain IV (R1448H/C/S/P), in intracellular S4-S5 loops of domain III or IV (e.g. F1473S), or in the C-terminus (F1705I) (37). During cooling to 27°C in vitro, paramyotonia muscle fibers slowly depolarize from −85 to about −45 mV, whereas normal muscle fibers depolarize by not more than 5 mV. The depolarization is associated with a long-lasting burst of action potentials, which ends when the membrane potential approximates values of −50 mV (2,41). At this voltage, most sodium channels are inactivated and therefore the muscle fibers become inexcitable and paralyzed. Functional expression of mutant channels revealed slowed fast inactivation and accelerated recovery from the inactivated state and an uncoupling of fast inactivation from activation (5,42). As also slow sodium channel inactivation should be incomplete to maintain depolarization-induced paralysis (43), several groups examined the effects of temperature on slow inactivation of the mutant channels (44–46). The results were not uniform and were difficult to interpret since entry into slow inactivation was already changed by the strikingly slowed fast inactivation. Bouts of long-lasting weakness in paramyotonia can be explained with an ongoing membrane depolarization in the majority of muscle fibers (i.e. a preference of fibers to stay in the P2 state).

**129.4.2.4 Epidemiology.** Paramyotonia is considered an extremely rare disorder, although little epidemiological work has been done. Prevalence is generally higher in Europe-derived populations and lower among Asians. Epidemiological estimates have been provided for the German population. Here, it was estimated that the prevalence is between 1:350,000 and 1:180,000 (47,48). It should be noted, however, that paramyotonia patients are not uniformly distributed across Germany. Many affected families stem from the Ravensberg area in northwest Germany, where a founder effect seems to be responsible for most cases (47,48). The prevalence in that area is estimated at 1:6000.

### 129.4.3 Hyperkalemic, Normokalemic and Hypokalemic Periodic Paralysis

Although in all the periodic paralysis—with the exception of the Andersen–Tawil syndrome (see later)—genes are mutated that encode channels conducting ions other than potassium, the level of serum potassium plays a decisive role on the symptoms, namely, by triggering attacks in the hyperkalemic and normokalemic forms and relieving paralysis in the hypokalemic form (Table 129-1, Figure 129-3). The situation with glucose is similar, because, in combination with insulin, it shifts potassium from the extracellular to the intracellular space and thus may likewise have triggering or therapeutic effects. The same is true for cortisone and drugs that modify the serum potassium level. In contrast, bodily and psychic stress, cold, infections, vaccinations,

operations and drugs always act adversely in all forms of channelopathies. This is even true for the myotonias except that with chloride myotonia cold does not objectively aggravate muscle stiffness, although patients subjectively report worsening. Muscle biopsy is indicated only if molecular genetics does not provide the diagnosis or when there is reasonable suspicion of additional neurogenic or inflammatory processes. Because of possible dangerous situations of hypoglycemia or hypokalemia, diagnostic tests using glucose/insulin loading should be performed only in exceptional cases. Stand-by anesthesia is required.

**129.4.3.1 Clinical Symptoms.** The typical symptom of the periodic paralysis is the experience of episodes of localized or generalized flaccid paralysis that may last for from a few minutes to several days. Respiratory and vocal muscles may be affected. Muscle reflexes might be reduced during an episode. At increased age of the patients, a permanent weakness may additionally develop and result in muscular dystrophy. Hyperkalemic, normokalemic and hypokalemic periodic paralysis are distinguished depending on the serum potassium during a full-fledged episode. The diagnosis between these variants is not always easy, as the result of a single measurement of serum potassium may be misleading. Additional information received from the determination of triggers and the duration of an episode as well as from the reaction to the administration of potassium, sodium or glucose may be helpful. Episodes of hyper- and normokalemic, but not of hypokalemic, periodic paralysis may be accompanied by myotonia. Between episodes, myotonia might be detectable only in the EMG, but at the beginning of an episode, myotonia may be present (49).

The symptoms set in during childhood or youth and only rarely during early adulthood. Frequency and severity of the paralytic episodes decrease from 50 years of age onward, but about 50% of cases then suffer from a chronic progressive muscular weakness with increased CK values, myopathic EMG changes, and histological alterations similar to a vacuolar myopathy or a limb girdle dystrophy. Cases with progressive atrophies have also been described (50). Females with incomplete penetrance for the paralytic attacks may well present with such a myopathy at a later age. In addition to the prevailing medical problems there are also psychosocial and judicial questions that arise during the course of these diseases (occupational suitability, military service, degree of disability, and possibility of therapy). Therefore, advocacy groups, social services, psychotherapists and paramedical services may be of essential help.

**129.4.3.2 Clinical Neurophysiology and Magnetic Resonance Imaging (MRI).** The dyskalemic periodic paralysis show a late decrement under repetitive nerve stimulation. To decide for the appropriate therapy it does not suffice to clarify the form of dyskalemia, one should also search for ictal or interictal edema and for dystrophy using MRI (6,51). The ischiocrural

musculature is particularly likely to be affected by fatty degeneration (52).

**129.4.3.3 Pathogenesis.** Dominant mutations in the gene encoding the muscular voltage-dependent sodium channel Nav1.4 are responsible for hyperkalemic periodic paralysis (53,54) (Figure 129-3). Hypokalemic periodic paralysis is caused by mutations in the protein segments containing the voltage sensors in either of two very homolog muscular ion channels: the sodium channel NaV1.4 and the L-type calcium channel CaV1.1 (encoded by *CACNA1S*) (55–57). In either channel, the mutations generate additional pores in the voltage sensor segments (58). These pores conduct aberrant sodium currents that induce the P2 state in the affected fibers (6). Determination of the specific mutant allows one to predict the minima of serum potassium levels adopted by a particular patient. These minima determine the severity of future paralytic attacks and of secondary cardiac disturbances. They are also responsible for the course of the progressive myopathy.

If the muscle fibers stay in the P2 state for a very long time (54), sodium ions will accumulate in the intracellular space, and by virtue of osmotic forces, this will be followed by accumulation of intracellular water (6). This disequilibrium plus the electrical inexcitability lead to a progressive myopathy that may develop vacuoles or may histologically look like a dystrophy.

**129.4.3.4 Epidemiology.** The periodic paralyses are rare diseases with dominant inheritance. The prevalence of all forms taken together is about 1:50,000. The hyperkalemic form is transmitted with full penetrance, a male to female ratio of 1:1, and a prevalence of 1:200,000 (25). Patients without interictal myotonia are much more prone to develop progressive myopathy and permanent weakness than individuals with myotonia. This is obvious with individuals having the most common T704M mutation: about half of them show myotonia in the EMG and they are the ones who do not develop permanent myopathy. The second most frequent mutation, M1592V, is always associated with myotonia in the EMG, and patients have never been reported to suffer from permanent myopathy.

The most frequent is the hypokalemic form with a prevalence of 1:100,000. It is transmitted with reduced penetrance in women (the male to female ratio is 3 or 4:1). The most common mutations are the Cav1.1 substitutions R528H and R1239H, which both lead to progressive myopathy.

The term normokalemic periodic paralysis was originally given to a variant described in the 1960s. The disorder resembled the hyperkalemic form in many aspects; the only real differences were the lack of increase in the concentration of serum potassium even during serious attacks, and the lack of a beneficial effect of glucose administration. The existence as a nosologic entity was questioned because of the potassium sensitivity of the patients and the identification of the most frequent

hyperkalemic periodic paralysis mutations T704M or M1592V in such families including the original family.

Recently, a potassium-sensitive type of periodic paralysis with normokalemia and episodes of weakness reminiscent of those in both hyperkalemic (initiation of an attack by potassium) and hypokalemic forms (duration of attacks) was reported (59). This phenotype, also named normokalemic periodic paralysis, is caused by *SCN4A* mutations at deeper locations of the voltage sensor of domain II at codon 675 (Figure 129-3). Functionally, R675 mutations generate a leak current with a reversed voltage dependence compared to mutations causing HypoPP-2 since this site is exposed to the extracellular space at stronger depolarizations. Future studies will show whether normokalemic periodic paralysis is a separate clinical entity. The diagnostics are as described for the two more common forms of the disease.

#### 129.4.4 Thyrotoxic Hypokalemic Periodic Paralysis

Thyrotoxic hypokalemic periodic paralysis resembles familial hypokalemic periodic paralysis with respect to changes in serum and urinary electrolytes during attacks and in its response to glucose, insulin, and rest after exertion. It differs from familial hypokalemic periodic paralysis as it occurs only in the course of hyperthyreosis, even though the latter does not need to be clinically obvious. While Graves' disease (hyperthyroidism only) shows a 5:1 female to male predominance with a prevalence of 2% in the general population, this ratio is about 1:6 in thyrotoxic hypokalemic periodic paralysis, at least in Asian patients. Statistically, the incidence of periodic paralysis in Asian men with Graves' disease has been estimated at between 13 and 24% (25). More than 75% of the cases occur in Asians, suggesting a predisposing racial factor (Chinese, Japanese, Korean, and Vietnamese). The attacks occur much more frequently in summer than in winter. A geographical component is not likely, because Chinese or Japanese immigrants in North or South America have the same disease frequency as in their country of origin. Reports of cases in Caucasians and Blacks indicate that the disease rarely occurs in non-Asians (60).

The onset of disease is usually after the age of 20 years. Forty-five percent of the patients develop the syndrome in the third decade, another 35% in the fourth, and the rest in the fifth decade of life. The thyrotoxicosis precedes or appears simultaneously with the periodic paralysis in more than 80% of the patients (61), but the thyrotoxic signs are relatively mild at the time of the initial attack (no palpitations, goiter, or exophthalmus). Typical are sudden paralytic attacks of proximal limb muscles after strenuous exercise or at rest following high-carbohydrate meals in the evening or during the night, and hypokalemia during the attacks. The serum potassium falls to levels below 3.5 mM in 80% of the patients. In some patients

it may be as low as 1.2 mM and cause life-threatening arrhythmias or sinoatrial block. As the hypokalemia is the result of an insulin-induced shift of potassium from the extracellular to the intracellular space of the muscles, potassium is released from the muscle at the end of an attack to cause rebound hyperkalemia. During an attack, both the arrhythmia and the acute paralytic attack are relieved by administration of potassium. The paralytic attacks cease when the euthyroid state is restored, but recurrence of the hyperthyroid state causes recurrence of the paralytic attacks.

*KCNJ18*, a gene encoding the inwardly rectifying potassium channel Kir2.6, was mutated in the four who were from Singapore of thirty unrelated Chinese patients (62) (Table 129-1). Kir2.6 is transcriptionally regulated by thyroid hormone.

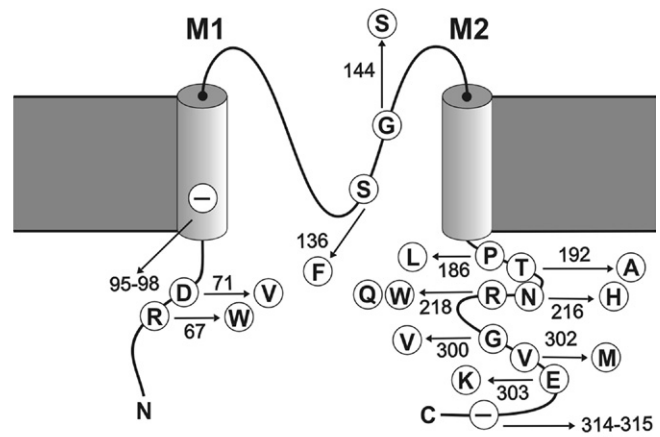
### 129.4.5 Andersen-Tawil Syndrome

This is a very rare disease, characterized not only by episodes of paralysis but also by life-threatening ventricular arrhythmias. During paralysis, the serum potassium level may be increased, normal or reduced (63). Accordingly, the response to oral potassium is unpredictable. The ECG should be monitored at rest and also in long-term observations. Some of these patients show skeletal abnormalities of the face, hands and/or feet. Mutations of the Kir2.1 potassium channel, an inward rectifier expressed in skeletal and cardiac muscle, are causative of the disorder (64) (Table 129-1, Figure 129-4). Kir2.1 channels are essential for maintaining the highly negative resting membrane potential of muscle fibers and accelerating the repolarization phase of the cardiac action potential. The mutations mediate loss of channel function by haploinsufficiency or by dominant-negative effects on the wild-type allele and may lead to long-lasting depolarization and membrane inexcitability. The prevalence is estimated to be <1:1,000,000.

### 129.4.6 Differential Diagnosis and Therapy of the Myotonias and Periodic Paralysis

**129.4.6.1 Differential Diagnoses.** When diagnosing myotonia patients one should always think of the myotonic dystrophies (DM), in particular if they have cataracts as in DM1 or atypical breast pain as in DM2. Patients having events of periodic paralysis may suffer from dyskalemia (e.g. accompanying Conn's or Addison's syndrome). In contrast to genuine periodic paralysis these conditions are accompanied by long-term pathologic alterations of muscle force and serum potassium. Of course, a so far compensated state can be decompensated by an acute additional change in serum potassium, and thus lead to an episode of paralysis.

**129.4.6.2 General Anesthesia.** Myotonia patients deserve special attention before and during narcosis. In particular, if the myotonia is not readily recognizable as



○ Andersen's syndrome — Deletion

**FIGURE 129-4** Membrane topology model of the inward rectifying Kir2.1 potassium channel of skeletal and cardiac muscle. Kir2.1 is a voltage-independent potassium channel encoded by *KCNJ2*. The functional channel is a homotetrameric protein. Mutations in the channel cause Andersen's syndrome.

in hyperkalemic periodic paralysis and myotonia fluctuans, the use of potassium and other depolarizing agents such as suxamethonium and cholinesterase inhibitors can provoke the myotonic reaction and thus compromise intubation and respiration. This can induce life-threatening incidents (65). Moreover, stress caused by the operation may lead to hypokalemia and may trigger a paralytic attack in patients with hypokalemic periodic paralysis. Development of hypokalemia during narcosis may evoke local generalized muscle weakness in all forms of periodic paralysis. Therefore, measures to retain body temperature should always obviate hypothermia of the patient during the operation.

**129.4.6.3 Therapy.** Therapeutic studies of the periodic paralyses have so far only been performed with dichlorophenamide (available only in Italy as Fenamide®) (66). Systematic studies for the therapy of the myotonias and periodic paralyses do not exist and, therefore, recommendations are not supported by evidence-based data (67–69).

In cases of benign myotonia no medication is required because patients learn to handle their disorder. Patients having a sodium channel myotonia should avoid strenuous ventures like hikes in the mountains. In cases of severe myotonia as in myotonia permanens or in cases of Becker myotonia, where medication seems appropriate, antiarrhythmic drugs have the best effect. By virtue of their “use-dependent” action they block in essence the pathologic after-activity without affecting the excitability per se. Their therapeutic window is, however, narrow. They not only affect the sodium channels but also improve chloride channel myotonia, although not so effectively as they do with sodium channel myotonia. For a long time the Ib-antiarrhythmic drug mexiletine was the drug of choice (70,71), but recently it has been taken off the market (with the exception of Japan) because of low cost-effectiveness. As alternatives, the Ic-antiarrhythmic



drugs flecainide and propafenone are available (72) (Table 129-1). Electro- and echocardiogram should be recorded before administration to avoid a heart bundle block. During use of one of the above antiarrhythmics, patients having heart insufficiency or arrhythmias should have no prolongation of the QRS-complex by more than 20% and the QT time should be no longer than 500 ms. The absolute QTc time should remain stable. Regular cardiologic controls are recommended with all patients. The control of serum potassium values and the monitoring of potential cardiac or central nervous side effects, as well as the avoidance of dehydration will permit a lifelong treatment, even with beginning in childhood. In the case of cardiac disturbances one may sidestep to the well-compatible drug lamotrigine. Carbamazepine or phenytoin should not be used because of their low anti-myotonic action.

With paramyotonia patients, a short-term therapy, e.g. for 2–3 days before the exposure to cold, is preferable to a long-term treatment. The same is true for sodium channel myotonia and extreme bodily stress. Patients with hyperkalemic periodic paralysis should avoid strong bodily stress and should have frequent and smaller meals. They should also avoid potassium-rich food (dried fruit, bananas, apricots, cauliflower, leguminous plants, etc.). For prophylaxis, medication with thiazide diuretics or carbonic anhydrase inhibitors is recommended (73). Both types of drugs stimulate the excretion of potassium, the latter ones, however, only to a small extent so that they can also be used for the hypokalemic form (see later). Beta-sympathomimetic drugs like salbutamol, when taken at the onset of an episode, may ease the symptom rather quickly. In addition, the simultaneous application of glucose and insulin may lower serum potassium rather quickly; in exceptional situations also a slow intravenous injection of calcium gluconate may be appropriate.

With normokalemic periodic paralysis patients, avoidance of dyskalemia and medication with carbonic anhydrase inhibitors are a suitable prophylaxis against paralytic episodes. Patients with hypokalemic periodic paralysis should avoid foods rich in carbohydrates and strong bodily work. They should eat foods rich in potassium (see earlier) and low in sodium. To avoid paralytic episodes, medication with carbonic anhydrase inhibitors like acetazolamide and dichlorphenamide and/or the aldosterone antagonist eplerenone is recommended. Potassium saving diuretics like triamterene or amiloride would also be suitable, but in certain countries they are available only in combination with potassium-excreting diuretics; this, of course, counteracts their effect. Mono-substances can be imported from abroad. The dose of the drugs should be slowly increased (e.g. eplerenone should start with 12.5 mg/day and be increased by 12.5 mg/day in the second week, etc.). A combination of carbonic anhydrase inhibitors, potassium-sparing diuretics and potassium, each at low dosage, may reduce the side

effects of the drugs. With this combination, the possibility of hyperkalemia with muscular weakness and cardiac arrhythmias exists, which persists until the hyperkalemia is reversed. The danger of this possibility to happen is, however, much lower than feared by many physicians, because most of the patients present even in the interval with serum potassium values at the lower norm. In fact, it turns out to be rather difficult to raise their serum potassium level to a persisting 4.8–5.2 mM, values that would suffice to let them experience less muscle weakness, less muscle pain and a reduced number of paralytic episodes.

In some patients, carbonic anhydrase inhibitors have an opposite effect: for reasons unknown they lead to an increase in the number and severity of paralytic attacks (74). In these cases aldosterone antagonists and potassium-sparing diuretics are helpful (6). At least in an acute episode potassium should be administered. The mentioned drugs are also effective in antagonizing the lasting muscle weakness, provided muscle tissue is still preserved (6). Since it has been shown that potassium may repolarize depolarized muscle fibers of patients with hypokalemic periodic paralysis, potassium should be given not only during a paralytic episode but also as a prophylactic measure. This may sound paradoxical, but often it works. Best suited are slow-retard potassium tablets (6).

A thyrotoxic hypokalemic periodic paralysis is best cured by treating the hyperthyreosis. In an acute case potassium and carbonic anhydrase inhibitors may be used (25). Some experts also advocate beta-blockers.

In Andersen-Tawil syndrome carbonic anhydrase inhibitors can be used as prophylactic means against paralytic attacks. Extrasystoles should not be treated at any cost; often the arrhythmias disappear with tachycardia, as with moderate bodily stress or fever. A treatment with blockers of the sodium or calcium channels or with beta-blockers is common but often not effective. Earlier investigators reported imipramine as useful. Amiodarone should be given only in severe cases because of its substantial side effects. For the long-QT syndrome this drug is even contraindicated (75). In cases of rhythmogenic syncope a pacemaker or a combined pacemaker/defibrillator may be indicated. All drugs that prolong the QT time are strongly contraindicated.

## 129.5 HEREDITARY CHANNELOPATHIES OF EXCITATION-CONTRACTION COUPLING

MH, central core myopathy and multiminicore myopathy are allelic diseases; they are all caused by mutations in the same gene *RYR1* (76,77). Most *RYR1* mutations result in a dominant predisposition to MH, which may occur as a life-threatening complication during an inhalation narcosis. Central core myopathy and multiminicore myopathy are diseases that can also be generated by mutations in other genes. For safety reasons, with

general anesthetics in patients with any kind of central core or multiminicore myopathy it is always assumed that there is an increased risk of a developing MH (78).

### 129.5.1 Malignant Hyperthermia (MH) and Malignant Hyperthermia Susceptibility (MHS)

MH is a dangerous condition even 50 years after its first description. Its dangerousness is evident from the fact that all over the world institutions of anesthesiology hold the antidote dantrolene in stock. Frequency of clinical crises, as well as lethality, have been substantially lowered over the past years by prevention, identification of susceptible persons, education of affected families and instruction of personnel. These measures have substantially contributed to the safety of patients in the area of anesthesiology.

The frequency of the genetic disposition to MH is estimated at 1:3000 in Caucasians. The clinical incidence is higher in children than in adults and estimated to be 1:50,000 or 1:100,000 for general anesthetics. These estimates, however, are based on clinical data collected at times when the classical trigger substances halothane and succinylcholine were frequently used in combination and at high doses. Nowadays, events of clinical crises must be assumed to occur at a much lower frequency. With the exception of NO and xenon, all volatile anesthetics and depolarizing muscle relaxants (the latter perhaps only in combination with the further) can elicit an MH crisis. Extreme bodily stress, a hot environment and infections may also trigger an “awake episode.” On the other hand, there are now a multitude of anesthetic drugs at hand for which safe applicability to persons susceptible to MH (MHS) has been proven. This permits the application of regional and general anesthetics also with MHS persons.

The clinical symptoms of an MH crisis (hypermetabolism, tachycardia, muscle spasms, increase in respiratory CO<sub>2</sub>, and rise in body temperature) are all based on an excessive release of Ca<sup>2+</sup> from the sarcoplasmic reticulum (SR). In about 50% of the families this excess is caused by a mutation in *RYR1*, the gene encoding the muscular Ca<sup>2+</sup> release channel, also called ryanodine receptor. In a few families, MH susceptibility is caused by mutations in *CACN1A5* (79), the gene encoding the muscular L-type calcium channel (i.e. the same channel that causes hypokalemic periodic paralysis when mutations alter its voltage sensors).

The diagnosis of MH susceptibility is still performed using the invasive in vitro contracture test (IVCT). This test is a functional, standardized procedure (80) in which the force developed in a contracture of the tested person's muscle biopsy is taken as an indirect marker for the sensitivity to contracture-triggering substances such as caffeine and halothane. In contrast to the IVCT test protocols primarily aimed at determining the clinical risk of

anesthesia-related events, diagnostic testing in Japan is performed by a functional test based on the quantification of calcium-induced calcium release (CICR) in saponized muscle fibers (81). The precision of this method and correlation to the other protocols is unknown. In addition to functional tests, a histological investigation may give evidence for central core myopathy or multiminicore myopathy, conditions that are frequently associated with MH susceptibility.

An a priori genetic investigation of a subject with potentially increased risk of MHS is costly because the *RYR1* gene consists of 106 exons. Usually MH susceptibility is first tested using IVCT and in the positive case the search for the mutation begins. For consanguineous relations of a proband with a causative mutation, a mere genetic investigation is sufficient. The number of mutations determined with respect to MHS has over the years increased to more than 100 (Figure 129-5). However, in only 30 mutations has a causative connection been established (10,82–84).

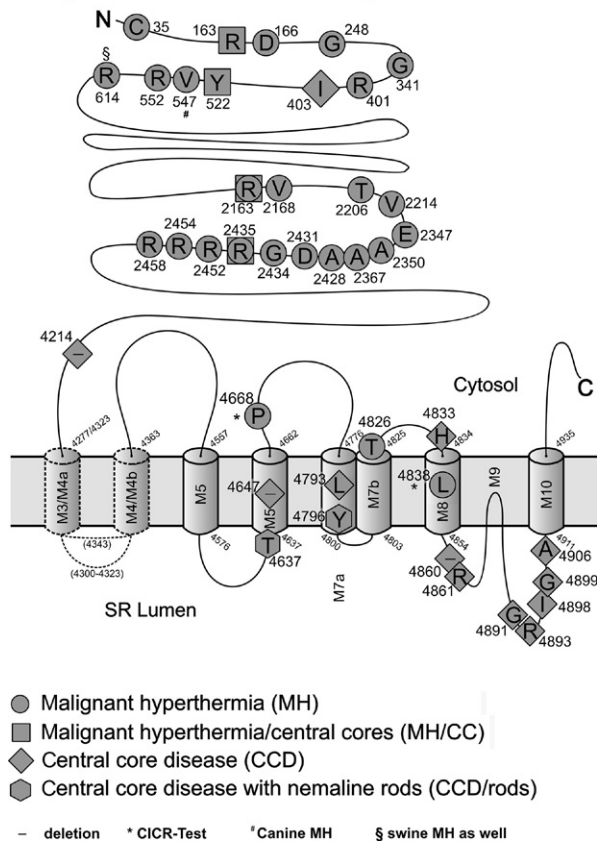
A person that has passed several general anesthetics without problems may not necessarily be taken to be MHS-negative for sure, because the likelihood of an MH crisis occurring during general anesthesia is only 1:5. It should be mentioned that MHS-positive persons should not undergo extreme bodily stress (marathon, triathlon, and competitive sports) or exposure to extremely high environmental temperature. Excessive alcohol consumption and abuse of drugs (ecstasy, amphetamines, or cocaine) may also elicit MH (85).

### 129.5.2 Central Core Myopathy

Patients with central core myopathy typically present already at birth with hypotrophy and hypotonia of the musculature (floppy infant) and later show delayed motor development. Quite often they have a congenital subluxation of the hip and later develop scoliosis. Weakness of the pelvic and humeral musculature is conspicuous. Usually there is neither restriction of the bulbar and respiratory muscles nor an external ophthalmoplegia. Most patients become stronger as they grow up and can walk independently. According to personal observations bodily endurance training seems to have positive effects. Only a minority of the children show a progressive course. Differential diagnosis to nemaline myopathy and multiminicore myopathy is sometimes difficult (86).

Central core myopathy is in most cases inherited as an autosomal dominant trait with alternating penetrance. Many family members of a patient are only diagnosed when they undergo genetic investigation. Since both central core myopathy and susceptibility to MH are caused by mutations in *RYR1*, it is assumed that persons having such a mutation are in any case susceptible to MH, no matter whether they show the disease or not (although exceptions to this rule have been reported). These exceptions pertain to mutations

## Ryanodine receptor RYR1



**FIGURE 129-5** The skeletal muscle ryanodine receptor (RyR1) forms a homotetrameric protein complex that is situated in the sarcoplasmic reticulum (SR) membrane and functions as calcium release channel. The cytosolic part, the “foot”, bridges the gap between the transverse tubular system and the SR. It contains binding sites for various activating ligands such as  $\text{Ca}^{2+}$  (in micromoles), ATP, calmodulin, caffeine and ryanodine (in nanomoles), and inactivating ligands such as dantrolene,  $\text{Ca}^{2+}$  ( $>10\mu\text{M}$ ), ryanodine ( $>100\mu\text{M}$ ), and  $\text{Mg}^{2+}$  (in millimoles). The transmembrane segments are numbered M3–M10. The first two cylinders with dashed lines indicate the tentative nature of the composition of the first predicted helical hairpin loop (M3–M4 or M4a–M4b). The long M7 sequence is designated as M7a and M7b. The proposed selectivity filter between M8 and M10 is designated as M9 even though it is clearly not a transmembrane sequence. Mutations causing susceptibility to malignant hyperthermia and/or central core disease are indicated.

in the C-terminal region of *RYR1*, which, in contrast to all other mutations, do not result in an increased, but a reduced,  $\text{Ca}^{2+}$  release. The reduced  $\text{Ca}^{2+}$  release could explain the muscle weakness. Mutations that lead to an increased open probability of the  $\text{Ca}^{2+}$  channel (“leaky channels”) could also generate the weakness by depleting the sarcoplasmic  $\text{Ca}^{2+}$  stores.

Serum CK is usually normal or only slightly increased. A conspicuous feature of the muscle biopsy is the dominance of type 1 fibers. NADH staining shows the characteristic centrally localized cores by the absence of an intermyofibrillar oxidative network. Longitudinal sections show that a core usually extends from one end to the other of type 1 fibers.

### 129.5.3 Multiminicore Myopathy

It is a recessively inherited congenital myopathy with genetic heterogeneity (87). The classic form of the disease usually leads to severe scoliosis. The affected muscles show an axial distribution, i.e. respiratory, bulbar and extraocular muscles are often struck. This form of the disease is caused by mutations in the gene *SEPN1*, encoding selenoprotein N, a protein that seems necessary for the release of  $\text{Ca}^{2+}$  from the SR.

Another form of multiminicore myopathy is caused by mutations in the *RYR1* gene (88). It is less severe, and scoliosis is little pronounced if at all. Patients show generalized muscle weakness with accentuation of the pelvic girdle and the hand muscles. Amyotrophy and hyperextensibility of the joints prevail (89). As with central core myopathy the course of the disease is rather benign and rarely progressive. Histology is also similar except that the cores do not extend over the whole fiber length and have fuzzy ends. In some patients having a core myopathy, mutations were found in *ACTA1*, a gene coding for alpha actin.

Monoallelic expression is the explanation for the question why the mode of inheritance of some of the core myopathies is difficult to explain (89,90). Several features of the silencing of the wild-type allele show characteristics of genomic imprinting; first, the sex of the nontransmitting parent is regularly female; second, the monoallelic silencing follows a tissue-specific pattern; and last, control fetuses generally show *RYR1* silencing in 10% of cases with tissue-specific expression (91).

## 129.6 CRITICAL POINTS IN FUNCTIONAL AND GENETIC STUDIES

### 129.6.1 In Vitro Functional Studies of Channel Mutants

Functional expression of mutations has contributed to the understanding of the molecular pathogenesis of almost all muscle channelopathies. However, there are undeniable problems of interpreting changes in function brought about by mutants in in vitro expression systems. The overexpression of introduced DNA in a heterologous cell system may lead to a nonphysiological localization of the encoded protein. This can lead to a false conclusion regarding channel significance. Second, the cells chosen for functional expression may have endogenous channel subunits that can potentially interact with or be upregulated by the introduced DNA. These can generate currents, which may be falsely assumed to appear because of the introduced DNA (compare (92) with (93) and (94)). Third, heterologous expression systems may secondarily modify the channels chemically, which may lead to misinterpretation of the functional significance of the channel subunits. An example of such a possibility is the skeletal muscle sodium channel Nav1.4, which exhibits more rapid



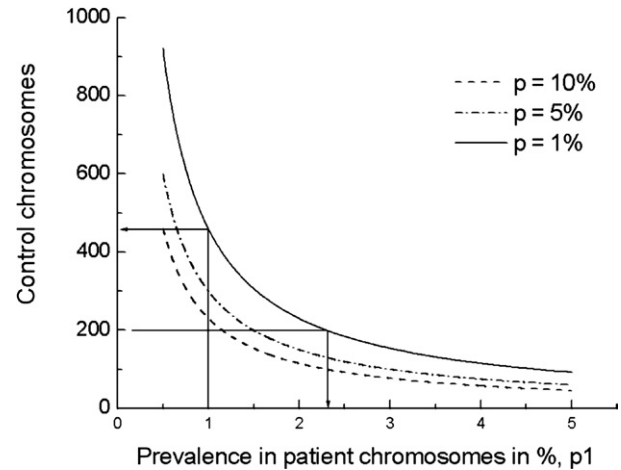
inactivation kinetics when expressed in human embryonic cell lines than in *Xenopus* oocytes. Originally, this finding was attributed to the lack of expression of the accessory  $\beta$  subunit in the oocytes; however, the rapid kinetics of the channel were also found when expressed in cells without endogenous  $\beta$  subunits. Therefore, post-translational modifications and association of sodium channels with other membrane proteins such as cytoskeletal components are now considered responsible for the differences in kinetics (5).

In short, the function of ion channels is highly dependent on the expression system used. The functional significance implied by these experiments may not necessarily be valid for the physiological situation in vivo. Moreover, the finding of a functional change may not necessarily indicate a mutation, but could instead be brought about by a polymorphism. In spite of inherent difficulties in the publication of such findings, several of these “functional polymorphisms” have been described. For example, S906T in Nav1.4 was found to segregate perfectly with periodic paralysis in several large pedigrees and was alleged to alter entry into and recovery from slow-channel inactivation. However, S906T occurs in 5% of the population without association with any disease (95). In the periodic paralysis families mentioned, it turned out that S906T happened to be linked to a much later identified causative change.

### 129.6.2 Genetic Screening of Control Populations

Owing to the shortcomings of the interpretation of functional studies, the genetic screening of large and adequately matched control populations for absence of the putative mutations is important to prove disease causality. Two reports have proposed the typing of 150–200 controls (300–400 chromosomes) for putative mutations with a prevalence of 1% by power analysis (96,97). A more general algorithm that recommends exclusion of the putative mutation in ethnically matched control chromosomes has recently been proposed (98). According to it, for a proposed maximally tolerable error of 1% and a mutation present on 1% of the tested patient chromosomes, at least 460 control chromosomes (230 control individuals; Figure 129-6) should be tested.

Therefore, the common laboratory practice to exclude a novel mutation in approximately 100 healthy controls is insufficient. An example for spurious conclusions is an R83H substitution in a potassium channel  $\beta$  subunit, MiRP2, suggested to cause dyskalemic periodic paralysis because it showed a loss of function in vitro and was found in two of 100 of such patients but in none of 120 unaffected controls (99). In later studies, the substitution was identified not only in one of 104 and one of 138 patients but also in eight of 506 and three of 321 controls (49,100). Taken together, the substitution is present in 1.17% of patients and in 1.16% of healthy controls, which does not support disease



**FIGURE 129-6** Number of required control chromosomes as proposed by a statistical equation to minimize the error. Let the prevalence of a mutation in a patient's chromosomes be  $p_1$  and the prevalence in control chromosomes be  $p_0$ ; then, the probability of an arbitrary control chromosome not to carry the mutation is  $(1 - p_0)$ . Because the world control population is large, the probability  $P$  of arbitrarily choosing  $n$  chromosomes thereof without the mutation may be approximated by  $P = (1 - p_0)^n$ . The null hypothesis would be that the mutation frequency is equal in patient and control chromosomes, i.e.  $p_0 = p_1$  and  $P = (1 - p_1)^n$ . The number of control chromosomes to be tested can be calculated by resolving the equation for the number  $n = \ln(P)/\ln(1 - p_1)$ . By setting an error probability  $P$  of 1%, the number of required control chromosomes is  $n = -4.6/\ln(1 - p_1)$  and  $n = 460$  for the example of  $p_1 = 1\%$ . The curve demonstrates that 100 control individuals (200 chromosomes) would be adequate for a  $p_1$  of 2.5%, a prevalence that is much higher than the most frequent monogenic disorder.

causality. Even though the difference between defining a putative mutation as being truly disease causing or as being a functional polymorphism may seem only marginal on a scientific level, this difference in definition has drastic consequences for an affected carrier whose diagnosis is made or confirmed by the finding and who is being medically treated. This problem will increasingly need to be addressed in future studies when the number of known mutations and putatively associated phenotypes continues to increase.

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## RELEVANT WEB PAGES

Hyperkalemic periodic paralysis in GeneReviews at GeneTests: [www.genetests.org](http://www.genetests.org)  
 Periodic Paralysis Association: [www.periodicparalysis.org](http://www.periodicparalysis.org)



### Biographies



**Frank Lehmann-Horn** has a Master's degree in mechanical engineering and is a Medical Doctor specializing in neurology and physiology. In 1986, he received the degree of Dr habil in neurology at the Technical University in Munich. Next to his scientific work and his teaching responsibilities, he takes care of many patients with myotonias, periodic paralyses, episodic ataxia, migraine and individuals susceptible to malignant hyperthermia. In 1992, Dr Lehmann-Horn became Head of the Institute of Applied Physiology at Ulm University. In 2005, Dr Lehmann-Horn was awarded the Dr honoris causa (Dr.h.c.) of the University of Debrecen in Hungary for his scientific collaborations on muscle excitation–contraction coupling and the coordination of two European networks sponsored by the European Union. In 2008, he was elected for the Endowed Research Senior Professor for Neurosciences of the Hertie-Foundation. In 2009, he became a member of the Heidelberg Academy of Sciences and was awarded the Art of Listening Award from the Genetic Alliance, Washington, DC. In 2010, as Hertie Senior Research Professor of Neurosciences, he became Head of the Division of Neurophysiology of Ulm University. Currently, he is Chairman of the Ulm Muscle Centre.



**Reinhardt Rüdel**, born in 1937, studied physics and medicine at the Universities of Erlangen, Vienna and Heidelberg. In 1965, he received a PhD in physics, and in 1970, the degree of Dr habil in physiology, both at the University of Heidelberg. His main academic teachers were Wolfgang Trautwein and Josef Dudel at Heidelberg, Sir Andrew Huxley at London and John R. Blinks at the Mayo Clinic in Rochester, MN. In 1971, he became interested in studying muscle diseases characterized by disturbed excitation, first with Erich Kuhn in rat model myotonia, and later with Kenneth Ricker and Frank Lehmann-Horn in excised intercostal muscle bundles from patients. From 1972 to 1979, he was professor of physiology at the Technical University of Munich; from 1979 to 2004 he was chairman and director of the Institute of General Physiology at the University of Ulm. There, he founded the Neuromuscular Center in 1993, always continuing with research into neuromuscular disorders. Being a wheelchair-bound muscle patient himself, he was very active in advocacy organizations for patients with muscle disorders serving as president of the German MDA from 1986 to 1992 and as president of the European MDA from 1987 to 1993. He was president of the VIIth International Congress on Neuromuscular Disorders in Munich and one of the three founders of the European Neuromuscular Centre (ENMC) at Baarn/the Netherlands. From 2000 to 2001 he was president of the German Physiological Society. At present, he is vice president of the Gaetano Conte Academy for the study of striated muscle at Naples/Italy.



**Karin Jurkat-Rott** is a Medical Doctor specialized in Physiology and working as deputy chair in the Division of Neurophysiology, Ulm University. In 2001, she received the degree of Dr habil in physiology at Ulm University. She is the scientific secretary of both the Ulm Muscle Center and the Ulm Center on Rare Diseases. Her main goal is to elucidate the pathogenesis of diseases that are caused by pathologically altered ion channels known as channelopathies. Examples are myotonia, periodic paralysis, ataxia and migraine. By genetic, functional and patient studies, she is developing models for pathogenesis and strategies for therapy. In addition, she is interested in various aspects of ion channels, e.g. their coding genes, the splicing of the RNA, the function of the channel proteins, and their modulation by exo- and endogenous ligands and toxins.



# CHAPTER

# 130

## Myotonic Dystrophies

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### 130.1 INTRODUCTION

Myotonic dystrophy (dystrophia myotonica 1, Steinert's disease; OMIM 160900) is an autosomal dominant disorder, clinically characterized by myotonia, dystrophic muscle degeneration, lens opacities, and variably associated other multisystemic phenotypes. The mutation responsible for the disease is an expansion of unstable CTG trinucleotide repeats in the 3' untranslated region (UTR) of the dystrophia myotonica protein kinase (*DMPK*) gene on chromosome 19q13.3. The identification of this mutation led to the recognition of autosomal dominant families whose clinical manifestations closely resemble this disease, but whose *DMPK* gene shows no CTG repeat expansion. The disease of these families was initially named proximal myotonic myopathy (PROMM; OMIM 602668) because of predominantly proximal distribution of muscle weakness and atrophy. The genetic mutation found in most of these families is an expansion of an intronic CCTG repeats in the zinc finger 9 (*ZNF9*) gene on chromosome 3q13.3–q24. The characteristics of this mutation are strikingly similar to that of myotonic dystrophy; both are large expansions of microsatellite repeats that are transcribed into RNA with repeated CUG motifs but not translated into the respective protein products. The pathogenic mechanisms of these two diseases have been speculated to be similar and predicted to be mediated by a “*trans*-dominant gain-of-function” involving repeat-containing RNA transcripts. At the Third International Myotonic Dystrophy Consortium meeting (1) the nomenclature of the genetic loci of these disorders was changed to *myotonic dystrophy type 1* (DM1) for myotonic dystrophy and *myotonic dystrophy type 2* (DM2) for PROMM. In 2003, the 115th European Neuromuscular Center (ENMC) workshop adopted the term myotonic dystrophy type 2 (DM2) as the clinical nomenclature for the entire progressive myotonic multiorgan disorders linked to the DM2 locus (Table 130-1) (2).

DM1 is the most common muscular dystrophy of adult life and presents with a variety of clinical challenges to all those involved with affected patients and

their families. The wide range of severities, age at onset and the multi-system involvement requires that a corresponding variety of specialists often need to be involved in the management of DM1 patients. Clinical geneticists encounter DM1 as a frequent cause for referral in relation to genetic counseling, presymptomatic detection, and prenatal diagnosis. As a result of their involvement with the whole family, clinical geneticists may be the first to make the diagnosis, especially in affected individuals at the extremes of life, when the disorder is often not recognized until it can be placed in the context of the family.

In contrast, DM2 presents with a milder, although potentially disabling, phenotype. The clinical features and the genetic mutation of DM2 resemble those of DM1, but the pattern of instability of the genetic mutation and of the genotype–phenotype correlation is very different between DM1 and DM2. Clinical geneticists may deal with these differences when genetic testing shows no DM1 mutation in patients suspected to have DM1.

The identification of the molecular basis of the mutation has made a significant impact in the management of patients with DM1 and DM2. This is true not only for the genetic aspects of the diseases, but increasingly for our understanding of the clinical variability. The cell biology and possible molecular processes involved in the pathogenesis of the disorder have proved to be complex and have enlightened our understanding of a broader group of diseases associated with gain-of-function mutations involving RNA. A more detailed account of the various clinical aspects of DM1 might be found in the recent edition of the monograph of Harper (3) and review by Turner and Hilton-Jones (4).

### 130.2 CLINICAL FEATURES

#### 130.2.1 Clinical Features of Myotonic Dystrophy Type 1

**130.2.1.1 Clinical Features and Differential Diagnosis of Myotonic Dystrophy Type 1.** DM1 was first recognized as a specific clinical entity in the

**TABLE 130-1 Nomenclature of Myotonic Dystrophies**

Disorder	OMIM#	Alternative Names	Disease Map Locus	Gene	Location	Mutation
Myotonic dystrophy type 1	#160900	Dystrophia myotonica 1 DM1 Steinert's disease	<i>DM1/19q13.2–q13.3</i>	<i>DMPK</i>	3'UTR	CTG expansion
Myotonic dystrophy type 2	#602668	Proximal myotonic myopathy Dystrophia myotonica 2 Ricker's disease PROMM DM2	<i>DM2/3q13.3–24</i>	<i>ZNF9</i>	Intron 1	CCTG expansion

**TABLE 130-2 Inherited Myotonic Disorders**

Disorder	Inheritance	Basic Defect
Myotonic dystrophy type 1 (DM1)	Autosomal dominant	CTG expansion in <i>DMPK</i> (chr.19)
Myotonic dystrophy type 2 (DM2/PROMM)	Autosomal dominant	CCTG expansion in <i>ZNF9</i> (chr.3)
Myotonia congenita (Thomsen's disease)	Autosomal dominant	Muscle Cl <sup>-</sup> channel ( <i>CLCN1</i> ) (chr.7)
(Becker's Disease)	Autosomal recessive	Muscle Cl <sup>-</sup> channel ( <i>CLCN1</i> ) (chr.7)
Paramyotonia congenita	Autosomal dominant	Muscle Na <sup>+</sup> channel ( <i>SCN4A</i> ) (chr.17)
Sodium channel myotonia	Autosomal dominant	Muscle Na <sup>+</sup> channel ( <i>SCN4A</i> ) (chr.17)
Periodic paralysis (Hyperkalemic)	Autosomal dominant	Muscle Na <sup>+</sup> channel ( <i>SCN4A</i> ) (chr.17)
Chondrodystrophic myotonia (Schwartz–Jampel)	Autosomal recessive	Perlecan ( <i>HSPG2</i> ) defect (chr.1)

descriptions by Steinert (5) and by Batten and Gibb (6), both in 1909. DM1 had previously been confused with the non-dystrophic myotonia, “myotonia congenita” which had been described earlier by Thomsen in his own family (reviewed by Harper (3)). The group of non-dystrophic myotonias is discussed fully in Chapter 129, although it is mentioned later in relation to the differential diagnosis of DM1.

Table 130-2 summarizes the principal inherited myotonic disorders. The multisystemic nature of DM1 was apparent from the earliest studies; Steinert's original paper mentioned testicular atrophy, and by 1912 Greenfield and Curschmann independently recognized that cataracts are part of the clinical picture. In 1918, Fleischer showed that cataracts may be the sole clinical feature and that such individuals may connect apparently separate families with DM1. These observations by Fleischer proved of particular importance, laying the foundation for the concept of “anticipation,” as discussed later (reviewed in Reference (3)).

Although DM1 may present initially to a wide variety of specialists, a neurologist, because of muscle weakness or myotonia, will see many. The distribution of muscle involvement is characteristic, and the combination of myotonia with significant weakness and wasting often permits a confident clinical diagnosis of DM1. The facial muscles, sternomastoids, and distal limb muscles are usually the earliest to be affected, exhibiting a pattern differing from most other neuromuscular disorders, and contrasting with that seen in the other primary muscular dystrophies, as summarized in Table 130-3. Facioscapulothoracic dystrophy, which also shows prominent facial

muscle involvement, has a much greater degree of shoulder girdle weakness, and lacks clinical myotonia. The facial features of DM1 are an important early and visible characteristic (Figures 130-1 and 130-2), and old photographs may be a valuable diagnostic aid.

Myotonia is the delay in relaxation of a muscle following contraction. Myotonia in DM1 is best elicited by asking the patient to grip firmly and to then let go, in order to elicit handgrip myotonia, or by firm percussion of the thenar eminence or the brachioradialis muscle to elicit thumb or wrist myotonia. Percussion of the tongue may show a persistent furrow. Myotonia of other muscles is rarely obvious in DM1. Myotonia may be minimal in cases with advanced wasting and is usually clinically undetectable in young children. Some younger adult patients may present solely with symptoms of handgrip myotonia.

When myotonia is the principal finding, DM1 must be distinguished from other myotonic disorders, in particular myotonia congenita. This is usually straightforward (Table 130-4), especially if other family members show typical features, but the occasional early case of DM1 with little weakness may be difficult to distinguish. The myotonia in DM1 and myotonia congenita usually improves after repetitive exercise of the muscle, which is known as the “warm-up phenomenon,” whereas myotonia in some sodium channelopathies exhibits paradoxical worsening by sustained muscle contraction (paramyotonia). Electromyography (EMG) shows myopathic changes including small-amplitude, short-duration motor units and electrical myotonia. The combination of myopathic changes and myotonia on EMG can help to distinguish between “non-dystrophic” myotonic conditions, such as

**TABLE 130-3 Muscle Involvement in Myotonic Dystrophy and Other Adult Muscular Dystrophies**

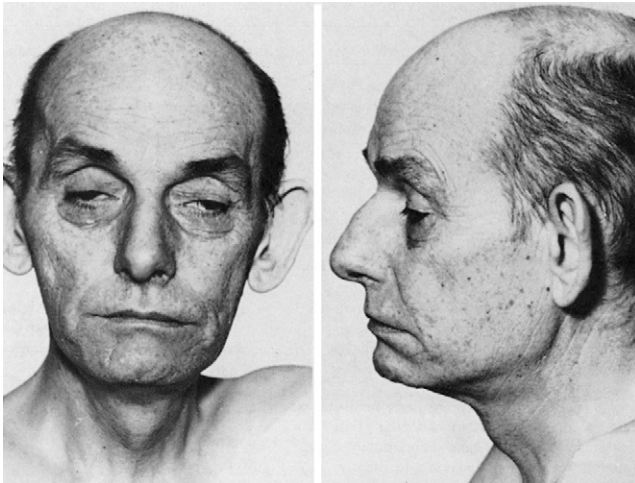
	Myotonic Dystrophy Type 1	Myotonic Dystrophy Type 2	FSHD	LGMD	BMD
Facial weakness	++	±	++	+	±
Ptosis	++	±	++	—	—
Jaw muscle	++	—	+	—	—
Sternomastoids	++	±	+	±	±
Shoulder girdle	±	+	++	++	+
Pelvic girdle	±	++	—	++	++
Proximal limb muscle	+	++	+	++	++
Distal limb muscle	++ <sup>a</sup>	± <sup>a</sup>	± <sup>c</sup>	±	±
Myotonia	++	± <sup>b</sup>	—	—	—
Pseudohypertrophy	±	—	—	±	++
Muscle pain	±	++	±	±	±

FSHD, faciohumeroscapular dystrophy; LGMD, limb girdle muscular dystrophy; BMD, Becker's muscular dystrophy; ++, prominent feature; +, may occur; ±, inconsistent or late feature; —, absent.

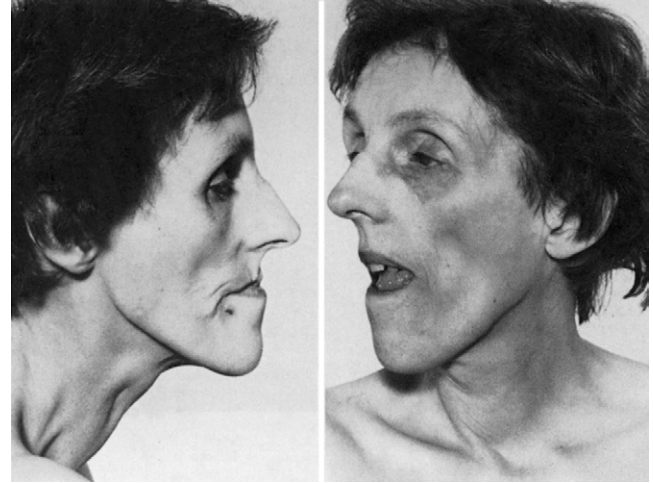
<sup>a</sup>Frequent in deep finger flexors.

<sup>b</sup>Electrical myotonia present in majority of patients but clinical myotonia may be subtle.

<sup>c</sup>Ankle dorsiflexion weakness is common in FSHD.



**FIGURE 130-1** Facial features of myotonic dystrophy. Note particularly the ptosis, facial weakness, and wasting of the jaw and sternomastoid muscles. (From Harper, P. S. *Myotonic dystrophy*. WB Saunders: Philadelphia, 1989, pp 17 (38))



**FIGURE 130-2** Facial features of myotonic dystrophy. Note particularly the ptosis, facial weakness, and wasting of the jaw and sternomastoid muscles. (From Harper, P. S. *Myotonic dystrophy*. WB Saunders: Philadelphia, 1989, pp 17. (38))

myotonia congenita, and “dystrophic” myotonias such as DM1 and 2. Muscle pathology shows a number of characteristic features in the classic adult form of the disease and is usually not needed to be performed to obtain a diagnosis. A confirmed genetic diagnosis based on clinical suspicion is better clinical practice.

In DM2, the distribution of muscle weakness is more proximal and myotonia is less severe. The striking resemblance between DM1 and DM2 warrants special consideration and DM2 is separately described later in the chapter.

**130.2.1.2 Smooth Muscle Involvement in Myotonic Dystrophy Type 1.** Smooth muscle, particularly of the gastrointestinal tract, is prominently involved in DM1 (Table 130-5). A study in Sweden has shown that for many patients gastrointestinal problems are the

most prominent symptom (7). The esophagus may show severely disturbed peristalsis in patients with relatively little weakness, which probably contributes to the aspiration pneumonia frequently seen in older patients, as well as in severely affected infants. Gastric emptying is delayed (8). Colonic involvement is common, and colicky abdominal pain is frequent in affected children, in whom megacolon may occur. Intestinal pseudo-obstruction may occur in both children and adults (9) mimicking a surgical emergency, but the condition normally responds to conservative treatment. Adult patients often have irritable bowel-type symptoms.

**130.2.1.3 Cardiopulmonary Involvement.** Cardiac involvement is an important clinical feature (see Reference (10) for a review; Table 130-6). Sudden death in adults is common and may have been underestimated in

**TABLE 130-4** Adult Clinical Features of Myotonic Dystrophies and Myotonia Congenita

	Myotonia Congenita	Myotonic Dystrophies (DM1 and DM2)
Onset of myotonia	Infancy or early childhood	Late childhood to adult life
Severity of myotonia	Often severe, generalized	Usually mild or moderate
Muscle weakness	Absent or late onset	Variable—mild to severe
Cardiac and smooth muscle	Absent	Common
Cataract	Absent	Diagnostic
Other systemic abnormalities	Absent	Widespread
Inheritance	AR, AD, sporadic	AD with anticipation

DM1, myotonic dystrophy type 1; DM2 myotonic dystrophy type 2. AR/AD autosomal recessive/dominant.

**TABLE 130-5** Gastrointestinal Tract Involvement in Myotonic Dystrophy Type 1\*

Anatomic Structure	Description
Pharynx	Delayed relaxation, retention of bolus, frequent tracheal aspiration
Esophagus	Reduced motility and pressure, dilation, dysphagia frequent
Stomach	Dilation, delayed emptying with food retention
Small bowel	Usually normal
Colon	Megacolon, fecal impaction, symptoms similar to irritable bowel syndrome; rarely volvulus
Anal sphincter	Myotonia demonstrable

**TABLE 130-6** Cardiac Abnormalities in Myotonic Dystrophies

Cardiac Abnormalities	DM1	DM2
Arrhythmias	+++	+
Atrioventricular block	+++	+ (11%; Day et al., 2003)
Cardiomyopathy	±	± (7%; Day et al., 2003)
Sudden death	++	± (reported)
Congestive heart failure	±	— (not reported)

DM1, myotonic dystrophy type 1; DM2, myotonic dystrophy type 2. "—" rare to "+++" common.

children (11). Conduction defects are seen on electrocardiography (ECG) in many affected adults, even in the absence of cardiac symptoms; varying degrees of heart block are the most common finding. More detailed studies with the His bundle ECG have confirmed these abnormalities (12) and suggested that widespread degeneration of the conducting tissue occurs when the myocardium as

a whole is relatively normal. Thus, all patients should receive regular ECG monitoring annually, supplemented when needed by 24-h monitoring and more detailed studies. It should also be noted that intra-Hisian conduction delay might be present in the absence of ECG abnormalities. Unfortunately, the role of electrophysiological testing in patients with normal ECG has not been established (13); however, in patients with symptoms such as syncope or with a family history of sudden death, or both, electrophysiological testing is strongly advised (14). The magnetic resonance imaging analysis of cardiac morphological and functional abnormalities may become useful for determining the necessity of electrophysiological studies (15). Insertion of a pacemaker should be seriously considered in patients with significant conduction problems (12,16). Drugs such as quinine, which may depress conduction, should be avoided. Although the prevalence of congestive heart failure is low among patients with DM1, tissue Doppler echocardiographic studies showed that up to 29% of asymptomatic patients have left ventricular systolic dysfunction (17,18). Bouts of various arrhythmias may occur, notably atrial flutter/fibrillation and ventricular tachyarrhythmias. These arrhythmias appear to be attributable to cardiomyopathy rather than abnormal autonomic regulation of the heart (19). Although the incidence of ventricular tachyarrhythmias may not be sufficiently high to justify implantable defibrillator therapy routinely when permanent pacing is indicated (13), defibrillator implantation has been proven to be life saving in some cases (20). The importance of sudden death in DM1 has recently been highlighted by Groh et al. (21), who found that patients with a severe abnormality on their ECG (rhythm other than sinus, PR interval of 240 ms or more, QRS duration of 120 ms or more, or second- or third-degree atrioventricular block) were at risk of sudden death.

In most cases, neither smooth nor cardiac muscle is significantly affected without some degree of skeletal muscle involvement; thus, these systems are not helpful as predictive tests or in genetic counseling, and they should not be used as such now that accurate mutational testing is possible. Although occasional patients have presented with smooth or cardiac muscle problems, they usually prove to have definite myotonia and weakness as well, even though the patient may not have complained of this.

Respiratory problems in DM1 are partly attributable to aspiration and to diaphragmatic involvement but may also reflect a central abnormality. Alveolar hypoventilation is well documented (22) and is frequently associated with hypersomnia. Use of domiciliary-assisted ventilation in patients with DM1 has been shown to be associated with prolonged survival and a sustained improvement in arterial blood gas tensions (23). Postanesthetic respiratory depression is a serious hazard; consequently, great care should be taken if anesthesia is required, even in young and mildly affected patients (24).



In spite of our awareness of the early mortality of DM1, with an average age of death at 53 and over 70% of patients dying from cardiorespiratory complications (25), there appears to be little impact on improving these devastating figures and a controlled clinical trial of cardiorespiratory intervention is sorely needed.

**130.2.1.4 Ocular Abnormalities.** Although cataracts have been well recognized as a complication of DM1, involvement of the eye is more extensive than cataract alone (Table 130-7).

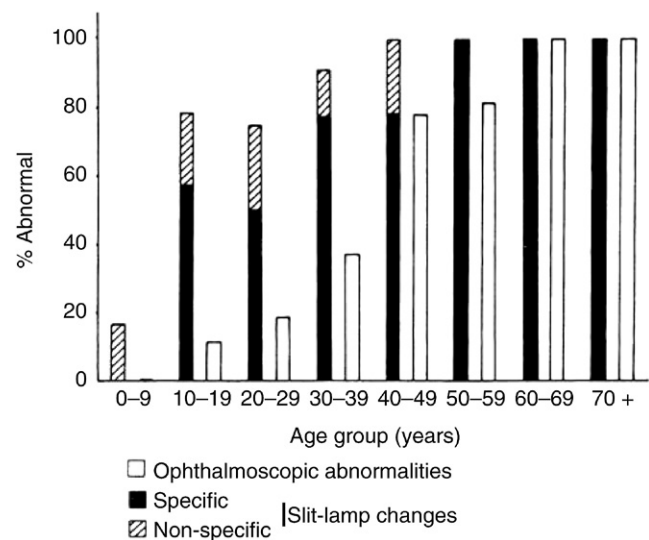
Lens opacities are rarely seen in young children, but this problem steadily increases with age, whereas clinically evident cataract is present by middle age in most patients (Figure 130-3). Lens opacities have a characteristic refractile, multicolored appearance when viewed with the slit lamp or “Christmas Tree” cataracts, although more mature cataracts may be difficult to distinguish from other types. Opacities are characteristically subcapsular rather than nuclear. No other myotonic disorder or primary muscle disease shows lens opacities of this type, with the exception of DM2; however, multicolored opacities can also occur in healthy individuals from the general population without the DM1 mutation (26). Although slit lamp examination for lens opacities is an important aid in the differentiation of atypical cases, it should not be used in presymptomatic detection. Retinal abnormalities are less well recognized and may be macular or peripheral. Electroretinography is a sensitive method of detecting changes, particularly when cataract obscures an ophthalmoscopic view. The retinopathy occurs independent of therapy with quinine or other drugs. The other ocular abnormalities are of less significance clinically, apart from ptosis, which is one of the most sensitive signs of the disorder in asymptomatic relatives or in old photographs. Clinically significant myotonia of the eyelids and ocular muscles is less common than in myotonia congenita. External ocular muscle weakness can also be seen.

**130.2.1.5 Brain Abnormalities.** Central nervous system (CNS) involvement is one of the most important and least well understood abnormalities in DM1. Since the earliest studies of DM1, abnormalities in the brain have been recognized, particularly when onset occurs early in life. Although major cognitive changes are unusual in adults, frontal executive dysfunctions including apathy, reduced initiative, stubbornness, and avoidant personality trait often render DM1 patients socially disadvantaged (27,28). Excessive daytime sleepiness is a frequent complaint of patients with DM1, and the condition cannot be fully attributable to central and obstructive sleep apnea, which are also common in DM1. Hypersomnolence in DM1 resembles narcolepsy by accompanying short sleep latency, the presence of sleep-onset rapid eye movement periods, and a dysfunction of the hypothalamic hypocretin system (29). Focal atrophy with progressive white matter lesions can be found on imaging studies in adults (30). Neurofibrillary tangles containing tau protein are increased in

**TABLE 130-7 Ocular Abnormalities in Myotonic Dystrophies**

Ocular Abnormalities	DM1	DM2
Cataract	~85%	80–90%
Retinal degeneration	>50%	Not reported
Low intraocular pressure, enophthalmos	60–80%	Not reported
Ptosis	~50%	Absent or mild
Corneal lesions	Occasional	Not reported
Extraocular myotonia	Infrequent	Absent
Extraocular muscle weakness	Infrequent	Absent

DM1, myotonic dystrophy type 1; DM2 myotonic dystrophy type 2.



**FIGURE 130-3** Incidence of lens opacities in myotonic dystrophy. (Data from Harper, P. S. *Myotonic dystrophy*. WB Saunders, Philadelphia, 1989, pp 171. (38))

number, and inclusion bodies have been noted in the brainstem (31,32). Abnormalities in auditory and other evoked potential studies (33), and in central oculomotor control further suggest widespread CNS dysfunctions (34). There has been recent increased interest in the CNS involvement in DM1. Weber et al. performed neuropsychological tests, structural cerebral MRI and 18F-deoxy-glucose PET (FDG-PET) in 20 DM1 and nine DM2 patients and matched healthy controls (35). DM1 and DM2 patients typically showed pronounced impairment of nonverbal episodic memory and a reduction of the global gray matter especially in the frontal and parietal lobes. A reduction in bilateral hippocampal volume was correlated specifically to both a clinical score and episodic memory deficits. White matter lesions were found in over 50% of patients, and were correlated to psychomotor speed. FDG-PET revealed a frontotemporal hypometabolism, independent of the decrease in cortical gray matter. All abnormalities were similar in both DM1 and DM2 patient groups but were more pronounced for DM1. This study confirmed that cognitive deficits tend to be more severe in DM1

than DM2 that decreases in cerebral gray matter may be independent of metabolism and that some of the characteristic cognitive changes are linked to specific structural changes. A comprehensive neuropsychological study and personality assessment, using the Millon Clinical Multiaxial Inventory (MCMI)-II on 121 patients with DM1 and 54 control patients, found that cognitive impairment particularly affected frontoparietal regions in DM1 and this deficit was correlated with CTG expansion in leucocytes (36). A further study investigated 50 DM1 and 14 DM2 patients and performed MRI to assess white matter lesions, neuropsychological profiling and SPECT analysis (37). The authors described a temporo-insular diffuse lesional pattern on MRI, which was specific for DM1. They also confirmed more severe cognitive involvement in DM1 than DM2. In conclusion, these studies confirm that there is significant cognitive involvement, especially in DM1, although a characteristic structural and neuropsychological profile has not been fully characterized.

**130.2.1.6 Endocrine Abnormalities.** Widespread endocrine disturbance exists in DM1 (Table 130-8 (3)), but the most significant abnormality observed clinically is testicular atrophy, which consists of a primary tubular degeneration with fibrosis and hyalinization and relative hypertrophy of Leydig cells. In spite of reduced spermatogenesis, patients may be fertile, even in the presence of clinical testicular atrophy. Male infertility is still a frequent occurrence. Elevation of blood follicle-stimulating hormone (FSH) is the main biochemical feature, with a lesser degree of luteinizing hormone (LH) elevation, whereas testosterone levels are commonly normal, in keeping with the conservation of Leydig cells and the normal secondary sex characteristics.

No directly comparable gonadal problem exists in women, but there is a high incidence of reproductive loss (one-third of all pregnancies in studies undertaken by Harper). Whereas losses late in pregnancy are probably

related to the congenital form of the disease (see later), the early pregnancy loss is more likely to reflect an endocrine disturbance, or possibly uterine smooth muscle involvement. This latter factor again becomes important in causing delayed and incoordinated labor.

Diabetes mellitus is a well-recognized problem, but clinical diabetes occurred only in 6% of the Harper's series (38). In contrast, biochemical studies have shown that abnormalities of insulin metabolism are almost universal. The main feature is insulin resistance, now recognized as resulting from reduced insulin receptor (39). Insulin resistance in DM1 is accompanied by abnormal levels of cytokines, such as leptin, tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), tumor necrosis factor II (TNFII) receptor, and testosterone, and by altered glucagon-like peptide-1 and adrenocorticotrophic hormone /cortisol responses (40,41). Additionally, insulin secretory dysfunctions may have a significant role (42).

Several other clinical features may reflect endocrine abnormalities, notably early balding, which is frequent in men, and the skeletal changes, which include cranial hyperostosis, enlargement of air sinuses, and frequently, a small pituitary fossa. Adrenocorticoid and mineralocorticoid metabolism are also impaired in DM1 (43,44).

**130.2.1.7 Congenital and Childhood-Onset Myotonic Dystrophy Type 1.** It is important to recognize that DM1 may also occur in neonates and young children, and that these forms, in particular congenital DM1, show marked clinical differences from the more classical 'adult' form (Table 130-9). Furthermore, unlike most genetic disorders, in which severe and mild forms run separately in families, the congenital and adult forms of DM1 are usually seen together, typically with the mother, who is often mildly affected, as the transmitting parent. An explanation for this phenomenon has come from our better understanding of the molecular

**TABLE 130-8 Endocrine Abnormalities in Myotonic Dystrophies**

Organ	DM1	DM2
Testis	Testicular atrophy (60–80% clinically); degeneration of tubular cells; hyperplasia of Leydig cells; serum testosterone slightly reduced	Testicular dysfunction (~65%) with low or low-normal testosterone levels, and oligospermia (59)
Ovary	No consistent evidence of abnormality; high fetal loss	Increased pregnancy loss reported (60)
Pituitary	Increased FSH levels; slightly increased LH levels; increased LHRH response Hyperresponsiveness to exogenous growth hormone <sup>a</sup>	Increased serum FSH
Pancreas	Increased insulin resistance Clinical diabetes <sup>a</sup>	Manifest diabetes (~25%; (59)) with insulin resistance
Thyroid	No evidence of abnormality; but myotonia sometimes found in hypothyroidism	Reported but probably coincidental
Adrenal; parathyroid	No consistent abnormality found	

DM1, myotonic dystrophy type 1; DM2, myotonic dystrophy type 2. FSH, follicle-stimulating hormone; LH, luteinizing hormone; LHRH, luteinizing hormone-releasing hormone.

<sup>a</sup>Rare or inconsistent abnormalities.

genetics of DM1 and is discussed in more detail later in the chapter.

Clinical features of congenital DM1 have been extensively reviewed in Harper's book (3). The hallmarks of the congenital form are generalized hypotonia and facial diplegia. Other features include jaw weakness with respiratory and feeding difficulty in neonates, and intellectual disability is common in those who survive the neonatal period. Talipes and arthrogryposis are frequent, and a variety of other features suggesting intrauterine onset of muscle dysfunction occur, such as hydramnios, diaphragmatic hypoplasia and thin ribs, as well as poor fetal movements. Many affected infants die soon after birth from respiratory problems and many neonates are not diagnosed rapidly during early life.

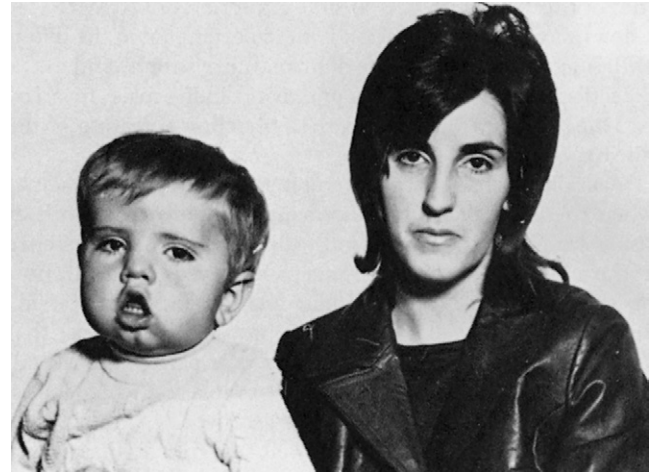
Infants and young children with the congenital form of DM1 do not show clinically evident myotonia, although some myotonic potentials are usually visible electromyographically. A young child with myotonia is much more likely to have one of the non-dystrophic myotonic disorders than DM1. The differential diagnosis of congenital DM1 includes other causes of infantile hypotonia, such as congenital myopathies, spinal muscular atrophies, Pompe's disease, and various CNS disorders, including cerebral palsy. The X-linked neonatal myotubular myopathy is particularly likely to be confused, although there are clear differences in muscle histology and molecular diagnosis is differentiating (see Chapter 127).

The facial diplegia and characteristic "tented mouth" should permit instant suspicion of congenital DM1 in many cases (Figure 130-4) and becomes even more characteristic as the child grows older (Figure 130-5). Some patients may have few neonatal symptoms but may present in later childhood with mental retardation or speech problems. By the second decade of life, myotonia is usually clinically apparent and the progressive "adult" features begin to appear. Many affected children appear to improve markedly during their first decade. In the original study by Harper (45), only two patients with congenital DM1 had died after the neonatal period and no survivor had failed to walk, although at least two-thirds were

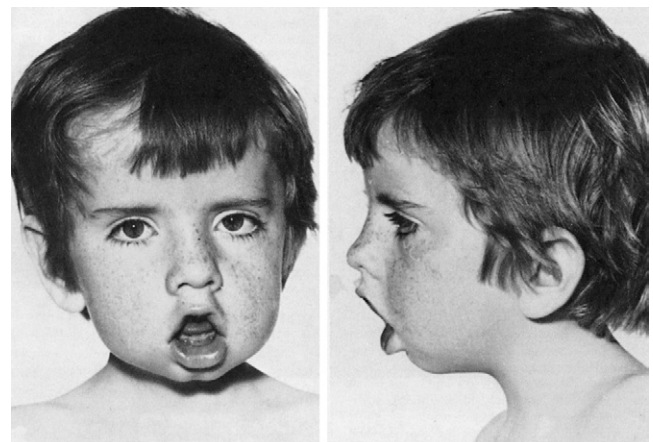
significantly cognitively impaired. Brain MRI of congenital DM1 patients typically shows ventriculomegaly and moderate/severe hyperintensity of the posterior white matter at all ages. The duration of assisted ventilation during the neonatal period is a significant prognostic factor for subsequent morbidity and developmental delay (46).

The combination of a relatively good prognosis for life with a poor prognosis for mental function requires careful consideration both when genetic counseling is given to affected women and when resuscitative measures are being attempted in a newly diagnosed congenital case. The long-term outlook for patients with congenital DM1 is poor for living an independent life and employment. Physical deterioration and complications may accelerate mortality in those older than 30 years.

The childhood onset DM1 is in many ways intermediate between the congenital and adult-onset types (47). Cognitive and behavioral problems are prominent in this



**FIGURE 130-4** Congenital myotonic dystrophy. Patient with his mildly affected mother. Note facial diplegia with "tented mouth."



**FIGURE 130-5** Congenital myotonic dystrophy. Same patient as in Figure 130-4, aged 6 years. Facial diplegia and jaw weakness persist.

**TABLE 130-9** Main Clinical Features of Congenital Myotonic Dystrophy Type 1<sup>a</sup>

Bilateral facial weakness
Hypotonia
Delayed motor development
Mental retardation
Neonatal respiratory distress
Feeding difficulties
Talipes
Hydramnios in later pregnancy
Reduced fetal movements

<sup>a</sup>Congenital form has not been reported in myotonic dystrophy type 2.



group, most notably with attention deficit hyperactive disorder and anxiety disorder (48), but the early perinatal problems are absent.

### 130.2.2 Clinical Manifestations of Myotonic Dystrophy Type 2/Proximal Myotonic Myopathy

Centers specializing in unusual myotonic disorders, notably, those of Ricker et al. in Germany (49) and Thornton et al. in the United States (50), were able to separate a group of patients with a DM1-like disorder, which does not show the CTG expansion. This disorder was named proximal myotonic myopathy (PROMM) initially because of conspicuous weakness and atrophy of proximal muscles (49). It soon became clear that the condition occurred in most Indo-European populations, although it seems to remain especially prominent in Germany (51).

There have also been reports of DM1-like families with no CTG repeat expansion, in which the distribution of muscle weakness and atrophy differed from PROMM. Families with one of these myotonic dystrophy-like diseases, designated myotonic dystrophy type 2 (DM2), were identified in Minnesota, and the DM2 locus was mapped to chromosome 3q (52). Subsequently, this locus was confirmed to be also the locus of PROMM in German families (53). Cross-examination of these families by German and American investigators led to the conclusion that DM2 is the same disease as PROMM. The genetic mutation of DM2/PROMM was found to be an expansion of CCTG repeats in intron 1 of the *ZNF9* gene (54), which accounts for the mutation in most PROMM and other atypical myotonic dystrophy-like families.

An autosomal dominant family with multisystemic myotonic myopathy with frontotemporal dementia was described with a new disease locus, proposed to be called “DM3”, and mapped to 15q21–q24 (55). These and other similar patients were subsequently found to be due to a mutation in the gene valosin-containing peptide (VCP) and mutations in VCP have been found to cause several myopathy phenotypes associated with a vacuolar myopathy and not myotonic discharges (56). There are also a handful of other families with multisystemic myotonic myopathies without DM1 or DM2 mutations and therefore “DM3” may still be described (2).

The main clinical features of DM2 are compared with DM1 in Table 130-3. In general, the disorder is relatively mild and slowly progressive, with proximal weakness and wasting predominant but not exclusive. There can be coexistent weakness in deep finger flexor muscles, mild facial involvement, and preservation of bulbar function and manual skills, and mild or clinically absent myotonia. Cardiac involvement is milder than in DM1, but arrhythmias are frequently detected. The atrioventricular block is infrequently found, but sudden cardiac deaths with histological cardiac abnormalities have been reported

(see Table 130-6) (2,57). Cataracts are present as with DM1 (see Table 130-7). In DM2, some endocrine abnormalities, such as testicular dysfunctions and insulin resistance, are as common as, but milder than, those found in DM1 (see Table 130-8). Frontal executive dysfunction (28) is reported, but the social and cognitive abilities are typically mild, and hypersomnolence and mental retardation are not prominent findings in DM2 (2). Severe muscle and joint pain, and calf muscle hypertrophy, may further differentiate DM2 from DM1. Gastrointestinal involvement has not been systematically studied, but DM2 patients often have similar but milder symptoms than in DM1. Muscle pathology is also comparable in some cases, although type 2 fiber atrophy, “denervation-like” changes, and the absence of sarcoplasmic masses are characteristic of DM2 (Table 130-10) (2,58). Serum creatine kinase and gamma glutamate transferase levels frequently show a mild increase (2,59). Wider features such as deafness and hyperhydrosis may occur (51,60), whereas some patients with the DM2 mutation show only subtle and nonspecific clinical signs.

Inheritance of DM2 is autosomal dominant, but anticipation may not occur and age of onset may not be related to the size of the quadruplet repeat expansion. No congenital or severe childhood cases have been recorded in families to date. It is noteworthy that even rare patients with homozygous CCTG repeat expansions show no congenital phenotype (61). There are no clear effects of the gender of the transmitting parent on the offspring’s phenotype (2,60).

### 130.3 DIAGNOSTIC INVESTIGATIONS OF MYOTONIC DYSTROPHY TYPES 1 AND 2

Molecular genetic analysis (see full description later) is now the most significant test in relation to both diagnosis and prediction, because it can be conveniently undertaken on a blood sample. Polymerase chain reaction (PCR) and Southern blot analyses are primarily

**TABLE 130-10 Muscle Histology in Myotonic Dystrophies**

Characteristic Features	DM1	DM2
Increased central nuclei	+	+
Nuclear chains	+	±
Ring fibers	+	+
Sarcoplasmic masses	+	–
Atrophy of type 1 fibers	+	–
Atrophy of type 2 fibers	–	+
Rimmed vacuoles	–	+
Small angular fibers	±	+
Moth-eaten fibers	±	+
Hypertrophy of type 2 fibers	±	±
Increased fibrosis	+	+

DM1, myotonic dystrophy type 1; DM2 myotonic dystrophy type 2.  
+, frequent; ±, infrequent; –, not typically seen.



used to detect *DM1* and *DM2* mutations. Repeat-primed PCR allows for quick detection of expansion mutations, although it does not determine the expansion size (62). In *DM2*, conventional PCR/Southern-blot protocols detect the *DM2* mutation in only 80% of subjects with known expansions. Thus, an additional *DM2* repeat assay (RA) that consists of amplifying the CCTG repeat by PCR and probing the resultant product with an internal probe is recommended to ensure greater than 99% specificity and sensitivity for known expansion (2,59). The diagnostic values of in situ hybridization-based analyses for the *DM2* mutations have also been investigated (63).

A clinical diagnosis should also be made in symptomatic patients, as some patients now achieve a genetic diagnosis before developing clinical symptoms. Muscle biopsy is no longer a primary means to diagnose *DM1*, although it provides valuable samples for research and may still have been performed by clinicians who suspected a myopathy but not *DM1/2*. A striking muscle immaturity seen in congenital *DM1*, with centrally placed nuclei and abundant satellite cells, provides a valuable distinction from other congenital neuromuscular disorders, especially X-linked myotubular myopathy, in which satellite cells are markedly reduced.

Stored frozen muscle samples should also be remembered as a useful source for DNA analysis in deceased patients when relatives are requesting prediction. Muscle is of special importance in allowing confirmation or exclusion of the congenital form in material from patients who have died.

## 130.4 GENETICS

### 130.4.1 Genetic Aspects of Myotonic Dystrophy Type 1

Early studies established an autosomal dominant inheritance pattern in *DM1* but highlighted the remarkable variability in phenotype and age at onset. Although our understanding of the genetics of anticipation and congenital *DM1* has remarkably increased, there are still unsolved questions with these phenomena. Furthermore, the genetics of *DM2* is distinct in many aspects from *DM1* in spite of the fact that both diseases are caused by repeat expansion mutations. Several detailed reviews are available (64,65).

**130.4.1.1 Gene Mapping of Myotonic Dystrophy Type 1.** The identification of the *DM1* gene was the result of pure gene mapping and positional cloning research, carried out over a period of almost a decade. After genetic linkage to the secretor and Lutheran blood group in 1971 and assignment to chromosome 19 in 1982, the gene locus had been narrowed down to a restricted region of 19q, which underwent progressive physical mapping and cloning (reviewed in reference (3)).

**130.4.1.2 Unstable DNA Sequence in Myotonic Dystrophy Type 1.** The specific change, a DNA expansion mutation, was recognized at the end of 1991 (66,67),

and was rapidly confirmed (68). After the discovery of the CAG expansion in Kennedy's disease and the CGG repeat in fragile X syndrome, the CTG repeat expansion in the 3'UTR of the *DMPK* gene was identified as the myotonic dystrophy mutation (69–71), which is now known as the *DM1* mutation.

#### 130.4.1.3 Anticipation and Myotonic Dystrophy

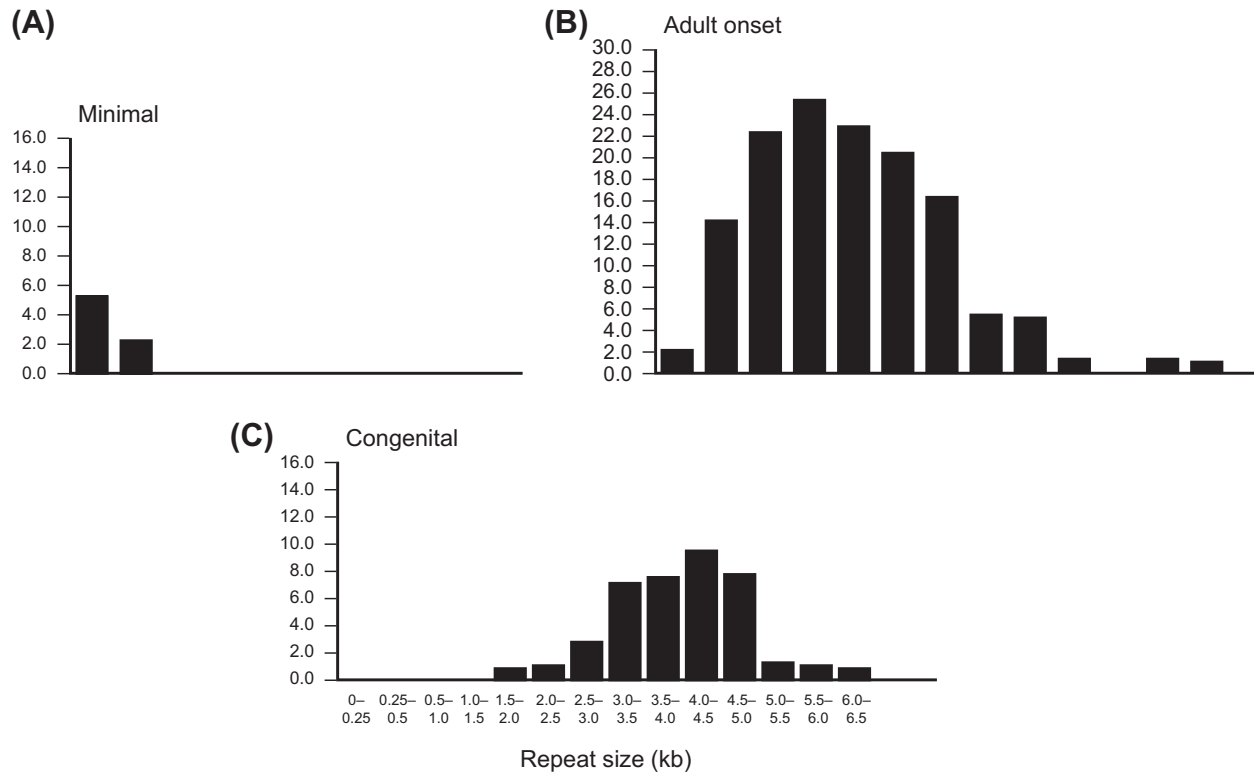
**Type 1.** The suggestion that *DM1* might occur with earlier onset and progressively more severe course in successive generations was proposed as long ago as 1918 by Fleischer, who found that patients with muscle disease could frequently be linked with past generations through individuals showing cataract as the only clinical manifestation. Although other clinical and family studies of myotonic dystrophy supported this, the analysis presented by Penrose (72) disputed anticipation as a result of the inherent ascertainment biases, together with the extreme intergenerational variability of myotonic dystrophy, which he suggested might result from effects of the opposite allele.

The fascinating story of how anticipation was recognized, forgotten, and then rediscovered was described by Harper et al. (73). By 1989, it had become clear from the work of Howeler that the biases postulated by Penrose were inadequate to explain anticipation (74), whereas the similarity with fragile X mental retardation had also been recognized. Anticipation is now recognized as a major criterion for any disorder attributed to an expanded trinucleotide repeat and has been validated in several independent analyses of *DM1*. Most of the apparent anticipation susceptibility reported in a wide range of other disorders may still reflect the biases documented by Penrose many years ago (75).

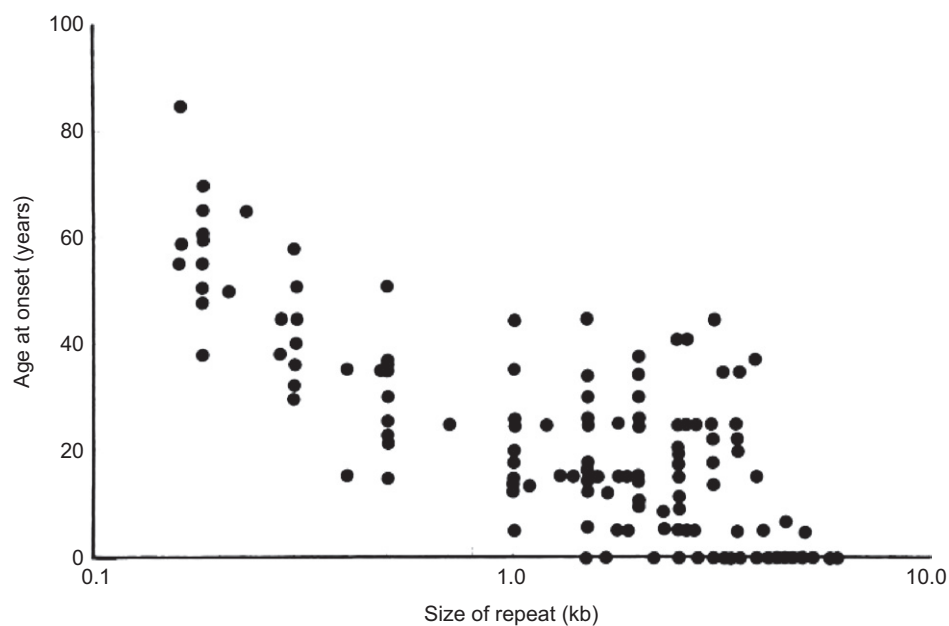
#### 130.4.1.4 The *DM1* Mutation: Clinical–Molecular

**Correlations.** Once the expanded CTG repeat sequence had been identified as specific for *DM1*, the question immediately arose as to what extent its variability could be correlated with severity and clinical phenotype. Correlations have been confirmed in numerous independent series (reviewed in references (3,65,76)). Figure 130-6 shows the relationship of severity and molecular defect when patients are classified into the three broad groups: severe childhood disease, classic adult-onset *DM1*, and minimal disease of later life. The overall difference between the groups is clear, but there is considerable overlap between the categories and the range of expansion for each group, making considerable caution necessary in using this information for prognosis in an individual case. Gennarelli et al. (77) have found that repeats of less than 100 were rarely associated with significant neuromuscular disease. When age at onset is used as the criterion instead of repeat size (Figure 130-7), a comparable correlation is seen. This finding would be expected in view of the close relationship between age at onset and severity of clinical disease.

The extremes of the distribution for age and severity are of particular interest, as among the most severe cases of congenital onset, expansions of a remarkable degree



**FIGURE 130-6** Correlation of phenotype and genotype in myotonic dystrophy. The three main clinical groupings in myotonic dystrophy are shown, together with the range of expansion in the CTG repeat. (A) Minimally affected individuals; (B) Classic phenotype with adult-onset; (C) Severe congenitally affected patients. Note the overlap between groups, even though the mean values are clearly distinct. (*Cardiff series; Data from Harley, H. G.; Brook, J. D.; Rundle, S. A., et al. Expansion of an Unstable DNA Region and Phenotypic Variation in Myotonic Dystrophy. Nature 1992, 355, 545–546 (67).*)



**FIGURE 130-7** Correlation of age at onset with size of expanded CTG repeat in myotonic dystrophy. (*Cardiff series; Data from Harley, H. G.; Brook, J. D.; Rundle, S. A., et al. Expansion of an Unstable DNA Region and Phenotypic Variation in Myotonic Dystrophy. Nature 1992, 355, 545–546 (67).*)

may occur, sometimes exceeding 2000 repeats (78). Among the minimally affected cases, particularly when these are restricted to those older individuals with cataracts alone, there is a consistently small expansion. The smallest expansions of all are seen in those parents in the older generation who are entirely normal clinically, but in whom one member of a couple consistently showed an expansion of 38–49 copies, compared with the normal range of 5–37 copies. Such individuals can be considered premutations, comparable to the normal male gene transmitters seen in fragile X syndrome. These cases are of particular significance in relation to the origins and persistence of the disease, as will be considered later. An extensive study of premutations from Spain has shown that no individual with less than 55 repeats showed any clinical abnormality, but that there was significant meiotic instability in the range of 30–55 repeats, especially when transmitted by men (79).

**130.4.1.4.1 Intergenerational Changes.** As already noted, major anticipation had long been recognized in DM1. The relationship between age at onset in parent and in offspring of DM1 families, noted by many previous studies extending back as far as that of Julia Bell in 1947, shows a corresponding relationship for the molecular defect (reviewed in References (3,65,76)). It is clear from these and similar data presented by others that the genetic instability of the DM1 mutation is likely to be the major factor underlying the observed anticipation.

For both the age at onset data and the molecular expansion, a small proportion of the parent–child pairs not only do not show anticipation but show later onset and smaller repeat sequence in offspring than in the parent. Pooled data from a number of centers have allowed this observation to be confirmed and extended (80), and it seems that about 6% of transmissions show a decrease in size of the repeat sequence, although the likely selection biases involved in the study of two-generation families make it difficult to attain precision. It is also of interest that in some of the cases in which the sequence decreased in size, anticipation was still observed in terms of earlier age at onset, although again this observation could be biased. A final point of relevance regarding those transmissions showing a decrease of repeat size is that they occur primarily during male meioses, an observation that leads to discussion of the important sex-related differences in transmission of the DM1 mutation.

**130.4.1.4.2 Parental Origin Effects.** Although DM1 behaves as a classic autosomal dominant trait in respect of equal incidence in, and transmission by, each sex, it has long been noted that this basic equality masks important sex differences in terms of transmission of the disorder. The most striking feature, mentioned earlier and discussed more fully later, is the observation that almost all congenital cases originate from an affected mother. Less obvious but of equal importance is the preponderance of men in the grandparental generation of

such congenital cases and in the earliest affected ancestors, usually showing only minimal disease (81,82).

These observations have now been confirmed and extended at the molecular level (reviewed by Harper (3)), although a number of aspects remain to be resolved. First, as already mentioned, it is the severe congenital cases that show the largest repeat sequences, some of which may be extreme. Paternal inheritance of congenital DM1 has been described but is very rare (83,84). Most cases in which the sequence has reduced in size are paternal in origin and putting these two observations together suggests an upper limit to expansion during male meiosis that is not present during female meiosis. This may be an important factor in explaining the maternal transmission of the congenital form. The study of those patients with minimal disease has confirmed its predominantly male origin in the earliest available ancestors (81,82). Most of the minimally affected progenitors have cataracts and are men. In those cases in which both parents are entirely normal, it has been the male parent in most instances (around 70%) who has shown the presence of a “pre-mutational” expansion. These data suggest a paradox at first sight such that there appears to be predominant male transmitted expansion and anticipation at the minimal end of the severity range, as compared with predominant female-transmitted expansion at the severe end (see Reference (65)).

The considerable degree of infertility and oligospermia found in many men with DM1 is a complicating factor, but it is clear that the degree of repeat sequence expansion seen in sperm differ considerably from that found in blood (85,86). Small pool PCR-based analysis of sperm from men with DM1 showed high levels of repeat-length variation. The length variation was heavily biased toward further expansion, with the largest length changes observed for premutation and protomutation alleles and the highest frequency of contractions was found in full mutation alleles (85). Embryos and gametes obtained during preimplantation genetic diagnosis have shown that there were significant increases in the number of repeats in embryos from female patients with DM1 and in their immature and mature oocytes, whereas, in spermatozoa and embryos from male patients, smaller increases were detected (87). These data are consistent with the parental origin effect observed in the intergenerational changes of the repeat size. Interestingly, the size of expanded allele in sperm in two male patients with DM1 did not change in 4 years. A study of siblings with DM1 showed that birth order, intergenetic interval, oldest sibling’s CTG repeat, parental age or parental CTG repeat size did not exert any significant influence on siblings’ genotype or phenotype (88). In oocytes, the enlargement of the repeat had occurred at the germinal vesicle stage, that is, either during premeiotic proliferation of oogonia or during prophase I of meiosis I (87). This implies DNA repair-based expansion of the repeat in these cells that have undergone a long period of quiescence, as proposed by Pearson (89).

**130.4.1.4.3 Molecular Basis of Congenital Myotonic Dystrophy Type 1.** The parent-of-origin effects described earlier go some way to explaining the severity and maternal transmission of the congenital form of DM1. On average, these cases show the largest expansions in the gene, whereas a combination of diminished male fertility and possible selection against sperm carrying very large expansions could explain the maternal transmission. A further aspect that has been clarified is why congenital cases almost always arise from mothers who either have, or will later develop, classical adult DM1, and only very rarely from those with mild late-onset disease such as cataracts alone (90). This can now be explained by the limitation of intergenerational change in the expanded sequence, so that it is unusual for a change sufficient to produce the congenital form to occur without an intervening generation. Congenital cases can be separated more clearly from others if the size of the parental repeat is taken into consideration, as well as that of the patient alone (reviewed by Harper (3)).

Despite these advances, a number of aspects of congenital DM1 remain without satisfactory explanation. The range of repeat size in this group shows extensive overlap with those showing no congenital or even childhood onset. The size of parental expansion may explain the origin but not the congenital onset itself, unless there is some more direct effect of parental phenotype on the disease in the offspring. The suggestion of an intrauterine maternal effect, proposed many years ago but never validated is still not entirely obsolete in this respect.

DNA from congenital DM1 patients showed complete methylation of restriction sites surrounding the CTG repeat (91), and methylation prevented binding of the zinc-finger protein CTCF to two sites on the DNA flanking the CTG repeat (92). These two sites formed insulators, and CTCF binding to these sites prevented promoter–enhancer interactions. Therefore, the DM1 locus methylation in congenital DM1 would disrupt insulator function. The effects of other loci interacting with the DM1 gene could also be relevant. Mitochondrial dysfunction has been suggested, but no molecular mitochondrial defect has been shown (93).

Defective muscle differentiation during the development has been well documented in studies of the skeletal muscle and myoblasts from subjects with congenital DM1 (92,94). However, the molecular mechanism through which the very large CTG repeat causes this muscle immaturity remains unknown (discussed later). In recent studies, cell-culture and transgenic mouse models overexpressing the 3'UTR of the *DMPK* gene showed defective muscle differentiation (95,96). Furthermore, this transgenic mouse model developed muscle atrophy and, later in life, hypertrophic cardiomyopathy, myotonic myopathy and hypotension traits (97). The overexpression of the *DMPK* 3'UTR may therefore cause features that resemble congenital DM1.

Further studies in muscle and other tissues will be needed to elucidate the molecular mechanisms leading to the congenital DM1 phenotype.

**130.4.1.4.4 Homozygosity for the DM1 Gene.** Molecular analysis has shown at least seven homozygotes for the minimal expansion mutation (98) that appear to be healthy. In contrast, two siblings carrying 43/180 and 43/500 CTGs showed an adult-onset phenotype (99). Neither of these cases had two fully expanded alleles, so it is not yet clear whether homozygotes generally will differ from heterozygotes, as has now been shown for Huntington's disease (100).

**130.4.1.4.5 Reversal of the Myotonic Dystrophy Type 1 Mutation.** Reduction of the DM1 mutation to the normal range has been observed in several individuals (81,82,101) who were detected as a result of discrepancy between a haplotype analysis suggesting transmission of the DM1 gene and a normal mutation analysis result. All cases so far have been of paternal origin, and it is not clear whether the process reflects a selection of sperm in the parent or some more direct molecular change in the early embryo. These cases are of practical importance in risk prediction for such individuals and their families, although it must be stressed that true non-penetrance of the gene in the offspring of an established case is exceptionally rare.

**130.4.1.4.6 Somatic Instability and Mosaicism.** From the earliest observations, it was clear that somatic instability was also a feature of the DM1 mutation, with analysis of blood leukocytes showing the expanded sequence as a “smear” or diffuse band (67). This suggests a range of fragment sizes, rather than the usual sharply defined band. This phenomenon has now been explored in greater detail (80,86,102) and occurs generally in trinucleotide repeat disorders, in particular those characterized by extreme expansions.

All the initial molecular studies relied on blood samples, and therefore the important question arose as to what extent tissue variation in the repeat sequence occurred. Autopsy studies have shown significant variation, whereas analysis of muscle biopsy samples has suggested that greater expansion of the repeat is generally seen in muscle than in blood (65); however, there is also greater variability. The repeat size in blood shows a closer correlation with clinical severity than that in muscle. This may reflect the inevitably localized origin of a muscle sample compared with a constantly circulating blood sample. It has the practical implication that blood probably remains the most secure guide to prognosis, insofar as it is wise to attempt this at all. In terms of prognosis, for the next generation, it may well be that sperm analysis will prove of practical relevance when methods of single sperm analysis become more generally available, given the differences between somatic tissues and germline.

A second field of relevance in relation to somatic instability is the question of change over time.



Those who show most spread in expansion are generally older and are relatively severe cases. It was noted early on that this was neither shown by older minimal cases, nor by chorion villus prenatal samples showing very large expansions (103). Analysis of blood samples taken at intervals of several years (104) has confirmed increased spread of repeat size in later samples, although no overall size increase was seen in muscle biopsy samples taken at intervals. These studies also illustrate a third relevant factor, that somatic variability is related to the initial size of the expansion, being greater for large expansions than for small expansions over a prolonged time period. What relationship such somatic change will have to the pathogenesis of the disease in different tissues and in different types of the disease is a point of considerable interest which future work should clarify.

True mosaicism usually implies the existence of clearly separate genetic cell lines in a single individual, as seen in cytogenetic disorders and in an increasing number of genetic conditions in which it is of germline origin, with a mixture of normal and abnormal tissue. Although individuals with fragile X syndrome showing clearly separate lines have been identified, this is not the main feature of trinucleotide repeat disorders, in which somatic instability is usually reflected in a more general spread of variation and in tissue heterogeneity. Any true mosaicism would be expected to result from early embryonic rather than germline variation. Monozygotic twins give some indication of the likely extent and role of such development differences and affected twin pairs generally show close similarity of both clinical features and repeat size.

**130.4.1.4.7 Mechanism of Instability of the CTG Repeat in Myotonic Dystrophy Type 1.** The molecular mechanism of changes in the CTG repeat length has been investigated by in vitro and in vivo models. Mouse models expressing a transgene containing expanded CTG repeats have proved to be particularly effective because they could replicate both germline and somatic instability with a strong bias toward expansions and the sex- and size-dependent characteristics (105–108). The size of expanded CTG repeats also showed age-dependent increases in somatic tissues of these animals as shown in humans (108), but there have been no mouse models exhibiting a “big jump” of the CTG repeat toward further expansion into the congenital DM1 range. Nevertheless, these mouse models provided data that cannot be easily obtained in human samples regarding the instability of the CTG repeat. Examinations of germline tissues in these animals showed that strong mosaicisms toward expansions were already observed in spermatogonia before meiosis, and no significant difference in mosaicism was detected between spermatogonia and spermatozoa, arguing against continued expansions during postmeiotic stages (109). These observations are in agreement with the human data suggesting that germinal expansions are produced at the beginning of spermatogenesis by a meiosis-independent mechanism. Another important finding

derived from these mouse models is that the repeat-length mutations appear to occur independently of DNA replication. There is no correlation with the levels of somatic mosaicism observed and cell turnover rates between tissues, and the brains of adult mice increase the degree of mosaicism in spite the post-mitotic state of neurons by four months of age (105). More recent studies using transgenic models have suggested that genes controlling DNA mismatch repair (such as Msh2, Msh3, and Msh6) play a critical role in the mutation process (110). Crossing mice transgenic for expanded CTG repeats with Msh2-deficient mice suggested that the absence of Msh2 shifts the instability toward contractions, both in tissues and through generations (111). Although Msh3 deficiency completely blocked the somatic instability, a lack of Msh6 resulted in a significant increase in the frequency of somatic expansions. Competition of Msh3 and Msh6 for binding to Msh2 in functional complexes with different DNA mismatch-recognition specificity may explain these data (110). CTG repeats are known to be able to adopt unusual secondary structures, such as “hairpins,” that differ from the usual double helical (B-DNA) structure (89). These unusual structures may be targets of mismatch repair.

**130.4.1.4.8 Origins of the Myotonic Dystrophy Type 1 Mutation.** Numerous family and population studies of DM1 were carried out before identification of the gene and mutation, which attempted to analyze mutation rate and related aspects such as genetic fitness, prevalence, and geographic variation. All were fraught with difficulty because of the extreme clinical variability of the disorder (38). Now that the unusual mutational mechanism underlying DM1 is understood, we can see why these early studies encountered such problems and can also begin to explain some of the apparent anomalies. The discussion here focuses on two separate but related questions: the origins of clinically identifiable cases of the disorder, and the ultimate origin of the initial expansion in the DM1 repeat sequence.

Regarding the origins of the clinical disorder, the validation of anticipation as a biological process has already been discussed. Clinically significant cases of DM1 do not arise de novo, but from a parent who has an expanded repeat sequence; usually, but not always, showing some minimal clinical features such as relatively early onset cataracts in comparison to senile cataracts. When the parents of patients with mild phenotypes are assessed and are found to be normal, one (usually the father) has always shown to have an expansion. It is these clinically normal individuals, usually with 38–49 repeats, who provide the link between different DM1 families so frequently shown by genealogical studies (81,82). The demonstration that unselected patients with cataracts do not show a markedly increased frequency of small expansions in the DM1 gene (112,113) makes it unlikely that those ancestors carrying small “premutation” expansions are or were clinically abnormal, which is a point

of considerable significance in relation to possible prevention in a population. The extent to which minimally affected individuals and normal premutation carriers are included in any study of genetic fitness and fertility will obviously have a profound influence on the results. It is also highly relevant to the question of why the disorder does not die out, as discussed later. How far back the instability of the expanded DM1 repeat extends, and to what degree, is an important question that remains largely unanswered.

Detailed analysis of a very large Dutch kindred studied over many years has shown that increased repeat size, clinical anticipation, and eventual elimination of the gene occurred in all branches within five generations (114). Premutation alleles identified both in distant relatives of probands and, more rarely, in unaffected spouses were found to be highly unstable and liable to expand in succeeding generations, particularly when transmitted by a man (79). These data suggest that premutation carriers are at high risk of having affected offspring within a limited number of generations, leading to elimination of the mutation by anticipation. Although there has been a report of a DM1 family in which the number of CTG repeats remained in the minimally expanded range through at least three, and possibly four, generations (115), premutation alleles within most DM1 families may not be the long-term source of new DM1 families. Interestingly, alleles within the high end of the normal size range have shown occasional expansion-biased instability during paternal transmissions (79). When they are put together, these data support the theory that the prevalence of DM1 is maintained by expansion of the high end of normal-size alleles. There is general agreement that the immediate origin of DM1 in families is incremental, with the disorder evolving gradually in successive generations across a threshold of repeat size producing clinical abnormalities.

Turning to the ultimate origin of the mutation, the situation is entirely different. Family and population studies in widely different countries, especially isolates or founding populations, such as those conducted in Quebec, northern Sweden, South Africa and Istria, had already shown an origin of the disease from one or very few ancestors—which is a conclusion supported by genetic marker studies. The discovery of linkage disequilibrium in more mixed populations (116) suggested that origin of the disease worldwide might be from a small number of ancestors. This view is supported by the finding that the DM1 expansion is not only the basis of the disorder worldwide but is exclusively associated with one allele of an insertion deletion polymorphism at this genetic locus.

Studies of the distribution of normal repeat sizes at the DM1 locus in different populations have shown considerable differences. It has been suggested that the frequency and origins of the DM1 mutation in a population might be related to such distributions, possibly with a higher frequency of larger sizes predisposing to a high

frequency. It has been shown that the haplotype associated with DM1 is also that found most commonly in individuals with a repeat number of five, together with those less common individuals showing repeat number in the upper range, but not those in the intervening range of repeat numbers. This has led to the hypothesis that the original mutational event was a jump from a repeat number of five to some value in the upper range, rather than a gradual increase through the intervening range (117). The shared worldwide haplotype indicates that this could have been a unique event (118), rather than the occurrence of a predisposition to instability on multiple occasions. This view has been challenged in the light of more general population studies of the distribution of CTG repeat lengths, suggesting that the occurrence of a group with high repeat numbers may antedate the origin of the insertion deletion polymorphism (119).

The geographic variations in current prevalence of DM1 are relevant to these questions. Although most differences can be attributed to relatively recent founder effect or to thoroughness of ascertainment, there is a striking absence of the disease in sub-Saharan African populations, even when carefully studied (120). This could reflect the different normal pattern of repeat sizes (121), in particular a relative deficiency of high repeat number alleles. It could also be due to the original mutation for DM1 having occurred after the divergence of African from Oriental and Caucasoid populations. The resolution of this question will be of considerable interest from a general anthropological angle, as well as for DM1. The re-evaluation (122) of a Nigerian patient, originally reported by Dada et al. (123), showing a CTG expansion on a different haplotype, is of considerable interest. In eastern (Asian) Indians patients with DM1, expanded CTG repeats have been found in yet another haplotype background, which is uniquely prevalent among the Indian population (124). The exceptional nature of these cases does not itself disprove a single origin of the mutation over most of the world. An issue that has repeatedly arisen in discussion of the origin and spread of DM1 is why, if its mutational origin has been so restricted and its subsequent effects in established cases are so deleterious, it has persisted as a relatively common disorder. In part, this is likely to be because of the considerable pool of normal individuals with repeat sizes near the upper limit of normal, who can act as a reservoir for the future origin of new cases through genetic instability, a process now validated in a recent study of premutations (79). In this context, the expansion into the upper range of normal repeat size, instead of the expansion into the lower abnormal range, must be considered the original mutational event of DM1 that took place after the migration out of Africa.

It is also possible, however, that additional factors are operating, one of which has been suggested to be “meiotic drive,” which is the term given to the preferential transmission at meiosis of a particular allele at a

given locus. Evidence for this has now been put forward in two studies, with preferential transmission in normal individuals of the chromosome carrying the larger repeat size at the DM1 locus (125,126). The data are inconsistent in regard to sex of transmitting parent, and reanalysis has not shown any evidence for abnormal segregation. Similar suggestions for an abnormal segregation of the expanded allele in DM1 families (83,127,128) and in transgenic mouse lines (107) have been reported; however, data from prenatal molecular studies, which are not subject to ascertainment bias, showed no evidence of meiotic drive (129).

The prevalence of the disorder in relation to other muscular dystrophies warrants mention. If the exceptionally high frequency in some isolated populations and the apparent deficiency in those of African origin are ignored, a range of estimates of 2.5–13.5 per 100,000 has been obtained in a series of European studies (summarized by Harper (130)). Given the difficulties of complete ascertainment, this suggests a broad prevalence for clinically significant cases of around one in 10,000, a higher figure than for other autosomal dystrophies, and comparable to that of Duchenne muscular dystrophy in prevalence, although lower in incidence. To explain the high prevalence of DM1, it now seems clear that the source of new cases lies principally in those clinically normal individuals with subclinical expansion in the gene, not in individuals with cataract or other mild clinical features where inter-generational disease transmission is self-limiting because of the severity of anticipation.

### 130.4.2 Genetics of Myotonic Dystrophy Type 2 (Proximal Myotonic Myopathy)

The clinical phenotype of DM2/PROMM closely resembles that of DM1. The identification of the DM2 mutation indicated that the genetic mutations are similar in these two disorders, in that both are caused by large expansions of an unstable repeat that contains transcribed but untranslated CUGs. The DM2 CCTG repeat varies in copy number in the general population, ranging from approximately 10–30 repeats. The range observed in patients is much larger than in DM1, spanning from 75 to more than 11,000 repeats, with an average of approximately 500 (54,59). Subsequent studies have suggested that DM2 shares at least a part of the pathogenic mechanism of DM1, such that the mutation exerts a *trans*-dominant RNA gain-of-function. The clinical and molecular genetics of DM2 have demonstrated some important distinctions. Anticipation has been described (59,131), but is not striking. Neither severe congenital form of DM2 nor obvious parental origin effects have been identified. Nonetheless, the DM2 repeat shows a high degree of intergenerational repeat-size instability with a tendency for contraction, striking somatic repeat-size mosaicism, and rapid expansions throughout the lifetime of the individual (54,59). The most consistent

correlation with repeat size was the age of the patient at the time of blood sampling. These very high levels of instability may have hampered attempts to analyze an intergenerational bias, parental origin effects, and the correlation between the repeat size and the age of onset.

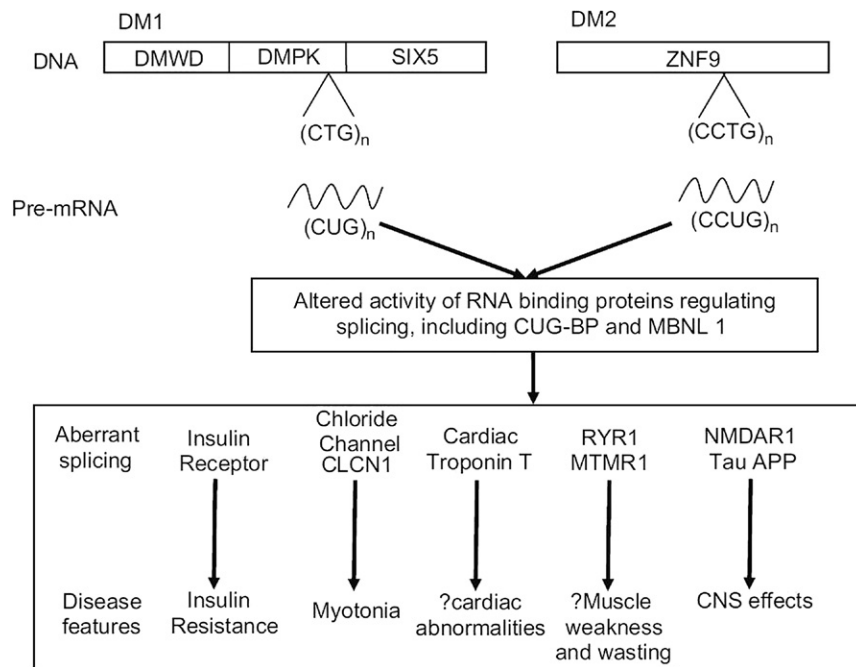
All DM2 families identified to date are from northern Europe, primarily Germany and Poland as well as American families with ancestors from these regions (59). An Afghan family has been described (61,132). The DM2 mutation has not been identified in sub-Saharan or east-Asian populations. The prevalence of the DM2 mutation in Germany has been estimated to be at least 1/100,000 (133). Recent haplotype and linkage-disequilibrium analyses of these families suggested that the DM2 expansion originated from a single or a few founder mutations (132,134). These studies estimated that the DM2 expansion mutation has arisen 200 to 540 generations ago (134), possibly before the Aryan migration of Indo-Europeans (132).

## 130.5 MOLECULAR AND CELL BIOLOGY

### 130.5.1 Molecular Pathogenesis of Myotonic Dystrophy Type 1

The mechanism through which the expanded CTG repeat leads to the multisystemic clinical phenotype of DM1 is still under investigation. The CTG repeat is located in the 3' untranslated region (3'UTR) of the *DMPK* gene and therefore the coding information of this gene remains intact in the mutant *DMPK* gene. The CTG repeat is transcribed into the messenger RNA (mRNA) as an identically sized CUG repeat. Although mechanisms of the majority of genetic diseases involve alterations of the protein product, this appears not to be so in DM1 and 2. The expanded CTG repeat gives rise to a mutant *DMPK* mRNA that contains an abnormally elongated CUG repeat. The expanded CTG repeat may also affect the mRNA levels of neighboring genes and the *DMPK* gene. Three major models of disease mechanism have emerged for DM1: (1) a model based on a gain of toxic function by the expanded CUG repeat in the mutant *DMPK* mRNA, (2) a model based on the loss-of-function of the genes in the vicinity of the CTG repeat, including *DMWD*, *SIX5*, and *DMPK* itself (Figure 130-8) and (3) haploinsufficiency of the *DMPK* protein, although there is little evidence for this hypothesis. The evidence for a toxic gain-of-function has been most compelling in recent years.

**130.5.1.1 Gain-of-Function by the Mutant *DMPK* mRNA.** DM1 is an autosomal dominant disease and therefore, even if the expanded CTG repeat totally suppressed the expression the *DMPK* protein from the mutant gene, the *DMPK* protein would still be produced at 50% of the usual level from the normal allele. For most genes, 50% is a sufficient amount to support normal cell functions. If the mutant *DMPK* gene makes



**FIGURE 130-8** Summary of the molecular pathogenesis of myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2), a “spliceopathy.” (Adapted from Turner and Hilton-Jones (4).)

a toxic product, it can produce cellular dysfunction regardless of the level of the normal DMPK protein. Therefore, a gain-of-function is an attractive pathogenic model to explain the dominant inheritance of DM1. The CTG repeat is located in the 3'UTR and therefore the gain-of-function model would not work for DM1 through the DMPK protein; however, the expanded CTG repeat is transcribed into the mutant DMPK mRNA as a noncoding CUG repeat, making the RNA-based gain-of-function most plausible. The first observation to support such an RNA gain-of-function model was that the mutant DMPK mRNA accumulates in nuclear foci in DM1 cells (135). Krahe and coworkers (136) postulated that abnormal processing of the mutant DMPK mRNA could be the basis of the gain-of-function theory in DM1 (see Figure 130-8).

Experimental evidence to support this hypothesis came from cell culture models. Mouse C2C12 myoblasts that express a transgene with expanded CTG repeats in the 3'UTR showed most of the characteristics of molecular pathology seen in DM1, including nuclear retention of the transgene mRNA and inhibition of myoblast fusion and differentiation (137–139).

Timchenko et al. (140) identified CUG-binding proteins (CUG-BPs) that bind to the 3'UTR of DMPK mRNA and CUG-BPs accumulated in muscle of a DM1 patient (141). Subsequently, CUG-BP was shown to regulate splicing of cardiac troponin T (*cTNT*) (142), insulin receptor (*IR*) (143), muscle chloride channel (*CLC-1*) (144,145), and myotubularin-related 1 (*MTMR1*) (146) gene transcripts, and splicing of these transcripts is altered in the muscle from patients with DM1. Furthermore, CUG-BP was shown to target *C/EBP* beta, which

regulates expression of many genes, and DM1 muscles show increased translation of an inhibitory isoform of *C/EBP* beta (145). CUG-BP appears to be involved in splicing regulation of many other gene transcripts that contain the “UG” repeat motif in the targeted intron (147). It was postulated that splicing abnormalities could lead to altered proportions of normally occurring isoforms or the production of aberrant isoforms. Widespread splicing dysregulation of mRNA from many genes, mediated through dysfunction of splicing factors such as CUG-BP, may therefore cause the multisystemic abnormalities of DM1. Since this suggestion, a group of other neurological diseases with a similar postulated mechanism have been described and named the “spliceopathies” (148).

Soon after the discovery of CUG-BP, it became clear that there are other CUG repeat binding proteins, which have been named CELF proteins (147). Miller et al. (149) identified a different class of proteins designated triplet repeat expansion (EXP) RNA-binding proteins. *Muscle-blind*, the *Drosophila* homolog of EXP, is required for terminal differentiation of skeletal muscle and eye, and EXP is expressed during muscle differentiation in mice. In humans, the EXP proteins consist of three paralogs (MBNL1, MBNL2, and MBNL3) coded by different genes on chromosomes 3, 13, and X, respectively (150). In contrast to CUG-BP, which binds to single-stranded CUG's, EXP binds to double-stranded CUG repeats, which are generated when CUG repeats form a hairpin structure (149). A longer CUG repeat tract is more likely to form a double-strand hairpin, and the EXP binding increases when the CUG repeat tract is longer. A study using yeast-three-hybrid analysis showed that MBNL1 interacts with both CUG and CCUG repeats, whereas



these repeats are not the primary targets of CUG-BP (151). Furthermore, the EXP proteins, but not CUG-BP, are detectable in nuclear foci in DM1 muscle, myoblasts, and fibroblasts, where the mutant DMPK mRNA is accumulated (150,152,153). MBNL1 has been found to be an important determinant of nuclear foci formation and aberrant IR splicing in DM1 (154), whereas CUG-BP and other CELF proteins regulate the equilibrium of splice-site selection by antagonizing the facilitatory activity of EXP proteins on splicing of IR and cTNT (154,155). The authors also showed that CUG-BP levels were elevated in DM1 cells by mechanisms that were independent of MBNL1 and MBNL2 loss.

In DM1 brain, cortical and subcortical neurons also show nuclear foci of mutant RNA, sequestration of EXP proteins, and dysregulated alternative splicing (156). In brains of patients with DM1 and of mice transgenic for the human myotonic dystrophy region with expanded CTG repeats, tau, a protein that is involved in many neurodegenerative diseases, shows abnormal isoforms that aggregate (31,157). Alternative splicing of the NMDA-R1 may also cause some of the functional neurological affects observed in DM1 (156). The SLITRK proteins are expressed primarily in neural tissue and have been implicated in affecting neurite outgrowth. Recently, reduced expression levels of SLITRK 2 and 4 have been described in adult and fetal post-mortem brain and in motor neurons differentiated from mutant DMPK human embryonic stem cells. These cells also demonstrated reduced synaptogenesis and increased neuritogenesis (158). These studies suggest that the RNA gain-of-function also contributes to the CNS pathophysiology in patients with DM1.

The gain-of-function mechanism mediated by the mutant DMPK mRNA through binding of these proteins to the expanded CUG repeat could explain both the dominant inheritance and multisystemic nature of this disease. Studies of two genetic mouse models have provided convincing evidence that this mechanism plays a central role in the pathogenic mechanism of DM1. First, Mankodi and coworkers (159) established a transgenic mouse line that expresses the skeletal actin gene engineered to include an expanded CUG repeat tract in the 3'UTR. The phenotype in these mice consisted of robust electrical myotonia and clinical and histopathological features of myopathy, very similar to those seen in human DM1. Furthermore, these transgenic mice showed the abnormal splicing pattern of muscle chloride channel, *Clcn1* gene in a pattern similar to that seen in human DM1 muscle (144,145). Second, disruption of the mouse *Mbnl1* gene leads to muscle and eye phenotypes, and RNA splicing abnormalities that are characteristic of DM1 (160). A summary of the changes in mRNA splicing which have been described in skeletal muscle, cardiac muscle and brain are detailed in Table 130-11 (adapted from 147). The toxic gain-of-function of mutant DMPK mRNA may be mediated by toxic effects other than the

**TABLE 130-11** List of Exons Shown to Have Misregulated Alternative Splicing in DM1 Skeletal Muscle Heart or Brain

Tissue/Gene	Target	Reference
<b>Skeletal muscle</b>		
ALP	Exon 5a, 5b	(230)
CAPN3	Exon 16	(230)
CLCN1	Intron 2, exon 7a, 8a	(126,145)
FHOS	Exon 11a	(230)
GFAT1	Exon 10	(230)
IR	Exon 11	(207)
MBNL1	Exon 7	(230)
MBNL2	Exon 7	(230)
MTMR1	Exon 2.1, 2.2	(146)
NRAP	Exon 12	(230)
RYR1	Exon 70	(163)
SERCA1	Exon 22	(163,230)
z-Titin	Exon Z4, Zr5	(230)
m-Titin M-line	Exon 5	(230)
TNNT3	Exon fetal	(160)
ZASP	Exon 11	(230)
<b>Heart</b>		
TNNT2	Exon 5	(142)
ZASP	Exon 11	(231)
m-Titin M-line	Exon 5	(231)
KCNAB1	Exon 2	(231)
ALP	Exon 5	(231)
<b>Brain</b>		
TAU	Exon 2, Exon 10	(31,156)
APP	Exon 7	(156)
NMDAR1	Exon 5	(156)

mis-splicing of pre-mRNA. There is recent evidence of RNA transcriptional effects with NKX2-5 (161). Post-transcriptional effects may also occur since CUGBP1 has been shown to play a role in RNA stability (162).

The molecular mechanisms causing muscle wasting in DM1 are poorly understood. The aberrant expression of the ryanodine receptor (*RyR1*) with exon 70 exclusion in adult DM1 skeletal muscle may alter excitation-contraction coupling and promote muscle degeneration (163). A mouse model expressing expanded CTG repeats in the 3'UTR of *DMPK* has been demonstrated to develop a muscle wasting phenotype that was temporally correlated with increased CUGBP1 expression, suggesting that CUGBP1 may contribute to muscle wasting in DM1 (164). A DM1 mouse model with skeletal muscle over-expression of CUGBP1 has been shown to reproduce many clinical and molecular defects of DM1 muscle including muscle wasting and dystrophic histology, which has added support for the hypothesis linking increased CUGBP and dystrophy (165). This has not explained the mechanism by which mis-splicing causes muscle loss. It has recently been demonstrated that elevated MBNL-3 levels disrupts Myocyte Enhancer Factor

2 (Mef2)  $\beta$ -Exon Splicing and this is a potential mechanism contributing to myodegeneration in DM1 (166).

**130.5.1.2 Loss of Dystrophia Myotonica Protein Kinase Function.** Although loss-of-function theories are less attractive than a gain-of-function to explain autosomal dominant diseases, there are autosomal dominant diseases caused by “haplo-insufficiency.” Thus, DMPK deficiency was proposed as the first pathogenic model soon after the identification of the DM1 mutation. However, studies have reported conflicting results regarding DMPK mRNA levels in skeletal muscle and other tissues from patients with DM1. Most studies, especially those using adult DM1 tissues, showed decreased levels of DMPK mRNA (reviewed in Reference (76)). The decrease in the DMPK mRNA has been correlated with the size of CTG repeat expansion, and attributed to the retention of mutant DMPK transcripts in nuclear foci in DM1 cells rather than impaired transcription of the mutant *DMPK* gene (see Figure 130-8) (136,167–169). Furthermore, Wang et al. (170) found that actual DMPK mRNA levels are below 50% of the normal level in some patients with DM1, potentially by dominant negative effect of the mutant DMPK mRNA on the wild-type DMPK mRNA.

DMPK is one of the myotonic dystrophy family of protein kinases (MDFPK). Although DMPK mRNA is strongly expressed in skeletal muscle, heart, brain, liver, and kidney (169), its exact physiological function is not fully understood. DMPK phosphorylates serine and threonine residues of myosin-binding subunit of myosin phosphatase and inhibits myosin phosphatase activity (171). Thus, DMPK may be involved in regulating cell size and shape like other MDPK. Several other proteins, such as the  $\beta$ -subunit of the L-type calcium channel (172), phospholemman (173), and CUG-BP (141) are also phosphorylated by DMPK. The DMPK protein appears to be preferentially localized in muscle and heart (174). In skeletal muscle, the DMPK protein has been found in sarcoplasmic reticulum (175).

The DMPK loss-of-function hypothesis came into focus in 1996, when initial observations of DMPK-deficient mice showed no robust abnormalities (176,177) except for mild muscle weakness. Further studies of homozygous and heterozygous *DMPK*-deficient mice suggested that haplo-insufficiency of DMPK does cause skeletal and cardiac muscle abnormalities through alterations of sodium and calcium channels. The abnormal sodium channel opening and repetitive discharges observed in the skeletal muscle membrane of these *DMPK*-deficient mice (178) may be in part caused by silencing of muscle Na channels (179). Skeletal muscle cells derived from *DMPK*-deficient mice also showed abnormal conductance of dihydropyridine receptor (180), which colocalized with DMPK at the sarcoplasmic reticulum (175) and was phosphorylated by DMPK in vitro (172). Cardiac conduction block found in homozygous and heterozygous *DMPK*-deficient mice (181,182) may be attributable to abnormal sodium channel gating

(183). Single cardiomyocytes derived from *DMPK*-deficient mice showed enhanced basal contractility, with evidence suggesting that increased  $\text{Ca}^{2+}$  uptake into the sarcoplasmic reticulum is the underlying cause (184). Thus, *DMPK* haploinsufficiency could account for some electrophysiological abnormalities in DM1 skeletal and cardiac muscles.

The *DMPK* isoforms may add an additional twist to the haploinsufficiency theory. Alternative splicing of *DMPK* transcript in a transgenic mouse model produces six major isoforms, with tissue-dependent expression (185). *DMPK* isoforms have cell-type- and subcellular location-dependent substrate specificities, which are attributable to an alternatively spliced VSGGG motif and C-terminal structures (186). Four RNA splicing factors have been shown to bind the *DMPK* transcript at the 3'UTR region downstream of the CUG repeat, yielding a novel mRNA isoform containing no CUG repeats, which was not retained in the nucleus in DM1 cells (187). Further studies are needed to gain additional insights regarding the role of these isoforms in the *DMPK* haploinsufficiency hypothesis.

**130.5.1.3 Effects on Adjacent Genes.** The CTG repeat expansion affects the chromatin structure and the expression of *DMPK* and neighboring genes, *SIX5* and *DMWD* (see Figure 130-8), in myotonic dystrophy. DNA triplet repeats mediate heterochromatin-protein-1-sensitive variegated gene silencing (188).

**130.5.1.3.1 Loss of *SIX5* Function.** *SIX5*, previously known as DM associated homeodomain protein (*DMAHP*) (189) is located immediately downstream of the *DMPK* gene (see Figure 130-8). *SIX5* shows homology to the *Drosophila* gene *sine oculis* and is involved in muscle and gonad development (133,190). Wang et al. noted that a DNase I sensitive site positioned immediately downstream to the CTG repeat is eliminated when the CTG repeat is expanded, and attributed this sensitivity loss to tight binding of nucleosomes to expanded CTG repeats (170). Thus, an expansion of the CTG repeat might impede the RNA polymerase procession through chromatin or the accessibility of transcription factors for the neighboring gene. The *SIX5* mRNA is found in tissues affected in DM1 such as eyes, skeletal muscle, heart, and brain (189,191). Subsequently, it was found that an expansion of the CTG repeat reduces expression of the *SIX5* gene, by impeding an enhancer element for the *SIX5* gene (see Figure 130-8) (192–194). This effect is expansion size-dependent and tissue-specific, with the greatest reductions found in muscle and liver (194,195). In contrast to these results, however, other groups reported no significant reduction in the *SIX5* expression level in DM1 tissues (196,197).

Klesert et al. (198) and Sarkar et al. (199) independently showed that *SIX5* deficiency causes cataracts in both homozygous and heterozygous “knockout” mice. *SIX5* regulates the transcription of the Na, K-ATPase 1 subunit gene (*Atpla1*), and the increased *Atpla1* mRNA

level in *SIX5* deficiency may alter the regulation of the osmotic balance within the lens and contribute to the development of lens opacity (199). Cardiac conduction system abnormalities were also found in *SIX5*-deficient mice (200). However, *SIX5*-deficient mice do not exhibit histopathological, contractile, or electrophysiological abnormalities in the skeletal muscle (201,202). Further studies are needed to determine whether other organs show abnormalities compatible with DM1 in these 'knockout' mice.

**130.5.1.3.2 Loss of DMWD Function.** An expansion of the CTG repeat may also influence the expression of the immediately upstream gene *DMWD* (see Figure 130-8) (203). The 5' end of the *DMPK* gene overlaps with the 3' end of the *DMWD* gene, and the 3' boundary of the *DMWD* has not been clearly defined. *DMWD* is highly expressed in brain and testis, which are known to be affected in DM1. The predicted protein product contains WD repeat sequences, which are found in several proteins engaged in control of cell division, transcription, mRNA processing, cytoskeletal assembly, vesicle fusion, and signal transduction (204). Decreased *DMWD* mRNA levels were reported in the cytoplasm of DM cell lines and adult DM1 skeletal muscle samples (203).

## 130.5.2 Molecular Pathogenesis of Myotonic Dystrophy Type 2

Mirroring the CTG repeat in *DMPK*, the CCTG tetranucleotide repeat in the first intron of the *ZNF9* gene (54) is transcribed into a CCUG repeat in the RNA but is not translated into the protein. The normal function of the *ZNF9* protein is unknown, although it is known to be capable of binding both DNA and RNA (205). No data have been reported on the transcription of the *ZNF9* gene from the mutant chromosome in DM2 patients. Therefore, whether haploinsufficiency of *ZNF9* plays any pathogenic role in DM2 remains unknown.

In addition to similarities in clinical phenotypes, significant similarities between DM1 and DM2 have also been demonstrated in the molecular mechanism of the disease at the RNA level (206). The muscleblind proteins accumulate in the ribonuclear foci containing CCUG repeats (151–153), and *CLC-1* and IR mis-splicing is also observed in patients with DM2 (145,207). These data suggest that the *trans*-dominant RNA gain-of-function mechanism may also have a central role in DM 2; however, one should be reminded that there are clinical differences between these two diseases, most notably the lack of the congenital form in DM2 (208). Whether the differences are due to the lack of loss-of-function of *DMWD/DMPK/SIX5* in DM2 or due to different gain-of-function effects by the CCUG repeat and/or loss-of-function of *ZNF9* remains to be investigated.

## 130.6 GENETIC COUNSELING AND RISK ESTIMATION

Genetic counseling for DM1 has always been difficult, owing to the extreme variability of the disorder, the parental origin effects, and the intergenerational differences. Although molecular advances have largely explained these phenomena, they have only partially helped in answering the practical questions. Approximate risk estimates based on the studies discussed earlier in this chapter are summarized in Tables 130-12 and 130-13. To a surprising extent, the guidelines remain based on premolecular work.

Full genetic counseling is particularly important in relation to presymptomatic testing (see later).

Although a full two-stage approach such as is generally used in Huntington's disease is probably required only in a minority of situations (209,210), presymptomatic testing is most certainly not a "laboratory only" procedure, and the complexities of genetic and family aspects require both time and experience. The International Myotonic Dystrophy Consortium (1) has established guidelines for genetic testing of DM1.

### 130.6.1 Molecular Analysis and Genetic Prediction

The feasibility of direct analysis for the DM1 mutation has become a routine approach to prediction and to genetic counseling in the disorder. Some of the tests for detecting minimal phenotype that were previously the mainstay of diagnostic testing, such as EMG and ocular slit-lamp examination, are obsolete. It is important to recognize that molecular analysis is also not without its pitfalls and inaccuracies, and that other evidence based on older tests should not be disregarded. The direct mutation analysis should also be used as the definitive diagnostic test for DM2 in prediction and genetic counseling.

In considering tests for prediction, several general points need to be borne in mind. First is the remarkable specificity and sensitivity of mutational testing in DM1 and DM2. If molecular analysis in an affected family member fails

**TABLE 130-12** Risk of Myotonic Dystrophy Type 1 for Clinically Normal First-Degree Relatives (Approximate)

Young Adults	10%	Risk of Carrying Mutation
(21–40)	5% Minimal	Risk of clinically significant disease Risk of congenitally affected child
Older adults (>40)	<5% >10%	Risk of clinically significant disease Risk of carrying mutation in sibship with cataract only
Children with congenitally affected sib	25% 10% 5%	Infancy Approximately age 16 Age >18

**TABLE 130-13 Risks to Offspring of Patients with Myotonic Dystrophy Type 1 (Approximate)<sup>a</sup>**

Parental Group	Approximate Risks to Offspring
<b>Men</b>	
Minimal disease, late-onset (usually <100 repeats)	No significant risk of congenital disease Risk of serious neuromuscular disease greater than offspring of corresponding females
Significant neuromuscular disease	Minimal risk of congenital disease Chance of reduced severity due to contraction of mutation greater than for female transmissions
Childhood onset	Reproduction rare. Congenital disease. Not expected
<b>Women</b>	
Minimal disease, late onset	No significant risk of congenital disease. Disease more likely to remain minimal (with unchanged repeat) than for male transmissions
Significant neuromuscular disease. Child already born with congenital onset	40–50% chance of congenital or severe childhood disease. Most of the remainder will remain unaffected
Significant neuromuscular disease. No congenitally affected child born	Uncertain. Chance of congenital or severe childhood disease 10–30% (see text)
Childhood onset	No systematic data, but chance of severe childhood disease high (probably 40–50%)

<sup>a</sup>NB: All groups have the same chance (50%) of offspring being genetically unaffected.

to show an abnormality, an alternative diagnosis is likely. On the other hand, if no such member is available, but the clinical diagnosis in the family is secure, molecular testing for DM1 and DM2 is still highly likely to give a definite answer. A second point, highly relevant for the laboratory, is the wide range of expansion that needs to be looked for in individuals who are homozygous for a normal allele on PCR-based analysis of DM1 and DM2 mutations. In DM1, Southern blot analysis is sufficient to supplement the PCR analysis, which may fail to amplify expanded allele. In DM2, however, the combination of routine PCR and Southern blot analyses is only 80% sensitive owing to the high degree of repeat size mosaicism. Additional repeat-primed PCR or long PCR should increase the detection of the DM2 mutation to 99% (59,211).

### 130.6.2 Prenatal Diagnosis

In DM2, no cases of prenatal DNA diagnosis have been reported, although it is technically feasible. This is probably because DM2 is generally presents as a milder disease than DM1, with no severe congenital or early childhood form. In contrast, prenatal diagnosis by direct mutation analysis is useful in DM1 families in which a severely affected child has been born, with a high risk of a subsequent child being similarly affected. The size of the expanded allele should be determined by Southern blot analysis, because repeat-primed PCR does not give the expansion size and long PCR may still have difficulty in amplifying very large alleles. Even extremely large bands are well defined on Southern blot analysis at this stage in DM1 fetuses (usually 10–12 weeks' gestation using chorion villus sampling) (103), reflecting the lack of somatic instability in early fetal life. The overlap between clinical groups makes it unwise to give

an accurate prognosis from the size of the expansion prenatally in DM1, although it is possible to distinguish the “minimal” phenotype from the severe in some cases. In any case, family studies alone have shown a close concordance in sibships once a severely affected child has been born (90).

When an affected man is the parent, a much greater range of expansion in the pregnancy is to be expected, with the possibility of reduction in size into the minimal, or rarely even into the normal range, as discussed earlier. The consequent uncertainty in likely severity needs to be made clear in genetic counseling beforehand.

Preimplantation diagnosis is now increasingly raised as a possibility for a range of genetic disorders, and there has been sufficient experience in DM1 to demonstrate it as an effective technique (212,213).

Although there is no evidence that pregnancy itself causes deterioration, it is important to recognize the possibility of complications during cesarean or other surgery and associated anesthesia. It is also important to discuss with patients that prenatal testing should not be performed if termination of the pregnancy is not considered as an option.

### 130.6.3 Presymptomatic Testing and Carrier Detection

Before undertaking presymptomatic testing and carrier detection, it is important to be clear as to what question is being asked. Is it related to the possible development of future disease (presymptomatic testing), to assessment of present symptoms in relation to the family disorder (diagnostic testing), or to concerns that the disorder might be transmitted in the absence of symptoms (carrier detection)? The answers to these questions will determine the



best combination of tests to be used. A symptomatic or asymptomatic young adult would be best offered genetic testing. In a young child at risk of DM1, clinical assessment remains the most helpful procedure, since genetic analysis in such a normal child raises many issues that may best be resolved when the child is older. There is no evidence that clinically normal mutation carriers are at any risk of surgical or other procedures.

A normal molecular result in an asymptomatic adult at risk now allows a high degree of reassurance in terms of both that individual's lack of risk of developing DM1 in the future and of transmitting the disorder (214), although confirmation of the mutation in an affected family member is always wise. This is a great advance on previous uncertainty resulting from the interpretation of lens-opacities and EMG, and from the possibility of recombination when linked DNA markers were being used. The uncertainty has now shifted to the prognosis for those who are clinically normal but shown to have an expanded repeat sequence. The correlations of phenotype available from current studies are retrospective and show broad variation; therefore any prognosis needs to be very cautious. For the "prematuration" expansion (38–49 copies), one can be reasonably confident that this will imply lack of significant neuromuscular disease, whereas the study by Gennarelli and coworkers (77) suggests that this can be extended to those with less than 100 repeats. Recently a comprehensive assessment of patients with less than 200 CTG repeats found that those with less than 100 repeats often only develop cataracts (215), although patients with symptomatic DM1 associated with less than 100 repeats can be seen. The psychosocial impact of presymptomatic DM1 testing was minor, although life is perceived as a change for the better by noncarriers and as a change for the worse by carriers (216). Presymptomatic testing is now feasible but no cases have been reported in DM2.

#### 130.6.4 Diagnostic Molecular Testing

The specificity of mutation testing now makes it extremely useful in assessing not only relatives with symptoms possibly related to the disorder but also those with no clear family history for whom DM1 or DM2 is suspected on clinical grounds. The differential diagnosis of congenital DM1, which is often difficult in relation to other neuromuscular disorders, is one such situation, whereas distinction of DM1 and DM2 from other myotonic disorders is easier in later life. The recognition of characteristic cataracts is a further situation in which molecular testing can be helpful.

As genetic testing is increasingly becoming a part of clinical practice, it is important that the genetic considerations be recognized and discussed with the patient to avoid problems. In this respect, DM1 and DM2 share many such issues with other late-onset disorders for which molecular testing is becoming an integral part

of practice and no longer the specialized preserve of geneticists.

### 130.7 MANAGEMENT

In spite of the profound changes in our understanding of DM during the past few years, we still have no specific therapy to alter the underlying course of the disorder. For the first time, however, we can look ahead to rational therapeutic approaches based on the relationship between the molecular defect and the pathological changes associated with the disease. As we discussed in the previous section, recent studies have shown increasingly convincing evidence for the trans-dominant RNA gain-of-function as the main pathogenic mechanism of DM1 and DM2. Experimental therapies using antisense oligonucleotides and ribozymes to counteract the RNA gain-of-function have been studied in cell culture and transgenic mice (217–220), and use of these strategies in human patients may become feasible in the future. Better understanding of the mechanism of the repeat instability may lead to the development of treatments to reduce the repeat size in patients (221,222). More recently, encouraging results using small molecules and antisense treatment has been published. Mulders (223) and Warf (224) have demonstrated reductions in ribonuclear foci and mRNA splicing defects using antisense oligonucleotides and small molecules in vitro and in animal models of DM1 by the complete elimination of expanded (CUG)*n* RNA transcripts or by the prevention of detrimental protein binding to thermodynamically stable (C/CUG)*n* hairpin structures. These developments offer new hope to patients with DM although obstacles such as toxicity and tissue-specific availability need to be overcome before small molecule and antisense treatments can be introduced into clinical practice (225).

There are several drug treatments for symptomatic management of DM1. The treatment of myotonia in DM1 is often not seen as a priority in the patient's management although there are probably a significant number of, especially younger, patients with otherwise minimal symptoms in whom treatment of myotonia could improve quality of life. Logigian (226) recently performed two randomized, double-blind, placebo-controlled crossover trials in 20 DM1 patients and found that mexiletine up to 200 mg 3 times daily was effective, safe, and well tolerated over 7 weeks as an anti-myotonia treatment. Phenytoin and quinine may also be helpful. The teratogenic possibilities for phenytoin and the cardiac conduction effects of all of these agents must be considered. Modafinil is useful in treatment of excessive daytime sleepiness (227,228). Bowel symptoms are often troublesome and may be helped with antibiotic treatment, such as norfloxacin, or a bile salt sequestrant such as cholestyramine (7,8).

Regular surveillance for cardiac conduction defects and tachyarrhythmias is important and a yearly ECG

should be performed as a minimum but there may be a role for electrophysiological testing in determining those patients who are most at risk.

The avoidance of unnecessary surgical and anesthetic hazards is vital. Unfortunately, many patients still experience avoidable post-operative complications. Mildly affected individuals with little physical disability are probably at greatest risk because the dangers are unappreciated. Conversely, helpful but necessary surgical measures, such as cataract extraction and correction of foot deformities, should not be withheld simply on the grounds of possible surgical hazard.

Major mobility aids, such as wheelchairs, are not needed by many patients or, if they are, only at a late stage, but thorough assessment of disability needs is important to avoid loss of mobility and independence. Light plastic ankle-foot orthoses to correct foot drop may make a considerable difference to stability and mobility.

The possibility of aspiration pneumonia resulting from swallowing dysfunction is possibly one of the most important complications of DM1. Regular assessment and advice by a speech and language therapist is important in conjunction with monitoring of vital capacity and sleep-disordered breathing with a home sleep study in the first instance. Many end-stage patients are malnourished, and discussion of possible parenteral feeding needs to be discussed earlier than is currently practiced.

Surveillance of patients should be an active process at each clinic visit and guidelines are being drawn up (4,229).

Finally, the importance of giving patients and families full details regarding their condition should not be underestimated. As with many genetic disorders, an informed patient is likely to know more about his or her own problem than most of the doctors and other professionals that they may encounter. Full written information produced in an easily readable style is probably the best way of ensuring that patients avoid most predictable complications of the disease. Helpful information of this type is available from the Myotonic Dystrophy Support Group and Muscular Dystrophy Campaign in the United Kingdom, from the Muscular Dystrophy Association of America, and from the Association Française contre les Myopathies in France, among others. A remarkable amount of information is also available on the Internet, but the sheer volume and its unselected nature are likely to give problems in interpreting the details. These issues have extensively been reviewed in the book “*Myotonic dystrophy: present management, future therapy*” (64).

## CROSS REFERENCES

Autosomally Inherited Muscular Dystrophies; Nondystrophic Myotonias and Periodic Paralysis; Congenital (Structural) Myopathies; Hereditary Muscle Channelopathies.

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## RELEVANT WEB PAGES

[www.myotonicdystrophysupportgroup.org/](http://www.myotonicdystrophysupportgroup.org/)  
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### Biography



**Dr Chris Turner** graduated from the University of Oxford Medical School in 1996 with the *Sir George Pickering Prize* for top of year. He then undertook medical house physician jobs in the Nuffield Department of Medicine in Oxford and subsequently trained at the Hammersmith Hospital and Queen Square. With a growing interest in degenerative mechanisms in the nervous system he undertook a PhD with Professor Tony Schapira at the Institute of Neurology, Royal Free campus, London into the “Molecular Pathogenesis of Huntington’s Disease.” His specialist neurology training was conducted at Queen Square, Chelsea and Westminster and St George’s Hospitals after which he took a Consultant Neurologist position in 2007 in the MRC Department for Neuromuscular Disease at Queen Square. He has developed a specialist interest in the Myotonic Dystrophies since his appointment and has spoken at national meetings of the MDSG as well as on lecture courses. He is currently involved in developing a National UK Myotonic Dystrophy Registry and Standards of Care for DM. He runs the Myotonic Dystrophy clinic at Queen Square.

# CHAPTER

# 131

## Hereditary and Autoimmune Myasthenias

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This article is a revision of the previous edition article by David Beeson and Nicholas Willcox, volume 3, pp 3072–3087.

### 131.1 INTRODUCTION

Myasthenia gravis (MG) and allied disorders of neuromuscular transmission are uncommon. They are characterized by weakness of voluntary muscle that is usually fatigable and often affects the extraocular and eyelid muscles at an early stage. Here, they are considered in order after a brief introduction to neuromuscular physiology.

### 131.2 NORMAL NEUROMUSCULAR TRANSMISSION

Motor nerve fibers are ensheathed by Schwann cells almost as far as the neuromuscular synapse, where they expand to form the nerve terminals, which are rich in both mitochondria and synaptic vesicles containing acetylcholine (ACh) (reviewed in Reference (1)). The nearby muscle membrane is highly folded and the ACh receptors (AChRs) are densely packed on the crests of these folds (Figure 131-1) (2). By contrast, the ACh esterase (AChE) is diffusely attached to the basal lamina throughout the intervening space, extending down into the clefts between the folds. The molecular anatomy and development of this exceptionally well-studied synapse are elegantly reviewed by Sanes and Lichtman (3), and its physiology by Vincent and Wray (4).

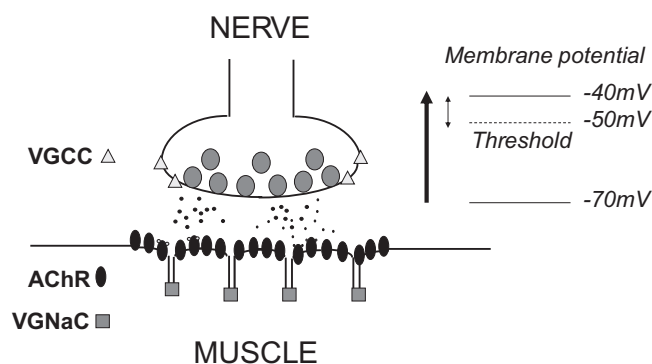
The vesicles can discharge spontaneously by fusing with the membrane, each releasing 5000 to 10,000 molecules of ACh. About 6000 of these bind to the AChRs and open their intrinsic cation channels, thus depolarizing the membrane locally; each resulting miniature endplate potential (mepp) is 0.8–1.0 mV in amplitude. By contrast, when a nerve impulse reaches the terminal, it causes voltage-gated calcium channels (VGCC), located in active zones in the nerve membrane, to open, and the resulting entry of  $\text{Ca}^{2+}$  ions triggers the discharge of about 50 vesicles. As the ensuing mepps summate, the endplate potential (epp) reaches threshold, and an action potential

is propagated in the muscle membrane by voltage-gated sodium channels. These are concentrated in the synaptic clefts but extend over the entire muscle fiber, so that the action potential spreads, ultimately triggering a contraction. After breakdown of ACh by AChE, residual choline is taken back into the nerve terminal and reincorporated into ACh by the enzyme choline acetyltransferase. The vesicles themselves are initially synthesized in the motor nerve cell body and transported down the axons to the synapse, where they are repeatedly recycled.

Normally, in humans, a nerve impulse depolarizes the muscle membrane from about  $-70$  mV to about  $-40$  mV, which only modestly exceeds the threshold of  $-50$  mV (Figure 131-1). This roughly 10 mV difference (the safety factor of transmission) is much smaller in humans than in some other species (e.g. mouse). The loss of this safety factor is fundamental to almost all the myasthenias and may result from an impaired transmitter release, a decrease in receptor numbers, altered receptor function, or altered synaptic structure (1).

### 131.3 DIAGNOSTIC METHODS

Standard electromyography (EMG) can readily detect both the impaired transmission and especially its partial recovery after maximal voluntary contraction (post-tetanic potentiation) that is characteristic of some presynaptic defects. Similarly, it may show the fatiguing decline in responses to repetitive stimulation (at 3 Hz)—“decrement”—typical of postsynaptic disorders, and single-fiber EMG may also reveal asynchronous responses of muscle fibers supplied by branches of the same axon—“jitter” (reviewed by Harper (5)). In specialist centers, electrophysiological studies of single endplates from biopsied muscle can quantify the number of packages of ACh released after a single impulse (“quantal content”), and so confirm a presynaptic disorder as in the Lambert–Eaton myasthenic syndrome (LEMS).



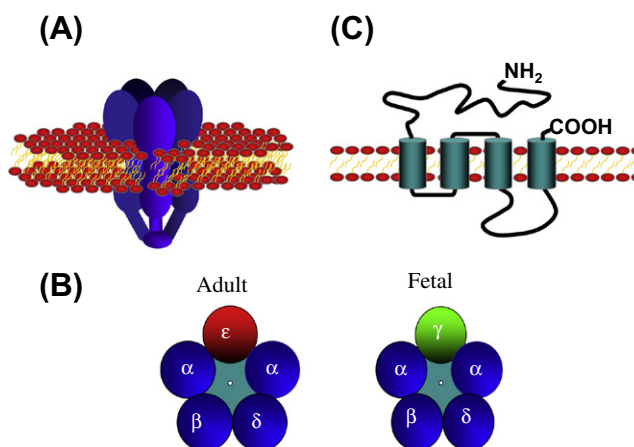
**FIGURE 131-1** The neuromuscular junction. Action potentials are propagated in the axon membrane by voltage-gated  $\text{Na}^+$  channels (and are terminated partly by the action of voltage-gated  $\text{K}^+$  channels). When they reach the nerve terminal, they open voltage-gated  $\text{Ca}^{2+}$  channels in the active zones (triangles). The resulting  $\text{Ca}^{2+}$  entry causes synaptic vesicles to discharge their ACh toward the crests of the folds in the muscle membrane opposite, where the AChRs are very densely packed (ovals). Voltage-gated sodium channels (squares) are activated once the membrane depolarization rises above  $-50\text{mV}$ .

The amplitudes of the mepps and epps, and their rise and decay times may suggest abnormalities in the numbers or kinetics of the AChRs (6). Straightforward staining for AChE gives an estimate of the endplate morphology and length (normally up to 8 sarcomeres) as well as of the AChE content (7). Binding of iodinated  $\alpha$ -neurotoxins (e.g.  $\alpha$ -bungarotoxin [ $^{125}\text{I}$ - $\alpha$ -BuTx]) quantifies any deficiency in AChR; normally in intercostal muscle, there are about  $1.2 \times 10^7$  binding sites per endplate (8). More specialized electron microscopy may also be invaluable (1). Once an initial diagnosis is made, genetic analysis has now become the gold standard for confirmation of an hereditary disorder.

If autoimmune MG is suspected, there is a highly sensitive and specific antibody assay that uses human muscle AChR prelabeled with  $^{125}\text{I}$ - $\alpha$ -BuTx, and gives positive results in more than 85% of patients with MG (9,10); another 5–10% instead have antibodies against muscle-specific tyrosine kinase (MuSK), which can be measured similarly (11). There are now equally valuable assays for antibodies to calcium channels for the LEMS (12). Finally, measurable clinical improvement up to 3 weeks after a course of plasma exchange may be an invaluable clue to an autoantibody-dependent pathogenesis (13).

### 131.4 ACETYLCHOLINE RECEPTOR

In the midterm fetus, the nicotinic AChR consists of  $\alpha_2$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  subunits (Figure 131-2B), but, in the last trimester, an  $\epsilon$  gradually replaces the  $\gamma$ . These two AChR isoforms differ both antigenically and in their channel kinetics. The subunits are all evolutionarily related and comprise 437 to 496 amino acids (summarized in Reference (14)). Each has about 210 amino acids of



**FIGURE 131-2** The acetylcholine receptor, viewed (A) from the side, (B) from the nerve terminal, illustrating the two isoforms of the AChR. (C) Topography of the  $\alpha$ -subunit.

extracellular sequence, three highly conserved transmembrane segments (M1–M3) almost in tandem, a more variable cytoplasmic loop, a fourth transmembrane segment, and a short extracellular tail (see Figure 131-2C). Together, they form a cylindrical structure (see Figure 131-2A) with the M2 segments of each subunit lining the central cation channel; charged residues forming a “funnel” at each end are thought to control its ion selectivity and conductance. The preference for  $\text{Na}^+$  is relative; about 6% of the normal current is due to  $\text{Ca}^{2+}$  entry. The M2 segments form amphipathic  $\alpha$  helices/ $\beta$  strands with (roughly) alternating hydrophobic and polar residues. At rest, the channel lumen is occluded by amino acid side-chains in the M2 segments of several subunits (15–17). These shift during the conformational change that follows ACh binding to sites at both the  $\alpha/\delta$  and  $\alpha/\epsilon$  (or  $\alpha/\gamma$ ) interfaces (18). This allows cations (mainly  $\text{Na}^+$ ) to enter the muscle and depolarize its membrane.

At the neuromuscular junction there are many potential target sites for genetic defects or for attack by autoantibodies or neurotoxins. They include the ion channels in the axons, nerve terminals and muscle membrane, the synthesis and recycling of the synaptic vesicles, the AChE and AChR molecules, and the numerous other molecules involved in their localization to the endplates, their anchorage there or in the synaptic structure itself (19). In particular, evidence from experiments in mice in which components of the neuromuscular junction were “knocked out” suggests that a core pathway is responsible for initiating and maintaining the specialized postsynaptic structures at the neuromuscular junction. In this pathway, agrin, released from the nerve terminal, activates MuSK located in the postsynaptic membrane; the MuSK signal is amplified through interaction with Dok-7 (20) and this leads to the precise aggregation and localization of the AChR through the association with the cytoplasmic anchoring protein rapsyn (3). Disruption of this core pathway is likely to result in a myasthenia (21).



### 131.5 CONGENITAL MYASTHENIC SYNDROMES

The “congenital” myasthenic syndromes (CMSs) are inherited disorders of neuromuscular transmission with no underlying autoimmune basis; they are reviewed by Engel and associates (6). They are heterogeneous, reflecting the diversity of underlying genetic defects, but share the characteristic fatigable muscle weakness with autoimmune MG. CMSs account for only around 5% of all myasthenias, but this number is increasing with improved diagnosis. Despite their name, in some syndromes there is great variation in the age of onset, with some not presenting until childhood or even after puberty. If neonates are affected, they typically show weakness on sustained exertion, for example, during crying and feeding, reflecting bulbar involvement. More seriously, there may be difficulties in respiration, requiring resuscitation. Any subsequent apneic attacks demand urgent therapy or even ventilation, both of which the parents must be trained to administer. If these measures are successful, the patients usually stabilize with therapy and they develop remarkably well in spite of some disability (22).

The phenotypes of the various CMSs are often superficially similar. Clinical, electrophysiological, cytochemical and morphological analysis has helped to discriminate postsynaptic, synaptic or presynaptic defects (reviewed in References (1) and (23)). The molecular genetic bases for many postsynaptic and synaptic syndromes (reviewed in Reference (6)) are now well understood and should help to improve their classification; however, other examples do not yet fall clearly into the categories listed in Table 131-1. Before describing these disorders, we now list some general conclusions.

First, AChR loss is a major cause of CMS. Second, apart from the slow-channel syndrome (see later), all the CMSs show recessive inheritance; many result from compound heterozygosity with distinct mutations in each allele (heteroallelism) or from parental consanguinity. Third, different molecular mechanisms can generate surprisingly similar syndromes from mutations in diverse regions of the AChR, Rapsyn, ChAT, ColQ or Dok-7. Fourth, different families with the same mutation can show remarkable differences in severity, implying other modifying influences; their identification might be turned to therapeutic advantage in the future. Fifth, the majority of known mutations in the AChR are in the adult-specific  $\epsilon$  subunit; presumably many patients survive through persistent low-level fetal ( $\gamma$ ) subunit expression that may vary between patients, whereas homozygosity for null alleles of other subunits might be lethal in utero. Sixth, the pathogenicity of many CMS mutations has been confirmed by comparing mutant protein function in transfected cells (biochemically or electrophysiologically) with that in patient muscle biopsies. Finally,

**TABLE 131-1 Major Categories of Congenital Myasthenias**

Classification of congenital myasthenic syndromes (CMS) and their genetic loci*	
Presynaptic CMS	Gene
CMS with episodic apnea	<i>CHAT</i>
Synaptic CMS	
Congenital endplate acetylcholinesterase deficiency	<i>COLQ</i>
CMS due to mutations in Agrin	<i>AGRN</i>
CMS due to mutations in the laminin $\beta 2$ chain	<i>LAMB2</i>
Postsynaptic CMS	
AChR deficiency syndromes	<i>CHRNA, CHRNB, CHRND, CHRNE</i>
AChR deficiency syndromes due to mutations in rapsyn	<i>RAPSN</i>
Slow-channel CMS	<i>CHRNA, CHRNB, CHRND, CHRNE</i>
Fast-channel CMS	<i>CHRNA, CHRND, CHRNE</i>
CMS due to voltage-gated sodium channel mutations	<i>SCN4A</i>
CMS due to mutations in MuSK	<i>MUSK</i>
CMS due to mutations in Dok-7	<i>DOK7</i>
Multiple pterygium/Escobar syndromes due to AChR $\gamma$ -subunit mutations	<i>CHRNA</i>
Fatal fetal akinesia deformation sequence due to NMJ protein mutations	<i>CHRND, RAPSN, DOK7</i>
Limb-girdle CMS with tubular aggregates	<i>GFPT1</i>

functional analysis of the mutated proteins is often required to determine pathogenicity for the many variants that are found in genes encoding neuromuscular junction proteins.

#### 131.5.1 Postsynaptic Defects

Mutations within the genes encoding the AChR or the AChR clustering proteins rapsyn and Dok-7 are the major cause of these abnormalities, although kinships with mutations in MuSK (24), Agrin (25) and the postsynaptic voltage-gated sodium channel have also been reported (26). More recently a CMS due to mutations in the ubiquitously expressed GFAT1 (*GFPT1* gene) has been identified (27), and it is not yet certain in these cases whether the primary defect is pre- or post-synaptic. The AChR subunits are encoded by separate genes of 10–12 exons; the *CHRNA*, *CHRNA* and *CHRND* loci (for  $\alpha$ ,  $\gamma$  and  $\delta$ ) are on chromosome 2, the *CHRNB*, and *CHRNE* ( $\beta$  and  $\epsilon$ ) loci are on chromosome 17 (14,28) and the *RAPSN* locus is on chromosome 11 (29). The various mutations may either increase or decrease the synaptic response to ACh; the clinical syndromes fall into five main groups, although rarely mutations are identified in other

neuromuscular junction proteins. Some of the mutations that cause AChR deficiency due to mutations in *CHRNAE* or *RAPSN* are illustrated in [Figure 131-3](#), although a more extensive table is listed in Reference (30).

### 131.5.2 Acetylcholine Receptor Deficiencies

AChR deficiency may arise from mutations within the AChR itself or through mutations in the clustering protein rapsyn. Currently, these are not distinguishable by EMG or muscle biopsies, but careful examination of the differing clinical features may help to predict the affected gene. The majority of AChR mutations causing AChR deficiency are located in *CHRNE* ( $\epsilon$  subunit), but rare severe examples have been identified in *CHRNA*, *CHRNA*, and *CHRNA* ( $\alpha$ ,  $\beta$  and  $\delta$  subunits).

### 131.5.2.1 AChR Deficiency due to Mutations in the AChR $\epsilon$ -Subunit.

**131.5.2.1.1 Clinical Features.** There is a characteristic reduction in endplate AChR numbers that results in reduced responses to ACh. These recessive disorders are the most common CMSs in the United Kingdom. Weakness is usually evident at birth or in the first few years of life and is characterized by feeding difficulties, ptosis, impaired eye movements, and delayed motor milestones. Ophthalmoplegia is a prominent and early feature that may help in differential diagnosis. Specialized EMG shows reductions in mEPSP amplitudes and, sometimes, a supranormal evoked quantal ACh release. Morphologically, the endplates are elongated with patchy endplate staining and simplified postsynaptic membrane folding (6). There is usually a good response to anticholinesterase medication; 3,4-diaminopyridine, which enhances ACh release, may be a useful alternative (31), but is available only from specialist centers.

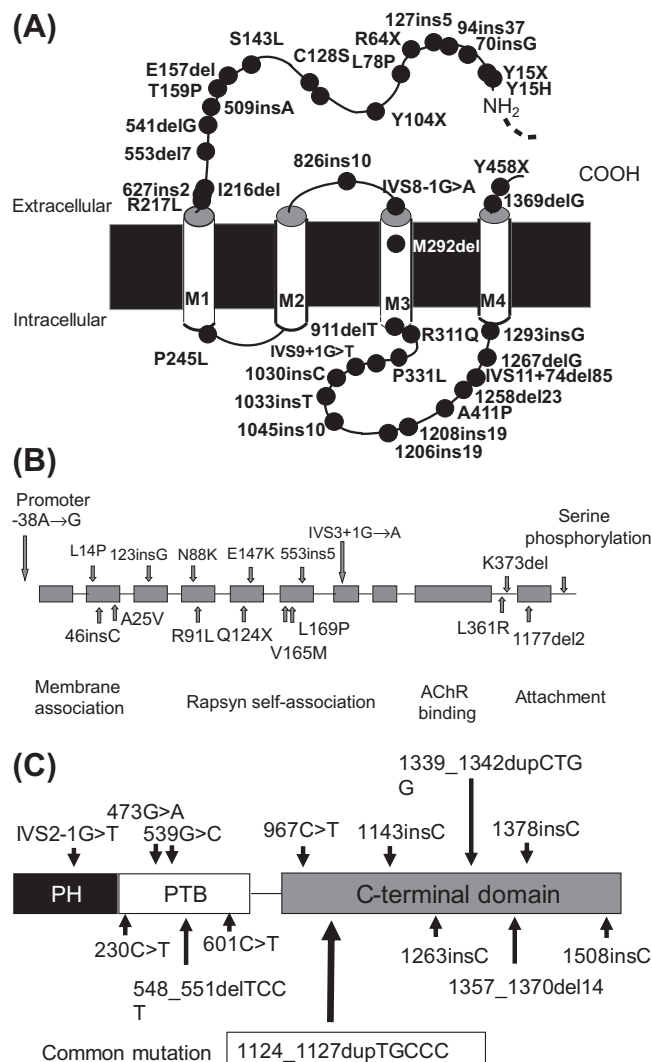
**131.5.2.1.2 Molecular Basis.** The vast majority of mutations within the AChR that cause an AChR deficiency syndrome are located in the  $\epsilon$ -subunit gene. More than 100 different mutations have been identified (6), and typical examples are shown in Figure 131-3A. In the UK there are few obvious founder effects, although  $\epsilon$ 1267delG is a common mutation in patients of gypsy ethnic origin (32), and  $\epsilon$ 1293insG in cases from north Africa (33). Several types of mutation have been identified along the gene (see Figure 131-3A);

- (i) promoter mutations affecting transcription (34);
- (ii) premature termination of the translated chain caused by frameshift, intron splice site or nonsense mutations (6);
- (iii) missense mutations in residues essential for folding/assembly, for example, in leader sequence, glycosylation site or cysteine loop (see Figure 131-3A) (6).

It has been shown that the presence of a cysteine residue three amino acids from the  $\epsilon$ -subunit C-terminus is required for surface expression and thus almost all mutations causing truncations will give rise to null alleles (35). In these cases, the residual neuromuscular transmission probably depends on low levels of the fetal form of the AChR ( $\alpha_2\beta\gamma\delta$ ).

### 131.5.2.2 AChR Deficiency due to Mutations in Rapsyn.

**131.5.2.2.1 Clinical Features.** The disorder may be divided into a more common “early-onset” phenotype that presents at birth or in early childhood and a “late-onset” phenotype that may present from early adulthood through to late middle age. By contrast with patients harboring  $\epsilon$ -subunit mutations, ophthalmoplegia is rarely seen in patients with rapsyn mutations, although they appear to have a high prevalence of strabismus. The early-onset phenotype usually presents at birth with hypotonia and bulbar dysfunction, and the patient may require assisted ventilation. Mild fixed joint



**FIGURE 131-3** Topological or linear representation for example mutations in the three most common forms of congenital myasthenic syndrome. (A) AChR deficiency syndrome mutations in the  $\epsilon$  subunit. (B) Rapsyn functional domains and the position of some of the identified mutations. (C) Dok-7 functional domains and positions of some of the identified mutations.

contractures (arthrogryposis multiplex congenita) of hands and ankles is a characteristic and frequent feature. Typically, there are severe exacerbations during early childhood, often presenting with respiratory failure, and often associated with minor infections; these usually resolve around the age of 6 years. Symptoms improve later in life, and the majority of patients experience little disability in adulthood. Similar episodic exacerbations have been described in CMS with episodic apnea, a much rarer disorder, caused by mutations in genes encoding presynaptic choline acetyltransferase and in fast channel syndromes (see later). The late-onset phenotype often follows a mild benign or episodic course and can easily be mistaken for seronegative immune-mediated MG or indeed not recognized at all. Patients frequently show marked weakness of ankle dorsiflexion (unusual in MG) in addition to proximal limb weakness. The majority of patients with rapsyn mutations (both early- and late-onset) respond extremely well to anticholinesterase medication, although some gain further benefit from the addition of 3,4-diaminopyridine. By contrast, even with the combination of anticholinesterase medication and 3,4-diaminopyridine, many patients with AChR- $\epsilon$  mutations remain moderately impaired (36,37).

**131.5.2.2 Molecular Basis.** By contrast with AChR deficiency due to AChR- $\epsilon$  mutations, nearly all rapsyn patients share the common missense mutation N88K (36). This greatly facilitates the genetic screen for this disorder. N88K is present in both Indian and European populations, and is thought to be derived from the same ancient founder (38). Patients may be homozygous for N88K or compound heterozygotes for N88K and a second mutated allele. A series of mutations have now been identified at the RAPSN gene locus (Figure 131-3B) (36,37). The precise mechanism through which each mutation caused the reduced number of AChR in the postsynaptic membrane has not been clearly defined, but it may involve the interaction of the AChR with rapsyn, the ability of rapsyn to self-cluster, and the stability of the rapsyn or the rapsyn-AChR clusters. In addition to mutations within the coding sequence, mutations have also been detected in the promoter region of the gene, and in particular  $-38A>G$  was observed at homozygosity and shown to arise from a common founder in near eastern Jews. Affected individuals with  $-38A>G$  show marked jaw and other facial malformations (39). The promoter mutations severely reduce the level of rapsyn mRNA transcription and may be found in the absence of N88K (39).

### 131.5.3 Fast-Channel Syndromes

**131.5.3.1 Clinical Features.** These recessive defects in the response to ACh are due to kinetic abnormalities of AChR function. Although the ultrastructure usually appears relatively normal, there may also be some reduction in endplate AChR numbers. Phenotype

may vary along with the particular molecular effects of the mutations, but these disorders may be severely disabling. Typically the patients present at birth with respiratory and/or bulbar problems, ophthalmoplegia and generalized muscle weakness. As is the case for RAPSN and CHAT mutations, apneic episodes are common in early life. In general they show a dramatic initial response to cholinesterase inhibitors but over time the benefit gradually reduces, and the addition of 3,4-diaminopyridine (3,4-DAP) is usually required.

**131.5.3.2 Molecular Basis.** The first defined examples were in compound heterozygotes with one low-expressor allele (40). In the other allele, an  $\epsilon$ L121P mutation caused reduced affinity for ACh and abnormally brief channel opening events. These kinetic effects were unmasked because of the concomitant null ( $\epsilon$ S143L) or low expressor ( $\epsilon$ G-8R) alleles (see iii earlier and Figure 131-3A). Other described fast-channel mutations are  $\alpha$ V132L,  $\delta$ E57K,  $\epsilon$ N182Y, and  $\epsilon$ D175N (41–43) and require the presence of a second null or low expressor mutation to unmask the fast channel phenotype (although  $\epsilon$ N182Y and  $\epsilon$ D175N were identified as heteroallelic in one patient; 42). A single example of dominant inheritance of a fast-channel syndrome caused by mutation  $\alpha$ F256L has been reported (44).

### 131.5.4 Slow-Channel Syndromes

**131.5.4.1 Clinical Features.** Usually, this disorder shows dominant inheritance. Age at onset and severity are strikingly variable, some carriers appearing unaffected. Most patients show selective severe involvement of wrist and finger extensor muscles. Frequently, there is a repetitive response to a single nerve stimulus and mepps and mep currents are prolonged. On microscopy, there is usually type I fiber predominance, and electron microscopy shows degenerative changes, sometimes with tubular aggregates that are termed an “endplate myopathy.” This also involves widening of the synaptic cleft, degeneration of the junctional folds, the subsynaptic mitochondria and nuclei, and vacuole formation. Unlike AChR deficiency and the fast-channel syndrome, this disorder is slowly progressive and anticholinesterase treatment or 3,4-diaminopyridine are ineffective or may even make the condition worse. Quinidine sulfate (serum levels of 0.7–2.5  $\mu$ g/mL) and fluoxetine, which both act to block the AChR ion channel when it is in the open state, are beneficial, although dose-related side effects may occur for both (45,46).

**131.5.4.2 Molecular Basis.** The slow-channel syndrome is caused by prolonged AChR channel activations. More than 18 different mutations have been reported (6). They occur not only in different subunits but also in different functional domains within them. They are all single amino acid changes and lead to a pathogenic gain of function in the AChR that explains their dominance. Mutations located in the M2 transmembrane segment

are thought to act predominantly by slowing channel closure (6). Others enhance the affinity of the AChR for ACh, which results in repeated openings of the channel during an extended period of ACh occupancy (47). These prolong the epp, causing the repetitive responses to single nerve stimuli. The pathogenicity of some of these mutations has been confirmed in transgenic mouse models (48).

Whatever the exact mechanisms, the prolonged openings apparently cause chronic, excessive entry of calcium ions through the open AChR channels resulting in an endplate myopathy. The myopathy may be exacerbated by activation of mutant channels by free local choline (49). In addition to this excitotoxic effect, persistent depolarization, resulting from summation of the prolonged endplate potentials, may cause muscle dysfunction at physiological rates of stimulation.

### 131.5.5 The Dok-7 Neuromuscular Junction Synaptopathy

**131.5.5.1 Clinical Features.** Dok-7 is a crucial molecule involved in the pathway that both clusters the AChR on the crests of the postsynaptic folds and also maintains the synaptic structure. Disorders resulting from mutations within Dok-7 are recessive (50). Age of onset varies, and some respiratory problems and stridor have been reported at birth or in early infancy (51). More commonly early motor milestones are normal and symptoms first present between 18 and 36 months with frequent falls. Thus, clinical onset is usually characterized by difficulty in walking after normal initial walking milestones. Ptosis may sometimes be present at birth but more usually develops in childhood. In general, proximal muscles are more affected than distal ones, leading to a typical “limb girdle” pattern of weakness, and, although ptosis was often present, extraocular muscles were rarely involved. Fluctuation in symptoms is common and patients may have been previously diagnosed with an unspecified congenital myopathy (52). There is a remarkable variation in disease severity with symptom onset ranging from birth to adolescence, or even into adulthood. Patients do not show long-term benefit from anticholinesterase medication and sometimes worsen; however, these patients generally show a remarkable improvement with ephedrine or salbutamol. The effect of these drugs is not seen immediately but rather there is a gradual but dramatic improvement over a period of months, and patients continue to improve for up to a year before the effect plateaus (53).

**131.5.5.2 Molecular Basis.** As is evident from the name, this disorder results from mutations in the adaptor protein, Dok-7. Dok-7 binds to the phosphotyrosine-binding site on MuSK located close to the postsynaptic membrane (20) and is thought to amplify the signal that results from Agrin binding. Mutations are located along the length of the Dok-7 protein. Surprisingly, Dok-7

polypeptides that are truncated in the C-terminal region remain partially active, and thus many of the mutations, including frame-shifting insertions or deletions are located in this region. This includes a common mutation c.1124\_1127dupTGCC that is found in around 60% of UK patients with Dok-7 mutations (50,52,54,55). In vitro cell culture experiments show that the Dok-7 mutations cause reduced agrin-induced AChR clusters, and that the form of these clusters is less complex, suggesting that Dok-7 is involved both in the maturation and maintenance of the neuromuscular junction structure.

### 131.5.6 Limb-Girdle CMS due to *GFPT1* Mutations

**131.5.6.1 Clinical Features.** These include fatigable weakness of the shoulder and hip girdle muscles, normal eye movements and minimal facial weakness, good response to esterase inhibitors, and evidence of tubular aggregates on muscle biopsy. As with most congenital myasthenic syndromes there is variability in severity, but in general onset is in childhood, usually slightly later than is seen for Dok-7 congenital myasthenic syndrome, and there is a deterioration in the strength of limb-girdle muscles over the following few years that limits sustained walking (27,52).

**131.5.6.2 Molecular Basis.** Classical linkage analysis and positional cloning have identified mutations in *GFPT1* to account for many of the cases of limb-girdle CMS associated with tubular aggregates on biopsy. Screening for *GFPT1* mutations in unsolved CMS cases with a wide range of different clinical phenotypes, but without tubular aggregates, was negative confirming that *GFPT1* mutations are associated with a distinct and recognizable CMS phenotype. *GFPT1* encodes the enzyme glutamine:fructose-6-phosphate amidotransferase 1, a key enzyme of the hexosamine biosynthetic pathway which yields the amino sugar UDP-N-acetylglucosamine (UDP-GlcNAc), which is an essential substrate for protein glycosylation. Thus, it may be that the glycosylation of components at the neuromuscular junction are impaired in this disorder (27).

### 131.5.7 Other Postsynaptic Syndromes

It seems probable that mutations in other key proteins involved in neuromuscular transmission will underlie additional CMSs (21). Examples described include reports of patients heteroallelic for mutations in MuSK (56), the postsynaptic voltage-gated sodium channel, *SCN4A* gene (26), or Agrin which stabilizes clustered AChR at the endplates (25). The patient with MuSK mutations had a fluctuating course of disease with episodes of severe respiratory distress in early childhood. Muscle biopsy revealed reduced endplate AChR levels; there was no clinical benefit from anticholinesterase medication on its own, but the patient did respond to



combined pyridostigmine and 3,4-diaminopyridine. A patient with a homozygous mutation in agrin, p.G1709R had evidence for disturbed neuromuscular junction structure although it did not directly affect AChR clustering in vitro. Agrin forms part of the core pathway, revolving around the synaptic organizer protein, MuSK, and from the few cases studied thus far it seems that  $\beta 2$ -adrenergic receptor agonists such as salbutamol or ephedrine will also be beneficial for these patients. In a severely affected patient with mutations in SCN4A, endplate potentials of normal amplitude failed to activate the postsynaptic voltage-gated sodium channels. This is due to the rapid inactivation of the  $\text{Na}_v1.4$  channels resulting from the aberrant V1442E. This mutation may show dominant inheritance; however, the other allele also harbored a clinically silent mutation S246L that causes small but detectable biophysical changes (26). In addition to mutations primarily affecting ion channels, it is possible that mutations in structural proteins disrupt neuromuscular transmission. An example is plectin, a structural protein of muscle and skin sometimes mutated in epidermolysis bullosa simplex. In one reported case, plectin expression was absent in muscle and severely reduced in skin. Striated muscle showed ultrastructural abnormalities with abnormal endplates and degeneration of the junctional folds (57), resulting in characteristic myasthenic weakness. Another case of heteroallelic mutations in  $\beta 2$  laminin, *LAMB2*, in a patient who had a kidney transplant has also been reported (58).

## 131.5.8 Synaptic Defects

### 131.5.8.1 Endplate Acetylcholinesterase Deficiency

**131.5.8.1.1 Clinical Features.** This recessive disorder usually presents with generalized weakness at birth or in infancy. Such symptoms together with delayed pupillary light reflexes may help to distinguish these cases from those with the slow-channel syndrome whom they often come to resemble in later life, both at EMG and in the muscle groups affected. Their disabilities probably again reflect the effects of prolonged ACh action, with AChR desensitization, persistent depolarization, and eventual endplate myopathy. Staining of endplates shows a loss or marked reduction in AChE that evidently prolongs the decay phase of the mepps. Morphological changes include small nerve terminals enveloped by Schwann cells, and degeneration of junctional folds. As expected, inhibitors of AChE have no effect or are detrimental, but both ephedrine and salbutamol may be beneficial (59), although the effects tend not to be quite as dramatic as seen in patients with Dok-7 CMS.

**131.5.8.1.2 Molecular Basis.** The AChE consists of enzymatic globular heads (arranged as tetramers or hexamers) that are encoded by the AChE gene on chromosome 7 (60). They are anchored to the basal lamina by the ColQ polypeptide (chromosome 3p24.2). It has a proline-rich attachment domain (PRAD), a central

collagen-like segment and a C-terminal region linking it to the basal lamina. Mutations, which may be homozygous or heteroallelic, have now been identified in each of the ColQ domains (59,61–63), but not yet in the catalytic globular heads. Thus, this disorder results from the failure to localize AChE at the endplate.

## 131.5.9 Presynaptic Defects

There are several reports of presynaptic defects in ACh release. They include inherited disorders resembling the LEMS (64) and also “familial infantile myasthenia,” which has recently been renamed as CMS with episodic apnea (73rd ENMC International Workshop: Congenital Myasthenic Syndromes), although this terminology is not ideal because many cases with RAPSN mutations show similar apneic episodes. The children with presynaptic defects may appear largely normal until a crisis develops, for example, during a bout of fever or stress. As in other presynaptic defects, the extraocular muscles are largely spared. Again the episodic crises, which may be life-threatening, become less frequent with age. On EMG, abnormal decrement is not found in resting muscle but is apparent after stimulation at 10 Hz for several minutes. Confirmation of diagnosis requires a normal-amplitude mepp in the resting state, abnormal decrease in mepp amplitude after 10-Hz stimulation for 5 min, and a normal number of AChR per endplate. These conditions usually respond to anticholinesterase medication.

**131.5.9.1 Molecular Basis.** Mutations in the gene encoding choline acetyltransferase (ChAT) have been shown to underlie some CMSs with episodic apnea (65). The mutations may markedly reduce ChAT expression or significantly impair its catalytic activity. It is not yet clear why there is selective vulnerability of the neuromuscular synapse to defective ACh resynthesis.

## 131.5.10 Fetal Akinesia Deformation Sequence (FADS)—Neuromuscular Junction Proteins

Neuromuscular transmission at nearly all normal adult muscle endplates is mediated by AChR consisting of  $\alpha 2\beta\delta\epsilon$  subunits, but for crucial periods of fetal development in utero transmission is mediated through the fetal form ( $\alpha 2\beta\delta\gamma$ ) of the AChR. Loss of fetal movement during these periods can lead to a series of developmental abnormalities.

**131.5.10.1 Clinical Features.** Multiple pterygia syndromes or Escobar syndrome is an autosomal recessive condition that manifests in orthopedic and cranial abnormalities. Characteristically, there is short stature, arthrogryposis multiplex congenita, pterygia of the neck and anomalies of the head including low-set ears, ptosis, a pointed and receding chin and high arched palate (66). Intrauterine death and stillbirths are common.

**131.5.10.2 Molecular Basis.** Mutations of the AChR  $\gamma$ -subunit gene *CHRNA7* have been found to underlie many cases of Escobar syndrome (Morgan; Hoffman). The loss of fetal AChR function associated with *CHRNA7* mutations are thought to result in fetal akinesia, which in turn causes the associated multiple developmental abnormalities. Surprisingly, some patients that harbor  $\gamma$ -subunit null alleles can survive, suggesting early expression of the  $\epsilon$ -subunit that partially compensates for loss of the  $\gamma$ -subunit might occur. Following birth, neuromuscular transmission is mediated by adult AChR and patients show little or no progression of their condition. Since the disorder results from lack of neuromuscular transmission at crucial developmental phases, it might be expected that recessive inheritance of loss of function mutations in other essential components of the neuromuscular junction, such as rapsyn, MuSK or DOK7 would also result in fetal akinesia, and indeed cases have been reported with mutations in RAPSN (67), DOK7 (68), and CHRNB1 and CHRNB2 (69).

The congenital myasthenic syndromes can be viewed as a spectrum of disorders, varying from lethal forms that cause death in utero through to those with only mild weakness. Definitive diagnosis of CMS often requires screening of candidate gene loci, followed by functional studies of the identified mutations in cellular systems. As reviewed by Engel and coworkers (1), experienced clinicians can often make a provisional diagnosis from the clinical and EMG features; also, it may be important to test affected muscles. Distinctive clinical features such as mild arthrogryposis at birth, the pattern of muscles affected, and response to anticholinesterase medication may suggest which gene is defective. In biopsied muscle, elongated, patchy or decreased AChE staining or reduced  $^{125}\text{I}$   $\alpha$ -neurotoxin binding may give further valuable diagnostic clues. Parental consanguinity or a positive family history (e.g. with affected siblings) are strongly suggestive of hereditary rather than autoimmune myasthenia, but of course are not essential criteria. Serum anti-AChR antibodies should be sought, although their absence does not exclude autoimmune MG because about 10–15% of cases are “seronegative” in the standard assay (see later). In neonates, it is particularly important to check both for these antibodies and for MG in the mother, partly for the sake of subsequent offspring as well as the proband. Finally, other causes of neonatal weakness, whether genetic (e.g. myotonic dystrophy) or acquired (e.g. botulism), should also be excluded.

## 131.6 ACQUIRED AUTOIMMUNE MYASTHENIA GRAVIS

### 131.6.1 Neonatal Myasthenia Gravis

The babies of about 10% of mothers with autoimmune MG have a transient and usually benign weakness, typically showing general hypotonia with difficulty in

feeding, swallowing, and crying but without prominent ocular signs (70–72). There is surprisingly little evidence of prenatal involvement, and weakness is usually noted within 1–3 days after birth. In more severe cases, there may be respiratory problems requiring assisted ventilation and even exchange transfusion. In most, however, treatment with anticholinesterases in advance of feeding suffices, and can be tapered to zero within the subsequent few weeks (13). It is probably wise to avoid breastfeeding. Only rarely does weakness persist for more than 6 weeks. With improved diagnosis and management, neonatal MG may now be less common than it was in the past.

Usually the maternal MG is the vital diagnostic clue, but the severity of the neonatal myasthenia often correlates poorly with it. Neonatal myasthenia is predicted better by high anti-AChR antibody titers in the mother, particularly against fetal AChR, and especially by the severity of her previous babies' myasthenia (70), which tends to be consistent from sibling to sibling. Conversely, in rare cases, the mother's MG may be diagnosed only because of the transmission to her baby; if so, anti-AChR assays on mother and baby are often invaluable. Occasionally, however, seronegative MG may be transmitted to a baby, supporting its autoimmune etiology. In other rare cases, recurrent severe fetal arthrogryposis occurs (73), sometimes even though the mother is asymptomatic. This suggests an idiosyncratic preference of the mother's antibodies for fetal AChR (74). This syndrome may result in abortion or stillbirth, but less severely affected babies may overcome their handicaps as their AChR matures to the adult form and their maternal antibody levels wane, leaving only mild residual disability.

### 131.6.2 Acquired Myasthenia Gravis

**131.6.2.1 Prevalence.** The overall prevalence of MG is approximately 1 per 5000 in Europe and North America, and does not differ greatly in other countries as far as is known (75). In the Far East, up to 30% of all cases show onset before 10 years of age, or even in infancy, and there are relatively fewer late-onset cases (after age 50 years). By contrast, in the children, the MG is mainly ocular and frequently associates with ophthalmic Graves' disease; whereas anti-AChR antibody titers are often low or negative in the standard assay, use of AChR from extraocular muscle increases the frequency of positivity substantially (76).

**131.6.2.2 Clinical Features.** As reviewed in Engel (77), acquired MG is characterized by weakness of skeletal muscle, which is typically fatigable and thus usually at its worst in the evenings. In all subgroups of patients (Table 131-2) the onset is often insidious but may coincide with infections or periods of stress. It commonly first affects movements of the eyes and, at a later stage, may involve bulbar, neck, hand, limb, or respiratory muscles. Tendon reflexes are usually brisk. In severe

**TABLE 131-2 Subgroups of Myasthenia Gravis Patients in the United Kingdom**

	Seropositive Generalized Myasthenia Gravis				Seronegative Generalized	
	Early-Onset	Late-Onset	Thymoma	Ocular MG	Anti-MuSK Pos	Neg
Prevalence	40%	40%	10–15%	10–20%	5–10%	~10% (% of total cases)
Onset age	<40 years	>40 years	15–90 years	0–90 years	10–50 years	10–50 years
Male: female	1:4	1.5:1	1.3:1	1.7:1	1:2	1:3
Serum anti-AChR: prevalence/titer	100% High	100% Low–medium	100% Medium	~60% Low	0% –	0% –
Thymic changes hyperplasia	Follicular hyperplasia	Atrophy hyperplasia	Thymoma	None or mild	Normal-for-age	Normal or mild
Striated muscle antibody	Rare	30–40%	100%	Rare	Rare	Rare
HLA association	(A1,B8,DR3)	(A3,B7,DR2) (n = 90)	Not detected (n = 60)	Not detected (n = 10)	Not detected (n = 10)	Not detected

cases, difficulties in swallowing and breathing can be life-threatening. Its mortality has declined greatly since the advent of assisted ventilation, plasma exchange, and particularly, corticosteroids. There are excellent handbooks on its natural history and therapy (77,78).

**131.6.2.3 Patient Subgroups and Diagnosis.** The classification and diagnosis of patients (see Table 131-2) have also been greatly helped by the highly specific assay for autoantibodies to the AChR (10,79). In whites, MG only occasionally begins before puberty. When it does, there may rarely be recurring apneic attacks/respiratory crises, and the parents should be trained in their management. Otherwise, the myasthenia in this juvenile MG subgroup resembles that in other early-onset cases, including the response to thymectomy (see later, and References (80) and (81)). Typical early-onset MG in the United Kingdom appears in the teens and 20s, and has a heavy female bias (see Table 131-2). The characteristic thymic abnormalities and human leukocyte antigen (HLA) associations distinguish these patients sharply from those of patients with the late-onset condition (see later). The clinical course in early-onset cases is very variable; 20–30% of patients experience long-term remissions, especially after thymectomy. The myasthenia may fluctuate, and in another 20–30%, the disease may progress and require long-term immunosuppressive therapy. Patients with late-onset disease often show more stable behavior, but MG associated with thymoma tends to deteriorate, especially after thymectomy. Patients with pure extraocular muscle involvement for at least 3 years rarely progress to generalized MG, and their serum antibody levels tend to be low or undetectable (10). In these cases, and in “seronegative” patients with typical generalized MG symptoms, there is good evidence for other pathogenic autoantibodies that are not measured in the standard assay (7). These recognize MuSK in some cases, especially those from southeastern Europe; their MG is often more difficult to control (82).

The diagnosis depends principally on the clinical picture, on the detection of anti-AChR antibodies by radioimmunoassay (10), and on EMG, which shows typical

decrement and jitter (5). A relatively sensitive and specific bedside test that distinguishes myasthenia from other causes of ptosis involves application of crushed ice in a latex glove to the eye. This leads to improvement of ptosis in MG and has been reported to have a sensitivity of 89% (83). In the edrophonium (Tensilon) test, strength is measured (e.g. by peak expiratory flow or degree of ptosis) before and after intravenous injection of short-acting anticholinesterase drugs. Because it carries some risk, this test is now mainly reserved for seronegative patients, in whom the diagnosis is reinforced by EMG, although neither test is totally specific for MG; similar abnormalities can occur both in congenital myasthenias and in the LEMS. MuSK antibodies are more prevalent in on the latitude of Mediterranean countries and are rarer further from the equator. MuSK positive patients are more likely to have early bulbar and respiratory symptoms with less severe limb involvement and only rare ocular symptoms (72). The clinical neurophysiology in MuSK usually shows abnormal SFEMG of facial muscles but RNS of limb muscles can be normal.

A small proportion of patients remain consistently negative for both AChR and MuSK antibodies. Clinical features and thymic pathology imply that seronegative myasthenia gravis (SNMG) is similar to MG caused by AChR antibodies. This has led to the hypothesis that the patients have antibodies to AChR that are not detected with conventional assays. Indeed a proportion of such patients can be shown to have antibodies that bind AChRs expressed in a cell lines in which AChR are aggregated on the cell surface by co-expression with the AChR-clustering protein, rapsyn (53). There are also reports of serum antibodies to the MuSK associated protein LRP4 (84), although this is rare and it is unclear whether not this antibodies are pathogenic.

**131.6.2.4 Pathology.** Pathological changes in striated muscle are not dramatic. Especially in thymoma cases, there may be sporadic foci of lymphocytes (lymphorrhages) not normally related to the endplates, which themselves are only mildly or minimally infiltrated by macrophages and occasional lymphocytes.

Immunolabeling has shown immunoglobulin G (IgG) and complement deposits on the postsynaptic membrane (85), and these include the membrane attack complex (i.e. even C9). The resulting damage greatly reduces the complexity of the synaptic folds, and thus the area of AChR-bearing membrane. Accelerated degradation of AChRs (by cross-linking and shedding) exaggerates this reduction in AChR numbers (86), which largely accounts for the myasthenic weakness. Direct blockade of AChR function is probably important only in a minority of cases (87).

### 131.6.3 Thymomas

Thymomas occur in 10–20% of myasthenics worldwide; although little is known of their etiology, mutations in the neoplastic epithelial cells vary strikingly between thymomas. In contrast with the other subgroups, these patients almost always have antibodies to striated muscle antigens as well as AChR (88). The neoplastic cells in the thymoma are usually mainly of cortical epithelial origin (89); this is the uniquely thymic cell type that normally “positively selects” developing T cells that can recognize foreign peptides when bound to self-HLA class I or class II molecules. The tumors usually resemble disorganized thymic cortex, both histologically and functionally, and frequently generate very many thymocytes and maturing T lymphocytes (89). Some of these might be aberrantly selected by AChR or striational epitopes expressed by the epithelial cells (88), and could subsequently initiate antibody responses to them after emigrating to the periphery (reviewed in Reference (90)). In theory, these tumors could instead be exporting T cells that have not been properly rendered self-tolerant in these disorganized tumors. Because MG develops in so many (30–50%), of all thymoma patients, and other autoimmune associations are rare (91), active selection seems more likely.

**131.6.3.1 Follicular Hyperplasia.** In the follicular hyperplasia seen in most early-onset cases, the changes are completely different, and are mainly medullary. The residual thymic epithelium is compressed by perivascular infiltrates of lymph node-type T-cell zones and germinal centers (reviewed in Reference (92)). Although these include mature/recirculating T and B lymphocytes specific for extrinsic antigens (e.g. influenza virus) (93), AChR-specific plasma cells are selectively activated in situ, and spontaneously secrete antibodies in culture. Indeed, these appear to be end-products of ongoing mutations in the germinal centers, which often include AChR-specific cells. They may be activated by the rare muscle-like myoid cells (94), found in both the normal and MG thymic medulla that express AChR of the fetal type. This might also explain the frequent improvement in these patients’ myasthenia after thymectomy, but it is still controversial (90). Similar changes are seen in some patients with neither anti-AChR nor anti-MuSK

antibodies, but they are uncommon in those with anti-MuSK.

## 131.7 IMMUNOGENETIC ASSOCIATIONS

In a summary of monozygotic twins with MG, only six of 18 pairs were concordant for symptoms and seven for anti-AChR antibody (95), and this figure is probably inflated by the usual biased reporting of concordant pairs. Nevertheless, all of 13 dizygotic pairs were discordant, implying a substantial genetic component to their susceptibility. Recurrence of MG in close relatives occurs in only about 2% of patients, who occasionally even have a thymoma (66,93,96). There is no obviously preferred transmission pattern (e.g. mother-to-daughter), and multiplex families are rare (97). There are also elevated frequencies of other autoimmune diseases and autoantibodies in MG (96,98), and in first-degree relatives of patients, especially on the mother’s side of the family but there have been no systematic immunogenetic studies such as those reported for thyroid disease (99).

A predisposition to the disorder is observed for certain alleles of some AChR subunit genes, especially the  $\alpha$ -subunit (100–102) and possibly the  $\delta$ -subunit (103,104). These associations are seen in both early- and late-onset MG. Because the  $\alpha$ -subunit association is almost confined to subjects with HLA-DQ5 (105), it suggests influences of AChR expression on induction of autoimmune T cell responses, or possibly on the course of the MG.

### 131.7.1 Early-Onset Myasthenia Gravis

In whites with early-onset MG, the strong association with HLA DR3, B8, and A1 (96), and its restriction to this subgroup were soon noted (98). Initially, the particularly strong linkage disequilibrium in this haplotype helped to highlight the association but has since made precise localization of the key susceptibility genes more difficult than in some other autoimmune diseases. MG is an antibody-mediated disease, and helper T cells, which recognize peptides presented by HLA-class II antigens, are widely held responsible for initiating the autoimmune response. Nevertheless, in northern Europeans, the associations have consistently been slightly stronger with HLA-B8 in the class I region than DR3 in class II (78,96,98). There may be predisposing loci in both class I and class II regions, or near the TNF/Lymphotoxin locus in class III, especially when anti-AChR antibody titers are high (101,106). There are likely to be further contributions from other immunological genes, for example, encoding cytokines or adhesion molecules (107). The female bias, the stronger HLA-DR3 and B8 associations in females, and their restriction to the child-bearing years, are all highly suggestive of hormonal interactions (108). Other autoimmune disorders are common in early-onset MG, especially on the mother’s side of the family.



### 131.7.2 Myasthenia Gravis in Chinese and Japanese

By contrast, in Chinese and Japanese MG patients, the most consistent associations are with the HLA-DR9-B46 haplotype (56,109), as in autoimmune thyroid disease. Interestingly, the association in MG is even stronger with DR9 than with B46, and it is most pronounced in the patients with earliest onset (109) in apparent contrast with the rare juvenile MG in northern Europeans. Again, familial recurrence is rare (109), and some distinctive provoking factor in the Far East seems likely.

### 131.7.3 Other Subgroups

In northern European men with late-onset MG, modest increases in HLA-DR7 (103) and in HLA-DR2 and -B7 replace those in DR3 and B8, although the associations and linkage disequilibrium are weaker (110). Finally, it is an intriguing puzzle that patients with thymoma or with pure ocular MG have shown no consistent HLA associations, even though the disorder is again clearly autoimmune. Perhaps the thymoma provides such a strong stimulus that it overrides genetic limitations.

### 131.7.4 Immunoglobulin Genes

In Japanese patients, there are also relatively strong associations with immunoglobulin heavy chain (IgH) constant region allotypes (111). They were noted in most subgroups of Japanese patients, especially in those of early onset or with thymoma, but have not been seen in the corresponding white or Chinese subgroups. There could be heritable differences in the variable region genes, perhaps encoding particularly pathogenic anti-AChR antibody specificities.

### 131.7.5 Conclusion

In conclusion, the striking differences, both in immunogenetics and in thymic histology, strongly suggest that there are different provoking factors in these subgroups (98). One particularly telling example is the typical seropositive generalized MG seen in about 1% of patients taking the drug d(-) penicillamine for rheumatoid arthritis or Wilson's disease. The antibodies and weakness disappear after withdrawal of this drug. These patients often have HLA-DR1 or DR7 with or without HLA-B8 (105), whereas other autoimmune complications of this therapy show different associations. Perhaps this very simple drug, with its reactive sulfhydryl group, modifies resident peptides in HLA-DR1 or linked molecules so as to provoke an AChR-specific T cell response.

## 131.8 MANAGEMENT

First-line therapy in MG is with anticholinesterases, especially the long-acting pyridostigmine, and in many

patients, this agent alone affords adequate control, although ocular weakness may respond poorly. In young patients with seropositive generalized MG, most neurologists favor early thymectomy, because about two thirds of patients can subsequently be maintained on pyridostigmine alone. The benefits of thymectomy have never been rigorously proved (e.g. in a randomized trial) and about 30% of patients require additional immunosuppressive therapy, for example, with alternate day corticosteroids, perhaps supplemented with azathioprine (to reduce the steroid dose). Although the thymomas are usually slow growing, their local spread can be dangerous and they should be sought and removed except in elderly patients and very frail cases. Invasive thymomas may recur without any change in either the anti-AChR antibodies or in the symptoms of the patients, who should therefore be regularly monitored radiographically. Perhaps surprisingly, the MG in these patients seldom resolves after surgery. Plasma exchange and intravenous immunoglobulin are usually reserved for crises and to improve strength preoperatively. Management strategies for MG are much more extensively reviewed in the handbooks cited earlier and by Newsom-Davis (112).

## 131.9 LAMBERT-EATON MYASTHENIC SYNDROME

LEMS is a rare disorder, characterized by proximal muscle weakness, diminished tendon reflexes, and autonomic symptoms, especially dry mouth, constipation, and impotence. The cardinal physiological defect is in ACh release at nerve endings, apparently caused by autoantibodies to voltage-gated  $\text{Ca}^{2+}$  channels (VGCC) (113). In particular, there is a reduced number of packages of ACh released after each nerve impulse (quantal content), and transmission is improved after maximal voluntary contraction of the relevant muscle (post-tetanic potentiation). The autonomic symptoms and EMG signs are helpful features in distinguishing LEMS from MG.

### 131.9.1 Etiopathogenesis

LEMS is a classic paraneoplastic syndrome (114). About 60% of cases have a small cell lung cancer (SCLC), and about 3% of patients with SCLC develop LEMS (although they may not always be recognized). These tumors are strongly associated with tobacco smoking, but little is known of their genetics. The onset of LEMS often precedes the diagnosis of the tumor by up to 5 years and can thus be a vital early warning. This condition tends to ameliorate if the tumors can be removed or destroyed (115), suggesting that they are driving the autoantibody response (116). In support of that, cultured SCLC cells express VGCC that are recognized by the patients' autoantibodies (117).

Equally in patients without SCLC, in whom there are no known provoking factors, LEMS symptoms improve

2–4 weeks after plasma exchange, and its physiological defects can also be passively transferred to mice with patients' Ig (118). Nerve terminals show disorganization of the active zone particles that are associated with the target  $\text{Ca}^{2+}$  channels, a disruption very similar to that seen in the patients (119). This effect probably depends mainly on cross-linking by divalent antibodies rather than on complement-mediated damage. Apart from striking type I fiber loss in some cases (120), there is no gross muscle pathology. Recent work on the different subunits and subtypes of VGCC shows that the  $\alpha 1A$  subunit of the P/Q channel type is the target in LEMS (121,122). The autoantibodies can be assayed using detergent-solubilized  $\text{Ca}^{2+}$  channels from human cerebellum prelabeled with appropriate toxins, especially from the marine snail *Conus magus*, assays that are now highly efficient and specific (12). As in MG, titers tend to decline after plasma exchange or immunosuppressive therapy (123).

### 131.9.2 Genetics

Because the autoimmune response must often begin when the tumors are small, these patients are presumably high responders. In a small study, they have shown modest IgH associations (with the Gml, 2, 21 haplotype) (124) that require confirmation. In the patients without SCLC, the onset age ranges from 10 to 70 years, and there is no strong sex bias or familial incidence. There is a considerably increased risk of other autoimmune diseases (especially vitiligo, alopecia, and thyroid disease) (124). There is also a significant increase in HLA-DR3 and B8 frequencies (125,126).

### 131.9.3 Management

In all cases an intensive search for lung tumors should be initiated at diagnosis and regularly thereafter. Patients with a history of smoking, cerebellar dysfunction and older patients with rapid progression of symptoms are more likely to have an underlying malignancy (117). In patients with SCLC, therapy for the tumor must obviously take priority, and often, by itself, resolves LEMS (115). Interestingly, the tumors apparently have a better prognosis in patients who also have LEMS (127). In any patients, the drug 3,4-diaminopyridine often enhances neuromuscular transmission (Versheuren et al.). Immunosuppression with corticosteroids with or without azathioprine is reliably effective, as is plasma exchange in crises. Therapy in LEMS has been reviewed (113).

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# CHAPTER

# 132

## Motor Neuron Disease

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### 132.1 INTRODUCTION

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig disease, motor neuron disease or Charcot disease, is an adult onset, progressive, neurodegenerative disorder involving the large motor neurons of the brain and the spinal cord. It produces a characteristic clinical picture with weakness and wasting of the limbs and bulbar muscles, leading to death from respiratory failure within five years. It was in 1874 that the French neurologist Jean Martin Charcot defined the clinical and pathological feature of the disease and gave it its name of amyotrophic lateral sclerosis. Sir William Osler recognized the hereditary component of the condition in 1880. More than a century later, the first gene linked to adult onset autosomal dominant ALS, Cu, Zn superoxide dismutase (*SOD1*) was identified and that led to the engineering of the first transgenic animal model of the disease. This mouse model became the backbone of ALS research and has been used worldwide to investigate pathogenic mechanisms and to test experimental therapies. In the past decade the list of genes causing motor neuron disease grew significantly, with the identification of *ALSIN*, which causes a rare form of recessive juvenile onset ALS with predominant upper motor neuron features, or *ALS2*, followed by the discovery of *SENATAXIN* (*SETX*) mutations causing *ALS4* and *VAPB* causing *ALS8*. The identification of TDP43 as the major disease protein in the ubiquitinated inclusions in ALS and frontotemporal dementia led to the detection of mutations in *TDP43* in a subset of patients with familial ALS or *ALS10*. The function of TDP43 as a DNA/RNA binding protein shifted ALS research to the role of DNA/RNA metabolism in motor neuron degeneration. Mutations in *FUS/TLS*, a gene that encodes a protein of similar function to TDP43, were found to cause *ALS6* and further implicated DNA/RNA fate in the pathogenesis of ALS. Mutations of the Optineurin (*OPTN*) and Valosin-Containing Protein (*VCP*) genes were recently linked to autosomal dominant ALS, whereas SGP11-associated gene *SPATACSIN* was found to cause autosomal recessive juvenile ALS or *ALS5*.

Recent breakthroughs in ALS genetics are the discoveries of ubiquilin2 (*UBQLN2*) as the causative gene of X-linked juvenile and adult-onset ALS and ALS with dementia, and the hexanucleotide repeat expansion in *C9ORF72* as the cause of ALS/Dementia and ALS linked to chromosome 9p21. Several other genes such as Angiogenin (*ANG*), *FIG4* and D amino acid oxidase (*DAO*) make small contributions to familial ALS, expanding the pathogenic mechanisms which lead to motor neuron death.

Although genetic studies of familial ALS have helped to better understand some of the molecular pathways involved in motor neuron degeneration, 84% of ALS is sporadic, without clearly defined etiology. The completion of the human genome project and the international Hap Map project together with the development of high throughput Genome-Wide Association Studies (GWAS), whole exome and whole genome sequencing technology have opened the door to the identification of genetic risk factors for sporadic ALS or provided important clues to disease pathogenesis. The interpretation of GWAS results and the translation of the genetic findings had proved to be challenging, since results were not replicated in different populations. Of all the susceptibility genes revealed by the different GWAS studies, the paraoxonase gene cluster has been extensively examined in the past years and has emerged as a robust genetic risk factor, although the result of the GWAS examination of its association with sporadic ALS is controversial. Interestingly, the ALS-FTD locus on 9p21 is also associated with sporadic ALS with a very high statistical probability, but the cohort of patients used initially may have been contaminated with familial cases. Recent studies show that about 5% of apparently sporadic cases have an expanded hexanucleotide repeat in their *C9ORF72* gene.

### 132.2 HISTORY

The term “Amyotrophic Lateral Sclerosis” was coined by Jean Martin Charcot in his 1874 clinico-pathological description of the disease; however Charcot was not



the first to describe ALS. In 1850, Aran published data from a series of eleven patients with progressive muscle weakness and atrophy of the limbs that he called progressive muscular atrophy (PMA). Duchenne was given some credit in the description of this disease, which was often referred to as Aran–Duchenne disease. Both Aran and Duchenne considered PMA a muscle disease, until Cruveilhier in 1853 studied the autopsy of one of Aran's patients, noted atrophy of the anterior spinal roots and the motor nerves and suspected a neurogenic etiology for the syndrome. Duchenne was the first to describe progressive bulbar palsy that he called “glosso–labial–laryngeal paralysis” and Erb was the first to describe primary lateral sclerosis (PLS) in 1875. Gowers was the first to group the three phenotypes under the same syndrome that was later named motor neuron disease by W. Russell Brain.

Although Aran may have alluded to the familial nature of ALS, it was Sir William Osler in 1880 that recognized that the Farr family of Vermont had a dominantly inherited form of ALS. It was not until a century later that genetic analyses of ALS kindreds, including the Farr family, led to the identification of *SOD1* as the first causative gene for ALS (1–4). Although genetic discoveries were initially slow, they quickly picked up in this past decade with the discovery of more than ten genes linked to familial motor neuron disease and the generation of a plethora of animal models from fruit flies and zebra fish to mice and rats. More recently, the tools of modern genetics, such as genome wide association studies (GWAS), have been applied to sporadic ALS in the quest for susceptibility genes that may help us better understand the complex genetic and environmental interactions that lead to this disease.

Because the etiology of this disease is mostly unknown, it is difficult to develop adequate therapies and despite all the genetic progress, clinical trials remain deceiving. Riluzole is the only FDA approved treatment, and has only a marginal effect on disease progression. The recent generation of patient specific motor neurons from induced pluripotent stem cells (iPS) obtained through the epigenetic reprogramming of skin fibroblasts (5) will open new horizons for in vivo disease modeling and drug discovery.

### 132.3 EPIDEMIOLOGY

ALS is the most common adult onset motor neuron disease; it has an incidence of about 2/100,000 and a prevalence of approximately 6/100,000 (6). The incidence of ALS is higher in men with a male to female ratio of 1.5:16, the incidence also increases with age, mainly between the ages of 55 and 65.

The frequency of ALS seems to be uniform around the world except for the western Pacific form of ALS. In 1950s, ALS in association with dementia and Parkinsonism was reported to occur 50–100 times more frequently in the Chamorro people of Guam, in the Kii peninsula of Japan and in the western part of New Guinea (7,8).

Although the incidence of this disease has significantly decreased in the past decades, its etiology remains elusive.

About 10% of ALS patients have either a first, second or third degree affected relative. Familial ALS can be transmitted as an autosomal dominant or recessive trait. Adult onset autosomal dominant transmission is more common than recessive transmission. Recessive transmission is usually limited to patients with juvenile onset ALS. Our group has reported five families with an unusual transmission of ALS as a dominant X-linked trait (9).

### 132.4 CLINICAL FEATURES

ALS presents in about two third of the patients with an asymmetric weakness and atrophy of one of the limbs (spinal onset ALS). Foot drop, difficulty in walking, loss of fine hand movements or shoulder weakness is often reported by patients as an early symptom, sometimes preceded by cramps. In the remaining one-third of the patients, weakness starts in the bulbar muscles (bulbar onset ALS), causing dysarthria followed by dysphagia. Physical examination reveals a combination of upper and lower motor neuron signs in the motor segment with features of weakness, atrophy and fasciculations in the affected muscle groups, together with brisk tendon reflexes and the absence of, or minimal sensory findings. The disease is progressive and usually spreads to anatomically connected segments of the neuroaxis, and most patients go on to develop bulbar and eventually respiratory symptoms that lead to death from respiratory muscle weakness. In a rare subset of patients, respiratory muscle weakness can be the presenting symptom. The extraocular muscles are usually not affected, while bladder and bowel sphincters are spared until late in the disease. Patient with ALS have normal cognition but neuropsychological testing may reveal frontal network dysfunction and personality traits coincident with perfectionism and elements of inflexibility with poor insight and judgment in about half the patients. Dementia of the frontal-temporal variant has been recognized to occur in association with ALS in a minority of cases.

The El Escorial criteria have been developed to standardize the diagnosis for clinical research (10). The diagnosis of ALS is based on history and an examination that shows progressive upper and lower motor neuron findings. The diagnosis is supported by results from electrophysiological study of peripheral nerve and muscle, MRI imaging of the central nervous system and laboratory tests that help exclude disorders mimicking ALS, such as multifocal motor neuropathy with conduction blocks, spinal spondylosis, syringobulbia, etc.

While ALS is incurable, symptomatic management provided in multidisciplinary ALS clinics improves patient quality of life and has a positive influence on disease progression. Unfortunately, the progression of the disease is relentless and most patients die within five years of disease onset.

### 132.5 CLINICAL VARIANTS OF AMYOTROPHIC LATERAL SCLEROSIS

Progressive muscular atrophy, a pure lower motor neuron syndrome, primary lateral sclerosis, a pure upper motor neuron syndrome, progressive bulbar palsy, brachial amyotrophic diplegia and monomelic atrophy are variants of ALS that have different clinical presentations, rate of progression and prognosis. It is not clear whether they represent entirely separate disease entities or a continuum of the same disorder. Family studies favor the latter.

### 132.6 PATHOLOGY

The pathology of ALS is characterized by the loss of pyramidal Betz cells in the motor cortex, as well as loss and degeneration of the large anterior horn cells of the spinal cord and lower cranial motor nuclei of the brainstem (11). The degenerating motor neurons display intracellular aggregates forming distinct inclusion bodies: ubiquitinated inclusions, Bunina bodies and hyaline conglomerate inclusions. Ubiquitinated inclusions are mainly found in the lower motor neurons of the brainstem and spinal cord. They can have a filamentous, skein-like or a rounded compact shape and are called Lewy body-like inclusions. TDP43 was recently found to be a major constituent of the ubiquitinated inclusions in sporadic ALS (12). Bunina bodies are small eosinophilic, hyaline intracytoplasmic inclusions that stain positive for cystatin C and transferrin and are considered specific to ALS (13). Hyaline conglomerates are highly immunoreactive for neurofilaments and are mainly found in the motor neurons of familial ALS patients.

Aside from the obvious selective neuronal loss and the morphological changes in the remaining motor neurons affected, ALS tissue also shows alteration of non-neuronal structures. Affected areas have been shown to be the site of a robust glial reaction composed of abundant activated microglia and astrocytes (14).

### 132.7 GENETICS OF FAMILIAL AMYOTROPHIC LATERAL SCLEROSIS

#### 132.7.1 ALS1 or SOD1-Linked ALS

One of the important breakthroughs in ALS research was the discovery in 1993 that mutations in the gene encoding the enzyme copper-zinc superoxide dismutase (*SOD1*) were associated with familial ALS (1,2). Since that report, more than 150 mutations in *SOD1* have been described, consisting mainly of amino acid substitutions, and more rarely of amino acid deletions or truncations. *SOD1* mutations account for 20% of familial ALS and for 2–7% of sporadic patients ([www.alsod.org](http://www.alsod.org)). The mode of inheritance is autosomal dominant with age dependent penetrance, except for the D90A mutation, which is transmitted in an autosomal dominant or recessive

fashion. In the US, the A4V mutation is the most common mutation, followed by the I113T mutations (15). In the UK, it is the I113T mutation (16), in Germany the R115G mutation (17) and the D90A is the most common mutation in patients of Scandinavian origin (18).

The clinical phenotype of patients with familial ALS due to *SOD1* mutations tend to show a preponderance of lower motor neuron features and more frequently presents with signs in the lower limb, with loss of the Achilles reflex (T. Siddique unpublished observation). This type of monomelic presentation can persist for many months before progressing and is reflected in the revised El-Escorial criteria (19).

The mean age of *SOD1* linked FALS is  $45.5 \pm 8.9$  (3) which is lower than that of sporadic ALS and the mean disease duration is of  $3.4 \pm 4.5$  years (3). There is a significant variability in age of onset and disease progression based on the type of the mutation and sometimes even in families with the same mutation. The A4V mutation has an aggressive, rapid course with a mean survival time of  $1.0 \pm 0.4$  years (3,4). In comparison, the G37R, G41D, H46R and G93C mutations have been associated with a more prolonged survival time of at least 10 years (3,15). The I113T, G93R and G85R mutations have been reported to cause a marked variability in disease progression from about two years to one or two decades (20). The I113T mutation has a reduced penetrance, (20) is one of the most common worldwide mutations, and is most frequently found in apparently sporadic ALS patients.

To explain the high prevalence of the A4V mutation in the United States, Saeed and colleagues determined that the North American A4V mutation arose from two founders, Native American (82%) and European (18%), about 400–500 years ago at the time of Jamestown and Plymouth landing (4).

The homozygous D90A mutation has a characteristic phenotype, with progression of symptoms in two phases: the preparetic phase characterized by low back pain, leg pain, stiffness and cramps in the legs for months or even years, followed by the paretic phase, with insidious onset of weakness in one leg with absent deep tendon reflex distally but brisk proximally. Patients sometime have atypical features such as intermittent ataxia and bladder disturbances. The disease has a slow progression, with mean survival of 13 years (18).

Unlike sporadic ALS, the neuropathology of *SOD1*-linked ALS is characterized by relative sparing of the motor cortex, slight or mild corticospinal tract involvement that contrasts with the severe atrophy of the anterior roots and the degeneration of lower motor neurons. Certain *SOD1* mutations such as the A4V, I113T and E100G mutations are also characterized by the involvement of the dorsal columns and the atrophy of the posterior roots (21,22). The cell bodies of surviving motor neurons contain hyaline conglomerate inclusions that are specific for certain *SOD1* mutations and Lewy body-like

inclusions. These inclusions are immunoreactive for SOD1 but not for TDP 43 or FUS (11,22,23). Bunina bodies are not a feature of SOD1 familial ALS (11).

Superoxide dismutases (SOD) are a group of enzymes that catalyze the dismutation of superoxide radicals ( $O_2^-$ ) to molecular oxygen ( $O_2$ ) and hydrogen peroxide ( $H_2O_2$ ), providing cellular defense against reactive oxygen species (24).

There are three isoforms of SOD in humans: cytosolic copper and zinc superoxide dismutase (SOD1, Cu, Zn SOD) encoded by chromosome 21q22, mitochondrial manganese superoxide dismutase (SOD2, MnSOD) encoded by chromosome 6q25 and extracellular superoxide dismutase (SOD3, EC-SOD) encoded by chromosome 4p15.

SOD2 or SOD3 mutations have not been described in ALS patients.

The *SOD1* gene contains five exons and four introns that are transcribed in five splice variants (25). SOD1 is a ubiquitously expressed and highly conserved 153 amino acid protein. It is composed of two identical subunits that form a 32 kDa homodimer, with their active site facing in opposite directions. Most of the protein is folded into an eight-strand beta-barrel and is stabilized by extensive hydrogen bonding. Each subunit contains a copper and zinc binding site and is held together by a disulfide bridge between cysteines 57 and 146 (26).

The role of *SOD1* mutations in familial ALS continues to be investigated. Most of our current understanding of the pathogenic role of mutant *SOD1* comes from the study of transgenic rodents that over-express mutant *SOD1*, and in particular from the widely used mouse model that over-expresses SOD1<sup>G93A</sup> mutation, originally developed by Gurney et al. in 1994 (27). The fact that mice deficient in *sod1* gene or over-expressing wild type *SOD1* do not develop motor neuron disease suggested early on that mutant *SOD1* acquired new toxic properties. The main toxicity of mutant *SOD1* is apparently independent of its enzymatic function, since the G93A mouse model develops progressive motor neuron degeneration despite high SOD1 activity levels, (27) while other rodents with the G85R mutation also develop an ALS-like phenotype but have reduced enzymatic activity (28). The development and the severity of the ALS-like phenotype in the transgenic animal depend on mRNA levels of mutant proteins, with gene dosage affecting the life span of the animal. Reduction of SOD1 expression using RNA interference in the G93A mouse model resulted in delayed disease onset and prolonged survival of the animal (29).

Mutation in *SOD1* causes conformational change and misfolding of the protein, which induces the formation of the aggregates observed in the spinal cord of familial ALS patients and the transgenic animals (30). Although the exact toxic mechanism by which the misfolded SOD1 causes the death of motor neurons is not fully known, many studies have demonstrated that the misfolded

protein causes mitochondrial dysfunction (31,32), disrupts axonal transport (33), overloads the unfolded protein response and causes ER stress (34).

SOD1 aggregates/inclusions in lower motor neurons are a prominent pathological feature in ALS patients with *SOD1* mutations and transgenic mouse models overexpressing mutant *SOD1*. These SOD1 aggregates appear to be specific to SOD1-linked, but not other types of ALS. Deng et al. proposed an oxidation-mediated protein aggregation hypothesis to explain the molecular mechanism of SOD1 aggregation. They hypothesized that the ALS-linked SOD1 aggregates are formed by SOD1 dimers and multimers, which are crosslinked through intermolecular disulfide bonds via oxidation of cysteine residues in SOD1. Consistent with this hypothesis, they have shown SOD1 dimers and multimers that are cross-linked by disulfide bonds in mutant *SOD1* transgenic mice. Moreover, they observed that wild-type *SOD1* could exacerbate the ALS phenotype or also convert an unaffected phenotype to an ALS phenotype in mutant *SOD1* transgenic mouse models. They demonstrated that the effects of wild-type *SOD1* on mouse phenotype are associated with a recruitment of wild-type SOD1 into aggregates by mutant *SOD1* reminiscent of prion-like behavior. This is consistent with the concept of the spread of the disease foci in the neural space. These data, therefore, have provided the first evidence that links protein oxidation and protein aggregation to neurodegeneration. The molecular mechanisms of protein aggregation in other types of ALS still remain elusive (31).

The role of non-neuronal cells in motor neuron degeneration and disease progression has also been the subject of intense debate. Mutant *SOD1*-specific expression in neurons is sufficient to induce motor neuron degeneration and paralysis in transgenic mice (35). It seems that the toxicity of mutant *SOD1* in motor neurons is necessary for disease initiation, and mutant *SOD1* damage in the glia may play a possible role in disease progression (36) through excretion of SOD1 and its uptake by motor neurons.

### 132.7.2 ALS2 or ALSIN-Linked ALS

In a large inbred Tunisian family with recessive juvenile ALS, Hentati et al. established linkages to chromosome 2q33–35 (37). The disease was characterized by early age of onset (3–23) and predominance of upper motor neuron signs. Homozygous deletions in the *ALSIN* gene were subsequently identified by two independent groups (38,39). *ALSIN* gene mutations were also found to cause juvenile primary lateral sclerosis (39) and infantile ascending hereditary spastic paraparesis (40).

The *ALSIN* gene has 34 exons and produces alsin protein, which is ubiquitously expressed and predicted to have a molecular mass of 184 kDa. It is alternatively spliced in two transcripts: a short 396 amino acid form and long 1657 amino acid form. The protein has multiple domains, including a guanine nucleotide exchange

factor homology domain that was shown to activate a small GTPase belonging to the Ras superfamily, a pleckstrin homology domain and a vacuolar protein sorting nine domain (38,39).

It was suggested early on that alsin may be involved in vesicle transport. Recently alsin was shown to be an important regulator of Rab5-mediated endosomal trafficking and degradation (41). Alsin knockout mice have been generated by independent investigators; they develop rather a mild phenotype characterized by poor performance on motor coordination tests and distal corticospinal tract degeneration without apparent motor neuron pathology, suggesting that ALS2 is predominantly a distal axonopathy (42).

### 132.7.3 ALS4 or SENATAXIN-Linked ALS

ALS4, also known as distal hereditary motor neuropathy with pyramidal features, is a rare, juvenile onset, autosomal dominant form of ALS characterized clinically by slowly progressing limb weakness, severe muscle wasting, pyramidal signs, the absence of overt sensory abnormalities or bulbar involvement and a normal life span (43).

Linkage was initially established to chromosome 9q34 in a large Maryland kindred (43) and heterozygous mutations were found in Senataxin gene (*SETX*) in 2004 in this family and in two additional ones that showed linkage to the same region (44). Homozygous mutations in the Senataxin gene lead to an unrelated disorder: ataxia-oculomotor apraxia 2 (45).

The Senataxin gene contains 26 exons and encodes a 302.8 kDa protein (44). The protein was named senataxin because of its yeast ortholog Sen1p—a splicing endonuclease. It contains a DNA/RNA helicase domain with strong homology to RENT1 and IGHMB2, two members of the superfamily I of helicases. RENT1 has been shown to be involved in non-sense mediated mRNA decay (46) and IGHMB2, a DNA binding protein that causes spinal muscular atrophy with respiratory distress (SMARD) (47).

Senataxin is a nuclear protein whose exact biological function remains unknown. It has been suggested that it is important for DNA repair, (48) and through its interaction with several proteins involved in transcription and mRNA processing it may also play a role in coordinating transcriptional events and in splicing regulation (49).

### 132.7.4 ALS 5 or Spatacsin-Linked ALS

Linkage of an autosomal recessive, slowly progressive, juvenile ALS or ALS5 was linked by Hentati and colleagues in 1998 to chromosome 15q15–q22 (50). Mutations in the *spatacsin* gene were identified by Orlacchio et al. in 2010 in 10 out of the 25 families studied with autosomal recessive juvenile onset ALS (51). The

*spatacsin* gene was previously reported to cause autosomal recessive spastic paraplegia with thin corpus callosum or SPG11 (52). The function of the protein is currently undetermined.

### 132.7.5 ALS6 or Fused in Sarcoma, Translocated in Liposarcoma (FUS/TLS)-Linked ALS

Mutations in *FUS/TLS* were initially reported independently but simultaneously by two groups of investigators in 2009. Kwiatkowski et al. reported 13 mutations in *FUS/TLS* in 17 different familial ALS kindreds linked to chromosome 16 or ALS6 (53) and Vance et al. reported 3 *FUS* mutations in nine additional families with ALS6 (54). Since these reports, about 35 pathogenic mutations have been reported to date in about 4% of all familial ALS and 5% in non SOD1-ALS and non TARDBP-ALS; however *FUS/TLS* mutations are rare (less than 1%) in sporadic patients with no apparent family history (55–57). It appears to be the second most frequent cause of familial ALS after SOD1. The inheritance pattern is dominant, except for one recessive mutation found in a family of Cape Verdean origin (53). Most mutations are missense mutations, except for few in-frame or frame-shift deletions or insertions, and all seem to cluster in exons four to six and 13 to 15 of the gene (55,57). Mutations in *FUS/TLS* are not restricted to any ethnic group, since they were found in European, African, Australian, European American, African American, Asian and Latino subjects (53–55,58–60).

Most patients with *FUS/TLS* mutation develop the classic ALS phenotype without cognitive impairment. In the series reported by Yan et al. patients with *FUS/TLS* had earlier age of onset, a higher rate of bulbar onset, and a shorter duration of symptoms than FALS patients with the SOD1 mutation (55). Other groups reported an atypical phenotype that consisted of symmetric proximal and axial weakness at onset with and without bulbar signs (58,61,62). About four *FUS/TLS* mutations have been associated with either FTD/MND or behavior variant FTD (55,61,63,64), and one mutation (R521C) was reported in an ALS/PD/DE complex index case (55). It is interesting that the R521C mutation is the most common *FUS/TLS* mutation reported and is also the one associated with atypical presentations.

*FUS/TLS* is a ubiquitously expressed 526 amino acid protein, encoded by 15 exons (65). *FUS/TLS* was initially isolated from the specific chromosomal translocation breakpoint of mixoid liposarcoma as the 5' part of a fusion gene with the transcription factor gene *CHOP* (66,67). *FUS/TLS* predominantly localizes to the nucleus and belongs to the FET/TET family of RNA/DNA binding proteins, together with Ewing's sarcoma protein (EWS) and the TATA-binding protein associated factor (TAF 15) (57,68).



The protein is characterized by an N-terminal domain rich in glutamine, glycine, serine and tyrosine residues, a glycine-rich region, an RNA recognition motif (RRM), multiple arginine/glycine/glycine (RGG) repeat in an arginine- and glycine-rich region and a C-terminal zinc finger motif (69–71). As in ALS caused by *TDP43* mutations, most ALS-causing mutations are clustered in the glycine-rich region and in the extreme C-terminal part of the protein. *FUS/TLS* was found to be essential for mice neonatal viability, since disruption of this gene in mice caused death of the animal within 16 h of birth (72). *FUS/TLS* is structurally close to the family of heterogeneous ribonucleoproteins (hnRNPs). It interacts with RNA, single and double stranded DNA, and is involved in a wide range of RNA-related cellular processes including transcription, pre-mRNA splicing, mRNA transport from nucleus to cytoplasm and micro RNA processing (55,56). *FUS/TLS* was found in RNA-transporting granules translocating to dendritic spines under excitatory stimulation (73). Cultured hippocampal neurons from *fus/tls* knockout mice displayed abnormal spine morphology and density, indicating that *FUS/TLS* participates in mRNA sorting to the dendritic spines and may regulate spine morphology to stabilize the synaptic structure (73). *FUS/TLS* is also a component of RNA stress granules (74).

Spinal cord sections from ALS patients with the *FUS/TLS* mutation show ubiquitin and p62 immunopositive skein-like cytoplasmic inclusions that are negative for *TDP43* but immunoreactive for *FUS/TLS* (54). *FUS/TLS* inclusions were initially only found in FALS patients with *FUS/TLS* mutations (53,54). Using a high pressure decloaking chamber technique for antigen retrieval, Deng et al. demonstrated that cytoplasmic *FUS* immunoreactive inclusions are not restricted to FALS due to *FUS* mutations, but were also found to colocalize with *TDP43*, ubiquitin and p62 in sporadic ALS, ALS/dementia, familial ALS due to *TDP43* and in non-SOD1 familial ALS tissue. Similar to *TDP43* inclusions, *FUS* immunopositive inclusions were absent in SOD1-linked ALS (23). *FUS* immunoreactive inclusions were also found in a subset of *TDP43* negative FTL-D-U cases (75), neuronal intermediate filament inclusion disease (76) and basophilic inclusion body disease (77).

How mutations in *FUS/TLS* cause disease remains poorly defined, but the discovery of *TDP43* and *FUS/TLS* mutations in familial ALS in RNA-DNA binding proteins shifted the focus of research to the role of RNA metabolism and processing in the pathogenesis of ALS.

### 132.7.6 ALS8 or Vesicle-Associated Membrane Protein-Associated Protein B: VAPB-Linked ALS

In a large Brazilian family of Portuguese extraction with autosomal dominant, slowly progressive, lower motor neuron disease or ALS8, Nishimura et al. established

linkage to chromosome 20q13 (78) and subsequently reported a missense P56S mutation in a vesicle-associated membrane protein-associated protein B gene (*VAPB*) in this family and in six additional families of the same heritage (79). This mutation resulted in three different clinical phenotypes: late onset SMA of Finkel Type, late onset, slowly progressive, atypical ALS with essential tremor, and typical ALS. Haplotype analysis of these seven families and a further Brazilian family of African origin showed a common founder effect arising around the middle of the fifteenth century at the time of Portuguese immigration to South America (80). This mutation has since been reported in two additional Brazilian families (81,82). The *VAPB* P56S mutation was also recently reported in a familial ALS patient of Japanese descent (60).

More recently a new missense mutation T46L was reported in a non-Brazilian familial ALS patient (83). Although segregation of this mutation with the disease phenotype in this family could not be demonstrated due to non-availability of DNA, functional *in vitro* and *in vivo* studies strongly suggest a pathogenic role for this mutation (83).

The *VAPB* gene contains six exons and encodes a 243 amino acid protein that is highly conserved and ubiquitously expressed. It contains three domains: a conserved N-terminal MSP (major sperm protein) domain shared by other vesicle-associated membrane proteins, a coiled-coil t-SNARE homology domain and C-terminal transmembrane domain (84). It is an intracellular membrane protein that is enriched in the endoplasmic reticulum and Golgi membranes; it is involved in the recruitment of FFAT motif containing proteins to the ER (85), vesicle trafficking (86), and plays a role in the unfolded protein response (87). In *Drosophila*, the fly homolog of *VAPB* binds to microtubule network and regulates bouton size at the neuromuscular junction (88). Introduction of the P56S mutation in cell cultures causes *VAPB* to lose its normal ER association and form dense cytoplasm aggregates (79,87). Many *Drosophila* models carrying either a P56S or a T46L corresponding mutation have been generated; they recapitulate many aspects of the disease, with a motor phenotype, neuronal cell death and aggregate formation (83), (89–91). The exact role of the *VAPB* mutation in motor neuron degeneration remains a mystery. Aside from its role in vesicular trafficking and the unfolded protein response, *VAPB* interaction with lipid binding proteins adds lipid metabolism dysfunction to the pathogenesis of ALS.

### 132.7.7 ALS9 or Angiogenin-Linked ALS

Greenway and colleagues established chromosome 14q11 as a new candidate region for autosomal dominant familial ALS or ALS9, and identified mutations in the Angiogenin gene in four familial and 11 sporadic ALS patients, mostly of Irish or Scottish descent (92,93).

Angiogenin gene mutation has since been reported in sporadic and familial patients from North America, France, Germany and Italy (60,94,95).

The Angiogenin gene (*ANG*) encodes a 14-kD angiogenic ribonuclease, a hypoxia responsive gene and a potent inducer of neovascularization in vivo. Wild type angiogenin was demonstrated to protect motor neurons from hypoxia-induced cell death, (96) and all the ALS-associated mutations showed a reduced neuroprotective activity against hypoxic injury, suggesting that angiogenin may be implicated in ALS pathogenesis through a loss of function (97).

### 132.7.8 ALS10 or TAR-DNA Binding Protein 43-Linked ALS

The identification of TAR-DNA binding protein 43 (TDP43) as the major disease protein of the ubiquitinated inclusions in ALS and in Frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) (12) led many authors to investigate the role of TDP43 in ALS pathogenesis, and to screen patients with familial ALS for mutations in the *TARDBP* gene. Sreedharan et al. screened 154 index familial ALS cases for mutations in *TARDBP*; they identified the M337V mutation in exon six of *TARDBP* gene in five affected individuals (four males and one female) from a two generation Caucasian family of English descent (98). They subsequently sequenced all six exons of *TARDBP* in a cohort of 200 British patients with sporadic ALS and identified another missense mutation, Q331L, in a man with limb onset ALS at the age of 72. No *TARDBP* mutation was found in a screen of 500 British controls. Screening of all six exons in an Australian sporadic ALS cohort identified a third mutation: G294A in a patient with limb onset ALS that started at the age of 65, with disease duration of 5 years (98). Since that report, a total of 38 mutations have been described in ALS patients with or without family history of ALS, corresponding to about 4% of familial ALS and about 1% of sporadic ALS cases (56). Most of the identified mutations are localized in the glycine rich region encoded by exon 6. They are all dominant missense mutations except for Y374X, a truncation at the extreme C-terminal of the protein (56). Most patients with *TDP43* mutations develop a classic ALS phenotype with bulbar or limb onset and without cognitive deficit. A few *TARDBP* mutations, some in exon 6, have also been identified in patients with behavior variant FTD or in FTD-MND (99,100) validating the fact that *TARDBP* mutations are not restricted to ALS alone.

The *TARDBP* gene is located on chromosome 1 (1p36), contains six exons and encodes the 43-kD TAR DNA-binding protein. Through alternative splicing, this gene could express 11 and may be more isoforms (101).

TDP43 belongs to the family of heterogeneous nuclear ribonucleoproteins (hn RNP) that bind RNA and DNA sequences through a common nucleotide binding domain

known as the RNA recognition motif (102). TDP43 is ubiquitously expressed, highly conserved throughout species (102) and predominantly localized to the nucleus (103). TDP43 was initially identified as the protein that binds specifically to pyrimidine-rich motifs of regulatory element in the HIV-1 long terminal repeat (LTR) known as TAR, and modulates HIV gene expression (104). TDP 43 is also involved in the regulation of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) exon 9 splicing (105). The protein is composed of two RNA recognition motifs (RRM1 and RRM2) flanked by an N-terminal and a C-terminal tail. The C-terminal region contains the glycine-rich domain that is important for protein-protein interaction (106) and the exon skipping activity of TDP43 (101). TDP43 is involved in many biological functions such as transcription and splicing regulation, mRNA stability (106) and micro RNA processing (107). TDP43 was also recently demonstrated to have multiple roles in the regulation of mRNA fate in neuronal cells, such as transcript stabilization and activity-dependent transport to dendrites (108,109).

TDP43 is also capable of responding to an environmental insult by assembling into stress granules (SGs), which are cytoplasmic ribonucleoprotein foci that sequester mRNAs, several RNA binding proteins and stalled translation initiation complexes to temporarily arrest protein synthesis as a protective response to cellular stress (110). In axotomized motor neurons in vivo, TDP43 was found to translocate to the cytoplasm, where it formed stress granules that dissolved after recovery, suggesting a role for TDP43 in shuttling mRNA between the nucleus and cytosol, during acute neuronal injury (111).

To investigate the role of TDP 43 in the pathogenesis of ALS several cell and animal models were developed:

Homozygous knock-down of *tdp43* is embryonically lethal in mice. Heterozygous *tdarbp* mutant mice exhibit signs of motor disturbance and muscle weakness without pathological changes in motor neurons (112). Knock-down of zebra fish *tdarbp* led to swimming deficit, which was rescued by co-expressing wild type, but not mutant, human *TARDBP* (113).

Overexpression of wild type *TDP43* in a transgenic mouse line led to a dose-dependent degeneration of cortical and spinal motor neurons and the development of spastic paraparesis reminiscent of ALS (114). Affected neurons showed accumulation of ubiquitinated and phosphorylated nuclear and, to a lesser degree, cytoplasmic TDP43 aggregates. The characteristic ~25-kDa C-terminal fragments were recovered from nuclear fractions and correlated with disease development and progression in wild-type TDP43 mice. Expression of human *TDP43* in various neuronal sub-populations of *Drosophila* also caused neurodegeneration, in particular expression in motor neurons led to motor neuronal loss with decreased locomotive abilities in the fly (115).

Overexpression of wild type or mutant *TDP43* in primary rat cortical neurons was toxic to primary neurons

and resulted in cytoplasmic mislocalization of the protein TDP43 (116). When mutant (A315T) *TDP43* was overexpressed in mice, it caused a progressive and fatal neurodegenerative disease reminiscent of ALS and FTLD-U without the presence of cytoplasmic TDP43 aggregates (117). In rats, however, overexpression of mutant *TDP43* (M337V) caused widespread neurodegeneration that predominantly affected the motor system, with degeneration of motor neurons and denervation atrophy of skeletal muscles recapitulating the ALS phenotype, with formation of cytoplasmic TDP 43 inclusions (118). Mutant *TARDBP* also caused a motor phenotype in zebrafish associated with shorter and disorganized axons with excessive branching as well as swimming deficits (113).

As stated above, in most cases of sporadic ALS and FALS due to *TDP43* mutation, the neuropathology is characterized by abnormal cytoplasmic accumulation of TDP43 that is associated with the formation of intracellular aggregate in the affected areas in these patients together with a reduction in the normal nuclear staining (57). In these aggregates TDP43 is phosphorylated, ubiquitinated and cleaved forming the 20–25 kDa C-terminal fragment that accumulates in detergent insoluble fractions derived from patients' tissues (12). Although it is clear from the animal models that TDP 43 is involved in neurodegeneration, the exact mechanism of TDP 43-induced neurodegeneration is yet to be resolved. Whether the toxicity of TDP 43 arises from the aggregate formation and the sequestration of essential proteins causing a toxic gain of function versus loss of its nuclear function or both is still debatable.

### 132.7.9 ALS11 or FIG4-Linked ALS

Mutations of the human *FIG4* gene on chromosome 6q21 are responsible for the recessively inherited disorder CMT4J, a severe early onset form of Charcot-Marie-Tooth disease. Chow et al. sequenced the *FIG4* gene in sporadic and familial ALS, and identified ten unique heterozygous variants of *FIG4* in nine patients: six with sporadic ALS and three with familial disease. These patients had upper motor neuron-predominant ALS of long duration and two patients had PLS (119). *FIG4* is a phosphoinositide 5-phosphatase that regulates the cellular abundance of phosphatidylinositol 3,5-bisphosphate. Phosphoinositides serve as molecular tags for intracellular vesicles and mediate vesicle trafficking. The etiological or pathogenic role of *FIG4* mutations has not been established.

### 132.7.10 ALS12 or Optineurin-Linked ALS

Maruyama et al. identified three mutations in the Optineurin (*OPTN*) gene in Japanese patients with familial or sporadic ALS (120). In four Japanese individuals from consanguineous families with ALS (ALS12) they found two homozygote mutations in the *OPTN* gene, one a

deletion of exon 5 in two siblings and the other a nonsense mutation (Q398X) in exon 12 in two individuals thought to be unrelated but who shared a common haplotype for a 0.9-Mb region containing the *OPTN* gene (120). They also found a heterozygous missense mutation (E478G) in exon 14 of *OPTN* gene in four individuals from two families with ALS. Although the inheritance pattern appeared to be autosomal dominant with incomplete penetrance, all affected individuals shared a common haplotype for 2.3 Mb on chromosome 10 around the *OPTN* gene suggesting a common founder effect.

Mutations in the *OPTN* gene were previously reported to cause primary open angle glaucoma and normal tension glaucoma (121). Both Q398X and E478G mutations were absent in a total of 5000 Japanese chromosomes, including subjects from the glaucoma study. The deletion mutation was also absent in 200 Japanese subjects, and not reported in over 6000 glaucoma individuals (120).

*OPTN* contains three noncoding exons in the 5'-untranslated region (UTR) and 13 exons that code for a 577-amino acid protein (121). It is a conserved protein that contains multiple coiled-coil domains, a leucine zipper, a ubiquitin-binding domain and a C<sub>2</sub>H<sub>2</sub> type zinc finger at its C-terminus (122). Although optineurin is ubiquitously expressed, it shows particularly high level of expression in certain tissues such as the retina, brain, heart, skeletal muscle, placenta, testis and kidney. Studies in various cell lines have shown that endogenous optineurin is present in the cytoplasm, Golgi and recycling endosomes (122).

Optineurin was first discovered as a binding partner of the adenoviral protein E3-14.7K and was shown to protect infected cells from TNF- $\alpha$ -induced cytolysis (123).

Optineurin shares a 53% amino acid homology with NF- $\kappa$ B essential modulator and was identified as an NF- $\kappa$ B essential modulator-related protein (124). It has been recently shown that *OPTN* is a negative modulator of NF- $\kappa$ B (125). *OPTN* links myosin VI to the Golgi complex and is involved in Golgi organization and exocytosis (126). It also interacts with Rab 8 and huntingtin and seems to be involved in the endosomal trafficking of the transferrin receptor (122).

The ability of optineurin to negatively modulate the NF- $\kappa$ B activity was lost when mouse neuroblastoma and spinal cord hybrid cell lines were transfected with Q398X and E478G mutant *OPTN* but not when transfected with wild type or glaucoma causing mutation in *OPTN* (120). Examination of the spinal cord from the subject with the E478G mutation, revealed intracytoplasmic eosinophilic inclusions in the motor neurons that were immunopositive for *OPTN*. Positive optineurin inclusions were also found in TDP43 and ubiquitin positive inclusions in patients with sporadic ALS. The SOD1-immunopositive Lewy body-like hyaline inclusions from cases with SOD1 linked familial ALS were also immunoreactive for optineurin (120).

It has been recently reported that optineurin positive inclusions are not restricted to ALS but have also been found in the ubiquitin-positive intraneuronal inclusions in ALS with dementia, in basophilic inclusions in the basophilic type of ALS, in neurofibrillary tangles and in dystrophic neurites in Alzheimer's disease, in Lewy bodies and in Lewy neurites in Parkinson's disease, in ballooned neurons in Creutzfeldt–Jakob disease, in glial cytoplasmic inclusions in multiple system atrophy, and in Pick bodies in Pick disease (127). Recently OPTN immunoreactive skein-like inclusions were found in the motor neurons from post-mortem spinal cords of patients with sporadic ALS and familial ALS without SOD1 mutations, but not in SOD1-linked ALS, or in the transgenic mice over-expressing SOD1<sup>G93A</sup> or SOD1<sup>L126Z</sup> (128). This finding implicates OPTN together with TDP43 and FUS in the pathophysiology of sporadic ALS and familial ALS not linked to SOD1. The exact role of OPTN in ALS is not known and, similar to TDP43 and FUS, could be related to a loss of function because of the sequestration of the protein in the inclusions or could be due to a toxic gain of function. Recently and most importantly, phosphorylated optineurin has been associated with autophagy; an emerging theme in neurodegeneration (147).

### 132.7.11 D-Amino Acid Oxidase ALS Linked to Chromosome 12q22

In a three generational familial ALS kindred with classic adult onset autosomal dominant ALS, Mitchell et al. reported an R199W mutation in the D amino acid oxidase gene (*DAO*). They demonstrated that this disease-associated mutation causes almost total loss of enzyme activity, impairs cell viability, promotes the formation of ubiquitin aggregates and increases apoptosis in neuronal cells (129). DAO is a peroxisomal flavoenzyme that catalyzes the oxidative deamination of D-amino acids such as D-alanine, D-serine, and D-proline to their corresponding keto-acids. In the brain, DAO controls the level of D-serine, a potent activator of N-methyl-D-aspartate-type glutamate receptor enhancing glutamate transmission that was shown to be elevated in the spinal cord of ALS patients and G93A transgenic mice (130).

### 132.7.12 Valosin-Containing Protein (VCP)-Linked ALS

Using whole exome sequencing, Johnson et al. recently reported five mutations in the *VCP* gene in patients with autosomal dominant familial ALS not linked to *SOD1*, *FUS* or *TDP43* (131). Two of these mutations (R191Q and R155H) were previously reported to cause Inclusion Body Myositis associated with Paget's disease of the bone and Fronto-Temporal Dementia (IBMPFD). The original IBMPFD was examined by one of us and no evidence of IBM was found; instead, greater proximal than

distal atrophy of spinal innervated muscles and respiratory failure was noted, along with mixed myopathic and neuropathic features on EMG (Siddique, unpublished). The obligate carrier of the R155H mutations comes from a large multigenerational family with IBMPFD and had a clinical diagnosis of ALS that was confirmed pathologically. In the nine reported patients with *VCP* mutation, three had dementia and five had rapid disease progression. Except for the family with the R155H mutation and one other relative of a patient with R191Q mutation that had bone disease, none had any personal or family history of muscle or bone disease.

*VCP* is a highly conserved, ubiquitously expressed, 97kDa protein that is a member of the AAA+ protein family of ATPases or ATPases associated with diverse cellular activity. These proteins are characterized by the presence of two ATPase domains, called the AAA domain. They are enzymatic machines that catalyze ATP hydrolysis and mediate many cellular functions, such as vesicle transport, organelle assembly, membrane dynamics and protein unfolding (132). Dysfunction in this family of proteins has been already implicated in hereditary spastic paraparesis due to spastin and paraplegin mutation.

The *VCP* protein has an N-terminal domain and two central D1 and D2 AAA+ domains. The N-terminal domain is necessary for substrate and cofactor association, whereas the D1 and D2 domains are needed for ATP binding and hydrolysis. A *VCP* monomer assembles into a functioning stable homo-hexamer with a central cylinder formed by the D1/2 domains surrounded by the N domains (133).

*VCP* has diverse biological functions. *Vcp* knockout is lethal in yeast, *C. elegans* and mice. It associates with many members of the ubiquitin-proteasome system, it is essential for endoplasmic reticulum associated degradation of misfolded ER, transmembrane and secreted proteins, (134) and participates in the delivery of degradation destined ubiquitinated cytosolic proteins to the 26S proteasome (135). *VCP* is also essential for autophagosome maturation and might be selectively required for autophagic degradation of ubiquitinated proteins, since overexpression of IBMPFD associated *VCP* mutation (R155H and A232E) in cells caused autophagy defect and accumulated autophagic vesicles contain ubiquitin-positive contents (136). *VCP* is now thought to be important for the coordination of protein degradation by both the ubiquitin-proteasome system and autophagy (133).

In normal muscle, *VCP* stains endomysial vessels and to a mild degree, muscle fiber's cytoplasm. In sporadic IBM, *VCP* staining was localized to debris in inclusions and vacuoles, but there was considerable staining in inflammatory cells and in regenerating muscle fibers. In muscles with IBMPFD, *VCP* was localized in large or small rounded aggregates in scattered muscle fibers, including those with no clear vacuoles or other



morphological changes (137). Patients with VCP dementia have ubiquitin positive intranuclear neuronal inclusions and rare intranuclear neuronal VCP inclusions (138,139). Both brain and muscle tissues from patients with IBMPFD showed the presence of TDP43 inclusions that co-localized with ubiquitin (138). Similar to Optineurin, VCP immunoreactivity was also observed in ubiquitin-positive intraneuronal inclusions in motor neuron disease with dementia, ballooned neurons in CJD, dystrophic neurites of senile plaque in Alzheimer's disease, and Lewy bodies in Parkinson's disease, thus implicating VCP in a wide array of neurodegenerative diseases.

The role of VCP in ALS pathogenesis is unclear, and according to Johnson et al., is responsible of about 1–2% of familial ALS, which is similar to that reported for TDP43. The discovery of VCP mutations causing ALS underscores the role of protein homeostasis, and the interface between the proteasomal system and autophagy in the pathogenesis of ALS, although the role of VCP in the proteasomal system is controversial.

### 132.7.13 ALS-FTD Linked to Chromosome 9p21

Many authors reported autosomal dominant familial ALS and frontotemporal dementia where motor neuron disease and the dementia exist as a spectrum with significant interfamilial variability (140–143).

Linkage analysis of our families with FTD-ALS revealed a LOD score of nine on chromosome 9p21 with a minimal candidate region of 3 megabases. We identified 8 KB of common disease haplotype in three of these families (Siddique unpublished). This locus was recognized as a major locus for FTD-ALS by several investigators (140,141,144).

Recently, two independent research teams reported the presence of a hexa-nucleotide repeat expansion GGGGCC located between non-coding exon 1a and exon 1b of *C9ORF72* gene that segregate with disease phenotype in FTD-ALS kindred (145,146).

*C9ORF72* is a gene that encodes an uncharacterized protein with no known domain or function but which is highly conserved across species. At least three alternatively spliced transcripts are expressed in all tissues, including the brain (146). The non-coding repeat expansion may act pathologically by reducing the translation of the *C9ORF72* long transcript or by sequestering RNA binding proteins, leading to dysregulation of alternative mRNA splicing as in myotonic dystrophy or in several spinocerebellar ataxias (146).

The neuropathology of familial ALS/FTD linked to chromosome 9p is characterized by the presence of ubiquitin and TDP43 immunoreactive neuronal cytoplasmic inclusions in the frontal cortex, dentate gyrus of the hippocampus and the hypoglossal nucleus (57,144). It is also interesting that hippocampal sections of patients

with ALS/FTD displayed prominent ubiquilin2 pathology (147). These ubiquilin2 inclusions were positive for ubiquitin and p62 but for the most part negative for TDP43 (147).

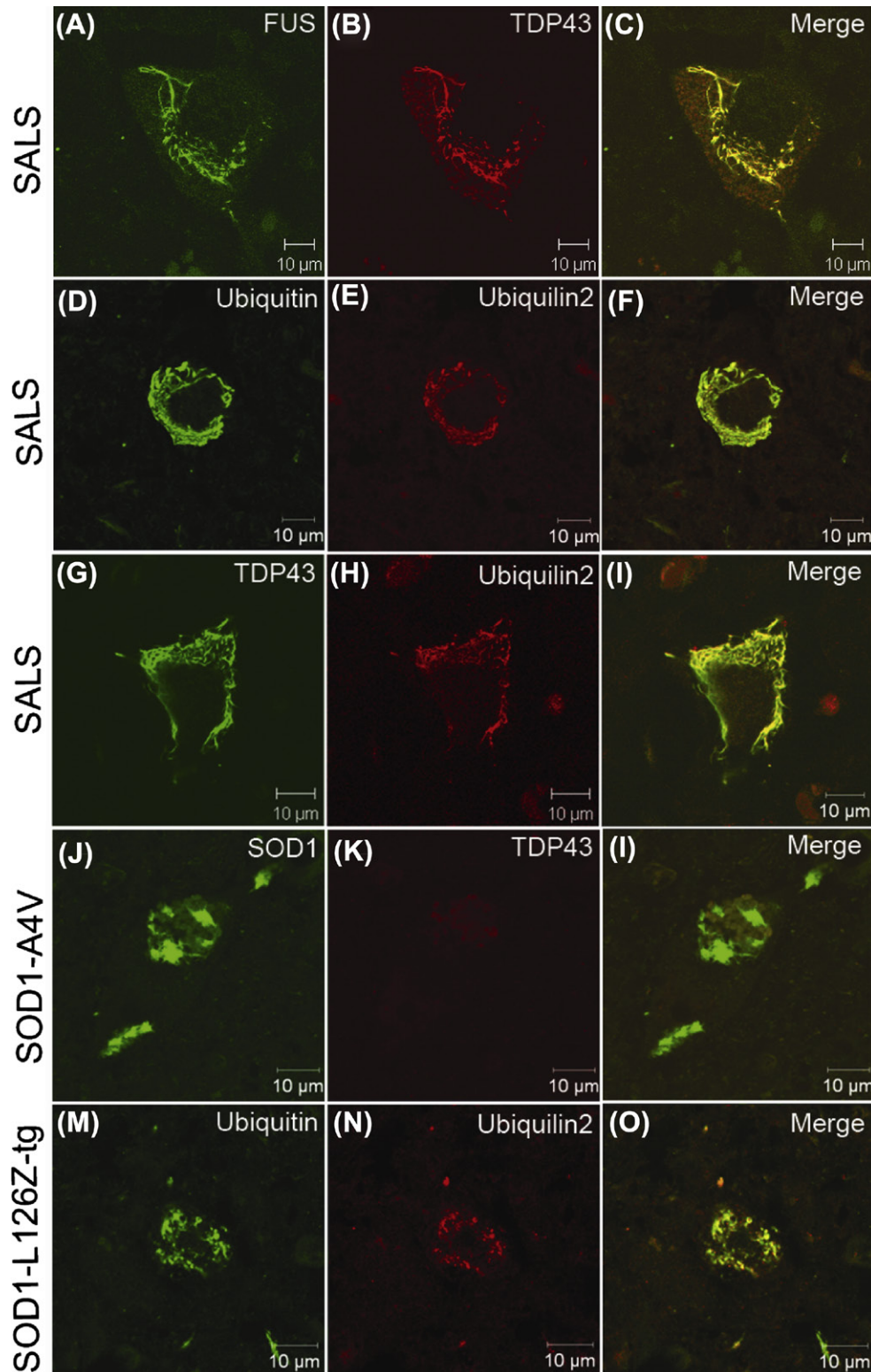
### 132.7.14 X-ALS or ALS/Dementia Linked to Ubiquilin2 Gene or *UBQLN2*

In a five generation family with X-linked dominant ALS and 19 affected individuals, we established linkage to a 21.3 Mb minimum candidate region on Xp11.23. Sequence analysis of 41 genes in the region revealed a missense mutation in *UBQLN2* gene which encodes ubiquilin2 in this X-ALS family. Four other *UBQLN2* mutations were also discovered in four unrelated families with ALS or ALS/dementia but without male-to-male transmission. None of these mutations were present in the SNP database or in the 928 ethnically matched control samples (147).

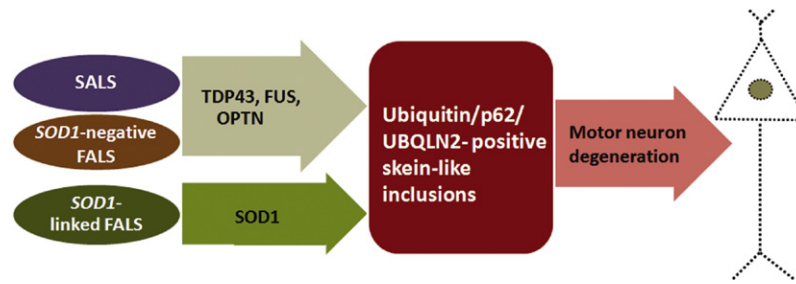
Clinically, patients presented with ALS or with ALS/dementia of the FTD variant. Age of disease onset was significantly younger in males compared to females, whereas disease duration was similar. Autopsy data were available from three patients with *UBQLN2* mutations. Pathological analysis of the spinal cord showed axonal loss in the corticospinal tract, loss of anterior horn cells and astrocytosis. Ubiquilin2 immunoreactive skein-like inclusions were observed in some remaining motor neurons. These inclusions were also immunopositive for ubiquitin, P62, TDP43, FUS and optineurin, but not for SOD1. The ubiquilin2-positive inclusions were also found in spinal cord sections of patients with sporadic ALS, ALS with dementia and FALS without SOD1 mutations (Figure 132-1). Spinal cord sections from patients with familial ALS due to SOD1 mutations did not show ubiquilin2-positive inclusions (Figure 132-1), supporting the hypothesis that SOD1-linked ALS has an independent pathway from the other forms of ALS (Figure 132-2) (147).

Apart from the spinal cord, widespread ubiquilin2-positive inclusions were also observed in the hippocampus of the ALS patients with dementia (Figure 132-3). But ALS patients without dementia did not show ubiquilin2-positive inclusions in the hippocampus, thus linking the hippocampal ubiquilin2 pathology to the cognitive decline in ALS patients with dementia.

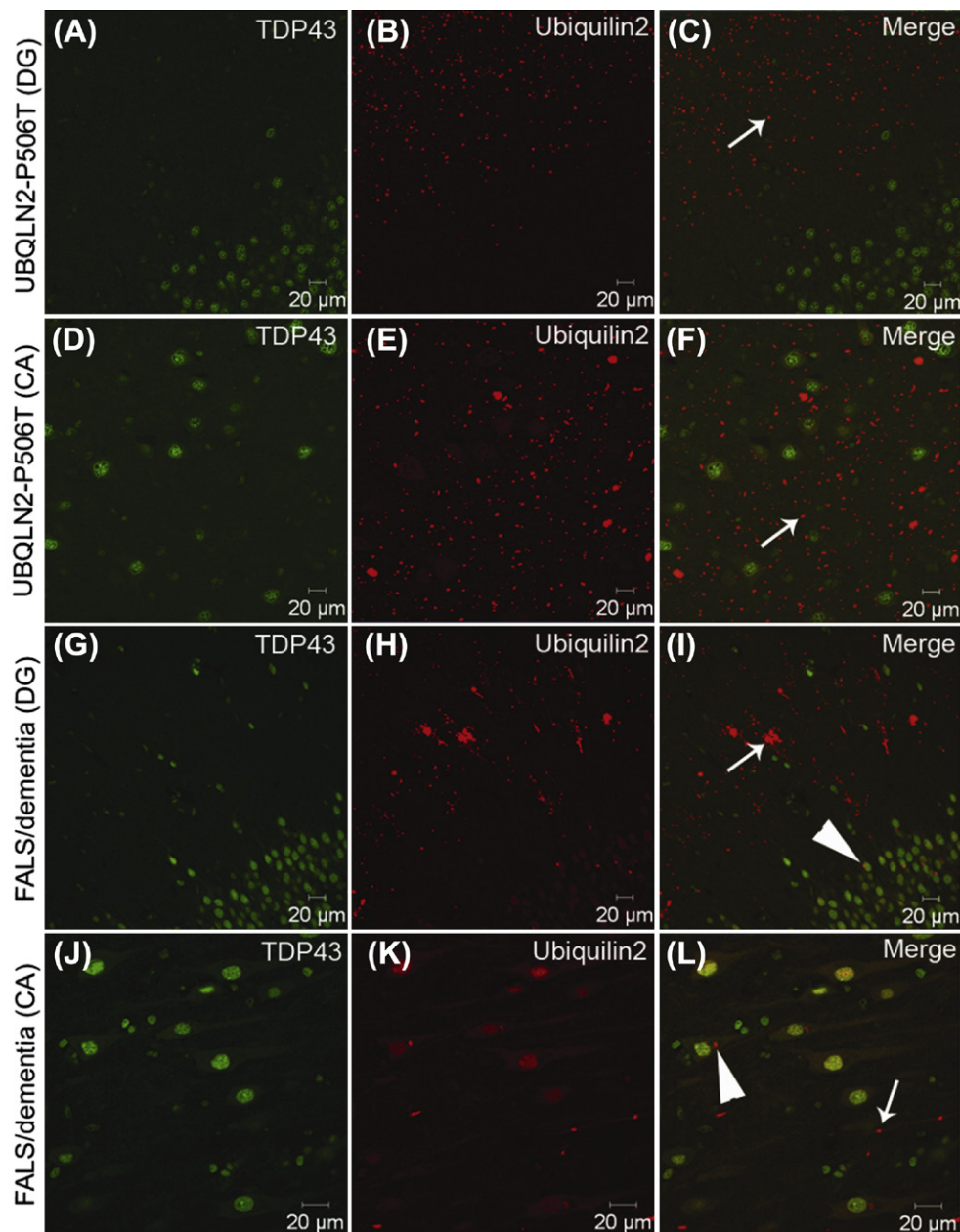
Ubiquilin2 is one of the four members in the Ubiquitin-like protein family that functionally link the ubiquitinated proteins to the protein degradation machinery (148,149). Expression of mutant ubiquilin2 results in an impairment of ubiquitinated protein degradation. These data, therefore, link mutations of ubiquilin2 to defects in the protein degradation pathway, abnormal protein aggregation and neurodegeneration, implying a common pathogenic mechanism underlying degeneration processes, not only in motor neurons but also other neurons as well (Table 132-1) (147).



**FIGURE 132-1** Differential involvement of ALS-associated proteins in skein-like inclusions in different types of ALS. Confocal microscopy was performed using immunofluorescent staining of the spinal cord sections from autopsy cases with sporadic and SOD1-linked ALS or a SOD1-ALS mouse model. Representative skein-like inclusions are shown to be immunoreactive for TDP43, FUS and ubiquilin2 in sporadic ALS (a–i). TDP43 is negative in the SOD1-positive inclusions in SOD1-linked ALS (j–l). However, skein-like inclusions are positive for ubiquilin2 in an ALS mouse model expressing mutant SOD1 (m–o). SALS, sporadic ALS; SOD1-A4V, ALS case with an A4V mutation of SOD1; SOD1-L126Z-tg, transgenic mouse expressing L126Z mutation of SOD1.



**FIGURE 132-2** Schematic model depicting two parallel pathways involved in the pathogenesis of ALS. UBQLN2, TDP43, FUS and optineurin are components of pathological inclusions in SALS and *SOD1*-negative FALS including *C9ORF72*-linked cases. *SOD1* aggregates are predominantly present in *SOD1*-linked FALS. The involvement of ubiquitin, p62 and ubiquilin2 may occur downstream and lead to formation of skein-like inclusions and ultimately motor neuron degeneration.



**FIGURE 132-3** Ubiquilin2 pathology in the hippocampus of ALS/dementia cases with or without UBQLN2 mutation. Confocal microscopy was performed using immunofluorescent staining of the hippocampal sections from an autopsy case with a UBQLN2-P506T mutation (a–f) and an ALS/dementia case without a UBQLN2 mutation (g–l). Representative neuritic inclusions that are ubiquilin2-positive, but TDP43-negative in the molecular layer of the dentate gyrus (DG) and CA region (CA) are indicated by arrows (a–f). Representative Neuritic and cytoplasmic ubiquilin2-positive inclusions in the molecular layer and dentate granule cells of the dentate gyrus (g–i), and CA region (j–l) are indicated by arrows and arrowheads, respectively.



**TABLE 132-1 Genetic Causes of Familial ALS**

Type	Locus	Gene	Phenotype	OMIM
ALS1	21q22	<i>SOD1</i>	AD-ALS	105400
ALS2	2q33	<i>ALSIN</i>	Juvenile AR-ALS Juvenile PLS Infantile onset spastic paraplegia	205100 606353 607225
ALS3	18q21	—	Incorrectly assigned to 18q21, a mutation in FUS (ALS6) has been identified in the family that was used for initial mapping	606640
ALS4	9q34	<i>SETX</i>	AD- Juvenile ALS	602433
ALS5	15q21	<i>SPATACSIN</i>	AR- Juvenile ALS SPG11	602099
ALS6	16p11	<i>FUS</i>	AD- ALS AD-ALS-FTD	608030
ALS7	20p13	—	AD-ALS	608031
ALS8	20q13	<i>VAPB</i>	AD distal SMA AD typical and atypical ALS	608627
ALS9	14q11	<i>ANG</i>	AD- ALS	611895
ALS10	1p36	<i>TDP43</i>	AD-ALS AD ALS-FTD	612069
ALS11	6q21	<i>FIG4</i>	AD-ALS CMT4J	612577 611228
ALS12	10p15	<i>OPTN</i>	AD-ALS	613435
ALS linked to chromosome 9	9p21	<i>C9ORF72</i>	AD ALS AD ALS-FTD	105550
ALS linked to chromosome 12	12q24	<i>DAO</i>	AD-ALS	—
ALS-VCP	9p13	<i>VCP</i>	AD-ALS IBMPFTD	— 167320
ALSX	Xp11.23	<i>UBQLN2</i>	X-juvenile ALS X-Adult ALS X-ALS/Dementia	

### 132.8 GENETICS OF SPORADIC AMYOTROPHIC LATERAL SCLEROSIS

Although the etiology of sporadic ALS is currently unknown, it is believed that the disease may arise from complex interactions between susceptibility genes and diverse environmental factors. Exploring the genetic basis of sporadic ALS and identifying these susceptibility genes has been a hot topic these past years, as the genes may provide clues to specific pathogenic pathways or indicate specific environmental risk factors.

The modern tools of molecular genetics has facilitated the genotyping of hundreds of thousands of single nucleotide polymorphisms (SNP) in each sample and allowed us to perform association studies using population-based case-control samples or family-based samples to determine whether a specific DNA sequence change will be found more frequently in disease cases than in controls.

To date, twelve genome-wide association studies (GWAS) and several candidate gene association studies have been published regarding sporadic ALS, with the first reported in 2007 (150). All these studies were hindered by the need for a large sample size; the small odds ratio measured that usually did not survive Bonferroni correction and the inability to replicate the results in different populations. Most samples were not matched to controls.

Nevertheless, these studies have revealed interesting genetic associations with sporadic ALS:

- FLJ10986 or FGGY was the first potential candidate gene to be discovered in a US population. It codes for a protein of unknown function that is expressed in the spinal cord and spinal fluid of patients and controls (151). Certain SNPs seemed to be associated with sex, age at onset, and site of onset of sporadic ALS. An SNP in this gene was also found to increase ALS susceptibility in a Chinese population (152); however, these findings were not replicated in a homogenous population from Northern Europe (153), from Germany (153), nor in a French or French Canadian cohort (154).
- ITPR2, or inositol 1,4,5-triphosphate receptor 2 gene, which encodes a calcium channel on the endoplasmic reticulum that is primarily responsible for controlling intracellular calcium concentrations in neurons, was identified as a susceptibility gene in a Dutch GWAS study that was replicated in Swedish and Belgian population with higher peripheral blood expression of ITPR2 in ALS patients compared to controls (155). Again these findings were not replicated in other GWAS studies involving individuals of European ancestry (156,157).



- DPP6 encodes a dipeptidyl-peptidase-like protein and was identified as a susceptibility gene in different populations of European ancestry (158). The identified variant was subsequently confirmed in two further cohorts of 221 Irish and 266 Italian sporadic cases (159,160) but failed to be replicated in additional Irish and Polish populations (161) and in a large two-stage GWAS of more than 2000 cases of US, Italian and German background (157) or in a large independent Italian population (162).

A recent study of more than 19,000 patients from nine countries identified an SNP in the UNC13A gene as being strongly associated with sporadic ALS (163). Members of the UNC13 family, such as UNC13A, are presynaptic proteins found in central and neuromuscular synapses that regulate the release of neurotransmitters (163). This SNP, however was not confirmed in an independent French population (164).

The Van ES study also identified 9p21 as susceptibility locus for sporadic ALS (163); this finding was also confirmed in another GWAS study including samples from the UK, USA, Netherlands, Ireland, Italy, France, Sweden and Belgium (165). In this last study, none of the previously associated SNPs in ITPR2, FGGY in DPP6 and in UNC13A reached genome-wide significance (165). Replication of the study of the two genetic variants, rs2814707 on 9p21.2 and rs12608932 on 19p13.3 that were reported to be most significantly associated with sporadic amyotrophic lateral sclerosis by van ES showed no evidence of association in Chinese and Japanese populations (166).

A Finnish GWAS study using familial and sporadic ALS samples and controls also found strong association with the 9p locus in the ALS patients; however the association was driven mainly by the familial cases and was much weaker in the sporadic cases (167). Although these findings suggest that the locus on 9p might be important not only to familial ALS-FTD but also for sporadic ALS, caution should be used in the interpretation of these results as it is not clear how careful the investigators were in excluding familial cases. Our study, using 500 sporadic ALS and 500 controls, did not reveal any association with the chromosome 9p locus for any single SNP; however haplotype analysis showed a robust association with the following SNPs: rs2814707, rs3849942 and rs903603 (Siddique, data not published).

In a pooled set of patients with ALS and controls from the UK, US and Belgium, the allelic variants of the RNA polymerase II component, ELP3, was found to be associated with ALS (168). Zebrafish knock down of ELP3 showed abnormal axonal branching similar to overexpression of SOD1 and knock down of ALSIN in zebra fish.

A candidate gene association approach was also used to investigate the role of several genes in sporadic ALS, such as APEX, VEGF, SMN1 and SMN2

copy numbers, HFE, DNCT1 and NEFL, with variable results or a mildly increased risk that was not replicated in repeat studies. These genes did not show any association in genome-wide association studies. A variant of the Angiogenin gene was associated with an increased risk of ALS in the Irish and Scottish population, (92) and mutations in these genes were found in sporadic and familial patients of Irish and Scottish descent (93). Peripherin gene mutations have been identified in two sporadic ALS patients, but it is not clear whether these variants are disease-causing or rare polymorphisms (169). Studies of APOE provided conflicting results: the E2 allele was shown to protect against early onset ALS (170), whereas the E4 allele may increase the risk of bulbar onset ALS in men (171). More recently, Ataxin-2 intermediate-length polyglutamine expansions were found to be associated with increased risk of ALS (172,173).

The paraoxonase (PON) gene cluster on chromosome 7q21 has been extensively examined in the past years, and has emerged as the most robust genetic risk factor for ALS. The PON genes, PON1, PON2 and PON3, code for enzymes involved in the detoxification of organophosphate pesticides and chemical nerve agents. A haplotype of large linkage disequilibrium spanning PON2 and PON3 was found to be associated with sporadic ALS in a large North American Caucasian family-based and case control cohort (174). Association with PON genes was also reported in Polish, Irish, French, French Canadian and Swedish populations (175–177). Seven missense mutations in the PON1 gene were identified in sporadic and familial ALS patients that were predicted to alter PON function, (178) further implicating the PON genes in the pathogenesis of ALS. It is of interest that mutation in a gene of similar function to PON (neuropathy target esterase (NTE)) causes autosomal recessive spastic paraplegia with distal muscle wasting, resembling Troyer Syndrome.

Lastly, Fecto et al. recently reported 10 heterozygote mutations in the P62 or Sequestosome1 (SQSTM1) gene in six individuals with familial ALS and in nine sporadic patients (179). These changes were absent in 724 controls, in the SNP database and in the 1000 genome database. Segregation studies were not performed due to a lack of DNA and predictive *in silico* analysis classified these mutations as pathogenic (179).

P62 is located on chromosome 5q35 and has eight coding exons. The protein is ubiquitously expressed, highly conserved in mammals, and has many functional domains (180). P62 plays an important role in protein degradation through the proteasomal pathway and the autophagic/lysosomal pathway (181,182). It is present in neuronal and glial ubiquitinated inclusions of many neurodegenerative diseases such as Alzheimer's, Parkinson's disease, and Dementia with Lewy bodies (183). It co-localizes with FUS and TDP43 in

ubiquitinated inclusions in the spinal motor neurons of sporadic ALS, non-SOD1 familial ALS and ALS with dementia (23,184).

It is of interest that three of the reported mutations located in the UBA domain have been previously identified in familial and sporadic Paget's disease of bone (185). We already know that at least two other genes, VCP and Optineurin, have been genetically linked to both ALS and Paget's disease of bone, suggesting that there might be a unifying point in the pathogenesis of the two diseases.

### 132.8.1 Amyotrophic Lateral Sclerosis and Parkinsonism Dementia Complex

Amyotrophic lateral sclerosis-parkinsonism/dementia complex of Guam is a fatal neurodegenerative disorder with unusually high incidence among the Chamorro people of Guam, in the Kii peninsula of Japan and in New Guinea. Clinically this syndrome is characterized by the co-occurrence of ALS with rigido-akinetic parkinsonism and early and severe dementia (186). Pathologically this syndrome is distinguished from classic ALS by the presence of widespread neurofibrillary tangles in the brain and in the spinal cord (186). The incidence of this disease was highest in the 1950s and has been in steady decline in the past decades. The familial clustering of ALS/PDC suggested a genetic basis for this syndrome early on, and segregation analysis supported an autosomal dominant inheritance with reduced penetrance that is possibly affected by environmental factors (187). No major locus for ALS/PDC has been identified; however a recent genome wide linkage analysis of a large complex pedigree and a series of smaller families from Guam, and a genome wide association analysis of Chamorro-based cases and controls revealed a strong association with three loci; two on chromosome 12 and one on chromosome 17 close to the MAPT locus (188).

### 132.8.2 Clinical Approach to the Genetic Diagnosis or Genotype–Phenotype Correlation

ALS is mainly a sporadic disease; familial cases are rare and occur only in about 10% of patients. Currently there are at least 10 genes that cause familial ALS, with *SOD1* being the most common followed by *FUS/TLS* and *TDP43*. The rest of the genes are very rare and sometimes restricted to certain ethnic backgrounds. A large number of ALS genes remain unknown.

A genetic etiology for the disease is readily suspected when there is a positive family history. Unfortunately, family history is not always available; it may be unknown to the patient if he is adopted or if one of the parents died at an early age before developing disease, if family history is not correctly collected, or if the information

obtained is restricted to the immediate patient's family. Incomplete penetrance may make familial ALS present like a sporadic disease.

In the absence of family history, certain clinical features may suggest a familial etiology, like an early age of onset, lower motor neuron presentation, the presence of atypical signs such as dementia, cerebellar features, or an unusually rapid or slow progression of symptoms.

Making the correct genetic diagnosis may help establish the diagnosis of ALS sooner and guide prognosis; however establishing an accurate phenotype–genotype correlation may not be easy, especially since some of the mutated genes harbor significant intra- and inter-familial variability.

These C9ORF72 expanded repeats are the most common genetic defect in ALS and ALS dementia. 50% of familial ALS patients, 5% of sporadic ALS patients and 61% of familial ALS patients with dementia carry the expanded allele of C9ORF72 (>23 repeats). For the most part, patients with *SOD1* mutations have a spinal onset of disease with predominance of lower motor neuron signs; cognition is usually spared with rare exceptions (189). Knowing the exact mutation can help predict disease duration. Disease progression is uniformly rapid in A4V and V148G patients, extremely variable with the I113T and slow with G37R, H46R and D90A. Scandinavian ancestry, slow progression and onset in the lower extremities suggest a D90A mutation.

The third most common gene implicated in familial ALS is *FUS/TLS*. Although most patient with the *FUS* mutation are indistinguishable from those with sporadic ALS, when compared to *SOD1* patients, *FUS* patients are found to have an earlier age of onset, a faster progression and a higher rate of bulbar disease (55). An atypical presentation with symmetrical proximal or axial weakness has been also reported by several groups for patients with *FUS* mutations (58,61,62). The presence of familial dementia should suggest *TDP43*, *FUS* mutation or familial FTD-ALS linked to 9p locus, whereas the presence of inclusion body myopathy or bone disease in the family should suggest *VCP* gene mutation. Optineurin and *SQSTM1* should be checked in a patient with ALS and Paget's disease of bone. Finally Angiogenin mutation should be checked in patients with Irish/Scottish background, while the *VAPB* gene should be evaluated in patients with Brazilian ancestors.

A very young age of onset or presence of disease predominantly in siblings should suggest a recessive inheritance. The predominance of upper motor neuron signs may point to an *ALSIN* gene mutation and the presence of mental retardation, dementia and atrophy of the corpus callosum and lower extremity spasticity may imply the presence of mutations in *SPATACSN* gene.

Finally the absence of male-to-male transmission should alert clinicians to the possibility of X-linked disease associated with ubiquilin2 mutation.

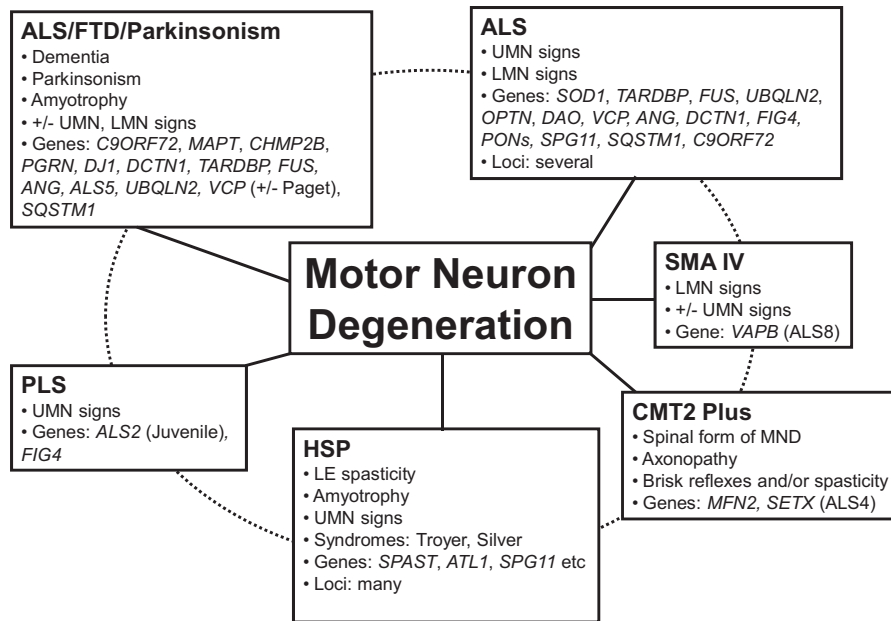


FIGURE 132-4 Phenome of ALS and related disorders.

## 132.9 CONCLUSION

ALS is a fatal disease of multiples etiologies; significant genetic progress has been made in the past decades with the discovery of several genes involved with familial ALS or related motor neuron degenerations (Figure 132-4). The discovery of these genes has allowed animal models of the disease to be engineered and helped in the understanding of pathogenic disease mechanisms and to test therapies.

ALS-associated genes have provided a unique opportunity to explore different pathways leading to motor neuron degeneration: from the role of oxidative stress, to protein aggregation, autophagy, abnormal RNA and DNA metabolism, and more recently defective protein and organelle degradation in the pathophysiology of ALS. In fact, recycling mechanisms may be at the heart of the molecular pathology not only of ALS, but also of several common neurodegenerative disorders.

The focus of genetic research has recently turned to sporadic ALS in order to discover susceptibility genes that will help identify specific environmental risk factors and to guide sporadic ALS modeling and therapy development for this incurable disease.

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## Biographies



**Teepu Siddique** earned his MBBS from the University of Karachi and trained in neurology at UMDNJ-NJ Medical School, thereafter specializing in neuromuscular medicine. He completed fellowships at the Hospital for Special Surgery-Cornell Medical Center and the NIH. He held faculty appointments at the University of Southern California and Duke University before coming to Northwestern University, where he is the Les Turner ALS Foundation/Herbert C Wenske Chair and director of the Division of Neuromuscular Medicine.

Dr Siddique's research has focused on the molecular genetics of ALS and related disorders, employing a range of strategies to identify a number of genes that cause the inherited form (SOD1, ALSIN, FUS & UBQLN2), and genetic loci. His group has developed several animal models for neurodegenerative diseases, including SOD1- and ALSIN-ALS. Genetic factors associated with the risk of sporadic ALS have been uncovered. His recent work with UBQLN2 has demonstrated a defect in the protein degradation pathways as a common mechanism of sporadic ALS, familial ALS and ALS/dementia, paving the way for targeted treatments in ALS.

Dr Siddique has received national and international recognition, including the first Shelia Essey Award in ALS from the American Academy of Neurology, the Hope through Caring Award from the Les Turner ALS Foundation and the Forbes Norris Award.



**Dr Han-Xiang Deng** received his MD and a PhD in medical cytogenetics from the Central South University, Xiangya School of Medicine, China, and a PhD in human genetics and molecular biology from Nagasaki University School of Medicine, Japan. He then joined the faculty in the Department of Neurology, Northwestern University Feinberg School of Medicine. His research focus is the causes and pathogenic mechanisms of neurodegenerative disorders. He contributed to the identification of several genetic defects in neurodegenerative disorders, including SOD1, alsin and UBQLN2 for amyotrophic lateral sclerosis (ALS),  $\alpha$ -TTP for vitamin E deficiency, and TRPV4 for scapuloperoneal spinal muscular atrophy (SPSMA) and hereditary motor and sensory neuropathy type IIC (HMSN IIC, also known as HMSN2C or Charcot-Marie-Tooth disease type 2C (CMT2C)). He and his collaborators have developed and characterized several mouse models of neurodegenerative diseases for further understanding the pathogenic mechanisms.

**Dr Senda Ajroud-Driss** received her medical degree from The Medical School of Tunis, Tunisia; she completed her Neurology Residency at the University of Illinois at Chicago and her Neuromuscular Fellowship at Northwestern University Feinberg School of Medicine. Prior to her residency, she worked as a post-doctoral fellow in Dr Teepu Siddique's Neurogenetic laboratory at Northwestern University, establishing linkage of an autosomal dominant mitochondrial myopathy and later identifying its gene (not yet published).

She is Board Certified in Neurology and in Neuromuscular Medicine. She is currently an Assistant Professor in the department of Neurology at Northwestern University. She is involved in research and the clinical care of patient with familial and sporadic ALS.

# CHAPTER

# 133

## Color Vision Defects

*Samir S Deeb and Arno G Motulsky*

















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### 133.1 INTRODUCTION

The human retina contains two classes of photoreceptors, rods and cones. Rods are responsible for vision in dim light, whereas cones mediate vision in bright light and enable the perception of color. Individuals with normal color vision have three types of cone photoreceptors; the short wave-sensitive or blue, middle wave-sensitive or green, and long wave-sensitive or red cones. Such individuals have normal trichromatic color vision. Red/green color vision defects are common among males. Individuals with severe color vision defects usually either have non-functional red (protanopes) or green (deutanopes) cone photoreceptors. Such individuals have dichromatic rather than trichromatic color vision. Those with milder color vision defects usually have either their red or green cone photoreceptor pigments replaced by an anomalous pigment with altered spectral sensitivity. Such individuals are classified as having protanomalous or deuteranomalous trichromatic color vision (Table 133-1).

A wide variation in the ability to discriminate between colors exists among individuals with defective color vision. Subtle variation in color perception also exists among individuals with normal color vision. Significant advances have been made during the last 20 years toward understanding the molecular and genetic bases of variation in both defective and normal color vision. In this chapter, genotype to phenotype relationships in normal color vision and in the various classes of defective color vision will be reviewed. Rare color vision defects include tritanopia (loss of function of the blue opsin), blue-cone monochromacy or incomplete achromatopsia (loss of function of both the red and green opsins), and complete achromatopsia (loss of function of all three cone types). Emphasis in this chapter will be placed on the common red/green color vision defects. Genetics plays a central role in red/green color vision defects. The common defects result from unequal homologous recombinations between the highly homologous and adjacent red and green opsin genes on the X-chromosome.

**TABLE 133-1** Classification of the Common X-linked Recessive Color Vision Defects

Class	Retinal Cones			Frequency (European Men)	Color Discrimination
Normal					
Protanopia			No	~1%	
Protanomaly				~1%	
Deutanopia		No		~1%	
Deuteranomaly				~5%	

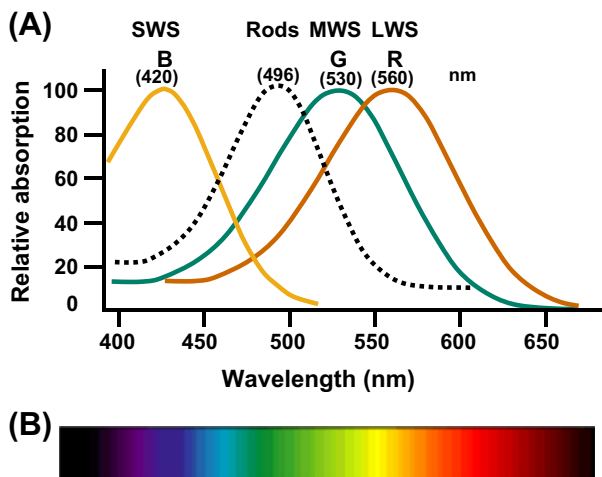
R', Red-like; G', Green-like types of cones. Color detection patterns in the visible spectrum are shown for normal trichromats and dichromats with protanopia or deutanopia. Anomalous trichromats (protanomalous and deuteranomalous) have a wide range of color discrimination capacity.

## 133.2 PHOTORECEPTORS

Each cone photoreceptor in the retina randomly expresses a single type of photopigment, which is fundamental to color perception. The wavelengths of maximal absorption ( $\lambda_{\max}$ ) of the photopigments of the blue, green, and red photoreceptors are at 420 nm, 530 nm, and 560 nm, respectively (Figure 133-1). The overlap between the absorption spectra of the three cone pigments allows color perception, by its ability to discriminate on the basis of wavelength. The sensation of various colors by the brain is based on comparisons of photon absorptions by the different cones.

The red and green cones are concentrated in the fovea, which is about 0.3 mm in diameter and contains about 10,000 cones. The fovea has no rods and very few blue cones, and has evolved as a specialized organ of high acuity and of red–green color vision. There are, on average, twice as many red cones as green cones in the fovea, with considerable variation among individuals. When the entire retina is considered, the average red/green pigment mRNA ratio is around 4.0, with a range of 1–10 (1). Variation in the red/green cone ratio makes little difference to color discrimination capacity. Color vision defects arise only at extreme ratios. The number of blue cones is 10%–20% of the total number of red and green cones. High-resolution imaging of the living human retina was accomplished using adaptive optics, and showed in two subjects that red and green cones are randomly arranged in the retina (2).

The pigment molecules are embedded within the membranes of the transverse disks of the outer segments of photoreceptors (Figure 133-2). There are about 1000 to 2000 transverse disks in a single photoreceptor, each containing about 100 pigment molecules.

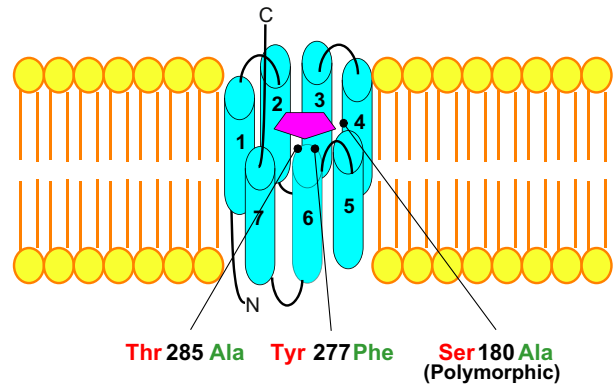


**FIGURE 133-1** (A) Absorption spectra of human rod (dotted) and blue (B, SWS), green (G, MWS) and red (R, LWS) cone photoreceptors. The wavelengths of maximal absorption ( $\lambda_{\max}$ ) in nanometers (nm) are indicated. (B) Colors of the visible spectrum that can be detected by trichromats with normal color vision by comparison of signals generated by the different cones.

## 133.3 MOLECULAR BIOLOGY OF THE PHOTOPIGMENTS

A characteristic structural motif of the family of photopigments is the hypothetical trans-membrane bundle (see Figure 133-2), within which the chromophore retinal, a derivative of vitamin A, is held. The visual pigments belong to an evolutionarily-related superfamily of trans-membrane G-protein-coupled receptors that include the adrenergic, serotonergic, dopaminergic, and muscarinic and olfactory receptors. The evolution of vertebrate photopigments and color vision has recently been reviewed (3). It has been proposed that color vision in primates evolved as an adaptation to feeding on colored fruits and leaves, and that the absorption spectra of the cones were tuned by the reflectance spectra of these objects (4).

The four human photopigments share varying degrees of sequence homology. The red and green photopigments are more closely related to each other (96% amino acid sequence identity) than to any other visual pigments (40%–45% identity) because their ancestral gene duplicated recently during evolution. The  $\lambda_{\max}$  values for the red and green photopigments differ by approximately 30 nm (see Figure 133-1). Most of this difference is accounted for by differences at three amino acid positions of the red and green pigment proteins: Ala 180 Ser (4 nm), Phe 277 Tyr (10 nm), and Ala 285 Thr (16 nm), with amino acids to the left (see Figure 133-2) being in the green pigment and those to the right being in the red pigment. Minor spectral contributions are made by



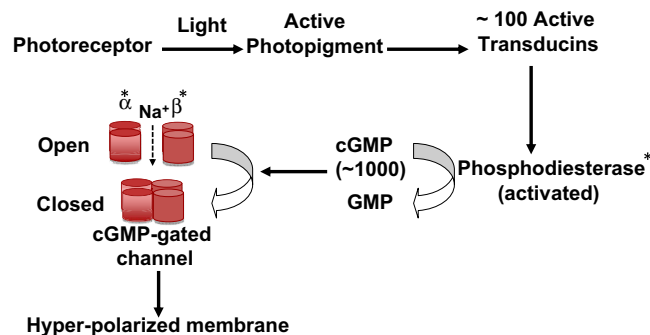
**FIGURE 133-2** Diagram of a photopigment molecule embedded within the cone photoreceptor outer segment membrane. The cylinders represent the 7 $\alpha$  helices that form a bundle that holds the chromophore 11-cis retinal (represented by a magenta pentagon). The N- and C-termini of the pigment protein (opsin) are indicated. The difference in spectral sensitivity between the red and green pigments depends to a large extent upon amino acid residues at positions 180, 277, and 285. The above amino acid residues are positioned to interact with the chromophore. Amino acids to the left of residue numbers interact with the chromophore giving it the  $\lambda_{\max}$  of the red pigment those to the right interact with the chromophore giving it the  $\lambda_{\max}$  of the green pigment. The Ser at position 180 in the red pigment is commonly polymorphic (Ser/Ala), and the Ala at 180 in the green pigment is less commonly polymorphic (Ala/Ser). The change from Ser to Ala at position 180 of the red pigment decreases the  $\lambda_{\max}$  by approximately 5 nm.

differences at Ile 65 Thr, Thr 230 Ile, Ser 233 Ala and Phe 309 Tyr.

### 133.4 PHOTOTRANSDUCTION

The sensation of color results from a comparison of the signal outputs from the three types of cone photoreceptors, and is referred to as trichromatic color vision. The overlap between the spectral sensitivity curves of these photoreceptors (see [Figure 133-1](#)) allows this comparison at each wavelength of visible light. Light of a certain wavelength has a characteristic ratio of probabilities for exciting the blue, green and red photoreceptors. Individuals with normal color vision are able to perceive approximately 2 million colors.

The absorption of a single photon of light causes the isomerization of the retinal chromophore of the photopigments from the 11-*cis* to an all-trans configuration. This isomerization results in the formation of an activated intermediate of the photopigment that triggers the signal amplification cascade. The first step in this cascade involves activation of hundreds of transducin which in turn activate about 70 cyclic GMP phosphodiesterase molecules. The resultant decrease in cGMP levels triggers closure of cGMP-gated channel blocking sodium and calcium influx. This in turn leads to hyperpolarization of the photoreceptor cell membrane and creation of electrical signals that are transmitted to the brain and



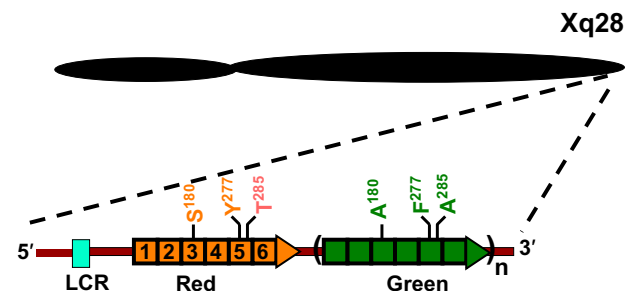
**FIGURE 133-3** A brief diagram of the phototransduction cascade. This pathway converts a photon to an electrical signal in the retina that is transmitted to the brain. This pathway involves major amplification of the energy from a single photon. An absorbed photon activates the photopigment that subsequently activates about 100 molecules of the regulatory protein transducin. Each activated transducin molecule, in turn, activates the enzyme phosphodiesterase. Phosphodiesterase then catalyzes the hydrolysis of about 1000 cGMP (cyclic guanosine monophosphate) molecules to GMP (guanosine monophosphate). Since cGMP keeps the  $\text{Na}^+$  ion channels in the photoreceptor membrane open, reducing its intracellular concentration closes the channels. As a result,  $\text{Na}^+$  ions can no longer enter the cell, thus hyperpolarizing the photoreceptor membrane (the inside of the membrane becomes more negative). The electrical signals due to this hyperpolarization are transmitted to the brain, resulting in light and color perception. \*Points to three proteins (phosphodiesterase and the alpha and beta subunits of the cGMP-gated channel) in which mutations have been shown to cause achromatopsia.

allow sensation of color ([Figure 133-3](#)). See phototransduction videos at: <http://wn.com/phototransduction> for an illustration of this process.

### 133.5 GENES ENCODING THE PHOTOPIGMENTS

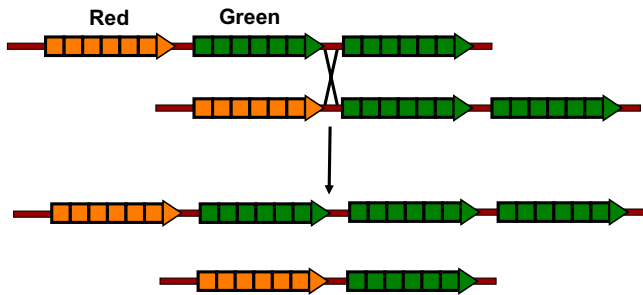
The cloning and characterization of the genes that encode the blue, red, and green photopigment apoproteins (opsins) have been largely due to the pioneering work of J. Nathans and his collaborators. The gene encoding the blue pigment (*OPN1SW*) is located on the long arm of chromosome 7. The genes encoding the green (*OPN1MW*) and red (*OPN1LW*) opsins are arranged in a head-to-tail tandem array ([Figure 133-4](#)) on the long arm of the X-chromosome (Xq28). The array contains repeat units (~40kb in length) starting with a red pigment gene, followed by one or more green pigment genes. The location of the three amino acids that contribute the major difference in the spectral properties of the red and green pigment genes are in exons 3 and 5 (see [Figure 133-4](#)). The two genes are almost identical in structure except for a 1.3kb insertion in intron 1 of the red pigment gene. This insertion was shown to be polymorphic among African Americans, 35%–45% of whom do not have this insertion. The red and green pigment gene repeats are 98% identical at the DNA sequence level. The intergenic regions (about 25kb in length) are also highly homologous in sequence. The high degree of homology between these repeat units predisposes the locus to relatively frequent unequal recombination and gene conversion contributing to the high frequency of color vision defects.

A master switch for expression of the red and green pigment genes, called the locus control region (LCR),



**FIGURE 133-4** Structure of the red and green pigment gene array on the X chromosome. Rectangles represent exons 1–6, and connecting solid lines represent introns. The red and green pigment genes are 15 and 13 kb respectively in length and are separated by approximately 25 kb of intergenic DNA. LCR, locus control region. The location of the three amino acid residues that contribute the majority of the 30 nm difference between wavelengths of maximal absorption ( $\lambda_{\text{max}}$ ) between the red and green pigments are indicated. Note the common Ser180Ala polymorphism in exon 3 of the red pigment gene. The number of green pigment genes in the array varies and is polymorphic. However, only the first two genes are expressed in the retina and, therefore, contribute to the color vision phenotype.





**FIGURE 133-5** Change in green pigment gene number due to unequal crossing over in women who have two X-chromosomes during meiosis. The red and green pigment gene arrays on the two X-chromosomes that are associated with normal color vision align out of register during metaphase of meiosis in females. A homologous unequal crossover occurring in the intergenic region produces gametes with altered numbers of green pigment genes. The lower position of the Figure shows the results of the crossover; a chromosome with three green genes and a chromosome with one green gene. Rectangles represent exons (1–6) of the red and green pigment genes, and connecting solid lines represent introns.

is located between 3.1 and 3.7kb 5' of the transcription initiation site of the red pigment gene. This control region is critical for ensuring mutually exclusive expression of the red and green pigment genes in individual cones, which is fundamental to color vision. It is thought that the LCR couples permanently in a photoreceptor progenitor cell with either the red pigment gene promoter and expresses the red pigment to form a red cone, or couples to the green pigment gene promoter to form a green cone.

It is believed that duplication of an ancestral red pigment gene occurred shortly after separation of the Old and New World primate lineages about 30–40 million years ago. This is in agreement with the observation that humans and Old World monkeys possess both red and green photopigment genes, whereas the majority of New World monkeys have a blue pigment gene on chromosome 7 plus a single red or green pigment gene on the X-chromosome. Interestingly, a New World monkey (*Alouatta*, the howler monkey) was found to have full trichromatic color vision due to the presence of both red and green photopigment genes on the X-chromosome due to a gene duplication that occurred independently in this species. Common polymorphisms in the single X-chromosome cone opsin genes were observed in the New World monkeys that are associated with the expression of either red or green pigments. Female heterozygotes for red and green encoding alleles were shown to have trichromatic color vision (3). Random X-chromosome inactivation during development of the retinas in such heterozygous females results in the formation of both red and green cone photoreceptors.

In individuals with normal color vision, the red pigment gene is located 5' of one or more green pigment genes (see Figure 133-4). The average number of green pigment genes in an array was shown to be 2 (with a

range of 1–6) in a population of European origin. The numerical polymorphism in the green pigment gene is due to unequal intergenic recombination (Figure 133-5). The number of green pigment genes in the array does not appear to influence either the number of green cones in the retina or the color vision phenotype since, as discussed later, only two genes (red and proximal green) of the array are expressed in the retina.

## 133.6 COLOR VISION TESTS

Many types of tests of color vision have been designed, some are simple and rapid for use in mass screening or in the clinical setting, and others are highly sophisticated and accurate for use in the laboratory setting. Four main categories of tests are briefly described: the plate tests, the arrangement tests, lantern tests, anomaloscopy and electroretinography (5).

For online testing see <http://www.TestingColorVision.com>

### 133.6.1 Pseudoisochromatic Plate Tests

The plate tests are designed in accordance with the color confusion characteristics of individuals with the various types of deficiencies. The plates contain designs in the form of numerals in various shades of color such that they are not seen (vanishing design) against the background by a color defective observer. In a second type of design (transformation), two figures are embedded in the background; one can be read by the color-defective observer and the other by the normal observer or vice versa. In the third design (the hidden design), the object can be seen by the color defective observer but not by the normal observer.

**133.6.1.1 The Ishihara Plates.** The Ishihara test (38 plates in the original version but also available as a standard test with 24 plates) is the most universally used test to screen for inherited red–green color vision defects. It is the most efficient plate test because it incorporates the vanishing, transformation, and the hidden designs as well as qualitatively diagnostic plates that allow differentiation of protan from deutan observers, and of the more severely affected dichromats from mild (but not from severe) anomalous trichromats. Inherited blue–yellow as well as acquired color vision defects (most of which are of the blue–yellow type) are not detected by the Ishihara test. Nevertheless, the Ishihara test is the most popular and reliable screening test that is widely available.

**133.6.1.2 The American Optical Company Hardy, Rand, and Ritter (AO HRR) Plates.** This test is used to screen for blue–yellow or tritan color vision defects but is less efficient in distinguishing normal from red–green color vision deficient subjects. Therefore, a combination of the AO HRR and the Ishihara tests would give the best complete assessment of color vision status that one could achieve using the plate method.

### 133.6.2 Screening Children for Color Vision Defects

The Ishihara and AO HRR plates can be used to screen for color vision defects in children from the age of seven years and older. Younger children are more difficult to screen but a specially devised eight-plate Ishihara test for unlettered persons based on shapes and tracing pathways has been successfully used on children as young as four years old.

### 133.6.3 Arrangement Tests

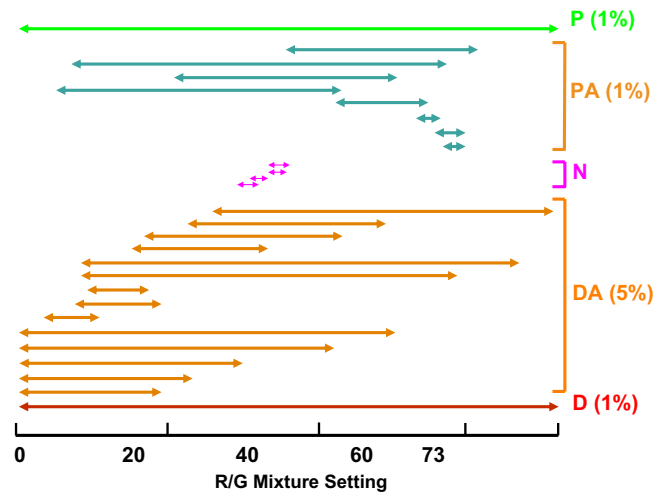
The arrangement tests involve asking observers to order a set of movable colored subjects according to either hue or saturation. This type of test assesses the color discrimination ability of both normal and color-defective individuals. The Farnsworth-Munsell 100-hue test (FM 100-hue test) consists of 85 color samples that span the entire natural color circle and are arranged in four boxes (21 or 22 colors each). In this test, the score for placement of each color sample is obtained by recording the absolute differences between the number designated for each sample (written on its reverse) and those of the adjacent samples. The scores are usually plotted to form a radial graph.

### 145.6.4 Lantern Tests

The US armed services (with the possible exception of the US Air Force), Coast Guard and Federal Aviation Agency medical examiners, use the “Farnsworth Lantern” (FALANT) as a color-naming test that simulates the identification of signal lights. This test is not used for clinical purposes. Nine pairs of colors (including red, green and white) are shown for initial assessment and are repeated twice if mistakes in naming of colors are made. The Pass/Fail level is based on the number of color naming errors. All dichromats and 75% of anomalous trichromats fail this test (2,9). There is no clear bimodality between passing and failing grades. Anomaloscopy or Farnsworth arrangement tests may identify anomalous trichromats who are likely to pass this lantern test.

### 133.6.5 Anomaloscopy

Anomaloscopy has been used widely for the detailed study and diagnosis of different types of color vision deficiencies. It is based on matching the color of a test light by mixing two other lights. Lord Rayleigh devised a simple test system to classify individuals with red-green color vision abnormalities. The observer views a pure yellow light (589–590 nm) on one half of a screen, whereas the other half of the screen projects a mixture of red (650 nm) and green (545–550 nm) lights. The brightness or intensity of the yellow light, as well as the proportion of the green and red lights, are adjusted by the subject until both hemi fields appear matched in color and brightness. Under the color conditions of



**FIGURE 133-6** Anomaloscopic Rayleigh match ranges of protan, deutan, and normal subjects. Horizontal bars represent the range of red and green light mixtures that each subject could match the standard yellow light. Numbers to the left of bars are for subject identification. P, Protanopia; PA, protanomaly; D, deuteranopia; DA, deuteranomaly; N, normal. (Data from Ref. 32).

the Rayleigh match, color detection by the short-wave sensitive or blue pigment cones is negligible. The most frequently used instrument is the Nagel Anomaloscope. The range of accepted matches of mixtures of green and red light against yellow is recorded, as is the mid-point of such matches. Figure 133-6 shows typical Rayleigh matches for normal and various color defective persons.

Normal observers accept a much narrower range of mixtures of red and green lights than color-defective individuals. Some color-defective individuals have both a wide match range and a shifted match midpoint, whereas others have only a shifted match midpoint. Dichromats such as protanopic and deuteranopic subjects will match yellow with any and all ratios of red and green, including red and green alone. Dichromatic deuteranopes require much more yellow to match pure red color than dichromatic protanopes, who need only a small amount of yellow to match the red field, which they perceive as of low intensity. Protanomalous subjects produce match ranges that are shifted to the red side of the spectrum, while the matches of deuteranomalous subjects are displaced to the green. Subjects with severe deuteranomaly and severe protanomaly tend to have relatively wide match ranges, while those with milder anomalous defects have narrower match ranges.

Definite diagnosis of protanopia, protanomaly, deuteranopia, and deuteranomaly requires anomaloscopy. This test is generally less available since anomaloscopy is not required for most clinical purposes in ophthalmology and optometry.

### 133.6.6 Electroretinography

In contrast to the various psychophysical methods of color vision assessment that rely on the observer's subjective perception of color, electroretinography (ERG)

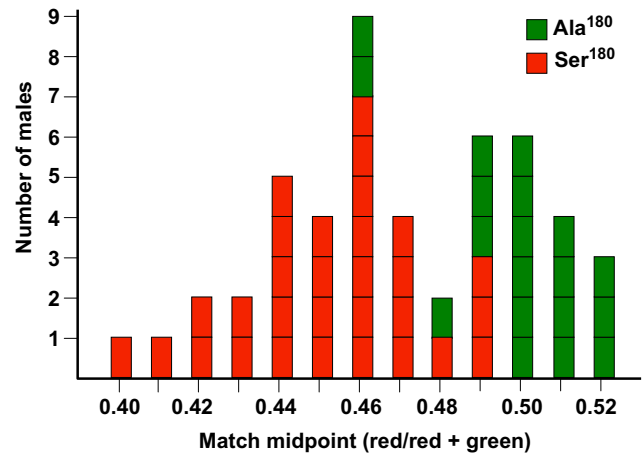
is an objective means of determining the color vision phenotype. In ERG, a corneal electrode placed on an anesthetized dilated eye records the retinal response to standardized flashes of light. Because of the somewhat invasive nature of the test, extensive studies of the various inherited color vision defect have not been reported. However, color vision defects characterized as deutan and protan could be discriminated by the log ratio of the sensitivity at short (480 nm) and long (620 nm) wavelengths (sensitivity quotient). The use of ERG for detection of female carriers of protan and deutan defects was particularly successful for deutan carriers and less so for protan heterozygotes.

### 133.7 THE MOLECULAR BASIS OF VARIATION IN NORMAL COLOR VISION

Subtle variation in color perception in the red–green region of the spectrum has been observed among individuals considered to have normal color vision. Rayleigh color matches determined by anomaloscopy in male subjects with normal color vision fell into two main groups. Women with normal color vision formed a third and larger group with intermediate values of Rayleigh match midpoints. A similar independently described Rayleigh match variability fitted transmission by X-linked inheritance in families. These observations pointed to the presence of two common spectrally different forms of the red pigment gene.

Eight relatively common amino acid polymorphisms were discovered in the red pigment gene and five in the green pigment gene. The substitutions at three (Ser180Ala, Ile230Thr, and Ala233Ser) of these sites involved a change from hydroxyl-bearing to non-polar amino acid residues and, therefore, are likely to alter the spectral characteristics of the red pigment. The Ser-180Ala polymorphism was of great interest because it was quite common in the red pigment, occurring at a frequency of 64% Ser and 36% Ala in the studied population of European origin (serine occurred at a frequency of 16% in the green pigment).

We investigated the possibility that the common Ser180Ala polymorphism could underlie the above-mentioned phenotypic distributions of color vision. Fifty white men with normal color vision were tested for the hypothesis that the two major groups in the distribution of color matching could be explained by the Ser/Ala polymorphism in the red pigment. The frequency distributions of Rayleigh match midpoints and of the deduced amino acid sequence of the red photopigment show that higher sensitivity to red light (i.e. requirement of less red in the mixture of red and green lights to match the standard yellow light) was highly correlated with the presence of Ser at position 180 (Figure 133-7). Therefore, these men have a different perception of red light to those having the Ala allele at this site. The retinas of females who are heterozygous for the Ala and Ser alleles would be expected to contain an additional cone type that is red-like. This is



**FIGURE 133-7** Frequency distribution of Rayleigh match midpoints as a function of the presence of serine (Ser) or alanine (Ala) at position 180 in the red pigment. Individual male subjects with Ser or Ala at position 180 are represented by red and green rectangles, respectively. Rayleigh match ranges were determined by asking individuals to combine various quantities of red and color lights to match a standard yellow light (see Figure 133-6). Individuals with Ser at position 180 required less red light in the mixture of red and green lights to match the standard yellow light than did those who had alanine (Ala). (Data from Ref. 33).

due to the random X-chromosome inactivation during retinal development that results in expression of either the Ser red in some cones or the Ala red in others. Thus the retinas of such heterozygous females will have four cone types: blue, green, red and red-like cones.

### 133.8 THE MOLECULAR BASIS OF COLOR VISION DEFECTS

#### 133.8.1 Color Vision Defects











The inherited types of color vision deficiencies have been classified into three major categories: The red–green (protan and deutan) deficiencies, the blue–yellow (tritan) deficiencies, and the achromatopsias. The most common defects are by far the X-chromosome-linked red–green defects, which occur at frequencies of about 8% in men and 0.4% in women of European extraction. The blue–yellow defects and achromatopsias are rare. The frequency, mode of inheritance and symptoms are given in Tables 133-1 and 133-2.

#### 133.8.2 Red–Green Color Vision Defects

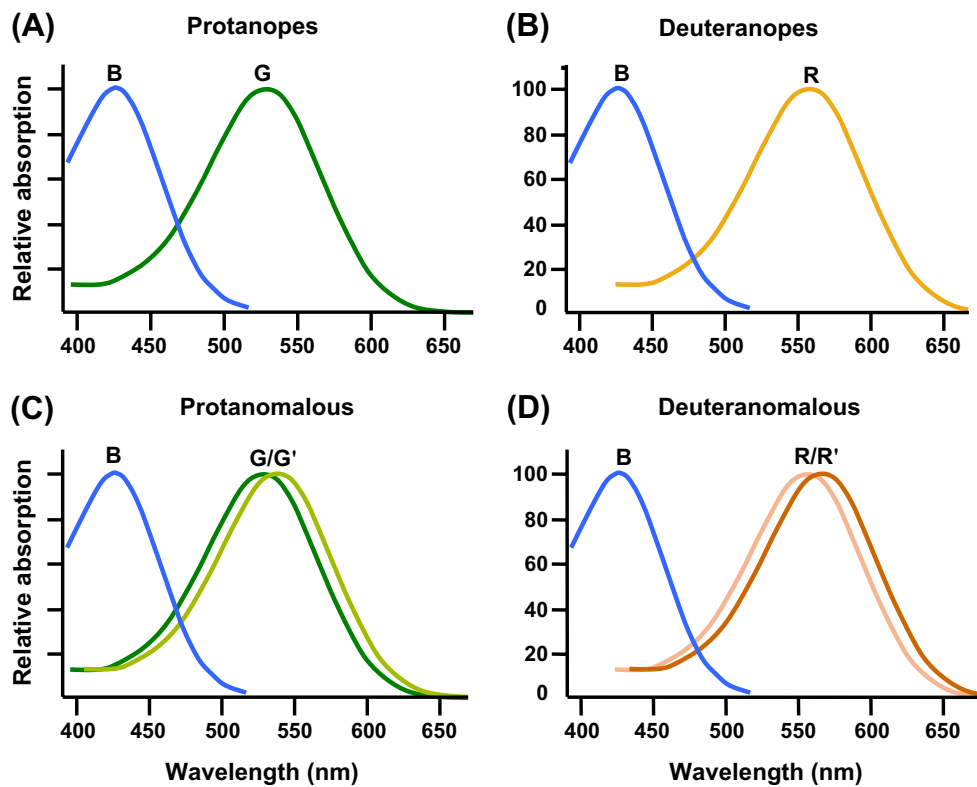
There is significant variation in the red–green color vision frequencies among populations of different ethnic groups.

**133.8.2.1 Subclasses.** Individuals with normal color vision can match the color of a test light of any wavelength composition by mixing the right proportions of three primaries, such as red, green and blue, or by mixing two of the primaries and adding the third to the test light. Therefore, they are referred to as having trichromatic

**TABLE 133-2 Classification of Rare Genetic Color Vision Defects**

Class	Retinal Cones			Frequency (European)	Color Discrimination
Normal					
Tritanopia (Aut dom)	No			~ 1/500	
Blue cone monochromacy (X-linked rec)		No	No	~1/100,1000	
Achromatopsia (Aut rec)	No	No	No	Very rare	

X-linked rec, X-linked recessive inheritance; aut dom, Autosomal dominant; aut Rec, Autosomal recessive. Color vision discrimination capacity includes simulated rainbow. B, Blue cone; G, Green cone; R, Red cone. The loss of B cones prevents discrimination of colors in the yellow, green and violet regions of the spectrum. Loss of R and G cones results in complete loss of color discrimination.



**FIGURE 133-8** Absorption spectra of cones of males with defective red-green color vision. The retina of a protanope has only blue (B) and green (G) cones (A), that of a deuteranope has blue and red cones (B) and both subjects have severe color vision defects. Protanomalous males have blue, green (G) and green-like (G') cones (C); deuteranomalous males have blue, red and red-like (R') cones (D) and both have milder color vision defects. The separation between the absorption spectra of normal and anomalous cone pigments is roughly inversely related to the severity of the color vision defect.

color vision. Individuals with severe color vision defects have lost one or more of the photopigments. In those with milder color vision defects, one of their normal pigments has been replaced by an anomalous pigment with altered spectral properties (Figure 133-8). Individuals who lack functional red or green cones are known as protanopes (P) or deuteranopes (D), respectively. They are able to match the color of a test light by mixing two primaries only

and therefore have dichromatic color vision. Individuals with only blue functional cones are classified as having monochromatic vision. A milder form of defective color vision among humans is anomalous trichromacy. Although anomalous trichromats appear to have three classes of photoreceptors, one of these classes has an anomalous spectral sensitivity. Protanomalous (PA) men have normal blue and green cones plus an anomalous red



cone with spectral sensitivity that is shifted significantly toward that of the green cone. Deuteranomalous (DA) individuals have normal blue and red cones, together with an anomalous green cone with a spectral sensitivity that is shifted toward that of the red cone.

### 133.8.3 Color Perception in Color Vision Deficiency

Color discrimination is a major component of our perceptual world. A large fraction (~8% of men and 0.5% of women) of the population of European origin has defective color vision and therefore perceives their environment differently. The frequency of the various color defects is less common in other continental populations (African males ~3% and East Asian males 3%–4%). Dichromats as a group have more abnormal color vision than anomalous trichromats. However, dichromats are said to have an advantage in penetrating military and natural scene camouflage. Dichromats were experimentally shown to be more proficient than observers with normal red–green color vision in detecting texture boundaries of target objects that are camouflaged by color.

A significant percentage (~15%–30%) of deuteranomalous trichromats (who constitute about two thirds of all color defectives) are not aware of their color vision defects. The most severely affected persons are protanopic

dichromats, followed by deuteranopic dichromats. Among the less severely affected trichromats, protanomalous men are more inconvenienced than deuteranomalous men. Protanopes and deuteranopes have problems discriminating red, yellow, and green but also deep red and deep brown. Colors of the red family may be perceived as black by protanopes. Most but not all dichromats have difficulty in discrimination and selection of colored articles, materials, and foods. Approximate simulations of how a natural scene appears to individuals with normal, protanopic, deuteranopic and tritanopic color vision are shown in Figure 133-9. The Web site (<http://jfly.nibb.ac.jp/html/colorblind>) describes various colors that color defective individuals have difficulty with, and includes instructions on how to prepare color presentations that are not confusing to color-deficient observers.

Recognition of red traffic light signals, barricade signal lights, and car or bicycle reflectors may be impaired, and reaction times may be slightly delayed among dichromats, particularly among protanopes. Poor lighting conditions may worsen color perception. However, color vision is not usually tested to obtain non-commercial driver licenses.

Having X-linked color vision defects has been used for many years to exclude applicants from a variety of employment, particularly among marine, air, rail, industrial and military occupations that require an ability to distinguish colors. Recent years have seen increasing use

**Normal**



**Protanope**



**Deuteranope**



**Tritanope**



**FIGURE 133-9** Simulation of the actual color vision of normals, protanopes, deuteranopes and tritanopes. Shown is a natural scene as it appears to a person with normal color vision and a simulation of how it appears to a protanope, deuteranope or a tritanope. Note that protanopes and deuteranopes are unable to distinguish red from green. Simulation was performed by the vischeck computer program that simulates human color vision and is freely available online at <http://vischeck.com/>.

of color-testing for a variety of practical applications that require color discrimination. Plate testing, such as the Ishihara test and the American Optical HRR plates, is usually employed for screening, and abnormal results may lead to rejection of job candidates. The use of a lantern test (such as FALANT [see earlier]) designed for practical testing by color naming may pass individuals such as those with deuteranomaly who fail plate-screening tests.

There are many occupations of all sorts including chemists, pharmacists, certain medical specialists, color printers, cartographers, commercial drivers and many others, in which color vision defects may be a handicap and may have significant consequences (6). In recent years, discrimination because of disability is increasingly condemned, particularly if such a “disability” has genetic causes; however, such “discrimination” may be justifiable if the color-defective person places others at risk or if he or she cannot adequately perform the required duties of employment. Therefore, rejection of color vision defectives for entry into certain occupations cannot be considered genetic discrimination, because the reason for not being hired has nothing to do with genetics per se but is based on job, public safety, or related issues. Decision making may not be entirely clear because, as an example, persons who are designated correctly as deuteranomalous by a plate test may be fully capable of carrying out practical tasks of color discrimination in certain jobs and may pass the lantern color test.

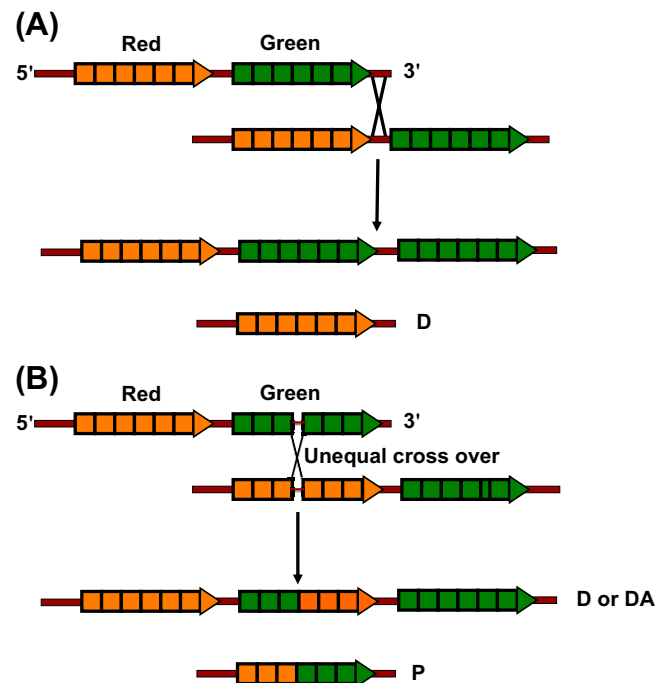
The relatively high frequency of color vision defects and the availability of reliable tests should lead to its wider detection among young people. Advice to avoid occupations that will cause problems for those with color vision abnormalities may be helpful and appropriate. At the same time, society and educational institutions should be aware of the extent of color vision defects and avoid colors on slides and computer screens that could be easily confused.

**133.8.3.1 Molecular Basis.** The molecular basis of the X-linked red–green color vision defects was first delineated by Jeremy Nathans and colleagues. They suggested that the various red–green color vision defects result from unequal homologous crossover or recombination between the red and green pigment gene units, leading either to deletions of the green pigment genes and/or to the formation of various types of full length red–green hybrid genes (Figure 133-10). The juxtaposition of the highly homologous red and green pigment genes during meiosis in females predisposed this locus to a relatively high frequency of such unequal recombination events. Subsequently, several studies have extended the delineation of the molecular bases of red–green color vision defects in various ethnic groups. The major findings of these investigations were:

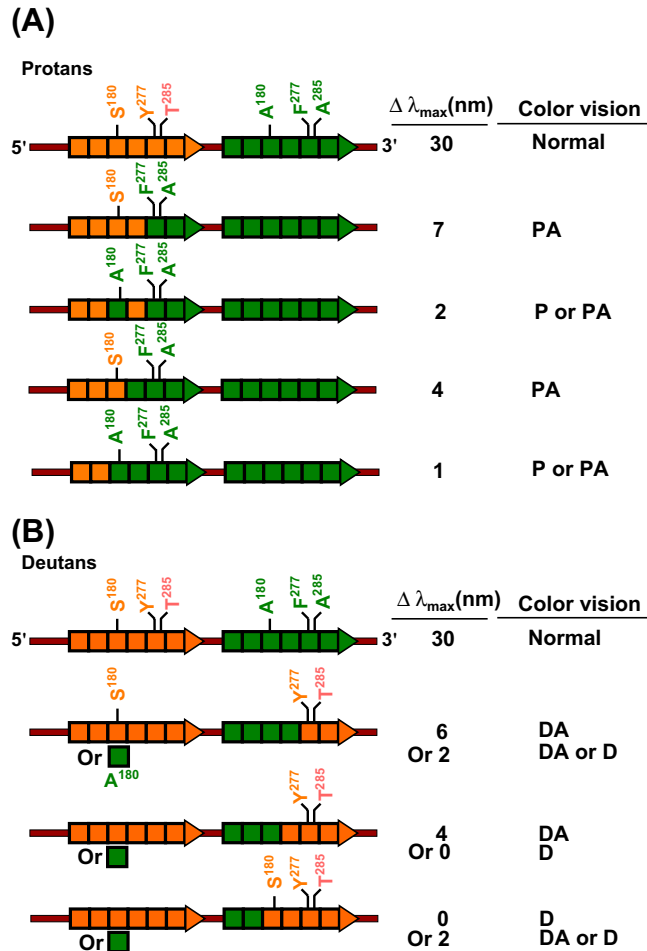
1. The majority of the *protan* red–green color vision defects are caused by conversion of the red pigment

gene to a variety of 5′ red–green 3′ hybrid genes that encode green-like pigments. The majority of *deutan* color vision defects are due to deletion of the green pigment gene or the conversion of the expressed green pigment gene to a variety of 3′ green–red 5′ hybrid genes that encode red-like pigments. In dichromats, *protanopia* is associated with gene arrays composed of a 5′ red–green 3′ hybrid gene found singly or together with normal green pigment genes, and *deuteranopia* was usually associated with gene arrays comprised of a single red pigment gene, and rarely with a red pigment gene together with a 5′ green–red 3′ hybrid gene. In anomalous trichromats, *protanomaly* was associated with gene arrays composed of a 5′ red–green 3′ hybrid and a normal green pigment gene, whereas *deuteranomaly* was associated with gene arrays composed of a normal red pigment gene, a 5′ green–red 3′ hybrid gene, in the absence or presence of a normal green pigment gene (Figure 133-11).

2. Exon 5 of the red and green color vision genes was found to play a major role in determining spectral sensitivity of the encoded photopigments. For example, replacement of exon five of the red pigment



**FIGURE 133-10** Unequal homologous crossing over in females during metaphase of meiosis leads to either gene deletion or the formation of red–green pigment hybrid genes. (A) Unequal homologous crossover in the intergenic region of the misaligned arrays of pigment genes leads to deletion of the green pigment gene(s), producing a single normal red pigment gene associated with deuteranopic (D) color vision deficiency, plus an array with one red and two normal green pigment genes. (B) Intragenic unequal crossover leads to the formation of 5′ red–green 3′ and the reciprocal 5′ green–red 3′ hybrid genes that are associated with either D or deuteranomalous (DA), and protanopic (P) Color vision defects, respectively.



**FIGURE 133-11** Genotype–phenotype relationships in men with protan and deutan color vision defects. Shown are examples of common gene arrays found among men with protan (Figure 133-11A) and deutan (Figure 133-11B) color vision defects. Rectangles represent exons (1–6) of the red and green pigment genes, and connecting solid lines represent introns. Amino acid differences at positions 180, 277 and 285 influence the major differences in  $\lambda_{\max}$  between the red and green pigments (A, alanine; S, serine; F, phenylalanine; T, threonine; Y, tyrosine). Differences ( $\Delta$ ) in  $\lambda_{\max}$  between the pigments encoded by the two genes in each array in males are indicated. The larger the  $\Delta \lambda_{\max}$  between the two pigments is generally associated with milder color vision deficiency. At low  $\Delta \lambda_{\max}$  (2 nm) the severity of the color vision defect is influenced by other factors, such as the density of the pigment in the photoreceptor. The hybrid genes were assigned the proximal position on the basis that distal hybrid genes are not expressed and therefore are not expected to cause color vision defects. Red–green and green–red hybrid genes are associated with protanopia (protanopic (P) or protanomalous (PA)) and deuteranopia (deuteranomic (D) or deuteranomalous (DA)). Color vision defects, respectively. Single gene arrays are associated with severe color vision defects (P) and (D) color vision defects. Multigene arrays are usually associated with milder defects unless the first two genes of the array encode pigment with identical or very similar (separated by 1–2 nm). The Ser180Ala polymorphism influences the  $\Delta \lambda_{\max}$  between the two pigments encoded by genes in the array and, therefore, the severity of color vision defects.

gene with that of the green pigment gene by unequal homologous recombination produced a hybrid pigment that was sufficiently green-like in its spectral

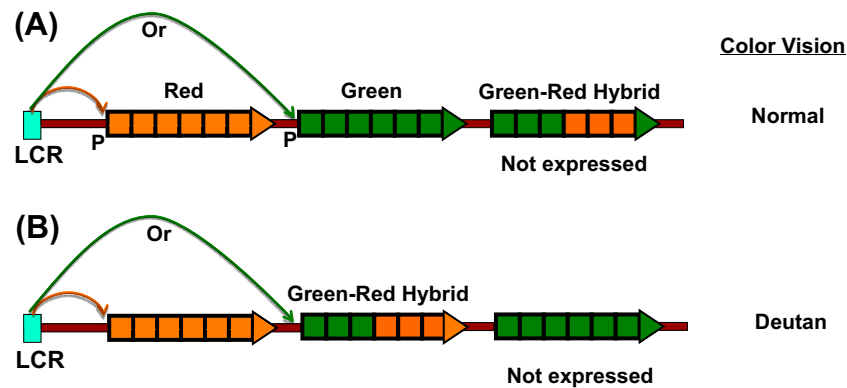
properties to make the subjects perform as *protans*. This result is consistent with the conclusions that differences at amino acid residues 277 and 285, which are encoded by exon 5, contribute the majority of the difference (24 of 30 nm) in wavelength of maximum sensitivity between the red and green pigments (see Figure 133-4).

3. Certain *deutan* men carried both green–red hybrid genes, as well as normal green genes. In theory, if all the genes were expressed in photoreceptors, these individuals would be expected to have normal color vision. In fact, about 6% of males with normal color vision carry such arrays. To explain this, we hypothesized that only the first two genes in the array are expressed in the retinal cone photoreceptors, as evidenced by analysis of red and green pigment mRNAs and by ERG on deutan subjects. Furthermore, in male eye donors who carry arrays typical of living *deutans* (normal red, normal green and a green–red hybrid gene), the green–red hybrid but not the normal green gene is expressed (7).
5. Based on studies of males with *deutan* and normal color vision, we determined, using long-range DNA amplification, that deutan color vision results only if the green–red hybrid gene occupies the second position in the array (Figure 133-12). We proposed that lack of expression of the green pigment genes that occupy third and more distal positions with respect to the locus control region (LCR) could be due to the very low probability of the LCR coupling to these genes and switching on their expression.
6. Point mutations were also found to cause relatively rare color vision deficiencies (8). For example, 2%–5% of *deutans* have a substitution of arginine for the highly conserved serine at position 203. The same mutation was also discovered as the cause of inactivation of a single red–green hybrid gene in about one third of probands with blue-cone monochromacy (see below). An association of an A→T substitution at position –71 of the promoter of the green pigment gene, located in the second position of the array, was found to be associated with *deutan* color vision in Japanese men (9). This is consistent with the observation that only the proximal green pigment gene of the array is expressed. The A-71T substitution has not yet been found among Europeans.

### 133.8.4 Relationship between Genotype and Severity of Color Vision Defects

The severity of color vision defects varies widely. A number of studies have addressed the question of how accurately the genotype at the red–green gene locus predicts the severity of the color vision defects. Because the presence of red–green hybrid genes is a common cause of color vision defects, these studies focused on determining





**FIGURE 133-12** Model for selective expression in the X-linked red/green pigment gene complex, and the importance of gene order in expression. Shown is a gene complex composed of a red, green and a 5' green–red 3' hybrid gene that can be associated with either normal or defective color vision (deutan) depending on the position of the hybrid gene. Note that only the first two genes in the array are expressed in the retina and, therefore, contribute to the color vision phenotype. (A) In the male carrying this gene complex, the locus control region (LCR) of the array turns on expression by activating the promoter (P) of the red pigment gene to form red cones, or the P of the proximal green pigment gene to form green cones. The green–red hybrid gene is not expressed because, most likely, it is much farther downstream from the activating LCR. Therefore, a male individual with this gene complex is expected to have normal color vision. (B) If the green–red hybrid gene occupies the second position in the array, the LCR expresses either the red or the green–red hybrid gene in a single cone photoreceptor. The normal green pigment gene, being located in the third position of this array, is not expressed. This gene arrangement would therefore be expected to be associated with deutan color vision defect in males because of the lack of normal green photoreceptors.

the structure of the gene arrays as well as the coding sequences of normal and hybrid genes they carry. Knowing the coding sequences of the genes allows inference of the spectral properties of the encoded pigments. For this purpose, we and others have developed a rapid polymerase chain reaction-based methodology to determine the sequence and number of genes in the red–green pigment array, as well as the presence, sequence, and in many cases, the position of hybrid genes in the array (10).

Theoretically, one would expect that men who carry a single-pigment gene or whose first two genes encode pigments with identical spectral properties to have the most severe color vision defects (dichromatic). This is what was generally observed (see Figure 133-11). There were a few exceptions, in that some of these men were severe anomalous trichromats instead of dichromats. These exceptions contradict fundamental color vision theory, because these subjects should have only blue and either red or green cones, yet they were able to make wavelength discriminations. Aside from differences in test performance, factors other than sequence of the pigments appear to influence the width of the Rayleigh match. These include variation in the ratio of red to green cones in the retina and in pigment optical density (quantity) (11) in photoreceptors.

Men whose first two genes of the array encode normal and anomalous pigments that differ in  $\lambda_{\max}$  would in general have milder color vision defects. The severity of such defects would be positively correlated to the  $\lambda_{\max}$  difference between the two pigments. Observations of both protan and deutan subjects roughly support this hypothesis (see Figure 133-11). The observed exceptions are those whose normal and anomalous pigments differ by 1–2 nm in  $\lambda_{\max}$  and are associated with either dichromatic or severe anomalous trichromatic color vision.

Again, other factors such as pigment optical density or retinal cone ratio, or both, may significantly influence the severity of the color vision defect when the difference between the two encoded pigments is small.

## 133.9 THE GENETICS OF RED–GREEN COLOR VISION IN WOMEN

### 133.9.1 Simple Heterozygotes

About two-thirds of female heterozygotes for red–green color vision defects are carriers of deuteranomaly, because this anomaly is the most frequent defect among men (5% of European male population). Random X-chromosome inactivation during early development allows expression of either allele of the red and green pigment genes on either chromosome in single cone cells. Psychophysical evidence is consistent with this prediction. The majority of protan and deutan heterozygotes have normal color vision by standard tests. Some heterozygotes have been found to have mild color vision deficiencies. In study of 31 obligate female carriers of color vision deficiency, Jordan and Mollon found that such subjects made significantly more errors on the Ishihara plates and had enlarged match ranges on Nagel anomaloscopy. The presence in the retinas of heterozygous women of four instead of three types of cone: blue, red, green and a fourth red–green hybrid, presents the potential for tetrachromacy. Tetrachromats possess four independent channels for processing signals from light resulting in a highly enhanced color discrimination capacity compared to trichromacy. We have recently tested this hypothesis in heterozygous carriers of protanomaly and deuteranomaly. The results showed that only one out of 24 carriers of deuteranomaly had



tetrachromatic color vision (12). A tetrachromat woman can see four distinct ranges of color instead of the three that trichromats can see. Three important factors seem to be important for having tetrachromatic color vision in female heterozygotes. One is a wide spectral difference between the normal and the additional anomalous pigment, the second is the relative ratio of cone types in the retina that vary considerably between individuals and is associated with color discrimination capacity, and the third is the role of optical density (the number of photopigment molecules per cone cell) in color discrimination. It may be possible that amino acid polymorphisms could alter the optical density of opsin molecules without changing their wavelength of sensitivity (13).

Rarely, a heterozygote may exhibit color vision deficiency, presumably as a result of extremely skewed X-chromosome inactivation of the normal allele, and thus express the defective allele. Skewed X-inactivation seems to be more common in one member of identical female twins. The frequency of this phenomenon among such heterozygote identical twin pairs is no more than 5%.

Molecular techniques usually cannot be used for the diagnosis of heterozygotes because such women carry both the normal and abnormal color vision gene arrays on their two X-chromosomes. However, heterozygotes for protan color vision defects can be identified because the red–green hybrid gene associated with such defects always occupies the first position in the array, and can be detected even in the presence of normal red pigment gene.

### 133.9.2 Compound Heterozygotes

Compound heterozygotes for red–green color vision defects have also been observed. Heterozygotes who carry an allele for deuteranomaly (presence of green–red hybrid gene) and an allele for deuteranopia (deletion of green pigment gene) on each of the two X chromosomes of different parental origin manifest with the milder phenotype of deuteranomaly. This is expected because their retinas have some cones with normal red pigment and others with the hybrid pigment. Similarly, protanomaly/protanopia compound heterozygotes manifest the milder phenotype of protanomaly.

In contrast, compound heterozygotes for protan and deutan defects on either the maternal or paternal X chromosomes have normal color vision, because some women have both normal red and green pigment genes and, therefore, are functionally heterozygotes for both protan and deutan defects.

### 133.9.3 Potential Gene Therapy in Red–green Color Vision Deficiencies

Improvement of color vision in dichromatic subjects (protanopic and deuteranopic) with severe color vision deficiency, being common among males, would be an important future accomplishment. The potential for

this improvement was tested in the dichromatic squirrel monkey that naturally has blue and green photoreceptors (14). Subretinal injection of a recombinant adeno-associated virus expressing the red pigment under the control of red pigment promoter and LCR was performed. The introduction of a third, randomly positioned, red cone photoreceptor into the retina resulted in trichromatic color vision behavior in this monkey. This provides the potential for similar gene therapy to improve color vision in humans in the foreseeable future.

## 133.10 BLUE–YELLOW (TRITAN) COLOR VISION DEFECTS

Inherited tritan color vision defects are much rarer than red–green defects and are characterized by selective loss of blue-sensitive photoreceptor function, causing greatly diminished or absent color discrimination in the blue–green region of the spectrum (450–480 nm). As in red–green defects, visual acuity is unimpaired. A survey in the Netherlands indicates that the frequency of tritan defects in the population may be as high as 1 in 500. The mode of inheritance is autosomal dominant, exhibiting variable severity of manifestation among affected members of a family.

Nine unrelated subjects with tritanopia have been reported. Heterozygosity for one of the following missense mutations in the gene encoding the blue pigment opsin, located on chromosome 7, has been shown to cause tritanopia: Gly79Arg in two Japanese subjects, Ser214Pro, and Pro264Ser and Arg283Gln. The retina of a subject with the Arg283Gln mutation was examined by adaptive optics technology. It was found that this mutation caused progressive blue cone dystrophy, as well as disruption of the regularity of the cone mosaic (15). The mutant alleles co-segregated with tritanopia in an autosomal dominant fashion; however, incomplete penetrance was observed in association with the Gly79Arg and Ser214Pro substitutions. The mutant gene(s) in two other known subjects with tritanopia remain unknown.

The dominant mode of inheritance suggests that accumulation of a defective opsin within photoreceptors causes either loss of function or cell death, reminiscent of the mutations in the rhodopsin and peripherin genes that cause a subset of autosomal dominant retinitis pigmentosa. Tritanopia has also been observed in association with some disorders of vision such as autosomal dominant juvenile optic atrophy.

## 133.11 THE ACHROMATOPSIAS

Achromatopsia refers to the complete absence of color discrimination capacity. Achromats can match any color to any other color by adjustment of the relative brightness. There are several types of inherited achromatopsia that fall into two major categories: typical complete and atypical incomplete (Table 133-2).

### 133.11.1 Typical Complete Achromatopsia: Total Color Blindness/Rod Monochromacy

Typical complete achromatopsia is inherited as an autosomal recessive trait with an incidence of approximately 1 in 30,000. In addition to loss of color discrimination, subjects suffer from reduced visual acuity from infancy, photophobia, and nystagmus. These symptoms reflect lack of function of all three classes of cone photoreceptors and vision is based on only rods. Adaptive optics technology was recently used to examine the status of cones in the living retina of a patient with achromatopsia. It was found that the size and density of cones in the fovea and parafovea were severely altered (16).

Phenotypic and genetic heterogeneities characterize this disorder have been reviewed (17). The first locus for this disorder was mapped to chromosome 2q11. The responsible gene at this locus is that encoding the  $\alpha$ -subunit of the cone photoreceptor cGMP-gated channel (*CNGA3*), located in the membrane of cone outer segment. This is a component of the signal transduction pathway in all three classes of cone photoreceptors (Figure 133-3). Mutations in this gene account for approximately 25% of all achromatopsia patients. *CNGA3* mutations were also observed among individuals with incomplete achromatopsia and in a few cases of cone dystrophy. The phenotypic heterogeneity is probably due to the extent to which the signal transduction cascade is affected by the mutation.

A second genetic locus for this disorder has been identified on chromosome 8q21–22 among patients from

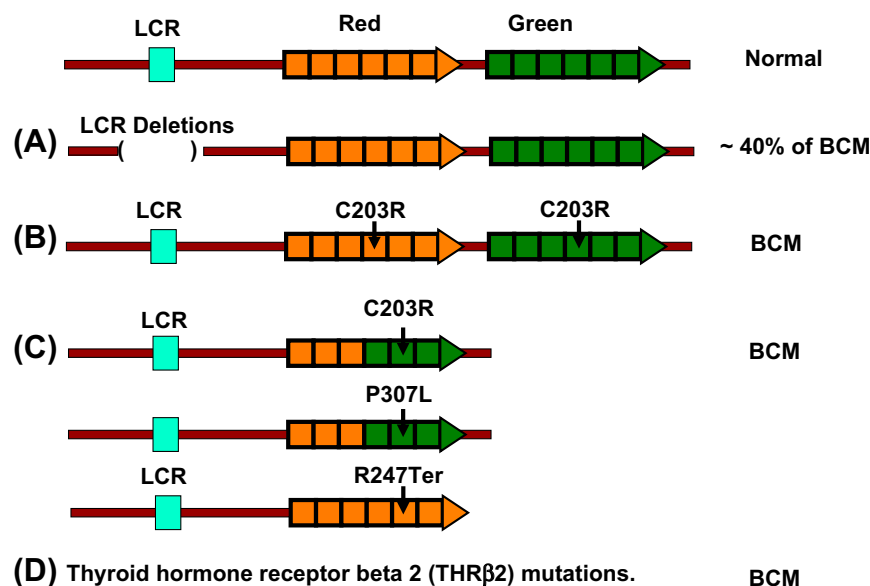
the Micronesian island of Pingelap and from European families. The incidence of achromatopsia on the island of Pingelap is about 10%, attributed to a founder effect. A description of the island of Pingelap and its inhabitants has appeared in the book *The Island of the Colorblind*. The responsible gene (*CGNB3*) encodes the  $\beta$ -subunit of the cone photoreceptor cGMP-gated channel (18). Mutations in this gene account for 40%–50% of all achromatopsia cases (19). It was demonstrated in the mouse retina that the proteins encoded by the *CNGA3* and *CGNB3* form the channel by heterotetrachromatic complex interactions (20).

Mutations in a third gene, a cone photoreceptor guanine nucleotide  $\alpha$ -subunit of the cone transducin gene (*GNAT2*) on chromosome 1p13, was shown to account for about 2% of these cases (21). The  $\alpha$ -subunit of the cone transducin is a key component of the signal transduction pathway in all classes of cone (Figure 133-2).

An achromatopsia network has recently been established: (<http://www.achromat.org/>).

### 133.11.2 Gene Therapy of Achromatopsia

The genetic causes of achromatopsia result in loss of function but not death of cone photoreceptors, providing an ideal model to re-establish functionality by gene therapy. This has been positively accomplished in knockout mouse models of the *GNAT2* (22) or the *CNGA3* (23) genes using sub-retinal injection of recombinant adeno-associated virus (AAV) gene therapy. A successful, stable restoration of cone function and day vision was also



**FIGURE 133-13** Four genetic mechanisms of causing blue-cone monochromatism (BCM). (A) Deletion of the locus control region (demarcated by brackets) that prevents expression of both red and green pigment genes. (B) Point mutations (C203R) that inactivate both the red and green photopigments. The C203R is the most common point mutation associated with BCM. (C) Gene deletion due to unequal recombination leaving the first gene in the array, followed by point mutations in the coding sequences (C203R, P307L or R247-to-Ter) with deleterious effects on the encoded photopigment. All three mutations cause BCM. (D) Homozygosity for mutations in the thyroid hormone receptor beta 2 gene (THRβ2).

accomplished in a canine model with achromatopsia due to a mutation in the *CNGB3* gene (24). The recombinant AAV used contained the promoter of the red pigment gene as well as the locus control region. The success and stability of cone function was higher in young-treated models (25), perhaps due to progressive loss of cones.

### 133.11.3 Atypical Incomplete Achromatopsia: Blue Cone Monochromacy

Blue cone monochromacy (BCM), also referred to as Pi1 monochromacy, or X-linked recessive incomplete achromatopsia, is an extremely rare disorder (less than 1 in 100,000) in which both red and green cone functions are absent. Therefore, using the remaining functional blue cones, the wavelength composition of light cannot be perceived since this requires comparisons between the outputs of signals from at least two cone types (see Table 133-2).

This disorder is characterized by severe reduction in visual acuity, central scotoma (corresponding to the blue cone-free fovea), infantile nystagmus and sometimes myopia. Progressive central retinal dystrophy has been reported in some patients with BCM, indicating that cone degeneration may result from the accumulation of an abnormally assembled photopigment in analogy with the mutations in rhodopsin found to underlie autosomal dominant retinitis pigmentosa. Bilateral macular atrophy has also been observed in a family with blue cone monochromacy due to a deletion of the locus control region.

There is a specific test that distinguishes between BCMs and rod monochromats. In this test, the subject is shown four plates, each containing three identical blue-green arrows and one purple-blue arrow. The plates differ from one another only in the chromaticity of the purple-blue arrow. Blue-cone monochromats can distinguish the purple-blue arrows in all four plates. Rod monochromats cannot.

Nathans and colleagues analyzed the structure of the red-green locus in individuals from 38 families with BCM. They found two mutational pathways that lead to this phenotype. One, found in 14 families, involved deletions (587–55 kb) that included a regulatory sequence located approximately 3.4 kb 5' upstream of the transcription initiation site of the red opsin gene (Figure 133-12). In some of these individuals, the red and green opsin genes were intact, whereas in others the deletions extended into the red opsin gene. This deleted regulatory region, referred to as a locus control region (LCR) (see earlier) was shown, in transgenic mice, to be essential for directing expression of both long and short wavelength-sensitive cones in the mouse retina.

The second pathway leading to BCM, found in 20 families, involved unequal homologous recombination between the green and red opsin genes that reduced the

gene array to only a single red or a 5' red-green hybrid gene (Figure 133-13). In 16 of these families, the green opsin portion of the hybrid gene had a Cys203Arg substitution, which rendered the encoded hybrid opsin nonfunctional (26). Interestingly, the same Cys203Arg mutation was found to be relatively common (2%) in the green opsin genes of white men (27b).

### 133.11.4 The Role of Thyroid Hormone in Color Vision

There is good evidence that thyroid hormone, by activating thyroid hormone receptor  $\beta 2$  (THR $\beta 2$ ), is critical for expression of the red and green pigment genes (27), leading to differentiation of red and green cones during development. THR $\beta 2$  also suppresses blue cone formation during development. Thr $\beta 2$ -null mice (28) were shown to have only blue cones due to loss of ability to differentiate green cones. A thyroid-resistant human subject was shown to have blue cone monochromacy (Figure 133-13) (28).

A thyroid-resistant patient who is compound heterozygote for mutations in THR $\beta 2$  had severely reduced red and green cone function (28).

### 133.11.5 Enhanced Blue-Cone (or S-Cone) Syndrome

Enhanced blue-cone syndrome is autosomal recessive disorder of photoreceptor cell fate. It is characterized by hyper-differentiation of blue cones at the expense of rods, and blindness in the late stages. Mutations in the orphan nuclear receptor *NR2E3* gene were shown to cause this syndrome (29). *NR2E3* (also called PNR) plays a critical role in differentiating rods and suppressing the cone pathway (30).

## ACKNOWLEDGMENT

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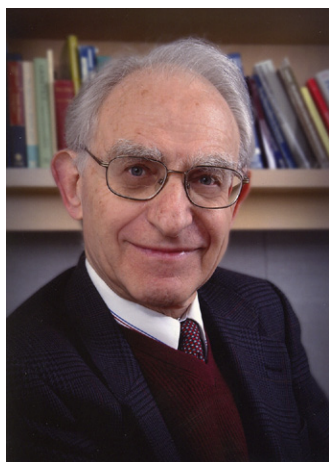
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## Biographies



**Dr Samir S Deeb** obtained his undergraduate degree in Agricultural Engineering in 1957 from the American University of Beirut, Lebanon. He then went to Colorado State University and graduated with a Master's degree in Nutrition in 1959. Subsequently, he spent four years at the University of Illinois, Champaign-Urbana, where he graduated with a PhD in Chemistry (major in Biochemistry) in 1964. Molecular genetics became of great interest to him, and he spent 2.5 years as a postdoctoral fellow with Dr Benjamin Hall in the Genetics Department at the University of Washington. From 1965–1983 he was a faculty member in the Biology Department at the American University of Beirut, Lebanon. He taught courses in cell biology, biochemistry, evolution, and genetics. His research focused on molecular genetics, particularly on the genetic basis of beta-thalassemia in Lebanese families. During this period, he was invited to the University of Illinois as a visiting faculty member in the Department of Biochemistry, spent one year on sabbatical and another year as a visiting faculty at the University of Colorado, Boulder. From 1976–1983 he was Chairman of the Biology Department, American University of Beirut. In 1983, he moved to the University of Washington, Seattle, where he is presently a Research Professor in the Division of Medical Genetics, Department of Medicine, and affiliate in the Department of Genome Sciences. His research at the University of Washington included the molecular genetic of dyslipidemias, coronary artery disease, type 2 diabetes, Alzheimer's disease, and color vision. In collaboration with Drs Arno Motulsky and David Teller (Department of Psychology), he contributed significantly to the molecule genetics of color vision. He has authored over 200 articles.



**Arno G Motulsky** is internationally recognized and honored for his achievements in research, training, and organization in the field of human genetics. He founded the Division of Medical Genetics at the University of Washington in 1957. This unit was among the first efforts that led to medical genetics becoming a key specialty of medicine. His research has spanned a wide variety of topics, including hematological genetics, clinical genetics, and pharmacogenetics. He is a founder of the field of pharmacogenetics. He initiated studies on the genetics of hyperlipidemia in coronary artery disease, which launched the spectacular career of Nobelist Goldstein. With Samir Deeb he contributed to the understanding of the molecular genetics of color vision and its defects. He has authored over 400 articles and several books, including the "Human Genetics" with Friedrich Vogel. The 4th edition with two additional editors and multiple co-authors appeared in 2010. He holds a variety of positions on editorial boards.

Dr Motulsky is an elected member of the National Academy of Sciences, the American Academy of Arts and Sciences, the American Philosophical Society, and the Institute of Medicine, as well as of the American Society of Clinical Investigation and the American Association of Physicians. Besides honorary doctorates, he has received several major prizes for his work in human and medical genetics.

# CHAPTER

# 134

## Optic Atrophy

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### GLOSSARY

**Dyschromatopsia** – difficulty or inability to discriminate colors, sometimes of a particular type (e.g. tritan deficiency is difficulty with discrimination along the blue–yellow axis).

**Ganglion cells** – the innermost neural retina cells whose axons form the optic nerve.

**Nystagmus** – an involuntary, rhythmic or wandering movement of the eyes that may be caused by early-onset visual dysfunction and/or brain pathology.

**Optic nerve** – the second cranial nerve, consisting of axons from ganglion cells of the retina and glial tissue. These axons largely synapse in the lateral geniculate nucleus of the thalamus, after partially decussating at the optic chiasm.

**Papillomacular bundle** – the mitochondria-rich ganglion cell axons which synapse with macular neural retinal.

**Scotoma** – a complete or relative inability to discriminate a light stimulus at a particular location of the visual field.

### 134.1 INTRODUCTION

The optic nerve is composed of over 1 million axons that originate in the retinal ganglion cells (the innermost cellular layer of the retina) and synapse largely in the lateral geniculate nucleus of the thalamus. These axons gather at the posterior portion of the eye, where—along with central nervous system glial tissue—they form the optic disc. It is largely from clinical examination of the optic disc that optic atrophy—a loss of retinal ganglion cell axons—is diagnosed. The optic nerve travels through the posterior orbit and through the optic canal of the skull. The two nerves meet at the anterior floor of the third ventricle to form the optic chiasm; there, the nasal fibers from each eye decussate to the opposite side of the brain, whereas the temporal fibers remain ipsilateral. In general, anything that causes harm to retinal ganglion cells or injures their axons along this course will lead to optic atrophy. Injury may be primary (e.g. the accumulation of metabolic products in some lysosomal storage diseases) or secondary (e.g. compression of the optic nerve by overgrowth of bone in the optic canal).

Hereditary optic atrophies may occur primarily as isolated disorders or as part of a systemic syndrome. Because some “primary optic nerve diseases” can sometimes include systemic features, this distinction is not always crisp. This chapter will focus on the two most common primary optic atrophies encountered in clinical genetics practice, namely, dominant optic atrophy (DOA, OMIM#165500, *OPA1*) and Leber hereditary optic neuropathy (LHON, OMIM#535000), although some attention will be given to rarer forms of optic atrophy. Although LHON and DOA differ in their clinical presentation and their genetics (Table 134-1), both result from abnormal mitochondrial function. Readers are referred to Chapter 11 for a discussion of basic mitochondrial physiology and genetics.

### 134.2 “PRIMARY” OPTIC ATROPHIES

#### 134.2.1 Dominant Optic Atrophy (Kjer Type) (MIM#165500, *OPA1*, \*605290)

Using Danish pedigrees, Kjer defined the clinical features of dominant optic atrophy (DOA), distinguishing it from Leber hereditary optic neuropathy (LHON) (1). Although he differentiated two dominant forms—one congenital with nystagmus and one infantile with no nystagmus—he questioned whether this division represented the phenotypic range for the same genetic condition. DOA is typically a bilateral, symmetric, primary optic atrophy, with an insidious onset in childhood. Progression is generally slow, resulting in variable visual acuity and field loss with a dyschromatopsia (classically, yellow-blue/tritan type) and optic nerve pallor (2). The prevalence of DOA is approximately 1:35,000 (English cohort) to 1:12,000 (Danish cohort), making it one of the most common forms of inherited optic atrophy (3).

**134.2.1.1 Clinical Features.** DOA typically has an insidious to subacute onset in the first decade of life. It has been described in children as early as age 2 years and is often detected when the child starts school; it can occasionally

**TABLE 134-1 Comparison of the Clinical Features of Dominant Optic Atrophy and Leber Hereditary Optic Atrophy**

	Leber Hereditary Optic Atrophy (LHON)	Dominant Optic Atrophy, Kjer Type
Inheritance	Mitochondrial DNA, maternal	Autosomal Dominant
Typical age of onset	2nd–3rd decade	1st–2nd decade
Presentation	Acute, painless visual loss	Chronic, painless, insidious visual loss
Gene(s)	<i>MTND4</i> (m.11778G>A) <i>MTND1</i> (m.3460G.A) <i>MTND6</i> (m.14484T>C) Collectively account for >90% cases	<i>OPA1</i> , 3q28–q29
End stage	Optic nerve pallor, legal blindness	Optic nerve pallor, legal blindness
Treatment	None	None

start later in life. A British study had, for example, two peaks of age of onset of the symptoms: 5 and 21–30 years (4). Similarly, Johnston et al. found that 58% of individuals had symptoms before the age of 10 (5). Since the identification of mutations in the nuclear-encoded *OPA1* gene in a significant percentage of patients with DOA (6) (see below), some clinical reports have focused on patients with confirmed mutations, whereas others have included both mutation-positive and mutation-negative patients. How (if at all) this affects the interpretation of these clinical data is still a subject of debate.

Bilateral loss of visual acuity is the main presenting symptom in DOA. The visual prognosis is relatively good: distance vision typically ranges from 20/70 to 20/100, but can be as good as 20/20 (subclinical), or as poor as light perception (although this is uncommon) (2,4). Visual acuity is usually symmetric, with a typical disparity between the impaired distance vision and the comparatively conserved near vision. DOA is typically slowly progressive, although this may not be universally true (3a,7). Given that DOA predominantly affects the papillomacular bundle (which serves central vision), visual field deficits are usually central or cecentral, with relative sparing of the retinal periphery. Although a tritan color deficit is classic for DOA, Votruba et al. found that over 80% of individuals have a mixed color deficit (4); color vision defects may precede loss of acuity. These same authors note that there is significant intra- and inter-familial variability in phenotype.

Pallor of the temporal optic nerve is the major finding on fundus examination (Figure 134-1). Eliott et al. found that there was no strict correlation between acuity and the degree of optic nerve pallor (8). Recently, Barboni and colleagues noted that the optic nerve heads of DOA patients are generally smaller than in age-matched controls (9). Retinal nerve fiber thickness, as measured with optical coherence tomography (OCT), is most severely affected in the temporal quadrant, with relative sparing of the nasal quadrant (10,11). The macular reflex may be unremarkable, decreased, or absent. In contrast to LHON, no vascular changes are described. Barboni et al. argue that the loss of nerve fiber layer thickness over time is similar to the normal aging process, although theirs was

a cross-sectional rather than a longitudinal study (11). The full field electroretinograms (ERG) is typically normal, but the pattern ERG shows a reduced N95 component, in agreement with primary ganglion cell dysfunction (4).

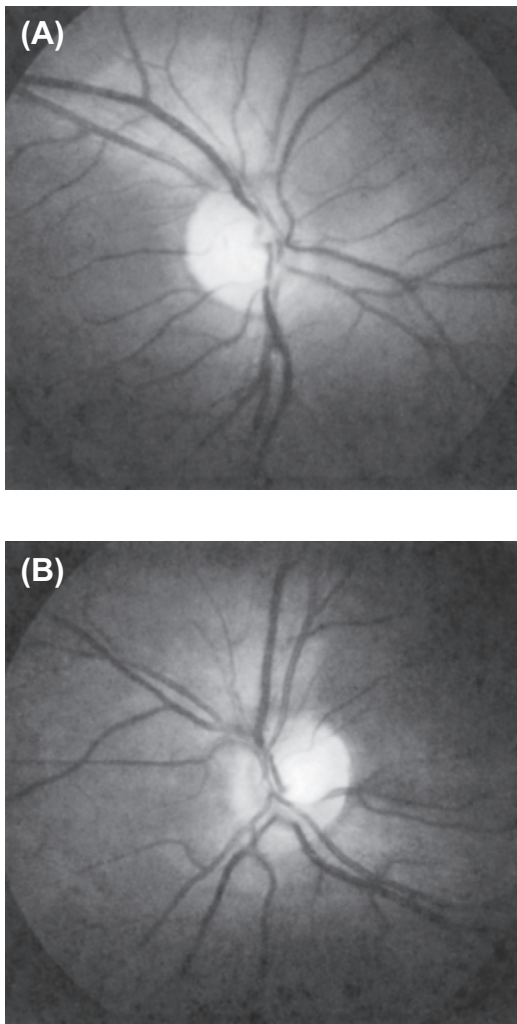
Approximately 20% of patients with *OPA1* mutations will develop a more severe disease variant, called “dominant optic atrophy plus” (DOA+), with additional neuromuscular features. In addition to cases featuring sensorineural deafness, ataxia, myopathy, peripheral neuropathy, and progressive external ophthalmoplegia (12), Yu-Wai-Man et al. uncovered two novel clinical presentations of DOA: spastic paraparesis was present in two families with DOA, while a phenotype similar to that of multiple sclerosis was exhibited in another family. Defective cytochrome C oxidative phosphorylation in skeletal muscle is a subclinical feature of patients with *OPA1*-related dominant optic atrophy, indicating a systemic expression of the *OPA1* defect (13). Retinal nerve fiber layer thinning is more pronounced in patients with DOA+ phenotypes (10). Yu-Wai-Man hypothesized that the involvement of other tissue types in DOA+ may be a direct consequence of the greater accumulation of secondary mitochondrial DNA abnormalities, the latter potentiating an already compromised mitochondrial oxidative reserve due to the mutant *OPA1* protein.

The differential diagnosis includes neuropathies and retinopathies with optic atrophy. LHON, recessive optic atrophy, toxic optic neuropathy, demyelinating diseases, hereditary macular dystrophies have to be considered (14).

Histopathologically, optic atrophy is characterized by general atrophy of the ganglion cell layer as well as partial atrophy of the optic nerve, without atrophy of the outer retinal layers. There is increased collagen in association with a decreased number of neurofibrils and myelin sheaths in the optic nerves, optic chiasm, and optic tracts (15).

**134.2.1.2 Genetics and Pathogenesis.** DOA is inherited in an autosomal dominant fashion (1); however, recent evidence has suggested that *OPA1* may actually result from semi-dominant inheritance (16). In 2000, two groups simultaneously reported on mutations in the *OPA1* gene in DOA (6). Although the penetrance of *OPA1* mutations approaches 100%, the expressivity of the phenotype is quite variable. Pathogenic *OPA1* mutations





**FIGURE 134-1** Dominant optic atrophy. (A) Right eye (OD). (B) Left eye (OS). This 37-year-old man has 20/30 vision in both eyes, a tritan color defect, and exhibits the characteristic temporal pallor and atrophy of the disc.

account for about 60% of all cases (17). The *OPA1* protein localizes to the mitochondria (1) and encodes a GTPase related to the dynamin family of proteins (6). In general, *OPA1* mutations are predicted to result in a loss of protein function, although dominant negative effects of missense mutation alleles have also been proposed. Several lines of evidence suggest that mutations in *OPA1* result in deficient mitochondrial function and bioenergetics. Skeletal muscle from patients with *OPA1* mutations showed reduced mitochondrial adenosine triphosphate production (13). Magnetic resonance spectroscopy similarly shows a reduced ATP peak in patients with *OPA1* mutations (18). Decreased mitochondrial DNA content is found in peripheral blood of some patients with *OPA1* mutations (19). Amati-Bonneau et al. show increased mitochondrial network fragmentation and decreased mitochondrial membrane potential in skin fibroblasts from patients with R445H mutations in *OPA1* (20). Elachouri et al. have recently presented evidence that *OPA1* is important in maintenance of the mitochondrial DNA,

as silencing of the *OPA1* gene in vitro resulted in mitochondrial DNA depletion due to deficient replication (21). In a *Drosophila* model, Yarosh et al. found that vitamin E and superoxide dismutase were able to reverse the dOpa1 phenotype, suggesting a role of reactive oxygen species in disease pathogenesis (22).

There is evidence of locus heterogeneity in DOA. *OPA1* is located on 3q28–29 (6,23). Kivlin et al. described a large North American family of German descent that showed a maximum lod score of 2 at a recombination fraction of 0.18 using the Kidd blood group antigen, which was later assigned to 18q12 mapped a locus (*OPA4*, OMIM #605293) (24). This locus was further refined by Kerrison et al. (25). Reynier and colleagues identified two different heterozygous mutations in the *OPA3* gene (see below) in two families with DOA and cataract (26). Barbet et al. have reported a three-generation French pedigree with DOA that maps to 22q12.1–q13.1 (*OPA5*, OMIM #610708) (27).

**134.2.1.3 Management.** No clinically proven treatment is available. Caution should be observed in translating findings from animal models into humans. Family screening, genetic counseling, and possible molecular diagnosis are indicated.

### 134.2.2 Leber Hereditary Optic Neuropathy (MIM 535000)

Leber hereditary optic atrophy (LHON) was first described by Theodor Leber in 1871 as a nosologically distinct optic neuropathy. It has long been postulated to be caused by a defect in mitochondrial metabolism, and the first mitochondrial DNA (mtDNA) mutation was found by Wallace's group in 1988 (see Chapter 11) (28).

LHON has to be distinguished from Leber congenital amaurosis, an autosomal recessive chorioretinal dystrophy that presents with poor vision at birth. This latter disorder can be associated with variable amounts of optic atrophy. Prevalence of LHON is estimated as 1 in 31,000 to 1 in 50,000, which is comparable to DOA.

**134.2.2.1 Clinical Features.** LHON is classically characterized by painless visual loss typically beginning in one eye, and eventually progressing to the other eye over an average interval of 2 months (29). Although rare unilateral cases have been reported (30), the rule is that the second eye will be affected within one year of initial presentation. Up to 25% of cases may present with bilateral visual loss. The deterioration of vision ranges from acute to subacute, and is followed by stability after 3–4 months. Most cases present in the second or third decade of life and approximately 90% of carriers who develop visual loss will do so by age 50 (31). Vision loss, however, can occur at any age and LHON should be included in the differential of acute sequential and/or bilateral optic neuropathy even in older and younger individuals. While approximately 20–60% of at-risk males experience visual loss, only 4–32% of at-risk

females develop visual loss (29). This incomplete penetrance and difference in gender susceptibility has been observed across multiple ethnic groups (32). The precise reason for these observations is unclear.

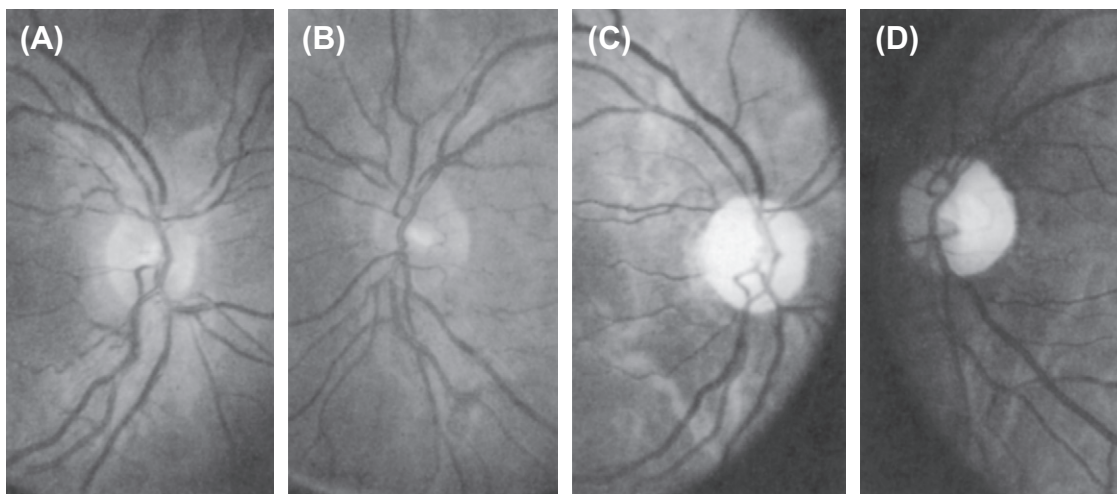
Careful electrophysiological and psychophysical testing has revealed abnormalities in color discrimination, subtle edema of the nerve fiber layer, subclinical central scotomas, abnormal multifocal ERGs, and abnormal visual evoked potentials prior to the onset of overt symptoms (33). Given the ethical and social consequences of presymptomatic diagnosis (e.g. inability to obtain disability or long-term care insurance), care should be taken in approaching the examination of asymptomatic family members of an affected individual.

Classically, the disease occurs as “thunder in a dry sky,” i.e. as an isolated vision loss without prodromal symptoms, although migraine-like headaches occur in up to 33% of the affected patients (34). During the acute phase, circum-papillary telangiectatic blood vessels, hyperemia of the disc, vascular tortuosity of the retinal blood vessels, and intracellular edema of the nerve fiber layer without leakage on fluorescein angiography are typical (Figure 134-2). Affected individuals develop a central or cecentral scotoma and impaired color vision. Pupillary responses may be relatively preserved for the degree of vision loss (35). However, some patients experience symptoms while presenting with of a normal eye exam, sometimes leading to an erroneous initial diagnosis of functional vision loss. Over time, the optic nerve becomes increasingly pale. The final visual outcome is variable, ranging from 20/50 to no light perception. Vision loss is generally permanent, although there may be some mild recovery of “islands” within the visual field over time. Kirkman et al. have documented significant effects of LHON on patients’ quality of life, as scored on the Visual Function Index (VF-14) questionnaire (36). Some genotype–phenotype correlation has been reported (see below).

With the advent of OCT, one can now correlate physical abnormalities of the retina and optic cup with the severity of visual acuity changes in patients with LHON. Spectral domain OCT allows for high resolution imaging and quantitative analyses of the retinal layers, thus allowing for sequential analysis of the progression of disease in LHON patients, as well as distinguishing between retinal disorders of the inner retinal layers and disorders of the outer retinal layers, such as LHON and DOA (37). The retinal nerve fiber layer (RNFL) is thickened in early (<6 months duration) LHON, and severely thinned in late (>6 months duration) LHON, with inferior and temporal fibers (papillomacular bundle) being the first and most severely affected (38). Additionally, males with LHON demonstrate a more diffuse retinal nerve fiber layer involvement than females on OCT imaging (33a). When assessed by Heidelberg Retina Tomograph (HRT), the eyes of LHON patients demonstrated significantly larger cup parameters, smaller rim volume, and thinner mean RNFL thickness than controls (39). Newman et al. found presymptomatic changes in static automated perimetry in patients with LHON (40).

Magnetic resonance imaging (MRI) of LHON patients typically demonstrates an acute enlargement of the anterior visual pathways without enhancement, as well as increased T2 signal from the optic nerves to the lateral geniculate bodies. Follow-up imaging a few months later often demonstrate persistent bright T2 signal in normal-sized or atrophied anterior visual pathways (41). LHON lesions may be distinguished from those of multiple sclerosis because they tend to be less than 5mm in diameter, lack sharp boundaries, and do not enhance with gadolinium (42).

Although limited reports of histopathology of the LHON optic nerve exist, Kerrison et al. noted optic atrophy and double-membrane bound inclusions consisting of calcium in retinal ganglion cells in tissue from a

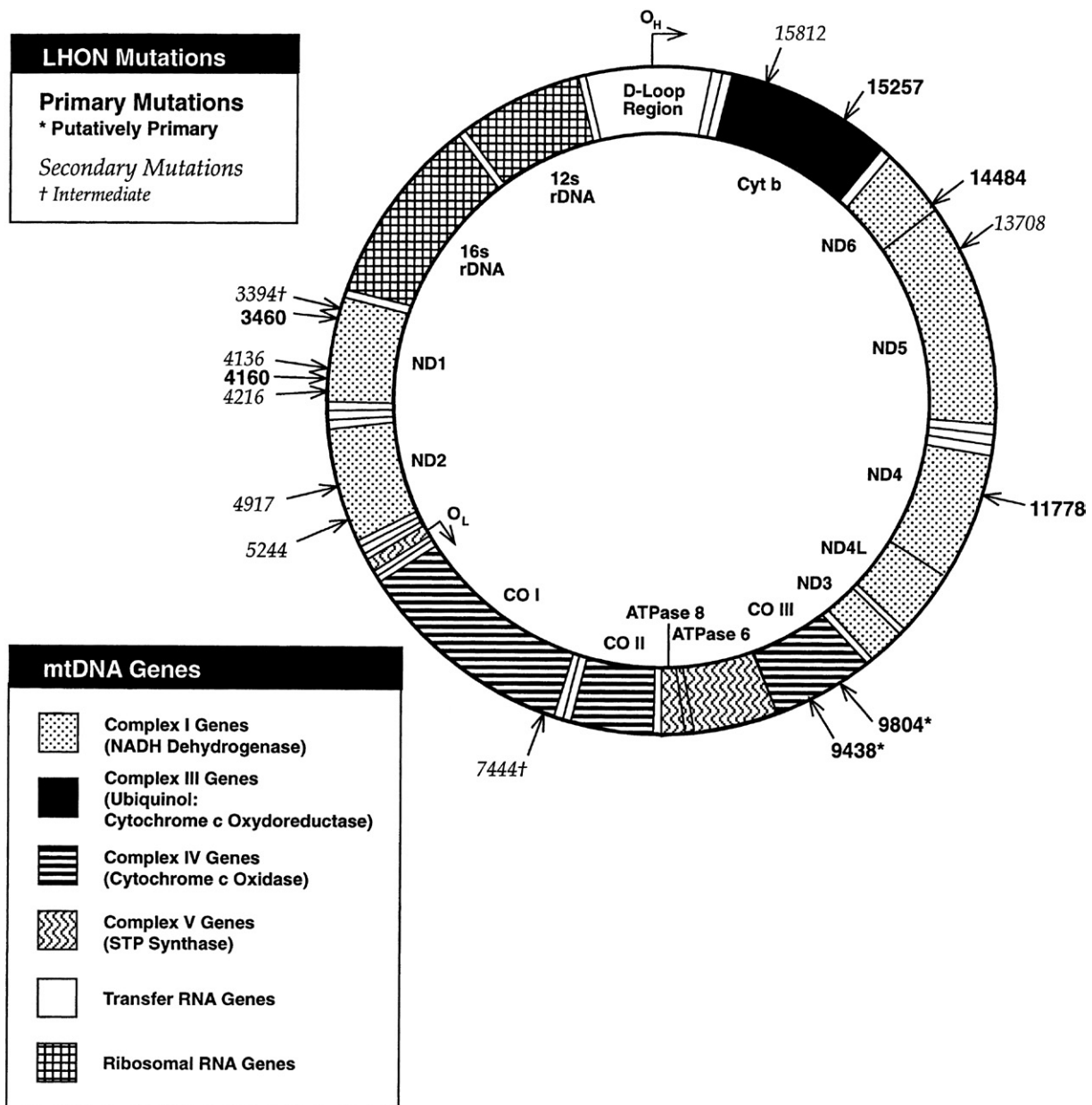


**FIGURE 134-2** Leber optic neuropathy. (A) OD. (B) OS. This 23-year-old man presented with a three-month history of decrease in vision when first seen. His initial examination disclosed hyperemic discs typical of the acute optic neuritis stage of the disease. The vision dropped to its lowest a few weeks later (20/200 OD and CF at 4ft OS). (C) OS. (D) OS. Two years later, fundus examination reveals the subsequent typical total bilateral optic atrophy, while his vision had increased to 20/25 in both eyes. He was found to have the 11778 mutation.

symptomatic 81-year-old woman (43). Molecular analysis confirmed homoplasmy for her mitochondrial mutations in ocular tissue.

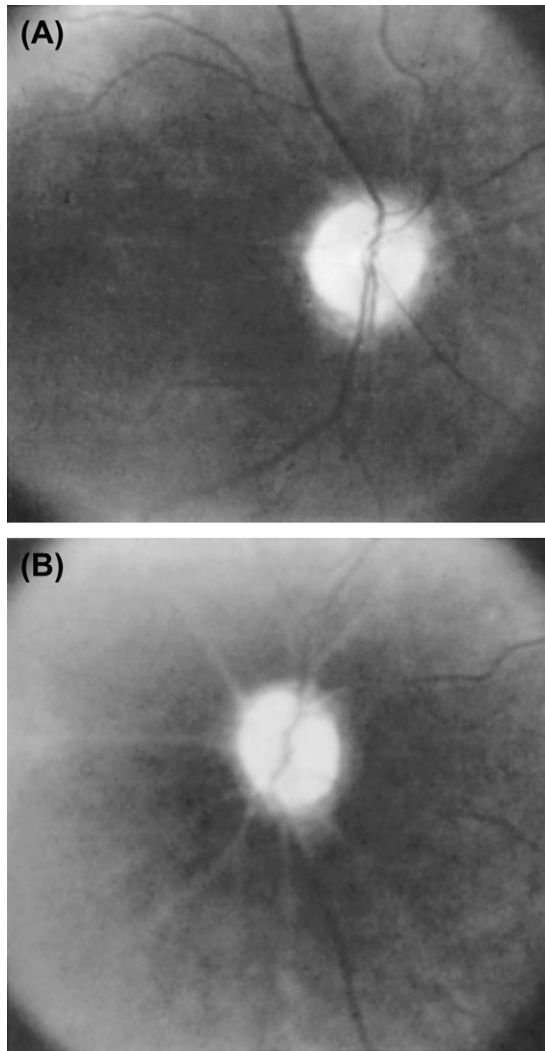
In most instances, LHON manifests as an isolated optic atrophy, but cardiac and neurologic manifestations may occur. Cardiac conduction abnormalities may become symptomatic and occasionally even life-threatening (44); they consist of pre-excitation syndromes, specifically Wolff–Parkinson–White and Lown–Ganong–Levine syndromes, or prolongation of the corrected QT interval, which has been observed in the female carriers. Because these conduction anomalies can be severe, an ECG should be obtained. The reported neurological abnormalities are uncommon and diverse in presentation (45). Nikoskelainen et al. performed neurological

exams on 38 men and 8 women with LHON and suggest a pathological relationship between LHON and various movement disorders, multiple sclerosis-like illness, and deformities of the vertebral column—a relationship dubbed “Leber’s plus” (46). The multiple sclerosis-like phenotype associated in some patients with LHON—particularly those with an m.11778 G>A mutation (see below)—is sometimes referred to as “Harding’s syndrome” (42,47). Vanopdenbosch et al. have suggested, in fact, that carrying a primary LHON-related mutation is a risk factor for developing MS (48). While no consensus exists on the utility of neuroimaging in patients with LHON, asking carefully about neurologic symptoms and/or documenting a systemic neurologic exam may be reasonable precautions.



**FIGURE 134-3** Mitochondrial DNA with primary and secondary point mutations responsible for Leber hereditary optic neuropathy. (Modified from Newman, N. J. *Leber’s Hereditary Optic Neuropathy: New Genetic Considerations*. Arch. Neurol. 1993, 50, 540–548, with permission.)





**FIGURE 134-4** Behr optic atrophy in a 24-year-old man with pallor of the optic nerve head and unexplained tremor since early childhood. A, OD. B, OS.

**134.2.2.2 Genetics and Molecular Mechanisms.** LHON is the prototypical disease caused by mutations in maternally-inherited mitochondrial DNA. As such, all children of a woman carrying an LHON mutation are expected to inherit the same mutation, whereas children of men with LHON are not at risk of inheriting the mutation. Despite this prediction, over half of patients present with no family history (49).

Between 90 and 95% of patients with LHON harbor one of three primary mutations in the mitochondrial DNA: m.3460G>A (in MTND1), m.11778G>A (in MTND4), or m.14484T>C (MTND6). All three mutations are in genes coding for subunits of Complex I in the electron transport chain. Numerous other rare mitochondrial DNA mutations have been reported, some of which have not been proven to be pathogenic (see Yu-Wai-Man et al. for a recent review) (14). The m.11778G>A mutation is the most prevalent change in most populations, although m.14484T>C accounts for nearly 90% of French Canadian cases of LHON.

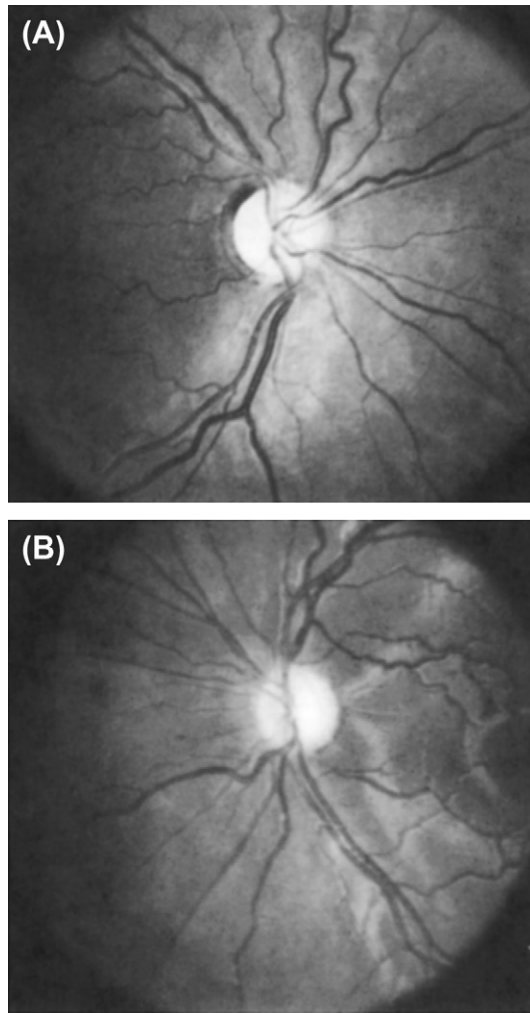
Molecular analysis has suggested a correlation between specific mitochondrial mutations and visual recovery (31). The 14484 mutation has the best prognosis, with a recovery rate, defined as a VA of 20/60 or better in both eyes, of 37%. The 3460 mutation recovery rate is variable but may reach 22%. The 11778 mutation has the worst prognosis with recovery seen in only 5% of patients.

Why do only a subset of males and a minority of females carrying a bona fide LHON mutation develop overt disease; and when they develop disease, why is it usually isolated to the optic nerve? While the precise answers to these questions are unclear, both environmental and genetic (nuclear and mitochondrial) factors may play a role. The optic nerve—particularly the papillomacular bundle—is highly dependent on ATP production from mitochondria, perhaps making it particularly susceptible to metabolic insult. Many other tissues, however, also have a strong reliance on mitochondrial ATP production, so this answer is, at best, incomplete. Some mitochondrial DNA mutations are present in only a fraction of a cell's mitochondria (heteroplasmy). A cell must accumulate some threshold of abnormal mitochondria—usually thought to be 60–80%—before it develops problems in bioenergetics (50). The most common mitochondrial DNA mutations are most often, however, homoplasmic (at least in the tissues assayed). Hudson et al. found that the haplogroup on which a mitochondrial mutation occurs (see Chapter 11) may influence the clinical outcome (51). Specifically, individuals with m.11778G>A and m.14484T>C were more likely to have significant visual loss when on the J haplogroup, while m.3460G>A were more susceptible to disease on the H haplogroup.

One attractive hypothesis that would help explain the proclivity of male carriers for developing disease is that a locus on the X-chromosome modifies the LHON phenotype. Bu and Rotter studied more than 1200 individuals from 31 large pedigrees to create a two-locus model for visual deficits in LHON, with the X-locus acting in synergy with the primary mitochondrial DNA mutation (52). Several groups have performed mapping studies and identified candidate loci on the X-chromosome—some of which overlap (53). To date, the precise gene (or genes) responsible for this process have not been identified and some groups have failed to confirm significant disease modification by an X-linked locus.

Another possibility is that the LHON phenotype is influenced by hormonal factors. Evidence suggests that estrogens may metabolically ameliorate mitochondrial dysfunction in this disease process (54). Giordano et al. found that LHON cybrid cells treated with 17 $\beta$ -estradiol activated the antioxidant enzyme superoxide dismutase 2, additionally resulting in decreased apoptotic rate, decreased production of reactive oxygen species, and decreased the number of mitochondria with hyperfragmented morphology. Additionally, 17 $\beta$ -estradiol induces a general activation of mitochondrial biogenesis,





**FIGURE 134-5** Hereditary optic nerve hypoplasia. Both sibs present with bilateral small and pale optic discs. A, 18-year-old young man (right eye). B, Nine-year-old brother (left eye).

and increases in vitro cell viability. Estrogen receptor  $\beta$  was also verified to localize to the mitochondrial network of human retinal ganglion cells via immunofluorescence and Western blot analyses.

Another variable affecting disease expression may be environmental factors, particularly those known or thought to be toxic to mitochondrial function. Kirman et al. studied 196 affected and 206 unaffected carriers, from 125 LHON pedigrees known to harbor one of the three most common LHON mitochondrial DNA mutations (55). Using a structured questionnaire, they found a strong, dose-dependent association between tobacco use and vision loss, with a clinical penetrance of greater than 90% in men who smoked. Alcohol intake tended—at least in its extreme—to predispose to vision loss, but this did not reach statistical significance. Case reports have suggested that cyanide (a known inhibitor of the mitochondrial respiratory chain), cocaine, MDMA (ecstasy), telithromycin (56), erythromycin (57), anti-tuberculosis medications (58), nutritional deficiencies (59), occupational solvent exposure (60), borreliosis (61), anemia

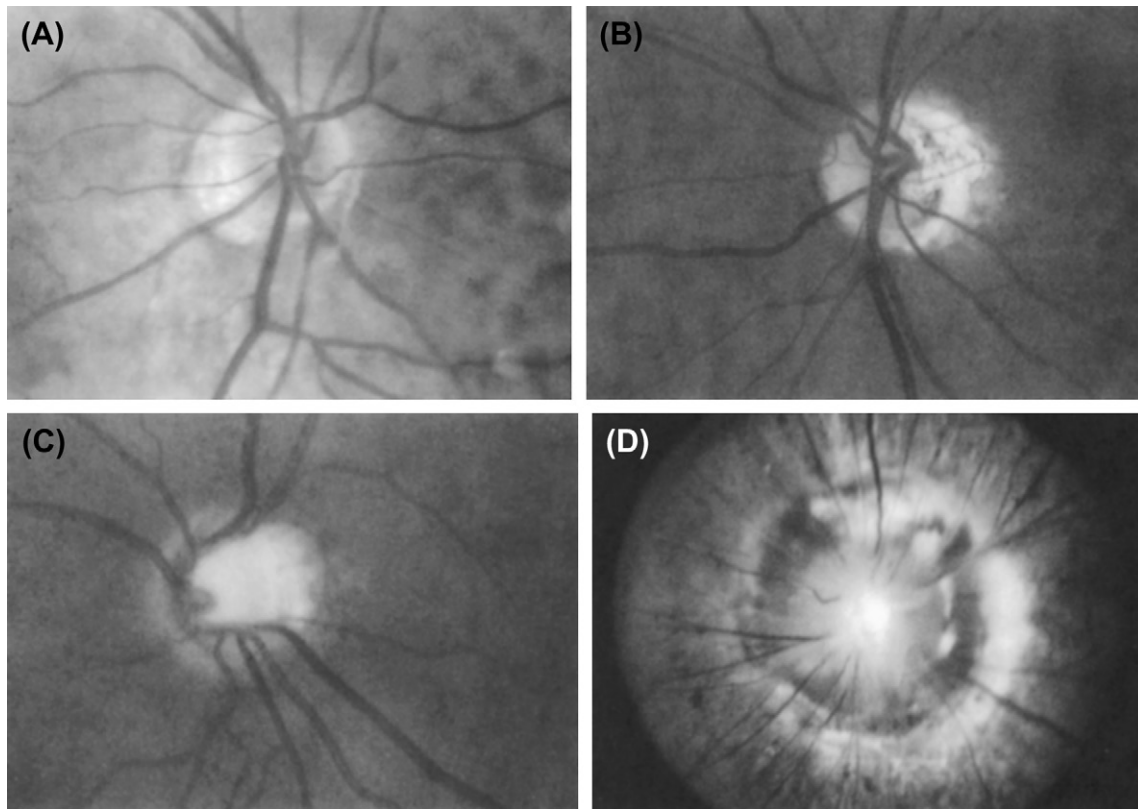
(62) and trauma (especially recent head trauma) may also affect disease expression.

**134.2.2.3 Treatment.** To date, there is no proven treatment for LHON. The timing and variable disease course associated with this disease makes the evaluation of any treatment through objective studies extremely difficult. Deciding what clinical outcome variables are most relevant (e.g. acuity, visual field, OCT findings) is not trivial. In the case of localized treatments such as gene therapy, deciding when and whether to treat the affected eye or the unaffected eye is also an important consideration.

Newman et al. evaluated the effectiveness of brimonidine purite eye drops in a nonrandomized, open label prospective pilot trial of nine patients (63). The goal of this trial was to determine whether the reported neuroprotective effects of brimonidine would reduce the rate of second eye involvement, after presentation with vision loss in one eye. The trial was complicated by the finding that seven patients had subtle findings of second eye involvement at the time of presentation (40). Based on their results, the authors conclude that brimonidine used at the concentration tested was not effective in preventing second eye involvement.

The short-chain synthetic benzoquinone, idebenone, is a potent antioxidant and inhibitor of lipid peroxidation that interacts with the mitochondrial electron transport chain, allowing the “bypass” of complex I. Mashima et al. first described the remission of LHON in a 10-year-old boy with idebenone administration in 1992 (64). A subsequent, small, noncontrolled trial has suggested that oral administration of idebenone, when coupled with vitamins B12 and C supplementation, may speed up visual recovery in affected patients (65). Since then, a multi-center randomized placebo-controlled trial of 900 mg idebenone per day in 85 LHON patients demonstrated that idebenone was safe and well-tolerated, and suggests that patients with discordant visual acuities are the most likely to benefit from idebenone treatment (66). A retrospective study of early and prolonged idebenone treatment in patients with acute LHON has similarly demonstrated that idebenone administration may significantly improve the frequency of visual recovery and possibly change the natural history of the disease (67).

Because most data suggest that the pathogenesis of LHON is due to a loss of function in electron transport chain proteins, gene replacement is an attractive therapeutic strategy. So long as the protein is targeted by the cell to the mitochondrial inner membrane, nuclear or episomal gene replacement (allotopic expression) would be predicted to be effective (68). In vitro studies suggest that gene replacement in cells from LHON patients improves mitochondrial function (68b,69). Animal studies have suggested that expression of wild-type complex I subunits is safe. Lam et al. have recently reported on the clinical characteristics of LHON patients with the G11778A mutation in anticipation of a gene therapy trial (70).



**FIGURE 134-6** Optic nerve malformations. A and B, Optic nerve hypoplasia associated with deMorsier syndrome. C, Marked optic nerve hypoplasia. D, “Morning Glory anomaly” of the disc.

Because tobacco and alcohol may act as mitochondrial toxins, avoiding smoking and excess alcohol intake is a reasonable precaution in patients who present with LHON or who are known/suspected carriers of an LHON mutation (55).

### 134.2.3 X-Linked Optic Atrophy (MIM %311050, *OPA2*)

A rare, early-onset, slowly progressive form of optic atrophy limited to males and accompanied by developmental delay and neurologic symptoms has been recognized since the 1970s. Using some of these original pedigrees, Assink et al. performed multipoint linkage analysis, placing the locus for this condition on Xp11.–p11.21 (71). The developmental delay and neurological abnormalities reported in these earlier studies may not be a consistent part of the phenotype, as Katz et al. describe a US family with similar ophthalmologic features but no neurological abnormalities, which potentially maps to the same locus (72).

### 134.2.4 Costeff Optic Atrophy Syndrome/ Type III 3-Methylglutaconic Aciduria (MIM\*606580, *OPA3*)

Type III 3-methylglutaconic aciduria (MGA, MIM 258501)—which is synonymous with *OPA3*—is an

autosomal recessive disease characterized by increased urine excretion of 3-methylglutaconic acid and 3-methylglutaric acid, early-onset bilateral optic atrophy and subsequent neurological and cognitive deficits. Anikster et al. used linkage analysis in 40 Iraqi Jewish patients to localize the *OPA3* locus to 19q13.2–13.3 (73). They identified a cDNA clone, FLJ22187, corresponding to a two exon gene in which a point mutation co-segregating with the phenotype was observed. The transcript of this gene is widely expressed, including in the skeletal muscle, brain and kidney. The *OPA3* protein is localized to the mitochondria (74). It is unclear whether patients who have 3-methylglutaconic aciduria, but not the full-blown Costeff syndrome, may harbor mutations in the *OPA3* gene (75).

Reynier et al. found that a form of autosomal dominant optic atrophy with cataract (MIM 258501) is allelic to *OPA3* (26). These authors posit that the two missense mutations they identify in their cohort may lead to disease via a mechanism other than haploinsufficiency, as the carrier parents of patients with Costeff optic atrophy syndrome—who appear to be haploinsufficient—are asymptomatic. This hypothesis is somewhat at odds, however, with the observation in a murine model containing a missense mutation in *Opa3* (76), where heterozygous mice are asymptomatic, but homozygous mice mirror the Costeff syndrome phenotype. Yu-Wai-Man et al. did not observe any *OPA3*

mutations in their cohort of 188 patients with either autosomal dominant or sporadic optic atrophy (77).

### 134.2.5 OPA4 (MIM %605293)

*OPA4* is the designation of autosomal dominant optic atrophy mapping to 18q12.2–12.3 described by Kerrison et al. (25) and previously linked to the Kidd blood group. These patients have a range of visual acuities from normal to legal blindness.

### 134.2.6 OPA5 (MIM %610708)

Barbet et al. describe a dominant optic atrophy in two unrelated French families that showed linkage to 22q12.1–q13.1 (27). The visual acuity loss in these patients began between the first and third decade of life and slowly progressed.

### 134.2.7 Autosomal Recessive Congenital/Early Infantile Optic Atrophy (MIM %258500, OPA6)

Barbet et al. reported on a consanguineous French family where four living individuals were affected by an early-onset, but slowly progressive, optic neuropathy (78). A genome-wide scan for homozygous regions identified a potential disease locus at 8q21–22. The authors exclude coding sequence changes in the *CNGB3*, *DECR1*, and *PDP1* genes in this region.

### 134.2.8 Non-Syndromic Autosomal Recessive Optic Atrophy; Optic Atrophy 7 (OPA7, MIM #612989)

This form of autosomal recessive juvenile-onset optic atrophy is characterized by severe bilateral visual acuity loss, optic disc pallor, and central scotoma. Identified by Hanein et al. (2009) in a large multiplex Algerian family and 3 other Maghreb families, onset usually occurs between 4 and 6 years of age. All but one of the affected individuals demonstrated strictly normal peripheral visual fields.

Affected individuals may or may not demonstrate partial deficiency in mitochondrial complex activity. One affected individual demonstrated partial deficiency of complex I, hypertrophic cardiomyopathy, mild hearing loss, and minor brain MRI alterations, all of which suggest mitochondrial dysfunction.

This form of optic atrophy is caused by a homozygous mutation in the transmembrane protein 126A gene (*TMEM126A*, MIM 612988). This mitochondrial protein, found in higher eukaryotes, is located at 11q14.1. The gene product contains 4 transmembrane domains, as well as a central domain conserved with *TMEM126B* (79). In contrast to *OPA1* mutations, cells carrying *OPA7* mutations do not demonstrate mitochondrial fragmentation and/or depletion of mitochondrial DNA,

thus suggesting that *TMEM26A* and *OPA1* are not functionally related.

## 134.3 COMPLEX OPTIC ATROPHIES

### 134.3.1 Behr Syndrome (MIM %210000)

Recessive bilateral optic atrophy of early onset with variable, severe neurologic signs, especially pyramidal, was described by Behr in 1909. This clinical entity is probably a heterogeneous group of disorders, whose root causes are mostly unknown; as such, a true “Behr syndrome” as a distinct clinical and genetic entity may not exist (see discussion below). Some have disputed that the cause of vision loss in some cases may not have been related to optic atrophy per se, as a retinal dystrophy was not ruled out. Alternative names are Behr complicated form of optic atrophy and complicated infantile optic atrophy.

The disorder has its onset in childhood, between the ages of 1 and 9 years, and is typically stable after an initial and variable period of progression. It is characterized by bilateral optic atrophy, seen on fundus examination as mild pallor of the disc, more marked temporally and rarely complete. The visual acuity is usually poor and measures about 20/400. There will be moderate to severe dyschromatopsia. Nystagmus is present in half the cases, and strabismus coexists in two thirds. Visual field defects can be present as central scotomas or temporal ones, which are rarely complete. The ERG is unremarkable in those patients where testing has been performed.

The neurological abnormalities resemble Friedreich ataxia: increased tendon reflexes, presence of Babinsky’s sign, hypertonia, mild ataxia and spasticity, and mental retardation.

Autopsy of one affected child showed optic atrophy as well as atrophy of the optic tracts. There were extensive degenerative changes in the lateral geniculate nuclei (LGN); disruption of the normal lamination of the LGN was present with dropout of neurons and gliosis. Changes in other thalamic nuclei and in the pallida were evident, but there was no demonstrable lesion in the cortex to correlate with the associated mental retardation. Horoupian and collaborators (80) interpreted the findings as showing a primary degeneration of the LGN with retrograde degeneration leading to optic atrophy.

Recessive inheritance is suggested by the involvement of siblings, with equal frequency in children of both sexes and by an increased frequency of consanguinity among the unaffected parents; however, an autosomal dominant inheritance pattern has also been described (81). The gene has not been mapped.

A major complexity in classifying Behr syndrome as a separate clinical and genetic entity is that the phenotypic expression is quite similar to other forms of optic atrophy with neurological signs. For example, some patients classified as having a “Behr-like”



syndrome were subsequently found to have type III 3-methylglutaconic aciduria and mutations in *OPA3* (73,82). The degree to which Behr syndrome overlaps with type III 3-methylglutaconic aciduria is made even more uncertain by the fact that the abnormal urine profile may not be picked up by routine urine organic analysis (83). No other genetic cause of Behr syndrome has been identified to date, although case studies of *OPA1* mutations in Behr syndrome patients may suggest that classical Behr syndrome should be re-classified under the umbrella of dominant optic atrophy-plus phenotypes (84).

As with other inherited optic atrophies, there is no proven effective treatment and care is supportive.

### **134.3.2 Wolfram Syndrome (WFS1: OMIM 222300; WFS2: OMIM 604928; Mitochondrial: OMIM 598500)**

DIDMOAD is an acronym for diabetes insipidus, juvenile-onset diabetes mellitus, optic atrophy and sensorineural deafness. Also known as Wolfram syndrome, the phenotypic variability of this autosomal recessive condition has led to other acronyms (e.g. DMOA) that include some, but not all, of the “classic” findings. In addition, patients may present with neurological deterioration (ataxia, peripheral neuropathy, myoclonus, psychiatric disease), gut dysmotility, primary and secondary hypogonadism, anterior pituitary dysfunction and urological findings (hydronephrosis, hydroureter, dilation of the urinary bladder) (85). The presence of optic atrophy and juvenile-onset diabetes mellitus are considered mandatory for the diagnosis. Barrett et al. estimated the population frequency to be about 1 in 770,00, with a 1 in 354 carrier frequency (85a).

Juvenile-onset diabetes mellitus may be the first manifestation of the syndrome, appearing between 2 and 20 years of age, usually within the first decade. Unlike classic type 1 diabetes, patients with Wolfram syndrome do not develop antibodies against islet cells; HLA-associated risk predictions are also different (86). The French Wolfram Group, headed by Cano et al, compared 26 Wolfram syndrome patients to 52 type-1 diabetes mellitus patients matched for age of diabetes onset. This study demonstrated a lower daily insulin requirement and lower hemoglobin A1c levels in Wolfram Syndrome patients, despite their use of a less intensive insulin regimen (87). Additionally, the study by Cano et al. demonstrated decreased prevalence of diabetic retinopathy, nephropathy, and other microvascular complications in patients with Wolfram Syndrome compared to type 1 diabetics. Fifty patients with Wolfram syndrome-related diabetes (WSD) were compared with the data of 24,164 patients with type 1 diabetes, and this demonstrated that WSD was diagnosed earlier than type 1 diabetes, with a lower prevalence of ketoacidosis (88). Additionally, this study demonstrated a correlation between WFS1 mutations (see below) and

age of diabetes onset, and identified glucose toxicity as an accelerating factor in disease progression. C-peptide stimulation indicated a small remaining insulin secretory reserve. Additionally, compared to wild-type beta cells, WFS1 deficient cells demonstrate impaired granular acidification, which is normally required for the priming of secretory granules preceding exocytosis. This may suggest a molecular mechanism for the profound impairment of glucose-induced insulin secretion in WFS1-knockout mouse models (89). WFS1-deficient beta cells exhibit increases in markers indicative of endoplasmic reticulum (ER) stress, thus causing beta cell loss through impaired cell cycle progression and increased apoptosis (90).

Progressive visual loss due to primary optic atrophy usually follows the diabetes mellitus symptoms and starts between the ages of 2 and 24 years, most often before age 15 years, with an average age of onset of 11 years. Visual impairment is severe, resulting in acuities of 20/2000–20/6000 (0.01–0.003), but ranges from 20/200 to hand motion. There is progressive, marked, diffuse pallor of the optic disc, which may be absent at the time of first complaints of vision loss. In a study of fifteen Wolfram syndrome patients, the prevalence of optic atrophy was 93.3%, color loss was 92.9%, cataract was 66.6%, pigmentary retinopathy was 30%, and diabetic retinopathy was 20% (91). Visual field testing in some cases demonstrates concentric and/or peripheral vision loss (92). In the retina, electrooculograms and ERGs are usually normal in the presence of reduced cone/rod dark adaptation, although Dhalla et al. report a case with pigmentary retinopathy (93). Neuropathological specimens demonstrate loss of retinal ganglion cells, myelinated axons in the optic nerve, chiasm, and tract, as well as neuron loss in the lateral geniculate nucleus (94). Diabetic retinopathy is extremely rare; the overall course is milder than that seen in isolated diabetes mellitus, with a lower prevalence of microvascular disease (87).

Hearing loss is present in about 66% of individuals with Wolfram syndrome, with the preferential involvement of high frequencies (95). Hearing impairment ranges from congenital deafness to a milder, progressive sensorineural hearing loss, and median age of onset is 12.5 years (85a). Among individuals with inactivating WFS1 mutations, five females demonstrated significantly greater hearing impairment than four males, thus suggesting a role for hormonal factors in hearing loss modulation (96). Neuropathological findings in Wolfram syndrome patients demonstrate loss of the organ of Corti in the basal turn of the cochlea, as well as focal atrophy of the stria vascularis, correlating well with high-frequency hearing loss (94).

Diabetes insipidus (DI), present in 51–87% of individuals (85b,97), is most often central in origin and usually presents in the second decade of life. Brain MRI may show absence of the typical T1-hyperintense signal from the posterior pituitary along with atrophy and gliosis in the supraoptic and paraventricular nuclei (PVN) of the hypothalamus (98). Necropsy has demonstrated similar



findings, as well as accumulation of vasopressin precursors in the PVN (99).

Neurological abnormalities, including neurodegeneration, are present in more than half of patients with Wolfram syndrome (85a). Widespread neurodegenerative changes, with a median age of onset of 15 years, are considered part of the syndrome and are supported by abnormal MRI findings, making Wolfram syndrome part of a multisystem neurodegenerative disorder. Progressive neurological findings are the result of generalized brain atrophy, with the involvement of cranial nerves, the brain stem, pons, cerebellum, and posterior hypothalamus (85a,98a,100). The most prominent atrophy was demonstrated in the cerebellum, medulla, pons, optic nerves, and posterior hypothalamus (101). Neuroimaging may also show atrophy of other brain regions in the setting of neurodegeneration, although this atrophy does not always result in patient symptoms (98a). Cognitive impairment, corresponding to cortical abnormalities on MRI, may also be observed in up to 32% of patients with neurological signs (102). DI and hearing loss have been correlated with degenerative damage of the hypothalamus and the vestibulocochlear nuclei (94). Ataxia has been correlated with Purkinje cell loss and gliosis in the cerebellar white matter (103). In a postmortem study, Hilson et al. demonstrate gross shrinkage and neuron loss in the pontine base and inferior olivary nucleus, as well as minimal neurohypophyseal tissue in the pituitary and decreased numbers of axons in the paraventricular and supraoptic nuclei, with relative sparing of the cerebellum (94).

Gastrointestinal complaints have also been noted in a large number of Wolfram syndrome patients, with 25% of patients complaining of constipation, chronic diarrhea, and other bowel dysfunctions. Some of these cases have been attributed to gluten intolerance, which is 20 times more frequent in patient populations who have had several years' history of diabetes mellitus (104).

Autosomal recessive inheritance with incomplete penetrance is supported by reports of families with more than one affected sibling, and consanguinity is noticed in 15%–32.5% of the cases. Many of the described cases are sporadic.

Wolfram syndrome is genetically heterogeneous and is currently subdivided into three genetic forms: WFS1 (MIM \*606201), WFS2 (MIM#604928), and a possible mitochondrial syndrome (MIM#598500). WFS1 is located on 4p16.1 and encodes a transmembrane ER glycoprotein called wolframin, which may function as a calcium channel or a regulator of calcium conductance in the ER (105). Khanin et al. found mutations in 90% of their patients with Wolfram syndrome (106). Wolframin is ubiquitously expressed, with particularly high levels in the pancreas, heart, brain and in insulinoma beta-cell lines (107). Disease likely results from loss of wolframin function (108). Diabetes mellitus development in DIDMOAD is attributed to impaired homeostasis of beta cells, and studies indicate that wolframin may help

fold proinsulin, a protein precursor of insulin, into the mature hormone that controls blood glucose levels (109). Additionally, WFS1 is up-regulated in mouse pancreatic beta cells during glucose-induced insulin secretion, whereas WFS1 knockdown in beta cells resulted in ER stress and cell dysfunction. These authors hypothesized that Wolfram syndrome thus involves chronic ER stress in pancreatic beta cells. Interestingly, a heterozygous mutation of WFS1 may explain some cases of autosomal dominant, non-syndromic sensorineural, low-frequency hearing loss (110). Sandhu et al. reported that two single nucleotide polymorphisms (rs10010131, rs6446482) are associated with risk for type 2 diabetes mellitus (111).

In contrast, WS2 localizes to the long arm of chromosome 4 (4q22–24) and is caused by mutations in the *CISD2* gene (MIM222300), which codes for an ER intermembrane protein (112). Unlike classic Wolfram syndrome, the patients—all from consanguineous Jordanian families—did not exhibit DI and also presented with severe upper gastrointestinal bleeding/ulceration (113). Chen et al. demonstrated that, in the mouse, *Cisd2* localizes to the outer mitochondrial membrane and is involved in the control of life span (114).

The third, a mitochondrial syndrome, was initially suggested by Rotig et al. in a patient with early-onset diabetes mellitus, optic atrophy and deafness, who harbored a 7.6 kb mitochondrial deletion (115). Barrientos et al. provided evidence that mutations in the 4p16 region predispose individuals to multiple mitochondrial DNA deletions in families with Wolfram syndrome (116), although Domenech et al. did not find mitochondrial DNA abnormalities in their Spanish cohort of six WS1 families (117). Hofmann et al. found, when comparing mitochondrial DNA variants identified in DIDMOAD patients with those found in LHON patients and healthy controls, that a high percentage of DIDMOAD patients harbored secondary LHON mutations, and that both DIDMOAD and LHON patients were concentrated in two different mitochondrial haplotypes (118). He thus concluded that different clusters of mitochondrial DNA variants may act as predisposing haplotypes, increasing the risk of either DIDMOAD or LHON. However, the characteristics distinguishing the three genetic subtypes have been questioned, as the *CISD2* gene of Wolfram syndrome 2 has been implicated in mitochondrial dysfunction as well (114a). As such, the relationship between either of the autosomal loci for Wolfram syndrome and mitochondrial mutations is unclear.

Treatment of Wolfram syndrome is symptomatic. The diabetes mellitus is treated with insulin. Physicians should monitor patients carefully for signs of urological complications. As wolframin is a transmembrane protein with multisystem localization, other tests to consider include dynamic pituitary gland tests, such as an insulin tolerance test and lutenising hormone releasing hormone (LH-RH) test, as well as a water deprivation test to determine posterior pituitary gland function (119). Depending

on the degree of hearing impairment, hearing loss may be managed with hearing aids or cochlear implantation. No treatment is available for the basic defect.

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### Biographies



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# CHAPTER

# 135

## Glaucoma

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### 135.1 INTRODUCTION

The glaucomas are a leading cause of blindness in the United States. One to two percent of Americans older than age 40 years are affected with glaucoma, and each year over 10,000 are blinded by the disease (1). In the United States, the glaucomas are the second most common cause of permanent blindness and the most common cause of blindness among blacks (2). The clinical diagnosis of glaucoma does not refer to a single, distinct condition, but rather to a collection of similar appearing diseases. The glaucomas all have the same basic phenotype, which is characterized primarily by a bilateral, progressive degeneration of the optic nerve. This glaucomatous optic neuropathy causes an irreversible loss of vision, which can lead to complete blindness without proper therapy. In most cases, glaucoma is associated with elevated intraocular pressure (IOP); however approximately one-third of cases have optic nerve degeneration despite intraocular pressures in the normal range. The anatomy and physiology of the optic nerve and the structures regulating aqueous humor dynamics are important elements in the biology of glaucoma and serve as a framework for investigating its pathogenesis. The optic nerve forms a link between the neurosensory retina and the brain, and is an important segment of the visual pathway. Light is detected by the photoreceptor cells of the retina and a signal is transmitted through complex interconnections with other retinal cells to the ganglion cell layer of the retina. The axons of the ganglion cells, which collectively form the optic nerve, project to the lateral geniculate nucleus and onto the occipital cortex, where the visual input is perceived.

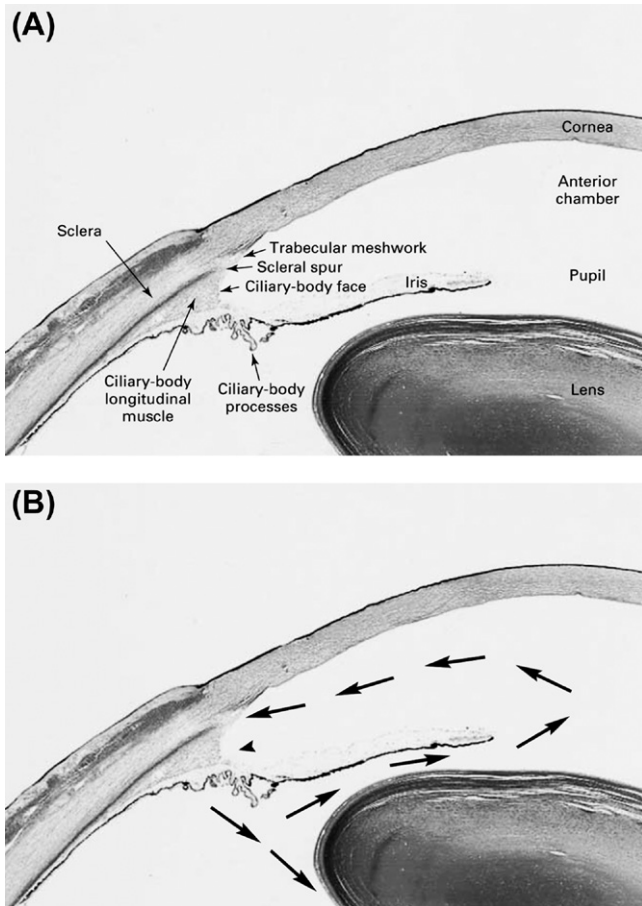
Elevated IOP is associated with most forms of glaucoma and is currently the only known modifiable risk factor for these diseases. IOP is determined by the balance between the rate of aqueous humor production and the rate of outflow from the eye. Aqueous humor is produced by the ciliary body, a tissue located posterior to the peripheral iris. The majority of the aqueous humor drains from the eye through the trabecular meshwork (TM),

a sponge-like tissue located at the iridocorneal angle, the junction of the iris and the cornea (Figure 135-1A). Current treatment strategies are all directed at lowering IOP, and in many cases, these interventions are effective at slowing or halting the progression of glaucoma. The early signs of the disease are often subtle and significant nerve damage and vision loss may occur before glaucoma is recognized by either patients or clinicians. Genetic studies of glaucoma could lead to the development of new diagnostic and therapeutic tools. The identification of glaucoma-causing genes could assist clinicians in diagnosing glaucoma before irreversible blindness occurs. Patients carrying alleles known to confer risk of severe disease could be targeted for aggressive therapy. Glaucoma genetic research could also produce gene-based therapeutic strategies and lead to the identification of alleles predicting treatment outcomes. A family history of glaucoma is well recognized as a major risk factor for the disease, (3), and both Mendelian and non-Mendelian forms of inheritance have been described. The most common adult-onset forms of glaucoma such as primary open-angle glaucoma (POAG), exfoliation syndrome glaucoma (EG) and closed-angle glaucoma have heritability consistent with that of a complex trait, while early onset forms typically exhibit Mendelian inheritance patterns (4).

This chapter is divided into four sections: (1) clinical presentation of heritable forms of glaucoma, including nomenclature and phenotypic descriptions; (2) identification of glaucoma genes using human linkage studies; (3) glaucoma genes identified from studying naturally occurring animal models; and (4) genes identified using genomic approaches.

### 135.2 CLINICAL FEATURES OF HERITABLE FORMS OF GLAUCOMA

The glaucomas are classified as two groups: primary and secondary glaucomas. Primary glaucoma is defined as isolated, idiopathic disease of the anterior chamber of the eye and the optic nerve, whereas secondary glaucoma



**FIGURE 135-1** The anterior segment of the eye. Panel A shows the major structures. Panel B shows the flow of aqueous humor through the anterior segment. Aqueous humor is produced by the ciliary body processes. It flows around the lens, then passes through the pupil and into the anterior chamber, where it nourishes the cornea before leaving the eye through the TM into the venous system. (From Alward, W. L. M. *Medical management of glaucoma*. *N. Engl. J. Med.* 1998, 339, 298–1307, with permission.)



**FIGURE 135-2** Gonioscopy. In order to view the structures of the iridocorneal angle, a gonioscopy contact lens is placed on the surface of the cornea. (Courtesy of Dr. WLM Alward, Department of Ophthalmology and Visual Sciences, The University of Iowa Hospitals and Clinics.)

is associated with known predisposing events including developmental abnormalities, systemic diseases, drug therapy, or trauma.

The primary glaucomas are further categorized by the anatomy of the junction of the iris and the cornea (the iridocorneal angle). The iridocorneal angle is the most important site of fluid drainage from the human eye. Primary glaucoma in an eye with a wide-open iridocorneal angle that allows unimpeded fluid outflow through the TM is termed primary open-angle glaucoma (POAG) (see Figure 135-1A). In glaucoma, the iridocorneal angle is narrow and aqueous humor outflow is obstructed. The vertex of the iridocorneal angle and the TM cannot be directly visualized under normal circumstances. Ophthalmologists may view this structure by placing a specialized lens directly on the cornea (Figure 135-2).

Open-angle glaucomas are further classified by the age of onset of disease (congenital; juvenile; and primary open-angle glaucoma [POAG]). Open-angle glaucoma with an onset during infancy (less than three years of age) is termed congenital glaucoma, whereas glaucoma with onset after 40 years of age is classified as POAG. Juvenile open-angle glaucoma (JOAG) has its onset at an age intermediate to that of congenital and primary open-angle glaucoma.

### 135.2.1 Primary Glaucomas

#### 135.2.1.1 Primary Open-Angle Glaucoma (POAG).

Of the many forms of glaucoma, POAG is the most common. In the Framingham Eye Study, 1.6% of subjects over 40 years of age were affected with POAG (5). The prevalence of POAG varies between ethnic populations. The prevalence of POAG in blacks is five times that in whites (2). It is a genetically complex trait that is likely to result from the effects of multiple genetic and/or environmental factors. Large pedigrees with apparent Mendelian inheritance have been reported, but typically POAG shows familial clustering without a recognized inheritance pattern (3). First-degree relatives of individuals affected with POAG have up to an eight-fold increased risk of developing POAG compared with the general public (6). Additionally, many of the clinical features of POAG are heritable quantitative traits, including cup to disc ratio and IOP (described later) (7).

**Classic Features of Primary Open-Angle Glaucoma.** In most cases, POAG is a slowly progressive and painless optic neuropathy with an associated insidious loss of visual field. Consequently, many patients are unaware that they are affected with this disease. The defining features of POAG (and of other forms of glaucoma) are characteristic optic nerve damage and loss of visual field. Many patients also have elevated IOP, although this feature is not required for the diagnosis of glaucoma. The defining clinical features of POAG are described in the following sections:

**Optic Nerve Disease.** Visual input from the retina reaches the brain through an intricate network of

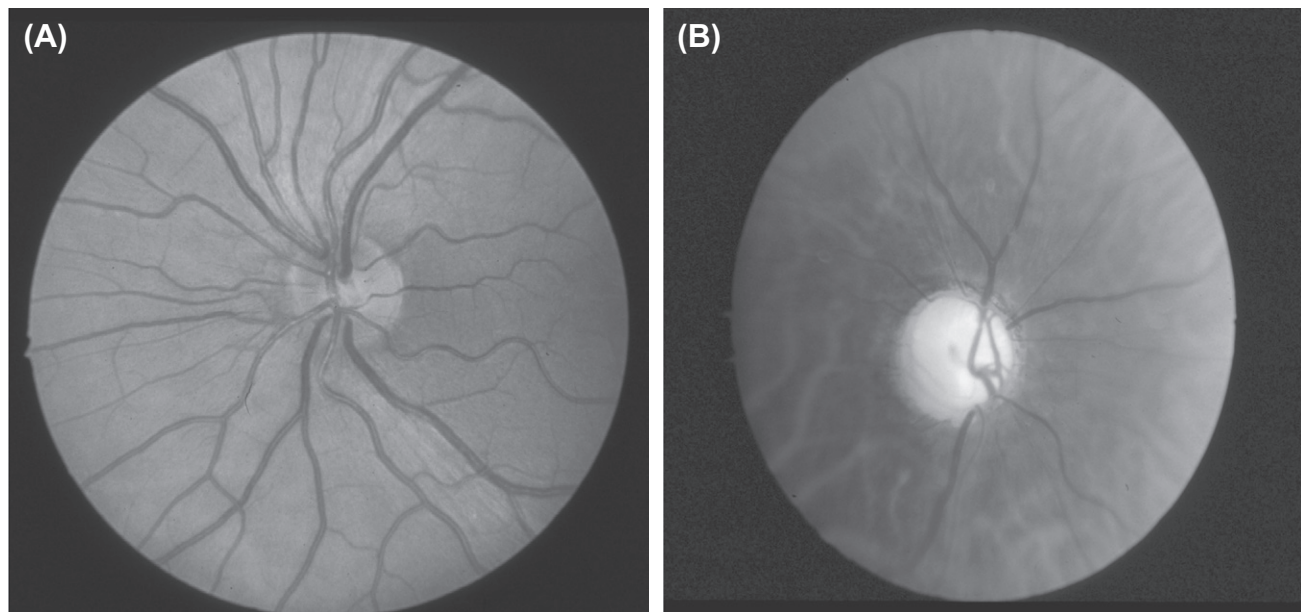
neuronal connections that begins with the axonal projections of retinal ganglion cells, which unite to form the optic nerve. The axons from the ganglion cells of the entire retina converge at the posterior pole of the eye forming the head of the optic nerve, the optic disc. As they come together in the plane of the globe, the ganglion cell axons turn posteriorly to exit the eye forming an indentation in the optic disc called the optic cup. Characteristics of the optic nerve (including the size of the optic cup) may be observed directly by looking through the pupil with an ophthalmoscope. A healthy optic disc is pink, and the ratio of the diameter of the optic cup to that of the optic disc averages 0.4 (Figure 135-3A). A defining feature of glaucoma is the progressive loss of the ganglion cell layer of the retina and the axons that form the optic nerve. As optic nerve tissue is lost, the optic cup grows larger, increasing the cup to disc ratio (Figure 135-3B).

**Loss of Visual Field.** Visual field is the area concurrently seen by a fixed eye. The topographical organization of the visual input is maintained during transmission from the retina to the visual cortex. The optic nerve damage caused by glaucoma destroys some of the connections between the retina and the brain, which causes defects in the visual field. The portion of the optic nerve that conveys peripheral and paracentral vision is preferentially affected by glaucoma. Consequently, individuals affected with glaucoma most commonly have constricted visual fields (peripheral blindspots with central vision spared). Because central vision is generally preserved until late in the course of glaucoma, patients with significant visual field losses frequently have good visual acuity.

Perimetry is the systematic measurement of visual fields. Patients fix one eye on a target directly forward, while the other eye is covered with a patch. Test objects, such as light sources or examiner's fingers, are then presented to the fixed eye at various positions. Patients signal when they see these objects, allowing their visual field to be mapped. Visual fields may be measured manually or with automated equipment using static or kinetic targets.

**Elevated Intraocular Pressure (IOP).** The anterior and posterior chambers of the eye are bathed in nutrient- and oxygen-rich aqueous humor, which provides nourishment to the avascular, translucent structures of the lens and cornea. Aqueous humor is secreted by the epithelium of the ciliary body and flows around the lens, through the pupil, and across the anterior surface of the iris to the angle formed between the cornea and iris, the iridocorneal angle (see Figure 135-1B). The aqueous humor drains from the eye through the TM (trabecular meshwork), which is located at the vertex of the iridocorneal angle. Aqueous humor passes through the TM to the venous circulation through a network of vessels beginning with Schlemm's canal. Although there are other pathways of drainage, the TM route accounts for the majority of outflow in human eyes.

The chief determinants of IOP are the rate of aqueous humor production by the ciliary body and the rate of aqueous humor outflow through the TM. In normal individuals the balance between aqueous humor production and outflow results in a mean IOP of approximately 15.5 mmHg. Two standard deviations above the mean IOP (20.5 mmHg) are generally accepted to be the upper limit of the normal range of pressure. There is



**FIGURE 135-3** Optic nerve head. (A) The optic nerve head of normal individuals has a characteristic pink color and an average cup to disc ratio of 0.4. (B) The optic nerve head of a patient with endstage glaucoma is excavated and has a cup to disc ratio approaching 1.0. There is a pale appearance in the areas where nerve tissue has been lost. (Courtesy of Dr. W.L.M. Alward, Department of Ophthalmology and Visual Sciences, The University of Iowa Hospitals and Clinics.)



a considerable overlap between the range of IOPs in glaucoma patients and the general population. Patients with IOPs above the normal range have an increased risk of developing glaucoma, yet an elevated IOP is not sufficient for a diagnosis of glaucoma. IOP also varies diurnally, with peak pressures usually occurring in the morning. IOPs above 21 mmHg are generally considered elevated.

Studies have shown that elevation of IOP in glaucoma is primarily due to increased resistance to aqueous humor outflow through the TM (8). The biological mechanisms of disease in glaucoma are poorly understood. The processes that lead to optic nerve damage and vision loss are unclear. It is generally accepted that IOP has a significant role in the pathogenesis of POAG and other forms of glaucoma. Currently, elevated IOP is the only modifiable glaucoma risk factor and all available glaucoma treatments attempt to slow the progress of disease by lowering IOP.

Elevated IOP is often associated with glaucoma. Some individuals develop optic nerve disease and visual field loss characteristic of glaucoma without ever having measurably high IOP ('normal tension' glaucoma). Conversely, others with elevated IOP may never develop the defining features of glaucoma. Elevated IOP in the absence of optic nerve disease and visual field loss is known as ocular hypertension. For most patients, high IOP is an important risk factor for the development of glaucoma and treatment of elevated IOP reduces this risk.

**135.2.1.2 Juvenile-Onset Primary Open-Angle Glaucoma (JOAG).** JOAG is a relatively rare subset of glaucoma representing less than 1% of total cases (9). JOAG has been defined as a severe form of POAG, which is characterized by onset of disease between three and 40 years of age and highly elevated IOPs, sometimes over 50 mmHg. The heredity of JOAG is much more evident than that of POAG; because of the early age of disease onset, several generations of affected family members are usually alive and available for examination. Consequently, many large JOAG pedigrees have been described which exhibit obvious autosomal dominant inheritance and a high, but incomplete penetrance.

**135.2.1.3 Normal Tension Glaucoma (NTG).** Some glaucoma patients develop optic nerve damage and visual field loss without ever having a high IOP measurement. This form of glaucoma is known as normal tension glaucoma (NTG). NTG patients share features of POAG (open iridocorneal angles, optic neuropathy, and visual field loss), but have IOP that consistently remains within the 'normal' range. A standard set of diagnostic criteria for NTG does not exist, however, the Collaborative Normal-Tension Glaucoma Study Group has used the following strict definition of NTG. Patients are diagnosed with NTG if they have;

1. optic disc abnormalities and visual field defects consistent with glaucoma,
2. no history of IOP over 24 mmHg, and
3. a diurnal IOP with a median of 20 mmHg or less with only one measurement of 23 or 24 mmHg (based on 10 measurements between 8 am and 6 pm over two days) (10).

Population-based prevalence studies have suggested that NTG is a common form of glaucoma, potentially accounting for up to 30% to 60% of cases in various populations (11).

At present, it is unclear whether NTG and high-pressure glaucoma represent different ends of a single spectrum of disease or are distinct conditions. Studies suggest that several clinical signs are more frequently found in NTG, including optic disc hemorrhages, specific changes in the appearance of the optic nerve head, and characteristic types of visual field defects (12). Other factors that have been investigated for an association with NTG include age, female gender and Asian ethnicity and vasospasm (13).

#### **135.2.1.4 Developmental Glaucomas.**

**135.2.1.4.1 Primary Congenital Glaucoma.** The congenital glaucomas are typically associated with severe disease and are usually diagnosed at birth. Primary congenital glaucoma (PCG) is characterized by isolated abnormalities of the iridocorneal angle that lead to decreased aqueous outflow and elevated IOP. PCG is a common form of childhood glaucoma and accounts for approximately 22% of total cases (14). The average prevalence of PCG is 1:10,000 births, although extreme variation with ethnic background (ranging from 1:1250 to 1:22,000) has been reported (14).

The classic signs of PCG are tearing, photophobia, and lid squeezing. The cornea may be cloudy or exhibit breaks in the corneal endothelium known as Haab's striae. These signs, which may be bilateral or unilateral, are all due to elevated IOP. The structure of the infantile eye is immature and it stretches in response to high IOP. Consequently, patients affected with congenital glaucoma often have markedly enlarged eyes, termed buphthalmos (Greek for 'ox eye', Figure 135-4). After three years of age, elevated IOP will no longer cause the eye to enlarge. The high IOP of PCG may cause severe optic neuropathy and profound vision loss unless treatment is urgently provided.

**135.2.1.4.2 Anterior Segment Dysgenesis Syndromes.** Most of the structures comprising the 'anterior segment' of the eye (iris, trabecular meshwork, anterior chamber, aqueous humor cornea and iridocorneal angle) arise developmentally from the cranial neural crest, and disruption of these developmental processes results in abnormal formation of the trabecular meshwork and other structures necessary for fluid removal, causing an early-onset form of glaucoma (typically between the ages of 10 to 20). These developmental disorders are usually inherited as autosomal dominant traits and may be associated with systemic abnormalities (15). For example, in addition to ocular dysgenesis, patients affected by Axenfeld-Rieger syndrome have abnormal





**FIGURE 135-4** Buphthalmos. Enlarged eyes caused by elevated IOP in congenital glaucoma. (Courtesy of Dr. W.L.M. Alward, Department of Ophthalmology and Visual Sciences, The University of Iowa Hospitals and Clinics.)

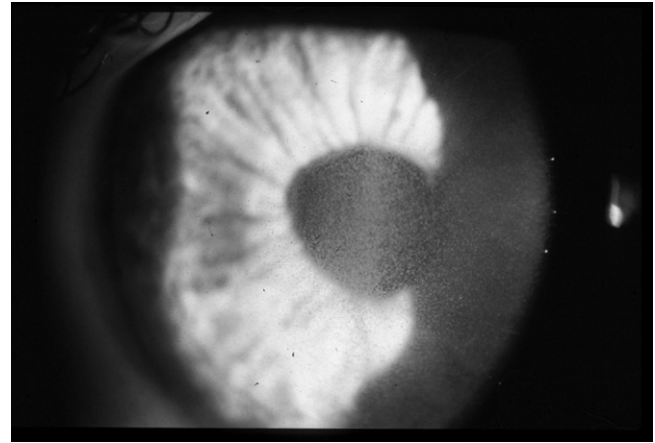
teeth and hearing loss (16). Other glaucomas of childhood are associated with additional ocular or systemic developmental abnormalities. Reviewed in Refs. (17–20).

## 135.2.2 Secondary Glaucomas

**135.2.2.1 Pigment Dispersion Syndrome (PDS)/ Pigmentary Glaucoma (PG).** Pigment dispersion syndrome (PDS) is an ocular condition in which pigment granules from the iris are released into the anterior chamber of the eye. A subset of PDS patients develop signs of glaucoma related to the dispersal of pigment. This secondary glaucoma is known as pigmentary glaucoma (PG) and shares many features with POAG (elevated IOP, optic neuropathy, and visual field loss). The clinical presentations of PDS/PG have been studied for over 100 years (21), and there is a rich history of observation and experimentation that has contributed to our knowledge of these conditions (22,23). The iris in most patients with PDS has a concave or ‘back-bowed’ shape, which appears to be an important factor in the pathogenesis of the disease (22). Liberation of pigment from the iris produces the classic features of PDS, including defects in the iris visible by transillumination and accumulation of released pigment on the corneal endothelium (Figure 135-5), trabecular meshwork, lens and zonules.

PDS is a common condition that affects up to 2.5% of the general population and may be more common in young nearsighted (myopic) men (24). PDS has a peak incidence before 30 years of age and becomes less common with age. Deeper anterior chambers, which are more common in myopic eyes, may promote an abrading configuration of the iris–lens surfaces. The normal, progressive growth of the lens tends to shallow the anterior chamber, which normalizes the configuration of the iris and reduces the risk of PDS with age.

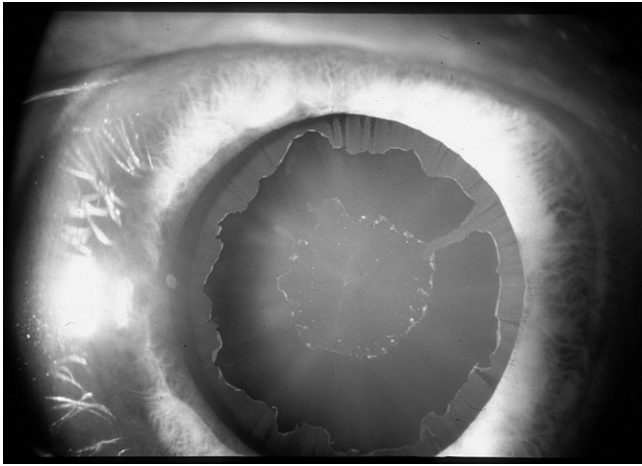
A significant fraction of PDS patients develop glaucoma secondary to pigment dispersion. The fraction



**FIGURE 135-5** Krukenberg's spindle. Dispersed iris pigment that accumulates on the corneal endothelium (known as a Krukenberg's spindle) is a sign of PDS/PG. (Courtesy of Dr. W.L.M. Alward, Department of Ophthalmology and Visual Sciences, The University of Iowa Hospitals and Clinics.)

of PDS patients that eventually progress to pigmentary glaucoma (PG) is as high as 35% to 50% in glaucoma clinics and tertiary care centers (25). A recent population based study from Olmsted County, Minnesota found that 10% of PDS patients had developed glaucoma over 5 years, and 15% over 15 years (26). The pathophysiological basis of the progression from pigment dispersion to glaucoma is largely unknown. The simplest explanations suggest that pigment accumulation in the TM impedes aqueous humor outflow, causing IOP elevation and subsequent glaucoma (27). Pedigrees with PDS segregating as an autosomal dominant trait have been described (28) and several loci have been identified, although causative genes have not yet been characterized.

**135.2.2.2 Exfoliation Syndrome/Exfoliative Glaucoma.** Exfoliation syndrome is a systemic condition in which fibrillar protein is deposited in tissues throughout the body, including the eye (29). Although the exfoliative material is also produced in many other organs, its pathology appears to be limited to the eye. The origin of the material is not completely understood, but may develop because of extracellular matrix degeneration and compromise of associated vasculature (30). In the eye, the material accumulates on all the structures of the ocular anterior segment. Exfoliative material on the lens is distributed in a characteristic ‘bull's-eye’ pattern (Figure 135-6). This annular configuration is generated by the iris as it rubs along the lens during normal dilation and constriction of the pupil. Exfoliative material is brushed away in a ring-shaped zone corresponding to the excursion of pupillary margin. Collections of exfoliative material may also be observed on the surfaces of the iris, cornea, and TM, along with pigment granules from the iris (probably released by abrasion between the iris and the lens). These signs of exfoliation syndrome are frequently extremely asymmetric.



**FIGURE 135-6** Lens appearance in exfoliation syndrome. Exfoliation material accumulates on the anterior lens capsule. The material is removed by abrasion with the iris during normal dilation and constriction of the pupil forming a 'bull's eye' pattern. (Courtesy of Dr. W.L.M. Alward, Department of Ophthalmology and Visual Sciences, The University of Iowa Hospitals and Clinics.)

The prevalence of exfoliation syndrome varies dramatically with ethnic background and increases with age (31). Exfoliation syndrome is common in Scandinavian and Mediterranean countries with a prevalence of up to 21% and is associated with a significant fraction of open-angle glaucoma in these areas. Recent studies indicate that residence in Northern latitudes is a risk factor for developing the syndrome (32).

Patients with exfoliation syndrome are at high risk of developing a secondary glaucoma (22% to 82%) (33). Exfoliative glaucoma is associated with similar pressure-related optic nerve damage and visual field loss to POAG. Patients affected with exfoliative glaucoma tend to be older than POAG patients and may present with IOP that becomes rapidly elevated.

In most cases of exfoliative glaucoma, the iridocorneal angles are open and elevated IOP is caused by changes in the TM related to the accumulations of exfoliative material. The amount of exfoliative material appears to correlate with the development of glaucoma (34), but its accumulation alone is not sufficient to cause glaucoma. The presence of exfoliative material may cause TM endothelial cells to become dysfunctional as they attempt to remove the exfoliative material by phagocytosis. The iridocorneal angle is generally open in exfoliation syndrome. Owing to an associated weakness in the suspension of the lens, patients with exfoliation syndrome have a predisposition for subluxation of the lens. Dislocated lenses may press the iris anteriorly and cause a form of closed-angle glaucoma-associated with exfoliation syndrome.

There is evidence from epidemiological studies that genetic factors significantly contribute to the pathogenesis of exfoliation syndrome as described earlier. Individuals of certain ethnic backgrounds are at higher risk

for developing exfoliation syndrome than others. Some families with inherited exfoliation syndrome have been described, and twin studies have provided support for a genetic basis of this disease (35,36).

### 135.2.3 Angle-Closure Glaucoma

The normal, flat configuration of the iris results in a wide-open iridocorneal angle allowing aqueous humor outflow through the TM (see Figure 135-1B). A number of anatomic abnormalities, however, can cause the iris to become apposed to the TM, resulting in a narrowed or closed iridocorneal angle and obstructed aqueous outflow. Angle-closure glaucoma is due to the elevated IOP associated with acute or chronic obstruction of aqueous outflow by a visible occlusion of the iridocorneal angle. The prevalence of angle-closure glaucoma varies greatly between ethnic backgrounds. Angle-closure glaucoma is extremely common among Eskimos and Asians (37).

**135.2.3.1 Mechanism of Angle Closure.** The relative position of the lens and the iris change as the pupil dilates and constricts. In susceptible individuals, the lens may become apposed to the pupil margin and obstruct the normal flow of aqueous humor through the pupil. This phenomenon, known as pupillary block, is the most common cause of angle-closure glaucoma. Pressure builds in the posterior chamber as aqueous humor collects behind the iris and eventually causes the iris to bow forward. This convex configuration of the iris brings the peripheral iris in close proximity to the TM causing a narrow or closed iridocorneal angle. Anatomic features of the eye may promote the development of angle closure. An enlarged lens may cause angle-closure by pushing the iris anteriorly against the TM (phacomorphic angle-closure). Similarly, abnormalities of the attachment of the iris to sclera at the iridocorneal angle may alter the configuration of the iris to favor the development of angle-closure.

Several environmental factors or physical characteristics of the eye may also predispose angle-closure by the pupillary block mechanism. In small, far-sighted (hyperopic) eyes, the anterior segment of the eye is 'crowded' with normal-sized lens and iris tissue. In such eyes, the relatively large lens is more likely to obstruct the pupil. With age, the lens gradually grows in size, further favoring the development of pupillary block. Some medications, such as topiramate, or other sulfa derivative drugs can in some individuals cause angle-closure glaucoma. In these drug-related cases both eyes typically develop symptoms, while in the lens-related cases using only one eye is affected at a given time.

Family and twin studies suggest that angle-closure glaucoma has a significant heritability (38), however genetic risk factors have not been identified for this condition.

### 135.3 IDENTIFICATION OF GLAUCOMA GENES USING LINKAGE ANALYSIS

Early-onset forms of glaucoma are amenable to linkage studies. Gene discovery using a positional cloning/linkage approach requires large affected pedigrees with well-defined inheritance. The early-onset glaucomas are inherited as either autosomal dominant or autosomal recessive traits. Disease onset during childhood results in affected pedigrees sufficiently large for a linkage-based approach to identify a genomic region harboring the causative gene. Some adult-onset (>age 40) glaucoma pedigrees have familial disease distribution suggestive of autosomal dominant or autosomal recessive inheritance patterns.

#### 135.3.1 Nomenclature of Glaucoma Loci

The nomenclature committee of the Human Genome Organization (HUGO) has designated a system for naming glaucoma gene loci (<http://www.gene.ucl.ac.uk/nomenclature>). The prefix 'GLC' indicates a glaucoma gene locus. The type of glaucoma is indicated by a subsequent number, where a '1' indicates open-angle glaucoma, a '2' indicates closed angle glaucoma, and a '3' indicates congenital glaucoma. Finally, a suffix letter is attached to each new glaucoma loci in chronological order of discovery. For example, GLC1A was the first open-angle glaucoma locus to be discovered. There is no systematic nomenclature for loci associated with secondary or developmental forms of glaucoma. The currently known primary and secondary glaucoma loci defined by genetic linkage studies are indicated in Table 135-1.

#### 135.3.2 Myocilin (MYOC, OMIM #601652); Juvenile Open-Angle Glaucoma

Using a large juvenile open-angle glaucoma pedigree, linkage analysis identified a chromosome 1q23 disease locus termed GLC1A, (39). Fine mapping and gene resequencing identified the MYOC gene as the GLC1A causative gene (40). MYOC encodes a protein (myocilin) previously identified as a steroid-responsive protein in TM cells challenged with glucocorticoids to simulate the conditions of steroid-induced ocular hypertension (OHT). Myocilin has an unknown function. Sequence analysis of the myocilin gene predicts a 57-kD protein with homology to olfactomedin, (41). The myocilin protein is targeted for secretion by a signal sequence and, the carboxy-terminus contains a consensus sequence, which may direct the protein to peroxisomes. Although Myocilin appears to be primarily associated with juvenile open-angle glaucoma, some mutations cause later onset primary open-angle glaucoma (42,43).

The glaucoma-associated mutations are concentrated in the olfactomedin-domain encoded by exon 3 of myocilin (44). The function of the olfactomedin domain is

unknown, and the significance of the clustering of myocilin mutations remains unclear.

More than 40 mutations have been identified in POAG patients. However, a single mutation (Gln368Stop) is associated with nearly half of all cases of myocilin-associated glaucoma (42). Haplotype analysis suggests that the Gln368Stop mutation arose in a single founder and patients with this mutation are distantly related to this common ancestor. If the prevalence of the Gln368Stop mutation in study populations is representative of its frequency in the general public, this defect in myocilin is one of the most common causes of eye disease with a known molecular defect. Myocilin mutations cause a dominant negative effect, and loss of myocilin function does not cause glaucoma (45).

#### 135.3.3 Optineurin (OPTN, OMIM #602432)

**135.3.3.1 Optineurin and Normal Tension Glaucoma.** In normal tension glaucoma, the optic nerve degenerates despite intraocular pressure in the normal range (less than 22mmHg). Using standard linkage approaches and a large pedigree affected by normal tension glaucoma, a gene for normal tension glaucoma was mapped to 10p14–p15 (GLC1E) (46). Screening candidate genes in this region revealed a mutation (Glu50Lys) that was responsible for the NTG affecting this pedigree (47). Subsequent studies have confirmed the association of the Glu50Lys optineurin variation with NTG, but other DNA sequence variants in this gene have not been consistently associated with the disease (48–50). Optineurin participates in the TNF- $\alpha$  mediated apoptotic pathways, which may explain its association with normal tension glaucoma, as this is a form of glaucoma with increased susceptibility to optic nerve degeneration caused by apoptosis.

#### 135.3.4 Cytochrome P450 (CYP1B1); Primary Congenital Glaucoma

Primary congenital glaucoma (PCG) is most commonly inherited as an autosomal recessive trait, although sporadic and autosomal dominant forms have also been reported (51).

Linkage studies using large pedigrees mapped a PCG-causing gene to the GLC3A locus on chromosome 2p21 (52). Subsequently, mutations in cytochrome P450 1B1 (CYP1B1), have been identified in families world-wide (53,54). Two other congenital glaucoma loci have been mapped (55,56) and one other disease causing gene, LTBP2 has been recently identified (57). LTBP2 maps to 14q24.1, 1.3 Mb from the boundary of GLC3C as it was originally defined. It is possible that a second congenital glaucoma gene resides within this region (Table 135-1).

**135.3.4.1 CYP1B1 Genotype–Phenotype Associations.** CYP1B1 mutations causing congenital glaucoma



TABLE 135-1 Human Glaucoma Loci

Locus	Human	OMIM	Clinical Phenotype	Gene	Reference
<i>Primary Glaucomas</i>					
GLC1A	1q23–q25	137750	POAG and JOAG	MYOC	(44)
GLC1B	2cen–q13	606689	NTG/POAG, low to moderate IOP	–	(112)
GLC1C	3q21–q24	601682	POAG	–	(113)
GLC1D	8q23	602429	POAG, modest IOP elevation	–	(114)
GLC1E	10p15–p14	137760	NTG	OPTN	(46,47)
GLC1F	7q35	603383	POAG	–	(115)
GLC1G	5q22	609887	POAG	WDR36	(116)
GLC1H	2p15–p16	611276	POAG	–	(117)
GLC1I	15q11–q13	609745	POAG	–	(118)
GLC1J	9q22	608695	JOAG	–	(119)
GLC1K	20p12	608696	JOAG	–	(119)
GLC1L	3p21–22	–	NTG	–	(120)
GLC1M	5q22.2–q33	610535	POAG	–	(121)
GLC1N	15q22–q24	611274	JOAG	–	(122)
GLC1O	19q33	613100	POAG	NTF	(123)
GLC1P	12p14	–	NTG	TBK1	(124)
GLC1Q	4q35.1–q35.2	–	POAG	–	(125)
<i>Secondary Glaucomas: Pigmentary</i>					
GPDS1	7q35–q36	600510	PDS/PG	–	(28)
GPDS2	18q22	–	PDS/PG	–	(126)
<i>Secondary Glaucomas: Developmental/Congenital</i>					
GLC3A	2p21	231300	Congenital	CYP1B1	(53,127)
GLC3B	1p36	600975	Congenital	–	(55)
GLC3C	14q24	–	Congenital	LTBP2 <sup>a</sup>	(56,57)
RIEG1	4q25–26	601542	Rieger's syndrome, irideodysgenesis	PITX2	(128)
RIEG2	13q14	601499	Rieger's syndrome	–	(129)
ASOD	10q25	602669	ASD	PITX3	(71)
IRID1	6p25	601090	Congenital hydrocephalus and ASD	FOXC1	(65,66)
WS2A	3p14	156845	Waardenburg syndrome type II	MITF	(130)

<sup>a</sup>LTBP2 maps 1.3 Mb from the originally defined GLC3C linkage interval and it is possible that a second congenital glaucoma gene resides in this region. ASD, anterior segment dysgenesis; JOAG, juvenile open-angle glaucoma; IOP, intraocular pressure; NTG, normal tension glaucoma; PDS, pigment dispersion syndrome; PG, pigmentary glaucoma; POAG, primary open-angle glaucoma.

cause a loss of protein function. Affected individuals with mutations in *CYP1B1* demonstrate significant variation in disease severity both among and between families that may be mutation specific, secondary to background haplotype effects, or due to effects of hypothetical modifier genes (58). Tyrosinase is a modifier of disease severity in mice (59), but tyrosinase gene variants do not appear to influence disease expressivity in humans (60).

**135.3.4.2 CYP1B1 Function.** The *CYP1B1* protein sequence contains several conserved domains that are likely to be functionally important, including heme-binding and hinge regions (61). Many of the *CYP1B1* mutations are clustered in conserved heme-binding and hinge regions, or cause premature termination that eliminates these structures (62).

*CYP1B1* is a member of a family of cytochrome P450 genes known to encode enzymes that metabolize and detoxify both endogenous and exogenous molecular, although their activity is not limited to detoxification. The cytochrome P450 enzymes have been postulated to have a role in development by influencing the metabolism of molecules

necessary for specific developmental processes (63). Two specific substrates of *CYP1B1* (estradiols and retinoic acid) could contribute to ocular development and specifically to the development of the ocular anterior segment.

#### 135.3.4.3 Anterior Segment Dysgenesis and Developmental Regulatory Genes.

**135.3.4.3.1 PITX2, FOXC1, LMX1B, PAX6; Developmental Glaucoma and Anterior Segment Dysgenesis.** The structures of the ocular anterior segment (iris, trabecular meshwork, anterior chamber, aqueous humor, and cornea) arise from the cranial neural crest. Abnormalities in genes controlling neural crest development can result in abnormal formation of the anterior segment structure involved in glaucoma. These developmental disorders are usually inherited as autosomal dominant traits and may be associated with systemic abnormalities (17). For example, patients affected by Axenfeld–Rieger syndrome have abnormal teeth formation in addition to ocular abnormalities (64). Several loci and two genes coding for transcription factors (paired-like homeodomain transcription factor 2 [*PITX2*] at



*RIEG1*, (15,16), and forkhead box C1 [*FOXC1*] at *RIEG3*, (65,66)) have been associated with Axenfeld–Rieger syndrome. A spontaneous *FOXC1* mutation is also the cause of the congenital hydrocephalus mouse (ch) that exhibits multiple defects of the skull and skeleton, kidneys, ureters and eyes (67,68). Other genes causing developmental glaucoma are *PAX6* (Aniridia) (69) and *LMX1B* (nail–patella syndrome and anterior segment dysgenesis) (70) and *PITX3* (anterior segment dysgenesis and cataracts) (71). Interestingly, the developmental glaucoma syndromes frequently exhibit variable expressivity, with glaucoma only occurring in about 50% of cases overall (72). The cause of the variable expressivity is not known; however, it may be secondary to modifier genes or stochastic effects (73).

## 135.4 GENES CAUSING GLAUCOMA IN ANIMAL MODELS

One approach for finding candidate genes for human glaucoma is to study animals with hereditary forms of glaucoma. The assumption of this approach is that genes important to animal forms of glaucoma will also contribute to human glaucoma. There are also valuable animal models relevant to glaucoma with inducible phenotypes. A full discussion of all of these models would be beyond the scope of this chapter. In the following sections the genes responsible for naturally occurring forms of glaucoma will be described in mice, dogs and zebrafish.

### 135.4.1 Mice

The DBA/2 lineage of mice, including DBA/2J (74–77), DBA/2NNia (78–80), and AKXD-28/TyJ (81) all develop age-related forms of glaucoma that include iris disease, increased IOP, and retinal ganglion cell loss. The disease-causing mutations have been identified in DBA/2J (74,75). The glaucomatous iris disease of DBA/2J mice results from a digenic interaction of mutations in two genes encoding melanosomal proteins, *Tyrp1* and *Gpnmb*. The features of DBA/2J eyes bear particular resemblance to human pigment dispersion syndrome and pigmentary glaucoma, and therefore, are excellent candidates for the causes of these diseases. Mutations in human *GNMB* or *TYRP1* have not been identified (74,82).

### 135.4.2 Dogs

A colony of Beagle dogs with the anatomic characteristics of POAG, including elevation of intraocular pressure and optic nerve disease, was used for a series of genetic linkage experiments that identified a homozygous missense change in *ADAMTS10* as the responsible mutation (83). In humans, homozygous mutations in *ADAMTS10* cause lenticular myopia, ectopia lentis, glaucoma, spherophakia and short stature, features of the Weill–Marchesani

syndrome (84). Mutations in *ADAMTS10* have not been associated with POAG in humans.

### 135.4.3 Zebrafish

A forward-genetic screen for adult ocular abnormalities in zebrafish identified *bugeye*, with phenotypic features related to glaucoma phenotype including enlarged eyes with myopia, elevated IOP, and damage to retinal ganglion cells. Using linkage analysis nonsense mutations in low density lipoprotein receptor-related protein 2 (*lrp2*) were identified in mutant fish (85). *Lrp2* is a large transmembrane protein of the low density lipoprotein (LDL)-receptor related protein (*Lrp*) family and participates in receptor-mediated endocytosis. A number of ligands bind the receptor, including Sonic hedgehog and Bone morphogenetic protein 4, vitamin and hormone binding proteins, apolipoproteins, (86). *Lrp2* is expressed in the zebrafish eye and particularly in the retinal pigment and ciliary epithelial cells (85). The ciliary epithelial cells have a critical role in production of aqueous humor and in intraocular pressure dynamics. In humans, mutations in *LRP2* cause the rare Donnai–Barrow syndrome (87), characterized by agenesis of the corpus callosum, diaphragmatic hernia, sensorineural deafness, hypertelorism, buphthalmia (enlarged eye globes) and high myopia.

Forms of glaucoma have also been described in a wide variety of other animal species, although genes have not yet been associated with these forms of glaucoma. These include the monkey (88), cat (89), horse (90), rabbit (91), quail (92), and turkey (93). With the increasing knowledge of a wide variety of animal genomes (94), the hereditary basis of these glaucomas is increasingly approachable.

## 135.5 IDENTIFICATION OF GLAUCOMA GENES USING GENOMIC APPROACHES

Most human patients with glaucoma are affected by the adult-onset forms of the disease, primary open-angle glaucoma (POAG), exfoliation glaucoma (EG), and angle-closure glaucoma. The genetic and phenotypic complexity of these conditions complicates genetic linkage approaches used to identify causative genes. Recent advances in molecular genetics and genomics have supported the genetic study of common forms of adult-onset glaucoma. In particular, the annotation of the human genome sequence and the development of the HapMap (95) have facilitated genome-wide association studies to identify genes contributing to ocular quantitative traits related to glaucoma pathogenesis (cup/disc ratio, CDR; optic nerve size and central corneal thickness, CCT), as well as genes associated with exfoliation syndrome and primary open-angle glaucoma. Table 135-2 lists the genes identified using genome-wide association studies as associated with common forms of glaucoma.

**TABLE 135-2** Recent Genome-Wide Association Studies Identifying Significant SNP Associations ( $p < 5 \times 10^{-8}$ ) for Common Forms of Glaucoma and Related Quantitative Traits

Disease or Trait	Gene <sup>a</sup>	Reference
Glaucoma-POAG (primary open angle glaucoma)	CAV1/CAV2	(106)
	TMCO1	(108)
	CDKN2BAS	(108,109)
Glaucoma-NTG (normal tension)	CDKN2BAS	(109)
	LRP12, ZFPM2	
Glaucoma-ES (exfoliation syndrome)	LOXL1	(102)
Central corneal thickness (CCT)	COL8A1	(101)
	ZNF469	(99)
	COL5A1	(100)
	AVGR8	
	AKAP13	
	PDE8A	
Optic nerve size	ATOH7	(97)
	SALL1	(131)
	CARD10	(132)
Optic nerve vertical cup-to-disc ratio	CDKN2B	(96)
	SIX1, SIX6	
	SCYL1	
	DCLK1	
	CHEK2	
	ATOH7	
	BCAS3	

<sup>a</sup>If the associated SNP(s) are found in an intergenic region the nearest gene(s) is listed.

### 135.5.1 Ocular Quantitative Traits (Cup/Disc Ratio); Optic Nerve Size and Central Corneal Thickness

Optic nerve parameters such as disc area and CDR are highly heritable and show substantial variation in human populations. Using normal populations, genome-wide analysis of ocular quantitative traits has led to the identification of *CDKN2BAS* and *SIX1SIX6* as genetic risk factors contributing to the CDR (96), and the *ATOH7* gene as an important determinant of optic nerve size (97). Using a similar approach, genetic factors contributing to central corneal thickness (CCT) have been identified in populations from around the world (98–101). CCT is one of the most heritable of the ocular quantitative traits. Many of the genes associated with CCT seem to be population specific, although several, including *ZNF469* and *COL8A2* have been identified in ethnically diverse populations (98,101).

**135.5.1.1 Exfoliation Syndrome.** Using a large case/control sample from Iceland, a genome-wide association study identified *lysyl oxidase-like 1* (*LOXL1*) as a major genetic risk factor for exfoliation syndrome (102), with a population-attributable risk of more than 99%. This result has been replicated in populations throughout the

world (29). *LOXL1* risk alleles are present in the majority of cases, but are also highly prevalent in controls, indicating that *LOXL1* genetic risk factors are necessary but not sufficient for the disease, and that other genetic and/or environmental factors are required for disease development (103). Recent studies suggest that the *LOXL1* genetic variants associated with disease cause decreased gene expression (104). *LOXL1* is a member of the lysyl oxidase family of proteins that catalyze the polymerization of tropoelastin to form the mature elastin polymer (105) and contribute to the spatial organization of elastogenesis.

**135.5.1.2 Adult Onset Primary Open Angle Glaucoma (POAG).** Recently, several genome-wide association studies for POAG have been completed. Using 1263 cases and over 34,877 controls from Iceland, DNA sequence variants in the *CAV1/CAV2* gene region associated with POAG (106), and this association has been replicated in a study of Caucasian cases and controls from the US (GLAUGEN) (107). A study of 590 advanced glaucoma cases and 3956 controls from Australia identified association between the SNPs located in the genomic regions containing the *CDKN2BAS* (previously associated with CDR) and *TMCO1* genes and POAG (108,109). These genes only account for a small proportion of POAG in the general population suggesting that additional POAG genes await discovery (110).

**135.5.1.3 Primary Angle-Closure Glaucoma.** Primary angle-closure glaucoma has a significant heritability, and the ocular quantitative traits related to angle-closure glaucoma, in particular axial length, are also highly heritable (38). These observations suggest that genetic factors could contribute to the development of this form of glaucoma, but genes have not yet been associated with common forms of this condition (111).

## 135.6 CONCLUSION

During the past decade, a number of new genes associated with glaucoma have been identified and future studies will likely identify many more. The identification of glaucoma genes will be the first step toward the development gene-based screening and diagnostic tests allowing for the identification of individuals at risk for disease before irreversible blindness occurs. The identification of genes contributing to glaucoma will also provide new insight into the molecular events underlying the disease pathophysiology suggesting novel therapeutic approaches. In particular genes that predispose to optic nerve disease in glaucoma could identify new targets for neuroprotective therapies that may delay or even prevent blindness associated with glaucoma.

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None.

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## Biography



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# CHAPTER

# 136

## Defects of the Cornea

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### GLOSSARY

**Corneal dystrophy** – With exceptions, a group of inherited corneal diseases that are typically autosomal dominant, are not usually associated with other ocular or systemic abnormalities, are bilateral, commonly involve the central cornea, and progress with advancing age.

**Glaucoma** – A group of diseases that have in common a characteristic optic neuropathy with associated visual field loss for which elevated intraocular pressure is one of the primary risk factors.

**Keratoconus** – A condition of conical protrusion of the cornea associated with central stromal thinning and irregularity of the corneal surface, as well as anterior stromal scarring and deep stromal fine striations. The abnormal shape can cause visual distortions.

**Laser-assisted in situ keratomileusis (LASIK)** – A surgery that uses an excimer laser to reshape the cornea of the eye in order to change focusing power and correct vision.

**Nystagmus** – An involuntary, rhythmic oscillation of the eyes.

**Penetrating keratoplasty** – Replacement of the host cornea with surgical transplantation from a donor eye. This term is now commonly used to refer to a full thickness corneal transplantation, whereas lamellar keratoplasty is used to refer to a partial thickness corneal transplantation.

**Phototherapeutic keratectomy (PTK)** – A surgery that uses an excimer laser to treat various ocular disorders by removing tissue from the cornea. It allows the removal of superficial corneal opacities and surface irregularities. The goal is to produce a smoother and clearer cornea, and not necessarily to reduce dependency upon eyeglasses or contact lenses.

**Strabismus** – A visual disorder where the eyes are misaligned and point in different directions. This can be caused by abnormalities in binocular vision or by anomalies of neuromuscular control of ocular motility. The misalignment may be intermittent or constantly present.

### 136.1 INTRODUCTION

Defects of the cornea can play an important role in clinical genetics, because of the relative ease with which abnormalities can often be detected using simple instruments such as a hand light or the ophthalmoscope. More subtle changes can be detected on examination using the slit lamp biomicroscope or other non-invasive imaging techniques allowing rapid screening, phenotypic categorization and correlation with the molecular genetic findings.

The following discussion reviews first the corneal dystrophies, followed by corneal changes associated with inherited systemic disorders, and finally isolated congenital corneal anomalies.

### 136.2 DYSTROPHIES

#### 136.2.1 Definition

As a group, the corneal dystrophies tend to be autosomal dominant in inheritance, are not usually associated with other ocular or systemic abnormalities, are bilateral, commonly involve the central cornea, and progress with advancing age. Some changes are usually present in the first few decades of life. Exceptions to all of these generalizations exist and are discussed as the specific entities are reviewed. On examination, these diseases are often limited to a single layer of the cornea, and are traditionally discussed in groups according to their layer of corneal involvement. Significant advances in the understanding of molecular genetic defects in many of these disorders allow for better grouping and classification (1,2).

These advances in genetic analysis have made it difficult to maintain the historical dystrophy classification system. In 2005, the International Committee for Classification of Corneal Dystrophies (IC3D) was formed to revise the nomenclature and include information regarding genetic analysis, histopathology, and phenotypic



description (Tables 136-1) (3). Their classification system continues to organize corneal dystrophies anatomically by the corneal layer that is chiefly affected. Depending on the evidence available to support its existence, each dystrophy is assigned to a category as follows:

- Category 1: A well-defined corneal dystrophy in which the gene has been mapped and identified and specific mutations are known.
- Category 2: A well-defined corneal dystrophy that has been mapped to one or more specific chromosomal loci, but the gene(s) remains to be identified.
- Category 3: A well-defined corneal dystrophy in which the disorder has not yet been mapped to a chromosomal locus.
- Category 4: This category is reserved for a suspected new, or previously documented, corneal dystrophy, although the evidence for it being a distinct entity is not yet convincing.

### 136.2.2 Epithelial and Subepithelial Dystrophies

Epithelial dystrophies affect the corneal epithelium and its basement membrane, most often causing painful recurrent epithelial erosions. Subepithelial erosions cause decreased vision and can also be accompanied by painful erosions.

Epithelial basement membrane dystrophy (including map-dot-fingerprint dystrophy, recurrent erosive corneal dystrophy, Cogan microcystic dystrophy) (a minority of cases are IC3D Category 1).

The hallmarks of epithelial basement membrane dystrophy (EBMD) are cysts and putty-like opacities in the corneal epithelium with irregular gray lines in a map-like configuration and/or fingerprint-like ridges in the deep epithelium (Figure 136-1). Often asymptomatic, they may still produce symptoms by making the corneal epithelial surface irregular and thus blurring vision. More commonly, they cause spontaneous erosion or sloughing of the epithelium, leading to sudden severe pain. Characteristically, affected patients report being awakened from their sleep by severe ocular pain and foreign body sensation. Examination at this time reveals a defect in the corneal epithelium that is often surrounded by loose, redundant epithelium, which may heal spontaneously over several hours. Onset of the disorder can be at any age but most commonly is after the second or third decade. Symptoms may occur frequently for months to years and then abate completely. Usually, no long-term visual difficulties occur and no scarring takes place unless secondary infection occurs during an erosive episode. The major differential diagnosis is between this disorder and a secondary form that occurs as an early sign of corneal edema from other causes. It may also be confused with Meesmann dystrophy. The disorder appears to be due to altered production of epithelial basement

membrane with multilaminated and irregular as well as abnormally positioned basement membrane and fibrillogranular material. Normal maturation of epithelium is prevented by this material, and the cells degenerate and form the visible cysts. Adherence of epithelium to this material is poor, leading to the recurrent erosions. Treatment consists of lubrication and antibiotic prophylaxis during acute episodes and the use of hypertonic ointments at night, which reduces the frequency of spontaneous erosions. Patching may be helpful, but many patients report increased discomfort when patched acutely. Pain relief can be achieved with topical non-steroidal anti-inflammatory drugs (NSAIDs). When surface irregularity reduces vision, the entire epithelium and abnormal basement membrane can be debrided to allow replacement by fresh epithelium. Soft contact lenses worn continuously may provide comfort to patients troubled with frequent erosions. Anterior stromal puncture of the cornea in areas not in the visual axis allows firmer adhesion of the corneal epithelium to the underlying layers, reducing the likelihood of recurrent erosions. Finally, excimer laser phototherapeutic keratectomy (PTK) has been shown to significantly decrease recurrences and improve vision. The occurrence of this disorder in the general population is quite frequent (4) and may represent a degeneration. An autosomal dominant inheritance with incomplete penetrance is suggested, with two studies identifying different point mutations in the TGFBI/BIGH3 gene in a subset of patients with EBMD (5,6). Per the IC3D classification system, most cases of EBMD are sporadic and may be degenerative with a minority of cases being Category 1.

**136.2.2.1 Epithelial Recurrent Erosion Dystrophy (Including Recurrent Hereditary Corneal Erosions) (IC3D Category 3).** This autosomal dominant dystrophy may appear at the age of six months, but is more typically noted around four to six years of age. It is characterized by recurrent corneal erosions in the absence of associated diseases and can be precipitated by minimal trauma, smoke, dust, or exposure to sunlight. The intensity and frequency of the recurrent attacks decline with time and tend to cease by the age of 50. Subepithelial haze or blebs may be seen with the erosions. Additionally, subepithelial opacities can be seen, which vary in appearance from fibrosis to protruding nodules consisting of keloid material based on Congo red staining. The morphological pattern (including an absent Bowman layer, progressive subepithelial fibrosis, abnormal sub-basal corneal nerves) likely reflects the healing response to recurrent epithelial erosions. Medical treatment concentrates on healing the epithelial defect and addressing the loosely adherent epithelium. Cycloplegics, topical antibiotics, lubricating ointments at bedtime, pressure patch, hypertonic saline, and bandage contact lenses can all play a role. The gene and chromosomal locus for this disorder is unknown and it is not genetically linked to any clinically similar corneal dystrophy with autosomal dominant inheritance (3,7–10).

**TABLE 136-1 Classification of Corneal Dystrophies in the International Committee for Classification of Corneal Dystrophies (IC3D)<sup>b</sup>**

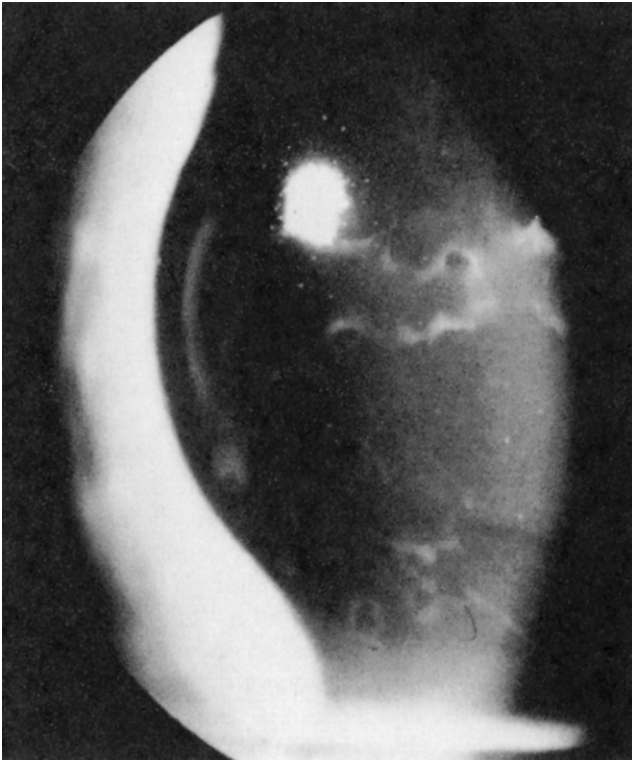
Dystrophy	MIM #	Inheritance	Gene Locus	Gene	IC3D Category
Epithelial basement membrane dystrophy	121820	Sporadic or AD	5q31	TGFBI in non-sporadic cases	1 in minority of cases
Epithelial recurrent erosion dystrophy	122400	AD	Unknown	Unknown	3
Meesmann corneal dystrophy	122100	AD	12q13 17q12	KRT3 KRT12	1 1
Lisch epithelial corneal dystrophy	None	X-linked dominant	Xp22.3	Unknown	2
Gelatinous drop-like corneal dystrophy	204870	AR	1p32	TACSTD2	1
Reis-Bucklers corneal dystrophy	608470	AD	5q31	TGFBI	1
Thiel-Behnke corneal dystrophy	602082	AD	5q31 10q24	TGFBI Unknown	1 2
Lattice corneal dystrophy type 1 and variants	122200	AD	5q31	TGFBI	1
Lattice corneal dystrophy type 2	105120	AD	9q34	GSN	1
Granular corneal dystrophy type 1	121900	AD	5q31	TGFBI	1
Granular corneal dystrophy type 2	607541	AD	5q31	TGFBI	1
Macular corneal dystrophy	217800	AR	16q22	CHST6	1
Schnyder corneal dystrophy	121800	AD	1p36	UBIAD1	1
Congenital stromal corneal dystrophy	610048	AD	12q21	DCN	1
Fleck corneal dystrophy	121850	AD	2q35	PIP5K3	1
Posterior amorphous corneal dystrophy	None	AD	12q21.33 <sup>c</sup>	Unknown	3 <sup>a</sup>
Central cloudy dystrophy of Francois	217600	Unknown	Unknown	Unknown	4
Pre-Descemet corneal dystrophy	None	Unknown	Unknown	Unknown	4
Fuchs endothelial corneal dystrophy	136800	Sporadic or AD	Early-onset: 1p34.3 Late-onset: 13pTel-13q12.13, 15q, 18q21.2-q21.32	Early-onset: COL8A2 Late-onset: unknown	Sporadic: 3 Late-onset: 2 Early-onset: 1
Posterior polymorphous corneal dystrophy	Type 1: 122000 Type 2: 609140 Type 3: 609141	AD	Type 1: 20p11.2-q11.2 Type 2: 1p34.3-p32.3 Type 3: 10p11.2	Type 1: unknown Type 2: COL8A2 Type 3: ZEB1 (TCF8)	Type 1: 2 Type 2: 1 Type 3: 1
Congenital hereditary endothelial dystrophy	Type 1: 121700 Type 2: 217700	Type 1: AD Type 2: AR	Type 1: 20p11.2-q11.2 Type 2: 20p13	Type 1: Unknown Type 2: SLC4A11	Type 1: 2 Type 2: 1
X-linked endothelial corneal dystrophy	None	X-linked dominant	Xq25	Unknown	2

MIM, Mendelian Inheritance in Man.

AD, autosomal dominant.

AR, autosomal recessive.

Unless otherwise noted, information is obtained from Weiss et al.<sup>b</sup><sup>a</sup>Based on the data from Aldave et al.,<sup>c</sup> the IC3D category would be updated to 2 from 3.<sup>b</sup>Weiss, J. S.; Moller, H. U.; Lisch, W.; Kinoshita, S.; Aldave, A. J.; Belin, M. W.; Kivela, T.; Busin, M.; Munier, F. L.; Seitz, B., et al. The IC3D Classification of the Corneal Dystrophies. *Cornea* 2008, 27 (Suppl 2), S1–83.<sup>c</sup>Aldave, A. J.; Rosenwasser, G. O.; Yellore, V. S.; Papp, J. C.; Sobel, E. M.; Pham, M. N.; Chen, M. C.; Dandekar, S.; Sripracha, R.; Rayner, S. A., et al. Linkage of Posterior Amorphous Corneal Dystrophy to Chromosome 12q21.33 and Exclusion of Coding Region Mutations in KERA, LUM, DCN, and EPYC. *Invest. Ophthalmol. Vis. Sci.* 51 (8), 4006–4012.



**FIGURE 136-1** Epithelial Basement Membrane Dystrophy. The gray lines and rings represent reduplications of epithelial basement membrane.

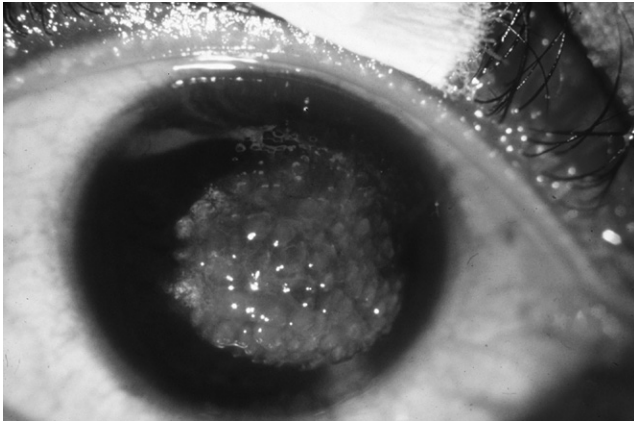
**136.2.2.2 Meesmann Corneal Dystrophy (Including Juvenile Epithelial Corneal Dystrophy and Stocker–Holt Dystrophy) (IC3D Category 1).** Meesmann corneal dystrophy (MECD) consists of multiple, small, round, clear cysts within the corneal epithelium, most prominently in the interpalpebral fissure. Most patients are asymptomatic, but some complain of mild recurrent ocular discomfort, presumably due to rupture of the cysts. Symptoms may occur very early in life but are usually milder than those seen in EBMD. Rarely is vision decreased and corneal sensation may be reduced. Treatment is the same as for EBMD, but aggressive therapy is less often required. Soft contact lens wear can improve comfort and lead to marked attenuation of the corneal cysts. MECD persists throughout life and will recur in regenerated epithelium if the pathologic epithelium is removed (10). The epithelium is disorganized with irregular cell sizes and the formation of cysts within the epithelium that may contain PAS-positive cellular degenerative material. A ‘peculiar substance’ of intracellular fibrogranular material is characteristically seen on electron microscopy of the epithelium. The inclusions in the epithelial cytoplasm have been identified as keratin aggregates, exhibiting irregular clumping of intermediate tonofilaments on electron microscopy. These findings are noted to be similar to other systemic keratin disorders where critical mutations disrupt normal intermediate

filament architecture. The keratins expressed in anterior corneal epithelium are K3 and K12. Both mutations in the keratin 3 (KRT3) gene at 12q13 (11,12) and various mutations in the keratin 12 (KRT12) gene at 17q12 have been described across numerous ethnicities (11,13–17). The mutations in KRT3 and KRT12 appear in the alpha helix rod domain. The numerous KRT12 mutations are in the highly conserved helix initiation and helix termination motifs and the 3 known KRT3 mutations are in the helix initiation motif. Mutations in these motifs can result in structurally fragile corneal epithelial cells that result in the clinical picture seen in this disorder. Autosomal dominant inheritance is well established.

Stocker-Holt corneal dystrophy bears similarities to Meesmann dystrophy in exhibiting a subtle, closely-packed, microcystic epithelium. Findings are more prominent in the interpalpebral zone and cysts may be clear or gray. Patients are usually mildly symptomatic with mild foreign body sensation and reasonable preservation of vision. Histopathology lacks the peculiar substance characteristic of Meesmann dystrophy. A mutation in the KRT12 gene (R19I) has been noted in the original family described (1).

**136.2.2.3 Lisch Epithelial Corneal Dystrophy (IC3D Category 2).** Lisch epithelial corneal dystrophy is characterized by gray bands of epithelial change that appear to be made up of microcysts. The opacities can also display other patterns including whorl-like, flame and feather shaped, radial, and club shaped. Patients complain of decreased vision. Pathological evaluation shows cytoplasmic vacuolization in the corneal epithelium. Treatment consists of corneal epithelial debridement, and there appears to be some benefit from wearing rigid gas-permeable contact lenses. Analysis of the original family indicates inheritance to be X-linked dominant, localizing to Xp22.3 and is distinct from K3 and K12 seen in Meesmann corneal dystrophy (18).

**136.2.2.4 Gelatinous Drop-like Corneal Dystrophy (Familial Subepithelial Corneal Amyloidosis, Subepithelial Amyloidosis, and Primary Familial Corneal Amyloidosis) (IC3D Category 1).** Gelatinous drop-like dystrophy is a form of corneal amyloidosis in which dense droplet-like amyloid deposits accumulate beneath the corneal epithelium during the first decade of life, assuming a ‘mulberry’ shape on the corneal surface (Figure 136-2). The disorder may take various forms, including band keratopathy (19). With time, stromal opacification and fusiform deposits can develop. Superficial vascularization can be seen. This disorder may cause significant visual disability, pain, photophobia, redness, tearing, and foreign body sensation. The deposits and stromal opacities are progressive and difficult to treat. Penetrating keratoplasty is usually unsuccessful because the recipient bed continues to be affected. A novel approach in transplanting



**FIGURE 136-2** Gelatinous Drop-like Dystrophy. Characteristic 'mulberry' appearance seen in the superficial cornea. Lattice corneal dystrophy. Typical ropy lattice lines are evident.

limbal corneal epithelial cells has met with short and intermediate term success (20). This implies that the disorder is at least in part of epithelial origin and is a promising new modality. The disorder is decidedly uncommon in the western hemisphere but is common in Asia. Inheritance is autosomal recessive. Mutations in the TACSTD2 (formerly M1S1) gene at 1p32 cause this disorder; over 20 distinct mutations have been discovered with the Q118X mutation being detected most often (3,10,21,22). Genetic heterogeneity may also be present, as there are reports of individuals with gelatinous drop-like dystrophy who were found to not have mutation in TACSTD2 (10,23).

### 136.2.3 Anterior Membrane Dystrophies

Anterior membrane dystrophies may present with recurrent erosions and epithelial changes but, in addition, demonstrate scarring at the level of Bowman layer – the 'anterior membrane' of the corneal stroma. These disorders have been characterized by their anatomical location. The recent finding of defects in the gene for keratoepithelin, a protein found in cornea, skin, and connective tissue, has led to the closer grouping of the anterior membrane and stromal dystrophies. In the cornea, keratoepithelin is found in greatest concentration in the Bowman layer, the anterior compacted portion of the corneal stroma, as well as in the deeper stroma and Descemet membrane. Ultrastructurally, some is present in the epithelial basement membrane as well (24). The gene for keratoepithelin is known as BIGH3 or TGFBI because transforming growth factor beta can induce its expression. Varying defects in this gene, especially the mutational hotspot codons Arg124 and Arg555, have been associated with some of the anterior membrane dystrophies as well as with granular and most forms of lattice corneal stromal dystrophies (25). Some defects may also be associated with EBMD, as described earlier (Tables 136-2).

**TABLE 136-2** Mutations of the Keratoepithelin Gene Described in Corneal Dystrophies<sup>a</sup>

Dystrophy	Gene Defect
Epithelial basement membrane dystrophy	Leu509Arg, Arg666Ser, Leu559Val <sup>b</sup>
Reis-Bücklers corneal dystrophy	Arg124Leu
Thiel-Behnke corneal dystrophy	Arg555Gln
Lattice corneal dystrophy, type 1	Arg124Cys
Lattice corneal dystrophy – variants	Over 30 exist, please refer to IC3D Classification for full list
Granular corneal dystrophy type 1	Arg555Trp
Granular corneal dystrophy type 2	Arg124His
Granular corneal dystrophy – variants	Val113Ile, Asp123His, Arg124Ser, Arg124Leu + Thr125_Glu-126del

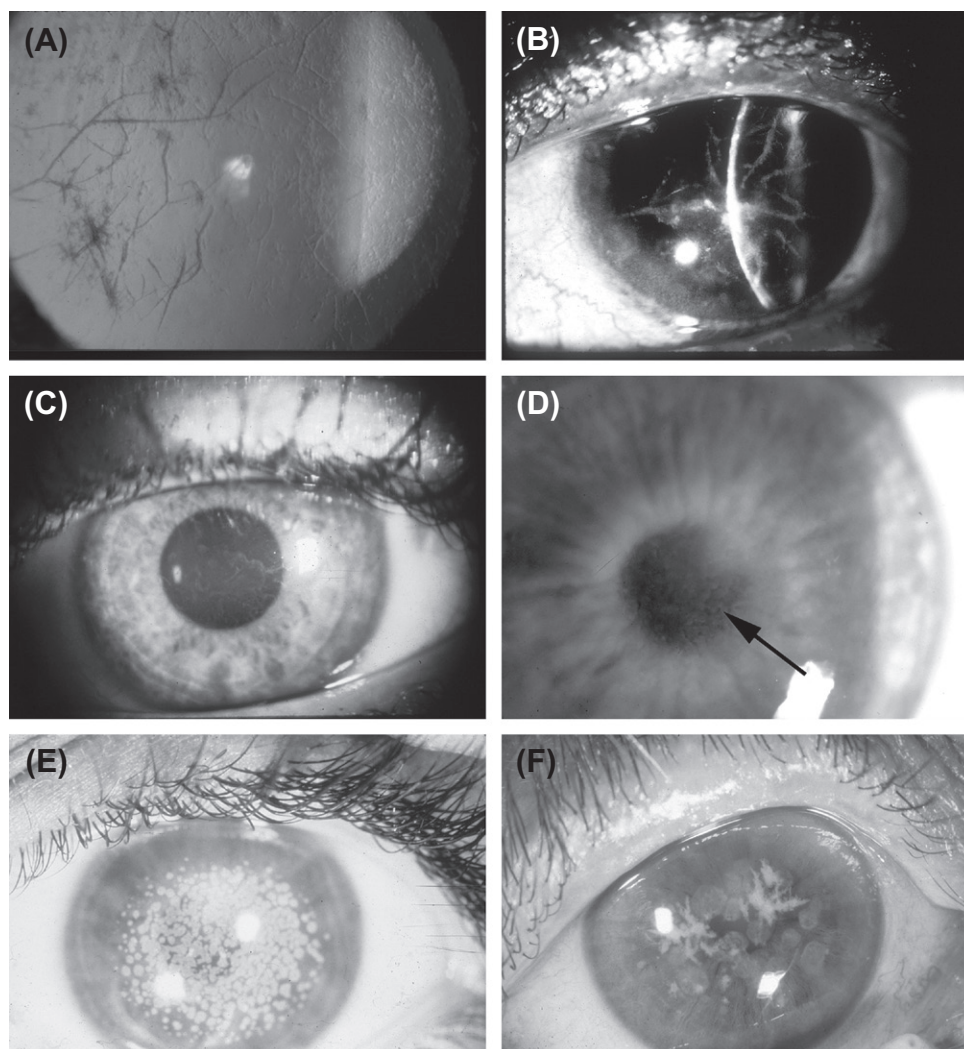
Unless otherwise noted, information is obtained from Weiss et al.<sup>a</sup>

<sup>a</sup>Weiss, J.S.; Moller, H. U.; Lisch, W.; Kinoshita, S.; Aldave, A. J.; Belin, M. W.; Kivela, T.; Busin, M.; Munier, F. L., et al. The IC3D Classification of the Corneal Dystrophies. *Cornea* 2008, 27 (Suppl 2), S1–83.

<sup>b</sup>Paliwal, P.; Sharma, A.; Tandon, R.; Sharma, N.; Titiyal, J. S.; Sen, S.; Kaur, P.; Dube, D.; Vajpayee, R. B. TGFBI Mutation Screening and Genotype–Phenotype Correlation in North Indian Patients with Corneal Dystrophies. *Mol. Vis* 16, 1429–1438.

**136.2.3.1 Reis–Bücklers Dystrophy (Including Granular Corneal Dystrophy Type III, Corneal Dystrophy of Bowman Layer Type I-CDBI, Superficial Granular Corneal Dystrophy, Geographic Corneal Dystrophy (Weidle), Atypical Granular Corneal Dystrophy, Anterior Limiting Membrane Dystrophy, Type I (ALMD I)) (IC3D Category 1).** Reis–Bücklers dystrophy (RBCD) is characterized by irregular gray-white reticular changes in the deep corneal epithelium and anterior stroma (Figure 136-3). These changes are evident in the first few years of life and are associated with painful recurrent corneal epithelial erosions, photophobia, and redness of the eyes. The episodes occur every few months for the first one to two decades of life but then become less frequent and often disappear by the age of 20–30 years. As time goes on, however, ring and net-like opacification and epithelial irregularity increase, causing decreased visual acuity. Corneal sensitivity to pain decreases as well. Treatment consists of patching and/or lubrication for the acute erosive episodes and mechanical dissection of the subepithelial and anterior stromal fibrous tissue later in life when visual acuity is significantly decreased. Alternatively, excimer PTK has been successful in reducing the incidence of recurrent erosions and improving visual acuity, but recurrence is common (26). The addition of adjuvant topical





**FIGURE 136-3** Dystrophies Associated with Defects in TGFBI. (A) Type I lattice corneal dystrophy exhibiting thin branching lines. (B) Lattice corneal dystrophy variant (formerly Type III lattice corneal dystrophy). Typical thicker, ropy lattice lines are evident. (C) Reis-Bücklers corneal dystrophy. Epithelial and anterior stromal changes lead to surface irregularity and recurrent erosion. (D) Thiel-Behnke corneal dystrophy. Note the more uniform 'honeycomb' configuration of the superficial opacities (arrow). (E) Granular corneal dystrophy type I. Note the clarity of the cornea between opacities and central location. (F) Granular corneal dystrophy type II. Characteristic snowflakes and discs seen in granular dystrophy with branching, stellate lines of lattice dystrophy.

mitomycin-C may be of benefit in reducing recurrence (27). A potential treatment for young patients may be corneal electrolysis. A group performed this procedure under topical anesthesia on an 11-year-old male with subepithelial opacities. It resulted in significantly improved vision eight months later, but further follow-up was not reported (28). Occasionally, scarring will be sufficient to warrant penetrating keratoplasty, although recurrence has been seen in some grafts. Pathological examination reveals irregularity of the epithelium with focal absence of epithelial basement membrane and disruption of basal epithelial cells and their attachments. Bowman layer, the anterior compacted portion of the corneal stroma, is diffusely disrupted and replaced with fibrous connective tissue with deposits staining for Masson trichrome that

projects into the subepithelial zone. Accumulation of keratoepithelin products have been shown between the epithelium and Bowman layer (29,30), and they are seen as characteristic rod-shaped bodies on electron microscopy that are immunopositive for keratoepithelin (24). This disorder is autosomal dominant in inheritance and caused by an Arg124Leu mutation in the TGFBI gene.

There have been reports suggesting that another TGFBI mutation, Gly623Asp, is causative of RBCD based on clinical examination, but histopathological exam was either not conducted for confirmation (31,32) or was inconsistent with the lack of Masson trichome staining (33). Other patients reported to have the Gly623Asp mutation had clinical phenotypes that were not similar to RBCD (34,35) and both

histopathological examination and electron microscopy on one of those patients was not compatible with RBCD (35), which suggest that this mutation could be causing a novel corneal dystrophy. Currently, the IC3D classifies this mutation as variant lattice corneal dystrophy (3). Another suspected mutation, a F540 deletion, for RBCD also lacks histopathological confirmation and is classified as variant lattice corneal dystrophy (3,10). Another study of a family with a clinical picture of RBCD showed an Arg124Cys mutation (which is normally associated with lattice corneal dystrophy) but also lacked histopathological confirmation (36).

**136.2.3.2 Thiel–Behnke Corneal Dystrophy (Including Corneal Dystrophy of Bowman Layer Type II-CDBII, Honeycomb-shaped Corneal Dystrophy, Anterior Limiting Membrane Dystrophy, Type II, Curly Fibers Corneal Dystrophy, Waardenburg–Jonkers Corneal Dystrophy) (IC3D Category 1, Potential Variant Is Category 2).** Thiel and Behnke described a similar clinical disorder in 1967 with honeycomb-like changes beneath the corneal epithelium (Figure 136-3), which is later in onset than Reis–Bücklers and has normal corneal sensation. The peripheral cornea is usually uninvolved. Vision gradually becomes impaired. Treatment is similar to Reis–Bücklers with good success (37). Thiel–Behnke corneal dystrophy (TBCD) is also known as curly fiber dystrophy, because characteristic electron dense ‘curly filaments’ replace Bowman layer. Clinical differentiation of RBCD from Thiel–Behnke is often difficult, making histological confirmation critical in assigning mutational defects. The curly fibers are immunopositive for keratoepithelin and pathognomonic in distinguishing this disorder from RBCD. While not considered to be definitive from a diagnostic standpoint, confocal microscopy has been used to distinguish TBCD from RBCD (38) and can be a useful tool when tissue is not available for histopathological analysis. TBCD has been mapped to TGFBI and it is thought that Arg555Gln may be specific for this disorder and that the two disorders are genotypically distinct (25,39,40). Some have diagnosed TBCD by clinical phenotype and the Arg555Gln mutation alone without obtaining ultrastructural information needed for confirmation. Genetic heterogeneity may exist, as some families with Thiel–Behnke have been linked to a defect at 10q24; the gene is unknown.

**136.2.3.3 Grayson–Wilbrandt Corneal Dystrophy (IC3D Category 4).** In 1966, Grayson and Wilbrandt described an autosomal dominant disorder in a single family that was similar to Reis–Bücklers dystrophy, with slightly later onset, less epithelial irregularity, less severe recurrent erosions and normal corneal sensation. Histopathologically, there is a homogenous eosin-staining material in between the epithelium and the Bowman layer. It stains with Periodic acid-Schiff, but not Alcian blue or Masson trichrome.

## 136.2.4 Band Keratopathy

Although quite rare, patients with primary band keratopathy have been reported. In its juvenile form, calcification presents early in life with whitish opacification in the interpalpebral region of the cornea, beginning at the limbus and progressing centrally. Sufficient central progression may reduce vision and fragmentation of the calcium may cause epithelial disruption and discomfort. In time, secondary corneal scarring and vascularization may occur. The adult form presents in a similar fashion late in life. Treatment consists of debridement and chelation of the calcium, with a combination of mechanical scraping and soaking with disodium EDTA. Excimer PTK is also an alternative as it is in most anterior stromal dystrophies. Differential diagnosis includes the numerous secondary causes of band keratopathy, including chronic ocular disease (e.g., uveitis, interstitial keratitis, Norrie disease), trauma, hypercalcemia, hypophosphatasia, and renal failure. In all forms of band keratopathy, calcium is deposited in Bowman layer of the cornea. Predisposition for the central cornea may relate to pH changes and evaporation from the tear film in the interpalpebral zone. Primary band keratopathy appears to be autosomal recessive in inheritance.

## 136.2.5 Stromal Dystrophies

Stromal dystrophies are highlighted by the accumulation of opaque substances in the corneal stroma, either within the keratocytes or between the stromal collagen fibers.

**136.2.5.1 Lattice Corneal Dystrophy.** Lattice dystrophy (LCD) has been the term used to describe a number of entities in which linear accumulations of amyloid are found in the corneal stroma. Types I, II, III, IIIA, and IV have previously been described. The IC3D classification system has reorganized lattice dystrophy as type 1, LCD variants (which includes former types III, IIIA, and IV), and type 2 (3).

**136.2.5.1.1 Lattice Corneal Dystrophy Type I (Including Classic LCD, LCD Type I, Biber–Haab–Dimmer) (IC3D Category 1).** In lattice dystrophy (LCD) type I refractile short rod-like lines, dense branchingropy lines, and smaller dot-like opacities are present in the corneal stroma at all levels (Figure 136-3). The corneal periphery is usually clear, and although a central haze may be present anteriorly in the stroma, most of the stroma between the opacities is clear. Anterior opacities may extend beneath the epithelium and lead to epithelial irregularity and corneal erosion. The corneal opacities become evident in the first and second decades of life and progress in extent and density. The earliest symptoms are usually due to recurrent epithelial erosions and blurring of vision caused by epithelial irregularity. As the central haze progresses in the fourth and fifth decades of life, visual acuity may decline further. Early therapy is directed to the recurrent erosions, whereas soft

contact lenses may improve acuity reduced by corneal surface irregularity. With more advanced opacification, penetrating keratoplasty successfully restores vision, although opacification may ultimately recur in the graft. Excimer laser ablation of superficial opacities may help some patients defer the need for keratoplasty (41). Moderate variability in degree of involvement exists, and unilateral cases have been reported (42,43). The major differential confusion comes from granular and macular dystrophies, which are discussed later. Histopathologically, amyloid deposits are demonstrated in the stroma in fusiform clumps that are most concentrated anteriorly. Normal stromal collagen lies adjacent to it. Inheritance of lattice dystrophy type I is autosomal dominant. The majority of reported cases indicate a R124C mutation (25,44) resulting in an accumulation of the N-terminal portion of the TGFBI (30). Phenotypic variation with the R124C mutation has been seen, with one group having reported five patients with features of both gelatinous drop-like dystrophy and LCD type I (45). Two studies reported patients who had clinical and histopathological features resembling Avellino corneal dystrophy (46,47). As mentioned previously, a group reported a family with a clinical picture of RBCD without histopathological confirmation (36). It is not yet known how this phenotypic diversity with the same mutation occurs, but one can speculate that other processes are involved, such as environmental influences or other corneal-specific genes that have not yet been elucidated.

**136.2.5.1.2 Lattice Corneal Dystrophy Variants.** Over 30 variants of LCD have been discovered. While there is phenotypic variability, almost all of these mutations lie in the Fas-1 domain 4 of TGFBI (the Arg-124Cys mutation is in domain 1). The mutations in this domain include conserved sequences and may affect protein structure leading to phenotypic abnormalities (48). This includes mutations outside of the 124 codon that result in LCD, type I such as Leu518Pro, Leu569Arg, Val539Asp, Val505Asp, and Ile522Asn (49–53).

In 1987, Hida et al. described the first cases of lattice dystrophy type III, which is now grouped under LCD variants. It is not associated with corneal erosions and has onset after age 40. The corneal lines are much thicker than in LCD type I (Figure 136-3). Unilateral cases have been reported. This disorder appears to be autosomal recessive. The localization of the gene is not conclusive, but an L527R mutation in TGFBI has been seen in cases of lattice dystrophy with histological features similar to LCD type III (54,55). One group reported a homozygous Val624Met mutation in 2 brothers, one of whom had a unilateral presentation similar to LCD type III and the other mild bilateral stromal opacities. The proband's daughter was heterozygous for the mutation and had no reported ophthalmic abnormalities (56).

Patients with findings similar to lattice type III, but with recurrent corneal erosion and dominant transmission, have been described as type IIIA, which is also now

classified under LCD variants. In Japan, a Pro501Thr mutation in TGFBI has been found in this disorder (57). Clinical LCD type IIIA has also been described with an Ala546Thr mutation (58), a deletion/frameshift mutation at Val627, Asn622Lys mutation (25) and a Phe-540Ser mutation (59).

Also classified under LCD variants is type IV lattice dystrophy. It is characterized by late onset of deep stromal opacities without corneal erosions and has been described with a Leu527Arg mutation (49,60) that has been suggested to be a founder mutation in a single Japanese ancestor (61). An atypical form of this variant has also been mapped to Asn544Ser (62), Val631Asp (25), and Gly594Val (51). One group reports a patient with a clinical picture of LCD type 4 as having an Arg496Trp mutation. This mutation is unique in comparison to the others by being located in the third Fas domain instead of the fourth (63).

LCD variants also include phenotypes with features of both type I and type IIIA. Schmitt-Bernard et al. (2000) have described such an entity, with late onset, asymmetric progression with lattice lines thicker than type 1, but thinner than type IIIA (64). This was associated with an Asn-Val-Pro629-30 insertional mutation (65). A similar intermediate dystrophy has been mapped to an H626R mutation in TGFBI in both Caucasian (40) and Asian families (66). Other mutations that result in a similar clinical picture include Asn622His (24), Thr538Arg, Leu518Arg, and His626Pro (25). As mentioned previously, Gly623Asp and a F540 deletion had been thought to be a cause of RBCD, but it is currently viewed as a LCD variant within the IC3D based on clinical diagnoses (25).

Varied phenotypes have been reported with the Ala546Asp mutation. Eifrig et al. reported on a family that had a clinical picture of polymorphic stromal opacities without a distinct lattice pattern and no corneal erosions. On histological analysis, amyloid was seen within the deep stroma. The authors designated this disorder as polymorphic corneal amyloidosis (67). Other studies have shown that such a designation may be too specific. Correa-Gomez et al. reported on another family with the same mutation and, while there were similarities in the morphology of the corneal opacities and amyloid deposition, there was a more distinct lattice pattern on clinical exam (68). Two groups reported families that had an Ala546Asp mutation along with a Pro551Gln mutation. One family was recognized to have a phenotypic LCD type I picture (69) while the other family's affected members had lattice lines with polymorphic stromal opacities and amyloid deposition (70). While the Ala546Asp mutation can result in different clinical findings, the P551Q mutation would also appear to have a role in modifying phenotype. Genetic heterogeneity with polymorphic corneal amyloidosis is also suggested by reports of a case with a F547S mutation (71) and cases without a pathogenic TGFBI mutation (72).



**136.2.5.1.3 Lattice Corneal Dystrophy Type II, Gelsolin Type (Familial Amyloid Polyneuropathy Type IV – Finnish Type, Meretoja Syndrome, Amyloidosis V) (IC3D Category 1).** Another form of lattice corneal change (LCD type II) has been noted in patients with familial amyloid polyneuropathy type IV (Finnish type, Meretoja syndrome). The opacities occur later in life, do not leave a clear periphery to the cornea, and there are fewer lattice lines and markedly fewer dot-like opacities. The lattice lines are more radially oriented compared to LCD type I. The central cornea is less severely involved, so that visual disturbance is less common and occurs later (in the sixth decade). Corneal sensitivity is decreased. After the age of 40, facial paresis and dermatochalasis are exceedingly frequent. Histopathology in these patients demonstrates amyloid, as does lattice dystrophy, with occasional suggestion of perineural distribution. The amyloid deposits are seen in the cornea, lacrimal gland, and scleral, choroidal, and adnexal blood vessels. A mutation in the gelsolin gene (GSN; 9q34) had been demonstrated in these families and a mutated 71 amino acid fragment of gelsolin makes up the amyloid seen in this disorder. A G654A missense mutation (resulting in Asp187Asn) has been found in Finnish, Portuguese, American, British, Japanese, Iranian, Spanish, and German families (73–78), while a G654T mutation (resulting in Asp187Tyr) is seen in Danish, Czech, and French families with LCD type 2 (79–81). The resulting accumulation of abnormal amyloidogenic gelsolin is the same with both mutations (74,80).

#### **136.2.5.2 Granular Corneal Dystrophy.**

**136.2.5.2.1 Granular Corneal Dystrophy, Type I (Including Corneal Dystrophy Groenouw Type I) (IC3D Category 1).** Granular corneal dystrophy Type I (GCD1) is characterized by very discrete white opacities in the central anterior corneal stroma. The opacities are moderately large (0.1–0.4 mm in diameter) and may take several shapes, although most commonly they are crumb-like (Figure 136-3). The corneal periphery is spared, and the corneal stroma is generally clear between opacities but may become slightly hazy as opacities coalesce. The opacities occur in the first decade of life but are mostly asymptomatic until later life, when there may be reduction in visual acuity. Corneal erosions are not common. Most patients require no treatment, but those with opacification sufficient to cause visual disability do well with corneal transplantation. Grafts usually do not have recurrence for at least 30 months, but opacities can be seen as soon as within a year at the graft–host interface or in the superficial donor tissue. Corneal electrolysis has been used to remove recurrent deposits after keratoplasty, and while the deposits recur within two to three years, this could delay the necessity of another transplant (82). Debridement or laser ablation (PTK) of the corneal epithelium in patients with early superficial opacities and visual symptoms or corneal erosions has been reported to improve symptoms (83,84). Recurrence

has been reported after PTK and in grafts (85). The use of soft contact lenses (86) and, because of its presumed epithelial origin, limbal stem cell transplants have been advocated (87). The most important clinical differential diagnosis is between this disorder and macular corneal dystrophy, which has a similar corneal appearance but less discrete opacities and markedly greater stromal haze that extends to the corneal periphery. Histological corneal specimens demonstrate aggregation of nodular hyaline material that stains with Masson trichrome throughout the stroma, but is more prominent anteriorly. The material is phospholipid and noncollagenous protein, which closely mimics a portion of TGFBI. Rod-shaped bodies that appear similar to those seen in RBCD are seen on transmission electron microscopy. Inheritance is autosomal dominant. A defect in TGFBI, Arg555Trp, is the most common mutation reported in GCD type I (88,89), but one case of a de novo mutation has been reported (90). Phenotypic variation is possible, with Arg555Trp, as shown with two patients displaying a vortex pattern in the granular deposits (91). Homozygous cases are more severe in appearance and disability and also respond more poorly to therapy (92).

#### **136.2.5.2.2 Granular Corneal Dystrophy, Type II (Including Avellino Corneal Dystrophy, Combined Granular-Lattice Corneal Dystrophy) (IC3D Category 1).**

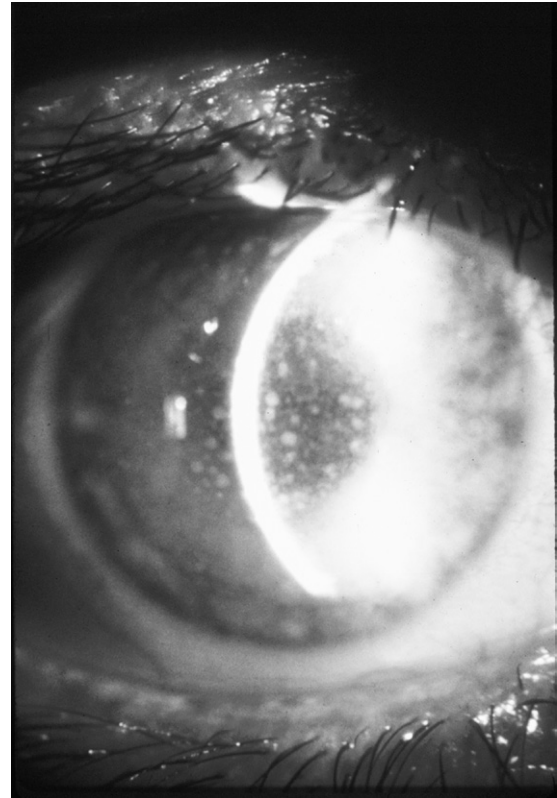
A variant of granular dystrophy with lattice-like amyloid deposits in addition to granular opacities has been described, and is now referred to as granular corneal dystrophy, type II (GCD2). The corneal findings include thin linear opacities similar to LCD type I, but are less prominent. Granular opacities may be more variable in appearance than GCD type I, described as snowflake or stellate. Onset is later than GCD1 and homozygous genotype results in a more severe form of the disease. There has been one report of a Chinese family with reduced penetrance of the GCD2 phenotype, which would suggest that environmental and other genetic factors may have an influence in the disease process (93). Histologically, there are hyaline and amyloid deposits that stain with Masson trichrome and Congo red respectively. Homozygotes have more severe histological findings. As in GCD1, penetrating keratoplasty can be performed, but recurrence can occur. One group published a study in which granular deposits were absent in areas of corneal neovascularization and suggested that identification of the responsible vascular factors might provide another treatment option (94). Refractive surgery is contraindicated in GCD2, since it can become exacerbated with increased corneal opacification (95–100). The first described patients originated from the Italian province of Avellino, which led to the term ‘Avellino dystrophy’. It is also known as combined granular-lattice dystrophy or R124H mutant TGFBI dystrophy. The majority of described pedigrees are, interestingly, from Japan, where it is the most common granular corneal dystrophy (57). GCD2 is also prevalent in Korea, where it is estimated



that 11.5 per 10,000 persons are affected (101). A mutation in the keratoepithelin gene, Arg124His, is virtually always found in this disorder (57,102). Similar to GCD type I, the cornea shows accumulation of TGFBI components, indicating the central role of the R124H mutation (30,103).

**136.2.5.2.3 Granular Dystrophy Type III and Granular Dystrophy Variants.** Granular dystrophy type III is superficial granular or Reis–Bücklers dystrophy, which is described above with the anterior membrane dystrophies. Granular corneal dystrophies have been described with clinical characteristics intermediate between type 1 and type III GCD, as well as exams not typical of either form. One variant has 2 mutations, R124L and a deletion of Thr125 and Glu126 (104), while another has a D123H mutation (89,105). These may represent an additional type of GCD or an intermediate variant. Another variant has a mutation at R124S and appears to be very similar to GCD1 with the exception of having a later onset (25,106). H626R, a mutation that has been classified as a LCD variant in the IC3D, was also reported in a patient with a clinical picture of GCD1 (107). More recently, two sisters in their second decade with a Val-113Ile mutation were reported to have a variant of GCD with anterior and midstromal granular lesions that had more peripheral than central involvement (108).

**136.2.5.3 Macular Corneal Dystrophy (Including Groenouw Corneal Dystrophy Type II, Fehr Spotted Dystrophy) (IC3D Category 1).** Macular corneal dystrophy differs from the preceding two classical corneal dystrophies in that it is autosomal recessive. Corneal opacification consists of diffuse stromal haze as well as focal gray-to-white opacities (Figure 136-4). Initially, central anterior opacities occur in the first decade of life and become deeper and more diffuse with significant ground-glass haze in the stroma between opacities. By the third or fourth decade, the opacities have extended into the deeper stroma and to the corneal endothelium with development of irregular guttae in Descemet membrane. Characteristically, involvement extends to the limbus, and the stroma between opacities is somewhat hazy, which allows this disorder to be distinguished from granular dystrophy. The corneal stroma is usually thinner, which seems to be explained by decreased interfibrillar spacing between collagen fibrils in diseased corneas compared to normal corneas (10). Photophobia, decreased corneal sensation, and recurrent erosions can occur. Visual acuity is often significantly reduced by the third decade of life, prompting keratoplasty. Success with surgery is high, but opacification may recur in grafts with varying degrees of visual impairment. While good results from lamellar keratoplasty have been reported (109), penetrating keratoplasty is the preferred choice when the pathology is not restricted to solely the anterior or posterior lamellae. Laser ablation may be helpful in early symptomatic cases with superficial involvement. Histopathologically, the Bowman layer, the corneal



**FIGURE 136-4** Macular Corneal Dystrophy. Diffuse clouding is present between the denser opacities.

stroma, Descemet membrane, and the corneal endothelium show excess accumulation of mucopolysaccharide (glycosaminoglycans) within lysosomes, Golgi vesicles, and endoplasmic reticulum as well as extracellularly between collagen fibrils (110). The accumulations are positive for stains for glycosaminoglycans, such as Alcian blue, colloidal iron, and periodic acid-Schiff. On electron microscopy, keratocytes and corneal endothelium show intracytoplasmic accumulations along with vacuoles and lamellar bodies. The extracellular matrix shows fibrillo-granular material that stains for glycosaminoglycans.

The mucopolysaccharide that accumulates is non-sulfated keratan sulfate related to a defect in a specific sulfotransferase, GlyNAc6ST (111). Studies of corneal explants suggest altered synthesis of keratan sulfate as well as other glycosaminoglycans. Furthermore, corneal explants from some patients with macular dystrophy have synthesized normal keratan sulfate proteoglycan but shortened dermatan sulfate chains. Keratan sulfate is normally found only in the cornea and cartilage. Cartilage from some macular dystrophy patients lacks normal keratan sulfate. Macular corneal dystrophy has been subdivided by the presence or absence of antigenic keratan sulfate in serum and in the cornea. Type I has none detectable in either the serum or cornea, type IA has antigenic keratan sulfate detectable in the cornea but not the serum, and type II has it detectable in both the serum and cornea. These immunophenotypes cannot be

differentiated on a clinical level and do not appear to have any clinical significance. The genes for types I and II were localized to 16q22 and have been fine mapped to a region containing a sulfotransferase gene, CHST6 (112). Since its description, a myriad of mutations have been identified in the CHST6 gene in macular dystrophy, indicating mutational heterogeneity leading to a common protein dysfunction (3,113–119). One case of macular corneal dystrophy could not be explained by CHST6 mutations, deletions or insertions in the upstream region, or by splice site mutations. This could indicate the existence of genetic heterogeneity or abnormalities in regulatory elements (120).

**136.2.5.4 Schnyder Corneal Dystrophy (Including Schnyder Crystalline Corneal Dystrophy, Central Stromal Crystalline Corneal Dystrophy, Schnyder Crystalline Dystrophy Sine Crystals, Hereditary Crystalline Stromal Dystrophy of Schnyder, Crystalline Stromal Dystrophy, Corneal Crystalline Dystrophy of Schnyder, Schnyder Corneal Crystalline Dystrophy) (IC3D Category 1).** In Schnyder corneal dystrophy (SCD), various patterns of lipid deposition are seen in the corneal stroma. Classically, central fine stromal crystals appear in the first year of life and progress. The crystals are also seen in Bowman layer. Eventually, corneal arcus and central stromal haze appear in the third or fourth decade of life. Fine crystals are deposited in a ring surrounding the central portion of the cornea, with less dense crystals and gray-white whorl-like lesions centrally. A dense lipid arcus at the corneal periphery and a girdle of anterior denser lipid at the nasal and temporal limbus are usually present. Only about half of the patients have the classic crystals with the remainder having non-crystalline lipid haze. Commonly, there is only mild disturbance of vision (121), with scotopic vision remaining good and photopic vision decreased. The decrease in photopic vision usually leads to a need for corneal transplantation sometime after the fifth decade (122). Recurrence has been reported but is infrequent and mild (123). Laser superficial keratectomy has also shown promise in improving vision (124,125). Two systemic findings have been associated with this disorder frequently enough to raise the question as to whether this is a true dystrophy or a systemic disorder. Genu valgum and other joint abnormalities have frequently been noted, but not in all families. Hyperlipemia with elevation of serum cholesterol and triglycerides has also been noted often, but families have been reported in which some members have both corneal changes and hyperlipemia, some only corneal changes and others only hyperlipemia. Previous work had failed to find a correlation between systemic disturbances in lipid metabolism and Schnyder dystrophy, suggesting that the metabolic abnormality is localized in the cornea. Others have found abnormal storage of lipid in skin fibroblasts in a family with this disorder. Histological examination of corneal specimens demonstrates the presence of cholesterol crystals, neutral

fats, and triglycerides in the corneal stroma. Esterified and unesterified cholesterol can be seen in basal epithelial cells, Bowman layer, stroma, and, rarely, endothelium. Positive staining is observed with lipid stains such as oil red O and Sudan black. On immunohistochemistry, apolipoprotein components of HDL can be detected. Crystalline dystrophy is an autosomal dominant disorder. The locus of the responsible gene, UBIAD1, is at 1p36, and multiple mutations can cause SCD (126–130). Recent work that has investigated protein homology, and molecular modeling suggests that mutations in UBIAD1 can cause a loss of function mutation in a mitochondrial prenyltransferase that affects cholesterol metabolism in an unknown manner (131).

A previous study (132) had proposed a novel dystrophy, central discoid corneal dystrophy (CDCD), based on examination of a family with clinical findings similar to SCD. Genetic analysis revealed a novel mutation in UBIAD1, suggesting that CDCD is a variant of a SCD (133).

**136.2.5.5 Congenital Stromal Corneal Dystrophy (Including Congenital Hereditary Stromal Dystrophy, Congenital Stromal Dystrophy of the Cornea) (IC3D Category 1).** Congenital stromal corneal dystrophy (CSCD) is an autosomal dominant disorder that begins at or shortly after birth and exhibits corneal haze with flaky opacities throughout the stroma in the entire cornea. These opacities become more numerous with time. Descemet membrane and the endothelium appear normal. Strabismus and nystagmus can be seen in affected patients. Penetrating keratoplasty may be needed and has resulted with clear grafts or minimal recurrence. It has been described in multiple families in Belgium, France, Norway, and Germany and is characterized on electron microscopy with unusually small, tightly packed collagen fibrils aligned in a random pattern (134). CSCD is caused by truncating mutations in the decorin (DCN) gene at 12q21 (135,136). Decorin is thought to play a regulatory role in collagen fibrillogenesis and matrix assembly. The stromal opacities seen on clinical exam are at least partially composed of accumulated truncated DCN protein (137).

**136.2.5.6 Central Cloudy Dystrophy of Francois (Including Posterior Crocodile Shagreen) (IC3D Category 4).** In central cloudy dystrophy cloud-like areas of haze occur centrally in the deep corneal stroma. Polygonal patches are separated by clear areas that form a pattern similar to the scales of a crocodile's skin (Figure 136-5), hence another of its descriptive names. It is virtually indistinguishable from posterior crocodile shagreen, which is a corneal degeneration and is not familial. This is usually seen as an incidental finding on routine examination at any age, although it is more common in later years. It produces no symptoms and requires no therapy. Histopathology of a single case showed extracellular mucopolysaccharide and lipid in the corneal stroma. Inheritance has been reported to be autosomal dominant



**FIGURE 136-5** Central Cloudy Dystrophy. The slit beam highlights the crocodile-skin opacities.

in a few families, but genotyping was not available at the time of these studies to confirm this. It is possible that these cases were instead posterior crocodile shagreen (3).

#### **136.2.5.7 Fleck Dystrophy (Including Speckled Dystrophy, Francois–Neetens Dystrophy) (IC3D Category 1).**

Fleck dystrophy is characterized by congenital, non-progressive fine opacities throughout the corneal stroma. The opacities are tiny discs or rings with clear centers. Patients are almost always asymptomatic and require no treatment, although photophobia and decreased corneal sensitivity have been reported. Asymmetry can occur and may occasionally be striking. Laser-assisted in situ keratomileusis (LASIK) does not appear to result in worsened vision based on a case report (138). A differential distinction must be made between this disorder and granular dystrophy, but the finer size and more diffuse distribution of opacities in fleck dystrophy usually create little difficulty with diagnosis. Patients with both fleck opacities and central cloudy dystrophy have been described, leading to the suggestion that these entities represent variations in expression of the same genetic defect. Histopathologically, most keratocytes are normal, whereas affected keratocytes demonstrate membrane-bound vacuoles containing acid mucopolysaccharide and lipid. The metabolic source of this accumulation is unknown. Inheritance is autosomal dominant and is due to mutations at PIP5K3, a phosphoinositide 3-kinase, at 2q35 (139,140).

**136.2.5.8 Posterior Amorphous Corneal Dystrophy (Including Posterior Amorphous Stromal Dystrophy) (IC3D Category 3).** Posterior amorphous corneal dystrophy is autosomal dominant and is characterized by

a gray sheet-like opacity in deep stroma near Descemet membrane. Transparent stromal breaks can be seen within the opacification. The lesions can be peripheral or centroperipheral, with the latter extending to the limbus and exhibiting corneal thinning with a flattened corneal curvature. Visual acuity is not greatly affected and usually requires no treatment. If necessary, a penetrating keratoplasty can be performed for severe visual impairment. Histologically, an irregular stromal architecture with attenuated corneal endothelium has been noted. On electron microscopy, abnormally oriented collagen fibers can be noted and some collagen fibers may interrupt Descemet membrane. This disorder has been observed as soon as 16 weeks after birth and may be a developmental disorder. Non-corneal findings are also present, including iris abnormalities such as corectopia, iridocorneal adhesions, papillary remnants, and pseudopolycoria. The responsible gene is currently unknown, but Aldave et al. have recently identified 12q21.33 as a locus. They were unable to detect mutations in candidate genes in the locus (KERA, LUM, DCN, EPYC) (141).

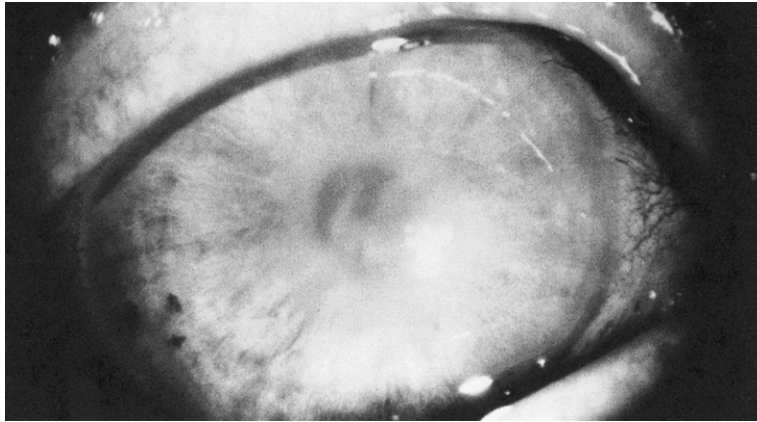
#### **136.2.5.9 Pre-descemet Corneal Dystrophy (IC3D Category 4).**

This is a disorder that is not well defined or associated with a definitive pattern of inheritance. It is unknown whether it is a hereditary or degenerative disorder. Onset is usually after 30 years, but the condition has been found in young children. Several subgroups exist. In one form gray opacities occur in the deep stroma just anterior to Descemet membrane with variable morphology, size, and distribution across the cornea. The punctiform and polychromatic subtype has more uniform and polychromatic changes. Similar lesions have been seen in association with systemic and other ocular disorders, such as X-linked and recessive ichthyosis, Fuchs endothelial corneal dystrophy (142,143), keratoconus, and pseudoxanthoma elasticum.

#### **136.2.5.10 Bietti Crystalline Corneoretinal Dystrophy and Other Stromal Dystrophies.**

Although it is not classified as a corneal dystrophy in the IC3D, we will briefly discuss Bietti crystalline corneoretinal dystrophy and its characteristic findings. In this disorder, very subtle fine crystals are seen in the cornea located in the anterior stroma. Also known as Bietti marginal corneal dystrophy, the lesions are marginal or paralimbal, as implied by the name. The disease is primarily characterized by a retinal degeneration with vascular sclerosis, night blindness and progressive visual field constriction. Crystal-like deposits have been reported on the anterior and posterior lens capsule of a patient with this disorder (144). Histopathology shows crystals and cytoplasmic inclusions in both corneal and conjunctival fibroblasts. The disorder has been mapped to chromosome 4 (4q35) (145) and, subsequently, the CYP4V2 gene (146) with numerous mutations reported (147–149). The function of the gene product is unknown, but the gene shares homology with genes involved in lipid metabolism, long suspected to be the defect in Bietti's corneal dystrophy (146).





**FIGURE 136-6** Fuchs Dystrophy. Epithelial and stromal edema as well as wrinkling of the posterior cornea are most prominent in the central cornea.

Several other disorders that cause opacities of various shapes and sizes in the deep stroma have been described. These include cornea farinata, deep filiform dystrophy, and polymorphic stromal dystrophy. Because these have not been well demonstrated to be inherited and may represent degenerative changes, the term *dystrophy* may not be warranted in these disorders. Deep filiform dystrophy and cornea farinata have been described in association with keratoconus and X-linked ichthyosis.

### 136.2.6 Endothelial Dystrophies

Endothelial dystrophies involve the corneal endothelium, which produces the dense Descemet membrane as its basement membrane. The endothelial cells actively maintain the cornea in a state of normal hydration. When endothelial dysfunction occurs, the corneal stroma and epithelium become overly hydrated, causing corneal edema, clouding, and decreased vision. Thus, this group of disorders is a frequent cause of visual disability.

**136.2.6.1 Fuchs Endothelial Corneal Dystrophy (Including Endothelial Corneal Dystrophy, Cornea Guttata, Endoepithelial Corneal Dystrophy) (IC3D Category 1 [Early-onset], Category 2 [Genetic Loci Known, Gene Unknown], Category 3 [No Known Inheritance]).** Fuchs dystrophy (FECD) initially is asymptomatic; fine guttate excrescences in Descemet membrane, often with overlying fine pigment dusting, are present on biomicroscopic examination. These excrescences are called corneal guttae. Later, stromal edema and clouding develop with wrinkling and thickening of Descemet membrane. At the same time, the corneal epithelium becomes edematous, initially with fine epithelial intracellular edema, then intercellular cyst formation, and ultimately with bleb-like separation of the epithelium (termed ‘bullous keratopathy’). All findings are most prominent centrally, but with prolonged disease the entire cornea eventually becomes edematous (Figure 136-6). The disorder is bilateral but often asymmetric, and females are more frequently affected

than males. Although corneal guttae may be noted on examination in asymptomatic patients in their thirties, symptomatic disease does not usually occur until the fifth or sixth decades. In certain families, patients may become symptomatic in their teens with smaller guttae. The first symptom is often hazy vision on arising in the morning, which abates as fluid evaporates from the cornea during the waking hours. As endothelial dysfunction increases, corneal edema becomes more persistent; eventually, vision remains blurred constantly. With the development of bullous epithelial edema, bullae may rupture and cause episodes of severe pain. With prolonged disease, subepithelial fibrosis, stromal vascularization, and fibrous thickening of Descemet membrane occurs. Treatment in the early stages consists of the use of hypertonic solutions administered as topical ocular drops to aid corneal dehydration. Painful bullae can be treated with the use of continuously worn soft contact lenses for palliation. Ultimate treatment requires corneal transplantation, more often a posterior lamellar transplant such as Descemet stripping automated endothelial keratoplasty (DSAEK), to restore both vision and comfort. Differential distinction must be made between Fuchs dystrophy and secondary causes of corneal endothelial dysfunction, including inflammatory diseases and trauma. Pathological examination shows decreased endothelial cell density; the remaining endothelial cells produce excess collagen posterior to Descemet membrane and become less effective as fluid barriers and pumps to dehydrate the corneal stroma. As noted previously, excess hydration of the corneal stroma and epithelium ensues. Electron microscopy reveals multiple layers of basement membrane-like material on the posterior part of Descemet membrane and immunohistochemistry has shown fibrinogen/fibrin in the posterior collagenous layer.

The basic pathogenetic mechanism of the cellular loss and dysfunction is unknown, but is likely multifactorial. Studies of Fuchs’ patients have noted an irregularity in the regulation of apoptosis (150,151). One study investigated the aqueous humor proteome in patients with



FECD and found altered levels of a few proteins involved in cell protection and survival, which could influence endothelial cell health (152). Additionally, one group discovered activation in the unfolded protein response in the corneal endothelium of FECD patients, suggesting a pathogenesis for cellular loss. This pathway manages improperly folded proteins and apoptosis occurs if the cell is overwhelmed with an excess of these proteins (153). Although most patients present as sporadic cases, described families strongly suggest a dominant pattern of inheritance, although there is a significant predominance of affected females. The prevalence of this disorder is lower in Asia than in North America. A patient with mitochondrial DNA missense mutation and multiple nonocular anomalies was also found to have Fuchs dystrophy, which may be coincidental or could implicate a role for mitochondrial DNA. A missense substitution, Gln455Lys, in the COL8A2 gene on chromosome 1, has been described in both Fuchs endothelial corneal dystrophy and posterior polymorphous dystrophy in a three-generation family with early Fuchs manifestations. It has also been seen in sporadic cases (154,155). Another COL8A2 mutation in early onset Fuchs, Leu450Trp, has been discovered in two families (156,157). This gene is involved in collagen VIII synthesis, which has been identified in Descemet membrane. Other studies of familial FECD as well as sporadic cases have not found mutations in COL8A2 (158,159). Genetic heterogeneity appears to exist, as late-onset FECD has been mapped to four loci on chromosomes 13 (FCD1) (160), 18 (FCD2) (161), 5 (FCD3) (162), 9 (FCD4) (163), and the SLC4A11 gene, which is also causative for congenital hereditary endothelial dystrophy type 2 (CHED 2) (164,165). The involvement of mutations in genes that cause other dystrophies (such as CHED2 or posterior polymorphous corneal dystrophy) could suggest that the endothelial dystrophies are on a spectrum of clinical disorders with genetic overlap.

**136.2.6.2 Congenital Hereditary Endothelial Dystrophy (Including Maumenee Syndrome) (IC3D Category 2 [CHED 1], Category 1 [CHED 2]).** Congenital hereditary endothelial dystrophy (CHED) refers to two entities with different patterns of inheritance and clinical courses. Ultimately, both forms have dense corneal opacification due to marked stromal edema, with stromal thickness increased to two or three times normal without vascularization. Fine epithelial edema without bullae is present. When visible, the region of Descemet membrane is thickened, but no guttate changes are present. The autosomal recessive form (type 2) presents with bilateral corneal clouding at birth or in the neonatal period. Symptoms of discomfort are absent and corneal clouding progresses very little with age. Nystagmus is often present. CHED 2 can be associated with hearing loss (Harboyan syndrome) (166). In the dominant form (type 1), photophobia and tearing are noted very early in life and biomicroscopic examination reveals corneal

stromal and epithelial edema, which progresses in the first to second year of life to milky corneal opacification. Nystagmus is uncommon. In both disorders, visual acuity is markedly reduced, but some useful vision may be retained. There are no consistent systemic abnormalities. On histopathology in both types, the number of endothelial cells is reduced and Descemet membrane can appear thick and laminated. Similar to posterior polymorphous corneal dystrophy (PPCD), the endothelial cells in CHED 1 express cytokeratin that is unlike normal endothelium (167). Corneal grafting has been carried out in some patients. The results were disappointing in older studies but more recently have been reported as yielding significant improvement in vision, even when grafting is carried out in adulthood. The use of topical hypertonic agents may provide some improvement in symptoms and acuity. Differentiation must be made between this disorder and others that present with corneal opacification early in life. Congenital glaucoma must be ruled out by the demonstration of normal intraocular pressure, lack of optic nerve cupping (if the nerve head can be seen), and lack of buphthalmic enlargement of the corneal diameter. Metabolic disorders can be ruled out by systemic evaluation and biochemical analysis. Birth trauma with rupture of Descemet membrane is unilateral, and the edema clears over time. Intrauterine inflammation is usually associated with other ocular abnormalities in addition to corneal edema. Posterior polymorphous dystrophy may present confusion and is discussed later. Pathogenesis appears to be dysfunction and focal absence of the corneal endothelium. The two genetic patterns, autosomal dominant and autosomal recessive, have been discussed above. Type I has been mapped to the pericentromeric region of chromosome 20 in the region identified for PPCD, while Type II has been found to be caused by mutations in the SLC4A11 gene which is at a different locus on chromosome 20 (168–174). CHED 2 may also have genetic heterogeneity as cases have been shown without any mutations in the SLC4A11 gene or its promoter region (169,175).

**136.2.6.3 Posterior Polymorphous Corneal Dystrophy (Including Posterior Polymorphous Dystrophy [PPMD], Schlichting Syndrome, Hereditary Deep Dystrophy, Posterior Grouped Vesicles) (IC3D Category 2 [PPCD 1], Category 1 [PPCD 2 and 3]).** Posterior polymorphous corneal dystrophy (PPCD) is characterized by broad pleomorphism, ranging from the presence of small (0.2–0.5 mm) vesicular-appearing opacities that extend posteriorly from Descemet membrane to ridge-like double linear excrescences, to more diffuse Descemet haze. The corneal stroma may be clear, mildly edematous or grossly edematous with overlying epithelial edema. The opacities may be present at birth and may progress over time. Most patients are asymptomatic; changes may be found on routine ophthalmological examination. Others may present with visually disabling edema at birth, or develop edema at any time later in life. Occasional

patients have broad iridocorneal adhesions and some develop open-angle glaucoma. The vast majority of patients require no treatment. Those with mild edema may respond to the use of topical hyperosmotic agents, whereas more extensive edema may prompt penetrating keratoplasty. When glaucoma is present it is treated as typical open-angle glaucoma. When edema is present in infancy, the differential diagnosis is important and is the same as for congenital hereditary endothelial dystrophy. The presence of polymorphous excrescences on Descemet membrane in the affected child and in asymptomatic family members confirms the diagnosis. The corneal endothelium in this disorder is abnormal and forms an epithelial-like multilayered covering to the posterior cornea, which produces abnormal Descemet membrane and may be deficient in barrier and pump function, leading to corneal edema. This may represent an abnormal differentiation of the mesenchymal anlage of the corneal endothelium. A review demonstrated a good prognosis for keratoplasty in patients without iridocorneal adhesions, but a poor prognosis and significant difficulty with glaucoma in grafts on patients with such adhesions. Histopathologically, multiple layers of collagen can be seen on the posterior surface of the Descemet membrane with nodular excrescences. Parts of the endothelium can be replaced by stratified squamous epithelial-like cells. On electron microscopy, thinning or absence of the posterior non-banded layer of the Descemet membrane may be apparent and multilayered epithelial-like cells with desmosomes and microcilia can be seen. Inheritance is usually autosomal dominant, although sporadic cases have been reported and occasional families have been described with autosomal recessive patterns of inheritance. Three loci have been identified for posterior polymorphous corneal dystrophy. A gene locus for PPCD 1 has been linked to 20q11 in the same region as congenital hereditary endothelial dystrophy type I but not type II. VSX1 was previously reported to be the responsible gene, which is also occasionally implicated in keratoconus (176), but a study performed on two large families showed that it was not involved (177). Recent work on mice with a PPCD phenotype showed a 78 base pair duplication in a locus that is syntenic to the human PPCD 1 interval on chromosome 20. It seems to lead to haploinsufficiency of the CSRP2BP gene and resulting PPCD clinical picture, suggesting that the corresponding human gene may have a role in PPCD 1 (178). The same mutation found in Fuchs dystrophy, a Q455K missense substitution in the COL8A2 gene, has also been linked to posterior polymorphous dystrophy (PPCD 2) (154,159). Numerous mutations in the ZEB1 (TCF8) gene in chromosome 10 have been identified as causative for PPCD 3 (179–181). Variable expression and incomplete penetrance has been observed with mutations in this gene (182).

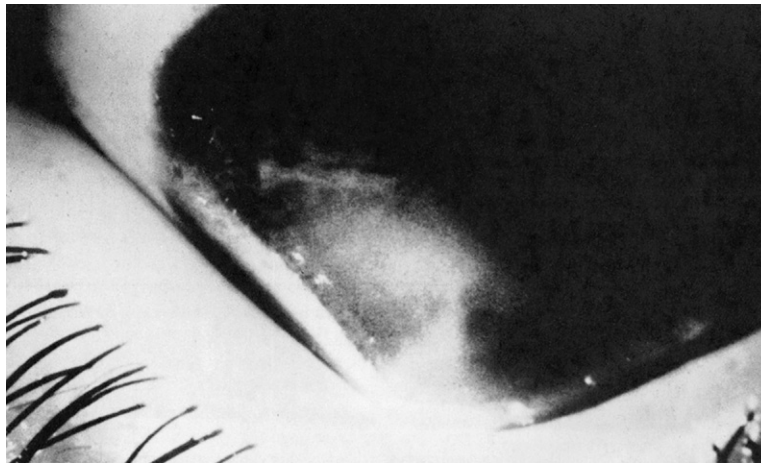
**136.2.6.4 X-linked Endothelial Corneal Dystrophy (IC3D Category 2).** This is a X-chromosomal dominant disorder with congenital onset that affects males more

than females. The male cornea displays corneal clouding that ranges from a milky ground glass appearance to a diffuse corneal haze. Subepithelial band keratopathy along with a moon crater-like appearance in the endothelium can occur. Males may experience nystagmus and often have blurred vision. Females are asymptomatic and only have the moon crater-like endothelial changes. The clinical course tends to be progressive in males and non-progressive in females. A corneal transplant may be needed and the graft usually remains clear, sometimes for as long as 30 years. Histopathology shows irregular thinning of the epithelium and Bowman layer, along with irregularly arranged collagen lamellae in the anterior stroma. Decreased numbers of endothelial cells and irregular thickening of the Descemet membrane, and small excavations and pits have been noted. Electron microscopy shows an abnormal anterior and posterior banded zone in Descemet membrane and a discontinuous endothelial layer. The responsible gene is unknown, but the genetic locus has been determined to be at Xq25 (183).

### 136.2.7 Ectatic Dystrophy

Ectatic dystrophy is added here because the disorder may affect all layers of the cornea and the primary anatomical layer of origin is unknown.

**136.2.7.1 Keratoconus.** In keratoconus there is conical protrusion of the cornea associated with central stromal thinning and irregularity of the corneal surface, as well as anterior stromal scarring and deep stromal fine striations (Figure 136-7). An iron pigment line surrounds the conical area at the level of the basal epithelium. Prevalence of this disorder in the general population is quite high, in one report as high as 1:2000. Typical onset is in the teenage years, with bilateral, often asymmetrical, progressive myopia and astigmatism. Initially, frequent spectacle changes are needed because of the refractive change, but eventually the irregular astigmatism produces inadequate acuity even with spectacles. At that point, rigid contact lenses allow good visual acuity by providing a regular refractive surface. As the conical protrusion of the cornea advances, however, contact lenses may not be well tolerated and acuity may be inadequate. Occasionally, as the disease advances, the Descemet membrane may spontaneously rupture, allowing rapid massive corneal hydration, hydrops, which persists for weeks to months until the Descemet membrane heals over. Although the vast majority of patients have no other associated problems, keratoconus has been described with a myriad of systemic diseases including atopic eczema, trisomy-21, Ehlers–Danlos syndrome, Marfan syndrome, Apert syndrome, and other disorders. Associated ocular disorders include vernal conjunctivitis, pigmentary retinopathy, Leber congenital amaurosis, and aniridia. Histopathological changes include disruption of the epithelial basement membrane and Bowman layer of the stroma with scarring. Confocal microscopy



**FIGURE 136-7** Keratoconus. Note the conical bulging and scarring of the cornea viewed with the patient in down-gaze.

shows an irregular structure of keratocytes in the anterior stroma and electron microscopy has revealed an abnormal electron dense material in the anterior stroma that has not been fully characterized (184).

The pathogenesis of this disorder has been investigated, but remains unknown. Enzyme and collagen cross-linking defects have been suggested as pathogenic. Studies of the biomechanical properties of corneas affected by keratoconus have shown that the stiffness of the anterior 200  $\mu\text{m}$  is decreased compared to a normal cornea (185) and that corneas with keratoconus have fewer cross links compared to normal corneas. Variations in collagen content and levels of protein synthesis in corneas removed at keratoplasty suggest strongly that keratoconus is a heterogeneous disorder. Increases in degradative enzyme levels and reduction in their inhibitor levels have been demonstrated in the epithelium. Exogenous factors, such as eye rubbing, have long been implicated as contributory factors in the development of keratoconus, further complicating genetic linkage studies (186,187). The increasing use of corneal topography, especially in young adults who undergo an evaluation for refractive laser surgery, and detection algorithms for detecting either subtle or forme fruste keratoconus has significantly improved screening and diagnosis.

Surgical intervention, usually in the form of penetrating keratoplasty, becomes necessary to restore good vision when contact lenses can't provide adequate vision, or can't be tolerated and other non-surgical approaches are not an option. To avoid the complications of endothelial rejection, alternatives to penetrating keratoplasty have included epikeratophakia, intracorneal rings, corneal collagen cross linking, and lamellar keratoplasty, with varying success. Deep anterior lamellar keratoplasty (DALK) is being utilized more often in keratoconus. When preparing the host corneal bed, a small amount of residual host stroma may remain on top of the Descemet membrane, resulting in an interface haze postoperatively after the donor button has been sutured into place. In

theory, if the entirety of Descemet membrane is bared, there should be no interface haze since there would be no host stroma remaining to touch the donor stroma. Whether the presence of residual stroma negatively affects the visual outcome is uncertain, with studies not yet providing a consensus (188,189). While the conventional wisdom has held that a penetrating keratoplasty will likely yield a better visual outcome than DALK because of the lack of an interface haze, studies have shown that the answer is still unclear, with some studies pointing to DALK (190,191) and others to PK or showing no significant difference (188,192–194). Corneal collagen cross linking involves the application of riboflavin and ultraviolet light A (UVA) on a de-epithelialized cornea in order to promote cross linking of corneal collagen to stiffen and provide more structural strength within the cornea. Kohlhaas et al. found that the anterior stroma of the human cornea absorbed 65–70% of the UVA light, which corresponded to the area of greatest stiffening (185). Another study showed that the diameter of the collagen fibers was increased in the treated areas, which provided further evidence that cross linking occurs with this treatment (195). European studies in humans have suggested that treatment could halt progression of keratoconus, reduce steepening of the cornea, and result in improved visual acuity with no major adverse events (196–199). Studies investigating the safety of the cross linking showed that corneas should be at least 400  $\mu\text{m}$  thick in order to prevent damage to the endothelium from UVA light. Keratocytes are destroyed down to a depth of approximately 300  $\mu\text{m}$  but repopulate after 6 months. Confocal studies have also shown a loss of corneal nerves anteriorly with regeneration occurring over 6 months (200,201). It has been shown that riboflavin penetrates deeper into the cornea with de-epithelialization (202), but treatment of intact epithelium with the addition of substances that increases epithelial permeability can provide a limited favorable result (203). It is not yet clear how long the effects of cross linking will last and whether retreatment will be needed.

Although the vast majority of cases appear to be sporadic, autosomal dominant and recessive pedigrees have been reported. A positive family history of keratoconus has been reported in 6–10% of patients, but may approach 23% of patients in some areas (204). Fourteen percent of patients in the Collaborative Longitudinal Evaluation of Keratoconus in the US reported a family member with keratoconus. Nonconcordance of keratoconus in identical twins has been infrequently reported. Through linkage studies, a number of loci have been linked to primary or isolated keratoconus (205). One study suggests that patients with Leber congenital amaurosis and a CRB1 mutation may be susceptible to keratoconus (206). As noted under PPCD, multiple mutations of the VSX1 gene on chromosome 20 have been associated with a form of autosomal dominant keratoconus (176). Whether VSX1 is involved in keratoconus (and to what degree) is still debatable, as there are studies that both support and refute a role for this gene (207–211). The SOD1 gene has also been proposed to be a candidate gene (212), but a more recent study did not reveal a pathogenic mutation (209). The SFRP1 gene encodes a protein involved in apoptosis that was recently found to be up-regulated in the corneal epithelium of four patients with keratoconus. Future work will be needed to determine whether this is a true pathogenic mutation (213). Other studies have implicated chromosomes 1 and 8, 2, 3, 5, 13, 14, 15, 16, 20 and 21 in patients with various ethnicities (204,205,214–221). No candidate genes have been identified. It is clear that the disease is complex, significantly heterogeneous, and a single locus is likely not responsible for all observed disease.

### 136.3 DEFECTS ASSOCIATED WITH SYSTEMIC DISEASE

Because defects associated with systemic disease are described elsewhere in much greater detail, attention here is directed to the ophthalmological and, in particular, the corneal abnormalities associated with them. The reader is advised to consult the appropriate chapters concerning the disorder discussed for information about pathogenesis and genetics.

#### 136.3.1 Metabolic Disorders

Metabolic disorders are generally associated with accumulation of an abnormal substance in the cornea. If the substance is produced by the corneal tissues, it may be found throughout the cornea. If it is found in elevated amounts in the blood, it is more commonly accumulated in the corneal periphery.

**136.3.1.1 Lysosomal Storage Diseases.** All three categories of disease described are associated with accumulation of excess material in the lysosomes of the corneal cells.

**136.3.1.1.1 Mucopolysaccharidoses.** Mucopolysaccharides form the ‘ground substance’ that makes up 4–4.5% of the normal cornea. Half of this is keratan sulfate I, which is not found elsewhere in the body. Chondroitin sulfate and chondroitin-4-sulfate account for 25% each. Dermatan sulfate is not present in the normal cornea but is found in corneal scars, rejected grafts and postviral opacifications. In the mucopolysaccharidoses, however, excess dermatan and keratan sulfates appear in the cornea, whereas heparan sulfate accumulates in the retina and central nervous system. Because of deficient lysosomal acid hydrolases, lysosomes accumulate material and cause characteristic pathological changes consisting of intracytoplasmic vacuolation in cells of all corneal layers. In addition, extracellular granular material and accumulation within the cytoplasm occur in both the corneal epithelium and stroma. The degree of corneal clouding characteristic of any given disease is a function of the degree of storage in the keratocytes. All mucopolysaccharidoses that have been studied, even those with clinically clear corneas, have shown the same basic abnormalities. Variations in corneal appearance are related to alterations in the fibrillar arrangement of the corneal stroma. Bone marrow transplant leads to only partial clearing of the cornea and some ocular findings have continued to progress despite bone marrow transplantation. Corneal transplants are usually technically successful, but recurrence is almost assured as early as a 1 year after surgery.

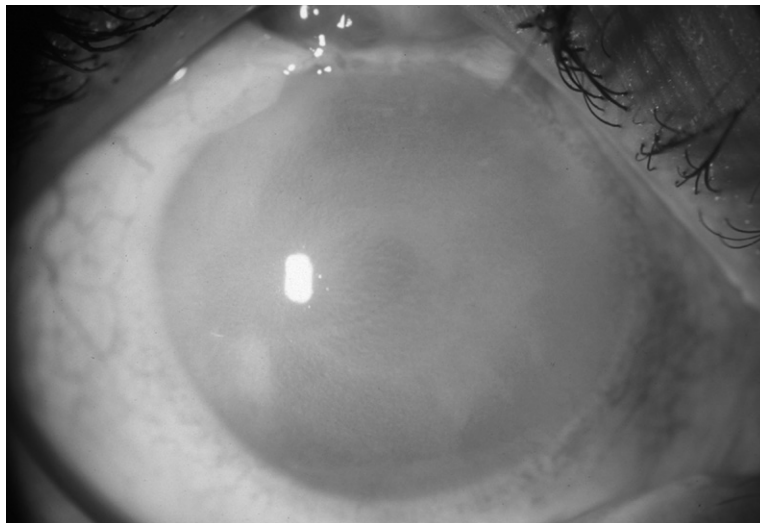
**Type I.** In the Hurler and Scheie syndromes, dermatan sulfate accumulates in the cornea. The corneas are clear at birth, but clouding begins shortly thereafter. The clouding consists of diffuse minute opacities, which give the cornea a ground-glass appearance (Figure 136-8). Although corneal transplants in patients with Scheie syndrome have occasionally remained clear, most have done poorly with time. Patients with clear grafts may still have poor vision because of retinal and/or optic nerve disease. Some studies have shown clear grafts as well as partial clearing of the host corneal rim after penetrating keratoplasty. Enzyme replacement therapy with  $\alpha$ -L-iduronidase has been investigated and its effects on corneal clouding are not yet clear (222).

**Type II.** Hunter syndrome patients with both the mild and severe form have clinically clear corneas, perhaps because of the lesser accumulation of dermatan sulfate. Histopathologically, however, accumulation is seen in the cornea; in the milder form, slit lamp biomicroscopy may demonstrate corneal clouding in older patients.

**Type III.** Sanfilippo syndrome does not accumulate dermatan sulfate, so the cornea is clear on clinical evaluation, although later in life mild stromal haziness may be seen at the slit lamp.

**Type IV.** Morquio syndrome accumulates keratan sulfate, allowing progressive corneal clouding that is similar to that seen in type I.





**FIGURE 136-8** Mucopolysaccharidosis Type I. Note the diffuse corneal clouding seen in a 19-year-old with Hurler–Scheie syndrome.

**Type VI.** Maroteaux-Lamy syndrome accumulates only dermatan sulfate, which leads to a progressive corneal clouding that may be milder than that seen with type I. The differential diagnosis of these disorders is made on the basis of systemic clinical findings and laboratory evaluation. As in type I, clear grafts with partial clearing of the host cornea after penetrating keratoplasty have been reported.

**Type VII.**  $\beta$ -Glucuronidase deficiency is associated with accumulation of both dermatan and keratan sulfates, and corneal clouding is reported to occur in 75% of cases. Corneal transplantation was reported to show good results in terms of clarity in one patient (223). Gene therapy in mice has shown promise in limiting accumulation and, when given locally, improving the clarity of affected murine corneas (224,225).

**136.3.1.1.2 Sphingolipidoses.** Sphingolipidoses, involving abnormalities in ganglioside degradation, are frequently associated with retinal and optic nerve abnormalities. Corneal findings are much less frequently found.

1. Fabry disease is a deficiency in lysosomal hydrolase  $\alpha$ -galactosidase A and has been associated with several ocular findings including retinal and conjunctival vascular abnormalities, lens changes, and papilledema (226). In the cornea, verticillata, a whorl-like haze, is seen in the epithelium of both affected males and female carriers. The opacity has no associated effect on vision. Differential should include drug effects from amiodarone, chloroquine and phenothiazines. Sphingolipid material is seen in the cytoplasm and lysosomes of the corneal epithelium on pathological examination.
2. Other sphingolipidoses, including Sandhoff disease and Niemann-Pick disease, may rarely show corneal clouding, although corneal involvement can be

demonstrated on pathological examination. Corneal opacities had been reported in Gaucher disease associated with calcific cardiac valvular disease (D409H variant).

**136.3.1.1.3 Mucolipidoses.** In muculipidoses (MLS), mucopolysaccharides and sphingolipids and/or glycolipids accumulate. Corneal clouding similar to that seen in the mucopolysaccharidoses may thus be seen in combination with the classic retinal cherry-red spot and central nervous system abnormalities. In GM<sub>1</sub> gangliosidosis, keratan sulfate accumulation in the cornea may lead to mild corneal clouding. In MLS II, corneal clouding may be seen occasionally clinically, whereas pathological evaluation reveals accumulation of granular material in the cornea even in clinically clear specimens. MLS IV has much more prominent corneal clouding, which may present early in the first year of life. MLS types I and III less commonly have corneal findings. The syndrome described by Goldberg et al. (1971) combines corneal clouding and a macular cherry-red spot. The variant form of metachromatic leukodystrophy also shows corneal clouding. Conjunctival biopsy may help to confirm the diagnosis with the demonstration of single membrane-limited vacuoles filled with fibrogranular material and lamellar bodies. Involvement of the corneal endothelium has also been reported (227). Recurrence after penetrating keratoplasty is rapid, likely because of the replacement of the donor epithelium with opaque recipient epithelium. In a patient with MLS IV, related unaffected donor conjunctival transplantation was reported to improve corneal clarity.

**136.3.1.2 Protein and Amino Acid Metabolic Abnormalities.**

**136.3.1.2.1 Cystinosis.** In the infantile form of cystinosis, multiple fine polychromatic needle-like crystals are seen in the anterior corneal stroma and in the

conjunctiva in the first year of life, whereas in the adolescent and adult forms they appear much later but have the same clinical appearance. Mild photophobia without visual deficit may occur in all forms, but is most common in the infantile form. Conjunctival biopsy with fixation in absolute alcohol may be used to confirm the diagnosis. Distinction must be made between these crystals and those seen in crystalline dystrophy, gout, and multiple myeloma. In patients with nephropathic cystinosis, renal transplantation does not appear to alter the progressive deposition of cystine crystals in the cornea and, as life expectancy has increased, so have the severity of ocular manifestations (228). Mutations in the cystinosis gene allelic with those of nephropathic cystinosis have been found in patients with ocular non-nephropathic cystinosis (229). Topical cysteamine eye drops promote clearing of crystals from the cornea (229,230). Unfortunately cysteamine oxidizes rapidly at room temperature to cystamine, which is ineffective, making this disorder more difficult to treat.

**136.3.1.2.2 Amyloidosis.** Amyloidosis is discussed in the section on lattice corneal dystrophy.

**136.3.1.2.3 Tyrosinemia Type II, Richner–Hanhart Syndrome.** The type II form of tyrosinemia has as a prominent feature marked photophobia and tearing with the development of branching central intraepithelial and subepithelial corneal opacities and ulcers. The lesions may present in the first decade of life and decrease over the next few decades. The lesions disappear on a diet low in phenylalanine and tyrosine and appear to be due to accumulation of tyrosine crystals within epithelial cytoplasmic membrane-bound inclusions. Untreated Type I form of tyrosinemia does not induce ocular symptoms because tyrosine is not accumulated at the same levels with the enzymatic defect well ‘downstream’ in the metabolic pathway. Treatment with NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3 cyclohexane dione), which inhibits the catabolism of tyrosine, is life-saving for these individuals, but can lead to a type II-like corneal presentation during childhood (231).

**136.3.1.2.4 Wilson Disease.** Although the ocular changes in this disorder are asymptomatic, they may alert the physician to the diagnosis. Yellow-brown, gold, or green deposition of copper occurs in the periphery of Descemet membrane and extends centrally for 1–3 mm in the Kayser–Fleischer ring. The ring may disappear with systemic treatment or liver transplantation, but Kayser–Fleischer ring reduction does not directly correlate with or predict clinical improvement in neuropsychiatric manifestations of Wilson disease. The same corneal findings can also occur in acquired liver disease with secondary copper transport impairment.

**136.3.1.2.5 Lowe Syndrome.** Although glaucoma and cataract are the classical ocular findings, dense corneal scarring with a dense white vascularized opacity in

the lower portion of the cornea occurs toward the end of the first decade of life. This may represent scarring secondary to corneal edema induced by the glaucoma; more likely, it is secondary to the prolonged digital-ocular manipulation seen in these children.

**136.3.1.2.6 Familial Dysautonomia, Riley–Day Syndrome.** Because of their severe tear insufficiency, corneal insensitivity to pain, and decreased frequency of blinking, these patients have trouble with recurrent corneal ulceration, with ultimate corneal scarring and vascularization. Tear replacement, lubricating ointments, punctal occlusion, and tarsorrhaphies may help to decrease the corneal complications.

### 136.3.1.3 Lipid Metabolic Abnormalities.

**136.3.1.3.1 Hyperlipoproteinemias.** In the hyperlipoproteinemias, corneal findings may aid in the initial diagnostic suspicion. *Corneal arcus* is the term used to describe the deposition of phospholipids, cholesterol esters, and triglycerides in the corneal periphery at all levels, but most markedly at the level of Descemet and Bowman layers. Most commonly, this is seen as a normal aging phenomenon, but its presence before the age of 30 or 40 should prompt evaluation of lipid metabolic function. Fredrickson types II–V may have arcus, whereas types I and II may have more extensive lipid infiltration of the cornea (termed ‘lipid keratopathy’). Xanthelasma may occur in types II–IV. All these changes may be due primarily to elevated cholesterol. Markedly elevated triglyceride, as can occur in all but type II, is associated with lipemia retinalis, the milky appearance of blood in the retinal vessels.

**136.3.1.3.2 Lecithin Cholesterol-Acyltransferase Deficiency.** Lecithin cholesterol-acyltransferase deficiency may be associated with an accumulation of diffuse gray dot-like opacities in the corneal stroma that is denser in the periphery. Bilateral corneal opacity becomes more evident with age and penetrating keratoplasty specimens exhibit secondary amyloidosis present throughout the stroma (232). A different defect at same locus leads to fish-eye disease, with much more extensive corneal opacification.

**136.3.1.3.3 Tangier Disease.** In  $\alpha$ -lipoprotein deficiency, the accumulation of cholesterol esters in the tissues, despite abnormally low serum levels, leads to the development of a corneal stromal haze that consists of many fine dots and is denser in the posterior stroma.

## 136.3.2 Corneal Abnormalities Associated with Other Inherited Syndromes

### 136.3.2.1 Cutaneous Disorders.

**136.3.2.1.1 Ichthyosis.** Corneal findings in ichthyosis vulgaris are uncommon. In X-linked ichthyosis, dense deep corneal stromal dot-like opacities can be seen on slit lamp examination; these are asymptomatic.

**136.3.2.1.2 Ectodermal Dysplasia.** Anhidrotic ectodermal dysplasia is associated with decreased tear production, secondary punctate epithelial corneal erosion and peripheral corneal vascularization. In some forms of ectodermal dysplasia, limbal stem cell dysfunction may lead to corneal opacification.

**136.3.2.1.3 Epidermolysis Bullosa.** Corneal scarring and pannus are frequent in the autosomal recessive dystrophic form and the junctional form. Peripheral corneal vascularization can be seen in the simplex form in a small proportion of patients.

**136.3.2.1.4 Xeroderma Pigmentosum.** Because of the failure of epithelial repair after actinic damage, these patients experience severe photophobia, and drying and scarring of the conjunctiva and cornea. At times, they may also develop anterior stromal corneal nodules. Malignant neoplasms frequently develop in the conjunctiva and cornea.

## 136.4 OTHER INHERITED CORNEAL ABNORMALITIES

Several congenital anomalies can involve the cornea. A few with inheritance are mentioned here.

### 136.4.1 Megalocornea

In megalocornea, non-progressive enlargement of the cornea to a diameter greater than 13 mm is seen in the absence of glaucoma. Occasionally, other anterior segment anomalies may be associated. It is important to differentiate this disorder from the progressive buphthalmos of congenital glaucoma. Inheritance is usually X-linked recessive, although autosomal dominant and autosomal recessive pedigrees have been reported.

### 136.4.2 Cornea Plana

In cornea plana, unusually flat and often smaller-than-normal corneas are present at birth. In addition, there is often sclera-like opacification of the cornea either peripherally or throughout. Anterior segment developmental abnormalities may also be present. Visual acuity is usually poor. Although commonly autosomal dominant, autosomal recessive inheritance has also been reported.

It can be seen in the MIDAS syndrome, which is an acronym for microphthalmia, dermal aplasia, and sclerocornea (233,234). The dermal aplasia usually consists of linear erythematous skin defects involving the face, scalp, neck and thorax. Non-ocular abnormalities such as cardiac anomalies, short stature, and absence of the corpus callosum can also be seen. This disorder is thought to be X-linked dominant, with deletions or unbalanced translocations involving Xp22.3 (235). It mainly occurs in females as it appears to be lethal in hemizygous males. Phenotypic heterogeneity in ocular findings have been reported (233).

### 136.4.3 Others

Many other anterior segment abnormalities with familial tendencies occur, including Axenfeld-Rieger, Peters, and other anterior chamber cleavage anomalies. Peters anomaly is a disorder with a congenital defect in the posterior cornea, corneal opacification, iris adhesions to the cornea, and, in some patients, lens developmental abnormalities. PAX6 gene mutations have been found in some patients with this disorder as well as in aniridia and dominantly inherited keratitis. Most patients with Peters anomaly, however, have normal PAX6 genes. Because of limitation of space here, the reader is referred to other articles the work of Waring, Rodrigues, and Laibson (1975) for a thorough discussion of these disorders (236–239).

## 136.5 CONCLUSION

Many inherited corneal defects can occur both as isolated findings and in association with systemic disease. An awareness of these is important to the general physician and geneticist as well as to the ophthalmologist.

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# Congenital Cataracts and Genetic Anomalies of the Lens

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## GLOSSARY

- Aphakia** – absence of the crystalline lens of the eye, either as a result of surgery or as a primary condition.
- Cataract** – a loss of clarity of the crystalline lens of the eye.
- Ectopia lentis** – displacement of the crystalline lens out of its usual position.
- Microspherophakia** – a lens that is smaller and rounder than normal.
- Autosomal dominant** – Mendelian inheritance pattern in which one abnormal copy of a gene causes disease.
- Autosomal recessive** – Mendelian inheritance pattern in which two abnormal copies of a gene cause disease.
- X-linked inheritance** – also called X-linked recessive. Inheritance of a gene on the X chromosome which is expressed in males more severely than females due to their lack of an additional X, rendering them hemizygous for the allele.

## 137.1 INTRODUCTION

The lens is a transparent crystalline structure responsible for transmitting light rays to the retina, which in turn transmits visual impulses to visual cortex via the optic nerve and visual pathways. It accounts for one-third the refractive power of the eye, the clear cornea accounting for the other two thirds. By far the most common anomalies of the lens are varying degrees of opacification, commonly referred to as cataracts. Other anomalies are much rarer; these include absence of the lens (aphakia), colobomas, abnormalities of the embryonic vascular system (persistent pupillary membrane, Mittendorf dot, persistent fetal vasculature), anomalies of shape (lenticonus, spherophakia), anomalies of size (microspherophakia), and dislocation of the lens (ectopia lentis). Anomalies of the lens can be divided into two major subgroups: (1) noncataractous anomalies and (2) cataracts.

## 137.2 NONCATARCTOUS ANOMALIES

The group of disorders known as noncataractous anomalies is summarized in [Table 137-1](#). Although the precise correlation between this group of disorders and the embryological development of the eye has not been demonstrated, it would be reasonable to assume that many of these anomalies can be explained by mechanisms that interfere with the embryologic development of the lens, as summarized in [Table 137-2](#) and depicted in [Figure 137-1](#).

While elongating, the anterior ends of cells in the developing lens move toward the anterior pole of the epithelial layer; the posterior ends move along the posterior capsule toward the posterior pole. Thus, the newer constituents of the lens surround the preexisting parts, and no cells formed in the lens are shed or extruded. The lens thus keeps a record of its complete cellular history, with the oldest portion innermost. This explains why lens opacities that develop during a limited period in the superficial (cortical) lens are later buried as opaque layers in the inner lens region, covered by a newly formed clear superficial layer (so-called nuclear or zonular cataracts). It is even possible to estimate the time of cataract formation from its relative depth within the lens. Cataracts may now be identified in utero by obstetrical ultrasound as early as 14 weeks gestation ([1](#)).

### 137.2.1 Congenital Aphakia

Congenital aphakia (absent lens) can result from lack of development of the lens anlage (primary aphakia) or pre-natal resorption of an abnormally developed lens (secondary aphakia). We have observed a case in a patient with Fraser cryptophthalmos syndrome at Cedars-Sinai Medical Center, in Los Angeles. We have also observed a child at the University of Iowa with bilateral lens

**TABLE 137-1 Noncataractous Anomalies**

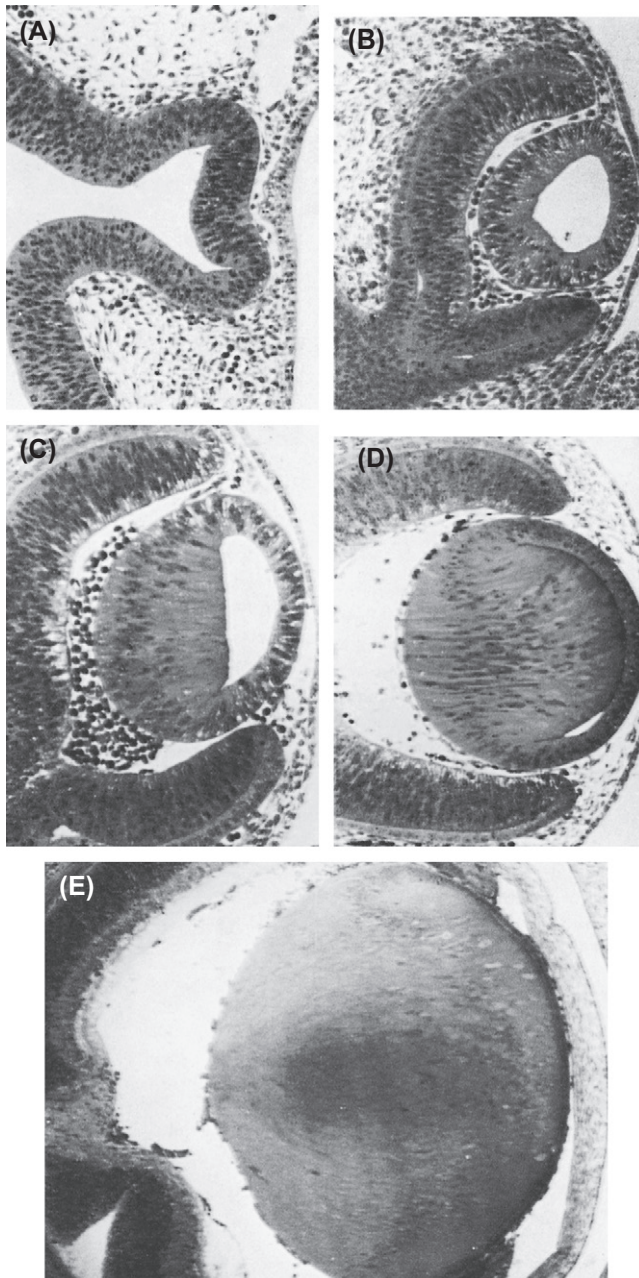
Aphakia
Autosomal recessive
Autosomal dominant
Coloboma
Simplex
Sporadic
Autosomal dominant
Tunica vasculosa lentis (remnants)
Pupillary membrane
Sporadic
Cibis anomaly
Mittendorf dot
Lenticonus
Anterior
Alport syndrome
Posterior
Sporadic/autosomal
Microspherophakia
Weill–Marchesani
Autosomal recessive
Autosomal dominant
Ectopia lentis
Isolated
Traumatic
Hereditary
Congenital dominant
Congenital recessive
Late-onset dominant
et pupillae
Syndromic
Marfan syndrome
Homocystinuria
Weill–Marchesani
Hyperlysinemia
Sulfocysteinuria
Molybdenum cofactor deficiency
Stickler syndrome
Cotlier syndrome (Wagner plus)
Branchio–Oculo–Facial syndrome
ADAMTSL4, FBN1 mutations

resorption in whom only an anterior capsule was present; the child's anterior segments appear normal other than small corneal diameters, and he has no other congenital anomalies. Recurrent phimosis of the capsular opening has occurred postoperatively. Congenital aphakia has been reported as a recessive disorder in a strain of mutant mice mapping to mouse chromosome 19. The responsible gene is *Pitx3*, due to a mutation in a promoter of the homeobox transcription factor; a mutation in the analogous *PITX3* gene in humans causes cataract and anterior segment dysgenesis (ASD) (2,3). Mutations in the *FOXE3* gene can cause autosomal recessive congenital aphakia in humans (4). Mutations or deletions in the *TFAP2A* gene in humans have been reported to cause autosomal dominant primary aphakia (5). These eyes also had other anomalies. The analogous gene in mice has been shown to be important in formation of the lens and in tissue–tissue interactions and development of the entire eye during embryogenesis (6). Because the lens plays a role in stimulating the growth and development of the anterior segment, these eyes are smaller than normal (microphthalmic). Lens coloboma consists of a circumscribed indentation of the lens equator (Figure 137-2). The zonule is usually defective in this region; uveal colobomata may be found in association. Dominant inheritance has been reported, occurring in individuals with associated ectopia lentis (7). c-Jun NH(2)-terminal kinase (JNK), a terminal kinase group of mitogen-activated protein kinases, initiates a cascade that causes Pax2 expression important in closure of the optic fissure (i.e. a disturbance in this cascade causes coloboma formation) (8). Colobomas of the eye are more common than previously thought, with an incidence of 1/2077 live births reported in Olmstead County (9). Lens coloboma may be associated with chorioretinal colobomas and commonly has an overlying iris coloboma and frequently cataract in the affected area of the lens. One in eight patients with ocular coloboma has CHARGE

**TABLE 137-2 Embryologic Development of the Human Lens (A Structure Entirely of Ectodermal Origin)<sup>a</sup>**

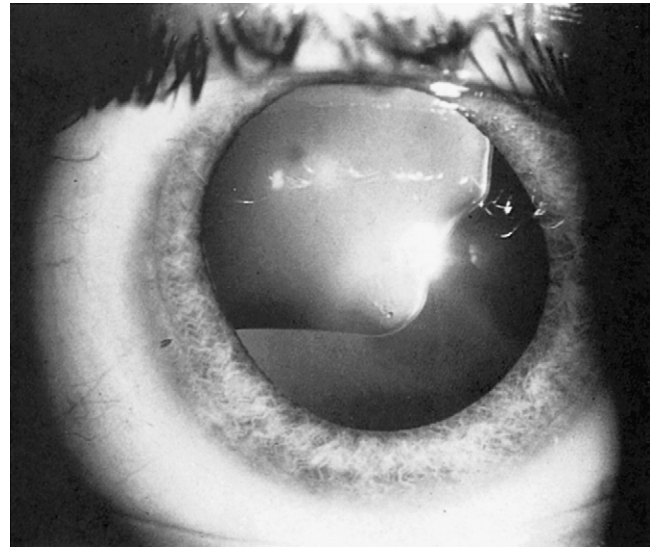
1. Week 3 (4-mm stage)—Formation of lens anlage begins with elongation of ectodermal cells at site where the optic cup touches the ectoderm to form the lens plate (Figure 137-1A).
2. Week 4 (7-mm stage)—Lens plate invaginates to form the lens pit.
3. Week 5 (11-mm stage)—Lens pit is sequestered from the surface ectoderm to form the lens vesicle (Figure 137-1B); The lumen of the lens vesicle is then obliterated by the elongation of the posterior cell layers into “fiber”-like cells (“primary” lens fibers (Figure 137-1C)).
4. Week 7 (20-mm stage)—“Primary” lens fibers reach from the posterior lens surface to the anterior cell layer, which maintains its epithelial character; an exclusively epithelial structure is thus formed covered on the outside by the basal membrane, which increases in thickness to form the lens capsule (Figure 137-1D).
5. Rest of fetal period and lens increases logarithmically in weight and volume by apposition of new lens material at the postnatal life equator; only the pre-equatorial region of the anterior epithelium divides and newly formed are pushed onto the lens equator, where they start to elongate into new (secondary) lens fiber cells (Figure 137-1E).

<sup>a</sup>While elongating the anterior ends of these cells move toward the anterior pole of the epithelial layer while the posterior ends move along the posterior capsule toward the posterior pole. Thus, the newer constituents of the lens surround the preexisting parts and no cells that have been formed in the lens are shed or extruded. The lens thus keeps a record of its complete cellular history, the oldest portion being innermost. This explains why lens opacities that develop during a limited period of time in the superficial (cortical) lens are later buried as opaque layers in the inner lens region, covered by a newly formed clear superficial layer (so-called nuclear or zonular cataracts). It is even possible to estimate the time of cataract formation from its relative depth within the lens (Koch et al., 1983) (53).

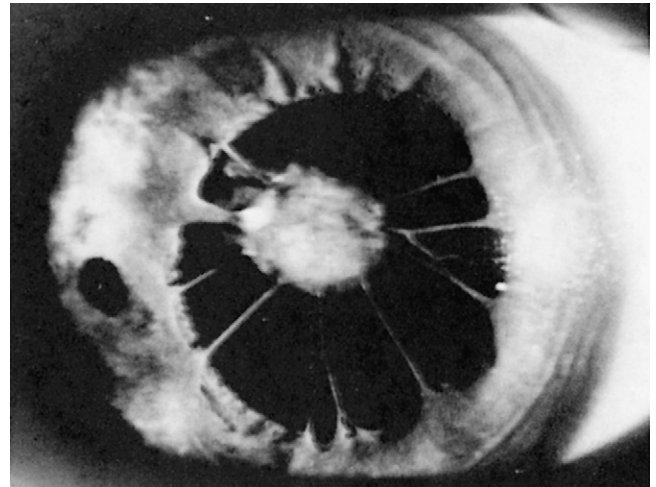


**FIGURE 137-1** Lens development in the rat. A, Formation of lens plate at day 11. B, Formation of lens vesicle at day 12. C, Beginning of elongation of posterior lens epithelium to form fiber cells at day 13. D, Obliteration of lens vesicle by elongated "primary" lens fibers at day 14. E, Appositional lens growth by formation of "secondary" lens fiber cells from the equatorial region at day 18. Human lens development is similar, except for timetable.

syndrome (9), which may be caused by mutations in the CHD7 gene (10). Persistent pupillary membranes (Figure 137-3) and Mittendorf dots (Figure 137-4) are remnants of the primitive vascular supply of the lens (tunica vasculosa lentis or posterior hyaloid vessels). All of this vascular system is usually resorbed, and these two entities remain as a result of interference with the resorption process (11). A Mittendorf dot is usually seen just behind the posterior lens capsule, inferonasal to the center of the



**FIGURE 137-2** Lens coloboma in a patient with Marfan syndrome.



**FIGURE 137-3** Persistent pupillary membrane with associated anterior polar cataract.



**FIGURE 137-4** Mittendorf dot, located behind the posterior capsule.



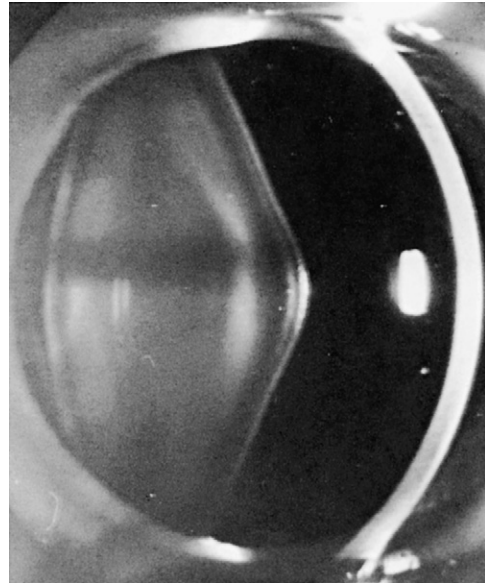
lens. Both conditions are sporadic and usually are of no visual consequence to the patient.

### 137.2.2 Lenticonus

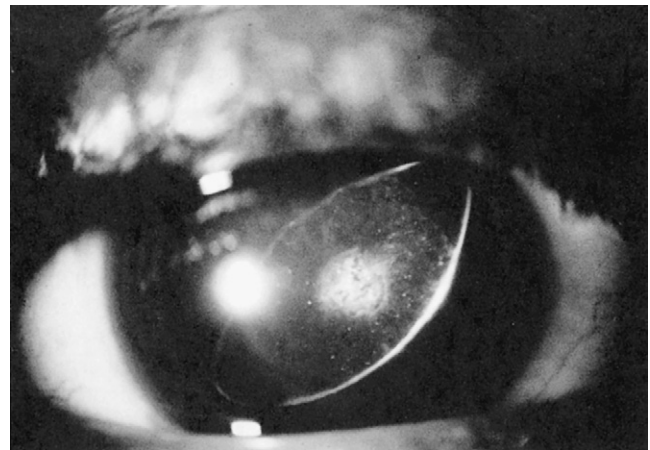
Lenticonus consists of a bulge in the front of the lens (anterior lenticonus) (Figure 137-5) or at the back of the lens (posterior lenticonus) (Figure 137-6). Anterior lenticonus is associated with Alport syndrome. This multisystem disorder has three major findings: chronic nephritis, nerve deafness, and specific ocular abnormalities. The common denominator is that basement membranes are affected in all three organs (12). Electron microscopy studies have shown abnormalities of the lens epithelium in Alport syndrome to include dilated Golgi complexes and rough endoplasmic reticulum, swollen mitochondria, increased tonofilaments, and absence of epithelial cells under the lens capsule (13). The anterior lenticonus is bilateral in 75% of patients, and typically, it is acquired, appearing in the 20- to 30-year age range with symptoms of increasing myopia (14). In addition to anterior lenticonus, other ocular abnormalities seen in Alport syndrome include arcus cornealis, posterior subcapsular cataracts (PSCs), myopia, and whitish gray dots in the macula. The inheritance pattern is complex, with X-linked, autosomal recessive and autosomal dominant forms. The X-linked form, found in 80% of patients, is due to a mutation in the gene *COL4A5* located at Xq22 encoding a type IV collagen a chain. The mutation causing autosomal recessive Alport syndrome was traced to *COL4A3* and autosomal dominant Alport syndrome to *COL4A4*, both on chromosome 2 (14,15). A variant of anterior lenticonus, known as anterior polar cataract, has been described in a patient with Ehlers-Danlos syndrome (16). Isolated, small flat anterior polar cataracts may be autosomal dominant and may not cause visual disturbance other than hyperopia. Posterior lenticonus is usually sporadic and of unknown pathogenesis (17). It is typically unilateral; however, there are reports of familial cases, in which it is bilateral with an autosomal dominant mode of inheritance, or possibly X-linked (18). Clinically, it appears as an “oil drop” phenomenon, with posterior cortical cataract found in front of the lenticonus in about 80% of cases. Increasing myopia is often an early sign. Visual acuity is usually decreased early in life, and associated amblyopia and strabismus are common. Posterior lenticonus has been reported with Alport syndrome and Pierson syndrome (15,19). Recently, bilateral posterior lenticonus in a child with a chromosomal translocation was found to be caused by disruption of the *TDRD7* gene; this gene was found to be expressed in the posterior lens capsule in chicks and mice and mutation causes cataract and glaucoma in mice (20).

### 137.2.3 Microspherophakia

The normal crystalline lens is thicker in the middle than at the edges. When instead it is round, it is known as



**FIGURE 137-5** Anterior lenticonus in a patient with Alport syndrome.

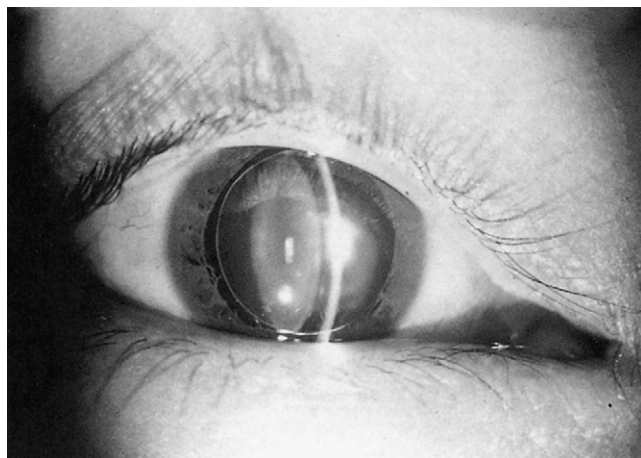


**FIGURE 137-6** Posterior lenticonus.

spherophakia, and if it is small as well, it is known as microspherophakia (Figure 137-7). Some ultrastructural studies do not show such lenses to be simply small and round but also wider posteriorly and narrower in front—the exact opposite of normal.

Microspherophakia is the typical manifestation of Weill-Marchesani syndrome; however, it may rarely be an isolated phenomenon or associated with another syndrome. The lens may be subluxated or dislocated. Ocular abnormalities reported with microspherophakia include lenticular myopia, angle-closure glaucoma, poor accommodative reserves, and tapetoretinal degeneration, while systemic associations include arachnodactyly, dwarfism, beaked nose, microstomia, micrognathia, and mental retardation (21,22). The mode of inheritance may be autosomal dominant or recessive. Isolated microspherophakia has been reported to be caused by mutations in the *LTBP2* gene (23).



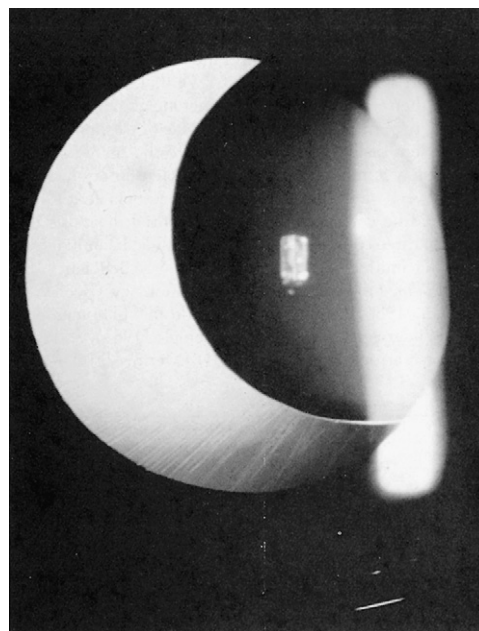


**FIGURE 137-7** Microspherophakia in a patient with Weill–Marchesani syndrome.

### 137.2.4 Ectopia Lentis

Ectopia lentis (subluxation of the lens) can appear as an isolated phenomenon, associated with an ectopic pupil only, or a manifestation of a number of multisystem disorders (see [Table 137-1](#)). A Danish review of ectopia lentis (EL) gave a prevalence rate of 6.4 per 100,000 live births. Etiology was determined in 69% of these cases with the following results: Marfan syndrome 68.2%, ectopia lentis et pupillae 21.2%, isolated dominant ectopia lentis 8%, and homocystinuria, sulfite oxidase deficiency, and Weill–Marchesani syndrome each comprising approximately 1%, with the remaining 31% of indeterminate etiology ([24](#)). Three different types of inherited bilateral isolated ectopia lentis can be distinguished: a congenital dominant form, in which most lenses are displaced upward; a congenital recessive form; and a late-onset dominant form, in which subluxation occurs between ages 20 and 65, with subluxation usually occurring inferiorly. Studies in multiple families with isolated ectopia lentis and lacking any clinical or echocardiographic signs of Marfan syndrome demonstrated a point mutation in *FBN1* (the gene for fibrillin) on chromosome 15 ([25–27](#)). Some of the more common syndromes which include ectopia lentis are discussed below. Other very rare conditions in which ectopia lentis occurs should be considered in the differential diagnosis if the preceding three conditions have been excluded are familial hyperlysinemia, sulfite oxidase deficiency, molybdenum cofactor deficiency, Ehlers–Danlos syndrome, Wildervanck syndrome, and Kniest dysplasia (metatropic dwarfism type II). In patients suspected of having these syndromes (e.g. sulfite oxidase deficiency) the ophthalmic examination is key, as dislocation of the lenses may be one of the early presenting signs ([28](#)).

Ectopia lentis et pupillae is a rare autosomal recessive disorder in which subluxation of the lens is associated with a displaced pupil (corectopia). The condition is congenital, usually nonprogressive, bilateral, and symmetric. In addition, patients have microspherophakia, miosis, and



**FIGURE 137-8** Ectopia lentis in a patient with Marfan syndrome (superotemporal dislocation).

poor pupillary dilation with mydriatics. Displacement of the lens may be in any direction, and the edge of the lens often bisects the pupil, causing diplopia and poor vision. There is often an associated increased transillumination of the iris periphery. All the affected ocular structures—the lens zonules, iris dilator muscles, and the posterior pigmented layer of the iris—have a common embryologic origin (i.e. the neuroectodermal layer). Mutations in the *ADAMTSL4* gene, as well as *FBN1*, are now known to cause ectopia lentis et pupillae ([29](#)).

Isolated ectopia lentis is relatively rare; more commonly, this is associated with a multisystem disease or an inborn error of metabolism. The common denominator appears to be a weakness of the zonules caused by an inherited disorder of connective tissue or an enzymatic deficiency. Studies have shown significantly decreased amounts of fibrillin in the lens capsules of patients with *Marfan syndrome*, as well as abnormal, disrupted fibrillin fibers in the lens capsule ([30](#)). The finding of a deletion in the fibrillin-1 (*FBN1*) gene of one autosomal dominant Weill–Marchesani syndrome family demonstrates that autosomal dominant Weill–Marchesani syndrome and Marfan syndrome are allelic conditions ([31](#)).

Although an extensive list of disorders reported to be associated with ectopia lentis is summarized in [Table 137-1](#), this discussion focuses only on the ocular manifestations of three most commonly associated multisystem diseases: Marfan syndrome, homocystinuria, and Weill–Marchesani syndrome.

In Marfan syndrome (see Chapter 153), a dominant disorder with skeletal, cardiovascular, and ocular anomalies, lens displacement occurs most commonly superiorly ([Figure 137-8](#)), although it can occur in any direction, including into the vitreous. Ectopia lentis occurs in

approximately 70% of Marfan patients and is often congenital, but it may occur later in life, progressing with the growth of the globe. Other ocular findings include enophthalmos, axial myopia, flat corneas, glaucoma, and peripheral retinal degenerative changes, with an increased incidence of retinal detachment in both phakia and aphakia. In rare cases, lens colobomas and spherophakia may be associated. Ocular manifestations tend to be bilateral and fairly symmetric. Many phenotypic variations of Marfan syndrome exist. Phenotypic variants of Marfan syndrome may be due to different mutations within the fibrillin-1 gene (32) or abnormal fibrillin microfibril morphology and assembly (33). These clinical variants may be referred to as type 1 fibrillinopathies, and the respective mutations are accessible via the international Marfan database (<http://www.umd.necker.fr>) (34).

The lens subluxation in *homocystinuria*, an inborn error of metabolism, occurs most commonly inferiorly (Figure 137-9) although it can occur in any direction. Ectopia lentis occurs in 90% of untreated homocystinuria patients. Other contrasting features to the ocular findings in Marfan syndrome include the following: (1) the zonules are weak, and dislocations occur more commonly into the anterior chamber; (2) lens displacement is not congenital but occurs during childhood or adulthood and may be progressive; and (3) patients have no abnormal angle structures. Glaucoma occurs in up to 25% of patients and is usually caused by a pupillary block mechanism secondary to lens dislocation. Other ophthalmic manifestations of homocystinuria include optic atrophy in 23% of patients, iris atrophy in 21%, anterior staphylomas in 13%, and lenticular and corneal opacities, each in 9% (35). Although the body habitus of patients with homocystinuria resembles that of Marfan syndrome, these patients may develop intellectual impairment, and increased levels of homocysteine and homocystine are found in their urine. The disease is commonly caused by an enzymatic deficiency of cystathionine b-synthetase.

In Weill–Marchesani syndrome, the lens dislocation is usually inferior. These patients are easily distinguished from patients with Marfan syndrome or homocystinuria because of their antithetical body habitus, consisting of short stature, short and wide fingers, and short and large chest (36,37). Other ocular abnormalities include angle-closure glaucoma due to peripheral anterior synechiae or to pupillary block from the lens dislocation. Myopia due to the small and round lens is also a common accompaniment (38). Weill–Marchesani syndrome has been described in both autosomal recessive and autosomal dominant forms. A locus for autosomal recessive Weill–Marchesani syndrome has been mapped to 19p13.3–p13.2, and a mutation in the fibrillin-1 gene (15q21.1) was found in two autosomal dominant Weill–Marchesani families. Differences between the autosomal recessive (AR) and autosomal dominant (AD) families were found in clinical presentation of microspherophakia (94% in AR vs 74% in AD) and ectopia lentis (64%



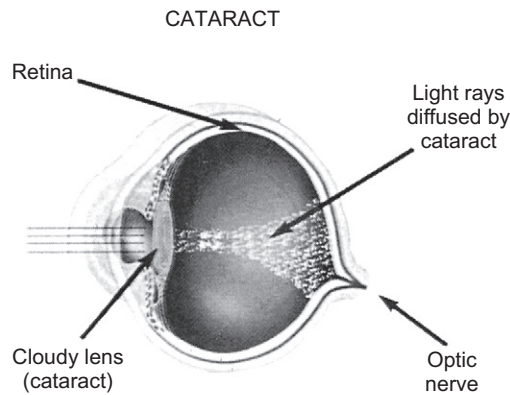
**FIGURE 137-9** Ectopia lentis in a patient with homocystinuria (inferior dislocation).

in AR vs 84% in AD) that were statistically significant, but not for myopia, glaucoma, or cataract (39). One gene associated with the autosomal recessive form is ADAMTS10, while FBN1 has been reported to be associated with the dominant form (40).

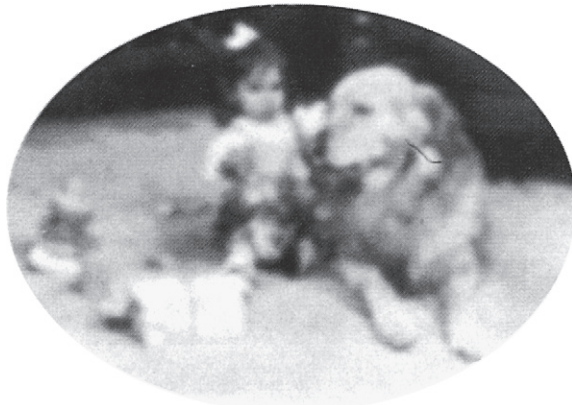
### 137.3 CATARACTS

A cataract is an opacification of the lens resulting in distorted and blurred vision (Figure 137-10). By far the most common cataract occurring in humans is the age related or senile cataract, caused by progressive opacification of the lens in adulthood. While genetic factors play a role in its development, it is a multifactorial and probably polygenic disorder. Environmental factors such as ultraviolet (UV) radiation from sunlight are thought to play an important etiologic role. Age-related cataract is so common that it is generally not regarded as a genetic disorder. Twin and sibling studies of age-related cataract have shown that genetic risk factors likely play a role in many cases of nuclear and cortical cataract. In addition, variations in at least eight genes known to cause inherited infantile and juvenile cataracts have been linked to age-related cataract (41).

From a genetic perspective, there are four important types of cataract: isolated hereditary congenital cataracts, cataracts associated primarily with ocular disorders, cataracts associated with genetic syndromes, and cataracts associated with metabolic disorders. The formal definitions of infantile, developmental, juvenile and congenital cataracts differ somewhat. It is useful to think



*A cataract is a clouding of the eye which causes vision to be distorted and blurred.*



**FIGURE 137-10** Illustration of the optical effects of a cataract.

of congenital and infantile cataracts as those that occur within the first year of life. This discussion uses the terms congenital and infantile interchangeably. Juvenile cataract generally means a cataract with onset in the first decade of life. Presenile cataracts are those that present before approximately 45 years of age and those that present after 45 years are considered age related or senile (42).

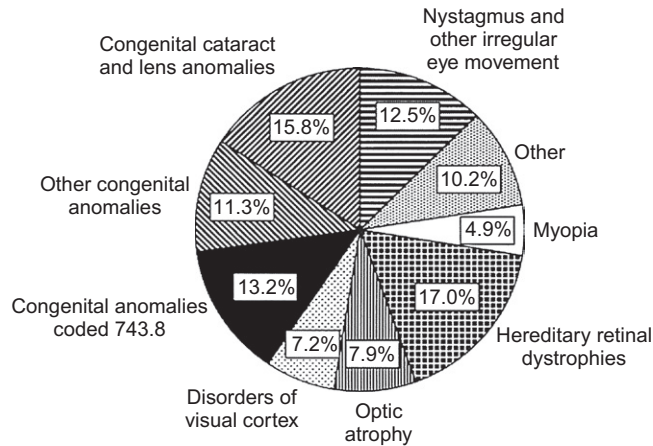
Congenital cataracts are one of the few true emergencies in pediatric ophthalmology. This is because in order to get a good visual outcome, surgery to remove the cloudy lens, and optical correction to replace it, must be achieved before 4 months of age. This requires early detection, preferably in the newborn nursery. Since virtually all bilateral congenital cataracts are genetic, either as isolated lens or ocular disorders, or part of a syndrome, there is also an urgent need for genetic counseling and guidance on surveillance, services, and prognosis for these families. We now know that almost 200 different genes and loci play a role in cataracts (41), yet we still do not have a means of routinely testing these children, making counseling a challenge. While most cases are autosomal dominant, an important minority are autosomal recessive and X-linked. The important finding that some mutations of the Nance-Horan Syndrome gene can cause isolated congenital cataract broadens the testing and counseling landscape. When there systemic

anomalies associated, the list of testable genes increases. This is true when there are specific related ocular findings as well. In some families, congenital cataract may be the most obvious sign of aniridia, caused by *PAX6* gene mutations. These eyes also have some degree of iris hypoplasia and foveal hypoplasia, as well as a high risk of corneal anomalies and glaucoma. Several other genes have also been implicated in anterior segment dysgenesis associated with congenital cataracts.

Congenital cataracts are responsible for approximately 10% of all blindness in children worldwide (43). One in 250 newborns has some form of congenital cataract, although some of these are not clinically significant. A national surveillance system in the United States that monitors hospital discharge data for birth defects (the Birth Defects Monitoring Program [BDMP]) demonstrated a prevalence of neonatal cataracts from 1988 to 1991 of 1.2 per 10,000 births (43). Some authors believe this statistic to be an underestimate (44). A Spanish study (45) yielded a figure of 6.31 per 100,000, in a study of over one million consecutive births between 1980 and 1995, as detected within the first three days of life. In contrast, a French study of 131,760 consecutive births yielded a figure similar to the US study, with 2.3 per 10,000 births (46). Studies in the United Kingdom (47) showed that a substantial proportion of congenital and infantile cataracts were initially missed, despite a national plan for routine screening. Other large studies from the United Kingdom and Australia both estimated the incidence to be between 2.2 and 2.49 per 20,000 live births (47,48). Variations in the figures demonstrate the difficulties in diagnosing neonatal cataracts. In the United States, the onus for diagnosing congenital cataracts usually falls on pediatricians, who are charged with assessing the red reflex of all newborns before discharge from the hospital. In one study, only 57% of congenital cataract patients were examined by an ophthalmologist by 3 months and 33% were not evaluated until after 1 year of life. In addition, documentation of surveillance and identification in large-scale studies is difficult, although it may be improved with capture-recapture type of data collection (49). A screening of 2447 4-year-olds yielded a prevalence of 7.7 cataracts per 10,000, indicating that symptoms leading to diagnosis or repeated examinations may increase the diagnostic yield. Of these cataracts identified, 40–55% were unilateral.

It was found that 15% of visually handicapped children have congenital cataract (Figure 137-11) (50). About 25–50% of congenital cataracts are hereditary, but a patient with a congenital cataract must undergo a complete workup to rule out other causes before this is determined (51). About 15% of congenital cataracts are associated with a multisystem syndrome, 70% are isolated, and another 15% are associated with abnormalities of other parts of the eye (42). A suggested clinical approach is outlined in Table 137-3.





**FIGURE 137-11** Causes of partial sight among new registrations aged 0–15 months. (From (50) with permission.)

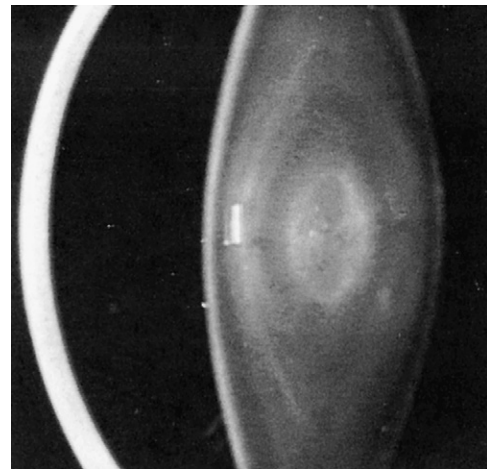
**TABLE 137-3 Diagnostic Approach to a Patient with a Congenital Cataract**

History
Trauma
Radiation exposure
Systemic disease
Maternal illness
Exposure to drugs
Family history of cataracts (family pedigree)
Eye exam
Slit-lamp evaluation of cataract
Retinoscopy
Ophthalmoscopy
Intraocular pressure
Gonioscopy
Examine family members for the same
Pediatric evaluation by pediatrician
Genetics evaluation by geneticist
Laboratory tests may include <sup>a</sup>
Antibody titers (rubella, CMV)
Urine sugars (galactosemia, galactokinase deficiency)
Karyotype/chromosome microarray (chromosomal anomalies)
Blood glucose
Urine microscopy (Alport syndrome)
Serum calcium, phosphorus, alkaline phosphatase (hypoparathyroidism, pseudohypoparathyroidism)
VDRL test
RBC galactokinase activity and RBC galactose-1-phosphate-uridylyltransferase (galactosemia)
Plasma amino acids (homocystinuria)
Urine protein (Alport syndrome)
Urine amino acid content (Lowe syndrome)
Urine copper level (Wilson disease)
Cholesterol pathway enzymes (Smith–Lemli–Opitz syndrome, cerebrotendinoxanthomatosis)
X-rays (Conradi’s syndrome)
Serum ferritin (hyperferritinemia cataract)

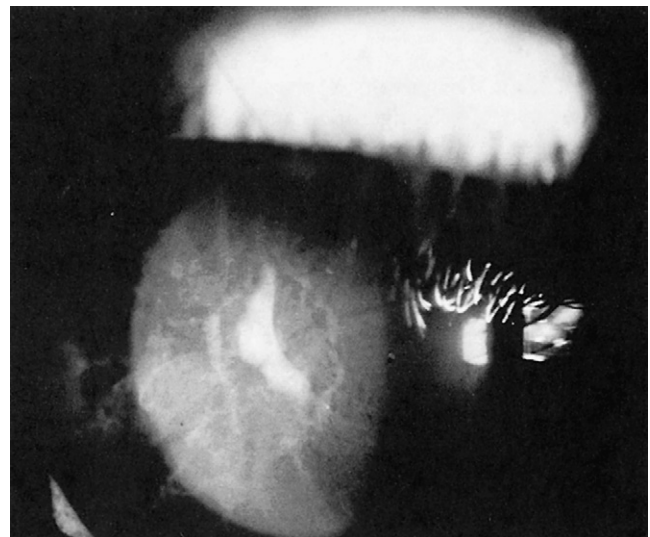
<sup>a</sup>Note the laboratory test performed will be dependent on the level of suspicion of a particular systemic disease.

### 137.3.1 Classification

Congenital cataracts can be classified according to morphology. For simplification, these can be divided into four broad categories: zonular, polar, total (mature), and membranous (51). Zonular cataracts involve one zone of the lens and can be subdivided into nuclear (Figure 137-12), lamellar (Figure 137-13), sutural (Figures 137-14 and 137-15), spear-like, coralliform, floriform, and capsular cataracts. The nuclear type is often bilateral and is usually associated with a significant decrease in visual acuity. The lamellar subtype (by far the most common type of congenital cataract) is characterized by an opaque layer surrounding a relatively clear nucleus. Lamellar cataracts are typically bilateral but slightly asymmetric, and generally have autosomal dominant inheritance (52). They may or may not severely affect vision. Sutural cataracts (involving the Y sutures) and small capsular cataracts often do not affect vision. Sutural cataracts are occasionally found



**FIGURE 137-12** Central pulverulent nuclear cataract.



**FIGURE 137-13** Lamellar cataract.



in asymptomatic relatives of affected patients. Autosomal dominant zonular cataracts with sutural opacities have been mapped to chromosome 17q11–12, near the beta A3/A1 crystallin gene (53). Aculeiform cataracts are punctate, needle-like cataracts, which are morphologically similar, but distinct from coralliform cataracts. They are usually bilateral with dominant inheritance, complete penetrance, and variable expressivity. The aculeiform cataract maps to the  $\gamma$ -crystallin gene (*CRYGD*) on chromosome 2q33–35, which lies in close proximity to *CRYBA2* (54).

Polar cataracts involve either the anterior (Figure 137-16) or posterior (Figure 137-17) pole of the lens and affect the capsule and underlying lens with limited visual detriment if they are less than 3 mm in diameter, although anterior polar cataracts may be associated with corneal astigmatism and hyperopia. Autosomal dominant anterior polar cataracts were associated with a 2;4 translocation in one family and were localized to 17p12–13 in another family (55). They may also occur sporadically. Anterior pyramidal cataracts are a subset of anterior polar cataracts, involving the capsule and lens in a hyperplastic, conical fashion. Reported in both

normals and Ehlers–Danlos patients, these cataracts are more visually significant than smaller, flatter anterior polar cataracts (16). They may be unilateral or bilateral (56). In total cataracts, the entire lens is opaque and is associated with poor visual acuity and nystagmus. Total cataracts may be found in Down syndrome, metabolic cataracts, congenital rubella, and rarely as a severe Mendelian cataract, as well as sporadically. Membranous cataracts involve an opaque, thin, fibrotic lens caused by resorption of lens protein and subsequent thinning in the anteroposterior direction of the lens. The anterior and posterior capsules fuse to form a dense white membrane. This condition may be associated with other ocular abnormalities, including PHPV, aniridia, and decompensated lenticonus, as well as with systemic syndromes including congenital rubella, Hallerman–Streiff, or Lowe syndromes. Cerulean cortical cataracts have been linked to mutations in the  $\beta$ -crystallin gene on human chromosome 22q (52). A second site for cerulean

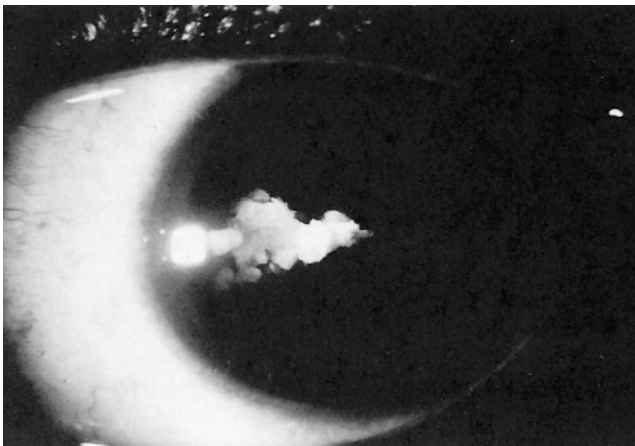


FIGURE 137-14 Sutural cataract (Y suture) female carrier.

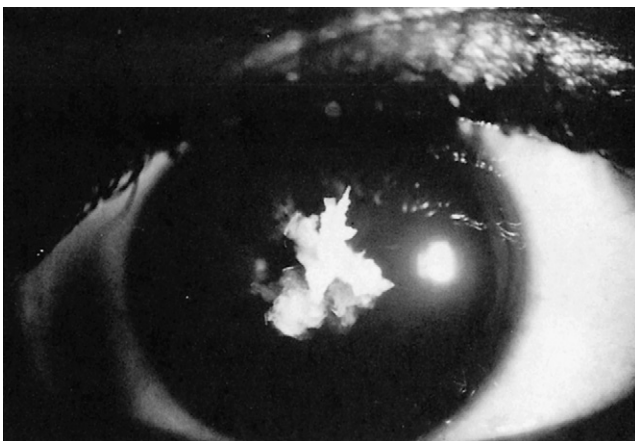


FIGURE 137-15 Sutural cataract (Y suture) male.

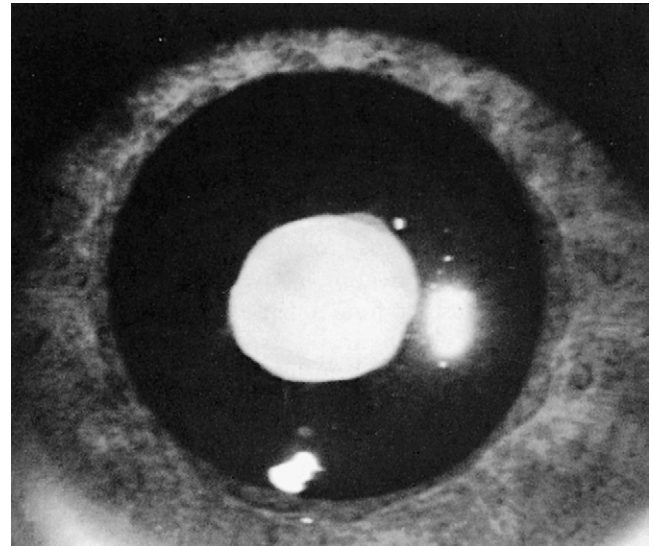


FIGURE 137-16 Anterior polar cataract.

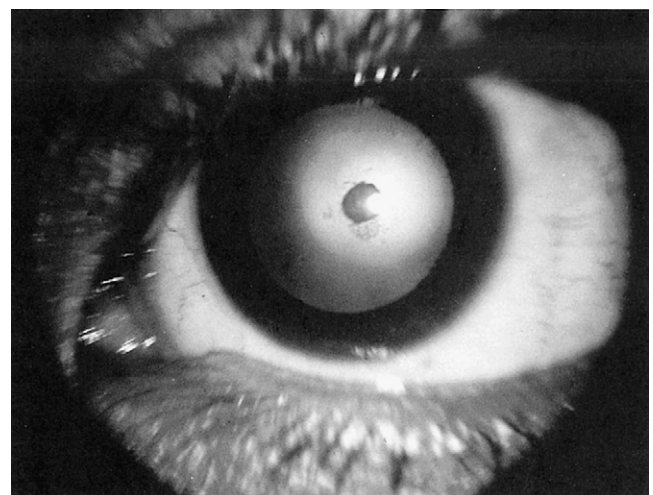


FIGURE 137-17 Posterior polar cataract.

cataracts was found on chromosome 17q24. A suture sparing congenital nuclear lenticular bilateral cataract phenotype was found in a large five-generation Swiss family, which was linked to chromosome 17, D17S1857 and found to be due to a *CRYBA3/A1* gene mutation (57). Morphological classification may help indicate age of onset, inheritance, etiology, or prognosis. Post-surgical visual outcomes have been found to portend a better prognosis in certain types of congenital cataract (specifically lamellar, pulverulent, polymorphic, coraliform, and cortical), which is important to address in clinical counseling (58). While age-related cataracts have a relatively narrow range of phenotypes—typically nuclear, cortical, posterior subcapsular, or combinations thereof—because they represent senescence of a normally formed crystalline lens, infantile cataracts are extremely polymorphic. They represent disruption of the normal formation of the lens.

### 137.3.2 Molecular Genetics

Isolated hereditary congenital cataracts can display any of the preceding morphologic subtypes (Figures 137-12 to 137-17). Although this classification is somewhat

artificial for congenital hereditary cataracts, and there is marked heterogeneity for both type and degree of cataract formation even within families, it is a useful clinical approach for identifying the various subtypes. Hereditary cataracts caused by the same gene can have very different morphology, even in members of the same family, while cataracts caused by different genes can appear similar.

Isolated congenital hereditary cataracts may be transmitted as autosomal dominant, autosomal recessive, or X-linked traits (Table 137-4). Most are inherited as autosomal dominant traits with a high degree of penetrance.

Hejtmancik has beautifully categorized the different types of genes currently known to be responsible for early-onset cataracts (42). A summary of the major genes and the mode of inheritance of the cataracts they cause may be found in Table 137-4. The genes currently known to play a role in non-syndromic cataract may be broadly grouped into three categories: those that code for crystallins, those that code for membrane/cytoskeleton proteins and those that code for transcription factors.

*Crystallin* gene mutation accounts for about 50% of isolated heritable cataract. Sixty mutations in 98 families have been reported in 10 human crystallin genes.

**TABLE 137-4 Human Cataract Genes and Loci**

Autosomal Dominant	Autosomal Recessive	X-linked	Sporadic
CCV (1p36)	GCNT2(6p24–p23)	CXN (Xp22)	
CTPP (1p34–p36)	CAAR (9q13–q22)	NHS (Xp22.13)	
FOXE3 (1p32)	CHX10 (14q24.3)		
GJA8 (1q21–25)	GJA8 (1q21–25)		
HSF4 (16q22.1)	HSF4 (16q22.1)		HSF4 (16q22.1)
2p24	3p22–p24.2		
CCNP (2p12)	LIM2 (19q)		
CRYGC (2q33–q35)	BFSP1 (20p11.23–p12.1)		
CRYGD (2q33–q35)	i (6p24)		
BFSP2 (3q21–q22)			
CRYGS (3q26.3)			
EYA1 (8q13.3)			
PITX3 (10q25)			
CRYAB (11q23.3)			
AQPO (12q12–14.1)			
GJA3 (13q11–13)			
CCSSO (15q21–22)			
MAF (16q23)			
CTAA2 (17p13)			
CRYBA3 (17q11–q12)			
CCA1 (17q24)			
19q13.4			
FTL (19q13.4)	19q13.4		
CPP3 (20p12–q12)			
CHMP4B (20q11.22)			
CRYAA (21q22.3)	CRYAA (21q22.3)		CRYAA (21q22.3)
CRYBB2 (22q11.2)			
CRYBB1 (22q11.2)	CRYBB1 (22q11.2)		
CRYBB3 (22q11.2)	CRYBB3 (22q11.2)		
CRYBA4 (22q11.2)			

Adapted from (42).

These typically cause nuclear or lamellar opacities. Most are isolated cataract; however, some are associated with microcornea or myopathy as well (41). Numerous mouse strains with various crystallin genes mutated also demonstrate cataract (41). Crystallins are the predominant protein family found within the lens, with multiple subtypes. One such site is a missense mutation near the  $\alpha$ -crystallin gene, *CRYAA* on chromosome 21q22.3 (59). Investigators identified the cause for a hereditary neuromuscular disorder, desmin-related myopathy, as due to a missense mutation in a highly conserved arginine (R120G) within *CRYAB*, the gene for  $\alpha$ B-crystallin on human chromosome 11q21–23 (60). The  $\alpha$ B-crystallin protein serves as a molecular chaperone and is a member of the small heat shock protein family, found in skeletal and cardiac muscle, in addition to the lens. This point mutation leads to an irregular structure and decreased chaperone function, which may be responsible for the phenotypes of autosomal dominant congenital cataracts and desmin-related myopathy (61,62).

Approximately 35% of isolated heritable cataracts have been found to be associated with mutations in genes for *membrane or cytoskeletal proteins* (41). These genes include connexins, major intrinsic protein (*MIP*), and lens intrinsic membrane (*LIM2*) protein. The connexins code for gap-junction proteins. Two of these genes, *GJA3* and *GJA8*, account for 20% of all isolated hereditary cataracts. Additional genes in this group include *TMEM114*, *CHMP4B*, *ESCRTIII* and *EPH receptor A2*, *BFSP1* and *BFSP2*.

A missense mutation in the *MIP* of the lens, encoded by the gene *MIP* on 12q14, was found to cause autosomal dominant “polymorphic” and lamellar cataracts. The mutations are postulated to interfere with water flow across the lens cell membranes (63). Other genes that may be disrupted and result in cataractogenesis include cytoskeletal proteins (e.g. *BFSP2*).

Mutations in transcription factors (*MIF*, *PITX3*, *HSF4*) often cause other ocular anomalies in addition to

cataract. *HSF4* mutation accounts for about 6% of all familial cataracts (41).

The most extensively studied autosomal dominant cataract is the pulverulent cataract (Figure 137-12). The name is derived from the Latin word *pulvis*, for “powdery” or “dust-like.” Two forms of this cataract have been described. In the zonular pulverulent cataract, dust-like particles are arranged in layers with a clear center in the lens; the lens nucleus is less opacified than the surrounding zone, which contains a high density of fine grained particles (64). This type of cataract was first reported in an extensive study of the Coppock family (34,65). The terms “Coppock” cataract and zonular pulverulent cataract are often used interchangeably in the literature. The Coppock cataract has been linked to mutations in *GJA8*, part of the connexin family, on chromosome 1q21–25 (66). A second site for zonular pulverulent cataracts was found on chromosome 13, near connexin 46 (67). The Coppock-like cataract is due to a mutation in the  $\gamma$ E-crystallin gene (*CRYGE*) on chromosome 2q33–35 (68). This causes increased expression of a truncated protein product and is the first example of a disease process associated with a pseudogene (69). Other genes for the Coppock-like cataract have been found on chromosome 22q11.2–12.2, with a mutation in *CRYBB2* (70) and in *CRYGC* (71). Aculeiform cataracts, Coppock-like cataracts, and polymorphic congenital cataracts (72), all map to 2q33, despite their phenotypic differences (54). The second type of pulverulent cataract described is a central pulverulent type, with similar dust-like opacities, involving only the embryonal nucleus (73) (Figure 137-12). Pulverulent cataracts are typically bilateral and symmetric between two eyes of the same individual. However, both forms of this cataract have been shown to occur within the same family, and even in the two eyes of the same individual (74). In summary, the three groups of genes most likely to be functional candidates in congenital cataract lens mutations include the *CRYG* cluster on chromosome 2 (*CRYGC* and *CRYGD* specifically), the *CRYBB2* on chromosome 22, and *GJA8* gene on chromosome 1 (75).

Variable expression in morphology of pulverulent cataracts in different members of a given family has been described, ranging from faint lens opacities that were difficult to detect, to opacities that obscured the view of the fundus (50); interocular phenotypic variability with seemingly random occurrence of both unilateral and bilateral cataracts in affected individuals in the same pedigree also occurs.

It is likely that the pulverulent cataract has more than one cause, because the genes responsible for this type of cataract have been linked to multiple loci; other studies have failed to demonstrate similar linkage. Isolated congenital cataract transmitted as an autosomal recessive trait is not as common as autosomal dominant; however, it does occur, especially in populations with a high percentage of consanguineous marriages,

**TABLE 137-5**     **Rodent Models of Cataract Mutations**

Name	Protein	Phenotype
Philly	$\beta$ 2-crystallin	Nuclear
ELO	$\gamma$ E-crystallin	
Fraser	MP-26	
LOP	MP-26	
To3	MP-10	Total
Hf1	MP-26	
No2	Connexin50	
Cat	MIP	Nuclear/total (ASOD)
Cat2	$\gamma$ -crystallin	
Cat4		
ak	<i>PITX3</i>	Aphakia
Cryba2	BetaA2 crystallin	Small lens, acquired cataract



and it constitutes a significant proportion of hereditary isolated cataracts (52). Mutations have been identified in the *LIM2* gene on 19q13.4 in one family with autosomal recessive pulverulent cataract and a mutation was found in a different consanguineous family with autosomal recessive cataract in the *CRYAA* gene. Other loci previously encountered to be responsible for autosomal recessive cataract include *i* on 6p24, 9q13–22, and 3p (41,42).

Many genes may cause or contribute to the occurrence of congenital cataracts (41,42). To date, genes causing autosomal dominant hereditary cataracts have been mapped to chromosomes 1, 2, and 16 using linkage analysis, and to every other autosome and X using linkage analysis, as well as a variety of other techniques (41,42, <http://cat-map.wustl.edu/>). Autosomal dominant congenital cataracts have been attributed to a missense mutation in the human  $\alpha$ -crystallin gene *CRYAA* on chromosome 21q22.3 (62). Mutations in the *CRYBB2* gene have been shown to cause nuclear cataracts, presumably by altering the folding, and hence the structure of the crystalline protein. The *CRYBB2* gene on chromosome 22 is one of the more important genes in determining the transparency of the lens. As of 2008 there were 39 mapped cataract loci and at least 26 of these locations have a known specific gene and mutation, with more than 100 different cataract mutants identified (42,76,77). A progressive early-onset cataract gene has been mapped to chromosome 17 (78). A locus for autosomal dominant anterior polar cataract has been linked to chromosome 17p, in a four-generation pedigree (79). In addition, autosomal recessive cataracts have been shown to be associated with the small *i* allele (80). Finally, cataracts have been associated with chromosomal translocations, including 3:4, 2:14, and 2:16 and a chromosome 9 inversion (20,81,82). In summary, although distinct morphologic types are identified and classification schemes are available, it is still important to recall that there is much genetic and morphological heterogeneity involved. Because of the genetic complexity of congenital cataracts, fee-for-service genetic testing is not available at this writing.

### 137.3.3 Animal Models

The etiology and pathogenesis of most cataracts is poorly understood. Because the organogenesis and physiology of the lens are essentially similar in various mammals, an understanding of cataract formation in animal models could enhance our knowledge of cataractogenesis in humans. Development of the lens is complex, with many growth factors involved, including fibroblast growth factors and transcription factors (e.g. *PAX6* and others). *PAX6*, in particular, has been identified in the lens vesicle by Western blot analysis as early as 6 weeks gestation (83). Heterozygous mutations in

*PAX6* are known to cause aniridia and microphthalmos, and cataract in some human patients, and homozygous *PAX6* mutations cause severe brain defects as well as congenital absence of the eye. While *PAX6* appears to be a major regulator of eye development, other homeobox genes are also involved, including *PITX3*, *SIX3*, and *OPTX2* (84). The galactose susceptibility of the rat lens has led to its use as a model for galactosemic cataracts (Figure 137-18). Hereditary cataracts have been described in rats, mice, fish, fowl, cattle, dogs (Figure 137-18), the degu, and guinea pig. The various models for inherited congenital cataracts in animals have been reviewed (85). The most commonly studied models are rodents. The main advantages of using rodent mutants include the well-researched genetics of the animals and the comparative ease of breeding large litters. In mice, the models include the Cts strain, Fraser mouse, lens opacity gene (*Lop*) strain, *Lop-2* and *Lop-3* strains, Philly mouse, Nakano mouse, *Nop* strain, Deer mouse, Emory mouse, Swiss Webster strain, Balb/c-net/net mouse, SAM-R/3 strain, and the *Aey2* mutant line, which involves a mutation in the *Crybb2* gene on chromosome 5 (86). Mouse mutants on chromosome 1 (*Cat2*) have mutations in the  $\gamma$ -crystallin gene (87). A mutant on mouse chromosome 3 (*No2*) corresponds with a missense mutation in the human connexin *Cx50* gene. Targeted ablation of *Cx50* in mice resulted in microphthalmos and zonular pulverulent cataracts. *Cx50* is an intercellular channel-forming protein, important for lens transparency as well as lens and overall ocular development (88). The associated human phenotype, the zonular pulverulent cataract, is transmitted in an autosomal dominant fashion, as studied using a missense mutation in the human connexin 50 gene (*P88S*) on chromosome 1q21–25. Only one *P88S* mutant is needed in each gap junction to abolish its proper function (89). The Philly mutant has a mutation in the  $\beta$ -crystallin gene on mouse chromosome 5. Cataracts due to a mutation in *MP10*, on chromosome 10 (*Cat*), are due to a mutation in the *MIP* corresponding to human 12q. The *ak* mutant on mouse



FIGURE 137-18 Cataract rat fed high-galactose diet.



chromosome 19 is due to a genetic defect in homeobox *PITX3*, corresponding to the human chromosome 10q25 (90). The mouse *cat4* mutant on chromosome 8 has anterior polar cataracts, central opacities, and lens–corneal adhesions, analogous to anterior segment ocular dysgenesis (ASOD), which may be due to a mutation in human chromosome 4q (91). A mouse with small lenses and a progressive cataract, which may be a model for age-related cataract, has mutations in *Cryba2* (92), and a mouse homozygous null for *TDRD7* develops cataracts as well as glaucomas (20). The mouse is a valuable model of human cataracts, but it also has an idiosyncrasy of the crystallin lens, which may hold answers in the future: when a mouse becomes very cold and/or when its cornea becomes very dry, the mouse lens rapidly becomes opaque. This opacity reverses quickly with increased body temperature and/or moisture. This reversible opacity of the lens is specific to mice and some other rodents, and even some young kittens.

Rat models include BUdr, ICR, Sprague–Dawley, and Wistar, spontaneously hypertensive rat (SHR), John Rapp inbred strain of Dahl salt-sensitive rat, as well as the WBN/Kob, Royal College of Surgeons (RCS), and Brown-Norway rats (Table 137-6). Because of the ease of making clinical observations in vivo and the subsequent availability of the intact lens for laboratory analyses at different stages of cataract formation, these animals provide excellent models for clinicopathological correlations, for monitoring the natural history of the aging process and metabolic defects, and for investigations on the effects of cataract-modulating agents and drugs, including the prospect of gene therapy (93). The miniature schnauzer (Figure 137-19), in which cataracts are inherited as an autosomal recessive trait, has served as a dog model for recent studies on congenital cataracts and microphthalmia (94).

### 137.3.4 Ocular Disorders Associated with Cataract

Cataracts associated primarily with ocular disorders are summarized in Table 137-7. For practical purposes, the eye is divided into two segments: an anterior segment, consisting of the cornea, angle structures, iris, and lens; and the posterior segment, consisting of the vitreous jelly and the retina. The anterior segment disorders listed in Table 137-7 are aniridia, anterior segment (mesenchymal) dysgenesis, Peter's anomaly, granular dystrophy, microcornea, and microphthalmia (which can also involve the posterior segment). The balance of the disorders is primarily of posterior segment origin. Embryologically, the anterior segment is derived from surface ectoderm and the neural crest. During embryological development of the anterior segment, neural crest cells separate the anterior one-third of the cornea and the lens (both surface ectodermal structures), which it invades in waves to form the posterior two thirds of the cornea, the angle structures, the anterior chamber, and the iris. Interference with this process of separation has been postulated to result in varying degrees of anterior segment abnormalities, such as aniridia (absent iris), which can be either partial (Figure 137-20) or complete; Rieger anomaly (iris hypoplasia and abnormal angle structures); Peters anomaly (central corneal leukoma and cataract) (Figure 137-21); microcornea (small cornea); and microphthalmia (small disorganized eye), all of which have been reported in association with cataracts. A recent report demonstrating a deletion at the *PAX6* locus (a homeobox gene) in a child with Peter's anomaly and heterozygosity in the R26 mutation in the *PAX6* paired box in family members with varying degrees of dominantly inherited anterior segment malformations, including Peter's anomaly, provides strong evidence in support of this theory (94). Many patients with *PAX6*

**TABLE 137-6** Cataracts Associated with Primarily Ocular Disorders

Aniridia
Anterior segment dysgenesis
Choroideremia
Cone-rod degeneration
Favre hyaloideoretinal degeneration
Granular corneal dystrophy
Leber's congenital amaurosis
Microcornea
Microphthalmia
Norrie disease
Peter's anomaly
Persistent hyperplastic vitreous
Retinitis pigmentosa
Snowflake vitreoretinal degeneration
Vitreoretinchoroidopathy
Wagner's hyaloideoretinal degeneration

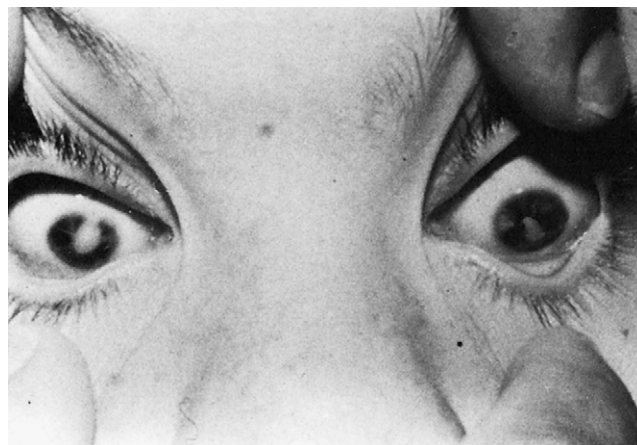


**FIGURE 137-19** Miniature schnauzer, an autosomal recessive cataract animal model.

**TABLE 137-7** Cataracts Associated with Autosomal Syndromic Genetic Diseases

Autosomal dominant
Chondrodysplasia punctata
Hallerman–Strieff syndrome
Incontinentia pigmenti
Kniest disease
Myotonic dystrophy
Nail patella syndrome
Neurofibromatosis type II
Oculodentodigital syndrome
Progeria syndrome
Stickler syndrome
Tuberous sclerosis
Branchio-oculo-facial syndrome
Autosomal recessive
Bardet–Biedl syndrome
Cerebrotendinous xanthomatosis
Chondrodysplasia punctata
Cockayne syndrome
Congenital ichthyosis
Galactosemia
Hallerman–Streiff syndrome
Homocystinuria
Mannosidosis
Marinesco–Sjögren syndrome
Neuraminidase deficiency
Refsum syndrome
Smith–Lemli–Opitz syndrome
Usher disease
Wilson disease
Zellweger syndrome
X-linked
Alport syndrome
Fabry disease
G6PD deficiency
Lenz dysplasia
Lowe syndrome
Nance–Horan syndrome
Pseudohypoparathyroidism
X-linked dominant chondrodysplasia punctata

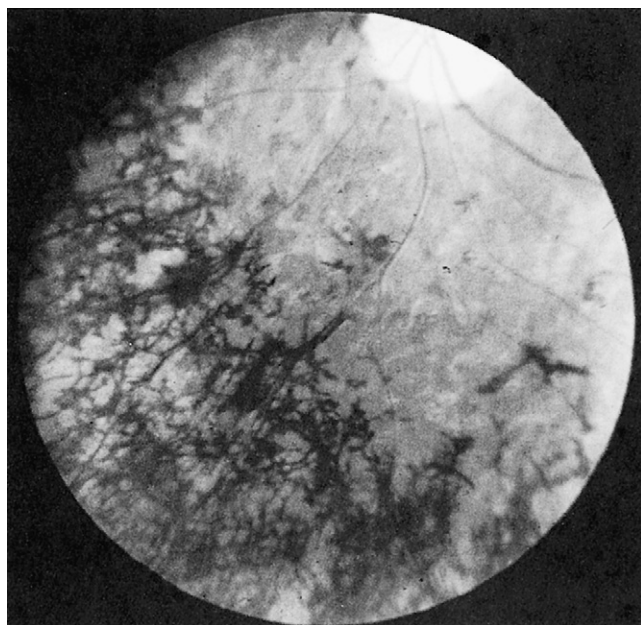
Adapted from Hejtmancik et al. (42) with permission.

**FIGURE 137-20** Cataract in aniridia (incomplete).**FIGURE 137-21** Cataracts in a patient with Peters anomaly. (Courtesy of Steven Orlin, MD, Scheie Eye Institute.)

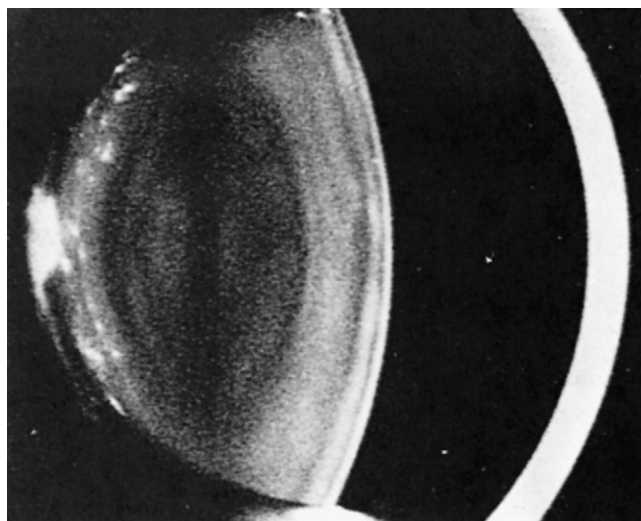
associated aniridia develop cataracts. Other pedigrees with autosomal dominant cataracts have excluded linkage to *PAX6*, indicating that multiple genes may be significantly involved in anterior segment development (95). *PITX3*, another homeobox gene on chromosome 10q25, was found to be mutated in families with autosomal dominant cataracts and anterior segment mesenchymal dysgenesis (ASMD). *PITX3* is part of the *RIEG/PITX* homeobox gene family, which has been found to be centrally involved in ocular development (2). Other cases of corneal anomalies associated with cataracts include several reports of microcornea and cataract inherited as an autosomal dominant trait (70).

Of the posterior segment disorders listed in Table 137-6, many of which are described in detail elsewhere in this text, retinitis pigmentosa (Figure 137-22) is the most common (1 in 3600 in the general population). Cataracts in this condition, as in other forms of retinal degeneration, typically develop in the third to fourth decade of life and are usually posterior subcapsular in nature (Figures 137-23 and 137-24). These cataracts appear to develop secondary to a cellular response in the vitreous (perhaps inflammatory), not as a primary part of the genetic disorder. Cataracts are also reported in Wagner syndrome, and in hereditary vitreoretinopathies, such as Stickler syndrome. A review of the original pedigree of Wagner syndrome demonstrated that all patients had cataracts by age 45 (96,97), and in most cases had required removal in the previous decade of life. In addition to cataracts and vitreoretinopathy, one such syndrome, which localized to a similar region within chromosome 5q13–14, included ectopia lentis, microphthalmia, persistent hypoplastic primary vitreous, anterior segment dysgenesis, and congenital glaucoma (98). This may be called Wagner plus syndrome and is also known as Cotlier syndrome. Cataracts occur in approximately 50% of patients with Stickler syndrome, or hereditary progressive arthroophthalmopathy, a connective tissue disorder in which the main associated ocular abnormality is retinal detachment, as well as high myopia. In one series, a distinctive wedge and fleck-shaped cataract was

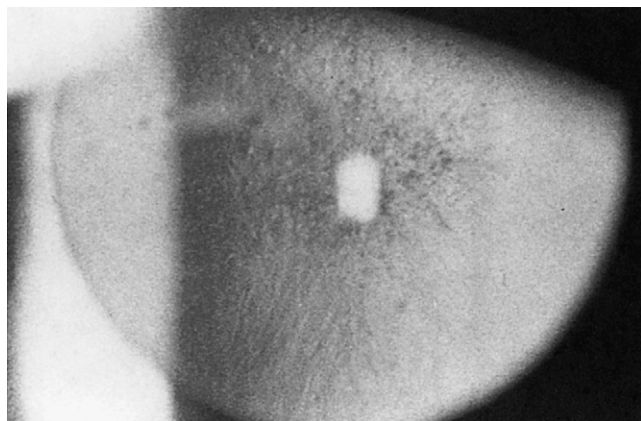




**FIGURE 137-22** Retinal photo showing bone spicule formation in a patient with retinitis pigmentosa.



**FIGURE 137-23** Slit-lamp photo demonstrating posterior location of PSC found in retinitis pigmentosa.



**FIGURE 137-24** Same cataract shown in Figure 137-3, with diffuse illumination.

found in 43% of patients studied; Seri et al. (99) suggested that these may serve as an early marker for the disease and facilitate early diagnosis. Stickler syndrome patients may be divided into two types. Those with mutations in the *COL2A1* gene tend to have the type 1 vitreous phenotype or *COL11A1* with type 2 vitreous changes. Those with a third mutation, *COL11A2*, have systemic features without any ocular findings (100).

### 137.3.5 Systemic Disorders Associated with Cataract

Cataracts associated with autosomal syndromic genetic syndromes overlap somewhat with cataracts associated with metabolic disorders (which are predominantly recessive); some of the more important disorders in both groups are listed together in Table 137-7.

Cataracts are a feature of several disorders, which are primarily skeletal in nature: chondrodysplasia punctata, pseudohypoparathyroidism, and Stickler syndrome (discussed previously), with which they are commonly associated; and Kniest dysplasia, Marfan syndrome, homocystinuria, and Majewski syndrome with which they are more rarely associated.

Chondrodysplasia punctata (Conradi–Hunermann syndrome) is characterized by asymmetric limb shortness, early punctate mineralization, and large skin pores resembling orange peel, as well as sparse coarse hair. Both dominant and X-linked dominant cases (lethal in males) have been described. Cataracts occur in up to 17% of patients. A recessive form (rhizomelic type) has also been described, characterized by short humeri and femora, coronal cleft in vertebrae, and punctate epiphyseal mineralization. Cataracts occur in up to 72% of patients in this group.

Pseudohypoparathyroidism (Seabright–Albright syndrome) is a defect in calcium phosphate metabolism. Both short stature and cataracts are related to the hypocalcemia that occurs in these patients. Other abnormalities include subcutaneous calcifications and brachydactyly. Because of a defect of kidney and skeletal adenylcyclase, serum calcium is low and serum phosphate elevated in spite of excessive secretion of parathyroid hormone.

In Hallermann–Streiff syndrome, or oculomandibulodyscephaly with hypotrichosis syndrome, cataracts occur in 94% of cases and microphthalmia occurs in 80% of cases. Other features comprising this syndrome are a small pinched or parrot-like nose, and hypotrichosis. Other occasional ocular abnormalities include blue sclera, nystagmus, strabismus, optic disc colobomata, glaucoma, and retinal pigment alterations.

Cataracts commonly occur in association with several skin diseases, including Werner syndrome, anhidrotic ectodermal dysplasia, hereditary ichthyosis, Rothmund–Thomson syndrome, and incontinentia pigmenti. Rothmund–Thomson syndrome, which includes poikiloderma, growth deficiency, premature aging, and predisposition to some malignancies, in addition to bilateral

cataracts, is attributed to mutations in the RECQL4 heliase gene (101,102). An important congenital malformation in incontinentia pigmenti is retinal dysplasia, but juvenile cataracts occur commonly in this disorder. Other ocular abnormalities include corneal opacities, strabismus, optic atrophy, uveitis, chorioretinitis, microphthalmos, nystagmus, and nasolacrimal duct obstruction.

Cataracts occur in many neurological syndromes. Congenital cataracts and microcornea are found in the autosomal recessive congenital cataract facial dysmorphism neuropathy (CCFDN) syndrome, which includes motor neuropathy, and moderate mental retardation with occasional pyramidal signs (103). A syndrome involving bilateral cataracts, spastic paraparesis with amyotrophy, and gastroesophageal reflux with persistent vomiting was mapped to chromosome 10q23.3–q24.2 (99).

Myotonic dystrophy is a dominant muscular dystrophy with a distinctive type of cataract. Typical lens changes occur in all patients in the second decade of life as multiple spots of opacity, with colored crystals localized in the anterior and posterior subcapsular layers in the form of a thin band, later progressing to maturity (104). Although characteristic opacities seen only by slit-lamp examination in the past constituted a principal method of presymptomatic detection of gene carriers, the discovery that the dystrophy mutation is an unstable DNA sequence, composed of varying numbers of CTG triplet repeats in the *DMPK* gene, allows for a specific molecular test for this disorder (105). Lens epithelial cells from patients with myotonic dystrophy were found to have decreased amounts of myotonic dystrophy protein kinase gene and decreased epithelial cell densities, perhaps leading to the iridescent opacities or PSCs observed in this syndrome (106). Experiments demonstrate that mice with deficiency in the *Six5* gene adjacent to the *DPMK* gene develop cataracts, and that the rate and severity of cataract formation is inversely related to the amount of *Six5* present (107,108). Congenital nuclear cataracts, with later developing anterior subcapsular cataracts, were reported in a patient with mild Becker muscular dystrophy due to a giant dystrophin deletion, similar to that found in the *mdx* mouse (109). It must be noted that the iridescent opacities characteristic of MD cataracts may also be found in the general population (110).

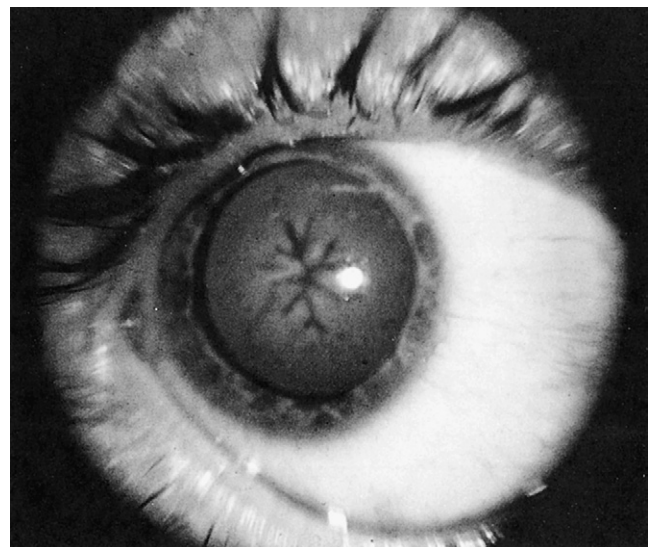
Neurofibromatosis 2 is an autosomal dominant disorder characterized by development of bilateral vestibular schwannomas and central nervous system (CNS) tumors (111). Its incidence is approximately 1 per 40,000 live births. In a recent survey of 49 patients with neurofibromatosis type 2, cataracts were found in 69% of patients. Cataracts can be the initial mode of presentation. The types of cataracts found in neurofibromatosis type 2 were posterior subcapsular or capsular (26.5%), cortical (26.5%), mixed (14.2%), and Mittendorf dots (8.2%) (112). Cataracts have also been reported in tuberous

sclerosis, another of the autosomal dominant inherited phakomatoses (113). Two rare dominant disorders in which cataracts also associated are nail patella syndrome and the oculodentodigital syndrome.

Several renal diseases have cataracts as part of their presentation. These include Lowe syndrome, Hunter-Jurenko syndrome, renal-dysplasia-cataract-blindness syndrome, Senior-Loken syndrome, Alport syndrome, Fabry disease, and Zellweger syndrome, as well as an autosomal recessive syndrome with immune complex glomerulonephritis and ophthalmic abnormalities (114).

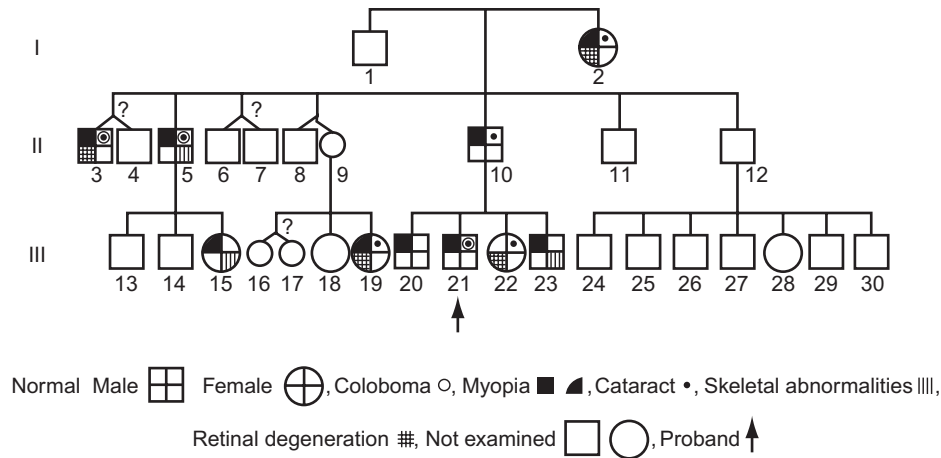
The most common in this group of diseases is Lowe syndrome, an oculocerebrorenal syndrome, with X-linked recessive inheritance. Affected hemizygous males have mental retardation, renal aminoaciduria, and ocular abnormalities, including congenital cataracts. Other ocular anomalies include glaucoma with dysgenic angles, pupillary abnormalities, and corneal keloids. Female carriers of Lowe disease have characteristic punctate spoke-like opacities in the lens cortex (115). The Lyon effect has been invoked in this disorder, with all lens fibers involved in affected hemizygotes, and only a proportion of fibers affected in female carriers, resulting in the characteristic spoke-like opacities. Since the gene for Lowe syndrome has been mapped on the X chromosome, suspected carriers can now be confirmed by molecular genetic studies (116). Different mutations within the *OCRL-1* gene may lead to different phenotypes (117). Lowe syndrome is a ciliopathy.

In Fabry disease, cataracts appear later in life. The female carrier has characteristic spoke-like lens opacities (Figure 137-25). Other ocular findings include vascular lesions of the conjunctiva, whorl-like opacities of the cornea, and vascular lesions of the retina (118). In Alport syndrome, described earlier, anterior lenticonus



**FIGURE 137-25** Spoke-like cataract in a patient with Fabry disease.





**FIGURE 137-26** Family pedigree showing autosomal dominant inheritance in Cotlier syndrome.

is the more prominent finding, with cataracts occurring only rarely.

Zellweger syndrome, or cerebrohepatorenal syndrome, is a multisystem disorder resulting from peroxisome malfunction, abnormal development of the skull, face, ear, hands, and feet, as well as some mental retardation, hepatic interstitial fibrosis, renal and cortical cysts, and ocular anomalies. Zonular cataracts are noted in association with this syndrome. Heterozygote carriers have curvilinear lens opacities, which can be a useful diagnostic aid if seen in a parent with a child who has the Zellweger facies and who is suspected of having the disorder. Other ocular abnormalities include cloudy cornea, optic atrophy, and retinal dystrophy with an extinct electroretinogram (ERG) (119).

Several other systemic diseases have retinal dystrophy and cataracts as part of the ocular manifestations. These include Refsum disease, Usher syndrome, Kearns–Sayre syndrome, gyrate atrophy, choroideremia, Bardet–Biedl syndrome, Cockayne syndrome, (see Chapter 147) and Cotlier syndrome. Cataracts have been identified in both early onset and classic Cockayne syndrome (120). Cotlier syndrome is an autosomal dominant disorder (Figure 137-26) with dense, punctate infantile cataracts (Figure 137-27), lens dislocation, retinal degeneration of the pigmentary perivascular type (Figure 137-28), retinal detachment, and skeletal Marfan-like dysmorphic features.

Cataracts associated with microcephaly and mental retardation are seen in Torsten Sjögren syndrome, Marinesco–Sjögren syndrome, Crome syndrome, Martosolf syndrome, CAMFAK syndrome, Lenz dysplasia, Smith–Lemli–Opitz syndrome, and Menkes syndrome. Micro syndrome encompasses autosomal recessive microcephaly, microcornea, congenital cataract, mental retardation, agenesis of the corpus callosum, retinal and optic atrophy, and hypogenitalism (121,122).

Marinesco–Sjögren syndrome consists of congenital cataract, mental retardation, and cerebellar ataxia (123) with lysosomal storage. In addition to cataract,



**FIGURE 137-27** Punctate infantile cataract seen in Cotlier syndrome.

Lenz dysplasia is associated with microcephaly and mental retardation, colobomatous microphthalmos, nystagmus, esotropia, short stature, kyphoscoliosis, anteverted simple pinnae, and dental anomalies (124). CAMFAK syndrome, an autosomal recessive trait, is an acronym for cataract, microcephaly, failure to thrive, and kyphosis (125). Nance–Horan syndrome is an X-linked disorder which may cause mental retardation, and which includes dental defects and severe congenital cataracts. Different mutations in the NHS gene on Xp have also been found to cause isolated



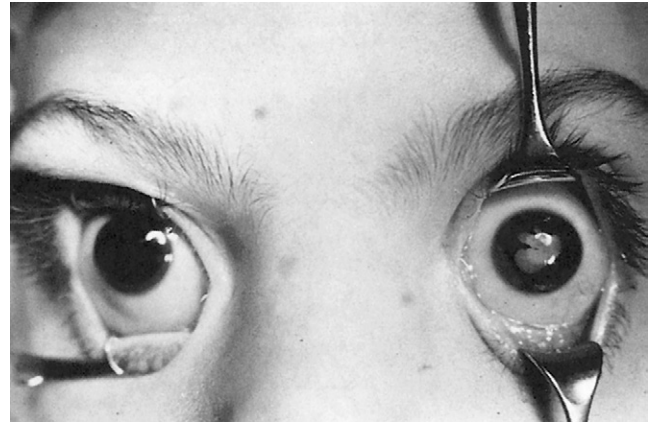
**FIGURE 137-28** Retinal photo showing perivascular pigmentary retinal degeneration in Cotlier syndrome.

congenital cataracts (42). Hyperferritinemia-cataract syndrome is an autosomal dominant disorder in which hyperferritinemia unassociated with iron overload leads to progressive cataract. L ferritin builds up in the lens (42).

### 137.3.6 Cataracts Associated with Biochemical Abnormalities

Cataracts associated primarily with metabolic disorders are found in disorders of cholesterol synthesis, galactosemia, mannosidosis, neuraminidase deficiency, phenylketonuria, and Wilson disease. Inherited defects in enzymes of cholesterol metabolism are associated with cataracts. The basis of this relationship apparently lies in the need of the lens to satisfy its sustained requirement for cholesterol by onsite synthesis; impaired synthesis of cholesterol can lead to alteration of the lens membrane structure. Lens membrane contains the highest cholesterol content of any known membrane. Smith–Lemli–Opitz syndrome, mevalonic aciduria (MAU), and cerebrotendinous xanthomatosis all involve mutations in enzymes of cholesterol metabolism; affected patients can develop cataracts (126). Cataracts have also been noted to occur in cystic fibrosis, with an increase noted as digestive function declines (127).

Smith–Lemli–Opitz syndrome is expressed in infancy by microcephaly, poor growth, dysmorphic features, genital and limb abnormalities, and mental retardation. Cataracts can also accompany the condition (128) (Figure 137-29). Patients with infantile cataract associated with pseudohermaphroditism should be tested for this



**FIGURE 137-29** Cataract in a patient with Smith–Lemli–Opitz syndrome.

disorder, as these findings typically occur together in this disorder. A buildup of 7-dehydrocholesterol and its metabolites largely replacing cholesterol occurs, resulting from a defective enzyme  $3\beta$ -hydroxycholesterol 7-reductase, which causes a block in the reduction of the double bonds between C-7 and C-8 in 7-dehydrocholesterol and in a precursor, desmosterol (129).

MAU is a rare autosomal recessive condition involving marked reduction in mevalonate kinase activity. It is expressed in infancy and carries a high rate of mortality. A defective form of the enzyme can result from a single base substitution in the coding region of this single-copy gene. The condition is characterized by developmental delay, dysmorphic features, hepatosplenomegaly, and progressive atrophy of the brain. The more severely affected individuals have cataracts. Serum and urinary levels of mevalonic acid can increase by more than 10,000-fold. The pathology, including the cataracts, could be a consequence of either reduced synthesis of cholesterol and its essential nonsterol precursors or more likely to direct toxicity from the accumulated mevalonic acid (126).

Cataracts plus Achilles tendon xanthomas before age 30 is the hallmark of cerebrotendinous xanthomatosis (130). The types of cataracts vary and are generally bilateral, with other ocular abnormalities, including optic atrophy being possible. Other features may include mental retardation and neurological abnormalities may also accompany this condition. Cerebrotendinous xanthomatosis can present in both children and adults, with 86% of affected patients having cataracts. This condition involves the accumulation of an unusual sterol, cholestanol in body sites, including the lens. It arises from a block in hepatic synthesis of bile acids due to a defect in the mitochondrial 27-hydroxylase required for oxidation of the cholesterol side chain.

Elevated serum ferritin and bilateral cataract formation have been identified in the hereditary hyperferritinemia-cataract syndrome (HHCR) which is autosomal dominant. The lens changes are dense

sutural and cortical opacities formed from amorphous mildly eosinophilic crystalline deposits (131). Affected individuals have normal serum iron and transferrin, but high serum ferritin. This elevation may be useful as a screening test for patients at high risk for this disease. It is interesting that the LIM2 structural gene is on human chromosome 19q13.4, with a protein product of 19.7 kD, which is similar to the size of the ferritin L-subunit (132).

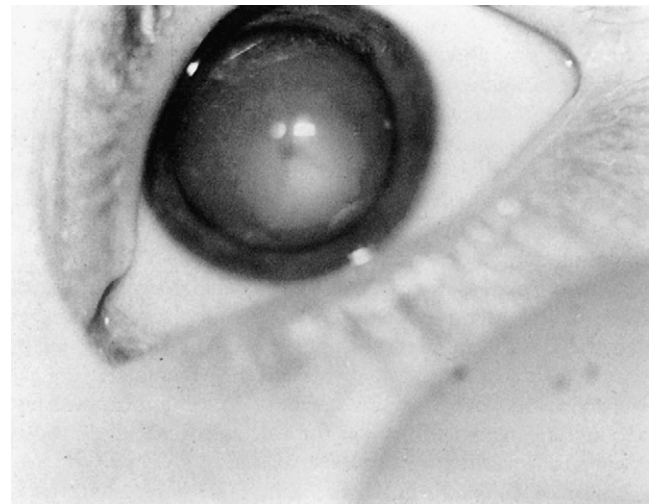
Galactosemia is a disorder caused by a deficiency of any one of three possible enzymes involved in the metabolism of galactose; galactokinase, transferase, or epimerase. Any single deficient enzyme can result in cataract through the accumulation of galactitol in the lens. It is a rare cause of congenital cataract but should be considered high on the list of differential diagnosis because of the salutary effect of early dietary therapy. The metabolism of galactose into glucose involves three steps, each assisted by an enzyme:

1. galactose + ATP to galactose-1-P (assisted by galactokinase);
2. galactose-1-P + UDP glucose to glucose-1-P (mediated by galactose-1-phosphate uridylyltransferase; and
3. UDP-galactose to UDP glucose (mediated by UDP GAL-4-epimerase).

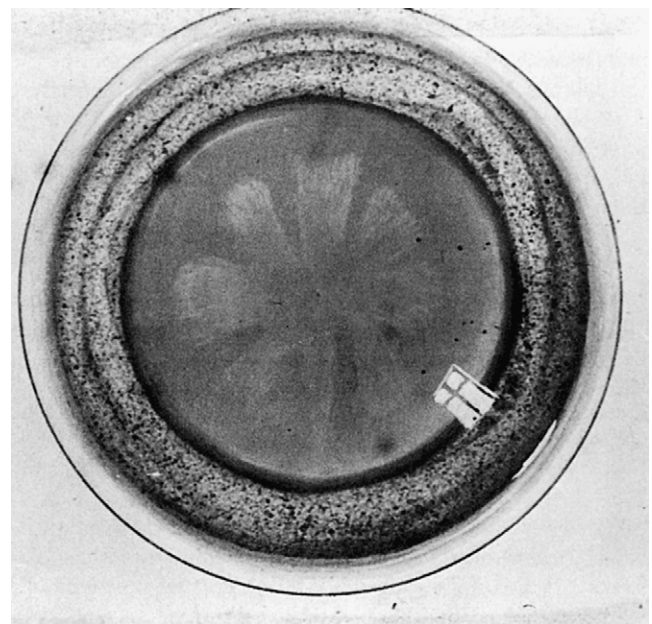
Deficiency in the enzyme galactokinase causes galactokinase deficiency and an absence of galactose-1-phosphate uridylyltransferase causes classic galactosemia in homozygotes. Both are autosomal recessive diseases. In both diseases, the accumulation of galactitol, a metabolite of galactose, causes the development of infantile cataracts in most untreated patients. Deficiency of the third epimerase enzyme was discovered incidentally in the evaluation of children screened for galactosemia. There are two variants of this disorder: a benign one without signs of galactosemia, and a more severe variant in which cataract formation has been reported.

In galactosemia, other abnormalities beside cataracts include nutritional failure, mental retardation, hepatosplenomegaly, and cirrhosis. In galactokinase deficiency, cataracts may be the sole manifestation in homozygotes. The morphological structure of the cataract in both diseases, if untreated, is similar. Initially a zone of lens becomes opacified. This can be nuclear, sutural, lamellar, posterior, cortical, or any combination of the above; the opacification ultimately progresses to mature cataract (Figure 137-30). The cataract is usually diagnosed in the first year of life, but an incomplete cataract may go unnoticed until the second or third decade of life.

In neuraminidase deficiency (sialidosis and galactosialidosis), ocular abnormalities include cherry-red spots in the macula, optic atrophy, corneal opacities, and lenticular opacities. There are two types, with cataracts more common in type I and corneal opacities found more commonly in type II.



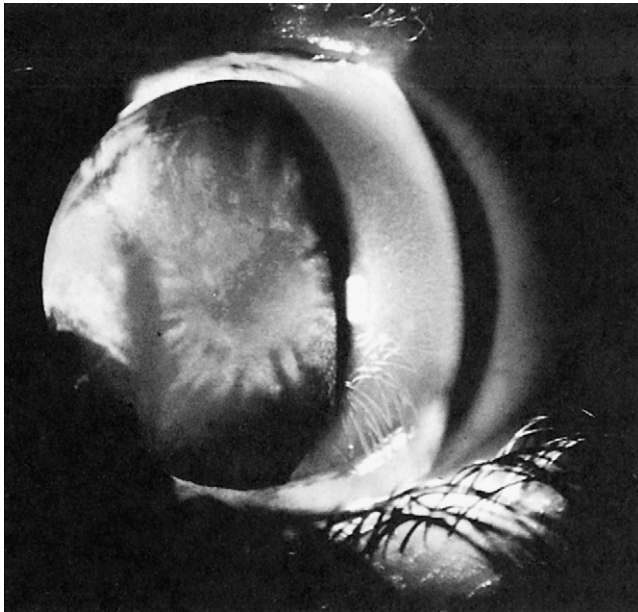
**FIGURE 137-30** Cataract in a patient with galactosemia.



**FIGURE 137-31** Artist impression of cataract noted in a patient with mannosidosis. (Courtesy of A. Linn Murphree, MD, Children's Hospital of Los Angeles.)

Other rare inborn errors of metabolism in which infantile cataracts have been reported are mannosidosis type I (Figure 137-31) and type II. Wilson's disease (hepatolenticular degeneration) is transmitted as an autosomal recessive trait and is characterized by juvenile cirrhosis of the liver and degeneration of the lentiform nucleus of the brain. It is caused by disturbed copper metabolism, with faulty or decreased synthesis of ceruloplasmin. Free serum copper is increased and copper deposits are not only found in the liver and brain stem but also in the eye. The cornea and lens accumulate copper, the latter in the form of a characteristic sunflower cataract (Figure 137-32), a yellowish star-like anterior subcapsular discoloration (133). Other rare case reports of congenital





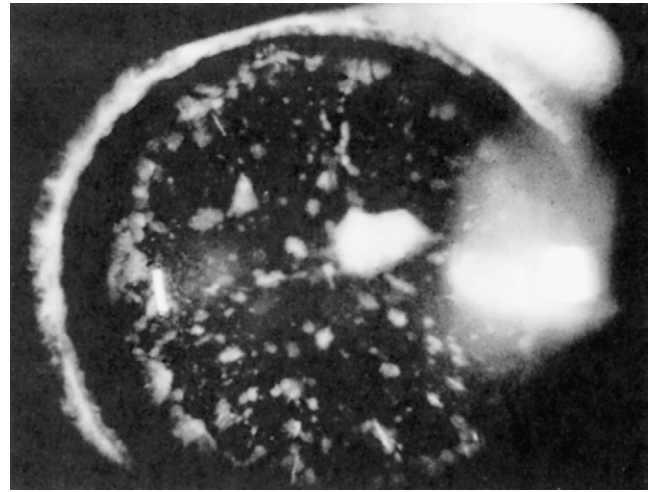
**FIGURE 137-32** “Sunflower cataract” in a patient with Wilson’s disease.

**TABLE 137-8** Cataracts Associated with Chromosomal Anomalies

Trisomy 10q
Trisomy 13
Trisomy 18
16q 18p-
18q-
Trisomy 20p
Trisomy 21
XO syndrome
Chromosome 3:4 translocation
Chromosome 2:14 translocation
Chromosome 2:16 translocation
Chromosome 9 inversion
Linkage studies
Dominant congenital—chromosome 1, 2, and 16
Early-onset progressive—chromosome 17
Nance syndrome—X chromosome (Xp21.1–22.3)
Hereditary hyperferritinemia cataract syndrome—chromosome 19 (bilateral nuclear)

cataracts have been reported in deranged proteoglycan (i.e. acid mucopolysaccharide–xylose–protein complex) metabolism with released xylose in the blood and urine and xylitol in the lens. Bilateral infantile nuclear sclerotic cataracts were described in a patient with a deficiency of complex I, a mitochondrial electron transport chain enzyme, which causes severe lactic acidosis and neurological dysfunction in the newborn (134). Sorbitol dehydrogenase deficiency may also cause congenital cataracts.

Cataracts associated with chromosomal anomalies are listed in Table 137-8. Of these the only morphologically characteristic cataract is the one found in Down syndrome, characterized by coronary and stellate cataracts in infancy or adolescence (Figure 137-33).



**FIGURE 137-33** Coronary cataract with stellate opacities typically seen in patients with Down syndrome between two eyes of the same individual. However, both forms of this cataract have been shown to occur within the same family, and even in the two eyes of the same individual (Girardet, 1943) (89).

### 137.4 THERAPY

Accurate diagnosis is the first step in the management of congenital lens anomalies. A rational approach to congenital cataract is outlined in Table 137-3. For congenital cataracts that interfere with vision, surgery is the only option. The one exception is the cataract in galactosemia, which may be reversed by dietary control. Although galactosemia is rare, the clinician should always have a high index of suspicion for this disorder. Once the diagnosis of galactosemia is confirmed, careful dietary management is required immediately, entailing the exclusion of all milk and milk-containing foods. Food containing galactose, such as organ meats, should also be avoided.

Management of congenital cataracts involves observation over time by a pediatric ophthalmologist if the opacities are minimal and do not interfere with vision. Specialized tests are needed to determine whether this is the case in young infants who cannot yet communicate. Opacities in the lens (cataracts) may result in deficient cell development of the visual cortex or other CNS centers such as the lateral geniculate body. The surgical removal of dense cataracts in infants before the age of 3 months has been advocated to prevent irreversible CNS damage. Studies have shown that only children who have a clear visual axis with optical correction in place before 4 months of age have a chance for normal vision. Aphakia (or lack of lens) results from surgical removal of cataracts. To correct for the optical strength of the lens, intraocular lenses, contact lenses, or glasses must be provided (135).

In patients with unilateral cataracts, amblyopia or poor vision due to lack of brain or retinal use can be prevented by a protocol of monitored occlusion of the sound eye to stimulate vision in the aphakic eye in



patients with unilateral cataracts. In addition, cataracts removed in infants with small eyes (microphthalmos) or large eyes (myopia) must be treated for the additional refractive error to prevent amblyopia (136). Corneal curvature problems (astigmatism) are another cause for amblyopia after cataract removal (137).

The surgical removal of bilateral cataracts in infants yield good visual results in the majority of cases (136), with intervention before 3 months of age usually yielding better results (139,140). Very early surgery (less than 2 weeks of age) has recently been reported to be associated with higher risk of developing glaucoma. Timing is still being investigated, but surgery performed between 2 and 6 weeks of age may offer the best visual results with the lowest increased risk of glaucoma. Follow-up of operated aphakic infants is paramount, as secondary glaucoma develops in many patients (141), and amblyopia remains a formidable obstacle following cataract surgery. Furthermore, the correction of aphakia with glasses or contact lenses requires continuous supervision as lenses are lost or glasses are scratched. Although controversy exists regarding the youngest age indicated for intraocular lens implantation, intraocular lenses are being increasingly implanted in patients with congenital cataracts. Results from a multicenter trial, the Infant Aphakia Treatment Study, which compared using contact lenses vs primary intraocular lenses in patients with congenital cataracts, found no difference in visual outcomes at 1 year of age, but the primary intraocular lens group required significantly more reoperations over the study period (142). Intraocular lens implantation after 2 years of age has become the standard of care for most practitioners in the USA. In the final analysis, the success of cataract surgery in infants depends on a close cooperation among physicians in various specialties, including pediatrics, neurology, genetics, and ophthalmology, and with their parents and caretakers who must do rigorous amblyopia treatment at least to the age of 9 years. Even with excellent care, some eyes will not have a good outcome, usually due to other ocular anomalies. Many of these might be predicted by the genetic cause of the cataract, however routine fee-for-service testing for isolated congenital cataracts is not available at the time of this writing. Some testing is available for cataracts which are part of a syndrome, such as PAX6. Availability of genetic tests, frequently updated, can be found online at [www.genetests.org](http://www.genetests.org). An excellent resource for understanding hereditary cataracts is the site *Cat-Map*. This site lists all currently known cataract genes and loci by chromosomes. It can be accessed at <http://cat-map.wustl.edu/>.

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### Biography



**Arlene V Drack, MD** is a clinician scientist specializing in juvenile inherited eye diseases. She is the inaugural Ronald V Keech Associate Professor in Pediatric Ophthalmic Genetics at the University of Iowa Department of Ophthalmology and Visual Sciences. Her research focuses on inherited eye diseases that affect children, particularly in the development of novel treatments. She codirects both the clinical and rodent electroretinogram services at the University of Iowa. Her clinical practice includes the full scope of pediatric ophthalmology and strabismus, in addition to running specialized genetic eye disease clinics.

# CHAPTER

# 138

## Hereditary Retinal and Choroidal Dystrophies

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### 138.1 INTRODUCTION

Inherited diseases of the retina and choroid cause significant visual morbidity with a number of them presenting during early childhood. There are over 1500 inherited diseases in which the term retina or choroid is mentioned in a search on the online Mendelian inheritance in man database ([www.omim.org](http://www.omim.org)). There are 184 retinal disease-causing genes identified so far, with a number of loci at which genes have yet to be identified (Retnet). With the rapid advances of technology in molecular genetics, gene discovery is accelerating. Whole exome and next generation sequencing has lead to the identification of several additional genes such as *GPR179*, *GNPTG*, *KIF11*, *MAK*, *HARS*, *LZTFL1*, *PLA2G5*, *TMEM237*, *C8ORF37* within the past 2 years (Table 138-1). However, as our ability for high through-put sequencing and identification of sequence variations is increasing, so are the challenges of translating this information from bench to the clinic—to individuals and families. Some laboratories in the United States are currently offering molecular genetic testing for a number of retinal and choroidal disease-causing genes as panels. A number of changes that can be missense, nonsense, splice altering variants, gene inactivating promoter mutations and copy number variations may be identified and can be disease-causing or benign variants. Given the difficulty in accurately determining the sequence variations as mutations or polymorphisms, and the increasing number of variants of unknown significance, the clinical exam remains very important. The classical diagnostic approach to retinal dystrophy patients is to correlate the fundus pattern (morphological appearance) and inheritance pattern with the results of electrophysiological and psychophysical tests such as the electroretinogram (ERG), electrooculogram (EOG), dark adaptation, and visual field test (1). These results are compared to the patterns seen in established

disease entities for the most accurate diagnosis. Frequently, the clinical appearance and test results clearly establish the clinical diagnosis, but because many retinal diseases have similar fundus appearances or nonspecific abnormalities, it may be necessary to observe the clinical course of the disease over many years and examine other affected family members in order to finalize the diagnosis. Hereditary diseases of the eye, with rare exceptions, have bilateral symmetrical involvement. When unilateral ocular disease is seen, other causes, such as birth defect, intrauterine or antenatal infection, trauma to the eye, and inflammatory diseases should be considered first, before giving the diagnosis of a hereditary etiology. If the asymmetry is in visual acuity without tissue changes, amblyopia may be the reason. Occasionally a patient will be seen with uniocular disease, which becomes bilateral after several years and, presumably, the uninvolved eye was in a subclinical state on the initial examination.

The genetic and phenotypic heterogeneity in retinal diseases are well established. The pleiotropy of single disease-causing gene, has been seen in a number of different retinal genes including *RDS*-peripherin2 where macular dystrophy, adult vitelliform lesions, retinitis pigmentosa (RP) like phenotype, central areolar choroidal dystrophy and fleck retinal phenotypes have been seen (2), *CRX* mutations where autosomal dominant and recessive Leber congenital amaurosis and cone-rod dystrophy have been observed and *ABCA4* mutations resulting in Stargardt's disease, cone-rod dystrophy and RP (3). Given this complexity, the clinical laboratories, ophthalmologists, geneticists and genetic counselors have to work together as a team to provide the most accurate diagnosis, one that is consistent clinically and molecularly, and to facilitate personalized therapeutic options. We will discuss the genetics and clinical presentation of a number of isolated retinal and choroidal diseases in this chapter.

**TABLE 138-1 Retinal Dystrophy Genes**

Retinitis Pigmentosa				
Inheritance Pattern	Gene	OMIM No.	Locus	Protein
AD/AR	RPE65	180069, 204000, 204100	1p31.2	Retinal pigment epithelium-specific 65 kD protein
AD	PRPF3	601414, 607301	1q21.2	Human homolog of yeast
AD	SEMA4A	600105, 604210, 607292	1q22	Semaphorin 4A
AD	SNRNP200	601664, 610359	2p11.2	Protein: small nuclear ribonucleoprotein 200 kDa (U5)
AD/AR	RHO	180,380	3q21–q24	Rhodopsin
AD	GUCA1B	602,275	6p21.1	Guanylate cyclase activating
AD	PRPH2	179605, 169150, 608133, 608161, 613105	6p21.2	Peripherin/RDS
AD	RP9	180,104	7p15.1–p13	PIM1-kinase associated protein 1
AD	KLHL7	268000, 611119, 612943	7p15.3	Protein: kelch-like 7 protein ( <i>Drosophila</i> )
AD	IMPDH1	146690, 180105	7q31.3	Inosine monophosphate
AD/AR	RP1	180100, 603937	8q11–q13	RP1 protein
AD	RP1L1	608,581	8q23.1	RP 1-like protein 1
AD	TOPORS	609507, 609923	9p21.1	Topoisomerase I binding arginine/serine rich protein
AD/AR	BEST1	153700, 268000, 607854, 613194	11q12.3	Bestrophin 1
AD	ROM1	180,721	11q13	Retinal outer segment membrane
AD/AR	NRL	162,080	14q11.2	Neural retina luciferase zipper
AD	RDH12	204000, 268000, 608830, 612712	14q24.1	Protein: retinol dehydrogenase 12

## 138.2 INHERITANCE PATTERNS AND MOLECULAR GENETICS

Most hereditary retinopathies follow conventional Mendelian patterns of inheritance; that is, autosomal dominant, autosomal recessive or X-linked recessive. However, more complex patterns are also known, such as X-linked recessive with expression in female “carriers” (4); digenic inheritance, which results from the sum of independent mutations, such as in *peripherin2/RDS* and *ROM1*; and mitochondrial mutations, which follow a maternal pattern of inheritance (Tables 138-1). A family in which expression follows an X-linked dominant pattern has been identified in which males are severely affected from early age, while females have a clear RP-like progression during adult years (4). Furthermore, many cases present as isolated or sporadic, that is, without other known affected relatives, so the mode of inheritance may be unclear.

Exceptional progress has been made in identifying genes and mutations causing inherited retinal and choroidal degenerations during the past decade. To date, 184 genes have been identified (the underlying gene and mutations are known) and an additional 41 loci mapped by linkage methods (assigned to a chromosomal site) (Table 138-1) (Retnet). This information has helped elucidate the various patterns of inheritance, but it has also demonstrated the extraordinary complexity of these disorders.

Based on these developments, inherited retinopathies are best characterized by the word “heterogeneity”—perhaps more so than for any other class of hereditary disease. There are three applicable types of heterogeneity.

First, there is genetic heterogeneity; that is, different genes can cause apparently similar conditions. For example, mutations in at least 22 different genes can cause autosomal dominant RP, some of which also cause autosomal recessive RP (Table 138-1). Although the clinical phenotypes caused by these 22 genes are similar, the biochemical pathways of the known proteins are distinct (5). One plausible reason that different pathogenic mechanisms might lead to similar clinical consequences is that in all cases studied to date, the end stage of retinal degeneration involves photoreceptor apoptosis (6).

Second, there is allelic heterogeneity; that is, different mutations in the same gene can cause different retinal diseases. For example, different mutations in *peripherin/RDS* can cause pattern dystrophies, adRP like phenotype, dominant macular dystrophy, or dominant pan-retinal degeneration (2). Also, different mutations in rhodopsin can cause adRP, dominant congenital stationary night blindness, or recessive RP (7). One reason for this variety of allelic phenotypes is that most proteins do not have just one “function,” but rather, multiple functional properties which reside in different portions of the molecule. Thus, *peripherin/RDS* is expressed in both rods and cones, but expression of *ROM1*, which binds to *RDS*, is limited to rods, so the pathology of some *RDS* mutations will be rod-specific, others cone-specific, and still others both.

Finally, there is clinical heterogeneity; that is, clinically distinct phenotypes caused by the same mutation, even within the same family. In general, a hallmark of dominant diseases with delayed onset is significant variation in age-of-onset and severity. This is particularly true

of dominant retinopathies, such as adRP. For example, different individuals with the Arg677Ter mutation in the RP1 gene can have early-onset adRP, late-onset adRP, or an apparent lack of clinical consequences; that is, a “skipped generation” (8). More strikingly, a splice site mutation in peripherin/RDS manifests as adRP, pattern dystrophies that progress to appear as atrophic macular dystrophies or dominant chorio-retinal disease (Figure 138-1a, b). A reasonable explanation for clinical heterogeneity is the modifying effects of environmental and additional genetic factors. However, environmental factors have not been identified in most cases (with the exception of sunlight exposure), and evidence for genetic factors is only suggestive (8).

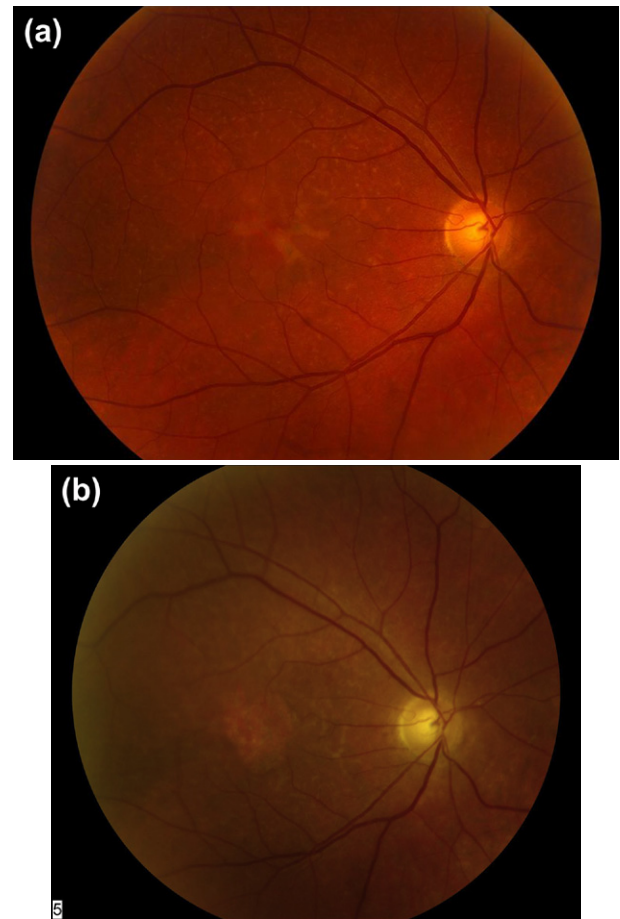
As a consequence of this heterogeneity, a thorough clinical evaluation and a complete family history, including pedigree construction, are necessary to identify the mode of inheritance and underlying mutation, but are often not sufficient. On many occasions the ultimate diagnosis depends on the results of genetic testing. The use of newer technologies such as next generation sequencing has resulted in the identification of disease-causing mutations in adRP to 65% (9). With newer gene and mutation discoveries, this is likely to increase.

### 138.2.1 Incomplete Penetrance

Incomplete penetrance in obligate cases of dominant RP has been documented on occasions in which the patient has no or very minimal retinal findings compared to affected age-matched relatives. The lack of obvious disease in an obligate carrier of the mutant dominant gene can be a source of confusion when first encountered, since it may initially mislead genetic counseling and cause errors in linkage analysis studies. Many of these patients when re-examined and tested a number of years after the initial visit will demonstrate signs of disease, but they are never as severely affected as fully penetrant relatives. The reason for the incomplete penetrance is not understood, but is presumed to be because of secondary gene interaction. The most striking example of a modifying factor is seen with PRPF31, mutations which cause the RP11 form of autosomal dominant RP. At least 10% of individuals carrying a disease-causing mutation in PRPF31 show no disease symptoms late in life, though other family members may be severely affected. Evidence suggests that the unaffected copy on the homologous chromosome can play a protective role in some cases (10). This is one of the best documented examples of secondary genetic effects controlling the consequences of primary mutations.

### 138.2.2 Variable Expressivity

Because the classification of these diseases relies heavily on the morphological appearance of the retina, the diagnosis may be confused by phenotypic variability. This



**FIGURE 138-1** a, b. Lipofuscin deposits in the macular region consistent with butterfly pattern dystrophy progressing over 10 years to cause atrophic lesion in the macula as in b.

can be seen as heterogeneity and variable expressivity of findings in autosomal dominant families, or instances of secondary expression, in which additional pathological conditions occur in a few patients when the majority of the patients with the same retinal degeneration do not have the finding. This is illustrated by cases of RP in which there is also Coats’ disease or retinal neovascularization. This phenomenon occurs rarely in RP and appears to represent one way that the retina responds in the degenerative process. The Coats’ reaction is more commonly seen in patients with RP12 due to mutations in the CRB1 gene where up to 30% of patients will develop this condition in one eye (11).

Variable expressivity that could be mistakenly called a new type of RP was exemplified in two Navajo sisters with autosomal recessive RP who were found to have unocular neovascularization; but on inspection of 40 other autosomal recessive Navajo RP patients, no other cases of retinal neovascularization were found. Presumably factors other than primary gene expression were responsible for the neovascularization in the two sisters. Another diagnostic problem are patients with distinctive patterns of retinal degeneration in which there are potentially multiple genetic etiologies for the fundus



appearance; as an example, white spots in the retina seen in retinitis punctata albescens, fundus albipunctatus, fundus flavimaculatus (Stargardt's disease), Malattia Leventinese, and vitamin A deficiency can look very similar on examination. Fortunately, the correct diagnosis can be confirmed by clinical electrophysiological and visual field testing and/or blood/DNA testing, as the gene has been found for all these entities. Another distinctive pattern that is presumably associated with multiple etiologies is a disorder called pigmented paravenous retinochoroidal atrophy (Figure 138-2) these patients demonstrate



**FIGURE 138-2** Pigmented paravenous retinochoroidal atrophy is a distinctive pigmentary degenerative pattern occurring next to retinal veins and sometimes caused by heterozygous mutations in *CRB1*.

pigmentary degenerative changes next to retinal veins. Some cases are progressively like RP, but most have a stable disorder. In the past, paravenous pigmentary patients have been regarded as having a distinct form of RP. However, this degenerative pattern has been documented to occur in many diseases; these include Wagner's and Stickler's vitreoretinal degeneration, rod-cone and cone-rod degenerations, as well as in sarcoidosis; the retinal degenerations in these cases are not likely to be due to the same etiology, although similar pathophysiological mechanisms may be occurring next to the retinal veins in these different disorders. In one family, heterozygous val162-to-met mutation in the *CRB1* gene has been reported as the causative mutation in six affected family members (12).

### 138.2.3 Visual Electrophysiological Testing

There are a number of tests that are useful in classifying the hereditary retinal and choroidal degenerations, which will be mentioned in summary fashion here since there are extensive reviews in other sources (Table 138-2) (1). The ERG is an evoked response in which a signal generated by the retina in response to a flash of light is recorded (after topical anesthesia) by a contact lens or foil electrode on the surface of the eye. Eyelid skin electrodes are occasionally employed in children but do not give as good quality tracings and should be avoided if possible. Careful standardization of test conditions is essential in order to obtain interpretable results. A kinetic (Goldmann) visual field is often needed to correctly interpret the results of the ERG testing and to evaluate visual function. When

**TABLE 138-2 Typical Electrophysiological Changes Seen in Retinal Diseases**

ERG Change	Disease (or Condition)
Nonrecordable ERG	Leber amaurosis congenita; retinal aplasia; RP; total retinal detachment
Abnormal or nonrecordable photopic ERG often mild rod ERG abnormalities	Cone degenerations-AD, AR; rarely, early RP which is progressive; Achromatopsia; X-linked blue monochrome monochromatism; X-linked cone dystrophy with tapetal-like sheen
Nonrecordable rod ERG, abnormal bright-flash dark-adapted ERG, normal to near normal photopic ERG	Congenital stationary night blindness; rarely, early RP which is progressive
Barely or nonrecordable scotopic ERG, abnormal photopic b-wave ERG	Rod-cone degenerations, typical RP; Leber's congenital amaurosis (juvenile form); choroideremia; chorioretinitis (variable); secondary RP including some storage diseases; progressive RPA
Abnormal cone and rod b-wave amplitudes, cones are relatively more affected than rods	Cone-rod degenerations, AD, AR, XLR; postinflammation (e.g. luetic chorioretinitis, which may also have a rod-cone pattern)
Negative waveforms: In the dark-adapted bright flash ERG the a-wave is normal to attenuated, but the b-wave does not return to the isoelectric point	X-linked retinoschisis; congenital stationary night blindness; enhanced blue-cone syndrome (Goldmann Favre disease); some autoimmune retinopathies
Nonspecific abnormalities	Metallic foreign bodies; chorioretinitis (acute or old); vasculitis/diabetic retinopathy; low serum taurine; early panretinal degeneration; partial retinal vascular occlusion

Standardized ERG testing isolates cone and rod function, as well as tests both rods and cones together. Comparison of each component against the others gives functional data that helps in arriving at the clinical diagnosis.

<sup>a</sup>X-linked rod-cone and cone-rod ERG patterns have been found within the same RP pedigree and appear to represent variable expressivity; however, these electrophysiological patterns have otherwise been consistent within pedigrees of autosomal dominant and recessive RP patients.

the test is performed in the light-adapted state, the test is called a photopic ERG and measures the cone system. The rods in the light-adapted state are bleached out, and do not respond to the light stimulus. Cone function also can be measured with a flickering stimulus, since rods do not respond over 20 cycles per second. After the patient is dark-adapted for at least 30 min, the test is repeated with a dim flash of light below cone threshold. This stimulus evokes a rod-isolated response. A mixed cone and rod response, testing both photoreceptor systems, is often performed with a bright flash in the dark-adapted state. The ERG is a mass response of the photoreceptors and does not correlate with visual acuity, which is a function of macular health. The macula only contributes a small fraction of the total ERG response.

As a general principle, it is important to separately test the cone and rod systems in a standardized fashion, since comparison of the relative changes is used to support or make clinical diagnoses. The first electroretinographic response to appear is a negative or downgoing waveform called the “a-wave,” representing the response of the receptor layer. The second waveform to appear is a positive upgoing or “b-wave” that is derived from bipolar and Mueller cells in the middle retina. Certain components of the ERG are lost or altered depending on the disease and severity of involvement. By comparing the cone-isolated, rod-isolated, and mixed cone and rod responses, diagnostic patterns are commonly seen in hereditary retinal disease states and are presented in [Table 138-2](#). A multifocal ERG has been developed for testing foveal function independent of the mass response type ERG. The Pattern ERG uses a check board pattern stimulus in which the total luminance never changes even though the checkerboard pattern changes, and this has been shown to measure function of inner retinal neuronal cells ([13](#)).

The EOG measures the standing potential that exists between the front and back of the eye, and is generated by the retinal pigment epithelial (RPE) layer, a continuous single cell layer under the retina. The EOG is altered in diseases affecting the RPE. The test is performed by placing skin electrodes medial and lateral to each eye. The patient looks alternately between two points 20° apart for periods of time in the light and then in the dark. The amplitudes of the response are quantified and expressed as a ratio, with the largest light-adapted value divided by the smallest dark-adapted value and the result multiplied by 100. This percentage value is called the Arden ratio. One hundred percent (100%) would indicate no response, and would be typical of advanced RP. Different normal values may be encountered depending on the measuring technique of the individual laboratory performing the test. The EOG is important for evaluating an individual suspected of having Best’s vitelliruptive degeneration, an autosomal dominant disorder in which the ERG is normal and the EOG is abnormal. Patients with the Best’s disease

gene and no fundus changes can be identified by the EOG. Typical normal values for the EOG are 180% or greater. The values are age-related and are altered in many retinal degenerative disorders.

The dark adaptation test measures the threshold of sensitivity of the retina to a small spot of light after the patient is placed in the dark and typically is measured over a 40 min period. The curve generated is bimodal, measuring the cone’s sensitivity first; at about 9 min the rods become desaturated and become progressively more sensitive. Maximum sensitivity is normally achieved by 40 min. Any panretinal disease, which causes rod damage or dysfunction may cause night blindness and can be quantitated by the dark adaptation test. Measuring the final rod threshold in several retinal locations after 40 min of dark adaptation is a good screening procedure for assessing night vision. This test is useful in functionally accessing complaints of night blindness in cases where dysfunction at night has vocational significance. The mechanisms of color vision are partly understood, and are based on pathways that involve specific cone receptors for red, green, and blue, whose signals are integrated by the visual system ([14](#)).

Researchers using molecular genetic techniques have confirmed that human trichromacy is determined by distinct cone photoreceptor cells; it was reported that red-green color blindness is caused by alterations in genes encoding red and green visual pigments, and anomalous or partial color blindness conditions appear to be related to hybrid and/or redundant coding ([15](#)). Also reported were the molecular structures of green, red, and blue pigment genes, and the chromosomal locations of rhodopsin (chromosome 3) and blue pigment (chromosome 7). The red and green pigment genes were previously known to be on the X-chromosome. Many retinal degenerations affect the cones, giving color vision abnormalities. These can be quantitated by color and intensity matching (Nagel anomaloscope), the Farnsworth-Munsell hue discrimination test, and color plates. In a broad sense, color vision defects may be divided into two groups: congenital and acquired. While hereditary congenital color vision defects are almost always red-green, affecting 8% of males and 0.5% of females, acquired defects are more often of the blue-yellow variety and affect males and females equally. There are a number of important clinical distinctions between the congenital and acquired color vision defects.

Patients with congenital color vision defects rarely misname colors, whereas patients with recent changes in color vision of an acquired nature will typically use incorrect color names or report that the color appearance of familiar objects has changed. Except in rare cases, congenital color vision defects affect the two eyes equally. In striking contrast, acquired color vision defects frequently affect one eye more than the other.

Congenital color vision defects are constant in type and severity throughout life except for changes that

occur with aging. Acquired color vision defects generally vary in type and severity, depending on the location and source of associated ocular pathology. Congenital color vision defects generally are not associated with observable retinal or optic nerve pathology, whereas acquired color vision defects frequently have macula changes.

The fluorescein angiogram is a good test for identifying retinal vascular and RPE abnormalities or alterations, since many retinal structures are normally impermeable to the fluorescein dye but will leak or stain if diseased, often in distinctive patterns. The diagnosis of some hereditary retinal diseases such as choroideremia, autosomal recessive preserved pararteriolar retinal pigment epithelial (PPRPE) RP12, early dominant RP with normal ERG, and X-linked carrier states may be supported by changes seen on the fluorescein angiogram, but are not apparent on clinical examination. Many of the hereditary retinal degenerations including a rare autosomal recessive cone degeneration, fundus flavimaculatus (Stargardt's disease), and some cases of RP will demonstrate a "dark choroid" or hypofluorescence of macular areas on fluorescein angiography. The reason for this appears to be a buildup of abnormal deposits within retinal pigment epithelium that blocks the choroidal fluorescence that is normally transmitted through this layer.

#### 138.2.4 Optical Coherence Tomography

Optical Coherence Tomography is a non-invasive cross-sectional imaging method using low-coherence interferometry to provide detailed morphometric information on retinal structure (16). It has become an important method in the diagnosis and follow up of RP, Stargardt disease (17) and albinism (18). This test is used increasingly in the diagnosis and management of inherited retinal diseases.

#### 138.2.5 Inheritance Patterns

The use of pedigree analysis has been an important discriminator in classifying the various hereditary retinal disorders. Many diseases such as RP, fundus flavimaculatus, and cone dystrophy, within each group, have a similar fundus appearance, yet multiple inheritance patterns are found for each retinal disorder type. Within the RP group, classification by inheritance pattern is a preliminary method to help select the correct laboratory approach for mutational analysis. If molecular diagnosis is not successful, then the clinical diagnosis based on phenotyping, electrophysiological results, and inheritance patterns is employed.

#### 138.2.6 Genetic Counseling

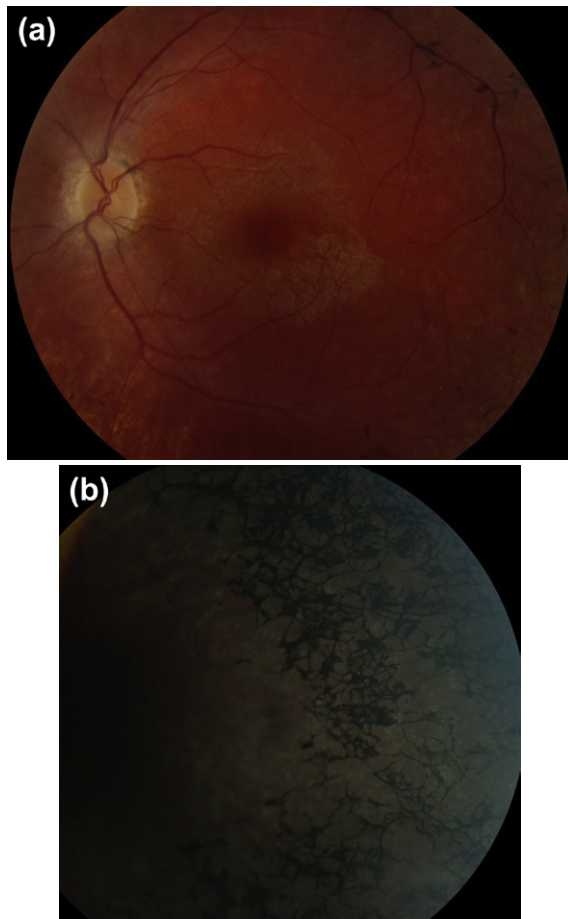
If the gene has not been found for a patient's retinal dystrophy, the two most important criteria in counseling patients with hereditary retinal degenerations are

a specific and correct diagnosis and an accurate pedigree that is interpreted correctly. This may take several patient visits to accomplish. Establishing a specific clinical diagnosis may require physiological or psychophysical testing, examining other family members, and the services of an ophthalmologist familiar with these diseases. Some of the retinal degenerations are inherited in established patterns, and if the diagnosis is unequivocally made, and gene testing is not available, then genetic counseling can be performed with reasonable certainty; for example, choroideremia (X-linked recessive), Best's disease (autosomal dominant), and Usher I (autosomal recessive) would be diseases in which counseling could be performed with reasonable certainty even if a family history was not available. However, other retinal degenerations such as the pigmentary retinopathies, fundus flavimaculatus, cone dystrophies, and vitreoretinal degenerations are documented to be inherited in more than one inheritance pattern, and mutation identification is needed, or if not available, a careful pedigree with examination and testing of other family members would be necessary in order to accurately counsel the patient. Clinical features usually are consistent within families, but can vary between pedigrees. Many of these diseases are pleiotropic, and counseling should rely heavily on the pedigree if the disease-causing gene is not known.

Occasionally, because complete pedigree or clinical information cannot be obtained, reliable genetic counseling cannot be given and it is best to carefully relate this to the patient. Unfortunately, misinformation is frequently given to this group of ophthalmological patients, with the most common misconception being that RP is always inherited through the female (X-linked recessive) when in the United States this is the least common mode of inheritance, representing approximately 10% of the RP population.

### 138.3 PIGMENTARY RETINOPATHIES/RETINITIS PIGMENTOSA

Retinitis pigmentosa (RP) is the name commonly given to a group of diseases characterized by progressive visual field loss, night blindness, and abnormal or nonrecordable ERG (Tables 138-1 and 138-2). Most patients will show pigment deposition in the retina along the blood vessels with optic nerve pallor and arteriolar narrowing in early stages advancing to characteristic 'bone spicule' pigmentary pattern in later stages (Figure 138-3a, b). Histological studies of RP retinas suggest that the source of the pigment is the retinal pigment epithelium (RPE). The retinal deterioration taking place is intrinsic to the disease process and does not appear to be related directly to the pigment deposition process, which is a secondary effect. Whether the pigment contributes to the deterioration of retinal function is doubtful. Localized retinal pigment deposition is commonly seen in processes injuring



**FIGURE 138-3** (a) Early stages of RP showing arteriolar narrowing and pigmentary deposits along the blood vessels. (b) Typical “bone-spicule”-like pigmentary deposits seen in RP. While RP comprises many different diseases, there is a common final pathway of degeneration leading to the retinal pigment deposition. (c) Optical coherence tomography showing cystoid macular edema in a patient with RP. (Courtesy: Dr Jiong Yan, Emory University.)

or destroying the RPE layer, and the RP pigmentary pattern can be regarded as a common degenerative pathway for a number of different acquired and hereditary retinal degenerations (Tables 138-1 and 138-2). Clinical classifications of RP are becoming less important as gene mutational analysis is now giving much clearer answers on the etiology of these disorders. Some clinicians reserve the term RP for the electroretinographically determined group with “rod-cone” degeneration in which patients have early night blindness and the ERG is abnormal, with the rod ERG being more abnormal or nonrecordable compared to the light-adapted cone ERG, which may show some response. However, recent reevaluation of classification by a number of RP centers shows that there are at least two RP groups based on whether the patient has diffuse rod loss or a mixed loss of cone and rod function. The latter group usually has electroretinographic changes in which the cone function is worse than rods, and the diagnostic term cone-rod degeneration has been used. These individuals generally have later night blindness and some have no nyctalopia until they have visual

fields less than 10°, and many of these patients have no retinal pigment deposits and could be termed “retinitis pigmentosa sine pigmento.” Cone-rod RP patients also have progressive visual field loss, retinal degeneration, vascular attenuation, optic atrophy, and RPE damage. The inheritance pattern can be autosomal dominant, autosomal recessive, X-linked or mitochondrial.

The pigmented retinopathies can be divided into two large groups, primary RP in which the disease process is confined to the eyes, with no other systemic manifestation; and secondary pigmentary retinopathy, a rarer occurrence in which the retinal degeneration is associated with single or multiple organ system disease. The list of RP syndromes, or diseases in which a panretinal degeneration is associated, is long (Retnet, OMIM), and the presence or absence of the retinal degeneration depends on the pathophysiological mechanisms and duration of the disease in question (19). The etiological relationship of the retinal degeneration to most of these syndromes is unknown. In some cases such as the mucopolysaccharidoses, mannosidosis, Batten’s disease, and Refsum’s disease, abnormal metabolic products are toxic to the retina and cause degeneration. Please find additional details in references discussing the individual diseases.

### 138.3.1 The Primary Pigmentary Retinopathies

Because over 90 forms of RP have been identified, either by gene or linkage identification, the classification is shifting to classification by gene diagnosis. However, it is still necessary to be aware of clinical classifications of the primary pigmentary retinopathies by inheritance type and subdivision, often using the pattern on the ERG or psychophysical measurement of rod function (Table 138-1). In general, morphological retinal appearance is not used to classify the entities in Table 138-1 because of nonspecificity of the patterns seen in the pigmented retinopathies and because of problems of secondary gene expression, which may alter the fundus appearance in patients with the same genetic type of pigmented retinopathy.

All forms of inherited RP are progressive, though “delimited forms” have been noted that appear clinically to be milder. One of these types is “sector RP” in which the apparent retinal degeneration is mainly localized to the inferior and inferior nasal areas of the retina. Many cases of sector RP have been found to be from rhodopsin gene mutations. In rhodopsin mutation rodent models light was found to cause retinal toxicity, and documentation of light toxicity in humans has been reported in rhodopsin Pro23His mutations, which are associated with sectoral RP. Studies in transgenic rodents with rhodopsin mutations have confirmed that this group is more sensitive to the toxic effects of light exposure.

Analysis of disease progression by inheritance type has been made, suggesting that as a group RP has a 4–5% loss per year of existing field, but all such analyses have



the handicap that multiple types of RP may be included within each inheritance type, each with its own genetic expression and each influenced by secondary factors. Another problem is that studies may focus on probands who may have the most severe expression of the disease.

The frequency of RP varies with country and race being studied. The highest known incidence is in the Navajo Indian, with 1 in 1800 being affected. The overall US frequency has been calculated to be 1 in 3700 with nearly 15–20% being autosomal recessive, 20–25% autosomal dominant, and 10–15% X-linked recessive (20). Up to 50% of cases presenting in the United States have no family history. It is generally assumed that most of these cases represent autosomal recessive RP, though undoubtedly a few are autosomal dominant with reduced penetrance or are X-linked recessive in which the last affected male was several generations past (Figure 138-4).

Rare cases of mitochondrial and X-linked dominant inheritance have been reported. The role of environmental (nongenetic) causes of RP is difficult to determine, but rare environmental causes may be found in a minority of isolated cases. Prenatal rubella or CMV infections may cause pigmentary retinopathies. Upon careful searching, many isolated cases find other relatives who had RP, night blindness, or blindness of unknown etiology. This discovery process may take several visits but allows for more accurate genetic counseling and potential gene diagnosis.

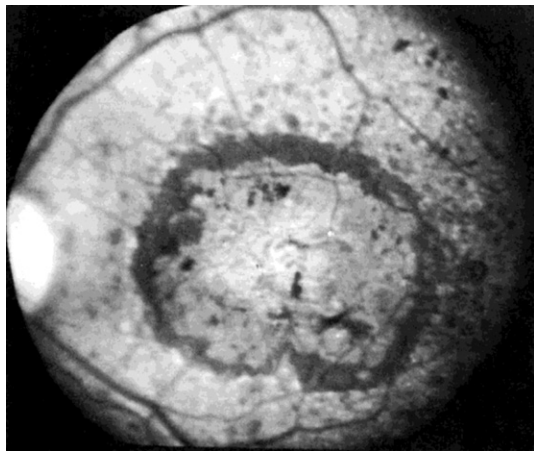
### 138.3.2 Management of Retinal Dystrophies

Most retinal dystrophies are relatively rare. Most ophthalmologists have limited experience in working with retinal dystrophy patients and may have limited experience in the principles of medical genetics. Most retinal dystrophy patients are anxious because of their uncertainty regarding possible blindness; it is therefore important to ensure that they are evaluated by specialists who are familiar

with these disorders. It is important to order the correct tests and to obtain a full pedigree. Once the information is assembled, the correct diagnosis must be related to the patient along with the significance of the findings, prognosis, and possible treatments. Most patients want to know about the latest research on their disease. Unfortunately, accurate information is not always supplied to them. The retinal dystrophy field is complicated, and it is desirable to refer these patients to specialists who are familiar with medical genetics and sophisticated electrophysiological testing. For example it is not unusual for RP patients to be told that they will go blind within one year, when in fact the disease is a chronic degenerative problem, and the majority of patients do very well for decades. Some RP patients are told not to have children, when in fact careful scrutiny of most pedigrees show that offspring are not at immediate risk unless the patient has an autosomal dominant disease or X-linked disorder.

Management of RP includes regular ophthalmological checkups and careful evaluation of the retinal degeneration to ensure that the type is diagnosed as specifically as possible. Not only is this important from a research point of view, but it also may help to identify the inheritance type in cases in which there is an inadequate family history. Frequently the most meaningful electrophysiological information is obtained early in the disease process. In addition, basic documentation by visual field and electrophysiological/psychophysical testing helps answer questions that invariably arise as to how severe the condition is and how fast it is changing. Many of these patients will benefit from counseling to deal with narrowed visual fields and night vision, and use of low vision aids. Advanced cases may need vocational rehabilitation and mobility training. While many patients will maintain reasonable central vision for years, some are legally blind (visual field  $<10^\circ$ ) and in the United States are entitled to more favorable tax treatment and governmental benefits due to this disability. Driving is often an issue with this group of patients. Many jurisdictions are aggressive about denying RP patients the right to drive. Interestingly, there have been studies that suggest that RP drivers are safer because they know their limitations. In many RP types, good central vision is maintained until advanced stages of the disease, and the issue is the size of the visual field. Patients must stop driving when their visual field is narrowed to the point where they will miss objects that will be in the path of their car. Common sense management works if the patient is realistic.

RP and some other retinal dystrophy patients commonly have additional problems such as headaches, light flashes, and light sensitivity, which should be treated symptomatically with standard headache medications and UV sun screening glasses. Careful studies of retinal degeneration have demonstrated that the retina remodels where surviving neurons develop new dendrites to adjacent cells (21). This may be the source of many aberrant flashes perceived by patients. Maintaining optimal



**FIGURE 138-4** Congenital RP and macular colobomata (staphylomata) is an autosomal recessive disorder. The macular area does not form, leaving a central “scooped-out” appearance; a diffuse pigmentary retinopathy is present from an early age.

vision with glasses and optical aids, and checking for treatable diseases such as cataract and glaucoma should be performed annually. If careful selection criteria are used, RP patients with cataracts may benefit from cataract surgery without fear of worsening the RP. In some patients, however, the visual symptoms from cataract become severe at about the same time that the visual field becomes noticeably narrowed; cataract surgery does not restore lost visual field (unless the cataract is severe), which is disappointing to some RP patients despite preoperative counseling that only the visual acuity is likely to be improved by surgery. Posterior chamber intraocular lenses give the broadest possible visual field and are strongly recommended for RP patients.

### 138.3.3 Treatment

A number of options for pigmentary retinopathies are currently under clinical trial. The use of ultraviolet absorbing lenses with or without tint should be encouraged. Vitamin supplements in adult patients (over the age of 18) with RP and in good general health are recommended; 15,000IU/day (palmitate form) of a vitamin A supplement under medical supervision is advised. It is suggested that they avoid high doses of vitamin E. The patient should be advised to avoid toxic levels of any vitamin or mineral (22). About 10% of RP patients will have retinal edema or cystoid macular edema during the course of their disease. There are several etiologies underlying this complication, including inflammatory reactions after cataract surgery, autoimmune components, and rarely vitreous-macular traction. Various therapies have been used with moderate success, including nonsteroidal anti-inflammatory drugs for postoperative cataract edema, and standard immunosuppressive agents for more extensive retinal edema (23). Anti-carbonic anhydrase inhibitors have shown some limited success in some patients also. Maintaining optimal vision with glasses and optical aids and checking for treatable diseases such as cataract and glaucoma should be performed annually. If careful selection criteria are used, RP patients with cataracts will benefit from cataract surgery without fear of making the disease worse. In some patients, however, the visual symptoms from cataract becomes severe about the same time that the visual field becomes noticeably narrowed; cataract extraction does not restore lost visual field (unless the cataract is severe), which is disappointing to some RP patients despite preoperative counseling that only the visual acuity is likely to be improved by the surgery. Posterior chamber intraocular lenses give the broadest possible visual field, and current knowledge would suggest that RP patients undergoing cataract surgery would be advised to have only this type of lens implant.

Several clinical trials, including therapy with valproic acid, DHA supplementation, retinal prosthesis, intelligent medical implant and genethrapy are underway and can be looked up at [www.clinicaltrials.gov](http://www.clinicaltrials.gov).

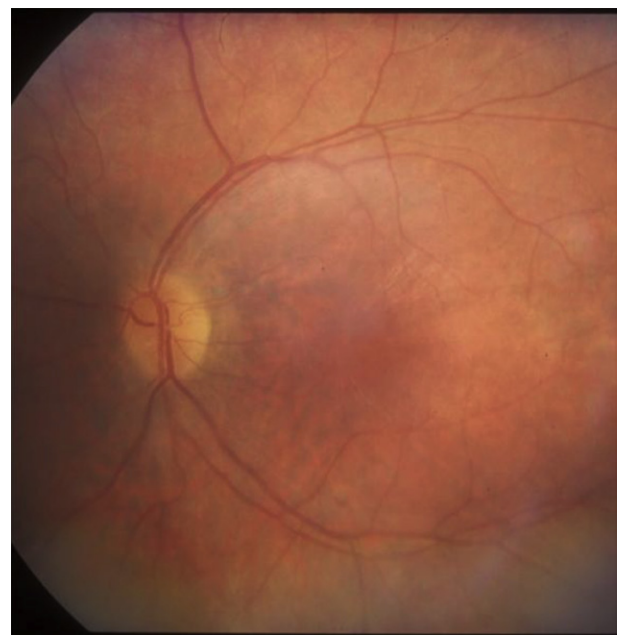
## 138.4 LEBER'S CONGENITAL AMAUROSIS

Leber's congenital amaurosis is characterized by an infant with poor vision since birth and has a nonrecordable ERG. The fundus examination may initially be normal, but over time pigmentary deposits usually develop (Figure 138-5). A searching nystagmus develops in early infancy and is common by two years of age.

A second form of Leber's is characterized by infantile night blindness, but the infant clearly can see. Symptoms of visual field loss are frequently noted by age 4. By this time an early pigmentary retinopathy may be noted, and the ERG is severely affected, but the child may maintain tubular fields with a visual acuity through middle adulthood in the 20/70–20/200 range. This form has been called Juvenile Leber's amaurosis, and a number of juvenile and LCA genes with less severe expression have been identified (Table 138-1).

Another form of congenital RP consists of pigmentary retinal degeneration and macular coloboma or staphyloma; occasionally keratoconus and mild skeletal abnormalities are found. All the previously mentioned forms of early RP are inherited in the autosomal recessive fashion, and all patients usually have varying degrees of hypermetropia. Some patients with ARRP have this phenotype, but the others remain unknown.

Finally, there is a rare form of autosomal dominant infantile onset RP that manifests early, but behaves in many ways like a very early onset, progressive cone-rod degeneration. These patients have normal appearing retinas until their adult years, but the visual fields progressively constrict and the patients become night-blind. These patients have been found to have overlapping



**FIGURE 138-5** Leber congenital amaurosis with an almost normal fundus appearance other than mild pigmentary changes.

segment of exons 3a and 3b in CRX gene resulting in a 12-bp deletion in codon 146.

### 138.4.1 Treatment

Gene replacement therapy is in clinical trial for Leber Congenital amaurosis caused by *RPE65* gene and has shown promising results (24).

## 138.5 THE PRIMARY CONE DEGENERATIONS

There are a number of different cone dysfunction syndromes that manifest from birth, or cone degenerations that occur in teenage or even later adult life. The group is diagnosed by an abnormal or nonrecordable photopic ERG and a normal scotopic ERG, while peripheral visual fields remain normal. All three inheritance patterns have been found in the cone degenerations. The genes causing this disorder are outlined in Table 138-1.

Cone degenerations occurring after infancy generally fall into two age ranges, with symptoms beginning ages 4–8 and then from the late teen years to age 30. The visual acuity may range from 20/50 to 20/400. Because the majority are autosomal recessive, there is seldom a family history; autosomal dominant pedigrees of cone degeneration have been documented and do not tend to become symptomatic until adolescence to the mid-1920s. There are likely multiple genetic forms of both the autosomal recessive and dominant cone degenerations. An adult-onset X-linked recessive cone dystrophy with a tapetal-like sheen (beaten metal appearance) and Mizuo–Nakamura phenomenon in which the fundus appearance changes with dark adaptation has been reported in several pedigrees, but the gene has not yet been determined.

Symptoms of cone dysfunction include loss of visual acuity, photophobia (light intolerance), and progressive color vision loss. Retinal pigment epithelial loss and pigment deposition may be seen in later stages. Mild to severe temporal optic atrophy is commonly seen. Demarcated circular macular lesions with or without tapetal reflexes or macular atrophy may be seen.

The cone degenerations should not be confused with congenital color blindness, in which there are color deficits for specific colors but no associated retinal degeneration. Patients with congenital color blindness (protanopia, deutanopia, and tritanopia) have normal visual acuity and do not show signs of progressive disease. The molecular mechanisms of color blindness have been well delineated by Nathans.

### 138.5.1 Achromatopsia

Achromatopsia (rod monochromatism) is a rare autosomal recessive form of cone dysfunction that is characterized by a near normal fundus appearance, nystagmus, a nonrecordable photopic ERG, and normal to near

normal scotopic ERG that remains stable. Vision is usually 20/200–20/400. Many patients demonstrate intense photophobia, increased reflexes from the retina surface, and may show temporal optic atrophy. Slight macular retinal pigment epithelial disturbances may be seen, but the appearance is essentially normal. The achromatopsia causing genes include *CNGA3*, that codes for the cone photoreceptor cGMP-gated cation channel, alpha subunit, cyclic nucleotide-gated channel, beta-3, *CNGB3* causing achromatopsia in the Pingelapese and *GNAT2* (25).

Dominant cone dystrophy linked to 6p21.1 was found to be due to mutations in guanylate cyclase activator 1A (*GUCA1A*), a calcium binding protein that is expressed in photoreceptor outer segments.

### 138.5.2 Blue Cone Monochromatism

X-linked blue cone monochromatism has similar features to rod monochromatism but is usually milder in expression. These patients are often diagnosed as having “congenital nystagmus,” and initially the correct diagnosis is missed until an ERG is performed. Two forms of blue cone monochromatism has been found by Nathans due to mutations in the green and red cone opsins, *OPN1LW*, and *OPN1MW*.

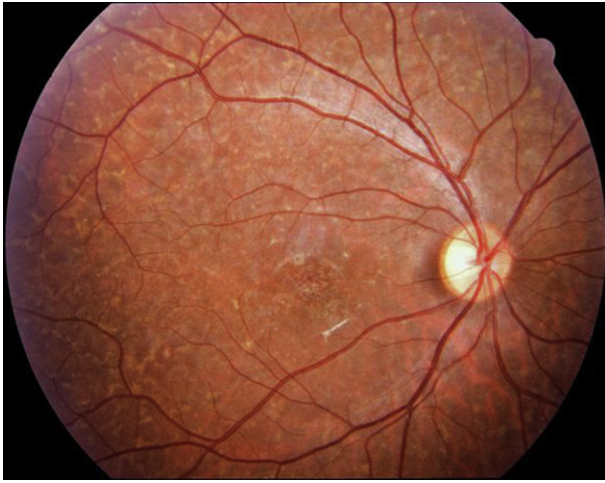
### 138.5.3 Fleck Retina Diseases (Fundus Flavimaculatus, Fundus Albipunctatus, Retinitis Punctata Albescens, Familial Drusen)

The clinical diagnosis of a specific fleck retina disease is made by a combination of ophthalmoscopy, electrophysiological and psychophysiological testing, clinical evidence of stationary or progressive disease, evaluating the visual field test. Photographs and the fluorescein angiogram may be particularly valuable in diagnosing Stargardt’s disease. These groups of disorders are characterized by discrete yellowish or yellow-white lesions at the level of the retinal pigment epithelium (RPE) that may be scattered throughout the fundus or confined to the posterior pole. These diseases are sometimes mistakenly called “fleck retina syndrome,” which is not correct as each disease is quite distinctive in appearance and/or clinical course. The genes for most of the fleck retinal diseases have been found.

### 138.5.4 Fundus Flavimaculatus (Stargardt’s Disease)

Fundus flavimaculatus or Stargardt’s disease is characterized by linear, or fish scale-shaped (pisciform) yellow lesions (Figure 138-6), which are most often seen in the macular region, but may extend out to the equator regions in distribution. Histological examination of donor eyes demonstrates lipofuscin deposits retinal pigment epithelial cells (RPE), which cause hypertrophy and





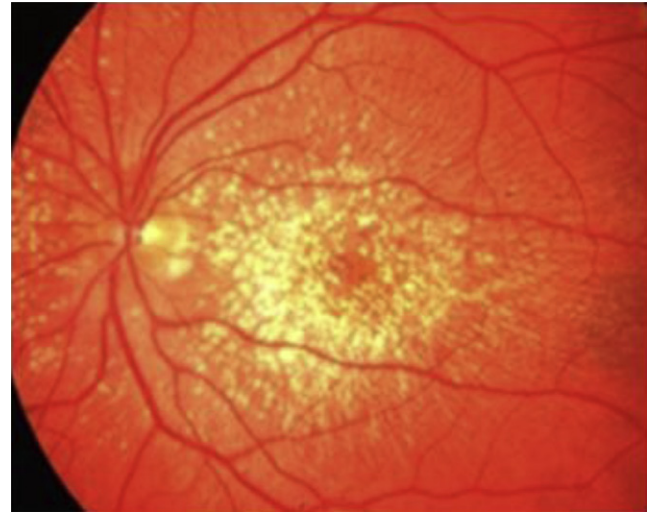
**FIGURE 138-6** Fundus flavimaculatus, one of the fleck retina diseases, is characterized by linear or fish scale flecks scattered throughout the posterior pole. Some patients develop atrophic macular and/or paramacular lesions. All cases are due to mutations in the ABCA4 gene.

clumping of localized RPE, which appear as “flecks” when compared to the adjacent normal-appearing RPE cells.

Fishman and Krill categorized fundus flavimaculatus into four stages of severity. Fundus flavimaculatus typically starts with a ring of yellow flecks surrounding the fovea with normal ERG and EOGs, and vision varying from 20/30 to 20/100. On fluorescein angiography the macula region is hypofluorescent (“dark choroid”) and the flecks may show focal hyperfluorescence (window defects) on fluorescein angiogram. There is a wide variety of expression, with varying age of onset and with some patients experiencing severe macular degeneration, and others remaining stationary. Some patients stabilize for a time with foveal islands of intact RPE with severe parafoveal RPE loss. Fundus flavimaculatus has been found in both autosomal dominant and recessive pedigrees (the latter most common). If the patient does not have macular involvement, the disease may be a relatively benign problem.

The ERG and EOG are normal to subnormal correlating with the amount of fundus involvement. Some patients may experience mild night blindness. The Goldmann visual field using smaller isopters may demonstrate a central scotoma, and a few patients may show some contraction of the peripheral field.

The Stargardt ABCA4 gene maps to chromosome 1p21–p13 and is part of the ATP-binding cassette (ABC) family, which is involved in energy-depending transport across membranes. The ABCA4 gene contains at least 50 exons and spans about 150 kb. The gene has high polymorphism. The gene has been implicated in fundus flavimaculatus, Stargardt’s disease, cone-rod dystrophy, and RP. The ABCA4 gene appears to have interaction with other genes to be disease-causing since many patients have been found to only have one allele with an ABCA4 mutation.



**FIGURE 138-7** Familial drusen are inherited in the autosomal dominant manner, characterized by accumulation or deposits of yellow material at the level of the retinal pigment epithelium. This fundus image shows radial distribution of drusen seen in Doyme honeycomb retinal dystrophy or Malattia Leventinese syndrome caused by mutation in the EFEMP1 gene.

### 138.5.5 Familial Drusen (Doyme Honeycomb Dystrophy, Malattia Leventinese)

The term “drusen” is used in ophthalmological nomenclature to refer to yellow–white deposits that are seen in the retina at the level of the retinal pigment epithelium with the ophthalmoscope (Figure 138-7). Retinal drusen should not be confused with golden yellow “drusen” of the optic nerve head, which are calcifications in the nerve substance inherited in the autosomal dominant fashion or an isolated occurrence. Unfortunately the retinal and optic nerve head types of drusen have been called with the same name.

Krill divided retinal drusen into hereditary and secondary degenerative forms. Familial forms are inherited in the autosomal dominant mode and occasionally may have incomplete penetrance. They typically start to appear at age 20–30, but may not become clinically significant for 10–20 more years. The late onset of symptoms increases the difficulty of establishing the autosomal dominant pattern, since many older relatives who were affected are deceased.

Initially the drusen begin as small round dots in the posterior pole, and over many years they increase in number and become more confluent. Pigment clumps may appear, and atrophy of the RPE may occur, leaving an atrophic macular scar in later years. A few cases have enough pigment clumps to entertain the diagnosis of RP. The extent of the drusen is variable, with most cases limited to the posterior pole. Retinal edema, subretinal hemorrhage, and neovascularization may occur in familial drusenosis, similar to the pathophysiological processes occurring in other types of macular degeneration. There is no known



effective treatment for this primary retinal degenerative process; secondary problems such as subretinal neovascularization occasionally may be treated with photocoagulation. The ERG is usually normal, though occasionally is mildly abnormal. The EOG is commonly abnormal.

The gene for Doyme honeycomb retinal dystrophy and Malattia Leventinese has been designated EFEMP1 for fibulin-like extracellular matrix protein 1, which maps to 2p21-p16.

### 138.5.6 Retinitis Punctata Albescens and Fundus Albipunctatus

Retinitis punctata albescens (RPA) and fundus albipunctatus (FA) are often compared because their phenotype can be identical. Both RPA and FA patients have numerous discrete white to yellow dots scattered throughout the fundus (Figure 138-8). RPA is progressive while FA is stationary. RPA is caused by mutations in RLBP1, a gene encoding cellular retinaldehyde-binding protein. FA is caused by mutations in RDH5, the gene encoding 11-cis retinol dehydrogenase.

Both conditions are inherited in the autosomal recessive fashion, although rare pedigrees of autosomal dominant FA have been reported. FA was classified as a form of congenital stationary night blindness by Krill. Clinically RPA acts like typical RP, with night blindness, progressive visual field loss, and retinal vessel attenuation. With time the focal yellow deposits may become pigmented.

In FA, patients are night-blind, but the visual acuity and visual fields remain normal. The ERG and EOG have been reported to be normal to abnormal. The retinal deposits can be stationary, increase, or regress. Marmor and others have reported patients with FA in which the ERG and EOG were abnormal initially and become



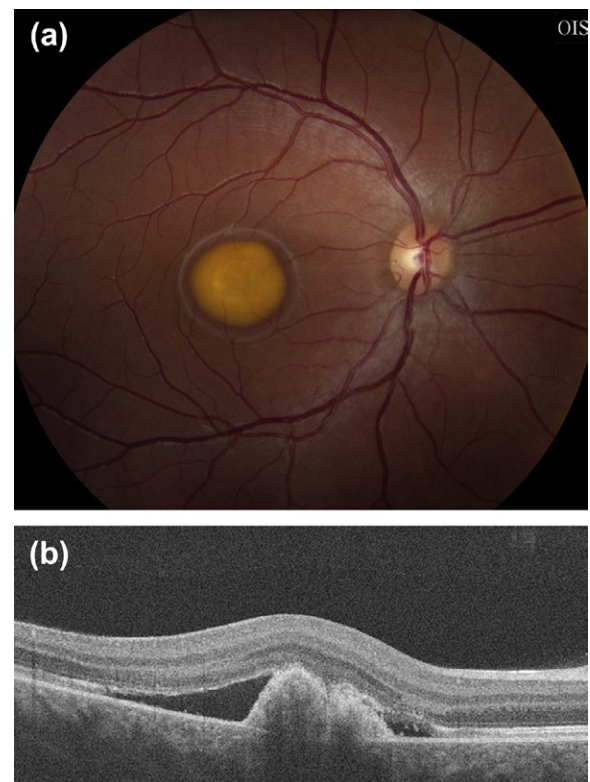
**FIGURE 138-8** Retinitis punctata albescens, characterized by discrete white-yellow deposits at the level of the retinal pigment epithelium seen throughout the retina. FA, a form of congenital stationary night blindness, has a similar appearance.

normal after 3 or 4 h of dark adaptation. These findings are consistent with abnormal photoreceptor pigment regeneration and help to distinguish FA from other types of congenital stationary night blindness.

Other conditions that can give scattered retinal spots include the fleck retina of Kandori, macular degeneration with cholesterol deposits, *peau d'orange* spots of pseudoxanthoma elasticum, vitelliruptive macular degeneration with multiple lesions, Bietti's crystalline retinopathy, the lacunae of Aicardi syndrome, the carrier state for X-linked albinism, punched-out lesions of presumed histoplasmosis syndrome, severe vitamin A deficiency, and scattered lesions of "birdshot retinochoroiditis." Many RP patients have fine scattered drusen as well as loss of the retinal pigment epithelium in discrete lobular patterns, which may give patterns somewhat similar to the fleck retina group.

### 138.5.7 Vitelliform Macular Dystrophy (Best Disease)

Best's disease is an autosomal dominantly inherited macular dystrophy that is known for its "egg yolk" macular lesion (Figure 138-9) that occurs early in the degenerative process, usually between 3 and 20 years of age. Krill reports having seen the lesion in a 16-month-old, and states that earlier lesions have been reported. Some family



**FIGURE 138-9** (a) Best disease, or vitelliruptive macular degeneration, has a distinctive "egg yolk" macular lesion in its early cystic phase. It is a dominant disease caused by mutations in bestrophin gene. (b) Optical coherence tomography showing subretinal lipofuscin deposits. (Courtesy: Dr Amy Hutchinson, Emory University.)

members do not develop the macular lesion, but always will have an abnormal EOG, which can be used to identify those patients at risk without findings who wish counseling. A natural history of this disorder has been published. Those affected have normal ERGs and abnormal electroculograms. The bestrophin gene is located on chromosome 11q13 and has been localized to the basement of the retinal pigment epithelium. The “egg yolk” lesions appear cystic, filled with a golden-yellow material. Vision is not as disrupted as might be thought from the appearance of the lesion, ranging from 20/30 to 20/60 in the early stages. Some patients will have multiple posterior pole lesions. However, if subretinal hemorrhage occurs, the vision may be poor. The natural course is for the cystic lesion to dissipate and leave an atrophic macular scar with mildly decreased central vision. Several histopathological reports have found increased cytoplasmic inclusions and lipofuscin granules in the retinal pigment epithelium; an abnormal fibrillar material was found in several retinal layers.

### 138.5.8 Albinism

Albinism is characterized by reduced pigmentation in the skin and hair with ocular involvement. Patients with any form of albinism may have ocular or visual disorders, ranging from severe to mild problems. In the past, albinism has been divided into oculocutaneous albinism and ocular albinism. In both there is a partial or total reduction of melanin deposition on melanosomes, due to mutations in genes involved in the biosynthesis of melanin pigment. There are multiple forms of oculocutaneous albinism.

Regardless of the type of albinism, the ocular involvement generally conforms to one of two clinical patterns: (i) congenitally subnormal visual acuity and nystagmus, and (ii) normal or only minimally reduced visual acuity and no nystagmus. The first pattern is true albinism, while the latter circumstance has been termed albinoidism because of the milder visual consequences. Both patterns share common clinical features of photophobia, iris transillumination, and hypopigmented eyes. They differ by whether the fovea develops; hypoplasia of the fovea is a sign of true albinism. Mutations in four genes OCA1, OCA2, OCA3 and OCA4 cause OCA, OA1 gene causes X-linked ocular albinism, and there are several genes causing Hermansky-Pudlak syndrome and Chediak-Higashi syndrome (26).

A common finding to all types of albinism is abnormal retinogeniculostriate projections, with many of the temporal hemiretinal nerve fibers decussating rather than projecting ipsilaterally to the geniculate body. These misprojections can be measured and diagnosed by visual evoked cortical potential testing. The first form of ocular albinism was described by Nettleship in a large X-linked recessive pedigree. The affected males had subnormal visual acuity, translucent irides, congenital nystagmus, photophobia, hypopigmentation of the fundus, and hypoplasia of the fovea. Pigmentary mosaicism is common in female carriers, who may be symptomatic. An

X-linked ocular form was reported in black patients who had moderately pigmented fundi and no transillumination of the iris, but patients with X-linked recessive ocular albinism had giant pigment granules on microscopic examination of skin biopsy.

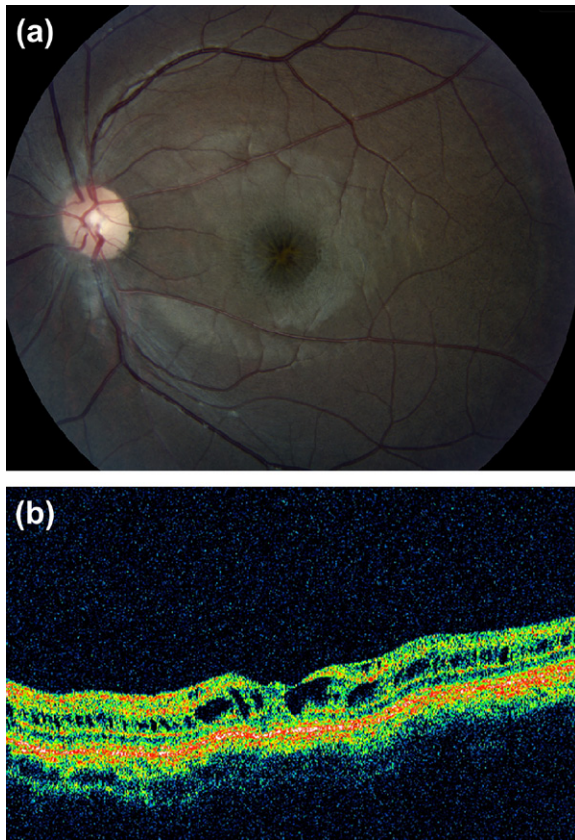
Autosomal recessive ocular albinism is relatively common, and females are as severely affected as are males. Patients have decreased visual acuities in the 20/100–20/400 range, translucent irises, congenital nystagmus, photophobia and strabismus. Patients are lightly pigmented at birth, and usually develop further pigmentation with age. In general, the more pigmentation that the patient demonstrates, such as around skin hair follicles and particularly in the retinal pigment epithelium in the posterior pole, the better is the visual prognosis. Many patients demonstrate improvement in their nystagmus and visual acuities as pigmentation develops with age. Genetic counseling depends on carefully establishing the inheritance pattern and gene testing of the OCA genes and OA1 gene that are clinically available.

### 138.5.9 X-Linked Retinoschisis

The term “retinoschisis” refers to a splitting of the neurosensory retina, which at times may mimic a retinal detachment when viewed with the ophthalmoscope. There are three forms of retinoschisis: senile onset with no known inheritance pattern, which involves a splitting of the nerve fiber layer of the retina; a secondary form that is mainly associated with tractional retinal detachments; and congenital X-linked recessive retinoschisis.

Congenital X-linked retinoschisis differs in significant ways from the senile form. The schisis occurs in the inner retina and may be slowly progressive. Eventually in some cases it may result in a panretinal degeneration. The phenotype is variable, even within families. The process also involves the macula, and one of the first signs that are seen is a microcystic elevation and radiating retinal folds of the macular area (Figure 138-9), but central vision may remain relatively good, typically in the 20/60 range. Several cases of macular schisis without peripheral involvement have been seen, but occur very infrequently. Peripheral schisis may or may not be present on a single examination, but usually it will be found with repeated examinations. Male children with the disease will frequently present with vitreous hemorrhages from broken retinal vessels in areas of schisis. Pigmentary deposits may develop in these areas destroyed in the disease process, so that advanced cases of X-linked retinoschisis can be mistaken for RP (Figure 138-10). The clinical diagnosis may have to be confirmed by viewing younger affected male family members.

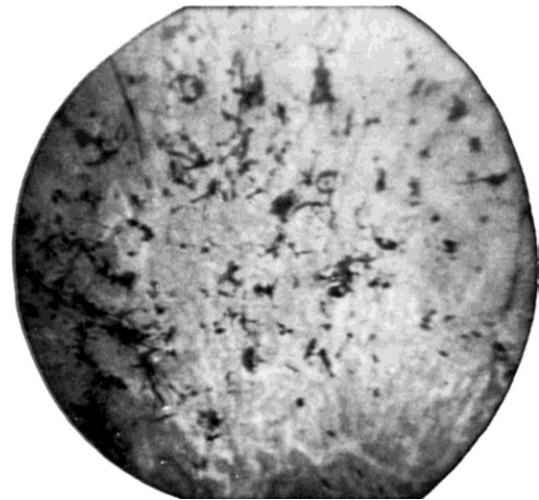
The retinal inner layer damage is reflected in the ERG; early in the disease the a-wave is normal or near normal, and the b-wave is attenuated in proportion to the amount of retinal schisis, typically giving a “negative



**FIGURE 138-10** (a) Congenital juvenile X-linked retinoschisis, with typical microcystic lesions of the maculae early in the disease. The retinoschisis may look similar to retinal detachment and usually develops later in childhood. The retinoschisis may be found during evaluation for vitreous hemorrhage, as the retinal vessels may break and bleed during the schisis process. (b) Optical coherence tomography showing cystic lesions in the neurosensory retina. (Courtesy: Dr Baker Hubbard, Emory University)

waveform” (see Table 138-2). Negative waveforms of the bright flash dark-adapted ERG occur in diseases in which the middle retina is affected and the photoreceptors are generally unaffected, resulting in an a-wave and no effective b-wave. The ERG may become extinguished in cases in which there is extensive retinal damage. The EOG and dark adaptation test are normal to abnormal depending on the stage of the disease.

The gene for the X-linked form of retinoschisis, retinoschisin, has been identified, and was initially found to be localized to the photoreceptor; however, subsequent studies have shown that the protein is transported to Müller cells. Presumably, retinoschisin is essential for Müller cell health, since mutations in its coding results in their degeneration. However, Wu reports that the mutational damage localizes to the bipolar synapse. Müller cells span the layers of the retina, with their endplates forming the inner limiting membrane and the distal ends forming the outer limiting membrane at the inner segments. Loss of the bridging extracellular retinoschisin matrix appears to be a key to the changes seen in retinoschisis.

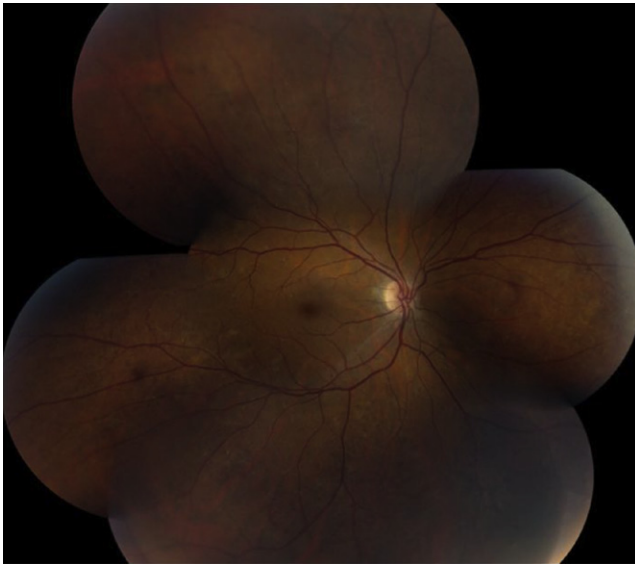


**FIGURE 138-11** Advanced juvenile retinoschisis in a 63-year-old man, demonstrating a pigmentary retinal degeneration with the appearance of RP.

### 138.5.10 Choroideremia

Choroideremia is an X-linked recessive retinal degeneration that clinically behaves like typical RP. Affected males develop symptoms of peripheral vision loss and night blindness from age 5–20 years. Occasionally a female carrier will develop symptoms and will have a mild retinal degeneration on ophthalmoscopic examination, though females are rarely seen with the disease. Affected female cases have been hypothesized to be extreme examples of lyonization. The majority of choroideremia carriers show some fundus signs, including subretinal pigment clumping and an appearance of granularity to the retinal pigment (Figure 138-11). When carriers are tested, usually the ERG, dark adaptation study and visual field examination are normal, but carriers with extensive fundus changes will demonstrate abnormalities on testing and may show progression with time. Usually the rate of progression does not functionally affect the carrier. Affected males have a lobular loss of the retinal pigment epithelium (RPE) and the underlying choriocapillaris, which starts in the equator region of the fundus with the retinal degeneration progressing toward the anterior retina and posterior pole (macula and surrounding retinal area) (Figure 138-12). Because the process is insidious, patients are frequently seen for the first time in a moderately advanced state in which the posterior pole has the only remaining normal tissue, allowing the patient good central but little side vision. The central retinal area appears to be more resistant to the disease process, which is also true for many other types of RP and central vision is often good even in advanced stages. After many years some patients will lose this central vision. The disease process may take 40 or 50 years to reach this tragic end point. Even with this extreme visual loss, many patients maintain some perception of light, or even hand motion vision. When recordable, the ERG





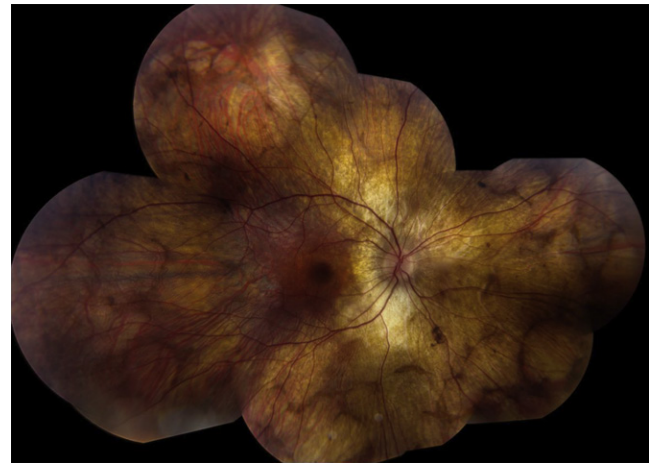
**FIGURE 138-12** Fundus images of Choroderemia carrier, an X-linked recessive retinal degeneration due to a mutation of geranyl transferase showing subretinal pigment clumping and an appearance of granularity to the retinal pigment.

shows a rod-cone degeneration. The EOG is abnormal, and dark adaptation testing shows final rod thresholds 2.4–4.0 log units elevated, proportional to the amount of retinal degeneration. The gene for choroideremia was described by Cremers, and has been described as a Rab escort protein defect (REP-1) resulting from mutations in the RAB27A gene. REP-1 acts in the prenylation of GTPases, regulators of intracellular protein trafficking. Clinically, the diagnosis is confirmed by the distinctive fundus pattern on the fluorescein angiogram in which there is frequently a scalloped pattern of RPE loss with no increased pigmentation to the RPE (Figures 138-13 and 138-14). Takki clearly demonstrated the biochemical difference between gyrate atrophy and choroideremia patients even though the fundus pattern is similar. The difference in inheritance patterns (AR vs. XL) in the two disorders also helps to differentiate them.

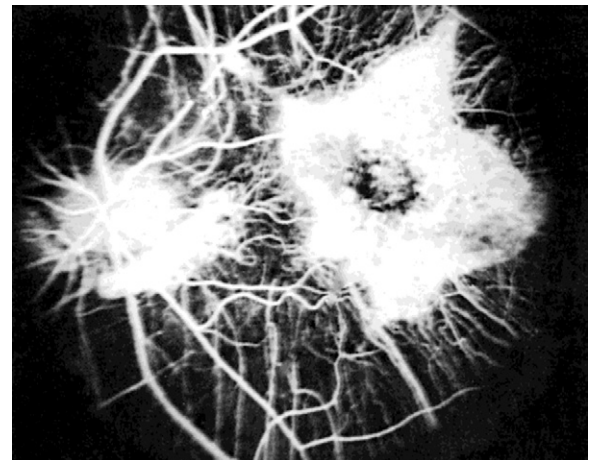
The initial diagnosis may be difficult because there are several different fundus appearances to choroideremia, and the RPE and choriocapillaris loss may not be obvious in all cases on ophthalmoscopy. If a patient clearly has X-linked recessive inheritance and choroideremia cannot be ruled out, then a fluorescein angiogram will clearly differentiate whether there is choriocapillaris loss and large choroidal vessel preservation.

Several syndromes with fundus features of choroideremia have been documented; van den Bosch reported an X-linked recessive kindred with mental retardation, acrokeratoses verruciformis, anhidrosis, and skeletal deformity. Ayazi reported a three-generation X-linked recessive family with choroideremia, congenital deafness, and obesity.

Differential diagnosis would include patients with Bietti's crystalline dystrophy, which is an autosomal



**FIGURE 138-13** Fundus images of Choroderemia affected individual with a mutation in the CHM gene with loss of retinal and choroidal layers outside of the macular region, scalloped appearance in mid periphery.



**FIGURE 138-14** Fluorescein angiogram of advanced choroideremia case, in which there is loss of retinal tissue outside the posterior pole with preservation of larger choroidal vessels. Fluorescein angiogram highlights the pattern of intact retinal pigment epithelium and choriocapillaris remaining in the macular area. This X-linked disease is due to mutations in the geranyl transferase Rab escort protein.

recessive disorder and will show posterior pole and occasionally equatorial choriocapillaris atrophy very similar to choroideremia; in these patients there are usually crystalline deposits in adjacent more normal retina and sometimes in the cornea. Patients who have thioridazine [Mellaril] retinal toxicity will have choriocapillaris dropout similar to choroideremia, but the psychiatric background is frequently found on taking the medical history.

Initially patients with gyrate atrophy and a few patients with other types of RP may be confused with choroideremia, since a scalloped pattern of retinal degeneration may be seen. However, distinguishing features are usually noted; gyrate atrophy and RP patients with this pattern do not have X-linked inheritance, and in gyrate atrophy the retinal pigment epithelium has a darker appearance



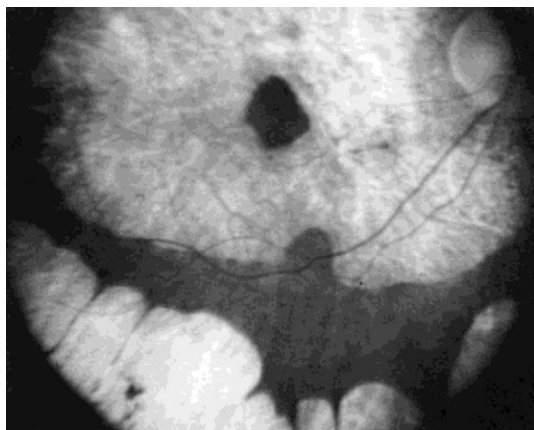
compared to the blonder fundus in choroideremia. The diagnosis of gyrate atrophy and Bietti's can be confirmed by mutational testing, and plasma ornithine levels or ornithine aminotransferase activity also will confirm if gyrate atrophy is present. RP patients with RPE and choriocapillaris dropout often have bone spicule formation in equator regions, and may demonstrate choroidal sclerosis, and relative preservation of anterior retinal RPE compared to the loss of these structures that is seen in choroideremia.

### 138.5.11 Gyrate Atrophy

A deficiency in ornithine amino transferase leads to ornithine plasma elevations, which are 6–10 times greater than normal and well documented to be associated with gyrate atrophy. Gyrate atrophy is an autosomal recessive disease that may manifest by eight years of age, and sometimes later. Patients develop a scalloped loss of retinal pigment epithelium (RPE) and choriocapillaris, and intact RPE has more pigmentation than normal (Figure 138-15). Myopia and cataracts are also commonly found. The ERG is abnormal to nonrecordable. There is progressive visual field loss and night blindness. It was originally thought that the effects of the hyperornithinemia were restricted to retinal degeneration, but mild systemic effects have been documented, including abnormal EEGs and changes in hair and muscle fibers; no obvious alteration in life span has been noted. Correction of ornithine accumulation prevents retinal degeneration in a mouse model of gyrate atrophy (27).

### 138.5.12 Usher Syndrome

Usher syndrome is the name most commonly given to the association of RP and congenital deafness, whether partial or profound. Some RP patients have acquired deafness in later adult years, and this is not termed Usher syndrome.



**FIGURE 138-15** Gyrate atrophy results from a mutation of ornithine amino transferase, leading to high serum ornithine. Patients with the disease typically have a scalloped loss of retinal pigment epithelium with progressive loss, myopia, and cataracts.

The one exception that confuses the rule is patients with Usher 3A, who have a progressive hearing loss with their RP, which is not typically noted until adult years. Usher 3A is due to clarin-1 a novel 4-transmembrane protein with a possible role in hair cell and photoreceptor synapses. Clarin-1 accounts for 40% of Usher syndrome in Finland; possible digenic deafness with *MYO7A*.

Both the partial and profound forms are inherited in the autosomal recessive manner. Currently there are 12 types of Usher syndrome in which the chromosomal location is known, of which ten have gene isolation (see RetNet).

*USH2A* is caused by mutations in Usherlin at chromosome 1q41, and encodes a protein that possesses laminin epidermal growth factor as well as fibronectin type III domains. A small number of patients with mutations have RP alone without deafness (28). *USH3A* is caused by mutations in *MYO7A*, a common component of cilia and microvilli. *USH1D* is caused by mutations in cadherin-like gene23 (*CDH23*), an intercellular adhesion protein. *USH1C* is caused by the Harmonin gene, a PDZ domain protein. In addition to this moderately well-described syndrome, a number of other genetic conditions and environmental insults may lead to pigmentary retinopathy and hearing loss. By careful study of individual patients and families, the diagnosis of Usher syndrome may be made with relative certainty.

The exact incidence of Usher syndrome has been difficult to determine, but surveys of RP patients suggest that about 10% are profoundly deaf, and ophthalmological examinations of children in deaf schools reveal that approximately 6% have RP. Vernon suggests that the prevalence of Usher syndrome is 3 cases per 100,000, and the carrier state is estimated to occur in about 1 per 100 individuals.

Four clinical subtypes of Usher syndrome have been defined by Merin. These consist of type I of RP with total deafness and no vestibular function; type II with RP, partial deafness, and intact vestibular function; type III with RP, complete deafness, vestibular ataxia, and sometimes psychosis (Hallgren syndrome); and type IV with RP, total deafness, and mental retardation. Studies with computed tomography have demonstrated that Usher syndrome patients have cerebellar atrophy. Patients with phytanic acid storage disease appear to meet the criteria for Merin type IV Usher's syndrome although it is not clear whether Merin described the same entity. Hallgren syndrome may be an environmentally induced problem occurring in Type I Usher patients since it has not been reported outside of Sweden, and the psychoses that some patients develop have been hypothesized to be related to the long hours of winter darkness.

Those affected with Usher syndrome are either congenitally deaf (deaf mutes) or they may be afflicted with partial deafness, which can be detected clinically by the speech impairment this causes. There is some variability in the progression in those patients who are partially deaf, and while not common, abrupt deterioration has been reported at puberty. The hearing defect is described as a

sensorineural loss, affecting mainly the higher range of frequencies bilaterally. The RP is slowly progressive over many years, and many of these individuals eventually will become blind.

### 138.5.13 Other Inherited Retinal and Choroidal Diseases

There are numerous hereditary primary and secondary retinal diseases, which cannot be covered in this overview, but the more common or well known ones are presented in Table 138-2. Sources for studying these diseases further would include McKusick's Mendelian Inheritance in Man (OMIM) at [www.omim.org](http://www.omim.org) and RetNet, the Retinal information Network, at <http://www.sph.uth.tmc.edu/RetNet>.

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### Biography



**Dr Suma Shankar** joined Emory University in January, 2010 with joint appointment in the Department of Human Genetics and the Department of Ophthalmology. She received her medical degree from Bangalore Medical College, India and did her Ophthalmology residency training and obtained the Membership of the Royal College of Ophthalmologists in London, and the fellowship of the Royal College of Surgeons in Edinburgh in the United Kingdom. She obtained a PhD, focusing on molecular genetics of eye diseases and completed a fellowship in pediatric ophthalmology at the University of Iowa. Following this she went to the University of California in San Francisco for another fellowship in medical genetics. Her research interests include the molecular genetic studies of ophthalmic diseases and ophthalmic manifestations in Ras/MAPK pathway syndromes. She has won the Western Scholar and Henry Christian Award for Excellence in Research from American Federation of Medical Research. She is leading the “Ocular Predictive Heath Initiative” project with a goal of establishing a database and DNA bank for inherited and complex eye diseases with genetic etiology at Emory.

# CHAPTER

# 139

## Strabismus

*J Bronwyn Bateman and Sherwin J Isenberg*

This article is a revision of the previous edition article by J Bronwyn Bateman and Sherwin J Isenberg, volume 3, pp 3228–3240.

### 139.1 INTRODUCTION

Strabismus, or ‘squint,’ is a misalignment of the visual axes of the eyes and occurs commonly. It may be termed ‘lazy’-, ‘wall’-, or ‘cross-eyed’ by the lay population. Nomenclature and classification systems are based on the amount (measured in prism diopters); direction of the deviation – horizontal, vertical, or torsional; laterality of deviating eye; frequency; age of onset; and the amount of deviation in the different fields of gaze (comitance or concomitance). The deviation can be latent (phoria; see below) or intermittent, both of which are based on fusional or binocular capabilities of the individual or, alternatively, it can be constant (tropia). The monofixation syndrome, a subset of strabismus defined as a small deviation (usually 10 prism diopters or less), may not be evident on external inspection and is associated with reduced but measurable fusion (binocularity; use of the two eyes together). Strabismus may be categorized on the basis of the age at onset of the deviation: ‘infantile’ is generally defined as occurring in the first 6 months of life, and ‘acquired’ develops at any age thereafter. Typically, as opposed to cranial nerve palsies or restrictive disorders such as dysthyroid ophthalmopathy, deviations that develop in childhood do not vary substantially in different fields of gaze (comitant or concomitant) such as right/left and up/down. The appearance of strabismus with aligned visual axes, as commonly occurs in infants and young children with epicanthal folds, is termed pseudostrabismus. Additionally, facial configuration may result in the appearance of strabismus even if the eyes are normally aligned.

Most forms of developmental delay are associated with a higher incidence of strabismus. Some forms of strabismus are associated with cranial nerve aplasia/malinnervation.

### 139.2 INCIDENCE AND PATHOPHYSIOLOGY

Strabismus occurs in 1.3–5.7% of all children (1–6). In some studies, strabismus occurs more frequently in females (7,8) and, in others, it occurs equally in males and females (9). For unknown reasons, normal males

have a stronger ability to maintain ocular alignment (10); evolutionary pressures may have favored human males with better binocularity.

Although refractive error is a variation of normal and has a genetic component, it is not a single gene phenotype. Both myopia (nearsightedness) and hyperopia (farsightedness) are important risk factors for the development of strabismus. The pathophysiological basis(es) of most cases are poorly understood. Clustering in families has been noted by many authors and dates to Hippocrates, who commented:

*‘We know that bald persons descend from bald persons; blue-eyed persons from blue-eyed persons and squinters from squinters, at least in the majority of cases’ (11).*

### 139.3 CLINICAL BACKGROUND

Historically, strabismus has been divided into phorias and tropias. A tropia is a ‘manifest’ deviation that is evident under binocular conditions (both eyes uncovered). Horizontal deviations are termed exotropia or exophoria (divergent), and esotropia or esophoria (convergent); vertical deviations are described as hyper- or hypotropias or phorias. As most individuals with strabismus have one eye that has better vision than the other, the deviating or non-fixating eye is designated. The horizontal and vertical deviations are quantified in prism diopters; a cyclotropia in which one or both eyes are torsionally deviated is measured in degrees. Horizontal and vertical deviations may be intermittent; namely, the strabismus may be evident at one moment but not another. A phoria is a deviation that develops under monocular viewing conditions; to identify a phoria or an intermittent (latent) strabismus (heterophoria), one eye is covered to eliminate the binocular stimulus to the central nervous system for ocular alignment. The deviation measured under the occluder is termed a phoria.

The causes of strabismus are poorly understood and the natural history differs in children compared to adults. Risk factors for the development of the most common forms of strabismus – the non-paralytic and



non-mechanical form with an onset in childhood – include reduced visual acuity uni- or bilaterally, refractive error of one or both eyes, reduction of the fusional (binocular) capabilities, mental retardation, neurological dysfunction, muscle disorders, including hypertonicity and hypotonicity, and craniofacial dysostoses. Less commonly, the disease can result from cranial nerve paresis, mechanical restrictions of the extraocular muscles, malformations of extraocular muscles, or supranuclear gaze disorders; such deviations are usually incomitant (difference in deviation depending on the field of gaze).

Sharp (focused on the retina) visual acuity in each eye and ocular alignment in early childhood fosters the central development of fusion (binocularity). Glasses/contact lenses may be necessary for sharp vision. Fusion is the ability to integrate the images from each eye and is the basis of depth perception; it develops early in life, during the stages of central nervous system plasticity in infancy. Ocular alignment is maintained by these fusional mechanisms, primarily stimulated by images on the peripheral retina. Children younger than 8–10 years of age have the ability to ‘suppress’ one of the images; thus, they usually do not have diplopia or double vision as a result of the ocular misalignment. Under binocular conditions, an individual with suppression will use one eye only; in an individual with normal fusion, the brain combines the slightly different images from each eye into a three-dimensional image. If central or peripheral vision is reduced in one eye, the risk for the development of strabismus is increased with the affected eye (reduced vision) deviating. Thus, organic causes of decreased vision, such as an asymmetrical decrease in central vision due to genetic diseases (e.g. Best disease) increases the risk of strabismus, as do decreases in peripheral vision, as occurs in retinitis pigmentosa. Likewise, decreased vision in an eye due to amblyopia increases the risk for strabismus. Curiously, the very gradual development of strabismus in a disease such as progressive external ophthalmoplegia may not cause double vision; the eye with reduced vision may be ‘ignored’.

The common forms of strabismus develop in childhood; congenital reduction of vision is associated with the onset of exotropia in infancy or early childhood. Individuals with a bilateral reduction of central or peripheral vision, for whatever reason (genetic or environmental), may have reduced binocularity, which constitutes an independent risk factor for strabismus.

The refractive status of the eyes, although a variation of normal in most instances, is the most common risk factor for the development of strabismus and is determined by the curvatures of the cornea and lens as well as the axial length (size) of the globe. These anatomical parameters form the basis of hereditary risk factors for strabismus. Hyperopia (farsightedness) predisposes to the development of acquired esotropia in childhood because the hyperopic individual must accommodate (focus) to clear the blurred image; accommodation and convergence

are synchronous. Normally, accommodation and accommodative convergence occur primarily when viewing near targets. However, hyperopic individuals must exert higher levels of accommodation both at a distance and close by in order to see clearly, and an esotropia may develop. Since accommodation is greatest for close targets, the deviation may be largest in these instances. Additionally, asymmetric refractive errors between the eyes may result in unequal visual acuity with and without glasses/contact lenses, which, in turn, may cause reduced binocularity, suppression, and an increased risk of strabismus. All children with strabismus are at risk of developing amblyopia (poor monocular vision related to reduced stimulation of the monocular visual pathways of the central nervous system in childhood) in the deviated eye. Myopia (near-sightedness) is a risk factor for exotropia.

Developmental delay, mental retardation, neurological dysfunction, and muscular disorders, including hyper- and hypotonicity, regardless of the cause, increase the likelihood of developing strabismus. Children with cerebral palsy are particularly prone to ocular misalignment. The mechanisms are poorly understood and may be similar to that in normal individuals; in particular, the neurological process of accommodative convergence and fusional ability may be poorly coordinated, predisposing to misalignment of the visual axes.

Craniofacial configuration is primarily determined genetically, and the interpupillary distance may influence the risk for the development of strabismus; a wide interpupillary distance increases the likelihood of exotropia (12,13). Craniofacial configuration may give the appearance of strabismus (pseudostabismus) when there is normal ocular alignment and, conversely, individuals with strabismus may appear to have normal ocular alignment. Furthermore, individuals affected with any of the craniofacial dysostoses are at increased risk of strabismus on the basis of altered (increased or decreased) interpupillary distance, exorbitism (shallow orbit), and/or malformations of the extraocular muscles. Strabismus is very common in the Crouzon, Apert, and Pfeiffer syndromes and in craniofacial malformations associated with midline defects.

### 139.4 ISOLATED STRABISMUS

The causes of non-paralytic strabismus, the most common form of strabismus, are many, and no single mechanism, environmental agent, and/or gene defect, has been identified as causative. An OMIM assignment has been given to a presumed susceptibility gene [OMIM %185100. There have been many, generally older, reports regarding the heritability of the common forms of strabismus and several excellent reviews have been published (8,14–17). Studies of the genetic bases of strabismus are difficult for many reasons. The definition of abnormal has a major influence on the conclusions of any study and the criteria for assignment of

affected status are variable and subject to debate. Phorias, particularly exophorias, are common in the general population, and the criteria for normalcy have not been established. Further complicating the assessment, many studies are retrospective and some combine multiple forms of strabismus, which may have different bases. As the pathogenic mechanisms of some forms of strabismus are unknown, the current classification system may not correlate with the causes. Additionally, documentation of cycloplegic refraction (pharmacological paralysis of accommodative or focusing mechanisms) is often absent in studies, making interpretations unreliable. There have been few, if any, studies to support the concept that environmental factors influence strabismus. Thus, strabismus is common and large populations would be required for definitive studies.

The incidence of strabismus in different cultural and genetic populations has been investigated and results vary considerably (18). Minor (19) believed that the incidence of strabismus was lower in African Americans as compared to Caucasians; Grover and Yaukey (20) confirmed this observation in a study in the 1940s, and similar findings were reported more recently (21). Holm (22) found that the incidence of exotropia was similar in African Americans and Caucasians but reported that esotropia is uncommon. The incidence of exotropia and myopia has been shown to be higher in Asian Indian children than Caucasians in England (23). Exotropia may be more common in Asians (18), as the prevalence of exotropia is increasing in Hong Kong (24). In Hong Kong, there are more individuals with exotropia than esotropia (24); in the United States and the United Kingdom, there are more individuals with esotropia than exotropia (21,25,26). These differences may be related to myopia (nearsightedness).

Experienced clinicians concur that strabismus is a heterogeneous disease and clusters in families. Population studies support a hereditary component (Figure 139-1) with a prevalence in siblings of an affected individual ranging from 11% to 70% (2,3,7–9,26–35). Esotropia tends to have a higher incidence in siblings in comparison to exotropia (7,32). The familial aggregation is greater than the clustering of known risk factors for strabismus (32). Chimonidou and colleagues (36) studied a Greek population and found strabismus, by report, in 55% of family members; they studied siblings with strabismus and found that over 95% were concordant for the type of deviation. Simpson and Alleslev (35) studied 311 siblings of 291 affected children from the Registry for Handicapped Children and Adults from British Columbia, Canada and found that strabismus, central nervous system disease, and stillbirths were more common in siblings of the proband than in the general population. Most studies combine forms of ‘comitant’ strabismus, and the conclusions may be unreliable because of heterogeneity.

Although many pedigrees of families with strabismus have been published, conclusions are inconsistent.



**FIGURE 139-1** Two brothers born with esotropia. They also had a sister born with the same condition.

Landon (37) reported a four-generation family with esotropia with multiple affected members in each generation. Czellitzer (38) found consanguinity in 6% of the parents of children with esotropia compared to a mean of 0.65% in the general population, and postulated two recessive loci causing strabismus (7,38); however, Waardenburg (8) believed that no conclusions could be inferred because consanguinity was relatively high in that Prussian community. As strabismus is relatively common in the general population, individual pedigree analyses generally do not establish Mendelian inheritance.

Dahlberg and Nordlow (39) studied two populations and obtained historical information regarding strabismus in parents, grandparents, and siblings of randomly selected children with esotropia and, based on simple Hardy–Weinberg calculations, concluded that the disease was not caused by a single autosomal dominant or an autosomal recessive gene. On the basis of his pedigrees, Waardenburg (8) postulated a ‘chief gene which may be supported by mainly dominant accessory genes’. Francois (40) reviewed his personal experience with esotropia and reported four pedigrees with members affected with strabismus; on the basis of vertical transmission through three generations in all his families, he concluded that autosomal dominant inheritance with reduced penetrance accounted for the disease. He postulated that heterophorias and heterotropias were caused by variable expressivity and that a heterophoria that predisposes to strabismus might be the trait, with environmental factors such as refractive error significantly influencing the outcome or likelihood of developing the disease.

More recently, Dufier and colleagues (9) retrospectively studied the families of 195 individuals with isolated esotropia. They found an affected family member of the proband, by history or examination, in over 50% and vertical transmission from parent(s) to child in 35%. They performed segregation analyses assuming a frequency of 3% of strabismus in the general population and concluded that an autosomal dominant model with incomplete penetrance was most likely for esotropia and

that an autosomal recessive model was most compatible with esotropia with amblyopia. Such conclusions are not fully supported because the assignment of affected status was based on historical information for some individuals.

Maumenee and colleagues (41) studied over 170 families with at least one member with infantile esotropia; some were ascertained by a parent with the disease and others through a young proband. Segregation analyses were performed and supported a Mendelian codominant model for a common allele. The standard errors were large and the authors suggested the existence of etiological heterogeneity with autosomal recessive, autosomal dominant and non-genetic cases.

Studies of families ascertained on the basis of a proband with exotropia are infrequent. Waardenburg (8) reported 18 families with more than one member with exotropia; 13 exhibited vertical transmission from parent to child. He postulated autosomal dominant inheritance with reduced penetrance and Francois (40) provided support with additional pedigrees.

Schlossman and Priestley (26) studied 88 families with both esotropia and exotropia, adding some families to 'enrich' the data with 'pertinent cases'; the authors calculated segregation ratios and concluded that both esotropia and exotropia were autosomal recessive diseases with one in four individuals carrying the gene for 'strabismus'. The study is not recent and their database may not be representative of current populations.

In an extensive and careful study by Richter (34), the siblings and parents of patients (probands=697; total=1509) and children with strabismus (probands=136; total=344) were ascertained at the time of vaccination. She combined the forms of strabismus and found affected siblings in 20–30% if neither parent was affected, in 30–44% if one parent was affected, and in 33–50% if both parents were affected. Thus, recurrence risks are increased if a strabismic proband has affected parent(s) or siblings. In a series of large quantitative genetic studies, Mash, Spivey, Hegmann, and their colleagues studied ocular alignment and other parameters in a group of strabismic patients and their families from the US state of Iowa. The studies assessed the heritability of ocular measurements that predispose to the development of strabismus. In 1972, Niederecker, Mash, and Spivey (10) found that parents of probands with either esotropia or exotropia had less ability to maintain ocular alignment (fusional amplitudes) than controls. In a subsequent study, Mash, Hegmann, and Spivey (42) found that certain vergence amplitudes (ability to align the eyes and fuse disparate images with artificial, prismatic misalignment) had higher heritability values than others and differed significantly among the strabismus populations. In 1974, Hegmann, Mash, and Spivey (43) performed regression analyses and calculated heritability for interpupillary distance; angle kappa  $\kappa$ , which is partially responsible for pseudostrabismus (the appearance of strabismus); and refractive error, including astigmatism,

in the families of affected individuals. They calculated a heritability estimate for interpupillary distance of 0.65 and for angle kappa  $\kappa$  of 0.44 but were unable to infer conclusions regarding refractive error because of large standard deviations. In a subsequent assessment of ocular deviation and the relationship of accommodative convergence to accommodation (AC/A ratio) (42), they found that ocular alignment (esodeviation or exodeviation) tended to be consistent within a family with substantial heritability from the female parent at 0.42. They also found that relatives of patients with esodeviations tended to have esodeviations and relatives of patients with exodeviations tended to have exodeviations; for unknown reasons, the mother's ocular alignment correlated best with that of the offspring. They calculated the heritability of the AC/A ratio at 0.38. These studies confirmed a heritable component, and support the concept that both anatomic features and binocular fusion (central nervous system) are factors in the development of strabismus.

A large family with accommodative esotropia was studied using linkage analysis, assuming an autosomal recessive model; linkage to a presumed susceptibility locus at 7p22.1 was demonstrated with a convincing log of the odds (statistical estimate of likelihood of genes being close to each other on a chromosome) score of 4.51 (44). Other families in the study did not demonstrate linkage to this region.

The monofixation syndrome or microtropia is a subset of strabismus characterized by a small deviation of ocular alignment associated with reduced fusion or binocularity; amblyopia may occur. Lang (45,46) studied over 20 families with at least one affected member and concluded that there was a hereditary tendency; he did not postulate a Mendelian pattern. Cantolino and von Noorden (47) studied 20 patients and family members and identified multiple asymptomatic relatives. They concluded that the hereditary pattern was most consistent with multifactorial inheritance on the basis of the absence of any 'simple Mendelian' pattern; six pedigrees were published and all showed vertical transmission of microtropia, strabismus, or both.

Twin studies further support the concept of a hereditary component predisposing to the development of strabismus. Waardenburg (8) combined previous reports of esotropia in twins with his cases and found the concordance rate of strabismus in monozygotic pairs to be approximately 80% (69 sets) and in dizygotic pairs to be approximately 12% (101 sets); he had no examples of exotropia in twins and there are few in the literature. The famous conjoined twins, Chang and Eng, each had exotropia (48). DeVries and Houtman (49) studied 17 pairs of monozygotic twins in which the proband developed esotropia within the first year of life and found concordance in eight. Rubin and colleagues (50) queried 50 ophthalmologists and compiled results on 22 sets of twins in which the proband had exotropia, and 122 pairs



in which one had esotropia; concordance was 77% in the monozygotic sets and 50% in dizygotic for exotropia and 75% in monozygotic and 53% in dizygotic for esotropia. Heritability was 0.54 for exotropia and 0.47 for esotropia. The authors analyzed esophoria and exophoria (deviation only present in the eye under an occluder and, thus, under monocular testing) separately and found the concordance to be relatively low for both (50). Richter (34) also studied strabismus (combining esotropia and exotropia) in twins and found concordance in 11 of 12 monozygotic pairs and 7 of 27 dizygotic pairs; based on all studies, including the twins, she concluded that strabismus was multifactorial. Hofstetter (51) studied 25 pairs of monozygotic twins and found a correlation coefficient of 0.67 for the AC/A ratio, which was higher than between pairs or families. Matsuo and colleagues (52) found the strabismus phenotype to be highly concordant in a relatively large study of Japanese twins. If genetic factors alone account for strabismus, the concordance of monozygotic twins should be 100%; conversely, if environmental influences alone cause the condition, 4% or less (the prevalence in the general population) of the dizygotic twin of an affected individual should be similarly affected.

Although no single intrauterine environmental agent has been shown to cause strabismus, the incidence of both esotropia and exotropia is higher in offspring of mothers who smoke during pregnancy; low birth weight was an additional risk for strabismus (21).

In conclusion, previous studies of the genetic bases of strabismus are difficult to interpret. Isolated strabismus is a common trait in the general population, and the causes are poorly understood. Without doubt, all forms of strabismus, esotropia, exotropia and the monofixation syndrome cluster in families, and there is limited evidence in support of an environmental basis aside from those factors that cause or predispose to mental retardation. However, strabismus does not appear to develop as a

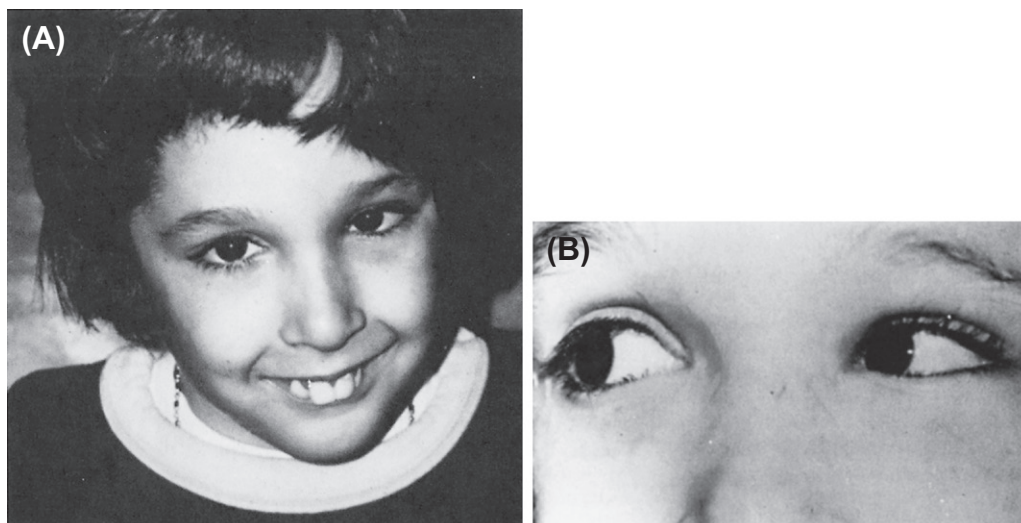
result of a single gene mutation. Some authors have postulated polygenic or multifactorial models (34,53–55) with a ‘threshold’ level of many factors, such as interpupillary distance, fusional ability, and refractive error, with strabismus becoming manifest if the individual has a combination of abnormalities.

Genetic counseling to an individual or parent of a child with strabismus regarding recurrence risk is not precise unless the ocular misalignment is secondary to an identifiable genetic disease. Although strabismus is common, recurrence risks have not been rigorously established. Cross (53) estimated the recurrence risk for a siblings of an affected individual to be 14–30% if neither parent was affected, 30% if one parent was affected, and 30–50% if both parents were affected, based on the work of Richter (34) and Czellitzer (7,38).

### 139.5 SINGLE GENE DEFECT DISORDERS

#### 139.5.1 Duane Anomaly/Syndrome

The Duane anomaly/syndrome (DURS1; OMIM %126800) (DURS2; OMIM %604356), initially described in 1905 (56), is caused by aplasia/hypoplasia of the sixth cranial nerve nucleus (57) with aberrant generation of the third nerve in many, but not all, cases (58). A peculiar combination of ocular motility anomalies is evident with narrowing of the vertical palpebral fissure on adduction (when the affected eye is directed toward the nose) and widening of the palpebral fissure on abduction (toward the temple); the ability of the eye to abduct is often reduced (Figure 139-2) (58). While many Duane anomaly/syndrome patients have no strabismic deviation in primary (ahead) gaze, other patients have an exotropia or esotropia and may compensate for the deviation by turning the head to achieve fusion. Subtypes, based on the strabismus, have been described but most clinicians



**FIGURE 139-2** Duane retraction syndrome. (A) Head turn to achieve fusion. (B) Narrowing of palpebral fissure in abduction.



currently describe the specific findings rather than categorize. Curiously, the profound atrophy of the lateral rectus (abducens) found in chronic VIth nerve palsy is not evident in Duane anomaly/syndrome (59a).

Although the isolated Duane anomaly disorder is usually sporadic, autosomal dominant inheritance occurs (DURS1)(60–64) in approximately 10% of cases (65). More than one of the clinical subtypes may be evident in a single family (60). Bilaterality occurs in 12–16% of both sporadic and autosomal dominant cases (60,66,67). A region (DURS1) (OMIM #126800) at 8q13 has been supported by several reports of chromosomal rearrangements (68–70).

A second locus (DURS2) (OMIM #604356) was mapped in a single large family (60) to 2q31 (71) and confirmed in additional families (72,73a). Seven mutations in the gene, *chimerin1* (*CHN1*; \*118423) that normally encodes two protein products,  $\alpha$ 1-chimerin (n-chimerin) or, a result of alternative splicing,  $\alpha$ 2-chimerin, a Rac guanosine triphosphatase-activating (RacGAP) signaling protein were reported in autosomal dominant cases (DURS2-DRS); all mutations result in abnormal  $\alpha$ 2-chimerin products with a gain-in-function (19a). These authors also reported that expression of mutant  $\alpha$ 2-chimerin constructs in chick embryos resulted in failure of oculomotor axons to innervate their target extraocular muscles (19a). Additional mutations have been reported (74a) and clinical variability within a family has been documented (75a).

In the sporadic cases, there is a preponderance of females (67). Concordance in monozygotic twins has been reported (76) with mirror-like laterality; however, discordance in monozygotic twins also has been documented (77). A germinal mutation in the *CHN1* gene is not a common cause of sporadic Duane anomaly/syndrome (19b). Systemic anomalies associated with sporadic Duane anomaly/syndrome include upper extremity anomalies (78), radial ray deformities (79,80), cardiac defects (81), deafness (79), blepharophimosis (82), and pseudotumor (83).

The Athabaskan brain stem dysgenesis syndrome/Navajo brain stem disorder/Bosley–Salih–Alorainy syndrome (#601536), autosomal recessive disorders of brain stem dysgenesis and characterized by global developmental delay, autism spectrum disorder, facial weakness, horizontal gaze palsy, deafness, central hypoventilation, seizures, and vascular malformations of the internal carotid arteries and cardiac outflow tract are caused by mutations in the *HOXA1* (\*142955) gene (84a). Affected members of several Middle Eastern families had Duane anomaly/syndrome and some had developmental delay, deafness and external ear defects (Bosley, 2009). Germinal mutations of the *HOXA1* gene are not common in Duane anomaly/syndrome.

Horizontal gaze palsy and early-onset, progressive scoliosis (#607313) is an autosomal recessive form of progressive external ophthalmoplegia (Dretakis, 1970;

66a, 86a)) and was mapped to 11q23-25 (66a). Nystagmus and synergetic convergence, asynchronous blinking and nystagmus may be evident (87a). The gene was mapped to 11q23-25 (66a) and mutations found in the *ROB3* gene (\*608630) (66b,74b,87a,88a,89a).

Wildervanck syndrome (OMIM #314600) consists of hearing loss, Klippel–Feil anomaly (fused cervical vertebrae), and Duane syndrome (90); it usually occurs in females and may be multifactorial and/or polygenic (91). Deafness may occur (92). Its basis is not known.

Duane anomaly/syndrome, deafness, and the Klippel–Feil syndrome can be inherited together as an autosomal dominant trait (63). An autosomal recessive form has been reported (93).

When associated with blepharophimosis (blepharophimosis/ptosis/epicanthus inversus (#110100) (94a,95b), mutations of the winged helix/forkhead transcription factor gene (*FOXL2*) (OMIM \*605597)) gene have been the basis (3a,61a,72a,82,96a,97a,b,c,98a,99a,c,100a).

The Duane-radial ray (DRRS) (OMIM #607323)/Okishiro (OMIM #607323)/IVIC syndrome (OMIM 147750) shows autosomal dominant inheritance, and consists of Duane syndrome (unilateral or bilateral) (89) and forearm anomalies ((78,80,81,88,89,96,101,102,103,104a); Temtamy 1978; (94d)). Not all affected individuals have Duane syndrome (105); some have renal disease (88,106), dental anomalies (94d), hearing loss (89c), thrombocytopenia (89c,107a) and anal stenosis (80). Colobomata (88) and optic nerve hypoplasia (102) have been reported. Multiple mutations of the gene encoding a zinc finger transcription factor *SALL4* (OMIM \*607343) have been reported to cause the disease (85, 85a,89,94c,105,106a,108,109,110a). Although there is clinical overlap between the DRRS syndrome and the Holt–Oram syndrome (OMIM #142900), the genetic defect is different; the mutated gene defect in the Holt–Oram syndrome is the *TBX5* (OMIM \*601620) which produces a DNA-binding protein that controls cellular differentiation.

The Townes–Brocks syndrome (#17480) is characterized by renal, aural and thumb anomalies and syndactyly, and its genetic basis is a mutation of *SALL1* (\*602218), containing a DNA-binding zinc finger domains and alanine- and glutamine-rich domains that are commonly found in transcription factors (105a); the disease is usually caused by premature codon termination with haplotype insufficiency. Less common manifestations include Duane syndrome (111a), chorioretinal colobomata (111a), iris Brushfield spots (111a), epicanthal folds (111a) facial nerve palsy (111a), deafness, hypothyroidism, gastroesophageal reflux, vaginal aplasia with bifid uterus, cryptorchidism, bifid scrotum without hypospadias scrotalis, hypoplasia of the corpus callosum, kyphosis, cardiac malformations and umbilical hernia (32a,49a,68a,95a,111a,112b,113a,114a); radial bone involvement has not been published and distinguishes the



**FIGURE 139-3** Congenital fibrosis syndrome. Chin is elevated to permit fusion with the eyes pulled downward by fibrotic inferior rectus muscles. Ptosis is present.

syndrome from the DRRS (OMIM #607323)/Okiihiro/IVIC syndrome (OMIM 147750). The possibility of a maternal anticipation has been raised (114a). Significant intra- and interfamilial variability has been documented.

### 139.5.2 Congenital Ocular Fibrosis Syndrome

Congenital ocular fibrosis syndrome, originally described in the late 1800s (115), is a group of diseases caused by inelasticity or fibrosis of some or all of the extraocular muscles. Affected patients usually have bilateral ptosis (droopiness of the eyelids) and strabismus with significant restriction of movement of the globes. The strabismus may be progressive (116). Many families with the autosomal dominant (111,117,118) and the autosomal recessive (119,120) forms have been described. Usually, both eyes are deviated downward due to more severe fibrosis of the inferior rectus muscles (Figure 139-3). Based on surgical findings, the muscles are fibrotic and may insert in an anomalous position (121); treatment is surgical (121,122). The disease, in at least one phenotype, is caused by absence of the superior division of the oculomotor (III) cranial nerve (73,58). This condition has been reported in association with the Prader-Willi syndrome (#176270) (59), Möebius syndrome (multiple cranial nerve palsies) (OMIM %157900) (123), optic nerve hypoplasia (123), Marcus-Gunn jaw winking (synchronous movement of the lid with chewing and/or jaw movement) (OMIM 154600) (124,125), and nervous system anomalies including agenesis of the corpus callosum, colpocephaly, hypoplasia of the cerebellar vermis, expansion of the ventricular system, pachygyria, encephalocele, hydrancephaly and spinal stenosis (124,126).

Congenital ocular fibrosis syndrome is usually inherited as an autosomal dominant trait (86,111,117,118)

(CFEOM1; OMIM #135700) (CFEOM3; OMIM %600638), with complete penetrance (CFEOM1) and variable expressivity; two forms were established on the basis of mapping to different chromosomes. Mapping of the autosomal dominant form by linkage analysis supported at least two loci, one on chromosome 12 (CFEOM1) (127) and another on chromosome 16q24.2–q24.3 (CFEOM3) (128). The expressivity is described as more variable in CFEOM3 (128). A recessive form (CFEOM2) (OMIM #602078) has been described and has been mapped to 11q13 (120); further genetic heterogeneity may exist (119).

The CFEOM1 form is caused by mutations of the gene entitled kinesin family member 21A (*KIF21A*) (OMIM \*608283) on chromosome 12q12. The protein product is a microtubule-dependent motor protein involved in the transport of cellular components along axonal and dendritic microtubules and multiple mutations in the coiled-coil domain have been described (116,117,123,129,130). A form with reduced penetrance has been mapped to this region (127) and mutations of the *KIF21A* gene reported (130). Thus, depending on the mutation, the heterozygote and the homozygote may exhibit clinical manifestations. Mutations of the *KIF21A* gene also may cause congenital ocular fibrosis syndrome associated with Marcus-Gunn jaw winking (125).

The second autosomal dominant form (CFEOM3) is less common and may be associated with reduced penetrance (128); it is a distinct form as it maps to chromosome 16q24.2–q24.3 (128,131). Some families with the CFEOM3 phenotype have mutations of the *KIF21A* gene (130).

Most cases of the autosomal recessive form are caused by mutations of the gene *ARIX/PHOX2A* (OMIM \*602753), on chromosome 11q13.3–q13.4, which regulates transcription of neurotransmitters such as the catecholamine biosynthetic genes. The families are clustered in the Middle East and several mutations have been reported (132,133).

A second autosomal recessive form (OMIM %609428) (Tukel syndrome) with ulnar hand deformities has been mapped to chromosome 21q22 in a large, consanguineous Turkish family (134).

The documentation of different genes causing CFEOM underscores the importance of a categorization based on the gene and specific mutations.

### 139.5.3 Möebius Syndrome

Initially described in the 1880s (135–137), individuals with the Möebius syndrome (OMIM %157900) usually have bilateral sixth and seventh nerve palsies; the sixth nerve palsies cause reduced abduction of the eyes and a large esotropia that may be difficult to treat surgically. The disease is a brain stem disorder (138,139) and hypoplasia of the sixth, seventh, and/or twelfth nerve palsies have been reported (95,114,140). Although ptosis may

be evident, abnormal neonatal sucking is usually the first symptom.

In a large study (139), Duane syndrome was evident (34%) and other manifestations such as hypesthesia of the cornea and face, autism, tongue hypoplasia, dysarthria with palatal and pharyngeal involvement other craniofacial abnormalities such as epicanthal folds, flattened nasal bridge, micrognathia, high-arched palate, external ear defects, dental anomalies and hypertelorism; malformations of the extremities, brachydactyly, clinodactyly, syndactyly, metacarpal abnormalities, pes planus, hypoplasia of the lower legs, talipes equinovarus, and arthrogryposis were common. Möebius may be associated with the Poland syndrome (141).

The disorder may be inherited in an autosomal dominant pattern (62b,142–144,145a) and has been reported to be concordant in twins (146) who had absent facial nerves at autopsy. Autosomal recessive inheritance also has been reported (142). A gene (MBS1) for the disorder has been mapped to 13q12.2–q13 and multiple chromosomal rearrangements have been reported (104,144,147–149). The disease has been transmitted vertically in a family, with affected members having a reciprocal translocation between chromosomes 1 and 13 (144). Linkage analyses have mapped genes for the disease to 3q21–q22 (112) and 10q21.3–q22.1 (150). Intra-uterine environmental agents such as thalidomide may cause the disease (151).

### 139.6 STRABISMUS ASSOCIATED WITH OTHER OCULAR DISEASE

Any genetic disorder that decreases vision in one or both eyes, particularly if asymmetrical, may be associated with strabismus. If the basis of the reduced vision is hereditary, the strabismus is a secondary effect of the gene defect. Thus, any genetic disease in which vision may be reduced is associated with a higher incidence of strabismus. Examples of such diseases include colobomatous microphthalmia, which may be inherited as an isolated

autosomal dominant disorder or be associated with single gene multisystem disease or chromosomal rearrangements (Figure 139-4), pediatric cataracts, which may be inherited as an autosomal dominant or recessive, or X-linked trait or be associated with multisystem or metabolic disorders, and retinal degenerations.

### 139.7 STRABISMUS ASSOCIATED WITH MULTISYSTEM DISEASE

#### 139.7.1 Mental Retardation

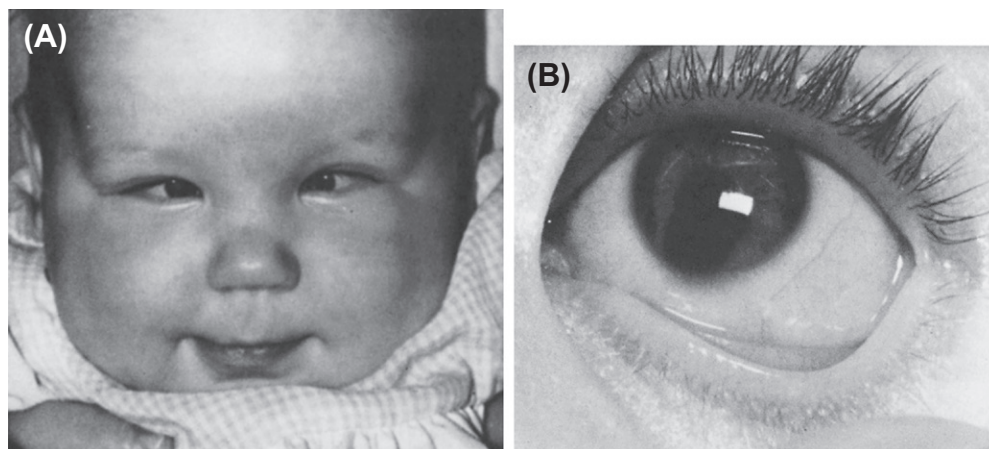
Any disease, regardless of its etiology, associated with mental retardation will have a higher incidence of strabismus – presumably related to either a reduced ability to achieve binocularity or an elevated AC/A ratio (see above) of the eyes. The mechanisms of strabismus in such individuals are poorly understood, but the increased incidence, although poorly documented, is readily apparent to ophthalmologists. As many disorders caused by single gene defects and chromosomal anomalies are associated with mental retardation, no single disorder is included in this chapter.

#### 139.7.2 Neuromuscular Disorders

Individuals with neuromuscular disorders, particularly cerebral palsy and ataxia, are commonly affected with strabismus. The cause is poorly understood but may be related to abnormalities in coordination that may alter accommodative convergence, fusional convergence, or both; such individuals may have difficulty in initiating and/or maintaining ocular alignment.

#### 139.7.3 Craniofacial Anomalies

The craniofacial synostoses are a group of disorders that are characterized by the premature closure of one or more cranial sutures (Figure 139-5). Such premature closure frequently alters configuration, including depth and



**FIGURE 139-4** Colobomatous microphthalmos. (A) Eyes are small and esotropic. (B) Defect in inferior iris extends to involve the optic nerve.



of the bony orbit, the relationship of the eye to the origin of the extraocular muscles at the apex of the orbits, and the distances between the pupils and the inner and outer canthi. Strabismus, shallow orbits with exposure of the cornea in some cases, or papilledema with subsequent optic atrophy may occur in any craniofacial anomaly; reduced vision increases the risk for strabismus. An exotropia commonly occurs; presumably because of the altered and divergent axes of the globe with respect to the orbit. Incomitance (different amount and/or direction of the ocular axes in different directions of gaze) is frequently encountered with variability of the deviation depending on the field of gaze; a V pattern exotropia is the most common form of 'incomitance', with the eyes diverging more in upward than downward gaze. Crouzon disease, Apert syndrome, and Pfeiffer syndrome, all of which are inherited in an autosomal dominant pattern, are commonly associated with strabismus for the aforementioned reasons; additionally, these disorders are associated with structural abnormalities of the extraocular muscles, including fusion, absence, or anomalous insertions of muscles (152,153). Other forms of premature closure of the sutures also are associated with an increased incidence of strabismus.

Most craniofacial dysostoses are caused by mutations of genes in the fibroblast growth receptor family and are tyrosine kinase receptors (145). The ocular features are similar in all and include shallow orbit with intermittent prolapse of the globe anterior to the lids, astigmatism, amblyopia, abnormal (fused or absent) extraocular muscles, and strabismus (100,153–155). The extraocular muscles can be absent, fused, and/or inserted on the globe in an abnormal location, resulting in a mechanical basis for at least some of the cases of strabismus (153). Stretching or traction on the optic nerve is a possible cause of decreased vision. However, hydrocephalus can lead to papilledema and optic atrophy (156).



**FIGURE 139-5** Child with craniofacial anomaly and exotropia.

Crouzon disease (CFP1) (OMIM #123500) has craniosynostosis as its major manifestation, and Apert syndrome (Apert) (ASC1) (OMIM #101200) is characterized by craniosynostosis and syndactyly of the hands and feet; both are caused by mutations of the. Individuals with Pfeiffer syndrome (ACS5) (OMIM #101600) have craniosynostosis and broad, short thumbs and large toes (157), and those with Saethre–Chotzen syndrome (OMIM #101400) have craniosynostosis, symmetrical syndactyly, ear anomalies, and a characteristic facies (158,159); blepharophimosis may be a feature (160).

Mutations of fibroblast growth factor receptor-2 (*FGFR2*) (OMIM \*176943) on chromosome 10q26 may cause Crouzon disease (161,162) as well as Apert (163), Pfeiffer (162) and Saethre–Chotzen (47,164) syndromes. The mutations causing Apert syndrome occur in a specific region of the *FGFR2* gene, and most cases represent one of the two common mutations (163,165,166) at the site.

Pfeiffer syndrome (OMIM #101600) is usually caused by heterozygous mutations of the *FGFR1* gene (\*136350) (75,167) on chromosome 8p11.2–p11.1, the *FGFR2* gene (OMIM \*176943) on chromosome 10q26.13 on chromosome 4p16.3; however, mutations on the *FGFR3* gene on chromosome 4p16.3 also may cause the phenotype of Pfeiffer syndrome. The identical mutation in the *FGFR2* gene can result in either Crouzon disease or Pfeiffer syndrome (162) (above), providing evidence that genetic modifiers influence the phenotype.

Although heterozygous mutations of the *FGFR3* (OMIM \*134934) gene, on chromosome 4p16.3, usually cause dwarfing syndromes such as achondroplasia (97), hypochondroplasia, and thanatophoric dysplasia; Muenke coronal craniosynostosis, lacrimoauriculodentodigital (149730) syndrome, Levy–Hollister syndrome, and the camptodactyly, tall stature, scoliosis, and hearing loss syndrome have been reported, Crouzon disease associated with acanthosis nigricans may be the phenotype (161a,110,89b). The mutation causing amino acid substitution Pro250Arg amino acid substitution in the *FGFR3* gene (97) in autosomal dominant craniofacial syndromes is similar to the mutation causing Apert syndrome in *FGFR2* and Pfeiffer syndrome in *FGFR1*.

Individuals with the Saethre–Chotzen syndrome (OMIM #101400) usually have mutations of the *TWIST1* gene (*TWIST1*) (OMIM \*601622) (47,94b, 112a,164,168,169) on chromosome 7p21, which encodes a nuclear transcription factor protein; less commonly, mutations in the *FGFR2* and *FGFR3* (above) cause the clinical features (47,164). A form with blepharophimosis is caused by a mutation in the *TWIST* gene (170).

Other craniofacial anomalies are associated with strabismus including clefting syndromes, frontonasal dysplasia, and the holoprosencephaly sequence.

Mandibulofacial dysostosis or the Treacher Collins–Francheschetti (TCOF) (OMIM #154500) syndrome, is a disorder of craniofacial development and is characterized



by malar and mandibular hypoplasia, downslanting lid slant, microtia, macrostomia and a defect in the outer third of the lower lid including absence of the lateral canthal tendon (171–175). Conductive hearing loss and cleft palate may be evident (176). Expression is variable (175,177). ‘V pattern strabismus’ (incomitant deviations) is common in this autosomal dominant disease (178). Additional ocular features include absence of the lacrimal puncta, congenital cataracts, uveal colobomata, and microphthalmia (67,178–180). The disease is caused by mutations of the treacle gene (*TCOF1*) (OMIM \*606847); the protein product is involved in ribosomal DNA gene transcription (181) and located on chromosome 5q32–q33.1. Multiple mutations have been described (176,182–186) and some regions appear to be ‘hot spots’ (184).

The Waardenburg syndromes are associated with pseudoesotropia. The disorders consist of lateral displacement of the medial canthi (dystopia canthorum); confluent eyebrows; partial depigmentation of the eyes, hair, and skin; and congenital deafness. Sensorineural hearing loss and heterochromia iridium are the two most diagnostic features (187). Because of the lid configuration, the eyes may appear to be esotropic (pseudoesotropia) despite alignment; approximately 20% have true esotropia (188).

Multiple forms have been described (188). The most discriminating clinical features include the inner intercanthal distance, which gives the appearance of esotropia (pseudoesotropia), philtrum length, lower facial height, and nasal bone length (189). There is a genotype–phenotype correlation with respect to mutation class and the coloration of the irides (190).

In Type 1 (WS1) (OMIM #193500), many mutations of the *PAX3* (OMIM \*606597) gene that activates the gene *MITF* (see WS2A) have been reported (191–193). Type 2AIA (WS2A) (OMIM #193510) does not have lateral displacement of the medial canthi (188); mutations of the gene *MITF* (OMIM \*15684), which encodes a basic helix-loop-helix (hHLH)-leucine zipper protein that influences the development of multiple cell types including neural crest-derived melanocytes and optic cup-derived retinal pigment epithelial cells, causes this form. The Type 2E (WS2E) (OMIM #611584) (194a)/Type WS4C (OMIM #613266) (124a)/Waardenburg-Shah syndrome (PCWH) (OMIM #609136) (18a) forms, like WS2A, does not have dystopia canthorum but may be associated with neurological signs is caused by heterozygous mutations of the *SOX10* (OMIM \*602229), a transcription factor with a DNA-binding motif high mobility group domain. Type 3 (WS3) (OMIM #148820) is characterized by the pigmentary anomalies, hearing loss/deafness and upper limb malformations and the basis is hetero- or homozygous mutations of the *PAX3* gene; dystopia canthorum and pseudoesotropia are found as occurs in WS1. The autosomal recessive (24a) and dominant (62a,127a) forms have been documented.

### 139.7.4 Chronic Progressive External Ophthalmoplegia

Chronic progressive external ophthalmoplegia is an acquired form of strabismus that may be associated with a mitochondrial myopathy and may be inherited in an autosomal dominant or recessive pattern. This disease overlaps with the mitochondrial myopathies, involving oxidative phosphorylation, including Kearns–Sayre which is caused by mitochondrial deletions (OMIM #530000). There is clinical overlap within this relatively diverse group of mitochondrial myopathies, and some mutations are somatic (180a). The five enzyme complexes (I, III, and IV) for oxidative phosphorylation are encoded by both nuclear and the mitochondrial DNA; complex II and complex V, adenosine triphosphate (ATP) synthase, are entirely nuclear.

In this group of disorders, ptosis and strabismus may develop at any age. The extraocular muscles become non-functional and the globe becomes progressively immobile; the eyes are usually misaligned. Although the patient may have diplopia (double vision), he/she may not as the onset is very gradual (personal experience). Additionally, retinal degeneration may develop and would be diagnosed on the basis of an abnormal electroretinogram. The retinal features are clinically variable and may precede the degeneration of the electroretinogram (94); evidence supports consistent changes in the retinal pigment epithelium in individuals with mtDNA defects (195).

The autosomal dominant forms are usually isolated (196) but may be associated with hypogonadism (197). The autosomal recessive form (198) (PEOB) (OMIM #258450) tends to be more severe and is usually associated with cardiopathy (199).

The bases are multiple; some families exhibit a Mendelian pattern of inheritance and others are compatible with mitochondrial inheritance (200).

One autosomal dominant form (PEOA1) (OMIM #157640) is caused by mutations of the gene (*POLG*) (OMIM \*174763), on chromosome 15q25, encoding DNA polymerase, gamma; the protein product is expressed in both the nucleus and the mitochondria. Mutations in the *POLG* gene disrupt the mtDNA by reducing the proofreading exonuclease activity of the enzyme, with accumulation of heteroplasmic levels of rare and recurrent point mutations in the skeletal muscle tissue and fibroblasts (201); multiple mutations in both the exonuclease domain (201) and other regions of the gene have been reported (202). Both hetero- (OMIM #157640) and homozygous mutations cause disease (203). Mutations in this gene also cause the form associated with hypogonadism (204).

The second autosomal dominant form of progressive external ophthalmoplegia (PEOA2) (OMIM #609283) is caused by mutations of the gene (*SLC25A4*; ANT; ANT1) (OMIM \*103220) encoding the predominant

mitochondrial protein ADP/ATP translocator, or adenine nucleotide translocator (solute carrier family 25 [mitochondrial carrier, adenine nucleotide translocator, member 4]); the gene is on chromosome 4q35. The reported clinical features are usually limited to the eye (205–207), but sensorineural hearing loss, thyroid disease, and an abnormal electromyogram have been described (205). Muscle weakness is not a feature. Few mutations have been reported (207–209).

The third autosomal dominant form (PEOA3) is caused by mutations (203,210) of the mitochondrial protein twinkle, the gene for which is entitled chromosome 10 open reading frame 2 (C10ORF2) (OMIM \*606075) and is on chromosome 10q24. The clinical features may be limited to the progressive external ophthalmoplegia or may include psychiatric disease with severe depression, hormonal dysfunction, cardiac disease, and generalized weakness (211) and may include dementia (9a); only ocular disease and muscle weakness may be evident (212). Multiple mutations have been reported (210).

The autosomal recessive form of chronic progressive external ophthalmoplegia, generally a more severe phenotype (204,213), is caused by mutations of the *POLG* gene that encodes DNA polymerase, gamma (202,203). Although individuals with Alper's syndrome, characterized by diffuse degeneration of gray matter, seizures, hepatic cirrhosis, and infantile demise, have mutations of the *POLG* gene, the only reported ophthalmological feature has been cortical visual impairment (214); strabismus has not been described.

The autosomal recessive disease SANDO is characterized by sensory ataxic neuropathy, dysarthria, and ophthalmoplegia (SANDO) (OMIM #607459) and is caused by mutations of the gene C10ORF2 (OMIM \*606075), above (215). However, the disease may be caused by mutations in the *POLG* gene (216,217). Thalamic lesions may be evident on MRI (218) and myopathy is not a feature. The mutations in the C10ORF2 gene (210,219) frequently result in deletions of mtDNA in the autosomal dominant form (211). In a sporadic case, heterozygous mutations were found in both the *POLG* and the C10ORF2 genes, a digenic mechanism (220).

Primary mutations of the mitochondrial DNA are not as common in progressive external ophthalmoplegia. However, deletions and primary mutations of the mitochondrial DNA are the bases of the Kearns–Sayre syndrome (KSS) (OMIM #530000). This disease is characterized by a progressive external ophthalmoplegia, cardiac disease and retinal degeneration (87). Endocrine disease is common (194,221). The bases are mitochondrial deletions and point mutations (222–224). A mutation in the mitochondrial tRNA for leucine (MTTL1) (OMIM \*590050), encoded by nucleotides 3230–3304, has been reported (225). A specific deletion has been reported with encephalopathy (226). These mitochondrial mutations may be inherited maternally (224) and, less commonly, paternally (227).

Progressive external ophthalmoplegia with myoclonus may be caused by mutations of the mitochondrial transfer RNA for lysine (encoded by mitochondrial nucleotides 8295–8364) gene (*MTTK*) (OMIM \*590060) (84,98,107). Thus, a mitochondrial mutation may be the basis of progressive external ophthalmoplegia and myopathy. A patient with myopathy, encephalopathy, sensorineural hearing loss, progressive external ophthalmoplegia, and retinopathy had a mutation of the mitochondrial tRNA for leucine is encoded by nucleotides 12266–12336 (228). Most reported mutations are somatic (98,225).

Some of the families with autosomal dominant inheritance of progressive external ophthalmoplegia may represent the congenital ocular fibrosis syndrome.

## 139.8 SUMMARY

Although increasing numbers of genetic are known to be responsible for strabismus, the common, pediatric varieties remain related to refractive errors, variations of normal.

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# Retinoblastoma and the RB1 Cancer Syndrome

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## 140.1 INTRODUCTION

A genetic syndrome is defined as a group of signs and symptoms that are characteristic of a specific individual or set of gene mutations. Retinoblastoma (Figure 140-1), an intraocular tumor of the developing retina, is the most often diagnosed malignancy of the *RB1* cancer syndrome, which results from mutations in the Retinoblastoma 1 (*RB1*) gene. The *RB1* syndrome is characterized by a lifelong predisposition to cancers of the retina, bone, soft tissue, skin and lung, amongst other tissues. Retinoblastoma can alternatively result from a somatic alteration of the *RB1* gene within the retina that has no heritable implications and which is not associated with a characteristic constellation of symptoms defining a syndrome. Regardless of its etiology, this solid childhood eye tumor has played a central role in illuminating the genetic mechanisms that regulate human cellular growth.

Retinoblastoma was the first tumor suppressor gene to be discovered and has defined the paradigm of the two-hit model of tumorigenesis (1). The *RB1* cancer syndrome exists when one allele of the retinoblastoma gene (*RB1*) is missing or inactivated in the germline, resulting in heterozygosity for the normal or wild-type *RB1* allele in all tissues. The development of each tumor focus, whether in the eye or elsewhere, then requires the loss or inactivation of the second *RB1* allele in a single retinoblast or other cell type, resulting in loss of heterozygosity and clonal expansion (Figure 140-2). Because the initial, predisposing *RB1* mutation, for genetic purposes termed 'M1', is present in all somatic cell types, multiple tissue types are at risk of tumor development following mutational events leading to loss of heterozygosity. Despite this general predisposition to tumor development, each individual tumor focus results from a separate second mutational event, termed 'M2'.

The initial M1 germline mutation may be inherited from either parent or may appear spontaneously during

embryonic development. Accordingly, the presence of mosaicism for M1 is a relatively common finding in the founder of a retinoblastoma pedigree. Regardless of whether M1 was inherited from a parent or occurred spontaneously, the presence of M1 in the germline constitutes the heritable retinoblastoma syndrome, as this mutation can be passed through the germline to offspring.

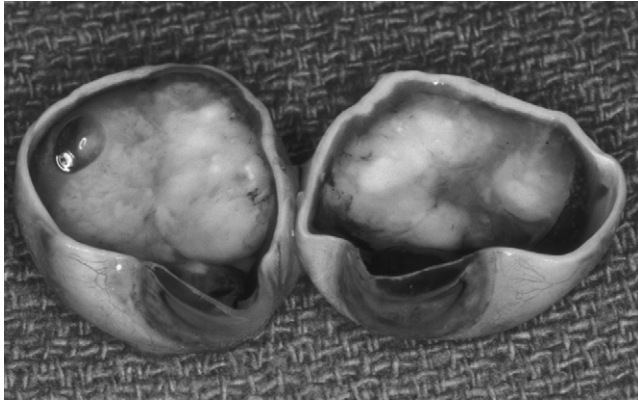
Alternatively, M1 may not exist in the germline, but may occur spontaneously in the developing retina. In this case, all other tissues, including the germline, do not possess *RB1* mutations and are not prone to tumor development. Intraocular tumors occur in these patients with acquisition of the second 'tumorigenic' mutation (M2) and are usually unifocal and unilateral. In these patients, both the *RB1* M1 and M2 mutations are not heritable and, therefore, are not passed on to offspring. The most common event associated with M2 in both heritable and non-heritable retinoblastoma is mitotic non-disjunction.

Because heritable retinoblastoma is associated with the *RB1* cancer syndrome and puts offspring at risk of retinoblastoma development, it is important for patients presenting with retinoblastoma to be seen by a clinical geneticist and to receive genetic counseling regarding the *RB1* mutation pattern underlying their disease.

The clinical geneticist must seek to answer two questions:

- (i) Is M1 present outside the eye in the patient or family being counseled, and
- (ii) If M1 is present outside the eye, when during the development of the embryo did it make its appearance and what tissues are affected?

The presence of retinoblastoma in both eyes can help answer those questions, since all patients with bilateral retinoblastoma have heritable disease. Genetic counseling for this disease would be simple and straightforward if all patients with unilateral retinoblastoma (Figure 140-3) had non-heritable disease. Unfortunately, that is not the



**FIGURE 140-1** Retinoblastoma is the grayish white mass that fills most of the vitreous cavity in this freshly enucleated and bisected eye. This tumor may fill the eye with little or no external sign except loss of vision in that eye, and that may never be noticed if the other eye is tumor-free. There is no evidence in this specimen of tumor extending outside the globe. Once the tumor has grown to this size, determining whether this tumor was of unifocal or multifocal origin is impossible clinically or histologically.



**FIGURE 140-2** Each of the white, round lesions is an independently arising intraretinal retinoblastoma in this image taken with a wide-angle retinal camera. The optic nerve is the orange disc just below the upper lesion. The fovea is the dark spot to the left of the optic nerve. The two larger lesions have recruited a blood supply. The faint small lesion at the lower right in the image is only 1.5 mm in diameter and has not recruited a blood supply. Each of these three lesions arose independently from the others as a result of inactivation of the normal allele at *RB1*. Multifocal retinoblastoma is clearly discernible only in early-stage disease, as in this image. Once the tumors grow to merge as a single tumor or intraocular ‘seeding’ of the tumor occurs, then multifocal disease cannot be separated clinically from unifocal disease.

case. Rather, 15 to 20 percent of patients with unilateral sporadic retinoblastoma possess a germline M1 mutation. Rarely, *RB1* mutations have been passed to offspring from people who never develop cancer in either eye. Alternatively, some patients with germline M1 mutations develop retinomas, a benign variant of retinoblastoma, or develop bone or soft tissue sarcomas without any ocular findings. These atypical presentations make it difficult



**FIGURE 140-3** Unilateral retinoblastoma in the right eye of a child whose pupils have been dilated pharmacologically. This is the classical ‘leukocoria’ or ‘cat’s eye’. The red reflex in the pupil in the child’s left eye can only be elicited with coaxial illumination from a direct ophthalmoscope or from the flash of a camera. A pen light will not elicit a red reflex. Small pupils will also prevent detection of this retinoblastoma.

to generate simple genotype–phenotype correlations, and patients without clear ocular findings may go undiagnosed for the *RB1* cancer syndrome. Identifying the *RB1* cancer syndrome in patients without bilateral retinoblastoma is one of the greatest challenges facing geneticists. The range of phenotypes associated with *RB1* mutations may be dependent on the specific molecular lesion present in the *RB1* gene. Certain mutations, such as the substitution of tryptophan for arginine at position 661, are associated with milder forms of disease, including unilateral benign retinoma and penetrance as low as 20%.

Since the discovery of *RB1* and its protein product pRb, significant research efforts have been undertaken to determine the role of pRb in the development of tumors and its intrinsic role in tumor suppression. Nevertheless, retinoblastoma itself is a very rare childhood tumor, with roughly 300–350 new cases diagnosed in North America each year. The majority of these patients are treated in one of five or six major retinoblastoma referral centers in the United States and Canada. It is not surprising, then, that most geneticists and pediatric ophthalmologists outside of these major retinoblastoma referral centers have little clinical experience with retinoblastoma patients and their families. Additionally, because referral of patients with suspected retinoblastoma comes from ophthalmologists, if a pediatric oncology unit is not widely recognized as an ocular oncology referral center, it will likely see and treat no more than five or six retinoblastoma patients annually. This is true of even the largest pediatric oncology programs. Most unilateral retinoblastoma patients are treated with primary enucleation for advanced disease and are not referred to a medical geneticist. Many pediatric ophthalmologists assume that sporadic unilateral retinoblastoma that presents relatively late in development, usually around two years of age, represents non-heritable disease. While this is often the case, this presentation is not sufficient to rule out heritable disease, due to the wide array of atypical presentations encountered with germline *RB1* mutations. For this reason, it is extremely important for all patients with retinoblastoma to undergo genetic testing, to determine



whether the patient harbors an underlying germline mutation that would put the second eye at risk of retinoblastoma, and the patient at risk of other life-threatening disease. The presence or absence of a germline mutation in a patient presenting with unilateral intraocular disease affects management of the eye, as isolated non-heritable cases often present late and are more frequently treated with enucleation. In contrast to this, cases with germline mutations are generally bilateral and patients present earlier with vision loss. These patients are treated most commonly with local therapy and chemoreduction. This has significant implications for patients, as chemotherapy and radiation, if required, have significant toxicities in these very young patients and may put these patients at higher risk of additional second tumor development.

## 140.2 INCIDENCE AND PREVALENCE<sup>1</sup>

Published incidence and prevalence figures for retinoblastoma assume that retinoblastoma represents a single disease. However, in 1971, Alfred Knudson separated this childhood tumor into heritable and non-heritable forms (1). Knudson's two-hit hypothesis, by which tumors develop from a single cell that has undergone two separate mutational events, M1 and M2, was based on the observed difference in the ages at diagnosis of bilateral and unilateral retinoblastoma patients and has provided the framework for our understanding of recessive tumor suppressor genes (1–4). For geneticists, the ultimate goal is to separate the two subgroups as distinct clinical entities. Unfortunately, incidence and prevalence figures are not available to compare the relative frequency of heritable and non-heritable retinoblastoma subgroups, regardless of their country of origin. This 'lumping' has tended to obscure the geographic differences in the relative frequency of the two subgroups.

The annual incidence of retinoblastoma, including both heritable and non-heritable subgroups, in North America is approximately 1 in 12,000–15,000 live births; in the Netherlands, the incidence is 1 in 17,000 (5). In Sweden and Finland, countries with comprehensive cancer registries and population data, the prevalence of retinoblastoma for the years 1958 through 1998 is reported at 11 cases for every million children younger than 5 years of age (6). The prevalence in Singapore is remarkably similar, at 11 cases for every million children in the under-5 age group (7).

Because most cases of heritable retinoblastoma result from new endogenous mutations, environmental influences have little role in their frequency and distribution (4). Geographic incidence differences appear to reflect

variance in the number of non-heritable, somatic cases. Sporadic, non-heritable retinoblastoma is more common in countries with a developing economy. Possible causes for this increased incidence of somatic retinoblastoma include socioeconomic, dietary, and infectious factors. Additionally, there is evidence to suggest that the real geographic differences in the relative incidence of heritable and non-heritable retinoblastoma are even larger than reported. The first evidence relates to the methods used to collect data. For example, the data for prevalence calculations in a study from Namibia came from children with retinoblastoma referred to and treated at a district or tertiary hospital (8). Among impoverished populations, many children with retinoblastoma die within the first few years of life from dissemination of their eye disease, due to lack of access to medical care. Therefore, prevalence data would tend to underestimate the frequency of disease, as relatively few patients are seen at the tertiary care centers where these studies are conducted. It is almost certain that some cases that are not seen at a tertiary or district hospital go undiagnosed. Nevertheless, the reported prevalence numbers in Namibia are twice those reported for more developed countries with lower poverty rates (6).

There are two additional factors that have an impact on the disparity in the distribution of retinoblastoma cases: world population distribution and birth rate. Because over 80% of the world's population lives in developing countries, the overall worldwide prevalence rates for retinoblastoma are likely to be significantly underreported due to the factors discussed above. Additionally, because the total fertility rate is significantly higher in developing nations relative to industrialized countries (4.0 relative to 1.4 children per woman) (9), statistically, there are at least 12 to 13 cases of retinoblastoma in developing countries for every case diagnosed in the developed world. Based on birth data from the 2002 Global Population Profile (10), and the incidence figures for the developed world, we estimate that approximately 1,000 new retinoblastoma cases occur each year in all of North America, Europe, Russia, Japan, Australia, and New Zealand combined, compared with 12,000 to 13,000 cases in developing countries.

## 140.3 THEORIES OF TUMORIGENESIS AND RB1

Nearly two decades ago, a group led by Weinberg, Albert, and Dryja cloned *RB1*, the first human cancer gene to be identified (11,12). Fung and coauthors published independent cloning of *RB1* three months later (13). The retinoblastoma tumor suppressor gene (*RB1*) is located on chromosome 13q14 and encodes the retinoblastoma tumor suppressor protein, pRB (14,15). Regardless of the genetic subgroup to which it may belong, retinoblastoma develops when an insufficient amount of the active form of pRB is available within a retinoblast to prevent repeated entry into the cell cycle (14–16). Inactivation

<sup>1</sup>Incidence is defined as the number of new cases of illness commencing, or of persons falling ill, during a specified time period in a given population. Prevalence is the proportion of persons with a particular disease in a given population at a given time. In referring to retinoblastoma, the proportion is usually expressed as the number of cases per 1 million children under the age of 5 years. Prevalence becomes an inaccurate measure of the occurrence of a disease if disease survival is low.

of pRB is universal in retinoblastoma cells, validating Knudson's two-hit hypothesis (17). Since its discovery, *RB1* has been shown to be a potent tumor suppressor in multiple cell types in addition to retinoblasts, and its central role in the suppression of human cancer is illustrated by the fact that almost all human cancers demonstrate pRB inactivation (18).

pRb plays a pivotal role in regulating the exit of cells from the cell cycle and in preventing continuous cell cycle progression. Significant research over the past two decades has helped to elucidate the molecular pathways responsible for suppression of cellular proliferation by pRb. pRb binds to pro-growth transcription factors of the E2F family, resulting in repression of the expression of their target genes. These E2F target genes encode enzymes responsible for DNA replication and nucleotide synthesis, as well as the growth-promoting cyclins A and E, and the proto-oncogenes b-Myb and c-Myc, amongst others (19–21). pRb also promotes G1 cell cycle arrest through the action of p27, a protein responsible for inhibiting the checkpoint cyclin-dependent kinase complexes that promote cell cycle progression at the G1/S transition. pRb promotes p27 activity by targeting the p27 inhibitor Skp2 for degradation by complexing it with the E3 ubiquitin ligase APC/C<sup>cdh1</sup>.

Because the intracellular deficiency of pRb protein is required for retinoblastoma development, the heterozygous genotype at *RB1* predisposes the retinoblast to become transformed into a cancer cell, but alone is insufficient for tumorigenesis. Transformation requires loss of function of both alleles at the *RB1* locus (22), and approximately 75 percent of retinoblastoma tumors demonstrate loss of heterozygosity (23) at chromosome 13 (24). As discussed, *RB1* analysis is now an essential part of the clinical evaluation of any child with unilateral, unifocal retinoblastoma, regardless of age at diagnosis. Meta-analysis of *RB1* mutations in 932 patients with retinoblastoma demonstrated that the predisposing mutation, M1, was most often a result of deletions and nonsense mutations, unlike other genetic diseases, in which missense mutations are most common (25). This analysis also identified 16 hot spots in the *RB1* gene that contained almost 40% of the total mutations observed. The remaining mutations were scattered throughout the length of *RB1*, but were most frequent in exons (26–32).

Examination of mutations by patients' country of origin identified two groups, demonstrating significant differences in the incidence of nonsense and splicing mutations (25). Valverde and colleagues noted a significant association between late age at diagnosis and splicing mutations. Their data suggest that there may be a genotype of *RB1* mutation that confers a delayed-onset phenotype. Most of the mutations in low-penetrance families were of three types:

- (i) Promoter mutations resulting in low expression of a normal *RB1* protein;
- (ii) Missense and in-frame deletions affecting non-essential sequence motifs, resulting in a partial inactivation of pRB that retains some functionality; and
- (iii) Splicing mutations leading to the reduction of normal messenger RNA splicing.

Lillington and coauthors employed comparative genomic hybridization (CGH) to identify chromosomal region alterations at sites other than 13q14 that are associated with histopathology, progression and patient outcomes (33). In an analysis of 49 tumors, chromosome imbalance was present in 96 percent of samples. In this series, as well as in a series from Germany (34), gains of 6p and 1q were the most frequent events. Alterations associated with metastasis included loss of 13q and 5q, while gain of 13q, monosomy of chromosome 16, and loss of 16q were more frequent in children in whom enucleation was performed at an older age (33). These authors found a significantly lower number of CGH alterations in eyes enucleated before one year of age relative to children enucleated at three years of age or above, with a median number of three chromosomal alterations found in the younger patients, as compared with 11 CGH changes in the older children.

#### 140.4 THE NATURAL HISTORY OF RETINOBLASTOMA TUMORIGENESIS

The process of tumorigenesis resulting in retinoblastoma involves the acquisition of several genetic alterations that occur sequentially (35,36). While the initiating event is the biallelic loss of *RB1* function, the subsequent genetic events, termed 'M3', which occur to accelerate tumorigenesis remain relatively unknown. Virtually all newly arising tumors that are smaller than 3 mm in diameter are perfectly round and symmetrical, the expected shape if all tumor cells were growing at the same rate (Figure 140-4). As clonal expansion proceeds, foci within the tumor clone show evidence of growth advantage. These foci develop into rapidly growing 'nodular excrescences' on the surface of the previously symmetric tumor (Figure 140-5) (37). Although there is no clearly documented connection between the ophthalmoscopically-observed focal growth changes in retinoblastoma tumors and specific chromosomal changes within the tumor, we know that, with time, complex chromosomal aneuploidy and associated genetic changes known to contribute to progression of malignancy take place within the tumor (38). Most of these changes involve loss of additional cancer suppressor loci or duplication of oncogene sequences. Regions of commonly observed chromosomal gains included in M3 include 6p22 and 1q31–2 (23). Candidate genes located in the amplified regions of 6p include the chromatin remodeling factor and histone chaperone protein DEK and the transcription factor E2F3 (39). DEK functions as a proto-oncogene through inhibition of apoptosis and promotion of cellular proliferation and de-differentiation



**FIGURE 140-4** Early intraretinal tumors are round because all cells are identical daughter cells of the original retinoblast that, by chance, developed M2, and was transformed into a retinoblastoma cell. No M3 events that bestow clonal growth advantage are yet manifest in this lesion. In the new international grouping system, this eye would be classified as group A disease. The tumors are 3 mm in size or less, and are further than 1.5 mm from the fovea and 3 mm from the optic nerve head (see Table 140-2).

(40–42). Its over-expression is also associated with inhibition of cellular senescence and repair of DNA double strand breaks (43,44). E2F3 has been found to be amplified in retinoblastoma patient samples, and its expression has been proposed to inhibit the pro-apoptotic function of the tumor suppressor p53 (23). Amplification of genes encoding the adhesion factor cadherin-11, p130 and MYCN have also been implicated in progression of retinoblastoma (32).

Candidate genes harbored in the region of amplification at 1q32 include those encoding the p53 inhibitors Mdm2 and MdmX. While most human cancers demonstrate mutations in the p53 tumor suppressor gene, which prevents them from undergoing apoptosis before clonal expansion can occur, p53 mutations are notably absent from the majority of retinoblastoma tumors. While the p53 inhibitor MDM2 is over-expressed in some forms of human cancer (45) and is located at the region of amplification observed at chromosome 1q32, mutations in Mdm2 have not been commonly observed in human retinoblastoma (46). Amplification of MdmX, however, has been observed in 65% of retinoblastoma cases. MdmX expression has been found to promote proliferation and to inhibit p53-mediated cell death in retinoblasts lacking *RB1* (47). While MDMX is an attractive target for new therapies to inhibit retinoblastoma, some reports have demonstrated a lack of correlation between the genomic amplification of MDMX and levels of its mRNA expression. Nevertheless, the MDMX inhibitor Nutlin-3 is currently under clinical investigation for the treatment of retinoblastoma, in combination with the p53 inducer, topotecan. This



**FIGURE 140-5** M3 (progression-of-malignancy) events have occurred in these two relatively small tumors, changing the earlier round shape (see Figure 140-4) to one that is more dumbbell-like. The nodular alterations of the initial shapes are postulated to be due to daughter clones of cells that contain a growth-advantage mutation compared to the original tumor cells. This eye would be classified as group B disease because of the larger size of the tumors and their close proximity to the optic nerve (see Table 140-2).

combination was found to produce an 82-fold reduction in tumor burden when injected subconjunctivally in murine models of retinoblastoma, and represents a promising new targeted therapeutic approach for treatment of these intraocular tumors (47).

## 140.5 THE *RB1* CANCER SYNDROME

Patients with heritable and non-heritable forms of retinoblastoma live with very different lifelong cancer risks, due to the presence or absence of the *RB1* mutation in all cells of the body. Patients with a germline *RB1* mutation possess the *RB1* cancer syndrome, putting them at risk of multiple types of tumors, in addition to retinoblastoma. These patients are heterozygous for one inactive or deleted *RB1* allele in every cell type of the body. These inactive *RB1* alleles may be inherited from either parent or may result from endogenous mutations occurring before, at the time of, or after conception. New, postzygotic *RB1* mutations are fairly common events and are the cause of germline mosaicism. If mutational mosaicism is not present, patients have a 45% risk to pass the causative mutation for the cancer predisposition syndrome to each of their children.

The two major consequences of the *RB1* cancer syndrome are the lifelong increase in risk of developing certain cancers, including retinoblastoma, bone, and soft tissue sarcomas, and the risk of transmitting this cancer predisposition to subsequent generations. Because of advances in the



diagnosis and treatment of retinoblastoma, most patients with the *RB1* cancer syndrome no longer die of their eye disease. Rather, premature deaths amongst patients with this syndrome occur from non-ocular cancers. Rapid progress in future, rapid progress in whole genome sequencing, epigenetics and proteomics may provide clinicians with tools for early identification of non-ocular cancers in an effort to reduce the mortality in this group of patients (48).

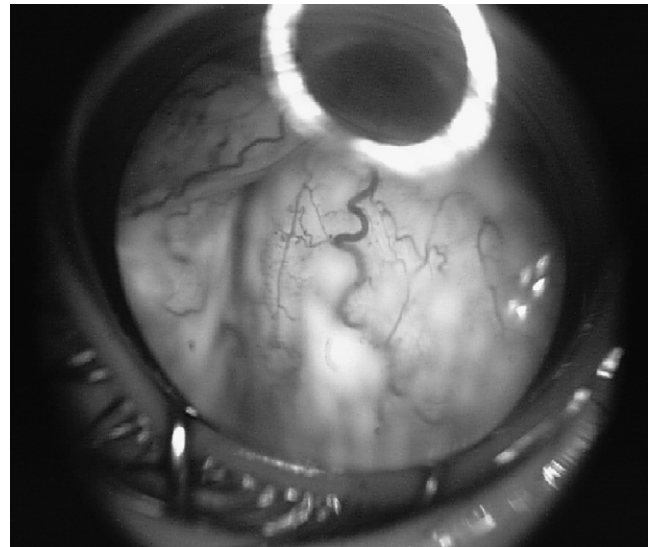
In more developed countries, patients with the *RB1* cancer syndrome are diagnosed at a relatively early age, usually around 12 months. It is important to refrain from using age at diagnosis to predict genotype. Early diagnosis does not necessarily equate with a germline M1 mutation or the presence of independently arising, bilateral, multifocal tumors (see Figures 140-2, 140-4 and 140-5). The observation of multifocal tumors is extremely helpful in interpreting the genotype of patients since multifocality is associated with the heritable form of disease. The example, shown in Figure 140-4, represents early findings observed during an eye examination of a newly born offspring of one affected parent.

Distinguishing between unifocal and multifocal origin of retinoblastoma is extremely difficult in patients with advanced intraocular disease (Figure 140-6; see also Figure 140-1). In fact, it may be impossible for the geneticist to use information from the ophthalmologist's description of the retinoblastoma to inform genetic counseling or risk assessment. As tumors enlarge, the normal loss of cellular adherence leads to shedding of small clumps of cells into the vitreous (Figure 140-7). In either the heritable or the non-heritable form of the disease, tumor cells can be 'seeded' from the primary tumor into the vitreous or subretinal space prior to treatment (Figure 140-8). Each tumor clump may be the origin of a suspected 'new' tumor. In most cases of advanced disease, the 'new' focus actually represents tumor dissemination from the primary site and not a newly arising tumor focus (see Figure 140-8).

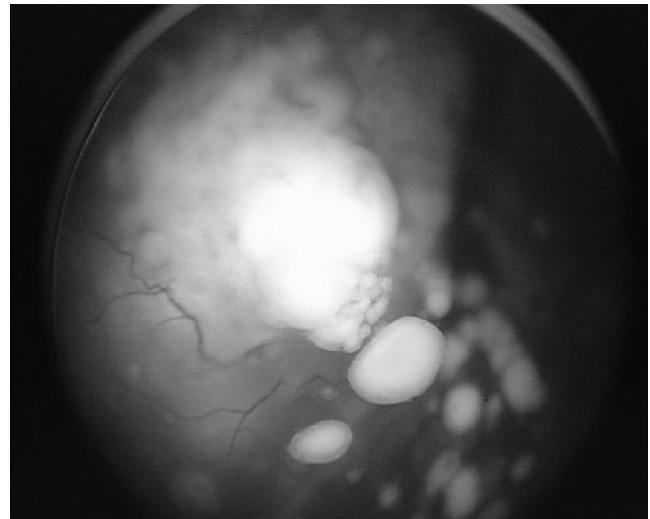
All retinoblastoma patients should be assumed to have a germline mutation until *RB1* genetic testing clarifies the genotype. Since grave consequences follow the failure to suspect or diagnose the presence of the *RB1* cancer syndrome, all newly diagnosed patients should be followed as if they have the heritable cancer syndrome. Most major retinoblastoma centers use examination under anesthesia (EUA) to monitor the retinas for evidence of newly arising retinoblastomas every four to six weeks until either the *RB1* status is clarified or the child reaches five years of age.

### 140.5.1 Retinal Tumors

Ninety-five percent of *RB1* cancer syndrome patients develop retinoblastoma in at least one eye. While the ocular tumors in the *RB1* cancer syndrome are usually bilateral and multifocal, roughly 15% of patients have tumors only in one eye (see Figure 140-3). In about 5% of patients with the *RB1* cancer syndrome, neither eye develops retinoblastoma. Since the transformation of the predisposed



**FIGURE 140-6** Advanced intraocular disease virtually fills the eye in this image. At this stage, multifocal disease cannot be clinically distinguished from unifocal disease. Any independently arising tumor foci have grown into one large tumor mass. (See also Figure 140-1).

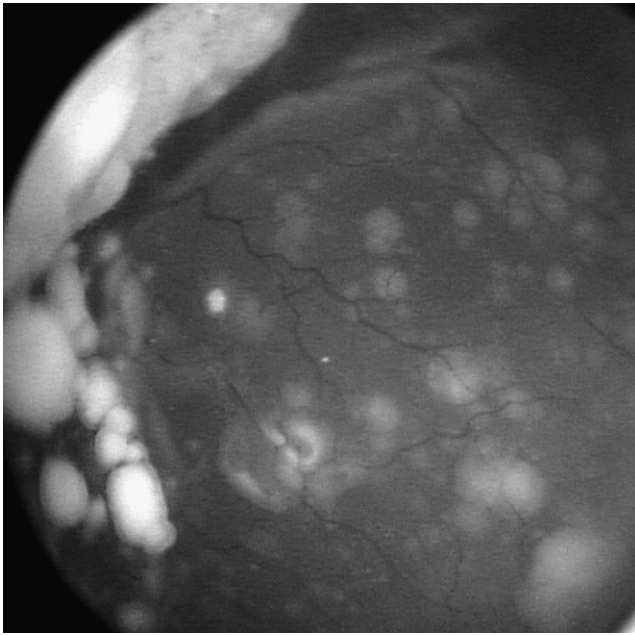


**FIGURE 140-7** Vitreous seeding of retinoblastoma in this image is seen as white avascular 'spherules' of tumor suspended in the gel-like vitreous behind the lens. The presence of vitreous seeding places this eye into a more advanced disease group because the cells in these seeds are difficult to destroy during the treatment process.

retinoblast requires a second spontaneous inactivating event at the *RB1* locus, it can be presumed that the remaining 5% who do not develop retinoblastoma never have a cell in either eye that acquires the chance mitotic inactivating event (M2) at the normal *RB1* allele. The age at diagnosis of isolated unilateral retinoblastoma does not identify those patients with the *RB1* cancer syndrome (49).

Notis and colleagues examined the clinical features of *RB1* cancer syndrome patients whose affected parent had unilateral retinoblastoma (50). Among 54 patients with retinoblastoma whose parent had unilateral disease, 91% developed bilateral retinoblastoma with a mean age at diagnosis of 10.5 months. These characteristics do not





**FIGURE 140-8** Digital image of a detached retina shows tumor seeding both in front of the retina (in the vitreous on the left of the image) and attached to the underside of the retina (the retinal vessels can be seen passing over the tumor plaques without distortion on the right side of the image). All of these tumor foci arose from clumps of tumor cells shed from the primary tumor(s) and are not newly arising retinoblastoma from M2 genetic events. Having more than 10 M2 mutations in a single eye is very rare.

differ from the expected ocular features in the whole group of *RB1* cancer syndrome patients.

When retinoblastoma affects both eyes or there is a positive family history of this disease, the patient demonstrates a heritable form. Unfortunately, unequivocal clinical features that are diagnostic of the *RB1* cancer syndrome (primarily bilateral retinoblastoma) are present in fewer than 40% of all retinoblastoma cases. The other 60% of patients with retinoblastoma present unilaterally and the genetics of the disease cannot be clinically deduced. Routine histological examination of the tumors in retinoblastoma patients provides little information about the genetic subgroup to which they belong. In order to detect the 15% of unilaterally affected patients who have the germline mutation and have the *RB1* cancer syndrome, genetic investigation is critical, as described later in this chapter.<sup>2</sup>

<sup>2</sup>The imprecise use of words such as bilateral and hereditary to refer to the group of patients with the *RB1* cancer syndrome contributes to ‘concept clutter’. If bilateral is chosen to describe this group of patients, then 15% of unilateral cases with the *RB1* mutation will be omitted. The word hereditary is an adjective that can be used to describe a disease. However, when referring to the biology of a characteristic capable of being transmitted from parent to offspring, the term heritable is more precise and appropriate. While most *RB1* cancer syndrome patients have bilateral retinoblastoma and may have inherited the predisposing mutation (M1) from a parent, neither of those features is a requirement for patients with the *RB1* cancer syndrome.

## 140.5.2 Non-Ocular Cancers

All patients with the *RB1* cancer syndrome, regardless of whether or not they developed retinoblastoma or were treated for this tumor, are at increased risk of succumbing to a non-ocular neoplasm (Table 140-1). A meta-analysis of 243 non-ocular tumors in retinoblastoma survivors found the following tumor types and relative frequencies: osteosarcoma, 37%; cutaneous melanoma, 7.4%; soft tissue sarcomas, 6.9%; brain tumors, 4.5%; fibrosarcoma, 3.3%; chondrosarcoma, 3.3%; and other sarcoma, 3.3% (51).

Although the terms non-ocular tumors and second malignant neoplasms (SMNs) are commonly used interchangeably, all non-ocular tumors in *RB1* cancer syndrome patients are not necessarily second primary malignancies. They may simply be the first malignancy to occur in a cancer predisposition syndrome patient who, by chance alone, did not develop retinoblastoma. The phrase ‘second malignant neoplasm’ is commonly used by pediatric oncologists and ocular oncologists, who are introduced to their patients by virtue of the patients having a ‘first’ malignant neoplasm, primarily retinoblastoma. Geneticists, on the other hand, deal primarily with family members of an affected patient or child, most of whom do not have a ‘first’ malignant neoplasm, but may be at some risk of the *RB1* cancer syndrome.

There is evidence that aggressive treatment of non-ocular cancer in *RB1* cancer syndrome patients is improving their survival (52). The long-term implications of this finding, however, remain unclear. Abramson and colleagues have documented that, if a patient survives the first non-ocular cancer, a second is subsequently diagnosed, and then a third, fourth and fifth until the patient eventually dies of a non-ocular cancer (53).

There are at least two major factors that contribute to the increased risk of non-ocular cancers in patients with the *RB1* cancer syndrome: (i) the presence of the germline *RB1* mutation and (ii) the type and intensity of retinoblastoma treatment utilized, especially external beam radiotherapy (EBR) (9,54–56). In very young patients, EBR has been noted to cause midface hypoplasia and to increase the risk of developing osteosarcomas, soft tissue sarcomas, and brain tumors. Etoposide, part of the three-drug chemotherapy regimen for retinoblastoma, may also be associated with the development of secondary leukemias (57).

**140.5.2.1 Impact of the Germline *Rb1* Mutation.** The *RB1* germline mutation alone confers an overall risk of development of non-ocular tumors of 6.9 (95% confidence interval [CI], 4.1–11) over what would be expected in the general population (55) (see Table 140-1). This standardized incident ratio (SIR<sup>3</sup>) for the *RB1*

<sup>3</sup>The SIR, or standardized incidence ratio, is the number calculated by dividing the number of observed cases (non-ocular tumors) by the number of non-ocular tumors expected in the general population. The larger the SIR, the greater the effect of the factor being measured.

**TABLE 140-1** Risks of Non-ocular Cancers in 1-Year Survivors of Heritable Retinoblastoma. Radiation ( $n = 849$ ; Person-yr at Risk, 21,706), No Radiation ( $n = 114$ ; Person-yr at Risk, 3602)

	Observed	Expected	SIR	95% CI	Observed	Expected	SIR	95% CI
All Sites	241	11	22	19–24	19	2.77	6.9	4.1–11
Heavily irradiated sites (>1 Gy)								
Bone	73	0.18	406	318–511	2	0.03	69	8.4–250
Soft tissue	33	0.23	140	96–196	1	0.04	23	0.6–131
Nasal cavities	32	0.02	1364	933–1925	0	0.01	0	0.0–688
Eye and orbit	17	0.05	312	181–499	0	0.01	0	0.0–392
Brain, CNS	10	0.62	16	7.7–29	0	0.11	0	0.0–33
Pineoblastoma	5	0.05	104	34–244	0	0.01	0	0.0–509
Buccal cavity	7	0.27	26	10–53	0	0.07	0	0.0–54
Thyroid	2	0.5	4	0.5–15	0	0.11	0	0.0–35
Moderately irradiated sites (0.4–1.0 Gy)								
Female breast	8	1.91	4.2	1.8–8.2	2	0.61	3.3	0.4–12
Skin melanoma	26	0.85	30	20–45	3	0.2	15	3.1–44
Lung	2	0.63	3.2	0.4–11	3	0.21	30	3.0–42
Leukemia	1	0.76	1.3	0.03–7.3	1	0.13	7.8	0.2–43
Lightly irradiated sites								
Uterus	5	0.25	20	6.4–46	2	0.1	20	2.5–74
Bladder	2	0.25	7.9	0.9–28	0	0.07	0	0.0–52
Excess absolute risk per	105.9				45.1	10,000 person-yr		

CI, confidence interval; CNS, central nervous system; SIR, standardized incidence ratio.

Adapted from Kleinerman, R. A.; Tucker, M. A.; Tarone, R. E., et al. Risk of New Cancers After Radiotherapy in Long-term Survivors of Retinoblastoma: An Extended Follow-up. *J. Clin. Oncol.* 2005, 23, 2272–2279.

germline mutation alone was calculated for all sites in 114 patients (3602 person-years at risk) with heritable retinoblastoma who were not treated with radiation. The most common sites of the non-ocular cancers among these 114 patients were bone (SIR 69), soft tissue (SIR 23), uterus (SIR 20), skin (cutaneous melanoma) (SIR 15), and lung (SIR 14).

A study from Great Britain suggests that the presence of the germline *RB1* mutation also increases the risk of smoking-associated cancers, including lung and bladder carcinoma, in older survivors of heritable retinoblastoma who were never treated with radiation (58). Among the 144 patients in this report, 58 developed subsequent cancers. Only 8 of the 58 cancers were sarcomas of bone or soft tissue origin, in marked contrast to the excess of sarcomas reported in the American literature. When compared to the general population, these middle-aged patients had a higher mortality from lung cancer, bladder cancer, and all other epithelial cancers combined. The authors concluded that survivors of heritable retinoblastoma who were not exposed to radiotherapy have a higher lifetime risk of developing late-onset epithelial cancer. They suggested that limiting exposure to tobacco, radiotherapy, and ultraviolet light might lessen that risk (58).

**140.5.2.2 Impact of Radiation Therapy.** The effect of adding the risk caused by radiation therapy to the risk of non-ocular cancer from the germline *RB1* mutation was elucidated (55) in a cohort of almost 1600 patients reported by Eng and colleagues, all of whom received

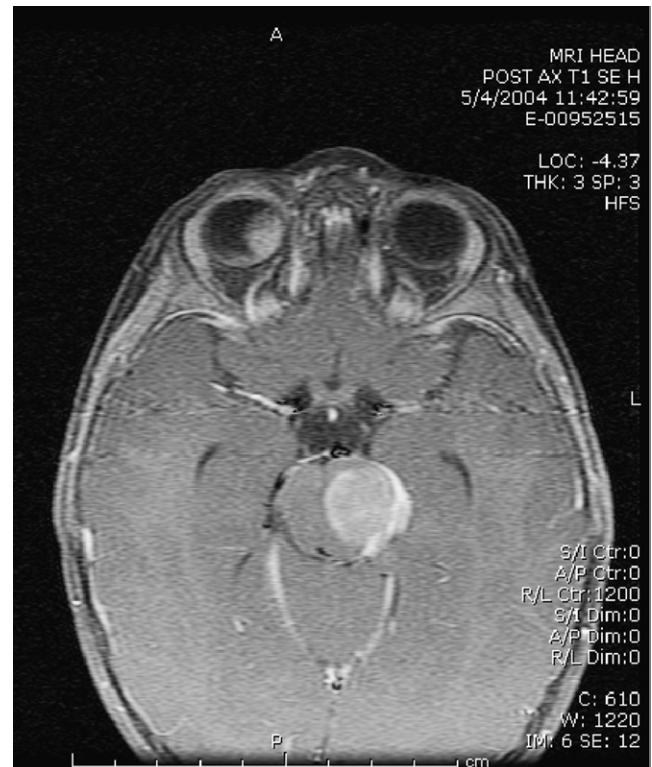
relatively high doses of radiation in the 1980s (54). The addition of radiation at all doses increased the overall risk of non-ocular cancer in those patients by a factor of 3.1, from 6.9 (95% CI, 4.1–11) to 22 (95% CI, 19–24) (see Table 140-1). In the 1506 New York patients in this series, radiation given before one year of age seemed responsible for the increased risk (59). Since current forms of radiation differ significantly from that applied in the 1980s, the data from the study must be interpreted cautiously and cannot be easily applied to radiation regimens used today. The same risks reported in these studies may not apply to newer approaches to radiation therapy, which involve reduced dosing and better treatment planning for patients.

Despite differences in radiation, the study does demonstrate an association between radiation and non-ocular tumor risk in patients with heritable retinoblastoma. The excess absolute risk per 10,000 person-years conferred by the germline *RB1* mutation alone is 45.1 versus 105.9 when radiation was used in the treatment of retinoblastoma in the *RB1* syndrome patient (55). The most common primary non-ocular neoplasm in *RB1* cancer syndrome patients is osteosarcoma, mostly of the head and neck, with a peak incidence during the growth years of 8–14, irrespective of whether or not radiation was received. Other relatively common non-ocular cancers include soft tissue sarcomas. Cutaneous malignant melanomas may appear during the teenage and early adult years, while midline brain primitive neuroectodermal tumors (PNETs) may occur from birth through age

3 years (Figure 140-9). Less common tumors that occur in the irradiated field include fibrous histiocytoma, leiomyosarcoma, angiosarcoma, rhabdomyosarcoma, meningioma, sinonasal small cell neoplasm, myoepithelioma, and schwannoma. Less common tumors that tend to occur outside the irradiated field include cutaneous malignant melanoma, renal cell carcinoma, Ewing sarcoma, carcinoma of the tongue, and medulloblastoma. Deletion of the chromosomal region 13q14 or mutations in *RB1* are common findings in some of these tumors (e.g. malignant fibrous histiocytomas (20)), even when they are found in patients without a history of retinoblastoma, suggesting that the development of some of these tumors may be more related to loss of *RB1* rather than to radiation exposure as treatment for retinoblastoma.

**140.5.2.3 Impact of Systemic Chemotherapy.** A less common type of second malignancy that occurs following the treatment of childhood cancers, including retinoblastoma, is alkylating agent- and topoisomerase II inhibitor-related acute myeloid leukemia and myelodysplastic syndrome (60). This relatively rare SMN in heritable retinoblastoma patients may be due to the presence of etoposide in the usual triple-drug treatment regimen (carboplatin, etoposide, and vincristine) that was first introduced about 20 years ago (57). Patients who have been found to have developed the topoisomerase II inhibitor-associated acute myelocytic leukemia have most commonly received 9 or 12 cycles of this three-drug regimen. In contrast to radiation, however, systemic chemotherapy is not known to increase the risk for tumors to which the *RB1* cancer syndrome patient is genetically predisposed. Because systemic chemoreduction with local therapy has been in use for only 20 years, the data on second tumor risk is only now being collected.

Due to the potential for increased tumorigenesis resulting from systemic chemotherapy, the use of intra-arterial chemotherapy is currently under investigation for treatment of intra-ocular retinoblastoma. Initially introduced by the Kaneko 20 years ago, Abramson and colleagues have recently reintroduced the use of melphalan when injected directly into the ophthalmic artery using interventional radiological techniques. Recently published results from this group describe 289 successful intra-arterial injections, with a median of three injections per eye, conferring a total event-free two-year survival rate of 70 percent (confidence interval 57.9–82.2%) (61). The long-term results regarding the efficacy of isolated intra-arterial chemotherapy, however, remain to be determined. Potential hazards include the inability to access subretinal tumor seeds that retain the potential for subsequent clonal expansion, as well as fibrosis of the ophthalmic artery, vascular spasms, and stroke. Debate is currently ongoing regarding the recommended use of intra-arterial chemotherapy and its efficacy when used in isolation versus as an adjunct to radiotherapy,



**FIGURE 140-9** This MRI scan shows a midline PNET in an *RB1* cancer syndrome patient who, by chance, had unilateral retinoblastoma. The ocular tumor can be seen as a white mass located nasally in the right eye which is the eye on the left in this image. PNET is a non-ocular neoplasm that is almost always diagnosed before age three years.

chemotherapy and/or enucleation. The very specific delivery of chemotherapy into the eye may also fail to treat patients with high-risk histopathological features for metastatic disease. These features, such as involvement of the optic nerve beyond the lamina cribrosa or choroidal invasion, may predispose children to the development of retinoblastoma at other sites and would be best treated with systemic chemotherapy or enucleation. Intra-arterial chemotherapy may not treat these high-risk features and may put children at higher risk of mortality from local disease extension.

**140.5.2.4 Lipomas in Patients with the *RB1* Cancer Syndrome.** There is some evidence that the presence of lipomas in *RB1* cancer syndrome patients might be associated with an increased probability of developing non-ocular malignancies (62). The observation of 13q14 deletions in lipomas had been reported before 1989, when Sreekantaiah and colleagues described three lipomas and summarized three from the previous literature with deletion of 13q14 (63). Rieder and associates also described loss of heterozygosity of *RB1* in lipomas from a retinoblastoma patient (64). Other groups have observed amplification of *Mdm2* in lipomas, malignant fibrous histiocytomas, and liposarcomas (65). In 2001, a report from Lohmann and colleagues described multiple lipomas linked to an *RB1* mutation in a large pedigree



with low-penetrance retinoblastoma (66). In 2003, that same laboratory reported that deletion of 13q14 distal to the *RB1* locus was present in a high percentage of lipomas (67). Interestingly, in that report, other regions of 13q were recurrently affected in lipomas, including regions near the *BRCA2* and *HMGIC* loci. As an interesting corollary, the related gene *BRCA1* is a transcriptional target of E2F4 and E2F5 transcriptional repressors, and E2F5 has been found to be amplified in some breast cancers.

**140.5.2.5 Trilateral Retinoblastoma.** The term trilateral retinoblastoma is used to describe patients with heritable retinoblastoma and midline brain tumors (see Figure 140-9) (68–72). The designation resonated in part because the pineal gland and pinealoblastoma morphology closely resemble the retina and retinoblastoma. In fact, these patients are *RB1* cancer syndrome patients who develop a second non-ocular neoplasm within the same time frame (0–3 years) as the diagnosis of their eye tumor(s). These midline brain tumors are primitive neuroectodermal tumors (PNETs) of uncertain origin. Patients with the *RB1* cancer syndrome who develop brain PNETs should be considered as having a second non-ocular tumor for both diagnostic and management purposes.

Kivela published a comprehensive meta-analysis of 106 children with trilateral retinoblastoma (69). The median age at diagnosis was five months, with a range from 0–29 months. The diagnosis of trilateral retinoblastoma was most commonly made in the second or third generation of affected patients. The mean time from the diagnosis of retinoblastoma to detection of the PNET was 21 months. Tumors in the region of the pineal gland (77%) were more common than suprasellar tumors (23%), but suprasellar tumors were diagnosed at a significantly earlier mean age (6.5 months vs. 32 months;  $P < 0.0001$ ). The tumors were detected earlier if neuroimaging was routine, but the age of death was the same. No child survived if the tumor was detected without screening, or if the tumor was larger than 15 mm in size at diagnosis. Cumulative 5-year survival was 27% if screening was done. These data suggest that routine magnetic resonance imaging (MRI) with early detection and aggressive treatment of small intracranial tumors might be associated with a better outcome. In our center, we order an MRI with 1-mm cuts, gadolinium enhancement, and fat suppression at diagnosis and every six months until four years of age.

### 140.5.3 Mosaicism of the Germline M1

The concept that new germline *RB1* mutations occur either in the gamete immediately prior to fertilization or in the fertilized egg has been overturned by the documentation of mosaicism in some new cases of heritable retinoblastoma (73–76). In fact, mosaicism

for the ‘founder’ *RB1* mutation is so common in sporadic bilateral retinoblastoma cases that, if the *RB1* mutation is being sought in a family with two affected generations, the search should be initiated in the second-generation individual to avoid confounding the result with mosaicism.

### 140.5.4 Chromosome 13 Deletion Syndrome

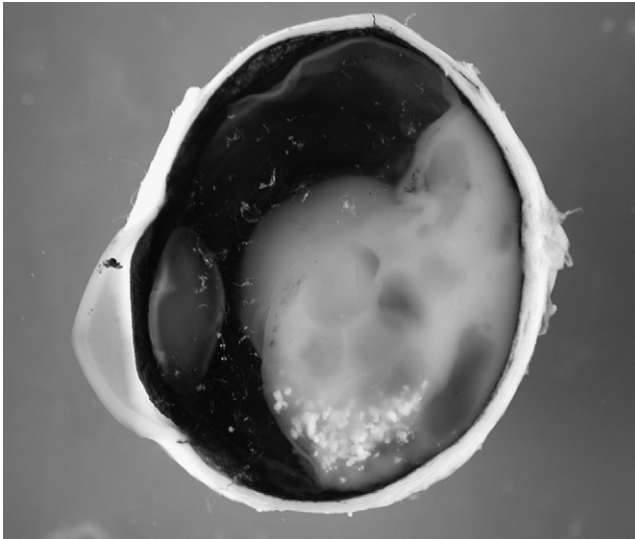
In 10–14 percent of *RB1* cancer syndrome patients presenting with clinical dysmorphism and neurologic impairment, chromosome 13 may be entirely absent through deletion. There appears to be an unexpectedly large number of patients with unilateral retinoblastoma in the chromosome 13 deletion subgroup of *RB1* cancer syndrome patients (28,77). It has been postulated that if a large deletion is the first genetic event and subsequent loss of the normal chromosome 13 occurs through non-disjunction, the most common spontaneous mitotic occurrence, the resulting cell would be nonviable. The result might be that fewer tumors arise in these patients, resulting in patients with only unilateral retinoblastoma. This topic is discussed further under the heading Genetic Counseling, later in the chapter.

### 140.5.5 Retinoma (Retinocytoma)

Retinoma (78) or retinocytoma (79) is a benign variant of intraocular retinoblastoma but bears the same genetic implications. Non-ocular cancer has been reported in patients in whom the only previous evidence of the *RB1* cancer syndrome was a retinoma (80,81). The clinical significance of the retinoma is that its presence in one or both eyes of the parent of a child with retinoblastoma may indicate that the parent carries the germline signature of the *RB1* cancer syndrome. This makes an ophthalmologic examination of the parents of a newly diagnosed retinoblastoma patient an important factor in the family history, which can influence genetic counseling.

If a retinoma is observed within the first few years of a child’s life, it may closely resemble type II regressed retinoblastoma lesions (grayish white lesions without prominent capillary vascularity or surrounding retinal pigment epithelial changes, but often containing cavitary spaces) (82). A retinoma is suspected when there is no significant reduction in tumor size following either systemic chemoreduction with local therapy or external beam radiotherapy (Figure 140-10). Retinomas tend to undergo regression and develop surrounding pigment epithelial changes throughout their natural history. Homogeneous gray masses are frequently centered in the region of pigment disruption (83) as described by Gallie and colleagues (78). These lesions are often stable over a long period of time (84,85).





**FIGURE 140-10** A large retinoma filling over half the intraocular volume. Obvious cavitory spaces and calcium deposits are present. The white flecks suspended in the vitreous are commonly described in eyes containing retinomas or retinocytomas.

However, retinomas pose a long-term risk to the patient, because they may undergo malignant transformation to retinoblastoma. The transformation of a retinoma may be the etiology of the rare and unusual finding of active intraocular retinoblastoma in a teenager or adult. Malignant transformation has been described in a 24-year-old woman (83) and a seven-year-old girl (86). The latter had a documented retinoma at age four years, but later developed retinoblastoma with vitreous seeding and eventually underwent enucleation. Singh suggests that this transformation event occurs in only about 4% of retinomas (84). In many centers, focal treatment is used to ablate the lesion in an effort to prevent malignant transformation.

## 140.6 NON-HERITABLE (SOMATIC OR POSTZYGOTIC) RETINOBLASTOMA

Worldwide, non-heritable retinoblastoma accounts for 70–80% of all cases of retinoblastoma. Non-heritable retinoblastoma was initially recognized to have a higher incidence in less-affluent populations more than a decade ago (87). This finding suggested an association with substandard living conditions. Unilateral, nonfamilial cases comprise almost 80% of total cases in less developed countries (30). In contrast, the percentage of unilateral cases in countries of the developed world is consistently reported at about 60%.

Patients who do not have a germline *RB1* mutation develop retinoblastoma only as a result of two rare events in a somatic cell that inactivate both alleles of the *RB1* gene. In this subgroup of the retinoblastoma population, there is no heritable predisposition to cancer, no shortened lifespan, and no risk to pass a genetic cancer predisposition on to subsequent generations.

### 140.6.1 Etiology

The emerging consensus is that environmental factors play a major role in the etiology of non-heritable retinoblastoma. Specific factors that are currently being explored include extreme poverty and poverty-associated dietary deficiencies during pregnancy. Another environmental factor with a possible etiologic role is human papillomavirus (HPV) infection during pregnancy.

**140.6.1.1 Poverty.** Evidence to support the contribution of poverty to the observed geographic excess of non-heritable retinoblastoma can be found in economically depressed, less developed countries. In North America, retinoblastoma occupies sixth place on the list of most common solid childhood cancers. However, the order of the list is markedly different in the tropical African country of Nigeria (88), where poverty and malaria are widespread and unilateral retinoblastoma is common. Retinoblastoma would top the list, if it were not for the slightly higher incidence of malaria-associated Burkitt lymphoma (88).

Namibia is an impoverished country on the arid southwest coast of Africa, where, in contrast to Nigeria, malaria is not a public health problem. The incidence of malaria-associated Burkitt lymphoma is very low; however, the prevalence of retinoblastoma is as high in Namibia as in Nigeria (8). The prevalence calculations in the Namibia study (5.8 cases per million children younger than 15 years) can be adjusted to approximately 19 cases per million children under 5 years of age and are nearly double those reported from Sweden and Singapore (11 cases per million children under 5 years of age).

Recent data from Orejula and colleagues suggest that the specific factor that is common among populations that live in poverty and may contribute to the increased number of patients with retinoblastoma is a deficiency of fruits and vegetables in the diet of pregnant mothers. Data from an isolated population in Mexico suggest that such a deficiency may increase the likelihood of having a child with the non-heritable form of retinoblastoma (89). In light of this new information, it is of more than passing interest that Alaskan natives, a population in which pregnant mothers have limited access to fresh fruits and vegetables for extended periods, likewise show an unexpectedly high incidence of retinoblastoma (90). The non-heritable form of the disease may be more common in situations in which maternal intake of folate and vitamins during pregnancy are inadequate. This association of folate deficiency and increased risk of retinoblastoma is discussed later in the chapter, in the section on prenatal assessment and diagnosis. Poverty also contributes to poor outcomes because of delayed access to treatment. A direct correlation between advanced retinoblastoma at diagnosis and poverty has been reported in Mexico (91).

**140.6.1.2 Human Papillomavirus.** HPV inactivation of pRb is the major cause of human cervical cancer. At least two groups have detected HPV DNA sequences in

retinoblastoma tumor tissue (92,93). The role of HPV-associated post-translational inactivation of pRB in the etiology of non-heritable human retinoblastoma is still unknown, but is under active investigation.

### 140.6.2 Diagnosis

The absolute diagnosis of non-heritable retinoblastoma cannot be achieved even with *RB1* mutation analysis, since it involves proving the absence of a mutation in any cell outside of the tumor focus. The residual risk after a laboratory has been unable to find an *RB1* mutation from a peripheral blood sample depends on that laboratory's mutation detection accuracy. Some laboratories can reduce the risk that sporadic unilaterally affected patients have the *RB1* cancer syndrome from about 15% to between 1% and 2% if they find no mutation in a blood sample. Other labs with a higher failure of detection rate will not accept blood only from patients with sporadic unilateral retinoblastoma. As a CLIA laboratory funded by the National Eye Institute, our laboratory has developed no cost, high-throughput techniques for whole gene exon sequencing and detection of chromosomal abnormalities through multiplex ligation-dependent probe amplification. These techniques allow the detection of point mutations, small and large insertions or deletions, truncations, inversions, and transpositions with greater than 95 percent sensitivity and specificity, returning results to the requesting clinician within one week.

Most patients with unilateral sporadic retinoblastoma present with advanced intraocular disease because vision is not affected in the other eye (see Figures 140-1, 140-3 and 140-6). In these cases, the eye is filled with tumor, preventing any determination of whether the tumor was originally unifocal or multifocal in origin. *RB1* analysis in these patients reveals that more than 10% will have an *RB1* gene mutation (94) and as many as 15% may have constitutional microdeletions of chromosome 13q14.

## 140.7 FEATURES OF RETINOBLASTOMA COMMON TO HERITABLE AND NON-HERITABLE CASES

### 140.7.1 Clinical Features and Age at Diagnosis

Retinoblastoma can be found at birth (20) or can be initially diagnosed as late as age five to seven years. Neonatal diagnosis has also been described. Classically, however, most cases are brought to medical attention before the age of three. Once the retina is terminally differentiated, the risk of newly arising retinal tumors has passed. Common presenting signs of retinoblastoma in the developed world include leukocoria (white pupil) (Figure 140-11) and strabismus (misalignment of the eyes). If the fovea (that part of the retina responsible for fine central vision) is replaced



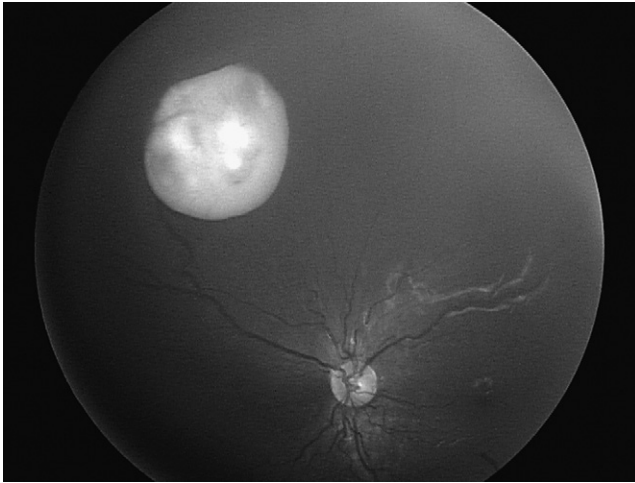
**FIGURE 140-11** Leukocoria is present in the left eye of this child. It is obvious in this flash photograph taken in a dimly lit kitchen when the child's pupils are naturally dilated. The light from the flash attachment on the camera is reflected by the white retinoblastoma in the left eye. This is the classical asymmetrical 'abnormal red reflex', sometimes referred to as 'cat's eye', 'animal eye', 'gleam', 'glow', 'glimmer', or 'something is not right with my baby's eye'. In bright ambient light, this child's pupil would be small and the leukocoria would not be seen. This is often the case in the pediatrician's office. This is also the reason that the parents or other family members see this sign before the pediatrician in over 85% of cases.

by the tumor in one eye, strabismus due to the loss of central vision in that eye may occur. Even total lack of vision in one eye of a child may go unrecognized by family members. Unilateral visual loss will not cause signs of vision impairment because of the normal vision in the fellow eye.

The tumor does not cause pain or discomfort as it enlarges and virtually never causes a red or inflamed eye except late in the course of the intraocular disease (see Figure 140-3). In very advanced intraocular disease, the tumor mass can interfere with the normal intraocular fluid outflow and result in glaucoma, redness, and pain. Proptosis (bulging of the eye from the orbit, usually due to the expansion of a tumor mass in the orbit) is a rare presenting sign of retinoblastoma in developed countries, but in a 2002 report, proptosis was the second most common presenting sign in Congolese patients (95). Extraocular disease at initial diagnosis is also common in Central and South America (30). In developed countries where there is relatively easy access to medical care, metastatic disease develops in fewer than 5% of patients with retinoblastoma. It is so rare at diagnosis that spinal tap and bone marrow aspiration/biopsy are no longer performed routinely unless there is other evidence of tumor outside the eye.

In the absence of a known family history of the disease, the early months of retinoblastoma growth in a child's eye usually go undetected by both family members and primary care physicians. Even if the pediatrician or family practitioner performs a red reflex test (Figure 140-12) or examines the optic nerve with a direct ophthalmoscope in the first year of the child's life, he or she may miss the tumor and not suspect its presence, unless it is large enough or located in a part of the retina where it will reflect the light entering the pupil.

Retinoblastoma shows no gender predisposition. The tumor occurs with equal incidence in right and left eyes, and is present in both eyes in about 20–40% of all



**FIGURE 140-12** This was one of two presumed retinomas in this child's eye. It did not respond at all to treatment with systemic chemotherapy but was successfully eradicated with repeated applications of transpupillary 532-nm laser energy. These presumed early retinomas have not yet developed the classical retinal pigment epithelial changes initially described for these lesions. It is very difficult to make the diagnosis prior to treatment. This lesion is grayer than might be expected, but the color separation from retinoblastoma is not reliable.

cases, depending on what region of the world is being sampled. In the United States, nearly 90% of diagnoses are made before the patient is five years old (96). Of 400 consecutive patients, only 34 (8.5%) were five years of age or older at the time of diagnosis, and 26 of these had active tumor (the remaining eight had retinoma or retinocytoma) (97). All 26 children had sporadic, unilateral disease; their average age was six years. A small number of older children and adolescents, and two adults, have been reported as having active retinoblastoma, most likely arising from a previous retinoma (see previous section on retinoma).

### 140.7.2 Survival

In bilateral patients, relatively small tumors often cause visual symptoms and lead to early detection and treatment. The overall survival of retinoblastoma exceeds 95% in developed countries. In contrast, survival of ocular tumors may be as low as 50% in some Latin American and African countries because of limited access to medical care (30). Unfortunately, early detection does not always equate with better treatment outcome. Advanced intraocular retinoblastoma has been diagnosed by prenatal ultrasound imaging at 21 weeks' gestation (98), documenting a very early inception and rapid growth prenatally. When retinoblastoma was diagnosed and the extent of disease evaluated in three-month-old patients, almost one third of patients had advanced disease (99).

In a recent Mexican study, there was a direct correlation between advanced retinoblastoma at diagnosis, reduced survival and poverty (91).

### 140.7.3 Natural History of Retinoblastoma in the Patient

Patients with the *RB1* cancer syndrome and those with the non-heritable, somatic form of the disease cannot always be distinguished on the basis of tumor morphology, growth rate, or extent of disease. If undiagnosed, retinoblastoma expands locally by filling the eye (see Figure 140-1) and extending into the optic nerve, orbit, and brain. Blood-borne distant metastases appear most commonly in bone marrow, bone, and brain. The development of metastases is most commonly associated with the length of time that active retinoblastoma is present in an eye (see Section 140.7.2). The profiles of patients at high risk of metastatic retinoblastoma fall into two broad groups. The first is the older child, age three to five years, who presents with advanced unilateral intraocular retinoblastoma and has had clinically apparent symptoms for months to years without diagnosis or treatment. The second group is characterized by the child with bilateral retinoblastoma in whom multiple intraocular recurrences have been treated without success over several years in attempts to salvage vision in an only eye. Parents who are reluctant to agree to enucleation of a second eye may be increasing their child's chance of the development of metastatic disease.

### 140.7.4 Natural History of Retinoblastoma in the Eye

Early intraretinal intraocular tumors are composed of cells which are identical daughter cells of the 'founder' retinoblast. Because all of the cells that make up early small tumors are identical, they have a common growth rate and expansion of the lesion is symmetrical (see Figures 140-2 and 140-4). As chromosomal aneuploidy gradually develops, daughter clones of tumor cells with a growth advantage develop (Figure 140-13; see also Figure 140-5). Irregular and nodular tumor growths (see Figure 140-13) are the clinical manifestation of progression-of-malignancy changes (M3) and almost always involve the largest tumors first.

In addition to being composed of identical tumor cells, early retinal tumors share two other characteristics: (i) the tumor cells are adherent to each other, and (ii) a blood supply is required for growth beyond a relatively small size. Anchorage independence eventually develops as this malignancy progresses. The clinical corollary of achieving anchorage independence is the breakup of tumor into small clumps of cells that seed the vitreous or subretinal space (Figure 140-14). The clinical observation that seeding takes place from a specific region of the growing tumor, most often an area that has shown rapid growth, suggests that this is a clonal process involving additional genetic alterations.





**FIGURE 140-13** This large tumor shows two sites where growth advantage mutations may account for rapid focal expansion of the tumor. Additional M3 events have occurred, bestowing likely gain-of-anchorage-independence mutations. The tumor cells from the new clone at site 1, indicated by the arrow, are about to be shed into the vitreous as local vitreous seeding. At site 2, the cells are beginning to spread beneath the retina into the subretinal space. This eye would be classified as having group C disease because of the localized seeding.



**FIGURE 140-14** This small tumor has a 'nipple' on the tip from which tumor cells will be shed into the vitreous as a presumed result of acquired anchorage independence M3 mutations. This would be classified as a group C eye because of the local vitreous seeding.

Once vitreous seeding has occurred, the free-floating clumps of tumor cells expand without an intrinsic blood supply (Figure 140-15). Advanced intraocular retinoblastoma is characterized by these very large avascular masses within both the vitreous and the subretinal space. Invasion into the optic nerve and into the choroid may subsequently develop and increase the risk of metastatic disease.



**FIGURE 140-15** Vitreous tumor seeds have spread diffusely into the vitreous. At this stage, salvage of the eye is questionable. The fine vitreous seeding is sequestered from systemically administered chemotherapy and may begin to grow when the chemotherapy is discontinued. Some have already settled on the retinal surface, where they will establish new tumor foci.

### 140.7.5 Classification of Retinoblastoma

Classification systems vary significantly from cancer to cancer. In general, staging systems look at the patient as a whole and attempt to assess the extent of disease involvement in the patient and to determine the likelihood of patient survival. Grouping systems evaluate the involvement of the organ of origin in an effort to assess the best treatment strategy for the organ's survival. 'Staging' uses the entire medical record as a source of information. In contrast, 'grouping' in retinoblastoma management is based on the findings in the eye, usually determined during an examination under anesthesia (EUA) of the eye.

Most patients with childhood solid tumors other than retinoblastoma are staged based on the extent of disease in the patient following evaluation of an incisional or excisional biopsy. Retinoblastoma is unique among the solid childhood tumors, in that a tissue diagnosis has never been a requirement before treatment initiation. Since retinoblastoma is frequently confined to the eye, biopsy is contraindicated and results in upstaging of the patient.

The Reese-Ellsworth grouping system (Table 140-2), which focuses on the likelihood of salvaging the eye, has been used since the early days of megavoltage radiation in the 1960s (100). In 2005, there was international collaboration to update the classification of eyes with retinoblastoma. A new grouping system based on the natural history of the intraocular disease rather than response to a specific treatment modality was proposed to replace the Reese-Ellsworth system (Table 140-3) (37). Chantada and colleagues presented a staging system similar to those that currently exist for other solid childhood malignancies as a tool to assist pediatric oncologists in



**TABLE 140-2** Reese-Ellsworth Group Classification of Eyes with Intraocular Retinoblastoma

Group I – Very Favorable	
Ia	Solitary tumor 4 DD at or behind the equator
Ib	Multiple tumors, none 4 DD, all at or behind the equator
Group II – Favorable	
IIa	Solitary tumor, 4–10 DD, all at or behind the equator
IIb	Multiple tumors, 4–10 DD, behind the equator
Group III – Doubtful	
IIIa	Any lesion anterior to the equator
IIIb	Solitary tumors larger than 10 DD behind the equator
Group IV – Unfavorable	
IVa	Multiple tumors, some larger than 10 DD
IVb	Any lesion extending anteriorly to the ora serrata
Group V – Very Unfavorable	
Va	Massive tumors involving over half the retina
Vb	Vitreous seeding

DD, disk diameter.

From Reese, A.; Ellsworth, R. The Evaluation and Current Concept of Retinoblastoma Therapy. *Trans. Am. Acad. Ophthalmol. Otolaryngol.* 1963, 67, 164–172.

treating the disease (Table 140-4) (29). The development of the new staging and grouping systems were coordinated so that they could be used together to completely describe the disease in any patient.

Murphree observed that the most significant feature of the intraocular tumor that impacted the likelihood of salvaging the eye was the presence or absence of intraocular tumor seeding (37,101) (compare Figures 140-4 and 140-5 with Figures 140-7 and 140-8). He divided all intraocular disease based on whether seeding had occurred. Eyes that had not developed seeding eventually became groups A (see Figure 140-4) and B (Figure 140-16; see also Figure 140-5) in the new international classification system. Eyes that had previously developed seeding became groups C (see Figure 140-13) and D (see Figures 140-7,140-8 and 140-15). Groups were separated to give ophthalmologists reliable predictors concerning final visual acuity outcomes and to allow consensus enrollment of retinoblastoma patients into multicenter clinical trials. Group A eyes are limited to those eyes containing small tumors (less than 3 mm in greatest diameter) that are also located at a sufficient distance from the fovea and optic nerve that local treatment of these lesions with laser is not likely to cause permanent visual impairment (Figure 140-4). Group B (see

Figures 140-2,140-5,140-12 and 140-16) is assigned to all other eyes with no seeding regardless of the location or size of the tumor.

Group C eyes are separated from group D eyes by the location and extent of the seeding. Group C eyes have minimal localized seeding (see Figures 140-13 and 140-14), whereas group D eyes have massive, diffuse seeding (see Figures 140-7,140-8 and 140-15). Group E includes those eyes that present at diagnosis with such extensive intraocular disease that their function and/or structure have been destroyed.

There is nothing in the classification systems for staging or grouping retinoblastoma that correlates with heritability. This is true whether using the older Reese-Ellsworth system or the newly developed international classification for grouping and staging.

### 140.7.6 Diagnosis and Treatment of Intraocular Retinoblastoma

The treating pediatric ophthalmologist must first assess whether or not the suspected signs present in the child referred because of the possibility of retinoblastoma are sufficiently strong to warrant an EUA. That first assessment is made in the office with a brief retinal exam and an ultrasound examination of the eye. If an intraocular mass that contains calcium is present, then a diagnosis of retinoblastoma is highly likely. A computed tomography scan can then be avoided (because of the unnecessary radiation) and the child can have a high-resolution MRI scan of the orbit and brain with thin sections, gadolinium enhancement, and fat suppression to exclude extraocular extension of disease or a concomitant PNET (see Figure 140-9).

At the staging EUA, a thorough eye exam is performed with assessment of intraocular pressure, a portable slit lamp examination, a retinal exam with scleral depression, and a retinal drawing. Digital images of the retina and ultrasound studies are also obtained. Grouping of each eye is performed according to the new International Group Classification of Retinoblastoma at most centers.

**140.7.6.1 Unilateral Retinoblastoma.** Primary treatment generally consists of primary enucleation (see Figure 140-1) for group E eyes. This is always recommended if the disease is unilateral. In most centers, it is recommended to have primary enucleation in unilateral eyes with very extensive intraocular seeding (group D). Systemic chemoreduction is difficult to justify for a young child when one eye is completely normal and the other has advanced disease (groups D and E).

**140.7.6.2 Bilateral Retinoblastoma.** For eyes in which salvage of some useful visual acuity is likely (bilateral groups A through D), systemic chemoreduction with local consolidation is commonly used. The Children's Oncology Group, a cooperative clinical trials group of the NCI, enrolls patients with Group B

**TABLE 140-3 International Group Classification of Eyes with Intraocular Retinoblastoma**

Group A – Very Low Risk	Discrete retinal tumors, none larger than 3 mm in diameter. All tumors located at least 3 mm from fovea and 1.5 mm from optic nerve. No vitreous or subretinal seeding. No retinal detachment allowed.
Group B – Low Risk	Discrete retinal tumors of any size or location. No vitreous or subretinal seeding. 5-mm-wide cuff of subretinal fluid around base of tumor allowed.
Group C – Moderate Risk	Discrete retinal tumors of any size or location. Any vitreous or subretinal seeding must be local, fine, and limited. Retinal detachment, current or past, 1 quadrant.
Group D – High Risk	Massive and/or diffuse endophytic or exophytic tumors. Massive and/or diffuse vitreous and/or subretinal seeding or masses. Retinal detachment, current or past, 1 quadrant.
Group E – Very High Risk (One or More)	Irreversible secondary glaucoma. Massive intraocular hemorrhage. Aseptic orbital cellulites. Tumor anterior to anterior vitreous face. Tumor touching lens. Diffuse infiltrating retinoblastoma. Phthisis or prephthisis.

From Murphree, A. L. The Case for a New Group Classification of Intraocular Retinoblastoma. *Ophthalmol. Clin. N. Am.* 2005, 18, 41–53.

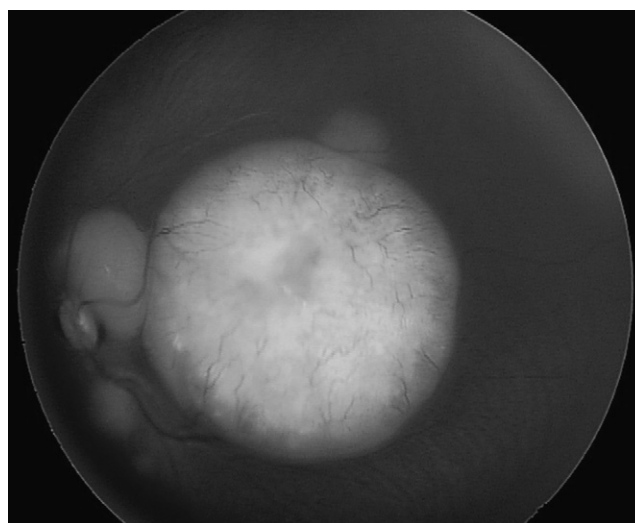
**TABLE 140-4 International Staging Classification of Patients with Retinoblastoma**

Stage 0	Patients treated conservatively without enucleation Eyes subject to group classification and systemic or local treatment
Stage I	Tumor completely resected by enucleation
Stage II	Microscopic residual tumor following enucleation
Stage III	Regional extension of disease
IIIa	Overt orbital disease
IIIb	Preauricular or cervical lymph node extension
Stage IV	Metastatic disease
IVa	Hematogenous metastasis
IVa1	Single lesion
IVa2	Multiple lesions
IVb	CNS extension
IVb1	CNS mass
IVb2	Leptomeningeal disease

CNS, central nervous system. Data from Chantada and colleagues (2005), Murphree (2005), and Reese and Ellsworth (1963).

through D disease into individual trials. In most eyes, there is a reduction in the volume of the tumor by 50–70% after the first cycle of systemic chemoreduction. We schedule the child for an EUA on day 0 of each cycle and perform focal laser consolidation prior to administering three agent chemotherapy (carboplatin, vincristine and etoposide).

The most commonly used duration of systemic chemotherapy is for six cycles over five to six months. Each cycle consists of administration of chemotherapy in an outpatient hospital setting and then allowing three to four weeks for the bone marrow to recover.



**FIGURE 140-16** In this group B eye, there are five separately arising retinoblastomas ranging from a barely detectable pinhead-sized lesion along the lower left edge of the image to the large central lesion located in the fovea. All are round except where structures such as the optic nerve or other tumor masses have distorted that shape. The posterior pole of the eye, where all these tumors are located, has the highest concentration of retinoblasts.

We treat each retinoblastoma lesion with laser consolidation at every EUA. Inadequate local consolidation can be associated with an increased risk of recurrence of treated lesions. The ideal treatment outcome is a chorioretinal scar that either is flat or contains a mass of calcified tumor debris (regression types 1 and IV). We make every effort to reduce the homogeneous type II regression pattern to a calcified scar with repeated laser application.

### 140.7.7 Cost Issues Associated With Treatment and *Rb1* Testing

The costs associated with treating a child with bilateral group D intraocular disease with systemic chemotherapy and laser consolidation in American pediatric specialty hospitals frequently often exceeds \$200,000. Patients traveling from other countries for treatment and who do not have American insurance are required by some American hospitals to deposit as much as \$150,000 before the hospital will see the child. Costs of this magnitude mean that, in developing countries and in the developed world, governmental health care policy should focus on prevention. Genetic counseling and early diagnosis are the key ingredients in such an effort.

In many developing countries, even those with access to chemotherapy for the treatment of children, parents are sometimes required to purchase the chemotherapeutic drugs and bring them to the hospital. Our current approach to primary treatment may not be a viable alternative for the majority of the world's retinoblastoma cases.

### 140.7.8 Regressed Retinoblastoma

The term regressed retinoblastoma has been misapplied to the lesion more correctly labeled retinoma or retinocytoma. Rarely, spontaneous massive necrosis of a very large intraocular tumor is associated with an aseptic orbital cellulitis and eventual shrinkage and disorganization of a blind eye (phthisis).

## 140.8 CLINICAL GENETICS AND GENETIC COUNSELING

The presence of a germline mutation in *RB1* has far-reaching implications for the retinoblastoma patient and his or her family. The discovery of a heritable mutation in *RB1* can impact the optimal treatment of the tumor, the risk of non-ocular cancer, and long-term survival as well as the occurrence risk of the *RB1* cancer syndrome in offspring and in other family members. Free testing for *RB1* mutations is currently available through eyeGENE, a program funded by the National Eye Institute. The American Society of Clinical Oncologists (ASCO) recommends genetic counseling for families because of the possibility of a heritable predisposition to retinoblastoma and other cancers. It is important to inform at-risk individuals about the availability of testing for constitutional mutations in *RB1* (ASCO policy statement).

### 140.8.1 Genetic Counseling

Genetic counseling is as important for the family of a child with retinoblastoma as it is for the adult survivor of this childhood cancer. Both families with clearly

hereditary disease and those with a single affected individual can benefit from genetic counseling.

When the geneticist participates in the early diagnosis and treatment stages of retinoblastoma, genetic testing and counseling can be facilitated. Because of the possibility of a chromosome 13 deletion, the child with retinoblastoma requires an assessment of his or her developmental progress as well as an examination for dysmorphic features. Parents of a child with retinoblastoma often have subsequent pregnancies that require genetic counseling and prenatal diagnosis.

Counseling the adult survivors of retinoblastoma can be challenging as well. Counseling becomes less straightforward in those circumstances in which DNA testing on a blood sample does not reveal an *RB1* mutation. A non-diagnostic test must be carefully explained to the patient since an undetectable, possibly mosaic mutation could yield an apparently normal result. When the adult retinoblastoma survivor has a blind spouse, the genetics professional should be prepared to address the risks of other hereditary causes of blindness. When both the proband and the spouse are blind, one with retinoblastoma and the other with another heritable cause of visual loss (e.g. retinitis pigmentosa), the counseling and testing process can become quite complex.

The primary issue that is of concern to the adult survivor of heritable retinoblastoma who seeks genetic counseling is the chance of blindness in his or her future affected children. Enucleation of one eye alone does not create significant visual impairment or legal blindness unless there is a tumor affecting the central retina in the fellow eye. In our experience, when newborns at risk of retinoblastoma are followed from birth with dilation of the pupil and scleral depression by an ophthalmologist looking for retinoblastoma, the likelihood of bilateral blindness is less than 10%. This information can be very useful in the setting of genetic counseling and is often reassuring to affected families.

It is also our observation that adult survivors of hereditary retinoblastoma who have not experienced a non-ocular cancer will minimize the risk of those cancers. The geneticist needs to raise this issue as well, so that the family understands the range of risks that any affected patient with the *RB1* cancer syndrome will face.

In order to counsel the family appropriately, the genetic counselor needs to assess the family's risk of a heritable mutation in *RB1*. A detailed three-generation pedigree should include the father's age, miscarriages, infant deaths, infertility, the use of assisted reproductive technology, other relatives with developmental delay or birth defects, and the history of blindness or other cancers and their age of onset in all grandparents and other first- and second-degree relatives.

Medical records and pathology reports of the tumor should be carefully reviewed to determine the nature and extent of the retinoblastoma—bilateral versus unilateral, multifocal versus unifocal. Even when this data

is complete and accurate, it does not provide a certain diagnosis of heritability.

Genetic counseling is particularly helpful when a baby is diagnosed in early infancy. For example, the counseling session may predate the diagnosis of a second focus of retinoblastoma in the same or the other eye. The risk of the development of multifocal disease is highest (16%) for children whose presenting tumor is diagnosed before one year of age, and lower (2.2%) when the diagnosis is made after age one (53,102). The mean time to the diagnosis of a new focus of retinoblastoma is 0.74 years. Therefore, when counseling a family with a child with sporadic unilateral retinoblastoma diagnosed in infancy, the counselor should qualify the risk assessment and encourage a follow-up visit for genetic risk reassessment at age two to three years.

Ophthalmology examinations on all first-degree relatives should be completed prior to genetic counseling. The presence of a retinoma in an asymptomatic parent has immediate implications for the patient and for the family. At the time of diagnosis, the family may be struggling to cope with this devastating news and overwhelmed with difficult treatment choices. Nevertheless, waiting even a few months to complete these exams can mean that another affected child might be conceived before the family is aware of the genetic risks.

Finally, the counselor needs to be aware of the proband's age, general health, and developmental status. Imaging studies of the brain are required to exclude extraocular extension of retinoblastoma or a concomitant PNET. The developmental status of the infant is an important clue to his or her overall well-being. Delayed motor and social milestones do not necessarily signal a problem in blind children. Normal children with visual impairment attain these skills at a later age than sighted children. Delayed speech, however, can represent a significant finding. If there is any concern about neurodevelopmental status, mental retardation, or dysmorphic features, a clinical genetic evaluation should be undertaken. The results of chromosome analysis and fluorescence in situ hybridization (FISH) studies for 13q14 should also be reviewed (26).

Although there is no known parent-of-origin effect in retinoblastoma, new germline mutations in *RB1* preferentially occur on the paternally inherited chromosome 13. Interestingly, patients with germline mosaicism also have more mutations on the paternally derived chromosome 13, suggesting that the high risk of mutation persists in the paternal genome even after fertilization. Advanced paternal age is a known risk factor in retinoblastoma.

## 140.8.2 Cytogenetics

Since 1976, when Franke and Kung proposed a relationship between bilateral retinoblastoma and 13q14 deletion (103), high-resolution cytogenetic analysis has become a

standard part of the evaluation of children with retinoblastoma. Deletions of varying sizes and other rearrangements of chromosome 13q14 are commonly identified. In a group of 203 patients with retinoblastoma, deletions were seen in approximately 5% of unilateral and 7.5% of bilateral retinoblastoma patients (28). As small deletions can be missed with conventional cytogenetic banding, FISH studies for the *RB1* locus are now recommended to augment the cytogenetic evaluation of the child with bilateral or unilateral retinoblastoma. Large numbers of cells should be examined to detect mosaic deletions, which is easily accomplished with FISH studies.

When a deletion is mosaic, which occurs in 1% of cases, the risk of retinoblastoma is not necessarily reduced. Only 7% of patients with 13q14 deletion mosaicism did not develop retinoblastoma in Kivela and colleagues' series (104). In the same report, the diagnosis of retinoblastoma was delayed because a mosaic 13q deletion was erroneously assumed to construe a lower risk of retinoblastoma. A recent study from a major retinoblastoma center in India reported that FISH studies have demonstrated constitutional deletion of chromosome 13q14 in almost 15% of unilateral sporadic retinoblastoma patients (38) without any clinical evidence that they might be *RB1* mutation carriers.

There are several reports of retinoblastoma in children with X; 13 translocations. Interestingly, the breakpoint need not involve 13q14. Even though the breakpoint involves another area on 13q, the intact *RB1* was disabled when inactivation from the X chromosome spread to the adjacent 13q14 region (105).

**140.8.2.1 Chromosome 13q Deletions.** Children with constitutional 13q14 deletions may have a particular recognizable pattern of facial dysmorphism, additional anomalies, and disabilities ranging from severe mental retardation to relatively mild speech delay. Typical facial features (26,106,107) include a broad high forehead, depressed and broad nasal bridge, bulbous nasal tip, anteverted earlobes, and a long prominent philtrum. Difficult intubation due to macroglossia has been reported in children with retinoblastoma due to 13q deletions (108). Although deletions are less common in familial retinoblastoma, we have seen an affected mother with borderline intelligence and a chromosome 13q deletion, whose affected child had similar learning problems.

**140.8.2.2 Retinoblastoma and Congenital Anomalies.** FISH studies should not be limited to children with facial dysmorphism or developmental delay. Some patients with small 13q deletions may have no discernible dysmorphism or developmental delay. Conversely, it is not safe to assume that patients with retinoblastoma who are developmentally delayed must have a chromosome 13 deletion. Aside from genetic counseling for retinoblastoma, some retinoblastoma patients will deserve a complete genetic assessment for other indications. We have several retinoblastoma patients who also have apparently unrelated genetic syndromes; one girl with



sporadic retinoblastoma, developmental delay, and a seizure disorder has fragile X syndrome. In our clinic, there are several retinoblastoma patients with isolated congenital anomalies (clubfoot, dysplastic ear) and others with autism or unexplained mental retardation. As yet, no relationship between retinoblastoma and other anomalies has been established outside of the deletion 13q syndrome.

### 140.8.3 *RB1* Analysis

*RB1* analysis can determine which retinoblastoma patients have heritable disease and can identify those individuals with an increased risk of non-ocular tumors. Functional domains and conserved sequences in *RB1* are hot spots for mutations. Many *RB1* mutations disrupt gene expression, and no functional protein product is produced. Because missense mutations allow formation of an *RB1* protein that may have some functional utility, a milder phenotype with a lower tumor-to-eye ratio and later onset can be seen in affected families. Missense mutations have been reported in several low-penetrance pedigrees.

Although gene sequencing is the mainstay of molecular diagnosis in retinoblastoma, this technique alone will detect only about 70% of mutations. Large deletions and gene rearrangements will not necessarily be detected by gene sequencing alone. Quantitative multiplex polymerase chain reaction (PCR) and MLPA can increase the detection rate 15% or more. Gene sequencing cannot reliably detect mosaicism of less than 20%. Epigenetic changes are also important, since hypermethylation of the promoter region disables *RB1* in approximately 10–11% of tumors from patients with unilateral sporadic disease. When a comprehensive testing protocol is carried out, the detection rate for *RB1* mutations exceeds 95%.

### 140.8.4 Testing Issues

Although it is rarely available, DNA from fresh frozen tumor can be useful in a genetic testing strategy. If a mutation is detectable in the tumor, a negative blood result is more reliable, although, mosaicism cannot be excluded.

**148.8.4.1 Bilateral Retinoblastoma.** When the patient has bilateral retinoblastoma, a mutation in *RB1* is a virtual certainty. DNA testing can be carried out on blood alone because, even if a mutation is not detected, the patient's status is unchanged. All patients with bilateral retinoblastoma are presumed to have a germline mutation, albeit sometimes mosaic or sometimes undetectable.

**140.8.4.2 Sporadic Unilateral Retinoblastoma with Fresh Tumor.** When the proband has sporadic unilateral retinoblastoma, a fresh frozen sample of the tumor tissue as well as a blood sample may be sent for analysis. In unilateral cases, the tumor tissue can be used first to identify both *RB1* mutations before the blood studies are

initiated. If one or both of the mutations cannot be identified in the tumor, then normal studies in the blood will not be useful for genetic counseling purposes, since the mutation may be present but not detectable.

**140.8.4.3 Sporadic Unilateral Retinoblastoma without Fresh Tumor.** When the proband has sporadic unilateral retinoblastoma and a fresh frozen tumor specimen is not available, it may be useful to test blood alone. Abnormal results in blood are definitive, allowing a diagnosis of heritable retinoblastoma. A non-diagnostic test for *RB1* gene mutation does not exclude the possibility of heritable disease. Patients need careful counseling to appreciate that they still have a risk of an undetected *RB1* mutation whenever there are normal results in blood, whether there are results for tumor tissue or not.

**140.8.4.4 Possible Uninformative or Unexpected Results.** Patients should be counseled about the possibility of uninformative results prior to analysis. The higher the detection rate, the less likely it is that patients will have to face the issue of uninformative results, which makes the selection of a testing laboratory of critical importance. Occasionally, completely unexpected results are obtained from *RB1* testing. In one unilateral sporadic patient, a normal unaffected parent was found to share a small 13q14 deletion with the affected child. This information was very difficult for the family to accept. Prior discussion of this possibility with the family before testing could have mitigated their distress.

### 140.8.5 Mosaicism

Mosaicism is common in retinoblastoma, and it is a factor that, because it can never be ruled out completely, must be thoroughly discussed before genetic testing. Mosaicism occurs in at least 10% of individuals with germline mutations in *RB1*, affecting both new unilateral and bilateral cases. Mosaicism should be suspected when a parent has unilateral retinoblastoma and his or her child has bilateral retinoblastoma.

Gene sequencing can miss low-level mosaicism. Mosaicism probably accounts for most of the non-detectable mutations in the blood of individuals with bilateral retinoblastoma. When the mutation can be identified in affected family members in subsequent generations, the founding affected member of the family may be tested with PCR and an allele-specific oligonucleotide probe, a more sensitive technique for the detection of mosaicism. Lohmann's group presented unpublished data at the XII International Retinoblastoma Symposium in 2005 indicating that, of 22 multiplex families in which the transmitting parent had unilateral retinoblastoma, mosaicism was detected in 4 of 22 affected parents.

### 140.8.6 Testing the Parents

When a child has a detectable *RB1* mutation, first-degree relatives should also be tested for the same mutation.

Parents may be unprepared for this step unless it is addressed early in the counseling process. Even with this preparation, it is a turning point that may be difficult for some parents who perceive, for the first time, that they are at risk, not just their children. Familial *RB1* mutations are identified in healthy unaffected parents. This may be most understandable with missense mutations in which the *RB1* gene remains capable of producing a gene product, but it has also been observed with other types of mutations. Some of these parents have no ocular findings. Their non-ocular cancer risk is difficult to assess, since no long-term studies of this group are available.

It must also be recognized that when *RB1* testing results are normal in a parent, germline mosaicism remains a possibility. For this reason, normal results in the parents of an affected child with an *RB1* mutation should be interpreted with caution. Prenatal diagnosis for the *RB1* mutation identified in the proband should still be offered for future pregnancies since germline mosaicism cannot be excluded in the parent.

### 140.8.7 Linkage Analysis

Linkage analysis of retinoblastoma families may be helpful in establishing an at-risk haplotype when direct gene analysis does not reveal the mutation in *RB1*. As an indirect test, linkage analysis is less reliable than direct mutation analysis, and it should never be used as the preferred method of testing. At first glance, linkage analysis may seem to be the more attractive option, because it is less costly and less time-consuming than complete gene analysis. Another advantage specific to retinoblastoma is the availability of intragenic probes within the *RB1* locus that substantially reduce the risk of undetected recombination events. However, there is a significant source of error that makes linkage analysis less attractive for most retinoblastoma families. Mosaicism for an *RB1* mutation is common in the founding member of a retinoblastoma family. This can confound the results of linkage analysis, creating the impression that the offspring of a mosaic parent, who has inherited the at-risk haplotype, are at risk of retinoblastoma when in fact that is not the case. Such a false-positive result could be devastating if linkage analysis were used for prenatal diagnosis purposes. To exclude the possibility of false positives due to undetected mosaicism, only very large pedigrees should be considered for linkage analysis. Ideally, the affected founder of a retinoblastoma family and any of his or her unaffected offspring should be excluded from the analysis.

### 140.8.8 Segregation Ratios in Offspring of Adults with Sporadic Bilateral Retinoblastoma

Mendelian segregation ratios dictate that half of the offspring of known *RB1* cancer syndrome patients will inherit the genetic defect in *RB1* from their affected

parent. About 90% of these individuals will develop retinoblastoma in one or both eyes. This is true when the parent's disease was due to an inherited gene defect passed on from an affected grandparent. Surprisingly, however, this is not the case for affected males with a new mutation. Among the offspring of males with bilateral sporadic disease, there is a sex ratio distortion that favors male offspring as well as a transmission ratio distortion that favors affected males (109).

Among all new cases of bilateral disease, there is also an excess of affected males. These observations are difficult to explain without invoking another underlying defect that affects the viability of female and male fetuses. Such an explanation was proposed by Naumova and Sapienza, who speculated that, in a certain percent of retinoblastoma (and by extension probably all) families, co-inheritance of a maternally inherited X-linked defect in imprinting affects a male's ability to erase the maternal imprint in the chromosomes he passes on (109). Thus female offspring from these males with two maternally imprinted X chromosomes would be nonviable. The observed excess of male offspring would in fact be due to a paucity of female offspring. This could explain the sex ratio distortion seen in many families, including those with retinoblastoma.

Since in this scenario most of the surviving fetuses would be male, it is also pertinent to this theory that the retinoblastoma gene is located on an imprinted chromosome 13. A man would transmit his maternally inherited chromosome 13 (which would not usually carry the new mutation for retinoblastoma) without erasing that maternal imprint. The resulting fetus, with two copies of chromosome 13, bearing a maternal imprint and unaffected by retinoblastoma, would not survive. A fetus would be viable when it inherited its father's paternally imprinted chromosome 13, the paternally derived chromosome 13 that usually contains the initial *RB1* mutation in a sporadic case. This viable fetus would also therefore be affected with retinoblastoma. In this situation, the retinoblastoma phenotype would be a marker for the grandpaternally inherited and properly imprinted chromosome 13. This explains the transmission ratio distortion seen in the offspring of males with sporadic bilateral retinoblastoma.

To date, this elegant theory, although persuasive, has not found a practical application in retinoblastoma genetic counseling. It is, however, useful to know that the typical Mendelian segregation ratios of affected and unaffected offspring and affected males and females may not apply to all families of males with sporadic bilateral retinoblastoma.

### 140.8.9 Clinical DNA Testing: Commercial Laboratories Offering DNA Testing

All *RB1* testing must be carefully coordinated so tumor and blood DNA specimens from the patient are collected

appropriately (110). The Internet is a helpful resource for those seeking genetic testing centers for retinoblastoma. GeneTests ([www.genetests.org](http://www.genetests.org)) provides updated information about many disorders, including retinoblastoma. A comprehensive review of pertinent clinical testing and counseling issues is available, as is a database of commercial and research laboratories that offer genetic testing for *RB1*. Another site, eyeGENE, provides free genetic testing for all inherited eye diseases including retinoblastoma. The genetics clinician or counselor must communicate directly with the laboratory before sending any samples. The costs, sample preparation, and detection rate vary with each laboratory.

## 140.9 APPROACHES TO PRENATAL ASSESSMENT AND DIAGNOSIS

### 140.9.1 Pre-implantation Genetic Diagnosis

The ability to provide testing of at-risk pregnancies has been available since the early 1990s (111,112). These include heritable cancers such as adenomatous polyposis coli, BRCA1, Li-Fraumeni syndrome, and von Hippel-Lindau syndrome, as well as Fanconi anemia and Wiskott-Aldrich syndrome (113). A method for detection of a known mutation in PGD of retinoblastoma was reported in 2000 (114). It was first reported as being used in the prenatal diagnosis of pregnancies at 50% risk of retinoblastoma in 2003 by Girardet and colleagues, who evaluated the amplification efficiency and allele dropout rates for two highly polymorphic microsatellite markers located within and close to *RB1*, respectively (115). Xu and colleagues subsequently reported the first liveborn after PGD for retinoblastoma in 2004 (116). This means of screening embryos for retinoblastoma was approved in Great Britain in 2005 (117).

PGD requires prior knowledge of the mutation in *RB1*. In in vitro fertilization (IVF), when the embryo is at the 8- to 10-cell stage, a blastomere, one cell from the developing embryo, is removed for genetic testing while the remaining mass of cells is returned to incubation. Those embryos not containing the mutation are deemed unaffected, and one or two, optimally, are implanted in the mother's uterus. Others that are unaffected may be frozen for future use. Due to the inaccuracies inherent in PGD, most protocols require that an amniocentesis be performed for prenatal diagnosis during the pregnancy to confirm the molecular analysis results and for cytogenetic analysis.

### 140.9.2 Concern Regarding Assisted Reproductive Technologies

Evidence is accumulating that assisted reproductive therapies (ART) may be associated with increased epigenetic disorders in babies conceived using these procedures. This has been reported in ART associated with an increased

incidence of Beckwith-Wiedemann syndrome and Angelman syndrome, for example. Epigenetic reprogramming is active during gametogenesis and at preimplantation and involves DNA methylation, imprinting, RNA silencing, covalent modifications of histones, and remodeling by other chromatin-associated complexes (118). Moll and colleagues report five patients diagnosed with retinoblastoma in the Netherlands between November 2000 and February 2002, all after IVF (119). The calculated relative risk of retinoblastoma is 7.2 for an IVF rate of 1% (95% CI, 2.4–17.0) or 4.9 for an IVF rate of 1.5% (95% CI, 1.6–11.3). Ben Ezra suggests that these five cases could represent clustering of cases reported by interested observers and one should use caution in assuming that IVF was the cause of the retinoblastoma (27).

### 140.9.3 Prenatal Genetic Diagnosis without PGD

Couples may elect to have prenatal genetic diagnosis without using PGD; this may be chosen if finances do not permit PGD or the family wishes to know the diagnosis but does not wish to intercede in the pregnancy. When the mutation is known, prenatal diagnosis can be performed via chorionic villus sampling or amniocentesis.

### 140.9.4 Fetal Ultrasound and Magnetic Resonance Imaging

Improved fetal imaging, fetal ultrasound, and fetal MRI add to the tests available for the diagnosis of prenatal retinoblastoma. Fetal ultrasound and fetal MRI are complementary technologies in the evaluation of fetal abnormalities. Ultrasound is widely available, relatively inexpensive, and safe, and it allows for real-time imaging (120). Ultrasound is limited, however, in women who are obese and in women whose pregnancies have reduced amniotic fluid. In addition, in later gestation it is difficult to evaluate intracranial anatomy through the more ossified fetal skull. Fetal position may also limit utility. The use of MRI in the fetus provides more standardized results and a larger field of view can be seen. With the advent of fast imaging techniques, MRI has been used to evaluate fetal anomalies since the mid-1990s (21). However, MRI has its own limitations including expense, claustrophobia on the part of the mother, and less spatial resolution. Most commonly, MRI has been used to evaluate central nervous system abnormalities, which in some centers have been found to influence or change management and counseling in up to 50% of cases (121). Some images can be seen in Figures 140-17–140-20.

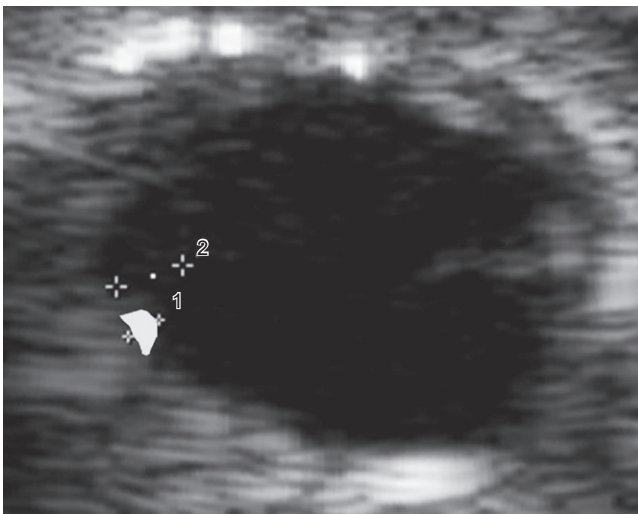
### 140.9.5 Factors Associated With Increased Risk of Sporadic Retinoblastoma

In studies of parental age and parity, it has been shown that there is a correlation between sporadic hereditary





**FIGURE 140-17** This child has an ocular prosthesis following enucleation for advanced unilateral retinoblastoma. The surgical removal of an eye for the definitive treatment of retinoblastoma can have very little impact on the cosmetically 'normal' appearance of a child. Enucleation without radiotherapy does not result in facial and bony deformity. Meticulous surgery and the use of as large an orbital implant as possible makes this treatment alternative a very attractive one, especially when advanced unilateral disease is present.



**FIGURE 140-18** This image is a fetal transabdominal ultrasound of the orbit at 20 weeks' gestation. Gray drawing designated by arrow represents what a 1-mm in height mass may look like. The dashed line represents how much a 2-mm lesion would protrude into the vitreous. (Image courtesy of David Miller, M.D., and Lisa Paquette, M.D.).

retinoblastoma and parental age. I Sivakumaran and colleagues demonstrated familial retinoblastoma was significantly associated with early para (52). Mean paternal age of sporadic bilateral cases was significantly higher than that of sporadic unilateral cases. There was no correlation with mean maternal age, so the conclusion was that advanced paternal age is associated with sporadic bilateral retinoblastoma. Dockerty and colleagues performed a case-control study of childhood cancers with regard to parental age, parity, and social class (122). Their results demonstrated that the odds ratio (OR) for retinoblastoma resulting from apparent new germ cell mutations among children of fathers age 45 or older was 3.0 (95% CI, 0.2–41.7).

As discussed earlier in this chapter, Orjuela and colleagues examined fruit and vegetable intake during pregnancy and risk of development of sporadic retinoblastoma (89). Using dietary recall questionnaires, these authors studied mothers of 101 children with retinoblastoma and 172 control mothers. The risk of having a child with retinoblastoma was increased for mothers consuming fewer than two daily servings of vegetables (OR 3.4; 95% CI, 2.0–6.0) or with a low intake of folate (OR 3.9; 95% CI, 2.1–7.3) or lutein/zeaxanthin (OR 2.6; 95% CI, 1.5–4.6) derived from fruits and vegetables.

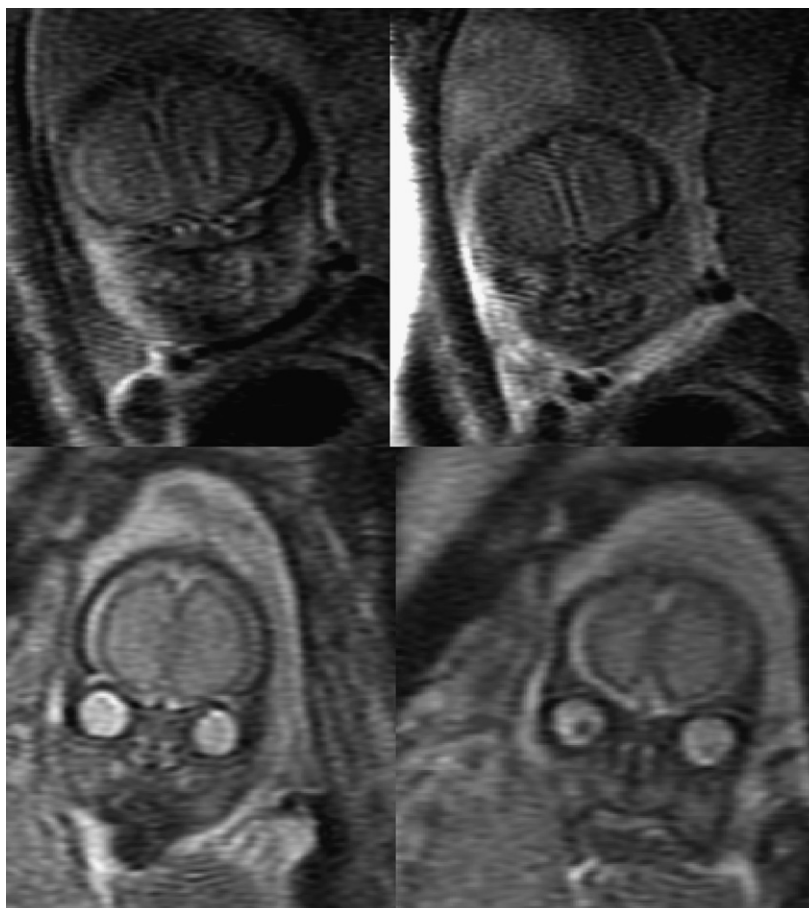
## 140.10 CONCLUSIONS

Retinoblastoma has played a pivotal in our understanding of the molecular genetics associated with tumorigenesis. This has led to improved screening and treatment options for patients and families. A thorough understanding of the genetics associated with retinoblastoma is critical for any practitioner who manages this disease. Today a genetics assessment is the standard of care for all families with retinoblastoma. Patients, parents, siblings and offspring benefit from this type of counseling. In the future, improved screening and directed treatments will translate into further reduction in patient and ocular morbidity.

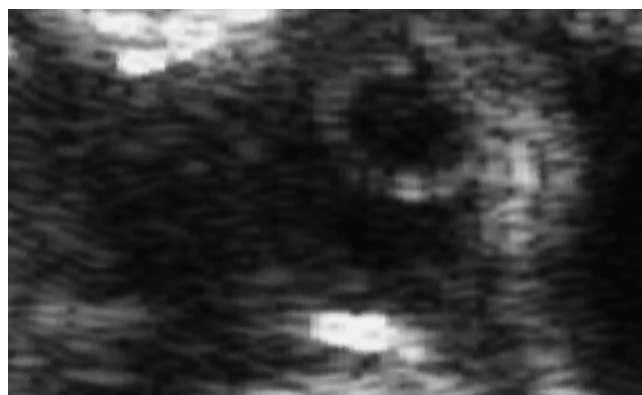
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17. The Molecular Biology of Cancer
21. Genetic Counseling and Clinical Risk Assessment
23. Diagnostic Molecular Genetics
26. Techniques for Prenatal Diagnosis
27. Neonatal Screening
45. Deletions and Other Structural Abnormalities of the Autosomes





**FIGURE 140-19** Fetal MRI. Top row, severe microphthalmia demonstrating absence of the fetal eye. Bottom row, Normal. The vitreous of the fetal eye appears bright in these sequences. The dark lucency in the lower right image represents the fetal lens. (Images obtained due to the courtesy of Ashok Panigrahy, M.D., and Lisa Paquette, M.D.).



**FIGURE 140-20** Fetal transabdominal ultrasound at 20 weeks' gestation. Circular shape indicates normal lens. (Image courtesy of David Miller, M.D., and Lisa Paquette, M.D.).

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# CHAPTER

# 141

## Anophthalmia, Microphthalmia, and Uveal Coloboma

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### GLOSSARY

- Anophthalmia** – absence of ocular tissue, most often on clinical examination
- Coloboma** – a general term implying a congenital loss of tissue in or around the eye
- Microphthalmia** – small eye; technically defined as have an axial length less than two standard deviations from the age-adjusted mean
- Optic cup** – a two-layered bilateral concave neuroepithelial structure that develops from the optic vesicle as it approaches the surface ectoderm
- Optic fissure** – a transient opening on the ventral surface of the optic cup that must close during the 5th to 6th week of human gestation in order for the eye to form a spherical structure
- Optic vesicle** – a bilateral evagination of the rostral neural tube that is the first major morphologic change in eye development
- Uveal coloboma** – a ventral defect in the iris, ciliary body, neural retina/retinal pigment epithelium/choroid, and/or the optic nerve caused by failure of the optic fissure to close

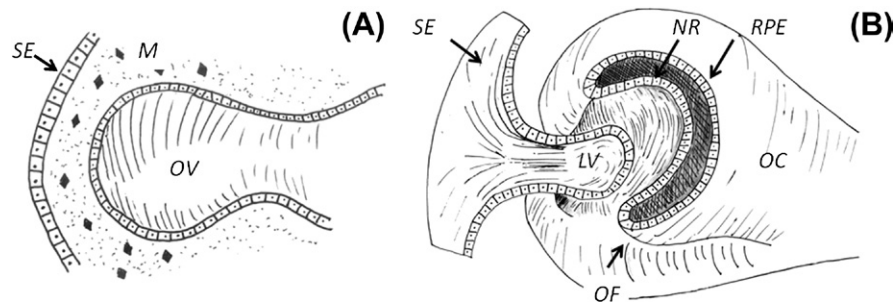
### 141.1 INTRODUCTION

The purpose of this chapter is to review several congenital ocular malformations—namely anophthalmia, microphthalmia, and coloboma—that are likely to be encountered in a clinical medical genetics practice. Emphasis will be placed on clinical diagnosis, molecular genetics, and developmental mechanisms. Readers are referred to Chapters 135, 136, 137, and 138 in this text for discussion of infantile cataracts, infantile glaucoma, infantile corneal abnormalities, and early-onset retinal degenerations.

Ocular malformations may have genetic and non-genetic (e.g. environmental) causes. Inherited ocular malformations may appear as familial or sporadic and they may be isolated or associated with a broader syndrome.

While significant strides have been made on understanding the developmental and genetic basis of these abnormalities, frequently no specific cause can be determined for a given malformation. Depending on the method of ascertainment, the population studies, and the definition of “ocular malformation,” published studies have found a birth prevalence of ocular malformations of 0.04–6.8/10,000 live births (1–7).

In order to understand the developmental mechanisms responsible for ocular malformations, it is helpful to briefly review early ocular development (8). During the fourth week of human gestation, rostral evaginations of the neuroectoderm (the optic vesicle) form bilaterally (Figure 141-1). The optic vesicle remains connected to the developing brain via the optic stalk, the primordium of the optic nerve. As this one-cell-thick sheet of neuroepithelium approaches the overlying surface ectoderm (SE), the distal optic vesicle invaginates to form a bilayered optic cup (OC), with the presumptive neural retina forming the inner layer and the presumptive retinal pigment epithelium (RPE) forming the outer layer. The invagination of the optic cup (OC), however, is asymmetric, such that there is a ventral gap (the optic fissure (OF)), which allows for periorbital mesenchyme that will form the primordial ocular vasculature to invade the developing eye and optic nerve. Beginning approximately in the fifth week of human gestation, the two edges of the OF approximate and fuse to form a circumferentially continuous OC. Coincident with the formation of the OC, the SE overlying the developing eye is induced to thicken into the lens placode. This specialized ectoderm then invaginates and detaches from the SE to form the lens vesicle (LV), the primordium of the crystalline lens. Around the time of OF closure, the cells of the presumptive neural retina adjacent to the optic stalk begin to differentiate into retinal ganglion cells. The optic nerve begins developing as these retinal ganglion



**FIGURE 141-1** Schematic of early ocular development. The eye develops as an evagination of the developing forebrain, called the optic vesicle (A). As the optic vesicle approaches the SE, the single layer of neuroepithelium invaginates to form the OC (B). The inner layer of this neuroepithelium is the presumptive neural retina, while the outer layer is the presumptive RPE. Formation of the OC is coincident with the invagination of the SE to form the LV. This invagination is asymmetric, such that there is a gap along the ventral surface of the OC called the OF. The edges of the fissure normally meet and fuse between the 5th and 6th weeks of human gestation; failure of OF closure leads to uveal coloboma.

cells send their axons through vacuolized areas of the optic stalk and into the developing brain (9,10). This differentiation process continues to move more peripherally through subsequent weeks of development. All these developmental processes are precisely orchestrated in space and time via a complex network of growth factors, transcription factors, and guidance molecules.

## 141.2 ANOPHTHALMIA, MICROPTHALMIA, AND UVEAL COLOBOMA

“Anophthalmia” implies the complete absence of ocular tissue. Patients who may appear anophthalmic clinically, may—on histologic and/or radiologic screening—have ocular remnants such as neural retina or lens tissue present. As such, anophthalmia likely lies on a phenotypic continuum with microphthalmia (small eye), leading to terms such as “true anophthalmia,” “extreme microphthalmia” (11), and “clinical anophthalmia” (12) in the literature.

Anophthalmia may be unilateral or bilateral and is often associated with short palpebral fissures and small orbits (Figure 141-2A). The developmental mechanisms for anophthalmia are incompletely understood but may include: (1) early eye patterning defects; (2) failure of the optic vesicle to form, perhaps due to reduced cell division of neural progenitor cells; and/or (3) regression of ocular structures that are initially formed.

The prevalence of anophthalmia may depend on the population studied (4), ranging from 0.2 to 0.4 per 10,000 live births in Western countries (7,13). Some reports group microphthalmia with anophthalmia. Gogate et al. have estimated that anophthalmia and microphthalmia comprise 41% of blindness in Maharashtra, India, but this is probably much larger than actual numbers in most countries (14).

Anophthalmia may be isolated or part of a broader syndrome (15). In isolated cases, inheritance may be autosomal dominant (AD) (16) or autosomal recessive (AR) (12). Note that in families with clear mendelian

inheritance, ocular pathology may be asymmetric or even unilateral. While some patients with syndromic forms of anophthalmia/microphthalmia often have delayed development and/or mental retardation, this is not always the case.

Reconstructive surgery, such as the use of spherical implants, orbital osteotomies, bone grafts, and/or orbital expanders, may improve the appearance of patients with anophthalmia (17). Serial expanders and buccal mucous membrane grafts may aid in conjunctival sac reconstruction. Lid reconstruction may require skin grafts and/or tissue flaps.

A microphthalmic eye is an eye that is two standard deviations below the age-adjusted mean axial length (18). Microphthalmia includes a range of phenotypes. Weiss has broadly broken microphthalmia into two categories—simple and complex (19,20). Simple microphthalmia refers to a small but otherwise structurally normal eye (Figure 141-2B), whereas complex microphthalmia refers to a small eye with other congenital ocular abnormalities (e.g. coloboma, cataract, or sclerocornea). Not all authors follow this convention in describing patients and some use the more general term “microphthalmia” rather than distinguish between these two subgroups. While we follow this distinction here, it is likely that “simple” and “complex” microphthalmia represent points along a phenotypic continuum in which anophthalmia is the most severe manifestation.

Simple microphthalmia may sometimes be used synonymously with the term “nanophthalmos,” especially when such eyes develop angle closure glaucoma due to anterior segment crowding and/or uveal effusions. The posterior segment of simple microphthalmic eyes is often proportionately shorter than the anterior segment (“posterior microphthalmia”) (19,21). Simple microphthalmia/nanophthalmos can be part of a systemic syndrome (e.g. the oculo-dento-digital syndrome, mucopolipidosis type III (22), and Kenny’s syndrome (23)), or inherited as an isolated trait. Yamani et al. have found that the sclera of nanophthalmic eyes contains irregularly arranged collagen lamellae, absence of elastic fibers, and abnormal



**FIGURE 141-2** (A) Patient with clinical anophthalmia due to a *SOX2* mutation, and (B) a patient with simple microphthalmia. (Pictures are courtesy of Dr Adele Schneider, Albert Einstein College of Medicine, Philadelphia, PA.)

glycogen-like deposits (24). This abnormal tissue architecture is accompanied by a decrease in scleral elasticity and an increase in scleral thickness, which may reduce blood flow through vortex veins and/or decrease trans-scleral flow of protein. It is likely that this abnormal fluid dynamics in the nanophthalmic eye contributes to the increased risk of uveal effusions and choroidal detachment, particularly after surgery (25–28). Sener et al. found that strabismus, particularly non-accommodative and refractive esotropia, was common in patients with nanophthalmos and that surgical dosing of muscle recessions did not have to be altered because of the small eyes of these patients (29). “Amblyopia,” they found, can also be refractory to treatment, perhaps because some of these patients may actually have subtle foveal hypoplasia on optical coherence tomography and an abnormal foveal avascular zone on angiography (30). Khan and Zafar have noted retinal degenerative and pigmentary changes in a subset of patients with nanophthalmos (31).

In 1998, Othman et al. mapped a locus (*NNO1*) for AD nanophthalmos to a 14.3-kb region on 11p (Online Mendelian Inheritance in Man (OMIM) #600165) (32). In this cohort, 22 patients were affected with high hyperopia (refractive error +7.25 to +13.00 D) and short axial lengths (17.55–19.28 mm). Of these, 12 had either a history of angle closure glaucoma or occludable anterior chamber angles on examination. More recently, Li et al. mapped a second AD nanophthalmos locus (*NNO3*) to 2q11–q14 in a Chinese family (OMIM #611897) (33). In addition to nanophthalmos, affected members of the family had enophthalmos, narrow palpebral fissures, mild ptosis, shallow anterior chambers, microcornea, and normal pupils. Hypermetropia ranged from +6.00 to +11.25 D.

AR nanophthalmos (OMIM #609549, also *MCOPS5*, #611040) has also been reported in an Amish family (34) to result from a mutation of the *MFRP* gene (OMIM #606227), which encodes a frizzled-related protein (35).

The phenotypic spectrum of this disorder has been extended by Ayala-Ramirez et al. and Crespi et al. to include retinal degeneration, foveoschisis, and optic disc drusen (36,37).

Complex microphthalmia refers to an eye that is small and accompanied by other congenital ocular malformations, such as uveal coloboma or persistence of the fetal vasculature (PFV). Like simple microphthalmia, complex microphthalmia can be isolated or part of a genetic syndrome. Loci/genes for both isolated and syndromic microphthalmia have been identified (Table 141-1) (38–58). Complex microphthalmia is sometimes observed in children with chromosomal abnormalities, most notably Wolf–Hirschhorn (4p-) syndrome, trisomy 13, cat eye syndrome, and 13q deletions (59,60). The most common form of syndromic microphthalmia is CHARGE syndrome (see Section 141.3.3). Because microphthalmia has been associated with over 100 different syndromes, an extensive review of the literature is beyond the scope of this text. However, selected prominent syndromes are detailed below.

### 141.2.1 Uveal Coloboma

The OF is a transient opening on the ventral surface of the developing OC that allows for the migration of periocular mesenchyme into the eye. Beginning approximately at 5 weeks of human gestation, the edges of the OF appose and fuse in order that the globe becomes a complete sphere. Failure of the OF to close results in uveal coloboma (61,62). The term “coloboma” has been used somewhat loosely in the ophthalmic literature to mean a developmental abnormality in or around the eye where a piece of tissue appears to be missing—hence the terms such as “eyelid coloboma” associated with Goldenhar syndrome or “macular coloboma” associated with Leber congenital amaurosis. Perhaps the most confusing



TABLE 141-1 Reported Loci/Genes for Microphthalmia

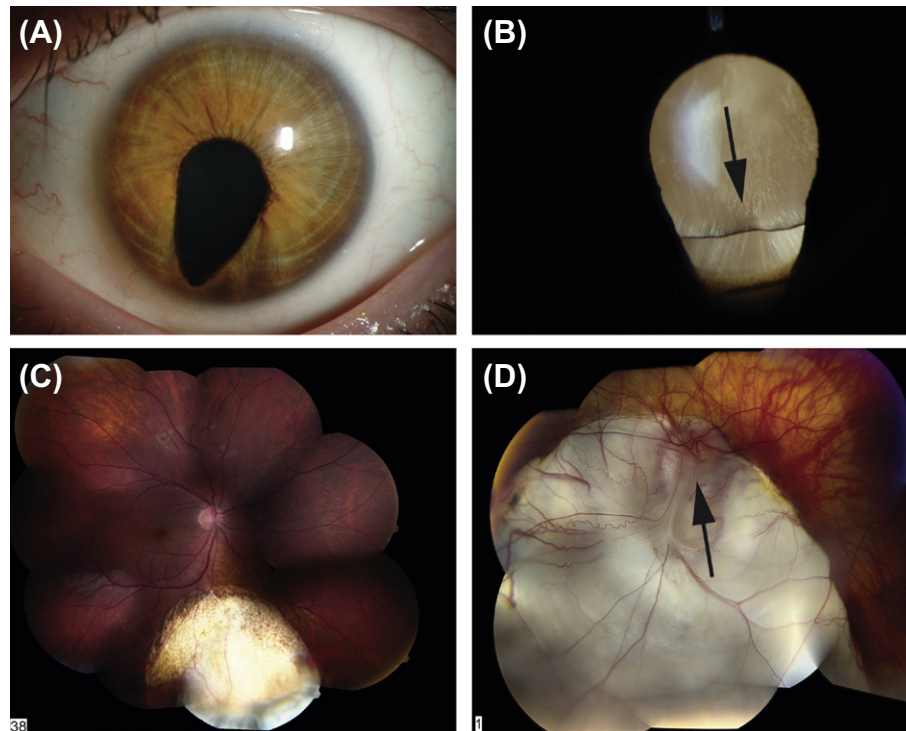
Designation	OMIM	Locus (Gene)	Inheritance	Associated Abnormalities
<i>MCOP1</i>	%251600	14q32	AR	
<i>MCOP2</i>	#610093	14q24.3 ( <i>CHX10/VSX2</i> )	AR	
<i>MCOPCB3</i>				
<i>MCOP3</i>	#611038	18q21.3 ( <i>RX/RAX</i> )	AR	
<i>MCOP4</i>	#613094	8q22.1 ( <i>GDF6</i> )	AD	Preaxial polydactyly, vertebral anomalies
<i>MCOP5</i>	#611040	11q23 ( <i>MFRP</i> )	AR	Posterior microphthalmia/nanophthalmos, retinal degeneration, foveoschisis, optic disc drusen
<i>MCOP6</i>	%613517	2q37.1	AR	Posterior microphthalmia
<i>MCOP7</i>	#613704	12p13.1 ( <i>GDF3</i> )	AD	Klippel–Feil
<i>MCOPCB1</i>				
<i>MCOPCB1</i>	%300345	X	XL	
<i>MCOPCB2</i>	%605738	15q12–q15	AD	
<i>MCOPCB4</i>				No locus or gene. Microphthalmia with colobomatous cyst
<i>MCOPCB5</i>	#611638	7q36 ( <i>SHH</i> )	AD	
<i>MCOPCT1</i>	%156850	16p13.3	AD	Mental retardation in some patients
<i>MCOPCT2</i>	#212550	14q23 ( <i>SIX6</i> )	AD	
<i>MCOPCT3</i>	302300		X (?)	
<i>MCOPCT4</i>	#610426	22q11.2–q13.1 ( <i>CRYBA4</i> )	AD	
<i>MCOPS1</i>	%309800	Xq27–q28	XL	Lenz microphthalmia (cleft lip/palate, dental abnormalities, syndactyly, abnormal ear helices, pectus excavatum)
<i>MCOPS2</i>	#300166	Xp11.4	XL	OFCD, Lenz microphthalmia (microcephaly, mental retardation, GU abnormalities, abnormal ear helices)
<i>MCOPS3</i>	#206900	3q26.3–q27 ( <i>SOX2</i> )	AD	Anophthalmia, developmental delay, CNS abnormalities, esophageal atresia, tracheoesophageal fistula, GU abnormalities
<i>MCOPS4</i>	%301590	Xq27–q28	XL	Ankyloblepharon, same region as <i>MCOPS1</i>
<i>MCOPS5</i>	#610125	14q21–q22 ( <i>OTX2</i> )	AD	Cataract, retinal degeneration, hypoplasia/aplasia of optic nerves, agenesis of corpus callosum, developmental delay, seizures, hypotonia
<i>MCOPS6</i>	#607932	14q21–q22 ( <i>BMP4</i> )	AD	Developmental brain abnormalities, hypopituitarism, polysyndactyly, retinal dystrophy
<i>MCOPS7</i>	#309801	Xp22 ( <i>HCCS</i> )	XL	MIDAS (microphthalmia, dermal aplasia, sclerocornea) syndrome, microphthalmia with linear skin defects, CHD/cardiomyopathy
<i>MCOPS8</i>	%601349	6q21	AD (?)	Mental retardation, borderline microcephaly, prognathism, cleft lip/palate, ectrodactyly, premature aging of skin
<i>MCOPS9</i>	#601186	15q24.1 ( <i>STRA6</i> )	AR	Matthew–Wood syndrome, diaphragmatic hernia with pulmonary hypoplasia, CHD, IUGR
<i>MCOPS10</i>	%611222		AR (?)	Microcephaly, progressive spasticity, seizures, mental retardation

Although many genes are associated with complex microphthalmia, at least two have been associated with simple microphthalmia. Note that although some loci are designated as “nonsyndromic,” systemic abnormalities have been reported in some of these patients. AD = autosomal dominant; AR = autosomal recessive; CHD = congenital heart disease; GU = genitourinary; IUGR = intrauterine growth abnormalities; MCOP = microphthalmia, nonsyndromic; MCOPCB = microphthalmia, isolated with coloboma; MCOPCT = microphthalmia, isolated, with cataract; MCOPS = microphthalmia, syndromic; XL = X-linked.

term is “optic nerve coloboma,” as this can refer to a uveal coloboma that affects the optic nerve, a congenital excavation of the optic nerve, or the morning glory disc anomaly. In this chapter, we will reserve the word “coloboma” to refer to conditions related to OF closure.

Uveal coloboma may accompany complex microphthalmia, but not all eyes with coloboma are microphthalmic. Similarly, many eyes with coloboma have small corneas (microcornea). Microcornea may exist, however, independent of coloboma and coloboma may exist independent of microcornea. When coloboma affects the anterior segment, the pupil appears

teardrop-shaped, pointing inferonasally (the direction of the OF, [Figure 141-3A](#)). If the coloboma extends slightly more posterior, it may affect the ciliary body and result in absent zonules in the inferonasal quadrant. The focal absence of zonules leads to focal flattening of the lens curvature, sometimes referred to as a “lens coloboma” ([Figure 141-3B](#)). This abnormality may result in significant astigmatism that should be optically corrected in children to avoid amblyopia. When a coloboma extends further posteriorly, it appears as a defect in the neural retina, RPE, and choroid, revealing bare sclera ([Figure 141-3C, D](#)). Sometimes these areas



**FIGURE 141-3** The clinical appearance of uveal coloboma. (A) Uveal coloboma of the iris appears as a teardrop-shaped pupil pointing inferiorly and nasally—the position of the OF. (B) Uveal coloboma affecting the ciliary body can lead to a defect in lens zonules, resulting in a focal flattening of the peripheral curvature of the crystalline lens—a so-called lens coloboma. This defect can lead to astigmatism and, in children, amblyopia. (C) Uveal coloboma that affects the posterior pole appears as a patch of bare sclera in the inferonasal quadrant. Because this coloboma does not involve the optic nerve and/or macula, the vision of this patient is normal. (D) Uveal coloboma can extend posteriorly to involve the optic nerve and macula. In such cases, the optic nerve is frequently small and dysplastic. Although visual prognosis is often guarded, attempts to improve vision with glasses and/or amblyopia treatment should still be attempted.

of colobomatous defects are focal and circumscribed; sometimes there are “skip areas” unaffected by the coloboma. Hyper- and hypopigmentation around the edges of the coloboma are common. When the coloboma extends posteriorly enough to involve the optic nerve and/or macula, vision can be affected. Maumenee and Mitchell have estimated that approximately 10% of childhood blindness results from coloboma (63). Optic nerves affected by coloboma are often dysplastic.

Although uveal coloboma is not a progressive condition per se, the sclera overlying the defect may become more excavated with time. Retinal blood vessels may initially bridge the surface of a coloboma. Over time, this stretching of the coloboma area may lead these vessels to bleed, resulting in a vitreous hemorrhage. In addition, uveal coloboma likely predisposes to retinal detachment (64,65) and choroidal neovascularization (abnormal blood vessel growth beneath the neural retina) (66) in some patients later in life. Presenile cataracts may also occur (63).

The term “microphthalmia with cyst” refers to a condition where neuroectodermal tissue protrudes through the unclosed OF to form a potential space adjacent to the developing eye. This potential space can fill with fluid, causing developmental disruption of the eye—hence, microphthalmia. Most cases are sporadic, although familial cases

have been reported (67). The cyst may push the eye superotemporally, resulting in a bulging mass behind the lower lid. Imaging of the affected orbit may show that the cyst is larger than the eye per se. The management of microphthalmia with cyst depends upon several factors: the visual potential of the eye, the growth of the cyst over time and the cosmetic appearance of the patient. Strategies include aspiration, observation, and excision (with or without enucleation) (68). In cases where enucleation is performed, socket expansion may improve the patient’s cosmetic appearance. Gupta et al. have reported on a syndrome of bilateral microphthalmia with cyst, and facial and limb anomalies (OMIM 607597) (69). Microphthalmia with cyst may be distinct from the condition “congenital cystic eye,” which likely results from failure of the optic vesicle to invaginate normally (70).

### 141.3 GENES AND SYNDROMES ASSOCIATED WITH ANOPHTHALMIA, MICROPTHALMIA, AND/OR UVEAL COLOBOMA

The nosology of anophthalmia, microphthalmia, and uveal coloboma (AMC) is complex and imperfect. In the broadest sense, AMC can be subdivided into “syndromic” and “nonsyndromic/isolated” forms, depending

on whether the developmental eye abnormalities are associated with systemic findings. While some patients may clearly fit into the “syndromic” category—for instance, patients with chromosomal abnormalities and multiple congenital anomalies—some patients may have relatively minor findings (e.g. the cutaneous syndactyly seen in some patients with mutations in *GDF6*). Approaching the patient with congenital eye anomalies therefore requires some appreciation of the continuum of phenotypes between these two extremes. Further complicating any classification scheme is the wide phenotypic spectrum reported in many patients with mutations in a developmentally regulated gene. Likely, this wide spectrum is at least partly due to noncoding sequence differences between individuals, sequence differences in other genes in the same developmental pathway, environmental factors, and stochastic factors. The major genes and syndromes associated with AMC are listed alphabetically below.

### 141.3.1 14q22–q23 Deletion Syndrome, *OTX2*, and Bone Morphogenetic Protein-4

Interstitial deletions of chromosome 14q22–23 cause a syndromic form of anophthalmia/microphthalmia, with brain, pituitary, digit, thyroid, and genital abnormalities (71). Ragge et al. found mutations in the bicoid-type homeodomain transcription factor gene, *OTX2* (OMIM #610125; *MCOPS5*), which lies in this region in series of individuals and families with a broad spectrum of phenotypes (54). Affected individuals may present with anophthalmia/microphthalmia, a retinal dystrophy resembling Leber congenital amaurosis and pigmentary retinopathy, and/or varying degrees of central nervous system pathology. Further complicating the phenotypic spectrum and the genetic counseling of such patients was apparent non-penetrance in mutation-positive individuals and gonadal mosaicism. Schilter et al. have recently proposed that the presence of pituitary anomalies and the absence of gastrointestinal manifestation may help distinguish *OTX2*-related from *SOX2*-related anophthalmia/microphthalmia (see Section 141.3.15) (72). Most mutations are predicted to result in premature truncation of the *OTX2* protein. While the predominant mechanism of disease is thought to be haploinsufficiency (73), a dominant-negative mechanism may play a role with at least some mutations (74). The presence of retinal degeneration in some patients with *OTX2* mutations may be related to its role in photoreceptor and bipolar cell development/maintenance (75,76).

The bone morphogenetic protein-4 gene (*BMP4*; OMIM #607932; *MCOPS6*) also lies within the 14q22–q23 region. *BMP* is a member of the transforming growth factor beta 1 (TGF- $\beta$ 1) family of secretory signaling molecules that play an essential role in embryonic development, including eye development (77). Mutations in the *BMP4* gene—like mutations in *OTX2*—lead to a broad spectrum of findings, including

anophthalmia/microphthalmia, retinal dystrophy, myopia, brain abnormalities, and polydactyly (55). The developmentally regulated genes *SHH* and *PTCH1* may act as modifiers of the *BMP4* phenotype. These findings, like those in patients with *OTX2* mutations, emphasize that mutations in developmentally regulated genes may result in both congenital malformations and a degenerative phenotype (e.g. retinal degeneration).

### 141.3.2 *BCOR*, Lenz Microphthalmia and the Oculofacialcardiodental Syndrome

Lenz microphthalmia (*MCOPS1*; OMIM #309800; and *MCOPS2*; OMIM #300166) is a phenotypically and likely genotypically heterogeneous, X-linked condition. In addition to (often colobomatous) microphthalmia or anophthalmia, patients may have intellectual disability and dental, ear, genitourinary, digital, and skeletal abnormalities (78). Two loci on the X chromosome, *MCOPS1* (Xq27–q28; OMIM #309800) and *MCOPS2* (Xp11.4; OMIM #300166), have been identified (51). Forrester et al. describe a pedigree mapping to Xq27–q28 that extends the phenotype to include cardiac anomalies (50). While Ng et al. identified a c.C254T (p.P85L) sequence change in the BCL-6-interacting corepressor (*BCOR*) gene in a family mapping to the *MCOPS2* locus (52), the gene responsible for the *MCOPS1* phenotype has not yet been identified. The nomenclature for Lenz microphthalmia is further complicated by the report of a family with clinical anophthalmia, preauricular skin tags, and cleft palate that maps to Xq27–q28 (*MCOPS4*; OMIM #301590), but this has been considered a distinct genetic entity (53).

Ng et al. also noted phenotypic overlap between Lenz microphthalmia and the X-linked oculofacialcardiodental syndrome (OFCD), which is lethal in males. Affected females often present with microphthalmia  $\pm$  cataracts, congenital heart anomalies, dysmorphic features (simple ears, elongated face, broad nasal tip), hammer-toe deformity, and dental abnormalities (e.g. persistent primary teeth and radiculomegaly) (79). Mutations predicted to result in *BCOR* haploinsufficiency were found in seven OFCD families, making it allelic to Lenz microphthalmia at the Xp11.4 locus. Morpholino knockdown of *BCOR* in zebra fish results in ocular, skeletal, and nervous system abnormalities, consistent with the human phenotype. In addition to describing a deletion of *BCOR* in a family with OFCDB, Horn et al. did not find any mutations in patients with Lenz microphthalmia, suggesting that the *MCOPS2* locus is a minor contributor to the Lenz microphthalmia phenotype (80).

### 141.3.3 CHARGE Syndrome

The most common cause of syndromic uveal coloboma is the CHARGE syndrome (OMIM #214800)—an acronym for coloboma (usually bilateral  $\pm$  microphthalmia),



congenital heart abnormalities, atresia or stenosis of the choanae (unilateral or bilateral), retardation of growth/development, genitourinary (including renal) abnormalities, and ear abnormalities (81). Patients may have hypomia/anosmia and hypogonadotropic hypogonadism, thus overlapping with the phenotype of Kallman syndrome. Mondini defects of the cochlea and/or absent/hypoplastic semicircular canals are found in nearly all patients on imaging. Patients may have other cranial nerve dysfunctions, including facial palsy and difficulty with swallowing. Orofacial clefting is observed in approximately 15–20% of individuals. Mutations in the chromodomain gene *CHD7* (OMIM \*608892) are found in approximately two-thirds of patients with CHARGE syndrome (82). A rare cause of CHARGE syndrome may be mutations in the *SEMA3E* gene (OMIM \*608166) (83). While the precise mechanisms by which mutations in *CHD7* result in coloboma and other systemic abnormalities is still unclear, recent evidence has supported a role of neural crest development (84), ribosomal synthesis (85) and stem cell gene expression (86).

#### 141.3.4 Growth-Differentiation Factor 3

In 2010, Ye et al. described six different missense mutations in growth-differentiation factor 3 (*GDF3* (OMIM \*606522); *MCOP7* (OMIM #613704); *MCOPCB6*, digenic (OMIM #613703)) in patients with variable microphthalmia and/or uveal coloboma accompanied by skeletal abnormalities (Klippel–Feil anomaly, vertebral fusion, scoliosis) (Figure 141-4) (87). Some individuals had both skeletal and ocular abnormalities, while others had one but not the other. In one case, an individual had a mutation in both *GDF3* and *GDF6*, raising the possibility that phenotypic variability may in part be explained by the “mutation load” in this gene family. *GDF3* is a member of the TGF- $\beta$ 1/bone morphogenetic protein family of growth factors that may also have *NODAL*-like activity (88–90).

#### 141.3.5 Growth-Differentiation Factor 6

Asai-Coakwell et al. identified a patient with an interstitial deletion of chromosome 8 (46,XX,del(8)(q21.2–q22.1)) with chorioretinal uveal colobomas, an atrial-septal defect, soft tissue syndactyly, and developmental delay (42). Included in this deletion interval is the growth-differentiation factor 6 (*GDF6*; *MCOP4* (OMIM #613094); *MCOPCB6*, digenic (OMIM #613703)), a member of the TGF- $\beta$ 1 family of growth factors. A subsequent screen of 489 patients with anophthalmia/microphthalmia/coloboma identified several different missense mutations (91). Klippel–Feil anomaly of the spine is observed in a subset of these patients, where incomplete penetrance and variable expressivity are common. Functional studies in vitro and in model organisms support a role of *GDF6/Gdf6/gdf6* in ocular



**FIGURE 141-4** Klippel–Feil anomaly of the cervical spine in a patient with a *GDF3* mutation. (Photo is courtesy of Dr Ordan Lehmann, University of Alberta, Edmonton.)

and skeletal development (92,93). A systemic screen of 50 patients with anophthalmia/microphthalmia/coloboma for mutations in *GDF6*, *OTX2*, *CHX10*, *RAX*, and *SOX2* found the highest prevalence of mutations in *GDF6* (8%) (94).

#### 141.3.6 HCCS, MIDAS Syndrome

MIDAS syndrome (microphthalmia, dermal aplasia and sclerocornea; *MCOPS7*; OMIM #309801) is an X-linked syndrome associated with unilateral or bilateral microphthalmia and linear, aplastic skin lesions, generally limited to the head and neck (95,96). With the exception of XX males (97,98), all reported cases have been in females, suggesting that this syndrome is lethal for males in utero. Numerous cytogenetic abnormalities leading to MIDAS syndrome have been reported in the region of Xp22 (95,99,100). Although there is some phenotypic overlap, MIDAS is genetically distinct from Golz syndrome (focal dermal hypoplasia (OMIM #305600), *PORCN* gene (OMIM \*300651)) and incontinentia pigmenti (OMIM #308300; *IKBK/NEMO* \*300248). In 2006, Wimplinger et al. identified one nonsense mutation (p.R197X) and one missense mutation (p.R217C) in the mitochondrial holocytochrome c-type synthase gene (*HCCS*; OMIM \*300056) (56). This enzyme acts as a heme lyase by covalently linking a



heme prosthetic group to cytochrome c and cytochrome c1. In addition, this group identified a mother–daughter pair who had a submicroscopic 8.6-kb deletion that involved the *HCCS* gene. These findings raise the interesting notion that mitochondrial genes may play a role in development (perhaps by regulating apoptosis) as well as their more established role in cellular energy metabolism.

### 141.3.7 *HMX1* and the Oculoauricular Syndrome

Studying the descendents of a consanguineous Swiss family originally described by Franceschetti and Valerio, Schorderet et al. identified a heterozygous 26-bp deletion in the *HMX1* transcription factor gene (OMIM \*142992, previously known as *NKX5-3*) (101). Affected individuals have congenital external ear abnormalities (with normal hearing) and a range of congenital ocular abnormalities including microphthalmia, microcornea, cataracts, iris adhesions to the lens and cornea, abnormal electroretinograms, and dysplastic optic nerves (oculoauricular syndrome; OMIM #612109). *HMX1* codes for a homeobox transcription factor protein expressed in the developing eye and ear. Knockdown of the zebra fish ortholog leads to microphthalmia and abnormal retinal lamination. The mouse mutant, Dumbo, results from a nonsense mutation in the *Hmx1* gene, which leads to high perinatal lethality, small size, abnormally positioned pinnae, and microphthalmia (102).

### 141.3.8 *MAF*

Using a family in whom a chromosome translocation, t(5; 16)(p.15.3; q23.2), cosegregated with anterior segment dysgenesis, cataract, and microphthalmia in both balanced and unbalanced forms, Jamieson et al. determined that the breakpoint disrupted the leucine zipper transcription factor gene, *MAF* (OMIM \*177075) (103). In addition, they identified a second family that exhibited AD pulverulent cortical and (later) posterior subcapsular cataract who had a missense mutation in a well-conserved residue in the DNA-binding domain of this gene (R288P, OMIM #610202). Two members of this family had microcornea and one had bilateral iris colobomas. Homozygous *Maf*<sup>−/−</sup> mice show cataract and microphthalmia, consistent with the human phenotype (104).

### 141.3.9 *PAX6*

The paired-box and homeodomain-containing transcription factor gene, *PAX6* (OMIM \*607108), plays a central role in ocular development. *PAX6* mutations are most often case aniridia (OMIM #106210)—an AD panophthalmic disease of ocular development characterized by iris hypoplasia, nystagmus, foveal hypoplasia, limbal stem cell deficiency, cataracts, and glaucoma

(105,106). Milder anterior segment phenotypes have also been described (OMIM #136520) (107,108). Although mutation of *Pax6* causes microphthalmia in mouse (109) and rat (110) models, *PAX6* mutations do not appear to be a major cause of microphthalmia/anophthalmia in humans. In a national study of anophthalmia, microphthalmia, and coloboma in Scotland, Morrison et al. noted no mutations in *PAX6* in 84 patients (3). However, rare cases of anophthalmia and congenital brain anomalies associated with mutations of both alleles of *PAX6* have been reported (111). Azuma et al. reported a patient with mental retardation, a complex developmental eye abnormality including uveal coloboma, and a *PAX6* missense mutation (F258S) (112).

### 141.3.10 Retina and Anterior Neural Fold Homeobox Gene

The retina and anterior neural fold homeobox gene (*RAX/RX* (OMIM \*601881); *MCOP3* (OMIM #611038)) is an evolutionarily conserved homeodomain gene that is essential for normal eye development (113). In mice, knockout of *Rax* function leads to failure of OC formation and anophthalmia. Other developmentally important genes such as *OTX2* and *SOX2* regulate *RAX* expression (114). Vororina et al. screened 75 individuals with anophthalmia or microphthalmia and found one boy with microphthalmia, sclerocornea, and autism who was a compound heterozygote for a Q147X nonsense mutation and a R192Q mutation, both within the DNA-binding domain of *RAX* (41). Although at least one other case of *RAX* mutation in anophthalmia has been reported (115), the prevalence of *RAX* mutations in patients on the AMC spectrum appears to be low (116).

### 141.3.11 *SIX3*

Mutations in the homeodomain gene *SIX3* (OMIM \*603714) cause holoprosencephaly (failure of the brain to develop into two complete, separate hemispheres) in humans, sometimes with ocular abnormalities such as microphthalmia and/or coloboma (117). Although lower vertebrate orthologs of *SIX3* are important in ocular development (118)—the designation “SIX” means “ortholog to *Drosophila sine oculis*”—the prevalence of *SIX3* mutations in nonsyndromic AMC patients appears to be low (3,119).

### 141.3.12 *SIX6*

*SIX6* (OMIM \*606326; *MCOPS6*; OMIM #607932), another member of the *SIX* family of homeodomain genes, has a sequence highly homologous to that of *SIX3* and is expressed in the developing retina and ventral optic stalk (120). As previously noted, interstitial deletions involving 14q23—where *SIX6* is located along with *OTX2* and *BMP4*—have been reported in individuals

with anophthalmia/microphthalmia and pituitary abnormalities (121,122). In 2004, Gallardo et al. identified a single missense mutation (T165A) in a patient with bilateral microphthalmia, nystagmus, and cataract (49); however, this sequence change was also observed in the patient's unaffected father. Aijaz et al., however, failed to identify any mutations in *SIX6* in 173 patients with AMC (123), raising the possibility that other genes in the 14q23 interval are likely responsible for the ocular and pituitary abnormalities in these patients.

### 141.3.13 Sonic Hedgehog

Sonic hedgehog (SHH) is an extracellular signaling molecule that is critical in multiple developmental processes (124), including the development of normal retinal lamination (125). Mutation of *SHH* (OMIM\*600725; *MCOPCB5*; OMIM #611638) in humans is generally associated with holoprosencephaly (126,127). Shh knockout mice exhibit cyclopia with abnormal retina-RPE patterning (128). SHH regulates the paired homeobox gene, *Pax2* (129,130), which is critical for OF closure in mice. Schimmenti et al. screened 53 probands with colobomatous microphthalmia for mutations in *SHH* and identified a novel 24-bp deletion that co-segregated in a two-generation family in which the proband had bilateral uveal colobomas. His mother, who has the same mutation, exhibited what is likely a *forme fruste* of coloboma (47).

### 141.3.14 *STRA6* ("Stimulated by Retinoic Acid 6")

Pasutto et al. reported a form of syndromic anophthalmia accompanied by a spectrum of congenital malformations including congenital heart defects, diaphragmatic hernia, alveolar capillary dysplasia, lung hypoplasia, and mental retardation (58,131). These patients have mutations in the *STRA6* gene (OMIM \*610745; *MCOPS9*; OMIM #601185), a member of the "stimulated by retinoic acid" genes. *STRA6* functions as a membrane receptor that mediates cellular uptake of vitamin A via the retinol-binding protein (132). This constellation of findings has overlap with the previously described "Matthew-Wood" syndrome (anophthalmia with pulmonary hypoplasia) (133). Although Golzio et al. did not find pathogenic mutations in a patient with Matthew-Wood syndrome, they reported truncating mutations in two fetuses with this syndrome (134). Vitamin A and its derivatives have major roles in the growth and differentiation of multiple embryonic tissues (135).

### 141.3.15 *SRY-BOX2* (*SOX2*)

Fantes et al. found that a child with bilateral anophthalmia and a cytogenetically balanced chromosomal translocation (46,XX,t(3; 11)(q27; p11.2)) had a deletion that eliminated the *SOX2* gene (OMIM \*184429; *MCOPS3*;

OMIM #206900) on chromosome 3 (136). Furthermore, four of eleven subjects with sporadic anophthalmia had *SOX2* nonsense mutations. Subsequently, other mutations, including deletions of the *SOX2* gene, have been reported (137–140). Hagstrom et al. note that *SOX2* mutations can cause not only anophthalmia, but also aplasia of the optic nerves, chiasm, and tracts (138). Mutation of *SOX2* generally produces a fairly severe, bilateral ocular disease and can be accompanied by brain malformations, learning disability, seizures, esophageal atresia, motor abnormalities, hypogonadotropic hypogonadism, male genital tract malformations, renal abnormalities, mild facial dysmorphism, and postnatal growth failure (141,142). *SOX2* mutations likely account for the largest proportion of the monogenic causes of anophthalmia/microphthalmia described to date.

*SOX2* codes for a member of SRY-like family of the high-mobility group (HMG) of transcription factors and lies within an intron of a non-expressed gene, *SOX2OT*. It is expressed in neuroectoderm early in development (143) and is important in maintaining self-renewing progenitor cells (144). (In fact, *SOX2* is one of the critical transcription factors used currently to create induced pluripotent stem cells (iPS cells) from differentiated cells (145).) Loss of *SOX2* function may result in an abnormally early exit of neural/ocular progenitor cells from their undifferentiated state, resulting in an overall loss of neural/ocular tissue. In addition, the transcription factors *SOX2*, *PAX6*, and *OTX2* display a complex network of interactions in ocular development and/or tissue maintenance (146), suggesting that *SOX2*-related phenotypes may also result from events later in development.

### 141.3.16 *VSX2* (*CHX10*)-Related Microphthalmia

The homeobox-containing transcription factor gene *VSX2* (*CHX10*; *MCOPCB3* (OMIM #610092); *MCOP2* (OMIM #610093)) has a critical role in the development of the neural retina (147). A nonsense mutation in the murine *Chx10* gene causes the microphthalmia, thin/hypocellular retina, and optic nerve aplasia found in the *ocular retardation* mouse (148). Studying a consanguineous Turkish pedigree with colobomatous microphthalmia and infantile cataract, as well as an Arab child with microphthalmia and no discernible pupil, Percin et al. found two independent homozygous mutations in the human *VSX2* (*CHX10*) gene (40). The prevalence of mutations in *VSX2* (*CHX10*) in patients with isolated anophthalmia/microphthalmia appears to be low in other populations (3,94).

## 141.4 A CLINICAL APPROACH TO THE PATIENT WITH AMC

Given the phenotypic and genotypic heterogeneity of patients with AMC, defining a routine approach to a

patient is difficult, if not impossible. What follows is based on the personal experience of the author.

In the setting of a newborn with AMC, the first distinction to make is between a “syndromic” and “nonsyndromic” or “isolated” phenotype. A careful examination of the child for any dysmorphic features is a reasonable first step. Further clinical work-up is, in part, dictated by the clinical exam. Patients who do not pass their newborn hearing screening must be carefully followed up. Even those who *do* pass their screening may require more detailed hearing testing later in life, if for no other reason other than to maximize the possibility for auditory learning in a child who may be visually compromised. Patients with an unexplained heart murmur may benefit from an echocardiogram. Patients with low tone, microcephaly, or other neurologic symptoms may require neurologic examination and/or neuroimaging. Renal ultrasound is a reasonable noninvasive screening test that does not require radiation. The clinical approach to an older child or adult with AMC can be informed by such factors as their cognitive development and physical symptoms/exam.

In providing recurrence risk counseling, a careful eye exam of the child’s first-degree relatives is important, as “microforms” of coloboma (Figure 141-5) and subclinical simple microphthalmia may occasionally be present and could influence genetic counseling. Morrison et al. found an approximately 10% empiric sibling recurrence risk in their cohort of AMC patients (3). While this is a helpful number, it may not directly change the genetic counseling for a specific, individual family; the sibling recurrence risk found in this study combines data from “high risk” and “low risk” families. Because—in the absence of other abnormalities in the family and/or

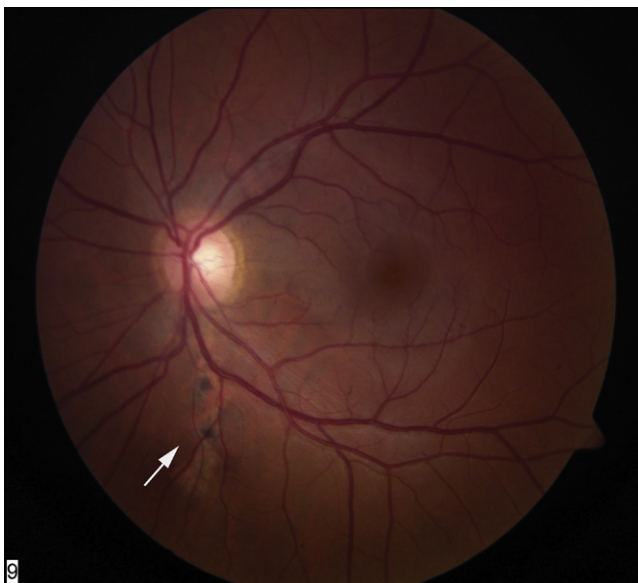
a defined syndrome in the proband—we currently have no good way to distinguish “high risk” from “low risk” families, we counsel that there is up to a 25% recurrence risk of AMC with each pregnancy. Because the severity of phenotype can vary even within a family that displays clear mendelian inheritance, predicting the visual potential of a second affected child is even more difficult.

A major concern for parents of a newborn with AMC is visual potential. Patients with unilateral visual loss adapt and generally require little in the way of visual aids, save for protective eyewear. Patients with bilateral abnormalities are more difficult to predict. In the setting of anophthalmia and extreme microphthalmia, we generally give a guarded visual prognosis. When uveal coloboma involves only the iris and/or the peripheral retina, vision is usually normal or near-normal. These children must still be followed regularly by a pediatric ophthalmologist as they are likely at risk for amblyopia due to astigmatism. Even in children with colobomas involving the optic nerve and/or macula, vision—although usually not 20/20—can be quite good. For this reason, we recommend careful, regular follow-up with a pediatric ophthalmologist, and have a low threshold for initiating spectacle correction and/or amblyopia treatment.

Molecular testing in AMC is also complicated. In the absence of other systemic findings and/or a defined syndrome, the yield of sequencing known genes remains low (3,94). Raca et al. report that clinically interpretable abnormalities on array comparative genomic hybridization is low in isolated AMC (149). As such, there is no single “high-yield” molecular test for nonsyndromic AMC at present, and clinical judgment tempered with the cost-benefit for the individual patient remains the guiding principle.

### 141.5 PERSISTENCE OF THE FETAL VASCULATURE/PERSISTENT HYPERPLASTIC PRIMARY VITREOUS

PFV—also known as persistent hyperplastic primary vitreous (PHPV)—is most often a sporadic unilateral congenital ocular malformation (150). During normal embryogenesis, periocular mesenchyme invades the center of the OC via the open OF. As the OF closes, this mesenchyme organizes to form the fetal vasculature of the developing eye. This hyaloid vasculature runs distal from its origin in the optic stalk, through the primary vitreous cavity, and encircles the developing lens. At the time of full-term birth, this primordial circulation has largely regressed, leaving the lens and vitreous cavity avascular. In PFV, the remnants of the hyaloid vasculature fail to completely regress. Often, PFV is associated with microphthalmia and a dense fibrotic plaque on the posterior surface of the lens. Adhesions between the iris and the anterior lens, glaucoma, and dragging of the ciliary body are also sometimes observed. Visual prognosis is usually guarded. While most PFV is sporadic, AR



**FIGURE 141-5** Uveal coloboma may be asymptomatic and undetectable without a detailed, dilated fundoscopic examination. Such “microforms” of coloboma as this focal RPE abnormality—when seen in the parent of a child with coloboma—help establish a pattern of inheritance and assist in genetic counseling.



(OMIM %611311) (151) and AD (OMIM %611308) (152) inheritance have been reported.

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- Chang, L.; Blain, D., et al. Uveal Coloboma: Clinical and Basic Science Update. *Curr. Opin. Ophthalmol.* 2006, 17 (5), 447–470.

## RELEVANT WEBSITES

- National Eye Institute information on uveal coloboma:  
<http://www.nei.nih.gov/health/coloboma/>.
- CHARGE syndrome support group/foundation:  
<http://www.chargesyndrome.org/>.
- Genetics Home Reference article on CHARGE:  
<http://ghr.nlm.nih.gov/condition/charge-syndrome>.
- International Anophthalmia/Microphthalmia Network:  
<http://www.anophthalmia.org/>.
- Gene reviews article on anophthalmia/microphthalmia:  
<http://www.ncbi.nlm.nih.gov/books/NBK1378/>.

## Biography



**Brian P Brooks** obtained his MD and PhD from the University of Pennsylvania. His residency training in ophthalmology and fellowship training in pediatric ophthalmology were completed at the University of Michigan. Dr Brooks completed a clinical genetics fellowship at the Metropolitan Washington DC Medical Genetics program centered at the National Human Genome Research Institute. Dr Brooks is one of the few physicians in the United States boarded in both ophthalmology and clinical genetics. He is currently Chief, Unit on Pediatric, Developmental, and Genetic Ophthalmology in the Ophthalmic Genetics and Visual Function Branch of the National Eye Institute. In 2010, he was awarded the Presidential Early Career Award for Science and Engineering by President Barak Obama, the nation's highest honor for young investigators.



## Hereditary Hearing Impairment

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**GLOSSARY**

**Aminoglycoside** – antibiotic used to treat Gram negative bacterial infection  
**Auditory neuropathy** – SNHI in which ABRs are absent or severely distorted but OAEs are present  
**Cochlea** – part of ear with sensory apparatus  
**Congenital** – present since birth  
**Eustachian tube** – connection between throat and middle ear  
**Hemosiderin** – breakdown byproduct of hemoglobin  
**Neural transmitter** – chemical involved in signal transduction at nerve endings  
**Otoacoustic emissions** – sounds produced in the cochlea by the mechanical activity of outer hair cells  
**Ototoxic** – damaging to the ear  
**Pathogenesis** – medical explanation for a particular finding  
**Penetrance** – likelihood that individuals with an abnormal gene will manifest the expected condition  
**Pinna** – external part of the ear  
**Retrocochlear** – auditory pathway from the cochlea to the brain, not involving the cochlea  
**Teratogen** – agent that causes damage to developing fetus when used during pregnancy  
**Syndrome** – recognizable pattern of findings reflecting a common pathogenesis  
**Vestibule** – part of ear with balance apparatus

**ABBREVIATIONS**

**ABR** – auditory brainstem response  
**ADNSHL or ADNSHI** – autosomal dominant non-syndromic hearing loss/impairment  
**ARNSHL or ARSNHI** – autosomal recessive non-syndromic hearing loss/impairment  
**AEP** – auditory evoked potentials  
**ARHI** – age-related hearing impairment (presbycusis)  
**AUNA/AUNB** – AD or AR auditory neuropathy  
**DFN** – deafness

**DFNA** – autosomal dominant non-syndromic hearing loss loci  
**DFNB** – autosomal recessive non-syndromic hearing loss loci  
**DFNX** – X-linked non-syndromic hearing loss  
**HI/HL** – hearing impairment/hearing loss  
**NSHL/NSHI** – non-syndromal hearing loss/impairment  
**OAE** – otoacoustic emissions  
**SNHL** – sensorineural hearing loss

**142.1 INTRODUCTION**

Hearing loss (HL) is the most common sensory abnormality in humans. In view of the complexity of the hearing mechanism, it is not surprising that HL can result from a wide variety of genetically determined aberrations, which involve virtually every type of biological function. These changes may be specific to hearing (non-syndromic) or can affect additional organs (syndromic), and sometimes require additional environmental triggers for phenotypic expression. We will discuss the normal anatomy and physiology of the hearing apparatus briefly, as well as the prevalence and classification of hereditary deafness. The embryology of the ear, although relevant to a full understanding of some of the developmental cases of syndromic deafness, is beyond the scope of this chapter. The clinical features associated with non-syndromic and syndromic HL, as well as the differential diagnosis, evaluation, and management of an individual with HL is described from a clinical genetics perspective. Recent advances in newborn hearing screening, as well as some of the problems, challenges, and culture of the deaf individual and community will be addressed. Throughout the chapter, current knowledge regarding the presentation, pathophysiology, and molecular aspects of hereditary HL will be summarized. Many genes involved in hereditary HL have been cloned, and the chromosomal locations

of many additional ‘deafness’ genes have been identified. Many of these findings have implications for clinical management and counseling, particularly in the diagnosis of autosomal recessive or X-linked nonsyndromic HL, in the prevention of aminoglycoside-induced ototoxicity in genetically susceptible individuals, and in the implementation of universal newborn hearing screening.

## 142.2 ANATOMY AND PHYSIOLOGY

The ear is divided into external, middle, and inner sections. The external part consists of the pinna and auditory canal, which ends in the tympanic membrane. The tympanic membrane represents the barrier between the external and middle ear. The middle ear is air filled; contains three bones, the malleus, incus, and stapes; and is linked to the oropharynx through the eustachian tube. The middle ear connects to the inner ear at the membranes of the oval and round windows. The inner ear contains the fluid-filled cochlear and vestibular systems, in which the receptors for hearing and equilibrium, the hair cells, are located. The cochlea is formed by the membranous cochlear duct, an elongated tube with  $2\frac{1}{2}$  turns containing the organ of Corti, the scala vestibuli, and the scala tympani, which surround the cochlear duct and connect at its apex.

Hearing occurs when sound reaches the tympanic membrane through the external acoustic meatus, vibrations are transmitted and amplified through the three middle ear bones, and the movement of the footplate of the stapes produces vibration of the oval window membrane. This causes pressure changes at the base of the scala vestibuli. The pressure wave travels first to the apex of the scala vestibuli, connects to the scala tympani, and the energy is then dissipated at the round window. The basilar membrane in the scala tympani vibrates as well, and presses the filaments of the hair cells of the organ of Corti against the tectorial membranes. This deflection of the stereocilia of the outer hair cells (OHC) begins the process of membrane depolarization, which transmits the acoustic signal to the inner hair cells, ultimately leading to auditory nerve activation via the cells of the spiral ganglion. The basal portion of the basilar membrane is maximally responsive to high-frequency sounds, while the apical portion is most sensitive to low-frequency sounds. Activation of the hair cells leads to release of neurotransmitters from the basal part of the hair cells, and activation of the afferent axon of the cochlear nerve. A short introduction to the extraordinary physiology of the 32,000 human cochlear hair cells, and their ability to transmit, by mechanical means, precise information about events taking place in microseconds, can be found in reviews by Hudspeth (1) and by Dror and Avraham (2). The cell bodies of the cochlear nerve are located in the petrous part of the temporal bone, with the afferent axons reaching to the hair cells, while the efferent ones enter the pons and split to go to the ventral and dorsal cochlear nuclei. At least two neurons are required

from here through the superior olive, lateral lemniscus nucleus, and inferior colliculus to the medial geniculate body in the midbrain (many but not all of the fibers cross to the contralateral side, mostly at the level of the pons). From there, the acoustic radiation extends to Heschl’s gyrus in the temporal lobe, where there is a point-to-point correspondence with the cochlea (low frequency to high frequency).

## 142.3 PREVALENCE AND CLASSIFICATION OF HL

### 142.3.1 Prevalence

HL is the most common neurosensory deficit, affecting around 270 million people worldwide. There are at least 36 million deaf and hearing-impaired people in the United States, 2 million of whom have severe-to-profound deafness (3). About 40–50% of individuals aged 65 and above have some degree of age-related hearing impairment (ARHI). Recent evidence suggests that ARHI (presbycusis) occurs due to a combination of environmental and hereditary factors (4,5), and variants in several genes, including the sodium acetyltransferase 2 gene (6), *GRM7*, encoding metabotropic glutamate receptor type 7 (7), and *GRHL2* (see DFNA28) (8) have been implicated in the process of age-related hearing loss (AR HL). Our discussion focuses on hereditary HI of childhood, which comprises most of the severe forms of inherited deafness, and includes examples of several common disorders associated with genetic hearing impairment in adults. It is estimated that 3 per 1000 children under the age of 3 years, and 4 per 1000 children under the age of 19 years, are hearing impaired. HL can be classified according to several parameters, each of which has important implications for diagnosis, prognosis, and therapy, as discussed in the later sections of this chapter.

### 142.3.2 Severity

The ‘loudness’ of a sound is described by the decibel (db), which is the logarithm to the base 10 of the intensity of the sound wave relative to a reference intensity, divided by 10. Most conversational speech occurs at 45–60 db. The severity of the HL is frequently divided into the following categories:

Mild	26–40 dB
Moderate	41–55 dB
Moderately severe	56–70 dB
Severe	71–90 dB
Profound	90+ dB

### 142.3.3 Anatomy/Physiology

The two major subgroups in this classification are conductive and sensorineural HL. In conductive deafness,

the defect resides in the external or middle ear, while in sensorineural deafness (also called perceptive or neural deafness), the abnormality lies somewhere between the hair cells of the cochlea and the auditory regions of the brain. SNHL is frequently subdivided into cochlear and retrocochlear deafness, depending on the localization of the defect. Mixed deafness includes both conductive and sensorineural components.

### 142.3.4 Associated Features

Deafness associated with abnormalities in other organ systems is called syndromic deafness, while deafness not associated with other abnormalities is classified as non-syndromic in nature.

**Time of onset:** If deafness is present at birth or during early infancy, it is considered congenital, and is frequently associated with an inability to acquire normal speech. Hearing impairment occurring subsequently is called either early-onset or late-onset HL, but no clear age brackets differentiate these types.

### 142.3.5 Etiological and Molecular Classification

Hearing impairment may be due to genetic or environmental causes. As described in the following sections, mutations in an ever-growing number of genes have been associated with nonsyndromic and syndromic HL. Molecular data will be used increasingly to define the nature of the hearing impairment, and, in some cases, to suggest anticipatory care strategies. When classifying childhood deafness using some of the criteria outlined here (Table 142-1), the proportion of affected individuals in each group varies among different studies. Although some variation may be related to ethnic and environmental differences, the manner in which patients were selected and the extent of their diagnostic evaluation influence the data. Many patients with apparently idiopathic deafness will eventually be classified more

specifically. Thus any etiological classification should be used only as a general guideline.

In addition to the previously noted difficulties in classifying HL patients, the following features may also distort the true percentages:

- Cases of acquired HL may occur in individuals who have a genetic predisposition to be adversely affected by an environmental factor.
- It is frequently difficult to differentiate between isolated HL due to a genetic defect and that due to an environmental cause, such as prenatal insult e.g. congenital CMV infection, or exposure to certain medications. Also, there is a tendency for parents and well-meaning physicians to attribute HI to some non-genetic cause. On the other hand, a causative insult may have been forgotten or unrecognized.
- Autosomal recessive nonsyndromic deafness may be overestimated, since autosomal dominant deafness with incomplete penetrance, or requiring interaction with environmental factors, might mimic a recessive trait in which both parents appear to be unaffected. Similarly, simplex ADSNHL cases may be misclassified as sporadic due to the lack of affected family members.
- Syndromic deafness may be underestimated, since the syndromic expression might be so mild as to be overlooked, or the syndromic component may develop only at a later time.

Lastly, it should be noted that many cases of congenital deafness are acquired, while many late-onset cases of deafness are genetic or reflect a genetic predisposition to an environmental factor.

## 142.4 ACQUIRED HEARING IMPAIRMENT

HL may be acquired through a variety of physical, chemical and infectious mechanisms. Genetics plays a role both in the occurrence and presentation of these factors, and, sometimes, in the susceptibility of the patient to the agent. Acquired HL may be caused by meningitis, mastoiditis or chronic otitis media, trauma and noise abuse, kernicterus, myxedema and exposure to various pharmaceutical agents (including aminoglycoside antibiotics, quinine, loop diuretics and cis-platinum) and teratogens. Kernicterus, which is mainly due to Rh and ABO blood group incompatibility, and hypothyroidism often have a genetic etiology. A provocative study in mice (9) documented an effect on cochlear development in mice deficient in type 2 iodothyronine deiodinase (D2). The authors suggested complex interaction of the thyroid hormones, D2, and thyroid hormone receptors in the development, onset, and regulation of auditory function. Aminoglycoside ototoxicity demonstrates genetic susceptibility in a significant proportion of cases, as discussed later in the

**TABLE 142-1** Proportion of Deafness by Etiologic Classification

I. Genetic: 35% <sup>a</sup>
1. Nonsyndromic: 60%
a. Autosomal recessive: 70–80%
b. Autosomal dominant: 20–30%
c. X-linked: 1%
d. Mitochondrial, multilocus: <1%
2. Syndromic: 40%
II. Acquired: 35%
1. Prenatal: 20%
2. Perinatal: 20%
3. Later onset: 60%
III. Idiopathic: 30%

<sup>a</sup>Genetic etiology accounts for 50% of prelingual SNHL.

section on mitochondrial inheritance. An early study suggested that HL due to rubella embryopathy occurs predominately in the genetically susceptible fetus (10). Dodson and colleagues (11) proposed a common mechanism for some forms of acquired, late-onset, progressive SNHL in both nongenetic and genetic conditions. They noted that superficial siderosis, representing localized deposition of hemosiderin following recurrent subarachnoid bleeding, may be the cause of HL in patients with bleeding of nongenetic etiology as well as in syndromes such as Alport syndrome and related conditions, hereditary amyloidosis (transthyretin amyloidosis and Muckle-Wells syndrome), Marfan syndrome, neurofibromatosis type 1, and hereditary hemorrhagic telangiectasia. They comment that such a pathogenesis, if proven, would represent a manifestation of deafness caused by genes that are not expressed in the cochlea.

Toxicity associated with a large number of drugs, particularly salicylates, loop diuretics, aminoglycosides and various chemotherapeutic agents, has been associated with SNHL and will not be discussed here (see mitochondrial inheritance for review of aminoglycoside-induced HI in the setting of genetic susceptibility). In addition, a recent study of chronic analgesic use in men showed that acetaminophen and other NSAIDs were associated with an increased risk of SNHL, particularly in younger individuals (12). Sudden-onset HL has been described in patients with chronic hepatitis C treated with pegylated interferon and ribavirin (13,14), and after recreational use of Vicodin (15,16). Otosyphilis (acute luetic hydrops) may be associated with acute onset HL, particularly in the setting of HIV (17), and shows improvement if treatment is initiated promptly (18). Persistent, localized measles virus infection is a major cause of adult-onset HL and is discussed in the section on otosclerosis. SNHL occurs as a secondary phenomenon in long-standing diabetes mellitus, most likely on the basis of chronic microvascular insult and sensory neuropathy. Other environmental exposures, especially cigarette smoking, may play a role in ARHL (19). Notably, a recent study implicates second hand smoke as a cause of low-frequency HL in adolescents (20). In addition to its contribution to presbycusis, noise abuse, in the form of personal stereo usage, has been associated with slight-mild HL in children (21), and may be an early precursor to later, progressive SNHL. Finally, SNHL occurs as a secondary phenomenon in long-standing diabetes mellitus, most likely on the basis of chronic microvascular insult and sensory neuropathy.

### 142.5 TERATOGENIC HEARING IMPAIRMENT

The effects of a number of human teratogens, ranging from infectious agents to rarely used therapeutic modalities, play a significant role in HL. The classical example of an intrauterine viral agent with teratogenic effects on

the human fetus is the rubella virus. Although rubella infection was highly prevalent, with a pandemic occurring in the United States between 1962 and 1965, the widespread use of childhood rubella vaccination, initiated in 1969, has helped control the congenital transmission of this virus in most Western populations. Though congenital cataracts comprised the first reported association between maternal rubella virus and fetal abnormality, the importance of deafness was soon documented. Rubella embryopathy presents a striking pattern of features, including cataracts, congenital cardiovascular defects, microcephaly, and mental retardation, as well as SNHL, which is the most frequent finding. The likelihood of finding an abnormality is closely related to the gestational age at maternal infection, and, although the likelihood of full-blown rubella embryopathy is quite low after 16 weeks, infection at any time in pregnancy may result in hearing impairment.

Congenital cytomegalovirus (CMV) infection, which has long been associated with HL, even in the case of inapparent infection, is now the leading cause of late-onset childhood HL (22,23). It is estimated that of the 20,000–40,000 infants born each year with congenital CMV infection in the United States, 90% have no detectable clinical abnormalities at birth and will not be detected by a clinical examination. Ear anatomy is usually normal, though a Mondini defect may be present occasionally. SNHL occurs in 10–15% of these asymptomatic infants, may present in early childhood and can be unilateral or bilateral. Since CMV-associated HL is often later onset and progressive, affected children may be missed by newborn hearing screening (24). Several studies document detection of CMV DNA using polymerase chain reaction in both Guthrie blood spots (25) and perilymph (26,27). A recent report of PCR-based evaluation of saliva suggested a strategy for targeted newborn screening for CMV (28). These techniques may be particularly important in light of evidence that early ganciclovir treatment of symptomatic children may prevent HL (29–31). The association of SNHL and congenital toxoplasmosis has been reviewed recently (32).

While its importance is muted by the other neurological sequelae, SNHL also occurs as part of the spectrum of untreated congenital syphilis, though it is unlikely to occur in children who are diagnosed at birth and treated appropriately (33).

Fetal alcohol syndrome is characterized by growth failure, microcephaly, typical facies, congenital heart defects, subtle skeletal anomalies, and variable mental retardation. Pinna anomalies and serous otitis media occur frequently, with consequent conductive HL adding to the developmental burden (reviewed by Mulvihill (34)). In a study of affected children referred for ear, nose, and throat evaluation, 4 of 14 demonstrated significant bilateral SNHL (35), suggesting a developmental effect of the inner ear. Though this observation was not



confirmed in a larger study (36), the authors emphasized the importance of early recognition and treatment for HI in children with FASD. Thalidomide has been associated with conductive HL secondary to hypoplasia or atresia of the external acoustic meatus, ossicular fusion or other defects, and inner ear anomalies (37–39). Of note, some cases previously attributed to thalidomide embryopathy represent the spectrum of SALL4-related disorders described later (40). Retinoic acid embryopathy also includes conductive HL secondary to microtia and malformation of the auditory canal (41), as does exposure to mycophenolate mofetil, which may result in craniofacial features overlapping those of Treacher-Collins syndrome or bilateral hemifacial microsomia but is also associated with major cardiac defects (42). Other human teratogens associated with HL include quinine, which induces inner ear abnormalities in rodents, trimethadione, fetal iodine deficiency, and fetal methyl mercury effects.

## 142.6 NONSYNDROMIC HEREDITARY HEARING IMPAIRMENT

About 70% of inherited deafness is not associated with any symptoms or signs apart from the HL, and is called nonsyndromic HL. This has been classified according to the pattern of inheritance into autosomal recessive (DFNB nomenclature assignment), autosomal dominant (DFNA), X-linked (DFNX), mitochondrial (maternal), and modifying loci (DFNM). While not clearly established, there is one report of purportedly Y-linked hearing impairment segregating in a large Chinese kindred (43). There is great clinical and genetic heterogeneity within each of the major categories. Clinical subdivision of each group according to age of onset, severity, and audiometric abnormalities has been attempted, but allows only a very general subclassification. The recent advances in molecular genetics have led to the identification of more than 127 chromosomal regions involved in nonsyndromic HL. At least 69 auditory genes have been identified – 24 autosomal dominant (AD), 40 autosomal recessive (AR), 2 X-linked, and 3 mitochondrial – in addition to a large number of genes for syndromic HL. This allows for a more precise classification of nonsyndromic deafness, for early diagnostic testing and intervention in some cases, and, rarely, for implementation of preventive measures. It should be noted that documentation of the genetic abnormality does not necessarily allow accurate prediction of the clinical phenotype. In addition, mutations in the same gene are known to cause both nonsyndromic and syndromic HL, can cause both AR HL and AD HL, or can cause more severe HI in some patients than in family members who carry the same mutation. Also, from a clinical perspective, it is important to stress the possibility that the initial diagnosis of nonsyndromic deafness may be revised if other symptoms appear later in life, as may occur, for example, in Pendred syndrome and Usher syndrome.

### 142.6.1 Autosomal Recessive Hearing Impairment

AR inheritance accounts for 75–80% of all cases of non-syndromic prelingual deafness (44). Most children with AR HI have congenital HI, are born to hearing parents, and seldom have a history of environmental exposure. Hearing impairment is always sensorineural, and severe, moderate, and early-onset varieties have been described. In general, AR HI is more severe than ADSNHL, and is typically nonprogressive. One of the earliest observations about AR deafness was its striking genetic heterogeneity, since the offspring of two unrelated partners with AR deafness usually have normal hearing. Recently, this concept has been substantiated with molecular tools. Thus far more than 50 different chromosomal regions have been found to contain genes leading to AR HL (45). These AR loci are denoted DFNB and are numbered in order of identification. For 40 of these regions, the genes responsible for the HL have been identified. To date, only 5 genes, *MYO3A* (DFNB30), *PJVK* (DFNB59), *LOXHD1* (DFNB77), and, occasionally, *TMPRSS3* (DFNB8/10) and *GJB2* (DFNB1), have been associated with progressive AR hearing impairment.

Because of its unexpectedly high contribution to non-syndromic hereditary HL, the most important gene is gap junction protein beta-2 (*GJB2*), which codes for the connexin (Cx) 26 protein:

- *GJB2* mutations account for approximately 50% of ARSNHI (DFNB1) and some cases of ADSNHI (DFNA3)
- *GJB2* mutation is the most common genetic cause of prelingual SNHL
- 35delG (a deletion of one in a string of six guanine nucleotides) comprises 70% of mutant alleles in ethnically diverse DFNB1 kindreds and represents a deletion hot spot (46)
- 35delG accounts for 80% of DFNB1 in Mediterraneans (47)
- *GJB2* mutations are found in 10% of ‘sporadic’ cases of nonsyndromic SNHI (48)
- The 35delG carrier rate is approximately 1 in 40 in the western hemisphere
- The 167delT mutation is common in Ashkenazi Jews with a carrier rate of 1 in 25 (49)
- The 235delC mutation occurs commonly in Asian populations

Mutations in *GJB2* are responsible for DFNB1, and account for half of all autosomal recessive deafness cases in various populations (49–51). *GJB2*-related HL is typically bilateral and nonprogressive, though progression has been noted. In over 80% of cases, the HI falls into the severe-profound range. Since the gene is very small, consisting of a single coding exon, and since two specific mutations, 35delG and 167delT, account for a significant proportion of the affected alleles in many populations,

effective screening for *GJB2* mutation is feasible. Due to the high prevalence of the 167delT mutation among Ashkenazi Jews, carrier screening has been suggested for this population. Mutations in *GJB2* make a smaller contribution to prelingual deafness in other populations and the mutation spectrum is different, with the 235delC mutation playing a prominent role in Asian populations (52–54). The genetic epidemiology, prevalence rates, and genotype–phenotype correlation of *GJB2* alleles were reviewed by Kenneson and colleagues (51). Genotype–phenotype correlations have also been described by Azaiez and coworkers (54), who suggested that at least one noncompensating mutation outside the coding region of *GJB2* contributes to HL associated with the DFNB1 locus. SNHL at the DFNB1 locus also may be caused by digenic interaction of a *GJB2* mutation and a deletion of *GJB6* (connexin 30). As described by del Castillo and colleagues (55), this 309-kb deletion is the second most common mutation found in nonsyndromic prelingual deafness in the Spanish population. This observation has been confirmed in other populations (56,57). The KID syndrome of keratitis, ichthyosiform erythroderma, deafness, and other findings is associated with progressive neovascularization of the cornea and of corneal grafts such that deafness-blindness may occur. KID syndrome has been associated with some mutations in *GJB2*, (58), providing a striking example of syndromic and nonsyndromic HL resulting from mutations in the same gene. Because a recurrent mutation (D50N) in *GJB2* is observed in Japanese patients with nonsyndromic HL (59) and in Austrian KID syndrome patients, Janecke and colleagues (60) suggested that the phenotype caused by this mutation depended on the genetic background of the affected individual.

DFNB2 and DFNB3 (see Table 142-2) are caused by mutations in the genes that encode the unconventional myosins 7A (61,62) and 15 (63), respectively. These myosins interact with actin in the hair cells of the inner ear, and affect stereocilia organization and hair cell contractility (see Usher syndrome). Myosin 7A is also associated with ADSNHL (64). DFNB4 is caused by mutations in a chloride-iodide-bicarbonate transporter (65,66) called *SLCA4*, which encodes pendrin (discussed in the section on Pendred syndrome). DFNB6 is caused by loss of function mutations in a novel gene, *TMIE*, which result in severe-to-profound prelingual deafness (67). Studies in Turkish families identified a founder mutation responsible for about 3% of ARSNHL in Turkish families and about 12% in families from southern Turkey (68). DFNB7 and DFNB11 are caused by mutations in a novel gene, *TMC1* (transmembrane cochlear expressed gene 1), which is required for physiological hair cell function (69). DFNB8 and DFNB10 are caused by mutations in a type II transmembrane serine protease [*TMPRSS3*], the first description of a protease involved in HL, manifesting both congenital HL (DFNB10) and childhood-onset, progressive HL (DFNB8) (70). *TMPRSS3* regulates the activity of an

epidermal sodium channel, ENaC, suggesting that it is an important regulator of sodium concentration in the cochlea (71).

DFNB9 is caused by mutations in *OTOF*, the gene encoding otoferlin (72), a protein involved in trafficking (exocytosis) synaptic vesicles of inner hair cells (73), which works in concert with myosin VI (DFNB37/A22) and is the proposed calcium sensor during inner hair cell exocytosis (74). A founder mutation in *OTOF*, Q829X, is responsible for 4–5% of sporadic and AR cases of non-syndromic SNHL in the Spanish population (75). HI associated with *OTOF* mutations is generally congenital and may present as an auditory neuropathy (see below) with early preservation and later loss of evoked otoacoustic emissions (OAE), raising a concern that neonatal screening utilizing transient evoked OAEs will miss such children (76). *OTOF* is also associated with later-onset HL at the first AR auditory neuropathy locus, AUNB1 (see below). DFNB12 is caused by an allelic mutation of the novel cadherin-like gene *CDH23* (77), which is also the gene implicated in Usher syndrome at the USH1D locus (see below). Mutations in *GIPC3*, which encodes the GAIP C-terminus-interacting protein 3, one of a protein family which interacts with a variety of other proteins involved in signal transduction, vesicular trafficking, endocytosis and neurotransmitter release, are responsible for HL at the DFNB15/72/95 loci (78,79). Mouse and human studies suggest that normal function of this gene, which is highly expressed in the inner hair cells and spiral ganglion, appears to be critical for the acquisition and propagation of acoustic signals. DFNB16 is caused by mutations in *STRC*, encoding a stereocillin expressed only in the sensory hair cells, and is associated with the stereocilia, the stiff microvilli forming the structure for mechanoreception of sound stimulation (80).

DFNB18 is caused by mutations in the alternatively spliced exons of *USH1C*, which is also a cause of Usher syndrome (81,82). DFNB21 is associated with mutations in the gene *TECTA*, encoding a protein constituent of the tectorial membrane,  $\alpha$ -tectorin, which is in contact with the sensory hair cells via the stereocilia (83). DFNB22, caused by mutations in the gene *OTOA*, which encodes the protein otoancorin, is involved in the attachment of the inner ear acellular gels to the apical surface of the underlying nonsensory cells (84). DFNB23 is caused by missense mutations of *PCDH15*, which encodes the protein protocadherin 15. This protein localizes to inner ear hair cell stereocilia and plays a role in the morphogenesis and cohesion of stereocilia bundles of the inner ear (85). Nonsense mutations in *PCDH15* are associated with another Usher syndrome locus, USH1F. Mutations in *RDX*, which encodes radixin, an ezrin-moesin-like protein critical for anchoring actin to sensory hair cell membranes, are associated with HL at the DFNB24 locus (86). Congenital severe-profound SNHL, sometimes associated with vestibular dysfunction is caused by mutations in

**TABLE 142-2 Autosomal Recessive (DFNB) Deafness Genes**

Locus	Gene	Protein/Function
DFNB1	<i>GJB2</i> <sup>a</sup>	Connexin 26; gap junction protein, major gene associated with prelingual ARSNHL
DFNB2 (DFNA11) (USH1B)	<i>MYO7A</i>	Myosin VIIA; stereocilia organization, hair cell contractility, transmembrane protein transport
DFNB3	<i>MYO15</i>	Myosin XV; interacts with actin and myosin VIIA
DFNB4	<i>SLC4A</i>	Pendrin; chloride-iodide-bicarbonate transporter
DFNB6	<i>TMIE</i>	Novel transmembrane inner ear expressed gene; needed for sensory hair cell development/maturation
DFNB7/11 (DFNA36)	<i>TMC1</i>	Transmembrane channel-like protein1; required for normal hair cell function
DFNB10	<i>TMPRSS3</i> <sup>b</sup>	Transmembrane serine protease 3, sodium regulator
DFNB9 (AUNB1)	<i>OTOF</i>	Otoferlin; synaptic vesicle trafficking, may present with auditory neuropathy
DFNB12 (USH1D)	<i>CDH23</i>	Cadherin 23; interacts with myosin VIIA and harmonin in USH supercomplex
DFNB15/72/95	<i>GIPC3</i>	GAIP PDZ domain protein 3; critical for IHC/OHC and spiral ganglion maintenance, signal transduction
DFNB16	<i>STRC</i>	Stereocillin; stiffens hair cell microvilli to assist in mechanoreception of sound
DFNB18 (USH1C)	<i>USH1C</i>	Harmonin; associates with a scaffold-like protein encoded by SANS, and all USH proteins
DFNB21 (DFNA8/12)	<i>TECTA</i>	$\alpha$ -Tectorin; tectorial membrane element
DFNB22	<i>OTOA</i>	Otoancorin; acellular gel component
DFNB23 (USH1F)	<i>PCDH15</i>	Protocadherin 15; morphogenesis and adhesion of stereocilia bundles, interacts with USH proteins
DFNB24	<i>RDX</i>	Radixin; anchors actin to sensory hair cell membrane
DFNB25	<i>GRXCR1</i>	Glutaredoxin, cysteine-rich 1; predicted protein modification via reversible protein S-glutathionylation
DFNB28	<i>TRIOBP</i>	TRIO and F-actin binding protein; needed for actin/stereocilia rigidity and flexible stereocilia rootlets
DFNB29	<i>CLDN14</i>	Tight junction claudin-14; structural support of auditory neuroepithelium in the organ of Corti
DFNB30	<i>MYO3A</i> <sup>a</sup>	Myosin IIIA; unconventional myosin, motor protein
DFNB31 (USH 2D)	<i>WHRN</i>	Whirlin; stereocilia elongation, critical USH protein, interacts with harmonin and SANS
DFNB35	<i>ESRRB</i>	Estrogen receptor-related receptor B; role in cochlear development and maintenance
DFNB36	<i>ESPN</i>	Espin; calcium-insensitive actin binding
DFNB37 (DFNA22)	<i>MYO6</i>	Myosin VI; unconventional myosin with multiple cellular functions
DFNB39	<i>HGF</i>	Hepatocyte growth factor; hair cell integrity
DFNB42	<i>ILDR1</i>	Insulin-like domain-containing receptor 1; unknown
DFNB49	<i>MARVELD2</i>	Tricellulin; tight junction protein critical for epithelial barrier maintenance in inner ear compartments
DFNB53 (DFNA13)	<i>COL11A2</i>	Type XI, alpha 2 collagen; fibrillar extracellular matrix component, tectorial membrane fibrils
DFNB59	<i>PJVK</i> <sup>a</sup>	Pejvak; Gasdermin family member, homology to DFNA5, cochlear hair cell and neuronal function
DFNB61	<i>SLC26A5</i>	Prestin; solute load carrier family, cochlear outer hair cell elongation, regulated by thyroid hormones
DFNB63	<i>LRTOMT</i>	Catecholamine-O-methyltransferase (COMT2); High cochlear expression, exact role unknown
DFNB67	<i>LHFPL5</i>	LHFP-like protein 5; transmembrane protein localized to mouse inner and outer hair cell stereocilia, function unknown
DFNB73	<i>BSND</i>	Barttin; Bartter syndrome gene, regulates potassium potassium recycling via chloride channel
DFNB74	<i>MSRB3</i>	Methionine sulfoxide reductase; recycles oxidized proteins, LOF may cause apoptosis
DFNB77	<i>LOXHD1</i> <sup>a</sup>	Lipoxygenase homology domain-containing 1 protein; highly conserved stereociliary protein
DFNB79	<i>TPRN</i>	Taperin; multiple functions, modulates actin dynamics
DFNB82	<i>GPSM2</i>	G-protein signaling modulator; maintains cell polarity
DFNB84	<i>PTPRQ</i>	Protein tyrosine phosphatase receptor Q; Interacts with myosin 6, stabilizes stereociliary actin ankle links
DFNB91	<i>SERPINB6</i>	Encodes a SERPIN peptidase inhibitor; may protect against stress-induced lysosomal leakage

<sup>a</sup>These genes may present with progressive hearing loss.

<sup>b</sup>DFNB8 onset is at age 8–10 with rapid progression; DFNB10 onset is congenital, stable.

*GRXCR1* at the DFNB25 locus (87). The glutaredoxin (GLX) domain is predicted to function in protein modification through the process of reversible S-glutathionylation, which affects protein localization and activity. This highly conserved gene is expressed primarily in embryonic cochlea. Pirouette (pi) mouse strains with spontaneous *Grxcr1* mutations have thin, foreshortened sensory hair cell stereocilia (88). *TRIOBP*, which encodes a filamentous actin-binding protein, is associated with HL at the DFNB28 locus (89,90). Kitahiri and co-authors (91) noted that deafness-causing mutations are located in exon 6 of *TRIOBP* and affect two of the three alternatively spliced forms of the protein,

*TRIOBP4* and *TRIOBP5*. They also described a mouse model, which demonstrated that normal *TRIOBP* promotes dense bundling of actin filaments, which allows mechanical rigidity of the stereocilia and ‘durable flexibility’ at the tapered end of actin. DFNB29 is caused by mutations in the gene encoding tight junction claudin-14 (*CLDN14*), which localizes to the sensory epithelium of the organ of Corti and likely provides structural support for the auditory neuroepithelium (92). DFNB30 is caused by mutations in *MYO3A*, encoding the unconventional myosin IIIA (93), another molecular motor protein, and one of the genes associated with progressive ARSNHL. DFNB31 results from



mutations in the gene *WHRN*, which encodes the scaffold protein whirlin, a PDZ domain molecule involved in stereocilia elongation (94), which also causes Usher syndrome, type 2D (95).

DFNB35 is caused by mutations in *ESRRB* (96), which encodes an estrogen-related receptor. Normal *ESRRB* expression appears to be important in both cochlear development and postnatal function. DFNB36 is caused by mutations in the gene *ESPN*, which encodes a calcium-insensitive actin-binding protein, espin, and presents as deafness with vestibular dysfunction and areflexia (97). DFNB37 is due to mutations in *MYO6*, which encodes the unconventional myosin VI protein. *MYO6* is involved in many processes in the inner ear, including membrane trafficking, recycling, cell movement and endocytosis (98), and has been proposed to interact with otoferlin (DFNB9) in the recycling of inner hair cell synaptic vesicles (74). Schultz and colleagues showed that mutations in *HGF*, a potent mitogen whose multiple isoforms are widely expressed in embryonic and adult tissues, result in profound, prelingual SNHL at the DFNB39 locus (99). They developed a conditional mouse *Hgf* knockout model which showed multiple cochlear anomalies including disorganization of the tectorial membrane, thin, flat stria vascularis, hypoplastic spiral ganglion and a graded pattern of outer hair cell degeneration ranging from complete loss of basilar OHC to milder involvement of apical cells. DFNB42 is caused by mutations in the insulin-like domain-containing receptor 1 gene, *ILDR1*, which encodes a transmembrane receptor of unknown function expressed in the vestibule and in cochlear hair cells and supporting cells during mouse embryogenesis (100).

Affected individuals carry truncating mutations, suggesting that the severe-profound, bilateral, prelingual SNHL is due to loss of function of the gene. Deafness at the DFNB49 locus results from mutations in *TRIC* (renamed *MARVELD2*, for MARVEL domain-containing protein 2), which encodes tricellulin, a tight-junction protein widely expressed in epithelia (101). Tricellulin appears to be an integral element in maintaining the structure and function of epithelial barriers in the various compartments of the inner ear. Some mutations in *COL11A2* (see DFNA13 and Stickler syndrome) result in AR SNHL at the DFNB53 locus (102). Molecular studies implicated *PJVK*, which encodes pejavakin, a member of the same family of highly conserved vertebrate proteins as DFNA5, in auditory neuropathy associated with the DFNB59 locus (103). DFNB59 is the first example of a human gene involved in neuronal HL. Missense mutations are associated with a cochlear phenotype, while nonsense mutations result in progressive SNHL as well as vestibular dysfunction (104). A motor protein, prestin, which is expressed dramatically in the cochlear outer hair cells, has been implicated as the cause of nonsyndromic SNHL in several families (DFNB61). The prestin gene is a member of the solute load carrier

superfamily (specifically, *SLC26A5*) and mitigates rapid elongation of the outer hair cells (105). Prestin expression is regulated by thyroid hormones, suggesting that HL associated with hypothyroidism may be mediated by inadequate prestin function (106).

DFNB63 results from mutations in the gene *LRTOMT*, a leucine-rich transmembrane O-methyltransferase (catecholamine-O-methyltransferase 2 or COMT2), which is highly expressed in the cochlear and vestibular hair cells, implicating catecholamine catabolism in the pathogenesis of the hearing impairment (107,108). Mutations in the lipoma HMGIC fusion partner-like 5 (*LHFPL5*), gene, encoding a transmembrane protein, are associated with SNHL at the DFNB67 locus (109,110). Although its homology to tmhs, the gene responsible for the ‘hurry scurry’ mouse phenotype, suggests an important role in normal cochlear function (111), the exact role of this gene is not well understood. *BSND*, the gene which causes Bartter syndrome, is associated with HL at DFNB73 (112). Though classified as a form of nonsyndromic ARSNHL, some affected individuals may manifest mild symptoms of Bartter syndrome. *MSRB3* encodes a methionine sulfoxide reductase, important in restoring oxidized proteins to their functional form. Ahmed and colleagues (113) demonstrated that functional null mutations of *MSRB3*, which encodes a methionine sulfate reductase, are responsible for human deafness DFNB74, and postulated that abnormal or absent *MSRB3* activity might lead to unprogrammed caspase-activated apoptosis, which may be particularly damaging to the mitochondria of cochlear cells. Along with *TIMM8A*, the gene responsible for the deafness–dystonia–optic atrophy syndrome (Mohr–Tranebjaerg syndrome), *MSRB3* provides an example of a nuclear-encoded gene with critical mitochondrial function. Progressive SNHL at the DFNB77 locus is caused by mutations in *LOXHD1* (114). A mouse model of *LOXHD1* demonstrated expression along the membranes of mature hair cells, which were normally formed, but showed functional perturbation and eventual cell death. *TPRN*, encoding taperin, is associated with HL at the +DFNB79 locus. Taperin localizes to the stereocilia ankle links, and may have multiple functions in the vestibule and organ of Corti, including modulation of actin dynamics (115). Walsh et al. (116) found that mutations in the gene *GPSM2*, which encodes G-protein signaling modulator 2, a G-protein activator, is associated with HI at the DFNB82 locus. Mutations in *PTPRQ*, which encodes the protein tyrosine phosphatase receptor Q, cause congenital, progressive, moderate-profound SNHL and vestibular dysfunction at the DFNB84 locus (117). Evidence suggests that myosin 6 and PTPRQ protein interact to stabilize the stereocilia actin ankle-link attachment to the overlying membrane in cochlear and vestibular hair cells (118). HL at the DFNB91 locus results from mutations in *SERPINF6*, which encodes an intracellular protease inhibitor expressed in inner hair cells. Mutation results in absent protease function, which



may lead to stress-induced lysosomal ‘leakage’ with resultant hearing impairment (119). *GPSM2*, the gene responsible for HL at the *DFNB82* locus (116), shows widespread cochlear expression in a mouse model and is likely involved in determination of cell polarity.

### 142.6.2 Autosomal Dominant

AD inheritance accounts for approximately 20–25% of all cases of nonsyndromic childhood deafness (44). The following clinical subdivisions have been described: congenital severe-profound, progressive early onset, unilateral, low frequency, mid-frequency, and high-frequency progressive. As a rule, unilateral or mild bilateral HL and progressive HL are much more likely to occur on an AD rather than an AR basis. The HL is usually sensorineural, but conductive HL due to otosclerosis may present in childhood (see below). AD hearing loss (ADHL) also shows striking heterogeneity, though no single locus accounts for a significant proportion of cases, in contrast to the common *GJB2* (connexin 26) mutations responsible for the major portion of AR hearing loss. More than 50 different chromosomal regions identified so far contain genes leading to ADHL (45). The ADHL loci are designated DFNA and encompass loci from DFNA1 to DFNA60. DFNA8/12, DFNA 20/26, and DFNA6/14 are attributed to three common genes, *TECTA*, *ACTG1*, and *WFS1*, respectively. Two of the regions, DFNA2 and DFNA3, contain at least two genes that can cause ADHL. To date, 24 different genes have been identified as causing ADHL. Three of these, *GJB2*, *MYO7A*, and *TECTA*, have been described as causes of AR hearing loss. Two additional gap junction proteins, connexin 31 (*GJB3*) and connexin 30 (*GJB6*), have been found to cause some cases of HI at the DFNA2 and DFNA3 loci, respectively (120,121). Interestingly, DFNA2 can also be caused by a mutation in a gene encoding a novel potassium channel, *KCNQ4* (122) in cochlear outer hair cells, and DFNA3 can be caused by mutations in *GJB2*. Mutations in *KCNQ4* show a dominant-negative effect resulting in abnormal channel trafficking at the outer hair cell surface (123) (Table 142-3).

DFNA1 was the first ADSNHL locus, and was notable because of the presentation of low-frequency hearing loss (Konigsmark syndrome). HI is caused by mutations in *DIAPH1*, one of a family of genes involved in cell movement and cell polarity (124), which regulates actin polymerization and the interactions of the actin cytoskeleton with microtubules in the periphery of cochlear hair cells. *DIAPH1* is regulated by *MITF*, one of the genes involved in Waardenburg syndrome (WS) (125), suggesting a mechanism by which *MITF* mutations lead to HI in WS. DFNA4 is caused by mutations in the nonmuscle myosin heavy-chain gene, *MYH14* (126), encoding an ATP-dependent molecular motor protein which helps regulate cell motility, polarity and cytokinesis via its interactions with the actin cytoskeleton (127). Although

truncating mutations have been associated with HI at the DFNA5 locus for some time (128), the function of DFNA5 protein remained elusive, despite observations that the gene is frequently silenced in a variety of common tumors. Recently, Op de Beeck and colleagues (129) summarized prior data and presented an elegant series of studies demonstrating that DFNA5 promotes apoptosis in both the wild type and mutant states, and plays a role in cell survival. HI at the DFNA6/14/38 locus is caused by mutations in the Wolfram syndrome 1 gene (*WFS1*), and is a common cause of low frequency, slowly progressive, SNHL (130,131). DFNA8/14 is associated with the gene *TECTA*, which also causes ARSNHL at the DFNB21 locus.

A recent study in Spanish and American subjects (132) showed that *TECTA* mutations comprise a common form of ADSNHL and found that, in addition to mutations in the ZP domain, mutations in the N-terminal region of  $\alpha$ -tectorin are associated with mid-frequency HI. DFNA9, discovered using a tissue-specific approach to gene discovery in the auditory system, is caused by mutations in the novel cochlear gene *COCH*. Cochlin, the *COCH* gene product, shows homology to molecules involved in a variety of functions ranging from hemostasis to immune regulation to extracellular matrix assembly. Mutations in *COCH* cause progressive SNHL with vestibular pathology (133). DFNA10 is due to mutations in the activator gene *EYA4* and causes late-onset HI (134). DFNA11 is caused by mutations in the gene *MYO7A* (see DFNB2 and USH1B, which are also attributed to mutations in *MYO7A*). DFNA13, caused by mutations in the gene *COL11A2* (135), affects the triple-helix domain of the collagen protein, localizes to the tectorial membrane in *COL11A2* knockout mice, and causes mid-frequency SNHL. DFNA15 (at the 5q31 locus also assigned to DFNA54) is caused by mutations in *POU4F3*, which encodes a transcription factor (136). DFNA17 is due to mutations in *MYH9*, a nonmuscle myosin heavy-chain gene, and causes a phenotype of progressive HL and cochleasaccular degeneration (137), most likely because of a dominant-negative effect on myosin filament assembly (138), although the pathogenesis of the HI unclear. *MYR9* mutations are also associated with a spectrum of syndromic features including congenital thrombocytopenia with large platelets, white cell inclusions, high-frequency SNHL, presenile cataracts and glomerulonephritis (variously labeled Fechtner–Epstein syndrome, Sebastian syndrome, May–Hegglin anomaly) (139), which must be distinguished from Alport syndrome.

DFNA20/26 is caused by mutations in the cytoplasmic  $\gamma$ -actin gene, *ACTG1*, which represents the first gene to be associated with HL that can be attributed to cytoskeletal (nonmuscle) actin (140,141). Morin and colleagues (142) provided data indicating that mutations in *ACTG1* caused progressive HI due to hair cell

**TABLE 142-3 Autosomal Dominant (DFNA) Deafness Genes**

Locus	Gene	Protein/Function
DFNA1	<i>DIAPH1</i> <sup>a</sup>	Drosophila diaphanous protein 1 analog; role in cell polarity and movement; may regulate actin polymerization
DFNA2	<i>GJB3</i> <i>KCNQ4</i>	Connexin 31; gap junction constituent Potassium channel protein
DFNA3 (DFNB1)	<i>GJB2</i> <i>GJB6</i>	Connexin 26; gap junction constituent Connexin 30; gap junctions
DFNA4	<i>MYH14</i>	Nonmuscle myosin heavy chain 4
DFNA5	<i>DFNA5</i>	DFNA5; novel protein, homologous to Pejvakin, promotes normal and unscheduled apoptosis
DFNA6/14/38	<i>WFS1</i> <sup>a</sup>	Wolframin; integral transmembrane protein, also associated with Wolfram syndrome
DFNA8/12 (DFNB21)	<i>TECTA</i>	$\alpha$ -Tectorin; tectorial membrane element
DFNA9	<i>COCH</i>	Cochlin; cochlear and vestibular function
DFNA10	<i>EYA4</i>	Threonine, tyrosine phosphorylase; required for postnatal organ or Corti function
DFNA11 (DFNB2) (USH1B)	<i>MYO7A</i>	Myosin VIIA; stereocilia organization and hair cell contractility
DFNA13 (DFNB53)	<i>COL11A2</i>	Collagen 11A2, fibrillar ECM protein
DFNA15 (overlaps DFNA54)	<i>POU4F3</i>	A POU domain gene; DNA transcription factor; involved in terminal neural cell differentiation
DFNA17	<i>MYH9</i>	Nonmuscle myosin heavy chain 9; interacts with actin cytoskeleton to regulate cell polarity and cytokinesis, causes cochleosaccular degeneration
DFNA20/26	<i>ACTG1</i>	$\gamma$ -Actin; cytoskeletal nonmuscle actin expressed in hair cells
DFNA22 (DFNB37)	<i>MYO6</i>	Unconventional myosin; membrane trafficking, recycling, endocytosis and cell movement
DFNA28	<i>GRHL2</i>	Grainyhead, Drosophila homologue 2; transcription factor expressed in cochlear epithelial cells
DFNA36 (DFNB7/11)	<i>TMC1</i>	Transmembrane channel-like protein 1; required for cochlear hair cell function
DFNA39	<i>DSPP</i>	Dentin sialophosphoprotein; noncollagenous matrix protein; some kindreds have dentinogenesis imperfecta
DFNA44	<i>CCDC50</i> <sup>a</sup>	Ymer; promotes EGF-mediated cell signaling
DFNA48	<i>MYO1A</i>	Unconventional myosin IA; molecular force sensor
DFNA50	<i>MIR96</i>	Micro RNA 96; proposed to regulate functionally necessary gene expression in cochlear hair cells
DFNA51	<i>TJP2</i>	Tight junction protein 2; organization of intercellular junctions, may interact with claudin
DFNA64	<i>SMAC/DIABLO</i>	Direct IAP-binding protein with low pI; promotes caspase activation in the mitochondrial cytochrome C apoptosis pathway

<sup>a</sup>These genes may be associated with low-frequency hearing loss.

cytoskeletal deterioration with age. DFNA22 is caused by a mutation in the gene *MYO6*, which encodes an unconventional myosin protein (143) involved in intracellular trafficking. DFNA28 is caused by a mutation of another transcription factor gene, *GRHL2* (originally called *TFCP2L3*) (144), which has also been implicated in AR HL (8). DFNA36 is allelic with DFNB7/B11 and is caused by mutations in a novel gene, *TMC1*, encoding a transmembrane protein required for cochlear hair cell function (71). Mutations in *DSPP*, which encodes dentin sialophosphoprotein, are responsible for nonsyndromic, second-third decade onset, high-frequency SNHL at the DFNA39 locus, though some kindreds show an association with dentinogenesis imperfecta, type 1 (145). *CCDC50*, which encodes a unique protein, Ymer, an effector of epidermal growth factor (EGF)-mediated cell signaling, is associated with postlingual, progressive HI at the DFNA44 locus (146). Ymer is widely expressed but may be particularly important in the microtubular cytoskeleton. *CCDC50* mutations result in cytoskeletal disorganization, possibly related to failure of normal downregulation of an EGF receptor. The HI involves the low-mid frequency range initially but progresses to include high-frequency HI over time. DFNA48 is caused by mutations in the *MYO1A* gene, which encodes an

unconventional myosin protein (147), and functions as an exquisitely sensitive tension meter, which is critical to the detection of auditory stimuli and in other important cellular functions (148).

HI associated with *MYO1A* mutations is postlingual, highly variable, typically moderate-severe high-frequency loss, which usually does not progress to profound deafness. Mutations in *MIR96*, which encodes a micro RNA expressed in vestibular and cochlear hair cells, cause HI at the DFNA50 locus because of altered expression profiles of genes critical to hair cell function (149). A mouse model of this disorder (150) demonstrated anomalous hair cells and progressive HL in heterozygotes and absent cochlear responses in homozygotes. An inverted genomic duplication including the entire wild type *TJP2* gene is responsible for HL at the DFNA51 locus (151), apparently on the basis of increased apoptosis of auditory hair cells, which may provide a model for age-related presbycusis. A recent report (152) describes HI at the DFNA64 locus resulting from mutation in *SMAC/DIABLO*, which encodes a proapoptotic mitochondrial protein. In addition to genes associated with specific DFNA loci, *CRYM*, which is expressed in the cochlea and vestibule, has also been implicated in AD SNHL (153). This gene encodes the human  $\mu$ -crystallin protein, which is involved in

high-affinity binding of thyroid hormones. HI is thought to be due to impaired Na,K-ATPase-mediated potassium recycling in cochlear fibrocytes (154).

Of great interest, mutations in many of the autosomal NSHL genes cause more than one type of hearing impairment. Mutations in *GJB2* (connexin 26) have been found in occasional families with nonsyndromic ADHL (155), and may also be associated with HI and dermatological findings including KID syndrome. Mutations in myosin VIIA (*MYO7A*) can cause AD nonsyndromic HL as well as Usher syndrome type IB; mutations in *USH1C* cause both DFNB18 and Usher syndrome type IC; mutations in *CDH23* also cause Usher syndrome ID, and some missense mutations are associated with presbycusis; and *PCDH15* mutations cause DFNB23 as well as Usher syndrome type IF. Genotype–phenotype correlation has been demonstrated with missense mutations generally causing the nonsyndromic, milder picture, and nonsense mutations causing Usher syndrome. Some mutations in *SLCA4* (PDS) cause Pendred syndrome. DFNB7/11 and DFNA36 result from mutations in *TMC1*. Mutations in *TECTA* (DFNB21) are also responsible for ADHL associated with the DFNA8/A12 locus (156), which also presents with variable phenotype. Mutations in *MYO6* cause AR HL (DFNB37) and AD HL (DFNA22). Mutations in *COL11A2* may cause AR (DFNB53), AD (DFNA13) or syndromic HL (Stickler syndrome, type III). Autosomal recessive *OTOF* (otoferlin) mutations are among the causes of nonsyndromic auditory neuropathy (76).

Finally, as noted above, dominant mutations in *MYR9*, which has been identified as the cause late-onset HL at the DFNA17 locus, have also been associated with the rare syndrome of congenital large platelets, thrombocytopenia, late-onset SNHL, cataracts and glomerulonephritis (139). In the case of *MYR9*-related disorders, as in the *USH* genes and in a number of other examples of mutations in a single gene causing both nonsyndromic and syndromic HL, clear genotype–phenotype correlations have been established. The importance of variable alleles and modifier genes in the phenotypic expression of deafness genes was reviewed by McHugh and Friedman using the genes encoding cadherin 23 and wolframin as representative examples (157).

### 142.6.3 X-Linked

X-linked inheritance, which is designated DFNX (previously DFN), accounts for only a small proportion (1%) of nonsyndromic deafness. For families in which two or more males have HL, X-linked inheritance should be considered. Because AR inheritance is so much more common than X-linked inheritance, the diagnosis of X-linked deafness should be made with caution, especially when providing genetic counseling. Most cases of X-linked nonsyndromic deafness are sensorineural with congenital, early, or second-decade onset. An important exception is the mixed type of hearing impairment

associated with fixation of the stapedial footplate and raised pressure in the perilymph, which is now designated as DFNX2 (158). Diagnosis of this condition is critical, since a perilymphatic ‘gusher’ with ensuing total HL and onset or worsening of vestibular symptoms occurs when stapedectomy is performed. The gene for this condition has been identified as the transcription factor *POU3F4* (159,160). It is important to note that heterozygous females in DFN kindreds may have later onset of milder, more stable HL than their affected male relatives. Females with DFNX2 are also at risk of perilymphatic gusher, and may receive a misdiagnosis of otosclerosis, leading to the possibility of a disastrous stapedectomy.

DFNX1 is caused by mutations in the gene *PRPS1*, which encodes phyosphoribosylpyrophosphate synthetase 1 (161), which is important for purine and pyrimidine biosynthesis. Abnormal expression of this gene is also associated with three syndromes with HL; X-linked recessive Charcot-Marie-Tooth with HL, ARTS syndrome, and PRPS superactivity syndrome, which have been reviewed by de Brouwer et al. (162), who also suggested the possibility of treatment with S-adenosylmethionine. DFNX4 (formerly DFN6) is caused by mutations in the gene *SMPX* (small muscle protein, X-linked), which is responsive to changes in physical force and may be important in the maintenance of inner ear cells (163,164). At least one other chromosomal locus involved in nonsyndromic hearing impairment, DFNX3, has been proposed to be on the X chromosome. Mutations in dystrophin have been suggested as the etiology of DFNX3, which maps within the Duchenne muscular dystrophy (DMD) gene (165); however, this postulate has not been confirmed, despite evidence from the *mdx* mouse model of DMD, in which SNHL loss is a component (166) (Table 142-4).

### 142.6.4 Auditory Neuropathy

Auditory neuropathy (also referred to as auditory neuropathy/auditory dys-synchrony, AN/AD) is defined as a HL associated with absent or abnormal auditory brainstem evoked responses and or pure tone audiometry in the presence of normal cochlear outer hair cell function, as indicated by preservation of OAEs. The finding of a normal tympanogram, persistent OAEs or CM (cochlear microemissions) and absent ABRs is consistent with a pathogenesis distal to the outer hair cells (i.e. involving IHC and/or auditory nerve). Hence, the lesion must be at the level of the inner hair cells (IHC), the auditory nerve, or the IHC/auditory nerve synapse. Although auditory neuropathy may accompany peripheral neuropathies such as Charcot-Marie-Tooth disease or Friedrich ataxia, it occurs most commonly as a sporadic or autosomal recessive condition. The most common known genetic cause of auditory neuropathy is mutation in the otoferlin gene, *OTOF*, described under DFNB9 and assigned the nomenclature AUNB1 (auditory neuropathy, AR, 1).

**TABLE 142-4 Nonsyndromic X-Linked Deafness (DFNX) Loci**

DFNX1	Postlingual, onset usually 5–15 years in males, fifth decade in females	<i>PRPS1</i>	Nucleotide biosynthesis pathway
DFNX2	Congenital mixed hearing loss associated with stapes fixation and perilymphatic gusher; females may have later onset hearing loss, gusher	<i>POU3F4</i>	Nuclear transcription factor
DFNX3	Congenital profound SNHL; mild-moderate high-frequency loss in adult females	Xp21.2	Gene not identified, may be DMD or is imbedded within the dystrophin gene
DFNX4	X-linked progressive deafness with onset about age 3–7 in males; second to third decade in females with initial high-frequency loss	<i>SMPX</i>	Pressure-responsive protein may protect/maintain cochlear cells exposed to mechanical stress

Recognition of this relatively common cause of SNHL is important and may be hindered in screening programs relying on evaluation of evoked OAEs (76). In addition to the typical congenital, prelingual SNHL associated with *OTOF* mutations, this gene has been associated with later onset, variable and even temperature-sensitive auditory neuropathy (167,168). The only other AR SNHL gene currently associated with auditory neuropathy is *PJVK*, encoding pejvakin, which causes HI at the *DFNB59* locus (see above). While less common, nonsyndromic autosomal dominant auditory neuropathy, *AUNA1*, was described in a large Caucasian kindred with average age of onset at 18 years in presumed heterozygotes and 8–9 years in homozygotes (169). *AUNA1* has been attributed to overexpression of *DIAPH3*, which encodes a member of the diaphanous-related formin family (DRF) of proteins involved in maintenance of cell polarity and cell shape, intracellular transport, and vesicular trafficking, including *DIAPH1* (*DFNA1*) (170). Affected individuals generally present with auditory neuropathy in the second decade, rapid progression to profound HL and loss of OAEs around the fifth decade, though some variability has been described. Although an X-linked auditory neuropathy locus, *AUNX1* has been described in a Chinese kindred (171), it appears to harbor a gene associated with peripheral neuropathy in addition. Finally, auditory neuropathy has been associated with mitochondrial mutation in Chinese patients (172).

### 142.6.5 Modifier Genes

A number of observations in human hearing impairment (especially intrafamilial variability of *GJB2*-related deafness) have suggested the possibility of modifier genes. Examination of mouse models has revealed that a number of genes influence the expression of auditory genes. Mouse genes that modulate HL include *tubby* (*tubby*) and *moth1* (modifier of *tubby* hearing) (173), and *dfw* (deaf waddler) and *mdfw* (modifier of deaf waddler) (174). In humans, a locus for a modifier gene (*DFNM1*) for hearing impairment associated with the *DFNB26* locus has been mapped to chromosome 1q24 (175). Schultz et al. (176) described the gene *ATP2B2*, which encodes the plasma membrane calcium pump protein PMCA2, as a modifier locus for deafness caused by at least two other genes (*CDH23* and *MYO6*) and,

possibly, by noise exposure. Modifier loci also appear to interact with the mitochondrial genome in the etiology of HL (see below).

### 142.6.6 Mitochondrial

Pedigrees with nonsyndromic sensorineural deafness and a maternal inheritance pattern are well known, though the proportion of mitochondrial HL among all nonsyndromic hearing impairment is not clear. While most cases manifest nonsyndromic HL, maternal inheritance of HL in association with diabetes, skin manifestations, neuromuscular symptoms, retinitis pigmentosa and other ocular findings has been reported as well. The most common mitochondrial gene defect leading to nonsyndromic HL is the homoplasmic A1555G mutation in the mitochondrial small ribosomal RNA gene (177). This mutation alone is not sufficient to cause the deafness phenotype, and additional nuclear modifier genes are thought to be necessary for phenotypic expression. Several of these modifier genes have been identified and appear to involve mitochondrial RNA processing (178,179). Importantly, the A1555G mitochondrial mutation also predisposes to aminoglycoside ototoxicity. About 15–30% of patients with aminoglycoside ototoxicity carry the A1555G susceptibility mutation. Hence, review of the pedigree and genetic testing can prevent exposure to aminoglycosides in at-risk maternally related family members of such patients. Four additional mutations in the same small ribosomal RNA gene have also been shown to predispose to aminoglycoside ototoxicity, but occur less commonly than the A1555G substitution (180). Three additional mitochondrial mutations in and around the tRNA<sup>ser</sup> (*UCN*) gene, which lead to nonsyndromic HL, have been identified in pedigrees from all over the world (reviewed by Fischel-Ghodsian (181)). These mutations appear to be nearly homoplasmic in the majority of cases, and the exact pathophysiological relationship between the mitochondrial mutations and HL remains unclear. Of note, a recent study (182) suggested that concomitant use of salicylates, chosen for their antioxidant effect, decreased the risk of aminoglycoside ototoxicity, though it did not address the question of genetic susceptibility in the study cohort. As noted above, some mitochondrial mutations have been implicated in the occurrence of auditory neuropathy. Finally, mitochondrial point mutations,



deletions and duplications have been described in kindreds with SNHL and diabetes mellitus, which occur with significant penetrance but not always together, with or without optic atrophy and/or retinitis pigmentosa, an observation confirmed in population studies of diabetic patients.

## 142.7 SYNDROMIC HEREDITARY HEARING IMPAIRMENT

HL occurs as a primary or occasional component of more than 400 syndromes, many of which occur rarely. The salient features and historical aspects of these conditions have been chronicled in numerous publications and will not be detailed here. The most comprehensive descriptions are provided in the text *Hereditary HL and Its Syndromes* (183) and in *Gorlin's Syndromes of the Head and Neck* (184). As is the case in nonsyndromic HL, syndromic HL is quite heterogeneous at both the phenotypic and the molecular level. This section reviews the major syndromes associated with HL, and provides a categorical approach to the evaluation of the patient with hearing impairment. The most common syndromes can be found in Table 142-5 and will be discussed below.

### 142.7.1 Overview

In general, conductive or mixed hearing impairment, and, in some cases, SNHL should be suspected in any disorder presenting with dysmorphic pinnae, external ear anomalies, known middle ear malformations or craniosynostosis (e.g. oculo-auriculo-vertebral spectrum (OAV), Treacher-Collins syndrome, oto-palatal-digital syndrome, Crouzon syndrome). Children with cleft palate generally develop middle ear effusions shortly after birth and are candidates for surgical ventilation of the middle ears. Those children with syndromic cleft palate are at increased risk of recurrent or persistent middle ear disease if the syndrome is also associated with oropharyngeal hypotonia (e.g. Down syndrome, velo-cardio-facial syndrome), as well as for secondary SNHL. Hence, early and periodic evaluation of hearing and tympanic membrane function is important if these children are to achieve maximum developmental progress. A particularly high frequency of HL is seen in the CHARGE syndrome, in which the HL varies from mild to profound. Temporal bone anomalies, including Mondini malformation and absence or hypoplasia of the semicircular canals, are seen commonly and have been proposed as a major diagnostic criterion (185). The etiology of this usually sporadic condition is heterozygous mutation in the gene *CHD7*, which encodes the chromo-domain helicase DNA-binding protein 7 (186).

Similarly, Kabuki syndrome, which is caused by usually de novo mutations in *MLL2* (187), is associated with HL in 27–45% of patients (188,189). HI in Kabuki

**TABLE 142-5 Common Childhood Deafness Syndromes**

Syndrome	Frequency	OMIM Numbers
Alport	1%	104200, 153640, 153650, 203780, 300194, 300195, 301050, 605249
Branchio-oto-renal (BOR)	2%	113650, 600237
Jervell & Lange-Nielson	1%	220400, 607542
Pendred	1–10%	274,600
Usher	3–5%	27600–276,904, 605,472
Waardenburg	2–5%	148820, 193500, 193510, 277580, 600193, 606662, 608890, 613265, 613266, 611584
Wolfram, WS-like disease (atypical WS)	≤1%	222300, 600965, 604928, 606201, 611507

syndrome may be conductive, sensorineural, or mixed, unilateral or bilateral, and varies in severity. Individuals with disorders of increasing bone density should be evaluated for hearing impairment. Various abnormalities may occur because of compression of cranial nerves as they exit the cranial foramina. Similarly, alterations in the bony labyrinth or ossicles may lead to sensorineural or mixed HL. Hence, hearing should be evaluated periodically in individuals with the severe AR form of osteopetrosis (Albers-Schönberg disease), sclerosteosis, Van Buchem disease, frontometaphyseal dysplasia, metaphyseal dysplasia of Pyle, craniometaphyseal dysplasia, craniodiaphyseal dysplasia, and other rare sclerosing conditions. Many skeletal dysplasias producing disproportionate short stature are regularly or occasionally associated with HL. Achondroplasia, the most common form of short-limbed dwarfism, is frequently complicated by otitis media, particularly in infancy and early childhood. While achondroplasia patients may have malformations of the ossicular chain, the likelihood and severity of conductive HL correlate best with the presence and chronicity of otitis media. SNHL has also been observed occasionally.

About 10% of patients with several types of osteogenesis imperfecta (primarily types III and IV) have severe SNHL, though most are more mildly affected (190). Also, a number of connective tissue disorders, of which the type II collagenopathies (especially Stickler syndrome, discussed below) are most prominent, are associated with SNHL (191). Craniosynostosis syndromes associated with *FGFR2*, *FGFR3* and *TWIST* mutations (Crouzon, Apert, Muenke and Saethre-Chotzen syndromes), and in *RAB23* (Carpenter syndrome) are often associated with mixed or SNHL (see review by Johnson and Wilkie) (192). Finally, hearing impairment occurs in many neurological conditions, inborn errors of

metabolism, conditions with endocrine dysfunction and in a variety of disorders with cutaneous or generalized pigmentary dysplasia, including many ectodermal dysplasias. Hence, hearing should be evaluated in any child with unexplained speech delay, particularly in the face of hypopigmentation of the skin, hair or iris, unusual hyperpigmentation (e.g. café-au-lait macules or lentiginos) or other ectodermal abnormalities.

### 142.7.2 Branchio-Oto-Renal Syndrome

Branchio-oto-renal syndrome (BOR) is an AD condition comprising variable features including preauricular pits or tags, pinna anomalies, lacrimal duct stenosis, branchial cleft cysts or fistulas, renal dysplasia, and sensorineural, conductive, or mixed HL. With an estimated incidence of 1 in 40,000, BOR accounts for about 2% of profound childhood deafness. The HL may have onset in childhood or later, may be progressive in nature, and is often associated with ossicular or cochlear defects, including Mondini malformation and dilated vestibular aqueduct (193–195). At least 6% of affected individuals have severe renal dysplasia, usually associated with hypoplasia (even aplasia), cysts, or structural anomaly of the kidney or collecting system and a glomerular lesion may occur. Thorough audiometric and renal evaluation of family members is indicated when this diagnosis is suspected. Diagnostic criteria for BOR have been established (196). The association of HL and preauricular pits without other findings of BOR has been called branchioto-otic syndrome (BO or BOS), which represents a variant of BOR in which renal disease is absent.

The BOR1 gene, which accounts for about 40% of BOR kindreds, was found to be *EYA1*, the human homolog of the *Drosophila* gene ‘eyes absent’, and one of a family of highly conserved genes (197) that function as both nuclear transcription factors and protein tyrosine phosphatases (198). Expression studies were consistent with a role in inner ear and kidney development. Chang and associates (196) reviewed mutations associated with BOR but were not able to demonstrate genotype–phenotype correlation in BOR/BO kindreds. BOR3, which accounts for about 2% of cases, has been attributed to mutations in the homeobox gene, *SIX1* (199,200), and mutations in *SIX5*, were found to cause BOR2, which accounts for 2.5–5% of cases (201) after a targeted evaluation of *SIX* genes which bind *EYA1*, as the interaction of *EYI1* with *SIX1* and *SIX5* is necessary for gene transcription.

### 142.7.3 Alport Syndrome

In addition to BOR, HL occurs in a number of syndromes in which renal malformation or disease is a major component. After BOR, the most important condition to consider is the Alport syndrome of SNHL and chronic nephritis, which comprises about 1% of all genetic HL

and over 2% of renal transplant patients. The earliest symptom is generally microscopic hematuria with or without proteinuria, followed by slow deterioration of renal function. Microhematuria is typically present in infancy or early childhood in affected males, and episodes of gross hematuria are frequent. Evaluation of the renal ultrastructure demonstrates irregular areas of thickening or attenuation of the basement membrane and interstitial changes. The condition is generally more severe in males, with childhood onset of nephritis and renal failure occurring as early as the second or third decade. Moderate, bilateral, high-frequency SNHL appears by adolescence in the majority of Alport patients, though severe, bilateral SNHL may occur in early childhood. Within pedigrees, nephritis and hearing impairment may occur individually or together, though hematuria precedes recognition of HL, confounding the diagnosis. HL occurs less frequently, has a later age of onset, and is milder in affected females in X-linked kindreds, while gender does not influence age of onset or severity of HL or hematuria in AR or AD families. Ocular defects including congenital cataracts and spherophakia have been noted in at least 40% of male patients, but occur infrequently in AD kindreds. Anterior lenticonus is a pathognomonic finding, and appears to be associated with fragility of the anterior capsule of the lens, which protrudes into the anterior chamber. Kashtan (202) provided a review of the molecular and clinical features of Alport syndrome, including strategies for diagnosis and treatment.

Although as many as five dominant and one recessive Alport syndrome phenotypes have been proposed on the basis of age of onset, presence or absence of HL and other features, X-linked inheritance accounts for about 85% of all cases. Alport syndrome is a type IV collagenopathy, specifically affecting COL4A3, COL4A4 and COL4A5, which are expressed in the cochlear basement membrane, part of the spiral ligament and stria vascularis. Defects in the  $\alpha$ -chain of type IV collagen (COL4A5), which maps to Xq22 and is a constituent of glomerular basement membrane, cause X-linked Alport syndrome (XLAS), and represented the first elucidation of a molecular defect in genetic deafness (203). Renieri and colleagues (204) detected mutations in only 45% of their patients using single-strand conformation polymorphism analysis, suggesting that some of the mutations occur in promotor regions, introns, or alternative splice sites. This observation was confirmed by a nearly 50% detection rate in 131 unrelated Alport patients evaluated for exonic mutations (205). Jais and associates (206) reviewed the natural history of XLAS and provided a review of the clinical features, as well as genotype–phenotype correlations in males from 195 families. Although nonsense mutations and some missense mutations have been associated with juvenile onset of renal disease and ESRD early in the third decade (207), genotype–phenotype correlation is limited for most missense mutations, and identification of most missense mutations is less useful in

interpreting the natural history of the disorder. Detailed genotype–phenotype correlations were reported recently by Bekheirnia and colleagues, who evaluated 681 male subjects from 175 kindreds, and noted that HL, ocular abnormalities and younger age of diagnosis of ESRD were associated with mutations at the 5′ end of the gene (208).

Two contiguous gene deletion syndromes involve the COL4A5 locus. In the association of Alport syndrome with leiomyomatosis of the esophagus and vulva, the COL4A5 and COL4A6 genes are deleted (209,210). The Alport syndrome, mental retardation, midface hypoplasia, and elliptocytosis association (AMME complex) involves deletion of COL4A5, the *FACL4* gene (211), encoding a long-chain acyl-CoA synthetase, and another highly conserved gene, *AMMECR1* (212). The association of Alport syndrome and mental retardation with a contiguous gene deletion in this region was further elucidated by Meloni and colleagues (213). The AR form of Alport syndrome has been associated with abnormalities of COL4A3 and COL4A4 (214) and accounts for up to 15% of cases (215). These genes have also been implicated in AD Alport syndrome, which accounts for fewer than 5% of cases (216,217), and in familial benign hematuria/thin basement membrane disease (218,219). The Fechtner-Epstein syndrome, originally included in the classification of Alport syndrome, has been attributed to mutations in the *MYH9* gene (a nonmuscle, class II myosin heavy-chain gene), which is also implicated in Sebastian syndrome, the May-Hegglin anomaly (220), and *DFNA17* (137,221). Individuals affected with one of these disorders, which are categorized as MYR9-related disorders, present with a variable history of congenital thrombocytopenia with giant platelets, typically postnatal-onset, high frequency, progressive SNHL, presenile cataract, glomerulonephritis with rapid deterioration of renal function and ESRD and neutrophil inclusions, which may be pathognomonic of this group of disorders (222). Striking genotype–phenotype correlations have been described such that the specific type and position of the mutation predicts the natural history of a MYR9-related disorder in a given kindred (139). From a medical standpoint, it is important to differentiate the MYR9-related disorders from Alport syndrome as several treatment modalities have shown promising results in the MYR-9 related nephropathy.

#### 142.7.4 Otosclerosis

The most common cause of HL in adults, otosclerosis, is a focal, immune-mediated disorder of unscheduled bone remodeling affecting the otic capsule. Clinically significant otosclerosis occurs in about 3–4/1000 whites, but is less common in blacks, and rare in Asians. There is a marked female preponderance and symptoms may exacerbate during pregnancy. Conductive, initially low-frequency, HL generally occurs in the third and fourth

decade, but may occur as early as adolescence, and is highly variable within pedigrees. Tympanometry and pure tone audiometry show characteristic patterns. Early in the course of the disease, pathological bone resorption occurs in the otic capsule, followed by formation of spongiform bone, collagen and vasculature. Subsequent progression to dense sclerosis with calcification of the annular ligament and fixation of the stapes in the oval window leads to moderately severe conductive HL. Cochlear involvement occurs in up to 10% of patients and presents with mixed or SNHL. While the medical benefit of sodium fluoride treatment has long been accepted, a critical review (223) found that the data supporting fluoride treatment is weak. Surgical stapedectomy provides improved hearing in greater than 90% of patients, but does not affect the sensorineural component.

Although AD inheritance with markedly diminished penetrance of 25–40% has been accepted historically, examples of kindreds with clear dominant segregation are rare, suggesting that otosclerosis is typically a complex genetic trait with genetic and environmental factors playing a role in its pathogenesis (224). Linkage studies have implicated ten AD loci, but only one potentially causative gene, encoding the T-cell receptor  $\beta$ , has been suggested (224). In addition, three susceptibility genes, which are all part of the transforming growth factor  $\beta$ 1 signaling pathway, *BMP2*, *BMP4*, and *TGF1*, have been identified (225). Recent data also implicates the *reelin* gene (*RELN*) as a susceptibility factor for otosclerosis (226). Although various factors such as estrogens, fluoride and traumatic injury have been associated with otosclerosis, the most important environmental factor is measles virus infection. Measles virus RNA has been isolated from surgically resected stapes footplates in the vast majority of subjects with typical otosclerosis. The importance of measles infection was demonstrated by a study, which showed a significant decrease in otosclerosis in Germany from 1993 to 2004 in individuals with prior measles virus vaccination (227). Sziklai and co-authors state that otosclerosis is an inflammatory disease due to chronic  $\text{TNF-}\alpha$  release in genetically susceptible individuals with persistent measles virus infection in the middle ear (228). They also cite evidence that SNHL may occur because of  $\text{TNF-}\alpha$ -mediated effects on gene expression in sensory epithelium, suggesting the possibility of treatment with anti- $\text{TNF-}\alpha$  antibody.

The differential diagnosis of otosclerosis includes skeletal dysplasias caused by mutations in the gene encoding *noggin* (*NOG*), an antagonist to the bone morphogenic protein gene family, including multiple synostosis syndrome (229), symphalangism type 1, and the syndrome of AD ankylosis of the stapes with broad thumbs, other skeletal anomalies and hyperopia (230). Of note, in the spectrum of conditions associated with abnormalities of *noggin*, stapes fixation is congenital. It is critical to differentiate common otosclerosis and the disorders associated with abnormal *noggin* from XR mixed HL with stapes

fixation and perilymphatic gusher (DFNX2 or Nance syndrome), as DFNX2 patients undergoing stapedectomy develop profuse, continuing drainage of perilymph with resultant increased HL and vestibular dysfunction. Finally, HL associated with osteogenesis imperfecta presents as typical otosclerosis.

### 142.7.5 Stickler Syndrome

Stickler syndrome (hereditary arthro-ophthalmopathy) is a common, extremely variable, AD condition characterized by high myopia, progressive liquefaction of the vitreous with associated risk of retinal detachment, joint laxity, mild spondyloepiphyseal dysplasia with marfanoid habitus and later degenerative arthropathy, characteristic facies, cleft palate, and conductive, mixed or SNHL. The palatal cleft may be of the Pierre-Robin type (U-shaped), and children with this condition are often seen in craniofacial clinics prior to the onset of significant vitreoretinal degeneration or HL. Therefore, it is particularly important to recognize the phenotype and implement an anticipatory care plan to screen for early retinal tears, as laser tacking may prevent retinal detachment. Progressive juvenile or adult-onset SNHL is an integral component of this condition, occurring in as many as 80% of cases.

Some Stickler families, designated vitreous type 1, demonstrate mutations in the type II collagen gene *COL2A1* (231). Another group is subdivided into type II Stickler syndrome, which is associated with a ‘beaded’ appearance of the vitreous and is due to mutations in the *COL11A1* gene, and type III or ‘nonocular’ Stickler syndrome, which is associated with *COL11A2* mutations. Wilkin and colleagues (232) presented evidence for a possible fourth Stickler syndrome locus. Work in mouse models showed that *COL11A1* and *COL11A2* are expressed in the basilar or tectorial membranes (233) and that *COL11A1* mutations act via a dominant-negative

effect (234). Annunen and colleagues (235) provided striking data regarding genotype–phenotype correlations. Compared to that reported for types II and III, mild, nonprogressive HL has been found in type I Stickler (236) syndrome. The auditory features have been reviewed by Admiraal and coworkers (237). Of note, mutations in *COL11A2* are also implicated in a nonsyndromic form of ADHL, DFNA13 (135), as well as otospondylomegaepiphyseal dysplasia (OSMED) and Weissenbacher–Zweymuller syndrome (238). The Stickler syndrome should be differentiated from Marfan syndrome, which has a small associated risk of sensorineural hearing impairment, possibly on the basis of superficial siderosis (11).

### 142.7.6 Waardenburg Syndrome

Waardenburg syndrome (WS) is the single most important disorder combining dermal abnormalities and hearing impairment. As many as 2–5% of deaf children have this condition, making it one of the most common recognizable syndromes associated with hearing impairment. Major features include partial albinism, manifested by poliosis (white forelock, which may acquire pigmentation), premature graying, patchy leukoderma, iris heterochromia (mixed blue-brown eye color in the same or separate eyes), and a relatively blond fundus; distinctive facies with synophrys, dystopia canthorum (lateral displacement of the inner canthi and lacrimal punctae, generally without orbital hypertelorism), an unusual nasal configuration with broad, high nasal root and hypoplastic nasal alae; and congenital SNHL, which is occasionally unilateral (239). Diagnostic criteria delineated by the WS Consortium include five major and five minor criteria (Table 142-6), as well as occasional findings (240).

WS is transmitted as a highly penetrant but variable AD trait, and has been classified into type I (WSI), which accounts for about 60% of WS cases, and type II (WSII)

**TABLE 142-6 Waardenburg Syndrome Type I**

Major Diagnostic Criteria	Minor Diagnostic Criteria
Sensorineural hearing loss <ul style="list-style-type: none"> <li>– Threshold at or greater than 25 dB at two or more frequencies (250–4000 Hz)</li> </ul>	Congenital leukoderma
Iris pigmentary abnormality <ul style="list-style-type: none"> <li>– Eyes of two different colors</li> <li>– Segmental iris heterochromia</li> <li>– Brilliant blue eyes</li> </ul>	Premature gray hair
Hair hypopigmentation <ul style="list-style-type: none"> <li>– White forelock at any age (may repigment)</li> <li>– Dystopia Canthorum</li> <li>– Lateral displacement of the inner canthi</li> <li>– Decrease in visible sclera</li> <li>– Affected first degree relative</li> </ul>	Synophrys or medial eyebrow flare
	Broad, high nasal root
	Hypoplasia of alae nasi
	Rare abnormalities <ul style="list-style-type: none"> <li>– Hirschprung disease</li> <li>– Sprengal anomaly</li> <li>– Spina bifida</li> <li>– Cleft lip and/or palate</li> <li>– Limb defects</li> <li>– Congenital heart abnormality</li> <li>– Vestibular dysfunction</li> <li>– Broad, square jaw</li> <li>– Low anterior hairline</li> </ul>



according to the presence or absence of dystopia canthorum. WSII has about a 50% risk of bilateral SNHL compared to a 25% risk in WSI patients. Absence of the organ of Corti and the stria vascularis, paucity of ganglion cells, and occasional vestibular abnormalities have been described. Madden and colleagues (241) found temporal bone abnormalities, including enlargement of the vestibular aqueduct, in all of their Waardenburg patients with HL. In addition, some patients experience a predominantly vestibular phenotype (242). Type III Waardenburg syndrome (WSIII, Klein-Waardenburg) includes the association of Waardenburg syndrome with musculoskeletal defects of the upper limbs. Cleft lip/palate occurs with increased frequency, as does Hirschsprung disease (aganglionic megacolon), consistent with abnormal migration of cells derived from neural crest ectoderm. The occurrence of WS and Hirschsprung disease with AR inheritance comprises type IV Waardenburg syndrome (also referred to as Shah-Waardenburg syndrome or Waardenburg-Hirschsprung disease). A recent review of molecular studies (243) found that loss of function mutations in the human paired-box gene *PAX3*, which is homologous to the mouse *Pax3* gene, cause WSI as well as the WSIII phenotype, though WSIII may be caused by homozygous or heterozygous mutations. Heterozygous *PAX3* mutations produce the mouse phenotype *Spotch* (Sp), which includes facial clefting, pigmentary abnormalities, and deafness. Of note, homozygous Sp mice have neural tube defects and other neural abnormalities, which are occasionally reported in WSI kindreds. As the *PAX* genes encode DNA-binding proteins, they play a critical role in regulation of transcription. Inner ear development in *Spotch* mice has been reviewed (244).

In contrast to WSI1, WSII demonstrates striking genetic heterogeneity. About 15–20% of cases are associated with heterozygous mutations in the human microphthalmia-associated transcription factor gene (*MITF*), which is analogous to the mouse microphthalmia gene, and is expressed in embryonic retina, otic vesicle, hair follicles, and skin. Mutant mice demonstrate a phenotype similar to WS. *MITF* fulfills several functions in the Wnt signaling pathway (245) and is involved in development and survival of melanocytes (246). *MITF* has also been shown to regulate *DIAPH1*, the gene associated with deafness at the *DFNA1* locus (125), in melanoma cells, suggesting that such regulation plays a role in the HI associated with *DFNA1* and WS2. Sanchez-Martin and colleagues (247) described WSII (labeled WSIIID) in two patients with homozygous deletions of *SNA12*, which encodes the human homolog of *SLUG*, a zinc finger neural crest transcription factor transactivated by *MITF*. *SOX10*, a co-transcription factor that functions in neural crest development, has been implicated as a cause of WSII (248) and WSIV (249). At least one other locus has been suggested by linkage analysis. In addition to *SOX10*, WSIV is caused by mutation in at least two other genes, the endothelin-3 gene (*EDN3*)

(250) and the endothelin-B receptor gene (*EDNRB*) (251). *PAX3* has been shown to regulate *MITF* expression (252), and synergistic interaction between *SOX10* and *PAX3* is required to activate *MITF* expression by binding to the proximal region of the *MITF* promoter (253).

Studies in the dominant megacolon (Dom) mouse document impaired *MITF* expression and subsequent aberrant development and survival of melanocytes in the face of aberrant *SOX10* transcripts (254). Of note, identification of a specific mutation is not usually helpful in predicting phenotype in a given individual, as all of the WS subtypes demonstrate variability within kindreds. WS associated with *SOX10* mutations may also manifest a more complex neurological picture including peripheral and central neuropathy (PCWH) (255). The syndrome of AR albinism, black lock, cell migration disorder causing Hirschsprung disease, and deafness (ABCD syndrome) has been associated with mutations in the *EDNRB* gene (256), and represents a variable presentation of Shah-Waardenburg syndrome. *PAX3* mutations have also been found in the craniofacial-deafness-hand syndrome (257,258) and in one patient originally described as having a mild form of the Yemenite deaf-blind syndrome (259). Molecular studies now allow clear differentiation of WSII from piebald trait, an AD condition caused by mutation in the *KIT* proto-oncogene (260), though some cases lacking *KIT* mutations have been caused by heterozygous *SLUG* deletions (261).

### 142.7.7 Usher Syndrome

By far the most important condition of ocular abnormality and HL is the Usher syndrome, a genetically heterogeneous phenotype in which SNHL, with or without vestibular dysfunction, is associated with later onset retinitis pigmentosa (RP). Usher syndrome was particularly frequent in the Jewish deaf population of Berlin, the French Acadian population of the United States and the Lapp population in Norway. As Usher syndrome may be underdiagnosed, prevalence estimates of 3.0–4.4 per 100,000 appear to be low, as suggested by a recent study, which estimated overall Usher syndrome prevalence to be 1/6000 (262). This common condition accounts for most cases of congenital deafness and RP, which comprise 3–5% of all profound childhood deafness (263,264). In a review of 600 patients from a registry of deaf-blind adults, Boughman and colleagues found that Usher syndrome accounted for 54% of the cases, though it was diagnosed in fewer than half of those individuals (265). There are two common Usher syndrome phenotypes, in which profound, congenital SNHL and vestibular dysfunction are features of type I (USH1), while normal vestibular function, later-onset, usually stable SNHL and progressive RP characterize type II (USH2). The rare type III Usher syndrome (USH3) presents with later onset, generally progressive but more variable deterioration of hearing and vision (Table 142-7).

**TABLE 142-7 Major Phenotypic Features of Usher Syndrome<sup>a</sup>**

Clinical Finding	Usher Type I	Usher Type II	Usher Type III
Hearing loss	Severe-profound, prelingual SNHL	Moderate-severe, nonprogressive	Postlingual, progressive
Vestibular function	Abnormal	Normal	Variable
Retinitis pigmentosa	Prepubertal onset	Pubertal or later	Latest onset

<sup>a</sup>Intermediate phenotypes have been described.

Although the type I phenotype (USH1) was considered to be more common, type II (USH2) accounts for up to 70% of all Usher syndrome patients (266). Genotype–phenotype correlations have been shown for several USH genes that are allelic to nonsyndromic DFN loci, and atypical USH presentations have been documented, often reflecting genotype–phenotype correlations in compound heterozygotes. Male infertility, mental retardation, and psychosis have been noted as well. Posterior cataract, which is a later component of the retinal degeneration, adds to the vision loss. Table 142-7 outlines the major features of Usher syndrome. An observation of abnormal sperm and photoreceptor axonemes led to speculation that defective connecting axonemes in cilia might lead to progressive loss of photoreceptors in Usher syndrome (267). The molecular pathogenesis of retinal degeneration in USH has been summarized recently (268). Because of the importance of this condition, and the difficulty in making an early diagnosis, the Usher Syndrome Consortium defined specific diagnostic criteria (269).

Molecular analysis confirmed marked heterogeneity for USH, with recent data indicating at least 12 loci, for which 10 genes have been identified (reviewed by Yan and Liu (270)). These include seven USH1 loci, four USH2 loci and one locus for USH3. USH1B, which is caused by mutations in the gene for myosin VIIA (271), comprises about 75% of USH1 cases. This unconventional myosin interacts with two other USH1 gene products, harmonin (USH1C) and cadherin 23 (USH1D), to promote transport of membrane-related proteins and intercellular adhesion (272–274). *MYO7A* mutations have also been described in two nonsyndromic forms of SNHL, DFNB2 (61,62) and DFNA11 (64) and in atypical USH cases. The *USH1C* gene product, harmonin, is a scaffold protein, which is a key element in the pathogenesis of Usher syndrome. Harmonin interacts with all of the known USH gene products and with other protein pathways to form a series of protein networks or interactomes involved in diverse functions, including morphogenesis of the hair cell stereocilia and calycal processes of photoreceptor cells, synaptic function; cell polarity and cell–cell interactions and signaling (274). USH1F results from mutations in another cadherin-like gene, *PCDH15*, which encodes a protocadherin and is the human analog of the Ames waltzer mouse. A specific mutation in *PCDH15*, R245X, has been shown to cause most USH1 in the Ashkenazi Jewish population (275). USH1G is caused by mutation in the novel scaffold

protein *SANS*, which is expressed in the cochlea and the eye, whose function is integrally related to that of harmonin (276). The *USH1A* locus has been withdrawn, as SNHL in the original kindreds given this designation has been shown to result from *MYO7A* mutations (277) and the genes for the *USH1E* and *USH1H* genes have not been identified.

USH2A is caused by mutation in a gene encoding an extracellular matrix protein (278,279) called usherin, which is expressed in the cochlea and the retina (280). A founder mutation in *USH2A* is responsible for most Usher syndrome in the Acadian population and French Canadians, illustrating the common origin of these populations (281). One *USH2A* mutation (now noted as 2299delG) accounts for the majority of mutant alleles in British and Chinese families (282), providing a basis for screening for USH2A. The *USH2B* locus has been mapped, but the genes remain unknown. *USH2C* has been attributed to mutations *GPR98* (*VLGR1*), a transmembrane G-coupled receptor (283) and to biallelic, digenic mutation in *GPR98* and *PDZD7*, whose gene product is homologous to the *USH1C* and *USH2D* proteins (284).

*WHRN*, the gene encoding the scaffold protein, whirlin, is implicated in USH2D and ARSNHL at the DFNB31 locus (95). USH3 is caused by mutation in the gene encoding the transmembrane protein, clarin (285), which may be involved in synaptic function of sensory hair cells. Using a mouse model, Geng and colleagues (286) documented expression of clarin in vestibular and cochlear hair cells, and observed postnatal changes implying a critical role for clarin in maintenance of hair cell function and neural transmission. Ocular findings are particularly variable in USH3, sometimes reflecting genotype–phenotype correlations, though marked intrafamilial variation has been observed. Although USH3 has been described primarily in the Finnish population, it occurs in other populations as well. Of note, several of the USH genes, including USH2A, USH2C and USH3, have also been associated with nonsyndromic retinitis pigmentosa (Table 142-8).

Congenital or later onset HL and progressive pigmentary retinopathy occur in a number of other conditions that should be considered in the differential diagnosis of Usher syndrome. Of particular interest to pediatricians is the Bardet–Biedl syndrome (reviewed by Beales and colleagues (287)), a highly heterogeneous AR condition characterized by mental retardation, retinal dysplasia

**TABLE 142-8 Genetic Heterogeneity of Usher**

Classification	Locus <sup>a</sup>	Gene/Protein
Type 1A (USH1A)	14q32	Gene withdrawn
Type 1B (USH1B)	11q13.5	MYO7A/Myosin VIIA
Type 1C (USH1C)	11.15.1	USH1C/Harmonin (Acadian)
Type 1D (USH1D)	10q2122	CDH23/Cadherin 23
Type 1E (USH1E)	21q21	Gene not identified
Type 1F (USH1F)	10q21–22	PCDH15/Protocadherin 15—R245X mutation accounts for most USH1 in the Ashkenazi Jewish population
Type 1G (USH1G)	7q24–q25	SANS/Sans, novel scaffold protein
Type 1H (USH1H)	15q22–23	Gene not identified
Type IIA (USH2A)	1q41	USH2A/Usherin, novel extracellular matrix or cell adhesion molecule
Type IIB (USH2B)	5q14.3–q21.3	Gene not identified yet
Type IIC (USH2C)	5q14	VLGR1/Very large G-coupled protein coupled receptor-1
Type IID (USH2D)	9q32	WHRN/Whirlin
Type III (USH3)	3q21–25	USH3A/Clarin-1, Finnish founder mutation PDZD7/PDZ domain-containing 7, shows digenic modification of USH3A

<sup>a</sup>Other loci are possible.

with or without RP, renal anomalies, polydactyly, hypogenitalism, obesity, and diabetes mellitus, in which HL is an occasional finding. This must be differentiated from the less common Alstrom syndrome of RP, SNHL, obesity, and diabetes mellitus, an AR condition, which is often misdiagnosed or underdiagnosed (288). Both conditions share overlapping features with Kearns–Sayre syndrome, ascribed to mutations in the mitochondrial genome, which presents with varying combinations of optic atrophy with or without pigmentary changes, progressive SNHL, diabetes, cardiomyopathy, generalized myopathy, and other findings. RP and SNHL are integral components of infantile Refsum disease (phytanic acid oxidase deficiency), a degenerative AR peroxisomal disorder characterized by infant-onset deafness and RP, macular dystrophy, peripheral neuropathy, severe developmental delay, hepatomegaly, and mild facial and skeletal dysmorphism; of classical Refsum disease (phytanic acid storage disease), which presents with the triad of later onset RP, peripheral neuropathy, and cerebellar ataxia associated with SNHL, ichthyosis, cardiac arrhythmias, abnormal renal function, and other features (both reviewed by Fournier and colleagues (289)); and of Cockayne syndrome, a heterogeneous AR phenotype comprising dwarfism, mental retardation, skeletal dysplasia, prematurely senile appearance, RP, optic atrophy, deafness, and severe sensitivity to ultraviolet light (reviewed by Nance and Berry, (290)) due to a DNA repair defect.

### 142.7.8 Syndromes with HL and Other Ocular Abnormalities

In addition to conditions resulting in HL plus RP, a large number of conditions combine HL with some other ocular abnormality. Chief among these is the Norrie syndrome, an XR condition characterized by congenital pseudoglioma, iris atrophy, cataracts, synechiae, and blindness, while later onset, progressive, high-frequency

SNHL (291), behavioral abnormalities and mental retardation are common but variable features. Norrie disease represents the most severe end of the spectrum of the group of retinopathies associated with mutations in the gene *NDP*. Milder presentations include some cases of persistent hyperplastic primary vitreous, familial exudative vitreoretinopathy (FEVR), Coats disease, and occasional cases of severe retinopathy of prematurity. *NDP*, which encodes the protein norrin, appears to be a cysteine knot growth factor (292). The ND knockout mouse shows abnormal retinal vascular development, as well as progressive loss of vessels in the cochlear stria vascularis (293) and abnormal signaling of the WNT pathway has been implicated in the pathogenesis of the ocular and auditory phenotypes (294).

### 142.7.9 Pendred Syndrome

Pendred syndrome (PS), an AR disorder combining SNHL variable vestibular dysfunction, temporal bone abnormalities and later onset goiter due to abnormal thyroid hormonogenesis, is the most common condition combining endocrine dysfunction and HL. Most cases of PS, which accounts for 1–10% of hereditary deafness, are caused by mutation in the gene *SLC26A* (formerly *PDS*) (295), whose product, pendrin, is a chloride-iodide-bicarbonate transporter responsible for chloride-bicarbonate exchange and maintenance of endolymphatic potential in the cochlea [reviewed in (296)]. Despite the stereotype of stable, severe-profound congenital HI, HL is variable, may develop in early childhood, and may be mild-moderate in severity, as well as progressive. An early review of 742 published cases found a striking female preponderance (297). Affected individuals virtually always demonstrate abnormalities on temporal bone CT or MRI. In subjects with molecular documentation of Pendred syndrome (PS), dilated vestibular aqueduct (DVA) is present in

80%, absence of the upper cochlear spiral in 75% and absent modiolus in 100% of ears (298). Profound deafness is generally associated with Mondini malformation of the cochlea. Unilateral or mild HL has been described occasionally. Vestibular dysfunction, usually characterized by episodic vertigo and fluctuating HI may occur occasionally and is especially associated with a specific mutation, H723A in *SLC26A4* (299). Three founder mutations in *SLC26A4*, p.Leu236Pro, accounting for 26% of alleles, p.Thr416Pro (15%) and c.1001 + G > A (14%), have been identified in Caucasians (300), and a founder mutation has also been identified in the Japanese population (301). Nonsyndromic deafness associated with EVA (DFNB4) is also caused by mutations in *SLC26A4* (65).

A second gene has been implicated in the etiology of Pendred syndrome. In nine patients with Pendred syndrome or nonsyndromic EVA, Yang and colleagues found heterozygous mutation in *FOXI1*, a transcriptional activator of *SLC26A4*. Causal mutations interfered with *FOXI1* binding, which compromised or completely abolished *FOXI1*-mediated transcriptional activation of *SLC26A4* (302). Furthermore, they described an EVA patient who was a compound heterozygote for mutation in *FOXI1* and *SLC26A4*, implicating digenic inheritance in Pendred syndrome and/or DFNB4. This finding was consistent with a mouse model of this pathway. The thyroid organification defect is mild in PS such that patients are often euthyroid with moderate thyroid enlargement developing in early adolescence. Mild hypothyroidism may precede or accompany the development of goiter, which is inconsistent and appears to be related to iodide intake. Although PS is easily recognized if goiter occurs, it cannot be diagnosed by clinical exam unless there is a known family history. Therefore, in the absence of molecular testing, children with apparently nonsyndromic deafness should be reevaluated periodically for the presence of thyroid enlargement or dysfunction.

### 142.7.10 Neurofibromatosis and Other Neurological Conditions

Although HL is an uncommon feature of neurofibromatosis type 1 (NF1), a formal hearing assessment is warranted, particularly in the face of any learning disability. Progressive, usually adult-onset SNHL and vestibular dysfunction are hallmark features of neurofibromatosis type 2 [NF2], and are associated with unilateral or bilateral vestibular schwannomas. Initial symptoms in NF2 commonly include tinnitus and vestibular dysfunction, which may precede significant HL. Typically, the HL has a subtle, asymmetrical presentation but may be fluctuating, with apparent intermittent improvement. Sudden deafness has been described. Although NF1 and NF2 both occur as AD conditions, it is important to distinguish NF2 (central neurofibromatosis), which is associated with abnormalities in merlin, a tumor suppressor

gene of the moesin–ezrin–radixin family of cytoskeletal proteins (303), from mild cases of NF1 (Von Recklinghausen disease), as the natural history and subsequent anticipatory management are quite different for these genetically distinct disorders. NF2. Diabetes insipidus, diabetes mellitus, optic atrophy, and deafness have been well described as the Wolfram or DIDMOAD (an acronym for the major features) syndrome, an AR condition in which other organ system involvement, progressive neurological deterioration and variable psychiatric symptoms occur as well. Although the onset of symptoms may be in early childhood, diabetes and progressive optic atrophy generally present in later childhood. Diabetes insipidus occurs in about one third of cases and has been attributed to vasopressin deficiency (304). Mild-moderate high-frequency SNHL is a variable feature with usual onset by the second decade, though congenital HI may occur. Most cases of Wolfram syndrome are caused by mutation in *WFS1*, the gene responsible for DFNA6/14/38 (130,131), an unusual nonsyndromic HL predominantly affecting the low frequencies. There is genotype–phenotype correlation with missense mutations associated with DFNA6/14/38 and loss of function mutations in Wolfram syndrome (305,306).

Charcot-Marie-Tooth disease, type 1A, is another important neurological condition in which HL typically occurs in the second decade, with severe hearing impairment noted by the end of the third decade. CMT1A is caused by point mutations in the peripheral myelin protein-22 gene (307). Of historical interest, the Mohr-Tranebjærg syndrome (DDP) of deafness, dystonia, and optic atrophy, which presents initially as nonsyndromic HI, was described originally as an apparently nonsyndromic form of progressive, postlingual SNHL, gaining the designation of DFN1 for the first X-linked deafness locus. DDP is caused by mutations in the gene *TIMM8A*, which encodes the deafness/dystonia peptide (308), a translocase involved in importing proteins into the mitochondria (309,310).

### 142.7.11 Jervell and Lange-Nielsen Syndrome

From an urgent diagnostic standpoint, the most important condition to consider in young children with persistent, profound, prelingual HI is the Jervell and Lange-Nielsen syndrome (JLNS, surdo-cardiac syndrome), a heterogeneous autosomal recessive disorder in which congenital, profound sensorineural deafness is associated with striking prolongation of the Q–T interval on ECG. Affected individuals typically develop arrhythmias, most notably *torsade de pointes* (polymorphic ventricular tachycardia), with resultant sudden death. Children with this condition may be misdiagnosed as having *petit mal* epilepsy or fainting spells, which decreases the likelihood of appropriate intervention, and sudden infant death syndrome may result in under-ascertainment of affected cases.



The syncopal episodes are particularly common in association with stressful activities and exercise, and deaths associated with swimming are not unusual. Although this condition occurs in only about 1% of congenitally deaf children (311), it must be considered in any child with AR or sporadic, profound, congenital SNHL.

Mutations in two related potassium channel genes, *KCNQ1* (312,313) and *KCNE1* (314,315), have been detected in most JLNS families, though there is a possibility of further heterogeneity. Tyson and colleagues (316) presented data indicating that *KCNQ1* mutations account for greater than 90% of cases. Four founder mutations in *KCNQ1* account for the increased prevalence in Norwegians (317). As heterozygosity for a mutation in either of these genes is among the known causes of isolated long QT syndrome (318,319), parents of JLNS children, while not deaf, are presumed heterozygotes for long QT syndrome and require appropriate molecular and clinical evaluation, counseling and treatment. Since JLNS cannot be diagnosed by clinical exam, ECG evaluation is strongly advised in any newly diagnosed child with apparently undifferentiated congenital SNHL, unless another gene (e.g. connexin 26) can be implicated on a molecular basis. Affected children may be candidates for cardiac pacing, which does not contraindicate placement of cochlear implants (320,321), though special attention should be paid to risk of arrhythmias associated with anesthesia (322,323). Although treatment with beta blockade and implantable pacemakers is successful in some cases, even aggressive management does not always prevent a cardiac event. Long term outcome has been reviewed by Swartz and colleagues (324), who also presented data supporting a milder phenotype in individuals with *KCNE1* mutations.

## 142.8 INBORN METABOLIC ERRORS

In addition to the endocrinological and neurological conditions described previously, HL occurs in a number of classical inborn metabolic errors. In particular, SNHL occurs very commonly in biotinidase deficiency, affecting as many as 75% of symptomatic children (325). This disorder of biotin recycling may present with developmental delay, optic atrophy, increased muscle tone, ataxia, seizures, and typical cutaneous manifestations, including alopecia, rash, and mucositis. Biotinidase deficiency is detectable via newborn screening, and its symptoms can usually be averted by prompt implementation of dietary management, including biotin supplementation. Heller and colleagues (326) characterized the CNS localization of biotinidase, and suggested that biotinidase and/or biotin play a significant role in normal hearing. Conductive HL associated with chronic middle ear disease is a regular feature of various lysosomal storage disorders, including mucopolysaccharidosis types I, II, IV, VI, and VII; mannosidosis; the sialidoses (neuraminidase deficiency); and multiple sulfatase deficiency. Progressive

SNHL is a feature of Tay-Sachs disease as well as Refsum disease, and may be a late manifestation of other storage disorders with CNS degeneration, including the disorders of peroxisome biogenesis (Zellweger syndrome spectrum). Progressive or sudden-onset SNHL is particularly prevalent in both males and females with Fabry disease (327,328). Current data suggests that enzyme replacement therapy may stabilize cochlear function and stabilize or improve vestibular function (329). It is particularly important to seek and aggressively treat HI in mild neurological disorders or CNS-sparing conditions as HL significantly increases the disability in these patients.

## 142.9 DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

The assessment of a child with hearing impairment is complex, and requires the interaction of several medical specialists, usually including at least an otolaryngologist, geneticist, audiologist, ophthalmologist, and pediatrician. It helps if the primary care physician serves as a medical home and takes the responsibility for coordinating the evaluation and communicating the overall implications of the findings to the family. The purpose of this multidisciplinary assessment is to identify the most likely cause for the HL, and to evaluate the severity and functional incapacitation associated with it.

The aims of diagnosing the precise cause of HL are:

- To prevent, if possible, further HL.
- To predict associated clinical manifestations, possibly prevent some of the medical complications by early intervention, and to identify associated features that might interfere with the normal social and professional adaptation to the HL.
- To provide genetic counseling for the parents of a deaf child and for deaf adults.

Providing an exact description of the nature and severity of the HL will hopefully allow optimal therapy.

### 142.9.1 History

The initial history should concentrate on the age of onset, progression, and quality of HL, as well as speech ability. In conductive HL, sounds appear quieter but are not distorted. The quality of speech is well maintained, since the patient hears his or her own voice well. By contrast, in sensorineural deafness, sound not only appears quieter but is also distorted. The most frequent distortion is loss of high frequencies, which diminishes the intelligibility of consonant sounds, with resulting difficulties in understanding speech. In severe sensorineural deafness, the patient does not hear his or her own voice, which leads to speech impairment. Discrimination of speech is better conserved with cochlear defects than with retrocochlear defects. The past medical history should document the

pregnancy, labor, delivery, and neonatal history, as well as previous hospitalizations, surgeries, and medications. The review of systems should be exhaustive, since deafness can be associated with signs and symptoms in many different organ systems. Having a check list with specific questions which pertain to the more common syndromes associated with HL ensures that all relevant points are elicited, for example asking directly for a white forelock at birth, premature gray hair, two differently colored eyes or constipation may lead to a diagnosis of Waardenburg Syndrome. Such findings are often quite subtle and may not be mentioned by the patient or parent without direct inquiry. Specific attention should be paid to the age at which a hearing-impaired child begins to walk, as a delay in achieving this milestone may be the first sign of a significant abnormality of vestibular function. Lastly, the family history should investigate the history and physical features of all relatives similar to the patient, and consanguinity should always be considered.

### 142.9.2 Physical Examination

A detailed physical examination of the patient and, sometimes, of close relatives is required, since many of the features of syndromic deafness can be missed in a cursory examination. Also, some critical features of specific syndromes may appear only with advancing age. Particular attention should be paid to the examination of the eyes, cranial nerves, neck and skin. Examination of the ears starts with an examination of the external ear, the auditory canal, and the tympanic membrane. Tuning fork tests are easily performed, and usually allow differentiation between conductive and sensorineural HL. The tuning fork is struck against a firm surface, and then either the fork is held in front of the external acoustic meatus (air conduction) or the base of the fork is held firmly against the skull (bone conduction). More detailed testing is rarely performed outside the otolaryngology office. Lastly, the physical examination should also assess the vestibular system, which is usually done by the Romberg test and an evaluation for nystagmus. The Romberg test requires the patient to stand with eyes closed and feet together, which is only possible with normal proprioception and vestibular system. Nystagmus is defined as an involuntary deviation of the eyes away from the direction of gaze. There are three main types of nystagmus: central, ocular, and vestibular. Vestibular nystagmus has a slow movement away from the line of vision, with quick return due to central correction. Vestibular nystagmus can be spontaneous, positional, or induced by rotational, visual, or caloric stimulation.

### 142.9.3 Laboratory Tests

Patients with childhood deafness require specialized laboratory testing. Most of the tests are performed to categorize the HL and to detect evidence of specific features

associated with the common deafness syndromes. In a minority of cases specific tests are done to detect evidence of acquired causes. These commonly used tests include:

- Otolological: audiometry (pure tone, speech, acoustic impedance), auditory brainstem response, temporal bone computed tomography scan or magnetic resonance imaging
- Vestibular: VEMP (Vestibular Evoked Potential)
- Ophthalmological: refraction; electroretinogram
- Cardiac: EKG
- Endocrine: thyroid function tests, other hormone analysis as indicated
- Renal: urinalysis, blood urea nitrogen, creatinine, renal ultrasound
- Infectious: viral cultures and serology or virus-specific DNA testing
- Genetic: DNA studies (e.g. *GJB2* and *GJB6* mutation analysis), evaluation of multiple genes using chip technology, chromosome analysis or chromosomal microarray as indicated

Since many of the nonaudiometric studies are familiar to the general physician, we describe only the audiology tests. This summary is not intended as an exhaustive description of the complex methodologies used, but only as an aid to assist the geneticist and pediatrician in the interpretation of the results obtained in a general audiological examination.

### 142.9.4 Pure Tone Audiometry

Pure tones of variable intensity and frequency are delivered to the ear by a headphone placed in the ear (air conduction) or on the mastoid (bone conduction). The patient signals whenever he or she hears a sound. It is important to use noise to mask the better hearing ear in both air conduction and bone conduction tests.

### 142.9.5 Speech Audiometry

A series of phonetically balanced words played from a tape recorder is presented to the patient at different intensities via headphones, and the patient repeats each word. The speech reception threshold is reached when 50% of the words are correct. In SNHL, there is often a loss of discrimination with increasing loudness.

### 142.9.6 Management

Management of the child or adult with HL varies depending on the presence or absence of additional findings, and the nature and severity of the HL. The goals of the physician provider should include distinguishing between syndromic and nonsyndromic or isolated deafness whenever possible, determining the etiology of the HL, developing and implementing a tailored anticipatory care plan designed to minimize morbidity related to

both HL and associated features, and providing appropriate genetic counseling to the patient and/or family. As noted previously, careful prenatal and postnatal history, detailed physical examination, and general or targeted examination of parents and/or siblings of an affected child are critical in detecting subtle historical or physical findings that are directive toward a diagnosis. Specialized testing (e.g. serological studies, echocardiogram, renal ultrasound, ophthalmological evaluation, skeletal or other radiographs) should be utilized whenever a specific syndrome is suspected, and should be considered in children with multiple malformations, unexplained dysmorphic features, or suspicious family history. Ideally, evaluation of the child with permanent HL should be accomplished in a multidisciplinary team (330). Recommendations for the genetic evaluation of children with congenital HL have been published in a policy statement from the American College of Medical Genetics (331). Molecular testing for a growing number of complex syndromes and nonsyndromic HL genes including GJB2 and GJB6 (connexin 26 and 30), SLC26A4 (Pendred syndrome and DFNB4), WFS1 (Wolfram syndrome and DFNA6/14/38), and for the various genes implicated in Usher syndrome is available on a service basis, and advanced molecular technology has facilitated evaluation of a larger number of genes in one test. Clinical laboratories also offer testing for known mitochondrial mutations associated with aminoglycoside ototoxicity. Several publications highlight the importance of testing for GJB2 and GJB6 (332,333), and include recommendations for a stepwise approach to molecular testing, though multigene panels may be more efficient and cost effective. SLC26A4 should be evaluated in children with an enlarged vestibular aqueduct or Mondini defect noted on a CT scan or MRI of the temporal bone region.

Since some common conditions such as the Pendred syndrome, JLNS, and Usher syndrome present initially as undifferentiated HL, at least in the absence of affected family members, a single ECG, imaging studies of the temporal bone and ophthalmological assessments can be justified, particularly for those patients whose molecular studies are negative for GJB2 and GJB6 mutations. Ophthalmological assessment is particularly indicated in children with profound SNHL and vestibular dysfunction, in whom the diagnosis of Usher syndrome type I is a consideration, as the genetic counseling and management issues are significantly different from those related to isolated nonsyndromic deafness. As infants with vestibular dysfunction are usually delayed in achieving their gross motor milestones, especially walking, such children may be referred for evaluation of 'developmental delay'. Children with clefting or other craniofacial malformations should be evaluated and followed by experienced personnel in a multidisciplinary craniofacial team setting. Genetic assessment and counseling should be provided as part of the team visit or by referral. Specific medical problems can be addressed as for any other patient and

will often require referral to additional specialists (e.g. referral of Alport patients to a nephrologist).

While the cost-effectiveness of early cochlear implantation has been established (334), complex issues such as the potential benefit of cochlear implantation in a given child, choice of the device, and surgical approach (335) should be deferred to an experienced otologist or to an appropriate subset of the craniofacial team. It is important to note that many forms of nonsyndromic HL, which are associated with normal cochlear development, are amenable to cochlear implant. This approach has become an important treatment modality for children with GJB2-associated HL and children with JLNS are candidates for cochlear implant even when they have pacemakers.

### 142.9.7 Screening for HL at Birth

Universal newborn screening (NBS), proposed as a means of early identification of the at-risk child, is now a standard practice in the United States and many other western world countries. Historically, NBS was supported by a National Institutes of Health Consensus Statement as early as 1993 (336). A later US Department of Health and Human Services publication describing the proposed screening process advocated the immediate implementation of widespread programs (337). Technological advances in automated newborn hearing screening technology, and a series of successful demonstration projects (338) resulted in the introduction of universal newborn hearing screening throughout the US in the mid- to late nineties. Early Hearing Detection and Intervention Programs (EHDI) in the United States are now characterized by reasonable per-infant costs for screening using noninvasive physiological tests, nursery-based screening rates of at least 95% of all infants, and referral rates for diagnostic testing of less than 6% (low false positive rates) (339). Children who 'fail' the newborn hearing screen are then tracked and audiological diagnostic testing is performed; the goal is to provide appropriate therapeutic and educational intervention for these infants by the age of six months. Since these children with HL are identified at a much earlier age, the incorporation of an etiological focus to the diagnosis of the HL, which includes genetic evaluation, testing and counseling, can be of great benefit to the family. The benefits of universal screening and early identification and treatment of the deaf child are well established (340,341). For newborn screening to be successful, crucial issues such as early hospital discharge, home birth, limitations of testing in small or geographically remote units, the need for retesting, referral, formal evaluation of children who fail the initial screen and lack of parental compliance must be addressed.

Establishing protocols for accurate diagnostic evaluation of the child with permanent HL and a laboratory basis for molecular evaluation of such children is critical. Appropriate intervention programs must be implemented

in a timely manner. With this in mind, the Joint Committee on Infant Hearing has set three goals: screening should be completed by one month of age, diagnosis of persistent HL should be made by three months, and intervention and treatment should commence by six months (342). Finally, educational materials, support services, and genetic counseling must be available for their parents. Although we are very successful in screening all infants by one month of age, recent data from CDC suggests that more than 50% of infants who fail hearing screening do not have a documented diagnosis of HL, and of those who do have a confirmed diagnosis of permanent HL, only 77% enroll for intervention by age six months (343). These findings underscore the need to establish a medical home for infants who do not pass their hearing screen, to help the families navigate through the follow-up and diagnostic processes. A workshop in January 2008 convened by the Agency of Healthcare Research and Quality and its federal partners reported on various aspects of the screening program, which could be enhanced with implementation of more evidence based practice for the benefit of children with early HL (344).

The Center for Disease Control and Prevention (345) has developed a manual that provides guidance for early identification and intervention (EHDI) programs. A review of EHDI programs in the United States (346) summarized their history, benefits, and current status, and noted that nearly 95% of newborns are now undergoing hearing screening in the United States. The review also summarized the experience of three relatively long-standing screening programs (Colorado, Hawaii, and Rhode Island), noting that less than 5% of children with permanent HL at entry to school were found to have passed their newborn hearing screen. Dent and colleagues (347) provided a summary of preliminary data from the Utah universal hearing screening program.

Similar reports describe early experiences with universal neonatal hearing screening in other parts of the world (348–350). Despite some successes, developing countries face multiple problems and limitations in the implementation of neonatal hearing screening (351). Finally, the efficacy of using dried blood spots to implement universal screening for common GJB2 and GJB6 mutations as well as for detection of congenital CMV infection is a topic of great interest and discussion (352–354). In addition to the benefits of early intervention for the affected child, a universal screening program offers the opportunity to identify and counsel at-risk couples prior to the birth of additional affected offspring. While the possibility of population screening for mutations causing SNHL exists, particularly with the detection of common connexin 26 mutations, many issues must be addressed before heterozygote screening can be implemented. The position that routine population screening is premature was underscored by a National Institute of Deafness and Other Communication Disorders (NIDCD)

Working Group (355), which felt that connexin 26 mutation screening was appropriate only in conjunction with a clinical trial and stressed the importance of including the deaf and hearing-impaired community in any effort to determine guidelines and recommendations for genetic testing. A survey of attitudes toward newborn screening and genetic testing for HL demonstrated wide acceptance of such testing among the deaf, hard-of-hearing, and normal-hearing populations (356). Taneja and colleagues (357) presented similar results in their survey regarding attitudes of deaf individuals toward molecular testing; however, they noted significant differences in responses of the deaf community versus the normal-hearing group.

### 142.9.8 Genetic Counseling and Prenatal Diagnosis

The process of genetic counseling for inherited deafness is often a daunting one. The consultand may be the hearing parent of a deaf child, a deaf individual, or even a deaf couple. Barriers to communication are often present, including the counselor's inability to sign and the counselee's muteness. Translators may not be easily available or may add an additional barrier between the counselor and the patient. Of great concern, the pedigree and medical history may contain inaccuracies, family myths, and instances of coincidental HI unrelated to the diagnosis in the patient. Even in the presence of a positive family history, the HI may be attributed to a variety of nongenetic circumstances such as trauma, fever, maternal rubella, or meningitis. As many syndromes demonstrate considerable intrafamilial variation, they may be difficult to diagnose without direct physical assessment of more distant family members. Likewise, late-onset conditions often escape detection or accurate diagnosis, and may present subsequent to the initial genetics evaluation of a given family member. Finally, the cultural fabric of the deaf community differs from that of the hearing world, and is generally poorly understood and underappreciated by hearing counselors. While some deaf patients would be relieved to learn that the risk of HL is low in their offspring, others would prefer deaf children or have little concern about this issue. As in any other situation, accurate genetic counseling depends on the accuracy of the diagnosis. In the presence of a clear-cut Mendelian diagnosis, risk assessment is relatively straightforward. In many instances a precise diagnosis cannot be established, and empirical risk figures must be used. For example, when a first affected child is born into a nonconsanguineous family with negative history for deafness, the empirical recurrence risk is about 10%, which reflects the recurrence risk of 25% if the inheritance is AR, or near zero if it represents a sporadic case due to a new mutation or an environmental exposure. Similarly, the empirical risk for a family with two deaf unrelated parents is 10%,



which reflects risks ranging from 100% if both parents have the same kind of AR deafness to 0% if they have different types of AR deafness, with many intermediate risk figures for different inheritance patterns. The empirical risk for families with only one deaf parent is around 5%. Another situation that occurs occasionally in genetic counseling is the normal-hearing couple who have two boys with deafness, raising the question of whether the inheritance is X-linked, and whether prenatal sex determination would be useful. An AR mode is more likely because AR deafness is so much more prevalent. With the demonstration that connexin 26 mutations represent the single greatest cause of AR HL and the availability of evaluating multiple genes at fairly reasonable cost, molecular testing has proved helpful in refining the risk assessment in a significant proportion of these cases.

Prenatal testing is possible in some instances of syndromic and nonsyndromic deafness. Improved ultrasound imaging of the fetus now leads to routine detection of fairly subtle malformations (e.g. microtia, abnormal hand posture), thus allowing diagnosis of specific syndromes that are not amenable to diagnosis by other means. Since molecular testing by direct mutation analysis can be used in those instances in which the precise gene defect has been identified, identification of a connexin 26 mutation, or a mutation in another deafness gene offers the possibility of molecular analysis of fetal cells. Some cases of both syndromic and nonsyndromic HL can be diagnosed by linkage analysis, which is especially applicable to large kindreds with multiple affected family members, especially if the causative gene has not been identified. Ideally, evaluation of the family should be completed prior to pregnancy. The availability of specific prenatal testing may or may not be important to the consultand, and implementation of testing remains a highly personal decision, which should be considered only after extensive genetic counseling.

### 142.9.9 Treatment

The treatment options for genetic HL depend on the precise type and severity of hearing impairment. Conductive HL can frequently be improved significantly by surgical repairs of the external ear and auditory canal, tympanic membrane, and ossicular chain. Hearing aids are the most commonly used treatment modality for SNHL, and have greatly improved performance through the use of more sophisticated circuitry, which allows some devices to fit within the external auditory canal. If conventional hearing aids are not sufficient, cochlear implants, which provide direct stimulation of the auditory nerve, have shown benefits in many patients with profound HI, and current practice includes cochlear implantation in many children with profound prelingual SNHL.

Treatment should always include the prevention of psychological, social, or academic difficulties associated

with HI. Training in cued speech, speech reading, sign language and use of sound and signaling devices plays a role in the prevention of complications due to HL. An understanding team of health professionals can provide important support for both the patient and the family in addressing these issues. Therapeutic intervention for associated findings may improve quality of life, particularly in those conditions in which deafness is associated with vision loss. Vitamin A therapy decreases the rate of retinal degeneration in hereditary retinal dystrophies (reviewed by Weleber and colleagues (358)), though specific efficacy in Usher syndrome is not well established. A report describing the benefit of a caspase-3 inhibitor in reducing retinal photoreceptor cell death in tubby mice, a suggested model for USH1, raises the possibility of a targeted treatment for retinal degeneration in human USH1 patients (359). Potential prophylactic treatment of otosclerosis with anti-TNF- $\alpha$  has already been described, as has S-adenosylmethionine supplementation in DFNX1 (see above). Reduction in the size of vestibular schwannomas and increased language discrimination has been achieved in some type 2 neurofibromatosis patients by treatment with bevacizumab (360). Optimistic early reports of possible regeneration of hair cells in the cochlea have not yet led to fruition. Izumikawa and colleagues (361) reported restoration of hearing by genetic manipulation of nonsensory cells in deafened mature guinea pigs. They documented ‘transdifferentiation’, which was induced by expression of the gene *Atoh1*, a critical regulator of hair cell development. While these investigations are still preliminary, the rapid rate of progress in gene identification is encouraging. The advances in hair cell regeneration and the role of gene therapy in treatment for deafness have been reviewed recently (362,363). The future holds hope that, with further elucidation of the molecular basis and specific pathogenesis of various causes of HL, improved screening and prevention strategies, as well as new therapies for specific defects, will become a reality.

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## FURTHER READING

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 GeneReviews <http://www.geneclinics.org>.  
 Hereditary Hearing Loss Homepage: <http://hereditaryhearingloss.org/>.  
 MRC Institute of Hearing Research: <http://www.ihr.mrc.ac.uk/index.php/research/>.  
 Online Mendelian Inheritance in Man (OMIM): <http://omim.org/>.  
 National Institute on Deafness and Other Communication Disorders: <http://www.nidcd.nih.gov>.

## Biographies



**Dr Falk** is a clinical geneticist who is board certified in Pediatrics, Clinical Genetics and Clinical Cytogenetics. Her major interests include the clinical and molecular aspects of classical chromosome disorders and microdeletion syndromes, genotype–phenotype associations in genetic disorders, prenatal diagnosis, teratogenesis and hereditary hearing loss. She has contributed to the understanding of mitochondrial mutation in aminoglycoside ototoxicity, to the identification of genetic loci for nonsyndromic clefting, Van der Woude syndrome and Stickler syndrome, and to the development of the ACMG Policy Statement providing genetic evaluation guidelines for the etiological diagnosis of congenital hearing loss. Currently, she is involved in studies of the clinical, ethical and social issues associated with use of donor gametes.



**Dr Pandya** is a physician scientist, board certified in Pediatrics, Clinical Genetics and Clinical Molecular Genetics. She has been involved in research on genetic hearing loss (HL) for more than a decade, and has participated in the development of national guidelines for the management of a child diagnosed with hearing loss. Her early career included studies of genetic modifiers of HL in individuals with Waardenburg syndrome. Subsequently, she was involved in identification of new recessive and dominant genes for nonsyndromic deafness, determining the molecular epidemiology of GJB2 deafness and establishing the largest national repository of DNA from deaf individuals. More recently she has focused on assessing the ethical impact of new discoveries in genetic hearing loss on deaf individuals and their families.

# CHAPTER

# 143

## Clefting, Dental, and Craniofacial Syndromes

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### 143.1 INTRODUCTION

Craniofacial anomalies comprise a large fraction of morbid human birth defects. They require surgical, nutritional, dental, speech, medical, and behavioral interventions and impose a substantial economic and societal burden (1). Clefting, or an aberrant space between normally fused tissues, usually occurs as either cleft lip with or without cleft palate (CL/P) or cleft palate only (CPO). Rare forms of clefts, such as lateral facial clefts extending into the orbit or laterally from the oral cavity, also occur. Clefts of the lip and/or palate can be divided into non-syndromic (isolated) and syndromic forms. In isolated clefts, affected individuals have no other physical or developmental anomalies. Studies suggest that about 70% of cases of CL/P and 50% of cases of CPO are non-syndromic (2,3). The syndromic cases can be subdivided into more than 500 Mendelian disorders (e.g. OMIM (4)), cases due to chromosome anomalies or to teratogens, and uncategorized syndromes. The complex etiology of clefting affords opportunities to identify gene–gene or gene–environment interactions that can shed light on human embryology and its disturbances (5–7).

### 143.2 DEVELOPMENT

In humans, an exquisitely choreographed cascade of gene expression, cell migration, cell transformation, and apoptosis between 14 and 60 days postconception creates the tissues of the face from the originating oropharyngeal membrane. Neural crest cell migration drives the swellings that form the frontonasal prominence and the paired maxillary and mandibular prominences. Fusion of the prominences results in normal facial relationships. By 48 days the upper lip is continuous, and by 60 days palatal shelf fusion completes facial embryogenesis (8–10). Disruption of any of the tightly regulated processes occurring in this time frame may predispose to cleft lip and/or palate. Genes with a demonstrated role in these

processes include those for transcription factors, growth factors, cell signaling molecules, and extracellular matrix proteins (7). Embryology suggests that clefts of the primary palate that involve the lip with or without the palate (CL/P) result from different disruptive mechanisms to clefts affecting only the secondary palate (CPO) (11). However, lumping of CL/P and CPO in etiological studies has recent support in that both CPO and CL/P are found in families segregating mutations of *MSX1* (12), *IRF6* (13), or *FGFR1* (14). In addition there is also support for separate mechanisms for clefts of the lip only, as distinguished from clefts involving the lip and palate together (15).

### 143.3 EPIDEMIOLOGY

CL/P affects about one in 700 births, with wide variability across geographic origin (16,17) and socioeconomic status (18). In general, Asian or American Indian populations have the highest birth prevalences, often as high as one in 500, with European-derived populations intermediate at about one in 1000 and African-derived populations the lowest at one in 2500. CL/P has remarkable differences in frequency by sex and side of clefting. There is a 2:1 male:female ratio and a similar 2:1 left-side:right-side ratio for unilateral clefts. CPO birth prevalence is more constant across groups of different ancestral origin at about one in 2500 but is compromised by ascertainment failure in studies that lack active surveillance programs.

### 143.4 GENETICS

#### 143.4.1 Human Genetics

For more than 200 years, scientists have suggested the importance of inheritance in the etiology of cleft lip and cleft palate. Trew (19) published the first description of a family with several affected members, and

Darwin (20) called attention to a publication (21) with “the transmission during a century of hare-lip with a cleft-palate.” Fogh-Andersen in Denmark was the first to collect a systematic data set of cleft families and to evaluate the observed inheritance patterns (22). His conclusions, that cleft families were consistent with the segregation of alleles at a single genetic locus with variable penetrance, were subsequently supported by segregation analyses (23) and twin studies (24). In the 1960s and 1970s, a particular statistical model termed the **multifactorial threshold model** was invoked to explain the familial patterns of oral-facial clefts, with recent support coming from a large, population-based study in Denmark (25). The identity of facial structures observed in normal monozygotic twins supports a strong genetic component to facial development. However, the lack of complete concordance for clefts in monozygotic twins (50% concordance versus 8%) in dizygotic twins argues that other factors (environment/stochastic) must play a role as well. Evaluation of sibling recurrence risk ratios estimates that  $\lambda$  for siblings is approximately 40. Schliekelman and Slatkin (26) estimated the number of loci contributing to the genetic component of CL/P as between three and six, although the confidence interval for the best estimate extended to 14 loci.

The emerging consensus of the genetic component of non-syndromic orofacial clefts is that relatively few genetic loci with common variants contributing to clefting are involved, with heterogeneity from family to family (or between populations), interacting genes, and rare variants and/or epigenetic factors also playing a role. Many investigators have used candidate gene, linkage and association approaches with new efforts now expanding to genome wide association and deep sequencing (candidate region, whole exome and whole genome) (7,27).

### 143.4.2 Linkage and Association Studies

Genetic investigation strategies for clefting have been recently reviewed (7). Linkage studies of clefting have led to the identification of one strong candidate, FOXE1, supported by the linkage, mutation identification and expression data (28–31). A few other candidate genes (32–35) continue to be of interest, based on candidate gene association work and/or sequencing studies, but other than IRF6 none has been replicated in genome-wide association studies GWAS (see below) so their specific contributions remain to be better defined.

Association studies using case-control or case-parent triads have also been used to examine candidate genes in CL/P for over 20 years (36–38). Only one gene, *IRF6*, that is etiological in the autosomal dominant Van der Woude syndrome (13) has consistently shown association in replicated studies (see below). Recently, GWAS have had striking success in finding new loci for clefting and have been recently reviewed (7,27).

### 143.4.3 Evidence from GWAS

Four published GWAS efforts have now made substantial contributions to our understanding of the loci/genes in which common variants contribute to non-syndromic clefting (39–42). They have each identified or confirmed a new locus at 8q24 as having a strong association with cleft lip and/or palate in European populations, strongly support a role for variants near the *VAX1* gene in clefting and identify new loci near the *MAFB* and *ABCA4* genes as having variants contributing to clefting seen most strongly in Asian populations. In one case (*MAFB*), additional functional and expression data support that gene as the best candidate (42). For the others, the specific gene and contributory SNP remain to be identified. It is also evident from other studies that it is likely that rare variants (as well as other genetic and environmental contributors) will also play a role in clefting, and currently applicable technologies such as whole exome or whole genome sequencing are likely to be used in studies of complex traits, including clefting.

### 143.4.4 Animal Models and Expression Studies

The strongest cleft candidate genes will include those in which normal expression of that gene takes place at the critical time and in the critical tissues for lip and palate development. Animal models and expression data can be powerful tools to identify candidate genes for a complex trait such as CL/P (43).

GWAS or linkage studies may identify regions containing tens or even hundreds of genes that could be causative, and so a priority list must establish the order in which to evaluate genes to confirm their etiological role. Animal models with spontaneously arising clefts or from knockout or ENU experiments can help to provide a list of genes on which direct sequencing can be carried out as confirmation steps. While cleft palate is a common phenotype in the mouse, cleft lip is rare. Two spontaneously arising cleft lip mutants, *Clf1* and *Clf2*, were identified in a genome-wide scan for susceptibility loci in the mouse using strain A/WySn (44), with an insertion into *Wnt9* as a likely mechanism affecting epigenetic regulation (45,46). Another mutant with high penetrance for cleft lip is *Dancer*, which arises from a translocation of p23 sequence into *Tbx10*, resulting in ectopic expression of *Tbx10* under the influence of the p23 promoter (47).

Global approaches to expression analysis of genes in craniofacial structures have begun to provide a broader view of gene function as well. First, the COGENE project ([hg.wustl.edu/COGENE/](http://hg.wustl.edu/COGENE/)) provides public web access to human gene expression data for 24 craniofacial-specific human tissues isolated from day 26 to day 60 embryos. The expression of genes relative to each other in both time and space is being visually represented using optical projection tomography (OPT) (48). OPT can be used



to evaluate the expression of new candidate genes and to demonstrate that the expression of existing genes is consistent with hypotheses about function. Expression profiling is now providing evidence for interacting genes and pathways in facial development (49,50). Finally, a newly supported effort by the NIDCR called FaceBase is providing a public website (<https://www.facebase.org/>) and compiling data to enable a common point source to interested users for data of many types (expression, imaging, phenotypic, genetic) across humans and a range of animal models (51).

#### 143.4.5 Environmental Factors and Gene–Environment Interactions

An environmental component of clefting was recognized when Warkany (52) associated nutritional deficiencies with cleft palate. More recent studies continue to support the importance of nutritional factors (53,54), as well as low socioeconomic status (18). Recognized teratogens that contribute to clefts include rare exposures, such as phenytoin and thalidomide, and also common environmental exposures, such as maternal alcohol or cigarette use (55–57). Increased risks from exposures can suggest metabolic pathways whose disruption may play a role in the development of CL/P. Common variables such as alcohol, smoking, or nutrition may have their effects amplified by pharmacogenetic variation. Studies of gene–environment interactions (58) for non-syndromic CL/P are in their infancy, but some interesting preliminary data support further studies of folate-metabolizing enzymes and genes in detoxification pathways (1,42).

### 143.5 CLINICAL EXAM AND PHYSICAL FINDINGS

Figure 143-1 shows examples of typical facial clefts as well as features associated with two of the more common syndromes: VDWS and ectrodactyly–ectodermal dysplasia and clefting.

#### 143.5.1 Associated Phenotypic Features

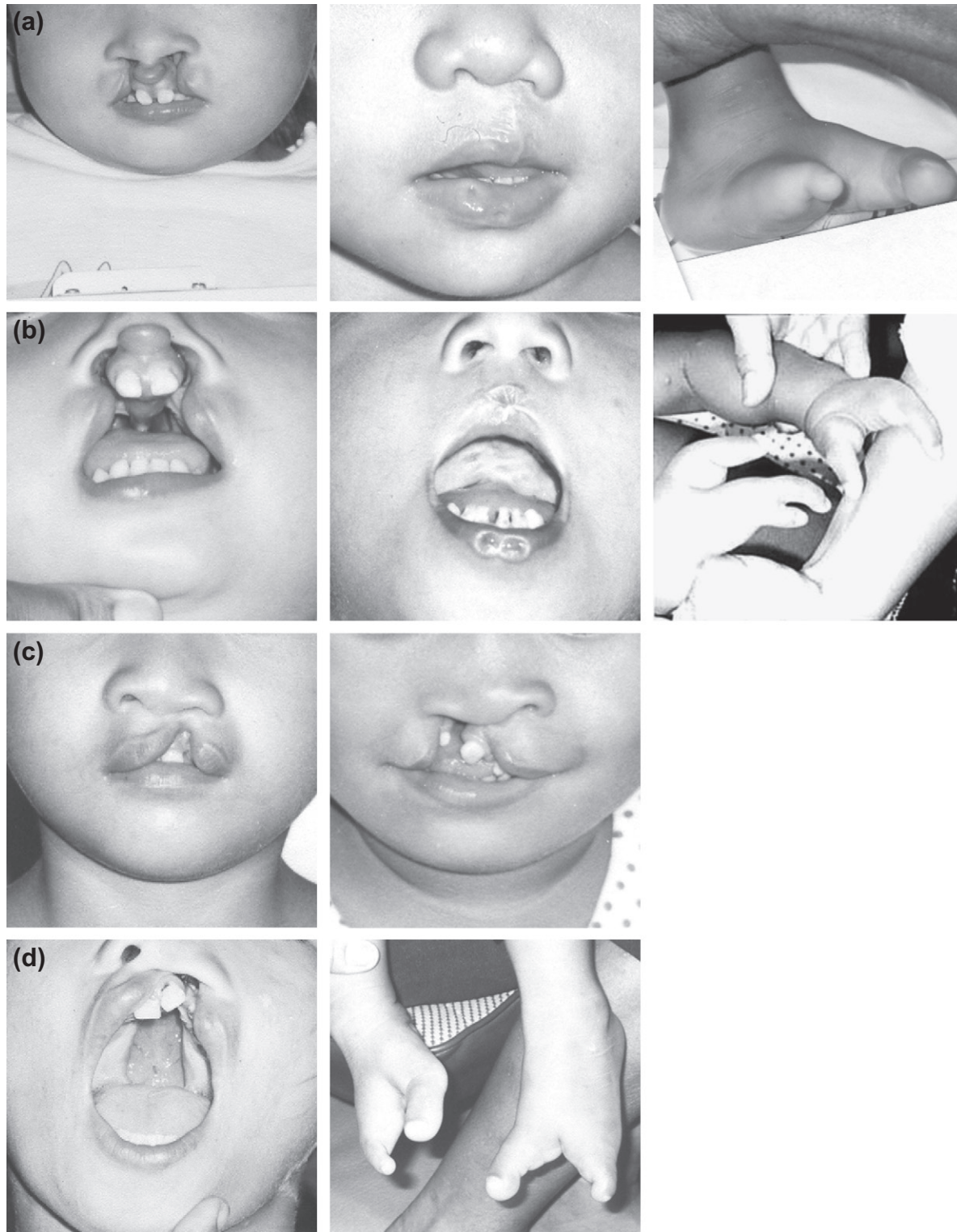
Accurate phenotyping is important for any human genetic study. To date, the cleft phenotype has generally been defined as a qualitative trait (i.e. affected or unaffected). However, some studies have begun to examine genetic differences for specific cleft phenotypes; e.g. genome-wide linkage results differ by the specific cleft phenotypes seen in multiplex families (30) and a specific putatively etiological variant in *IRF6* shows greater statistical significance in CL alone than in CLP (15). The spectrum of cleft anomalies includes visible microforms of the lip (e.g. notches or grooves in the upper lip) and palate (e.g. cleft uvula and submucous CP). Of note, microform CL is increased in individuals with *BMP4* mutations (59).

In addition to genetic differences by overt and microform cleft types, it now appears that the spectrum of phenotypes due to orofacial cleft risk factors includes a variety of associated subclinical phenotypic features, sometimes termed endophenotypes, as recently reviewed (60). Such subclinical phenotypes may allow researchers to identify noncleft individuals at increased risk to be carrying cleft genes, or to divide families into more homogeneous subsets, leading to improved power for genetic studies of clefting, and improve ability to provide effective genetic counseling, particularly recurrence risk information.

Subclinical phenotypic features are within the range of normal variability but are seen at increased frequency in individuals with OFC or their relatives, as opposed to controls with no family history of OFC (60). This idea has been present in the literature for many years, with the earliest subclinical phenotype studies focused on laterality measures such as handedness, due to the preponderance of left-sided unilateral clefts. More recent studies have found a range of subclinical phenotypes that are increased in OFC cases and relatives: orbicularis oris muscle defects (61–64); dental anomalies (65); lip dermatoglyphics (66); facial measurements (60,67,68); and brain variants on MRI (69–72). Furthermore, examination of such phenotypes is beginning to blur the historical distinction between CL/P and CP in some cases; e.g. in a small study there was a significant proportion of CP cases with orbicularis oris defects (73).

#### 143.5.2 Clinical Examination

The clinical examination of a newborn or an older child with a cleft of the lip or palate has as its primary focus the determination as to whether isolated clefting or a syndromic form of clefting is present. As is true for any clinical genetic examination, family history, prenatal history, prenatal testing, and postnatal testing are important considerations in addition to the physical exam itself. The clinical exam focuses on both dysmorphology of the head and other regions, and on the parents or siblings when available. Identifiable structural abnormalities such as congenital heart malformations, limb anomalies (ectrodactyly syndactyly, etc.), or central nervous system malformations make syndromic identifications straightforward in some cases. In others the findings may be far more subtle. In addition, there may be overlap between syndrome definitions. For example, mutations in *MSX1* can present with isolated dental anomalies outside the region directly affected by the clefts (e.g. in the lower jaw). Whether cases of clefts with dental anomalies should be considered as isolated clefts can be debated, but the presence of such findings has implications for recurrence risks in families. *MSX1* and *FGFR1* nonsense mutations may present as isolated clefts, but the sibling recurrence risks may be well above the 3%–5% used for isolated clefts in general.



**FIGURE 143-1** (A) A child with bilateral cleft lip and palate. (B) A child with unilateral cleft lip and palate. (C) Lip pits in the lower lip as seen in two cases of van der Woude syndrome. (D) Facial, limb, and hand defects seen in the EEC syndrome.

The distinction between syndromic and non-syndromic is evolving, and a complete exam and family history will increasingly be coupled to molecular analysis. Some specific considerations that are of primary concern would include the possibility of the deletion 22q11.2 syndrome, which can commonly have clefts; VDWS, in which lip

pits may be absent in up to 15% of individuals with mutations; *MSX1* mutations, which can have isolated dental anomalies; and *FGFR1* mutations, which in classic cases cause features of Kallmann syndrome that may be absent in some carrier individuals within an affected pedigree.

### 143.5.3 Genetic Counseling

One of the main concerns expressed in a genetic counseling session is recurrence risk (RR) estimation, that is, the chance that a disorder will recur in a family given the presence of a previous affected individual, with the most common scenario being parents with an affected child. For orofacial clefts, RR estimates can be made with precision for some syndromic forms if they are Mendelian or due to a recognized chromosomal anomaly, but the majority of orofacial clefts are isolated or non-syndromic. As reviewed earlier in the chapter, much recent progress has been made toward identifying specific genetic determinants of orofacial clefts, but as yet there are no genetic tests that could identify carriers of genes that increase the risk of having an offspring with non-syndromic clefts. For such cases, there is still a reliance on empiric RR figures. Empiric RRs are determined by surveying a large number of families with affected individuals and cataloging the percentages of affected relatives (74,75). Table 143-1 summarizes the ranges on RR for non-syndromic CL/P and CPO.

Cleft severity (e.g., bilateral CL/P vs. isolated cleft lip only) and family history can result in an increased risk in offspring as well (25). Interestingly, RR estimates can also be modified by the presence of subclinical phenotypic features in unaffected relatives, for example orbicularis oris discontinuities (76).

### 143.6 CONCLUSION

In summary, clefts of the lip and palate are common and complex human birth defects that have both genetic and environmental causes. Seventy percent of clefts involving the lip appear to be isolated, but we are increasingly recognizing the contribution of single genes to such cases, with *IRF6*, *MAFB*, *VAX1* and a locus at 8q24 as recently identified examples. Molecular genetic tests are not yet available for non-syndromic clefting, so recurrence estimates currently are based on family history and associated clinical features; however, we may soon reach a point at which molecular analyses are appropriate for a broad spectrum of individuals with apparently isolated clefts.

**TABLE 143-1** Empiric Recurrence Risk Estimates (in Percentages) for Isolated Orofacial Clefts

Cleft Lip with or without Palate	Affected Family Member	Cleft Palate
One sibling	1.2–5.1	2–5
Two siblings	9.0–14.0	10–20
Mother	1.9–6.8	–
Father	2.0–4.5	–
One parent (not specified)	1.7–5.0	3.0–7.0
First cousin	0.2–0.5	–
Aunt/uncle	0.4–0.8	–
Grandparent	0.2–0.8	–

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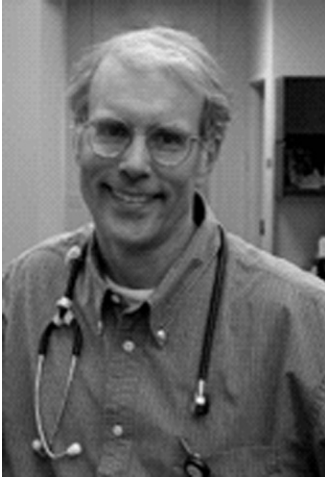


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### Biographies



Jeffrey C Murray, MD has a longstanding career commitment in understanding the genetic and environmental causes of complex pediatric diseases. He is a trained pediatrician and geneticist in the area of human molecular genetics. He has used human gene mapping approaches and epidemiology to discover the underlying causes of cleft lip and palate. He is the co-director of the NIDCR FaceBase consortium, directs the Craniofacial Anomalies Research Center and oversees the projects on cleft etiology in Denmark, Iowa and the Philippines. These efforts use genome-wide association, linkage and sequencing approaches to identifying genetic causes and then coupling these to environmental covariates.



Mary L Marazita, PhD has many years of experience in applying the techniques of statistical genetics and genetic epidemiology to multiple complex human traits (craniofacial birth defects, oral health traits, and others), including many studies of the genetics and phenotypes in non-syndromic orofacial clefting families. She is also engaged in cross-disciplinary clinical research, and serves as a professor in the University of Pittsburgh Clinical and Translational Science Institute (CTSI). She has made major contributions in identifying genes for CL/P, dental anomalies and understanding the role of subphenotypes in CL/P.

# CHAPTER

# 144

## Craniosynostosis

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### GLOSSARY

**Craniosynostosis** – craniosynostosis is premature fusion of the sutures leading to abnormal morphology of the skull and can prevent normal brain and neurologic development requiring surgical intervention.

**Fibroblast growth factor receptors** – fibroblast growth factor receptors (FGFR1, FGFR2, and FGFR3) are a group of tyrosine kinase receptors with multiple isoforms, activated by a wide range of ligands and heparin sulfate proteoglycans, and are involved in developmental processes of differentiation, cell growth and migration, and bone growth. Mutations in these receptors are commonly found in coronal craniosynostosis.

**Sutures** – sutures of the skull consist of undifferentiated mesenchymal cells between ossifying fronts of bones. These cells differentiate to lay down osteoblasts at the leading edge of growing bones. The major sutures of the neurocranium include the coronal, metopic, sagittal, and lambdoid sutures, which separate the frontal, parietal, and occipital bones.

**Transcription factor** – transcription factor proteins bind to regulatory regions of genes and require the recognition of RNA polymerase to regulate gene expression. Transcription factors TWIST1, MSX2, RAB23, and RUNX2 are involved in normal embryonic skull development as well as the pathogenesis of craniosynostosis.

### 144.1 INTRODUCTION

Craniosynostosis is defined as the premature fusion of skull bones. This malformation is usually isolated, but can be found in association with human disorders that are etiologically and pathogenetically heterogeneous. More than 100 syndromes with craniosynostosis as a feature have been clinically delineated (1,2). The genetic basis of some isolated cases as well as craniosynostosis syndromes such as Crouzon, Apert, Jackson–Weiss,

Pfeiffer, Muenke, Crouzonodermoskeletal, Beare–Stevenson, Antley–Bixler with or without genital abnormalities and disordered steroidogenesis, Saethre–Chotzen, Baller–Gerold, craniosynostosis Boston-type, craniofrontonasal, Carpenter, and CRSDA (craniosynostosis and dental anomalies) syndromes have been identified (3–11). Mutations have been found in a variety of genes coding for the fibroblast growth factor receptors (FGFRs), TWIST1, RECQL4, MSX2, ephrins, p450 oxidoreductase, ras-associated protein RAB23, and interleukin 11-receptor alpha. These discoveries have elucidated not only the biology of craniosynostosis, but also the biology of normal skull and sutural development.

### 144.2 SKULL AND SUTURAL DEVELOPMENT

The early development of the human skull is marked by three phases: membrane formation, chondrification, and ossification (12–15). Skull formation begins by the third to fourth week of gestation, and the calvarium is distinguished clearly from the vertebral column by the fifth week. The membrane, derived from mesenchyme of the neural crest and paraxial mesoderm, encapsulates the developing brain and ultimately gives rise to the neurocranium. At the sixth week of gestation, chondrification initiates in both the body of the sphenoid and basiocciput and continues until the seventh week, when the dura mater becomes evident. Subsequently, isolated ossification centers come together to form the individual bones of the skull. Direct ossification of the membrane at the top of the developing skull gives rise to the cranial vault, and ossification of chondrocranium gives rise to the base of the skull. Further development of the skull is associated with rapid brain growth, differentiation and vascularization.

At birth, calvarial bones overlap as the human head is compressed through the birth canal. The resultant

molding normalizes during the first week of life by cranial expansion and widening of the sutural areas. These sutures are articulations in which contiguous margins of cranial bone approximate each other and are united by a thin layer of fibrous tissue. As cranial sutures develop during infancy, they adjust to the growing brain with bone deposition at the sutural margins. The sutures prevent separation between bones by external forces such as trauma, but allow for slight adjustments to absorb minor trauma during infancy and childhood. Sutures function to permit growth of the skull yet provides a firm bond between adjacent bones. Patent sutures eventually close with bony bridging.

Head shape depends in part on the developmental stage when fusion of individual sutures occurs. With premature fusion of sutures or synostosis, the head shape is altered (1). Premature closure of the sagittal suture results in dolichocephaly or an oval-shaped skull with an elongated anterior-posterior length. When the coronal sutures are involved, brachycephaly results with a reduced anterior-posterior length of the skull. Unilateral involvement of the coronal or lambdoidal suture produces plagiocephaly, an asymmetric head shape. With unilateral coronal involvement, there is anterior flattening on the side of the synostosis with a compensatory posterior bulge on the contralateral side. With unilateral lambdoidal synostosis, there is asymmetric posterior flattening with a contralateral anterior bulge. Metopic fusion produces trigonocephaly with prominence in the midregion of the forehead. Multiple sutural synostoses may also occur.

### 144.3 GENETIC EPIDEMIOLOGY OF CRANIOSYNOSTOSIS

Craniosynostosis is among the most common human malformations, with a birth prevalence of approximately one in 2500 newborns in European, African, and Asian populations (1). Craniosynostosis most often occurs as an isolated feature although it can also appear in association with other malformations or diseases. The etiological and pathological factors are both environmental and genetic. Conditions with secondary craniosynostosis include metabolic conditions (hyperthyroidism, rickets, mucopolysaccharidoses), hematological disorders (thalassemia, sickle cell anemia), malformations (holoprosencephaly, microcephaly, encephalocele), and disorders of teratogenic (diphenylhydantoin, valproic acid, retinoids) and iatrogenic (rapid decompression of hydrocephalus) causation.

Sagittal synostosis is the most common isolated craniosynostosis due in part to intrauterine constraint and has a birth prevalence of one in 5000 (1). Seventy-two percent of cases are sporadic with a male to female ratio of 3.5:1. Six percent of cases are familial and predominantly of autosomal dominant inheritance with 38% penetrance. Isolated coronal synostosis occurs half as

frequently as isolated sagittal synostosis. Sixty-one percent of cases are sporadic with a male to female ratio of 1:2 and show an association with advanced paternal age. Ten to 14% of cases are familial with autosomal dominant inheritance and 60% penetrance. Isolated metopic synostosis accounts for almost as many cases as isolated coronal synostosis (16,17) and has a birth prevalence of 0.8–1.9 per 10,000. Epidemiological studies have reported an increase in the occurrence of metopic synostosis by as high as four-fold after the last decade compared to the early 1900s, perhaps due to improvement in the recognition of those cases, especially those requiring an early surgical procedure (18,19). There have been other etiological theories, involving genetic and environmental factors, mechanical constraints, pharmaceutical effects, and social factors (20–22). Ten percent of cases are familial primarily with autosomal dominant inheritance. The male:female ratio is approximately 3.3:1, and no advanced maternal or paternal age has been reported (17). All other single suture synostosis, including lambdoid synostosis, and multiple sutural synostosis are much less frequent. Associated anomalies, especially congenital heart, limb, and ear defects, occur more often with coronal synostosis, and there is more known about the genetic bases of craniosynostosis involving the coronal suture than other sutures.

## 144.4 CRANIOSYNOSTOSIS SYNDROMES

### 144.4.1 Crouzon Syndrome

Crouzon syndrome (OMIM #123500) is one of the most common, autosomal dominant conditions involving coronal synostosis. Other common features include midface hypoplasia and shallow bony orbits resulting in ocular proptosis although there is significant variability in expression (Figure 144-1). It is not unusual for more mildly affected family members to go undiagnosed until the birth of a more severely affected child. The birth prevalence is estimated at one in 25,000 births and accounts for 4.5% of all cases of craniosynostosis. Over half of Crouzon syndrome cases occur as new, spontaneous mutations. Germline mosaicism has also been proposed in families with two unaffected parents who had more than one affected child (23,24).

The clinical findings of Crouzon syndrome are generally limited to the head and neck region. This is in contrast to several other craniosynostosis syndromes, in which abnormalities of the hands, feet, or both are common. Crouzon syndrome is characterized by brachycephaly resulting from coronal synostosis. The forehead is often high and prominent. Other common features are hypertelorism, strabismus, midface hypoplasia, a prominent beaked nose, high arched palate, mandibular prognathism, and dental malocclusion (see Figure 144-1). Ocular proptosis is a consistent finding and is due to the





**FIGURE 144-1** Crouzon syndrome. Brachycephaly, ocular proptosis, beaked nose, and midface hypoplasia. (From Tewfik, T. L.; Teebi, A. S.; der Kaloustian, V. M. *Selected Syndromes and Conditions. In Congenital Anomalies of the Ear, Nose, and Throat, 1st ed.*; Tewfik, T. L.; der Kaloustian, V. M., Eds.; Oxford University Press: Oxford, 1997; pp 461.)

development of shallow bony orbits. In extreme cases, subluxation of the globe occurs. The placement of the ocular musculature is affected as well, and strabismus is a common feature. Cervical spine abnormalities, especially fusion of vertebral bodies has been described. The fusion of C2–C3 occurs most frequently. Fusion of tracheal arches (tracheal cartilaginous sleeve) has also been reported (25). Conductive hearing loss occurs in a significant percentage of Crouzon syndrome patients. Intelligence is usually normal. However, in cases where the craniosynostosis leads to significant and prolonged increase in intracranial pressure, intellectual compromise and vision loss may result.

The majority of cases with Crouzon syndrome are caused by missense mutations in fibroblast growth factor receptor 2 (*FGFR2*)(OMIM \*176943)(26).

#### 144.4.2 Jackson–Weiss Syndrome

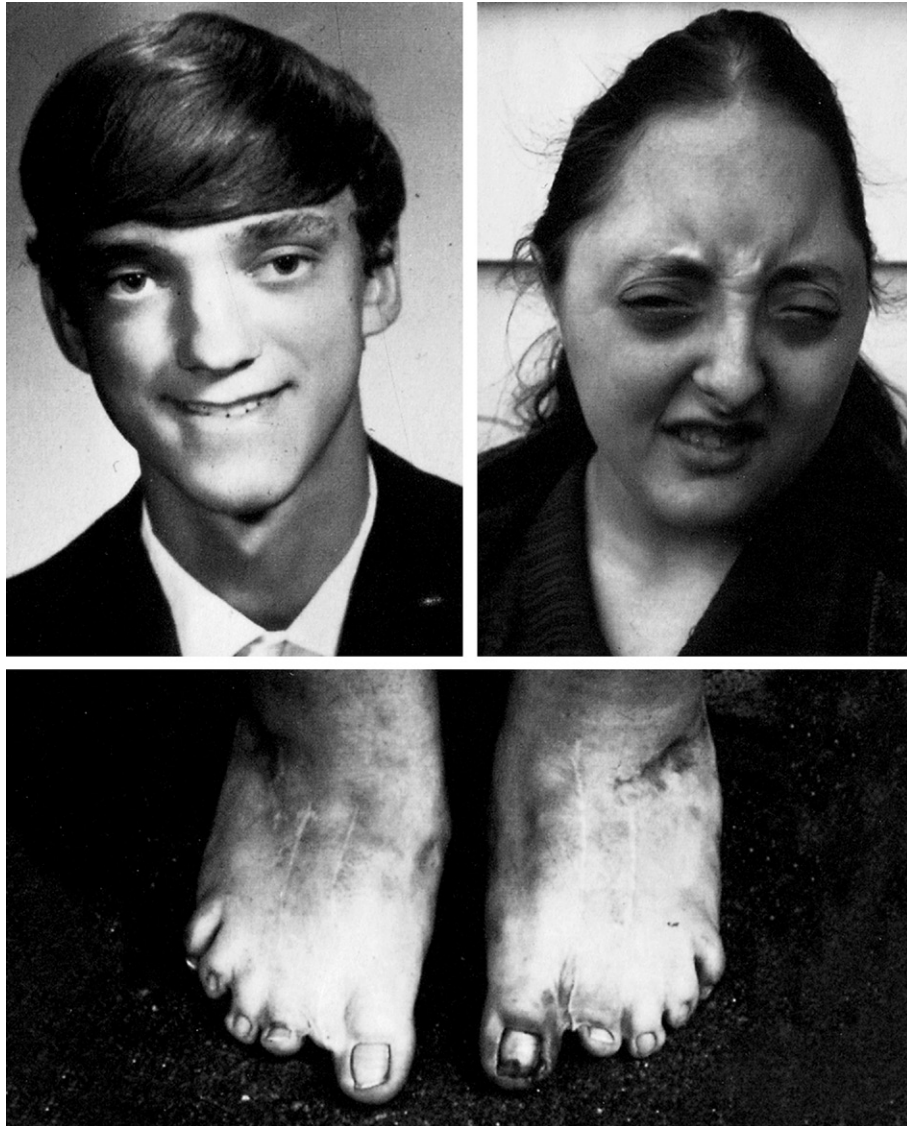
Jackson–Weiss syndrome (OMIM #123150) is an autosomal dominant disorder of craniosynostosis and bony foot deformities. It was initially described in very large Amish kindred in 1976 (27). The cranial features were quite variable. In fact, the authors felt that within this extended family, the entire range of the cephalosyndactyly syndromes (with the exception of Apert syndrome) appeared to be represented. Midfacial hypoplasia was common, and the skull manifestations ranged from mild prominence of the forehead to severe acrocephaly (Figure 144-2). Radiographic evidence of craniosynostosis was obtained in several affected individuals with apparently normal skull shape. Even in those individuals with severe cranial deformity, intelligence was unaffected.

A common feature in all affected family members was bony abnormalities of the feet. These include broad, medially deviated great toes, and fusion of tarsal bones, with calcaneo-cuboid fusion occurring commonly (see Figure 144-2). Some individuals had no outwardly apparent foot abnormality, yet had bony fusion or other defect revealed on radiograph. Others were noted to have cutaneous syndactyly of the second and third toes. An unrecognized branch of the original family was recently studied, and additional features were noted in affected members, including a leg-length discrepancy and unilateral absence of the fifth digital ray of the foot (28). No individual was found to have bony abnormalities of the hands or thumbs, a feature, which was used to distinguish Jackson–Weiss syndrome from other craniosynostosis conditions.

Jackson–Weiss syndrome is due to missense mutations in *FGFR2* (29). Because this syndrome is allelic with Crouzon syndrome and some patients with either of these two diagnoses can have the identical mutations in the same or unrelated families, these phenotypes are now thought to belong to the clinical spectrum of *FGFR2*-craniosynostosis syndromes.

#### 144.4.3 Pfeiffer Syndrome

Pfeiffer syndrome (OMIM #101600) is an autosomal dominant disorder of craniosynostosis, broad thumbs, and great toes, and many cases are sporadic. Craniofacial features include synostosis of the coronal sutures producing brachycephaly, midface hypoplasia, and relative prognathism (Figure 144-3 and Figure 144-4). Hypertelorism, prominent/beaked nose, and high arched palate are also frequent findings. The hands and feet show a

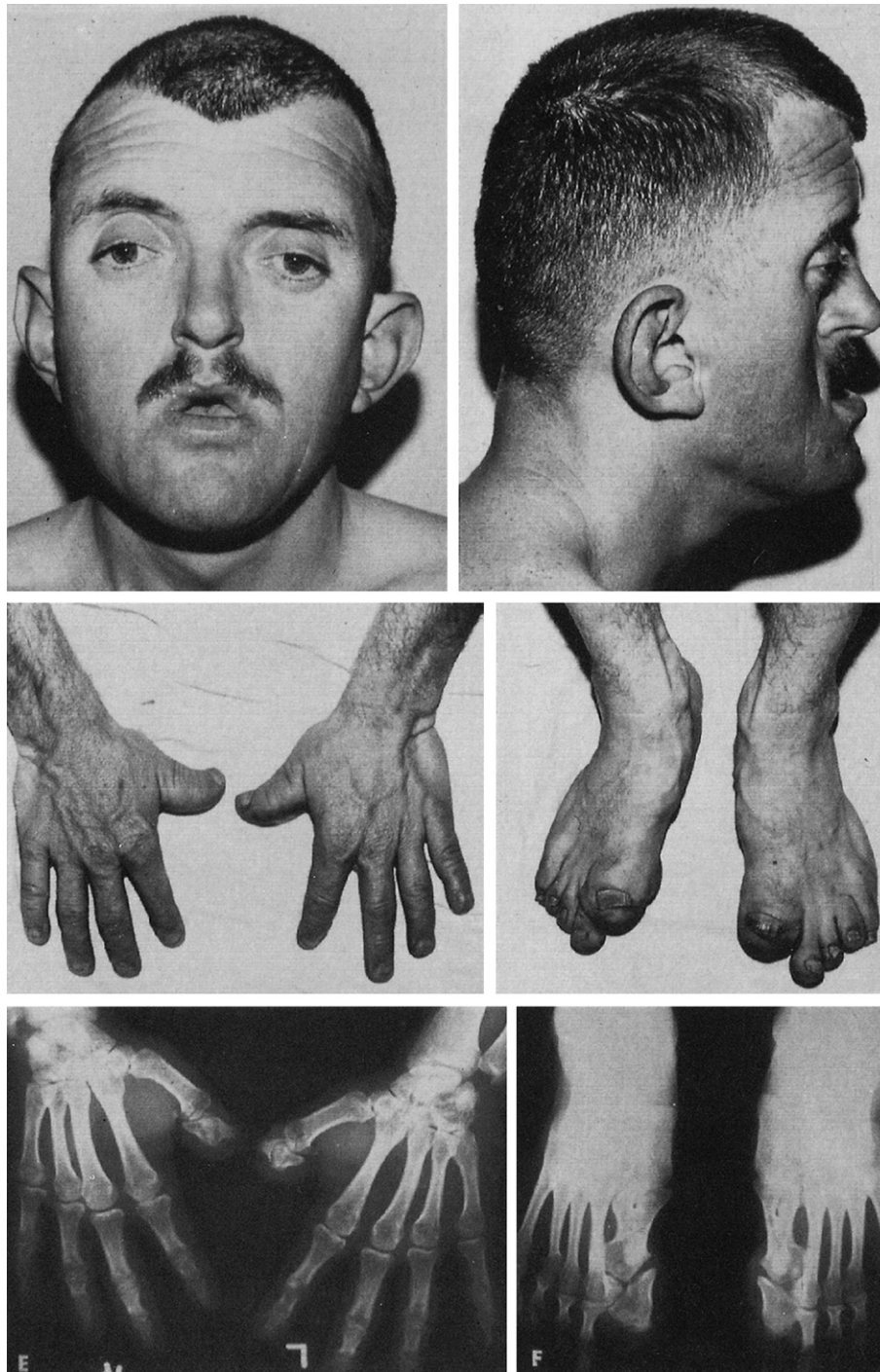


**FIGURE 144-2** Jackson-Weiss syndrome. Variable craniosynostosis with brachycephaly (left) and acrocephaly (right) and large great toes and syndactyly. (From Jabs, E. W.; Li, X.; Scott, A. F., et al. *Jackson-Weiss and Crouzon Syndromes Are Allelic with Mutations in Fibroblast Growth Factor Receptor 2*. *Nat. Genet.* 1994, 8 (3), 275–279; Jackson, C. E.; Weiss, L.; Reynolds, W. A., et al. *Craniosynostosis, Midfacial Hypoplasia and Foot Abnormalities: An Autosomal Dominant Phenotype in a Large Amish Kindred*. *J. Pediatr.* 1976, 88 (6), 963–968.)

characteristic pattern of malformation (see [Figure 144-3](#)). Brachydactyly is common, and due to a shortening of (and in some cases, to a complete absence of) the middle phalanx. Thumbs and great toes are broad, and often show a varus deformity. There may be fusions between phalanges, or among various tarsal or carpal bones. Fusions of the proximal ends of metatarsals and metacarpals have also been described. Other bony abnormalities include fusion of cervical or lumbar vertebrae, radiohumeral and radioulnar synostosis (see [Figure 144-4](#)). Additional rare abnormalities associated with the Pfeiffer syndrome include ptosis, optic nerve hypoplasia, choanal atresia, preauricular tags, bifid uvula, supernumerary teeth, pyloric stenosis, and anteriorly placed anus (2).

As with several other dominant craniosynostoses, there is marked variability in expression. The wide spectrum

prompted Cohen (30) to propose a clinical classification of Pfeiffer syndrome into types I, II, and III. Type I is considered the “classic” Pfeiffer syndrome with the previously mentioned characteristics. This type may be inherited, and intelligence is generally normal. Type II is characterized by a cloverleaf (kleeblattschädel) skull deformity, the broad thumbs and great toes typical of Pfeiffer syndrome, and ankylosis of the elbows (see [Figure 144-4](#)). Type II is usually a sporadic occurrence with poor prognosis for intelligence and long-term survival. Type III is similar to type II without a cloverleaf skull. Proptosis is often marked. This type is also generally a sporadic occurrence within a family, and has a poor prognosis for survival and function. Types II and III are more likely to be associated with various internal anomalies as well, including abnormalities of the central nervous system. The classification is not absolute, and



**FIGURE 144-3** Pfeiffer syndrome type I. Brachycephaly, midface hypoplasia, mandibular prognathism, brachydactyly, broad thumbs that deviate radially, and short broad great toes with varus deformity and partial cutaneous syndactyly. (From Cohen, M. M., Jr. *An Etiologic and Nosologic Overview of Craniosynostosis Syndromes*. In *Malformation Syndromes, Birth Defects Original Article Series*; Bergsma, D., Eds.; 1975, 11 (2); pp. 137–189.)

there is overlap between the subtypes, yet was felt useful for classification and prognosis for an affected individual.

Pfeiffer syndrome is due to mutations in either fibroblast growth factor receptor 1 (*FGFR1*, OMIM \*136,350) or *FGFR2* (31). Patients with mild features of Pfeiffer syndrome have Type I and generally have *FGFR1* mutations. Patients with this syndrome can share identical *FGFR2* mutations with Crouzon and

Jackson–Weiss syndromes. Therefore, Pfeiffer syndrome is also considered to belong to the clinical spectrum of *FGFR2*-craniosynostosis syndromes.

#### 144.4.4 Apert Syndrome

Apert syndrome (OMIM #101200) is perhaps the most easily recognizable of the craniosynostosis syndromes.





**FIGURE 144-4** Pfeiffer syndrome type II. Brachyurricephaly, ocular proptosis, hypertelorism, depressed nasal bridge, midface hypoplasia, and low-set posterior rotated ears. Radiographs show mild cloverleaf skull deformity, increased digital markings, and radioulnar synostosis. (From Okajima, K.; Robinson, L. K.; Hart, M. A., et al. *Ocular Anterior Chamber Dysgenesis in Craniosynostosis Syndromes with a Fibroblast Growth Factor Receptor 2 Mutation*. *Am. J. Med. Genet.* 1999, 85 (2), 160–170.)

In addition to craniosynostosis and typical facial features, the hands and feet are characterized by a ‘mitten type’ syndactyly of the digits (Figure 144-5). Apert syndrome is also more likely to be associated with internal organ and central nervous system abnormalities as well as mental impairment. This syndrome is rare and occurs less than 1 in 65,000 births. Most cases are sporadic and only a few familial cases of autosomal dominant inheritance have been documented.

The pathogenesis of the craniosynostosis in Apert syndrome is somewhat unique. While the coronal sutures are typically fused at the time of birth, the sagittal suture generally does not form at all. Instead, there is typically a wide-open gap along the top of the skull at birth beginning at the glabella. Over the first 2–4 years of life, this gap is filled in by bony islands that eventually coalesce to form an intact calvarium. However, no sagittal or metopic suture is ever formed to allow for continued skull growth. This process has been described by Cohen and Kreiborg (32) as sutural agenesis, as opposed to

craniosynostosis in the sagittal region. Due to the large congenital “pressure valve” afforded by the gap in the bones, it is rare for an Apert syndrome patient to have difficulty with increased intracranial pressure in the first few years of life. However, the incidence of mental deficiency is still higher in these patients, due to a higher frequency of central nervous system defects.

The brain is typically megalencephalic, and the shape is also affected by the skull abnormalities. Due to the constriction of the area behind the fused coronal sutures, the developing brain is forced to grow upward and bulge forward, where the bony gap affords more room. This may lead to an appearance similar to that seen with an encephalocele. Other primary central nervous system defects include absent or abnormal corpus callosum, gyral abnormalities, white matter hypoplasia, heterotopic gray matter, and limbic structure defects (33). These primary abnormalities are likely to contribute to the finding that early surgical intervention does not appear to significantly improve intellectual outcome (34).

The facial appearance of an Apert syndrome patient is characterized by the high and prominent forehead, midface hypoplasia giving the appearance of prognathism, and a prominent, beaked nose (see Figure 144-5). There may be horizontal grooves over the supraorbital ridges in infancy that tend to disappear with age. The palate is typically high arched, and quite narrow, giving a “Byzantine arch” appearance. Crowding of the dentition is common. Of all the features of Apert syndrome, it is clearly the hands and feet that are most characteristic. There is severe and symmetric syndactyly of the digits, which at a minimum involves the second through the fourth fingers and toes, and may or may not involve the first and/or fifth digits as well. Nails may be distinct or continuous over several digits (see Figure 144-5).

Apert syndrome is also associated with an increased incidence of internal organ malformations, most commonly in the cardiovascular system (10%) and genitourinary system (9.6%). A smaller percentage was found to have respiratory and gastrointestinal malformations (each at 1.5%) (35). Certain dermatological conditions, such as hyperhidrosis, oily skin, and acneiform lesions are frequent in Apert syndrome. Acne is not only found on the face, but commonly extends to the arms, chest, and back (36).

Ninety-nine percent of Apert syndrome patients have either of two mutations, Ser252Trp or Pro253Arg, in FGFR2 (37).

#### 144.4.5 Muenke Syndrome

This craniosynostosis syndrome is defined by a Pro250Arg mutation in FGFR3, rather than a distinct phenotype (OMIM #602849) (7). The birth prevalence of this condition has been estimated to be one in 30,000 and may account for as much as 5% of all craniosynostosis cases (38). The associated clinical findings are highly variable,





**FIGURE 144-5** Apert syndrome. High prominent forehead, downslanting palpebral fissures, midface hypoplasia, and mitten type syndactyly of digits and severe syndactyly of toes. (From Lessard, M.-L.; Mulliken, J. B. *Major Craniofacial Anomalies. In Congenital Anomalies of the Ear, Nose and Throat, 1st ed.*; Tewfik, T. L., der Kaloustian, V. M., Eds.; Oxford University Press: Oxford, 1997; pp 307; Mann, T. P., Ed. *Colour Atlas of Paediatric Facial Diagnosis*; Kluwer Academic Publishers: London, 1989; pp 88.)



**FIGURE 144-6** Muenke syndrome. Brachycephaly, ptosis and downslanting palpebral fissures (left), mild ocular proptosis, hypertelorism, and prognathism (right). (From Paznekas, W. A.; Cunningham, M. L.; Howard, T. D., et al. *Genetic Heterogeneity of Saethre–Chotzen Syndrome, due to TWIST1 and FGFR Mutations*. *Am. J. Hum. Genet.* 1998, 62 (6), 1370–1380.)

with bicoronal or unicoronal synostosis, midface hypoplasia, downslanting palpebral fissures, and ptosis (Figure 144-6). Some patients with this mutation have a Crouzon-like, Pfeiffer-like, Jackson–Weiss-like, or Saethre–Chotzen-like phenotype. Large craniosynostosis families have been described with variable craniofacial morphology, including severe pancraniosynostosis (39–41). One such family has been referred to as having “Craniosynostosis, Adelaide type” (OMIM %600593). Other patients have isolated or non-syndromic craniosynostosis and still others have only macrocephaly or even a normal cranium. Developmental

delay and hand and foot anomalies are seen in some individuals. Radiographs can detect brachydactyly, distal and middle phalangeal hypoplasia, thimble-like middle phalanges, coned epiphyses, carpal bone malsegmentation, hallux valgus, and tarsal, calcaneal and navicular fusions. Although the phenotypic spectrum is highly variable, coronal craniosynostosis with brachydactyly and carpal/tarsal coalition have been suggested as the most characteristic features associated with this mutation (42). Patients also show significant, but incompletely penetrant and predominantly low frequency, sensorineural hearing loss (43).

### 144.4.6 Crouzonodermoskeletal Syndrome

There are patients who have features that resemble Crouzon syndrome, but also have a dermatological disorder of acanthosis nigricans and melanocytic nevi (OMIM #612247, Crouzon syndrome and acanthosis nigricans) (44) (Figure 144-7). The association of these skeletal and skin findings breeds true as a distinct autosomal dominant condition (45). Isolated acanthosis nigricans is characterized by verrucous hyperplasia of the skin and is usually found in flexural areas, but in this craniosynostosis condition it is present in the perioral, periorbital, and nasolabial areas as well as the neck, axillae, chest, breast, and abdomen. Additional findings include female preponderance, late childhood onset of dermatological features, severe choanal atresia, hydrocephalus, and cementomas of the jaw. Vertebral anomalies and conductive hearing loss are present with less frequency than in classic cases of Crouzon syndrome (46). Subtle forms of skeletal dysplasia associated with achondroplasia (OMIM #100800), the most common form of *FGFR3*-related short stature, such as brachydactyly and widening of the interpediculate distances of the spine, have been found in Crouzonodermoskeletal syndrome patients (47).

These phenotypic findings are consistent with different functional changes at the same transmembrane domain of *FGFR3* due to the Ala391Glu mutation in Crouzonodermoskeletal syndrome and the common Gly380Arg mutation in achondroplasia.

### 144.4.7 Beare–Stevenson Cutis Gyrata Syndrome

Beare–Stevenson cutis gyrata syndrome (OMIM #123790) is a rare condition with less than 20 reported patients (48). These sporadic cases are associated with

advanced paternal age, and therefore presumed and later proven through molecular studies to be heterozygous for autosomal mutations. The syndrome is marked by furrowed skin (*cutis gyrata*), acanthosis nigricans, craniosynostosis with craniofacial dysmorphism, digital anomalies, umbilical and anogenital anomalies, and early death (Figure 144-8). The *cutis gyrata* presents with fine ribbing and deep folds on the scalp, forehead, face, neck, chest, palms, soles, preauricular region, and perianal/genital region. The craniofacial features are similar to other craniosynostosis syndromes with midface hypoplasia, ocular hypertelorism and proptosis, choanal atresia, and palatal anomalies. In severe cases, a cloverleaf skull deformity may be present. A prominent umbilical stump can be noted at birth and is characteristic of this condition.

Beare–Stevenson syndrome is caused by missense mutations in *FGFR2*.

### 144.4.8 Osteoglophonic Dysplasia

Osteoglophonic dysplasia (OMIM #166250, Fairbank–Keats syndrome) is a rare, autosomal dominant skeletal dysplasia also associated with advanced paternal age. The major features are craniosynostosis, unerupted teeth, platyspondyly with anterior projection, brachydactyly, and rhizomelic dwarfism (Figure 144-9). The craniofacial anomalies are similar to those of other craniosynostosis syndromes with frontal bossing, ocular proptosis, and maxillary hypoplasia. Its most characteristic features are multiple lucent metaphyseal defects for which the name of the dysplasia was derived from a Greek word meaning “hollowed out.” The bones have generalized osteoporosis and are susceptible to fractures. Patients have demonstrated renal phosphate wasting and osteomalacia (1,49).

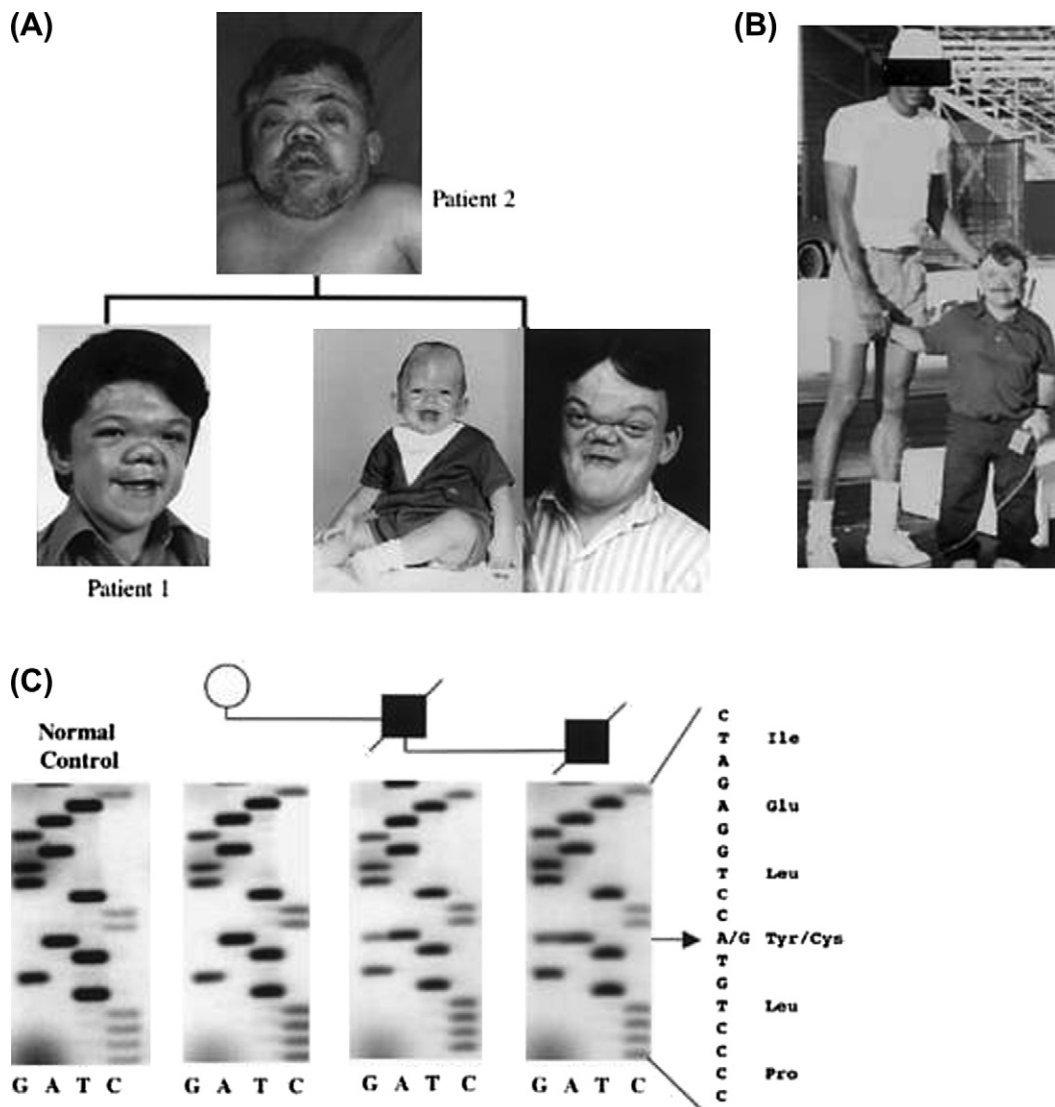
Osteoglophonic dysplasia is caused by missense mutations in *FGFR1*.



**FIGURE 144-7** Crouzonodermoskeletal syndrome. Ocular proptosis, midface hypoplasia, and hyperpigmentation and thickening of the neck. (From Meyers, G. A.; Orlow, S. J.; Munro, I. R., et al. *Fibroblast Growth Factor Receptor 3 (FGFR3) Transmembrane Mutation in Crouzon Syndrome with Acanthosis Nigricans*. *Nat. Genet.* 1995, 11 (4), 462–464.)



**FIGURE 144-8** Beare–Stevenson cutis gyrata syndrome. Coarse and fine wrinkling of the skin with facial features of ocular proptosis and mid-face hypoplasia (**right**; From Przylepa, K. A.; Paznekas, W.; Zhang, M., et al. *Fibroblast Growth Factor Receptor 2 Mutations in Beare–Stevenson Cutis Gyrata Syndrome*. *Nat. Genet.* 1996, 13 (4), 492–494.; **left**; From Hall, B. D.; Cadle, R. G.; Golabi, M., et al. *Beare–Stevenson Cutis Gyrata Syndrome*. *Am. J. Med. Genet.* 1992, 44 (1), 82–89.)



**FIGURE 144-9** Osteoglophonic dysplasia. Severe craniofacial abnormalities including craniosynostosis, telecanthus, and facial hypoplasia (infant and adult photo of the same patient). (From White, K. E.; Cabral, J. M.; Davis, S. I., et al. *Mutations that Cause Osteoglophonic Dysplasia Define Novel Roles for FGFR1 in Bone Elongation*. *Am. J. Hum. Genet.* 2005, 76 (2), 361–367.)



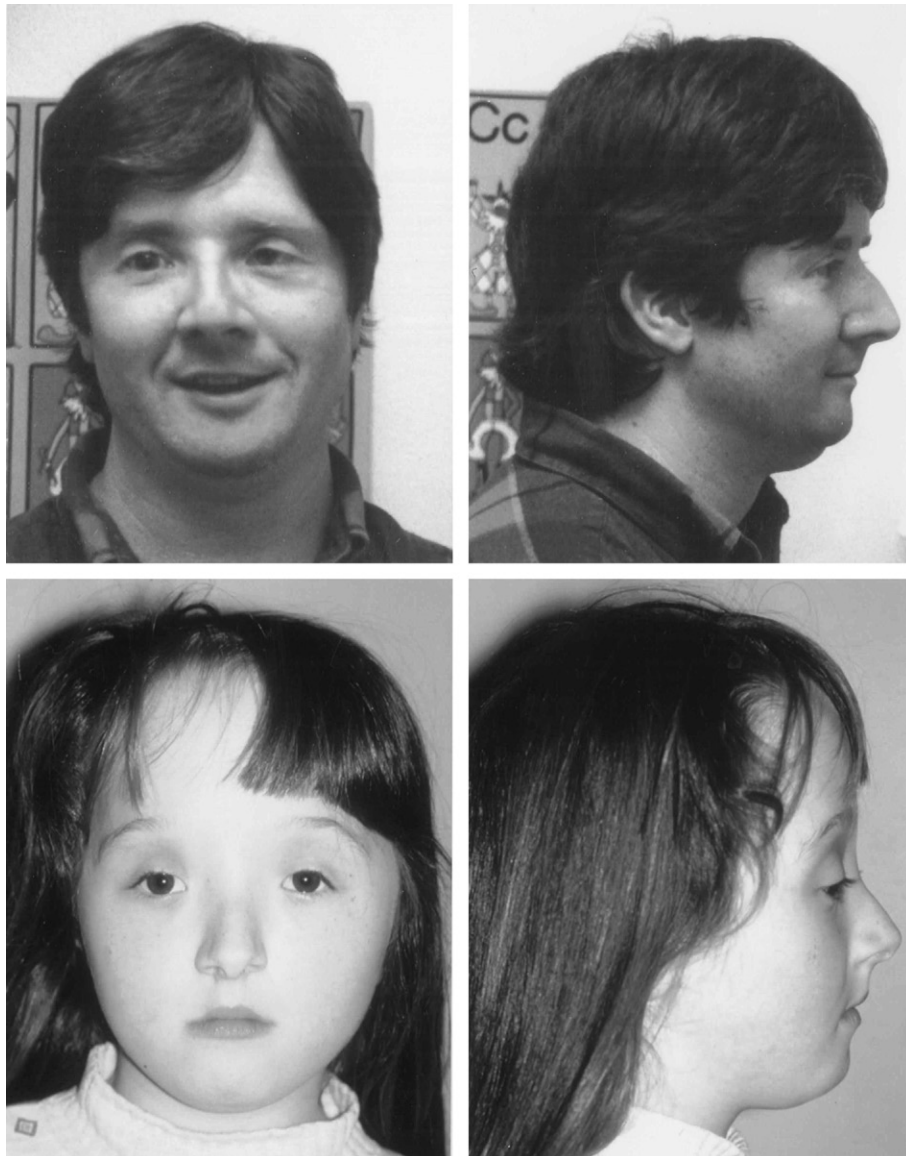
### 144.4.9 Saethre–Chotzen Syndrome

Saethre–Chotzen syndrome (OMIM #101400) is a common autosomal dominant, craniosynostosis condition. In general, however, the features are most often mild, and individuals only rarely require surgical intervention to address the cranial shape. It is felt to occur at a greater frequency than the other dominant craniosynostosis syndromes, and due to its milder nature, is likely to be under-diagnosed.

As craniosynostosis is not a constant feature, there is a wide variation in the appearance of affected individuals (50). Early closure may affect the various sutures, producing brachycephaly, acrocephaly, or trigonocephaly. The degree of sutural synostosis often differs between the right and left sides, producing both plagiocephaly and

facial asymmetry. In some cases, the fontanels may be late in closing, and there may be persistent parietal foramina. In addition to the asymmetry, the most common facial features are ptosis, a low frontal hairline, and ear abnormalities (Figure 144-10). Specifically, the ears tend to be small, and posteriorly rotated. The crura are often prominent. There is an increased incidence of hearing loss.

Hand and foot abnormalities are less dramatic than in other related syndromes. Often, there is brachydactyly with partial cutaneous syndactyly. Most often, the syndactyly occurs between the second and third fingers or toes, although other digits have also been reported to be affected occasionally. Bifid halluces or duplication of the large toe in association with features of Saethre–Chotzen syndrome had been designated as Robinow–Sorauf syndrome (OMIM #180570). Mutations in the same gene,



**FIGURE 144-10** Saethre–Chotzen syndrome. Facial asymmetry, midface hypoplasia, ptosis, and hypertelorism. (From Paznekas, W. A.; Cunningham, M. L.; Howard, T. D., et al. *Genetic Heterogeneity of Saethre–Chotzen Syndrome, due to TWIST1 and FGFR Mutations*. *Am. J. Hum. Genet.* 1998, 62 (6), 1370–1380.)



*TWIST1* (OMIM \*601622), have been found in both conditions, which suggest that Robinow–Sorauf represents cases of clinical variability in Saethre–Chotzen syndrome (51,52).

#### 144.4.10 Craniosynostosis, Boston-Type

Craniosynostosis, Boston-Type (OMIM #604757) is an autosomal dominant condition described in a single New England family (53). The affected family members have variable cranial morphology with craniosynostosis and the consistent finding of recession of the supra-orbital region in relation to the anterior surface of the cornea (Figure 144-11). Skull malformations included

forehead retrusion, frontal bossing, turribrachycephaly, and the cloverleaf skull anomaly. Most affected individuals were myopic or hyperopic. Several affected members suffered from severe headaches, and four had a seizure disorder. Intelligence was normal. No hand or foot abnormalities were noted on inspection, but radiographic examination demonstrated short first metatarsals in three of the four individuals evaluated. A cleft of the soft palate and a triphalangeal thumb were each noted in one member.

Craniosynostosis, Boston-Type is caused by a Pro-148His mutation in *MSX2* (OMIM \*123101) (54). It was the first mutation discovered to be associated with a craniosynostosis syndrome.



**FIGURE 144-11** Craniosynostosis, Boston-type with brachycephaly and frontal orbital recession (**top**; From Müller, U.; Warman, M. L.; Mulliken, J. B.; Weber, J. L. Assignment of a Gene Locus Involved in Craniosynostosis to Chromosome 5qter. *Hum. Mol. Genet.* 1993, 2 (2), 119–122.), and parietal foramina (**bottom**; From Wilkie, A. O. M.; Tang, Z.; Elanko, N., et al. Functional Haploinsufficiency of the Human Homeobox Gene *MSX2* Causes Defects in Skull Ossification. *Nat. Genet.* 2000, 24 (4), 387–390.) The two conditions: 1) brachycephaly and frontal orbital recession 2) parietal foramina, are caused by *MSX2* gain and loss of function mutations, respectively.



**FIGURE 144-12** Craniofrontonasal syndrome. Hypertelorism, divergent squint, central nasal groove (**left**, From Twigg, S. R.; Kan, R.; Babbs, C., et al. *Mutations of Ephrin-B1 (EFNB1), a Marker of Tissue Boundary Formation, Cause Craniofrontonasal Syndrome*. Proc. Natl. Acad. Sci. U.S.A. 2004, 101 (23), 8652–8657.), and orbital asymmetry (**right**, From Wieland, I.; Jakubiczka, S.; Muschke, P., et al. *Mutations of the Ephrin-B1 Gene Cause Craniofrontonasal Syndrome*. Am. J. Hum. Genet. 2004, 74 (6), 1209–1215.)

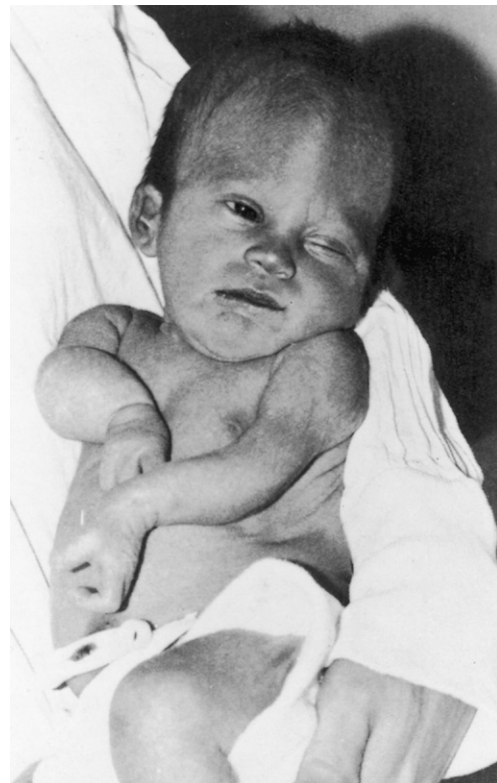
#### 144.4.11 Craniofrontonasal Syndrome

Craniofrontonasal syndrome (OMIM #304110) describes a subgroup of patients with frontonasal dysplasia. This syndrome is an X-linked condition associated with unilateral or bilateral coronal synostosis (55). Affected females have a severe cranial defect with hypertelorism and a bifid nasal tip (Figure 144-12). Other midline abnormalities are present including anterior cranium bifidum, corpus callosum hypoplasia, cleft lip and palate, omphalocele, unilateral breast hypoplasia, and diaphragmatic and umbilical hernias. There can also be craniofacial asymmetry and other skeletal malformations. Extracranial features include sloped shoulders with dysplastic clavicles, pectus excavatum, mild cutaneous syndactyly, and characteristic longitudinal groove of the nails. Less commonly, duplication of the first digit, joint abnormalities, axillary pterygium, and asymmetric lower limb shortness are present. Paradoxically, obligate transmitting males with this X-linked condition are more mildly affected with only hypertelorism and cleft lip and palate.

Craniofrontonasal syndrome is due to loss of function mutations in the ephrin B1 gene (*EFNB1*, OMIM \*300035)(56).

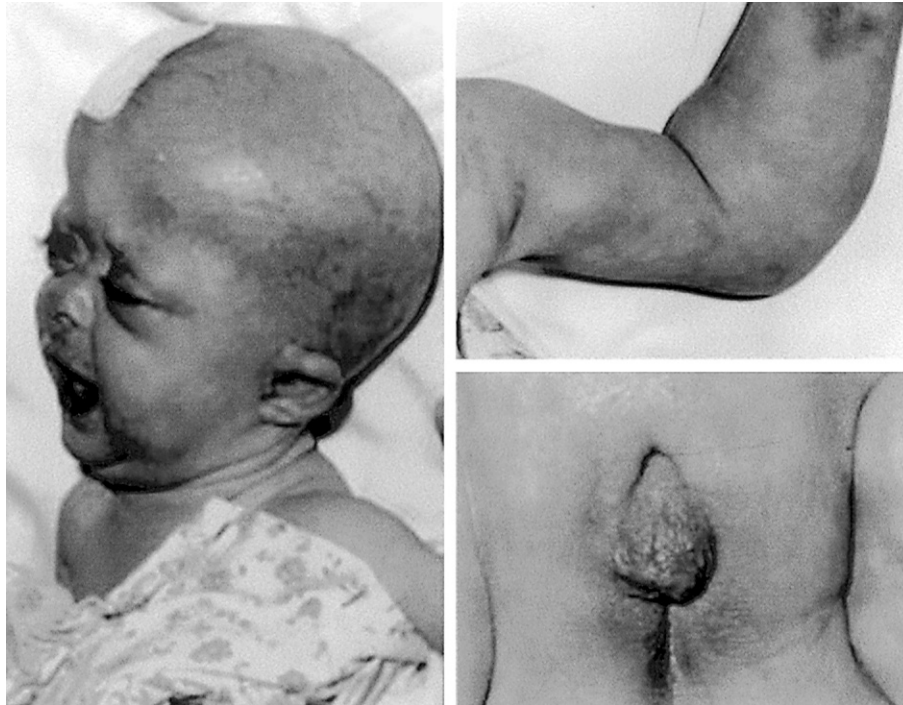
#### 144.4.12 Baller–Gerold Syndrome

Baller–Gerold syndrome (OMIM #218600) is a rare condition involving craniosynostosis and radial ray defects (Figure 144-13). The coronal suture is most often affected, but synostosis of the lambdoid, metopic, and sagittal sutures have been reported as well (2,57). There is also variability in the radial ray defects, which may include hypoplasia or aplasia of the radii and/or thumbs. Other upper extremity abnormalities, such as a short and curved ulnar bone are common.



**FIGURE 144-13** Baller–Gerold syndrome. Craniosynostosis with metopic ridge and radial aplasia with shortened forearms. (From Gorlin, R. J.; Cohen, M. M., Jr.; Hennekam, R. C. M., Eds.; *In Syndromes with Craniosynostosis; Syndromes of the Head and Neck*, 4th ed.; 2001; Oxford University Press: Oxford; pp 654–703.)

A long list of associated anomalies other than those of the skull and upper limbs has been reported in patients with Baller–Gerold syndrome. These include congenital defects of the heart, kidneys and vertebrae. Anteriorly placed or imperforate anus has also been reported, as



**FIGURE 144-14** Antley-Bixler syndrome. Craniosynostosis, fixed elbow joint, clitoromegaly and hooded prepuce. (From Reardon, W.; Smith, A.; Honour, J. W., et al. Evidence for Digenic Inheritance in Some Cases of Antley-Bixler syndrome? *J. Med. Genet.* 2000, 37 (1), 26–32.)

have perineal and rectovaginal fistulae. Short stature is common, and individuals with both mental retardation and normal intelligence have been noted. Due to the presence of these multiple additional features, other genetic conditions must be considered in individuals with craniosynostosis and radial ray defects. These would include Saethre-Chotzen, VACTERL (Vertebral, Anal, Cardiac, TE fistula, Renal, Limb) association (OMIM #192350), Fanconi's anemia, Roberts phocomelia (OMIM #268300), Rothmund-Thompson (OMIM #268400), Holt-Oram (OMIM #142900) and Thrombocytopenia-Absent Radius (TAR, OMIM #274000) syndromes. Effort should be made to exclude each of these conditions before assigning a diagnosis of Baller-Gerold syndrome.

Baller-Gerold syndrome is due to heterozygous mutations in the *RECQL4* gene (OMIM \*603780), which codes for a DNA helicase protein and increases predisposition to cancers and premature aging (9). Baller-Gerold syndrome could be integrated in a clinical spectrum that encompasses Rothmund-Thomson and RAPADILINO (OMIM #266280; RAdial ray defect, PAtella hypoplasia and aplasia and cleft or high arched PAlate, DIarrhea and DIsllocated joints, Little size and LImb malformations, NOse slender and NOormal intelligence) syndromes that are associated with radial ray defects, but not craniosynostosis. All three conditions are allelic as they can be caused by mutations in the *RECQL4* (9). Baller-Gerold and Rothmund-Thomson syndromes have overlapping features of poikiloderma and anterior displacement of the anus, while all three conditions have facial dysmorphisms, patellar abnormalities, growth deficiency, and

gastrointestinal disturbances in common. A patient with Baller-Gerold and a rare midline NK/T lymphoma was reported to have two different truncating mutations in the *RECQL4* gene (58).

#### 144.4.13 Antley-Bixler Syndrome with Genital Abnormalities and Disordered Steroidogenesis (POR Syndrome, P450 Oxidoreductase)

Antley-Bixler syndrome (OMIM #207410) was first described in a child with “trapezoidocephaly” from coronal and lambdoid synostosis, midface hypoplasia, humeroradial synostosis, bowing of femora, fractures, and other abnormalities (59) (Figure 144-14). Most cases have also been sporadic. Affected individuals may have additional skeletal and visceral features such as ocular proptosis, dysplastic ears, depressed nasal bridge, choanal stenosis, camptodactyly, and malformations of the heart and kidney. Respiratory obstruction with early demise has been reported in about 55% of cases.

Patients with Antley-Bixler syndrome have been associated with heterozygous, missense mutations in *FGFR2* (60). Of interest, patients with these mutations have features similar to a severe form of Pfeiffer syndrome with elbow synostosis, but do not have urogenital problems or recessive mutations initially attributed to some cases also called Antley-Bixler syndrome. Chun et al. (60) and Reardon et al. (61) proposed that Antley-Bixler syndrome patients with ambiguous genitals may represent a digenic or distinct condition (see below).





**FIGURE 144-15** Carpenter syndrome. Metopic ridging and lateral bulge with multiple suture synostosis, broad thumbs and syndactyly, brachydactyly, clinodactyly, and polydactyly. (From Jenkins, D.; Seelow, D.; Jehee, F. S., et al. *RAB23 Mutations in Carpenter Syndrome Imply an Unexpected Role for Hedgehog Signaling in Cranial Suture Development and Obesity*. *Am. J. Hum. Genet.* 2007, 80, 1162–1170.)

Patients with autosomal recessive inheritance of skeletal features of Antley–Bixler syndrome, fractures, bowing of the femurs, and ambiguous genitalia such as vaginal atresia, fused labia minora, and hypoplastic labia majora in females (OMIM #201750, see Figure 144-14) were subsequently found to have biochemical abnormalities in steroidogenesis with decreased activities of 17- $\alpha$ -hydroxylase, 17,20-lyase, and 21-hydroxylase (61). Virilization of affected females occurs due to disturbed androgen metabolism (62). Patients can also have abnormalities in sterol metabolism with decreased activity of lanosterol 14 $\alpha$ -demethylase (63). Furthermore, offspring of mothers taking the antifungal agent, fluconazole (an inhibitor of lanosterol 14 $\alpha$ -demethylase), during early pregnancy have Antley–Bixler-like features (64). All of these enzymes require cytochrome P450 oxidoreductase, and the *POR* gene (OMIM \*124015) coding for this cofactor was found to contain homozygous or compound heterozygous mutations in patients now designated with POR syndrome (3).

#### 144.4.14 Carpenter Syndrome (Acrocephaly Type II)

Carpenter syndrome (OMIM #201000) is a rare disorder with craniosynostosis, obesity, polydactyly, and soft-tissue syndactyly (65). Multi-suture craniosynostosis and polysyndactyly are present in all patients described to date, and abnormal external genitalia occur in all males. It is unlike the other inherited craniosynostoses mentioned above in two aspects. First, it is an autosomal recessive condition and secondly, the synostosis involves the midline metopic and sagittal sutures as well as the coronal sutures (Figure 144-15). Other characteristics include brachydactyly with shortening or absence of the middle phalanges, molar agenesis, genu valgum, hypogenitalism, congenital cardiac defects, umbilical hernia, and learning disability.

Carpenter syndrome is caused by mutation in the *RAB23* gene (OMIM \*606144), which encodes a member of the RAB guanosine triphosphatase (GTPase) family of vesicle transport proteins, and acts as a negative regulator of hedgehog signaling which is known to be involved in craniofacial development of the midline (5).

#### 144.4.15 Craniosynostosis and Dental Anomalies Syndrome (CRSDA)

This rare autosomal recessive, craniosynostosis syndrome is also referred to as Kreiborg–Pakistani syndrome (OMIM #614188) as it was described in Pakistani and English families. Affected individuals have metopic, coronal, sagittal, and/or lambdoid sutures, papilledema, maxillary hypoplasia with class III malocclusion, and variable dental anomalies of delayed tooth eruption of the canine and premolars and as many as seven supernumerary teeth in the incisor, canine, and premolar regions (Figure 144-16). Variably present were features like Crouzon syndrome and short stature, abnormal cranial base angle, prominent ears, constricted nasopharynx, broad first toes, hallux valgus, mild 2–3 toe syndactyly, clinodactyly, short phalanges, and dysplastic fingernails.

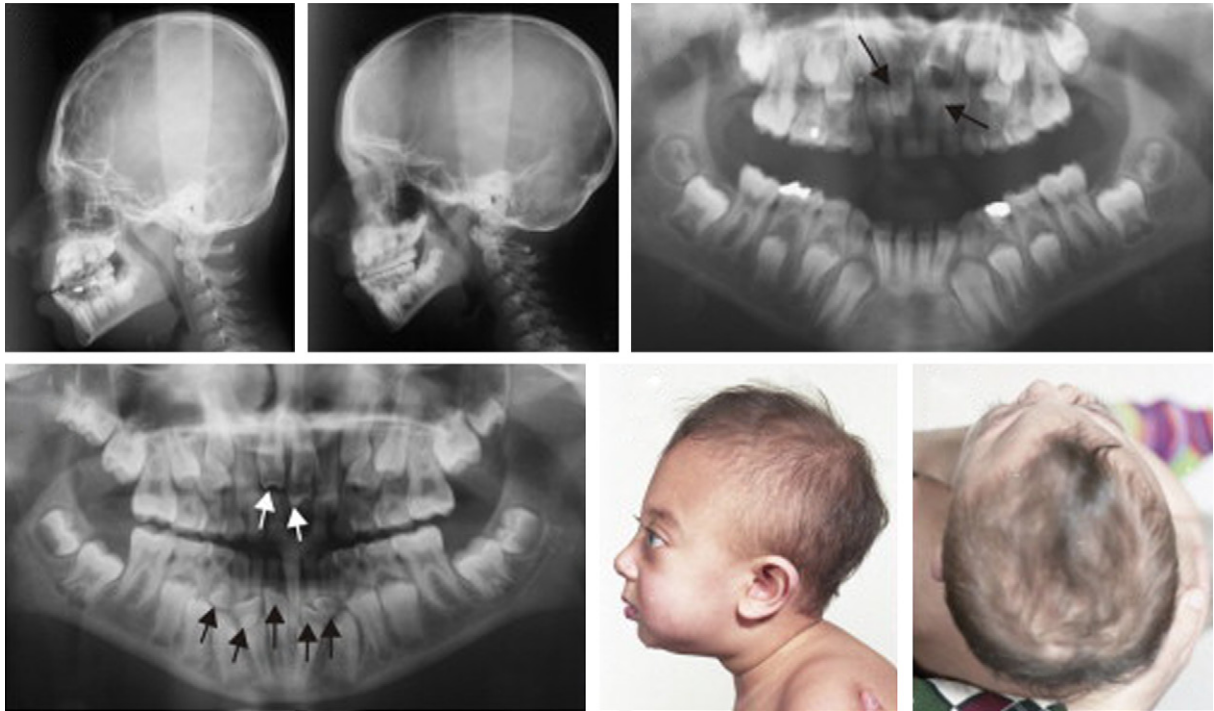
Interleukin 11 receptor, alpha (*IL11RA*, OMIM \*600939) gene mutations were identified using homozygosity mapping in three consanguineous families (8).

### 144.5 GENES RESPONSIBLE FOR CRANIOSYNOSTOSIS

#### 144.5.1 Fibroblast Growth Factor Receptors

**144.5.1.1 Biology of FGFRs.** The *FGFR* genes code for a family of proteins consisting of an extracellular





**FIGURE 144-16** CRSDA syndrome. Turribrachycephaly with exorbitism, trigonocephaly, and two supernumerary teeth indicated by arrows. (From Nieminen, P.; Morgan, N. V.; Fenwick, A. L. *Inactivation of IL11 Signaling Causes Craniosynostosis, Delayed Tooth Eruption, and Supernumerary Teeth*. *Am. J. Hum. Genet.* 2011, 89, 67–81.)

domain with three immunoglobulin-like domains (IgI, IgII, and IgIII), a transmembrane domain, and an intracellular domain with tyrosine kinase activity (66,67) (Figure 144-17). The protein conformation of the immunoglobulin-like domains is in part dependent on disulfide bonds, which form between cysteine residues (68). Immunoglobulin-like domains II and III (IgII and IgIII) are sufficient for ligand binding. In humans there are four known receptors and multiple alternative isoforms. Each receptor and each of its isoforms has its specific spatiotemporal pattern of expression during development.

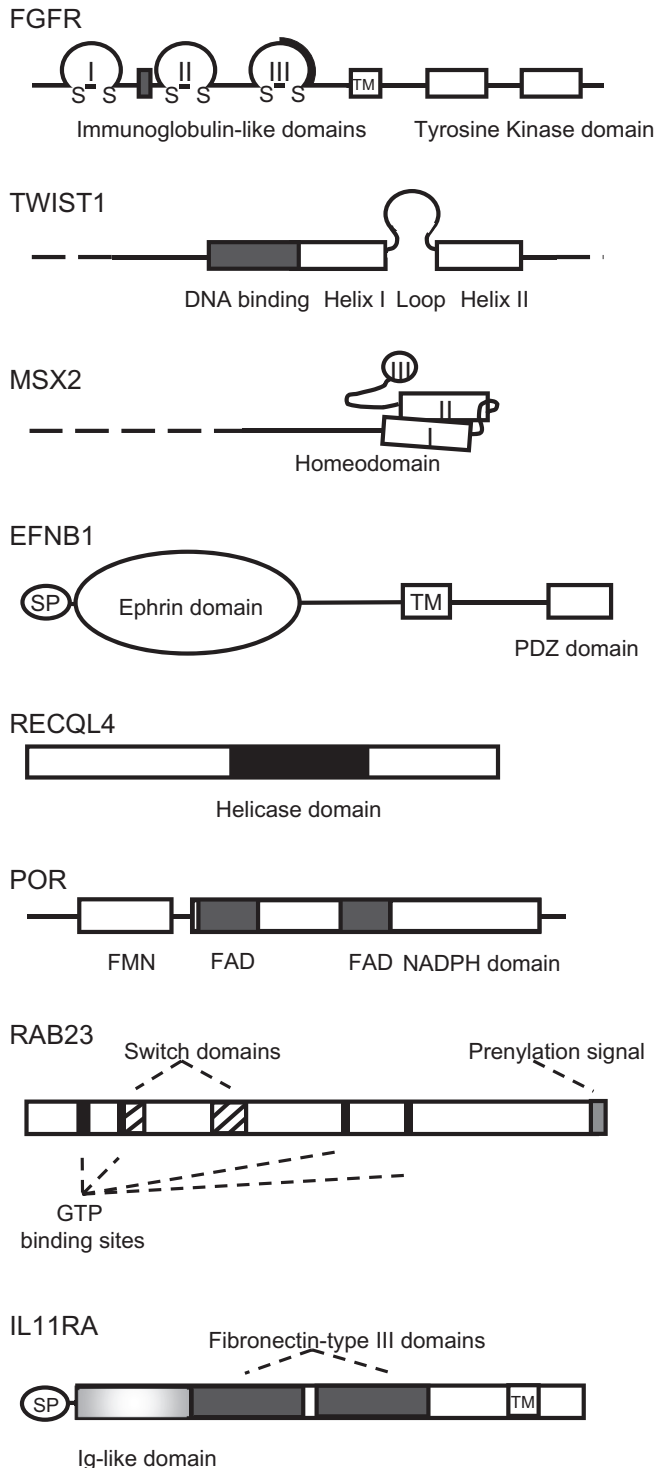
Fibroblast growth factors (FGFs) act as ligands to these receptors (69,70). In humans there are 22 known FGFs, and each of these is expressed in a specific spatiotemporal pattern. These ligands bind to the receptors with differing affinity and specificity and induce the FGFRs to homodimerize or heterodimerize. FGFs are monomeric and are unable to activate FGFRs without heparan sulfate-containing proteoglycans (71). Formation of this complex results in transautophosphorylation of the receptor's intracellular tyrosine residues, which then stimulate the intrinsic catalytic activity of the receptors and recruit sites for downstream signaling proteins involved in distinct developmental pathways, such as MAPK, PLC $\gamma$ 1, and PI3K (72–74).

The expression of FGFRs 1–3 during calvarial ossification provides insight into the pathogenesis of craniosynostosis syndromes caused by mutations in these receptors. Before the ossification stage of human skull

development, FGFR1 is expressed in the head mesenchyme; FGFR2 is strongly expressed in the epidermis and the prebone mesenchyme condensations and the FGFR3 IIIc isoform is found at low levels in the epidermis and basal head mesenchyme (75,76). During membranous ossification of the skull vault, FGFR1 and FGFR2 and to a lesser extent FGFR3 are expressed in the prebone mesenchyme, prior to osteoblastic secretion of osteoid, around the mineralized bone. When sutures appear in the periphery of the ossification centers, high levels of FGFR1 and FGFR2 and low levels of FGFR3 IIIc are present at the osteogenic front of sutures in the prebone mesenchyme, osteoid, and periphery of the bone trabeculae. Expression of the mouse homolog of FGFR4 has been noted in the developing calvaria (76).

**144.5.1.2 FGFR Mutations.** More than 100 different mutations in FGFR1, FGFR2, and FGFR3 have been found in more than 1000 reported cases of Crouzon, Jackson–Weiss, Pfeiffer, Apert and other craniosynostosis syndromes usually involving the coronal suture (77–80). FGFR mutations have been identified in at least nine different craniosynostosis syndromes. There is more than one mutation for each FGFR–craniosynostosis syndrome, except Muenke and Crouzonodermoskeletal syndromes. Both Crouzon and Pfeiffer syndromes are associated with more than 50 different mutations.

To date all of the FGFR mutations that have been identified occur in FGFR2 with the exception of some occurring in FGFR3 and FGFR1. Most FGFR2 mutations occur in the extracellular domain, primarily in the



**FIGURE 144-17** Schematic protein diagrams. FGFR has an extracellular domain composed of three immunoglobulin-like domains (I, II, III) with disulfide bonds (S-S) and heparin binding sequence of heparan sulfate-containing proteoglycans binding domain (gray box), transmembrane domain (TM), and intracellular split tyrosine kinase domain (two rectangular boxes). The second half of IgIII is coded by a variably spliced exon (thickened portion of IgIII). TWIST1 has a DNA binding domain and helix-loop-helix region. The MSX2 homeodomain is composed of a flexible N-terminal extension followed by helices I, II, and III. EFNB1 has an extracellular domain composed of a signal peptide (SP) and ephrin domain, TM, and intracellular tyrosine residues and PDZ domain. RECQL4 has a helicase domain. POR has an electron donating flavin mononucleotide

IgII and IgIII domains coded by exons IIIa (also referred to as exon 8 and exon U) and IIIc (exon 10 and exon D). The recurrent mutations in these domains occur at amino acid residues Ser252, Pro253, Cys278, and Cys342, and account for more than 80% of all cases. No FGFR4 mutations have been detected in association with craniosynostosis.

FGFR mutations are usually amino acid substitutions. Many of these mutations replace or introduce a cysteine residue, resulting in aberrant disulfide bonds and alteration in the protein conformation of the ligand-binding region of IgII and IgIII. Identical missense mutations coding for FGFR1 Pro252Arg, FGFR2 Pro253Arg and FGFR3 Pro250Arg paralogs are observed in three different craniosynostosis conditions (81). There are some splicing mutations, small insertions, and deletions that are in-frame.

**144.5.1.3 FGFR In Vitro and Animal Models.** The mechanism by which FGFR2 mutations cause craniosynostosis is gain of function, and it has been studied extensively by microinjection of mutant FGFR2 mRNA in *Xenopus* oocytes/embryos or transfection of mammalian cells. The data show that some FGFR2 mutations cause ligand-independent constitutive activation by creating unpaired cysteine residues participating in intermolecular disulfide bonding (82,83). Other mutations, involving amino acid residues with side chains such as glutamic acid, aspartic acid and glutamine, create ligand-independent hydrogen-bonded FGFR dimers (84). A change in conformation in the kinase activation loop has been suggested to result in increased tyrosine kinase activity. The two Apert syndrome mutations, FGFR2 Ser252Trp and Pro253Arg, have been demonstrated to cause loss of ligand specificity and retained ligand dependence for receptor activation (85). An interesting animal model supports these findings. The model is a heterozygous mutant mouse, *Bey*<sup>+/-</sup>, with features that resemble Crouzon syndrome (86). The mice were created by retroviral insertional mutagenesis of ES cells, and the insertion was found in the intragenic region between *Fgf3* and *Fgf4*. The transcript analysis demonstrated that the expression of both FGF ligands is increased in the cranial sutures of the *Bey* mice and most likely causes secondary receptor overactivation. The various means by which the gain of function mutant mechanism is mediated for each of the different mutant receptors may in part explain the phenotypic variability.

Transgenic Crouzon syndrome *Fgfr2c*<sup>C342Y/+</sup> mice, having a mutation analogous to a common one found in patients, developed the typical characteristics of rounded calvaria, proptotic eyes, and shortened midface (87).

domain (FMN), flavin adenine dinucleotide domains (FAD), and nicotinamide adenine dinucleotide phosphate (NADPH) binding domain. RAB23 has GTP binding sites, switch domains, and prenylation signal. IL11RA has an SP, immunoglobulin-like domain, fibronectin-like III domains, and transmembrane.

Fusion of the coronal sutures occurred bilaterally with decreased cell proliferation and enhanced differentiation of osteoprogenitor cells. Transgenic Apert syndrome *Fgfr2*<sup>S252W/+</sup> and *Fgfr2*<sup>P253R/+</sup> mouse models have premature fusion of the coronal suture resulting from decreased proliferation, abnormal and increased differentiation, and accelerated bone formation at late embryonic stages as compared to controls. Along with the abnormal osteogenic process, there is aberrant chondrogenesis in these mice, with the presence of ectopic cartilage in the developing middle suture of the skull and long bones as well as a cartilaginous tracheal sleeve also observed in humans (88,89). Downstream of FGFR2, the ERK1/2 and p38 signaling pathways have been found to be activated and in turn, small molecule inhibition of these pathways ameliorates the skull phenotype (88,90,91).

The mechanism of the FGFR1 Tyr372Cys mutation in osteoglophonic dysplasia has been shown to be ligand-independent activation (49). This is in contrast to loss of function, FGFR1 mutations in Kallmann syndrome 2 (OMIM #147950), an autosomal dominant condition without craniosynostosis and characterized by hypogonadism and anosmia (92). Of interest, the characteristic features of osteoglophonic dysplasia overlap with those of FGFR2-craniosynostosis and FGFR3-short-stature conditions caused by activating mutations. The FGFR3-short-stature spectrum includes achondroplasia (OMIM #100800), hypochondroplasia (OMIM #146000), thanatophoric dysplasia types I and II (OMIM #187600, #187601), and SADDAN dysplasia (OMIM #134934.0015). Thanatophoric dysplasia type II is associated with a cloverleaf skull deformity, as well as severe dwarfism and typically the FGFR3 Lys650Glu mutation in the intracellular tyrosine kinase domain.

**144.5.1.4 Phenotype–FGFR Genotype Observations.** Some phenotype–genotype correlations have been delineated and therefore are useful for prenatal and postnatal diagnosis (Table 144-1). The most consistent correlation is the presence of either the FGFR2 Ser252Trp or Pro253Arg mutations in the linker region between IgII and IgIII and the clinically distinct Apert syndrome phenotype (37,93). These two mutations account for more than 99% of more than 400 cases of Apert syndrome reported in the literature. The Ser252Trp mutation is present in the majority (67%) of cases. Of interest, a single case of somatic mutation of FGFR2 Ser252Trp was described in a phenotypically normal male with severe acne; the mutation was found only in his affected skin and not his normal skin or blood (94). Severe acne can be a feature of Apert syndrome.

The FGFR1 Pro252Arg mutation, in the paralogous region of the less common FGFR2 Pro253Arg mutation of Apert syndrome, is associated with the Pfeiffer syndrome type I phenotype (31), whereas the more severe Pfeiffer syndrome types II and III are more often associated with FGFR2 mutations.

FGFR2 Ser351Cys or Trp290Cys mutations in the IgIII domain are found in severe cases of craniosynostosis with the clinical diagnosis of Pfeiffer syndrome that resemble Antley–Bixler syndrome, a diagnosis initially distinguished from the former by significant limb abnormalities of upper limb synostosis, lower limb bowing, and fractures (60,95).

The FGFR3 transmembrane mutation, Ala391Glu, is associated with Crouzonodermoskeletal syndrome (44). The presence of this FGFR3 mutation, rather than an FGFR2 mutation, predicts that a young patient will have acanthosis nigricans as well as craniosynostosis. FGFR2 juxta- or trans-membrane mutations, Ser372Cys and Tyr375Cys, are associated with Beare–Stevenson cutis gyrata syndrome (48). FGFR1 mutations Tyr372Cys, Cys379Arg, and Asn330Ile, in the analogous region of the IgIII domain cause osteoglophonic dysplasia (49).

Although traditionally many of the craniosynostosis syndromes have been considered clinically distinct, molecular data have shown that some are allelic conditions and can share identical mutations (79). Several FGFR2 mutations are present in patients with different clinical diagnoses. For example, FGFR2 Cys278Phe, Cys342Arg, and Cys342Ser mutations are each associated with Crouzon, Pfeiffer, and Jackson–Weiss syndromes. The FGFR2 Ser252Trp and the *FGFR2* IVS8-2A->G splicing mutation have been observed in cases with the diagnoses of Pfeiffer and Apert syndromes (96). FGFR2 Ser351Cys and Ala344Ala are seen in patients with atypical craniosynostosis cases and either Pfeiffer or Crouzon syndromes. Furthermore, the FGFR3 Pro250Arg mutation has been associated with several clinical diagnoses including Crouzon, Jackson–Weiss, Pfeiffer, Saethre–Chotzen, and unclassified syndromes. This specific mutation has also been identified in several affected individuals with non-syndromic unilateral coronal synostosis from a large prospective series, some of which were previously thought to be caused by intrauterine constraint (97). These conditions represent a spectrum of overlapping phenotypes caused by human FGFR2 and FGFR3 mutations and are now considered FGFR-related craniosynostosis syndromes. Furthermore, the fact that identical FGFR mutations may or may not result in craniofacial or other anomalies suggests that other genetic or stochastic factors modulate the effects of aberrant FGFR signaling on craniofacial, as well as limb and skin development.

Curiously, mutant FGFRs and their inappropriate expression in tumor cells of stem cell myeloproliferative disorder, multiple myeloma, and carcinomas such as breast, bladder, or cervix have been reported (98–101). Surprisingly none of the FGFR-craniosynostosis conditions caused by germline mutations are known to be associated with increased risk of tumors.

**144.5.1.5 Paternal Origin of Sporadic FGFR Mutations.** More than 20 autosomal dominant disorders including Crouzon, Apert, and Pfeiffer syndromes are

**TABLE 144-1    Craniosynostosis Conditions and Mutations<sup>a</sup>**

Condition	Gene	Protein			
Diagnosis OMIM Number	Symbol and Location OMIM Number	Nucleotide Change	Type	Region	Amino Acid Change
FGFR-craniosynostosis syndromes					
Crouzon syndrome #123500 Jackson–Weiss syndrome #123150 Pfeiffer syndrome #101600	FGFR2 Exon IIIa (8 or U) Exon IIIc (10 or D) *176943	Missense, splice site, in-frame insertion and deletion	Tyrosine kinase receptor	Extracellular IgII–IgIII domain	p.C278F, p.Q289P p.T320GfsX5, p.C342R, p.C342S, p.C342W, p.C342Y, p.A344A, p.A344G, p.S347C, p.S351C, p.S354C, c.940-2A>G
Pfeiffer syndrome #101600	FGFR1 Exon IIIa *136350	Missense	Tyrosine kinase receptor	Extracellular IgII–IgIII linker	p.P252R
Apert syndrome #101200	FGFR2 Exon IIIa *176943	Missense, splice site, Alu repeat insertion, intragenic deletion	Tyrosine kinase receptor	Extracellular IgII–IgIII domain	p.S252W, p.P253R, p.S252F, c.1041-1042insAlu, c.940-2AA>G, 1372 bp deletion resulting in chi- meric IIIb and IIIc domain
Muenke syndrome; Craniosynostosis, Adelaide type #602849; %600593	FGFR3 Exon IIIa #134934	Missense	Tyrosine kinase receptor	Extracellular IgII–IgIII linker	p.P250R
Crouzonodermoskeletal syndrome #612247	FGFR3 Exon IIIa #134934	Missense	Tyrosine kinase receptor	Transmembrane domain	p.A391E
Beare–Stevenson, cutis gyrata syndrome #123790	FGFR2 Exon 11 *176943	Missense	Tyrosine kinase receptor	Juxta- or trans- membrane domain	p.S372C, p.Y375C
Osteoglophonic dysplasia #166250	FGFR1 *136350	Missense	Tyrosine kinase receptor	Extracellular IgIII domain, juxta- or trans-membrane domain	p.N330I, p.Y372C, p.C379R
Thanatophoric dysplasia type II #187601	FGFR3 #134934	Missense	Tyrosine kinase receptor	Intracellular tyrosine kinase domain	p.K650E
Saethre–Chotzen syndrome; Robinow–Sorauf syndrome #101400; #180750	TWIST Exon 1 and non- coding region *601622	Nonsense, in-frame duplication, missense, micro- deletion, large chromosome dele- tion 7p15.3–p22	Helix–loop–helix transcription factor	Entire protein	p.Q71X, p.Y103X, p.P139dup7, p.R154fsX237

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Craniosynostosis, Boston type #604757	MSX2 Exon 2 *123101	Missense	Homeobox domain transcription factor	Homeodomain	p.P148H
Craniofrontonasal syndrome #304110	EFNB1 *300035	Missense, splice site, frameshift insertion and deletion, nonsense	Transmembrane protein with ephrin and PDZ domains	Entire protein	p.R66X, p.P119H, p.G151S
Baller–Gerold syndrome #218600	RECQL4 *603780	Splice site, missense, frameshift deletion	DNA helicase		p.R102W, p.D779LfsX57, c.2335del22, g.2886delT, IVS17-2A>C
Antley–Bixler syndrome with genital abnormalities and disordered steroidogenesis #207410	POR *124015	Missense, frameshift insertion, in-frame deletion and insertion, splice site, nonsense	Multidomain with FMN, FAD, NADPH binding domain	Entire protein	p.R457H in Japanese
Carpenter syndrome #201000	RAB23 *606144	Missense, frameshift insertion or deletion, nonsense	RAB guanosine triphosphatase		p.L145X
Craniosynostosis and dental anomalies syndrome (CRSDA) #614188	IL11RA *600939	Missense, duplication, nonsense	Cell surface receptor of interleukin 11	Fibronectin-type domain III	p.Gln159X, p.Pro221Arg, p.Ser245Cys, p.Arg296Trp, c.916_924dup
Greig cephalopolysyndactyly #175700	GLI3 *165240	Large chromosomal deletion 7p13, intragenic deletion, in-frame insertion and deletion, missense, splice site; frameshift, nonsense in first or third of gene	Zinc finger transcription factor	Entire protein	p.R290X, p.R792X
Alagille syndrome #118450	JAGGED1 *601920	Frameshift, nonsense	Ligand for NOTCH receptors		p.E553X, c.delGAAAG
Sphrintzen–Goldberg syndrome #182212	FBN1 *134797	Missense, splice site	Extracellular microfibril protein	Repeat EGF-like domain	p.C1223Y, p.C1221Y
Furlong syndrome #609192	TGFBR1 *190181	Missense	TGFB receptor superfamily protein	Kinase domain	p.S241L
C syndrome, Opitz trigonocephaly syndrome #211750	CD96 *606037	Missense	Immunoglobulin superfamily protein	IgIII domain	p.T280M
9p- syndrome with trigonocephaly #158170	FREM1, contiguous gene syndrome *608944	Large chromosome deletion 9p22–p24, missense	Extracellular matrix protein		p.Y285C, p.R498Q, p.G1500V
11q- syndrome with trigonocephaly; Jacobsen syndrome #147791	Contiguous gene syndrome	Large chromosome deletion 11q23–q24			

TABLE 144-1    Craniosynostosis Conditions and Mutations <sup>a</sup> —cont'd					
Condition	Gene	Protein			
Diagnosis OMIM Number	Symbol and Location OMIM Number	Nucleotide Change	Type	Region	Amino Acid Change
Hypophosphatasia, infantile type #241500	ALPL *171760	Missense, frameshift deletion, splice site	Alkaline phosphatase	Entire protein	p.M45I, p.M45L, p.M45V
Craniosynostosis, anal anomalies, porokeratosis syndrome (CAP) %603116	Pathway including RUNX2 *600211				
Non-syndromic coronal synostosis, associated with Crouzon; or normal phenotype	FGFR2 *176943	Missense	Tyrosine kinase receptor	Extracellular and intra-cellular domains	p.S252L, p.A362S, p.K526E; p.A315S, p.A337T
Non-syndromic coronal synostosis, associated with Muenke syndrome; or normal phenotype	FGFR3 *134934	Missense	Tyrosine kinase receptor	Extracellular IgII–IgIII linker	p.P250R
Non-syndromic coronal synostosis	TWIST1 *601622	Missense	Helix–loop–helix transcription factor	TWIST1 Box domain	p.A186T
Non-syndromic coronal synostosis	EFNA4 *601380	Missense, frameshift	Ephrin ligand	Frameshift mutation in C-terminus	p.H60Y, p.P117T, c.471_472delCCinsA
Trigonocephaly, Metopic synostosis with facial skin tags #190440	FGFR1 *136350	Missense	Tyrosine kinase receptor	Extracellular IgIII domain	p.I300W
Non-syndromic sagittal synostosis	FGFR1 *136350	Missense	Tyrosine kinase receptor	Extracellular domain	p.T261M <sup>b</sup>
Non-syndromic sagittal and/or unilambdoid synostosis	FGFR2 *176943	Missense	Tyrosine kinase receptor	Extracellular domain	p.C278W, p.A315T <sup>b</sup>
Non-syndromic sagittal synostosis	TWIST1 *601622	Missense	Helix–loop–helix transcription factor	TWIST1 Box domain	p.S188L, p.S201Y

<sup>a</sup>For some disorders, there is no definitive information in the literature for each heading, to date. Either the most common mutations if there are multiple mutations associated with mutations if there are only a few mutations associated with a condition are listed.

<sup>b</sup>There are also variations in the noncoding regions of these genes in non-syndromic sagittal synostosis and their significance are unknown.

associated with advanced paternal age (102,103). Of interest, paternal origin of *FGFR2* mutations was molecularly proven for more than 100 sporadic cases of Crouzon, Apert, Pfeiffer, and Muenke syndromes. At least eleven different *FGFR2* mutations (nine missense, two splicing mutations) in Crouzon and Pfeiffer syndromes (104), the two common *FGFR2* mutations, Ser252Trp and Pro253Arg, in Apert syndrome (105); and the *FGFR3* Pro250Arg mutation in Muenke syndrome (106) have been shown to occur exclusively in the paternally derived gene. No cases of de novo mutations of maternal origin have been identified for any of these conditions. It has been suggested that *FGFR* genes, specifically *FGFR2* and *FGFR3*, may be predisposed to the mutational events and that the high frequencies of mutations in several of these nucleotides (e.g. Apert syndrome Ser252Trp mutation) may be due to a selective advantage of the mutated germ cells. Studies on the frequency of mutations in the male germline all agree that the frequency of the mutations in sperm increases with age. These results suggest that older men may be at an increased risk for having children with these craniosynostosis conditions (102,107).

### 144.5.2 TWIST1

The *TWIST1* gene (OMIM \*601622) codes for a transcription factor with a basic DNA binding and helix-loop-helix domains (see Figure 144-17). It is expressed during embryonic mesodermal development of the head and limbs. Early in murine development it is present in the mesoderm outside the primitive streak (108), and later it is found in the somites, head mesenchyme, first aortic arches, second through fourth branchial arches, limb buds, and the mesenchyme beneath the epidermis (109). *Twist1* is expressed in the undifferentiated cells committed to muscle and cartilage development and in primary osteoblastic cells from the newborn murine calvaria (110). The expression of *Twist1* precedes that of *Fgfr* genes at the time of initiation of the coronal suture.

More than 20 Saethre–Chotzen syndrome cases are associated with chromosome 7p15.3–p22 translocations, deletions, and other rearrangements outside the coding region of *TWIST1* (111,112). These chromosome 7 alterations are presumed to lead to loss of *TWIST1* function secondary to the loss of important regulatory regions or position effects. Partial duplication of chromosome 7p containing the *TWIST1* gene leads to delayed closure of a large anterior fontanelle, which is the opposite phenotype of craniosynostosis (113,114). This suggests that calvarial development is sensitive to *TWIST1* dosage.

More than 80 different *TWIST1* coding mutations have been identified in approximately 80% of patients with Saethre–Chotzen syndrome (51,111,115–119). The Tyr103STOP and 416–417dup21 are among the few recurrent mutations. The mutations can be missense, nonsense, duplication, deletion and insertions.

The latter types of mutations lead to a premature STOP codon and unstable mutant transcript by the mechanism of nonsense-mediated decay. Point mutations are also predicted to cause a loss of *TWIST1* function by altering the ability of the protein to dimerize or to bind to its DNA target(s). In vitro experiments have suggested that missense mutations involving the helical domains lead to loss of *TWIST1* heterodimerization with the E12 basic helix–loop–helix protein and altered ability of the *TWIST1* protein to localize in the nucleus (120).

Mice heterozygous for loss of the *Twist1* gene, with skull and limb anomalies similar to those in Saethre–Chotzen patients, further support the theory of haplo-insufficiency as being the mechanism of the *TWIST1* mutations. This loss of *TWIST1* function causes premature osteoblast differentiation (121). Furthermore, the dependence of cranial phenotype on genetic background in these heterozygous mice resembles the variable expressivity reported for Saethre–Chotzen syndrome (122). The significant interfamilial and intrafamilial variability of Saethre–Chotzen syndrome is found for patients with different *TWIST1* mutations as well as unrelated patients sharing identical mutations. Physical findings in patients with large gene deletions do not differ from those with point mutations. However, a correlation has been suggested between large deletions including *TWIST1* and developmental delay (111,112).

Patients with phenotypes that more closely resemble Saethre–Chotzen syndrome than other craniosynostosis conditions were found to have the *FGFR3* Pro250Arg mutation (40,50,81). The molecular data that mutations in different genes, such as *TWIST1* and *FGFRs* can cause very similar craniosynostosis phenotypes, suggest that these genes are involved in the same molecular developmental pathway. In fact, mice heterozygous for the loss of *Twist1* demonstrated ectopic expression of *FGFR2* in the midsutural mesenchyme of the developing sagittal suture of the skull (123). Therefore, based on both clinical and molecular data from humans with craniosynostosis and mice with loss of the *Twist1* gene, loss of function of *Twist1* leads to gain of function or activation of *FGFRs*. Thus it is not surprising that mutations in these genes both cause the common phenotype of craniosynostosis.

### 144.5.3 MSX2

The *MSX2* gene (OMIM \*123101) codes for a transcription factor protein containing a homeobox domain (see Figure 144-17). Murine *Msx2* is expressed in the neural crest derived from mesenchyme of first through fourth branchial arches, limb buds and calvaria. A mutation in the homeobox domain, Pro148His, was found in a large family with variable skull involvement ranging from fronto-orbital recession to cloverleaf deformity (54). This amino acid substitution is a gain of function mutation and confers enhanced DNA binding affinity (124).

A patient who was partially trisomic for chromosome 5q where the *MSX2* gene is located was reported to have craniosynostosis of the sagittal and lambdoid sutures, craniofacial abnormalities, cardiac defects, and growth and developmental delay (125). Mice with *Msx2* two-fold or less overexpression developed premature cranial suture fusion and ectopic bone formation (126). Mice with 13–22 copies of the transgene died around birth with severe craniofacial malformations, but with no evidence of craniosynostosis (127).

*Msx2*-deficient mice have defects of skull ossification and persistent calvarial foramen (128). This phenotype results from defective proliferation of osteoprogenitors at the osteogenic front during calvarial morphogenesis, and closely resembles that associated with human *MSX2* haploinsufficiency in the condition of parietal foramina (OMIM #168500) (129,130). These effects are opposite to those of the *MSX2* Pro148His homeodomain mutation, associated with craniosynostosis, demonstrating that the variation in skull phenotype is dose-dependent. Of interest, parietal foramina are genetically heterogeneous and can also be caused by loss of function mutations in the transcription factor, *ALX4* (OMIM #609597, \*605420; (131)). In mice, *Alx4* and *Msx2* are partially redundant in function (132). It is likely that *MSX2* and *ALX4* are components of the same molecular pathway of calvarial osteogenic proliferation and differentiation as *TWIST1* and *FGFRs*.

#### 144.5.4 Ephrin B1

Ephrin ligands and their tyrosine kinase receptor complexes play an important role in embryonic tissue morphogenesis through regulating cell shape and movement (133,134). The ligands themselves can also act as receptors, and in turn, the receptors can act as ligands so that bidirectional signaling is a mechanism by which ephrins control processes of cell–cell communication. There are two classes of ligand; ephrin A, which are anchored to the membrane by a glycosylphosphatidylinositol linkage, and ephrin B, which are transmembrane proteins. There are three members of the ephrin B protein family, each with an intracellular region of multiple tyrosine residues and a PDZ domain (see Figure 144-17). The ephrin B ligands play a role in the patterning of the developing skeleton, nervous system, intestine, and blood vessels. This transduction system is involved in neuronal growth and cell migration, mainly by acting as regulators through extracellular signal regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and integrin cell surface interactions.

The mouse *Efnb1* gene is expressed in the frontonasal crest, and the human homolog was found to be mutated in craniofrontonasal syndrome patients (10,56,135). More than 80 different *ENFB1* mutations have been identified in cases of craniofrontonasal dysplasia (OMIM \*300035, #304110). Most are missense and others are nonsense,

frameshifts, or splicing mutations. Arg66STOP, Pro119His, and Gly151Ser are recurrent and an intragenic deletion has been detected. These mutations cause loss of function by affecting receptor–ligand interaction, oligomerization, and reverse signaling by the ligand.

Ephrin B1 knockout mice display a shortened skull, cleft palate, omphalocele, and malformations of the axial and appendicular skeleton similar to those found in craniofrontonasal syndrome patients (136). The mice have asymmetric attachment of ribs, lack of joints, and polydactyly, which are thought to be secondary to missing or ectopic segmentation of mesenchymal condensations during early skeletal development. The heterozygous female mice have additional features and lower viability compared to hemizygous males, similar to the paradoxical phenotypic observations in humans with this X-linked condition. No skewing of X-inactivation was observed in blood or cranial periosteum of patients (135) and no Y homolog was found to explain the severity in females. It has been postulated that “metabolic interference” or a disruptive effect of the mutant allele of the wildtype allele might be the mechanism by which females are more severely affected than males.

There is genetic evidence that Ephrins function together with *Twist1* and *Msx2* in boundary formation and the pathogenesis of coronal synostosis (137). In *Twist1*<sup>+/-</sup> mice with coronal synostosis, the frontal-parietal boundary is defective. Specifically, neural crest cells invade the undifferentiated mesoderm of the mutant coronal suture. This boundary defect is accompanied by an expansion in *Msx2* expression and reduction in ephrin-A4 distribution. Reduced dosage of *Msx2* in the *Twist1* mutant background restores the expression of ephrin-A4, rescues the suture boundary and inhibits craniosynostosis. Thus, mutations in the above genes are involved in the same molecular pathways in coronal synostosis.

#### 144.5.5 RECQL4

*RECQL4* is a member of the RecQ helicase family of proteins known to be involved in maintaining genome stability (138) (see Figure 144-17). There are at least three RECQ homologs, *RECQL2* (OMIM \*604611), *RECQL3* (OMIM \*604610), and *RECQL4*, known to be associated with five autosomal recessive disorders characterized by genomic instability and cancer predisposition including Werner (OMIM #277700), Bloom (OMIM #210900), Rothman–Thomson, RAPADILINO, and Baller–Gerold syndromes. Only one of the more than 24 reported Baller–Gerold syndrome patients was noted to have lymphoma (58).

It is known that *RECQL4* forms a stable complex with *UBR1* (OMIM \*605981) and *UBR2* (OMIM \*609134), which are ubiquitin ligases of the N-end rule pathway (139). Although *UBR1* and *UBR2* mediate polyubiquitylation and subsequent degradation of their



substrates, UBR1 and UBR2-bound RECQL4 were neither ubiquitinated in vivo nor degraded in HeLa cells. Further, the RECQL4-UBR1/2 complex was shown to have DNA-stimulated ATPase activity. Processing of aberrant DNA structures that arise during DNA replication and repair is a major role of ATP-dependent DNA helicase.

In Baller–Gerold syndrome patients, at least six mutations of RECQL4 have been identified with an Arg1021Trp substitution, splice site and frameshift mutations (9,140). Several knockout embryonic lethal and viable Recql4-mutant mouse models with exons 9–13 deleted have been generated. The latter mutant mice exhibited distinctive skin abnormality, birth defects of the skeletal system, genomic instability and increased cancer susceptibility on a sensitized genetic background, similar to Rothmund–Thomson syndrome (141). Cells from the mice had high frequencies of premature centromere separation and aneuploidy. Recql4 may have a role in sister chromatid cohesion leading to chromosomal instability, cancer predisposition and birth defects in the mutant mice. A mouse model with a Baller–Gerold specific mutation has not been generated to date.

### 144.5.6 P450 Oxidoreductase

P450 oxidoreductase (POR) is the obligatory flavoprotein intermediate that transfers electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH) to all microsomal cytochrome P450 enzymes (see Figure 144-17). The *POR* gene (OMIM \*124015) has a ubiquitous expression pattern. In humans, there are at least 57 genes for cytochrome P450 enzymes with 50 encoding microsomal P450s; including 20 genes encoding P450s involved in the biosynthesis of steroids, cholesterol, fatty acids, and eicosanoids. One would predict that loss of function mutations in P450 oxidoreductase would have significant multisystem consequences. In fact, POR knockout mice are embryonic lethal with neural tube, cardiac, eye, and limb abnormalities (142,143). However, in humans, POR recessive mutations have been identified with partial, combined deficiencies of two steroidogenic microsomal P450 enzymes: P450c17, which catalyzes steroid 17 $\alpha$ -hydroxylation and 17,20-lyase activity, and P450c21, which catalyzes steroid 21-hydroxylase activity; and deficiency of the sterol P450 enzyme, lanosterol 14 $\alpha$ -demethylase (3). Over 30 different mutations have been found in more than 40 cases (62,144–146). Most of the mutations are missense, but there are also nonsense, frameshift, splicing, small in-frame deletions and insertions. The Arg457His mutation is the most common in Japanese individuals because of a founder effect (145). The mutations that severely reduce the enzyme activity correlated most with the skeletal phenotype, while the milder mutations occurred in individuals with disorders of steroidogenesis, but normal skeletal development.

### 144.5.7 RAS-Associated Protein RAB23

RAB23 is a member of the RAB family of small guanine triphosphatases (GTPases) that regulate intracellular trafficking of membrane-associated proteins (OMIM \*606144) (147–149) (see Figure 144-17). Given the evidence that RAB23 regulates the hedgehog pathway, it is not surprising that some aspects of the phenotype of Carpenter syndrome resemble other human disorders associated with disturbed hedgehog signaling and midline structural abnormalities and limb defects. The most notably involved sutures are those in the midline, metopic and sagittal sutures. The combination of postaxial polysyndactyly of the hands and preaxial polysyndactyly of the feet is very similar to the pattern that occurs in Greig syndrome (OMIM #175700), which is due to haploinsufficiency of *GLI3*, a downstream component of the sonic hedgehog signaling pathway (150) and is consistent with decrease in *Gli3* repressor in *Rab23* mutant mouse embryos (151). The brachydactyly present in Carpenter syndrome, characterized by hypoplasia or absence of the middle phalanges, resembles brachydactyly type A1 (OMIM #112500), which is caused by heterozygous missense mutations in Indian hedgehog (152).

### 144.5.8 Interleukin 11 Receptor, Alpha

The ligand for the IL11RA receptor is interleukin-11. The binding of IL11 to IL11RA leads to the formation of a hexameric cell–surface complex of two ligands, two alpha-receptors, and two gp130 signaling co-receptors (beta receptors) (153) (see Figure 144-17). The formation of the signaling complex enables phosphorylation of the intracellular tyrosines of gp130 to trigger intracellular signal transduction, through either the *JAK/STAT1/3* or the *SHP2/MAPK/ERK* phosphorylation pathways (154,155). IL11 is widely expressed in mesenchymal and stromal cells and has been associated with diverse biological functions, including the regulation of bone formation and remodeling (156). The causative role of impaired IL11 signaling in the craniosynostosis phenotype of CRSDA patients was supported by phenotypic similarities between the human condition and *Il11ra* null mutant mice with the maxillary hypoplasia and class III malocclusion. Many missense mutations in CRSDA are located in the second fibronectin-type domain III (8).

## 144.6 OTHER SYNDROMES WITH CRANIOSYNOSTOSIS AND THEIR GENES

Disease genes have been identified for a few syndromes for which craniosynostosis is not a major feature. Greig cephalopolysyndactyly syndrome (OMIM #175700) is an autosomal dominant condition associated with preaxial and postaxial polydactyly of the hands and feet with variable syndactyly and hypertelorism. Craniosynostosis is observed in about 5% of cases. It is caused by

deletions of chromosome 7p13 region and loss of function mutations of *GLI3* (OMIM \*165240), a transcription regulator in the sonic hedgehog pathway during embryonic development. Mutations have been identified to be chromosomal translocations or large deletions, exonic deletions and duplications, small in-frame deletions, missense, and splicing mutations. Frameshift and nonsense mutations generally occur in the first and last thirds of the gene (157). The location of these mutations differs from those for the allelic condition, Pallister–Hall syndrome (OMIM #146510), which does not present with craniosynostosis. This latter condition is characterized by hypothalamic hamartoblastomas, craniofacial anomalies, bifid epiglottis, postaxial polydactyly, cardiac and renal defects and endocrine dysfunction. *GLI3* mutations are usually in the middle third of the gene for this syndrome. Other cases of Greig cephalopolysyndactyly with metopic and sagittal synostosis may resemble Carpenter syndrome and diagnostic testing for *GLI3* should be considered when no *RAB23* mutations are detected (158).

Alagille syndrome is an autosomal dominant condition with variable abnormalities of the liver, heart, skeleton, eyes and facial structures (OMIM #118450). A few cases have been reported with unilateral coronal craniosynostosis (159). Approximately 80% of patients with Alagille syndrome have mutations in the *JAGGED1* gene (OMIM \*601920). Jagged1 is a ligand in the Notch signaling pathway, which has been shown to regulate the formation of tissue boundaries. Jagged1 functions downstream of *Twist1* as an effector in the development of the coronal suture and in the formation of a boundary between osteogenic and non-osteogenic cells in mice (160).

Shprintzen–Goldberg craniosynostosis syndrome (SGS, OMIM #182212) is associated with craniosynostosis, exophthalmos, midface hypoplasia, arachnodactyly, camptodactyly, other connective tissue related problems and developmental delay (161). Furlong syndrome (OMIM #609192) is a similar Marfanoid disorder with craniosynostosis, which differs from SGS by the absence of mental retardation (162). It has been suggested that genetic variation in the fibrillin 1 (OMIM \*134797) and transforming growth factor beta-receptor 1 (OMIM \*190181) genes may play a role in the pathogenesis of craniosynostosis for these conditions.

C syndrome, also known as Opitz trigonocephaly syndrome (OMIM #211750), is associated with not only trigonocephaly, but also severe mental retardation, hypotonia, variable cardiac defects, redundant skin, abnormalities of joint and limbs, visceral anomalies, and dysmorphic facial features, including upslanted palpebral fissures, epicanthal folds, depressed nasal bridge, and low-set, posteriorly rotated ears (163). In a patient having C syndrome with a balanced chromosomal translocation t(3; 18), the *CD96* gene (OMIM \*606037) was disrupted at the 3q13.13 breakpoint (164). A Thr280Met mutation

in the *CD96* gene was found in one patient with severe features of the C syndrome phenotypic spectrum (165).

Several reports show an association of metopic synostosis or trigonocephaly with chromosomal abnormalities, more significantly with del(9)(p22p24) and del(11)(q23q24) (166). Deletions in these two chromosomal regions are associated with syndromic phenotypes and almost invariably include trigonocephaly. For the chromosome 9p region, more than 100 patients were assessed for copy number variations (CNVs). Five de novo CNVs involving *FREM1*, *FRAS1*-related extracellular matrix protein one gene (OMIM \*608944) were found and then three additional mutant alleles were identified by resequencing (167). Monosomy of chromosome 11q23 is associated not only with metopic synostosis, but also mental retardation, craniofacial anomalies, congenital heart defects, and blood dyscrasias and has been defined as Jacobsen syndrome (OMIM #147791). The haploinsufficiency of one gene in these regions could theoretically lead to either syndromic or non-syndromic cases of craniosynostosis. Less frequently, other chromosomal deletions such as those of 3q, 7p, 13q (1,168) and 22q11 (169) have also been associated with craniosynostosis.

Craniosynostosis may be observed in hypophosphatasia, particularly in the infantile type (OMIM #241500) if the affected child survives infancy. It is an autosomal recessive disorder of bone mineralization characterized by rachitic changes, failure to thrive, and seizures in infancy. Hypophosphatasia is caused by many different mutations in the alkaline phosphatase gene, *ALPL* (OMIM \*171760).

Craniosynostosis, anal anomalies, and porokeratosis syndrome (CAP syndrome, CDAGS; OMIM #603116) is another autosomal recessive condition of craniosynostosis, delayed closure of the fontanel, cranial defects, clavicular hypoplasia, anal and genitourinary malformations, and skin eruption. At least seven unrelated individuals have been reported, and it has been hypothesized that the gene defect in this condition causes abnormal regulation of multiple signaling pathways, including *RUNX2* (OMIM \*600211), during osteoblast differentiation and craniofacial morphogenesis (170).

### 144.7 NON-SYNDROMIC CRANIOSYNOSTOSES AND THEIR GENES

Little is known about the genetics of non-syndromic craniosynostosis (171). Initially a candidate gene approach was used. The *FGFR3* Pro250Arg (P250R) mutation is found in patients with non-syndromic coronal synostosis (97). Four to 12% of isolated unilateral and 25–40% of isolated bilateral coronal synostosis cases in clinic or hospital-based studies have the *FGFR3* P250R mutation (172,173). Cassileth et al. described poorer postoperative cranial morphology in patients with unicoronal synostosis and the *FGFR3* P250 mutation (174), and Arnaud et al. observed a trend for patients with bicoronal

synostosis and the FGFR3 P250 mutation to have revision surgery to correct cranial morphology more often than patients without the mutation (175). Thomas et al. conducted a retrospective study of patients with isolated coronal synostosis that were operated on at a single unit, and found that the FGFR3 P250R mutation was significantly associated with transcranial reoperation and specifically a 20.7% reoperation rate to relieve increased intracranial pressure (176). They also observed that cases with the mutation had early intervention to prevent excessive frontal bulging at around 6 months of age.

A FGFR2 Ala315Ser (A315S) mutation was reported in a patient with unicoronal synostosis and a birth history of breech presentation and skull compression (177). EFNA4 mutations were reported in a three patients with non-syndromic coronal synostosis (137). Mutations Ala186Thr, Ser188Leu, and Ser201Tyr in the TWIST1 Box domain were found in one case of isolated left coronal synostosis and two cases of isolated sagittal synostosis (178,179). In a family with autosomal dominant sagittal synostosis and metopic ridging, a FGFR3 Ala334Thr mutation was found (180). A FGFR1 Ile1300Trp mutation was found in a girl with non-syndromic metopic fusion who had two facial skin tags, but no other anomalies (181). A FGFR2 A315T mutation was reported in one case of non-syndromic sagittal synostosis (182).

Of note, a genome-wide association study was performed on non-syndromic sagittal craniosynostosis and identified risk loci on chromosomes 20 and 7 in the European American population (183). Preliminary results suggested the strongest associations were found with a  $p \leq 1.13 \times 10^{-14}$  (OR = 4.58) in the 3' UTR of BMP2 (OMIM \*112261) on chromosome 20 and  $p \leq 1.61 \times 10^{-10}$  (OR = 0.19) in an intron of BBS9 (OMIM \*607968) on chromosome 7. Furthermore, these significant associations were replicated in an independent American Caucasian population. Future studies will focus on these regions to identify disease-causing sequence variations and additional high-throughput technologies will be applied to discover disease genes for non-syndromic craniosynostosis.

## 144.8 EVALUATION OF CRANIOSYNOSTOSIS

### 144.8.1 Dysmorphology Examination

A multidisciplinary team approach is required for the evaluation and management of the patient's skeletal, audiological, ocular, oral, dental, respiratory, skin, and endocrine abnormalities (184). A genetic examination of a child with potential craniosynostosis or other craniofacial anomaly requires a thorough and detailed evaluation of appearance and structure. One must pay particular attention to cranial shape, symmetry, and facial features, as well as extracranial structures (such as hands and feet) that may also be involved in craniosynostosis syndromes.

The skull shape is best assessed from a “bird’s eye view,” usually best achieved with the examiner standing and the child on a seated parent’s lap. This will allow an overall assessment of the symmetry and shape of the cranium. In this way, one can look for areas of flattening or prominence of the skull. The head shape may be described as dolichocephalic, brachycephalic, or plagiocephalic. Additional features of skull shape can be assessed from a frontal view, such as a high or “tower” forehead referred to as acrocephaly, or whether the forehead is sloping backward or narrowed at the temples. Placement of the ears, whether at the same level, or one located more posteriorly than the other when seen from the top can also provide information as to the likelihood of a prematurely synostosed suture. Head circumference should also be obtained to document if there is restriction of normal skull growth.

Facial features are also very important in the evaluation of a child with craniosynostosis. Noting abnormalities of bony development, such as underdevelopment of the malar bones, shallow bony orbits leading to ocular proptosis, prognathism or retrognathism may be critical in helping to arrive at the correct diagnosis. Left/right asymmetry is a significant feature, and may involve either bony or soft tissues. For example, ptosis may affect one or both eyes, or both eyes to varying degrees, especially in Saethre–Chotzen syndrome. The shape of the nose should be noted as well, as a “beaked” appearance is common in several craniosynostosis disorders.

The oral cavity should be assessed for integrity and characteristics of the palate. Clefting is important, as is a particularly narrow and high arched (“Byzantine”) palate, as might be seen in the Apert syndrome. Dentition may be affected by the anatomy, such as missing teeth with alveolar clefting, or dental crowding in micrognathia. Ears should also be carefully described. The shape of the ear may be typical of a particular syndrome such as prominent ear crura in the Saethre–Chotzen syndrome.

Craniosynostosis syndromes may also involve dysmorphisms in extracranial regions. Most important, abnormalities of hands and feet can help to distinguish between different craniosynostosis syndromes, which may have similar facial appearances. Mitten-type syndactyly of all or almost all digits, as seen in the Apert syndrome is perhaps the most recognizable anomaly. Other syndromes may have more subtle and variable features. While bony or soft tissue fusions may be evident on physical examination, radiographs are helpful in assessing bony abnormalities not immediately apparent. Other organs should also be examined such as the heart and kidneys, which can be screened by ultrasonography. Evidence for genitalia abnormalities or endocrine dysfunction should be considered in making the diagnosis of POR syndrome.

Lastly, in performing a dysmorphology examination on a patient with possible syndromic craniosynostosis, the skin should be evaluated for texture or pigmentary

changes. Acanthosis nigricans, a darkened, raised, velvety textured patch is not only associated with Crouzon syndrome, it may help to identify the causative gene, *FGFR3* versus *FGFR2*, in that patient. Severe acneiform lesions on the arms or chest are often seen in the Apert syndrome. Cutis gyrata, an unusual, ridged texture to the skin is characteristic of the Beare–Stevenson cutis gyrata syndrome.

### 144.8.2 Radiographic Evaluation

If craniosynostosis is suspected on a clinical basis, the patient should be evaluated radiographically to assess the patency of the sutures. Plain skull films are often used as a screening device, but are not diagnostic. If the sutures are clearly seen to be open, the evaluation may end there. However, if the sutures are either felt to be synostosed or not well visualized, and then further evaluation is warranted.

A computed tomography (CT) scan is a more accurate way of assessing the patency of the sutures. This can be done in conjunction with a three-dimensional reconstruction of the individual images of the skull and skull base. This allows for a highly accurate assessment of sutural patency and bony contours. In addition, it allows the surgeon to “virtually reconstruct” the skull using the computer images. This exercise, when done preoperatively, gives the surgeon a better idea of the extent of skull remodeling that will be needed at the time of surgery.

Plain radiographs of the patient’s hands and feet as well as spine, hips, or long bones should also be considered at the time of workup, to potentially aid in the detection of subtle features and diagnosis of one of the many craniosynostosis syndromes.

### 144.8.3 Genetic Evaluation

A proper genetic evaluation of a patient with potential craniosynostosis begins with a thorough history. The patient’s mother should be questioned regarding any complications or medical conditions that may have impacted the pregnancy. Specific questioning should be directed at detecting any potential source of fetal crowding, such as uterine anomalies (large fibroids or bicornuate uterus) or a multiple gestation that may have led to compression of the baby’s head and secondary synostosis. History of medications such as anticonvulsant or retinoid use by the mother during pregnancy may be significant.

Family history is also quite important. Parents should be questioned regarding any congenital anomalies in family members. This includes not only abnormalities of head shape, but also visual problems, hearing loss, heart defects, digital anomalies, short stature, and early infant death. It is helpful if the parents are asked to bring family photos with them, so more subtle features of a mildly affected relative may be detected. Obviously, if

there is a documented family history of craniosynostosis, one would wish to know this information. However, this may not be volunteered by the parents, and should be specifically asked.

The patient’s medical history should be reviewed. Often, the patient is a young child with a relatively short medical history; yet important facts would include the length of gestation (premature infants often have a dolichocephalic shape to the skull), and the presence or absence of any medical conditions that may predispose to early sutural synostosis, such as hyperthyroidism. In young infants, sleeping position is also an important factor. As the medical profession has turned to promoting sleeping in a supine position for new babies, there has been a significant rise in referrals for, and evaluation of, positional plagiocephaly (185–187).

The physical examination, as detailed previously, is directed at identifying any abnormality of cranial shape as well as significant dysmorphic features that may be of diagnostic importance. As part of the evaluation, both parents should also be examined to rule out an inherited disorder of head shape and abnormalities of the hands and feet. The parent may thus be diagnosed with a mild form of craniosynostosis, whether syndromic or non-syndromic. An important distinction to be understood is that non-syndromic craniosynostosis does not mean noninherited. There are many families in which non-syndromic craniosynostosis is inherited in an autosomal dominant manner. This obviously will have important implications for the parents regarding their risk of having affected children. It is also important to inform parents that there can be phenotypic variability among affected family members with most autosomal dominant craniosynostosis conditions. Therefore, a mildly affected parent may have an offspring who is more or less severely affected.

### 144.8.4 Genetic Testing

If a craniosynostosis syndrome is suspected and a clinical diagnosis can be made, the risk for an affected parent to have an offspring with the same diagnosis is 50% for most of the craniosynostosis conditions discussed previously because they are autosomal dominant conditions. The exceptions are POR, Carpenter, or CRSDA syndromes and hypophosphatasia, which are autosomal recessive conditions and carry a recurrence risk of 25%. If both biological parents are unaffected and recessive conditions are excluded, their recurrence risk is less than 5%, which is an increased risk over that for the general population because of the possibility of gonadal mosaicism.

Chromosomal analysis or comparative genomic hybridization array may be useful in a child with craniosynostosis, other malformations, and development delay. Deletions at chromosome 9p22–p24 and 11q23–24 have been associated with trigonocephaly. In the case of Saethre–Chotzen patients with additional features and



developmental delay, a karyotype should be performed to rule out a chromosome 7p15.3–p22 rearrangement or deletion. Ninety percent of patients with a deletion involving the *TWIST1* gene have mental retardation.

To make a specific diagnosis or to predict the outcome of a pregnancy, gene testing can be ordered for a given syndrome (see Table 144-1). A single gene can be tested for some autosomal dominant conditions. *FGFR2* exons IIIa can be analyzed for the mutations that code for Ser252Trp and Pro253Arg and will be detected in 99% of Apert syndrome cases. *FGFR2* juxta-transmembrane mutations can be found with Beare–Stevenson cutis gyrata syndrome. A *FGFR3* mutation in the exon that codes for the juxta-transmembrane will be found in 100% of cases of Crouzonodermoskeletal syndrome. *FGFR1* can be tested for mutations in osteoglophonic dysplasia patients. *MSX2* Pro148His mutation can be tested for Craniosynostosis, Boston-Type. The coding region of the *EFNB1* gene can be screened for mutations that will be found in 87% of craniofrontonasal dysplasia patients.

More than one gene should be tested for in patients with the clinical diagnosis of Crouzon, Pfeiffer, Jackson–Weiss, Antley–Bixler, and Saethre–Chotzen syndromes, as they have significant overlapping clinical features and genetic heterogeneity. *FGFR2* exons IIIa and IIIc and *FGFR3* exon IIIa should be tested in patients that have features of Crouzon or Jackson–Weiss syndrome, and a mutation will be detected in more than 90% of cases. *FGFR1* exon IIIa (Pro252Arg), *FGFR2* exons IIIa and IIIc, and *FGFR3* exon IIIa (Pro250Arg) should be tested for patients with features of Pfeiffer syndrome, and a mutation will be detected in more than 85% of cases. Mutations in *FGFR2* exons IIIa and IIIc and the coding regions of the *POR* gene can be found in the majority of patients with features of Antley–Bixler syndrome. *POR* gene testing should be prioritized in cases with ambiguous genitalia and abnormalities of steroidogenesis.

Saethre–Chotzen syndrome patients should first be tested for *TWIST1*, then the *FGFR3* exon IIIa (Pro250Arg mutation). A microdeletion can be detected in about 11% of cases by dosage analysis using genotype markers, fluorescent in situ hybridization (FISH), or other dosage-detection methods such as real-time PCR, multiplex ligation-dependent probe amplification (MLPA), or comparative genomic hybridization (CGH) (111,157). Using this combination of genetic tools, more than 70%–80% of mutations can be detected in Saethre–Chotzen syndrome patients. Patients in whom the diagnosis of Baller–Gerold syndrome is possible should undergo *RECQL4* gene analysis, but may also require testing to rule out Fanconi’s anemia, Thrombocytopenia–Absent Radius, and Roberts syndrome or imaging studies for possible VACTERL.

To detect rare mutations in the spectrum of craniosynostosis syndrome above, one can screen *FGFR2* exons 3, 5, 11, and 14–17; and search for heterozygous gene

deletions (MLPA kit including *TWIST1*, *MSX2*, *ALX4*, *EFNB1*, and *RUNX2*) (188). Non-syndromic coronal craniosynostosis patients can be screened for the *FGFR3* exon IIIa for the Pro250Arg mutation and the *FGFR2*, *TWIST1*, and *EFNA4* genes, and non-syndromic sagittal synostosis may have *FGFR2* and *TWIST1* changes. Non-syndromic metopic synostosis patients can be screened for mutations in the *FGFR1* gene to detect the Ile300Trp mutation.

At one craniofacial unit, targeted molecular genetic and cytogenetic testing was performed on 326 children born from 1993 to 2002 who required surgery for craniosynostosis and were monitored until the end of 2007 (38). Eighty-four children were screened for pathological genetic alterations in *FGFR1*, 2, 3, *TWIST1*, *EFNB1* and *CGH*. Eighty-six percent had single-gene mutations and 14% chromosomal abnormalities. Genetic diagnoses were made for 21% of all craniosynostosis cases, and the *FGFR3* P250R mutation was the single most common mutation, accounting for 5% of all cases. Those with genetic diagnoses were associated with increased rates of many complications. Children with the clinical diagnosis of non-syndromic unicoronal or bicoronal synostosis were more likely to have a causative mutation than those with sagittal or metopic synostosis. Repeat craniofacial surgery was required for 58% of children with single-gene mutations and 17% of those with chromosomal abnormalities.

Gene testing is particularly useful in cases of prenatal diagnosis where ultrasound monitoring cannot detect subtle features associated with craniosynostosis syndromes. Postnatal diagnosis is important when the affected status of a relative is not clinically obvious. Testing offered to first-degree relatives can assess the risk to their offspring. Knowledge of whether a newborn will be affected with craniosynostosis and of known phenotype–genotype correlations will allow health care professionals to plan treatment, anticipate and potentially avoid complications of increased intracranial pressure, hydrocephalus, deafness, visual problems, cleft palate, choanal atresia, dental malocclusion, respiratory problems, dermatological disorders, psychosocial difficulties, and mental retardation in children with craniosynostosis.

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### Biographies



**Ethylin Wang Jabs** is currently the Vice Chair and a Professor of the Department of Genetics and Genomic Sciences, Professor of Pediatrics, and Professor of Developmental and Regenerative Biology at Mount Sinai School of Medicine. She has an adjunct appointment as Professor of Pediatrics, Medicine, and Surgery at Johns Hopkins University. Dr Jabs is a member of the Society for Pediatric Research, American Association of Physicians, and the American Society for Clinical Investigation and is a medical advisory board member of The Smile Train and Moebius Syndrome Foundation. She served as President of the Society of Craniofacial Genetics. She has been on the editorial board of the *Journal of Craniofacial Genetics and Developmental Biology*, *Pediatric Research*, and *Genetics in Medicine*. Dr Jabs is a clinically active pediatric geneticist who is board certified in clinical genetics, clinical cytogenetics, and molecular genetics. The major research interests of the Jabs' laboratory include developmental genetics of craniofacial disorders. These studies are elucidating the pathogenetic mechanisms of mutations, signaling pathways involved in abnormal processes and phenotype-genotype correlations. Population association studies are being conducted on non-syndromic cleft lip with or without cleft palate and craniosynostosis. Based on these findings, therapeutic strategies are being tested in transgenic mouse models for craniosynostosis to ameliorate abnormal craniofacial morphology.



**Dr Amy Feldman Lewanda** is a pediatric medical geneticist at Children's National Medical Center in Washington, DC. After receiving her MD degree from the Mount Sinai School of Medicine in New York, NY, she completed an internship and residency in Pediatrics at Mount Sinai Hospital. This was followed by a fellowship in Medical Genetics at Johns Hopkins Hospital in Baltimore, MD. Her research focused on identifying genes responsible for craniosynostosis syndromes. After her fellowship, Dr Lewanda has been in clinical practice with a particular interest in craniofacial genetics, prenatal diagnosis, and congenital malformations.



## Abnormalities of Pigmentation

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**145.1 INTRODUCTION**

Because of their visually striking phenotypes, disorders of pigmentation were the first genetic diseases ever documented, with clinical descriptions in some cases dating back hundreds or even thousands of years. Similar phenotypes have been observed in many different mammalian species, and during the Victorian era the breeding of mice with interesting coat pigmentation patterns became a popular hobby, eventually forming the basis of one of the most important animal models of modern scientific research. With the rediscovery of Mendel's principles of genetics early in the twentieth century, albinism was one of the original diseases proposed by Garrod as an "Inborn Error of Metabolism" (1,2), and the inheritance of normal human pigmentary variation was one of the first traits studied, though with limited success.

The processes involved in regulating normal pigmentation (also termed melanogenesis) occur at many levels and are under complex control. Pigment cells, known as melanocytes, must develop and migrate to the correct tissue locations and have all the components needed to produce melanin in a specific subcellular organelle, known as the melanosome. These processes are under strict genetic control, with many gene loci involved. Variations in and interactions of many of those loci regulate constitutive levels of pigmentation, including skin, hair and eye color. Furthermore, pathological mutations in many of these same genes can more drastically affect pigmentation in humans, resulting in various pigmentary diseases which involve reduced pigmentation (hypopigmentation), increased pigmentation (hyperpigmentation), or abnormal pigmentary patterning.

In the mouse, over 370 loci are currently known to affect melanocyte development, migration, and melanogenesis (3–5), and over half of these genes have been

identified, as have their human homologs (<http://www.espcr.org/micemut/>; The Albinism Database, <http://www.cbc.umn.edu/tad/genes.htm>; The Human Gene Mutation Database, <http://www.hgmd.org>). The products of these genes include melanosomal enzymes, melanosomal structural proteins, and proteins that regulate gene expression in melanocytes, either as DNA-binding proteins or as members of signal transduction pathways (see Table 145-1).

Genetic disorders of pigmentation can be generally categorized as disorders of melanocyte development, differentiation, or migration; disorders of melanocyte function; disorders of melanosome biogenesis/transport; and disorders of melanocyte survival (for reviews, see References (6–9)). Disorders of melanocyte development, differentiation, or migration typically result in abnormal pigmentary patterning, with congenital patches of hypopigmented skin and overlying hair that largely lack melanocytes, such as in piebaldism or Waardenburg syndrome. Disorders of melanocyte function typically result in global hypopigmentation, with reduced melanin levels in the skin, hair, ear and eye. This may be due to the reduced ability of melanocytes to make melanin, such as in classical oculocutaneous albinism, or due to more complex defects of melanosomes as well as other organelles, such as in Hermansky–Pudlak syndrome, with consequent abnormalities of other organ systems, such as the thrombopoietic and immune systems. Disorders of melanocyte survival involve the abnormal destruction of functional melanocytes, such as in generalized vitiligo. Disorders of hyperpigmentation also occur, such as melasma and post-inflammatory pigmentation, which result in abnormal increases in levels of melanin that can be temporary or permanent. Recent work on these disorders has provided important insight into fundamental components that

TABLE 145-1 Genes and Diseases of Pigmentation

Gene	Human Disease	Inheritance	Murine Locus	Protein
<b>Disorders of melanocyte development, differentiation, or migration</b>				
<i>KIT</i>	Piebaldism	Dominant	Dominant white spotting ( <i>W</i> )	Receptor tyrosine kinase
<i>KITLG</i>	Familial Progressive Hyperpigmentation?	Dominant	Steel ( <i>Sf</i> )	KIT ligand
<i>PAX3</i>	Waardenburg syndrome types I,III	Dominant	Spotch ( <i>Sp</i> )	Transcription factor
<i>MITF</i>	Waardenburg syndrome type IIA, Tietz syndrome	Dominant	Microphthalmia ( <i>mi</i> )	Transcription factor
Unknown	Waardenburg syndrome type IIB	Dominant	Unknown	
Unknown	Waardenburg syndrome type IIC	Dominant	Unknown	
<i>SNAI2</i>	Waardenburg syndrome type IID	Dominant	<i>Snai2</i>	Transcription factor
<i>SOX10</i>	Waardenburg syndrome types IIE, IVC, PCWH syndrome	Dominant	Dominant megacolon ( <i>Dom</i> )	Transcription factor
<i>EDNRB</i>	Waardenburg syndrome type IVA, ABCD syndrome, HSCR2	Dominant	Targeted; WS4-like	Endothelin-3 receptor
<i>EDN3</i>	Waardenburg syndrome type IVB, HSCR4	Dominant	lethal spotting ( <i>ls</i> )	Endothelin-3
<i>ADFN</i>	Albinism–Deafness syndrome		Unknown	Unknown
<i>ADAMTS20</i>	Unknown		Belted ( <i>bt</i> )	Metallopeptidase
<i>LMX1A</i>	Unknown		dreher ( <i>dr</i> )	Transcription factor
<b>Disorders of melanocyte function</b>				
<i>TYR</i>	Oculocutaneous albinism type I	Recessive	albino ( <i>c</i> )	Tyrosinase
<i>OCA2</i>	Oculocutaneous albinism type II	Recessive	pink-eyed dilute ( <i>p</i> )	OCA2 melanosome protein
<i>TYRP1</i>	Oculocutaneous albinism type III	Recessive	brown ( <i>b</i> )	DHICA oxidase
<i>SLC45A2</i>	Oculocutaneous albinism type IV	Recessive	underwhite ( <i>uw</i> )	MATP
<i>GPR143</i>	Ocular albinism type 1	X-Linked recessive	Oa1	G-coupled protein receptor
<i>PMEL</i>	Unknown		silver ( <i>si</i> )	PMEL17, GP100
<i>DCT</i>	Unknown		slaty ( <i>st</i> )	DOPAchrome tautomerase
<i>ASIP</i>	Unknown		nonagouti ( <i>a</i> )	Agouti signal protein
<i>ATRN</i>	Unknown		mahogany ( <i>mg</i> )	Attractin
<i>MC1R</i>	Red hair		extension ( <i>e</i> )	Melanocortin 1 receptor
<i>POMC</i>	Red hair		<i>Pomc</i>	Pro-opiomelanocortin
<b>Disorders of melanosome biogenesis/transport</b>				
<i>HPS1</i>	Hermansky–Pudlak syndrome type I	Recessive	pale-ear ( <i>ep</i> )	BLOC-3 component
<i>AP3BP1</i>	Hermansky–Pudlak syndrome type II	Recessive	pearl ( <i>pe</i> )	AP-3 adaptor $\beta$ 3A subunit
<i>HPS3</i>	Hermansky–Pudlak syndrome type III	Recessive	cocoa ( <i>coa</i> )	BLOC-2 component
<i>HPS4</i>	Hermansky–Pudlak syndrome type IV	Recessive	light-ear ( <i>le</i> )	BLOC-3 component
<i>HPS5</i>	Hermansky–Pudlak syndrome type V	Recessive	ruby-eye-2 ( <i>ru2</i> )	BLOC-2 component
<i>HPS6</i>	Hermansky–Pudlak syndrome type VI	Recessive	ruby-eye ( <i>ru</i> )	BLOC-2 component
<i>DTNBP1</i>	Hermansky–Pudlak syndrome type VII	Recessive	sandy ( <i>sdy</i> )	Dystrobrevin binding protein 1 (Dysbindin); BLOC-1 component
<i>BLOC1S3</i>	Hermansky–Pudlak syndrome type VIII	Recessive	reduced pigmentation ( <i>rp</i> )	BLOC-1 component
<i>PLDN</i>	Hermansky–Pudlak syndrome type IX	Recessive	pallid ( <i>pa</i> )	BLOC-1 component
<i>LYST (CHS1)</i>	Chédiak–Higashi syndrome	Recessive	beige ( <i>bg</i> )	Lysosomal trafficking regulator?
<i>MYO5A</i>	Griscelli syndrome type I	Recessive	dilute ( <i>d</i> )	Myosin 5A molecular motor complex
<i>RAB27A</i>	Griscelli syndrome type II	Recessive	ashen ( <i>ash</i> )	Myosin 5A molecular motor complex
<i>MLPH</i>	Griscelli syndrome type III	Recessive	leaden ( <i>ln</i> )	Myosin 5A molecular motor complex
<i>RABGGTA</i>	Unknown		gunmetal ( <i>gm</i> )	Rab geranylgeranyltransferase $\alpha$ subunit
<i>AP3D1</i>	Unknown		mocha ( <i>mh</i> )	AP3 Adaptor $\delta$ subunit
<i>VPS33A</i>	Unknown		buff ( <i>bf</i> )	Organelle protein trafficking
<i>CNO</i>	Unknown		cappuccino ( <i>cno</i> )	BLOC-1 component
<i>MUTED</i>	Unknown		muted ( <i>mu</i> )	BLOC-1 component
<b>Disorders of melanocyte survival</b>				
<i>RERE</i>	Generalized vitiligo	Complex		Immune cell apoptotic regulator
<i>FOXD3</i>	Generalized vitiligo	Dominant		Neural crest melanoblast differentiation regulator
<i>PTPN22</i>	Generalized vitiligo	Complex		Immune cell transcriptional regulator
<i>IFIH1</i>	Generalized vitiligo	Complex		Innate immune regulator
<i>FOXP1</i>	Generalized vitiligo	Complex		Immune cell transcriptional regulator
<i>CD80</i>	Generalized vitiligo	Complex		Lymphoid membrane receptor; primes T cells

**TABLE 145-1 Genes and Diseases of Pigmentation—cont'd**

Gene	Human Disease	Inheritance	Murine Locus	Protein
<i>LPP</i>	Generalized vitiligo	Complex		Immune system regulator?
<i>CLNK</i>	Generalized vitiligo	Complex		Regulator of immunoreceptor signaling
<i>TSLP</i>	Generalized vitiligo	Complex		Immune system regulator
<i>HLA-A</i>	Generalized vitiligo	Complex		Presents peptide antigens
<i>MHC class II</i>	Generalized vitiligo	Complex		Gene unknown
<i>BACH2</i>	Generalized vitiligo	Complex		B cell transcriptional repressor
<i>CCR6</i>	Generalized vitiligo	Complex		Immune cell cytokine
<i>SLA</i>	Generalized vitiligo	Complex		Immune system regulator of antigen signaling
<i>IL2RA</i>	Generalized vitiligo	Complex		Immune system cytokine receptor
<i>CLNK</i>	Generalized vitiligo	Complex		Mast cell immunoreceptor signal transducer; regulator of immunoreceptor signaling
<i>CD44</i>	Generalized vitiligo	Complex		T cell regulator
<i>TYR</i>	Generalized vitiligo	Complex		Tyrosinase
<i>Chr11q21</i>	Generalized vitiligo	Complex		Gene desert
<i>IKZF4</i>	Generalized vitiligo	Complex		T cell transcriptional regulator
<i>SH2B3</i>	Generalized vitiligo	Complex		B and T cell differentiation regulator
<i>GZMB</i>	Generalized vitiligo	Complex		Granzyme B; immune function effector
<i>OCA2-HERC2</i>	Generalized vitiligo	Complex		OCA2 melanosome protein
<i>MC1R</i>	Generalized vitiligo	Complex		Melanocortin 1 receptor
<i>NLRP1</i>	Generalized vitiligo	Complex		Innate immune regulator
<i>TICAM1</i>	Generalized vitiligo	Complex		Innate immune regulator
<i>UBASH3A</i>	Generalized vitiligo	Complex		Regulator of T cell receptor signaling
<i>XPB1</i>	Generalized vitiligo	Complex		Immune system transcriptional regulator
<i>C1QTNF6</i>	Generalized vitiligo	Complex		Immune system regulator?
<i>TOB2</i>	Generalized vitiligo	Complex		T cell regulator
<i>FOXP3</i>	Generalized vitiligo	Complex		Immune system transcriptional regulator

regulate melanocyte functions, including development, proliferation, survival and differentiation.

This chapter describes our current understanding of mechanisms and genes involved in regulating the pigmentary system and disorders that result when their functions are disrupted (summarized in Table 145-1). Brief overviews of melanocyte biology and the biochemistry and genetics of melanogenesis are included, to provide a background for understanding the mechanisms involved in pigmentary disorders. Many of the genes discussed function at multiple levels (e.g., development, migration and differentiation), and in such cases the genes are summarized at first mention in this chapter.

## 145.2 THE PIGMENTARY SYSTEM

Melanocytes constitute only a minor subpopulation of cells in mammalian tissues, but they are the only cells that can produce the melanin needed to pigment the skin, hair and eyes (reviewed in References (10,11)). The melanin produced has a variety of important and distinctive functions:

1. As a cosmetic entity—participating in protective coloration and in sexual attraction within species
2. As a barrier protecting against ultraviolet (UV) radiation

3. As a potent scavenger of cytotoxic radicals and intermediates
4. As a participant in developmental processes—particularly relating to optic/neurologic development

Melanocytes that populate the skin (epidermis and hair bulbs) originate as melanocyte precursors (termed melanoblasts) in the neural crest during embryologic development (reviewed in References (12,13)). Melanocytes that reside in other locations, e.g., the choroid of the eye and the stria vascularis of the inner ear, also originate from the neural crest, whereas the retinal pigment epithelium (RPE) of the eye is of neuroectodermal origin. Melanoblasts migrate throughout the developing organism to their eventual destinations, then must stop migrating, survive, proliferate and differentiate into melanocytes. The melanin produced is a large pigmented biopolymer synthesized within specific unique subcellular organelles termed melanosomes. The melanins produced, and the melanosomes wherein they are deposited, can be of several types, with differing visible colors and presumably with distinct functional properties. The major classes of melanins are brown-black eumelanins and yellow-red pheomelanins. In the epidermis and hair bulbs, the visible colors produced also depend on transfer of pigment-bearing melanosomes from melanocytes to neighboring keratinocytes, where

they are further distributed in the upper layers of the epidermis and into hair shafts.

Mammalian pigmentation is regulated at many different levels and is influenced by many genes, either directly or indirectly. In mice, more than 700 different mutations have been identified that affect pigmentation, and these map to more than 370 distinct genetic loci (reviewed in References (3,14) and the curated Coat Color Genes website, <http://www.espcr.org/micemut/>). Many of these pigment-related genes have now been identified and are known to regulate human pigmentation as well, operating at the tissue, cellular, or subcellular level.

### 145.2.1 Development of Melanocytes

As noted above, in vertebrates, all melanocytes (except those in the RPE) originate from melanoblast precursors derived from the dorsal neural tube during embryonic development (reviewed in References (12,13)). For melanoblasts to reach their eventual destinations in the body, cues must be provided to initiate migration of these precursor cells from the neural tube along defined pathways. They must continue to migrate along those pathways to their targeted destinations, and then they must stop migrating and begin to transition into functional melanocytes (the latter process being termed specification). Each of those steps is under the control of many independent genetic and environmental factors, and the overall timing of these processes is critical for achieving the correct adult pigmentation patterns in a given individual of any species.

The genes that function at this level encode, among other things, transcription factors (e.g. *MITF*, *PAX3* and *SOX10*), receptors and their ligands (e.g. *EDNRB/EDN3* and *KIT/KITL*), and other factors important to the start/continue/stop signals for migration, specification, etc. (e.g. *ADAMTS20* and *SNAI2*). Mutations in these genes and/or disruptions of their functions may perturb normal development, leading to developmental diseases that affect pigmentation, and in many cases also affect other tissues that in some way depend on cells derived from the neural tube. Regarding pigmentation, these diseases typically involve white spotted areas where melanoblasts failed to populate (typically on the forehead and abdomen) that are congenital and relatively stable throughout life. Examples of such developmental diseases include Waardenburg Syndrome (WS), Hirschsprung's disease (HSCR) and Piebaldism (reviewed in References (9,12,13,15–19)). A summary of genes involved in melanoblast development, migration and specification, along with the functions of their encoded proteins and associated diseases (when known) is provided in Table 145-1.

### 145.2.2 Distribution and Survival of Melanocytes

The eventual distribution and survival of melanocytes in a given tissue such as the skin is an important factor in

determining the pigmented phenotype. Melanocytes in the eye are localized in the choroid, iris and retina, where they function essentially as a photoprotective barrier. Ocular melanocytes are relatively dormant, and their rates of melanin production are low following fetal development. Unlike other melanocytes, in ocular melanocytes pigment granules remain within the cells. In contrast, in follicular melanocytes (i.e., those within hair bulbs), melanosomes are transferred to evolving hair shafts, thus giving the hair visible color. As they age, hair bulb melanocytes often decrease or even cease production of pigment, resulting in the characteristic graying of hair associated with aging (5,20–22). Similarly, skin melanocytes reside stably at the dermal–epidermal border and proliferate extremely slowly, if at all. Their melanosomes are transferred to neighboring keratinocytes, the predominant cell type in the epidermis. Melanosomes transferred to keratinocytes are then processed, being further degraded and redistributed, in smaller pieces and/or in larger complexes that eventually result in visible skin color as the proliferative keratinocytes migrate towards the skin surface (23). The number of melanocytes per unit area in the skin is remarkably similar between races, and the phenotypic differences of skin colors, ranging from white to black, depend principally on the quantity and distribution of the melanin “packets” or dust in the keratinocytes near the skin surface rather than melanin within the melanocytes (reviewed in References (23–27)).

As discussed above, the appropriate distribution of melanocytes in the skin, hair, and eyes depends on proper development of melanoblasts during embryological development, their appropriate migration to distant sites in the developing organism, and their differentiation to functional melanocytes once in place. It has become clear that many of the pigment-specific genes are involved in regulating human pigmentation at multiple stages, and many of these same genes, when mutant, result in pigmentary disorders. To date, a large number pigment-related genes involved in melanocyte signaling events that regulate melanoblast distribution and survival have been identified, characterized, and associated with pigmentary diseases. These include many of the same genes discussed above that are involved in melanoblast development, such as piebaldism (*KIT*), WS (*PAX3*, *SOX10*, *MITF*, *SOX10*, *EDNRB*, *EDN3*), and Hirschsprung disease (*EDNRB* and *EDN3*).

Once melanoblasts have arrived at their final destinations in the tissues, they must survive and proliferate to populate the tissue to achieve the correct distribution and density required for normal function. A relatively large number of genes are involved in those processes, and their disruption can lead to decreased melanocyte function in the adult. Again, the genes involved in these processes encode, among other things, transcription factors (e.g. *MITF* and *FOXD3*), receptors and their ligands (e.g. *EDNRB/EDN3* and *KIT/KITLG*), and other factors important to the signals that regulate proliferation, survival, etc. (e.g. *BCL2* and *SEMA3C*). Many of these genes function at multiple stages, ranging from developmental processes (as noted



above) through differentiation processes (as discussed below). Regarding effects on pigmentation, mutations in those genes typically lead to hypopigmented areas where melanoblasts failed to differentiate or survive that are progressive; pigmentation may be normal at birth, but pigmentation abnormalities become more severe with age. Examples of such diseases include Vitiligo and Reticular pigmentosum (for reviews, see References (7,28–35)).

### 145.2.3 Differentiation and Function of Melanocytes

Many of the precursors and intermediates in the melanin biosynthetic pathway are cytotoxic quinones and derivatives, and that is probably a major reason why the biochemical machinery to produce melanin is confined within the membrane-bound melanosomes. Melanosomes are lysosome-related organelles (LROs), and many of their components are shared with other LROs (36–38). The specific structure and function of melanosomes relates to the specific expression of a limited number of melanosomal proteins that are produced only in melanocytes. Melanosomal biogenesis begins with the blebbing of membrane-bound vesicles from the smooth endoplasmic reticulum, initially containing only an amorphous matrix and termed stage I melanosomes. Stage I melanosomes do not contain any of the melanogenic enzymes required to produce melanin but do contain PMEL17 protein which is required to make the fibrillar matrix (36,39). Depending on the type of melanin to be produced, the melanosomes then reorganize from amorphous structures either into characteristic fibrillar patterns oriented along the longitudinal axes of the organelles (for eumelanin production) or into particulate spherical organelles (for pheomelanin production), at which time they are termed stage II melanosomes. Tyrosinase and other melanogenic determinants, such as the tyrosinase-related proteins (TYRP1 and DCT), are synthesized on ribosomes and are processed through the endoplasmic reticulum to the Golgi apparatus, where they are glycosylated en route to the active face of that network (reviewed in References (23,40)). They are then secreted within coated vesicles into the cytoplasmic milieu and are transported specifically to stage II melanosomes, where they fuse with its limiting membrane, with their catalytic domains facing inward, and active melanogenesis begins (stage III melanosomes). Fully pigmented stage IV melanosomes are filled with melanin, and no internal architecture remains visible.

Ultrastructural histochemistry has clearly demonstrated that tyrosinase is functionally competent while in transit through the Golgi apparatus and in coated vesicles, and it is not clear how melanogenesis is delayed *in vivo* until tyrosinase arrives at the melanosome (40,41). The mechanism involved is of great interest because it may play a role in some types of abnormal hypopigmentary conditions in which active tyrosinase is present yet no melanin is produced. Once melanogenesis has begun, the melanin produced is deposited in characteristic patterns within

melanosomes. If eumelanin is produced, it accumulates uniformly on internal fibers of melanosomes, and the opacity of the organelles gradually increases until eventually all substructure is obscured. If pheomelanin is produced, it accumulates in a heterogeneous fashion within particulate spherical melanosomes, which do not become fibrillar due to the lack of PMEL17 expression. Regardless of which type of melanin is produced, as melanosomes mature they steadily move away from the perinuclear region to the peripheral dendritic areas of melanocytes using the molecular motor machinery of melanocytes (reviewed in References (42–44)). As melanosomes accumulate at the termini of the melanocyte dendrites in the skin, they are pinched off and engulfed by surrounding keratinocytes. Disruptions in melanosomal transport (e.g. due to disrupted function of RAB27A, MYO5A or MLPH) and/or transfer result in Griscelli Syndrome (GS) (reviewed in References (44,45)).

Melanosomes are unique organelles in that they contain the pigment-specific catalytic and structural proteins involved in melanin production, such as tyrosinase. However, melanosomes belong to the LRO family, which also includes lysosomes, platelet dense bodies, etc. This relationship is highlighted by the fact that several other types of pigmentary disorders, including Chédiak–Higashi syndrome (CHS) and Hermansky–Pudlak syndrome (HPS), involve not only the disruption of melanosome structure and function, but also the disruption of lysosomes, platelets and other LROs (reviewed in References (38,46–49)). More than 15 different genes in mice have mutant phenotypes similar to these human lysosomal/pigmentary disorders, and to date nine of those have been definitively associated with various types of human HPS. Studies of these mouse and human phenotypes have shown that factors involved in the sorting/targeting of “lysosomal” proteins are also involved in melanosome biogenesis, and in fact this system has provided an ideal model to study organelle biogenesis. Many of the following proteins function in complexes important to the trafficking of proteins to LROs. Those complexes are known as BLOC-1, BLOC-2 and BLOC-3, although how their function is not yet clear (reviewed in References (48–52)).

### 145.2.4 Regulation of Melanosome Structure and Function

Although the chemical reactions involved in the production of different types of melanins from the amino acid tyrosine have been known for some time, it is only recently that the underlying complexity of the controls involved in producing melanins has been unraveled. There are many unusual properties of the melanin biopolymer that make it resistant to most approaches of chemical and physical analysis; in addition, the extremely reactive nature of melanogenic intermediates and their ability to rapidly and spontaneously form melanin *in vitro* has made the chemical pathway difficult to characterize. Tyrosinase is the only enzyme absolutely essential for melanin formation.

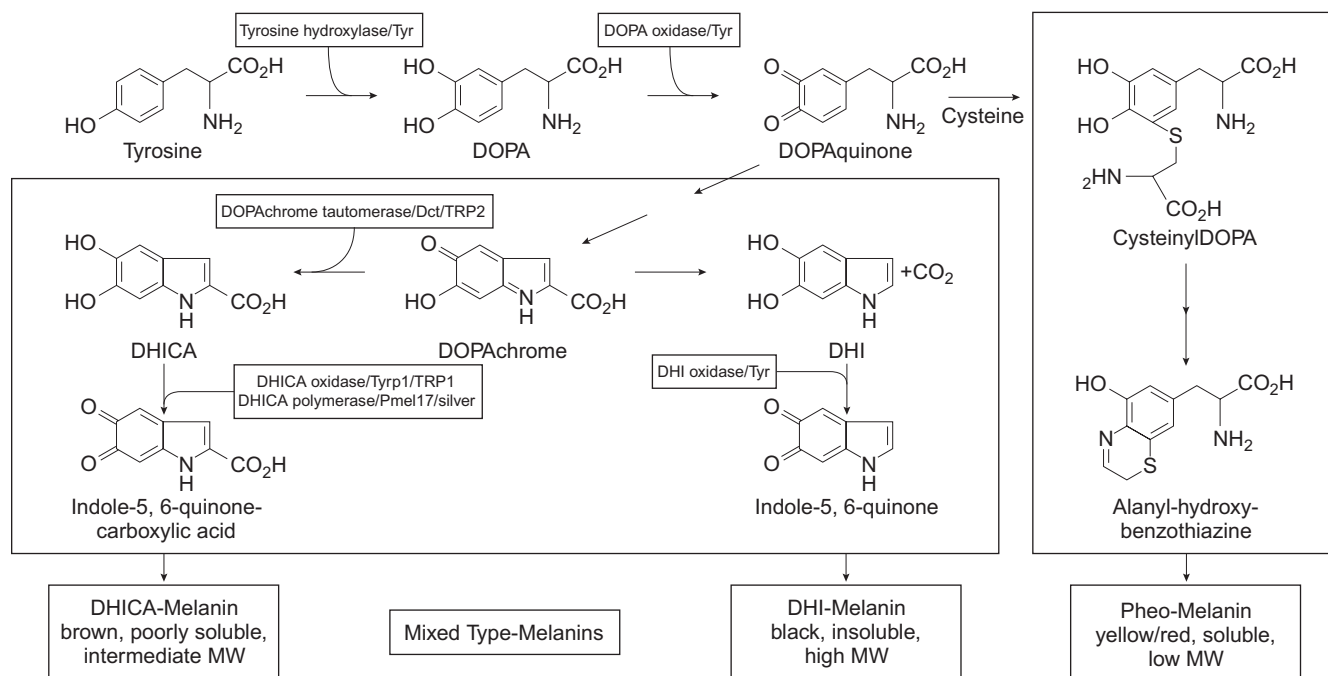
The early view of the regulation of melanin synthesis was that it depended directly on the amount of tyrosinase produced in melanocytes, because the substrate tyrosine was ubiquitous. However, it is now clear that the activity of tyrosinase and the production of melanin are regulated at several levels within melanocytes, and that the type of melanin produced is also actively regulated at several steps distal to the action of tyrosinase.

*In vitro*, the basic biochemical pathway for melanin formation from tyrosine depends directly on the function of tyrosinase. Tyrosinase (encoded by the *TYR* locus) has three distinct catalytic functions in melanin biosynthesis (53,54) (Figure 145-1). It is essential to melanin formation by virtue of its ability to catalyze the hydroxylation of tyrosine, the first and rate-limiting step in the melanin biosynthetic pathway, though tyrosinase can also utilize DOPA, DHI, or DHICA as substrates. This initial hydroxylase reaction is especially critical because there is negligible spontaneous hydroxylation of tyrosine in the absence of tyrosinase, particularly at the acidic pH within melanosomes. DOPAquinone, once formed, will continue through the melanogenic pathway by quickly cyclizing to form the indole ring structures known as leukoDOPAchrome and DOPAchrome. In turn, DOPAchrome will spontaneously decarboxylate *in vitro* to produce 5,6-dihydroxyindole (DHI), which will then rapidly oxidize to indole-5,6-quinone. Melanin was originally thought to consist simply of a homogeneous polymer of indole-5,6-quinone and related derivatives, but it is now clear that melanins are much more heterogeneous than that, containing mixtures of at least several additional intermediates in the pathway, some of them carboxylated (55–58).

Tyrosinase is synthesized as a *de novo* protein of approximately 60 kDa and is glycosylated en route to melanosomes to a final size of approximately 75 kDa. The catalytic functions of tyrosinase are highly dependent on its binding of two copper atoms per molecule at highly conserved metal ligand-binding motifs (59,60). The processing of tyrosinase (and the other melanogenic proteins) is tightly regulated in the Golgi apparatus and endoplasmic reticulum (ER) by the chaperone system for glycosylation and metal ligand binding (61–63). Many tyrosinase amino acid substitutions result in the retention of virtually all corresponding tyrosinase polypeptides in the ER (63–65), resulting in their premature proteolysis by proteasomes. The processing and trafficking of tyrosinase through subcellular compartments to melanosomes also requires correct intracellular pH (66,67); reviewed in (23,68,69).

*In vivo*, other biological constraints are put on the melanin biosynthetic pathway by factors that function downstream of tyrosinase and significantly alter the flow of the pathway. These factors include other enzymes (such as DCT/DOPACHrome tautomerase and TYRP1/5,6-dihydroxyindole-2-carboxylic acid [DHICA] oxidase), the availability of reactive sulfhydryl groups (e.g. cysteine), melanogenic inhibitors, and perhaps even other regulatory elements, such as intracellular pH and sorting proteins responsible for the trafficking of melanosomal proteins.

Two basic types of melanin can be produced in mammalian melanocytes: eumelanin, which is black or brown in color, and pheomelanin, which is red or yellow in color (reviewed in References (70–72)). The commitment



**FIGURE 145-1** Melanin biosynthetic pathway. The melanin pathway presents a summary of the known reactions and regulatory enzymes in eumelanogenesis and pheomelanogenesis.

to produce either type of melanin is made as soon as DOPAquinone is produced. If sulfhydryl groups, especially cysteine (73,74), are available in melanosomes, they will stoichiometrically capture DOPAquinone as it is produced, resulting in the production of cysteinylDOPAs. The nascent cysteinylDOPAs then undergo a series of poorly understood reactions that result in the cyclization of a second ring and the polymerization of those intermediates into a polymer called pheomelanin. Pheomelanins have many properties distinct from eumelanins in addition to their visible differences; they are significantly more soluble and of lower molecular weight, and presumably their functional properties are also quite distinct.

However, if sulfhydryl groups are absent/depleted in melanosomes, the eumelanogenic pathway will be followed. The DOPAquinone will be quickly converted to DOPACHrome, which represents yet another key regulatory point in the pathway (reviewed in References (75–77)). In the absence of other factors, DOPACHrome will spontaneously decarboxylate to form DHI, but in the presence of divalent metal cations and/or a melanogenic enzyme called DOPACHrome tautomerase (encoded by the *DCT* locus), the carboxylated intermediate DHICA will be produced. DHI and DHICA are not thought to be interconvertible chemically, so once this step is reached, the carboxyl content of the resulting melanin will be determined. If DHI is produced, it will be rapidly oxidized to indole-5,6-quinone and then quickly polymerized to large-molecular-weight biopolymers. However, if DHICA is produced, since it is relatively stable *in vivo* at the acidic environment of the melanosome, it will only slowly oxidize within melanosomes unless another enzyme is present to push the reaction further. In mice, an enzyme known as DHICA oxidase (encoded by the *Tyrp1* locus) has such an oxidase activity towards DHICA (78,79), and in the presence of Tyrp1, DHICA will be oxidized to the carboxylated indole-quinone and then to a melanin biopolymer. Although murine tyrosinase can oxidize DHI, it cannot utilize DHICA as a substrate. In humans, however, TYRP1 (encoded by the *TYRP1* locus) is not able to oxidize DHICA, whereas human tyrosinase is able to do this (80). TYRP1 has a more critical function in regulating melanogenesis via its stabilization of tyrosinase trafficking, as detailed below. DHICA-melanins appear to be of lower molecular weight, more soluble and of a brownish color when compared with DHI-melanins, which are very highly polymerized, insoluble and black (81). Melanins produced in mammalian melanocytes contain both carboxylated and decarboxylated intermediates, with the former typically constituting 50% or more of the total (82). In humans, melanins usually consist of mixtures of eumelanins and pheomelanins, termed mixed melanins. The exact ratio of the eumelanins and pheomelanins in mixed melanins probably varies with the individual. As noted above, it is not yet completely known what environmental factors influence which type

of melanin is made, or even whether human melanocytes can produce both types of melanins at the same time, or are similar to lower mammals, which produce only one or the other at any given time (for review, see Reference (27)). Characterizing the importance of incorporating these various intermediates (i.e. with or without sulfhydryl and/or carboxyl groups) to the structure and function of the melanins produced should provide important insights into understanding the regulatory controls over this pathway and their functional significance.

In addition to enzymatic components, several other melanocyte-specific proteins are critical for the unique structural arrangement of melanosomes, while others are involved in regulating the melanosomal environment that in turn regulates melanin production. The most important structural component is Pmel17 (also known as gp100), encoded by the *PMEL* locus. The processing of Pmel17 protein is critical to producing the myeloid-like fibers that transform melanosome structure from homogeneous to fibrillar, and on which melanins are deposited. MART1 is also crucial to the processing and trafficking of Pmel17, and disruption of MART1 function affects pigmentation. Other pump-like proteins thought to be present in melanosomes (or in key trafficking vesicles) include P/OCA2, SLC24A5, and SLC45A2, all of which are critical to the regulation of pigmentation. Expression and functions of these proteins are involved in regulating normal variation in the constitutive pigmentation of the hair, skin and eyes. These functions are also important in responding to environmental factors, and abnormal increases in their functions can cause various hyperpigmentary diseases, such as melasma and post-inflammatory hyperpigmentation (reviewed in References (23,24,83,84)).

### 145.2.5 Regulation of Melanin Production

The regulation of melanocyte differentiation *in vivo* is primarily under the control of the melanocortin 1 receptor (MC1R), its agonist  $\alpha$ -melanocyte stimulating hormone (MSH), and its antagonist agouti signal protein (ASIP). A large number of other receptor–ligand interactions also control melanocyte functions (differentiation, survival, migration, etc.), many of which are discussed above (e.g. EDNRB and EDN3, KIT and KITL); for reviews see References (23,24,84). The interactions of MC1R, MSH, and ASIP determine whether black/brown eumelanin versus red/yellow pheomelanin is synthesized, and how much of these melanins will be produced by melanocytes (85–90). The unstimulated state of melanocytes (i.e. in the absence of MSH) results in minimal production of melanin and predominant production of pheomelanin; the addition of MSH leads to a dramatic stimulation of eumelanin production in melanocytes, due to the increased expression of most melanosomal proteins (91). ASIP serves as an antagonist of MSH that prevents the activation of MC1R even in the presence of MSH, which in turn causes a decrease in pigment

production and a switch from eumelanin to pheomelanin synthesis. The human genes encoding MC1R, MSH, and ASIP are partially responsible for normal variation in the color and/or intensity of skin, hair, and eye pigmentation in humans, including the regulation of eumelanin/pheomelanin production (43,69).

Many additional loci also participate in the regulation of constitutive color, including a number of receptors expressed by melanocytes and the corresponding ligands produced by neighboring cells (keratinocytes, fibroblasts, etc.) (92–94) and by the environment (e.g. UV radiation) (25,26,95). The complex interactions involved in these processes are reviewed in References (10,11,23,24). The sum of those factors produces the full range of colors seen in tissues under normal conditions and responses, and over-activation of many of them have been associated with various hyperpigmentary diseases, e.g. melasma (96–100) and post-inflammatory hyperpigmentation (96). Most of those conditions are not inherited pigmentary disorders and thus are not within the scope of this chapter. Interested readers are referred to the reviews cited above for more detailed information.

### 145.2.6 Interactions and Regulation

It has been known for many years that there is a direct response of the pigment system in melanocytes to specific factors such as MSH and UV irradiation. Although the overall upstream sequences of the tyrosinase gene family genes (*TYR*, *TYRP1*, and *DCT*) are very different (101), each contains both shared transcriptional regulatory elements as well as functional promoter elements unique to each gene (102–104). Distally regulatory regions have been identified as far downstream as 15 kb. The most important regulatory element shared by the upstream regions of all three members of the tyrosinase gene family is the M-box (4), which is bound by the M-box binding factor MITF (105–108). *TYR* and *TYRP1* are thought to be coordinately regulated by melanotropic agents via cAMP-dependent protein kinase and protein kinase C (109). There are several lines of evidence indicating that cAMP is a key regulatory component, along with MITF, in the regulation of melanin biosynthesis (105,110). Nitric oxide (NO) is also thought to play a role in the induction of *TYR* gene expression via the cGMP pathway (111).

If expression of these genes is coordinately regulated, such regulation is more complex than simple activation of the three genes. In the mouse, tyrosinase enzymatic activity does not always correlate with *Tyr* mRNA levels or the amount of melanin produced (112), whereas levels of *TYRP1* protein do seem to directly correlate with the amount of eumelanin (113). In many (though not all) mouse melanocyte lines, *Dct* mRNA levels correlate with *DCT* activity (114). *Dct* is expressed very early in developing mouse embryos, usually around 10 days post-conception, whereas *Tyrp1* is expressed at

11.5 days post-conception, and *Tyr* transcripts at 13.5 days post-conception (115).

## 145.3 GENETICS AND DISORDERS OF HUMAN PIGMENTATION

### 145.3.1 Disorders of Melanocyte Development, Differentiation, or Migration—Piebaldism and Waardenburg Syndrome

Disorders of melanocyte development, differentiation, or migration typically result in abnormal pigmentary patterning, with congenital patches of hypopigmented skin and overlying hair that largely lack melanocytes, such as in piebaldism or in the various forms of Waardenburg syndrome (WS). Pigmentation in uninvolved areas is normal. Because of the visually evident phenotype, piebaldism was one of the first genetic disorders to be recognized, and is said (116) to be the first disorder for which a pedigree was presented.

A total of seven genes have thus far been associated with disorders of melanocyte development, differentiation, or migration, including *KIT* (piebaldism; OMIM 172800); *PAX3* (Waardenburg syndrome type I; WS1; OMIM 193500 and Waardenburg syndrome type III; WS3; OMIM 148820); *MITF* (Waardenburg syndrome type 2A; WS2A, OMIM 193510), *SNAI2* (WS2D, OMIM 608890), *EDNRB* (Waardenburg syndrome type 4A; WS4A; OMIM 277580), *EDN3* (Waardenburg syndrome type 4B; WS4B; OMIM 613265), and *SOX10* (Waardenburg syndrome types 2C; WS2C; OMIM 606662 and Waardenburg syndrome type 2E; WS2E; OMIM 611584).

**145.3.1.1 Piebaldism.** Piebaldism (OMIM 172800) presents with a characteristic pattern of white forelock and multiple symmetrical, irregularly shaped, white or depigmented macules. This condition has been reported in the literature under a variety of names, including partial albinism, familial white spotting, white forelock and piebaldism (117,118). The white forelock is usually present at birth or early in life, and may be only a few strands or a large patch of white hair. The underlying skin is white. The white macules occur on the face, trunk (typically ventral), and extremities, and the hair growing from them is white. The white forelock may be the only manifestation, and both hair and underlying skin may be involved. Areas of hyperpigmentation are often present within and along the borders of a hypopigmented macule. Melanocytes are typically absent in the white patches of skin and hair follicles of the white hair. Piebaldism is autosomal dominant in inheritance (118). A child born to affected parents was presumed to be homozygous for piebaldism; the child had complete absence of cutaneous pigment and blue eyes (119).

Piebaldism results from mutations or deletions of *KIT* (117,120–129). This was first suspected on the basis of



the identification of *Kit* deletions in mice with dominant white spotting (W) (130–133), the localization of human *KIT* at chromosome 4q11–q13 (134,135), and the identification of a patient with piebaldism associated with an interstitial deletion at chromosome 4q12 (136). The W locus of the mouse was shown to represent the *Kit* proto-oncogene, which encodes a transmembrane receptor tyrosine kinase (130) that is expressed in mast cells, melanoblasts/melanocytes, hematopoietic stem cells, intraepithelial lymphocytes, interstitial cells of Cajal, and germ cells. With respect to the pigment-related phenotype, *KIT* loss of function mutations may affect the development and/or migration of melanocytes, producing the phenotype of piebaldism.

The human *KIT* gene has 21 exons spanning 34 kb (135,137,138), with evidence of alternative mRNA splicing (138). In a human family with piebaldism, a *KIT* missense mutation was identified, which exhibited linkage with piebaldism in this family with a lod score of 6.02 at  $\theta = 0$  (120). Another case was shown to have deletion of both *KIT* and the adjacent *PDGFRA* genes (139). As in the mouse, different alleles of the human *KIT* gene result in a spectrum of phenotypes ranging from mild to severe forms of piebaldism (140). A missense mutation, R796G, located in a highly conserved region of the tyrosine kinase domain, has been associated with piebaldism and deafness (128). Other mutations in the *KIT* gene can result in mastocytosis or in gastrointestinal stromal tumors (141).

The ligand for the *KIT* receptor is *KIT* ligand (*KITL*) (142–144), encoded by the *KITLG* gene located at 12q22 (145). In the mouse, *Kitlg* gene mutations at the steel (*Sl*) locus prevent its binding to the receptor, resulting in a spotted phenotype identical to that of mutations in the W locus (142,144,145). The *Kitlg* mutant phenotype also affects erythropoiesis and germ cell development, as observed with the W locus. While mutations in human *KITLG* might be expected to result in a phenotype similar to that of piebaldism (145), no patients with a piebaldism-like phenotype have been identified with mutations in *KITLG* (140), though a missense substitution in this gene has been associated with familial progressive hyperpigmentation (146).

Another potential human piebaldism gene is *ADAMTS20*, which encodes a metalloprotease that is secreted and is involved in degradation of the extracellular matrix and in tissue remodeling (reviewed in References (16,147)), and which is essential for melanoblast migration and survival (148). In the mouse, *Adamts20* mutations cause belted (*bt*) white-spotting (149).

**145.3.1.2 Waardenburg Syndrome (WS).** The classic phenotype of Waardenburg syndrome consists of the combination of lateral displacement of the inner ocular canthi with a broad nasal ridge (hypertelorism), white forelock (poliosis), different colors of the two irides (heterochromia irides), white eyelashes, white areas of skin (leukoderma), hyperplasia of the medial portion of the eyebrows, and

congenital sensorineural (cochlear) hearing loss (150–152). In fact, four main types of WS are distinguished: WS type 1 (WS1) with lateral displacement of the ocular inner canthi (dystopia canthorum) (OMIM 193500); WS type 2 (WS2) without dystopia canthorum (OMIM 193510); WS type 3 (WS3) with limb muscle hypoplasia and joint contractures (Kline–Waardenburg syndrome; OMIM 148820); and WS type 4 with Hirschsprung disease (Waardenburg–Shah syndrome; OMIM 277580) (150,151,153). WS1, WS2, and WS3 are autosomal dominant, whereas WS4 is autosomal recessive in inheritance, as reviewed in References (150,151,153). However, the genetics of WS are complex, involving a number of different genes, with mutant phenotypes and patterns of inheritance that do not correspond perfectly to the four clinical WS types. Thus, WS1 and WS3 result from mutations of *PAX3* (154); WS2 results from mutations of *MITF* (155,156) and *SNAI2* (157); and WS4 can result from mutations of *EDN3*, *EDNRB*, or *SOX10* (150).

**145.3.1.2.1 WS1 and WS3.** WS1 is an autosomal dominant disorder characterized by congenital piebald-like hypopigmentary patches of skin and overlying hair, heterochromia irides, congenital sensorineural hearing loss and dystopia canthorum. WS3, also known as Kline–Waardenburg syndrome (158), is similar, but also includes upper limb anomalies (147).

Initial identification of the gene associated with WS1 was based on similarity to the mouse splotch (*Sp*) phenotype, which involves spotty areas of hypopigmentation. The region of mouse chromosome 1 containing the *Sp* locus is syntenic to the region of human chromosome 2 to which WS1 had been mapped (2q35–2q37) (159–161). *Pax3* mutations were identified in *Sp* mice (162), and human *PAX3* (previously called *HuP2*) was then shown to contain mutations in patients with WS1 (163,164). In fact, mutations in *PAX3* result in both WS1 and WS3, as well as in Craniofacial–deafness–hand syndrome (CDHS) (147).

*PAX3* consists of 10 exons spanning approximately 100 kb (165) at chromosome 2q35. A large number of *PAX3* mutations have been identified in patients with WS1 or WS3, typically with specific private mutations being found in a specific family (147,152,161,166–169). Many *PAX3* mutations appear to result in complete loss of function, the dominant nature of WS1 and WS3 suggesting haploinsufficiency for dosage of functional *PAX3* protein (164). Mutations that produce an activity level of greater than 50% of normal result in only dystopia canthorum. Null mutations that reduce the level of active protein to 50% in the heterozygote also affect melanocyte migration, resulting in patches of depigmentation. A few mutations produce a level less than 50% and are thought to act as a dominant negative. These individuals have limb muscle defects and neural tube defects and are representative of WS3.

*PAX3* encodes a member of the paired box domain family of transcription factors that are homologous to

the *Drosophila* *prd* homeobox gene, in which mutations result in the gooseberry phenotype that affects segmentation and segment polarity (159,161,163). The highly conserved PAX3 protein contains three distinct functional domains: a paired box, a conserved octapeptide domain, and a homeobox domain. The mouse *Pax3* gene (previously known as *splotch*; *Sp*) plays a role in pattern formation in the embryonic neural crest, and the high degree of homology between mouse Pax3 and human PAX3 proteins suggests that the human PAX3 plays a similar role in the neural crest development (170,171). PAX3 regulates the differentiation of melanocytes by modulating transcription of *MITF*, which in turn regulates downstream transcription of many melanocyte-specific genes (17,168,172).

**145.3.1.2.2 WS2.** The phenotype of WS2 is similar to that of WS1, but lacks dystopia canthorum. There are several genetic subtypes of WS2. WS2A (OMIM 193510) results from mutations in *MITF* (152,156,173–176). WS2B (chromosome 1p21–13.3; OMIM 600193) and WS2C (chromosome 8p23; OMIM 606662) have been mapped in individual families, but the corresponding genes have not yet been identified. WS2D (OMIM 608890) results from mutations in *SNAI2* (also known as *SLUG*) (157). WS2E (OMIM 611584) results from mutations in *SOX10*, which is also associated with WS4 (OMIM 277580).

Mutations of *MITF* cause WS2A, as well Tietz syndrome (OMIM 103500), and contribute to digenic inheritance in a form of ocular albinism with sensorineural deafness WS2-OA; (OMIM 103470); (177). The microphthalmia (*Mitf*) gene was first identified in mice, and encodes a protein containing a basic helix-loop-helix-zipper (bHLH-ZIP) motif, which is associated with a family of transcription factors (178). The human *MITF* gene is located at chromosome 3p12.3–p14.1 (156), and consists of nine exons, including four different alternative first exons, each having unique promoter sequences (179). Depending on which promoter is used to initiate transcription, a different protein isoform, MITF-A, -H, -B or -M, is produced. Promoter M is melanocyte-specific, promoter A directs expression in the RPE, cervical cancer and melanoma cells, while promoter H directs expression in the RPE and cervical cancer cells. The MITF protein is a transcription factor that regulates processes involved in melanocyte development, differentiation, function and survival (180). MITF binds to the M-box and E-box, two melanocyte-specific promoter motifs found in the 5' region of genes encoding tyrosinase-related proteins and many other pigmentation-related genes (reviewed in (30,147,181)). Many different human *MITF* mutations have now been identified, and appear to produce WS2 by means of haploinsufficiency for MITF function (147). Some mouse *Mitf* alleles, when homozygous, result in a completely unpigmented, deaf mouse with defective eye development, whereas other *Mitf* alleles have a less severe effect in homozygotes.

Rare patients have been identified with WS2D, resulting from deletions of 8q11 that include the *SNAI2* gene (also known as *SLUG*). *SNAI2* consists of three exons that encode a 268-amino acid protein with a molecular mass of approximately 80 kDa. In two of 38 unrelated patients with WS2 and no mutation in the *MITF* gene, homozygous deletions of the *SNAI2* gene were identified (157). Similarly, *SNAI2* deletions were also observed in patients with piebaldism who lacked *KIT* mutations (182), but who presumably should be reclassified as having WS2D. *SNAI2* is a transcription factor containing five zinc finger regions (183). *SNAI2* is important in the initial stages of cell migration from the neural tube (184), and is thought to function by inducing the epithelial–mesenchymal transition that is involved in development, more specifically migration and/or survival, and other cellular processes (reviewed in (147)).

WS2E is similar to WS2A, but may additionally include neurological abnormalities such as mental impairment, myelination defects, and ataxia. WS2E results from mutations of *SOX10* (185–188). *SOX10* is also associated with WS4 and PCWH syndrome (see below) (147).

**145.3.1.2.3 WS4.** WS4 (OMIM 277580) is similar to WS1 but additionally includes Hirschsprung disease. WS4 is divided into three genetic subtypes (185,189–192), resulting from mutations in *EDNRB* (WS4A; OMIM 277580), *EDN3* (WS4B; OMIM 613265), or *SOX10* (WS4C; OMIM 613266).

Mutations in *EDNRB*, encoding the receptor for ligand endothelin-3, result in WS4A, ABCD syndrome (previously known as albinism and black locks syndrome), and genetic susceptibility to Hirschsprung disease (HSCR2; aganglionic megacolon) (193). *EDNRB* is located at chromosome 13q22 (194). Targeted mutations of mouse *Ednrb* result in a phenotype similar to human WS4A, including coat color spotting (195).

Mutations in *EDN3*, encoding endothelin-3, result in WS4B, as well as in a genetic susceptibility to Hirschsprung disease (HSCR4) and idiopathic congenital central hypoventilation syndrome (CCHS; OMIM 209880). *EDN3* is located at chromosome 20q13.2. In the mouse, targeted *Edn3* mutations result in a phenotype similar to that resulting from disruption of *Ednrb*, providing an early suggestion for a second locus associated with WS4 in humans (196), and indicating that the EDN3-EDNRB ligand–receptor interaction is important for the development of epidermal melanocytes and enteric neurons.

Mutations in *SOX10* cause WS4C, as well as WS2E, and PCWH syndrome (147,197,198). *SOX10* is located at chromosome 22q13.1 (191). *SOX10* belongs to the SOX family of transcription factors, and is involved in regulating the early development of the neural crest and the migration of melanoblasts (199,200). *SOX10* also participates in regulating transcription of *MITF*, and also directly regulates transcription of *DCT* and *EDNRB* (201,202).

**145.3.1.3 Albinism–Deafness Syndrome (ADFN).** A large Moroccan Sephardic Israeli family has been described that includes multiple males with piebald-like white spotting of skin and overlying hair and profound congenital deafness inherited in an X-linked recessive pattern (OMIM 300700), with evidence of hearing defect in carrier females (203,204). The *ADFN* gene has been mapped to chromosome Xq24–q26 (205), but has not yet been identified. It remains uncertain whether other reports of similar clinical findings in other patients in fact represent the same entity (206,207).

### 145.3.2 Disorders of Melanocyte Function—Albinism

Albinism is a heterogeneous group of genetic disorders, almost all autosomal recessive, which are characterized by congenital global reduction of pigmentation of the skin, hair, and eyes, either alone or with other systemic abnormalities (for reviews, see References (208,209)) (Table 145-1). Because of the visually evident phenotype, albinism was the first genetic disorder ever recognized, with clear descriptions in many ancient texts (Figure 145-2). The most severe form, oculocutaneous albinism (OCA) is estimated to occur with a prevalence of approximately 1 in 18,000 to 20,000 in the US population, though accurate population-based surveys have never been done. The ranges of clinical phenotypes of the different forms of albinism overlap substantially, and, with the exception of CHS, the different forms of OCA cannot be distinguished on clinical grounds and require molecular analysis.

Patients with albinism can have little or no integumentary pigment ranging all the way to near-normal, and in general the degree of clinical severity is directly related to the amount of melanin pigment. Thus, lack of melanin pigment in the skin can make patients subject to severe sunburn and, over time, skin damage and susceptibility to skin cancer, particularly in parts of the world with high rates of UV exposure. It is therefore essential that patients with all forms of albinism avoid unnecessary sun exposure and make liberal use of sunscreen and other forms of sun protection.

In addition to deficient integumentary pigmentation, all forms of albinism also include reduced melanin pigmentation of the iris (Figure 145-3) and retina (Figure 145-4). Melanin plays key (albeit unknown) roles in development of the optic tracts, and patients with albinism thus manifest a stereotypic set of optic abnormalities that occur independently of the specific gene involved, and instead appear to relate in severity to the degree of melanin deficiency during optic tract development during fetal development and postnatal life (210–219). These defects are not observed in obligate heterozygotes for these recessive diseases. Deficient iris pigmentation results in photophobia, and deficient retinal pigmentation results in foveal hypoplasia and



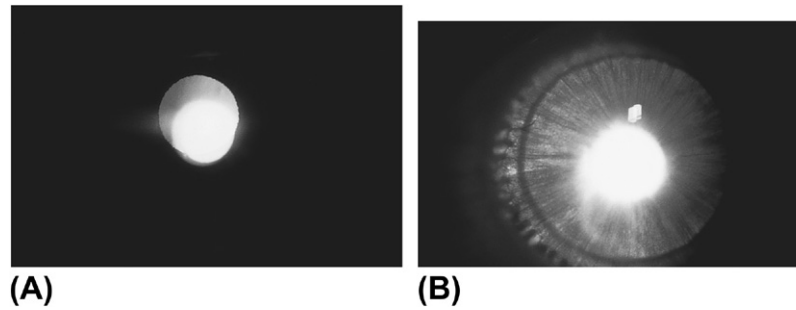
**FIGURE 145-2** Adult European-derived female with OCA1A.

consequent reduced visual acuity that cannot be corrected to normal (“low vision”) (76,219–221), with eventual Snellen visual acuity ranging from 20/80 to 20/400. In general, clinical severity correlates with the amount of melanin pigment in the eye (222). Foveal hypoplasia does not interfere with color vision (223–225). Aberrant neural projections from the retina to the brain, with neuronal misrouting at the optic chiasm, lateral geniculate nucleus and motor nuclei (223,226–229) (for review, see References (230,231)) (Figure 145-5), result in stereoscopic depth perception, horizontal pendular nystagmus, and often strabismus (221,222,232). The response to flash or pattern-onset stimuli in a visual evoked response test is often used to establish a diagnosis of albinism in children (including neonates) (233), the magnitude of the visual evoked potential (VEP) changes generally correlate with the clinical features of albinism (234). Frequently, photophobia and low vision are among the most important impediments to normal daily life in persons with albinism. While most children with albinism function well in regular school, attention should be paid to special needs for vision, including large-print reading materials and preferential seating in the classroom.

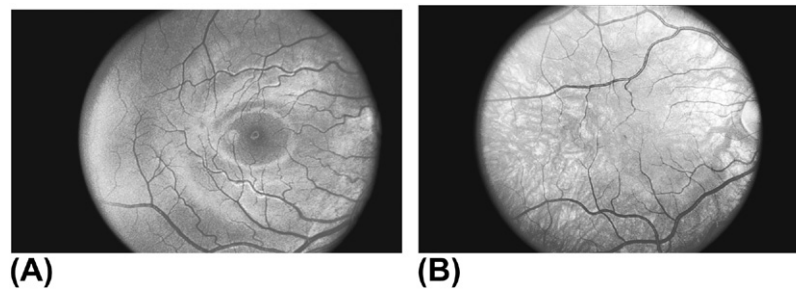
Pigmented melanocytes occur in the inner ear; in the stria vascularis, which provides the driving current for the hair cells, the sensory receptors in the inner ear. While the absence of melanin pigment in the inner ear makes animals with albinism more susceptible to noise-induced hearing loss (235,236), in humans albinism is not associated with auditory abnormalities.

In addition, the visually evident lack of skin and hair pigmentation can result in significant psychosocial morbidity due to real or perceived social stigmatization and isolation, particularly in individuals derived from ethnic groups in which dark skin and hair pigmentation is the

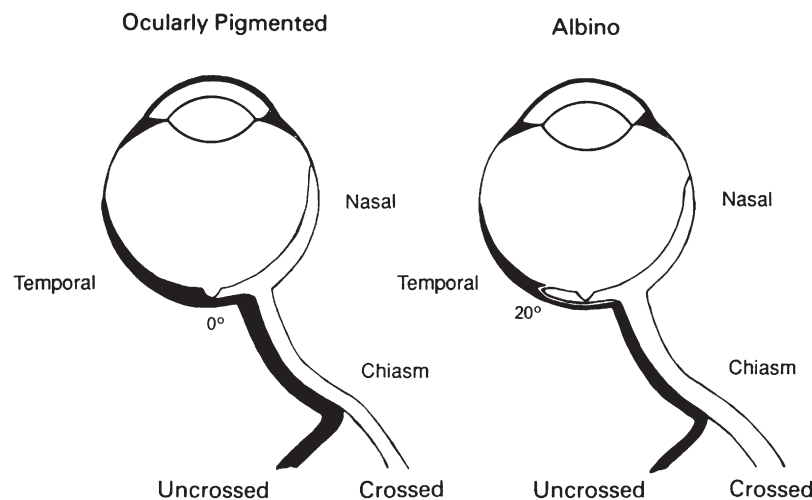




**FIGURE 145-3** Iris transillumination. A) Normally pigmented iris with no transillumination on slit-lamp examination; B) Grade 4 (full) iris transillumination in an individual with OCA1A.



**FIGURE 145-4** Fundus abnormalities in OCA. A) Normal retinal pigment from a normal individual; B) Absence of retinal pigment, with visualization of the choroidal vessels, from an individual with OCA1A.



**FIGURE 145-5** Distribution of retinal ganglion fibers in an eye with normal pigment (left) and an eye in albinism (right). With normal ocular pigment, the nasotemporal border corresponds to the fovea, with the temporal fibers projecting to the ipsilateral lateral geniculate nucleus. With albinism, the nasotemporal border is shifted 20° or more into the temporal retina, resulting in most retinal ganglion fibers crossing at the chiasm and projecting to the contralateral lateral geniculate nucleus. (From Creel, D. J.; Summers, C. G.; King, R. A. *Visual Anomalies Associated with Albinism*. Ophthalmic Paediatr Genet. 1990, 11 (3), 193–200, with permission.)

norm. An extreme and particularly horrific manifestation of this in recent years has been the systematic murder and harvesting of body parts from people with albinism for their imagined magical properties in Kenya, Tanzania, Burundi, and other parts of east Africa, where ironically, the prevalence of albinism is perhaps the highest in the world. Useful information for individuals with albinism is available from the National Organization of Albinism and Hypopigmentation (NOAH) (<http://www.albinism.org>).

The classification of the different types of albinism has evolved considerably over the past several decades.

Previously, classification was based on clinical grounds and on biochemical testing using the hair bulb incubation test, distinguishing OCA as “tyrosinase-negative” and “tyrosinase-positive” types (237–240). Both of these approaches are now known to be unreliable. Diagnostic classification is now based on molecular analysis, with a total of 18 genes thus far identified as being responsible for different types of albinism in humans, involving deficient melanin biosynthesis, defective melanosome biogenesis, or defective melanosome transport. Four genes are associated with oculocutaneous albinism



(OCA), in which the recessive phenotypes are largely due to reduced biosynthesis of melanin pigment, while the number, distribution, and structure of the melanosomes and melanocytes in the skin, hair follicles, and eyes remains normal: *TYR* (oculocutaneous albinism type 1; OCA1; OMIM 203100), *OCA2* (oculocutaneous albinism type 2; OCA2; OMIM 203200), *TYRP1* (oculocutaneous albinism type 3; OCA3; OMIM 203290), and *SLC45A2* (oculocutaneous albinism type 4; OCA4; OMIM 606574) (see Table 145-1). Mutations in these same genes can also result in clinically milder phenotypes known as “autosomal recessive ocular albinism” (AROA), in which the optic defects are similar to in OCA, though less severe, while there may be relatively apparent integumentary hypopigmentation. One additional gene, *GPR143*, is associated with an X-linked recessive form of ocular albinism (OA1; OMIM 300500). In addition, a total of 13 different genes are associated with three different syndromic disorders in which oculocutaneous albinism occurs in concert with life-threatening systemic manifestations: Hermansky–Pudlak syndrome (HPS; OMIM 203300), Chédiak–Higashi syndrome (CHS; OMIM 214500), and Griscelli syndrome (GS). To date, mutations in nine genes have been associated with HPS, one with CHS, and three with GS. Some individuals with albinism who do not have detectable mutations in any of these genes may represent other types of albinism or other pigmentation disorders associated with mutations in genes that have yet to be identified.

#### 145.3.2.1 Oculocutaneous Albinism (OCA).

**145.3.2.1.1 OCA1.** Oculocutaneous albinism type 1 (OCA1; OMIM 203100, 606952) is the classic form of albinism, and because of its visually evident phenotype was the first genetic disorder to be recognized and studied (241–243). The clinical phenotype includes absent or greatly reduced pigmentation of the skin (Figure 145-2), hair, and eyes (Figures 145-3 and 145-4), predisposing to sunburn, long-term skin damage from ultraviolet radiation, and skin cancers, as well as reduced visual acuity, nystagmus, strabismus, and photophobia, as described above.

OCA1 results from mutations of the *TYR* gene (OMIM 606933), encoding tyrosinase, which catalyzes the rate-limiting initial steps of melanin biosynthesis (described above). Human OCA1 is thus homologous to the classic *c*-albino phenotype of the mouse (5). The human *TYR* gene is located at chromosome 11q14.3 (244), and encodes a 529 amino acid polypeptide with molecular weight 62.6 kDa. The *TYR* gene consists of five exons spanning approximately 120 kb (245,246). More than half of the coding region is within exon 1 (819 bp), with exons 2 to 5 ranging from 148 to 225 bp. A second locus containing tyrosinase-related sequences is a partially duplicated, non-transcribed pseudogene on chromosome 11p11.2–cen (*TYRL*) (245,247), consisting of the distal portion of IVS3 through sequences downstream of exon 5. *TYR* and *TYRL* have 98% nucleotide sequence identity within exons 4 and 5, complicating molecular diagnostics

of OCA1 using polymerase chain reaction (PCR) based approaches, and requiring the use of PCR primers that differentially amplify *TYR* and not *TYRL*. *TYRL* exists in the gorilla but not the chimpanzee, indicating that the *TYRL* duplication occurred relatively recently in primate evolution (248). Furthermore, the *TYR* gene is a member of a multi-gene family that also includes *TYRP1* and *DCT*, although these related genes do not interfere with specific PCR amplification of *TYR* in practice.

OCA1 has been estimated to occur with a prevalence of about 1 in 40,000 in the EUR population; however, no accurate population-based surveys have ever been done. Recent comprehensive sequence-based mutation surveys of the known OCA genes have demonstrated unambiguously that OCA1 is the most frequent type of OCA in the EUR population (249) and in Japanese (250), whereas OCA2 is the most common form of OCA in African-derived individuals. Many pathological *TYR* mutations have been identified, the great majority being point mutations—missense, nonsense, frameshift, and splice junction mutations (The Albinism Database, <http://albinismdb.med.umn.edu/>). Large gene deletions of human *TYR* have been observed (251), but are very rare. The majority of patients with OCA1 are compound heterozygotes with different mutant maternal and paternal alleles, with many families having “private” mutations, but a few common mutations do exist (249,252,253); in the EUR population these are missense alleles T373K and P81L. A complete list of *TYR* mutations and the ethnicity of individuals in which they were observed can be found on the Albinism Database (<http://albinismdb.med.umn.edu/>).

The major determinant of a patient’s OCA1 phenotype is the amount or type of residual tyrosinase catalytic activity, although in patients who do have residual activity, the constitutional pigment background of the affected individual also plays a role (254). Thus, the range of clinical variation spans so-called “OCA1A” (“tyrosine-negative”: Figure 145-2; (76,253,255–259), “OCA1B” (“yellow albinism”): (237,254,260–263), to “autosomal recessive ocular albinism” (see below). The majority of the mutations reported result in no residual tyrosinase activity. There is also a subset of mutations associated with about 5–10% residual activity that, in a homozygous or heterozygous dose, result in OCA1B with varying amounts of cutaneous and ocular pigmentation. Several of the *TYR* missense substitutions result in temperature-sensitive, thermolabile forms of tyrosinase (261,264) that are misfolded, retained in the endoplasmic reticulum, and are prematurely degraded by proteosomes (61,63–65,67,265,266); these are thus analogous to the Siamese cat and the Himalayan mouse, both of which are the result of a temperature-sensitive enzyme. An unusual mechanism by which *TYR* mutations might affect enzyme function is by interfering with binding its two copper (Cu) atoms, and it has been noted that *TYR* missense substitutions associated with OCA1 cluster

in the Cu-A and Cu-B binding regions of the protein (54,257,263), perhaps affecting  $\alpha$ -helical regions that ligand the copper atoms (267), rendering the enzyme inactive (60,267).

One important special case involving *TYR* (or occasionally other OCA loci) is so-called “autosomal recessive ocular albinism” (AROA), in which the ocular manifestations of OCA predominate (though they are less severe than in typical OCA), while the integumentary manifestations are minimal or absent (268). AROA was described before the availability of molecular analysis. These have subsequently shown that virtually all cases of AROA are associated with mutations in *TYR* (269,270). Specifically, almost all patients with clinical AROA are compound heterozygotes for an OCA1 mutant allele and a non-OCA1 allele containing a common missense polymorphism of *TYR* that is frequent in the EUR population, R402Q (245). The R402Q variant tyrosinase polypeptide is temperature-sensitive (264) and thus is retained in the endoplasmic reticulum and degraded (65). However, this variant retains approximately 25% residual catalytic activity even at 37 °C; thus, patients with AROA have about 13–25% of residual tyrosinase activity, depending on the other allele. It seems likely that residual activity might be less at the elevated temperature of the fetus, affecting the developing optic tracts, but that residual activity might be much higher at the relatively cool temperature of postnatal skin, resulting in a fixed neurological defect but near-normal integumentary pigmentation. While the prevalence of *TYR*-related AROA is not known, the prevalence of the OCA1/R402Q genotype is estimated at about 1 per 300 individuals in the EUR population.

The *TYR* promoter region specifies four transcriptional start sites, with the major 5' start site –79 bp 5' to the ATG translation initiation codon, and minor 5' start sites at –75, –46, and –42 bp (245,271). Analyses of transgenic mice with different lengths of the *Tyr* promoter have shown that as little as 270 bp of the proximal promoter sequence produces cell-specific and temporal-specific expression, but not wild-type levels of pigmentation (272,273), while production of transgenic mice using a 250-kb yeast artificial chromosome containing over 150 kb of the 5'-flanking sequence conferred wild-type levels of pigmentation (274). Within the proximal –270 to –80 nt region, several positive and negative transcriptional regulatory elements have been identified (246,271,275–277). In human *TYR*, these include TATA (–106 bp) and CAATT (–199 bp) motifs, as well as a ~230-bp (GA)<sub>n</sub> polymorphic microsatellite at about –713 nt, the functional significance of which is unknown (271,278,279), and which has not been associated with normal pigmentary variation. In mouse *TYR*, a critical 11-bp regulatory element, AGTCATGTGCT, is located –104 nt upstream of the transcriptional start site, termed the “M-box” (106,275,280). A second M-box, sometimes termed the “E-box,” is located at –12 nt (281,282).

M-box elements occur in the promoters of all three members of the *TYR* gene family both in mice and in humans and are essential for melanocyte-specific expression (101,103,106,283). Within the 11-bp M-box motif, a 6-bp motif, CANNTG, binds a family of helix–loop–helix transcription factors (178); in melanocytes, the most important of these is the microphthalmia-associated transcription factor (MITF), encoded by the *MITF* gene (see above). MITF binds both the M-box and the TDE, a cis-acting enhancer element located approximately 1.86 kb upstream of the transcriptional start site (107,246,282,284,285). The 39-bp TDE core element contains the 6-bp M-box core motif, and directs melanoma cell-specific expression of a reporter gene (107) and in fact is the strongest M-box in the *TYR* promoter (285). In the mouse, MITF directs specific transcription of the *Tyr* and *Tyrp1* genes in melanin-producing cells (286). Nevertheless, the M-box alone cannot confer cell-specific expression of pigment genes in melanocytes (287,288), as MITF is expressed in cells that express and do not express pigment-specific genes (101,106). The M-box can activate transcription of reporter genes in multiple cell types, and deletion of the M-box does not completely abolish *TYR* transcription. A second, ubiquitous transcription factor also binds the M-box, although its identity and role in melanocyte-specific transcription are unknown (106). Other transcription factors expressed in the melanocyte, including Brn2, TBX2, PAX3, SOX10, N-Oct-3 and N-Oct-5, also are involved in controlling transcription of pigment-specific genes (110,289) and bind other regulatory elements in the proximal *TYR* promoter.

A more distal transcriptional regulatory element is located –12 to –15 kb upstream of the transcriptional start site (290–292), in a 200-bp region containing two DNase I hypersensitive sites embedded within a scaffold/matrix attachment region (S/MAR) (290,293). This region is rearranged in the chinchilla-mottled (*cm*) *Tyr* mutant allele of mouse, resulting in hypopigmentation, indicating that this region plays an important role in *Tyr* expression (291). DNase I footprinting at this site revealed a binding site containing the palindrome TGACTTTGTCA, which resembles the binding motifs for both a cAMP-responsive element-binding (CREB) transcription factor and the activator protein-1 (AP1) binding factor (290). A similar sequence occurs in human *TYR* –9 kb upstream of the *TYR* transcriptional start site (294–296), which also exhibited DNase hypersensitivity in humans. The core sequences of this region were shown to specifically increase transcription of a reporter gene in melanocytes in conjunction with the *TYR* proximal promoter (294–296).

**145.3.2.1.2 OCA2.** Individuals with albinism having yellow hair and white skin, or brown hair and skin, have been documented in many historical records, particularly those related to the European exploration of the African continent (242,297–301). The first major insight into

the separation of OCA into different types was provided by the hair bulb incubation test which, while unreliable, seemed to distinguish individuals with “tyrosine-negative” versus “tyrosine-positive” OCA (237–239). The fact that this represented true genetic locus heterogeneity was demonstrated by finding normally pigmented offspring from a mating between one individual with tyrosinase-negative OCA and another with tyrosinase-positive OCA (238,302).

Individuals with oculocutaneous albinism type 2 (OCA2; OMIM 203200) exhibit a recessive phenotype very similar to that of OCA1 (Figure 145-6), though on the average slightly less severe in all respects, particularly in patients of African descent (242,297–301,303–306). Hair may be pigmented at birth or early in life, and many individuals with OCA2 develop localized (nevi, freckles, and lentigines) skin pigment, often in sun-exposed regions of the skin. Many affected individuals accumulate pigment in their hair and eyes during their lifetime, and this is more marked in individuals from darker ethnic groups. OCA2 is found in all populations, and it is the most prevalent type of OCA in equatorial Africa (300,304,307–313), with an estimate prevalence of 1 in 3900 in southern African blacks (313) and 1 in 1400 in Tanzania (311). Unfortunately, the prevalence of skin cancer is very high in patients from these regions, due to very high rates of UV exposure. Several smaller isolated populations have also been reported to have a high frequency of OCA2, the apparent result of founder effects (240,314,315). The estimated carrier frequency for this deletion in African Americans is 1 in 200 to 1 in 500 (316).

OCA2 results from mutations of the OCA2 gene (OMIM 611409) on chromosome 15q11.2–q13 (317,318), a region that had been shown to be linked to OCA2 (319). OCA2 is the human homolog of the pink-eyed dilute (*P*) locus of the mouse (317,318,320–325). The human OCA2 gene is quite large, consisting of 24 exons, with the translational initiation codon in exon 2, and encodes an 838 amino acid polypeptide with molecular mass of 92.8 kDa (318). OCA2 is a transmembrane protein containing 12 membrane-spanning regions (318,325), and it shares homology with several small molecule transport proteins of lower species. It has been suggested that OCA2/P might function as a transporter within melanosomes (318,326), but as yet there is no conclusive evidence of its function (327–331). Studies of OCA2/P localization and function indicate that the protein functions in the sorting of TYR and TYRP1, and in the mouse, *Oca2* mutations disrupt the trafficking of those enzymes to melanosomes, leading to the hypopigmented phenotype (332). It has also been suggested that, given the importance of organellar pH in the correct sorting of proteins (333,334), particularly those trafficking to melanosomes (66,67), that the function of the OCA2 protein may be to regulate the pH of sorting vesicles involved in intracellular trafficking and subcellular



FIGURE 145-6 Female, age 4 years, with OCA2.

localization of tyrosinase (68,250,318,335). There is no direct functional assay for the OCA2 protein, though distinction between pathogenic mutations and polymorphisms of OCA2 can be accomplished by transfection assay of *p*-deficient mouse melanocytes (336).

OCA2 is the most common type of OCA in individuals of African descent (316,337), and is the second most common type of OCA in EUR individuals (249). The most common OCA2 gene mutation is a 2.7-kb deletion that accounts for 60–90% of mutant alleles in individuals from southern and sub-Saharan African descent (311,316,323,337), and which also occurs in the Brandywine isolate of Maryland as a founder mutation (240). Mutations in OCA2 also are the cause of so-called “brown” oculocutaneous albinism (BOCA) (338). A large number of OCA2 mutations have been identified in patients from other populations, including many missense mutations, as well as frameshift, nonsense, and splice junction mutations (249,252,253,269,315,323,338–343). As for OCA1, a small number of patients with AROA have mutations in the OCA2 gene (270,323), suggesting that these patients likely have substantial residual function. Other OCA2 variants act as the major determinant in blue eye color (68,344–348) and as a major risk factor for malignant melanoma (349).

The discovery of the OCA2 gene also depended on its involvement in hypopigmentation observed in many patients with the Prader–Willi (PWS) and Angelman (AS) syndromes (317,318,350–354). PWS is a developmental syndrome that includes neonatal hypotonia, hyperphagia and obesity, hypogonadism, small hands and feet and mental retardation



associated with characteristic behavior (355–359). Approximately 70% of PWS patients have an interstitial deletion on the long arm of the paternally-derived chromosome 15, and most PWS patients without a deletion of the chromosome 15q(pat) have uniparental disomy for the maternal chromosome 15. Many PWS patients with 15q deletions have clinically apparent hypopigmentation (350,353,354,360,361); moreover, about 1% of PWS patients have concomitant OCA2 (318,322).

Similarly, AS is a complex developmental disorder that includes developmental delay and severe mental retardation, microcephaly, neonatal hypotonia, ataxic movements, and inappropriate laughter (362–364). Most cases are the result of interstitial deletion of chromosome 15q11–q13. Hypopigmentation occurs in more than 50% of AS patients (352), correlating with the presence of a deletion of 15q(mat) (365,366), and again about 1% of AS patients have concomitant OCA2. Together, these findings suggested that PWS and AS patients with concomitant OCA2 might be hemizygous for OCA2 mutant alleles on the intact chromosome, or possibly might have uniparental isodisomy for the mutant allele. This hypothesis has been confirmed by the demonstration of hemizygous OCA2 mutations in patients with PWS (318,322) and AS (367,368).

**145.3.2.1.3 OCA3.** Oculocutaneous albinism type 3 (OCA3; OMIM 203290) results from mutations in *TYRP1*, located at chromosome 9p23 (369–371), and which encodes a 537 amino acid TYRP1 polypeptide with molecular weight of approximately 60 kDa (372,373). Human OCA3 thus corresponds to the brown (*b*) phenotype of the mouse (374,375), which results from mutations of *Tyrp1* and is associated with a brown rather than a black coat (4). It seemed likely that a human OCA phenotype would be associated with mutations of human *TYRP1*, and an African-American child with generalized hypopigmentation was identified with a homozygous *TYRP1* frameshift mutation (376). The boy, one of dizygotic twins, had light brown skin and hair and blue-gray irides at birth, whereas his twin had darker skin and hair consistent with normal pigmentation for an African-American newborn. Melanocytes cultured from the affected twin contained no TYRP1 protein or *TYRP1* mRNA, and DHICA oxidase activity was greatly reduced. These cells produced reduced amounts of insoluble melanin and appeared brown rather than the black color found with normally pigmented African-American control melanocyte cultures.

Genetic linkage studies subsequently showed that OCA3 corresponds to “rufous” or “red OCA” (OMIM 278400) which mapped to the *TYRP1* locus on chromosome 9p in the South African population. The same *TYRP1* frameshift mutation (1104delA) observed in the original African-American patient was found to account for 50% of the mutant alleles in the study population (377). The phenotype of OCA3 in South African individuals includes red or reddish brown skin, ginger or

reddish hair, and hazel or brown irides (377–379). All of the ocular features of albinism are not always present, however, as many patients do not have iris translucency, nystagmus, strabismus, or foveal hypoplasia. Individuals who have OCA and also have red hair and reddish brown pigmented skin have been reported in Africa and in New Guinea (301,377,378,380,381), though it should be noted that some individuals with OCA1 or OCA2 also have red hair, and they should not be confused with individuals with OCA3.

The number of different *TYRP1* mutations identified in patients with OCA3 remains relatively small. Because *TYRP1* plays a role in the distal pigment eumelanin pathway, and loss of DHICA oxidase enzymatic activity does not lead to a loss but only a change in the amount or biochemical character of eumelanin (and in mouse a change in coat color), it is unsurprising that the phenotype of OCA3 is generally less clinically severe than that of OCA1, OCA2, or OCA4 (see below).

Murine *Tyrp1* is thought to encode the DHICA oxidase function of the eumelanin biosynthetic pathway (78), although human *TYRP1* does not seem to have this same catalytic function (80). DHICA is a relatively stable intermediate and, in the acidic milieu of the melanosome, its half-life is quite long. Melanogenic enzymes are present in a complex within the melanosome, sometimes termed a “metabolon” (382–385). *In vivo*, the most important role of murine and human *TYRP1* in pigment production may be its ability to stabilize tyrosinase and to act as a chaperone escort for tyrosinase trafficking to melanosomes (386). *Tyrp1* mutations significantly decrease the half-life and catalytic function of tyrosinase, which may be the major reason that *Tyrp1* and *TYRP1* mutations have dramatic effects on the pigmentation phenotype. *TYRP1* physically associates with tyrosinase during their post-translational processing, and *TYRP1* missense substitutions in the recognition of both proteins as abnormal, which leads to their retention in the ER and degradation by proteasomes (63,65). Nevertheless, in the presence of mutant *TYRP1* protein, some tyrosinase does escape the ER and is sorted to melanosomes; hence, the phenotype of OCA3 is less severe than that of OCA1. For recent reviews, see References (23,68,69).

The *TYRP1* gene consists of eight exons, with the translational start codon residing in exon 2 (101,103). *TYRP1* is a member of the tyrosinase gene family that also includes *TYR* and *DCT*. Sequences in the first exon appear to be important for the efficiency of *TYRP1* gene expression, but are not sufficient to confer pigment cell-specific expression (387). The minimum promoter of the mouse *Tyrp1* gene is between –44 and –107 bp from the transcriptional start site, located 20 bp downstream of the TATAAA box (289). Within this region, several cis-regulatory sequences, along with several regulatory factors that bind to these sequences, have been identified. As for the *TYR* promoter region,



the M-box motif appears to be a major pigment gene-specific regulatory element (388). In the mouse, MITF binds to the *Tyrp1* M-box, activating *Tyrp1* transcription, with a consequent increase in the levels of cAMP. As for the *TYR* locus, the M-box cannot be the only element to confer melanocyte-specific transcription of *TYRP1*.

Another protein, melanocyte-specific factor (MSF), may play the role of a melanocyte specific trans-activating factor, acting both as a positive-acting transcription factor and as an anti-repressor (288). MSF appears to bind to two negative regulatory elements in the *Tyrp1* promoter, one at -237, called MSEu, and the other located next to the initiation site, called MSEi (288). Both of these sites contain a 6-bp motif, GTGTGA, and bind the brachyury-related transcription factor (Tbx2), a repressor of *Tyrp1* transcription. Binding of MSF to these two elements competes with the binding of Tbx2, allowing MSF to act both as an anti-repressor and promoter of *Tyrp1* transcription (389). A complex model of *Tyrp1* transcriptional regulation includes MITF binding to the M-box, oct binding to the octamer motif, and other transcription factors binding to the TATA element (288). In this model, the presence of MSF promotes *Tyrp1* transcription. In the absence of MSF, Tbx2 binds both the MSEi and MSEu regulatory sequences, repressing *Tyrp1* transcription. This repression has been demonstrated in melanoma cells and in melanocytes. If MSF and Tbx2 also interact with the promoters of other pigment-specific genes, modulation of MSF and Tbx2 expression may be a means by which the melanocyte controls melanin pigment biosynthesis. *TYRP1* is only expressed in cells containing eumelanin, whereas *TYR* is expressed in cells containing either pheomelanin and/or eumelanin, indicating that *TYR* and *TYRP1* can also be differentially expressed, adding an additional layer of complexity to the regulation of the expression of pigment genes (113).

**145.3.2.1.4 OCA4.** Oculocutaneous albinism type 4 (OCA4; OMIM 606574) results from mutations of *SLC45A2*, the human homolog of the mouse underwhite (*uw*) gene (390). Underwhite-mutant mice manifest reduced pigmentation, so *uw* was considered a candidate gene for human OCA. After the murine *uw* gene was identified as *Slc45a2*, sequencing of the human *SLC45A2* gene in patients with OCA with no mutations in *TYR*, *OCA2*, or *TYRP1* identified a Turkish patient homozygous for a G-to-A transition in exon 2 (c.386-1 GA), resulting in a splice site mutation. A number of additional *SLC45A2* gene mutations were subsequently identified in other patients with OCA (249,270,391,392). OCA4 is the third most frequent type of OCA in the EUR population (249), and is the second most common type of OCA in Japanese (391,393), the apparent result of a founder mutation in the Japanese and Korean populations (393).

The phenotype of OCA4 is similar to that of OCA2. Individuals are often born with some hair pigment, but

this can be minimal (390-392). Hair color is white to white/yellow to blond to brown. Those with darker hair can have lightly pigmented skin that develops some tan with sun exposure. Typical ocular features of albinism are present.

The *SLC45A2* gene product, termed membrane-associated transporter protein (MATP), consists of 530 amino acids with a molecular weight of 58.3 kDa. The *SLC45A2* gene is located at chromosome 5p13.3, and consists of seven exons spanning approximately 40 kb, with two alternatively spliced transcript variants encoding different protein isoforms (394). The function of MATP is unknown, but in mouse *uw*-mutant melanocytes, tyrosinase processing and intracellular trafficking to the melanosome are disrupted and the enzyme is abnormally secreted from the cells in immature melanosomes, disrupting the normal maturation process of those organelles (395).

*SLC45A2* polymorphisms have also been associated with normal variation in human pigmentation (396). Two variant alleles, 272K and 374L, are associated with dark hair, skin, and eye color in the EUR population, and the 374L variant allele is a population marker for EUR (397).

**145.3.2.2 X-Linked Recessive Ocular Albinism.** X-linked recessive ocular albinism (OA1; Nettleship-Falls ocular albinism; OMIM 300500; Table 145-1) is an X-linked recessive disorder in which affected males manifest many of the ocular features of albinism, but in which skin and hair pigmentation is generally normal (398,399). The irides are blue to brown, and optic changes of albinism include nystagmus, reduced retinal pigment with foveal hypoplasia, altered crossing of the optic nerves at the chiasm, and variable iris transillumination (268,400-405). In African-American and Japanese males, iris color is often brown and there is little iris translucency (268,405). In EUR patients, the skin typically appears normally pigmented, whereas in African-American patients with darker skin there may be scattered hypopigmented macules; these are rarely seen in the skin of EUR individuals. The melanocytes in the skin, hair follicles, and RPE are normal in size, shape, and number, but melanocytes in the skin, hair follicles, iris and retina contain giant melanosomes (called macromelanosomes), along with some normal melanosomes (268,403,406). The systemic nature of the melanosome defect in OA1 suggests that this is really a type of OCA in which the major manifestations are in the eye.

Heterozygous females exhibit ocular pigmentation changes that result from X-inactivation (401,407-410). A variegated or spotty pattern of retinal pigmentation (sometimes described as a mud-splattered mosaic pattern), which progressively becomes more coarse, and punctate areas of iris translucency are seen in 80-90% of obligate heterozygous females (401,410). Macromelanosomes are seen in the melanocytes of obligate

heterozygous females. A few heterozygous females have more evident ocular changes of albinism, including nystagmus and reduced visual acuity, and these are thought to be the result of non-random X-inactivation (411).

The gene for OA1 was initially localized to the short arm of the X chromosome in linkage with the Xg blood group, and was subsequently localized to Xp22.32 (412,413) and then more precisely to Xp22.3–Xp22.2, between the markers DXS237 and DXS143 (414). The OA1 gene was subsequently identified as *GPR143* (415,416), encoding G protein-coupled receptor 143. The coding sequence consists of nine exons and encodes a 404 amino acid protein that has several possible transmembrane domains, possibly with multiple isoforms (417). The mouse *Gpr143* gene encodes a protein with a high degree of similarity to the human protein, including the presence of isoforms (418).

OA1 mutations consist of missense, frameshift, and splicing mutations, as well as a number of different deletions involving all or part of the *GPR143* locus (419–428). Many of these deletions involve exon 2, most likely caused by unequal crossing-over between flanking *Alu* repeats (two *Alu* repeats in intron 1 and one *Alu* repeat in intron 2) (426,429). Other exons are also involved in deletions (419,427), and the frequency of large intragenic deletions is higher in Northern American than in European cases that have been studied (419). The missense mutations are located throughout the central coding region with some clustering between the first and second transmembrane regions in exons 1, 2, and 3. Some individuals with missense mutations also have other features, including X-linked ichthyosis and developmental delay (427).

The GPR143 protein is thought to be associated with the melanosome membrane in the melanocyte (417). GPR143 is a G protein-coupled receptor, representing a novel member of the GPCR superfamily, and has specific interactions with heterotrimeric G proteins (430). OA1 may therefore be caused by a defect in an intracellular signal transduction system. The gene is expressed at high levels in the retinal pigment epithelium and at lower levels in the brain and adrenal glands. Part of the pathogenesis of OA1 is the formation of giant macromelanosomes in melanocytes. Analysis of *Gpr143*-knockout mice suggests that the macromelanosomes are produced by the abnormal growth of individual melanosomes, instead of fusion of several smaller melanosomes (431). The GPR143 protein may be involved in sorting or trafficking the vesicles to developing melanosomes (432,433), with a role in reorganization of the late endosomal compartment, including enlargement of the late endosomes and a redistribution of the mannose 6-phosphate receptors as important steps in melanosome biogenesis (433). GPR143 may function as a stop signal for melanosome growth, its absence resulting in macromelanosome formation. In some respects, OA1 may thus be considered

a defect of melanosome biogenesis/maturation (see below).

One large Afrikaner family has been reported with X-linked OA co-segregating with late-onset sensorineural deafness (434). Macromelanosomes were demonstrated in skin from affected males and obligate heterozygous females, and the clinical features were consistent with OA1. The locus for this condition maps to chromosome Xp22.3, suggesting overlap with OA1 (434,435), and it is unclear whether this OA-deafness phenotype is part of the OA1 spectrum or is related to a contiguous gene defect in this family. A family that contained three generations of males and females affected with congenital deafness and OA has been described, but little information on this family is available (436).

**145.3.2.3 Autosomal Dominant OCA.** “Autosomal dominant OCA” has been described in several families (437,438). One French family had hypopigmentation in three generations, including one family member with hypopigmentation and PWS (438), suggestive of OCA2. Indeed, all families with multigenerational OCA that have been subjected to molecular analysis have proven to be examples of pseudodominance, with mutations of *TYR* or *OCA2* involving affected individuals in sequential generations.

#### 145.3.2.4 Other OCA Candidate Genes.

**145.3.2.4.1 PMEL.** In mice, *Pmel* corresponds to the silver (*si*) locus, and in humans is located at chromosome 12pter–q21 (439). The *PMEL* gene encodes a 668 amino acid protein (known as Pmel17, GP100, and silver) with molecular weight of 70.9 kDa including the putative signal peptide. The protein has several potential glycosylation sites and a hydrophobic region at the carboxyl-terminal end, indicating that it may be membrane bound. Pmel17 protein was initially characterized as “stablin” (440), which was shown by transfection experiments to be associated with “indole-blocking factor” or “stablin” activity, which binds DHICA and DHI (440), associated with polymerization of melanogenic intermediates (441,442). Pmel17 is a relatively abundant matrix component of both melanosomes and premelanosomes (36,61,443), with extensive sequence similarity to a chicken melanosomal matrix protein (444,445). The Pmel17 protein may function as a component of the melanosomal matrix, limiting melanogenesis to that intracellular compartment and perhaps protecting melanocytes from harmful intermediates of melanin biosynthesis (79). In that respect, Pmel17 functions as a solid-state surface for melanin binding and polymerization, fulfilling the polymerase function ascribed to it (23,442,446,447).

In the mouse, *si* mutations are characterized by premature graying (“silvering”) of coat hair due to melanocyte loss (79). *Si*-mutant melanocytes grown in culture produce pigment but have a much slower growth rate than normal melanocytes (448).

The Pmel17 protein has also been identified as a melanoma-specific antigen. The melanoma-specific antibody, HMB50, recognizes a glycoprotein released by neonatal foreskin melanocytes and by melanoma cells but not by adult melanocytes, independent of the amount of pigment produced by the cells (449). It was hypothesized that the protein related to the proliferative state of the cells, with only growing cells producing the molecule, thus correlating with *si* mutations that affect melanocyte viability and cause premature graying. It was subsequently shown that the protein recognized by HMB50 is Pmel17 (450,451). Human Pmel17 was also isolated as a melanocyte lineage-specific antigen (gp100) (452) recognized as a melanoma antigen by a cultured T-cell line established from the tumor-infiltrating lymphocytes (TILs) of a patient with advanced metastatic melanoma (453). It has been suggested that Pmel17/gp100 may be a tumor-rejection antigen that could be useful for the development of immunotherapy for melanoma (453).

*PMEL* has been screened as a candidate gene in OCA and AROA patients who lack mutations in *TYR*, *OCA2*, *TYRP1*, and *SLC45A2* (249,270). However, thus far no apparently pathological *PMEL* mutations have been identified.

**145.3.2.4.2 DCT.** The *DCT* gene, which encodes DOPachrome tautomerase, is located at chromosome 13q32.1. *DCT* mutations have not yet been associated with pigmentary disease in humans; however, given the relatively minor effects on pigmentation of *Dct* mutations in mice and its specific function in the melanogenic pathway, it might be expected to be involved with a very mild form of OCA. DOPachrome tautomerase converts DOPachrome to its carboxylated derivative DHICA; in the absence of this enzyme, DOPachrome will be spontaneously decarboxylated to produce DHI, which dramatically affects the properties of the melanosomes produced. Although the metal-binding domains found in tyrosinase are conserved in *DCT*, copper is not the bound ligand, and zinc fulfills the role as the active metal ligand, consistent with the distinctly different reactions catalyzed by tyrosinase and *DCT* (59). The size of *DCT* (about 75–80 kDa) is slightly larger than tyrosinase or *TYRP1*. Expression of *DCT* during development occurs earlier than does expression of *TYR* and *TYRP1*; the implications of this are not known, but have been suggested to perhaps relate to its role in minimizing the cytotoxicity of melanogenic intermediates. The production of DHICA rather than DHI may have cytotoxic implications to melanocytes (454); DHI is relatively cytotoxic *in vivo* because it is quickly metabolized to indole-5,6-quinone and then to melanin. The production of DHICA rather than DHI may be of primary importance in minimizing the cytotoxic effects of melanogenic intermediates, rather than the nature and color of melanin per se. For recent reviews, see References (23,68,69).

## 145.4 DISORDERS OF MELANOSOME BIOGENESIS/TRANSPORT—HERMANSKY-PUDLAK SYNDROME, CHÉDIAK-HIGASHI SYNDROME, AND GRISCELLI SYNDROME

### 145.4.1 Hermansky–Pudlak Syndrome (HPS)

Hermansky–Pudlak syndrome (HPS; (455–457)) is a group of rare autosomal recessive genetic disorders characterized by OCA of variable severity (221,458–460), a bleeding diathesis, and a poorly defined ceroid-lipofuscin lysosomal storage disease (Figure 145-7). Most patients develop life-threatening complications during adulthood, including progressive pulmonary fibrosis, granulomatous colitis, and occasionally cardiomyopathy and renal failure (456,457,459,461–463). Unlike in CHS and Griscelli syndrome, discussed below, there is no immune deficit in HPS (464). There is currently no specific or effective treatment for HPS, and average survival is only 30–50 years, death usually resulting from restrictive lung disease (68%), hemorrhage (17%), or colitis (15%). HPS is rare in most populations, but is relatively common in Puerto Rico, where the prevalence has been estimated at approximately 1 in 1800 (456,465).

In HPS, skin hypopigmentation is associated with quantitatively reduced and qualitatively abnormal melanosomes in skin melanocytes (466,467). Bleeding is usually clinically mild, and is associated with virtual absence of platelet dense granules, “storage pool deficiency,” and defective platelet aggregation (456,468–470). Lysosomal



**FIGURE 145-7** A Puerto Rican patient with HPS1.



storage in HPS is characterized by accumulation of ceroid-lipofuscin-like material in the lysosomes of reticuloendothelial cells, bone marrow, lung macrophages, and many other cell types (456,463,471–474), suggesting lysosomal dysfunction. However, HPS patients exhibit none of the other typical findings of lysosomal storage disease.

In the mouse, there are 15 loci that, when mutated, result in phenotypes similar to that of human HPS (see Table 145-1), including hypopigmentation due to a reduction of melanosomes; platelets with an absence of dense bodies, which results in a prolonged bleeding time; and ceroid deposition in a number of organ tissues (475–477). Most of the protein products of these loci appear to be involved in protein sorting and trafficking, resulting in abnormal biogenesis of lysosome-related organelles, including melanosomes, platelet dense bodies, and type II pneumocytes (460,478–481).

The identification of these mouse loci with HPS-like phenotypes has facilitated the identification of human genes associated with other types of HPS, although the first HPS gene was identified in humans by use of labor-intensive positional cloning techniques (482). As these HPS genes have been identified, a common theme has emerged; all of the corresponding proteins are involved in the formation of lysosomes and other related organelles, including melanosomes (47). Furthermore, all of these HPS genes are ubiquitously expressed in humans and in mice. Three genes—*AP3B1* (encoding the  $\beta$  subunit of adaptor protein 3 complex; APC-3); *AP3D1* (encoding the  $\delta$  subunit of APC-3); and *VPS33A* (encoding vacuolar protein sorting protein 33a; VPS33A) have orthologs in yeast. APC-3 is a protein complex involved in protein trafficking of integral membrane proteins from the trans-Golgi network or late endosomes (460). VPS33A is involved in vacuolar protein sorting (483). In contrast, the other HPS proteins do not have orthologs in yeast and have no significant homology to other proteins (50,479). These other HPS proteins form protein complexes termed biogenesis of lysosome-related organelles complexes (BLOCs). There are at least three such BLOC complexes, BLOC-1, BLOC-2, and BLOC-3, ranging in size from from 175 kDa (BLOC-3) to 340 kDa (BLOC-2) (50,479,484,485). These complexes contain different numbers of proteins, with BLOC-1 containing at least eight protein subunits (50). The exact functions of the BLOCs remain unknown, but they most likely are involved in the fusion events involving endosomal membranes and organelle biogenesis. In the mouse, mutations involving protein subunits of BLOC-1 have more severe phenotypes than BLOC-2 or BLOC-3, suggesting that BLOC-1 may be involved in early events in melanosome formation. However, this is less evident in humans. There are reports of other BLOCs (BLOC-4 and BLOC-5), but these may be alternatives to BLOCs 1–3.

There are currently nine human HPS genes known, all of which are homologous to these mouse HPS-like loci. *HPS1* is the human homolog of mouse pale ear (*ep*) (482,484,486–489). *AP3B1* (HPS2) is the human homolog of mouse pearl (*pe*) (490–492). *HPS3* is the human homolog of mouse cocoa (*coa*) (493–495). *HPS4* is the human homolog of mouse light ear (*le*) (484,496). *HPS5* is the human homolog of mouse ruby-eye 2 (*ru2*) (497). *HPS6* is the human homolog of mouse ruby-eye (*ru*). *DTNBP1* (HPS7) is the human homolog of mouse sandy (*sd*) (498). *BLOC1S3* (HPS8) is the human homolog of mouse reduced pigmentation (*rp*) (499,500). *PLDN* (HPS9) is the human homolog of mouse pallid (*pa*) (501).

**145.4.1.1 HPS1.** HPS is generally rare. However, two isolated populations in which Hermansky–Pudlak syndrome type 1 (HPS1; OMIM 203300) is frequent—one in Puerto Rico (Figure 145-7) and the other in Switzerland (502)—provided an opportunity to map the first HPS gene, *HPS1* (OMIM 604982) to chromosome 10q24.2 (489,502,503), on the assumption of autozygosity based on a hypothesized common founder for the mutated gene in these two population isolates. *HPS1* was mapped a chromosome region syntenic to that containing the pale ear (*ep*) and ruby-eye (*ru*) loci of the mouse, two of the candidate HPS models. Further fine mapping, subsequent isolation of the *HPS1* gene, and DNA sequencing led to identification of pathological gene mutations (482). Furthermore, *HPS1* was found to be orthologous to the mouse pale-ear (*ep*) (482,487,504) gene. The human *HPS1* gene consists of 20 exons spanning 30.5 kb (486). A partial *HPS1* pseudogene comprised of exon 6 is located on chromosome 22q12.2–12.3 (505). The major 3.6 kb *HPS1* mRNA encodes a 700 amino acid, 79.3 kDa protein (482,486). A minor 1.5 kb mRNA encoding a 324-amino acid protein results from alternative splicing (488). The 3.6 kb mRNA encodes an HPS1 polypeptide containing two transmembrane domains, whereas the HPS1 protein encoded by the 1.5 kb mRNA lacks the carboxyl transmembrane domain. Confocal immunofluorescence analysis shows both cytoplasmic and membrane-associated distributions of HPS1 protein (485,506), suggesting the possible existence of soluble and insoluble isoforms, as would be predicted by the two alternative HPS1 polypeptides (476). Primer extension analysis identified a single major transcription start site and two minor sites (486). There are two TATA boxes, with the proximal site most likely being the active one. Several other putative transcriptional motifs occur in this area, but none are melanocyte-specific (486).

A founder 16-bp duplication frameshift mutation occurs in Puerto Rican HPS1 patients, and a 1-bp duplication frameshift was found in the Swiss HPS1 patients (482). Many other *HPS1* mutations have been identified, most of which constitute protein-null alleles (458,475,482,507–511). Among the most



important *HPS1* mutations is a splice consensus mutation (IVS5+5 GA) (507) that is common in Japan and appears to derive from an ancestral founder (510,511).

Several tissues are affected by *HPS1* mutations, and *HPS1* mRNA is expressed in a number of different cell types (482), including melanocytes, megakaryocytes (progenitors of platelets), and several other tissues, including kidney, liver, and lung, the sites of ceroid accumulation in patients with HPS. The HPS1 protein is a component of the BLOC-3 protein complex which is important for protein trafficking, although the precise mechanism is not yet defined (484,485).

**145.4.1.2 HPS2.** Two individuals of Dutch origin who showed clinically more severe symptoms than typical HPS1, including the usual features of OCA and bleeding together with additional features of neutropenia and immune deficiency, were identified who did not have a detectable mutation in HPS1. It was therefore hypothesized that these individuals had a mutation in another locus. Mutations in the human ortholog of the mouse pearl (*pe*) gene (491), *AP3B1*, located at chromosome 5q14.1, encoding the  $\beta$ 3A subunit of adaptor complex AP-3, were found to be responsible for HPS2 (OMIM 608233) in these individuals (491,512), and additional cases were identified subsequently (513,514). Fibroblasts from these patients had normal messenger RNA (mRNA) levels for all four AP-3 subunits,  $\delta$ ,  $\beta$ 3A, 3A, and  $\alpha$ 3, but had lower protein levels when compared to normal fibroblasts or individuals with mutations in *HPS1*. The clinical severity of the HPS2 phenotype roughly correlates with the mutations that are present (515).

AP-3 is critical for protein trafficking (492,516,517), including targeting tyrosinase to melanosomes (518), and functions with AP-1, BLOC-2, RAB38, and RAB32 to mediate protein trafficking to lysosome-related organelles (519).

**145.4.1.3 HPS3.** HPS3 (OMIM 614072) results from mutations in *HPS3* (493), the human ortholog of the mouse cocoa (*coa*) (495) gene, located at chromosome 3q24. HPS3 was initially described in a Puerto Rican isolate (493) and subsequently has been identified in individuals from a variety of other populations (520). A founder mutation (3904-bp deletion of exon 1 and adjacent intron) was identified in the Puerto Rican isolate (465,493), and has subsequently been identified in patients from other groups (520). The HPS3 phenotype is somewhat less severe than that of HPS1, with less hypopigmentation, less severe ocular features, and less bleeding, and may not include the complication of pulmonary fibrosis (520,521). It is of interest that prevalence of two different forms of a disorder as rare as HPS occurs in Puerto Rico, suggestive of possible heterozygote advantage, though this has not yet been proven. *HPS3* encodes a component of the BLOC-2 complex (493–495).

**145.4.1.4 HPS4.** HPS4 (OMIM 614073) results from mutations in *HPS4*, the human ortholog of the mouse

light ear (*le*) (496) gene, located on chromosome 22q12.1. HPS4 is perhaps the second most common form of HPS in EUR individuals (496,522,523). HPS4 is clinically similar to HPS1, with an OCA phenotype generally similar to that of OCA1.

The *HPS4* gene encodes a 708 amino acid, 76.9 kDa polypeptide. It is a component of the BLOC-3 complex (484,485,524) and interacts with RAB9 (525).

**145.4.1.5 HPS5.** HPS5 (OMIM 614074) results from mutations in the *HPS5* gene, which consists of 23 exons located at chromosome 11p15.1. The initial patient, born to consanguineous Turkish parents, was homozygous for a 4-bp frameshift deletion (497). *HPS5* is the human ortholog of the mouse ruby-eye-2 (*ru2*) (497) and *Drosophila* pink (*p*) (526,527) genes, and encodes a member of the BLOC-2 complex (497). The 1129 amino acid HPS5 polypeptide contains a number of predicted phosphorylation sites for protein kinases A and C and casein kinase II, and a predicted myristoylation site, and binds to  $\alpha$ -integrin chains.

**145.4.1.6 HPS6.** HPS6 (OMIM 614075) results from mutations in the *HPS6* gene, which consists of a single exon located at chromosome 10q24.32, and is the human orthologue of the mouse ruby-eye (*ru*) (497) gene. A number of gene mutations have now been identified in different patients with HPS6 (497,528,529). The 775 amino acid HPS6 protein is a component of the BLOC-2 complex (497).

**145.4.1.7 HPS7.** HPS7 (OMIM 614076) results from mutations in *DTNBP1*, located at chromosome 6p22.3, and is the human ortholog of the mouse sandy (*sdv*) (498) gene. *DTNBP1* has also been genetically associated with susceptibility to schizophrenia (530), although findings have been inconsistent (531,532). The original HPS7 patient, an adult female born of consanguineous parents, was homozygous for a nonsense mutation, and had typical HPS with no psychiatric symptoms. The 352 amino acid, 40 kDa gene product is named dystrobrevin-binding protein 1 (also dysbindin) (533) and is a component of the BLOC-1 complex (498).

**145.4.1.8 HPS8.** HPS8 (OMIM 614077) results from mutation in *BLOC1S3*, located at chromosome 19q13.32 (499) and is the human ortholog of the mouse reduced pigmentation (*rp*) (500) gene. Homozygous mutations were identified in members of a large, inbred Pakistani family with OCA but inconsistent history of bleeding diathesis (499). *BLOC1S3* is a component of the BLOC-1 complex (534).

**145.4.1.9 HPS9.** HPS9 (OMIM 614171) results from mutations in *PLDN*, located at chromosome 15q21.1 (501), and is the human ortholog of the mouse pallid (*pa*) gene. An Indian child born of consanguineous parents was homozygous for a nonsense mutation. *PLDN* encodes pallidin, a 172-amino acid protein that is a component of the BLOC-1 complex (535,536). *PLDN* consists of seven exons, subject to alternative mRNA

splicing that results in two somewhat different protein isoforms, PLDN-1 and PLDN-2 (501).

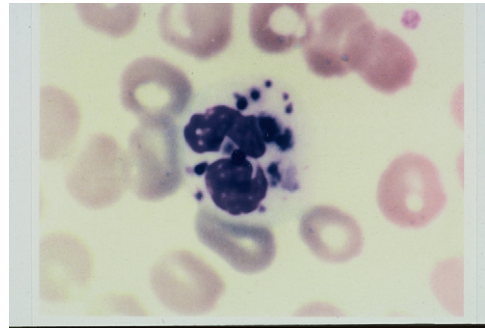
### 145.4.2 Chédiak–Higashi Syndrome

Chédiak–Higashi syndrome (CHS; OMIM 214500) is a rare autosomal recessive disorder that consists of OCA, immune defects with neutropenia and susceptibility to recurrent bacterial infections, mild bleeding diathesis, and progressive neurological abnormalities; for reviews, see (537,538). Skin, hair, and eye pigment is reduced or diluted in CHS, but some affected individuals do not exhibit obvious albinism and the hypopigmentation may only be noted when compared to other family members. Hair color is light brown to blond, with a metallic silver-gray sheen. Iris pigment is reduced, and nystagmus and photophobia may be present or absent (539). Most patients with CHS eventually develop an “accelerated” lymphoproliferative phase, with generalized lymphohistiocytic infiltrates with erythrophagocytosis (540), fever, jaundice, hepatosplenomegaly, lymphadenopathy, pancytopenia and bleeding. There is no specific treatment for CHS, and management of the accelerated phase is quite difficult. Chemotherapy provides only transient benefit, and most patients die prior to seven years of age unless given bone marrow transplantation (541–543), though this does not correct the hypopigmentation and does not prevent eventual neurological deterioration (542).

About 5–15% of CHS patients have a milder clinical course, with less severe hypopigmentation, fewer significant infections, and no accelerated phase. Most of these patients eventually develop neurological manifestations, including seizures, modest progressive intellectual decline, and progressive peripheral neuropathy, with tremor, muscle weakness, clumsiness and wide-based gait (537,544,545).

The ultrastructural hallmark of CHS is the presence of large eosinophilic peroxidase-positive inclusion bodies in granulocytes and other tissues (546). The diagnosis of CHS is thus readily established on clinical grounds together with examination of leukocytes in a standard peripheral blood smear (Figure 145-8), and indeed sometimes is diagnosed incidentally on this basis.

The gene for CHS was localized to chromosome 1q42.3 by homozygosity mapping (547,548). At the same time, the gene (named *Lyst*) for a similar murine phenotype, beige (*bg*), was identified and the homologous human *LYST* (also called *CHS1*) gene was mapped to chromosome 1q43 using mouse *Lyst* as a hybridization probe (549,550). The human *LYST* gene was subsequently cloned and sequenced, and mutations were identified in individuals with CHS (551–553). A large number of *LYST* mutations have now been identified (554–556), and there is a clear genotype–phenotype correlation, with patients with typical, severe CHS being homozygous for protein-null alleles and patients with milder forms of CHS being compound heterozygotes or



**FIGURE 145-8** Prominent cytoplasmic inclusions in a polymorphonuclear leukocyte in a patient with Chediak–Higashi syndrome.

homozygotes for alleles containing missense mutations (555).

The human *LYST* gene expresses two transcripts, a major 13-kb mRNA that encodes the major 3801 amino acid, 429kDa *LYST* protein and a minor 6-kb mRNA that encodes a 1990 amino acid polypeptide (551,552,557). *LYST* mRNA is ubiquitously expressed in all cells and tissues tested in both in humans and in mice, and alternative splicing produces transcripts ranging from 3–4kb to 12–14kb in size (551,552). The major *LYST* protein contains a stathmin-like sequence as well as several HEAT, ARM, and WD40 repeats (551,552). The HEAT and WD40 repeats are associated with proteins involved with vesicle trafficking and protein transport (558). *LYST* also contains a so-called BEACH (Beige and CHs) domain (552). Immunohistochemistry analysis shows that the *LYST* protein is cytosolic, with no apparent membrane association (550,559). The specific function of the *LYST* protein is not known, but it is thought to be a lysosomal trafficking regulator (*LYST*), perhaps accounting for the abnormal trafficking of melanogenic proteins found in melanocytes from individuals with CHS (551,556,560,561).

### 145.4.3 Griscelli Syndrome

Griscelli syndrome (GS) is associated with pigment dilution of the skin and the hair, with large clumps of pigment in hair shafts and accumulation of melanosomes in melanocytes (562). Most patients also develop an uncontrolled T-lymphocyte and macrophage activation syndrome (known as hemophagocytic syndrome), leading to death in the absence of bone-marrow transplantation (562).

Griscelli and colleagues initially reported two girls with hypopigmentation characterized as silver-gray hair and scattered hypopigmented areas surrounded by normal or hyperpigmented skin (563). No ocular features of albinism were present, even though the term partial albinism was used in the title of the paper. Both had neutropenia, thrombocytopenia, absent delayed hypersensitivity and hypogammaglobulinemia with repeated infections, and one died with sepsis. Parents and sibs were normal, and inheritance appeared to be autosomal

recessive. Melanin was irregularly distributed in the skin. Melanocyte number and size were normal. Melanocytes were full of normal-sized pigmented melanosomes, but only a few were passed to keratinocytes. Hair shafts contained large clumps of pigment, primarily in the medullar zone, interspersed with normal-sized pigment granules, and these produced the silver-gray sheen.

There are now three types of GS (for review, see Reference (44) and see Table 145-1), all of which include hypopigmentation (“hypomelanosis”) of the skin and hair, with large clumps of pigment granules in the hair shafts and an accumulation of melanosomes in cutaneous melanocytes, due to defects of melanosome transport and inability to distribute melanosomes to keratinocytes in the hair shafts and skin (564). Clinical manifestations other than melanosome clumping and hypopigmentation include a primary neurological deficit (GS1), immune impairment (GS2), or solely hypopigmentation of the skin and hair (GS3). The two original GS patients are now classified as having GS2.

**145.4.3.1 GS1.** Griscelli syndrome type 1 (GS1; OMIM 214450) results from mutations in *MYO5A* (565); reviewed in References (44,45,564). *MYO5A* consists of 35 exons located on chromosome 15q21.2. *MYO5A* is the human ortholog of the mouse dilute (*d*) gene. GS1 is characterized by hypopigmentation and a primary neurological abnormality but does not include immunological deficiencies or hemophagocytic syndrome manifestations (566). Neurological impairment can be marked early in life, with hypotonia, seizures, motor delay, and mental retardation (565,567–571). Similar cases have been described as Elejalde syndrome (OMIM 256710) (572–574). *MYO5A* encodes myosin 5A, a dimeric 160 kDa protein containing an actin-binding domain and an ATP-binding site, which is involved in short-range movement of melanosomes along actin filaments. Together with *RAB27A* and *MLPH*, *MYO5A* plays a role in moving melanosomes to the dendrites.

**145.4.3.2 GS2.** Griscelli syndrome type 2 (GS2; OMIM 607624) results from mutations in *RAB27A* (562,575–578); reviewed in References (44,45,564). *RAB27A* consists of six to nine exons, depending on alternatively mRNA splicing, located on chromosome 15q21.3. *RAB27A* is the human ortholog of the mouse ashen (*ash*) gene. GS2 is characterized clinically by hypopigmentation and an immune deficiency (562,575–578). Affected individuals develop a hemophagocytic syndrome similar to that in CHS, with secondary T-lymphocyte and macrophage activation that leads to lymphohistiocytic infiltration of the central nervous system (576,579), and most patients will die unless treated by bone marrow or stem cell transplantation (579,580). *RAB27A* is a GTPase and part of the Ras oncogene family. *RAB27A* acts as a *MYO5A* receptor and functions as part of a molecular motor complex required for the movement of melanosomes from the perinuclear area to the dendrites, which is

necessary for their eventual transfer to neighboring keratinocytes (334).

**145.4.3.3 GS3.** Griscelli syndrome type 3 (GS3; OMIM 609227) results from mutations in *MLPH* (581,582); reviewed in References (44,45,564). *MLPH* consists of 16 exons (15 coding exons) located on chromosome 2q37, and is the human orthologue of the mouse leaden (*ln*) gene. GS3 is characterized clinically by hypopigmentation without neurological or immunological problems (583). *MLPH* is also involved in melanosome movement, perhaps functioning to tether melanosomes to the myosin motor transport complex containing *MYO5A* (581).

## 145.4.4 Rare Conditions with Albinism and Hypopigmentation

**145.4.4.1 Generalized Cutaneous Hypopigmentation (Albinoidism).** The term albinoidism refers to generalized cutaneous hypopigmentation in the absence of ocular changes of albinism (OMIM 126070) (584). This is not a very precise term, and it is confusing because of its similarity to the term albinism, and probably has no place in the modern clinical lexicon; a more correct term would be generalized cutaneous hypopigmentation. Families with generalized cutaneous hypopigmentation have been described, and have the potential of representing new genetically distinct syndromes.

**145.4.4.2 Oculocerebral Syndrome with Hypopigmentation (Cross Syndrome).** Cross syndrome presents with generalized cutaneous hypopigmentation and neurological abnormalities that include developmental delay, mental retardation, spasticity, athetoid movements, muscle atrophy, growth retardation and other changes (OMIM 257800) (585–587). The features of this syndrome are variable, and all cases may not represent the same entity. The cutaneous hypopigmentation has been generalized but variable in degree, and the hair has been noted to have a white, silver, or silver-gray appearance (588,589). Some of the affected individuals have had ocular changes of albinism, suggesting a diagnosis of OCA1 or OCA2 rather than hypopigmentation limited to the hair and skin (590).

## 145.5 DISORDERS OF MELANOCYTE SURVIVAL—VITILIGO

Acquired loss of integumentary melanocytes is termed vitiligo, one of the most striking of all human disease phenotypes, and perhaps the most common pigmentary disorder. Vitiligo is characterized clinically by patchy loss of pigmentation of the skin and overlying hair, in most cases with tendency towards a somewhat symmetrical distribution (Figure 145-9). In some respects, vitiligo appears similar to piebaldism, and many patients with piebaldism are initially misdiagnosed as vitiligo. The marked contrast between patches of involved and uninvolved skin particularly impacts persons of color,



with social consequent stigmatization and negative impact on quality of life (591). Indeed, the first Prime Minister of India, Jawaharlal Nehru, ranked vitiligo with malaria and leprosy as the principal medical “curses” of his country.

There are two main types of vitiligo: generalized vitiligo (GV; includes non-segmental vitiligo, acrofacial vitiligo, vitiligo universalis; Figure 145-9) and segmental vitiligo (SV) (592–595). In GV, which represents approximately 90% of cases, depigmentation tends to be slowly progressive, waxing and waning but sometimes eventually involving virtually the entire integument. In SV, depigmentation tends to have rapid onset, but then stabilizes and remains quite localized. In both GV and SV, involved areas of skin generally lack melanocytes (596). It is now clear that GV is an autoimmune disease, and while SV has been much less studied, there is some recent evidence that it may also have an autoimmune basis that for some reason only acts locally.

### 145.5.1 Generalized Vitiligo (GV)

GV accounts for >90% of cases of vitiligo, is usually adult-onset ( $23.9 \pm 16.0$  years), is progressive and multifocal. In about 30% of cases, GV co-occurs with other autoimmune disorders, particularly autoimmune thyroid disease, type 1 diabetes, rheumatoid arthritis, pernicious anemia, psoriasis, Addison’s disease and lupus, both in GV patients themselves and in close family members (597,598), suggesting that these disorders share common genetic risk factors. There is extensive evidence that GV has an autoimmune pathogenesis (for review, see Reference (599)), including its co-occurrence with other autoimmune diseases, the frequent occurrence of new lesions at sites of skin injury (“Koebner phenomenon”), autoantibodies to melanocyte components in some patients (600), and perilesional infiltrates of cytotoxic T-lymphocytes (601).

The occurrence of frequent clustering of GV cases among close relatives (602,603) was initially interpreted as suggesting autosomal dominant inheritance (592). However, it is now clear that GV is in most cases a complex, polygenic, multifactorial disorder, involving numerous different susceptibility genes, most encoding proteins that regulate or mediate recognition or destruction of melanocytes by the immune system, as well as unknown environmental triggers (35,604,605). Many GV susceptibility loci are shared with other autoimmune diseases, most the human ortholog of likely underlying the observed epidemiological association among these diseases.

One family has been identified in which GV exhibits a pattern of autosomal dominant inheritance with reduced penetrance (606), suggestive of a rare causal variant with high penetrance. Genetic linkage analysis was used to map this locus (AIS1) to chromosome 1p31.3 (OMIM 607836) (606), and fine-mapping and DNA sequencing subsequently identified a private mutation in the promoter of *FOXD3* in affected family members that



**FIGURE 145-9** A patient of African-derived origin with generalized vitiligo.

up-regulated transcription in cells that express endogenous *FOXD3* mRNA (607). The *FOXD3* protein is an embryonic transcription factor essential for neural crest development and specification of the melanoblast lineage, and mutations that up-regulate its functions cause hypopigmentation (608–612).

Genetic linkage analysis has also been used to study families with more typical GV in the EUR and Chinese populations. Linkage analysis of EUR multiplex GV families identified *NLRP1* (613,614), encoding a key regulator of the innate immune system, particularly in skin dendritic (Langerhans) cells (615). Genetic linkage analysis of Chinese multiplex GV families identified the MHC (OMIM 193200) (616); *XBP1* on chromosome 22q12 (616,617), encoding a transcription factor which regulates transcription of MHC class II alleles (618,619); as well as a locus on chromosome 4q13–q21 that has not yet been identified (620).

Genome-wide association studies (GWAS) have also been used to identify GV susceptibility loci. In EUR subjects, two GWAS (621–624) have identified at least 29 loci, including *PTPN22*, *RERE*, *IFIH1*, *FOXP1*, *CD80*, *LPP*, *CLNK*, *TSLP*, *HLA-A*, *MHC class II* (the specific gene has not yet been identified), *BACH2*, *CCR6*, *SLA*, *IL2RA*, *CASP7*, *CD44*, *TYR*, an unknown locus on chromosome 11q21, *IKZF4*, *SH2B3*, *GZMB*, *OCA2*, *MC1R*, *UBASH3A*, *C1QTNF6*, *TOB2*, *FOXP3*, probably *TICAM1*, and have confirmed *XBP1*, altogether accounting for about 18 percent of the heritability of GV in EUR individuals (624). In Chinese subjects, a GWAS (625) identified two loci; the MHC class III region and *CCR6*. All but three of these GV susceptibility genes



encode proteins involved in regulation of the immune system and/or have been genetically associated with susceptibility to other autoimmune diseases. However, these three encode melanocyte components: *TYR* encodes tyrosinase; *OCA2* encodes the OCA2 protein; and *MC1R* encodes the melanocortin receptor. Each of these genes has been associated with normal pigmentary variation of the skin, hair, or eyes. For *TYR*, DNA sequencing has identified the causal (protective) variants as two missense substitutions common in the EUR population but virtually absent in other groups, S192Y and R402Q (626). For *OCA2*, the causal (protective) variant appears to be the same as that which is associated with blue eyes in the EUR population (624). In both cases, these GV-protective variants likely reduce the amount of the corresponding *TYR* or *OCA2* peptide antigens available for presentation by HLA-A2 (for which the causal allele has been identified as *HLA-A\*02:01*) (626). Furthermore, for both *TYR* and *OCA2*, the variants that are protective for GV are likewise high-risk for malignant melanoma (621,626). For these loci, GV and melanoma are thus genetic opposites, suggesting that GV may represent a dysregulated normal process of immune surveillance for malignant melanoma (627).

### 145.5.2 Segmental Vitiligo (SV)

SV usually occurs in childhood (up to 30% of pediatric vitiligo), and has rapid onset but remains localized and unilateral, often on the face (593,594,628). SV is not epidemiologically associated with co-occurrence of the autoimmune diseases that are associated with GV (595). Nevertheless, some evidence has recently emerged that immune phenomena may contribute to the pathogenesis of SV (629), including observation of a lymphocytic infiltrate of CD8+ and some CD4+ T cells at the lesional margin (630). Furthermore, GV and SV occasionally co-occur in the same families (595), suggesting that they may share some causal risk factors. As yet, there has been no systematic analysis of the genetic basis of SV.

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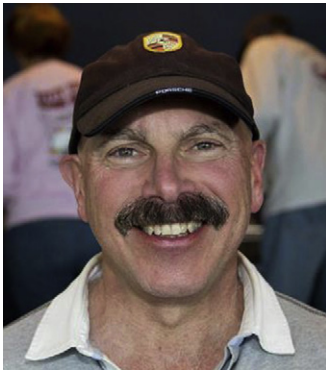
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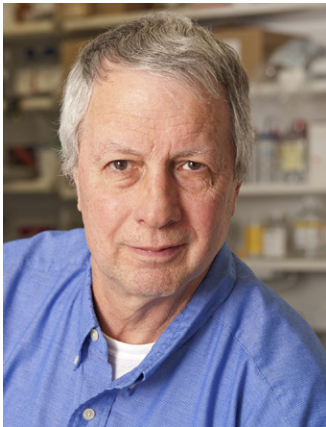
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### Biographies



**Dr Richard A Spritz** is Professor of Pediatrics and Director of the Human Medical Genetics and Genomics Program at the University of Colorado School of Medicine. Dr Spritz's work in the field of human genetics began in the "pre-molecular" era, and in the mid-1970s he took part in the earliest work on recombinant DNA and human genes, including the first cloning of human genes and identification of the first human disease gene mutation. Over the past 30 years, Dr Spritz and his colleagues have studied the genes involved in causing many different human diseases, including hemoglobin disorders, albinism, piebaldism, Hermansky–Pudlak syndrome, Chediak–Higashi syndrome, and other skin diseases, autoimmune diseases such as vitiligo and thyroid disease, and cleft lip/palate, and he has published over 225 scientific papers on these investigations. For the past several years, Dr Spritz has led an international team that carried out genomewide association studies of generalized vitiligo, establishing the autoimmune basis of the disease and its inverse relationship with malignant melanoma.



**Dr Vincent J Hearing** obtained his PhD from the Catholic University of America and continued his studies on mammalian pigmentation initially as a postdoctoral fellow, then as a staff fellow in the Dermatology Branch at the NCI, moving to the Laboratory of Cell Biology in 1983 where he now is a Senior Biomedical Research Scientist. He has served as President of the PanAmerican Society for Pigment Cell Research, President of the International Federation of Pigment Cell Societies, Editor of the journal *Pigment Cell Research* and Organizer of the 19th International Pigment Cell Conference. He serves as Deputy Chief of the Laboratory of Cell Biology and Chief of the Pigment Biology Section at the NCI, where he continues his research.



# CHAPTER

# 146

## Ichthyosiform Dermatoses

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### 146.1 INTRODUCTION

Ichthyosis, derived from the Greek word meaning fish, is a descriptive name for a group of inherited disorders of keratinization (cornification) in which the skin has large amounts of scale. Most of these patients have one of four major types of ichthyosis (see Table 146-1), the remainder fall into several less common forms.

### 146.2 APPROACH TO DIAGNOSIS OF ICHTHYOSIS

Ichthyosis can be hereditary or acquired. Hereditary forms can present at birth or occur later in life. The approach to diagnostically focusing down toward a specific type of ichthyosis involves consideration of a number of factors including clinical dermatological features, age of onset, family history and pedigree and associated abnormalities (1). The clinical presentation is important. For example, the diagnoses most likely to be encountered when assessing a collodion newborn are different to those when assessing an adult with mild ichthyosis, atopic eczema and asthma. Some ichthyoses appear as primarily skin disorders (ichthyosis vulgaris, congenital autosomal recessive ichthyosis, epidermolytic hyperkeratosis, etc.). Others are characterized by involvement of additional specific organ systems which may be focused (e.g. Sjögren–Larsson syndrome with neurological involvement) or broad (e.g. trichothiodystrophy with a wide spectrum of multisystem involvement). Some are considered part of syndrome complexes (e.g. Keratitis-Ichthyosis-Deafness (KID) syndrome). While some ichthyoses can be diagnosed by skin biopsy (e.g. epidermolytic hyperkeratosis), for most ichthyosiform disorders, routine histology shows a variable pattern of hyperkeratosis with or without inflammation which is at best suggestive

but not diagnostic. Occasionally, the diagnosis of an ichthyosiform dermatosis may need to be distinguished from other disorders, such as the clinical presentation of a newborn with exfoliative erythroderma, where epidermolytic hyperkeratosis (bullous congenital ichthyosiform erythroderma) is clinically similar to staphylococcal scalded skin syndrome.

### 146.3 THE MORPHOLOGY OF NORMAL SKIN

The epidermis is a multilayered tissue which is firmly attached to the dermis by a series of interlocking structures, some of which are formed by the epidermal cells and others by the fibroblasts. The lowermost layer of cells is called the basal cell layer, which is the primary site of cell division. As these cuboidal cells move up they become more flattened in appearance and show increasing numbers of keratin intermediate filaments that traverse through the cells. The uppermost layer of living cells is the granular layer; this contains blue granules when histological sections are stained by hematoxylin and eosin. The cells die and cornify at the top of this layer to form the highly compact and flattened cells of the stratum corneum. The thickness of the epidermis and its various layers differ in various areas of the body, but is generally about 100 µm except for the palms and soles.

In most areas of normal skin, the epidermis is organized into units which consist of a single stack of stratum corneum cells surmounting a large number of less flattened, more undifferentiated viable cells. The cells of each stack interdigitate with neighboring cells to form a highly cohesive membrane. The epidermis renews itself about every month as a result of cell division in the basal layer, with a cell cycle time of several hundred hours.

**TABLE 146-1 Classification of Most Common Ichthyosiform Dermatoses**

	Mode of Inheritance	Age of Onset	Clinical Appearance	Associated Features	Histology
Ichthyosis vulgaris	Autosomal semi-dominant	Childhood	Fine, light scales; flexures spared; hyperlinear palms and soles; keratosis pilaris	Atopy	Decreased to absent granular layer
X-linked ichthyosis	X-linked recessive	Birth or infancy	Large, dark scales; lateral face and neck commonly involved; flexures variable involved; palms and soles normal	Corneal opacities (do not affect vision); steroid sulphatase deficiency	Normal granular layer
Congenital Autosomal Recessive Ichthyosis (lamellar ichthyosis and congenital ichthyosiform erythroderma)	Autosomal recessive rarely autosomal dominant	Birth	Lamellar Ichthyosis: large plate-like scales, severe ectropion, variable erythroderma. Congenital Ichthyosiform Erythroderma: fine white scales, pronounced erythroderma. Both forms have hyperkeratotic palms and soles, flexural involvement	Ectropion, eclabium; collodion presentation	Thickened granular layer
Epidermolytic hyperkeratosis	Autosomal dominant, high frequency new mutations	Birth	Blisters and erosions at birth and during childhood. Coarse, verrucous scales, particularly in flexures	Pungent odor; frequent skin infections	Vacuolar degeneration of suprabasilar epidermis

Some of the cells in the basal layer are stem cells and remain there.

Differentiation of epidermal cells is called keratinization or cornification and involves a number of metabolic pathways. The cells contain the usual complement of subcellular organelles essential for energy production, synthesis of proteins, etc. In addition, one finds tonofilaments, desmosomes, keratohyalin granules and cornified envelopes which are essential for full expression of epidermal differentiation.

Desmosomes are attachment units between cells which consist of a modified region of the cell membrane and an intercellular component. These must be broken down and remade as the cell moves through the different layers, but the mechanism for this has not been elucidated. The tonofilaments run from the attachment plaque of the desmosome to lamin which surrounds the nucleus. The basic subunit of the tonofilament is a double stranded protein with a unique X-ray diffraction pattern suggesting a supercoiled  $\alpha$ -helical configuration. These molecules aggregate side-to-side and end-to-end to give rise to keratin intermediate filaments. The filaments contain free sulfhydryl groups in the living layers but these become oxidized when the cell cornifies.

Keratohyalin granules consist of at least two components, one of which gives rise to the cornified cell envelope, the other acts as a matrix for the filaments. The matrix material is not essential for filament packing and may not be present in all epidermal cells, but the formation of a cornified envelope is a critical part of keratinization. Transglutaminase 1 is an enzyme that is important

in the formation of this insoluble envelope through calcium dependent cross-links between proteins, including loracrin and involucrin.

Lamellar granules appear in the cytoplasm of granular cells and their contents are extruded into the intercellular space. They are rich in lipid and protein and contribute to the barrier properties of the stratum corneum and possibly act as an additional intercellular glue for the cornified cells (2). As cells cornify at the base of the stratum corneum they die, and their subcellular organelles and most of their soluble proteins are broken down and reabsorbed. Most of their water is lost and the cells assume a flat and compact appearance. The desmosomes remain but are modified in appearance.

## 146.4 ICHTHYOSIS VULGARIS

### 146.4.1 Clinical Features

Ichthyosis vulgaris (MIM ID #146700) is the commonest form of the ichthyosiform dermatoses, comprising about 95% of all types, with at least 1% of the population involved to some degree.

The disease almost never starts at birth but appears sometime after the first 3 months of life. The scales are fine, white and adherent but may be coarser over the lower extremities (Figure 146-1). The turned-up margins of the scales give a rough feel to the skin and account for the 'pasted on' appearance. The extensor surfaces of the extremities are most severely involved, while the flexural and intertriginous areas are usually spared. The



**FIGURE 146-1** Ichthyosis vulgaris with fine, white, adherent scales.

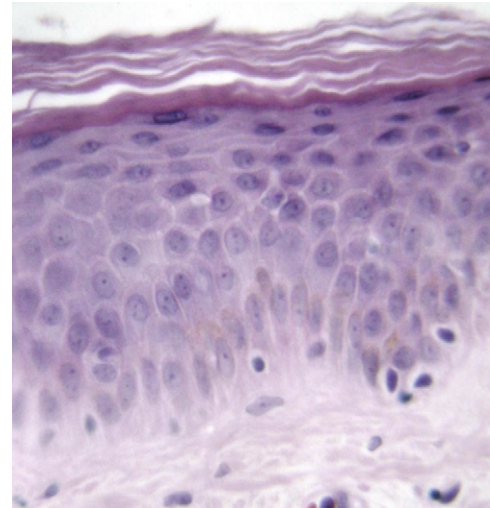


**FIGURE 146-2** Ichthyosis vulgaris showing hyperlinear palms.

face and scalp may be involved but usually clear in later life. Unlike X-linked ichthyosis, scaling of the neck is uncommon, occurring in a reported 8.5% of patients with ichthyosis vulgaris. There is accentuation of the palmar and plantar markings (Figure 146-2). Follicular hyperkeratosis (keratosis pilaris), especially of the arms, thighs and buttocks, is a frequent feature. The nails and mucous membranes are free of lesions. The disorder is worse in cold, dry weather, when fissuring may occur, while in humid, warm weather there may be almost complete clearing.

Atopy is frequently associated with ichthyosis vulgaris and in some patients eczema is a major problem, often causing more symptoms than the hyperkeratosis itself. Eczema herpeticum may be a complication, and patients are more susceptible to fungal infections.

Histopathologically, skin of ichthyosis vulgaris shows decrease to absence of keratohyalin granules (Figure 146-3), and this can also be seen by electron microscopy (3). Profilaggrin is a polypeptide that contains a variable number of filaggrin repeats, the main protein of keratohyalin granules, and important in the formation of the epidermal barrier. Filaggrin binds to keratin intermediate filaments and is involved in their aggregation during the formation of the stratum corneum. The breakdown of filaggrin is



**FIGURE 146-3** Ichthyosis vulgaris histopathology. There is thickening of the stratum corneum and absence of granular layer.

thought to be involved in maintaining skin hydration and water retention within the stratum corneum (4).

Kinetic studies have shown normal values for epidermal cell transit time from basal layer to stratum corneum. This suggests that the excessively thick stratum corneum is due to retention of scale rather than to hyperproliferation. The barrier function of the epidermis is reduced in ichthyosis vulgaris which allows enhanced penetration into the skin (4-6).

#### 146.4.2 Genetics

The pattern of inheritance of ichthyosis vulgaris was originally thought to be autosomal dominant and the variation in the degree of involvement among family members was attributed to varying expression of the gene. It is now known that mutations in the gene encoding profilaggrin cause ichthyosis vulgaris and that the disease follows a semi-dominant inheritance pattern (3). The ichthyosis is mild in individuals who have one mutated allele, and more severe in the presence of two mutated alleles. Filaggrin abnormality is also a predisposing factor for atopic dermatitis (7). Two mutations (R501X and 2282del4) are common in the European population but others have been described (8). The filaggrin-null phenotype has been found in approximately one in 90 children in the north of England (9). Filaggrin mutations can be genetic modifying factors exacerbating other types of skin disorders, since filaggrin mutations are not uncommon. Individuals with other types of ichthyosis (e.g. X-linked ichthyosis) who also carry a filaggrin mutation can have a more severe phenotype (10).

#### 146.4.3 Management

There is no specific treatment for this lifelong disorder of cornification. The primary goal of therapy is removal of the excessively thick stratum corneum which lacks

sufficient moisture on its outer surface due to a lack of water binding compounds which are derived from filaggrin. However, one may achieve satisfactory improvement by hydrating and softening the skin surface with a lubricating cream. This can be particularly effective when a moisturizing cream is applied to wet skin after bathing or showering. Keratolytic agents which contain salicylic or lactic acid, ammonium lactate or urea are useful in removing scale (11,12), and often results in normal-appearing skin in a few days (13). Commercially available 12% ammonium lactate lotion by prescription is widely used and avoids the inconsistency of topically compounded preparations.

## 146.5 X-LINKED ICTHYOSIS

### 146.5.1 Clinical Features

Approximately one in every 6000 males is affected with X-linked ichthyosis (XLI, MIM ID #308100). In one large study (Wells and Kerr, 1966), the disease was present at birth in 17%, while 84% were affected by three months of age. The onset has not been reported to occur after one year of age.

Although some texts state that affected individuals may be born as collodion babies, most congenital presentations of XLI are milder. Subsequently, the skin develops a generalized involvement with the extensor surfaces most severely affected. The lateral face, neck and scalp may also be markedly involved, but the flexural and intertriginous surfaces are less so. The palms and soles are usually normal and keratosis pilaris ordinarily does not occur. The scales tend to be large, thick, adherent and dark (Figure 146-4), and have been described as having an unwashed appearance. This is a particular problem on the lateral sides of the neck.

Corneal opacities are frequently associated with this disorder (Sever et al., 1968; Jay et al., 1968; Costagliola et al., 1991), and female carriers may also show the opacities, which usually are not manifest before adolescence. The corneal opacities resemble gray-white filaments, commas or dots, and are located on the posterior capsule of Descemet's membrane. They are detected only by slit lamp examination and do not affect visual acuity. They are rarely seen in normal persons or other forms

of ichthyosis. Males with XLI may have cryptorchidism and cancer of the testes has been reported.

The histological pattern of the skin is that of orthokeratotic hyperkeratosis with a normal or thickened granular layer. There are no abnormalities of the adnexal structures, and follicular plugging is not present. Kinetic studies of epidermal cell renewal are normal, the same as ichthyosis vulgaris.

### 146.5.2 Genetics

The mode of inheritance of XLI is X-linked recessive. Thus, the disorder is usually evident only in males. Carrier females may show both minimal scaling of the legs, which resembles that of ichthyosis vulgaris, and corneal opacities. A deficiency of steroid sulfatase has been demonstrated in XLI, and this abnormality is specific and not observed in the other types of ichthyosis except multiple sulfatase deficiency. In most patients, the loss of steroid sulfatase results from deletion of a segment of DNA on the X chromosome, where the gene for the enzyme is located (14,15). Furthermore, it has been found to be genetically heterogeneous (Conary et al., 1987; Gillard et al., 1987). In addition, some families have been found which appear to have point mutations of the gene rather than deletion of a chromosome segment.

The locus for XLI has been assigned to Xp22.32. Patients have mutations or deletions of the steroid sulfatase gene (<http://www.ncbi.nlm.nih.gov/omim/300747>). Some patients have larger deletions which may include adjacent genes, and these continuous deletions syndromes may present with additional clinical features. These include the chondrodysplasia punctata gene (CDPX1; MIM #302950) (16), the gene for Kallman's Syndrome (KAL1; MIM ID +308,700) (17), the short stature gene (SHOX; MIM ID \*312,865) and a gene whose deletion is associated with mental retardation (18,19).

Placental steroid sulfatase deficiency and low serum and urine estriol levels had been found in pregnancies associated with difficulty in initiation of labor due to failure of cervical dilation. Subsequently this was recognized to occur in male fetuses that developed X-linked ichthyosis. Shapiro reported the association of X-linked ichthyosis with steroid sulfatase deficiency using cultured fibroblasts and labeled steroid substrates and later measured steroid sulfatase in 25 patients the enzyme deficiency. Patients with ichthyosis vulgaris and lamellar ichthyosis had normal values of the enzyme.

Although the sulfation of steroids may in part be a detoxification, it has been appreciated that some sulfated steroids may have a physiological role. Sulfated precursors appear to be important for testosterone production in the testes. Dihydroepiandrosterone sulfate (DHEAS) secreted by the adrenal gland and cholesterol sulfate by the liver are present in significant amounts in the blood, and these could be used as substrates in the production of other hormones or metabolic products. Placental



**FIGURE 146-4** X-linked ichthyosis with large, thick, dark scales.



steroid sulfatase appears to be important for the latter part of pregnancy. DHEAS secreted from either maternal or fetal adrenal glands must be desulfated prior to its aromatization in forming estrogen, and this occurs primarily in the placenta.

Several disorders of lipid metabolism have been shown to be associated with ichthyosis-like changes of the skin. In hypothyroidism one observes such alterations in the skin, and ingestion of pharmacological doses of nicotinic acid and triparanol, which decrease blood cholesterol, cause similar changes. In Refsum disease (MIM #266500), where there is an accumulation of phytanic acid in the body due to a deficient alpha-oxidation, ichthyosis is one of the multisystem abnormalities which are observed. Finally, in multiple sulfatase deficiency (MIM #272200), where arylsulfatases A, B and C are all reduced, and which is caused by a mutation in a sulfatase-modifying factor-1 gene, (20,21) ichthyosis is a constant feature (22).

Steroid sulfatase is present in the lamellar bodies, which transport and secrete it into the intercellular spaces where it provides cholesterol for barrier function (22,23). In the normal epidermis, cholesterol sulfate decreases at the epidermal surface facilitating desquamation of the terminally differentiated corneocytes. As a result of steroid sulfatase deficiency, cholesterol sulfate accumulates in the epidermis leading to failure of normal corneocyte desquamation and a retention hyperkeratosis. A recent study has investigated the role of steroid sulfate as a protease inhibitor. It has been previously reported that desmosomes play a key role in the adhesion of corneocytes, and their digestion by two types of serine proteases leads to desquamation. A build-up of steroid sulfate, a protease inhibitor would thus retard desquamation in X-linked ichthyosis (24). Elias (23) confirmed this inhibition and further showed that there is a non-lamellar phase separation in the interstices of the stratum corneum, Nemes showed that cholesterol sulfate altered the function of transglutaminase 1 (25). Cholesterol sulfate has also been found to be a transcriptional regulator of transglutaminase 1 and involucrin expression.

It has been reported that the diagnosis of X-linked ichthyosis can be established by a determination of steroid sulfatase in specimens of callus and scale (26). Steroid sulfatase has also been studied in peripheral blood leucocytes with similar results, and affected individuals show more rapid mobility by electrophoresis of lipoproteins (27). Biochemical diagnosis of X-linked ichthyosis consists of demonstrating steroid sulfatase deficiency by lack of enzymatic activity (direct biochemical assays) or by showing an increase in substrates. Direct biochemical techniques work by demonstrating either steroid sulfatase or arylsulfatase C deficiency in tissues where they are usually found, which includes placenta, skin, fibroblasts, leukocytes and keratinocytes. Fluorescent in situ hybridization is commonly used for diagnosis as biochemical testing is not widely available.

### 146.5.3 Management

The treatment of X-linked ichthyosis is similar to that described for ichthyosis vulgaris. It is exacerbated by dry climate and improved by humid weather. Topical lubrication and keratolytics such as urea and lactic acid usually provide substantial relief.

## 146.6 AUTOSOMAL RECESSIVE CONGENITAL ICHTHYOSIS

### 146.6.1 Clinical

Autosomal recessive congenital ichthyosis (ARCI) is a heterogeneous group of disorders which present at birth and share autosomal recessive inheritance. The typical presentation is that of a baby born enveloped within a casing of thick stratum corneum resembling collodion, hence the name collodion baby. Other newborns may have generalized erythroderma. The incidence is about one per 300,000 live births. Tension from the collodion membrane may result in pulling out of the eyelids (ectropion) and lips (eclabium) (Figure 146-5). Constriction from the membrane may restrict breathing or compromise blood flow to the limbs or digits. Over days, the membrane begins to fissure and peel, leaving an erythematous skin. Complete desquamation may take several weeks. During the neonatal period, imperfect barrier characteristics of the stratum corneum and breaks in the skin lead to an increased susceptibility to bacterial infection. Therefore, these infants should be monitored for infection. Hydration and lubrication can facilitate desquamation. In cases where the membrane is causing constriction of limbs or breathing, systemic retinoids have been used to facilitate desquamation.

Over time, the skin may develop into different clinical phenotypes, with the most common being lamellar ichthyosis (LI, MIM ID #242300) and congenital ichthyosiform erythroderma (CIE, MIM ID #242100). In older literature, before LI and CIE were recognized as different entities based on clinical features, both were lumped under the name non-bullous congenital ichthyosiform erythroderma (NBCIE). CIE is characterized by skin with fine white scale and erythroderma, which may



**FIGURE 146-5** Autosomal recessive congenital ichthyosis with collodion presentation. Newborn with ectropion, eclabium and collodion membrane which is cracking.



**FIGURE 146-6** Congenital autosomal recessive ichthyosis-lamellar ichthyosis. Large, thick scales over the skin of the face with ectropion. (A) Showing large sheets of scale on legs. (B) Verrucous hyperkeratosis on flexural surfaces of the arm and trunk.

improve at the time of puberty. In contrast, the skin of LI is brown in color with thick, large, plate-like scale.

In LI, the erythematous, thick skin of the newborn becomes less red over time and the skin becomes covered with large, thick scales, which may form a mosaic pattern (Figure 146-6). The scale often tends to be most severe over the lower extremities. The palms and soles are usually hyperkeratotic and show increased markings. Fissuring of the hands and feet is a common problem. Hair is present but the scalp is often covered with adherent scale, and bacterial infection may occur and in conjunction with traction from the tight skin, may result in scarring alopecia. Ridges and grooves form in the nails. Ectropion is usually present, particularly in dry weather, and may be severe in later life. Decreased sweating is common and associated heat intolerance may be severe during exercise or in hot weather.

In CIE, the erythema present during the newborn period persists as generalized erythroderma and the skin is covered with fine, white scale (Figure 146-7). There may be varying severities of ectropion, eclabion, hypohidrosis with heat intolerance and alopecia.

Histological examination in LI shows hyperkeratosis, a thickening of the stratum corneum. In CIE, there is usually parakeratosis, a retention of the nuclei in the stratum corneum. This may reflect the finding that the rates of epidermal proliferation in the skin of CIE are much higher compared to LI (28).

While many patients can be clinically classified as LI or CIE, others have clinical features intermediate between these two presentations. These intermediate phenotypes have made a genotype/phenotype correlation difficult to define.

### 146.6.2 Genetics

Linkage analysis done in a number of families with the severe phenotype of classic LI (large plate-like scale,



**FIGURE 146-7** Congenital autosomal recessive ichthyosis – congenital ichthyosiform erythroderma. There is generalized redness and fine, white scales. In contrast to severe LI, this patient has minimal ectropion.

ectropion, and eclabion) mapped the diseased gene to chromosome 14q11 near the transglutaminase 1 gene (*TGM1*; 190,195) (29). Subsequently, in some families the disease was shown to result from mutations in the gene encoding transglutaminase 1 (30,31). This is an enzyme that is important in cross-linking proteins during the formation of the fully differentiated keratinocyte, the corneocyte. Loricin and involucrin are major precursor proteins to the cornified cell envelope expressed late in epidermal differentiation. Involucrin expression starts in the upper spinous layer and precedes loricin expression, which is restricted to the granular layer. Both proteins become cross-linked by the activity of transglutaminases as major components of the cornified cell envelope by  $\epsilon$ -( $\gamma$ -glutamyl) lysine bonds.

The molecular heterogeneity of ARCI has been confirmed with the finding of mutations in a variety of genes whose function relates to epidermal differentiation. In the United States, about half of patients with ARCI were found to have germline mutations in *TGM1* (32). In other families, mutations have been found in *ALOX12B*, (603,741), *ALOXE3* (607,206) (lipoxygenases), *ABCA12*, (MIM ID \*607,800, ATP-binding cassette transporter), *ichthyin* (MIM ID \*609,383, divalent cation transporter), and *CYP4F22* (MIM ID \*611,495, cytochrome P450 family) (33). Mutation types include missense, nonsense, deletion, splice-site, and insertion (34). While a definitive genotype/phenotype correlation has been elusive, *TGM1* mutations in ARCI has been associated with collodion presentation at birth, ectropion, plate-like scale and alopecia (32).

The usual pattern of inheritance is autosomal recessive, but an autosomal dominant type has been reported (35).

### 146.6.3 Management

Treatments are based on managing symptoms, which can include thickening of the skin with cracking and fissuring. Decreased sweating may cause heat intolerance. While the abnormal skin may be thick, the skin barrier function is abnormal leading to increased transepidermal water loss and there may be enhanced penetration of drugs. In addition to removing thick scale (keratolysis), hydration and lubrication are the main goals of treatment. Soaking softens the thick stratum corneum which can then be more easily removed with textured sponges or abrasives. Lubricating bath oils and topical lubricants help seal in the moisture. Topical keratolytics such as urea and lactic acid help thin hyperkeratotic areas. Topical salicylic acid is also used but caution is necessary because with an abnormal barrier there may be enhanced absorption and risk of toxicity. The oral retinoids, isotretinoin and acitretin, can produce a marked improvement (36–38). Gene replacement with retroviral vectors bearing the transglutaminase 1 gene has been successful in restoring function of keratinocytes cultured from patients with lamellar ichthyosis, but clinical utility has not been established (39).

## 146.7 EPIDERMOLYTIC HYPERKERATOSIS

### 146.7.1 Clinical Features

Epidermolytic hyperkeratosis (EHK, MIM ID #113800) was previously called bullous congenital ichthyosiform erythroderma. It occurs in one per 200,000–300,000 live births and usually presents at birth (Figure 146-8) with generalized erythema and blistering. This may result in extensively denuded areas. These moist, raw surfaces may lead to secondary infections and sepsis. The

diagnosis may be confused with other blistering diseases, such as epidermolysis bullosa or staphylococcal scalded skin syndrome. The erythema gradually subsides a few months after birth, and the bullae appear less frequently.

The clinical appearance of the skin varies between families, and six clinical phenotypes have been described (40). In some patients with generalized involvement, after the newborn period the disease may evolve into thick brown verrucous scale which often has a cobblestoned appearance accentuated at the flexures and over the knees and elbows (Figure 146-9). In others there may be generalized erythroderma with scaling. Some individuals have limited involvement with primarily palmar plantar hyperkeratosis and scaling limited to flexural areas. The skin in EHK may have a strong odor, thought to be due to bacterial overgrowth.

The histological picture of EHK is distinctive with hyperkeratosis, papillomatosis and acanthosis (41). There is a vacuolar degeneration of the epidermis above the basal layer which leads to blistering. Large clumped keratohyalin granules are present in a thickened granular layer. There is hyperproliferation of the keratinocytes within the epidermis as in lamellar ichthyosis, but in spite of its thickness, the epidermal barrier is defective,



**FIGURE 146-8** Epidermolytic hyperkeratosis. A newborn with peeling of the epidermis leaving erosions. (Courtesy of Dr. Joseph McGuire.)



**FIGURE 146-9** Epidermolytic hyperkeratosis. Thick verrucous hyperkeratosis and scaling over the knees and legs with erosions where hyperkeratosis has blistered off.



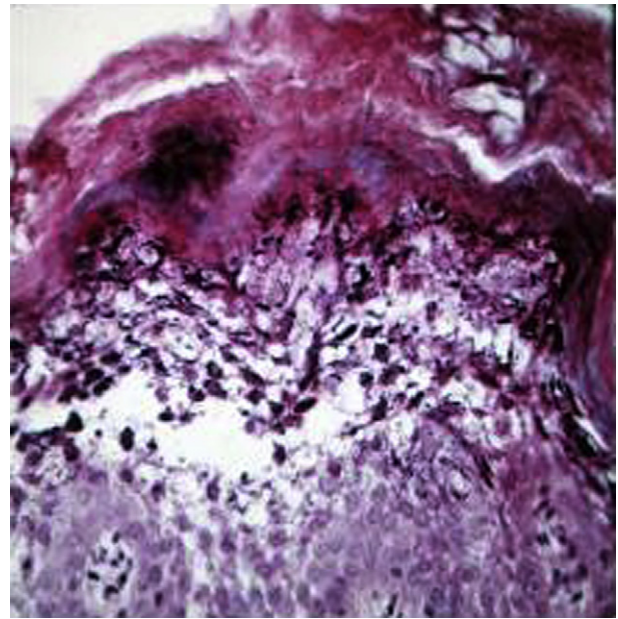
resulting in increased water loss through the abnormal stratum corneum (Figure 146-10). Ultrastructural studies demonstrate clumping of keratin intermediate filaments beginning in the first suprabasilar layer. These clumps contain keratin 1 and 10 (42). This and the histopathology of vacuolar degeneration of the suprabasilar epidermis are the result of destabilization of the cytoskeleton of differentiated keratinocytes. Keratins 1 and 10 pair as heterodimers to form the keratinocyte intermediate filaments which make up the cytoskeleton of suprabasilar keratinocytes. The cytoskeleton of the epidermal cells in the basal layer is composed of keratins 5 and 14. In EHK the cytoskeleton of the suprabasilar keratinocytes is fragile and prone to disruption. Mutations in the genes *KRT1* (MIM ID \*139,350) or *KRT10* (MIM ID \*148,080) coding for either keratin 1 or 10, respectively, have been found in families with EHK (42,43). These mutations occur in regions of the keratin molecule which are important for filament assembly. A genotype/phenotype correlation has been identified in that most families with mutations in *KRT1* have involvement of the palms and soles, whereas those with mutations in *KRT10* tend to have absence of severe palm and sole hyperkeratosis (40).

Several disorders resembling EHK have been described. Ichthyosis bullosa of Siemens (MIM ID #146800) is a rare autosomal dominant skin disorder with clinical features similar to EHK, but which occur in the more superficial layers of the epidermis. It is characterized by a mild epidermolytic ichthyosis which tends to localize to the flexures. Affected individuals are born with extensive superficial blistering, which develops into hyperkeratotic plaques over the extremities (44). However, the hyperkeratosis and blistering is milder than in EHK. Histological exam shows that lysis of keratinocytes is restricted to the upper spinous and granular layer. This results in peeling that occurs superficially, and leaves an area under the peeled layer that is dry and this has been called molting (Figure 146-11). The term epidermolytic ichthyosis has been suggested for this group of disorders (45).

### 146.7.2 Genetics

EHK is inherited as an autosomal dominant trait and due to mutations in the genes encoding keratin 1 or 10. But there is a high frequency of spontaneous mutation, so many cases have no family history (46). Prenatal diagnosis, previously accomplished by histology of fetal skin biopsy, is now done by direct gene sequencing (47).

In ichthyosis bullosa of Siemens, mutations are found in (*KRT2E*; 600,194), the gene encoding keratin-2, a keratin that is expressed only in the more superficial layers of the epidermis. A form of EHK confined to only the palms and soles was described by Vöerner. This disorder is associated with mutation in (*KRT9*; 607,606) which encodes keratin 9, a protein that is expressed only in the palms and soles. Ichthyosis hystrix of Curth



**FIGURE 146-10** Epidermolytic hyperkeratosis. Histopathology showing balloon degeneration of the epidermis above the basal layer.



**FIGURE 146-11** Ichthyosis bullosa of Siemens. Peeling of the superficial epidermis leaving a dry base, which has been called molting.

and Maklin (IHCM) has variable expression from limited to volar skin to generalized involvement and clinically resembles EHK, but blistering does not occur. In one family with IHCM a mutation in the variable tail region of the keratin 1 gene (MIM ID \*139,350) was found which leads to a retraction of the cytoskeleton from the nucleus and failed translocation of loricrin to desmosomal plaques (48).

### 146.7.3 Management

The skin of EHK has thickened areas of hyperkeratosis but also has a propensity toward blistering. Treatment includes hydration, lubrication and keratolytics, and



varies with the clinical presentation. The skin of EHK is often covered with thick hyperkeratotic horn, which may be subject to traction that may lead to blistering. For individuals with thick, hyperkeratotic skin, keratolytics to remove the thick horn can help prevent blistering. Oral retinoids may be very helpful to minimize the thick scale. Because they may enhance blistering, particularly at higher doses, therapy with systemic retinoids should be started at a low dose. Systemic and topical antibiotics are used when infection is present. Sodium bicarbonate or bleach baths may help to control the odor.

## 146.8 ICHTHYOSIS OF THE NEWBORN

Ichthyosis of the newborn describes a group of disorders present at birth or shortly after that may have different genetic defects. The diagnosis may be established by the appearance of the infant, the course of the disorder and biopsy of the skin.

### 146.8.1 Collodion Baby

**146.8.1.1 Clinical Features.** The most common of these disorders is the collodion baby; in which the infant is enclosed in a parchment-like membrane at birth. The tightly fitting brownish membrane (see [Figure 146-5](#)) distorts the features of the infant. The membrane dries rapidly and begins to fissure and split. The babies may have difficulty in nursing and breathing and exhibit problems in thermoregulation and are at risk of infection. The skin of these babies may evolve over time into one of the characteristic ichthyosis phenotypes, such as lamellar ichthyosis or congenital ichthyosiform erythroderma, or to a less specific intermediate phenotype (see [Table 146-2](#)). Alternatively, the skin may improve during infancy as occurs in the self-healing collodion baby ([49,50](#)), in which affected children clear shortly after birth or have very mild scaling of the skin for some months (Van Gysel et al., 2002). This has been reported in siblings with unaffected parents and is likely to be inherited as an autosomal recessive trait. The genetic basis of self-healing collodion baby has been reported to include mutations in *ALOX12B*, *ALOXE3* and *TGM1* ([51–53](#)). Patients with trichothiodystrophy may present at birth with taut, shiny membrane, or with just erythroderma.

Congenital ichthyosis may present with redness and scaling at birth rather than as a collodion baby and can be confused with the congenital ichthyoses associated with involvement of other organs, and this should make the physician search more carefully for other abnormalities. These may not be apparent immediately but may take some months to appear, as, for instance, in the Sjögren-Larsson syndrome.

Collodion presentation has also been reported in trichothiodystrophy, ankyloblepharon-ectodermal defects-cleft lip/palate (AEC) syndrome, chondrodysplasia punctata,

Gaucher disease, loricrin keratoderma, neutral lipid storage disease and X-linked hypohydrotic ectodermal dysplasia ([54](#)). Although some texts state that X-linked ichthyosis and epidermolytic hyperkeratosis may start as a collodion baby (Beare and Rook, 1972), this is likely not to be the case. The latter may present with blistering at birth or shortly after, and the peeling epidermis is confused with a collodion membrane.

**146.8.1.2 Management.** The collodion babies are best managed by placing them in a high-humidity, temperature-controlled incubator and carefully monitoring their vital signs. The membrane has a composition similar to stratum corneum and is pliable when kept moist. Topical petrolatum based creams and emollients (preferable sterile) are often used to keep the skin hydrated and soft but it has been suggested that they may contribute to infection ([55](#)). Hand washing and measures to limit exposure to infection should be implemented. The membrane should not be mechanically removed since this may tear off the epidermis, causing an increased risk of infection and resulting in scarring. However, the membrane sometimes restricts respiration or can cause constriction of extremities or digits. Constrictions can be released with mechanical means ([56,57](#)) and rarely systemic retinoids are used. Clear fluids should be given for the first few days until it is determined that the child can swallow properly and is not aspirating. Evidence for infection should be looked for and, if present, treated promptly and aggressively.


### 146.8.2 Harlequin Ichthyosis

**146.8.2.1 Clinical Features.** The harlequin ichthyosis is a distinct genetic entity in which the newborn has such a strikingly grotesque appearance that it is usually not confused with the other types of ichthyosis ([Figure 146-12](#)). It is considered the most severe presentation of congenital ichthyosis. The skin has a thick armour-like covering with an off-white color which has deep fissures running in different directions. There is marked ectropion, the palpebral conjunctiva obscuring the globe, which is normal. There is a degree of eclabium which gives a fish-mouth appearance. The external ears are poorly developed or absent. The hands and feet have a grayish white color and the fingers and toes may look necrotic, perhaps from interruption of the blood supply by constriction. Clinical evaluation and post-mortem examination have not revealed a consistent abnormality of other organ systems.

In the past this was called harlequin fetus, because most were born premature and succumbed shortly after birth, but with enhanced management individuals can survive, hence the term harlequin baby. Subsequently the skin evolves into an ichthyosiform erythroderma with redness and fine scaling ([58](#)).

**146.8.2.2 Genetics.** Previously there was some confusion about classification of the harlequin fetus, with some

**TABLE 146-2 Autosomal Recessive Congenital Ichthyosis: Collodion and Harlequin Presentations in the Newborn**

	Disease		Characteristic Clinical Features	Mutated Gene	Protein
HARLEQUIN	HARLEQUIN	MOST SEVERE	Premature birth, severe ectropion/eclabium, thick armor-like skin with deep fissures.	ABCA12	ATP-binding cassette subfamily A member 12
COLLOIDON	LAMELLAR ICHTHYOSIS (LI)		Ectropion/eclabium at birth. After shedding of collodion membrane skin develops large, brown, plate-like scale.	TGM1 in most; ABCA12 in some	Transglutaminase 1
	INTERMEDIATE PHENOTYPES BETWEEN LI AND CIE *		Variable congenital presentation (collodion or only erythroderma) and subsequent skin manifestations	CYP4F22	Cytochrome P450 family 4 subfamily F, polypeptide 22
	CONGENITAL ICHTHYOSIFORM ERYTHRODERMA (CIE)*		After shedding of collodion membrane skin appears red (erythroderma) with fine, white scale.	ALOXE3 & ALOX12B in some NIPAL4 (ICHTYN) reported TGM1 reported	Arachidonate lipoxygenase 3 & arachidonate 12-lipoxygenase; Ichthyin Transglutaminase 1
	SELF HEALING COLLODION BABY	MILDEST	Erythroderma or collodion membrane at birth. Clears, often within weeks leaving minimal redness and scaling	ALOXE3; ALOX12B I TGM1	Arachidonate lipoxygenase 3 & arachidonate 12-lipoxygenase; Transglutaminase 1

\*Genotype/phenotype correlation poorly defined.



**FIGURE 146-12** Harlequin fetus. Infants are usually born premature, with marked ectropion and eclabium, and skin broken into large plaques separated by deep fissures.

reports suggesting that the disorder may consist of phenotypically similar but genetically distinct entities. Dale and Karn (1993) and colleagues had classified harlequin fetus ichthyosis based on the electron microscopic appearance of keratohyaline granules, keratin expression, presence or absence of profilaggrin/filaggrin and possible defective protein phosphatase 2A expression. All individuals had abnormal lamellar granules and absent lipid lamellae. Harlequin fetus is an autosomal recessive trait. There is now strong evidence that mutations in the *ABCA12* gene (MIM ID \*607,800), which codes for a protein involved in lamellar granule secretion and lipid transport, are the genetic basis of the disorder (59,60). Most mutations in harlequin ichthyosis are truncation mutations which lead to loss of function of the *ABCA12* peptide. Mutations in the same gene in other families have been associated with the other clinical phenotypes of ARCI, including lamellar ichthyosis and congenital ichthyosiform erythroderma (61).

Previously, prenatal diagnosis had been accomplished by biopsy of fetal skin and possibly by sonographic features (62). Characteristic morphological abnormalities of harlequin ichthyosis can be detected as early as 17 weeks gestation. Clumps of abnormally keratinized cells with an increased amount of large lipid deposits were detected in amniotic fluid cell pellets indicating that the fetus was affected with harlequin ichthyosis. These results were confirmed by fetal skin biopsy, which was consistent with harlequin ichthyosis at 21 weeks gestation (63). Prenatal

diagnosis and carrier detection is currently available by molecular methods.

**146.8.2.3 Management.** There are several reports of successful treatment with intensive management in the neonatal period in conjunction with systemic retinoid treatment, such as etretinate or acitretin (64–66). After the newborn period, the skin develops the appearance of congenital ichthyosiform erythroderma (64).

## 146.9 HYPERKERATOSIS OF THE PALMS AND SOLES

Hyperkeratosis of the palms and soles accompanies many of the diseases in this chapter, and those in which hyperkeratosis of the palms and soles is a major clinical manifestation or are well known palmar plantar keratoderms (PPK) are listed in Table 146-3 (for a general review, see Ratnaveil and Griffiths, 1997 (67)).

These hereditary PPKs are a group of genodermatoses characterized by abnormal thickening of the epidermis of the palms and soles. They are distinguished from each other by severity (mutilating vs. non-mutilating), distribution (punctate vs. focal vs. diffuse), involvement of other areas of the skin, associated abnormalities, histopathology (epidermolytic vs. nonepidermolytic) and inheritance. Three different clinical phenotypes of autosomal dominant PPK are shown. The Unna Thost type of PPK has diffuse involvement (Figure 146-13). In contrast, punctuate keratoderma (Figure 146-14) has small discrete areas of thickening. In the striate form of PPK (Figure 146-15) hyperkeratosis is arrayed in linear bands.

The keratins are the more common genes mutated in the PPKs. Keratins are expressed as a pair of acidic and basic proteins which map to a cluster on chromosome 17q and 12q respectively, with 5/14 and 1/10 the major pairs of the basal and super basal layers. Since obligate heterodimers make up the keratin filaments, a mutation in either the acidic or basic type of keratin protein of a pair may cause disease. In addition to the main expression pairs, there are a number of accessory keratins expressed in particular epithelial tissues. Keratin 9 is specifically expressed in the suprabasal cells of the palms and soles. Keratin 2e is localized in the more differentiated epidermal cells. Keratins 16/6a and 17/16b are expressed in the upper outer hair root sheath, nail bed and palm and sole epidermis. Striate PPK has been associated with mutations in desmosomal proteins including desmoglein 1 (DSG1 gene 125,670, 18q11–12) and desmoplakin as well as keratins 1 and 16. Naxos disease (MIM ID #601214), caused by mutations in the plakoglobin gene (JUP; 173,325, 17q21), is a striate PPK with woolly hair and cardiac abnormalities with a risk of sudden death. Carvajal syndrome (MIM ID #605676), with striate PPK, woolly hair and left ventricular cardiomyopathy is caused by mutations in the gene encoding desmoplakin (DSP; 125,647, 6p24).

**TABLE 146-3    Palmar Plantar Keratodermas (PPK)**

Disease	Inheritance	Onset	Appearance	Associated Features	Mutated Gene [Locus]	Abnormal Protein	Abnormal Function
Cardiofaciocutaneous syndrome	Autosomal dominant or sporadic	First year	Thick, yellow plaques on palms, soles, and extensor surfaces	Sparse hair, mental retardation; cardiac abnormalities; distinctive facies; growth failure	KRAS [12p12.1] BRAF [7q34] MEK1 [15q21] MEK2 [19q13.3]	KRAS BRAFMEK1 MEK2	Proteins that interact commonly with ERK participate in regulating different proliferative and apoptotic pathways
Carvajal syndrome	Autosomal recessive	1st year	Epidermolytic PPK	Woolly hair, electrocardiogram abnormalities; dilated cardiomyopathy	DSP [6p24]	Desmoplakin	Adhesion protein
Erythrokeratoderma variabilis (EKV)	Autosomal dominant	Infancy	In addition to PPK, generalized or localized hyperkeratosis and migratory red patches.		GJB3 [1p35.1] GJB4 [1p35.1]	Connexin 31 Connexin 30.3	Gap junction protein Gap junction protein
Focal PPK with oral mucosa hyperkeratosis	Autosomal dominant	First decade	Calluses develop primarily over plantar pressure points	Oral leukokeratosis; nail changes; epidermolysis is a variable histologic feature	KRT16 [17q]	Keratin 16	Keratinocyte cytoskeleton
Howel-Evans Syndrome	Autosomal dominant	Age 5–15 years	Diffuse, symmetric, non-transgradient keratoderma, involving primarily plantar pressure points	Esophageal carcinoma; oral leukokeratosis; normal nails	[17q25]		
KLICK (keratosis linearis with ichthyosis congenita and sclerosing keratoderma) syndrome	Autosomal recessive	Birth	Diffuse keratoderma with sclerosis, deformities, pseudoainhum	Multiple keratotic papules, in symmetrical cordlike array over flexures	POMP [13q12.3]	Proteosome maturation protein	Protein degradation



Mal de Meleda	Autosomal recessive	Infancy	Diffuse, transgradiens PPK with painful fissures and malodor; hyperkeratotic plaques over elbows & knees	Koilonychia and subungual hyperkeratosis; leukokeratosis; hyperhidrosis	SLURP1 [8qter]	Secreted Ly6/plaur domain-containing protein 1	Suggested as a secreted protein
Naxos disease	Autosomal recessive	Infancy	Striate PPK	Arrhythmogenic right ventricular dysplasia/cardio-myopathy, woolly hair; risk of sudden death	JUP [17q21]	Plakoglobin	Cytoplasmic protein associated with desmosomes; intermediate junctions
Pachyonychia congenita (Type 1, PC1)- Jadassohn-Ledwadowsky type	Autosomal dominant	Infancy	Painful focal hyperkeratotic calluses	Pincer or other nail dystrophy, oral leukokeratosis; follicular hyperkeratosis	KRT16 [17q12–q21] or KRT6A [12q13], but may be KRT17 or KRT6B	Keratin 16 or 6a, but may be keratin 17 or 6b	Keratinocyte cytoskeleton
Pachyonychia congenita (Type 2, PC2)- Jackson-Lawler type	Autosomal dominant	Infancy	Painful focal hyperkeratotic calluses	Pincer or other nail dystrophy, multiple steatocystomas; minimal oral involvement; natal teeth; pili torti	KRT17 [17q12] or KRT6B [12q3], but may be KRT16 or KRT6a	Keratin 17 or 6b, but may be keratin 16 or 6a	Keratinocyte cytoskeleton
Papillon-Lefèvre syndrome	Autosomal recessive	Infancy to 5 years	Diffuse, transgradiens PPK with spread to Achilles tendon areas, elbows & knees	Severe periodontitis; dural calcification; deafness; pyogenic infections	CTSC (DPPI) [11q14.1–q14.3]	Cathepsin C	Lysosomal protease
Punctate PPK	Autosomal dominant	2nd or 3rd decade	Multiple punctate keratoses over entire palmoplantar surfaces	Punctate keratoses induced by trauma; variable nail changes	[15q22–q24]		
Striate PPK Type 1	Autosomal dominant	Infancy	Linear hyperkeratotic plaques along plantar aspect of fingers. May have more diffuse involvement of soles.		DSG1 [18q12.1–q12.2]	Type 1: Desmoglein 1	Adhesion protein

TABLE 146-3 Palmar Plantar Keratodermas (PPK)—cont'd							
Disease	Inheritance	Onset	Appearance	Associated Features	Mutated Gene [Locus]	Abnormal Protein	Abnormal Function
Striate PPK Type 2	Autosomal dominant	Infancy	Linear hyperkeratotic plaques along plantar aspect of fingers. May have more diffuse involvement of soles		DSP [6p24]	Type 2: Desmoplakin	Adhesion protein
Striate PPK Type 3	Autosomal dominant	Early childhood	Linear hyperkeratotic plaques along plantar aspect of fingers. May have more diffuse involvement of soles		KRT1 [12q13]	Type 3: Keratin 1	Keratinocyte skeleton
Unna-Thost Type	Autosomal dominant	1st 2 years	Diffuse, waxy PPK	Parrot-beaking of fingernails; secondary dermatophyte infection; nonepidermolytic histopathology	KRT1 [12q13]	Keratin 1	Adhesion protein
Vohwinkel syndrome (keratoderma hereditaria mutilans; mutilating keratoderma with ichthyosis)	Autosomal dominant	Infancy	Diffuse, honeycombed PPK	Pseudo-ainhum; starfish shaped keratoses on extensor surfaces of elbows, knees and knuckles	LOR [1q21]	Loricrin	Cornified form
With hearing loss					GJB2 [13q11–q12]	Connexin 26	Gap junction
Vörner Type	Autosomal dominant	Infancy	Diffuse, 'dirty' PPK	Clinically similar to Unna-Thost; epidermolytic histopathology	KRT9 [17q12–q21]	Keratin 9	Keratinocyte skeleton; refined to plantar

Striate PPK Type 2.  
Striate PPK Type 3.



**FIGURE 146-13** Hereditary palmar plantar keratoderma (PPK). Unna Thost type of autosomal dominant PPK.



**FIGURE 146-14** Hereditary PPK. Punctate type of PPK.



**FIGURE 146-15** Striate PPK. Linear bands of hyperkeratosis along length of fingers.

In addition to the diseases listed in the [Table 146-3](#), there are a large number of disorders referenced in OMIM in which there is some form of keratoderma, but the major feature in most is involvement of some other tissue or organ. Some are quite common disorders such as the ichthyoses, while many others are not. The titles are clues to their other manifestations, but not completely so.

The current management of the palmoplantar keratodermas involves the regular use of topical keratolytics and manual paring of the hyperkeratoses. Oral retinoids may be helpful in some patients. Associated dermatophyte infections are common and can be treated with topical or oral antifungal agents.

### 146.9.1 Pachyonychia Congenita

Pachyonychia congenita is an autosomal dominant keratoderma characterized by painful hyperkeratotic callosities and plaques of the plantar surfaces of the feet, hypertrophic nail dystrophy, follicular hyperkeratosis, oral leukokeratosis and cutaneous cysts. Cutaneous features are highly variable. Nail changes start in infancy with an upward growth of the nail plate with prominent subungual hyperkeratosis. Palmar callosities may occur with physical labor. Plantar callosities, which may begin in childhood, are painful, may be accompanied by blistering and hyperhidrosis and are usually the most disabling aspect.

Two types have been described (see [Tables 146-2](#)). Type I (Jadassohn–Lewandowsky type, MIM ID #167200) includes oral white plaques (non-premalignant leukokeratosis) and hyperhidrosis of the hands and feet. Follicular hyperkeratoses may occur. The oral leukokeratosis may be the earliest sign, and laryngeal involvement, manifested by hoarseness, has been described. Type II (Jackson–Lawlor type, MIM ID #167210) may include natal teeth, steatocystomas and pili torti.

**146.9.1.1 Genetics.** PC is caused by mutations in one of four keratin genes. Keratins encode the cytoskeleton of epithelial cells. Previously, mutations in *KRT6A* (MIM ID \*148,041) or its expression partner *KRT16* (MIM ID \*148,067) have been found in type 1 and mutations in *KRT6B* (MIM ID \*148,042) or *KRT17* (MIM ID \*148,069) in type 2. These mutations result in an abnormal keratinocyte cytoskeleton, which leads to cell fragility. Data from the International PC Research Registry ([www.pachyonychia.org](http://www.pachyonychia.org)) shows phenotypic overlap between these types and a new classification is being developed to link the mutation and clinical phenotype (68).

### 146.9.2 Trichothiodystrophy

Trichothiodystrophy (MIM ID #601675) is an autosomal recessive disorder characterized by sulfur deficient, brittle hair and a broad spectrum of clinical manifestations. Newborns usually present with erythroderma or a collodion-like membrane, and may have low birth weight and congenital cataracts. An extensive review of the literature has identified a wide spectrum of clinical features, including abnormalities at birth, pregnancy complications in mothers carrying an affected fetus, a high frequency of recurrent infections, and a high mortality in children (69). The pregnancies of mothers carrying

affected fetuses may be complicated by abnormalities such as preterm labor or pre-eclampsia (70). Clinical features include photosensitivity, intellectual impairment, short stature, microcephaly, recurrent infections, bone abnormalities of osteosclerosis and osteopenia, ichthyosis and nail dystrophy. Magnetic resonance imaging of the brain usually shows dysmyelination. Children typically have a very pleasant, outgoing personality and most have some degree of growth failure.

The term trichothiodystrophy (TTD) was proposed by Price in 1980, who recognized the fact that despite the broad spectrum of clinical abnormalities, all patients have sulfur deficient, brittle hair. When examined under light microscopy, the short, brittle, broken hairs of the patients appear to have hair shaft abnormalities of trichoschisis (a sharp transverse break), ribboning, and trichorrhexis nodosum-like defects (71,72). Under polarizing microscopy the hair shafts exhibit a distinctive pattern called tiger-tailed banding (Figure 146-16) which is diagnostic. Once the diagnosis is suspected, examination of hair shafts provides a quick and easy diagnostic test. The hair shafts are prone to breakage because of a reduction in high sulfur matrix proteins. Amino acid analysis of hair shafts shows reduced levels of cysteine and cystine.

TTD encompasses patients who have been described as having Tay syndrome, Amish brittle hair syndrome, Sabinas brittle hair syndrome and Pollitt syndrome. About half of the patients with TTD may have clinical photosensitivity, which can range from mild to severe. Patients with TTD may have mutations in the same DNA repair genes that cause xeroderma pigmentosum, which is associated with a large increase in the risk for development of skin cancer. In contrast, TTD is not associated with an increase in skin cancer risk.

**146.9.2.1 Genetics.** TTD is inherited in an autosomal recessive pattern and can be caused by mutations in genes involved in nucleotide excision repair including XPD (*ERCC2*, MIM ID \*126,340), XPB (*ERCC3*, MIM ID \*133,510) or TTDA (*TFB5*, MIM ID \*608,780). Most patients have mutations in the *XPD* gene. The proteins

made by these genes are components of the transcription factor TFIIH (*TFIIH*, MIM ID \*189,972) that is involved in both DNA repair and transcription. Some patients have mutations in *TTDN1* (MIM ID \*609,188), a gene whose function is not known. The clinical manifestation of TTD and xeroderma pigmentosum are very different, but may be caused by different mutations in the same genes. TFIIH is a helicase involved in both DNA repair and transcription. It is thought that mutations causing xeroderma pigmentosum affect the repair function while those causing TTD affect the transcription related function (73).

**146.9.2.2 Management.** Monitoring for infection, developmental delay, cataracts, bone abnormalities and growth difficulties, and providing appropriate support for learning, adequate nutrition, and ambulation are important. Awareness of the possibility of photosensitivity and if present, provision of protective measures with sunscreen and protective clothing is necessary. In addition to sun exposure, some photosensitive patients may be sensitive to ultraviolet A, which can be encountered from common light sources, such as unshielded fluorescent bulbs.

## 146.10 RARE ICHTHYLOSES

There are several very rare syndromes that include ichthyosis as a variable feature. The skin in these disorders is often the least involved feature.

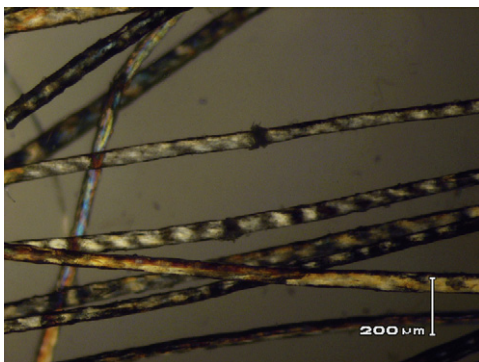
### 146.10.1 Rare Ichthyoses Associated with Metabolic Disease

#### 146.10.1.1 Phytanic Acid Storage Disease.

**146.10.1.1.1 Clinical Features.** Phytanic acid storage disease Refsum disease was first described by Refsum in 1946, and the specific biochemical defect was first reported by Klenk and Kahike in 1963. The principal clinical features of the disease are early onset retinitis pigmentosa, peripheral motor and sensory polyneuropathy, cerebellar ataxia and high cerebrospinal fluid protein without cells. The onset is usually before 10 years of age but can occur later. Weakness and difficulty with walking, and failing vision, particularly night vision, are the most common initial complaints. Other clinical findings include cranial nerve involvement (anosmia, neurogenic hearing loss, abnormal papillary reflex), and skeletal abnormalities.

The skin is usually not severely involved, showing small white scales primarily of the extensor aspect of the trunk and extremities. The appearance most closely resembles ichthyosis vulgaris.

The disease has a progressive course with gradual deterioration, although many patients may show periods of remission. Severe exacerbation followed by partial recovery has been noted. Several deaths have been reported without known cause, and it has been speculated that a cardiac arrhythmia was responsible, since



**FIGURE 146-16** Trichothiodystrophy (TTD) hair shafts. Under polarized light microscopy TTD hair shafts show tiger tail banding pattern and shaft defects. Note the sharp breaks (trichoschisis).



ECG changes accompany the disease. Respiratory failure appears to be an important cause of death.

**146.10.1.1.2 Genetics.** The mode of inheritance of phytanic acid storage disease is autosomal recessive. It is caused by inherited defects in the metabolic pathway for phytanic acid, a dietary branched-chain fatty acid. The poorly metabolized phytanic acid accumulates in fatty tissues including myelin sheaths and in organs such as the kidney and liver. The enzyme defect in Refsum disease (MIM ID #266500) has been discovered to be phytanoyl-coenzyme A hydroxylase deficiency (Jansen et al., 1997), (*PHYH*, or *PAHX*, MIM ID \*602,026) or the gene encoding peroxin-7 (*PEX7*, MIM ID +601,757). Heterozygotes have about half the capacity for oxidizing phytanic acid as measured by tissue culture study although plasma levels are ordinarily normal. The infantile form (MIM ID #266510) of the disease is caused by mutations in (*PEX1*, MIM ID +602,136) or (*PEX2*, MIM ID +170,993), or (*PEX26*, MIM ID +608,666) peroxisome proteins.

**146.10.1.1.3 Management.** Therapy of the condition has been based on reduction of phytanic acid in the diet with a concurrent decrease in body phytanate levels (74). This assumes that the phytanate is responsible for the abnormalities. Patients so treated have shown an improvement in ichthyosis and neurological status symptomatically and by objective criteria, although it has not been possible to induce neurological changes in animals by loading with phytanates. Diets with less than 5 mg of phytanic acid are most effective. Care must be exercised to avoid weight loss which may rapidly mobilize tissue phytanates and raise blood levels. Plasma exchange has been reported to be effective in combination with diet since it allows removal of large amounts of phytanic acid.

#### **146.10.1.2 Chanarin–Dorfman Syndrome (Neutral Lipid Storage Disease with Ichthyosis).**

**146.10.1.2.1 Clinical Features.** Chanarin–Dorfman Syndrome (MIM ID #275630) is a rare inborn error of metabolism which presents as congenital ichthyosiform erythroderma and is characterized by intracellular accumulation of lipid droplets composed of triacylglycerol in most tissues. In addition to ichthyosis, multiple organs are involved. Manifestations include hepatosplenomegaly, myopathy, ataxia, neurosensory hearing loss, cataracts and mental retardation. The disease may present as a collodion baby with ectropion and eclabion. Peripheral blood smear shows lipid droplets in granulocytes (Jordans anomaly). Lipid droplets may also be seen in other cells including the basal keratinocytes of the epidermis.

**146.10.1.2.2 Genetics.** The pattern of inheritance is autosomal recessive and the disorder has been found to be caused by mutations in the CGI58 gene (abhydrolase domain-containing 5, *ABHD5*, MIM ID \*649780) (75). Adipose triglyceride lipase (ATGL) is a triacylglycerol hydrolase that promotes the catabolism of stored fat.

ATGL requires activation by CGI58. Therefore CGI58 functions as a lipolysis activator (76).

### **146.10.2 Rare Ichthyoses Associated With Neurological Disease**

#### **146.10.2.1 Sjögren-Larsson Syndrome.**

**146.10.2.1.1 Clinical Features.** Sjögren-Larsson syndrome (MIM ID #270200) was described in a group of 28 patients from a small area of Sweden in 1957 by Sjögren and Larsson. However, patients of various nationalities have been described. Infants tend to be pre-term, and at birth or within the first few months of life there are hyperkeratotic lesions, often generalized, with erythema clearly noted in some cases. While large lamellar scales are observed in some patients, in many there is skin thickening with marked accentuation of the ridge-like pattern of the skin reminiscent of lichenification (Figure 146-17) (77). The scalp, palms and soles are variably involved, the face sometimes, with ectropion rarely present. Itching is a frequent complaint. Intertriginous areas have been clear in some individuals. The ichthyosis is associated with a permeability barrier abnormality which has been attributed to abnormal lamellar body formation and secretion (78).

The neurological symptoms often begin as a delay in reaching motor milestones during the first few years of life due to spastic diplegia of the legs and less severe changes in the arms. Cognitive defects are common and vary from mild to severe. Characteristic retinal lesions which appear as glistening white dots around the fovea have been reported in most affected individuals and may



**FIGURE 146-17** Sjögren Larsson syndrome. Dark, ridge-like pattern of the skin.

appear as early as the second year. White matter disease may be present on brain MRI.

**146.10.2.1.2 Genetics.** Sjögren–Larsson Syndrome (SLS) is inherited as an autosomal recessive trait. The patients have deficient activity of the aldehyde portion (fatty aldehyde dehydrogenase (FALDH)) of the fatty alcohol: nicotinamide adenine denucleotide (NAD<sup>+</sup>) oxidoreductase (FAO) complex. FALDH catalyzes aldehydes derived from fatty alcohol metabolism and FAO catalyzes the overall oxidation of fatty alcohol to fatty acid. SLS patients are deficient in both FALDH and FAO activity. The gene encoding the human fatty aldehyde dehydrogenase (*ALDH3A2*; MIM ID \*609,523) is located on 17p 11.2 and mutations in this gene cause SLS (79). There is striking heterogeneity in the mutations in the FALDH gene which include deletions, insertions, missense mutations, splicing defects and rearrangements. Diagnosis can be made by measuring FALDH or FAO activity in cultured fibroblasts or leukocytes, or by DNA based methods such as screening for common mutations in selected populations or gene sequencing. Prenatal diagnosis can be made using DNA based or enzymatic studies of amniocytes or chorionic villous cells (80).

**146.10.2.2 Erythrokeratodermias.** The erythrokeratodermias are a heterogeneous group of disorders that are characterized clinically by hyperkeratosis and localized erythema. Two distinct clinical phenotypes are recognized, erythrokeratoderma variabilis and progressive symmetric erythrokeratoderma.

**146.10.2.2.1 Clinical Features.** Erythrokeratoderma variabilis (MIM #133200) is a rare disorder which usually presents during the first year but may occur later as ichthyosiform plaques and migratory red patches. The ichthyosiform plaques have different clinical character among different patients. Some patients have generalized involvement with persistent hyperkeratotic plaques over most of the body. Others have localized, sharply demarcated plaques (see Figure 146-18A) which may remain fixed for years. Both types have migratory red patches (see Figure 146-18B) which vary in size and may migrate over minutes to hours. The red areas may be triggered by trauma or temperature change and are symptomatic in

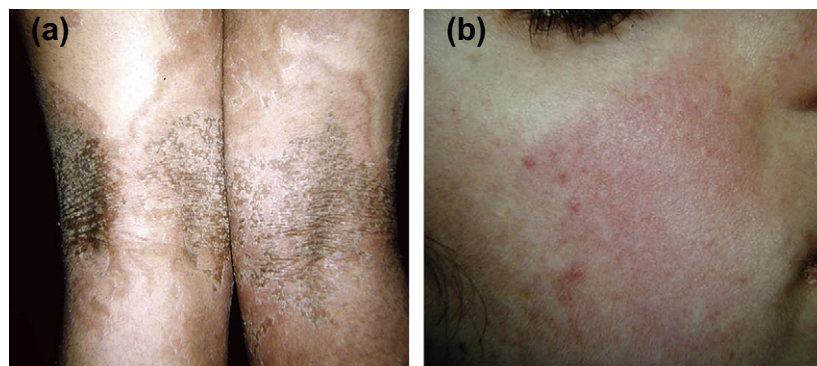
some patients, who often complain of burning. Hyperkeratosis of the palms and soles may be present. Erythrokeratoderma with a progressive spinocerebellar ataxia (Giroux-Barbeau type, MIM ID %133,190) has been described.

In progressive symmetric erythrokeratoderma (MIM #602036) the migratory red patches do not occur and only persistent erythematous hyperkeratotic plaques are present and they are for the most part limited to the extremities. They appear shortly after birth and progress slowly during childhood. They may regress after childhood.

Histological examination of the skin shows hyperkeratosis with a basket-weave appearance at the top and a compact one in the lower zone. There is hypergranulosis, papillomatosis, variable acanthosis and in some areas there may be a ‘church spire’ appearance. There is hyperplasia of the rete ridges with elongated capillaries and a peri-vascular lymphohistiocytic infiltrate in the dermis.

**146.10.2.2.2 Genetics.** Erythrokeratoderma variabilis is inherited as an autosomal dominant trait. The genetic locus for erythrokeratoderma variabilis was mapped to a locus on chromosome 1p32–34 (81), in a region of candidate connexin genes involved in epidermal differentiation. Mutations have been found in *GJB3* (*GJB3*; MIM ID +603,324) and *GJB4* (MIM ID \*605,425), the genes encoding connexins 31 and 30.3 respectively (82,83). However, the disorder is clinically heterogeneous even within families.

In a Japanese family with progressive symmetric erythrokeratoderma, a frameshift mutation in the loricrin gene on chromosome 1q21 was found (84). Loricrin is a major structural component of the cornified cell envelope, formed beneath the plasma membrane of stratified squamous epithelial cells during terminal differentiation. The progressive symmetric erythrokeratoderma is associated with keratoderma. This finding suggests that erythrokeratoderma variabilis and progressive symmetric erythrokeratoderma are distinct entities. However, in a more recent study, the same mutation in *GJB4* has been found in different families with either



**FIGURE 146-18** Erythrokeratoderma variabilis. (a) Sharply margined erythematous plaques with scaling accentuated at the borders. (b) Patients develop red patches, as shown here, which move over periods of minutes to hours.

erythrokeratoderma or progressive symmetric erythro-keratoderma (85).

### 146.10.3 Rare Ichthyoses Associated with Bone or Skeletal Disease

#### 146.10.3.1 Chondrodysplasia Punctata.

**146.10.3.1.1 Clinical Features.** Chondrodysplasia punctata is a heterogeneous group of disorders, first described by Conradi (1914), which has punctate calcifications that show up as stippling in radiographs. This may also be observed in other cartilagenous tissues such as the larynx and trachea. The disease has been classified into the Conradi-Hunermann type and a rhizomelic type, but several other forms have been described (see Ch. 165 Chondrodysplasias).

Cutaneous abnormalities are observed in about one fourth of the individuals and appear in the first few months of life. The eruption has a distinctive appearance (Figure 146-19) with a whorled pattern of thick white adherent scales on a background of erythematous skin. There is palmar and plantar hyperkeratosis, but nails and hair are normal. The cutaneous eruption disappears spontaneously by 3–6 months of age. Follicular atrophoderma and cicatricial alopecia have been observed in the site of the scaling.

The Conradi-Hünemann-Happle syndrome (CDPX2) (MIM #302960) is characterized by ichthyosis, chondrodysplasia punctata, cataracts and short stature. Clinically unaffected mothers or mothers with very mild disease expression may have somatic mosaicism or X-inactivation (86).

Histological examination of the skin shows marked hyperkeratosis and focal parakeratosis with invagination into the ostia of the pilosebaceous units. There is irregular elongation of the rete pegs with dilation of the dermal papillae and a relatively normal-appearing dermis.

**146.10.3.1.2 Genetics.** The Conradi-Hunermann syndrome is inherited as an X-linked dominant trait and mutations have been found in the emopamil binding protein (EPB, MIM ID \*300,205) (87,88). It has been demonstrated that there is an increased amount

of 8(9)-cholesterol and 8-dehydrocholesterol in tissue samples in X-linked dominant Conradi-Hunermann Syndrome, because of a mutation in *EBP*. An autosomal dominant (MIM %118650) type of chondrodysplasia punctata has also been described.

The rhizomelic type of chondrodysplasia punctata is a peroxisomal disorder. The rhizomelic type 1 (RCDP1) is autosomal recessive (MIM #215100) and caused by mutations in the peroxisomal biogenesis factor-7 gene, (*PEX7*, MIM ID +601,757). Type 2 (RCDP2, MIM ID #222765) is associated with a deficiency of dihydroxyacetonephosphate acyltransferase (MIM ID \*602,744). Type 3 (RCDP3, MIM #600121) is caused by mutations in the alkylidihydroxyacetonephosphate synthetase (alkyl-DHAP synthetase) gene. (*AGPS*, MIM ID \*603,051).

#### 146.10.3.2 CHILD Syndrome.

**146.10.3.2.1 Clinical Features.** CHILD Syndrome (MIM#308050) is an acronym that stands for Congenital Hemidysplasia with Ichthyosiform erythroderma and Limb Defects. The syndrome is characterized by unilateral erythema and scale, with a distinct demarcation in the middle of the trunk (Figure 146-20). The skin manifestations are present at birth or within the first week of life. Ipsilateral limb defects may vary from hypoplasia of fingers to a complete absence of an extremity. Ipsilateral hypoplasia of other parts of the skeleton along with defects in the brain and viscera are also seen. Punctate ipsilateral epiphyseal calcifications have also occurred.

**146.10.3.2.2 Genetics.** The ratio of female to male cases is approximately 19:1. The mode of inheritance is X-linked dominant. As a rule, it should affect exclusively girls because the underlying X-linked gene exerts a lethal effect on male embryos. Boys with the disease are assumed to have an early somatic mutation which allows them to survive. There is a mutation in the gene encoding NAD(P)H steroid dehydrogenase-like protein *NSDHL* (MIM ID \*300,275) (89,90), which affects cholesterol synthesis. Patients have also been described with mutations in the gene encoding EPB, the gene that also causes CDPX2 (90).



**FIGURE 146-19** Chondrodysplasia punctata. A newborn with a whorled pattern of scales on a red base. (Courtesy of Dr Nancy Esterly.)



**FIGURE 146-20** CHILD Syndrome. Unilateral erythema and scale with ipsilateral limb defects.



### 146.10.4 Rare Ichthyoses Associated with Hair Disease

#### 146.10.4.1 Netherton Syndrome.

**146.10.4.1.1 Clinical Features.** This autosomal recessive disorder (MIM ID #256500) is characterized by localized or generalized congenital ichthyosis, hair shaft abnormalities, immune deficiency and elevated IgE levels. The disease is usually present at birth with erythroderma and scaling. Infants may have failure to thrive or growth retardation and are at risk of postnatal dehydration, enteropathy and systemic infection, which can be fatal. Skin involvement can range from ichthyosis linearis circumflex (ILC), which is distinctive and appears as polycyclic and serpiginous erythematous lesions bordered by a double-edged scale on the trunk and proximal extremities (Figure 146-21) to a generalized ichthyosis resembling lamellar ichthyosis.

Patients have a specific abnormality of the hair called trichorrhexis invaginata (bamboo hair). The affected hairs show swelling in which there is a ball and socket invagination of the hair into itself, with the wider socket being more proximal (Figure 146-22). These hairs are fragile and break at the nodal swellings. Some patients are said to have normal hair or trichorrhexis nodosa or pili torti. Making a diagnosis in the neonatal period may be challenging since the pathognomonic features of trichorrhexis invaginata and ichthyosis linearis circumflexa are usually delayed until after infancy.

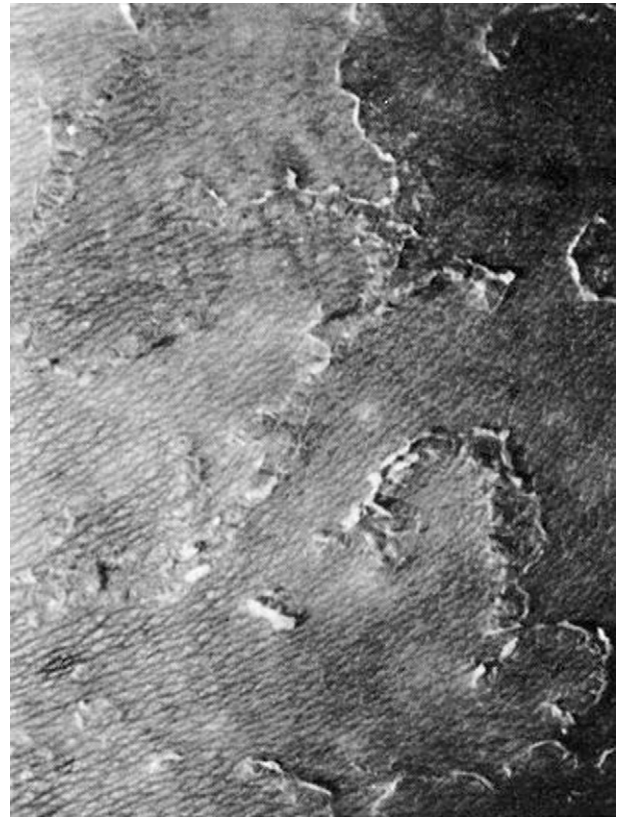
Some patients have an atopic dermatitis-like involvement of the skin with lichenification of the popliteal and antecubital fosse and the dorsum of the wrist. This may be associated with pruritus, allergic reactions and asthma.

**146.10.4.1.2 Genetics.** Netherton syndrome is caused by a mutations in the (*SPINK5*, MIM ID \*605,010) gene which encodes the serine protease inhibitor Kazal-type 5 protein (LEKTI) (91,92).

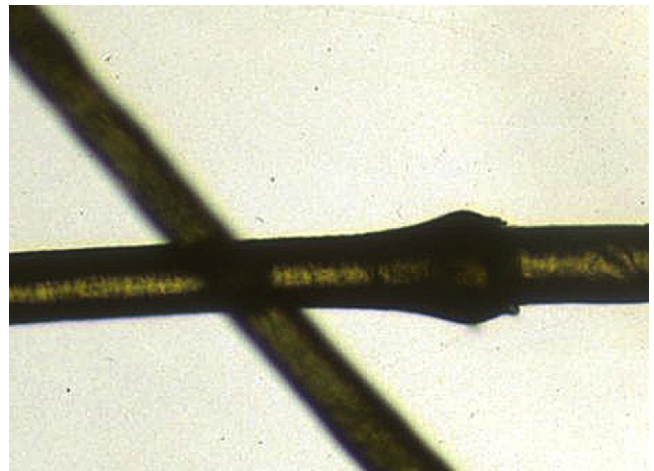
### 146.10.5 Rare Ichthyoses Associated with Eye and Ear Disease

#### 146.10.5.1 Keratitis–ichthyosis–Deafness (KID) Syndrome.

**146.10.5.1.1 Clinical Features.** KID syndrome (MIM ID #148210) is an ectodermal dysplasia characterized by a vascularizing keratitis, sensorineural hearing loss and an ichthyosiform disorder called erythrokeratodermia. Corneal neovascularization is progressive and may lead to visual impairment. Rather than the scaling typical of ichthyosis, skin involvement is often thickened with accentuated markings and localized to fixed plaques with a coarse surface (see Figure 146-23). These occur symmetrically and typically involve palmar and plantar surfaces. Patients may also have hair, nail and tooth abnormalities. There is increased susceptibility to mucous membrane and skin infections, which may be fatal in the newborn period. Chronic mucocutaneous



**FIGURE 146-21** Ichthyosis linearis circumflexa of Netherton syndrome. Serpiginous lesions with double-edged scale. (Courtesy of Dr James Stroud.)



**FIGURE 146-22** Netherton syndrome. (A) Trichorrhexis invaginata or 'bamboo' hair. (B) histopathology shows intussusception of hair in follicle. (Courtesy of Dr James Stroud.)

candidiasis may occur. Over time, chronic infection of hair bearing areas may lead to draining sinus tracts and scarring alopecia. Some patients have severe acneform eruptions and abscesses. Chronic paronychia may lead to nail dystrophy.

In some patients, there is a generalized mild hyperkeratosis of the skin with discrete erythematous plaques of the face (see Figure 146-24), extremities





**FIGURE 146-23** KID syndrome. Thickened, fixed plaques with a coarse surface.

and trunk with exaggerated skin markings, a variable verrucous appearance and some crusting. Rare patients have developed squamous cell carcinoma of the tongue.

**146.10.5.1.2 Genetics.** Most cases are inherited in an autosomal dominant pattern, but cases consistent with recessive inheritance have rarely been reported. The disorder has been found to be caused by dominant mutations in *GJB2* (MIM ID \*121,011), the gene that encodes connexin-26 (93). Connexins are transmembrane proteins that assemble to form gap junctions, which allow intercellular movement of molecules and ions.

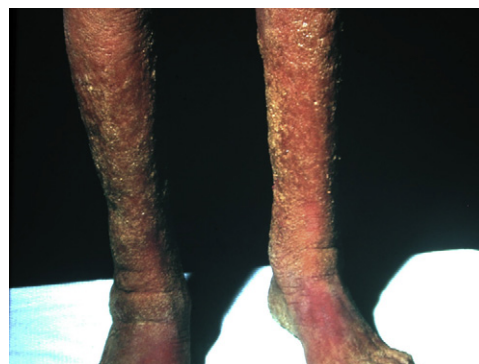
**146.10.5.1.3 Management.** Infections of the skin, nails and mucous membranes are treated with appropriate antimicrobials, but are often resistant to clearing. Chronic infection of hair bearing areas may lead to scarring alopecia. Skin plaques of erythrokeratoderma are often resistant to treatment with agents typically used to treat ichthyosis.

## 146.10.6 Very Rare Ichthyosiform Disorders

There are a number of very rare ichthyoses which have a tissue or organ other than the skin which appears to be a singular feature. They have other abnormalities which can be found in OMIM under Clinical Synopsis. We have selected the disorders listed here because they have an identified mutated gene. Others can be found in OMIM. The disorders which we would like to identify are listed.

**146.10.6.1 Hair Defects.** #607626. Ichthyosis, leukocyte vacuoles, alopecia, and sclerosing cholangitis 3q28-q29, *CLDN1* gene (603,718).

#610765. Ichthyosis with hypotrichosis, autosomal recessive 11q24-q25, *ST14* gene (606,797).



**FIGURE 146-24** KID syndrome. Symmetrical fixed plaques on the face and scarring alopecia.

**146.10.6.2 Nail Defects.** #601952. Keratosis linearis with ichthyosis congenita and sclerosing keratoderma (KLICK syndrome), 13q12.3, *POMP* gene (613,386).

**146.10.6.3 Skin Fragility.** #173650. Kindler syndrome, 20p13, *FERMT1* (*KIND1*) gene (607,900).

**146.10.6.4 Neurological and Sensory Defects.** #308205. Ichthyosis follicularis, atrichia, and photophobia syndrome (IFAP syndrome), Xp22.12-p22.11, *MBPS2* (300,294) gene.

#609528. Cerebral dysgenesis, neuropathy, ichthyosis, and palmoplantar keratoderma syndrome (cednik syndrome) 22q11.2, *SNAP29* (604,202) gene.

#610768. Congenital disorder of glycosylation, type im; *CDG1M* 9q34.11, *TMEM15* gene (610,746).

#607721. Noonan-like syndrome with loose anagen hair 10q25, *SHOC2* gene (602,775).

#182600. Spastic paraplegia 3, autosomal dominant; *SPG3A* 14q11-q21, *SPG3A* gene (606,439).

**146.10.6.5 Multiple Organs Consistently Affected.** #608013. Gaucher disease, perinatal lethal, 1q21, glucocerebrosidase gene (*GBA*; 606,463).

#115150. Cardiofaciocutaneous syndrome 12p12.1 (*KRAS*, 190,070) 7q34 (*BRAF*, 164,757), 15q21 (*MEK1*, 176,872), 19p13.3 (*MEK2*, 601,263). These proteins are involved in a common RAS/ERK pathway regulating cell differentiation, proliferation and apoptosis. May have palmar plantar keratoderma (see Table 146-2).

#208085. Arthrogryposis, renal dysfunction, and cholestasis 15q26.1, *VPS33B* gene (608,552).

## 146.11 PHENOTYPE BY FUNCTION: ADVANCES IN UNDERSTANDING

### 146.11.1 Pathophysiological Mechanisms Underlying Ichthyoses

The past few decades have brought tremendous progress in identifying mutations causing a spectrum of ichthyosiform disorders. This has led to a better framework for understanding the pathophysiology that causes specific diseases. A relationship between different components of keratinocyte biology and keratinocyte pathology is emerging and may provide in a more mechanistically

**TABLE 146-4 Functional Classification of Selected Ichthyosiform Dermatoses**

Functional Abnormality	Disease
<b>Structural proteins</b>	
Cytoskeleton	
Filaggrin	Ichthyosis vulgaris
Keratins	Epidermolytic hyperkeratosis Ichthyosis bullosa of Siemans Pachyonychia congenita Palmar plantar keratodermas: Focal PPK with oral mucosa hyperkeratosis Striate PPK Type 1 & 3 Unna-Thost Type Vöerner Type
<b>Cornified Envelope Formation</b>	
Transglutaminase 1	ARCI/Lamellar ichthyosis
Loricrin	Progressive symmetric erythrokeratoderma Vohwinkel syndrome (mutilating keratoderma with ichthyosis)
<b>Intercellular junctions</b>	
Desmoplakin	Carvajal syndrome (PPK, woolley hair, cardiomyopathy)
Plakoglobin	Naxos disease
Desmoglein 1	Striate PPK Type 1
Desmoplakin	Striate PPK Type 2
<b>Lipid metabolism</b>	
Lipoxygenases	ARCI
Lipolysis activator	Chanarin-Dorfman
Steroid dehydrogenase	CHILD syndrome
Sterol isomerase	Conradi-Hunerman-Happle syndrome (chondrodysplasia punctata)
Phytanic acid catabolism	Refsum disease
Fatty alcohol & Fatty aldehyde metabolism	Sjögren-Larsson syndrome
Steroid sulfatase	X-Linked Ichthyosis
<b>Intercellular communication</b>	
Gap junction proteins (connexins)	Erythrokeratoderma variabilis Keratitits-Ichthyosis-Deafness syndrome Progressive symmetric erythrokeratoderma Vohwinkel syndrome with hearing loss
<b>Protein metabolism</b>	
Protease	IFAP (Ichthyosis follicularis, atrichia and photophobia syndrome)
Proteasome	KLICK
Protein trafficking	MEDNIK
Protease inhibitor	Netherton syndrome
Protease	Papillon-Lefèvre syndrome

focused classification of these disorders. Table 146-4 shows a classification of ichthyosiform dermatoses arrayed by functional abnormality. Further clarification of the aberrant mechanisms underlying these disorders may lead to more directed therapeutic approaches and

will undoubtedly lead to a better understanding of normal keratinocyte biology.

### 146.11.2 Prenatal Diagnosis

Prenatal diagnosis of several types of ichthyosis is widely available. Molecular diagnosis, which is best performed early in pregnancy, is preferable over the older methods of fetoscopy and fetal skin biopsy, which are now rarely used. For ichthyoses where the specific mutation in the family has been determined, chorionic villous sampling can be done in the first trimester or amniocentesis in the second trimester. FISH analysis can be used for the prenatal diagnosis of X-linked recessive ichthyosis, where most patients have a complete deletion of the STS gene. Prenatal diagnosis of TTD has been reported using defective DNA repair capacity (94). Prenatal diagnosis of Sjögren-Larsson Syndrome can be performed by molecular methods using fresh or cultured chorionic villi and in the past, has been done by using an enzymatic assay for fatty aldehyde dehydrogenase activity (FALDH) in cultured amniocytes or chorionic villus fibroblasts (95,96). In the setting of an autosomal recessive disorder where the molecular defect is known and relatives may be at risk, carrier detection can be performed. Genetests ([www.ncbi.nlm.nih.gov/sites/genetests/](http://www.ncbi.nlm.nih.gov/sites/genetests/)) is a source of available testing resources.

### A LIST OF WEBSITES OF USE TO THE READER IN REGARD OF THE TOPIC

Foundation for Ichthyosis and Related Skin Types ([www.scalyskin.org](http://www.scalyskin.org)).

Gene Tests: [www.ncbi.nlm.nih.gov/sites/genetests/](http://www.ncbi.nlm.nih.gov/sites/genetests/)

### CROSS REFERENCE

Peroxisomal Disorders; Chondrodysplasias; Disorders of DNA Repair and Metabolism; Disorders of the Hair.

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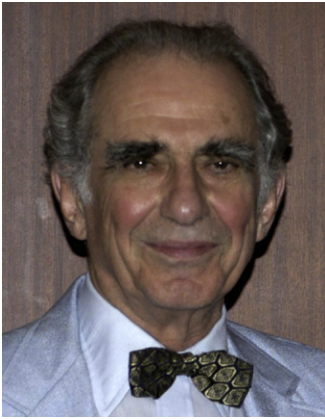


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### Biographies



**Howard Baden** is a professor of Dermatology at Harvard Medical School, and a Dermatologist at the Massachusetts General Hospital. He is a member of the Cutaneous Biology Research Center located in the Massachusetts General Hospital, which was established in 1989. His research was concerned with differentiation of epidermis and the skin appendages. He was responsible for defining the basis of a number of human genetic disorders. As a clinician Dr Baden was recognized for his expertise with genetic disorders of the skin, most particularly of the epidermis and hair and nails. He has published a number of papers and reviews in this field and was an author in the prior issue of the *Principles and Practice of Medical Genetics*.

**Dr DiGiovanna** is currently in the Dermatology Branch of the National Cancer Institute, NIH, serves as a consultant to the Division of Dermatological and Dental Drugs of the Food and Drug Administration, and since 1980 has held various positions at the NIH. He was previously the Director of Dermatopharmacology and Professor in the Department of Dermatology at the Warren Alpert Medical School of Brown University. His areas of interest include the efficacy and toxicities of systemic retinoids, and the clinical phenotypes and underlying causes of inherited skin disorders. He has worked extensively on the clinical characterization and underlying genetic causes of the disorders of cornification, skin cancer predisposition syndromes, and the disorders of DNA repair. He has published over 125 original articles, 25 book chapters and lectured widely at national and international conferences.

# CHAPTER

# 147

## Epidermolysis Bullosa

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### GLOSSARY

- Desmosomes** – (Greek: desmos, band; soma, body)  
Localized adhesion complexes on the lateral sides of plasma membranes in simple and stratified squamous epithelia. They are the major cell–cell adhesion complexes in the epidermis, anchoring keratin intermediate filaments to the cell membrane.
- Hemidesmosomes** – A structure that stabilizes the attachment of epidermis to the basement membrane. The molecular components of desmosome and hemidesmosome are different.
- Focal adhesions** – Specific types of large macromolecular assemblies through which both mechanical force and regulatory signals are transmitted between the extracellular matrix and the cell.

### 147.1 INTRODUCTION

Epidermolysis bullosa (EB) is a clinically and genetically heterogeneous group of genodermatoses characterized by mucocutaneous blistering and chronic epithelial fragility (1). The characteristic symptoms, skin blistering and erosions, result from intraepidermal or dermo-epidermal tissue separation caused by minor friction or trauma. EB occurs across all populations throughout the world, but no precise numbers exist on its global prevalence or incidence. Data from a number of European countries suggest a prevalence of  $24\text{--}49 \times 10^{-6}$  (References (2,3), and J. Bauer, Salzburg and M. Jonkman, Groningen, personal communications), and an incidence of 1 in 39,000 has been reported (German EB Network registry, [www.netzwerk-eb.de](http://www.netzwerk-eb.de)). In the US National EB Registry, a prevalence of  $8.22 \times 10^{-6}$  was calculated for the year 1990 (4). This figure was believed to be low due to the fact that the registry did not capture the mildest forms of EB. Similarly, other studies have emphasized the fact that a large number of patients with relatively mild forms of EB were found only when family members of more severely

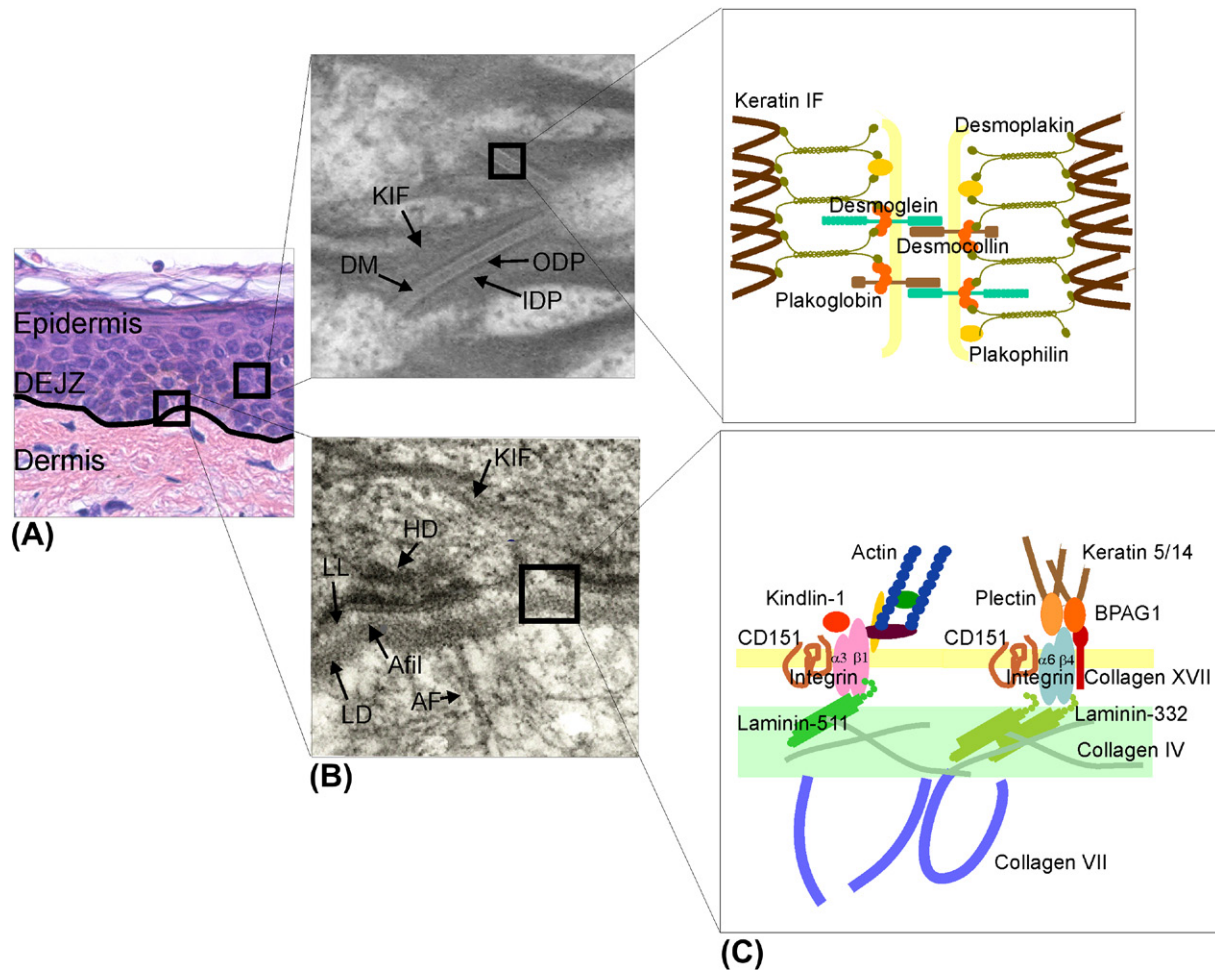
affected patients were examined. Many of them had gone unrecognized, since these patients regarded their skin as quite normal and had never seen a dermatologist.

### 147.2 MOLECULAR BASIS OF EPIDERMAL AND DERMO-EPIDERMAL ADHESION

The cutaneous basement membrane zone, which attaches the lower layer of epidermis to the underlying dermis in the skin, is structurally and functionally altered in EB. In rare cases, cell–cell adhesion in the upper layers of epidermis is perturbed. Electron microscopic studies have been instrumental in visualizing the ultrastructural characteristics of the cutaneous basement membrane zone and the adhesion complexes that attach the skin layers, including the desmosomes, hemidesmosomes, the basement membrane proper, anchoring filaments and anchoring fibrils (5). They lend the skin its resistance against environmental factors, including mechanical, physical and microbial insults (6). The desmosomes anchor cytoskeletal intermediate filaments to cell–cell junctions, and at the cutaneous basement membrane zone, the hemidesmosome-anchoring filament complexes link the cytoskeleton within basal keratinocytes with the extracellular basement membrane. This, in turn, interacts with anchoring fibrils, which attach the lower part of the basement membrane to the underlying dermis (7). At least 16 genes encoding proteins of the epidermis or the cutaneous basement membrane zone are involved in the pathogenesis of EB (Table 147-1). In order to understand the molecular basis of the epidermal and dermo-epidermal adhesion and their abnormalities in EB, a summary of these structures is given below and in Figure 147-1.

#### 147.2.1 Epidermal Cell–Cell Adhesion

The desmosomes are intercellular junctions in tissues exposed to mechanical stress, such as the skin,



**FIGURE 147-1** Morphologic and ultrastructural features of human skin. (A) HE staining of normal skin with epidermis, dermis and the dermo-epidermal basement membrane zone (DEJZ). Details of the marked areas are shown in panel B as transmission electron micrographic pictures and in panel C as schematic representations of the molecular components. (B) TEM depicting the desmosomes (upper panel) and DEJZ (lower panel). The critical structures are indicated by arrows: keratin intermediate filaments (KIF), outer dense plaque (ODP), inner dense plaque (IDP), dense midline (DM), hemidesmosomes (HD), lamina lucida (LL), and, anchoring filaments (Afil), lamina densa (LD), anchoring fibrils (AF). (C) The critical disease relevant molecular components of desmosomes (upper panel) and DEJZ (lower panel) are depicted schematically. (EM courtesy of Dr Ingrid Hausser, Heidelberg).

myocardium, bladder and gastrointestinal mucosa (8). In the epidermis, desmosomes are located at keratinocyte plasma membranes tethering the keratin intermediate filaments to the plasma membrane (9). Ultrastructurally, they consist of an outer electron-dense plaque adjacent to the plasma membrane, an electron-lucent zone, and an inner dense plaque (10,11) (Figure 147-1B). The adjacent cells are separated by an extracellular domain, ~30nm wide, which contains a dense midline. Each plaque consists of a supramolecular protein complex of components belonging to three protein families: cadherins, armadillo proteins, and plakins (Figure 147-1C). The desmosomal cadherins, desmogleins and desmocollins, are transmembrane proteins. The extracellular domain of these proteins consists of cadherin repeats separated by calcium binding motifs, and the transmembrane stretch is followed by an intracellular domain at the cytoplasmic face of the plasma membrane (8). The

extracellular domains interact to form the adhesive interface, whereas the cytoplasmic tails bind to the armadillo proteins, plakoglobin ( $\gamma$ -catenin) and plakophilins 1–4. These proteins regulate the assembly of the desmosomal components and attach desmoplakin and keratin filaments to the desmosome.

Plakoglobin is found at both desmosomes and adherence junctions; it functions as a scaffold for a number of binding partners, including desmosomal cadherins, E-cadherin and desmoplakin (12). Plakophilins are expressed in a tissue- and differentiation-specific manner, and plakophilin 1 and 2 are also found in the nucleus, suggesting specific regulatory roles for these proteins. The N-terminus of plakophilin 1 binds desmoglein 1, desmoplakin and keratin intermediate filaments, and it plays a key role in the assembly of the desmosomes (13). Desmoplakin links the keratin cytoskeleton to the armadillo proteins and cadherins. It is the most abundant



component of the desmosomes and consists of a central  $\alpha$ -helical coiled-coil rod domain, which is flanked by globular N- and C-termini. The N-terminal domain binds plakoglobin and plakophilins. The stable molecular interactions between desmosomes and intermediate filaments provide a high degree of resistance to mechanical forces. Apart from the adhesion function, emerging *in vivo* data suggest that desmosomal molecules also play a role in epidermal cell signaling (10).

### 147.2.2 Cell–Matrix Interactions

The hemidesmosomes mediate the attachment of the basal keratinocytes of the epidermis to the underlying basement membrane (Figure 147-1B). At the molecular level, they represent supramolecular assemblies of distinct intracellular and transmembrane proteins (Figure 147-1C). Two intracellular plakin homologs, BPAG1 (bullous pemphigoid antigen 1, also known as BP230 or the 230-kD bullous pemphigoid antigen) and plectin are components of the hemidesmosomal inner plaque. The homology of these plakins implies that these proteins bind keratin intermediate filaments. Different plectin isoforms expressed in epithelia and muscle tissues have been characterized (14,15). The two transmembrane components of the hemidesmosomes are collagen XVII (BPAG2 or BP180, the 180-kD bullous pemphigoid antigen) and  $\alpha 6 \beta 4$  integrin. Collagen XVII is a prototype of the transmembrane collagens, i.e. type II transmembrane proteins with a collagenous ectodomain (16). This cell surface receptor can interact with several binding partners. The intracellular ligands of collagen XVII are plectin, BPAG1 and  $\beta 4$  integrin, and its extracellular ligands are  $\alpha 6$  integrin and laminin-332 (17). The ectodomain of collagen XVII binds laminin-332 and is essential for the adhesion of the hemidesmosomes to the underlying basement membrane (17). The ligands of  $\alpha 6$  integrin include CD151, collagen XVII and laminin-332 (18).

Apart from hemidesmosomes, other adhesion molecules at the basolateral plasma membrane of basal keratinocytes include proteins found in focal adhesions *in vitro*, e.g. integrin  $\alpha 3 \beta 1$ , transmembrane collagen XIII, and kindlin-1 (also known as fermitin family homolog 1) (Figure 147-1C). These are involved in tethering the actin cytoskeleton to cell-matrix adhesions as alternative means to anchor basal epithelial cells to the basement membrane (18). A stabilizer of integrin functions at the cutaneous basement membrane zone, CD151, is a member of the tetraspanin family of cell surface proteins. It forms complexes with  $\alpha 3 \beta 1$  and  $\alpha 6 \beta 4$  integrins at the basolateral surface of basal keratinocytes (19).

The cutaneous basement membrane zone provides firm attachment between the epidermis and the reticular dermis. Underneath the plasma membrane of basal keratinocytes is the basement membrane proper, which

ultrastructurally consists of two layers, as shown by electron microscopy (Figure 147-1B). The zone adjacent to the epidermal basal cells is the 25–50 nm wide lamina lucida. This zone is traversed by anchoring filaments, which originate in the plasma membrane of basal cells and insert into the lamina densa and thus secure the epithelial cells to the underlying basement membrane. The anchoring filaments consist of laminin-332 and collagen XVII. The lower zone, the lamina densa, appears as an electron-dense amorphous structure of 20–50 nm in width. Its major molecular components are collagen IV, nidogens, perlecan, and laminins, which all can polymerize to network structures (20,21).

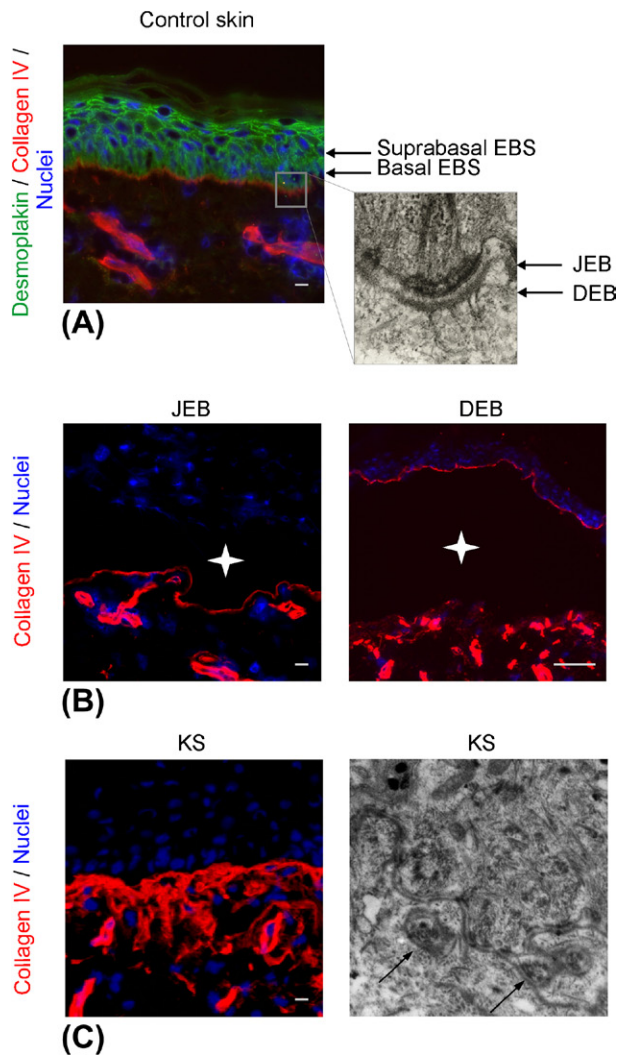
From the lamina densa, anchoring fibrils, which by electron microscopy appear as cross-striated condensed fibrous aggregates with frayed ends, extend into the dermis (Figure 147-1B, C). The proximal end inserts into the lamina densa, and the distal end is integrated into the fibrous network of the dermis or loops back to the basement membrane, thus providing anchorage of the basement membrane to the reticular dermis.

The detailed knowledge on the molecular components and their suprastructures in the epidermis and the cutaneous basement membrane zone is very helpful for diagnostic evaluation of EB skin biopsy specimens by immunofluorescence (IF) staining or transmission electron microscopy (TEM). An IF staining with antibodies to the individual marker proteins indicates the localization of these molecules at the cutaneous basement membrane zone and allows determination of the blistering level in EB (Figure 147-2). Furthermore, the intensity of the IF signal can be indicative of an altered or missing protein and thus helpful for determination of a candidate gene and the subtype of EB (see below).

### 147.3 CLASSIFICATION

The latest international consensus classification defines four major EB types based on the ultrastructural level of tissue separation (1): EB simplex (EBS), junctional EB (JEB), dystrophic EB (DEB), and the Kindler syndrome (KS) (Figure 147-2). These can be further separated into subtypes. In addition to the level of tissue separation, clinical severity and the inheritance pattern were applied as criteria for the major subtypes (Table 147-1). Further clinical and molecular genetic characteristics could distinguish more than 30 minor subtypes of EB (1), although many of these represent allelic variants and it remains questionable whether they should be considered as distinct subtypes.

In EBS, the cleavage occurs within the epidermis. This category is subdivided into two major subtypes: (a) suprabasal with cleavage within the upper epidermal layers and (b) basal with cleavage within in the basal keratinocytes at the bottom of the epidermis. Mutations in the genes encoding desmoplakin, plakophilin-1 and plakoglobin underlie suprabasal EBS. In the majority of



**FIGURE 147-2** The classification of EB is based on the level of skin cleavage, and relies on IF mapping and TEM findings. (A) In the left panel, indirect IF staining of control normal human skin with antibodies to desmoplakin (green) and collagen IV (red) is shown. Details of the demarked area are shown in TEM in the right panel. The levels of cleavage in the main EB types are indicated by arrows. (B) Skin biopsies from patients with JEB and DEB were immunostained with antibodies to collagen IV. The blister is marked by an asterisk. Note the staining in the floor and the roof of the blister, respectively. (C) Note the irregular staining pattern of collagen IV in KS skin (left), consistent with the reduplication of the basement membrane shown in TEM (right). Bars = 50  $\mu$ m. (TEM courtesy of Drs. Ingrid Hausser, Heidelberg, and Ivelina Yordanova, Pleven).

basal EBS cases, keratin 5 and 14 are mutated, and, consequently, keratin intermediate filaments are structurally and functionally altered. In more rare forms of EBS, intracellular proteins or protein domains within the hemidesmosomes are mutated or missing, including plectin, BPAG1, the intracellular domain of integrin  $\beta$ 4, or collagen XVII.

In JEB, dermo-epidermal separation takes place along the lamina lucida within the basement membrane zone, and the hemidesmosome-anchoring filament complex appears altered. Transmembrane and extracellular proteins of the hemidesmosomes and the anchoring filaments

are affected: collagen XVII, laminin-332 or integrin  $\alpha$ 6 $\beta$ 4. The separation into major JEB subtypes relies on the severity of the disorder. JEB-Herlitz, caused by lack of laminin-332, has a lethal course due to extreme fragility of the skin and mucous membranes, whereas JEB-non-Herlitz (also called JEB-other) exhibits milder, yet variable degree of chronic skin fragility but essentially a normal lifespan.

In DEB, blistering takes place at the level of the anchoring fibrils in the uppermost dermis. The anchoring fibrils can be either completely absent, reduced in number or, if present, ultrastructurally altered. Mutations in the gene for collagen VII, *COL7A1*, underlie both dominant and recessive forms of DEB.

KS is considered to be a distinct EB subtype because, in contrast to the other major types of EB described above, there are typically multiple cleavage planes— intraepidermal, junctional, or sub-lamina densa (1). Mutations in the *FERMT1* gene, which encodes kindlin-1, a keratinocyte protein in the focal adhesion complexes, are the underlying cause of KS.

## 147.4 CLINICAL FEATURES

### 147.4.1 EB Simplex

#### 147.4.1.1 Suprabasal EBS

**147.4.1.1.1 Lethal Acantholytic EB.** Lethal acantholytic EB (MIM 609638) is an extremely rare subtype caused by loss-of-function mutations in the desmoplakin gene, *DSP*. Severe mucocutaneous fragility and epidermolysis are already evident during delivery and rapidly progress to extensive erosions (22). The phenotype in four published cases is somewhat variable but includes universal alopecia, nail loss or neonatal teeth (23–25). Massive transcutaneous fluid loss leads rapidly to early demise (23–25). Histology reveals suprabasal clefting and acantholysis throughout the spinous layer of the epidermis. Immunostaining for desmoplakin is negative, or a punctuated intercellular pattern may be observed. TEM demonstrates keratin intermediate filaments severed from desmosomes.

It should be noted that mutations in the *DSP* gene can cause a spectrum of phenotypes with or without skin fragility (26). Two patients were described with trauma-induced skin blistering, palmoplantar keratoderma, alopecia and early onset cardiomyopathy (27,28). These phenotypes were due to loss-of-function or truncating desmoplakin mutations. In contrast, compound heterozygosity for nonsense and missense mutations was shown to cause skin fragility, woolly hair and palmoplantar keratoderma, with or without associated cardiomyopathy (29,30). In all cases, morphological examination of the skin revealed adhesion defects involving the entire stratum spinosum.

**147.4.1.1.2 Lethal Congenital EB.** This new entity belonging to the suprabasal EBS subtype is characterized by extensive areas of superficially eroded skin at birth,

**TABLE 147-1 Classification of EB, Causative Genes, Affected Proteins and Mode of Inheritance**

Major EB Types	Major EB Subtypes	Gene, MIM	Protein	Inheritance
EBS	Suprabasal	<i>DSP</i> , 125647	Desmoplakin	AR
		<i>PKP1</i> , 601975	Plakophilin 1	AR
		<i>JUP</i> , 173325	Plakoglobin	AR
	Basal	<i>KRT5</i> , 148040	Keratin 5	AD
		<i>KRT14</i> , 148066	Keratin 14	AD, AR
		<i>PLEC</i> , 601282	Plectin	AR, AD
		<i>DST</i> , 113810	BPAG1	AR
		<i>ITGB4</i> , 147557	$\alpha 6\beta 4$ integrin	AR
		<i>COL17A1</i> , 113811	Collagen XVII	AR
JEB	Herlitz	<i>LAMA3</i> , 600805	Laminin-332	AR
		<i>LAMB3</i> , 150310		
		<i>LAMC2</i> , 150292		
	Non-Herlitz (also called JEB-other)	<i>LAMA3</i> , <i>LAMB3</i> , <i>LAMC2</i>	Laminin-332	AR
		<i>COL17A1</i> , 113811	Collagen XVII	AR
		<i>ITGA6</i> , 147556	$\alpha 6\beta 4$ integrin	AR
		<i>ITGB4</i> , 147557		
	With renal and respiratory involvement	<i>ITGA3</i>	Integrin $\alpha 3$ subunit	AR
DEB	Dominant	<i>COL7A1</i> , 120120	Collagen VII	AD
	Recessive			AR
KS	—	<i>FERMT1</i> , 607900	Kindlin-1	AR

AR, autosomal recessive; AD, autosomal dominant.

absence of the scalp hair and onycholysis, and has been reported in only one individual so far (31). The condition rapidly progressed to a generalized epidermolysis with massive fluid loss through the epidermis, which apparently lacked any barrier function, and produced a lethal outcome within two weeks. Null mutations in the *JUP* gene encoding plakoglobin cause this suprabasal EBS subtype. Other mutations in the *JUP* gene are known to be associated with cardiac abnormalities (26), however, in this case ultrasound revealed normal morphology and function of the heart. Possibly, the early demise of the patient prevented clinical manifestations of cardiac anomalies. Histopathology of the skin showed pronounced acantholysis and cleavage in the suprabasal epidermis, with loss of upper spinous, granular and horny layers. Basal keratinocytes were attached to the basement membrane, but had little or no contact to neighboring and suprabasal cells. TEM revealed absence of desmosomes on the lateral aspects of basal cells and around all spinous cells.

Similar to the cases of desmoplakin deficiency, *JUP* mutations have also been associated with a spectrum of phenotypes. A phenotype consisting of mild skin fragility, accompanied by woolly hair and palmoplantar keratoderma, caused by homozygous mutations in the 5' region of *JUP* was recently described in three unrelated patients (32). Trauma-induced skin blistering, predominantly on the extremities, was noted during the first month of life (32,33). Nails were normal at birth but subsequently became dystrophic. No cardiac anomalies were disclosed by the age of 14 years. IF staining demonstrated marked reduction of plakoglobin expression, whereas TEM revealed widening

of intercellular spaces between keratinocytes extending from the suprabasal layer to the granular layer, and hypoplastic desmosomes which were reduced in number.

**147.4.1.1.3 Ectodermal Dysplasia–Skin Fragility Syndrome.** The ectodermal dysplasia–skin fragility syndrome (MIM 604536) is caused by loss-of-function mutations in the *PKP1* gene encoding plakophilin 1 (34,35). Clinical features include trauma-induced or spontaneous erosions of the skin, hypotrichosis or woolly hair, dystrophic nails, and painful fissuring with palmoplantar keratoderma (35). Typically, affected children have growth abnormalities. The morphological characteristics include dysadhesion of the cells in the suprabasal layers of the epidermis or a complete separation of the epidermis above the upper spinous layers. IF staining with plakophilin 1 antibodies is negative. TEM reveals loss of cell–cell adhesion in the lower suprabasal layers as well as few, relatively small desmosomes. A very similar phenotype associated with cardiomyopathy was reported to be caused by truncating desmoplakin mutations (28).

#### 147.4.1.2 Basal EBS

**147.4.1.2.1 Basal EBS Caused by Keratin 5 or 14 Mutations.** Basal EBS is the most common EB subtype, accounting for about one half of all cases (36). Blisters result from disintegration of the basal keratinocytes in the lowermost epidermis as a result of friction and trauma to the skin. Most cases are inherited in an autosomal dominant manner. Blistering starts at birth or soon thereafter, and the clinical spectrum ranges from mild localized to severe generalized blistering (Figure 147-3). The blisters heal without scarring, although secondary trauma and infection can cause milia,





**FIGURE 147-3** Clinical features of EBS basal. (A) EBS localized in a 45-year-old man with the heterozygous *KRT14* mutation p.V268D. (B) EBS Dowling–Meara due to *KRT14* mutation p.R125C in an infant. Note disseminated herpetiform blisters. (C) The 33-year-old of the girl shown in panel B has the same mutation, but only mild toenail dystrophy as a clinical symptom. (D) Severe generalized blistering in a 40-year-old patient with EBS Dowling–Meara caused by the *KRT5* mutation p.E477K. (E) Severe plantar keratosis in a 35-year-old patient with EBS Dowling–Meara caused by the *KRT14* mutation p.Y129D. (F) Generalized blistering in a patient with autosomal recessive EBS caused by the homozygous *KRT14* mutation p.W305X. (G) EBS with mottled pigmentation caused by the *KRT5* mutation p.P25L in a young woman.

mild scars and nail dystrophy. Particularly in cases with generalized blistering, painful palmoplantar hyperkeratosis can develop. The most common subtypes are EBS localized (MIM 131800), EBS Dowling–Meara (MIM 131760), and EBS–other generalized (MIM 131900). In addition, EBS with mottled pigmentation (MIM 131960) and migratory circinate EBS (MIM 609352) represent clinically distinct but rare subtypes. Autosomal recessive EBS (MIM 601001) due to *KRT14* null mutations is rare in Western populations, but accounts for about one-third of EBS cases in the Middle East (37).

In all forms of EBS, IF staining of skin with antibodies to keratins and cutaneous basement zone proteins demonstrates splitting within the lowermost epidermis. However, in about 60% of cases, no skin cleavage is found in the biopsy specimens, and the IF is not conclusive. Negative keratin 14 staining is indicative of recessive EBS. Mutations in either the gene for keratin 5, *KRT5*, or for keratin 14, *KRT14*, underlie most cases of basal EBS (1). It should be noted that keratin 5 and 14 mutations may cause a spectrum of disorders manifesting with reticulate pigmentation, such as Dowling–Degos disease (MIM 179850), dermatopathia pigmentosa reticularis (MIM 125595), or

the Naegeli–Franceschetti–Jadassohn syndrome (MIM 161000), all without skin blistering (38).

**147.4.1.2.2 Basal EBS Caused by Plectin Defects.** Plectin deficiency may cause three different, rare EBS subtypes: EBS with muscular dystrophy (MIM 226670), EBS with pyloric atresia (MIM 612138), both inherited in an autosomal recessive manner, and EBS Ogna (MIM 131950), which has an autosomal dominant inheritance (39).

EBS with muscular dystrophy manifests with congenital generalized blistering accompanied with late-onset progressive muscular dystrophy (Figure 147-4). The skin lesions heal without scarring, but atrophy and nail dystrophy are present. Additional phenotypic features include mild palmoplantar keratoderma, dental anomalies, corneal involvement, extensive mucosal erosions and urethral strictures. Age of onset and the extent of muscular dystrophy are variable, but in most cases the patients become wheelchair-bound in their twenties and can die prematurely. Thus far, only a few patients with EBS with pyloric atresia due to plectin mutations have been reported (39–41). All exhibited widespread congenital absence of skin together with pyloric atresia noted at birth and died from complications of the disease shortly thereafter.





**FIGURE 147-4** EBS with muscular dystrophy. Hemorrhagic blisters and involvement of the toenails in a 3-year-old patient with EBS with muscular dystrophy caused by compound heterozygosity for *PLEC* mutations c.4643\_4667dup and c.7120C>T. (Photo courtesy of Dr Slobodna Murat-Susic, Ljubljana).

Both EBS with muscular dystrophy and EBS with pyloric atresia are caused by mutations in the *PLEC* gene. The most severe forms of EBS with muscular dystrophy are associated with premature termination codon mutations, while a definitively milder form has been encountered as a result of an in-frame 9-bp deletion mutation (42). IF staining with plectin antibodies can show complete absence of this protein, or the staining is reflecting the type of mutations in the *PLEC* gene. TEM of skin reveals cleavage within basal cells just above the hemidesmosomes, with hypoplastic attachment plates and impaired keratin filament insertion into the inner hemidesmosomal plaque. Recently, it was suggested that mutations within *PLEC* exon 31 and loss of the full-length plectin with residual expression of the rodless plectin isoform leads to EBS with muscular dystrophy. In contrast, mutations located outside exon 31 lead to complete loss or marked attenuation of both the expression of full-length and rodless variants of plectin and underlie the more severe EBS with pyloric atresia phenotype (43). The same authors reported the first case of EBS associated with both pyloric atresia and muscular dystrophy, caused by compound heterozygous premature termination codon-causing mutations located within exon 32 of *PLEC* (44).

Only a few families with EBS Ogna have been reported. All patients carry the same heterozygous *PLEC* mutation, p.R2000W (45). EBS Ogna manifests with seasonal acral blistering with onset in infancy, generalized tendency to skin bruising and onychogryphosis (45). IF staining with most plectin antibodies is positive, whereas particular monoclonal antibodies recognizing epitopes within the rod domain do not immunoreact with skin samples of EBS Ogna patients (43).

Recently, a mutation in exon 1f of *PLEC*, leading to disruption of the plectin isoform 1f was associated with

autosomal recessive limb-girdle muscular dystrophy (MIM 613723), without skin manifestations (46).

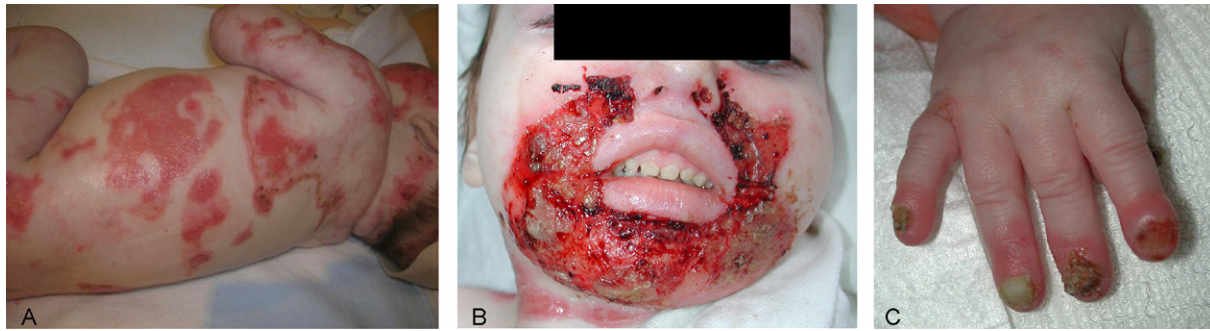
#### 147.4.1.3 Rare EBS Subtypes

**147.4.1.3.1 Recessive EBS Caused by *BPAG1* Mutations.** In one patient, recessive EBS was caused by a homozygous nonsense mutation in the dystonin gene (*DST*) that encodes the epithelial isoform of bullous pemphigoid antigen 1, BPAG1-e (also known as BP230) (47). Clinical features included lifelong generalized blistering and erosions. In addition, the patient had mild episodic neurological symptoms, but it remained unclear whether these were a manifestation of the *DST* mutation, or reflected a concomitant disease of the patient, a cerebral arteriopathy, due to a heterozygous mutation in *NOTCH3* gene. The *DST* mutation led to loss of hemidesmosomal inner plaques and a complete absence of immunostaining for BPAG1-e, as well as reduced labeling with antibodies to plectin,  $\beta 4$  integrin, and collagen XVII (47).

**147.4.1.3.2 EBS Caused by Mutations in the Intracellular Domain of Transmembrane Proteins.** Rare cases of EBS have been shown to result from mutations altering the intracellular domain of hemidesmosomal transmembrane proteins,  $\beta 4$  integrin and collagen XVII. Deletions in the *ITGB4* and *COL17A1* genes have been shown to cause intracellular cleavage low in the basal keratinocytes due to hampered attachment of keratin intermediate filaments to the hemidesmosome (48–50). The clinical features are consistent with EBS, and there was no history of pyloric atresia (48–50).

### 147.4.2 Junctional EB

**147.4.2.1 JEB-Herlitz.** JEB-Herlitz (MIM 226700) is the most severe JEB subtype and usually leads to death within the first two years of life. Extreme mucocutaneous fragility, erosions and blistering from birth are characteristic (Figure 147-5A). Extremities, the scalp and the face are usually affected early on, together with oral and respiratory mucous membranes (Figure 147-5B). Blisters heal without scars, but mild atrophy may occur after recurrent lesions. At some stage the healing ceases and chronic erosions and exuberant granulation tissue dominate the skin phenotype. Nails are lost and paronychia-like lesions with granulation tissue at the fingertips are characteristic (Figure 147-5C). Enamel defects, particularly pitting and hypoplasia, are common, but dental growth is normal. Laryngeal involvement with blisters and strictures causing upper airway obstruction may become life threatening. Eventually, fluid and protein loss, combined with poor feeding, cause severe systemic complications and, ultimately, organ failure. IF staining of the skin reveals absence or significant reduction of laminin-332. Null mutations in the *LAMB3*, *LAMC2* and *LAMA3* genes, which encode the three constituent polypeptide chains of the laminin-332 molecule, underlie this subtype.



**FIGURE 147-5** Clinical presentation of JEB-Herlitz. (A) A newborn with JEB-Herlitz. Severe involvement of areas exposed to mechanical trauma. (B) Extensive, poorly healing granulomatous facial lesions in JEB-Herlitz. (C) Typical nail involvement.

**147.4.2.2 JEB-Non-Herlitz (JEB-Other).** The most common JEB subtype, collectively called JEB-non-Herlitz (or JEB-other) (MIM 226650), encompasses a spectrum of phenotypes ranging from localized to generalized skin blistering, with onset at birth or later in life (51) (Figure 147-6). In addition to blisters, typical clinical symptoms include hypopigmentation at the sites of blistering, mild skin atrophy, and dystrophy or loss of nails (Figure 147-6A–C). In some cases, parietal or even total alopecia develops in the course of the disease (Figure 147-6D). Dental abnormalities are typically observed in form of generalized enamel hypoplasia (Figure 147-6E,F) leading to susceptibility to caries and loss of teeth early in life. Extracutaneous involvement is not common. TEM demonstrates tissue separation along the lamina lucida and the presence of poorly developed hemidesmosomes. IF staining of the skin reveals junctional blistering and positive, yet frequently attenuated, laminin-332 staining, and/or reduced or absent collagen XVII staining. This JEB subtype is genetically heterogeneous: mutations in the laminin-332 genes *LAMB3*, *LAMA3* and *LAMC2*, or in the collagen XVII gene, *COL17A1*, can underlie these phenotypes.

EB nevi are large, often irregular melanocytic lesions initially described in JEB-non-Herlitz (52). They may manifest clinical, histological and dermatoscopic features suggestive of melanoma (53). Although it is not clear whether EB nevi are melanoma precursor lesions, regular follow-up is recommended (52). It should be noted that EB nevi might occur in patients with all EB variants (52) (Figure 147-7).

**147.4.2.3 JEB with Pyloric Atresia.** JEB with pyloric atresia (MIM 226730) is a relatively rare EB subtype, characterized by skin blistering and pyloric atresia, which may be suggested prenatally by gestational hydramnion (54). The severity of the disease can be variable. At birth, the condition can be dramatic and perinatally lethal, in spite of surgical correction of pyloric atresia, while mild JEB with pyloric atresia has a favorable prognosis after surgery. In some cases, skin involvement can be minimal, sometimes with late onset of acral blistering, nail dystrophy and/or enamel



**FIGURE 147-6** Clinical features of JEB-non-Herlitz. (A) A 16-year-old male compound heterozygous for the *LAMA3* mutations p.Q738X and p.A1484V. Note the partial dystrophy of the finger nails. (B) The same patient exhibits linear scars in traumatized areas of the skin. (C) Blistering, hypopigmentation, nail loss and dystrophy in a 37-year-old woman with JEB-non-Herlitz due to *COL17A1* mutations p.M1T and p.R1226X. (D) Extensive non-scarring alopecia in the patient shown in panel C. (E) Enamel pitting in the patient shown in panel A. (F) Careful examination of his healthy mother revealed involvement of the teeth, but no skin or nail manifestations.

hypoplasia. Disease-causing mutations have been discovered in the genes coding for  $\beta 4$  and  $\alpha 6$  integrin, *ITGB4* and *ITGA6*, with the majority of mutations





**FIGURE 147-7** EB nevi. (A) A 7-year-old patient with EBS. (B) A 28-year-old patient with JEB-other, and (C) in a 5-year-old patient with DEB.

involving the integrin  $\beta 4$  subunit (54,55). IF staining of the skin reveals junctional blistering, and attenuated staining for  $\beta 4$  and/or  $\alpha 6$  integrin (54). Interestingly, the severity of pyloric atresia may also be variable and can be absent in some cases with skin manifestations but with a family with history of JEB with pyloric atresia (56). It is apparent, therefore, that factors other than the mutations in the  $\alpha 6\beta 4$  integrin genes are likely to contribute to this phenotype (57).

**147.4.2.4 JEB with Renal and Respiratory Involvement.** Recently mutations in the integrin alpha3 gene (*ITGA3*) were identified in three children with a multi-system disorder comprising skin, renal and respiratory involvement. The disease first manifested with respiratory distress which was compatible with the diagnosis of interstitial lung disease. The nephrotic syndrome was in all cases a laboratory finding and was treated with peritoneal dialysis. Skin blistering started between 2 to 4 months of age and was rather mild. Mucosal membranes were not affected. After trauma, nails became dystrophic. All patients survived the neonatal period but died because of multiorgan failure at 4–19 months of age (100).

### 147.4.3 Rare Subtype: LOC Syndrome

Laryngo–oculo–cutaneous (LOC) syndrome (MIM 245660) is a rare autosomal recessive disorder characterized by cutaneous erosions and granulation tissue in the conjunctival and laryngeal mucosa, without overt skin blistering (58). The condition is confined to the Punjabi Muslim population (59,60). Many patients do not survive childhood, the most common cause of premature death being acute or chronic respiratory obstruction with secondary pulmonary infection and sepsis. Additional symptoms derive from conjunctival granulation tissue which can lead to extensive palpebral occlusion and blindness. Dental deformities are also common. The molecular basis of LOC syndrome is a frame shift mutation in the *LAMA3* gene, which leads to an N-terminal deletion of the  $\alpha 3B$  laminin isoform (59).

### 147.4.4 Dystrophic EB

**147.4.4.1 Dominant DEB.** The clinical spectrum of the dominant DEB (DDEB) (MIM 131750) is broad and can range from localized acral blistering to generalized involvement. Blisters always heal with scarring, and albopapuloid, scar-like lesions on the trunk, prurigo-like nodules on lower legs, nail dystrophy and involvement of the gastrointestinal tract can be features of DDEB (Figure 147-8). IF of the skin shows normal or reduced collagen VII staining at the roof of a trauma-induced blister (61), and TEM shows qualitatively or quantitatively altered anchoring fibrils (62).

**147.4.4.2 Recessive DEB.** Recessive DEB (RDEB) (MIM 226600) can exhibit a broad spectrum of clinical manifestations and degrees of severity (62). It is subdivided into two main subtypes, severe generalized RDEB, and generalized RDEB-other.

Severe generalized RDEB is associated with profound disability, and has a major impact on the quality of life of the patients and their families. Generalized blistering is present at birth and increases progressively, leading to poorly healing wounds and extensive scarring (Figure 147-9A).

Mitten deformities of hands and feet develop early in life (Figure 147-9B, C). Oral and gastrointestinal involvement with blistering, erosions, scarring and strictures leads to malnutrition, which impairs food intake and, in combination with protein loss through the wounds, results in anemia and growth retardation (Figure 147-9D). Squamous cell carcinoma is a feared complication of severe generalized RDEB (63), with a cumulative risk of 70% by the age of 35 years (64). IF of the skin shows absence or significant reduction of collagen VII, and all other marker antibodies map to the blister roof. TEM demonstrates cleavage below the lamina densa and absence or significant reduction of the anchoring fibrils.

The group of RDEB-other comprises a wide range of clinical manifestations, from localized manifestations to generalized blistering of skin and mucous membranes



**FIGURE 147-8** Clinical spectrum of DDEB. (A) Localized DDEB in a 27-year-old man caused by *COL7A1* mutation p.G2064V and manifesting with occasional blisters in areas of mechanical trauma and (B) toenail dystrophy. (C) and (D) Generalized DDEB with nodular and albo-papuloid lesions in a 42-year-old patient with *COL7A1* mutation p.G1755D. Her sister and mother are similarly affected.



**FIGURE 147-9** Clinical features of severe generalized RDEB. (A) Chronic non-healing wounds and scars on skin areas exposed to mechanical trauma on the back of a 30-year-old man with *COL7A1* loss-of-function mutations. (B) and (C) Mitten deformities of hands and feet in severe generalized RDEB. (D) Microstoma and dental caries are common in severe generalized RDEB.





**FIGURE 147-10** Clinical spectrum of RDEB-other. (A) Mild localized disease in a 44-year-old man, homozygous for the *COL7A1* mutation p.G1719R. (B) Generalized RDEB-other in a 56-year-old woman compound heterozygous for *COL7A1* mutations p.R2777X and p.R2069C. Note diffuse scarring alopecia. (C) Blisters and scars on sacral skin areas exposed to mechanical trauma. (D) Palmar scarring, finger contractures and loss of nails in the same patient. Her main complications are related to the esophageal strictures which require yearly dilatation.

(Figure 147-10). Scarring, mucosal involvement, dental and nail dystrophy, or loss of nails are common, however, mitten deformities of hands and feet do not develop. IF of the skin demonstrates a reduced collagen VII signal at the blister roof, and TEM shows reduced number of anchoring fibrils often with altered morphology (61). The molecular bases of all DEB subtypes are mutations in the *COL7A1* gene encoding collagen VII, the major component of the anchoring fibrils. Nonsense mutations are typical in severe generalized RDEB; missense mutations, particularly glycine substitution mutations in the collagenous domain, usually underlie DDEB; the phenotypes in the group of RDEB-other are caused by homozygosity or compound heterozygosity for nonsense, missense and splice site mutations, or small deletions and/or insertions (65,66).

#### 147.4.5 Kindler Syndrome

KS (MIM 173650) is a rare autosomal recessive genodermatosis characterized by congenital skin blistering and mild photosensitivity, which improve with age, and a progressive generalized poikiloderma with extensive skin

atrophy (67). Palmoplantar keratoderma, nail dystrophy, webbing of the fingers and joint contractures may occur (Figure 147-11). Oral, ocular, esophageal, intestinal, anal, urethral, and genital mucous membranes are severely affected in the majority of the patients, leading to impaired quality of life (68,69). Squamous cell carcinoma is a relatively common complication of KS in adults (67,69). IF staining with antibodies to cutaneous basement membrane zone proteins demonstrate discontinuous, branched staining patterns, while TEM reveals reduplication of the basement membrane and different levels of blister formation (see also Figure 147-2C). Mutations in the *KIND1* (*FERMT1*) gene, encoding kindlin-1, cause the KS, with more than 45 different loss-of-function mutations reported to date (69).

#### 147.4.6 Extracutaneous Symptoms and Complications in EB

Extracutaneous symptoms can be associated with EB because the basement membrane zones of the eye, the mucous membranes of the orifices, the gastrointestinal, respiratory and urogenital tracts are similar to the skin. Also the basement membranes in the muscle bear molecular similarities to this zone in the skin. Furthermore, secondary changes, such as scarring and fibrosis can cause symptoms in several organs (Table 147-2) (70). Complications related to skin and mucosal membranes mainly arise in severe EB variants, and may impede the well being and the quality of life (e.g. dysphagia, urethral strictures, pseudosyndactyly), or become life threatening (e.g. systemic infections, skin cancers). These manifestations and the associated EB types are summarized in Table 147-2. Anemia is a frequent feature in severe forms of DEB and JEB. It has a complex pathogenesis and a significant impact on the overall well being of the patients (71). Cardiomyopathy is a rare, or perhaps under-recognized, complication of severe generalized DEB with multifactorial causes, and potentially severe outcome (71).

Rare associations of skin blisters with other organ manifestations have been reported in isolated cases. The association between hereditary nephritis, sensorineural deafness and pretibial epidermolysis bullosa caused by a homozygous single nucleotide insertion in the gene encoding CD151 was described in two sibs (72). Single cases of JEB caused by *LAMB3* and *ITGB4* mutations, respectively, were associated congenital nephrotic syndrome (73,74).

#### 147.5 DIFFERENTIAL DIAGNOSIS

Although EB should be included in the differential diagnosis of a newborn with blisters, the clinical features in a newborn are not characteristic for any subtype (Figure 147-12), and sub-classification on the clinical basis may be difficult even for an experienced pediatrician or dermatologist. Distinct secondary symptoms, such as scarring,



**FIGURE 147-11** Clinical features of KS. (A) Acral skin blistering in a child with KS, homozygous for the *FERMT1* mutation c.676dup. (B) Skin atrophy begins on the dorsal aspects of the hands in childhood and is usually the first cue for the clinical diagnosis. (C) Progressive skin atrophy associated with sclerotic changes on the hand of a 28-year-old man with KS homozygous for the same duplication mutation c.676dup. (D) Webbing of the fingers and pseudoainhum in a 27-year-old man with KS caused by the homozygous *FERMT1* mutation c.1718+1G>A. (E) In children with KS, mild photosensitivity and reticular erythema in sun-exposed areas are common (the same patient as in panel A). (F) Poikiloderma develops in young adults and extends over the entire skin surface (the same patient as in panel C). (G) Gingivitis, periodontitis and cheilitis angularis in a young male with KS caused by the homozygous *FERMT1* mutation c.456dup.

alopecia, nail dystrophy, enamel defects or pigmentary changes, appear later in the course of the disease, which when combined with biopsy data and mutation analysis will then allow appropriate sub-classification.

The differential diagnosis of a newborn must include, apart from all EB types, other genetic skin diseases manifesting with skin erosions or blisters (Table 147-3). In the postnatal period, infections (herpes simplex, staphylococcal infections, candidiasis) should be excluded, as well as blistering due to maternal autoimmune diseases (transplacental antibody transfer in herpes gestationis and pemphigus vulgaris). The differential diagnosis of acquired skin fragility in children and adults is quite broad.

### 147.5.1 Diagnostic Tests

Clinical diagnosis of EB, with sub-classification, is often possible in adults based on the fully developed phenotype, but this may be difficult in a newborn because of the absence of typical secondary symptoms (Figure 147-12) (7). Careful investigation of the family history must be accompanied by a systematic clinical examination of the entire integument and mucosal surfaces, nails, hair and dentition. The involvement of other organs, e.g. digestive, urogenital, or musculoskeletal systems, must also be considered (Table 147-2).

Histopathological analysis of skin biopsies is usually the first diagnostic step. Classical dermatopathology using hematoxylin–eosin staining can distinguish intraepidermal cleavage, but its resolution is not sufficient to distinguish between the junctional and subepidermal tissue separation.

IF mapping is the method of choice for first-line EB diagnostics. This method employs a panel of antibodies against the integral components of the cutaneous basement membrane zone and of the cell–cell contacts as it reveals the level of tissue separation within the blistering areas (Figure 147-2), and can often identify the defective gene due to absent or attenuated staining for the corresponding protein (75). In the majority of cases it is sufficient for the diagnosis of the EB type and may indicate the candidate gene. As a general rule, the degree of the molecular defect correlates with the clinical severity, e.g. complete absence of a cutaneous basement membrane zone protein implies the presence of a severe form of EB. Thus, IF may also be useful for prognostication (76). KS represents a particular challenge, since this condition is characterized by gross abnormalities of the cutaneous basement membrane zone, and multiple components exhibit abnormal staining patterns: collagen XVII,  $\alpha 6\beta 4$  integrins and plectin display interrupted staining patterns at the cutaneous basement membrane zone, whereas

**TABLE 147-2 Main Complications and Extracutaneous Manifestations in EB**

Symptom	Mainly seen in	Comments
Cutaneous complications		
• Infections	All	Sepsis in JEB and lethal congenital EB
• EB nevi	All	
• Webbing of fingers and toes, pseudosyndactylies	DEB, KS	In KS only partial webbing, but pseudoainhum possible; loss of dermatoglyphics
• Skin cancer at a later age	DEB, KS	In DEB after the age of 20, in KS after the age of 40 years
Ocular	JEB, DEB, KS	Can cause severe pain
• Blisters		
• Blepharoconjunctivitis		
• Corneal erosions		
• Exposure keratitis		
Upper respiratory airways		
• Blisters, erosions, scarring of the oropharynx	JEB, DEB	
• Hoarse cry or voice	JEB	
Gastrointestinal tract		
• Oral blistering and erosions	All	May impede food intake
• Oral scarring, microstoma	DEB, KS	May impede food intake, speech and dental care
• Dysphagia	DEB, KS	Impedes swallowing
• Esophageal stenosis	DEB, KS	Impedes swallowing
• Pyloric atresia	EBS and JEB w. PA	
• Colitis	KS	Rare
• Constipation	JEB, DEB, KS	
Genitourinary tract		
• Urethral strictures	JEB, DEB, KS	
Dental		
• Enamel defects	JEB	
• Increased prevalence of dental caries	JEB, DEB	
Musculoskeletal system		
• Mitten deformities of hands and feet	DEB sev. gen.	
• Joint contractures	DEB	
• Osteopenia and osteoporosis	DEB	
• Muscular dystrophy	EBS w. MD	Late-onset muscular dystrophy
Cardiomyopathy	DEB sev. gen.	
Anemia	JEB, DEB	

JEB, junctional EB; DEB, dystrophic EB; KS, Kindler syndrome; JEB w. PA, junctional EB with pyloric atresia; EBS w. MD, EB simplex with muscular dystrophy; DEB sev. gen., dystrophic EB severe generalized.

laminin-332, collagens IV and VII show a broad and branched deposition in the upper dermis. This constellation is highly suggestive of the diagnosis of KS. IF with antibodies to kindlin-1 delivers variable results, including positive, reduced or absent staining, and is therefore not always conclusive.

TEM indicates the level of skin cleavage and reveals the ultrastructure of desmosomes, keratin filaments, hemidesmosomes, anchoring filaments and anchoring fibrils. It is particularly useful in the recognition of EBS Dowling-Meara characterized by clumped keratin filaments and of the KS characterized by reduplications of the lamina densa and multiple levels of cleavage (1).

Depending on the availability of technical equipment and proficient expertise (1,75), either IF or TEM can be employed for EB diagnostics. However, the IF is faster and less costly, and a comparative study showed it to be more sensitive and more specific than TEM in the diagnosis of EB (77).

## 147.6 MOLECULAR GENETICS OF EPIDERMOLYSIS BULLOSA

### 147.6.1 Complexity of the Cutaneous Basement Membrane Zone

Critical for understanding the molecular basis of EB is the recognition of attachment complexes at the dermo-epidermal junction required for stable association of epidermis to the underlying dermis. As indicated earlier in this chapter, initial ultrastructural studies demonstrated the presence of hemidesmosome-anchoring filament complexes that are required for stable association of the basal keratinocytes to the lamina lucida of the underlying basement membrane. Similarly, on the dermal side of the basement membrane zone, there are prominent structures—anchoring fibrils—which secure the association of the lower part of the lamina densa to the upper papillary dermis. Thus, there is a network





**FIGURE 147-12** Clinical features of different EB forms can be very similar in newborn and infants. For example, aplasia cutis may occur in almost any EB form. (A) EBS; (B) JEB-non-Herlitz; (C) JEB-Herlitz; (D) RDEB.

**TABLE 147-3 Differential Diagnosis of EB: Other Genetic Skin Diseases**

Disease	Mutated Genes
Acral peeling skin syndrome <sup>a</sup>	<i>TGM5</i>
Keratinopathic ichthyosis <sup>a</sup>	
Epidermolytic ichthyosis	<i>KRT1, KRT10</i>
Superficial epidermolytic ichthyosis	<i>KRT2e</i>
Pachyonychia congenital	<i>KRT16, KRT6A, KRT6B, KRT17</i>
Incontinentia pigmenti <sup>a</sup>	<i>NEMO</i>
Hereditary porphyries	<i>UROD, UROS, PPOX, HMBS, FECH</i>
AEC syndrome <sup>a</sup>	<i>p63</i>
Hailey–Hailey disease	<i>ATP2C1</i>
Darier disease	<i>ATP2A2</i>

<sup>a</sup>In newborn.

structure which extends from the intracellular milieu of basal keratinocytes through the dermo-epidermal basement membrane to the upper papillary dermis. Such an intact network is required for a stable association of the two principal layers of the skin, the epidermis and the dermis, with the intervening basement membrane. Perturbation of this network structure through genetic mutations which lead to the absence of one of the structural components of these attachment complexes, or which perturb critical protein–protein interactions within these complexes, was initially predicted to result in fragility of the skin, in a manner that exposure to minor trauma results in blisters and erosions, characteristic of EB (78).

## 147.6.2 Candidate Genes and Mutation Analysis

Identification and characterization of the genes encoding the structural components of the dermo-epidermal attachment complexes, coupled with immunohistochemical characterization of the corresponding proteins, reveal that hemidesmosomes are composed of proteins encoded by four distinct genes (*COL17A1*, *ITGA6*, *ITGB4*, and *PLEC*) encoding type XVII collagen,  $\alpha 6\beta 4$  integrin, and plectin, respectively. These hemidesmosomal complexes interact with basal cell specific keratin intermediate filaments consisting of keratins 5 and 14, encoded by *KRT5* and *KRT14*. The anchoring filaments traversing the lamina lucida consist primarily of laminin-332 (previously known as laminin 5), a trimeric protein complex comprised of  $\alpha 3$ ,  $\beta 3$  and  $\gamma 2$  subunits encoded by the corresponding genes, *LAMA3*, *LAMB3* and *LAMC2*. Finally, the anchoring fibrils consist predominantly, if not exclusively of type VII collagen encoded by *COL7A1*. Thus, there are 10 distinct candidate genes which can possibly harbor mutations in the classic forms of EB. Subsequently, mutations in these 10 different genes have been identified in different forms of EB, and a large number of mutations have now been identified in different families (55,65). More recently, a consensus panel (1) concluded that three additional clinical conditions displaying fragility of the skin with blistering tendencies should be included in the epidermolysis bullosa diagnostic category. These include lethal acantholytic EB with suprabasal blistering, the KS with mixed level of tissue separation, and ectodermal dysplasia-skin fragility syndrome, with suprabasal tissue separation.



**TABLE 147-4 Diagnostic Laboratories**

Laboratory, Country, Website	Available Test
Laboratory of Molecular Therapy, eb house Austria, <a href="http://www.eb-haus.eu">www.eb-haus.eu</a>	IF, mutation analysis
Necker Enfants Malades Hospital, France, <a href="http://www.magec.eu/magec/pages/soins.html">www.magec.eu/magec/pages/soins.html</a>	IF, TEM, mutation analysis
EB Center Freiburg, Germany, <a href="http://www.netzwerk-eb.de">www.netzwerk-eb.de</a>	IF, mutation analysis
Laboratory of Molecular Dermatology, Tel Aviv, Israel	Mutation analysis
Hereditary Research Laboratory, Jerusalem, Israel	Mutation analysis
Laboratory of Molecular and Cell Biology, IDI-IRCCS Rome, Italy	IF, TEM, mutation analysis
Department of Dermatology Hokkaido University, Japan <a href="http://www.derm-hokudai.jp/en/index.html">www.derm-hokudai.jp/en/index.html</a>	IF, TEM
Center for Blistering Diseases, Groningen, The Netherlands	IF, TEM
Department of Genetics, Groningen, The Netherlands	Mutation analysis
Laboratory of Regenerative Medicine, Madrid, Spain <a href="http://www.ciberer.es">www.ciberer.es</a>	IF, mutation analysis
The National Diagnostic Epidermolysis Bullosa Laboratory, UK, <a href="http://www.guysandstthomas.nhs.uk/services/dash/dermatology/specialties/specialistlabs/ebtab.aspx">www.guysandstthomas.nhs.uk/services/dash/dermatology/specialties/specialistlabs/ebtab.aspx</a>	IF, TEM, mutation analysis
DeBRA Molecular Diagnostics Laboratory, Thomas Jefferson University, USA	Mutation analysis
Gene Dx, USA, <a href="http://www.genedx.com">www.genedx.com</a>	Mutation analysis
Stanford School of Medicine, USA, <a href="http://dermatopathology.stanford.edu/services/epiderm.html">dermatopathology.stanford.edu/services/epiderm.html</a>	IF, TEM
Beutner Laboratories, USA, <a href="http://www.beutnerlabs.com">www.beutnerlabs.com</a>	IF

IF, immunofluorescence; TEM, transmission electron microscopy.

These three conditions have been shown to harbor defects in the *DSP*/desmoplakin, *FERMT1*/kindlin-1, and *PKP1*/plakophilin 1 gene/protein systems (see Clinical Features section). An additional case study suggested that a previously unrecognized autosomal recessive form of EBS is caused by mutations in the *DST* gene encoding the epithelial isoform of the 230-kD bullous pemphigoid antigen (BPAG1-e) (47). Finally, lack of plakoglobin as a result of homozygous nonsense mutation in the *JUP* gene was shown to lead to lethal congenital EB (31).

Thus, collectively, there are as many as 16 genes that can harbor mutations in different variants of EB (79). The location of the gene products within the cutaneous basement membrane zone, the types and combinations of mutations, and the consequences of the mutations at the mRNA and protein levels explain the existence of different variants. Furthermore, this genetic heterogeneity, when placed on the individual's genetic background and exposure to external trauma provides an explanation for the considerable phenotypic variability even within a family with the same mutations.

### 147.6.3 Implications of Molecular Genetics for the Management of EB

**147.6.3.1 Genetic Counseling.** Identification of mutations in distinct genes in different variants of EB has made a significant impact on the management of patients with EB through improved diagnosis and subclassification with prognostic implications. Specifically, examination of the mutations in the candidate genes in a newborn can predict, in general terms, the overall prognosis of the affected individual. For example, stop codon mutations in both alleles of the type VII collagen

gene predict a severe phenotype characterized by extensive mutilating scarring and development of aggressive, rapidly metastasizing squamous cell carcinomas. In contrast, the presence of a glycine substitution missense mutation in one allele is likely to result in a significantly milder form of dystrophic form of EB with essentially normal lifespan but with lifelong blistering tendency (65).

Identification of the mutations in a newborn can also have profound consequences for genetic counseling concerning the risk of recurrence in the same or subsequent generations in families with no prior history of EB. This information is particularly useful in the dystrophic forms of EB that can be inherited either in an autosomal dominant or autosomal recessive pattern, occasionally with clinically indistinguishable phenotypes (80).

**147.6.3.2 Prenatal Diagnosis of EB.** Identification of the genes and specific mutations has made a major impact on prenatal testing for EB in families at risk for recurrence. Early on, prenatal diagnosis was based on fetal skin biopsy which required invasive procedures during the latter part of the second trimester, around 17–20 weeks gestation. The interpretation of the fetal skin biopsy by immunohistochemical evaluation or by ultrastructural analysis also required special expertise and personnel that was available only in a few centers globally. DNA-based testing from chorionic villus sampling is now readily available as early as the 10th week of gestation, or from amniocentesis at around 15 weeks (81,82). This testing can be based on either direct mutation analysis or genetic linkage utilizing informative markers within the flanking candidate genes. Such early DNA-based prenatal testing is now in routine use and available in research laboratories or in commercial gene testing laboratories (Table 147-4).

In order to avoid the complications of invasive procedures, including the risk of fetal loss, in chorionic vilus sampling or in amniocentesis, new approaches for prenatal testing from maternal blood have recently been developed. These are based on previous demonstrations of fetal cells and free fetal DNA in maternal circulation (83). Regarding the use of fetal cells as a source of DNA in maternal blood, the estimates of the number of such cells vary widely. It has been estimated, however, based on Y-chromosome specific markers, that there are 2–6 fetal cells per ml of maternal blood in mid-gestation. A number of different cell types have been identified, including trophoblasts, mesenchymal stem cells, nucleated erythrocytes (CD71<sup>+</sup>), and hematopoietic progenitor cells (CD34<sup>+</sup>). These cell populations can be enriched by different techniques, including flowcytometry, magnetic bead separation, density-gradient centrifugation and laser capture microscopy. While the use of fetal cells isolated from the maternal blood for prenatal diagnosis is still being developed, a significant concern relates to the fact that fetal cells can persist in maternal tissues for years after pregnancy (84). This would complicate the use of this technique in subsequent pregnancies. However, prenatal diagnosis for predicting the fetal genotype from fetal cells in maternal circulation has been successful in case of a number of diseases, including  $\beta$ -thalassemia, sickle cell anemia, and lamellar ichthyosis (83).

The maternal plasma also contains free fetal DNA that is easily detectable by PCR (85,86). The concentration of fetal DNA in maternal blood progressively increases during pregnancy, and it accounts for ~3–6% of total free DNA at term. This DNA is likely a reflection of trophoblast breakdown, as fetal free RNA, which has been shown to be placentally derived, can also be detected in maternal blood (87). The advantage of assaying the free fetal DNA in plasma is that it disappears very rapidly after the birth, and at 24 h post-partum very little, if any, fetal DNA can be detected. A limitation of prenatal testing by analysis of fetal free DNA in maternal circulation relates to the fact that the maternal allele in fetal DNA is masked by maternal DNA. Thus, the feasibility of utilizing this approach is limited to screening for a paternal allele of a paternally-derived dominant mutation or in compound heterozygotes. Nevertheless, this approach has been successfully used for prenatal diagnosis of cystic fibrosis, achondroplasia, congenital adrenal hyperplasia, and other conditions. Furthermore, the fetal *RHD* gene locus in rhesus negative mothers can be analyzed by this approach (83).

**147.6.3.3 Preimplantation Genetic Diagnosis.** An extension of DNA-based prenatal testing, preimplantation genetic diagnosis, can now be performed in connection with in vitro fertilization. This technology has advantages in terms of avoiding the ethical issues related to the termination of a pregnancy, if elected, but its utility has been somewhat limited by relatively low success of in vitro fertilization procedures in general, and the costs of this procedure. Nevertheless, the feasibility

of this approach has already demonstrated in case of patients with EB (88,89).

## 147.7 TREATMENT AND THERAPEUTICS

### 147.7.1 Prevention and Wound Care

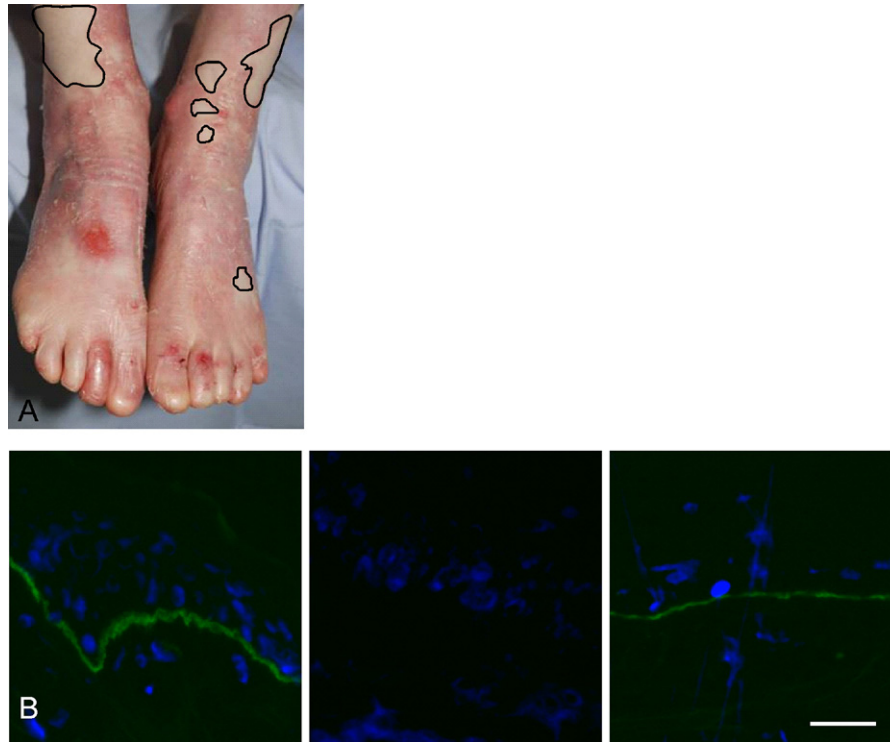
So far, no cure exists for EB, and the treatment is primarily supportive and symptomatic. The best practice encompasses protection from mechanical friction and trauma, combined with meticulous wound management and skin care. Strongly adhesive bandages should not be used, since they cause blistering in all EB subtypes. New blisters should be opened to reduce fluid pressure, but the blister roof not removed; it acts as a protective layer during re-epithelialization. Wound care consists of cleansing and application of appropriate non-adhesive dressings, such as hydroactive colloid gels or silicon-based dressings (90). Crèmes that further re-epithelialization are useful, and topical corticosteroids may reduce itch caused by secondary eczematization. Topical antibiotics are required for the treatment of secondary infections. In general, the wound management should aim at reducing pain and complications, such as infection, and at enhancing quality of life. In severe forms of EB, the secondary involvement of many other organs requires multi-disciplinary management in specialized centers.

### 147.7.2 Development of Molecular Therapies for EB

Not until very recently, there has been relatively little progress in developing effective and specific treatments for EB. However, identification of specific mutations in the candidate genes and elucidation of the consequences of such mutations have provided a basis for development of novel therapeutic approaches, taking advantage of the progress in molecular and cell biology in general areas. These approaches consist of gene therapy, protein replacements, or cell based therapies (79). Some of these approaches, such as allogeneic bone marrow transfer and fibroblast therapy, are in early clinical trials for patients with RDEB (91,92).

## 147.8 REVERTANT MOSAICISM

Revertant mosaicism is a naturally occurring phenomenon involving spontaneous correction of a pathogenic mutation in a somatic cell, and this has been described in different forms of EB, particularly in junctional, due to *COL17A1* mutations (93,94). Clinically, revertant mosaicism manifests with patches of normal appearing, nonblistering skin (see Figure 147-13). Besides providing a useful model system to study revertant mosaicism in general, revertant mosaicism may provide an opportunity for autologous cell therapy through propagation of keratinocytes from the revertant area. These cells can



**FIGURE 147-13** Revertant mosaicism in EB. (A) Normal appearing skin areas (outlined with black) surrounded by affected skin with erosions and scars in a patient with RDEB. (B) If staining with antibodies to collagen VII (green) of a control skin sample (left), a skin sample from a patient with severe generalized RDEB (middle), and biopsy from a revertant area of skin of the same patient (right). Nuclei are stained in blue with DAPI; bar = 50  $\mu$ m (Courtesy of Dr Dimitra Kirts, Freiburg).

then be incorporated into epithelial sheets, with subsequent grafting to the blistered and eroded areas (95).

### 147.9 ANIMAL MODELS

The value of animal models in advancing our understanding of the disease mechanisms in EB has been amply demonstrated by the development of genetically modified mice that recapitulate features of different forms of this disease (96). In addition, a number of naturally occurring mutations resulting in blistering phenotypes in larger animals, such as dogs, sheep, and horses, have been identified (97). These animal models serve now as a platform to develop molecular therapies for EB.

### 147.10 THE ROLE OF PATIENT ADVOCACY ORGANIZATIONS

Disease-specific advocacy organizations (also known as “support groups”), provide support and education to affected individuals and their families regarding their disease, its treatment, and the latest developments in research (98). These organizations are increasingly becoming involved in research through fund raising and grant support, as well as soliciting patients for clinical trials (99). The premiere patient advocacy organization for EB is the Dystrophic Epidermolysis Bullosa Research Association (DebRA) International, a network

**TABLE 147-5 Patient Organizations**

DebRA Web sites	
<a href="http://www.debra-international.org">www.debra-international.org</a>	<a href="http://www.debra.org.au">www.debra.org.au</a>
<a href="http://www.debra.org">www.debra.org</a>	<a href="http://www.debra.nl">www.debra.nl</a>
<a href="http://www.debra.org.uk">www.debra.org.uk</a>	<a href="http://www.debraitaliaonlus.org">www.debraitaliaonlus.org</a>
<a href="http://www.ieb-debra.de">www.ieb-debra.de</a>	<a href="http://www.ebae.org">www.ebae.org</a>
<a href="http://www.debra-austria.org">www.debra-austria.org</a>	

of national organizations representing the patients’ interests in the community and public policy arenas. New patients should be encouraged to contact such national patient advocacy organizations. The website information for DebRA International and Debra of America is included in Table 147-5.

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Chapters: Abnormalities of Pigmentation; Ichthyosiform Dermatoses; Diagnostic Molecular Genetics; Heterozygote testing and carrier screening; Techniques for Prenatal Diagnosis.

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## LIST OF WEBSITES RELAVANT TO EB

### Web Addresses

[www.debra-international.org](http://www.debra-international.org)  
[www.orpha.net/consor/cgi-bin/index.php?lng=EN](http://www.orpha.net/consor/cgi-bin/index.php?lng=EN)  
[www.guysandstthomas.nhs.uk/services/dash/dermatology/specialties/genetics.aspx](http://www.guysandstthomas.nhs.uk/services/dash/dermatology/specialties/genetics.aspx)  
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### Description

A worldwide network of patient support groups on EB  
 Rare Disorders  
 Information on disease and diagnostic laboratories  
 Genetic Dermatology  
 Information on disease, patient care and diagnostics  
 German EB Center  
 Information on disease, patient care and diagnostics  
 French National Center for Genetic Skin Disorders  
 Austrian EB Center  
 Information on disease, patient care and diagnostics  
 Australasian Blistering Disease Foundation  
 DebRA of America  
 Information, news and support for patients with EB  
 Mutation database  
 Human Intermediate Filament Database  
 International DEB patient registry  
 COL7A1 gene variants database  
 Clinical trials



### Biographies



**Cristina Has, MD**, studied medicine from 1985 to 1991 at the Medical University Cluj-Napoca, Romania. She received her MD degree, and completed her residency training in dermatology at the same university. She continued her postdoctoral work in genetics at the University of Münster, Germany and the National Center of Genotyping Evry, France. In 2003, Dr Has joined Dr Bruckner-Tuderman in the Department of Dermatology, managing the molecular genetic diagnosis of EB, and became Professor in 2012. Dr Has has experience in genetic testing for a number of inherited diseases, and is the author of numerous scientific articles on the genetics of EB and other genodermatoses. Her clinical and scientific specialties are blistering skin diseases, genodermatoses and molecular genetics of the skin.



**Leena Bruckner-Tuderman, MD**, has been Professor and Chair of the Department of Dermatology at the University Freiburg Medical Center in Freiburg, Germany since 2003. She studied medicine in Oulu, Finland, and after an experimental dissertation in molecular medicine, continued her postdoctoral work in biochemistry in Piscataway, NJ, USA, and in structural biology in Basel, Switzerland. She specialized in dermatology at the University of Zurich, Switzerland. She has held prestigious fellowships in Switzerland and Germany and has been Visiting Professor at the University of Hong Kong and at Harvard Medical School, Boston. The internationally recognized research of Leena Bruckner-Tuderman focuses on molecular genetics and disease mechanisms of skin fragility syndromes, biology of basement membranes and the extracellular matrix, epithelial-mesenchymal communication, and cell-matrix interactions, and more recently on development of cell-, gene- and protein-based therapies. Her publications include more than 300 original articles in peer-reviewed journals. Since 2007 Dr Bruckner-Tuderman has been a fellow and director of the School of Life Sciences–LifeNet of the Freiburg Institute for Advanced Studies, FRIAS and since 2009 she has been a founding member and one of the directors of Freiburg Center for Rare Diseases. She is a board member of many national and international foundations, programs, committees and editorial boards for journals in dermatology and venerology, as well as a member of the German Academy of Sciences Leopoldina, and a scientific advisor to the German Research Foundation DFG and the Federal Ministry for Education and Research, BMBF.



**Jouni Uitto, MD, PhD**, has been Professor of Dermatology and Cutaneous Biology, and Biochemistry and Molecular Biology, and Chair of the Department of Dermatology and Cutaneous Biology at Jefferson Medical College, in Philadelphia, Pennsylvania, since 1986. He is also Director of the Jefferson Institute of Molecular Medicine at Thomas Jefferson University. He received his MD and PhD degrees from the University of Helsinki, Finland, and completed his residency training in dermatology at Washington University School of Medicine, St. Louis, Missouri.

Dr Uitto is internationally recognized for his research on connective tissue biology and molecular genetics in relation to cutaneous diseases. Dr Uitto's publications include 623 original articles in peer-reviewed journals, 298 textbook chapters and review articles, and 920 abstracts on presentations in national and international meetings (7/2011). Dr Uitto has been the recipient of numerous national and international awards, including Honorary Doctoral degrees from the University of Kuopio, University of Oulu and University of Turku, all in Finland, as well as Honorary Professorship at China Medical University, Shenyang.

Dr Uitto has held office in several scientific and professional societies, including President of the Society for Investigative Dermatology; and President and Chairman of the Board of Trustees of Dermatology Foundation. Dr Uitto is also section editor of the *Journal of Investigative Dermatology*, associate editor of the *American Journal of Pathology*, and he is on the editorial boards of numerous peer-reviewed journals.

# CHAPTER

# 148

## Ectodermal Dysplasias

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### GLOSSARY

#### Ankyloblepharon

**Pronunciation** – āng'kə-lō-blēf'ə-rŏn'

**Function** – noun

**Definition** – Adhesion of the eyelids to each other; also called blepharosynechia

#### Eccrine

**Pronunciation** – ěk'rĭn

**Function** – adjective

**Definition** – of or pertaining to certain sweat glands, distributed over the entire body, which secrete a type of sweat important for regulating body heat

#### Ectoderm

**Pronunciation** – ek-tuh-durm

**Function** – noun

**Definition** – The outer germ layer of an animal embryo, which gives rise to epidermis and nervous tissue

#### Hypodontia

**Pronunciation** – hĭ'pō-dŏn'shē-ə, -shə

**Function** – noun

**Definition** – A congenital condition of having fewer than the normal number of teeth.

#### Hypohidrosis

**Pronunciation** – hĭ'pō-hĭ-drō'sĭs

**Function** – noun

**Definition** – Abnormally diminished sweating

#### Hypotrichosis

**Pronunciation** – hĭ'pō-trĭ-kō'sĭs

**Function** – noun

**Definition** – A less than normal amount of hair on the head or body.

is generally accepted that two or more systems must be affected in order for a condition to be defined as an ectodermal dysplasia syndrome. The ectodermal dysplasia syndromes may be associated with defects of additional organ systems as well. The mammary gland, thyroid gland, adrenal medulla, anterior pituitary, central nervous system, external ear, cornea, conjunctiva, lacrimal gland and lacrimal duct are other structures that are derived from the embryonic ectoderm. Congenital malformations of teeth, hair, nails, or sweat glands may also occur as single isolated malformations. Developmental disorders involving only one type of structure (teeth, hair, nails, or sweat glands), even if associated with other malformations, have arbitrarily been classified as not constituting an ectodermal dysplasia syndrome.

Almost 200 conditions have been classified as ectodermal dysplasias (1). Various classification schemes have been proposed over the past 30 years. Freire-Maia and Pinheiro published a monograph in 1984 (with later updates) that classified the disorders according to clinical features and associated defects (2–4). They divided the ectodermal dysplasias into groups, based on which of the basic ectodermal structures of hair, teeth, nails, or eccrine sweat glands were involved. Group A conditions had at least two ectodermal structures affected, while group B conditions had only one of the four major ectodermal structures involved, but had other ectodermal defects of the ears, lips, palms or soles. All possible combinations of two or more structures were described, resulting in 11 subgroups. However, the investigators realized that this taxonomic system is arbitrary, and did not take into account the pathogenesis or genetics of the specific disorders. Identification of the genes responsible for some of these disorders in humans and other animals, and the understanding of the developmental biology of such structures as hair, skin and teeth have advanced markedly during the past 15 years (5–7). These scientific advances have led to efforts to reclassify the ectodermal dysplasias on basis of molecular and developmental biology (8–13).

### 148.1 INTRODUCTION

The ectodermal dysplasias are a complex and heterogeneous group of disorders characterized by anomalies of ectodermal structures, including abnormalities of the teeth, hair, nails, sweat glands or other eccrine glands. These malformations result from developmental defects in tissues in which progenitor cells were originally derived from the ectoderm of the developing embryo. It

The ectodermal dysplasias can be grouped according to their molecular basis, including the categories of defects in the NF- $\kappa$ B signaling pathway genes, transcription factors, gap junctions, structural molecules and adhesive molecules. The development of epithelial appendages depends on intricate networks mediating epithelial–mesenchymal interactions during development. Genetic studies of the ectodermal dysplasias have helped define these networks, and have led to the elucidation of developmental pathways. Hopefully, these scientific discoveries will lead to molecularly-based therapies, with the possibility of treating or replacing defective and missing teeth, hair follicles, or eccrine sweat glands. In the future, new tissue engineering techniques using adult stem cells may be able to treat hypodontia postnatally (14). Gene delivery and therapy to existing hair follicles and skin is also being actively explored (15,16). Finally, even in utero therapy may be possible in the future to replace deficient proteins or genes needed for normal development. This has recently been done for the X-linked form of hypohidrotic ectodermal dysplasia in the *tabby* mouse (17).

All of the ectodermal dysplasias appear to be genetic in etiology, with every mode of Mendelian inheritance encountered among the syndromes. The actual number of distinct, non-allelic disorders is unclear. Although almost 200 conditions have been described, many have been reported in only one or two families, and there is

often significant clinical overlap among the conditions. Ultimately, a full understanding of the basic defects in these disorders will help determine how many individual disorders exist. Already a number of separately classified ectodermal dysplasia syndromes associated with orofacial clefting have been shown to be allelic and due to p63 mutations. There are no accurate population-based studies of the prevalence of this group of disorders. The most common condition among the ectodermal dysplasias is hypohidrotic ectodermal dysplasia. Individuals with this disorder, usually inherited as an X-linked recessive trait, account for 80% of families registered with the National Foundation for Ectodermal Dysplasias, an organization for families with individuals affected with ectodermal dysplasia in the United States. Various other ectodermal dysplasia syndromes account for the remaining membership, including ectrodactyly–ectodermal dysplasia–clefting (EEC) syndrome and other p63-related conditions, Clouston syndrome (hidrotic ectodermal dysplasia), incontinentia pigmenti, tricho–dento–osseous syndrome, Goltz syndrome and Witkop (tooth and nail) syndrome. A discussion of all of the ectodermal dysplasias is beyond the scope of this chapter, but the most common and classic ectodermal dysplasia syndromes are reviewed, with a discussion of their clinical features, genetics, diagnosis, and management. (See summary in Table 148-1.)

**TABLE 148-1 Genes and Chromosomal Regions for Selected Ectodermal Dysplasias**

Chromosome	Gene	Protein or Gene Product	Inheritance	Ectodermal Dysplasia (ED)	Protein Function	OMIM Number
Xp11.23	<i>PORCN</i>	Five isoforms (PORCA-PORCE)	XLD	Focal dermal hypoplasia (Goltz syndrome)	Membrane targeting and secretion of Wnt proteins necessary for embryonic tissue development	305600
Xq12–q13.1	<i>EDA1</i>	Ectodysplasin-A	XLR	XLHED	Triggering ligand molecule	305100
Xq28	<i>IKBKG (NEMO)</i>	IKK- $\gamma$ (NF-kappa-B essential modulator)	XLD XLR XLR	Incontinentia pigmenti 2 OL–HED–ID syndrome Hypohidrotic ED with immune deficiency	NF- $\kappa$ B cytoplasmic inhibitor	308300 300301 300291
1q32	<i>PKP1</i>	Plakophilin 1	AR	ED/skin fragility syndrome	Desmosomal plaque accessory protein	604536
1q42.2–q43	<i>EDARADD</i>	Ectodysplasin-A receptor adapter	AD or AR	ADHED and ARHED	Intracellular molecule adaptor of EDAR death domain	129490, 224900
2q11–q13	<i>EDAR</i>	Ectodysplasin-A receptor	AD or AR	ADHED and ARHED	Transmembrane receptor of EDA	129490, 224900
2q35	<i>WNT10A</i>	Wingless-type MMTV integration site family, member 10A	AR	Odonto–Onycho–Dermal dysplasia (OODD)	$\beta$ catenin-mediated specific intracellular signaling	257980
3q27	<i>TP63</i>	p63	AD AD	ADULT syndrome Ectrodactyly, ED, cleft lip/palate syndrome 3 (EEC3)	Transcription factor	103285 604292



**TABLE 148-1 Genes and Chromosomal Regions for Selected Ectodermal Dysplasias—cont'd**

Chromosome	Gene	Protein or Gene Product	Inheritance	Ectodermal Dysplasia (ED)	Protein Function	OMIM Number
			AD	Limb–mammary syndrome		603543
			AD	Ankyloblepharon—ectodermal defects—clefting (AEC)		106260
			AD	SHFM4 syndrome		605289
			AD	Rapp–Hodgkin syndrome (RHS)		129400
4p16.1	<i>MSX1</i>	Msx1	AD	Witkop syndrome	Transcription factor	189500
6q21–q23.2	<i>GJA1</i>	Connexin 43	AD	Oculo–Dento–Digital dysplasia (ODDD)	Connexin protein, intercellular junction	164200
7q11.2–q21.3	<i>EEC1</i>	Unknown	AD	Ectrodactyly, ED, cleft lip/palate syndrome 1 (EEC1)	Unknown	129900
11q23–q24	<i>PVRL1</i>	Nectin 1	AR	Cleft lip/palate–ED syndrome (CLPED1)	Tight junction cellular membrane stability	225060
			AR	Rosselli–Gulienetti syndrome		225000
12q13	<i>KRT6A</i>	Keratins 6A and 6B	AD	Pachyonychia congenita 1 and 2	Structural component of hair and nails	167200
	<i>KRT6B</i>				Structural component of hair and nails	167210
12q13	<i>KRTHBS</i>	Keratin 85	AD	ED, 'pure' hair–nail type	Structural component of hair and nails	602032
13q11–q12	<i>GJB2</i>	Connexin 26	AD	Palmoplantar keratoderma, with deafness	Connexin protein, intercellular junction	148350
			AD	Keratitits–Ichthyosis–Deafness syndrome, AD (KID, AD)		148210
			AD	Ichthyosis, hystrix-like, with deafness (HID syndrome)		602540
13q12	<i>GJB6</i>	Connexin 30	AD	Clouston syndrome	Connexin protein, intercellular junction	129500
14q13	<i>IKBA</i>	IκBα	AD	Hypohidrotic ED with immune deficiency	NFκB cytoplasmic inhibitor	164008
16q22.1	<i>CDH3</i>	Cadherin-3	AR	ED, ectrodactyly, and macular dystrophy (EEM)	Adhesion molecule cell–cell binding function	225280
17q12–q21	<i>KRT14</i>	Keratin 14	AD	Naegeli–France–schetti–Jadassohn syndrome	Structural component of hair and nails	161000
17q12–q21	<i>KRT16</i>	Keratins 16 and 17	AD	Pachyonychia congenita 1 and 2	Structural component of hair and nails	167200
	<i>KRT17</i>				Structural component of hair and nails	167210
17q21.3–q22	<i>DLX3</i>	Homeobox protein DLX-3	AD	Tricho–Dento–Osseous syndrome	Transcription factor	190320
19	<i>EEC2</i>	Unknown	AD	Ectrodactyly, ED, cleft lip/palate syndrome 1 (EEC2)	Unknown	

## 148.2 HYPOHIDROTIC ECTODERMAL DYSPLASIA

### 148.2.1 Clinical Features

X-linked hypohidrotic ectodermal dysplasia (XLHED), also known as anhidrotic ectodermal dysplasia or Christ–Siemens–Touraine syndrome (MIM 305100), is the most common ectodermal dysplasia syndrome. The majority of individuals with hypohidrotic ectodermal dysplasia have the X-linked form. It is characterized by hypotrichosis (sparseness of scalp and body hair), hypohidrosis (reduced ability to sweat), and hypodontia (congenital absence of teeth). This condition was first recognized by Dr John Thurnam in 1848 in two male cousins. In 1875, Charles Darwin discussed it in “The variation of animals and plants under domestication” (2nd ed.), saying:

*I may give an analogous case, communicated to me by Mr. W. Wedderburn, of a Hindu family in Scinde, in which ten men, in the course of four generations, were furnished, in both jaws taken together, with only four small and weak incisor teeth and with eight posterior molars. The men thus affected have very little hair on the body, and become bald early in life. They also suffer much during hot weather from excessive dryness of the*

*skin. It is remarkable that no instance has occurred of a daughter being thus affected.*

The gene was assigned to the X chromosome in 1921 by Thadani. In 1929, Weech reported additional cases and expanded the clinical description. Subsequently, it has been determined that, in addition to the common X-linked form of HED due to mutations in the EDA1 gene (XLHED), there are clinically indistinguishable autosomal recessive forms (ARHED) (MIM 224900) and milder autosomal dominant forms (ADHED) (MIM 129490) due to mutations in the EDAR and EDAR-ADD genes (18,19). The exact prevalence of HED is not known, but it is estimated that at least one in 5000–10,000 newborns has the disorder.

The majority of affected males with XLHED and males and females with ARHED have significant dental abnormalities. They have hypodontia and are missing most of their primary and secondary dentition. Occasionally, there may be complete anodontia. Nine permanent teeth are present on average (20). The central incisors and canines may have a conical or peg shape (Figure 148-1) and the molars may have hooked cusps. The alveolar ridges are noticeably hypoplastic, which can be a clue to absence of the underlying dentition in infants and young children.

There is hypotrichosis (Figure 148-2), with sparse scalp hair in childhood and subsequent premature balding in adolescents and adults. The hair tends to be hypopigmented, fine and short. There may be slow growth and excessive breakage of the hair shafts. Body hair and eyebrows are also sparse, and eyelashes may be thin to absent. However, beard and mustache hair are near normal in adults, and axillary and pubic hair may be present.



**FIGURE 148-1** Boy with XLHED showing conical teeth and sparse hair and eyebrows.



**FIGURE 148-2** Boy with XLHED.

Variable degrees of hypohidrosis are present, and significant episodes of hyperthermia frequently occur in infancy and early childhood. The eccrine sweat glands are sparse to absent, while apocrine glands are more normal. Sebaceous glands are also hypoplastic to absent. The skin is generally smooth, dry and soft and appears fragile. There are fine wrinkles around the eyes with persistent periorbital hyperpigmentation. Affected individuals are also susceptible to eczema and atopic dermatitis. There may be mild nail dystrophy, but it is less significant than in other forms of ectodermal dysplasia. There is often lack of dermal ridges.

The craniofacial appearance is distinctive, with frontal bossing, prominent supraorbital ridges, short nose with depressed nasal bridge (saddle nose), small alae nasi, maxillary hypoplasia, full lips and mild prognathism (Figures 148-3 and 148-4). The ears may be prominent. There is absence of tear production due to involvement of lacrimal glands. There is also a hoarse or raspy voice. Abnormal development of the nasal and respiratory mucosal glands leads to frequent respiratory infections in early childhood. Atrophic rhinitis with ozena (foul-smelling nasal discharge) and nasal concretions (solidified secretions in the nasal air passages) are problematic throughout life. Physical growth and psychomotor development are usually normal. Some individuals have difficulty gaining weight in infancy and early childhood, and maintain a thin body habitus.



**FIGURE 148-3** Boy with XLHED.

Although some affected individuals may have extensive peeling of the skin as newborns, most babies appear normal at birth, creating difficulties in the early diagnosis of the disorder. If the disorder is unrecognized, it can cause morbidity and even mortality (ranging from 2 to 20% based on varying reports) during infancy and early childhood, mainly due to the neurologic sequelae of unrecognized episodes of hyperthermia (21). Febrile seizures may occur in 5.9% of infants with XLHED and 17% of infants with other forms of HED. The diagnosis of HED is usually delayed until the teeth fail to erupt on time, or the teeth that do appear are noted to have an abnormal conical shape.

Female carriers of XLHED can have a broad range of clinical involvement; some may appear completely normal, while others may exhibit any or all of the characteristic features, including clinically significant hypodontia, hypotrichosis, and unilateral or bilateral hypoplasia of the breasts with deficient milk production during lactation. They may have patchy distribution of sweat function. In families with no affected males, on the basis of chance alone, there may be one or more manifesting female carriers, which can cause difficulties in diagnosis. Many mildly affected females are not diagnosed with the disorder until after a fully affected male is born in their family. The wide clinical variability seen in carrier females is due to the process of X-inactivation (Lyonization), and significantly complicates carrier detection based on physical examination. Female carriers of XLHED are mosaics of functionally normal and abnormal cells, with their clinical findings dependent on the percentage of abnormal cells in the progenitor cells that gave rise to their hair, teeth, and glands. Rarely, fully affected females may be observed due to a balanced X;autosome translocation,



**FIGURE 148-4** Girl with HED showing characteristic facies and sparse hair.

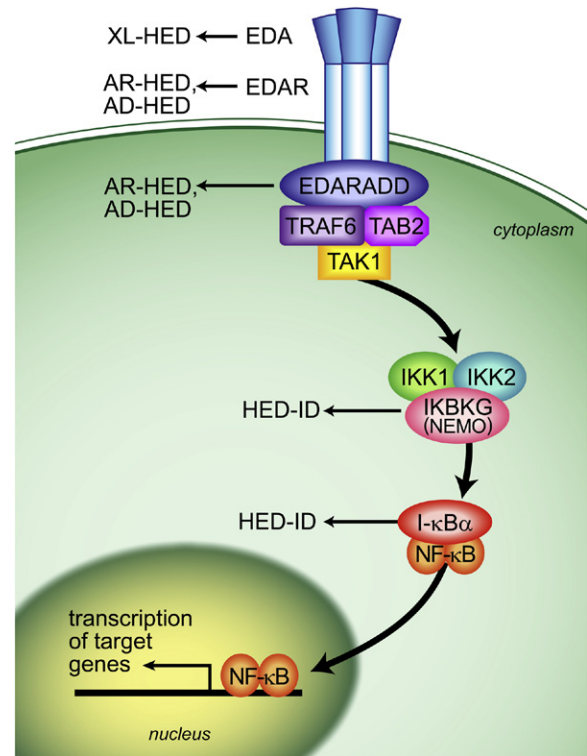


chromosomal aneuploidy (such as coexisting Turner syndrome), or extreme skewing in random X-inactivation. Males and females with ADHED typically have milder features of HED.

### 148.2.2 Genetics

XLHED is caused by mutations in the gene for ectodysplasin (EDA). The initial clue to location of the HED locus on the X chromosome came from the description of a female patient with complete manifestations of HED and an apparently balanced chromosomal rearrangement between one of her X chromosomes and chromosome 9. At least three other affected females with X;autosome balanced translocations have been identified, all with breakpoints in the Xq12–q13.1 region. Genetic mapping studies using linkage analysis confirmed the localization of the gene to the Xq12–q13.1 region. Positional cloning of the EDA gene was accomplished by identification of closely linked markers flanking the locus. The isoform of the gene isolated initially contained only two exons, and mutations were found in only 10% of affected individuals. However, the subsequent isolation of additional isoforms led to the identification of mutations in 95% of XLHED families. The active ectodysplasin isoform (EDA-A1) encodes a 135 amino acid protein, with a single transmembrane domain, and includes a collagen-like domain within the extracellular region. The protein is a member of the tumor necrosis factor (TNF) ligand super-family. The protein is expressed in the epithelial cells of developing skin and hair follicles. Ectodysplasin is the ligand in a developmental signaling pathway named the EDA signal transduction pathway (Figure 148-5) (22).

Mutations at three separate loci within the EDA pathway are responsible for clinically indistinguishable forms of hypohidrotic ectodermal dysplasia. The pathway consists of a ligand, ectodysplasin, its receptor (EDAR), and a receptor associated intracellular protein (EDARADD). The receptor complex transduces a signal to the nucleus by means of the NF- $\kappa$ B transcription factor, which ultimately leads to the appropriate cellular response (23). The ectodysplasin pathway shares several common downstream elements (NF- $\kappa$ B, IKK complex) with other TNF signal transduction pathways (24). Understanding of the role of the ectodysplasin pathway in both tooth and hair follicle development has been aided by experiments in wild-type and mutant mice (25–27). Of great potential future therapeutic importance in humans, treatment of pregnant *tabby* mice with a recombinant form of EDA1 engineered to cross the placental barrier permanently rescued the *tabby* phenotype in the offspring (17). Treatment of *tabby* mice and dogs with XLHED within days after birth with recombinant ectodysplasin has also been



**FIGURE 148-5** Ectodysplasin signal transduction pathway. (Adapted from Mikkola, M. L. *Molecular Aspects of Hypohidrotic Ectodermal Dysplasia*. Am. J. Med. Genet. A. **2009**, 149A (9), 2031–2036.)

shown to correct many of the symptoms of the disorder (17,28). The gene for the *downless* locus in the mouse was found to be the receptor (EDAR) for the EDA-A1 ligand. Mutations within the human homolog are responsible for both ARHED, and the milder ADHED (29) (Figure 148-6). Missense mutations within different domains of the receptor can result in different patterns of inheritance for the disorder. Missense mutations within the extracellular ligand-binding domain (C87R; R89H) cause autosomal recessive hypohidrotic ectodermal dysplasia. The mutations abolished or severely diminished EDAR *in vitro* binding to EDA-A1 (30). Other mutations in EDAR have also been identified (18). An even rarer autosomal recessive disorder also produces an identical phenotype to XLHED, and mutations have been found in *crinkled* mice and humans in an adaptor protein (EDARADD) that interacts with the EDAR receptor (31,32). The ectodysplasin pathway regulates NF- $\kappa$ B signaling through a key regulatory protein IKK-gamma (IKBKG, previously known as NEMO). Hypomorphic mutations affecting IKBKG function cause a closely related disorder hypohidrotic ectodermal deficiency with immunodeficiency (HED-ID) (33) (see further discussion below). Complete loss of function of IKBKG causes prenatal lethality in affected males, and causes the allelic disorder incontinentia pigmenti in females (34).





**FIGURE 148-6** Boy with autosomal recessive HED due to mutations in the EDAR gene. (With permission from Naeem, M.; Muhammad, D.; Ahmad, W. *Novel Mutations in the EDAR Gene in Two Pakistani Consanguineous Families with Autosomal Recessive Hypohidrotic Ectodermal Dysplasia*. *Br. J. Dermatol.* **2005**, 153 (1), 46–50.)

### 148.2.3 Diagnosis and Differential Diagnosis

X-linked hypohidrotic ectodermal dysplasia can be identified beyond infancy by physical signs and symptoms. The issue of genetic heterogeneity is important because, as noted previously, rare autosomal recessive and dominant forms of hypohidrotic ED, as well as HED-ID must be considered in the differential diagnosis. If families have only a single affected male individual or sibship,

autosomal recessive inheritance cannot be distinguished from X-linked recessive inheritance. The risk of having an affected male in the offspring of aunts, sisters, and daughters of an affected individual would be negligible if the condition in the particular family is inherited as an autosomal recessive trait, but may be as high as 25% if the trait is X-linked. It appears that the majority of families with hypohidrotic ectodermal dysplasia have mutations in the EDA1 gene located at Xq12–q13.1. However, the possibility of genetic heterogeneity should be included in the genetic counseling of families when only a single individual or sibship is affected, especially families with consanguinity or those with fully affected females. Fully affected females should have a karyotype performed to rule out an X;autosome translocation. If there is a history of significant or recurrent infections in a male with HED, the related disorder of hypohidrotic ectodermal dysplasia with immunodeficiency (HED-ID) due to IKBKG mutations must be considered (see further discussion below).

Clinical tests were developed in the past to help identify carrier females on the basis of dental and sweat pore analyses, but most had significant limitations in administration and subjective interpretation, as well as overlap of the findings between the normal and carrier populations. Diagnostic mutation analysis of the EDA gene can overcome the problems presented by the phenomenon of lyonization in carrier testing.

Molecular testing can help distinguish the different types of HED, and assist in determining whether females are carriers of XLHED. Direct mutation analysis of EDA is clinically available for the X-linked form, including sequencing of the EDA gene as well as deletion analysis. The EDA gene would usually be analyzed first, unless autosomal recessive inheritance was suggested by the presence of a severely affected female or consanguinity within the pedigree. Several groups, surveying over 100 patients with presumed XLHED, have identified numerous EDA mutations (30,35,36). Mutation detection rates varied from 63% to 95%, due to the detection methods utilized and patient selection. Mutation detection rates were higher in typically affected male patients, and in families with more than one affected generation. There are a few recurrent mutations, especially in codons 155 and 156, and specifically the R156H mutation, but many families have private mutations. Deletions are identified in at least 10% of cases. Clinical molecular testing of the receptor (EDAR) is also available for families with possible autosomal recessive or dominant forms of the disorder. EDARADD mutations have only been described in a few families, and clinical testing for this form of the disorder is not yet available. As with most clinical molecular testing, inability to find a mutation in a family reduces the probability that the family has a specific form of HED, but does not eliminate it.

### 148.2.4 Management and Counseling

There is intrafamilial and interfamilial variability in the degree of hypohidrosis and hypodontia. The identification of affected individuals at an early age is extremely important to avoid episodes of uncontrolled hyperthermia due to the inability to sweat, and to prevent potential central nervous system damage or even death. Hot environments and fevers must be managed with cooling measures or antipyretics. Affected individuals must have access to an adequate supply of water and a cool environment. Wearing a “cooling vest” or a wet T-shirt and using a spray bottle of water when outdoors can help maintain a cool body temperature. With appropriate measures, normal life expectancy and intelligence can be achieved. Dry skin and eczema are seen in most affected individuals and require skin care products and possibly dermatological consultation. The nasal mucosa is also involved with problems of crusting and ozena, and this can be treated with the application of nasal solutions. Regular visits with an ENT physician may be necessary to manage the nasal and aural concretions (37). Dental treatment must begin at an early age and is needed throughout childhood, due to the absence of most of the deciduous and permanent dentition. Bonding of the conical shaped teeth in young children may be done to improve the appearance and chewing ability. Hypodontia is treated with dentures beginning early life, as early as three years of age, and dental implants are a treatment option in older individuals (38–40).

Families can benefit from a medical genetic evaluation for both diagnostic and reproductive counseling purposes. Affected individuals may have a prior family history of the disorder, but in many cases there is only a single affected individual representing a new mutation in the gene. Females who carry an EDA gene mutation have a 25% risk of having an affected son and a 25% risk of having a carrier daughter with each pregnancy. Affected males cannot pass the trait on to their sons, but all of their daughters are carriers of the disorder and may show variable clinical expression.

Identification of the carrier status of at-risk females is possible by physical examination in some female carriers, when the findings are unambiguously abnormal, with examination of the dentition being the most reliable approach. Subclinical tests of sweating can be misleading, and subjective assessment of heat intolerance and thin hair should be avoided. Clinical availability of direct mutation analysis allows diagnosis and carrier testing for XLHED. Such testing and counseling of at-risk females would best be deferred until reproductive age.

As with many X-linked disorders, female carriers of EDA mutations may request prenatal diagnosis of the disorder, while others have chosen testing of their newborn males postnatally to see if they are affected. Prenatal diagnosis of EDA or EDAR mutations using direct

DNA-based diagnostic testing is feasible. Pre-implantation genetic diagnosis may be available for families in whom the specific mutations have been identified.

## 148.3 ODONTO-ONYCHO-DERMAL DYSPLASIA (OODD) SYNDROME (MIM 257980)

### 148.3.1 Clinical Features

Odonto-onycho-dermal dysplasia (OODD) syndrome, a form of hypohidrotic ectodermal dysplasia, has been shown to be caused by mutations in the WNT10A (wingless-type MMTV integration site family, member 10A) gene (41). There is a broad spectrum of clinical features in these patients, typically including significant hypodontia as well as abnormal fingernails and toenails (42). Most cases are inherited in an autosomal recessive fashion, associated with homozygous or compound heterozygous WNT10A mutations, but up to 50% of heterozygotes may display some clinical features.

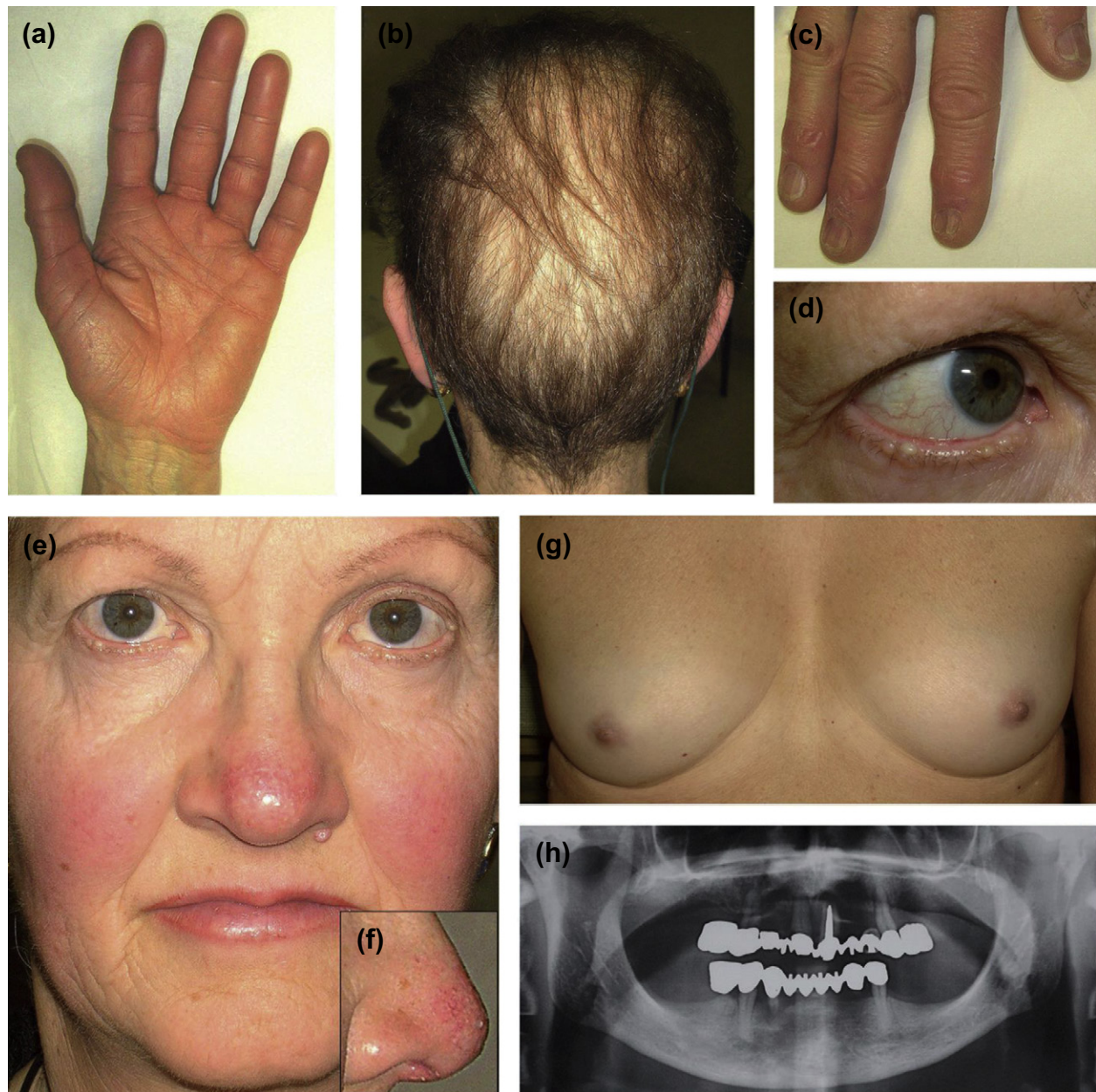
The most consistent clinical feature is severe hypodontia of the permanent teeth. Some patients may have anodontia. The deciduous teeth are often nearly normal in number, but may be small and widely spaced. There may be a smooth tongue with reduced fungiform and filiform papillae. Hair is absent at birth, and older individuals have dry and thin hair and may have sparse eyebrows. When examined by electron microscope, the hair shaft has longitudinal depressions. Skin manifestations include palmar erythema and keratoderma of the palms and soles. Hyperhidrosis involving the palms and soles is commonly present. Some affected individuals may have hypohidrosis and keratosis pilaris elsewhere on the body. Histologically, the skin shows orthokeratosis, hyperkeratosis, hypergranulosis and mild acanthosis in the epidermis. The fingernails and toenails are dystrophic (onychodysplasia) and there can be congenital absence of nails (anonychia).

Schopf-Schulz-Passarge syndrome (SSPS) is a rare disorder characterized by eyelid hidrocystomas (eyelid cysts) in association with other findings of ectodermal dysplasia similar to those seen in the OODD syndrome (43) (Figure 148-7). Recently, this disorder was also shown to be due to mutations in the WNT10A gene (44,45).

### 148.3.2 Genetics

Mutations in the WNT10A gene have been reported in up to 9% of individuals with ectodermal dysplasia and in 25% of individuals with hypohidrotic ectodermal dysplasia who do not have a mutation in the EDA gene (46,47). A recent report indicates that WNT10A mutations may be more common than previously suspected (48). Sixty-five cases of HED were analyzed for mutations in EDA, EDAR, EDARADD and WNT10A; 58% had EDA mutations, 16% had EDAR mutations and 16% had WNT10A mutations for a total detection rate of 90%, and only one





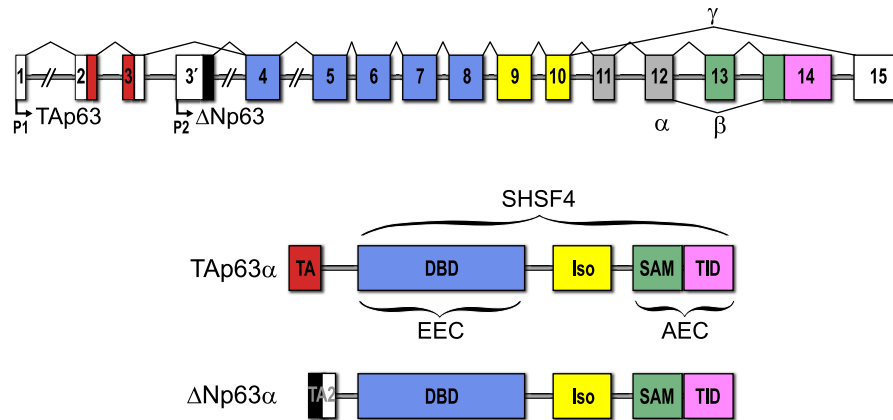
**FIGURE 148-7** Woman with Schopf-Schulz-Passarge syndrome due to WNT10A mutations. (a) Palmo-plantar keratoderma. (b) Sparse, thin scalp hair. (c) Onychodystrophy. (d) Opalescent and translucent cyst-like lesions of the eyelid margins. (e) Telangiectatic rosacea. (f) Dis-seminated milia over the nasal tip and columella. (g) Bilateral hypoplastic nipples. (h) Panoramic radiograph showing severe oligodontia. (With permission from Castori, M., et al. Schopf-Schulz-Passarge Syndrome: Further Delineation of the Phenotype and Genetic Considerations. *Acta Derm. Venereol.* 2008. 88(6): p. 607-612.)

case had an EDARADD mutation (48). Molecular genetic testing is clinically available for WNT10A.

#### 148.4 P63-RELATED ECTODERMAL DYSPLASIA SYNDROMES

There are multiple well-recognized ectodermal dysplasia syndromes that are associated with mutations in the p63 gene (49). These disorders are a heterogeneous group with some overlapping clinical features, but there are also distinctive features that allow them to be separated into different syndromes. The disorders include ectrodactyly-ectodermal

dysplasia-clefting (EEC) syndrome (MIM 604292), ankyloblepharon-ectodermal defects-cleft lip/palate (AEC) or Hay-Wells syndrome (MIM 106260), limb-mammary syndrome (LMS) (MIM 603543), acro-dermato-ungual-lacrimal-tooth (ADULT) syndrome (MIM 103285), Rapp-Hodgkin syndrome (RHS) (MIM 129400) and split hand-split foot malformation (SHFM4) syndrome (MIM 605289). The differences in the phenotypes are linked to the domain of the protein in which the mutation is located. The p63 gene, which is located at 3q27, encodes a transcription factor that has a critical role in epithelial appendage development (50) (Figure 148-8). At least six different



**FIGURE 148-8** p63 gene structure and two of the protein isoforms, indicating the locations of the typical mutations for EEC, AEC and SHFM4 syndromes.

transcripts can be generated by alternative promoter usage and splicing. The protein domains include an amino-transactivating domain, a core DNA-binding domain, a carboxy-oligomerization domain, a sterile alpha motif (SAM) domain and a transactivation inhibitory domain (TID). The DNA binding domain and an oligomerization domain are shared by all isoforms, while only a subset of isoforms has a transactivation inhibitory domain or a SAM domain. The SAM domain is devoted to protein–protein interaction and is implicated in developmental processes. The p63 protein has been shown to be localized to the nuclei of basal cells in stratified epithelium, including the skin, oral mucosa, cervical and vaginal epithelium, urothelium and breast (51,52).

## 148.5 ECTRODACTYLY, ECTODERMAL DYSPLASIA, AND CLEFT LIP/PALATE SYNDROME (MIM 604292)

### 148.5.1 Clinical Features

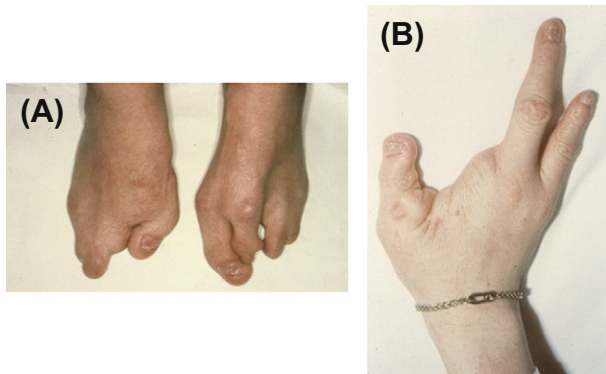
Ectrodactyly–ectodermal dysplasia–clefing (EEC) syndrome is the most common ectodermal dysplasia syndrome associated with mutations in the p63 gene. In addition to abnormalities of hair, skin and teeth, patients with classic EEC syndrome have cleft lip and palate and ectrodactyly (split hands or feet). There is a characteristic facies with maxillary hypoplasia, a broad nasal tip and a short philtrum (Figure 148-9). The scalp hair is sparse and fine, and there may be patches of alopecia. The eyebrows and eyelashes are sparse as well. The nails may be thin and brittle, or may have pits. Dental abnormalities may include hypodontia, conical-shaped teeth, and enamel dysplasia leading to an increased risk of caries. The skin may be thin and dry resembling dermatitis and with an increased susceptibility to eczema. However, sweating is relatively normal in this condition. The majority of cases have clefting, usually cleft lip and palate, although either isolated cleft lip or isolated cleft palate can be seen. Blepharitis, keratitis, and dacryocystitis due to absent or hypoplastic lacrimal



**FIGURE 148-9** Boy with EEC and repaired cleft lip and palate.

puncta can occur. The characteristic limb defects include ectrodactyly (split hands and feet) and syndactyly, but the presentation is variable (Figure 148-10). All four extremities may be affected, only one or two, or none at all. Central nervous system malformations have been reported in EEC syndrome, with growth hormone deficiency due to hypothalamic–pituitary insufficiency in a few cases, and semilobar holoprosencephaly associated with hypogonadotropic hypogonadism and central diabetes insipidus in another case. Choanal atresia has been reported occasionally. Genitourinary anomalies are frequently found and





**FIGURE 148-10** A and B. EEC feet and hand.

can include hydroureter, hydronephrosis, and renal agenesis. Mental retardation is rare in EEC syndrome. There is significant intrafamilial and interfamilial variability in the associated malformations.

### 148.5.2 Genetics

EEC syndrome has an autosomal dominant inheritance pattern with highly variable expression. EEC syndrome has non-allelic heterogeneity and has been localized to three different loci, EEC1 (MIM 129900), EEC2 (MIM 602077) and EEC3 (MIM 604292), but only the p63 gene at 3q27 has been identified thus far as the molecular basis of EEC3, and p63 mutations are found in 98% of classic EEC syndrome cases (53). EEC1 has been mapped to 7q21 based on translocations and deletions in patients with syndromic split hand and split foot and by linkage analysis (54,55). EEC2 has been mapped to chromosome 19 in one family. The predominant mutations seen in the EEC syndrome are amino acid substitutions in the DNA-binding domain of the protein while mutations in other parts of the protein cause related clinical conditions (56).

### 148.5.3 Management and Counseling

Treatment for EEC syndrome involves surgical correction of the cleft lip and palate, as well as management of any associated speech, dental, or hearing problems. Treatment for lacrimal duct anomalies is also frequently needed. Orthopedic or plastic surgery management of the hand and foot malformations, and occupational therapy intervention, should be employed as needed. Hypodontia can be treated with appropriate prosthodontic care, including dental implants. All patients should be evaluated for clinically significant genitourinary anomalies, and if growth disturbance or any other endocrine abnormality is present, an evaluation of the hypothalamic–pituitary axis would be indicated. In families with a clear clinical diagnosis, autosomal dominant inheritance should be presumed. Incomplete expression or even non-penetrance may occur, the latter perhaps due to gonadal mosaicism, which should be considered in the genetic counseling.



**FIGURE 148-11** Boys with AEC syndrome showing the scarring alopecia.

Direct molecular diagnosis is available to detect p63 mutations. Prenatal diagnosis could be done by molecular diagnosis. It has also been accomplished by ultrasonographic detection of the involved structures. However, the wide degree of clinical variability should be stressed.

## 148.6 ANKYLOBLEPHARON, ECTODERMAL DEFECTS, CLEFT LIP/PALATE (MIM 106260)

### 148.6.1 Clinical Features

Ankyloblepharon, ectodermal defects, and cleft lip/palate (AEC) syndrome, also known as Hay–Wells syndrome, is another disorder caused by p63 mutations. Affected individuals may have coarse, wiry, sparse hair, or may have scarring of the scalp with almost total alopecia (Figure 148-11). Almost all newborns have superficial skin erosions that can be patchy, or can involve large areas of the body. The erosions are most often on the scalp. Nails are absent or dystrophic. Sweating is subjectively decreased in this condition, but does not cause hyperthermia as in HED. The teeth may be conical in shape and reduced in number, and maxillary hypoplasia may be present. It has been reported that adults with AEC have an average of 4.75 permanent teeth (57). Cleft lip and/or palate are frequently associated, and alveolar synechiae (congenital adhesions between the upper and lower jaws) are occasionally seen. Fusion of the eyelids (ankyloblepharon filiforme adnatum) is a distinctive feature of the disorder seen in about 70% of cases and is a clue to the diagnosis in affected newborns. Lacrimal puncta may be absent with associated chronic blepharitis (58). The eyelashes may also be absent.

### 148.6.2 Genetics, Diagnosis, and Differential Diagnosis

AEC syndrome is an autosomal dominant disorder, allelic to the EEC3 locus, and caused by mutations in

the p63 gene (59–61). About 80% of the p63 mutations in AEC syndrome result in amino acid substitutions in the sterile alpha motif (SAM) domain and about 20% are in the transactivation inhibitory domain (TID) of the protein. The mutations are predicted to affect protein–protein interactions. Rapp–Hodgkin syndrome (RHS) is an allelic disorder with the AEC syndrome; these two disorders are very similar clinically and are primarily differentiated by the presence of ankyloblepharon in AEC syndrome (60,62). Some authors have suggested that AEC and RHS should be considered as the same syndrome, and that RHS is part of the spectrum of AEC syndrome. Clinical molecular testing of the p63 gene for AEC syndrome is available. It is generally suggested that sequencing of exons 13 and 14 should be done first, since these exons encode the SAM and TID domains where the vast majority of mutations are found. Sequencing of the rest of the p63 gene could be done if no mutation is found in exons 13 and 14, since rare AEC/RHS-like cases have had mutations outside these exons, and because there is quite a bit of clinical overlap among the other syndromes caused by p63 mutations. Deletion and duplication testing could also be done, although thus far, this has not been reported in AEC or RHS. Somatic and/or germline mosaicism has been suggested in a few families with multiple affected children and normal parents.

### 148.6.3 Management and Counseling

Dermatological evaluation and ongoing management of the skin erosions and other skin problems is required. Early attention should be given to the potential occurrence of serious scalp infections and sepsis in affected infants and children. Referral to plastic surgery for surgical correction of the cleft lip and/or cleft palate is needed, with follow-up treatment for any associated speech or hearing problems. Surgical treatment of the ankyloblepharon may be necessary, although some of these lyse spontaneously. Many individuals need ophthalmological care for lacrimal duct obstruction, dry eyes and blepharitis. Dental anomalies and hypodontia should be treated with appropriate prosthodontic care. Some infants and young children have growth and nutritional problems, and the involvement of a gastroenterologist and dietitian may be needed (63).

Genetic counseling should be provided regarding the autosomal dominant inheritance of the disorder. About 30% of affected infants have an affected parent and 70% represent new mutations. Somatic and germline mosaicism has been suspected in several families, and this should be discussed with regard to recurrence risks for future pregnancies. Prenatal molecular genetic diagnosis for the disorder is possible if a p63 mutation has been identified in the family.

## 148.7 RAPP–HODGKIN SYNDROME (MIM 129400)

### 148.7.1 Clinical Features

Rapp–Hodgkin syndrome (anhidrotic ectodermal dysplasia with cleft lip/palate) is another ectodermal dysplasia syndrome with orofacial clefting, which is also inherited as an autosomal dominant trait. However, ectrodactyly is not seen in this disorder. The Rapp–Hodgkin syndrome was originally described in a family with anhidrotic ectodermal dysplasia, cleft lip and palate, a narrow nose, and a small mouth. Trichodysplasia is present, with the scalp hair in childhood coarse and wiry, with microscopic descriptions of the hair reporting both pili torti (twisted hair) and pili canaliculi (canal-like depressions running the length of the shaft) (Figure 148-12). Alopecia of the scalp occurs in adulthood in affected females and males, as well as sparseness of the lateral eyebrows and eyelashes. Hypodontia, conical shaped teeth, and dysplastic nails occur. Although the numbers of sweat glands are reported to be reduced, clinical hyperthermia is usually not a problem. Submucous cleft or cleft of the uvula may be present, rather than frank clefting, along with maxillary hypoplasia. Occasional findings have included agenesis/atresia of the lacrimal puncta, hypospadias, atretic ear canals, and malformed auricles.

### 148.7.2 Genetics

Rapp–Hodgkin syndrome is inherited as an autosomal dominant trait with variable expression. This condition has significant clinical overlap with both the EEC and AEC syndromes, and they are all allelic disorders associated with mutations of the p63 gene. Mutations occur mainly in the SAM domain or the TID domain of the p63 protein (60,62,64–66). As mentioned above, RHS is considered by some authors to be part of the spectrum



**FIGURE 148-12** Girl with AEC/RHS syndrome showing the abnormal hair.

of AEC syndrome, and not a separate entity. Please refer to the molecular genetic testing strategy outline for AEC syndrome above.

### 148.7.3 Management and Counseling

The treatment for this disorder is essentially the same as for AEC. Management involves surgical correction of the cleft lip and palate and treatment of any speech or hearing problems. Surgical correction for atresia of the lacrimal puncta may be required. Hypodontia requires appropriate prosthodontic care, including dental implants in severe cases. Severe alopecia and balding can be treated with a wig. Families should be counseled that the disorder is inherited as an autosomal dominant trait with variable expression. At-risk individuals should be examined for any minimal involvement with the disorder. Molecular genetic diagnosis is possible by sequencing of the p63 gene, beginning with exons 13 and 14, and prenatal diagnosis could be performed if the mutation in the family is known.

## 148.8 ADULT (ACRO-DERMATO-UNGUAL-LACRIMAL-TOOTH) SYNDROME (MIM 103285)

ADULT syndrome resembles EEC syndrome but also displays excessive skin freckling (67). As with other syndromes allelic to EEC, mutations in the p63 gene have been described in ADULT syndrome, one in the DNA binding domain (DBD) of the gene that results in a possible unique gain-of-function (68,69) and others in the TA2 domain and just before the DBD (70).

## 148.9 LIMB-MAMMARY SYNDROME (MIM 603543)

Limb mammary syndrome (LMS) was originally described with major features of severe hand and foot anomalies with hypoplasia/aplasia of the mammary glands (56,71). However, minor features affecting the ectodermal appendages were also noted. Mutations of the p63 gene were subsequently described in families with LMS within the p63 SAM domain (53).

## 148.10 SPLIT HAND/SPLIT FOOT MALFORMATION (SHFM4) (MIM 605289)

Isolated ectrodactyly (split hand/split foot malformation) can be inherited as an autosomal dominant trait without other malformations. Five separate genetic loci have been identified in isolated split hand/split foot malformation. p63 plays an important role in the regulation of the formation and differentiation of the apical ectodermal ridge (AER), which is critical for limb development. p63

mutations were found in 4 of 35 families with SHFM in one study (53).

### 148.10.1 Differential Diagnosis

Roselli–Gulienetti (MIM 225000) syndrome is an ectodermal dysplasia syndrome with associated clefting but can be distinguished by the associated features. Odontotrichomelic (cleft lip/palate, tetrapromelia, and deformed pinnae), Bowen–Armstrong, and LADD syndromes all have some phenotypic overlap with EEC syndrome and the other p63-related disorders. The EEM (ectodermal dysplasia, ectrodactyly, and macular dystrophy) syndrome is inherited as an autosomal recessive trait and has been shown to be due to mutations at a separate genetic locus (CDH3) (72). Curly hair–ankyloblepharon–nail dysplasia syndrome (CHANDS) has some overlapping features, but does not have the skin changes or clefting seen in p63-related disorders. Ankyloblepharon filiforme adnatum can be inherited as a separate autosomal dominant trait. Some infants with AEC syndrome are misdiagnosed as having epidermolysis bullosa because of the skin erosions, and an incorrect diagnosis of ichthyosis might be made initially if a newborn with AEC has erythroderma or a collodion membrane.

## 148.11 HIDROTIC ECTODERMAL DYSPLASIA (CLOUSTON SYNDROME) (MIM 129500)

### 148.11.1 Clinical Features

Hidrotic ectodermal dysplasia, also known as Clouston syndrome, is an autosomal dominant disorder that affects the hair, nails, and skin. This condition was first reported in a large kindred that was originally from France and emigrated to Canada, the northeastern United States, and Scotland. However, Clouston syndrome has been reported in diverse ethnic and racial groups as well. There is complete penetrance with variable expressivity.

Individuals with Clouston syndrome have normal sweat and sebaceous gland function and normal teeth. The hair is abnormal and is fine, brittle, and slow-growing (Figure 148-13). Females may have total alopecia, while males may have patchy alopecia, typically with more hair present than affected females. The eyebrows and eyelashes are sparse. Axillary and pubic hair may be absent or sparse. The hairs have reduced tensile strength and a disorganized fibrillar structure with decreased cysteine and disulfide bonds and reduced birefringence in polarized light. Another characteristic feature is palmo-plantar hyperkeratosis. The palms and soles are thick and dyskeratotic. The nails are typically severely dystrophic. Nails can be discolored, thickened, and dystrophic,





**FIGURE 148-13** (A–D) Clouston syndrome.

or hypoplastic with a cone-shaped or triangular nail. The distal ends of the fingers are thickened and may appear clubbed. The skin of the knuckles, elbows, axillae, areolae and pubic area may be hyperpigmented. Breast development in females is normal. Ophthalmological defects include cataracts, photophobia, strabismus, conjunctivitis or blepharitis.

### 148.11.2 Genetics, Diagnosis and Differential Diagnosis

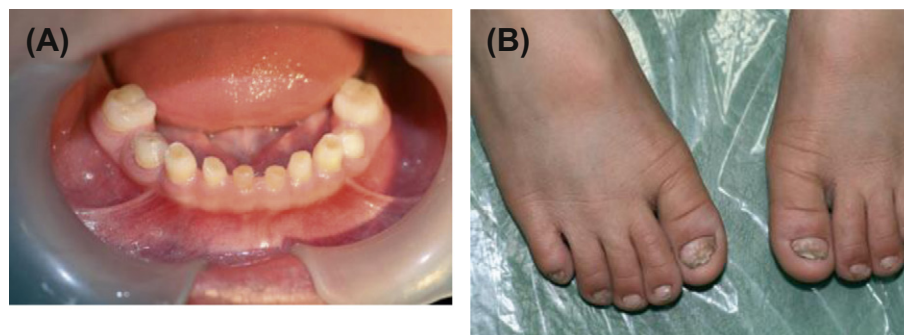
Clouston syndrome is caused by mutations in the gap junction protein beta-6 gene (GJB6). In 1996, Kibar mapped the locus for this disorder to the pericentromeric region of chromosome 13q, using linkage analysis in eight French-Canadian families. Lamartine et al. (2000) demonstrated mutations in the gene encoding connexin-30. One mutation, Gly11Arg, was found in individuals from several areas of Europe. More than 20 different connexin proteins have been identified. These proteins form connexons and allow communication between adjacent cells at gap junctions (73). Connexons are intercellular channels that regulate the exchange of ions, small metabolites, and secondary messengers between the cytoplasm of adjacent cells. Cell culture transfection studies of GJB6 mutations in Clouston patients demonstrated impaired trafficking of the protein to the plasma membrane, preventing the formation of functional Cx30 gap junctions. Mutations in different connexins are associated with hearing loss, and other dermatological and neurological disorders (74). Another missense mutation in the GJB6 gene causes autosomal dominant nonsyndromic

sensorineural deafness (DFNA3; MIM 601544). Mutations in GJB2 (connexin 26) cause non-syndromic deafness, palmoplantar hyperkeratoderma and the autosomal dominant syndrome Keratitis–Ichthyosis–Deafness (KID) (see further discussion below). Deafness is an uncommon feature in Clouston syndrome, although it has been reported.

The diagnosis of Clouston syndrome is based on the clinical findings and observation of an autosomal dominant pattern of inheritance. Molecular genetic testing of the GJB6 gene is clinically available. Targeted mutation analysis for the four common mutations, Gly11Arg, Ala-88Val, Val137Glu and Asp50Asn, should be performed first, followed by full sequencing if one of these mutations is not found (75–77). All individuals with typical features of Clouston syndrome have been found to have mutations in GJB6.

The condition may be distinguished from a number of other types of ectodermal dysplasias by its major involvement of the hair and nails, but with both normal teeth and sweat gland function. It should be distinguished from other conditions with alopecia and nail dysplasia, including onychodystrophy and alopecia inherited as simple autosomal dominant traits. Similar skin involvement of the palms and soles can be seen keratosis palmoplantaris with periodontopathia (MIM 245000). Pachyonychia congenita types 1 and 2 have focal palmoplantar hyperkeratosis and hypertrophic nail dystrophy, but the hair is not affected. Autosomal dominant and recessive hypohidrotic ectodermal dysplasia may have overlapping hair characteristics, but are associated with hypodontia and hypohidrosis, not seen in Clouston syndrome.





**FIGURE 148-14** A and B. Witkop syndrome with abnormal nails and teeth. (With permission Memarpour, M.; Shafiei, F. *Witkop Tooth and Nail Syndrome: A Report of Three Cases in a Family*. *Pediatric Dermatology* **2011**, 28 (3), 281–285.)

### 148.11.3 Management and Counseling

Dermatological treatment for the skin and nail involvement is recommended. Emollients can be used to treat the palmoplantar hyperkeratosis, which tends to worsen with age. The use of artificial nails may improve the appearance of the hands and feet. Total alopecia can be treated with wigs or hair pieces as appropriate. Families should be counseled that the condition is inherited as an autosomal dominant trait with variable expression. At-risk individuals should be examined closely for signs of the disorder because of the variable expression. Molecular diagnosis of mildly affected family members may be helpful if the GJB6 mutation is known. Pre-implantation and prenatal diagnosis are potentially available, again if the GJB6 mutation has been previously identified in the family.

## 148.12 TOOTH AND NAIL SYNDROME (WITKOP SYNDROME) (MIM 189500)

### 148.12.1 Clinical Features

Witkop syndrome is an autosomal dominant ectodermal dysplasia syndrome, first described by Dr. Carl Witkop in 1965. It is also called tooth and nail syndrome, since affected individuals have hypodontia and nail dysplasia (Figure 148-14). The incidence is about 1–2 per 10,000. The hair is usually normal, but is sometimes thin or fine. Heat tolerance and sweat gland function are normal. The nails are described as being small, spoon-shaped and slow-growing, and may fracture easily. Longitudinal ridges or pits in the nails may be seen. The nail dysplasia is usually more severe in childhood and may improve with age, such that there may be no obvious nail abnormalities in affected adults (78). Also, the toenails often have more significant abnormalities than the fingernails. Dental abnormalities include congenital absence of primary and/or permanent teeth. Sometimes, the abnormalities of the primary dentition are very subtle, and the condition is not recognized until the permanent teeth fail to erupt normally. The most common missing teeth are the mandibular incisors, maxillary canines and second molars. The teeth may be malaligned, conical in shape, or widely spaced. The teeth are described as being

parallel-sided with narrow crowns, or tapering toward the incisal edge. It is unusual for affected individuals to have more than 20 missing teeth, but this has been seen occasionally; more often, there are only a few missing teeth.

### 148.12.2 Genetics, Diagnosis, and Differential Diagnosis

Witkop syndrome is autosomal dominant with variable expression, caused by heterozygous mutations in the *MSX1* gene, which is the homolog of the *Drosophila* muscle segment homeobox 1 gene and is located at 4p16.1. In 2001, Jumlongras et al. studied a three generation family with Witkop syndrome using linkage analysis and a candidate gene approach and they identified a S202X nonsense mutation in the *MSX1* gene that cosegregated with the phenotype (79). They predicted that the mutation caused the phenotype via haplo-insufficiency. They then generated *Msx1*-knockout mice and demonstrated that both tooth and nail development were disrupted in the *Msx1*-deficient mice. *MSX1* mutations have also been found in patients with isolated tooth agenesis (80), with tooth agenesis and oral clefting (80), and with non-syndromic cleft lip and palate (81,82). *MSX1* interacts with other regulatory proteins, including *DLX* (distalless homeobox protein), *MSX2* (muscle segment homeobox 2 protein) and *TBP* (TATA-binding protein), and Jumlongras et al. (2001) postulated that the clinical variability of Witkop syndrome and other patterns of malformation associated with *MSX1* mutations may be related to the effects of these or other modifier genes.

With regard to the differential diagnosis, Witkop syndrome can be distinguished from hypohidrotic ectodermal dysplasia by its pattern of inheritance and by the lack of involvement of sweat glands. Hidrotic ectodermal dysplasia, or Clouston syndrome, has nail involvement but lacks dental involvement, and therefore, should be easily distinguished from Witkop syndrome. There may be an autosomal recessive form of tooth and nail syndrome; Fried reported first cousins from a consanguineous family with absent and conical-shaped teeth, thin concave toenails, and fine, slow-growing scalp hair (83). Simple oligodontia without nail involvement can be

inherited as a distinct autosomal dominant trait. PAX9, another transcription factor, is mutated in familial oligodontia with primarily molar involvement (84,85).

### 148.12.3 Management and Counseling

The main treatment for Witkop syndrome involves developing an appropriate dental treatment plan for the hypodontia (86). Families should be counseled regarding the autosomal dominant inheritance pattern with variable expression. At-risk family members should be examined carefully for any evidence of mild involvement with the disorder. Mutation analysis of the MSX1 gene can be performed in several clinical laboratories.

## 148.13 IKBKG GENE-RELATED DISORDERS

### 148.13.1 Incontinentia Pigmenti, Familial Male-Lethal Type (IP2) (MIM 308310)

**148.13.1.1 Clinical Features.** Incontinentia pigmenti (IP) is a complex X-linked disorder that can affect multiple ectodermally derived structures, including the central nervous system (CNS) and the eyes. The clinical features of IP have been reviewed in detail (87). The vast majority of affected individuals are female, although rare affected males have been reported (88). Ectodermal involvement is primarily of the teeth, hair, and skin, with minor involvement of the nails. Normal sweat gland function is present. Dental anomalies are seen in 80% of cases and may include hypodontia conical-shaped teeth, and delayed dental eruption, affecting both the deciduous and permanent dentition. Onychodystrophy is seen in 40% of individuals, usually consisting of mild ridging and pitting, but severe nail dysplasia occasionally occurs. Skin involvement occurs early during the first weeks of life and evolves over time through four stages (Figure 148-15). Stage I is seen at birth or in the first weeks of life, with erythema and

blistering of the skin, accompanied by eosinophilia. Stage II is the verrucous stage, manifested by warty or hyperkeratotic lesions that form in the area of the healing blisters. The verrucous lesions usually resolve with time. Stage III is associated with hyperpigmentation, with swirling gray or brown pigmentation frequently following the lines of Blaschko. The pigmentation usually fades with time and is undetectable in individuals over 20 years of age. Stage IV is the atretic stage, during which hypopigmentation and streaky alopecia are seen on the trunk and extremities. Patches of alopecia can also occur on the scalp, as well as thin sparse hair in some children. Breast and nipple hypoplasia are sometimes seen. Eye involvement is characterized by retinal vessel proliferation, similar to retinopathy of prematurity, which may proceed to retinal scarring and detachment (89). Other eye problems include cataracts, myopia, strabismus, microphthalmia and optic atrophy (90,91). Approximately 15–25% of affected individuals have been reported to have CNS problems such as developmental delays, seizures, spastic paresis and MRI abnormalities (87,90–92).

**148.13.1.2 Genetics, Diagnosis, and Differential Diagnosis.** Incontinentia pigmenti is an X-linked dominant trait, typically with prenatal lethality in males. This is supported by a high female-to-male ratio in affected families and an increased incidence of miscarriages. Rare affected males with an otherwise “lethal” mutation can be explained by somatic mosaicism or associated Klinefelter syndrome (47,XXY). The disorder was mapped to Xq28 and was subsequently shown to be due to mutations in the NF- $\kappa$ B essential modulator gene (NEMO), also known as the I- $\kappa$ B kinase-gamma (IKBKG) gene (93). Eighty percent of new mutations involve an 11.7kb deletion of exons 4–10, but point mutations and smaller deletions and duplications can occur (24,34,92). Mutations in IKBKG disrupt the NF- $\kappa$ B signaling pathway and may affect both developmental pathways and susceptibility to cellular apoptosis (34). IP cells with the common IKBKG 11.7kb deletion lack NF- $\kappa$ B activation, which normally protects against apoptosis; therefore, IP cells are susceptible to proapoptotic signals, and they die easily, which offers an explanation for the embryonic male lethality. In affected females, early death of the cells with an active X bearing the IP mutation and their replacement by cells with an active normal X leads to a skewed X-inactivation pattern.

The diagnosis of IP in infancy is usually straightforward if a typical rash is present, and skin biopsy can demonstrate the associated eosinophilia. The condition should be distinguished from other neonatal skin disorders such as epidermolysis bullosa and bullous impetigo. Diagnosis in older children and adults is more difficult and should be distinguished from hypomelanosis of Ito, which can have both pigmentary abnormalities (depigmentation) and CNS involvement. Hypomelanosis of Ito has been associated with chromosomal mosaicism.



**FIGURE 148-15** Typical skin lesions in incontinentia pigmenti.

**148.13.1.3 Management and Counseling.** Referral to a dermatologist in the neonatal period is recommended for appropriate care of the blistering skin lesions and to manage any skin infections or cellulitis. Serial ophthalmological examinations are needed during infancy to monitor for neovascular changes and to identify other associated eye anomalies. Ocular screening exams have been suggested at birth, monthly until three to four months, every three months until one year of age, and then biannually until three years of age (94). There have been reports of successful treatment of the retinopathy by both cryotherapy and laser photocoagulation (89). Referral to a neurologist should be made if necessary for seizures, spasticity or focal deficits and an MRI scan of the brain should be performed if there are retinal changes or neurological abnormalities (95,96). Appropriate dental care should be provided for hypodontia or conical-shaped teeth.

Affected females should be counseled that one half of their daughters will be carriers and that one half of their male offspring will result in a spontaneous abortion. Since most skin changes may have resolved or could be very minimal, determining the carrier status of adult females by clinical examination can be difficult. Careful dental and retinal examination should therefore be performed. Molecular testing of the IKBKG gene is available for clinical diagnosis, as well as for carrier testing and prenatal diagnosis. Approximately 65% of affected females have the 11.7kb deletion of exons 4–10. Other intragenic deletions and duplications as well as substitutions have been reported, and IKBKG gene sequencing can be performed if the common deletion is not found. X-inactivation studies on leukocytes to look for skewing can be done on females in whom no mutation can be found, but this is not specific for IP.

## 148.13.2 Hypohidrotic Ectodermal Dysplasia with Immune Deficiency (HED-ID) (MIM 300291)

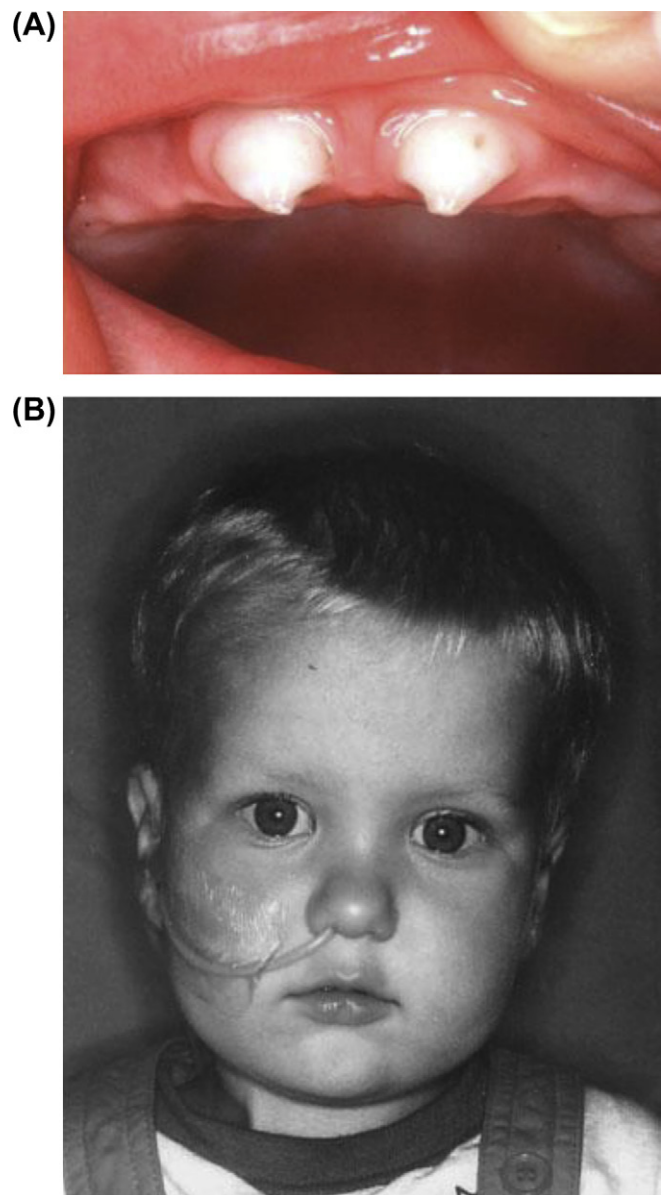
Hypomorphic mutations in the IKBKG gene cause a related X-linked disorder, hypohidrotic (anhidrotic) ectodermal dysplasia with immunodeficiency. There is also a rare disorder, hypohidrotic ectodermal dysplasia with immunodeficiency, osteopetrosis, and lymphedema (OL-HED-ID) caused by a IKBKG mutation (MIM 300301) (33,97).

**148.13.2.1 Clinical Features.** Male individuals with clinical features typical of XLHED were identified who had severe recurrent infections, including miliary tuberculosis, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pneumoniae*. Subsequently, they were found to have hypomorphic mutations in the NKBKG (NEMO) gene. Affected males have severe hypogammaglobulinemia and impaired antibody response to polysaccharides. Impaired natural killer (NK)-cell activity is seen in some cases. The abnormalities of hair, teeth, and eccrine sweat glands can be either identical to or milder than findings in individuals with XLHED or ARHED

(Figure 148-16). A few patients have been reported with osteopetrosis and lymphedema in addition to the other features; they were found to have a mutation in the stop codon (X420W) that adds 27 extra amino acids to the IKBKG protein and decreases its stability (98).

**148.13.2.2 Diagnosis.** Diagnosis is suggested by the clinical phenotype in combination with a history of recurrent infections. Mutation analysis of the NKBKG gene is clinically available to confirm the diagnosis.

**148.13.2.3 Management and Counseling.** Management of the HED-related medical problems is the same as for XLHED. Involvement of an immunologist and other



**FIGURE 148-16** (A and B) Boy with HED-ID syndrome due to hypomorphic IKBKG mutation. (With permission from Orstavik, K. H.; Kristiansen, M.; Knudsen, G. P.; Storhaug, K.; Vege, A.; Eiklid, K.; Abrahamsen, T. G.; Smahi, A.; Steen-Johnsen, J. Novel Splicing Mutation in the NEMO (IKK-gamma) Gene with Severe Immunodeficiency and Heterogeneity of X-chromosome Inactivation. *Am. J. Med. Genet.* 2006, 140 (1), 31–39.)



subspecialists as needed are required to manage the serious and recurrent infections.

### 148.13.3 Autosomal Dominant Hypohidrotic Ectodermal Dysplasia with Immune Deficiency (MIM 164008)

A boy with clinical features of HED-ID was reported in whom a heterozygous gain-of-function mutation was found in the *IKBA* gene (99). This gene encodes the  $\text{I}\kappa\text{B}\alpha$  protein in the EDA signal transduction pathway. The mutation enhanced the inhibitory capacity of  $\text{I}\kappa\text{B}\alpha$  and resulted in impaired NF- $\kappa\text{B}$  activation. The patient had a severe T-cell immunodeficiency in association with sparse scalp hair and conical teeth.

### 148.13.4 Tricho–Dento–Osseous Syndrome (MIM 190320)

**148.13.4.1 Clinical Features.** Tricho–dento–osseous (TDO) syndrome is a rare autosomal dominant disorder which involves the teeth, hair, and nails (100). Affected individuals also have increased bone density. The hair is kinky and curly, especially during the neonatal period. The nails may show thick cornification and splitting of the superficial layers. Dental findings include enamel hypoplasia and hypocalcification, with taurodontism (enlargement of the body of the tooth and the pulp chamber) of both the deciduous and permanent molars. Affected individuals with this disorder have both normal sweat gland function and normal skin. Skeletal findings include mild to moderate increase in bone density of the long bones and skull, with dolichocephaly of the cranium due to sagittal synostosis (101). There is considerable phenotypic variability in this disorder.

**148.13.4.2 Genetics, Diagnosis, and Differential Diagnosis.** TDO is inherited as an autosomal dominant trait. It was mapped in four kindreds from North Carolina to chromosome 17q21, and a 4-bp deletion in the human distal-less homeobox gene (*DLX3*) was found which correlated with the TDO phenotype in six families (102). The mutation is predicted to cause a frameshift and premature termination codon, resulting in a functionally altered *DLX3* protein. Other mutations in *DLX3* are associated with more severe phenotypes (103,104). The *DLX3* protein is a putative transcriptional activator implicated during development and differentiation of epithelial tissues (105). *DLX3* has been shown to regulate hair follicle differentiation and cycling (106). Transgenic mice expressing mutant *DLX3* were shown to have disruption of odontoblast polarization and defective dentin (107).

TDO syndrome has the same dental findings seen in another autosomal dominant disorder with only dental involvement, amelogenesis imperfecta (hypomaturational–hypoplasia type) with taurodontism (AIHHT) (MIM 104510). Sequencing of the *DLX3* gene in a family with AIHHT identified a two base pair deletion within the

homeodomain of *DLX3*. Thus at least some families with AIHHT are allelic with TDO and a phenotype–genotype difference may exist.

**148.13.4.3 Management and Counseling.** Management includes dental care for the enamel hypoplasia. Genetic counseling regarding the autosomal dominant inheritance pattern with variable expression should be provided. No clinical molecular testing is available yet for either confirmation of the diagnosis or prenatal diagnosis.

### 148.13.5 Goltz Syndrome (Focal Dermal Hypoplasia) (MIM 305600)

**148.13.5.1 Clinical Features.** Goltz syndrome, also known as focal dermal hypoplasia, is a rare X-linked dominant disorder associated with defects in tissues of both ectodermal and mesodermal origin (108). The characteristic feature is patchy dermal hypoplasia with herniation of fat through the dermal defects. The phenotype is highly variable, but many patients with Goltz syndrome have significant craniofacial dysmorphic features and complex ocular anomalies in addition to ectodermal abnormalities (Figures 148-17 and 148-18). They may have mild microcephaly and facial asymmetry with a pointed chin. The ears may be lowset, protruding or with a simplified helical shape, and the auditory canals may be narrow. Mixed sensorineural and conductive hearing may be present. There are frequent ophthalmological complications, including microphthalmia (15%), anophthalmia, aniridia (3%), or ectopia lentis (6%). Strabismus or nystagmus can occur. There may be iris and/or choroidoretinal colobomas, or optic atrophy. The nasal bridge may be narrow with a broad nasal tip or notched alae nasi. Cleft lip, with or without cleft palate, and papillomas of the gingiva or lips have been described. Papillomas of the larynx and esophagus have been reported as well. Arborescent papillomas of the axillae, periumbilical area, anus and vulva have also been seen. With regard to the teeth, hypodontia, enamel hypoplasia and delayed eruption are common. Notched incisors may be present. There may be supernumerary nipples, asymmetry of the breasts or athelia. A variety of diaphragmatic and abdominal wall defects may be associated with Goltz syndrome, including diaphragmatic hernia or hiatal hernia, diastasis recti, umbilical hernia, or even omphaloceles. Anteriorly displaced anus, intestinal malrotation, and renal anomalies such as horseshoe kidney or hydronephrosis have also been reported. Recently, Pentalogy of Cantrell has been identified in a patient with Goltz syndrome (109).

Skin findings include linear or reticular hyperpigmentation or skin atrophy with localized deposits of superficial fat. There may be hypoplastic fingertip epidermal ridges, absent fingernails and toenails, or dystrophic nails that are spoon shaped or grooved. Other digital anomalies include syndactyly, polydactyly or ectrodactyly. Hair abnormalities include sparse or brittle hair, often with patchy alopecia of the scalp. Intellectual disability is



found in about 15% of cases, and short stature in some. Brain defects can include hydrocephalus, agenesis of the corpus callosum or Arnold–Chiari malformation. The majority (90%) of cases are seen in females; about 95% are sporadic and 5% are inherited from a parent, usually the mother. Affected liveborn males (10% of cases) are all the result of a new somatic mosaic mutation; it is presumed that hemizygous non-mosaic males would be non-viable.

**148.13.5.2 Genetics and Diagnosis.** Goltz syndrome is due to mutations or deletions of the *PORCN* gene which is located at Xp11.23. In 2007, Grzeschik et al. studied

a group of patients with Goltz syndrome, including six individuals from one family and 10 additional unrelated individuals, using comparative genomic hybridization; they found deletions in two patients at Xp11.23 that shared four genes within an 80 kb overlapping region, including the *PORCN* gene (110). At the same time, Wang et al. amplified and sequenced all coding exons of the *PORCN* gene in 15 girls with confirmed or suspected Goltz syndrome, and they identified heterozygous mutations in 10 of the cases (111). In one of these cases, low level signal for the mutation was identified in the girl's father suggesting somatic mosaicism, but all other parental samples were negative for the mutation, consistent with new mutations in the affected girls. Analysis of additional cases by Grzeschik, Leoyklang and others has identified a variety of missense, nonsense and splice site mutations in the *PORCN* gene, confirming that this is the gene responsible for Goltz syndrome (110,112).

The *PORCN* gene encodes the human homolog of *Drosophila* porcupine. There are five isoforms of the gene product that are derived from alternative splicing, and are expressed in multiple tissues. In mice and *Drosophila*, the protein has been shown to be required for secretion of WNT proteins needed for activation of target genes in many developmental pathways. In mouse embryos, *Porcn* is expressed in most of the same tissues that are affected by anomalies in humans with Goltz syndrome.



**FIGURE 148-17** Goltz syndrome.



**FIGURE 148-18** Goltz syndrome features. (With permission from Maas, S. M., et al. Phenotype and Genotype in 17 Patients with Goltz–Gorlin Syndrome. *J. Med. Genet.* **2009**, 46 (10), 716–720.)

Clinical molecular testing is available for the PORCN gene. Mutations in affected individuals have included nonsense, frameshift, missense mutations, as well as deletions of the entire gene (113). Sequencing of the PORCN gene should be performed in females, followed by deletion testing by FISH or array CGH if no mutation is found. Males suspected of having Goltz syndrome should have sequencing of the gene; no affected male has been found to have a deletion. Mosaicism for PORCN mutations has been reported. Parents of affected girls should be examined for subtle signs of the disorder. Prenatal and pre-implantation diagnostic testing are both available if the disease-causing mutation is known in the family.

**148.13.5.3 Management and Counseling.** Because of the potential for multiple anomalies, a full evaluation should be completed including imaging of the brain, skeleton, and chest and abdominal organs, ophthalmology examination and audiological evaluation. Syndactyly and split-hand/foot malformation may require surgical correction or other intervention. Affected individuals should also be monitored for scoliosis. Follow-up with an otolaryngologist is often needed to evaluate for the papillomas in the oropharynx and airway, especially prior to endotracheal intubation for surgery. Dermatology consultation for long-term management of the skin lesions is required.

Genetic counseling regarding the X-linked dominant inheritance pattern should be given to the family. Since the majority of cases represent sporadic new mutations, the recurrence risk for most families will be low, but careful evaluation of the parents in combination with molecular diagnostic testing is necessary to provide the most accurate recurrence risk information.

### 148.13.6 KID (Keratitis–Ichthyosis–Deafness) Syndrome (MIM 242150)

**148.13.6.1 Clinical Features.** KID (Keratitis–Ichthyosis–Deafness) syndrome is a rare autosomal dominant condition associated with keratitis, ichthyosiform skin lesions and sensorineural hearing loss (114). There is generalized thickening of the skin, palmoplantar keratoderma, and erythematous verrucous skin lesions (Figure 148-19). The erythematous plaques are sharply demarcated and are seen on the face and limbs. The thickening of the skin appears in infancy. About half of affected individuals have recurrent skin infections, including bacterial and fungal infections. Alopecia is seen in at least 80% of cases, and can range from total scalp alopecia to minimal loss of eyebrows or eyelashes. Some individuals have sparse fine hair without alopecia. Nail dystrophy is also a very frequent feature. Sweating may be reduced. The teeth can be normal, but may have delayed eruption, brittleness or tendency for caries. Eye problems result from keratoconjunctivitis sicca with corneal vascularization and decreased vision. The hearing loss is congenital and non-progressive.

**148.13.6.2 Genetics.** KID syndrome is caused by mutations in the gene for connexin 26 (GJB2). 78–87% of cases have the Asp50Asn mutation, but other missense mutations have been described, possibly associated with a more severe phenotype. The Ser17Phe mutation was associated with development of squamous cell carcinoma of the tongue (114). About 65% of affected individuals represent new mutations, but familial cases occur as well. Germline mosaicism was suspected in a family with two affected children and normal parents (114).

**148.13.6.3 Management and Counseling.** Dermatology consultation with continued follow-up is required for the skin manifestations. Affected individuals should be monitored for squamous cell carcinoma of the skin or tongue, which has been seen in at least 10% of cases. Ophthalmology follow-up for progressive eye manifestations is required. Corneal transplant has been tried to treat the corneal vascularization. Audiology evaluation is needed for hearing aids and possible cochlear implants to treat the hearing loss.

Molecular diagnostic testing is available clinically. Genetic counseling regarding the autosomal dominant inheritance pattern should be provided.

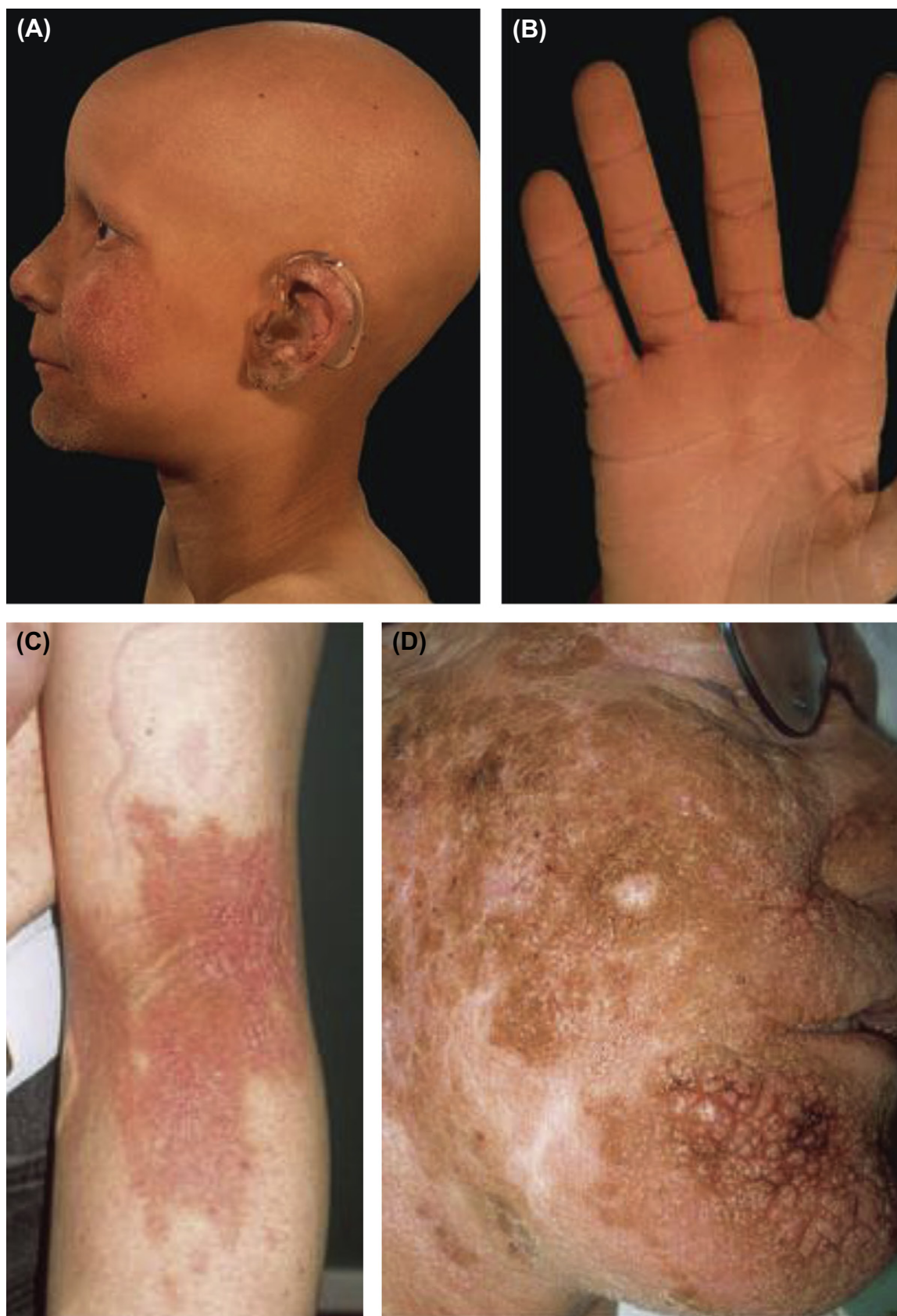
### 148.13.7 Oculo–Dento–Digital Dysplasia (MIM 164200)

**148.13.7.1 Clinical Features.** Oculo–Dento–Digital dysplasia (ODDD) is an autosomal dominant disorder with variable expression. Affected individuals have sparse hair, enamel hypoplasia, camptodactyly and distinctive facial features with small eyes. The scalp hair is dry, sparse and slow-growing. Eyebrows and eyelashes may be absent or sparse. Both the deciduous and permanent teeth have enamel hypoplasia and are prone to decay. They are small, yellowish and friable. Sweat glands, skin and nails are normal. The eyes have short palpebral fissures, with microcornea and or micropthalmia, although the vision is usually normal. There is a narrow nose with hypoplastic ala nasi. Bony abnormalities include camptodactyly of the fourth and fifth fingers in over 80% of case, hyperostoses of the skull, broad ribs and clavicles, and abnormal trabeculation of the long bones. Syndactyly of the fourth and fifth fingers and toes may be present.

**148.13.7.2 Genetics.** ODDD is caused by mutations in the connexin-43 gene (GJA1) localized to chromosome 6q22–q23 (115–117).

**148.13.7.3 Management and Counseling.** Dental care for the enamel hypoplasia is necessary. Vision is usually normal, but 10–15% of affected individuals develop glaucoma, so they should be followed by an ophthalmologist periodically.

Genetic counseling regarding autosomal dominant inheritance with variable expression should be provided. Clinical molecular genetic testing is available for the GJA1 gene.



**FIGURE 148-19** (A–D) KID syndrome features. (With permission from Mazereeuw-Hautier, J., et al. *Keratitis–Ichthyosis–Deafness Syndrome: Disease Expression and Spectrum of Connexin 26 (GJB2) Mutations in 14 Patients*. *Br. J. Dermatol.* **2007**, 156 (5), 1015–1019.)



### 148.13.8 Ectodermal Dysplasia–Ectrodactyly–Macular Degeneration (EEM) Syndrome (MIM 225280)

**148.13.8.1 Clinical Features.** Ectodermal dysplasia–ectrodactyly–macular degeneration (EEM) syndrome is a rare autosomal recessive disorder. It is characterized by sparse scalp hair, eyelashes and eyebrows, small, widely spaced teeth, agenesis of some teeth, limb anomalies including ectrodactyly, syndactyly and camptodactyly and macular dystrophy. Affected individuals have normal sweating. The visual acuity gradually decreases with progression of the retinal degenerative abnormalities. The presence of macular dystrophy distinguishes it from other ectodermal dysplasia syndromes with limb malformations.

**148.13.8.2 Genetics.** Mutations in the CDH3 gene for cadherin-3 have been identified in several families with EEM (72,118). CDH3 is expressed in the apical ectodermal ridge and the interdigital mesenchyme in mice, which is compatible with the limb abnormalities seen in EEM. A related disorder, congenital hypotrichosis with juvenile macular dystrophy (HJMD), is also caused by CDH3 mutations, but the mutations are distinct in the two syndromes. Clinical molecular genetic testing is available in Europe for the CDH3 gene.

**148.13.8.3 Management and Counseling.** Management of the dental, limb and hair concerns are the same as for the other ED disorders discussed above. Long-term ophthalmological care will be needed for the visual concerns. Genetic counseling with regard to the autosomal recessive inheritance of the disorder should be provided.

## CROSS REFERENCES

Other relevant articles could include #152 (hair), #155 (clefting defects) and #182 (ichthyoses).

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## FURTHER READING

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# CHAPTER

# 149

## Skin Cancer

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### 149.1 INTRODUCTION

The three structural components of the skin are the epidermis, the dermis and the skin appendages. The epidermis is the most cellular layer and the main cell population is composed of keratinocytes dedicated to terminal differentiation and the production of stratum corneum. Within the epidermis there are also melanocytes, a dendritic cell population usually found in the basal layer of the epidermis in a ratio of around one melanocyte to every 10 basal keratinocytes. In the mid-epidermis there is a significant population of the second dendritic cell component of the epidermis, the antigen presenting Langerhans cell. The Merkel cell is seen in and around the basal layer of the epidermis. Keratinocytes give rise to squamous cell carcinoma, and precursor lesions (such as actinic keratoses, and Bowen's disease). Although basal cell carcinomas arise in the epidermis, the cell of origin remains controversial (1). Melanocytes give rise to melanoma and benign melanocytic nevi. Langerhans cells give rise to Langerhans cell histiocytosis and Merkel cells to Merkel cell carcinoma.

The dermis contains fibroblasts and mast cells, and other structures such as the vasculature, smooth muscle and neural tissue all have their own cell population, each with the potential for malignant change.

The third major components are the skin appendage structures. These include the hair follicle and the sebaceous gland, frequently referred to as the pilosebaceous unit. The apocrine gland and erector pili muscles may also be found in association with this structure. The eccrine sweat glands provide a separate discrete appendageal cell population.

All the cell types present in the epidermis, dermis, and skin appendages have the capacity for malignant change (2), and skin cancer is common and becoming increasingly so. Sixteen to forty four percentage of Americans were predicted to develop skin cancer in their lifetime at the beginning of this century (3). By far the most common cutaneous malignancy is the basal cell carcinoma, while the type responsible for the majority of deaths is that arising from the melanocyte, melanoma.

Tumors arising from the dermis are much less frequent, but may arise from, for example, the vasculature (such as angiosarcomas and Kaposi sarcoma). A wide variety of tumors arise from the skin appendages, but all are rare, and are usually locally expansive without metastatic potential. They are of interest to geneticists, however, in that a number of rare genetic conditions are associated with specific skin appendage tumors, such as sebaceous gland tumors in the Muir–Torre syndrome.

As for most cancers, rare individuals and families exist in which there is a markedly increased risk of skin cancer due to inherited so-called highly penetrant cancer genes. These families are said to have a family cancer syndrome, and such syndromes are typified by family members having cancers earlier in life than average, and having multiple primary cancers as well as having more than expected numbers of cancers in the family. Most increased susceptibility however results as a result of a combination of inherited, less penetrant genes (which each increase the risk only a little but the individual may have inherited several different low penetrance genes) and causal environmental exposures.

In this chapter we will predominantly discuss the three most common types of skin cancer, basal cell carcinoma, squamous cell carcinoma and melanoma. We will describe something of their clinical features, then we will explore first what is known of the common lower penetrance susceptibility genes and second the family cancer syndromes associated with each type of skin cancer.

### 149.2 BASAL CELL CARCINOMA

Basal cell carcinomas (BCC) arise from the epidermis and the BCC is the commonest form of cutaneous malignancy, found mainly on the face. The cell of origin surprisingly remains controversial. The observation discussed below that these cancers occur in individuals with the Bazex–Dupre–Christol syndrome in which the hair follicle is clearly abnormal might suggest that the cell of origin may be follicular, as has recently been argued by Seykora et al. on the basis of cell biology (1). Very rarely however these

tumors have been reported on the palm (which is clearly not hair bearing) which has given rise to the suggestion that the cell of origin was a progenitor of an eccrine cell (sweat gland) or an epidermal stem cell (4). Variation in the incidence of basal cell carcinoma geographically suggests that the main etiological factor is sun exposure (in that it is most common where fair skinned people live in hot countries such as Australia), although the evidence for a clear linear relationship between the incidence of basal cell carcinomas and cumulative lifetime sun exposure is not as obvious as it is for squamous cell carcinoma. Indeed there is a view that basal cell carcinomas share with melanoma an association with intermittent (holiday) rather than cumulative sun exposure, and there is a corresponding higher risk in individuals of higher socioeconomic status who have more access to holiday sunshine (5). Basal cell carcinomas are becoming more common over time. A study from New Hampshire reported an incidence per 100,000/year of 310 for males and 166 for females for 1993–1994, with a 4% rise per year for both sexes over a 15-year period (6). In Europe there is similar evidence for marked increases in incidence as above, especially in females on the body, which was attributed to changed patterns of intermittent sun exposure (7,8).

### 149.2.1 Clinical Features and Pathology

Basal cell carcinomas (BCC) develop as slow-growing nodules mainly on the central part of the face, although they may occur on the trunk and on the limbs. The lesions appear initially as small round translucent papules, often with telangiectatic blood vessels on the raised rolled edge. The lesion expands slowly over months and years and may break down to develop an ulcerated center. Left untreated, these lesions will spread locally and destroy soft tissue cartilage and even bone, hence the alternative name *rodent ulcer* but metastases are fortunately very rare (Figure 149-1).

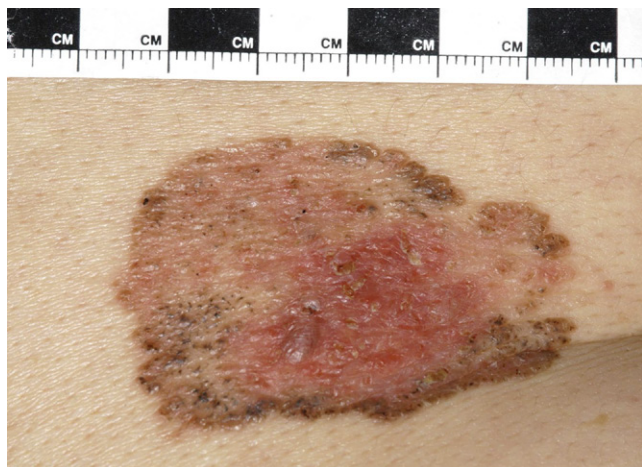
The pathology is that of tongues of epidermal cells invading the dermis but retaining a connection with the

overlying epidermis; the lateral border of these invading clusters of cells has a margin of palisaded cells. In addition, there is a surrounding stroma, which is an integral part of the tumor and which is particularly prominent in the morpoeic (scarring) variant.

### 149.2.2 Genetics of BCC

BCC are usually sporadic although there is genetic variation between individuals in terms of susceptibility. The strongest determinant of risk is skin color, as BCC are seen predominantly (but by no means exclusively) in white skinned peoples. Attempts are being made to identify common low to medium penetrance susceptibility genes using genome wide association studies (GWA) and candidate gene approaches. Given that BCC are most common in fair skinned people, GWA and candidate gene studies have identified genes which determine skin color (pigment genes) as BCC susceptibility genes. Red haired individuals are particularly sun-sensitive and therefore it is not surprising that variants in genes associated with red hair are associated with increased risk—such as the melanocortin 1 receptor gene (*MC1R*) (9–12) and the agouti signaling protein locus (*ASIP*) (13). The *MC1R* variants result in increased proportions of red/yellow pigment in the skin and hair and this is associated with increased risk of sunburn. Polymorphisms in other pigment genes are also associated with risk of BCC, such as the oculocutaneous albinism A2 gene *OCA2* (13) and the locus *SLC45A2* (14) and most recently the interferon regulator gene 4, *IRF4* (15). A recent meta-analysis suggested that both pigment genes and other genes not associated with pigmentation are associated with risk (16). Loci not known as yet to be associated with pigmentation but which have been shown to be associated with susceptibility in GWA include a gene coding for keratin 5, a locus near to the *CDKN2A* locus at 9p and *TERT* which codes for telomerase and therefore impacts on telomere length (14,17). A GWA also identified a signal at chromosome 1p36 which contains a number of candidate genes (*PAD14*, *PAD16*, *RCC2* and *ARHGEF10L*), and another at 1q42 in the region of the ras-homolog *RHOA* (18). There are a number of relatively small candidate gene studies reported to have shown a relationship with risk of basal cell carcinoma, but these are difficult to evaluate given their size and lack of adequate validation and therefore will not be listed here.

There are two inherited syndromes in which there is a marked propensity to BCC: the nevoid basal cell carcinoma syndrome and the Bazex–Dupre–Christol syndrome, which are described here (Figure 149-2). Progress in understanding these syndromes has led to an increased understanding of the somatic genetic events in BCC. A third, called Rombo syndrome, is a very rare entity characterized by the presence of atrophoderma vermiculatum of the face, multiple milia, telangiectases, acral erythema and a propensity to develop basal cell carcinomas (19). The authors have not seen this condition



**FIGURE 149-1** A pigmented superficial basal cell carcinoma arising on the chest.

and do not know its relationship, if any to the Bazex–Dupre–Christol syndrome.

### 149.3 NEVOID BASAL CELL CARCINOMA SYNDROME (GORLIN–GOLTZ SYNDROME)

The nevoid basal cell carcinoma syndrome, transmitted as an autosomal dominant trait, was described by Howell and Caro (20) and by Gorlin and Goltz (21). The disease has nearly complete penetrance and variable expressivity (22). In the UK, around 1 in 30,827 people are affected (prevalence) (23). Gorlin estimated

that about 40% of cases represent new mutations [see OMIM (Online Mendelian Inheritance in Man)] and a more recent estimate was 26% (23). The median age of diagnosis for non-familial cases tends to be later (23 years, range 3–39 years) than for familial cases (12 years, range 0–44 years) (23). Farndon reported that 1 in 200 patients with BCC had Gorlins, falling to 1 in 5 with first diagnosis under the age of 19 years (24).

#### 149.3.1 Clinical Features

Affected individuals have a combination of congenital skeletal defects plus a tendency to develop tumors,

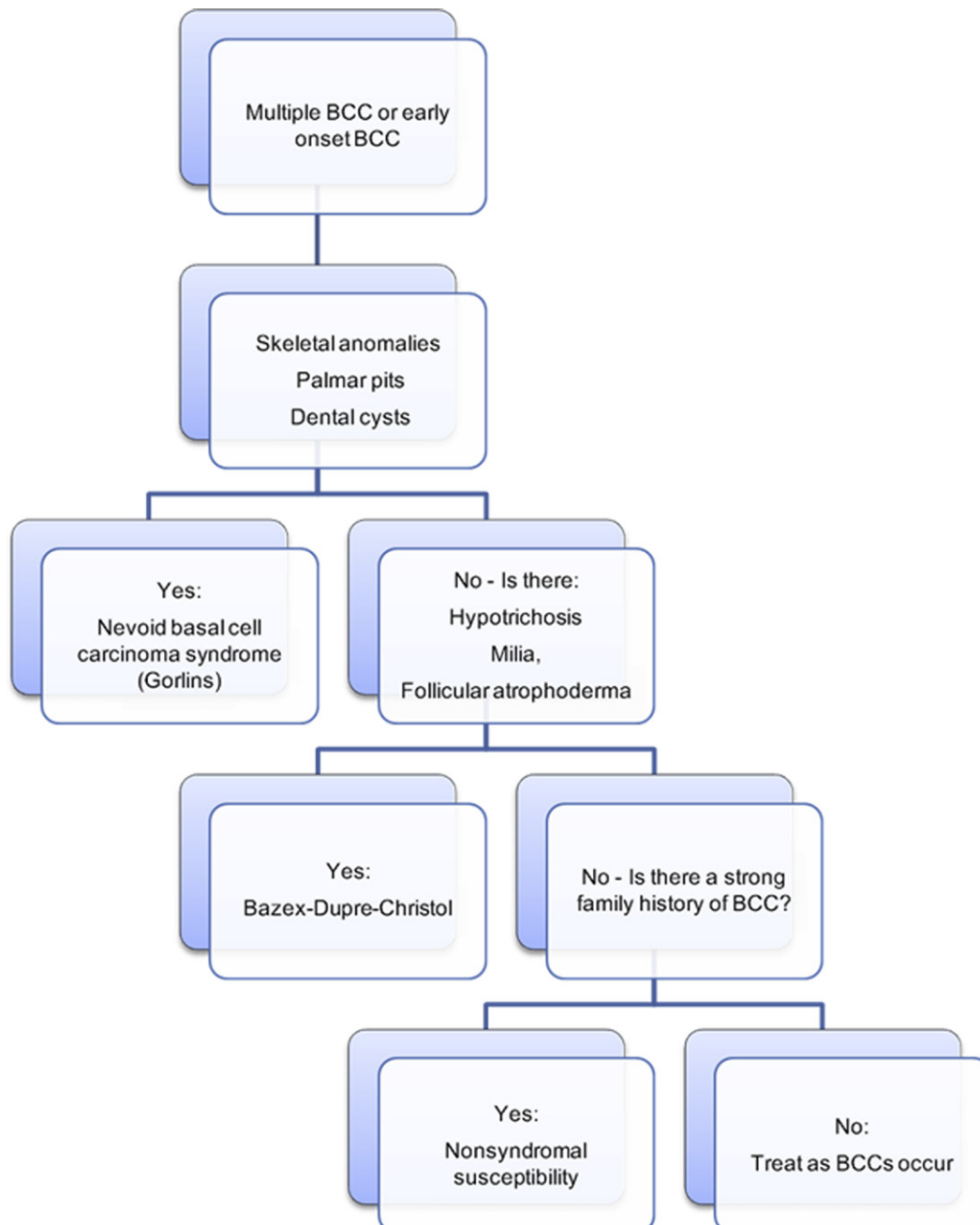


FIGURE 149-2 Investigation of early onset or multiple basal cell carcinomas.

including multiple basal cell carcinomas, ovarian fibromas (25), and medulloblastomas in affected children. Rarely, other tumors develop such as fibrosarcomas but the more common ovarian fibromas are of low malignant potential (26). Developmental anomalies include mild mandibular prognathism, lateral displacement of the inner canthi, frontal bossing, kyphoscoliosis, short metacarpals, abnormal ribs and calcification of the falx cerebri.

Hamartomatous cysts are well described in multiple sites such as the lungs and intestine. Affected patients have radiosensitivity so that multiple BCC have been seen in cutaneous radiation fields and multiple meningiomas have been reported in patients with this syndrome treated with radiotherapy for medulloblastoma (27). Radiotherapy is therefore to be avoided in affected patients where possible.

The most common first symptomatic presentation is to dental surgeons as a result of mal-aligned teeth due to jaw cysts (keratocysts), although the majority of cysts are small and are only picked up on X-ray. 75% of cases in the series reported by Kimonis had jaw cysts, 80% arising before the age of 20 years (28). The cysts are usually locally removed and examined pathologically, with more radical surgery used for locally recurrent tumors.

The skin signs are of small pits in the skin of the palms and soles, which often look pink especially after immersion in water, and when viewed with the dermatoscope. 90% of patients have pits (28) although the number of pits is very variable. Basal cell nevi start to arise around puberty and are initially tiny: 1–2 mm diameter lesions, often pigmented and most numerous in sun-exposed areas of skin. Only a small proportion of these lesions evolve into BCC, generally beginning in the second decade (Figure 149-3). The basal cell carcinomas are initially unusually small and may be missed if careful examination is not performed at regular supervision visits. Although the lesions in sporadic basal cell carcinoma are concentrated on the face, a high proportion of lesions in Gorlin syndrome are found on the trunk. Gene-environment (sun exposure) interaction is illustrated by the lower penetrance of skin cancers in black patients with the syndrome (29) and higher penetrance in families living at lower latitudes where sun exposure is more intense (30). Protection from the sun is therefore an important part of management, with a consequent need for vitamin D supplementation.

### 149.3.2 Molecular Genetics of Familial and Sporadic Basal Cell Carcinomas

Mutations in the *PTCH* gene at 9p 22.3 have been shown to be the cause of the nevoid basal cell carcinoma syndrome (31,32). Wicking reported 28 mutations in the *PTCH* exons in 71 probands, but genotype/phenotype correlations are poor and it seems likely



**FIGURE 149-3** A BCC arising on the eyelid of a patient with nevoid basal cell carcinoma syndrome.

that modifier genes exist (33,34). Patched is the protein product of *PTCH*, and is a key component of the Hedgehog signaling pathway driving cell proliferation. It is the cell membrane receptor for the Hedgehog family of proteins which are important in embryological development, drawing parallels with developmental anomalies in the nevoid basal cell carcinoma syndrome. *PTCH* has 23 exons encoding a transmembrane glycoprotein of 1447 amino acids. Most reported germline mutations have been frameshift or nonsense mutations leading to truncated Patched proteins. A French paper reported 29 mutations detected in 65 patients with the syndrome and, in common with other studies, the majority were frameshift resulting in loss of the last group of six transmembrane domains of the Patched protein (22). It is assumed that the syndrome is an example of Knudsen's two hit hypothesis wherein germline mutations of *PTCH* represent the first "hit" on the tumor suppressor gene, and that somatic changes in the second *PTCH* allele represent the second "hit," although in a recent report only 13/44 cases fit the two hit model (35).

Subsequently somatic mutations in *PTCH* and associated genes have been reported commonly in sporadic BCC, (36–38) and many of these mutations have a characteristic "UV signature" (39) so that investigation of the nevoid basal cell carcinoma syndrome has contributed much to understanding the carcinogenesis of BCC in general. Stone and colleagues (40) subsequently observed that human Patched forms a physical complex



with the vertebrate homologue of the drosophila protein, called Smoothed (SMO), and suggested that SMO was a signaling component linked to *PTCH* and in control of Sonic Hedgehog function. Their observations were confirmed by Xie et al. (41) who reported activating *SMO* mutations in sporadic basal cell carcinomas, showing that wild type *PTCH* and *SMO* genes both act as tumor suppressor genes for basal cell carcinoma, and that mutated *SMO* could function as an oncogene in basal cell carcinoma. The specific *PTCH* mutations for basal cell carcinomas and their absence in other skin tumors except for the very similar tricoepithelioma has been confirmed (42). Interestingly, an animal model for the nevoid basal cell carcinoma syndrome exists, in which one copy of *PTCH* is inactivated, hypersensitivity to ionizing radiation is demonstrable and chronological development of tumors from basaloid proliferations towards infiltrative tumors can be demonstrated and are presumed to result from sequential multi-step genetic events (43).

In drosophila, Hedgehog signaling requires the presence of the transcription factor cubitus interruptus, which shows homology to the Gli family of transcription factors in vertebrates (for a review, see Dicker et al. 2002 (39)). The induction of the transcription factor Gli 1 has been observed in basal cell carcinomas (44) and Gli 1 protein has been reported to be expressed in the cytoplasm of basal cell carcinomas (45). Thus there is a clear pathway of molecular genetic events implicating Sonic Hedgehog, *PTCH*, *SMO* and Gli 1 leading to development of familial and sporadic basal cell carcinomas.

### 149.3.3 Diagnosis of Nevoid Basal Cell Carcinoma Syndrome

A diagnosis can be made if two major or one major and two minor criteria are fulfilled.

#### Major Criteria.

- Multiple (>2) basal cell carcinomas (BCC) or BCC occurring under age 30 years or >10 basal cell nevi.
- Odontogenic keratocyst (histologically proven) or polyostotic bone cyst.
- Three or more palmar or plantar pits.
- Ectopic calcification: lamellar or early (<20 years) falx calcification.
- First degree relative with nevoid basal cell carcinoma syndrome.

#### Minor Criteria.

- Congenital skeletal anomaly: bifid, fused, splayed or missing rib or fused vertebrae.
- Occipitofrontal circumference >97th centile with bossing.
- Cardiac or ovarian fibroma.
- Medulloblastoma.
- Lymphomesenteric cysts.

- Congenital malformation: cleft lip and/or palate, polydactyly, eye anomaly (cataract, coloboma, microphthalmia).

### 149.3.4 Management

**149.3.4.1 Suggested Screening in Children from a Known Family** (developed by Dr Carol Chu, Clinical Genetics, St James's Hospital, Leeds UK, from published data (30,46,47)).

If the family is known to have nevoid basal cell carcinoma syndrome, mutation analysis can be offered if the family is seen and counseled by a clinical geneticist. If a mutation is found in an affected family member, a pre-symptomatic test may be offered to children after appropriate counseling. In a family with a known mutation, if the gene test does not show a mutation in the child, then no further surveillance needs to be undertaken. In a family with a known mutation, if a gene test shows the same mutation in an at-risk family member, then the following screening is recommended:

- Sun avoidance.
- As sun avoidance leads to vitamin D deficiency and sub-optimal levels are generally common in healthy people around the world (48) and specifically in patients with this syndrome (49), supplementation with vitamin D is desirable to achieve levels in the range 60 nmol/L to 85 nmol/L. It is difficult to predict levels therefore measurement may be indicated.
- Baseline chest, skull and spine X-ray.
- Echocardiogram.
- Annual dental screening including orthopantomogram from 8 years.
- Annual skin examination by dermatologist from puberty.
- Six monthly neurological examinations from birth to 3 years and then yearly until age 7 years to promote the early detection of medulloblastoma.

If no mutation is found in the family then at-risk children should have the screening as suggested in the section below as for families with suspected nevoid basal cell carcinoma syndrome.

**149.3.4.2 Investigation in Cases of Suspected Nevoid Basal Cell Nevus Syndrome with No Family History.** In a child who presents with jaw cysts:

- If single cyst and no minor criteria, observe only.
- If more than one cyst:
  - Family history taken.
  - Clinical examination noting clefting or eye abnormalities.
  - Head circumference measurement.
  - Skin examination.
  - Examination of palms and soles of feet for pits.
  - Baseline chest and spine X-ray.
  - Baseline skull X-ray.

- If no abnormalities on tests above, 2 yearly dental screening until 16 years and repeat clinical examination and skull X-ray at 12 years and 20 years.
- If abnormalities at baseline screen or 12 or 20 year screen consistent with diagnostic criteria refer to clinical genetics for full family assessment and possible mutation analysis.

**149.3.4.3 Treatment.** Jaw cysts are excised conservatively except after local recurrence where a more aggressive excision is carried out including removal of a cuff of bone. Basal cell nevi on the skin are observed, but basal cell carcinomas are removed as is usual using cryosurgery, excision, photodynamic therapy (PDT) or Moh's surgery for extensive tumors.

The treatment of cutaneous lesions was reviewed most recently by Lo Muzio and van der Geer (50,51). Radiotherapy must be avoided wherever possible, as affected patients are very sensitive to ionizing radiation; most visibly as the development of more numerous and more aggressive BCC (52) but probably also in other sites (27). Alternate therapies for patients with extensive tumors include photodynamic therapy (53) and most encouragingly for patients with very advanced disease, resolution of multiple tumors has been reported after use of a small molecule inhibitor of the Hedgehog signaling pathway via interaction with Smoothened (54).

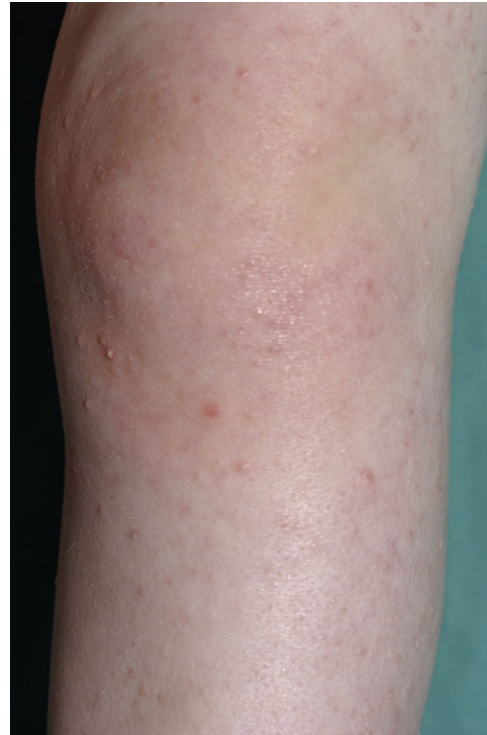
#### 149.4 BAZEX-DUPRE-CHRISTOL SYNDROME

This very rare syndrome is a disorder of the hair follicle. The condition was first described by Bazex (55) and has remained most common in France (56). Affected children have hypotrichosis (thin and abnormal hair) (Figure 149-4) and milia (Figure 149-5). Subsequently, deep and lax hair follicles become obvious particularly on the dorsae of the hands (known as follicular atrophoderma) which have been likened to “ice-pick marks” (57)



**FIGURE 149-4** Hypotrichosis in the Bazex–Dupre–Christol syndrome.

(Figure 149-6). Hypohidrosis is a feature, and in a family previously reported by Gould and Barker (58) we have reported the occurrence of hidradenitis suppurativa and the development of multiple perineal trichofolliculomata (59), emphasizing that the pathology in this condition is indeed centered on the hair follicle. In one large pedigree, the clinical picture was recognizably more severe in males (60) although the authors have not seen this in the two families in their care. The early development of multiple basal cell carcinomas in sun exposed areas is a key



**FIGURE 149-5** Milia on the knee of a two-year-old with Bazex–Dupre–Christol syndrome.



**FIGURE 149-6** Follicular atrophoderma on the dorsum of the hand of an adult with Bazex–Dupre–Christol syndrome.

component of the syndrome, but for affected families the phenotypic effects on hair growth and the skin in general are often more symptomatic. The age of onset of the BCC is variable between and within families, from early childhood to middle age. The cell of origin of BCC overall remains controversial but the association of BCC with the Bazex–Dupre–Christol syndrome, which is clearly a disorder of the hair follicle, supports the view that BCC may arise from the hair follicle. Inheritance of Bazex–Dupre–Christol syndrome is by the X-linked dominant route (60), and the gene has been mapped to Xq24–27.1 (61). The critical interval has been recently narrowed to an 11.4Mb interval which includes 101 genes (57).

#### 149.4.1 Non-Syndromal Familial Basal Cell Carcinomas

There are sporadic reports of familial clustering of multiple basal cell carcinomas (62) without the characteristic features of either the nevoid basal cell carcinoma or Bazex–Dupre–Christol syndromes, which may result from the inheritance of as yet unidentified highly penetrant susceptibility genes, or possibly the inheritance of multiple lower penetrance genes in individuals who have had a significant amount of sun exposure.

### 149.5 SQUAMOUS CELL CARCINOMA

#### 149.5.1 Epidemiology

Squamous cell carcinoma (SCC) is a malignancy derived from the epidermal keratinocyte. Most are postulated to result from excessive sun exposure and there is a linear relationship between cumulative lifetime exposure to ultraviolet (UV) radiation and the development of squamous cell carcinoma. Chronic inflammation/ulceration is causal occasionally, the human papillomavirus may predispose and carcinogens are also important, albeit much less frequently. Sir Percival Pott recognized this first, describing an increased incidence of scrotal cancer in chimney sweepers due to chronic exposure to coal tar products.

A study from New Hampshire reported a 235% rise in the incidence of squamous cell cancers in males and a 350% rise in squamous cell carcinoma in females between 1979 and 1994, with 1994 figures at 97/100,000/year for males and 32/100,000/year for females (6). This increase in incidence has been seen in white populations at many latitudes and was attributed to sun exposure and iatrogenic immunosuppression (which increases susceptibility to skin cancer) (56,63). A Swedish study reported in 2003 confirmed this increased incidence, with time showing a strong cohort effect which they attributed to intentional sun bathing. Clustering in families indicates the role of genetic susceptibility, and the study reported familial standardized incidence ratios of 2.25

(95% CI 1.93–2.59) for exposed sites and 2.60 (95% CI 1.38–4.20) for usually covered sites (64).

One squamous cell carcinoma is seen for every three or four basal cell carcinomas in a healthy population. However, this ratio is reversed in individuals receiving therapeutic immunosuppression after organ transplantation, so that squamous cell carcinoma is more common in this group than is basal cell carcinoma (65,66). This observation would suggest that a degree of immunosurveillance is operative in the skin and that this is more important for squamous cell carcinoma than for basal cell carcinomas, although the incidence of both is increased in immunosuppressed patients. The human papillomavirus (HPV) also has a role in SCC carcinogenesis as evidenced in epidermodysplasia verruciformis (EV) described below, and by the identification of epidermodysplasia verruciformis HPV subtypes as a risk factor for skin cancer in immunosuppressed renal transplant patients (67). It is clinically apparent in transplant recipients that sun exposure and latent infection with some HPV subtypes cooperate in SCC carcinogenesis (68) but a causal role for HPV in SCC remains controversial, see review (69).

A number of rare genodermatoses predispose to squamous cell carcinoma and in these syndromes SCC tends to develop earlier in life than in the sporadic variety, as discussed below.

#### 149.5.2 Clinical Features and Pathology of Squamous Cell Carcinoma

Squamous cell carcinomas (SCC) arise predominantly on sun-exposed skin in white skinned outdoor workers, although they may also occur in chronically inflamed skin or scars (Figure 149-7). Males have therefore been affected more frequently than females in the past but, with increasing recreational sun exposure and a decrease



**FIGURE 149-7** Squamous cell carcinoma on the dorsum of the hand in a patient with damage resulting from chronic sun exposure.



in the number of males occupationally sun-exposed, the incidence is now becoming more equal between the sexes. SCC may arise on sun-damaged skin *ab initio* or may arise on in situ disease (Bowen's disease) or sun induced precursors, called actinic keratoses. Actinic keratoses are small scaling or crusted lesions which arise on sun-damaged skin. The exact risk of malignant change in these actinic keratoses is debated, but is of the order of 1–16% per year (70).

The clinical appearance of the classic SCC is that of a non-healing nodule on any body site. Ulceration is relatively common, and the history is frequently that of months or even years of slow growth. Although SCC can be suspected on clinical grounds, a diagnostic excision is usually essential to confirm the clinical impression. The pathology is that of invasive tongues of epidermal keratinocytes which show varying degrees of dedifferentiation and invasion into the underlying dermis. For most tumors surgery is curative, although metastases may occur from primaries which are very thick, arise in particular sites such as the ear and lip, or when the tumor arises in scar tissue and especially in the immunosuppressed.

### 149.5.3 Management

Management of SCC is preferably surgical although radiotherapy may also be used.

### 149.5.4 Genetics of SCC

There is individual variation in susceptibility to SCC. Fair skinned people are more susceptible, and an extreme of this is seen in people with oculocutaneous albinism who are very susceptible to SCC, especially when they live at low latitudes. In most populations, fair skin associated with red hair and freckles is the principal phenotypic marker of risk of SCC, as for other forms of skin cancer. This phenotype is in turn controlled by the pigmentation genes and these are clearly the most important low penetrance susceptibility genes in terms of prevalence. Thus, genes which control the quantity and sub-cellular localization of melanin in the skin are important, such as polymorphisms in the oculocutaneous albinism gene *OCA-2* (71). Also influential are genes which control the ratio of eumelanin to pheomelanin produced, namely variants in the melanocortin receptor gene *MC1R* (10,12,72). Polymorphisms in the *IRF4* gene (15,73) and *SLC45A2* gene (14,16) have also been associated with SCC risk. Both of these genes influence pigmentation, however the additional role of *IRF4* in the immune response may contribute to its role in susceptibility to SCC (15). There are some candidate gene studies reported, for example there is some interest in genes coding for detoxifying enzymes (74) as susceptibility genes but the data are as yet preliminary (75,76). DNA repair genes are obvious candidate susceptibility genes, and a number of studies are emerging in which

this has been addressed (77–80). The vitamin D receptor polymorphisms *Bsm1* and *Fok1* are reported to be associated with risk (81), presumably by modifying cell responses to vitamin D. This risk interacts with vitamin D intake for *Bsm1* polymorphisms (81). Genome wide association studies will further assist in identification of relevant genes associated with SCC.

There are in addition a number of rare hereditary conditions predisposing to squamous cell carcinoma of the skin which we will discuss below:

- Xeroderma pigmentosum
- Self-healing epithelioma of Ferguson Smith
- Epidermodysplasia verruciformis
- Dystrophic epidermolysis bullosa (Hallopeau–Siemens type)
- Porokeratoses

The incidence of non-melanoma skin cancer is also increased in additional genetically determined conditions, which will not be described further such as those listed in Table 149-1.

In terms of somatic genetic events, in both actinic keratoses and squamous cell carcinomas, there is clear evidence of mutation of the *p53* gene (82), which is present in nearly half of tumors (83–85). The function of wild type *p53* is to prevent progress of cells into the cell cycle and thus allow time for unscheduled DNA repair post-UV exposure. Without this checkpoint, cells with ultraviolet (UV) induced signature mutations, (frequently C-T and CC-TT mutations) proliferate and predispose to both actinic keratoses and subsequently to squamous cell carcinomas (82). Mutations with the typical UV signature are also seen in *HRAS* particularly in SCC developing in psoriatics treated with PUVA light treatment (86). Allelotype studies show numerous abnormalities of chromosomes 3p, 9p, 13q, 17p and 17q (38,87). There is some evidence that loss of the 9p tumor suppressors at the *CDKN2A* locus also plays a role in SCC carcinogenesis, as loss of heterozygosity and loss of the entire of 9p are frequently observed in SCCs (88,89). However, conflicting results from immunohistochemical studies of p16<sup>INK4</sup> expression (reviewed by Boukamp et al. (90)) demonstrate that the role of *CDKN2A/p16<sup>INK4a</sup>* in the development of SCCs is currently not understood. The complex karyotype found in SCCs is not found in BCCs, suggesting UV exposure is not the only cause (90).

## 149.6 XERODERMA PIGMENTOSUM

Xeroderma pigmentosum (XP) is one of a group of diseases characterized by defective DNA repair (nucleotide excision repair defects) (91–93), the others being trichothiodystrophy and Cockayne syndrome. There is a remarkable heterogeneity in phenotypes of these syndromes despite a common defect in excision repair. Only patients with classic XP are susceptible to skin cancer (91–93), although patients with trichothiodystrophy are



**TABLE 149-1 Genetic Conditions Predisposing to BCC and SCC**

Condition	Mode of Inheritance	Pathway	Susceptibility
Bazex–Dupre–Christol syndrome	Sex linked dominant	Unknown	BCC
Basal cell nevus syndrome	Autosomal dominant	Hedgehog signaling pathway	BCC
Rombo syndrome	Autosomal dominant	Perhaps DNA repair and/or cell-cycle regulation <sup>a</sup>	BCC
Brooke–Spiegler syndrome	Autosomal dominant	Apoptosis regulation, <sup>b</sup> cell cycle <sup>c</sup>	BCC
Multiple hereditary infundibulocystic BCC	Autosomal dominant	Unknown	BCC
Schopf–Schultz–Passarge syndrome	Autosomal recessive	Unknown	BCC
Xeroderma pigmentosum (groups A to G)	Autosomal recessive	Nucleotide excision repair	BCC, SCC and melanoma
Xeroderma pigmentosum variant	Autosomal recessive	DNA polymerase	SCC
Multiple self-healing squamous epithelioma (Ferguson Smith syndrome)	Autosomal dominant	TGF- $\beta$ signaling <sup>d</sup>	SCC
Epidermodysplasia verruciformis	Autosomal recessive usually but also sex linked and dominant postulated	Unknown but associated with increased susceptibility to viral infection	SCC
Oculocutaneous albinism types 1A, 1B, 1I, 1II and 1IV	Autosomal recessive	Melanin synthesis	SCC
KID (keratitis, ichthyosis and deafness)	Autosomal dominant	Mutation in connexin-26 gene, <i>GJB2</i>	SCC
Dystrophic epidermolysis bullosa	Autosomal recessive	Connective tissue	SCC, small increase melanoma
Junctional epidermolysis bullosa	Autosomal recessive	Connective tissue	SCC
Fanconi anaemia	Autosomal recessive	DNA repair	SCC
Rothmund–Thomson	Autosomal recessive	Chromosomal stability	SCC
Bloom syndrome	Autosomal recessive	Chromosomal stability	SCC
Dyskeratosis congenita (Autosomal dominant and sex linked types)	Autosomal dominant Sex linked	Telomere maintenance	SCC
Werner syndrome	Autosomal recessive	Chromosomal stability	SCC and melanoma
Hermansky–Pudlak syndrome and Hermansky–Pudlak syndrome, type 2	Autosomal recessive	Melanosome and lysosome storage	SCC
Griscelli syndrome (type 1–3)	Autosomal recessive	Pigment granule transport	SCC
Elejalde disease	Autosomal recessive	Pigment granule transport	SCC
Chediak–Higashi syndrome	Autosomal recessive	Lysosome transport regulation	SCC

Madan, V.; Lear, J. T.; Szeimies, R. M.; Non-Melanoma Skin Cancer. *Lancet* **2010**, 375 (9715), 673–685.

([www.cancer.gov/cancertopics/pdq/genetics/skin/HealthProfessional/Table4](http://www.cancer.gov/cancertopics/pdq/genetics/skin/HealthProfessional/Table4)), ([www.cancer.gov/cancertopics/pdq/genetics/skin/HealthProfessional/Table2](http://www.cancer.gov/cancertopics/pdq/genetics/skin/HealthProfessional/Table2)), mode of inheritance from OMIM.

<sup>a</sup>van Steensel, M. A.; Jaspers, N. G.; Steijlen, P. M. A Case of Rombo Syndrome. *Br. J. Dermatol.* **2001**, 144, 1215–1218.

<sup>b</sup>Brummelkamp, T. R.; Nijman, S. M.; Dirac, A. M.; Bernards, R. Loss of the Cyclinomatosis Tumor Suppressor Inhibits Apoptosis by Activating NF- $\kappa$ B. *Nature* **2003**, 424, 797–801.

<sup>c</sup>Stegmeier, F.; Sowa, M. E.; Nalepa, G.; Gygi, S. P.; Harper, J. W.; Elledge, S. J. The Tumor Suppressor CYLD Regulates Entry into Mitosis. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, 104, 8869–8874.

<sup>d</sup>Goudie, D. R.; D'Alessandro, M.; Merriman, B., et al. Multiple Self-healing Squamous Epithelioma Is Caused by a Disease-Specific Spectrum of Mutations in TGFBR1. *Nat. Genet.* **2011**.

sun sensitive. Eight subtypes of XP, groups A–H, are characterized by defective unscheduled post ultraviolet (UV) DNA repair of keratinocytes (94,95) and the ninth (xeroderma pigmentosa variant or pigmented xerodermod) is a defect of post replication UV repair (96). XP is inherited as an autosomal recessive disease and is characterized by rapid development of persistent erythema in the first year of life when the child is exposed to ultraviolet radiation. If sun protection is not introduced, premature aging of the skin is followed by the development of malignancy in the first decade (97). Progressive neurological degeneration occurs in a number of patients, specifically those with subtypes A, B, D and G (98).

The loci of the XP genes and their function are itemized in Table 149-2. Groups A–G range from individuals with the most defective DNA repair in group A to the least defective in group G (99). XP is more common in the Middle East and in Japan than in Europe and the United States (100).

### 149.6.1 Clinical Features

Affected individuals may be diagnosed during their first year of life because of the observation that exposure to natural sunlight gives rise to persistent and prolonged erythema. If not protected from further UV exposure,

**TABLE 149-2** Clinical Variants of Xeroderma Pigmentosum and Genes Responsible

Type	Gene Symbol	Chromosomal Locus	Role of Gene in DNA repair
A	<i>XPA</i>	9q22.3	Maintains single stranded DNA during repair <sup>a</sup>
B	<i>ERCC3</i>	2q21	3'–5' DNA helicase <sup>b</sup>
C	<i>XPC</i>	3p25	Recognition of DNA damage and global genome repair <sup>c</sup>
D	<i>ERCC2</i>	19q13.2–q13.3	5'–3' DNA helicase <sup>b</sup>
E	<i>DDB2</i>	11p12–p11	Recognition of UV-induced DNA damage <sup>d</sup>
F	<i>ERCC4</i>	16p13.3–p13.13	DNA endonuclease 5' to lesion <sup>e</sup>
G	<i>ERCC5</i>	13q33	DNA endonuclease 3' to lesion <sup>f</sup>
H	<i>ERCC1</i>	19q13.2–q13.3	Forms a complex with ERCC4 to form DNA endonuclease 5' to lesion <sup>e</sup>

Chromosomal locus from OMIM.

<sup>a</sup>Volker, M.; Mone, M. J.; Karmakar, P., et al. Sequential Assembly of the Nucleotide Excision Repair Factors In Vivo. *Mol. Cell* **2001**, *8*, 213–224.

<sup>b</sup>Reardon, J. T.; Sancar, A. Nucleotide Excision Repair. *Prog Nucleic Acid Res. Mol. Biol.* **2005**, *79*, 183–235.

<sup>c</sup>Sugasawa, K.; Ng, J. M.; Masutani, C., et al. Xeroderma Pigmentosum Group C Protein Complex Is the Initiator of Global Genome Nucleotide Excision Repair. *Mol. Cell* **1998**, *2*, 223–232.

<sup>d</sup>Scriba, A.; Konickova, R.; Czyzewski, B. K., et al. Structural Basis of UV DNA-Damage Recognition by the DDB1–DDB2 Complex. *Cell* **2008**, *135*, 1213–1223.

<sup>e</sup>Staresinic, L.; Fagbemi, A. F.; Enzlin, J. H., et al. Coordination of Dual Incision and Repair Synthesis in Human nucleotide Excision Repair. *EMBO J.* **2009**, *28*, 1111–1120.

<sup>f</sup>Evans, E.; Moggs, J. G.; Hwang, J. R.; Egly, J. M.; Wood, R. D. Mechanism of Open Complex and Dual Incision Formation by Human Nucleotide Excision Repair Factors. *EMBO J.* **1997**, *16*, 6559–6573.

the affected child will have mottled, dry skin similar to that found on sun-exposed areas of an elderly person, with extensive freckling. Before the introduction of rigorous sun avoidance, these children developed cutaneous malignancies during their first decade. Most serious are melanoma and SCC, and many died of metastatic malignancy before the age of 20.

The introduction of effective sun-screening regimens has brought about a significant change in the outlook for these children. With vigorous sun avoidance, the use of protective clothing, and appropriate sunscreens, these children now live to reproductive age (100,101).

A proportion of patients with xeroderma pigmentosum have both ocular and neurological problems in addition to cutaneous problems. Ocular problems include UV-induced keratitis, retinopathy and UV-induced cataract, while neurological problems may include progressive mental retardation, microcephaly and deafness.

## 149.6.2 Management

Management of families with XP includes a regimen of vigorous sun avoidance. The use of highly protective sunscreens, clothing, sun avoidance and UV protective film on windows is required. There must be careful cutaneous surveillance with excision of all possible early cutaneous malignancies and histological examination. Vitamin D supplementation with monitoring of blood levels to achieve those in the range 60–85 nmol/L is therefore essential. The incidence of squamous cell carcinoma is very high indeed in these families; there is also a higher-than-expected incidence of basal cell carcinoma, malignant melanoma, and vascular tumors (102). Tumors of the tip of the tongue are reported more commonly than would be expected. High dose isotretinoin is reported to prevent new neoplasms in those with multiple skin cancers (103). As progressive neurological abnormalities can occur in a minority, routine neurological examination may be indicated.

## 149.7 MULTIPLE SELF-HEALING EPITHELIOMAS OF FERGUSON SMITH

In 1934, John Ferguson Smith described two large kindreds in Ayrshire in the west of Scotland with a history in several generations of multiple self-healing epitheliomas (104): keratoacanthoma like lesions (Figure 149-8). Affected family members generally developed their lesions after puberty, and onset of the condition may be as late as age 40, or older. The clinical picture is that of the development of many rapidly expanding hyperkeratotic lesions over time, usually on sun-exposed skin. Affected patients are therefore advised to avoid sun exposure without becoming vitamin D depleted. These lesions, despite a pathological picture indistinguishable from invasive squamous cell carcinoma, do not usually progress, but spontaneously regress over a period of weeks or months frequently leaving an unsightly or crenated scar (Figure 149-9). SCC may however occur rarely even in sites associated with greater risk of metastasis such as the anus (Figure 149-10), lip or vulva. The syndrome is rare and therefore data are limited, but there is a view that affected patients are radiosensitive (although the use of radiotherapy has been reported (105)) and therefore that radiotherapy should be avoided.

Family studies indicate clearly that transmission is by autosomal dominant inheritance. The gene for multiple self-healing epitheliomas of the Ferguson Smith type has been mapped to chromosome 9q. Haplotype studies indicated that the disease locus was most likely to lie between D9S29 (9q31) and D9S1 (9q22.1–q22.2) (106), but it took many years to identify the causal gene. It was when it became possible to sequence many hundreds of genes with exon capture using high capacity shotgun sequencers that mutations in the *TGFBR1* gene, (being outside the 4-Mb interval originally proposed (107)), was identified as the



**FIGURE 149-8** Exuberant keratoacanthoma-like lesion on the ear of a patient with self-healing epitheliomas of Ferguson Smith.



**FIGURE 149-9** Crenated scars left on the skin of the nose after spontaneous involution of a keratoacanthoma-like lesion in self-healing epitheliomas of Ferguson Smith.

causal gene. It was established that the families with self-healing epitheliomas of Ferguson Smith had loss-of-function mutations in the same *TGFBR1* gene as underlies Loeys–Dietz syndrome in which condition the mutations are missense mutations. It is surprising (given that this condition was previously supposed to be a founder mutation arising in Ayrshire) that multiple mutations were identified in the *TGFBR1* gene although c.154G>C was the most frequent (107). It may be therefore subsequently prove to be that the syndrome is more common in parts of the world other than Scotland than had been supposed.



**FIGURE 149-10** Anal tumor in self-healing epitheliomas of Ferguson Smith: such tumors are rare in this syndrome but do occur and may in this site behave more aggressively than in other sites in this condition.

### 149.8 EPIDERMODYSPLASIA VERRUCIFORMIS

Epidermodysplasia verruciformis (EV) is a condition in which there is increased susceptibility to infection with the human papillomavirus (HPV) leading to multiple warts, and where particular HPV subtypes are present, also to squamous cell carcinoma. The HPV subtypes associated with EV are now known as betaPV types (108). Unlike high-risk mucosal HPV subtypes which cause cervical cancer, betaPV types do not integrate into cellular DNA and appear additionally to require exposure to ultraviolet light for carcinogenesis (108). A very large number of HPV types may be involved in EV, but types 5 and 8 are the most consistently associated with a risk of developing squamous cell carcinoma. The betaPV HPV types are ubiquitous (being detected in excess of 80% of people without skin cancer), but may then proliferate as a result of loss of immunity when the host is immuno-suppressed either genetically, as in EV or as a result of immunosuppressive drug treatment. There is recent evidence also for a small role for other oncogenic viruses such as the Merkel polyomavirus (109).

Although EV may be acquired, as a result of HIV infection for example (110,111) or haematological malignancies (112), usually it is manifest as a rare, recessively inherited genodermatosis. Recessively inherited EV was mapped to chromosome 2p21–p24 (EV2) and 17q25 (EV1). On chromosome 17, 2 adjacent related genes—*EVER1/TMC6* (113) and *EVER2/TMC8* (114)—were identified. Nonsense mutations in the genes *EVER1* and *EVER2* have been identified in over 75% of cases (115). It is thought that mutations at these loci result in susceptibility to the viral infection as a result of impaired cell-mediated immunity but the precise mechanisms remain



unclear. There are reports of more occasional X-linked inheritance (116,117). A family from India was reported in which the mode of inheritance appeared to be more consistent with a dominant gene although consanguinity is another possible explanation, (118) and more recently a mother and affected sons were reported from Iran (119). A lack of *EVER1* and 2 mutations was recently reported in a family in which EV appeared to be dominantly inherited (115), so that the genetic basis of all cases of EV is not yet understood.

### 149.8.1 Clinical Features

Affected individuals develop large numbers of flat and plane warts, most abundant usually on the face and neck, in childhood. These warts tend to coalesce to form large plaques. Premalignant lesions such as actinic keratoses and Bowen's disease start to develop in the third and fourth decades and squamous cell carcinoma may develop in up to 20% of such lesions, most commonly on sun-exposed sites, implying a role for ultraviolet radiation as a co-carcinogen (118).

## 149.9 RECESSIVE DYSTROPHIC EPIDERMOLYSIS BULLOSA (OF THE HALLOPEAU-SIEMENS TYPE)

Squamous cell carcinoma may complicate different forms of epidermolysis bullosa (EB) (120–122) but the association is particularly with the recessive dystrophic type. This autosomal recessive condition is the most severe variant of dystrophic epidermolysis bullosa (EB), and is due to mutations in the gene *COL7A1* which encodes the anchoring fibril protein of type 7 collagen. This is a very large gene with 118 exons located on 3p21. The Hallopeau–Siemens type of EB is an autosomal recessive disease due to mutations in both alleles of *COL7A1*, leading to reduction in mRNA and impaired synthesis of collagen 7 peptides for assembly into anchoring fibrils (123,124).

Affected infants can be diagnosed at birth by the presence of fragile skin and rapid development of blisters at sites of trauma which then scar. Nails, teeth, hair and oral mucosa are also abnormal. Children with this variant of EB are at greatly increased risk of squamous cell carcinoma, which frequently develops in the second decade on sites of blister-induced scarring. The carcinomas are unusually aggressive and metastasize early, so death results from SCC in 78.7% of affected individuals by the age of 55 years (122). Tumors arise predominantly at the sites of maximal scarring rather than sun exposure (121) and chronic inflammation/continuous wound healing is probably causal. A small study reported no evidence for a role for the human papillomavirus in these patients any more than in SCC occurring in healthy individuals (122). There does not seem to be an excess of non-cutaneous cancer (125) but there is a small excess of melanoma (122). That the tumors behave in a very much

more aggressive way than do sporadic SCC suggests that they are biologically different and although attempts are being made to understand this (126) the nature of these differences is not yet understood.

## 149.10 POROKERATOSES

The term porokeratosis describes a group of disorders of keratinization associated with an unstable clone of keratinocytes which give rise to a characteristic clinical and pathological picture. One variety of this condition, porokeratosis of Mibelli, is inherited by autosomal dominant transmission, and is a precursor to squamous cell carcinoma in 7%–20% of cases. Many tumors in these patients appear to have followed radiotherapy and immunosuppression (127). The gene responsible has been provisionally mapped to the short arm of chromosome 3 (128).

### 149.10.1 Clinical and Pathological Features

Affected patients have large plaques on any body site, characterized by a raised rim and central mildly atrophic epidermis. The characteristic “rim” is reflected in the pathology which shows a raised so-called cornoid lamella with loss of granular layer and parakeratosis around the edge of the lesion.

## 149.11 MUIR–TORRE SYNDROME

Patients with the autosomal dominant hereditary non-polyposis colon cancer syndrome (HNPCC) may also develop skin cancers, and this sub-type, or phenotypic variant of the HNPCC syndrome is known as the Muir–Torre syndrome (129). The syndrome is defined by a combination of sebaceous tumors of the skin and one or more internal malignancies, most often colon cancer. The types of skin tumor classically include sebaceous adenoma, sebaceous epithelioma, sebaceous carcinoma, and keratoacanthoma (130,131) but may also include actinic keratoses and squamous cell carcinomas. HNPCC is characterized by germline mutations of DNA mismatch repair genes (including *bMSH2*, *bMLH1*, *bPMS1* and 2, *bPMS* and *bMSH6*) (129), and corresponding microsatellite instability may be demonstrated in resected skin tumors from Muir–Torre families. Germline mutations in the HNPCC associated mismatch repair genes, particularly *bMSH2* (130) (not so far in *bPMS1* or 2), have been reported. It is said that a family history consistent with the Muir–Torre syndrome should additionally be excluded when patients have multiple non-sebaceous tumors, tumors in relatively sun-protected sites or tumors occurring in people who also have sebaceous hyperplasia (129), but the validity of this is unclear.

Where skin tumors suggestive of Muir–Torre syndrome occur with no personal or family history of HNPCC, the genetic basis and indeed the risk of visceral tumors in the future is difficult to assess. It is reported however that



sebaceous tumors overall are associated with Muir–Torre syndrome in only 14% of cases (132). Screening tumors for microsatellite instability (by immunohistochemistry) or colonoscopy may be appropriate but advice from genetics services may be the best way to proceed.

## 149.12 MELANOMA

Melanoma is responsible for the great majority of skin cancer deaths. The incidence has increased dramatically in the United States, the United Kingdom and Australasia during this century (133). The highest rates currently occur in New Zealand (134,135). The rate of increase has been faster than for any other malignancy with the exception of lung cancer in women, but there is evidence of a leveling off in incidence in some countries (135), but not in others (136).

Melanoma is predominantly a cancer of people who have skin which tends to burn, and higher incidences occurring where pale-skinned peoples live in hotter countries indicates that sun exposure is the main cause. Rare cases occur however in all skin types in sun-protected sites such as the sole of the foot, under the nail or on genital skin, and the etiology of these rarer subtypes is as yet unclear. The type of sun exposure which is causal for the majority of melanoma however has been controversial, but there is clear evidence for a role of intermittent sun exposure resulting in sunburn (137,138). This increased incidence has therefore been attributed to intense sun exposure on holiday in white skinned (and therefore vulnerable) peoples. A current hypothesis however is that there may be two routes to the development of melanoma, via acute, intermittent sun exposure (for which increased numbers of melanocytic nevi are a risk factor) and via chronic cumulative overexposure (139,140). Evidence for this ‘two route’ hypothesis has come from molecular studies in primary tumors, which have suggested that mutations in the *BRAF* gene (141) typify superficial spreading melanomas which arise on intermittently sun-exposed body sites and for which melanocytic nevi are a risk factor, but are much less common in melanomas arising on chronically sun-exposed or in non-sun-exposed sites (142). Melanomas are therefore genomically complex and although common inherited susceptibility genes have been identified, the relationship between these genes and the genomically different primary tumors remains unclear.

### 149.12.1 Clinical Features and Pathology

Melanoma commonly arises from benign proliferations of melanocytes: melanocytic nevi, or moles, but it may arise from normal appearing skin. Most commonly the tumor grows horizontally in the epidermis for some time (radial growth phase) before growing vertically (vertical growth phase). There are different clinico-pathological subtypes of melanoma which start as radial growth phase.

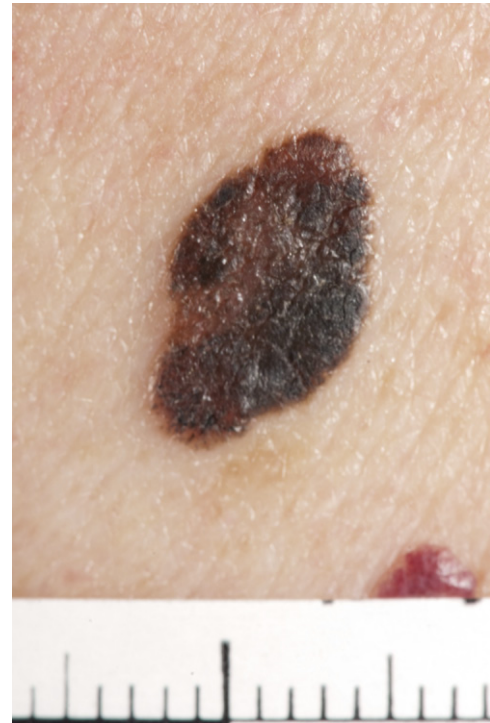
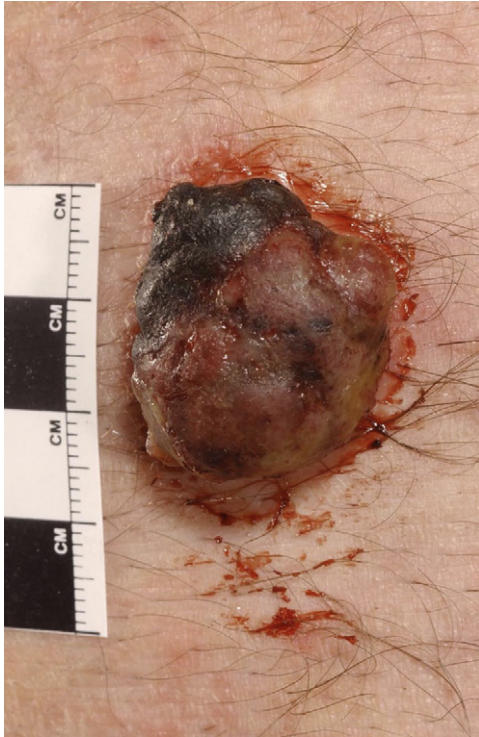


FIGURE 149-11 Superficial spreading melanoma.

The commonest is the superficial spreading melanoma (Figure 149-11). Lentigo maligna is a variety seen most commonly on the face and acral lentiginous melanoma on the hand or foot. The nodular melanoma appears to go into vertical growth phase from the beginning, although in some tumors the radial growth phase is probably just destroyed by the growing vertical phase tumor (Figure 149-12). It is thought that the progression from benign nevus through atypical nevus (dysplastic nevus), radial growth phase melanoma to vertical growth phase is a continuum in which multiple genetic events occur: in a similar way to that described in the colon as the tumor evolves from a polyp.

### 149.12.2 The Genetics of Susceptibility to Melanoma

The most common genetic determinants of susceptibility to melanoma (and non-melanoma skin cancer) are the skin pigmentation genes, as melanoma is predominantly a cancer of white skinned peoples. Thus genes which produce darker skin are protective, and therefore the genes which govern the amount of melanin produced by melanocytes and the way that melanin is packaged in the skin are major determinants of susceptibility. Additional genes control the type of melanin produced. The *MC1R* gene, for example, codes for the melanocortin receptor to which melanocyte stimulating hormone (MSH) binds, and *MC1R* variants exist which modulate the ratio of black pigment (eumelanin) to red pigment (pheomelanin) produced in response to MSH. These variants increase the amount of red pigment produced and therefore



**FIGURE 149-12** A friable nodular melanoma difficult to distinguish from other forms of skin cancer.

inheritance of these results in red hair and fair skin (72), and freckles (143), all of which are risk factors for melanoma (144) and skin cancer in general. These *MC1R* variants are established as melanoma susceptibility genes (145,146) even in the presence of dark hair (147). In terms of common low penetrance melanoma susceptibility genes, *MC1R* variants are the first genes clearly identified as significant. A number of small, candidate gene studies have been reported which have provided some evidence of association with melanoma risk but these are difficult to evaluate and therefore will not be listed here. GWA has now identified 11 susceptibility genes (including confirming the role of *MC1R*) (Table 149-3). These genes include pigment genes, genes associated with increased numbers of nevi and additional genes not associated with a particular phenotype (see Table 149-3). The genes of biological interest include *CASP8* (which is in an apoptosis pathway) and the ataxia telangiectasia gene *ATM* (Barrett et al. (2011)—submitted).

**149.12.2.1 Melanoma in Family Cancer Syndromes.** The majority of families in which melanoma clusters are at increased risk of melanoma alone or melanoma and pancreatic cancer and the genetic basis of these susceptibilities is discussed below.

Melanoma does, however, also occur in other genetically determined family cancer syndromes. Melanoma is a second malignancy for example in familial retinoblastoma, accounting for a proportion of second malignancies in affected individuals occurring particularly in the radiation field (148). A cohort study was reported in

1993 in which a statistically significant excess of mortality was demonstrated from melanoma in retinoblastoma survivors (149). Anecdotal reports of both retinoblastoma and melanoma in families have been made (150), but a cancer registry review showed no increased risk of melanoma in parents of affected children (151).

There is thought to be an increased risk of melanoma in families with the Li-Fraumeni syndrome (152), in which the majority of cancers are sarcomas, brain tumors or bilateral breast cancers, and which are attributed to inherited mutations in the gene *p53*. Certainly a registry study from Sweden showed an increased risk of childhood osteosarcoma in children of mothers with melanoma (153) and there is an excess of melanomas in survivors of childhood soft tissue sarcomas (154), but as for all second cancers it is difficult to differentiate genetic from treatment effects.

Melanoma is also reported to occur in a relatively small proportion of families with an increased risk of bowel cancer due to inherited mutations in mismatch repair genes (hereditary non-polyposis coli) (HNPCC). In one recent series, nine family members from eight families developed melanoma in a series of 60 with HNPCC (155).

Melanomas have been described in the Birt-Hogg-Dube syndrome (156) and in one report these were multiple desmoplastic melanomas (157). We have seen acral lentiginous melanoma in one case in Leeds, but the strength of the association is unknown. Birt-Hogg-Dube syndrome is a rare genodermatosis in which there are hair follicle hamartomas on the face, renal cancer, and lung cysts associated with pneumothoraces (158). The syndrome is associated with germline *FLCN* mutations (159) which code for a putative tumor suppressor gene called folliculin.

**149.12.2.2 High Penetrance Genes/Familial Melanoma.** Progress has been made in understanding rare, high penetrance, susceptibility genes by studying families with multiple cases of melanoma but without the above listed cancer family syndromes. This progress has resulted from work carried out by many groups around the world and as a result of the activities of the Melanoma Genetics Consortium ([www.genomel.org](http://www.genomel.org)).

Approximately 5% of melanoma patients have a clear history of melanoma in a first-degree relative. Patients in multiple case melanoma families present with their first primary melanoma on average two decades earlier than sporadic cases, and have a higher than expected incidence of multiple primary melanomas. As with sporadic melanomas, the commonest subtype is the superficial spreading melanoma.

An early study on 14 US families with both familial melanoma and also large numbers of atypical or dysplastic nevi suggested that a melanoma susceptibility gene was located at chromosome 1p36 (160), but this report has not been confirmed, and no candidate gene has emerged.

Forty percentage of the families with three or more cases of melanoma carry causal mutations in the

**TABLE 149-3 Low Penetrance Susceptibility Genes Identified for Melanoma from Genome-wide Association Studies**

Gene	SNP	Risk of Melanoma for Each Allele <sup>i</sup>	Associated Phenotype	References of Studies also Showing Association
MC1R	rs258322	1.70 (1.54, 1.87)	Red hair	a,b,c
TYR	rs1393350	1.30 (1.21, 1.39)	Fair skin	a,c
ASIP	rs4911414	1.20 (1.12, 1.28)	Red hair	c,d
TERT	rs401681	1.20 (1.12, 1.28)	None known	e
SLC45A2	rs35390	0.36 (0.23, 0.53)	Darker hair and skin	f,g
IRF4	rs872071	1.07 (1.00, 1.14)	Darker skin and more nevi	h
CDKN2A/MTAP	rs7023329	0.83 (0.78, 0.88)	Number of nevi	a,i
PLA2G6	rs6001027	0.85 (0.79, 0.91)	Number of nevi	i
CASP8	rs13016963	1.14 (1.09, 1.19)	No associated phenotype	j
ATM	rs1801516	0.84 (0.78, 0.90)	No associated phenotype	j
MX2	rs45430	0.88 (0.85, 0.92)	No associated phenotype	j

<sup>a</sup>Bishop, D. T.; Demenais, F.; Iles, M. M., et al. Genome-wide Association Study Identifies Three Loci Associated with Melanoma Risk. *Nat. Genet.* **2009**, *41*, 920–925.

<sup>b</sup>Han, J.; Kraft, P.; Nan, H., et al. A Genome-wide Association Study Identifies Novel Alleles Associated with Hair Color and Skin Pigmentation. *PLoS Genet.* **2008**, *4*, e1000074.

<sup>c</sup>Gudbjartsson, D. F.; Sulem, P.; Stacey, S. N., et al. ASIP and TYR Pigmentation Variants Associate with Cutaneous Melanoma and Basal Cell Carcinoma. *Nat. Genet.* **2008**, *40*, 886–91.

<sup>d</sup>Brown, K. M.; Macgregor, S.; Montgomery, G. W., et al. Common Sequence Variants on 20q11.22 Confer Melanoma Susceptibility. *Nat. Genet.* **2008**, *40*, 838–840.

<sup>e</sup>Nan, H.; Qureshi, A. A.; Prescott J.; De Vivo I.; Han J. Genetic Variants in Telomere-Maintaining Genes and Skin Cancer Risk. *Hum. Genet.* **2011**, *129*, 247–253.

<sup>f</sup>Stacey, S. N.; Sulem, P.; Masson, G., et al. New Common Variants Affecting Susceptibility to Basal Cell Carcinoma. *Nat. Genet.* **2009**, *41*, 909–914.

<sup>g</sup>Duffy, D. L.; Zhao, Z. Z.; Sturm, R. A.; Hayward, N. K.; Martin, N. G.; Montgomery, G. W. Multiple Pigmentation Gene Polymorphisms Account for a Substantial Proportion of Risk of Cutaneous Malignant Melanoma. *J. Invest. Dermatol.* **2010**, *130*, 520–528.

<sup>h</sup>Duffy, D. L.; Iles, M.; M., Glass, D., et al. IRF4 Variants Have Age-Specific Effects on Nevus Count and Predispose to Melanoma. *Am. J. Hum. Genet.* **2010**, *87*, 6–16.

<sup>i</sup>Falchi, M.; Bataille, V.; Hayward, N. K., et al. Genome-Wide Association Study Identifies Variants at 9p21 and 22q13 Associated with Development of Cutaneous Nevus. *Nat. Genet.* **2009**, *41*, 915–919.

<sup>j</sup>Barrett, J. H.; Iles, M. M.; Hartland, M., et al. Genome-Wide Association Study Identifies Three New Melanoma Susceptibility Loci. *Nat. Genet.* **2011**, *43*, 1108–1113.

*CDKN2A* gene at 9p21 which codes for a cell cycle control protein called p16. This protein binds to and inhibits the cyclin-dependent kinase 4 (CDK4). 9p21 is deleted in a high proportion of melanoma cell lines (161) and *CDKN2A* is also an important suppressor gene in other malignancies, particularly in pancreatic cancer (162). The gene is composed of two exons (1 $\alpha$  and 2) which code for p16 and one alternatively spliced exon (exon 1 $\beta$ ), which codes for a completely unrelated protein called p14ARF. Insertions, deletions and missense or nonsense mutations have all been recorded in *CDKN2A* in familial melanoma patients in all three exons of the gene, and functional studies have confirmed the loss of suppressor activity in a proportion of these mutations. Some mutations impact on p16 alone, some on p14ARF alone (2–3% of families overall) (163,164) and others on both, but in all families the susceptibility is predominantly to melanoma. GenoMEL maintains a data base of these mutations (eMelanobase) which is now part of LOVD (Leiden Open Variation Database) <http://chromium.liacs.nl/LOVD2/home.php>.

A number of founder mutations in *CDKN2A* have been reported. p.M53I mutations appear to be particularly common in Australian and Scottish families, but not in US families. Table 149-4 summarizes mutations

found across families in Australia, Europe and North America in a GenoMEL study reported by Goldstein et al. (164). The percentage of families investigated who have *CDKN2A* mutations ranges from 20% to 50% depending on geographical location with an overall prevalence of 38% in 465 families (164). In general, families with three or more affected family members have a higher incidence of mutations. In lower incidence areas such as Italy, the proportion of two case families with identified mutations is higher than elsewhere (165). A high proportion of melanoma families have family members with multiple primary melanomas, and *CDKN2A* mutations have also been reported in patients with multiple primary melanomas who have no identifiable positive family history of melanoma (166). A possible gene environment interaction is suggested by the observation that the incidence of melanoma in families with the 9p21 mutation has increased 21-fold since 1952, and the suggested reason for this increase is greater exposure to ultraviolet radiation (167), although there may be some biases here. A consensus statement published following a review of the available literature stated that, except in regions of high melanoma incidence, for example Australia, higher rates of *CDKN2A* mutation are found in those with three or more primary invasive melanomas and/or families with at least one invasive melanoma and



**TABLE 149-4 Melanoma Families with *CDKN2A* Germline Mutations from the Melanoma Genetics Consortium (GenoMEL)**

Reference	Country	Number of families investigated	Number with mutations (%)	Commoner mutations (number)
Goldstein et al. 2006	Australia	164	32 (20)	p.M53I (5) p.R24P (3) p.L32P (3) c.458-105A>G (3)(3)
	Europe	214	106 (50)	c.225_243del19 (19) p.G101W (12) p.R112_L113insR (11) c.458-105A>G (7) p.M53I (10) p.R24P (6)
	North America	87	40 (46)	c.-34G>T (8) p.V126D (6) p.G101W (4) p.M53I (4)
		465	178 (38)	p.R24P (5)(6)

Goldstein, A.; Chan, M.; Harland, M., et al. High-Risk Melanoma Susceptibility Genes and Pancreatic Cancer, Neural System Tumors, and Uveal Melanomas Across GenoMEL. *Cancer Research* 2006, 66, 9818–9828.

two or more other diagnoses of invasive melanoma and/or pancreatic cancer among first or second degree relatives on the same side of the family (168). Individuals who satisfy these criteria will benefit most from genetic counselling and possible testing, although the value of clinical testing is debated (169,170).

Rare families have been described in which susceptibility appears to be due to inherited mutations in the *CDK4* gene at the p16 binding site. Worldwide, only 2% of families have been reported with a *CDK4* mutation (164). This is clearly excessively rare in melanoma families, but does appear to be mutated in some of the families originally reported as having chromosome 1 abnormalities. This gene maps to chromosome 12q14 and the mutant acts as a dominant oncogene by preventing inhibition by wild type *CDKN2A* (171,172).

The fact that not all melanoma families investigated show *CDKN2A* or *CDK4* mutations suggests that other melanoma susceptibility genes exist, and a number of families have been identified linked to 9p21 who do not currently have identifiable *CDKN2A* mutations, implying the presence of a possible second tumor suppressor gene near this locus. Strenuous efforts are being made to identify the “missing” genes.

A high proportion of melanoma families also have large numbers of melanocytic nevi which are clinically and pathologically atypical—so-called dysplastic nevi (173) (Figure 149-13). These nevi are larger than banal benign nevi, and may have an irregular edge and histological evidence of a stromal reaction. They reflect the increased proliferation seen in melanocytes in at-risk individuals after sun exposure. Although it was initially suggested that only those members of melanoma families with such nevi were at risk of melanoma, other studies have shown that this is not the case and that family

members with a normal complement of banal melanocytic nevi may also be *CDKN2A* mutation carriers with associated melanoma risk (174).

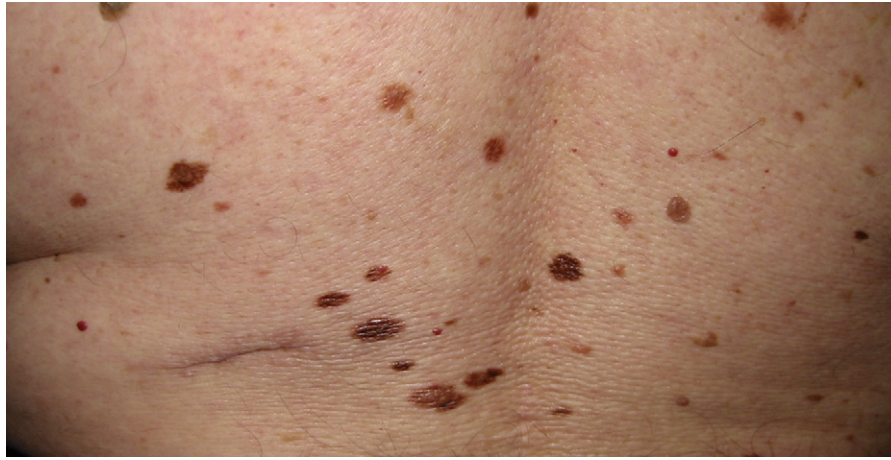
In some geographical areas of the world (not in Australian (164) and UK families to date at least) inherited *CDKN2A* mutations also increase the risk of pancreatic cancer (175–177), although clustering of both cancers may occur also in families without such mutations (178). A study of Dutch families with melanoma demonstrated a significantly increased risk of pancreatic cancer, cancers of the respiratory system, lip, oral cavity and pharynx, non-melanoma skin tumors and tumors of the eye/brain (uveal melanoma and brain tumors) in patients carrying the p16-Leiden *CDKN2A* mutation (177). There is some further anecdotal evidence for an increase in squamous cell carcinomas of the head and neck, (179) and for sarcoma (180).

### 149.12.3 Management

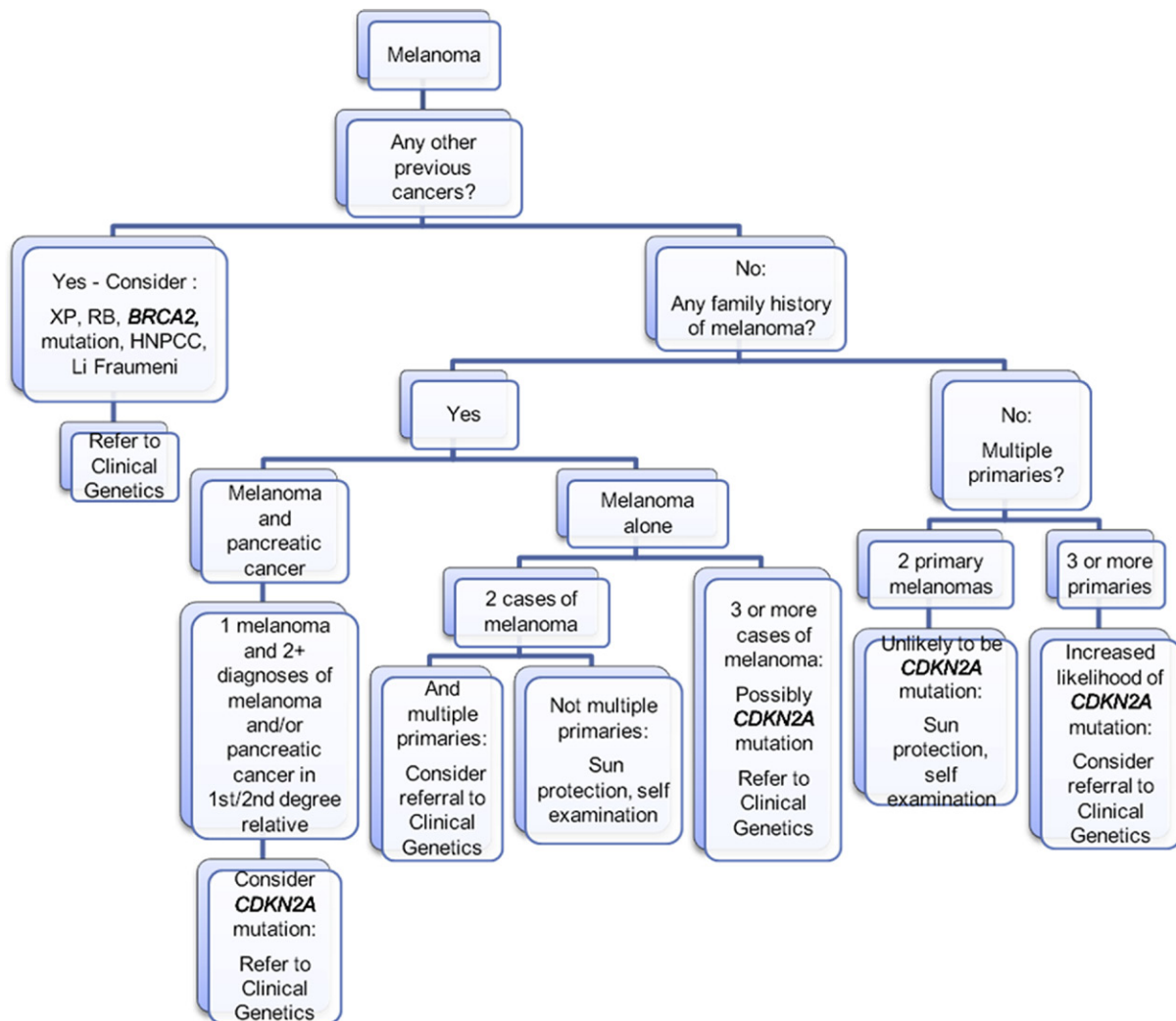
All patients with suspected early melanomas should have a family history taken, and corroborative pathological evidence sought for lesions said to be melanomas in family members. If confirmed, family members should be offered sun protection advice (without inducing vitamin D depletion), a total body skin examination to identify early melanomas or atypical nevi but most particularly, self-examination training.

All suspected melanomas should be excised with a narrow margin of surrounding normal skin and sent for pathological confirmation. The thickness of the tumor measured from the overlying granular layer to the deepest underlying invasive cell will determine whether a wider excision is needed and will also determine the likely prognosis. Consider referral to





**FIGURE 149-13** Multiple nevi in the atypical mole syndrome, associated with risk of melanoma within families at risk of melanoma in the general population.



**FIGURE 149-14** Indications for referral to clinical genetics in cases of malignant melanoma.

clinical genetics for counseling and genetic testing for **CDKN2A** mutations for those individuals with multiple (three or more) primary melanomas, and patients with one melanoma and a family history of two or

more other cases melanoma or pancreatic cancer (168) (Figure 149-14).

Patients in whom a primary melanoma has been excised should be regularly followed-up, particularly

members of melanoma families, as there is a greatly increased risk of second and subsequent primary melanomas in these families. Detailed photography at the first visit of all melanocytic nevi may be useful for identifying subsequent early change in these nevi. Protection from sunburn without inducing vitamin D depletion is crucial, and families with *CDKN2A* mutations should also be advised to avoid smoking given the relationship between smoking and pancreatic cancer.

## FURTHER READING

Information for genetic counselors working with melanoma families is available at [www.genomel.org](http://www.genomel.org).

## CROSS REFERENCES

Nature and Frequency of Genetic Disease; Genomics and Proteomics; Genome Structure and Gene Expression; Mutations in Human Disease: Nature and Consequences; Mendelian Inheritance; Multifactorial Inheritance and Complex Traits; Population Genetics; Genetic Epidemiology; The Molecular Biology of Cancer; Genetic Counseling and Clinical Risk Assessment; Diagnostic Molecular Genetics; Heterozygote Testing and Carrier Screening; Genetic Counseling; Ethical and Social Issues in Clinical Genetics; Deletions and Other Structural Abnormalities of the Autosomes; Vitamin D Metabolism or Action; Disorders of DNA Repair and Metabolism; Retinoblastoma; Disorders of the Hair; Clefting, Dental, and Craniofacial Syndromes; Abnormalities of Pigmentation; Epidermolysis Bullosa.

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### Biographies



**Professor Julia Newton-Bishop** is Professor of Dermatology at the University of Leeds, UK. She manages a skin cancer screening service at St James's Hospital in Leeds and leads a research group designed to identify the causes of melanoma and the determinants of survival. She is the chairman of the international melanoma genetics consortium GenoMEL ([www.genomel.org](http://www.genomel.org)) and a new consortium called BioGenoMEL addressing the genetics of melanoma relapse.



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# CHAPTER

# 150

## Psoriasis

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### GLOSSARY

**Psoriasis** – Chronic inflammatory disease of the skin characterized by red scaly plaques on the body and thought to be autoimmune in nature

### 150.1 INTRODUCTION

Psoriasis is a chronic inflammatory skin disease that affects around 2% of the Caucasian population. The majority of cases arise in individuals younger than 30 years of age, with more than 10,000 cases per year arising in children less than 10 years old (1). The cutaneous manifestations of psoriasis are visibly obvious, with a strong negative impact on quality of life (2). Up to 40% of patients develop an associated arthritis, which can be severe and deforming (3). The disease has a strong genetic component (4) and tends to have a fluctuating course that can sometimes be linked to environmental factors (5). In recent years, considerable progress has been made in unraveling the genetic complexity of psoriasis, mostly through the use of genome-wide association studies, with over 20 susceptibility loci identified to date.

### 150.2 CLINICAL FEATURES AND SUBTYPES

Psoriasis is clinically characterized by erythematous scaly plaques, which are sharply demarcated from normal skin and predominantly located on the scalp and extensor surfaces of the extremities, although in some cases they can involve the entire skin surface. Several different forms of cutaneous psoriasis can be observed in the same person, including chronic plaque, guttate, inverse, seborrheic, and pustular types (Figures 150-1 and 150-2). Although it has been demonstrated that the clinical phenotypes of psoriasis may be genetically determined to a large extent, the clinical manifestations of psoriasis can change over time in any given person (6).

Chronic plaque psoriasis (psoriasis vulgaris) is the most common type of psoriasis, seen in approximately 90% of patients. This is characterized by red, scaly

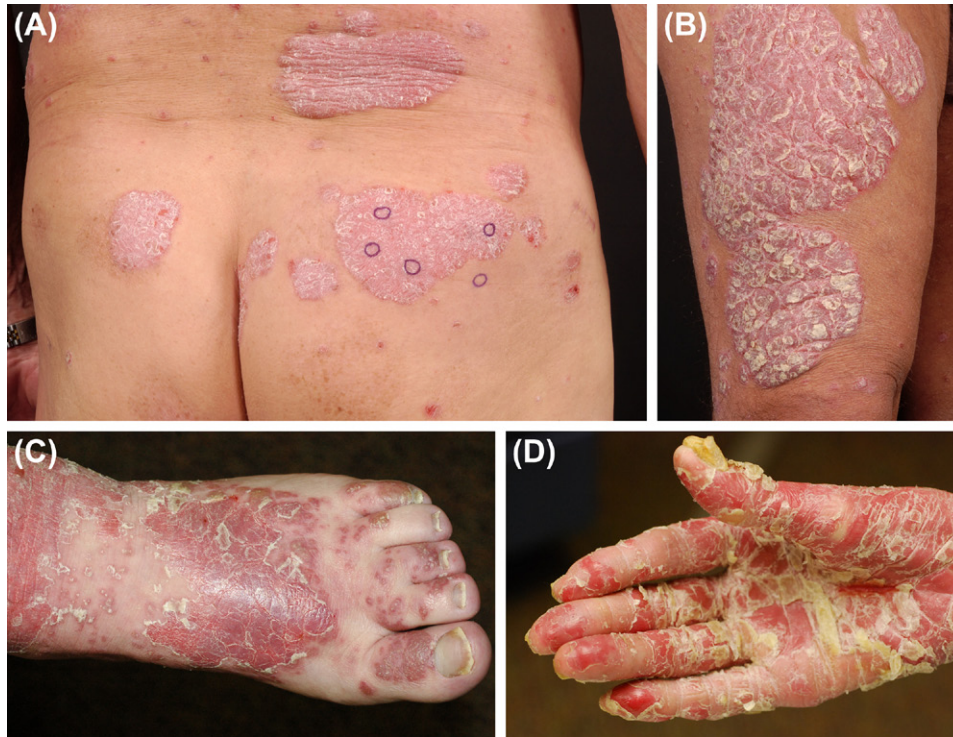
plaques, sharply demarcated from normal skin, located primarily on the elbows and knees (Figure 150-2). The severity and extent of the disease varies widely from patient to patient, although there is consistently constant production of large amount of scale.

Guttate (drop-like) psoriasis is characterized by eruption of many small (0.5–1.0 cm in diameter) scaly lesions over the trunk and proximal extremities. It typically occurs in young adults and has a strong association with preceding or concomitant streptococcal pharyngitis (7). The eruption is typically self-limited, lasting up to 12–16 weeks, although around one-third to one-half of patients go on to develop chronic plaque psoriasis (8). It has a very strong association to human leukocyte antigen (HLA) variant HLA-Cw6 (9).

Other clinical subtypes of psoriasis are less common. In inverse psoriasis, the lesions are located in the major skin folds such as the axillae, inguinal folds, genital region, and the neck. Scaling is usually minimal or absent. Seborrheic psoriasis is characterized by erythematous plaques with greasy scale localized to seborrheic areas such as the scalp, glabella, nasolabial folds and presteral areas. In the absence of typical psoriasis findings elsewhere, it may be hard to distinguish from seborrheic dermatitis. Several different types of pustular psoriasis exist, including localized, generalized, and palmoplantar pustulosis. Generalized pustular psoriasis can present abruptly with high fevers, diffuse redness, and waves of pustules on the skin and is the only form of psoriasis that can be life-threatening.

Finally, erythrodermic psoriasis is the generalized form of the disease that affects all body sites, including the face, hands, feet, trunk, and extremities. Instead of thick, adherent, white scale there is diffuse erythema with superimposed fine scale. Patients tend to lose excessive heat because of generalized vasodilation and this can lead to high-output cardiac failure.

Nail changes are frequently observed in psoriasis and are found in up to 40% of patients. Several distinct changes have been described and these correlate with age, duration, and extent of disease, and with the presence of psoriatic arthritis (10).



**FIGURE 150-1** Chronic plaque psoriasis—the most common form of psoriasis (A, B, C, D). It is characterized by red, scaly lesions, sharply demarcated from normal skin. Psoriatic lesions are most commonly located on the extensor surfaces of the extremities, such as the knees and elbows, but can involve any part of the skin. With extensive involvement, or localized such as on the hands and feet (C and D), it can be severely debilitating.

Psoriatic arthritis is a common manifestation of psoriasis seen in 10–40% of patients (3,11). Psoriatic arthritis is rheumatoid-factor negative and usually presents a few years after the onset of skin disease, between the ages of 35 and 45 years (12). It has a prominent genetic component and several different subtypes have been described, as originally defined by Moll and Wright (13). These subtypes involve distal interphalangeal arthritis of the hands and feet, symmetric polyarthritis, symmetric oligoarticular arthritis, predominant axial spondylitis, and/or arthritis mutilans. The clinical subtype of psoriatic arthritis is not fixed and the clinical manifestations in any given patient have been shown to change considerably over time (14).

### 150.3 PREVALENCE, AGE AT ONSET AND SEX RATIO

Psoriasis is found worldwide, although its frequency varies widely among different ethnic groups, with reported prevalence ranging from 0 to 11.8% (15). The reasons for this wide range in prevalence ratios are multiple. Many of these studies had several confounding variables, most notably the method of ascertainment (clinic-based or population-based, examination-based, or questionnaire-based). Nevertheless, examination of available population-based studies show prevalence ranging from 0.2 to 4.8%, with the average for northern Europe being around 2% (16) and in the United States ranging from 2.2

to 2.6% (17). Incidence of psoriasis is lower in Asians, around 0.3 (18) to 1.2% (19), and in a population-based examination of more than 12,500 Samoans and 26,000 South American Indians, not a single case of psoriasis was observed (20). Based on several small investigations from Africa there appears to be a difference in prevalence of psoriasis in East Africans (2.0%) versus West Africans (0.3%) (20a), potentially explaining the low frequency of psoriasis in African-Americans, most of whom originated from West Africa (17).

Prevalence of psoriatic arthritis varies widely between studies, ranging from 0.04 to 0.4% (3). A recent population-based survey determined that the prevalence of psoriatic arthritis in the United States is 0.25%, with 11% of psoriasis patients having concomitant arthritis (11).

Psoriasis is equally common in males and females (20a,21) and there is no evidence for clinical differences between males and females (16). It may first appear at any age, from birth to the eighth or ninth decade, although it is most likely to appear between the ages of 15 and 30 years (16). Age of onset correlates with HLA-Cw6 carriage (22) and with a higher likelihood of positive family history, and this was the major reason for the previously used classification of psoriasis into type I and type II disease, with type I having strong association with HLA-Cw6 and onset before the age of 40 years, and type II onset after the age of 40 and lacking HLA associations (22a).





**FIGURE 150-2** Different clinical manifestations of psoriasis. Psoriasis can present in several different forms. It can sometimes present as annular plaques (A), or pustular (B), although this presentation is much less common. Some lesions can present with marked hyperkeratosis (C, D). Often with resolving pustular psoriasis, the lesions progress to chronic plaque psoriasis or diffuse erythroderma (E). Nail changes are common in psoriasis and several types of nail changes are recognized, including dystrophy with marked subungual hyperkeratosis (F) and loosening of the nail plate from the nail bed (onycholysis) (G).

#### 150.4 GENETIC EPIDEMIOLOGY

Psoriasis has been appreciated as a genetic disease for nearly 100 years (23). However, only about one-third of patients have an affected first-degree relative (20a). The strongest evidence for the genetic basis of psoriasis was previously provided from twin studies (16). As identical twins have all their alleles in common, in contrast to fraternal twins where the sharing is only about half, if genes play an important role the disease concordance should be greater in monozygotic than dizygotic twins. This is indeed the case in psoriasis, where concordance in monozygotic twins has been shown to range from 35 to 73% in various studies (24). This is about three times higher

than that observed in dizygotic twins (16), and as this concordance is less than 100%, it suggests involvement of environmental factors in addition to genetic predisposition. Interestingly, twin studies have also indicated that many of the clinical features observed in psoriasis are also determined by genetic factors (24a).

Using recurrence risk analysis it has been demonstrated that psoriasis is a polygenic disease. Recurrence risk analysis involves calculating the risk ratio,  $\lambda_r$ . This parameter describes how much more common a disease is in relatives of index case compared with the general population (25). Application of this type of analysis to three major population-based epidemiological studies

(26) demonstrated that the excess recurrence risk of psoriasis for relatives of degree  $r$  dropped by a factor of 6–7 as  $r$  increased from 1 to 2, as opposed to the factor of 2 predicted for monogenic disorders (27), indicative of polygenic inheritance. In accordance with such a model, the risk of psoriasis in an offspring has been estimated to be 41% if both parents are affected, 14% if one parent is affected, and 6% if one sibling is affected, compared with 2% when no parent or sibling is affected (28).

Twin studies and pedigree studies have also been used to calculate the heritability ( $h^2$ ) of psoriasis, which measures the proportion of the variability of a multifactorial trait that is due to genetic factors. Various estimates have calculated this to be in the range of 52–90%, which is among the highest of any multifactorial disorder (20a,27).

The only study to analyze concordance of psoriatic arthritis in twins found a concordance rate of 10% in monozygotic versus 3.7% in dizygotic twins, lower than that observed for cutaneous psoriasis (24d). However, this study was far too small to draw definite conclusions. Importantly, numerous other studies have reported familial aggregation of psoriatic arthritis (29) and the risk for affected first-degree relatives ( $\lambda_1$ ) is in the range of 25–55 (24d,30) which is substantially higher than that observed for cutaneous psoriasis (ranging from 4 to 10) (27).

Parent-of-origin effects have also been proposed for psoriasis and psoriatic arthritis (31). In one study, the birth-weight of children of parents with psoriasis was found to be influenced by the sex of the parent with psoriasis, with offspring from fathers with psoriasis being heavier than children from mothers with psoriasis (32). Excessive paternal transmission in psoriatic arthritis has been described (33), consistent with the findings of a linkage study showing predominant transmission of an allele of paternal origin on chromosome 16 (34).

## 150.5 ENVIRONMENTAL FACTORS

Multiple environmental factors have been implicated in the pathogenesis of psoriasis (16) and the impact of these factors is demonstrated by the less-than-perfect disease concordance in monozygotic twins, as discussed earlier. The specific roles of the various environmental factors are likely to differ, with some being important in triggering the disease while others exacerbate or modify the disease (16). Streptococcal throat infections are the best characterized environmental factor in psoriasis (35) and have been shown to both trigger the disease as well as exacerbate preexisting chronic plaque psoriasis (36). The first report of this association was made in 1916 (37) and has since then been confirmed in multiple studies (7,8). Interestingly, primary skin infections by streptococci generally do not trigger or exacerbate psoriasis (16). The association of streptococcal throat infections and HLA-Cw6 is of particular interest as it has been

hypothesized that psoriasis is caused by cross-reactive epitopes between human skin keratins (KRT16 and KRT17) and streptococcal M-protein, a rod-like extracellular virulence factor of streptococci with sequence homology to epidermal keratins (35). Consistent with this hypothesis, skin homing CD8+ T-cells taken from HLA-Cw6+ patients have been shown to respond to antigens common to both KRT17 and M-protein, whereas non-psoriatic HLA-Cw6+ controls respond only to the M-protein peptides (38). Another line of evidence for this mechanism is that T cells carrying the same T-cell receptor gene rearrangement have been identified in the tonsils and lesional skin of psoriatic patients (39). This would suggest that in genetically susceptible (HLA-Cw6+) individuals, T cells react against streptococcal antigens in the tonsils and subsequently home to the skin where they encounter cross-reactive KRT17 and KRT16 derived antigens. Thus guttate and streptococcal-induced psoriasis may be caused by cross-reactive T-cells activated by streptococcal infections of the tonsils, whereas in chronic plaque psoriasis inflammation is maintained by autoreactive T cells that continue to respond to keratin peptides long after the streptococcal infection has been cleared (16,40).

HIV infection has been associated with severe active psoriasis (41). However, the prevalence of psoriasis in HIV infection is about the same as in the general population (~2%) (42), suggesting that HIV infection is not a trigger but a modifying factor in psoriasis. Thus as the immunodeficiency in HIV progresses, psoriasis gets progressively more active and severe, but interestingly, remits in the terminal phase (43). Likewise, with active antiretroviral treatment, psoriasis improves (44). This exacerbation has been speculated to be due to loss of regulatory CD4+ T cells and subsequent uncontrolled increase in the activity of the CD8+ T-cell subset (40,45). Consistent with this scenario, other autoimmune diseases that are CD8+ T-cell driven predominate in HIV (45). Another possibility is that the HIV virus has a more direct role in the pathogenesis, either by increasing the activity of infected T cells prior to their death and/or by activation of dendritic cells, which are known hosts for HIV. Consistent with this are the findings that HIV transcripts have been identified in the skin of patients with HIV-associated psoriasis and within both CD4+ T cells and dendritic cells (46).

Known as the Koebner phenomenon or the isomorphic response, induction of psoriasis at sites of trauma is a well-known reaction seen in about 25% of psoriasis patients (47). This is an all-or-none type of a response, such that if psoriasis occurs at one site of injury, it will occur at all sites. It is typically seen in patients with active disease or during periods of flare, and manifests around 7–14 days after the injury.

Several other factors have been implicated in the onset of psoriasis. One prominent factor is stress, particularly acute stressful events (48). Patients with high stress have



been found to have more severe disease (48b), which is perhaps not surprising as psoriasis can be emotionally disabling, particularly in those severely afflicted (48d). Psoriasis typically develops or flares from 2 days up to a month after the initial stressful event (16).

Finally, smoking (49) and several drugs have been implicated in triggering flares of psoriasis; these include medications such as antimalarials, beta-blockers, lithium, nonsteroidal anti-inflammatory drugs, imiquimod (16), and, curiously, anti-TNF (tumor necrosis factor) agents, which are highly effective against psoriasis (50). The majority of patients who experience flares following anti-TNF treatment have worsening or new onset of chronic plaque psoriasis, but around 40% present with pustular flares of the palmar and plantar surfaces, often in association with chronic plaque psoriasis elsewhere on their skin (50b). The pathologic mechanisms underlying these flares could involve cross-regulation of interferon alpha (IFN- $\alpha$ ) and TNF (51). The non-uniformity of this phenomenon also suggests an element of heterogeneity in the inflammatory network in psoriasis.

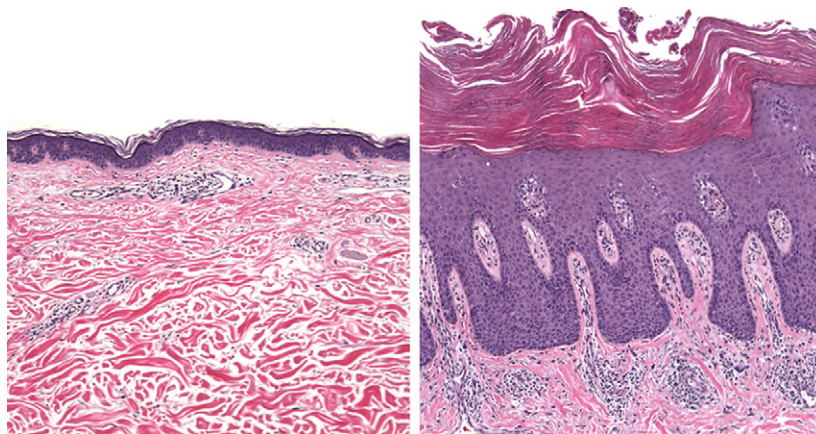
## 150.6 COMORBIDITIES

Psoriasis, particularly severe disease, has been shown to be associated with an increased risk of myocardial infarction (52) and death (53). Psoriasis patients also have increased prevalence of the metabolic syndrome (54), being more likely to have diabetes, hyperlipidemia and higher body-mass index (48c,55), with severe psoriasis being more likely in obese individuals (49). Interestingly, the increased risk of myocardial infarction persists even after adjusting for all these covariates (52), as has been observed in other systemic inflammatory diseases (56). These observations suggest that a state of systemic inflammation, perhaps promoted by shared genetic susceptibility variants, may predispose to atherosclerosis in these disorders.

## 150.7 HISTOPATHOLOGY AND LESIONAL EVOLUTION

Psoriasis is characterized by markedly increased epidermal growth and altered differentiation in association with immunological, inflammatory, biochemical, and vascular abnormalities, along with a poorly understood relationship to nervous system function (16). Fully developed psoriatic lesions are characterized by uniform elongation of the rete ridges of the epidermis with thinning of the epidermis overlying the dermal papillae (Figure 150-3). There is altered differentiation in the epidermis with confluent parakeratosis (retention of nuclei) in the stratum corneum and accompanied loss of the granular layer. Nearly all basal keratinocytes are proliferating in psoriatic skin compared to less than 10 percent in normal skin (57), and this is accompanied by a large increase in the metabolic activity of epidermal cells (58). Dilated, tortuous capillaries appear in the dermal papillae with a markedly increased number of lymphocytes, macrophages, dendritic cells and mast cells. Neutrophils tend to collect within the epidermis, either in the stratum corneum where they form microabscesses (of Munroe) or smaller collections in the spinous layer (spongiform pustule of Kogoj).

The transition from uninvolved skin to fully developed psoriasis proceeds through several stages. It is of interest that normal-appearing skin of patients with psoriasis manifests subtle morphologic and biochemical changes involving lipid biosynthesis (58a,59) and innate immunity (60). The earliest changes noted in a developing lesion are marked edema and infiltration of mononuclear cells (61). The overlying epidermis begins to demonstrate edema between the keratinocytes (spongiosis) and there is focal loss of the overlying granular layer. Accompanying these changes is dilatation of the superficial blood vessels. In developing lesions there is gradual increase in the thickness of the epidermis accompanied



**FIGURE 150-3** Histopathology of psoriasis. Comparison of uninvolved (left) versus involved (right) skin from the same individual. Psoriatic lesions are characterized by marked increased thickness of the epidermis with prolongation of the rete pegs, altered differentiation with loss of the granular layer, and overlying confluent parakeratosis in the stratum corneum. There is dilatation and increase tortuosity of dermal blood vessels in the dermal papillae and mononuclear infiltrate that is composed of macrophages, dendritic cells, and T cells.

with increased metabolic activity and DNA synthesis (58a,62). There is an increased number of T cells and dendritic cells in the dermis (63) along with increased number of degranulated mast cells and dermal macrophages (58a,62). In the center of the lesions, rete ridges begin to elongate with more confluent parakeratosis eventuating into a fully developed psoriatic plaque.

## 150.8 IMMUNOPATHOGENESIS

Multiple lines of evidence suggest that psoriasis is a T-cell mediated disease, driven at least in part by a positive feedback loop from activated T cells to antigen-presenting cells, with contributions from innate immune mechanisms involving the epidermis, macrophages (64), vascular epithelium, and possibly mast cells (65). Psoriasis was for many years thought to be mediated by T helper 1 cell (Th1) IFN- $\gamma$ -secreting cells (66), but recently the focus has shifted toward a novel subset of CD4+ T lymphocytes producing interleukin 17 (IL-17) (67) and/or IL-22 (65), and therefore named Th17 and Th22 respectively. These cells are increased in psoriatic lesions (65,67) and while their differentiation from naïve CD4+ T cells is induced by the cytokines IL-1 and IL-6 (68,69), their maintenance and expansion is dependent on IL-23 (70), which is derived from dendritic cells, macrophages, and keratinocytes, the latter likely at a lower level (71). Th17 cells are characterized by expression of the IL-23 receptor and produce and release the cytokines TNF- $\alpha$ , IL-17, IL-21, and IL-22, which sometimes is attributed to a separate Th22 subset (72). The importance of these cells in the pathogenesis of psoriasis is supported by the rapid decrease in Th17 responses seen with effective anti-TNF treatment (73). There is considerable plasticity in this system and Th17 cells have been shown to be able to change into IFN- $\gamma$ -producing Th1 cells (74). Given the increased number of both Th1 and Th17 cells in psoriasis (70), it is notable that the Th1-derived cytokine IFN- $\gamma$  programs myeloid-derived dendritic cells to induce Th17 cells via IL-1 and IL-23 (70). In psoriatic skin there is a distinct compartmentalization of T cells, with CD4+ T cells predominating in the upper dermis while CD8+ T cells mostly localize to the epidermis (75). Importantly, entry of T cells into the epidermis appears to be a crucial event in the development of psoriatic lesions (76). CD8+ T cells have a functional division similar to CD4+ T cells, with the majority producing TNF- $\alpha$  along with IFN- $\gamma$  (Tc1), IL-17 (Tc17), and/or IL-22 (Tc22), all of which are found in increased numbers in the psoriatic epidermis (65,77). These subsets have similar cytokine requirements as the T helper cells, with the Tc17 being similarly dependent on IL-23 for maintenance and expansion (78). Some CD8+ T cells in psoriasis are clonally expanded (79), suggesting that they may be responding to a limited set of antigens in the context of HLA class I molecules, notably HLA-Cw6. Although CD8+ T cells are thought to be primarily cytotoxic in nature, the injury might be

sublethal as psoriatic keratinocytes are relatively resistant to apoptotic damage (80). Thus, instead of causing cytotoxic death, these cells may promote the hyperplastic and pro-inflammatory psoriatic epidermal response through the release of cytokines (65). The CD8+ T cells are likely to be primed and activated through a process called cross-presentation (81). Cross-presentation involves the update of intracellular proteins from other cells by antigen-presenting dendritic cells, which in turn present them in the context of HLA class I on their surface (81). Cross-presentation is dependent upon CD4+ T-cell support (82), which might explain the observed dependence in psoriasis on CD4+ T cells in a xenograft mouse model (83). Keratinocytes contribute to the inflammatory response by producing effectors of innate defense known to be highly overexpressed in psoriasis, including the defensins hBD-2 and hBD-3, CCL20, CXCL9 and CXCL10, S100A7, S100A8, S100A9, and S100A15 (6,70,84). In addition to antimicrobial properties, many of these proteins have chemotactic activities, such as CCL20 for Th17 cells (85), and CXCL9 and CXCL10 for Th1 cells (86), which can amplify the inflammatory cascade. Other examples include induction of Th17 cytokines by the closely related S100A7 and S100A15 proteins (87) and beta-defensin 2, which increases production of IFN- $\gamma$  and TNF- $\alpha$  in T cells (88).

In addition to T cells and keratinocytes, other cell types play important roles in the inflammatory network in psoriasis. Macrophages are important cells of the innate immune system and are capable of antigen presentation and activating T cells during stimulation of the adaptive arm of the immune system (89), particularly after IFN- $\gamma$  priming as is observed with these cells in psoriasis (89). Macrophages are increased in psoriatic skin (89) and are located just under the basement membrane, adjacent to proliferating keratinocytes (90). These cells co-express inducible nitric oxide synthase (iNOS) and contribute to the inflammatory network in psoriasis through production and release of TNF- $\alpha$  and IL-23 (89). Based on findings in psoriasis-like mouse models, it has been suggested that macrophages may play a key role in the pathogenesis of psoriasis, at least in part through their TNF- $\alpha$  production (91).

Dendritic cells (DCs) may also play major role in psoriasis. Two main types of DCs are present in psoriatic lesions: plasmacytoid DCs and myeloid-derived DCs (92). Plasmacytoid dendritic cells are a specialized subset of DCs that are increased in number in psoriatic lesions and characterized by the ability to make IFN- $\alpha$  (93). Intravenous administration of IFN- $\alpha$  has been shown to worsen psoriasis in some patients (94). Similar exacerbations have been observed with the use of imiquimod, which acts through toll-like receptor (TLR) 7 located within plasmacytoid dendritic cells (95). IFN- $\alpha$  has multiple different actions including inducing cross-presentation of antigens and cross-priming of CD8+ T cells (96). IFN- $\alpha$  also induces the expression of major

histocompatibility complex (MHC) class I molecules on the surface of nucleated cells and can promote dendritic cell maturation (97). In a xenograft mouse model, it was shown that plasmacytoid dendritic cells initiate psoriasis through the production of IFN- $\alpha$  (98). A major factor in the activation of the plasmacytoid dendritic cells is thought to be a complex between DNA and the antimicrobial peptide cathelicidin, which is overexpressed in psoriasis lesions (99,100). However, the role of IFN- $\alpha$  in stable chronic plaque psoriasis has been questioned, based on the results of a clinical trial of a neutralizing anti-IFN- $\alpha$  monoclonal antibody (mAb) (101). Myeloid DCs include epidermal Langerhans cells and inflammatory dendritic epidermal cells (IDECs), as well as resident and inflammatory dermal DCs (92,102). In psoriatic lesions there is a marked increase in the number of inflammatory DCs expressing iNOS and/or key inflammatory cytokines such as TNF- $\alpha$  and IL-23 that in turn induce generation and expansion of Th17 T cells and activation of the keratinocytes (70,92).

Beyond the several cellular players described above, mast cells, neutrophils, and endothelial cells have all been suggested to play a role in the inflammatory network. Mast cells are increased in developing psoriatic lesions (58a) and have prominent degranulation in active lesions (58b). In addition to large numbers of inflammatory mediators, mast cells have been shown to be able to produce both IFN- $\gamma$  (103) and IL-17 (65). Neutrophils are commonly seen in the upper epidermis of the psoriatic lesions, where they may accumulate in small aggregates (spongiform pustule of Kogoj) or microabscesses in the stratum corneum (Munro microabscesses) and can be a source of IL-17 (65). The relevance of IL-17 production by mast cells and neutrophils in comparison to the production and release from activated T lymphocytes (65) is still unclear. Suggestive of an important role for mast cells, c-kit mutant  $W^{sash}/W^{sash}$  mice, which are nearly devoid of mast cells (104), display markedly defective early inflammatory and immune responses in the imiquimod-treated mouse model of psoriasiform inflammation (105). Endothelial cells are also likely to be active participants in the pathogenic process. Endothelial cells are strongly activated in developing and mature psoriatic lesions (58a,106) and play a major role in controlling the influx of inflammatory cells and serum proteins into psoriatic lesions.

### 150.9 PSORIASIS SUSCEPTIBILITY LOCI AND THEIR ROLES IN DISEASE PATHOGENESIS

Motivated by the favorable genetic epidemiology of psoriasis described earlier, several groups embarked on a search for genetic determinants of psoriasis in the 1990s (107). These studies relied on the property of genetic linkage (i.e. either consistent co-segregation of a particular genetic marker with disease, or sharing of alleles in

affected sibling pairs). However, except for the *PSORS1* locus, these studies yielded no consistent evidence for linkage to specific non-MHC loci (reviewed in (108)). The same problem has been encountered in a variety of other complex genetic disorders (109), mainly due to the high population frequency and low penetrance of disease alleles in many complex genetic disorders (110). In this setting, tests of association are much more powerful than tests of linkage, provided causal variants or proxies for them can be genotyped (111). However, in contrast to linkage studies, population-based association studies comparing allele frequencies in cases and controls require at least 100,000 genetic markers to comprehensively survey the genome (112). Thus, genome-wide association studies (GWAS) were not feasible in the 1990s, and genetic association studies were limited to candidate genes or regions. Since then, however, millions of genetic markers have become available in the form of single nucleotide polymorphisms (SNPs) (113). Concurrently, technologies were developed for high-throughput genotyping, allowing 100,000–1,000,000 SNPs to be typed in thousands of individuals at a reasonable cost. These developments have led to major advances in the genetics of psoriasis as well as many other complex genetic disorders (114). While there are large remaining gaps in our ability to account for all of the heritability known to exist in such disorders (115), these studies have provided major insights into the genetic architecture of many common yet complex diseases.

Because of the large number of markers being tested, GWAS require fairly stringent criteria for declaring statistical significance, as well as having a requirement for independent replication (116). Using a fairly generous significance criterion of  $p < 5 \times 10^{-7}$ , 25 psoriasis susceptibility loci have been reported at genome-wide levels of significance as of this writing. Using the more stringent criterion of  $p < 5 \times 10^{-8}$ , this number declines to 21 (Table 150-1). While in most cases the true causal variants responsible for these association signals remain to be identified, it is intriguing that many of these loci map close to genes that broadly map into a few distinctive biological pathways. Most of these are immunologic in nature and have to do with antigen processing and presentation, the Th1/Th17 inflammatory axes, and TNF- $\alpha$  and nuclear factor kappa B (NF- $\kappa$ B) signaling, as well as dendritic cell and macrophage activation. However, additional loci appear to be pertinent to epidermal barrier function and innate immune defense. Below, we will discuss these loci in the context of their hypothesized roles in psoriasis pathogenesis (Figure 150-4). While we group these genes into several axes, we would emphasize that these axes interact in many important ways.

#### 150.9.1 Antigen Presentation

It has been known for almost four decades that a major risk gene for psoriasis is located within the major



TABLE 150-1 Psoriasis Susceptibility Loci

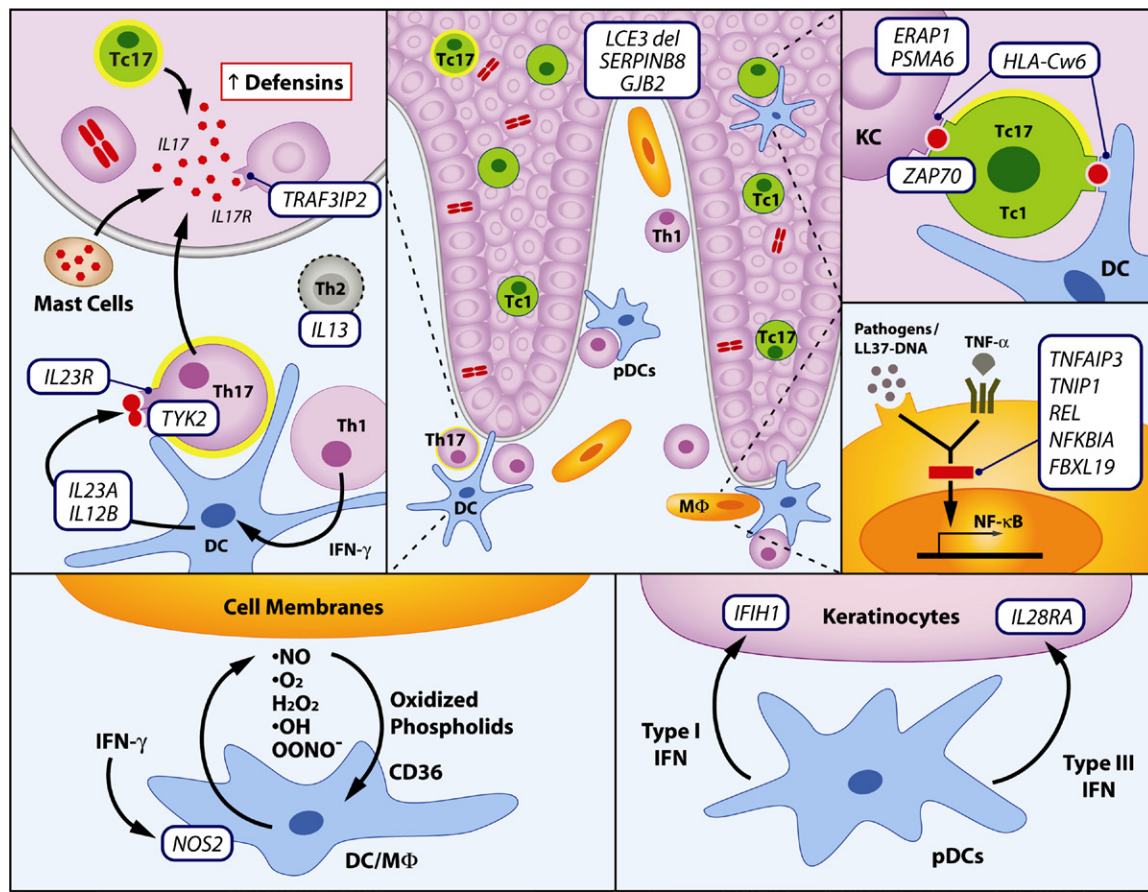
Chromosome	Nearby Gene or Locus	OR (95% CI)	Risk Allele	Cases	Controls	P-Value	Association Reported with Other Inflammatory Phenotypes	Reference
1p31	<i>IL23R</i>	1.49 (1.27–1.74)	T	0.35	0.29	$3 \times 10^{-08}$	Cr, UC, AS, PBC, BD	(126a)
1q21	<i>LCE3</i> cluster deletion	1.38 (1.19–1.61)				$1.38 \times 10^{-08}$		(164)
1p36	<i>IL28RA</i>	1.13 (1.05–1.22)	A	0.77	0.73	$6.89 \times 10^{-08}$		(129)
2p16	<i>REL</i>	1.12 (1.04–1.20)	G	0.62	0.56	$3.59 \times 10^{-09}$	RA, CD	(129)
2q24	<i>IFIH1</i>	1.29 (1.17–1.43)	A	0.90	0.86	$1.06 \times 10^{-13}$	T1D	(129)
5q15	<i>ERAP1</i>	1.130 (1.05–1.22)	A	0.40	0.36	$2.56 \times 10^{-11}$	AS	(129)
5q31	<i>IL13</i>	1.12 (1.02–1.24)	G	0.83	0.78	$5E \times 10^{15}$	Asthma	(126a)
5q33	<i>TNIP1</i>	1.27 (1.14–1.44)	A	0.83	0.78	$1 \times 10^{-20}$	SLE	(126a)
5q33	<i>IL12B</i>	1.39 (1.26–1.53)	G	0.86	0.79	$2 \times 10^{-28}$	SLE, Asthma, UC, Cr	(126a)
5q33.3	<i>PTTG1</i>	1.20 (1.13–1.28)	C	0.22	0.18	$1.11 \times 10^{-08}$		(127)
6p21	<i>HLA-C</i>	4.66 (4.23–5.13)	T	0.31	0.14	$<10^{-100}$		(126a)
6q21	<i>TRAF3IP2</i>	1.7 (1.3–2.22)	G	0.10	0.06	$7.31 \times 10^{-09}$		(129,139)
6q23	<i>TNFAIP3</i>	1.22 (1.13–1.32)	G	0.37	0.32	$9 \times 10^{-12}$	SLE, RA, SS, Cr, CD, T1D	(126a)
8p23.2	<i>CSMD1</i>	1.17(1.11–1.23)	C	0.22	0.17	$3.78 \times 10^{-08}$		(127)
12q13	<i>IL23A</i>	1.49 (1.28–1.73)	A	0.96	0.93	$1 \times 10^{-09}$		(126a)
13q12.11	<i>GJB2</i>	0.87 (0.84–0.91)	C	0.46	0.51	$8.57 \times 10^{-08}$	KID	(127)
14q13	<i>NFKBIA/PSMA6</i>	1.19 (1.11–1.27)	C	0.61	0.57	$1.52 \times 10^{-11}$		(129)
16p11.2	<i>FBXL19</i>	1.16	G	0.39	0.35	$9 \times 10^{-10}$		(131)
17q11.2	<i>NOS2A</i>	1.20	G	0.40	0.35	$4 \times 10^{-11}$		(131)
18q22.1	<i>SERPINB8</i>	0.87 (0.83–0.91)	C	0.22	0.28	$5.92 \times 10^{-09}$		(127)
19p13	<i>TYK2</i>	1.40 (1.23–1.61)	A	0.93	0.90	$4.04 \times 10^{-11}$	Cr, HIES, T1D, SLE, MS	(129)
19q13.41	<i>ZNF816A</i>	0.88 (0.84–0.92)	C	0.31	0.36	$2.11 \times 10^{-09}$		(127)
20q13	<i>ZNF313/IRNF114</i>	1.20 (1.11–1.30)	A	0.90	0.87	$1.4 \times 10^{-08}$		(179)

Loci yielding  $p < 5 \times 10^{-8}$  in at least one study, and two loci (*IL28RA* and *GJB2*) closely approximating this threshold, are shown. AS = ankylosing spondylitis; BD = Bechet's disease; CD = celiac disease; Cr = Crohn's disease; HIES = hyper-IgE syndrome; KID = keratitis ichthyosis deafness syndrome; MS = multiple sclerosis; PBC = primary biliary cirrhosis; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus; SS = Sjogren syndrome; T1D = type 1 diabetes; UC = ulcerative colitis.

histocompatibility complex (MHC) region on chromosome 6 (117), and more recently that it resides in the class I end of the MHC (118). With the advent of GWAS to provide an unbiased view of the entire genome, the pre-eminence of the MHC's genetic influence has become clear in psoriasis, as it has in other complex immune/inflammatory disorders, including systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis (119). However, for all of these disorders, the genetic dissection of the MHC has proven to be quite a challenge. The MHC is characterized by strong linkage disequilibrium (LD), as first reported as early as 1968 (120). Early studies suggested that LD extended more than 2 Mb in some cases, but not all (121). This differs from the LD pattern reported for other regions in the genome, where strong LD exists in segments averaging approximately 22 kb (122). However, closer inspection reveals that the local structure of LD in the MHC is similar to

that found across the rest of the genome, except that a higher amount of long-range LD is observed between shorter segments of more typical LD, resulting in extended or ancestral haplotypes (121,123). Although the psoriasis MHC risk variant had been mapped to the vicinity of human leukocyte antigen (HLA)-C through case-control association and family studies (118,124) this extensive linkage disequilibrium along with the high number of immune response genes in this region caused a great difficulty in identifying the true risk gene in this region, which was not achieved until 2006 (125). In that study, through the use of recombinant ancestral haplotypes along with DNA sequencing of critical intervals, HLA-Cw\*0602 was identified as the most likely disease allele in this region (125). However, it was recently shown, using logistic regression analyses adjusting for HLA-Cw\*0602 status, that there are at least two additional loci within the MHC region. One of them





**FIGURE 150-4** Integrating the genetics and immunology of psoriasis. Genes identified as psoriasis-associated are italicized in boxes. Upper mid panel—Most of CD4+ T cells are localized in the dermis (purple), whereas CD8+ T cells are predominantly localized in the epidermis (green). Around 5% of CD8+ T cells in the epidermis and CD4+ T cells in the dermis produce IL-17 (yellow halo). Macrophages (MΦ), plasmacytoid dendritic cells (pDCs), and myeloid dendritic cells (DC) all participate in the inflammatory network of psoriasis. Psoriatic epidermis is hyperproliferative, characterized by increased number of mitoses and marked elongation of the rete ridges. The *LCE3B* and *LCE3C* deletion is thought to be involved in epidermal repair, whereas the role of *SERPINB8* in the epidermis is not fully known. Left upper panel—IFN-γ is able to prime DCs to increase production of IL-23, which in turn can signal through the IL-23 receptor on T cells to increase production of IL-17. IL-17 acts primarily on the epidermis, with increased production of pro-inflammatory cytokines and antimicrobial peptides such as beta-defensins, which are highly expressed in psoriatic lesions. Another source of IL-17 can be mast cells (purple). Several genes involved in this inflammatory axis have been identified as psoriasis-susceptibility genes. IL-13 may influence Th1/Th17 polarization or may have other immunomodulatory effects on this inflammatory axis. Right upper panel—Antigen presentation now appears to be central to the pathogenesis of psoriasis. Several genes involved in antigen processing and presentation have now been identified as risk factors. Right middle panel—NF-κB pathway regulation is central to many inflammatory processes. The NF-κB pathway is downstream of multiple cytokines, such as TNF-α, as well as pattern-recognition receptors such as toll-like receptors. Several genes involved in modulating and regulating NF-κB signaling seem to have a major role in psoriasis. Left lower panel—*NOS2*, which encodes for inducible nitric oxide synthase, is primarily located in DCs and MΦ in psoriatic lesions. It is strongly induced by IFN-γ and can amplify immune responses through generation of oxidized phospholipids. Right lower panel—pDCs may have a central role in pathogenesis of psoriasis and are the major source of type I and type III interferons. Two genes involved in this pathway were recently identified as risk genes in psoriasis. (Modified with permission from Nair et al. (197).)

is located within an intron of the open reading frame *C6orf10* and the second one is located between HLA-B and the *MICA* gene (126). The function of *C6orf10* is not known, but *MICA* is a distant homolog of the MHC class I proteins and can be induced by cellular stress in epithelia and interact with T cells (126b).

The class I human leukocyte antigens are expressed on the surface of all cells and have a critical role in host defenses against intracellular pathogens through the presentation of peptide antigens (typically but not always of intracellular origin) to CD8+ T cells (127) (Figure 150-4). Consistent with a role for HLA-Cw\*0602 in the

pathogenesis of psoriasis, CD8+ T cells are the predominant cell type in the epidermis of psoriatic lesions (75). Some of these cells are clonally expanded (79), and their entry into the epidermis is essential for the triggering of disease in a mouse xenograft model (76). HLA-C can also serve as a ligand for killer immunoglobulin-like receptors (KIRs), which can either inhibit or stimulate natural killer (NK) cells (6). The *KIR* locus has been associated with psoriatic arthritis (128) and would be a plausible candidate in the pathogenesis of psoriasis. However, our current understanding of HLA-C-KIR interaction involves a dimorphic allotype of HLA-C (Asn80/Lys80).

*HLA-Cw6* is one of several “group 2” alleles carrying Lys 80. Thus, if this mechanism were responsible for the observed association of *HLA-Cw6* with psoriasis, it would be expected that a combination of all “group 2” alleles would provide a stronger association signal in individuals carrying the cognate inhibitory KIR genotype than does *HLA-Cw6*, but this was not the case for psoriatic arthritis (125). Thus, the role of *HLA-Cw6* as a genetic regulator of NK cell activity in psoriasis remains to be clarified.

A recent genome-wide association study provided further evidence for the autoimmune basis of psoriasis through the identification of a gene involved in antigen recognition by T cells. This gene, *ZAP70*, which encodes for a tyrosine kinase that is involved in CD8+ T-cell responses, was recently recognized as a likely risk gene for psoriasis, but only in those who also carry *HLA-Cw6* (129) (Figure 150-4). This kinase is closely associated with the T-cell receptor and has a major role in setting the response threshold of the T cell, which is critical for avoidance of autoreactivity (129).

Psoriasis has also been associated with the *ERAP1* gene, which encodes an endoplasmic reticulum aminopeptidase that is involved in the trimming of peptides for presentation by MHC class I molecules (129,130). Interestingly, this association is only associated with disease in *HLA-Cw6+* individuals, further highlighting the likely functional importance of MHC class I antigen presentation in general, and of *HLA-Cw6* in particular (129). Another recently identified susceptibility locus contains *PSMA6*, a proteasomal subunit involved in MHC class I antigen processing that is overexpressed in psoriatic lesions (131) (Figure 150-4). However, this region also contains *NFKB1A*, which is an attractive candidate gene for psoriasis because it encodes I $\kappa$ B $\alpha$ , a major inhibitor of NF- $\kappa$ B signaling (129,131).

### 150.9.2 The IL-12/IFN- $\gamma$ and IL-23/IL-17 Axes

T lymphocytes have been shown to have a major role in psoriasis pathogenesis, best exemplified by the remarkable clinical efficacy of cyclosporin A (132). The most prominent subsets of T cells are of the Th1 and Th17 lineages, and although it was initially thought that these two axes had inhibitory effects on each other (133), it has recently been shown that under some circumstances, such as those that exist in psoriatic lesions, there is synergistic interaction (70) and plasticity between the two, as Th17 cells have been shown to be able to change into IFN- $\gamma$ -producing Th1 cells (74), although not vice versa. Th1 cells are the main source of IFN- $\gamma$ , whereas Th17 cells are characterized by expression of the IL-23 receptor and produce and release the cytokines TNF- $\alpha$ , IL-17, IL-21, and IL-22, which in some circumstances appears to be attributable to a separate Th22 subset (72). Other cells may contribute to this, such as mast cells, which, as

described earlier, have been demonstrated to be a source of both IL-17 and IFN- $\gamma$  in psoriasis (65,103); neutrophils; and macrophages, which may contribute to local IL-22 production (65). Through the production of these cytokines, they have profound effects on the epidermis, with activation of keratinocytes and elicitation of other pro-inflammatory cytokines, growth factors, and antimicrobial peptides (134).

Genetic studies have demonstrated that polymorphisms close to or within several of the genes involved in these immunological pathways increase the risk of psoriasis. Thus, polymorphisms in the *IL12B* gene (126a,135), which encodes the p40 subunit that is shared by IL-12 and IL-23; the *IL23A* gene (126a), encoding the p19 subunit of IL-23; and *IL23R* (135), encoding the unique subunit of the IL-23 receptor, have all been shown to increase the risk of psoriasis (Figure 150-4). When considered alongside recently published functional studies implicating IL-23 in the spontaneous development of psoriasis lesions in a xenograft model (136) and the remarkable clinical efficacy of anti-p40 treatment in psoriasis (137), these genetic associations strongly reinforce the biological significance of the Th1 and Th17 inflammatory axes in the pathogenesis of psoriasis. The clinical overlap between psoriasis and other chronic inflammatory diseases such as ankylosing spondylitis and Crohn's disease may be explained, at least in part, by shared risk alleles such as in the *IL23R* gene (138). Other loci close to or within genes involved in these pathways have recently been published. Thus, polymorphisms in the *TRAF3IP2* gene, which encodes CIKS/ACT1, have been shown to increase risk of psoriasis (139). ACT1 is a signaling adapter for IL-17-mediated cellular responses (139b), binding to the cytoplasmic tail of the IL-17 receptor (140) and linking it, through TNF-receptor-associated factors (TRAF) 3 and TRAF6, to downstream signaling of the MAPK and NF- $\kappa$ B pathways (140,141). Perhaps surprisingly, the psoriasis-associated polymorphism resulted in a near complete loss of ability of ACT1 to interact with TRAF6 (139b). TYK2 is a member of the Janus family of kinases (142), was recently implicated as a susceptibility gene in psoriasis (129) and encodes for a protein tyrosine kinase that has a central role in the signaling of multiple cytokines including IL-12 and IL-23, thus affecting both generation of Th1 and Th17 cells (142), and IFN signaling (129).

### 150.9.3 TNF- $\alpha$ and NF- $\kappa$ B Signaling

The transcription factor nuclear factor kappa B (NF- $\kappa$ B) is ubiquitously expressed, is a key player in regulation of both innate and adaptive immune responses (143), and has a major role in regulation of epidermal differentiation and proliferation (144). Given the central importance of this signaling pathway, it is tightly controlled with a large number of positive and negative regulators. Dysregulation of this pathway, as was demonstrated in mice with epidermis-specific deletion of *Ikk2*, which

leads to inhibition of NF- $\kappa$ B activation, results in severe inflammatory skin disease (145). Interestingly, the transgenic mice were indistinguishable from their littermates at birth but developed the inflammatory phenotype spontaneously after few days and this phenotype was TNF- $\alpha$ -dependent (145). NF- $\kappa$ B also plays a major role in the immune cell infiltrate in psoriasis, perhaps most prominently in macrophages (146). Macrophages (89), along with dendritic cells (147), are a significant source of TNF- $\alpha$ , which expression is dependent on the activation of NF- $\kappa$ B signaling, and TNF- $\alpha$  in turn is a potent activator of NF- $\kappa$ B itself (148a,b,c). This suggests that NF- $\kappa$ B signaling is necessary for normal epidermal homeostasis and that dysregulation of this system can lead to skin inflammation that is dependent on TNF- $\alpha$ , which may be primarily derived from the inflammatory infiltrate.

Several loci involved in this pathway have been implicated as susceptibility genes for psoriasis (Figure 150-4). *TNIP1* and *TNFAIP3* (126a) encode for the A20 and ABIN1 proteins, respectively. These proteins interact with each other, and may act at multiple steps in the NF- $\kappa$ B signaling pathway (149), with a net result of pathway inhibition (150). In most cell types, baseline A20 expression is very low, but its transcription is rapidly induced upon NF- $\kappa$ B activation. A20 is also able to inhibit TNF-induced NF- $\kappa$ B activity (151) and may also interact with other signaling molecules such as TRAF6 (152), which is downstream of the IL-17 receptor (149). Polymorphisms near *TNFAIP3* yield genome-wide significant associations with rheumatoid arthritis (153), Crohn's disease (138a), and systemic lupus erythematosus (154). As these polymorphisms are not the same or in linkage disequilibrium with those associated with psoriasis, it suggests that different alleles of *TNFAIP3* can lead to different chronic inflammatory diseases (6). It is of interest that susceptibility to atherosclerosis maps to the *Tnfaip3* locus in mice (155), particularly as atherosclerosis is a major comorbidity of psoriasis (52), systemic lupus erythematosus (156), and rheumatoid arthritis (b). *REL* (129) encodes c-Rel, one of the five principal members of the NF- $\kappa$ B family of transcription factors. It has been implicated in the development of Foxp3+ regulatory T cells (157) and is also expressed in the epidermis, where it has a role in epidermal development and homeostasis (158). The *NFKB1A* (129,131) locus has also been shown to increase risk of psoriasis, and the product of this gene, I $\kappa$ B $\alpha$ , is one of three proteins that associate with NF- $\kappa$ B dimers in their inactive state in the cytoplasm (159). I $\kappa$ B $\alpha$  is a part of a negative feedback loop where nuclear NF- $\kappa$ B drives I $\kappa$ B $\alpha$  expression that in turn inhibits NF- $\kappa$ B activation (159). In the absence of I $\kappa$ B $\alpha$ , the termination of NF- $\kappa$ B activation in response to stimuli such as TNF- $\alpha$  is significantly delayed (145,160). *FBXL19* (131) is another psoriasis-susceptibility candidate that has been proposed to have a role in NF- $\kappa$ B signaling (131). FBXL19 is structurally related to FBXL11, an F-box family member shown to inhibit

NF- $\kappa$ B (131). However, its exact role in regulating this signaling pathway is presently unknown.

#### 150.9.4 Dendritic Cell/Macrophage Function

Two of the major sources of key cytokines in psoriasis, including IL-1, IL-12, IL-23, and TNF- $\alpha$ , are macrophages and dendritic cells (89,147), which are found in increased numbers in psoriatic lesions in spatial proximity with both CD4 and CD8 T cells (63a,161). As described earlier, a subset of inflammatory dendritic cells, often referred to as TIP-DC, are thought to play a key role in the pathogenesis of psoriasis (147). This is supported by the rapid down-modulation of their products, including TNF- $\alpha$  and IL-23, with effective treatment of psoriasis (73). A prominent feature of TIP-DC is the expression of the inducible isoform of nitric oxide synthase (iNOS) (89,147), which when expressed rapidly liberates large amounts of nitric oxide (NO). A variant in the *NOS2* gene locus, which encodes iNOS, was recently shown to increase risk of psoriasis (131). Nitric oxide plays a vital role in the regulation of inflammation (162). IFN- $\gamma$  is a very potent inducer of iNOS and in addition to producing NO, iNOS can also be a source of superoxide, hydrogen peroxide, and peroxynitrite (163). These free radical species react rapidly with proteins and lipids in their vicinity, generating oxidized phospholipids that in turn can activate macrophages, with a resulting increase in pro-inflammatory cytokine production (Figure 150-4). Interestingly, besides IFN- $\gamma$  signaling, iNOS is also regulated through NF- $\kappa$ B pathway activation (143).

#### 150.9.5 The Epidermal Barrier and Antimicrobial Defenses

The epidermal barrier itself may play a role in psoriasis susceptibility (Figure 150-4) as deletion of two genes, *LCE3C* and *LCE3B*, encoding two structural epidermal proteins, increases the risk of psoriasis (164,165). The expression of these genes is low-to-undetectable in normal skin (164) but they are highly expressed in lesional skin in those patients that do not carry the deletion (164). Interestingly, there is an epistatic interaction between the *LCE3C* and *LCE3B* deletion and the *HLA-Cw6* locus, although this appears to differ between populations (165,166). Although the mechanism behind the increased susceptibility with this deletion is still unclear, it has been suggested that these two proteins are involved in barrier repair after epidermal injury or inflammation (167), although this incompletely addresses the epistasis with the HLA-C locus. Other recently implicated loci in psoriasis include one harboring the connexin 26 (*GJB2*) and serine protease inhibitor B8 (*SERPINC8*) genes (129,130). Connexins form a part of gap junctions, but these are highly specialized membrane structures that form channels between the cytoplasm of adjacent



cells, providing a means for the exchange of ions, second messengers, and small metabolites (168). At least nine connexin genes are expressed in skin and these play a crucial role in keratinocyte growth and differentiation (168). Mutations in the connexin 26 gene are associated with several distinct skin disorders, most of which are characterized by markedly thickened epidermis, often on the palms and soles, in association with sensorineural deafness (168,169). Interestingly, connexin 26 has been shown to be upregulated in psoriatic epidermis (170) and persistent expression of connexin 26 in a transgenic mouse model led to epidermal barrier defects and development of a psoriasis-like phenotype with increased proliferation of keratinocytes and immune infiltration (171). Serine protease inhibitors (serpins) are a large superfamily of structurally related proteins that regulate the activity of proteases involved in diverse biological processes. Little is known about the exact biological function of serpin protease inhibitor B8, but its localization in the epidermis has been described (172). Finally, the number of copies an individual carries of a gene cluster encoding three epidermally expressed antimicrobial peptides (human beta-defensins 2, 3, and 4), has been shown to influence psoriasis risk, with a higher number of copies conferring a higher risk (173). This interesting finding still requires independent confirmation.

### 150.9.6 Other Genetic Risk Factors

Several other genes have been implicated as susceptibility genes for psoriasis. These include variants close to or within the *IL4/IL13* gene cluster on chromosome 5 (126a). IL-4 and IL-13 are Th2-related cytokines that influence immune responses, and abnormalities in this system may polarize the immune response away from Th2 toward Th1- or Th17-mediated responses (70). The polymorphism associated with psoriasis is non-synonymous, resulting in amino acid change of the IL-13 cytokine. Interestingly, the A allele of IL-13 (R130Q) is associated with asthma and atopy (174), whereas the G allele is associated with psoriasis (126a). The asthma-associated R130Q variant has been shown to signal more effectively through the IL-13R $\alpha$ 1/IL-4R $\alpha$  heterodimeric receptor, as measured by STAT6 phosphorylation, and binds less well to the inhibitory IL-13R $\alpha$ 2 receptor (175). Thus, the psoriasis-associated variant may have an opposite effect with greater signaling through the IL-13R $\alpha$ 2 receptor and decreased affinity for the IL-13R $\alpha$ 1/IL-4R $\alpha$  heterodimeric receptor. This is of interest as IL-13 has been shown to be able to induce production of TGF $\beta$ 1 through the IL-13R $\alpha$ 2 receptor on macrophages (176), and overexpression of TGF $\beta$ 1 in a transgenic mouse model has been shown to result in a psoriasis-like skin disorder (177). Several loci located close to genes involved in interferon signaling were recently implicated in psoriasis (129). These include the interleukin 28 receptor, alpha (*IL28RA*), and the interferon-induced helicase

C domain-containing protein 1 (*IFIH1*) (129), which also has been implicated as a risk factor for type 1 diabetes (178). Currently, not much is known about the function of these candidate genes in psoriasis. In addition, a putative regulatory variant within the *ZNF313-RNF114* gene has been associated with psoriasis in two independent genome-wide association studies (131,179). *ZNF313* belongs to a family of E3 ubiquitin ligases (179). E3 ligases have been associated with the pathogenesis of several autoimmune conditions (180) but the role of this variant in psoriasis is unknown.

A GWAS performed in Han Chinese clearly implicated *HLA-Cw\*0602*, *IL12B*, and the *LCE3B/LCE3C* indel associations (165), and a subsequent follow-up study implicated *ERAP1* and confirmed the *TNIP1* association described earlier. An additional set of loci, including *PTTG1*, *CSMD1*, and *ZNF816A* in addition to the *GJB2* and *SERPINB8* genes described above (127), has thus far been identified only in Chinese populations, possibly due to allelic heterogeneity.

Taken together, the identification of these susceptibility loci has dramatically changed our view of the pathogenic mechanisms operating in psoriasis. Instead of looking at psoriasis as a purely T-cell mediated disease, it is now evident that defects in multiple different cellular compartments play a role in its pathogenesis (Figure 150-4). The identification of *HLA-Cw6* as one of the major susceptibility genes, the interaction with *ERAP1*, and the association with *ZAP70*, strongly implicates HLA class I antigen presentation to CD8+ T cells as a central element in psoriasis pathogenesis. In addition, *IL12B*, *IL23A*, *IL23R*, and *TYK2* are likely to have a major role in amplifying the Th17 response in psoriasis, and *TRAF3IP2* is likely to regulate intracellular signaling downstream from the IL-17 receptor. The NF- $\kappa$ B regulatory variants may play active roles downstream of cytokines such as TNF- $\alpha$  and IL-17. As endorsed by *IFIH1*, interferon signaling also appears to be central in causing or triggering the disease. The *IL4/IL13* locus may influence the Th2 vs Th1/Th17 balance, either via IL-4 (181), or via IL13 (182). The importance of the epidermal compartment is suggested by the fact that several susceptibility loci harbor genes thought to be involved in epidermal barrier formation, repair, and defenses. All of these findings are consistent with the hypothesis that psoriasis is maintained by a T-cell response against a limited set of self-antigens, likely due to an underlying defect in immunologic tolerance maintained by cross-presenting macrophages and dendritic cells (81), and amplified by type I interferons such as IFN- $\alpha$  (183).

### 150.10 MANAGEMENT

There is a broad range of both topical and systemic agents available for the treatment of psoriasis. Mild to moderate disease is typically treated with topical agents, often along with ultraviolet phototherapy. As topical



treatments are often cosmetically unacceptable and time-consuming to apply, non-compliance is high (184). When choosing a treatment regimen for patients with psoriasis, several different factors need to be taken into account. The extent and the measurable severity of the disease needs to be considered along with the patient's own perception of his or her disease. As psoriasis is a chronic disease it is important to consider the long-term safety of the treatment along with patient's comorbidities, which may increase their risk of toxicity. Finally, treatment efficacy may diminish with time, a well-known phenomenon in psoriasis, termed tachyphylaxis.

The most common topical therapy used is topical steroids. Improvement is usually achieved within 2–4 weeks, with maintenance treatment consisting of intermittent applications. Long-term treatment may lead to thinning of the skin (atrophy), telangiectasia and prominent stretch marks (striae). Other topical agents used include vitamin D3 analogs (185), retinoids, topical calcineurin inhibitors, dithranol, and coal tar (186). Phototherapy is a very effective way to control mild to moderate psoriasis and often works synergistically with topical agents. The mechanism of action of phototherapy appears to involve selective depletion of T cells, predominantly those that reside in the epidermis (40,187). Several systemic oral agents are available for treatment of psoriasis and are usually reserved for patients with more severe disease. Methotrexate is highly effective for chronic plaque psoriasis (188) and is also used for management of other subtypes of psoriasis such as erythrodermic and pustular psoriasis (189). It is also very effective in the treatment of psoriatic arthritis (190). Although methotrexate inhibits dihydrofolate reductase, it is its activity on AICAR transformylase, an enzyme involved in purine metabolism, that is thought to be the main mechanism of its anti-inflammatory activity (191). This leads to accumulation of extracellular adenosine, which has potent anti-inflammatory activities (191). Acitretin is a systemic retinoid that is used for psoriasis although its mechanism of action is not fully understood, and is particularly effective for generalized pustular and erythrodermic psoriasis (189). Cyclosporine A is another T-cell immunosuppressant that was in common use prior to the advent of biologics and is very effective in the treatment of severe psoriasis, although side-effects limit it to short-term use. Other oral systemic agents available but not commonly used include sulfasalazine, fumaric acid esters, and mycophenolate mofetil (189).

The treatment of severe psoriasis has changed dramatically in the past decade, with the introduction of biologic agents. Currently, three anti-TNF agents are available for use in psoriasis—infliximab, etanercept, and adalimumab. Given the central role of TNF- $\alpha$  in psoriasis, as described before, it is perhaps not surprising that these agents are highly effective in treating both psoriasis and psoriatic arthritis and may also reduce the risk of cardiovascular events in patients

with chronic inflammatory disorders (192). Recently, a new biologic, ustekinumab, was approved for the treatment of psoriasis. This medication binds the p40 subunit of IL-12 and IL-23 and prevents interaction of these cytokines with their receptors (193). As described earlier, this treatment blocks IL-12, which is central to the development of Th1 cells and is inhibitory for Th17 (71,194) and Th22 (195) cells through its neutralizing effect on IL-23. Thus, it affects a biologic pathway that maps many of the susceptibility loci in psoriasis, including *IL12B*, *IL23A*, *IL23R*, *TRAF3IP2*, and *TYK2*. Clinical studies have demonstrated that this treatment is highly effective in psoriasis and has slightly greater effect than etanercept (196). A large number of systemic agents are currently in development for the treatment of psoriasis, several of which block pathways previously identified from genetics, and it is foreseeable that the next few years are going to be very exciting in terms of our understanding of the central disease processes in psoriasis as well as our ability to target them therapeutically.

## 150.11 CONCLUSION

With the recent successes in mapping out susceptibility loci and genes, we now have the beginnings of a genetic blueprint that can help point us toward molecular pathways that are central to the pathogenic process. Although it is foreseeable that this blueprint is going to become even more comprehensive with the elucidation of additional causative variants, we are now finally able to begin to understand how such a distinctive pattern of cutaneous inflammation develops in this fascinating yet enigmatic disease.

## CROSS REFERENCES

Multifactorial Inheritance and Complex Traits; Inflammatory Bowel Disease; Autoimmunity: Genetics and Immunological Mechanisms; Rheumatoid Disease and Other Inflammatory Arthropathies.

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## RELEVANT WEBSITE

<http://www.psoriasis.org>. response.

### Biographies



**Johann E Gudjonsson, MD, PhD**, is an assistant professor at the Department of Dermatology at the University of Michigan. Dr Gudjonsson graduated from the University of Iceland Medical School, receiving his medical degree in 1998 and a PhD in immunology and genetics in 2003. He has been the recipient of several awards and honors, including the Young Investigator Award of the American Academy of Dermatology in 2007, and the Frances and Kenneth Eisenberg Emerging Scholar of the A. Alfred Taubman Medical Research Institute in 2010. He is the director of Inpatient and Consultation Dermatology at the University of Michigan Health System, and has been listed on the Best Doctors in America since 2010.

Dr Gudjonsson's research is focused on the genetic basis of psoriasis and his work has contributed to the isolation of several risk genes. The main focus of his work is on how specific risk alleles influence inflammatory pathways, and creating tools to determine how combinations of different risk genes influence inflammatory processes in the skin, and effectively map out these processes.



**James T Elder, MD, PhD**, was named in 2008 as the Kirk D. Wuepper Professor of Molecular Genetic Dermatology. Dr Elder received MD and PhD degrees in molecular biophysics and biochemistry from Yale University. After a molecular biology postdoctoral fellowship at the University of California (Berkeley), he completed an internship in internal medicine and a dermatology residency and fellowship at the University of Washington, followed by a senior research fellowship at the University of Michigan. Board-certified in dermatology.

Dr Elder directs an active research laboratory at the University of Michigan in addition to his clinical duties. Dr Elder's laboratory utilizes tools of molecular biology and genetics to better understand several human skin diseases. The lab is a world leader in the use of genetic linkage and association techniques to learn more about how the immune system activates the epidermal wound healing mechanism in psoriasis, and triggers joint destruction in psoriatic arthritis. In 2006, his laboratory identified HLA-Cw6 as the disease allele at *PSORS1*, the major psoriasis-susceptibility locus in the major histocompatibility complex (MHC). His current efforts in this area are focused on identification of psoriasis susceptibility genes outside the MHC, with 20 loci confirmed thus far. His laboratory is also carrying out a combined analysis of global gene expression and DNA variation in psoriasis, as well as a genome-wide scan for psoriatic arthritis genes.

# CHAPTER

# 151

## Cutaneous Hamartoneoplastic Disorders

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### 151.1 INTRODUCTION

Cutaneous hamartoneoplastic disorders encompass a wide variety of syndromes, among which are neurofibromatosis types 1 (NF1) and 2 (NF2), schwannomatosis, tuberous sclerosis complex (TSC), nevoid basal cell carcinoma syndrome (NBCCS) or Gorlin syndrome, PTEN hamartomatous syndrome (encompassing Cowden disease and Bannayan–Riley–Ruvalcaba syndrome), hereditary leiomyomatosis and renal-cell cancer (HLRCC), and Birt–Hogg–Dubé syndrome (BHD) (Table 151-1). These syndromes are linked as each is associated with susceptibility to tumors and has characteristic dermatologic features, in some cases neoplastic and in some non-neoplastic. All of these syndromes are addressed in detail in other chapters, except HLRCC and BHD, which are covered in detail below.

### 151.2 HEREDITARY LEIOMYOMATOSIS AND RENAL-CELL CANCER

#### 151.2.1 Genetics of HLRCC

Hereditary leiomyomatosis and renal-cell cancer (HLRCC; Online Mendelian Inheritance in Man (OMIM) 150,800) is a syndrome characterized by the development of cutaneous and uterine leiomyomas and renal cancer. Multiple cutaneous and uterine leiomyomas (MCUL) was initially described in 1973 by Reed et al. as an autosomal dominant syndrome (1). In 2001, two families were reported with renal cancer as well as multiple cutaneous and uterine leiomyomas by Aaltonen et al. and termed HLRCC (2). In their report, they speculated that this syndrome is a variant of that described earlier. They also noted the aggressiveness and the distinct histology of the renal cancers in patients with HLRCC (3). The region containing the gene was mapped using linkage to 1q42–44. The gene in which mutations were responsible for HLRCC was identified quickly as fumarate hydratase (*FH*) by an international consortium (4).

Individuals with a single mutation in *FH* develop HLRCC; those with biallelic mutations (homozygous or compound heterozygotes) develop fumarate hydratase deficiency (FHD). FHD is a rare metabolic disease in which patients develop fumaric aciduria, progressive encephalopathy, hypotonia, failure to thrive, and seizures (5–12). Patients with FHD usually do not survive beyond the first few months of life, although some more mildly affected individuals have been described (11,12). Relatives of these patients have been reported to develop cutaneous and uterine leiomyomas, and as such are at risk to develop renal cancer (4,11).

Over 300 families have been described with HLRCC (13–15). The percentage of patients and families that have personal or familial medical histories (e.g. multiple cutaneous leiomyomas or type two papillary renal cancer) identified with mutations in *FH* has varied substantially depending on the study. In the initial reports, encompassing 56 HLRCC families seen at the US National Cancer Institute (NCI), 52 (93%) were found to have mutations in *FH* (16,17). Expanding the phenotype, the percentage has decreased, as in the French National Cancer Institute “Inherited predisposition to kidney cancer” network’s study, which found mutations only in 71% (40/56) (13). However, there has not been the suggestion that additional predisposition genes exist for HLRCC. The group based at the NCI found mutations scattered across the gene, whereas the Multiple Leiomyoma Consortium initially reported clustering of mutations in the 5′ region of the gene, but that has changed with the identification of additional families (4). All types of point mutations have been reported, with missense mutations by far the most predominant (57%; 191/337) in the *FH* mutation database ([http://chromium.liacs.nl/lovd\\_sdh/home.php?select\\_db=FH](http://chromium.liacs.nl/lovd_sdh/home.php?select_db=FH)) (15). Large genomic deletions have been described but are rare (4,16,18,19). There have been some recurrent mutations reported in *FH*, including c.905-1G>A in families of Jewish Iranian background; p.R58P, which has been seen in several families; and nucleotide 698 appears to be

TABLE 151-1 Cutaneous Hamartoneoplastic Disorders

Syndrome	Gene	Protein	Neoplastic Dermatologic Disease	Non-Neoplastic Dermatologic Findings	Associated Cancer Types
Birt–Hogg–Dubé syndrome	<i>FLCN</i>	Folliculin	–	Fibrofolliculomas	Renal cancer—oncocytic, chromophobe
Hereditary leiomyomatosis and renal-cell cancer	<i>FH</i>	Fumarate hydratase	–	Cutaneous leiomyomas	Renal cancer—papillary type 2
Neurofibromatosis type 1	<i>NF1</i>	Neurofibromin	–	Café au lait macules Dermal, intradermal, subdermal neurofibromas	Malignant peripheral nerve sheath tumors Optic gliomas Pheochromocytoma Brain tumors Leukemia (CML, MDS)
Neurofibromatosis type 2	<i>NF2</i>	Merlin	–	Cutaneous schwannoma	Vestibular schwannomas Cranial and peripheral nerve schwannomas Meningiomas Ependymomas Medulloblastoma
Nevoid basal cell carcinoma syndrome	<i>PTCH</i>	Patched	Basal-cell cancers	Facial milia Palmar/plantar pits	
PTEN hamartomatous syndrome	<i>PTEN</i>	PTEN	Melanoma	Trichilemmomas Acral keratoses Papillomatous papules Mucosal papillomas	Breast cancer Thyroid cancer Endometrial cancer
Tuberous sclerosis complex	<i>TSC1</i> <i>TSC2</i>	Hamartin Tuberin	–	Facial angiofibroma Hypomelanotic macule Connective tissue nevus Forehead plaque Ungual and periungual fibromas	Angiomyolipomas Subependymal giant cell astrocytomas

CML = chronic myelogenous leukemia; MDS = myelodysplastic syndromes.

a mutational hotspot leading to p.R233H and p.R233C (4,18,20). Missense mutations have been postulated to have a dominant negative effect. As FH exists as heterotetramer, only one in 16 subunits would be composed entirely of wild-type protein if one allele encoded an altered protein. This paradigm would be similar to that seen in other classic genetic syndromes, such as collagen disorders. However, recent studies have demonstrated similar reductions of FH activity in patients with both missense and truncating mutations, decreased by at least 50%, suggesting that the dominant negative hypothesis is not correct in vivo (13). The lower fumarate hydratase enzymatic activity in affected patients has been proposed as a method for screening of family members, however genetic testing remains a more efficient method to detect affected individuals (21).

No genotype–phenotype correlations have been reported in multiple studies; in fact, intrafamilial heterogeneity has been observed in multiple cases, despite similar decreases in FH activity (13). The penetrance for the complete phenotypic manifestations of HLRCC has yet to be fully defined, although similarly to many cancer susceptibility syndromes, as more families are

tested, individuals with mutations but no manifestations of disease have been identified. This finding has led to the consideration of modifiers of penetrance, and as such a family-based study was done to attempt to identify other linked genes, but did not identify a modifier (22). However, this study was limited in design and lower, and modifiers of penetrance, either genetic or environmental, may exist.

Mutation screening has been suggested for all sporadic cases of papillary type II renal cancer with the appropriate histologic subtype, particularly in the setting of a positive family history and early-onset disease (23). Several studies have looked at patients with isolated papillary type II, identifying mutations in 17%; however, pathologic screening should be done to select patients (13). A series of patients with early-onset uterine leiomyosarcoma (diagnosed under the age of 45) were screened, and only 1/67 (1.5%) found to have mutations; genetic testing for *FH* mutations would not be cost-efficient in that population (24). However, mutation testing also needs to be considered in families with multiple women with aggressive uterine leiomyomas (25).



### 151.2.2 Fumarate Hydratase Biology

The fumarate hydratase gene, *FH*, spans 22kb, with ten exons, and encodes two fumarate isoenzymes, cytosolic and mitochondrial. FH catalyzes the conversion of fumarate to malate in the tricarboxylic acid (TCA) cycle. Consistent with a postulated role as a tumor suppressor gene, loss of the wild-type allele is observed in cutaneous and uterine leiomyomas, as well as renal cancer, from individuals with *FH* mutations (2–4,18,26). The active form of FH is a homotetramer, in which three of the four chains combine to form the enzymatic active site (27). Loss of FH results in accumulation of fumarate and other TCA intermediates. Along with mutations in the succinate dehydrogenase complex (SDH), leading to susceptibility to pheochromocytoma and paraganglioma, and *VHL*, leading to von Hippel–Lindau disease, mutations in *FH* have been long thought to lead to a “pseudohypoxic” state.

Hypoxia sensing is primarily mediated through the hypoxia-inducible factors (HIFs), which are transcription factors that regulate adaptation to tissue hypoxia. HIF is a heterodimer that is composed of an unstable  $\alpha$ -subunit (e.g. HIF1 $\alpha$ ) and a stable  $\beta$ -subunit (e.g. HIF1 $\beta$ /ARNT). There are three known HIF $\alpha$  genes, with the best characterized being *HIF1 $\alpha$*  and *HIF2 $\alpha$* . HIF1 $\alpha$  and HIF2 $\alpha$  have shared and independent target genes. HIF1 $\alpha$  activates genes associated with glycolysis and BNip3, while HIF2 $\alpha$  activates cyclin D1, vascular endothelial growth factor (VEGF), transforming growth factor alpha (TGF $\alpha$ ), and Oct4 (28–31). Under normoxic conditions, VHL binds to the hydroxyproline residue on the HIF- $\alpha$  subunits, targeting them for ubiquitination and proteosomal degradation (32). The generation of the hydroxyproline is catalyzed by the prolyl hydroxylase domain-containing proteins (PHDs), a subclass of 2-oxoglutarate (2OG) oxygenases, which use O<sub>2</sub> and 2-ketoglutarate ( $\alpha$ -KG) as substrates. PHD2 is inhibited under hypoxic conditions, allowing transcription of HIFs (33,34). Dysregulation of HIF has long been known to be important in the development of von Hippel–Lindau-associated tumors. Mutations in SDH-associated tumors also have linked to stabilization of HIF1 $\alpha$ , in a study demonstrating that elevated levels of succinate inhibit its prolyl hydroxylation by the PHDs (35). Upregulation of Hif1 $\alpha$  and its target genes has also been shown many times, including in mouse embryonic fibroblasts from Fh1-deficient mice (36); accumulation of fumarate also inhibits the PHDs, even more potently than succinate (37). Both fumarate and succinate act as competitive inhibitors of PHDs, although the mechanism of action may differ between the two TCA cycle intermediates (38,39). With the loss of FH, and thus TCA cycle function, several studies have explored energy production in these cells. FH-deficient tumors take up large amounts of glucose and shift to aerobic glycolysis (40,41). They maintain partial TCA cycle function through the uptake of glutamine, and dispose

of excess carbon through the synthesis of heme, and its excretion from the cell as bilirubin (42). Gottlieb et al. demonstrated that the inhibition of heme oxygenation was synthetically lethal with FH loss, suggesting it could be a potential therapeutic target (42).

Despite the role that excess fumarate plays in creating a “pseudohypoxic” environment through HIF stabilization, how it leads to tumor formation has not been well defined. A recent study using back crosses in mice has shown that neither the presence of *Hif* nor the absence of *Phds* is required for renal cyst formation in an Fh1-deficient background; in fact, loss of *Hif1 $\alpha$*  exacerbates renal cystic development (43). Two recent studies have demonstrated that FH loss results in activation of Nrf2-dependent activation of antioxidant pathways (43,44). NRF2, a transcription factor, is a key regulator of the antioxidant response, with multiple target genes that contain NRF2 response elements (45). Cellular levels of NRF2 are regulated by KEAP1 (Kelch-like ECH-associated protein 1), which is the substrate recognition subunit of a Cul3-based E3 ubiquitin ligase. Through tandem mass spectrometry, Ooi et al. and Adam et al. showed that fumarate modifies critical cysteine residues (Cys155 and Cys288) within KEAP1, so that it is unable to bind to NRF2 and target it for degradation (43,44). Upregulation of NRF2 may be an alternative pathway, other than through “pseudohypoxia,” which may lead to FH-deficiency-associated tumorigenesis, although the exact mechanism remains to be elucidated.

### 151.2.3 HLRCC Disease Manifestations

**151.2.3.1 Renal Cancer.** The predominant pathologic type of renal cancer associated with HLRCC is papillary type II; however, other types of renal cancers are observed, including collecting duct and clear-cell cancers (16,18,46). Independent of underlying architecture, cells in the renal cancers associated with HLRCC have a characteristic pathologic appearance, with large nuclei with inclusion-like orangeophilic or eosinophilic nucleoli surrounded by a clear halo (47).

As many of the mutations leading to HLRCC in *FH* are missense, leading to stable but inactive protein, immunohistochemistry for FH is not a reliable marker to detect those renal papillary type II tumors associated with HLRCC. Fumarate reacts spontaneously with cysteine sulfhydryl groups to chemically modify proteins, in a process termed succination. Recently, immunohistochemistry for S-(2-succinyl) cysteine (2SC) has been proposed as a marker of FH loss, and thus mutations in *FH* (48). Pollard et al. validated it as an immunohistochemistry marker on over 1000 specimens, correctly predicting the presence of *FH* mutations (48,49). Thus, use of immunochemistry to identify patients who need evaluation for HLRCC and subsequent genetic testing for mutations in *FH* may become part of clinical practice. In particular, this methodology may be helpful, as standard

immunohistochemistry markers have not identified a characteristic pattern of staining (47). Array-based comparative genomic hybridization (aCGH) has been done to characterize FH-deficient renal cancers. Loss of chromosome 1q was found, as expected, consistent with the tumor suppressor role of *FH*, as were gains of chromosomes 2, 7, and 17, and losses of 13q12.3021.1, 14, 18, and X, suggesting a distinct genetic profile for these renal tumors (50). However, specific genetic associations have not yet been identified.

Overall, approximately 20% of families with HLRCC have renal cancer, usually a single proband (15,51). However, there is significant variability in the reported prevalence of renal cancer in families and patients with HLRCC, depending on how the proband was ascertained. In the series from NCI, 62% of families with MCUL/HLRCC had an individual with renal cancer; most probands were ascertained through a hereditary renal cancer program (16). In studies ascertaining on the basis of multiple cutaneous leiomyomas, the frequency of renal cancer is low, at 1–2% and 16% in two series (17,18). The standardized incidence ratio (SIR) has been calculated at 6.5 for renal cancer in HLRCC, comparing Finnish families with *FH* mutations with the general population (230-fold in the 15–29-year-old age group, and 45-fold in the 30–44 age group) (26).

Renal cancers associated with HLRCC tend to be early onset, high grade and aggressive. Patients can also have predominantly cystic lesions, as well as the more common solid tumors (26). The incidence of renal cysts has been reported to be as high as 36% in patients with HLRCC, and they are observed in *Fh1*-deficient mice (26,52). Metastatic renal cancer in individuals as young as 17 has been reported, with the youngest age of onset reported as 11 years (53,54). The average age of diagnosis is approximately 40 years, substantially younger than sporadic renal cancer (46). In general, renal cancer in HLRCC is solitary, however there have been a few reports of bilateral and multifocal disease (46,47). In one series of 40 renal tumors, all of the tumors were characterized as high grade (Fuhrman grade III or IV) based on the aberrant nuclei (47). In the initial study describing HLRCC, all six patients with renal cancer presented with metastases and died of their disease (2). While the absolute numbers have decreased in more recent studies, a similar pattern has been seen, with 10 of 19 (53%) patients either dead or with advanced disease with a median follow-up of 34 months (46). Many reports of malignant disease exist, and most patients die within 5 years after diagnosis (51). Thus, the natural history of renal cancer in HLRCC is different than for other hereditary renal cancer syndromes, which tend to exhibit multifocal disease with a relatively benign natural history, leading to differences in screening guidelines, reviewed below.

**151.2.3.2 Uterine Leiomyomas.** Uterine leiomyomas (fibroids) are benign tumors that arise from the smooth

muscle cells of the uterus. They are extremely common; in the United States, by the age of 50, they are found in the 70% of white women and over 80% of women of African ancestry (55). They also are the most frequent non-renal manifestation of HLRCC, and develop in 75–98% of female patients (2,4,16). The leiomyomas tend to be early onset and severe, diagnosed on average 10 years earlier than in sporadic disease, with 68% diagnosed before the age of 30 in one series (16,56). The histopathology of the uterine leiomyomas associated with HLRCC appears to be quite similar to that of the renal tumors, in particular the nuclear features, with prominent eosinophilic nucleoli surrounded by a clear halo (57). Comparing women with *FH* mutations with those without mutations in the same family, the risk was statistically significantly increased, with an odds ratio of 7.6 (95% confidence interval (CI) 2.9, 20) (56). Most women have surgical treatment of their leiomyomas, with many having hysterectomies before the age of 30 (56). The early age of hysterectomy in women with HLRCC may have an adverse impact on their ability to bear children, however that has not been universally reported (16,56). Uterine leiomyosarcomas have been observed in the European population and in Finland; the SIR was calculated at 71 (26). Leiomyosarcomas have not been reported in the North American population, potentially as hysterectomy is more frequent. However, recent studies suggest that the risk of uterine leiomyosarcoma was overestimated, as when the uterine tumors in the Finnish families were re-reviewed according to the latest World Health Organization (WHO) criteria, they were classified as benign mitotically active or atypical leiomyomas; additionally, no tumor necrosis was seen, which is an essential component of leiomyosarcomas (51). Thus, only one true malignant uterine leiomyosarcoma has been reported. As such, the risk of sarcomatous transformation is low in patients with HLRCC.

**151.2.3.3 Cutaneous Leiomyomas.** Cutaneous leiomyomas (piloleiomyomas) are painful pink-purplish nodules that affect individuals in a disseminated or segmental distribution. They are benign tumors that arise from the piloerector apparatus (17). Cutaneous leiomyomas occur in 80–100% of individuals with HLRCC, with a mean age of presentation of 25 years (range 10–47 years), but can develop later into the 40s (58). In a study from The Netherlands, all patients with HLRCC over the age of 40 had cutaneous leiomyomas (14). The appearance and symptoms associated with the cutaneous disease can vary widely. They are usually 5–20 mm in diameter, and can range from flesh-colored to pink or brown (58). Patients have a variable number of lesions, from 1 to 100, usually on the extremities and trunk, rarely on the face. They can be clustered or widely scattered all over the body. Mild presentations are seen in 40%, with five or fewer lesions (14,16–18). However, some patients have very severe symptomatic presentations, associated with pain, usually in response to temperature change and touch. There have

been two case reports of skin leiomyosarcoma in association with *FH* mutations (16,17).

**151.2.3.4 Other Tumor Manifestations.** Wilm's tumor has been reported in two pediatric patients with *FH* mutations, suggesting a possible associated predisposition (14,59). Leydig-cell tumors have also been reported in a patient with HLRCC. Screening of sporadic Leydig-cell tumors also identified a second male with a germline mutation, suggesting that patients with Leydig-cell tumors should be asked about pertinent family history (60). Gastrointestinal stromal tumors (GIST), adrenocortical disease, and ovarian cystadenomas have also been described in patients with HLRCC (61–63).

### 151.2.4 Medical and Surgical Management of HLRCC

The greatly increased risk of early onset aggressive renal cancer in HLRCC necessitates screening and quick intervention. While there are no standard guidelines for renal cancer screening, patients have been diagnosed as young as 11 years; however, it is the only report of a patient below the age of 15 (53). Thus, screening should start at age 15, with abdominal/pelvic computed tomography (CT) or magnetic resonance imaging (MRI) annually. Given the aggressive nature of these lesions, radical nephrectomy at diagnosis (regardless of tumor size) is the recommended treatment strategy as soon as lesions are identified. Cutaneous and uterine leiomyomas are much more common than renal cancer in HLRCC. The cutaneous leiomyomas can be quite painful and have cosmetic implications; they are treated with standard dermatologic methods. Women with uterine leiomyomas should be advised about the options in terms of uterine preservation and potential impact on child bearing. The leiomyomas themselves should be followed on an annual basis.

## 151.3 BIRT-HOGG-DUBÉ SYNDROME

### 151.3.1 Genetics of Birt-Hogg-Dubé Syndrome

Birt-Hogg-Dubé syndrome (BHD) is an autosomal dominant syndrome characterized by the development of fibrofolliculomas, renal cancer, predominantly hybrid oncocytic tumors, and lung cysts and pneumothorax. An inherited syndrome associated with fibrofolliculomas in two siblings, and a description of similar skin lesions in their father, along with intestinal polyps, was initially described by Hornstein and Knickenberg in 1975 (64). Subsequently, a large Canadian family was described by Birt, Hogg, and Dubé, three dermatologists, in which 15 of 70 family members had lesions they called fibrofolliculomas, abnormal dilated hair follicles with a hyperplastic mantle of connective tissue (65). This family also had trichodiscomas and acrochordons. Since the initial

description of these families, it has been recognized that they constitute one syndrome—Birt-Hogg-Dubé (BHD). Although renal cancer was not included in the initial descriptions of BHD, the association between renal cancer and BHD was subsequently noted, first in 1993 by Roth et al. (66). In 1999, Toro et al. enlarged upon the association, describing 7 of 13 patients with renal cancer (67). The gene for BHD was mapped to 17p12–17q11.2 using genome-wide linkage analysis by two groups in 2001 (68,69). Quickly thereafter, the gene in which mutations are associated with BHD was identified and named folliculin (*FLCN*) (70). The protein had no homology to previously identified proteins, but was found to be conserved across species and expressed in a wide spectrum of normal tissues, including skin, lung, and kidney.

Most mutations in *FLCN* are truncating, nonsense, and frameshift, although missense mutations and large genomic rearrangements have been described (71,72). Multiple groups have noted that mutations are most frequently found within a C<sub>8</sub> tract in exon 11, and are either insertions or deletions (73–75). Of note, when the two most common mutations, c.1285dupC and c.1285delC (previously 1733insC and 1733delC), were compared, the insertion was associated with a higher rate of renal cancers ( $p=0.03$ ) (73). An association of mutations in exon nine with lung cysts, and thus pneumothorax ( $p=0.0002$ ), has been suggested (76). Missense mutations and in-frame deletions in *FLCN* have been investigated for pathogenicity, and found to disrupt protein stability (77). The same study also found a highly conserved domain between amino acids 100 and 230, identified by computational analysis. There are two *FLCN* mutation databases (<http://grenada.lumc.nl/LOVD2/shared1>, <http://www.skingenetdatabase.com/home.php>), which can be used for reference for mutation characterization and single nucleotide polymorphism (SNP) identification; however, only the former is currently maintained (78,79).

Over multiple series of families with clinically defined mutations in BHD, mutations in *FLCN* are found in approximately 85% of families (73,76,80,81). Although some series have not included large genomic rearrangement testing, which may increase the percentage of patients with clinically defined BHD carrying *FLCN* mutations, a recent series from The Netherlands including such screening only identified 22/27 (81%) families with mutations (81). Of note, however, two of the remaining five families carry likely pathogenic *FLCN* exon one deletions, which if included would have increased the mutation detection rate to 88%. Most families described with BHD due to mutations in *FLCN* have the three classic manifestations of lung cysts, renal cancer, and fibrofolliculomas, although each individual may not have all three manifestations. However, mutations in *FLCN* also have been found in familial isolated spontaneous pneumothorax and patients with multiple lung cysts, without

other evidence of BHD (82–85). BHD is likely the most common cause of familial isolated pneumothorax (86). Rarely, familial isolated non-clear-cell renal cancer has also been associated with mutations in *FLCN* (87). Thus, phenotypically differing conditions are associated with *FLCN* mutations, so genetic testing may be indicated in the absence of classic BHD.

Criteria for the diagnosis of BHD have been proposed, and include major criteria of (1) at least five fibrofolliculomas, at least one histologically confirmed, of adult onset; or (2) pathogenic *FLCN* mutation. Minor criteria are (1) multiple lung cysts: bilateral basally located lung cysts with no other apparent cause, with or without spontaneous pneumothorax; (2) renal cancer: early onset (<50 years) or multifocal or bilateral renal cancer, or renal cancer of mixed chromophobe and oncocytic histology; and (3) a first-degree relative with BHD (88). Patients should have one major or two minor criteria for diagnosis. BHD is certainly vastly underdiagnosed. As BHD has become increasingly recognized by pulmonologists as a cause of lung cysts and pneumothorax, referrals to many cancer or medical genetics clinics, and thus diagnoses of BHD, have seen a significant rise in the past 5 years. Furthermore, BHD is not associated with a decrement in reproductive fitness, so many large families with multiple affected members have been observed.

### 151.3.2 *FLCN* Biology

*FLCN* was validated by several studies as a tumor suppressor gene lost in renal cancer associated with BHD. Unusually, the second allele of *FLCN* is most frequently inactivated by point mutation rather than loss (89). Somatic mutations in *FLCN* are infrequently identified in sporadic renal cancers (90,91). However, neither loss of heterozygosity nor *FLCN* mRNA expression is observed in fibrofolliculomas, so it is possible that haploinsufficiency is enough to allow their development (92,93).

Two animal models of BHD have occurred naturally, in the rat and dog (94,95). In the “Nihon” rat, which contains a point mutation in the rat homolog of *BHD*, homozygosity leads to early fetal demise, and heterozygosity is associated with early onset of renal cancers, predominantly clear cell (94). The syndrome of hereditary multifocal renal cystadenocarcinoma and nodular dermatofibrosis (RCND) was described in German shepherds prior to the identification of *FLCN* as the causative gene for BHD. The gene was mapped into a region syntenic to that also mapped for BHD, and a point mutation was found in the canine homolog of *FLCN* (95). Similarly to the rat model, homozygosity is embryonically lethal, and heterozygotes develop bilateral multifocal renal cystadenocarcinoma, uterine leiomyomas, and dysplastic hair follicles.

*FLCN* does not have homology to any other known protein and its function has been difficult to elucidate. Since heterozygous loss gives rise to such a diverse

spectrum of phenotypes, its function has not been clarified through an understanding of the disease process. Initially, several studies suggested that *FLCN* was part of the mTOR signaling pathway (96–99). Baba et al. linked loss of *FLCN* and upregulation of mTOR signaling as possibly mediated through an *FLCN*-interacting protein (FNIP1) and AMP-activated kinase (AMPK) (96,100). Studies in mouse models found downregulation of p-S6 with loss of *FLCN*, so the association between *FLCN* and mTOR signaling may be context dependent (101). Perhaps not surprisingly given the somewhat contradictory results, subsequent functional work has suggested that aberrant mTOR signaling may be associated with downstream effects, not directly with those mediated by *FLCN* (102). Two recent studies have implicated *FLCN* in TGF $\beta$  signaling (102,103). Profiling in UOK257, an *FLCN*-null renal cancer cell line derived from a BHD patient, in which wild-type *FLCN* was expressed, found differential expression of many genes involved in TGF $\beta$  signaling (103). Levels of TGF $\beta$ 2, INHBA, and SMAD7 were reduced in the *FLCN*-null line, and in BHD-associated renal tumors as compared with normal kidney. In *Bhd*<sup>-/-</sup> embryonic stem (ES) cells, resistance to apoptosis associated with decreased expression of Bim was observed, with the defects observed appearing similar to those seen in TGF $\beta$  receptor and ligand mutants (102). This observation was confirmed by showing that *Bhd*<sup>-/-</sup> ES cells have hypoacetylation of TGF $\beta$  target gene promoters. Although much further work needs to be done to fully elucidate the function of *FLCN*, these studies are the first steps to understanding the function of a protein that, when mutated, leads to a disparate set of phenotypes.

### 151.3.3 Disease Manifestations of BHD

The three major manifestations of BHD are fibrofolliculomas of the skin, renal tumors, and lung cysts/pneumothorax. As discussed above, most individuals with BHD do not have all three phenotypes, but within families all can be observed. Patients with *FLCN* mutations can be ascertained through dermatology (fibrofolliculomas), or pulmonary (lung cyst and pneumothorax) or urologic oncology (renal cancer). In each case, patients who present with isolated findings have been found to have *FLCN* mutations. Interestingly, a positive family history for renal cancer or pneumothorax is associated with a higher risk of those disease manifestations, but the converse is not true, in that a family history of renal cancer is not associated with an increase in risk of pneumothorax (71). This finding suggests that modifiers of BHD disease phenotype may exist, which may account for the variability in phenotype. In addition, the clustering of phenotypes within families may account for observed ascertainment biases; patients and families identified through dermatology clinics are likely to have lower rates of renal cancer than those seen in urologic clinics.



**151.3.3.1 Fibrofolliculomas.** Fibrofolliculomas of the skin appear in the 20s to 30s and are considered characteristic of BHD (74,93). Patients can have from only a few fibrofolliculomas on their cheeks to extensive involvement of the face, neck, and upper back in a “cape-like” pattern. Fibrofolliculomas are abnormal growths of the hair follicles with epithelial strands extending into the surrounding stroma. Generally, they are lighter than skin color, but can be skin-colored or slightly erythematous, and are dome shaped. In BHD, oral papules have also been described in up to 43% of patients, mostly localized on the lips and commissural folds, also arising after the age of 30 (67,104). Prior to the identification of *FLCN*, a clinical diagnosis of BHD was made based on having 10 lesions consistent with fibrofolliculomas, at least one biopsy-positive (80). In addition to fibrofolliculomas, acrochordons and trichodiscomas have been reported in some studies of BHD patients (65,74). However, other studies suggest that these lesions are incorrectly diagnosed fibrofolliculomas (105–107). Interestingly, loss of the second allele of *FLCN* is not observed in fibrofolliculomas and as such it is postulated that haploinsufficiency is sufficient for predisposition (93). The lesions do not cause any problems for patients beyond cosmetic. Successful treatment of the fibrofolliculomas with CO<sub>2</sub> laser treatment has been reported (108).

Confusion between angiofibromas and fibrofolliculomas has been reported, as they are both skin-colored papules and can be found across the cheeks. Angiofibromas are associated with the inherited diagnoses of tuberous sclerosis complex (TSC) and multiple endocrine neoplasia type 1 (MEN1). Angiofibromas tend to be flesh-colored or erythematous, and clustered in the nasolabial folds and across the cheeks and chin. In addition, patients with TSC can have forehead plaques and hypomelanotic macules on the face that extend around the neck and upper back. Familial multiple trichodiscomas (familial multiple discoid fibromas) has been described as a “look-alike” for Birt-Hogg-Dubé syndrome (74,109). The families are clinically distinct with early onset of skin lesions, involvement of the ear, both pinna and helix, and discoid fibromas without the follicular epithelial component characteristic of the fibrofolliculoma/trichodiscoma papules seen in BHD, and do not carry mutations in *FLCN* (109). The families do not have renal or pulmonary disease, as seen in BHD.

**151.3.3.2 Renal Cancer in BHD.** In a large series of patients studied with BHD, 20% (38/187) of individuals from 24 families, representing 45% of total families, had renal cancers (73). The number of renal cancers in each patient ranged from a solitary lesion to multifocal bilateral cancers. In this series of patients with BHD from the US National Institutes of Health (NIH), the renal cancer was male-predominant (27 males, 11 females) and the average age of diagnosis was 48 (range 31–71) (73). A follow-up series of 51 independent families found a higher rate, in that 34% of individuals and 49% of

families had renal cancer; 41% of families ascertained based on dermatologic findings (71). Many families have only a solitary individual with renal cancer (71). Other series of patients have observed slightly lower rates of renal cancer, consistent with the earlier reports, with lifetime risks (at age 70 years) of 16% (95% CI 6, 26%) (81). In the series with higher rates of renal cancer, the relative risk is estimated to be increased sevenfold (80). Several families described with multiple individuals with oncocytomas were found to have fibrofolliculomas and subsequently mutations in *FLCN*, suggesting that BHD accounts for familial oncocytoma in some cases (67,110).

A wide spectrum of renal cancers has been observed in patients with BHD, even within the same kidney. The most common type of tumor is an unusual hybrid oncocytic tumor (mixed oncocytoma and chromophobe). Observation of a hybrid oncocytic tumor in any patient should prompt an evaluation for BHD, as it is so characteristic of this disease. It has been suggested that oncocytomas and chromophobe tumors share a common cell of origin and have similar gene expression profiles (111,112). In a series of 84 tumors from 10 patients with BHD, 67% were hybrid oncocytic, 23% chromophobe, 7% clear-cell tumors, and 3% oncocytomas (113). Not only can multiple types of renal cancer be observed within a single kidney, within a BHD-associated renal cancer there can be multiple cell types. Small nodules of tumor similar to the large hybrid oncocytic tumors seen have been observed throughout the kidney, consistent with cancer-susceptibility syndrome with all cells predisposed to develop disease (114). While the malignant potential of the hybrid tumors has not been entirely elucidated, it appears to be low if present (112,113,115). The various studies have not noted features suggestive of metastatic behavior, such as the presence of distant metastases, vascular invasion, or tumor necrosis. Unfortunately, this benign behavior does not appear to be the case for the clear-cell renal cancers that arise in patients with BHD. As noted above, clear-cell renal cancer has been described in patients with BHD, along with the multiple other tumor types. The clear-cell tumors have been shown to have loss of 3p and/or mutations in *VHL* (114).

**151.3.3.3 Pulmonary Disease.** Lung cysts and pneumothorax are a major component of BHD and likely have the highest morbidity. They were initially described by Toro et al. in 1999 (67). A large series of 198 patients from 89 families were evaluated for lung cysts and pneumothorax (76). A total of 187 (89%) and 48 (24%) patients had lung cysts and pneumothorax, respectively. Not surprisingly, the presence of lung cysts ( $p=0.006$ ), number of cysts, cyst volume, and largest cyst volume and diameter (all  $p<0.0001$ ), were all related to risk of pneumothorax. The relative risk of pneumothorax has been estimated to be 50.3 (95% CI 6.4–352), decreasing to 32-fold when adjusted for other covariates (80). The pulmonary cysts in BHD are found predominantly in the lower medial zone of the lung, tend to be irregularly

shaped, can be multiseptated and located close to the pleura (116,117). When compared with the cysts seen in women with lymphangiomyomatosis, they are larger and less circular, as well as being found in a different distribution through the lungs (118). The pulmonary cysts are lined with epithelial cells, and include type II pneumocyte-like cells (119). Furuya et al. suggest that they may not be bullae or blebs, but hamartomatous cysts that grow slowly and rupture. Interestingly, they also stain positively for FLCN, suggesting that haploinsufficiency is enough to allow cyst formation (119).

**151.3.3.4 Other Manifestations.** Colorectal adenomas have been reported in association with BHD, as well as other cancers (74,120). However, it is not clear that any of these are found at rates higher than would be expected based on population-based incidence. Colonoscopy in 45 patients with BHD did not show an increased frequency of adenomas (80). Parotid oncocytomas have been reported as a manifestation of BHD, and FLCN has been found to be expressed in the acinar cells of the parotid (92,121). Larger studies, preferably prospective, will need to be done to evaluate the question of risk for other associated cancers.

### 151.3.4 Medical and Surgical Management of BHD

Patients do not necessarily have all of the three major manifestations of BHD; they can have any combination of findings. This feature of BHD is of particular importance to note when counseling family members of a proband with BHD. All family members need to have genetic testing for mutations in *FLCN*, whether or not they have evident manifestations of disease.

Currently, there are no standard-of-care consensus guidelines for screening for the manifestations of BHD in families with known disease. Both the risk of renal cancer and pneumothorax are of concern. Based on screening recommendations for other hereditary renal cancers, annual to biannual screening of the kidneys by MRI starting in the early 20s, given the earliest age of cancer diagnosis in patients with BHD, seems reasonable. Screening using ultrasound is too insensitive for small lesions (122) and annual CT may give too high cumulative radiation doses. Pavlovich et al. described the management of BHD-associated renal tumors in fourteen patients over a median follow-up of 38 months (113). Four patients with lesions less than 3 cm were observed without need for intervention and without evidence of distant metastasis. Of the ten patients with lesions larger than 3 cm, one patient underwent bilateral nephrectomy and renal transplant, unilateral nephrectomy with contralateral partial nephrectomy was performed in two patients, and seven underwent bilateral partial nephrectomy. Local recurrence in retained renal remnants was observed in three patients and two patients died from metastatic renal-cell cancer (RCC) (both of whom had

conventional RCC). The treatment of renal masses in patients with BHD follows a similar strategy as VHL: lesions smaller than 3 cm are observed and those larger than 3 cm undergo immediate surgical resection.

As the number and volume of lung cysts are associated with risk of pneumothorax, a baseline chest CT is helpful. Screening after the initial study depends on the initial findings, with those with multiple cysts needing regular (annual CT) scanning and pulmonary consultation. A lower threshold for pleurodesis may be warranted in patients with pneumothorax. Of importance, as patients are at increased risk of pneumothorax, they need increased intraoperative monitoring when having surgery. In addition, they should be advised against scuba or other deep-sea diving due to the risk of pneumothorax. In general, they are not advised against flying on commercial flights as cabins are pressurized. As smoking is a risk factor for both pneumothorax and renal cancer, patients are strongly encouraged not to smoke.

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## RELEVANT WEB PAGES

FH mutation database: [http://chromium.liacs.nl/lovd\\_sdh/home.php?select\\_db=FH](http://chromium.liacs.nl/lovd_sdh/home.php?select_db=FH)

FLCN mutation databases: <http://grenada.lumc.nl/LOVD2/shared1>, <http://www.skengenedatabase.com/home.php>

### Biography

**Katherine (Kate) L Nathanson** is Associate Professor of Medicine in the Division of Translational Medicine and Human Genetics, and the co-leader of the Abramson Cancer Center's Cancer Control Program. She trained in internal medicine and clinical genetics. Dr Nathanson is currently a physician–scientist who focuses on cancer genetics and genomics as a tool to improve to improve patient care. She maintains both an active clinical practice and laboratory research program. Her clinical practice focuses on inherited cancer susceptibility syndromes, predominantly focusing on those associated with hamartomatous syndromes, renal cancer, and endocrine tumors. In her research program, she investigates both the inherited and somatic genome in human cancers, specializing in testicular cancer, familial breast cancer, renal cancer, melanoma, and pheochromocytomas/paragangliomas.

## Inherited Disorders of the Hair

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Columbia University, NY, USA**GLOSSARY**

**Acanthosis nigricans** – an ill-defined, velvety, hyperpigmented plaque involving the fold of the skin, including the axilla, neck and groin

**Atrichia** – absence of hair

**Atrophoderma** – atrophy of the skin

**Blepharitis** – inflammation of the eyelids

**Cicatricial alopecia** – scarring alopecia

**Copy number variations** – DNA segments with 1 kb or more of gains or losses

**Corneodesmosin** – a protein found in corneodesmosomes that is highly expressed in the upper layers of the epidermis, contributing to the skin membrane barrier. Moreover, corneodesmosin is highly expressed in the inner root sheath of the hair follicle

**Desmocollins** – specialized cadherin proteins that interact with themselves and with other proteins contributing to the formation of desmosomes

**Desmogleins** – specialized cadherin proteins that interact with themselves and with other proteins contributing to the formation of desmosomes

**Desmoplakins** – specialized proteins that connect the intermediate filaments to the desmosomal plaque, contributing to cellular integrity

**Desmosomes** – specialized intercellular structures that function in cell–cell adhesion

**Ectodermal dysplasia** – a group of disorders characterized by abnormal development of at least one of the following structures: hair, nails, sweat glands, and teeth

**Ectrodactyly** – also known as split hand foot. It is characterized by loss or defective central hand or foot digits, giving the appearance of lobster claws

**Forelock** – the area above the central part of the forehead

**Hemophagocytosis** – phagocytosis of blood components by histiocytes. It can occur in the bone marrow and other tissues

**Heterochromia** – a difference in color. Most commonly used to describe different iris colors

**Ichthyosis** – dry, thickened, scaly and flaky skin that resembles the skin of fish

**Ichthyosis linearis circumflexa** – migratory polycyclic and serpiginous erythematous plaques with double-edged scale at the margins, which are characteristic of Netherton syndrome

**Keratodermas** – disorders characterized by thickening of the skin of the palms and soles

**Keratosis follicularis** – in the text we used this term to describe erythematous papules arising in follicular regions; distinct from another genetic condition also known as keratosis follicularis, Darier's disease

**Milia** – white, keratin-filled papules that can appear on skin and mucous membranes

**Moniliform hair** – hair where several regular nodes are formed secondary to defects in the hair cortex

**Pachyderma** – thickening of the skin and particularly of the dermis resembling the thick skin of the elephant

**Pili torti** – twisted hair

**Plakoglobins** – belong to the family of armadillo proteins and are constituents of desmosomes and intermediate junctions

**Plakophilins** – belong to the family of armadillo proteins and are constituents of desmosomes

**Placode** – embryonic thickening of the epithelial layer from which the hair develops

**Steatocystomas** – epidermal cystic lesions that lack a granular layer and contain yellowish oily liquid and frequently hair structures and sweat glands

**Synophrys** – confluence of the eyebrows

**Trichomegaly** – excessive growth of the eyelashes

**Trichorrhexis invaginata** – also known as bamboo hair. A hair shaft anomaly due to intussusception of the hard keratinized part of the hair follicle into the softer non-keratinized portion

**Trichorrhexis nodosa** – weakening of the hair follicle leading to hair follicle splitting, giving the hair the appearance of a broom

**Trichoschisis** – a transverse break of the hair shaft at a region that is devoid of cuticle

**Vesicle** – a fluid-filled membrane-enclosed elevation in the skin



**Woolly hair** – is characterized by hair shaft anomalies that clinically present with tightly curled hair. Woolly hair is distinct from the tightly curly hair in African populations in that woolly hair shows hair shaft anomalies which can lead to hair loss and hair depigmentation

## 152.1 INTRODUCTION AND HAIR ANATOMY

The hair follicle (HF) is an important epidermal structure that plays a role in maintaining adequate internal body temperature control for several species during temperature extremes, and provides the first line of defense against environmental changes, toxins, and infectious agents. The hair is composed of several layers in cross section that work together in maintaining its integrity. The layers from the inside to the outside (Figure 152-1) are as follows. First is the hair shaft, which is made up of three layers: the medulla in the center is surrounded by the hair cortex and the hair shaft cuticle externally. The hair shaft is then surrounded by the inner root sheath (IRS), which itself is made up of three layers: the innermost part is referred to as the IRS cuticle, the middle part Huxley's layer, and the external part is Henle's layer. The IRS is then surrounded by a one-cell layer known as the companion layer, which separates the IRS from the next layer, the outer root sheath (ORS) of the HF. During

growth and differentiation of the several layers, keratin proteins are being expressed abundantly and differentially, providing a rigid structure for the HF (1). These keratins interact with the desmosomes formed between the cells, contributing to the integrity of the HF (2). Importantly, several mutations in keratins, desmosomes, and other proteins regulating their expression have been linked to several phenotypes in human hair disorders.

Longitudinally, the hair can be roughly separated into four regions (Figure 152-2): the bulb, which contains the matrix, the site where melanocytes reside, and which surrounds the dermal papilla (DP); the suprabulbar region, extending up to the site of arrector pili muscle insertion (the area of the bulge that contains the HF stem cells); the isthmus region, extending between the arrector pili muscle and the sebaceous gland; and the infundibular region that extends from the sebaceous gland to the skin surface. All the layers of the HF are derived from the hair matrix except for the ORS, which is contiguous with the basal interfollicular epidermis (1).

## 152.2 HF MORPHOGENESIS AND CYCLING

The formation of mammalian HFs occurs during embryogenesis through a series of reciprocal interactions between the ectoderm (epidermis) and the underlying

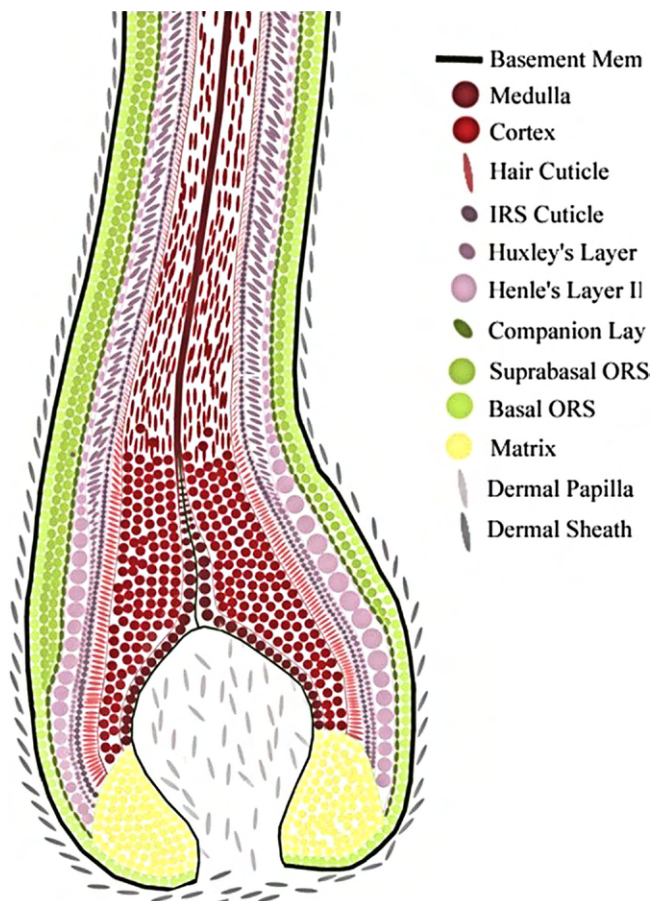


FIGURE 152-1 Structure of the hair follicle.

mesoderm (dermis) (3). The initial signal for hair development originates from the dermis, which signals to the overlying epithelium to form the placode. It is widely believed that the first signal is related to the Wnt signaling pathway (4). Wnt in the epidermis leads to abundance of  $\beta$ -catenin, which forms the placode (5). The placode then signals to the underlying mesoderm, forming the dermal condensate. Reciprocal signaling between the two compartments leads to the downgrowth of the placode (6,7). The placode then surrounds the dermal condensate, which will later on become the DP, and the cells in the placode proliferate and differentiate to make the several layers of the HF. Wnt/ $\beta$ -catenin expression is required for the activation of its direct transcriptional target, EDA receptor (EDAR), and therefore nuclear factor kappa B (NF- $\kappa$ B) signaling (8). EDAR functions in suppressing the placode inhibitory factors, mainly Dkk1, and thus indirectly potentiates the activity of the Wnt/ $\beta$ -catenin pathway (9).

Once the HF is formed, it begins to cycle. The hair cycle is divided largely into three phases (Figure 152-3), in addition to a recently described fourth phase, exogen (10). The growth phase, regression phase, rest, and shedding phases are termed anagen, catagen, telogen, and exogen, respectively. During cycling, the portion of the HF below the level of the isthmus goes through cyclic growth and regression, while the portion above the isthmus is permanent. The anagen phase is the longest phase, with variable duration depending on the body site. Generally, the anagen phase in the human scalp (longest hair anagen phase in humans) lasts 4–6 years. During the anagen phase, the lower part of the permanent HF (bulge) grows downward and forms the matrix of the HF, which differentiates into the various HF compartments (1). During the telogen phase, the club hair is encased by the

permanent portion of the HF, while during catagen the lower part of the HF undergoes apoptosis and regresses toward the permanent portion.

### 152.3 THE HAIR KERATINS AND THEIR REGULATION

The human genome has 54 functional annotated keratin genes, among which 28 belong to the type I keratin gene cluster and 26 belong to the type II gene cluster. All type II keratin genes cluster together on chromosome 12q13.13, while type I keratin genes cluster on chromosome 17q21.2, with the exception of keratin K18, a type I keratin, which is located in the type II keratin gene domain (11).

Among type I genes, 11 code for type I hair keratins, designated K31–K40 (previously known as Ha1–Ha8, Ka35, Ka36), while 17 code for type I epithelial keratins. Likewise, six of the type II genes encode hair keratins designated K81–K86 (previously known as Hb1–Hb6) while the remaining 20 code for epithelial keratins (12,13).

The main difference between hair and epithelial keratins is that hair keratins possess a greater amount of sulfur in their head and tail domains, which enables them to form tight cross-linking with keratin-associated proteins (KAPs) (14) that contribute to the hard structure of both hair and nails. Hair keratins are mainly expressed in the hair cortex and cuticle, and are structurally divided into three functional regions: the head domain, close to the N-terminus; the  $\alpha$ -helical rod domain, which includes the helix initiation and termination motifs; and the tail domain, which is close to the C-terminus. The rod domain is essential for the correct assembly with keratins of the opposite type, and therefore functional intermediate filaments. The head and tail domains are required for the interaction of the keratins with the KAPs (11).

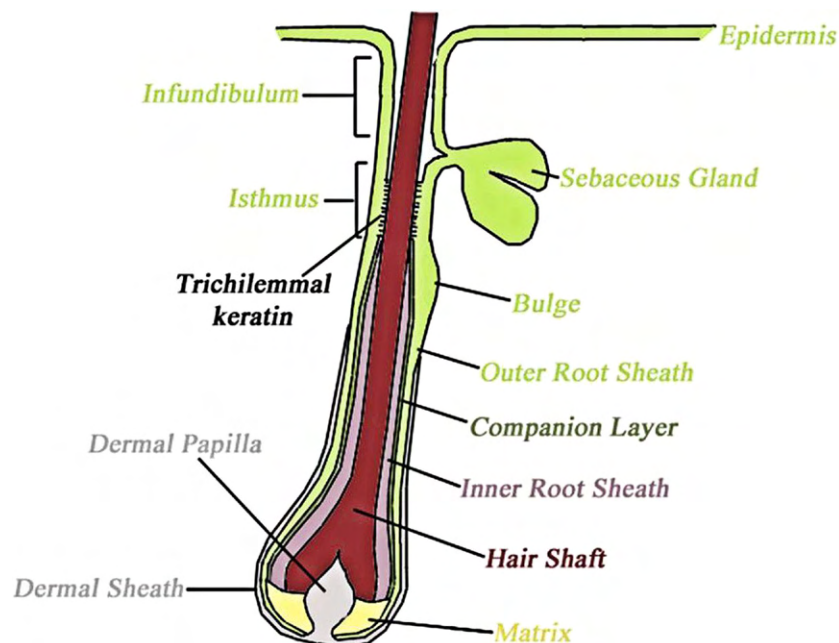


FIGURE 152-2 The four longitudinal regions of the hair.

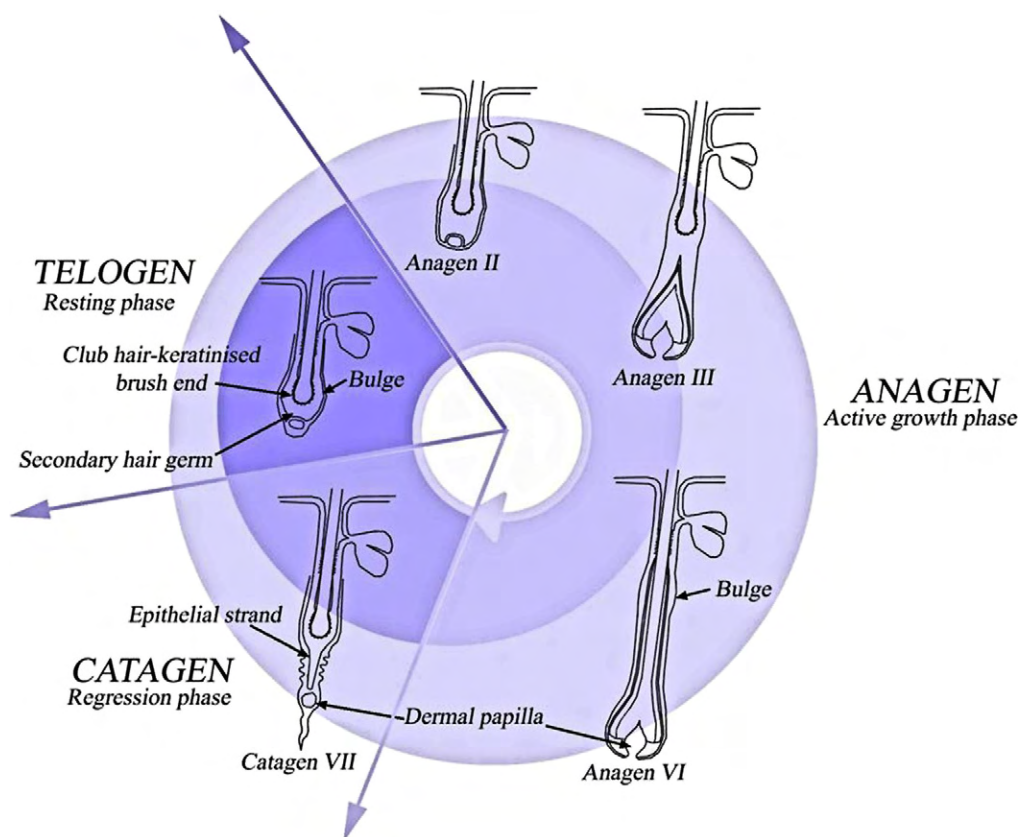


FIGURE 152-3 The hair cycle.

The differentiation of the hair and hair compartments involves the coordinated spatial and temporal expression of a large number of keratin genes. The production of the different keratins is a tightly regulated process that involves several transcription factors and proteins. FOXN1 plays a crucial role in the activation of genes involved in cortex and IRS differentiation. The most commonly affected keratins are keratin 33 and keratins 81, 85, and 86 (1). Moreover, downstream molecules of the Wnt signaling pathway trigger matrix cells toward cortex differentiation (6). HOXC13 is another transcription factor that is implicated in the regulation of hair keratins. The expression of HOXC13 overlaps with that of keratins 32, 35, 82, and 85. DLX3, a homeobox transcription factor, is another factor which regulates early signaling within the matrix, regulating the expression of hair keratin genes in the hair shaft and IRS (15). Several human hair phenotypes and syndromes with mutations in keratins or their regulatory proteins have been identified and will be discussed later in the chapter.

#### 152.4 DESMOSOMES OF THE HF

Desmosomes are intracellular adhesion structures that are critical for cell–cell attachment in the epithelial and myocardial tissues and in other tissues (16). The desmosomal cadherin family is a major structural unit of the

desmosome. It is composed of desmogleins (DSGs) and desmocollins (DSCs). DSGs and DSCs are glycoproteins containing a single transmembrane domain, through which they interact extracellularly, contributing to intercellular adhesion. Intracellularly, the cadherins bind to plakoglobins, plakophilins, and indirectly to desmoplakin, forming the desmosomal plaque. The desmosomal plaque interacts with the keratin intermediate filaments (KAFs). These interactions impart resilience and strength, and allow the distribution of physical impacts throughout tissues (17).

DSG(1–4) are all expressed in the human HF. *DSG4* is highly expressed in the precortex, keratinizing zone of the cortex, lower hair shaft cuticle, and the upper IRS cuticle (18). *DSG4* expression is regulated by several transcription factors, including HOXC13 and FOXN1 (2). Thus it is perhaps not surprising that mutations in any of these components would be associated with hair disorders.

#### 152.5 GENETICS OF HYPOTRICHOSIS AND OTHER STRUCTURAL HAIR ABNORMALITIES

Hypotrichosis denotes loss or reduction of HF density or integrity, and can affect any site on the body in both men and women. We and others have identified several genes associated with different types of hypotrichosis, where





**FIGURE 152-4** Generalized hereditary hypotrichosis simplex.

hypotrichosis occurs as an isolated finding or in the setting of a syndrome.

### 152.5.1 Disorders Involving the Wnt/ $\beta$ -Catenin Pathway

**152.5.1.1 Odonto-Onychodermal Dysplasia and Schopf–Schulz–Passarge Syndrome.** Odonto-onychodermal dysplasia (OODD; Online Mendelian Inheritance in Man (OMIM) 257980) is a rare autosomal recessive condition, described first in Lebanese families, and is characterized by hypotrichosis, onychodysplasia, palmar erythema and hyperhidrosis, palmoplantar keratoderma (PPK), oligodontia, and diffuse follicular hyperkeratosis. This condition is associated with deactivating mutations in the *WNT10A* gene, which produces an activator ligand of the Wnt/ $\beta$ -catenin signaling pathway (19). In addition to being crucial in hair morphogenesis, the Wnt/ $\beta$ -catenin pathway is also critical in the formation of other ectodermal structures. Not all patients with mutations in the *WNT10A* gene will present with hypotrichosis, perhaps due to the presence of several Wnt activator ligands that may compensate for its absence. Schopf–Schulz–Passarge syndrome (SSPS; OMIM 224750) is allelic to OODD, with mutations in the *WNT10* gene. SSPS has a similar presentation to OODD but a distinguishing feature is apocrine hidrocystomas (benign tumors of the sweat glands) that occur around the eyelids (20).

**152.5.1.2 Generalized Hereditary Hypotrichosis Simplex.** Hereditary hypotrichosis simplex (HSS; OMIM 605389) is an autosomal dominant condition characterized by a progressive hair loss involving the scalp, face, and body hair (Figure 152-4) (21). Patients

generally start to lose hair starting in the second half of the first decade, and progress to complete hair loss by the third decade of life. We have recently identified the gene mutated in this condition, *APCDD1*, in Pakistani and Italian families (22). We have demonstrated that *APCDD1* is a membrane-tethered protein abundantly expressed in the DP, the matrix, and the hair shaft of human HFs. Unlike WNT10, which functions as an activator of the Wnt/ $\beta$ -catenin pathway, *APCDD1* is an inhibitor of Wnt/ $\beta$ -catenin. Mutations in *APCDD1* are dominant negative against the wild-type allele. Therefore, we suggest that when *APCDD1* is mutated, Wnt/ $\beta$ -catenin is continuously activated, leading to depletion of HF stem cells. This may explain why people initially have normal hair and subsequently develop progressive hair loss.

### 152.5.2 Human Hair Disorders Involving the EDA-A1/EDAR/EDARADD Signaling Pathway

The EDA-A1/EDAR/EDARADD signaling pathway is part of tumor necrosis factor (TNF) superfamily pathway, and is crucial in hair morphogenesis since it functions to potentiate the activity of Wnt/ $\beta$ -catenin (see Section 152.2). Moreover, the TNF pathway is crucial for the development of other ectodermal structures, including teeth and sweat glands. EDA is a member of the TNF family and is a type II transmembrane protein that consists of three regions. In order to become functionally active, EDA must be cleaved and released out of cells, where it forms a trimer and binds to the EDAR (23). EDAR is a type I transmembrane protein and a member of the TNF receptor superfamily, with a



cysteine-rich domain in the extracellular region as well as a potential death domain in its intracellular region (18). Usually, EDA-A1 binds to EDAR and activates it, which then interacts with EDARADD through its death domain, resulting in the activation of NF- $\kappa$ B (24). Activation of NF- $\kappa$ B is essential for the development of the ectodermal structures and therefore mutations in genes along the TNF pathway lead to ectodermal dysplasia syndromes with overlapping clinical features, including sparse hair.

**152.5.2.1 Hypohidrotic Ectodermal Dysplasia.** Hypohidrotic ectodermal dysplasia (HED) is a disorder affecting the hair, teeth, and sweat glands. Clinically, patients show hypotrichosis and characteristic facies with a saddle nose, periorbital wrinkling with hyperpigmentation, conical teeth, and oligodontia (Figure 152-5), (see Chapter 148). Due to the impaired development of sweat glands, patients present with unexplained bouts of recurrent fevers. HED is most commonly inherited in an X-linked pattern (OMIM 305100) due to mutations in the EDA-A1 gene, although autosomal dominant (OMIM 129490) and autosomal recessive (OMIM 224900) inheritance due to mutations in the autosomal EDAR and EDARDD genes have been reported (18).

**152.5.2.2 X-linked Anhidrotic Ectodermal Dysplasia with Immunodeficiency and Incontinentia Pigmenti.** X-linked anhidrotic ectodermal dysplasia with immunodeficiency (XL-EDA-ID; OMIM 300291) is an X-linked recessive disorder that occurs mainly in males. This condition is caused by hypomorphic mutations in the *IKBKG* (inhibitory  $\kappa$ B kinase  $\gamma$  gene), which encodes NEMO (NF- $\kappa$ B essential modulator). The activity of NEMO is compromised, along with the TNF signaling pathway. Therefore, patients present with many features overlapping those of HED including the characteristic facies, atrichia or hypotrichosis, hypohidrosis,

oligodontia, and recurrent bouts of unexplained fevers. The main feature that distinguishes XL-EDA-ID from HED is the recurrent infections beginning early in life, since NF- $\kappa$ B is required for the maturation of the immune system (25). Incontinentia pigmenti (IP; OMIM 308300) is an X-linked dominant condition which is allelic to XL-EDA-ID. Unlike XL-EDA-ID, IP results from complete loss-of-function mutations in the NEMO gene and therefore this condition occurs almost exclusively in females because most males, except for those who are mosaic or are 47XXY, will die in utero (26). Clinically, patients present with perturbations in skin pigmentation with a variety of malformations affecting the skin, hair, teeth, eyes, heart, and central nervous system. The characteristic skin signs occur in four highly stereotyped stages: at birth, patients are born inflammatory vesicles; these change few months later into verrucous patches; later on, the verrucous patches will be replaced with a distinctive pattern of hyperpigmentation; and the fourth stage is characterized by dermal scarring (27).

### 152.5.3 Human Hair Disorders Involving the Keratins and Their Regulators

**152.5.3.1 Monilethrix.** Monilethrix (OMIM 158000) is a condition that affects the hair and sometimes the nails, and is most commonly inherited in an autosomal dominant pattern, though autosomal recessive patterns have recently been reported. It is clinically characterized by dystrophic alopecia that involves most commonly the occipital region of the scalp, but in more severe cases can involve the entire scalp, eyebrows, eyelashes, body hair, and nails (Figure 152-6). Patients may also develop keratosis follicularis and perifollicular erythema. Monilethrix is mostly caused by mutations in the type II hair keratins K81 and K86, and less commonly K83



FIGURE 152-5 Hypohidrotic ectodermal dysplasia.

(28,29). The most common site of mutations was localized to the conserved helix termination motifs of K81, K86 and K83, followed by the helix initiation motifs. These keratins are highly expressed within the hair cortex, and are critical to the normal development of the cortex. Mutations in these keratins result in hairs with a beaded appearance because of a periodic decrease in diameter along the hair shaft (28,29). To date, no mutations linked to monilethrix have been associated with mutations in type I hair keratins.

**152.5.3.2 Pure Hair and Nail Ectodermal Dysplasia.** Several types of pure hair and nail ectodermal dysplasias (OMIM 602032) with either autosomal recessive or autosomal dominant pattern of inheritance have been reported. To date, only mutations in K85, a type II hair keratin that is critical for the normal development of the HF, have been linked to recessive pure hair and nail ectodermal dysplasia. Patients with autosomal recessive pure hair and nail ectodermal dysplasia are characterized by total alopecia and dystrophic nails (Figure 152-7). K85 is mainly expressed in the matrix of the follicle, which might explain the severe phenotype in these patients (30,31).

**152.5.3.3 Autosomal Dominant Woolly Hair.** We and others have recently identified keratin 74, a type II IRS

keratin, as the cause of autosomal dominant woolly hair (ADWH; OMIM 194300) in a four-generation Pakistani family. Patients with ADWH report a slowly growing hair which stops growing at few inches. Clinically they are characterized by dry and coarse woolly hair with normal hair density (32) (Figure 152-8). More recently, Wasif et al. reported mutations in keratin 74 in the setting of hypotrichosis (33). Therefore, within the same family, keratin 74 can cause either woolly hair or hypotrichosis.

**152.5.3.4 Loose Anagen Hair Syndrome.** Loose anagen hair syndrome (LAHS; OMIM 600628) is a sporadic hair disorder but an autosomal dominant pattern of inheritance has been suggested. LAHS is clinically characterized by anagen hairs that are easily plucked from the scalp with no pain. Generally, hair shafts have reduced caliber but are not fragile and not easily broken. It has been proposed that defects in keratin 75, a type II keratin in the companion layer, may underlie LAHS (11,34).

**152.5.3.5 Pachyonychia Congenita Type II (Jackson–Lawler Syndrome).** Pachyonychia congenita type II (PCII; OMIM 167210) is a rare genodermatosis characterized by thickened nails, PPK, natal teeth, and steatocystomas. Although not commonly associated with



FIGURE 152-6 Monilethrix.



FIGURE 152-7 Autosomal recessive pure hair and nail ectodermal dysplasia.





**FIGURE 152-8** Autosomal dominant woolly hair.

a hair phenotype, patients can develop pili torti when keratin 17, an ORS keratin, is mutated (1).

**152.5.3.6 T-Cell Immunodeficiency with Congenital Alopecia and Nail Dystrophy.** T-cell immunodeficiency with congenital alopecia and nail dystrophy (OMIM 601705) is an extremely rare autosomal recessive condition first identified in southern Italy. Patients present with congenital alopecia, nail dystrophy, and severe infections due to T-cell immunodeficiency. Patients usually succumb to the infections during their first year of life. Mutations have been identified in the *FOXN1/WHN* gene, a regulator of keratin expression (35). *FOXN1* plays a critical role in the development of the thymus, the site where T cells mature and become competent, and is a transcriptional regulator of many hair keratin genes.

**152.5.3.7 Tricho-Dento-Osseous Syndrome.** Trichodento-osseous syndrome (TDO; OMIM 190320) is an autosomal dominant condition caused by mutations in the *DLX3* gene, a regulator of keratin expression. This condition is associated with very tightly curled hair, enamel hypoplasia, and diffuse bony abnormalities (36).

## 152.5.4 Human Hair Disorders Involving the Desmosomes

**152.5.4.1 Localized Autosomal Recessive Hypotrichosis/Monilethrix.** Localized autosomal recessive hypotrichosis (LAH; OMIM 607903) is a rare nonsyndromic hair disorder in which patients present with hypotrichosis involving the scalp, chest, arms, and legs. The eyebrows and beard are partially affected, and the axillary hair, pubic hair, and eyelashes are normal. We and others have identified mutations in the desmoglein 4 (*DSG4*) gene as the cause of LAH (37). We and others subsequently identified *DSG4* mutations in the setting of autosomal recessive monilethrix (38). *DSG4* is the only desmoglein member expressed in the keratinizing zone of

the hair shaft cortex, overlapping with the expression of the hair keratins K81, K83, and K86 (18). We postulated that abnormal *DSG4* proteins alter the switch from the proliferation to the differentiation of trichocytes, leading to abnormal and premature keratinization of the hair shaft, resulting in beaded hair as part of the phenotype in some cases (37).

**152.5.4.2 Ectodermal Dysplasia/Skin Fragility Syndrome.** Ectodermal dysplasia/skin fragility syndrome (OMIM 604536) is an autosomal recessive genodermatosis that presents clinically with skin fragility (with trauma-induced erosions and blistering), short and sparse hair, PPK, thickened and dystrophic nails, and occasionally hypohidrosis. This condition is caused by mutations in the plakophilin 1 (*PKP1*) gene (38). Examination of patients' epidermis under electron microscopy demonstrated aberrant cytoplasmic distribution of desmoplakin (DSP) and perinuclear aggregation of KIFs, which indicated that loss of *PKP1* led to disruption in DSP distribution and abnormal aggregation of KIFs. Moreover, *PKP1* affects the size and number of desmosomes, keratinocyte cell migration, and the calcium stability of desmosomes (39).

**152.5.4.3 Cardiocutaneous Diseases: Naxos Disease and Carvajal Syndrome.** *DSG2* and *DSC2* are predominantly expressed in the myocardium of the heart. Heterozygous mutations in the *DSG2* or *DSC2* genes have been reported to underlie arrhythmogenic right ventricular cardiomyopathy (ARVC) as their only clinical manifestation (OMIM 107970) (40,41). Disruption of the desmosomal plaque components PKG or DSP results in several hereditary diseases involving not only the heart, but also the skin and hair. Naxos (OMIM 601214) and Carvajal (OMIM 605676) syndromes are autosomal recessive human disorders characterized by woolly hair, PPK, and cardiomyopathy, which result from protein truncating mutations in the desmosomal components PG and DP, respectively (42,43). Two homozygous

mutations in the gene encoding PG were also found to underlie skin fragility accompanied by diffuse PPK and woolly hair, without heart abnormalities. Interestingly, one of these mutations resulted in sparse and woolly hair, whereas patients harboring the other mutation had abundant woolly hair (44). Over 40 human mutations in the DSP gene have been shown to cause either skin or heart disease, or a combination of skin, hair, and heart abnormalities, underscoring its importance for the development and integrity of these tissues. Like PG, DSP mutations can be associated with hair abnormalities, with both woolly hair and alopecia being described (45).

**152.5.4.4 Hypotrichosis and Recurrent Skin Vesicles.** Hypotrichosis with recurrent skin vesicles (OMIM 613102) is a recently described condition with an autosomal recessive pattern of inheritance, first identified in the Pakistani population. Clinically, affected individuals present with sparse scalp, facial, and body hair with fragile hair shafts, as well as recurrent diffuse vesicles involving the scalp and body and non-mucosal surfaces of the body that heal without scarring. The disease was mapped to the DSG–DSC gene cluster on chromosome 18q12.1, and a homozygous nonsense mutation in the DSC3 gene was subsequently identified in all affected individuals (46).

**152.5.4.5 Hereditary Hypotrichosis Simplex of the Scalp.** Hereditary hypotrichosis simplex of the scalp (HHSS; OMIM 146520) is an autosomal dominant disorder with heterozygous nonsense mutations in the CDSN gene (47). Clinically, patients present with hypotrichosis limited to the scalp. CDSN is expressed in the IRS of the HF. Aggregates of abnormal CDSN can be detected around the HF, as well as in the papillary dermis in patients' skin, suggesting that they are toxic to the HF. Thus, the mutant CDSN protein appears to function in a dominant negative manner (47). Recently, homozygous mutations in CDSN have also been reported in the setting of generalized inflammatory skin peeling syndrome, an autosomal recessive skin disorder characterized by superficial splitting of the upper layer of the epidermis (48) (OMIM 270300).

### 152.5.5 Woolly Hair Related to the LIPH/LPAR6 Signaling Pathway

Lipase H (LIPH) encodes a member of the phospholipase A1 family and is required for the synthesis of lysophosphatidic acid (LPA) (49). LPAR6 (also known as P2RY5) encodes the receptor P2Y5, a seven transmembrane G-protein-coupled receptor (GPCR). Recently, it was found that LPA is a ligand for the receptor P2Y5, which explains the similar phenotypes in patients with either P2RY5 or LIPH gene mutations. Moreover, the expression of P2Y5 overlaps with that of LIPH in the HF (50). These data underscore the important role of the LPA/P2Y5 signaling pathway in the normal development of the HF (51). P2Y5 is mainly expressed in the

inner root hair sheath of the HF that arises from the hair matrix and differentiates before the keratinocytes of the central hair matrix. This provides support for the normal development of the hair shaft, and perhaps explains why the hair becomes twisted when LPA/P2Y5 is disrupted, resulting in a woolly hair phenotype. In addition to the scalp hair abnormalities, patients can develop sparse eyebrows, eyelashes, axillary, and body hair, but usually the beard hair is normal.

**152.5.5.1 Autosomal Recessive Woolly Hair and Autosomal Recessive Hypotrichosis.** We and others have recently shown that mutations in LPAR6 and Lipase H (LIPH) are associated with autosomal recessive woolly hair (ARWH; OMIM 278150) and/or autosomal recessive hypotrichosis (OMIM 611452) (50,52). Mutations in both genes result in a clinically indistinguishable phenotype, which can range from woolly hair to hypotrichosis and complete loss of hair (Figure 152-9) (50). Generally, patients initially present with woolly hair, after which approximately 50% will progress to develop hypotrichosis of variable severity with age. Histology is consistent with decreased number of HFs with miniaturization (53).

### 152.5.6 Hair Disorders Related to the Hair Cycle Regulator, Hairless (HR)

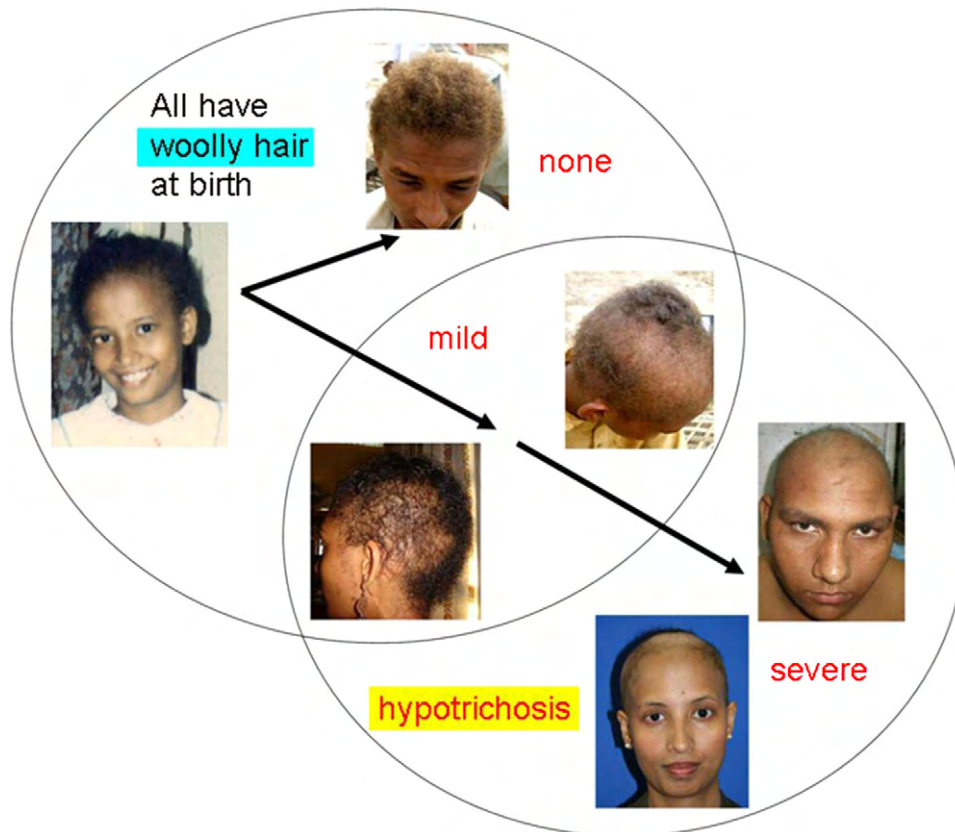
**152.5.6.1 Atrichia with Papular Lesions.** Atrichia with papular lesions (APL; OMIM 209500) is a recessive condition due to mutations in the hairless gene on chromosome 8 (54). Patients initially are born with normal hair density and hair loss occurs few months later, leading to complete alopecia without regrowth of the hair. Patients will later develop milia-like papules involving the face, arms, and knees (54) (Figure 152-10). This condition is frequently misdiagnosed as alopecia areata (AA; see Section 152.9.1). A main distinguishing feature from AA is the presence of milia-like papules in APL. Hairless is expressed in the HF, where it plays a pivotal role in the hair cycle (55). During the regression phase (catagen) the lower part of the HF is degenerated, and under certain stimuli probably related to a cross talk between the DP and the bulge, HF stem cells are activated to regenerate the hair and go back to the anagen phase. Hairless appears to mediate the cross talk between the DP and bulge compartments, leading to the progression of the hair cycle from catagen to anagen. Thus, failure of communication between the DP and the bulge will lead to the arrest of hair growth after the first cycle (56). Mutations in the VDR have been associated with vitamin-D-resistant rickets (OMIM 277440) where patients develop hair loss and milia-like papules similar to APL in association rachitic bone changes during mid to late infancy (57).

**152.5.6.2 Marie–Unna Hypotrichosis.** Marie–Unna hypotrichosis (MUH; OMIM 146550) is a nonsyndromic hereditary hair disease with an autosomal



dominant pattern of inheritance. MUH is clinically characterized by three stages: at birth, affected individuals present with sparse scalp and facial hair, with subsequent development of coarse, wiry, and twisted

hairs in early childhood. Later on during adolescence, affected individuals develop complete hair loss, resembling androgenetic alopecia (AGA). Recently, Wen et al. (58) reported that the 5'-untranslated region



**FIGURE 152-9** Autosomal recessive woolly hair or autosomal recessive hypotrichosis: range of phenotypes and progression of the condition.



**FIGURE 152-10** Atrichia with papular lesions.

(5'-UTR) of the *HR* gene has four potential upstream open reading frames (uORFs), designated U1HR–U4HR. Sequencing of these four uORFs of 19 families with MUH revealed that affected individuals in all 19 families carried heterozygous nucleotide changes within the U2HR (58).

### 152.5.7 Hair Disorders with Mutations in the P-Cadherin (*CDH3*) Gene

**152.5.7.1 Hypotrichosis with Juvenile Macular Dystrophy and Ectodermal Dysplasia with Ectrodactyly and Macular Dystrophy.** Mutations in the *CDH3* gene, encoding P-cadherin, have been shown to underlie two distinct autosomal recessive hereditary diseases in humans. Mutations in *CDH3* were identified in families with hypotrichosis with juvenile macular dystrophy (HJMD; OMIM 601553), which is characterized by sparse hair and weak eyesight due to progressive macular dystrophy of the retina (59). Patients will, on average, be completely blind by the age of 40 years. More recently, mutations in *CDH3* were also identified in ectodermal dysplasia and ectrodactyly with macular dystrophy, termed ectodermal dysplasia and ectrodactyly with macular dystrophy (EEM) syndrome (OMIM 225280) (Figure 152-11) (60). For the same mutation, members within the same family will either have HJMD or EEM. Affected individuals with EEM syndrome show common hair and eye phenotypes with HJMD; however, EEM patients also have the additional clinical manifestation of split-hand/foot malformation (SHFM), suggesting crucial roles for P-cadherin in the development not only of hair and the retina but also of the limb. Mutations in p63, a major transcription factor in epithelial tissues, cause several autosomal dominant

diseases, such as ectodermal dysplasia, ectrodactyly, and cleft lip/palate (EEC) syndrome, which shows phenotypic overlap with P-cadherin mutations in hair and limbs (61). We have shown that p63 colocalizes with P-cadherin in developing HF placode and limb buds during mouse embryogenesis. Furthermore, we have demonstrated that *CDH3* is a direct target gene of p63 (60). Our data underscores the critical role of P-cadherin in the HF, as well as in limb development.

### 152.5.8 Disorders of Pigmentary Hair

**152.5.8.1 Griscelli Syndrome.** Griscelli syndrome is divided into three types: Griscelli syndrome I (GS 1; OMIM 214450) is an autosomal recessive condition characterized by light skin, silvery hair, and neurologic impairment. Mutations occur in the *MYO5A* gene (62). GS 1 is also known as Elejalde syndrome. Griscelli syndrome type 2 (GS 2; OMIM 607624) is the most severe among the three types, inherited in an autosomal recessive pattern, and characterized by light skin, silvery hair, and severe immunodeficiency and lymphohistiocytotic hemophagocytosis, requiring bone marrow transplantation (63). GS 2 is associated with mutations in the *RAB27A* gene. Griscelli syndrome type 3 (GS 3; OMIM) is the most benign of the three types and is characterized exclusively by light skin and silvery hair. Mutations occur either in the *MYO5A* or the melanophilin gene (64). The skin color defect in GS is not due to impaired synthesis of melanin but is secondary to the impaired delivery of the pigment to the appropriate location in the skin and HF.

**152.5.8.2 Chédiak–Higashi Syndrome.** Chédiak–Higashi syndrome (CHS; OMIM 214500) is an autosomal recessive condition associated with mutations in the



FIGURE 152-11 EEM syndrome.

*LYST* gene (65). Clinically, patients present with light-colored skin, silvery hair, photophobia, recurrent infections, and neuropathy. Most individuals will develop an accelerated phase of an uncommon lymphoproliferative disorder characterized by lymphohistiocytic infiltrates, hepatomegaly, hypersplenism, jaundice, and pancytopenia. If not treated with bone marrow transplantation, most patients will die in their first decade of life (66). The most common cause of death is either infection or severe bleeding due to thrombocytopenia. Patients who survive past the first decade will go on to develop progressive neurologic impairment. The gene mutation in CHS affects the synthesis of granules in different types of cells, including immune cells and melanocytes, which explains the clinical features of CHS.

**152.5.8.3 Cross Syndrome.** Cross syndrome (OMIM 257800), also known as Kramer syndrome or oculocerebral syndrome with hypopigmentation, is an extremely rare autosomal recessive condition with unknown genetic defect. Clinically, patients present with light-colored skin, silvery hair, microphthalmia, corneal clouding, spastic paraplegia, and developmental delay (67).

## 152.5.9 Disorders Associated with Light-Colored Hair

**152.5.9.1 Albinism.** Albinism is divided into four major subtypes according to different genetic basis and clinical features. Type I oculocutaneous albinism (OCA1; OMIM 606952) and type II oculocutaneous albinism (OCA2; OMIM 203200) most commonly present with light-colored hair. OCA1 occurs secondary to mutations in the tyrosinase gene which is required for the synthesis of melanin, while OCA2 occurs secondary to mutations in the *P* gene which is required for regulating the PH for the normal functioning of tyrosinase. Clinical features associated with albinism include decreased pigment in the eyes, decreased visual acuity, and nystagmus (68).

**152.5.9.2 Hermansky–Pudlak.** Hermansky–Pudlak syndrome (HPS; OMIM 203300) is a rare autosomal recessive disorder, most commonly occurring in the Puerto Rican population, that clinically resembles oculocutaneous albinism, with recurrent episodes of bleeding, granulomatous colitis, cardiomyopathy, kidney failure, and progressive fatal pulmonary fibrosis. At least eight types of HPS exist; HPS3 being the most common, with mutations in the *HPS3* gene (69).

**152.5.9.3 Waardenburg Syndrome.** Waardenburg syndrome (WS) is divided into four types: WS1 (OMIM 193500), WS2 (OMIM 193500), WS3 (OMIM 148820) and WS4 (OMIM 277580). WS1 and WS3 are caused by mutations in the *PAX3* gene, type 2 is caused by mutations in the *MITF* gene, and type 4 is caused by mutations in *SOX10* or the endothelin-B receptor gene (*EDNRB*). All forms of WS are inherited in autosomal dominant pattern. The common clinical features among

all types of WS include a characteristic white forelock with premature graying of the hair, heterochromic irides, and sensorineural deafness (70).

**152.5.9.4 Piebaldism.** Piebaldism (OMIM 172800) is a rare autosomal condition characterized by mutations in the *KIT* gene. Affected individuals are clinically characterized by a white forelock and depigmented skin patches involving one side of the body that do not cross the midline. The gene defect is presumed to cause abnormal migration of melanocytes from the neural crest during development (71).

**152.5.9.5 Phenylketonuria.** Phenylketonuria (PKU; OMIM 261600) is an autosomal recessive condition caused by mutations in phenylalanine hydroxylase. Phenylalanine hydroxylase is required for the conversion of phenylalanine to tyrosine that is required for melanin synthesis. Patients usually have light-colored skin and hair, secondary to melanin deficiency, eczema, epilepsy, and severe neurologic and developmental abnormalities (72).

## 152.5.10 Disorders Associated with Trichorrhesis Nodosa

**152.5.10.1 Netherton Syndrome.** Netherton syndrome (NS; OMIM 256500) is an autosomal recessive genodermatosis caused by mutations in the *SPINK5* gene, which encodes the serine protease inhibitor LEKTI that functions in the formation of the skin membrane barrier. Affected individuals are born with nonspecific generalized erythroderma covered with fine scales. Patients then develop severe atopic dermatitis, hair loss, a characteristic ichthyosis known as ichthyosis linearis circumflexa, and rarely hypernatremic dehydration and failure to thrive. The most common hair abnormality in NS is trichorrhesis nodosa, although trichorrhesis invaginata is diagnostic. The preferred site to examine the hair abnormalities is the eyebrows (73).

**152.5.10.2 Menkes Kinky Hair Syndrome.** Menkes disease (MNK; OMIM 309400) is an X-linked recessive condition caused by mutations in the *ATP7A* gene, encoding a copper-ATPase transporter. Patients with MNK have copper deficiency and clinically present with growth retardation, cerebral and cerebellar degeneration, dementia, seizures, and kinky hair that on light microscopy shows pili torti (see below), moniliform hairs (see below) and trichorrhesis nodosa (74).

**152.5.10.3 Argininosuccinic Aciduria.** Argininosuccinic aciduria (OMIM 207900) is an autosomal recessive condition caused by mutations in the gene encoding argininosuccinate lyase, one of the enzymes in the urea cycle. Affected individuals become symptomatic early after birth and show hyperammonemia, encephalopathy, respiratory alkalosis, convulsions, liver enlargement, dry skin, and brittle hair that reveals trichorrhesis nodosa under light microscopy. It is presumed that the brittle hair is secondary to deficiency in arginine (75).



**152.5.10.4 Citrullinemia.** Citrullinemia (OMIM 215700) is an autosomal recessive condition caused by mutations in the gene encoding argininosuccinate synthetase, which also forms part of the urea cycle. Patients have similar features to those with argininosuccinic aciduria (76).

### 152.5.11 Disorders Associated with Pili Torti

**152.5.11.1 Bjornstad Syndrome.** Bjornstad syndrome (BJS; OMIM 262000) is an autosomal recessive disorder caused by mutations in the *BCS1L* gene. Clinical features include progressive sensorineural deafness, hair loss secondary to pili torti, and hypogonadism. Mutations in *BCS1L* lead to accumulation of reactive oxygen species, to which the hair and ears are sensitive (77).

### 152.5.12 Disorders Associated with Trichoschisis

**152.5.12.1 Trichothiodystrophy.** Trichothiodystrophy (TTD) is a rare autosomal recessive condition in which the hair is brittle, with trichoschisis and a low sulfur and cysteine content. On polarized microscopy, the hair displays an alternating dark and light banding pattern, which is referred to as tiger tail banding. TTD can be clinically divided into several types. To date, three genes have been implicated in the pathogenesis of TTD: *XPB*, *XPD* (mutated in photosensitive TTD), and *TTDN1* (mutated in non-photosensitive TTD). *XPB* and *XPD* are genes which are implicated in xeroderma pigmentosum, but unlike xeroderma pigmentosum, TTD is not associated with increased risk of skin cancer. TTD clinically presents with variable manifestations including cutaneous, neurologic, and growth abnormalities. Several acronyms are used to describe the clinical manifestations of TTD. PIBIDS (OMIM 278730), IBIDS (OMIM 242170), and BIDS (OMIM 234050) stand for: photosensitivity, ichthyosis, brittle hair, intellectual impairment, decreased fertility, and short stature. Other disorders that are listed under TTD include Sabinas syndrome (OMIM 211390), which is an autosomal recessive condition characterized by hair and nail deformities in association with mental retardation; Pollitt syndrome (OMIM 275550), an autosomal recessive condition characterized by brittle hair and nails, mental retardation, and delayed bone age, with or without dental caries; and Itin syndrome (OMIM 258360), also known as TTD with immunodeficiency, an autosomal recessive disorder characterized by brittle hair and nails, and mental retardation in association with neutropenia and immunoglobulin deficiency. The last subgroup of TTD is TTD with intrauterine growth retardation (IUGR), which is characterized by hair abnormalities, severe IUGR with failure to thrive,

developmental delay, recurrent infections, cataracts, and hepatic angioendotheliomas (78,79).

## 152.6 HAIR ABNORMALITIES IN ASSOCIATION WITH METABOLIC DISEASES

### 152.6.1 Acrodermatitis Enteropathica

Acrodermatitis enteropathica (OMIM 201100) is a rare autosomal recessive disorder caused by mutations in the *SLC39A4* gene, encoding an intestinal zinc transporter. It is clinically characterized by intermittent diarrhea; exfoliative dermatitis that is more pronounced periorally, over the finger and toe tips, and in the inguinal area; and alopecia affecting the scalp, eyebrows, and eyelashes (80).

### 152.6.2 Biotinidase Deficiency

Biotinidase deficiency (OMIM 253260) is a rare autosomal recessive condition caused by mutations in the biotinidase gene. Clinically, patients present with seizure as the most common symptom, in association with ataxia, hearing loss, and skin rash with alopecia resembling that of acrodermatitis enteropathica. Early-onset multiple carboxylase deficiency (OMIM 253270) is a similar disorder, caused by mutation in the holocarboxylase synthetase gene (81).

## 152.7 OTHER DISORDERS ASSOCIATED WITH HAIR PHENOTYPES

### 152.7.1 Trichorhinophalangeal Syndrome

Trichorhinophalangeal syndrome type 1 (TRPS1; OMIM 190350) is an autosomal dominant condition caused by mutations in the *TRPS1* gene. TRPS1 patients have sparse scalp hair, bulbous tip of the nose, long flat philtrum, thin upper vermilion border, and protruding ears. Skeletal abnormalities include cone-shaped epiphyses at the phalanges, hip malformations, and short stature (82). Similarly, TRPS type 2 (OMIM 150230) and TRPS type 3 (OMIM 190350) are associated with sparse scalp hair.

### 152.7.2 Keratosis Follicularis Spinulosa Decalvans

Keratosis follicularis spinulosa decalvans (KFSD; OMIM 308800) is an X-linked recessive disorder that presents in early childhood with keratotic follicular papules; progressive alopecia of the scalp, eyelashes, and lateral third of the eyebrows; photophobia; corneal dystrophy; blepharitis in association with hyperkeratosis over the elbows, knees, palms, and soles; and nail dystrophy. Recently, mutations have been identified in the *MBTPS2* gene, involved in lipid regulation in the skin membrane barrier. Ichthyosis follicularis atrichia photophobia (IFAP; OMIM 308205)



is allelic to KFSD and is clinically characterized by noninflammatory spiny excrescences, hyperkeratosis, and non-cicatricial alopecia and photophobia (83).

### 152.7.3 Hidrotic Ectodermal Dysplasia

Hidrotic ectodermal dysplasia (HED; OMIM 129500), also known as Clouston syndrome, is an autosomal dominant condition caused by mutations in the connexin 30 gene. Patients usually present with total alopecia and nail dystrophy. Unlike patients with anhidrotic ectodermal dysplasia, patients with HED have normal sweating (84).

### 152.7.4 Bazex–Dupre–Christol Syndrome

Bazex–Dupre–Christol syndrome (OMIM 301845) is an X-linked dominant disorder of unknown genetic basis. Clinically, it is characterized by congenital hypotrichosis; follicular atrophoderma affecting the dorsa of the hands and feet, the face, and extensor surfaces of the elbows or knees; and the development of basal cell neoplasms by the second decade of life (85).

### 152.7.5 Hypotrichosis–Lymphedema–Telangiectasia Syndrome

Hypotrichosis–lymphedema–telangiectasia syndrome (HLTS; OMIM 607823) in both dominant and recessive forms has been described, caused by mutations in the *SOX 8* gene. Clinically, patients present initially with hypotrichosis with subsequent development of telangiectasias and lymphedema (86).

### 152.7.6 Chondrodysplasia Punctata Type 2

Chondrodysplasia punctata type 2 (CPXD; OMIM 302960), also known as Conradi–Hunermann syndrome, is an X-linked dominant condition caused by mutations in the *EBP* gene, coding for sterol isomerase emopamil-binding protein. Affected individuals present with coarse hair, scarring alopecia, striate keratoderma, atrophic whorls of hyperpigmentation, cataracts, skeletal abnormalities including short stature, and epiphyseal stippling and rhizomelic shortening of bones (87).

## 152.8 HYPERTRICHOSIS

Hypertrichosis describes all forms of hair growth that are excessive for the body and age of the individual, which occur at any site where HFs are present, and are not under the influence of androgens. Inherited hypertrichoses are very rare human disorders, whose incidence as a group has been estimated to be as low as one in 1000 million (18). Here we will divide hypertrichosis conditions into two main groups: the first are conditions

with generalized hypertrichosis and the second are inherited conditions with patchy hypertrichosis.

### 152.8.1 Disorders with Generalized Hypertrichosis

**152.8.1.1 Ambras Syndrome.** Ambras syndrome (AS; OMIM 145701) is a rare form of generalized CH. Affected individuals present with generalized hypertrichosis that is more pronounced over the upper part of the body, face, and ears, in association with abnormal facial features, such as a triangular, coarse face and long palpebral fissures (Figure 152-12). We studied three patients with AS, and defined an 11.5-Mb candidate interval for AS on chromosome 8q, which includes the *TRPS1* gene (82). One of the three patients with AS carries a pericentric inversion with a breakpoint in 8q23.1, which lies 7.3 Mb downstream of *TRPS1*. We found that the expression of *TRPS1* transcript was markedly downregulated in lymphoblasts of the patient (82). We have also detected a significant downregulation of *Trps1* expression in Koala mice. The Koa mice are characterized by excess hair on the muzzle and ears, and carry a 51.5-Mb inversion on mouse chromosome 15, of which the proximal breakpoint lies 791 kb upstream of the *Trps1* gene (82). Taken together, our data implicate a position effect on the expression of *TRPS1*, which underlies the hypertrichosis phenotype in both human and mouse.

**152.8.1.2 Hypertrichosis with Gingival Hyperplasia.** Hypertrichosis with gingival hyperplasia (OMIM 135400) is most commonly inherited in an autosomal dominant pattern. Affected individuals present with generalized hypertrichosis and coarse facial features, including thick lips, wide and flat nasal bridge, large ears, and gingival hyperplasia (Figure 152-13). The genetic basis of hypertrichosis with gingival hyperplasia is unknown, though recently copy number variations (CNVs) in the genome were reported to be implicated in the pathogenesis of the disease. Affected individuals from three Chinese families with autosomal dominant generalized CH with or without gingival hyperplasia were shown to have a heterozygous deletion on chromosome 17q24.2–q24.3 (88). Moreover, a sporadic case of CH with gingival hyperplasia was heterozygous for a duplication of the same region on chromosome 17q (88). Importantly, the *SOX9* gene, a crucial transcription factor for HF development (89), is located approximately 2 Mb downstream of these CNVs. The data raise the possibility that the CNVs close to the *SOX9* gene may also show a position effect on its expression.

**152.8.1.3 Cantu Syndrome.** Cantu syndrome (OMIM 239850) is a very rare genetic disorder of unknown etiology and is characterized clinically by congenital hypertrichosis, cardiomegaly, and bone abnormalities. The inheritance pattern is unclear, although autosomal dominant, autosomal recessive, and spontaneous occurrence



FIGURE 152-12 Ambras syndrome.



FIGURE 152-13 Hypertrichosis with gingival hyperplasia.

have been reported. We have recently identified a 375-kb duplication on chromosome 4q26–27. The region of duplication encompasses three genes: *MYOZ2*, *USP53*, and *FABP2*. *MYOZ2* and *USP53* are known to be highly expressed in cardiac muscle, and we found that *USP53* is expressed in the HF (90).

**152.8.1.4 Barber–Say Syndrome.** Barber–Say syndrome (OMIM 209885) is a rare congenital condition characterized by severe hypertrichosis, mainly on the back and forehead; atrophic skin with hyperlaxity and redundancy; and facial dysmorphism with hypertelorism, ectropion, telecanthus, abnormal and low-set ears, bulbous nasal tip, small teeth, nystagmus, low frontal hairline, and high arched palate (91).

**152.8.1.5 X-linked Hypertrichosis.** X-linked hypertrichosis (OMIM 307150) is a form of hypertrichosis inherited in an X-linked recessive or dominant form, characterized by congenital generalized hypertrichosis that is associated with dental anomalies and deafness (Figure 152-14). The gene mutated in this condition is still unknown but lies on chromosome Xq24–q27.1 (92).

**152.8.1.6 CAHMR Syndrome.** CAHMR (OMIM 211770) is an autosomal recessive condition reported in an Egyptian family. Affected individuals present with congenital cataracts and congenital hypertrichosis that is more pronounced on the upper part of the body, in association with mental retardation (93).

**152.8.1.7 Amaurosis Congenita, Cone-Rod Type with Congenital Hypertrichosis.** Amaurosis congenita, cone-rod type with congenital hypertrichosis (OMIM 204110) is an autosomal recessive form of hypertrichosis characterized by severe retinal dystrophy with visual impairment since birth and profound photophobia in the absence of night blindness. The underlying ophthalmologic pathology suggested a cone-rod type of congenital amaurosis. The ocular defects are accompanied by trichomegaly, bushy eyebrows with synophrys, and excessive facial and body hair with hypertrophied circumareolar hair on the breasts (94).

**152.8.1.8 Hypertrichosis and Acromegaly.** Hypertrichosis and acromegaly is an autosomal dominant form of generalized hypertrichosis. Affected individuals

characteristically present with generalized hypertrichosis and acromegaloid facies with no intraoral lesions or gingival hyperplasia (95).

### 152.8.2 Disorders Associated with Patchy Hypertrichosis

**152.8.2.1 H Syndrome.** The H syndrome (OMIM 612391) is an autosomal recessive disorder characterized by cutaneous hyperpigmentation, patchy hypertrichosis, hepatosplenomegaly, heart anomalies, progressive sensorineural hearing loss, hypogonadism, short stature, hyperglycemia, hallux valgus, and fixed flexion contractures of the toe and finger joints. The clinical diagnosis relies mainly on the presence of hyperpigmentation and hypertrichosis in the thighs. Mutations occur in the *SLC29A3* gene (96).

**152.8.2.2 Winchester Syndrome.** Winchester syndrome (OMIM 259600) is an autosomal recessive condition characterized by multicentric osteolysis, predominantly over the hands and feet, associated with generalized osteoporosis. Radiographically, the osteolysis is accompanied by a characteristic widening of the metacarpal and metatarsal bones. In addition, affected individuals display coarse facial features, corneal opacities, and generalized hypertrichosis. Mutations have been identified in the *MMP2* gene, encoding matrix metalloproteinase-2 (97).

**152.8.2.3 Porphyrrias.** The four types of porphyria that are characterized by patchy hypertrichosis are porphyria cutanea tarda (PCT; OMIM 176100), hepatoerythropoietic porphyria (HEP; OMIM 176100), variegate porphyria (VP; OMIM 176200), and erythropoietic protoporphyria (EPP; OMIM 177000). Both PCT and HEP are caused by mutations in urogen decarboxylase, with the former inherited as an autosomal dominant condition while the latter is inherited in an autosomal recessive

pattern. VP is inherited as an autosomal recessive condition caused by mutations in protoporphyrinogen oxidase. EPP is an autosomal dominant condition and occasionally autosomal recessive, caused by mutations in ferrochelatase (98).

**152.8.2.4 Schinzel–Giedion Syndrome.** Schinzel–Giedion (SGS; OMIM 269150) syndrome is a condition caused by dominant mutations in the *SETBP1* gene. Affected individuals present with severe mental retardation, hypertrichosis, midface retraction, macroglossia, low-set ears, skeletal abnormalities, genitourinary and renal malformations, and cardiac defects (99).

**152.8.2.5 Berardinelli–Seip Syndrome.** Berardinelli–Seip syndrome (OMIM 608594), also known as congenital generalized lipodystrophy, is a rare autosomal recessive metabolic disease characterized by a near absence of adipose tissue from birth or early infancy and severe insulin resistance in association with acanthosis nigricans, hypertrichosis, muscular hypertrophy, hepatomegaly, altered glucose tolerance or diabetes mellitus, and hypertriglyceridemia. Berardinelli–Seip syndrome is caused by mutations in the *AGPAT2* gene, encoding 1-acylglycerol-3-phosphate O-acyltransferase-2 (100).

**152.8.2.6 Warburg Micro Syndrome.** Warburg micro syndrome (OMIM 600118) is an autosomal recessive syndrome characterized by facial hypertrichosis, microcephaly, microcornea, congenital cataract, optic atrophy, hypogenitalism, mental retardation, agenesis of the corpus callosum, hypotonia, and spastic palsy. Mutations have been identified in the *RAB3GAP* gene, encoding the catalytic subunit of the RAB3 GTPase-activating protein complex (101).

**152.8.2.7 Gorlin–Chaudhry–Moss Syndrome.** Gorlin–Chaudhry–Moss syndrome (OMIM 233500) is an autosomal recessive condition characterized by craniosynostosis, flat midface, microphthalmia, conductive hearing loss, hypertrichosis, coarse hair



FIGURE 152-14 X-linked hypertrichosis.



and low frontal hairline, and short metacarpals and phalanges (102).

**152.8.2.8 Ramon Syndrome.** Ramon syndrome (OMIM 266270) is characterized by maxillary fibrous dysplasia, gingival fibromatosis, epilepsy, neurodevelopmental delay, hypertrichosis, and growth retardation (103).

**152.8.2.9 Zimmermann–Laband Syndrome.** Zimmermann–Laband syndrome (ZLS; OMIM 135500) is an autosomal dominant inherited condition characterized by a coarse facial appearance, gingival fibromatosis, hypoplasia of the terminal phalanges and nails of hands and feet, hypertrichosis, hepatosplenomegaly, and mental retardation. The genetic basis of ZLS is unknown but has been reported in the setting of translocations including t(3;8) and t(3;17) (104).

**152.8.2.10 Cornelia de Lange Syndrome.** Cornelia de Lange syndrome (CDLS1; OMIM 122470) is an autosomal dominant multisystem malformation syndrome characterized by microcephaly, long philtrum, facial dysmorphism, bushy eyebrows with synophrys, anteverted nares, maxillary prognathism, thin lips, upper limb defects, and hypertrichosis, in association with prenatal and postnatal growth and mental retardation. Mutations have been found in around 50% of individuals and they occur in the *NIPBL* gene, which encodes a component of the cohesin complex (105).

**152.8.2.11 Leprechaunism.** Leprechaunism (OMIM 246200), also known as Donohue syndrome, is an autosomal recessive condition characterized by elfin facial appearance; absence of subcutaneous tissue; low-set ears; large hands, feet, and genitalia; decreased muscle mass; acanthosis nigricans; hypertrichosis and pachyderma; and hypoglycemia, with most patients dying within the first year of life. Mutations have been reported in the insulin receptor gene (106).

**152.8.2.12 Cervical Hypertrichosis with Kyphoscoliosis.** Cervical hypertrichosis with kyphoscoliosis (OMIM 117850) is an autosomal dominant condition characterized by excessive hair growth localized to the cervical area, associated with underlying kyphoscoliosis.

Hypertrichosis may also occur at other places along the spine, including the thoracolumbar and sacral regions (107).

**152.8.2.13 Michelin Tire Baby Syndrome.** Michelin tire baby syndrome (OMIM 156100) is an autosomal dominant condition characterized by multiple circumferential deep skin folds on the extremities and gyrus-like on the back, in association with craniofacial anomalies, seizures, mental retardation, esotropia, hemiplegia, congenital heart defect, nevus lipomatosus, and smooth muscle hamartoma, with or without overlying hypertrichosis (108).

**152.8.2.14 Coffin–Siris Syndrome.** Coffin–Siris syndrome (OMIM 135900) is an autosomal recessive condition also known as fifth digit syndrome. Affected individuals show developmental delay, coarse facial features, hypertrichosis, and hypoplastic or absent fifth distal phalanges (109).

## 152.9 GENETICS OF POLYGENIC DISEASES

### 152.9.1 Alopecia Areata

AA (OMIM 104000) is the second most common cause of hair loss in humans, with a lifetime risk of 1–2%. AA affects about 5.3 million people in the United States alone and shows a broad range of phenotypes from a limited (patchy) non-scarring hair loss on the scalp only to complete hair loss on the whole body, known as alopecia universalis (Figure 152-15). There is no permanent organ destruction, and regrowth of the hair remains possible. Histologically, all types of AA are characterized by the presence of a diffuse lymphocytic infiltrate around the HF bulb sparing the bulge, the site where HF stem cells reside. AA is a tissue-specific autoimmune disease, but its molecular mechanism remains largely unknown. Under normal circumstances, the HF is an immune-privileged organ that expresses low levels of major histocompatibility complex (MHC) proteins (110). The genetic basis



**FIGURE 152-15** AA. Left: Limited (patchy) non-scarring hair loss on the scalp. Right: Complete hair loss on the whole body (alopecia universalis).





**FIGURE 152-16** Androgenetic alopecia.

of AA is largely unknown. We recently performed a genome-wide association study (GWAS) on patients with AA and identified several loci that are implicated in the pathogenesis of AA (111). A region of strong association resided in a region of linkage disequilibrium containing genes, the ULBP gene cluster, that encode activating ligands of the natural killer cell receptor NKG2D. We discovered that these ligands are expressed in AA lesional scalp and are markedly upregulated in the HF dermal sheath during active disease. Our findings place AA within the context of shared pathways among autoimmune diseases and implicate a novel disease mechanism, the upregulation of NKG2D ligands, in triggering autoimmunity. Other loci we identified include: *CTLA4*, *IL2/IL21*, *IL2RA* (CD25), *Eos* (*IKZF4*), *ERBB3*, syntaxin 17 (*STX17*) and peroxiredoxin 5 (*PRDX5*). Interestingly, many of these genes have been implicated to play some role in immune regulation.

### 152.9.2 Androgenetic Alopecia

AGA (OMIM 109200) is characterized by shortened anagen phase and HF miniaturization, and is the most common form of hair loss, affecting approximately 40% of men and women (Figure 152-16). AGA is also known as male-pattern baldness in men and female-pattern hair loss in women. Although genetic variations in or close to the androgen receptor have been linked to AGA (112), until recently not much else was known about this common condition. It was recently shown that the single nucleotide polymorphism (SNP) rs1160312 is associated with AGA. This variant lies on chromosome 20 between the *PAX1* and *FOXA2* genes (113). It is still yet unclear whether this variant itself or some other variant nearby is affecting the expression of either gene leading to AGA. With the advance of genetic techniques, such as whole-genome sequencing, it will be possible to pick up many rare variants that were missed with other techniques, and such variants may help us better understand common conditions such as AGA.

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### Biographies



**Dr Kurban** is Assistant Professor of Dermatology, Biochemistry, and Molecular Genetics at the American University of Beirut, Lebanon (as from July 2011). He achieved his MD in 2004, qualified as a dermatologist in 2008, after which he worked with Dr Christiano for 3 years; in parallel he has gained another American-board-accredited fellowship in clinical molecular genetics. He has more than 25 peer-reviewed articles.



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# CHAPTER

# 153

## Marfan Syndrome and Related Disorders

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### 153.1 INTRODUCTION

Several hundred distinct heritable disorders of connective tissue (HDCT) have been described phenotypically. Each is presumed to be due to a mutation in a single gene because of the inheritance pattern in families, or evidence that a component of the extracellular matrix (ECM) is abnormal. Mutations could occur in a structural gene for a macromolecule, a gene specifying one of the many post-translational modifications that components of the ECM undergo, a gene encoding a growth factor or a growth factor receptor, and so forth. The upper bounds of the number of HDCT and the number of constituents of the ECM are unknown but are clearly not identical; numerous clinically distinct disorders are due to different mutations in the same gene. Some of these conditions are discussed in this chapter and others are reviewed elsewhere.

For much of the twentieth century, knowledge of the ECM evolved at a seemingly leisurely pace that belied the amount of effort expended by anatomists and biochemists. Application of the techniques of molecular genetics markedly hastened the pace of discovery. Not so long ago, the ECM was parsed simply into fibrous components and ground substance (essentially everything that was not fibrous). Classification schemes have evolved toward structure or function, but remain unsatisfactory.

Elastic and collagen fibers are common to both approaches. Proteoglycans comprise much of what was previously called ground substance. Many of the components are glycoproteins, a term that encompasses all of the collagens, the fibrillins, the nectins or integrins, and much more. In recent years, the most extraordinary realizations have been how intimately and precisely these macromolecules interact, as well as how much variation there is both in interactions among components and among the ECM and cells in different tissues and

at different stages of development. This chapter reviews the composition, structure, function, and pathobiology of one class of components of the ECM, the extracellular microfibrils.

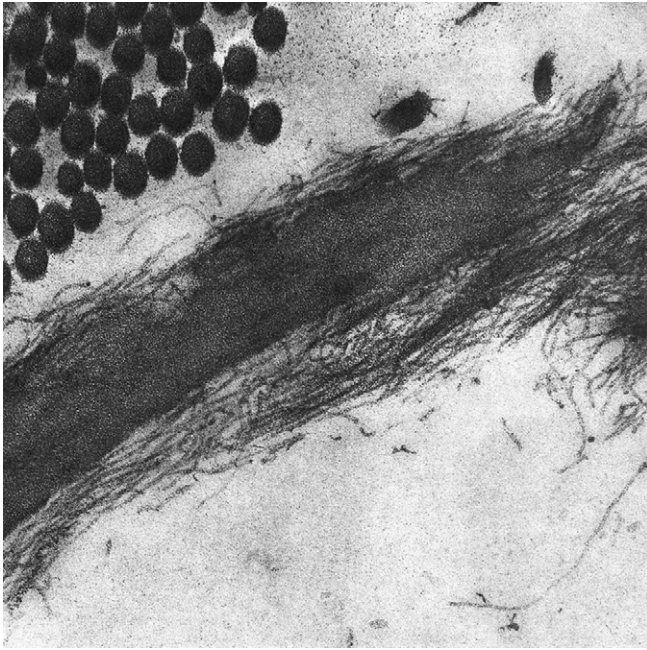
The classification scheme is completely arbitrary. As more is learned about individual components of the microfibril, it is becoming clear that microfibrils are not simply structural moieties. Some mutations in their major component, fibrillin, seem to have a much greater effect on developmental regulation than on structure.

### 153.2 STRUCTURE AND COMPOSITION OF EXTRACELLULAR MICROFIBRILS

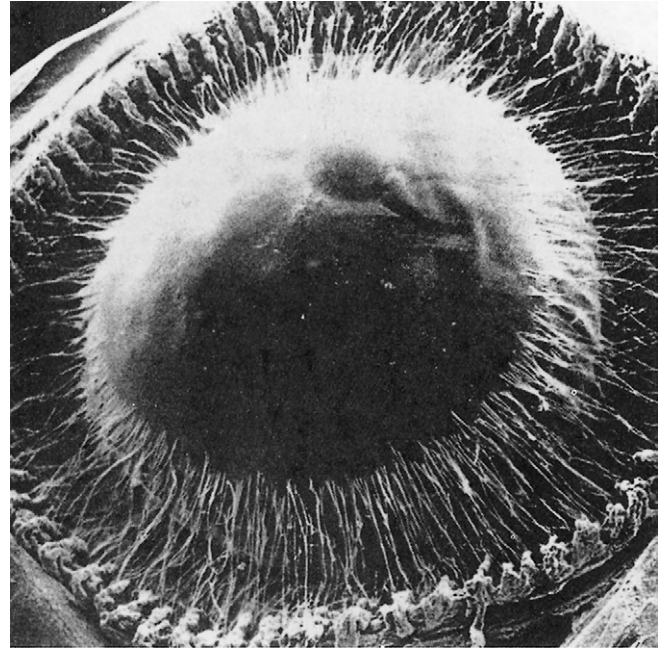
Because of their size and characteristic periodicity, the major collagen molecules were first emphasized in ultrastructural studies of the ECM. Elastic fibers also garnered early attention because of their apparent lack of periodicity and peculiar histological staining properties. However, noncollagen linear aggregates have been recognized in electron micrographs of ECM from diverse tissues for nearly four decades (1). A long structure of 10–14 nm in diameter was consistently found to be associated with elastic fibers from vasculature, lung, and skin (2,3) and was called the microfibril (4) (Figure 153-1).

Microfibrils appeared early in tissues undergoing elastogenesis and seemed to be the scaffolding on which tropoelastin is deposited. Subsequently, structures of similar appearance were found in nonelastic tissues, including the superficial dermis (5) and the ocular zonule (6,7) (Figure 153-2). Were these structurally similar but functionally distinct components of ECMs the same?

The answer began to emerge from the studies conducted by Gibson, Cleary, and colleagues in Adelaide, Australia, and by Sakai and colleagues in Portland,



**FIGURE 153-1** Elastic fiber in longitudinal section. In the upper left are collagen fibers in cross section. The amorphous core of the elastic fiber consists of cross-linked elastin. The microfibrils surround this core. (Courtesy of Douglas Keene, PhD.)



**FIGURE 153-2** Scanning electron micrograph in which the cornea and iris have been removed. The zonules emanating anteriorly and posteriorly from the ciliary bodies are clearly seen. The anterior insertions of the zonules on the lens are also shown to good effect. (x25.) (From Streeten, B. W. (6).)

Oregon. Both groups extracted proteins from ligament and other elastic tissues, using methods designed to eliminate collagen and elastin. They then separated the remaining proteins by gel electrophoresis and prepared antibodies to these potential components of microfibril. Immunohistology of various tissues showed that the 10–14-nm microfibrils<sup>1</sup> from the ECM of all tissues bore compositional as well as morphological similarities (8,9).

Subsequently, numerous protein components associated with the microfibril have been characterized (Table 153-1). Some, such as amyloid P and fibronection, are clearly not integral components of the structure. Part of the difficulty in assigning components is that microfibrils differ depending on the tissue source and stage of development. The fibrillins are the only confirmed components of all microfibrils. Monomers of fibrillin associate head-to-tail to form the basic structure of the microfibril. The latent transforming growth factor- $\beta$  (TGF- $\beta$ )-binding proteins are likely to be integral components of some but not all microfibrils. Microfibril-associated glycoprotein-1 seems to be associated with all microfibrils (Lynn Sakai, personal communication).

<sup>1</sup>The diameter of the microfibril depends on the method of fixation and examination, hence the 10–14-nm designation. Many investigators have settled on 12 nm as the characteristic diameter. These structures are also called elastin-associated or elastic fiber-associated microfibrils, but this ignores their important anchoring function independent of the elastic fiber. In this chapter, the term microfibril refers to this 12-nm microfibril.

### 153.3 FIBRILLINS

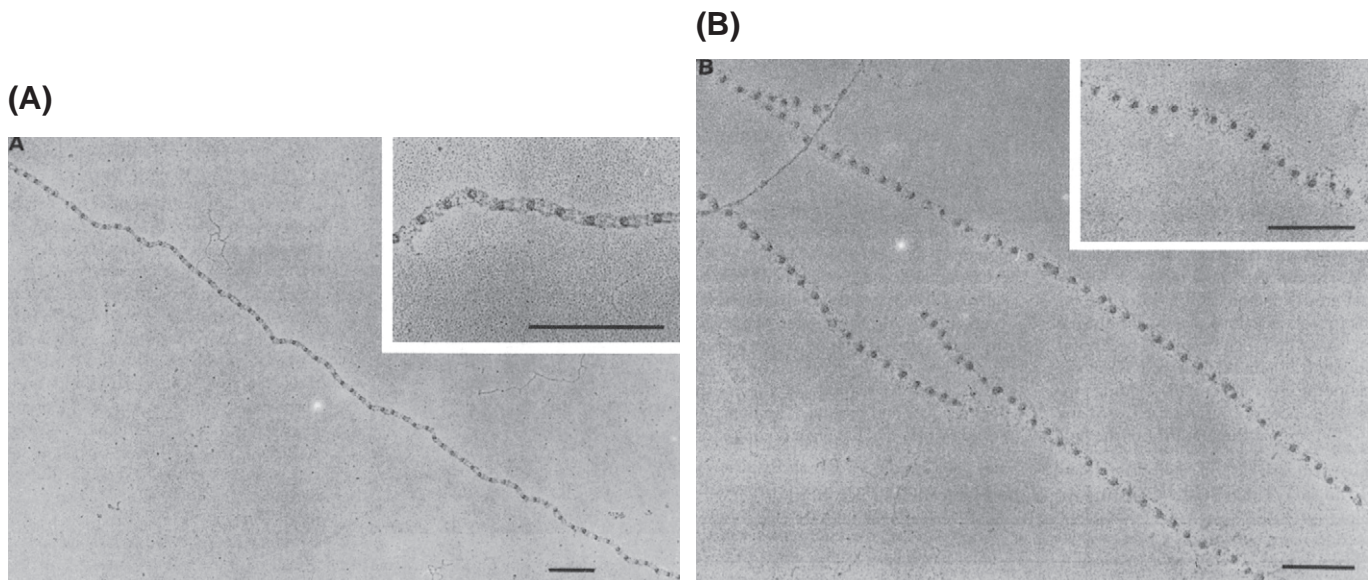
Sakai and colleagues (9) isolated a glycoprotein of about 350 kDa and called it fibrillin. Monoclonal antibodies to fibrillin bound to 12-nm microfibrils wherever they occurred in the ECM and did not associate with any other macromolecular structure. Fibrillin has emerged as the principal component of microfibrils from all tissues studied (10). Purified monomers of fibrillin, when allowed to aggregate, attain the beads-on-a-string appearance characteristic of microfibrils (Figure 153-3) (11–13). The biology and biochemistry of fibrillin have been difficult challenges because of the size of the monomer and its high content of cysteine residues and consequent intra- and interchain disulfide bonds. The monomer is synthesized by many cells of the ECM, including fibroblasts, smooth muscle cells, osteoblasts, and perichondrocytes. The initial protein has a leader sequence and a propeptide, which are cleaved before microfibrillar assembly occurs outside the cell. The molecule binds calcium, and removal of calcium by chelation alters the morphology of the interbead domain of purified microfibrils, an effect that is reversible (14,15). Fibrillins are conserved through evolution and are found in primitive organisms. Expression of fibrillins begins early in embryogenesis (16–19).

A gene for fibrillin was partially cloned by independent groups led by Sakai and Ramirez in 1991 (20). The messenger RNA (mRNA) was about 10 kb in length, consistent with a protein of 350 kDa. Both groups mapped a fibrillin gene (*FBN1*) to human chromosome 15q21. Lee



**TABLE 153-1** Constituents of the Extracellular Microfibril

Protein	Gene	Map Locus
Structural components confirmed for all microfibrils		
Fibrillin-1	<i>FBN1</i>	15q21.1
Fibrillin-2	<i>FBN2</i>	5q23.q31
Structural components of some microfibrils		
Latent transforming growth factor- $\beta$ -binding protein 1	<i>LTBP1</i>	2p12–q22
Proteins likely associated with some microfibrils		
Latent transforming growth factor- $\beta$ -binding protein 2	<i>LTBP2</i>	14q24
Fibulin-2	<i>FBLN2</i>	3p25–p24
Microfibril-associated protein-2	<i>MFAP2</i>	1p36.1–p35 (formerly designated MAGP1)
Proteins possibly associated		
Microfibril-associated	<i>MAGP2</i>	12p13.1–p12.3 glycoprotein-2
Microfibril-associated protein-3	<i>MFAP3</i>	5q32–q33.3
Lysyl oxidase (found in elastic fibers but not isolated microfibrils)	<i>LOX</i>	5q23.3–q31.2
Microfibril-associated protein-1	<i>MFAP1</i>	15q15–q12
Latent transforming growth factor- $\beta$ -binding protein 3	<i>LTBP3</i>	11q12
Latent transforming growth factor- $\beta$ -binding protein 4	<i>LTBP4</i>	19q13.1–q13.2



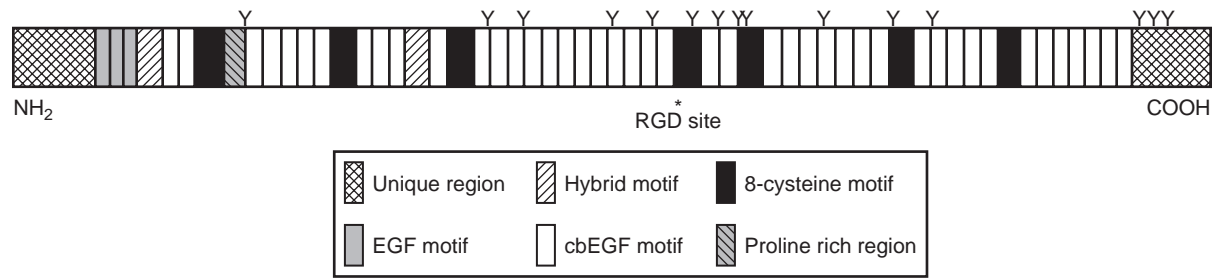
**FIGURE 153-3** Transmission electron microscopy of microfibrils purified from dermal fibroblasts and rotary shadowed. (A) Normal control. (B) Cells from a patient with Marfan syndrome. Note the differences, which are especially striking in the interbead regions. (Courtesy of Cay Kielty, PhD.)

and coworkers (20) also found a second locus for fibrillin (*FBN2*) that mapped to human chromosome 5q23–q31. Corson and colleagues (21) recently identified a third fibrillin locus on chromosome 19p13.3–p13.2. Fibrillin-2 (OMIM 121050) and fibrillin-3 (OMIM 628529) are highly homologous to fibrillin-1 (OMIM \*134797) and co-localize to all microfibrils, with a preference for elastin-associated microfibrils (18).

The entire genomic organization of *FBN1* has been elucidated (22). Its 65 exons are spread over 200kb of chromosome. The open reading frame contains 8,616 nucleotides surrounded by 134-nucleotide 5′- and 916-nucleotide 3′-untranslated regions. The regulatory sequences have not yet been defined in detail. The amino acids of fibrillin-1 are highly conserved among mammals.

The three fibrillins have distinct regions and repeating sequences consistent with the mosaic organization of most structural proteins of the ECM (Figure 153-4). The most extensive region consists of two types of motifs. One comprises 47 repeats, each containing six cysteine residues and most of which are encoded by a single exon (numbers 11–63). The motif of these repeats is highly homologous to epidermal growth factor (EGF); 43 of the repeats contain a consensus sequence for calcium binding, a known function of one class of EGF-like repeats. The second most prevalent motif contains eight cysteine residues and is homologous to a motif in transforming growth factor- $\beta_1$ -binding protein (TGF $\beta_1$ -BP). These sequences are interspersed with the EGF-like motifs along the C-terminal three-fourths of the protein. A





**FIGURE 153-4** Structural and functional motifs of fibrillin-1.

region of high proline content is an unusual motif, as are a number of repeats that diverge slightly from the consensus EGF-like and TGF $\beta$ <sub>1</sub>-BP-like motifs. Additionally, 11 potential sites for glycosylation and one for cell attachment (RGD sequence) occur.

Fibrillin-2 and -3 share this same organizational mosaic. The fibrillins are thus similar to other ECM proteins in certain respects. The EGF-like motifs, in particular, are found in laminin, perlecan, the fibulins, nidogen, and tenascin; the known characteristics of these molecules shed light on the structure of fibrillin. The six cysteine residues form three specific disulfide bonds, creating a stable conformation of three antiparallel  $\beta$ -sheets. Serial repetitions of these units results in a rodlike conformation (23). In other molecules, calcium binding is important for stabilizing this motif and for enabling protein-protein interactions, and evidence suggests this to be the case for fibrillin (15,23–25). Furthermore, evolutionary conservation of many of the noncysteine residues and the occurrence of a preponderance of mutations discovered thus far in the EGF-like repeats lend additional weight to their functional importance [(26); *vide infra*]. There is also accumulating evidence that fibrillins promote cell-matrix interactions (27,28), in part through the TGF $\beta$ <sub>1</sub>-BP motif (29).

In mammals, fibrillin-2 and fibrillin-3 tend to be expressed earlier than fibrillin-1, especially at sites of elastogenesis. The exception is the cardiovascular system, in which fibrillin-1 is deposited first (19). Fibrillin-1 is also expressed preferentially at sites where elastogenesis is minimal or absent. However, fibrillin-1 and -2 are widely expressed in mouse embryos (30,31). Both fibrillins have N- and C-propeptides that require cleavage (32), are extensively modified post-translationally, and participate in complex series of polymerizations and cross-linkings to form the microfibril (33,34). Fibrillin-3 has been inactivated in the mouse (21), which needs to be considered in interpreting the various mouse models of Marfan syndrome (MFS) (*vide infra*).

### 153.3.1 Microfibril-Associated Glycoprotein

Gibson and Cleary purified a number of peptides from microfibrils; some probably represent cleavage fragments of fibrillin and other constituents, but several are clearly

intrinsic components. One, termed microfibril-associated glycoprotein (MAGP; OMIM \*156790), has a predicted molecular weight of 31 kDa based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, sequencing the bovine complementary DNA (cDNA) for MAGP showed that the actual amino acid sequence predicts a peptide of only 21 kDa. The C-terminal region is rich in cysteine and basic residues, whereas the N-terminus contains acid residues and proline. The human cDNA for MAGP, encoded by *MFAP2*, has eight coding exons and two alternatively spliced 5'-untranslated exons. *MFAP2* maps to the region 1p36.1–p35.

### 153.3.2 Microfibril-Associated Protein-1

The size of microfibril-associated protein-1 (MFAP1; OMIM 600215), when partially purified from microfibrils, was 56 kDa. Subsequently, the cDNA was cloned, and the amino acid sequence predicted a protein of 54 kDa. The 2.1-kb mRNA is expressed widely in chick embryo tissues, especially those producing elastic fibers. The human counterpart maps to 15q12–q21, intriguingly close to *FBN1*.

### 153.3.3 Microfibril-Associated Protein-3

This protein has no homologies with known proteins. Monoclonal antibodies against the recombinant protein react with ocular zonules (35). The map location in the human for this simple gene (only two translated exons) is 5q32–q33.2, close to but clearly distinct from *FBN2*.

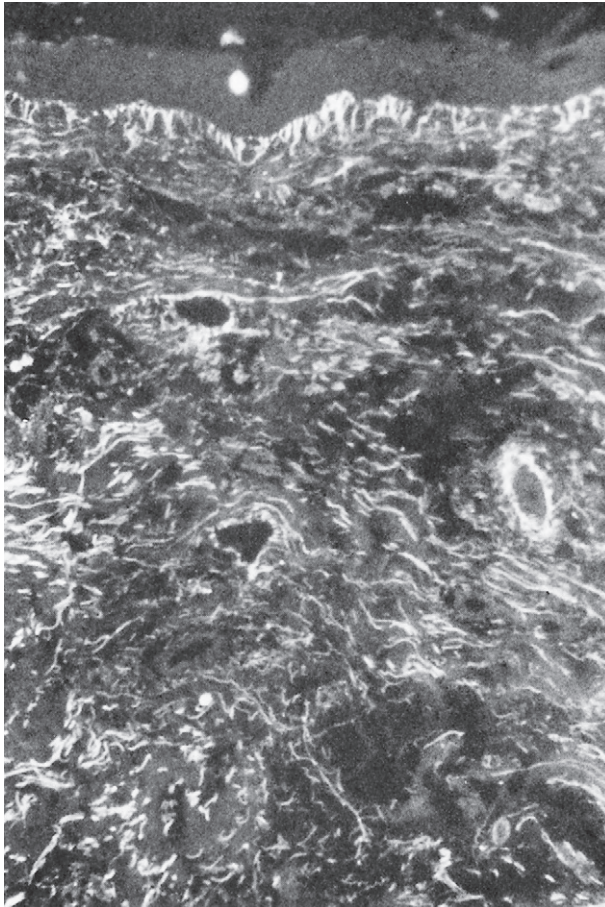
### 153.3.4 Fibulin-2

Fibulin-2 immunolocalizes to microfibrils. Whether it is an integral structural component or only associated remains unclear. If the latter, the association seems to have some specificity; fibulin-2 binds to particular cleavage fragments of fibrillin-1 (36). The gene for fibulin-2, *FBLN2*, maps to 3p24.2–p25 in the human.

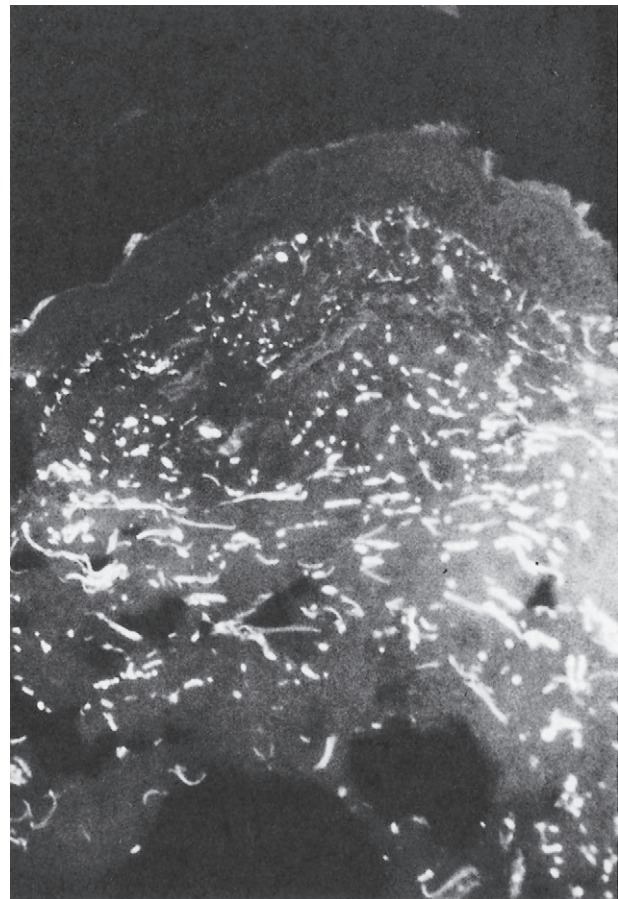
### 153.3.5 Other Components of the Microfibril

Lysyl oxidase (OMIM \*153455), a copper-requiring enzyme crucial to interchain bonding of elastin, is one of

(A)



(B)



**FIGURE 153-5** Immunofluorescent histopathology of the dermal-epidermal junction (top of each figure) and dermis (x100). (A) Biopsy of the posterior upper arm from a 35-year-old woman with no evident connective tissue disorder. (B) Biopsy of the posterior upper arm from a 33-year-old woman with classic Marfan syndrome. The section was incubated with a mouse monoclonal antibody to fibrillin-1 (gift of D. Lynn Sakai) and washed, and the bound antibody visualized with goat antimouse IgG conjugated with FITC. (Sections prepared by Grant Anhalt, MD.) SD, standard deviations; US/LS, upper segment-to-lower segment ratio.

the amorphous components of the elastic fiber and probably has a specific association with microfibrils. Emilin (OMIM \*130660), a 115-kDa glycoprotein, may have a role in the interaction of elastin and the microfibrils. It was originally called gp115. In addition, peptides of apparent molecular weights 25 and 78 kDa have been isolated from microfibrils but remain poorly characterized.

#### 153.4 FUNCTIONS OF MICROFIBRILS

Microfibrils have at least four major functions:

- (1) Microfibrils are formed in sites of elastogenesis (37). However, the mechanisms of elastogenesis are far from clear; for instance, mice that synthesize no fibrillin-1 still form elastic fibers of relatively normal appearance (38), and elastin appears to be deposited in some sites lacking any evidence of microfibrils (LY Sakai, personal communication).
- (2) Because of their location in mature elastic fibers exterior to the amorphous elastin component, microfibrils

also likely play a role in linking elastic fibers to each other, to other components of the ECM, and to cells.

- (3) Microfibrils unassociated with elastin perform anchoring functions of several types. They are the principal component of the ocular zonule and thus hold in place a relatively large structure, the lens (39) (see Figure 153-2). In addition, microfibrils are the major constituent of the so-called oxytalan fibers, which extend perpendicularly from the dermal-epidermal junction into the papillary dermis (Figure 153-5). Along with type VII collagen and other macromolecules, they most likely have a role in stabilizing that important interface.
- (4) Finally, microfibrils bind the latent TGF- $\beta$  complex, serving as a sump to regulate the level of active TGF- $\beta$  at any stage of development and postnatal life.

A variety of other functions are possible. The roles of microfibrils in wound healing, fibrosis and neoplasia have not been explored.

## 153.5 MARFAN SYNDROME (OMIM \*154700): THE PROTOTYPE OF DISORDERS OF MICROFIBRILLOGENESIS AND THE FIBRILLINS

### 153.5.1 Historic Perspective

In 1896, the French pediatrician A. B-J. Marfan described a nearly 6-year-old girl with long, thin fingers and limbs, which he termed *dolichostenomelia* (40). This girl also had multiple joint contractures and developed scoliosis. Several years later, Achard (41) described a patient who had loose-jointedness of the hands, as well as hypognathism and *dolichostenomelia*; this condition was called *arachnodactyly*. In retrospect, neither of these patients may have been affected by what now is called Marfan syndrome (42). Over the next 40 years, other features of the syndrome were coupled with the skeleton. In 1914, subluxation of the ocular lenses was associated with the *dolichostenomelic habitus* (43), although two tall, loose-jointed sibs with *ectopia lentis* had been described many years before (44). The heritable nature of the condition and primary involvement of tissue derived from embryonic mesoderm were first noted by Weve (45), who also associated Marfan's name with the phenotype for the first time, calling the syndrome *dystrophia mesodermalis congenita, typus Marfanis*. The aortic complications of dissection and dilatation were clearly associated with the skeletal findings by Baer and colleagues (46) and by Etter and Glover (47), although reports of congenital heart disease and *arachnodactyly* had appeared previously (48,49). McKusick (50) drew wider attention to the spectrum of the cardiovascular problems encountered in living patients and postmortem specimens. Of more fundamental importance was his labeling of MFS as a heritable disorder of connective tissue, one of the first of a long line of conditions to be so designated. Beals and Hecht (51) described the congenital contractural *arachnodactyly* syndrome and proposed that it was the condition that had affected Marfan's original patient.

Debate continues over whether Abraham Lincoln, who was tall, *dolichostenomelic*, and loose-jointed, was affected by MFS (52–55). The violinist Paganini was unquestionably asthenic and loose-jointed, features he used to enhance his showmanship and virtuosity; whether he had MFS is also uncertain (56). In recent years, several young athletes have died from aortic complications of MFS, some of which were diagnosed, unfortunately, only in retrospect. The death in 1986 of US Olympic Volleyball star Flo Hyman increased awareness of the condition among the general public, athletic coaches and trainers and physicians throughout the world.

Since publication of the 5th edition of this text, considerable progress has been achieved in understanding the etiology, pathogenesis, clinical history, and management of MFS. This chapter and the references herein emphasize the developments of the past few years.

### 153.5.2 Prevalence

Based on crude calculations of the size of the catchment area and the number of Marfan patients in the files of Johns Hopkins Hospital, the prevalence was estimated as 4–6 per 100,000. Since the manifestations of MFS may extend from the limits of normal to the floridly 'classic', in which the diagnosis is unquestionable, the actual prevalence of MFS clearly exceeds this estimate.

MFS occurs in all races and in all major ethnic groups residing in the United States. Relative prevalences in ethnic groups elsewhere are unknown, but cases of MFS have been reported from around the world.

### 153.5.3 Marfan Phenotype and Natural History

MFS can usually be defined on the basis of clinical features and mode of inheritance, despite discovery of the fundamental defect (57). Increasingly, clinicians resort to searching for mutations in the gene encoding fibrillin-1 (*FBN1*). While this can be useful, especially in families in which the specific mutation has been defined, or in which linkage of the classic phenotype to the fibrillin-1 locus (*FBN1*) on chromosome 15 can be confirmed, molecular analysis can also complicate the diagnosis, as will be discussed subsequently. Although many systems are affected in MFS, the cardinal features defining the classic phenotype appear in four systems; the skeletal, the ocular, the cardiovascular and the central nervous system (58). Table 153-2 summarizes the most prevalent manifestations observed in 50 consecutive patients, published before the dura was known to be affected.

In 1972, the records of 257 Marfan patients at Johns Hopkins Hospital were examined for life expectancy and causes of death (59). The study was performed when medical and surgical therapy had virtually no beneficial impact on patient survival. Survival had fallen to 50% for men at age 40 years and for women at age 48 years, a reduction in expected life span of about 30–40% for both sexes. The mean age of death of the 72 deceased patients was 32 years. The immediate cause of death in more than 90% of cases was a cardiovascular complication. Dissection or rupture of the aorta and chronic aortic regurgitation with congestive heart failure accounted for the vast majority of deaths.

**153.5.3.1 Skeletal Manifestations.** Mean height in MFS is greater than that of either unaffected sibs or the population average for similar sex, age, ethnicity and cultural background (Figure 153-6). At birth, affected children tend to be longer than normal, a discrepancy that persists, although the growth rate is no greater than that of their unaffected peers. Both puberty and the pubertal growth spurt occur about 2 years earlier in MFS (60).

The limbs are disproportionately long compared with the trunk (*dolichostenomelia*). The increased length of the limbs may be estimated if the lower segment length



**TABLE 153-2** Features of Marfan Syndrome in 50 Consecutive Clinic Patients

	No. of Patients Demonstrating Clinical Feature
<i>Ocular</i>	35/50
Ectopia lentis	30/50
Myopia	17/50
<i>Cardiovascular</i>	49/50
Mid-systolic click only	15/50
Mid-systolic click and late systolic murmur	9/50
Aortic regurgitant murmur	5/50
Mitral regurgitant murmur only	3/50
Abnormal echocardiogram	48/50
Aortic enlargement	42/50
Mitral valve prolapse	29/50
Prosthetic aortic valve	5/50
<i>Musculoskeletal</i>	50/50
Arachnodactyly	44/50
US/LS 2 SD below mean for age	36/47
Pectus deformity	34/50
High, narrow palate	30/50
Height >95th percentile for age	29/50
Hyperextensible joints	28/50
Vertebral column deformity	22/50
Pes planus	22/50
<i>Family history</i>	40/47
Additional documented cases of syndrome	40/47
Sporadic cases (presumed new mutations)	7/47
Unclear or unknown pedigree	3/50

SD, standard deviation; US/LS, upper segment-to-lower segment ratio. Adapted from Pyeritz, R. E & McKusick, V. A (386).

(top of the pubic ramus to floor) is divided into the upper segment length (height minus lower segment). This US/LS ratio varies with age during normal growth but, in the person affected by MFS, is usually at least two standard deviations below the mean for age, race, and sex (Figure 153-7). The US/LS ratio may be exaggerated by scoliosis or abnormal kyphosis. Similarly, the arm span-to-height ratio, which usually exceeds 1.05 in MFS, can be confounded by contractures of the elbows.

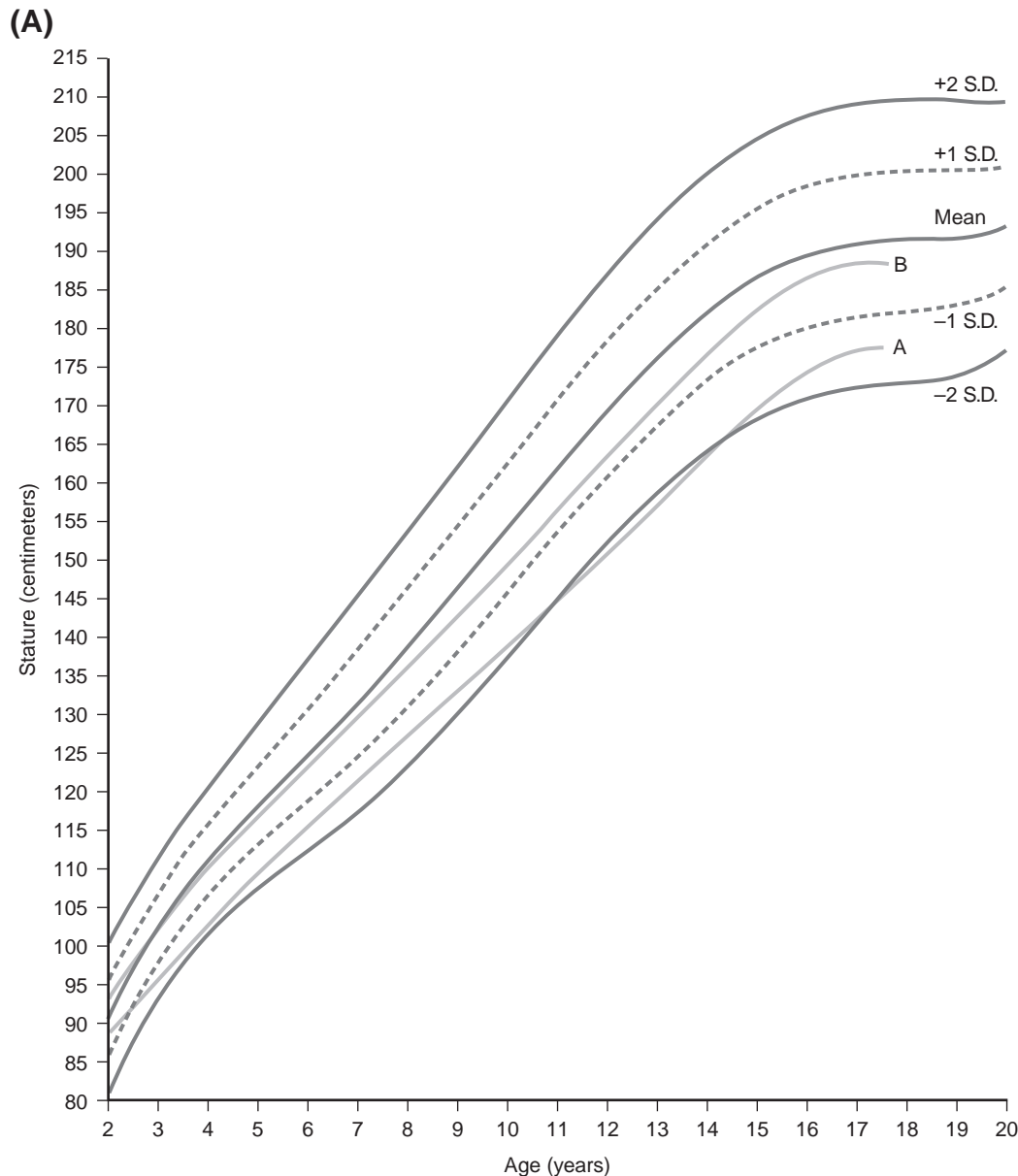
Arachnodactyly appears in numerous other syndromes and remains largely a subjective feature. Attempts to provide a radiographic criterion by means of the length-to-width ratio of hand bones (metacarpal index) have not demonstrated enough improvement in diagnostic power to justify the time, cost and radiation exposure (61). Simple maneuvers such as the thumb sign (positive if the thumb, when maximally opposed within the clenched hand, projects beyond the ulnar border) (Figure 153-8A) and the wrist sign (positive if the distal phalanges of the first and fifth digits of one hand overlap when wrapped around the opposite wrist) (Figure 153-8B) are helpful

when positive. However, these maneuvers are subject to observer interpretation and may reflect the longitudinal laxity of the hand rather than arachnodactyly.

Longitudinal overgrowth of the ribs produces anterior chest deformity, either depression (pectus excavatum or funnel chest) (Figure 153-9A) or protrusion (pectus carinatum or pigeon breast) (Figure 153-9B) of the sternum. In these cases, the chest is often asymmetrical, with one set of costochondral junctions (usually the left) protruding more than the contralateral set. The anterior chest deformity can change markedly during the course of growth of the ribs. A mild pectus excavatum in an infant can worsen in a matter of a few years, become asymmetrical, or convert to a carinatum defect. Joint laxity is frequently present but has little diagnostic specificity. The fingers, wrists, elbows, and knees (genu recurvatum) are commonly hyperextensible. Laxity of the carpal ligaments produces flat feet (pes planus with or without calcaneoplanovalgus) in at least one quarter of patients (62) (Figure 153-10). Some patients demonstrate limited extension or unequivocal congenital contractures, usually of the fingers or elbows, which may coexist with laxity of other joints. Joint laxity can lead to recurrent dislocation, most commonly of the first metacarpal-phalangeal joint and the patella. Laxity of the ankle and foot produces instability and various foot deformities, in addition to pes planus. While most patients have adequate foot function, if severe deformity is ignored at an early age, lifelong gait disturbances can result. Pes cavus may occasionally develop. Joint laxity of the fingers, elbows, and knees often lessens with age. In later life, degenerative arthritic changes are commonplace in joints that were once particularly lax. Complaints of joint discomfort are common in patients with MFS of all ages (63).

Scoliosis may occur at one or multiple sites along the vertebral column and generally worsens during periods of rapid growth, such as early adolescence (64). From a study of a relatively unbiased clinic population, the prevalence of severe scoliosis is about 10% (64), considerably less than was suggested in earlier studies. Scoliosis first noted in infancy tends to progress relentlessly (65). Mild degrees of curvature can best be appreciated clinically by observation of erect patients from behind as they bend forward at the hips with arms at full length and palms in contact; either the curve of the vertebral column will be more evident, or one shoulder will be higher than the other (Figure 153-11). A leg-length discrepancy of greater than 2cm occurs in about 5% of patients and correlates with severity of scoliosis (66,67). Thoracic scoliosis is usually obvious on the routine chest radiograph. Kyphosis of the thoracic or thoracolumbar region often accompanies scoliosis, but in many patients, even in the absence of scoliosis, a straightening of the usually mild thoracic kyphosis (straight-back syndrome) or even a thoracic lordosis occurs, resulting in a reduced anteroposterior diameter of the chest (Figure 153-12).





**FIGURE 153-6** Growth curves for stature in the Marfan syndrome. Plots of height versus age of males (A) and females (B) not treated with hormones. The curves are fifth-degree polynomial least-squares regressions from the mean  $\pm$  1 and 2 SD. The thin curves are for comparison to the general population's 50th (A) and 95th centile (B). (From Erkula, G., et al. (60).)

Cervical scoliosis and kyphosis can be associated with neck pain (68). Spondylolithesis in the lumbosacral region occurs in some young patients at a higher frequency than expected in the general population (64). Atlantoaxial rotatory instability has led to subluxation in a few cases (69).

Congenital dislocation of the hip is probably of increased frequency, but is not a common problem (70). An abnormally deep socket of the hip joint (protrusio acetabuli) occurs in about 30–50% of Marfan patients (71,72) (Figure 153-13), occasionally causing disability (64). As patients survive longer, degenerative arthritis of the hip, knees, and other joints will become more common complaints.

The occurrence of several of the above musculoskeletal features in the same individual should prompt consideration of MFS (73), although the sensitivities and specificities of various combinations of features have not been investigated.

The hard palate is often narrow and highly arched and causes tooth crowding. Retrognathia is common and contributes to dental malocclusion and a predisposition to obstructive apnea and sleep disturbance (74–77).

Studies of bone mineral density in MFS have come to conflicting conclusions. Observations before the early 1970s are unreliable because of potential confusion with homocystinuria, in which osteoporosis clearly occurs.

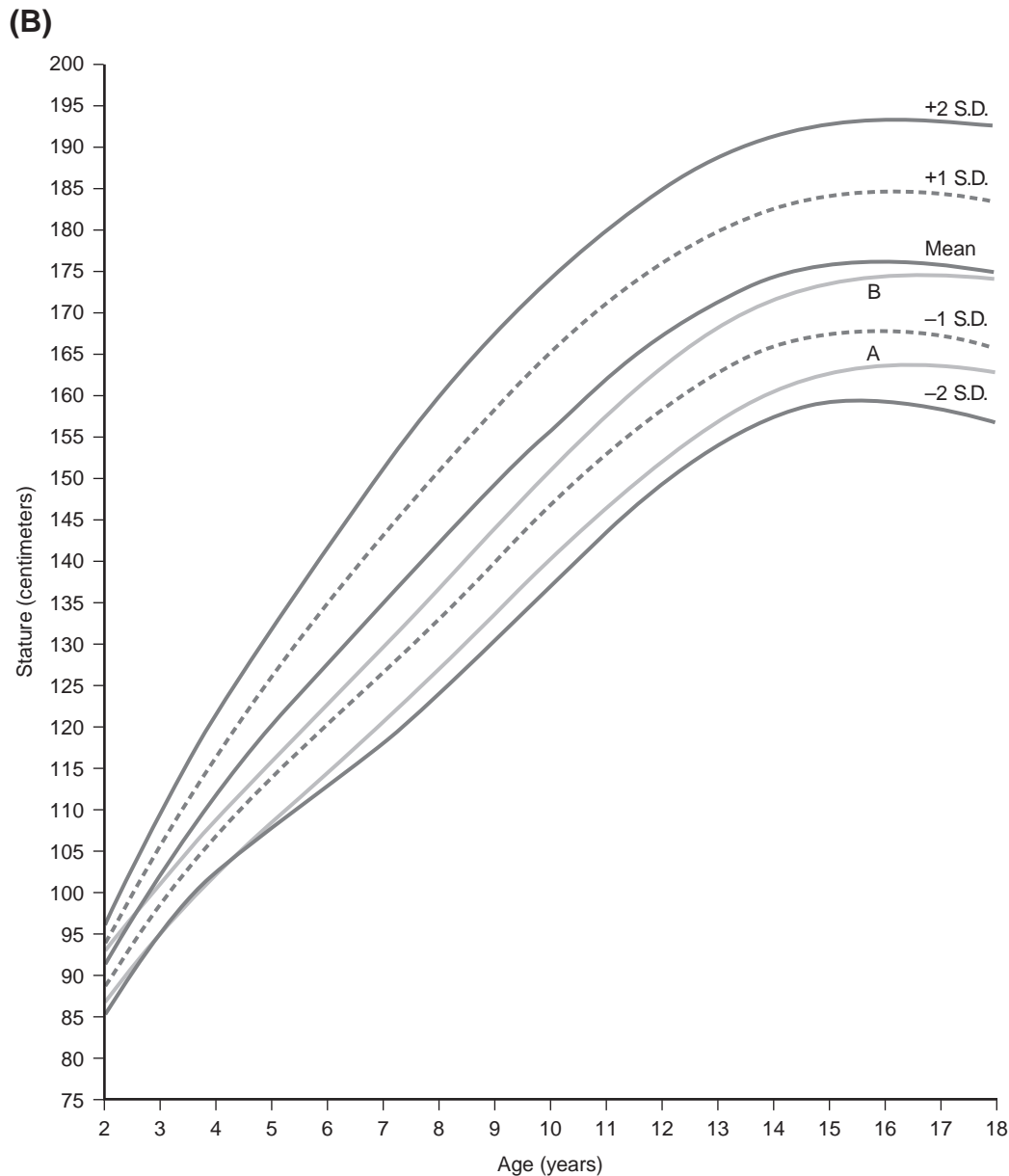


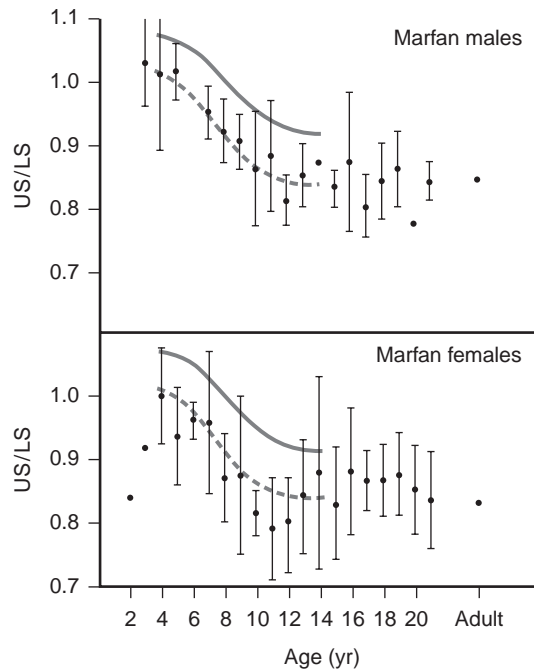
FIGURE 153-6—cont'd

One investigation used dual-energy X-ray absorptiometry of the lumbar spine, proximal femur and total body in 17 premenopausal women with MFS. The conclusion was that women with MFS have abnormally low total body and proximal femoral bone mineral density (78). In a study of only the distal forearm by single photon absorptiometry of 10 men and 4 women with MFS, the opposite conclusion was reached (79). However, two women in the latter study had abnormal results. A more recent study found osteopenia in adult males with MFS (78). In another study, dual energy X-ray densitometry showed no difference in bone density at the lumbar spine and the femoral neck between adult women with MFS (mean age 51 years) and controls matched for height and age (80). More recent studies have favored decreased bone mineral density as a pleiotropic feature of MFS (81–83).

As people with MFS live longer, the possibility of an increased risk of fracture should be explored, especially in women.

**153.5.3.2 Ocular Manifestations.** On inspection, patients with Marfan syndrome may have megalocornea and a miotic pupil. The eyes are often deeply set because of lack of retrobulbar fat. Strabismus occurs in 20%, one half of whom have exotropia (84). Studies of corneal shape (keratometry) show that most people with MFS have relatively flat corneas (Table 153-3). The anterior surface of the iris has a smooth, velvety appearance and transilluminates in about 10% of cases. Typically, the dilator muscle is poorly developed leading to a miotic, difficult-to-dilate pupil.

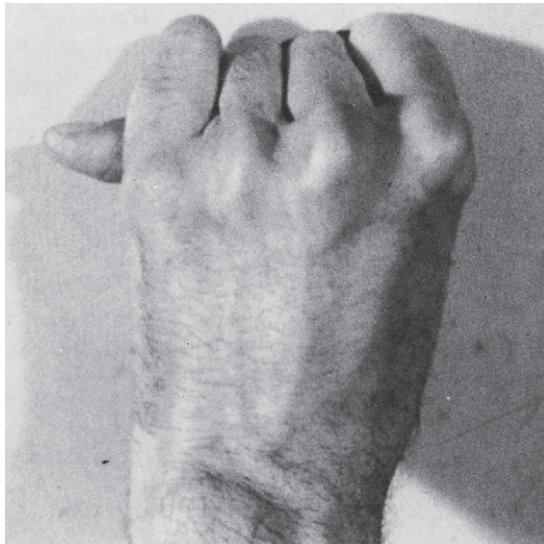
The diameter of the lens is often smaller than normal; a dislocated, microspherophakic lens occurs in 3%



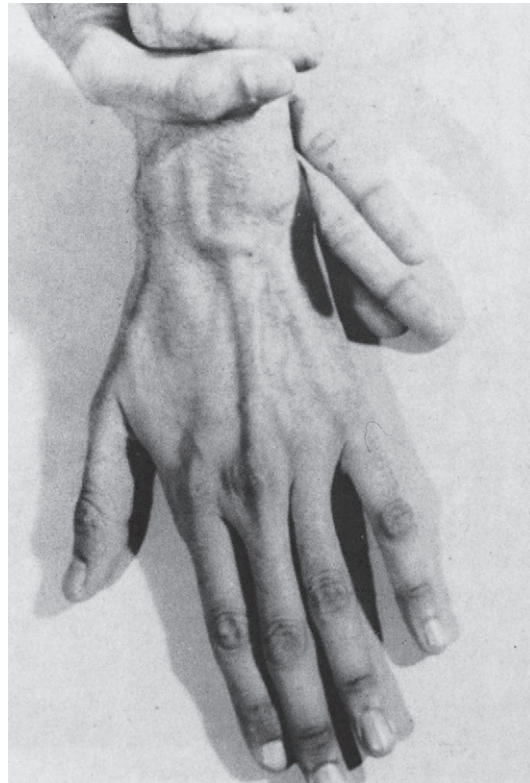
**FIGURE 153-7** Upper segment-to-lower segment ratios (US/LS) in Marfan syndrome. The US/LS falls with increasing age through early puberty. Points show the means for white patients grouped in 1-year intervals. Bars show  $\pm 1$  SD. The solid curve is the mean and the dashed curve 2 SD below the mean for unaffected whites. (Adapted from McKusick, V. A. (85).)

of patients and is associated with severe high myopia. Dislocation of the lens (ectopia lentis) is progressive and occurs in a proportion of cases variously estimated at 50–80%; it is usually bilateral, but may be asymmetrical. Ectopia lentis is often not evident at birth (85). The lens early in life is most commonly displaced superotemporally (Figure 153-14). Most of the zonules remain intact as visualized on slit lamp examination, and accommodation is possible. Subluxation tends to progress early in childhood (age 2–4 years) or early in adolescence (age 9–12 years) (I. H. Maumenee, unpublished observations). Posterior subluxation occurs and is easily missed. The lens in MFS will only rarely dislocate into the anterior chamber; anterior luxation is presumably prevented by the miotic pupil. Detection of minimal lens subluxation at the bedside is enhanced by performing direct ophthalmoscopy two to three feet in front of the subject. Subluxation is suggested by fluttering of the iris (iridodonesis), enhanced by rapid alternating right and left gaze. Any patient suspected of having MFS must undergo an annual slit-lamp examination with the pupils fully dilated. However, because of hypoplasia or aplasia of the dilator muscle, mydriasis may be difficult to achieve, making assessment of minor degrees of lens dislocation difficult. A predisposition to cataracts is becoming more evident as people live longer, with development in the fifth decade common (I. H. Maumenee, unpublished observations).

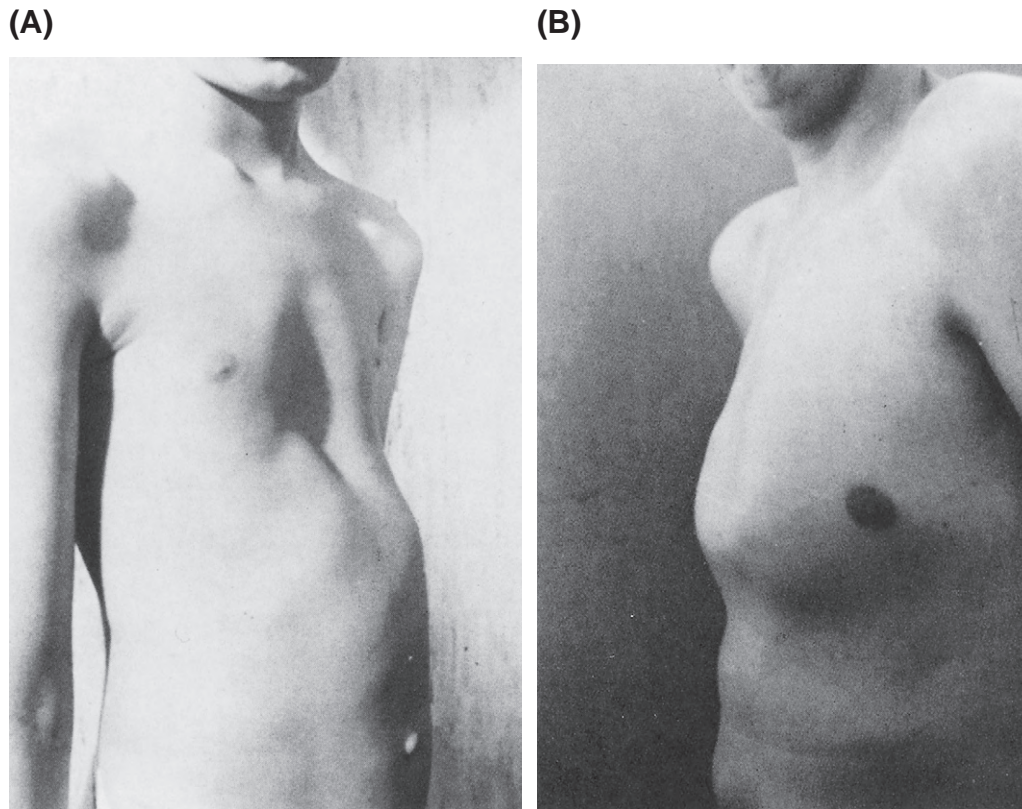
(A)



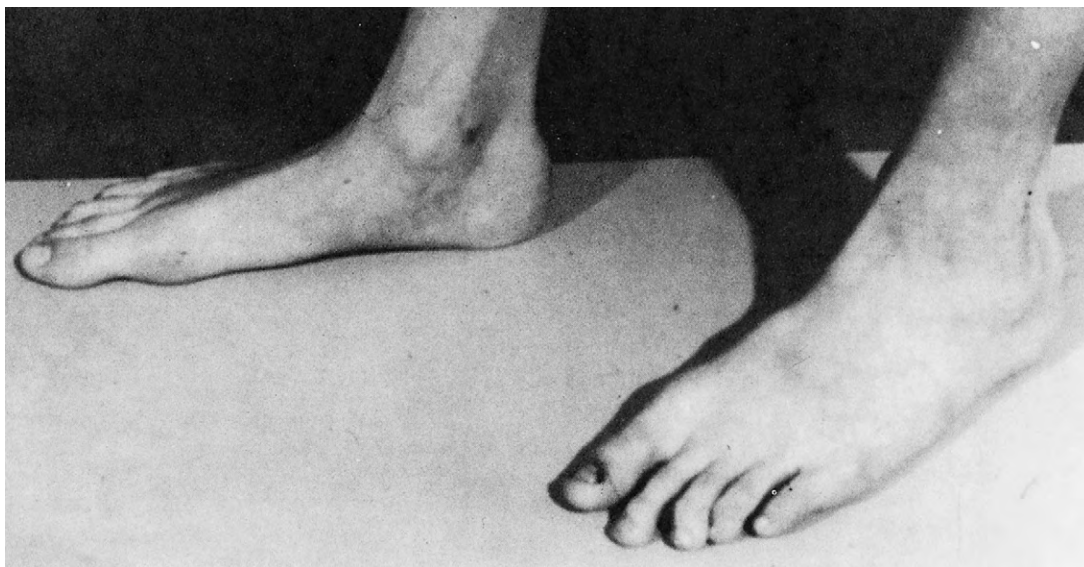
(B)



**FIGURE 153-8** Positive thumb (A) and wrist (B) signs in a 30-year-old man with Marfan syndrome. Arachnodactyly is evident.



**FIGURE 153-9** Pectus excavatum (A) and carinatum (B) in two young adolescents with Marfan syndrome.

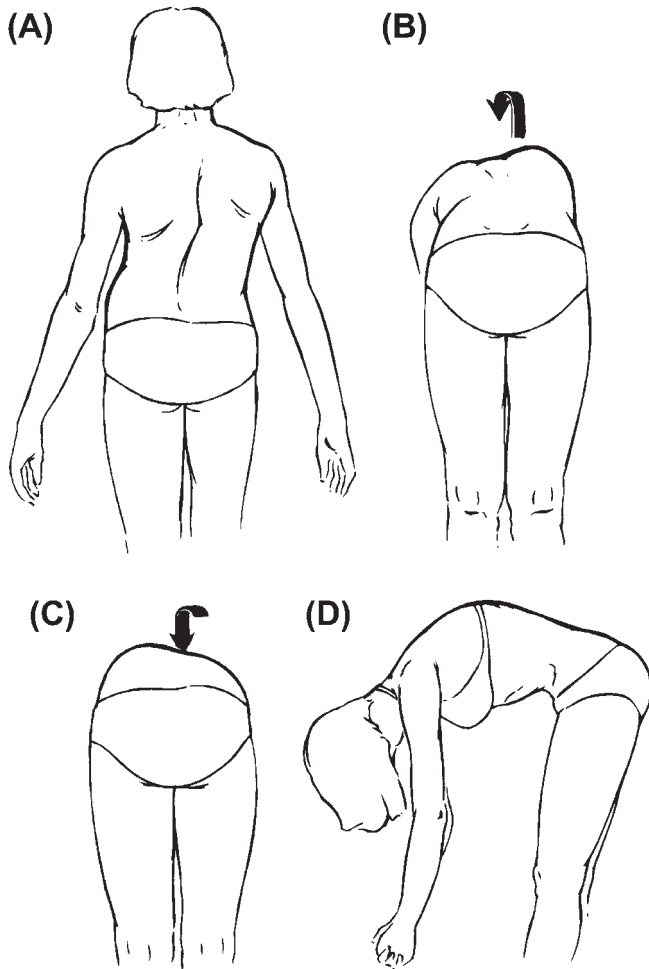


**FIGURE 153-10** Pes planus in a man with Marfan syndrome. Note the arachnodactyly and the long narrow foot.

The axial length of the globe may be increased, contributing to myopia, increased risk of retinal detachment (especially with axial lengths of greater than 25 mm), and lens subluxation. Spontaneous retinal detachment has been observed before age 5 years, and the lifetime occurrence risk is around 10%. The prevalence of retinal detachment increases following lens extraction (86).

The range of refractive errors detected in MFS is extremely broad and is not limited to myopia (86). However, myopia is frequent, and may appear early and be severe. While increased axial length of the globe and a dislocated spherophakic lens lead to myopia, a flat cornea leads to hyperopia, counteracting the myopia. Astigmatism is common and tends to be high and oblique.





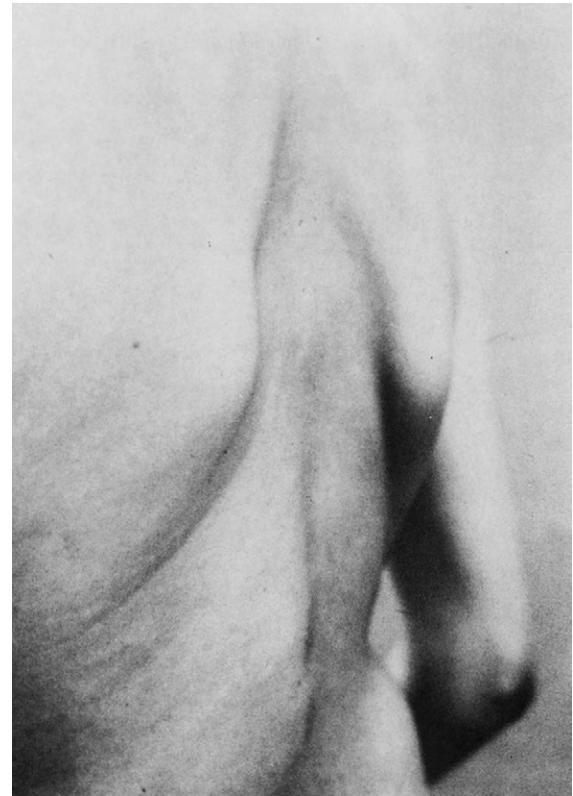
**FIGURE 153-11** Bedside diagnosis of abnormal curvature of the vertebral column. (Courtesy of Paul Sponseller, MD.)

High degrees of astigmatism may remain incompletely corrected at refraction and lead to reduced visual acuity.

Previously, most cases of glaucoma followed lens surgery; the prevalence in MFS with ectopia lentis was 7.7% and increased to 14.8% when the lens had been removed. In the largest series of MFS reported, glaucoma was present in 2.4% of patients younger than 40 years, and in 12.9% of those 40 years and older. Open-angle glaucoma occurred in nearly half of the younger group but only 30% of the older (87). Primary trabeculodysgenesis occurs in infants with severe manifestations of MFS and is a cause of congenital glaucoma (88).

**153.5.3.3 Cardiovascular Manifestations.** The two most common cardiovascular features of MFS are mitral valve prolapse and dilatation of the proximal ascending aorta. The former may result in mitral regurgitation, while the latter may result in aortic regurgitation and predispose to aortic dissection and rupture. The mean age at death is reduced by 30–40% in persons with MFS: nearly all the precocious deaths result from a cardiovascular complication (59).

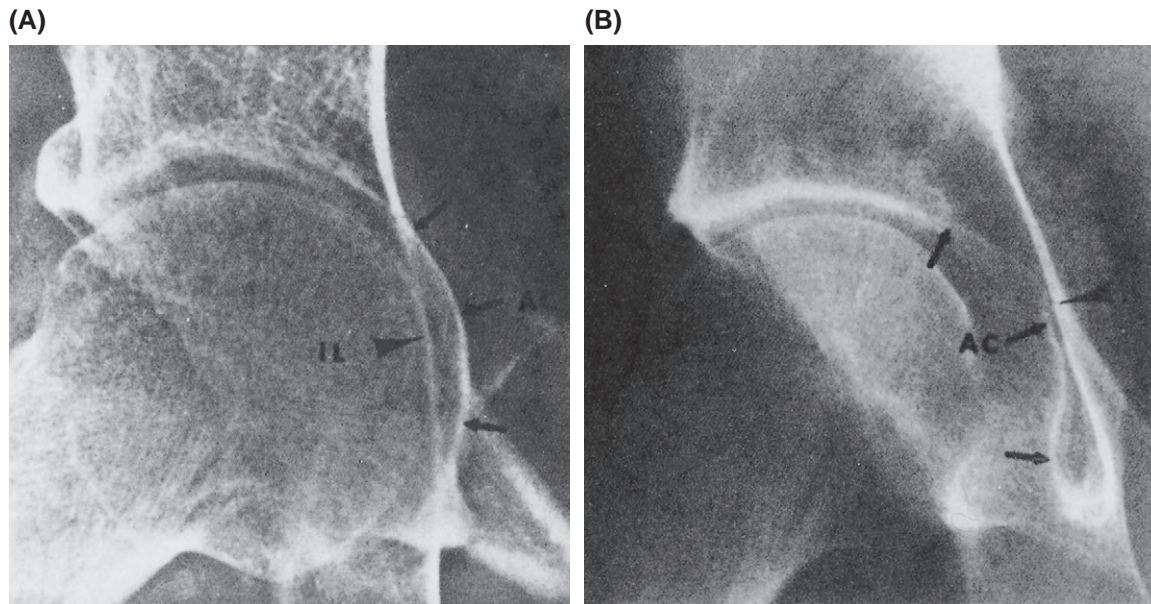
**Mitral Valve Prolapse.** About 60% of patients have auscultatory signs of mitral or aortic valve pathology,



**FIGURE 153-12** Thoracic lordosis in Marfan syndrome. This adolescent boy has a reversal of the usual thoracic kyphotic curve, resulting in a reduction of the anteroposterior diameter of the thorax. This type of deformity is present in the 'straight-back syndrome'.

but the rest may have normal cardiovascular physical findings (see Table 153-2). Echocardiography greatly enhances detection of the cardiovascular abnormalities, with concomitant improvement in the ability to diagnose MFS. For example, whereas about one third of these patients have single or multiple systolic clicks or systolic murmurs of presumed mitral origin, echocardiography shows that 50–80% of all Marfan patients have prolapse of at least the posterior mitral leaflet (89,90). The prolapse is often pansystolic, with exaggerated leaflet excursion and thickness, suggesting redundancy of valvular tissue. Dilation of the mitral annulus is common, and calcification occurs in a minority. Mitral valve prolapse may not be clinically or echocardiographically present during infancy but may be noted several years later. The degree of prolapse may worsen with age, and mitral regurgitation may appear and progress hemodynamically in some patients who initially had only prolapse (89,91). Even in children, the mitral regurgitation can become severe enough to warrant valve repair or replacement (92).

**Aortic Root Dilatation.** Transthoracic echocardiography of the Marfan aorta (Figure 153-15) in both children and adults readily shows the diameter of the proximal aorta ('root'). Dimensions that are measured routinely include the aortic annulus, the sinuses of Valsalva (SoV), the supra-aortic ridge, and the proximal ascending aorta.



**FIGURE 153-13** Moderate protrusio acetabulae. (A) Right hip of a patient with Marfan syndrome is shown. Arrows point to the acetabular line (AC) and the arrowhead to the ilioischial line (IL). Note the medial displacement of AC. (B) Normal hip. (From Kuhlman J. E., et al. (72).)

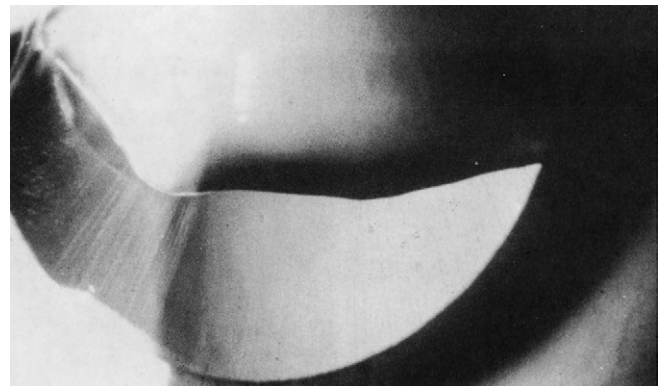
**TABLE 153-3** Corneal Shape in Marfan Syndrome<sup>a</sup>

Age (Yr)	No. of Eyes Measured	Mean Keratometer Reading (Diopter $\pm$ SD)
1–8	37	40.9 $\pm$ 2.4
9–14	28	40.8 $\pm$ 2.3
15	72	41.9 $\pm$ 1.7

<sup>a</sup>Normal adult eyes have mean keratometer readings of 43.7  $\pm$  0.2.

The diameter of the SoV, measured at the level of the aortic valve cusps, is positively associated with age and body surface area in both normal controls and people with MFS (93). In MFS, the SoV diameter is usually, but not always, greater than the upper limit of the normal range (94,95), even in young children (92,96–98). In MFS, dilatation rarely involves the annulus, begins in the SoV, and may remain confined there or progress into the proximal ascending aorta after effacement of the sinotubular junction. Except when dissection occurs, dilation rarely progresses as far as the innominate artery.

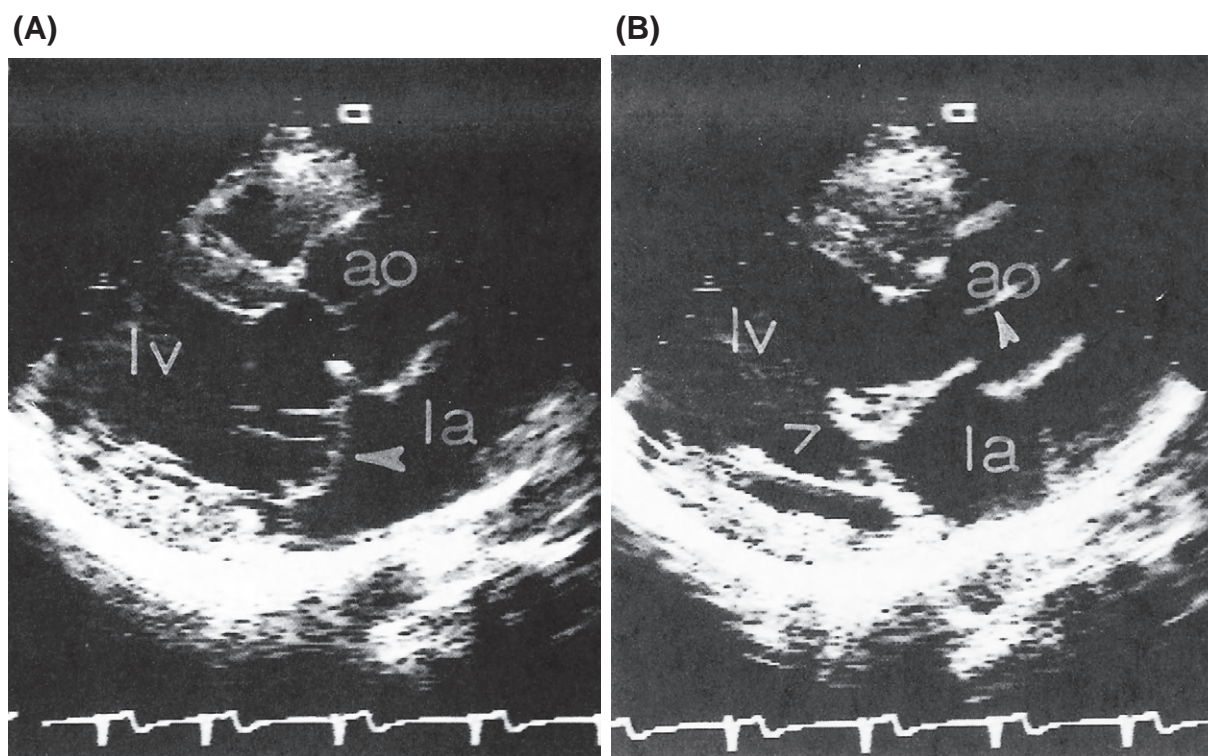
Rarely will a person in whom the diagnosis of MFS is strongly suspected on other grounds (skeletal, ocular, and family history) have none of these echocardiographic abnormalities. In a few patients, a technically satisfactory transthoracic echocardiogram is unobtainable because of a severe pectus deformity or emphysema. In these patients, transesophageal echocardiography, computed tomography (CT), and magnetic resonance imaging (MRI) are reasonable alternatives for following the aortic diameter.



**FIGURE 153-14** Ectopia lentis in Marfan syndrome, shown by slit-lamp photography of the lens of a young girl. The lens is dislocated supratemporally. Note the zonules, which are stretched but intact. (Courtesy of Dr. Irene Maumenee.)

The caliber of the proximal aorta increases during childhood in MFS because of both physical growth and dilation. After skeletal maturity, it is safe to assume that further root enlargement in young adulthood is due to pathologic dilation. Remodeling of the aortic root occurs during mid- to late-adulthood, associated with age, gender, blood pressure and body size (99), and this needs to be considered when MFS subjects are followed over the course of their lives. The predictors of the rate of dilation are uncertain. Peripheral blood pressure seems to have little impact, especially since most young people with MFS have brachial blood pressure measurements in the normal range. One study suggested that the pulse pressure determined by applanation tonometry at the carotid artery was positively associated with the diameter of the SoV (100); however, the association was weak and the





**FIGURE 153-15** Cross-sectional echocardiograms, parasternal long-axis view, obtained from a 10-year-old boy. In both frames, the aortic root (ao) in the sinuses of Valsalva is dilated. (A) In systole, the mitral valve (closed arrowhead) is closed and prolapsed into the left atrium (la). (B) In diastole, the open arrowhead points to the thickened anterior mitral leaflet. The aortic valve (closed arrowhead) shows a thin closure line. LV, left ventricle.

methodology is not readily available. When such studies are performed, children with MFS do have increased pulse wave velocity and aortic stiffness (101). The aortic annulus may dilate to a modest degree; but dilation in the region of the sinotubular ridge results in eventual failure of the cusps of the aortic valve to appose. As the aorta continues to dilate, the regurgitant flow increases. The left ventricular response to aortic regurgitation in MFS is qualitatively similar to that with other causes of chronic aortic regurgitation, but the following sequence of events may evolve more rapidly in MFS. The left ventricle dilates to compensate for the increased stroke volume required. Eventually the myocardium begins to fail, and irreversible myopathic changes follow. The end stage, if dissection or rupture of the aorta does not supervene, is death from congestive heart failure (59).

**Aortic Dissection.** This is the most feared complication of MFS. About 90% of acute dissections in MFS begin in the aortic root, just distal to the coronary ostia. Many dissections of the ascending aorta extend, either immediately or in a stuttering pattern over hours or days, to involve the arch and descending aorta, often extending into the iliac vessels. The dissection event is usually sudden, with all of the classic signs and symptoms reflected in series of patients with MFS. However, some dissections occur with few or atypical symptoms, and if the patient survives the acute event, he or she will be left with a chronic dissection of some portion of the

aorta. Cross-sectional echocardiography often displays the ascending aorta for a distance of 4–6 cm above the aortic valve and may be the first clue that a clinically silent dissection has occurred. However, if a dissection is suspected, transesophageal echocardiography, CT, or MRI is the preferred noninvasive approach to diagnosis. Furthermore, the time saved in reaching a diagnosis by noninvasive means enhances survival for patients who require surgery. Roman, Devereux, and colleagues suggested that dilation above the sinotubular junction is a strong predictor of aortic complications (102). In children, the initial aortic diameter was a predictor of both growth rate and complications (96). Other studies suggest that the strongest predictor of dissection of the ascending aorta, aside from the actual caliber of the root, is a history of a dissection in a relative (103).

According to data from the International Registry of Aortic Dissection, half of all aortic dissections in patients less than 40 years of age are due to MFS (104).

The elastic properties of the Marfan aorta are distinctly abnormal. The wall is considerably stiffer than expected from an early age; this is associated with heightened systemic pulse wave velocities (96,97,105,106). MRI and tissue Doppler imaging enable determination of aortic distensibility (107,108). The more stiff the aorta, and the larger the diameter, the more likely that the aortic root will dilate progressively (109). Investigations are needed to define which indices of aortic

elasticity are both prognostically useful and easily determined noninvasively.

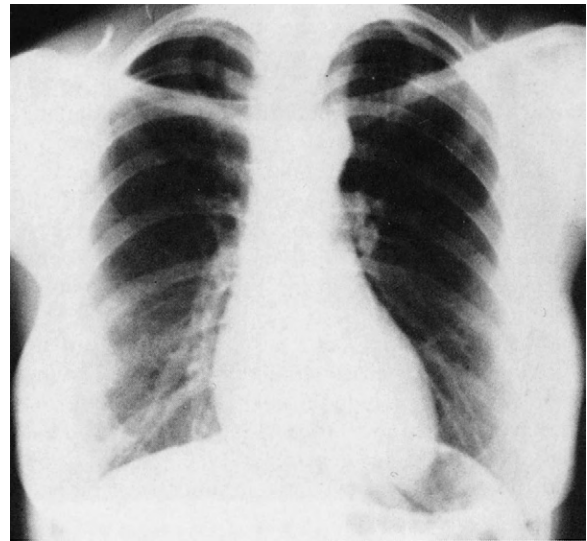
The dilated ascending aorta is more susceptible than the undilated aorta to traumatic dissection or rupture. Numerous cases of dissection or rupture, often associated with sudden death, have occurred in persons with MFS while they were exercising (110) or when they were involved in relatively minor deceleration injuries in automobile accidents.

The risk of severe cardiovascular complications during pregnancy is unquestionably increased in a woman with MFS. The major issue for counseling and management is to define the risk for the individual patient. Data are derived from two principal sources: case reports and observational series. The latter can be retrospective or prospective. As with many other medical conditions, case reports tend to over-represent complications (111). A summary of 57 publications reporting 99 patients showed that 50% suffered severe events (112). On the other hand, several observational series document over 300 pregnancies in more than 120 women, and the occurrence of severe maternal cardiovascular complications varied between 1% and 6% (112–116). The increased cardiac output that occurs during the midtrimester places greater strain on the dilated ascending aorta than in the non-pregnant condition. The largest retrospective survey from a single institution examined 105 pregnancies in 26 women with MFS (114). Only one death occurred, due to endocarditis in a woman with severe mitral valve disease that predated the pregnancy (85). At the same institution, 21 women were followed through 45 pregnancies and thereafter (116). None with minimal aortic dilation (less than 42mm) had any vascular complication during pregnancy, and echocardiography every 6 weeks showed no further aortic dilation. During mean follow-up evaluation of 6 years in these women, and compared to a group of nulliparous Marfan women of similar severity, none suffered apparent cardiovascular worsening attributable to pregnancy. Two women (one with aortic regurgitation and moderate aortic dilation and one with a pre-existing dissection) suffered acute dissection of the descending aorta during pregnancy.

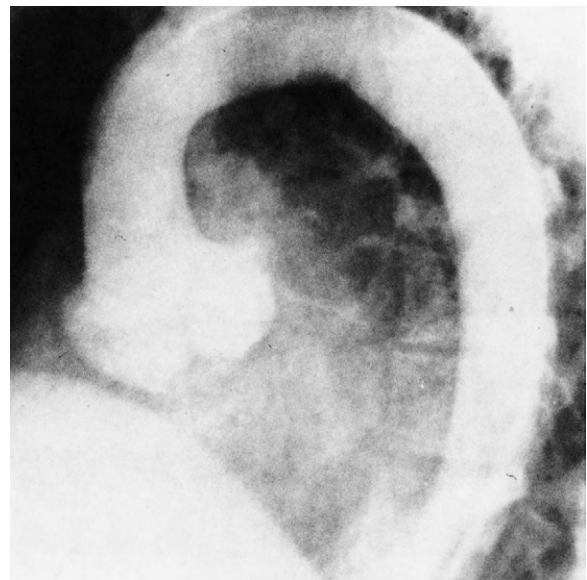
The chest radiograph is an insensitive technique for detecting early aortic root enlargement or dissection. Dilation of the proximal ascending aorta is visible on the frontal radiograph only when substantial; mild to moderate enlargement is frequently hidden by the vertebral column and cardiac silhouette (Figure 153-16).

On aortography, the dilated Marfan aorta is characteristic (Figure 153-17). The enlargement is symmetrical and begins in the sinuses of Valsalva. Rarely does the dilation extend as far as the innominate artery, and the ascending aorta has a gourd-like appearance when contrast material is injected.

MRI provides superb visualization of the entire aorta and is useful for detecting dilation, dissection, and coarctation (no increased prevalence in MFS) anywhere along



**FIGURE 153-16** Normal chest radiograph in the presence of aortic root dilation. The proximal aorta of this 28-year-old woman affected by Marfan syndrome measured 4.8cm, nearly 50% greater than normal. This enlargement is hidden by the cardiac silhouette.

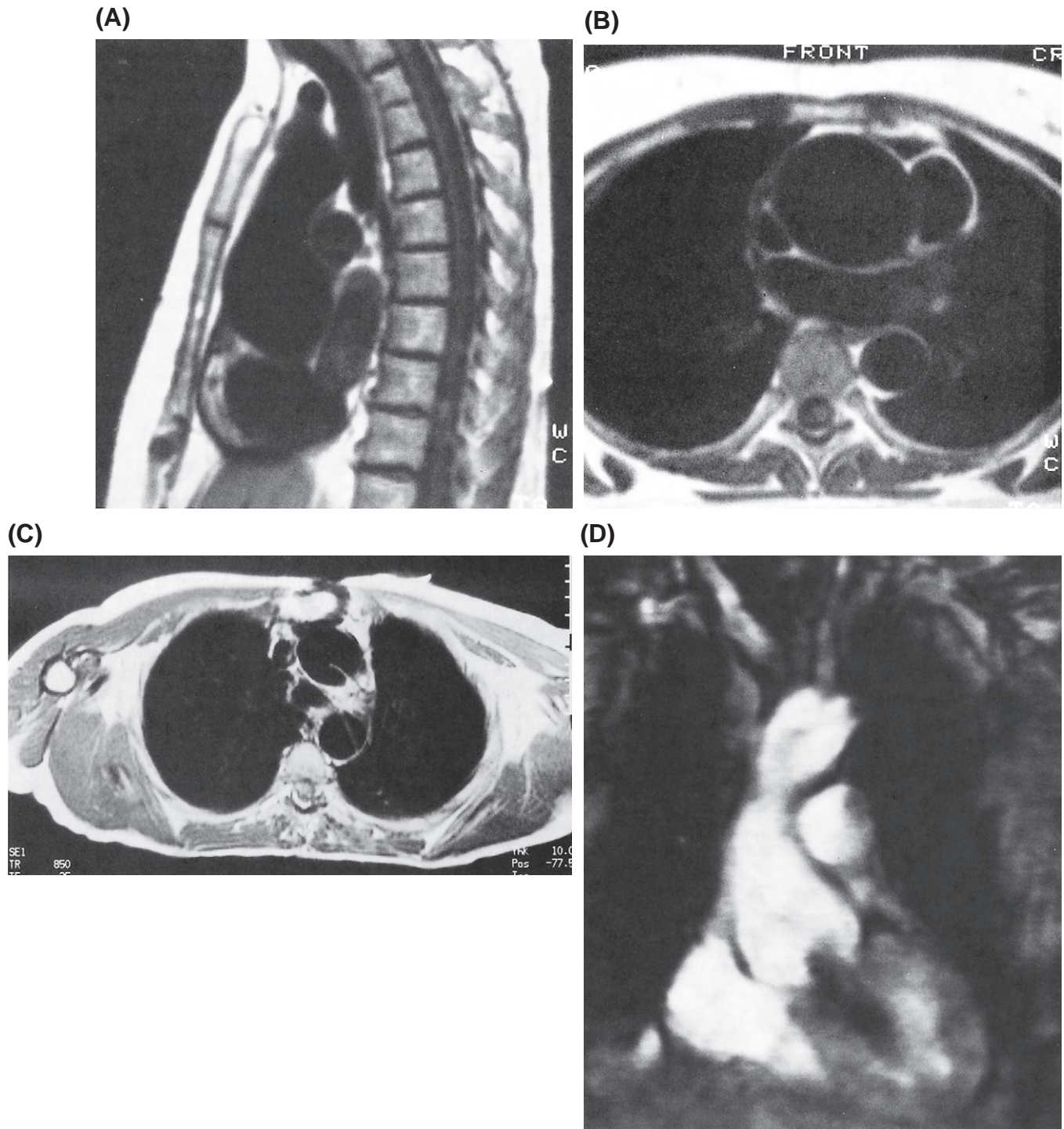


**FIGURE 153-17** Aortic root dilation in Marfan syndrome. The sinuses of Valsalva are symmetrically dilated to a moderate degree in this 37-year-old woman.

the vessel and its major branches (Figure 153-18). As a method of following the course of aortic problems (other than simple dilation of the root), especially postoperatively, CT and magnetic resonance angiography are optimal, but magnetic resonance (MR) avoids repeated radiation exposure.

**Other Arterial Involvement.** Elastic arteries besides the aorta can be clinically affected in MFS. Involvement of cerebral arteries may rarely produce intracranial hemorrhage from aneurysm rupture (117), but asymptomatic aneurysms are certainly more common, but of unknown prevalence (118,119). One study from a large



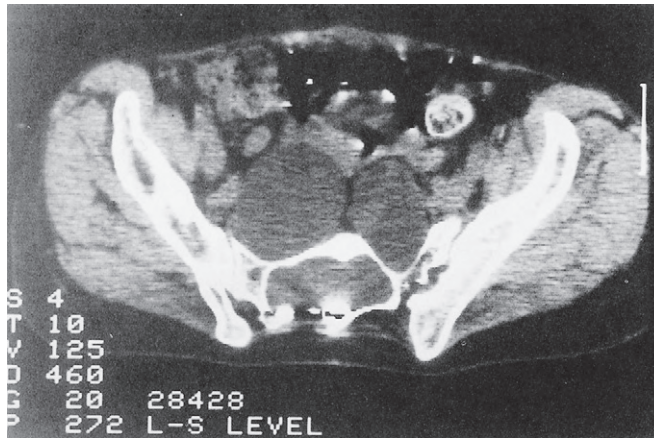


**FIGURE 153-18** Magnetic resonance imaging of the Marfan aorta. T1-weighted images (A–C) show that flowing blood gives no signal, and the lumen of vessels is black. (A) Parasagittal view, showing the gourd-shaped aortic root and ascending aorta. (B) Axial view through the aortic root and mid-descending thoracic aorta; the aortic root is somewhat elliptical and measures 53 mm 61 mm. (C) Axial view through the proximal aortic arch and proximal descending thoracic aorta, showing a type I aortic dissection. (D) Coronal section through the ascending aorta and left ventricle collecting data gated to the cardiac cycle; flowing blood gives a light image. Turbulence from aortic regurgitation in this patient generates a dark image into the left ventricle.

Marfan clinical program found no examples of symptomatic intracranial aneurysm (120). Dissection of both intracranial and extracranial cerebral vessels does occur in Loeys–Dietz syndrome and other disorders of TGF $\beta$  receptors, which have been confused phenotypically

with Marfan syndrome in the past (121). Similarly, coronary, pulmonary, and peripheral arteries can show medial degenerative changes similar to the aorta (122), but reports of complications are few (123–129). Whether the abdominal aorta will show a heightened

(A)



(B)



**FIGURE 153-19** Dural ectasia in Marfan syndrome. (A) Axial computed tomogram through the sacrum and ilia showing considerable enlargement of the neural canal and thinning of the vertebral bone. In addition, large meningoceles emanate from the neural foramina bilaterally. The patient was a 34-year-old man. (B) Magnetic resonance image in the sagittal plane showing ectasia of the sacral neural canal and a large anterior meningocele. The patient was a 23-year-old woman.

predisposition to dilation as patients with MFS age is an open question.

**Ventricular Function.** Anecdotal experience shows that some patients with MFS, who do not have abnormalities of valve function or any other risk factor, do have diminished left ventricular function. Fibrillin is present in the myocardium, and certain mutations could predispose to impaired systolic, diastolic, or generalized contractility. Alternatively, some patients with MFS might also harbor a separate mutation for dilated cardiomyopathy. Studies addressing these points have involved relatively few subjects, and the results are conflicting (130,131).

**Electrophysiology.** No typical electrocardiographic (ECG) abnormalities occur. Changes may result from chronic valvular regurgitation, but usually after these lesions are clinically recognizable. Axis deviations occur because of rotation of the heart by severe pectus excavatum or thoracic lordosis. Some patients have dysrhythmia, usually supraventricular but occasionally ventricular in origin. Mitral regurgitation promotes left atrial enlargement, which in turn predisposes to atrial fibrillation. Patients should always be questioned about palpitations and light-headedness or near syncope, at rest and with exercise. Any symptoms of more than trivial

nature should prompt further evaluation, generally by 24-hour or event ECG monitoring.

**153.5.3.4 The Dura.** Ectasia of the caudal dural sac is a common finding, evident on radiographs of the lumbosacral spine as bony erosions of the neural foramina and anteroposterior scalloping of vertebrae, and on axial CT or MRI scans as a widened neural canal (132–137). An extreme manifestation is an intrapelvic meningocele (Figure 153-19), which may present as a pelvic mass and be confused with an ovarian cyst or tumor (138). Dural ectasia is positively associated with reports of back pain in MFS (132,133,139). Criteria for detecting dural ectasia by MRI or CT include a wider dural sac below L5 than above L4; the presence of anterior sacral meningocele; in the adult, a diameter of the L5 nerve root of greater than 6.5mm; and scalloping of the S1 vertebral body (132,133). The frequency of dural ectasia is 70–90% (134,140). The signs of dural ectasia are often present in childhood, and the severity increases with age (140). An increased size of the dural sac at L3 and S1 was 95% sensitive and 98% specific for MFS (141).

Enlargement of the cranial subarachnoid spaces is common in MFS and is evident as enlarged perivascular (Virchow-Robin) spaces, cranial cisterns, and optic nerve



sheaths. The dura may be extremely thin and fragile when manipulated. Dural ectasia is usually asymptomatic but should be in the differential diagnosis of a Marfan patient with low back pain, lower radicular pain, or leg weakness (142). Chronic headache, especially one that worsens on upright posture, should suggest either a large pelvic meningocele, which acts as a sump for cerebrospinal fluid (CSF), or a dural tear with persistent leakage of CSF (143–145). The presence of dural ectasia should be considered when administering spinal or epidural anesthesia to any patient with Marfan syndrome (146).

**153.5.3.5 The Skin and Integument.** The predominant abnormality of the skin is the stria atrophica, most commonly found over the anterior shoulders, lumbar region, and lateral hips (Figure 153-20). Striae gravidarum can be marked in women with MFS. The skin is otherwise not unusually fragile or susceptible to bruising or poor healing, but may be hyperextensible. Hernias frequently occur, especially in the inguinal region (147). They may appear in early childhood, and a history of multiple repairs is not uncommon. Congenital diaphragmatic and paradiaphragmatic hernias have been reported (148,149).

**153.5.3.6 The Pulmonary System.** Some patients, usually those with severe MFS evident as infants, have cystic lung disease. If the mouse model of MFS is analogous to the human condition, these cysts are not degenerative, but developmental, due to overactivity of TGF- $\beta$  (150). Usually, the cysts, or bullae, are not detected until a pneumothorax occurs. Spontaneous pneumothorax occurs in about 5% of Marfan patients (151). Symptoms are typical of pneumothorax in the general population, specifically ipsilateral chest pain, sudden dyspnea, and tachycardia (152).

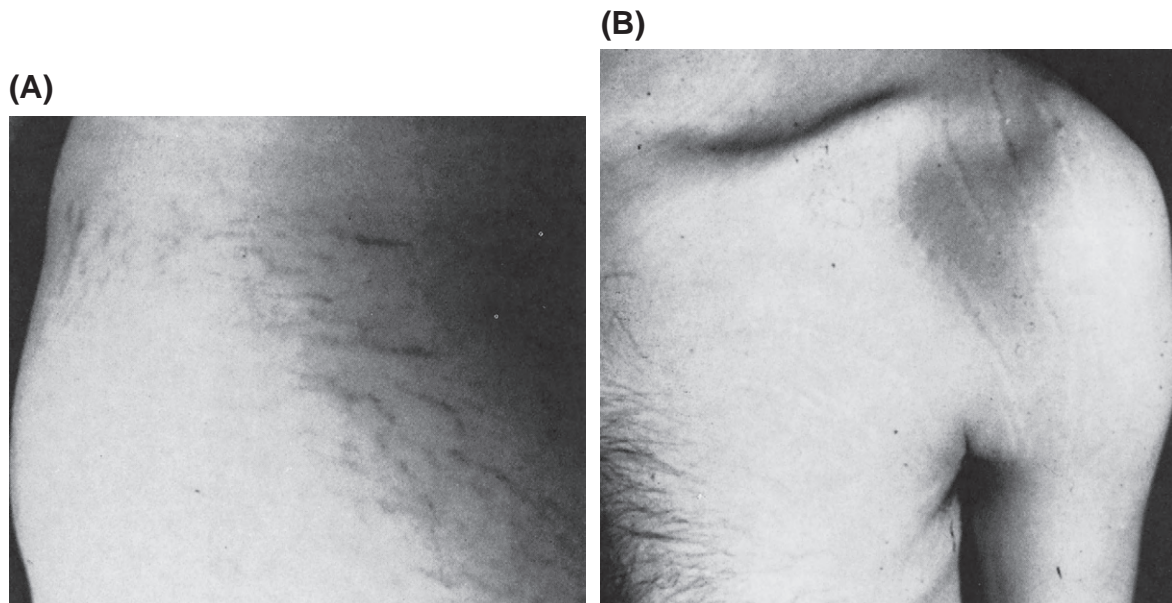
Some patients have markedly reduced total lung capacity and residual volume ascribable to deforming

kyphoscoliosis or pectus excavatum. Even in patients without thoracic distortion, forced vital capacity is consistently less than predicted based on height. However, the use of a more accurate predictor of thoracic size, such as the sitting height, shows that static lung volumes in the absence of severe scoliosis and pectus excavatum are not markedly abnormal (153). One study of a small number of young patients suggested an increased prevalence of bronchial hyperreactivity in the absence of clinical signs of asthma (154).

The prevalence of sleep-disordered breathing is probably not as high as suggested (74,75), but the patient (or spouse) should be questioned directly about snoring, nocturnal periodic breathing, and daytime hypersomnolence, especially in the presence of marked retrognathia (77,155). The sleep disturbance is usually obstructive and can be due to craniofacial restriction and laxity of the upper and middle airway that becomes evident only with relaxation of the skeletal muscles.

**153.5.3.7 The Skeletal Musculature.** Many, but by no means all, patients with MFS have a paucity of skeletal muscle, which contributes to their asthenic habitus. Typically, strength is not abnormal. My personal observation is that many of these children and adolescents cannot hypertrophy their muscles with exercise (not that this is ever encouraged!) and the Marfan mouse model clearly has a skeletal myopathy (154). Rarely, muscle weakness can affect respiratory function (156).

**153.5.3.8 The Central Nervous System.** It has been assumed that people with MFS have no impairment of cortical function. Indeed, 30 consecutive school-age clinic patients had average intellectual and gross motor development. However, one-half of these young patients had one or more neuropsychological deficits, including



**FIGURE 153-20** Striae atrophicae of (A) the hip of an 8-year-old girl, and (B) of the anterior shoulders in a 27-year-old man with Marfan syndrome.

learning disability, attention-deficit disorder with or without hyperactivity, neuromaturational immaturity, and verbal-performance discrepancy (157). Joint laxity of the hand and wrist contributed to the latter deficit, but the pathogenesis of the rest of the problems is obscure. Others have found problems with tasks requiring sustained visual attention and visual construction abilities; difficulties could not be explained simply on the basis of reduced visual acuity (158).

A number of single cases of psychiatric disturbance in patients with MFS have been published (159), but the likelihood is that these occurrences are coincidental. Many patients have difficulty dealing psychologically with the complications of MFS, their management, and their effect on lifestyle (160).

### 153.5.4 Pathology

**153.5.4.1 Gross Pathology.** Numerous reports of autopsies of patients affected by MFS appear in the older literature (see McKusick (85) for review). The aorta dilates in its most proximal region, the sinuses of Valsalva. The dilation is symmetrical and may extend above the sinotubular junction. The diameter of the ascending aorta usually returns to normal before the innominate artery, unless a dissection is present. Dilation of the sinotubular junction, at the upper aortic valve attachments, results in failure of the cusps to coapt.

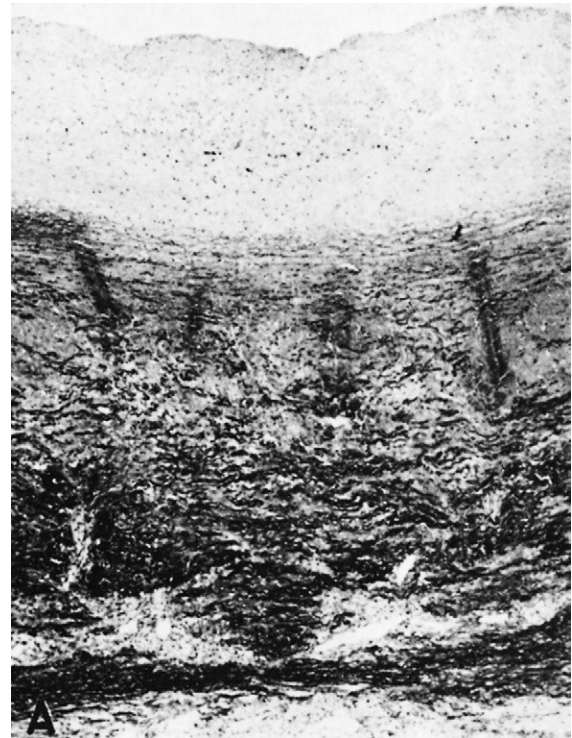
When dissection of the aorta occurs, the entry tear in the intima is frequently several centimeters above the aortic annulus, in the area of greatest dilation. The dissection can either progress antegrade no farther than the arch, progress through the arch into the descending aorta, or extend retrograde. In the latter case, attachments of the valve cusps may be torn, coronary ostia may be occluded, or rupture into the pericardial sac producing tamponade may occur. Any dissection can undergo rapid or gradual evolution. Intimal tears in the absence of dissection are common in the dilated proximal ascending aorta.

The valve cusps are usually diaphanous and redundant, and the mitral valve and annulus are susceptible to calcification. Fenestrations occur in a minority of aortic and mitral valve cusps. The aortic and mitral valves are susceptible to the development of bacterial endocarditis. The proximal pulmonary artery is usually mildly dilated, but pulmonic regurgitation is uncommon. Aneurysmal dilation of coronary arteries has been reported but is uncommon. The coronary ostia may be abnormally high in the aortic root because of root dilation.

No specific changes occur in the atria, ventricles, or myocardium that are not attributable to the effects of chronic valvular regurgitation. Several patients have had clinical congestive heart failure out of proportion to their valvular disease; in these cases, the existence of a cardiomyopathy associated with MFS has been suggested. Histopathological studies of such patients are lacking.

Reports of gross ocular pathology are few, and little can be said other than that the lenses tend to dislocate superiorly, the globes tend to be elongated in the anteroposterior direction, and the corneas are flatter than normal. Published reports of gross pathology of bone, ligaments, and tendons show no particular abnormalities.

**153.5.4.2 Histology and Ultrastructure.** The aortic and mitral valves usually show myxomatous degenerative changes. The aortic wall characteristically shows changes in the medial layer. The elastic fibers become progressively swollen, fragmented, and reduced in number as the aortic diameter increases (Figure 153-21). Lacunae appear in the media, which are filled with basophilic proteoglycan. The term *cystic medial necrosis* has been applied to these histological changes, but it is a misnomer and its use should be discouraged. Neither necrosis nor cysts are present. These histopathological features are also seen in aortas from non-Marfan patients who had hypertension or aortic valve disease, and to a lesser degree may be a concomitant of aging. The most reasonable explanation is that the histopathology indicates injury and repair regardless of the cause of the injury (161). The changes are similar to those seen in experimental copper deficiency and lathyrism in animals (162). Electron microscopic examination confirms focal elastin fiber degeneration and abnormalities of collagen fibers. The medial smooth muscle fibers appear shrunken, and their basement membrane is greatly thickened. Arteries elsewhere in the body may show evidence



**FIGURE 153-21** Medial degeneration of the aorta in Marfan syndrome. Note the fragmentation and disarray of the darkly stained elastic fibers (von Geisen stain).



of medial disorganization, but less than that present in the ascending aorta. Mouse models of MFS has enabled an investigation of how abnormalities in arterial walls evolve over time (39; *vide infra*). The early changes of microcalcification, intimal hyperplasia, and disorganized ECM – all associated with signs of adventitial inflammation – prompted re-examination of human pathological specimens. Dietz and colleagues (27) observed similar histopathological changes in all elastic arteries in autopsy specimens of patients with MFS. ‘Osmiophilic elastolysis’, reported years previously in MFS (122,163), represented the same pathological process. Cartilage biopsies from the iliac crest in three adolescent patients were examined by transmission electron microscopy. The chondrocytes had dilated endoplasmic reticulum, cytoplasmic vacuoles, and increased glycogen stores. Elastic fibers in the skin are seen to be abnormal by ultrastructure and immunohistopathology (164). Ocular zonules show abnormal immunohistological staining for fibrillin-1 (39). Few reports of histological changes in other tissues exist.

### 153.5.5 Genetics

In the vast majority of reported families, segregation of MFS is consistent with autosomal dominant inheritance. Reports of multiple affected sibs with ostensibly normal parents are rare. Based on pedigree analysis, germinal mosaicism is extremely uncommon, but has been confirmed by molecular studies (165–167).

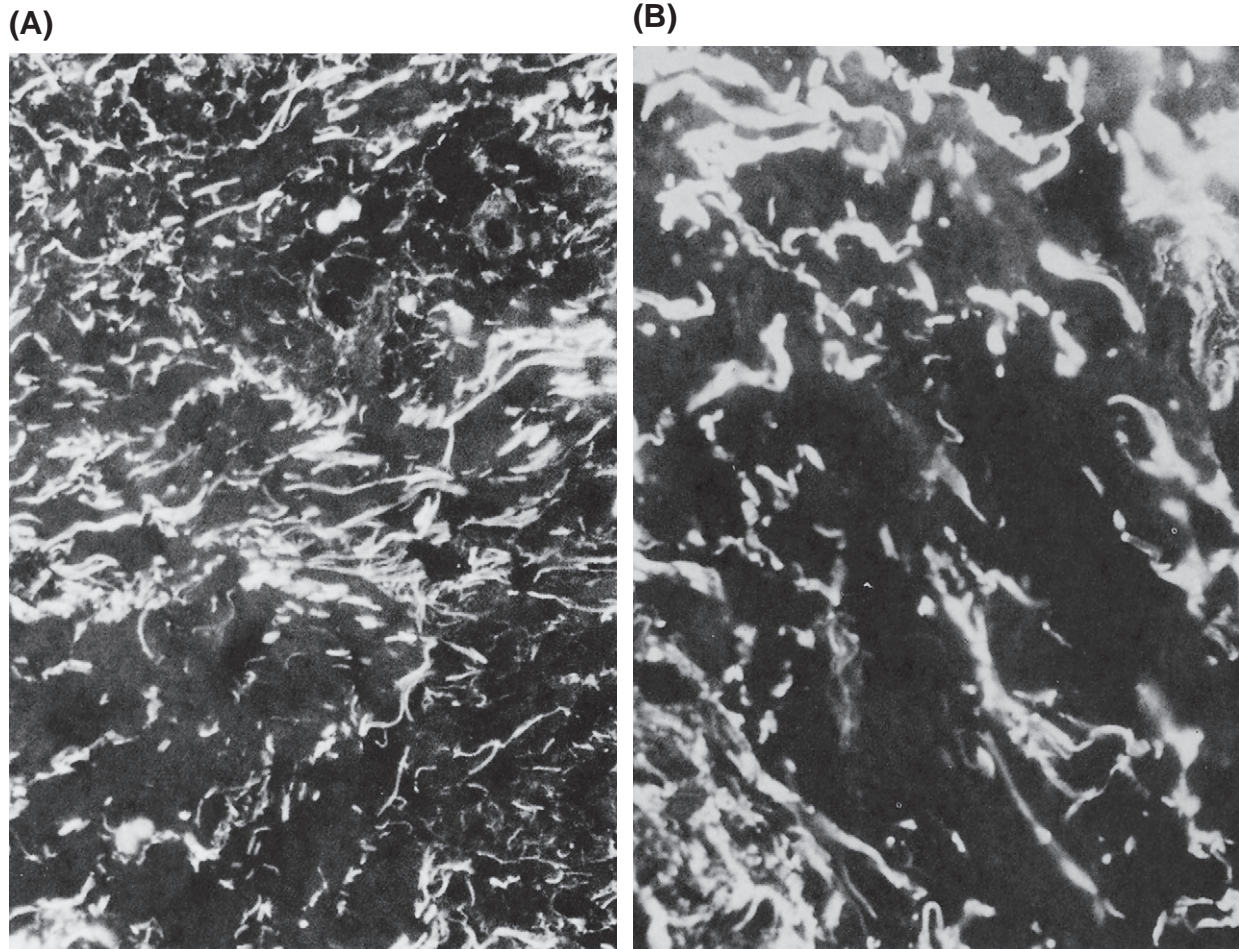
No formal studies of the frequency of sporadic cases or of the evolutionary (or genetic) fitness of MFS have been published. McKusick (85) estimated that 15% of patients had unaffected parents and most likely developed MFS from *de novo* mutation in a parental germ cell. At our medical genetics clinic, neither parent was affected for 41 of 138 consecutive Marfan patients. While various biases influence which patients attend a clinic, these data suggest that sporadic cases account for 15–30% of all patients. The average age of the fathers of sporadic cases exceeds by some 7 years that of fathers in the general population (168); the average age of the mothers of sporadic cases is not as advanced. This paternal age effect has been described in other autosomal dominant disorders, such as achondroplasia.

The extreme interfamilial variability in the Marfan phenotype largely reflects the extensive genetic heterogeneity in mutations of *FBN1* (169). However, intrafamilial variability can be marked; this undoubtedly explains the claims of ‘non-penetrance’ that appear in the older literature. When presumed cases of ‘non-penetrance’ or ‘*formes frustes*’ in families in which other, well-documented cases of MFS occur are examined by sensitive methods (e.g. echocardiography), the Marfan phenotype can usually be diagnosed with confidence. The biological basis of intrafamilial variability in MFS is unclear and is probably multifactorial. Several large families in which

the *FBN1* mutation has been discovered provide opportunities for analysis of mutant allele expression, epistasis, and other explanations (170).

### 153.5.6 Etiology

More than 70 years ago, MFS was hypothesized to be a generalized disorder of the mesenchyme (45). It was one of the original group of conditions classified as a heritable disorder of the connective tissue (50). Peltonen and colleagues in Finland discovered the first linkage relationship, between MFS and anonymous DNA markers on human chromosome 15q (171). Dietz and Francomano and colleagues (172) at Johns Hopkins then refined this linkage through cooperation of the largest pedigree of MFS described in the literature; the strongest linkage, at  $\Theta=0$ , occurs with marker D15S1, which maps to 15q21.1 (172). Subsequently, linkage studies of families throughout the world confirmed this locus. There is no convincing evidence for intergenic heterogeneity in the cause of MFS. Recent reports of mutations in *TGFBR2* in a French family (173) and in several other patients with some features of MFS (174) is intriguing because of the clear role of abnormal TGF- $\beta$  signaling in classic MFS (*vide infra*). However, when clinical descriptions of these subjects are available, they do not warrant a diagnosis of classic MFS. Contemporaneous with the linkage studies, Hollister and Sakai and colleagues pursued the possibility that abnormalities of microfibrillogenesis underlay MFS. Using a monoclonal antibody developed by Sakai against fibrillin, skin from patients with MFS was examined by immunofluorescent microscopy (164). Patients usually had evidence of decreased microfibrils, both at the dermal-epidermal junction (where microfibrils are unassociated with elastin) and coating elastic fibers in the deeper dermis (Figure 153-22). In addition, fibroblast monolayers, grown from the skin specimens, often produced an ECM deficient in microfibrils (Figure 153-23). Byers and Milewicz and collaborators examined the synthesis, secretion, and matrix incorporation of fibrillin in fibroblast specimens from patients with MFS and found one or another defect in all but a few cases (175). Taken together, these results pointed strongly at fibrillin as a candidate protein (and gene) for the cause of MFS. The cloning of *FBN1* (20) and its mapping to the same region of chromosome 15q as MFS (20,176,177) provided additional circumstantial evidence. However, it was the discovery of a *de novo* point mutation in *FBN1* in two unrelated patients with classic severe MFS that clinched the assignment of cause (177). This first mutation, called R239P at the time, substituted a proline for asparagine at a highly conserved residue in a calcium-binding EGF repeat (Figure 153-24). Over the subsequent years, the technical capabilities for identifying mutations in *FBN1* have improved considerably. In the best hands, in more than 95% of cases of classic MFS, a mutation can be found (121,178–180).



**FIGURE 153-22** Elastic fibers of the reticular (deep) dermis from age-matched subjects (x200). (A) Normal control. (B) Person with Marfan syndrome. Note the diminished number of elastic fibers 'coated' with fluorescence, many of which are shortened. Multiple reports of a single French pedigree with 'Marfan syndrome' (28) that maps to chromosome 3p (45) are not inconsistent with this conclusion, because this family does not have Marfan syndrome as defined by international standards (61). (Sections prepared as in Figure 153-5. Antifibrillin monoclonal antibody courtesy of Lynn Sakai, PhD.)

Most mutations in *FBN1* are missense, with those causing premature termination next in frequency. There are relatively few large deletions (181). Genotype-phenotype correlations have been few, with the most well-established being missense mutations of the calcium-binding EGF-like domains in the middle region of the coding sequence, which are more likely to cause a severe phenotype evident at birth. An international database of mutations in *FBN1* is available (182) (<http://www.umd.be/FBN1>; [www.biobase-international.com](http://www.biobase-international.com)). More than a thousand mutations have been reported, most of which are novel.

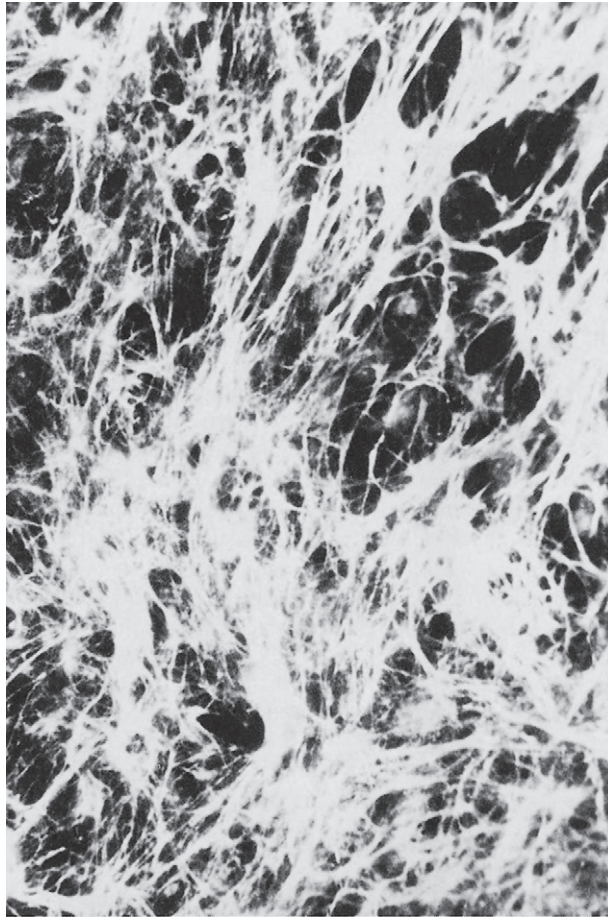
### 153.5.7 Molecular Pathology

Since 1991, a wide variety of mutations that cause MFS (and related disorders) have been found in *FBN1* (169,183). Initial mutation detection based on single-strand conformational polymorphism or denaturing gradient gel electrophoresis analyses of cDNA proved inefficient (184). Next, genomic DNA was examined by

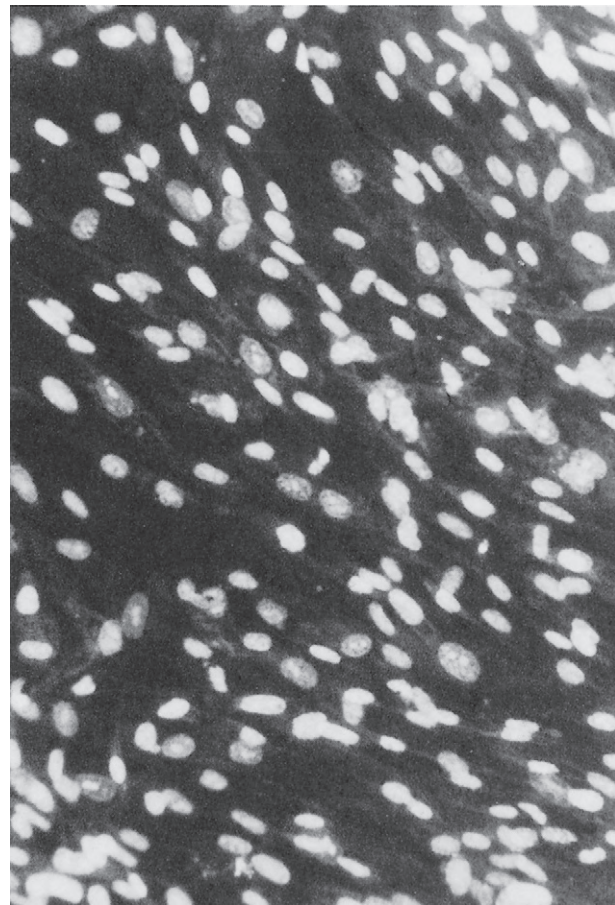
polymerase chain reaction amplification of each exon, followed by mutation detection enhancement gel heteroduplex analysis. This approach contributed greatly to the total mutations reported to the international database. Most mutations are private (169). Thus there is extensive intragenic heterogeneity at *FBN1*, and no common mutation will emerge. Mutations in regulatory regions of *FBN1* or deep within introns are possible explanations for the failure to identify pathological changes in coding sequences. A few observations are possible. There have been few large deletions. The most common mutation is missense, and most affect calcium-binding EGF-like motifs. A frequent class of mutation is substitution for one of the cysteine residues of the EGF-like motifs. Little can be deduced about genotype-phenotype correlations. Mutations near the C-terminus were initially predicted to cause a milder phenotype, even isolated ectopia lentis. However, severe MFS can result from mutations in this region (185). Severe MFS evident in infancy tends to involve missense mutations in calcium-binding EGF-like motifs encoded by exons 24 through 27, and 31 through



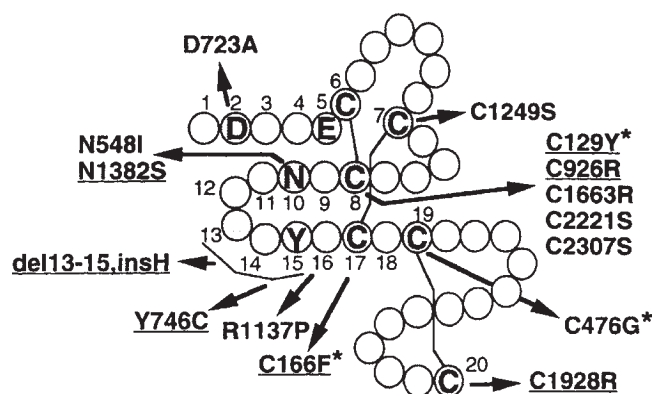
(A)



(B)



**FIGURE 153-23** Extracellular matrix of cultured dermal fibroblasts stained with a mouse antifibrillin monoclonal antibody and counterstained with FITC-labeled antimouse IgG. (A) Culture from a normal subject. (B) Culture from a person with the Marfan syndrome. The nuclei are stained with propidium iodide to demonstrate equal confluency of the cells. (Antifibrillin antibody courtesy of Lynn Sakai, PhD.)



**FIGURE 153-24** Some mutations identified in EGF-like motifs of fibrillin-1 in DNA from patients with Marfan syndrome. Note the proclivity for substitutions of cysteine residues and residues involved in calcium binding. (Courtesy of Harry Dietz, MD.)

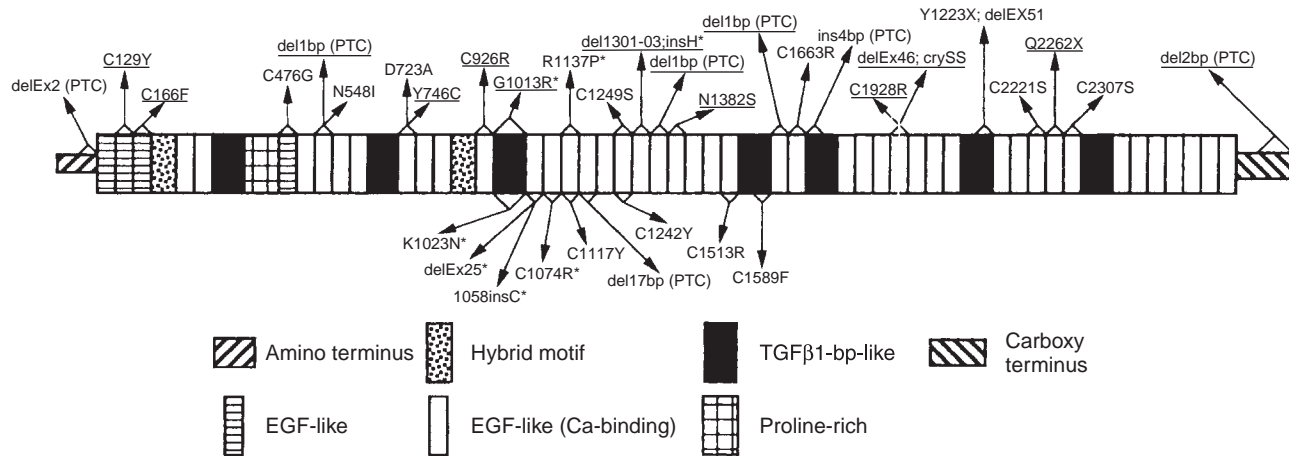
cause a less severe disorder, including the MASS (mitral valve, aorta, skin, and skeleton) phenotype.

### 153.5.8 Pathogenesis

Pathogenesis can be considered at progressively more complex levels, from molecules through tissues to the whole organism (see Chapter 14). This is the most important challenge facing investigators, because only through understanding how this pleiotropic phenotype arises will more effective methods of treatment be devised (189). Studying pathogenesis at complex levels, such as how mutations in *FBN1* cause overgrowth of long bones, is quite difficult in humans. The development of animal models greatly facilitates this process.

**153.5.8.1 Molecular Pathogenesis.** Although the structure of microfibrils is still far from clarified, they are aggregates of multiple structural proteins, the most important of which – the fibrillins – are organized in multimeric fibers composed of dozens and perhaps hundreds of monomers. From a structural perspective, the Marfan phenotype could arise from a defect in any component

32 (186–188). However, mutations in the same exons cause much less severe forms of MFS. Mutations that likely result in early degradation of the mutant protein (e.g. premature chain termination mutations) tend to



**FIGURE 153-25** Some of the published *FBN1* mutations that cause Marfan syndrome. Those labeled above the schematic of the fibrillin-1 cDNA were first identified by collaborators at Johns Hopkins University School of Medicine. (Courtesy of Harry Dietz, MD.)

of the microfibril, in its post-translational processing, or in the construction of the mature structure. As emphasized earlier, there is no genetic or molecular biological evidence that any molecule other than fibrillin-1, or any defect other than a structural mutation, is causative of classic MFS. The mouse model of MFS has markedly altered the understanding of the pathogenesis of a growing number of the features of this condition. Microfibrils play an important role in regulating the activity of TGF- $\beta$ , and abnormal or deficient fibrillin-1 appears to lead to increased TGF- $\beta$  activity during pre- and postnatal development (19,190).

The biochemical impact of *FBN1* mutations in humans has been characterized, primarily in cultured dermal fibroblasts, by:

- (1) The size of the mutant monomer,
- (2) How much of the mutant monomer is synthesized,
- (3) How much is secreted,
- (4) How much fibrillin is deposited in the ECM, and
- (5) The ultrastructure of microfibrils (12,175,191–193).

Most mutations in *FBN1* are of the missense variety and therefore do not reduce the size of the fibrillin monomer (Figure 153-25). A dominant-negative effect seemed a plausible mechanism for addressing the fundamental question of why heterozygosity for a fibrillin mutation should be so severe, when 50% of the normal complement of monomers is being synthesized.

Initially, Dietz and colleagues provided further evidence of dominant-negative pathogenesis by transfecting normal mouse fibroblasts with a DNA construct for a human *FBN1* mutation that produces a truncated N-terminal fragment of the protein. The transfected fibroblasts showed a marked reduction of incorporation of normal mouse fibrillin into the ECM (194).

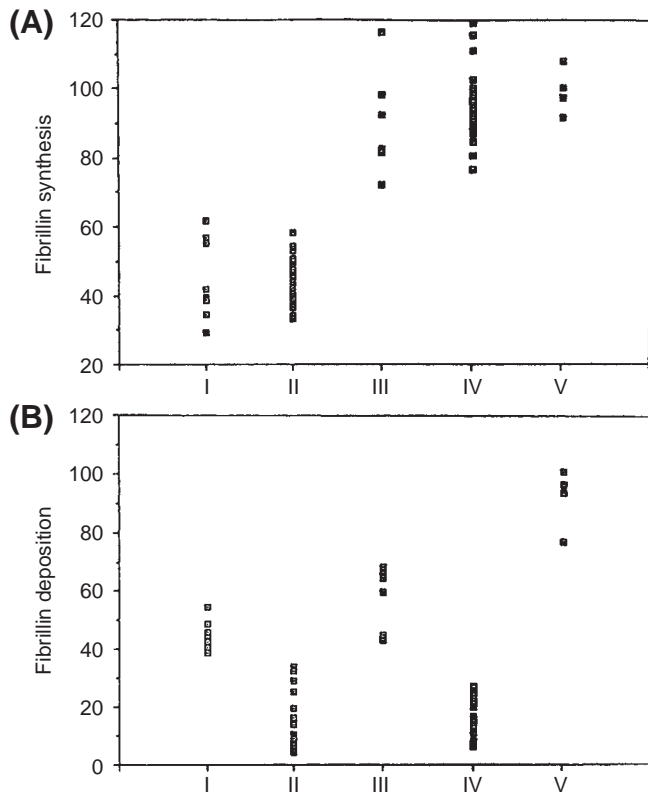
Kielty and colleagues have investigated the ultrastructure of microfibrils from patients and controls and correlated findings with biochemical characteristics. Microfibrils isolated from postconfluent fibroblast

cultures of most patients have distinct differences from control cell lines (Figure 153-26). No discernible microfibrils can be isolated from fibroblast cultures of some patients (12).

But what of the mutations that do not appreciably diminish fibrillin deposition in the ECM? One mutation results in a premature peptide chain termination; only 6% of this mutant mRNA is expressed, and the amount of mutant fibrillin-1 monomer is therefore markedly reduced. As a result, most of the monomers present are normal, the microfibrils are little affected, and the phenotype is mild. The stoichiometry of monomer interaction is apparently crucial, because premature termination mutations that produce slightly more transcript (16% and 23% mutant mRNA) are associated with a severe phenotype. By extension of these findings, a mutation resulting in a failure to produce any fibrillin from one *FBN1* allele (a null mutation) should produce an extremely mild phenotype, and this has been observed in some patients with the MASS phenotype (*vide infra*). However, a patient with no detectable mutant *FBN1* protein has severe MFS. So what constitutes a null mutation in this scheme? In searching for answers, an important consideration is the possibility that a mutant peptide or mutant mRNA might interfere with intracellular trafficking or processing of the normal monomer or mRNA. The dominant-negative effect would result in a severe phenotype, but the actual mutant product would not be detected and would be called a null phenotype. In this case, the amount of fibrillin-1 deposited in the ECM might be key to the severity (195). However, work with the mouse model of MFS, described in the next section, calls dominant-negative pathogenesis into question.

Apparently, failure to produce any normal fibrillin-1 has a major consequence on phenotype. Two parents with MFS, the father with a W217G substitution and the mother heterozygous for G2627R, produced a child who was the compound heterozygote (196). The child died of cardiac failure at age 4 months; he had previously





**FIGURE 153-26** Categorization of how mutations in *FBN1* affect synthesis and deposition of fibrillin-1 in cell culture. A, Amount of fibrillin synthesized by dermal fibroblasts, as a percentage of that synthesized by control cells. B, Amount of fibrillin incorporated into the extracellular matrix is shown. Samples in class V were indistinguishable from controls in both measures. (From Aoyama, T.; Francke, U.; Dietz, H.; Furthmayr, H. Quantitative Differences in Biosynthesis and Extracellular Deposition of Fibrillin in Cultured Fibroblast Distinguish Five Groups of Marfan Syndrome Patients and Suggest Distinct Pathogenetic Mechanisms. *J. Clin. Invest.* **1994**, 94, 130–137.)

been reported as a probable ‘homozygote’ because of the parental phenotypes and his severe features.

**153.5.8.2 Animal Models.** A model for MFS was found in nature, but unfortunately, not in the most useful of experimental animals. Potter and Besser and colleagues struggled to preserve and breed a mutation that occurred spontaneously as a germinal mosaic in a Limousin bull (197). Multiple offspring, most produced by artificial insemination, had rather classic features of MFS. Affected animals have abnormal fibrillin metabolism, but no mutation has yet been found in a gene for fibrillin, which maps to bovine chromosome 10.

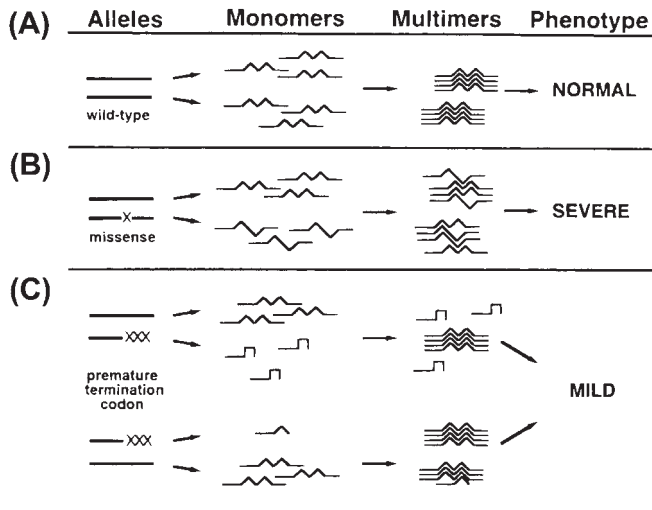
Ramirez, Dietz and colleagues (198) produced two mutant strains of mice made heterozygous for mutations in *FBN1* by targeted in-frame deletions. The strain heterozygous for a mutant allele expressed at a level 5–10% of the normal allele developed only subtle skeletal changes at advanced age. The homozygotes generally died in infancy of aortic rupture. While the histopathology of the aortic lesions was similar to those in humans with MFS, the areas of disrupted elastic fibers were quite focal. This suggested that minimal expression of *FBN1* is necessary

for elastic fiber formation in the mouse aorta (198). A second mouse mutation, a targeted intron insertion, resulted in reduced expression (15%) of normal protein from the mutant allele (38). The heterozygous animal had a normal phenotype throughout life. The homozygote, however, showed both aortic and skeletal features similar to MFS. Long bones overgrew. Life expectancy was only 3–6 months, with death due to aortic rupture. Most striking was early-onset calcification of the media of all elastic arteries. The regions of calcification became more extensive with age and were associated with proliferation of vascular smooth muscle cells and inflammation of the adjacent adventitia. The advanced vascular changes were associated with expression of matrix metalloproteinases and marked elastolysis. The diffuse calcification and intimal hyperplasia were subsequently found on retrospective review of autopsy specimens of humans with MFS (27).

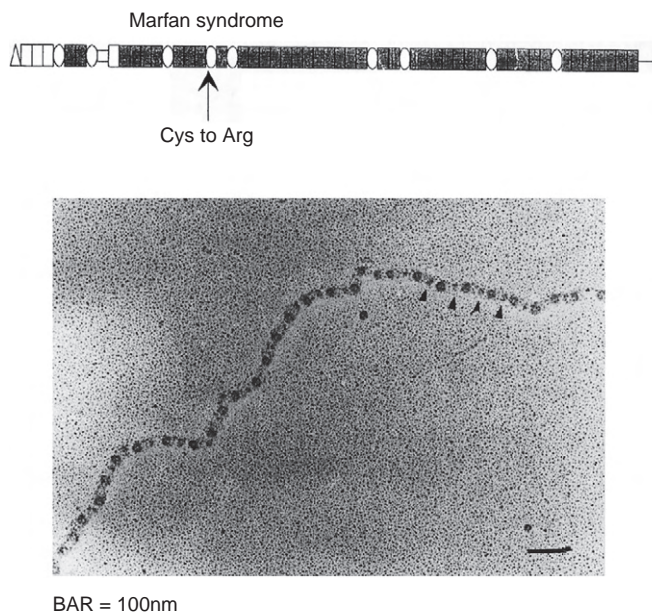
Mice carrying one or two copies of mutations known to cause human MFS are even better models (199,200). One strain, which carried two normal mouse alleles of fibrillin-1 and variable numbers of the aberrant human *FBN1* allele, showed no abnormal phenotype (200). In fact, these mice showed that mutant fibrillin-1 was incorporated into microfibrils. When the same ‘human’ mutation was introduced into the mouse *fbn1* locus, the MFS phenotype was produced. Introduction of the normal *FBN1* transgene onto this mutant mouse background normalized the phenotype. These results were contrary to the ‘dominant-negative’ pathogenetics mechanism, and supported haplo-insufficiency of normal fibrillin-1 as the most important determinant of the MFS phenotype.

Investigations using peptides of human fibrillin-1 carrying a mutation known to cause MFS support the notion that microfibrils are more sensitive to proteolytic degradation, by increasing the susceptibility to degradation (201), up-regulating matrix metalloproteinases (202), increasing apoptosis, or combinations thereof.

The strongest evidence for the role of altered TGF- $\beta$ -signaling (Figure 153-27) came from studies of development of the lung and the mitral valve in mice bearing MFS mutations (203). Neptune and colleagues (150) showed that there was a failure of distal alveolar septation, leading to an emphysematous-like pathology. The abnormal lung showed abnormally high levels of active TGF- $\beta$ ; treatment of perinatal MFS mice with an anti-TGF- $\beta$  antibody prevented the abnormal lung phenotype. Mice deficient in fibrillin-1 showed postnatal development of mitral valve prolapse, associated with abnormally increased TGF- $\beta$  signaling (204). As in the lung, inhibition of TGF- $\beta$  activity ‘rescued’ the Mitral valve prolapse (MVP) phenotype (Figure 153-28). Mice homozygous for a hypomorphic allele (*Fbn1*<sup>mgR/mgR</sup>) showed marked dural attenuation and elastic fiber disorganization. Increased activated TGF- $\beta$  and matrix metalloproteinase-2 was also present (67). Enhanced TGF- $\beta$  signaling through both the canonical (Smad-facilitated) and noncanonical pathways occurs (205,205a).



**FIGURE 153-27** Dominant-negative model of molecular pathogenesis of the fibrillinopathies. A, Normal synthesis, secretion, and processing of fibrillin-1 monomers leads to normal microfibrillar aggregates and a normal phenotype. B, A mutation that alters primary structure but does not interfere greatly with synthesis and secretion produces a pool of abnormal fibrillin-1 monomers that interfere with microfibrillar assembly, resulting in a severe phenotype. C, Mutations that result in either a marked reduction of transcript or a mutant peptide incapable of aggregating with the normal monomers lead to a reduced number of relatively normal microfibrils, and a mild phenotype. (From Dietz, H. C.; Pyeritz, R. E. *Molecular Genetic Approaches to Investigating Cardiovascular Disease*. Ann. Rev. Physiol. 1994, 56, 763–796, with permission.)



**FIGURE 153-28** Effect of a mutation causing Marfan syndrome of the ultrastructure of microfibrils produced by cultured dermal fibroblasts. Microfibrils from a patient with a missense mutation substituting arginine for cysteine at residue 890, in one of the two regions sharing homology for both EGF and TGF- $\beta$ 1 binding protein motifs in fibrillin-1. (Bar = 100 nm.) (From Kielty C. M., et al. (147).)

Serum from MFS mice and humans with MFS contains increased levels of circulating TGF- $\beta$  (206). Aging MFS mice develop osteopenia in association with up-regulation of Rankl in response to excess TGF- $\beta$  signaling (207).

Additional evidence for the role of excessive TGF- $\beta$  signaling in Marfan syndrome derives from the therapeutic efficacy of angiotensin receptor blockade in preventing, and even reversing, the cardiovascular phenotype in the mouse model of MFS (208). Losartan, which is in widespread use as an antihypertensive due to its antagonism of the angiotensin II type 1 receptor, also interferes with the action of TGF- $\beta$ . Prenatal treatment of mice with MFS normalizes the aortic histopathology. Additionally, and surprisingly, MFS mice who are not treated until 2 months of age (adolescence in human terms) have little aortic or lung pathology when sacrificed at 8 months of age. Mice lacking signaling through the angiotensin II type 2 receptor have accelerated aortic disease, and signaling through the type 2 receptor is required for the protective effect of losartan. Losartan, as opposed to angiotensin converting enzyme inhibition, blocked enhanced signaling through the ERK pathway (noncanonical pathway) (208a). Treatment of MFS mice with losartan does not improve osteopenia, but treatment with the bisphosphonate, alendronate, does (207). Human trials of angiotensin receptor blockade are in progress (209).

Mice carrying one copy of the tight skin (*Tsk*) mutation, which occurred spontaneously, have thickened skin firmly adherent to underlying tissue, excessive accumulation of collagen and glycosaminoglycan in many tissues, increased size due to overgrowth of cartilage and bone, and normal life span (210). The *Tsk/Tsk* genotype results in embryonic lethality. The mutation was mapped to chromosome 2, in a region syntenic with human chromosome 15q21. This prompted examination of the mouse fibrillin-1 locus, which was shown to harbor a large genomic duplication (211). The mutation causes a 14-kb, as opposed to the normal 11-kb, transcript. The molecular pathogenesis remains unclear. Of interest is the development in *Tsk/+* mice of autoantibodies to fibrillin-1. Humans with scleroderma also develop antibodies to fibrillin-1 (212). Furthermore, scleroderma is especially common in the Choctaw Native Americans, and a candidate gene-association study showed linkage between the disease and polymorphic markers near *FBN1* (213).

### 153.5.9 Diagnosis

Each of the clinical features of MFS occurs with variable frequency in the general population. Occasionally, several will occur together by chance alone. In determining which of these individuals are affected by MFS, some other systemic connective tissue disorder, or no clear syndrome, more diagnostic reliance is placed on the presence of manifestations that are at once uncommon in the population but common in MFS (subluxed lenses, aortic dilation or dissection, dural ectasia) than on soft features (myopia,

**TABLE 153-4 Diagnostic Criteria for Marfan Syndrome: Revised Ghent Criteria**

In the absence of a family history	
Marfan syndrome is present if:	
[1] The aorta is dilated ( $Z \geq 2.0$ ) and ectopia lentis is present;	
[2] The aorta is dilated and a mutation in <i>FBN1</i> known to cause MFS is present;	
[3] The aorta is dilated and the systemic score* totals 7 points or more <sup>a</sup> ; or	
[4] Ectopia lentis and a mutation in <i>FBN1</i> known to be associated with a dilated aorta are present.	
In the presence of a family history	
Marfan syndrome is present if:	
[5] Ectopia lentis is present;	
[6] The systemic score* totals 7 points or more; or	
[7] The aorta is dilated ( $Z \geq 2.0$ if 20 years old or older; $Z \geq 3.0$ less than 20 years).	
*Systemic score	
Points	Sign
3	Wrist AND thumb sign
1	Wrist OR thumb sign
	Chest deformity
2	Pectus carinatum, or
1	Pectus excavatum or chest asymmetry
	Foot deformity
2	Hindfoot deformity, or
1	Pes planus
2	Pneumothorax
2	Dural ectasia
2	Protrusio acetabulae
1	Reduced US/LS AND increased arm span/height AND no severe scoliosis
1	Scoliosis or thoracolumbar kyphosis
1	Reduced elbow extension
1	Facial features—3 of the following 5: dolicocephaly, enophthalmos, downslanting palpebral fissures, malar hypoplasia, retrognathia
1	Skin striae
1	Myopia $\geq 3$ diopters
1	Mitral valve prolapse

Maximum total=20; having 7 or more points indicates systemic involvement US/LS = upper segment/lower segment.

<sup>a</sup>In the absence of discriminating features of Loays–Dietz syndrome or vascular Ehlers–Danlos syndrome.

Adapted from Loays, B. L., et al. (57).

mitral prolapse, tall stature, joint laxity, and arachnodactyly). The clinical diagnostic criteria last proposed (2010) by an international committee (57) and referred to as the ‘Revised Ghent Criteria’ are summarized in Table 153-4. These criteria make it less likely that, for example, the familial occurrence of scoliosis, pectus excavatum, and mitral valve prolapse, while meriting consideration of MFS, warrants that diagnosis (214), and more likely that these features may well represent the MASS phenotype (94). In comparison with the original Ghent criteria (215), more emphasis is placed on aortic dilatation, ectopia lentis and a mutation in *FBN1*. Less emphasis is placed on dural ectasia, mainly because a separate radiological imaging study is necessary to detect it. The major weakness of

the new criteria is the reliance on the ‘Z-score’ to define aortic dilatation, with a score of  $>+2.0$  being required; this represents a measurement greater than two standard deviations above the mean adjusted for age, sex and body surface area. Without any change in the aortic diameter, the interpretation of whether it is dilated or not in the same patient can vary as the patient grows, or gains or loses weight. Thus there is the possibility that the diagnosis of MFS can vary for purely trivial reasons. Additionally, very large individuals can have an aortic Z-score less than 2 despite the actual diameter being larger than 40 mm, which is considered pathologically dilated in the minds of most cardiologists (216).

Two specialized studies are required of any person in whom MFS is suspected: slit-lamp examination of the eyes and echocardiography. The former establishes whether ectopia lentis is present. Echocardiography is needed to determine whether MVP and aortic root dilation are present.

Conditions that must be excluded when considering the diagnosis of MFS include homocystinuria, contractural arachnodactyly, a number of the Ehlers–Danlos variants, and familial mitral valve prolapse or MASS phenotype. A variety of other conditions include one or more of the clinical features of MFS but would rarely be confused; these are listed in Table 153-5. The ‘marfanoid hypermobility syndrome’ (OMIM 154750) and the ‘neonatal MFS’ (217) are not separate entities; rather they represent exceptional variants of the classic phenotype.

Homocystinuria (see Chapter 92) must be excluded in any patient thought to have MFS because the pleiotropic manifestations of the two conditions include many of the same organ systems. Homocystinuria is ruled out effectively by a negative quantitative amino acid analysis of plasma (218). Patients with the pyridoxine-responsive form of homocystinuria may have a mild phenotype and escape detection until adolescence or adulthood, especially if they unwittingly take regular multivitamin supplements containing folate and B<sub>6</sub> (219). Such cystathionine  $\beta$ -synthase-deficient patients may have a negative qualitative urine screen for disulfides.

Congenital contractural arachnodactyly (CCA) is an autosomal dominant condition characterized by features recognizable at birth, including contractures of the digits, elbows, and knees; folding or ‘crumpling’ of the superior helix of the ear; and arachnodactyly (51). This condition is much less common than MFS. Some patients who clearly have MFS also have congenital contractures and abnormal ears. Thus any patient suspected of having CCA should have echocardiography and detailed ophthalmological examination. The joint contractures in CCA usually respond well to physical therapy begun early in life. Considerable risk of developing scoliosis requires persistent attention during childhood and adolescence in any person with CCA (220). Linkage of CCA to the *FBN2* locus has been clearly established (20), and point mutations occur in the EGF-like and TGF $\beta$ <sub>1</sub>-BP-like motifs of this gene (221).



**TABLE 153-5 Other Conditions Sharing Clinical Features with Marfan Syndrome**

<i>Skeletal</i>
Homocystinuria
Congenital contractural arachnodactyly
MASS phenotype (includes mitral valve prolapse syndrome)
Shprintzen–Goldberg syndrome
Osteogenesis imperfecta
Pseudoxanthoma elasticum
Eunuchoidism or delayed puberty
Klinefelter syndrome (47,XXY)
Trisomy 8 (47, XX or XY, 8)
Goodman camptodactyly syndrome B
Stickler syndrome
Syndrome of nerve deafness, eye anomalies, and marfanoid habitus
Nemaline myopathy
Syndrome of pigmentary degeneration, cataract, microcephaly, and arachnodactyly
Myotonic dystrophy
Multiple endocrine adenomatosis, type 2B
Achard syndrome
<i>Ocular</i>
Homocystinuria
Familial ectopia lentis
Weill–Marchesani syndrome
Ehlers–Danlos syndrome, type VI
Stickler syndrome
<i>Cardiovascular</i>
Shprintzen–Goldberg syndrome
Contractural arachnodactyly
Ehlers–Danlos syndromes, types I, II, and VI and other variants
Bicuspid aortic valve, aortic coarctation, and aortic aneurysm
MASS phenotype (includes mitral valve prolapse syndrome)
Osteogenesis imperfecta, types I and IV
Familial aortic aneurysm and familial aortic dissection (includes “Erdheim cystic medial necrosis”)
Syphilitic aortitis
Relapsing polychondritis
Ankylosing spondylitis
Reiter syndrome
<i>Skin</i>
MASS phenotype
<i>Dural ectasia</i>
Neurofibromatosis, type I
Lateral meningocele syndrome (387)

Patients with Ehlers–Danlos syndrome of the classic or hypermobility types (previously termed types I, II, or III) may be asthenic and have skeletal deformity (see Chapter 154). Mitral valve prolapse occurs frequently, and occasionally the aortic root may dilate somewhat (222). Generally, the body proportions are not disturbed, as in MFS. Severe joint laxity and skin hyperelasticity argue against MFS.

Persons with the MASS phenotype may have mitral valve prolapse, scoliosis, thoracic lordosis, asthenia, striae, and pectus excavatum, but they lack lens subluxation or severe aortic involvement. These features are heritable as an autosomal dominant trait (94).

The first steps in establishing the diagnosis should always be a careful medical history, family history and physical examination. Determination or rejection of the diagnosis of MFS on purely clinical grounds often must await the results of the detailed ophthalmological examination and echocardiogram. Understanding the molecular basis of MFS has greatly aided diagnosis in specific instances (166), but *FBN1* mutations cause conditions other than MFS, often the very conditions that clinicians are interested in excluding (Table 153-6).

Diagnosis by linkage analysis is rarely necessary today. Similarly, immunofluorescence assays of fibrillin incorporation into the ECM of cultured fibroblasts – so important in pointing to the cause of MFS (164) – have no value in diagnosis of patients with equivocal clinical findings.

Prenatal diagnosis is possible by mutation detection using DNA from amniocytes or chorionic villi (223). Mutation detection using DNA from single cells removed from preimplantation blastocysts is also feasible (194), and is being clinically applied (224,225).

### 153.5.10 Management

No specific therapy exists for the underlying defect in MFS. Therapeutic efforts are directed at first establishing an accurate diagnosis, determining which problems are present at diagnosis, anticipating the problems that will probably arise in the future, and pursuing certain prophylactic measures for specific problems. These patients should have one physician who is knowledgeable about the syndrome, who serves as the primary caregiver, and who refers to specialists as the need arises. One approach for guiding the overall management is an ‘integrated care pathway’ (226). While this approach may help the primary care physician who is unfamiliar with MFS, and does reduce inter-practice variation, no formal assessment of effectiveness has been performed.

Encouraging and facilitating adolescents with MFS to transition to self-management is a crucial aspect of long-term care (227–229).

**153.5.10.1 Skeletal.** All children with any evidence of scoliosis and all adults with a progressive deformity should be evaluated by an orthopedic specialist regularly, perhaps semiannually. Scoliosis of greater than 10° in children should be pursued aggressively if the curve progresses, with physical therapy and mechanical bracing. Bracing is not beneficial for curves greater than 40–50° and is unlikely to be effective for scoliosis of the thoracic spine in the face of concomitant thoracic lordosis (230). Spinal surgery is necessary when bracing fails to halt progression and in curves of greater than about 40° (64,230,231). In the past, spinal surgery in children stunted further growth of the segments of the vertebral column instrumented, and resulted in excessive trunk disproportion. Extensible spinal growing rods mitigate this problem (232). Issues to address in advance of surgical correction include the patient’s cardiopulmonary



**TABLE 153-6 Mutations in *FBN1* Associated with Phenotypes Other than Marfan Syndrome**

Amino Acid Change	Exon	Exon Type	Phenotype	OMIM No.	Reference
R122C	4	EGF(ncb)	Atypical skeletal, no cardiovascular	134797	(388)
G1127S	27	EGF(cb)	Familial aortic aneurysm	134797	(389)
C1223Y <sup>a</sup>	29	EGF(cb)	Shprintzen–Goldberg syndrome	182212	(358)
Frame shift	41	TB5 domain	MASS	604308	(184)
Deletion R-S-L-C-Y-R-N-Y	41	TB5	Weill–Marchesani	608328	(378)
T1696C	41	TB5	Geloephysic dysplasia	231050	(211)
A1728T	42	TB5 domain	Acromiric dysplasia	102370	(211)
E2447K	59	EGsdF(cb)	Familial ectopia lentis	129600	(308)
R2726W	64	CT	Familial tall stature	134797	(193)
W2756X	65	CT	MASS	604308	(390)

cb, calcium-binding; CT, C-terminus; EGF, epidermal growth factor–like; ncb, non–calcium-binding.

<sup>a</sup>This mutation also causes classic Marfan syndrome.

status, the adequacy of the bone to withstand fixation, and the presence of dural ectasia (66,233).

Scoliosis tends to progress most during rapid growth, especially in early adolescence. If this adolescent growth spurt can be shortened, the scoliosis may not progress so far. A second benefit of decreasing the length of time that the bones have to elongate is a reduction in adult body height. Girls can be treated before menarche with estrogen on a daily basis, to induce puberty and epiphyseal closure. Progestagen is added for five days each cycle to prevent dysfunctional bleeding. Alternative schemes for hormonal therapy also work. No conclusive data are yet available to show whether scoliosis is abated by this approach. Adult height has clearly been reduced in women who begin therapy before the menarche (234) (Figure 153-29).

Other orthopedic problems associated with joint instability should be managed by an orthopedic surgeon familiar with connective tissue disorders. The major indication for repair of anterior chest deformity is cardiopulmonary compromise, and repair should be deferred until late adolescence if possible (235). Repair early in life provides many years for rib growth to depress the sternum and re-establish the deformity. The so-called minimally invasive technique for repair of pectus excavatum has not been formally studied in MFS (236).

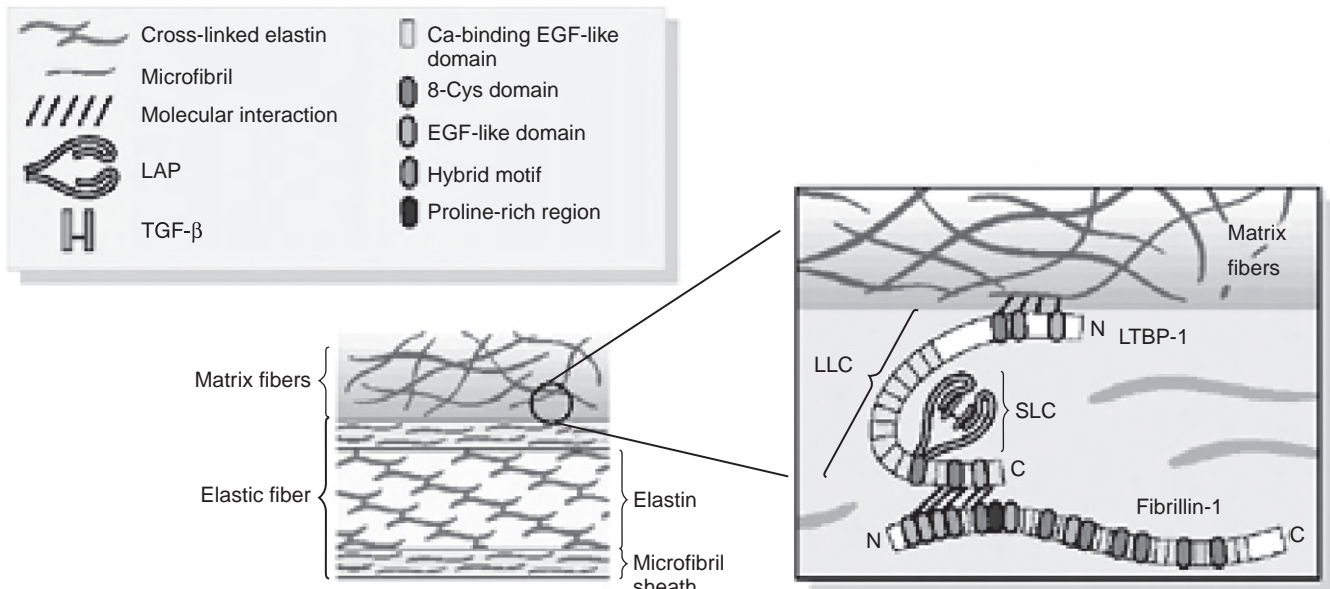
Even though many people with MFS complain of pain in the feet, few need aggressive therapy (237). Finding shoes to fit the long, narrow feet properly is a difficult and expensive chore, but one well worth the effort.

**153.5.10.2 Ocular.** The patient should be evaluated annually by an ophthalmologist experienced in connective tissue disorders. Emphasis should fall on correction of refractive errors and management of amblyopia; documentation of the direction and degree of lens subluxation; and detection of anterior chamber abnormalities, cataracts, glaucoma, and retinal detachment. Greater than 90% of patients can be refracted to vision better than 20/40 without lens surgery. Full correction should be pursued as early as possible and amblyopia treated aggressively.

All patients and their families should be counseled about the earliest symptoms of retinal detachment and the necessity of seeking evaluation immediately. Advances in the techniques of intraocular surgery have markedly improved the risk of postoperative retinal detachment to less than 5%, even in children (238,239). Similarly, the chances of a successful operative repair for retinal detachment have improved (240). The subluxed lens usually does not require surgery, except when adequate correction of the refractive error is impossible because of complex refractive errors, or because of phacodonesis, cataracts, phacolytic glaucoma or impending total lens dislocation (81). When the displaced lens is left in situ, optical correction can be achieved through the lens (phacically) or around the lens (aphakically). An aphakic correction can be facilitated by chronic use of atropine eye drops. Optical correction can be achieved through the use of glasses, contact lenses, or both. When the lens is removed, implantation of an artificial lens is a reasonable alternative. An artificial lens should not be implanted into the anterior chamber because of the high risk of endothelial cell loss secondary to excess lens mobility due to disparity in size.

Several approaches to surgical correction of myopia have appeared over the past decade. All involve sculpting the cornea to make it flatter, thereby counteracting the effects of the lens and the increased length of the globe. Typically in MFS, the cornea is already abnormally flattened (but not thin). The progressive nature of lens dislocation may prevent achieving long-term adequate correction. Thus corrective corneal surgery should not be performed in children. The adult patient desiring corneal surgery for myopia should be counseled by an ophthalmic surgeon well versed both in MFS and the techniques available; the diagnosis of MFS may be a contraindication.

**153.5.10.3 Cardiovascular. Routine Evaluation.** The frequency of cardiological evaluation depends on the severity of the manifestation. If the patient has only mitral valve prolapse and a mildly dilated aorta without any valvular regurgitation, an annual examination including

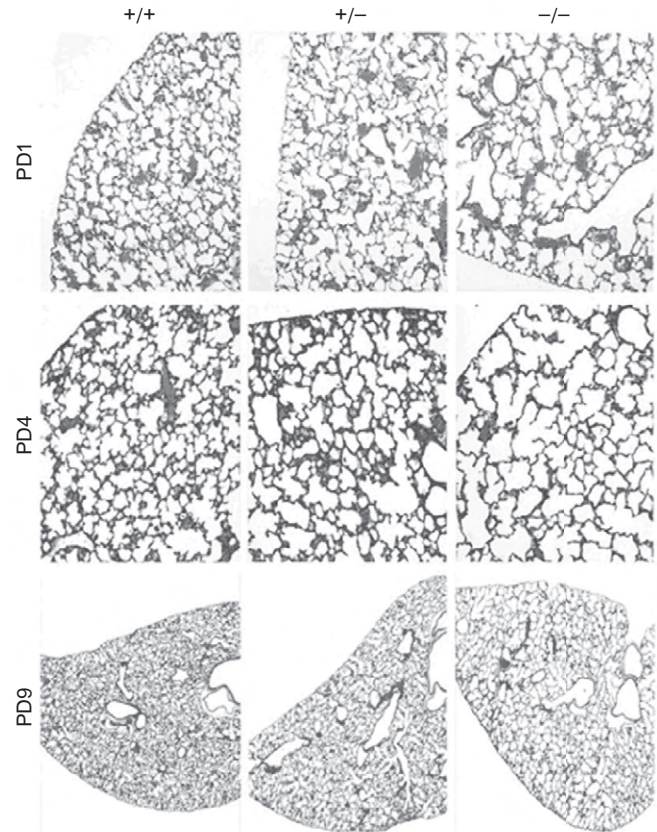


**FIGURE 153-29** Schematic interaction between TGF- $\beta$  and fibrillins. (From Kaartinen, V. & Warburton, D. (384), with permission.)

an echocardiogram is sufficient. As the aorta dilates and as valvular regurgitation appears and progresses, more frequent examinations are indicated. A periodic ECG is warranted for patients with known dysrhythmia, coronary artery disease, or ventricular dysfunction. Children, adolescents, and young adults with classic MFS and no dissection are very unlikely to have dilation elsewhere in their vasculature other than the aortic root, so routine angiography by CT or MR is not warranted.

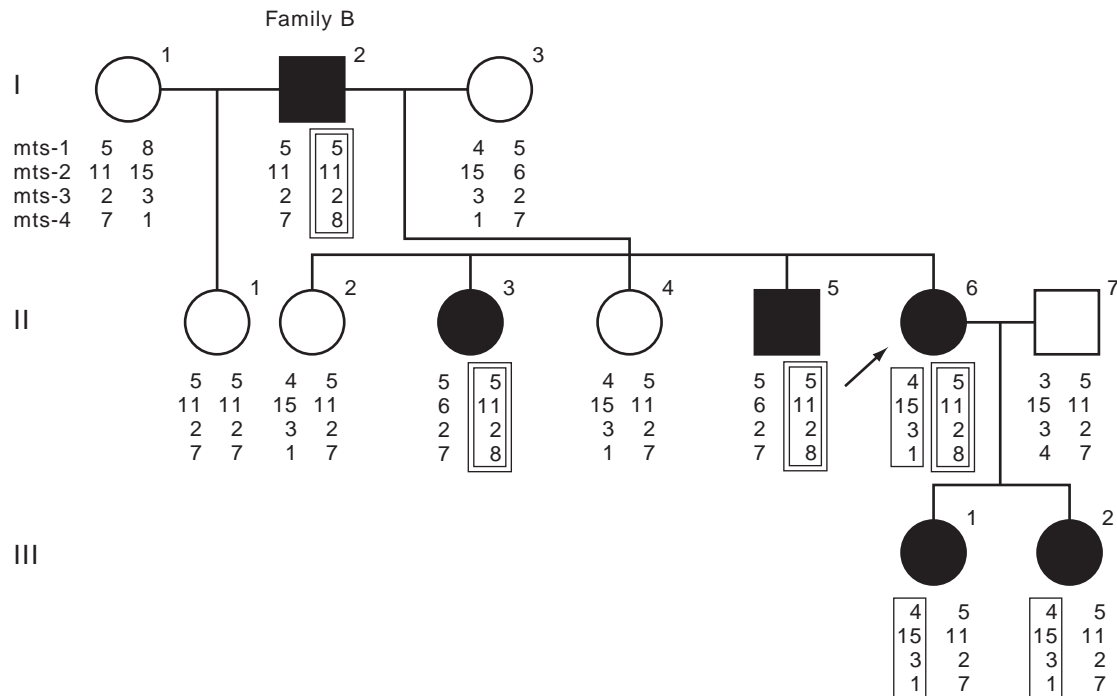
**Activity Modulation.** The dilated aorta is susceptible to rupture or dissection, either spontaneously or following modest trauma. The shearing forces of ventricular ejection may be the driving forces behind dissection and dilation. For these reasons, some restriction of patient activity seems warranted (241). Patients should not engage in contact sports, activities requiring maximal exertion, or isometric exercise. Most prepubertal children do not require stringent restrictions. However, their interests and activities should be channeled away from competitive athletics.

**$\beta$ -Adrenergic Blockade.** The use of  $\beta$ -adrenergic blockade to delay or prevent severe aortic complications was suggested four decades ago (242,243), but convincing evidence of its utility appeared much later (244–246). In a prospective randomized trial of propranolol in patients with mild to moderate aortic dilation, two beneficial effects of chronic  $\beta$ -blockade occurred: the rate of aortic root enlargement was lessened (Figure 153-30) and fewer patients developed aortic regurgitation or dissection during the course of the study (246) (Figure 153-31). Any patient with classic MFS should be considered for prophylactic therapy. Atenolol is the drug we currently use first; it is relatively cardioselective, is less lipophilic (so central nervous system side effects are minimal), and has a longer half-life than propranolol. In adults, we begin treatment at 50mg in the



**FIGURE 153-30** Pathology of lung development in a mouse model of MFS. The three rows represent 1, 4, and 9 days postnatal. The three columns are lung tissue from wild type, heterozygous, and homozygous animals. Note the larger air spaces in mice carrying a mutation, due to impaired differentiation of terminal alveolar septae. (From Neptune, E. R., et al. (150) Copyright Nature Publishing Group, with permission.)

morning and advance the dose (giving an evening dose as the next step), to keep the resting heart rate at less than 60beats/min and the heart rate during moderate



**FIGURE 153-31** Pedigree in which both classic Marfan syndrome and a milder connective tissue disorder segregate. Individuals II-6, III-1, and III-2 have Marfan syndrome, linked to *FBN1* (haplotypes of polymorphic markers shown below pedigree symbols). Individuals I-2, II-3, and II-5 have not inherited or passed on the same *FBN1* allele that occurs in Marfan relatives, but have mild skeletal and cardiovascular features that suggested Marfan syndrome to their physicians. Whether these individuals also have a mutation in *FBN1* or another component of the ECM is still under investigation. (From Pereira, L.; Levrin, O.; Rameriz, F., et al. *Diagnosis of Marfan Syndrome: A Molecular Approach for Stratification of Cardiovascular Risk within Families*. N. Engl. J. Med. **1994**, 331, 148–153.)

exercise at less than 100beats/min. Because of marked variation in individual responsiveness to all  $\beta$ -adrenergic blocking agents, the dose must be titrated for patients of any age. We begin treatment in children as soon as possible. As noted above, circulating levels of TGF- $\beta$  are increased in people with MFS. Chronic treatment with a  $\beta$ -blocker (or an angiotensin receptor blocker) reduces the serum level of TGF- $\beta$  (206).

Chronic treatment with  $\beta$ -adrenergic blockade reduces left ventricular inotropy, which in turn reduces  $dP/dt$  in the aorta (247). In addition, we and others (96,248) have shown that chronic  $\beta$ -adrenergic blockade tends to normalize the compliance of the aortic root in people with MFS. Presumably this response is beneficial, but this notion has not been tested in long-term follow-up. Moreover, the improvement in stiffness is not uniform in all patients (248), and patients with less aortic root dilation may respond best (249,250).

The adverse effects of long-term therapy with  $\beta$ -adrenergic blockers must be considered and potentially monitored. Serum high-density lipoprotein levels are reduced slightly. There is an increased risk of developing type 2 diabetes mellitus, although the risk is small, it can be monitored, and it has been documented only in patients taking  $\beta$ -blockers because of hypertension. People with MFS often complain of fatigue and diminished libido (160); the contribution of medications to these symptoms is unclear.

**Other Medical Therapy.** Not all people tolerate  $\beta$ -adrenergic blockade. Whether alternative medications have salutary effects on the aortic root remains an open question. In the past, we and others used verapamil, a calcium channel blocker of the non-dihydropyridine class, with modest negative inotropic activity, as a first alternative to  $\beta$ -blockade. Recently, utilizing mouse models of MFS, Dietz and colleagues demonstrated convincingly that treatment from early in life with any of the classes of calcium channel blocker greatly accelerated aortopathy [Dietz HC. Personal communication]. A study is currently underway to investigate this issue retrospectively in humans. Until that study produces results, it would be prudent to avoid calcium channel blockers in MFS for any reason.

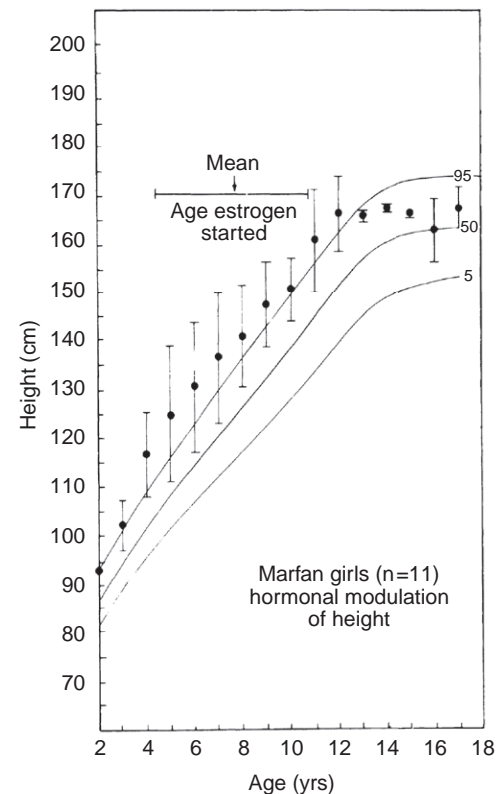
Losartan, an angiotensin receptor blocker (ARB), has several additional effects that reduce local effects of TGF- $\beta$ . Because of the evidence of excess TGF- $\beta$  activity in the pathogenesis of MFS, losartan was given to neonatal Marfan mice, with beneficial reduction in aortic medial pathology (208). When therapy with losartan was delayed until the MFS mice were 2 months old (adolescence to a mouse), the already dilated aorta reverted to normal caliber and histopathology. Dietz and colleagues treated 18 severely affected young MFS patients with an ARB. These patients were on optimal doses of  $\beta$ -adrenergic blockade, but had progressive dilatation of their aortic roots. Treatment with an ARB



(losartan in 17 instances) resulted in stabilization of dilatation in all cases (251). These results have stimulated half a dozen human trials of ARBs of various designs around the world (252). The largest and most advanced is supported by the National Heart, Lung and Blood Institute and completed recruitment of subjects in early 2011. Young subjects with considerable dilatation of the aortic root were randomly assigned to take atenolol or losartan for 3 years (209). Until the results of this and other clinical trials of ARBs in humans with MFS are completed, caution should be used before recommending this class of drug as an alternative to  $\beta$ -adrenergic blockade.

**Cardiovascular Surgery.** The proximal aorta was recognized as being at risk for dilation and dissection in MFS in 1943. Disease of the aorta was documented as a major problem in 1955, and as the leading cause of death in MFS by far in 1972. Until relatively recently, however, no reliable surgical approach to correcting aortic pathology was available. The first attempts to repair the aortic root in any condition were not reported until 1964. When applied to MFS, both short-term (operative) and, for the fortunate few who left the hospital, long-term results were discouraging. The aneurysmal tissue was difficult to repair, all of the sinus of Valsalva could not be resected, and the tissue was friable. A major advance occurred in 1968 with the report of Mr. Hugh Bentall, a surgeon in London (253). He devised the ‘composite graft’, in which a prosthetic aortic valve is sutured into one end of a Dacron conduit; this permitted resection of the entire aortic root aneurysm and aortic valve, with suturing to the tough aortic annulus and the distal ascending aorta. While nearly a decade was required for this technique to be widely applied in MFS in the United States and Europe, the short-term results were so encouraging that no controlled trial evaluating different techniques was ever performed (Figure 153-32).

Surgical correction of the proximal aorta and aortic valve is required in several situations: in an acute emergency due to rupture, dissection, or severe left ventricular decompensation; when early signs of left ventricular strain appear in the presence of severe regurgitation; and for enlargement of the aortic root even if the aortic regurgitation is mild or absent. Use of the composite graft (Figure 153-33) resulted in a dramatic decrease in perioperative mortality of aortic surgery in MFS (254,255). Through early 2011, none of more than 350 patients who underwent elective repair of the aortic root for MFS at the Johns Hopkins Hospital failed to leave the hospital (D. Cameron, personal communication). A prosthetic valve requires lifetime anticoagulation. In young children and for those with a strong contraindication to coumarin derivatives, the use of a fixed homograft (aortic valve and proximal aorta, from a human cadaver) works well; the major concern is loss of pliability of the aortic cusps with time. Pulmonary autografts (the so-called Ross



**FIGURE 153-32** Hormonal modulation of height in girls affected by Marfan syndrome. In all cases, treatment with ethinyl estradiol (0.05 mg/10 kg/day) and conjugated estrogen (Premarin, 10 mg, days 25–28, each menstrual cycle) was begun before menarche. Therapy was continued until the time menarche was likely to have occurred naturally or until bone maturation was well advanced, whichever occurred first. Comparison with Figure 153-6B shows early cessation of growth and reduction in height of the treated girls.

procedure) (256) are not recommended because the main pulmonary artery contains the same medial degeneration as does the dilated aortic root.

Beginning in 1983 with the work of Sir Magdi Yacoub in London and greatly advanced by Dr. Tirone David in Toronto, various techniques to preserve the native aortic valve while replacing the dilated sinuses of Valsalva and proximal ascending aorta have emerged (257–259). Results of valve-sparing employing the ‘reimplantation’ approach have been quite encouraging (258,260–264). The notion of preserving the aortic valve is appealing for anyone faced with lifelong anticoagulation, but particularly so for young women who wish to attempt pregnancy and for anyone with a relative contraindication to warfarin. However, no randomized trial has been performed (and would be difficult in any event), and even comparing the results among centers and surgeons is difficult because of different techniques and indications. However, a registry of aortic surgical results was established by the National Marfan Foundation in the USA; analysis of the intermediate-term results of several hundred patients found that root repair by the reimplantation technique (David V approach) was an excellent alternative to the composite graft (265). The best candidates



are those with no or minimal aortic regurgitation and an aortic root dimension of 45–55 mm. If a patient has a family history of aortic dissection, the criterion for prophylactic repair should be 50 mm or lower. For children, two criteria are useful: when the aortic root dimension exceeds twice that predicted for the age and body surface area (93,95) or twice the diameter of the distal ascending aorta or proximal descending aorta. Reimplantation remodeling is effective in children with MFS (266).

Occurrence of aortic dissection before repair of the aortic root, whether the initial dissection involved the entire aorta (type A) or just the descending portion (type B), decreases life expectancy (255,267). The reduced life expectancy is due to multiple factors, including the morbidity and mortality of further dissection and that of subsequent aortic surgery. On a positive note, the techniques for repairing extensive dissections involving the aortic arch, which involve deep hypothermic total circulatory arrest (268,269), and the abdominal aorta (270) have greatly improved. In a number of instances, patients have had the entire aorta replaced by prosthetic material (271). Recently, endovascular stents have been used in abdominal aortic aneurysms (AAA) and thoracic dissections due to inflammatory and atherosclerotic disease (272). While such an approach is of potential benefit because of the avoidance of major surgery, its use in MFS is not to be recommended at this time, despite rare reports of short-term success (273).

Some patients require mitral valve surgery alone, or in addition to aortic surgery. In most cases, repair rather than replacement of the mitral valve is possible, with acceptable long-term outcome (274,275).

After any repair of the aorta, patients should be maintained on chronic  $\beta$ -adrenergic blockade. Additional medications should be employed aggressively to control blood pressure in the normal or low-normal range. As noted above, calcium channel blockers may have long-term deleterious effects, although this needs to be documented in humans. Likewise, a disturbing number of MFS patients who had prophylactic root repair and were treated post-operatively with an ARB have subsequently developed acute dissection of the descending thoracic aorta (type B) (Craig Miller & David Liang, personal communication). This anecdote requires confirmation, but brings into question what additional medications might be employed. An inhibitor of angiotensin-converting enzyme might be the next choice.

Function of the prosthetic aortic valve and the left ventricle should be monitored by echocardiography at least annually. The entire aorta should be examined by CT or magnetic resonance angiography before surgery (276). If no occult dissection or dilation beyond the root is present, examination every 2 years is warranted. In one study, 43% of patients who had had prophylactic aortic root repair developed some degree of dilation of one or both coronary ostia (277); these tended not to progress, but exceptions are reported (278). The risk is

lower when the ostia are reimplanted with the button technique rather than the original Bental wrap approach (279). Following aortic root repair, the rate of dissection of the distal ascending aorta is low, so that routine, concomitant replacement of the transverse arch may not be warranted (280). Complications requiring reoperation are higher in any patient who had a type A dissection at the time of initial repair (255,281).

Only a small number of persons with MFS have undergone cardiac transplantation; survival was about 50% after nearly 3 years (282).

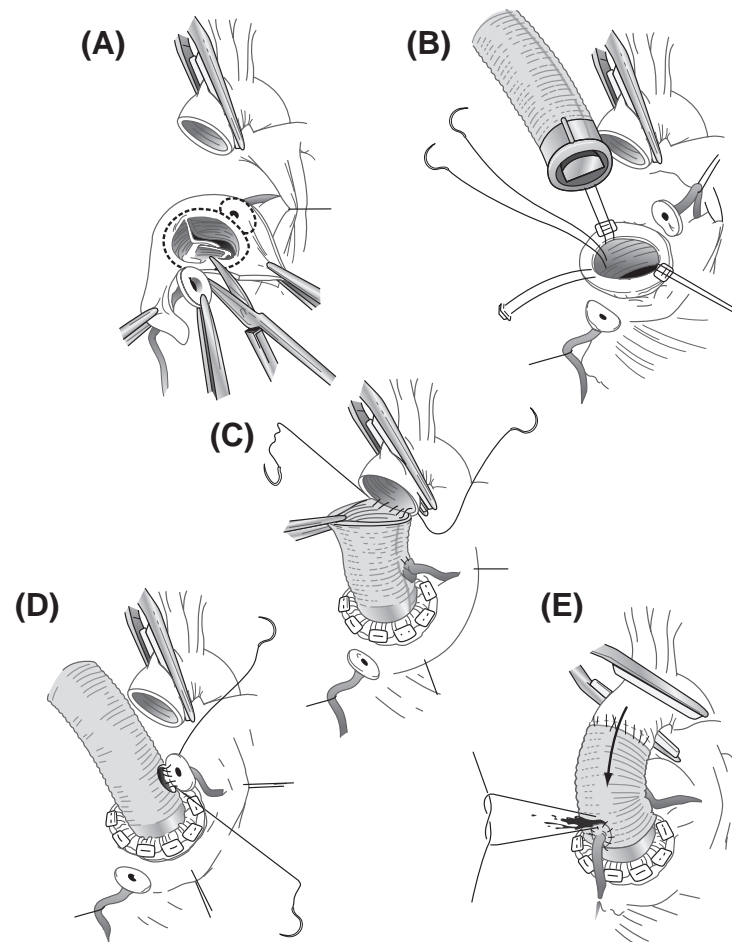
**Endocarditis Prophylaxis.** All persons with MFS are potentially at increased risk of endocarditis because of the abnormal ECM of the cardiac valves, regardless of whether demonstrable valvular abnormalities exist. However, at this time only patients with mild or worse valvular dysfunction should be instructed to practice routine (oral) antibiotic prophylaxis at the time of dental, genitourinary, and selected other procedures (257). Patients who have undergone uncomplicated composite graft repair or valve-sparing surgery may not be at any more risk for endocarditis than preoperatively. More aggressive prophylaxis involves parenteral antibiotics, such as injected ampicillin plus gentamicin; for those allergic to penicillins, vancomycin can be substituted.

**Pregnancy.** In the general population, most dissection of the aorta in young people occurs in pregnant women; all the factors are not certain, but hormonal changes, increased blood volume, increased cardiac output, and the sudden volume surge of autotransfusion as the uterus contracts postpartum probably contribute. All women with MFS are at further increased risk of aortic dissection during pregnancy, especially if the aortic root dimension is greater than 40 mm (114). However, most women with MFS suffer no major complication (*vide supra*) (112,114,116,283). What criteria might define women at the highest risk? We suggest the issues listed in Table 153-7 be explored in any woman with MFS of childbearing age. We perform echocardiography and cardiovascular assessment every 6–8 weeks during pregnancy.

Whether to keep a pregnant woman on  $\beta$ -adrenergic blockade as she attempts to conceive and during pregnancy is not settled (79). My personal preference is not to expose the early fetus to medication unless a strong reason, such as supraventricular tachydysrhythmia, occurs.  $\beta$ -Blockade can be instituted during the second half of gestation, with virtually no teratogenic risk. At delivery, the effects of fetal bradycardia and hypoglycemia can be managed by the neonatologist.

Although clinical series have arrived at different conclusions, cervical incompetence occurs with an increased frequency in women with MFS, and this, in large part, accounts for the reports of spontaneous fetal loss and preterm delivery (114).

Delivery should be by whatever approaches to anesthesia, volume support, and parturition are least hemodynamically stressful to mother. Cesarean section is



**FIGURE 153-33** Technique of composite graft repair of the aortic root. A, Button of aortic wall surrounding each coronary ostium is trimmed. B, Composite graft is sutured to the aortic annulus after excision of the native aortic valve. C, Distal anastomosis is constructed to the ascending aorta proximal to the innominate artery. D, The coronary ostial buttons are next sutured to holes cut in the woven Dacron of the conduit. E, The aortic clamp is released, permitting retrograde flow of blood, and air is evacuated from the graft. The patient can then be weaned from cardiopulmonary bypass. (From Coselli, J. S.; Crawford, E. S. *Composite Aortic Valve Replacement and Graft Replacement of the Ascending Aorta Plus Coronary Ostial Reimplantation*. Semin. Thorac. Cardiovasc. Surg. 1993, 5, 55–62, with permission.)

not indicated for other than standard obstetric reasons. Excessive postpartum hemorrhage associated with uterine inversion can occur (115).

**153.5.10.4 Pulmonary System.** Because of the risk of pneumothorax, people with MFS should be counseled to avoid rapid changes in atmospheric pressure such as occur during scuba diving and flying in unpressurized aircraft. Management of pneumothorax follows standard guidelines (284) with the caveat that recurrence is highly likely and more aggressive initial therapy should be considered. Because MFS predisposes to developmental anomalies of the lung that resemble emphysema, all patients should be counseled even more rigorously than is typical against smoking. The potential for atlantoaxial rotatory instability should be considered during tracheal intubation and positioning for surgery (69); however, this is more of a problem for patients with Loeys–Dietz syndrome (285).

**153.5.10.5 The Dura.** Dural ectasia can cause radicular pain in the buttocks and legs; nerve root paralysis affecting bowel and bladder function and motor

**TABLE 153-7 Risk Factors for Cardiovascular Complications of Pregnancy**

Aortic root dilation (measures of >40–42 mm carry increasing risk)
Rapid rate of increase of aortic root dimension (>5 mm/6 mo)
Aortic regurgitation
Preexisting aortic dissection
Mitral regurgitation of moderate severity or worse
Left ventricular dysfunction, especially dilated cardiomyopathy
Any other structural cardiovascular abnormality
History of dysrhythmia
Hypertension or preeclampsia
Family history of aortic dissection
Risk of aortic dissection greatest during second half of pregnancy and for several months postpartum

function in the legs; and dull, unremitting discomfort in the lower abdomen and pelvis. In a woman with the latter complaints, pelvic ultrasound may show a cystic mass that is interpreted as an ovarian cyst. Gynecological surgery should never be performed in a woman with

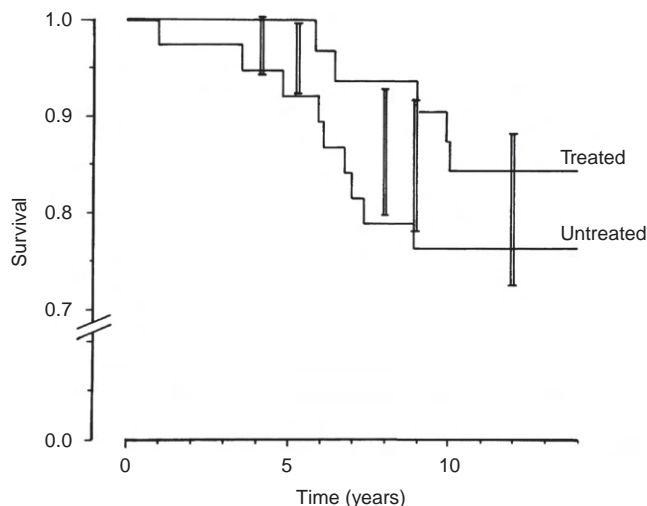
MFS before considering the presence of anterior meningoceles. A variety of treatments have been attempted for symptomatic dural ectasia, and none is consistently effective. Pain relief can be obtained sometimes by non-traditional analgesics, such as antidepressants and antiepileptics. Surgical placement of shunts draining CSF into the peritoneum can decompress meningoceles and reduce stress on nerve roots. Rarely can the neural sacs be tied off or resected because of the nerve roots that track over the dura. Medications that reduce CSF pressure, such as acetazolamide, have not been subjected to clinical trial.

### 153.5.11 Counseling

One major issue in genetic counseling is straightforward, each affected person having a 50% probability of passing the allele to any offspring. When MFS is diagnosed in a young child and the family history is negative, the parents must be carefully examined to ensure that neither has signs of MFS. If both parents are phenotypically normal, considerable reassurance can be given that the risk of future affected offspring is slight (see discussion of germinal mosaicism, *vide supra*).

Women affected with MFS must deal with two concerns. The first is the 50% risk that any offspring will inherit the syndrome. The second concerns the risk of cardiovascular problems during pregnancy. Parents should be counseled about the range of intrafamilial variability in MFS. Affected offspring may be more or less severely involved than their parents.

The complex issues in transitioning responsibility of care from parent to affected child should be addressed longitudinally over a period of years (227–229).



**FIGURE 153-34** Effectiveness of chronic propranolol therapy in reducing cardiovascular events in Marfan syndrome. Subjects were followed until the study was terminated by death, developing aortic regurgitation, or dissection, or need for aortic surgery.  $\beta$ -Adrenergic blockade was associated with consistent protection, especially in the middle years of the trial.

Genetic counseling sessions are also an opportunity to address a person's perception of MFS – its seriousness and effects on lifestyle, occupation, and psyche. Patients have to grapple with the prospects of lifelong medication and modulation of exercise and lifestyle (286–288). One study found compliance with recommendations about medication and activity approached 80% and began to identify issues associated with noncompliance (160,287). Patients may be more willing to discuss compliance with medication and adverse effects of medication with a counselor (250).

Support groups for MFS have been established in many countries. The National Marfan Foundation in the United States maintains web-based resources (<http://www.marfan.org>), and sponsors national meetings for patients and families and periodic international gatherings of investigators. The International Federation of Marfan Syndrome Organizations (<http://marfanworld.org>) also exists.

### 153.5.12 Life Expectancy

Compared to the natural history study of nearly three decades ago (59,168), an examination of contemporary clinical history shows a marked improvement of about 25% in life expectancy for men and women (288–290) (Figure 153-34). This gain is due primarily to both better survival after cardiovascular surgery and early, prophylactic surgical intervention (255). Viewed another way, over the past 30 years, life expectancy has increased by about 30 years (291).

## 153.6 DISORDERS RELATED TO MARFAN SYNDROME THROUGH PHENOTYPE, ETIOLOGY OR PATHOGENESIS

### 153.6.1 MASS Phenotype (OMIM \*157700)

Concern about the possible diagnosis of MFS is a common reason for referral to medical genetics clinics (292). Many such probands have one or more features of MFS but fail to meet strict criteria (see Table 153-4). We conducted a systematic examination of such referrals to one clinic and found that many patients have clear evidence of a systemic disorder of connective tissue that merges with the mild end of the Marfan phenotypic spectrum (94). The acronym MASS represents common (but not essential) involvement of the mitral valve, the aorta, the skin and the skeleton.

MVP as an echocardiographic entity occurs in perhaps 2.5% of the general population (293), is more common among women, and is more common in adolescence and young adulthood than in childhood or old age. MVP is an autosomal dominant trait in many families. Based on standard transthoracic echocardiographic evaluation, two classes of MVP can be defined: those with abnormal

leaflet motion but normal leaflet morphology; and those with both prolapse and thickening of the leaflets, which correlates with the degree of myxomatous degeneration (294). The latter is sometimes termed **primary MVP**; is typical of the MVP in most heritable disorders of connective tissue, including MASS phenotype; and is more likely to be associated with clinical complications. In some people, MVP is associated with deformity of the chest wall or the vertebral column, or both. In our study, patients also often had an aortic root dimension near the upper limit of normal for age and body surface area (but no dissection or apparent progressive enlargement), myopia (but by definition no ectopia lentis), joint hypermobility (in addition to pectus excavatum and scoliosis), and striae atrophicae. Although inheritance was not specifically explored, some probands had a family history of similarly affected relatives. Subsequently, pneumothorax has been seen in a number of patients. Thus, the MASS phenotype is not MFS, although in some instances it certainly resembles what one would expect a mild variant of MFS to be. The MASS phenotype seemingly has a good prognosis, with the worst manifestation being a predisposition to progressive MVP and mitral regurgitation. Whether and how ‘familial MVP’ and MASS phenotype should be distinguished have not been clarified (183).

The MASS phenotype is likely heterogeneous in etiology, hence the term **phenotype**, rather than syndrome. A very few patients with a mutation in *FBN1* qualify for a MASS phenotype diagnosis (187,195). One example is a patient with a mutation that results in little abnormal fibrillin monomer and is close to a null allele. In general, mutations near the 3'-terminus of *FBN1*, little expression of the mutant allele, and in vitro deposition of fibrillin in the ECM greater than 70% of normal are all associated with a milder MFS-like phenotype (183,187,195). However, other patients with missense mutations near the 3' end of *FBN1* have had a severe, classic MFS phenotype. Meanwhile, other pedigrees that could be characterized as MASS are not linked to *FBN1* (295,296). Moreover, three loci for familial MVP have been mapped, Xq28, 13q31.3–q32.1, and 16p11.2–p12.1 (297–299). Finally, a family with MVP inherited as an autosomal dominant trait showed linkage to 10q32 (Yuan, unpublished). Some of the relatives, including a few with documented MVP, developed idiopathic dilated cardiomyopathy; a previous family with autosomal dominant cardiomyopathy had been mapped to the same locus (300).

### 153.6.2 Ectopia Lentis (OMIM \*129600)

Pedigrees with autosomal dominant ‘isolated’ ectopia lentis without deformity of the pupil have been described for many years. Pleiotropic manifestations suggestive of a systemic connective tissue disorder were not noted. In addition, ectopia lentis exists as an autosomal recessive trait without signs of a systemic connective tissue disorder (OMIM 225100) (301). Recently, however, attention

has been redirected to identifying families and to determining etiology because of the discovery of *FBN1* mutations in MFS. Linkage to *FBN1* was found (20,302) and mutations reported (303–306). Interestingly, most of the families reported with mutations in *FBN1* come close to qualifying for the Marfan diagnosis, in that relatives with ectopia lentis have tall stature, scoliosis, joint laxity, or pectus deformity. In several families, a mutation in *FBN1*, R240C, was found associated with ectopia lentis (307), while the same mutation was found in a family with classic MFS (308). Faivre and colleagues examined 146 adults who did not meet diagnostic criteria for MFS (using the old Ghent criteria) but who had an apparently pathological mutation in *FBN1* (309). Of those whose only major manifestation of MFS was ectopia lentis, most had missense mutations involving cysteine, none were truncating, and few occurred in the middle exons of the gene. This latter study emphasizes that at least some families ascertained through ectopia lentis should be followed for the possible development of cardiovascular features.

Mutations in *ADAMTSL4*, which encodes a metalloproteinase, occur in a consanguineous family with recessive ectopia lentis (310).

### 153.6.3 Loeys–Dietz Syndrome (OMIM 609192)

Some patients originally diagnosed as MFS were investigated from a molecular perspective because they lacked mutations in *FBN1* and showed features atypical of MFS, such as hypertelorism, bifid uvula or actual cleft palate, intellectual delay, and tortuosity (unwinding) of the thoracic aorta and other arteries with dilation and dissection (121,285,311). This condition is due to mutations in two of the receptors for TGF- $\beta_1$ , *TGFBR1* and *TGFBR2*.

Skeletal features that are particularly common include talipes equinovarus, atlantoaxial instability, scoliosis, spondylolithesis and dural ectasia (312). Patients do not have or develop ectopia lentis. Some develop autoimmune disorders of the gastrointestinal system. Aortic dissection tends to occur at smaller aortic root diameters than in MFS, so patients need to undergo prophylactic repair at younger ages (if children) and at diameters around 40mm in adults (266,313).

### 153.6.4 Familial Aortic Aneurysm and Dissection (OMIM 132900, 607086, 607087, 611788, 613780)

Familial, non-syndromic disease of the thoracic aorta, whether predisposing to aneurysm or dissection Thoracic aortic aneurysms and dissections (TAAD), is much more common than generally recognized (314,315). While inheritance may not be clear from the small pedigrees that come to attention clinically, in many families



predisposition to thoracic aortic disease is an autosomal dominant trait (316). Analysis of fibroblast cultures of skin or aorta in some patients has revealed mutations of *FBN1* in a few cases (183,316,317). However, mutations in *FBN1* and *COL3A1* account for a small minority of such families (318,319). Linkage analyses in families has identified a number of loci (320–323), including *TGFBR1* and *TGFBR2* (Table 153-8). For these two genes, the most severe end of the phenotypic spectrum is the Loeys–Dietz syndrome (*vide supra*), but some families have features largely restricted to the thoracic aorta. In families with *TGFBR1* mutations, men died earlier from TAAD, whereas females tended to develop aneurysms and dissections of branch arteries. In families with *TGFBR2* mutations, while the genders behaved similarly, type A dissection occurred in aortic roots dilated less than 50 mm (324).

In a large Dutch family, in which arterial aneurysms and dissections co-segregated with osteoarthritis of early onset, a mutation in *SMAD3* was found (325). This mutation, and two others in two additional families with the same phenotype, all disrupted the MH2 domain of the *SMAD3* protein, which is crucial for proper oligomerization with *SMAD4*. Presumably, TGF- $\beta$ -mediated gene activation is impaired.

In an important change in the way we think about these conditions, Dr. Dianna Milewicz and colleagues recognized that mutations in three genes (*ACTA2*, *MYH11*, and *MYLK*) disrupt intracellular cytoskeletal molecules and their interactions with integrins and the ECM in vascular smooth muscle cells (326,327). Many mutations predispose not only to dilatation of the thoracic aorta and occasionally cerebral arteries, but non-atherosclerotic occlusion of coronary, cerebral and visceral arteries (327).

### 153.6.5 Arterial Tortuosity Syndrome (OMIM#208050)

The condition initially was described in infants, often from consanguineous families. Some of the young patients had features of cutis laxa, and many died in early childhood from a variety of cardiovascular problems including stroke and severe peripheral pulmonic stenoses. Eventually adults were recognized with the same, albeit milder, phenotype including marked elongation and tortuosity of the aorta and its major branches, and a variety of signs of a systemic connective tissue disorder (joint hypermobility, soft, doughy skin) (328). The typical facial features include an elongated face, retrognathia, down-slanting palpebral fissures, blepharophimosis and a beaked nose (329).

Arterial tortuosity was mapped in consanguineous families to 20q13 (330,331). Mutations in the gene (*SLC2A10*) encoding a nuclear glucose transporter, *GLUT10*, were found in several patients (329). Obligate heterozygotes have normal vasculature. The pathogenesis

seems to involve transcriptional regulation of several ECM proteins that regulate TGF $\beta$  signaling; with the result that TGF $\beta$  activity is accentuated in the vascular wall.

### 153.6.6 Phace(s) Association (OMIM 606519)

Another condition with multiple anomalies, known by the acronym PHACE(S) association (posterior fossa abnormalities of the brain, hemangiomas, arterial, cardiac, and eye defects, and sternal clefts), predisposes to arterial tortuosity, aortic dilation, and aortic rupture (332,333). The association overwhelmingly affects females, no familial instances are reported, and the etiology is unknown.

### 153.6.7 Bicuspid Aortic Valve (OMIM 109730)

The tetrad of bicuspid aortic valve, coarctation of the aorta, MVP, and ascending aortic aneurysm is increasingly well recognized as autosomal dominant with variable expression (334). In some families, one or more of the aortic findings occur in relatives of a proband with a more severe, congenital left-sided flow defect, such as hypoplastic left heart. In patients ascertained through a bicuspid aortic valve, defects of the ascending aortic wall should be considered (335–338). The dilatation typically spares the sinues (in distinction to MFS), and is most pronounced in the mid-ascending aorta and into the arch. Because transthoracic (i.e. standard) echocardiography often does not visualize the mid-ascending aorta, patients with a bicuspid aortic valve should have a CT or MR of the thorax to establish if dilatation is present, and if so, to be followed by an adequate imaging modality. The histopathology of the aortic wall typically shows medial degeneration, indistinguishable from MFS. In aortic media in both conditions, the C-terminal fragment of filamin A is abundant, likely due to increased activity of the protease, calpain A (339).

Bicuspid aortic valve clearly predisposes to type A dissection because of the involvement of the ascending aorta, but the criteria for when to operate solely for the aneurysm (rather than a dysfunctional valve) are not well established. In many instances of relatively normal valve function, a valve-sparing ascending aortic repair, typically involving hemi-arch replacement, is feasible. The long-term outlook of such cases, especially with regard to function of the aortic valve, remains to be determined. Similarly, any patient who is to undergo surgery for a dysfunctional bicuspid aortic valve should be considered for concomitant aortic root repair, especially if the maximal diameter exceeds 45 mm (340,341). Because the embryological derivation of the pulmonary and aortic trunks is the same, the Ross procedure is not the best treatment for patients who require surgery for their aortic valve and aortic root disease (342,343).

**TABLE 153-8 Human Hereditary Aneurysm Conditions and Murine Models of Aneurysm**

Gene (Protein)	Human Aneurysmal Syndrome	Animal Model Phenotype	Pathway Implicated
<i>Extracellular matrix protein</i>			
<i>FBN1</i> (fibrillin-1)	Marfan Syndrome – Fully penetrant ascending aortic aneurysm	KO: Perinatal lethality, Pulmonary hypoplasia, arteriopathy Hypomorphic: Arteriopathy, aneurysm, dissection	TGFβ
<i>EFEMP2</i> (fibulin-4)	Cutis Laxa with Aneurysm – Ascending aortic aneurysm and tortuosity	KO: Ascending aortic aneurysm, defective elastogenesis, perinatal lethality Sm22-Cre (smooth muscle) KO: Ascending aortic aneurysm	TGFβ
<i>ELN</i> (Elastin)	Cutis Laxa with Aneurysm- low penetrance ascending aortic aneurysm and dissection	Haploinsufficient: Obstructive arterial disease with increased VSM proliferation, increased lamellae number KO: Accentuated phenotype	Unknown
<i>COL1A1</i> (Collagen 1 alpha-1)	Osteogenesis Imperfecta – extremely rare aortic aneurysm Ehlers Danlos Syndrome, type 7A- dissection of medium size arteries	KO: Adult onset aortic aneurysm and dissection	Collagen Metabolism
<i>COL1A2</i> (Collagen 1 alpha-2)	Osteogenesis Imperfecta – extremely rare aortic aneurysm Ehlers Danlos Syndrome–cardiac valvulodystrophy type 7B– borderline aortic root enlargement with aortic regurgitation	Homozygous LOF: Decreased body weight, bony abnormalities, no arterial phenotype reported	Collagen Metabolism
<i>COL3A1</i> (Collagen 3 alpha-1)	Ehlers–Danlos, type 4 – frequent arterial dissection with infrequent aneurysm	KO: Frequent neonatal mortality, aortic rupture, intestinal rupture	Collagen Metabolism
<i>COL4A1</i> (Collagen 4 alpha-1)	Hereditary angiopathy, nephropathy, aneurysms, and muscle cramps – infrequent aneurysms	KO: Embryonic lethal (E10.5-11.5), basement membrane failure	Collagen Metabolism
<i>COL4A5</i> (Collagen 4 alpha-5)	X-linked Alport syndrome – ascending aortic and abdominal aneurysms and dissections	Nonsense mutation: No overt aortic disease noted	Collagen Metabolism
<i>LOX1</i> (lysyl oxidase 1)	No human phenotype described	KO: Low penetrance aortic aneurysm, perinatal lethality	Collagen Metabolism TGFβ
<i>PLOD1</i> (lysyl hydroxylase 1)	Ehlers–Danlos, type 6–rare aneurysm	KO: Spontaneous aneurysm and dissection, gait abnormalities	Collagen Metabolism
<i>PLOD3</i> (lysyl hydroxylase 3)	Bone Fragility with Contractures, Arterial Rupture, and Deafness – frequent medium sized arterial aneurysms	KO: Embryonic lethality (E9.5) and basement membrane fracture	Collagen Metabolism
<i>Transmembrane protein</i>			
<i>TGFBR1</i> (tgfb1)	Loeys–Dietz Syndrome – highly penetrant root and diffuse large and medium arterial aneurysm	KO: Midgestational death with yolk sac defects M318R heterozygous knock in: Aortic Root and diffuse aneurysm (D. Loch, unpublished observations)	TGFβ
<i>TGFBR2</i> (tgfb2)	Loeys–Dietz Syndrome - highly penetrant root and diffuse large and medium arterial aneurysm FTAAD - highly penetrant root and medium arterial aneurysm	KO: Defects in hematopoiesis and vasculogenesis, lethal (E10.5), <i>TGFBR2</i> flox: Impaired elastogenesis, decreased lysyl oxidase in aorta G457W heterozygous knock in: Aortic Root and diffuse aneurysm (D. Loch, unpublished observations)	TGFβ
<i>SLC2A10</i> (Glucose transporter 10)	Arterial Tortuosity Syndrome – diffuse arterial tortuosity, stenoses, aneurysms	Homozygote missense: Arterial Thickening with increased elastin deposition, elastin fractures at advanced age	TGFβ
<i>NOTCH1</i> (notch1)	Bicuspid Valve with ascending aortic aneurysm	KO: Embryonic lethal (E9.5) Required for somite segmentation, defects in angiogenesis.	NOTCH/ Jagged1
<i>JAG1</i> (jagged1)	Alagille Syndrome – intracranial aneurysms, coarctation of the aorta, aortic aneurysm	KO: Embryonic lethal (E9.5) with diffuse hemorrhages	NOTCH/ Jagged1
<i>PKD1</i> (polycystin-1)	Polycystic Kidney Disease with intracranial aneurysms	KO: Embryonic lethal (E14.5) with polycystic kidneys Hypomorphic expression: Adult onset aortic aneurysm and dissection	Unknown
<i>PKD2</i> (polycystin-2)	Polycystic Kidney Disease with intracranial aneurysms	KO: Defects in cardiac septation and left-right axis determination, kidney and pancreatic cysts	Unknown

Continued

**TABLE 153-8 Human Hereditary Aneurysm Conditions and Murine Models of Aneurysm—cont'd**

Gene (Protein)	Human Aneurysmal Syndrome	Animal Model Phenotype	Pathway Implicated
<i>GJA1</i> (connexin 43)	Hypoplastic Left Heart Syndrome (HLHS)	Nonsense (W45X): Coronary artery aneurysms	Unknown
<i>ENG</i> , <i>ACVRL1</i>	Hereditary hemorrhagic telangiectasia— dilated ascending aorta in some adults; arteriovenous malformations common	<i>Eng</i> and <i>Acvrl1</i> KO: aorta not evaluated	? BMP
<i>Cytoplasmic proteins</i>			
<i>ACTA2</i> ( $\alpha$ -smooth muscle actin)	Familial Aortic Aneurysm with levido reticularis and iris flocculi	KO: Viable offspring with normal life span and impaired vascular contractility	IGF-1, Ang2
<i>MYH11</i> (smooth muscle myosin)	Familial Aortic Aneurysm with patent ductus arteriosus	KO: Neonatal lethality, urinary retention, dilated cardiomyopathy	IGF-1, Ang2
<i>FLNA</i> (Filamin A)	Periventricular Nodular Heterotopia with Ehlers–Danlos features – ascending aortic aneurysm and valvular dystrophy	KO: Neonatal lethality, Persistent Truncus Arteriosus, endothelial cell-cell contact defects	Unknown
<i>NF1</i> (Neurofibromin 1)	Neurofibromatosis - medium sized arterial aneurysm and stenosis	KO: Enlarged head, pale liver, cardiac malformations	Ras/MEK/ ERK
<i>PTPN11</i> (SH2 domain-containing protein tyrosine phosphatase-2)	Noonan and LEOPARD Syndrome – coronary artery aneurysms and rare ascending aortic aneurysm	KO: Embryos die preimplantation Missense (D61G): Cardiac defects, defective valvulogenesis, skeletal anomalies, myeloproliferative disorder	Ras/MEK/ ERK
<i>NPHP3</i> (Nephrocystin-3)	Nephronophthisis	KO: Low penetrance intracranial aneurysms	Unknown
<i>NOS3</i> (Nitric Oxide Synthetase 3)	Refractory hypertension	KO: Abnormal aortic development with BAV, in combination with ApoE–/–, mice show abdominal arterial aneurysm and dissections	NO
<i>TSC2</i> (tuberin)	Tuberous Sclerosis – Diffuse thoraco- abdominal aneurysms	Heterozygous KO: Increased proliferation of VSMCs upon injury	mTOR/ AKT
<i>GAA</i> ( $\alpha$ -1, 4-glucosidase)	Acid Maltase Deficiency, adult onset – intracranial aneurysms	KO: Lysosomal accumulation in heart, aorta, skeletal muscle	Unknown
<i>S100A12</i> ( <i>S100A12</i> )	No Human Phenotype, increased <i>S100A12</i> expression in human MYH11 aneurysmal tissues	Sm22 $\alpha$ promoter-S100A12 Transgenic mouse: VSM disarray, elastin fragmentation, thoracic aneurysm	TGF $\beta$ , IL-6
<i>Nuclear proteins</i>			
<i>MED12</i> (mediator complex subunit 12)	Lujan–Fryns Syndrome—extremely rare aneurysm	Hypomorphic mutants: Embryonic lethal (E10), defects in neural tube closure, somatogenesis, heart formation	Wnt/ $\beta$ - catenin, Wnt/PCP
<i>KLF15</i> (Kruppel-like factor 15)	No Human Phenotype, <i>Kruppel-like factor</i> 15 downregulated in human abdominal aortic aneurysm	KO: Aortic aneurysm and cardiomyopathy	TSP-1, p53
<i>KLF2</i> (Kruppel-like factor 2)	No Human Phenotype	KO: Embryonic aortic aneurysm and dissection	Unknown
<i>SMAD3</i>	Aortic aneurysm, osteoarthritis	KO: colorectal adenocarcinoma; immune dysfunction	TGF $\beta$
<i>Inborn errors of metabolism</i>			
<i>GLA</i> ( $\alpha$ -galactosidase A)	Fabry disease—dilatation of ascending aorta common	Gla-deficient mice	Unknown
<i>GAA</i> ( $\alpha$ -1, 4-glucosidase)	Pompe disease, adult onset – intracranial aneurysms	KO: accumulation of glycogen in lysosomes in heart, aorta, skeletal muscle	Unknown
<i>Chromosomal anomaly</i>			
45 X,0	Turner Syndrome – Bicuspid aortic valve, coarctation of the aorta, ascending aneurysm	XO mice demonstrate no phenotypic heart disease	Unknown
<i>Chemical models</i>			
	No human phenotype	Ang2 infusion model	Ang2, TGF $\beta$ MCP-1, IL-6
	No human phenotype	Elastase infusion model	Unknown
	No human phenotype	Periarterial calcium application	JNK1

Adapted from Lindsay et al. (142) with permission and appreciation.

In two families with multiple members having bicuspid aortic valve as well as other congenital heart defects, mutations in *NOTCH1* were found (344). Screening many additional subjects with a bicuspid valve, with or without an aneurysm or a family history, has failed to detect other mutations in *NOTCH1*. A genome-wide linkage study in 353 individuals from 38 families found evidence for linkage to 18q, 5q and 13q, with the strongest signal at 18q22 (345).

### 153.6.8 Familial Intracranial Aneurysm (OMIM 105800)

Intracranial (berry) aneurysms have long been known to be familial. In some cases, the underlying cause is adult polycystic kidney disease (345). In other pedigrees, the phenotype was linked to 19q13.3 (346).

### 153.6.9 Abdominal Aortic Aneurysm (OMIM 100070)

In most cases, the pathology of aneurysms of the abdominal aorta (AAA) is quite distinct from aneurysm and dissection of the thoracic aorta, with evidence of inflammation being much more prominent in the abdomen. There is a substantial literature showing the familial occurrence of AAA, but most studies have concluded that multifactorial inheritance is most consistent with the pedigrees (347). However, some pedigrees suggest autosomal dominant inheritance and some pedigrees showed linkage to 4q31 and 19q13 (348). Interestingly, this latter locus is the same region identified for intracranial aneurysms. More recently, a genome-wide association study found strong association between AAA and markers at 3p12.3 (349).

### 153.6.10 Rare Familial Cardiovascular Disorders

Familial occurrence of calcific aortic disease (OMIM 114065) and fibromuscular dysplasia (OMIM 135580) (330) are of unclear etiology but could well be attributable to abnormalities of the elastic fiber, microfibrils, or the small molecules they influence. Supravalvular aortic stenosis (OMIM \*185500) is due to mutations in the *ELN* locus at 7q11.2 encoding tropoelastin. A contiguous gene deletion in this region causes Williams syndrome (OMIM 194050; see Chapter 45).

### 153.6.11 Familial Tall Stature

Tall stature unassociated with other skeletal abnormalities (except in the proband) is linked to *FBN1* in one pedigree (193). A point mutation, causing R1828W, seems to result in failure to cleave the C-terminal propeptide, and segregates with tall stature in this family. Milewicz and colleagues suggested that this mutation creates, in essence, a null allele, and that the patients have simply a

50% reduction in fibrillin-1 in the ECM. This interesting hypothesis needs to be confirmed by both biochemical analysis and sequencing of the other *FBN1* allele.

### 153.6.12 Familial Kyphoscoliosis

A point mutation in *FBN1*, G1796E, co-segregated with tall stature, pectus excavatum, kyphoscoliosis, and mild vertebral dysplasia in one family, all of whose members lacked ocular or cardiovascular evidence of MFS (350).

### 153.6.13 Familial Pneumothorax

To complete the spectrum of autosomal dominant traits involving predominantly one of the cardinal systems of classic MFS, the uncommon disorder familial pneumothorax was examined in three pedigrees for linkage to the *FBN1* locus. Each family showed autosomal dominant inheritance, with one instance of nonpenetrance in one family. None of the affected individuals showed any outward evidence of a connective tissue disorder. A common *FBN1* haplotype failed to segregate with the phenotype in all three families, indicating that mutations in *FBN1* do not account for this disorder (351). In a large Finnish pedigree, spontaneous pneumothorax was inherited as an autosomal dominant trait with complete penetrance and no other clinical features. Linkage analysis localized the gene to 17p11, and screening of candidate loci for mutations identified a 4-bp deletion in *FLCN* (352), mutations in which had previously been found in Birt-Hogg-Dubé (BHDS) syndrome (OMIM #135150). In BHDS, cystic lung disease is associated with fibrofolliculomas of the skin, and renal carcinoma.

### 153.6.14 Shprintzen–Goldberg Syndrome (OMIM \*182212)

As originally described, the phenotype consists of craniosynostosis (associated with exophthalmos), mandibular and maxillary hypoplasia, arachnodactyly, camptodactyly, hemias, muscular hypotonia, and developmental delay (353). Additional cases of this rare disorder have emphasized both the range of skeletal abnormalities (highly arched palate, pectus carinatum or excavatum, dolichostenomelia) and involvement of the cardiovascular system (354–357). Several patients with this phenotype, clearly well beyond the limits of MFS, have developed marked aortic dilation or dissection (355,358). A mutation in *FBN1* has been found in two patients (358,359), and some of the other reported patients might well have Loeys–Dietz syndrome (359).

### 153.6.15 Congenital Contractural Arachnodactyly (OMIM \*121050)

CCA is defined primarily by abnormalities of the skeletal system inherited as an autosomal trait.



Contractures, most severe at birth, typically affect the digits of the feet and hands, knees, and elbows. The contractures tend to improve with age and with physical therapy, unless pterygia are present. Scoliosis, often with accentuated kyphosis, develops during childhood and adolescence and can become severe. The only consistent, non-skeletal manifestations are mild hypoplasia of the calf muscles and ‘crumpling’ of the ear helix (360). In one pedigree, CCA was associated with intraocular colobomas. Ectopia lentis, aortic dilation, and myxomatous degeneration of the mitral valve, if present, suggest a diagnosis of MFS. Indeed, numerous patients have been reported as ‘contractural arachnodactyly’ with cardiovascular disease who undoubtedly had classic MFS (361,362). On the other hand, aortic dilation can occur in patients with CCA and mutations in *FBN2* (363). In the ultimate irony, Gabrielle P., Marfan’s first patient may have had CCA (42). Mild to moderate folding with foreshortening of the helix is fairly common in the general population and may be increased in MFS. As noted earlier, congenital contractures of the digits and elbows (but rarely the knees) are also common in MFS. One distinction may be the observation that the contractures of CCA improve, especially with physical therapy, whereas those associated with MFS usually do not. No comprehensive studies of anthropometrics have been performed in CCA. Thus description of dolichostenomelia, arachnodactyly, and ‘marfanoid’ habitus are based on clinical perception and not objectivity.

Several pedigrees were linked to *FBN2* on chromosome 5q23–31 (20). Several dozen mutations have been found in unrelated patients (221,364,365) and are cataloged at: [www.umd.be/FBN2](http://www.umd.be/FBN2). Two sibs with especially severe manifestations were the products of a double second-cousin mating, raising the possibility of an autosomal recessive form.

### 153.6.16 Lujan–Fryns Syndrome (OMIM 309520)

One of the most commonly – and incorrectly – used descriptors in clinical genetics is the term **marfanoid**. Typically, the word is used for anyone who appears tall and slender with an elongated face and dolichostenomelia. The appearance of ‘marfanoid’ in a consultant’s report is often enough to prompt the primary care physician to request a genetic evaluation; in my experience, few such individuals have Marfan syndrome, so the predictive value of a ‘marfanoid habitus’ seems quite low (292). There are good reasons to suggest that the term should be discarded altogether.

‘Marfanoid’ was used to describe males who were initially ascertained on account of mental retardation (366). Those affected were not actually tall, but were slender, had elongated arms, and had characteristic craniofacial features of a long narrow face, retrognathia,

and a highly arched palate. Inheritance was consistent with X-linkage. Fryns (367) reported several cases, and the condition is commonly called Lujan–Fryns syndrome. For the reasons noted previously, this is preferable to the other name, X-linked mental retardation with marfanoid habitus. In one institutionalized population of mentally retarded adults, 5% of males had a form of X-linked mental retardation, and 10% of them had Lujan–Fryns syndrome (368). In addition to retardation, most, but not all, patients have neurobehavioral abnormalities (369). Subsequently, the phenotype has been reported in occasional female relatives of probands. Few patients have had comprehensive cardiovascular evaluation, which should be performed given the report of ventricular septal defects and aortic root dilation in two relatives with Lujan–Fryns (370).

Several probands have mutations in the *MED12* gene, and the mutation segregates with the condition in the families (371). Lujan–Fryns syndrome is thus allelic with Opitz–Kaveggia syndrome (OMIM 305450).

### 153.6.17 Weill–Marchesani Syndrome (OMIM \*277600; 608328)

Weill–Marchesani syndrome (WMS) is a condition long classified as an autosomal recessive trait because of multiple affected sibs of consanguineous parents and its occurrence among inbred populations (85). However, an increasing number of pedigrees consistent with autosomal dominance are being observed (372–374). The phenotype is characterized by ocular and skeletal changes that are in many respects the antithesis of MFS. The lens is ectopic, but usually spherophakic. Stature is below average, the digits are short, and the joints are stiff. A few cardiovascular problems have been noted, including pulmonic stenosis and subvalvular aortic stenosis, but these findings have been inconsistent. Reduced ability to open the mouth and maxillary hypoplasia can impair anesthetic management of the airway (375). Review of the phenotypes in reported families that showed both recessive and dominant inheritance detected no important distinctions (373).

By homozygosity mapping in an inbred Lebanese/Saudi pedigree, a locus causing recessive WMS was mapped to 19p13.3–p13.2 (376). Mutations in *ADAMTS10*, a locus in this region that encodes a disintegrin and metalloproteinase, were found in several families with recessive WMS (377).

After staining for fibrillin, decreased immunofluorescence of the dermal-epidermal junction and the deep dermis was found in skin from a patient of the family described by Gorlin and colleagues (375). This same biopsy showed an apparent increase in elastic fibers. Linkage studies found the autosomal dominant form to map to 15q, close to *FBN1*, and an in-frame deletion was found (373,378,379).

### 153.6.18 Stiff Skin Syndrome (OMIM #184900)

Rare patients have hardened, thickened skin over their entire body; autosomal dominant inheritance is clear in some families. The skin changes can limit ambulation and cause flexion contractures. Skin biopsy analysis showed increased deposition of both fibrillin and elastin (380). Sequencing of *FBN1* found mutations in a number of patients, including one who had stiff skin and ectopia lentis.

The tight skin mouse is heterozygous at the *Tsk* locus, which is syntenic with human chromosome 15. The original mouse strain has a large genomic duplication within the *Fbn1* locus (381).

### 153.6.19 Multiple Self-Healing Squamous Epithelioma (MSSE) (OMIM #132800)

This condition was first described by a British dermatologist in a Scotsman, and most cases have been found in that country (382). This autosomal dominant disorder is characterized by benign but invasive squamous epitheliomas that arise spontaneously (especially on sun exposed skin) and regress over a period of weeks to months. Depressed scars, which can be cosmetically unappealing, result. Variable expressivity and even apparent non-penetrance occur. The dermatologist's son, the noted human geneticist Malcom Ferguson-Smith, worked assiduously over several decades to map the locus. These investigations culminated with the discovery of mutations in *TGFBR1*, but of a type distinct from those that cause Loeys-Dietz syndrome and familial thoracic aortic aneurysm (383). The mutations in patients with MSSE occur predominantly in the receptor domain with some in the kinase domain (the latter being truncating mutations), whereas those causing Marfan-related disorders occur solely in the kinase domain and are predominantly missense. Interestingly, analysis of the DNA from lesions of MSSE shows loss of the normal allele. Thus, *TGFBR1* functions as a tumor suppressor.

### 153.6.20 Acromicric (OMIM#102370) and Geleophysic (OMIM#231050) Dysplasias

These two conditions share phenotypic features but acromicric dysplasia is autosomal dominant while geleophysic dysplasia is autosomal recessive. Both have marked short stature, prominent shortening of the hands and feet, thick skin and limited joint range of motion. About half of all patients with geleophysic dysplasia have mutations in *ADAMTSL2* (OMIM#612277). Mutations in exons 41 and 42 of *FBN1* affect the TGF $\beta$ -binding protein-like domain 5, and cause several syndromes (Table 153-6), including both of these skeletal dysplasias (211). Along with Weill-Marchesani syndrome, they represent the antithetical phenotype of the Marfan syndrome caused by mutations in the same gene.

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### Biography



Reed Pyeritz completed his PhD and MD at Harvard and trained in internal medicine at the Peter Bent Brigham Hospital in Boston and medical genetics at the Johns Hopkins Hospital in Baltimore. He is a professor of Medicine and Genetics at the Raymond and Ruth Perelman School of Medicine of the University of Pennsylvania in Philadelphia, where he directs the Center for the Integration of Genetic Healthcare Technologies and serves as vice-chair for academic affairs of the Department of Medicine. He is a senior fellow in both the Center for Bioethics and the Leonard Davis Institute for Health Economics.

His research has focused on several areas, including clinical investigations of heritable disorders of connective tissue and cardiovascular diseases, especially those affecting the thoracic aorta. Currently he is studying how more refined methods of investigating genetic variation, such as cytogenomic arrays and whole genome sequencing, actually increase the level of uncertainty in applying the results clinically.

# CHAPTER

# 154

## Ehlers–Danlos Syndrome

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### 154.1 INTRODUCTION

Ehlers–Danlos syndrome (EDS) is a group of disorders characterized by abnormalities of skin, joints, and other connective tissues (1–3) (Table 154-1). Early descriptions of these disorders emphasized joint laxity and skin hyperextensibility (4,5), but as more patients were identified, skin fragility, easy bruising, and the occasional complication of arterial and bowel rupture were recognized (6,7a,8). This clinical heterogeneity has been explained, in part, by biochemical and molecular genetic dissection of the underlying mechanisms. An attempt, in 1997, to integrate the clinical, biochemical, and molecular genetic studies provided a more descriptive classification than the previous numeric one (9). Additional studies since that time suggest that further amplification of the nosology is warranted. Table 154-1 provides the older (numerical) and current (descriptive) classification, a summary of the molecular genetic studies, and proposal for additional types of EDS based on recent studies.

This chapter summarizes the clinical, genetic, and biochemical features of recognized EDS types, provides a summary of the details of collagen structure, biosynthesis, and tissue distribution that serves as the basis for understanding the molecular mechanisms of some of these disorders, and discusses recent findings that expand the clinical and molecular realms of EDS.

### 154.2 COLLAGEN GENES AND PROTEINS

The collagens form a family of evolutionarily related and structurally similar proteins that have a signature amino acid sequence (the repeating triple helical sequence of Gly-Xaa-Yaa, in which Xaa and Yaa can be almost all other amino acids but are often proline), the characteristic amino acids hydroxyproline and hydroxylysine that result from post-translational modification, and form

trimeric molecules that contain the same chains or closely related chains that form a collagen triple helix. The genes that encode collagens are under tight tissue- and cell-specific control so that each collagen has a characteristic distribution (10a,11,12a). The family of collagen genes is divided into several distinct lineages that encode proteins with different distributions and structures. The major lineage is the fibrillar collagens, which are encoded by a set of nine closely related genes (*COL1A1*, *COL1A2*, *COL2A1*, *COL3A1*, *COL5A1*, *COL5A2*, *COL5A3*, *COL11A1*, and *COL11A2*) that form three clades (13,14). These genes encode the chains of types I, II, III, V, and XI collagens. Types II and XI are expressed in cartilage and mutations in those, unlike mutations in the other genes, do not give rise to EDS phenotypes.

Ehlers–Danlos syndrome phenotypes are known to result from mutations in the *COL1A1* and *COL1A2* genes of type I collagen, the *COL3A1* gene of type III collagen, and the *COL5A1* and *COL5A2* genes of type V collagen. Mutations in genes that encode proteins responsible for post-translational modification of some collagens produce EDS phenotypes. These genes include *PLOD1*, which encodes the modifying enzyme lysyl hydroxylase 1, and *ADAMTS2*, which encodes the pro-collagen I N-proteinase. In addition, it has become clear that mutations in other genes that express proteins found in the extracellular matrix or modify matrix proteins can also produce similar clinical phenotypes.

The genes for all fibrillar collagens are complex and contain between 50 and 66 exons. Each gene encodes a protein that contains a signal sequence, an amino-terminal propeptide, a triple helical domain, and a carboxyl-terminal propeptide. The triple helical domain is characterized by a repeating Gly-Xaa-Yaa motif (in which glycine (Gly) is in every third position and Xaa and Yaa can be any amino acid except tryptophan and cysteine). In each chain of the fibrillar collagens, there are

**TABLE 154-1 Clinical Features, Mode of Inheritance, and Biochemical Disorder in Ehlers–Danlos Syndrome**

Descriptive Type	Clinical Features	Inheritance <sup>a</sup>	Molecular Defect
Classical type	Soft, velvety, hyperextensible skin; easy bruising; joint hypermobility; “cigarette paper” scars; prematurity	AD	<i>COL5A1</i> and <i>COL5A2</i> mutations (EDS type I and II)
	Soft hyperextensible skin, joint hypermobility, abnormal scars, cardiac valvular abnormalities, aortic dilatation	AR	<i>COL1A2</i> null alleles
	Soft hyperextensible skin, joint hypermobility, abnormal scars	AD	<i>COL1A1</i> R136C (rare)
	Soft hyperextensible skin, normal scarring, joint hypermobility	AR	<i>TNXB</i> null mutations
Hypermobile type	Soft skin, no scarring, marked large and small joint hypermobility	AD	Most not known (EDS type III). Heterozygosity for <i>TNXB</i> null mutations (rare: <i>COL3A1</i> G637S)
Vascular type	Thin, translucent skin with visible veins; marked bruising; skin and joints have normal extensibility; arterial, bowel, and uterine rupture	AD	Mutations in the <i>COL3A1</i> gene (EDS type IV) that alter type III collagen synthesis, secretion, and structure
Kyphoscoliotic type	Soft, velvety, hyperextensible skin; hypermobile joints, scoliosis; ocular fragility and keratoconus	AR	Lysyl hydroxylase deficiency due (EDS type VI) to mutations in the <i>PLOD1</i> gene
Arthrochalasia type	Congenital hip dislocation, joint hypermobility; soft skin with normal scarring	AD	A: <i>COL1A1</i> exon 6 splice site (EDS type VIIA) mutations that delete N-proteinase cleavage site from the protein B: <i>COL1A2</i> exon 6 splice site mutations that delete N-proteinase cleavage site from the protein
Dermatosparaxis	Very soft, fragile, bruisable skin, marked joint hypermobility, blue sclera, small jaw, hypertrichosis	AR	<i>ADAMTS2</i> mutations that result in (EDS type VIIC) loss of procollagen N-proteinase activity
Other Types of Ehlers–Danlos Syndrome			
X-linked (EDS type V)	Soft, velvety, hyperextensible skin, hypermobile joints, bruising with muscle hemorrhage, abnormal scars	XLR	Not known (single family)
Periodontal type	Soft hyperextensible skin, mild scarring, mild joint hypermobility, pretibial bruising and heme pigmentation, generalized periodontal loss with early tooth loss	AD	One locus at 12p13 (EDS type VIII); probably genetically heterogenous
Fibronectin deficiency	Soft skin, mild hypermobility, bruising, platelet aggregation defect	?AR	Defect in fibronectin (single family)
Progeroid type	Progeroid facies, multiple nevi, mild mental retardation, skin hyperextensibility, bruisability, moderate skin fragility, joint hypermobility principally in digits, with radio-ulnar synostosis and developmental delay	AR	Mutations in <i>B4GALT7</i> that encodes galactosyltransferase-I
EDS with brain heterotopias	Soft, hyperextensible skin, joint hypermobility, seizures, arterial aneurysms	XLD	Mutations in <i>FLNA</i> gene that encodes the filamin A protein

<sup>a</sup>AD = autosomal dominant; AR = autosomal recessive; XLD = X-linked dominant; XLR = X-linked recessive.

just over 1000 amino acid residues in the triple-helical motif that has no interruptions in the triplet structure.

### 154.3 BIOSYNTHESIS OF COLLAGENS

The biosynthesis of collagen molecules involves many steps beyond gene transcription. The 50 or more exons of the fibrillar collagen genes are arrayed over 18–500 kbp. The *COL1A1* is the smallest of the fibrillar collagen while the *COL5A1* gene is the largest. The majority of

the others have gene sizes between 40 and 60. The genes are transcribed and spliced in the nucleus; the mature mRNAs of 4800–7200 nucleotides are transported to the cytoplasm where they are translated on membrane-bound polysomes. Each chain is initiated with a signal sequence that facilitates cotranslational insertion into the lumen of the rough endoplasmic reticulum (RER) and is cleaved shortly after entry into the lumen. The precursor chains (pro $\alpha$  chains) of the fibrillar collagens are 1350–2000 amino acids in length.

As cotranslational and post-translational events, certain lysyl residues in the Y-position of the triple helix, and virtually all Y-position prolyl residues, are hydroxylated by the enzymes lysyl hydroxylase (encoded by *PLOD1*), and prolyl-4-hydroxylase (which is a heterotetramer made up of two chains each of the  $\alpha$  and  $\beta$  subunits that are encoded by *P4HA* and *P4HB*, respectively) (15). These enzymes are encoded by distinct genes but share cofactors (ferrous iron, ascorbate or other reducing compounds, and  $\alpha$ -ketoglutarate). Prolyl-4-hydroxylase is located within the lumen of the RER and the  $\beta$ -subunit is identical to disulfide isomerase, a protein that catalyzes interchain and intrachain disulfide bond formation during protein folding and chain association. Lysyl hydroxylase is located in the membrane of the RER. The degree of both prolyl and lysyl hydroxylation varies in different collagen types and may vary from tissue to tissue for the same collagen. Following hydroxylation, some hydroxylysyl residues are glycosylated to form glucosyl-galactosylhydroxylysyl or galactosylhydroxylysyl residues (16), and oligosaccharide is added to a single asparagine residue in the C-terminal propeptide of each chain.

When the synthesis of each pro $\alpha$  chain is complete, the carboxyl-terminal propeptide of each chain folds and is stabilized by intrachain disulfide bonds. Each chain has a specific region that appears to function as a chain recognition domain (17), which determines the manner in which chains assemble into the procollagen molecule of each type. The three constituent chains of each procollagen first interact with each other at sites within the C-terminal propeptide, the association is stabilized by interchain disulfide bonds, and triple helix is propagated toward the N-terminal end of the molecules. Post-translational hydroxylation and glycosylation of residues in the triple-helical core domain cease when a stable triple helix is achieved. In turn, the stability of the triple helix is dependent on complete hydroxylation of the Y-position prolyl residues. Upon formation of the stable triple helix, the procollagen molecule is transported to the Golgi and then to the cell surface. Once secreted, fibrillar procollagen molecules are processed to collagen by proteolytic cleavage with specific proteases that remove the amino-terminal and carboxyl-terminal propeptides.

The extracellular molecules assemble into fibrils and other higher-order structures, where they become covalently cross-linked to form highly stable polymers. It now appears likely that the major collagen fibrils in the extracellular matrix of skin and many other tissues are largely type I collagen, and depend on a small core of type V collagen molecules for initial formation (18). The cross-links are formed following oxidative deamination of lysyl or hydroxylysyl residues located just outside the triple-helical domains, to produce highly reactive aldehydes (19). Lysyl oxidase, a copper-containing enzyme, initiates this process (20a,21a). The activated residues can form multifunctional cross-links that stabilize fibrils and higher-order aggregates (19). The structure of the

collagen fibril, its aggregation into bundles, and the mechanical properties of tissues depend on cross-links and on interactions with several other macromolecules, most notably proteoglycans and other glycoproteins.

## 154.4 CLASSICAL EHLERS–DANLOS SYNDROME (TYPES I AND II—GRAVIS AND MITIS)

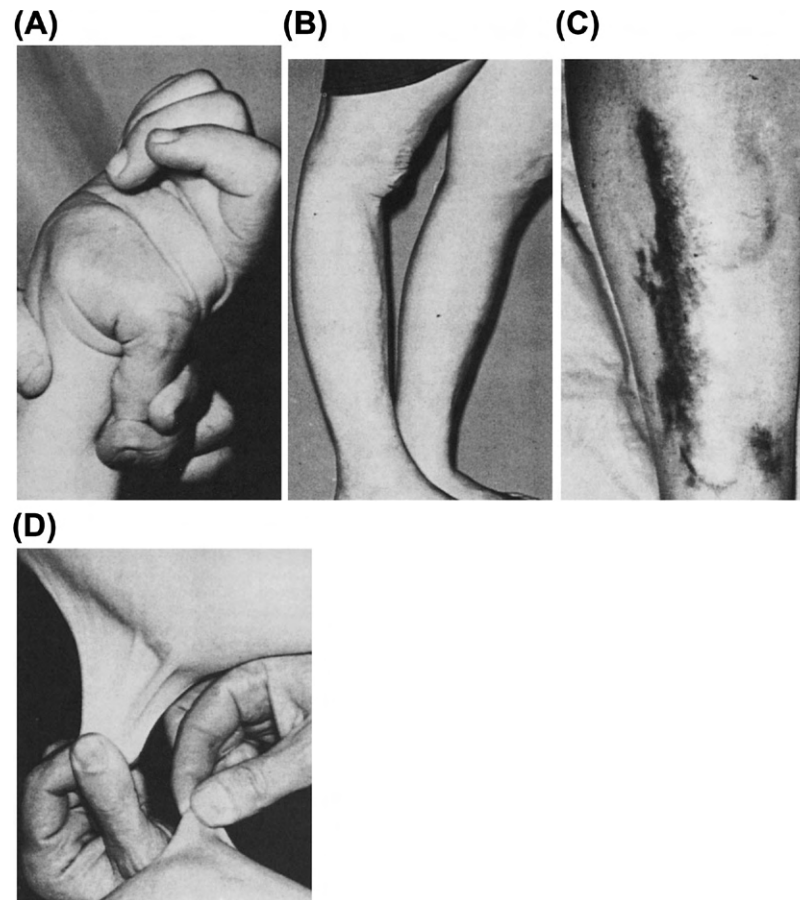
### 154.4.1 Clinical Aspects

The revised classification of EDS groups the previously distinct EDS type I and EDS type II as the “classical” variety. This clinical group clearly represents a genetically heterogeneous group of disorders. The clinical distinctions among the groups that have mutations in different genes are not yet well defined. The majority of individuals within this group appear to have mutations that affect the expression or structure of type V collagen with most in the *COL5A1* gene and a smaller number in the *COL5A2* gene. A second group has “null” mutations in the *COL1A2* gene, for which they are either homozygous or compound heterozygotes. A third set has mutations in the *TNX* gene that encodes the protein tenascin X, a large extracellular matrix glycoprotein that interacts with collagens. Finally, there is a small set that has mutations that do not alter the triplet structure of type I collagen proteins but introduce usually disallowed amino acids in the sequence. These different groups are described separately, although for most the clinical descriptions are not as robust as for the others and distinguishing features are not always clear.

The most common form of dominantly inherited, or sporadic, classic EDS results from mutations in the type V collagen genes. The classical variety of EDS is usually inherited in an autosomal dominant fashion, although new mutations are common. Typically in the classical forms, the skin is soft and velvety in texture and can be extended several centimeters away from attachment sites (Figure 154-1). The skin has increased compliance but returns to its original shape promptly and is not usually lax. It is fragile, and there is easy bruising. Trauma results in gaping wounds that may bleed very little but heal with atrophic “cigarette-paper” scars. Areas of repeated trauma, such as elbows, knees, and shins, often have marked pigment deposition in addition to the characteristic scars. Small accumulations of connective tissue, “molluscoid pseudotumors,” may form in the skin and may be calcified.

Although it was initially reported that as many as 50% of infants with classical forms of EDS are born prematurely by 4–8 weeks (22), this claim was based on a relatively small study. Although there are no recent series in which this issue has been examined critically, clinical experience suggests that prematurity is less common than once thought. Bruising during the newborn period is unusual and most often begins as children start to crawl and stand. At this time, skin fragility also becomes evident, and characteristic scars may appear on the





**FIGURE 154-1** Clinical features of classical EDS (EDS type I). (A) Joint hypermobility, (B and C) “cigarette paper” scars and hyperpigmentation, and (D) skin hyperextensibility.

forehead, under the chin, and on the knees and elbows. Motor development may be slower than normal because joint hypermobility limits stability until muscle strength is sufficient to overcome ligamentous laxity. Intellectual development is normal in the absence of other complicating conditions.

There are no studies available to examine longevity of individuals with classical forms of EDS. The clinical impression is that life span is normal and not compromised by the condition. It has been estimated that up to half the people with classical EDS may have mitral valve prolapse, again on the basis of clinical experience, but few supporting data are available. A recent study of aortic diameter in individuals with classical and hypermobile EDS suggested that those individuals had aortic root and proximal aortic diameters that were distributed asymmetrically with more clustered toward the upper normal ranges. None of the adults in the group had diameters that appeared to put them out of the normal range but several individuals were excluded from the study because of ascertainment through aortic surgery (23). The frequency of surgery appears low, but one individual with classical EDS and a defined mutation in *COL5A1* was known to have had cardiac surgery (24) during adolescence. Other cardiac structural abnormalities are also

seen but the frequency is uncertain as this group of patients was recruited in a cardiac clinic (25).

Pes planus is common and moderate scoliosis is seen in some individuals. Ligamentous laxity appears to be associated with earlier than usual degenerative arthritis, although an underlying biochemical defect common to the ligament and cartilage has not been excluded.

**154.4.1.1 Other Forms of Classical Types of Ehlers–Danlos Syndrome.** There is a rare form of EDS that appears similar in initial presentation to the classical forms but is recessively inherited and may be accompanied by aortic dilation and cardiac valvular involvement (26–28). Individuals with this condition may have joint hypermobility and skin hyperextensibility but bruising is generally not striking. Aortic dilatation is a common feature in the older affected individuals and multiple valvular insufficiency can be seen. This group of individuals is distinguished from those with classical EDS by the underlying mechanism of disease, in that all have been found to be homozygous or compound heterozygotes for mutations that inactivate the *COL1A2* gene of type I collagen. The characteristic abnormalities in collagen fibril structure (see later discussion) are not seen.

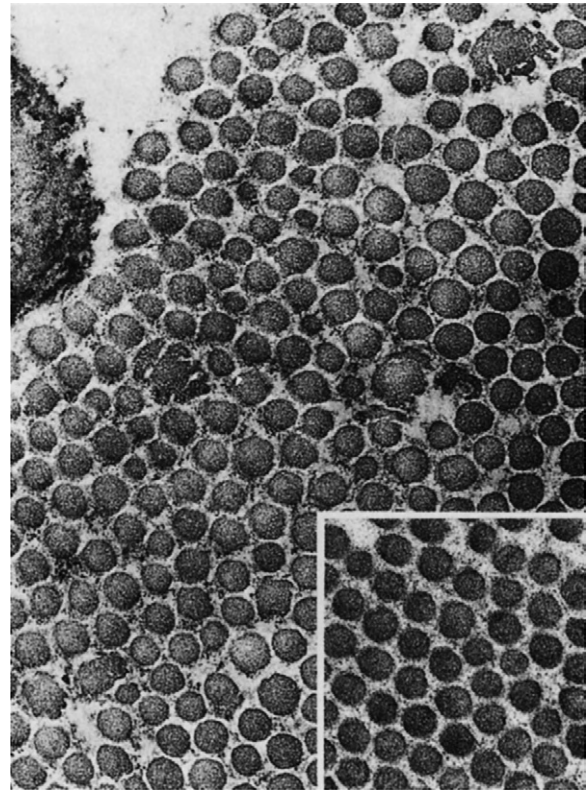
An additional distinguishable group of individuals with a form of classical EDS is characterized by joint

hypermobility, skin hyperextensibility, and easy bruising, but they do not form abnormal scars (in contrast to both previous groups) (10,29,30). Mitral valve disease may be more common in this group than in other forms of classical EDS. In contrast to the most common classical form, this condition is inherited in an autosomal recessive fashion and the underlying mutations are in the *TNX* gene. One striking finding is that heterozygotes, particularly women, are likely to have significant joint hypermobility (31).

### 154.4.2 Biochemical and Molecular Genetic Bases of Classic Ehlers–Danlos Syndrome

**154.4.2.1 Type V Collagen Genes.** In the majority of individuals with classical forms of EDS, the underlying mutations are in the type V collagen genes, *COL5A1* and *COL5A2* (24,32–34). The pathway to this discovery was unexpectedly long. It has been known for many years that collagen fibrils in the dermis of individuals with classical EDS are 10–40% larger than normal (35–37), are irregular in outline, and “composite” structures are common (Figure 154-2). These altered fibrils, which consist largely of type I collagen, generally do not reflect alterations in type I collagen genes as linkage studies excluded them in some families (38–40). In the more common forms of classical EDS, biochemical studies of collagens synthesized by cultured fibroblasts were largely unhelpful (24,32,34) but linkage studies (41–43) served to strongly implicate type V genes in molecular pathogenesis. Segregation studies and direct mutation analyses make it clear that mutations in two type V collagen genes (*COL5A1* and *COL5A2*) do not underlie the clinical phenotype in, perhaps, a third of individuals with classical EDS (32,44), although problems in definitive ascertainment of phenotype could give similar results.

The identification of mutations provided the definitive demonstration of the role of these collagens in the clinical picture of classical EDS (44–48). The initially identified point mutation in the *COL5A2* gene was not published as a complete description for several years (49). The demonstration of mutations in the *COL5A1* (50a) gene began with identification of a chromosomal translocation that interrupted the gene on chromosome 9 (48). A second mutation that resulted in loss of the amino acids encoded by exon 65 of the *COL5A1* gene was the consequence of a 4-bp deletion from the intron 65 donor site (44). In another family, compound heterozygosity for a mutation that results in substitution for a glycine within the triple helical domain of the  $\alpha 1(V)$  chain (G1489D, indicated with respect to the first coding amino acid of the prepro $\alpha$  chain rather than of the triple helix, which is the anchor point for the other mutations cited) and a substitution amino-terminal to the triple helix (G530S), raises the question of the basis of moderate intrafamilial variability in this disorder (51). The recent finding of homozygosity for the *COL5A1* G530S



**FIGURE 154-2** Electron micrograph of collagen fibrils in skin from an adult with classical EDS. The normal fibrils (inset) are round and about equidistant. The fibrils in skin from the affected individual are larger than the controls, have irregular borders, and in some instances are markedly irregular. (Courtesy of Dr Karen Holbrook, University of Florida.)

variant in two individuals with classical EDS raises the question of whether it, too, could cause the phenotype in homozygotes (51). Within the *COL5A2* gene, both splicing mutations (46) and alterations in the triple helical sequences (33,49) have been identified that appear to alter the post-translational modification of type V collagen molecules and presumably alter their secretion and interactions in the matrix. Within the *COL5A1* gene, the initial identification of mutations that would alter the amount of the product synthesized (45,48) suggested that both qualitative and quantitative effects on type V collagen production could result in similar phenotypes. Mutation searching among *COL5A1* and *COL5A2* transcripts proved to be frustrating and in only the minority of affected individuals could mutations be identified (44,46).

This apparent paradox may have two resolutions. First, it is clear that a significant proportion of individuals with classic EDS (20–30%) have mutations in the *COL5A1* gene that lead to marked instability of the mRNA expressed from that allele (24,32–34). In all cases, the mRNAs appear to undergo “nonsense-mediated decay” and are not present or present in very low abundance in the cytoplasm (52,53). Search for mutations in the mRNA/cDNA (where most studies were done) would

not identify abnormalities because of the instability of the mutation-bearing sequences. It is now clear as well that other genes harbor mutations that result in the phenotype.

**154.4.2.2 A Recessive Form of Classic Ehlers–Danlos Syndrome that Results from Mutations in the *TNXXB* Gene.** Homozygosity for null mutation in the *TNXXB* gene, which encodes tenascin-X, gives rise to a clinical picture that shares much with classical EDS (29). *TNXXB* and a pseudogene (*TNXXA*) flank the active *CYP21B* gene, homozygous loss of which results in 21-hydroxylase deficiency. The first patient identified came to clinical attention because of 21-hydroxylase deficiency and was noted to have clinical findings of the classical variety of EDS. Notably, the characteristic alterations of collagen fibril structure in skin, characteristic of the classical forms of EDS, were missing. Following the initial identification of this condition, several additional families and affected individuals have been found (30,54). The protein is secreted by matrix cells and appears to be related to the formation of the elastic fiber network (55). Although the same defect in mice recapitulates most of the human phenotype (56), the molecular pathogenesis of the clinical outcome remains uncertain. A rearrangement that inactivates the *TNXXB* gene is thought to be present in about 10% of the carriers of 21-hydroxylase deficiency in some populations (57). Heterozygosity for null alleles results in significant joint hypermobility (31), making it a candidate gene for the hypermobile form of EDS. At present, the frequency of classical EDS that is the result of homozygosity or compound heterozygosity for *TNXXB* null alleles is not known. Surprisingly, no missense mutations in the gene have yet been identified.

**154.4.2.3 A Recessive Form of Classic Ehlers–Danlos Syndrome due to Null Mutations in the *COL1A2* Gene.** As indicated previously in this chapter, rarely, mutations in the *COL1A2* gene appear to lead to a specific variant of a recessively inherited classical form of EDS, but only a few such individuals have been identified (26–28,58). Cultured dermal fibroblasts from these individuals synthesize type I procollagen that contains only pro $\alpha$ 1(I) chains. Mutations have now been characterized in four affected individuals and in each case the mutation leads to introduction of a premature termination codon in the mRNA encoded by both *COL1A2* alleles. As a consequence the mRNA is unstable and rapidly degraded so that no protein is encoded. In two of the four individuals, the mutations are homozygous and the parents are consanguineous. In the remaining two, the alleles have different mutations and no parental consanguinity was known. These mutations differ from those seen in a child with a form of osteogenesis imperfecta (OI) whose cells made only pro $\alpha$ 1(I) chains and homotrimers (59–61). In those cells, homozygosity for a 4-bp deletion in exon 52 of the *COL1A2* gene led to a frameshift and a change in the last 48 amino acids of the chain. The difference in the phenotype between these two sets of individuals has been puzzling and the explanation for the variance is not yet

clear. In the cells from the child with OI, the protein is very unstable and probably degraded by the proteasome, an effect that might alter the cellular protein metabolism.

**154.4.2.4 Substitutions for Non-Glycine Residues in the Triple Helical Domain of  $\alpha$ 1(I) Chains.** In two unrelated patients with classical EDS, a mutation in one *COL1A1* allele results in substitution of a conserved arginine in the triple helical domain by cysteine (R134C within the triple helical domain) (62). Cysteine is absent from the triple helical domain of the normal pro $\alpha$ 1(I) chain, but when present can link two altered pro $\alpha$ 1(I) chains in the same molecule or, if only a single altered chain is in the molecule, leave a free sulfhydryl group for interaction in the matrix. Several other substitutions of cysteine for arginine within the triple helical domain have been identified that do not appear to result in the classical EDS phenotype (unpublished data). In contrast, the R836C mutation was recently associated with Caffey disease (infantile cortical hyperostosis) in unrelated families (63). As these individuals get older, their bone disease goes into remission but they develop joint hypermobility and skin hyperextensibility.

Another candidate for mutations in classical EDS is the fibronectin gene, which is thought to be altered in function in a single family with a form of EDS similar to a mild classical form and previously classified as having EDS type X (64). A mutation has never been identified.

## 154.5 HYPERMOBILE TYPE (EDS TYPE III—FAMILIAL HYPERMOBILITY)

### 154.5.1 Clinical Aspects

The hypermobile type of EDS, familial hypermobility, is an autosomal dominant disorder with variable expression in which the major clinical features are large and small joint hypermobility (9). There is considerable variability in the clinical expression both within and among families. The skin is generally of normal character. The major clinical problem is marked joint laxity, which may be accompanied by recurrent joint dislocation. Joint discomfort is often out of proportion to the apparent signs of mobility and puzzling to both the physician and the affected individual (65,66). Surgical repair of dislocation may be helpful in some people but the pain may be very difficult to ameliorate without use of significant pain medications and an integrated approach to management.

### 154.5.2 Biochemical and Molecular Genetic Aspects

One of the most disappointing aspects of the study of individuals with hypermobility forms of EDS is the relative lack of progress toward identification of the underlying mechanisms of the disorder. Several considerations probably contribute to the paucity of findings. First is the relative scarcity of large families in which to use linkage studies to identify candidate regions. In part, this



probably reflects the dynamic nature of the disorder, in which hypermobility is much more apparent in childhood and adolescence than in older life. Thus the identification of affected older individuals in families depends on historical considerations rather than clinical signs. Second is the likely heterogeneity of the disorder and the difficulty in distinguishing subtypes by clinical presentation.

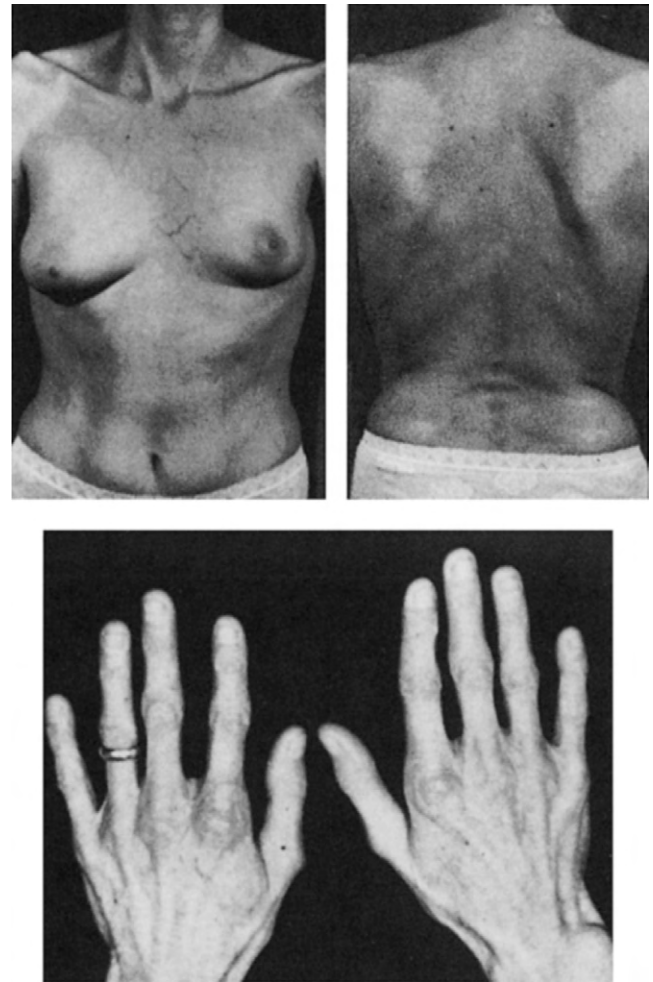
The ultrastructural findings in skin are similar to those in EDS types I and II (35,67). In one family, however, a mutation in the *COL3A1* gene (Gly637Ser, in reference to the first glycine of the triple helical domain) was found (68). It is not clear if this family has a mild form of the vascular form of EDS (EDS type IV), in which the major manifestations of arterial and bowel rupture are delayed to a later age (69,70).

The most significant finding with respect to the genetic basis was the recognition that individuals who are heterozygous for null mutations in the *TNX* gene have significant hypermobility, consistent with the diagnosis of the hypermobility type of EDS (31,71,72). Strikingly, in this group the frequency of hypermobility is far higher in women than in men, consistent with the clinical impression that women have greater underlying joint mobility than men. In those families in which heterozygotes were studied, variability of the clinical presentation was marked and mimicked that seen in some other families in which mutations in *TNX* were not identified, suggesting the gender modifies the clinical presentation and must be considered in family studies and in counseling.

## 154.6 VASCULAR, ECCHYMOTIC TYPE (EDS TYPE IV)

### 154.6.1 Clinical Aspects

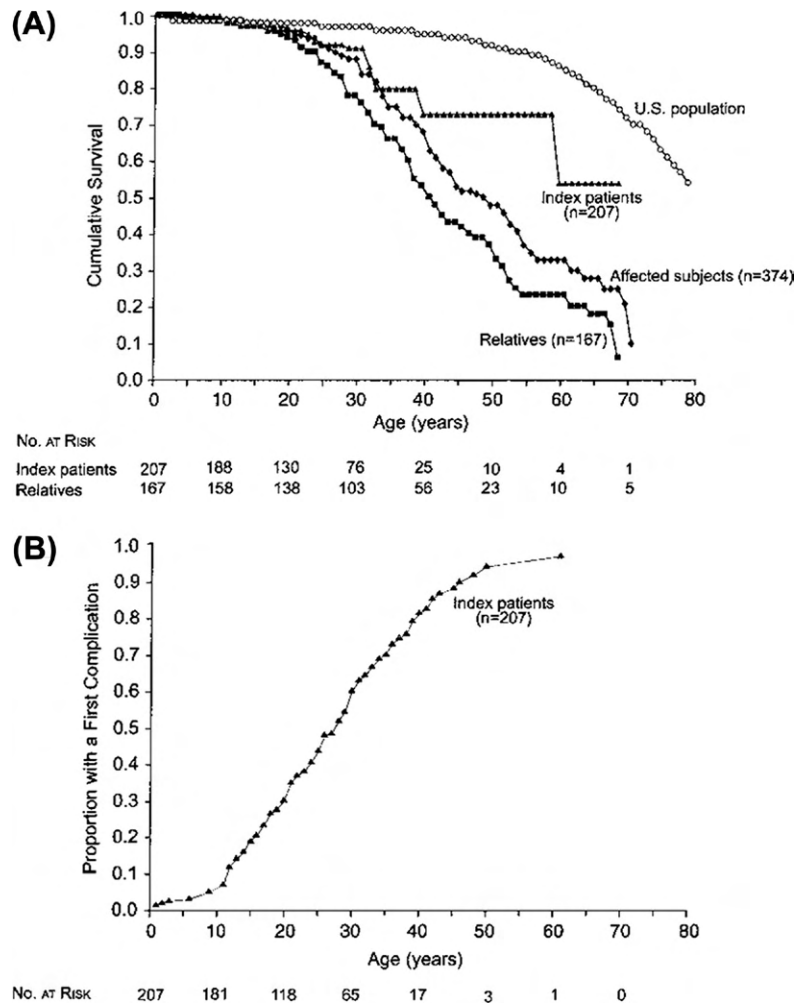
Ehlers–Danlos syndrome type IV, the vascular or ecchymotic variety, was recognized as a distinct entity by Barabas (46), although Sack (8) and Gottron (7a) had probably described the same condition. Many individuals with the vascular type of EDS have fragile, thin, or translucent skin through which the venous pattern is readily visible (Figure 154-3). They generally bruise easily and often have unexplained ecchymoses in well-protected areas. Some have a “characteristic” faces with tight skin, thin nose, and a “stare” (73,74) but, increasingly, as more individuals are identified by molecular genetic or biochemical studies, it is clear that clinical diagnosis on the basis of facial findings is not common. Large joint mobility is generally normal with hypermobility limited to the small joints of the hands. In those with the “acrogeric” form, the skin over the distal extremities has an aged, thin, and atrophic appearance (73,75,76). Venous varicosities are common and may be severe. In addition, congenital hip dislocation may be more common than expected (70), and keratoconus is a complication that may occur as early as 20 years of age (77) but can be treated successfully with corneal transplantation.



**FIGURE 154-3** Clinical features of the vascular type of EDS (EDS type IV). This 26-year-old woman's skin is thin so that the venous patterning shows readily over the chest. Her hands have a particularly aged appearance (acrogeria). She had a dissection of her right carotid artery in her late thirties without neurologic complications and died at age 48 years as a consequence of bowel rupture and sepsis.

The major clinical complications of the vascular type of EDS are arterial, bowel, and uterine rupture, emphasizing that although arterial complications are very common, other organs are involved as well. Because of these dramatic complications, life expectancy is shortened to a mean of less than 50 years for affected males and females (70) (Figure 154-4). This type of EDS is inherited in an autosomal dominant fashion. New mutations in the causative gene, *COL3A1*, are common and about one half of identified individuals with the condition have no apparent family history of the disorder. Although autosomal recessive inheritance was first thought to be the mode of inheritance (73,78) the supporting evidence was not strong and all the recent molecular genetic studies are consistent with heterozygosity for mutations in the *COL3A1* gene as the cause of the phenotype. There are no convincing examples of recessive inheritance for mutations in the *COL3A1* gene and similar phenotypes probably result from mutations in other genes (see, for example, Reference (79)).





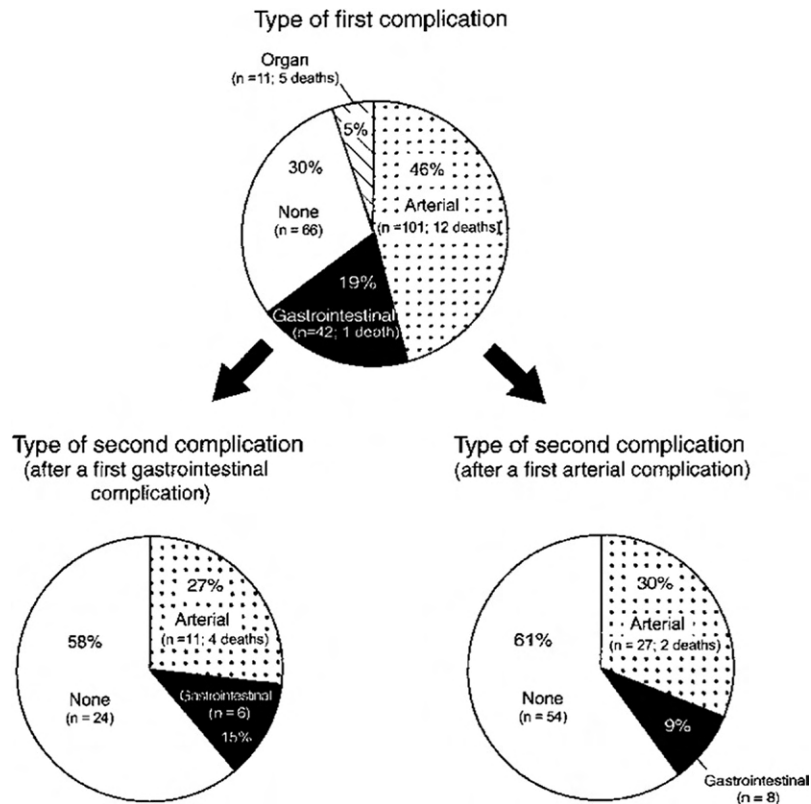
**FIGURE 154-4** (A) Survival curve and (B) age of penetrance with a major complication, for individuals with the vascular form of EDS. (From Pepin, M.; Schwarze, U.; Superti-Furga, A.; Byers, P.H. *Clinical and Genetic Features of Ehlers–Danlos Syndrome Type IV, the Vascular Type*. N. Engl. J. Med. **2000**, 342, 673–680.)

The most common complication is arterial rupture (70). The clinical presentation depends on the location of the ruptured artery. Hemorrhagic stroke (80), intrathoracic hemorrhage, intra-abdominal and retroperitoneal bleeding, and compartmental syndromes may all result (Figure 154-5). The cause of death in most individuals with EDS type IV is related to arterial bleeding. Bowel rupture, often rupture of the sigmoid colon, is the next most common complication of the disorder (70). Uterine rupture (70,81,82), as well as vascular rupture (83–86) and other vascular difficulties, which can include coronary artery dissection (87), may complicate pregnancy. These complications may be fatal and it appears that as many as 10% of woman who have one or more pregnancies may die of pregnancy-related complications (70).

The clinical features of the disorder are variable. In some individuals, the diagnosis can be made early in infancy because of the marked bruising and thin skin. In others, including some with a family history of the disorder, the diagnosis may be suspected in infancy because of bruising or, less commonly, some of the usual

complications. However, in many individuals the diagnosis is not suspected until a significant complication has occurred and even then, the vascular form of EDS might not be considered in the differential diagnosis. Major complications of the condition are uncommon in childhood, with less than 1% of affected individuals having identifiable events prior to puberty. About 25% have their first complication by age 20 and more than 80% have serious complications by 40 years of age (70). Despite this, there are individuals who live into their sixties and seventies.

The major cause of death is arterial rupture, accounting for more than 90% of deaths in men and a somewhat smaller proportion in women. Surgery is often complicated by the fragility of vessels, making repair difficult. Nonetheless, most people appear to survive the first episode of vascular rupture and that likelihood is increased if the diagnosis is known beforehand. Surgical intervention may be quite successful and lead to years extended life (88). One of the major controversies revolves around identification and treatment of aneurysms. Currently



**FIGURE 154-5** Distribution of complications among index cases with the vascular form of EDS and the outcome of those complications. (From Pepin, M.; Schwarze, U.; Superti-Furga, A.; Byers, P.H. *Clinical and Genetic Features of Ehlers–Danlos Syndrome Type IV, the Vascular Type*. N. Engl. J. Med. **2000**, 342, 673–680.)

there is no agreed-upon protocol by which to follow the vasculature in affected individuals. While molecular resonance or computerized tomographic imaging with contrast and three-dimensional reconstruction are very effective means to identify aneurysms, the friable nature of the vessels has made elective surgical intervention the less frequently followed option. In the past few years, the use of endovascular stents has encountered some success in apparently stabilizing aneurysmal regions of vessels (89–92) but failure is also encountered (93). This practice remains an unsettled question but clear evidence of success would be a welcome addition to the relatively limited armamentarium to be used in this setting.

Rupture of the bowel with sepsis accounts for only a small proportion of deaths in both men and women. As the major site of rupture is in the sigmoid colon (accounting for more than one third of the reported ruptures in our series), surgical removal of the distal colon has been used in an attempt to prevent additional episodes, apparently successfully in some individuals. Prompt surgical intervention is important, and the usual approach to bowel perforation is appropriate (94).

Other complications of EDS type IV include carotid cavernous sinus fistula formation (54a,54b,84a,91a,95a,96a,97a,98a), which has been treated successfully by embolization and arterial surgery.

There is currently no medical treatment known to decrease the risk of complications or to increase life span

predictably. There can be considerable variation in expression of a *COL3A1* mutation within the same family, making evaluation of therapies difficult. Surgical intervention, particularly for bowel rupture, may have a significantly beneficial effect (99). It is unclear whether removal of aneurysmal sections of major arteries is similarly helpful, as complications of surgery often ensue (100).

Although the vascular form of EDS is inherited in an autosomal dominant fashion and results from heterozygosity for mutations in the *COL3A1* gene, the recurrence rate following birth of an affected infant to unaffected parents is not zero (101–103). Recurrence results from parental mosaicism for the mutation, but at present the risk of recurrence is unknown. The rate of parental mosaicism could approach 15% (Schwarze, U. and Byers, P.H., unpublished). Prenatal identification of an affected fetus (or exclusion of the diagnosis) is possible by analysis of intragenic *COL3A1* markers in familial forms (12), by analysis of type III procollagen synthesized by cultured CVS cells (104), or by direct DNA analysis in families in which the mutation has been identified.

### 154.6.2 Biochemical and Molecular Genetic Aspects

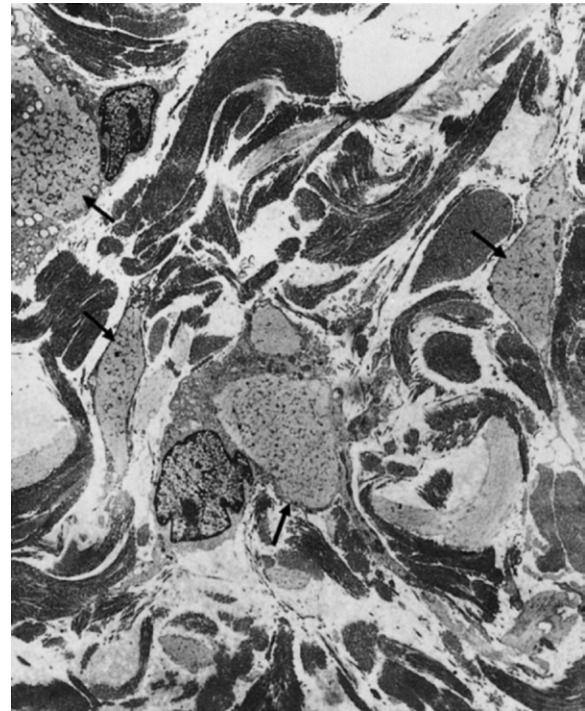
The biochemical basis of this disorder, alterations in the synthesis and secretion of type III procollagen, was recognized in 1975 (78). The clinical diagnosis of EDS type

IV is generally suspected in the presence of marked bruising or following a major arterial or bowel complication in a young person, if there is no family history of the condition. The diagnosis can be confirmed biochemically by the demonstration of decreased type III procollagen production by cultured dermal fibroblasts. When examined by electron microscopy, skin from individuals with EDS type IV has characteristics that appear to depend on the nature of the mutation (21,35,37). With some mutations there is very marked intracellular storage of the abnormal type III procollagen with pooling of the material in the rough endoplasmic reticulum (Figure 154-6). In those individuals, collagen fibrils tend to be small and uniform in diameter. In others, intracellular pooling of procollagen appears to decrease, and fibril diameter becomes more variable.

More than 200 mutations in the *COL3A1* gene have now been identified in individuals with the vascular form of EDS (summarized in the Database of Type I and Type III Collagen Mutations—<http://www.le.ac.uk/genetics/collagen/>; (105,106)). Approximately two thirds of the mutations are single nucleotide substitutions that result in substitutions for glycine residues in the triple helical domain of the  $\alpha 1(\text{III})$  chain. Most of the rest are splice site mutations that result in exon-skipping, although some have more complex outcomes (107,108). Most mutations in the *COL3A1* gene that produce the vascular EDS phenotype are private to the family. There are two notable exceptions, both of which occur at CpG dinucleotides. The first results in substitution of serine for glycine at position 16 of the triple helix (Gly16Ser) and has been seen more than a dozen individuals (70). The second results in a mutation at the splice donor site of intron 24 and results in skipping of exon 24; this mutation has been seen in almost two dozen unrelated families (70). There are a small number of other mutations that have been seen in more than one family. There are a small number of multi-exon deletions (102,109,110) and some small genomic deletions that remove a portion of an exon (70,111).

The substitutions for glycine in the triple helix are not seen in the proportion expected (112) on the basis of glycine codon usage in the *COL3A1* gene. The small substituting residue alanine is seen far less often than predicted. It is possible that this low frequency could be related to a less severe phenotype that escapes clinical detection, or less effect on the protein folding that leads to less dramatic abnormalities in biochemical studies. Perhaps as direct genetic testing increases in frequency and the clinical range in which testing is used, this discrepancy may be explained by identification of a milder phenotype in which substitutions of glycine by alanine are found more frequently.

To date, *COL3A1* mutations that result in loss of a stable transcript are also seen far less frequently than anticipated based on mutation frequency in other collagen genes. For example, the mild osteogenesis



**FIGURE 154-6** Electron micrograph of fibroblasts in skin from patient depicted in Figure 154-3. The rough endoplasmic reticulum of each fibroblast in the field is markedly dilated (arrows) and filled with abnormal type III procollagen molecules. This patient is heterozygous for a point mutation in the *COL3A1* gene that results in substitution of the glycine at position 1018 in the triple helix by valine. (Micrograph courtesy of Dr Karen Holbrook.)

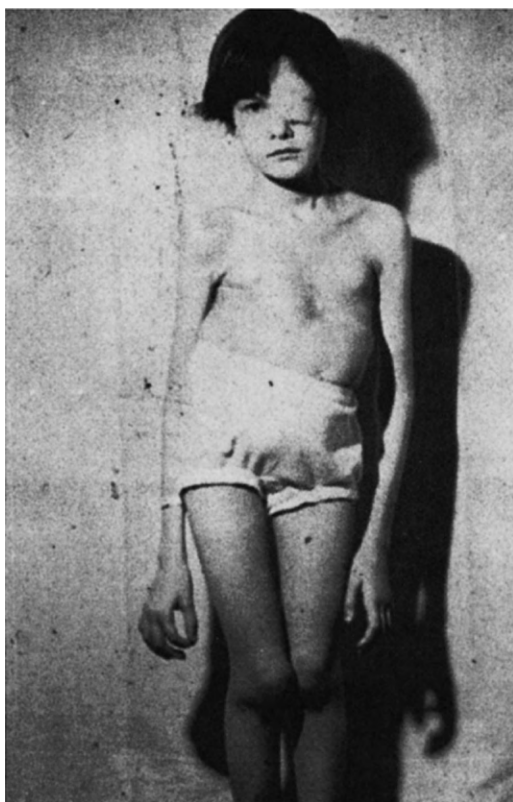
imperfecta (OI) type I phenotype almost always results from null mutations in the *COL1A1* gene and leads to half the normal production of type I procollagen. To date, only a handful of null mutations in the *COL3A1* gene have been identified (113), and although the phenotype is milder in some families there are others in which the clinical presentation is indistinguishable from that seen with other classes of mutation. As direct gene testing expands, it is likely that this group will be identified more often.

## 154.7 KYPHYOSCOLIOTIC EHLERS–DANLOS SYNDROME (EDS TYPE VI)

### 154.7.1 Clinical Aspects

The kyphoscoliotic type of EDS (EDS type VI) was the first true molecular disorder of collagen metabolism to be recognized in humans (98). The first patients described were two sisters who had smooth, hyperextensible, velvety skin; moderate scarring; bruising; large and small joint laxity; a marfanoid habitus with moderately severe thoracic kyphoscoliosis; and keratoconus with ocular globe fragility (Figure 154-7). Since the original report, more than 20 individuals with EDS type VI have been described (57a,92a,96,114,115,116,116a,116b,117–122). Many of these reports are linked





**FIGURE 154-7** Clinical features of the oculo-scliotic type of Ehlers–Danlos syndrome (EDS type VI). The young girl in the photograph is one of the first children recognized to have this condition. She has a marfanoid habitus and scoliosis and ruptured her ocular globe following minor trauma. (Courtesy of Dr Sheldon Pinnell, Duke University.)

to mutation analysis and, unfortunately, there has been no attempt to define a natural history of the disorder from all the available patients. The major clinical clue to early diagnosis is profound hypotonia in the newborn period and progressive scoliosis. It is not uncommon for children with this form of EDS to be evaluated for neurological disorders before the diagnosis of EDS is reached.

The disorder is inherited in an autosomal recessive fashion. It is not clear if there is an ethnic or racial predisposition, although from the reported patients almost all are of white background. The frequency of the kyphoscoliotic type of EDS is not known. In a recent survey of allele frequency, the most common allele—a 7-exon duplication involving exons 10–16—was found in 1 of 582 normals studied (121). This allele, which appears to represent a single mutational event, accounts for about half of the mutant alleles so far identified in affected individuals (119) but about 20% of all mutant alleles (123). If these represent approximate frequencies, then the kyphoscoliotic form of EDS could affect as many as 1 in 60,000 individuals. This frequency seems much higher than that recognized at present, so the estimate could be biased by the population assayed for the common mutation.

In two compilations of the clinical features of reported individuals (2,118,124) identified through biochemical screening, it was striking that vascular complications, including intracerebral and other arterial hemorrhage, were more common than had been anticipated and contributed to morbidity in at least three individuals. If scoliosis is severe, cardiopulmonary compromise can shorten lifetime (125).

As with other recessively inherited disorders, the recurrence risk for an additional affected sibling, following the birth of a child with the type of EDS, is 25%. Prenatal identification of an affected fetus or exclusion of the disorder can be accomplished by direct mutation detection in appropriate families (120), or by measurement of enzymatic activity in cultured amniocytes or chorionic villus cells (126).

Treatment with ascorbic acid in some individuals enhances lysyl hydroxylation in collagens as measured by excretion of hydroxylysine in the urine (127) and may ameliorate the clinical phenotype over a period of several months. Ascorbate appears to act by inducing the enzyme and by acting as a cofactor (114,127–129).

Treatment of scoliosis with bracing appears to be unsuccessful, and even with surgery the outcome apparently is uncertain. Ocular globe fragility can lead to loss of vision although surgical techniques are being proposed that may preserve vision (130).

A small number of individuals with phenotypic features that appear compatible with the clinical diagnosis of the oculo-scliotic type of EDS do not have evidence of biochemical abnormalities in the enzyme (2,20), and this is referred to as the type B form of the syndrome. The molecular and biochemical basis of this condition is uncertain.

### 154.7.2 Biochemical and Molecular Genetic Aspects

The kyphoscoliotic form of EDS (EDS type VI) results from mutations in the *PLOD1* gene (119) that encodes the enzyme lysyl hydroxylase 1, which hydroxylates triple helical lysyl residues in collagens. The *PLOD1* gene is located at chromosome 1p36.2–p36.3 (131). Two other lysyl hydroxylases, *PLOD2* and *PLOD3*, have been identified (132) but all the mutations known to produce the kyphoscoliotic form of EDS are in the *PLOD1* gene (Table 154-2). This enzyme does not appear to hydroxylate the lysyl residues in the telopeptide regions (just external to both ends of the triple helix) (103a). Modification of the telopeptide lysyl residues involved in cross-link formation appears to be the domain of the *PLOD2* gene (133). Mutations in that gene result in Bruch syndrome, a form of osteogenesis imperfecta with joint contracture (133,134). Mutations in *PLOD1* decrease the activity of the enzyme to about 10% or less of the normal level, leading to decreased lysyl hydroxylation within the triple helical domains of



type I and III collagens. Type II collagen appears to be normally hydroxylated, presumably because one of the other enzymes is expressed with greater abundance in that tissue. The consequence of decreased lysyl hydroxylation is altered production of the stable intermolecular cross-links that provide tissues with tensile strength (114a). Although alterations in enzymatic activity are readily demonstrated in cultured dermal fibroblasts, the assay is cumbersome and not performed in many places. The alteration in the collagen cross-links produced is reflected in the altered ratio of hydroxylated to unhydroxylated cross-links excreted in the urine, providing a relatively simple, rapid, and inexpensive diagnostic test for the condition (135,136).

As indicated above, the most common mutant allele of the *PLOD1* gene is a duplication of approximately 8.9kb of the gene that contains seven exons (9,13,29,55,83,100,137) as a result of Alu–Alu recombination (115,117,123). Analysis of linked polymorphic variants in the mutant allele suggests that the majority of the alleles are the progeny of a single mutational event (119,123); an alternate but less likely possibility is that the alleles identified by the variants contain a predisposing sequence and, so, the mutation has occurred several times. The duplication involved Alu elements located in introns 9 and 16 (117). The mutation results in an in-frame duplication of 260 amino acid residues. The mRNA appears to be stable

but the enzyme is nonfunctional. Of 53 known mutant alleles compiled in 2000 (119), 27 are represented by the duplication allele. This frequency clearly represents a bias of ascertainment as the allele can be readily recognized and thus tested for. One other allele, 1557C>G (Y511X), has been found in 7 alleles, and two others (see Table 154-2) have been identified in more than one individual. The remaining 11 mutant alleles have been seen in single individuals. In the majority of individuals in which the duplication has been identified, the second allele has not yet been described.

The mutations include those in which premature termination codons lead to very unstable mRNA (see Table 154-2) and others in which the mRNA appears to be stable but the resulting protein lacks enzymatic activity. There does not appear to be a recognizable phenotype–genotype correlation at this point.

## 154.8 ARTHROCHALASIS TYPES OF EHLERS–DANLOS SYNDROME (EDS TYPES VIIA AND VIIB)

### 154.8.1 Clinical Aspects

The former EDS type VII has now been divided into two groups—one referred to as the arthrochalasis types and the other as dermatosparaxis (see later discussion) (9). Arthrochalasis refers to very marked joint laxity

**TABLE 154-2** *PLOD1* Mutations in the Oculo-Scoliotic Form of Ehlers–Danlos Syndrome (EDS Type VI)

Exon	Mutation <sup>a</sup>	Effect on Protein	References
2	c.154insC	Frameshift with PTC in exon 4; unstable mRNA	Heikkinen et al., 1999 (186)
2	c.145C>T	Q49X	Yeowell and Walker, 2000 (119)
4	c.426T>A	Y142X	Yeowell and Walker, 2000 (119)
IVS 4	IVS4-2delA	Splice variants, unstable	Heikkinen et al., 1999 (186)
IVS 5	IVS5+1G>A	Exon 5 skip produces frameshift and PTC in exon 7	Yeowell and Walker, 1997 (121)
10–16	Duplication	Duplication of amino acid residues 326–585	Hautala et al., 1993; Pousi et al., 1994; Heikkinen et al., 1997; Feshchenko et al., 1998; Yeowell and Walker, 2000 (95,115,117,119,123)
9	c.955G>A	R319X	Hyland et al., 1992 (116)
IVS9	IVS9+3insTT	Exon 9 skip	Pajunen et al., 1998 (187)
10	c.979C>T	Q327X	Yeowell and Walker, 2000 (119)
11	c.1099–1113del	Del367–371 (DLCRQ)	Yeowell and Walker, 2000 (119)
	c.1336T>G	W446G	Walker et al., 2005 (188)
14	c.1534C>G	Y511X	Yeowell and Walker, 2000 (119)
15	c.1594delGAG	E532del	Ha et al., 1994 (96)
IVS15	IVS15-2delA	Exon 16 skip	Pousi et al., 1998 (132)
IVS16/exon17	3 kb deletion	Exon 17 skip	Pousi et al., 1998 (132)
17	c.1760AC>GA	N587R	Heikkinen et al., 1997 (123)
17	1772–1785del	Frameshift with PTC in exon 17	Heikkinen et al., 1997 (123)
17	c.1838G>C	W612C	Feshchenko et al., 1998 (95)
18	c.2008C>T	R670X	Yeowell and Walker, 2000 (119)
19	c.2032G>A	G678R	Ha et al., 1994 (96)

<sup>a</sup>The nucleotide number of the mutation is in reference to the A of the initiator methionine residue in the coding sequence. This differs from the sequence of the cDNA (GenBank M98252) in which the cDNA sequence has an additional 24 nucleotides. Many of the mutations in the literature have used the GenBank sequence as the basis of the description so the nucleotide numbers here may be 24 less than those cited but the amino acid numbering remains as before in which the initiator methionine is residue 1 of the sequence.

characteristic of two groups of EDS (the former types VIIA and VIIB) but, unfortunately, is not a term in general use in the medical community, although the term arthrochalasis multiplex congenita is used in the orthopedic literature. It is likely that only a small group of individuals identified in that literature actually have the molecular defects that define this type of EDS.

Ehlers–Danlos syndrome types VIIA and B (used here for clarity) are characterized by marked joint laxity, bilateral congenital hip dislocation, and relatively normal skin (Figure 154-8) (21,138,139). The hip dislocation may be recalcitrant to routine therapy and even to surgical repair. Short stature is seen in some, and some individuals have mild midface hypoplasia and a small nasal bridge. Wormian bones in the skull and long bone fractures have been seen some individuals with both forms (Table 154-3). There is a surprising degree of heterogeneity such that some individuals have lifelong difficulties with joint mobility that limit their ability to ambulate, while others do well following surgery to stabilize their hips.

Although the majority of reported individuals are the first affected in their family, biochemical and molecular genetic studies (see later discussion) confirm that the disorder results from heterozygosity for mutations in the *COL1A1* and *COL1A2* genes of type I collagen. The majority of affected individuals represent new mutations, but the small number of families in which the disorder is inherited through multiple generations confirms the nature of inheritance (138,140).

### 154.8.2 Biochemical and Molecular Genetic Aspects

When EDS type VII was first distinguished from other forms of EDS (141), the condition was thought to be due to alteration in the enzymatic processing at the N-terminal end of the type I procollagen molecule, analogous to

the, then recently identified, disorder dermatosparaxis in cattle (141a). It was subsequently shown (142) that there was a defect in the structure of type I procollagen molecules, synthesized by cells cultured from some of the same affected individuals. It soon became clear that mutations at the splice donor and splice acceptor sites of exon 6 of the *COL1A1* and *COL1A2* genes, or deletion of the exon itself, result in the phenotypes (Figure 154-9; see also Table 154-3) (21,108,143–153). In general, mutations in the *COL1A1* gene give rise to more striking joint laxity, although it is unlikely that the clinical features alone would distinguish disorders in the two genes.

Exon 6 of both genes contains the N-proteinase site as well as a lysine residue that is hydroxylated and involved in cross-link formation. Mutations in the splice donor site of both genes result in skipping exon 6. As a consequence, chains that lack the sequences encoded by exon 6 cannot be cleaved by the normal proteinase and molecules contain one or more abnormal pro $\alpha$ 1(I)-chains or an abnormal pro $\alpha$ 2(I) chain (see Figure 154-9). Mutations at the splice acceptor site have slightly different effects that appear to depend on the nature of the substituting nucleotide and its position. At positions 14 and 15 of each exon, there is an AG dinucleotide that can serve as a splice acceptor site if the constitutive site is lost. As a result, with some splice acceptor mutations the cryptic site is often used, which results in deletion of 15 nt from the mature mRNA. These nucleotides encode the first five amino acid residues of the exon, which includes the N-proteinase site. The lysine residue involved in cross-link formation is lost if the entire exon is deleted. In both instances, the formation of collagen fibrils is disturbed (150,154). The effect of these mutations on bone integrity (i.e. the occasional fracture and wormian bones seen in some individuals) may reflect interference with mineralization of collagen fibrils by the retention of the propeptide in bone.



**FIGURE 154-8** Clinical features of arthrochalasis multiplex congenita (EDS type VIIA). This infant was born with bilateral congenital hip dislocation and had marked joint laxity as well as a large inguinal hernia. This child has a *COL1A1* acceptor splice site mutation. (Courtesy of Dr Louanne Hudgins, Stanford University.)

## 154.9 DERMATOSPAXIS (EDS TYPE VIIC)

### 154.9.1 Clinical Aspects

Dermatosparaxis, EDS type VIIC, is a recessively inherited disorder that results from absence of the activity of procollagen N-proteinase (now known as *ADAMTS2*) (155), the enzyme that cleaves the N-terminal propeptide from type I procollagen. About a dozen children with this disorder have now been identified (155–162). Affected children all exhibit striking joint laxity; extremely soft, fragile, and extensible skin; easy bruising; mild hirsutism; a small chin; and blue sclera (Figure 154-10). One child was noted to have inguinal tears at birth (160,162) and one was born prematurely (156). One of the first children reported (162) had a perinatal cerebral hemorrhage but was intellectually normal at age 2. The oldest

**TABLE 154-3 Clinical Features of Ehlers–Danlos Syndrome Type VIIA and VIIB that Result from Splice Site Mutations Around Exon 6 of the COL1A1 and COL1A2 Genes**

Reference	CHD <sup>a</sup>	Dislocation	Scoliosis	Fractures	Bruising	Scars	History Family	Gene	Mutation
Byers et al., 1997 (A)	+	+		–			+	COL1A2	Intron 5 –2AØG
Byers et al., 1997 (B)	+	+	+	–			–	COL1A2	Intron 5 –1GØA
Chiodo et al., 1992	+	+	+	+	+	+	+	COL1A2	Intron 5 –1GØC
Byers et al., 1997 (E)	+			–			NA <sup>a</sup>	COL1A2	Exon 6 –1GØA
Weil et al., 1989 <sup>b</sup>	+	+					–	COL1A2	Exon 6 –1GØA
Vasan et al., 1991	+	+	+		+		–	COL1A2	Intron 6 +1GØA
Nicholls et al., 1991	+	+	+				+	COL1A2	Intron 6 +1GØA
Weil et al., 1990 <sup>c</sup>	+	+					–	COL1A2	Intron 6 +1GØA
Nerlich et al., 1994	+					+	–	COL1A2	Intron 6 +1GØA
Watson et al., 1992 <sup>d</sup>	+	+		+			+	COL1A2	Intron 6 +1GØA
Giunta et al., 1999	+	+	+				–	COL1A2	Intron 6 +1GØA
Byers et al., 1997 (C)	+						–	COL1A2	Intron 6 +1GØT
Byers et al., 1997 (D)	+	+		+			–	COL1A2	Intron 6 +1GØT
Weil et al., 1988 <sup>e</sup>	+	+					–	COL1A2	Intron 6 +2TØC
Ho et al., 1994	+	+			+		–	COL1A2	Intron 6 +2TØC
Giunta et al., 1999	+	+			+	–	–	COL1A2	Intron 6 +2TØC
Byers et al., 1997 (F)	+	+	+	+	+	+	–	COL1A2	Exon 6 deletion
Byers et al., 1997 (G)	+						–	COL1A1	Intron 5 –1GØA
D'Allesio et al., 1991	+	+	+	+			–	COL1A1	Exon 6 –1GØA
Weil et al., 1989 <sup>f</sup>	+	+	+				–	COL1A1	Exon 6 –1GØA

(Modified from Byers, P.H.; Duvic, M.; Atkinson, M.; et al. Ehlers–Danlos Syndrome Type VIIA and VIIB Result From Splice-Junction Mutations or Genomic Deletions that Involve Exon 6 in the COL1A1 and COL1A2 Genes of Type I Collagen. *Am. J. Med. Genet.* **1997**, 72, 94–105 (138).)

<sup>a</sup>CHD = congenital hip dislocation; NA = not available.

<sup>b</sup>Clinical information is also contained in Steinmann et al. (142) and Lichtenstein et al. (141).

<sup>c</sup>Clinical information is also contained in Eyre et al. (146).

<sup>d</sup>Clinical information is also contained in Viljoen et al., 1987 (189).

<sup>e</sup>Clinical information is also contained in Wirtz et al., 1987; Wirtz et al., 1990 (153, 190) and Steinmann et al., 1985.

<sup>f</sup>Clinical information is also contained in Cole et al., 1987 (154).

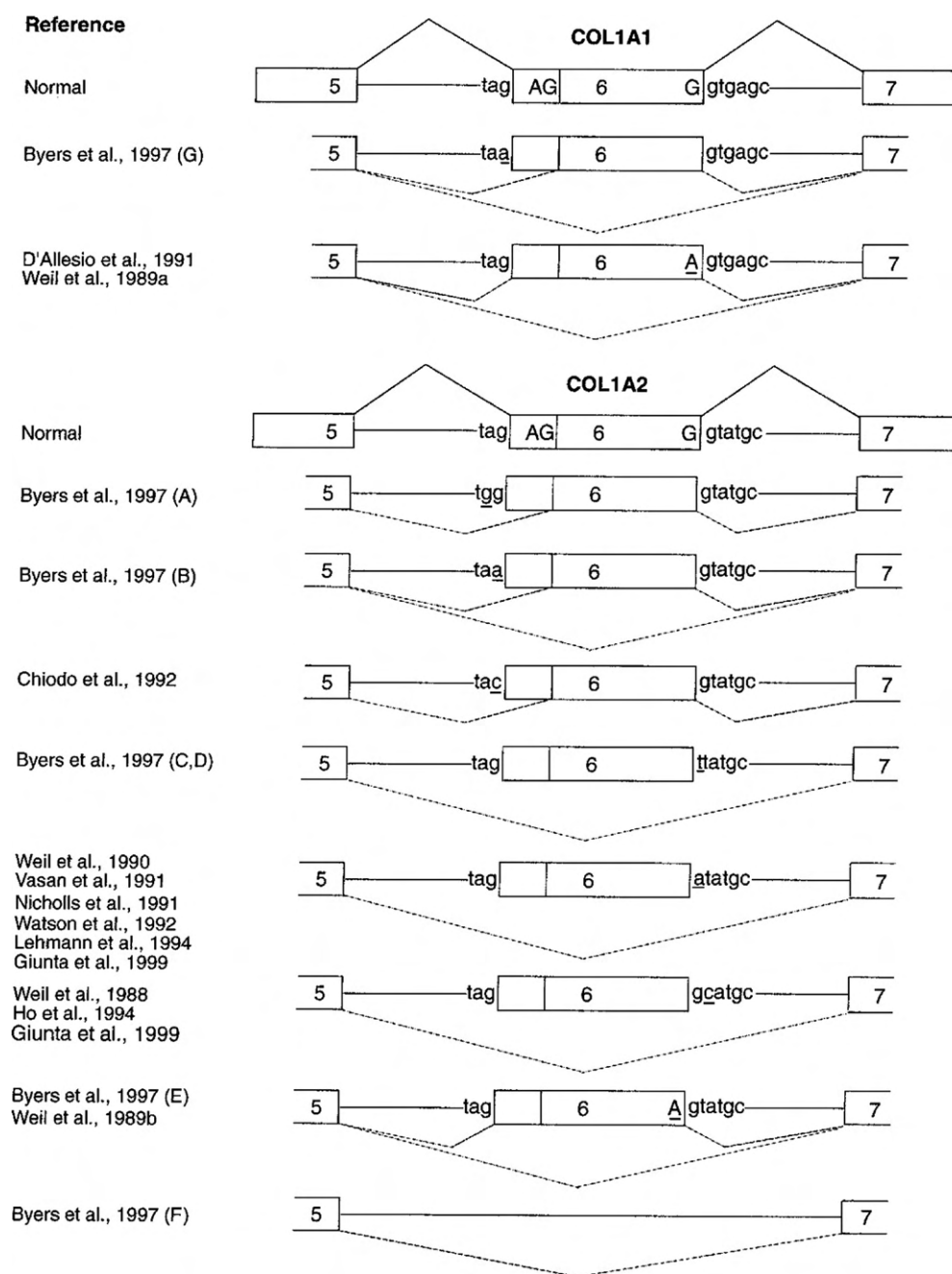
of the known affected individuals is now in her early twenties (161). At age 16 she had normal intellect, and very marked wrinkling and redundancy of the skin over her hands and feet, and of the skin of her face. No other major complications had ensued although joint laxity, easy bruising, and skin fragility persist.

The activation of residual enzyme, if present, or replacement of enzymatic activity would be the most beneficial treatment. There is no consensus on therapy at this point, and because the natural history is not yet well defined, the objectives of therapy are not well delineated. The natural history of dermatosparaxis in animals is not well studied although the disease is known in several species (163–167). It remains to be determined if the vascular wall is well maintained even when skin is dramatically altered. Such studies could be helpful in planning therapeutic intervention.

### 154.9.2 Biochemical and Molecular Genetic Aspects

Cultured dermal fibroblasts from children with dermatosparaxis fail to remove the amino-terminal propeptide from type I procollagen (159,162). In skin from affected individuals there is a small amount of conversion, but

it is not clear if the site used is the normal cleavage site (162). Collagen fibrils in skin from these children have a unique “hieroglyphic” pattern when viewed in cross-section and appear ribbon-like in longitudinal section (Figure 154-11). The altered packing is a consequence of failure to remove the N-terminal propeptide (168). This condition was known in cattle, sheep, dogs, and cats for many years before the human variant was identified. In cattle and humans it is clear that mutations in the gene that encode the procollagen I N-proteinase are responsible for the phenotype (156). Five of the six children who were first identified were homozygous for the same mutation that resulted in a premature termination codon (Q225X). Three of these children were from Ashkenazi Jewish families, although there was no known consanguinity in the families. A fourth was of Hispanic origin and the fifth of undefined white background. Four of the five were homozygous at three polymorphic sites downstream from the mutation while the fifth was heterozygous at one of the sites. We found no examples of the allele in 192 Ashkenazi chromosomes (Schwarze, U. and Byers, P.H., unpublished). The sixth child was born to first cousins and was homozygous for a different mutation, W795X. In both instances the major product of the gene appeared to be very unstable, consistent with



**FIGURE 154-9** Mutations in the *COL1A1* and *COL1A2* genes that result in the EDS type VIIA and EDS type VIIB phenotypes, respectively. The mutation in each instance is underlined, and the effect of the mutation on mRNA splicing is shown (dashed lines). The bibliographic citations to the left indicate the origin of the data for the mutations.

nonsense-mediated decay of the mRNA. Subsequent analysis has identified a series of other mutations that may not destabilize the mRNA, as would be expected for premature termination codons in central exons (155).

With a known mutation, prenatal diagnosis is feasible and can be used to exclude or confirm the diagnosis in subsequent pregnancies.

The bovine mutation is a 17-bp deletion that results in a frameshift and premature termination codon. The mutations have not been identified in the other animals with the condition.

## 154.10 OTHER FORMS OF EHLERS–DANLOS SYNDROME

### 154.10.1 X-linked Ehlers–Danlos Syndrome (EDS Type V)

Beighton (1,3) described a family in which affected males had skin hyperextensibility similar to that seen in EDS type II. Joint mobility and cutaneous bruising were thought to be less extensive, but intramuscular hemorrhage was more common. There was no evidence of





**FIGURE 154-10** Clinical features of human dermatosparaxis (EDS type VIIC). (A and B) A 15-year-old girl with very marked wrinkling of the skin of her face and hands. (From Reardon, W.; Winter, R.M.; Smith, L.T.; et al. *The Natural History of Human Dermatosparaxis (Ehlers–Danlos Syndrome Type VIIC)*. Clin. Dysmorphol. **1995**, 4, 1–11.)

abnormalities in lysyl oxidase function or in collagen cross-link formation (169), as have been found in cells cultured from individuals with occipital horn syndrome (170). Linkage to the Xg blood group and color blindness were excluded in the family (171). It is unclear whether this family, instead, has an autosomal dominant form of EDS, probably a variety of the classical form, with variable expression. Until additional linkage studies can demonstrate an X-chromosomal location for this disorder, the uniqueness of the condition remains uncertain.

### 154.10.2 Periodontal Form of Ehlers–Danlos Syndrome (EDS Type VIII)

The periodontal form of EDS was first proposed as a separate entity on the basis of two families in which periodontal disease was accompanied by marked bruising, joint hypermobility, and skin hyperextensibility (172). The

condition was inherited in an autosomal dominant fashion. In those families, affected individuals had lost most of their teeth by their early twenties as a result of gingival recession that did not appear to be primarily inflammatory. A small number of additional families have been described (97,137,173–177). Pretibial bruising is often more pronounced than expected with other forms of EDS.

Biochemical studies have failed to identify abnormalities in type I or III procollagen (174,175). The condition should be distinguished from EDS type IV, in which periodontal disease is common. The treatment of the noninflammatory gingival recession is not clear but assiduous attention to dental hygiene may be helpful. Orthodontic manipulation may lead to early loss of teeth (97). Gene mapping studies now suggest that EDS type VIII, the periodontal type, may be genetically heterogenous. One locus was mapped to a 4.5 Mb region on the short arm of chromosome 12, but analysis of several candidate genes in the region was not fruitful. On the basis of those studies, there appears to be at least one additional locus and perhaps more.

### 154.10.3 Fibronectin Defect Form of Ehlers–Danlos Syndrome (EDS Type X)

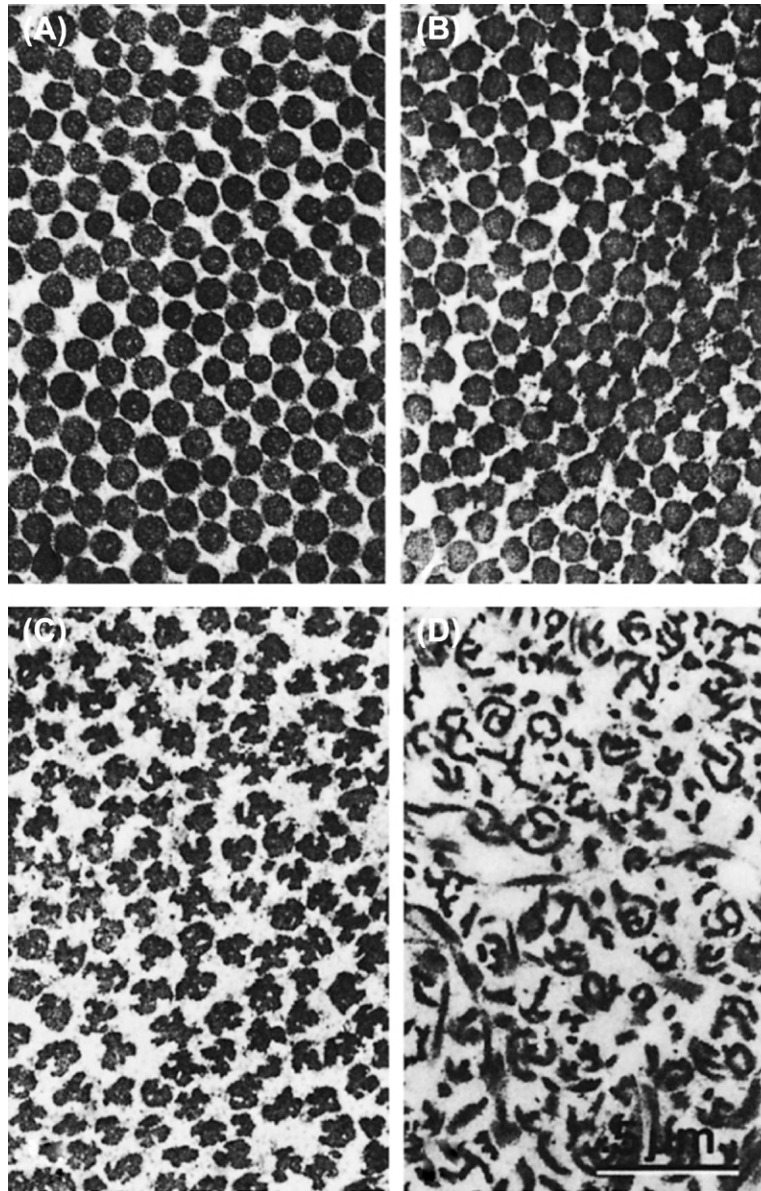
This disorder was described in a single family, in which mild features compatible with EDS type II or III were associated with a variable disorder of platelet aggregation that could be corrected with heterologous plasma or purified fibronectin (64). Skin from one affected individual had alterations in fibril morphology. No biochemical defect in fibronectin has yet been identified. In as much as platelet aggregation defects may be a concomitant finding in some individuals with different forms of EDS (178), it is unclear that this condition warrants a unique identity.

### 154.10.4 Progeroid Type of Ehlers–Danlos Syndrome

Several children have now been described with progeroid facies, multiple nevi, mild mental retardation, skin hyperextensibility, bruisability, moderate skin fragility, and joint hypermobility principally in digits, with radio-ulnar synostosis and developmental delay (179,180). There is moderate variation in the clinical presentation. Inheritance is autosomal recessive and mutations have been identified in the *B4GALT7* gene that encodes galactosyl-transferase-I (179,181,182). The enzyme is responsible for the transfer of galactose to the xylose residue on serines in the formation of heparin sulfate and chondroitin sulfate.

### 154.10.5 Ehlers–Danlos Syndrome with Periventricular Heterotopia

Two reports from 2005 describe an X-linked dominant disorder, with probably male lethality, characterized



**FIGURE 154-11** Electron micrograph of skin from individuals with EDS type VIIA, EDS type VIIB, and dermatosparaxis. Collagen fibrils in normal skin have a uniform diameter and regular spacing (A). In skin from patients with EDS type VIIB (B) and EDS type VIIA (C) there is increasing irregularity of the fibrils, while the skin from a child with dermatosparaxis has very disorganized fibrils (D). (From Byers, P.H.; Duvic, M.; Atkinson, M.; et al. *Ehlers–Danlos Syndrome Type VIIA and VIIB Result from Splice-Junction Mutations or Genomic Deletions that Involve Exon 6 in the COL1A1 and COL1A2 Genes of Type I Collagen*. *Am. J. Med. Genet.* **1997**, 72, 94–105.)

by joint hypermobility, skin extensibility but without significant scarring, presentation with seizures, and the radiological finding of brain heterotopia, often in the periventricular region (7,183). Some of the individuals had arterial aneurysms, including aortic enlargement. Two similar patients had been reported previously (184,185). Surprisingly, the mutations in the families were found in the *FLNA* gene that encodes the protein filamin A, a protein that cross-links the actin fiber cytoplasmic network in cells. Mutations in the gene are known to cause otopalatodigital syndrome types 1 and 2, frontometaphyseal dysplasia, and Melnick–Needles syndrome. The clinical differences are no doubt related to specific mutations in the gene.

### 154.11 SUMMARY

Some forms of EDS are common, while others are rare. It is important to identify those individuals with EDS type IV because they are at risk of significant medical complications. In addition, it is important to identify those with EDS type VI because the genetic risk in the families is different than it is for those with the far more common dominantly inherited forms. Many individuals have some of the clinical features of EDS, including joint laxity and soft extensible skin, yet do not fit clearly into the major categories of EDS. Further biochemical and molecular genetic studies of such individuals and families will be important to identify the underlying molecular defects and the natural history of the disorders.



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# CHAPTER

# 155

## Heritable Diseases Affecting the Elastic Fibers: Cutis Laxa, Pseudoxanthoma Elasticum, and Related Disorders

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### 155.1 INTRODUCTION

The elastic fiber system of connective tissues forms a network that is responsible for resilience and elasticity of various organs. The relative concentration and distribution of elastic fibers in different tissues varies (Table 155-1). Their relative concentration is highest in the aorta and arterial blood vessels as well as in the lungs, while lower concentrations are present in the skin and various tendons. An important specialized tissue location of the elastic structures is Bruch's membrane, an elastin-rich sheath in the eye within the retina. Elastin is synthesized in arterial tissues by vascular smooth muscle cells, and the likely cell type responsible for elastin synthesis in the skin is the fibroblast (Figure 155-1A).

Examination of the elastic connective tissues in human dermis reveals an interconnecting fiber structure consisting of an intricate network of fibers of varying diameters (Figures 155-1B, C, and 155-2A). Transmission electron microscopy reveals that mature elastic fibers consist of two distinct components, elastin, a well-characterized connective tissue protein, and elastin-associated microfibrils (1,2) (Figure 155-2B). In mature connective tissue, elastin forms the core of the fiber while the microfibrils are relatively minor components at the periphery of the fibers. However, in elastin fibrillogenesis during fetal development or during elastic fiber regeneration, the first components being synthesized are the microfibrils, which form a scaffold in which the elastin molecules align. With increasing fetal age and maturation of the elastic fibers, elastin becomes the predominant component.

In fact, electron microscopic estimations of the fully matured elastic fibers suggest that the elastin protein represents over 90% of the total content of the fibers. Thus, the microfibrils appear to play a critical role during early fibrillogenesis, while the physiologic properties of mature elastic fibers are primarily attributable to the elastin network. It should also be noted that microfibrils devoid of elastin can be identified in a number of tissues (see also Chapter 153). These microfibrils may consist of some of the same components as noted in association with elastic fibers, as for example the fibrillins and the fibulins, or they may be composed of unrelated proteins, such as type VI collagen.

**TABLE 155-1** Relative Concentrations of Elastin and Collagen in Various Tissues<sup>a</sup>

Tissue	Percentage of Dry Weight	
	Elastin	Collagen
Skin	0.6–2.1	71.9
Lung	3–7	10
Aorta	28–32	12–24
Ligamentum nuchae	74.8	17
Achilles tendon	4.4	86.0
Liver	0.16–0.30	3.9

<sup>a</sup>The values represent determinations from different animal species; the tissue origin and the assay methods are described in Grant, M. E.; Prockop, D. J. The Biosynthesis of Collagen. *N. Engl. J. Med.* **1972**, *286*, 194–199; 242–249; 291–300.

The biochemical characterization of elastin and associated microfibrillar proteins was hampered for many years by their insolubility. However, with the advent of techniques of molecular biology, many of the structural components of elastic fibers are now well characterized, and critical features of their basic structure and expression of the corresponding genes are well known (1,3,4) (Table 155-2). Interest in understanding the elastic fiber network has recently increased with the realization that changes in the structure and metabolism of these fibers are associated with a number of heritable and acquired diseases (Table 155-3).

## 155.2 BIOLOGY OF ELASTIN

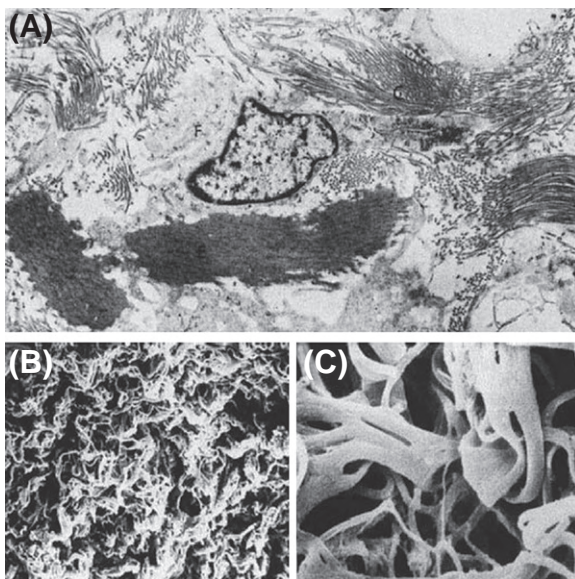
### 155.2.1 Protein Structure

The basic molecular unit of elastin is a linear polypeptide, also known as tropoelastin, with an approximate molecular mass of 70 kDa. The precise amino acid sequence of human tropoelastin was originally deciphered from cloning of full-length cDNA and the corresponding gene (*ELN*) (5,6) (Figure 155-3). Amino acid composition of elastin is rich in hydrophobic residues, and about one-third of the total number of amino acids is composed of glycine residues. The distribution of glycine is different, however, from that in collagen, where glycine occupies every third residue in a repeating Gly-X-Y sequence. Instead, in elastin the glycine residues are clustered within hydrophobic domains, which

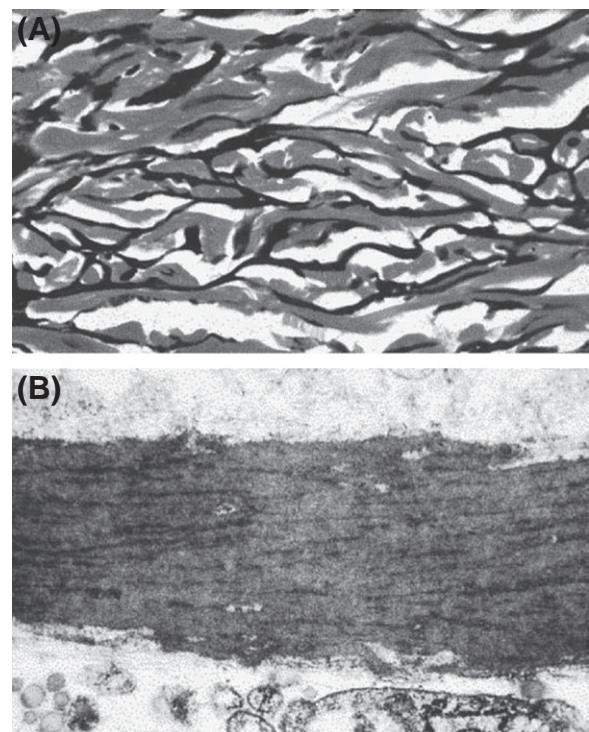
alternate with alanine- and lysine-rich sequences, so-called cross-link domains (Figure 155-4). It is of interest to note that elastin also contains some hydroxyproline, but the relative content of this amino acid is considerably lower than in collagen and the values for hydroxyproline are variable.

One of the characteristic features of elastic fibers is the presence of cross-links, which render the individual tropoelastin molecules highly insoluble (see Figure 155-4). The two major cross-link compounds, known as desmosine and isodesmosine, are apparently unique to elastin (7). The content of desmosine in various elastin preparations has been shown to be fairly constant, ~1.5 residues per 1000 amino acids, and assay of these cross-links can therefore provide a quantitative measure of the elastin content in tissues (8).

The elastic fibers display a resilient nature, providing elasticity to various organs owing to the presence of alternating hydrophobic and cross-link domains. When stretched, the hydrophobic domains are exposed to the surrounding aqueous milieu, and the energy for contraction of the fibers is derived from return of these hydrophobic groups to the nonaqueous environment. Thus, alterations in the primary structure of tropoelastin, or in the cross-linking process, which is enzymatically mediated by lysyl oxidase (see later discussion), could perturb



**FIGURE 155-1** Ultrastructure of elastic fibers in human skin. (A) Transmission electron microscopy demonstrates a fibroblast (F) surrounded by elastic sheets (E) and collagen fibers (C). (B and C) Scanning electron microscopy reveals the presence of interconnecting fibers of variable diameters. (Modified from Uitto, J.; Rosenbloom, J. *Elastic Fibers*. In *Dermatology in General Medicine*; 5th edn; Freedberg, I. M., Eisen, A. Z., Wolff, K., et al., Eds.; McGraw Hill: New York, 1999; p. 260.)



**FIGURE 155-2** Morphology of the elastic fibers in human dermis. (A) Elastic fibers, visualized by histopathology using an “elastin-specific” stain (Verhoeff–van Gieson), form an interconnecting network (black). (B) Transmission electron microscopy of an individual elastic fiber reveals the presence of an electron-lucent central core consisting of elastin, and electron-dense (darker) elastin-associated microfibrils, which are superimposed on the core.

**TABLE 155-2 Clinical Features, Histopathology, Inheritance, Associated Biochemical Findings, Gene Defects, and Predisposing Conditions in Diseases with Elastic Fiber Abnormalities**

Disease	Inheritance <sup>a</sup>	Clinical Manifestations	Histopathology of Elastic Fibers	Gene Defects, Biochemical Findings, and Predisposing Clinical Conditions
Pseudoxanthoma	AR, NH	Yellowish papules coalescing into plaques Inelastic skin Cardiovascular and ocular abnormalities	Accumulation of pleomorphic and calcified elastic fibers in the mid-dermis	Deposition of calcium apatite accumulation of glycosaminoglycans MRP6/ABCC6 gene mutation β-thalassemia; D-penicillamine treatment
Buschke–Ollendorff syndrome	AD	Dermatofibrosis lenticularis disseminata and osteopoikilosis	Accumulation of interlacing elastic fibers in the dermis	Increased desmosine content
Cutis laxa	AR, AD, or NH	Loose, sagging, inelastic skin Pulmonary emphysema Urinary and gastrointestinal tract diverticuli	Fragmentation and loss of elastic fibers	Decreased desmosine content; FBLN5 gene mutation increased elastase activity D-penicillamine treatment skin lesions (e.g. drug reaction)
Copper deficiency syndromes	XR	A spectrum of clinical manifestations—Menkes syndrome; Occipital horn syndrome (previously X-linked cutis laxa)	Paucity of the central amorphous component of elastic fibers while the microfibrillar material is normal. Frayed and split arterial intima reflecting defect in elastin and collagen cross-linking	Reduced activity of lysyl oxidase copper-dependent enzyme ATP7A gene
DeBary syndrome	AR	Cutis laxa-like skin changes Mental retardation Dwarfism	Rudimentary, fragmented elastic fibers	Reduced elastin mRNA levels
The wrinkly skin syndrome	AR	Decreased elastic recoil of the skin Increased palmar and plantar creases	Decreased number and length of elastic fibers	Variant of cutis laxa?
Mid-dermal elastolysis	NH	Fine wrinkling of the skin, primarily in exposed areas	Fragmentation and loss of elastic fibers in the mid-dermis	Sun exposure
Anetoderma	NH	Localized areas of atrophic, sac-like lesions	Loss and fragmentation of elastic fibers in the dermis	Reduced desmosine content due to inflammatory reactions
Elastosis perforans	NH	Hyperkeratotic papules, commonly on the face and neck	Accumulation and transepidermal elimination of elastic fibers	D-penicillamine-induced septal cross-linking
Elastoderma	Unknown	Loose and sagging skin with loss of recoil	Accumulation of pleomorphic elastotic material without calcification in the mid and lower dermis and the subcutaneous tissue	Variant of pseudoxanthoma

**TABLE 155-2 Clinical Features, Histopathology, Inheritance, Associated Biochemical Findings, Gene Defects, and Predisposing Clinical Conditions in Diseases with Elastic Fiber Abnormalities—Cont'd**

Disease	Inheritance <sup>a</sup>	Clinical Manifestations	Histopathology of Elastic Fibers	Gene Defects, Biochemical Findings, and Predisposing Clinical Conditions
Isolated elastomas	NH	Dermal papules or nodules	Accumulation of thick elastic fibers in dermis	
Elastofibroma	NH	Deep subcutaneous tumor, usually on subscapular area	Accumulation of globular elastic structures encased in collagenous meshwork	Trauma on the lesional area; chronic sun exposure
Actinic (solar) elastosis	NH	Thickening and furrowing of the skin	Accumulation of irregularly thickened elastic fibers in dermis	Chronic sun exposure
Marfan syndrome	AD	Skeletal, ocular, and cardiovascular abnormalities, hyperextensible skin; striae distensae	Fragmentation of the elastic structures in aorta	Mutations in the FBN1 gene
Congenital contractural arachnodactyly	AD	Joint contractures		Mutations in the FBN2 gene
Williams syndrome	AD	Supravalvular aortic stenosis; velvety skin; dysmorphic facies	Disruption of smooth muscle and matrix relationship affecting blood vessels	Allelic deletion of the elastin gene
Costello syndrome	Unknown	Multiple developmental defects; soft skin with excess wrinkling and deep creases on hands and feet	Impaired deposition of insoluble elastin in different tissues	Improper assembly of elastin; 67 kDa elastin-binding protein defect in fibroblasts
Variants of corneal dystrophy	AD, AR	Progressive opacification of the cornea leading to severe visual handicap	Progressive accumulation of corneal deposits (amyloid)	Mutations in the gene encoding the corneal protein

AD = autosomal dominant; AR = autosomal recessive; XR = X-linked recessive; NH = not a heritable disease.

<sup>a</sup>Most of these conditions represent a group of diseases with clinical, genetic, and biochemical heterogeneity.

<sup>b</sup>The genetic defects and biochemical abnormalities have been demonstrated in only a limited number of patients in each group, and it is not known whether they are the same in all patients. The biochemical findings listed are those related to elastic fibers.

<sup>c</sup>Most cases are AR or sporadic with unknown inheritance. Rare cases with a distinct acquired form of pseudoxanthoma elasticum have been described.



**TABLE 155-3 Components of Elastic Fibers**

Protein	Characteristic Features	Human Gene Locus
Elastin MICRO-FIBRILLAR PROTEINS	~68 kDa, several splice variants; insoluble, highly cross-linked; provides elasticity to tissues	7q11.2
<b>Fibrillins</b>	350 kDa	
FBN1	Contain EGF and TGF- $\beta$ -binding protein motifs	15q15–q21
FBN2	Contain EGF and TGF- $\beta$ -binding protein motifs	5q23–q31
<b>Latent TGF-<math>\beta</math> Binding Proteins (LTBP)</b>		
LTBP1	150–205 kDa	2p12–q22
LTBP2	Contain EGF and TGF- $\beta$ -binding protein motifs	14q24
LTBP3	Secreted as a complex with latent TGF- $\beta$ ; bone defects in null mice	11q12
<b>Fibulins</b>		
FBLN1	100–240 kDa	22q13.3
FBLN2	Contain EGF and anaphylatoxin motifs	3p24–p25
FBLN5		
<b>Microfibril-Associated Glycoproteins (MAGP)</b>		
MAGP1 (MFAP2)	31 kDa; widely distributed in microfibrils	1p36.1–p35
MAGP2 (MP25)	25 kDa	12p12.3–p13.1
<b>Microfibril-Associated Proteins</b>		
MFAP1	Very acidic	15q15–q21
MFAP3	Expressed in microfibrils in ocular zonules	5q32–q33.2
MFAP4	Frequently deleted in Smith–Magenis syndrome	17p11.2
<b>Other Components</b>		
MP78/70 (big-h3)	Mutated in different variants of corneal dystrophy	5q31
Lysyl oxidase	Probably not a structural component	5q23–q31
Lysyl oxidase like 1 (LOXL1)	Cross-linking and scaffold for spatial deposition of elastin	15q24
Emilin (gp115)	At least two isoforms	2p23.3–p23.2

EGF = epidermal growth factor; TGF- $\beta$  = transforming growth factor beta. Data from Uitto, J., Rosenbloom, J. Elastic Fibers. In Freedberg, I. M., Eisen, A. Z., Wolff, K., et al. (Eds); *Dermatology in General Medicine*, 5th edn; McGraw Hill: New York, p. 260.

the functional elasticity of the fibers and manifest as a disease.

### 155.2.2 The Elastin Gene (*ELN*)

The human elastin gene, localized to the long arm of chromosome 7, consists of 34 exons spanning ~45 kb (5) (see Figure 155-3A). These exons correspond to a full-length cDNA of ~3.5 kb, which encodes approximately 800 amino acids in a polypeptide of 70 kDa (6).

A remarkable feature of the human elastin gene is that its pre-mRNA undergoes extensive alternative splicing, a number of exons being post-transcriptionally deleted (6,9). Since these physiological deletions of individual exons are in frame, the alternative splicing results in synthesis of an ensemble of tropoelastin polypeptides with slight variations in their primary sequences. The physiological or developmental significance of the alternative splicing in elastin is unknown, but the observations suggest that small insertions or deletions in the gene, if in frame, are unlikely to result in pathology.

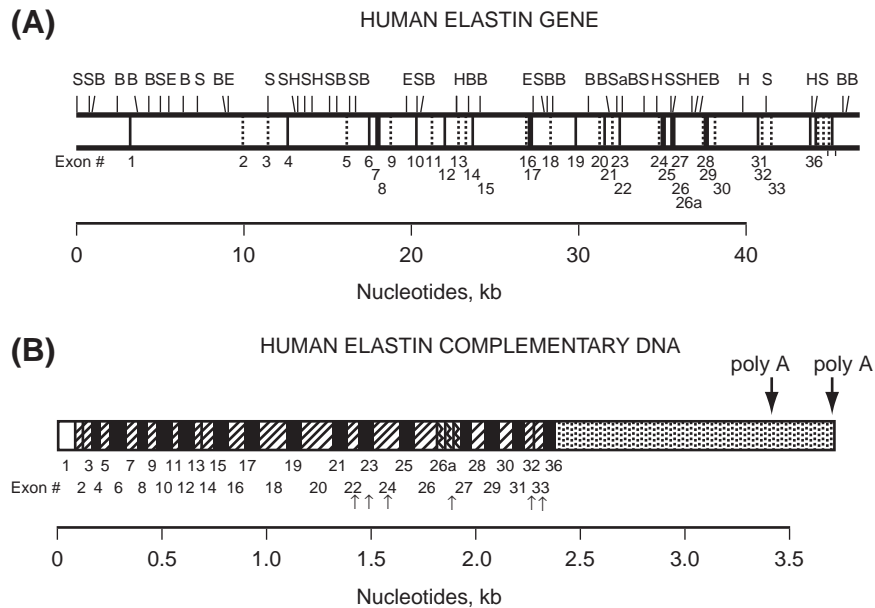
### 155.2.3 Degradation and Remodeling

Although the metabolic turnover of elastin is very slow as compared with proteins in general, a portion of the body's elastin is continuously degraded and may in part be replaced by newly synthesized fibers (10). In addition, degradation of elastin is markedly increased in a variety of pathologic conditions. Thus, the tissues containing elastin must contain proteolytic enzymes that are capable of degrading elastic fibers. Evidence for a specific elastolytic enzyme, elastase, was first obtained from study of the pancreas, and the properties of pancreatic elastase have since been extensively investigated. Subsequently, elastolytic enzymes were detected in several other tissues as well as in a variety of cell types, including polymorphonuclear leukocytes, monocyte/macrophages, and platelets (11).

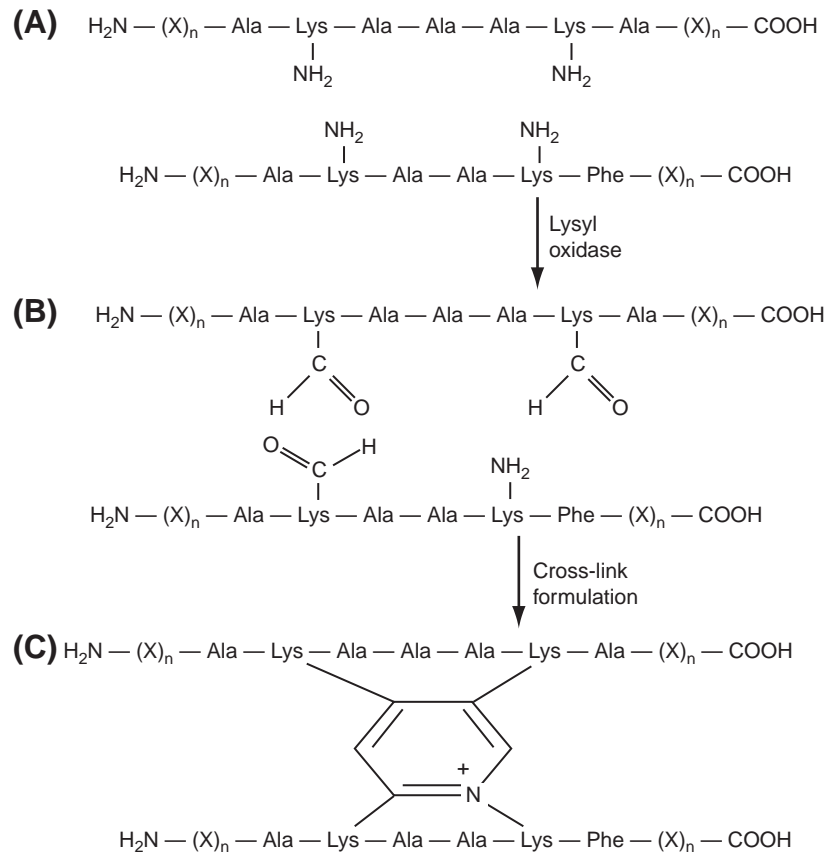
The classic elastases are serine proteases that degrade insoluble elastic fibers at neutral or slightly alkaline pH. The activity of these enzymes is inhibited by serum factors such as  $\alpha$ 1-antitrypsin and  $\alpha$ 2-macroglobulin. Other elastases have been shown to be metalloenzymes requiring calcium for their activity (12). One such enzyme is secreted by macrophages isolated from peritoneal inflammatory exudates, and this metalloenzyme is different from leukocyte or pancreatic elastases in that it cleaves elastin at peptide bonds at the amino side of leucyl or isoleucyl residues. A similar  $\text{Ca}^{2+}$ -dependent metalloenzyme has been demonstrated in human skin fibroblast cultures (11).

### 155.2.4 Pathology of the Elastic Fibers

The biosynthetic pathways involved in the fibrillogenesis and degradation of the elastic fibers must be carefully controlled to allow maintenance of an optimal level of functional elastic fibers in tissues. If the balance in the rate of biosynthesis or degradation, or both, of the elastic fibers is perturbed, the steady-state level of such fibers could be altered, manifesting as a disease. Furthermore, structural alterations due to genetic mutations in the genes encoding any of the components of the elastic fibers or in the enzymatically mediated post-translational events, such as the cross-linking of elastin, could lead to pathology. In fact, several heritable and acquired



**FIGURE 155-3** Schematic representation of the human elastin gene (A) and the human elastin complementary DNA (B). (A) The gene (*ELN*) consists of 34 exons numbered starting from the 5' end. (Note that the last exon is numbered 36 to be consistent with the bovine elastin gene structure.) The exons are drawn to scale and span ~45 kb of genomic DNA on the long arm of chromosome 7. (B) The human elastin cDNA, ~3.5 kb, has a 2.4-kb coding region corresponding to the 34 exons in the gene. The exons subjected to alternative splicing are indicated by arrows.



**FIGURE 155-4** Formation of covalent intermolecular cross-links, desmosines, between two tropoelastin polypeptides. (A) Amino acid sequences at a potential cross-link region within the elastin polypeptides. Note the presence of paired lysine residues separated by two or three alanine residues and depicting free  $\epsilon$ -amino groups ( $\text{NH}_2$ ). (B) Three of these  $\epsilon$ -amino groups are converted to corresponding aldehydes, allysine residues, by the action of lysyl oxidase, a copper-dependent enzyme. (C) Three of the aldehyde residues and a lysine residue with an unmodified  $\epsilon$ -amino group fuse to form a stable ring-like structure, desmosine, which covalently links the two polypeptides into an insoluble elastic structure. (Modified from Uitto, J.; Rosenbloom, J. *Elastic Fibers. In Dermatology in General Medicine, 5th edn; Freedberg, I. M., Eisen, A. Z., Wolff, K., et al., Eds.; McGraw Hill: New York, 1999; p. 260.*)

connective tissue diseases have been shown to affect the elastic fibers (see Table 155-3). In some of these conditions, mutations can be found in the genes encoding the elastic fiber components, i.e. elastin and the associated microfibrils, and such diseases could be considered as primary heritable elastic fiber disorders. In other heritable conditions, the primary defect may reside in genes other than those encoding the components of the elastic fibers. In such conditions, even though the elastic fibers are clearly abnormal, the pathology is secondary to an unrelated primary event. Finally, in a number of acquired connective tissue disorders, elastic fibers are altered in such a manner that the changes contribute to the clinical presentation, thus producing a phenocopy of their heritable counterparts (13,14).

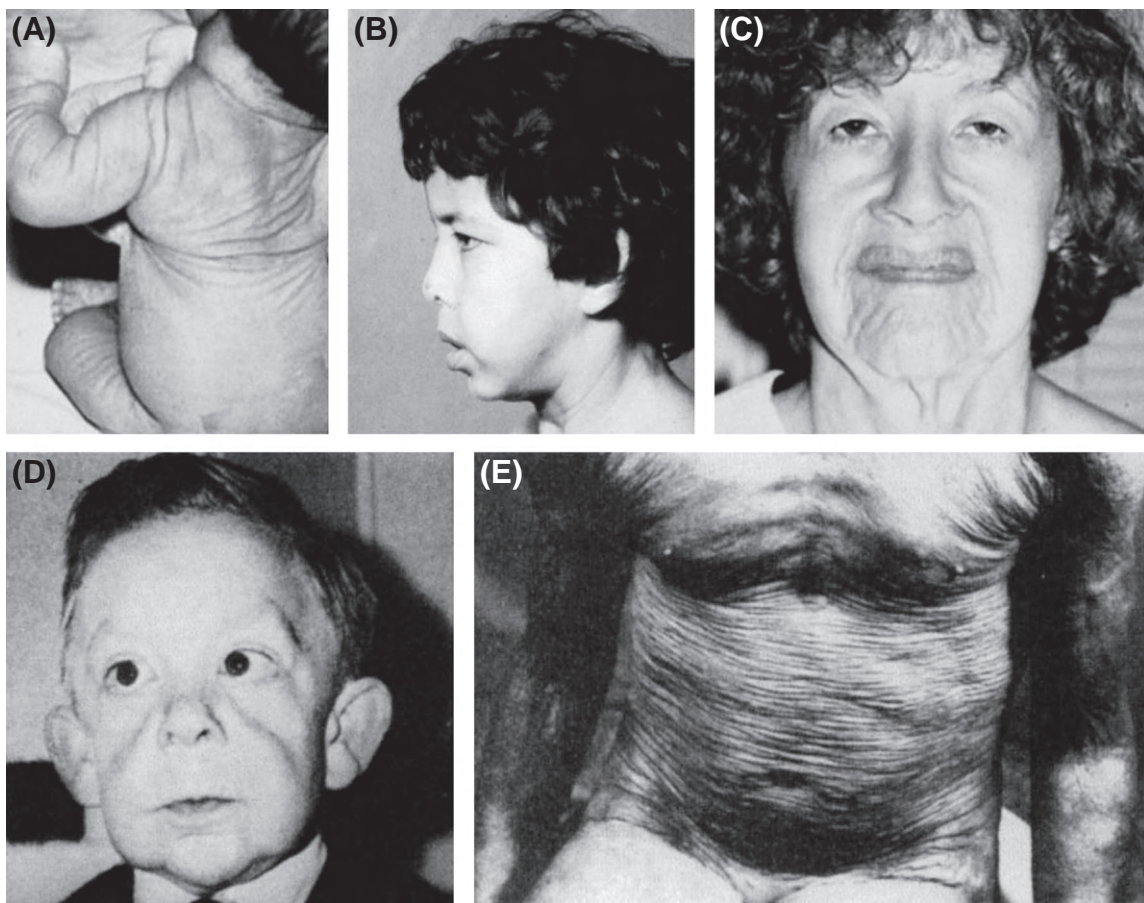
In this overview, the heritable disorders affecting the elastic fibers have been divided into three broad categories, based primarily on clinical findings and histopathological observations of elastic fibers. These three groups include: (1) diseases manifesting primarily with lax skin and associated features due to loss of functional elastic fibers, such as the cutis laxa syndromes; (2) those where elastin accumulation in tissues is a predominant

histopathologic feature, resulting in generalized or localized lesions, as exemplified by cutaneous elastomas and pseudoxanthoma elasticum; and (3) those in which abnormalities in elastin fibrillogenesis can explain the clinical phenotypes as due to lack of functional elastic fibers, as in copper deficiency syndromes.

### 155.3 ELASTIN DEFICIENCY DISORDERS

#### 155.3.1 Cutis Laxa and Other Lax Skin Syndromes

**155.3.1.1 Diagnostic Features and Inheritance.** Cutis laxa (Online Mendelian Inheritance in Man (OMIM) 123700, 219100) is clinically defined as loose and sagging skin that demonstrates reduced elasticity and resilience (Figures 155-5 and 155-6). In some patients the clinical findings are limited to the skin, the phenotype being primarily of cosmetic concern. In other cases, however, the cutaneous findings are associated with a number of extracutaneous manifestations, including pulmonary emphysema (see Figure 155-6D), vascular malformations, umbilical and inguinal hernias, and gastrointestinal and



**FIGURE 155-5** Clinical features of cutis laxa. Note loose and folded skin in an infant (A), sagging jawline in a 4-year-old boy (B), premature aging appearance in a 30-year-old female (C), characteristic facial features in a patient with autosomal recessive cutis laxa (D), and generalized mid-truncal wrinkling as a consequence of an inflammatory urticarial reaction (E). (D is from Pope, F. M. *Pseudoxanthoma Elasticum, Cutis Laxa, and Other Disorders of Elastic Tissue*. In *Principles and Practice of Medical Genetics*, 3rd edn; Rimoin, D. L., Connor, J. M., Pyeritz, R. E., Eds.; Churchill Livingstone, New York, 1997; p. 1083. E is from Verhagen, A. R., Woerdeman, M. J. *Post-inflammatory elastolysis and cutis laxa*. *Br. J. Dermatol.* 1975, 92, 183–190; with permission.)



vesico-urinary tract diverticuli, which cause considerable morbidity and mortality. In fact, in the most severe cases, cutis laxa is lethal during the early postnatal period, primarily from pulmonary complications.

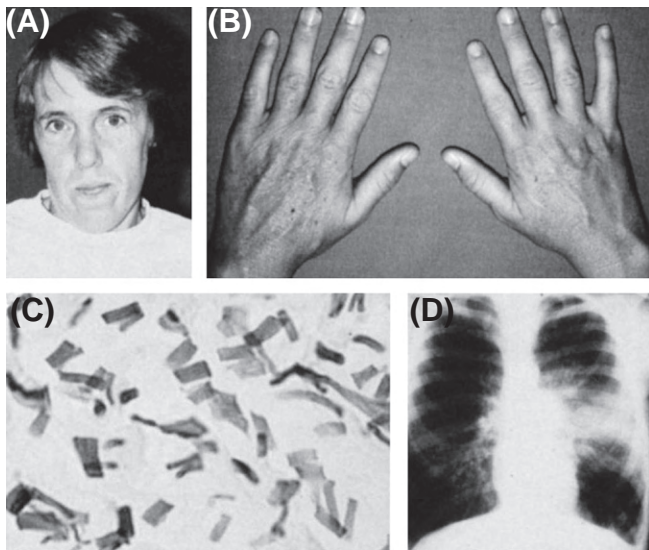
Clinical findings consistent with cutis laxa can also be part of the normal aging process affecting the skin or associated with other heritable connective tissue disorders, particularly some variants of Ehlers–Danlos syndrome (15) (see Chapter 154). In the latter patients, the primary pathology resides in the collagen meshwork, and they should not be diagnosed as having cutis laxa. This diagnosis should be reserved specifically for those conditions in which abnormalities either in the structure or quantity of elastic fibers can be demonstrated by histopathological and ultrastructural means.

The inheritance of cutis laxa is variable, and both autosomal dominant (OMIM 123700) and autosomal recessive (OMIM 219100) patterns occur (16–19). In some cases, typical phenotypic findings are present at birth or will be noted shortly thereafter, while in many cases the onset is delayed even by several decades (20). It should also be noted that many cases with late-onset cutis laxa are, in fact, a distinct acquired variant that results from inflammatory tissue reaction (14,21). Such inflammatory reactions can be due to allergic drug eruption or they initially manifest with urticarial lesions in the skin. Phenotypically, these late-onset acquired forms of the disease can be indistinguishable from milder heritable forms of cutis laxa, in which the findings are limited to the skin.

Histopathology of skin in patients with cutis laxa, using special “elastin-specific” stains (Verhoeff–van

Gieson and orcein), reveals loss, fragmentation, or both, of elastic fibers. In particular, in the newborns with severe cutis laxa, the elastic fibers can be essentially absent. In many cases with late-onset cutis laxa, the elastic fibers are present but fragmented, with absence of contiguous functional elastic fibers (see Figure 155-6C). In these cases, electron microscopy similarly demonstrates irregular fragmentation of the fiber structure. In addition to elastic fiber abnormalities, ultrastructural alterations in collagen fibers have also been described in families with cutis laxa (22). The collagen changes, however, are likely to reflect the overall perturbation of the extracellular matrix of connective tissue, as a result of altered mechanical properties in the skin.

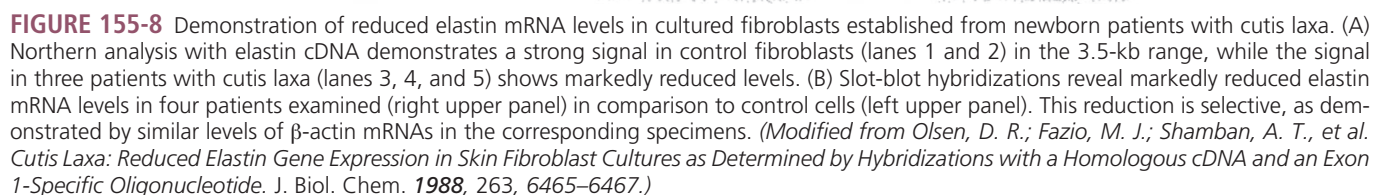
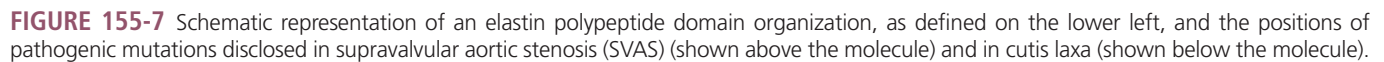
**155.3.1.2 Molecular Mechanisms.** The biochemical and genetic events leading to loss, fragmentation, or both, of the elastic fibers in most cases with cutis laxa are unknown. Consistent with the notion that the elastin gene expression is primarily perturbed in some cases of cutis laxa, it has been demonstrated that elastin mRNA steady-state levels can be reduced in patients with severe congenital forms of the disease (23,24). Specifically, quantitation of elastin mRNA in these patients, either by northern analysis or by slot-blot hybridizations in fibroblast cultures, has revealed that elastin gene expression may be essentially undetectable or clearly less than 10% of the level noted in healthy unrelated controls (Figure 155-7). Although the reasons for reduced steady-state levels of mRNA could be multiple (e.g. reduced rate of transcription or decreased stability of the transcript), the end result would be the same, i.e. less elastin protein being synthesized in the skin and possibly other tissues affected in these patients. Subsequently, there have been reports to suggest the presence of specific mutations in the elastin gene, presumably giving rise to autosomal dominant forms of the disease. One of the first of these patients, a 37-year-old female, was noted as a newborn to have heavy folds under her chin and across her chest, abdomen, groin, and thighs (25). She had multiple facial cosmetic surgeries from 4 years of age on. She also had multiple peripheral pulmonary stenoses; bilateral herniorrhaphies were performed at the age of 7 years, and right ventricle hypertrophy was noted at 19 years of age. Her father had similarly loose skin on his chest, forearms, and abdomen, in addition to facial drooping. Histopathology revealed markedly reduced quantities of dermal elastic fibers, and these fibers appeared fragmented. Sequencing of the elastin gene (*ELN*) revealed deletion of a single adenine in codon 748 within exon 32 (Figure 155-8). This mutation was also present in the proband's father, but not in her mother. Scanning of the second elastin allele did not reveal the presence of any pathogenetic mutations. The mutation causes a frame-shift, which results in replacement of 37 carboxy-terminal amino acids in the elastin polypeptide by a novel 62 amino acid missense sequence. Analysis of mRNA and



**FIGURE 155-6** Clinical, histopathological, and radiological findings in a patient with autosomal recessive cutis laxa. (A) Loose and sagging facial skin in this 15-year-old male. (B) His hands show fine wrinkling giving the premature aging appearance, acrogeria. (C) Histopathology of skin with orcein stain reveals fragmentation of elastic fibers. (D) Chest radiograph reveals findings consistent with severe pulmonary emphysema. Note the infiltrate in the left lung.



Another study similarly identified two frameshift mutations (c.2012delG and c.2039delC) in exon 30 of



the elastin gene (26) (see Figure 155-8). Transcripts from these mutated alleles were unstable, but complicating the interpretation of the data was the suggestion that exon 30 undergoes alternative splicing in fibroblasts. Nevertheless, these mutations were interpreted to be “dominant-acting” and responsible for qualitative and quantitative defects in elastin, resulting in the cutis laxa phenotype. Collectively, these two reports suggest that primary mutations in the elastin gene can be responsible for certain forms of cutis laxa, the mechanism being potentially dominant-negative, and similar observations have been subsequently made in additional cases with an autosomal dominant mode of inheritance (19,27,28).

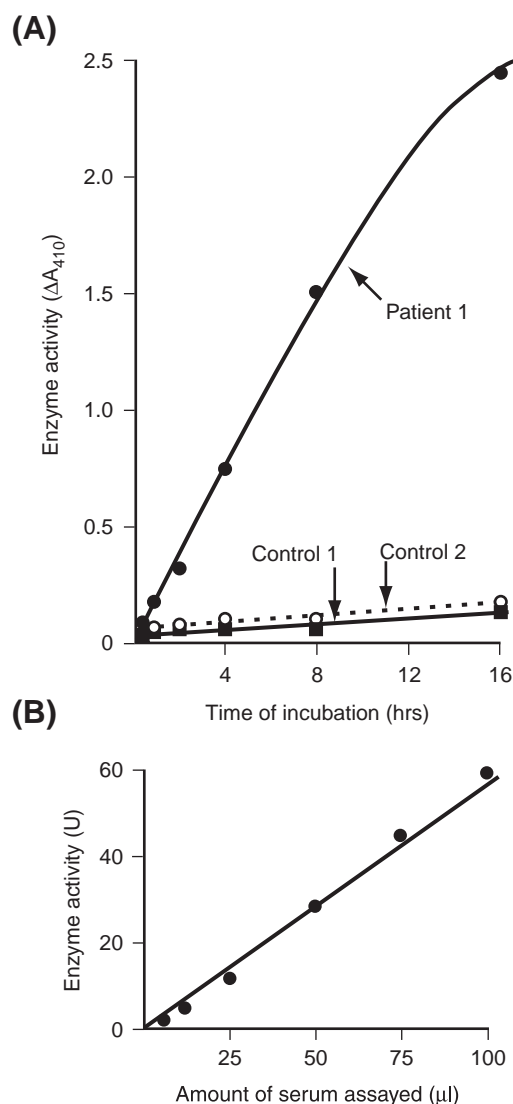
An alternate mechanism for the development of the cutis laxa phenotype is enhanced degradation of the elastic fibers, as illustrated by an intriguing case with cutis laxa and severe pulmonary emphysema (OMIM 235360). This patient also had severe congenital hemolytic anemia of unknown type (29,30). At the age of 15 years, pulmonary emphysema was documented by radiography and pulmonary function tests (see Figure 155-6). Most notably, the vital capacity was 63%, the total lung capacity 183%, and the residual volume 630% of the predicted age-matched values, consistent with severe pulmonary emphysema (29). At the same time, the patient was noted to appear much older than his chronologic age, with sagging skin on the face and neck, as well as premature wrinkling appearance on the backs of his hands (acrogeria) (see Figure 155-6A and B). Histopathologic examination of the skin revealed paucity and fragmentation of the elastic fibers (Figure 155-6C). Laboratory values, specifically  $\alpha$ 1-antitrypsin, as well as serum copper and ceruloplasmin levels, were within the normal range. The patient died at the age of 20 years from the complications of pulmonary emphysema.

The patient had two affected siblings, an older sister and a younger brother, who had died of complications of hemolytic anemia at the ages of 3 and 7 years, respectively. The autopsies revealed emphysematous lung changes in both siblings. A 9-year-old sister and both parents were phenotypically normal, and there was no other family history of similar clinical problems. Thus, the inheritance of cutis laxa, pulmonary emphysema, and hemolytic anemia in this family was consistent with an autosomal recessive pattern.

A possible mechanism leading to cutis laxa and pulmonary emphysema in this individual was provided by observations that the activity of an elastase-like enzyme, measured by degradation of a synthetic substrate, succinyl-(L-alanyl)<sub>3</sub>-p-nitroanilide (SAPNA) (31), was markedly elevated as compared to healthy unrelated controls (Figure 155-9). Specifically, the enzyme activity in this patient's serum was more than 80-fold higher than in the controls. Further characterization of this enzyme indicated that the activity was highest at a neutral or slightly alkaline pH, around 7.6, and that the activity could be inhibited by Na<sub>2</sub>EDTA, suggesting that the enzyme

requires a divalent cation for its activity. The activity was not inhibited by serum protease inhibitors (30). It is conceivable, therefore, that enhanced elastase-like activity was responsible for degradation of elastic fibers both in the skin and the lungs of this patient, resulting in a combined phenotype of cutis laxa and pulmonary emphysema.

In addition to elastin, a limited number of mutations in cutis laxa have been disclosed in the *FBLN5* gene encoding fibulin-5, a microfibrillar component of the elastic fibers. Among them, a recurrent homozygous missense mutation (p.S227P) was reported in two



**FIGURE 155-9** Demonstration of markedly elevated elastase-like enzyme activity, determined by degradation of synthetic small molecular peptide (SAPNA; see text) in serum of the patient with cutis laxa presented in Figure 155-6, as compared to unrelated healthy controls (A). The second graph demonstrates that the enzyme activity assay is linear with respect to the amount of serum assayed (B). (Modified from Anderson, L. L.; Oikarinen, A.; Ryhanen, L., et al. Characterization and Partial Purification of a Neutral Protease from the Serum of a Patient with Autosomal Recessive Pulmonary Emphysema and Cutis Laxa. *J. Lab. Clin. Med.* 1985, 105, 537–546.)

independent families of Turkish and Iranian ancestry, both with severe autosomal recessive cutis laxa (32,33). In another patient with relatively mild cutis laxa with redundant, loose skin and mitral valve regurgitation, a heterozygous *FBLN5* mutation was discovered. The mutation consisted of an internal 483-bp duplication in the fibulin-5 mRNA, resulting in the synthesis and secretion of a mutant fibulin-5 protein with four additional tandem calcium-binding epidermal-growth-factor-like motifs. The mutation arose from a 22-kb tandem gene duplication, encompassing the sequence from intron 4 to exon 9 (34). In addition to fibulin-5, the fibulin-4 gene has been suggested to harbor mutations in patients with autosomal recessive cutis laxa. The first described case had a missense mutation (p.E57K) resulting in cutis laxa associated with multiple bone fractures at birth, vascular abnormalities, developmental emphysema, inguinal and diaphragmatic hernias, joint laxity, and pectus excavatum (35). Detailed examination of additional patients with fibulin-4 deficiency has revealed the presence of aortic aneurysms, arterial tortuosity, and vascular stenosis, potentially reflecting the involvement of altered transforming growth factor beta (TGF- $\beta$ ) signaling (36).

Adding to the molecular heterogeneity of cutis laxa are demonstrations of mutations in the *LTBP4* gene associated with an early lethal phenotype of autosomal recessive cutis laxa and severe gastrointestinal and vesico-urinary malformations in four patients (37). This gene encodes latent TGF- $\beta$  binding protein 4, and its absence in the extracellular matrix causes increased TGF- $\beta$  activity, leading to defective elastic fiber assembly in affected tissues. In some patients with autosomal recessive cutis laxa, but without catastrophic pulmonary emphysema, mutations in the *ATP6V0A2* gene have been reported (38). The corresponding protein, the A2 subunit of a vacuolar H<sup>+</sup>-ATPase, located in cell and organelle membranes, is responsible for glycosylation of proteins important for their proper function. It has been suggested that mutations in this proton pump prevent N- and O-glycosylation of extracellular matrix proteins and impaired secretion of tropoelastin, resulting in cutis laxa phenotype (39,40). The clinical phenotype ranges from generalized wrinkled skin to presentation of characteristic cutis laxa, with pre- and postnatal growth delay and skeletal abnormalities.

Mutations in the *PYCR1* gene have also been detected in families with autosomal recessive cutis laxa and progeroid features, as well as in one individual who was believed to have de Bary syndrome (41–43). The corresponding protein, pyrroline-5-carboxylate reductase 1, a mitochondrial enzyme, is critical for proline biosynthesis. Its deficiency causes abnormal mitochondrial morphology in fibroblasts of the affected individuals. Proline is involved particularly in the biosynthesis of extracellular matrix proteins, as well as in the oxidative cell cycle, and it is plausible that these activities contribute to the progeroid changes in the affected individuals.

Finally, the cutis laxa phenotype has been encountered in patients with MACS syndrome (macrocephaly, alopecia, cutis laxa, and scoliosis). This syndrome is caused by mutations in the *RIN2* gene, encoding a ubiquitously expressed Ras- and Rab-interactor protein involved in the regulation of endocytic trafficking (44,45). *RIN2* deficiency was found to be associated with paucity of dermal microfibrils and deficiency of fibulin-5, which may underlie the cutaneous phenotype in these patients. Collectively, these findings attest to considerable molecular heterogeneity of cutis laxa.

### 155.3.2 Acquired Cutis Laxa

The proteolytic mechanisms in the patient with increased elastase-like enzyme activity may be analogous to some cases with acquired forms of cutis laxa, demonstrating extensive sagging of the skin with little or no evidence of internal organ involvement. However, the skin involvement can be generalized or localized (14). Frequently, these individuals experience an inflammatory reaction in their skin, sometimes associated with allergic drug eruption, which within ensuing weeks or months can result in rapidly progressing premature aging and sagging of the skin, resulting in a phenocopy of the heritable forms of cutis laxa. The acquired cutis laxa phenotype can be associated with a number of conditions, including multiple myeloma, Sweet's syndrome, and urticarial vasculitis. In the cases with inflammatory cutaneous reaction, a possible explanation is that the inflammatory cells, such as polymorphonuclear leukocytes or monocyte-macrophages, release powerful elastases that become activated and result in proteolytic degradation of the elastic fibers. As a consequence, the elastic fibers become progressively degraded and reduced in number, with phenotypic consequences of sagging skin.

The inflammatory destruction of elastic fibers in acquired cutis laxa has been suggested, in some cases, to be associated with missense alleles in the elastin and fibulin-5 genes (46). Specifically, a patient who developed acquired cutis laxa following *Toxocara canis* parasite infection was heterozygous for a fibulin-5 mutation (p.G202R) and compound heterozygous for elastin alleles p.A55V and p.G773D. Western analysis revealed abnormal proteolytic processing of tropoelastin in the patient's fibroblasts, suggesting that the observed sequence variants render the elastic fibers susceptible to inflammatory destruction mediated by proteases.

### 155.3.3 Anetoderma

A pathomechanistically similar, yet phenotypically distinct, situation may involve anetoderma, usually an acquired condition with clinical, histopathological, and electron microscopic features of cutis laxa within the isolated cutaneous lesions in the skin in a limited distribution. Familial occurrence of anetoderma has also been



reported in a few cases, and both dominant and recessive inheritance patterns have been suggested (47–49). Individuals with anetoderma develop numerous protruding sac-like cutaneous lesions (Figure 155-10). Histopathology and electron microscopy of the lesional skin demonstrate that the elastic fibers are irregularly fragmented (see Figure 155-10). The concentration of elastin, determined by radioimmunoassay of desmosine, an elastin-specific cross-link compound, is markedly reduced in the lesional skin, as compared with unaffected skin from the same patients, as well as with normal skin from unrelated control subjects (50). Histopathology and immunohistochemical studies showed that the lesional areas of skin often contain inflammatory cell infiltrates in association with immune complexes and complement (51). It

is conceivable, therefore, that these inflammatory cells release proteases responsible for degradation of elastic fibers in the lesional areas of skin. Traditionally, anetoderma has been divided into inflammatory (Jadassohn) and noninflammatory (Schweninger-Buzzi) types. This distinction is not very clear, however, and it appears that anetoderma represents a continuum of the degree of inflammation, with the unifying pathomechanism of proteolytic degradation of the elastic fibers.

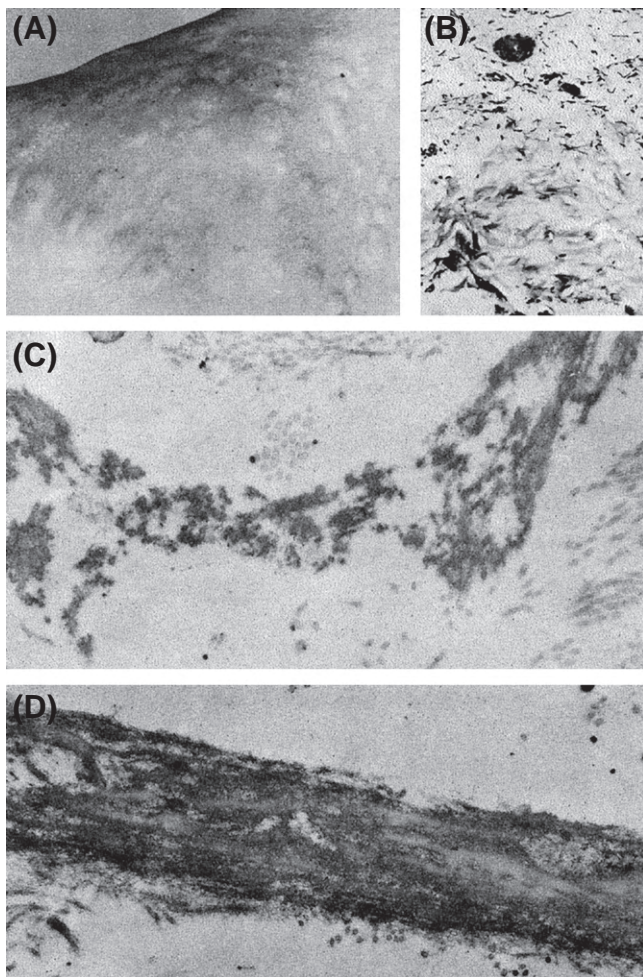
### 155.3.4 Wrinkly Skin Syndrome

A number of conditions with cutaneous features of cutis laxa have been reported as distinct clinical entities. One of them is wrinkly skin syndrome (OMIM 278250), a rare autosomal recessive disorder primarily reported in Middle Eastern ethnic groups. Cutaneous features in these patients are reminiscent of those in cutis laxa. Specifically, skin over the abdomen and dorsum of the hands and feet appears wrinkled, the elasticity of the skin in the affected areas is reduced, and there is an increase in palmar and plantar creases (52,53). In fact, it has been suggested that wrinkly skin syndrome and cutis laxa represent the same disorder (54). However, unlike in cutis laxa, mental retardation, microcephaly, hypotonia, and musculoskeletal connective tissue abnormalities seem to be regular features of this syndrome. Nevertheless, similar to cutis laxa, the elastic fibers in the mid-reticular dermis are fragmented and distorted in structure (52).

The molecular basis of wrinkly skin syndrome is currently unknown, although it has been observed that manifestations of this syndrome are associated in some patients with deletions in the q32 region of chromosome 2 (55). It should be noted that two collagen genes, *COL3A1* and *COL5A2*, reside in this region of chromosome 2, perhaps making them candidate genes for this condition.

### 155.3.5 Mid-Dermal Elastolysis

This condition was initially reported as an acquired non-inflammatory disease in young adults with a pathognomonic histopathologic finding: band-like loss of elastic fibers in the mid-dermis (56). Clinically, these individuals manifest with fine wrinkling, usually affecting large areas of skin on the upper arms and the upper back, primarily in young women (57,58). There is usually no family history, and the cardinal clinical finding (i.e. very fine wrinkling of the skin) is often accentuated on the sun-exposed areas. In analogy to anetoderma, a suggestion has been made to divide this condition into inflammatory and noninflammatory variants (59). However, since preceding inflammation may have already subsided, and only the sequelae of the inflammatory reaction are evident at later stages, this distinction may not have any etiologic or pathomechanistic significance. Finally, these individuals, as a rule, do not demonstrate internal organ



**FIGURE 155-10** Clinical, histopathologic, and electron microscopic observations in a patient with anetoderma. (A) The patient demonstrates numerous protruding sac-like lesions on the upper back. (B) Histopathology reveals loss and fragmentation of elastic fibers in the papillary dermis (Verhoeff–van Gieson stain). Transmission electron microscopy shows markedly fragmented elastic fibers in the papillary dermis (C) and the deep reticular dermis (D) of the lesional skin. (Modified from Oikarinen, A. I.; Palatsi, R.; Adomian, G. E., et al. Anetoderma: Biochemical and Ultrastructural Demonstration of an Elastin Defect in the Skin of Three Patients. *J. Am. Acad. Dermatol.* 1984, 11, 64–72.)



involvement. Thus, mid-dermal elastolysis appears to be an acquired skin condition limited to the mid-dermis, displaying features of an autoimmune process (60). This condition should be recognized in differential diagnosis of heritable forms of cutis laxa.

### 155.3.6 De Barsy Syndrome

Another condition characterized by defective development of elastic fibers in the skin with accompanying cutis-laxa-like features is de Barsy syndrome (OMIM 219150), an autosomal recessive disorder characterized by progeria-like appearance with distinct facial features (61,62). In addition to skin findings, this complex disorder is characterized by severe developmental problems affecting a number of organs, such as the eyes (corneal opacities). The syndrome includes dwarfism and extensive neurological abnormalities, including hypotonia, mental retardation, and congenital bilateral athetosis. Elastic fibers in the skin have been shown to be frayed and reduced in number and density, and examination of the elastin mRNA levels by northern hybridizations has suggested reduced gene expression (63). Thus, these patients have associated features of cutis laxa, but the underlying molecular defects in this multisystem disorder are currently unknown. However, as noted above, a patient with cutis laxa due to mutations in the *PYCR1* gene has been suggested to have de Barsy syndrome (43).

### 155.3.7 Michelin Tire Baby Syndrome

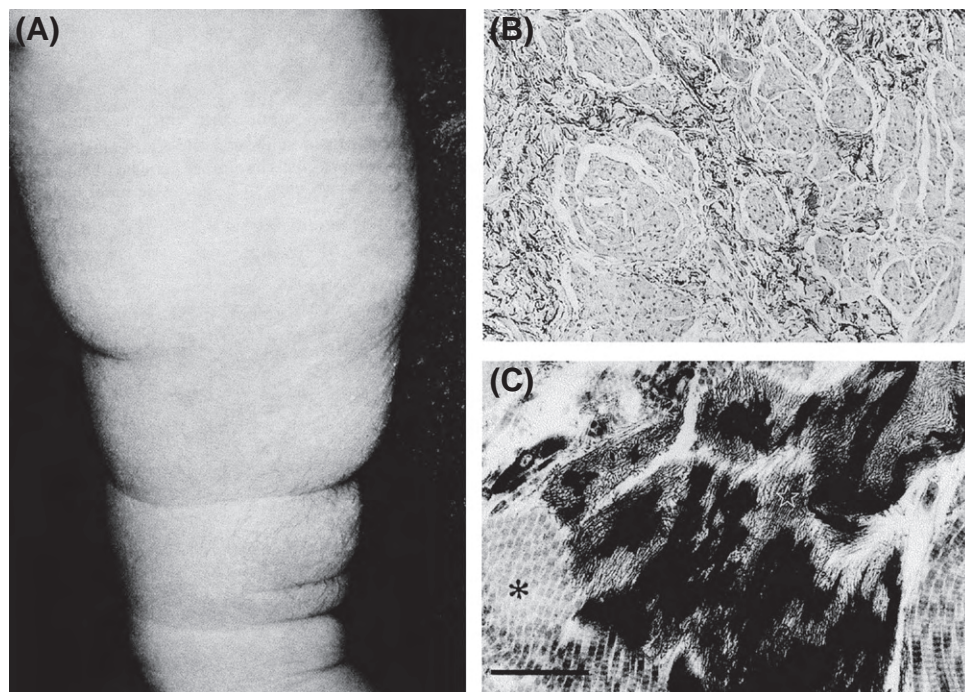
This is another rare condition with fragmentation and paucity of elastic fibers (OMIM 156610). Specifically, these patients demonstrate large skin folds, and histopathology has revealed fragmented elastic fibers, with the amount of elastin being decreased (64) (Figure 155-11). However, the primary pathology in these cases appears to be unrelated to elastin, and the peculiar appearance in most cases is due to an underlying nevus lipomatosus and/or generalized smooth muscle hamartomas (65–67). A child with features of Michelin tire baby with smooth muscle hamartomas, and in addition, multiple anomalies, had a familial paracentric inversion of chromosome 7q (68). It has been suggested that this disease is inherited as an autosomal dominant trait (69,70).

## 155.4 ELASTIN ACCUMULATION DISEASES

### 155.4.1 Pseudoxanthoma Elasticum

#### 155.4.1.1 Diagnostic Features and Inheritance.

Pseudoxanthoma elasticum (PXE; OMIM 264500) has been traditionally considered as a systemic connective tissue disorder affecting the extracellular matrix in various organs of the body (71,72). PXE was initially delineated as a clinical entity distinct from xanthomas (hence pseudoxanthoma) over a century ago (73). During



**FIGURE 155-11** Clinical and morphologic presentations of Michelin tire baby syndrome. (A) A 15-month-old patient demonstrates prominent folds on the leg. (B) Histopathology reveals fragmented elastic fibers surrounding smooth muscle bundles (Weigert's stain). (C) Transmission electron microscopy reveals fragmentation of elastic fibers and paucity of elastin (star), while collagen fibers appear normal (asterisk). (Modified from Sato, M.; Ishikawa, O.; Miyachi, Y., et al. *Michelin Tire Syndrome: a Congenital Disorder of Elastic Fiber Formation?* Br. J. Dermatol. 1997, 136, 583–586; reproduced with permission.)

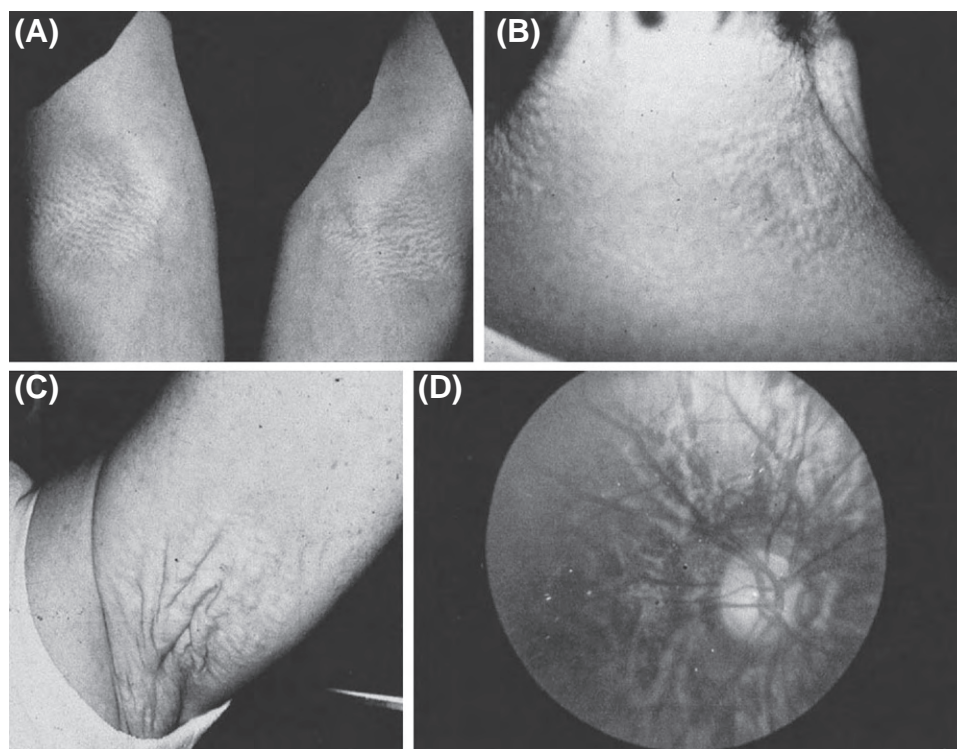
the ensuing decades, various clinical observations, histopathology, and ultrastructural findings suggested that the primary pathology resides in elastic fibers. Consequently, PXE was considered as a prototype of heritable connective tissue disorders with primary involvement of the elastic fiber system. Recent studies have clearly shown that the classic PXE is caused by mutations in the *ABCC6* gene, a putative transmembrane efflux transporter expressed primarily in the liver and the kidneys (74). Based on these and related observations, PXE is now considered to be a metabolic disorder (see below).

The clinical manifestations of PXE center on three major organ systems of the body, the skin, the eyes, and the cardiovascular system. The presenting manifestations are usually evident in the skin as small yellowish papules, which tend to coalesce into larger plaques, and eventually involve the entire skin (Figure 155-12). The affected skin becomes progressively lax, redundant, and inelastic, causing an appearance of premature aging and eventually having considerable cosmetic impact. Histopathologic evaluation of skin reveals accumulation of pleomorphic elastotic material, as revealed by “elastin-specific” stains (such as Verhoeff–van Gieson and orcein) (Figure 155-13) (75). More remarkably, these pleomorphic elastotic structures become calcified in a progressive manner over the lifetime of the affected individual (see Figure 155-13). Thus, skin biopsy demonstrating

mineralization of aberrant elastic fibers in the mid-dermis is essential in confirming the diagnosis of PXE. This diagnosis can be further supported by demonstration of mutations in the *ABCC6* gene.

The eyes are involved, with characteristic appearance of angioid streaks, which result from fractures in Bruch’s membrane, an elastin-rich sheath within the retina (see Figure 155-12D). As a result of fragmentation of this membrane, there is breakage of retinal blood vessels and neovascularization sets in from the adjacent choroid plexus, associated with bleeding in the retina. Consequently, the affected individuals experience progressive loss of visual acuity, which can eventually lead to legal blindness, although entire loss of vision is rare (72).

The vascular involvement may manifest as elevated blood pressure, intermittent claudication, weakened peripheral pulses, and infrequently, internal bleeding primarily from gastric arterial blood vessels (76). In some families, the cardiovascular manifestations predominate and can lead, although rarely, to early myocardial infarcts of the affected individuals in their 1930s or 1940s. There is considerable phenotypic variability, both intrafamilial and interfamilial, and the involvement of any given organ system may predominate in certain families. This variability has presented a diagnostic challenge to accurately identify individuals with PXE on the basis of clinical manifestations (77). Furthermore,



**FIGURE 155-12** Characteristic clinical findings in pseudoxanthoma elasticum. Note small papular lesions on the antecubital fossa (A) and on the site of the neck (B). These primary lesions tend to coalesce into large plaques rendering skin redundant and inelastic (C). Ophthalmologic examination reveals the presence of angioid streaks (D). (Modified from Uitto, J.; Pulkkinen, L.; Ringpfeil, F. *Molecular Genetics of Pseudoxanthoma Elasticum—A Metabolic Disorder at the Environment/Genome Interface?* Trends Mol. Med. 2000, 7, 13–17.)



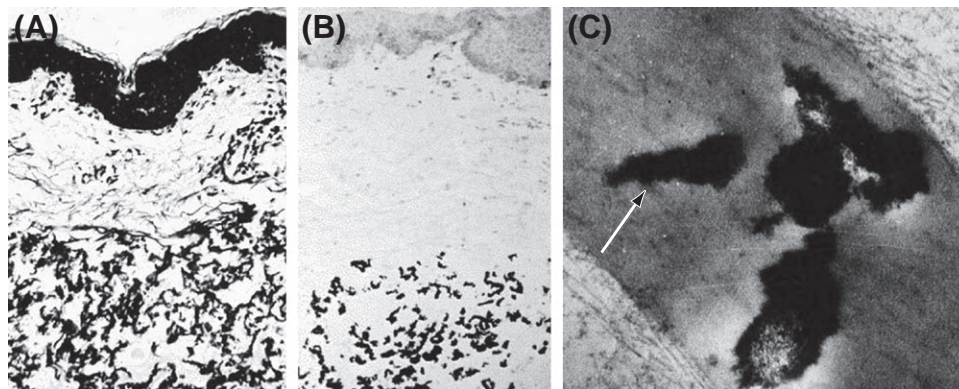
the clinical manifestations are rarely present at birth and often become evident not until the second or third decade of life, thus complicating and prolonging the process leading to correct diagnosis.

Adding to the complexity and the diagnostic challenge of PXE were early suggestions that both autosomal dominant and autosomal recessive forms of the disease exist. Recent advances in molecular genetics of PXE have, however, clearly established that PXE is exclusively an autosomal recessive disorder (78,79).

**155.4.1.2 Molecular Pathology.** Since the primary pathology of PXE resides in the elastic fibers, the genes involved in the synthesis and assembly of the elastic fiber network in the body were initially considered as the prime candidate genes (80). These include elastin on chromosome 7q, elastin-associated microfibrils, such as fibrillin 1 and fibrillin 2 on chromosomes 15 and 5, and lysyl oxidase, a copper-dependent enzyme necessary for cross-linking of the elastic fibers, on chromosome 15. In addition, members of the microfibrillar protein family on chromosomes 1, 2, 5, and 15 were considered as potential candidate genes (see later discussion). However, early genetic linkage analyses excluded these chromosomal regions (80). Subsequent studies, utilizing positional cloning approaches, provided strong evidence for linkage of the inherited forms of PXE to the short arm of chromosome 16 (81,82), and the critical candidate region was eventually narrowed by the use of informative recombinants to consist of ~500 kb (83,84). Examination of the existing genome database revealed that this region contained four genes, none of which had an obvious connection to the extracellular matrix of connective tissue in general or to the elastic fibers in particular. Systematic sequencing of these candidate genes resulted in identification of mutations in *ABCC6* (OMIM 603234), which encodes ABCC6 (also known as MRP6) protein, belonging to a family of genes encoding multidrug-resistance-associated proteins (85). A large number of pathogenic mutations have since been reported in

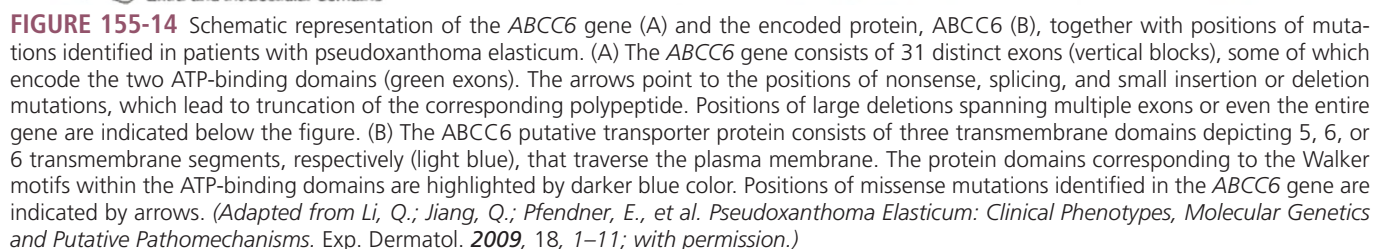
this gene (Figure 155-14) (74,86–88). These mutations can now be utilized for confirmation of the diagnosis, for prenatal diagnosis, and for presymptomatic testing in individuals at risk for inheritance of the disease. The latter consideration is particularly important considering the late onset of the manifestations (89). Genetic linkage analyses in various multiplex families have failed to suggest locus heterogeneity in classic forms of the disease, and thus far *ABCC6* seems to be the only gene underlying the PXE phenotype.

The *ABCC6* gene consists of 31 exons dispersed within ~73 kb of DNA on chromosome 16p13.1, and the corresponding mRNA, 4.5 kb, encodes a polypeptide of 1503 amino acids (90,91). The ABCC6 protein is predicted to consist of three transmembrane-spanning domains, composed of five, six, and six transmembrane segments, respectively (see Figure 155-14). The majority of the mutations disclosed thus far reside in the carboxy-terminal half of the protein, affecting primarily the intracellular domains, the nucleotide-binding folds (NBF1 and NBF2) necessary for the function of this protein as a putative transmembrane transporter (see Figure 155-14). Many of the genetic lesions are nonsense mutations or small insertions or deletions resulting in premature termination of translation, or mutations affecting the consensus splice sites and predicted to result in out-of-frame deletion of the mRNAs (92). The premature termination codons, besides predicting synthesis of a truncated polypeptide, may also result in downregulation of the corresponding transcript through nonsense-mediated mRNA decay. In addition, a number of missense mutations, particularly those replacing critical arginine residues within the cytoplasmic domains of ABCC6, have been disclosed. Finally, large recurrent deletions involving the *ABCC6* locus have been found in some families, reflecting genomic instability surrounding the *ABCC6* locus on chromosome 16p.13.1 (93–95). Collectively, these mutations predict markedly reduced or absent activity of ABCC6.



**FIGURE 155-13** Pathology of cutaneous elastic fibers in pseudoxanthoma elasticum. (A) Histopathology with orcein stain of a skin biopsy reveals accumulation of elastotic material in mid-dermis. (B) Staining of an adjacent section demonstrates that these elastotic structures are calcified (von Kossa stain). (C) Transmission electron microscopy reveals depositions of calcium within the elastic fibers (arrow). (Modified from Uitto, J.; Pulkkinen, L.; Ringpfeil, F. *Molecular Genetics of Pseudoxanthoma Elasticum—A Metabolic Disorder at the Environment/Genome Interface?* Trends Mol. Med. 2000, 7, 13–17.)

Particularly intriguing observations with potential pathomechanistic implications for PXE have been made in patients with PXE-like cutaneous findings in





association with vitamin-K-dependent multiple coagulation factor deficiency (96,99,100). These patients show primarily cutaneous lesions similar to those seen in PXE, i.e. small yellowish papules and excessive folding and sagging of the skin and loss of recoil. These patients have been described as having combined clinical features of both PXE and cutis laxa (99). However, cutaneous lesions in these patients depict characteristic mineralization of the elastic structures in the dermis similar to PXE, a finding that is not present in patients with cutis laxa.

The patients with PXE-like cutaneous findings and vitamin-K-dependant coagulation factor deficiency have mutations in the *GGCX* gene encoding  $\gamma$ -glutamyl carboxylase, an enzyme responsible for  $\gamma$ -glutamyl carboxylation of Gla-proteins, including vitamin-K-dependent coagulation factors and matrix Gla-protein (MGP) (101). Most of these patients show inactivating missense mutations in both alleles of *GGCX*, and the absent enzyme activity does not allow activation of the vitamin-K-dependent coagulation factors through  $\gamma$ -carboxylation, thus explaining the bleeding tendency in these patients. The explanation for ectopic connective tissue mineralization in these patients may involve the role of MGP. This protein, in its fully carboxylated form, is a powerful anti-mineralization factor that has the capacity to prevent ectopic mineralization under normal homeostatic levels of calcium and phosphate, as shown by the development of the corresponding knockout mouse, *MGP*<sup>-/-</sup>, characterized by extensive mineralization of connective tissues (102). However, the under-carboxylated form of MGP is inactive, potentially allowing the mineralization process to ensue. Examination of skin lesions in patients with PXE with specific antibodies that distinguish the fully carboxylated and under-carboxylated forms of MGP has suggested that this protein is under-carboxylated in the lesional skin (103,104). A potential explanation for under-carboxylation of MGP in classic PXE involves the possibility that the *ABCC6* protein participates in transmembrane transport and distribution of vitamin K, specifically its reduced form (KH<sub>2</sub>), an obligatory cofactor of  $\gamma$ -glutamyl carboxylase (74,105). In this context, it should be noted, however, that feeding *Abcc6*<sup>-/-</sup> mice with vitamin K does not counteract the mineralization process (106).

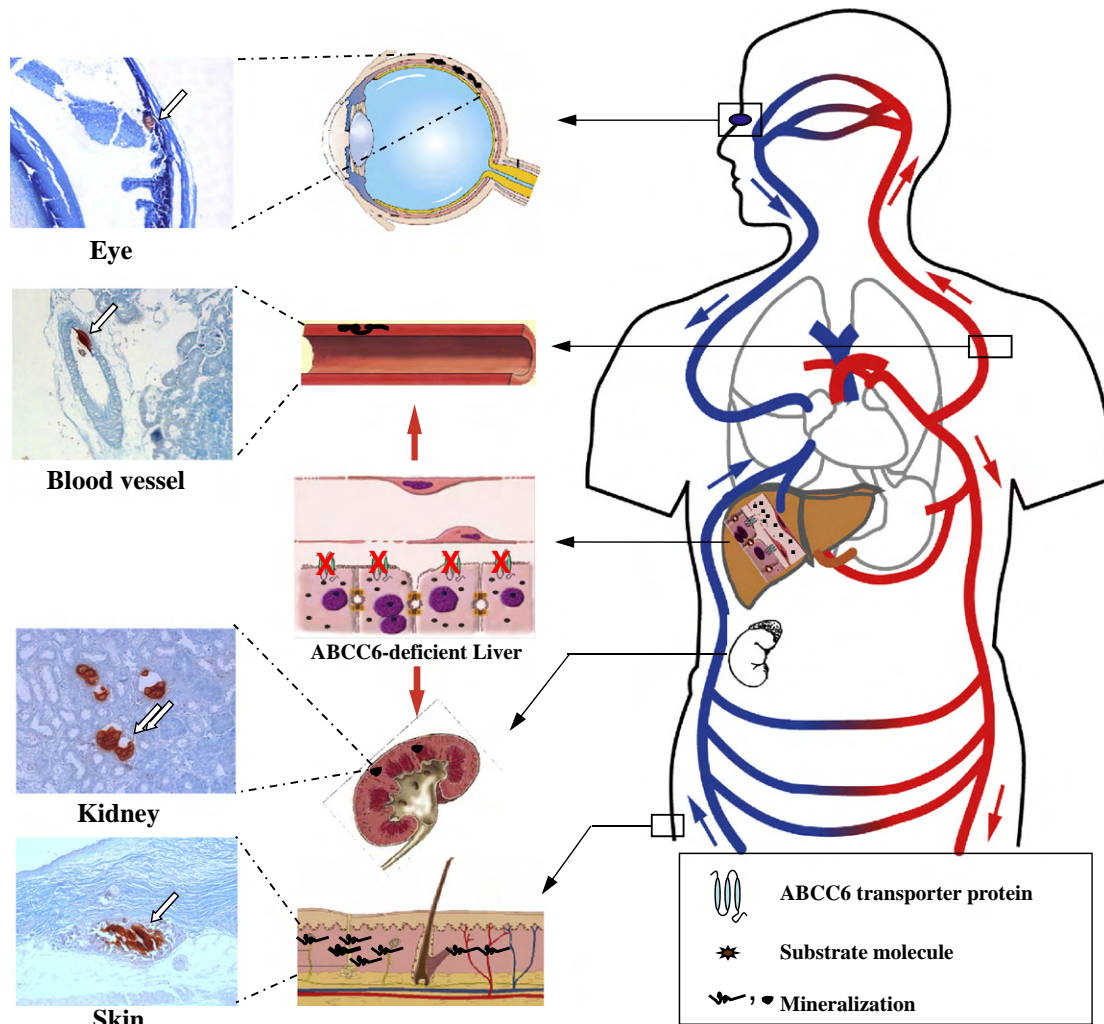
Entering to the differential diagnosis of PXE are patients with vascular calcification due to mutations in the *NTSE* gene. These patients present with progressive calcification of arterial blood vessels particularly in lower extremities, associated with calcium phosphate deposits in joint capsules in the hands and feet (107). The histopathology of the calcified arteries is somewhat similar to those in patients with PXE; *NTSE* encodes CD73, a 5' exonucleotidase critical for synthesis of adenosine. It was, therefore, postulated that adenosine has a role in inhibiting aberrant vascular calcification under physiologic conditions. Thus, considering the similarity between PXE and the CD73 deficiency, it was suggested

that adenosine may be one of the molecules physiologically transported by *ABCC6* (108).

A remarkable observation is that *ABCC6* is expressed primarily, if not exclusively, in the liver and the kidneys (90), organs not known to be clinically affected in PXE. This observation raises the question of the relationship between the *ABCC6* mutations and the manifestations in PXE affecting the elastic fibers in various organs. Thus, PXE could be considered as a metabolic disorder rather than a primary connective tissue disease (109,110) (Figure 155-15). In support of the “metabolic hypothesis” are observations that serum from patients with PXE is deficient in anti-mineralization capacity, an observation that has been confirmed with *Abcc6*<sup>-/-</sup> mice. Since *ABCC6* protein in the liver is considered to be an efflux transporter, it is conceivable that in the absence of transport activity, factors contributing to the anti-mineralization capacity of proteins, such as MGP, would be deficient in circulation, allowing the ectopic mineralization to ensue. This mechanism would also explain the delayed onset of the clinical manifestations and the progressive nature of the calcification process. At the same time, it should be noted that while most elastic structures demonstrate histopathological and ultrastructural evidence of mineralization, not all organs rich in elastin are clinically affected in PXE. Specifically, the lungs, tissues rich in elastin, are not affected in PXE (72).

It has been suggested that low level of expression of *ABCC6* can be detected by RT-PCR in cells derived from mesenchymal tissues, such as dermal fibroblasts or vascular smooth muscle cells (86,111). If confirmed, an alternate possibility would be that the absent expression of *ABCC6* modifies the biosynthetic capacity of “PXE cells” in a manner that would result in synthesis of aberrant elastic structures with affinity for calcium. Nevertheless, the critical role of mutations in *ABCC6* resulting in the PXE phenotype has been confirmed by the recent development of a mouse models in which *ABCC6* has been deleted by targeted inactivation (112,113). These mice develop extensive tissue calcification, including skin, retina, and arterial blood vessels, thus recapitulating the histopathologic and ultrastructural features of PXE.

**155.4.1.3 Molecular Diagnostics.** The observations on the mutations in the *ABCC6* gene have raised several intriguing questions regarding the genetics of PXE. One of the issues relates to the mode of inheritance. Traditionally, PXE has been reported to be inherited either in an autosomal recessive or autosomal dominant pattern, and a number of sporadic cases with uncertain inheritance have also been noted. However, there is no molecular evidence of autosomal dominant inheritance in any of the families examined thus far. In some cases, a pseudodominant pedigree structure can explain the inheritance of the disease in two generations, with a mistaken clinical impression of autosomal dominant inheritance (114). Another explanation for the purported autosomal dominant inheritance may reside in the fact that many of the

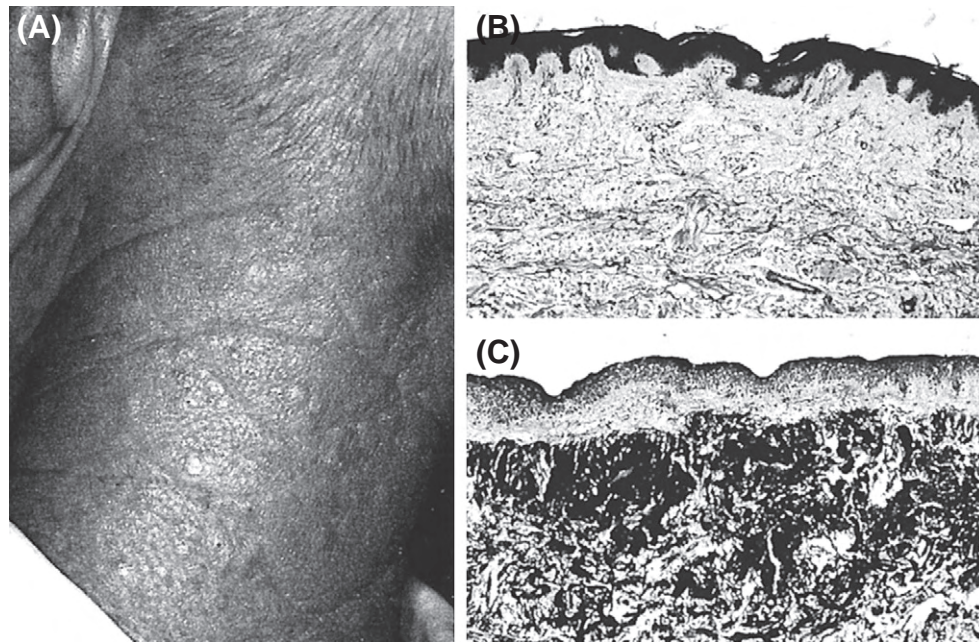


**FIGURE 155-15** Illustration of the proposed “metabolic hypothesis” of pseudoxanthoma elasticum (PXE). Under physiologic conditions, the ABCC6 protein is expressed in high levels in the liver and serves as an efflux pump on the baso-lateral surface of hepatocytes, transporting critical metabolites from the intracellular milieu to the circulation (right side of the panel). In the absence of ABCC6 transporter activity in PXE, reduced concentrations of such substrate molecules, which may serve as physiologic anti-mineralization factors, are found in the circulation, resulting in mineralization of connective tissues in a number of organs, such as the eye, the arterial blood vessels, the kidneys, and the skin (middle panel). The presence of mineralization is illustrated by histopathologic examination (Alizarin red stain) of tissues in *Abcc6*<sup>−/−</sup> mice that recapitulate the features of human PXE. (Adapted from Uitto, J.; Li, Q.; Jiang, Q. *Pseudoxanthoma Elasticum: Molecular Genetics and Putative Pathomechanisms*. *J. Invest. Dermatol.* **2010**, 130 (3), 661–670; with permission.)

clinical findings in PXE are rather common in the general population, particularly at an older age. Specifically, cutaneous findings mimicking PXE are frequently encountered in older individuals in sun-exposed areas of skin, with characteristic accumulation of elastic material in the mid-dermis (13,115) (Figure 155-16). Furthermore, other diagnostic manifestations of PXE, such as the cardiovascular involvement, are prevalent in the elderly (76). Thus, such individuals may have been thought to be affected with PXE, particularly in families with history of PXE, conveying the impression of autosomal dominant inheritance. At the same time, it has been suggested that heterozygous carriers of PXE mutations may manifest with features of PXE (116–118). However, molecular screening of many such families has revealed *ABCC6* mutations on both alleles of these presumed heterozygous carriers (79).

Although molecular analysis has not yet been supportive, it is conceivable that partial deficiency of ABCC6 function could lead to haploinsufficiency, which, particularly under certain environmental or dietary conditions, would manifest as a milder form of the disease. In this context, it should be noted that the function of the ABC transporter proteins and/or their substrate specificity and flux could be altered by a number of dietary, hormonal, or environmental factors. For example, high intake of dairy products rich in calcium and phosphate during childhood and adolescence has been statistically associated with the severity of PXE (72,119). The role of diet has been experimentally addressed using the *Abcc6*<sup>−/−</sup> mice as a model system. These studies have shown that the mineral content of their diet can clearly modulate the ectopic mineralization process (120–122). However, calcium did not modulate





**FIGURE 155-16** Clinical features and histopathology of skin in sun-damaged area on the neck. Note that the clinical appearance has some resemblance to pseudoxanthoma elasticum (PXE). (A) Histopathology of the skin on the neck reveals massive accumulation of elastotic material (C), as compared to skin in the sun-protected area (buttocks) from the same individual (B). Note that these elastic structures are not calcified, as in PXE (see Figure 155-13).

the degree of mineralization; instead, magnesium, when added to the mouse diet in amounts that increased the magnesium concentration by five-fold over the standard diet, was able to prevent the ectopic mineralization noted in these mice (121). Conversely, an experimental diet with low magnesium was shown to accelerate the mineralization process in *Abcc6*<sup>-/-</sup> mice, as compared with the corresponding mice kept on a standard diet (123). These findings support the notion that changes in the diet can alter the age of onset and extent of mineralization in PXE, and they suggest that dietary magnesium might be helpful for treatment of patients with PXE (124). Similarly, hormonal influence on manifestations of PXE can be inferred from the predominance of female patients with PXE, as well as from reported deterioration of the disease as a result of multiple pregnancies (72). Finally, certain lifestyle variables, such as cigarette smoking, can clearly modify manifestations of PXE, as exemplified by worsening of intermittent claudication.

#### 155.4.2 Elastoderma

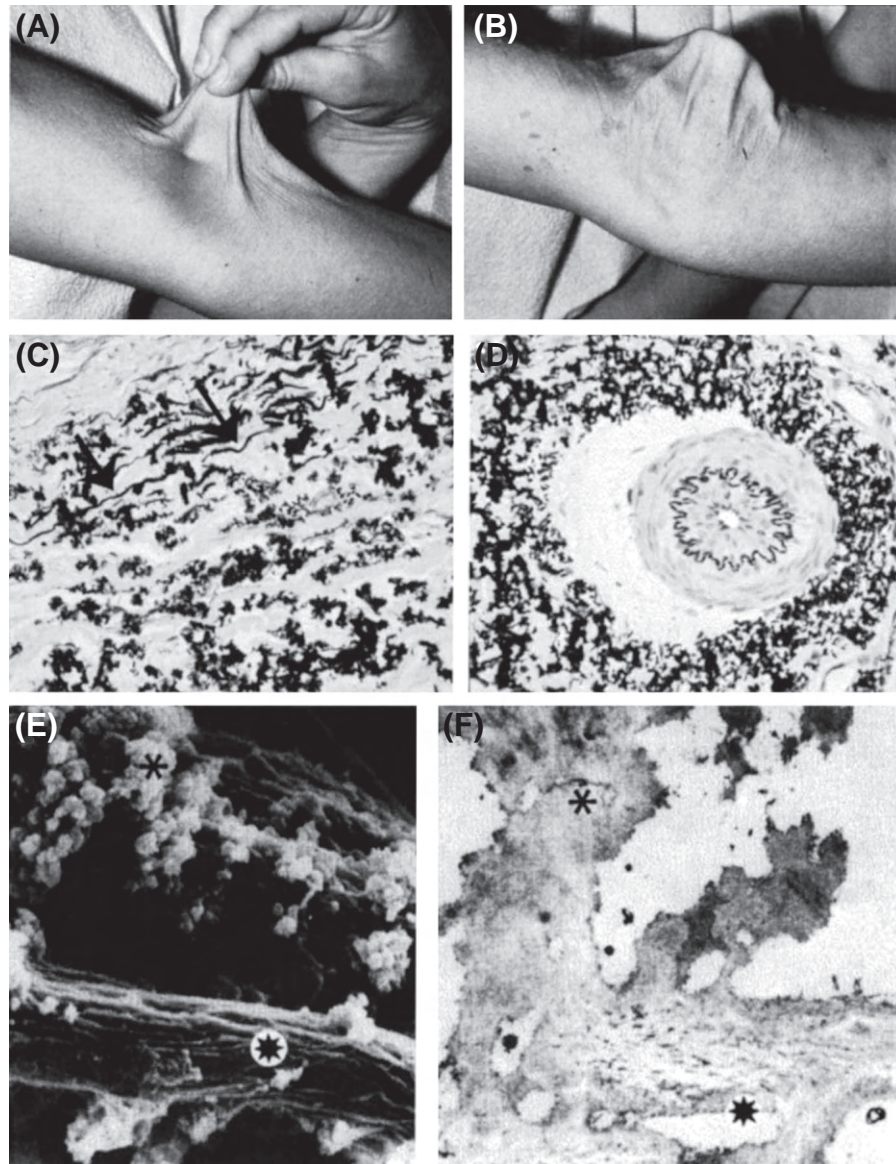
This is an extremely rare condition with striking clinical similarity to PXE. Specifically, Kornberg et al. (125) described a 33-year-old female who had an approximately 4-year history of progressive laxity of the skin, primarily on the elbows and knees. Physical examination revealed loose and sagging skin, which upon stretching displayed incomplete and delayed recoil (Figure 155-17A and B). The patient was otherwise in good health, and there was no evidence of internal involvement or eye problems. Examination of a skin biopsy specimen

revealed accumulation of pleomorphic elastotic material in the mid-dermis, as well as surrounding deeper blood vessels on the lower parts of the dermis (Figure 155-17C and D). Both scanning and transmission electron microscopy revealed abnormal elastic structures in association with, if not in direct extension of, normal-appearing elastic fibers (Figure 155-17E and F). The excessive, abnormal-appearing material was more electron dense than the normal elastic fibers.

As a quantitative biochemical measure of the cross-linked elastic fibers, desmosine concentration was also measured in the affected and unaffected skin by radioimmunoassay. The mean concentration of desmosine in the lesional skin was  $260.2 \pm 4.9$  ng/mg wet weight of skin; the corresponding value in the unaffected skin of the same patient was  $70.2 \pm 1.3$  ng/mg, while the desmosine concentration in the skin of nine healthy age-matched controls was  $79.3 \pm 22.7$  ng/mg (125).

The accumulation of abnormal elastic material, as demonstrated by histopathological staining and by desmosine assay, together with the clinical features, is reminiscent of PXE. Remarkably, however, the elastic fibers were not calcified, thus excluding definitive diagnosis of PXE. Furthermore, there was no evidence of generalized elastin problems affecting the internal organs or ophthalmologic findings characteristic of PXE.

Similar clinical, histopathological, and ultrastructural findings were subsequently reported in a 27-year-old male (126). Thus, elastoderma appears to be a rare, yet distinct, entity with accumulation of pleomorphic elastin in the skin. The molecular and genetic bases of this condition remain unknown.



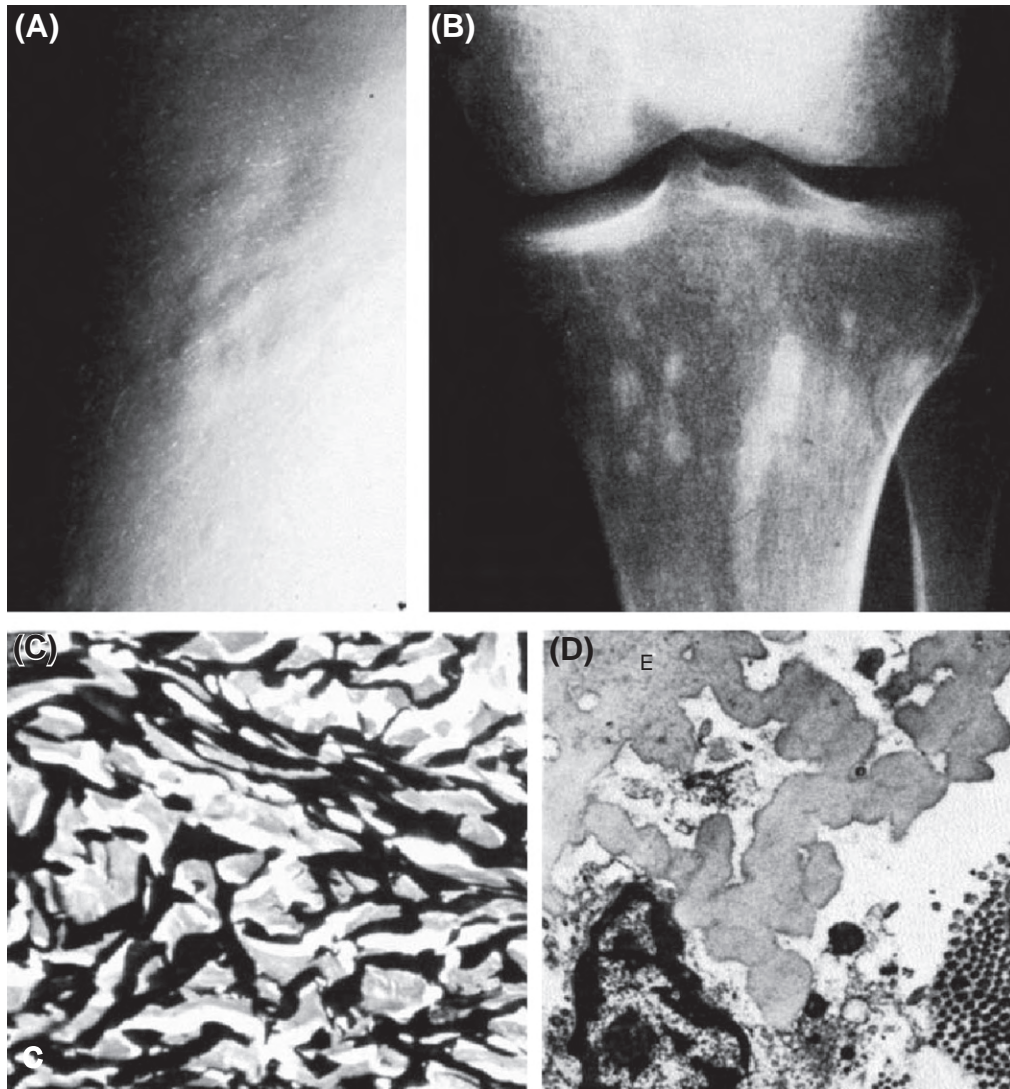
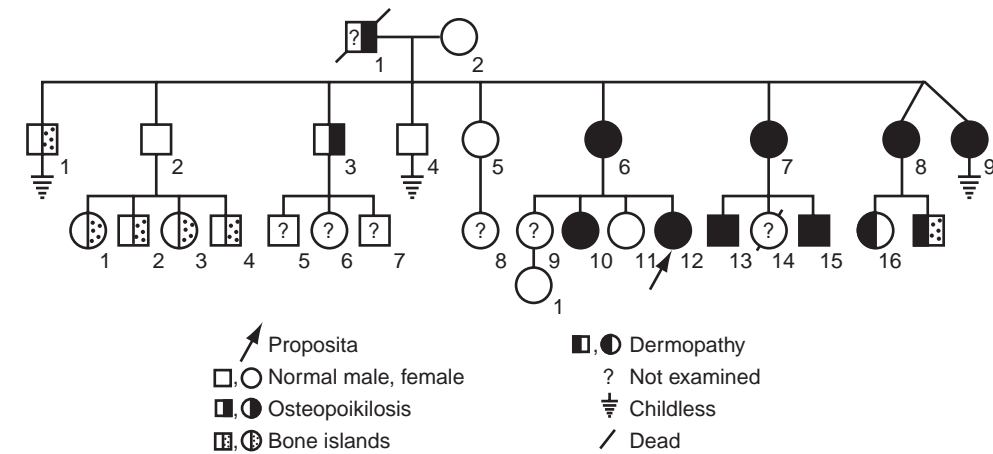
**FIGURE 155-17** Clinical, histopathologic, and ultrastructural features in elastoderma. Note that the patient's skin on the elbow is hyperextendable (A); however, stretching of the skin results in incomplete and delayed recoil (B). Histopathology with elastic stains (Verhoeff–van Gieson) reveals accumulation of pleomorphic elastic structures in the mid-dermis (C) and surrounding a small blood vessel in the deeper dermis (D). The presence of normal-appearing elastic fibers is indicated by arrows in (C). Scanning electron microscopy (E) and transmission electron microscopy (F) demonstrate morphologically altered, electron-dense structures (asterisks) in association with normal-appearing elastic fibers (stars). (A–C, E, F modified from Kornberg, R. L.; Hendler, S. S.; Oikarinen, A., et al. *Elastoderma—Disease of Elastin Accumulation Within the Skin*. N. Engl. J. Med. 1985, 312, 771–774.)

### 155.4.3 Buschke–Ollendorff Syndrome

Buschke–Ollendorff syndrome (OMIM 166700) is characterized by cutaneous findings, known as dermatofibrosis lenticularis disseminata, together with radiologically detectable bone lesions, osteopoikilosis (Figure 155-18A and B) (127,128). This condition is inherited in an autosomal dominant pattern (see top of Figure 155-18), although a few sporadic cases with the syndrome have also been reported. The cutaneous lesions appear as small papules or discs, most commonly found on the lower trunk and extremities. The onset of the skin lesions is usually before puberty, but rarely they may be present at

birth. Pathologic examination of the skin lesions reveals accumulation of elastic structures that are, however, unusually broad and interlacing (see Figure 155-18C). No fragmentation of the fibers is noted. Electron microscopy reveals that these elastic structures are peculiarly branching, and the diameter of individual fibers is highly variable. The accumulation of elastic fibers in the skin has been shown to be associated with elevated production of elastin by cultured dermal fibroblasts (129). Collagen is, for the most part, normal, and the cutaneous lesions appear to be primarily due to accumulation of elastotic material. The accumulation of elastin has also





**FIGURE 155-18** Clinical, histopathologic, and ultrastructural findings in Buschke–Ollendorff syndrome. A large pedigree with characteristic cutaneous lesions and bone findings, with inheritance consistent with autosomal dominant pattern, is shown on the top. The cutaneous findings manifest as groups of small yellowish papules typically on the lower back (A), while the bone changes manifest as radiologically detectable densities at the ends of long bones (B). Histopathology of the skin from the lesional area reveals accumulation of interlacing broadened elastic fibers (C), and transmission electron microscopy reveals, next to a fibroblastic cell (F), irregularly shaped elastic fibers (E), which are deficient in the microfibrillar component (D). (Modified from Uitto, J.; Santa Cruz, D. J.; Starcher, B. C., et al. *Biochemical and Ultrastructural Demonstration of Elastin Accumulation in the Skin Lesions of the Buschke–Ollendorff Syndrome*. *J. Invest. Dermatol.* 1981, 76, 284–287.)

been confirmed by assaying the desmosine content, which indicated that the elastin concentration in the lesions is increased three- to seven-fold (130). Thus, the cutaneous lesions are connective tissue nevi of the elastin type (131).

An integral part of Buschke–Ollendorff syndrome is the presence of osteopoikilosis, which can usually be demonstrated at the ends of long bones (see Figure 155-18B) as well as in the hands of the affected individuals. The relationship between the skin findings and the osteopoikilotic bone lesions is not clear, and the biochemical nature of the osseous lesions is unknown. Histopathology of the osteopoikilotic bone lesions has been reported to consist of foci of compact bone (132). Ordinarily, bone does not contain elastin, and accumulation of elastin at the end of long bones is unlikely. It is perhaps more likely then that the skin findings and the bone lesions result from biochemically unrelated, but genetically closely linked aberrations in these tissues. In support of this interpretation are the observations in a large family with autosomal dominant inheritance of Buschke–Ollendorff syndrome (see Figure 155-18, top). In this family, most affected individuals had complete manifestations of the syndrome (i.e. the presence of skin findings and osteopoikilosis). However, careful examination of a male with osteopoikilotic bone changes failed to reveal the presence of any cutaneous lesions (individual no. 3, second generation). At the same time, another individual (no. 16) clearly demonstrated the presence of skin findings but a complete skeletal radiographic survey failed to find evidence of osteopoikilosis. It should be noted that in a number of individuals (nos. 1–4, third generation), no cutaneous findings were present, and the bone findings consisted of “bone islands,” potential precursors of osteopoikilosis. Collectively, the cutaneous lesions in Buschke–Ollendorff syndrome are asymptomatic, and primarily of cosmetic concern. However, correct recognition of the osteopoikilotic bone lesions as benign and distinction of the cutaneous lesions from those in PXE is critical.

The gene underlying Buschke–Ollendorff syndrome has been identified as *LEMD3*, which encodes an inner nuclear membrane protein (133), and the presence of heterozygous loss-of-function mutations in this gene have been confirmed in several families with Buschke–Ollendorff syndrome or osteopoikilosis alone, while studies on some families have failed to detect exonic mutations in this gene (134–136). The *LEMD3* gene antagonizes bone morphogenic protein and activin–TGF- $\beta$ -receptor-activated Smad signaling pathways, but how these interactions result in accumulation of elastic fibers in the Buschke–Ollendorff patients’ skin remains unclear.

#### 155.4.4 Elastomas

A number of cases with connective tissue nevi with elastin accumulation in the skin, in the absence of

osteopoikilosis, have also been described (130). Although some of these appear to be familial, a number of them are isolated cases with no family history. Some lesions are present at birth or develop at the early stages of life (juvenile elastoma) while others develop later in life. Most of these conditions are asymptomatic and only of cosmetic concern; however, considering the differential diagnosis of other infiltrative processes, a skin biopsy should be performed to confirm the diagnosis of connective tissue nevi of the elastin type.

In some patients, connective tissue nevi of both elastin and collagen type can be found, and some of the lesions seem to be of mixed type, with generalized proliferation of these two fibrillar components of the connective tissue. In some cases, the lesions consist, in addition to extracellular matrix components, of lipids and have been described on histological basis as elastofibrolipomas (137).

A well-defined variant of soft-tissue tumors with distinct accumulation of abnormal elastin is elastofibroma dorsi, a benign tumor characteristically located in the subscapular region (138). Histologically, these lesions consist of collagen and elastin admixed with lipids, and contain an infiltrate of fibroblasts and adipocytes (139). The elastic fibers have a peculiar appearance of globules, either singular or in the form of beaded strings, arranged in a linear fashion. The incidence of elastofibroma dorsi is not known, but this condition, as judged from autopsy findings (140), is relatively common in individuals over 50 years of age. Most cases appear to be sporadic and the mode of inheritance is unknown. However, physical trauma to the subscapular area may play a role in the development of these subcutaneous, poorly defined lesions. Of interest is the observation that elastofibroma dorsi is particularly prevalent in an area of Okinawa, Japan, and many of these patients have a family history of similar lesions (141).

A particular variant of the connective tissue nevi of the elastin type is acrokeratoelastoidosis of Costa (OMIM 101850; 20, 36). This condition manifests with multiple papules on the hands and feet, and histopathology demonstrates accumulation of elastic fibers which are frequently fragmented (elastorrhexis), a finding that can also be detected to a certain extent in the adjacent, clinically unaffected area of skin. The cutaneous lesions also demonstrate foci of hyperkeratosis or acanthosis, which have been considered to be secondary (142). Thus, this condition is regarded as a primary elastic tissue disorder, which can be divided into the hereditary and the acquired type (143). In some families, autosomal dominant inheritance has been suggested (144).

### 155.5 DISORDERS OF ELASTIN FIBRILLOGENESIS

As indicated previously in this chapter, the fibrillogenesis of elastic fibers requires a number of carefully controlled steps. One of the early events is the assembly of

the microfibrillar scaffold into which the elastin molecules align so as to provide directed growth of elastic fibers with a regular, uniform diameter. This process is assisted by a number of elastin-associated microfibrils belonging to different protein families that have been characterized by biochemical and molecular means (see Table 155-3) (1,145–147). Subsequent to their assembly into fibrillar organization, the elastin molecules become extensively cross-linked in a complex reaction initiated by lysyl oxidase, a copper-dependent enzyme expressed in fibroblasts and other elastin-producing cells (see Figure 155-4) (148). Thus, perturbations in the microfibrils, as well as alterations in the activity of lysyl oxidase, can result in abnormalities in the elastic structures. In a number of complex disorders, elastin fibrillogenesis is altered, and the elastic fiber abnormalities are part of a broader spectrum of pathology. At the same time, specific genetic mutations have been described in the microfibrillar proteins, fibrillin 1 and fibrillin 2, resulting in clinical phenotypes of Marfan syndrome and congenital contractural arachnodactyly, respectively. (For discussion of these two conditions, see Chapter 153) Furthermore, mutations in the gene encoding  $\beta$ ig-h3, a component of the elastin-associated microfibrils, have been disclosed in variants of corneal dystrophy (149,150).

Menkes syndrome (see Chapter 100), a complex X-linked disorder, is characterized by developmental delays in maturing neural functions, and associated with a number of features, including connective tissue and hair abnormalities (151,152). In a characteristic case, the skin is easily stretchable and loose, reminiscent of the features in cutis laxa. The hair is brittle and has hair shaft abnormalities, mostly pili torti. Electron microscopic examination of the elastic fibers in the skin has revealed paucity of the central amorphous component consisting of elastin, while the microfibrillar material appears to retain normal morphology (153,154). Menkes syndrome is caused by mutations in the gene encoding a copper transport protein (*ATP7A*) resulting in low serum copper and ceruloplasmin levels and affecting a number of copper-dependent enzymes in different tissues (155–157). In cases with essentially complete absence of *ATP7A* function, the copper levels are extremely low and a multitude of clinical findings can be seen in patients affected with the classic form of Menkes syndrome, including abnormal hair, multiple joint dislocations, seizures, hypotonia, and motor delay with mental retardation. The multitude of neurological, connective tissue, and hair abnormalities can be explained by reduced activities of copper-dependent enzymes, including dopamine  $\beta$ -hydroxylase, cytochrome C oxidase, superoxide dismutase, tyrosinase, and lysyl oxidase (158).

The understanding of Menkes syndrome pathology has been advanced by study of animal models of genetic copper deficiency syndromes. Perhaps the closest animal model for Menkes disease is the brindle mouse mutant

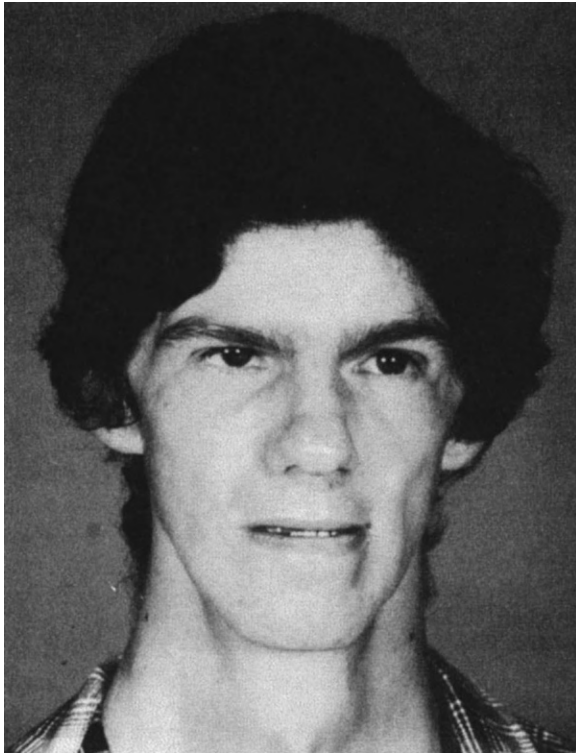
(MObr), which has a two-amino-acid deletion in the highly conserved region of the mouse gene homologous to *ATP7A* (159).

A number of distinct conditions allelic with Menkes syndrome, but with differing phenotypes, have been described. Specifically, a condition known as occipital horn syndrome, previously classified as X-linked cutis laxa or Ehlers–Danlos syndrome type IX, is also due to mutations in *ATP7A*. The primary manifestations of occipital horn syndrome relate to skeletal and connective tissue abnormalities, which could conceivably result from reduced lysyl oxidase activity as a result of low serum copper concentration (160,161). Examination of the mutations and their consequences on the level of expression of *ATP7A* has suggested that partial expression of the gene can occur, and the most severe manifestations of Menkes syndrome are avoided by the residual copper transport activity. A number of cases manifesting the spectrum between full-blown Menkes disease and milder connective tissue manifestations of occipital horn syndrome have also been noted (162,163). For example, in specific families with splice-site mutations in *ATP7A*, approximately 20% of the Menkes gene product has been predicted, and the individuals survive but manifest a number of clinical features associated with Menkes syndrome (163). Thus, these allelic conditions seem to form a continuous spectrum of severity depending on the level of expression of *ATP7A*, as a result of different kinds of mutations in this particular gene.

### 155.5.1 Williams Syndrome and Supravalvular Aortic Stenosis

Another complex developmental abnormality involving the elastic structures is Williams syndrome (OMIM 194050). This disease manifests with a number of developmental and central nervous system problems, as well as characteristic cardiovascular findings (164,165). Specifically, the manifestations include mental retardation, characteristic personality traits, specific cognitive profile, dysmorphic facial features (Figure 155-19), and connective tissue abnormalities. Most patients with Williams syndrome have supravalvular aortic stenosis (SVAS; OMIM 185500), hemodynamically significant narrowing of the aorta, although other arterial blood vessels can also be affected (166). It has been demonstrated that Williams syndrome is a contiguous gene deletion syndrome on chromosome 7q, and the multiplicity of the deleted genes may give rise to the spectrum of phenotype. In case of elastin gene (*ELN*) deletions within the critical interval, cardiovascular manifestations in the form of SVAS accompany the syndrome. This notion is supported by the findings that either familial autosomal dominant or isolated cases with SVAS, without signs of other features of Williams syndrome, are due to mutations in *ELN* (see Figure 155-8) (167). These mutations include nonsense and splice-site mutations, as well as 1-bp deletions





**FIGURE 155-19** Typical facial features of a patient with Williams syndrome. (From Pope, F. M. *Pseudoxanthoma Elasticum, Cutis Laxa, and Other Disorders of Elastic Tissue*. In *Principles and Practice of Medical Genetics*, 3rd edn; Rimoin, D. L., Connor, J. M., Pyeritz, R. E. Eds.; Churchill Livingstone: New York, 1997; p. 1083; reproduced with permission.)

or insertions (168–171), as well as larger deletions and translocations (172–175).

### 155.5.2 Costello Syndrome

This is a complex developmental disorder syndrome characterized by mental retardation, loose skin, coarse facies, skeletal deformities, cardiomyopathy, and predisposition to malignancies (OMIM 218040) (176–179). The main cutaneous findings are loose skin on the hands, feet, and neck, but not on the trunk, as well as deep palmar and plantar creases, palmoplantar hyperkeratosis, and pigmented nevi of the palms and soles. The assembly of elastic fibers in this condition has been shown to be abnormal, in spite of adequate synthesis of elastin and normal deposition of the microfibrillar scaffold (180,181). These observations suggest, therefore, that the elastin abnormalities in Costello syndrome may result from improper interaction between the microfibrillar component and elastin. In support of this interpretation, it has been suggested that the 67-kD elastin-binding protein, which has been suggested to chaperone elastin polypeptides through the secretory pathways for extracellular assembly, is functionally deficient. It has also been postulated that Costello syndrome may result from accumulation of chondroitin sulfate, which causes shedding of this elastin-binding protein from cells, thus contributing to the connective tissue abnormalities in this

complex disorder (180). The genetic basis of Costello syndrome has been shown to be germline mutations in the *HRAS* proto-oncogene (182). In most cases, the genetic lesions are missense mutations in codons 12 or 13, and a potential correlation between the mutation (such as p.G12A) and the risk for malignancy has been suggested (183,184). The details as to how the mutations in *HRAS* might result in elastic fiber abnormalities remain unresolved.

### ACKNOWLEDGMENTS

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### Biography



**Jouni Uitto**, MD, PhD, has been Professor of Dermatology and Cutaneous Biology, and Biochemistry and Molecular Biology, and Chair of the Department of Dermatology and Cutaneous Biology at Jefferson Medical College, in Philadelphia, Pennsylvania, since 1986. He is also Director of the Jefferson Institute of Molecular Medicine at Thomas Jefferson University. He received his MD and PhD degrees from the University of Helsinki, Finland, and completed his residency training in dermatology at Washington University School of Medicine, St. Louis, Missouri.

Dr Uitto is internationally recognized for his research on connective tissue biology and molecular genetics in relation to cutaneous diseases. Dr Uitto's publications include 602 original articles in peer-reviewed journals, 278 textbook chapters and review articles, and 901 abstracts on presentations in national and international meetings (April 2011). Dr Uitto has been the recipient of numerous national and international awards, including honorary doctorate degrees from the University of Kuopio, University of Oulu, and University of Turku, all in Finland, as well as honorary professorship at China Medical University, Shenyang.

Dr Uitto has held office in several scientific and professional societies, including as President of the Society for Investigative Dermatology and President and Chairman of the Board of Trustees of Dermatology Foundation. Dr Uitto is also Section Editor of the *Journal of Investigative Dermatology*, Associate Editor of the *American Journal of Pathology*, and he is on the editorial boards of numerous peer-reviewed journals.

# CHAPTER

# 156

## Osteogenesis Imperfecta (and Other Disorders of Bone Matrix)

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### GLOSSARY

**Dual-Energy X-ray Absorptiometry (DXA)** – This two-dimensional, quantitative technique uses an X-beam with two distinct energies, which get attenuated differently in bone and soft tissue. By measuring the posterior–anterior X-ray absorption profile, bone density ( $\text{g}/\text{cm}^2$ ) and tissue fat is determined. Bone area ( $\text{cm}^2$ ), bone mineral content, and lean tissue can also be derived. The values are compared with those of normal age-, sex-, and height-matched controls and reported as standard deviation or z-scores. The usual sites measured are the total body, lumbar spine, forearm, and proximal femur.

**Platyspondyly** – A general technical term describing reduction in height (flattening) of vertebrae seen in the sagittal plane.

**Peripheral Quantitative Computerized Tomography (pQCT)** – pQCT uses computer tomography to perform a three-dimensional measurement of bone structure. This enables the separate determination of trabecular and cortical bone density ( $\text{g}/\text{cm}^3$ ), bone mineral content, cross-sectional area, and strength-strain indexes in discrete regions of the peripheral skeleton. Currently, the common sites measured are the 4% and 65% sites of the forearm and tibia.

**Wormian bones** – Small plate-like bones along cranial sutures, particularly lambdoid and parieto-occipital bones identified by the physiologist Olaus Wormius. Found in higher frequency in certain dysplasias of the mineralized skeleton and premature aging disorders.

### 156.1 INTRODUCTION

This large group of genetic disorders of the skeleton consists of diseases characterized by decreased bone density and abnormal biomechanical properties of bone, e.g. bone fragility and impaired longitudinal growth. Notwithstanding this definition, there is considerable variability in all these disorders reflecting different manifestations at different ages, and the impact of different classes of mutations in the same molecules.

Decreased bone density, as measured by dual-energy X-ray absorptiometry (DXA), may result from reduced production, defective mineralization, or increased breakdown of normal or defective bone, or a combination of these. Osteomalacia (i.e. undermineralized bone) characterizes hereditary rickets and other disorders leading to defective mineralization. Osteopenia (insufficient bone) characterizes the hereditary osteoporoses and many other genetic and acquired diseases of childhood. Osteoporosis (i.e. the clinical syndromes resulting from osteopenia) is characterized by liability to fractures, particularly crush fractures of the vertebrae. The osteogenesis imperfecta (OI) syndromes are numerically the most frequent of the heritable syndromes with osteoporosis.

### 156.2 OSTEOGENESIS IMPERFECTA

The term osteogenesis imperfecta was proposed by Vrolik in 1840 to explain the origin of a hereditary skeletal

condition leading to susceptibility to fracture and severe skeletal deformity. Also known as fragilitas ossium, osteopsathyrosis, and Ekman–Lobstein or Vrolik disease, OI is characterized by increased bone fragility and decreased bone mass. There is significant variability in the clinical features and severity within OI. The extreme variability in OI results in part from genetic and biochemical heterogeneity.

Various nosologies and classification schema have been proposed over the past 200 years, but a numerical nosology has been widely used during the past 30 years (1). This grouped the various types of OI into five subgroups, OI types I–V, with the addition of a number of extremely rare syndromes. In an attempt to account for recent new insights into the pathogenesis of OI, the International Nomenclature Committee for Constitutional Disorders of the Skeleton (INCCDS) in 2010 proposed a descriptive clinical and molecular nosology for the groups (see Table 156-1) that combines molecular diagnoses with the former descriptive and numerical nomenclature (2).

The newer disorders are encompassed as subtypes of these five groups of disorders (see Tables 156-2–156-4). In addition to these syndromes, there are a large number of syndromes with osteoporosis and features in common with osteogenesis imperfecta, and a number of premature aging syndromes with overlapping clinical features.

### 156.2.1 Pathogenesis of Osteogenesis Imperfecta Syndromes

The vast majority of individuals with OI in European populations have mutations in collagen type I genes, either *COL1A1* or *COL1A2* (3). In other populations, such as in southern Africa, Southern Asia, or Samoa, evidence suggests that mutations in other genes may be more prevalent as causes of moderate to severe OI.

Collagen type I has a triple helical structure consisting of two  $\alpha 1$  chains and one  $\alpha 2$  chain. For the triple helix to fold correctly, every third amino acid residue must be a glycine, with the remaining amino acids rich in proline and hydroxyproline (3,4). The  $\alpha$ -chains are initially synthesized as pro- $\alpha$ -chains with polypeptide extensions at either end. The carboxy propeptide extension is essential for pro- $\alpha$ -chain association prior to triple helix assembly, which occurs in a carboxy to amino direction.

Four classes of COL1 mutation have been described (3): (1) those that reduce the amount of procollagen type I produced; (2) those that disrupt the triple-helical structure of procollagen type I—substitution of the glycine residue, small deletions or duplications, or exon skipping; (3) those that disturb chain association by alteration of the carboxy-terminal propeptide residues; and (4) those that reduce the amount of pro $\alpha 2$ (I), thus altering chain composition.

**TABLE 156-1** The Recommended Nomenclature of OI Syndromes INCCDS 2010

Syndrome Names <sup>a</sup>	Equivalent Numerical Type	Subtypes
Classic non-deforming OI with blue sclerae	I	2
Common variable OI with normal sclerae	IV	2
OI with calcification in interosseous membranes	V	1
Progressively deforming OI with normal sclerae	III	10
Perinatally lethal OI	II	5

<sup>a</sup>The syndromes are listed roughly in order of increasing severity of fracture tendency and skeletal deformity.

Using COL1 mutation analysis alone, it is difficult to make genotype/phenotype correlations in OI. In general, however, mutations resulting only in a quantitative defect in collagen type I production result in a milder phenotype than those leading to a qualitative defect (3,5). The most predictable outcome is from premature termination of transcription, which results in mRNA instability, reduced procollagen type I production, and a mild OI type I phenotype. Most instances at the molecular level are the result of nucleotide deletions that result in a frameshift in transcription and premature termination at a new stop codon. In such cases, normal collagen type I chains are produced, but the amount of collagen is reduced.

The phenotypic outcome of glycine mutations in the triple helix is less certain (5). In an attempt to link disease severity with the site and type of glycine mutation, severity rules have been formulated. *The chain rule*: a glycine mutation in  $\alpha 1$ (I) would result in a more severe phenotype than an equivalent mutation in  $\alpha 2$ (I) because there are twice as many  $\alpha 1$ (I) chains; *the position rule*: a glycine mutation nearer the carboxy-terminus of either  $\alpha 1$ (I) or  $\alpha 2$ (I) would disturb the triple helix formation more than the equivalent mutation nearer the amino terminus and result in a more severe phenotype; *the substitution rule*: a charged amino acid (aspartic acid, glutamic acid, or arginine) or a large amino acid (tryptophan) substitution of glycine would have a more severe consequence than small neutral amino acid (alanine or serine) substitution, because they cause the greatest alteration to the configuration of the triple helix. Numerous exceptions to the severity rules have been noted. This is in part because the two assumptions upon which these rules were based—that each segment of the Gly-X-Y repeat of the  $\alpha$ -chains has an equal role in triple helix formation and stability, and that the stability of mRNA, procollagen, and collagen were similarly effected by different mutations—do not hold true in all circumstances. This led to the formulation of the regional model, which suggests that at certain positions within the collagen triple helix there is



**TABLE 156-2** Progressively Deforming OI With Normal Sclerae (OI Type III)

Disorder—Gene	Mode of Inheritance <sup>a</sup>	MIM <sup>b</sup> of condition	Chromosome Locus	Gene Product
OI type III— <i>COL1A1</i>	AD AR	259420	17q 17q	Collagen α1(I)
OI type III— <i>COL1A2</i>	AD AR	259420	7q22.1	Collagen α2(I)
OI type III— <i>CRTAP</i>	AR	610682	3p22.3	Cartilage-associated protein
OI type III— <i>P3H1/ILEPRE1</i>	AR	610915	1p34.1	Prolyl-3-hydroxylase
OI type III— <i>PIPB</i>	AR	259440	15q21	Peptidylprolyl isomerase B
OI type III— <i>FKBP10</i>	AR	610968	17q21	FK506-binding protein
OI type III— <i>SERPINH1</i>	AR	613848	11q13.5	Collagen chaperone-like protein
OI type III— <i>SP7/OSX</i>	AR	606633	12q13	SP7/osterix
OI Type III— <i>SERPINF1</i>	AR	613982	17p13.3	Serpin peptidase inhibitor F1
OI Type III— <i>BMP1</i>	AR	112264	8p21.3	Bone morphogenetic protein 1

<sup>a</sup>AD = autosomal dominant; AR = autosomal recessive.<sup>b</sup>MIM = Mendelian Inheritance in Man.**TABLE 156-3** Perinatally Lethal OI (OI Type II)

Disorder—Gene	Mode of Inheritance <sup>a</sup>	MIM <sup>b</sup> of condition	Chromosome Locus	Gene Product
OI type II— <i>COL1A1</i>	AD	166210	17q	Collagen α1(I)
OI type II— <i>COL1A2</i>	AD AR	166210 259400	7q22.1	Collagen α2(I)
OI type II— <i>CRTAP</i>	AR	610854	3p22.3	Cartilage-associated protein
OI type II— <i>P3H1/ILEPRE1</i>	AR	610915	1p34.1	Prolyl-3-hydroxylase
OI type II— <i>PIPB</i>	AR	259440	15q21	Cyclophilin B

<sup>a</sup>AD = autosomal dominant; AR = autosomal recessive.<sup>b</sup>MIM = Mendelian Inheritance in Man.**TABLE 156-4** Syndromes With Phenotypic Features Overlapping OI

Disorder	Mode of Inheritance <sup>a</sup>	MIM <sup>b</sup> of condition	Chromosome Locus	Gene	Gene Product
Osteoporosis pseudoglioma	AR	259770	11q12–13	<i>LRP5</i>	Low density lipoprotein-related protein 5
Osteoporosis, cataracts, and retinal dysplasia (spondylo-ocular)	AR	605822			
OI with congenital joint contractures type 1 (Bruck syndrome)	AR	259450	17q21.2	<i>FKBP10</i>	FK506-binding protein
OI with congenital joint contractures type 2 (Bruck syndrome)	AR	609220	3q23–q24	<i>PLOD2</i>	Procollagen-lysine, 2-oxoglutarate S-dioxygenase 2
Osteopenia with radiolucent lesions of the mandible	AD	166260	—	—	
Calvarial donut lesions with bone fragility	AD	126550	—	—	
Osteogenesis imperfecta with craniosynostosis (Cole–Carpenter syndrome)	Sp	112240	—	—	

<sup>a</sup>AD = autosomal dominant; AR = autosomal recessive; SP = sporadic.<sup>b</sup>MIM = Mendelian Inheritance in Man.

mutation tolerance that results in a less severe phenotype than would otherwise be expected (6). It is also clear that less severe phenotypes can arise from increased mRNA or protein degradation, with a resultant decrease in the amount of mutant collagen.

Apart from glycine, type I collagen proteins are rich in proline and lysine. Proline residues are hydroxylated at the 4' position, except for proline 986 in collagen α1(I)

and proline 707 in collagen α2(I), which are hydroxylated at the 3' position (7). The extent of 3' hydroxylation of these proline residues is directly correlated with the amount of type I collagen secretion, so that deficiency states result in a wide spectrum of phenotypic expression of OI ranging from Perinatally Lethal OI type II through severe Progressively Deforming type III. Prolyl 3-hydroxylation in the 3' position is carried out by a multiprotein

complex consisting of prolyl-3-hydroxylase 1 (leprecan) (8), cartilage-associated protein (CRTAP) and peptidylprolyl isomerase B (PPIB), which is also known as cyclophilin B (9,10).

Defective lysyl hydroxylation resulting from mutations in procollagen-lysine, 2-oxoglutarate S-dioxygenase 2 (PLOD2) results in the syndrome of OI and congenital joint contractures, or Bruck syndrome type 2 (11).

Recently, five further genes whose products play a role in type I collagen trafficking have been implicated in the pathogenesis of autosomal recessive types of OI, including *FKBP10* (12), serpin H1 (coding for HSP47) (13), and serpin F1 (14,15). *SP7/OSX* (osterix) (16) is a novel gene, not yet shown to have a role in collagen trafficking.

The majority of molecular studies into collagen type I have utilized skin fibroblasts. Little work has been performed using osteoblasts, which may differ from fibroblasts in respect to post-translational modification of mutant collagen and the incorporation of abnormal collagen molecules into the extracellular matrix of bone (17). Furthermore, other matrix proteins such as proteoglycans, hyaluronan, decorin, fibronectin, and thrombospondin may be abnormally expressed in osteoblasts with a mutation of collagen type I, further modifying the phenotype. The molecular pathology of the specific OI types is discussed below.

The organic abnormalities described above also result in disruption to the inorganic (mineral) phase of OI bone. Human OI bone has a higher average mineralization density than normal bone, and the murine model of moderate to severe OI (OIM mouse) has smaller and less well aligned mineral crystals than the wild-type mouse (18). It is the combination of the organic and inorganic abnormalities of OI bone that alters its biomechanical properties and makes it brittle. On an organic level, OIM mouse collagen has reduced tensile strength compared to its wild-type counterpart (19).

Somewhat surprisingly, OI bone is harder than normal bone, with an increased material density. Despite this, it breaks more readily and accumulates fatigue damage more easily. In addition, there is a reduction in the amount of bone in OI. Histomorphometric studies have shown a decrease in core width, cortical thickness, and trabecular number and thickness in OI bone (20). Individual osteoblasts produce a reduced amount of bone in OI, but owing to their increased number, the bone formation rate is increased. However, this does not lead to a net gain in bone mass, because osteoclastic activity is also increased. Together, these findings indicate a high turnover state with minimal net gain in bone mass. The increase in bone turnover is reflected in increased serum and urinary levels of markers of bone resorption (deoxypyridinoline and N-telopeptide) and bone formation (alkaline phosphatase and osteocalcin) (21). The reduction in core width seen on trans-iliac bone biopsies translates into thinner long bones with a reduced polar moment of inertia, further increasing the propensity to fracture.

## 156.3 MANAGEMENT OF OSTEOGENESIS IMPERFECTA

### 156.3.1 Densitometric Evaluation of Bone

Fracture frequency alone is unreliable for evaluating the efficacy of therapy in osteogenesis imperfecta, as the frequency of fractures varies widely from year to year in one patient and widely among patients. Its usefulness can be improved, however, by comparing the fracture frequency of radiologically confirmed fractures for the three years before treatment with that during treatment. Even so, it is necessary to combine this measure with other outcomes when evaluating therapeutic regimes. To this end, bone mineral density has frequently been employed. The assessment of bone density by standard X-ray films is highly dependent on a number of variables and is not reliably quantifiable. Thus, other investigative techniques, most notably dual-energy X-ray absorptiometry (DXA), have been employed to evaluate changes in bone density when subjects with OI were treated with therapeutic agents such as calcitonin and bisphosphonates.

DXA technology to assess bone mineral content and density in children with OI has been used increasingly over recent years. The skin entrance dose for total body, femur, and lumbar spine DXA is less than 50  $\mu$ Sv, which compares favorably with other investigative techniques and background radiation. However, the use and interpretation of DXA data in children, and especially those with OI, pose certain difficulties which must be taken into account.

There are two main measurements obtained when DXA is performed: bone mineral content (BMC) and bone mineral density (BMD). BMC represents the mass of mineral in the bone being evaluated and is measured in grams. BMD, as measured by DXA, does not represent the true volumetric density ( $\text{g}/\text{cm}^3$ ) of the bone, as would normally be measured in physics. Rather, it is the mass of bone mineral per projection area ( $\text{g}/\text{cm}^2$ ) and is given the term “areal BMD.” Areal BMD is a size-dependent measure. Shorter children therefore have a reduced areal BMD compared to age-matched controls, not because there is anything abnormal with the composition or structure of their bones, simply that the bones are smaller. Because the majority of children with OI have short stature, correcting for height when interpreting areal BMD is essential.

Further confounders that need to be considered are movement artifact from an uncooperative child, positioning difficulties due to spine and limb deformities, and erroneous projection areas due to excessively osteopenic bones.

Given these caveats, serial DXA measurements 6–12 months apart may be beneficial in evaluating bone mineral accrual in children with OI. In a growing bone, BMC and areal BMD should increase, therefore static

or decreasing values may indicate that an abnormality in bone development exists. Serial BMC and areal BMD assessment can also be used to evaluate the effectiveness of bone-specific interventions. Many therapeutic trials in OI (e.g. intravenous and oral bisphosphonates) assume that by increasing BMC and areal BMD bones are made stronger, and therefore have DXA measures as a primary outcome variable. It remains to be seen whether DXA data can successfully guide treatment decisions or duration. Research is also underway into the utility of peripheral quantitative computer tomography (pQCT) to evaluate the volumetric density of OI bone. At present, this method remains a research tool, but may prove valuable in clinical use.

### 156.3.2 Severity Grading in Osteogenesis Imperfecta Syndromes

Internationally agreed criteria for grading severity between affected individuals were proposed and adopted by the International Nosology group in 2009 (see [Figure 156-1](#)). They rely on clinical and historical data, fracture frequency, bone densitometry, and level of mobility. Confusion has resulted in the past because the numerical nomenclature of OI syndromes does not relate to severity but to the order in which the syndromes were identified and described.

### 156.3.3 Treatment of Osteogenesis Imperfecta

The aim of treatment in OI is to reduce fracture frequency and maximize mobility and other functional capacities (22). The optimal treatment approach involves an interdisciplinary team consisting of bone and mineral physicians, clinical geneticists, orthopedic surgeons, rehabilitation specialists, physiotherapists, and occupational therapists (22–24).

Despite optimal surgical and rehabilitative care, extreme bone fragility and deformity persists in many patients. Because of this, many treatments have been used in an attempt to improve bone quality and the quality of life of children and adults with OI. Albright, in 1981, reviewed 96 reports of 20 different treatments in OI, including hormones (calcitonin, cortisone, estrogen/androgen, growth hormone, parathyroid hormone, thyroxine, and thymus extract); vitamins A, C, and D; and minerals (aluminum, calcium, fluoride, magnesium, phosphate, and strontium); as well as arsenic, radiation, dilute hydrochloric acid, calf bone extract, and ovariectomized goat's milk. The majority of the authors reported a beneficial response to their treatment intervention. However, these therapies have fallen out of favor as none has delivered sustained benefit (25).

The major medical advance has come from the introduction of bisphosphonate therapy, and treatment with cyclic intravenous pamidronate or neridronate is now

viewed as the “gold standard” for the treatment of children and adults with OI of moderate to severe degree and its Cole–Carpenter and Bruck variants (26–29). To date, little data is available on the treatment of mild OI (two or fewer fractures per year, no vertebral crush fractures, and no long-bone deformities) or the use of oral bisphosphonates in OI. Bisphosphonates are potent antiresorptive agents that disrupt osteoclastic activity by interfering with the mevalonate pathway of cholesterol biosynthesis (30). Bisphosphonates bind avidly to the hydroxyapatite crystal of bone, which results in locally active concentrations within bone. While bound to the hydroxyapatite crystal, bisphosphonates are biologically inert. As bisphosphonate-containing bone is resorbed, the drug is released, once again becoming active against the osteoclast. This reservoir effect results in bisphosphonates having an extended half-life of many years.

### 156.3.4 Bisphosphonate Therapy for Children with Osteogenesis Imperfecta

The most extensively studied bisphosphonate in OI is Pamidronate®, a moderately potent amino-bisphosphonate. To date, the majority of published studies on bisphosphonate use in OI have been in children with moderate to severe disease, and all but one (31) have been observational in design.

The first reported use of a bisphosphonate in OI was in 1987, when Devogelaer et al. noted a “striking radiological and clinical improvement” in a 12-year-old girl treated for 12 months with oral pamidronate (32). It was not until 1998, however, that Astrom and Soderhall, and Glorieux et al. published systematic evaluations of cyclical intravenous pamidronate in children with severe OI (26,27). In summary, pamidronate in children with OI has been reported to decrease bone pain, enhance well-being, improve mobility and muscle strength, reduce fracture incidence, increase long-bone cortical thickness, increase vertebral size with vertebral reshaping, and increase bone mass and BMD. In an attempt to prevent growth disturbance and spine and limb deformity, cyclical intravenous pamidronate has also been used in babies and infants with OI (28). The treatment response in the younger children was more pronounced than in the older cohort, but further follow-up is needed to evaluate if function, growth, scoliosis, and limb deformities are improved. A multicenter international trial of the potent bisphosphonate zoledronate was carried out to assess its dosage, efficacy, and safety in OI compared to pamidronate. This study showed a similar response in terms of improvements in serial bone density and quality of life. An increased frequency of fracture was seen in the zoledronate treatment group, which may have been due to inclusion of some children with more severe OI. Zoledronate has the benefits of being able to be administered more rapidly and of having a longer dosing interval than pamidronate,

**(A) Mild Severity (of OI)**

- Annualized fracture rate of less than or equal to 1
- Straight long bones, i.e. no intrinsic long-bone deformity
- Minimal vertebral crush fractures
- Absence of chronic bone pain or minimal pain controlled by simple analgesics
- Normal or near-normal growth velocity and height
- Fully ambulant other than at times of acute fracture
- Regular school attendance, i.e. does not miss school owing to pain, lethargy, or fatigue
- Lumbar spine bone mineral density z-score usually  $\geq -2.0$



Children with Mild OI most often have OI type I or IV

**(B) Moderate Severity (of OI)**

- Annualized fracture rate greater than 1
- Bowing of long bones related to immobilization for recurrent fractures
- Vertebral crush fractures
- Anterior bowing of legs and thighs
- Lumbar spine bone mineral density z-score  $\leq -2.5$ .
- Absent from school owing to pain more than 5 days per year

**(C) Severe Disease (Progressively Deforming OI)**

- Progressive deformity of long bones and spine (unrelated to fractures)
- Multiple vertebral crush fractures
- Marked impairment of linear growth
- Cases intermediate between severe and extremely severe have few rib fractures but crumpled long bones

**(D) Extremely Severe Disease**

- Thighs held in fixed abduction and external rotation with limitation of movement of most joints
- Clinical indicators of severe chronic pain (pallor, sweateness, whimpering or grimacing on passive movement)
- Continuously beaded ribs due to multiple fractures before birth
- Crumpled (concertina-like) long bones
- All vertebrae hypoplastic/crushed
- Respiratory distress leading to perinatal death
- Perinatally lethal course



**FIGURE 156-1** Showing patients representative of levels of severity. (A) Mild severity—girl aged 7 years with OI but no skeletal deformity; (B) Moderate severity—girl aged 4 years after 12 months therapy with bowing right leg; (C) Severe—teenage male aged 14 years; (D) Extremely severe/perinatally lethal.



both of which may prove advantageous to patients and health-care facilities.

There is growing interest in the utility of oral bisphosphonates in OI. In a placebo-controlled trial of oral Olpadronate® in 34 children with OI (16 olpadronate), Sakkars et al. reported that olpadronate was associated with an increase in lumbar spine BMD, but did not improve muscle strength, mobility, function, or vertebral height (31).

A small randomized controlled study of the oral bisphosphonate risedronate showed an improvement in spine bone density and decrease in bone turnover, but no change in bone biopsy or pQCT data (33). A large multinational placebo-controlled trial is currently underway to further evaluate oral bisphosphonate use in mild OI. A large multicenter double-blind randomized placebo-controlled trial of alendronate in children with moderate to severe OI showed that while there was an improvement in bone density and decrease in bone turnover, there was no improvement in fracture rate, bone pain, vertebral height, bone histomorphometry, or physical activity with treatment (34). These data would suggest that, until there is data to the contrary, oral bisphosphonates should not be used in favor of intravenous bisphosphonates in children with OI. Oral therapy may, however, be of benefit as maintenance therapy in children with moderate to severe OI, or as initial therapy in mild OI. Studies are currently underway addressing both these issues.

Once bisphosphonates are stopped, they have little, if any, effect on new bone produced with growth and modeling. As such, it is usually necessary to give children with OI a maintenance dose of bisphosphonate following their acute treatment regimen. The best maintenance therapy is uncertain but is likely to be 30–50% of the acute treatment dose.

Histomorphometry has provided valuable insight into the actions of pamidronate in children with OI (35). The major effects of pamidronate were to increase cortical thickness and trabecular number. Trabecular thickness was not enhanced. Bone turnover was significantly reduced, with a decrease in both bone resorption and formation below that of age-matched normal controls. There was also an increase in residual calcified cartilage within the bone.

In adults with OI, pamidronate and a similar compound, neridronate, have been shown to increase spine and hip areal BMD and decrease fracture rates (36,37). The results in adults have not been as marked as those in children, suggesting that bisphosphonate therapy should be instigated during childhood to obtain maximal benefit.

The safety of bisphosphonate therapy continues to be of concern to many clinicians (38). To allow for this issue to be systematically evaluated, it is of paramount importance that children and adolescents only receive bisphosphonate therapy as part of well-run clinical trials. Pamidronate lowers serum calcium concentrations and this is most marked following the first infusion cycle (39). In

vitamin-D-replete individuals receiving the recommended calcium intake, the hypocalcemia is self-remitting. The majority of children have an acute phase reaction (fever, muscle pain, headache, and vomiting) 12–36 h following initial exposure to pamidronate. This appears to be due to a pamidronate-provoked release of osteoclast-specific interleukin 6, mimicking the type of febrile response seen from macrophage-released interleukin 6 in influenza (40). These side-effects can be minimized by the administration of acetaminophen (paracetamol) or the anti-inflammatory medication ibuprofen (41).

Infants with severe OI and pre-existing respiratory compromise have been reported to experience an acute respiratory distress in associated with the first pamidronate infusion (42). The etiology remains unclear, but may relate to cytokine release, hemodynamic compromise from fluid administration during the first infusion, or both. This report stresses the often fragile state of young children with severe OI and the need for close monitoring of this cohort during treatment.

Animal studies have shown that high-dose bisphosphonates can suppress growth, and concerns have been raised of this possibility in children. Counter to these concerns, Zeitlin et al. showed that pamidronate significantly improved the growth of children and adolescents with moderate to severe OI compared to historical controls over a 4-year treatment period, by preventing limb and spine deformity (43). In the same report, these authors noted rapid weight gain in a number of children with severe OI. The etiology of the weight gain remains unclear, but excessive weight could have a detrimental effect upon function and increase fracture risk. Transient uveitis occurs in approximately 1% of children who receive pamidronate (24).

As mentioned above, pamidronate suppresses bone turnover in children with OI to well below that of normal aged-matched controls (35). As highlighted recently, at high doses, pamidronate can interfere with bone modeling and result in undertubularization of long bones. In the growing skeleton, a reduction in bone remodeling results in the accumulation of mineralized cartilage within the bone, which contributes to the increase in bone density seen with pamidronate treatment (44). Further, acute reduction in remodeling and the persistence of calcified cartilage in bone account for the characteristic sclerotic metaphyseal lines seen on long-bone radiographs of children receiving pamidronate therapy (45).

Osteonecrosis of the jaw, or non-healing dental extractions, has emerged as a major issue in patients treated with high-dose or potent bisphosphonate (BRON). It was first reported by Marx in 2003 (46). It is most simply described as non-healing, painful jaw wounds following dental extraction or other dental procedures. At the present time, the risk from normal exfoliation of deciduous teeth in children is not quantified but presumed to be extremely small. Notwithstanding reports in adults, there has been no report of children treated with bisphosphonates long

term developing osteonecrosis of the jaw. A large study of 64 young people with OI from Sweden treated with bisphosphonates for up to 12 years revealed no instance of BRON (47). The risk of BRON in carefully supervised treatment of children is also believed to be very small.

Bisphosphonates are contraindicated during pregnancy, and all females of reproductive age should have a negative pregnancy test before each pamidronate treatment cycle or before commencing oral bisphosphonates. Because bisphosphonates persist in mineralized bone for many years, concern has also been expressed that bisphosphonates administered before conception could be released from the maternal skeleton during the pregnancy and affect the fetus (38,48). Munns et al. published a report in 2004 of two women with OI who became pregnant after 5 years of pamidronate therapy. No pamidronate was administered following conception. Both pregnancies went to term and there were no maternal complications noted. It could not be excluded, however, that the adverse events noted in the babies, hypocalcaemia and talipes equinovarus, were related to maternal pamidronate therapy (49). Clearly, further systematic follow-up of pregnancy outcome in this cohort is required and females should be counseled about the uncertainty surrounding this aspect of bisphosphonate treatment.

Other issues that can only be addressed through the continued systematic evaluation of bisphosphonate therapy in children and adults with OI are: the long-term benefits of treatment, the optimal treatment regime to maximize benefit and minimize potential long-term side-effects, and the outcome following cessation of therapy.

In summary:

- (1) Cyclic intravenous pamidronate has good short-term safety and efficacy in children and adolescents with moderate to severe OI and related disorders.
- (2) Pamidronate therapy should be offered to children with moderate to severe OI as defined by: two or more long-bone fractures per year, and/or vertebral crush fractures, and/or long-bone deformities, and/or children with OI type III or IV.
- (3) In severe cases, treatment can be started during infancy, but these children need to be monitored very closely, especially during the first infusion cycle.
- (4) Treatment continues to be effective in older teenagers and the upper age limit of responsiveness still remains to be defined.
- (5) Several dosage regimens appear to be effective:
  - (a) Low-dose frequent administration—0.5–1 mg/kg/month (50)
  - (b) High-dose infrequent administration—9 mg/kg/year, with a dose and treatment interval that varies with age (39).
- (6) All children treated with a bisphosphonate should be part of a well-run clinical trial. This will enable the safety and efficacy of these compounds to be adequately evaluated.
- (7) After completion of the acute treatment phase, most children will require ongoing maintenance therapy.
- (8) Further study is required before bisphosphonates can be administered to all patients with OI on the basis of low BMD alone.

### 156.3.5 Other Medical Therapies

Intermittent recombinant human parathyroid hormone (rhPTH) is a potent bone anabolic agent that increases bone mineral density and reduces vertebral fractures in postmenopausal and glucocorticoid-induced osteoporosis (51). By increasing bone formation, it was hoped that rhPTH could be a useful adjunct to bisphosphonate therapy. However, the occurrence of osteosarcomas in a significant proportion of treated young rats (52), and the possibility of this occurring in humans, has meant the risks of its use in children outweigh any potential benefit (53).

Recombinant human growth hormone (rhGH) has been shown to increase bone turnover in children with OI (54). As bone turnover is already increased in OI, any further increase may be detrimental to the bone health of these children. Antonazzi et al. reported that rhGH in combination with bisphosphonate increased BMD and growth compared to bisphosphonates alone (55).

Both bone marrow transplant (BMT) and gene therapy offer a cure for OI. Despite reported success from preliminary studies of BMT in children with severe OI (56,57), bone disease specialists remain skeptical of the benefit (58). As there is the potential for cure with BMT, it has been suggested that this therapy not be discarded, but that the basic science and technician details be thoroughly evaluated in animal models before further human studies are undertaken.

Many obstacles exist to successful gene therapy in OI. The most significant is the requirement to firstly knock out the abnormal collagen gene from which mutant molecules are synthesized that interfere with collagen macromolecular assembly and cause the most severe phenotypes; only then can the gene coding for the missing protein be replaced. It is encouraging to note that *in vitro* experiments have shown that hammerhead ribosomes (small RNA molecules that can cut mRNA in the absence of protein cofactors) can reduce the amount of mutant *COL1A1* mRNA by about 50% (59). This may be the first step on the path to a genetic cure for OI, but introduction of these therapies into the clinical setting remains some time off.

## 156.4 CLINICAL FEATURES OF THE OI SYNDROMES

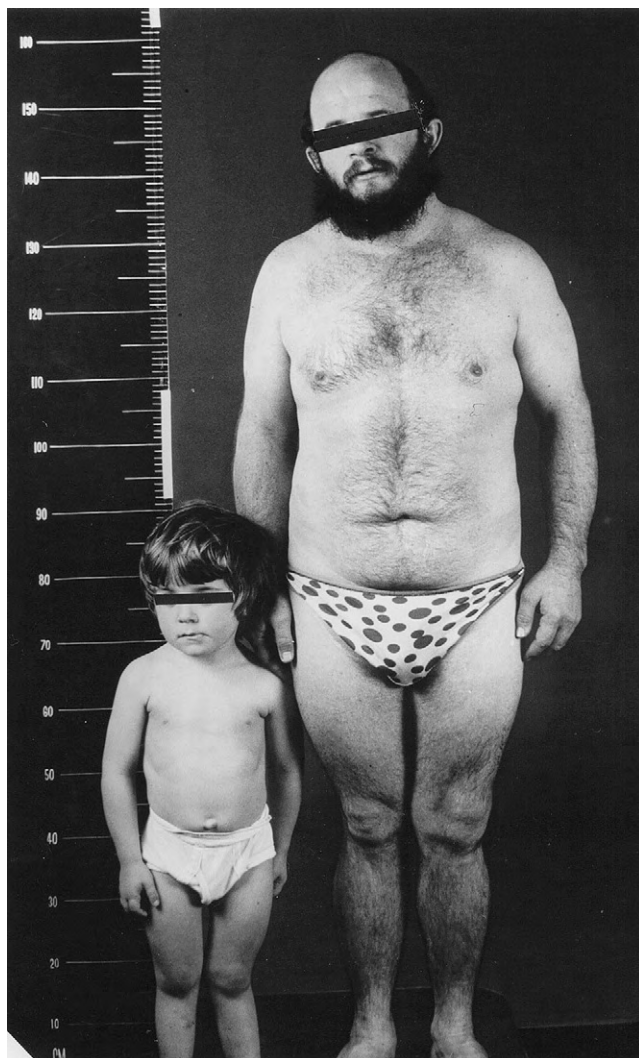
### 156.4.1 Non-deforming Osteogenesis Imperfecta with Blue Sclerae—OI Type I

This type of OI is characterized by autosomal dominant inheritance of excessive bone fragility, which is usually associated with low bone mass, distinctly blue sclerae,

and susceptibility to conductive hearing loss commencing in adolescence and adult life. This is the most common type of OI and has a birth frequency in the order of 1:25,000 live births and a similar population frequency (60–62). In most European communities, the majority of people with OI have affected relatives. (Synonyms: Van der Hoeve syndrome, trias fragilitis ossium.)

**156.4.1.1 Clinical Features.** The sclerae are of a deep blue-gray hue and remain so throughout life. Fractures characteristically result from minimal trauma. Despite the tendency to bone fragility, accidental trauma does not always lead to fractures.

Opalescent dentine (OD), also known as dentinogenesis imperfecta (DI), is observed in some families with this trait but not others. OD produces a distinctive yellowing and apparent transparency of the teeth, which are often worn prematurely or broken. Some teeth with OD may have a particularly grayish hue. Radiologic study of these teeth shows that they have short roots with constricted corono-radicular junctions (see Figure 156-2).



**FIGURE 156-2** Classic osteogenesis imperfecta type I. Father and son both with numerous fractures, intensely blue sclerae, and mild shortening of stature. Father wears a hearing aid.

Paterson et al. (61) have shown that patients with OI type I and normal teeth differ from patients with OI type I with OD. The latter group is more likely to have fractures at birth (25% vs 6%) and have a higher fracture frequency, more severe short stature, and skeletal deformity. Both groups have a similar frequency of joint hypermobility, bruising, hearing loss, and joint dislocations.

When fractures occur at birth, these are usually only a few in number. Individuals with fractures at birth subsequently have no more deformity, handicap, or number of fractures than other individuals who have their first fracture after 1 year of age. Deformities of the limbs in this group are usually the result of fractures, but bowing, particularly of the lower limbs, is common and is associated with low bone mass. Some adults have progressive kyphoscoliosis, which may be of a severe degree. Kyphosis alone is common in older adults with OI type I, but rarely seen in children. There is usually excessive hypermobility in the small joints of the hands and feet, but this feature is less marked in adults (62). Some patients have marked hypermobility, and in clinical features, show significant overlap with the hypermobile type of Ehlers–Danlos syndrome. Adult patients with OI type I frequently suffer from chronic fatigue or fibromyalgia related to their hypermobility. Hearing impairment and vestibular dysfunction are troublesome complications (63,64).

There is a high frequency of arcus cornea in adults with OI type I. No other visual abnormalities are associated with OI. Patients with OI type I frequently have easy bruising (62), which in children may be mistaken for child abuse, when associated with unexplained fractures. Ankle sprains and dislocation occur rarely.

Bone densitometry determined by dual-energy X-ray absorptiometry falls into the low normal range in many children with OI type I. However, in late childhood and adolescence, areal bone density in the long bones and spine frequently may fall to two standard deviations below the mean.

Radiographic studies in most patients in this group show generalized osteopenia, evidence of previous fractures, and normal callus formation at the site of recent fractures. Deformities are usually the result of angulation at the site of previous fractures. However, bowing of the femora, tibiae, and fibulae and deformity in the bones of the feet, particularly metatarsus varus, are observed. Severe osteoporosis of the spine is rarely seen in these patients, and most have normally formed vertebral bodies with some wedging and flattening. Wormian bones are not necessarily present in the skull and are found in no more than 70% of subjects. Spontaneous improvement is observed during adolescence, with a marked reduction in the frequency of fractures. This reflects the resistance to fracture of larger-diameter long bones. There is exaggerated postmenopausal bone loss, and adults who have not had fractures for many years may commence having fractures again in their late 40s–50s.



**156.4.1.2 Genetics.** Inheritance is autosomal dominant with variable expressivity. Penetrance for fractures approximates 90% and for blue sclerae 100%. Hearing impairment is age related (63). Patients with OI type I and OD have earlier and more marked manifestations than those with OI type I and normal teeth, and this feature seems to be inherited within families.

**156.4.1.3 Molecular Pathology.** At the cellular (in vitro) level, these patients show approximately 50% reduction in synthesis of type I procollagen. The mutations are heterozygous, but there is no evidence for deletion of one or other allele. Post-translational modification of type I procollagen appears to be normal in contradistinction to all other forms of OI. The commonest mutational mechanism in OI type I is mutation arising from premature termination of transcription and translation (PTT) from one *COL1A1* allele, as a result of sense-to-nonsense mutation, out-of-frame deletions, or insertions that result in generation of a new stop codon downstream or splice mutations. *COL1A2* mutations are far less commonly encountered than *COL1A1* mutations (65,66).

**156.4.1.4 Differential Diagnosis.** *Blue sclerae and keratoconus:* In this autosomal recessive syndrome, blue sclerae are associated with keratoconus, middle ear bone conduction defect, joint hypermobility, and spondylolysis without liability to fractures (67).

#### 156.4.1.5 Management.

**156.4.1.5.1 Therapy.** Children and adults with OI type I have less tendency to progressive skeletal deformity than in other types of OI and thus they maintain better mobility. Serial bone densitometry should be possible as these patients have little skeletal deformity. If there are recurrent long-bone fractures or vertebral crush fractures in children and adults, cyclic intravenous pamidronate (see section 156.3.4 above) can be used with a very favorable response to therapy. A multidisciplinary approach to caring for these children and adults is also important because of the associated issues of joint hypermobility (occupational therapy), progressive hearing impairment, and vertigo (audiologist and ENT specialist). Where OD is present, 6-monthly dental reviews should be undertaken. It may be valuable to monitor the clinical state and bone density in adult women planning a pregnancy before and immediately after the pregnancy. Given the caveats discussed above, it may be advisable to institute therapy following delivery with a bisphosphonate for those women whose pregnancy-related bone loss has resulted in symptomatic osteopenia and/or reduced bone mineral density that falls into the symptomatic/fracture range.

**156.4.1.5.2 Genetic Counseling and Prenatal Diagnosis.** For an affected adult mating with a normal partner, there is a 50% chance of an affected child. Where two adults with OI type I mate, there is a 25% chance for a homozygous affected infant, which is likely to be perinatally lethal or severely affected. Ultrasound

and radiographic studies prenatally will occasionally differentiate a normal from an affected fetus. Prenatal prediction of OI type I is feasible when a specific mutation has been identified. Preimplantation genetic diagnosis has been reported (68).

### 156.4.2 Common Variable Osteogenesis Imperfecta with Normal Sclerae—OI Type IV

OI type IV is characterized by osteoporosis leading to bone fragility without the characteristic features of the OI type I syndrome (i.e. blue sclerae and early onset deafness) (69). See Figure 156-3. (Synonyms: Ekman-Lobstein disease, osteopsathyrosis idiopathica.) There are many families found to be affected in population studies of OI and there is phenotypic overlap with osteoporosis resulting from other causes.

**156.4.2.1 Clinical Findings.** The sclerae may be bluish at birth but become progressively less blue as the patient matures. Adults usually have normal sclerae and there is no tendency to develop arcus cornea. These individuals have variable ages of onset of fractures, which may be present at birth (approximately 25%) or may not occur until adult life. Significant bowing of the lower limbs has



**FIGURE 156-3** Common variable osteogenesis imperfecta type IV. Patient aged 2 years (not walking) with mild bowing of the lower limbs and sclerae of normal hue; her father is similarly affected.



been present at birth as the only feature of this syndrome. Some patients improve with age in that bowing lessens. Just as in OI type I, these patients appeared to show a spontaneous improvement at the time of puberty, and generally fewer fractures are encountered in adolescents and adults. However, the large majority of patients have short stature of postnatal onset and measurement of bone density by DXA may reveal persistent osteopenia (62,70).

A small proportion have severe progressive deformity of lower limb bones and spine that is out of proportion to their severity as judged by fracture frequency. It is clear that this is mainly the result of progressive osteoporosis and can be prevented by early institution of bisphosphonate therapy. Those so affected show more skull deformity and more severe short stature.

Opalescent dentine has been observed in some families and not others, suggesting OI type IV should be divided into two subgroups: OI type IV with normal teeth, and OI type IV with OD, which is associated with a greater frequency of fractures, and more fractures at birth.

Hearing impairment and vestibular dysfunction, although less frequent than in patients with non-deforming OI and blue sclerae, are troublesome complications. These complications are discussed in detail at the end of this section.

Radiographically, this group is defined by generalized osteopenia. Although multiple fractures may be observed in the skeleton at birth and throughout life, as a group, these patients have less osteopenia and fewer fractures than infants with OI type III. The skull does not always show multiple Wormian bones, which are absent in 30–50% of subjects. This group has an increased frequency of progressive deformity of the skull base, known as basilar impression. The presence of OD is associated with a five times increase in risk of basilar impression (71).

**156.4.2.2 Genetics.** Inheritance is autosomal dominant. As in OI type I, families are observed either with normal teeth or with OD.

**156.4.2.3 Molecular Pathology.** Mutations recognized to date in OI type IV have been evenly distributed between *COL1A1* and *COL1A2*. These mutations, which are usually missense mutations in the glycine residues, reduce collagen triple helix formation and stability and thus presumably reduce the amount of collagen available for bone formation (3,72).

#### 156.4.2.4 Differential Diagnosis.

(1) *Juvenile idiopathic osteoporosis (IJO)*: IJO is a rare, self-limiting disorder that may be difficult to distinguish from mildly affected cases of OI type IV (with normal teeth). Features of IJO not associated with OI are: onset of symptoms 2–3 years prior to the onset of puberty and usually self-remitting. In addition, there is a negative family history, metaphyseal fracture, neo-osseous ossification, straight long bones with normal cortical thickness, no extra-skeletal manifestations as in OI, and decreased bone turnover.

Osteopenia in IJO is most marked in the axial skeleton (73). All patients in this group should be investigated for mutations in *LRP5* (74).

(2) *Osteogenesis imperfecta with calcification in interosseous membranes*: People with OI type V also have normal sclerae (75).

(3) *Other Mendelian disorders and inborn errors of metabolism characterized by osteopenia*: Osteopenia is usually also a feature of progeria (Hutchinson–Gilford syndrome) an autosomal dominant disorder; Winchester, Cockayne, and Rothmund–Thompson syndromes; and Fanconi pancytopenia; all autosomal recessive syndromes. It is also frequently recognized in homocystinuria, methylmalonicacidemia, dibasic aminoaciduria, prolidase deficiency, glycogen storage disease type I, Menkes syndrome, and Lowe oculocerebrorenal syndrome.

(4) A number of autosomal recessive syndromes (see Table 156-4) are complicated by osteoporosis and may present because of osteoporosis-related complications.

#### 156.4.2.5 Management.

**156.4.2.5.1 Monitoring.** Regular monitoring of fracture frequency and bone densitometry by DXA is useful to predict the need for bisphosphonate therapy. Screening for basilar impression for practical purposes can be commenced after 5 years (71). If not present then screening can be repeated every 3 years. When basilar impression is detected, annual monitoring for progression is indicated. Audiology should be undertaken from early adolescence onwards.

**156.4.2.5.2 Therapy.** Treatment is as for OI in general. Where there are recurrent long-bone fractures, vertebral crush fractures, or skeletal deformity, cyclic intravenous pamidronate should be offered as the first line of treatment. The response in OI type IV is particularly good. In osteopenic but asymptomatic children with BMD  $\leq 2.0$  SD for age, one can make the case to treat before the first vertebral or femoral fracture occurs, as the immobilization following a fracture in an osteopenic OI child is usually worsened and can rarely be reversed by physical activity alone. Further studies are required before this approach can be routinely recommended. Where dentinogenesis imperfecta is present, 6-monthly dental reviews should be undertaken.

**156.4.2.5.3 Genetic Counseling and Prenatal Diagnosis.** For the offspring of an affected parent and normal parent, there is a 50% chance of affected offspring. Ultrasound prenatal diagnosis is unlikely to be definitive, although fractures and leg bowing may be detected in the third trimester. Prenatal prediction of OI type IV has been achieved by direct sequencing when a specific mutation has been identified. Preimplantation genetic diagnosis has been reported in OI type IV (68).

### 156.4.3 Osteogenesis Imperfecta with Calcification in Interosseous Membranes—OI Type V

OI type V is a moderate to severe bone fragility disorder, and accounts for approximately 5% of individuals with OI seen in the hospital setting and up to 10% with severe to moderately severe OI (76). Calcification (possibly ossification) of the interosseous membrane in the forearms and the legs commences early in life (Figure 156-4). This leads to restriction of pronation and supination, and eventual dislocation of the radial heads (75,77,78). The sclerae are white, and OD and Wormian bones are not present. Those affected tend to have higher serum alkaline phosphatase values and have an increased risk of developing hyperplastic callus following a fracture or orthopedic surgery. Rarely, the hyperplastic callus has been mistaken for an aggressive osteosarcoma of bone.

Familial recurrence is consistent with autosomal dominant inheritance (75).

This disorder was first recognized as a separate entity 90 years ago and has been known as the “hyperplastic callus” type of OI. These subjects have ossification in the interosseous ligaments, and it is now accepted that this latter feature is the most penetrant feature and that many affected individuals do not develop hyperplastic callus. This disorder has a distinct pattern of bone histomorphometry with coarse mesh-like lamellation, which distinguishes it as an entity from OI type IV.

Hyperplastic callus following long-bone fracture is a frequent finding in patients with OI type V. On occasion, a massive callus develops, leading to swelling and pain at the site of the fracture, which can mimic osteosarcoma but may be distinguished from it on magnetic resonance imaging (MRI) and computerized tomography (CT). Its progress may be prevented by the prompt use of indomethacin, an anti-inflammatory prostaglandin inhibitor. Collagen studies exclude the involvement of type I collagen genes.

#### 156.4.3.1 Management.

**156.4.3.1.1 Therapy.** Treatment is as for OI in general. Where there are recurrent long-bone fractures, vertebral crush fractures, or skeletal deformity, cyclic intravenous pamidronate should be offered as the first line of treatment. As with OI types III and IV, OI type V

responds well to cyclical intravenous pamidronate (79). Anti-inflammatory medication is of benefit to reduce the severity of hypertrophic callus development. An overuse syndrome in the shoulder abductors frequently results from the need to abduct the shoulders to achieve pronation of the hands. Consultation with an occupational therapist is important to minimize the disability arising from the upper limb and shoulder symptoms.

**156.4.3.1.2 Genetic Counseling.** For the offspring of an affected parent and a normal parent, there is a 50% chance of being affected. Until the causative mutation is identified, prenatal diagnosis remains problematic.

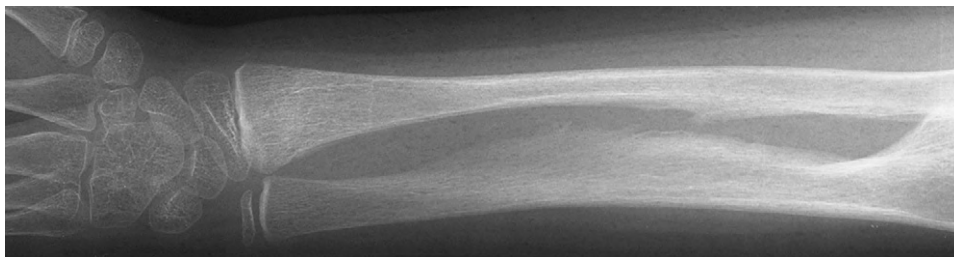
### 156.4.4 Progressively Deforming Osteogenesis Imperfecta with Normal Sclerae—OI Type III

Progressively deforming OI was originally characterized by autosomal recessive inheritance of usually non-lethal OI with severe bone fragility leading to progressive deformity of the skeleton and usually light blue or normal sclerae (80) (Figure 156-5). Because the “progressively deforming OI” phenotype is not specific to this form of OI, individuals with those forms of OI with moderately severe expression and fresh heterozygous mutation in type I collagen genes have been classified in this group in addition to multiple recessive disorders. The group is subdivided into two subgroups:

- (1) OI type III—autosomal dominant
- (2) OI type III—autosomal recessive types.

For practical purposes, at the present time these are phenotypically indistinguishable and the distinction is made on unequivocal pedigree grounds or on molecular genetic evidence.

**156.4.4.1 Clinical Features.** These individuals have newborn or infant presentation with severe bone fragility and multiple fractures leading to progressive deformity of the skeleton. They are generally born at or near term and have normal birth weight and often normal birth length, although this may be reduced because of deformities of the lower limbs at birth. Fractures are present in most cases at birth and occur frequently during childhood. Although the sclerae may be blue at birth, observation of many patients with this syndrome reveals that



**FIGURE 156-4** Osteogenesis imperfecta type V. Plain radiograph of forearm showing ossification extending into the interosseous ligament from the medial borders of the radius and ulna.

the sclerae become progressively less blue with age. The presence of clubfeet at birth should raise the possibility that the child has one of the Bruck syndromes or that the disorder is due to homozygous mutations in *FKBP10*. All patients have poor longitudinal growth and fall well below the third percentile in height for age and sex. Progressive kyphoscoliosis develops during childhood and progresses into adolescence. Hearing impairment has not been reported in children with this syndrome. Hearing loss has been variously reported in 16–80% of adults. In view of the severe osteopenia and liability to fractures, it is likely that there is significant involvement of the ossicular chain, leading to hearing defect. OD is a variable feature.

At birth, there is usually overmodeling of the shafts of the long bones, with widening of the femoral metaphyses and angulation of the tibiae. There is generalized osteopenia and multiple fractures. Within weeks to months, in most infants, the shafts of the long bones show undermodeling, producing a “broad-bone” appearance. From several years of age, progressive disruption and repair of osseous trabeculae in the metaphyses may produce a “popcorn” appearance similar to that seen in various forms of enchondromatosis. The ribs are thin, osteopenic, and progressively crowded as platyspondylia increases. The skull shows multiple Wormian bones, although these may not be evident until several weeks to months of age.

In the past, approximately only one-third of the patients survived long term, reflecting not only the severity of the disorder but also the heterogeneity within the group. Death usually resulted from the complications of severe bone fragility and skeletal deformity, including kyphoscoliosis, pulmonary hypertension, and cardiorespiratory failure. Given the present therapeutic options, specifically treatment with cyclic intravenous pamidronate commenced in infancy, it can be expected that the majority of patients with OI type III will survive into adult life. Furthermore, early institution of

bisphosphonate therapy results in less skeletal deformity and more normal growth.

#### 156.4.4.2 Genetics.

**156.4.4.2.1 Autosomal Dominant OI Type III.** The great majority of cases of OI type III in North America and Europe are dominantly inherited and result from heterozygous missense mutations in type I collagen genes (3,5). Sibling recurrence results from parental germinal cell mosaicism, so that an empiric recurrence risk of approximately 6% should be given (81).

**156.4.4.2.2 Autosomal Recessive Forms of OI Type III.** Autosomal recessive forms may be the commonest cause of OI in black Africans (82). In North America it is estimated that 15% of children with a phenotype with severe progressive deformity result from autosomal recessive inheritance. There is some evidence that there are regional differences in the prevalence of distinct autosomal recessive disorders between western and southern African populations. In eastern Mediterranean and Pakistani patients affected with OI, recessive inheritance has been confirmed at the molecular level in a high percentage of those affected. Recurrence in siblings and parental consanguinity point to autosomal recessive inheritance except in populations such as western and southern Africa and Samoa, where there is a high heterozygote frequency for recessively inherited types of OI.

#### 156.4.4.3 Molecular Pathology.

- Autosomal dominant OI type III

Heterozygous missense mutations account for the majority of cases of OI type III in European populations. These mutations predominantly involve the first glycine of the Gly-X-Y repeat and result in delayed triple helical assembly. This is associated with increased degradation of type I collagen polypeptides. The bone pathology results from an excess of bone turnover and abnormal bone architecture (see section 156.2.1).

- Autosomal recessive forms of OI type III

Homozygous or doubly heterozygous mutations in at least nine genes have been shown to result in a pattern of OI with progressive deforming of long bones, spine, or pelvis (protrusio acetabulae).

- *COL1A1* and *COL1A2* mutations

In one well-documented child with OI type III born to normal but consanguineous parents, the collagen gene mutation, a 4-nucleotide frameshift deletion, alters the pro $\alpha$ 2(I) chains, which are not incorporated into the collagen triple helix. The collagen present in tissues such as bone and skin was composed of  $\alpha$ (1)I trimers (83).

However, there had been very few reports of homozygosity for mutations in *COL1A1* or *COL1A2* during some 35 years of research into type I collagen mutations in OI, but many reports of pedigrees pointing to autosomal recessive inheritance. During the past 10 years, eight further genes have been characterized in which



**FIGURE 156-5** Progressively deforming osteogenesis imperfecta type III. A 14-year-old boy with severe short stature and progressive deformity of long bones and spine.



mutations result in either perinatally lethal and/or severe OI (84) (Tables 156-2 and 156-3).

- Genes regulating 3'-prolyl hydroxylation

Three of these genes code for a protein which comprises one of the three components of the multiprotein enzymatic unit responsible for prolyl 3-hydroxylation. These are *LEPRE1*, coding for prolyl-3-hydroxylase (8); *CRTAP*, coding for cartilage-associated protein (7); and *PPIB*, coding for peptidyl-prolyl isomerase B (10). *CRTAP* and *PPIB* proteins have chaperone functions and it is therefore interesting to note that the proteins encoded by *FKBP10* (coding for FK506-binding protein) and *SERPINH1* (coding for the collagen chaperone protein HSP47) also have important collagen chaperone functions. There is some variability in the severity of phenotypes.

- *FKBP10*, coding for FK506-binding protein

Approximately 4% of children with moderately severe OI with normal scleral hue and normal teeth have a phenotype previously known as OI type VII, characterized by a distinctive pattern of bone histomorphometry with excessive osteoid on the trabecular margins and a “fish-scale-like lamellar” appearance on polarized light examination. These patients, previously designated OI type VI in Online Mendelian Inheritance in Man (OMIM), have the identical phenotype to patients described in a large Turkish kindred discovered to have a mutation in the *FKBP10* gene, a molecular chaperone coding for FK506-binding protein, which has peptidyl-prolyl isomerase activity (12). Subsequently, mutations in *FKBP10* were shown to be common in the Saudi Arabian, southern African, and Samoan populations (85,86), where OI may manifest as congenital clubfeet and progressive protrusio acetabulae in young adult life.

- Serpin H1 and serpin F1

Severe progressively deforming OI phenotypes result from mutations serpin H1, coding for heat shock protein 47 (HSP47) (13). Mutations in serpin F1 result in a phenotype previously known as OI type VI (14). Both these genes encode important chaperone functions for type I collagen polypeptide modification and processing in the endoplasmic reticulum prior to secretion.

- Osterix (OSX)

A further form of OI with autosomal recessive inheritance has been reported in an Egyptian proband with moderately severe OI. This results from mutations in *OSX*, which encodes an osteoblast-specific transcription factor SP7/*OSX*, which belongs to the specificity protein (Sp) subgroup of the Kruppel-like family of transcription factors. In mice, *Osx* is essential in regulating the differentiation of preosteoblasts to osteoblasts and operates downstream from *RunX2* in the mouse. Mutations in *RUNX2* in humans result in cleidocranial dysplasia,

where there is also a propensity to fracture in some patients (16).

- *BMP1* and carboxy-terminal procollagen cleavage

Homozygous mutations in bone morphogenetic protein (gene *BMP1*), responsible for cleavage of the carboxy-terminal peptide of type I collagens, have also been reported to result in a severe progressively deforming OI phenotype. This patient had increased bone density but none of the features of an osteopetrotic bone disorder. The mutation interferes with processing in type I collagen fibrillogenesis (87).

#### 156.4.4.4 Differential Diagnosis.

- (1) *OI type II*: There is an overlap in phenotypic severity with infants with OI type II, but infants with OI type III usually have better ossification of the skull and the hips are not usually held in fixed abduction and external rotation.
- (2) *Severe OI type I and type IV*: As infants with OI type III are severely affected, phenotypic features alone at birth do not allow distinction from other severely affected infants with OI type I or OI type IV. Patients with OI type I and IV with OD are more likely to have fractures at birth (25%) and short stature with deformity of long bones and spine. Family history and natural history of skeletal radiographic abnormalities are the features by which these patients may be distinguished from other cases with a progressively deforming phenotype with blue (type I) or normal sclerae (type IV). However, novel genetic mechanisms such as double heterozygosity or isodisomy should be considered when considering recurrence risk.
- (3) *Cole-Carpenter syndrome*: This syndrome is characterized by severe bone fragility and metaphyseal and rib fractures similar to those seen in non-accidental injury. In addition, there is proptosis, craniosynostosis, and prominent vascular markings over the anterior cranium (see below).
- (4) There are many other rare syndromes with osteoporosis that must be distinguished from OI type III (see Table 156-4).

#### 156.4.4.5 Management.

**156.4.4.5.1 Therapy.** Intensive rehabilitation and orthopedic care should be instituted to prevent progressive deformity and provide for as normal a development as possible. This is best delivered in a center of expertise by a multidisciplinary team or in consultation with a regional team. Intramedullary rodding may improve mobility and prevent fractures, and is also used to correct and prevent progressive deformity. Various orthoses can be provided or designed that assist in the rehabilitation of these children; these orthoses improve mobility and normalize activities of daily living. Cyclic intravenous pamidronate has been used in children with OI type III resulting in decreased fracture frequency, increased mobility, and improved quality of life and should be



offered from infancy. In centers of expertise, consideration should be given to commencing therapy in the first year of life. Where dentinogenesis imperfecta is present, 6-monthly dental reviews should be undertaken (88).

### 156.4.5 Perinatally Lethal Osteogenesis Imperfecta Syndromes—OI Type II

OI type II is characterized by extreme bone fragility, leading to intrauterine or early infant death. The group is clinically and biochemically heterogeneous (89–91). Three subgroups useful for prognostic counseling are distinguished on a radiographic basis: (1) subgroup with continuously beaded ribs and crumpled long bones (see Figure 156-6); (2) similar long bones but relatively normal ribs with few if any rib fractures; and (3) long, narrow dysplastic but beaded ribs with inadequately modeled long bones and multiple fractures. Collagen and DNA studies show that within each subgroup there is further biochemical and molecular heterogeneity.

Babies in each of the three subgroups have been demonstrated to have abnormal brain neuropathology consisting of perivenous microcalcifications and impaired neuroblast migration and periventricular leukomalacia (92). Of seven babies with OI type II, only one fell into

subgroup C, but that baby had hippocampal malrotation as well as the abnormal features seen in babies with the other types of OI type II.

#### 156.4.5.1 Clinical Features.

- Subgroup A

Affected infants are commonly premature, and except where hydropic, with mean birth weight and birth length less than the fiftieth percentile for gestation (89). One-fifth are stillborn, and the remainder die within hours or days of birth (90% deceased by 4 weeks). General connective tissue fragility is present, and dismemberment may occur during delivery.

The thighs are characteristically held in fixed abduction and external rotation (see Figure 156-6), the chest is small, and limbs are markedly short and frequently angulated and bowed. The cranium often appears disproportionately large for the face, and there is commonly mild micrognathia with a small narrow nose. Dark blue sclerae are present in virtually all those affected.

Radiographic study shows a small thorax with slightly shortened ribs, which are thickened with continuous beading or wavy contours. The femora appear broad and rectangular with fine wavy margins (like a concertina) or are broad, undermodeled, and very hypoplastic. Long and short tubular bones are demineralized, shortened, and sometimes crumpled with multiple fractures. The tibiae are usually angulated. Vertebrae appear flattened and hypoplastic to a variable degree. The skull and face show diminished mineralization and multiple ossification centers (Wormian bones) throughout the calvaria. The pelvis is hypoplastic, with flattening of acetabular roofs and iliac crests.

- Subgroup B

Subgroup B patients appear phenotypically similar to subgroup A at birth, but death from respiratory failure is not as likely. Survival for weeks to months and even years is observed (89). Radiographically, the ribs are shortened with few or no fractures. The long bones are broad and crumpled (accordion-like) as in subgroup A. In those infants who survive the newborn period, a typical broad bone appearance develops in the first few weeks of life.

- Subgroup C

Subgroup C syndrome is extremely rare. All babies have been very small for gestational age and were stillborn or died in the newborn period (81). Skeletal radiographs show slender, not so uniformly beaded ribs, slender long bones with fractures, twisting of the shafts of the femora, and angulation deformities. There is extreme demineralization of the skull and face.

**156.4.5.2 Genetics.** It is now clear from collagen biochemical and DNA studies that sibling recurrence in the majority of families of western and Central European



**FIGURE 156-6** Osteogenesis imperfecta type II. Stillborn infant showing short, deformed limbs with broad thighs fixed at right angles to trunk, and relatively large head.

background can be explained by parental germline mosaicism. At least 18 such pedigrees have been identified (3). Furthermore, clinical and molecular investigations suggest that in most of such families, one or other parent can be shown to have mild features of OI due to high-level somatic mosaicism (93).

True autosomal recessive inheritance is common in a number of populations, but the phenotype will often be similar to the second prognostic group (B) with less severe involvement of ribs yet nevertheless a high perinatal mortality (8,84).

Where the proband has findings of subgroup B, the empiric recurrence risk in a United Kingdom population was 7.7% (81), presumably reflecting the mixture of historic European and immigrant populations from areas with a high risk of consanguinity. The finding of sibling recurrence in association with consanguinity confirms that a proportion of cases arise from autosomal recessive inheritance.

Subgroup C may represent an autosomal recessive disorder based on the observation of sibling recurrence and parental consanguinity (89), although few cases have been reported and a genotype correlation has not yet been established.

**156.4.5.3 Molecular Pathology.** OI type II, like OI type III, is genetically heterogenous. Generally those babies with a phenotype that conforms to the subgroup A with continuous fractures of the ribs, studied at the biochemical or DNA level, have been shown to have mutations in type I collagen genes, both *COL1A1* and *COL1A2*. Diminished production of type I collagen results from abnormal intracellular processing, and abnormal type I collagen is found in their bone (3,8,84). The majority of mutations in *COL1* genes in these cases have been heterozygous. The most common mutations are missense and occasional multi-exon deletion mutations in the helical domain of type I collagen genes and rarely mutations in carboxy-terminal propeptides. The substitution rules apply (3,4). These mutations disrupt triple helical assembly of the two type I collagen polypeptides. Point mutations in glycine codons, such as glycine to cysteine, which lead to disulfide bridging, and glycine to arginine substitutions, disrupt helix formation. Delayed triple helical assembly results in overmodification of type I collagen pro $\alpha$ 1(I) and pro $\alpha$ 2(I) polypeptide chains which are prematurely degraded. Severe tissue collagen deficiency leads to structurally weak bone and skin. The skeleton is progressively crushed and broken by uterine contraction during intrauterine life.

In families where the phenotype conforms more to the subgroup B pattern with a paucity of rib fractures, homozygous or doubly heterozygous mutations in genes resulting in defective prolyl 3-hydroxylation have frequently been encountered. Thus, the most commonly encountered mutations are in *LEPRE1*, *CRTAP*, or *PPIB* (84,90). Despite the high perinatal lethality, there are occasional long-term survivors who can be grouped

with those patients with OI type III. Here we observe a continuum of phenotypes.

#### 156.4.5.4 Differential Diagnosis.

- (1) *OI with microcephaly and cataracts*: This syndrome has clinical and radiographic features similar to OI type II, but all affected have microcephaly and cataracts (94). On the basis of sibling recurrence, autosomal recessive inheritance was postulated, but germinal mosaicism in a parent for a contiguous gene deletion might also explain the pedigree.
- (2) *OI type III*: The distinction between these two syndromes is based on the clinical and radiographic findings at the time of birth. In OI type III, the femora do not show a crumpled concertina appearance at birth. Infants with OI type II, from the second severity subgroup, who survive to several months of age will show skeletal radiographic findings indistinguishable from OI type III at that time.
- (3) *Neonatal hyperparathyroidism*: Both primary and secondary neonatal hyperparathyroidism may result in rib and long-bone fractures. Periosteal cloaking (subperiosteal calcification) should be distinguished from the crumpled accordion-like appearance of long bones in OI type II (95).
- (4) *Infantile cortical hyperostosis (perinatal form)*: The clinical and radiographic findings in the infantile cortical hyperostosis (Caffey disease) should be distinguished from OI type II, although thickening of ribs and beading and thickening of the long bones is frequently confused (see below). One form of infantile cortical hyperostosis results from a specific mutation, R836C, in *COL1A1* (96).

#### 156.4.5.5 Management.

**156.4.5.5.1 Therapy.** Therapeutic intervention will not usually lead to survival. An occasional infant with milder subgroup B disease will survive and require intensive management (see OI type III, section 156.4.4.5). In general, infants with perinatally lethal crumpled bone OI should not be treated with cyclic intravenous pamidronate because survival is very unlikely despite therapy and there is a high likelihood of non-skeletal complications such as neurologic involvement.

**156.4.5.5.2 Genetic Counseling and Prenatal Diagnosis.** When possible, recurrence risks following a single case of OI type II in a family are approximately 6–7% (81). However, certain populations have an increased prevalence of autosomal recessive forms of perinatally lethal OI, and these include west African and south Asian populations where there is a higher rate than usual of parental consanguinity. Byers et al. (1988) have studied 65 complete families of OI type II and found an empiric recurrence risk of approximately 6% (97). This may reflect an admixture of these rare recessive forms as well as families where there is germline mosaicism for heterozygous mutations. Serial ultrasound studies from the fourth to fifth gestational month are indicated in all

future pregnancies, with a high likelihood of diagnosis of OI type II based on limb shortening, fractures, or polyhydramnios (98).

In families with one or more previously affected offspring, direct DNA analysis on chorionic villus biopsy tissue may be performed in subsequent pregnancies when a DNA mutation has been detected in a previously affected infant. Commercial testing for type I collagen structural mutations as well as for mutations in the collagen-processing and chaperone genes can be performed.

### 156.4.6 Cole–Carpenter Syndrome

This is a rare syndrome characterized by severe bone fragility and metaphyseal and rib fracture often presenting in the first 9 months of life. The radiographic features in the skeleton may be similar to those reported in non-accidental injury. In addition, there is proptosis, craniosynostosis, and prominent vascular markings over the anterior cranium which become more evident in the second 6 months of life. Deformity of the long bones and spine is progressive. The subsequent course is very similar to that seen with progressively deforming OI with marked growth failure. Computerized tomographic 3D reconstruction of the skull confirms craniosynostosis with a number of unusual patterns. One such pattern shows sagittal fusion but parasagittal extension of the anterior fontanelle posteriorly. The mode of inheritance is unknown but presumably results from fresh dominant mutation (99). Type I collagen biochemical screening for mutations has proven negative.

**156.4.6.1 Management.** Cole–Carpenter syndrome should be managed as for OI type III. Neurosurgical intervention may be required for hydrocephalus. Osteoporosis in Cole–Carpenter syndrome appears to be responsive to treatment with cyclic intravenous pamidronate.

### 156.4.7 Osteogenesis Imperfecta with Congenital Joint Contractures (Bruck Syndrome)

Bruck syndrome (BS) was delineated in 1989 by Viljoen et al. (100). It is a recessively inherited disorder characterized by bone fragility and joint contractures, which may be present at birth or develop during childhood and are progressive in nature. In some cases, the major joints of the upper and lower limbs show webbing (pterygia). Wormian bones are present, but the sclerae are white and the teeth are normal.

**156.4.7.1 Molecular Pathology.** Genetic studies indicate that at least two types of Bruck syndrome can be distinguished. One type maps to chromosome 17q and results from mutations in *FKBP10*. In some instances of progressively deforming OI resulting from mutation in *FKBP10*, talipes and congenital joint contractures are

present, but in other patients mutations result in brittle bones alone (86). The second type of Bruck syndrome is not linked to 17q but to mutations in the specific lysyl hydroxylase 2 gene (*PLOD2*) which is located on chromosome 3q23–q24 (11,101). To date, all cases have had congenital joint contractures. Thus there are at least two genes where mutations result in OI syndromes with congenital joint contractures: BS type I resulting from mutations in *FKBP10* and BS type 2 resulting from mutations in *PLOD2*.

**156.4.7.2 Management.** The Bruck syndromes should be managed as for severe OI. Management of the osteoporosis and bone fragility may be necessary before serial splinting can be safely undertaken. The osteoporosis of BS is responsive to treatment with cyclic intravenous pamidronate, although experience is limited.

## 156.5 OTHER MANIFESTATIONS OF OSTEOGENESIS IMPERFECTA

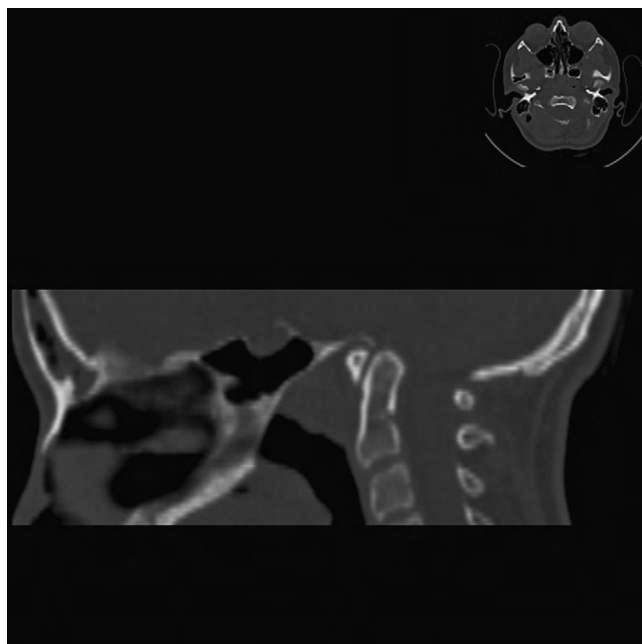
### 156.5.1 Hearing Impairment and Vestibular Function in OI

Hearing impairment is a frequent finding in adults with OI types I, III, and IV and is progressive with age (63,64,102). In a large population study in Finland, almost 58% of patients had audiometrically demonstrable hearing loss. Hearing loss commenced in the second to fourth decades and was initially conductive but progressive such that mixed hearing loss was the most frequent finding in older patients. In most population studies, hearing loss occurs more frequently in OI type I.

Vestibular dysfunction resulting in episodic vertigo was reported in 52% of a Finnish cohort of adult patients (>19 years). Patients with hearing loss had more vertigo (62.5%) than adults without hearing loss.

**156.5.1.1 Management.** Almost 13% of subjects in the Finnish study had hearing loss without recognizing it. Regular audiometry should be performed commencing from adolescence onwards, to diagnose onset of hearing loss and monitor its progress. Rarely, hearing loss due to a combination of factors occurs prior to 12 years so that if hearing loss is suspected in younger patients, audiometry should be performed. Hearing aids should be supplied with satisfactory restoration of hearing. For the majority of patients with bilateral hearing loss, fitting both ears gives a better functional result than fitting one ear. The mixed loss in OI is progressive with age so that in time the benefit may diminish (64). Stapedectomy may restore hearing in young patients with conductive hearing loss. The best outcomes are achieved in centers with special expertise in stapedectomy in subjects with OI. The pathology of hearing loss poses certain surgical difficulties. In OI type I, there may be a thick vascular mucosa with excessive bleeding tendency (103).





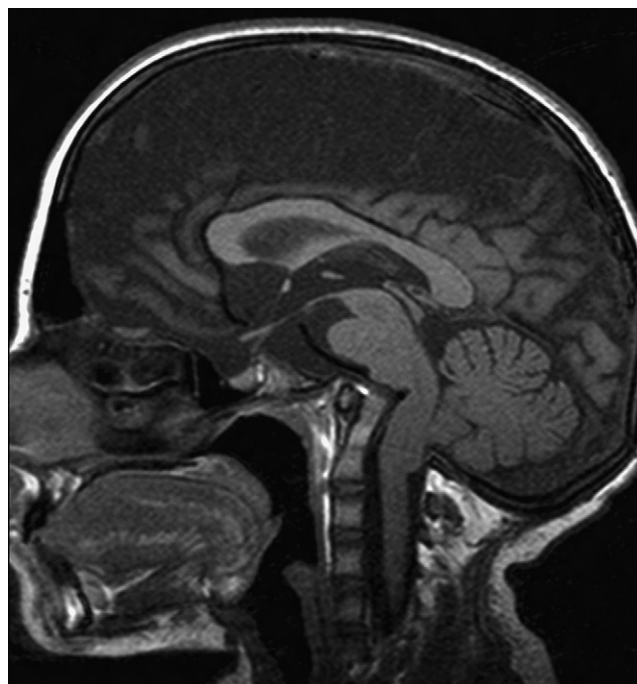
**FIGURE 156-7** Severe basilar impression. CT sagittal midline reconstruction of skull base showing odontoid 18mm above Chamberlain's line (tip of hard palate to midpoint and posterior lip of foramen magnum).

### 156.5.2 Craniocervical Anomalies and Basilar Impression

Prior to the era of bisphosphonate therapy, craniocervical anomalies and basilar impression of the skull base occurred in 25% of subjects with OI (71). Basilar impression results from the combination of ligamentous laxity and soft base of skull. It remains to be determined if bisphosphonate therapy decreases the frequency of this complication. Basilar impression is mainly confined to adults with moderate to severe OI type III or IV and is more common in those with dentinogenesis imperfecta. The process of basilar impression, defined as protrusion of the tip of the odontoid process 5 mm above Chamberlain's line or 7 mm above McGregor's line, is associated with characteristic symptoms and signs. The headache is characteristically induced by coughing or sneezing, and radiates to the occiput. Trigeminal neuralgia, vertigo, and weakness in arms and legs may occur. Localizing features on neurologic examination include upbeat or downbeat nystagmus, facial spasm or paresis, and pyramidal signs (104). These signs should be sought in all individuals with OI. Investigation includes lateral base of skull radiographs and serial CT with midline reconstruction.

Subjects with basilar impression on CT (see Figure 156-7), or symptoms and signs, should go on to MRI of the craniocervical junction (see Figure 156-8). Hydromyelia of the upper cervical cord has been reported.

**156.5.2.1 Management.** Occipitocervical fusion does not prevent the progression of basilar impression. The definitive operation is trans-oral or transnasal



**FIGURE 156-8** Severe basilar impression. Midline sagittal MRI T1-weighted image showing upward and posterior displacement of the clivus with angulation and compression at the pontomedullary junction.

anterior clivectomy and decompression of the craniocervical junction (105,106). Reclined seating of babies and young infants with moderate to severe forms of OI and early treatment with cyclic intravenous pamidronate may prevent the development of basilar impression (71).

## 156.6 SYNDROMES WITH OSTEOPOROSIS AND EYE DISEASE

### 156.6.1 Osteoporosis–Pseudoglioma Syndrome

Osteoporosis-pseudoglioma (OPG) syndrome is rare and characterized by generalized osteoporosis leading to fractures and deformity of long bones and spine, and progressive abnormalities of the anterior and middle segments of the eye (107). (Synonym: ocular form of OI.) Histology of bone biopsy specimens demonstrates decreased core width and trabecular bone volume.

The eye findings include microphthalmia, microcornea, corneal opacities, shallow anterior chamber, iris atrophy, and lens opacity. The changes in the eye result from persistence of the hyaloid remnant and are initially characterized as pseudotumors then progress to phthisis bulbar in infancy or childhood (108). Sadly, some patients have had enucleation of the eye as a result of misdiagnosis. Mental retardation, hypotonia, and joint hypermobility and ventricular septal defect are variable manifestations.



When strictly defined, this autosomal recessive syndrome is due to mutations in LDL-receptor-related protein 5 (LRP5) (109). Obligate carriers have a reduced bone mineral density and may have an increased incidence of osteoporotic fractures (109). LRP5 is intrinsic to the WNT signaling pathway and bone formation. The full extent of the clinical and molecular heterogeneity in OPG syndrome is still to be elucidated.

### 156.6.2 Geroderma Osteodysplasticum

This disorder is a mixed connective tissue disorder with features of both OI and Ehlers–Danlos syndrome. Affected children have osteopenia with platyspondylia and multiple Wormian bones in the skull. Fractures occur uncommonly. Eich et al. reported that radiographs of the knees show a metaphyseal peg indenting the distal femoral epiphyses (110). These patients have both hyperelasticity of their skin and hypermobility of joints. The skin is thin and transparent, and on the hands finely wrinkled similar to the appearance in Ehlers–Danlos syndrome type IV and dominantly inherited cutis laxa. Geroderma osteodysplasticum is an autosomal recessive disorder and results from mutations in the *GORAB* gene on chromosome 1q24 (111). A similar but distinct disorder with cutis laxa and progeroid features, including multiple Wormian bones in the skull, results from mutations in *PYCR1* (112).

## 156.7 HYPOPHOSPHATASIA

Hypophosphatasia includes several conditions with overlapping phenotype characterized by bowing deformities of the skeleton of varying severity and a reduced serum alkaline phosphatase due to deficiency of the tissue non-specific alkaline phosphatase (TNSALP). There is also elevation of serum and urinary phosphoethanolamine (113).

Seven clinical phenotypes can be distinguished: perinatal lethal, perinatal benign, infantile, childhood, adult, odontohypophosphatasia, and pseudohypophosphatasia (114). Clinical and radiographic findings in each group show a significant correlation with TNSALP residual activity, site-directed mutagenesis, and computer-assisted modeling studies of TNSALP mutations (115).

### 156.7.1 Perinatal Lethal Hypophosphatasia

Neonates with perinatal lethal hypophosphatasia show disproportionately short limbs with bowing or angulation deformity. The skull vault is thin and membranous. Radiographic studies show extremely poor ossification throughout the skeleton, with thin ribs, hypoplastic vertebrae, demineralized facial bones, and markedly reduced ossification of the skull vault. The metaphyses

of the long bones are frayed and splayed, sometimes with a moth-eaten appearance.

Occasional absence of ossification of one or more vertebral centers or ribs is a characteristic feature. There are usually angulation deformities of the leg and forearm bones. Occasionally ossified “spurs” protrude laterally from ulnae and fibulae.

In the most severe cases death occurs prenatally or in the newborn period, due to respiratory distress associated with seizures and encephalopathy. The serum alkaline phosphatase is low, and the TNSALP measured in cultured fibroblasts is extremely low or undetectable.

### 156.7.2 Perinatal Benign Hypophosphatasia

There are patients who are ascertained perinatally by ultrasound examination who, following birth, appear to have gradual resolution of their skeletal manifestations. Follow-up of these patients suggests that they follow either an infantile or later onset course.

### 156.7.3 Infantile Hypophosphatasia

This form presents before 6 months of age with failure to thrive and signs of rickets, or with pyridoxine-responsive seizures in the first few weeks of life (116). While the cranial sutures are wide, there may be functional craniosynostosis, which progresses if an infant with this form survives infancy. Long-bone deformity and fractures frequently occur. Hypercalcemia and hypercalciuria cause recurrent vomiting, nephrocalcinosis, and renal failure. Spontaneous remission of the bone disease may occur.

Radiologically, there is severe generalized osteopenia with bowing of long bones, lucent defects particularly around growth plates, and fraying of metaphyses. There is frequently a transition from calcified diaphysis to uncalcified metaphyses. Sequential studies show progressive changes of rickets and demineralization.

### 156.7.4 Childhood Hypophosphatasia

Diagnosis is usually made in the second year of life because of an abnormal gait or fractures. There is premature loss of deciduous teeth. Rachitic deformities with enlargement of wrists, knees, and ankles contribute to an abnormal “painful” gait. Radiographically there is metaphyseal fraying with focal bone defects. Functional craniosynostosis occurs and the skull may develop a copper-beaten appearance.

### 156.7.5 Odontohypophosphatasia

In these subjects there is premature loss of deciduous and permanent teeth and biochemical evidence of a raised

level of phosphoethanolamine. Serum alkaline phosphatase (total) may be normal, as a low TNSALP may be masked by other alkaline phosphatase (ALP) isoenzymes, e.g. intestinal ALP.

### 156.7.6 Adult Hypophosphatasia

This usually presents in adult life. There is a frequently a history of premature loss of deciduous teeth and/or rickets. Laboratory findings include raised phosphoethanolamine, and densitometric (DEXA) and radiographic studies confirming osteopenia. Pseudofractures in the lateral cortices of the proximal femora are characteristic of hypophosphatasia.

### 156.7.7 Management

**156.7.7.1 Investigations.** Diagnosis is usually suspected as a result of the clinical presentation and radiographic studies demonstrating metaphyseal fraying, osteopenia, and lucent lesions in the skeleton. Lethal cases may have a moth-eaten appearance. In nonlethal cases, measurements of serum calcium, phosphate, alkaline phosphatase, and urinary phosphoethanolamine may strongly support the diagnosis. An elevated quantitative urinary phosphoethanolamine may be diagnostic. Where biochemical and molecular diagnosis is feasible, a skin biopsy for cultured skin fibroblasts should be collected from affected children. TNSALP activity can be measured in skin fibroblasts and there is then a ready source of DNA for molecular studies. Diagnosis may also be made by direct analysis of the *TNSALP* gene from peripheral blood.

**156.7.7.2 Therapy.** There has been, until recently, no definitive treatment for severe hypophosphatasia (113). However, enzyme replacement therapy with ENB-0040, a bone-targeted recombinant human TNSALP, has been shown to reverse the bone pathology in some severely affected infants (117). Calcitonin, glucocorticoid, calcium restriction, and chlorothiazide may also correct the hypercalcemia and improve bone mineralization in children with perinatal or infantile disease (118). Load-bearing intramedullary rods have proven beneficial in the management of fractures and pseudofractures in adults with hypophosphatasia (114).

**156.7.7.3 Genetics.** In the vast majority of perinatally lethal, infantile, and childhood cases, the disorder is inherited as an autosomal recessive trait. In adult cases, both autosomal dominant and autosomal recessive modes of inheritance have been observed. Apparent autosomal dominant inheritance may result from pseudo-dominance arising from compound heterozygosity for mild mutations (119).

**156.7.7.4 Molecular Genetics.** The alkaline phosphatase in chondro-osseous tissue is important in the hydrolysis of phosphate esters to release phosphate ions for normal calcification. In congenital hypophosphatasia, a

deficiency in the production or stability of bone alkaline phosphatase enzyme leads to a deficiency in the availability of free phosphate necessary for calcification. The tissue alkaline phosphatase gene (*TNSALP*) is located on chromosome 1 close to the Rh locus at 1p36.1–34.

The majority of mutations in patients with perinatal and infantile forms result in significant reduction in levels of TNSALP. These include the majority of nonsense, frameshift, and splicing mutations, and mutations which affect the active site of the TNSALP enzyme predicting a severe phenotype (115,120).

**156.7.7.5 Genetic Counseling and Prenatal Diagnosis.** There is a 25% recurrence risk for hypophosphatasia in sibs. Prenatal diagnosis can be achieved by measurement of the TNSALP in cultured chorionic villus cells. When specific mutations can be found, direct DNA analysis is possible (119).

**156.7.7.6 Differential Diagnosis.** Pseudohypophosphatasia is the term used by Scriver and Cameron (1969) to describe patients with clinical and radiographic findings of hypophosphatasia in the presence of a normal serum alkaline phosphatase, but elevated urinary phosphoethanolamine (121).

## 156.8 OTHER BONE FRAGILITY DISORDERS

### 156.8.1 Chronic Recurrent Multifocal Osteomyelitis and SAPHO Syndrome

Chronic recurrent multifocal osteomyelitis (CRMO) is a disorder characterized by recurrent episodes of painful osteolytic lesions in children, with radiographic appearances suggesting sub-acute or chronic osteomyelitis (122). It is frequently associated with recurrent episodes of pustular lesions on hands and feet. The disorder is multifocal and most often seen in the spine and pelvic bones, and the tubular bones particularly around the knees and clavicles. In the spine, there may be destructive changes with complete disruption of vertebrae, reminiscent of eosinophilic granuloma. Mandibular involvement has also been reported. Spontaneous remission of osteolytic lesions may be observed. These patients often have generalized osteopenia and may present with vertebral crush fractures in adjacent vertebrae (123,124).

The SAPHO syndrome (synovitis, acne, pustulosis, hyperostosis, and osteitis) may be a related disorder in adults. In SAPHO syndrome, there is peripheral arthritis, hyperostosis of the anterior chest wall involving sternocostal or sternoclavicular joints, and destructive lesions of the thoracolumbar vertebrae.

**156.8.1.1 Molecular Pathology.** Majeed syndrome comprises chronic recurrent multifocal osteomyelitis and congenital dyserythropoietic anemia with palmar-plantar pustulosis. It is due to mutations in Lipin 2 (*LIPIN2*), localized to chromosome to 18p. The chronic

multifocal osteomyelitis (CMO) mouse, which has autosomal recessive inheritance of a disorder similar to that in man, results from homozygous mutations in the proline-serine-threonine phosphatase-interacting protein 2 gene (*Pstpip2*) (125).

**156.8.1.2 Therapy.** Non-steroidal anti-inflammatory medications alone, or in combination with oral glucocorticoids, are generally effective in this condition (122). However, treatment with cyclic intravenous pamidronate not only corrects the osteopenia in CRMO but may result in healing of the focal destructive lesions in vertebrae, and given the significant correction of bone pathology, may be the treatment of choice at the present time (123). Cyclic intravenous pamidronate has also been used with effect in SAPHO syndrome.

### 156.8.2 Singleton–Merten Syndrome

This syndrome of generalized osteoporosis is characterized by unusual changes in hand bones with expansion of the marrow space. It is further characterized by progressive calcification of the thoracic aorta and calcific aortic stenosis and dental dysplasia. The disorder may result from autosomal dominant inheritance (126). The disorder bears some similarities to idiopathic infantile arterial calcification (IIAC), which has recently been shown to result from homozygous mutations in ecto-nucleotide pyrophosphatase/phosphodiesterase 1E (NPPI) activity (127). Patients with IIAC have spontaneous periarticular as well as aortic calcifications in early life.

### 156.8.3 Neonatal Hyperparathyroidism

Infants with neonatal hyperparathyroidism generally present with a narrow chest, simulating thoracic dysplasia (128). Leg bowing is often present. Chest radiographs show rib fractures and severe osteopenia, but long bones show periosteal cloaking with periosteal calcification and subperiosteal lucency. The mandible is osteopenic and the metaphyses irregular. The radiographic findings must be distinguished from OI type II where the femora are crumpled (accordion-like), and perinatally lethal infantile cortical hyperostosis (Caffey disease) where the mandible and clavicles are hyperostotic. Neonatal hyperparathyroidism is usually a self-limited disorder with spontaneous regression over the first four months of life.

Mucopolipidosis II (MLII) is a group of disorders resulting in defective lysosomal phosphotransferase, which may disturb signal transduction through the calcium-sensing receptor. The perinatal hyperparathyroidism commonly seen in MLII is similarly self-limited and remits in the first 4–6 months, although dysostosis multiplex is progressive. After the first year, patients with MLII manifest radiographic and histopathologic features

characteristic of hyperparathyroidism but with normal serum parathyroid hormone, which has been designated pseudohyperparathyroidism (129).

**156.8.3.1 Management.** Neonatal hyperparathyroidism is usually a self-limiting disorder. Severely affected infants may die as a result of thoracic insufficiency. Hypercalcemia should be managed conservatively.

### 156.8.4 Infantile Cortical Hyperostosis (Caffey Disease)

Infantile cortical hyperostosis (ICH) presents in fetal or early infantile life with clinical evidence of a systemic bone disorder (96). Prenatal onset may result in limb bowing and irregularity of ribs, simulating multiple rib fractures (130). Radiographs show massive subperiosteal new bone in ribs, long bones, and mandible. The affected areas are typically inflamed with fever and hot tender swelling.

**156.8.4.1 Genetics.** Autosomal dominant and autosomal recessive inheritance and a sporadic form have been postulated based on clinical and pedigree information. In the dominantly inherited form, the disorder is self-limiting. A postulated autosomal recessive form is usually perinatally lethal. The sporadic “epidemic” form is now rare.

**156.8.4.2 Molecular Pathogenesis.** In three unrelated families with autosomal dominant Caffey disease (ADCD) a heterozygous 3040C>T transition was detected in exon 41 of *COL1A1*, resulting in a R836C mutation in the triple helical domain of pro $\alpha$ (1) chains of type I collagen. Penetrance was not complete, such that 20% of heterozygous carriers of the disease-causing mutation had not manifested Caffey disease. The pathogenesis of ADCD is not completely explained by the *COL1* mutation.

**156.8.4.3 Management.** Corticosteroids and indomethacin have been employed with resolution of symptoms in the sporadic and autosomal dominant forms of ICH. In the forms with fetal onset, there is a high frequency of fetal hydrops and survival is rare although not unknown with supportive therapy (130).

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### Biographies



**Prof. David Sillence**, MBBS, MD (Melb), FRACP, FRCPA, FAFPHM, FAFRM, FFSc(RCPA) is a professor of Medical Genetics in the Sydney Medical School and a consultant physician to the Connective Tissue Dysplasia Clinic and Centre for Children's Bone Health in the Sydney Children's Hospital Network (Westmead) since 1980. He trained both at the Murdoch Institute with David Danks and at the Cedars–UCLA International Skeletal Dysplasia Registry with David Rimoin. He has worked in the field of genetic disorders of bone and mineral metabolism for four decades. Since 1980, with colleagues from Westmead, Professor Sillence has developed a comprehensive multidisciplinary program for the skeletal investigation and medical treatment of children with connective tissue dysplasias encompassing genetic skeletal dysplasias, genetic bone and mineral disorders, Ehlers–Danlos syndromes, and related disorders.

Prof. Sillence and his collaborators pioneered the evaluation of bone density by dual-energy X-ray absorptiometry in children, and treatment of osteoporosis with various regimens of the class of drugs known as bisphosphonates, in Australia. He has been a member of the International Nomenclature Committee for Constitutional Disorders of the Skeleton since 1986. With Dr Jenny Ault he commenced the Osteogenesis Imperfecta Clinic in 1987 as part of the larger Connective Tissue Dysplasia Service in the Sydney Children's Hospital Network (Westmead Campus).

**Assoc. Prof. Craig Munns**, MBBS, PhD, FRACP is a Senior Staff Specialist in Bone and Mineral Medicine at the Children's Hospital at Westmead. He completed his pediatric and endocrinology training at The Royal Children's Hospital, Brisbane, Australia, and was then appointed as Clinical Associate in Genetic and Metabolic Bone Disorders at the Shriners Hospital for Children, Montreal, Canada, under Dr Francis Glorieux and Dr Frank Rauch.

Assoc. Prof. Munns obtained his PhD through the University of Queensland. He has expert knowledge in the diagnosis and management of pediatric bone disorders. At The Children's Hospital at Westmead, Associate Professor Munns has established collaborative links with many groups throughout the hospital and is uniquely placed to investigate and manage the care of children with bone disorders. He has also established a strong relationship with basic bone scientists at the hospital. He has established collaborative links throughout the hospital and introduced pQCT, bone biopsy for histomorphometry, functional muscle assessment, additional bone turnover markers, and vibration platforms into the hospital.

Assoc. Prof. Munns' major research interests are in novel therapeutic approaches to children with primary and secondary bone disorders and he has published in this area.



# CHAPTER

# 157

## Disorders of Bone Density, Volume, and Mineralization

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### 157.1 INTRODUCTION

More than 30 disorders are known with generalized or localized increase in the density or size of the skeleton or individual skeletal elements.

### 157.2 OSTEOPETROSIS GROUP OF DISORDERS

Osteopetrosis has traditionally been subdivided on the basis of the age at presentation. However, the situation is considerably more complex, and an increasing number of genetically distinct disorders have been described with an overlapping spectrum of clinical and radiographic features. The systematic study of spontaneously occurring and transgenic mouse mutants has predicted more than 18 genetic defects that might result in osteopetrosis (1). These studies provide a broad grouping of osteoclast defects into:

- (1) Defects of early osteoclast differentiation
- (2) Defects in receptor activator of nuclear factor  $\kappa$ B (NF- $\kappa$ B) and related proteins
- (3) Defects in osteoclast function
- (4) Defects in acidification by the osteoclast.

#### 157.2.1 Defects in Early Osteoclast Differentiation

In the mouse, osteoclast differentiation depends on M-CSF (mouse-colony stimulating factor) and its receptor, the transcription factor PU.1, and the microphthalmia basic helix-loop-helix zipper transcription factor (Mitf). Mutations in each of these genes results in a type of murine osteopetrosis.

#### 157.2.2 Defects in Receptor Activation of Nuclear Factor $\kappa$ B and Related Proteins

Osteoclast differentiation is also dependent on the interaction of osteoprotegerin with RANKL (receptor activator of nuclear factor  $\kappa$ B ligand). The interaction between RANKL and RANK is a major determinant of bone mass. RANK signaling is influenced directly by c-fos, NF- $\kappa$ B, and TRAF-6 (tumor necrosis factor receptor-associated factor 6). Murine osteopetrosis has been observed in murine knockout of RANK, RANKL, c-fos, NF- $\kappa$ B subunits, and TRAF-6.

#### 157.2.3 Defects in Osteoclast Function

A large number of proteins are important for osteoclast function, including tyrosine kinase c-src, osteopetrosis-associated transmembrane protein (OSTM1; the protein product of the *Gl* gene), the enzyme cathepsin K, and tartrate-resistant acid phosphatase (TRAP), which has a role in intracellular transport. Mutations in their genes also result in murine osteopetrosis. Anchoring of osteoclasts in bone is important, as demonstrated by the observation of osteopetrosis in mice with mutations in  $\alpha$ V $\beta$ 3 integrin.

#### 157.2.4 Defects in Acidification

This group of disorders has been the most productive for the delineation of mouse–human homology. Mice mutant for defects in the ATP-dependent osteoclast-specific vacuolar proton pump (ATP6i or TCIRG1) and mice with mutations in the CLCN7 chloride channel gene develop a form of osteopetrosis. Human carbonic anhydrase II deficiency results in renal tubular

acidosis and infantile onset of osteopetrosis, fractures, and developmental delay.

The observation that mammalian osteopetrosis can be cured by parabiosis has led to successful cures with bone marrow transplantation in laboratory animals and in the human disorder (2). Knowledge from animal models should hopefully guide treatment in human disorders in the future. The full spectrum of osteopetrosis in humans will have to await the molecular characterization and genotype/phenotype correlation in these rare disorders, but at present the clinical grouping includes:

- (1) Osteopetrosis with precocious manifestations (several forms, including autosomal recessive osteopetrosis (ARO), severe neonatal, and malignant forms)
- (2) Osteopetrosis with usually delayed onset (several forms, including Albers–Schonberg; autosomal dominant osteopetrosis type II (ADOII or osteopetrosis late onset); and osteopetrosis, intermediate type (IAO))
- (3) Osteopetrosis with autosomal recessive inheritance (mild type)
- (4) Osteopetrosis with renal tubular acidosis.

### 157.2.5 Osteopetrosis with Precocious Manifestations

The precocious form of the disease is most frequently discovered during the first few months of life but may present as failure to thrive, malignant hypocalcemia, or anemia with thrombocytopenia (leukoerythroblastic anemia), or even because of severe, perhaps overwhelming infection. Rarely, fractures lead to medical attention (Synonyms: osteopetrosis congenita).

**157.2.5.1 Clinical Findings.** Generalized hyperostosis may be recognized at birth, but usually develops rapidly after birth and leads to crowding of the marrow cavity resulting in anemia and extramedullary hemopoiesis, hepatosplenomegaly, and thrombocytopenia, leading to purpura and ecchymosis. Anemia results from excessive hemolysis. A defect in macrophage killing of bacteria may account for a tendency to severe and overwhelming infection. Progressive encroachment on the optic foramina may lead to optic atrophy and blindness. In some cases, evidence of optic nerve encroachment is present at birth. Hypocalcemia is not an uncommon finding, and serum phosphorus may also be low. Elevated serum alkaline phosphatase is a constant finding. Radiologically, the diagnostic findings are a generalized increase in bone density combined with defective metaphyseal modeling and a “bone-in-bone” appearance, most marked in the vertebral bodies. Diffuse hyperostosis leads to loss of demarcation of the cortex and medullary cavities. Irregular deposition of bone at the metaphyses may produce the appearance of parallel plates of dense bone at the ends of long bones. The skull shows a dense base with normal-to-increased density of the vault and markedly increased density in the orbital margins.

**157.2.5.2 Genetics.** Inheritance is generally autosomal recessive, although some cases with newborn presentation are due to heterozygous mutations resulting in autosomal dominant inheritance or fresh dominant mutation.

**157.2.5.3 Molecular Pathology.** Following the discovery of the murine defect in the osteoclast-specific vacuolar proton pump ATP6i subunit, also known as TCIRG1, on chromosome 11q12, mutations were discovered in 50%–60% of children with severe osteopetrosis (3–5). In Costa Rica, where there is a 10 times expected frequency of osteopetrosis, two missense mutations account for all the known mutations (3). In bone, osteoclasts are present and normal in appearance, and the resorptive defect results from the resulting acidification defect. Aker et al. showed SNX10 to interact with ATP6i (V-ATPase). They found homozygous mutations in the SNX10 gene in eight molecularly negative patients with infantile malignant osteopetrosis (6).

In a further 15% of cases in the same series, mutations were detected in the *CLCN7* gene on chromosome 16p12, which codes for a chloride channel also important in acidification. Although the majority of the patients were homozygous/doubly heterozygous for *CLCN7* mutations, a few patients were heterozygous with only one mutant allele. These presumably represent dominant negative mutations, as the chloride channel protein exists as a multimer and a defect in only 50% of the secreted proteins would disrupt assembly and function of the channel. Homozygous mutations in the human *GL* gene, encoding osteopetrosis-associated transmembrane protein (OSTM1), on chromosome 6q21 (homologous to mouse *gl/gl*) have been reported in 2% of individuals with autosomal recessive osteopetrosis (7,8). OSTM1 protein and *CLCN7* protein colocalize in late endosomes and lysosomes of various tissues forming a molecular complex (8). The *gl/gl* mutant phenotype in the mouse results in a very severe osteopetrosis phenotype with prenatal or perinatal death, so this mutation should be looked for in babies with perinatally lethal osteopetrosis. Mutations such as that in the mouse colony stimulating factor 1 (*MCSF1*) gene, responsible for the *op/op* mouse phenotype (9), or mutations in *PU.1*, have not yet been discovered in human osteopetrosis.

Mutations in the *TNFSF11* gene (*RANKL*) on chromosome 13q14.11 result in a mild form of autosomal recessive osteopetrosis that clinically resembles the autosomal dominant form with a mild T-cell defect (10). Mutations in *TNFRSF11A* (*RANK*) on chromosome 18q21.44 result in osteopetrosis with hypogammaglobulinemia (11).

#### 157.2.5.4 Differential Diagnosis.

(1) **Perinatally lethal osteopetrosis.** Siblings with severe osteopetrosis with in utero fractures were described with hyperdensity and skeletal lesions on ultrasound at 18 weeks' gestation (12). The parents were first cousins. Brain histopathology showed gliosis and axonal swelling with heterotopic islets of immature cells and occasional foci of calcification.

- (2) **Ectodermal dysplasia, anhidrotic with immunodeficiency, osteopetrosis, and lymphedema** (OL-EDA-ID, or X-linked osteopetrosis lymphedema, anhidrotic ectodermal dysplasia, and immunodeficiency). The report of a hemizygous male patient with nonsense-to-sense (TER420TRP) mutation in the NF- $\kappa$ B essential modulator (IKBKG) gene on chromosome Xq28 is the first report of a mutation in this X-linked gene resulting in osteopetrosis. The phenotype is predicted given the importance of NF- $\kappa$ B in RANK signaling and osteoclast proliferation. This is not surprising since osteoclasts and cells of the hematopoietic system have a common origin (13). Heterozygous mutations in *IKBKG* (NEMO) result in incontinentia pigmenti type II in female patients. Mutations in *CalDAG-GEF1* and *Kindlin-3* have been identified in autosomal recessive osteopetrosis variants with leukocyte adhesion deficiency (14,15).
- (3) **Raine dysplasia**. Newborns with Raine dysplasia have diffuse osteosclerosis, wide fontanelles, and a distinctive face.
- (4) **Blomstrand dysplasia**. This perinatally lethal osteosclerosis disorder is characterized by advanced skeletal maturation. It is diagnosable prenatally with features of a short limb skeletal dysplasia and rib and long-bone features, which may be misinterpreted as perinatally lethal osteogenesis imperfecta. It results from autosomal recessive inheritance of inactivating mutations in the parathyroid hormone/parathyroid-hormone-related peptide (PTH/PTHrP) receptor localized to chromosome 3p21–p22 (16).

**157.2.5.5 Management.** *Therapy:* Treatment is aimed at decreasing or arresting progressive hyperostosis and correcting anemia and thrombocytopenia. General supportive measures including prompt and vigorous treatment of infections and minimizing neurologic complications, particularly progressive optic atrophy due to encroachment of the optic foramina. Care should be taken in restricting calcium intake in the subgroup of children who have changes of rickets at the ends of their long bones, so called osteopetro-rickets. Prednisone may ameliorate the progress of the anemia and thrombocytopenia. However, this regimen is rarely helpful in malignant osteopetrosis. Neurosurgical unroofing of the optic foramina has been helpful in some patients, with improved vision postoperatively. In other patients, vision is preserved. Optic atrophy is usually established by the time the disorder is diagnosed. At best, surgical intervention can only ameliorate the visual disability and prevent its progression. Bone marrow transplantation of appropriately human leukocyte antigen (HLA)-matched donor marrow has been curative in some patients (17). The experimental animal data indicates that most types of osteopetrosis, but not all, will be responsive depending on the underlying defect (1). The present data show a 50% survival at a median of 15 months post-transplant.

Families need to be aware that vision is rarely restored following the transplant. Generally, without successful bone marrow engraftment the prognosis for survival is poor, and death from complications such as anemia, bleeding, or overwhelming infection is not uncommon in the first few months or years.

*Genetic counseling and prenatal diagnosis:* For normal parents with one or more infants with congenital osteopetrosis, the risk approximates 25% for a subsequently affected infant. Radiographic prenatal diagnosis has been generally unsuccessful in detecting increased bone density during the second trimester. Direct molecular genetic diagnosis requires the characterization of the molecular defects in the various types of osteopetrosis with precocious manifestations.

### 157.2.6 Osteopetrosis with Later Onset and Juvenile Types

Apart from cases with congenital or infantile presentation of osteopetrosis, there is a group of patients in whom the onset of the disease is recognized later (osteopetrosis tarda or Albers-Schonberg disease). The prevalence was 1 per 18,000 in Denmark (18).

**157.2.6.1 Natural History.** Patients may present in childhood, adolescence, or young adult life because of fracture (greater than 75%), mild craniofacial disproportion, mild anemia, complications arising from neurologic involvement, or osteitis with osteonecrosis, usually of the mandible (19) (Figure 157-1). Encroachment on optic nerves results in visual disability in approximately 5% of patients, but hearing impairment resulting from petrous bone sclerosis occurs in some 25% of individuals with late onset. Increased bone density may be discovered incidentally on routine radiologic study for some non-skeletal problem. Biochemically, there is normal serum calcium, parathyroid hormone (PTH), and calcitonin. Serum acid and alkaline phosphatases may be elevated, the latter with healing fractures.

Skeletal radiographs show generalized increased density of cortical bone with defective metaphyseal modeling of the long bones, resulting in a club-shaped appearance. There is longitudinal and transverse osteodense striation at the ends of the long bones in more than half the patients. The vertebral column shows osteodense vertebral endplates. The base of the skull is usually dense and thickened, but the face and vault are generally less severely involved. There is considerable clinical variability even within the same family. Some affected individuals have been reported that do not exhibit radiographic findings (20). Increased levels in serum of BB-isoenzyme of creatine kinase (CK) and TRAP help in making the diagnosis of autosomal dominant osteopetrosis (21).

**157.2.6.2 Genetics.** In most cases, this milder presentation of osteopetrosis is inherited as an autosomal dominant trait, although families with autosomal recessive inheritance have been reported. Heterogeneity has been



**FIGURE 157-1** Osteopetrosis (later-onset type). Eight-year-old boy with frontal bossing and recurrent fractures.

suggested on phenotypic grounds (22). Several subtypes were proposed but the disorder designated type I is synonymous with endosteal hyperostosis of the Worth type, and due to gain-of-function mutations in *LRP5* (low-density lipoprotein-related protein 5).

**157.2.6.3 Molecular Pathology.** Osteopetrosis of later onset primarily results from heterozygous missense mutations in the *CLCN7* chloride channel gene on chromosome 16p13 (23). Several mutations representing single exon deletions have been reported. Autosomal dominant (AD) osteopetrosis is allelic with severe autosomal recessive (AR) osteopetrosis due to *CLCN7* mutations. Intermediate forms of juvenile osteopetrosis with AR inheritance have been reported also to show homozygosity for missense mutations in the *CLCN7* gene in 40%–60% of intermediate forms of osteopetrosis phenotype (5). Mutations in *CLCN7* have been found in approximately 75% of AD osteopetrosis type II (5).

**157.2.6.4 Management.** Therapy should be directed at recognition and treatment of complications. Bone fragility is a leading complication. Children with osteopetrosis should be educated to avoid body-contact sport and other situations which have a high probability of fracture. These children are likely to have unusual fractures, such as of the femoral neck and hangman fracture of the cervical spine. Transfusion may be required for anemia, and splenectomy may be necessary or useful in some patients with refractive anemia. Splenectomized

patients, however, require antibiotic prophylaxis against encapsulated organisms as they are in double jeopardy first from the disorder and second from splenectomy. Regular, usually annual, testing of visual fields and acuity and baseline radiographs of optic foramina should be carried out during childhood. Regular audiometry should also be performed once the children can be tested by free field audiometry. On the whole, treatment with low-calcium diet, PTH, calcium chelating agents, calcitriol, corticosteroids, and heparin has not shown clear therapeutic advantages.

### 157.2.7 Osteopetrosis with Renal Tubular Acidosis

Osteopetrosis with renal tubular acidosis is an autosomal recessive type of infantile osteopetrosis that presents with fractures and/or short stature, visual impairment, and mental retardation in the first few years of life. (Synonyms: Guibad–Vainsel syndrome, carbonic anhydrase II deficiency, marble brain disease.)

**157.2.7.1 Clinical Findings.** These children present with an intermediate form of osteopetrosis with symptoms of visual disability, mental retardation (some have normal intelligence), and fractures resulting from osteosclerosis. Short stature increases with age. Radiology reveals intracranial ossification, generalized osteosclerosis, and narrowing of the optic foramina. Hematologic investigation demonstrates anemia and evidence of extramedullary hemopoiesis. Serum electrolytes are consistent with a mixed proximal and distal renal tubular acidosis.

**157.2.7.2 Genetics.** This is an autosomal recessive disorder with relatively high gene frequency in Middle Eastern families. More than half the known cases have been in families from Kuwait, Saudi Arabia, and north Africa. Consanguinity was present in 75% of the families reported by Hu et al. in 1992 (24). Prenatal diagnosis may be possible in families with a defined mutation (25).

**157.2.7.3 Biochemistry and Molecular Genetics.** The biochemical defect is in carbonic anhydrase II, an enzyme whose integrity is important for acidification by osteoclasts in the process of mineral resorption and in the renal tubules. Patients generally show a mixed (proximal and distal) renal tubular acidosis. Several different mutations have been described in the carbonic anhydrase II gene, which is localized to chromosome 8q22 (24). The predominant mutation in patients from the Middle East and Africa is a novel splice junction mutation at the 5' end of intron 2 of the carbonic anhydrase II gene, known as the Arabic mutation. In an Italian boy with a splice donor mutation of exon 6, the disorder was unusually mild and the renal tubular acidosis had only a distal component (25).

**157.2.7.4 Therapy.** Management is as for children with moderately severe osteoporosis. Regular monitoring of visual fields, review for cranial nerve encroachment, and developmental/psychometric testing are



important. Vitamin D supplementation is important if rickets develop. Hypokalemia should be corrected with oral potassium. Correction of renal tubular acidosis with oral bicarbonate may aggravate the bone disease.

### 157.3 RAINE DYSPLASIA

Raine syndrome is an autosomal recessive disorder that combines diffuse osteosclerosis with distinctive facial dysmorphism and intracranial calcification (26,27). The dysmorphism includes puffiness of periorbital tissues (Figure 157-2), microcephaly with herniation through anterior or posterior fontanelles, gum hypertrophy, prominent tongue, cleft palate, and low-set ears. Radiographs show osteosclerosis of the facial bones and cranial vault and subperiosteal thickening of ribs, clavicles, and diaphyses of the long bones. Brain ultrasound or magnetic resonance imaging (MRI) reveals calcification unevenly distributed throughout the brain but mainly in periventricular white matter in parietal and occipital lobes. Detailed neuropathology demonstrates single and clumped calciospherites mainly in neuropil but also in perivascular tissues (27). The disorder was thought to be perinatally lethal but an attenuated phenotype has been reported (28).

#### 157.3.1 Genetics

Since the first case was described in 1989, approximately 24 cases have been reported. Consanguinity has



**FIGURE 157-2** Raine syndrome. Newborn showing characteristic puffiness of periorbital tissues and proptosis with short nose and tented upper lip.

been observed and sex distribution has been normal, suggesting autosomal recessive inheritance. There has been a predominance of cases from Middle Eastern families. Mutations in *FAM20C* on chromosome 7p22 have been identified in 11 affected patients (29). *FAM20C* codes for DMP4, a dentin matrix protein that probably plays a role in mineralization (30). Osteopetrosis with renal tubular acidosis appears to be a distinct entity. There is phenotypic overlap between Raine syndrome and desmosterolosis; however, these appear to be distinct entities as intracranial calcification is not observed in desmosterolosis and measurement of the hepatic desmosterol/cholesterol ratio was normal in one case of Raine syndrome (27).

### 157.4 PYKNODYSTOSIS

Pyknodysostosis is a rare generalized hyperostotic bone disease recognized from infancy by short limbs, short stature, characteristic facies, and wide anterior fontanelle persistent into adult life (31). (Synonyms: osteopetrosis acro-osteolytica, pycnodysostosis.)

#### 157.4.1 Clinical Findings

The skull, which appears large with frontal and occipital bossing and a wide anterior fontanelle, may bring the patient to attention. The hands and feet are short and broad, and the nails may be deformed and brittle. The sclerae are often bluish, and this, in combination with a tendency to fractures with minimal trauma, may lead to confusion with osteogenesis imperfecta.

Radiographically, increased bone density is recognizable by 3 years of age. There is a generalized increase in bone density without long-bone or metaphyseal striation. In older patients, the hands characteristically show progressive osteolysis of the distal phalanges, which appear small and fragmented. The characteristic findings in the skull are wide sutures and Wormian bones, and in the face, a small mandible with an obtuse mandibular angle.

#### 157.4.2 Genetics

This disorder is inherited as an autosomal recessive trait. An increased frequency of parental consanguinity has been described in several populations.

#### 157.4.3 Molecular Pathology

Homozygosity by descent was used to map pyknodysostosis to 1q21 (32). Gelb et al. then demonstrated that mutations in the cathepsin K gene were responsible for pyknodysostosis. Nonsense, missense, and stop codon mutations have all been reported. Cathepsin K is a lysosomal protein responsible for proteolytic degradation

in the osteoclast. Homozygosity for ala277val resulting from paternal uniparental isodisomy for chromosome 1 has been reported (33). In mice, cathepsin K transcription is regulated by a number of transcription factors in *Mitf*.

#### 157.4.4 Genetic Counseling

For normal parents, there is a 25% risk for further affected offspring. Ultrasound prenatal diagnosis is not feasible. If a mutation in the cathepsin K gene can be demonstrated, direct DNA diagnosis in chorionic villus could be performed.

### 157.5 DYSOSTEOSCLEROSIS

Dysosteosclerosis is a rare autosomal recessive skeletal dysplasia characterized by generalized increase in bone density and short stature of postnatal onset. It is differentiated from osteopetrosis and pyknodysostosis by radiologic evidence of platyspondylia with superior and inferior irregularity of vertebral ossification, and clinical findings of a high incidence of development defects of the teeth with delayed eruption of primary dentition, severe hypodontia, and early loss of the teeth (34). Secondary dentition may fail to erupt. Otherwise, the complications consisting of fractures, visual and hearing loss, and recurrent infections of mandible and paranasal sinuses are very similar to those encountered in osteopetrosis. The etiology of this disorder remains unknown.

### 157.6 OSTEOPOIKILOSIS, BUSCHKE–OLLENDORF SYNDROME, AND MELORHEOSTOSIS

Osteopoikilosis and melorheostosis are most commonly asymptomatic and are usually recognized during routine radiologic study including skeletal films. Occasionally, patients are seen with both types of lesions.

#### 157.6.1 Osteopoikilosis

In osteopoikilosis, many small, round or oval osteodense foci are seen in the skeleton, most commonly in the epiphyses and carpal and tarsal centers. The lesions represent foci of old remodeled bone with lamellar structure (35). The disorder is associated with joint pain in some 20% of cases and with skin lesions in an unknown proportion. Osteopoikilosis can occur in isolation or in association with connective tissue nevi of the skin, a condition termed Buschke–Ollendorf syndrome. These consist of slightly elevated whitish-yellow fibrocollagenous nevi (dermatofibrosis lenticularis disseminata) or elastic-type nevi (juvenile elastoma). There is an increased incidence of keloid formation.

#### 157.6.2 Melorheostosis

In melorheostosis, irregular linear osteodense lesions are seen along the axis of the tubular bones. The radiologic appearance of the osteodense lesions has been likened to candle wax flowing down the side of a candle. Single or multiple areas of the skeleton may be involved. The pattern of lesions may be correlated with the sensory scleromeres. It has been suggested that melorheostosis may be the late consequence of lesions of the sensory nerve supply to various skeletal elements (36). The lesions may be associated with shortening of certain bones leading to discrepancy in limb length, soft tissue contractures of the joints or palmar and plantar fasciae, and intermittently painful swelling of affected joints, muscle atrophy, hemangiomas, or lymphedema.

#### 157.6.3 Genetics of Osteopoikilosis and Melorheostosis

Osteopoikilosis can occur either as an isolated anomaly or as part of the Buschke–Ollendorf syndrome, which is inherited as an autosomal dominant disorder with variable expressivity (see above). Melorheostosis usually occurs sporadically but may occur in families inheriting osteopoikilosis and dermatofibrosis lenticularis.

#### 157.6.4 Molecular Genetics

The LEM-domain-containing 3 gene (*LEMD3*) on chromosome 12q13 functions to regulate BMP and transforming growth factor beta (TGFβ) signaling in bone and connective tissue by antagonizing both signaling pathways (35). Loss-of-function mutations have been demonstrated in three families with osteopoikilosis who simultaneously also segregated Buschke–Ollendorf syndrome (35).

#### 157.6.5 Management

Management is directed at the connective tissue, skin, and orthopedic complications.

### 157.7 OSTEOPATHIA STRIATA

In osteopathia striata, which also may demonstrate autosomal dominant inheritance, linear regular bands of increased density are seen throughout the skeleton, radiating vertically from metaphyses and with fan-like array in the iliac wings. These should be distinguished from similar striations seen in osteopetrosis, which are associated with metaphyseal modeling defects, and transverse bands of osteodensity at the ends of the long bones. Typical changes of osteopathia striata are seen in diverse syndromes.

In the syndrome of focal dermal hypoplasia (Goltz syndrome), lesions consisting of dermal hypoplasia with

herniation of the adipose tissue are combined with skeletal defects of the limbs (hypoplasia, aplasia, and syndactyly) are inherited in an X-linked dominant fashion. In SPONASTRIME dysplasia (**spondylar-nasal-striated-metaphyses**), the osteopathia striata is not evident until late childhood. This is an autosomal recessive spondylo-metaphyseal dysplasia with severe short stature.

### 157.7.1 Osteopathia Striata with Cranial Sclerosis

Osteopathia striata may also be inherited along with cranial sclerosis (OSCS) as an X-linked dominant syndrome (37). In this syndrome there is associated psychomotor retardation (17%), hearing deficiency (80%), and congenital heart defects. Macrocephaly is seen with frontal bossing in virtually all patients and radiographic study shows cranial hyperostosis. The appendicular skeleton can also be involved. Although there is a high frequency of deafness, the progressive encroachment on cranial foramina is not as marked as in other craniotubular remodeling disorders (see Section 157.8). Nevertheless, facial palsy and palsy of other cranial nerves has been reported. Spontaneous remission of some cranial nerve palsies occurs.

### 157.7.2 Genetics

There is an excess of affected females, and female patients are more likely to have more severe disease-related complications. Mutations in the *WTX* gene on chromosome Xq11.1 causes OSCS. *WTX* is a tumor-suppressor inhibitor of canonical WNT signaling (38). *WTX* protein contains an acidic domain (AD), a  $\beta$ -catenin binding site, and three binding sites for adenomatous polyposis (ADC). Jenkins et al. attributed the cellular mechanism underlying the hyperostotic phenotype to osteoblast activation, inhibition to osteoclast differentiation, or redirection of pluripotent stem cell differentiation. Nonrandom X-inactivation has been demonstrated in an affected female with a severely affected son (39).

## 157.8 CRANIOTUBULAR REMODELING DISORDERS

Craniotubular remodeling disorders are a large group of hyperostoses characterized by abnormal modeling as well as increased density of bone. A distinction has been drawn between the craniotubular dysplasias (e.g. craniodiaphyseal dysplasia), in which modeling abnormalities are prominent (40) although there is also severe sclerosis of the skull, and the craniotubular hyperostoses (e.g. endosteal hyperostosis (van Buchem's syndrome)), in which cranial and tubular bones are deformed by overgrowth of osseous tissue, rather than by a defect in bone remodeling. This distinction is somewhat arbitrary, as all these disorders result from excess bone deposition

versus resorption with specifically different patterns of skeletal involvement. In essence, they are all disorders in which there is generally minimum involvement of the spine compared with osteopetrosis, pyknodysostosis, and dysosteosclerosis, in which increased osteodensity is seen throughout the spine and the rest of the skeleton with minimal changes in the cranial vault.

In diaphyseal dysplasia (Camurati-Englemann disease; CED), craniodiaphyseal dysplasia, the craniometaphyseal dysplasias, frontometaphyseal dysplasia, and pachydermoperiostosis, sclerosis in the region of optic foramina may lead to visual impairment, papilledema, and optic atrophy. Sclerotic narrowing of internal acoustic foramina and the middle ear may lead to various patterns of conductive or sensorineural hearing loss; encroachment on the facial foramina may lead to facial paresis and encroachment on the foramen magnum to long tract signs, hyper-reflexia, weakness, and even sudden death or paraplegia.

### 157.8.1 Diaphyseal Dysplasia (Camurati-Englemann Disease)

Diaphyseal dysplasia is a rare craniotubular remodeling disorder (also known as progressive hereditary diaphyseal dysplasia) with significant neuromuscular involvement (41).

**157.8.1.1 Clinical Findings.** Symptoms usually begin between 4 and 10 years of age, but the onset of symptoms has been described as early as 3 months and as late as the sixth decade. Failure to thrive or gain weight, fatigability, and abnormal gait are frequent presenting symptoms. Pain in the legs of progressively increasing severity may occur. The gait is characteristically wide based and waddling, with reduced muscle mass and poor muscle tone. Flexion contractures may develop at the elbows and knees. Bowleg or knock-knee deformity may be seen in the lower limbs, and the feet may be flat and pronated. Deep tendon reflexes in some cases have been hypoactive and in others hyperactive, with occasional ankle clonus. Increased lumbar lordosis and scoliosis may occur with variable degrees of back pain. Symptoms and signs of encroachment on cranial nerves may be present.

The radiographic features include symmetrical fusiform enlargement of the diaphyses of the long bones, with normal epiphyses and metaphyses. In the diaphyses, there is enlargement of the cortex by endosteal and periosteal accretion of mottled new bone. The lesions are often first recognized centrally in the long bones and progress proximally and distally with gradual involvement of adjacent normal bone. In the skull, there may be sclerosis of the base and frontal areas.

Serum calcium, phosphorus, and serum alkaline phosphatase are characteristically normal. Muscle histology has been reported to show loss of individual muscle fibers with replacement by adipose tissue, atrophic muscle fibers, and slightly pyknotic sarcolemmal



cell nuclei with hyalinization and decrease in the prominence of cross-striations. There is an increased frequency of autoimmune complications, which includes vasculitic pathology, anemia, neutropenia, and thrombocytopenia in some patients. The response to steroid therapy further suggests an immune contribution to the pathogenesis (42).

**157.8.1.2 Genetics.** Camurati–Engelmann dysplasia is inherited as an autosomal dominant trait with variable penetrance and wide expressivity. There is considerable variation in the signs, symptoms, and severity between affected individuals within the same family.

**157.8.1.3 Molecular Genetics.** *TGFβ1* has been shown to stimulate bone formation and suppress bone resorption under physiological conditions (43). A cluster of mutations in *TGFβ1* in the region of the carboxyl terminus of the latency-associated peptide (LAP) domain has been delineated (44,45). The multiple tissue regulatory functions of *TGFβ* may explain the wide variety of extra-skeletal manifestations observed in patients with CED, including the muscle weakness. The pathogenesis of CED is yet to be completely elucidated. *TGFβ1* on chromosome 19q13.2 is the only gene known to be associated with CED. Mutations which inhibit secretion of *TGFβ1* caused upregulation of the signaling pathway (46). The majority of mutations are sense-to-missense in the signaling and LAP domain, resulting in intracellular retention of mutant nascent peptide with decreased secretion.

#### 157.8.1.4 Differential Diagnosis.

- (1) **Camurati–Engelman disease type II.** Nishimura and colleagues reported two patients with clinical features of progressive diaphyseal dysplasia but striations of bones. The disorder appeared to be clinically distinct from hyperostosis generalisata with striations of the bones and mutations that were not detected in the LAP protein region of *TGFB1* (47).
- (2) **Hyperostosis generalisata with striations of the bones (HGS).** This appears to be a disorder distinct from osteopathia striata with cranial sclerosis. It is characterized by cortical thickening of tubular bones and thick bony trabeculae of tubular bones, spine, and skull (48).

**157.8.1.5 Management.** Therapy for this condition should be aimed at maximizing the mobility of the patient. Orthopedic correction of deformity of lower limbs by appropriate osteotomy has been reported to help in the habilitation of these patients. There have been reports of good symptomatic response to low-dose steroid therapy. Deflazacort has been used with a good response and few adverse effects (49).

## 157.8.2 Craniodiaphyseal Dysplasia

Craniodiaphyseal dysplasia is a rare craniotubular remodeling disorder characterized by massive hyperostosis and sclerosis of the skull and facial bones (Figure 157-3), and hyperostosis and defective remodeling of the shafts of the



**FIGURE 157-3** Craniodiaphyseal dysplasia. Boy aged 13 years showing massive hyperostosis of cranium, facial bones, and mandible (leontiasis ossea).

tubular bones. The epiphyses and metaphyses are only mildly affected or spared. The diaphyses are shaped like a policeman's night stick. The early symptoms may be related to respiratory difficulty due to narrowing of the nasal passages (50,51).

**157.8.2.1 Natural History.** Flattening of the nasal root may be noted at birth, and symptoms may occur as early as 3 months of age. Hyperostosis of the cranial and facial bones is progressive in the first years of life, and frank prominence of nasal and adjacent maxillary bones is usually recognized by 1–2 years of age. Symptoms and signs produced by encroachment on cranial foramina including the foramen magnum are marked.

Skeletal radiographs show massive hyperostosis of the cranial bones which develops rapidly during infancy and completely obscures the normal anatomical landmarks. The spine, ribs, clavicles, and scapulae appear hypermineralized but normal in shape. The metaphyses of the long bones are broad and undermodeled. These patients are often of normal-to-tall stature. Serum calcium and phosphorus appear to be normal, but serum alkaline phosphatase is markedly increased.

**157.8.2.2 Genetics.** This disorder is inherited as an autosomal dominant trait in most cases. Autosomal recessive inheritance has been suggested in some cases but the nature of the defect is not known.

**Molecular genetics:** In the autosomal dominant form of craniodiaphyseal dysplasia, heterozygous mutation in the *SOST* gene has been documented in two affected



children. The *SOST* mutations are in the secretion signal of the gene, preventing sclerostin secretion (52).

**157.8.2.3 Management.** There is no effective medical or surgical treatment to prevent the progressive craniofacial hyperostosis and sclerosis and its complications. Some amelioration may be possible with surgical decompression but because of the extreme hyperostosis it is very difficult (53). Special attention should be given to amelioration of hearing and visual impairment and to psychosocial counseling for affected children and their families with this cosmetically disfiguring disorder. As with other craniotubular remodeling disorders, special care must be taken near water as the hyperdense skeleton may contribute to drowning.

### 157.8.3 Endosteal Hyperostosis, Van Buchem Disease, and Sclerosteosis

Endosteal hyperostosis and sclerosteosis are a group of disorders characterized by marked accretion of osseous tissue at the endosteal (inner) surface of bone, leading to narrowing of the medullary canal or obliteration of the medullary space.

**157.8.3.1 Worth Type of Endosteal Hyperostosis.** Worth type is a rare dominantly inherited variety, frequently associated with the presence of a torus palatinus. The torus is a prominent midline ridge of the hard palate in the mouth and is noted in 5% of the population as a normal variant (54). Endosteal hyperostosis predominates in the long bones, but marked hyperostosis of the cranium and lower jaw may occur. This disorder does not always run a benign course, and neurologic involvement may occur (55). Serum alkaline phosphatase may be markedly elevated.

*Molecular genetics:* The disorder can be caused by mutations in *LRP5* on chromosome 11q13.2. Gain-of-function mutations in the amino terminal region of the LDL receptor-related protein 5 (*LRP5*) are responsible (56). Other gain-of-function mutations also result in osteopetrosis indistinguishable from the Albers-Schonberg type apart from an absence of bone fragility. Loss-of-function mutation in *LRP5* is responsible for the osteoporosis-pseudoglioma syndrome.

**157.8.3.2 Van Buchem Type of Hyperostosis.** Hyperostosis corticalis generalisata, or van Buchem disease, is a recessively inherited variety of endosteal hyperostosis, characterized by progressive mandibular enlargement from childhood, and in adult life by signs and symptoms resulting from sclerotic encroachment of optic and acoustic foramina. Serum alkaline phosphatase is markedly elevated. Radiographically, there is marked thickening of the skull, starting in the base and extending to the vault, and increased density of the mandible after puberty. There is increased density of the cortices of tubular bones with narrowing of the marrow cavity.

*Molecular Genetics:* The disease is caused by a 52-kb deletion approximately 35 kb of the sclerostin (*SOST*) gene that removes a *SOST*-specific regulatory element (57). An autosomal dominant form of endosteal hyperostosis can be caused by mutation in *LRP5* (56).

**157.8.3.3 Sclerosteosis.** Sclerosteosis is an autosomal recessive disorder clinically and radiologically almost indistinguishable from van Buchem disease. Sclerosteosis has been differentiated by a high frequency of hyperostosis in the nasal and facial bones, producing a broad flat nasal bridge and ocular hypertelorism with minor hand malformations. The latter consist of cutaneous syndactyly, radial deviation of the second and third fingers, and absent or hypoplastic nails.

**157.8.3.4 Genetics of Van Buchem Disease and Sclerosteosis.** Both van Buchem disease and sclerosteosis result from autosomal recessive inheritance. These are disorders of osteoblast hyperactivity. Genetic studies mapped both to chromosome 17q12–q21. *SOST* is the only gene known to cause sclerosteosis and van Buchem disease (58,59).

Sclerosteosis results from homozygosity for mutation in the *SOST* gene, on chromosome 17q12–q21, which encodes a protein with sequence homology to members of the dan family of secreted glycoproteins. These have been shown to be antagonists of the TGF $\beta$  superfamily (60,61). In a large Dutch family with van Buchem disease, sclerostin downregulation is thought to result from a 52-kb deletion approximately 35 kb downstream of the *SOST* gene which in some way interferes with *SOST* transcription (57).

### 157.8.4 Pachydermoperiostosis

Pachydermoperiostosis, also known as primary hypertrophic osteoarthropathy, is an unusual condition characterized by progressive thickening of the skin and clubbing of the fingers with usual onset in adolescence, although presentation in childhood has been observed. Radiographic findings are similar to those observed in hypertrophic osteoarthropathy associated with chronic pulmonary disease (62).

**157.8.4.1 Clinical Findings.** Those affected develop a massive appearance of the limbs, which may be disproportionately long, and thickening of the facial skin with seborrheic hyperplasia. Complaints of easy fatigability, joint pain, and blepharitis are frequent. The skeletal changes include cortical thickening and sclerosis of the tubular bones, and thickening of the calvaria and base of the skull, which may lead to conductive and/or sensorineural hearing loss, and narrowing of other intervertebral foramina, resulting in neurologic symptoms. Acromegaly has been described in association with pachydermoperiostosis (63).

**157.8.4.2 Genetics.** Pachydermoperiostosis may be genetically heterogenous. Families with vertical transmission consistent with autosomal dominant inheritance

have been observed. The molecular basis of the autosomal dominant form is still unknown. Autosomal recessive inheritance has been documented (64). The autosomal recessive form is molecularly heterogeneous. Homozygous mutations in *HPGD* on 4q34.1 and *ALCO2A1* on 3q22.1–q22.2 have been reported. *HPGD* (NAD(+)-dependent 15-hydroxyprostaglandin dehydrogenase) is the main enzyme of prostaglandin degradation. Homozygous affected individuals develop primary hypertrophic osteoarthropathy secondary to chronically elevated prostaglandin E2 levels (65). Heterozygote relatives also show milder biochemical and clinical manifestations. Mutations in the *HPGD* gene have also been reported in cranio-osteoarthropathy. Using exome sequencing, homozygous mutation in solute-carrier organic-anion-transporter family member 2A1 (*SLCO2A1*), which encodes a prostaglandin-transporter protein, has been identified in a single individual with primary hypertrophic osteoarthropathy (66).

### 157.8.5 Craniometaphyseal Dysplasias

The craniometaphyseal dysplasias (CMDs) are a genetically heterogeneous group of disorders. Both autosomal dominant (Figure 157-4) and recessive inheritance have been described, both groups of patients have overlapping clinical and radiographic findings. In general, recessive CMD has more severe hyperostosis and complications than dominantly inherited CMD.

**157.8.5.1 Natural History.** There is wide variability in the onset of symptoms and signs, but some cases

have been recognized in infancy. Clinically, both dominant and recessively inherited varieties show progressive facial dysmorphism with broad osseous prominence of the nasal root extending across the zygoma (Figure 157-4). Difficulty with breathing and encroachment on the nasal passages may be recognized in the first 6 months of life. Signs and symptoms of sclerotic encroachment on cranial foramina vary in severity from patient to patient but may be unusually severe.

The essential radiologic features are hyperostosis of the skull, nasal, and maxillary bones extending bilaterally across the zygoma with failure of pneumatization of the paranasal sinuses and mastoids and hyperostosis of the mandible. The long bones show flaring and decreased density of the metaphyses (Erlenmeyer flask deformity) due to failure of remodeling of the metaphyses during growth. Hyperostosis and sclerosis of the mandible develop but are less severe than in craniodiaphyseal dysplasia.

**157.8.5.2 Genetics and Molecular Genetics.** The autosomal dominant form of CMD was localized to 5p15.2–p14.1 (67). Dominantly inherited CMD families have mutations in the *ANK* gene, the human homolog of the mouse “progressive ankylosis” (*Ank*) gene. The *ANK* gene codes for a transmembrane protein that regulates the transport of pyrophosphate, an anion which is an important intermediate in bone formation (68). The autosomal recessive form of CMD has been mapped to 6q21–22 (69).

**157.8.5.3 Management.** As in craniodiaphyseal dysplasia, the cosmetic and neurologic problems in craniometaphyseal dysplasia may be considerable. Plastic surgery for the facial hyperostosis and neurosurgery for foramen magnum compression have been successfully performed (70,71).

#### 157.8.5.4 Differential Diagnosis.

##### (1) Craniometadiaphyseal dysplasia, Wormian bone type.

There are several reports of this autosomal recessive syndrome characterized by a large forehead, prominent parietal eminences, and evidence of a mild craniotubular remodeling disorder with sclerosis of the cranial base (72). Serum alkaline phosphatase levels are moderately elevated. In the skull, multiple Wormian bones are present and there is under-tabulation of broad long bones and ribs. This disorder is one in which there is mixed osteosclerosis (skull) and osteopenia. Previous cases of Schwartz–Lelek syndrome are now thought to represent severely affected cases of craniometadiaphyseal dysplasia.

**(2) Oculodentodigital dysplasia (ODD).** This autosomal dominantly inherited disorder combines characteristic facial appearance with microphthalmia hypotrichosis, dental anomalies, fifth finger camptodactyly, and syndactyly between the fourth and fifth fingers with evidence of a mild craniotubular remodeling disorder. There is undermodeling of broad metacarpals



**FIGURE 157-4** Craniometaphyseal dysplasia (autosomal dominant) in woman aged 42, showing hyperostosis of nasal process of frontal bone and adjacent maxilla and mandible.

and phalanges and broad, long tubular bones. ODD results from mutations in the connexin 43 (gap junction protein alpha-I) gene on chromosome 6q21–6q23.2 (73). Autosomal recessive oculodentodigital dysplasia has been identified, due to homozygous *GJA1* gene mutation (74).

### 157.9 CRANIOTUBULAR REMODELING DISORDERS RESULTING FROM MUTATIONS IN FILAMIN A

Vèrloes, in 2000, confirmed the phenotypic overlap noted by previous authors in:

- (1) Frontometaphyseal dysplasia (FMD)
- (2) Otopalatodigital syndrome type I (OPDI)
- (3) Otopalatodigital syndrome type II (OPDII)
- (4) Melnick–Needles syndrome (MNS).

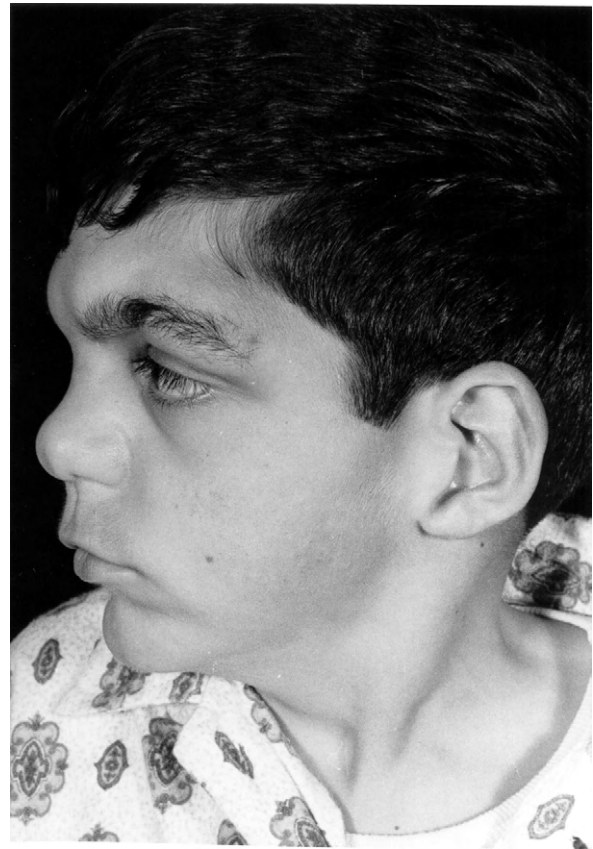
All four disorders, also referred as the otopalatodigital (OPD) spectrum disorders, are characterized by sclerosis of the skull base, and to a varying extent, facial bones. X-linked inheritance has been demonstrated in all four disorders (75). These disorders were mapped to Xq28 and represent a distinct skeletal dysplasia family due to mutations in filamin A (76).

**Molecular pathogenesis:** The filamins are a diverse group of proteins with sequence homology to spectrin, dystrophin, and actinin. Filamin A encodes a 280 kDa protein characterized by actin-binding domains (ABD), which interact with F-actin, and two calponin homology domains (CHD1 and CHD2), and regulates reorganization of the actin cytoskeleton. Mutations that lead to the OPD spectrum disorders occur in defined regions of the filamin A protein, suggesting that very specific functions are being altered in these disorders (56). Heterozygous nonsense and frameshift mutations in the filamin A gene can result in periventricular nodular heterotopia, an X-linked dominant male lethal seizure disorder.

#### 157.9.1 Frontometaphyseal Dysplasia

Frontometaphyseal dysplasia produces a clinically striking facial appearance with a pronounced supraciliary ridge resulting from a torus-like bony overgrowth of the frontal bones (Figure 157-5).

**157.9.1.1 Clinical Findings.** The prominent supra-orbital ridge, which extends across the entire frontal bone, may be recognized at birth, although in some cases not until later. It is associated radiographically with poor development of the frontal and other paranasal sinuses and with mandibular hypoplasia. The pelvis shows an unusually abrupt flare. There is marked frontal hyperostosis and variable hyperostosis of long tubular bones. Metaphyses of the long bones are under-modeled resulting in an Erlenmeyer flask appearance. The bones of the hands and feet are under-tubulated.



**FIGURE 157-5** Frontometaphyseal dysplasia. Boy aged 12 years showing torus-like hyperostosis of frontal bone extending across cranium.

Frontometaphyseal dysplasia should be distinguishable from Sphrintzen–Golberg syndrome, in which there are similar modeling defects in short tubular bones. Hirsutism, obstructive uropathy, congenital stridor, and conductive deafness have been observed. Scoliosis with various alterations of the modeling of the tubular bones may be present.

**157.9.1.2 Genetics.** The disorder is inherited as an X-linked dominant trait (77). Females are more mildly affected than males and have features similar to osteodysplasty. There is significant phenotypic overlap between patients with frontometaphyseal dysplasia and otopalatodigital syndrome (OPD).

Frontometaphyseal dysplasia results from missense mutations in the filamin A gene. Sequence analysis of the *FLNA* gene detects mutations in 57% of affected individuals with FMD (56).

#### 157.9.2 Otopalatodigital Syndrome Type I

Otopalatodigital syndrome is characterized by short stature, and characteristic face with prominent supra-orbital ridges and broad flattened nasal bridge. There is a high frequency of conductive hearing loss. The feet are characteristic, with a short, broad first toe, wide first interdigital space, and varus of all toes that



are terminally short and expanded. Sequence analysis detects mutations in the *FLNA* gene in 43% of individuals with OPDI (56).

**157.9.2.1 Molecular Genetics.** In this group of patients, missense mutations in the filamin A gene are clustered in the CHD2 domain. Again, a number of recurrent missense mutations, such as P207L, have been observed.

### 157.9.3 Otopalatodigital Syndrome Type II

Short stature is of prenatal onset and recognized on prenatal ultrasound. Dysmorphic features include hypertelorism, frontal bossing, broad nasal bridge, and usually wide anterior fontanelle present with cleft palate. The hands are distinctive, with broad, short thumbs and often overlapping fingers. Radiographs show broad undermodeled phalanges and abnormal metacarpals that have geometric segmental shapes reminiscent of atelosteogenesis III (a disorder that results from mutations in filamin B). There is variable syndactyly of fingers and toes. The femora are bowed laterally and tibiae are bowed medially with hypoplasia of fibulae. Osteosclerosis is present in the skull base.

**157.9.3.1 Molecular Genetics.** The recurrent mutation, E254K, in the filamin A gene has been found in all cases of OPDII with omphalocele. This mutation is predicted to interfere with a conserved salt bridge with lysine 169. Other missense mutations have been found in cases without omphalocele in regions of filamin important in actin binding. Sequence analysis of the *FLNA* gene detects 69% of individuals with OPDII (56).

### 157.9.4 Osteodysplasty (Melnick–Needles Syndrome)

Melnick–Needles syndrome (MNS) is characterized by abnormal thinning and irregular narrowing of ribs and long bones.

**157.9.4.1 Clinical Findings.** The age at diagnosis is variable, and affected individuals usually present because of an abnormal gait with bowing of the extremities. Occasionally, dislocation of hips or delayed closure of the anterior fontanelle occurs. On the whole, these patients do not have short stature, and psychomotor development and adult height are normal. Facial appearance is somewhat typical, with slight exophthalmos, protruding cheeks, a high narrow forehead, prominent orbital rims, micrognathia, and misaligned teeth. The lower thorax is narrow. There is incurving of the distal segments of the thumbs. Congenital urinary tract anomalies and heart defects occur relatively frequently and are consistently found in affected males.

Radiographically, there is uneven thickening of the cortex of long bones, which have irregular contours and multiple constrictions, producing a wavy border. The diaphyses are slightly curved and show metaphyseal modeling defects. There is sclerosis of the cranial

base. Coxa valga and dislocation of the hips is frequent. The ribs appear wavy, the pelvis is triangular, and the iliac wings appear to be narrowed in their lower third.

**157.9.4.2 Genetics.** Osteodysplasty is inherited as an X-linked dominant trait with severe disease or infant lethality in males. Robertson et al. (1997) described a family with male otopalatodigital syndrome type II, in which female heterozygotes had skeletal changes characteristic of mild Melnick–Needles syndrome (78).

**157.9.4.3 Molecular Genetics.** Two recurrent mutations within exon 22, A1188T and S1199L, were reported in 12 unrelated families with Melnick–Needles syndrome. Both mutations resulted from separate de novo mutation events (56). Sequence analysis of the *FLNA* gene detects 100% of affected individuals (56).

**157.9.4.4 Differential Diagnosis.** Frank-ter-Haar syndrome. This autosomal recessive (AR) disorder has been referred to as a severe AR osteodysplasty in view of the findings of caliber variation of ribs and bowed long bones similar to those seen in MNS. Males and females are affected. Dysmorphic features include megalocornea/congenital glaucoma, large anterior fontanelles, prominent coccyx with a skin fold in the natal cleft, and congenital heart defects (79). Homozygous mutations in *TKS4* (podosome adaptor gene) on chromosome 5q35 have been associated with Frank-ter-Haar syndrome (80).

## 157.10 TUBULAR STENOSIS (KENNY–CAFFEY SYNDROME)

Tubular stenosis is characterized by narrowing of the medullary cavity and hypermetropia or myopia. In some patients, tetanic seizures due to hypocalcemia occur in infancy (81,82).

### 157.10.1 Clinical Findings

Medullary stenosis may be recognized at an incidental radiographic study or as part of the investigation of the infant with clinical manifestations of hypocalcemia. Other clinical features include delayed closure of the anterior fontanelle and early-onset hypermetropia, myopia, or optic atrophy.

Radiographically, there is a widening of the cortex in the long bones and short tubular bones of the hands and feet, without overall widening of the diaphyses, leading to reduction of the medullary cavity. Rarely, vertebrae, pelvis, carpals, tarsals, and skull may show increased density.

### 157.10.2 Genetics

Both an autosomal dominant syndrome of medullary stenosis and hypocalcemia and an autosomal recessive syndrome have been reported.



### 157.10.3 Molecular Genetics

Autosomal recessive Kenny–Caffey syndrome (AR-KCS) results from mutations in the tubulin-specific chaperone E (TBCE) gene, mapped to 1q42 (83). Mutations in *TBCE* result in abnormal tubulin assembly. Mutations in *TBCE* also account for the Sanjad–Sakati syndrome of autosomal recessive congenital hypoparathyroidism (83).

### 157.10.4 Differential Diagnosis

Tubular stenosis is regularly seen in the Down syndrome newborn skeleton, where it is misinterpreted as osteosclerosis.

## 157.11 HYPERPHOSPHATASEMIA WITH OSTEOECTASIA

In hyperphosphatasemia with osteoectasia, progressive skeletal deformation is associated with marked elevation of alkaline phosphatase. As the clinical and radiologic findings resemble Paget disease, it has sometimes been known as juvenile Paget disease, hyperostosis corticalis deformans juvenilis or familial idiopathic hyperphosphatasia.

### 157.11.1 Clinical Findings

The disease usually has its onset between 2 and 3 years of age, when painful deformity of the extremities develops and leads to gait abnormalities and sometimes fractures. The clinical findings are similar to those of Paget disease in adults but are more generalized and symmetric in distribution. Short stature ultimately results. The skull is large, and radiographically, the diploe is widened and there is loss of normal calvarial structure. Bony texture is variable with dense areas (showing a teased cotton–wool appearance) interspersed with lucent areas. Marked demineralization is seen throughout the remainder of the skeleton. The long bones appear cylindrical, even fusiform, and deformed with the loss of normal metaphyseal modeling. Pseudocysts with a dense bony halo may be seen throughout the long bones. Clinical response has been observed with experimental treatment with recombinant osteoprotegerin (84).

### 157.11.2 Genetics and Molecular Genetics

The disorder is inherited as an autosomal recessive trait. Juvenile Paget disease results from homozygous mutations in the tumor necrosis factor receptor superfamily, member 11B (*TNFRSF11B*) gene (85,86). Mutations in the *TNFRSF11B* gene on chromosome 8q24 that encodes osteoprotegerin, a regulator of osteoclastogenesis, account for the majority of cases with idiopathic

hyperphosphatasemia (87). In normal bone, osteoprotegerin suppresses bone turnover by acting as a decoy receptor for RANK ligand (osteoclast differentiation factor) and this function is disrupted by the known mutations in osteoprotegerin.

### 157.11.3 Management

Clinical and radiographic amelioration of the skeletal lesions after treatment with intravenous pamidronate (similar to the regimen used for osteogenesis imperfecta) has produced encouraging results.

## 157.12 DISORDERS OF PARATHYROID HORMONE RESISTANCE

It is usual to subdivide pseudohypoparathyroidism into the following disorders, which have significant overlap in phenotypic features, but distinctive endocrine and biochemical findings:

- (1) Pseudohypoparathyroidism type I
  - (a) Type Ia
  - (b) Type Ib
  - (c) Type Ic
- (2) Pseudo-pseudohypoparathyroidism
- (3) Pseudohypoparathyroidism type II

Synonyms: pseudohypoparathyroidism (PHP), pseudo-pseudohypoparathyroidism (PPHP), parathyroid resistance syndromes.

### 157.12.1 Clinical Findings

Pseudohypoparathyroidism (PHP) describes a group of disorders characterized by biochemical hypoparathyroidism (i.e. low serum calcium and elevated serum phosphate), increased serum concentrations of parathyroid hormone (PTH), and end-organ resistance to the biological actions of PTH (88).

The clinical findings commonly associated with PHP disorders are disproportionate short stature with acrodysplasia (selective distal shortening of tubular bones, predominantly of metacarpals but also of metatarsals and phalanges), and a round face and obesity. Collectively, these features are termed Albright's hereditary osteodystrophy (AHO). There is also a high frequency of mental retardation and ectopic, usually subcutaneous, calcification (88).

In pseudo-pseudohypoparathyroidism (PPHP) (89), subjects have the somatic features of AHO in the presence of normal serum chemistries and normal end-organ responsiveness to exogenous PTH.

As PHP and PPHP are often both found in the same family, it is likely that they represent variability within the same dominant genetic disorder (see Section 157.12.3).

### 157.12.2 Natural History

In pseudohypoparathyroidism type I, symptomatic hypocalcemia may be the presenting sign. Seizures, tetanic episodes, and evidence of ectopic or intracranial calcification may also bring a child to attention. The serum calcium may be normal in childhood and decrease with age. Mental retardation or a family history of short stature and mental retardation may be recorded. Affected individuals develop short stature with moderate obesity and dental anomalies. These consist of delayed eruption, enamel hypoplasia, dentin hypoplasia, and teeth with short blunt roots.

In PHP type Ia and PPHP, short digits arise from early closure of the epiphyses of the short tubular bones of the hands. The fourth and fifth digits are most often involved and the second least of all. The commonest phalanx affected is the distal first, due to premature closure of its epiphysis. The findings are virtually identical to those seen in brachydactyly type E and some females with Turner syndrome. Cone-shaped epiphyses may be seen at the base of both first metacarpals. Many subjects will also have unusual forms of spinal canal stenosis affecting any region of the spine, but particularly the cervical and/or lumbar spine. These features are similar to those encountered in cheirolumbar dysostosis and there may again be overlap in pathogenesis between subjects with PPHP and cheirolumbar dysostosis.

Other skeletal anomalies include scoliosis, radius curvus, cubitus valgus, coxa valga and vara, and genu valgum and varum. Ectopic calcification usually appears in infancy and may be located in any site, but most commonly in the extremities around large joints.

Mental retardation is present in some 75% of cases with PHP. The mean IQ is in the order of 60. Mental retardation is strongly correlated with the finding of hypocalcemia at presentation, occurring in 70% of affected with hypocalcemia, but only 30% of normocalcemic patients. Lenticular opacities have been reported in 44% of patients.

Endocrine abnormalities associated with PHP include hypothyroidism, hypogonadism, growth hormone deficiency, and diabetes mellitus.

### 157.12.3 Genetics and Pathogenesis

Many of the actions of PTH on kidney and bone are mediated through cAMP. In normal subjects, the administration of exogenous PTH significantly increases renal cAMP production and urinary cAMP levels. The PTH infusion test allows for the various forms of PHP to be distinguished on biochemical grounds. In PHP type I, exogenous PTH fails to cause an increase in urinary cAMP or phosphate, whereas in PHP type II, PTH causes normal urinary cAMP but impaired phosphate excretion (88).

In PHP type I, serum and urinary cAMP generation by endogenous PTH is defective, which initially suggested a defect in PTH binding or transduction at the PTH/PTHrP receptor in the pathway of generation of cAMP. Coupled transduction at the PTH/PTHrP receptor is mediated by guanine nucleotide-binding regulatory proteins (G-proteins). There are both G<sub>s</sub> (stimulatory) and G<sub>i</sub> (inhibitory) proteins. The characterization of the G proteins has shown that in most patients with PHP type I, it is the G<sub>s</sub>- $\alpha$  protein subunit of the adenylate cyclase receptor complex that is defective. The gene that encodes this protein, *GNAS1*, is located on chromosome 20q13.3. Studies into the activity of the G<sub>s</sub>- $\alpha$  protein subunit have allowed for subjects with PHP type I to be subdivided into two distinct groups: PHP type Ia (those with G<sub>s</sub>- $\alpha$  protein subunit deficiency) and PHP type Ib (those with normal levels of G<sub>s</sub>- $\alpha$  protein subunit). The G<sub>s</sub>- $\alpha$  protein subunit receptor complex is also important for the transduction of other hormones. As such, in addition to PTH resistance, patients with PHP type Ia may have resistance to thyroid-stimulating hormone (TSH), gonadotropins, glucagon, calcitonin, and growth hormone. Subjects with PHP type Ib have isolated PTH resistance (occasional mild TSH resistance) without the features of AHO. Subjects with PHP type Ic have the biochemical and clinical features of PHP type Ia, but lack a demonstrable abnormality of G<sub>s</sub> or G<sub>i</sub> (88). Linglart et al. found functional defects of G<sub>s</sub>- $\alpha$  in subjects with PHP type Ic, which would not be apparent on current in vitro assays (90).

PHP type II is a rare disorder with an uncertain etiology and inheritance. Subjects with PHP type II have a reduced phosphaturic response, but a normal cAMP response to exogenous PTH (88). This disorder may result from the inability of cAMP to activate downstream targets within the kidney, or be secondary to PTH resistance in patients with severe and unsuspected vitamin D deficiency (88).

### 157.12.4 Molecular Pathogenesis of PHP Type Ia, PPHP, and PHP Type Ib

Subjects with PHP type Ia have (1) the biochemical findings of PHP, (2) the phenotypic features of AHO, and (3) multiple endocrine resistances. This disorder is caused by mutations of *GNAS1* on chromosome 20q13.3, which results in a reduction in G<sub>s</sub>- $\alpha$  protein subunit activity. The same mutation also results in PPHP, a disorder characterized by AHO but normal sensitivity to PTH and other hormones (91). Both disorders are frequently observed within the same kindred. *GNAS1* is a highly imprinted gene, with the development of PHP type Ia or PPHP being dependent on the gender of the parent transmitting the mutant allele (91). If the *GNAS1* mutation is inherited from a male with either PHP type Ia or PPHP, the affected offspring will all have PPHP. If the same mutation is

transmitted from a female with either of these disorders, then the affected offspring will all have PHP type Ia. Patients with PPHP generally coexist with PHP type Ia in the same family but never in the same sibship (92). Despite the Gs- $\alpha$  subunit being a ubiquitously expressed signaling protein, the hormone resistance associated with PHP type Ia is limited to only a few tissues. These observations, together with the imprinting described above, suggest that Gs- $\alpha$  mRNA from one parental allele is transcribed in certain hormone-responsive cells/tissues (93). It is postulated therefore, that hormone resistance only results from heterozygous Gs- $\alpha$  mutations in cells/tissues where there is monoallelic Gs- $\alpha$  subunit expression (93). In other tissues, 50% reduction in Gs- $\alpha$  levels result in haploinsufficiency; this AHO develops regardless of the gender of the parent transmitting the defect (92). Further work is required to clarify this.

To add further complexity, *GNAS1* has multiple transcriptional units (91). The Gs- $\alpha$  subunit is encoded by exons 1–13. A second transcript, *XL<sub>2s</sub>*, has a novel first exon (XL) that splices onto exons 2–13 and is transcribed only from the paternal allele. In vitro it functions as a stimulatory G protein. A third transcript, *NESP55*, is located upstream of exon XL and the Gs- $\alpha$  subunit exons. *NESP55* is expressed from the maternal allele and is a chromogranin-like neuroendocrine secretory protein. Two other transcripts A/B and AS have been identified. A/B is expressed on the paternal allele. Its promoter and first exon is 2.5 kb upstream of Gs- $\alpha$  (94).

PHP type Ib is an autosomal dominant disorder characterized by renal resistance to PTH (occasional mild TSH resistance) without any features of AHO. Some individuals may show the skeletal features of hyperparathyroidism (91). PHP type Ib does not result from mutations in the exons encoding Gs- $\alpha$  or the PTH/PTHrP receptor (93). The genetic defect in PHP type Ib appears to be paternally imprinted and inherited in a manner similar to the PTH-resistance of PHP type Ia and PPHP. Family studies suggest that PHP type Ib results from a mutation in a putative *cis*-acting element involved in the methylation imprinting at *GNAS1* exon A/B (93).

### 157.12.5 Differential Diagnosis

- (1) **Causes of hypoparathyroidism and hypocalcaemia.** These include both primary and secondary hypoparathyroid states and are excellently reviewed by Bastepe et al. (94).
- (2) **Syndromes of mental retardation, obesity, short stature, and brachydactyly.** These include Turner syndrome (45, X gonadal dysgenesis), basal cell nevus syndrome (Gorlin's syndrome), Gardner syndrome, and type E brachydactyly (95).
- (3) **Chromosome deletion 2q37.3 syndrome.** A subgroup of patients with deletion 2q37 detected

by subtelomeric fluorescence in situ hybridization (FISH) have mild intellectual disability and features of AHO with short metacarpals. This subgroup commonly has deletion 2q37.3. This chromosome region contains the gene for GPR35, a member of the G protein coupled receptor superfamily (96).

- (4) **Czech dysplasia—metatarsal type.** Progressive shortening of metacarpals and metatarsals is found in adults and presumably commences during childhood. Otherwise this bone dysplasia shows a severe spondyloarthropathy with marked narrowing of joint spaces (97). The thoracic spine shows marked platyspondylia and lumbar spine is relatively spared. Markers of bone formation are elevated. Czech dysplasia is inherited as an autosomal dominant trait. It is thought to be a common bone dysplasia in Czechoslovakia and neighboring countries. A heterozygous (R75C) mutation in the *COL2A1* gene on chromosome 12q13 has been identified in studied individuals (98,99).

### 157.12.6 Management

The basic principles of treatment are the same as those for the management of hypoparathyroidism. The aim is to maintain low-normal serum calcium concentrations with supplementary dietary calcium and vitamin D, thus avoiding symptoms of tetany while minimizing hypercalciuria. With treatment, the serum phosphorus usually returns to the high normal range. The serum calcium can fluctuate, which can result in periods of lethargy, especially during childhood. This often improves with calcitriol therapy. Treatment does not prevent the progression of lenticular opacities but may improve mental functioning.

Estrogen may significantly influence serum calcium concentration in females with PHP (88). Decreased serum calcium is associated with estrogen therapy and at the time of menses in women with both PHP and hypoparathyroidism. During pregnancy, however, the requirement for vitamin D therapy in some women with PHP decreases significantly. This may be due to placental production of 1,25-dihydroxyvitamin D (88).

Patients with PHP type Ia will frequently have multiple hormone resistance. As such, their endocrine status requires regular monitoring and if indicated, replacement therapy started.

## 157.13 X-LINKED HYPOPHOSPHATEMIC RICKETS

X-linked hypophosphatemic rickets (XLH) is the most common heritable form of rickets, with a prevalence of 1:20,000 (100). It is a renal phosphate wasting disorder characterized by rickets resistant

to vitamin D therapy, a low serum phosphate, and an inappropriately normal 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D). (Synonym: X-linked vitamin D-resistant rickets.)

### 157.13.1 Clinical Findings

Children with XLH exhibit short stature, rickets, lower extremity deformity (genu varum or valgum), and dental abscesses. Growth delay is often present within the first few months of life, but the lower extremity bowing may not develop until the child commences weight bearing. Adults typically have osteomalacia and may develop bone pain, joint stiffness, and overgrowth of bone at the site of muscle attachments (enthesopathy). Enthesopathy may result in joint limitations and various neurologic compressive syndromes.

There is significant variability in the phenotype, ranging from patients with only hypophosphatemia to those with a combination of hypophosphatemia, short stature, and rickettic bone changes. Affected females may have less severe radiological and dental abnormalities and tend to respond better to therapy (100). Although growth rate is consistently disturbed, following the commencement of therapy the growth of children with XLH generally parallels the normal growth curves (101). In some mildly affected individuals, growth may accelerate at puberty and cross the third percentile. In others, the growth spurt of puberty may aggravate bowing of the legs.

Dentition may be late, and abnormal development of the maxillofacial region has been reported. Spontaneous dental abscesses occur in the primary dentition of 25% of children (102), and 85% of adults reporting dental problems (103).

In adults, bowing of the legs and short stature may persist. In some patients, evidence of active osteomalacia may be present. Overgrowth of bone at the site of muscle attachments (enthesopathy) may lead to joint limitations and various neurologic compressive syndromes.

### 157.13.2 Genetics

XLH is inherited as an X-linked dominant trait. Because there is no father-to-son transmission, it is expected that there would be twice as many females as males affected (104). Males with XLH have been reported as having more severe radiological and dental abnormalities than females (102,105). This was attributed to random X-chromosome inactivation in females (the Lyon hypothesis), with a gene dose effect. Subsequent clinical and animal studies have questioned these initial findings (104,106). In a review of 30 children with XLH, Whyte et al. found no evidence to support a gene dose effect and suggested that the different severity was secondary to such factors as sex hormones and physical activity (104).

### 157.13.3 Molecular Pathology

XLH results from impaired renal phosphate reabsorption, inadequate 1,25-dihydroxyvitamin D response to hypophosphatemia, and an intrinsic osteoblast defect (100). Mutations in the phosphate-regulating endopeptidase homolog gene (*PHEX*) on chromosome Xp22.11 are associated with XLH (100). Numerous mutations have been reported in *PHEX* (101). The spectrum of mutations includes a majority of missense (34%), nonsense (27%), deletion (16%), and splicing mutations (15%). Despite much research, the exact mechanism by which *PHEX* mutations result in XLH remains to be elucidated.

The human gene *PHEX* encodes a 749-amino-acid polypeptide with 96% homology to its murine counterpart. It is a membrane-bound zinc metallopeptidase expressed predominantly in fetal bone (osteoblasts), teeth (odontoblasts), and growth-plate cartilage (chondrocytes) (100). It is not, however, expressed in the proximal renal tubule, the site of phosphate reabsorption. These data lead to the hypothesis that a circulating factor (a phosphatonin) regulated by *PHEX* influences renal phosphate homeostasis and impairs 1,25(OH)<sub>2</sub>D metabolism. This hypothesis was supported by parabiosis and cross-transplant experiments between wild-type and Hyp mice (107–109).

The most likely candidate phosphatonin is fibroblast growth factor 23 (FGF-23), which both in vivo and in vitro has been demonstrated to inhibit renal phosphate transport, inhibit the formation of 1,25-dihydroxyvitamin D, and is associated with tumor-induced osteomalacia (74). Other possible phosphatonins include matrix extracellular phosphoglycoprotein (MEPE) and secreted frizzled related protein 4 (sFRP4) (100).

There is also an intrinsic osteoblast defect present in XLH, which is not resolved by treatment with phosphate and calcitriol (100). This may explain why adequate treatment does not completely normalize the growth and skeletal deformity of subjects with XLH.

### 157.13.4 Management

The goal of treatment in XLH is to maximize growth and minimize skeletal deformity. Oral supplementation with phosphate combined with vitamin D is effective in achieving these goals, although the normalization of stature is not always possible (110). An acidic oral phosphate supplement has been recommended as this is better tolerated. For maximum benefit, phosphate must be given in five divided doses at 3–4 h intervals both day and night. This is an arduous treatment regime, but the benefits gained justify the approach. Phosphate therapy alone leads to the development of secondary hyperparathyroidism (110). Calcitriol (1,25-dihydroxyvitamin D<sub>3</sub>) has been shown to be particularly effective in correcting the growth disorder in XLH and is the vitamin D of choice in the management of XLH.



Although rachitic changes and reduced growth are noted within the first year of life, treatment with phosphate and calcitriol is often delayed until 12 months of age, when weight bearing begins. The treatment regime is phosphate 100 mg/kg/dose, five times a day, and calcitriol 0.25 µg twice daily. The phosphate dose can be reduced within a month or so. Bloods should be drawn for calcium, alkaline phosphatase, and PTH levels 2 weeks after starting treatment. The urine calcium:creatinine ratio should also be monitored. Children should be seen every 3 months to monitor their serum and urine biochemistry and growth. A renal ultrasound should be performed annually to evaluate for nephrocalcinosis. Renal impairment is a rare complication of nephrocalcinosis when it does occur. An annual bone age is useful to monitor for rachitic changes and skeletal maturity.

Surgical osteotomy has a valuable role in correcting residual deformity of the lower limbs, usually during puberty.

### 157.13.5 Differential Diagnosis

- (1) **Autosomal dominant hypophosphatemic rickets (ADHR).** Like XLH, ADHR is disorder associated with isolated renal phosphate wasting and inappropriately normal 1,25-dihydroxyvitamin D concentrations (111). There is, however, significant phenotype heterogeneity with regards to age of onset and severity of skeletal deformities and incomplete penetrance in ADHR (111). Further, two patients have been reported who appeared to lose their renal phosphate defect, making ADHR the only genetic hypophosphatemia disorder where this has been described (112). ADHR results from mutations in the *FGF23* gene on chromosome 12p13 (113), which result in increased biological activity of FGF-23 (114).
- (2) **Autosomal recessive hypophosphatemic rickets (ARH1 and ARH2).** Two autosomal recessive types have been described. Type 1 is caused by mutations in the *DMP1* gene on chromosome 4q22.1 and type 2 is caused by mutations in *ENPP1* gene on chromosome 6q23.3 (115,116).
- (3) **Tumor-induced osteomalacia (TIO).** TIO has similar clinical and biological features to XLH. This disorder is, however, acquired, and often manifests with significant bone and muscle pain, out of keeping with the physical and radiological findings. The serum alkaline phosphatase may be more elevated than observed in XLH and ADHR (100). Unlike in XLH and ADHR, the 1,25-dihydroxyvitamin D in TIO is frequently low, which results in hypocalcemia and secondary hyperparathyroidism (100). The most common tumor associated with TIO is a phosphaturic mesenchymal tumor, mixed connective tissue variant (PMTMCT). These are slow growing benign tumors that are otherwise asymptomatic, and are often difficult to identify due to their small size and obscure location (100). Removal of the tumor results in rapid resolution of the disorder. Patients with fibrous dysplasia, neurofibromatosis, and linear nevus sebaceous syndrome may also develop significant hypophosphatemia and osteomalacia, which may be mediated by FGF-23 (117).
- (4) **Vitamin-D-dependency rickets (VDD).** This syndrome appears to be biochemically heterogenous, with two forms presently delineated (see Chapter Disorders of the Body Mass).
  - (a) **Autosomal recessive VDD rickets.** These patients have early infancy onset of hypocalcemia, normal or low serum phosphorus, elevated serum alkaline phosphatase, generalized aminoaciduria, and clinical and radiographic findings of severe rickets. Serum 25-hydroxyvitamin D is normal, but serum 1,25-dihydroxyvitamin D is below the limits of detection (118). The disorder results from defective 1-hydroxylation of 25-hydroxyvitamin D due to mutations in the 1-α-hydroxylase gene (119) (see Chapter Disorders of the Body Mass).
  - (b) **Target organ resistance to 1,25-dihydroxyvitamin D.** Brooks et al. (1978) reported a 22-year-old black patient with symptomatic osteomalacia, hypocalcemia, and hyperaminoaciduria from the age of 15 years (120). In this patient, plasma 25-hydroxyvitamin D was markedly increased. Osteomalacia in the face of elevated serum 1,25-dihydroxyvitamin D is thought to be due to impaired target organ responsiveness (see Chapter Genetic Lipodystrophies).

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# CHAPTER

# 158

## Chondrodysplasias

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### 158.1 INTRODUCTION

The skeletal dysplasias are a heterogeneous group of disorders associated with abnormalities in the size and shape of the limbs, trunk, and/or skull that frequently result in disproportionate short stature. Until the 1960s, most disproportionate dwarfs were considered to have either achondroplasia (those with short limbs) or Morquio disease (those with a short trunk). It is now apparent that there are well over 450 distinct skeletal dysplasias, which have been classified primarily on the basis of their clinical or radiographic characteristics (see [Table 158-1](#) and [Further Reading](#)).

### 158.2 CLASSIFICATION AND NOMENCLATURE

Current nomenclature for the chondrodysplasias is somewhat confusing and is based on (1) the part of the skeleton that is affected radiographically (e.g. the metaphyseal dysplasias) or clinically and/or radiographically (e.g. the acromesomelic dysplasias); (2) a Greek term that describes the appearance of the bone or the course of the disease (e.g. diastrophic (twisted) dysplasia, thanatophoric (death-seeking) dysplasia); (3) an eponym (e.g. Kniest dysplasia, Ellis-van Creveld syndrome); or (4) a term that attempts to describe the pathogenesis of the condition (e.g. achondroplasia, osteogenesis imperfecta) or the gene implicated (e.g. SEMD, Aggrecan type).

The extent of the heterogeneity in these disorders and the variety of methods used for their classification have resulted in further confusion. Clinical classifications have divided the skeletal dysplasias into those with short-limbed dwarfism, and those with short-trunk dwarfism. The short-limbed varieties have been further subdivided on the basis of the segment of the long bones that is most severely involved. Other clinical classifications have been based on the age of onset of the disorder, and those disorders that manifest themselves at birth (achondroplasia)

versus those that first manifest in later life (e.g. pseudoachondroplasia). Although this information is occasionally still useful in describing various skeletal dysplasias and in arriving at a diagnosis, the advent of nearly universal prenatal ultrasound examinations has altered our perception of the timeline and revealed this criterion to be often subjective in nature. Associated clinical abnormalities have also been used to subdivide these disorders. Examples are the myopia of spondyloepiphyseal dysplasia congenita, the cleft palate of Kniest dysplasia, the fine hair of cartilage-hair hypoplasia, and the polydactyly and congenital heart disease of the Ellis-van Creveld syndrome. Still other disorders have been classified on the basis of their apparent mode of inheritance, for example the dominant and X-linked varieties of spondyloepiphyseal dysplasia.

The most widely used method of differentiating the skeletal dysplasias has been the detection of skeletal radiographic abnormalities. Radiographic classifications are based on the different parts of the long bones that are abnormal (epiphyses, metaphyses, or diaphyses) ([Figures 158-1 and 158-2](#)). Thus there are epiphyseal and metaphyseal dysplasias, which can be further divided depending on whether or not the spine is also involved (spondyloepiphyseal dysplasias, spondylometaphyseal dysplasias). Furthermore, each of these classes can be further divided into several distinct disorders based on a variety of other clinical and radiographic differences.

In an attempt to develop a uniform nomenclature and classification system for these disorders, an International Nomenclature of Constitutional Diseases of Bone was proposed in 1970 and updated in 1977, 1983, 1992, 1997, 2001, 2006, and 2010.

As short stature is a frequent finding in these disorders, the term “dwarfism” has been historically used for them. However, “dwarfism” is thought to result in stigmatization and is popularly unappealing, and for these and other reasons, the term “dwarfism” has been dropped from the current nomenclature and replaced by the term dysplasia. This latter term, which means “disordered growth,” reflects the probable pathogenesis of the majority of the chondrodysplasias. In contrast, malformations

<sup>†</sup>Deceased.

**TABLE 158-1 Clinical, Radiologic, Morphologic, and Genetic Features in the Chondrodysplasias (Associated with Defects of Growth of Tubular Bones and/or Spine)**

Dysplasia	Head and Neck	Chest and Trunk	Limbs	Other	Skull	Ribs	Vertebrae
<b>Achondroplasia group</b>							
Thanatophoric dysplasia, type I (includes platyspondylic lethal, San Diego type)	Large, bulging forehead, prominent eyes, depressed nasal bridge, wide fontanelles and sutures	Small, narrow, pear-shaped	Micromelia	Hydrocephalus, congenital heart and CNS defects	Large calvaria, short base, small foramen magnum; infrequent craniosynostosis	Short, cupped and splayed anteriorly	Hypoplastic, inverted U-shaped (AP), marked flattening with round anterior end (lateral)
Thanatophoric dysplasia, type II	Cloverleaf skull	Small, narrow, pear-shaped	Micromelia		Cloverleaf skull	Short, cupped and splayed anteriorly	Platyspondylia less severe than type 1
Achondroplasia	Large head, bulging forehead, low nasal bridge, prominent mandible	Slight rib flaring	Rhizomelic shortening, fatty folds of skin in infancy, genu varum	Early otitis media, spinal stenosis	Large calvaria, short base, small foramen magnum	Short, cupped anteriorly	Decreased lumbosacral interpedicular distance, short pedicles
Hypochondroplasia	Normal head to slight prominence of forehead	Normal-mild lumbar lordosis	Rhizomelic shortening of extremities, short broad hands, limited extension at elbow	Mild short stature, muscular appearance	Normal	Normal to slightly flared anteriorly	Lumbosacral interpedicular narrowing, short pedicles, posterior scalloping
SADDAN (severe achondroplasia with developmental delay and acanthosis nigricans)	Severe midface hypoplasia, frontal bossing	Small chest	Rhizomelic shortening, fatty folds of skin persisting to childhood	Developmental delay, structural CNS anomalies, acanthosis nigricans	Large calvaria, no craniosynostosis	Short	Platyspondylia, decreased lumbosacral interpedicular distance, short pedicles
Osteoglophonic dysplasia	Prominent supra-orbital ridge, depressed nasal bridge, short neck	Normal	Rhizomelic shortening	Delayed tooth eruption	Craniosynostosis	Progressive rib expansion	Normal
<b>Type II Collagenopathies</b>							
Achondrogenesis II/ hypochondrogenesis (Langer-Saldino)	Round flat face, short neck	Short, barrel-shaped	Very short	Distended abdomen, fetal hydrops	Large calvaria, posterior ossification defect	Very short ribs	Absent or minimal vertebral ossification (thoracolumbar)
Kniest dysplasia	Flat face, prominent wide-set eyes, broad nasal root	Short trunk	Short, prominent knees, joint contractures	Myopia, retinal detachment, hearing loss	Frontal flattening, maxillary hypoplasia, shallow orbits	Short	Diffuse flattening, coronal clefts
Spondyloepiphyseal dysplasia congenita, type Spranger-Wiedemann (SEDC)	+/- Round flat face, short neck, prominent eyes, +/- cleft palate	Short barrel chest, pectus carinatum	Mild rhizomelic shortening, normal hands, +/- club feet	+/- Myopia, retinal detachment, hearing loss, subluxation of C1-C2	Relatively normal	Short	Flattened, dorsal wedging (pear-shaped); odontoid hypoplasia

Pelvis	Limb Bones	Chondro-osseous Morphology	Mode of Inheritance	Chromosomal Locus	Gene	Gene Product	MIM No.
Small, short, flat spiculated acetabulum; small sacrosciatic notch	Short, bowed femora +/- bowing of other bones, metaphyseal flaring (with medial spike-femora)	Generalized disruption of growth plate, poor columns, fibrous bands and fibrous ossification; large RER <sup>a</sup> inclusion bodies in fetus	AD	4p16.3	<i>FGFR3</i>	FGFR3	187600
Small, short, flat spiculated acetabulum; small sacrosciatic notch	Straight femora	Generalized disruption of growth plate, poor columns, fibrous bands and fibrous ossification	AD	4p16.3	<i>FGFR3</i>	FGFR3	187601
Squared-off ilia, small sacrosciatic notches	Short, broad; oval radiolucency in proximal femora in infancy; relative overgrowth of fibulae	Chondrocytes normal and growth plate regular, periosteal overgrowth	AD	4p16.3	<i>FGFR3</i>	FGFR3	100800
Slightly short basilar segments of ilia	Rhizomelia with short, wide bones, prominent deltoid tubercles, elongated fibulae	Normal growth plate	AD	4p16.3	<i>FGFR3</i> some families not linked to <i>FGFR3</i>	FGFR3	146000
Similar to achondroplasia	Severe rhizomelic shortening, neonatal tibial and fibular bowing	Short proliferative and hypertrophic zones, increased vascularity of cartilage, overgrowth of cortical bone	AD	4p16.3	<i>FGFR3</i>	FGFR3	134934 <sup>a</sup> (MIM no. For FGFR3; no separate no. for SADDAN)
Small sacrosciatic notches	Multiple lucent defects metaphyses rhizomelia		AD	8p11	<i>FGFR1</i>	FGFR1	166250
Short ilia, flat acetabular roofs; unossified pubic bones	Short broad, mild/moderate metaphyseal changes, long fibulae, absent talus and calcaneus	Large, ballooned chondrocytes with matrix, growth plate hypercellular and irregular, sclerosed vascular channels	AD	12q13	<i>COL2A1</i>	Type II collagen	200610
Small ilia, flat, irregular acetabular roofs	Clublike metaphyses, delayed ossification of femoral heads, cloud effect in epiphyseal plate regions	Swiss cheese cartilage with abnormal vacuolated matrix, perilacunar foaminess, dilated RER	AD	12q13	<i>COL2A1</i>	Type II collagen	156550
Delayed ossification of pubic bones	Delayed epiphyseal ossification and deformity of hips and knees, retarded ossification of carpal and tarsal centers, coxa vara	Dilated RER in chondrocytes, microcysts in proliferative zone, columns short	AD	12q13	<i>COL2A1</i>	Type II collagen	183900

Continued

**TABLE 158-1 Clinical, Radiologic, Morphologic, and Genetic Features in the Chondrodysplasias (Associated with Defects of Growth of Tubular Bones and/or Spine)—cont'd**

Dysplasia	Head and Neck	Chest and Trunk	Limbs	Other	Skull	Ribs	Vertebrae
Spondyloepime-taphyseal dysplasia, Strudwick type	Cleft palate	Pectus carinatum, scoliosis	Genu valgum	Resembles SEDC at birth	Normal	Splayed, bulbous	Early: like SEDC; later: platyspondylia, endplate irregularity, scoliosis, C1–C2 subluxation
Spondyloperipheral dysplasia	Hypoplastic midface	Short trunk, lumbar lordosis	Brachydactyly	Myopia, retinal detachment	Normal	Normal	Similar to SEDC
Arthro-ophthalmopathy (Stickler syndrome; heterogenous; also caused by defects in type IX and XI collagens)	Cleft palate, mandibular hypoplasia, midface hypoplasia		Hypotonia, hyperextension of joints, later pain and morning stiffness	Marfanoid habitus, myopia, retinal detachment, conductive hearing loss	Normal	Normal	Wedging of thoracic vertebrae and Schmorl disease
Type XI collagenopathies							
Stickler dysplasia (heterogenous)							
Otospondylo-mega-epiphyseal dysplasia (OSMED)	Cleft palate, flat face	Back pain	Short limbs, large knees and elbows, decreased joint mobility in adults	Normal stature, neurosensory deafness	Midface hypoplasia, mandibular hypoplasia	Short	Coronal clefts, platyspondylia
Weissenbacher–Zweymuller (heterozygous OSMED)	Cleft palate, flat face		Early rhizomelia, followed by catch-up growth	Normal stature, sensorineural deafness	Midface hypoplasia, mandibular hypoplasia	Short	Coronal clefts
Fibrochondrogenesis	+/- Unusual facies with hypertelorism	Narrow chest +/- omphalocele	Short +/- contractures of hands and clubbed feet	+/- Patent foramen ovale	Midface hypoplasia	Short, cupped 11 ribs	Platyspondylia, coronal clefts
Sulfation disorders group							
Achondrogenesis IB (Fraccaro)	Round face, soft skull, short neck	Short	Very short	Polyhydramnios common	Poorly ossified	Short, thin ribs, cupped ends, no fractures, no beading	Unossified vertebral bodies
Atelosteogenesis type II (including de la Chapelle dysplasia)	Unusual	Narrow, scoliosis	Short, dislocations, equinovarus, gap 1–2 digits	Cleft palate, patent foramen ovale, early death, laryngeal stenosis	Relatively normal	Short ribs	Infrequent coronal and sagittal clefting



Pelvis	Limb Bones	Chondro-osseous Morphology	Mode of Inheritance	Chromosomal Locus	Gene	Gene Product	MIM No.
Delayed ischial and pubic ossification	Delayed epiphyseal ossification, peripheral ossification CFE, metaphyseal and epiphyseal changes, dappling of metaphyses with fragmentation, (fibula>tibia, ulna>radius)	Inclusion bodies in chondrocytes, hypocellular growth plates	AD	12q13	<i>COL2A1</i>	Type II collagen	184250
Delayed ischial and pubic ossification	Similar to SEDC, brachydactyly	Irregular distribution of chondrocytes, Inclusion bodies	AD	12q13	<i>COL2A1</i>	Type II collagen	271700
Normal	Mild epiphyseal dysplasia, especially CFE and distal tibiae, irregular articular surfaces, early-onset degenerative arthrosis, especially of hips		AD	12q13	<i>COL2A1</i>	Type II collagen	108300
			AD	1p21	<i>COL11A1</i>	Type XI collagen	604841
Square iliac wings	Short long bones rhizomelia, especially femora, wide prominent metaphyses; late-enlarged (mega) epiphyses		AR	6p21.3	<i>COL11A2</i>	Type XI collagen	215150
Relatively normal	Rhizomelia with dumbbell-shaped femora and humeri, resolution of bone changes		AD	6p21.3	<i>COL11A2</i>	Type XI collagen	277610
Hypoplastic	Short femora, dumbbell shaped, metaphyseal flare, very short fibulae, ectopic ossification along long bones	Densely fibrous collagenous matrix; growth plate markedly disorganized	AR	1p21.1	<i>COL11A1</i>	Type XI collagen	228520
Hypoplastic crenated ilia	Short with trapezoid femora, crenated tibiae, unossified fibulae	Matrix devoid of collagen fibrils, dense collagenous ring around cells, lack of columns, fibrous zone between resting cartilage and woven bone	AR	5q31.3–q34	<i>DTDST</i>	DTD sulfate transporter	200600
Normal sacrosclatic notch	Short dumbbell humeri (bifid distal), short dumbbell femora, enlarged 2 + 3 metacarpals, round and small middle phalanges, small fibulae	Dense collagen bundles around chondrocytes	AR	5q31.3–q34	<i>DTDST</i>	DTD sulfate transporter	256050

Continued

**TABLE 158-1 Clinical, Radiologic, Morphologic, and Genetic Features in the Chondrodysplasias (Associated with Defects of Growth of Tubular Bones and/or Spine)—cont'd**

Dysplasia	Head and Neck	Chest and Trunk	Limbs	Other	Skull	Ribs	Vertebrae
Diastraphic dysplasia	Acute swelling of pinnae of ears in infancy; later, cauliflower ears	Scoliosis	Short with club feet, hitchhiker thumbs, synphalangism	Cleft palate	Ossified ear pinnae	Precocious ossification of costal cartilage	Scoliosis and lumbar interpedicular narrowing
Multiple epiphyseal dysplasia with club feet (rMED)	Normal, no cleft palate	Scoliosis	Club feet, brachydactyly				Scoliosis
Spondyloepiphyseal dysplasia, Pakistani type	Normal	Kyphoscoliosis	Bowed lower limbs, brachydactyly	Early degenerative joint disease (hands, spine)			Platyspondylia, irregular endplates
Dyssegmental dysplasia group							
Dyssegmental dysplasia, Silverman–Handmaker type	Short neck, micrognathia, hypoplastic orbits, flat facies, cleft palate	Narrow chest	Severe micromelia	Encephalocele, +/- hirsutism, patent ductus, stillborn or neonatal death	Severe midface hypoplasia, micrognathia	Very short flared ribs, round malformed scapulae	Anisodisondyly with both coronal and sagittal clefting
Dyssegmental dysplasia, Rolland–Desbuquois type	Short neck, micrognathia, hypoplastic orbits	Narrow chest	Short limbs, decreased mobility	+/- Hirsutism; +/- hemia, survival post newborn	Midface hypoplasia, mild micrognathia	Short, flared	Coronal clefting, oversized vertebrae
Filamin-related disorders							
Atelosteogenesis type I	Abnormal facies, cleft palate, micrognathia	Narrow	Severe shortening, bowing, talipes	Stillborn or neonatal death	Relatively normal	Short ribs	Platyspondylia
Atelosteogenesis type III	Midface hypoplasia, micrognathia	Narrow	Rhizomelic shortening, deviation of fingers, joint dislocations	Neural tube defect	Hypoplastic maxilla and mandible	Normal	Cervical spine: severe scoliosis/segmentation defects
Larsen syndrome	Prominent forehead, flattened face, hypertelorism, +/- cleft palate	Soft, collapsing thorax	Multiple dislocations	Severe joint laxity, +/- dysraphism of spine/neurologic impairment	Craniofacial disproportion with midface hypoplasia	Normal	Fusion defects of cervical spine
Otopalatodigital syndrome, type II	Prominent forehead, large fontanelle, midface hypoplasia, hypertelorism, micrognathia, cleft palate	Narrow chest, scoliosis	Rhizomelic shortening, dislocation of elbows and hips, camptodactyly, equinovarus deformity		Normal	Thin, posteriorly pinched	Early: slight cervical and thoracic platyspondylia

Pelvis	Limb Bones	Chondro-osseous Morphology	Mode of Inheritance	Chromosomal Locus	Gene	Gene Product	MIM No.
Relatively normal	Short with broad metaphyses, delayed epiphyseal ossification, short and/or oval first metacarpals	Chondrocytes enlarged, clustered, degenerating, surrounded by dense collagen, cystic areas with fibrovascular tissue and intracartilaginous ossification	AR	5q31.3–q34	<i>DTDST</i>	DTD sulfate transporter	222600
	Multiple small epiphyses, double-layered patella, brachydactyly		AR	5q31.3–q34	<i>DTDST</i>	DTD sulfate transporter	226900
	Delayed epiphyseal ossification CFE and knees, mild metaphyseal abnormalities		AR	10q23–q24	<i>ATPSK2</i>	ATP sulfurylase/APS kinase	603005
Small round dense, amorphous ilia	Very short, broad angulated long bones, hypoplastic first metacarpals	Mucoid degeneration of cartilage, disorganized growth plate, large, unfused calcospherites	AR	1q36	<i>HSPG2</i>	Perlecan	224410
Wide flared ilia, small sacrosciatic notches	Short, broad tubular bones; dumbbell femora; enlarged first metatarsals, accelerated ossification in newborn	Resting cartilage contains patches of broad collagen fibers, growth plate normal	AR	1q36	<i>HSPG2</i>	Perlecan	224400
Small iliac wings, absent/hypoplastic ischium/pubis	Very short to absent humeri and/or femora, bowed short tibiae, absent fibulae, dyschondrocytic ossification of the hands	Acellular areas of cartilage, areas containing cell clusters and large "giant" cells	AD	3p14.3	<i>FLNB</i>	Filamin B	108720 (AO1) 112310 (Boomerang)
Vertical ischia	"Tombstone"-shaped proximal phalanges, bifid digits	Normal	AD	3p14.3	<i>FLNB</i>	Filamin B	108721
Normal	Slender, multiple joint dislocations, duplication of calcaneus		AD	3p14.3	<i>FLNB</i>	Filamin B	150250
Normal	Early bowing of the long bones, joint subluxation, carpal fusion, abnormal hypoplastic metacarpals and metatarsals, round hypoplastic phalanges	Abnormal bone	XLR	Xq28	<i>FLNA</i>	Filamin A	304120

Continued

**TABLE 158-1 Clinical, Radiologic, Morphologic, and Genetic Features in the Chondrodysplasias (Associated with Defects of Growth of Tubular Bones and/or Spine)—cont'd**

Dysplasia	Head and Neck	Chest and Trunk	Limbs	Other	Skull	Ribs	Vertebrae
Osteodysplasty, Melnick–Needles	Exophthalmos, micrognathia			Malaligned teeth	Skull base sclerosis; delayed fontanelle closure	Irregular or ribbon ribs	Tall bodies, anterior–posterior concavity of bodies
Metatropic dysplasia/TRPV4 group							
Metatropic dysplasia	Normal	Tail-like sacral appendage, trunk appears long and narrow at birth, develops severe scoliosis	Short with prominent joints	Long in length at birth, appears short-limbed in infancy, short-trunked later (scoliosis)	Normal	Short, flared, cupped anteriorly	Early: markedly flattened (wafer), wide intervertebral spaces. Late: platyspondylia and scoliosis
Spondylometaphyseal dysplasia, type Kozlowski		Kyphoscoliosis, pectus carinatum	Waddling gait, knee and hip pain in childhood, limitation of large joint motion	Short trunk, short stature	Normal	Normal	Platyspondylia with open staircase appearance on AP view
Brachyolmia (AD type)		Scoliosis	Normal upper to lower segment ratios	Clinodactyly	Normal	Normal	Severe platyspondylia with flattening in cervical spine
Short rib dysplasia (SRP) (+/– polydactyly) group							
Short rib-polydactyly syndrome type I/III (Saidino–Noonan/Verma–Naumoff)	Round, flattened face	Hydropic appearance, narrow thorax, protuberant abdomen	Markedly short hands and feet, postaxial polydactyly	+/– Defects of heart, kidneys, lungs and GI tract	Normal	Very short, horizontal	+/– Flat with wide intervertebral disc spaces
Short rib-polydactyly syndrome type II (Majewski), type IV (Beemer—without polydactyly)	Short flat nose, low-set ears, cleft lip or palate	Hydropic appearance, narrow thorax, protuberant abdomen	Moderately short, pre- or +/– postaxial polydactyly	+/– PDA, dysplastic kidneys, respiratory tract anomalies; +/– OFD (1) (Mohr–Majewski)	Normal	Very short, horizontal	Relatively normal
Asphyxiating thoracic dysplasia (ATD) (Jeune)	Normal	Long narrow, prominent rosary, respiratory distress	Variable shortening, short broad hands and feet, (rare postaxial polydactyly)	Respiratory insufficiency, progressive nephropathy	Normal	Very short, cupped anteriorly	Normal
Chondroectodermal dysplasia (Ellis–van Creveld dysplasia)	+/– Midline puckering of upper lip, +/– natal teeth	Long narrow chest	Postaxial polydactyly of hands +/– of feet, acromesomelic shortening, nail dysplasia	Congenital heart disease, epispadias	Normal	Short ribs	Normal
Thoracolumbar hypoplasia (Barnes)	Normal	Narrow chest	Normal	Laryngeal stenosis	Normal	Short, horizontal orientation	Normal
Multiple epiphyseal dysplasia and pseudoachondroplasia							
Multiple epiphyseal dysplasia, types fairbanks and ribbing		Thoracic kyphosis +/– back pain	Pain and stiffness in knees hips, and ankles, waddling gait	Presents age 2 years or older	Normal	Normal	Endplate irregularity and Schmorl nodes (adult)



Pelvis	Limb Bones	Chondro-osseous Morphology	Mode of Inheritance	Chromosomal Locus	Gene	Gene Product	MIM No.
Flared iliac wings, tapered ischia	Flared metaphyses of long bones; S-shaped tibiae, bowed radii, long femoral necks		XLD	Xq28	<i>FLNA</i>	Filamin A	309350
Hypoplastic crescent-shaped ilia, low-set anterior iliac spines	Short, broad club-like halberd femur	Chondrocytes vacuolated with inclusions, growth plate irregular vascularization	AD	12q24.11	<i>TRPV4</i>	TRPV4	156530
Narrow sacrosciatic notches, broad horizontal irregular acetabular roofs	Metaphyseal irregularity, hips widened epiphyseal plate, coxa vara, marked delay of carpal ossification	Proliferative zone irregular columns, fibrous appearance to matrix on EM	AD	12q24.11	<i>TRPV4</i>	TRPV4	271660
Normal	Clinodactyly		AD	12q24.11	<i>TRPV4</i>	TRPV4	113500
Small ilia, flat acetabulum	Very short, medial and lateral metaphyseal spurs, polydactyly	Disorganized growth plate, broad short trabeculae	AR	3q25	<i>IFT80</i>	IFT80	263530 263510
Normal	Short, ovoid tibiae, polydactyly, premature ossification of epiphyses	Short irregular columnization	AR	4q33	<i>NEK1</i>	NIMA-related kinase	263520 269860
Square, short ilia, flat acetabulae, spurs at margins of acetabulae	Premature ossification capital femoral epiphyses, broad proximal femoral metaphyses	Variable findings, lipid inclusions in chondrocytes, cartilage islands in metaphyses	AR	3q25 11q22.3 2q24.3	<i>IFT80</i> <i>DYNC2H1</i> <i>TTC21B</i>	IFT80 DYNC2H1 TTC21B	208500
Squared ilia with hook-like spurs at acetabulae, similar to ATD	Acromesomelic shortening with cone epiphyses, hamate-capitate fusion, slanting proximal tibial metaphyses	Variable, vascularity of cartilage, cartilage islands in metaphysis, irregular columns	AR	4p16	<i>EVC1</i> , <i>EVC2</i>	EVC protein	225500
Small pelvic outlet	Normal		AD				187760
Normal	Small irregularly ossified epiphyses involving many areas, especially hips, +/- hands and feet	Variable: normal to disturbed growth plate, inclusion bodies in chondrocytes, dilated RER	AD	6q12-14 1p33-32.2 20q13.3 2p24-23 19p13.1	<i>COL9A1</i> <i>COL9A2</i> <i>COL9A3</i> <i>MATN3</i> <i>COMP</i>	Type IX collagen Matrilin 3 COMP	120210 600204 600969 607078 132400

Continued

**TABLE 158-1 Clinical, Radiologic, Morphologic, and Genetic Features in the Chondrodysplasias (Associated with Defects of Growth of Tubular Bones and/or Spine)—cont'd**

Dysplasia	Head and Neck	Chest and Trunk	Limbs	Other	Skull	Ribs	Vertebrae
Pseudoachondroplasia	Normal skull and face	Trunk appears disproportionately long	Very short limbs with genu varum or valgum, hypermobility of joints, small broad hands	Not manifested until at least 2 years of age	Normal	Normal	Platyspondylia, anterior tongue-like protrusion, endplate irregularity
Metaphyseal dysplasias							
Metaphyseal dysplasia, type Jansen	Prominent forehead		Rhizomelic shortening, enlarged joints		Reticulate pattern in calvaria, sclerosis of base	Splayed and cupped anteriorly	Normal
Eiken dysplasia	Normal	Normal	Short limbs, brachydactyly short fibulae		Normal		Platyspondylia, delayed ossification sacrum
Blomstrand dysplasia	Unusual facies, prominent eyes, cataract, depressed nasal bridge	Narrow chest	Very short limbs	Neonatal lethal, polyhydramnios, overall increased bone density and advanced maturation	Midface hypoplasia	Thick and short	Irregular contours
Metaphyseal dysplasia, type Schmid			Waddling gait, bowed legs, generalized shortening of limbs		Normal	Normal	Irregular endplates only in early childhood
Metaphyseal dysplasia, type McKusick (cartilage-hair hypoplasia)	Fine, sparse, lightly pigmented hair and eyebrows	Harrison grooves	Short, lax ligaments, short pudgy hands, telescoping fingers	Megacolon, immune defects, propensity to skin cancer	Normal	Splayed and cupped anteriorly	Normal
Metaphyseal dysplasia with pancreatic insufficiency and cyclic neutropenia (Shwachman–Diamond)		+/- Small narrow chest	+/- Short	Pancreatic insufficiency and malabsorption, neutropenia	Normal	+/- Short, flared	Normal

Pelvis	Limb Bones	Chondro-osseous Morphology	Mode of Inheritance	Chromosomal Locus	Gene	Gene Product	MIM No.
Acetabular irregularity, hypoplastic ischium and pubis	Generalized epiphyseal and metaphyseal dysplasia, hands: short tubular bones with cupped metaphyses, miniepiphyseal at CFE	Prominent inclusions in chondrocytes showing lamellar organization on EM	AD	19p13.1	<i>COMP</i>	COMP	177150
Demineralized (otherwise normal)	Metaphyses wide, splayed and frayed with cortical erosion, abnormal phalanges	Chondrocytes large, matrix fibrillar, cluster of hypertrophic cells at growth plate, irregular line of ossification with tongues of cartilage in metaphyses	AD	3p21–p22	<i>PTHrP</i>	G protein trans-membrane receptor for PTH and PTHrP	156400
Delayed ossification pubis and cap femoral epiphyses	Unusual, delayed ossification of metacarpals, metatarsals and phalanges		AR	3p21–p22	<i>PTHrP</i>	G protein trans-membrane receptor for PTH and PTHrP	600002
	All tubular bones short with wide metaphyses, +/- bowing, advanced bone maturation	Some chondrocyte vacuolization, hypertrophic and proliferative zones narrow with irregular columns	AR	3p21–p22	<i>PTHrP</i>	G protein trans-membrane receptor for PTH and PTHrP	215045
Normal	Metaphyseal splaying and cupping in many long bones (especially hips), coxa vara, normal metaphyses of hands and feet	Same as Jansen	AD	6q21–q22	<i>COL10A1</i>	Type X collagen	156500
Normal	Metaphyseal flaring and irregularity, especially knees, long distal fibulae	Same as Jansen	AR	9p21–p13	<i>RMRP</i>	RNA component of mitochondrial RNA-processing endoribonuclease	250250
Normal	Mild generalized metaphyseal irregularity	Same as Jansen	AR	7q11	<i>SBDS</i>	SBDS protein	260400

Continued

**TABLE 158-1 Clinical, Radiologic, Morphologic, and Genetic Features in the Chondrodysplasias (Associated with Defects of Growth of Tubular Bones and/or Spine)—cont'd**

Dysplasia	Head and Neck	Chest and Trunk	Limbs	Other	Skull	Ribs	Vertebrae
Adenosine deaminase (ADA) deficiency		Rachitic rosary	Short	Severe combined immunodeficiency	Normal	+/- Short, cupped anteriorly	Normal
Spondyloepimetaphyseal dysplasias							
Spondyloepiphyseal dysplasia tarda (X-linked)	Normal	Sternal protrusion, back pain	Osteoarthropathy of hips and knees	Mild short trunk, short stature	Normal	Normal	Platyspondylia with hump-shaped central portion
Progressive pseudorheumatoid dysplasia		Kyphoscoliosis	Progressive arthropathy, joint stiffness/swelling		Normal	Normal	Platyspondylia, anterior endplate erosions
Dyggve–Melchior–Clausen dysplasia	Short neck	Thoracic kyphoscoliosis, scoliosis, lumbar lordosis, flared ribs, protruding sternum, short trunk	Waddling gait, enlarged joints with restriction, small claw hands	Mental retardation (most)	+/- Microcephaly	Mild flaring	Anterior pointed platyspondylia, vertebral notching
Wolcott–Rallison dysplasia	Normal	Normal	Joint pain, difficulty walking	Infantile onset diabetes mellitus, renal insufficiency, hepatosplenomegaly	Normal	Normal	Platyspondylia
Acrocapitofemoral dysplasia	Relatively large head size	Narrow thorax	Short limbs, brachydactyly			Mild metaphyseal cupping	Ovoid vertebral bodies, anterior notching
Schimke immunosseous dysplasia	Low nasal bridge, bulbous nasal tip	Short neck and trunk, hyperpigmented macules		Thyroid dysfunction, progressive renal failure, immunodeficiency, cerebral ischemia	Normal	Normal	Ovoid vertebrae
SPONASTRIME dysplasia	Prominent forehead, flat midface and nasal bridge, prognathism	Scoliosis, increased lumbar lordosis	Short limbs, joint laxity		Flattened maxilla	Normal	Lumbar vertebral changes: early: platyspondylia, child: anterior of body taller, late: vertebral body normal height
Spondyloepiphyseal dysplasia with joint laxity (SEMDJL)	Oval prominent eyes; blue sclerae; long upper lip	Kyphoscoliosis	Short limbs, spatulate nails, club foot; dislocated hips	Cleft palate; joint laxity; skin soft and doughy	Normal	Relatively normal	Kyphoscoliosis, platyspondylia, oval vertebrae



Pelvis	Limb Bones	Chondro-osseous Morphology	Mode of Inheritance	Chromosomal Locus	Gene	Gene Product	MIM No.
Normal	Long bones: irregular and splayed metaphyses, spurs	Absent columns and cells; irregular calcified cartilage, poor trabecula formation	AR	20q13.11	<i>ADA</i>	Adenosine deaminase	102700
Hypoplastic iliac wings	Early: ossification delay of epiphyses, later: large epiphyses and premature osteoarthritis of hips	Fairly normal with clustering of proliferative cells	XLR	Xp22	<i>SEDL</i>	Sedlin	313400
Acetabular irregularity	Narrow joint spaces, widened metaphyses, flattened epiphyses	Clustering of chondrocytes, pyknotic nuclei	AR	6q22–q23	<i>WISP3</i>	Wnt-1 inducible signaling pathway protein 3	208230
Lacy iliac crest, hypoplastic ilia, tibiae, ischia, and acetabular roof, small sacrosciatic notches	Small epiphyses (esp. CFE), brachydactyly, cone epiphyses, small carpal centers	Foci of multiple degenerating chondrocytes surrounded by dense fibrous capsule	AR	18q12–21.1	<i>FLJ90130</i>	<i>FLJ90130</i>	223800
	Epiphyseal delay, small fragmented epiphyses, resorption of CFE	Paucity of chondrocytes; lack of columns; dilated RER; thick collagen fibers	AR	2p12	<i>EIF2AK3</i>	Eukaryotic translation initiation factor 2-alpha kinase 3	226980
Egg-shaped femoral heads, short femoral necks	Cone epiphyses, large distal femoral epiphyses, premature epimetaphyseal fusion		AR	2q35–36	<i>IHH</i>	Indian hedgehog	607778
Platyspondylia, shallow acetabular roof	Hypoplastic ilia, abnormalities, especially of CFE; occasional metaphyseal	Nests of epiphyseal chondrocytes in resting cartilage, hypocellular resting cartilage	AR	2q34–36	<i>SMARCA1</i>	SMARCA-like protein 1	242900
	Metaphyseal changes, especially distal femur, proximal tibia; striations		AR				271510
Flared iliac wings, narrow sacrosciatic notches	Epiphyseal delay; metaphyseal irregularity; radial head dislocation; short-ended tubular bones of hands and feet		AR				271640

Continued

**TABLE 158-1 Clinical, Radiologic, Morphologic, and Genetic Features in the Chondrodysplasias (Associated with Defects of Growth of Tubular Bones and/or Spine)—cont'd**

Dysplasia	Head and Neck	Chest and Trunk	Limbs	Other	Skull	Ribs	Vertebrae
Severe spondylodysplastic dysplasias							
Achondrogenesis 1A (Houston–Harris)	Round face, soft skull, short neck	Short	Very short	Polyhydramnios common	Poorly ossified	Short thin ribs, cupped ends, fractures, beaded	Unossified vertebral bodies
Opsismodysplasia	Frontal bossing, short nose, long philtrum, abnormal ears	Narrow thorax, C1–C2 subluxation	Short stubby hands and feet	Hypotonia, susceptibility to respiratory infections	Large ossification defect	Short, severe anterior and posterior cupping	Absent; hypoplastic; platyspondylia
Spondylometaphyseal dysplasia, type Sedaghatian	Abnormal		Severe rhizomelic shortening	Neonatal lethal	Relatively normal	Occasional 11 pairs, short	Platyspondylia,
Schneckenbecken dysplasia	Large head, short neck, flat midface, +/- cleft palate	Narrow chest, distended abdomen	Very short long bones, hands and feet less severely affected	Generalized edema +/- cryptorchidism	Flat midface	Short, splayed	Platyspondylia, round anterior ossification
Spondylometaphyseal							
Spondylometaphyseal dysplasia, type corner fracture (Sutcliffe)			Waddling gait, progressive coxa vara		Normal	Normal	Increased endplate convexity, anteriorly wedged (ovoid) appearance
Brachyolmia spondylodysplasias							
Brachyolmia (Hobaek type) (Toledo type)		Short trunk, back pain in adult, mild scoliosis		Punctate corneal opacities (Toledo type)	Normal	Precocious costochondral calcification	Platyspondylia; irregular vertebral endplates, lateral extended vertebral bodies, rectangular in shape
Chondrodysplasia punctata							
Chondrodysplasia, punctata, rhizomelic type	Severe flat face, depressed bridge and tip of nose (Binder's facies)		Proximal shortening	Cataracts, ichthyosiform erythroderma, joint contractures	Midface hypoplasia	Shortened	Wide coronal clefts
Chondrodysplasia punctata, Conradi–Hünemann type	Flat face, depressed bridge and tip of nose	+/- Scoliosis	Asymmetrical shortening	Cataracts, ichthyosiform erythroderma, alopecia, joint contractures	Flat facial bones	Relatively normal	+/- Coronal clefts, marked stippling of spinal region

Pelvis	Limb Bones	Chondro-osseous Morphology	Mode of Inheritance	Chromosomal Locus	Gene	Gene Product	MIM No.
Hypoplastic crenated ilia	Short trapezoid femora, crenated tibiae, unossified fibulae	Matrix devoid of collagen fibrils, dense collagenous ring around cells, lack of columns, fibrous zone between resting cartilage and woven bone	AR	14q31	<i>TRIP11</i>	TRIP11	200600
Square iliac bones; spurs; hypoplastic ischium and pubis	Short, stocky long bones, severe metaphyseal cupping; marked epiphyseal delay; hands: short bones with metaphyseal flare	Enlarged widened hypertrophic zone with wide septae	AR				258480
"Lacy" iliac crests	Short long bones, metaphyseal cupping and irregularity, epiphyseal delay, brachydactyly	Long irregular columns	AR				250220
Snail-shaped ilia	Dumbbell-shaped femora, shortened wide fibulae, prematurely ossified tarsals	Resting cartilage hypocellular, round chondrocytes with round central nucleus, growth plate short, spicules	AR	1p31.3	<i>SLC35D1</i>	SLC35D1	269250
Normal	Developmental coxa vara, small triangular fragments originating from the metaphyses ("corner fractures")		AD				184255
Normal	Normal	Chondrocytes unevenly distributed, surrounded by dense staining material, growth plate short with clusters of hypertrophic cells	AR				271530
Trapezoid ilia, stippling of ischiopubic area	Short femora, humeri, stippled calcification in epiphyses and periarticular areas	Vascularization of cartilage with dysplastic myxoid or fibrotic areas, irregular growth plate	AR	6q22–q24 1q42 2q31	<i>PEX7</i> <i>DHAPAT</i> <i>AGPS</i>	PTS2 receptor DHAP acyltransferase AGP synthase	215100 222765 600121
Stippling of ischiopubic area	Asymmetrical shortening, stippled epiphyses in carpal and tarsal centers	Cystic myxoid degeneration of cartilage, fibrous scarring at growth plate	XLD	Xp11.2	<i>EBP</i>	Sterol-delta8-isomerase	302960

Continued

**TABLE 158-1 Clinical, Radiologic, Morphologic, and Genetic Features in the Chondrodysplasias (Associated with Defects of Growth of Tubular Bones and/or Spine)—cont'd**

Dysplasia	Head and Neck	Chest and Trunk	Limbs	Other	Skull	Ribs	Vertebrae
Hydrops-ectopic calcifications-moth-eaten bones (HEM) (Greenberg dysplasia)	Flat face	Small chest	Severe micromelia, polydactyly	Neonatal lethal, hydrops	Deficient ossification	Short with anterior stippling	Platyspondylia, marked stippling of spinal region, tracheal stippling
Chondrodysplasia punctata, brachytelephalangic type	Binder's facies		Hypoplastic distal phalanges, short limbs	Tracheal calcifications, hearing loss	Flat facial bones	Relatively normal	Coronal clefts, stippling of spinal region
Chondrodysplasia punctata, tibio-metacarpal type	Binder's facies		Short limbs		Midface hypoplasia and micrognathia	Relatively normal	Coronal clefts
Rhizomelic and mesomelic dysplasias							
Omodysplasia			Rhizomelic shortening, upper and lower limbs	Nevus flammeus, occasional congenital heart disease	Normal	Normal, occasionally 13 pairs	Normal
Dyschondroseosis (Leri Weill)			Mesomelic shortening, dorsal subluxation distal ulnae	Mild short stature	Normal	Normal	Normal
Mesomelic dysplasia, type Langer	Micrognathia		Severe mesomelic shortening		Mandibular hypoplasia	Normal	Normal
Mesomelic dysplasia, type Nievergelt			Severe mesomelic shortening, equinovarus, brachydactyly, clinodactyly		Normal	Normal	Normal
Mesomelic dysplasia, type Robinow	Prominent forehead, hypoplastic mandible, hypertelorism, down-slanting palpebral fissures, short, flat nose		Mesomelic shortening, hypoplastic nails	Genital hypoplasia +/- cryptorchidism	Mandibular hypoplasia	Normal	+/- Posterior osseous fusion; hemivertebrae
Mesomelic dysplasia, type Reinhardt			Moderate mesomelic shortening, radial bowing, ulnar deviation of hands, lateral bowing of legs (cutaneous dimple)		Normal	Normal	Normal
Mesomelic dysplasia, type Wemer	Normal		Polydactyly, triphalangeal thumb, bowed lower limbs	Syndactyly	Normal	Normal	Normal



Pelvis	Limb Bones	Chondro-osseous Morphology	Mode of Inheritance	Chromosomal Locus	Gene	Gene Product	MIM No.
"Moth-eaten" iliac wings	"Moth-eaten," very short long bones	Chondrocytes with dilated RER and inclusion bodies	AR	1q42.1	<i>LBR</i>	Lamin B receptor	215140
Stippling of ischiopubic areas	Similar to Conradi–Hunermann, plus distal phalangeal hypoplasia and deformed 2nd proximal phalanx		XLR	Xp22.32	<i>ARSE</i>	ARSE	302940
Relatively normal	Short tibiae; short (2,3,4) metacarpals; stippled epiphyses (sacrum, tarsal, carpal areas)		SP				118651
Minor iliac wing hypoplasia	Short humeri and femora with distal hypoplasia, dislocated radial heads		AR	13q31	<i>GPC6</i>	Glypican 6	258315
Normal	Radii and tibiae short in relation to ulnae and fibulae, Madelung-like deformity		XLD (pseudo-autosomal)	Xp22.3	<i>SHOX</i>	SHOX	127300
Normal	Short and thick hypoplastic fibulae and distal ulnae		XLR (homozygous) (pseudo-autosomal)	Xp22.3	<i>SHOX</i>	SHOX	249700
Normal	Rhomboid-shaped radii, ulnae, tibiae, fibulae, radioulnar and tarsal synostoses		AD				163400
Normal	Hypoplastic distal ulnae +/- radial head dislocations		AD AR	9q22	<i>ROR2</i>	Receptor tyrosine-kinase-like orphan receptor 2	180700 268310
Normal	Short radii and ulnae, hypoplasia of distal ulnae and proximal fibulae		AD				191400
Normal	Absent/hypoplastic tibiae, pre-axial polydactyly		AD				188770

Continued

**TABLE 158-1 Clinical, Radiologic, Morphologic, and Genetic Features in the Chondrodysplasias (Associated with Defects of Growth of Tubular Bones and/or Spine)—cont'd**

Dysplasia	Head and Neck	Chest and Trunk	Limbs	Other	Skull	Ribs	Vertebrae
Mesomelic dysplasia, Kantaputra type	Normal	Normal	Severe mesomelic shortening, with bowing of upper limbs	Camptodactyly	Normal	Normal	Normal
Acromelic and acromesomelic dysplasias							
Acromicric dysplasia	Unusual		Small hands and feet, limited finger flexion		Relatively normal	Normal	Normal
Geleophysic dysplasia	"Happy" facies		Small hands and feet with tight skin, joint contractures, splenomegaly	Tracheal narrowing, mitral/tricuspid stenosis, hepatosplenomegaly	J-shaped sella	Slightly broad ribs	Normal
Trichorhinophalangeal dysplasia, type I	Pear-shaped bulbous nose, unusual philtrum, sparse thin, slowly growing hair		Brachydactyly, proximal interphalangeal swelling, thin nails		Normal	Normal	Normal
Trichorhinophalangeal dysplasia, type II	Same		Same plus exostoses		Normal	Normal	Normal
Acrodysplasia with retinitis pigmentosa and nephropathy (Saldino–Mainzer)			Brachydactyly		Normal	Normal	Relatively normal
Acrodysostosis	Saddle-nose, hypertelorism		Short hands	Mental retardation	Brachycephaly, midface hypoplasia, prognathism, absent nasal bone ossification	Normal	Irregular end-plates, decreased interpediculate lumbar distances
Grebe dysplasia	Normal	Normal	Severely shortened limbs, postaxial polydactyly		Normal	Normal	Normal
Acromesomelic dysplasia, type Hunter–Thompson		Small thorax, mild truncal shortening	Meso- and acromelia with mild rhizomelia, square short hands, elbow deformities, upper extremities more severe than lower		Normal	Mildly short ribs	Infancy and childhood: oval-shaped; adult: posterior wedging

Pelvis	Limb Bones	Chondro-osseous Morphology	Mode of Inheritance	Chromosomal Locus	Gene	Gene Product	MIM No.
	Short radii and ulnae, carpal-tarsal synostoses, malformed talus, calcaneus		AD	2q24–32			156232
Normal	Short metacarpals and phalanges, mild proximal pointing, short long bones	Abnormal growth plate	SP	15q21	<i>FBN1</i>	Fibrillin	102370
Normal	Short, plump tubular bones in hands and feet, small capital femoral epiphyses, short long bones	Lysosomal vacuolization	AR	9q34.2 15q21	<i>ADAMTSL2</i> <i>FBN1</i>	ADAMTS-like protein 2 fibrillin	231050
Normal	Type 12 cone epiphyses of hands and feet, metaphyseal widening of femoral necks with sclerosis and hypoplastic-appearing CFE associated with Legg–Perthes-like changes		AD	8q24.12	<i>TRPS1</i>		190350
Normal	Same plus multiple exostoses		AD	Deletion 8q24.11–q13	<i>TRPS2</i>		150230
Normal	Cone epiphyses of hands and feet, metaphyseal widening of femoral necks with sclerosis and hypoplastic-appearing CFE		AR				266920
Normal	Hands and feet: short tubular bones, cone-shaped shaped epiphyses		AD				101800
Normal	Proximal to distal increasing severity: mildly short humeri and femora to absent metacarpals/metatarsals, absent proximal and middle phalanges		AR	20q11.2	<i>CDMP1</i> (homozygous) Brachydactyly C (heterozygous)	Cartilage-derived morphogenic protein 1	200700
Hypoplasia of iliac base and irregular acetabulae	Progressive shortening with metaphyseal flare, especially acro- and mesomelia, mild epiphyseal delay, brachydactyly with cone epiphyses		AR	20q11.2	<i>CDMP1</i> <sup>a</sup> mutations in <i>CDMP1</i> also cause brachydactyly C and Grebe	Cartilage-derived morphogenic protein 1	201250

Continued

**TABLE 158-1 Clinical, Radiologic, Morphologic, and Genetic Features in the Chondrodysplasias (Associated with Defects of Growth of Tubular Bones and/or Spine)—cont'd**

Dysplasia	Head and Neck	Chest and Trunk	Limbs	Other	Skull	Ribs	Vertebrae
Angel-shaped phalango-epiphyseal dysplasia (ASPED)			Early osteoarthritis	Abnormal dentition	Normal	Normal	Normal
Acromesomelic dysplasia, type Maroteaux	Slightly flat mid-face	Thoracic kyphosis	Short forearms, hands, legs and feet		Scaphocephalic skull	Mildly short	Infancy: oval vertebral bodies, adult: anterior wedging
Dysplasias with prominent membranous bone involvement							
Cleidocranial dysplasia	Large, prominent forehead, wide persistent fontanelles and sutures	Drooping shoulders and narrow chest, +/- scoliosis	Hyperextensibl, +/- coxa vara; fingers short and square	Abnormal dentition	Decreased ossification, multiple Wormian bones	Absent or hypoplastic clavicles	+/- Retarded ossification of bodies, posterior wedging
Bent-bone dysplasia group							
Camptomelic dysplasia, classic long-limbed type (includes acamptomelic camptomelic)	Large calvaria, small flat face, low-set ears, micrognathia	Small, narrow	Bowed femora and tibiae with dimple at maximum convexity	Respiratory distress, multiple internal anomalies, sex reversal	Enlarged, dolichocephalic; narrow and shallow orbits	Narrow and wavy, often 11 pairs, hypoplastic scapulae	Hypoplastic cervical bodies, others flattened, thoracic vertebral hypoplasia (pedicles and bodies)
Stuve-Wiedemann dysplasia	Micrognathia, +/- short neck		Bowing of femora and tibiae; joint contractures	Respiratory distress, hypotonia, hyperthermic episodes, swallowing difficulties	Hypoplastic mandible	Mildly short	Normal
Multiple dislocations with dysplasias							
Desbuquois syndrome	Round, flat face, micrognathia, long upper lip	Neck short, thorax small	Multiple dislocations, fingers deviated radially; hitchhiker's thumb	Joint laxity, +/- mild mental delay	Micrognathia, mid-face hypoplasia	Mildly short ribs	Coronal clefts, high vertebral bodies
Pseudodiastrophic dysplasia	Large cranium, hypertelorism, flat nasal bridge, large malformed earlobes, cleft palate	Scoliosis	Rhizomelic shortening, club feet, finger and elbow dislocations	Bluish sclerae	Normal	Slightly short, anterior flare	Ovoid platyspondylia, scoliosis
Spondyloepiphyseal dysplasia with joint laxity—leptodactylic form	Midface hypoplasia	Scoliosis	Multiple dislocations, short limbs	Laryngotracheomalacia	Normal		Platyspondylia

AD = autosomal dominant; AP = anteroposterior; AR = autosomal recessive; ATD = asphyxiating thoracic dysplasia; ER = endoplasmic reticulum; MIM = Mendelian Inheritance in Man; RER = rough endoplasmic reticulum; SEDC = spondyloepiphyseal dysplasia congenita; SP = sporadic; XLD = X-linked dominant; XLR = X-linked recessive.

\*See type II collagenopathies; those with *COL11A1* mutations have a different vitreous phenotype compared to those with *COL2A1* mutations, while those with *COL11A2* mutations do not have any ocular changes.



Pelvis	Limb Bones	Chondro-osseous Morphology	Mode of Inheritance	Chromosomal Locus	Gene	Gene Product	MIM No.
Normal	Angel-shaped middle phalanges of hands +/- feet, generalized delay in epiphyseal ossification		AD				105835
Relatively normal	Short tubular bones, especially forearms, very short tubular bones of hands and feet, cone epiphyses of metacarpals and phalanges	Normal	AR	9p21-12	<i>NPR-B</i>	Natriuretic peptide receptor B	602875
Absent ossification of or hypoplastic pubis, hypoplasia of ilia	Pseudoepiphyses of metacarpals and metatarsals, retarded ossification of carpals and tarsals	Mild decrease in growth plate height, decreased periosteal ossification	AD	6p21	<i>CBFA1</i>	Core-binding factor alpha-1 subunit	119600
Tall, narrow ilia; increased acetabular angles, vertical ischia, hypoplastic ischiopubic rami	Comparatively long, slender, bowed femora, short tibiae	Cartilage normal to slightly irregular, periosteal trabeculae converge at point of angulation	AD	17q24.1-q25.1	<i>SOX9</i>	SOX9	114290
Normal	Mild bowing of upper limbs, more pronounced bowing of lower limbs, large metaphyses, cortical thickening		AR	5p13.1	<i>LIFR</i>	Leukemia inhibitory factor receptor	601559
Flared iliac wings flat acetabular roof	"Monkey wrench" femora, delta phalanx index finger, knee dislocation, club feet	Large chondrocytes, dilated ER	AR	17q25	<i>CANT1</i>	CANT1	215200
Horizontal acetabular roofs	Upper limb rhizomelia, elbow dislocations, multiple interphalangeal	Resting cartilage normal, mild shortening of growth plate	AR				264180
Small cap femoral epiphyses	Dislocations, generalized epiphyseal dysplasia, gracile metacarpals, distally shortened ulna		AD				603546

of single bones or groups of bones, which presumably do not reflect a generalized disorder of the skeleton, have been referred to as dysostoses.

Although the International Nomenclature provides a uniform standard for referring to specific disorders, so that the same disease is called the same entity by all authors, many of the names are inaccurate. For example, achondroplasia and achondrogenesis are inaccurate terms in defining the pathogenesis of these conditions, but are so well entrenched in the literature that they persist. As the morphology, pathogenesis, and especially the basic biochemical and molecular defect in each of these disorders is unraveled, this nomenclature has been updated to refer to the specific pathogenetic or metabolic defect. The etiologic or pathogenetic nomenclature is used for certain skeletal dysplasias, such as the mucopolysaccharidoses, mucopolysaccharidoses, and disorders of mineralization (e.g. b-glucuronidase deficiency, fucosidosis, hypophosphatasia).

### 158.3 CLINICAL EVALUATION

The osteochondrodysplasias are syndromes that represent generalized disorders of the skeleton and usually result in disproportionate short stature. Affected individuals usually present with the complaint of disproportionate short stature, and the abnormality in stature must first be documented by the use of the appropriate growth curves with adjustment for ethnic background and parental heights (1). In general, patients with disproportionate short stature have skeletal dysplasias, and those with relatively normal body proportions have endocrine, nutritional, prenatal, or other non-skeletal defects. There are exceptions to these rules, as congenital hypothyroidism can lead to disproportionate short stature, and a variety of skeletal dysplasias, such as osteogenesis imperfecta and hypophosphatasia, may result in normal body proportions.

A disproportionate body habitus may not be readily apparent on casual physical examination. Thus anthropometric measurements, such as upper to lower segment ratio, sitting height, and arm span, must be obtained

before the possibility of a mild skeletal dysplasia, such as hypochondroplasia or multiple epiphyseal dysplasia, can be excluded. Although sitting height is a more accurate measure of head and trunk length, it requires special equipment for consistent accuracy. Upper to lower segment ratios (U/L), on the other hand, provide a reasonably accurate measure of body proportions and can be easily obtained. The lower segment measure is taken from the symphysis pubis to the floor at the inside of the heel, and the upper segment is obtained by subtracting the lower segment value from the total height. McKusick (2) has published standard U/L curves for both white and black Americans, which are quite useful for rapid assessment of proportion (2). For example, a white infant has an upper/lower segment ratio of approximately 1.7; it reaches 1.0 at approximately 7–10 years, and then falls to an average U/L of 0.95 as an adult. Blacks, on the other hand, have relatively long limbs, and reach a U/L of approximately 0.85 as adults. Another index of limb

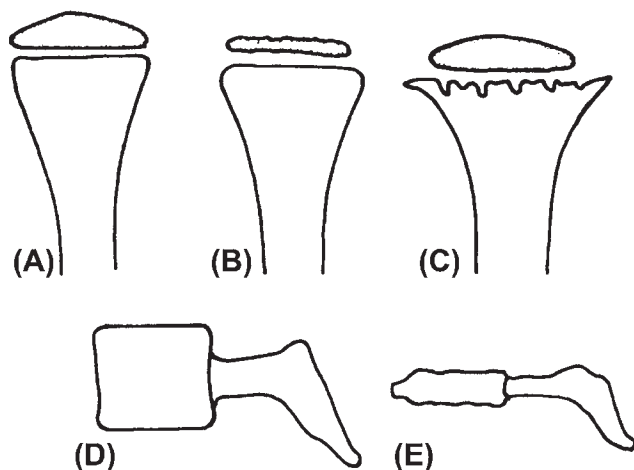


FIGURE 158-1

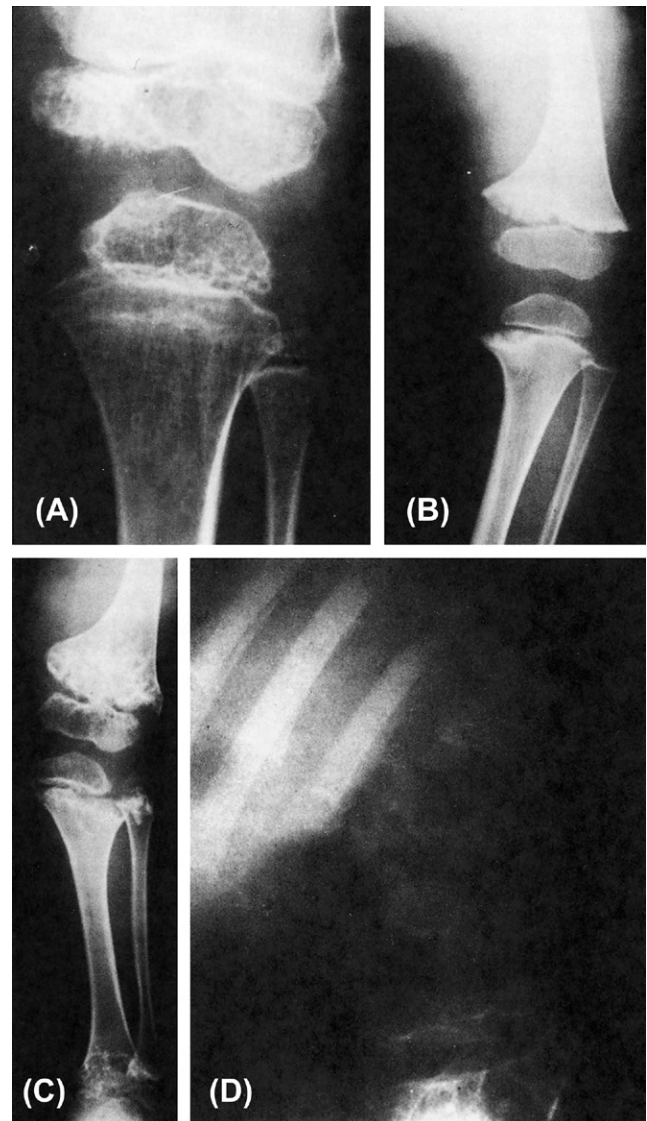


FIGURE 158-2

versus trunk length is based on the arm span measurement, which usually falls within a few centimeters of total height. These measurements are most useful in determining whether or not an abnormally short individual is proportionate or not, and the type of disproportion present. For example a short-limbed dwarf will have an abnormally high U/L ratio and an arm span that is considerably shorter than his height (Figure 158-3).

As in the differential diagnosis of most other disorders, an accurate history, family history, and physical examination may lead one to the correct diagnosis. Certain skeletal dysplasias have prenatal onset and the more severe forms are detected by fetal ultrasound and are evident at birth. However, others may not manifest until late infancy or early childhood. Thus a child who was normal until 2 years of age and then develops disproportionate short-limbed dwarfism is more likely to have pseudoachondroplasia or multiple epiphyseal dysplasia

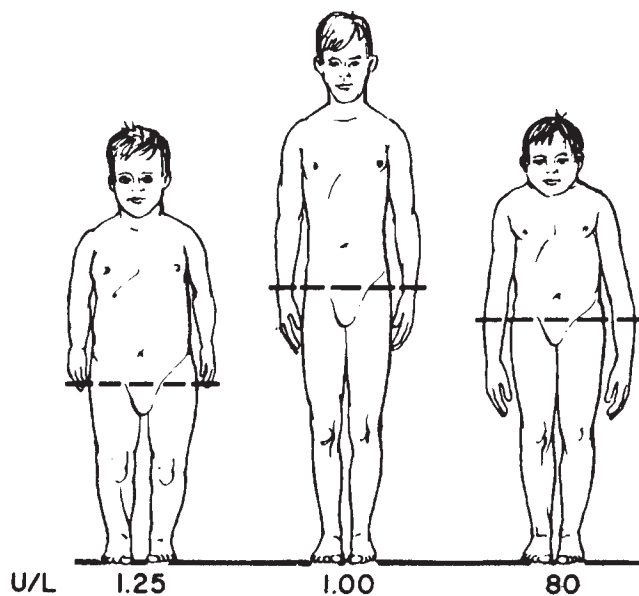


FIGURE 158-3

than achondroplasia or spondyloepiphyseal dysplasia congenita. Furthermore, many parents may not notice short stature until 1 or 2 years of age, but, in reality, it existed from the time of birth.

A detailed physical examination may reveal the correct diagnosis or point to the likely diagnostic category (Table 158-1). First, one must establish whether the disproportionate shortening affects primarily the trunk or the limbs and, if the latter, whether it is proximal (rhizomelic), middle segment (mesomelic), or distal (acromelic), or a combination of these (Figure 158-4). The term rhizomelia is frequently inadvertently abused and should not be applied without measuring.

A variety of head and neck dysmorphisms and malformations can be seen in the skeletal dysplasias. A disproportionately large head with frontal bossing and flattening of the bridge of the nose suggests achondroplasia or thanatophoric dysplasia (TD). Cloverleaf skull is sometimes associated with TD and, rarely, with camptomelic dysplasia and a variety of malformation syndromes. Congenital cataracts suggest a form of chondrodysplasia punctata (CDP). Myopia may be found associated with any of the type II collagen disorders. Complete or partial cleft palate, bifid uvula, or high-arched palate may be found in Kniest dysplasia, spondyloepiphyseal dysplasia congenita, or diastrophic dysplasia. The upper lip is short and tethered in chondroectodermal dysplasia. Acute swelling of the pinnae of the ears occurring shortly after birth, followed by cauliflower ears, is characteristic of diastrophic dysplasia. The latter is probably one of the few truly pathognomonic findings in the skeletal dysplasias.

Postaxial polydactyly is characteristic of chondroectodermal dysplasia and of the lethal short rib-polydactyly syndromes, and may also be seen in asphyxiating thoracic dysplasia (Figure 158-5). Preaxial polydactyly is frequently observed in short rib-polydactyly syndrome II (Majewski), and, rarely, in short rib-polydactyly syndrome I (Saldino-Noonan). In diastrophic dysplasia, the hands are short and broad, the thumbs are hypermobile,

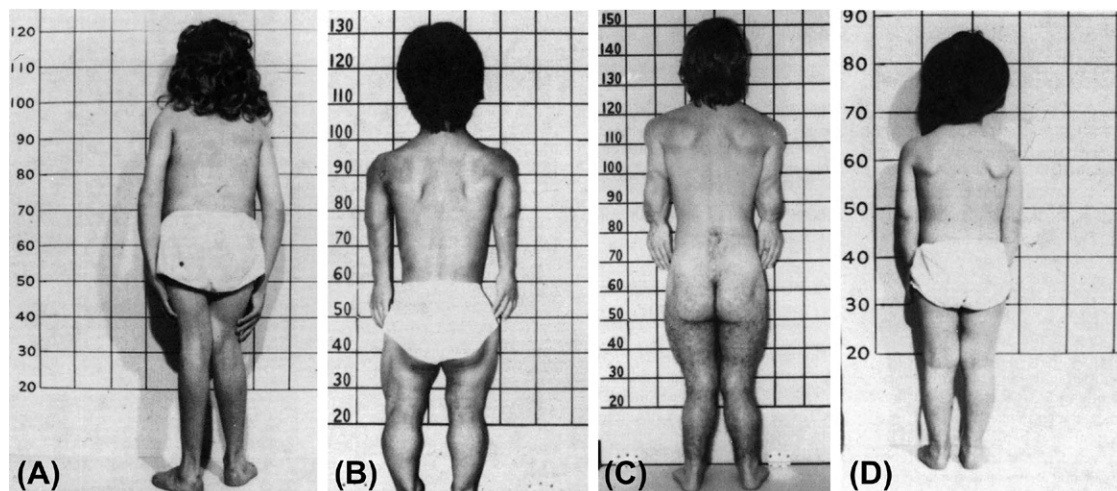


FIGURE 158-4



proximally inserted, and abducted leading to the “hitchhiker thumb” configuration, and flexion creases in the fingers are frequently absent (Figure 158-5). In achondroplastic children, the hand has a trident appearance (Figure 158-5). Hypoplastic nails are characteristic of chondroectodermal dysplasia, while the nails may be short and broad in the McKusick type of metaphyseal dysplasia (cartilage-hair hypoplasia). Shortening of the distal phalanges and second proximal phalanx is characteristic of the dysplasias named CDP, brachytelephalangic type. Club feet may be seen in infants with Kniest dysplasia, spondyloepiphyseal dysplasia congenita, and osteogenesis imperfecta, but are most characteristic of diastrophic dysplasia. Multiple joint dislocations suggest Larsen syndrome, Ehlers–Danlos syndrome type VII, or otopalatodigital syndrome; less severe degrees of joint laxity, particularly of the hands, may be seen in other types of skeletal dysplasia (e.g. cartilage-hair hypoplasia and pseudoachondroplasia). Bone fractures may occur in all of the osteogenesis imperfecta syndromes and several types of hypophosphatasia, osteopetrosis, dysosteosclerosis, and achondrogenesis type IA (Houston–Harris).

In the neonate or infant, a long, narrow thorax suggests asphyxiating thoracic dysplasia, chondroectodermal dysplasia, or metatropic dysplasia. A very small thorax is also seen in TD, the short rib-polydactyly syndromes, and homozygous achondroplasia. In some neonates with spondyloepiphyseal dysplasia congenita, the sternum and neck are short, and the chest may be small

with pectus carinatum, producing early respiratory distress. In the child or adult, scoliosis is frequently seen in metatropic dysplasia and diastrophic dysplasia.

Congenital cardiac defects are seen in several of the skeletal dysplasias. In chondroectodermal dysplasia the most common lesion is an A-V cushion defect with a common atrium. In short rib-polydactyly syndrome I (Saldino–Noonan), a variety of very complex lesions involving the great vessels, transposition or double outlet right or left ventricle, and ventricular septal defect have been reported, and in short rib-polydactyly syndrome II (Majewski) the most common cardiac defect is transposition of the great vessels.

Gastrointestinal manifestations are not common in the skeletal dysplasias, but congenital megacolon can be seen in cartilage-hair hypoplasia, malabsorption secondary to pancreatic insufficiency in the Schwachman–Diamond syndrome, and anorectal anomalies in short rib-polydactyly syndrome. Omphalocele can be a feature of otopalatodigital syndrome type II.

In the older child or adult, the complications associated with specific disorders may aid in the diagnosis. Genu varum is seen in a number of skeletal dysplasias, but in achondroplasia it is associated with lateral curvature primarily of the middle segment of the limb with overgrowth of the fibula proximally, while in cartilage-hair hypoplasia, there is generalized bowing with marked overgrowth of the fibula distally (Figure 158-6). In pseudoachondroplasia, genu varum, valgum, or a windswept abnormality may be seen, and is associated

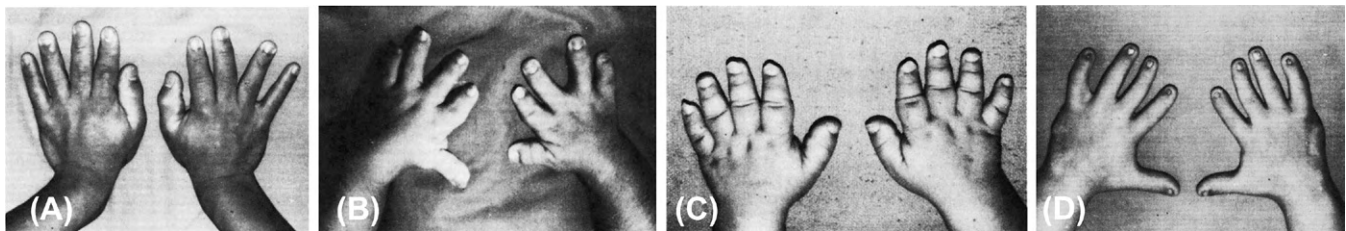


FIGURE 158-5

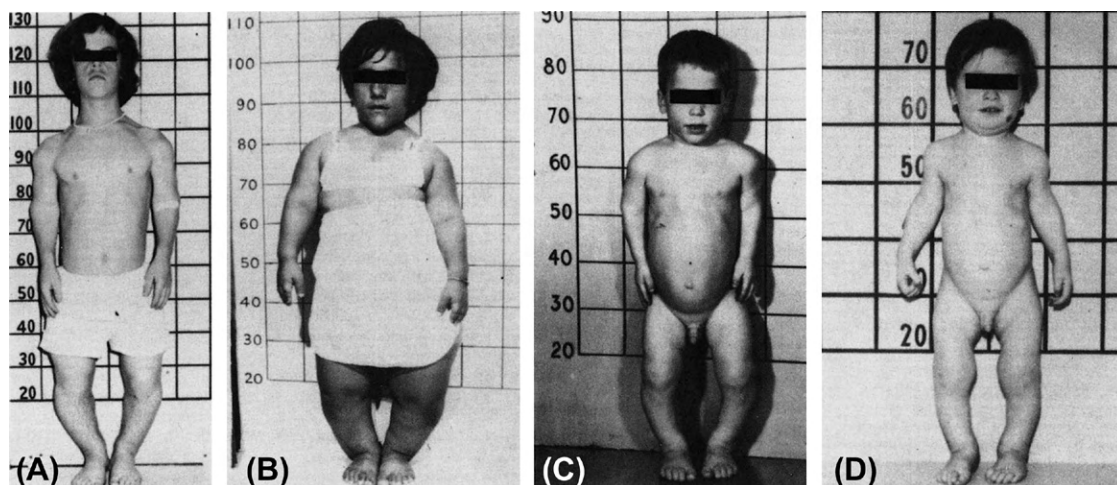


FIGURE 158-6



with severe instability and ligamentous laxity at the knees (Figure 158-6). Thus, careful physical examination with delineation of all the skeletal and non-skeletal abnormalities can be quite helpful in arriving at a diagnosis.

A complete family history, including details of still-born children and parental consanguinity, should be obtained. Parents should always be closely examined, looking for evidence of a dysplasia in a partially expressed form. This is especially important in dominantly inherited disorders with wide variability of expressivity, where a parent may be mildly affected without knowing it (e.g. osteogenesis imperfecta). The appearance of a new autosomal dominant skeletal dysplasia, such as achondroplasia, is frequently associated with advanced paternal age. The health of parental sibs, especially male sibs and male relatives of the mother, should be questioned, as some conditions are inherited in an X-linked fashion. Since each of the skeletal dysplasias most frequently presents as an isolated case in the family, an isolated instance of a skeletal dysplasia in a family cannot provide information as to the mode of inheritance of the particular disorder. However, the type of familial aggregation, when it occurs, can be most helpful. For example, a pedigree with affected sibs and normal parents suggests a recessive type of disease and argues against autosomal dominant disorders, such as achondroplasia and hypochondroplasia. However, germ cell mosaicism can also account for this situation, as in osteogenesis imperfecta II and pseudoachondroplasia, and this phenomenon may be much more common than previously appreciated. If two achondroplastic parents produce a severely affected offspring, it is most likely homozygous achondroplasia, rather than TD. However, different modes of inheritance have been observed in disorders that are difficult to distinguish clinically, such as the autosomal dominant and recessive forms of multiple epiphyseal dysplasia and osteogenesis imperfecta type 3.

#### 158.4 RADIOLOGIC EVALUATION

The next step in the evaluation of the disproportionately short patient is to obtain a full set of skeletal radiographs. A full series of skeletal views, including anteroposterior (AP) and lateral views of the skull, AP and lateral views

of the spine, and AP views of the pelvis and extremities, with separate views of the hands and feet, is optimal. A lateral radiograph of the knees can be helpful in diagnosing the recessive form of multiple epiphyseal dysplasia (MED). Lateral views of the foot are particularly helpful in identifying punctate calcification of the calcaneus, which may be a clue to the diagnosis of the milder forms of CDP, in confirming the absence or hypoplasia of the calcaneus and talus in newborns with spondyloepiphyseal dysplasia congenita, or in delineating the double ossification centers of the calcaneus in Larsen syndrome and asphyxiating thoracic dystrophy. However, owing to their intrinsic natural variability, radiographs of the feet are not generally helpful. An abridged skeletal survey should include at a minimum AP view of the pelvis and AP views of the knees, left hand, and lateral spine. Skeletal radiographs alone are often sufficient to make an accurate diagnosis, since the classification of skeletal dysplasias has been based primarily on radiographic criteria (Table 158-1). Attention should be paid to the specific parts of the skeleton involved (spine, limbs, pelvis, skull), and within each bone, to the location of the lesion (epiphysis, metaphysis, diaphysis) (Figures 158-1 and 158-2). The skeletal radiographic features of many of these diseases change with age, and it is usually beneficial to review radiographs taken at different ages when possible. In some disorders, the radiographic abnormalities following epiphyseal plate fusion are nonspecific, so that the accurate diagnosis of an adult disproportionate dwarf may be impossible unless prepubertal films are available.

Radiologic diagnosis is also based upon recognition of unique patterns of abnormal skeletal ossification, such as the total lack or marked reduction of ossification of the vertebral bodies in the achondrogenesis syndromes (Figure 158-7). Some radiographic features characterize certain disorders. For example in achondroplasia the acetabulae are flat with tiny sacrosciatic notches, rather square iliac wings with rounded corners, and an oval translucent area in the proximal femora and humeri in infants. The finding of a decreasing interpediculate distance from L1 to L5 is seen nearly uniformly in people with achondroplasia and hypochondroplasia. In Kniest dysplasia and metatropic dysplasia, the long

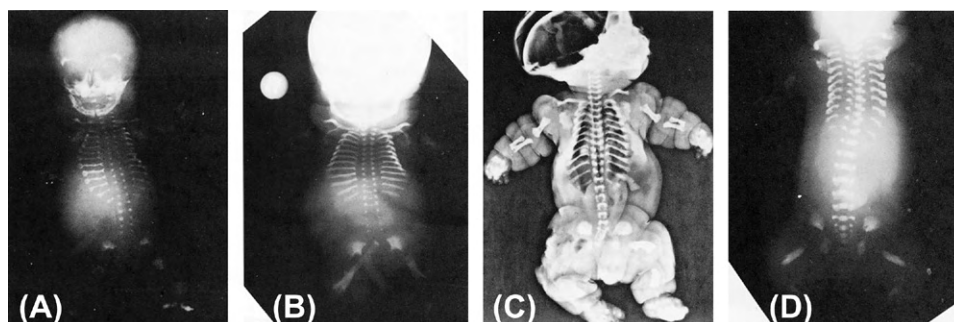


FIGURE 158-7

bones, and femora in particular, have a dumbbell-shaped appearance in the newborn period. Bowing of the limbs (camptomelia) is observed in camptomelic dysplasia, osteogenesis imperfecta, congenital hypophosphatasia, TD, and a heterogeneous group of disorders with broad, bent long bones.

There are calcified projections or spikes on the medial borders of the metaphyses of the femora in TD, and medial and lateral borders in short rib-polydactyly syndrome I/III (Figure 158-7). Cupping of the ends of the ribs and the long bones and metaphyseal flaring are features of a large number of dysplasias, including achondroplasia, the metaphyseal dysplasias, asphyxiating thoracic dysplasia, and chondroectodermal dysplasia.

While fractures in the newborn suggest one of the osteogenesis imperfecta syndromes, they may also be seen in congenital osteopetrosis and severe hypophosphatasia. In achondrogenesis type IA (Houston–Harris), the ribs are thin and wavy with beading, suggesting fractures, but the long-bone and vertebral findings readily distinguish this dysplasia from osteogenesis imperfecta. In the older individual, fractures may also be seen in a variety of osteopetrotic syndromes, including dysosteosclerosis and pyknodysostosis.

Retarded ossification manifest by absence of epiphyseal centers or marked delay in their ossification, is found in the various forms of type II collagen disorders and other spondyloepimetaphyseal and multiple epiphyseal dysplasias. Although less common, advanced carpal ossification is seen in certain skeletal dysplasias, such as Desbuquois syndrome. Stippling of the epiphyses is characteristic of the various forms of CDP, but may also be seen with Zellweger syndrome, warfarin-related embryopathy, and occasionally, lysosomal storage diseases, Smith–Lemli–Opitz syndrome, and congenital infections.

Rib shortening is most severe in the short rib-polydactyly syndromes and TD, but may also be marked in patients with asphyxiating thoracic dysplasia, chondroectodermal dysplasia, and metatropic dysplasia. It is also quite marked in children with compound skeletal dysplasias like homozygous achondroplasia.

Marked decrease in or absence of ossification of the vertebral bodies suggests a diagnosis of achondrogenesis (Figure 158-7). Deficient ossification of several vertebral bodies with good preservation of the pedicles is fairly specific for hypophosphatasia. Marked reduction in ossification of the cervical, upper thoracic, and lower lumbosacral vertebral bodies may also be seen in spondyloepiphyseal dysplasia congenita, Kniest dysplasia, and other types of spondyloepiphyseal dysplasias. Severe platyspondylia is characteristic of metatropic dysplasia, lethal perinatal osteogenesis imperfecta type II, TD, Morquio disease, and spondylometaphyseal dysplasia (Kozlowski type), among others. In TD, the vertebrae have a characteristic ossification defect so that they appear U-shaped (pedicles taller than vertebral bodies) in the thoracic spine, but with an H and inverted U shape

in the lumbar spine (Figure 158-7). In spondylometaphyseal dysplasia (Kozlowski type), the AP view of the spine is characteristic, with a central core and widened platyspondylic bodies that overhang the overfaced pedicles, reminiscent of an open staircase. Similar overfaced pedicles are seen in brachyolmia and metatropic dysplasia. Coronal clefts of the vertebrae can be seen in Kniest dysplasia, dyssegmental dysplasia Rolland–Desbuquois type, Weissenbacher–Zweymüller syndrome, atelosteogenesis, short rib-polydactyly syndrome type I, and various types of CDP.

These examples are representative of but a few of the many typical radiographic features seen in the skeletal dysplasias (Table 158-1). In many instances, an accurate diagnosis can be made by simply examining the skeletal radiographs, but in other disorders only the general type of dysplasia, such as spondyloepiphyseal dysplasia, can be readily classified, and further information may be required to diagnose its exact form. Furthermore, only part of the heterogeneity of the skeletal dysplasias has been delineated to date and there are many disorders that will require morphologic, biochemical, or molecular studies for their exact delineation.

## 158.5 CHONDRO-OSSEOUS MORPHOLOGY

Morphologic studies of chondro-osseous tissue have revealed specific abnormalities in many of the skeletal dysplasias (3–7) (Table 158-1). In certain of these disorders, histologic examination of chondro-osseous tissue may be useful in making an accurate diagnosis of the specific skeletal disorder. In other disorders, no histopathologic alterations are present, or they are nonspecific, and in these cases pathologic examination is useful only in ruling out a diagnosis.

On morphologic grounds, the chondrodysplasias can be broadly divided into those disorders (1) that show no qualitative abnormality in endochondral ossification, (2) in which there are abnormalities in cellular morphology, (3) that have abnormalities in matrix morphology, and (4) in which the abnormality is primarily localized to the area of chondro-osseous transformation. In certain disorders, abnormalities in two or more of these areas can be seen.

Conditions with minimal disturbance of endochondral ossification include achondroplasia and hypochondroplasia, where endochondral ossification is qualitatively normal, but where there are some abnormalities in the height and arrangement of proliferative columns, particularly in the center of the large growth plates. In asphyxiating thoracic dysplasia, where several workers have shown prominent lipid inclusions in chondrocytes, the growth plate organization is essentially normal, but calcified cartilage cores are seen far into the metaphysis.

In the achondrogenesis syndromes, defects in cellular morphology, matrix, and/or chondro-osseous

transformation can be seen. In achondrogenesis 1A (Houston–Harris) the chondrocytes are large and contain prominent periodic acid-Schiff (PAS)-positive inclusions. Endochondral ossification is markedly disturbed with absence of columns of proliferative cells and lack of cellular hypertrophy. In achondrogenesis II (Langer–Saldino) there is complete disruption of endochondral ossification with large chondrocytic lacunae and little intervening matrix (Figure 158-8).

In TD (Figure 158-8), and short rib-polydactyly type I and type II, there appears to be defective maturation of chondrocytes with reduced and disorganized columnization. Consequently, vascular invasion and chondro-osseous trabeculae are short and deformed with bridging between the trabeculae. Hypertrophic chondrocytes are irregularly arranged at the zone of chondro-osseous transformation and lack columnization. Bands of mesenchyme-like fibrous tissue extend from the perichondrial area into the growth plate. Periosteal bone extends up over the cartilage peripherally.

A group of conditions show dilatation of the chondrocyte rough endoplasmic reticulum (RER), consistent with defective synthesis or abnormal processing of matrix proteins that accumulate in the RER. These include pseudoachondroplasia, where the inclusions are prominent and in some cases show a highly regular RER inclusion (Figure 158-8), consisting of alternating electron-dense and electron-lucent lamellae reflecting the accumulation of cartilage oligomeric matrix protein (COMP) and other structural components of the cartilage extracellular matrix. Dilatation of the RER is seen also in spondyloepiphyseal dysplasia, spondylometaphyseal dysplasia (Kozlowski), some forms of multiple epiphyseal dysplasia, and Kniest dysplasia, among others. Thus dilatation of the RER is not a diagnostic finding, but identification of the accumulated material will lead to the biochemical and molecular basis of the disorder.

The matrix pathology in Kniest dysplasia is striking. Endochondral cartilage with paraffin processing shows dehiscence of matrix leading to the Swiss cheese cartilage appearance. With plastic embedding, these are areas of relatively acellular matrix surrounded by attenuated chondrocytes with a bubbly appearance to the perilacunar matrix.

Matrix abnormalities are also seen in diastrophic dysplasia, CDP (various types) and the Dyggve–Melchior–Clausen syndrome. In diastrophic dysplasia, the matrix of the reserve zone cartilage develops a particularly fibrillar appearance and shows areas of microscar formation (Figure 158-8). Chondrocytes, both by light microscopy and by electron microscopy, are surrounded by dense corona of large collagen fibers. The observation of similar perichondrocytic rings in atelosteogenesis II and achondrogenesis IB led to the successful search for a common pathogenetic mechanism in these three disorders; that is, mutations in the diastrophic dysplasia sulfate transporter gene. In Dyggve–Melchior–Clausen syndrome, chondrocytes by light microscopy appear to be arranged around a relatively large common lacuna, with up to 10 chondrocytes clustered around each lacuna. In CDP of both the rhizomelic recessive and X-linked dominant Conradi–Hunermann varieties, there appears to be an alteration in epiphyseal and reserve zone cartilage matrix with areas of dystrophic (nonendochondral) ossification, fibrous dysplasia, and even areas of fat deposition.

Syndromes with striking histopathologic abnormalities have been reported by Stanescu et al. (6), (fibrochondrogenesis) and Greenberg et al. (8). Achondrogenesis IA (Houston–Harris) and IB (Fraccaro) have been distinguished one from the other by morphologic as well as radiographic differences (9,10); however, achondrogenesis type II (Langer–Saldino) and hypochondrogenesis

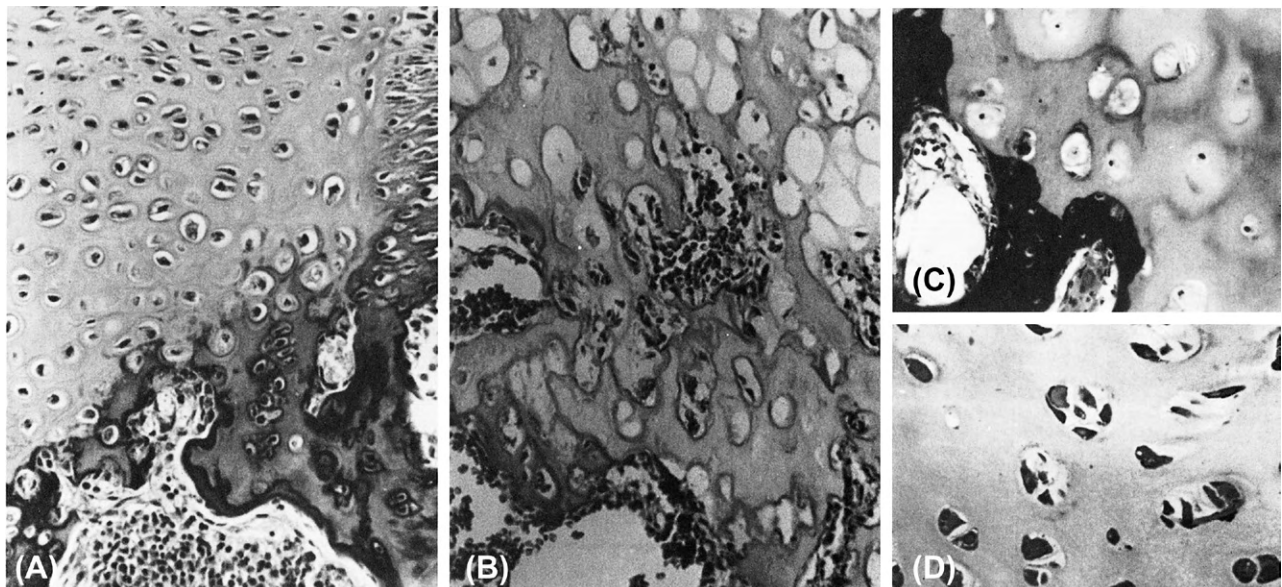


FIGURE 158-8



have identical chondro-osseous morphology and represent variability in a single disorder (type II collagen disorders). Thus pathologic analysis of chondro-osseous tissue can be of great help in the diagnostic evaluation of the chondrodysplasias, in the delineation of new syndromes, and the lumping of related disorders.

## 158.5 BIOCHEMICAL AND MOLECULAR ABNORMALITIES

Based on similarities in clinical, radiographic, and morphologic features, the chondrodysplasias have been grouped into bone dysplasia families, which Spranger had hypothesized share common pathophysiologic mechanisms. In recent years, the great progress that has been made in our knowledge of the basic biology of skeletogeny, and the advances of the human genome initiative have resulted in an explosion of knowledge concerning the biochemical and molecular defects in the skeletal dysplasias. Through various methods, including the candidate gene approach, positional cloning, human–mouse synteny, and whole-exome sequencing, specific gene defects that produce the skeletal dysplasias have been identified. These may be classified into several general categories, which are designed to reflect the pathogenesis of the disorders: (1) defects in extracellular structural proteins, (2) defects in metabolic pathways (including enzymes, ion channels, and transporters), (3) defects in folding and degradation of macromolecules, (4) defects in hormones and signal transduction mechanisms, (5) defects in nuclear proteins and transcription factors, (6) defects in oncogenes and tumor suppressor genes, (7) defects in RNA and DNA processing and metabolism, and (8) defects in intracellular structural proteins (11,12). There remain many dysplasias whose underlying molecular mechanism has yet to be discovered and may require the addition of new categories. The following are examples of each group but do not represent a complete listing in each category.

### 158.5.1 Defects in Extracellular Structural Proteins

**158.5.1.1 Type II, IX, and XI Collagens.** Since type II collagen is found primarily in cartilage, the nucleus pulposus, and the vitreous of the humor of the eye, it was postulated that type II collagen defects would be found in those disorders in which these specific tissues are affected (13,14). Indeed, biochemical and molecular defects in type II collagen have now been found in a large group of patients with phenotypes ranging from the lethal achondrogenesis II and hypochondrogenesis, to the various spondyloepiphyseal dysplasias and spondyloepimetaphyseal dysplasias (SEMD), through Kniest dysplasia, Stickler syndrome, and mild spondyloepiphyseal dysplasia, resulting in “precocious” familial osteoarthropathy (10,11). These

disorders can be grouped together under the term “type II collagenopathies” and run a continuous phenotypic spectrum. They have in common heterozygous mutations of the type II collagen gene (*COL2A1*). The majority of these patients also have electrophoretically detectable abnormalities in cartilage type II collagen. There appears to be a direct correlation between the ratio of type I to type II collagen in cartilage and the clinical severity of the disorder. Normally, type I collagen is not found in cartilage; however, in achondrogenesis type II, which is the most severe of these disorders, only type I collagen is seen in cartilage. In hypochondrogenesis, which is less severe radiographically, cartilage contains both type I collagen and post-translationally overmodified type II collagen. In the spondyloepiphyseal dysplasias, type I collagen is not seen at all, and both overmodified and normal type II collagen can be found in cartilage.

Mutations that result in a substitution for a triple helical glycine residue appear to be the most common type of mutation (11). In most cases of achondrogenesis II/hypochondrogenesis in which mutations have been defined, there are nucleotide substitutions for a glycine residue, which are clustered toward the carboxy-terminal end of the molecule. In a few cases, small deletions or splice site mutations have been found in achondrogenesis II/hypochondrogenesis (15). In the spondyloepiphyseal and SEMD, a variety of single nucleotide substitutions have been described throughout the molecule, in addition to deletions and insertions. Kniest dysplasia appears to be somewhat unique in having splice junction mutations clustered around the amino-terminal end of the molecule (16,17). Interestingly, a variant with brachydactyly (spondyloperipheral dysplasia) is associated with truncating mutations in the c-propeptide domain of *COL2A1* (18,19).

Stickler syndrome is genetically heterogeneous, with mutations described in *COL2A1*, *COL9A1*, *COL9A2*, and *COL11A1*, and there is a non-ocular form with mutations in *COL11A2*. Approximately half the patients have been found to have mutations in type II collagen, and all have resulted in premature termination of translation, presumably leading to decreased synthesis of type II collagen (20). Mutations that result in the synthesis of qualitatively abnormal type II collagen chains lead to more severe phenotypes, and mutations that result in reduced synthesis of structurally normal type II collagen produce the milder Stickler phenotype.

Type XI collagen is a heterotrimer composed of three distinct alpha chains encoded by *COL11A1*, *COL11A2*, and *COL2A1*. In Stickler syndrome, the majority of the mutations reported in *COL11A1* result in premature end of translation (21). It appears that those patients with *COL11A1* mutations have more severe and earlier hearing loss as well as a different vitreous phenotype, as compared with those patients with a *COL2A1* mutation. A variety of mutations have now been described in *COL11A2* in other cases of Stickler syndrome (22).



These appear to be distinct from the other Stickler cases, in that they do not have the severe myopia and vitreoretinal degeneration, consistent with the fact that *COL11A2* is not expressed in the vitreous humor.

OSMED (otospondylomegapeiphyseal dysplasia) is an autosomal recessive skeletal dysplasia caused by loss-of-function mutations in *COL11A2* (23). It has many similarities to the type II collagenopathies, particularly Kniest and Stickler syndromes, but is associated with profound sensorineural hearing loss and lack of ocular involvement. Another phenotype, referred to as Weissenbacher–Zweymüller syndrome or heterozygous OSMED, is essentially a form of non-ocular Stickler syndrome with skeletal radiographic findings that resolve with age. It is caused by a heterozygous glycine substitution in *COL11A2* (24). Mutations in *COL11A1* have also been described in fibrochondrogenesis.

Type IX collagen is a heterotrimeric nonfibrillar collagen composed of three  $\alpha$  chains encoded by three distinct genes: *COL9A1*, *COL9A2* and *COL9A3*. Some families with multiple epiphyseal dysplasia have specific splice site mutations predicted to cause exon skipping, which would interfere with one of the collagenous domains of type IX collagen and thus disturb its interaction with other matrix proteins. This type of mutation, and only this type, has been described for *COL9A1* (25), *COL9A2* (26,27), and *COL9A3* (28). The patients with multiple epiphyseal dysplasia (MED) due to type IX collagen gene mutations tend to have severe changes at the knees and relative hip sparing. A *COL9A2* allele causing a tryptophan substitution for a glutamine residue within the second collagenous domain of  $\alpha 2(\text{IX})$  has been associated with intervertebral disc disease in a few Finnish families (29). Homozygous loss-of-function mutations leading to Stickler syndrome phenotype have been described for *COL9A1* and *COL9A2* (30,31). This is perhaps not surprising given the overlap in expression patterns with *COL2A1* and type X1 collagen genes. Of note though, neither the heterozygous carriers of the loss-of-function mutations nor the homozygotes suffer from MED, clearly indicating that the MED-associated mutations are acting via a dominant negative mechanism.

**158.5.1.2 Type X Collagen.** Mutations in *COL10A1* have been defined in the Schmid type of metaphyseal chondrodysplasia (32,33) and presumably lead to a reduced amount of type X collagen in the matrix. This correlates with the presence of type X collagen exclusively in the hypertrophic zone of the growth plate. All the mutations appear to map to the same region of the gene, which encodes the carboxypropeptide required for triple helical formation. Other forms of metaphyseal dysplasia, however, do not have mutations in this molecule.

**158.5.1.3 Cartilage Oligomeric Matrix Protein.** Cartilage oligomeric matrix protein (COMP) is coded for by a gene on the short arm of chromosome 19 where the pseudoachondroplasia phenotype had been mapped. A variety of mutations have now been defined in the *COMP*

gene in cases of pseudoachondroplasia, as well as in a number of cases of MED (34,35). COMP is a member of the thrombospondin family of extracellular calcium-binding proteins, and the majority of mutations occur in the calmodulin-like repeat regions of the molecule, with a minority found in the globular carboxyl-terminal region (36). Some MED patients also have mutations in the gene for another cartilaginous structural protein, matrilin 3 (37,38), thus there are at least six different causative genes for MED (*COMP*, *COL9A1*, *COL9A2*, *COL9A3*, *MATN3* and *DTDST*) making this relatively mild skeletal dysplasia one of the most genetically heterogeneous.

## 158.5.2 Defects in Metabolic Pathways

### 158.5.2.1 Diastrophic Dysplasia Sulfate Transporter.

Diastrophic dysplasia was mapped to chromosome 5q by linkage disequilibrium mapping. Hästbacka et al. (39), identified a gene that closely resembled a sulfate transporter gene previously described in the rat. Reduced sulfate transport appears to have a dramatic effect on sulfation of chondroitin sulfate-containing proteoglycans, a family of highly expressed and heavily sulfated proteins that participate in the function of cartilage in supporting compressive loads. Although diastrophic dysplasia sulfate transporter (*DTDST*) is also expressed in other tissues, cartilage is hypothesized to be disproportionately affected because of the cartilage's high demand for sulfate and its avascular nature. A number of mutations in the *DTDST* gene have been described in both Finnish and non-Finnish patients with this disorder (39).

Superti-Furga et al. (40) described similar defects in sulfate uptake in cartilage from patients with the more severe disorder known as achondrogenesis 1B. Based on the similar morphologic characteristics of cartilage in these three disorders (i.e. rings of collagen around the chondrocytes), mutations in *DTDST* were sought in atelosteogenesis type II, and eventually identified. Most mutations are located within the coding region of the *DTDST* gene. There appears to be some genotype–phenotype correlation with the most severe disease—achondrogenesis 1B—due to homozygosity or compound heterozygosity for mutations resulting in a null phenotype or amino acid substitutions in the transmembrane domains. When one of these mutations is paired with a mutation having a less dramatic effect, e.g. an amino acid substitution within a cytoplasmic domain, the phenotype is less severe; i.e. either atelosteogenesis II or diastrophic dysplasia (41). The allele R279W, first reported in atelosteogenesis type II, has been shown to cause a recessive type of multiple epiphyseal dysplasia when homozygous. This form of MED shows the clinical features of brachydactyly and club foot and the interesting radiographic feature of double/multi-layer patella on lateral knee radiographs (42).

**158.5.2.2 Arylsulfatase E.** The X-linked recessive form of CDP was mapped to the short arm of the X

chromosome (Xp22.3), near the boundary of the pseudoautosomal region close to the steroid sulfatase locus. Franco et al. (43) identified three adjacent genes that encoded previously unrecognized sulfate enzymes in this region of the chromosome, which were named arylsulfatase (ARS) D, E, and F. Missense mutations in the *ARSE* gene were found in a number of male patients with CDP and in several male patients given the diagnosis of brachytelephalangic CDP. Subsequently nonsense mutations and large deletions have been reported in multiple patients with brachytelephalangic CDP (44).

**158.5.2.3 Sterol Biosynthesis.** Smith–Lemli–Opitz syndrome (SLOS) is a multiple congenital anomaly syndrome characterized by the accumulation of 7-dehydrocholesterol due to deficiency in 7-dehydrocholesterol reductase, the final step in the enzymatic synthesis of cholesterol. A small minority of patients with SLOS have stippled epiphyses. This suggested that blocks in other steps of the cholesterol biosynthesis pathway might be responsible for some forms of CDP. Investigations subsequently showed that patients with Conradi–Hunermann CDP had increased levels of 8-dehydrocholesterol and cholest-8(9)-en3bol, which suggested that the enzyme delta(8)-delta(7)-sterol isomerase was deficient. Mutations have now been found in the gene for this enzyme in several patients (45,46). Abnormal sterol elevations have also been detected in patients with hydrops-ectopic calcification-moth-eaten bones (HEM) skeletal dysplasia. Subsequently, mutations have been identified in the lamin B receptor (*LBR*) gene (47) which is now recognized as the primary sterol delta(14)-reductase, with heterozygotes having the Pelger–Huet anomaly in granulocytes as their only clinical sign (48).

### 158.5.3 Defects in Folding and Degradation of Macromolecules

**158.5.3.1 Other Lysosomal Enzyme Defects.** Mutations in a large number of genes coding a variety of lysosomal enzymes have been described in the mucopolysaccharidoses and mucopolipidoses (see Chapter 102). There is a great degree of variability in the severity of the skeletal changes, but they fall under the general heading of dysostosis multiplex (beaking of lumbar vertebrae, proximal pointing of metacarpals, J-shaped sella turcica, paddle ribs, acetabular changes) (49).

### 158.5.4 Defects in Hormones and Signal Transduction Mechanisms

**158.5.4.1 Fibroblast Growth Factor Receptor 3.** In 1994, the achondroplasia gene was mapped to the short arm of chromosome 4 (4p16.3) close to the Huntington disease locus (9,50). During the long search for the Huntington disease gene, a potential candidate for achondroplasia was identified; that is, fibroblast growth factor receptor 3 (*FGFR3*). Within a few months of the

linkage report, mutations in *FGFR3* were found to be responsible for achondroplasia (51). Of great interest was the finding that over 98% of the cases analyzed were due to the same amino acid substitution (Gly380Arg). Almost all of the cases carry the same mutation, a G to A transition at nucleotide 1138, and the remaining cases had a G to C transversion at the same nucleotide, resulting in the same amino acid substitution. Since over 80% of cases of achondroplasia represent new mutations, this represents the single most common mutation known in man.

A number of mutations in *FGFR3* have now been found in TD (52). Individuals who have thanatophoric dysplasia type II (TDII), with straight femora and severe cloverleaf skull, all had mutations on codon 650, causing a lysine to glutamic acid substitution in the intracellular TK domain. Severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN) is a rare form of achondroplasia which is also due to a mutation at codon 650 (lysine to methionine) (53). SADDAN is often compatible with survival to adulthood, and lacks the cloverleaf skull seen in TDII.

In cases of thanatophoric dysplasia type I (TDI) with curved femora, with or without cloverleaf skull, most of the mutations have been found in the extracellular domain and have in common a substitution of a cysteine for another amino acid; for example, arg248cys, ser249cys, ser371cys, tyr373cys. The French group has also described mutations in a stop codon (807) in five patients with TDI (54).

Heterozygosity for *FGFR3* mutations has also been detected in hypochondroplasia. A lysine to asparagine substitution at codon 540 accounts for roughly 50% of hypochondroplasia (55,56). A few patients have been found to have different substitutions in *FGFR3*, and many cases of hypochondroplasia are apparently unlinked to *FGFR3*.

**158.5.4.2 Parathyroid Hormone-Related Peptide Receptor.** Jansen-type metaphyseal chondrodysplasia is an autosomal dominant skeletal dysplasia with rickets-like changes in the metaphyseal areas of the bones. Schipani et al. (57) defined a mutation in the parathyroid hormone (PTH)/parathyroid hormone-related peptide (PTHrP) receptor (*PTHrP1*) (57). The heterozygous mutation caused a histidine to arginine substitution at position 223 in the PTHrP receptor protein. This mutation has now been found in several patients with Jansen metaphyseal dysplasia and a few other mutations have also been identified (58). These mutations lead to constitutive ligand-independent activation of the PTH/PTHrP receptor and consequently hypercalcemia, hypophosphatemia, and the radiographic abnormalities.

Blomstrand dysplasia is a lethal chondrodysplasia characterized by sclerosis and advanced endochondral ossification; that is, the radiographic abnormalities are the opposite of those seen in Jansen metaphyseal dysplasia. Recently, mutations resulting in absence of

functional PTH/PTHrP receptors have been identified in patients with Blomstrand dysplasia (59). A second recessive skeletal dysplasia, Eiken syndrome, has been shown to result from truncating mutations in the C-terminal cytoplasmic tail (60). This rare dysplasia has severely retarded epiphyseal ossification and long-bone changes that are somewhat similar to Jansen-type metaphyseal dysplasia (61).

### 158.5.5 Defects in Nuclear Proteins and Transcription Factors

**158.5.5.1 SOX9.** Camptomelic dysplasia of the classic long-bone variety is one of the few disorders associated with sex reversal. A substantial number of XY individuals have genital abnormalities that range from minor abnormalities of the external genitalia to complete sex reversal. Chromosomal rearrangements in cases of camptomelic dysplasia localized the gene responsible to 17q24.1–q25.1. High-resolution mapping of the candidate region positioned a breakpoint in one patient close to the *SOX9* locus. *SOX9* is a transcription factor gene structurally related to the *SRY* (sex-determining region Y gene), which encodes the factor necessary for testicular development in mammals (62) and cartilage development (63). Dosage of this transcription factor is critically important for normal development. Mutations of *SOX9* leading to haploinsufficiency have now been reported in a number of patients with classic camptomelic dysplasia who are chromosomally normal, including 46,XX and 46,XY females, as well as a 46,XX male (63,64). Several patients with camptomelic dysplasia due to chromosomal rearrangements have breakpoints outside the coding region of the gene (65) indicating that disruption of the control region is sufficient to produce the camptomelic phenotype. All of the mutations to date have been heterozygous, negating the previously proposed autosomal recessive inheritance of this disorder. Thus, camptomelic dysplasia of the classic type is an autosomal dominant trait, and intergenerational transmission has been documented (66). The gene is expressed in condensing mesenchyme, but not in mature chondro-osseous tissue. Genomic changes in the regions surrounding the *SOX9* locus can cause milder phenotypes, including isolated Pierre–Robin sequence and the brachydactyly-anonychia syndrome (67).

**158.5.5.2 SHOX.** Dyschondrosteosis was mapped to the short arm of the X chromosome following the observations of XY translocations in some patients with Leri–Weill dyschondrosteosis and the occurrence of a Madelung deformity in some Turner syndrome patients. The short stature homeobox-containing gene (*SHOX*) maps to the pseudoautosomal region of Xp and encodes a homeodomain-containing transcription factor. This gene escapes X-inactivation. Several mutations causing haploinsufficiency of *SHOX* (nonsense mutations and deletions) have been found in patients with

dyschondrosteosis, and those patients with homozygous mutations or deletions have the more severe phenotype of Langer mesomelia (68,69).

### 158.5.6 Defects in Oncogenes and Tumor Suppressor Genes

**158.5.6.1 Multiple Exostoses, Trichorhinophalangeal Syndromes.** Hereditary exostoses (EXT) is an autosomal dominant disorder that has been shown to be heterogenous on molecular grounds. *EXT1* on 8q and *EXT2* on 11p have both been cloned, and mutations in these genes account for approximately 70% of patients with multiple exostoses. There is a third locus at 19p. The Langer–Giedion syndrome is a contiguous gene disorder caused by a microdeletion on chromosome 8 with features of the trichorhinophalangeal syndrome (sparse hair, bulbous nose, short stature) and multiple exostoses. Haploinsufficiency for the transcription factor *TRPS1* is responsible for the trichorhinophalangeal phenotype (70) and haploinsufficiency for *EXT1* is responsible for the multiple exostoses phenotype.

### 158.5.7 Defects in RNA and DNA Processing and Metabolism

**158.5.7.1 Cartilage-Hair-Hypoplasia.** Cartilage-hair-hypoplasia (CHH) is an autosomal recessive condition more prevalent among the Amish and the Finnish. Radiographically, it is a metaphyseal dysplasia with brachydactyly. The pleiotropy of this disorder is often emphasized, with manifestations ranging from sparse hair to aganglionic megacolon to immune deficiency. However, variability is huge and even a skeletal-only form exists (71). Mutations in the gene *RMRP* have been reported in patients of various ethnicities. The gene product is not a protein but rather a functional RNA, which forms part of a complex dedicated to cleavage of RNA in mitochondrial DNA synthesis and processing of pre-rRNA (72).

**158.5.7.2 Shwachman–Diamond Syndrome.** Shwachman–Diamond syndrome shares cardinal features with CHH: recessively inherited metaphyseal dysplasia with immune deficiency. It has the additional feature of pancreatic insufficiency, which can be helpful in arriving at the diagnosis (73). The dysplasia is caused by mutations in the *SBDS* gene (74), which is transcribed to protein, but like the *RMRP* product, is involved in RNA metabolism (75).

### 158.5.8 Defects in Intracellular Structural Proteins

**158.5.8.1 Filamin-A-Related Disorders.** Filamin A is an actin-binding protein which forms an integral part of the cytoskeleton. Loss-of-function mutations in the X-linked gene coding for this protein were originally described in girls with periventricular nodular



heterotopias who did not have any skeletal phenotype. It came as a surprise when, several years later, gain-of-function or altered-function mutations were described in the same gene in the otopalatodigital (OPD) family of disorders: OPDI, OPDII, Melnick–Needles syndrome, and frontometaphyseal dysplasia (76). There is considerable variability in severity in this group but also enough similarity to allow their grouping prior to molecular proof. These skeletal phenotypes did not have an associated neurologic component. However, in 2004, one patient has been described with features of both forms of filamin-A-related disorders. A girl with a single missense mutation (L2439M) had both bilateral periventricular nodular heterotopias and frontometaphyseal dysplasia (77), indicating that the phenotypes are not always so separate.

**158.5.8.2 Filamin-B-Related Disorders.** Filamin B is structurally similar to filamin A and probably has a similar role within the cellular cytoskeleton. Mutations were identified in a spectrum of skeletal dysplasias with predominant spine and articular involvement (78). Recessive loss-of-function mutations were found in families with spondylcarpotarsal syndrome (a phenotype characterized by vertebral, carpal, and tarsal fusions) whereas dominant missense mutations were identified in individuals with Larsen syndrome, atelosteogenesis I, and atelosteogenesis III (78). This confirmed the clinical impression that atelosteogenesis III and Larsen syndrome were allelic but also demonstrated once more that apparently unrelated conditions such as spondylcarpotarsal and atelosteogenesis could arise from different mutation effects on the same protein, as had been seen with filamin A.

## 158.6 SUMMARY

In addition to clinical and radiographic tools, the evaluation of skeletal dysplasias often requires morphologic, biochemical, and molecular investigations. Diagnosis of the specific form of skeletal dysplasia can be of great importance in the prognosis, prevention, and treatment of these disorders, and in the provision of accurate genetic counseling. Since space constraints prevent independent discussion of each of the many chondrodysplasias in this chapter, a few of the extremely rare disorders have been left out and for the majority, the salient clinical, radiographic, pathologic, and genetic characteristics are outlined in Table 158-1. The reader is referred to the classified Further Reading at the end of this chapter for further details concerning each of these syndromes.

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## Biographies

**Dr Sheila Unger** is a Canadian-born geneticist with a longstanding interest in the skeletal dysplasias. She completed her medical genetics training at the University of Toronto and then did a 2-year fellowship with Professor Dan Cohn at UCLA in conjunction with the International Skeletal Dysplasia Registry at Cedars-Sinai hospital. She currently lives and works in Lausanne, Switzerland, and serves as the clinical and radiographic coordinator for the European Skeletal Dysplasia Network (ESDN). She is a founding member of the International Skeletal Dysplasia Society.

**Dr Ralph S Lachman, MD**, completed his pediatric residency at the Mount Sinai Hospital and served in the US Army as base pediatrician in Bad Kreuznach, Germany. The next 4 years were occupied with a diagnostic radiology residency at the New Mount Sinai Hospital. This was followed by a pediatric radiology fellowship at Harvard's, Boston Children's Hospital. He is boarded in both diagnostic radiology and pediatrics. He is also "Subspecialty boarded" in pediatric radiology, for which he was one of the original candidates. He became the first pediatric radiologist at Harbor-UCLA Medical Center, joining the faculty of medicine at UCLA. Another fateful event occurred in 1970, the simultaneous arrival of Dr Lachman and Dr David L Rimoin. Dr Rimoin was to become his co-investigator for the next 40 years, but even more so, his good friend. They both realized that they had common interests in syndromes, the evolving field of genetics, and especially in the radiological-oriented area of skeletal dysplasias. This commonality of interests led to the evolution of the International Skeletal Dysplasia Registry. Dr Lachman has published over 250 research papers in peer-reviewed journals. He has authored several books and book chapters. Recently, he solely completed the 5th edition of Taybi and Lachman's *Radiology of Syndromes, Metabolic Disorders and Skeletal Dysplasias*, which has recently been released. Dr Lachman has been on the UCLA faculty for the last 40 years. He has been Professor Emeritus since 1999. Recently, he was appointed Visiting Scholar and then Clinical Professor at Stanford University. He has served for many years as medical advisor for the Little People of America.



**David L Rimoin MD, PhD** During the final preparation of this book, we lost a major pioneer in the field of Medical Genetics: David L Rimoin, MD, PhD. Dr. He died May 27th, 2012 following a diagnosis of pancreatic cancer only a few days earlier, having seen patients even on the Tuesday before he died. Dr Rimoin's achievements as Steven Spielberg Chair and Director of the Medical Genetics Institute at Cedars-Sinai, as the former Chairman of the Department of Pediatrics and Professor of Medicine and of Pediatrics at UCLA and founding director of the multi-campus UCLA Genetics Training Program, were profound. Children, adults and families from across the country and the world who turned to him for his medical expertise have lost a uniquely skilled and caring physician who was singularly devoted to their health and wellbeing. His medical contributions will continue to bring healing for generations --through the books he authored, his leadership and mentoring, the papers he presented, his lectures and speeches, and through the research that gave birth to lasting medical progress. We honor David Rimoin's passion for excellence, scholarship, innovation, his contributions to transformational medicine and his ceaseless kindness and integrity.

## Abnormalities of Bone Structure

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Oregon Health and Science University, Portland OR, USA**GLOSSARY**

**Endochondral ossification** – a type of bone formation in which bone is first generated as a cartilage template, which is subsequently replaced by bone proper. It is responsible for the formation and growth of most of the skeleton.

**Loss of heterogeneity** – this occurs when there is loss of normal function of one allele of a gene in a cell in which the other allele is already inactivated. In genetic disease, loss of the first allele is usually inherited, while loss of the second allele results from somatic mutation.

**Membranous ossification** – a type of bone formation in which bone is generated directly from fibrous mesenchymal or bone tissues with no cartilage template. The calvaria, clavicles, body of the mandible, spinous processes of the vertebrae, and part of the pelvis arise in this fashion.

**Somatic mosaicism** – when gene mutations cause genetic disease, they typically arise in the sperm or egg and are present in all cells of the affected individual. However, mutations can sometimes occur later in embryologic development and only in somatic cells (non-germ cells). In this case, the individual has two populations of somatic cells, normal and mutant, and disease occurs only in the tissues and cells derived from the mutant cells.

**159.1 INTRODUCTION**

In the past few decades, many disorders have been delineated that are characterized by abnormal skeletal development, collectively termed the skeletal dysplasias. Many of these, designated the osteochondrodysplasias, are thought to result from disturbances in the normal ossification process. The osteochondrodysplasias have been subdivided into three categories: defects of growth of tubular bones and/or spine, disorganized development of cartilage and fibrous components of the skeleton, and abnormalities of density of cortical diaphyseal structure and/or metaphyseal model (1). The disorders comprising the second category are the subject of this chapter. They can best be understood in the context of the normal ossification process.

The skeleton normally develops and grows through a combination of two distinct forms of ossification:

endochondral and membranous (2). In the latter form, bone develops directly from fibrous tissue. The calvaria, clavicles, body of the mandible, spinous processes of the vertebrae, and part of the pelvis arise in this fashion. In addition, diaphyseal widening of individual bones occurs in this manner. The remainder of the skeleton develops through endochondral ossification, a more complex process in which a cartilage model for each bone is formed and is subsequently transformed into true bone. The cartilage anlagen arise early in embryonic development, and by mid-pregnancy, ossification has spread throughout each bone, leaving only the epiphyses as cartilaginous structures. Continued proliferation and hypertrophy of the cartilage bordering the ossification front (the endochondral growth plate) is responsible for linear growth of the bone. Highly organized zones of proliferative and hypertrophic cartilage can be identified within the growth plate. As the slowly progressing ossification front penetrates the cartilage at the cartilage–bone interface, true bone is laid down. Initially, it is immature or woven bone, but with modeling it is replaced by mature or lamellar bone (The maturation process occurs in membranous ossification as well.) Secondary centers of ossification, which exhibit a similar sequence of events, also develop in the epiphyses during late fetal life and throughout childhood. With completion of puberty, growth plate activity ceases and the structure is transformed into bone.

Certain generalizations can be made about the disorders discussed in this chapter. Several of them, such as hereditary multiple exostoses or enchondromatosis, are characterized by aberrant growth plate activity. The lesions in these disorders are restricted to bones that arise by endochondral ossification. The activity of these lesions tends to parallel that of the normal growth plate (i.e. growth during childhood, quiescence after puberty). By contrast, bone maturation is disturbed in some of the other disorders, such as fibrous dysplasia of bone. Puberty seems to have little effect on these lesions, which may affect all bones. Thus, the clinical features in these disorders are often determined by the relationship of the specific abnormality to the normal ossification process.

## 159.2 DYSPLASIA EPIPHYSEALIS HEMIMELICA

Dysplasia epiphysealis hemimelica (DEH) is a developmental disorder of childhood characterized by asymmetrical growth of epiphyseal cartilage. Originally described as tarsomegalie in 1926, several designations have been used: tarsoepiphyseal aclasis, chondrodys-trophy epiphysairi, benign epiphyseal osteochondroma, carpal osteochondroma, osteochondroma of the distal femoral epiphysis, epiphysealis hyperplasia, and intra-articular osteochondroma of the astragalus (3,4). The term DEH was introduced by Fairbank (1956) to distinguish this condition from multiple epiphyseal dysplasia and chondrodysplasia punctata. DEH has been extensively reviewed by several authors (3–7).

Males are affected approximately three times as often as females. Symptoms usually arise between the ages of 2 and 14 years but have been described as early as 18 months (8). In a few cases, the diagnosis has been established during adulthood. Joint deformity, especially at the knee and ankle, restricted motion, and occasionally pain call attention to the condition. Bony hard swelling is found at the sites of the lesions, which are usually confined to one side of a limb. The medial side is mainly affected in the leg, whereas in the arm, which is involved infrequently, the radial side predominates. The most common sites in order of decreasing frequency are talus, distal femoral epiphysis, distal tibial epiphysis, proximal tibial epiphysis, tarsal navicular, median cuneiform, and distal fibular epiphysis. The axial skeleton is rarely involved, but lesions of the pubis (3), acetabulum (9), and scapula (10) have been reported. Multiple lesions occur in about two-thirds of the patients.

Skeletal radiographs reveal irregular enlargement of the affected epiphyses and tarsal and carpal bones. There is usually a lobulated multicentric mass adjacent to one side of the epiphysis or bone. In young children, multiple ossification centers may be seen within this mass, but with time these fuse to form a single ossified mass that eventually becomes part of the adjacent bone. Mild widening of the metaphyses of affected bones may also be seen. When the talus is affected, the ossification centers may appear prematurely. Magnetic resonance imaging (MRI) may be useful to define the extent of the lesions (11).

Histologic examination of the lesions shows nests of proliferating and hypertrophic chondrocytes surrounding ossification centers. The appearance resembles that seen at secondary ossification centers; it is also indistinguishable from the pattern observed in osteochondral exostoses.

The lesions and their secondary deformities tend to increase during the first few years of life, after which they become somewhat quiescent and enlarge only slightly as the child continues to grow. Both shortening and lengthening of the affected limbs compared with unaffected limbs has been described. New ossification centers may

appear radiographically; however, as described earlier, these fuse with each other and the normal portion of the bone. After puberty, there is little change. Treatment must be individualized and usually involves excision of the lesions that contribute to deformities and interfere with normal function. Malignant degeneration of the lesions has not been described.

All cases of DEH reported to date have been sporadic. Moreover, in one instance, one of a pair of monozygotic twins was affected (12). Hensinger et al. (1974), however, reported the autosomal dominant transmission of DEH together with intracapsular chondromas, extraskeletal chondromas, and osteochondromas (13).

## 159.3 HEREDITARY MULTIPLE EXOSTOSES

The formation of many cartilage-capped exostoses that give rise to deformities of the growing skeleton characterizes hereditary multiple exostoses. The syndrome has been recognized as a familial entity for more than a century. Many terms, including diaphyseal aclasis, multiple osteochondromas, multiple osteochondral exostoses, hereditary deforming osteochondrodysplasia, and multiple exostoses, have also been applied to it.

The clinical and radiographic features have been delineated by Solomon and Crandall et al. (14–16). The vast majority of patients are discovered during the first decade of life, often by the age of 2 years. Bony lumps of the scapula and tibia are usually noted first, probably because of the conspicuous nature of these areas. Skeletal radiographs at this time, however, usually show lesions in other bones. Palpable masses have been detected soon after birth in affected infants known to be at risk for the condition. The lesions characteristically appear and increase in size during childhood. After completion of puberty, no new lesions form, and the activity of existing lesions ceases. Asymptomatic ones may be detected by radiograph at any age. In addition, some lesions may actually disappear with time. The lesions are juxtaepiphyseal in origin and most frequently reside at the ends of tubular bones, vertebral borders of the scapula, iliac crest, and ribs. Involvement of vertebral bodies, patella, and carpal and tarsal bones is rare; however, the lesions can arise in any bone that develops by endochondral ossification. The radiographic appearance of the individual lesions varies considerably. In general, they appear as projections of the bone from which they come, the overlying cortex and inner marrow cavity are continuous with those of the parent bone.

The earliest lesion viewed radiographically is an asymmetrical overgrowth of the metaphyseal cortical bone, which lies immediately adjacent to the growth plate. As the parent bone lengthens, two patterns may evolve. Normal growth of the juxtaepiphyseal metaphyseal bone may resume, so that the exostosis appears to migrate toward the diaphysis as the bone elongates (Figure 159-1).



**FIGURE 159-1** Radiograph showing exostosis of the diaphysis of the humerus in a 16-year-old boy with hereditary multiple exostoses.

Alternatively, the exostosis may continue to expand at the metaphysis, producing an irregular club- or sometimes cauliflower-shaped end of the bone ([Figure 159-2](#)). Pedunculated lesions, which point away from the joint, may also be seen near the metaphysis. The behavior of the lesions is unpredictable, varying from one bone to another within the same individual and even within the same bone ([14](#)).

In two-thirds of patients, the clinical picture is dominated by skeletal deformities distinct from the actual exostoses. They result from reduced linear growth of the affected long bones. In a study of 76 patients, Solomon (1961) found that forearm deformities including bowed radius, conical ulna, and radiohumeral dislocations were present in 50% of patients, whereas genu valgum, valgus deformities of the ankles, and deformities of the hands were present in 21%, 45%, and 17%, respectively ([17](#)).



**FIGURE 159-2** Radiograph showing exostosis of the proximal humerus in an 18-year-old woman with hereditary multiple exostoses.

Except for the valgus deformities of the ankles, these deformities were asymmetrical. Scoliosis and pelvic and thoracic deformities were occasionally found as well. Short stature due to shortened extremities was common (41%) but was rarely severe. Shapiro et al. (1979) noted frequent limb length discrepancies ([18](#)). Crandall et al. (1984) estimated that half of affected persons are moderately or severely handicapped ([16](#)).

The most serious complication of this syndrome is malignant degeneration of the exostoses. Although development of chondrosarcoma has been reported in as many as 25% of patients ([19](#)), the actual incidence is probably much lower, in the range of 3–10% of patients ([20–22](#)). Some families may be more prone to malignant degeneration than others ([16](#)). The tumors tend to occur in the pelvic girdle, most commonly arising from the ilium or proximal femur, and less often in the shoulder girdle. The diagnosis is most frequently made in the early 30s, and the first signs are usually swelling and, rarely, pain or neurologic symptoms ([21](#)). The tumors generally grow slowly and metastasize late. Because the



exostoses do not normally enlarge after completion of puberty, any swelling or pain associated with the lesion, especially in the pelvic or shoulder region, should suggest malignant change. Other rare complications include large pelvic exostoses that cause urinary obstruction and renal failure, malposition of a pregnant uterus, intestinal obstruction, and spinal cord compression (14,23).

Treatment depends on the particular deformities and complications that occur, although most patients require surgery. The most common procedures include removal of exostoses that interfere with function, contribute to deformity, produce compression, or are suspected of undergoing malignant degeneration; epiphysiodesis to compensate for reduced growth of affected bones; excision of the radial head in cases of humeroradial dislocation; and correctional osteotomies for specific deformities (18).

Examination of an exostosis histologically shows a projection of trabecular bone covered by a cartilage cap. In children and adolescents, columns of normally appearing proliferating and hypertrophic chondrocytes are found along the bony margin. The appearance is very similar to a normal growth plate except that the cartilage–bone interface is irregular, and collections of hypertrophic chondrocytes are found in the bone (24). In the adult, the cartilage is reduced to a thin rim or is absent altogether.

Hereditary multiple exostoses is inherited as an autosomal dominant trait. When studied radiographically, there is essentially complete penetrance. Males and females are equally affected, but there is slight tendency toward similarity in the distribution and type of lesions and deformities within families (15). Three genetic loci have been identified to date (25–28). In most families, the condition maps to chromosome 8q24.11–q24.13, the region to which the Langer–Giedion syndrome has been localized (see section 159.4), which has been designated *EXT1*. A number of mutations have been found in *EXT1* and to lesser extent in *EXT2*, which maps to chromosome 11p11–p13 (29–37). The risk of malignant degeneration is greater for patients with mutations of *EXT1* compared to those with *EXT2* mutations (38). The third locus maps to chromosome 19p, but it is a minor locus for hereditary multiple exostoses (39,40).

The mechanism by which *EXT* mutations cause exostoses is not well understood. However, the detection of loss of heterozygosity for the *EXT* loci in tumors, combined with the loss-of-function in the reported mutations, suggests that the *EXT* gene products function as tumor suppressors (31,33,37,41,42). *EXT1* and *EXT2* encode type II transmembrane glycoproteins that were first identified in the endoplasmic reticulum in association with glycosyltransferase activity (43,44). It appears that complexes containing products of both *EXT1* and *EXT2* and localized to the Golgi apparatus constitute the active glycosyltransferase enzyme(s), which are involved in the synthesis and polymerization of heparan sulfate (45,46). Disturbances in cell surface heparan sulfate may interfere with signaling

involving hedgehog proteins and potentially other growth factors (47). In fact, Koziel et al. have proposed that development of exostoses in this syndrome reflects local activation of Indian hedgehog signaling (48). Mouse modeling experiments have established that clonal homozygous inactivation of *Ext1* in growth plate chondrocytes leads to osteochondromas typical of human exostoses (49,50). These findings implicate clonal loss of heterozygosity for *EXT1* or *EXT2* in these cells as the mechanism responsible for exostoses in humans. Presumably the resulting loss of glycosyltransferase enzyme activity and disturbance of heparan sulfate synthesis disturbs the local regulatory circuits that modulate the fate of these cells.

#### 159.4 LANGER–GIEDION SYNDROME

In Langer–Giedion syndrome, multiple exostoses occur as a component of a multisystem disorder. Also known as the trichorhinophalangeal (TRP) syndrome type II and acrodysplasia with exostoses, this syndrome is rare. The first two cases were described independently by Langer (1968) and Giedion (1969) (51,52). Hall, together with Langer, Giedion, and others, reported five additional patients and delineated the syndrome in 1974 (53). Several more cases have been added to the literature (54–62). Heavy eyebrows, large bulbous nose with thickened alae and septum, prominent elongated philtrum, and thin upper lip together with mild microcephaly, large poorly developed protruding ears, and sparse scalp hair give rise to a characteristic craniofacial appearance. Other consistent features have included mental retardation, delay in the onset of speech, short stature, multiple exostoses, cone-shaped epiphyses, and loose skin. The mental retardation is generally mild to moderate in degree. In one patient who was initially considered to be mentally retarded, intelligence was eventually determined to be normal after a profound hearing deficit was found (57). A hearing loss has been detected in half the patients tested. The delay of speech development has been observed in at least two patients with normal audiograms, however, and it appears to be out of proportion to the degree of mental retardation.

The multiple exostoses are similar in clinical behavior and radiographic appearance to those seen in hereditary multiple exostoses. Diminished linear growth of affected bones and secondary deformities occur as well. No cases of malignant degeneration have been reported, but most of the patients described to date have been children.

Several types of cone-shaped epiphyses have been described by Giedion (1969) (52). All patients with this syndrome have had the type 12, in which the distal epiphyses of the metacarpals and proximal epiphyses of the phalanges of the hand are affected. Small conical-shaped epiphyses appear to invaginate into the adjacent metaphyses, often with fusion and widening of the metaphyses (53). These abnormalities are not visible radiographically before the age of 3–4 years, however, because ossification

of the epiphyses in the hand bones is insufficient before this age. Epiphyseal irregularities have been found in other parts of the skeleton. In particular, Perthes-like changes in the capital femoral epiphyses have been seen in half the patients. This generalized epiphyseal disturbance is probably responsible for the short stature exhibited in all the cases.

The occurrence of multiple fractures has been mentioned as a component of the syndrome; however, it has been demonstrated in only three of the thirteen patients. Moreover, one patient had only a single traumatic fracture of the humerus (63), and the two others were identical twins who showed generalized skeletal demineralization. In fact, Hall et al. (1974) questioned whether this feature was a part of the syndrome or simply a second abnormality restricted to the twins (53).

Although most of the children have cutaneous involvement, it has varied with age. The loose skin seems to be most striking during early childhood and regresses or even disappears between the ages of 6 and 14 years. Small, brown to black maculopapular nevi are found on the face, scalp, neck, and upper trunk of the older children but have not been seen before age 4 years.

Small deletions of the long arm of chromosome 8 (q24.11–q24.13) have been identified in most patients with Langer–Giedion syndrome in whom high-resolution banding has been done (58,60,62,64). Such studies initially confused the nosology of the TRP syndromes, however, because deletions typical of TRP type II have been detected in two patients with clinical and radiographic features of TRP type I (61,65). Ludecke et al. (1991) showed that 15 of 16 patients had loss of genetic material; the shortest region of deletion overlap was estimated to be less than 2 Mb (66). It was subsequently shown that both the *EXT1* gene and the gene mutated in TRP syndrome types I (*TRPS1*) and III reside within the region deleted in Langer–Giedion syndrome (30,67–70).

### 159.5 ENCHONDROMATOSIS

Enchondromatosis is another rare disorder of the developing skeleton. It was originally described by Ollier, who called it dyschondroplasia, but it has been variably referred to as Ollier disease, multiple enchondromatosis, multiple enchondromas, and internal enchondromatosis (71). It must be distinguished from a similar but yet distinct disorder, Maffucci syndrome, in which the combination of multiple enchondromas and cutaneous hemangiomas and other tumors is found.

The manifestations of the disorder result from the occurrence of cartilaginous tumors in the metaphyses of bones that are formed in cartilage. They have been best described by Fairbank (1948) (71). Both long and short tubular bones are preferentially involved, and the more rapidly growing ends of these bones are the most frequently affected sites. For example, lesions in the region of the knee joint and at the lower ends of the

radius and ulna are particularly common sites, whereas the phalanges and the pelvis are somewhat less common. The scapula, ulna, ribs, sternum, base of the skull, and facial bones are rarely affected, and the cuboid bones (e.g. the vertebrae, carpal, and tarsal bones) usually escape. Tumors do not occur in the calvaria. By definition, more than one lesion must be present; however, the involvement may vary considerably from enchondromas affecting a single limb to tumors throughout the skeleton. In the latter instance, the lesions are asymmetrical and bilateral in most cases (72).

The characteristic deformities result from direct expansion of the tumors and from reduced linear growth of the affected bones. The most common deformities include phalangeal enlargement, asymmetrical shortening of the limbs, bowing of the long bones, ulnar deviation of the wrist, dislocation of the radial head, and genu valgum. In rare instances when the base of the skull is involved, facial asymmetry and cranial nerve compression may occur. Fractures of the affected bones are uncommon. Considerable variability has been noted regarding the severity of the deformities, ranging from asymptomatic lesions detected only by radiographs to extensive disfigurement and disability (e.g. massive swelling of fingers and toes).

The disorder is usually detected during childhood but has been identified at birth in an infant who exhibited asymmetrical limb shortening (72). The appearance of new lesions as well as tumor growth and progression of deformities occurs in an unpredictable fashion during childhood. The lesions often regress and deformities stabilize after puberty. Renewed growth during adulthood suggests sarcomatous degeneration. The occurrence of this complication is probably less than in Maffucci syndrome (see Section 159.6), but actual figures are not known.

Radiographically, the lesions vary from minute foci of incompletely calcified epiphyseal cartilage extending linearly from the growth plate into the metaphysis of the bone to large tumorous masses of cartilage that produce extensive metaphyseal enlargement (Figure 159-3). Irregular calcifications are often found within the tumor. Thinning and disruption of the cortex of the overlying bone may occur; and there may be abnormal metaphyseal modeling. In addition, radiolucent defects often extend into the shaft of the bone (73). The radiographic changes of enchondromatosis may be influenced by age (72). For example, despite shortening of a bone, typical lesions may not be seen during infancy. Furthermore, there may be a gradual “filling in” of the lesions with normal-appearing bone after puberty.

The tumor pathologically consists of lobulated masses of irregularly dispersed chondrocytes encased within bone. Proliferative and hypertrophic cells are found, and some areas resemble the normal endochondral growth plate. In tissue from older patients, intracartilaginous ossification may be seen (24).

Enchondromatosis has occurred in a sporadic fashion in almost all cases reported to date. Both sexes



**FIGURE 159-3** Radiograph showing enchondromas in distal femur and proximal and distal tibia and fibula in a 12-year-old boy with enchondromatosis.

are affected, but it is more common in males (71). The source of the metaphyseal enchondromas is not known, although an activating mutation of the parathyroid hormone/parathyroid-hormone-related protein (PTH/PTHrP) type I receptor (*PTHr1*) has been identified in a few patients or tumors (74,75). Expression of the mutant receptor in vitro and in transgenic mice constitutively activated Indian hedgehog signaling and produced enchondromal-like lesions, raising the possibility that enchondromas could result from somatic *PTHr1* mutations.

### 159.6 MAFFUCCI SYNDROME

In 1891, an Italian, Maffucci, described a patient with enchondromas and superficial hemangiomas. Today, the syndrome in which this combination occurs bears his name, however, it is recognized that the manifestations are much more extensive. The skeletal manifestations are similar to those seen in isolated enchondromatosis. Expanding cartilaginous tumors in the metaphyses of tubular bones, primarily, develop during childhood. The metacarpals and phalanges of the hand are the most common sites, although lesions are frequently observed in the tibia, fibula, femur, radius, ulna, and humerus as well (76). The tumors tend to be asymmetrical and bilateral and cannot be distinguished radiographically or histologically from those found in patients with enchondromatosis. Likewise, tumor expansion and shortening of involved bones lead to a similar array of deformities. Spontaneous fractures through areas of advanced rarefaction have been reported in 26% of patients in one series (77). Cranial nerve palsies due to involvement of the base of the skull have also been described (78).

The major non-skeletal manifestation of the disorder is the occurrence of simple or cavernous cutaneous hemangiomas (77,79). Usually located on the limbs, they lie in the deep layers of the skin and subcutaneous tissues. Their size varies from a few millimeters in diameter to many centimeters. They are not limited to the skin but may also be found throughout the viscera. There is a slight tendency for the hemangiomas and enchondromas to show a similar distribution with regard to laterality, but no direct relationship exists between the two. Phlebectasia is commonly observed, and thrombosis and subsequent calcification often occur within vascular spaces. In fact, phleboliths seen on radiography are found in nearly half the patients with this condition (77). In reporting a patient with enchondromas and fibromuscular dysplasia of cerebral arteries, Slagsvold et al. (1977) speculated that this arterial lesion might represent another vascular manifestation of the disorder (80). Lymphangiomatosis has been described in several cases (76,78). Other non-skeletal manifestations seen in the Maffucci syndrome include vitiligo, hyperpigmentation, and nevi (78). Most soft tissue lesions are painless, although mild discomfort as well as increased skin temperature may accompany the hemangiomas (76).

The skeletal abnormalities do not usually present until early or mid-childhood, but the hemangiomas are often detected at or shortly after birth. The clinical picture through puberty is dominated by the skeletal lesions; as with enchondromatosis, it is unpredictable. After the completion of puberty, however, the enchondromas do not usually progress, although this is not invariable (77).

A predisposition to neoplasia during adulthood is well established. The greatest risk is for sarcomatous degeneration of the enchondromas, which has been estimated to occur in 15–30% of patients (76,77,79,81,82). The



risk does not correlate with the severity of involvement. Malignant degeneration of hemangiomas and lymphangiomas also occurs, and patients may develop multiple primary tumors. There are also reports of many other malignant and benign tumors occurring in patients with Maffucci syndrome. These include osteosarcoma, cartilaginous hamartoma, fibrosarcoma, glioma, mesenchymal ovarian carcinoma, carcinoma of the pancreas, uterine polyps and fibroids, adrenal cortical adenomas, thecoma of the ovary, multiple fibromas, and leukemia (75,76,81–85). Chromophobe adenoma of the pituitary has been noted in seven of 114 reported cases (86). Because of the isolated nature of many of these reports, it is not clear if these associations are significant or simply coincidental. Sudden enlargement of either skeletal or non-skeletal tumors during adulthood, however, should make one suspicious of malignant degeneration.

The treatment consists of orthopedic and surgical intervention to minimize deformities and for cosmetic purposes. Careful surveillance for malignant degeneration of both skeletal and non-skeletal tumors, especially in the brain and abdomen, is essential.

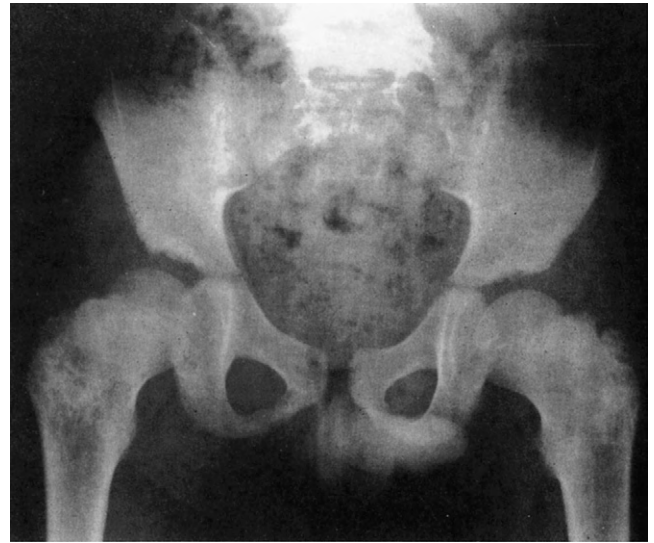
Maffucci syndrome occurs in all races with equal sex distribution. All cases have been sporadic, and affected women have produced unaffected offspring (76). Normal karyotypes have been obtained in several instances (76). To explain the many mesenchymal tumors, it is generally thought that the syndrome results from a generalized defect in the mesodermal tissues (76–78); however, the nature of this defect is unknown.

### 159.7 METACHONDROMATOSIS

Metachondromatosis is a distinct syndrome in which both exostoses and enchondromas are found. Only a few cases have been reported. Thirteen were members of three families in which the trait showed autosomal dominant transmission (87–89). Other cases have been described by Lachman et al. (1974), Keret and Bassett (1990), Wenger et al. (1991), Ikegawa et al. (1992), and Wittram and Carty (1995) (90–94).

Clinically, patients may be short and usually present with exostoses, preferentially affecting the tubular bones of the hands and feet. In contrast to the lesions seen in hereditary multiple exostoses that point away from the epiphyses, the exostoses in this syndrome point toward the joint. In addition to the exostoses, irregularly calcified lesions that are sometimes separated from bone have been observed near the epiphyses (88). An unusual feature of the syndrome is the tendency for the tumors to regress and actually disappear in adulthood (87–89).

The enchondromas are found in the metaphyses of long bones and in the iliac crest, which is an unusual location for the tumors in enchondromatosis and Maffucci syndrome (Figure 159-4). Irregularity of the endplates of the vertebral bodies has also been seen. Presumably, this defect, together with the metaphyseal involvement



**FIGURE 159-4** Anteroposterior radiograph of pelvis showing enchondromas in both iliac crest and exostoses of femoral necks in an 8-year-old with metachondromatosis. (Courtesy of R. S. Lachman, Los Angeles, CA.)

by enchondromas, is responsible for the short stature. Avascular necrosis of the capital femoral epiphysis has been reported in a few cases (91–93). The risk of malignant degeneration of the cartilaginous tumors is not known.

Loss-of-function mutations of *PTPN11* (protein tyrosine phosphatase, non-receptor type 11), which encodes the protein tyrosine phosphatase SHP2, have been detected in two multigenerational families with metachondromatosis (95). *PTPN11* functions as a regulator of the RAS/MAPK pathway downstream of many receptor tyrosine kinases. Mutations of *PTPN11* have been identified in the Noonan, Noonan-like, and LEOPARD syndromes.

### 159.8 FIBROUS DYSPLASIA OF BONE

Fibrous dysplasia of bone is characterized by the replacement of bone by dysplastic fibrous tissue. Although initially confused with osteitis fibrosa cystica of hyperparathyroidism, it was recognized as a separate entity in 1937 by Albright et al., who described the skeletal lesions in association with increased skin pigmentation and endocrine disturbances (96). Five years later, Lichtenstein and Jaffe (1942) delineated the pathologic features of the skeletal lesions, which they termed fibrous dysplasia of bone, and observed that the extraskeletal abnormalities did not occur when only a single bone was involved (97). The disorder has since been defined further by several reviews in which the clinical and radiographic features of nearly 300 patients have been examined (98–104). Although there has been a tendency to classify fibrous dysplasia on the basis of whether extraskeletal features are found, it appears more appropriate to divide it on the basis of whether the lesions involve one or more than one



bone, because the extraskeletal features occur only in the latter instance.

Hypotheses that the lesions of this condition result from autonomous hyperplasia of multiple endocrine glands and other tissues due to disturbed regulation of cyclic adenosine monophosphate (cAMP) (105–109) and that they reflect mosaicism of a dominant gene mutation (110) evolved in the 1970s and 1980s. Both proved to be correct. It is now clear that the disorder results from mutations of the gene encoding the heterotrimeric signal transducer guanine nucleotide-binding protein, alpha-stimulating activity polypeptide 1 (GNAS1), which couples cell surface receptors to adenylyl-cyclase-dependent downstream pathways (111–115). Substitution of either histidine or cysteine for arginine at position 201 of GNAS1 leads to ligand-independent activation of relevant signaling pathways. It should be noted that loss-of-function mutations of *GNAS1* are found in patients with Albright hereditary osteodystrophy.

Current dogma holds that all fibrous dysplasia patients with activating *GNAS1* mutations exhibit somatic mosaicism (115,116). If the mutation occurs early in life, mutation-bearing cells are likely to reside in many locations, giving rise to polyostotic fibrous dysplasia and extraskeletal lesions of McCune–Albright syndrome. Monostotic lesions reflect isolated somatic mutations in bone cells that occur later in life.

### 159.8.1 Monostotic Fibrous Dysplasia

Patients with monostotic disease are thought to be much more common than those with the polyostotic form. All cases have occurred on a sporadic basis. Males and females are equally affected. The most frequently affected sites in monostotic fibrous dysplasia are the craniofacial bones, including the skull, maxilla, and mandible; ribs; femur; tibia; and humerus. The pelvis, other long bones, vertebrae, and tarsal bones are occasionally involved (99,103,104). The lesions in the extremities usually present during adolescence with pain, swelling, and pathologic fractures. The craniofacial lesions are often heralded by swelling, asymmetrical growth of the skull or face, and occasionally unilateral proptosis; they tend to occur in the second and third decade. Rib lesions are often asymptomatic and may be discovered at any age, often as an incidental finding on a chest X-ray (104).

The earliest radiographic change consists of a loss of density at the site of the lesion. Later, there is expansion of the bone with erosion and thinning of the cortex from within. The shaft may exhibit a “ground-glass” appearance, on which prominent trabecula are superimposed. In long bones, the lesions appear to begin in the metaphysis and extend into the diaphysis (101). Sclerosis may be associated with involvement of the facial bones.

The lesions tend to grow slowly before adolescence; however, their activity is variable (117,118). After

puberty, they usually become inactive, but Henry (1969) observed that several patients developed symptoms, often pathologic fractures of long bones, beyond this age (104). He also noted fibrous dysplasia may become activated or reactivated during pregnancy. Malignant degeneration does occur but rarely. Schwartz and Alpert (1964) calculated the incidence to be approximately 0.4% of patients (119). Osteogenic sarcoma was the predominant tumor and occurred at an average age of 32 years, following a mean lag time of 13.5 years after the initial presentation. Several of the patients in whom malignant degeneration occurred have received previous radiation therapy (99,119,120).

Although the radiographic appearance of fibrous dysplasia is characteristic, it is not pathognomonic. Therefore, in the monostotic form, a biopsy is necessary to confirm the diagnosis. Histologically, the lesions consist of poorly defined, partially calcified trabecula of bone embedded within dense cellular fibrous tissue. The bone is immature (woven) in type; no lamellar (mature) bone is found. The trabecula are rimmed by only a few osteoblasts, and there is a paucity of osteoclasts (24,98,99,102). These changes seem to vary little with age. Cysts, dense fibrosis, islands of cartilage, and lamellar transformation of woven bone have also been described. Although some of these changes may be the consequence of previous surgery and trauma (102), they largely reflect the effects of constitutive activation of cAMP-dependent signaling pathways in bone cells. The effects include enhanced proliferation, and reduced and inappropriate osteoblastic differentiation (114,121,122).

Conventional treatment of monostotic fibrous dysplasia involves surgery to remove abnormal tissue. If this is not possible, curettage of the lesion and packing it with bone chips is indicated. Treatment is successful when the lesion is completely removed. However, if not, recurrence is common. Infusion of the bisphosphonate pamidronate may provide an effective alternative treatment (123).

### 159.8.2 Polyostotic Fibrous Dysplasia

In polyostotic fibrous dysplasia, McCune–Albright or Albright syndrome, bone lesions that are identical to those found in the monostotic form of the disease occur in multiple bones, in association with abnormal skin pigmentation and a variety of endocrine disturbances. The bone lesions are the only invariable component of the syndrome as classically defined. They are found throughout the skeleton, although the most frequent sites are the femur, tibia, pelvis, phalanges, ribs, humerus, and base of the skull (99). The radiographic appearance of the extracranial lesions is essentially the same as seen in monostotic fibrous dysplasia (Figure 159-5). Cranial involvement is usually characterized by diffuse sclerosis of the base of the skull, often involving the sphenoid, sella turcica, and roof of the orbit, together with thickening



**FIGURE 159-5** Radiograph showing fibrous lesions in the proximal tibia in a 14-year-old girl with polyostotic fibrous dysplasia.

of the occiput and obliteration of the paranasal sinuses. Radiolucent areas may be scattered through these areas of increased density.

The bone lesions are usually evident by the age of 10 years, and patients most often present with a limp, leg pain, or fracture (99). Deformities are common; they include leg length discrepancy, coxa vara, shepherd's-crook deformity of the femur, bowing of the tibia, Harrison's groove, and protrusio acetabuli. Most patients have at least one fracture, and many have repeated ones. Extensive craniofacial involvement may produce facial deformities as well as cranial nerve compression, hearing loss, sinusitis, and lacrimal duct obstruction (100). Spinal cord compression has been associated with vertebral involvement (124). The progression of the deformities is often associated with the extension of existing lesions. Puberty seems to have no effect on such extension or on the incidence of fractures. New lesions may appear, usually after puberty, and spontaneous improvement rarely occurs (99). An elevation of serum alkaline phosphatase may be found.

Malignant degeneration has been reported more often in polyostotic than in monostotic fibrous dysplasia (119). It is thought that the higher incidence in the former is due to the greater number of lesions; the risk per lesion is the same in both (118). The risk is relatively low, 0.4% of patients, and the complication occurs less often in the craniofacial region than in other parts of the skeleton (100). Several of the patients with this complication have received prior radiation therapy (118).

The extraskeletal manifestations of polyostotic fibrous dysplasia involve the skin and endocrine glands. The cutaneous lesions consist of flat patches of brown pigmentation. They follow an irregular contour and are frequently evident at birth. They may be extensive and may, but not necessarily, overlie the bone lesions (125).

Sexual precocity occurs in about one-third of patients, mostly females. In contrast to the sequence of events seen in normal girls undergoing puberty and in most types of sexual precocity, vaginal bleeding usually occurs first and may precede breast development and the appearance of axillary and pubic hair by many years (126). It may appear as early as 3 months of age. The early bleeding is usually scant and irregular, and normal menstrual periods begin at the time of expected puberty. Moreover, fertility appears to be unaffected (125). Accelerated skeletal maturation accompanies the sexual precocity. Laboratory findings in patients have been difficult to interpret. Girls with precocious puberty have larger than normal ovaries containing cysts (107). Gonadal and adrenal steroids have been found to be elevated in the plasma (105,107); however, both low and high levels of circulating gonadotropins have been observed (105,107,126,127). Benedict (1962) noted that in three such cases in which ovarian tissue had been examined, no evidence of ovulation was found (126). However, active spermatogenesis was seen in a testicular biopsy from a 6-year-old boy with this condition (128).

Hyperthyroidism is a common feature, occurring in 30% of patients in one series (126). It may also contribute to the accelerated skeletal maturation. The thyroid abnormality is mild and distinct from Graves' disease in that ocular changes are lacking, and histologically there is no lymphocytic infiltration in the thyroid tissue. Instead, diffuse hyperplasia is found (128). Thyroid-stimulating hormone levels have been determined as being low on three occasions (129). Features of acromegaly and pituitary gigantism have been described several times, and an elevation of growth hormone has been detected at least once (127,128). Cushing syndrome due to bilateral adrenal hyperplasia has been documented at least five times (105,130,131). Hyperparathyroidism has been observed twice (103,132).

As discussed earlier, this condition usually results from postzygotic activating mutations of arginine 201 in *GNAS1* leading to a mosaic distribution of cells bearing constitutively active adenylate cyclase activity. With the delineation of its genetic basis, the clinical spectrum of this condition has been expanded to include severely ill infants with hepatobiliary disease, cardiac disease, isolated peripheral precocious puberty and other nonendocrine and non-skeletal manifestations. These infants and children would not ordinarily be suspected to have McCune–Albright syndrome but were found to have the same mutation observed in other patients with typical features of the syndrome

(115,133). In a 2004 assessment of 113 patients presenting with the classic triad of polyostotic fibrous dysplasia, café-au-lait skin pigmentation and precocious puberty (24%), two of the triad (33%) or only one feature (40%), the activating mutation was detected in 43%. Mutation detection increased to 90% when affected tissue was available for analysis, although skin lesions were positive for mutation in only about one-fourth of instances. The mutation was found in 46% of blood samples from patients presenting with the classic triad of manifestations, 21% of patients with two signs, and 8% of patients with a single feature (115). These observations indicate that affected tissue should be analyzed for mutation if possible.

Other abnormalities have rarely been observed in patients with polyostotic fibrous dysplasia and may be components of the syndrome. Multiple intramuscular myxomas have been noted in 11 patients (134,135). They tend to develop in clusters, especially in the thigh region, and usually present during adulthood. Hyperplasia of reticuloendothelial tissue and both lymphoid and myeloid metaplasia have also been described (129).

Surgical correction may be useful for skeletal deformities (136). Although still somewhat controversial, voral alendronate, appear to be effective in reducing bone turnover and alleviating pain in this disorder (137–140).

### 159.9 CHERUBISM

In 1933, Jones reported four sibs with an unusual facial appearance; they appeared to be looking toward the heavens. He coined the term cherubism. Despite the introduction of many descriptive designations, including familial multilocular cystic disease of the jaws, familial fibrous dysplasia of the jaws, familial fibrous swelling of the jaws, familial bilateral giant-cell tumor of the jaw, familial intraosseous fibrous swelling of the jaw, disseminated juvenile fibrous dysplasia of the jaws, and familial osseous dysplasia of the jaws, the term cherubism seems to be firmly established (141).

By 1970, more than 70 cases had been described. The clinical features of cherubism vary considerably (141–143). In general, the affected children present with painless symmetrical swelling of the jaws between the ages of 18 months and 7 years. The swelling progresses rapidly over the next 2–3 years, after which it slows until puberty. Depending on the severity, the swelling can range from little more than broadening of the lower jaw to marked fullness of the lower face associated with thickening of the maxilla. In the most severe cases, maxillary expansion pushes the floor of the orbit upward. The cherubic look results from the combination of the displaced orbit and poorly supported lower eyelid; the altered facial contour permits the rim of sclera to be exposed above the lower eyelid (142). It is uncommon.

Maxillary involvement occurs only when mandibular involvement is severe. When present, it may be viewed

intraorally. The alveolar processes may become thickened and the vault of the palate obliterated to the extent that speech is impaired. Dental abnormalities including delayed eruption, missing or displaced teeth, premature loss of deciduous teeth, and absence of permanent molars have been observed. Enlargement of submandibular lymph nodes has also been described frequently. After puberty, there is a gradual normalization of the facial appearance, although in most cases there is some degree of residual enlargement (142). Treatment varies with the degree of involvement and may often not be needed because of the tendency toward spontaneous improvement.

Radiographs of the mandible taken during childhood reveal bilateral symmetrical well-defined multilocular radiolucent areas associated with expansion of bone and cortical thinning (Figure 159-6) (144,145). The mandibular rami are always involved. The entire mandible may become involved except for the condyles, which are always spared (143). Similar changes are seen in the maxilla when it is affected. The maxillary sinuses may be obliterated. In adults, the radiolucent areas fill in with granular bone and become dense and sclerotic. The radiographic changes of the young are pathognomonic, but those seen in adults are not.

Histologically, the bone is replaced by cellular fibrous tissue containing scattered trabecula of woven bone and many collections of giant cells that resemble osteoclasts. Interestingly, the bone affected by the pathologic process is derived from the first branchial arch.



**FIGURE 159-6** Lateral radiograph of the jaw showing the multilocular lesions of the mandible in an 8-year-old girl with cherubism.



Cherubism is an autosomal dominant trait. A review of 21 families showed that the penetrance is 100% in males and 50–70% in females (146). There is considerable variability, however, and radiographs may be needed to detect mildly affected individuals, especially during adulthood. A few patients have been reported with both cherubism and Noonan syndrome (147–149). The cherubism locus maps to chromosome 4p16.3 (Mangion et al., 1999; Tiziani et al., 1999). Mutations have been detected in a gene that encodes the adapter protein SH3BP2 (7,150,151). Mouse modeling of cherubism has revealed that the mutations cause gain of SH3BP2 function, which promotes inflammatory bone loss mediated substantially through tumor necrosis factor alpha (152).

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## RELEVANT WEBSITES

Online Mendelian Inheritance in Man: <http://www.ncbi.nlm.nih.gov/omim>.

### Biography



Dr Horton received AB and MD degrees from the University of Kansas, and completed a residency in Internal Medicine at the University of Kansas School of Medicine and fellowship training in Medical Genetics at UCLA-Harbor General Hospital with additional genetics training at the NIH. He has held faculty positions at the University of Kansas School of Medicine, University of Texas Medical School-Houston and Oregon Health & Science University in Portland, where he has also been Director of the Shriners Hospital for Children Research Center since 1993. He has a long-standing research interest in skeletal biology with special emphasis on inherited skeletal dysplasias and their molecular pathogenesis. He has published well over 150 articles and book chapters on both the clinical and basic science aspects of these conditions. He is active in many relevant medical and scientific organizations, sits on a number of advisory and review committees and is considered an expert in the field of skeletal dysplasias.



# CHAPTER

# 160

## The Dysostoses

Deborah Krakow

### 160.1 INTRODUCTION

The dysostoses constitute a group of disorders in which the skeletal involvement is predominantly manifested as abnormalities of individual bones or in a group of bones. These abnormalities can occur singly or in combinations, and frequently occur with other congenital anomalies. They differ from the osteochondrodysplasias, in which there is a generalized abnormality in bone or cartilage, though there can be significant overlap between the dysostoses and the skeletal dysplasias, and among the individual dysostoses. Thus, many of the dysostoses are classified within the *Nosology and Classification of Genetic Skeletal Disorders* (1). The classification of dysostoses can be challenging, though now with increasing knowledge regarding the molecular pathogenesis of these disorders. Unlike the osteochondrodysplasias, the dysostoses have not been officially classified into specific groups. Therefore, a discussion of dysostoses is a description of the specific conditions in which abnormalities of individual bones are seen. Thus, the dysostoses can be grouped into categories of those primarily concerned with craniofacial involvement, those with predominantly axial involvement, and those affecting only the extremities. These categories are purely arbitrary for the convenience of discussion and clinical categorization and do not necessarily reflect underlying or overlapping pathogenesis.

The dysostoses with craniofacial involvement are discussed elsewhere. The well-described dysostoses with predominantly axial involvement or appendicular involvement are included here, though by no means is this list inclusive of all the dysostoses. With the increase in the number of newly described genetic and dysmorphic disorders, there are many new syndromes that have dysostoses as part of the constellation of findings. Many excellent textbooks on syndromes include detailed discussions on dysostoses (2–12).

The dysostoses include conditions in which there is an abnormality in a bone or group of bones. Limb and skeletal patterning and development occur before the 8th week of gestation; consequently, the predominant features of most dysostoses have been determined early in development. Therefore, early developmentally expressed genes

remain excellent candidate genes for these disorders. In many cases, anomalies determined early in gestation can be visualized by prenatal ultrasound; therefore, genetic counseling and prenatal diagnosis should be offered for those disorders with a recurrence risk based on autosomal recessive inheritance or germ line mosaicism.

### 160.2 DYSOSTOSES WITH PREDOMINANTLY AXIAL INVOLVEMENT

#### 160.2.1 Vertebral Segmentation Defects, Including The Klippel–Feil Anomaly (Klippel–Feil Sequence)

Abnormal segmentation can involve any of the vertebral bodies (Figure 160-1) but is most frequently seen in the cervical area (Figure 160-2). Cervical vertebral segmentation anomalies are referred to as the Klippel–Feil anomaly (Klippel–Feil sequence; Online Mendelian Inheritance in Man (OMIM) 148900) whether they involve fusion of two segments or the entire cervical spine. There are actually several distinct subcategories, as originally defined by Gunderson et al. (13). Dominant inheritance, recessive inheritance, and no simple genetic basis for the disorder are all possible. The mechanism that leads to the malformations seen in the Klippel–Feil anomaly appears to be a failure of the normal segmentation and fusion processes of the mesodermal somites, which occurs between the 3rd and 7th weeks of gestation. Bouwes Bavinck and Weaver (14) have proposed that this occurs on the basis of a vascular accident early in development, although there is disagreement on this hypothesis. Familial cases have been reported (15), though the vast majority of cases occur sporadically, or as part of other genetic syndromes.

Clinically, Klippel–Feil syndrome consists of a triad: (1) short neck, often with the presence of pterygium colli; (2) low hairline; and (3) painless limitation of head movement. The scapula is frequently displaced. Neurologic compromise is occasionally present, and it may imply that the spinal cord has been compressed or that there is a congenital structural anomaly of the spinal cord. The most severe form of cervical segmentation defect is discussed

under spondylocostal dysplasia. Hemivertebrae, defective posterior elements (spina bifida occulta), clefting of the vertebral body anteriorly, reduction in the number of vertebrae, and anomalies at the occipital-atlantal articulation may be seen (4). Segmentation abnormalities in the C2–3 region may lead to subluxation and secondary cord compression. Patients with the Klippel–Feil anomaly should have flexion/extension radiographs to evaluate the cervical vertebrae. Subluxation of the thoracic or lumbar vertebrae can also occur, leading to spinal nerve compression. Abnormal segmentation in the thoracic and lumbar areas leads to scoliosis, either congenitally or developing during early childhood. In addition to the vertebral anomalies, extra, fused, or missing ribs and Sprengel anomaly (upward displacement of the scapula) are often seen.

Other congenital anomalies may be seen frequently with multiple vertebral fusion defects. Congenital heart disease, vascular abnormalities, and cleft palates may be present. Hearing loss (80%) with structural anomalies of the ossicles (16), renal dysgenesis ranging from hypoplasia to bilateral agenesis, internal genital anomalies including vaginal atresia and bicornuate uterus, limb anomalies, partial facial paralysis, and ptosis have also been reported (13,17).

In 1967, Gunderson et al. distinguished three types of cervical vertebral fusion defect (13):

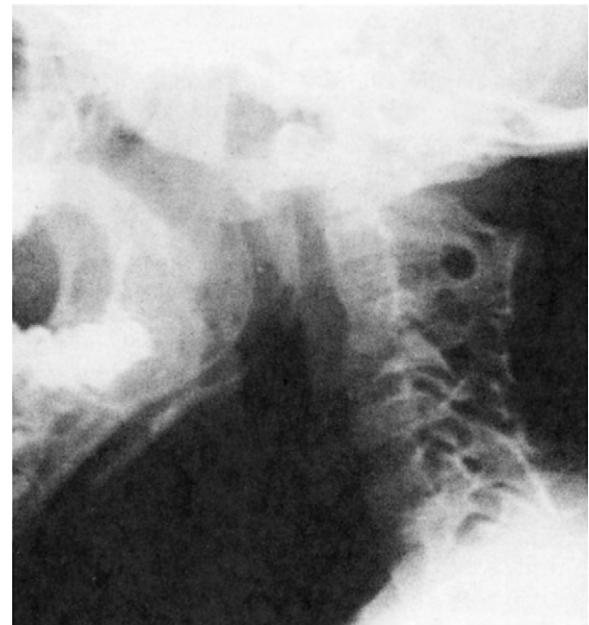
- Type I—massive fusion of many cervical and upper thoracic vertebrae into bony blocks (Figures 160-1 and 160-2)
- Type II—fusions of only one or two interspaces, usually C2–3 or C5–6; however, there can be intrafamilial variability, hemivertebrae and occipital-atlantal fusion, and other congenital anomalies
- Type III—multiple vertebral segmentation anomaly, including both cervical fusion and lower thoracic or lumbar fusion

A fourth type of Klippel–Feil anomaly has been suggested and is associated with sacral agenesis (18). This attempt to sort out familial types of vertebral fusion and the presence or absence of other system involvement is very helpful, though not all cases fit the classification.

In type I, most reported cases are sporadic, and may have multiple other congenital anomalies. There are isolated case reports of a second affected sib or a sib with multiple congenital anomalies, raising the possibility of a rare recessive gene or somatic mosaicism being responsible for the anomalies (19). In type II, other congenital anomalies are rarely noted; type III is often associated with multiple organ anomalies and subsequent neurologic compromise. Usually, it is a sporadic occurrence in the family. However, Wynne-Davies (19) recognized families with an apparent autosomal dominant inheritance with marked variability in vertebral anomalies. Clarke et al. (15) described a four-generation Klippel–Feil syndrome family with an autosomal dominant form of the disorder. The vertebral fusion was confined to the cervical spine and



**FIGURE 160-1** Klippel–Feil anomaly. Radiograph of the thoracic spine. Note right sixth thoracic hemivertebrae, and associated changes in T5.



**FIGURE 160-2** Klippel–Feil anomaly. Radiograph of cervical spine. Note the fusion of the posterior elements of C1 to the occiput and fusion and partial rotation of C2–5.

occurred in association with malformation of laryngeal cartilages, mild to severe vocal impairment, and microtia. Some members of the family had a history of mild conductive hearing impairment and bilateral restricted supination and limited flexion of the elbow. The syndrome co-segregated with a pericentric inversion on chromosome 8, inv(q22.2.q22.3), and demonstrated a mutation in the gene that encodes growth and differentiation factor 6 (*GDF6*) produces the phenotype (15b). Some other sporadic cases also had mutations in *GDF6*, though these changes account for a small number of affected individuals with Klippel–Feil disorder. Further, Ye et al. in 2010 identified two individuals from one family with isolated Klippel–Feil abnormality and heterozygosity for mutations in *GDF3* (20). Though this represents a limited number of cases in which the molecular basis of Klippel–Feil has been isolated, the findings demonstrate the importance of alterations in BMP signaling affecting the cervical spine.

A subdivision of vertebral segmentation anomalies has been designated the Wildervanck or cervical-oculoacoustic syndrome (OMIM 314600), in which there is a preponderance of females (21). It is characterized by congenital perceptive deafness, abducens paralysis with retraction of the bulb of one or both eyes (Duane syndrome), facial hypoplasia and asymmetry, fusion of cervical vertebrae, and occasionally elbow hypoplasia.

Both the MURCS (OMIM 601076) and VATER (OMIM 192350) associations have vertebral anomalies with multiple other congenital abnormalities that occur together more frequently than would be expected by chance (8). The MURCS association consists of müllerian duct aplasia; renal hypoplasia, dysgenesis, or ectopia; and cervical-thoracic somite dysplasia. The VATER association consists of vertebral anomalies, anal anomalies, and tracheal, esophageal, and radial ray defects. The original observations—renal, cardiac, and limb anomalies and single umbilical artery—were also noted to be seen with this group, giving rise to the acronym VACTERLS. Therefore, some cases with Klippel–Feil anomaly may have the aforementioned associations, and consideration should be given to screening those patients for other congenital anomalies. Recent advances in genome analysis may uncover the underlying mechanism of these disorders and it is possible that these associations are genetically complex in their origin.

The pathogenesis of isolated Klippel–Feil is not well understood. This probably results from genetic and allelic heterogeneity, and in some cases, contributing nongenetic causes. PAX1, a developmentally expressed transcription factor, has been implicated as a possible candidate gene (22). The mouse *Pax1* mutant phenotype “undulated” shows vertebral segmentation defects similar to human Klippel–Feil syndrome (23). Analysis of a cohort of individuals with Klippel–Feil syndrome identified some sequence changes not seen in control patients, suggesting an association with the disorder, but no conclusive data implicated PAX1 as causative. As mentioned above, some cases of Klippel–Feil result from heterozygosity for changes in the gene that encodes GDF6 and GDF3.

### 160.2.2 Spondylocostal and Spondylothoracic Dysostoses

There are several types of spondylocostal and spondylothoracic dysostoses (OMIM 277300; OMIM 608681), in which abnormal spinal segmentation and malformations of the ribs occur. Spondylocostal dysostoses (SCDOs) show considerable clinical and radiographic overlap with spondylothoracic dysostoses. Usage of varying terms to describe vertebral segmentation disorders that include costovertebral/spondylocostal/spondylothoracic dysostosis/dysplasia has led to significant confusion in the literature. Further, there are some cases that defy classification. Clinically, these patients have a short neck and/or trunk.

Other visceral malformations are not generally seen, though when seen, they frequently correspond to the site of the vertebral segmentation defects (24). This group of dysostoses falls into two major types: (1) mild varieties, which may be inherited as a dominant condition (SCDO type 5); and (2) more severe varieties, which may be inherited as autosomal recessive traits and the types are now based on gene identification (SCDO types 1–4) (23a–30).

Dominantly inherited multiple segmentation anomalies of vertebrae (SCDO5) are characterized by hemivertebrae, fused vertebrae, butterfly vertebrae, and various rib anomalies, and the genetic etiology remains unknown (19,31). Because of marked variability within these families, detailed radiographs are needed to establish the possibility of subclinical hemivertebrae or fusion. Individuals with the recessively inherited types of multiple segmentation anomalies of the vertebrae have marked shortening of the trunks, and diagnosis is readily apparent because the disproportion of the short trunk is dramatic when compared with the relatively long limbs. However, there appear to be several forms and genetic heterogeneity has been established. In the Jarcho–Levin spondylothoracic dysostoses (occipito-facial-cervicothoracic-abdomino-digital dysplasia, described in 1938), the severe disproportion of the trunk and limbs gives a crablike appearance on radiographs, resulting from marked platyspondylia of the fused vertebrae (32). Many of these children succumb secondary to respiratory difficulties. Jarcho–Levin syndrome is more frequently seen in individuals of Puerto Rican ancestry and the eponym is used when the disorder manifests in Puerto Rican individuals of Spanish descent (33). The gene responsible for Jarcho–Levin in the Puerto Rican population is *MESP2*. A homozygous nonsense mutation is responsible for approximately 80% of spondylothoracic dysostosis cases in the Puerto Rican population, suggesting a founder effect (33a). In mouse models, *Mesp2* has been shown to be expressed in presomitic mesoderm and plays a critical role in vertebral body formation (33b). Recessively inherited mutations in *MESP2* that occur in individuals of non-Puerto-Rican inheritance produce spondylocostal dysostoses of type 2 (SCDO2) (33a).

There are other identified genes that produce autosomal recessive SCDOs (34). Using homology of synteny (the “pudgy” mouse) and linkage analysis, the gene for SCDO1 was identified as *DLL3*, or delta-like-3 gene (35). *DLL3* is a notch ligand, therefore revealing the role of the notch-signaling pathway in patterning of the human skeleton. Mutations in lunatic fringe (*LFNG*) lead to SCDO3, and *HES7* produces SCDO4 (36). *MESP2*, *DLL3*, *LFNG*, and *HES7* are all components in notch signaling, demonstrating the importance of notch signaling in axial skeletal development. However, the gene defect has not been established in all cases of spondylocostal and spondylothoracic dysostoses. Within this group of disorders, there is significant phenotypic and genetic heterogeneity.



### 160.2.3 Sprengel Deformity

Sprengel deformity (Sprengel sequence; OMIM 184400) is characterized by a congenital upward displacement of the scapula. It usually occurs as a sporadic event (unilateral or bilateral) or in association with a variety of abnormalities. Most cases of Sprengel deformity are sporadic; however, a few families have been described with autosomal dominant transmission (37,38). Sprengel deformity presumably results from a failure of the normal embryologic descent of the scapula from the neck to the thorax during the second month of gestation.

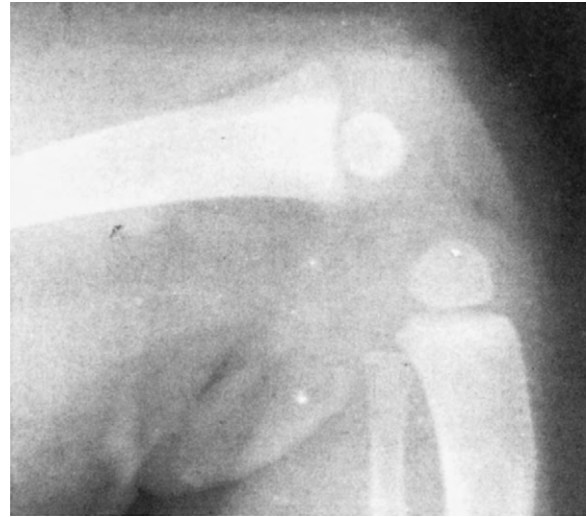
The scapula is usually hypoplastic and abnormal in shape. As a result of its abnormal shape and position, it may produce a lump in the upper back and lead to restricted movement of the shoulder. In 20–50% of cases, there is accumulation of connective tissue or even bony fusion between the scapula and ribs or vertebrae (39). Associated skeletal anomalies occur in more than half of cases, and Sprengel deformity can be seen in Klippel-Feil syndrome. Scoliosis, hemivertebrae, fused vertebrae, spina bifida occulta, cervical ribs, missing ribs, fused ribs, clavicular anomalies, and hypoplasia of the muscles of the shoulder girdle can also co-segregate with Sprengel deformity. Association with situs inversus has been described, as have chest deformities and limb anomalies (40). Many cases of Sprengel deformity seem to be only one manifestation of a more generalized disturbance of the shoulder girdle and upper thorax (11). Surgery may be needed, both to improve function of the shoulder and back and to improve cosmetic appearance. Surgery usually involves removal of the scapulovertebral communication. Without surgery, exercise and stretching seem to be of little value.

### 160.2.4 Osteo-Onychodysostosis (Nail-Patella Syndrome)

Osteo-onychodysostosis (OMIM 161200) is a dominantly inherited condition with four major clinical features: dysplasia of the nails, absence or hypoplasia of the patella (Figure 160-3), abnormalities of the elbow, and the presence of iliac horns (pyramidal spurs just outside the sacroiliac line) (Figure 160-4). The tendency to develop nephritis can be a serious complication and seems to have a familial aggregation (6).

The disorder is recognized at different ages, depending on the degree of involvement (41). Some patients have difficulty walking because of instability of the knee. Occasionally, infants present with contractures of hips, knees, elbows, feet, or fingers and may even have pterygium across the joints. Other individuals are basically asymptomatic and only recognized as a part of family studies. Height is usually within normal limits, but affected individuals are mildly short for the family.

The nails are abnormal, and the thumb and index fingers are most often affected. They may be small and concave,



**FIGURE 160-3** Nail-patella syndrome (osteonychodysostosis). Radiograph of the knee. Note absent patella and proximal tibial distortion.



**FIGURE 160-4** Nail-patella syndrome (osteonychodysostosis). Radiograph of the pelvis. Note flaring of the iliac wings with definitive iliac horns.

longitudinally grooved, abnormally split, pitted, softened, discolored, or brittle with triangular-shaped lacuna or central hypoplasia. The toes are rarely involved. The patellae are absent or small and laterally dislocated and may be bipartite (Figure 160-3). They may subluxate or dislocate. Prominence of the medial femoral condyle, hypoplasia of the lateral femoral condyle, and proximal tibia distortion are all seen. Hypoplasia of knee tendons and hypoplasia of the quadriceps and vastus medialis may contribute to instability of the knee. At the elbow, hypoplasia of the capitulum and relative hypertrophy of the medial condyle with hypoplasia of the radial head are present. Lateral or posterior subluxation of the radial head results in decreased pronation/supination and lack of full extension. Webbing at



the elbow may be seen, and the carrying angle is increased. Deltoid, triceps, and brachioradialis hypoplasia occur (4). Hypoplasia of the lateral humeral condyle and head of the fibula may also be seen. Iliac horns arising from the central area of the outer surface of the iliac wing are usually pyramidal in shape and are diagnostic of this disorder (Figure 160-4). The iliac horns occur bilaterally but may be asymmetrical. They are palpable in many patients, but they are rarely clinically relevant. Other reported skeletal anomalies include foot changes (equinovarus, calcaneovalgus, or most frequently, pes planus), dislocated hips, coxa valgus, and contractures of other major joints. Another significant abnormality in nail–patella syndrome is the presence of open-angle glaucoma (42).

The renal lesion in nail–patella syndrome resembles chronic glomerulonephritis without infection (3). Clinically, the renal involvement presents with proteinuria. Uremia develops in 30% of patients. Accumulation of abnormal collagen fibers and thickening of the basement membrane can be found in the glomeruli on autopsy. Taguchi et al. (43) demonstrated the characteristic changes in the glomeruli in nail–patella syndrome patients without clinical renal involvement. These changes may represent a basic structural defect present as part of the disease, rather than a phenomenon that is secondary to glomerular sclerosis (44). All patients should be screened for renal disease, especially those in families with a history of renal involvement.

The gene localization for nail–patella syndrome had been known since 1976, when it was linked to the ABO blood group on chromosome 9q34. The location of the gene was reduced to a small chromosomal interval (45), and the gene defect identified in *LMX1B*, a LIM-homeodomain transcription factor (46). The majority of mutations in *LMX1B* are premature stop codons, resulting in haploinsufficiency for *LMX1B*. *LMX1B* plays a critical role in dorsal/ventral patterning of the vertebral limb. Targeted disruption of *Lmx1b* in mice produces a phenotype similar to that seen in humans, including absence of the nails and patella, as well as abnormal renal pathology (47).

Differential diagnosis for nail–patella syndrome consists of syndromes associated with absent/small patella and nail changes. These include small patella syndrome (OMIM 147891), patella aplasia-hypoplasia (OMIM 168860), familial recurrent dislocation patella (OMIM 169000), Meier–Gorlin syndrome (OMIM 224690), genitopatellar syndrome (OMIM 606170), DOOR syndrome (deafness, onychodystrophy, osteodystrophy, mental retardation; OMIM 220500), Coffin–Siris syndrome (OMIM 135900), and RAPADILINO syndrome (radial ray defect, patellar hypoplasia, diarrhea and dislocated joints, limb malformations, long slender nose and normal intelligence; OMIM 255280). Senior syndrome (OMIM 113477) and ectodermal dysplasias have similar nail abnormalities but also have skin changes that are distinct and not seen in nail–patella syndrome.

### 160.2.5 Cerebrocostomandibular Syndrome (Rib Gap)

Rib gaps (consisting of uncalcified fibrous or cartilaginous tissues in the posterior part of the rib causing “flail chest”), Pierre–Robin anomaly (micrognathia, cleft palate, glossoptosis), and vertebral dysplasia are the constellation of findings in this syndrome (OMIM 117650). Abnormalities of the heart and brain, extra skin at the neck, collapsing trachea, deafness, mental retardation, hydrocephaly, spina bifida, subluxation of the elbows, and polycystic kidneys have also been reported (48,49). There can be marked variability of expression within families.

In infancy, radiographic gaps in the dorsal portions of the ribs with fragmented ossification and absence of normal costovertebral articulations are seen. Lesions are bilateral but not necessarily symmetrical. Most affected infants die in the newborn period of respiratory insufficiency due to a flail chest. Frequently, affected individuals fail to thrive, and many have feeding problems because of the micrognathia and may require feeding gastrostomy.

Both autosomal dominant ((50), Morin et al., 2001a) and autosomal recessive inheritance have been reported, though the recessive inheritance pattern may represent germ line mosaicism. Zeevaert et al., 2009 (50b) reported homozygosity for mutations in *COG1* (component of oligomeric Golgi complex 1) in two patients with a cerebrocostomandibular-like syndrome, but additional patients with cerebrocostomandibular syndrome (CCMS) mutations were not identified. Rib gaps may be seen in several conditions (e.g. diaphanospondylodysostosis; OMIM 608022) and are thus not pathognomonic for the diagnosis of CCMS.

## 160.3 PREDOMINANT LIMB INVOLVEMENT

### 160.3.1 Acheiropodia

Acheiropodia (absence of the hands and feet; OMIM 200500) is an autosomal recessively inherited condition with absence anomalies of both upper and lower limbs, usually with symmetrical involvement. Acheiropodia has only been seen in inbred Brazilian kindreds of Portuguese origin (51,52). The limb malformation involves a terminal transverse hemimelia (below the elbow) in the upper limbs and a terminal transverse hemimelia of the distal third of the lower limbs. Some affected individuals have an elongated small bone at the tip of the stump (Bohomoletz bone) parallel to the humerus. This inherited condition can be differentiated from other terminal transverse hemimelia conditions in that the latter usually have unilateral involvement of the upper limbs and the lower limbs are rarely involved, whereas the defects in acheiropodia are usually uniform and bilateral and involve both upper and lower limbs (53). In addition, there are no other associated abnormalities in acheiropodia. The chromosomal

location of the gene defect, chromosome 7q36, was established (54), and the disease gene identified: *C7ORF2*, the human homolog of the *Lmbr1* gene (55). The differential diagnoses for acheiropodia include thalidomide syndrome, amniotic bands, and Hanhart syndrome.

### 160.3.2 Tetraphocomelia–Cleft Palate Syndrome (Roberts Syndrome, Pseudothalidomide Syndrome, SC Syndrome)

This is a striking syndrome with autosomal recessive inheritance (OMIM 268300). Limb reduction anomalies are seen in all four extremities. The limb abnormalities include shortening, fusion, or absence of the long bones (tetraphocomelia), often with reduction in the number of fingers and occasionally toes (missing or fused metacarpals or phalanges). These dysostoses are seen together with cleft lip and palate (usually bilateral, but occasionally only cleft palate or high palate). Midline facial hemangioma, mental retardation, intrauterine growth retardation, ocular hypertelorism, hypoplastic alae nasi, cloudy cornea, and fine thin silvery-blond hair are often present. Occasionally, relatively large clitoris or penis, cystic renal changes, or central nervous system structural changes (encephalocele, hydrocephaly) are seen (56,57).

Chromosome studies show puffing and premature separation of the centromeres in the heterochromatic C-banded regions of most chromosomes, but particularly chromosomes 1, 9, 16, and Y (58). It has been demonstrated that some Roberts syndrome patients have an abnormality in constitutive heterochromatin and show cellular hypersensitivity to DNA-damaging agents such as mitomycin C. Roberts syndrome results from mutations in the *ESCO2* gene (59), the protein product of which is required for the establishment of sister chromatid cohesion during S phase.

### 160.3.3 Oro-acral Syndrome (Hanhart Syndrome, Aglossia Adactylia, Ankyloglossia Superior)

Limb reduction (usually all four limbs with transverse amputations) and intraoral anomalies (usually a small or vestigial tongue, sometimes with adhesions of the tongue to the hard palate) comprise a distinctive syndrome that appears to be sporadic (OMIM 103300) (60). The etiology is unknown.

The mandible is usually severely micrognathic (61), which can lead to feeding abnormalities. Although the tongue may be rudimentary, speech is usually possible. The lower incisors may be absent, and occasionally bony adhesions of the jaw and cleft palate occur. The type of limb defect varies from just absent digits to complete transverse absence below the elbow and knee (62). The humerus and femur are always preserved, and lower limbs are usually spared when compared with the upper limbs (4,63,64). There is never complete lack of either

tongue or limbs. Intelligence is usually normal. Occasionally, Möbius syndrome (OMIM 157500) is seen in association with oro-acral syndrome (12).

### 160.3.4 Radioulnar and Humeroradial Synostoses

Radioulnar and humeroradial synostosis (bony fusion at the elbow joint; OMIM 179300; OMIM 143050) can occur as an isolated anomaly or in association with several specific syndromes. Early in life, there may appear to be a joint space on radiographs, but cartilaginous synostosis may already have occurred.

In isolated radioulnar synostosis, a proximal bony fusion of the radius and ulna is seen radiographically, and there is impaired pronation/supination of the arm. Consequent limitation of function is usually minimal. Several families have been reported with dominant inheritance (65). Radioulnar synostosis is also seen in several chromosomal disorders, particularly involving abnormal numbers of X and Y chromosomes (e.g. 47,XXX). In addition, radioulnar synostosis can be seen in Poland syndrome, SC or pseudothalidomide syndrome, Pfeiffer syndrome, and Permian syndrome.

Clinically, patients with humeroradial synostosis have an immobile elbow joint with decreased muscle in the upper arm, and bony fusion is seen on radiographic examination. Surgical manipulation may be necessary to improve function. Isolated humeroradial synostosis can be inherited as an autosomal recessive trait (Keutel et al., 1970) or as a dominant (66), or frequently as a sporadic occurrence. Of note, humeroradial synostosis is often a component of multiple synostosis and other genetic syndromes.

### 160.3.5 Brachydactyly

Brachydactyly, or shortening of the digits due to abnormal development of either the phalanges or metacarpals, is seen either as an isolated malformation (in a variety of different forms) or in conjunction with anomalies of one or more other systems (67). Bell classified the isolated brachydactylies into 10 definitive groups (A1–6, B, C, D, and E) (Table 160-1) on the basis of her review of 1336 individuals affected with brachydactyly. Familial reports of each type of isolated brachydactyly have shown an autosomal dominant pattern of inheritance.

Brachydactyly A is also called brachymesophalangia because there is a shortening of the middle phalanges. In brachydactyly type A1 (Farabee type, BDA1; OMIM 112500), all middle phalanges are short or hypoplastic, and the proximal phalanges of the thumbs and halluces may be shortened. In the most severe cases, fingers are approximately one-half their normal length. The third digit is usually least affected. Severe cases may have both cosmetic and functional problems. In two large families with BDA1, the gene has been mapped to chromosome

**TABLE 160-1** Listing of Brachydactyly Syndromes and Responsible Genes

Brachydactyly Type (BD)	Gene(s)
A1 (BDA1)	Indian hedgehog ( <i>IHH</i> )
A2 (BDA2)	Bone morphogenetic protein receptor 1B ( <i>BMPR1B</i> )
A3 (BDA3)	Unknown
A4 (BDA4)	Unknown
A5 (BDA5)	Unknown
A6 (BDA6)	Unknown
B (BDB1)	<i>ROR2</i>
C (BDC)	<i>GDF5</i>
D (BDD)	<i>HOXD13</i>
E (BDE)	<i>HOXD13</i>

2q35–36 (20a). Gao et al. (61a) showed that heterozygosity for mutations in the Indian hedgehog (*IHH*) gene were responsible for the disorder. Indian hedgehog belongs a family of conserved genes involved in signaling. Another locus for BDA1 (*BDA1B*) has been identified on chromosome 5p13.2 (68), and the possibility of a third locus has been suggested (69).

In brachydactyly type A2 (BDA2; OMIM 112600), the middle phalanges are shortened; however, the shortening is predominantly in the second digits. The middle phalanx has a characteristic rhomboid or triangular shape as a result of a continuous epiphysis from proximal to distal ends on the shortened side. Consequently, growth of the phalanx is outward and results in radial angulation. Heterozygosity for mutations in the bone morphogenetic protein receptor 1B gene (*BMPR1B*) and growth and differentiation factor 5 (*GDF5*) leads to BDA2 (70,70a). Further, alterations (deletions or duplications) in the gene for bone morphogenetic protein 2, *BMP2*, lead to BDA2 (70c,d). Type A3 (BDA3; OMIM 112700) is characterized by shortening of the middle phalanx of the fifth finger. The epiphyses may be cone shaped, and there is usually radial curving of the little finger, resulting in clinodactyly. It is seen with increased frequency in individuals of Asian descent (71). In type A4 (BDA4; OMIM 112800), the middle phalanges of digits 2 and 5 are shortened. If digit 4 is affected, there is radial deviation. Clubfoot can be seen in these families. Finally, type A5 has shortening of all middle phalanges but is also associated with nail dysplasia and bifid distal phalanges of the thumb and hallux (72). Brachydactyly A5 can be confused with brachydactyly B; however, the latter is distinguished by hypoplasia of the distal phalanges, according to Temtamy and McKusick (10). Brachydactyly A5 has not been reported as a distinct entity in the literature. Osebold et al. (73) reported a sixth form of brachymesophalangia (BDA6; OMIM 112910) with mesomelic shortening of the limbs and radial deviation of the index finger, though it has been suggested that the findings in this family represented a form of chondrodysplasia punctata (74).

In brachydactyly type B (BDB1; OMIM 113000), the first brachydactyly to be described in the literature (69a), there is shortening of middle phalanges and shortening or complete absence of the terminal phalanges. Digits on the radial side of the hand are usually less severely affected than digits on the ulnar side (10). Abnormalities are consistently symmetrical, and the feet are less severely affected. Soft tissue syndactyly, symphalangism, carpal and tarsal fusions, and shortening of metacarpals and metatarsals may be additional features. The additional abnormalities can be severely debilitating. A distinctive facies has been associated with BDB1, including wide-spaced, down-slanting palpebral fissures and a bulbous nose (75). Some authors have referred to brachydactyly B as symbrachydactyly or apical atrophy because of the syndactyly, fusion of phalanges, and lack of terminal phalanges. Heterozygosity for mutations in the receptor tyrosine kinase-like orphan receptor gene, *ROR2*, is responsible for BDB1 (76). Mice homozygous for the defects in *Ror2* were found to have abnormal patterning of the digits, similar to those seen in BDB1 (77).

Brachydactyly type C (BDC; OMIM 113100) has marked variability. However, characteristically, the middle and proximal phalanges of digit 5 are shortened, although anomalies also occur in the index and middle fingers. It is striking that the fourth digit is basically normal and extends beyond the other digits. There may be marked ulnar deviation of the proximal phalanx of the index finger. Short metacarpals and symphalangism are occasionally seen, and there may be bifid phalanges and/or radial projections. There is only minimal involvement of the feet. Short stature and clubfeet may be associated features. Radiographic studies show delayed maturation of ossification centers and hypoplasia, aplasia, or early fusion of the epiphyses (10). BDC results from heterozygosity for mutations in the growth differentiation factor V gene (*GDF5*) or *CDMP1*, a member of the bone morphogenetic proteins (78). While genetic heterogeneity has been suggested in BDC, in a large family that showed linkage to chromosome 12q24 (79), it was demonstrated that the disease was produced by heterozygosity for an insertion in *GDF5* (80).

Brachydactyly type D (BDD; OMIM 113200) is characterized by a shortened, broad terminal phalanx of the thumbs and great toes, usually expressed bilaterally. The abnormality is attributed to an early closure of the epiphysis at the base of the distal phalanx of the thumb (10). Reynolds et al. (81) reported the familial association of BDD and Hirschsprung disease. Gray and Hurt (82) found incomplete penetrance in males but complete penetrance in females. In at least some individuals with BDD, heterozygosity for mutations in *HOXD13* have been identified, though the affected individuals had an overlap phenotype between BDD and BDE (83).

Finally, brachydactyly type E (BDE; OMIM 113300) is characterized by shortening of one of or all the metacarpals or metatarsals. Terminal phalanges are often short,

and hyperextensibility of the hands is common. The fourth digit is most frequently affected. There may be associated mild short stature (84) or mild generalized spondyloepiphyseal changes (85). Families with BDE associated with one of the following features have been reported: microcornea, keratoconus, and hypertension. BDE with short stature and hypertension has been mapped to chromosome 12p12.2–p11.2 (86). As noted earlier, individuals with an overlap BDD/BDE phenotype have been shown to be heterozygous for *HOXD13* mutations.

### 160.3.6 Symphalangism

Symphalangism, or fusion of the phalanges, can be seen as an isolated finding (often post-traumatically) or inherited as an autosomal dominant trait. Both proximal (87) and distal (88) forms have been described. Families with distal symphalangism are rare, whereas proximal symphalangism is a much more common phenomenon. In addition, distal symphalangism tends to be an isolated anomaly and proximal symphalangism tends to occur with other associated abnormalities.

In dominantly inherited proximal symphalangism (OMIM 185800), usually more than one joint or finger is involved (Figures 160-5 and 160-6), but variability in the age of onset and severity is seen within families. Involvement of other bones can be seen, such as coalition or fusion of the tarsal and carpal bones, radial head dislocation, radiohumeral synostosis, vertebral synostosis, scoliosis, craniosynostosis, and deafness in affected family members. Therefore, careful evaluation of all joints, spine, and skull and hearing should be performed in family members of a patient affected with symphalangism. Symphalangism may be present at birth; however, radiographic examination at birth may not show complete bony fusion but simply a narrowing of the interzone. In contrast to other types of contractures and dysostotic processes, individuals with symphalangism may actually be made worse (i.e. have more rapid fusion) by vigorous physical therapy.

Multiple synostoses syndrome (OMIM 186500), considered a distinct syndrome from proximal symphalangism, has many overlapping features. In multiple synostoses syndrome, the symphalangism follows a pattern similar to proximal symphalangism, but is usually more extensive. Several kindreds with autosomal dominantly inherited proximal symphalangism and multiple synostosis syndromes have conductive deafness, with onset usually at the age of 2 or 3 years and progression throughout childhood. A bony fusion of the stapes and petrous part of the temporal bone has been demonstrated at surgery in several patients. In multiple synostosis families, affected individuals tend to have long, narrow faces and a prominent nose with a broad bridge and hypoplastic alae nasi. Fingers can be short and may even be missing the distal phalanx and nail.

Whether proximal symphalangism and multiple synostosis syndromes are allelic has been a long-standing

question. Linkage analysis in both proximal symphalangism (89) and multiple synostoses syndrome (89a) mapped both disorders to the identical chromosomal location, 17q21–22. In both of these disorders, heterozygosity for mutations in the *NOGGIN* gene was demonstrated (89b,90). *NOGGIN* is a developmentally expressed gene that is a regulator of the bone morphogenic proteins (3a) that are involved in chondrogenesis. *Noggin* null mutant mice fail to initiate normal joint development and exhibit extensive cartilaginous fusions. Further mutations in *GDF5* and *FGF9* also produce multiple synostoses syndrome, implicating both BMP and FGF signaling in the formation and maintenance of joints.

### 160.3.7 Polydactyly

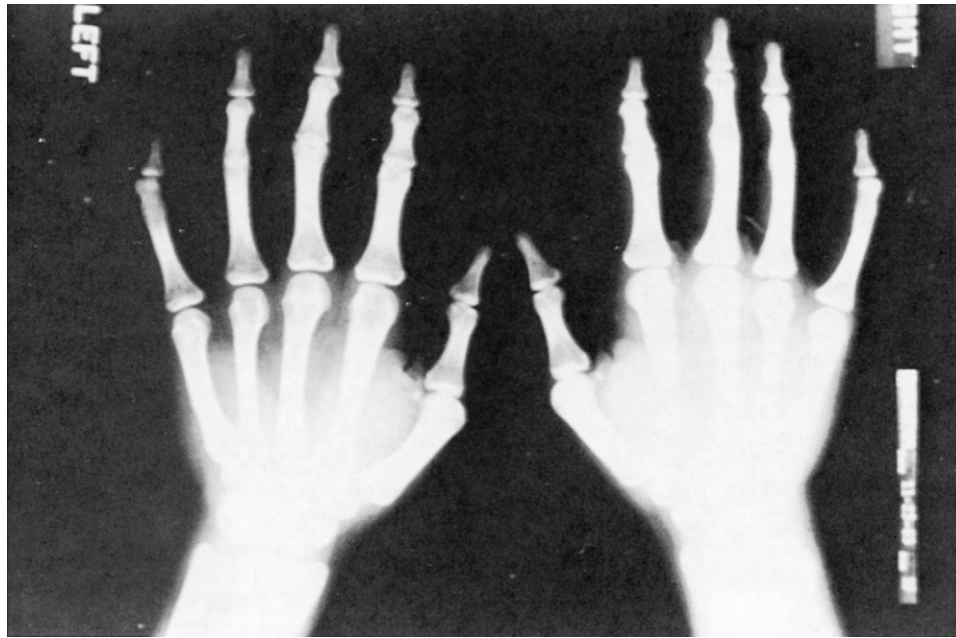
Patients with polydactyly, or the presence of a supernumerary digit or a portion of an extra digit(s), can be classified into two major groups: those with preaxial addition and those with postaxial addition (4,91).

Isolated postaxial polydactyly has long been recognized as a specific entity, is more common in some populations, and is inherited as a dominant trait. In the same family with apparent dominant inheritance, variability of expression can be so marked that the gene is clinically manifested as fully developed extra digits (type A1; OMIM 174200) in some family members and simply as rudimentary supernumerary digits or postminimi (type B; OMIM 174200) in others (10). In an Indian family with type A polydactyly, Radhakrishna et al. (92) identified heterozygosity for a mutation in *GLI3*, one of the vertebrate orthologs of the *Drosophila* cubitus interruptus gene. This expands the phenotype of *GLI3* mutations from Greig cephalopolydactyly syndrome to Pallister–Hall syndrome to isolated postaxial polydactyly types A1 and B. Postminimi can be removed at birth by occluding the skin tag with a tight thread or string; the tag subsequently drops off. Adults so treated may often be unaware that they had polydactyly, and a careful family history should therefore be taken from parents. By contrast, extra digits involving bone or cartilage must be excised surgically to avoid osteomyelitis.

Preaxial polydactyly is much less common than postaxial polydactyly and has been subdivided into four different categories by Temtamy and McKusick (10):

- Type 1—preaxial thumb polydactyly (OMIM 174400)
- Type 2—polydactyly of a triphalangeal thumb (OMIM 174500); linkage has been established to chromosome 7q36 (93) and has been shown to be due to point mutations in a sonic hedgehog (*SHH*) gene regulatory element that resides in intron five of *LMBR1* (50a)
- Type 3—polydactyly of an index finger (OMIM 174600)
- Type 4—preaxial polydactyly and syndactyly, or crossed polydactyly type I (OMIM 174700).





**FIGURE 160-5** Proximal symphalangism. Radiograph of the dorsal view of the hands. Note fusion of proximal phalanges on digits 4 and 5 and partial fusion of the proximal phalanges on digits 2 and 3.



**FIGURE 160-6** Proximal symphalangism. Hands are held in tight fist. Note the absence of flexion creases over the proximal phalanges on digits 3, 4, and 5 and the partial function of the proximal joint of digit 1 bilaterally.

Heterozygosity for mutations in *GLI3* has been shown to produce this type of poly- and syndactyly (94). All types are inherited as autosomal dominant traits, but variability and reduced penetrance have been reported (7).

Polydactyly is associated with several specific syndromes, the most notable of which are the chondrodysplasias, such as Ellis–van Creveld (chondroectodermal dysplasia), the acrocephalosyndactylies, hypothalamic hamartoblastoma syndrome (95), Meckel syndrome, trisomies 13 and 18, Laurence–Moon–Biedl syndrome, Biemond syndrome type II, orofaciocigital syndrome types II and III, polydactyly with dental and vertebral anomalies (96,97), syndrome of polydactyly with myopia, polydactyly with imperforate anus and vertebral anomalies

(98), scalp defects and polydactyly (99), Kaufman syndrome, and several chromosomal anomalies. For the specific dysostotic changes found in other bones in these syndromes, consult Taybi and Lachman (9).

### 160.3.8 Syndactyly

Syndactyly, or webbing of the fingers, can be complete or partial; it can be simple, with only skin binding the fingers together, or complicated, when there are fused bones, extra bones, or shared structures, such as nerves, vessels, or nails (4). Isolated syndactyly has at least five forms. All five are inherited as autosomal dominant traits and are usually bilaterally symmetrical. Variability of expression is seen (100).

Syndactyly type 1 (SD1, zygodactyly; OMIM 185900) is a cutaneous fusion of the third and fourth fingers and/or second and third toes. The webbing is usually to the nail base. Occasionally, other digits can be affected. In one large family, the SD1 locus has been identified on chromosome 2q34–q36 (101), as confirmed by Ghadami and colleagues (102).

Syndactyly type 2 (synpolydactyly, central polydactyly, SPD; OMIM 186000) is also complete cutaneous webbing of the third and fourth fingers, with a partially duplicated digit hidden in the web. In the feet, there is syndactyly of the fourth and fifth toes, with a sixth toe in the web. Heterozygosity for mutations in *HOXD13* is responsible for the phenotype (103).

Syndactyly type 3 (ring finger, SDTY; OMIM 186100) is complete cutaneous webbing between the fourth and fifth fingers. The middle phalanx of the fifth fingers may be hypoplastic. The feet are usually normal. Heterozygosity for mutations in the gap junction protein alpha-1 (*GJA1*) gene has been demonstrated (104). This is also the gene responsible for oculodentodigital dysplasia (OMIM 164200).

Syndactyly type 4 (Hass; OMIM 186200) involves syndactyly of all fingers and the thumb, with finger flexion creating a cuplike hand. The feet may have lateral polydactyly. Syndactyly type 5 (MC/MT synostosis; OMIM 186300) involves cutaneous syndactyly of the third and fourth fingers with synostosis of the metacarpals of digits 4 and 5. In the foot, there is syndactyly of toes 2 and 3 and metatarsals 4 and 5. In two families, duplications in limb region 1, mouse, homolog (*LMRB1*) lead to the phenotype (85a,105). These findings implicate an allelic series that includes acheiropodia, olydactyly, preaxial type II, syndactyly, type IV, triphalangeal thumb, type I, and triphalangeal thumb-polysyndactyly syndrome.

Syndactyly can also be seen in an X-linked syndrome of syndactyly with mental retardation. Syndactyly also occurs in Smith–Lemli–Opitz, Apert, Pfeiffer, Carpenter, Saethre–Chotzen, orofacioidigital, Goltz, and multiple pterygium syndromes. The genes for many of these disorders have been identified.

### 160.3.9 Camptodactyly

Camptodactyly is clinically manifested by soft-tissue flexion contractures of the fingers at one or several joints. Contractures may be present at birth or develop in childhood or even adulthood; they may be stationary or progressive or may improve with physical therapy. Camptodactyly may be seen as an isolated anomaly, inherited as an autosomal dominant trait (91,106), or occur as a feature in many syndromes. Camptodactyly does not usually interfere with function unless it is very severe at birth or vigorous physical therapy is not begun early enough.

There are probably several different etiologies of camptodactyly, one of which is misplaced, hypoplastic,

or absent tendons (107,108). The types of contractures seen in camptodactyly often benefit from surgical repair, by reattaching tendons or soft-tissue release, and from physical therapy with stretching exercises (109). Both fingers and toes can be affected. Some forms may represent early aging, inflammatory response, or deterioration of connective tissue, which would lead to fibrosis and contracture of tendons.

Recently, several syndromes that include finger contractures along with many other features have been given a primary designation of camptodactyly, which may be confusing. These include Tel Hashomer camptodactyly syndrome (camptodactyly, distinctive facial features, dermatoglyphic changes, and musculoskeletal anomalies; OMIM 211960), which is inherited in inbred Arab and Brazilian families as an autosomal recessive (110); Guadalajara camptodactyly I (camptodactyly, intrauterine growth retardation, mental retardation, unusual facies, and musculoskeletal anomalies; OMIM 211910), which is inherited as an autosomal recessive (111); and Guadalajara camptodactyly II (camptodactyly, intrauterine growth retardation, mental retardation, short second toe, and musculoskeletal changes; OMIM 211920), which is inherited as an autosomal recessive disorder (112). In addition, camptodactyly has been reported as a recessive disorder with ichthyosis (113), and as a dominant with scoliosis (114), with symphalangism (115), and with brachydactyly (116).

Camptodactyly is seen as a feature of more than 50 conditions and is frequently associated with chromosomal anomalies.

### 160.3.10 Ectrodactyly

The term ectrodactyly (from the Greek “*ektromo*,” denoting abortion, and “*daktylos*,” finger) theoretically should be reserved for a specific hand anomaly characterized by transverse terminal aphalangia or partial to total absence of the distal segments of fingers. However, the term has been used for the split-hand or lobster-claw anomaly and the EEC syndrome (ectrodactyly, meaning split hand, ectodermal dysplasia, clefting) and so has come to be misleadingly associated with split hand.

The “true” ectrodactyly anomaly may involve one or more phalanges (aphalangia), one or more digits (adactylia), or the full hand (acheiria), and even part of the upper arm. More severe manifestations are hemimelia or amelia. All these abnormalities are considered to represent various degrees of severity of the same anomaly and may be due to an intrauterine vascular occlusion. True ectrodactyly is usually sporadic and unilateral. Reports of familial ectrodactyly probably represent brachydactyly B. In a study by Birch-Jensen (117), 19 individuals affected with true ectrodactyly had 51 normal children. However, a higher incidence of associated malformations in patients and their families has been reported (118). Certainly, “true” ectrodactyly can be seen with

a wide variety of additional anomalies. Several families have been reported with more than one individual affected with terminal transverse amputations of digits or the whole limb; however, no typical pattern of inheritance has emerged (36,119).

The autosomal dominantly inherited form of isolated ectrodactyly (split hand, lobster claw, or cleft hand/foot) can be quite variable within a family and can even involve absence of the long bones. Typically, there is a medial cleft of the hand, the hand being divided into two portions. Often, there is syndactyly of the remaining fingers, and there can be medial clefting and absence analogous to the hand. Occasionally, polydactyly or triphalangeal thumbs are seen. The limbs can be asymmetrically involved (120). This form of ectrodactyly/split hand is remarkable for skipping generations, and some pedigrees demonstrate reduced penetrance. Not only are there variable features and reduced penetrance within a family, six loci for split hand/split foot have been mapped: *SHFM1* (autosomal dominant; OMIM 183600) on chromosome 7q21 (121); *SHFM3* (autosomal dominant; OMIM 600095) on chromosome 10q24 (122–124); *SHFM4* (OMIM 605289) on chromosome 3q27, caused by mutations in *TP63* (125); *SHFM5* (OMIM 606708), localized to chromosome 2q31 (126,127); *SHFM6* (OMIM 225300), caused by homozygosity for mutations in *WNT10B* (118a,38a); and *SHFM2* (X-linked), localized to a 5.1-Mb interval on chromosome Xq26 (128,129).

The combination of ectodermal dysplasia (involving teeth, hair, and nails), ectrodactyly (split hand), and cleft lip/palate (EEC) is striking (130,131). In addition to this triad, genitourinary and lacrimal duct anomalies and choanal atresia can be seen. The features can be variable between affected members of the same family. The degree of keratosis and urinary tract involvement is also inconsistent among affected family members (132). There are at least four chromosomal loci for EEC. EEC1 (OMIM 129900) maps to chromosome 7q11.2–q21.3 based on a number of patients with chromosomal aberrations in this region (133). EEC3 and formerly EEC2 (OMIM 604292) result from heterozygosity of mutations in the tumor protein p63 (*TP63*) gene on chromosome 3 (115,134).

Adams–Oliver syndrome (OMIM 100300) is a syndrome of limb absence anomalies (“true” ectrodactyly) and scalp defects. Autosomal dominant inheritance with variable expression has been reported in several families (135). Scalp defects (cutis aplasia congenita) may vary in size and may occasionally overlie skull defects. The distal limb defects are transverse and may involve only the distal portion of the digits or may involve more of the limb, with absence of hands or feet. Polydactyly has been reported (99). Some cases have remarkable cutis marmorata telangiectasia (136) or cutis aplasia on other parts of the body. Brain anomalies (structural and functional), as well as cardiac and renal abnormalities (137), may be seen. The manifestations are so variable that it is easy to

miss an affected relative unless there is careful examination. The features of this syndrome are very suggestive of a vascular disruptive sequence.

## 160.4 PREDOMINANT LIMB INVOLVEMENT WITH OTHER ASSOCIATED ABNORMALITIES

### 160.4.1 Catel Manzke Syndrome

The combination of Pierre–Robin sequence (cleft palate, glossoptosis, and micrognathia) together with bilaterally short second metacarpal and accessory proximal phalanx of the index finger, giving radial clinodactyly of the index finger, has come to be known as the Catel Manzke syndrome (OMIM 302380) (138). Affected individuals may also have knee defects and congenital heart defects. There is an excess of affected males, suggesting that the inheritance may well be X-linked recessive (139).

### 160.4.2 The Poland Anomaly

The two major components of the Poland anomaly (OMIM 173800) are unilateral symbrachydactyly and ipsilateral aplasia of the sternal head of the pectoralis major muscle. Clinically, there is unilateral absence of the normal anterior axillary fold and shortening or reduction of the limb (140). Most reports of the Poland anomaly indicate that cases are sporadic, though familial cases have been reported (10,141). Etiology is unknown, but early in utero vascular compromise has been proposed (14,142).

The isolated malformations of either symbrachydactyly or unilateral aplasia of the sternal head of the pectoralis major muscle are also sporadic. However, David and Winter (143) recently reported familial absence of the pectoralis major and other chest muscles. Poland anomaly has been found to be more common in males (3:1), and the right side is more commonly involved than the left (2:1) (142). Analogous malformations have been seen in the lower limb. Several reported cases have been associated with the use of vascular constricting agents by the mother in early pregnancy (140). The question of an increased risk for leukemia in the Poland anomaly has been raised (144).

The Poland anomaly has also been seen in association with other skeletal anomalies such as radioulnar synostosis, Sprengel deformity, and coalition of the carpal bones, camptodactyly, polydactyly, skin dimples, deficiencies of the rib cage, scoliosis, and kyphosis. Other skeletal anomalies include cervical ribs, clubfoot, metatarsus adductus, and syndactyly of the toes. Visceral anomalies reported include dextrocardia, herniation of the lungs, inguinal and umbilical hernias, cryptorchidism, ipsilateral hypoplasia of the kidney, coloboma of the optic disc, encephalocele, and microcephaly (142). Features of Poland syndrome also have been seen in individuals with Möbius, Goldenhar, and Adams–Oliver syndromes (145–147).



### 160.4.3 Rubinstein–Taybi Syndrome

Individuals affected with Rubinstein–Taybi syndrome (OMIM 180849) typically have broad toes and thumbs and characteristic facies with a beaked nose (148). The facies is unusual even in infancy, and in addition to the prominent (beaked or pinched) nose, the columella is long and protrudes below the alae nasi. There is an antimongoloid slant to the eyes, with arched eyebrows and a highly arched palate. The ears may be malrotated and usually have thickened helices (149). The broad thumbs and toes are very characteristic and appear spatulate, short, stubby, flattened, and wide. The broad-toed appearance is the result of both soft-tissue and bony changes, which can be seen on radiographs of the terminal phalanx. Duplication of the proximal or distal phalanx of the thumb has been reported (150). All reported patients have been mentally retarded (with IQ estimates less than 50), and microcephaly is seen in more than half the cases. Electroencephalographic abnormalities have been reported, as has absence of the corpus callosum. Short stature is a common feature. In a study of 105 patients, approximately 80% were less than the 3rd percentile for height (148). Other features that have been noted are cardiac malformations, joint laxity, increased deep tendon reflexes, stiff awkward gait, severe keloid formation, cryptorchidism, increased propensity toward malignancy, duplicated kidneys or ureters, absence of the kidney, and hydronephrosis or hydroureter (8). There are many abnormal radiographic findings; however, none are pathognomonic for Rubinstein–Taybi syndrome. They include short and wide terminal phalanges of the thumbs and great toes; short, wide, and tufted terminal phalanges in most fingers; flaring of the ilia; and retardation of skeletal maturation (9).

Breuning et al. (151) identified the candidate region for Rubinstein–Taybi syndrome to chromosome 16 (16p13.3) by studying those patients with the disorder and reciprocal translocations. Petrij et al. (152) then showed that the breakpoints at 16p13.3 that were demonstrated in Rubinstein–Taybi patients were restricted to a region containing the gene for the human CREB-binding protein (*CREBBP*). They showed, furthermore, that point mutations in *CREBBP* could produce Rubinstein–Taybi syndrome. A certain level of *CREBBP* is essential for normal development. It is a nuclear protein that participates as a coactivator in cyclic-AMP-regulated gene expression and thus can affect various signal transduction pathways. In addition to those in *CREBBP*, mutations have also been identified in *EP300* (153). Both *CREBBP* and *EP300* are potent histone acetyltransferases, and part of the pathogenesis of this disorder may be due to abnormal chromatin regulation of an extensive number of genes.

### 160.4.4 Coffin–Siris Syndrome

Sparse scalp hair, generalized hirsutism, intrauterine and postnatal growth retardation, mental retardation, coarse

facial features, full lips, and hypoplastic distal phalanges and nails, particularly of the fifth finger and lateral toes, characterize Coffin–Siris syndrome (OMIM 303600) (154). Affected individuals have recurrent respiratory problems and feeding difficulties (22). Structural brain anomalies, partial gastric outlet obstruction, and cardiac anomalies have also been reported (155). The disorder was mapped to Xp22.2 (156), and deletion, nonsense, and missense mutations have been identified in *RKS2* (157). Interestingly, *RKS2* has been shown to activate *CREBBP* in vitro, suggesting that a similar pathway produces both Coffin–Siris and Rubinstein–Taybi syndrome (158).

### 160.4.5 Fanconi Anemia (Pancytopenia Dysmelia)

Fanconi anemia (OMIM 227650) is an autosomal recessive disorder characterized by pancytopenia, hyperpigmentation, short stature, and radial and renal defects. In some cases, not all these clinical features are present, and the severity of the features present can be extremely variable (159).

The pancytopenia is usually progressive. Average age of onset has been estimated as 8 years, and all marrow elements are involved. However, anemia may precede a decrease in white blood cells or platelets or vice versa. Reports of the onset of hematologic symptoms have varied from 17 months to 22 years. Fetal hemoglobin has been recorded as elevated, and in some cases there have been reduced numbers of erythrocytes and leukocytes and decreased levels of platelet hexokinase (160). Hyperpigmentation of the skin is probably the most consistent clinical feature and appears as fine generalized hypermelanosis, often presenting before hematologic manifestations and increasing with age. Café-au-lait spots are also seen. Pre- and postnatal growth deficiency is frequent, also associated with microcephaly. About 20% of affected individuals are mentally retarded. The most common bony defects are radial ray abnormalities with hypoplastic, absent, digitalized, or supernumerary thumbs; hypoplasia of the first metacarpal; and hypoplasia or absence of the radius and greater multiangular and navicular bones. Bifid thumbs, retarded skeletal maturation, osteoporosis, microcephaly with thick calvaria, syndactyly, and hip dislocations have been reported (161,162). Patients should be evaluated for renal abnormalities, and those with external ear anomalies should also be evaluated for associated deafness. Other reported abnormalities include spina bifida, Klippel–Feil anomalies, scoliosis, rib abnormalities, Sprengel deformity, and flat or clubbed feet.

Complications usually result from pancytopenia with bleeding, pallor, and recurrent infections. Therapy with steroids, bone marrow transplantation, and granulocyte colony-stimulating factor have been helpful for the pancytopenia, although life expectancy is reduced. Affected individuals have a predisposition to leukemia and other



tumors. Cancer deaths may be increased for the heterozygous carriers, though this is not completely clear (163). In patients with Fanconi anemia, chromosomal aberrations are seen and include a number of chromatid breaks, gaps, exchange figures, and endoreduplication, suggesting a defect in DNA repair.

Fanconi anemia is inherited as an autosomal recessive trait, except for one X-linked locus (*FANCB*; OMIM 300514), and genetic heterogeneity has been clearly established, with at least 13 genetic subgroups (164). Six genes responsible for the phenotype have been identified. Almost 80% of cases of Fanconi anemia can be accounted for by defects in either *FANCA* or *FANCC*. The Fanconi anemia (FA) nuclear complex is composed of the FA proteins A, C, E, F, and G, and is essential for protection against chromosome breakage. This complex activates the downstream protein *FANCD2* by monoubiquitination. This action then produces an association with the *BRCA1* (OMIM 113705) protein at sites of DNA damage (165). Thus this complex and process are essential for maintaining genomic stability.

#### 160.4.6 Aase Syndrome (Blackfan-Diamond with Thumb Anomalies)

Triphalangeal thumbs with hypoplastic anemia and growth deficiency have been reported in several sets of sibs (1a). The syndrome (OMIM 105650) also includes hypoplastic (normochromic, normocytic) anemia, present from infancy. Leukocytes and thrombocytes are normal, although fetal hemoglobin may be increased (166). Cardiac anomalies, cleft lip, and mild mental retardation can occur. Steroid therapy may be effective in some cases (167). In some patients, heterozygosity for mutations in *RPS19* on chromosome 19q13.2 have been identified (168). A second locus for the disease has been localized to chromosome 8p23.3 (169).

#### 160.4.7 Thrombocytopenia–Absent Radius Syndrome (Thrombocytopenia Radial Aplasia Syndrome)

Thrombocytopenia–absent radius (TAR; OMIM 274000) syndrome is considered to be an autosomal recessive and dominant trait, although consanguinity has not been truly reported (170). The cardinal clinical features are thrombocytopenia (usually with symptoms in the newborn period) and bilateral absence of the radius with the presence of both thumbs (171).

The thrombocytopenia is congenital and has a hypomegakaryocytic basis. Thrombocytopenia gradually improves over the first 2 years, and platelets may even be in the normal range in adulthood. Viral illnesses, particularly gastrointestinal viral illness, will often aggravate the thrombocytopenia. Leukemoid reactions are seen in 60–70% of patients during the first year. Eosinophilia has been reported in 50% of the patients. Anemia is

thought to be the result of blood loss but may be due to hypoplasia of red blood cells during the first year (171). The radius is absent bilaterally in all cases (Figures 160-7 and 160-8). The ulna is absent bilaterally in about 20% of the cases and is frequently hypoplastic in other cases. The humerus may be short and dysplastic. Thumbs are always present. Anomalies of legs and hips are occasionally seen (e.g. dislocated hips, tibial torsion, abnormal tibiofibular joints, stiff knees, dislocation of the patella, and camptodactyly of the toes with occasional clubfoot) (172). Congenital cardiac abnormalities are seen in one-third of the cases, the most frequent being tetralogy of Fallot and septal defects. Slightly short stature is the rule.

Complications are related to thrombocytopenia and symptomatic bleeding. This could be prevented by proper platelet transfusion. There may be delayed motor function because of hand anomalies; however, eventual good function can be expected. Other complications of the skeletal anomalies include nerve compression of the wrist and arthritis. TAR patients seem to have an increased incidence of allergy to cow's milk, which precipitates episodes of diarrhea and low platelets. The molecular etiology appears to be complex, thus explaining what



**FIGURE 160-7** Thrombocytopenia–absent radius syndrome. Radiographs of chest and upper limbs. Note absence of radius bilaterally, presence of thumb, and shortening of ulna.



**FIGURE 160-8** Infant with TAR syndrome. Note curved short forearm, ulnar deviation of the hand, and presence of the thumb bilaterally.

appeared to be both autosomal recessive and dominant forms of the disease. Klopocki et al. (172a) found that TAR syndrome results from a 200-kb microdeletion on chromosome 1, but the deletion itself is not sufficient to produce disease; another genetic modification is probably necessary to produce TAR syndrome.

Differential diagnosis should include Nager syndrome, pseudothalidomide syndrome, and Fanconi anemia. Ultrasound demonstration of absence of the radius and cordocentesis for platelet counts have resulted in accurate prenatal diagnosis.

### 160.4.8 Orofaciodigital Syndromes

The orofacioidigital (OFD) syndromes have been subdivided into five types, though seven types have been described. They are somewhat similar clinically but have different patterns of inheritance and different associated anomalies (173,174). In all types, the clinical manifestations of the face are striking, with hypertelorism, epicanthal folds, micrognathia, epidermoid cysts, hypotrichosis, and hypertrophic frenula with clefts of the alveolar margin, lips, and tongue. There is dystrophia canthorum, a broad nasal root, and hypoplasia of the alar cartilage and malar bone (6).

OFD type I (Papillon–Leage; OMIM 311200) is clinically distinguished by cleft palate, frenular hypertrophy, thick alveolar bands, aplasia of the nasal alae, ventral clefts and hamartomas of the tongue, dental anomalies, alopecia, and evanescent milia in infancy. Mental retardation is seen in approximately 50% of the cases and is apparently associated with central nervous system malformations, frequently agenesis of the corpus callosum. Polycystic renal anomalies have been seen in the kidneys of several autopsied cases. On radiographs, OFD type I is characterized by brachydactyly (due to short metacarpals, metatarsals, and phalanges and usually showing an irregular distribution), clinodactyly, syndactyly, camptodactyly, and cone-shaped epiphyses. Preaxial polydactyly has been seen in a few cases. OFD type I is an X-linked dominant disorder, which maps to chromosome Xp22.3–p22.2 (175), and hemizygosity for mutations in the gene *CXORF5* have been identified (176).

OFD type II (Mohr; OMIM 252100) is clinically distinguished by normal intelligence, normal hair, normal teeth, midline pseudocleft of the upper lip with associated midline hypertrophic frenula, and no lateral oral frenula. Affected individuals have bilateral hallucal polysyndactyly and conductive hearing loss. There is polysyndactyly of the toes, usually symmetrical with partial reduplication of the hallux, and postaxial polydactyly, primarily of the hand. Clinodactyly, brachydactyly, metaphyseal irregularity and flaring, and supernumerary sutures of the skull are also seen (9).

OFD type III (OMIM 258850) is very similar to type I but in addition has alternately see-saw winking of the eyelids and abnormal extraocular muscles. This entity

has been described only rarely. OFD type IV (OMIM 258860) is very similar to type II but in addition has short ribs, postaxial polydactyly, talipes, and severe tibial dysplasia. Both pre- and postaxial polydactyly are present. This syndrome overlaps the Majewski type of short rib-polydactyly syndrome (type II). OFD type VI (OMIM 277170), or Varadi–Papp syndrome, is distinguished from the other OFD types by the metacarpal abnormalities with central polydactyly and cerebellar abnormalities (177). There is some phenotypic abnormality with Joubert syndrome.

### 160.4.9 Holt–Oram Syndrome

Holt–Oram syndrome (HOS; OMIM 142900) is characterized by autosomal dominant inheritance (with penetrance of at least 90%), congenital heart disease, and radial ray defects. The characteristic hand malformation is digitalization of a triphalangeal thumb so that the thumb is attached in the same plane as other fingers. However, in some cases, the thumb is absent or rudimentary. In addition, the more severe cases show absence or hypoplasia of the radius or even the ulna and humerus. Wrist radiographs are particularly important. Abnormal scaphoid bone and an abnormal first metacarpal-to-first distal phalanges ratio may identify otherwise apparently normal gene carriers (178). Cardiovascular abnormalities are variable. The most common cardiac defect is an atrial septal defect of the ostium secundum type. Other described defects are patent ductus arteriosus, pulmonary hypertension, ventricular septal defect, and transposition of the great vessels with a prolonged P–R interval. Marked variability of limb and heart malformations may occur within the same family. The bony changes that may be seen radiographically are triphalangeal, hypoplastic, proximally placed, or absent thumb; an abnormally shaped scaphoid bone; additional carpal bones or lack of ossification of the carpals, particularly the os centrale; a long ulnar styloid; carpal fusions; an apparent increase in length of the first metacarpal and shortening of the fifth middle phalanx with clinodactyly; a prominent or posterior projection of the medial epicondyle; clavicular hypoplasia; deformed humeral head; and small rotated scapulae (179).

Terrett et al. (180) studied seven families with HOS and established linkage to chromosome 12q in five families. In 1997, both Li et al. (181) and Basson et al. (182) demonstrated that HOS is due to heterozygosity in the *TBX5* gene. *TBX5* is one of the T-box genes involved in DNA binding. It is known to bind to NKX2-5 and synergistically promote cardiomyocyte differentiation. In HOS, there are some genotype–phenotype correlations (183). Defects producing null alleles are predicted to produce both limb and heart defects; however, specific missense mutations produce either significant cardiac defects with minor skeletal anomalies, or the converse.

Clinically, HOS is distinct from other radial ray defect syndromes in that there are no abnormalities of the kidneys or gastrointestinal tract, no deafness, no ear malformations, no mental retardation, and no specific hematologic disorder.

#### 160.4.10 Focal Femoral Hypoplasia (Proximal Femoral Focal Deficiency)

In focal femoral hypoplasia, variable deficiency of the proximal segment of the femur either unilaterally or bilaterally occurs with preservation of the distal part of the limb. There is a continuum of involvement starting most proximally, involving just the femoral head, and extending to involving the lower limb with complete absence of the femur. Severely involved cases may even fall into the femur–fibula–ulna (FFU) complex. Some cases have unusual facies (see later). Typically, most cases of proximal femoral focal deficiency (PFFD) are sporadic with no known etiology.

When the pattern of long bone loss in the lower limb is analyzed, two specific patterns emerge: FFU complex and femur–tibia–radius (FTR) complex. Among PFFD patients, 50% also have a fibular deficiency and 25% have an ulnar deficiency (FFU). Among these FFU patients, usually all four limbs are involved, but there may be marked asymmetry. Variations include coxa vara to complete femur absence, shortened ulna to humero-radial synostosis, and small fibula to complete absence. Usually no spine or visceral anomalies are present. The hand and foot are relatively spared, but anomalies have been reported (4).

In the FTR pattern, the deficiency at the distal end of the femur is most important. Loss of the tibia may be most striking, but any combination of loss may occur. The complex tends to be seen more in genetic forms of lower limb deficiency as compared with PFFD and FFU, which appear to be sporadic (184). The FTR pattern was also seen in thalidomide embryopathy (4).

#### 160.4.11 Femoral Hypoplasia–Unusual Facies Syndrome

A distinct pattern of malformation characterized by Daentl et al. (185) was designated femoral hypoplasia–unusual facies syndrome (OMIM 134780) (Figure 160-9). Clinical features include femoral hypoplasia, usually on the severe end of the spectrum, and the unusual facial features of up-slanting palpebral fissures, short nose with broad nasal tip, long philtrum, thin upper lip, micrognathia, and cleft palate. In addition, renal anomalies, lower vertebral anomalies, and deformed pelvis have been reported. With multiple surgeries and the use of prosthetic devices, most patients are ambulatory and functional. Intelligence is completely normal. Occasionally, with severe micrognathia, feeding and respiratory problems occur.

Shortening of the humerus, restricted motion at the elbows, and Sprengel deformities found in some infants would suggest that this syndrome is not confined to the lower limbs and that a similar mechanism could be acting on both upper and lower limbs. All reports so far have been of sporadic cases; however, an association with maternal diabetes has been suggested.

#### 160.4.12 Hand–Foot–Uterus Syndrome (Hand–Foot–Genital Syndrome)

The hand–foot–uterus syndrome (OMIM 140000) is an autosomal dominantly inherited condition with variable expressivity (186). Clinical features include small feet with unusually short halluces and short, abnormal thumbs. Females with the disorder have duplication anomalies of the genital tract and decreased fertility. Genital tract anomalies are variable but are thought to represent different degrees of expression of the same underlying developmental problem. Duplication of the uterus varies in severity from a full double uterus to a uterus duplex unicornis. In addition, some patients have duplication of the cervix with a septate vagina. These structural anomalies result in fertility complications; however, normal pregnancies are possible. Males have been reported with cryptorchidism and hypospadias; however, these seem to be distinct embryologically from the female genital duplication anomalies (187). Congenital heart disease has been reported in some patients; therefore, a careful cardiovascular examination is warranted.

Radiographic findings include short first metacarpals and metatarsals, short fifth fingers with clinodactyly, trapezium–scaphoid fusion in the wrist, cuneiform–navicular fusion in the foot, an os centrale, and a long ulnar styloid (186).

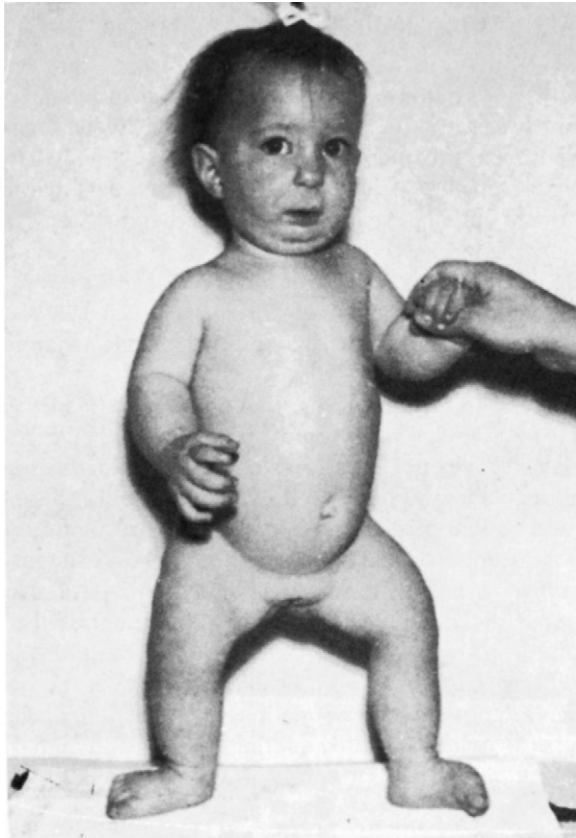
The gene defective in hand–foot–genital syndrome is *HOXA13* (188). Identified mutations in *HOXA13* have included stop codons, expansion of polyalanine tracts, and missense mutations in a highly conserved domain of the protein (189).

#### 160.4.13 Focal Dermal Hypoplasia (Goltz Syndrome)

Focal dermal hypoplasia (FDH; OMIM 305600) has been observed almost exclusively in females; however, there are reported cases of affected males (190,191). It appears to be an X-linked dominant condition with increased lethality in males. Clinical features include cutaneous changes, skeletal defects, digital malformations, ocular anomalies, and oral/dental anomalies (192).

The most striking clinical features in all the cases of this syndrome are the widespread foci of dermal hypoplasia with herniation of fat (appearing as yellow-brown nodules), red streaking of the skin, and papillomas of the lips, gums, vulva, and anus. The areas of dermal hypoplasia appear as atrophy or absence of skin, but the epidermis is





**FIGURE 160-9** Focal femoral hypoplasia with unusual facies syndrome. Note the short upper leg, short nose with broad nasal tip, long philtrum, thin upper lip, micrognathia, and left equinovarus deformity of the foot.

intact. Telangiectasia and hypo- and hyperpigmentation are seen. Surgery is indicated for removal of lesions (e.g. angiofibromas) that are in areas prone to trauma. The hair is sparse, sometimes blonde or almost white in color, with patchy areas of alopecia. Nails may be absent, hypoplastic, dystrophic, spoon-shaped, or grooved. Teeth are congenitally absent or malformed (193).

Digital anomalies include syndactyly, polydactyly, camptodactyly, and absence deformities. Most individuals have short stature and can have vertebral anomalies leading to kyphosis and scoliosis. There is asymmetry of the face, trunk, and limbs. Several different ocular changes have been described: microphthalmia, colobomas, strabismus, and nystagmus. There is occasional mental retardation.

The degree of clinical severity varies widely. Some patients may have only skin changes or simply syndactyly, whereas others may show full manifestations of the syndrome with microphthalmos, bilateral coloboma of the iris, ectopia lentis, hypoplasia of the teeth, and severe cutaneous, skeletal, and digital malformations. Happle and Lenz suggested the patchiness represents mosaicism with death of the cells that have the affected X activated (194). Mutations producing Goltz syndrome have been identified in the porcupine, drosophila, homolog of, (POCN) gene (195).

#### 160.4.14 Ulnar–Mammary Syndrome

The combination of ulnar ray defects, hypoplasia or aplasia of mammary glands, hypoplasia of nipples, hypoplasia of apocrine glands with reduction in body odor, and genital anomalies in males is an autosomal dominant disorder (OMIM 181450) (196). The ulnar ray defect may be so mild as to involve only stiffness of the distal interphalangeal fifth finger joint or hypoplasia of the fifth fingernail, or may present with complete absence of one to three ulnar rays of fingers (197). Other reported visceral anomalies include dental abnormalities, subglottic stenosis, pyloric stenosis, anal stenosis, imperforate hymen, kidney malformations, hypodontia, and pectoralis muscle hypoplasia.

Carpals and tarsals may also be involved, postaxial polydactyly has been seen, and feet may show hypoplasia of toes. Axillary and body hair is sparse, but pubic hair is normal. Nipple hypoplasia may lead to the inability to breast-feed. Affected males may have cryptorchidism, small penis, and small testes with decreased sperm counts and infertility. In a large family, the chromosomal location for ulnar-mammary syndrome was identified on chromosome 12q24.1 (198). Further analysis demonstrated that the defective gene is *TBX3* (199), a T-box gene.

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# CHAPTER

# 161

## Arthrogryposes (Multiple Congenital Contractures)

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### 161.1 INTRODUCTION

Arthrogryposis multiplex congenita (AMC) is a term that has been used for almost a century to describe conditions with nonprogressive multiple congenital joint contractures. The conditions that have been called arthrogryposis range from well-known syndromes to nonspecific combinations of joint contractures. The term has become descriptive rather than diagnostic and is now used in connection with a very heterogeneous group of patients and disorders, all of which have in common multiple congenital joint contractures. We have begun to realize that over 350 specific disorders are or maybe associated with multiple contractures in the newborn, and that, although most improve over time, some are progressive.

Arthrogryposis has been said to be a rare condition, but in fact it has an incidence somewhere between 1 in 3000 and 1 in 5000 live births, much more common than most genetic disorders and congenital anomalies (1–4). Many kinds of congenital contractures, such as clubfeet and dislocated hips, are relatively quite common. One in 100 to 200 infants is born with some type of congenital contracture. The term arthrogryposis implies a more generalized involvement, with multiple joints having congenital contractures, and is most often reserved for conditions that involve more than one part of the body.

The medical literature on arthrogryposis (multiple congenital joint contractures) is very confusing. Over the past 50 years, more than 3000 articles have been published describing various types of arthrogryposis, but the term has been used very loosely, initially as a diagnostic term but more recently as a clinical sign or as a general category of disorders. In addition to the imprecise use of the term, the medical literature on arthrogryposis is confusing, because many authors fail to fully describe the clinical features of their cases, or to separate subgroups. In the past, authors have often lumped together several

patients with congenital contractures, who in actuality represent different specific entities, and have then made generalizations about recurrence, management, prognosis, and treatment. More recently, responsible genes have been identified for many specific types of arthrogryposis and the range of variability is being defined (see [Tables 161-7 and 161-8](#)). This chapter attempts to sort out some of the major known clinical entities with congenital contractures, to describe a clinical approach to distinguishing heterogeneity, to discuss the investigation of the individual with congenital contractures, and to make some comments about genetics, recurrence risk, prenatal diagnosis, and therapy (2,5–8).

It has become increasingly apparent, both from animal studies (9–12) and from human work (13–22), that anything that leads to decreased movement *in utero* may also lead to congenital contractures or fixation of joints that will then be present at birth. Even prolonged hypotonia may lead to congenital contractures.

The early studies of Drachman and Coulombre (11) demonstrated that temporary paralysis by curarization of chick embryos at various times in development resulted in multiple congenital contractures. The immobilized chick developed fixation of different joints, depending on the time in morphogenesis that immobilization occurred. DeMyer and Baird (10) have shown that removal of amniotic fluid during gestation in rats could lead to limitation of movement of joints and intrauterine growth retardation, with the development of generalized contractures by birth. Most recently, early amniocentesis has also been associated not only with congenital contractures of the foot, but with arthrogryposis (23).

Moessinger (12) noted that rat fetuses that had been paralyzed *in utero* by curare developed a set of anomalies he designated the “fetal akinesia deformation sequence.” He noted that these findings were similar to anomalies seen in the human Pena–Shokeir syndrome (type I),

including multiple joint contractures, pulmonary hypoplasia, micrognathia, intrauterine growth retardation, short umbilical cord, and polyhydramnios. These clinical features are seen in several specific conditions (16,24,25). When any one of them is present, the others should be looked for.

It appears that any process that limits movement during development of the fetus or embryo may lead to congenital contractures. The *in utero* process may well be similar to the postnatal process of wearing a cast for a broken bone. When the cast is taken off, there is usually residual limitation of movement in joints that were immobilized. Swinyard (18) has called this a “collagenic response,” with thickening of the joint capsule and fibrous development in muscle tissue. Undoubtedly, lack of movement either produces a set of cytokines or fails to produce a set of cytokines that ultimately leads to this connective tissue response. The growing embryo or fetus that has superimposed limitation of movement may develop even more marked contractures because the process of growth may compound the contractures leading to additional deformation. In this regard, most congenital contractures may be considered deformations rather than primary malformations (1003), since the limbs are usually formed normally and then develop secondary changes. The timing during development probably plays a critical role in the severity of contractures, in the position of joints, and in the secondary changes that may occur in other organ systems (16,25,84). It would appear that lack of movement or hypotonia takes over a month to produce contractures in the third trimester, but the length of time may be less in the first and second trimesters.

The potential causes of limitation of movement *in utero* include:

1. Myopathic processes, including myopathies and abnormal muscle structure or function, such as absence or loss of muscle tissue and congenital myopathies
2. Neuropathic processes, including abnormalities in nerve structure or function, either central or peripheral; failure of nerves to form, migrate, or myelinate; and congenital neuropathies
3. Neuromuscular end-plate abnormalities, including abnormal structure receptors and transmitters
4. Abnormal connective tissue, including bone, joint, and tendon abnormalities; skin abnormalities (such as restrictive dermopathy); and abnormal tendon attachments
5. Limitation of space or restriction of movement within the uterus, as in the case of twins, structural anomalies of the uterus, amniotic bands, fibroids, or decreased amniotic fluid, as with leakage
6. Problems related to maternal illness, including maternal infections, interference with fetal neurotransmitters as by antibodies, etc.
7. Maternal exposures related to medications, drugs, and environmental factors, such as muscle relaxants, high temperature, fever, etc.
8. Compromise of the blood supply to the placenta and/or the embryo/fetus.

## 161.2 APPROACH

An approach to sorting out various types of congenital contractures (Table 161-1) includes taking a careful history of the pregnancy and delivery; a full family history; a detailed physical examination with documentation of what parts of the body are involved in the process; photographs at different ages and measurements including range of movement of various joints; a natural history of complications and response to therapy; laboratory data, such as muscle biopsies; imaging studies; autopsy results, including central nervous system (CNS) histopathology; chromosome studies; and molecular studies as appropriate, including comparative genomic hybridization (CGH) microarray. This kind of evaluation has proven to be important and necessary in distinguishing different types of arthrogryposis. Thus, in the course of the investigation of a patient with congenital contractures, definition of all these areas will be helpful in arriving at a specific diagnosis.

### 161.2.1 Histories

Pregnancy history, delivery history, family history, and natural history over time are all extremely important, and several particular points should be kept in mind in their analysis.

**161.2.1.1 Pregnancy History.** The pregnancies of infants with multiple congenital contractures do have an increased frequency of complications (27). The question of intrauterine infections should always be considered in the evaluation of infants with congenital contractures, particularly those with neurologic impairment. Not infrequently, the mother has had an infection that could have led to secondary fetal CNS damage, and she may not even be aware of it. It is appropriate to do immunoglobulin M studies in the newborn with congenital contractures, looking for increased levels that would indicate intrauterine infection, and in addition, specific titers can be measured (coxsackievirus, enterovirus, Akabane virus, etc.). Seasonal and geographic variables may be indicators of the particular viral infection that should be investigated. Maternal fever greater than 39°C for an extended period may lead to CNS damage. Secondary contractures may occur in the fetus because of abnormal nerve growth or migration association with maternal hyperthermia (28). Chronic maternal illness such as diabetes mellitus, multiple sclerosis, myotonic dystrophy, or myasthenia gravis are important because they have been associated with arthrogryposis in the newborn due to secondary effects, such as abnormal glucose metabolism, increased triple



**TABLE 161-1 Clinical Evaluation****History**

Pregnancy (anything decreasing *in utero* movement leads to congenital contractures)

- Illness in mother, chronic or acute (diabetes, myasthenia gravis, myotonic dystrophy, etc.)
- Infections (rubella, rubeola, coxsackie, enterovirus, Akabane, etc.)
- Fever ( $>39^{\circ}\text{C}$ , determine timing in gestation)
- Nausea (viral encephalitis, position of baby, etc.)
- Drugs (curare, robaxin, alcohol, dilantin, addictive drugs, misoprostol, etc.)
- Fetal movement (polyhydramnios, fetal kicking in one place, "rolling," decreased)
- Oligohydramnios, chronic leakage of amniotic fluid
- Polyhydramnios, hydrops
- Trauma during pregnancy (blow to the abdomen, attempted termination, car accident, etc.)
- Other complications during pregnancy such as bleeding, abnormal lie, threatened abortion, etc.
- Prenatal diagnosis (early amniocentesis, ultrasound studies, etc.)

**Delivery history**

- Presentation (breech, transverse, etc.)
- Length of gestation
- Traumatic delivery (limb position, CNS, fracture, etc.)
- Intrauterine mass (twin, fibroid, etc.)
- Abnormal uterine structure or shape
- Abnormal placenta, membranes, or cord length or position
- Time of year, geographic location

**Family History**

- Marked variability within family
- Change with time – degenerate vs improve
- Increased incidence of congenital contractures in second- and third-degree relatives
- Hyperextensibility or hypotonia present in family member
- R/O myotonic dystrophy, myasthenia gravis in parents (particularly mother)
- Consanguinity
- Advanced parental (mother or father) age
- Increased stillbirths or miscarriages
- If more than one consecutively affected child, consider maternal antibodies to fetal neurotransmitter

**Newborn Evaluation****Description of contractures**

- Which limbs and joints
- Proximal vs distal
- Flexion vs extension
- Amount of limitation (fixed vs passive vs active movement)
- Characteristic position at rest
- Severity (firm vs some give)
- Complete fusion or ankylosis vs soft tissue contracture

Other anomalies (contractures are most obvious, look carefully for other anomalies)

**Deformities**

Genitalia (cryptorchid, lack of labia, microphallus, etc.)

Limbs (pterygium, shortening, webs, cord wrapping, absent patella, dislocated radial heads, dimples, etc.)

Jaw (micrognathia, trismus, etc.)

Facies (asymmetry, flat bridge of nose, hemangioma, movement, etc.)

Scoliosis and kyphosis (fixed or flexible)

Dimples (over specific joints or bones)

Skin (hemangioma, defects, hirsutism)

Dermatoglyphics (absent, distorted, crease abnormalities, etc.)

Hernias, inguinal and umbilical, abdominal wall defect

Other features of fetal akinesia sequence

- Intrauterine growth restriction
- Pulmonary hypoplasia
- Craniofacial anomalies (hypertelorism, cleft palate, depressed tip of nose, high bridge of nose)
- Functional short gut with feeding problem
- Short umbilical cord

**Malformations**

Eyes (small, corneal opacities, malformed, ptosis, strabismus, etc.)

CNS (structural malformation, seizures, MR, etc.)

Palate (high, cleft, submucous, etc.)

Limb (deletion anomalies, radioulnar synostosis, etc.)

GU (structural anomalies of kidneys, ureters, and bladder)

Skull (craniosynostosis, asymmetry, microcephaly, etc.)

Heart (congenital structural anomalies vs cardiomyopathy)

Lungs (hypoplasia vs weak muscles or hypoplastic diaphragm)

Tracheal and laryngeal clefts and stenosis

Changes in vasculature (hemangiomas, cutis marmorata, blue cold distal limbs, etc.)

Other visceral anomalies

**Other Features**

Neurologic examination (detailed)

- Vigorous vs lethargic
- Deep tendon reflexes (present vs absent, slow vs fast)
- Sensory intact or not

**Muscle**

- Mass (normal vs decreased)
- Texture (soft vs firm)
- Fibrous bands
- Normal tendon attachments or not
- Changes with time

**Connective tissue**

- Skin (soft, doughy, thick, extensible)
- Subcutaneous (decreased fat, increased fat)
- Hernias (inguinal, umbilical, diaphragmatic, or eccentric)
- Joints (thickness, symphalangism, etc.)
- Tendon attachment and length

**Course****Changes with Time**

Developmental landmarks (motor vs social and language)

Growth of affected limbs

Progression of contractures

Lethal vs CNS damage vs stable vs improvement

Asymmetry (decreases or progresses)

Trunk vs limb changes

Intellectual abilities

Socialization

Feeding problems

**Response to Therapy**

Spontaneous improvement

Response to physical therapy

Response to casting

Which surgery at which time

Development of motor strength proportionate to limb size

Abnormal reaction to drugs

*Continued*

TABLE 161-1 Clinical Evaluation—Cont'd

**Laboratory Evaluation***Tests*

Documentation of range of motion and position with photographs

Radiographs if:

- Bony anomalies (gracile, fusions, extra or missing carpals and tarsals, etc.)
- Disproportionate
- Scoliosis
- Ankylosis
- Dislocation (hips, radial head, patella, etc.)

CT scan, MRI, or functional MRI to evaluate CNS or muscle mass obscured by contractures

Ultrasonic evaluation of CNS, other anomalies, or to establish potential muscle tissue

Chromosome studies or CGH if:

- Multiple system involvement
- CNS abnormality (eye, microcephaly, MR, lethargic, degenerative)
- Streaky or segmental involvement
- Consider fibroblasts if lymphocytes were normal and patient has MR with no diagnosis
- Gene testing if fits known disorder in which gene testing available

Video of movement including facial, range of movement, strength – repeat at regular intervals

Viral culture ± specific antibodies or IgM levels in newborn

Muscle biopsy in normal and affected areas at time of surgery to distinguish myopathic from neuropathic (do special histopathology and electron micrographic studies) – if CPK or unusual muscle response, do muscle biopsy earlier, examine mitochondria

EEG - if seizures or episodes

EMG in normal and affected area

Nerve conduction in normal and affected area

CPK if:

- Generalized weakness
- Doughy or decreased muscle mass
- Progressively worse

Eye examination (opacities, retinal degeneration, etc.)

Maternal antibodies to neurotransmitters, if myasthenia gravis or recurrent affected pregnancies without diagnosis

Spinal muscular atrophy (SMN) DNA testing if accompanying hypotonia

Mitochondrial DNA if other suggestions of mitochondrialopathy

Metabolic screening – see Table 161-16

*Autopsy*

Visceral anomalies

CNS – brain neuropathology

Spinal cord (number and size of anterior horn cells, presence or absence of tracts at various levels)

Ganglion, peripheral nerve

Eye (neuropathology)

Muscle tissue from different muscle groups (EM and special stains, R/O ragged red fibers)

Diaphragm for thickness or hernia

Fibrous bands replacing muscle

Cartilaginous or bony fusion

Tendon attachments

Other malformations, deformations, or disruptions

CGH array if multiple congenital anomalies

Save DNA for molecular testing

CGH = comparative genomic hybridization; CNS = central nervous system; CPK = creatine phosphokinase; CT = computed tomography; EM = electron microscopy; EMG = electromyography; GU = genitourinary; IgM = immunoglobulin M; MR = mental retardation; MRI = magnetic resonance imaging; R/O = rule out.

repeat expansion, or inhibiting fetal neurotransmitters (21) (Table 161-6). A history of nausea in the mother is of importance as a high frequency of pregnancies that result in congenital contractures have been noted to be associated with excessive nausea at various times in pregnancy (27). It is not known whether the nausea is due to a maternal viral infection, to abdominal discomfort, to irritation because of an abnormal positioning of the fetus, or to something else. Use of drugs by the mother during the pregnancy that might lead to decreased movement of the fetus, such as muscle relaxants, drugs that may lead to potential CNS damage and secondarily affect fetal movement (e.g., alcohol), prostaglandins (e.g., misoprostol), and vasoconstrictors, should all be queried about specifically when taking a pregnancy history (18,29). Trauma or surgery during the pregnancy may be associated with the development of multiple contractures. An increased occurrence of congenital contractures associated with failed attempted termination of pregnancy has been observed (30a,30b). Early amniocentesis appears to be associated with a small increased risk for multiple congenital contractures (23). A careful pregnancy history frequently indicates abnormal fetal movement (decreased movement, movement in only one area, or a “swimming

motion”). Polyhydramnios during the pregnancy may indicate fetal compromise (e.g., that the fetus is not swallowing normally). Together with hydrops of the fetus, polyhydramnios is a poor prognostic sign for viability. Long standing oligohydramnios or chronic leakage of fluid may lead to fetal constraint and secondary deformational contractures. An abnormal fetal position may be a clue to intrauterine joint contractures. Both transverse lie and breech positioning are seen with increased frequency in pregnancies that result in arthrogryposis. Prenatal diagnosis is possible for most types of arthrogryposis using real-time ultrasonic evaluation of the fetus looking for movement and range of movement of joints; however, routine prenatal diagnosis usually does not pick up congenital contractures. Because management of the pregnancy may be altered if arthrogryposis is recognized prenatally, it should be considered in situations of an abnormal fetal position, polyhydramnios, or when the mother reports decreased movement (85).

**161.2.1.2 Delivery History.** Delivery history is usually abnormal in arthrogryposis, either because of an abnormal presentation or difficulty in delivery due to the fixed joints. Breech position and even spinal hyperextension are often seen. It is not unusual for a fracture to

occur during delivery (5–10% of the time (31)) or after birth iatrogenically when working with or evaluating the contractures. The presence of fractures in the newborn with arthrogryposis does not specifically imply a skeletal dysplasia since affected individuals often have thin, gracile, osteoporotic long bones (32). Babies with congenital contractures have been erroneously diagnosed as having osteogenesis imperfecta by inexperienced individuals simply on the basis of fractures. However, a form of arthrogryposis and osteogenesis imperfecta (Bruck syndrome) does exist. The length of gestation is usually normal, although certain conditions with congenital contractures, such as trisomy 18, may go past the delivery date, and other factors, such as congenital infections and rupture of membranes, may lead to early delivery. The length of labor is often prolonged because of difficulty in actually delivering due to the unusual position of the fetal joints. Breech and transverse positioning are relatively common, leading to cesarean section. Cesarean section is also often performed due to fetal distress. Both CNS and limb trauma may occur during delivery. Infants in a hyperextended position *in utero* should be delivered by cesarean section to avoid damaging the spinal cord (31).

Careful examination of the uterus for structural uterine anomalies or a uterine mass such as a fibroid is important if cesarean section is performed. The placenta, membranes, and cord insertion need to be examined looking for amniotic bands or signs of vascular compromise. The umbilical cord may be shortened related to decreased *in utero* movement, or may actually be long when wrapped around a limb. In such a case, vascular or mechanical compression of that limb may occur. Ectopic pregnancies are usually associated with congenital contractures.

**161.2.1.3 Family History.** A careful family history is important. There may be marked variability within families as to severity of contractures; congenital contractures in the parents of an affected child may actually have worked themselves out by the time the parents reached adulthood. The lack of normal flexion creases in the adult is a clue that there was lack of normal *in utero* movement. Frequently, family members have other kinds of connective tissue abnormalities such as hyperextensibility, dislocated joints, clubfeet, and dislocated hips (2). A surprisingly large number of children (15–20%) with arthrogryposis have multiple congenital contractures secondary to specific complications, such as hypotonia *in utero*, CNS structural anomalies, or lax connective tissue occurring with a known genetic disorder. For instance, congenital contractures are occasionally seen in association with tuberous sclerosis and neurofibromatosis. Infants with multiple congenital contractures are born more often than by chance to mothers affected with myotonic dystrophy, myasthenia gravis, multiple sclerosis, and arthritis. Thus careful family histories with special attention to relevant disorders should be taken. Consanguinity, multiple stillbirths or miscarriages, and advanced parental (both mother and father) age should be asked about.

## 161.2.2 Physical Examination

Careful newborn examination and documentation is important. Photographs at birth and regularly thereafter are extremely helpful in identifying a specific type of arthrogryposis. Position of contractures as well as active and passive range of motion should be carefully measured and described. It is important to document whether the contractures are proximal or distal, and in flexion or in extension, the amount of limitation, and the characteristic position at rest. Some conditions usually only involved the arms and other usually only the legs (Table 15). Spine and jaw contractures should be looked for. Flexion creases, particularly of the hand, should be documented. The presence of dimples overlying joints with contractures is frequent. Skin webs across joints with limitation of movement are common and may be present at birth or develop with time. The findings may reflect something about the time of onset of the limitation of movement of the affected joint. Assessment of muscle function and strength should be documented at regular intervals. It is important to describe tendon attachments, because “misplaced” tendons and abnormal or missing sesamoid bones in the tendons are frequently seen (33,950). For instance, the patella is often missing or underdeveloped. Limitation of opening of the jaw (trismus) should be defined specifically, because it has significance for feeding and intubation as well as diagnosis (34). Care should be taken when examining the range of motion since iatrogenic fractures may occur during examination in the newborn period.

A variety of additional anomalies, including disruptions, deformations, and malformations, have been seen in children with congenital contractures. These anomalies may well give some clue to the underlying basic process and to the differential diagnosis. Careful documentation of the neurologic status, including the presence of hypotonia or hypertonia as well as the amount and texture of the muscle tissue present, is important. Range of eye movements is important. Eye examination by an ophthalmologist should be done at least once. Other connective tissue findings, such as loose or tight skin, hyperextensibility, and the like, should be documented. Pigmentary changes in the skin, abnormal hair pattern, and the presence of edema or hirsutism should be recorded.

## 161.2.3 Course

The natural history and response to therapy are different in different forms of arthrogryposis. These differences are helpful in distinguishing specific entities but may also indicate the need for different types of therapy. For instance, physical therapy to stretch contractures is usually indicated, but in diastrophic dysplasia this may actually lead to ankylosis of joints. Many forms of arthrogryposis respond well to simple physical therapy and use (e.g., the hands in distal arthrogryposis), whereas others usually require surgical procedures in addition to

physical therapy (e.g., most cases of amyoplasia). Prognosis for children with normal intelligence may be very good and enable independent living and productive lives despite severe handicaps. A rare form of arthrogryposis is prone to malignant hyperthermia, so anesthesia should be undertaken with care in all children with congenital contractures (35). Trismus leads to difficulty with feeding and intubation. Seizures may develop with age (36). Some rare forms include deterioration of mental status. All children with arthrogryposis will have motor developmental delay.

Parents should be encouraged to keep photographic or video records over time. They should keep a notebook of photographs, procedures, consultations, diagnostic tests, growth, response to therapy, and so forth, to illustrate changes and accomplishments over time.

### 161.2.4 Therapy

Treatment of arthrogryptic joints is a controversial topic, probably because there is no one completely successful approach (8,20,37–43,44b,45–52,53b,54,1004,1017). It has become clear that vigorous physical therapy early is very important to avoid muscle atrophy. Splinting, combined with physical therapy, may be preferable to continuous casting. Night splints after surgical procedures are used to maintain the increased range of movement and position. Recently, Botox has been used in mild cases. If surgery is undertaken, muscle biopsy should be performed at least once from an affected area and from a normal area of muscle for comparison. Malignant hyperthermia occurs rarely in association with anesthesia (particularly in myopathies), so care should be taken to monitor temperature during surgery (35,56). Children with arthrogryposis frequently develop otitis (secondary to spending excessive time lying on their backs) leading to decreased hearing and middle ear fluid retention, but rarely have deafness on the basis of sensorineural abnormalities or bony anomalies of the middle ear (57a). Some affected children have feeding problems early, related to uncoordinated swallowing and weak muscles. They often improve over time, but may require G or J tubes initially. Some children need speech therapy. The lungs may be hypoplastic and respiratory support may be needed in the newborn period. Stem cell therapy (involving both muscle and nerve) is not possible at this time, but considering the advances in tissue specific growth factors, may be possible in some combination in the future. Most recently, therapeutic doses of acetylcholine-like drugs may be useful in multiple pterygium syndromes related to receptor subunit deficiencies.

### 161.2.5 Laboratory Tests

On the whole, laboratory tests have not been extremely useful in making a diagnosis in cases of congenital contractures. Electromyography (EMG), muscle biopsies,

nerve conduction studies, and muscle enzyme studies are quite often interpreted as showing nonspecific or disuse changes; however, if they indicate a myopathy (58,59), that may be very helpful in making a diagnosis. Muscle biopsies often show fatty and connective tissue replacement of muscle fibers, variation in muscle fiber size, or decreased fiber diameter; all nonspecific signs of muscle atrophy. However, ragged red fibers and specific myopathic and inclusion changes should be looked for. Radiographs are useful in ruling out specific bone dysplasias and in documenting disproportion, osteoporosis, and scoliosis. Ultrasound studies may help to document the amount of muscle present (51). Magnetic resonance imaging (MRI) and computerized axial tomography (CAT) scans also help to define the amount of muscle present as well as CNS structural anomalies (60). Maternal antibodies to fetal neurotransmitters is a useful test if recurrence is unexplained (21). Many specific genes have been found for specific disorders (Tables 161-7 and 161-8 list known responsible genes and their chromosome locations). Linkage or DNA studies may be warranted depending on clinical features. The genes for over 120 disorders with multiple congenital contractures have been identified. The genes responsible for an additional over 70 disorders with multiple congenital contractures have been found since the last edition. However, no screening DNA chip is available. Interestingly, many genes related to muscle proteins are being identified, even in disorders that had previously been thought to be neuropathic disorders. Chromosome studies and CGH array should be performed in affected individuals with additional structural anomalies (other than deformations) or when mental disability is present, since many microdeletions and duplication syndromes have multiple congenital contractures (Table 161-5). Several metabolic and storage disorders may have multiple congenital contractures at birth making appropriate screening important (Table 161-16).

### 161.2.6 Autopsy

If a child with multiple congenital contractures dies, it is very important to do a complete autopsy. Information on the changes seen in arthrogryposis on autopsy, as well as in normal tissue, demonstrates a wide range of possibilities by present techniques (58–60,62–65). The pathogenesis that is associated with particular patterns of abnormalities is often hard to interpret. Spinal cord sections at multiple levels and muscle pathology from several sites need to be evaluated (62). The finding of specific changes that allow diagnosis is very important to the family if it can establish the risk for recurrence. Structural CNS abnormalities are frequent, including migration and myelination abnormalities. Increased size of ventricles and decreased brain mass as well as structural anomalies of the brain are not uncommon. “Misplaced” tendons are frequently seen; whether these are primary



or secondary changes is not yet clear (950). Thickening of joint capsules and fibrous fatty replacement of muscle are frequent. Major or specific structural abnormalities of organs or brain help to delineate specific conditions. Examination of the eye may be helpful. DNA should be saved for studies or future use, depending on findings.

### 161.2.7 Genetics

Some generalizations can be made about the genetic aspects of arthrogryposis. In the past, when a child with arthrogryposis or congenital contractures was born to normal parents with an unremarkable family history, the parents were given an empiric “polygenic/multifactorial” 5% recurrence risk that they might have another affected child. However, with careful documentation, a specific diagnosis can be reached in at least half of the patients seen with arthrogryposis (2). For instance, amyoplasia appears to have no recurrence risk at all (31,66). Distal forms of arthrogryposis may have as much as a 50% recurrence risk. Multiple forms of X-linked recessive arthrogryposis exist (67–69). In the category of individuals with CNS involvement, consideration of chromosome anomalies, particularly submicroscopic, and mosaicism must be excluded; this sometimes involves fibroblast chromosome studies (70) and microarray studies on two or more tissues. Among children with arthrogryposis and CNS involvement, the recurrence risk may be as high as 25%, with an average risk of 10–15%. (This implies, of course, that there is a high incidence of autosomal recessive disorders or maternal factors within this category.) Recognition that maternal antibodies against embryonic or fetal proteins related to movement can lead to arthrogryposis suggest new avenues of diagnosis. Every effort to achieve a specific diagnosis should be made; however, for those families in which a specific diagnosis cannot be made, the empiric recurrence risk to unaffected parents of an affected child or to the affected individual with arthrogryposis continues to be in the 3–5% range (2).

Prenatal diagnosis is possible in many conditions with congenital contractures, using real-time serial ultrasound studies to evaluate fetal movement (71–80a,81–84). Real-time ultrasound studies at 16, 20, 24, and 32 weeks are recommended, and require 45–60 min of observation related to various joint movement and structural evaluations (84). Nuchal edema and gracile osteoporotic bones may be seen. Chest and lung size should be evaluated since many forms of Pena–Shokeir phenotype have markedly reduced lung size (25,84). There are many reported cases of failure to make the diagnosis in an infant with multiple joint contractures using real-time ultrasound during the second trimester (85). In our experience, the presence and timing of contractures in the second trimester is dependent on the specific condition. In several genetic forms of arthrogryposis, contractures are easily seen by 14 weeks, whereas in others they do not occur until 20 or 24 weeks, or even later. Depending on the condition,

marked intrafamilial variability can be seen (86). Care must be taken to look at each major joint for full range of motion. However, the timing of onset of *in utero* limitation of movement is probably different for different conditions. Nevertheless, congenital contractures are frequently missed prenatally. More than half of cases of amyoplasia, in which contractures are already present at 14 weeks, are missed in spite of having had prenatal ultrasound. The presence of or absence of flexion creases may give clues as to when the movement or failure of movement in the limb occurred. For instance, flexion creases of the fingers and hand are established by 14 weeks of development. Norms for *in utero* movement have now been established (87). If arthrogryposis is diagnosed prenatally, consideration of therapy to increase fetal movement in the second and third trimester should be considered (increased maternal activity, maternal deep breathing, caffeine, etc.), to possibly increase lung volume and lessen the severity of contractures in the limbs at birth, and must be weighed against expected outcome. Early delivery may be considered if lungs are mature since the longer the lack of movement is present *in utero*, the more severe the contractures will be. In the situation of maternal antibodies against fetal neurotransmitters, suppressive steroid therapy is promising. If a family is at risk for recurrence, prenatal diagnosis by real-time ultrasound should be offered in subsequent pregnancies in order to provide appropriate management of an affected pregnancy (84).

### 161.2.8 Developmental Considerations

In humans, the limb bud first becomes obvious during the fifth to sixth weeks of embryonic development. There is a cranial–caudal progression with the upper limb bud development, ahead of the lower limbs. Movement begins as early as 8 weeks in the upper limbs, and can be seen proximally by 9 weeks and distally by 10 weeks. Movement requires an intact neuromuscular unit (84,87). During this time, there is a shifting vascular supply in the CNS, possibly making anterior horn cells vulnerable to hypoxia. Normal joint development in the limbs apparently requires embryo/fetal movement (88) undoubtedly involving embryonic/fetal muscles. Flexion creases in the fingers and palms develop secondary to movement between 12 and 14 weeks.

Rare cases of ongoing pregnancy after attempted termination of pregnancy (30a,30b) or maternal trauma secondary to motor vehicle accidents that would be expected to lead to maternal hypotension and/or compromised vascular supply to a fetus who is later born with congenital contractures allows the establishment of the timing of events. These cases suggest a timing such that events at 8 weeks are associated with limitation of jaw opening and possibly involvement of all four limbs, those at 9 weeks may have involvement of only the upper limbs or all four limbs, those at 10 weeks usually have involvement of all four limbs, and those at 11 weeks

have involvement of only the lower limbs. Some of these cases also have CNS compromise, but some seem to only involve contractures (both extension and flexion) of limbs with fibrous fatty change in muscle. The pathogenesis is still unclear, but these cases of trauma appear to be relevant to amyoplasia and distal IIE (distal arthrogryposis with trismus) as well as the contractures associated with CVS and early amniocentesis (23).

Lung and gastrointestinal tract fetal *in utero* development clearly requires movement (use). In the normal fetus, breathing motions begin around 12 to 14 weeks and become regular by 20 weeks (89). They involve both diaphragmatic and intercostal muscles. In various forms of arthrogryposis, these respiratory movements are decreased leading to suppressed alveolar development, surfactant maturation, and lung size (25,90). Some forms even have poor development or eventration of the diaphragm. Fetal swallowing begins early in the second trimester. Failure to initiate regular swallowing or the presence of blockage of the intestinal tract (such as in gastroschisis or bowel atresia) may lead to polyhydramnios and to delay in maturation of coordinated peristalsis, growth of the length of the bowel, and maturation of gastrointestinal secretions. This kind of delayed development of the gastrointestinal tract is likely to lead to feeding problems in the newborn in infants with arthrogryposis, and they often require tube feeding. However, interestingly, many affected infants “outgrow” the problem.

There seems to be a 3- to 4-month period after birth during which normalization can occur: “catch-up” growth in the lungs may occur so that near-normal respiratory function may be gained; the intestinal motility and secretions may mature and regular feeding may be established; and increased range of motion of joints may be accomplished by physical therapy, casting, and splinting. However, by about 4 months, usually no further gains occur and surgery may be considered.

As genes involved in various types of inherited arthrogryposis are being identified to be components of different types of muscle, consideration of which types of muscle (striated, smooth, cardiac, facial, diaphragm, etc.) are affected in specific forms of arthrogryposis should be undertaken. Furthermore, the issue of primary myopathy versus secondary myopathy (disuse atrophy) must be considered.

### 161.2.9 Prevention and Therapy

Only a few disorders with multiple congenital contractures are preventable or directly treatable at this time. Several maternal (Table 161-6) metabolic disorders (Table 161-16) predispose to CNS damage that can be associated with multiple congenital contractures. If mother is under good metabolic control before becoming pregnant and during pregnancy, this should lead to prevention. Several drugs have been associated with multiple congenital contractures if taken by the mother

during pregnancy. These include muscle relaxants, misoprostol, cocaine, and alcohol. Obviously, avoidance is important, although there may be genetic predispositions in both mother and fetus. Certain maternal diseases predispose to arthrogryposis in the fetus, including maternal myasthenia gravis and maternal antibodies to fetal neurotransmitters (21). Steroid therapy seems to improve fetal outcome when there are maternal antibodies to fetal neurotransmitters (21,91,92). Finally, several inborn errors in metabolism in the fetus can lead to arthrogryposis (glycogenosis type VII; phosphofructokinase deficiency; ARC; and metabolic situations leading to edema, such as storage disorders). *In utero* therapy for some of these may be available in the future.

Early initiation of physical therapy and splinting to stretch affected joints (rather than initial casting) has markedly improved outcomes, and led to less atrophy of the muscle that is present. Treatment often requires orthopedic intervention (37–50), etc. During surgery, seizures (36,653) and malignant hyperthermia may occur (35,56).

## 161.3 DIFFERENTIAL DIAGNOSIS

The differential diagnosis of multiple congenital contractures is extensive. Tables 161-2–161-6 outline most of the more common conditions to consider. Tables 161-9 and 161-11 through Table 161-18 may help identify disorders to consider. Table 161-10 lists conditions known to often have multiple congenital contractures; however, either due to limitation in space or knowledge, they are only listed. Overall, 5–10% of cases of arthrogryposis have been considered to be myopathic (58,98) and 90–95% to be neuropathic (58). However, recently many muscle proteins have been found to be involved in conditions previously thought to be neuropathic, such as the distal arthrogryposes (86,99–105a,106). The neuropathic group include amyoplasia, the etiology for which is unclear. With true malformations of the CNS and with *in utero* disuse, muscle biopsies may appear neuropathic. However, if a myopathy or end-plate abnormality occurs early in the *in utero* development, there may be retrograde loss of the developing neuron. Thus pathologic changes can be confusing. Many specific conditions can be recognized by particular clinical features and/or by laboratory tests.

The clinical approach found to be most useful has been to first distinguish three categories of congenital contractures on a clinical basis: (i) primarily limb involvement; (ii) musculoskeletal involvement plus other system malformations or anomalies; and (iii) musculoskeletal involvement plus lethality, CNS dysfunction, and/or significant mental retardation/mental disability. Tables 161-2–161-4 are organized on the basis of this clinical approach. In addition, there is a table (Table 161-6) on maternal illness and exposure. Table 161-5 represents some of the many chromosomal anomalies, including microdeletions and duplications,

TABLE 161-2 Primarily Limb Involvement							
Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Absence of finger prints	136000 612761	Absent dermal ridges Mild webbing Congenital milia Dystrophic dry skin	Fingers Toes	Flexed Camptodactyly	Milia: transient (disappear at 6 months)	Chromosomes: normal Skin biopsy: normal	AD <i>SMARCAD1</i> at 4q22
Absence of DIP creases		Absent DIP creases Palmar contractures	Fingers Hands	Camptodactyly Flexed	Progressive	Radiographs: no bone fusion Chromosomes: normal	AD
Amyoplasia (see also transient neonatal arthrogryposis)		Loss of muscle tissue and replacement with fat and fibrous tissue Usually symmetrical contractures, always equinovarus feet and extended elbows Round face, midline capillary malformation Normal intelligence Abdominal wall defects and bowel atresia (10%) Increased in one of monozygous twins	Wrists Elbows Shoulders Hips Knees Feet	Flexion Extension Internal rotation Flexion, ± CDH Extension or flexion Equinovarus	Improves with therapy Usually need surgery Normal IQ Classically symmetric with all four limbs involved Some individuals have only arms or legs involved	Spinal cord: decreased size of anterior horn cells Muscle: variation in fiber diameter, small fibers, replacement of muscle with fat and connective tissue	Apparently sporadic with increase of anomalies related to vascular compromise
Angulation of long bone with overlying dimples and shortening of soft tissue		Angulation of long bones Dimple at apex of angulation Shallow flexion creases Short flexion tendon Short stature	Elbows Wrists Ankles	Limitation of dorsiflexion Limitation of extension Limitation of extension	Angle decreases Limited movement decreases	Radiographs: show bone angulation	AR
Antecubital pterygium (Shin Shun)	178200	Elbow dysplasia Antecubital web ± carpal wrists anomalies	Elbows ± Wrists	Carpal fusion Flexion	Nonprogressive	Radiographs: ± fusion of humerus and ulna, ± dysplasia of condyle, ± trochlear dysplasia	AD

TABLE 161-2    Primarily Limb Involvement—Cont'd							
Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Bruck syndrome (also see angulation of long bone and osteogenesis imperfecta: see Table 161-4)	259450	Osteoporosis and fractures	Elbows	Limitation of extension	Inability to walk	Normal collagen I studies	AR, consanguinity
	607063	Bowing of long bones			Decreased muscle strength		<i>PLOD2</i> mutations at 17p12
	609220	Scoliosis secondary to vertebral flattening and wedging anomalies; may lead to pulmonary restriction	Hips	Lateral rotation	Intramedullary rodding helps	Radiographs: bowing, osteoporosis, and wormian bones	<i>FKBP10</i> at 17q21.2
	610698	No blue sclera, deafness, or dentinogenesis	Knees	Flexion	Pulmonary function compromise	Cystic changes at fracture sites	Also linkage to 3q23–24
		Short stature	Ankles	Equinovarus	Increasing deformity		
		Hypermobility of small joints	Feet		Progressive thoracic kyphosis		
					Progressive webbing		
Camptodactyly (see also Table 161-3 and Table 161-13)	114200	Camptodactyly Not always present at birth	Fingers ± Toes	Flexion Flexion	May be progressive	Shortness of deep flexor tendons	AD
Camptodactyly with arthropathy	208250	Camptodactyly Joint effusions ± arthritis	Fingers Toes	Stiff Curved	Slowly progressive	Arthritis changes	AR
Clasped thumbs, congenital (see also adducted thumbs and clasped thumbs with MR, Table 161-4)	314100	Extensor muscles and tendons of thumb weak or absent	Thumbs (bilateral) Occasionally 1st finger	Flexion at MP and DIP joints Radial deviation	Surgical treatment, tendon transplant	Hypoplastic tendon Peripheral neuropathy	AD <i>PRG4</i> gene mutation at 1q25–q31
Coalition(s)	186400	Calcaneus–navicular	Ankles	Fusion ± Contractures		Radiographs: synostosis Differential diagnosis: peroneal spastic foot	AD Some related to 17q22
	186570	scaphoid–astragalus	Toes				
	186750	Talus–navicular					
	602991	Calcaneus–scaphoid					
Contractures, continuous muscle discharge, and titubation	160120	Contractures	Hands	Fixed	Worsening contractures	Abnormal muscle fibers	AD <i>KCNA1</i> at 12p13.3
	170260	Myokymia	Feet	Flexion	Pulmonary compromise		
		Ataxia Titubation	All extremities	Stiffened			



Distal arthrogryposis (type 1)	108120	Clenched hand with overlapping fingers at birth; opens to ulnar deviation (90%)	Fingers	Clenched, overlap, then open and ulnar deviation	Good therapy response, improves with time Variable within families	Radiographs: hip dislocation, mild scoliosis, misplaced or hypoplastic tendons	AD (variable) <i>TPM2</i> ( $\beta$ tropoysin) 9p11.3 <i>MYBPC1</i> at 12q23.2 <i>TNN12</i> at 11p15.5 <i>TNNT3</i> at 11p15.5
	160794	Usually calcaneovalgus, but all combinations (80%) of clubfeet Other major joint contractures	Knees	Often uninvolved			
	190990		Elbows	Often uninvolved			
			Ankles	Calcaneus valgus or equinovarus			
			Toes	Overlap camptodactyly			
Humeroradial synostosis (HRS)	143050 236400	Familial AD: 100% are bilateral AR: 91% are bilateral Sporadic: 62% have hypoplasia of hand and 77% have involvement of ulna	Elbow	Fixed	Surgery may improve function	Radiographs: humeroradial synostosis	46% are sporadic 38% are familial 25% are AR 50% are AD
Liebenberg syndrome (primarily upper limbs)	186550	Prominence of radial head Unusual slope of olecranon process Brachydactyly Stebломicrodactyly Intercarpal fusion and synostosis Dislocation of radial head	Mainly upper limb flexion contractures		Nonprogressive	Radiographs: fusion of triquetrum and pisiform	AD
<b>Lower Limbs Only</b>							
Lower limb only Amyoplasia		Only lower limbs involved Markedly decreased muscle Slightly short legs	Hips Knees Feet	May be dislocated Flexed or extended Equinovarus severe	Nonprogressive Responds to PT and surgery	Fibrous fatty replacement of muscle	Sporadic Increased in one of twins
Lower limb only arthrogryposis Fleury type (spinal muscular atrophy)	600175	Lower limbs only involved Lumbar lordosis Thoracic scoliosis $\pm$ kyphosis Muscle weakness or paralysis in lower limbs with wasting Absent reflexes No dimpling	Hips  Knees Feet	Flexion, subluxation Flexion Variable	Feet respond well to surgery Scoliosis progressive Nonprogressive limb involvement No fasciculations	Radiographs: spine – WNL or spina bifida occulta Blood and CSF chemistry: WNL EMG and nerve conduction: WNL Biopsy: variation in fiber diameter and fatty replacement CT scan: variable muscle size	AD Variable expression <i>TRPV4</i> at 12q23.24

TABLE 161-2    Primarily Limb Involvement— <i>Cont'd</i>							
Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Lower limb only arthrogryposis (Ray/Sarralde type)	602484	Pelvic dysplasia Muscular hypotrophy of lower limbs Prenatal growth retardation, particularly lower segment Lumbar lordosis Winged scapulae Muscle hypoplasia in legs	Hip  Knee Feet	Pelvis hypoplastic with contractures Flexion Variable	Hypoplastic lower limb muscles Hypertrophy of neck and paraspinous muscles Continue to be below 5th in percentile height Feet resistant to therapy	Radiographs: pelvic dysplasia, small pelvic wings, acetabular dysplasia, iliac hypoplasia, notched acetabulae osteoporosis, vertebrae cuboid and wedged, slender long bones, lumbar lordosis Lab: WNL EMG: WNL, normal nerve conduction	AR, consanguinity
Meningomyelocele with spinal dysplasia	182940	Neural tube defects interfering with cord development		Variable lower limb congenital contractures		Can be diagnosed prenatally	Multifactorial Part of NTD spectrum
Lower limb only arthrogryposis, X-linked (type 6) (See also Kuskokwim syndrome Table 161-3)	301830	Thin tendons Lower limbs affected in males		Generalized flexion of lower limb	Lower limbs responsive to therapy Ambulatory with impaired gait Nonprogressive		X-linked, mapped to Xq23–27
Mesomelic dysplasia	156232	Short middle segment Coalitions of carpals and tarsals	Wrist Feet	Flexed Equinovarus	Able to function independently	Radiographs: bony fusion and short middle segment	AD Linked to 2q24–q32
Patella aplasia-hypoplasia (See also genitopatellar syndrome)	168860	Occasional congenital contractures Absent or small patellae			Functional	Radiographs: missing or small patellae	AD <i>PTLAH</i> gene mutation at 17q21–22

Poland anomaly	173800	Absent pectoralis major (costal head) Ipsilateral limb deficiency	Flexion contractures of affected arm		Nonprogressive	Radiographs: hypoplasia of missing or absent phalanges, rib deficiencies, synostoses	Sporadic, AR? Probably vascular
Radioulnar synostosis (RUS)	179300	Radioulnar synostosis Short forearm	Hand Elbow	Fixed in extension Limitation of movement	Nonprogressive	Radiographs: synostosis of proximal radius and ulna seen in chromosomal anomalies involving X and Y	Sporadic and AD
Saul–Wilson type skeletal dysplasia		Short stature Bone dysplasia Characteristic facies Seizures	Generalized flexion and limitation of movement Feet	Clubfeet	Nonprogressive	Radiographs: short phalanges with cone-shaped and sclerotic epiphyses, overtubulation of long bones, hypoplasia of vertebral bodies	
Symphalangism “Cushing” (see also multiple synostosis, Table 161-3)	186500 602991	Symphalangism of PIP joints Deafness variable Variable carpal and tarsal ankle bone fusion	Fingers Lower limbs	Fusions Clinodactyly Fusions of toes	Fusions and contractures become worse with time	Radiographs: PIP fusions ± DIP fusion, ± talus and navicular fusion	AD NOG at 17q21–22 GDF5 of 20q
Symphalangism distal	185700	Symphalangism of DIP joint Synostosis of metacarpals and metatarsals	Generalized limitation of movement of hands, feet, and limbs		Nonprogressive	Fusions of phalangeal bones	AD
Symphalangism/brachydactyly (see also multiple synostosis, Table 161-3)	113450	Brachydactyly Symphalangism (proximal and distal) ± Clubfeet ± Craniosynostosis ± Scoliosis just before menarche Deafness Missing distal fingers or nails	Hands Hips Feet	Limited movement Flexed Talipes equinovarus	Nonprogressive	Lab data normal Radiographs: carpal fusion ± Flat vertebrae ± craniosynostosis	AR AD

**TABLE 161-2**   **Primarily Limb Involvement—Cont'd**

Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Symphalangism/brachydactyly, Nievergelt–Pearlman type	163400	Radioulnar synostosis Coalitions in tarsals and carpals Symbrachyphalangism of fingers	Hands Elbows  Feet	Clinocamptodactyly Radioulnar synostosis Talipes, tarsal synostoses	Nonprogressive	Radiographs: radioulnar synostoses, carpal and tarsal synostoses	AD
Transient neonatal arthrogryposis		Flexion contractures Muscles firm and rigid	Generalized flexion contractures, except elbows usually extended		Contractions resolve over first week  Muscles then become normal texture	Elevated muscle enzyme and WBC count	Sporadic
Vertical tibial crease syndrome		Vertical crease down anterior shin, usually bilateral	Generalized flexion contractures		Nonprogressive Responsive to therapy Half have DD		AR
Upper Limb Only (See also Table 161-3 and Liebenburg syndrome)							
X-linked resolving arthrogryposis	301830	Flexion contractures Camptodactyly Anterior rotation of shoulders	Generalized flexion contractures Feet	Flexed ankle	Resolving		X-linked

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AD = autosomal dominant; AR = autosomal recessive; CDH = congenital dislocation of the hip; CSF = cerebrospinal fluid; CT = computed tomography; DIP = distal interphalangeal; EMG = electromyography; FDS = flexor digitorum superficialis; FPL = flexor pollicis longus; PIP = proximal interphalangeal; WBC = white blood cell; WNL = within normal limits.



TABLE 161-3 Musculoskeletal Involvement Plus Other System Anomalies							
Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Aase–Smith syndrome (see also distal arthrogryposis with ophthalmoplegia DAV/2B)	147800	Camptodactyly Dandy–Walker malformation Agenesis of corpus callosum Hydrocephalus Cleft palate Scoliosis Thin fingers with hypoplastic dermal ridges Mild craniofacial features	Hands Elbows Knees Hips Feet	Flexion Usually flexion Flexion or extension Flexion Equinovarus	Nonprogressive except one died as newborn	Autopsy: neuroblastoma, ventriculoseptal defect	AD
Alkuraya arthrogryposis syndrome (Perthes and ophthalmoplegia, see also distal arthrogryposis DA5/2B)	614051	Ophthalmoplegia (downward gaze) Congenital heart disease Perthes of femoral heads Pyloric stenosis	Generalized flexion contractures		Progressive hip disease Develop loose joints	MRI of eye and brain within normal limits X-rays: collapse of epiphyseal of femoral head	AR
Camptodactylies							
Camptodactyly, arthropathy, coxa vara, pericarditis, synovitis (ARC)	208250 604283	Thickened synovia Pericarditis Distal contractures	Fingers  Knees Hips	Camptodactyly  Flexion contractures Flexion	Progressive contractures Thickening of synovia Pericarditis may be lethal Swelling of joints progressive	Biopsy: hyperplasia of synoviocytes, hyperplasia of pericardium	AR <i>PRG4</i> at 1q24–q25
Camptodactyly, Guadalajara (two types)	211910	Camptodactyly Short stature IUGR Microcornea Microphthalmia Microcephaly, MR Pectus excavatum	Hands  Hips Feet	Flexion  Dislocated Equinovarus	Nonprogressive	Radiographs: dislocated hips, scoliosis	AR
Camptodactyly, Kilic (see also distal DAV/2B)	602612	Camptodactyly Myopia Fibrosis of medial rectus Mild scoliosis Ptosis High palate	Fingers  Knees Feet	Camptodactyly  Flexion Pes caves	Progression, functional	Radiographs: coalition of carpals	AR

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**TABLE 161-3    Musculoskeletal Involvement Plus Other System Anomalies—Cont'd**

Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Camptodactyly, London	211965	Camptodactyly	Fingers	Flexion, ulnar deviation	Nonprogressive		AR
		Facial immobility	Feet	Vertical talus			
		Ichthyosis					
		Short stature					
		Scoliosis					
Camptodactyly, Tel Hashomer	211960	Camptodactyly	Fingers	Spindle shaped	Nonprogressive	Muscle hypoplasia, type 2b fibers	AR
		Long, thin nose with high, broad bridge; prominent forehead	Toes	Contractures		Mild skeletal changes	
		Short stature					
		Scoliosis					
		Cardiac anomalies					
Caudal deficiency and asplenia (see also maternal diabetes)		Caudal deficiency	Hips	Flexion	Nonprogressive	Radiographs: absent sacrum, dextrocardia	Some are X-linked
		Asplenia	Knees	Flexion			
		Agenesis/hypoplasia of corpus callosum					
		Meningomyelocele					
		Cardiac anomalies					
Congenital fiber type disproportion with congenital contractures (most cases of arthrogryposis have fiber disproportion from disuse)	102610	Generalized contractures	Distal flexion contractures		Nonprogressive	Often related to disuse	Sporadic AR
	255310	Disuse atrophy					
							<i>ACTA1</i> at 10q24
Conradi–Hünemann syndrome (chondrodysplasia punctata)	300205	Hypertelorism, prominent forehead	Generalized flexion contractures		Deformities may increase	Radiographs: punctuate calcifications, tubular bones mildly short, scoliosis – abnormal vertebrae	AD; X-linked dominant lethal in males
	302960	Cataracts (17%)	Feet	Talipes equinovarus			
		Limb contractures (27%)					
		Patchy skin –Atrophoderma –Follicularis/alopecia					
					Boney changes lead to progressive scoliosis	Pathology: epiphyses abnormal	<i>EBP</i> at Xp11p33
Contractural arachnodactyly	121050	± Congenital heart anomalies	Generalized flexion contractures		Improves with age (knees the worst)	Radiographs: bones gracile, kyphoscoliosis, advanced bone age	AD
	612570	Chest deformities	Elbow	Limitation of supination and pronation			
		Long thin limbs			About 10% get worse with age		
		Generalized contracture	Feet	Dorsiflexion			
		Crumpled ear					<i>FBN2</i> mutation at 5q23–31
							12q13

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Diastrophic dysplasia	222600 606718	Dwarfism with contractures Joint contractures Hitchhiker thumb becomes fused Scoliosis/vertebral instability Abnormal ears Cleft lip and palate ± Abnormal tracheal ring	Thumbs  Fingers  Hips Feet	Adducted  Ankylosed at PIP joint Dislocated Severe talipes equinovarus	Progressive (probably due to joint trauma) Fusion of many joints Calcium deposits throughout	Radiographs: precocious calcification of cartilage Pathology: abnormal epiphyses, hypertrophic auricular cartilage and calcifications, specific histology	AR <i>SLC26A2</i> at 5q31
<b>Distal Arthrogryposes</b> (See also Gordon syndrome DAI, Table 161-2, Table 161-4)							
Distal arthrogryposis with deafness and camptodactyly (Bamshad type 6)	602782	Sensorineural deafness Camptodactyly without fusion Proximally placed thumbs Dry skin	Generalized flexion contractures		Nonprogressive	Muscle mass decrease distally Histiocytosis	AR check correct location <i>CATSAL</i> , <i>CATSH</i> at 11q25
Distal arthrogryposis with facial involvement (Sheldon–Hall, Bamshad type 2B)	160720 190990 191043 600692 601680	Down-slanting palpebral fissures Prominent chin Small mouth Cervical webbing Variability in family	Generalized flexion contractures		Nonprogressive Functional hands Ambulatory		AD fast twitch muscle apparatus <i>TNNT3</i> at 9q21 <i>TNNI2</i> at 11p15 <i>TNNT3</i> at 11p15.5 <i>MYH3</i> at 17p13
Distal arthrogryposis, ophthalmoplegia, and firm muscles (Hall type IIB, Bamshad type 5)		Firm muscles Ophthalmoplegia Deep-set eyes Ptosis Stiff spine Decreased facial expression Short stature	Generalized contractures in flexion Usually talipes equinovarus		Progressive, particularly pulmonary restriction Ophthalmoplegia which may develop over time Ambulatory, remains stiff Muscle firm	Muscle biopsy; ragged red fibers, disuse atrophy	AD One family with retinitis pigmentosa and MR
Distal arthrogryposis with cleft lip/palate (Hall DA IIC)		Distal contractures Cleft lip ± palate variable in family members	Generalized flexion contractures Feet	In various positions	Responds to therapy		AD

**TABLE 161-3    Musculoskeletal Involvement Plus Other System Anomalies—Cont'd**

Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Distal arthrogryposis with scoliosis (Hall type 2D, Bamshad type 4)	609128	Distal contractures Severe scoliosis ± vertebral anomalies	Fingers Feet Back	Clenched Variable Stiff and scoliotic	Scoliosis Progressive	Occasional hemivertebrae	AD
Distal arthrogryposis with trismus (Hall type DA IIE may be part of amyoplasia spectrum) DA Type 7B	121070	Distal contractures Trismus Mild MR in 1/3	Generalized flexion contractures Hands  Feet	Wrist flexion, MCP extended, fingers flexed Variable	Responds to therapy Jaw often continues to have limitation		Sporadic Seen in one of MZ twins May often have vascular compromise
Distal arthrogryposis Shalev type, mainly uppers DA Type 16		Ptosis Arched eyebrow Webbing of neck 65% Brachydactyly Umbilical hernia Decreased facial movement	Upper limbs flexion contractures Hands Elbow  Lower limbs less frequent 35%  Knee Foot	flexion contractures Camptodactyly Flexion contractures   Extended Clubfoot, vertical talus	94% nonprogressive		AR
Distal arthrogryposes absent teeth, distinct face DA Type 13		Absent bicuspid teeth Otosclerosis Depressed coronal suture Broad nose Long philtrum Short stature	Generalized flexion contractures Fingers  Feet	flexion contractures Camptodactyly, adducted thumb Camptodactyly and bunion	Progression of contractures ADHD		AD
Duane's retraction syndrome and multiple contractures (see DA IIB/5)		Inability to abduct eye Narrow of palpebral fissure	Generalized flexion contractures		Nonprogressive		Mainly sporadic
Dundar–Sonada distal arthrogryposis	601776	Hypertelorism MR Blepharophimosis Down-slanting palpebral fissures Arachnodactyly Renal anomalies Cardiac anomaly Broad forehead	Hands Generalized flexion contractures Feet	Adducted thumbs Clubfoot	Nonprogressive	Hydronephrosis	TARP at AR



Ectodermal dysplasia with contractures	601701	Dry skin Hyperkeratosis Sparse hair, pili torti ± Cataract Brittle nails Enamel anomalies Enlarged joints	Hands Generalized flexion contractures Feet	Camptodactyly Generalized flexion contractures Equinovarus	Short stature Normal development Develop diabetes Develop scoliosis		AR Consanguinity
Ectodermal dysplasia and cleft lip/palate with contractures		Blepharophimosis Cleft lip and palate Micrognathia Sparse hair Nail hypoplasia Developmental delay Glandular hypospadias Dental abnormalities	Fingers Generalized flexion contractures Hips Feet	Camptodactyly Dislocation Metatars adductus	Developmental delay Small but WNL Develop hyperopia and amblyopia Delayed dental eruption		
Ectodermal involvement, caudal appendage with contractures		Absent primary teeth Caudal appendage Slow-growing hair Ocular hypertelorism, blepharophimosis Bulbous nose Broad gums Shield chest	Generalized flexion contractures		Secondary teeth appear late	Radiographs: wormian bones Chromosomes: normal	Consanguinity
Ehlers–Danlos VIII	13008	Arachnodactyly Periodontitis Arthropathy Hyperextensible Aortic and mitral regurgitation Skin wrinkling and striking atrophic scars	Mild generalized flexion contractures		Progressive arthropathy and loss of teeth		AD 12p13 linkage
Ehlers–Danlos-like VIB-2	601776 608429	Progressive laxity of skin and joints Arthropathy Straight spine	Hands Feet	Adducted thumbs Clubfeet	Progressive arthropathy and contractures		AR <i>CHST14</i> at 11p
Focal femoral dysplasia (included femoral facial syndrome, FFS, FH, UHS)	134780	Cleft palate, long philtrum, short nose with hypoplastic alae nasi, micrognathia Shortened limbs: lower > upper Renal anomalies ± Unusual facies	Elbows Femur Hips Feet	Fixed in flexion Shortened flexion Dislocated Variable	Nonprogressive	Vertebral anomalies	Sporadic (associated with maternal diabetes)

TABLE 161-3 Musculoskeletal Involvement Plus Other System Anomalies—Cont'd							
Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Freeman–Sheldon syndrome (craniocarpotarsal dystrophy; whistling face syndrome; see also distal arthrogryposes DA-2)	193700 601680	Pursed lips, small mouth Cleft palate Immobile face, ptosis Contractures with ulnar drift Notched alae nasi “H” on chin Scoliosis	Hands  Hips Feet	Flexion with ulnar deviation Dislocated Calcaneovalgus	Nonprogressive Displaced tendons Fibrotic facial muscles		AD <i>MYH3</i> at 17p13 ?AR form
Gordon syndrome (Hall type IIA, Bamshad type 3) (see also distal arthrogryposes)	114300 193700	Distal contractures Cleft palate (40%) Ptosis Short stature Variable in families	Hands  Feet	Clenched, overlapping fingers Variable, calcaneovalgus or equinovarus	Respond well to therapy		AD <i>MYH3</i> at 17p13
Hand–foot–uterus syndrome	140000 142959	Contractures of hands and feet Coalitions Small thumbs and great toes Structural anomalies of uterus, particularly duplication	Hands Feet	Camptodactyly Talipes equinovarus	Nonprogressive	GU anomalies including renal	AD <i>HOXA13</i> at 7p15.2
Hanhart syndrome (aglossia adactyl/hypoglossia/hypodadylia)	103300	Limb deficiency with transverse loss Small or absent tongue ± Möbius sign Fusion of knee or elbow joints	Hands Elbows Knees Feet	Fused digits Fused Fused Fused digits	Nonprogressive		Sporadic, probably vascular
Holt–Oram syndrome	142900 601620	Abnormalities of shoulders, hands, and wrists Cardiac defects (structural) Abnormal joint structure (rare)	Hands Knees Feet	Usually hypoplasia, synostosis Flexed Talocalcaneal synostosis	Nonprogressive	Pathology: congenital heart defects, particularly septal, lung agenesis	AD <i>TBX5</i> at 12q24
Hoepffner syndrome	233805	Characteristic facial features with pinched, beaked nose Slim hands Mild pectus excavatum	Large joint flexion contractures		Nonprogressive	Abnormal lipids and cholesterol, reduced stimulation of RNA synthesis	AR
Juvenile hyaline fibromatosis (see Poretic–Murray syndrome)							

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Knies dysplasia	120140 156550	Short-trunk dwarfism Short stature Kyphoscoliosis Joint enlargement with limitation Myopia, deafness, cleft palate	Generalized flexion contractures		Progressive weakness	Lab: some with high keratin sulfate excretion Radiographs: irregular ossification of epiphyses	AD, heterogenous <i>COL2A1</i> at q13
King–Denborough syndrome, includes Lumbee (see also multiple pterygium syndrome with hyperthermia, Table 161-3)	145600 180901 255993	Noonan-like facies with ptosis Webbed neck Kyphosis, scoliosis Cleft palate – high arch Pterygium with progressive weakness Malignant hyperthermia Seizures Pectus excavatum Cryptorchidism	Generalized flexion contractures Hips Feet	Dislocated Talipes equinovarus	Does respond to therapy Beware of anesthesia Progressive trunk deformities May have MR	Muscle biopsy does not always show myopathy CPK: high, low, or normal Several families have central core myopathy	AR Linked to genes for malignant hyperthermia <i>RYR1</i> at 19q13.3 <i>MHS3</i> at 17q
Kuskokwim syndrome (see also lower limbs only distal arthrogryposis, Table 161-2)	208200	Multiple joint contractures ± Pigmented nevi ± Corneal reflexes Normal intelligence	Elbows Knees  Feet	Flexed ± webs Flexed, fixed with webs Various positions		Pathology: muscle atrophy Radiographs: ± patella migration Normal muscle and nerve function	AR <i>FKBP10</i> at 17q21.2
Larsen syndrome	150250 (AD) 245600 (AR) 603381 606374 613165	Prominent forehead, hypertelorism, depressed nasal bridge, flat round face Multiple joint dislocations, particularly anterior knees, elbows Hand anomalies: long cylindrical fingers, broad at end Scoliosis ± vertebral anomalies Short stature ± Cleft palate ± Midcervical kyphosis/subluxation Cardiac septal defects Laryngeal and tracheal collapse	Generalized flexion contractures		Diminished cartilage Rigidity at birth, improves with time Continue to subluxate many joints	Radiographs: dislocated patella, poor ossification of phalanges, CDH, extracalcaneal accessory bone, hypoplasia of humerus distally †, carpal ossification, cervical vertebral subluxation Hydrocephalus	AR more severe <i>IMPAD1</i> at 8q12.1 <i>CANT1</i> at 17q25.3 <i>DTDST</i> at 5q32 AD <i>FLNB</i> at 3p14.3 <i>COL7A1</i> at 3p21.31 <i>B3GAT3</i> at 11q12.3

TABLE 161-3 Musculoskeletal Involvement Plus Other System Anomalies—Cont'd							
Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Marfan syndrome (severe neonatal)	134797	Marfanoid body habitus	Generalized flexion contractures		Usually lethal	One case homozygous for fibrillin 1 mutation	<i>FBN1</i> at 15q21 New mutations
	154700	Aortic incompetence Micrognathia Anterior chest deformity Pulmonary emphysema Ocular abnormalities Hypermobility fingers					
MASP mutations (3 M syndrome, Carnevale syndrome)	265050	Blepharophimosis	Hands	Camptodactyly		X-rays coalitions and synostosis	AR <i>COLEC11</i> at 2p25.3 <i>MASP1</i> at 3q27
	600521 612502	Ptosis Cleft lip and palate Deafness Developmental delay Craniosynostosis Congenital heart disease Radial ulnar synostosis Caudal appendage Omphalocele Diaphysis rectus	Hips Back	Dislocated Scoliosis			
Metaphyseal dysplasia (Jansen)	156400	Small thorax	Generalized flexion contractures		Progressive	Radiographs: hypercalcemia, hyperostosis of calvaria, lack of metaphyseal ossification gives gross irregular cyst-like areas	AD <i>PTHR</i> at 3p27.3
	168468	Characteristic facies Flexion joint deformities Wide, irregular Short stature					
Metatropic dysplasia	156530	Disproportionate short stature	Generalized flexion contractures		Progressive changes from epiphyseal dysplasia to metaphyseal dysplasia  Spinal changes severe	Radiographs: platyspondylia, kyphoscoliosis, pelvic hypoplasia, short limbs – metaphyseal flaring, epiphyseal irregularity, hyperplastic trochanters	AD? AR <i>TRPV4</i> at 12q24
	605427	Kyphoscoliosis Prominent joints with restricted mobility, but ± finger extensibility Pelvic hypoplasia					
Möbius syndrome	157900	Facial diplegia (Vth and VIth nerves)	Hands	Camptodactyly	Nonprogressive	EMG: frontalis orbicularis oculi, facial and external rectus – no response  Pathology: muscle hypoplasia and absent muscle	Most sporadic Also linked to 13q12.2–q1
		Joint contractures, reduction anomalies Difficulty swallowing Chest and trunk: absent muscles (e.g., pectoralis muscle)	Knees Feet	Flexed Talipes equinovarus			



Multiple pterygium syndrome (Escobar type) (See also maternal antibodies against neurotransmitters receptors)	100730 253290 265000	Multiple webs develop over time Cryptorchidism Vertebral anomalies ± scoliosis ± cleft palate Deafness Eyelids: antimongoloid slant, ptosis Progressive thoracic lordosis Restrictive lung disease Short stature	Generalized flexion contractures  Fingers Hips Feet	  Camptodactyly with hyperextensibility Lumbar lordosis Rockerbottom or calcaneovalgus	Two forms: nonprogressive and progressive Pterygia increase with time Camptodactyly quite functional Finger webs develop	Pathology: skin, spinal cord, and brain normal Muscle hypoplasia, fatty replacement; Progressive respiratory dysfunction	AR <i>CHRNA1</i> at 2q37 <i>CHRNA1</i> at 2q24–32 <i>RAPSN</i> <i>DOK7</i> at 4p16.3
Multiple pterygium syndrome (autosomal dominant)	178110	Pterygium across large joints	Generalized flexion contractures with multiple webs Camptodactyly		Fairly functional		AD
Multiple pterygium and malignant hyperthermia syndrome (see also King–Denborough syndrome, Table 161-3)	217150	Decreased facial movement Neck webs Crouching stance Scoliosis	Generalized flexion contractures		Respond to preventative treatment Progressive scoliosis Monitor during anesthesia for possible malignant hyperthermia episodes	Low CPK Biopsy for muscle pathology	AR <i>RYR1</i> ?
Multiple synostosis (severe symphalangism, WL syndrome; also see synphalangism)	186500 601146	Symphalangism Deafness from ossicular fusion – progressive Long nose with broad bridge Synostosis of elbows Coalition of tarsals and carpals Distal digit defects	Generalized fusion and flexion contractures		Increasing fusions Progressive deafness	Fusion of bones, hemivertebrae, fixed ossicles	<i>NOG</i> at 17q21–22 Also <i>GDF5</i> at 20q.11.2 <i>FGF9</i> at 13q11q12
Nail–patella syndrome (hereditary onycho-osteodysplasia) (see also absent patella and genitopatella syndromes)	161200 602575	Nail dysplasia Absent or hypoplastic patella Joint contractures ± kidney disease Occasional elbow and knee webbing Glaucoma Dislocation radial heads	Generalized flexion contractures Fingers Feet	Ulnar deviation Pes Planes, talipes equinovarus	Nonprogressive except renal disuse	Radiographs: pathognomonic iliac horns, absent patella, subluxation of radial heads	AD <i>LMX1B</i> at 9q34 Renal failure (nephritis) mapped to 9q34 and <i>COL1A5</i>

**TABLE 161-3    Musculoskeletal Involvement Plus Other System Anomalies—Cont'd**

Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Nemaline myopathy (see central core and Table 161-4 myopathies)	161650 256030	Hypotonic Respiratory problems Congenital heart disease Short 1st metacarpal Adducted thumbs Micropenis	Generalized flexion contractures Feet	Talipes equinovarus		Autopsy and biopsies shows nemaline bodies	AR <i>NEB</i> at 2q21.2–22ACTA at 1q42.1
Neurofibromatosis	162200 613113	Neurofibromas Café-au-lait spots (multiple) Congenital contractures ±	Generalized flexion contractures		Can be progressive	Histopathology: neurofibromas When contractures often CNS structural abnormality	AD <i>NF1</i> at 17q11–q12 New mutations frequent
Neuropathic Israeli–Arab arthrogryposis	208100	Marked variability in family Hypoplastic muscles around affected joints ± CHD	Generalized flexion contractures Feet	Talipes equinovarus	Individuals with mild symptoms cope well Most adults are employed Normal intelligence	Marked decreased nerve conduction	AR Mapped to 5q33 (near <i>SMN</i> ) Females less affected than males
Nevo syndrome (see also Ehlers–Danlos VIA)	153454 601451	Kyphosis Hyperbilirubinemia Hypotonia Prominent forehead Hyperlordosis Cervical spine Cryptorchidism	Feet Generalized flexion contractures	Talipes	Increasing laxity	MRI: myopathic changes	<i>PLOD1</i> at 1p36.22
Oculo–dento–digital syndrome	121014 164206	Small sunken eyes Thin nose Severe hypoplasia of enamel, microdontia Phalangeal hypoplasia, camptodactyly of 4th and 5th digits Syndactyly Microphthalmia	Fingers Feet	Camptodactyly Hallux abductis		Chromosomes: normal Radiographs: absence of middle phalanges of toes, widening of long and short tubular bones and ribs and clavicles, metaphyseal changes, white matter changes in CNS	AD <i>GJA1</i> at 6q21–q23.2

Oral–canial–digital syndrome (Juberg–Hayward syndrome)	216100	Cleft lip and palate Hypoplastic, inflexible, distally placed thumbs Bilateral elbow deformities Microcephalus, mild MR Pituitary dysfunction Prominent forehead	Thumbs Elbows  Toes Renal anomalies	Inflexible Limited extension, dislocated radial heads Camptodactyly		Radiographs: 1st metacarpals small, radius dislocated, vertebral anomalies Chromosomes: normal Low growth hormone	AR
Parastremmatic dysplasia	168400 605427	Multiple contractures Twisted legs Bowed long bones Kyphoscoliosis	Elbows Hips Knees Elbows  Feet	Flexed Flexed Dislocated ± Radioulnar synostoses ± Calcaneovarus	Slowly progressive	Radiographs: coarse trabeculations, dense “flocked wool” areas, lacy border to pelvis	AD <i>TRPV4</i> at 12q24
Pfeiffer cardiocranial syndrome (FGFR syndrome)	101600 136350 176943	Craniosynostoses, brachycephaly, syndactyly ± Contractures ± Humeroradial synostosis CHD Often webbing	Generalized flexion contractures Thumb	Adducted and broad		Radiographs: broad distal phalanges	AD <i>FGFR1</i> at 8p11.23 <i>FGFR2</i> at 10q26
Popliteal pterygium syndrome (facio–genital–popliteal, Gorlin type)	119500 607199	Popliteal web with cord at edge Cleft palate, cleft lip, lip pits, frenula, intraoral web, ± ankyloblepharon Syndactyly Nail anomalies; absence, deformities of digits Short stature Genitals with web and scrotal anomaly	Knees Feet	Flexion with webs Equinovarus	May require surgery to release – watch for nerve and vessel in web	Pathology: free edge of web is cord-like and web contains sciatic nerve and blood vessel Variable within family	AD <i>IRF6</i> at 1q32.2
Proteus syndrome with distal arthrogryposis	164730 176920	Hamartoses Congenital lipomas Lymphangiomas and hemangiomas Hemihypertrophy Skin anomalies	Fingers  Elbows Feet	Camptodactyly with ulnar deviation Flexion Metatarsus varus	Patchy overgrowth Hemihypertrophy Improving contractures Variable Sudden death	Quite variable pathology of tumors	AD Mainly new mutations <i>AKT1</i> at 14q32.3

**TABLE 161-3    Musculoskeletal Involvement Plus Other System Anomalies—Cont'd**

Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Puretic–Murray syndrome (juvenile hyaline fibromatosis)	228600 608041	Joint contractures Face/skull deformities Gingival hypertrophy Multiple fibromatosis Skin lesions Infections: skin, eyes, nose, ears Other tumors	Generalized flexion contractures		Progressive contractures from early infancy with pain	Histopathology: skin ↑ collagen, ↑ soluble protein, tumors, ↑ fat content, altered connective tissue	AR <i>CMG2</i> or <i>ANTX</i> at 4q21
Rigid spine muscular dystrophy (also see stiff man/stiff baby Table 161-3)	602771 606210	Stiff spine Dislocated radial heads Feeding problems Heart failure related to RVH Scoliosis Bilateral diaphragmatic and inguinal hernias	Elbows  Neck Ankles	Limitation of movement Flexion Limitation of movement	Not progressive, however respiratory failure and heart failure	Myopathic muscle biopsy Cytoplasmic bodies Mini multicore myopathy EMG: exaggerated response	AR Consanguinity <i>SEPN1</i> at 1p36
Sacral agenesis	142994 Rarely 176450	Lower limb aplasia with contractures ± Others	Shoulders Hips Feet	Sprengel deformity Flexion Talipes equinovarus		Presacral tumor Radiographs: absence of sacrum Mostly due to maternal diabetes Bony abnormalities in 1% of diabetic mothers	Mostly sporadic Currarinio syndrome (AD) <i>MNX1</i> at 7q36
Schwartz–Jampel syndrome	142461 255800	Small stature Myotonia Fixed facial expression, pinched; pursed lips, small mouth Contractures; pectus carinatum Blepharophimosis Myopia Normal intelligence	May have generalized flexion contractures		Progressive: very mild at birth, gets worse during childhood  Variable expression: the worse the bony changes, the worse the progression	EMG: characteristic myotonic pattern Flattened epiphyses Dislocated radial head	AR <i>HSPG2</i> at 1p36
Spondyloepiphyseal dysplasia congenita	120140 183900	Short trunk Myopia Lag in epiphyseal mineralization including vertebrae Deafness	Elbows Hips Knees	Joint limitation Dislocation Flexion contractures	Progressive	Radiographs: flat epiphyses, ± mineralization of pubis, talus, calcaneus, knee centers, flat vertebrae	AD <i>COL2A1</i> at 12q13.11



Stiff man/stiff baby syndrome (see also rigid spine, Table 161-3)	138491 138492 184850	Alert, tense facial expression Hiatal, diaphragmatic, and umbilical hernias Feeding problems Intermittent generalized contractures	Generalized flexion contractures		Improves	EMG: exaggerated response	AD <i>GLRA1</i> at 5q33 and <i>GLRB</i> at 4q32.1 and antibodies against the
Trismus pseudo-camptodactyly syndrome (Dutch Kentucky syndrome, Hecht Beals syndrome)	158300 160741	Trismus, inability to open mouth fully Limited movement of fingers when wrist dorsiflexed Dislocated hips Generalized contractures Short stature	Jaw Fingers Hips Feet	Tight Camptodactyly Dislocated Varus	Nonprogressive	Pathology: shortened flexor tendons TMJ abnormally flat	AD <i>MYH8</i> at 17p12–p13
Tuberous sclerosis	191092 191100 605284	Glioma–angioma lesions, phacomata Seizures Adenoma sebaceum, ± shagreen patch Pigment and depigmented patches ± Joint contractures Cardiac and renal tumors	Fingers Feet Toes	Camptodactyly Equinovarus Camptodactyly	Can be progressive	Autopsy: brain – tuberous sclerosis lesions Radiographs: no bone changes	AD Variable, most without contractures <i>TSC1</i> at 9q <i>TSC2</i> at 16q
Ullrich congenital muscular dystrophy	120250 254090	Weakness Hypotonia, motor delay Proximal contracture Distal hyperextensibility Scoliosis, torticollis Rough skin	Shoulders Elbows Hips Knees Ankles	Tight Flexion Dislocated Flexion Flexion	Progressive	MRI: CNS normal EMG: myopathic Muscle biopsy: dystrophic and sclerotic	AR AD <i>COL6A3</i> at 2q33 <i>COL6A1</i> at 21q22.3 <i>COL6A2</i> at 21q22.3
VATER association	192350	Vertebral/vascular defects Anal atresia TE fistula Esophageal atresia Radial/renal defects	Hands Arms Leg	Camptodactyly Flexion contractures Flexion contractures		Chromosomes: normal Pathology: CHD, anal atresia, TE fistula, renal anomaly	Mostly sporadic <i>HOXD13</i> at 2q31.1
Van den Ende–Gupta syndrome	600920 613619	Blepharophimosis Arachnodactyly Prominent forehead Triangular face Skeletal anomalies Hypoplastic alae nasi Microstomia, beaked nose Large ears Everted lower lip	Generalized flexion contractures Short stature Dislocated radial head Fingers Toes	Camptodactyly Camptodactyly	Nonprogressive Self-limited contractures	Mild skeletal changes Bowed long bones	AR Consanguinity <i>SCARF2</i> at 22q11.21

**TABLE 161-3 Musculoskeletal Involvement Plus Other System Anomalies—Cont'd**

Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Waardenburg–Klein syndrome (WS3)	148820 606597	White patch or early graying hair Pigmentary anomalies Blepharophimosis Deafness Displaced inner canthus Heterochromia of the iris Syndactyly and finger contractures Carpal fusions	Generalized flexion contractures Fingers Wrists Elbows	Camptodactyly Flexion Flexion	Nonprogressive	Neural crest anomaly	AD <i>PAX3</i> at 2q35–q37.1
Weill–Marchesani syndrome	277600 608990	Short stature Stiff joints Carpal tunnel Small/dislocated lenses	Generalized flexion contractures Fingers	Camptodactyly	Slowly progressive	Carpal and tarsal tunnels from excessive connective tissue	AR <i>ADAMS10</i> at 19p13.3–p13.2
Winchester syndrome	259600 120360	Craniofacial asymmetry and coarsening Brachydactyly and contractures Faint corneal opacities Malar flush, thick facial skin Osteoporosis Gum thickening Arthropathy	Generalized flexion contractures		Progressive	Lab: not MPS Pathology: swelling and degeneration of mitochondria, dilation of endoplasmic reticulum, osteolysis of tarsals, osteoporosis	AR <i>MMP2</i> at 16p11.2
X-linked arthrogryposis, moderately severe (type 3)	301830	Generalized contractures Dimples over shins Small mouth May have mild MR	Generalized flexion contractures		Slowly improves	Short hypoplastic tendons	X-linked recessive Xp11.3–q11.2

Ach = acetylcholine; AD = autosomal dominant; ADHD = attention deficit/hyperactivity disorder; AR = autosomal recessive; CDH = congenital dislocation of the hip; CHD = congenital heart disease; CK = creatine kinase; CPK = creatine phosphokinase; EMG = electromyography; GU = genitourinary; IUGR = intrauterine growth restriction; MCP = metacarpophalangeal; MPS = mucopolysaccharidosis; MR = mental retardation; MRI = magnetic resonance imaging; MZ = monozygotic; PIP = proximal interphalangeal; TE = tracheoesophageal; TMJ = temporomandibular joint.

**TABLE 161-4     Musculoskeletal Involvement Plus Central Nervous System Dysfunction and/or Mental Retardation and/or Le**

Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Acrocallosal syndrome (see also Aicardia– Goutières syndrome)	200900 611254	MR Agenesis corpus callosum Dandy–Walker malformation Postaxial polydactyly Duplication of hallux Hypertelorism Macrocephaly	Generalized flexion contractures			Ciliopathy	AR <i>KIF7</i> at 15q26 <i>GLI3</i> at 7p13
Adducted thumbs (see also clasped thumbs, MASA, Table 161-4 and clasped thumbs Table 161-2	201550	Thumbs flexed Developmental delay Micrognathia, cleft palate Craniosynostosis Microcephaly Dysmyelination Pectus Excavatum Hydrocephaly	Thumbs  Wrists Elbows Knees Feet	Flexion/ adduction Limited extension Adducted Flexion Talipes equinovarus, varus	Do poorly	Chromosomes: normal EMG: abnormal Cine-esophagoscopy: abnormal Lab: proteinuria Pathology: displaced tendons, dysmyelination Degeneration of AHC of spinal cord	AR
Adenylosuccinate lyase deficiency	103050	Microcephaly IUGR Hypotonia Self mutilation MR	Generalized flexion contractures		Progressive early lethal respiratory failure	Pachygyria Cerebral atrophy White matter gliosis Urinary ↑ S-Ado and/or SAIC Ar	AR <i>ADSL</i> at 22q13.1
Aicardi–Goutières syndrome (Cree encephalitis)	225750 606609 606754 610333 610329 610181 610326	Encephalopathy Intracranial calcifications Severe MR Mimics CNS infection	Generalized contractures		Do poorly May lose digits	CNS with multiple vascular scarring and loss of tissue CSF lymphocytes	AR <i>TREX1</i> at 3p2 <i>SAMHD1</i> at 20q11.2 <i>RNASEH2A</i> at 19p13.1 <i>RNASEH2B</i> at 13q14.3 <i>RNASEH2C</i> at 11q13.1
Al-Awadi–Raas– Rothschild syndrome	276820 601570	Upper and lower limb deficiency Hypoplastic pelvis TE fistula Imperforate anus Cleft palate Renal agenesis	Generalized flexion contractures		Lethal	Osteopenia	AR <i>WNT7A</i> at 3p

**TABLE 161-4    Musculoskeletal Involvement Plus Central Nervous System Dysfunction and/or Mental Retardation and/or Le**

Entity	MIM#	Primary Features	Contractures			Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position	Progression		
Antley–Bixler syndrome (also phenocopy maternal fluconazole, two subtypes related to ± steroidogenesis)	124015	Unusual facies with frontal bossing and midface hypoplasia	Fingers	Flexion	DD when O <sub>2</sub> deprived or severe	ECG, EMG, lab: WNL	AR
	176750		Wrists	Flexion at 190, carpal synostosis	May do well	Radiographs: radiohumeral synostoses, tarsal synostoses, choanal atresia, occasional radial ulnar synostosis	<i>POR</i> at 7q11.2
	201750	Bent long bones			cranosynostosis		steroidogen defect
	207410	Craniosynostosis and proptosis	Elbows	Radio-humeral synostosis	May have Arnold–Chiari		<i>FGFR2</i> at 10q
		Contractures – camptodactyly (70%) Radiohumeral synostosis (100%) Scoliosis	Ankles  Toes	Tarsal synostosis, flexed Camptodactyly	Normal intelligence Subtypes related steroidogenesis and genital anomalies		without steroidogen defect Phenocopy with maternal fluconazol
ARC (Neselo of syndrome) (arthrogryposis, renal dysfunction, cholestasis syndrome – two subtypes)	208085	Joint contractures	Hands	“Clubbed”	Lethal	Autopsy: rarefaction of anterior horn cells	AR
	608552	Liver disease (jaundice and biliary stasis)	Feet	Calcaneus and talipes equinus		Pigmentary storage disease in liver cells, nephrocalcinosis	<i>VPS33B</i> at 15q
	613401	Renal dysfunction with nephrocalcinosis				Renal tubular cell degeneration 10% cardiac defect	<i>VIPAR</i> at 14q
Bartsocas–Papas syndrome (lethal popliteal pterygium)	119300	Contractures with marked webs	Flexion of all joints (particularly knee to foot), webs		Flexion, webs	Usually lethal; 1/4 survive to 20 months	AR
	263560	Perioral and perianal and distal limb skin dysplasia Syndactyly leading to mitten hand and foot Cleft lip, nose, and palate Microcephaly IUGR Ankyloblepharon					
Blepharophimosis, joint contractures, MR, Dandy–Walker malformation syndrome		Blepharophimosis Dandy–Walker malformation Auricular pits Cerebellar abnormalities MR	Hips Knees	Flexion Flexion	Nonprogressive	CT: absence of corpus callosum, Dandy–Walker malformation, aplasia of cerebellar vermis	AR



Bohring–Opitz syndrome	605039 612990	Severe MR (100%) Microcephaly Trigonocephaly Prominent eyes IUGR (83%) Palate high or cleft Contractures (60%) Cardiac (50%)	Hands Wrists Elbows Hips Knees Ankles	Clasped Flexed Flexed Extended Extended Extended	Lethal (40% infancy)	R/O Opitz C-BOS mutation R/O trisomy 3p	AR <i>ASXL1</i> at 20q
Bowen–Conradi syndrome	211180 611531	IUGR Microcephaly Micrognathia Prominent nose Cloudy corneas Large ears Clinodactyly and rockerbottom feet	Hands Hips Feet	Ulnar deviation Dislocated Valgus	Lethal	Absent vermis, hypoplastic cerebellum	AR <i>EMG1</i> at 12p1
Campomelic dysplasia	114290 608160	Curvature and shortening of long bones, particularly femur and tibia  Pretibial dimpling over curves Cleft palate, flat face Hypoplasia of facial bones, scapulae, and fibulae ± Ambiguous genitalia ± Craniosynostosis XY may have genital defects	Elbows Hips Feet	Synostosis CDH Calcaneovalgus or equinovarus	Lethal, perinatal or death in infancy observed  2/3 males present as females	Radiographs: bowing of long bones, fractures, platyspondylia, abnormal enchondral ossification, cervical vertebral anomalies Pathology: ambiguous genitalia Chromosomes: normal or may have translocation involving 17q	AR <i>SOX9</i> gene mutations 17q23  Some cases of mutations campomelic
Carbohydrate-deficient glycoprotein syndrome (includes congenital disorders of glycosylation)	212065 601785	Ataxia Hypotonia MR Severe neonatal onset Hydrops Retinitis pigmentosa, esotropia Peculiar subcutaneous fat Nipple retraction Hypogonadism	Elbows Knees	Flexion Flexion	Often lethal Axial hypotonia Bleeding tendency Peripheral neuropathy Severe MR Liver disease Ascites	Lab: low cholesterol, high triglycerides, transferin low CT scan: cerebellar atrophy, demyelination Autopsy: liver with fatty changes and cirrhosis Renal cysts Cerebellar hypoplasia Cardiomyopathy	AR <i>PMM2</i> gene mutations 16p13.3 <i>PM1</i> gene m at 15q22.3
Central core disease– congenital onset (see also congenital myopathies)	117000 180901	Contractures Hypotonia Kyphoscoliosis Fetal akinesia	Generalized flexion contractures  Hips	Dislocated	Maybe lethal May be at risk for malignant hyperthermia	Muscle shows characteristic eccentric cores	AR (includes minicore d AD (sarcomer disorganiz <i>RYR1</i> gene mu at 19q13.1

TABLE 161-4

Musculoskeletal Involvement Plus Central Nervous System Dysfunction and/or Mental Retardation and/or Let

Entity	MIM#	Primary Features	Contractures			Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position	Progression		
Cerebro-oculo-facio-skeletal (COFS) syndrome (Pena-Shokeir II) Types 1-4	126340	Microcephaly, IUGR	Fingers	Flexion, medially deviated	FTT, lethal	Dysmyelination, cerebral atrophy	AR
	126380	Cataracts, microphthalmia,			Progressive	Chromosomes: normal	<i>ERCC6 (CSB)</i> at 10q11.23
	133530	deep-set eyes, retinal	Elbows	Flexion	degeneration	Radiographs:	<i>ERCC2</i> at 19q
	214150	pigmentary changes	Hips	Flexion	Brain atrophy	platyspondylia,	<i>ERCC5</i> at 13q
	278780	Micrognathia	Knees	Flexion	Severe DD	membranous cranial bones, decreased ossification,	<i>ERCC1</i> at 19q
	609413	Abnormal large ears	Feet	Calcaneovalgus, rockerbottom		osteoporosis; renal anomalies, osteopetrosis can be seen	
	610756	Overlapping flexed fingers					
	610758	Prominent nasal root Kyphoscoliosis Hypotonia					
Chondrodysplasia punctata (rhizomelic dysplasia, R/O maternal warfarin)	215100 601757	Symmetrical rhizomelic shortening of limbs Stippled epiphyses particularly centrally Characteristic facial features, flat face Microcephaly Cataracts CHD Severe MR Mottled irregular skin	Generalized flexion contractures Feet	Talipes equinovarus	Lethal or severe MR	Peroxisomal disorder Radiographs: punctuate calcifications, flared metaphyses with epiphyseal stippling, vertebral clefing	AR <i>PEX7</i> gene mutation at 6q22-q
Clasped thumbs and MR syndrome (also see adducted thumbs and MASA syndrome, Table 161-4)	303350	Adducted thumb ± camptodactyly ± Moderate to severe MR Microcephaly to hydrocephaly ± aqueductal stenosis	Hands Feet	Camptodactyly Camptodactyly and clubfoot	Lethal to long life	Malmigration of neurons, hypoplastic thumb	X-linked recessive Related to X-linked hydrocephalus
Congenital disorders of glycosylation (see carbohydrate-deficient glycoproteins syndrome)							
Contractural	121050	Arachnodactyly	Fingers	Flexion	Often lethal	Autopsy: absence of subcutaneous fat	AD
arachnodactyly (severe congenital)	612570	Camptodactyly Crumpled ear Microphthalmia Micrognathia	Elbows Spine Knees	Flexion Scoliosis Flexion			<i>FBN2</i> mutation 5q23-q31

Crisponi syndrome (cold-induced sweating syndrome; Sohar Crisponi syndrome)	601378 604237	Paradoxical contractures Hyperthermia Hypersalivation Cold-induced sweating Feeding difficulties Camptodactyly Pinched face Trismus	Generalized flexion contractures		Usually death in first few years Feeding difficulty Respiratory Scoliosis progresses	Normal muscle biopsy CT, MRI: brain within normal limits	AR Mutation in C at 19p13.1
Dandy–Walker, mental retardation, basal ganglia disease and seizures (Pettigrew syndrome) see also Ohdo syndrome and blepharophimosis AR syndrome	304340	Hypotonia Spasticity Contractures Seizures Choreoathetosis Coarse facial features	Elbows Knees Feet	Flexion Flexion Talipes equinovarus	Severe MR and seizures	Cystic enlargement of fourth ventricle Cerebral atrophy Cerebellar hypoplasia Dandy–Walker Iron in basal ganglia	X-linked Xq25–q27
Dyggve–Melchior–Clausen dysplasia	223800 607461	Camptodactyly Short trunk MR Short long bones Short neck	Fingers Hips	Stiff, claw-like Dislocated	Progressive Spinal cord compression at C1	Radiographs: platyspondylia, irregular lacy iliac crest, odontoid hypoplasia, vertebral notches ER changes in biopsy Urine shows MPS	AR <i>DYM</i> gene mu at 18q12–
Dyssegmental dysplasia (Rolland–Desbuquois)	224400	Short trunk Thick, broad long bones Cleft palate Stiff joints Variable size vertebrae Limitation of full extension throughout	Vertebrae  Generalized flexion contractures	Segmental dysplasia	Lethal	Segmental spinal defects	AR Two subtypes
Encephalopathy, edema, hypsarrhythmia, optic atrophy syndrome (PEHO syndrome)	260565	Progressive encephalopathy Edema Hypsarrhythmia Optic atrophy Severe hypotonia/hyperreflexia Characteristic facial features with pear-shaped face	Fingers  Feet	Camptodactyly, tapering Talipes equinovarus	Lethal by age 6 yrs Profound MR	Autopsy: small cerebellum, spongy vacuolation in cerebral cortex, loss of Purkinje cells and granule cells with proliferation of Bergmann glia	AR
Eagle–Barrett syndrome (prune-belly syndrome)		Renal anomaly Prune belly Pulmonary hypoplasia Potter facies Imperforate anus	Multiple flexion contractures, particularly lower limb		Often lethal	Various GU and GI anomalies Arthrogryposis common	Usually sporadic

TABLE 161-4     **Musculoskeletal Involvement Plus Central Nervous System Dysfunction and/or Mental Retardation and/or Let**

Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
FG syndrome (Opitz–Kaveggia; also see Lujans Fryns syndrome)	300188 305450	MR Large head, broad tall forehead with cowlick Hypotonia ± joint contractures Imperforate anus and other GI abnormalities, including constipation ± CHD Seizures; CNS anomalies ± Contractures Hyperactive	Fingers Knees Wrists Ankles  Feet	Ulnar deviation Radial deviation Limited extension Lateral displacement Contractures	Nonprogressive	Chromosomes: normal Pathology: CNS abnormal, partial agenesis of corpus callosum Radiographs: ± agenesis of corpus callosum	X-linked recessive <i>FGS1</i> at Xq13 <i>MED12</i> <i>FLNA</i> at Xq28
Fowler-type hydranencephaly	225790 610865	Proliferative vasculopathy Hydrocephalus Hydranencephaly Fetal akinesia	Generalized flexion contractures		Lethal Prenatally diagnosable	Proliferative vasculopathy Glomerular vasculopathy Diffuse ischemic lesions in CNS with calcifications	AR <i>FLVCR2</i> at 14q24.3
Fryns syndrome	229850	Coarse face, cloudy corneas Cleft palate, micrognathia Hypoplasia/aplasia of lungs Digitalization of thumbs Distal limb deformities and hypoplasia GU anomalies ± absent diaphragm Omphalocele	Fingers  Thumbs	Flexion of PIP joints Digitalization	Lethal	Chromosomes: normal, occasional translocation Radiographs: hands and feet – rudimentary digit development Pathology: hypoplasia of lungs and diaphragm, broad clavicles, CNS abnormality, hydro- cephalus CGH normal	AR
Fukutin mutations includes (cerebro-oculo muscle syndrome, HARD ± E, muscle eye brain (MEB), Walker– Warburg syndrome)	236670 253280 253800 606596 606612 607155 607423 607439 607440 613153 613154 613150	Muscular dystrophy Cerebral atrophy/ heterotopia/myelin abnormalities Lissencephaly/ polymicrogyria Optic atrophy MR	Generalized flexion contractures		Lethal Progressive	CT: cerebral atrophy, dilated cerebral ventricles, cerebral cysts, lissencephaly, and polymicrogyria EEG: abnormal Lab: elevated CPK Muscle biopsy: dystrophic changes	AR <i>FKRP</i> at 19p11 <i>FCMD</i> at 9q31 <i>POMT1</i> at 9q31 <i>POMT2</i> at 14q24 <i>FKTN</i> at 9q31 <i>POMGNT1</i> at 10p15 <i>LARGE</i> at 22q13



Gaucher disease, perinatal lethal	606463 608013	Hydrops Hepatosplenomegaly Ichthyosis Facial storage	Generalized flexion contractures		Progressive lethal	Glucocerebrosidase deficiency	AR <i>GBA</i> at 1q21
Gelophysic dysplasia	231050 612277	Short stature and short bones Campto- and brachydactyly Aortic stenosis Happy facial appearance MR	Fingers Feet Hips	Camptodactyly Talipes equinovarus Dislocated	Progressive	Radiographs: dysostosis multiplex, inclusions in muscle cells	AR <i>ADAMTSL2</i> at 9q34.2 <i>FBN1</i> at 15q2
Genitopatellar syndrome	606170	Severe MR Microcephaly Absent corpus callosum Absent patella Genital anomalies	Dislocations and flexion contractures		Poor outcome	CNS anomalies	AR <i>KAT6B</i> at 10q
German syndrome	231080	Generalized contractures Edema Fetal akinesia sequence Hypotonia Hypotonic facies Cleft or high palate Large ears MR	Generalized flexion contractures		Nonprogressive Edema improves	Muscle shows microscopic glycogen, brain apparently normal	AR
Hypomyelination (see myopathies)							
Ives microcephaly, micromelia syndrome	251230	IUGR Microcephaly Fused elbows Short forearm ± 1 bone Hypoplastic hand missing whole digits	Generalized flexion contractures		Lethal	Pathology: renal dysplasia, decreased myelin	AR
Lenz–Majewski syndrome (hyperostotic dysplasia)	151050	Progressive osteosclerosis or osteopetrosis Synostosis and symphalangism Enamel abnormalities Broad ribs and clavicles Pinched nose, large ears, prominent eyes ± Choanal atresia MR Thin skin with prominent veins Delayed closure of fontanelle	Generalized flexion contractures		Progressive		?AD

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TABLE 161-4 Musculoskeletal Involvement Plus Central Nervous System Dysfunction and/or Mental Retardation and/or Let							
Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Leprechaunism (Donohoe syndrome)	147670 246200	IUGR Specific elfin facies Hirsutism Loose, dry skin Contractures	Hands Feet	Flexion Talipes varus	Lethal Spinal muscular atrophy	Associated with insulin receptor gene Autopsy: renal hyperplasia, focal changes in liver, calcified deposits in kidneys, aberration of endocrine system, brain normal	AR <i>INSR</i> gene mutation at 19p13.2
Lethal arthrogryposis with anterior horn cell disease (Finnish) (LAAHD)	253310 603371 611890	Severe hydrops IUGR Hypoplastic muscle Thin spinal cord Early <i>in utero</i> onset Hypoplastic lungs Anterior horn cell loss	Generalized flexion contractures Occasional extended knee Kyphosis		Lethal	Early onset <i>in utero</i> Autopsy shows loss of anterior horn cells Severe neurogenic changes	AR Allelic to LCCS2 <i>GLE1</i> at 9q34
Lethal congenital contracture syndrome 1 (Finnish)	253310 603371	Generalized contractions Hypoplastic lungs Edema Marked fetal hydrops Mild webbing	Generalized flexion contractures		Lethal	Nonspecific changes of fetal akinesia found at autopsy Pulmonary hypoplasia Decreased AHC with degeneration Thin bones	AR <i>GLE1</i> at 9q34
Lethal congenital contracture syndrome 2 (Israeli Bedouin)	190151 607598	Lethal congenital contractures IUGR No edema Hydramnios Extended bladder	Wrists, elbows, hips, knees, ankles	Flexion contractures	Lethal	Nonspecific	AR <i>ERBB3</i> at 12q
Lethal congenital contracture syndrome 3	611369	Lethal congenital contractures IUGR No edema Hydramnios	Generalized flexion contractures May have extended legs		Lethal	Similar to LCCS2	AR <i>PIP5K1C</i> at 19
Lissencephaly with fetal akinesia sequence (3 types)	257320 600514 601160	Fetal akinesia with lissencephaly Microcephaly Hydrocephaly Hydrops	Flexion contractures		Lethal Pulmonary hypoplasia	Lissencephaly Absence of corpus callosum and vermis Cystic cerebellum Stippled epiphyses	AR, consanguinity Type 1: <i>PAFAH1B1</i> 17p13.3 Type 2: <i>RELN</i> 7q22.1 Type 3: with brain dysplasia

Marden–Walker syndrome (heterogenous with an excess of males)	248700	Hypertelorism, blepharophimosis, ptosis Apparent arachnodactyly Joint contractures Developmental delay Kyphosis, scoliosis Hypotonia Characteristic facies: immobile, depressed nasal bridge	Fingers Generalized flexion contractures Feet	Camptodactyly Equinovarus or rockerbottom		Pathology: atrophic muscles, CDH, microcysts in kidneys, ± muscle fiber size Hematology and urine: normal CT: Dandy–Walker malformation, hypoplastic corpus callosum	AR
Martsolf syndrome (includes Warburg Micro syndrome)	212720 609275	Short stature MR Hypogonadism (small penis) Malocclusion Cataracts, microphthalmia Hypermobility tapering fingers Microcephaly Arnold–Chiari malformation Diplegia	Adducted thumbs Clubfeet	Flexion contractures	Nonprogressive	Cerebral atrophy Dilated ventricles	AR <i>RAB3GAP2</i> mutations 1q41 <i>RAB3GAP1</i> at <i>RAB18</i> at 10p
MASA syndrome (see also adducted thumbs, clasped thumbs, Table 161-4)	303350 308840 600118 602536	Adducted thumbs Aphasia Shuffling gait Short stature MR Spastic paraplegia	Generalized flexion contractures		Nonprogressive	CNS apparently normal	X-linked <i>LICAM</i> at Xq2
Megalocornea and skeletal anomalies		Megalocornea Usual-shaped hand Prominent forehead, saddle nose, large ears, micrognathia Gibbus/kyphoscoliosis Contractures distally MR IUGR	Fingers Feet	Camptodactyly Talipes equinovarus		Chromosomes: normal Radiographs: kyphoscoliosis, thin cortical bones of skull	AR
Meningomyelocele (see Table 161-3)		Spinal lesion Paralysis ± joint contractures ± Others	Usually lower limbs, depending on lesion position Flexion	Clubfeet	Can be lethal	X-rays show bony defect often associated with CNS problems	Multifactorial





Myelinopathies with multiple congenital contractures	129010 145900 159440 601097 605725	Hypotonia Decreased facial movement Ophthalmoplegia Decreased muscle mass PDA Feeding problems Scoliosis	Generalized flexion contractures		Nonprogressive Pulmonary problems Early death	Markedly decreased myelin and white matter	AR Mutations may include <i>ERG2</i> at 10q23.3 <i>MPZ</i> at 1q23.3 <i>PMP22</i> at 17p11.2 <i>PRX</i> at 19q13.32
Myhre contractures with muscular hypertrophy syndrome		Distal contractures Muscle hypertrophy Hearing loss Short stature MR CHD	Generalized flexion contractures			Radiographs: thickened calvaria, broad prominent mandible, broad ribs, flattened vertebrae	AD
Myopathies with multiple congenital contractures (see also nemaline myopathy and central core disease)	102610 161650 161800 180901 255310 256030 600016 603590 608441 612540	Hypotonia, weakness Decreased facial movement $\pm$ Ophthalmoplegia Decreased muscle mass PDA Feeding problems Scoliosis IUGR Pulmonary hypoplasia	Generalized flexion contractures		Nonprogressive Pulmonary problems, respiratory failure	CNS variable anomalies May have muscle biopsies with intranuclear rods, central core rods, nemaline rods Markedly decreased myelin and white matter	Mutations may include: <i>BIN1</i> at 2q14.3 <i>CNTN1</i> at 12q24.31 <i>SYNE-1</i> at 6q25.1–q25.3 <i>LARGE</i> at 22q13.31 <i>ACTA1</i> at 1q42.1 <i>RYR1</i> at 19q13.32 <i>NEB</i> at 2q23.32
Myotonic dystrophy, severe congenital	160900	Mother with myotonic dystrophy Poor suck, difficulty swallowing Generalized hypotonia Facial diplegia Cataracts Ptosis MR Polyhydramnios	Generalized flexion contractures		Progressive, limited life expectancy Prenatally diagnosable clinically and molecularly	Biopsy: muscular dystrophy Pathology: abnormal CNS, mitral valve prolapse EMG may be positive	AD with anticipation Triple repeat expansion greater expansion when transmitted from mother <i>PMPK</i> at 19q13.32
Neu–Laxova syndrome	256520	Lissencephaly Microcephaly Absence of corpus callosum Short neck Hypertelorism Micrognathia Exophthalmos Syndactyly Edema IUGR Open eyes Ichthyotic skin changes	Fingers Wrists Elbows Hips Knees Feet	Overlapping Flexion Flexion Flexion Rockerbottom	Lethal Prenatally diagnosable	Chromosomes: normal Pathology: lissencephaly, absent corpus callosum, cerebellar hypoplasia, small placenta, short umbilical cord, skin with ichthyotic changes	AR

TABLE 161-4 Musculoskeletal Involvement Plus Central Nervous System Dysfunction and/or Mental Retardation and/or Lethality							
Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Neuromuscular disease of the larynx		IUGR Abnormal CNS Pierre–Robin facies Respiratory distress, absent arytenoid cartilage No visceral malformations	Hands 3rd finger Feet	“Clubbed” Flexion Talipes equinovarus	Lethal	Autopsy: absent arytenoid cartilage, brain abnormal, neuromyopathic changes in limb and laryngeal intrinsic muscles  Normal chromosomes	Sporadic AD 5q31
Osteogenesis imperfecta, congenital lethal, “crumpled bone type” (type II)	120160	Short limbs	Wrists	Flexion	Lethal, often stillborn	Radiographs: wormian bones, fractures, cystic changes – long bones, flattened vertebrae, poor mineralization, crumpled bones Type 1 collagen defect	AD <i>COL1A2</i> at 7q <i>COL1A1</i> at 17q <i>FKBP10</i> at 17q
	120150	fractures – poor	Elbows	Flexion			
	166210	mineralization, wormian bones	Knees	Webbing			
	610698	Blue sclera, shallow orbits, small nose ± Contractures, hydrocephalus	Feet	Fixed			
Otopalatodigital syndrome, type II	300017	Deafness, conductive	Fingers	Broad distal phalanges		Radiographs: facial bones hypoplastic; secondary ossification center at base of metacarpals, broad distal phalanges, pectus excavatum	X-linked recessive <i>FLNA</i> at Xq28
	304120	Dwarfism/bone dysplasia					
		Adontia, soft cleft palate	Wrists	Limited supination			
		Characteristic facies: hypertelorism, frontal bossing Mild MR Broad distal phalanges	Elbows Toes	Limited extension Clinodactyly, broad distal phalanges			
Pena–Shokeir phenotype (ankylosis, facial anomalies, and pulmonary hypoplasia) (type 1)	208150	IUGR CNS abnormalities Ankylosis of joints Pulmonary hypoplasia Polyhydramnios, small placenta, short umbilical cord Hypertelorism, micrognathia Fetal akinesia sequence	Hands Elbows Hips Knees Feet	Camptodactyly Flexion Ankylosis Flexion Rockerbottom	Lethal in almost all cases Prenatally diagnosable	Autopsy: congenital myopathy, abnormalities of cerebral cortex and cerebellum Pathology: lung hypoplasia Chromosomes: normal	Some sporadic AR Many subtypes with familial recurrence reported At least 18 different familial forms on basis of changes

Phosphofructokinase deficiency, infantile (glycogen storage VII)	232800 610681	Myopathy High-arched palate Microretrognathia Developmental delay Seizures Corneal clouding	Hands Wrist Hips Knees Feet	Flexion Camptodactyly Flexion Flexion Talipes equinovarus	Progressive respiratory failure Treat with ketogenic diet Most die without treatment	EMG: low amplitude Lab: CPK normal, aldolase elevated, PFK absent, myophosphorylase normal Muscle biopsy: variation in fiber size, subsarcolemmal vacuoles, excess glycogen accumulation	AR <i>PFKM</i> at 12q13
Potter syndrome	164761 191830 611559	Renal agenesis Flattened face Large floppy ears Micrognathia Skin crease under eyes Wrinkled sin Joint contractures Oligohydramnios Lung hypoplasia Broad hands, clubfeet	Hands Wrists Elbows Feet	"Spade-like" Flexion Flexion Talipes equinovarus	Lethal	Chromosomes: normal Autopsy: ± absent uterus and vagina, pulmonary hypoplasia, kidneys and ureters absent or rudimentary, fetal akinesia sequence	Sporadic, occasional AR/AD <i>RET</i> at 11q11.2 <i>UPK3A</i> at 22q11.2
Prader-Willi habitus, osteoporosis, hand contractures syndrome (Urban, Rogers, Meyer)	264010	MR Short stature Obesity Genital abnormalities Hand and foot contractures	Hands Feet	Flexion Fixed		Radiographs: wormian bones, osteoporosis Thyroid: normal	AR
Restrictive dermopathy	150330 275210 606480	Flexion contractures Tight, thin skin Open eyes and mouth Microcephaly ± CNS anomalies	Hands Elbows Hips Knees Ankles	Flexion Flexion Flexion Flexion Flexion	Lethal Prenatally diagnosable	Skin histology: hyperkeratotic stratum corneum with hyperkeratotic keratinization, hypoplastic sebaceous structures and eccrine sweat glands, absence of dermal ridges and thin dermis May have CNS abnormalities, particularly posterior columns	AR <i>DOK7</i> at 4p16.3 <i>RAPSN</i> at 11p15.5

TABLE 161-4 Musculoskeletal Involvement Plus Central Nervous System Dysfunction and/or Mental Retardation and/or Learning Disability							
Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Ritscher–Schinzel syndrome (cranio–cerebro–cardiac, 3C syndrome)		Prominent forehead Ocular coloboma Cleft palate Cerebellar vermis hypoplasia Dandy–Walker Hydrocephaly Cardiac – multiple structural defects Deafness Scoliosis	Hands Generalized flexion contractures	Camptodactyly	D/D Multiple organ anomalies	Vertebral anomalies Renal anomalies	AR
Roberts syndrome (pseudothalidomide syndrome, SC syndrome)	268300 609353	Microbrachycephalia, ± craniosynostosis	Elbows	Radiohumeral synostosis	Usually lethal	Chromosomes: can have “puffing” phenomenon Pathology: CHD, renal anomalies	AR <i>ESCO2</i> at 8p21
		Limb reduction ±	Knees	Femorotibial fusion			
		Humeroradial synostosis ± Cleft lip and palate Characteristic facies Moderate to severe MR ± IUGR	Feet	Talipes calcaneovalgus			
Schinzel–Giedion syndrome	269150 611060	Hypertrichosis CHD	Fingers Feet	Camptodactyly Talipes equinovarus	Usually lethal	CNS: small brain, hypoplasia of corpus callosum Fatty liver Neuroectodermal sacrococcygeal tumor	AR <i>SETBP1</i> at 8p11
		High, prominent forehead Hydronephrosis MR Camptodactyly Split sternum Choanal atresia Abundant skin	Generalized flexion contractures				
Smith–Lemli–Opitz syndrome – severe	270400 602858	Camptodactyly and syndactyly ± polydactyly CNS structural anomalies Renal agenesis/cysts/dysplasia Pulmonary segmentation Hypotonia	Generalized flexion contractures		Lethal when severe	Multiple internal anomalies of all systems Abnormal cholesterol metabolism	AR <i>DHCR7</i> at 11q13
Sotos-like syndrome		Large size	Elbows	Flexion	Awkward Peculiar speech	Neonatal jaundice NSD1 neg	AR
		Large head	Wrists	Flexion			
		MR	Hips	Flexion			
		Kyphoscoliosis	Knees	Flexion			
		Edema	Ankles	Flexion			

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Spastic paraplegia (Goldblatt syndrome)	184260	Spastic paraplegia MR Optic atrophy Mild ataxia	Feet	Talipes equinovarus	Progressive		X-linked recessive Mapped to Xq21–Xq22
Spinal muscular atrophy (SMA1, see also chromosome 5q deletion)	253300 600354	Hypotonia Respiratory insufficiency	Generalized flexion contractures		Progressive Lethal		AR <i>SMN</i> at 5q13 Usually all four alleles abnormal or absent
Spondylospinal–thoracic dysostosis	601809	Short spine with defects Fusion of and missing vertebrae Pterygia ± Cleft palate Crablike thorax	Fingers Wrists Elbows Hips Knees Feet	Camptodactyly Flexion Flexion Flexion Flexion Contractures	Usually lethal Pulmonary failure	Radiographs: vertebral anomalies	AR
Trigonocephaly (C) syndrome	211750	Trigonocephaly (triangular shape) Metopic suture fusion Nose with broad root Anteverted nares Hypotelorism MR	Fingers  Elbows Hips Knees Feet	Campto- and clinodactyly  Flexed Dislocated Flexed Clubbed	Often lethal	Chromosomes: normal EEG: abnormal	AR <i>CD96</i> at 3q13.13–c
Weaver syndrome	277590 606681	Macrosomia Accelerated skeletal maturation Camptodactyly Unusual facies	Fingers Feet	Camptodactyly Talipes equinovarus		Radiographs: broad distal femora and ulnae	Sporadic <i>NSD</i> mutations 5q35
Wieacker muscular atrophy and contractures	314580	Clubfeet Progressive distal atrophy Dyspraxia of eye and tongue muscles Mild MR	Feet	Talipes equinovarus	Progressive		X-linked recessive
X-linked arthrogryposis type 1, anterior horn cell loss	301830 314370	Severe contractures Hypertelorism Camptodactyly Hypoplastic and thin nails Small jaw Seizures Severe kyphoscoliosis	Hands Elbows Hips Knees Ankles	Camptodactyly Flexion Flexion Flexion Flexion	Decreased movement <i>in</i> <i>utero</i> Lethal by 2 yrs Often breech	Autopsy: anterior horn cell disease, infantile spinal muscular atrophy EMG: neurogenic	X-linked <i>UBE1</i> at Xp11.23
X-linked arthrogryposis type 2		Clasped thumbs Wide alveolar ridges Bilateral ptosis “Saddle bag” scrotum Small penis	Knees	Extension	Severe but not lethal	Muscle biopsy: abnormal muscle fibers	X-linked

TABLE 161-4     Musculoskeletal Involvement Plus Central Nervous System Dysfunction and/or Mental Retardation and/or Lethality

Entity	MIM#	Primary Features	Contractures			Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position	Progression		
X-linked arthrogryposis type 5	300158	Profound MR Microcephaly, asymmetrical facies Hypogonadism Joint hypermobility, excess fingertip arches, long palms Esotropia Scoliosis	Fingers Feet Toes	Camptodactyly Rockerbottom Camptodactyly, hallux valgus	Delayed bone growth Short	Fragile X negative Radiographs: thick calvaria, bifid sternum	X-linked Mapped to Xq23–q27
Zellweger syndrome (cerebro–hepato–renal syndrome)	214100	CNS impairment, IUGR Hypotonia ± joint contractures Myopathic facies Hepatomegaly High forehead with delayed closure of sutures	Generalized flexion contractures		Lethal	Chromosomes: normal Radiographs: calcification density over ischium and hip joints, stippling of patella and hands, agenesis of corpus callosum Pathology: lissencephaly, polymicrogyria, renal cysts, abnormal liver function with iron deposits, peroxisomal disorder ECG, EEG: abnormal	AR Peroxisomal abnormalities <i>PEX1</i> gene mu- tations at 7q21–q <i>PEX2</i> gene mu- tations at 8q21.1 <i>PEX3</i> gene mu- tations at 6q21.1 <i>PEX5</i> gene mu- tations at 12p13.3 <i>PEX6</i> gene mu- tations at 6p21 <i>PEX12</i> gene mutations <i>PEX14</i> gene mutations at 1p36.2 <i>PEX26</i> gene mutations at 22q11.21 Chromosome abnormalities

AR = autosomal recessive; AD = autosomal dominant; CDH = congenital deformity of the hip; CHD congenital heart defect; CNS = central nervous system; CPK = creatine phosphokinase; CT = computed tomography; ECG = electrocardiography; EEG = electroencephalography; EMG = electromyography; ER = endoplasmic reticulum; FTT = failure to thrive; GI = gastrointestinal; GU = genitourinary; IUGR = intrauterine growth retardation; LCCS1/2 = lethal congenital contracture syndrome 1/2; MPS = mucopolysaccharidosis; MR = mental retardation; MRI = magnetic resonance imaging; PDA = patent ductus arteriosus; PIP = proximal interphalangeal; TE = tracheoesophageal; WNL = within normal limits.

**TABLE 161-5** Chromosomal Syndromes Known to be Associated with Multiple Congenital Contractures

Chromosomal Syndrome	References
47XXY/48XXXY	(863,864)
48XXX/49XXXY	(504)
Xq28 duplication	(865)
Trisomy 1q	(866,1007)
1p36 deletion	(866)
1p4	
1q23.1–31.1	(610,866)
1q42.3	(867)
Trisomy 2	(866)
3p duplication	(866)
Trisomy 3q	(866)
4p duplication	(866,1007)
4q duplication	(866)
5q deletion	(44a,178,868,869)
6p deletion	(870)
6q duplication	(871–874)
6q21–23	(873)
6q24–25.3	(874)
Trisomy 8	(108,504,866,891)
8q	(866)
8 – TRP syndrome	(477a,875–878)
Trisomy 9	(504,866)
9p deletion	(504,866)
Trisomy 9q	(866,1007)
10p isochromosome	(504)
Trisomy 10p	(504,866,1016)
Trisomy 10q	(504,866)
10q25 deletion	(879)
Trisomy 11q	(866)
11q deletion	(504,866)
Trisomy 13	(504,866)
Trisomy 14q	(504,866)
Trisomy 15q/15q duplication	(3,866)
16p11.2	(880,1007)
17p – Miller–Dieker	(866)
Trisomy 18	(3,866)
18q deletion	(866)
20q isochromosome mosaic	(882)
20q11.2–q12 deletion	(883)
22q11.2 deletion	(232)

TABLE 161-6 Multiple Congenital Contractures Related to Maternal Environment							
Entity	MIM#	Primary Features	Contractures		Progression	Lab Tests, Radiographs, Autopsy Findings	Inheritance
			Body Area	Position			
Attempted termination of pregnancy and/or trauma		Multiple congenital contractures Vascular accidents with secondary loss of tissue	Generalized or localized Mostly flexion		Many involved CNS destruction May be hypotonic	Imaging of brain may show areas of loss Muscle biopsy may show fiber disproportion and fatty replacement	Environmental
Maternal acidosis		Growth restriction Developmental delay Hydrocephaly Axial skeletal malformations	Generalized flexion contractures		Progressive Prenatally diagnosable		Maternal effect
Maternal alcohol consumption – fetal alcohol syndrome		Microcephaly, IUGR Characteristic facial features Joint contractures, palmar creases (73%) ± CHD (50%) MR and behavioral problems	Generalized flexion contractures		Nonprogressive	Pathology: CNS abnormal, CHD	Teratogenic effect of alcohol on fetus
Maternal antibodies against fetal neurotransmitter receptor subunits (see multiple pterygium, Escobar type, Table 161-3; multiple pterygium, lethal; myasthenia gravis, maternal and congenital)		Hypotonia Polyhydramnios	Generalized flexion contractures		Usually stillborn Steroid therapy and plasmapheresis may help Increasing severity with parity	Fetal ACh receptor radioimmunoassay for antibodies	Autoantibodies
Maternal diabetes		Multiple congenital anomalies Sacral agenesis	Any type of combination of multiple congenital contractures		Hypoglycemia early Metabolic disturbance	Maternal glucose control	DMPK gene mutation 19q13.2– Maternal poor control of diabetes



Maternal fluconazole		Bony fusions Radiohumeral synostosis Craniosynostosis	Generalized flexion contractures	May have CNS structural anomalies	Maternal history of consumption	
Maternal hyperthermia		History of maternal fever > 39 °C for several hours Microcephaly ± Microphthalmia ± Cleft palate MR ± Seizures	Generalized flexion contractures	Nonprogressive but may have cerebral palsy	CNS structural anomalies EEG: abnormal	Environmental
Maternal misoprostol ingestion or vaginal suppositories		Terminal limb defects Constriction rings Möbius syndrome CNS abnormalities Hypoplastic muscle Anterior horn cell deficiency Microcephaly	Generalized flexion contractures or amyoplasia-like	Nonprogressive, but often CP and MR	CNS anomalies, including hydrocephaly	Environmental
Maternal multiple sclerosis		Multiple congenital contractures	Generalized flexion contractures	Nonprogressive	Maternal testing	Maternal effects
Maternal muscle relaxant		Fetal akinesia signs	Generalized flexion contractures			History of ingestion during pregnancy
Maternal myasthenia gravis (see also congenital myasthenia gravis and AR myasthenia gravis, Table 161-4)	254200 254300	Pulmonary hypoplasia Kyphoscoliosis Micrognathia Hypotonia	Generalized flexion contractures	May be lethal but may markedly improve with tensilon therapy	May have low ACh receptor antibodies	About 12% of infants born to affected mothers are affected themselves <i>DOK-7</i> at 4p
Prenatal diagnosis by early amniocentesis or CVS		Early CVS or amniocentesis	Lower limb contractures	Nonprogressive	Responds well to PT	

ACh = acetylcholine; AD = autosomal dominant; AR = autosomal recessive; ATP = adenosine triphosphate; CHD congenital heart defect; CNS = central nervous system; EEG = electroencephalogram; ER = enteric reduction; Hb = hemoglobin; IUGR = intrauterine growth retardation; MR = mental retardation.

**TABLE 161-7 Chromosomal Localization of Genes for Disorders with Multiple Congenital Contractures**

Disorder	Chromosomal Localization
<b>Primarily Limb Involvement</b>	
Absence of finger prints	4q22.3 ( <i>SMARCA1</i> )
Bruck syndrome	17p12 ( <i>TLH</i> ) ( <i>PLOD2</i> ), 17q21.2 ( <i>FKBP10</i> ) and 3q23–24 linkage
Clasped thumbs	1q25–q31 ( <i>PRG4</i> )
Continuous contractures	12p13.3 ( <i>KCNA1</i> )
Distal arthrogryposis type 1 and 2B	9p11.3 ( <i>TPM2</i> ), 11p15.5 ( <i>TNN12</i> ), 11p15.5 ( <i>TNNT3</i> ) 17p13MYH3 12q23.2 ( <i>MYBPC1</i> )
Lower limb DA – Fleury type	12q23–24 ( <i>TRPV4</i> )
Mesomelic dysplasia	Xq24–32
Patella aplasia-hypoplasia	17q21–22 ( <i>PTLAH</i> )
Symphalangism/synostosis, coalitions	17q21–22 ( <i>NOG</i> ), 20q ( <i>GDF5</i> )
X-linked arthrogryposis, lower limbs only	2q24–q23
<b>Musculoskeletal Involvement Plus Other System Anomalies</b>	
Camptodactyly–arthropathy–coxa vara–pericarditis syndrome (ARC)	1q24–q25 ( <i>PRG4</i> )
Congenital fiber disproportion	1q42–13 ( <i>ACTA1</i> )
Conradi–Hunermann	(Xp11.23) ( <i>EBP</i> )
Contracture arachnodactyly	5q23–31 ( <i>FBN2</i> ) 12q13
Craniosynostosis syndrome	4p16.3 ( <i>FGFR3</i> ), 8p11.23 ( <i>FGFR1</i> )
Deafness, camptodactyly (DA 6)	11q25 ( <i>CATSAL</i> , <i>CATSHL</i> )
Diastrophic dysplasia	5q32 ( <i>SLC26A2</i> )
Digital arthrogryposis I	9q21.2 ( <i>TNNT3</i> ), 11p15.5 ( <i>TNN12</i> , <i>TNNT3</i> ), and 17p13 ( <i>MYH3</i> )
Distal arthrogryposis type 2B (Sheldon–Hall)	9q21.2 ( <i>TNNT3</i> ), 11p15.5 ( <i>TNN12</i> , <i>TNNT3</i> ), and 17p13 ( <i>MYH3</i> )
Dundar–Sonoda syndrome TARP	15q15.1 ( <i>CHST14</i> )
Ehlers–Danlos IV	2q32.2 ( <i>COL3A1</i> )
Ehlers–Danlos VIII	12p13
Ehlers–Danlos VIB-2	15q15 ( <i>CHST14</i> )
Epidermolysis bullosa dystrophica	3p21.31 ( <i>COL7A1</i> )
Freeman–Sheldon syndrome (DA 2A)	17p13 ( <i>MYH3</i> )
Genitopatallar syndrome	10q22.1 ( <i>KAT6B</i> )
Gordon syndrome (DA IIA, DA3)	17p13.1 ( <i>MYH3</i> )
Hand–foot–uterus syndrome	7p15.2 ( <i>HOXA13</i> )
Holt–Oram syndrome	12q24.1 ( <i>TBX5</i> )
Inclusion-body myopathy	17p13.1 ( <i>MYH2</i> , <i>MYH3</i> )
Kniest dysplasia	12q13.1 ( <i>COLA21</i> )
King–Denborough syndrome/multiple pterygium and malignant hyperthermia	Genes for malignant hyperthermia in 19q13.1 ( <i>RYR1</i> ) and 17q ( <i>MHS3</i> )
Kuskokwim syndrome	17q21.2 ( <i>FKBP10</i> )
Larsen syndrome	3p14.3 ( <i>FLNB</i> ), 3p21.31 ( <i>COL7A1</i> ), AR 8q12.1 ( <i>IMPAD</i> ), 17q25.3 ( <i>CANT</i> ), 5q32 ( <i>DTDST</i> )
Marfan syndrome (severe neonatal)	15q21.1 ( <i>FBN1</i> ), 3p24.2–p25?
MASP1 (3M, Carnevale)	3q27.3 ( <i>MASP1</i> ), 2p25.3 ( <i>COLEC11</i> )
Metaphyseal dysplasia (Jansen)	3p21.31 ( <i>PTH1R</i> )
Metatrophic dysplasia	12q24.1 ( <i>TRPV4</i> )
Möbius syndrome	13q12.2–q13
Moore–Weaver	11p15 ( <i>TNN12</i> )
Multiple pterygium (Escobar type)	2q31.1 ( <i>CHRNA1</i> ), 2q37.1 ( <i>CHRNA1</i> , <i>CHRNA2</i> )
Multiple synostosis, symphalangism	17q21–22 ( <i>NOG</i> ), 20q11.22 ( <i>GDF5</i> ), 13q11 ( <i>FGF9</i> ), 4p16.3 ( <i>DOK7</i> )
Myelinopathies	1q23.3 ( <i>MPZ</i> ), 10q21.1 ( <i>ERG2</i> ), 17p12 ( <i>PMP22</i> ), 19q13.2 ( <i>PRX</i> )
Myopathies	22q12.3 ( <i>LARGE</i> ), 6q25 ( <i>SYNE1</i> ), many others
Nail–patella syndrome	9q34.1 ( <i>LMX1B</i> )
Nemaline myopathy	2q22 ( <i>NEB</i> ), 1q42.13 ( <i>ACTA1</i> )
Neurofibromatosis	17q11–q12 ( <i>NF1</i> )
Neuropathic Israeli-Arab (autosomal recessive)	5qter
Nevo syndrome	1p36.22 ( <i>PLOD1</i> )
Oculo–dental–digital syndrome	6q22.31 ( <i>GJA1</i> )
Otospondylomegapiphyseal dysplasia	6p21.32 ( <i>COL11A2</i> )
Parastremmatic dwarfism	12q24.1 ( <i>TRPV40</i> )
Pfeiffer cardiocranial syndrome	10q26 ( <i>FGFR2</i> ), 8p11.23 ( <i>FGFR1</i> )
Popliteal pterygium syndrome	1q32–q41 ( <i>IRF6</i> )
Proteus syndrome	14q32.32 ( <i>AKT1</i> )

**TABLE 161-7 Chromosomal Localization of Genes for Disorders with Multiple Congenital Contractures—Cont'd**

Disorder	Chromosomal Localization
Puretic–Murray syndrome (infantile hyalinosis)	4q21 ( <i>ANTXR2</i> )
Rigid spine muscular dystrophy	1q36.11 ( <i>SEPN1</i> )
Robino Syndrome	3p14.3 ( <i>WNT5A</i> )
Sacral agenesis	7q36 ( <i>MNX1</i> , Currarino syndrome)
Schwartz–Jampel syndrome	1p36.1 ( <i>HSPG2</i> )
Sheldon–Hall syndrome (DA2)	9p13.3 ( <i>TPM2</i> ), 17p13.1 ( <i>MYH3</i> )
Spondyloepiphyseal dysplasia congenita	12q13.1 ( <i>COLA21</i> )
Stiff man	5q33.1 ( <i>GLRA1</i> ), 4q32.1 ( <i>GLRAB</i> )
Trismus-pseudocamptodactyly	17p12–p13.1 ( <i>MYH8</i> )
Tuberous sclerosis	9q ( <i>TSC1</i> ), 16q ( <i>TSC2</i> )
Ullrich congenital muscular dystrophy	2q37 ( <i>COL6A3</i> ), 21q22.3 ( <i>COL6A1</i> ), 21q22.3 ( <i>COL6A2</i> )
VATER Association	2q31.1 ( <i>HOXD13</i> )
Van den Ende–Gupta syndrome	22q11.21 ( <i>SCARF2</i> )
Waardenburg–Klein	2q25–q37.12 ( <i>PAX3</i> )
Weill–Marchesani syndrome	19p13.3–13.2 ( <i>ADAMS10</i> )
Winchester syndrome	16q13 ( <i>MMP2</i> )
X-linked moderately severe	Xp11.3–q11.2
<b>Musculoskeletal Involvement Plus Central Nervous System Dysfunction and/or Mental Retardation</b>	
Acrocallosal syndrome	7p13 ( <i>GLI3</i> ), 15q26.1 ( <i>K1F7</i> )
Adenyl succinate lyase deficiency	22q13.1–13.2 ( <i>ADSL</i> )
Aicardi–Goutierres syndrome	3p21 ( <i>TREX1</i> ), 11q13.2 ( <i>RNASEH2C</i> ), 13q14.3 ( <i>RNASEH2B</i> ) 19p13.13 ( <i>RNASEH2A</i> ), 20q11.23 ( <i>SAMHD1</i> )
Al-Awadi syndrome	3p25 ( <i>WNT7A</i> )
Antley–Bixler syndrome	7q11.2 ( <i>POR</i> ), 10q26 ( <i>FGFR2</i> )
ARC (Nezelof syndrome)	15q26.1 ( <i>VPS33B</i> ), 14q24.3 ( <i>VIPAR</i> )
Bartsocas–Papas syndrome	20q11.21 ( <i>ASXL1</i> )
Bohring–Opitz syndrome	21q22.3 ( <i>RIPK4</i> )
Bowen–Conradi syndrome	12p13.3 ( <i>EMG1</i> )
Campomelic dysplasia	17q24.3–q25.1 ( <i>SOX9</i> )
Carbohydrate-deficient glycoprotein syndrome	16p13 ( <i>PMM2</i> ); 15q22–qter ( <i>PM11</i> )
Central core disease	19q13.1 ( <i>RYR1</i> )
Cerebro–oculo–facial–skeletal (COFS) syndrome	19q13.2 ( <i>ERCC1</i> ), 13q33.1 ( <i>ERCC5</i> ), 10q11.23 ( <i>ERCC6</i> ), 19q13.32 ( <i>ERCC2</i> )
Chondrodysplasia punctata (rhizomelic)	6q22–q24 ( <i>PEX7</i> )
Clasped thumb and mental retardation	Associated with X-linked hydrocephaly
Cocoon syndrome	10q24.31 ( <i>CHUK</i> )
Congenital muscular dystrophy (merosin deficient)	6q22–23 ( <i>LAMA2</i> )
Contractural arachnodactyly	5q23–31 ( <i>FBN2</i> )
Crisponi syndrome	19p13.11 ( <i>CRLF1</i> )
Dandy–Walker, MR, basal ganglion seizures	Xq25–27
Dyggve–Melchior–Clausen syndrome	18q12–q21.1 ( <i>DYM</i> )
Episodic ataxia/myokymia syndrome	12p13.32 ( <i>KCNA1</i> )
FG syndrome	Xq13 ( <i>MED12</i> ), Xq28 ( <i>FLNA</i> )
Fowler-type hydranencephaly	14q24.3 ( <i>FLVCR2</i> )
Fukuyama congenital muscular dystrophy	9q31.2 ( <i>FKTN</i> ) 9q34.1 ( <i>POMT1</i> ); 14q24.3 ( <i>POMPT2</i> ), 9q31–33 ( <i>FCMD</i> ), 19q13.2 ( <i>FKRP</i> )
Gaucher disease – perinatal lethal	1q22 ( <i>GBA</i> )
Geleophysic dysplasia	9q342 ( <i>ADAMTSG2</i> ), 15q21.1 ( <i>FBN1</i> )
Glycogen storage IV	9q34 ( <i>GLE1</i> )
Leprechaunism	19p13.2 ( <i>INSR</i> )
Lethal arthrogryposis with AHC disease	9q34.11 ( <i>GLE1</i> )
Lethal congenital contractures syndrome (Finnish) I	9q34.11 ( <i>GLE1</i> )
Lethal congenital contractures syndrome (Finnish) II	12q13 ( <i>ERBB3</i> )
Lethal congenital contractures syndrome (Finnish) III	19p13.3 ( <i>PIP5K1C</i> )
Lissencephaly with FADS	7q22.1 ( <i>RELN</i> ), 17p13.3 ( <i>PAFAH1B1</i> )
Martsof syndrome, Warburg micro syndrome	1q41 ( <i>RAB3GAP2</i> ), 2q21.3 ( <i>RAB3GAP1</i> ), 10p12.2 ( <i>RAB18</i> )
MASA syndrome	Xq28 ( <i>L1CAM</i> )
Miller–Dieker syndrome	17q13.2 ( <i>L1S1</i> )
Multiple pterygium syndrome – lethal	2q31.1 ( <i>CHRNA1</i> ); 2q37.1 ( <i>CHRNA1</i> , <i>CHRNA2</i> )
	22q12.3 ( <i>LARGE</i> )

Continued

**TABLE 161-7 Chromosomal Localization of Genes for Disorders with Multiple Congenital Contractures—Cont'd**

Disorder	Chromosomal Localization
Muscular dystrophy, congenital, rigid spine	1p35–36 ( <i>SEPN1</i> )
Myasthenia, congenital	11p11.2–p11.1 ( <i>RAPSN</i> ), 17p12–p11 ( <i>CHRNA1</i> ), 17p13–p12 ( <i>CHRNA2</i> )
Myelinopathies	1q23.3 ( <i>MP2</i> ), 10q21.1 ( <i>ERG2</i> ), 17p12 ( <i>PMP22</i> ), 19q13.2 ( <i>PRX</i> )
Myopathy centronuclear	2q14.2 ( <i>BIN1</i> )
Myotonic dystrophy	19q13.3 ( <i>DMPK</i> )
Myotubular myopathy	Xq28 ( <i>MTM1</i> )
Neuromuscular disease of larynx	5q31
Nevo syndrome	1p36.2 ( <i>PLOD1</i> )
Oculodentodigital syndrome	6q21–23.2 ( <i>GJA1</i> )
Osteogenesis imperfecta	7q21.3 ( <i>COL1A2</i> ), 17q21.23 ( <i>COL1A1</i> )
Otopalatodigital II	Xq28 ( <i>FLNA</i> )
Pfeiffer syndrome	10q26 ( <i>FGFR2</i> ), 8p11.23 ( <i>FGFR1</i> )
Phosphofructokinase deficiency infantile (glycogen storage VII)	12p13.3 ( <i>PFKM</i> )
Potter syndrome	11q11.2 ( <i>RET</i> ), 22q13.31 ( <i>UPK3A</i> )
Raine syndrome	7q22.3 ( <i>FAM20C</i> )
Renal adysplasia	10q11.21 ( <i>RET</i> ), 22q13.31 ( <i>UPK3A</i> )
Restrictive dermopathy	4p16.3 ( <i>DOK7</i> ), 11p11.2 ( <i>RAPSN</i> ), 1p34.2 ( <i>ZMPSTE24</i> ), 1q22 ( <i>LMNA</i> )
Robert syndrome	8p21.1 ( <i>ESCO2</i> )
Schinz–Gideon syndrome	18q12.3 ( <i>SETBP1</i> )
Schwartz–Jampel syndrome	1p36.12 ( <i>HSPG2</i> )
Smith–Lemli–Opitz syndrome	11q12–q13 ( <i>DHCR7</i> )
Spinal muscular atrophy	5q13.2 ( <i>SMN</i> )
Spastic paraplegia (Goldblatt syndrome)	Xq21–q22
Spinal muscular atrophy	5q13.2 ( <i>SMN</i> )
Trigonocephaly (C syndrome)	3q13.3–q13.2 ( <i>CD96</i> )
Waardenburg Shah syndrome	22q13.1 ( <i>SOX10</i> )
Walker–Warburg syndrome	3p22.1 ( <i>GTDC2</i> )
Weaver syndrome	5q35 ( <i>NSD1</i> )
X-linked lethal arthrogryposis 1	Xp11.23 ( <i>UBE1</i> )
Zellweger syndrome	Multiple <i>PEX</i> genes
5q deletions (spinal muscular atrophy)	5q13.3 ( <i>SMN</i> ) microdeletions

DA = distal arthrogryposis; MR = mental retardation.

**TABLE 161-8 Types of Arthrogryposis by Chromosome Location**

Chromosome	Short Arm	Long Arm
1	1p34.2: Restrictive dermopathy ( <i>ZMPSTE24</i> ) 1p36.11: MD, congenital, spinal rigidity ( <i>SEPN1</i> ) 1p36.1: Schwartz–Jampel syndrome ( <i>HSPG2</i> ) 1p36.13: Stiff man/baby syndrome ( <i>SEPN1</i> ) 1p36.22: Nevo syndrome ( <i>PLOD1</i> )	1q21: Gaucher perinatal lethal ( <i>GBA</i> ) 1q22: Restrictive dermopathy ( <i>LMNA</i> ) 1q23.3: Myelinopathy ( <i>MP2</i> ) 1q24–q25: Camptodactyly arthropathy coxa vara pericarditis ( <i>PRG4</i> ) 1q25–q31: Camptodactyly–arthropathy–coxa vara–pericarditis ( <i>CACP</i> ) 1q31: Multiple joint stiffness, AR 1q32.2: Popliteal pterygium syndrome 1q41: Martsolf syndrome ( <i>RAB3GAP</i> ) 1q42.13: Congenital fiber disproportion, Nemaline myopathy ( <i>ACTA1</i> ) 2q14.3: Central core myopathy ( <i>BIN1</i> ) 2q21.3 Martsolf syndrome ( <i>RAB3GAP1</i> )
2	2p25.3: MASP1 syndrome ( <i>COLEC11</i> )	2q23.3: Nemaline myopathy ( <i>NEB</i> ) 2q24–32: Mesomelic dysplasia 2q31.1: VATER association ( <i>HOXD13</i> ) 2q25: Waardenburg–Klein ( <i>PAX3</i> ) 2q32.2: Ehlers–Danlos 2q37.1: Multiple pterygium Escobar ( <i>CHRNA1</i> , <i>CHRNA2</i> ) 2q37: Ullrich congenital muscular dystrophy ( <i>COL6A3</i> )



TABLE 161-8 Types of Arthrogryposis by Chromosome Location—*Cont'd*

Chromosome	Short Arm	Long Arm
3	3p14.3: Larsen syndrome ( <i>FLNB</i> ) 3p14.3: Robinow syndrome ( <i>WNT5A</i> ) 3p21.31: Larsen syndrome, Epidermolysis bullosa dystrophica ( <i>COL7A1</i> ) 3p21: Aicardi syndrome ( <i>TREX1</i> ) 3p22: Metaphyseal dysplasia ( <i>PTHR1</i> ) 3p25: Al-Awadi-Raas-Rothchild ( <i>WNT7A</i> ) 3p27.3: MASP1 syndrome ( <i>MASP1</i> )	3q13.3-2: Trigonencephaly/C syndrome ( <i>CD96</i> ) 3q23-24: Bruch syndrome 2
4	4p16.3: Restrictive dermopathy, Multiple pterygium syndrome ( <i>DOK7</i> ) 4p16.3: Craniosynostosis syndrome, Deafness and camptodactyly ( <i>FGFR3</i> )	4q21: Poretic-Murray syndrome, Infantile hyalinosis ( <i>ANTXR2</i> ) 4q22.3: Absence of fingerprints ( <i>SMARCA1</i> )
5		5qter: Neuropathic Israeli-Arab (Shohat type) 5q13 microdeletion including SMN complex: arthrogryposis with SMN 5q23-q31: Contractural arachnodactyly ( <i>FBN2</i> ) 5q31: Neuromuscular disease of the larynx 5q32: Diastrophic dysplasia, Larsen syndrome ( <i>SLC26A2</i> ) 5q33.1: Stiffman Syndrome ( <i>GLRA1</i> ) 5q35: Weaver syndrome ( <i>NSD1</i> )
6	6p21.32: Ootospondylomegaepiphyseal dysplasia ( <i>COL11A2</i> )	6q22.31: Oculodentodigital syndrome ( <i>GJA1</i> ) 6q22-23: Congenital MD (merosin deficient) ( <i>LAMA2</i> ) 6q22-24: Chondrodysplasia punctata (rhizomelic) ( <i>PEX7</i> ) 6q25: Myopathy ( <i>SYNE</i> )
7	7p13: Acrocallosal syndrome ( <i>GLI3</i> ) 7p15.2: Hand-foot-uterus ( <i>HOXA13</i> )	7q11.2: Antley-Bixler syndrome ( <i>POR</i> ) 7q21.3: Osteogenesis imperfecta ( <i>COL1A2</i> ) 7q22.1: Lissencephaly ( <i>RELN</i> ) 7q22.3: Raine syndrome ( <i>FAM20C</i> )
8	8p11.23: Pfeiffer cardiocranial syndrome ( <i>FGFR1</i> ) 8p21.1: Roberts syndrome ( <i>ESCO2</i> )	8q12.1: Larsen syndrome ( <i>IMPAD1</i> )
9	9q11.3: Distal arthrogryposis type 1 ( <i>TPM2</i> )	9q: Tuberous sclerosis ( <i>TSC1</i> ) 9q: Symphalangism 9q21.2: Sheldon-Hall distal arthrogryposis ( <i>TNNT3</i> ) 9q31-q33: Fukuyama congenital MD ( <i>FKRP</i> ) 9q31.3: Myasthenia congenita ( <i>MUSK</i> ) 9q34.1: Fukuyama congenital muscular dystrophy ( <i>POMT1</i> ) 9q34: (Finnish) Lethal congenital contractures syndrome I, AHC disorders ( <i>GLE1</i> ) 9q34.1: Nail-patella syndrome ( <i>PNS, LMX1B</i> ) 10q11.21: Renal adysplasia ( <i>RET</i> )
10	10p12.1: Martsolf syndrome ( <i>RAB18</i> )	10q11.23: COFS syndrome ( <i>ERCC6</i> ) 10q21.1: Myelinopathy ( <i>ER62</i> ) 10q22.2: Genitopatellar syndrome ( <i>KAT6B</i> ) 10q24.31: Cocoon syndrome ( <i>CHUK</i> ) 10q26: Pfeiffer cardiocranial syndrome, Antley-Bixler syndrome ( <i>FGFR2</i> )
11	11p11.2-1: Congenital myasthenia restrictive dermopathy ( <i>RAPSN</i> ) 11p15.5-pter: Distal arthrogryposis Sheldon-Hall 2B and Moore-Weaver ( <i>TNNI2</i> ) ( <i>TNNT3</i> ) 12p11.2-p13.3 Acrocallosal syndrome	11q11.2: Potter syndrome ( <i>RET</i> ) 11q12-q13: Smith-Lemli-Opitz syndrome ( <i>DHCR7</i> ) 11q13.2: Aicardi syndrome ( <i>RNASEN2C</i> ) 11q14-q23: Tuberous sclerosis 11q25: Deafness, histiocytosis and arthrogryposis

Continued

TABLE 161-8 Types of Arthrogryposis by Chromosome Location—Cont'd

Chromosome	Short Arm	Long Arm
12	12p13: Ehlers–Danlos VIII linkage 12p13.3: Phosphofructokinase deficiency (PFKM) 12p13.3: Bowen–Conradi (EMG1) 12p13.3: Episodic ataxia/Continuous contractures (KCNA1)	12q13: Contractural arachnodactyly (FBN2) 12q13: LCCS II (ERBB3) 12q13.1: Spondyloepiphyseal dysplasia congenita; Kniest dysplasia (COLA2T1) 12q13.3: Phosphofructokinase (PFKM) 12q24.1: Holt–Oram syndrome (TBX5) 12q24.1: Metatropic (TRPV4) 12q13: Israeli Bedouin lethal congenital contractures 2 12q23.2: Distal Arthrogryposis (MYBAC1) 12q23–24: Fleury type lower limb (TPRV4) 12q24.1: Holt–Oram (TBX5) 12q24.1: Parastremmatic dwarfism (TPRV4) 13q11: Multiple synostosis (FGF9) 13q12.2–q13: Möbius syndrome (MBS1)
13		13q33.1: COFS syndrome (ERCC5)
14		14q24.3: ARC syndrome (V1PAR) 14q24.3: Fowler syndrome (FLYCR2) 14q24.3: Fukuyama congenital muscular dystrophy (POMPT2) 14q32.32: Proteus syndrome (AKT1)
15		15q15.1: Dundar–Sonoda syndrome (CHST14) 15q15: Ehlers–Danlos VIB-2: (CHST14) 15q21.1: Marfan syndrome, Geleophysic dysplasia (FBN1) 15q26.1: ARC syndrome (VPS33B) 15q26.1: Acrocallosal syndrome (KIF7)
16	16p13.3–p13.2: Carbohydrate-deficient glycoprotein (PMMZ)	16q: Tuberous sclerosis (TSC2) 16q13: Winchester syndrome (MMP2)
17	17p12: Bruck syndrome (PLO2) 17p12: Myelinopathy (PMP22) 17p12–13: Trismus–pseudocamptodactyly (MYH8) 17p13: Freeman–Sheldon, Sheldon–Hall, Gordon syndrome (MYH3) 17p13: Miller–Dieker syndrome (deletion) 17p13.1: Inclusion myopathy (MYH2 and MYH3) 17p13–12: Congenital myasthenia (CHNRB, CHRNE) 17p13.3: Lissencephaly (PAFAH1B1)	17q1: King–Denborough syndrome (MHS3) 17q11–q12: Neurofibromatosis (NF1) 17q13.3: Miller–Dieker syndrome (L1S1) 17q21.2: Bruck syndrome (FKBP10) 17q21.2: Kuskokwim syndrome (FKBP10) 17q21.23: Osteogenesis imperfecta (COL1A1) 17q21–q22: Multiple synostosis syndrome; patella aplasia-hypoplasia (PTLAH) 17q22: Symphalangism, Multiple synostosis, coalition (NOG) 17q24.3–q25.1: Campomelic dysplasia (SOX9) 17q25.3: Larsen syndrome (CANT1)
18	19p13.11: Crisponi syndrome (CRLF1) 19p13.13: Aicardi syndrome (RNASEH2A)	18q12–21.1: Dyggve–Melchior–clausen syndrome (DYM) 18q12.3: Schinzel–Gideon (SETBP1)
19	19p13.2: Leprechaunism (INR) 19p13.3–p13.2: Weill–Marchesani syndrome (ADAMS10) 19p13.3: LCCSIII (P1P5K1C)	19q13.1: King–Denborough syndrome (RYR1) 19q13.2: Myelinopathy (PRX) 19q13.2: Cerebro–oculo–facial–skeletal syndrome (ERCC1) 19q13.2: Fukuyama muscular dystrophy (FKRP) 19q13.2–19q13.3: Myotonic dystrophy (DMPK) 19q13.3: Central core (RYR1) 19q13.32: Cerebro–oculo–facio–skeletal syndrome 2 (ERCC2)
20		20q11.22: Multiple synostosis (GDF5) 20q11.23: Aicardi syndrome (SAMHD1) 20q11.21: Bohring–Opitz syndrome (ASXL1)
21		21q22: Ullrich congenital MD (COL6A2) (COL6A1)
22		21q22.3: Bartsocas–Papas syndrome (RIPK4) 22q11.21: Van den Ende–Gupta syndrome (SCARF2) 22q12.3: Myopathy (LARGE) 22q13.1–13.2: Adenyl succinate lyase deficiency (ADSL) 22q13.31: Potter syndrome (UPK3A)

**TABLE 161-8** Types of Arthrogryposis by Chromosome Location—*Cont'd*

Chromosome	Short Arm	Long Arm
X	Xp11.23: Conradi–Hunermann ( <i>EBP</i> ) Xp11.23: X-linked lethal arthrogryposis ( <i>UBE1</i> )	Xq13: FG syndrome ( <i>MED12</i> ) Xq21–q22: Spastic paraplegic (Goldblatt syndrome) Xq23–q27: X-linked arthrogryposis, lower limbs only; clasped-thumb syndrome; Xq25–27: Dandy–Walker basal ganglion Xq28: MASA syndrome ( <i>LICAM</i> ) Xq28: Oto–palatal–digital II ( <i>FLNA</i> ) Xq28: FG syndrome ( <i>FLNA</i> ) Xq28: Myotubular myopathy (MTM1)

ARC = arthrogryposis, renal dysfunction, cholestasis syndrome; AR = autosomal recessive; COFS = cerebro-oculo-facio-skeletal syndrome; LCCS II/III = lethal congenital contracture syndrome 1/2; MD = muscular dystrophy.

**TABLE 161-9** Specific Organ or Area of Involvement in Various Disorders with Arthrogryposis (Congenital Contractures)

<b>Adducted thumbs</b> Adducted thumb syndrome Clasp thumb syndrome Contractural arachnodactyly Dundar–Sonoda syndrome Freeman–Sheldon syndrome Martsolf syndrome MASA syndrome Multiple pterygium – lethal syndrome Nemaline myopathy X-linked arthrogryposis type 2 X-linked hydrocephaly <b>Arachnodactyly</b> Aase–Smith I syndrome – long fingers with short distal phalanges Antley–Bixler syndrome – long thin Blepharophimosis–arachnodactyly syndrome – dislocated radial heads Congenital contractural arachnodactyly Congenital Marfan syndrome Dundar–Sonoda syndrome Ehlers–Danlos syndrome VIII Hoepffner syndrome – slim hands Larsen syndrome – long cylindrical fingers Marden–Walker syndrome Marfan syndrome – severe neonatal van Benthem syndrome van den Ende–Gupta syndrome <b>Arms Only</b> Amyoplasia Antecubital pterygium (Shin Shun) Autosomal dominant pterygium syndrome Baraitser–London camptodactyly Guadalajara camptodactyly III Hunter–MacDonald camptodactyly Leibenberg syndrome Lin–Gettig syndrome Rozin and Kilic camptodactyly Shalev-type distal arthrogryposis Urban–Rogers–Meyer syndrome X-linked resolving arthrogryposis <b>Arthropathy</b> Camptodactyly and arthropathy Camptodactyly, coxa vara, pericarditis, and synovitis (ARC)	Ehlers–Danlos VIII Ehlers–Danlos VIB-2 <b>Bone Abnormalities</b> Aarskog syndrome – short distal phalanges Aase–Smith I syndrome – small epiphyses, tall vertebrae Angulation of long bone – apex of angulation has dimples Antley–Bixler – radiohumeral fusion, angulation long bone Bruck syndrome – easily fractured, angulated Campomelic dysplasia – curved long bones CAD – agenesis coccyx or sacrum Caudal deficiency and asplenia – lack lower vertebrae Chondrodysplasia punctata, rhizomelic – symmetric stippling Coalitions – many types Conradi–Hünemann syndrome (chondrodysplasia punctata) – symmetric stippling Diastrophic dysplasia Dyggve–Melchior–Clausen syndrome – platyspondylia, irregular boney pelvis Dyssegmental dysplasia Ehlers–Danlos syndrome VIB Freeman–Sheldon dysplasia Geleophysic dysplasia – dysostosis multiplex Humeroradial synostosis Jequier syndrome – advanced BA, spicules, broad long bones Kniest syndrome – epiphyseal dysplasia, flat vertebrae Lenz–Majewski dysplasia – progressive scoliosis and thickening of all bones, AF remains open, symphalangism of proximal phalanges Lerman–Sagie syndrome – osteopetrosis Liebenberg syndrome Lower limb and pelvic dysplasia (AR) Megalocornea and skeletal anomalies Mesomelic dysplasia Metaphyseal dysplasia (Jansen) Metatropic dysplasia – progressive changes, metaphyseal flare Myhre muscle hypertrophy – thickened bones including calvaria Nevo syndrome – osteoporosis, advanced bone age, kyphoscoliosis Osteogenesis imperfecta – crumpled bone, wormian bones, cystic metaphyses Teebi–Shaltout syndrome – osteolysis Oto-onycho-peroneal syndrome – absent/hypoplastic fibula, fusion clavicle, and scapular spine Parastremmatic dysplasia
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**TABLE 161-9 Specific Organ or Area of Involvement in Various Disorders with Arthrogryposis (Congenital Contractures)—Cont'd**

<p>Patella aplasia–hypoplasia syndrome Prader–Willi-like – wormian bones Pseudodiastrophic dysplasia Radioulnar synostosis Saul–Wilson dysplasia Spondyloepiphyseal dysplasia congenita – flat epiphyses and vertebrae Spondylothoracic dysostoses Symphalangism – many types van den Ende–Gupta syndrome Weill–Marchesani syndrome Winchester syndrome Zellweger syndrome – stippling of epiphyses and patellae, calcifications</p> <p><b>Boney Fusions</b> Antley–Bixler – radiohumeral synostosis, coalitions Coalitions Diastrophic dysplasia Hanhart – fused knee or elbow Humeroradial synostosis Ives microcephaly micromelia syndrome – elbows Lenz–Majewski syndrome Liebenburg syndrome Mesomelic dysplasia Multiple synostosis – multiple fusions Ophthalmomandibulomelic syndrome – fusion TMJ Pfeiffer cardiocranial syndromes – humeroradial synostosis Radioulnar synostosis Roberts syndrome – humeroradial synostoses Symphalangism (many types) Waardenburg–Klein syndrome – carpal fusion</p> <p><b>Bowel Abnormality</b> Aarskog syndrome – Hirschsprung malformation (rare) Aase–Smith syndrome – VSD (rare) Al–Awadi syndrome – TEF, IA Alkuraya syndrome – pyloric stenosis Amyoplasia – gastroschisis, bowel atresia, abdominal wall defects Bowen syndrome – omphalocele, umbilical hernia BRESHECK – Hirschsprung “C” syndrome variant – malrotation Distal (2E Hall) – bowel atresia Eagle–Barrett – 1A FG syndrome – imperforate anus Fryns syndrome – omphalocele Lin Gettig – small bowel atresia, omphalocele, umbilical pigmentation Miller–Dieker syndrome – duodenal atresia Mitochondrial defects – atresias Shalev-type distal arthrogryposis – umbilical hernia VATER association – TEF, EA, BA</p> <p><b>Cardiology</b> Aarskog syndrome – ASD, PS (rare) Aase–Smith syndrome – VSD (rare) Acrocallosal syndrome Alkuraya syndrome Antley–Bixler syndrome – ASD, PDA Bohring–Opitz syndrome Bowen syndrome – ASD</p>	<p>“C” syndrome variant – frequent variety of anomalies, VSD, ASP, PDA Camptodactyly, pericarditis, synovitis–pericarditis Camptodactyly, Tel–Hashomer – congenital heart disease Carbohydrate-deficient protein syndrome – cardiomyopathy Cardiac deficiency and asplenia Cardiomeic syndrome Contractural arachnodactyly – congenital heart anomalies Dundar–Sonoda syndrome – CHD, ASD Ectodermal dysplasia and cardiomyopathy Ehlers–Danlos VIII – aortic and mitral regurgitation FG syndrome Geleophysic dysplasia – aortic stenosis Holt–Oram syndrome – structural defects, particularly septal Larsen syndrome – septal defects Lerman–Sagie – murmur, enlarged interventricular septum, PDA Lin–Gettig – VSD Marfan syndrome, congenital – aortic incompetency Miller–Dieker (lissencephaly II) – PDA Mitochondrial defects Myhre muscle hypertrophy Myopathies – PDA Nemaline myopathy – congenital heart disease Neuropathic Israeli–Arab – congenital heart PHAVER syndrome – VSD, ASD, double outlet, coarctation of aorta, pulmonary atresia Pfeiffer cardiocranial syndrome – congenital heart Ritscher–Schinzel – multiple structural defects Robert syndrome Schinzel–Giedion syndrome Stiff man syndrome – heart failure</p> <p><b>Caudal Appendage</b> Ectodermal dysplasia with caudal appendage Gul syndrome Teebi–Shaltout syndrome 3C Carnevale syndrome</p> <p><b>Central Nervous System Structural Anomaly</b> Aarskog syndrome – polymicrogyria (rare) Aase–Smith I syndrome – Dandy–Walker with secondary hydrocephalus and cerebellar anomalies, agenesis corpus callosum Acrocallosal – agenesis corpus callosum, macrocephaly, cerebral and temporal lobe atrophy Adenylosuccinate lyase deficiency – pachygyria, cerebral atrophy, white matter gliosis Adducted thumbs syndrome – dysmyelination Aicardi–Goutieres – calcification, scarring white matter disruption Attempted termination of pregnancy Basal ganglion syndrome – choreoathetosis Bixler microcephaly Blepharophimosis, mental retardation, Dandy–Walker malformation – cerebellar anomalies, absence of corpus callosum Bohring–Opitz – microcephaly Bowen–Conradi syndrome – agenesis of corpus callosum, microcephaly, hypoplastic cerebellum, absent vermis BRESHECK – micro-hydrocephaly CAD – hydrocephaly, microcephaly Carbohydrate-deficient glycoprotein syndrome – cerebellar hypoplasia, dysmyelination</p>
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**TABLE 161-9 Specific Organ or Area of Involvement in Various Disorders with Arthrogryposis (Congenital Contractures)—Cont'd**

<p>Caudal deficiency and asplenia – agenesis of the corpus callosum, meningocele</p> <p>Clasped thumb syndrome – malmigration, microcephaly, hydrocephaly, aqueductal stenosis</p> <p>Coffin–Siris syndrome</p> <p>COFS – dysmyelination, degenerative brain atrophy</p> <p>Dandy–Walker syndrome – cystic enlargement of 4th ventricle, cerebral atrophy, cerebellar hypoplasia, iron in basal ganglion</p> <p>Dundar–Sonoda syndrome – mild hydrocephalus, tethered cord, absent septum pellucidum</p> <p>Fetal alcohol syndrome</p> <p>FG syndrome – agenesis of the corpus callosum</p> <p>Fowler syndrome – proliferative vasculopathy, hydranencephaly, hydrocephaly, diffuse ischemic lesions with calcifications</p> <p>Fukuyama muscular dystrophy – heterotopias, cerebral atrophy, lissencephaly, polymicrogyria, myelin abnormalities</p> <p>Genitopatellar syndrome – absent corpus callosum</p> <p>Illium syndrome – cystic brain, old scars</p> <p>Ives microcephaly, micromelia syndrome – myelin dysplasia</p> <p>Johnson hyperkeratosis and decreased posterior columns</p> <p>Lethal arthrogryposis with AHC disease (Finnish) – loss AHC, thin spinal cord</p> <p>Lin–Gettig – agenesis of corpus callosum</p> <p>Lenz–Majewski – white matter dysgenesis, hydrocephalus, facial palsy (all rare)</p> <p>Lissencephaly with fetal akinesia – lissencephaly, agenesis of corpus callosum and vermis, cystic cerebellum</p> <p>Marden–Walker – Dandy–Walker malformation, hypoplastic corpus callosum</p> <p>Martsolf syndrome – Arnold–Chiari malformation, microcephaly, cerebral atrophy, dilated ventricles</p> <p>Maternal fever – microcephaly</p> <p>Maternal misoprostol – microcephaly, AHC deficiency, structural CNS abnormality</p> <p>Maternal acidosis – microcephaly, hydrocephaly</p> <p>Meningomyocele – cord disruption, +/- CNS structural anomaly</p> <p>Miller–Dieker syndrome – lissencephaly, microcephaly, large ventricles</p> <p>Mitochondrial defects – cerebellar defects, calcifications of basal ganglia, cerebellar anomalies</p> <p>Möbius syndrome – facial diplegia</p> <p>Multiple pterygium syndrome, lethal type – cerebellar hypoplasia</p> <p>Myelinopathies – markedly decreased myelin, white matter</p> <p>Neurofibromatosis – neurofibromas and CNS structural</p> <p>Neu–Laxova syndrome – lissencephaly, agenesis of the corpus callosum, microcephaly</p> <p>Neuromuscular disease of larynx – CNS changes</p> <p>Osteogenesis imperfecta – hydrocephaly</p> <p>PHAVER syndrome – meningocele</p> <p>PEHO syndrome – small cerebellum, spongy vacuolation of cortex, loss of Purkinje cells</p> <p>Pena–Shokeir phenotype – various</p> <p>Restrictive dermopathy – posterior columns</p> <p>Ritscher–Schinzel – cerebellar vermis hypoplasia, Dandy–Walker anomaly, hydrocephaly</p> <p>Schinzel–Giedion syndrome – hypoplastic corpus callosum</p> <p>Smith–Lemli–Opitz syndrome</p> <p>Tuberous sclerosis – structural anomalies</p> <p>van den Ende–Gupta syndrome – cerebellar hyperplasia</p>	<p>Walker–Warburg syndrome – lissencephaly, dysmyelination</p> <p>X-linked anterior horn cell disease</p> <p>Zellweger syndrome – lissencephaly, polymicrogyria, agenesis corpus callosum</p> <p><b>Chest Deformity</b></p> <p>Aarskog syndrome – pectus excavatum (freq)</p> <p>Adducted thumbs syndrome</p> <p>Camptodactyly, Guadalajara – pectus excavatum</p> <p>Carbohydrate-deficient glycoprotein syndrome – inverted nipples</p> <p>Congenital Marfan syndrome – pectus carinatum, excavatum</p> <p>Contractural arachnodactyly – pectus excavatum, carinatum</p> <p>Dundar–Sonoda syndrome – thin ribs, pectus (oce)</p> <p>Ectodermal dysplasia with cardiomyopathy – chest deformity</p> <p>Ectodermal involvement with caudal appendage – shield chest</p> <p>Fryns syndrome – absent diaphragm</p> <p>Hoepffner syndrome – pectus excavatum</p> <p>King–Denborough syndrome – pectus excavatum</p> <p>Lerman–Sagie – ↑ AP diameter, bell-shaped, wide-spaced nipples</p> <p>Lin–Gettig – pectus, widespread nipples</p> <p>Marfan syndrome, severe congenital – pectus excavatum</p> <p>Moebius – absent muscles</p> <p>Multiple pterygium syndrome, Escobar type – restrictive lung disease</p> <p>Nevo syndrome – pectus</p> <p>Pena–Shokeir phenotype – ↑ AP diameter</p> <p>Schinzel–Giedion – split sternum</p> <p>Schwartz–Jampel syndrome – pectus carinatum</p> <p>Spondylothoracic dysostosis – fusion of ribs</p> <p>van Bentham syndrome</p> <p>van Biervliet syndrome</p> <p>X-linked arthrogryposis type 5 – bifid sternum</p> <p><b>Choanal Atresia</b></p> <p>Antley–Bixler syndrome</p> <p>Lenz–Majewski syndrome</p> <p>Schinzel–Giedion syndrome</p> <p><b>Cleft Lip</b></p> <p>Bartsocas–Papavasiliou and cleft nose</p> <p>Diastrophic dysplasia</p> <p>Distal arthrogryposis (Hall IIC)</p> <p>Ectodermal dysplasia with cleft lip/palate</p> <p>Oral cranial digital syndrome – cleft lip and palate</p> <p><b>Cleft Palate</b></p> <p>Aase–Smith syndrome – hard palate, also soft with bifid uvula</p> <p>Adducted thumbs syndrome</p> <p>Bartsocas–Papavasiliou syndrome – cleft nose, lip palate</p> <p>Bixler microcephaly</p> <p>BRESHECK – submucous</p> <p>Bohring–Opitz – cleft, high arched</p> <p>Campomelic dysplasia</p> <p>Diastrophic dysplasia</p> <p>Distal arthrogryposis (2C Hall)</p> <p>Dyssegmental dysplasia</p> <p>Ectodermal dysplasia and cleft lip/palate</p> <p>Focal femoral dysplasia and long philtrum</p> <p>Fryns syndrome</p> <p>German syndrome</p> <p>Gordon syndrome – 40%, familial variability</p> <p>King–Denborough syndrome</p> <p>Kniest syndrome</p> <p>Larsen syndrome</p>
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Continued

**TABLE 161-9 Specific Organ or Area of Involvement in Various Disorders with Arthrogryposis (Congenital Contractures)—Cont'd**

<p>Lin-Gettig – cleft palate</p> <p>Maternal hyperthermia</p> <p>Multiple pterygium syndrome, Escobar type</p> <p>Oculo-auriculovertebral spectrum</p> <p>Oral-cranial-digital dysplasia – cleft lip and palate</p> <p>Otopalatodigital syndrome II – soft palate</p> <p>Popliteal pterygium syndrome, Gorlin type – frenula, intraoral web</p> <p>Ritscher–Schinzel syndrome</p> <p>Robert syndrome</p> <p>Rudiger syndrome</p> <p>Sonoda syndrome</p> <p>Spondylothoracic dystrophy</p> <p>VSR syndrome</p> <p><b>Coarse Face</b></p> <p>Dandy–Walker – MR, basal ganglion disease</p> <p>Fryns syndrome</p> <p>Gaucher syndrome</p> <p>Phosphofructokinase deficiency</p> <p><b>Craniosynostosis</b></p> <p>Aarskog syndrome – rare</p> <p>Adducted thumbs syndrome</p> <p>Antley–Bixler syndrome – lambdoid-coronal 100%</p> <p>Bohring–Opitz syndrome – trigonocephaly</p> <p>“C” syndrome variant – 100% metopic suture</p> <p>Campomelic dysplasia</p> <p>Dundar–Sonoda – sagittal (occ.), large AF 100%</p> <p>Lin–Gettig – craniosynostosis metopic, sagittal, lambdoid (all)</p> <p>Pfeiffer cardiocranial syndrome</p> <p>Roberts syndrome</p> <p>Symphalangism/brachydactyly</p> <p>Trigonocephaly syndrome – metopic suture fusion</p> <p><b>Deafness</b></p> <p>Antley–Bixler – ext. auditory stenosis 30%</p> <p>BRESHECK</p> <p>Diastrophic dysplasia – ossicular deformation</p> <p>Distal arthrogryposis and deafness DA6</p> <p>Distal arthrogryposis (724) (absent teeth) – otosclerosis</p> <p>Kniest syndrome</p> <p>Lin–Gettig – neurosensory</p> <p>Multiple pterygium – Escobar</p> <p>Multiple synostosis syndrome – ossicular fusion</p> <p>Myhre muscle hypertrophy</p> <p>Oculo-auriculovertebral spectrum</p> <p>Ohno blepharophimosis syndrome</p> <p>Oto-onycho-peroneal syndrome</p> <p>Otopalatodigital syndrome II – conductive</p> <p>Ritscher–Schinzel syndrome</p> <p>Waardenburg–Klein syndrome</p> <p><b>Dental</b></p> <p>Aarskog syndrome – large incisors, hypoplastic enamel, natal teeth</p> <p>“C” syndrome variant – wide alveolar ridge, extra frenulum</p> <p>Distal arthrogryposis with absent teeth type (724) (Beals)</p> <p>Ectodermal dysplasia with cardiomyopathy – hypoplastic teeth</p> <p>Ectodermal dysplasia with contractures – enamel abnormalities, delayed eruption</p> <p>Ectodermal dysplasia with cleft palate – dental anomaly</p> <p>Ectodermal involvement with caudal appendage – absent primary teeth, broad gums</p> <p>Ehlers–Danlos VIII – periodontics</p>	<p>Jequier syndrome – broad irregular alveolar ridge</p> <p>Lenz–Majewski syndrome – abnormal enamel, microdontia</p> <p>Nevo syndrome – high arched, micrognathia</p> <p>Oculodentodigital syndrome – hypoplastic enamel, microdontia</p> <p>Ohno blepharophimosis – hypoplasia</p> <p>Otopalatodigital syndrome II – adontia</p> <p><b>Diaphragm Abnormality/Hernia</b></p> <p>Fryn syndrome</p> <p>Rutledge syndrome</p> <p>Stiff man/stiff baby syndrome</p> <p><b>Dislocated/Abnormal Radial Head</b></p> <p>Aase–Smith I syndrome – freq. in surviving individuals</p> <p>Antecubital pterygium – fused dysplasia elbows</p> <p>Antley–Bixler syndrome</p> <p>Blepharophimosis–arachnodactyly syndrome</p> <p>“C” syndrome variant – AD, probably 100%</p> <p>Diastrophic dysplasia</p> <p>Dundar–Sonoda syndrome</p> <p>Humeroradial synostosis</p> <p>Ives microcephaly</p> <p>Liebenberg syndrome</p> <p>Meiten syndrome</p> <p>Nail–patella syndrome</p> <p>Nievergelt–Pearlman syndrome</p> <p>Ophthalmocranial–digital syndrome</p> <p>Oto-onycho-peroneal syndrome – abnormally formed, limited pterygia</p> <p>Oral–cranial–digital syndrome</p> <p>Radiohumeral synostosis</p> <p>Roberts syndrome</p> <p>Rutledge syndrome</p> <p>Schwartz–Jampel syndrome</p> <p>Stiff man/stiff baby syndrome</p> <p>Symphalangia/brachydactyly syndrome</p> <p><b>Dislocations Generalized Prominent</b></p> <p>Bruck syndrome – hypermobility, small joints</p> <p>Ehlers–Danlos VIII</p> <p>Ehlers–Danlos VIB2</p> <p>Larsen syndrome</p> <p>X-linked arthrogryposis 5</p> <p><b>Dysmyelination</b></p> <p>Adducted thumbs syndrome</p> <p>Aicardi syndrome</p> <p>Carbohydrate-deficient glycoprotein syndromes</p> <p>COFS</p> <p>Fukuyama muscular dystrophy</p> <p>Ives microcephaly</p> <p>Lenz–Majewski syndrome</p> <p>Walker–Warburg syndrome</p> <p><b>Ears Abnormal</b></p> <p>Aase–Smith I syndrome – large</p> <p>Antley–Bixler – truly low-set, small, over-folded</p> <p>Blepharophimosis arachnodactyly – large</p> <p>Blepharophimosis syndrome – Dandy–Walker malformation – auricular pits</p> <p>Bowen–Conradi syndrome – ears poorly folded, large</p> <p>BRESHEK – large ears</p> <p>COFS – large ears</p> <p>Contractural arachnodactyly – over-folded top, crumpled</p>
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**TABLE 161-9 Specific Organ or Area of Involvement in Various Disorders with Arthrogryposis (Congenital Contractures)—Cont'd**

<p>Diastrophic dysplasia – calcified cartilage</p> <p>Dundar–Sonoda syndrome – posterior rotation</p> <p>Jequier syndrome – large, crumpled</p> <p>Lin–Gettig – small, external canal stenosis (all)</p> <p>Megalocornea skeletal anomalies – large</p> <p>Nevo syndrome – large, low-set</p> <p>Oculo-auriculovertebral spectrum</p> <p>Ohdo blepharophimosis</p> <p>Oto-onychoperroneal syndrome – unfolded ears, prominent antihelix and cues, no lobule</p> <p>PHAVER syndrome – simple, over-folded, notch, prominent antihelix, conductive deafness</p> <p>van den Ende–Gupta syndrome – large ears</p> <p><b>Eye Anomaly (see Ophthalmology)</b></p> <p>Aarskog syndrome – ptosis, hypertelorism (freq.)</p> <p>Aase–Smith I syndrome – ptosis, decreased eye movement</p> <p>Alkuraya syndrome – ophthalmoplegia</p> <p>Antley–Bixler syndrome – proptotic</p> <p>Bartsocas–Papavasiliou syndrome – ankyloblepharon</p> <p>Blepharophimosis, MR, Dandy–Walker – blepharophimosis</p> <p>Bohring–Opitz syndrome – prominent</p> <p>Bowen–Conradi syndrome – cloudy cornea, congenital glaucoma, large cornea</p> <p>BRESHECK – microphthalmia</p> <p>Camptodactyly, Guadalajara type I – microcornea, microphthalmia</p> <p>Camptodactyly, Kilic type – myopia, ptosis, medial fibrosis</p> <p>Carbohydrate-deficient glycoprotein syndrome – retinitis pigmentation</p> <p>Chondrodysplasia punctata, rhizomelic – cataract</p> <p>COFS syndrome – cataracts, microphthalmia, retinal pigmentation</p> <p>Conradi–Hünemann syndrome – cataracts, hypertelorism</p> <p>Contractural arachnodactyly – microphthalmia</p> <p>Distal arthrogryposis 2B – ophthalmoplegia, deep-set, ptosis</p> <p>Duane's retraction syndrome with contractures – inability to abduct the eye, narrow palpebral fissure</p> <p>Dundar–Sonoda syndrome – blepharophimosis, deep-set, short palpebral fissure</p> <p>Ectodermal dysplasia with cleft palate – blepharophimosis, develop hyperopia, amblyopia</p> <p>Ectodermal involvement with caudal appendage – blepharophimosis</p> <p>Fetal alcohol syndrome – short palpebral fissure</p> <p>Freeman–Sheldon syndrome – ptosis</p> <p>Fryns syndrome – cloudy cornea</p> <p>Fukuyama congenital muscular dystrophy – optic atrophy</p> <p>Jequier syndrome – hypertelorism, puffy eyelids, coloboma of eyelid</p> <p>King–Denborough syndrome – ptosis</p> <p>Kniest syndrome – myopia</p> <p>Larsen syndrome – hypertelorism</p> <p>Lerman–Sagie syndrome – deep-set eyes</p> <p>Lenz–Majewski – hypertelorism, lacrimal duct stenosis, prominent eyes</p> <p>Lin–Gettig – hypertelorism, small palpebral fissures (all)</p> <p>Mietens syndrome – corneal opacity, strabismus, nystagmus</p> <p>Marden–Walker syndrome – blepharophimosis, ptosis</p> <p>Marfan syndrome – dislocated lens, myopia</p> <p>Martolf syndrome – cataracts, microphthalmia</p> <p>Maternal hyperthermia – microphthalmia</p>	<p>Megalocornea and skeletal anomalies – megalocornea</p> <p>Möbius syndrome – lack of eye movement</p> <p>Moore–Weaver syndrome – antimongoloid slant, hypertelorism, short palpebral fissures</p> <p>Multiple pterygium syndrome–Escobar – antimongoloid slant, ptosis</p> <p>Myelinopathies – ophthalmoplegia</p> <p>Myopathies – ophthalmoplegia, ptosis</p> <p>Myotonic dystrophy congenital – cataracts, ptosis</p> <p>Neu–Laxova syndrome – open eyes, hypertelorism, exophthalmos</p> <p>Nevo syndrome – short palpebral fissures, deep-set, myopia, squint</p> <p>Oculodentodigital syndrome – sunken eyes, small, microphthalmia</p> <p>Ohdo blepharophimosis</p> <p>Ophthalmomandibulomelic dysplasia – corneal opacities</p> <p>Ophthalmoplegia, retinitis pigmentosa, mental retardation</p> <p>Osteogenesis imperfect – blue sclera</p> <p>Oto-onychoperroneal – persistent membrane, coloboma of retina</p> <p>PEHO syndrome – optic atrophy</p> <p>Phosphofructokinase deficiency – cloudy cornea</p> <p>Popliteal pterygium syndrome – ankyloblepharon</p> <p>Restrictive dermopathy – open eyes</p> <p>Ritscher–Schinzel – ocular coloboma</p> <p>Schwartz–Jampel syndrome – myopia, blepharophimosis</p> <p>Shalev-type distal arthrogryposis – ptosis</p> <p>Spastic paraplegia (Goldblatt syndrome) – optic atrophy</p> <p>Spondyloepiphyseal dysplasia – myopia</p> <p>van den Ende–Gupta syndrome – blepharophimosis</p> <p>Waardenburg–Klein syndrome – blepharophimosis, heterochromia iris, displaced inner canthus</p> <p>Walker–Warburg syndrome – retinal aplasia, microphthalmia, cataract</p> <p>Wieacker muscular atrophy – ocular dyspraxia</p> <p>Weill–Marchesani syndrome – dislocated lenses</p> <p>Winchester syndrome – corneal opacity</p> <p>X-linked arthrogryposis 2 – ptosis</p> <p>X-linked arthrogryposis 3 – esotropia</p> <p><b>Facial Movement Decreased</b></p> <p>Bartsocas–Papavasiliou syndrome – perioral webs</p> <p>Camptodactyly, Baraitser–London-type</p> <p>Distal arthrogryposis with facial involvement (Sheldon–Hall 2B)</p> <p>Distal arthrogryposis with ophthalmoplegia (Hall IIB, Bamshad 5)</p> <p>Freeman–Sheldon syndrome – pursed mouth, “11” on chin</p> <p>Hanhart syndrome – Möbius (occasional)</p> <p>King–Denborough syndrome – Noonan-like</p> <p>Marden–Walker syndrome</p> <p>Maternal misoprostol – Möbius</p> <p>Maternal myasthenia gravis</p> <p>Möbius syndrome – facial diplegia</p> <p>Multiple pterygium Escobar</p> <p>Multiple pterygium malignant hyperthermia</p> <p>Myelinopathies</p> <p>Myopathies</p> <p>Myotonic dystrophy congenita</p> <p>Schwartz–Jampel syndrome – pursed lips, small mouth</p> <p>Shalev-type distal arthrogryposis</p> <p>Stiff man/stiff baby – alert, tense face</p> <p>Zellweger syndrome</p> <p><b>Feeding Problems – Major</b></p> <p>(most CNS involved types of arthrogryposis have feeding problems)</p> <p>Crisponi syndrome</p>
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**TABLE 161-9 Specific Organ or Area of Involvement in Various Disorders with Arthrogryposis (Congenital Contractures)—Cont'd**

<p>Hanhart syndrome – swallowing difficult</p> <p>Möbius syndrome – swallowing difficulty</p> <p>Myelinopathies</p> <p>Myopathies</p> <p>Myotonic dystrophy congenital</p> <p>Rigid spine muscular dystrophy</p> <p>Stiff man/stiff baby syndrome</p> <p>Wieacker syndrome – dyspraxia of tongue muscles</p> <p><b>Forehead</b></p> <p>Acrocallosal syndrome – prominent</p> <p>Antley–Bixler syndrome – frontal bossing</p> <p>Camptodactyly, Tel-Hashomer – prominent</p> <p>Conradi–Hünemann syndrome – prominent</p> <p>Dundar–Sonoda syndrome – frontal bossing</p> <p>FG – high, broad, and cowlick</p> <p>Larsen syndrome – prominent</p> <p>Megalocornea and skeletal anomalies prominent</p> <p>Nevo syndrome</p> <p>Osteogenesis imperfecta – shallow orbits, wormian bones</p> <p>Otopalatodigital II – frontal bossing</p> <p>Ritscher–Schinzel syndrome</p> <p>Schinzel–Giedion syndrome – high, prominent</p> <p>Zellweger syndrome – high forehead</p> <p><b>G-U Anomaly</b></p> <p>Aarskog syndrome – shawl scrotum (very freq.), cryptorchidism (moderate), hypospadias (rare)</p> <p>Amyoplasia – cryptorchidism, hypospadias, hydronephrosis (rare)</p> <p>Antley–Bixler – female masculinized, small fused labia, clitorimegaly</p> <p>Bowen syndrome – prominent large clitoris</p> <p>BRESHECK – cryptorchidism</p> <p>Campomelic dysplasia – XY looks XX</p> <p>“C” syndrome variant – cryptorchidism</p> <p>Dundar–Sonoda syndrome – cryptorchidism</p> <p>Ectodermal dysplasia and clefts – hypospadias</p> <p>Genitopatellar syndrome</p> <p>Hand–foot–uterus – uterine anomalies</p> <p>Lenz–Majewski – hypospadias, cryptorchidism</p> <p>Lerman–Sagie syndrome – cryptorchidism</p> <p>Lin–Gettig – small penis, cryptorchidism, uretal reflux (all)</p> <p>LCCS2 – extended bladder</p> <p>Martolf syndrome – micropenis</p> <p>Multiple pterygium syndrome, Escobar type – cryptorchidism, webs</p> <p>Nail–patella syndrome – renal disease</p> <p>Popliteal pterygium syndrome, Gorlin type – cryptorchidism, webs, scrotal anomaly</p> <p>Prader–Willi-like</p> <p>X-linked arthrogryposis 2 – saddlebag scrotum, small penis</p> <p><b>Hair – See Skin</b></p> <p><b>Hand</b></p> <p>Bartsocas–Papas syndrome – mitten hand</p> <p>BRESHECK – polydactyly</p> <p>Smith–Lemli–Opitz – polydactyly</p> <p><b>Hydrops</b></p> <p>Carbohydrate-deficiency glycoprotein syndrome – hydrops</p> <p>Gaucher disease – perinatal lethal hydrops</p> <p>German syndrome</p> <p>Glycogen storage IV</p> <p>LAAMD</p> <p>Lissencephaly syndromes</p>	<p>Mitochondrial disorders</p> <p>Myelinopathies</p> <p>Myopathies</p> <p>Neu-Laxova syndrome – hydrops</p> <p>Phosphofructokinase deficiency</p> <p>Sotos-like syndrome – edema</p> <p><b>Hypotonia</b></p> <p>Adenylosuccinate lyase deficiency</p> <p>Anterior horn cell disease (Finnish)</p> <p>ARC</p> <p>Bowen syndrome – underdeveloped lung one side</p> <p>COFS</p> <p>Dandy–Walker, MR, basal ganglion disease</p> <p>Dundar–Sonoda syndrome</p> <p>Eagle–Barrett syndrome</p> <p>FG syndrome</p> <p>Fetal akinesia sequence</p> <p>Finnish anterior horn cell</p> <p>Fetal akinesia deformation sequence</p> <p>Fowler syndrome</p> <p>Fukutin abnormalities</p> <p>German syndrome</p> <p>Lerman–Sagie syndrome</p> <p>Lethal congenital contracture syndrome 1–3</p> <p>Lin–Gettig syndrome</p> <p>Marden–Walker syndrome</p> <p>Miller–Dieker syndrome</p> <p>Mitochondrial defects</p> <p>Myelinopathies</p> <p>Myopathies</p> <p>Myotonic dystrophy congenita</p> <p>PEHO syndrome</p> <p>Smith–Lemli–Opitz syndrome</p> <p>SMA deletions</p> <p>Ullrich myopathy</p> <p>Zellweger syndrome</p> <p><b>Jaw Anomaly – Micrognathia (see also Trismus)</b></p> <p>Aase–Smith I – micrognathia</p> <p>Bartsocas–Papas – perioral webs</p> <p>Cardiomelic syndrome</p> <p>Distal arthrogryposis 2E – trismus</p> <p>Blepharophimosis–arachnodactyly syndrome</p> <p>Distal arthrogryposis with facial involvement (Sheldon–Hall)</p> <p>Dundar–Sonoda – micrognathia, microstomia at rest</p> <p>Freeman–Sheldon – small mouth, contracted around</p> <p>Lin–Gettig – micrognathia</p> <p>Moore–Weaver syndrome – microstomia</p> <p>Ophthalmomandibulomelic syndrome – fusion of TMJ</p> <p>Schwartz–Jampel syndrome – small mouth, pursed lips</p> <p>Trismus–pseudocamptodactyly syndrome – trismus</p> <p>van den Ende–Gupta syndrome – microstomia</p> <p><b>Large</b></p> <p>Prader–Willi-like syndrome</p> <p>Sotos-like syndrome</p> <p>Weaver syndrome</p> <p><b>Laryngeal/Tracheal Anomalies</b></p> <p>Acrocallosal syndrome – laryngomalacia</p> <p>Diastrophic dysplasia – calcification trachea</p>
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**TABLE 161-9 Specific Organ or Area of Involvement in Various Disorders with Arthrogryposis (Congenital Contractures)—Cont'd**

<p>Larsen syndrome – laryngeal/trachea collapse  Neuromuscular disease of the larynx  Richieri–Costa/Pereira syndrome – tracheolaryngeal abnormalities</p> <p><b>Legs Only Affected</b>  Amyoplasia – legs only  Angulation of long bone syndrome  Autosomal dominant, Fleury  Autosomal recessive, Sarralde, Ray  Fuhrman syndrome  Genitopatellar syndrome  Kuskokwim syndrome  Meningomyelocele, spina bifida  Prenatal early diagnosis, CVS, amnio  X-linked arthrogryposis, Zori</p> <p><b>Limb Deficiency or Extra</b>  Acrocallosal syndrome – polydactyly  Amyoplasia – distal loss, transverse limb loss – rare  Attempted termination of pregnancy  CAD – nubbins of digits  Focal femoral dysplasia – proximal loss  Hanhart syndrome – transverse loss, fusions of digits  Holt–Oram syndrome – radial ray loss  Ives microcephaly – missing digits  Liebenburg syndrome – distal phalanges loss  Maternal misoprostol exposure – terminal digit loss  Mietens syndrome – short middle segment  Moebius – reduction anomalies  Multiple synostosis syndrome – distal loss  Oculodentodigital syndrome – hypoplasia distal digit  Oto-onychoponeal syndrome – fibular aplasia  PHAVER syndrome – radiology, missing thumb, digitalized broad thumb  Poland anomaly – distal loss  Popliteal pterygium syndrome, Gorlin type  Roberts syndrome  Smith–Lemli–Opitz syndrome – polydactyly  Symphalangism/brachydactyly syndrome – distal loss  VATER – radial ray defects</p> <p><b>Liver</b>  ARC – jaundice, biliary states  Carbohydrate-deficient glycoprotein syndromes – ascites fatty changes, cirrhosis  Gaucher disease, infantile type – hepatosplenomegaly, hydrops  Jequier syndrome – liver fibrosis, portal hypertension  Leprechaunism, focal changes  Nevo syndrome – bilirubinemia  Phosphofructokinase deficiency  Zellweger syndrome</p> <p><b>Malignant Hyperthermia</b>  Central core disease  King–Denborough syndrome  Maternal hyperthermia  Multiple pterygium and malignant hyperthermia  Myopathic types of arthrogryposis</p> <p><b>Maternal Affect</b>  Antley–Bixler syndrome – virilization</p> <p><b>Muscles</b>  CAD – hypoplastic, replaced with fat  Central core disease</p>	<p>Congenital fiber type disproportion  Continuous muscle discharge and titubation  Distal arthrogryposis 2B – firm muscles, ragged red fibers  Fukuyama congenital muscular dystrophy  Lerman–Sagie syndrome – disproportion, fibril and fiber abnormalities  Myhre muscle hypertrophy</p> <p><b>Myopathies</b>  Nemaline myopathy  Neuromuscular disease of larynx  Schwartz–Jampel syndrome – myotonia  Stiff man/stiff baby syndrome  Transient neonatal – firm muscle</p> <p><b>Myopathies</b>  Central core disease – hypotonia  Mitochondrial defects – ragged red fibers  Nemaline myopathy – hypotonia (occasional central cores)  Noonan-type arthrogryposis – night cramps  Schwartz–Jampel – myotonia  Ullrich congenital myopathy – weakness, hypotonia  Wieacker muscular dystrophy</p> <p><b>Neurologic</b>  Adenylosuccinate lyase deficiency – self mutilating  “C” syndrome variant – truncal hypotonia, limbs hypertonic  Carbohydrate-deficient glycoprotein syndrome – ataxia, hypotonia, dysmyelination  Contractures continuous – ataxia  Crisponi syndrome – autonomic dysfunction  COFS – progressive degeneration, hypotonia  Dandy–Walker, MR, basal ganglion disease – choreoathetosis  Dyggve–Melchior–Clausen – C1 compression  FG – hypotonic  Larsen syndrome – C1 compression  Lerman–Sagie – hypotonia  Lin–Gettig – hypotonic to hypertonic  Martsolf syndrome – diplegia  MASA – aphasia, shuffling gait, spastic paraplegia  Meningomyelocele – spinal lesion, paralysis  Myotonic dystrophy congenital – facial diplegia  Neurosensory syndrome – sensory defect with arthrogryposis  PEHO syndrome – hypersarhythmia, hypotonia, hyperreflexia, progression encephalopathy  Sacral agenesis – deficiency  Spastic paraplegia syndrome – ataxia, spastic  Wieacker muscular dystrophy – dyspraxia of the eye and tongue muscles</p> <p><b>Nose</b>  Aase–Smith I syndrome – small flat  Antley–Bixler – pear-shaped, short, depressed, short columella  Bixler microcephaly – large nose  Bowen syndrome – small short nose with flat bridge and short columella  “C” syndrome variant – broad nasal root  Camptodactyly Tel–Hashomer – high bridge  Distal arthrogryposis (724) – absent teeth, broad nose  Ectodermal involvement with caudal appendage – bulbous nose  Emery–Nelson – not protruding, flat mid-nose  Focal femoral dysplasia – hypoplastic alae nasae  Freeman–Sheldon syndrome – notched alae nasi</p>
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**TABLE 161-9 Specific Organ or Area of Involvement in Various Disorders with Arthrogryposis (Congenital Contractures)—Cont'd**

<p>Hoepffner syndrome – pinched nose</p> <p>Jequier syndrome – short, short columella</p> <p>Larsen syndrome – depressed nasal bridge</p> <p>Lenz–Majewski – short, choanal atresia, pinched nose</p> <p>Lerman–Sagie syndrome – high bridge</p> <p>Lin–Gettig – hypoplastic alae, short columella</p> <p>Megalocornea and skeletal abnormalities – saddle nose</p> <p>Multiple synostosis – long nose, broad bridge</p> <p>Nevo syndrome – high bridge</p> <p>Oculodentodigital syndrome – thin nose</p> <p>Oto-onychoponeal syndrome – prominent</p> <p>Schinz–Giedion syndrome – choanal atresia</p> <p>Trigonocephaly syndrome – broad base, hypertension</p> <p>van den Ende–Gupta syndrome – hypoplastic alae</p> <p><b>Ophthalmologic (see Eye)</b></p> <p>Aarskog syndrome – ophthalmoplegia retinal vessel</p> <p>Aase–Smith I syndrome – ophthalmoplegia</p> <p>Blepharophimosis–arachnodactyly</p> <p>COFS syndrome – cataract, optic nerve hypoplasia</p> <p>Distal arthrogryposis 2B – ophthalmoplegia</p> <p>Marden–Walker syndrome – ptosis, blepharophimosis</p> <p>Marfan syndrome – dislocated lens</p> <p>Möbius syndrome – lack of eye movement</p> <p>Oculo-auriculovertebral spectrum</p> <p>Oculodentodigital syndrome</p> <p>Ophthalmomandibulomelic dysplasia</p> <p>Ophthalmoplegia – retinitis</p> <p>Weill–Marchesani syndrome – dislocated lens</p> <p><b>Osteoporosis – Severe</b></p> <p>Bruck syndrome</p> <p>Osteogenesis imperfecta</p> <p><b>Other Organs</b></p> <p>Caudal deficiency – asplenia</p> <p>Ectodermal involvement with caudal appendage</p> <p>Stiff man/stiff baby syndrome – diaphragmatic hernia</p> <p>VATER – esophageal atresia, anal atresia, TE fistula</p> <p><b>Peroxysms</b></p> <p>Crisponi syndrome – sweating, hyperthermia</p> <p>PEHO syndrome</p> <p>Rigid spine syndrome</p> <p>Stiff man/stiff baby syndrome</p> <p><b>Patella Abnormality</b></p> <p>Campomelic dysplasia</p> <p>Coffin–Siris syndrome</p> <p>Ear–patella–short stature syndrome</p> <p>Genitopatellar syndrome</p> <p>Guadalajara type II camptodactyly</p> <p>Nail–patella syndrome</p> <p>Patella aplasia hypoplasia syndrome</p> <p><b>Pelvis</b></p> <p>Maternal diabetes – sacral agenesis</p> <p>Meningomyelocele – pelvis dysplasia</p> <p>Metatrophic dysplasia – pelvis hypoplasia</p> <p>Nail–patella – pelvic horn</p> <p>Parastremmatic – Lacey borders</p> <p>Sacral agenesis – no sacrum</p> <p>X-linked – pelvis hypoplasia</p> <p><b>Pterygium (See Webbing)</b></p> <p>Acrorenal-mandibulo-uterine syndrome</p>	<p>Oto-onychoponeal syndrome</p> <p>PHAVER syndrome – elbow shoulder</p> <p><b>Pulmonary Change (see also Chest)</b></p> <p>Anterior horn cell disease (Finnish) – pulmonary hypoplasia</p> <p>Bowen syndrome – lung underdeveloped one side</p> <p>CAD</p> <p>Distal arthrogryposis, ophthalmoplegia – progressive, restrictive, lung disease</p> <p>Eagle–Barrett syndrome – pulmonary hypoplasia</p> <p>Fetal akinesia sequence – pulmonary hypoplasia</p> <p>Fowler syndrome – pulmonary hypoplasia</p> <p>Fryn syndrome – hypoplasia, diaphragmatic, abnormality</p> <p>LCCS I, II, III – all have pulmonary hypoplasia</p> <p>Marfan syndrome congenital severe – pulmonary emphysema</p> <p>Multiple pterygium, Escobar – progressive pulmonary restrictive</p> <p>Multiple pterygium lethal – pulmonary hypoplasia</p> <p>Myasthenia gravis congenital – pulmonary hypoplasia</p> <p>Myopathies – progressive failure</p> <p>Nemaline myopathy – respiratory distress early</p> <p>Pena–Shokeir syndrome – pulmonary hypoplasia</p> <p>Potters syndrome – pulmonary hypoplasia</p> <p>Smith–Lemli–Opitz – lung segmentation</p> <p>Spondylospinal thoracic dystrophy – crablike chest</p> <p><b>Renal</b></p> <p>Al-Awadi syndrome – renal agenesis</p> <p>Antley–Bixler – 40% renal agenesis, dysplasia, duplicated collecting, abnormal ureter</p> <p>ARC – renal dysfunction, nephrocalcinosis</p> <p>BRESHECK – hypoplasia, cystic kidney, small bladder</p> <p>Carbohydrate-glycoprotein deficient syndrome – renal cysts</p> <p>Dundar–Sonoda syndrome – renal anomalies, hydronephrosis, horseshoe kidney</p> <p>Eagle–Barrett syndrome – renal anomaly, prune belly</p> <p>Fowler syndrome – glomerular vasculopathy</p> <p>Freeman–Sheldon syndrome</p> <p>Genitopatellar – hydronephrosis</p> <p>Ives microcephaly micromelia syndrome – renal dysplasia</p> <p>Leprechaunism – calcifications in kidneys, renal hyperplasia</p> <p>LCCS II – extended bladder</p> <p>Lin–Gettig – ureteral reflux severe</p> <p>Nail–patella syndrome – renal disease</p> <p>Marden–Walker syndrome – renal cysts</p> <p>Miller–Dieker syndrome – renal agenesis</p> <p>Potter syndrome – agenesis</p> <p>Ritscher–Schinzel syndrome</p> <p>Rudiger syndrome – hydronephrosis, urethral stenosis</p> <p>Schinzel–Giedion syndrome – hydronephrosis</p> <p>Smith–Lemli–Opitz syndrome – renal agenesis</p> <p>Tuberous sclerosis – tumors</p> <p>Zellweger syndrome – renal cysts</p> <p><b>Scoliosis (Significant)</b></p> <p>Antley–Bixler syndrome – kyphosis</p> <p>Bruck syndrome – kyphosis</p> <p>Contractural arachnodactyly – kyphosis</p> <p>Distal AMC with scoliosis (Hall 2D Bamshad 4)</p> <p>Camptodactyly London</p> <p>Camptodactyly Tel-Hashomer</p> <p>COFS – kyphosis</p>
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**TABLE 161-9 Specific Organ or Area of Involvement in Various Disorders with Arthrogryposis (Congenital Contractures)—Cont'd**

<p>Diastrophic dysplasia Ectodermal dysplasia with cardiomyopathy – kyphosis King–Denborough syndrome Larsen syndrome – vertebral defects Marden–Walker – kyphoscoliosis Maternal myasthenia gravis Megalocornea and skeletal anomalies – gibbus Metatrophic dysplasia Multiple pterygium syndrome Escobar – lumbar lordosis Multiple pterygium syndrome malignant hyperthermia – scoliosis crouching stance Myelinopathies Myopathies Nevo syndrome – cervical hypolordosis Parastremmatic dysplasia Ritscher–Schinzel syndrome Sotos-like – kyphosis Stiff man/stiff baby syndrome X-linked arthrogryposis I X-linked arthrogryposis 5 <b>Seizures</b> Aase–Smith I syndrome – rare Basal ganglion disease Bixler microcephaly BRESHECK “C” syndrome variant – frequent CAD – 1/3 Dandy–Walker, MR, basal ganglion disease FG syndrome King–Denborough syndrome Maternal hyperthermia Miller–Dieker syndrome Mitochondrial defects PEHO syndrome – hypsarrhythmia Phosphofructokinase deficiency Saul–Wilson skeletal dysplasia Tuberous sclerosis X-linked arthrogryposis, type 1 (anterior horn cell loss) <b>Shin Dimples</b> Angulation of long bones Autosomal recessive arthrogryposis, inherited type Campomelic dysplasia X-linked arthrogryposis, type 3 <b>Skin, Hair, and Nails</b> Aarskog syndrome – scalp defects rare Absence of dermal ridges – milia, dry skin, dystrophic nails Amyoplasia – stork mark, hemangiomas (5%) Bartsocas–Papas syndrome – abnormal hypoplastic skin around the mouth and anus BRESHECK – alopecia, hyperkeratosis, with normal sweat glands “C” syndrome variant – redundant skin, hirsute, midline facial hemangioma Camptodactyly, London – ichthyosis Carbohydrate-deficient glycoprotein syndrome – abnormal fat distribution Conradi–Hünemann syndrome – alopecia, folliculitis, atrophoderma, patchy skin Deafness and camptodactyly (DA6) – dry skin</p>	<p>Ectodermal dysplasia and cardiomyopathy – woolly, sparse hair; dry skin; brittle nails Ectodermal dysplasia and contractures – hyperkeratosis, pili torti, dry skin Ectodermal dysplasia and clefting – sparse hair, nail hypoplasia Ectodermal involvement with caudal appendage – slow-growing hair Ehlers–Danlos VIII – wrinkling skin, atrophic scar Ehlers–Danlos VIB2 – hyperextensible, bruisable, ichthyosis Gaucher, infantile – ichthyosis Kuskokwim syndrome – pigmented nevi Johnson hyperkeratosis and decreased posterior column Lenz–Majewski syndrome – thin skin with prominent vessels, wrinkles Leprechaunism – loose, dry skin; hirsutism Nail–patella syndrome – nail dysplasia Neurofibromatosis – café au lait, neurofibromas Neu–Laxova syndrome – constrictive, ichthyotic skin Nevo syndrome – edema, soft skin, fragile Oto–onychoponeal syndrome – absent or dysplastic nails Popliteal pterygium syndrome, Gorlin type – absent nails and lip webs Potter syndrome – redundant, wrinkled, increased creases Proteus syndrome – lipomas, hamartomas, hemangiomas, mottling Puritic–Murray syndrome – fibromatosis, skin lesions, skin infections Restrictive dermopathy – tight, thin Schinzel–Giedion syndrome – hypertrichosis, abundant skin Tuberous sclerosis – adenoma, depigmentation, shagreen patches Ullrich congenital myopathy – rough skin Waardenburg–Klein syndrome – white patches, early graying, pigmentary anomalies Winchester syndrome – malar flush, thick facial skin <b>Syndactyly</b> Aarskog syndrome – mild in hands and feet, very frequent Absence of dermal ridges Amyoplasia Bartsocas–Papas syndrome – mitten hand Hanhart syndrome Neu–Laxova syndrome Pfeiffer syndrome Poland syndrome Smith–Lemli–Opitz syndrome <b>Teeth (see Dental)</b> <b>Thumbs – Proximal or Abnormal</b> Adducted thumbs syndrome Clasped thumb and mental retardation Clasped thumb syndrome Deafness and camptodactyly Diastrophic dysplasia – “hitchhiker” thumb Dundar–Sonoda syndrome – adducted thumbs Fryns syndrome – digitalization of thumb Hand–foot–uterus syndrome – small and small big toes Holt–Oram syndrome – small Jequier – broad thumbs Martsolf syndrome MASA syndrome Nemaline myopathy – short first metacarpal</p>
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Continued

**TABLE 161-9 Specific Organ or Area of Involvement in Various Disorders with Arthrogryposis (Congenital Contractures)—Cont'd**

Oral–cranial–digital syndrome (Juberg–Hayward syndrome) – hypoplastic, distal thumb

Pfeiffer cardiocranial synostosis – broad distal phalanges

PHAVER syndrome – missing, digitalized

X-linked arthrogryposis, type 2

#### **Tongue**

Hanhart syndrome – small or absent

Möbius syndrome – hypoplastic

Wieacker syndrome – muscular atrophy, dyspraxia

#### **Trismus**

Aase–Smith I syndrome – frequent

Amyoplasia – rarely

Carney variant

Crisponi syndrome

Distal arthrogryposis IIE

Distal arthrogryposis – Sheldon–Hall

Freeman–Sheldon syndrome

Trismus–pseudocamptodactyly

#### **Tumors**

Aase–Smith – neuroblastoma

Hunter–MacDonald camptodactyly – meningiomas

Proteus – hamartomas, lipomas, hemangiomas

Puretic–Murray – multiple fibromas, other tumors matoses

Neurofibromatosis – neurofibromas

Sacral agenesis – presacral tumor

Schinz–Giedion – neuroectodermal sacrococcygeal tumor

Tuberous sclerosis – glioma angioma, phacoma

#### **Umbilical**

Aarskog–Scott syndrome – defect

Lin–Gettig – pigmentation, hernia

Shalev-type DA – long

#### **Uterine Anomaly**

Acrorenalmandibular syndrome

Hand–foot–uterus – duplications, structural anomalies

#### **Vascular Defects**

Amyoplasia – GI, distal digits

Attempted termination of pregnancy

Fowler syndrome – proliferative vasculopathy

Hanhart syndrome

Liebenburg syndrome – distal digit loss

Maternal misoprostol

#### **Vertebral Anomaly**

Aarskog syndrome – particularly cervical (freq.)

Antley–Bixler syndrome – fused, probably

“C” syndrome variant – segmentation (occ.)

CAD – agenesis coccyx and sacrum, flattening

Diastrophic dysplasia – cervical instability

Dyssegmental dysplasia – variable size, segmental dysplasia

Focal femoral dysplasia – vertebral anomalies

Larsen syndrome – cervical instability, vertebral defects

Metatrophic dysplasia – cervical instability, progressive change

Mitochondrial defects

Multiple pterygium syndrome, Escobar type – segmentation defects

PHAVER syndrome – butterfly, hemi-, webbing

Ritscher–Schinzel syndrome

Sacral agenesis

Spondylothoracic dysostosis – crablike segmentation defect

VATER association

X-linked arthrogryposis, Zori

#### **Webbing, Includes Pterygium (see also Syndactyly)**

Absence of fingerprints – mild finger webbing

Acrorenal–mandibulo–uterine syndrome

Amyoplasia – mild

Antecubital pterygium (Shin Shun)

Bartsocas–Papas – popliteal

Bruck syndrome – progressive

Distal arthrogryposis with facial involvement (Sheldon–Hall) – mild

King–Denborough syndrome

Kuskokwim syndrome

Moore–Weaver syndrome – neck webbing

Multiple pterygium syndrome, Escobar type – progressive

Multiple pterygium syndrome lethal – severe

Shalev-type of DA

Multiple pterygium syndrome with malignant hyperthermia – neck webbing

Nail–patella – occasional elbow webbing

Oto–onychoperroneal syndrome

PHAVER syndrome – elbow, shoulder

Popliteal pterygium syndrome, Gorlin type – knee, along popliteal edge with neurovascular bundle, intraoral web

Spondylothoracic dystrophy

AD = autosomal dominant; AF = anterior fontanelle; AR = autosomal recessive; ARC = arthrogryposis, renal dysfunction, cholestasis syndrome; ASD = atrial septal defect; BA = bowel atresia; CHD = congenital heart defect; CNS = central nervous system; DA = distal arthrogryposis; GI = gastrointestinal; IA = intestinal atresia; LCCS = lethal congenital contracture syndrome; PS = pulmonary stenosis; TE = tracheoesophageal; TEF = tracheoesophageal fistula; TMJ = temporomandibular joint; VSD = ventricular septal defect.



**TABLE 161-10 Disorders not Included for Lack of Space, Time, and/or Adequate Data**

<p><b>Primarily Limbs</b> (Table 161-2)</p> <p>Impaired pronation and supination</p> <p>Neurosensory contractures, Cyprus</p> <p><b>Limbs Plus Other Body Areas</b> (Table 161-3)</p> <p>Aarskog syndrome</p> <p>Abruzzo syndrome</p> <p>Alves syndrome (924)</p> <p>Distal arthrogryposis with plantar flexion creases</p> <p>Ectodermal dysplasia and cardiomyopathy</p> <p>Freeman–Sheldon-like syndrome (318)</p> <p>Guadalajara III camptodactyly</p> <p>Hunter–MacDonald camptodactyly</p> <p>Megalocornea and skeletal anomalies</p> <p>Middleton syndrome (974)</p> <p>Oculo-auriculovertebral spectrum (911)</p> <p>Ohno blepharophimosis syndrome</p> <p>Oto-onycho-peroneal syndrome</p> <p>Patella aplasia–hypoplasia syndrome</p> <p>Pointer camptodactyly</p> <p>Rozin and Kilic camptodactyly</p> <p>Shin crease/Dimple (799a and 799b)</p> <p>TARP syndrome</p> <p><b>Limbs Plus Lethal or Neurologic Dysfunction</b> (Table 161-4)</p> <p>Abruzzo syndrome</p> <p>Acrorenal-mandibulo-uterine syndrome</p> <p>Agamanolis syndrome (921)</p> <p>Al-Gazali arthrogryposis with coloboma and renal anomalies</p> <p>BRESHECK</p> <p>Bixler microcephaly</p> <p>Blenthem syndrome (913)</p> <p>BMR syndrome</p> <p>Braddock syndrome</p> <p>"C" syndrome variant (412b)</p> <p>CAD</p> <p>CASK syndrome</p> <p>Carey–Fineman–Ziter syndrome</p> <p>Cardiac deficiency and asplenia</p> <p>Cardiomelic syndrome</p> <p>Catel–Manzke syndrome</p> <p>Cerebro-oculogential-syndrome</p> <p>Cerebellar hypoplasia with contractures (984,985)</p> <p>Chitayat distal arthrogryposis syndrome</p> <p>Christian syndrome</p> <p>Christianson syndrome</p> <p>Cohen syndrome (936)</p> <p>Costello syndrome</p> <p>Cutis laxa and contractures (914)</p> <p>Digito-renal-cerebellar syndrome</p> <p>Ear, patellar, craniosynostosis syndrome (910)</p> <p>Emery Nelson camptodactyly</p> <p>Facio-cardio-melic syndrome</p> <p>Fine–Lubinsky syndrome</p> <p>Fitch syndrome (943)</p> <p>Fuhrmann syndrome</p> <p>Galloway–Mowat syndrome</p> <p>Guion Almeida camptodactyly</p>	<p>Guadalajara I camptodactyly</p> <p>Guadalajara II camptodactyly</p> <p>Haspelagh syndrome (915)</p> <p>Gustavson syndrome</p> <p>Heyn syndrome</p> <p>Homfrey syndrome</p> <p>Illum syndrome</p> <p>Jequier syndrome</p> <p>Juberg Marsidi Brooks syndrome</p> <p>Johnson hyperkeratosis and decreased posterior columns (959)</p> <p>Juberg Howard syndrome (577a)</p> <p>Keipert syndrome</p> <p>Laurin–Sandrow syndrome</p> <p>Lenz microphthalmia syndrome (916)</p> <p>Lerman–Sagie syndrome (969)</p> <p>Lin–Gettig syndrome (agenesis of corpus callosum, MR)</p> <p>KBG syndrome</p> <p>Miles–Carpenter syndrome</p> <p>NAA10 syndrome</p> <p>Nievergelt–Pearlman syndrome</p> <p>Neurosensory syndrome</p> <p>Oculo-auriculovertebral spectrum (911)</p> <p>Ophthalmomandibulo-melic syndrome (912, 990)</p> <p>Orocraniodigital syndrome</p> <p>Pagon–Gallop camptodactyly</p> <p>Palant syndrome</p> <p>Pena–Shokeir phenotype familial subtypes (90)</p> <p>Perisylvian polymicrogyri</p> <p>Perlman syndrome</p> <p>Pettigrew syndrome</p> <p>PHAVER syndrome</p> <p>Potter syndrome</p> <p>Proud syndrome (645)</p> <p>Raine syndrome</p> <p>Richieri–Costa I camptodactyly (macrocephaly, hypoplastic digits, absent ribs)</p> <p>Richieri–Costa II camptodactyly (prominent lower lip, cleft lip, ptosis, mottled pelvis bones) (549b)</p> <p>Richieri–Costa/Pereira syndrome (cleft mandible, hand anomalies, laryngeal anomalies, hypertelorism, short middle segment)</p> <p>Rudiger syndrome</p> <p>Schimke syndrome</p> <p>Shkalin microcephaly, clubfeet, agenesis of corpus callosum syndrome (304b)</p> <p>Simpson–Golabi–Behmel syndrome</p> <p>Sprintzen–Goldberg syndrome</p> <p>Teebi–Shaltout syndrome</p> <p>Tiemann syndrome</p> <p>Tricho-oculo-dermo-vertebral syndrome (742a)</p> <p>Urban–Rogers–Meyer syndrome (816)</p> <p>van Benthem syndrome (917,918)</p> <p>van Biervliet syndrome (53a)</p> <p>Walker–Warburg syndrome (675,676,678,725,1010)</p> <p>Weyer acro-dental dysostosis</p> <p>Winter pachygyria syndrome</p> <p>Weidemann–Rautenstrauch syndrome</p>
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TABLE 161-11 Distal Arthrogryposes: Differential Diagnosis with Additional Types of Distal Arthrogryposis (Not Including Camptodactyly Syndromes – See Table 161-13)				
Hall Classification	Bamshad Classification			Genes
I	1.	Distal (AD, simplex), some families mainly involve hands (includes digitotarsal dysmorphism)	1A	<i>TPM2, MYBPC1, TNN12, MYH3</i>
IIA	2.	Gordon's syndrome (AD, short stature +/- CP)	3	
IIB	3.	AD with ophthalmoplegia – may not be congenital; one family included retinal pigment changes and MR; may have Dandy-Walker in family	5	
IIC	4.	Clefting, AD, trismus, may be Gordon syndrome		
IID	5.	Scoliosis (may include Goodman syndrome)	4	
IIE	6.	Trismus and unusual hand ~ amyoplasia spectrum		
	7.	Freeman-Sheldon syndrome	2A	<i>MYH3</i>
	8.	Sheldon-Hall (includes Moore-Weaver syndrome)	2B	<i>TNNT3, TNN12, MYH3, TPM2</i>
	9.	Sheldon-Hall look-alike	2C	
	10.	Deafness, camptodactyly syndrome	6	<i>FGFR3</i>
	11.	Trismus pseudocamptodactyly (Beal's syndrome, Kentucky Dutch syndrome)	7	<i>MYH8</i>
	12.	AD, multiple pterygium	8	
	13.	Contractural arachnodactyly	9	Fibrillin 2
	14.	Distal with plantar flexion contractures	10	
	15.	Dundar-Sonoda syndrome (MR, unusual face)		
	16.	Absent teeth and abnormal facies (may be Sheldon-Hall)		
	17.	Chitayat syndrome (AR, MR, hypopituitary)		
	18.	X-linked distal arthrogryposis		
	19.	Shalev syndrome (AR, mainly upper limbs, ptosis)		

AD = autosomal dominant; AR = autosomal recessive; CP = cleft palate; MR = mental retardation.

**TABLE 161-12 Multiple Pterygium Syndromes**

1. Antecubital Pterygium Syndrome (Shin Shun) AD
2. Popliteal Pterygium Syndrome (IRF6) AD
3. Pterygium Autosomal Dominant (also DA 8)
4. Bartsocas-Papas Syndrome (RIPK4) AR
5. Lethal Multiple Pterygium Syndrome (Gillin-Pryce-Davies Type) (Embryonic neuroreceptor unit mutations ) AR
6. Myasthenia Gravis Congenital - AR
7. Antibodies to embryonic neuroreceptor subunits - maternal
8. Multiple Pterygium Syndrome Escobar (CHRNA7, 2q37, etc.) AR
9. Multiple Pterygium Syndrome with Malignant Hyperthermia AR
10. Pterygium with Ectodermal Dysplasia AR
11. Skin Webbing with Cutis Laxa AR
12. X-linked variety of Pterygium Syndrome
13. Shalev type involving mainly upper limbs with ptosis AR
14. Other syndromes with pterygia
  - a. Chromosomal disorders
  - b. Escobar with creased tongue AR
  - c. Fetal akinesia sequence disorders
  - d. Haspelagh syndrome
  - e. Kuskokwim syndrome (FKBP10) AR
  - f. Median report chin webs
  - g. Mietens syndrome
  - h. Myopathies
  - i. Pfeiffer syndrome (FGFR2) AD
  - j. Sacral agenesis
  - k. Severe Amyoplasia
  - l. Williams

AD, autosomal dominant; AR, autosomal recessive; DA, distal arthrogryposis

Hall, J.G. Revisiting limb pterygium syndromes (Paper submitted for Festschrift in honour of Professor Christos Bartsocas). For the sake of science. 2012, ZHTA Medical Publications, Athens, Greece, Honorary volume for Christos S. Bartsocas, 373-385.

**TABLE 161-13** Types of Camptodactyly Which are Variable Within Families and Often Involve Lower Limbs

<b>Autosomal Dominant Syndromes</b>
Welch and Temtamy (classic, isolated)
Baraitser, Lizcarno (mainly uppers, scoliosis, torticollis, cervical vertebral fusion)
Christian (platyspondylia, vertebral fusions, carpal and tarsal fusions)
Deafness and camptodactyly (tall stature)
Emery Nelson (flat face, abnormal nose)
Guadalajara III (spinal defects, hypertelorism, brain structural anomaly)
Hunter MacDonald (scoliosis, deafness, congenital heart defects, and meningioma)
<b>Autosomal Recessive Syndromes</b>
ARC (arthropathy, pericarditis)
Guion arthropathy, Almeida (MR, skin tag, CP, and arachnodactyly)
Guadalajara I (facies unusual, thoracoskeletal abnormalities, microcephaly)
Guadalajara II (short neck, hypoplastic patellae, DD, microcephaly)
Ichthyosis and Windmill–Vane (Baraitser) (ichthyosis, seizures – possibly two types)
Lin–Gettig syndrome (agenesis corpus callosum, craniosynostosis, MR)
Pagon Gallop (MR, obesity, osteoporosis, large epiphyses)
Pointer syndrome (extended second digit)
Richeiri–Costa I (lethal, hypoplastic digits)
Richeiri–Costa II (MR, long face, cleft lip)
Rozin and Kilic (scoliosis, lateral rectus fibrosis)
Tel–Hashomer (short stature, normal IQ, prominent forehead, hypertelorism)
van den Ende–Gupta Syndrome (arachnodactyly, blepharophimosis, “surprised” facies)
<b>X-linked Syndromes</b>
Aarskog syndrome

ARC = arthrogryposis, renal dysfunction, cholestasis syndrome; CP = cleft palate; DD = developmental delay; IQ = intelligence quotient; MR = mental retardation.

**TABLE 161-14** Fetal Akinesia Sequence

(1)	Classic Pena–Shokeir syndrome
(2)	Lower motor neuron disorder with generalized decrease in anterior horn cells (Chen type)
(3)	Lethal congenital contractures syndrome type 1
(4)	Lethal congenital contractures syndrome type 2
(5)	Lethal congenital contractures syndrome type 3
(6)	Lethal lower motor neuron deficiency with degeneration
(7)	Families with apparent increase in monozygotic twinning
(8)	Normal <i>in utero</i> growth, macrocephaly and Pena–Shokeir phenotype (Lammer type)
(9)	Absence of pyramidal cells, immature CNS development, adducted thumbs, kyphoscoliosis, and severe pulmonary hypoplasia (Biscegli type)
(10)	CNS dysgenesis and degeneration, seizures, trismus, endocrine hyperplasia, and abdominal wall herniation (Erdl type)
(11)	Skeletal muscle maturation defect
(12)	Pyramidal tract degeneration
(13)	<i>In utero</i> seizures, scoliosis, together with cerebral and cerebellar hypoplasia in males (Persutte type)
(14)	Microphthalmia, microtia, and normal birth size (Thomas type)
(15)	Olivo–ponto–cerebellar hypoplasia
(16)	Failure to myelinate peripheral nerves – many genes
(17)	Holoprosencephaly with hypokinesia and congenital contractures in an X-linked recessive pattern of inheritance
(18)	Hydranencephaly, calcification of basal ganglion and proliferative vasculopathy (Fowler type)
(19)	Calcification of leptomeninges, the surface of cerebral convolutions, neurons, muscles, and vessels (Illum type)
(20)	Familial intrauterine anoxia and/or ischemia.

CNS = central nervous system.

(Hall, J. G., Pena–Shokeir Phenotype (Fetal Akinesia Deformation Sequence Revisited). *Birth Defects Res. A Clin. Mol. Teratol.* **2009**, 85, 677–694).



**TABLE 161-15 Arthrogryposis Syndromes Which Usually or Often Present with Only Upper or Lower Limb Involvement****Upper Limbs**

Agenesis of corpus callosum, severe MR, camptodactyly (Lin–Gettig Syndrome)  
 Amyoplasia – upper limb only  
 Antecubital pterygium syndrome (Shin Shun)  
 Autosomal dominant pterygium  
 Baraitser–London camptodactyly  
 Guadalajara camptodactyly III  
 Hunter–MacDonald syndrome  
 Liedenbug syndrome  
 Rozin and Kilic camptodactyly (ptosis, ophthalmoplegia)  
 Shalev-type arthrogryposis (ptosis, umbilical hernia)  
 Urban–Rogers–Meyer syndrome  
 X-linked resolving arthrogryposis

**Lower Limbs**

Amyoplasia lower limbs only  
 Angulation of long bone syndrome  
 Fuhrmann syndrome  
 Genitopatellar syndrome  
 Kuskokwim syndrome  
 Lower limb AD (Fleury type)  
 Lower limb AR (Ray/Sarralde)  
 Lower limb X-linked, caudal dysplasia  
 Meningomyelocele/spina bifida/spinal dysraphism  
 Prenatal early amniocentesis or CVS

AD = autosomal dominant; AR = autosomal recessive; CVS = chorionic villus sampling; MR = mental retardation.

**TABLE 161-16 Metabolic Disorders Presenting with Arthrogryposis**

Name	Gene
Adenylosuccinate lyase deficiency – AR (microcephaly, hypotonia, self-mutilation)	<i>ADSL</i>
ARC (arthrogryposis, renal dysfunction, cholestasis syndrome; Nezeloff syndrome) – AR (cholestasis, renal (Fanconi), hypotonia)	<i>VPS33B, VIPAR</i>
Carbohydrate-deficient glycoprotein syndrome – AR (hydrops, unusual fat, liver anomalies)	<i>PMM2, PM11</i>
Gaucher disease – perinatal lethal – AR (hydrops, hepatosplenomegaly, ichthyosis)	<i>GBA</i>
Glycogen storage IV – AR (hydrops, fetal akinesia, muscle deposits)	<i>GLE1</i>
Juvenile hyaline fibromatosis (Puretic–Murray syndrome) – AR (gingival hypertrophy, fibromas, infections, pain)	<i>CMG2, ANTXR2</i>
Phosphofructokinase deficiency (glycogen storage VII) – AR (seizures, corneal cloudy, hepatosplenomegaly)	<i>PFKM</i>
Zellweger syndrome – AR (FIT, hypotonia, prominent forehead)	Many <i>PEX</i> genes

AR = autosomal recessive; also mitochondrial disorders, myopathies, myelinopathies, malignant hyperthermia disorders, skeletal dysplasias, and other storage disorders.

**TABLE 161-17 X-linked Syndromes with Arthrogryposis**

Definite			
Entity	MIM#	Location	Gene
XAMC-I; Severe spinal muscular atrophy (SMA2)	301830	Xp11.23	<i>UBE1</i> [now <i>UBA1</i> ]
Aarskog syndrome (Aarskog–Scott syndrome, Faciodigitogenital syndrome)	305400	Xp11.22	<i>FGD1</i> ( <i>Rho/Rac</i> )
CASK syndrome	300749	Xp11.4	<i>CASK</i>
Conradi–Hunermann syndrome	302960	Xp11.23	<i>EBP</i>
Dundar–Sonoda syndrome (X-linked)	601776	15q15.1	<i>CHST14</i>
Christianson syndrome	300243	Xq26.3	<i>SLC9A6</i>
FG syndromes			
FG1 (Opitz–Kaveggia syndrome)	305450	Xq13.1	<i>MED12</i>
FG2 (part of the FLNA spectrum)	300321	Xp28	<i>FLNA</i>
VACTERL with or without hydrocephaly	314390	Xq26.3	<i>Z1C3</i>
Juerg–Marsidi–Brooks syndrome	300612	Xp11.22	<i>HUWE1</i>
X-linked lissencephaly	300067	Xq23	<i>DCX</i>
MASA or X-linked Aqueductal Stenosis	307000	Xq28	<i>L1CAM</i>
MEHMO	300148	Xp21	<i>EIF2S3</i>
Myopathies			
Central nuclear myopathy	310400	Xq28	<i>MTM1</i>
Myotubular-related Protein 1	300171	Xq28	<i>MTMR1</i>
Reducing body myopathy	300717	Xq26.3	<i>FHL1</i>
NAA10 syndrome	300013	Xq28	<i>NAA10</i>
Otopalatodigital syndrome II	304120	Xq28	<i>FLNA</i>
Proud syndrome	300004	Xp21.3	<i>ARX</i>

Continued

**TABLE 161-17 X-linked Syndromes with Arthrogryposis —Cont'd**

Definite			
Entity	MIM#	Location	Gene
Simpson-Golabi-Behmel			
Simpson-Golabi-Behmel Type 1	312870	Xq26.2	<i>GPC3</i>
Simpson-Golabi-Behmel Type 2	300209	Xp22.2	<i>OFD1</i>
TARP syndrome	311900	Xp11.23	<i>RBM10</i>
Probable			
Entity	MIM#	Location	
Abruzzo and Erickson 1977	302905	X-linked	
Adductor laryngeal paralysis and mental retardation in an X-linked pattern	308850	X-linked	
Aicardi syndrome	304050	Xp22	
XAMC-II; Arthrogryposis type II	301830		
XAMC-III; Arthrogryposis type III	301830		
XAMC-IV; Arthrogryposis type IV	300158	Xq23-q27	
Belpharophimosis mental retardation (BMR) – (OHDO)	249620	X-linked	
Braddock et al. 1993	123155	X-linked	
Catel-Manzke syndrome	302380	X-linked	
Caudal deficiency and asplenia	208530	Excess of males	
Facio-cardio-melic dysplasia	227270		
Fiber-type disproportion	300580	Xq13.1-q22.1	
Greenblatt et al. 1989			
Heyen et al. 2008			
Homfrey et al. 1995			
Johnson et al. 1993			
Keipert et al. 1973	255980		
Ladda type 1993	301815		
Miles and Carpenter 1991	309605	Xq13-q22	
X-linked Pena-Shokeir phenotype (fetal akinesia sequence)	306990	X-linked	
Pena-Shokeir phenotype (fetal akinesia sequence), Lammer 1989	300073		
Perisylvian polymicrogyri	300388	Xq27.2-q28	
Pettigrew et al. 1991	304340	Xq25-q27	
Podder et al. 1995			
X-linked Multiple pterygium of the lethal type	312150	X-linked	
Multiple pterygium syndrome X-linked dominant			
Tiemann et al. 2005	610001		
Van Bethem et al. 1970			
Weiacker-Wolff syndrome	314580	Xq13-q21	

**TABLE 161-18 Skeletal Dysplasias that Usually Have Arthrogryposis (Multiple Congenital Contractures)**

Antley-Bixler syndrome ( <i>POR</i> )
Atelosteogenesis II ( <i>DTDST</i> )
Atelosteogenesis III ( <i>FLNB</i> )
Boomerang syndrome (Atelosteogenesis I) ( <i>FLNB</i> )
Campomelic dysplasia ( <i>SOX9</i> )
Chondrodysplasia punctata – Conradi-Hunermann type ( <i>EBP</i> )
Chondrodysplasia with joint laxity ( <i>IMPAD1</i> )
CHST-3 disorders
CINA (NOMID syndrome) ( <i>CIAS1</i> )
Debuquois syndrome ( <i>CANT1</i> )
Diastrophic dysplasia and other DTDST disorders
Dygge-Melchior-Claussen dysplasia ( <i>DYM</i> )
Dyssegmental dysplasia (Rolland-Desbuquois type) ( <i>HSPG2</i> )
FGFR2/FGFR1 disorders (Pfeiffer syndrome)
FGFR3 disorders (Achondroplasia, Hypochondroplasia, Thanatophoric dysplasia)
Frank-Ter Haar syndrome ( <i>SH3PXD2B</i> )
Gelophysic dysplasia ( <i>ADAMTSLA</i> )

**TABLE 161-18 Skeletal Dysplasias that Usually Have Arthrogryposis (Multiple Congenital Contractures)—Cont'd**

Grebe dysplasia ( <i>CDMP1</i> )
Jansen metaphyseal dysplasia ( <i>PTHR1</i> )
Kneist dysplasia ( <i>COL2A1</i> )
Larsen syndrome ( <i>FLNB</i> )
Lenz-Majewski hyperostotic dysplasia
MED (AR) (COMP related)
Melnick-Needles osteodysplasty ( <i>FLNA</i> )
Mesomelic dysplasia Kantaputra type ( <i>HOXD</i> )
Metatrophic dysplasia ( <i>TRPV4</i> )
OPD I ( <i>FLNA</i> )
OPD II ( <i>FLNA</i> )
Pseudodiastrophic dysplasia
Rhizomelic chondrodysplasia punctata ( <i>PEX7</i> )
Schwartz-Jampel syndrome ( <i>HSPG2</i> )
Spondyloperipheral dysplasia ( <i>COL2A1</i> )
Trichorhinophalangeal II (deletion 8q24.11-q24.13)

Spranger, J. W.; Brill, P. W.; Nishimura, G.; Superti-Furga, A.; Unger, S. *Bone Dysplasias: An Atlas of Genetic Disorders of Skeletal Development*, 3rd ed.; Oxford University Press: New York, NY, 2012.

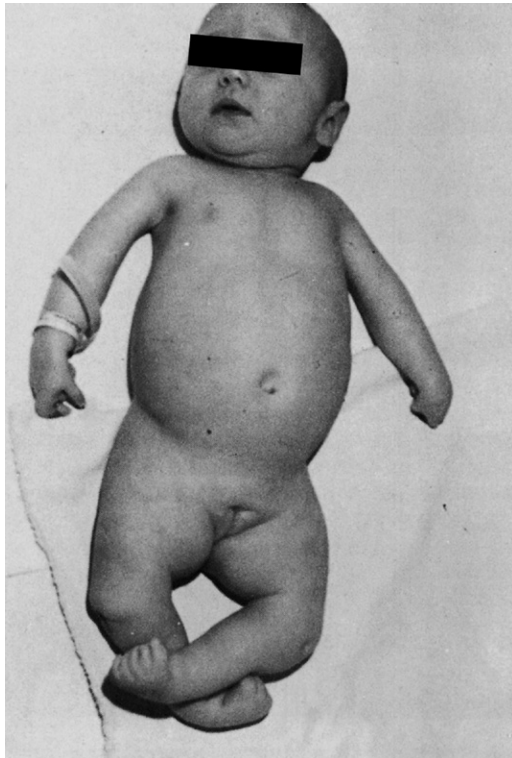
that are associated with congenital contractures. Chromosome studies or better yet CGH array should be done in any child with arthrogryposis and mental disability. There is obvious overlap between these categories because some individuals with a condition may only have involvement of the limbs while others in the same family or with the same condition may have more widespread involvement. Similarly, in some conditions with congenital joint contractures, a characteristic feature is mental disability, but there may be affected individuals who do not have mental disability. Nevertheless, we have found this approach to be the most useful for the clinician. It is impossible to describe in detail all the conditions with congenital contractures, but it seems appropriate to emphasize several of the frequently observed conditions under the three categories. As gene pathways are being identified, conditions may be classified as being related to muscle proteins, muscle metabolism, and homeostasis proteins, proteins involved in development and function of lower motor neurons, proteins involved in skeletal and cartilage systems and differentiation, proteins involved in nervous system growth, regulation and differentiation, proteins involved in peroxisome organization, excision repair proteins, proteins involved in cellular and developmental functions, proteins involved in metabolic pathways and “others” (84). Alternatively, if the structural area of abnormality can be identified, multiple congenital contractures can be classified as cerebral, spinal, neuromuscular connection, myopathy, connective tissue, or limitation of movement. Classification is now possible for over half of cases of arthrogryposis. Understanding etiology enhances the chance for prevention and therapy. However, for now, a clinical approach is necessary.

## 161.4 FREQUENTLY OBSERVED CONDITIONS

### 161.4.1 Primarily Limb Involvement

**161.4.1.1 Amyoplasia.** Amyoplasia is the most common condition with severe multiple congenital contractures and is referred to as “classical arthrogryposis” by most orthopedic specialists. One-third of all patients in our large study of congenital contractures had amyoplasia (31,368,370,752). Many forms of arthrogryposis have decreased muscle mass, hypoplastic muscle, or loss of muscle; however, when the term Amyoplasia is used in this chapter, it is meant to refer only to this specific condition. Care should be exercised when using this diagnostic term since it implies a specific natural history, recurrence risk, and etiology.

Amyoplasia is characterized by very specific positioning, usually with symmetrical limb involvement, and usually involves all four limbs; however, occasionally only the arms or only the legs are involved. Affected individuals have fibrous bands and fatty tissue where muscles would normally be, suggesting that the muscle or muscle anlage was formed embryologically but failed to develop in a normal way. These patients usually have firmly fixed joints (Figure 161-1) with a fusiform or cylindrical shape to the limbs in the newborn period, suggesting that the limitation of movement has been present for many months. Usually flexion creases are shallow or not present, suggesting lack of movement occurs earlier than 14 weeks of embryologic development. The feet are almost always in equinovarus position, the wrists are almost always flexed, the shoulders are internally rotated and sloping, and the elbows are extended and fixed at 180 degrees at birth, but may develop some flexion with



**FIGURE 161-1** Amyoplasia in a newborn. Note alert expression, characteristic internally rotated shoulders with decreased muscle mass, extended elbows, wrist and hand contractures, equinovarus deformities of the feet, dimples and flexion contractures at the knees. The face is round, the nose short and upturned; there is a midline facial hemangioma.

growth and physical therapy. Rarely, the loss of functional muscle is so severe that some elbow flexion occurs *in utero* (the bones grow in length, but the fibrous muscles do not, leading to elbow flexion), but these individuals are always severe with marked muscle hypoplasia. There may be slight webbing of skin across the hips, knees (if flexed), and axillae. The hips may be flexed or extended in abduction or adduction and are often dislocated. The knees may be fixed in extension or in flexion. Sensation is intact. Deep dimples are most often present over joints. The affected limbs appear mildly shortened.

Early physical therapy is extremely important to loosen contractures that are present at birth and thereby give whatever muscle is present a chance to strengthen, rather than atrophy. The best results of mobilizing contractures in Amyoplasia seems to be in the first 3 to 4 months. However, of course, care must be taken not to fracture bones that are osteoporotic. Surgery is important to align the limbs in positions of function (8,38,52,188,1004). Magnetic resonance or ultrasound (112) imaging prior to surgery may provide an indication of the muscle mass present. In many cases, an unusual angle of the joints or flattening of the joint surface needs to be taken into consideration when doing physical therapy.

About 90% of individuals affected with Amyoplasia have no other system involvement; however, a variety of

other anomalies can be seen in Amyoplasia. Ten percent of patients have abdominal wall defects (gastroschisis, lateral wall hernias) or bowel atresia (66,187). There is a very high frequency of “stork mark”-type birthmarks over the midface. The face is usually somewhat round and flat with mild micrognathia. Mild trismus may be present.

About 5% of the cases have had amniotic bands or digit reduction anomalies on one or more limbs. A few children have a decrease in size of the distal digits; many have mild syndactyly or webbing of the digits. Often at birth, but definitely with time, there is undergrowth of an affected limb, much like the disuse atrophy seen in polio. Intelligence is usually within normal limits, unless there was trauma and/or anoxia at birth. In fact, families often feel that affected children are brighter than average. Rare cases of growth hormone deficiency have been seen.

There appears to be an increased incidence of Amyoplasia in one of monozygotic twins (with one normal twin and one affected twin). Often the affected twin has only arms or only legs involved (186).

It appears that all cases of Amyoplasia have been sporadic, and many affected individuals have reproduced, having unaffected children. Since Amyoplasia occurs in approximately 1 in 10,000 live births, recurrence could occur by chance alone. Prenatal diagnosis in subsequent pregnancies can be offered for reassurance using serial real-time ultrasound to detect normal limb movement. Most probably, the condition is secondary to some type of vascular compromise during the first trimester. It is likely that the timing is related to the degree of involvement.

**161.4.1.2 Distal Arthrogryposes.** Many types of arthrogryposis have distal involvement (113,114) (Table 161-11). Distal arthrogryposis type 1 is characterized by a very specific positioning of hands in the newborn period and primarily distal contractures of the limbs (114,115) (Figure 161-2). Other conditions have distal contractures, but this particular type has autosomal dominant inheritance and is characterized by being quite responsive to physical therapy. The hand positioning in the newborn is similar to that seen in trisomy 18, with a clenched fist and overlapping fingers (Figure 161-3). With physical therapy, the hand usually opens up, but there is often some residual ulnar drift of the fingers. Foot positioning is variable. Both equinovarus and calcaneovalgus feet have been seen within the same family, as well as in a single individual. Both the hand and foot abnormalities appear to have misplaced tendons; there are several cases in which this has been documented at surgery. Occasionally, affected family members have contractures of the hips, knees, and elbows. There can be marked variability in the involvement within a family.

Some individuals with primarily distal involvement of the limbs have been reported as cases of familial campodactyly, (Table 161-13) contractural arachnodactyly,





**FIGURE 161-2** Distal arthrogryposis type I in a newborn. Note pre-dominant distal contractures, with overlapping finger contractures, ulnar deviation, and clubfeet.



**FIGURE 161-3** Distal arthrogryposis type I newborn hand. Note clenched fist and overlapping fingers similar to hand position in trisomy 18.

or Freeman–Sheldon syndrome because of the similarity of distal limb involvement and positioning. Several sporadic cases have been associated with advanced paternal age, suggesting new dominant mutations. Family studies are very helpful because observing the variability within a family allows proper classification (113,116–123a). Molecular testing is available for many of these families related to sarcolemmal proteins.

Bamshad et al. (116) have suggested a division of distal arthrogryposes into several categories because specific gene mutations involving components of fast twitch muscle have been identified in families (Table 161-11). Some families tend to have facial

muscle involvement with limitation of facial movement and/or ptosis (Bamshad type 2B) while other families lack facial involvement (Hall type I (114), Bamshad type 1 (99)).

Most affected individuals with distal arthrogryposis type 1 have no other nonorthopedic anomalies; however, several subcategories of distal arthrogryposis may have additional physical abnormalities. It is not clear whether these are totally distinct categories or whether several subtypes can be seen within the same family (121). We list them under Tables 161-2 and 161-3. One of the subtypes is often referred to as Gordon syndrome (122) (Hall type IIA, Bamshad type 3) and has the additional features of cleft palate and short stature in some affected family members (~30–50%). The degree of short stature and severity of clefting in an affected individual seems to be proportionate to the severity of involvement of the joints.

Another category of distal arthrogryposis (Hall type IIB, Bamshad type 5) (24,113,114) has very firm muscles that may show ragged red fibers on biopsy. Ophthalmoplegia develops in this condition during childhood. Eyes are deep set and the faces are somewhat elfin. Autosomal dominant inheritance has been seen in several families with male-to-male transmission (114,324,325,328,329,995). Dimples and lack of flexion creases on the fingers are also seen. It does not appear to be progressive or to have degeneration; however, pulmonary involvement may be progressive.

Patients with another type of distal arthrogryposis primarily involving distal limbs (Hall type IIC) (113,114,121,123a) also have cleft lip. Severe scoliosis is seen in some families with distal contractures (Hall type IID, Bamshad type 4) (113,114,124).

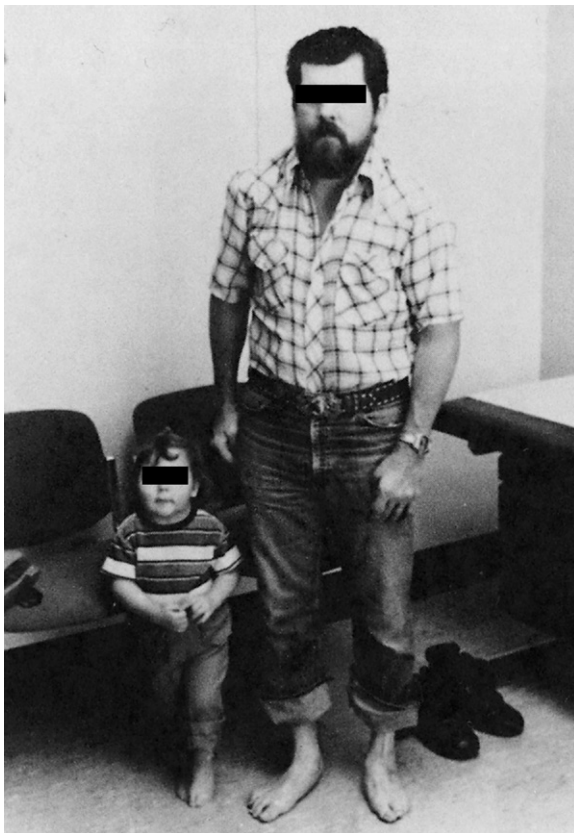
Still another subcategory of distal arthrogryposis (Hall type IIE), is important to identify because it has limitation of jaw movement (trismus) with or without micrognathia in association with more generalized congenital contractures (114). The hand contractures are in an unusual position with flexion of the wrist, hyperextension of the metacarpophalangeal joint, and flexion of the other finger joints. Approximately one-third of these cases have mild mental retardation; all of these cases appear to be sporadic. The trismus can be severe, leading to feeding problems and possibly respiratory compromise. There is a higher than expected incidence of other joint anomalies within the family, and it is not entirely clear whether the condition is sporadic. It has been designated distal arthrogryposis with trismus (Hall type IIE) for want of a better term (114).

All forms of distal arthrogryposis seem relatively responsive to physical therapy and surgical procedures. Some individuals and families are so mildly affected that one is not able to recognize any residual deformity, and only by talking to the grandparents is it found that there were mild contractures at birth.

### 161.4.2 Limb Involvement with Other System Abnormalities

The second major category of conditions with congenital contractures is that in which the contractures of the limbs are in association with anomalies or malformations of other parts of the body (see Table 161-3). Some of the distal arthrogryposes fit in this category because of other system involvement.

**161.4.2.1 Freeman–Sheldon Syndrome.** Perhaps the best known of these is Freeman–Sheldon syndrome, or whistling face syndrome (also known as craniocarpotarsal dystrophy), in which there are congenital contractures, primarily of the hands and feet with overlapping fingers and ulnar deviation, in association with pursed mouth and limitation of facial movement secondary to fibrosis of facial muscles (Figure 161-4). There may be a characteristic H-shaped connective tissue band on the chin. Affected individuals tend to have long faces with markedly decreased facial movement. Individuals with Freeman–Sheldon syndrome may also have scoliosis, midface hypoplasia, lateral coloboma of the alae nasi, ptosis, and an antimongoloid slant of the eyes. Freeman–Sheldon syndrome may be more resistant to therapy than other forms of distal arthrogryposis (125) even though



**FIGURE 161-4** Freeman–Sheldon or whistling face syndrome in father and daughter. Note hand and foot contractures; more severe in the father, who has a very small left foot. The eyes are deeply set with an antimongoloid slant, the mouth is small, and there is a mild groove in the chin. Both have short stature and short necks.

the responsible genes overlap. It is clear that there is interfamilial and intrafamilial variability, but that autosomal dominant inheritance is the most common mode of inheritance. The gene for the autosomal dominant type has been identified to be MYH3, the embryonic form of the myosin heavy chain (106).

**161.4.2.2 Contractural Arachnodactyly and Congenital Marfan Syndrome.** Contractural arachnodactyly is a well-defined condition with autosomal dominant inheritance characterized by congenital contractures, long thin extremities, crumpling of the top of the helix of the ear, and kyphoscoliosis (126a). Various chest deformities are seen as well, with pectus excavatum or carinatum. Marked inter- and intra-familial variability is seen. Less than 10% of cases appear to be severely involved with progressive scoliosis. The other 90% of cases seem to improve with age. There has been a suggestion of overlap with congenital severe Marfan syndrome, in that some individuals with contractural arachnodactyly may have structural abnormalities of the heart with mitral valve prolapse and/or aortic aneurysms (128) (see Chapter 153). This is not surprising since both conditions involve fibrillin genes: fibrillin I (15q21.1) in Marfan syndrome and fibrillin II (5q23–31) in contractural arachnodactyly (129). Severely affected sporadic newborns have been reported with advanced bone age and additional gastrointestinal or vertebral anomalies (130).

**161.4.2.3 Pterygium Syndromes.** Many newborns and children with arthrogryposis have webs (or pterygia) across the joints that have limited movement. However, several specific conditions with marked webbing (131,132) are seen as specific entities with consistent features in different families (Table 161-12). Many of the responsible genes have been identified.

**Multiple pterygium syndrome (Escobar type)** is characterized by flexion contractures at birth. At birth, webs may not be present or prominent, but with aging, webs develop at the neck, elbows, knees, and intercrural areas. Cleft palate, deafness, scoliosis, and short stature are frequently present, as well as segmentation anomalies of the vertebrae. Many cases are sporadic, but most appear to have autosomal recessive inheritance (132–134) and involve the embryonic neurotransmitter receptor subunits (135–141a). A few families have been reported with marked variability and possible dominant inheritance (142–144). This may be a progressive condition as about 20% of individuals seem to get worse as they enter puberty, with decreasing pulmonary capacity and increasing thoracic lordosis (132,134). Myopathic changes have been reported in a fairly typical case (145). The possibility of therapy now exists utilizing therapeutic doses of acetylcholine-like drugs and have worked in animal models.

**Lethal multiple pterygium syndrome (Gillin–Pryse–Davis type)** is characterized by intrauterine growth retardation, severe flexion contractures, and webs *in utero*, frequently with cystic hygromas and hydrops. Lungs are



severely hypoplastic, so that survival usually does not occur. Polyhydramnios, ocular hypertelorism, cryptorchidism, cardiac hypoplasia, and ambiguous genitalia in males are frequently seen (146). Subtypes related to bony fusions and the time during gestation when severity becomes obvious may exist (131). Apparently, most cases are autosomal recessive and can be diagnosed prenatally (147,148). However, there may be an X-linked recessive form (149). The genes involved in embryonic neurotransmitter receptor subunits are also involved in some cases, suggesting *in utero* therapy may be possible (138,150a,151).

**Popliteal pterygium syndrome (Gorlin type)** is characterized by dense webs in the popliteal area (often with the nerve and blood vessel in the edge of the web), cleft lip and palate, syndactyly, nail anomalies, reduction deformities of digits, and other contractures. It is an autosomal dominant condition with marked variability in expression (152,153). Recently, the IRF6 gene on chromosome 1q32–q41 has been found to be responsible, so prenatal diagnosis should be possible (154).

**Bartsocas–Papas syndrome (lethal popliteal web syndrome)** is characterized by severe popliteal webs, cleft lip and palate with facial clefts, hypoplasia of the nasal tip and genital area, syndactyly with missing distal digits, sparse hair, and hypoplastic teeth. It is almost always lethal, but if the newborn period is survived, intelligence appears normal. Autosomal recessive inheritance seems likely, and consanguinity, particularly with Mediterranean ancestry, has been observed (155–157a,158a). Mutations have been found in the RIPK4 gene (159).

**Multiple pterygium with malignant hyperthermia** has been observed in several families. Scoliosis, torticollis, myopathic facies, and cleft palate have been present (35,160,161). Creatinine phosphokinase (CPK) levels are not necessarily elevated, and muscle biopsy is compatible with disuse atrophy rather than a myopathy. The relationship to King syndrome is not clear because King syndrome has a Noonan phenotype with malignant hyperthermia and elevated CPKs, but congenital contractures can be seen (162).

**161.4.2.4 Chondrodysplasias.** A variety of chondrodysplasias (including campomelic dysplasia, diastrophic dysplasia, Dyggve–Melchior–Clausen dysplasia, dyssegmental dysplasia, Kniest dysplasia, Larsen syndrome, metatropic dysplasia, and osteogenesis imperfecta) also fall into this second category of congenital contractures associated with other system anomalies (163) (see Chapter 157).

### 161.4.3 Congenital Contractures with CNS Anomalies or Dysfunction and/or Lethality

The third category of conditions with congenital contractures is that in which there are congenital contractures of the limbs associated with CNS malformations

or dysfunction, and/or lethality (Figure 161-5; see Table 161-4). Many relatively rare autosomal recessive lethal conditions as well as many chromosomal anomalies fall into this category.

**161.4.3.1 Pena–Shokeir Phenotype.** Pena and Shokeir described the combination of short fixed limbs and pulmonary hypoplasia. Subsequently, many other families have been reported (16,90,164), with the additional features of intrauterine growth retardation, polyhydramnios, short umbilical cord, and unusual craniofacies being noted. This combination of features seems to be due to fetal akinesia (12), suggesting the fetus must move *in utero* to have normal development of a variety of structures. Many familial subtypes can be distinguished on the basis of CNS pathology and clinical features (see Table 161-14), suggesting that the features represent a phenotype rather than a specific diagnosis. Prenatal diagnosis is possible in these families (25,84,165,839).

**161.4.3.2 Cerebro–Oculo–Facial–Skeletal Syndrome.** Pena and Shokeir also described cerebro-oculo-facio-skeletal syndrome (COFS) (166). This has led to a great deal of confusion because abnormal eye involvement is common in lethal conditions with contractures. COFS is characterized by structural abnormalities of the brain, dysmyelination, and eye anomalies, including microphthalmia and cataracts (167). In the original family, the excision repair gene XPD has been found to be involved



**FIGURE 161-5** Congenital contractures associated with central nervous system dysfunction. Note all limbs have flexion contractures; there is microcephaly with an abnormally shaped head and an unusual facies. The infant has micro-ophthalmia, abnormally shaped ears, a thin upper lip, long philtrum, micrognathia, mild webbing, and a large inguinal hernia.

(168,169) and other excision repair gene mutations may give a similar phenotype (particularly CSA). The natural history of this degenerative condition is variable even within a family. In some families, some affected individuals are born with contractures and others are spared at birth but develop contractures later in life. Renal anomalies, other visceral anomalies, and variations in bone density can be seen. An X-linked recessive form may exist.

**161.4.3.3 Neu-Laxova Syndrome.** Neu-Laxova syndrome is a striking phenotype with dramatic contractures, intrauterine growth retardation, microcephaly, open eyes, tight ichthyotic skin, and severe CNS anomalies (170). Kyphosis, syndactyly, and hydrops are often seen. It is a lethal autosomal recessive condition and can be diagnosed prenatally (171).

**161.4.3.4 Restrictive Dermopathy.** Several recent reports describe a syndrome with contractures in which it appears that fetal skin fails to grow normally and thereby restricts movement, leading to secondary contractures. All families are compatible with autosomal recessive inheritance (172–174) and several genes have been found (175–177).

**161.4.3.5 Chromosome Anomalies.** Many chromosome anomalies can have congenital contractures (70) (Table 161-5). With the advent of micro CGH arrays, many new microdeletion and microduplication syndromes are being identified, many of which have associated multiple congenital contractures. Some encompass genes already identified to be responsible for particular known types of arthrogryposis. Others effect CNS development to the detriment of *in utero* function, leading to fetal akinesia and/or hypotonia sufficient to develop secondary joint contractures. Every autosome has been implicated to be associated with arthrogryposis. There are some chromosomal disorders which seem to always have severe congenital contractures, such as trisomy 8 mosaicism and trisomy 18.

Any child with mental disability and arthrogryposis should have CGH array studies and consideration given to doing CGH on fibroblasts looking for mosaicism (which seems to be quite common in children with mental disability and arthrogryposis) (24). It should be noted that all children with arthrogryposis will have motor developmental delay due to the contractures, but if social and intellectual delay is present, then the disorders listed in Tables 161-4 and 161-5 should be considered.

**Trisomy 18** is probably the most frequent condition in this category, characterized by intrauterine growth retardation, visceral anomalies with extremely high incidence of heart disease, radial limb anomalies, short sternum, small pelvis, facial paralysis, and a typical positioning of fingers with overlapping fingers, clenched fists, and rocker-bottom feet.

**Trisomy 8 mosaicism** also frequently presents with congenital contractures and is often characterized by

absence of the patella and deep furrows in the palms or soles. Individuals with trisomy 8 mosaicism may only be identified with fibroblast karyotyping.

**Deletion 5q.** The genes for spinal muscular atrophy (SMA; Werdnig Hoffman disease) have been identified. Classical SMA has hypotonia without contractures at birth. However, deletions of 5q that involve the SMA gene may produce *in utero* hypotonia with contractures at birth. These children tend to do poorly (178).

## 161.8 SUMMARY

Multiple congenital contractures are relatively frequent and often part of recognizable syndromes. Marked heterogeneity does exist, as seen in Tables 161-2–161-7. Careful investigation should lead to a specific diagnosis in more than half the cases, allowing more specific prognostication, counseling, and therapy.

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## RELEVANT WEBSITES

Arthrogryposis: A Text Atlas. [http://www.globalhelp.org/publications/books/book\\_arthrogryposis.html](http://www.globalhelp.org/publications/books/book_arthrogryposis.html).  
Avenues. A National Support Group for Arthrogryposis Multiplex Congenital. <http://www.avenuesforamc.com/>.  
Arthrogryposis Multiple Congenital Support, Inc. <http://www.amcsupport.org/>.

## Biography



**Dr Judith Hall**, Professor Emerita in the Departments of Pediatrics and Medical Genetics at the UBC and Children's and Women's Health Centre of BC, BC's Children's Hospital, Vancouver, is a pediatrician and clinical geneticist who has worked on birth defects and non-traditional mechanisms of disease. She has conducted clinical research for the past 35 years with a major interest in the description of natural history and identification of syndromes. Dr Hall has contributed to the understanding of the tissue-specific and time-specific nature of gene expression as well as the changes of gene expression during embryonic, fetal, and childhood development. She has served in many leadership roles, including Chair of the UBC and BC Children's Hospital Departments of Pediatrics, presidency of the American Society of Human Genetics, the Western Society of Pediatrics, and the American Pediatrics Society, as well as on numerous national and international committees and boards. She became an Officer of the Order of Canada in 1998, a Founding Fellow of the Canadian Academy of Health Sciences in 2005, and a Fellow of the Royal Society of Canada in 2011.

## Common Skeletal Deformities

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Oregon Health and Science University, Portland, OR, USA**162.1 INTRODUCTION**

Familial aggregation of common skeletal deformities is well known. Often this is because the deformity is a component of a simply inherited syndrome, such as the scoliosis that usually occurs in diastrophic dysplasia or the clubfoot that often accompanies spondyloepiphyseal dysplasia congenita. However, even after these syndromes are excluded and only the idiopathic variety of the deformities is considered, familial aggregation still exists. Much interest has been generated in attempting to define the role of genetic and nongenetic factors in these instances. Twin studies, family surveys, and analysis of individual pedigrees have been used, and many potential environmental causes have been investigated. These various approaches have indicated that genetic factors play an important role in the etiology of certain of the deformities, and in several instances, specific modes of inheritance have been identified. Genetic heterogeneity has frequently been uncovered. In certain cases, environmental factors have been incriminated and often their relationship to genetic predisposition defined. For some of them, however, the contribution of genetic factors remains undefined, and in virtually all cases, the precise mechanism by which these factors predispose to the deformities is poorly understood. The goal of this chapter is to examine the current knowledge concerning the inheritance of these common skeletal deformities.

**162.2 IDIOPATHIC SCOLIOSIS**

Scoliosis, curvature of the spine, results from a multitude of causes. Examples of specific causes are poliomyelitis with trunk paralysis, myelomeningocele, neurofibromatosis, and radiation therapy to one side of the trunk (1). Scoliosis is also frequently associated with congenital anomalies, such as hemivertebrae or abnormally segmented vertebral bodies, and with certain of the skeletal dysplasias in which the spine is involved, such as the spondyloepiphyseal dysplasias and spondylometaphyseal dysplasias (2–4). In the vast majority of cases, however, the etiology is obscure, and the term *idiopathic scoliosis* is used.

The incidence of idiopathic scoliosis in the population varies considerably, ranging from 0.2 to 2–8% (1,5). It usually appears and shows the greatest progression during periods of rapid growth, infancy and adolescence. The process may begin during the juvenile period as well but worsens during adolescence. In so-called infantile scoliosis, the curve usually appears during the first year of life, is typically to the left side, often resolves without treatment, and occurs most frequently in boys. It is common in Great Britain, accounting for almost half of all cases of idiopathic scoliosis, but is uncommon in North America. Idiopathic scoliosis of the adolescent variety usually appears during the pubertal growth spurt, is to the right side, and occurs predominantly in girls. Curves developing after the age of 6 years show the characteristics of adolescent scoliosis (6).

Familial aggregation of idiopathic scoliosis has long been recognized. Many forms of inheritance have been postulated, including autosomal dominant, X-linked dominant, and multifactorial inheritance (5–11). Environmental factors have also been proposed to explain this phenomenon (12).

There have been many reports of twins with at least one member of the pair having scoliosis. Fisher and DeGeorge (1967) questioned the validity of those reported before 1967 because of insufficient radiographic studies (13). A total of 24 pairs of monozygous twins and 20 pairs of dizygous twins have been reported since then (6,13–15). The scoliosis detected in one or both twins was almost always the adolescent type. Over 90% of the monozygous twins were concordant for scoliosis, in contrast to 55% concordance for dizygous twins. All of the monozygous twin group were girls, whereas the dizygous twin group contained over one-third boys. Of the dizygotic, unlike-sex group, the girl was most often affected. Thus, the substantially higher rate of concordance for monozygotic compared to dizygotic twins illustrates the importance of genetic factors in the etiology of idiopathic scoliosis. However, the marked preference for girls indicates that sex is a strong determinant as well.

Several surveys have examined the incidence of scoliosis in family members (Table 162-1). They are difficult to compare because different methods and criteria



TABLE 162-1 Scoliosis Surveys in Families

Author	DeGeorge and Fisher	Wynne-Davies	Filho and Thompson	Cowell et al.	Riseborough and Wynne-Davies	Bonaiti et al.	Czeizel et al.	Connor et al.
Year	1967	1968	1971	1972	1973	1976	1978	1987
Location	New York	Edinburgh	Toronto	Wilmington	Boston	Paris	Budapest	Glasgow
No. probands	446	114	201	110	208	241	116	36
Method	Questionnaire	Examination	Radiograph	Radiograph	Radiograph	Interview	Photofluorography	Examination
Criteria		"Rib hump"		10° curve	20° curve		10° curve	
Predominant type surveyed	Adolescent and infantile	Adolescent	Adolescent	Adolescent	Adolescent	Adolescent	Adolescent	Infantile
Incidence (%)								
Parent	19	3	6	35	11	7	3.4	
(Male/female)	(6/13)			(29/42)	(3/18)	(5/9)	(3/4)	
Siblings	9	5	7	36	12	8	8	0
(Male/female)					(4/17)	(5/12)	(7/10)	
All relatives								
1st-degree			6.9	6.8	11.1	7.4	5.8	
2nd-degree			3.7	1.6	2.4	1.9	1.4	
3rd-degree			1.6	1.0	1.4	0.9	0.8	

for diagnosis were used. For example, the diagnosis was made by a questionnaire in the survey of DeGeorge and Fisher (1967), whereas radiography was used by Cowell et al. (1972) and photofluorography by Czeizel et al. (1978) (8,10,12). Moreover, although most of the surveys focused on adolescent scoliosis, some included substantial numbers of only children with infantile scoliosis (6,16). Despite these differences, the surveys showed similar trends. For adolescent scoliosis, the parents and siblings of patients with scoliosis were affected much more frequently than the general population, the incidence ranging from 3 to 35% for parents and from 5 to 36% for siblings. The percentage of affected relatives was much lower in infantile scoliosis: no affected siblings in the study of Connor et al. (1987) and 2.6% of first-degree relatives of infantile scoliosis patients versus 12% in adolescent scoliosis in the survey of Wynne-Davies (1968) (6,16). More recently, Purkiss et al. observed idiopathic scoliosis in 17.3% of offspring of patients with congenital scoliosis (17). When the incidence of scoliosis among second- and third-degree relatives was examined, the surveys showed a dramatic drop-off. Furthermore, girls were consistently affected more often than boys in all categories of relatives, and the frequency of affected relatives was the same for male and female probands (5,10). A recent analysis for adolescent scoliosis among twins from the Danish Twin Registry confirmed the higher concordance for monozygotic twins but suggested that the risk for scoliosis when the other twin has scoliosis is smaller than previously believed (18).

Several other observations were made in these surveys. For instance, plagiocephaly (molding of the head) was common in patients with infantile scoliosis, particularly those with resolving scoliosis (16). It was on the same side as the curve and was transient in almost all cases (6). Mental retardation was found in one-third of patients with progressive infantile scoliosis, where additional malformations were observed in infants with congenital scoliosis (5,6,16). However, except for the high incidence of scoliosis already mentioned, the frequency of the additional anomalies in relatives was the same as in the general population. A substantial elevation of maternal age was found for adolescent scoliosis in three of the surveys (5,6,12), but it was noted to be normal by Filho and Thompson (1971) (7). Finally, Wynne-Davies (1968) observed several instances in which typical infantile and adolescent scoliosis occurred in the same family (6).

The surveys confirm that genetic factors are important in the etiology of idiopathic scoliosis, primarily of the adolescent variety (onset beyond 6 years). Moreover, the incidence figures in first-degree relatives and rapid drop-off in second- and third-degree relatives indicate multifactorial inheritance. According to this model, the highest frequency of scoliosis should be found in the relatives of boys, the least affected sex (19). However, the incidence of affected relatives was found to be

approximately the same for affected boys and girls. The model also predicts that the frequency of affected relatives is related to the severity of the condition in the proband. This was not assessed in most of the surveys because of the many variables that influence the degree of scoliosis, such as age and treatment, although Czeizel et al. (1978) showed a trend in this direction (10). In a few patients, they also observed that the scoliotic curve was slightly greater in offspring of affected fathers than those of affected mothers and greater yet when both parents were affected.

There have also been many families reported in which members in several generations exhibited adolescent scoliosis (8,20–23). Because no instance of male-to-male transmission was seen in 17 families studied in depth, Cowell et al. (1972) proposed X-linked dominant inheritance (8). Riseborough and Wynne-Davies (1973), however, challenged this interpretation because father-to-son transmission had been observed in the families described by the other authors, and it had also been noted in one of their cases (5). Thus, families exhibiting autosomal dominant inheritance of scoliosis do exist.

In summary, genetic factors appear to play a definite role in the causation of idiopathic scoliosis. The observation that infantile and adolescent scoliosis appear within the same families suggests that common genetic factors are involved in both, although they seem to be much more important in the latter. Genetic heterogeneity exists with regard to adolescent scoliosis. In some families, it appears to be transmitted as an autosomal dominant trait. In most, however, it seems to be inherited in a multifactorial manner. The female sex is a strong determinant in converting the genetic predisposition into clinical disease.

The mechanism through which genetic factors operate is unknown. An association between HLA-A19 and scoliosis was reported by Bradford et al. (24). Several investigators have observed abnormalities in collagen and proteoglycan (25–30). In most of the studies, the biochemical alterations were most marked at the region of greatest spinal curvature, making it difficult to determine if they were primary or secondary in nature. Moreover, Carr et al. (1992) showed discordant segregation of adolescent idiopathic scoliosis and the two type I collagen genes (*COL1A1*, *COL1A2*) in four families, making linkage to these loci very unlikely (31). Similarly, Miller et al. (1996) excluded linkage to genes encoding fibrillin 1 (*FBN1*) and elastin as well as *COL1A2* in eleven families displaying apparent autosomal dominant transmission of idiopathic scoliosis (23).

Genome-wide linkage analyses of idiopathic scoliosis have pointed to many locations, including regions on chromosomes 3, 6, 10, 12, 17, 18, 19, and X (32–36). Genome-wide association studies have provided evidence for involvement of the estrogen receptor, matrix metalloproteinase-3 and interleukin-6 in the pathogenesis of idiopathic scoliosis (37–39).

### 162.3 SPONDYLOLISTHESIS

The slippage of a vertebral body forward over the one below it is called spondylolisthesis. It most commonly involves the fifth lumbar vertebra, although the fourth lumbar and occasionally other vertebrae may be affected. Patients are usually symptomatic but may exhibit low back pain, stiffness, or even neurologic symptoms (40). In most instances, the displacement is associated with, and thought to be due to, a defect in the pars interarticularis (posterior inferior process) of the vertebral arch (41). This defect is designated spondylolysis, and five types have been defined: dysplastic (congenital), isthmic, degenerative, traumatic, and pathologic, the most common being the isthmic type (42). Spina bifida occulta often accompanies the dysplastic type (43).

Spondylolysis occurs in 4–8% of the general population over 6 years of age, whereas spondylolisthesis is found approximately half as often (40,44). Familial aggregation of spondylolysis with and without displacement has been observed on several occasions. The dysplastic and isthmic forms of spondylolysis are found in these families. In reviewing spondylolysis, Wynne-Davies and Scott (1979) noted that in most radiographic surveys of family members, about 27% of near relatives were affected (44). In their study, which was restricted to spondylolisthesis of the fifth lumbar vertebra, 19% of relatives had spondylolysis. However, when subdivided according to the type of spondylolysis in the proband, 33% and 15% of relatives of patients with the dysplastic and isthmic forms, respectively, were affected. They also noted that the relatives sometimes had the opposite type of spondylolysis to that found in the index case.

Several individual families have been reported containing multiple affected members (40,41,45,46). Wiltse (1962) postulated autosomal recessive inheritance with incomplete penetrance, but most authors have concluded that autosomal dominant inheritance is more likely (41). In one study, the penetrance was 75% for spondylolysis, and approximately 30% of those patients showed some degree of slippage (40).

The inherited abnormality appears to be a defect in the pars interarticularis of the vertebral arch. It can be either the dysplastic or isthmic type, although the latter is more common (44). Similarly, different vertebral bodies can be affected as evidenced by monozygotic twins who exhibited defects at different levels (41). Radiographically, the defects become evident between the ages of 5 and 7 years (41). The process of fatigue fracture due to repeated stress and trauma rather than an acute traumatic event is thought to be responsible (47). The slippage, if it occurs, usually appears during adolescence, concomitantly with the pubertal growth spurt. The displacement is greater if spina bifida occulta or other vertebral anomalies are present (43). The occurrence of spina bifida seems to be etiologically independent, however, because the incidence of spina bifida and other neural

tube defects is no greater in the patients' relatives than in the general population (44).

Two type IX collagen polymorphisms that introduce tryptophan residues into the triple helical domain of the collagen chain have been linked to an increased risk of lumbar disc disease (48,49). In a study of 107 patients undergoing surgery of the lumbar spine, Matsui et al. (2004) found that a disproportionately high number of patients with a tryptophan polymorphism had spinal stenosis with spondylolisthesis (50).

### 162.4 CONGENITAL DISLOCATION OF THE HIP

Congenital dislocation of the hip (CDH), sometimes referred to as developmental dysplasia of the hip, is characterized by the displacement of the femoral head outside the acetabulum before or slightly after birth. When lesser degrees of displacement occur so that the femoral head articulates with the outer margin of the acetabulum, the term *congenital subluxation* is used. Acetabular dysplasia refers to the development of an abnormally shallow acetabulum without actual displacement (51). CDH is a common birth defect, but the incidence varies considerably throughout the world (Table 162-2), ranging from 0.6 per 1000 whites living in Birmingham, England, to 38 per 1000 North American Indians living in Arizona (52). Although much of the discrepancy is due to different methods of ascertainment or criteria for diagnosis, etc., environmental and genetic factors are thought to be important as well. For example, CDH is more common in infants born in the winter months (53,54). Presumably, the wrapping of the child for warmth keeps the hips in the extended position in which dislocation is more likely. The practice of swaddling an infant to a cradle board with hips extended and adducted for the first few months of life is thought to account for the high incidence of CDH in certain American Indian groups (55). Intrauterine posture is very important as well; both breech presentation and being the first infant born to a mother predispose to CDH (53,54). The female sex is perhaps the major determining factor. CDH occurs approximately six times as often in girls as in boys. It is thought that the production of estrone by the fetal ovary and possibly relaxin by the

**TABLE 162-2 Incidence of CDH**

Location	Incidence (per 1000)
Birmingham, England	0.6
Oslo, Norway	1.0
Salford, England	1.6
New York City, USA	1.6
Malmo, Sweden	1.7
Salt Lake City, USA	9.1
Jerusalem, Israel	9.8
Budapest, Hungary	27.5
Arizona, USA (Navajo Indian)	38.0

fetal uterus, both of which increase ligamentous laxity, account for this sex predilection (52,55).

Familial aggregation of CDH has long been recognized. Twin studies have consistently shown a higher concordance rate for monozygotic than for dizygotic twins (Table 162-3). There have been several large surveys in which the incidence of CDH in relatives of probands with CDH has been determined (Table 162-4). In two of the recent studies, the patients were divided into two groups: those having neonatal and those having late onset, depending on whether the diagnosis was made before or after the age of 4 weeks. This was because before 1960 it was not appreciated that CDH could be diagnosed in the neonatal period; most cases were detected when the child began to walk. Thus, the prior studies had dealt primarily with the late-onset type, and it was not known if the patients diagnosed in the neonatal period represented the same or perhaps an etiologically different group. Most recently, an analysis of birth records for infants born Utah yielded relative risk figures of 12.13, 11.85, and 1.74 for first degree relatives, siblings, and first cousins, respectively (56).

TABLE 162-3 Twin Studies in CDH			
Authors	Year	Concordance	
		Monozygotic	Dizygotic
Idelberger	1951	10/29	3/109
Kambara and Sasakawa	1954	15/21 <sup>a</sup>	3/6 <sup>b</sup>
Wynne-Davies	1970	1/2	1/3
Czeizel et al.	1975	3/6	0/11

<sup>a</sup>Twins classified as monochorionic.

<sup>b</sup>Twins classified as biovular.

From Czeizel, A., Szentpetery, J., Tusnady, G., Vizkelety, T. Two Family Studies on Congenital Dislocation of the Hip after Early Orthopaedic Screening in Hungary. *J. Med. Genet.* 1975, 12 (2), 125–130.

The incidence of CDH was found to be much higher in relatives of patients with CDH than in the general population in all the surveys. For example, the incidence in siblings ranged from 2.2 to 14%, the highest being in Hungary, where the population incidence is high. In the two studies in which neonatal and late-onset CDH were separated, there were several instances in which both types were observed within the same family (53,54). Moreover, in one pair of dizygotic twins, both types of CDH were found.

The sex preference for girls was observed in affected sibs. For instance, the 5% of affected siblings reported by Record and Edwards (1958) comprised 1% brothers and 10% sisters (57). Similarly, the series of Carter and Wilkinson consisted of 4% brothers and 7% affected sisters (55). In general, the incidence of affected siblings was slightly greater when the proband was male (52,54,58), as would be expected in polygenic inheritance. However, in the two surveys in which the data were subjected to statistical analysis, no significant difference was found (53,54). A dramatic drop-off in the incidence of CDH in second- and third-degree relatives compared with first-degree relatives was observed by Wynne-Davies (1970), Bjerkreim and van der Hagen (1974), and Czeizel et al. (1975) (53,54,58).

The genetic contribution to CDH appears to have two separate components. Based on the observation that the configuration of the acetabulum is determined by a multiple gene system (57), Carter and Wilkinson (1964) postulated that acetabular dysplasia inherited in this fashion interacted with ligamentous laxity, possibly transmitted as an autosomal dominant trait, to predispose an infant to CDH (55). Subsequent studies by Wynne-Davies (1970) (53) and Czeizel et al. (1975) confirmed that “normal” parents of children with CDH have acetabula that measure radiographically as being more shallow than normal (53,59). In the first study, the association was noted only for the late-onset type

TABLE 162-4 Family Surveys of CDH					
Author(s)	Year	Type	Incidence (%)		
			Parents	Siblings	Children
Muller and Seddon	1953	NS <sup>a</sup>	1.3	2.2	3.4
Record and Edwards	1958	NS		5.0	
Carter and Wilkinson	1964	NS		5.7	
Woolf et al.	1968	NS	16	4.3	
Wynne-Davies	1970	Neonatal	0.8	13.5	
		Late onset	0.8	5.0	12.1
Bjerkreim and van der Hagen	1974	Neonatal	1.8	6.0	
		Late onset	2.7	8.5	
Czeizel et al. <sup>b</sup>	1975	NS	2.1	14.0	
		NS	2.3	14.0	

<sup>a</sup>NS = not specified.

<sup>b</sup>Surveys at two locations reported.

From Czeizel, A., Tusnady, G., Vaczo, G., Vizkelety, T. The Mechanism of Genetic Predisposition in Congenital Dislocation of the Hip. *J. Med. Genet.* 1975, 12 (2), 121–124. Carter, C.O., Wilkinson, J. A. Genetic and Environmental Factors in the Etiology of Congenital Dislocation of the Hip. *Clin. Orthop.* 1964, 33, 119–128.



of CDH; however, it was observed in both types in the latter investigation. Beals (2003) reported a family with apparent autosomal dominant transmission of acetabular dysplasia (60).

Several studies have demonstrated the occurrence of generalized joint laxity in infants with CDH (53,55,59,61). This was observed especially for boys, and particularly in the neonatal form. Furthermore, these studies showed that it was more common in family members than in the general population; but the mode of inheritance was difficult to determine (i.e. joint laxity is difficult to assess and quantify and tends to decrease with age). Carter and Wilkinson (1964) postulated that this common form of joint laxity was an autosomal dominant trait (55,61). The basis for this speculation appears to have been a few families exhibiting autosomal dominant transmission of generalized joint laxity frequently associated with CDH (62,63). Horton et al. (1980), however, demonstrated that these particular families probably had a separate autosomal dominant trait, which they termed *familial joint instability syndrome* (64). This syndrome can be distinguished from simple joint laxity as seen in CDH by its tendency to present at birth and its association with dislocation of several major joints in addition to the hip. Moreover, the incidence of CDH is approximately equal in boys and girls with this syndrome. Thus it is not clear if the joint laxity in typical CDH is truly an autosomal dominant trait as postulated or may be simply the extreme of normal joint mobility, possibly inherited in a polygenic fashion. In either case, simple familial joint laxity is very common. It occurs in 5% of the normal population and conveys a small but definite risk for CDH (53,61,65).

Thus, CDH appears to be inherited as a multifactorial trait. Acetabular dysplasia transmitted in a polygenic manner and ligamentous laxity inherited in a polygenic or autosomal dominant fashion interact with several factors to bring about CDH. The other factors include female sex (which probably indicates it has a hormonal basis) and others, as yet undefined, that influence the position of the hip before birth and during infancy. Although increased joint laxity may be associated with an early diagnosis and a shallow acetabulum with a later one, the two seem to act additively. Genome-wide studies applied to CDH on a limited basis to date have identified associations of CDH with two developmental regulators of early skeletal development, *TBX4* and *GDF5* (66,67).

## 162.5 CLUBFOOT

Clubfoot is a relatively common congenital anomaly occurring at a rate of approximately 1–3 per 1000 live births (68). It occurs as a component of several syndromes, especially those involving the nervous and connective tissue systems, but also as an isolated developmental anomaly, idiopathic congenital clubfoot. The idiopathic variety shows familial aggregation, but its

inheritance is confusing. Many types of transmission including autosomal recessive, X-linked recessive, autosomal dominant, and multifactorial have been proposed (69,70). Part of the confusion is due to the inclusion of patients with unrecognized syndromes in surveys of idiopathic congenital clubfoot. In addition, many investigators have considered clubfoot as a single entity, when in fact it is composed of three distinct anomalies: talipes equinovarus, talipes calcaneovalgus, and metatarsus varus. The limited number of genetic studies in which this heterogeneity has been appreciated has indicated that the three forms are separate entities (69,71,72).

### 162.5.1 Talipes Equinovarus

Talipes equinovarus is characterized by adduction of the forefoot, inversion of the heel, and plantar flexion of the forefoot and ankle. It is seen in males approximately twice as often as in females, and about 18% of patients exhibit additional minor abnormalities of connective tissues such as joint laxity and hernias (72). In a study of 174 twin pairs, Idelberger (1939) reported the concordance to be 3% in monozygotic twins and 3% in dizygotic twins (73). In a survey of 110 families in 1964, Palmer described 43 families in which at least two members had the deformity (71). Noting instances in which multiple members and three generations of a family were affected, together with the occurrence of male-to-male transmission and an equal ratio of affected males to females, he suggested that autosomal dominant transmission with reduced penetrance was responsible for the deformity at least in certain families. A segregation analysis of data from many of these families and others 10 years later (Palmer et al., 1974) suggested that multifactorial inheritance was more likely (71). However, the incidence in second- and third-degree relatives was higher than expected. This mode of transmission was supported by studies performed by Carter (1965) in which he found that the incidence of talipes equinovarus was 2.1, 0.61, and 0.20% in first-, second-, and third-degree relatives, respectively (19). In addition, a survey of relatives of 340 patients revealed that 2.9% of relatives had talipes equinovarus, whereas talipes calcaneovalgus and metatarsus varus were very infrequent (72). Male relatives of affected females showed the highest rate.

Rebbeck et al. (1993) carried out complex segregation analysis of this deformity in 143 white families from Iowa (74). Their results were most consistent with the hypothesis that talipes equinovarus results from mendelian segregation of a single gene with two alleles plus the effects of other unmeasured factors shared by siblings. Thus, it appears that in most cases, talipes equinovarus is inherited in a manner influenced by both mendelian and non-mendelian factors. The former may predominate in some families. However, for sporadic cases, the recurrence risk to sibs of approximately 3%, as has been used for many years, seems reasonable (71,72).

A microduplication of chromosome 17q23.1q23.2 was recently reported in three families with isolated clubfoot; this region contains the T-box *TBX4* that has been implicated previously in clubfoot (75). There is also evidence for contributions from genes encoding HOX proteins and proteins involved in apoptosis in the etiology of clubfoot deformities (76).

### 162.5.2 Talipes Calcaneovalgus

In talipes calcaneovalgus, there is dorsal flexion of the forefoot and the plantar surface of the foot faces laterally. It is mild, often correcting spontaneously, and it occurs more often in girls (male/female ratio 0.61:1). It is frequently seen in first-born children, suggesting that uterine constraint might be an etiologic factor. Like talipes equinovarus, other minor connective tissue abnormalities occur in approximately 18% of patients, especially congenital dislocation of the hip, which was present in nearly 5% of patients. The incidence of affected sibs is 4.5%, suggesting multifactorial inheritance (72).

### 162.5.3 Metatarsus Varus

Inversion and adduction of the forefoot are found alone in metatarsus varus. It resembles talipes calcaneovalgus in many respects. Often mild, it may go unnoticed. Girls are affected slightly more frequently than boys, and it is observed in approximately 4.5% of sibs. Again, multifactorial inheritance is suggested. It differs, however, in that patients do not exhibit additional minor connective tissue abnormalities, nor does there appear to be any excess of first-born infants (72).

Metatarsus varus is a manifestation of diastrophic dysplasia, which is caused by mutations of the sulfate transporter gene, *DTDST*, also designated solute family 26 (sulfate transporter), member 2 (*SLC26A2*). Since this family of autosomal recessive disorders displays a wide range of severity, it was proposed that “mild” *DTDST* mutations could account for some cases of isolated metatarsus varus. Huber et al. detected homozygosity for the Arg279Trp mutation of *DTDST* in such a case (77). Bonafe et al. found no evidence of such mutations in talipes equinovarus (78).

## 162.6 JUVENILE OSTEOCHONDROSES

The juvenile osteochondroses are a group of disorders in which localized noninflammatory arthropathies result from regional disturbances of skeletal growth (Table 162-5). There is ischemic necrosis of either primary or secondary endochondral ossification centers (79). Most of the abnormalities occur sporadically, but familial forms have been described. Legg–Perthes disease, osteonecrosis of the capital femoral epiphysis, has received the greatest attention. It has been reported as an autosomal dominant trait (80), as a sex-influenced dominant trait

with reduced penetrance (81), and as a multifactorial trait (82). However, these studies were done, or, in the case of Gray et al., data recorded, before the delineation of several simply inherited skeletal dysplasias that exhibit abnormal development of the capital femoral epiphyses. It seems likely that patients with these conditions may have been included in the studies, especially patients with the mild form of multiple epiphyseal dysplasia (Ribbing), an autosomal dominant trait in which involvement may be restricted to the capital femoral epiphyses (3,4). In the recent surveys in which attempts were made to exclude such patients (83–85), the frequency in relatives was found to be very low; approximately 1% of first-degree relatives were affected and the incidence in second- and third-degree relatives approached that of the general population. Moreover, in the three pairs of unselected monozygotic twins reported, all were discordant for Legg–Perthes disease (86). Thus in the vast majority of cases, this condition is not inherited.

Blount disease, a growth disturbance of the medial aspect of the proximal tibial growth plate, occurs in both infancy and adolescence. Pedigrees consistent with autosomal dominant transmission have been reported in the infantile form (87). However, Bathfield and Beighton (1978), in a survey of 231 sibs of 110 patients with the infantile form, found that only 10 were affected (88). They concluded that common environmental factors were largely responsible for the familial aggregation.

Osteochondritis dissecans involving multiple sites, especially the knees, hips, elbows, and ankles, has been

**TABLE 162-5 Juvenile Osteochondroses**

Region Affected	Eponym	Inheritance
Capital femoral epiphysis	Legg–Perthes disease	–
Tibial tubercle	Osgood–Schlatter disease	–
Os calcis	Sever disease	–
Tarsal of navicular bone	Kohler disease	–
Head of second metatarsal	Freiberg disease	–
Vertebral bodies	Scheuermann disease	–
Medial aspect of proximal tibial epiphysis	Blount disease, tibia vara	AD <sup>a</sup>
Subchondral areas of diarthroidal joint (particularly knee, hip, elbow, and ankle)	Osteochondritis dissecans	AD
Capitellum of humerus	Panner disease	–
Patella	Larsen–Johansson disease	–

<sup>a</sup>AD = autosomal dominant.

reported as an autosomal dominant trait in several families (89–91). The condition is characterized by the separation of a small piece of articular cartilage and underlying bone to form a loose body within the joint. Overlap with other of the osteochondroses has been observed. For example, osteochondritis dissecans has been seen in patients with involvement of the tibial tubercle (Osgood–Schlatter disease), spine (Scheuermann disease), the medial aspect of the proximal tibial epiphyses (adolescent Blount disease), patella (Larsen–Johansson syndrome), and the capital femoral epiphyses (Legg–Perthes disease) (90,92). Thus it appears to be a generalized disorder affecting growing epiphyses and may be inherited in some families.

The prevalence of Scheuermann disease was recently studied in a large Danish twin cohort (93). The proband-wise concordance was 0.31 for monozygotic twins compared with 0.13 for dizygotic twins, supporting a genetic component to this condition.

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## RELEVANT WEBSITE

Online Mendelian Inheritance in Man: <http://www.ncbi.nlm.nih.gov/omim>.

## Biography



**Dr Horton** received AB and MD degrees from the University of Kansas, completed a residency in internal medicine at the University of Kansas School of Medicine and fellowship training in medical genetics at UCLA–Harbor General Hospital, with additional genetics training at the NIH. He has held faculty positions at the University of Kansas School of Medicine, University of Texas Medical School–Houston and Oregon Health and Science University in Portland, where he has also been director of the Shriners Hospital for Children Research Center since 1993. He has a long-standing research interest in skeletal biology with special emphasis on inherited skeletal dysplasias and their molecular pathogenesis. He has published well over 150 articles and book chapters on both the clinical and basic science aspects of these conditions. He is active in many relevant medical and scientific organizations, sits on a number of advisory and review committees and is considered an expert in the field of skeletal dysplasias.

# CHAPTER

# 163

## Hereditary Noninflammatory Arthropathies

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### 163.1 INTRODUCTION

Noninflammatory arthropathy is a major manifestation of a number of genetic disorders. There is a great variety of phenotypes, which present a diagnostic challenge in the clinical setting. In some of these conditions this arthropathy is generalized, while in others it predominates in the hip joints, with or without significant involvement of the spine.

Degenerative osteoarthropathy (OA) is the end result of a number of different pathologic processes that culminate in cartilage degeneration, with remodeling and proliferation of new bone. The underlying mechanisms may involve disturbances in skeletal development; minor abnormalities of the cartilage matrix itself, notably, genetic defects of type II collagen; infiltration, as in certain storage disorders; and aseptic necrosis, as in the hemoglobinopathies. In addition, neuropathic joints develop in some hereditary neurologic conditions. Environmental factors may also play a role, and the interaction of primary defects in cartilage and abnormal external forces may be the pathogenetic determinants in some bone dysplasias and hypermobility syndromes. The pathogenetic situation was summed up by Mitchell and Crues (1), who stated “articular disease may result from either an abnormal concentration of force across a joint with normal cartilage matrix or a normal concentration of force across an abnormal joint.”

The general categories of hereditary noninflammatory arthropathy are listed in Table 163-1. Degenerative OA, especially of the weight-bearing joints, is a common complication in a large number of genetic skeletal dysplasias and disorders (2). Many of these conditions have been reviewed elsewhere in this book, but those that have not been covered in the context of OA are outlined in this chapter.

### 163.2 SPONDYLOEPIPHYSEAL DYSPLASIAS

In the 1997 version of the *International Nomenclature and Classification of Osteochondrodysplasias* (3),

several conditions in the spondyloepiphyseal dysplasia (SED) category, in which the underlying defect has been elucidated, were listed as type II collagenopathies. This category was further refined in the 2002 update (4), the 2006 update (5) and most recently, in the 2010 update (6). Other disorders in this group, such as Langer-Saldino dysplasia (achondrogenesis type II), platyspondylic dysplasia, Torrance type, and hypochondrogenesis, present as lethal neonatal dwarfism and do not enter into a discussion of degenerative arthropathy. Equally, Kniest and Stickler syndromes, which are also linked to type II collagen in some affected families (7), and in which degenerative OA occurs together with other significant manifestations, have been fully reviewed in a previous chapter and are not considered further in this section.

SEDs are characterized by predominant involvement of the vertebral bodies and epiphyses of the proximal joints, as well as considerable phenotypic and genetic heterogeneity; in some forms dwarfism with a characteristic shortened trunk is severe, while in others stature approaches normality. Myopia and hearing loss are variable syndromic components. The classical severe form of SED congenita (SEDC) is inherited as an autosomal dominant trait, and is recognizable at birth, but other, milder, autosomal dominant forms may only become evident in late childhood or early adulthood.

Osteoarthropathy of the hip joint, which may be an end result of coxa vara of the hip and/or avascular-necrosis-like changes in the capital femoral epiphysis, is a major cause of handicap in several of these disorders, to the extent that prosthetic joint replacement may be necessary at a comparatively young age. This complication is also of considerable diagnostic significance as it may be the presenting feature of an otherwise mild generalized skeletal dysplasia. Genu valgum and increased laxity of the medial collateral ligament may also be present resulting in pain and degenerative changes in the knee.

Linkage to the type II collagen gene (COL2A1) has been demonstrated in some families with classical SEDC

**TABLE 163-1 The Hereditary Noninflammatory Arthropathies**

<b>Defects of the Cartilage Matrix</b>
SED group of disorders, including Kniest and Stickler syndromes, and the familial hip joint dysplasias ( <i>Proven or possible defects of type II collagen</i> )
Alkaptonuria ( <i>Abnormal binding of polymers of homogentisic acid to cartilage collagen</i> )
Gout and pseudogout ( <i>Deposition of calcium pyrophosphate and hydroxyapatite crystals in the cartilage matrix</i> )
<b>Infiltration and/or Aseptic Necrosis (Femoral Head)</b>
Storage disorders
Wilson disease
Hemochromatosis
Gaucher, Fabry, and Farber diseases
Hemoglobinopathies
<b>Mechanical Collapse due to Interaction of Primary Defects in Cartilage and External Forces</b> ( <i>notably the femoral head</i> )
Skeletal dysplasias
Hypermobility syndromes
<b>Primary Hip Joint Dysplasias</b>
Perthes disease
Slipped femoral capital epiphyses
<b>Neuropathic Arthropathy</b>
Amyloidosis
Charcot-Marie-Tooth syndrome
Déjérine-Sottas syndrome
Familial dysautonomia

SED = spondyloepiphyseal dysplasia.

but not in others (8–14,81). This gene is situated on chromosome 12.q13.1–q13.2. In the same way, some of the milder, late-onset forms of autosomal dominant SED have been shown to be linked to type II collagen. For instance, in Namaqualand hip dysplasia, which has been diagnosed in 45 persons in five generations of a South African family (Figures 163-1–163-4), a logarithm of the odds (LOD) score of 7.98 indicates linkage to *COL2A1* (15). More recently, a three-generation family in the United States with mild SEDC was reported with a novel Y-position proline substitution in the triple helical domain (Gly-X-Y) of the pro $\alpha$ 1(II) in *COL2A1* (12). Conversely, a family of British stock with SEDC and generalized OA, living in Kimberly, does not show linkage (LOD 2.26) (16). The determinant gene in this kindred has been reported to be a null mutation in aggrecan (17,18). A mutation in aggrecan was also described in an autosomal recessive form of spondyloepimetaphyseal dysplasia (SEMD), aggrecan type (19). This was a missense mutation affecting the C-type lectin domain of the protein, and was detected in a Mexican family distinguished by severe short stature and a novel group of radiographic findings (19). A similar mutation was recently reported in a Swedish family with autosomal

dominant familial osteochondritis dissecans with mild short stature and early-onset osteoarthritis (20). Characterization of mutations in *COL2A1* has been undertaken, and the Cardiff University Human Gene mutation database (21) lists at least 33 human mutations that have been described. There is great phenotypic variation in SED, making genotype–phenotype correlations more challenging (22) and it is possible that there are hot spots in this gene associated with some forms of mild SED in which OA occurs (23).

Degenerative OA of the hip joint predominates in other dominant mild chondrodysplasias of SED type. In South Africa, Beukes hip dysplasia (Figure 163-5), named after a family of Dutch stock in which 47 persons in six generations are affected, is a disorder of this type (24). This condition has distinctive radiologic stigmata, and there is little doubt concerning its syndromic identity. Beukes hip dysplasia was shown to be not linked to *COL2A1* (25), and the disease-associated gene was subsequently mapped to chromosome 4q35 (26). The osteochondrodysplasias that are of special importance in South Africa have been reviewed by Beighton (27).

In the context of SED, it is noteworthy that in pseudoachondroplasia (PSACH) and some forms of multiple epiphyseal dysplasia (AD-MED), the disease-associated genes are allelic on chromosome 19. The phenotype in these conditions is the result of defective cartilage oligomeric matrix protein (COMP). Considerable intragenic heterogeneity is present, but the phenotypes are fairly consistent (28,29). AD-MED may also result from mutations in matrillin-3 (MAT3) and type IX collagen (30). It has now been shown that an unstable trinucleotide expansion in the *COMP* gene may be the causative mechanism (31). Contraction as well as expansion can occur in the PSACH/MED disorders, with either process producing the disease phenotype. Previously, expansions of this type had largely been recognized in genetic neurologic disorders, and the fact that they can also underlie skeletal disorders has important implications.

### 163.3 FAMILIAL OSTEOARTHROPATHY

The major problems in the elucidation of the genetic determinants of OA are the considerable heterogeneity of the disorder and the great difficulty in precise phenotypic delineation of autonomous entities within this general category. Although OA is common, large families with apparent mendelian transmission of the condition are few and far between.

Primary OA, or degenerative arthropathy, is a very common disorder of middle and old age. More than 80% of all persons over the age of 65 years have radiologic stigmata; of these individuals, about 25% are symptomatic. Risk factors include obesity and trauma, but it is not solely an age-related disorder and there is some evidence for a genetic component (32). The findings of the large-scale Framingham offspring investigation (33), and





**FIGURE 163-1** Namaqualand hip dysplasia in a branch of an affected South African family. The major manifestations are premature degenerative osteoarthropathy of the hip joint.



**FIGURE 163-2** Namaqualand hip dysplasia. Anteroposterior radiographic view of an affected girl, showing early flattening and fragmentation of the femoral capital epiphyses.



**FIGURE 163-3** Namaqualand hip dysplasia. Anteroposterior radiographic view of an affected adult. The hips show advanced osteoarthropathy, with loss of joint space, irregularity, and distortion of the femoral heads, and patchy sclerosis and lucency.

the Baltimore longitudinal study (34) have also indicated that genetic factors are involved. Twin studies have been suggestive of a significant genetic component in OA of the hand and knee (35). It is possible, though unproven, that some “normal polymorphisms” of type II collagen might convey an increased propensity to the development of OA. It is also possible that subchondral bone may be primarily involved in the pathogenesis (36).

The best example of familial OA is probably Heberden’s arthropathy. The influence of heredity in hand OA has been observed and studied in a variety of ways, including the assessment of relative risk in siblings, aggregation in families, and disease concordance in twins. Stecher in 1941 noted a hereditary disposition for

hand OA expression, with a twofold excess of disease in mothers and a threefold excess in sisters of patients with Heberden’s nodes compared with unrelated controls. (37). It is of historical interest that William Heberden (1710–1801), an English physician, gave the following account of the characteristic “nodi digitorum”:

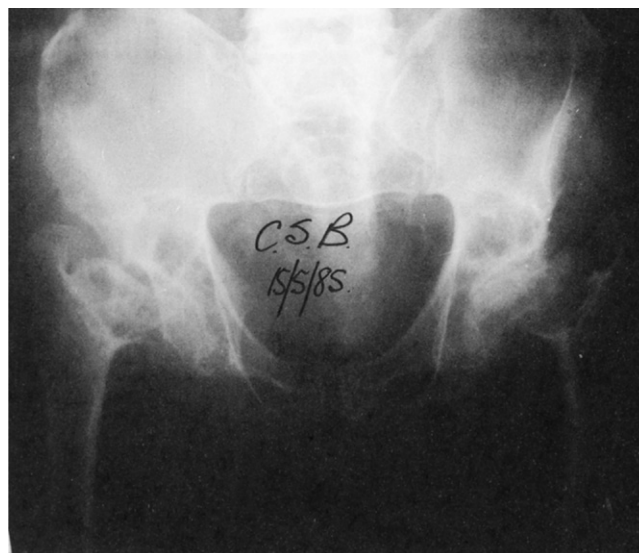
What are those little hard knobs, about the size of a small pea, which are frequently seen upon the fingers, particularly a little below the top, near the joint? They have no connexion with the gout, being found in persons who never had it; they continue for life; and being hardly ever attended with pain, or disposed to become sores, are rather unsightly, than inconvenient, though they must be some little hindrance to the free use of the fingers.





**FIGURE 163-4** Namaqualand hip dysplasia. Lateral radiographic view of the spine of a girl aged 10 years. The vertebral bodies show mild flattening and end-plate irregularity.

The form of generalized OA that is accompanied by Heberden's nodes may represent a sex-influenced or sex-limited autosomal dominant trait (34). It has been suggested that females have a greater genetic liability to OA in general than men (38). A study by Jonsson et al. noted that sisters of patients with interphalangeal joint and first carpometacarpal (CMC1) joint OA have relative risks of 5.0 and 6.9, respectively, to develop OA in the same joint (39). The results of familial aggregation and twin studies provide strong evidence for an inherited predisposition to hand OA. The exact mode of inheritance remains unclear and multiple studies have resulted in reports of association or linkage with a variety of genetic loci (40).



**FIGURE 163-5** Beukes hip dysplasia. Anteroposterior radiographic view of an affected adult. The hips show gross osteoarthritis and a valgus deformity of the femoral necks.

Although this condition is common, the comparatively late onset poses difficulties for family studies and for linkage investigations with respect to the *COL2A1* locus on chromosome 12 (41). An association between an aggrecan polymorphic allele and OA of the fingers has been proposed by Horton et al. (42), and the heritability of OA of the peripheral joints has been discussed by Bijkerk and associates (43). Kalichman et al. (44) undertook a clinical and radiologic investigation of OA of the hands in 1190 persons in 295 nuclear families in a homogenous population of Russian stock. In a sophisticated statistical analysis of their findings, the authors found some evidence of the activity of a major genetic determinant in OA. They could not, however, provide confirmation for a putative gene at 11q12 (45). Associations with *MATN3* mutations have been reported for CMC1 joint involvement in an Icelandic population with familial hand osteoarthritis and also in a separate population with CMC1 OA of the hand with spine OA (46,47). Mutations in *MATN3* have also been reported in AD-MED (48) and matrillin-3 polymorphisms have been associated with spinal disc degeneration and hand osteoarthritis (47). OA at the distal interphalangeal (DIP) and CMC1 joints can occur independently or in the same patient, and it has been suggested that CMC1 OA may need to be treated distinctly from interphalangeal joint OA, as susceptibility linkages may be joint-specific (39,49,50). A genetic linkage study by Hunter et al. found the highest heritabilities at CMC1 and DIP joints as well as the highest LOD scores (51). These investigators also concluded that a joint-specific approach to hand OA genetics may provide greater linkage, as evaluating hand OA as a general entity may decrease the strength of association (51).

The type II collagen gene is a good candidate for the basic defect in primary OA, and linkage was reported more than a decade ago (52,53). This observation would have been of immense importance, in view of the very high frequency of OA in the population as a whole. On further study, however, it emerged that the condition in question was a mild chondrodysplasia with involvement of the vertebrae and epiphyses, and it could thus be categorized in the SED group of disorders (54,55).

The pathogenesis of primary OA is still the subject of intensive investigation. A candidate gene approach to autosomal dominant nonsyndromic OA in a large Dutch family involved 14 collagen or collagen-related candidate genes, of which 10 were excluded (56). Following a radiographic survey in middle-aged persons in Holland, these authors undertook haplotype analysis and identified associations between generalized OA and three polymorphisms in the *COL2A1* gene (57). Loughlin et al. (50) carried out a genomic screen and identified possible OA-related loci on chromosomes 4, 6, and 16. In a subsequent investigation that was focused on chromosome 6, they demonstrated linkage in females but not in males between primary OA of the hip and a locus at 6p12.3–q13, close to the *COL9A1* gene. Further studies of this region using single nucleotide polymorphisms (SNPs) did not support the contention that *COL9A1* was associated with primary OA (58). Possible linkage of OA to a locus on chromosome 2q has been reported (45,59,60). In an investigation in 69 persons in 22 Tasmanian families with digital OA and Heberden's nodes, no linkage with the putative locus on 2q could be demonstrated (61).

It is apparent that there is considerable nonallelic heterogeneity in generalized OA and that the genes that are involved differ in the degree to which they confer susceptibility. In view of the rapid progress in molecular genetics and the importance of OA, however, it seems very likely that determinant genes will be identified in the foreseeable future.

#### 163.4 PRIMARY OSTEOARTHROPATHY OF THE HIP

In the context of primary OA, the question arises as to whether or not OA of the hip joint, in the absence of significant involvement of other joints, is an independent genetic entity. This common disorder in middle age and advancing years is important, as prosthetic joint replacement is often required. Risk factors such as trauma, obesity, and possibly sporting activities apply but are by no means absolute; there is no obvious simple mode of inheritance, but genealogic studies are difficult because of the late onset. Family clustering has been documented (32,62,63), and twin studies have yielded positive results (64). In a comparative study of pelvic radiographs of 135 monozygous and 277 dizygous sets of healthy female

twins, concordance of OA hip was greater in the monozygous twins. The severity of OA was also greater in the monozygous pairs. The authors concluded that genetic factors made a significant contribution to OA of the hip in females (65).

Populations of different genetic stock living in the same environment may provide clues; for instance, in Hawaii, total hip replacement rates are higher in whites than in Asians (66). Similarly, there is a very low prevalence of primary OA of the hip in Asian, black, and East Indian populations (67).

In a large investigation in Iceland, using a database of total hip joint replacements undertaken between 1972 and 1996, family clustering was identified (68). Thereafter, a susceptibility locus on chromosome 16p was identified in a large family (LOD 2.58) (69). At the clinical, radiologic, histologic, and phenotypic levels, this familial OA of the hip was indistinguishable from the idiopathic, nonfamilial OA of the hip (70).

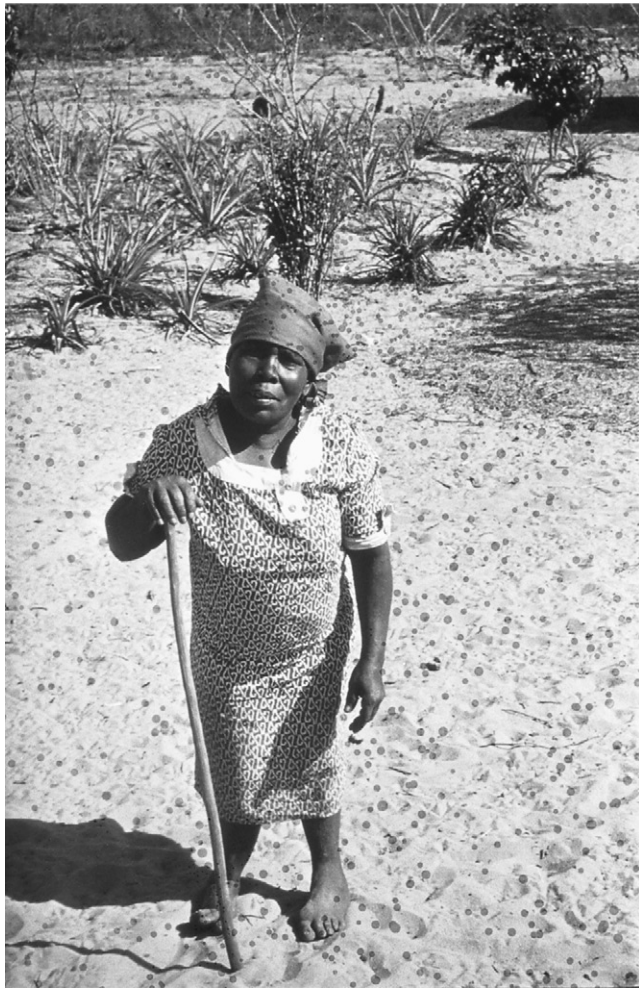
The determinant genes for the  $\alpha_1$  chain of type II collagen and the vitamin D receptor are adjacent loci on 12q. Granchi et al. (71) investigated polymorphic sites in these genes in 143 persons in whom hip joint replacement had been undertaken for primary or secondary OA. The findings were interpreted as providing evidence for a genetic component for the risk of OA in persons with severe hip dysplasia.

Part of the heritability of hip OA may be explained by the hip morphology produced by many of the identified susceptibility genes, which are active during skeletal development (72). On the other hand, as with generalized OA, it is possible that collagen polymorphisms might be involved in the pathogenesis of OA of the hip. The Genetics, Osteoarthritis and Progression (GARP) study identified the deiodinase, iodothyronine, type II (D2) gene (*DIO2*) as a susceptibility gene for hip OA, and found that a mutation in this gene is more likely to increase the vulnerability of cartilage to non-optimal hip morphology instead of causing these shapes (73).

#### 163.5 MSELENI JOINT DISEASE

Mseleni joint disease (MJD) is a remarkable disorder that presents with widespread generalized degenerative OA in late childhood and causes severe crippling handicap in adulthood (74). The condition occurs in high frequency in an isolated area in northern KwaZulu-Natal, South Africa, near the border with Mozambique, and was first described in 1970 (75). Many persons are affected, and the condition has a major socioeconomic impact; the only option for management is prosthetic hip joint replacement. A condition very similar to MJD, Kasin–Beck disease, occurs in the Urov valley of Siberia, parts of China, and North Korea (76). Selenium deficiency, aflatoxins in foodstuffs, and hypoxia (13) have been incriminated in the latter, but despite more than

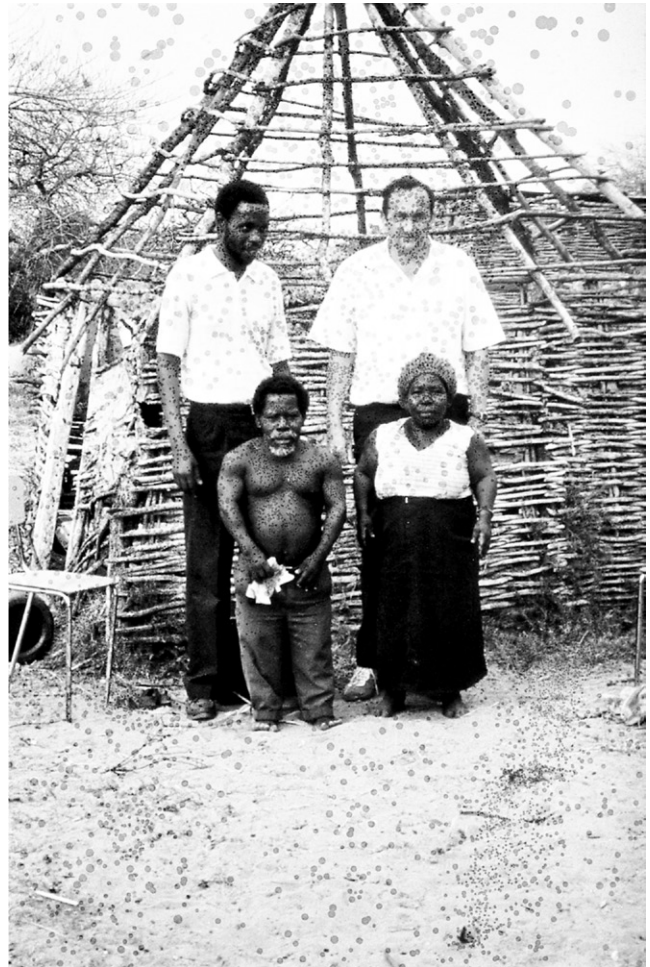




**FIGURE 163-6** Mseleni joint disease. An affected woman in her home environment. (From Agarwal, S. S.; Phadke, S. R.; Fredlund, V., et al. *Mseleni and Handigodu Familial Osteoarthropathies: Syndromic Identity?* Am. J. Med. Genet. **1997**, 72, 435–439.)

two decades of intensive investigations, no environmental determinants have been identified for MJD. It is of interest that a disorder that resembles MJD, bearing the geographic designation “Handigodu joint disease,” has been documented in several villages in the Shimoga district of Southern India (77).

To date, extensive research has yet to uncover the etiology of MJD. There is no evidence for mendelian inheritance patterns and epigenetic changes in response to the environment have been postulated (78). Type VI collagen has been shown to be overabundant in hip joint cartilage in affected adults undergoing joint replacement, but this finding may represent a secondary phenomenon (79). Among affected persons are dwarfed individuals (Figures 163-6 and 163-7) with the characteristic clinical and radiographic stigmata in severe degree (80). These small persons do not represent a continuum with their affected relatives, in whom stature is essentially normal. Equally, genealogic data do not indicate that they are homozygous for the faulty



**FIGURE 163-7** Mseleni joint disease. Two affected adults with severe stunting of stature. (From Agarwal, S. S.; Phadke, S. R.; Fredlund, V., et al. *Mseleni and Handigodu Familial Osteoarthropathies: Syndromic Identity?* Am. J. Med. Genet. **1997**, 72, 435–439.)

gene. The status of the brachydactylous dwarfs of Mseleni thus remains uncertain. By the end of 2004, there was anecdotal evidence that the incidence of MJD in the Mseleni region was diminishing rapidly. This observation, if substantiated, would be suggestive of the fluctuation of the influence of an unrecognized environmental agent.

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### Biography

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# CHAPTER

# 164

## Pathways—Cohesinopathies

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### 164.1 INTRODUCTION

The group of diagnoses linked together by their common disruption of the chromosomal cohesion complex, cohesin, or its regulators have been collectively termed “cohesinopathies.” Cornelia de Lange syndrome (CdLS; OMIM #122470, #300590, and #610759), the first disorder found to be caused by alterations in a cohesin regulatory protein, is currently the best recognized and most well studied. While this chapter will discuss a number of the cohesinopathies, the major focus will be on CdLS.

CdLS, also known as Brachmann–de Lange syndrome, is a rare genetically heterogeneous disorder affecting multiple organs and systems during development. Vrolik and Brachmann reported severely affected individuals in 1849 and 1916, respectively (1,2), and de Lange, describing two unrelated individuals, subsequently proposed diagnostic criteria in 1933 (3). CdLS has been estimated to occur in about 1:10,000 to 1:50,000 (4) individuals. However, the actual incidence is likely higher as the clinical presentations are quite variable. With recent molecular diagnostic capabilities, we now realize a higher prevalence of very mildly affected individuals that have been underappreciated and historically were less likely to be diagnosed as CdLS (5). Almost all cases are sporadic, resulting from dominantly acting *de novo* mutations, although recurrence in siblings due to parental (germ line) mosaicism has been reported (6,7), as has direct transmission from mildly affected individuals to their children (8–10). Somatic growth, central nervous system, craniofacial, musculoskeletal, and gastrointestinal systems are the most commonly affected. Additional systemic involvement includes auditory, genitourinary, cardiac, integumentary, hematopoietic, and ophthalmologic. Mutations in three genes, *NIPBL* on chromosome 5p13, *SMC1A* on chromosome Xp11, and *SMC3* on chromosome 10q25, can be identified (collectively) in 70% of individuals with clinically diagnosed CdLS

(8,9,11,12), with *NIPBL* being the major contributor. All of the identified causative genes to date are structural or regulatory components of cohesin.

### 164.2 CLINICAL FEATURES OF CdLS

#### 164.2.1 Growth and Development

Individuals with CdLS have proportionate small stature of prenatal onset, usually identified late in the second trimester. At birth, the measurement parameters are typically below the 10th percentile, and fall to below the 5th percentile by early childhood, although growth parallels standard curves. CdLS-specific growth curves are available (13). In adulthood, average heights and weights are almost universally below the 3rd percentile, and significant microcephaly persists (13). Improved growth parameters may be observed in individuals with *SMC1A* mutations compared to those with *NIPBL* mutations (11). Developmental delay and intellectual disability are typically observed. Speech and language are most significantly affected, while perceptual organization and visual–spatial memory are more preserved. The average IQ ranges from mild to moderate mental retardation; however, both borderline normal and severe mental retardation are commonly reported. Learning continues throughout life without evidence of regression. Early intervention has proven helpful in improving developmental outcomes and should be continued for as long as possible (14). Genotype–phenotype correlations are observed (see below).

#### 164.2.2 Behavior and Neurological Involvement

Almost all individuals with CdLS have some degree of behavioral issues that may be caused or aggravated by

physical complications. Self-injurious behavior, obsessive-compulsive behaviors, attention deficit disorder with or without hyperactivity (ADHD), short attention span, depression, and autistic features have all been consistently reported. Behavioral issues, particularly self-injury and anxiety, usually increase during adolescence. Social and environmental interactions can be achieved to variable degrees (15–18). Seizures are the primary neuropathological manifestation, although only seen in a minority of individuals. No specific electroencephalogram (EEG) pattern has been described and the seizures are generally well managed with standard medical intervention. Sleep disturbances commonly manifest as multiple night awakenings (15). Neuroradiological findings are uncommon but may include enlarged ventricles particularly at the basal cisterns, atrophy of white matter particularly at the frontal lobes, and hypoplasia of the brainstem and cerebellar vermis (19). Gyral structural abnormalities, myelination defects, and neurofibrillary tangles inside neurons have also rarely been reported on autopsy (20,21). Both hypertonia and hypotonia can occur.

Probands consistently have a high pain threshold that may be due in part to a poorly characterized peripheral neuropathy (19).

### 164.2.3 Facial Dysmorphism

The facial features are the most clinically consistent and recognizable finding in CdLS (Figure 164-1). Common features include a short neck, low posterior hairline, hirsute forehead, arched eyebrows, thick and long eyelashes, synophrys, ptosis, low-set ears, flattened midface, short nose, long philtrum, a thin upper lip with down-turned corners, a high palate, widely spaced teeth, and micrognathia. Cleft palate, including submucous clefts, are present in approximately 20% of probands (22,23).

### 164.2.4 Limb Involvement

Typical extremity findings range from small hands and small feet to more severe reduction defects of the upper limbs (Figure 164-2). Disproportional shortening of the



**FIGURE 164-1 Facial Characteristics in Cornelia de Lange Syndrome.** (A) Typically affected 1-year-old male, and (B) 19-year-old male, both with severe truncating mutations in *NIPBL*. Note characteristic facial features (arched eyebrows, synophrys, ptosis, anteverted nares, long philtrum, thin upper lip with down-turned corners and micrognathia) and severe asymmetrical defects of the forearms. (C) More mildly affected 3-year-old boy and (D) 19-year-old girl with missense mutations in *NIPBL*. Note characteristic but more subtle facial features. (E) 7-year-old girl and (F) 5-year-old boy with *SMC1A* mutations and mild facial characteristics.



first metacarpal, proximally placed thumb, brachydactyly, and clinodactyly can be seen in the majority of individuals, and single palmar creases in over half (23). Nearly one third of the probands have upper limb malformations, with severity ranging from various forms of oligodactyly to ulnar deficiency to absent forearm (Figure 164-2). Individuals with CdLS can also have radial head dysplasia with or without radioulnar synostosis resulting in incomplete elbow extension or dislocation (23). The lower extremities are less significantly involved (23) but are generally small, with 2–3 syndactyly of the toes and a short 4th metatarsal being commonly seen. In general, individuals with more significant limb involvement tend to also have more severe cognitive impairment.

### 164.2.5 Visceral Involvement

Almost all organ systems can be involved in CdLS to varying degrees. Gastroesophageal reflux disease (GERD) is nearly universal in CdLS and contributes to feeding problems in infancy and early childhood. Medical treatment is almost always indicated and surgical intervention is often required (17). Ongoing surveillance for GERD throughout the lifetime of an individual with CdLS is important as increased rates of esophageal damage and Barrett's esophagus have been noted consistently (22,24). Pyloric stenosis, diaphragmatic hernia, malrotation, and increased risk for volvulus formation have also been frequently reported (25).

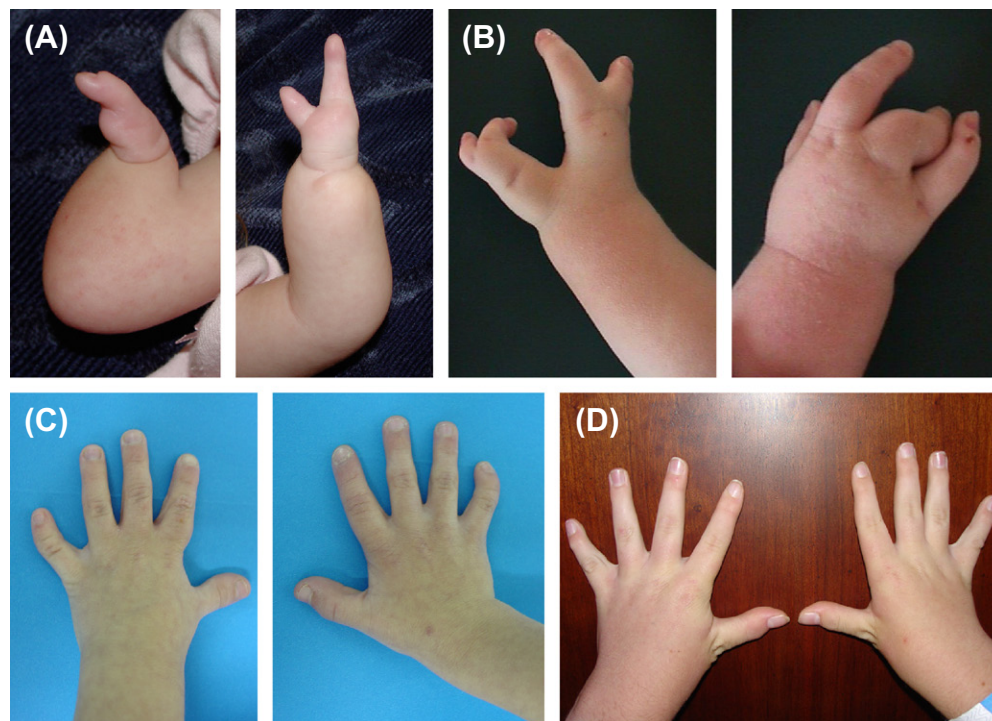
One-quarter to one-third of probands have congenital heart defects, most commonly represented as ventricular or atrial septal defects, although any lesion may be seen (26,27). Renal malformations and dysfunction can be seen as well, commonly represented by vesiculoureteral reflux, pelvic dilatation, and renal dysplasia (28). Major causes of morbidity and mortality in CdLS are gastrointestinal complications including diaphragmatic hernia in infancy, followed by aspiration pneumonia complicated by GERD and volvulus at older ages (23).

### 164.2.6 Additional Systemic Manifestations

**164.2.6.1 Skin.** Hypertrichosis is a common manifestation and is seen mainly on the face, back, and extremities. Cutis marmorata can also be seen in half of the probands (23).

**164.2.6.2 Ophthalmology.** Almost all the probands have peripapillary pigmentation. High myopia, ptosis, nasolacrimal duct obstruction, blepharitis, and mild forms of microcornea are most common. Nystagmus is less commonly seen. Cataract, glaucoma, and other eye malformations are rare (29).

**164.2.6.3 Audiology.** Auditory and vestibular anomalies include both sensorineural and conductive hearing loss, recurrent otitis media, and sinusitis (30,31). Hearing loss may be seen in up to 80% of probands (30,32).



**FIGURE 164-2 Upper Limb Involvement in Cornelia de Lange Syndrome.** (A and B) The more severe reduction defects/oligodactyly typical of the more severe form of CdLS, in this case in two children with severe *NIPBL* mutations. Note typical ulnar side involvement in (A) and characteristic asymmetrical limb involvement in both children. (C) Milder involvement of the hands in a child with a missense mutation in *NIPBL*, manifesting as small hands with 5th finger clinodactyly and short first metacarpal (proximal thumbs). (D) Similar mild hand findings to (C) in a child with an *SMC1A* mutation.

**164.2.6.4 Orthopedic.** Orthopedic manifestations, beyond the upper limb deficiencies described above, include hip dislocation or dysplasia, scoliosis, tight Achilles tendons, and delayed maturation of bone (10,33).

**164.2.6.5 Genitourinary.** In addition to the renal involvement noted above, genitalia are generally hypoplastic with cryptorchidism, micropenis, and hypospadias common in males, and small labia majora in females.

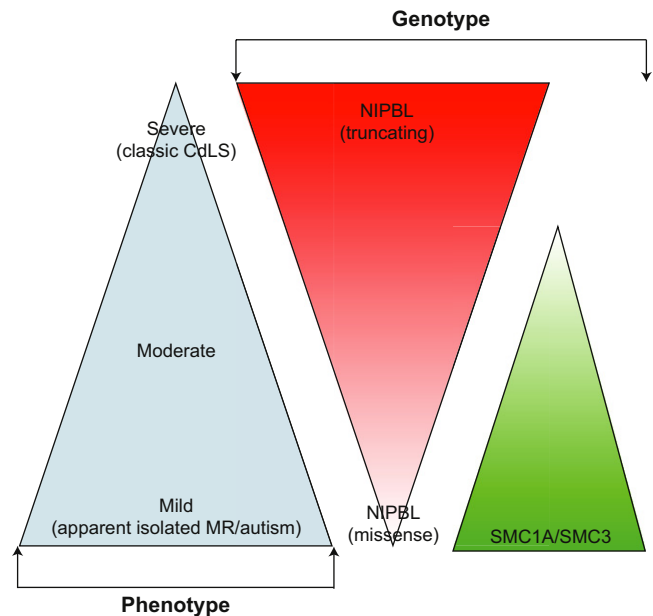
**164.2.6.6 Other.** Normal puberty occurs, although slightly delayed in some (22,23). Fertility is normal amongst less severely affected probands (34). Although less is known about the natural history of CdLS, a few reports describe large cohorts of affected adults. Premature aging has been suggested (22). Other than Barrett's esophagus, as described above, as a result of undiagnosed or poorly managed GERD, there is no obvious increased risk of cancer, although rare cases of liver hemangioendothelioma and Wilms' tumor were reported in autopsies from individuals with features of CdLS (35). Thrombocytopenia has also been consistently reported (36,37).

## 164.3 DIAGNOSIS

Diagnostic criteria and severity scales for CdLS have been proposed (19,38,39), although there is no single set of pathognomonic features of CdLS and diagnosis is based on a constellation of dysmorphism, cognitive, and growth parameters. Identification of a pathogenic mutation in *NIPBL*, *SMC1A*, or *SMC3* is considered diagnostic.

Approximately 60% of CdLS probands have a heterozygous mutation in *NIPBL*. Genotype–phenotype correlations amongst a large cohort of CdLS probands indicate that presumably haploinsufficient *NIPBL* mutations (truncating mutations, splice-site mutations, or frame-shifting indels) often result in a more severe cognitive and structural phenotype than do missense mutations (40). Approximately 5% of probands with a clinical diagnosis of CdLS were found to have missense or small in-frame deletion mutations in *SMC1A* (11,41,42), and one individual was found to have an in-frame 3-bp deletion in the *SMC3* gene (11). Individuals with *SMC1A* and *SMC3* mutations tend to have mild to moderate mental retardation without significant impairments in growth or structural abnormalities of the limb or other organ systems (11). Individuals with *SMC1A* mutations tend to have a more prominent nasal bridge than is typically seen in patients with *NIPBL* mutations (43), and the majority have normal growth parameters at birth and even later in life (Figure 164-1). For most individuals with *SMC1A* mutations, walking and speech are often acquired, and overall this group exhibits a much milder level of cognitive involvement (11). Figure 164-3 summarizes the genotype–phenotype correlations seen in CdLS.

Overall, approximately 65% of CdLS probands with a confident clinical diagnosis have mutations in one of the cohesin-associated genes (*NIPBL*, *SMC1A*, or *SMC3*). Amongst individuals with a more severe, or



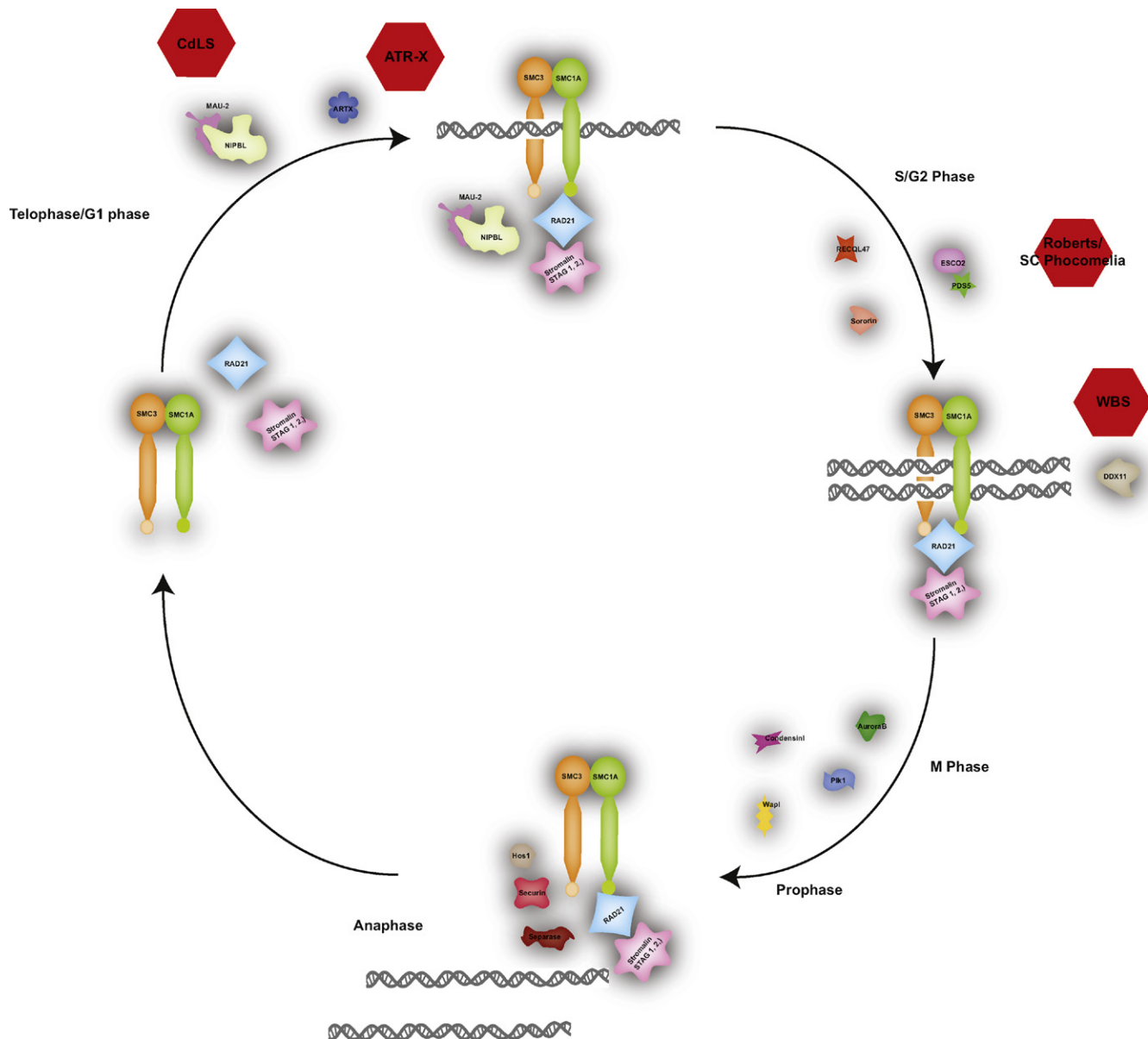
**FIGURE 164-3 Genotype–Phenotype Correlations in CdLS.** Left triangle represents distribution of severity seen in CdLS with the classic/severe phenotype being seen in the minority of probands (~30%) and the mild phenotype representing the majority of ascertained probands (some of which have been ascertained as apparent isolated mental retardation and/or autism, who upon referral to genetics were recognized as having subtle features of CdLS). The middle inverted triangle represents the distribution of *NIPBL* mutations identified with the majority of probands, with the classic/severe phenotype having truncating mutations and the majority of probands with a mild phenotype having missense or other frame-preserving mutations. The triangle on the right represents the distribution of mutations seen in *SMC1A* and *SMC3*, with the majority being seen in mildly to moderately affected probands and not seen in the severe/classic form of CdLS.

“classic,” CdLS phenotype, the mutation detection rate is even higher (~80%) and much more likely to involve the *NIPBL* gene. The molecular etiology of the remaining 35% of probands is unknown at this time. Mutations in the regulatory sequences of these three genes may account for a small percentage of cases and are not routinely screened for in clinical testing. Additional cohesin pathway genes are likely to be implicated as causative of CdLS when mutated, and with over 25 known genes implicated in the cohesin complex and its regulation there are many potential candidates.

## 164.4 GENETICS

### 164.4.1 Cohesin Biology

While mitotic chromosomes were first described in the late 1800s (44), it was not until 1985 that the first yeast mutant involved in maintaining the stability of chromosomes through mitosis was described. Termed *SMC1* for stability of mini chromosomes, (45), the gene was subsequently cloned in 1993 (46). With the identification of additional structurally related proteins, “SMC”



**FIGURE 164-4 Cohesinopathies.** Human disorders associated with mutations in cohesin and its accessory proteins. SMC1A, SMC3, RAD21, and either SA1 or SA2 are the four major subunits of cohesin in somatic vertebrate cells. RAD21 crosslinks the head domains of SMC1A and SMC3, in an ATP-dependent manner, whereas RAD21 also binds to the fourth cohesin subunit, SA. NIPBL and MAU-2 form a complex and facilitate cohesin loading and unloading. Sister chromatid cohesion is established during S phase, mediated by sororin and ESCO1/ESCO2, after which PDS5 maintains cohesion through G2 phase. The removal of cohesin from the chromosome arms starts at prophase and is regulated by PLK1, aurora B kinase, condensin I, and WAPL. During the metaphase-to-anaphase transition, the separase inhibitor securin is degraded by APC thereby activating separase, which in turn cleaves centromeric cohesin as well as residual cohesin on the chromosome arms. Recently, ESCO1 and its yeast homolog Eco1 were identified as acetyltransferases required for the acetylation of human SMC3, a necessary step to establish the cohesiveness of chromatin-loaded cohesin during S phase. This acetylation counteracts the effects of WAPL and PDS5, two additional regulatory proteins. SMC3 is subsequently deacetylated during anaphase by yeast Hos1. Cohesin cleavage by separase at the onset of anaphase triggers SMC3 deacetylation, and SMC3 molecules that remain acetylated after mitosis due to Hos1 inactivation are unable to generate cohesion during the subsequent S phase. Human disorders are listed corresponding to their disease-causing genes in the cohesin pathway and at which point in the cell cycle these respective genes are active.

proteins were renamed “structural maintenance of chromosome” proteins (47). The term “cohesins” was coined (48) to clarify the involvement of yeast Smc1, Smc3 and Scc1(Rad21) in a “common cohesion apparatus for all eukaryotic cells in mitosis and meiosis.” Since that time, over 25 proteins have been implicated in cohesin’s

canonical role of controlling appropriate sister chromatid segregation during the mitotic cell cycle.

Cohesin is a dynamic complex regulated at various cell cycle stages by multiple mechanisms (Figure 164-4) (49). The canonical role of cohesin, as first described in yeast, is to control sister chromatid segregation during



both mitosis and meiosis. Four evolutionarily conserved subunits form the core structural component of the cohesin complex, two SMC proteins, SMC1A and SMC3, a kleisin protein RAD21 (also known as MCD1 or SCC1), and STAG1/STAG2 (also known as SA1/SA2). Paralogs SMC1B, REC8, and STAG3, respectively, are cohesin subunits with specialized roles in meiosis (50). Homologs of the cohesin complex and its regulatory genes have been identified in all eukaryotic model systems, including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Xenopus laevis*, mouse, and in humans (51).

The protein structures of SMC1A and SMC3 are very similar; each spans 1000–1500 amino acids and contains, at the N- and C-terminal, globular domains each separated by alpha helical structures from a globular “hinge” domain in the middle. The molecule folds around the mid-portion hinge domain to form an antiparallel coiled coil, bringing together the N- and C- domains to form a “head” domain. This cohesin head domain contains three highly conserved motifs: Walker A, Walker B, and a signature motif, that form an ATPase domain (52,53). SMC1A and SMC3 dimerize at the hinge domains to form a V-shaped structure. The N terminus of RAD21 interacts with the head domain of SMC3 and the C terminus interacts with the head domain of SMC1A, with ATP being required for the RAD21 and SMC1 association (54). RAD21 crosslinks the SMC heterodimer, lies in close proximity to the ATPase active sites in cohesin head domains, and binds to the fourth subunit of the cohesin ring structure, STAG1/STAG2 (55). The two prevailing models of cohesin’s mechanism of action in sister chromatid cohesion include the “topological” and “handcuff” models. The “topological” model proposes that the cohesin ring opens to allow sister chromatids to enter the ring and become topologically entrapped when RAD21 relocks the ring (54,56). The “handcuff” model proposes specific interactions of DNA–protein and protein–protein, with one cohesin ring binding one single chromatid after which inter-cohesin complex oligomerization occurs (56–58).

Cohesin has been demonstrated to bind to chromatin. AT-rich cohesin-associated regions (CARs) are distributed at roughly 15-kb intervals at transcriptional convergent sites during G1/S phase in budding yeast (59,60). By contrast, in vertebrates, binding happens during telophase of the preceding cell division. Cohesin also binds at G2/M phase when a double-strand DNA break is created (61). Removal of cohesin from chromosome arms commences in prophase and is completed by early anaphase (Figure 164-4). Although no consensus DNA sequence for cohesin binding has been identified, cohesin binding is enriched at heterochromatin (62) and the chromatin surrounding a DNA double-strand break (DSB) (63).

NIPBL heterodimerizes with hSCC4 (MAU-2) and is required for loading of cohesin onto chromatin in

mitosis, and to all chromosome regions currently under study, such as heterochromatin, CARs, centromeres, and DSBs (63,64). Despite its importance, the mechanism of NIPBL loading of cohesin onto DNA is poorly understood. The NIPBL/MAU-2 complex seems to be involved in all cohesin activities, including SMC ATPase activation, hinge dimerization, chromatin binding, and chromatin remodeling (56). Cells elaborately regulate chromatin binding of cohesin, both temporally and spatially, although most of these mechanisms are unknown at present. This complex regulation enables cohesin to perform diverse biological functions and it is anticipated that mutations in associated proteins will contribute to multiple human diseases, both known and unknown. Cohesin-independent mechanisms of cohesion (e.g. condensin complexes (65), ORCs (origin recognition complexes) (66), centromere complexes (67), and DNA catenation (68)) are also suggested to exist, as complete unpairing of sister chromatids cannot be achieved by knocking down cohesin function alone.

Removal of cohesin from chromosomes is equally complex, with disassociation from the chromosome arms during prophase, while the pericentric cohesin is protected, until the onset of anaphase. In vertebrates, both cohesin subunits STAG2 and RAD21 are substrates of polo-like kinase 1 (Plk1). A functioning Wapl is also required for prophase removal (69–71). Shugoshin/MEIS-322/Sgo1 (72) and sororin (73) protect centromeric cohesins. An evolutionarily conserved protein, Pds5/BimD/Spo76, interacting with Wapl, sororin, and Eco1, is also involved in maintaining pericentric cohesion in prophase (74). Inactivation of cohesion and the complete dissolution of cohesin at the onset of anaphase enable faithful sister chromatid segregation, regulated by securin and separase. Securin binds and inhibits protease activity of separase (Esp1) before anaphase (75). At the beginning of anaphase, securin is degraded by APC (anaphase-promoting complex) and separase is activated (76). The active separase cleaves RAD21 and the cohesin complex is further degraded (75).

Human ESCO1 and its yeast homolog Eco1 are acetyltransferases required for the acetylation of human SMC3 at lysines K105 and K106, a necessary step to establish the cohesiveness of chromatin-loaded cohesin during S-phase (77–79). This acetylation counteracts the effects of WAPL and PDS5, two additional regulatory proteins, which together play roles in dynamically counteracting cohesion (77,78,80–82). SMC3 is subsequently deacetylated during mitosis, and yeast HOS1, a class I histone deacetylase, was identified as the enzyme effecting this role during anaphase (83–85). Cohesin cleavage by separase at the onset of anaphase triggers SMC3 deacetylation, and SMC3 molecules that remain acetylated after mitosis due to Hos1 inactivation are unable to generate cohesion during the subsequent S phase.



### 164.4.2 Cohesin Function and the Etiologies of CdLS and Other Cohesinopathies

With the identification of mutations in cohesin regulatory and structural components that resulted in human developmental disorders (Table 164-1) it was initially speculated that cohesion or cell cycle defects were the cause of the developmental deficits. An early study in a large cohort of CdLS samples suggested that precocious sister chromatid separation (PSCS) was identified in a small percentage of cells in 41% of probands' lymphoblastoid cell lines (LCLs) versus 9% in healthy controls (86). Although, the PSCS noted in these probands was present in a small percentage of any individuals cells and did not involve all sister chromatids. However, subsequent studies have not found obvious cohesion defects in CdLS cells (87). In contrast, in Roberts syndrome (see Section 164.5.1), cells from probands show "heterochromatic repulsion," which was demonstrated by premature sister-chromatid separation primarily at the heterochromatic regions on prophase and metaphase chromosomes (88). It is unclear whether or not the sister chromatid defects seen to varying degrees (or not all) in the cohesinopathies contribute to the phenotypic manifestations of these diagnoses.

Accumulating evidence is suggesting that the non-canonical role of cohesin in regulating gene expression may play an important pathogenic role in CdLS and other cohesinopathies (Figure 164-5). The initial critical evidence for cohesin-mediated gene regulation came from screens that identified the *Drosophila* *Scs2* homolog, *Nipped-B*, to be required for long-range activation of the homeobox genes *cut* and *Ultrabithorax* (*Ubx*) (89,90). The heterozygous *Nipped-B* null alleles reduced *Nipped-B* mRNA only by 25% (a very similar pattern is observed in human (91) and mouse (92) expression studies) with presumed upregulation of the non-mutant allele. Further reduction to 50% wild-type levels by in vivo RNA interference (RNAi) is lethal; however, cohesion defects were not observed. Cohesion defects are seen in the homozygous *Nipped-B* mutant flies; however, these result in

early embryonic lethality (90). Whole-genome mapping of cohesin and Nipped-B chromatin binding sites in *Drosophila* has revealed strong co-localization with cohesin, suggesting that Nipped-B and cohesin remain associated on the chromatin after the loading process (93).

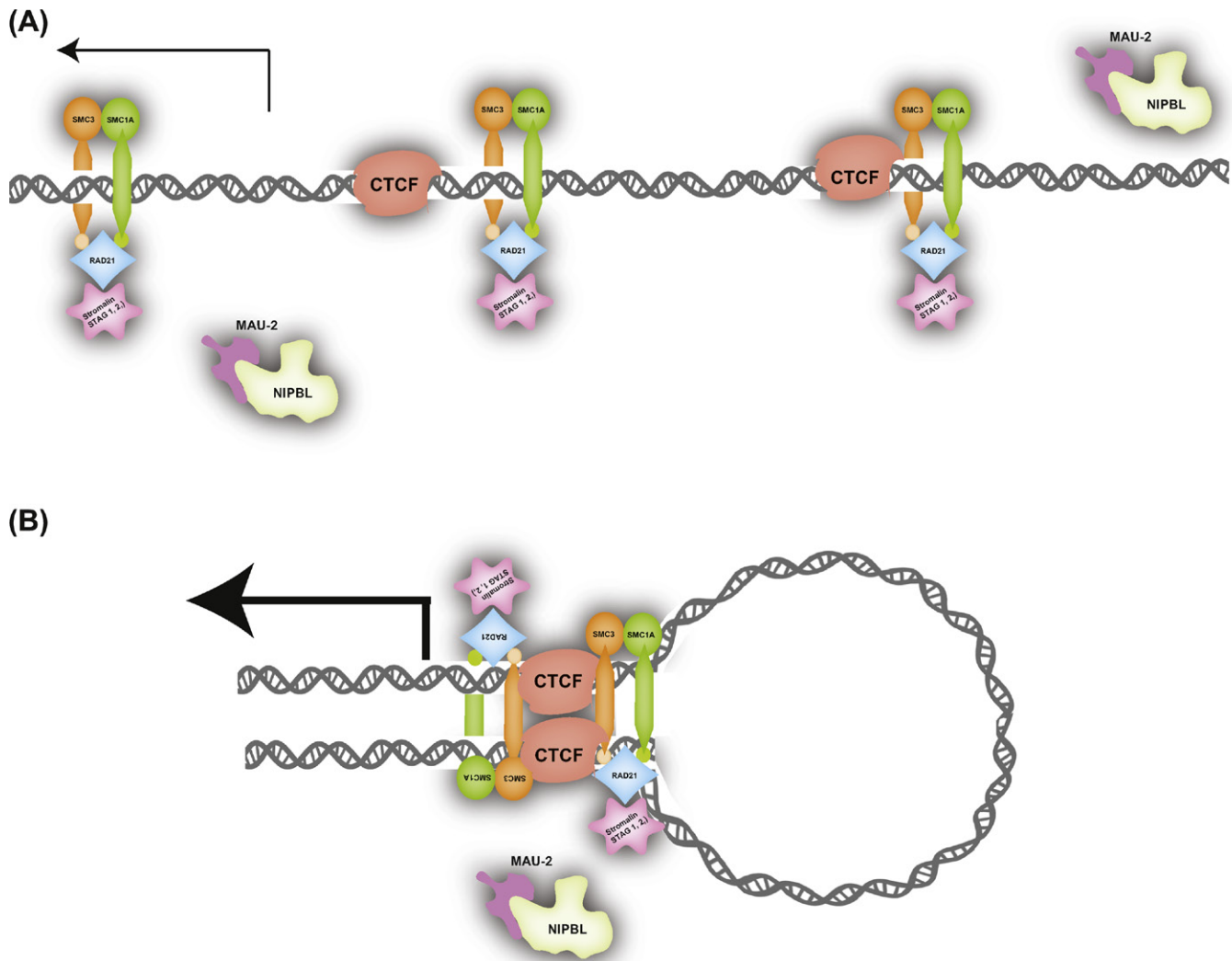
Further support for a role of cohesin in gene regulation has been demonstrated in settings that are independent of sister chromatid cohesion. Homozygous *Drosophila* mutants of *Smc1*, *STAG*, and *Rad21* have shown defective axon pruning of the postmitotic gamma neurons in the developing mushroom body (94,95). As these observations occurred in postmitotic (non-dividing) cells, chromosome segregation and cell cycle regulation are clearly not involved. In zebra fish, the functions of *Rad21* and *Smc3* are needed for proper expression of *runx1* and *runx3* genes in early embryonic development (96).

In addition, a growing body of evidence indicates that cohesin interacts with multiple transcriptional regulatory complexes. Genome-wide chromatin immunoprecipitation experiments in human and mouse cells have identified co-localization of cohesin and CTCF, a zinc-finger protein with enhancer blocking/boundary activities (97–99). Knockdown of either cohesin or CTCF would influence genome-wide gene expression levels, and knockdown of cohesin alone resulted in dysregulated gene expression of CTCF targets (97,98).

Genome-wide gene expression and chromatin immunoprecipitation (ChIP) array studies in lymphoblastoid cell lines have revealed a highly conserved transcriptional profile in CdLS probands that tightly correlates with cohesin binding status (91). In addition, the cohesin complex is necessary for proper function of the transcriptional regulatory mediator complex to maintain expression of pluripotent markers in mouse embryonic stem cells (100). Furthermore, recent work has also demonstrated that the  $\beta$ -globin complex is regulated by cohesin and its chromatin interactions (101). Collectively, data suggest that an important mechanistic action of cohesin complex mutations involves a defect in transcriptional regulation.

TABLE 164-1 Cohesinopathies

Diagnosis	Gene	Chromosome Location	Phenotype Features	Inheritance
Cornelia de Lange syndrome	<i>NIPBL</i>	5p13.1	Severe, moderate and mild CdLS	Autosomal dominant
	<i>SMC1A</i>	Xp11.2	Physically milder CdLS, significant cognitive impairment	X-linked dominant
	<i>SMC3</i>	10q25	Physically milder CdLS, significant cognitive impairment (only one proband identified)	Autosomal dominant
Roberts syndrome/SC phocomelia	<i>ESCO2</i>	8p21.1	No established genotype–phenotype correlation	Autosomal recessive
Warsaw breakage syndrome	<i>DDX11</i>	12p11	Only one proband identified to date	Autosomal recessive
Alpha-thalassemia/mental retardation syndrome, X-linked	<i>ATR-X</i>	Xq13		X-linked recessive



**FIGURE 164-5 Model Depicting Cohesin's Role in Regulation of Gene Expression.** Cohesin and its regulatory proteins have been implicated in regulation of gene expression across multiple species. The disruption of its gene regulatory function is likely the cause of many of the specific developmental features seen in the cohesinopathies. (A) DNA strand with low basal levels of expression (indicated by arrow) across a region where cohesin and CTCF are bound. (B) At specific genomic regions where cohesin and CTCF interact, DNA looping is facilitated and gene expression is altered (in this case upregulated) by the apposition of regulatory elements with promoters.

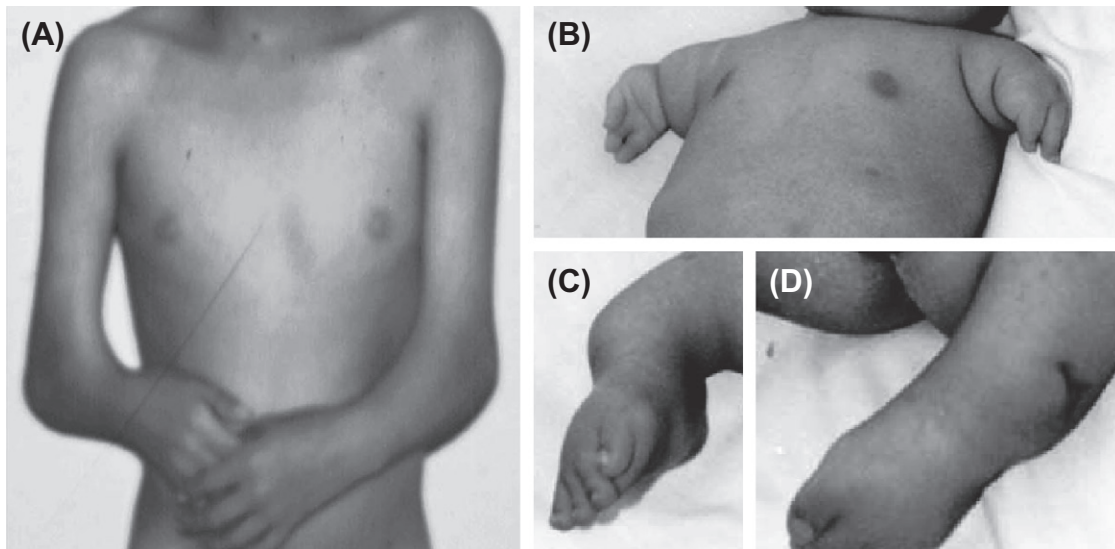
Currently, 35% of clinically diagnosed CdLS probands have no identifiable mutation in one of the three implicated genes. This suggests other disease-causing genes remain to be discovered or regulatory regions of known genes may be involved. Clear candidates for CdLS or similar disorders would include the other components of the cohesin ring: RAD21 and STAG1/STAG2, or other proteins that interact with or regulate cohesin and modulate its chromatin association dynamics, for example, PDS5A/PDS5B, WAPAL, and sororin.

## 164.5 OTHER DISORDERS OF COHESIN AND SISTER CHROMATID COHESION

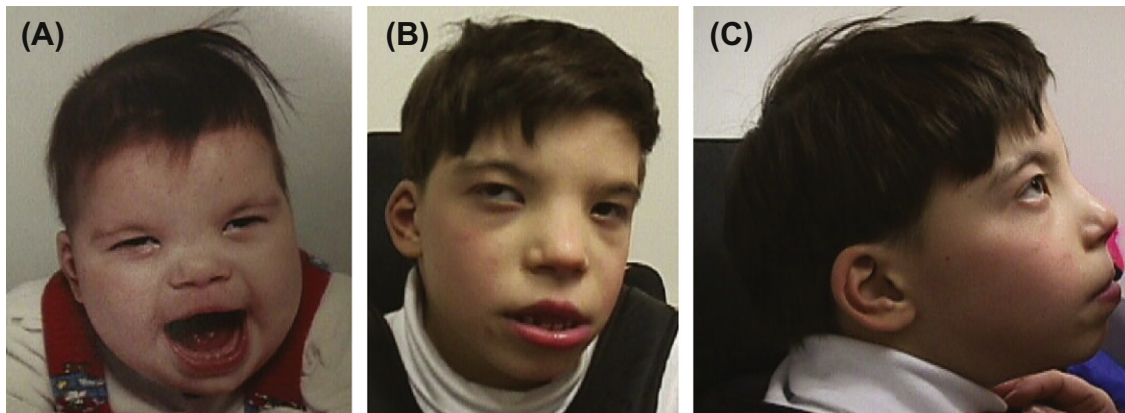
### 164.5.1 Roberts/SC Phocomelia Syndrome

Roberts/SC phocomelia syndrome (RBS/SCP; OMIM #268300) is an autosomal recessive genetic disorder

caused by homozygous or compound heterozygous mutations in the *ESCO2* gene (102,103). The clinical features of this syndrome are distinct from CdLS but display systemic overlap (104). The consistent features seen in RBS include pre- and postnatal growth retardation, microcephaly, bilateral cleft lip and palate, symmetric mesomelic limb shortening ("phocomelia") (Figure 164-6), genitourinary abnormalities, and congenital heart defects. The yeast homologous gene *Eco1*/Ctf7 acetyltransferase is required for the establishment and stabilization of sister chromatid cohesion but not for binding of cohesin to chromosomes (105,106). In fission yeast, *Eco1* physically interacts with *Pds5* to counteract its inhibition of cohesion (106). In budding yeast, *Eco1* acetylates *Smc3*'s head domain in a cell-cycle-dependent manner to promote sister chromatid cohesion (77,78). The *Drosophila* *Eco1* ortholog specifically establishes cohesion at centromeres, and not along the chromosome



**FIGURE 164-6 Limb Involvement in Roberts/SC Phocomelia Syndromes.** (A) Shortening of long bones of arms with contractures seen in SC phocomelia. (B, C, and D) More severe involvement of the upper (B) and lower (C and D) extremities of a child with Roberts syndrome. (Photos courtesy of Dr Hugo Vega).



**FIGURE 164-7 Facial Features in ATR-X.** Facial features in a young boy with ATR-X. (A) at 1 year of age, and (B) full face and (C) profile at 8 years of age.

arms (107). Chromosome cohesion defects are seen in cells from RBS/SCP probands demonstrating “heterochromatic repulsion” by premature sister chromatid separation, primarily at the centromeric regions on prophase and metaphase chromosomes (88). A new study has shown that most mutations in *ESCO2* identified in RBS probands result in disruption of the acetyltransferase domain. This results in faulty cohesion and other cellular events in RBS cell lines, indicating the acetyltransferase activity contributes to the development of the major organ systems affected in RBS (108,109). Of interest, studies in zebra fish demonstrated a very different pattern of gene dysregulation in RBS/SCP from that seen in CdLS, with many dysregulated genes involved in cell cycle and apoptotic pathways (110). This suggests that cell proliferation defects and apoptosis may play a significant role in the RBS/SCP phenotype rather than gene dysregulation as suggested in CdLS (110).

### 164.5.2 Disorders Demonstrating Cohesion Defects

Some disorders have associated cohesion defects but the causative proteins have not themselves been directly linked to the cohesin complex or its regulation but clearly interact at some level.

**164.5.2.1 Alpha-Thalassemia/Mental Retardation Syndrome, X-Linked.** Alpha-thalassemia/mental retardation syndrome, X-linked (ATRX; OMIM #301040), is a multisystem disorder characterized by postnatal growth and mental deficiency, microcephaly, dysmorphic craniofacial features (hypertelorism, midface hypoplasia, anteverted nares, and full lips with protruding tongue) (Figure 164-7), lack of speech, seizures, and abnormal genitalia in males. Affected individuals usually have a mild form of hemoglobin H (Hb H) disease. ATRX is caused by mutations in *ATRX* on the X chromosome (111).



*ATRX* encodes a chromatin remodeling enzyme that associates with the chromoshadow domain of HP1 $\alpha$  (as does NIPBL) and preferentially localizes to the pericentromeric heterochromatin in mouse and human cells (112). *ATRX* was suggested to have a role in loading cohesin onto chromatin during S phase and recruiting cohesin to specific chromosome loci (112). Mitotic progression is disrupted in cultured human cells with depleted *ATRX*; defective sister chromatid cohesion and chromosome movement to spindle equator were present at the same time. Similar findings were seen in embryonic mouse brains with no *ATRX* protein (112). The impaired cohesin targeting or transportation due to mutations in *ATRX* may therefore contribute to the clinical phenotypes in *ATRX* syndrome.

**164.5.2.2 Warsaw Breakage Syndrome.** A single male child with severe microcephaly, pre- and postnatal growth retardation, and abnormal skin pigmentation was found to have mitomycin C (MMC)-induced chromosomal breakage in fresh T-lymphocyte cultures, as well as in Epstein-Barr virus (EBV)-immortalized B lymphoblasts (113). Striking centromeric cohesion (“railroading”) and premature chromatid separation (PCS) defects were seen in 50–60% of cells. Reduced levels of the helicase DDX11 were identified and sequencing revealed compound heterozygous mutations. DDX11 is the ortholog of yeast Chl1, and short interfering RNA (siRNA) experiments in human cells point to a role for DDX11 in sister chromatid cohesion.

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### Biographies



**Matthew A Deardorff, MD, PhD**, is Assistant Professor of Pediatrics, in the Division of Human Genetics, at The Children's Hospital of Philadelphia and The Perelman School of Medicine at the University of Pennsylvania.

Dr Deardorff trained as a pediatrician and clinical geneticist with a focus in dysmorphology. He has extensive research experience in developmental biology and human developmental disorders. Clinically, Dr Deardorff's pediatric genetics practice focuses on the diagnosis and management of children with rare genetic disorders, with an emphasis on skeletal dysplasias, overgrowth, limb defects, Cornelia de Lange syndrome, and related diagnoses. His research has focused on identifying the molecular etiology of human developmental disorders and understanding the molecular and developmental mechanism by which these identified causative gene changes result in specific phenotypes.



**Ian D Krantz, MD**, is Professor of Pediatrics, in the Division of Human Genetics, at The Children's Hospital of Philadelphia and The Perelman School of Medicine at the University of Pennsylvania.

Dr Krantz trained as a pediatrician and clinical geneticist with a focus in dysmorphology. His clinical interests lie in the diagnosis and management of individuals with multisystem developmental diagnoses. Towards this end, Dr Krantz has established multispecialty clinics to care for individuals with Cornelia de Lange syndrome, Alagille syndrome, Pallister–Killian syndrome, and related developmental diagnoses. His research interests focus on understanding the molecular controls of syndromic human developmental disorders (with a focus on Cornelia de Lange syndrome and Pallister–Killian syndrome) as well as isolated birth defects and sensory deficits (such as congenital heart defects and hearing impairment), and the development of therapeutic strategies for these diagnoses. Dr Krantz is currently the director of the Medical Genetics Training Program at the Children's Hospital of Philadelphia.



Genes and Mechanisms in  
Human Ciliopathies*Dagan Jenkins and Philip L Beales*

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**GLOSSARY**

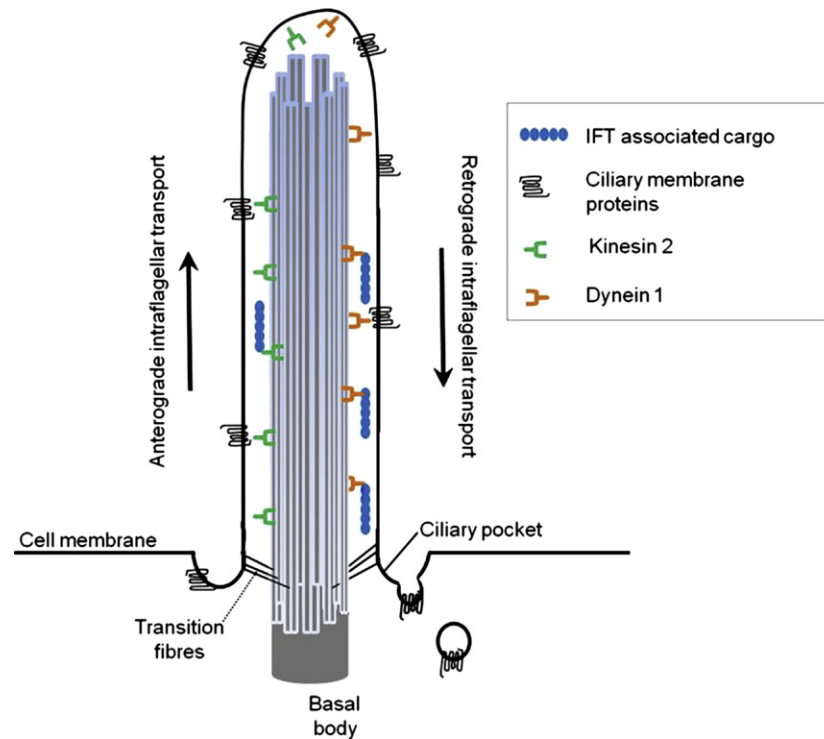
- Brachydactyly type A1** – shortening/absence of the middle phalanges in the hands or feet
- Molar tooth sign** – a midline keyhole-shaped defect in the occipital bone dorsal to the foramen magnum commonly found in patients with Joubert syndrome and other ciliopathies
- Polydactyly** – formation of supernumerary digits in either the hands or feet
- Primary ciliary dyskinesia** – a group of disorders defined by primary ultrastructural defects in motile cilia, leading to phenotypes such as brachiectasis, situs inversus and infertility
- Retinitis pigmentosa** – progressive retinal dystrophy caused by degeneration of the photoreceptors in the retinal pigmented epithelium, typically leading to legal blindness in the fourth or fifth decades of life
- Short-rib polydactylies** – several dwarfism syndromes which constitute a subgroup of ciliopathies, characterized by severe shortening of the ribs causing constriction of the thorax and often leading to difficulties with breathing (asphyxiating thoracic dystrophy)
- Situs inversus** – complete mirror image reversal of organ asymmetries. The normal arrangement is known as situs solitus

**165.1 CILIA STRUCTURE AND FUNCTION**

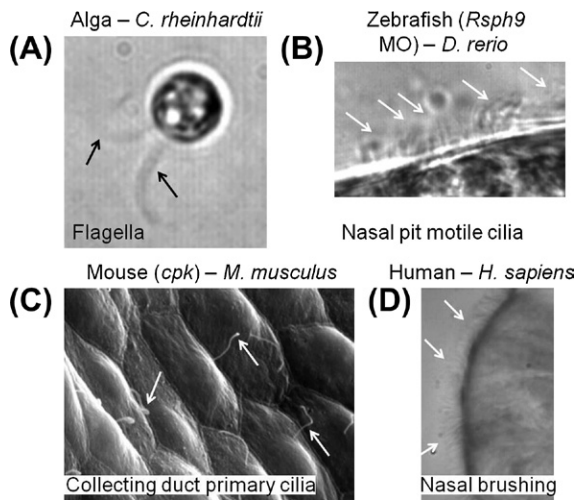
Cilia are microtubular protrusions present on the apical surface of most cells. They consist of a ciliary axoneme, composed of nine outer microtubule doublets (1,2) (Figure 165-1). Some cilia also have a central pair of microtubules, and are therefore known as “9+2” cilia, while others do not have a central pair and are referred to as “9+0” cilia. Classically, “9+2” cilia are motile, exhibiting very regular patterns of beating to generate fluid flow in structures such as the respiratory epithelium (Figure 165-2). The outer doublets are connected by both inner and outer dynein arms that hydrolyze ATP to generate the force necessary for

movement of the cilium (Figure 165-3). Furthermore, radial spoke proteins link the inner and outer doublets in motile cilia, and are essential for proper cilia motility, with mutations in radial spoke proteins causing primary ciliary dyskinesia (PCD). In contrast, “9+0” cilia are generally non-motile, and are referred to as primary cilia to account for the observation that almost all cells within the body will at some point form a single “9+0” cilium. Although generally true, it must also be noted that variations on this grouping into “9+0” and “9+2” cilia have been described (3).

The ciliary axoneme is anchored within the cell by a cytoplasmically located structure known as the basal body, that consists of a mother and a daughter centriole, which are complex microtubule-based structures linked by a fibrous mesh. The centrioles organize a pericentriolar matrix (PCM), which comprises a heterogeneous group of proteins that serve to nucleate microtubules, thereby acting as the major microtubule-organizing center within the cell (4,5). Cilia formation is intimately linked to the cell cycle. The centrosomes are essential for formation of the mitotic spindle, and the centrioles themselves undergo duplication in G2 phase to ensure that both daughter cells have two centrosomes following cell division (6). Cilia only form in quiescent cells in G0 phase, where the centrosomes are recruited to the cell surface to form a basal body and to nucleate the ciliary axoneme. The number of cilia can also vary in different cell types. Whereas most cells will form a single primary cilium at some point, some cells, such as those in the center of the embryonic node, form a single motile cilium. By contrast, some specialized cells, such as respiratory epithelia in the mammalian lung or cells adjacent to mucus-secreting cells on the epidermis of *Xenopus* embryos, can have >50 motile cilia. Whatever the type of cilium, each has its own basal body. Therefore, the formation of many cilia in certain cell types is preceded by massive duplication of centrioles and their subsequent apical docking.



**FIGURE 165-1** The cilium. Schematic representation of a cilium demonstrating the microtubular axoneme surrounded by membrane. Anterograde and retrograde transport via kinesin 2 and dynein 1 are shown on the left and right of the cilium, respectively. Also shown are the ciliary pocket, transition fibers and basal body (see text for details), and as well as vesicles fusing with and budding from the basal membrane.

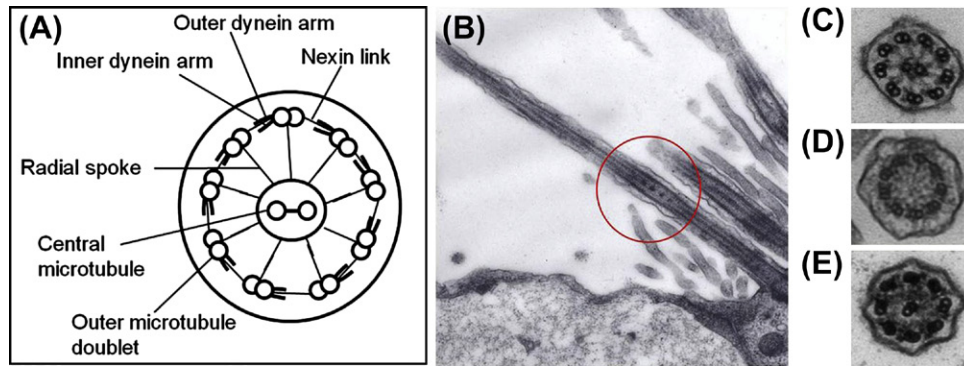


**FIGURE 165-2** Cilia in diverse epithelia and organisms. (A) Flagella in the free-swimming alga, *Chlamydomonas reinhardtii*. (B) Motile (9+2) cilia in the nasal pit of zebra fish (shown is an embryo injected with a morpholino targeting *Rsp9*; cilia do form in these “morphants,” but cilia motility is perturbed.) (C) Scanning electron microscopy showing primary cilia (9+0) in the collecting duct epithelia of *Cpk* mutant mice (these cilia form normally although signaling is perturbed.) (D) Motile (9+2) cilia of normal human nasal scrapings. (A and B reproduced from Castleman et al. (14), with permission; C reproduced from Jenkins and Winyard 2011, with permission; D—picture courtesy of Amelia Shoemark, Royal Brompton Hospital.)

Cilia/flagella are ancient organelles found in most eukaryotes. Two organisms, which have illuminated the biology of cilia formation are *Chlamydomonas reinhardtii* and the nematode worm, *Caenorhabditis elegans*. *C. reinhardtii* consists of only two cells, each of which

has a single flagellum used for swimming (Figure 165-2; Videos 165-2, 165-3). These flagella have essentially the same structure as mammalian cilia. Indeed, transport of the proteins needed to build the cilium occurs by the same processes as required to build flagella, hence it is termed intraflagellar transport (IFT). The evolutionary conservation of this process is exemplified by the *Ift88* gene, which encodes polaris in mice, and which leads to a complete loss of cilia when inactivated in either mouse, *C. reinhardtii*, or *Ca. elegans* (7–10). Following docking of the basal body and initial nucleation of the ciliary axoneme, so-called “anterograde” movement of various cargo to the tip of the cilium is driven by kinesin-2, a molecular motor that travels toward the “minus” end of microtubules; a second molecular motor known as dynein travels toward the “plus” end of microtubules, toward the cytoplasm, to drive retrograde IFT (11) (Figure 165-1). *Ca. elegans* has specialized ciliary axonemes in certain sensory neurons, and speeds of IFT of around 1  $\mu\text{m/s}$  have been recorded in these cells and also in cultured mammalian cells (12,13). A large and growing family of IFT proteins has been identified, which are essential for cilia formation. IFT proteins are also grouped into those that regulate either anterograde (group B) or retrograde (group A) IFT.

Although the membrane encasing the cilium appears to be contiguous with the rest of the cell membrane, it is now evident that there is regulated movement of both proteins and lipids into the cilium, which means that membrane composition is different in these two areas (15). A critical component of this diffusion barrier at the base of the cilium may be septin 2, a member of the



**FIGURE 165-3** Structure of the ciliary axoneme. (A) Diagram showing the structure and various components of a 9+2 motile cilium in cross-section. (B) Transmission electron microscopy showing a longitudinal section through a motile cilium from a patient with primary ciliary dyskinesia caused by mutation in *RSPH9*. Circle indicates intermittent loss of the central microtubular pair owing to loss of this radial spoke protein. (C) Transverse section through a normal cilium, showing the various components. (D) Patient with *RSPH9* mutation—note absence of central pair. (E) Patient with *RSPH4A* mutation showing abnormal 8+1 structure. (Reproduced from Castleman et al. (2009) (14), with permission.)

guanosine triphosphatase (GTPase) family conserved from yeast (16). Loss of this factor disrupts ciliary membrane protein localization (16,17). Recent evidence has also identified vesicle trafficking proteins with highly specific roles in trafficking various components to cilia. Rab proteins are archetypal vesicle transport proteins, and a screen of 46 Rabs identified only three that were necessary for cilia formation (18). A ciliary necklace (5) and “ciliary pore complex” (19) have also been reported in various species, functioning as barriers through which only selected proteins are allowed passage into the ciliary compartment. The corollary of this is that selective transport will increase the concentration of specific proteins within cilia, which may facilitate targeted interactions. More recently, Molla-Herman et al. described the ciliary pocket, a depression of the plasma membrane in which the primary cilium is rooted (20); the pocket shares many morphologic features with the flagellar pocket of Trypanosomatids, which is a trafficking-specialized membrane domain at the base of the flagellum. The pocket is observed around virtually all primary cilia in mouse retinal pigment epithelial cells but it cannot be absolutely essential for function since it is not universal among kidney cells for example (20).

## 165.2 CLINICAL ASPECTS OF CILIOPATHIES AND CILIOPATHY-LIKE DISORDERS

### 165.2.1 Definition of Ciliopathy

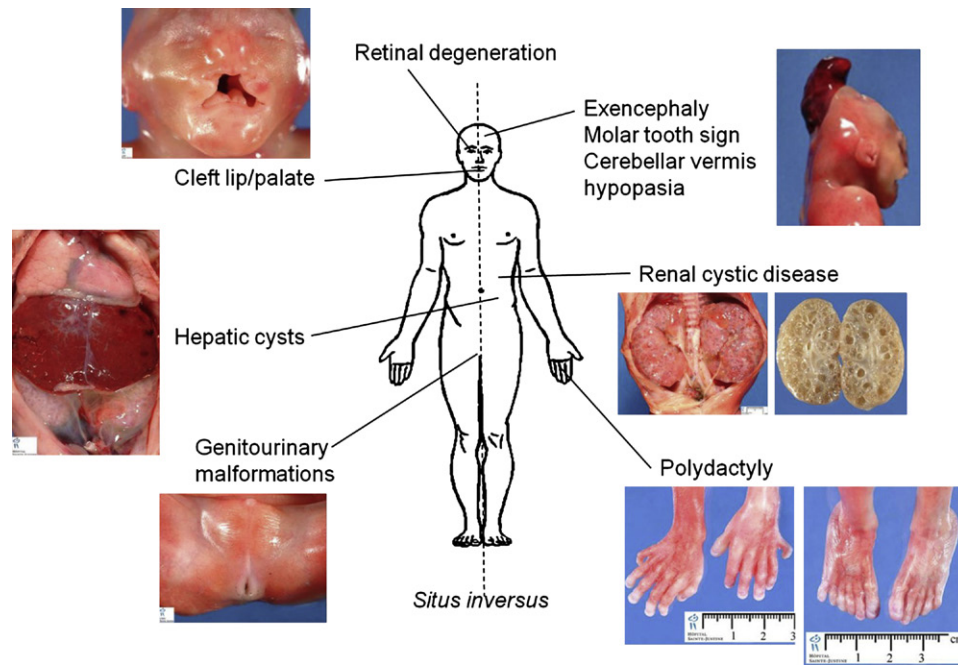
The term “ciliopathy” was first coined in 1984 by Corniliet et al. (21) to describe the varied ultrastructural abnormalities of motile (9+2) cilia observed in the respiratory tracts of children with recurrent respiratory tract infections. This term was subsequently adopted by Badano et al. (3) in its current form, in reference to the varied phenotypes caused by mutations that disrupt cilia or basal body formation. These authors noted a subset of clinical features shared by each of five clinically distinct

syndromes that fall into this category, implicating ciliary dysfunction in their pathogenesis. Since then, the term ciliopathy has variably been used to describe both individual clinical features as well as entire syndromes. The term ciliopathy has also been used rather more speculatively either to describe syndromes featuring defects characteristic of ciliary dysfunction, without evidence that the causative gene is a ciliary protein, or for diseases in which the causative gene has a putative ciliary function even though the clinical features are not characteristic of ciliary dysfunction. To illustrate this point, patients with mutations in *XPNPEP3* develop nephronophthisis, a cardinal ciliopathy (see later), yet this gene encodes a mitochondrial protein that does not appear to function within cilia (22). This more speculative usage has detracted from the original intent of grouping together these disorders.

In this chapter, we will therefore restrict use of the term ciliopathy only to describe phenotypes that can be caused by mutations in several different genes that have been shown to function in cilia, and where there is experimental evidence from model systems that the disease can be caused by ciliary dysfunction (Figure 165-4). While the term ciliopathy is sometimes used more widely to describe whole syndromes, it is likely that some syndromic features are unrelated to ciliary dysfunction, because many ciliary proteins are also likely to have additional functions independent of cilia.

### 165.2.2 An Overview of the Data Summarized in This Chapter

A large repository of information regarding the ciliopathies has accrued from a variety of sources over the last two decades, including human genetics, basic cell biology, proteomics, and biochemical studies. Whereas previous reviews about ciliopathies have variously used databases such as the Online Mendelian Inheritance in Man database or the London Dysmorphology Database to predict ciliopathy disorders on the basis of shared phenotypes, these databases were constructed before the



**FIGURE 165-4** Summary of ciliopathies. Range of ciliopathies are summarized using pictures of Meckel syndrome fetuses to exemplify a severe ciliopathy disorder. (Clinical photographs reproduced from <http://www.humpath.com>, Sainte-Justine Hospital, Montreal, QC, Canada, with permission.)

advent of molecular diagnosis for most disorders, with a primarily clinical nosologic purpose. The predictive capacity of these databases has been reviewed elsewhere (23). Similarly, proteomics databases (in particular the Ciliary Proteome Database) have proven to be a useful guide in identifying new ciliary proteins and new genes mutated in ciliopathies. Nonetheless, this database is likely to include many false-positives, and so proof of ciliary function requires experimental confirmation.

For these reasons, we have limited our analysis of ciliopathies (summarized in Tables 165-1, 165-2, and 165-3) to those for which the causative gene(s) has been identified. We also limit our description of the phenotypes associated with each gene to the actual phenotypes listed for molecularly proven cases in original publications, and we have made every effort to document individual clinical features precisely, and to give some indication about the frequencies with which they occur. For further information, in cases where the same gene is mutated in different syndromes we have also separated the clinical summary for each disorder. We will now describe the broad categories of ciliopathies (Tables 165-1, 165-2), and we will outline the experimental evidence supporting ciliary dysfunction in these disorders. We will then describe the defining clinical aspects of notable ciliopathies.

### 165.2.3 Ciliopathy-Like Disorders

**165.2.3.1 Bardet–Biedl Syndrome.** Bardet–Biedl syndrome (BBS) is diagnosed using a combination of major and minor features. Major criteria are rod–cone

dystrophy, polydactyly, obesity (typically truncal), learning difficulties, hypogenitalism in males, and renal anomalies. A clinical diagnosis of BBS is accepted only if a patient has four major criteria, or three major criteria and two or more secondary criteria. Secondary features are speech disorder, brachydactyly/syndactyly, developmental delay, polydipsia/polyuria, ataxia, diabetes mellitus, abnormal dentition/palatal defects, congenital heart disease, and hepatic fibrosis (109,110). Causative mutations have been identified in 17 different genes in BBS. Many of the encoded proteins are uncharacterized biochemically, and are defined by the structural domains that they contain, such as tetratricopeptide repeats (BBS8) and tripartite motifs (BBS11). A subset of these proteins (BBS1/2/3/4/5/7/8/9 together with BBIP10, PCM-1, and RAB8a) form a protein complex known as the BBSome (discussed later) (111–114). BBS3/ARL6 is a small GTPase involved in vesicle trafficking to the cilium, while BBS6, 10, and 12 are members of the type II chaperonin superfamily that also form a complex (25,26,115).

Several lines of evidence suggest that many BBS proteins function within the basal body. First, comparative analysis of the proteomes of two distantly related ciliated organisms, *Homo sapiens* and *C. reinhardtii*, identified 668 proteins that were shared by these species but that were absent from the unciliated plant *Arabidopsis thaliana*, thereby defining the flagellar apparatus basal body (FABB) proteome. BBS proteins were significantly enriched in the FABB proteome and BBS genes were transcriptionally upregulated following deflagellation of *C. reinhardtii* (31). This analysis, in combination



TABLE 165-1 Causative Genes in Ciliopathies and Ciliopathy Disorders

Gene ID	Syndrome	Type of Protein Encoded	Function in Cilia Biology	First Discovery (Reference)	Other Refs
<i>AIP1</i>	Leber congenital amaurosis			(24)	
<i>ARL6 (BBS3)</i>	BBS	Ras superfamily, small GTPase	Localizes to ciliated cells in <i>Ca. elegans</i> and undergoes IFT; localizes to basal body and ciliary gate in mammalian cell lines; component of BBSome	(25)	(26)
<i>ARL13B</i>	Joubert syndrome (classical form)	Ras superfamily, small GTPase	Localizes to ciliary axoneme in mouse and human tissues	(27)	
<i>ATXN10</i>	Nephronophthisis		Interacts with NPHP5	(28)	
<i>B9D2</i>	Meckel syndrome	B9-domain-containing protein	Tagged B9D2 localizes to the base of cilia and <i>B9d2</i> −/− mice have fewer and abnormal cilia in the embryonic node and neural tube	(190)	
<i>BBS4</i>	BBS		Targets cargo to pericentriolar material; component of BBSome	(29)	(30)
<i>BBS5</i>	BBS	Uncharacterized	Localizes to ciliated cells in <i>Ca. elegans</i> and mammalian tissues; knockdown causes loss of flagella in <i>Chlamydomonas</i> ; component of BBSome	(31)	
<i>BBS7</i>	BBS	Six-bladed β-propeller structure; sequence similarity to BBS1 and BBS2	Component of BBSome	(32)	
<i>BBS10</i>	BBS	Type II chaperonin	–	(33)	
<i>BBS12</i>	BBS	Type II chaperonin	–	(34)	
<i>CCDC39</i>	PCD	Coiled-coil domain containing protein	Bobtail dogs and human patients with <i>ccdc39</i> /CCDC39 mutations have variably abnormal central microtubular pairs within cilia; endogenous CCDC39 localizes to cilia	(35)	
<i>CCDC40</i>	PCD	Coiled-coil domain containing protein	<i>ccdc40</i> −/− zebra fish and patients with CCDC40 mutations have abnormal shorter cilia and variably abnormal central microtubular pairs within cilia	(36)	
<i>CC2D2A</i>	Joubert syndrome	Coiled-coil and C2-domain-containing	Tagged/overexpressed form localizes to basal body in mammalian cell lines; cilia absent in patient fibroblasts	(37)	(38)
<i>CC2D2A</i>	Joubert-spectrum disorders	Coiled-coil and C2-domain-containing	Tagged/overexpressed form localizes to basal body in mammalian cell lines; cilia absent in patient fibroblasts	(38)	
<i>CC2D2A</i>	Mental retardation + RP	Coiled-coil and C2-domain-containing	Tagged/overexpressed form localizes to basal body in mammalian cell lines; cilia absent in patient fibroblasts	(39)	(38)
<i>C20R71</i>	RP	Uncharacterized; proline-rich domain	Tagged/overexpressed form localizes to a pericentrosomal location and ciliary axoneme; downregulated in <i>Bbs4</i> −/− mouse retinas	(40)	
<i>CEP41</i>	Joubert syndrome	Centrosomal protein	Endogenous CEP41 localizes to the ciliary axoneme, and knockdown leads to reduced polyglutamination of cilia and abnormal outer microtubular doublets of cilia	(191)	
<i>CEP290</i>	Joubert-syndrome-related disorders	Uncharacterized	Tagged/overexpressed form localizes to centrosomes and ciliary base in mammalian cell lines	(41)	
<i>CEP290</i>	Leber congenital amaurosis	Uncharacterized	Tagged/overexpressed form localizes to centrosomes and ciliary base in mammalian cell lines	(42)	(41)

Continued

TABLE 165-1 Causative Genes in Ciliopathies and Ciliopathy Disorders—cont'd

Gene ID	Syndrome	Type of Protein Encoded	Function in Cilia Biology	First Discovery (Reference)	Other Refs
<i>CEP290</i>	Meckel syndrome	Uncharacterized	Tagged/overexpressed form localizes to centrosomes and ciliary base in mammalian cell lines	(43)	(41)
<i>CRB1</i>	Leber congenital amaurosis			(44)	
<i>CRX</i>	Leber congenital amaurosis			(45)	
<i>DYNC2H1</i>	Jeune syndrome	Dynein-heavy-chain protein	<i>Dync2hc1</i> —/— MEFs have abnormal cilia with swollen tip; abnormal cilia in patient chondrocytes	(46,47)	(48)
<i>DYNC2H1</i>	Short-rib polydactyly Type III	Dynein-heavy-chain protein	<i>Dync2hc1</i> —/— MEFs have abnormal cilia with swollen tip; abnormal cilia in patient chondrocytes	(46,47)	(48)
<i>DNAI1</i>	PCD	Dynein-intermediate chain	Ultrastructurally abnormal cilia in patients	(49)	
<i>DNAI2</i>	PCD	Dynein-intermediate chain	Endogenous <i>DNAI2</i> localizes to ciliary axoneme; ultrastructurally abnormal cilia in patients	(50)	
<i>DNAH5</i>	PCD	Dynein-heavy-chain protein		(51)	
<i>DNAH11</i>	PCD	Dynein-heavy-chain protein		(52)	
<i>EVC</i>	Ellis-van Creveld syndrome	Uncharacterized; contains leucine zipper, nuclear localization signals, putative transmembrane domains	Endogenous <i>Evc</i> localizes to the basal body in mouse chondrocytes	(53)	(54,55)
<i>EVC2</i>	Ellis-van Creveld syndrome	Uncharacterized	—	(54)	
<i>EVC2</i>	Weyer acrocardial dysostosis	Uncharacterized	—	(54)	(56)
<i>GLIS2</i>	Nephronophthisis	Kruppel-like zinc finger transcription factor	Endogenous <i>Glis2</i> localizes to the ciliary axoneme in MDCK cells	(57)	
<i>GUC2YD</i>	Leber congenital amaurosis			(58)	
<i>HYLS1</i>	Hydroletharus	Uncharacterized; putative transcription factor with nuclear localization signal	<i>HYSL1</i> is only found in organisms with centrioles, it localizes to centrioles in worms and frogs, it is required for cilia formation in <i>C. elegans</i> ciliated neurons and <i>X. laevis</i> mucociliary epithelium	(59)	(60)
<i>IFT80</i>	Jeune asphyxiating thoracic dystrophy	Intraflagellar transport protein	Endogenous <i>ift80</i> localizes to basal bodies and ciliary axoneme in ATDC5 cells; required for normal ciliogenesis in <i>T. thermophila</i>	(61)	
<i>IFT122</i>	Sensenbrenner syndrome	Intraflagellar transport protein	Reduced cilia length in patient fibroblasts and zebra fish <i>ift122</i> morphants	(62)	
<i>IFT43</i>	Sensenbrenner syndrome	Intraflagellar transport protein	<i>ift88</i> and <i>ift57</i> accumulate at the ciliary tip of patient fibroblasts	(63)	
<i>INPP5E</i>	(MORM—mental retardation, truncal obesity, retinal dystrophy, micropenis)	Inositol phosphatase	<i>Inpp5e</i> —/— mice have reduced number and shorter cilia in cystic renal epithelia, and <i>Inpp5e</i> —/— MEFs have fewer cilia when tyrosine kinase receptor signaling is activated; endogenous <i>Inpp5e</i> localizes to ciliary axonemes in MEFs	(64)	
<i>INPP5E</i>	Joubert syndrome	Inositol phosphatase	<i>Inpp5e</i> —/— mice have reduced number and shorter cilia in cystic renal epithelia, and <i>Inpp5e</i> —/— MEFs have fewer cilia when tyrosine kinase receptor signaling is activated; endogenous <i>Inpp5e</i> localizes to ciliary axonemes in MEFs	(65)	
<i>INPP5E</i>	CORS	Inositol phosphatase	<i>Inpp5e</i> —/— mice have reduced number and shorter cilia in cystic renal epithelia, and <i>Inpp5e</i> —/— MEFs have fewer cilia when tyrosine kinase receptor signaling is activated; endogenous <i>Inpp5e</i> localizes to ciliary axonemes in MEFs	(65)	

INVS	Nephronophthisis type II		Endogenous Invs is present in a punctate pattern along the ciliary axoneme of MDCK cells	(66)
KIF7	ACLS	Kinesin motor protein	Kif7 localizes to the base of cilia in the absence of Shh, and to the tip in presence of Shh; cilia are longer in patient fibroblasts	(67) (68,69)
KIF7	Hydroletharus	Kinesin motor protein	Kif7 localizes to the base of cilia in the absence of Shh, and to the tip in presence of Shh; cilia are longer in patient fibroblasts	(67) (68,69)
KIF7	Joubert syndrome	Kinesin motor protein	Kif7 localizes to the base of cilia in the absence of Shh, and to the tip in presence of Shh; cilia are longer in patient fibroblasts	(70) (68,69)
KTU	PCD			(71)
LCA5/CORF152	Leber congenital amaurosis	Lebercilin; uncharacterized; coiled-coil domain-containing	Endogenous lebercilin localizes to the ciliary axoneme in RPE and IMCD3 cells	(44)
LRA7	Leber congenital amaurosis			(72)
LRRCS0	PCD	Leucine-rich-repeat protein homologous to C reinhardtii outer dynein arm protein 7	Regulates vesicle trafficking to cilia	(73) (74)
MKS3	Joubert syndrome			(75)
NEK1	Short-rib polydactyly Majewski type (Type II)	Kinase domain, nuclear localization and export signals, coiled-coil domains	Endogenous NEK1 localizes to the basal body, and Nek1 is required for ciliogenesis	(76) (77,78)
NEK8	Nephronophthisis	Rcc1-domain-containing; related to never in mitosis A (NIMA)	GFP-NEK8 localizes to centrosomes and cilia; cilia are longer in mice with Nek8 mutations (jck mouse)	(79) (80)
NPHP1	Joubert syndrome			(81)
NPHP4	Nephronophthisis			(82,83)
NPHP5 (IQCB5)	Senior-Løken syndrome	IQ domain protein	Endogenous NPHP5 localized to the ciliary axoneme	(84)
NPHP6 (CEP290)	Joubert syndrome	13 coiled-coil domains, SMC ("structural maintenance of chromosomes" domain), nuclear localization signal, six KID motifs, homology with tropomyosin, ATP/GTP-binding motif (P-loop)	Endogenous CEP290 localizes to the centrosome	(85)
OFD1	Oral-facial-digital syndrome Type 1	Uncharacterized	Localizes to centrosome and interacts with pericentriolar material components	(86,87)
RDH12	Leber congenital amaurosis			(88,89)
RPF65	Leber congenital amaurosis			(90)
RPGR	PCD with RP			(91)
RPGRIP1	Leber congenital amaurosis			(92,93)
RPGRIP1L	CORS	3 N-terminal coiled-coil domains, C-terminal RPGR-interacting domain, two central C2 motifs		(82)
RPGRIP1L	Meckel syndrome	3 N-terminal coiled-coil domains, C-terminal RPGR-interacting domain, two central C2 motifs		(82)

Continued

TABLE 165-1 Causative Genes in Ciliopathies and Ciliopathy Disorders—cont'd

Gene ID	Syndrome	Type of Protein Encoded	Function in Cilia Biology	First Discovery (Reference)	Other Refs
<i>RSPH9</i>	PCD	Radial-spoke heavy-chain protein	Connects the inner and outer microtubule doublets to regulate coordinated beating of cilia	(14)	
<i>RSPH4A</i>	PCD	Radial-spoke heavy-chain protein	Connects the inner and outer microtubule doublets to regulate coordinated beating of cilia	(14)	
<i>SDCCAG8</i>	Senior-Løken syndrome	N-terminal globular domain, nuclear localization signal, 8 coiled-coil domains	Endogenous SDCCAG8 is localized adjacent to the centrosome, in centrosomal appendages	(94)	
<i>SDCCAG8</i>	BBS	N-terminal globular domain, nuclear localization signal, 8 coiled-coil domains	Endogenous SDCCAG8 is localized adjacent to the centrosome, in centrosomal appendages	(94)	
<i>TCTN2</i>	Joubert syndrome		Abnormal ciliogenesis in Tctn2 <sup>-/-</sup> mouse embryonic fibroblasts and neural tubes; interacts with MKS1	(28)	
<i>TMEM67</i>	COACH syndrome	Transmembrane protein (predicted)		(95)	
<i>TMEM138</i>	Joubert syndrome	Transmembrane protein (predicted)	Endogenous TMEM138 present in ciliary axoneme as well as cytoplasmic vesicle; knockdown of Tmem138 resulted in fewer cilia	(192)	
<i>TMEM216</i>	Joubert-related syndromes	Transmembrane protein (predicted)	Endogenous TMEM216 present in/adjacent to the basal body/ciliary base; knockdown of Tmem216 resulted in fewer cilia and disrupted apical docking of centrosomes	(96)	
<i>TMEM216</i>	Meckel syndrome	Transmembrane protein (predicted)		(96)	
<i>TMEM237</i>	Joubert syndrome-related disorders	Transmembrane protein (predicted)	Endogenous TMEM237 localizes to the ciliary transition zone and knockdown leads to fewer ciliated cells	(193)	
<i>TTC21B</i>	Nephronophthisis	Intraflagellar transport protein 139	Ttc21b <sup>-/-</sup> mice have abnormal nodal cilia; retrograde IFT is impaired and cilia are shortened, following knockdown of Ttc21b in IMCD3 cells	(97)	(13)
<i>TULP1</i>	Leber congenital amaurosis	Tubby-like protein 1; regulates G-protein-coupled phospholipid signaling		(98)	(99)
<i>TXNDC3</i>	PCD	Thioredoxin enzyme	Ultrastructurally abnormal cilia in patients	(100)	
<i>WDR35</i>	Sensenbrenner syndrome	WDR-repeat-containing and orthologous to IFT121; tubby-like protein 4; binds phosphoinositides	lft88 and lft57 accumulate at the ciliary tip of patient fibroblasts; WDR35 forms part of an IFT-A complex involved in transport of signaling proteins to cilia	(101)	(63,102)
<i>WDR35</i>	Short-rib polydactyly	WDR-repeat-containing and orthologous to IFT121; tubby-like protein 4; binds phosphoinositides	Overexpressed Wdr35 localizes to the basal body, and ciliogenesis is perturbed in Wdr35 <sup>-/-</sup> mice; lft88 and lft57 accumulate at the ciliary tip of patient fibroblasts; WDR35 forms part of an IFT-A complex involved in transport of signaling proteins to cilia	(103)	(63,102)



with genetic linkage data, served to identify mutations in *BBS5*. Secondly, all *Ca. elegans bbs* genes are expressed exclusively in ciliated neurons where they regulate IFT (116,117). Finally, several BBS proteins have also been shown to localize to centrosomes and basal bodies in mammalian cells and tissues (26,30,116,118), and a subset of BBS proteins interact physically to form the so-called BBSome that regulates vesicle transport to cilia (111–114).

### 165.2.3.2 Meckel–Joubert Spectrum Disorders.

Meckel and Joubert syndromes are allelic disorders, and a number of causative genes have been identified. Meckel syndrome is embryonic lethal, whereas Joubert syndrome patients survive past birth. Meckel syndrome is typically caused by complete loss-of-function mutations, whereas Joubert syndrome is often associated with likely hypomorphic mutations. Therefore, in genetic terms, the Meckel–Joubert syndromes represent a spectrum of phenotypic severity. Typically, patients with Joubert syndrome have cerebellar vermis hypoplasia together with a characteristic brain stem malformation on magnetic resonance imaging known as the molar tooth sign (MTS; see Section 165.3.6). Additionally, fetuses with Meckel syndrome have occipital meningoencephalocele, postaxial polydactyly, renal cystic dysplasia, and hepatic disease. Several other disorders overlap clinically with Joubert syndrome, with patients exhibiting the MTS. These include cerebello–oculo–renal syndrome (CORS), with patients also having retinal degeneration and renal cystic disease. A subset of patients also have Joubert syndrome in association with renal disease, and these patients have mutations in *NPHP1* or *NPHP6*, a gene also mutated in patients with nephronophthisis with or without retinal degeneration (the latter known as Senior–Løken syndrome). Collectively, the term “Joubert syndrome-related disorders” has been used to describe this clinical heterogeneity, although the MTS is a pathognomonic feature as shown in Tables 165-1 and 165-2.

### 165.2.3.3 Short-Rib Polydactyilies and Related Disorders.

A number of ciliopathy-like disorders have now been described in which skeletal defects are particularly striking, and patients frequently exhibit very severe defects of the rib cage leading to compression of the thorax. These disorders include the short-rib polydactyilies, Jeune asphyxiating thoracic dystrophy (JATD), and Sensenbrenner and Ellis–van Creveld syndromes, which collectively define a subgroup of ciliopathy disorders. The genes that have currently been found to cause these disorders are *DYNC2H1*, *EVC*, *EVC2*, *IFT43*, *IFT80*, *IFT122*, *NEK1*, and *WDR35* (46,47,53,54,61–63,76,101,104,119); (Tables 165-1 and 165-2). As described below, mouse models deficient for *Dync2h1*, *Evc*, *Ift80*, and *Ift122* have implicated defective hedgehog signaling in skeletal and limb malformations (brachydactyly type-A1 and polydactyly). These patients also exhibit ciliopathies that are not characteristic of defective hedgehog signaling, such as renal

cystic disease, supporting more general roles for these proteins in ciliary function. Indeed, several of these proteins (*IFT122*, *TULP3*, and *WDR35*) have been shown to form a complex, which regulates transport of several G-protein-coupled receptors to cilia in conjunction with *THM1/TTC21B*, which is mutated in patients with nephronophthisis (97). Furthermore, *IFT122* is required to transport *DYNC2H1* into cilia. Interestingly, patients with Sensenbrenner syndrome sometimes also exhibit craniosynostosis. This phenotype is not characteristic of defective hedgehog signaling, although there is growing evidence that elevated hedgehog signaling may contribute to this phenotype in conjunction with alterations in other molecular pathways (120–123), possibly reflecting the regulation of several signaling pathways (including Wnt and platelet-derived growth factor signaling) by the cilium. Finally, it should be noted that the striking rib phenotype in many of these patients is also not typical of defective hedgehog signaling (124). Instead, this phenotype could relate to defects in the sclerotome and dermomyotome, which are somite-derived tissues.

**165.2.3.4 Primary Ciliary Dyskinesia.** PCD is defined by ultrastructural defects in cilia as seen by transmission electron microscopy and altered patterns of cilia beating (Figures 165-2 and 165-3). Ultrastructural defects include absence of various components of the ciliary axoneme. Mutations in several genes (*DNAH5* and 11, and *DNAI1* and 2) lead to loss of outer dynein arms (ODA), whereas only the inner dynein arms (IDAs) are absent in patients with mutations in *CCDC39* (35,49–52). In contrast, *CCDC40* mutations lead to abnormal positioning and/or supernumerary pairs of central microtubules, and *CCDC39* and 40 mutants additionally have abnormal nexin links and abnormal radial spokes (35,36). Therefore, mutations in different components can cause distinct ultrastructural defects. While *DNAH5* and 11 and *DNAI1* and 2 encode dynein chain proteins, which makes sense in light of the ODA defects associated with mutations in these genes, the functions of *CCDC39* and 40 are less clear, although it has been proposed that these genes regulate the assembly of various components of the ciliary axoneme (35,36). *TXNDC3*, another gene mutated in PCD, encodes a thioredoxin enzyme that is expressed as two different mRNA isoforms, the relative levels of which seem to be important for cilia formation (100). Both of the encoded proteins are able to bind microtubules, and so *TXNDC3* may also regulate synthesis of axonemal components. Although *LRRCS0* encodes an uncharacterized protein, it has been speculated that mutations in this gene in PCD affect trafficking from the cytoplasm to cilium (73,74). Finally, mutations have been identified in *RSPH9* and *A4*, which encode radial spoke head proteins that connect the radial stalk and outer microtubules to the central pair, and regulate the bend waveform of cilia as they beat (14). Both *TXNDC3* and *RSPH9/A4* mutant cilia exhibit variable/intermittent

**TABLE 165-2 Clinical Features in Ciliopathy Disorders**

Gene (Syndrome)	Neurologic	Liver	Kidney	Lungs	Heart	Obesity
<i>BBS1-17</i>	(++) Learning difficulties; (+) speech disorder, developmental delay, ataxia		(++) Renal anomalies; (+) polydypsia, polyuria	(+) Fibrosis	(+) Congenital heart defects	(++) Truncal obesity; (+) diabetes mellitus
<i>B9D2 (Meckel syndrome)</i>	(++) Encephalocele	(+) Ductal plate malformation	(+) Renal cysts			
<i>ARL13B</i>	(++) MTS, hypotonia/ataxia, psychomotor delay, mental retardation, oculo-motor apraxia (+) occipital encephalocele					
<i>ATXN10</i>	(–) Seizures	(+) Fibrosis	(++) Nephronophthisis			
<i>CCDC39</i>				(++) Chronic respiratory infections		
<i>CCDC40</i>				(++) Chronic respiratory infections		
<i>CC2D2A (Joubert only)</i>	(++) Occipital encephalocele; (+) anencephaly, hydrocephaly	(++) Fibrotic/cystic	(++) Large cystic kidneys; (+) horseshoe kidney	(++) Hypoplastic lungs		
<i>CC2D2A (Joubert and COACH)</i>	(++) MTS; (+) encephalocele	(+) Fibrosis, hepatosplenomegaly	(+) Abnormal ultrasound/function			
<i>CC2D2A (mental retardation + retinitis pigmentosa)</i>	(++) Mental retardation					
<i>C2ORF71</i>						
<i>CEP41 (Joubert syndrome)</i>	(++) MTS, mental retardation, psychomotor delay					
<i>CEP290 (Joubert syndrome-related disorders)</i>	(++) MTS, hypotonia/ataxia, psychomotor delay, mental retardation, oculomotor apraxia, breathing abnormalities		(+) Nephronophthisis, cortical cysts			
<i>CEP290 (Leber congenital amaurosis)</i>	(+) MTS, hypotonia, mental retardation, ataxia, autism					
<i>CEP290 (Meckel syndrome)</i>	(++) Dandy–Walker malformation, (+) occipital meningocele, occipital encephalocele, hydrocephaly, cerebellar vermis hypoplasia, arhinencephaly; (–) corpus callosum hypoplasia	(++) Bile duct proliferation	(++) Cystic kidney disease			

Limb	Eye	Skeletal	Craniofacial	Laterality defects	Genitourinary	Other	References
(++) Polydactyly; (+) brachydactyly, syndactyly	(++) Rod/cone dystrophy		(+) Abnormal dentition, palatal defects		(++) Hypogenitalism (males)		(25)
(++) Polydactyly							(190)
	(+) Retinopathy						(27)
							(28)
				(+) Situs solitus, situs inversus, heterotaxia; (–) polysplenia			(35)
							(36)
(++) Polydactyly (hands and feet); club foot			(+) Cleft lip/palate		(+) Genitourinary malformations (variable)		(37)
	(++) Abnormal eye movements; (+) retinal dystrophy, coloboma						(38)
	(++) Retinitis pigmentosa, astigmatism						(39)
	(++) Retinitis pigmentosa						(40)
(+) Polydactyly	(–) Retinopathy				(+) Micropenis	(–) Growth hormone deficiency	(191)
	(++) Blindness/ RP		(–) Cleft palate				(41)
	(++) Leber congenital amaurosis						(42)
(+) Polydactyly			(–) Cleft lip/palate				(43)

TABLE 165-2 Clinical Features in Ciliopathy Disorders—cont'd

Gene (Syndrome)	Neurologic	Liver	Kidney	Lungs	Heart	Obesity
<i>DYNC2H1</i> (Jeune asphyxiating thoracic dystrophy)						
<i>DYNC2H1</i> (short rib polydactyly Type III)		(–) Hepatic biliary hyperplasia	(–) Tubular microcysts			
<i>DNAH5</i>				(++) Chronic respiratory infections		
<i>DNAH11</i>				(++) Chronic respiratory infections		
<i>DNAI1</i>				(++) Bronchiectasis, nasal polyps		
<i>DNAI2</i>				(++) Bronchiectasis, sinusitis, rhinitis, pneumonia		
<i>EVC1</i> (Ellis–van Creveld syndrome)					(++) Atrial septal defect	
<i>EVC2</i> (Ellis–van Creveld syndrome)					(++) Atrial septal defect	
<i>EVC2</i> (Weyer's acroental dysostosis)						
<i>GLIS2</i> (Nephro- nophthisis)			(++) Nephronophthisis			
<i>HYLS1</i> (Hydroletharus)	(++) Hydrocephalus, absent upper midline structures of the brain			(+) Respiratory defects	(+) Congenital heart defects	



Limb	Eye	Skeletal	Craniofacial	Laterality defects	Genitourinary	Other	References
		(++) Short ribs and thoracic constriction, small hands and feet, short long bones	(++) Trident acetabular roof				(46)
(+) Polydactyly		(++) Short ribs and thoracic constriction, small hands and feet, short long bones; (+) trident pelvis, metaphyseal spikes	(++) Trident acetabular roof		(-) Genitourinary malformations		(46,47)
				(+) Situs inversus			(51)
				(+) Situs inversus, dextrocardia			(52)
				(+) Situs inversus			(49)
				(+) Situs inversus, situs solitus			(50)
(++) Polydactyly, dysplastic nails		(++) Short limbs; (+) short ribs, genu valgum	(++) Oral frenulae, dysplastic teeth				(53,54,56,104)
(++) Polydactyly, dysplastic nails		(++) Short limbs; (+) short ribs, genu valgum	(++) Oral frenulae, dysplastic teeth				(53,54,56,104)
(++) Polydactyly, syndactyly, dysplastic nails			(++) Dysplastic teeth				(56)
							(57)
(++) Polydactyly			(++) Micrognathia; (+) keyhole-shaped defect in occipital bone		(++) Duplicated uterus in females, variable genitourinary malformations		(59,194)

Continued

**TABLE 165-2 Clinical Features in Ciliopathy Disorders—cont'd**

Gene (Syndrome)	Neurologic	Liver	Kidney	Lungs	Heart	Obesity
<i>IFT80</i> (Jeune asphyxiating dystrophy)						
<i>IFT122</i> (Sensen- brenner syndrome)		(–) Hepatomeg- aly, fibrosis				(++) Renal failure
<i>IFT43</i> (Sensen- brenner syndrome)		(+) Liver disease	(++) Renal disease			
<i>INPP5E</i> (MORM— mental retar- dation, truncal obesity, retinal dystrophy, micropenis)	(++) Mental retardation				(+) Heart disease	(++) Truncal
<i>INPP5E</i> (Joubert syndrome)	(++) MTS, oculomotor apraxia, hypotonia/ apraxia, psychomotor delay, mental retarda- tion		(–) Renal cysts			
<i>INPP5E</i> (CORS)	(++) MTS, oculomotor apraxia, hypotonia/ apraxia, psychomotor delay, mental retarda- tion					
<i>INVS</i> (Nephro- nophthisis type 2)			(++) Nephronophthisis; (+) renal cysts, end- stage renal failure		(+) Hypertension; (–) ventricular septal defect	
<i>KIF7</i> (ACLS)	(++) Anencephaly; (+) MTS, hydrocephaly; (–) arhinencephaly					
<i>KIF7</i> (Hydroletha- lus)	(++) MTS, absent corpus callosum				(–) Ventricular septal defect	
<i>KIF7</i> (Joubert syndrome)	(++) MTS, mental retarda- tion; (+) ataxia					
<i>LCAS/CORF152</i> (RP)						
<i>LRRC50</i> (PCD)				(++) Bronchiectasis, bronchitis, sinusitis; (+) otitis		

Limb	Eye	Skeletal	Craniofacial	Laterality defects	Genitourinary	Other	References
		(++) Narrow chest/short rib cage, short femora with bowing; (+) polydactyly, brachydactyly					(61)
(++) Brachydactyly	(-) Retinal dystrophy	(++) Short narrow thorax, short limbs	(++) Dolichocephaly, abnormal dentition, sparse hair			(+) Inguinal hernia, lax skin	(62)
(+) Short limbs, brachydactyly, syndactyly, polydactyly			(++) Abnormal dentition; (+) craniosynostosis				(63)
	(++) Retinal dystrophy				(++) Micropenis in boys		(64)
	(+) Retinopathy		(-) Plagiocephaly, microcephaly				(65)
	(++) Retinopathy						(65)
				(-) Situs inversus			(66)
(++) Polydactyly			(++) Cleft lip/palate; (-) micrognathia				(67)
(++) Polydactyly	(-) Optical atrophy		(++) Macrocephaly, hypertelorism; (-) cleft lip/palate, dental anomalies				(67)
(-) Polydactyly	(-) Coloboma		(++) Hypertelorism				(70)
	(++) Leber congenital amaurosis leading to blindness						(44)
							(73)

Continued

**TABLE 165-2 Clinical Features in Ciliopathy Disorders—cont'd**

Gene (Syndrome)	Neurologic	Liver	Kidney	Lungs	Heart	Obesity
<i>MKS3/TMEM67</i> (Joubert and COACH syndromes)	(++) Cerebellar ver- mis hypoplasia; (+) MTS, ataxia, mental retardation, enlarged ventricle IV, hypotonia, hetertopias		(+) Microcysts	(+) Fibrosis, bile duct proliferation		
<i>NEK1</i> (short rib polydactyly Type II)			(–) Cystic kidneys	(++) Hypoplastic lungs	(–) Ventricular septal defect	
<i>NEK8</i> (Nephro- nophthisis)			(++) Nephronophthisis			
<i>NPHP1</i> (Joubert syndrome)	(++) MTS, developmental delay		(+) Nephronophthisis, end-stage renal disease			
<i>NPHP4</i> (Nephro- nophthisis)			(++) Nephronophthisis, end-stage renal disease			
<i>NPHP5</i> (Senior– Løken syndrome)			(++) End-stage renal disease			
<i>NPHP6/CEP290</i> (Joubert syndrome)	(++) Cerebellar vermis aplasia, ataxia, mental retardation; (+) occipi- tal meningoencephalo- cele		(++) End-stage renal disease			
<i>OFD1</i> (Oral- facial-digital syndrome Type 1)	(++) Mental retardation		(+) Polycystic kidneys			
<i>RPGRIP1L</i> (CORS)	(++) Cerebellar ataxia, mental retardation, MTS		(++) End-stage renal disease, nephro- nophthisis			
<i>RPGRIP1L</i> (Meckel syndrome)	(++) Occipital encephalo- cele, anencephaly; (+) microphthalmia	(++) Bile duct proliferation	(++) Cystic kidneys			
<i>SDCCAG8</i> (Senior–Løken syndrome)			(++) Nephronophthisis			



Limb	Eye	Skeletal	Craniofacial	Laterality defects	Genitourinary	Other	References
	(-) Oculomotor apraxia						(75,95)
		(++) Extreme short ribs, narrow thorax, disproportionate dwarfism (especially short tibiae), squared scapulae, elevated clavicles	(++) Cleft lip/palate				(76)
	(-) RP						(79)
	(++) Oculomotor apraxia						(81)
	(-) RP						(82,83)
	(++) RP						(84)
	(+) Tapetoretinal degeneration, congenital amaurosis, nystagmus; (-) retinal coloboma						(41)
(+) Syndactyly; (-) polydactyly, brachydactyly			(++) Oral frenula; (+) cleft lip/palate, dental anomalies, alopecia				(86)
	(++) Oculomotor apraxia; (+) ptosis, nystagmus; (-) retinitis pigmentosa	(+) Scoliosis; (-) genu valgum					(82,83)
(++) Polydactyly		(+) Long bone bowing	(++) Cleft lip/palate				(82)
	(++) Retinal degeneration						(94)

Continued

**TABLE 165-2 Clinical Features in Ciliopathy Disorders—cont'd**

Gene (Syndrome)	Neurologic	Liver	Kidney	Lungs	Heart	Obesity
<i>SDCCAG8</i> (BBS)	(++) Mental retardation; (–) arachnoid cyst		(++) Nephronophthisis; (–) end-stage kidney failure			(++) Obese
<i>TCTN2</i> (Joubert syndrome)	(++) MTS; (–) cerebellar vermis hypoplasia		(–) Nephronophthisis			
<i>TTC21B</i> (Nephronophthisis)			(++) Nephronophthisis			
<i>TMEM138</i> (Joubert syndrome)	(++) Molar tooth sign; (–) Dandy–Walker malformation, encephalocele		(+) Nephronophthisis, cystic kidneys			
<i>TMEM216</i> (Joubert-related disorders)	(++) Molar tooth sign; (+) Dandy–Walker malformation; (–) polymicrogyria	(–) Bile duct proliferation	(+) Nephronophthisis; (–) cystic kidneys, abnormal cortico-medullary differentiation			
<i>TMEM216</i> (Meckel syndrome)	(+) Meningocele, anencephaly, encephalocele; (–) Dandy–Walker malformation	(++) Bile duct proliferation	(++) Cystic kidneys		(–) Ventricular septal defect	
<i>TMEM237</i> (Joubert-syndrome-related disorders)	(+) Molar tooth sign, encephalocele, Dandy–Walker malformation; (–) corpus callosum hypoplasia, cerebral vermis hypoplasia, hydrocephalus		(++) Cystic kidneys			
<i>WDR35</i> (Sensenbrenner syndrome)				(–) Recurrent infections		

(++) = phenotypes commonly found in patients (>60%); (+) = phenotypes observed in multiple affecteds, but not commonly (30–60%); (–) = phenotypes only observed in a single patient or <10% where many patients described

loss of outer dynein arms or central pairs, respectively. Therefore, as well as regulating cilia motility, each component of the ciliary axoneme is also required to maintain structural integrity.

As outlined in Table 165-2, patients with PCD suffer from chronic recurrent respiratory tract infections caused by failed mucociliary clearance. Other associated features include bronchiectasis, sinusitis, and pneumonia. Laterality defects are also common in PCD patients, including situs inversus (known as Kartagener's syndrome), situs solitus, dextrocardia, and polysplenia, which can be explained by the requirement for motile cilia to generate directional fluid flow within the embryonic node (see later). Finally, males are often infertile, owing to impaired sperm flagella function. In summary, PCD represents a distinct class of motile ciliopathies, which is defined by ultrastructural defects in cilia, and

emphasizes the physiologic and developmental importance of cilia motility.

### 165.3 CLINICAL ASPECTS OF CILIOPATHIES

#### 165.3.1 Retinitis Pigmentosa

In the vertebrate retina, the ratio of rods to cones varies among species. The human retina contains about 120 million rod cells and 5 million cone cells but their distribution across the retina is uneven. A third class of photoreceptor cells, the photosensitive ganglion cells, do not contribute to vision directly, but are thought to affect circadian rhythms and the pupillary reflex. Rods are extremely sensitive, and function well in low light levels, being triggered by a very few photons. Contrast

Limb	Eye	Skeletal	Craniofacial	Laterality defects	Genitourinary	Other	References
					(+) Hypogenitalism		(94)
	(-) Nystagmus						(28)
							(97)
(-) Polydactyly	(+) Oculomotor apraxia, retinal degeneration, chorioretinal coloboma		(-) Hypertelorism				(194)
(+) Polydactyly	(+) Oculomotor apraxia, nystagmus; (-) chorioretinal coloboma, microcornea	(+) Camptodactyly/long-bone bowing					(96)
(++) Polydactyly		(+) Long bone bowing	(+) Cleft palate		(-) Hypoplastic external genitalia	(++) Inguinal hernia	(96)
	(+) Nystagmus, strabismus; (-) coloboma, optic atrophy, morning glory disc anomaly						(193)
(++) Short limbs, brachydactyly, syndactyly, polydactyly		(++) Pectus excavatum, narrow thorax	(++) Cranio-synostosis, hypertelorism, abnormal dentition				(101)

this with cones, which require significantly brighter light to produce a signal.

Photoreceptor cell death is central to the inherited retinal degenerations more commonly termed retinitis pigmentosa (RP). Abnormalities of the photoreceptors (rods and cones) or the retinal pigment epithelium (RPE) of the retina lead to progressive visual loss and may occur in isolation (non-syndromic) or together with additional clinical features (syndromic). Of the non-syndromic forms, RP can be inherited in autosomal dominant, autosomal recessive, or X-linked patterns. More than 170 genes have been associated with RP pathogenesis (source: Retnet database), the most common of which are *RHO*, *RP1*, and *RDS*, accounting for approximately 25–30%, 5–10%, and 5–10% of autosomal dominant RP cases respectively (125,126). In contrast, autosomal recessive RP is rare, with mutations in *RPE65*, *PDE6A*, and *PDE6B*

(phosphodiesterase subunits in the phototransduction cascade) causing 2–5% of cases. *RPGR* (also known as *RP3*) and *RP2* mutations are the most common causes of X-linked RP.

Several gene mutations impact upon the phototransduction cascade, consisting of light-induced conformational change of 11-*cis*-retinal to all-*trans*-retinal, which in turn activates opsins (e.g. rhodopsin). These molecules activate transducin and cGMP-PDE6, with a consequent lowering of intracellular cGMP levels. In one of the best-characterized models of RP, the *rd1* mouse, exon 7 is mutated in the beta subunit of the rod *Pde6* gene, culminating in accumulation of cGMP.

Retinal degeneration is a hallmark of many ciliopathies such as Bardet-Biedl, Alström, and Senior-Løken syndromes in humans and several ciliary gene mouse mutants (e.g. Oak Ridge polydactyly (*Orpk*)). As with forms of Usher syndrome (sensori-neural deafness

TABLE 165-3 Documented Cases of Possible Triallelic/Digenic Inheritance

Major Gene	Putative Modifier Gene	No. Independent Families Reported	Further Evidence for Functional Interaction	Reference	Phenotype
BBS1 (hom)	BBS2 (het)	3	—	(105)	BBS
BBS3 (hom)	BBS2 (het)	1	—	(105)	BBS
BBS6 (hom)	BBS2 (het)	1	—	(105)	BBS
BBS2 (hom)	BBS6 (het)	4	—	(105)	BBS
BBS4 (hom)	BBS2 (het)	1	—	(29)	BBS
BBS1 (hom; M390R)	BBS2 (het missense)	1	—	(106)	BBS
BBS1 (compound het; M390R + frameshift)	BBS6 (het missense)	1	—	(106)	BBS
BBS2 (hom nonsense)	BBS1 (het splice)	1	—	(106)	BBS
BBS1 (hom; M390R)	BBS2 (het missense)	1	—	(107)	BBS
BBS1 (hom; M390R)	BBS4 (het missense)	1	—	(107)	BBS
BBS1 (compound het; M390R + nonsense)	BBS6 (het; missense)	2	—	(107)	BBS
BBS2 compound het)	BBS1 (M390R het)	1	—	(107)	BBS
BBS1 (hom; missense)	BBS1 (het; E234K)	1	—	(107)	BBS
BBS1 (hom; M390R)	BBS3 (het)	1	—	(25)	BBS
BBS1 (hom; M390R)	BBS5 (het; N184S)	1	—	(31)	BBS
BBS7 (hom; T211I)	BBS1 (het; E234K)	1	—	(106)	BBS
BBS10 (hom)	BBS1 (het)	5	Knockdown of both bbs1 and bbs10 showed a modest/additive interaction in zebra fish	(33)	BBS
BBS10 (hom)	BBS4 (het)	2	Knockdown of both bbs4 and bbs10 showed a strong/synergistic interaction in zebra fish	(33)	BBS
BBS10 (hom)	BBS6 (het)	4	—	(33)	BBS
BBS10 (hom)	BBS7 (het)	1	—	(33)	BBS
BBS12 (hom)	BBS3 (het)	1	—	(34)	BBS
BBS1 (hom; M390R)	C2ORF71 (het)	1	—	(40)	This patient had BBS incl RP
BBS10 (hom; frameshift)	MKS1 (het; missense)	1	—	(108)	BBS
BBS10 (het; frameshift)	MKS1 (het; missense)	1	—	(108)	BBS
BBS1 (hom; nonsense)	MKS1 (het; missense)	1	—	(108)	BBS
BBS9 (hom; splice)	MKS3 (het; missense)	1	—	(108)	BBS
CEP290 (het; nonsense)	MKS3 (het; missense)	1	Knockdown of both cep290 and mks3 in zebra fish showed a modest/additive interaction	(108)	BBS
BBS12 (compound het; splice/missense)	C2ORF86/FRITZ (het missense)	1	—	(17)	BBS
MKS6 (compound het; splice/frameshift)	C2ORF86/FRITZ (het missense)	1	—	(17)	Meckel syndrome
NPHP5 (compound het; 2 nonsense mutations)	NEK8 (het)	1	—	(79)	



<i>BBS1 (M390R hom or M390R het + nonsense)</i>	<i>KIF7 (het)</i>	2	Knockdown of both <i>bbs1</i> and <i>kif7</i> showed a modest/additive interaction in zebra fish	(67)	
<i>BBS9 (hom; nonsense)</i>	<i>KIF7 (het)</i>	1	Knockdown of both <i>bbs9</i> and <i>kif7</i> showed a modest/additive interaction in zebra fish	(67)	
<i>KIF7 (hom; nonsense)</i> <i>TTC21B (hom)</i>	<i>TMEM67 (hom; missense)</i> <i>NPHP4 (compound het missense variants)</i>	1 1	– Knockdown of both <i>ttc21b</i> and <i>nphp4</i> showed a modest/additive interaction in zebra fish	(192) (97)	Joubert syndrome Nephronophthisis
<i>TMEM216 (hom)</i>	<i>TTC21B (het)</i>	1	Knockdown of both <i>ttc21b</i> and <i>tnmem216</i> showed a modest/additive interaction in zebra fish	(97)	Meckel syndrome
<i>CC2D2A (hom)</i>	<i>TTC21B (het)</i>	1	Knockdown of both <i>ttc21b</i> and <i>cc2d2a</i> showed a modest/additive interaction in zebra fish	(97)	Meckel syndrome
<i>BBS12 (hom)</i>	<i>TTC21B (het)</i>	2	Knockdown of both <i>ttc21b</i> and <i>bbs12</i> showed a modest/additive interaction in zebra fish	(97)	BBS
<i>BBS4 (hom)</i>	<i>TTC21B (het)</i>	1	Knockdown of both <i>ttc21b</i> and <i>bbs4</i> showed a modest/additive interaction in zebra fish	(97)	BBS
<i>BBS10 (hom)</i>	<i>TTC21B (het)</i>	1	Knockdown of both <i>ttc21b</i> and <i>bbs10</i> showed a modest/additive interaction in zebra fish	(97)	BBS
<i>BBS7 (hom)</i>	<i>TTC21B (het)</i>	1	Knockdown of both <i>ttc21b</i> and <i>bbs7</i> showed a modest/additive interaction in zebra fish	(97)	BBS
<i>BBS6 (hom)</i>	<i>TTC21B (het)</i>	1	Knockdown of both <i>ttc21b</i> and <i>bbs6</i> showed a modest/additive interaction in zebra fish	(97)	BBS
<i>BBS1 (hom)</i>	<i>TTC21B (het)</i>	1	Knockdown of both <i>ttc21b</i> and <i>bbs1</i> showed a modest/additive interaction in zebra fish	(97)	BBS

het = heterozygous; hom = homozygous.

with RP) and Leber congenital amaurosis, many of the encoded proteins localize to the inner segment or connecting cilium of the photoreceptor where their role is, at least in part, associated with transport of cargo from the inner to outer segments. Disruption to this process leads to inner segment accumulation of opsins and other proteins required for the phototransduction cascade, ultimately triggering cell death.

The clinical presentation of retinal degeneration varies according to the disease and its etiology. For example, in BBS, most children have few signs of eye disease at birth; however, by 6–8 years of age on average, many will have difficulty seeing at low light levels, the so-called night blindness. This will progress to severe daytime loss of visual acuity over the ensuing two decades. BBS-related visual loss is thought to arise from a predominance of rod dysfunction (followed inevitably by cone destruction); hence the night blindness. Contrast this with Alström syndrome (AS), in which most children are born with horizontal pendular nystagmus and have early photophobia owing to disturbance of cones. This leads to a progressive loss of acuity so that it is often difficult to distinguish between AS and BBS later in life.

Despite the ever-increasing number of newly identified RP genes, the pathomechanisms leading to photoreceptor degeneration remain poorly defined. Prior studies suggest that photoreceptor cell loss in RP is driven by apoptosis (126–129) but more recent studies point to alternative mechanisms (130–132).

### 165.3.2 Cystic and Non-Cystic Kidney Disease

Exploitation of the evolutionary conservation of ciliary and flagellar proteins has contributed immeasurably to our understanding of the etiology of ciliopathic disease. The study of model organisms including *C. reinhardtii*, *Ca. elegans*, zebrafish, and mice has been central in the dissection of key phenotypes, renal anomalies chief amongst them. The role for cilia in renal disease came from the initial observation that mutation of the IFT protein IFT88/polaris in the *Tg737* mouse model led to shortened renal tubular cilia (10). Later, proteins encoded by genes mutated in human autosomal dominant polycystic kidney disease, such as polycystin 1 (PC-1) and polycystin 2 (PC-2), localized to the primary cilia membrane in renal epithelial cells (133,134).

Several cilia- or centrosomally localized proteins, when disrupted, cause renal tubular or interstitial disease. Polycystic kidney disease (PKD) is divided into autosomal dominant (ADPKD) and autosomal recessive (ARPKD) types. ADPKD is caused by heterozygous mutations in either *PKD1* (85%) or *PKD2* (15%) whereas ARPKD arises from mutations in *PKHD1* (encodes fibrocystin). ADPKD is the most common ciliopathy, occurring in up to one in 500 persons, and can occur at any age but more commonly manifests in adulthood. By contrast, ARPKD affects around one

in 20,000 persons and onset is always in childhood, and it causes significant mortality in the first months of life.

BBS and AS patients do not invariably have renal disease but can enter renal failure at any point in their life. Around one-third of BBS patients will develop chronic renal disease with tubular cystic degeneration but also with a mixed histologic picture. This may include glomerular cystic disease and tubulointerstitial cell infiltrates with interstitial fibrosis. Many BBS patients also have developmental structural kidney defects with persistence of fetal lobulation, blind ending tubular cysts and calyceal dilation (135). In AS, renal function declines with age, and end-stage renal disease is a common cause of death in AS patients (136).

The autosomal recessive nephronophthisis (NPHP) is the most common cause of inherited end-stage renal failure (ESRF) in children and young adults. NPHP is subdivided clinically, based on the median age of onset of ESRF, into infantile (1 year), juvenile (13 years) and adolescent (19 years). Early clinical signs include polyuria, polydipsia, and secondary anemia. In contrast to ADPKD, NPHP kidneys are either normal or reduced in volume, with much smaller cysts at the corticomedullary junction, tubular basement membrane disruption, periglomerular fibrosis, and tubulointerstitial cell infiltrates with interstitial fibrosis. Twelve NPHP genes have thus far been identified (*NPHP1–11* and *NPHPL1*), accounting for just 30% of cases. Deletion of *NPHP1* was initially discovered in juvenile NPHP (81). The *NPHP2* gene encodes *inversin* and is mutated in infantile NPHP (66) leading to slightly enlarged kidneys that more closely resemble PKD kidneys. *Inversin* mutant (*Inv*) mice develop *situs inversus totalis*, as do a small proportion of *NPHP2* patients.

Joubert and Meckel syndromes are caused by mutations in genes shared with NPHP (e.g. *NPHP1*, *NPHP3*, *NPHP6*, and *NPHP8*). Meckel syndrome fetuses characteristically develop large multicystic kidneys.

Even chondrodysplasia patients with conditions such as short-rib polydactyly, Jeune asphyxiating thoracic dystrophy and cranioectodermal dysplasia who are mutated in key ciliary genes encoding transport proteins (*IFT80*, *IFT122*, *IFT43*, *DYNC2H1*) develop renal cysts.

Finally, oral-facial-digital Type 1 patients (mutated in X-linked *OFD1*) also develop cystic kidney degeneration.

### 165.3.3 Hepatic Disease

Fibrocystic disease of the liver is commonly associated with ciliopathies such as ADPKD, ARPKD, Joubert, Bardet–Biedl, Meckel–Gruber, and oral–facial–digital syndromes. The ductal plate malformation, a developmental abnormality of the portobiliary system, is the basis of the liver disease in ciliopathies that manifest congenital hepatic fibrosis, Caroli syndrome, and polycystic liver disease (137). Hepatocellular function is relatively well preserved in ciliopathy-associated liver diseases.

### 165.3.4 Situs Inversus and Laterality Defects

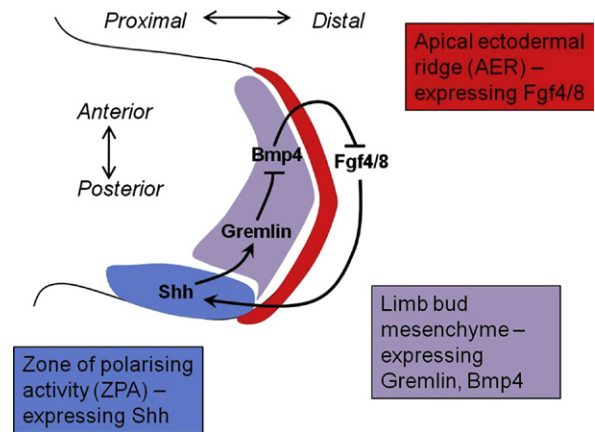
A broad range of laterality defects are frequently found in many ciliopathies including primary ciliary dyskinesia, BBS (116), OFD1 syndrome, and NPHP (NPHP2). Situs solitus refers to the normal, expected organ configuration, whereas heterotaxy, or situs ambiguus, is the abnormal arrangement of the thoracoabdominal organs resulting in congenital abnormalities. Although Baillie first described situs inversus in 1793, the first link between cilia function and heterotaxy was made in the 1970s by Afzelius when he reported a number of male patients with infertility and the Kartagener's syndrome triad (a form of PCD) (138). Heterotaxy (which can include asplenia or polysplenia) is usually accompanied by multiple congenital abnormalities, the most severe of which are congenital heart malformations (accounting for about 3% of congenital heart defects) such as transposition of the great vessels. The mechanism of nodal signaling is described later in this chapter but many genes (>20) when mutated have now been implicated in the etiology of heterotaxy. Those genes associated with PCD usually encode structural protein members of the motile cilium, such as dynein arms or radial spokes, thus impacting on the function of nodal cilia during early embryogenesis.

### 165.3.5 Polydactyly

Polydactyly is a common manifestation of several human ciliopathies such as BBS, OFD1, JATD, and MKS. It is also present in many mouse models such as *Orpk/polaris/lft88*, *Kif3a* and *Ofd1*. The underlying mechanism associating supernumerary digits with cilia dysfunction was first shown by the Anderson lab in two mouse mutants, *flexo* and *wimble*. These mice displayed up to five additional digits on each limb, which was shown to arise from disruption of hedgehog signaling (described later). They subsequently showed Hh signaling in cells to be dependent on an intact primary cilium, the purpose of which is to regulate Gli activators, which in turn play the major role in limb pattern formation. See Section 165.4.1 for further details (Figures 165-5 and 165-6).

### 165.3.6 Brain Dysgenesis and Malformations

A frequent association with many complex ciliopathies is the presence of structural brain anomalies. In Joubert syndrome where infants present with hypotonia, abnormal jerky eye movements known as oculomotor apraxia, intermittent hyperventilation, and severe cognitive impairment, there are typical posterior fossa lesions such as underdevelopment of the cerebellar vermis, an area of the brain that controls balance and coordination. The



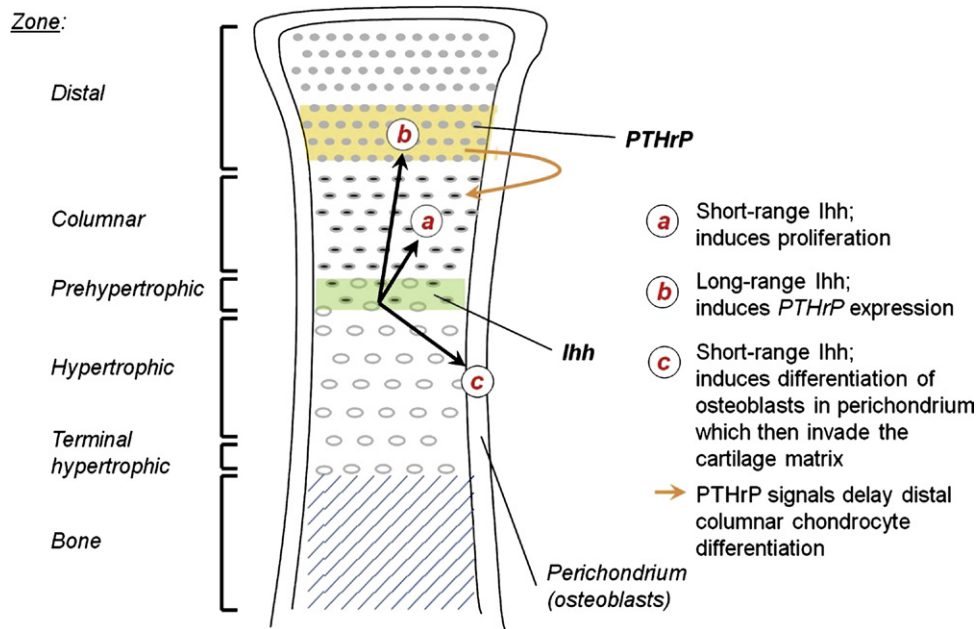
**FIGURE 165-5** Early limb bud development. Scheme showing the early limb bud (~4 weeks in human fetuses), with proximo-distal (what will become shoulder-to-finger-tip) and anteroposterior (little-finger-to-thumb) axes indicated. Shh is secreted from the zone of polarizing activity (ZPA) where it signals to the limb bud mesenchyme and sets up a posterior-to-anterior gradient of positional information. Antagonism between Shh and BMP signaling (by regulating expression of the BMP antagonist Gremlin) modulates expression of Fgf ligand in the apical ectodermal ridge (AER), which in turn signals back to the ZPA and mesenchyme.

MTS is seen on transverse magnetic resonance imaging (MRI) taken at the level of the midbrain in patients with Joubert syndrome. It describes an enlarged and horizontal tubular structure on each side of the midline emerging from the midbrain, reminiscent of a molar tooth, and is caused by a lack of normal decussation of superior cerebellar peduncular fiber tracts. Other posterior fossa defects in Joubert syndrome include Dandy-Walker malformation. Dandy-Walker syndrome is frequently associated with other central nervous system (CNS) disorders including underdevelopment/absence of the corpus callosum, the bundle of axons connecting the two cerebral hemispheres.

Acrocallosal syndrome (ACLS), recently established as a ciliopathy, is characterized by corpus callosum agenesis, polydactyly, multiple dysmorphic features (macrocephaly with prominent forehead and occiput, and hypertelorism) and severe learning difficulties. Mutations in *KIF7* and *GLI3* are causative (67).

Hydroletharus was first distinguished from Meckel syndrome in Finland. There is usually severe polyhydramnios and perinatal lethality. Fetuses have hydrocephalus, micrognathia, and polydactyly. There is hydrocephalus where the corpus callosum and septum pellucidum are absent, often accompanied by cerebellar vermis hypoplasia. A midline keyhole-shaped defect in the occipital bone dorsal to the foramen magnum is characteristic. Unlike Meckel syndrome, cases do not have cystic kidneys or liver, and the CNS anomaly is hydrocephalus rather than posterior encephalocele. The first mutations in Finland where hydroletharus is prevalent were reported in *HYLS1* and more recently, *KIF7* (67).

Subtle changes in the volume of the hippocampus and cerebellum have also been described using structural and



**FIGURE 165-6** Endochondral bone development. Endochondral bones (such as long bones of the limb) form in layers, including the distal growth plates, columnar cells, and hypertrophic zones. These cells progressively differentiate to form a cartilage matrix, which is subsequently invaded by osteoblasts in the perichondrium to form bone. *Ihh* is secreted from the pre-hypertrophic zone, where it signals (a) to columnar cells to allow this population to expand by cell proliferation; (b) to induce *PTHrP* expression in distal cells, which signals to distal columnar cells to delay their differentiation into chondrocytes; and (c) to induce osteoblast differentiation in the perichondrium (these osteoblasts subsequently invade the cartilage matrix to form bone).

functional MRI, in BBS patients (23). Conditional *Kif3a* mouse mutants, as well as mutants of *Ift88* and *Ftm*, all showed defective adult hippocampal neurogenesis, indicating the importance of the primary cilium in transducing sonic hedgehog signal for neuronal development.

## 165.4 MOLECULAR AND DEVELOPMENTAL MECHANISMS IN CILIOPATHIES

### 165.4.1 Role of Cilia in Hedgehog Signaling

Disrupted hedgehog signaling is associated with a characteristic set of birth defects, including agenesis of the corpus callosum, polydactyly, and heart and skeletal malformations, as well as gonadal dysgenesis in boys (124). Hedgehog signaling is currently the clearest example of the mechanisms by which cilia regulate intracellular signal transduction. The basic paradigm of this pathway is conserved from flies to humans, and has been reviewed in detail elsewhere (139). However, there are significant differences between vertebrate and invertebrate hedgehog signaling, and recent evidence has shown that this relates to evolved differences in the role of cilia in this pathway (139–141). Hedgehog signaling proceeds via a series of repressive interactions; three essentially equivalent hedgehog morphogens, sonic (Shh), Indian (Ihh) and desert hedgehog (Dhh), bind predominantly to a single cell-surface receptor, patched 1 (Ptch1). In the absence

of morphogen, Ptch1 represses a second transmembrane protein, smoothened (Smo), which leads to the proteolytic cleavage of the Gli transcription factors, Gli2 and 3, into their transcriptional repressor forms. In contrast, binding of Shh, Ihh, or Dhh to Ptch1 leads to the derepression of Smo, and the accumulation of Gli transcriptional activators.

Although this pathway is highly conserved, both the mechanisms by which Ptch1 represses Smo and those leading to the activation of the Gli transcription factors have remained unclear, and recent evidence suggests that this may involve cilia. In 2007, Rohatgi et al. showed that Ptch1 and Smo exhibit mutually exclusive patterns of ciliary localization—in unstimulated fibroblasts Ptch1 is present within cilia but Smo is not, whereas Smo becomes localized to cilia following application of Shh protein to cells, and Ptch1 concomitantly becomes excluded from cilia (142). Shh was shown to bind directly to Ptch1 at the ciliary membrane, and Smo was constitutively localized to cilia in *Ptch1*<sup>−/−</sup> cells. Further work has suggested that Smo is trafficked directly from the plasma membrane to the ciliary membrane via a process of lateral transport, independent of dynamin-mediated endocytosis (143).

β-arrestins are also essential for trafficking of Smo into cilia following Shh treatment (144). β-arrestins are key regulators of receptor internalization in clathrin-coated vesicles (145), and this work has suggested a model whereby Smo is internalized from the plasma membrane and recycled into the cilium through interaction with the type-II kinesin motor, Kif3a. Smo is



constitutively localized to cilia in cells deficient for the dynein-heavy-chain protein, DYN2CH1 (orthologous to the gene mutated in asphyxiating thoracic dystrophy and short rib polydactyly) (46,47), although these cells fail to elicit a hedgehog response (48,146). Therefore, Smo translocation into cilia is necessary, but not sufficient, for hedgehog signaling. Instead, it seems that IFT of Smo within cilia is essential for active signaling, because a study correlating hedgehog pathway activity with the subcellular localization of Smo in the presence of various agonists/antagonists has suggested the existence of both active and inactive conformations of Smo within the cilium (147). Much less is known about the way in which Smo is transported away from cilia, although Rab23, a key vesicle transport protein involved in early stages of endocytosis, which is mutated in Carpenter syndrome, is one such molecule (120,148–150).

### 165.4.2 Hedgehog Signaling—Limb and Skeletal Malformations

As mentioned in the previous section, some of the ciliopathies are also associated with defective hedgehog signaling, and there is now direct evidence from mouse models of ciliopathy disorders supporting a role for defective hedgehog signaling in pathogenesis. The best evidence for this comes from analysis of skeletal and limb malformations, although there is also evidence that defective hedgehog signaling causes agenesis of the corpus callosum, a cardinal feature of human hedgehog pathway mutations and a ciliopathy (120,124). Mutations in the hedgehog pathway cause a characteristic set of human birth defects, and there is clear phenotypic similarity to ciliopathies (124). Indeed, mutations in *GLI3*, which encodes a transcription factor involved in hedgehog signaling, have occasionally been found to cause oral–facial–digital syndrome Type-1 (151). Furthermore, mutations in *KIF7*, the human ortholog of *Costal 2* that regulates hedgehog signaling in fruit flies, cause ciliopathies as part of ACLS and hydrolethalus (67). Inactivation of each of the hedgehog morphogens gives rise to distinct defects, reflecting their different sites of action. For example, inactivating mutations in *IHH* cause a particular type of dwarfism, acro–capito–femoral dysplasia, owing to its essential role in endochondral ossification. Within the hands, these patients characteristically exhibit absence/shortening of the middle phalanges (brachydactyly type A1). By contrast, *SHH* mutations cause polydactyly, reflecting its indispensable role during early patterning of the limb buds (124,152).

The roles of Shh and Ihh in skeletal and limb development are outlined in Figures 165-5 and 165-6. Briefly, Shh is secreted from the posterior margin of the early limb bud, in a region known as the zone of polarizing activity (ZPA). This establishes a posterior-to-anterior gradient of Shh morphogen, which interacts with other signaling pathways to establish the identities of different

digits. In contrast, Ihh acts somewhat later within the forming endochondral skeleton. Ihh is expressed in the prehypertrophic zone from which it is secreted to activate hedgehog signaling in several cell types—it induces cell proliferation in adjacent chondrocytes, expression of parathyroid-hormone-related protein in distal chondrocytes, and promotes osteoblast differentiation in the perichondrium (152). Owing to these complex roles in endochondral ossification, both gain- and loss-of-function mutations in *IHH* cause shortening of these bones (153–155). Mutations causing either enhanced or reduced Shh signaling within the limb bud also cause polydactyly, suggesting that this is also a default phenotype following loss of positional information.

Consistent with these observations, both skeletal and limb malformations are a common aspect of a variety of ciliopathy disorders (Table 165-2). This is especially striking in the short rib polydactylies, as well as the related Ellis–van Creveld, Jeune, Sensenbrenner, and Weyer’s syndromes. These disorders are characterized by shortening of the long bones, brachydactyly, and severe shortening of the ribs, sometimes leading to asphyxiating thoracic dystrophy, together with polydactyly. To date, causative mutations have been identified in eight genes, *DYNC2H1*, *EVC*, *EVC2*, *IFT80*, *IFT122*, *NEK1*, and *WDR35* (46,47,53,54,61–63,76,101,103,104). *Evc* is expressed in various skeletal structures in the mouse, where the protein localizes to the ciliary base in chondrocytes (55). Mouse mutants of *Evc*, *Ift80*, or *Wdr35* have been analyzed in detail (55,146,156–158), and all three exhibit abnormal skeletal development, including abnormal costochondral junctions within the ribs and shortening of the long bones, associated with abnormal growth plates (55,156).

Several lines of evidence support a role for defective hedgehog signaling in pathogenesis. *Dync2h1* regulates intraflagellar transport, and *Dync2h1*–/– mice exhibit accumulation of material at the ciliary tip (48,159). Although cilia form normally in *Ift80* and *Evc* null-mutant mice, the response to hedgehog morphogen is diminished in fibroblasts derived from all three models (48,55,156,159). Furthermore, overexpression of mutant versions of *EVC2* that are encoded by mutations causing Weyer acroental dysostosis inhibit hedgehog signaling in a dominant-negative manner when overexpressed in wild-type cells (56). Within the tibia of *Evc*–/– mice, reduced expression of hedgehog target genes was also observed in both perichondrium and proliferating chondrocytes (55). Mice lacking either *Tulp3* (the mouse ortholog of *WDR35*) or *Ift122* also exhibit skeletal defects and polydactyly, although in contrast to *Dync2h1*, *Evc*, and *Ift80*, both encode negative regulators of hedgehog signaling (158,160). Emphasizing the generality of disrupted hedgehog signaling in skeletal ciliopathies, knockout in bone of *Ofd1*, the gene mutated in oral–facial–digital syndrome type-1, also causes loss of hedgehog signaling (161).

### 165.4.3 Sensing Fluid Flow and Calcium Signaling—Laterality Defects and Renal Cystic Disease

Both left–right patterning defects and renal cystic disease are key components of ciliopathies. Analysis of these seemingly contrasting phenotypes has implicated primary cilia in the sensation of fluid flow and calcium signaling. This has largely resulted from work on the polycystins PC-1 and PC-2, which are mutated in ADPKD (162,163). Both PC-1 and PC-2 have been shown to localize to the primary cilium of various cell types, where they form a likely receptor–ion channel complex (133).

As well as ADPKD, both mice and zebrafish lacking PC-2 develop laterality defects (164,165). Left–right patterning is initiated by the embryonic node in mice (known as Kupffer’s vesicle in zebrafish), an epithelial invagination in the posterior of the embryo. Two types of cilia decorate the node—motile cilia are present within the node, whereas non-motile primary cilia are located peripherally (166). The motile cilia generate leftward fluid flow, which is thought to bend the primary cilia on the left side of the node (167). Bending of these cilia has been shown to induce calcium influx in the paraxial mesoderm to the left of the node in a polycystin-dependent manner (164,167–169,189). It is important to note that cilia form normally in the absence of PC-2, emphasizing its role in cilia-mediated calcium signaling.

Fluid flow has also been shown to induce calcium influx in cultured Madine–Darby canine kidney collecting duct cells (MDCK), and requires both PC-1 and -2; this calcium influx leads to calcium-induced calcium release from the endoplasmic reticulum by the ryanodine receptor (133). Polycystins also interact with the inositol triphosphate receptor, which also releases calcium from intracellular stores (170,171). High levels of intracellular calcium in renal epithelial cells may lead to the clearance of intracellular cAMP, levels of which are elevated in renal cystic epithelia (172). Fluid flow has recently been suggested to activate mTOR signaling (173), and to regulate gene expression by phosphorylation and nuclear export of the transcriptional co-repressor HDAC5, leading to upregulation of *MEF2C* (174). *Foxj1a*, a master regulator of cilia formation, and its downstream targets are also upregulated following cystic distention of zebrafish pronephric ducts and in *Ift88*<sup>−/−</sup> renal cystic epithelia, leading to increased ciliary beating (175). Therefore, fluid flow and calcium signaling link cilia to the formation of renal cysts. It must be noted that cilia may also regulate other processes that contribute to renal cyst formation, and this has recently been reviewed in detail elsewhere (176).

### 165.4.4 Motile Cilia—Laterality Defects, Infertility, Brachiectasis

Motile cilia are important in a variety of physiologic processes, and disruptions to motile cilia lead to several

characteristic ciliopathies. Cardinal motile ciliopathies include brachiectasis, hydrocephalus, laterality defects, and infertility. Generally, there are three configurations of motile cilia. While some cells, such as those within the embryonic node, carry only a single motile cilium (monociliated), other cell types, such as those lining the respiratory tract, are multiciliated. In contrast, sperm possess flagella, which are specialized motile cilia that propel them into the female reproductive tract. Interestingly, whereas most motile cilia have a 9+2 configuration, motile cilia within the node are unique in having a 9+0 structure.

Clearance of mucus in the respiratory tract is necessary to prevent infection (177), and ultrastructurally abnormal cilia have been reported in children with recurrent respiratory tract infections (21). Furthermore, motile cilia on ependymal cells lining the ventricles of the brain are necessary for cerebrospinal fluid flow. A crucial determinant of directed ciliary beating is the orientation of cilia. Within the multiciliated epithelium of the respiratory tract, all cilia on the epithelial surface point in the same direction, which allows the coordinated movement of the cilia in a wave-like motion to move mucus in one direction (Video 165-2). At the tissue level, correct orientation is achieved by planar cell polarity (PCP)—that is, all cells have intrinsic polarity within the plane of the epithelium. PCP is achieved by specific PCP-related proteins that are distributed asymmetrically within each cell, which is established by extracellular cues such as Wnt ligands (178).

At the cellular level, the asymmetric orientation of cilia results from the positioning of basal bodies (178,179). Similar mechanisms regulate the positioning of motile monocilia within the embryonic node, which point to the left and posterior of the node so that the different forces generated by the up- and down-strokes collectively generate leftward fluid flow (180,181). In the previous section, the importance of cilia in activating calcium signaling specifically on the left side of the node was discussed. In contrast, the importance of leftward fluid flow within the node was elegantly demonstrated by experiments using a flow culture system to reverse flow to the right hand side—this resulted in signal transduction to the right of the node (167). This has also been confirmed by the characterization of many mutants that perturb motile cilia within the node, leading to randomization of signaling lateral to the node and randomized organ laterality.

Although generally identical in structure, cilia and flagella exhibit very different patterns of movement. While cilia generally exhibit back-and-forth bending, flagella generate a propeller-like circular motion, allowing them to propel individual cells through liquid. The motility of both flagella and conventional 9+2 motile cilia relies on the integrated structure of the ciliary axoneme. In particular, the inner and outer dynein arms that interconnect the outer microtubule doublets generate the shear force

required to bend the cilium, and this is regulated by the nexin link (Figure 165-3). The radial spokes are crucial to translate this movement to the central microtubules. In particular, this connection translates the force generated by the dynein arms into regular and coordinated movement. It is likely that regulatory/accessory proteins determine the exact pattern of movement that different cilia display. Defects in these coordinated movements lead to the motile ciliopathies described, including male infertility owing to impaired movement of sperm. Interestingly, fathers who are heterozygous for mutations in PCD genes do not display reduced fertility. This is an important point, not only for genetic counseling, but because if this was the case, one might expect the mutation never to be passed on to children owing to selection of sperm carrying the wild-type allele. The reason this does not occur is likely to be because diploid spermatogonial stem cells provide long-lived mRNA necessary for the subsequent development and function of haploid sperm, and because sperm themselves seem capable of sharing these transcripts subsequently (182).

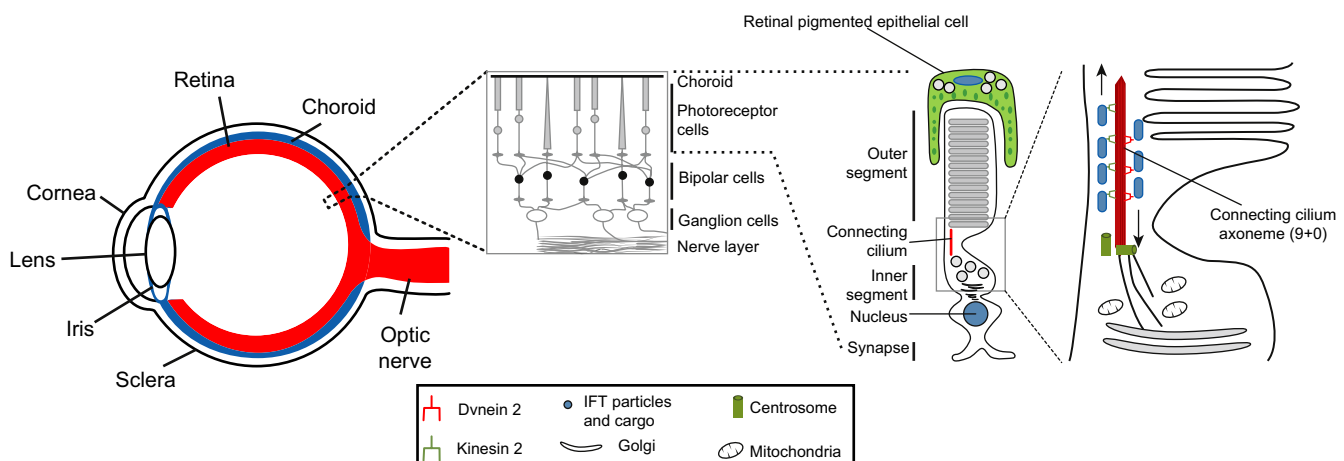
### 165.4.5 Specialized Sensory Cilia—Retinal Degeneration and Deafness

Photoreceptor cells in the retina are one example of specialized sensory cilia. They consist of a synaptic terminus, a nucleus, and inner and outer segments (Figure 165-7). While the membrane stacks that contain all of the photosensitive pigment are present in the outer segment, all of the cellular machinery for biosynthesis is present in the inner segment. All of the cellular material that makes up the outer segment must therefore be transported to that location, and this is achieved by dynein- and kinesin-mediated transport along the connecting cilium that connects the two segments. Likewise, the absence of biosynthetic machinery within more conventional cilia

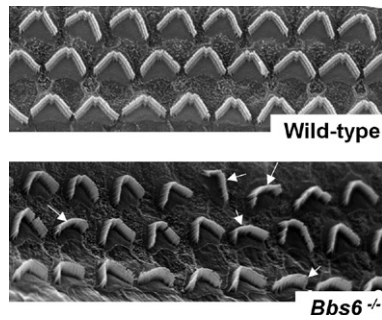
means that proteins and lipids must be transported to cilia by both vesicle transport and IFT. The connecting cilium of photoreceptors consists of microtubule doublets arranged in a 9+0 configuration, and is essentially identical to the ciliary axoneme of primary cilia.

*RPGR* is mutated in approximately 20% of patients with X-linked retinitis pigmentosa (183), a severe form of retinal degeneration similar to that seen in many patients with Bardet–Biedl and Senior–Løken/nephronophthisis syndromes. *RPGR* localizes to the connecting cilium and interacts with several IFT proteins, and probably regulates transport along the connecting cilium (184). *RPGR*-interacting protein 1 (*RPGRIP1*) also localizes to the connecting cilium, and interacts with a very similar protein encoded by *RGRIP1L*, which is mutated in both *CORS*, which is clinically similar to Joubert syndrome and Meckel syndromes, sometimes in combination with *RP*. *RPGRIP1L* also interacts with nephroretinin, which is encoded by the *NPHP4* gene that is also mutated in *RP* and nephronophthisis (82,83). Therefore, retinal degeneration can be caused by defective transport along the specialized connecting cilium of photoreceptor cells.

Specialized ciliary axonemes, so-called kinocilia, are also a striking feature of sensory hair cells within the inner ear of mammals. Each kinocilium (one per cell) is associated with many stereocilia, actin filament bundles encased by membrane. Collectively, kinocilia and stereocilia form a chevron-shaped bundle of cytoskeletal components that sense auditory vibrations (185) (Figure 165-8). Defects in their formation can cause sensorineural hearing loss and balance disturbance. As previously discussed, motile cilia are often orientated asymmetrically within the plane of an epithelium to generate fluid flow. This PCP is also apparent in the organ of corti, where the hair cells reside, and PCP proteins are also required for the proper orientation of kinocilia and



**FIGURE 165-7** Photoreceptors in the eye represent specialized cilia. From left to right: schematic showing the overall structure of the eye; a section of the retina (boxed region) expanded to show the different cell layers, including photoreceptor cells (rods and cones); expanded photoreceptor cell (together with associated retinal pigment epithelium cell) showing inner and outer segments adjoined by the connecting cilium; expanded view of the connecting cilium showing dynein- and kinesin-mediated anterograde and retrograde transport.



**FIGURE 165-8** Specialized kinocilia on hair cells in the inner ear, and planar cell polarity. Scanning electron micrographs of the 6-week-old mouse cochlea showing that each hair cell possesses stereociliary actin bundles arranged in a chevron shape. Note that the single kinocilium that was present at the apex of these stereociliary bundles has degenerated by this stage. In the wild-type, the orientation of these actin bundles is organized within the plane of the epithelium, whereas several are abnormally rotated in *Bbs6*<sup>-/-</sup> mutants (white arrows).

stereocilia. This is achieved following migration of the basal body to the abneural side of each hair cell, where it nucleates microtubules. BBS proteins are required to maintain the correct planar cell polarity of hair cells in mice, in conjunction with PCP proteins (186,187).

Usher syndrome is the most common cause of coexisting hereditary blindness and deafness. Several Usher syndrome proteins maintain connections between stereocilia, and also localize to the connecting cilium of photoreceptor cells. Some patients with this condition also exhibit sinusitis and bronchiectasis, further suggesting a role in mucociliary clearance and thus a wider role in ciliary function. Therefore, it has been suggested that some subtypes of Usher syndrome may be considered ciliopathies. On the other hand, mouse models of Usher syndrome do not exhibit altered planar polarity of hair cells, but instead have fewer and disorganized stereocilia, and so Usher proteins may function independently of other ciliopathy proteins.

### 165.5 DIRECT INTERACTIONS BETWEEN CILIOPATHY PROTEINS AND EVIDENCE FOR COMPLEX INHERITANCE IN A SUBSET OF FAMILIES

As listed in Table 165-1, causative mutations in at least 70+ genes have been identified in ciliopathy disorders. All ciliopathy disorders follow an autosomal recessive pattern of inheritance, with the single exceptions of oral-facial-digital syndrome Type I, which is X-linked recessive, and Weyer acroental dysplasia, which can be caused by heterozygous dominant-negative mutations in the Ellis-van Creveld syndrome gene, *EVC2* (53,86,87,104). There is also extensive genetic heterogeneity, with as many as 17 genes mutated in BBS, and 10 genes causing Leber congenital amaurosis, for example. Given that this large number of

genes are associated with overlapping phenotypes, it is perhaps not surprising that many of the encoded proteins interact, and some form multiprotein complexes. One example is the so-called “BBSome,” formed by a subset of BBS proteins, that has been shown to regulate vesicle trafficking to the basal body in conjunction with small GTPases, Arl6 (encoded by *BBS3*) and Rab8a (111–114). Another complex that traffics G-protein-coupled receptors into cilia involves associations between WDR35 and IFT122 (both of which are mutated in Sensenbrenner syndrome/short rib polydactyly) (101,103), as well as THM1/TTC21B (mutated in nephronophthisis) (97), TULP3, WDR19, and IFT140 (102). Interestingly, several of these proteins have been shown to negatively regulate hedgehog signaling, consistent with skeletal defects in Sensenbrenner syndrome and suggesting that formation of this complex is functionally relevant in pathogenesis (13,157,158,160,188). *INV/NPHP2* and *LCA5*, which are mutated in nephronophthisis and Leber congenital amaurosis, respectively, are further examples of proteins that have multiple interacting partners (44,66) and references therein. Therefore, the exclusive recessive inheritance and extensive genetic heterogeneity is likely to reflect complex interactions between networks of ciliopathy genes, involving many specific protein–protein interactions.

Molecular genetic findings also support functional interactions between ciliopathy proteins, because several examples of allelism between distinct ciliopathy disorders have been described. For example, several genes mutated in nephronophthisis, including *SDC-CAG8* (94), also segregate pathogenic mutations in BBS families. Meckel–Grüber and Joubert/Joubert-related syndromes have also been shown to be allelic, with mutations in *CC2D2A*, *CEP290*, *INPP5E*, *MKS3*, *RPGRIP1L*, *TMEM67*, or *TMEM216* causing both conditions (37,38,41–43,64,65,75,82,95,96). Indeed, identical missense mutations in *TMEM216* can cause either condition, and both phenotypes were found to coexist in at least one sibship (96). There is also a clear genotype–phenotype correlation—whereas homozygous/compound heterozygous nonsense or frameshift mutations cause the more severe embryonic-lethal Meckel–Grüber syndrome, patients with Joubert syndrome caused by mutations in *CC2D2A*, *INPP5E*, *MKS3*, or *RPGRIP1L* always carry at least one missense mutation, which are likely to represent hypomorphic alleles (37,38,43,64,65,82,95,96). A similar scenario is also found in patients with mutations in *DYNC2H1*, *IFT80*, *IFT122*, or *WDR35*, causing either short rib polydactyly or Sensenbrenner syndrome, who also always carry at least one missense/point mutation, suggesting that homozygous/compound heterozygous nonsense or frameshift muta-



tions would be lethal or cause a more severe phenotype (46,47,61,62,101,103).

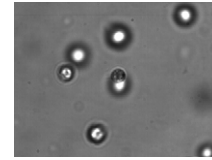
These observations clearly show that a spectrum of clinical severity exists in ciliopathies, dependent on the mutational load. The existence of large complexes of ciliopathy proteins further raises the possibility that mutations in multiple ciliopathy genes may participate in complex inheritance. The first evidence supporting this possibility came from examples of possible triallelic inheritance in BBS, whereby three mutant alleles in two genes are required to cause disease (105,107). Resequencing of *BBS* genes in large numbers of patients identified a small number of families with mutations in more than one *BBS* gene, and in some families, patients with three mutant alleles had a more severe clinical presentation than relatives with only two mutations in a single gene. Furthermore, in other families individuals were very occasionally described who carried two known pathogenic mutations in a single *BBS* gene, but who were clinically normal (a notable example is the M390R mutation in *BBS1*, the most common mutation in BBS) (24,29,106). Since these initial studies, families segregating three mutations in two genes have been described for other pairs of ciliopathy genes in nephronophthisis, Leber congenital amaurosis, and Joubert, Meckel, and Senior-Løken syndromes also (listed in Table 165-3). It has also been suggested that some of these “third” alleles may act as tissue-specific modifiers. For example, the R830W polymorphism in the *AHI1* gene was found more commonly in, and was over-transmitted to, patients who had two mutant alleles in *NPHP* genes and presented with nephronophthisis and retinitis pigmentosa, in comparison to patients with nephronophthisis alone.

Collectively, the many examples of possible triallelic inheritance, together with the extremely low frequencies of the identified mutations in control populations, provide overwhelming evidence in support of oligogenic inheritance in ciliopathy disorders. However, there are two cautionary notes for the clinician. First, it must be noted that it is not possible to say definitively that triallelic/oligogenic inheritance is in operation in any single family segregating three or more mutant alleles, and so this information is of limited value for genetic counseling. Secondly, the relative contribution of triallelic/oligogenic inheritance to ciliopathy disorders is likely to be relevant in only a minority (~5–8%) of families. Therefore, segregation of two mutations in a single gene is still likely to be the prevailing mode of inheritance in most patients.

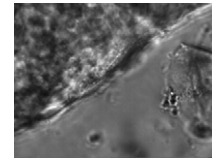
## SUPPLEMENTARY DATA

Supplementary video related to this article can be found at <http://dx.doi.org/10.1016/B978-0-12-383834-6.00165-8>.

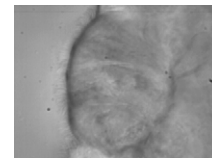
The following are the supplementary data related to this article:



**VIDEO 165-1:** Video showing a single alga (*Chlamydomonas reinhardtii*) swimming. (Reproduced from Castleman et al. (14) with permission.)



**VIDEO 165-2:** Video of the nasal pit of a live wild-type anesthetized zebra fish. Beating cilia can be seen, and flow of material within the nasal pit can also be appreciated. (Reproduced from Castleman et al. (14) with permission.)



**VIDEO 165-3:** Video of a primary nasal epithelial scraping from a normal human, showing cilia beating in a coordinated manner. (Video courtesy of Amelia Shoemark, Royal Brompton Hospital, and a still from the video is shown in Figure 165-2D.)

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## RELEVANT WEBSITE

RetNet database <http://www.sph.uth.tmc.edu/Retnet/>.

## Biographies



**Dagan Jenkins** is currently a postdoctoral research fellow at the UCL Institute of Child Health (2010–present). He previously completed his PhD at the same institute with Prof. Adrian Woolf (2002–2005), working on the genetics of human renal tract malformations, where he discovered *de novo* *UPK3A* mutations in patients with renal dysplasia. Dr Jenkins subsequently undertook postdoctoral training with Prof. Andrew Wilkie at the University of Oxford (2005–2010), where he discovered *RAB23* mutations in Carpenter syndrome, as well as making other contributions to the molecular genetics of human craniosynostosis. His current work uses zebra fish and mice, together with other functional approaches, to understand how *RAB23* and other cilia-related proteins regulate signal transduction, and how this modulates human disease phenotypes, including craniosynostosis and congenital heart disease.



**Philip Beales** is currently Professor of Medical and Molecular Genetics and Wellcome Trust Senior Research Fellow at the UCL Institute of Child Health. He is also honorary consultant in clinical genetics at both Great Ormond Street Hospital for Children and Guys and St Thomas' NHS Trust in London. Following general medical and pediatric training at the Royal London Hospital, he went on to specialty training in clinical and molecular genetics at Guy's Hospital. He completed a postdoctoral post at Baylor College of Medicine, Houston, Texas, following which he set up his lab at the Institute of Child Health in London. He has general interests in the genetic causes of rare diseases and childhood obesity, but has focused on delineating the etiology of Bardet–Biedl syndrome, which he co-discovered lies with dysfunctional primary cilia. He has contributed to a greater understanding of the newly emerging category of ciliopathies and the field of cilia biology, by defining a group of chondrodysplasias, including Jeune asphyxiating thoracic dystrophy, Sensenbrenner syndrome, and acrocallosal syndrome, as ciliopathies. In addition to functionally characterizing ciliopathies, his lab is now pursuing therapeutic targets for these conditions. Prof. Beales heads the Cilia Disorders Laboratory at UCL, is director of GOSGENE, theme lead for the Biomedical Research Centre and an NIHR investigator. He is an editor/board member of several journals and co-editor in Chief of the BMC open access journal, CILIA. He sits on several grant committees including the Wellcome Trust, and is a fellow of the Academy of Medical Sciences.